สมบัติของไซแลนเนสจาก *Aureobasidium pullulans* PBU109 และการประยุกต์ในขั้นตอนก่อนฟอกเยื่อกระดาษจากฟางข้าว

นางสาววิชาณี แบนคีรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

PROPERTIES OF A XYLANASE FROM *Aureobasidium pullulans* PBU109 AND ITS APPLICATION IN PREBLEACHING OF RICE STRAW PULP

Miss Wichanee Bankeeree

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biological Sciences
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Chulalongkorn University
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Thesis Title	PROPERTIES OF A XYLANASE FROM *Aureobasidium pullulans* PBU109 AND ITS APPLICATION IN PREBLEACHING OF RICE STRAW PULP
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Field of Study	Biological Sciences
Thesis Advisor	Associate Professor Hunsa Punnapayak, Ph.D.
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วิชาณี แบนคีรี : สมบัติของไซแลนเนสจาก *Aureobasidium pullulans* PBU109 และการประยุกต์ในขั้นตอนก่อนฟอกเยื่อกระดาษจากฟางข้าว (PROPERTIES OF A XYLANASE FROM *Aureobasidium pullulans* PBU109 AND ITS APPLICATION IN PREBLEACHING OF RICE STRAW PULP) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. หรรษา ปุณณะพยัคฆ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. พงศ์ธาริน โล่ห์ตระกูล, หน้า.

ไซแลนเนสที่ผลิตจาก *Aureobasidium pullulans* PBU 109 ถูกทำให้บริสุทธิ์ เพื่อศึกษาสมบัติ และประเมินศักยภาพในการนำไปใช้เป็นสารปรับสภาพก่อนฟอกสำหรับเยื่อกระดาษจากฟางข้าว ไซแลนเนสขนาดมวลโมเลกุล 75 กิโลดาลตันสามารถผลิตจาก *A. pullulans* PBU 109 ได้ในปริมาณสูงสุด 10.09 ± 0.27 U/mL เมื่อเลี้ยงในอาหารที่มีซังข้าวโพด 3.9 % (โดยน้ำหนักต่อปริมาตร) เป็นเวลา 72 ชั่วโมง ไซแลนเนสถูกทำให้บริสุทธิ์ขึ้น 17.3 เท่าและได้ผลผลิตเท่ากับ 13.6 เปอร์เซ็นต์ของปริมาณเริ่มต้น ไซแลนเนสบริสุทธิ์สามารถทำงานได้ดีในช่วงค่าความเป็นกรดด่าง 4-10 และอุณหภูมิ 50-80 องศาเซลเซียส โดยภาวะที่เหมาะสมที่สุดต่อการทำงานอยู่ที่ค่าความเป็นกรดด่าง 6 และอุณหภูมิ 70 องศาเซลเซียส ไซแลนเนสบริสุทธิ์ค่อนข้างไม่เสถียรที่ 70 องศาเซลเซียสโดยสูญเสียแอคติวิตีไปมากกว่าครึ่งหนึ่งเมื่อเวลาผ่านไป 1 ชั่วโมง ความเสถียรของเอนไซม์สามารถทำให้เพิ่มขึ้นได้ด้วยการเติม 0.75 มิลลิโมลาร์ซอร์บิทอลซึ่งสามารถเพิ่มค่าครึ่งชีวิตของเอนไซม์สูงขึ้น 10 เท่าที่อุณหภูมิ 70 องศาเซลเซียส ที่ความเข้มข้น 10 มิลลิโมลาร์ Mg^{2+} Co^{2+} และ Ca^{2+} มีผลกระตุ้นไซแลนเนสบริสุทธิ์ในขณะที่ Fe^{2+} และ Cu^{2+} มีผลยับยั้ง ทั้งไซแลนเนสหยาบและบริสุทธิ์สามารถย่อยสลายไซแลนที่แตกต่างกันได้ แต่ไม่สามารถย่อยสลายแอลฟาเซลลูโลส กระดาษกรอง ซีเอ็มซี และ p-nitrophenyl-β-D-xylopyranoside โดยค่า *K*m และ *V*max ของไซแลนเนสบริสุทธิ์ในการย่อยสลายไซแลนจากไม้บีช ข้าวโอ๊ต และฟางข้าว มีค่าใกล้เคียงกัน เมื่อนำไซแลนเนสหยาบไปใช้เป็นสารปรับสภาพเยื่อกระดาษจากฟางข้าวก่อนการฟอกด้วยไฮโดรเจนเพอรอกไซด์ พบว่าจะมีประสิทธิภาพสูงสุดเมื่อเติม 0.75 มิลลิโมลาร์ซอร์บิทอล และเมื่อนำเยื่อไปขึ้นแผ่น กระดาษที่ได้มีค่าความขาวสว่างเพิ่มขึ้น 13.5% และมีค่าต้านทานแรงดึงและค่าต้านทานแรงฉีกสูงขึ้น 1.16 และ 1.70 เท่าตามลำดับ เมื่อเปรียบเทียบกับเยื่อที่ไม่ได้ผ่านการปรับสภาพด้วยเอนไซม์ จากผลการทดลองสรุปได้ว่าไซแลนเนสจาก *A. pullulans* PBU 109 มีศักยภาพในการนำไปใช้เป็นสารปรับสภาพก่อนการฟอกเยื่อกระดาษจากฟางข้าวโดยเฉพาะเมื่อใช้ร่วมกับซอร์บิทอล

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5273924523 : MAJOR BIOLOGICAL SCIENCES
KEYWORDS: ENDOXYLANASE / THERMOSTABILITY / AUREOBASIDIUM PULLULANS / BLEACHING

WICHANEE BANKEEREE: PROPERTIES OF A XYLANASE FROM *Aureobasidium pullulans* PBU109 AND ITS APPLICATION IN PREBLEACHING OF RICE STRAW PULP. ADVISOR: ASSOC. PROF. HUNSA PUNNAPAYAK, Ph.D., CO-ADVISOR: ASST. PROF. PONGTHARIN LOTRAKUL, Ph.D., pp.

A xylanase from *Aureobasidium pullulans* PBU 109 was produced, purified, and studied for its properties including the potential use as a prebleaching agent of rice straw pulp. The 75 kDa xylanase was produced from *A. pullulans* PBU 109 at a maximum yield of 10.09 ± 0.27 U/mL after cultivated in an optimized production medium containing 3.9% (w/v) corncob for 72 hours. The xylanase was purified 17.3-fold to apparent homogeneity with a recovery yield of 13.6%. The purified xylanase was active over a broad pH range from 4 to 10 and at the temperature between 50 and 80°C. The optimal condition for activity of the purified enzyme was at pH 6.0 and 70°C. However, the enzyme was relatively unstable at 70°C, losing more than half of its original activity after 1-h incubation. Thermostability of the enzyme was improved by the addition of 0.75 mM sorbitol that prolonged its half-life up to 10-fold at 70°C. At 10 mM, the metal ions from $CaCl_2$, $MgCl_2$, and $CoCl_2$ enhanced the xylanase activity, while the ions from $FeSO_4$ and $CuCl_2$ displayed an inhibitory effect. Both crude and purified xylanases were active on different xylans but not on α-cellulose, filter paper, CMC and p-Nitrophenyl-β-D-xylopyranoside. The *K*m and *V*max of the purified enzyme were comparable among the reactions on xylans from beech wood, oat spelt and rice straw. When the crude enzyme was used to pretreat rice straw pulp, the greatest efficiency was obtained from a mixture containing xylanase and 0.75 mM sorbitol. Pretreatment of the rice straw pulp with the enzyme prior to H_2O_2 bleaching increased the ISO sheet brightness and the tensile and tear indexes up to 13.5%, 1.16- and 1.71-fold, respectively. This xylanase from *A. pullulans* PBU 109 has a high potential to be used as a pretreatment agent of rice straw pulp, especially in the presence of sorbitol.

Field of Study: Biological Sciences Student's Signature .....
Academic Year: 2014 Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I
INTRODUCTION

1.1 Rationale

The pulp and paper industry is rapidly growing in the last several years and becoming one of the worst offenders in environmental terms (Savitha et al., 2007). Increasing demand for paper products, the raw materials, i.e. woody pulp, have to be very cost effective and it leads to deforestation at the alarming rate. In view of diminishing forest resources and limitation of expanding commercial forest areas, non-woody plants have gained much interest as an alternate source of pulp fibers. Therefore, the use of non-woody plants and/or agricultural residues for papermaking is being recommended by different world organizations due to changes in agricultural policies, wood supply issues and environmental aspects (Anwar et al., 2014). Among many agricultural materials used for pulp manufacture, rice straw is widely used in some countries of the southern and eastern Asia due to its ready availability, abundance and certain fiber properties (Squires, 2014). Rice straw is also preferred for special paper production including glassine and greaseproof paper, duplex and triplex paper, corrugating medium, strawboard and wrapping paper (Zahran & El-Ameir, 2014). It is due to the suitable average fiber length and fiber width in 1.4 mm and 0.009 mm, respectively that make the rice straw fibers more suitable than the hardwood fibers for such papers (Biermann, 1996). In addition, low lignin content in rice straw requires less energy and chemicals consumption during pulping and bleaching process (Marja-Sisko, 1995).

The release of large amounts of organic wastes, chlorinated organics, ash and heavy metals to water used during the pulping and bleaching processes is the major problem of the pulp and paper industry (Bajpai, 2012). Therefore, a number of alternative processes for bleaching of pulp have been suggested including enzymatic method. Since the goal of pulp bleaching is the complete removal of residual lignin and colored extractives in cooked pulps, several different enzymes have been trialed to reduce the amount of chemicals used. The delignification with oxidative enzymes

including lignin peroxidases, manganese-dependent peroxidases and laccases have been widely studied for the prebleaching step (Arias et al., 2003; Karigar & Rao, 2011). However, the requirement for an expensive mediator precludes it from practical application (Kieliszek & Misiewicz, 2014) and the structural complexity of lignin and other polymers in cell wall has limited the success of most trials using enzymatic lignin degradation system (Taşpinar & Kolankaya, 1998). In fact, the physical barrier during the bleaching step is related to the lignin-hemicellulose complex (LHC) in pulp. Several types of lignin-hemicellulose bonds have been proposed and most of them are easily cleaved under the alkaline pulping conditions (Harun et al., 2013). However, the benzyl ether type linkages between the α-hydroxyl group of lignin phenylpropane unit and hydroxyl group of xylan are resistant to such process and most are remained after pulping. The residual LHCs do not only form a barrier against the subsequent bleaching reagent on surface of fibers but are also perceived as amphipathic substances or chromophores in pulp (Techapun et al., 2003). Therefore, another potential candidate for the enzymatic delignification is the group of xylanolytic enzymes, especially endo-1,4-β-D-xylanases (EC 3.2.1.8). Such xylanases selectively hydrolyze the main chain of xylan on the fiber surfaces, resulting in the removal of LHCs which makes the structure of the fibers more permeable to subsequent chemical extraction (Nagar et al., 2013). This suggests that the xylanase digestion step is most beneficial if applied before chemical bleaching. Moreover, xylanase pretreatment enhances the removal of transition metal ions, such as iron, manganese, magnesium and calcium, which cause the formation of colored metallic complexes in the carbohydrates (Bahar et al., 2011). Therefore, the pre-treatment of pulp with xylanase may provide a cost-effective method that can decrease the chemical consumption, reduces the discharge of toxic organic compounds and consequently, lowers environmental pollution impact (Khandeparkar & Bhosle, 2006).

In order to use a xylanase for pre-bleaching treatment, more attention has been focused on the enzyme stability under different processing conditions such as high temperature, neutral to mild alkali pH and inhibitory ions. In addition, the enzyme must not show any cellulolytic activity since it will adversely affect the quality of paper pulp. Fungal xylanases that are cellulase-free are generally preferred

for pretreatment and bleaching of paper pulp due to their easy-to-harvest in extracellular form, higher yield compared to bacterial xylanases and the presence of several readily produced auxiliary enzymes that are necessary for de-branching of the substituted xylans (Akhavan Sepahy et al., 2011; de Alencar Guimaraes et al., 2013b). The interest for such fungal xylanases and their applications in the pulp and paper industries has increased significantly during last several years (Addleman et al., 1995; Bajaj et al., 2011; Kulkarni et al., 1999; Vicuña et al., 1997). However, most reported fungal xylanases are generally unstable at high temperature for a long period. To improve the thermostability of the fungal enzymes, several methods such as chemical modification, cross-linking, immobilization, treatment with additives and protein engineering, have been investigated with more or less success (Gupta et al., 1999; Sarath Babu et al., 2004). Addition of certain compounds to a protein solution that change its microenvironment provides a simple but practical means of increasing the stability of enzyme. The addition of polyols, for example, reportedly improves the thermostability of a number of fungal enzymes (Belloco et al., 2005; Eremin et al., 2001; Haouz et al., 2001; Meng et al., 2004), including xylanases from *Trichoderma reesei* QM 9414 (López & Estrada, 2014) and *Aspergillus niger* DFR-5 (Pal & Khanum, 2010). Several studies have also suggested that the molecular size and number of hydroxyl groups per polyol molecule play a crucial role in mediating the protection against thermal denaturation. However, to select an appropriate polyol, the nature of that specific enzyme must be included for consideration.

From a number of studies, some tropical isolates of *Aureobasidium pullulans* were found to produce thermophilic xylanase without cellulolytic activity (Ohta et al., 2001b; Tanaka et al., 2006). Since a genetically diverse group of *A. pullulans* has been reported from Thailand (Manitchotpisit et al., 2009), it is of interest to find a good thermiphilic xylanase-producing strain that can be used for the pulp biopretreatment. Therefore, the objectives of this study were to (i) produce a cellulose-free, thermophilic xylanase from a Thai *A. pullulans* strain (PBU-109) using agricultural wastes (ii) characterize the biochemical properties of the purified xylanase from *A. pullulans* PBU-109, (iii) determine the effect of polyols on the thermostability of the enzyme, and

(iv) investigate the potential application of the xylanase in the prebleaching of rice straw pulp.

1.2 Objectives of this study

1. To produce a xylanase from from *A. pullulans* PBU-109 using cheap raw material
2. To characterize the properties of the purified xylanase *A. pullulans* PBU-109
3. To improve the thermostability of the enzyme by selected polyols
4. To investigate its potential application of xylanase in the pulp prebleaching of rice straw pulp

1.3 Key words

Endoxylanase, Thermostability, Sorbitol, Paper production, Black yeast, Color variants, *Aureobasidium pullulans*

1.4 Anticipated benefits

Industrial bio-prebleaching process of rice straw pulp will be improved.

CHAPTER II

LITERATURE REVIEWS

2.1 Hemicellulose

Wood is an important renewable material used by humans for a variety of downstream applications. The basic subcellular structure in wood is the cell wall, mainly consisting of the cross-linked polymers cellulose, hemicellulose, and lignin. Within the cell wall structure, all three constituents interact via covalent and non-covalent linkages, with the hemicellulose being found at the interface between the lignin and cellulose where it is important for fiber cohesion and plant cell wall integrity (Beg et al., 2001). Hemicellulose is the second abundant heteropolysaccharide family comprising a mixture of monosaccharide including D-xylose, L-arabinose, D-glucose, D-galactose, D-mannose, D-glucuronic acid, D-galacturonic acid, minor amount of L-rhamnose and a variety of O-methylated neutral sugars (Mosier et al., 2005).

2.1.1 Structure and distribution

The backbone structure of hemicellulose composes with polymeric chains of β-(1⟶4)-linked β-D-xylopyranosyl units that called xylan. In different plant species, the structure of xylan varies due to the different type of side chains including predominantly acetyl, arabinofuranosyl and glucuronic acid (Yang et al., 2007). Following are some of the structures of xylan commonly found in plants:

2.1.1.1 Homoxylans

These types of molecules are linear and un-substituted xylans. The homopolymeric chains of β-(1⟶3) linkage of D-xylose units (Fig. 2.1A) have been reported in esparto grass (Chanda et al., 1950) and tobacco (Eda et al., 1976) while mixed β-(1⟶3, 1⟶4)-glycosidic linkages (Fig 2.1B) found in seaweeds.

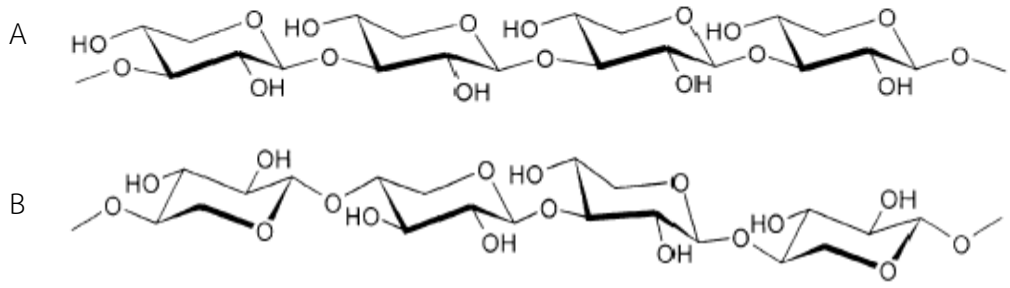

Figure 2.1 Primary structure of β-(1⟶3) linkage (A), and mixed β-(1⟶3, 1⟶4)-glycosidic linkages (B) (Vodenicarova et al., 2006)

2.1.1.2 Glucuronoxylans

These structures are referred to 4-O-methyl-D-glucurono-D-xylan (MGX). The main backbone chains are substituted with the single 4-O-methyl-α-D-gluco-pyranosyl uronic acid residues (MeGlcA) at position O-2 and often methylated at position O-4 (Fig. 2.2). In hardwoods, MGX have a high rate of substitution in the ratio of Xyl:MeGlcA from 4:1 to 16:1 while average ratio being around 10:1 (Vodenicarova et al., 2006).

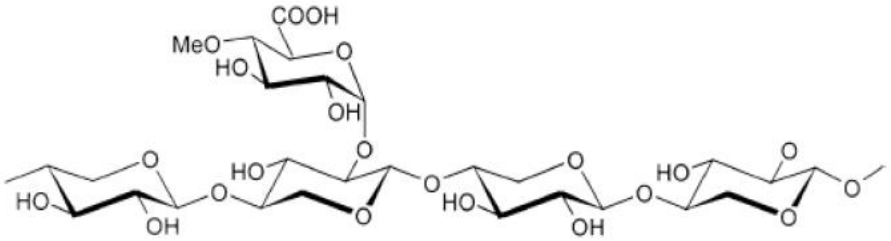

Figure 2.2 Primary structure of 4-O-methyl-D-glucurono-D-xylan (Vodenicarova et al., 2006)

2.1.1.3 Arabinoxylans

These structures of xylan consist of L-arabinofuranose residues attached on O-2 or/and O-3 position to polymeric backbone chains. The presence of arabinose side chains reduce interaction between chains that refer to more flexible conformations. In addition, the arabinose side chains can also be esterified to ferulic acid that, in some species, provides a chemical link between hemicellulose and lignin

(Levigne et al., 2004). Arabinoxylans are commonly found in cereals of wheat, rice, rye and oat (Vodenicarova et al., 2006).

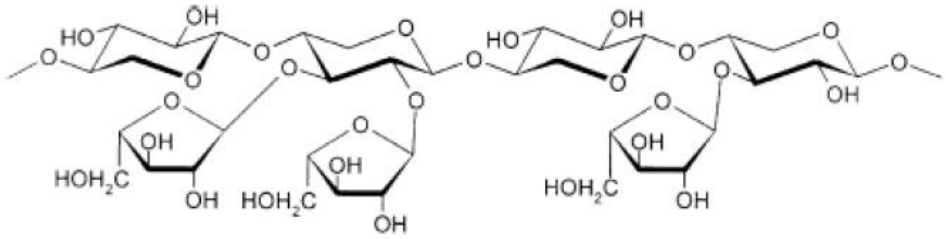

Figure 2.3 Primary structure of water-soluble L-arabino-D-xylan (Vodenicarova et al., 2006)

2.1.1.4 (Arabino)glucuronoxylan and (glucurono)arabinoxylan

In softwoods and cereals, xylan chains are substituted with 4-O-Methyl-D-glucuronic acid (MeGlcA) and L-arabinofuranose group at position O-2 and O-3, respectively (Fig. 2.1). AGX structure is frequently substituted with MeGlcA and can be found in coniferous species, grasses and cereals. For GAX structure, it is more substituted with L-arabinofuranose and can be found in softwoods, wheat, corn and rice bran.

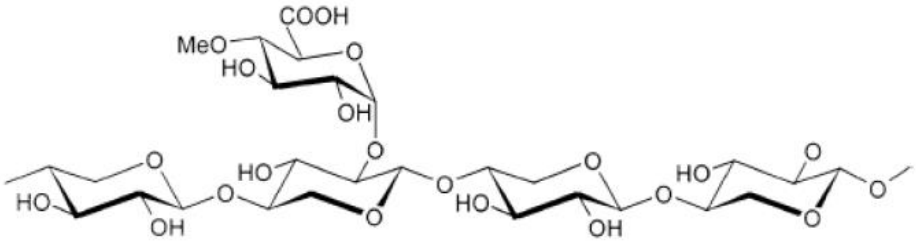

Figure 2.4 Primary structure of (L-arabino)-4-O-methyl-D-glucurono-D-xylan (AGX) (Vodenicarova et al., 2006)

2.1.2 Enzymatic hydrolysis of hemicellulose

Due to the diversity in the chemical structures of xylan derived from the cell walls of plants, the complete hydrolysis of hemicellulose requires a large variety of cooperatively acting enzymes. Endo-1,4-β-D-xylanases (EC 3.2.1.8) plays a major role in the degradation of xylan by cleaving the xylosyl backbone and releasing short xylooligosaccharides, which are further hydrolyzed into xylose units by β-D-xylosidases

(EC 3.2.1.37) (Fig 2.5). However, endo-1,4-β-D-xylanases do not cleave glycosidic bonds between xylosyl units if substituted (Lee & Forsberg, 1987). Therefore, it is necessary to cleave side chains by several accessory enzymes including α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73) and *p*-coumaric acid esterases (EC 3.1.1.-) for completely hydrolysis

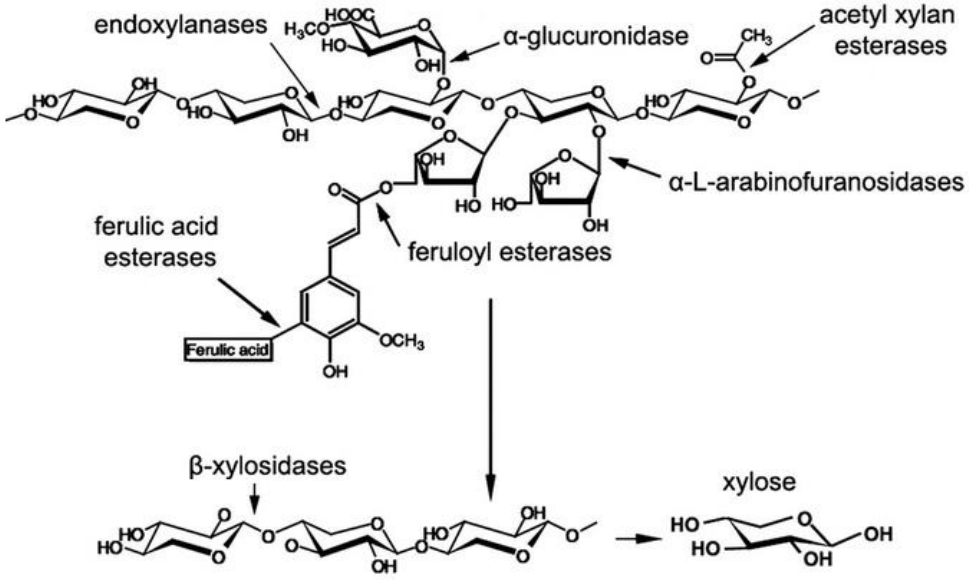

Figure 2.5 Enzymes involved in hemicellulose degradation (Sun et al., 2012)

2.2 Endo-1,4-β-D-xylanase

Among all xylanolytic enzymes, endo-1,4-β-D-xylanases are the most important that have been extensively studied due to its function and applications. Endo-1,4-β-D-xylanases are glycoside hydrolases (EC 3.2.1.8) which catalyze the hydrolysis of the glycosidic bonds in the xylan backbone resulting in the low degree of polymerization of the substrate. The hydrolysis of xylan by xylanase may be written as follows:

$$H(C_5H_8O_4)_n\,OH + H_2O \longrightarrow H(C_5H_8O_4)_{n-p}\,OH + H(C_5H_8O_4)_p\,OH$$

This equation shows the stoichiometry of a single reaction in a xylan molecule that may occur at many points in the chain (Polizeli et al., 2005). The official name is endo-1,4-β-xylanase, but commonly used synonymous terms include xylanase,

endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4-β-D-xylanase, β-1,4-xylanase and β-xylanase (Collins et al., 2005).

2.2.1 Classification and mode of action

The heterogeneity and complexity of xylan has resulted in an abundance of diverse xylanases with varying specificities, primary sequences and folds. The classification of xylanase has been done in several ways based on the substrate specificity and product profile (Wong et al., 1988), the molecular weight and isoelectric point (pI) (Collins et al., 2005; Wong et al., 1988), the crystal structure and amino acid sequences (Henrissat & Bairoch, 1996). Xylanase hydrolysis is a specific reaction that also depends on the nature of the substrate molecule, i.e. on the chain length, the degree of branching, and the presence of substituents (Reilly et al., 1997). Therefore, the end products released from xylan hydrolysis are in different forms e.g. xylose, xylobiose, xylotriose and arabinose. The first classification of these enzymes using substrate specificity and end-product profile separated xylanases into two types of non-debranching and debranching enzymes (Wong et al., 1988). Non-debranching enzymes are the group of xylanase which do not hydrolyze at the 1,3-α-L-arabino-furanosyl branch-points of arabinoxylans, and thus do not liberate arabinose while debranching enzymes hydrolyze these side-branches, liberating arabinose. Although this criterion was classified xylanase in a number of fungal species, but some fungi were capable to produce xylanase in both functions leading to the limitation of enzyme classification. The correlation between xylanases and their physicochemical properties, in terms of molecular weight and pI, was secondly proposed to classify these enzymes by (Wong et al., 1988). Approximate 70% of xylanases were demonstrated to divide into two groups of basic enzyme (low pI) with low molecular weight (< 30 kDa) and acidic xylanases (high pI) with high molecular weight (> 30 kDa) (Collins et al., 2005). However, it was not sufficient for all xylanases, in particular fungal xylanase.

The multiplicity of xylanases producing in each individual strain was found to relate with the differential processes of mRNA, post-translation and proteolytic digestion (Chavez R & 2002; Sun et al., 2012). Therefore, the efficient classification system was introduced based on the primary structure comparisons of the catalytic domains in

both structural and mechanistic features (Henrissat & Bairoch, 1996). Xylanases from different producing strains that exhibited the related amino acid sequences, three-dimensional structure, similar molecular mechanism and functional information were grouped in the same family. This system became the standard criterion for the classification not only for xylanase, but also with other glycosidases including cellulase. The information on the classification of enzymes could be found in the Carbohydrate-Active Enzyme (CAZy) database. According to this database, xylanases are classified to glycoside hydrolase (GH) families 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 and 62. The group of families 16, 51 and 62 presented the bi-functional enzymes containing two catalytic domains while families 5, 7, 8, 10, 11 and 43 have a truly distinct catalytic domain with endo-1,4-β-xylanase activity (Collins T., 2005). Within this classification system, xylanases are normally reported as being confined to families 10 and 11 (Jeffries, 1996; Subramaniyan & Prema, 2002a; Sunna A, 1997) (Table 2.1).

Enzymes in glycoside hydrolase (GH) family 10 consist of endo-1,4-β-xylanases (EC 3.2.1.8) and endo-1,3-β-xylanases (EC 3.2.1.32) that have high molecular mass with low pI (Bhardwaj et al., 2012). The major enzymes of this GH family are endo-1,4-β-xylanases that are capable to hydrolyze short xylooligosaccharides including xylobiose and xylotriose at the aglyconic bond and also active on low molecular mass cellulose substrates (Biely et al., 1997). This indicates that these enzymes have small substrate-binding sites and low substrate specificity. According to the crystal structure, kinetic of the activity on various sizes of xylo-oligosaccharides and end product, it was found that GH10 xylanases typically have four to five substrate-binding sites (Biely et al., 1997). GH 10 xylanases are able to hydrolyze xylosidic linkages close to the substituted residues or 1,3-β bond (Gilkes et al., 1991). Moreover, the cleavage sites on the non-reducing end are more specific than the reducing side. The structure of enzyme has been likened to a salad bow, with one face of the molecule having a large radius (approximately 45A°) due to the elaborate loop architecture, while the opposite surface has a radius of approximately 30A° (Collins et al., 2005; Derewenda et al., 1994) (Fig. 2.6A).Xylanases in this GH family 11 are true xylanases as they compose with one type of endo-1,4-β-xylanases (EC 3.2.1.8) and active on xylose containing substrates (Collins et

al., 2005). GH11 xylanases have high pI, low molecular weight, double-displacement catalytic mechanism and two glutamates that act as the catalytic residues (Krengel & Dijkstra, 1996; Sabini et al., 2001). Although GH11 enzymes present in the same capability with GH10 xylanases to hydrolyze the aryl β-glycosides of xylobiose and xylotriose at the aglyconic bond, but GH 11enzymes were inaction with cellobiosides. In addition, these enzymes are preferentially active with long chain xylans (Katapodis et al., 2003) due to the high amount of large substrate-binding sites (Bray & Clarke, 1992). Therefore, xylanases in GH11 family display several interesting properties including high substrate selectivity and high catalytic efficiency. The hydrolysis of GH11 xylanases are often occur at the non-substituted regions of the arabinoxylan backbone while GH10 enzymes are able to hydrolyze xylose linkages closer to the substituted regions (Biely et al., 1997). The structure of GH11 xylanases is the β-jelly roll fold that composes with two layers of β-pleated sheets surrounding the catalytic site (Torronen & Rouvinen, 1997) (Fig. 2.6B).

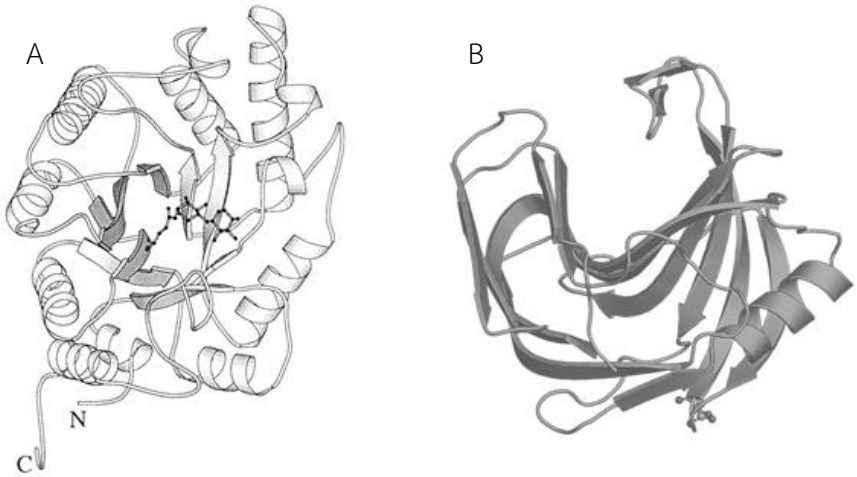

Figure 2.6 Ribbon representation of xylanase structures from glycoside hydrolase family 10 and 11. (A) Structure of the family 10 xylanase, Xyl10A, from *Streptomyces lividans* (Ducros et al., 2000). (B) Structure of the family 11 xylanase, rXynA, from *Bacillus subtilis* (Murakami et al., 2005)

Table 2.1 Characteristic of xylanases in glycoside hydrolase (GH) family 10 and 11

Organisms	Proteins	Mw (kDa)*	pI	T_{opt} (°C)	pH	References
Acrophialophora nainiana	Xyn6-11	19.0	-	60-65	7.0	(Salles et al., 2007)
Aspergillus nidulans FGSC A4	XlnC10	34.0	-	52	4.9	(Bauer et al., 2006)
Aspergillus nidulans	XlnB11	20.0	4.2	50-55	6.0	(Perez-Gonzalez et al., 1996)
Aureobasidium pullulans NRRL Y-2311-1	XynA11	25.0	-	45-50	5.0	(Li & Ljungdahl, 1994)
Aureobasidium pullulans var. *melanogenum*	XynI11	24.2	6.7	50	2.0	(Ohta et al., 2001a)
Aureobasidium pullulans ATCC 20524	XynII10	39.0	8.9	70	6.0	(Tanaka et al., 2006)
Fusarium oxysporum CK3-1	Xyl2-10	31.6	8.2	55	5.5	(Ito S., 2002)
	Xyl2-11	25.7	-	60	4.0	
Fusarium fujikuroi IMI 58289	XylA11	45.0	3.8	50	5.0	(Wiemann et al., 2013)
Irpex lacteus NK-1	Xyn10A	38.0	7.2	60	5.0	(Bauer S, 2006)
Penicillium funiculosum	XynD10	41.0	-	80	5.0	(Lafond et al., 2011)
Penicillium oxalicum B3-11-2	XynB11	21.3	-	50	4.0	(Wang et al., 2013)
Rhizopus oryzae 99-880	XynII11	22	-	55	4.5	(Xiao Z., 2014)
Paenibacillus sp. DG-22	XynC10	156.7	4.8	65	6.5	(Lee & Lee, 2014)
Streptomyces olivaceoviridis E-86	XynG10	20.5	8.5	60	6.0	(Kaneko S., 2004)
Talaromyces emersonii	Xyn10	47.9	4.4	75	4.4	(Sunna A, 1997)
Talaromyces versatilis	XynF11	23.0	4.5	60	4.0	(Lafond et al., 2014)

* Mw = molecular weight, pI = isoelectric focusing, T_{opt} = optimum temperature

2.2.2 Xylanase producing strains

Since hemicellulose represents the second main component in lignocellulosic material, microorganisms that play an important role in biomass degradation were considered as the potential source of xylanase production. The abilities to produce xylanase and enzyme properties from several strains of bacteria and fungi isolated from different habitats including soil compost, animal manure or decaying woods have been intensely reported within the last decades. To use xylanase in the development of bio-bleaching in paper industry, the process requires xylanases which should be stable at high temperature (>70℃) and active in alkaline condition (pH >8.0) due to the nature of the cooked pulp (Raj et al., 2013). Bacteria were well known to produce enzyme with the wide range of properties such as active at high temperature, acidic or alkali condition. The bacterial strains that produce thermostable and alkalophilic xylanase are mostly belong to the genera *Bacillus, Clostridium* and *Streptomyces* (Prakash et al., 2012). For instance, *Bacillus subtilis* ASH 7414 isolated from soil was able to produce xylanase in high amount (506 IU/mL) with low level of cellulase. These enzymes were stable over the pH range 6.0-9.0 (Sanghi et al., 2010). Kumar and Satyanarayan (2014) reported the thermoalkalophilic xylanase from *Bacillus halodurans* TSEV1. The optimum temperature and pH for the enzyme activity were 80℃ and pH 9.0 (Kumar & Satyanarayana, 2014). The facultative bacterium, *Bacillus licheniformis* JK7, isolated from the rumen of goat exhibited the optimum temperature at 70℃ for xylanase (0.44 U/mL) and was stable at a temperature range of 20 to 50℃ (Seo et al., 2013). Wide properties of bacterial xylanases are well suited for such industrial processes; however, the level of xylanase production from bacteria was considerably lower than those of fungi (Singh et al., 2003).

Even through the maximum activity of fungal xylanase was often found in acidic or neutral condition (pH 5.0-8.0) and at temperature below 50℃ (Prakash et al., 2012) which make fungal xylanases less suitable for pulp and paper industry. Filamentous fungi are particularly interested as producers of xylanases due to the high production level, extracellular secretion of enzyme, and production of auxiliary enzymes for xylan debranching (Bakri et al., 2010; Polizeli et al., 2005). Among the filamentous

fungi, the genera *Aspergillus* and *Trichoderma* are dominant strains for commercial xylanase preparation because high amount of xylanases were produced in the range of 700-1,500 U/mL (Subramaniyan & Prema, 2002b). Commercial xylanase preparations from fungal sources are manufactured by companies worldwide as shown in Table 2.3. One of interesting xylanase producer was *Schizophillum commune* that has been reported to produce high level of xylanase (1,244 U/mL) after cultivated in optimized medium using avicel and peptone as main substrates (Steiner et al., 1987). White rot fungi such as *Phanerochaete chrysosporium* also has been reported for xylanase production produced in the corn stalk medium with xylanase activity of 102-118 U/mL (Dobozi et al., 1992; Sato et al., 2007).

Most of fungi produced a number of extracellular enzymes in term of isoform that might have diverse in physicochemical properties, structures, specific activities and yields (Collins et al., 2005). For instant, *Trichoderma reesei* produced at least four xylanases (XYN I, II, III and IV) with different molecular weight (20-30 kDa) and pI (4.0-9.0) (Ilmén et al., 1997; Schuster & Schmoll, 2010). *Aspergillus niger* also produced at least two xylanases (XynA I and II) with different molecular weight (30-70 kDa), pI (4.0-7.5) and optimum temperature (40-70°C) (Amore et al., 2013; von Gal Milanezi et al., 2012). The multiple enzymes might be occurred by various factors including multi-independent genes or single copy of the gene with different alleles or post-secretional modification (Wong et al., 1988). Moreover, the secretion of xylanase was often found to associate with low or high amount of cellulases and might adversely affect to reduce the quality of the paper pulp after applied for bio-bleaching. These characteristics fall short of the required criteria for industrial applications. To fulfil the requirement of more robust xylanases, extensive exploitation of fungal diversity for cellulase free xylanase that active at high temperature and alkaline condition is still ongoing.

Several isolates of non-cellulolytic *Thermomyces lanuginosus* also have been reported to produce xylanases that were stable at high temperatures in the range of 50-80°C and over a broad pH range between 3.0 and 12.0 (Singh et al., 2000b). The molecular mass and pI of purified xylanase was varying from 23-29 kDa and 3.7-4.1, respectively. The crude enzyme of *T. lanuginosus* strain SSBP showed the high cellulase-

free xylanase (3,005 U/mg substrate) when grown in culture medium using corncobs as main substrate (Singh et al., 2000a). In 2007, the alkali tolerant fungus *Penicillium citrinum* isolated from soil have been reported to produce alkalophilic and moderate thermostable xylanase (Dutta et al., 2007). This enzyme was endoglucanase free xylanase that was stable over a wide pH range from 4.0 to 10.0 while the optimum condition was at 60°C and pH 8.5. This xylanase can be produced under solid substrate fermentation using wheat bran as main substrate. Sharma et al (2010) reported two xylanase with the activity around 15,000 units/g dry weight substrate from *Malbranchea flava* MTCC 4889 using rice straw as main substrate. These purified xylanases (MFX I and II) were optimally active at 70°C and pH 9.0. Enzymes were relatively stable with the half-life of 4 hours at 60°C and pH 9.0 (Sharma et al., 2010).

The major problem in scale-up process of xylanase production by fungi is the cell viscosity and shearing force. The propeller is normally used to maintain the medium homogeneity in fermenter, whereas it also affects to damage fungal mycelium in high speed of agitation and leading to reduce the xylanase yield (Bakri et al., 2011; Cai et al., 2014). Therefore, the xylanase producers that represent in unicellular cell or yeast-like morphology are considerable as the appropriated resource due to the overall homogeneity, low cell damage by shearing force of agitation and low viscosity of the cultivation broth. Although cellulase-free xylanases that were active in high temperature and alkaline condition have been found in filamentous fungi, but the report of these enzymes in yeast was limited.

One strains of yeast-like morphology that have been reported to produce cellulase-free xylanase in remarkable yield was *Aureobasidium pullulans* (Leather et al., 1989; Leathers, 1986; Li & Ljungdahl, 1994). The prominent xylanase (20 kD) from *A. pullulans* strain NRRL Y-2311-1 was purified and characterized (Leathers, 1989). The purified xylanase exhibited a pI of 8.5 and specific activity for polymeric xylan was 2100 IU/mg under optimal conditions (pH 4.5 and 45°C).

Table 2.2 Commercial xylanases produced from fungi and its applications.

Company	Product brand	Strains	Applications
Alltech, Inc, (USA)	Allzym PT	*Aspergillus niger*	Upgrading animal feed
Alltech, Inc, (USA)	Fibrozyme	*Aspergillus niger*	Upgrading animal feed
		Trichoderma viride	
Amano Pharmaceutical	Amano 90	*Aspergillus niger*	Pharmaceutical, food
Co,Ltd			and feed industry
Danisco Ingredients	Grindazym PF	*Aspergillus niger*	Supplementation of
(Denmark)	Grindazym GP5000		poultry and piglet feed
Genecor International	Multifect XL	*T. longibrachiatum*	Food industry
Europe Ltd (Finland)			
Ciba-Geiby Ltd (Switzerland)	Irgazyme 40	*T. longibrachiatum*	Pulp and paper industry
Iogen Corp. (Canada)	Xylanase GS35	*Trichoderma reesei*	Pulp bleaching, pulp cleaning and animal feed processing
Novozymes	Bio-Feed-Plus	*Humicola insolens*	Animal feed
(Denmark)	Novozym 431	*T. longibrachiatum*	Animal feed
Primalco Ltd Biotec	Ecopulp X-200	*Trichoderma reesei*	Improve the
(Finland)			bleachability of softwood and hardwood kraft pulps

Data obtained from (Beg et al., 2001) and (Haltrich et al., 1996)

2.2.3 *Aureobasidium pullulans*

Aureobasidium have been described as yeast-like fungus that was classified in phylum Ascomycota, class Euascomycetes, order Dothideales, and family Dothideaceae (de Hoog & Yurlova, 1994). Recently, *A. pullulans* has been revised in a new family of Aureobasidiaceae (Thambugala et al., 2014). The morphology of this specie was found in different forms including branched septate filaments, large chlamydospores, and smaller elliptical yeast-like cells that showed in Figure 2.7. The different morphology of *A. pullulans* has been reported to depend on the environmental conditions, particularly the carbon and nitrogen sources of the culture medium and it plays an important role with the production of its bioproduct such as exopolysaccharide called pullulans (Simon et al., 1993).

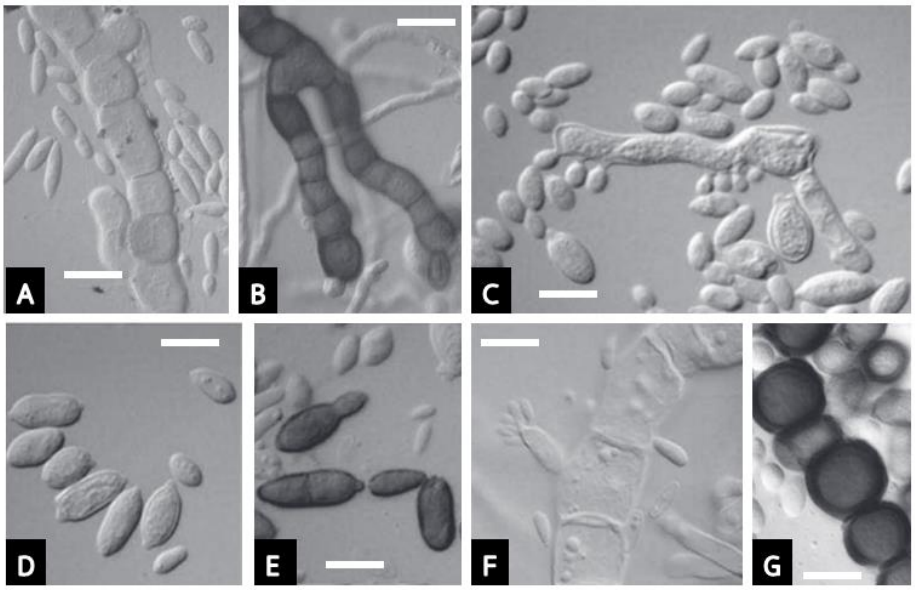

Figure 2.7 Polymorphic forms of *Aureobasidium pullulans*. A: vegetative hyphae, B: melanized hyphae, C: short hypha synchronously producing conidia, D: conidia, E: dark brown conidia, F: terminal and intercalar conidiophors performing synchronous conidiation G: chlamydospores. Scale bar: as marked on A–G = 10 μm (Zalar et al., 2008).

A. pullulans is commonly known as black yeast due to the production of black pigment which is the melanin-like compound (Chi et al., 2009; de Hoog & Yurlova, 1994). The colony colors of this species are varying while often found in pale pink,

cream, light brown or yellow in the early stage of cultivation and then become dark olivaceous or blackish after chlamydospore production in late stage. Out of this group, the color- variant strains have been reported by Wickerham and Kurtzman (1975) to exhibit the brilliant pigment including red, yellow, orange, or purple instead of typically pigmented strains and these strains were commonly found from tropical and subtropical zones (Wickerham & Kurtzman, 1975). The occurrence of A. *pullulans* has mostly been reported in temperate (such as Europe and America) and some isolates from polar areas (Vaz et al., 2011) that referred to the cosmopolitan species. A wide variety of habitats for *A. pullulans* have been reported including soil, rock surface, lime stone, coastal hypersaline water (Gunde-Cimermana et al., 2000), painted walls, bathroom surfaces (Lotrakul et al., 2009) and frequently found to associate with plant leaves (Prasongsuk et al., 2005; Urzi et al., 1999).

Since this species is widespread in tropical, a large number of *A. pullulans* have been isolated from different tropical habitats in various areas of Thailand during the last decade. The first report was by Punnapayak et al. (2003) that isolated *A. pullulans* from airborne spores in different area in Thailand including Loei province, Chiangmai province, and shady area in Bangkok. Three obtained isolates were identified as *A. pullulans* by similarity of the internal transcribed spacer (Manitchotpisit et al., 2009) sequences of the rDNA with databases and the character of exopolysaccharide, called pullulans.

In attempt to find the potential pullulans-producing strain, *A. pullulans* were isolated from latex-painted, bathroom cement-wall surfaces and leaves of mango, tamarind and Asoka in Thailand (Prasongsuk et al., 2005). The polymorphic forms, infrared spectra of exopolysaccharide, and ITS sequences were confirm all five isolates as *A. pullulans.* Furthermore, isolates NRM2 and SK3 presented the characteristic of color-variant strains to produce a pink and a yellow pigment, respectively. The highest pullulans content was found in isolate BK4 at 97%.

Lotrakul et al. (2009) reported ten *Aureobasidium* isolates that were collected from bathroom surfaces in Bangkok and vicinities. Out of these ten isolates, cell extract of four isolates including BM1, HKW1, HKW2, and KT1 exhibited a significant

inhibitory effect against hyphal growth and spore germination of *Aspergillus* in selected species. The extracts of BM1, KT1, HKW1 and HKW2 inhibited *Aspergillus terreus*, whereas KT1 and BM1 extracts inhibited *Aspergillus fumigatus*. In addition, only BM1 extract inhibited *Aspergillus flavus*. Interestingly, all extracts presented the same Rf value with that of aureobasidin A from TLC analysis.

To categorize tropical isolates of *A. pullulans*, the multilocus phylogenetic trees from 5 loci sequences, including internal transcribed spacer, intergenic spacer 1, translation elongation factor-1 alpha, beta tubulin, and RNA polymerase II, were reported by (Manitchotpisit et al., 2009). Forty five new isolates of *A. pullulans* from 15 provinces in Thailand were classified into 12 clades. Color-variants were found in 9 among all isolates and grouped into clade 5 and 8. Interestingly, almost all color variant isolates, especially in clade 8, produced high xylanase activity. This result was similar with the previous report of (Leather, 1984) that color variant was found to produce xylanase in high yield when compared with typical strain. Therefore, it suggested that Thailand is an appropriated source for color variant strains of *A. pullulans* that might be the potential species of xylanase production.

Since Leathers (1986; 1984) reported the extremely high levels of xylanolytic enzymes into culture media without cellulase activity from color variant strain of *A. pullulans* NRRL Y-2311-1, this species has gain much attention as a commercial xylanase producing source. The properties of xylanase in different isoform producing by this strain were reported in later years. Two extracellular xylanase (APX-I and APX-II) with similar molecular weight were reported and the dominant xylanase (APX-I) in molecular weight of 20 kDa highly exhibited specific activity of 2,100 IU/mg with polymeric xylan under optimal condition at pH 4.5 and 45°C (Leathers & Timothy, 1989).

Another strain in this species that was reported to produce acidophilic xylanase was *A. pullulans* var. *melanigenum* strain ATCC 20524 (Ohta et al., 2001b). The purified xylanase appeared the single band protein in a molecular weight of 24 kDa on SDS-PAGE analysis and had isoelectric point of 6.7. This xylanase was active in acidic condition (pH 2-4) while the optimum condition was at pH 2.0 and 50°C. The open

reading frame of this xylanase gene (*xynI*) consisted of 663 bp, encoded a presumed prepropeptide of 34 amino acids and a mature protein of 187 amino acids with a calculated molecular weight of 19.9 kDa and a deduced pI of 5.05. The xylanase gene was present as a single copy in the genome from southern blot analysis. The deduced amino acid sequence of XynI from this typically pigmented strain was found to closely relate with XynA from the color-variant strain NRRL Y-2311-1 in 95% identity. The distribution of Asp-152 and the additional of Glu-153 and -157 presented in the XynI that might be responsible for its lower pH optimum and pI value, without affecting the temperature optimum when compare with the color-variant strain NRRL Y-2311-1. In addition, sequence alignment and phylogenetic analysis showed that the XynI enzyme was related to the glycosyl hydrolase family-11 xylanases.

Another xylanase isoform (XynII) from the same strain, ATCC 20524, were reported by (Tanaka et al., 2006). The purified xylanase presented molecular weight of 39 kDa on SDS-PAGE analysis and pI of 8.9. The optimum condition of xylanase activity was found at 70°C and pH 6.0 while this enzyme retained more than 80% of the original activity in a wide range of pH (4.0-10.0). Moreover, the xylanase was free of activity toward carboxymethyl cellulose. The *xynII* gene encoded 361 amino acids of a precursor protein and 335 amino acids of mature protein that was calculated in molecular weight of 37.34 kDa and a deduced pI of 8.44. Sequence alignment and phylogenetic analysis suggested that the XynII belongs to glycosyl hydrolase family-10. This study was the first report of this family-10 xylanase from *A. pullulans*. In this report, it indicated that *A. pullulans* was the potential species for xylanase production due to the enzyme properties, especially family-10 xylanase that was active in alkaline condition and at high temperature. Furthermore, cellulase-free xylanase from this species also present the advantage for using in bleaching of pulps without the damage of cellulose fiber. Therefore, the exploration of *A. pullulans* that produces xylanase in high level and/or novel properties is still ongoing.

2.2.4 Xylanase applications

Xylanases become a major group of industrial enzymes due to their potential application in various industries. For instance, using of xylanase for xylan

hydrolysis may result in xylooilgosaccharide (XOs) production which can be used as prebiotic to stimulate growth and/or activity of probiotic bacteria in colon and thus health improving in human (Roberfroid, 1997; Vázquez et al., 2000). Xylose resulting from xylan hydrolysis can be used as substrate to produce xylitol, valuable sweetener in pharmaceutical and food industries (Parajó et al., 1998) and other fermented products including bioethanol (Viikari et al., 2012). The addition of xylanase in agricultural silage and grain feed will help to improve the digestibility and nutrition of animal feedstock (Cowieson et al., 2010). Xylanase can be applied for beverage industry to pretreat the arabinoxylans in cereal grain (e.g. rye, wheat, malt) leading to enhance the clarification and reduce the viscosity of fruit juice, wine and beer (Dervilly et al., 2002).

One of the most important biotechnological applications of xylanase is in pulp bleaching (Viikari et al., 1994). The purpose of this process was to increase the pulp brightness by using selective chemicals with multi-stage technology. The bleaching of pulp associates with the removal of lignin and degradation of the residual chromophores on the fibers (Subramaniyan & Prema, 2002b). Chlorine-based chemicals are usually requires for the conventional method of pulp bleaching, which generate the mutagenic organic substrates and forming a major pollution with environment. Therefore, the enzymatic bleaching as alternative method has drawn much attention in this respect.

Lignin is the complex component that consists of coniferyl alcohol and/or sinapyl alcohol with high amount of heterogeneous branches. In general, lignin has a strong chemical and physical interaction between hemicellulose, especially glucomannan and xylan to form the lignin-carbohydrate complex (LCC) (Bjorkman, 1954). The types of LCCs linkage that frequency found in cell wall of plant are benzyl ester and ether (Fig 2.8) while only benzyl ether linkages of LCCs are remaining after alkaline pulping (Wegener & Fengel, 1977). Moreover, the regenerated LCCs are also occurred by aldol condensation and benzylic acid rearrangement during the alkaline pulping process and become the barrier for chemical penetration in bleaching (Bjorkman, 1954). The formation of chromophores, brown color substance in pulp, also has been reported to generate from a series of lignin derivatives and also polysaccharide component (SjÖStrÖM, 1993). Therefore, using of xylanase might be plays an important role to reduce the recalcitrant of

LCCs, increase the permeability of bleaching chemicals and decrease the amount of chromophores.

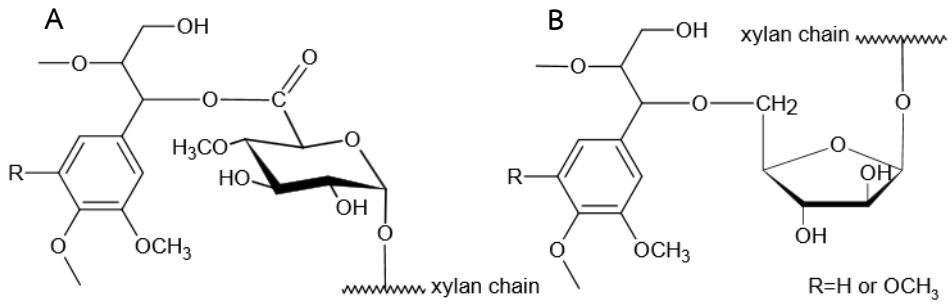

Figure 2.8 Proposed structures of lignin-carbohydrate complexes: a) benzyl ester type and (b) benzyl ether type (RunCang et al., 2003).

The applications of fungal xylanase for bio-bleaching have been reported in last two decades. To apply for bleaching of Eucalyptus kraft pulp, cellulase-free xylanases from *Acrophialophora nainiana, Humicola grisea* var. *thermoidea* and two strains of *Trichoderma harzianum* were used in prebleaching and subsequently bleached with chlorine dioxide (Medeiros et al., 2002). All xylanase treatments were efficient in the reduction of kappa number indicating in the higher removal of lignin when compared with the control without the xylanase pretreatment. Xylanase producing from *T. harzianum* presented the highest effective among all xylanases due to the reduction of pulp viscosity and chlorine consumption, and improvement of brightness. In addition, xylanase pretreatment also reduced the amount of chlorine dioxide in 12%.

As the best producing strains of xylanase activity have been reported from *Penicillium corylophilum*, *Aspergillus niger* and *Trichoderma longibrachiatum*, all of these strains were used for xylanase production (Medeiros et al., 2007). Crude enzymes were applied for the prebleaching of commercial pulp prior to bleach with chlorine dioxide. Xylanase preparations from *T. longibrachiatum* and *P. corylophilum* were

effective to reduce pulp kappa number while the highest release of reducing sugar was found in xylanase from *P. corylophilum*. On scanning electron microscopy, oxygen-bleached pulp after xylanase treatment revealed morphological changes, including holes, cracks, filament forming and peeling.

In recent year, the efficient in pulp bleaching of xylanase from two different strains of *Aspergillus niger* and *Aspergillus flavus* was reported by de Alencar Guimaraes et al. (2013). These strains were grown in several kinds of agricultural residue while wheat bran was presented as the best carbon source of xylanase production. Moreover, xylanase activity in the combination medium of wheat bran and corncob was higher than using xylan alone. The xylanase produced by *A. niger* was more stable than from *A. flavus* showing a half-life of more than 45 minutes at 55°C while the *A. flavus* xylanase ehibited the excellent stability only at high pH (6-8). On biobleaching assay the xylanases from *A. flavus* were more effective in comparison to xylanases from *A. niger* in term of lower kappa number and higher chromophores release. It indicated that alkalotolerant xylanase are important for bleaching process of pulp.

The study on effect of enzyme combination between xylanase (X) derived from *Aureobasidium pullulans* and laccase-mediator system (LM) using *Trichophyton* sp. LKY-7 laccase (TrL) with N-hydroxy-2-pyridone analogue (NHP) as a mediator in bleaching of hardwood kraft pulp was propose by Yoon & Jung (2013). The parameter of pulp after bleaching in each individual enzyme was not in efficient especially for non-chemical bleached pulp. Three steps of pulp bleaching by X, LM and hydrogen peroxide (XLMH) was presented in the most efficient method by exhibit the highest value of brightness (56.3 % ISO) and lowest of kappa number (8.2) among other treated pulps.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and Equipment

- Autoclave: Ta Chang Medical Instrument Factory, Taiwan
- Autopipette: Pipetman, Gilson, France
- Bright-field microscope: Model CH30, Olympus, Japan
- Centrifuge, refrigerated centrifuge: Hettich, Germany
- Centrifuge, microcentrifuge: Model Denville 260D, Denville Scientific Inc., USA
- Electrophoresis unit: Model mini-protein cell, Bio-Rad Applied Biosystem Company, USA
- Fiber quality analyzer: FQA LDA02, OpTest Equipment, Canada
- Fluorescence spectrophotometer: Model LS 55, Perkin-Elmer, UK
- Sephacryl S-200 High Resolution Column: GE Healthcare Bio-Sciences, Sweden
- HiTrap DEAE-sepharose Fast Flow (FF) column: GE Healthcare Bio-Sciences, Sweden
- Laminar flow: Model BV 123, ISSOC, Thailand
- Membrane filter: Whatman No.1, GE Healthcare Bio-Sciences, Sweden
- Moisture balance: FD-600, Kett Electric Laboratory, Japan
- Optical tester: Color Touch PC, Technidyne, U.S.A
- pH meter: Model PP-50, Sartorius, Germany
- Power supply: Model Power PAC1000, Bio-Rad Laboratories, USA
- Pulping autoclave digester: UEC-2017A, Universal Engineering, India
- Rapid-Köthen sheet former: RK-2A KWT, PTI, Austria
- Reflectance spectrophotometer: Macbeth, USA
- Shaker: Model SPL15, Labcon, The Republic of South Africa
- Spectrophotometer: Model UV-2800, Unico, USA
- Tear strength tester: Protear, Thwing-Albert, U.S.A.
- Tensile strength tester: Strograph E-S, Toyo Seiki, Japan
- Thermo Nicolet Nexus 670 spectrophotometer: GMI, Inc., USA

- UV transluminater: Model ECX-26-M, Vilber Lourmat, France
- Vivaflow 50, Polyethersulfone membrane, MWCO 10 kDa membrane cut-off, Sartorius AG, Germany
- Weigh balance, 2 digits: Model BL610 Sartorius, Germany
- Weigh balance, 4 digits: Model TC-205 Denver Instrument Company, USA

3.2 Chemicals

- 3, 5-dinitrosalicylic acid (DNS): Sigma-Aldrich Inc., USA
- Acetic acid: Ajex Finechem, New Zealand
- Ammonium sulfate: Ajex Finechem, New Zealand
- Bacto peptone: Difco, USA
- Beechwood xylan: Fluka, Switzerland
- Bovine serum albumin (George et al.): Ajex Finechem, New Zealand
- Calcium chloride dihydrate: Ajex Finechem, New Zealand
- Copper sulfate: Carlo Erba, Italy
- Ethylene diamine tetra-acetic acid (EDTA): Ajex Finechem, New Zealand
- Ferrous sulfate: Fluka, Switzerland
- Hexadecyltrimethyl ammonium bromide (CTAB): Sigma-Aldrich Inc., USA
- Hydrochloric acid: Carlo Erba, Italy
- Isopropanol: Fisher Scientific, UK
- Precision Plus Protein™: Bio-Rad Laboratories, USA
- Potassium chloride: Ajex Finechem, New Zealand
- Potassium hydrogen sulphate: Ajex Finechem, New Zealand
- Magnesium chloride: Scharlau, Spain
- Magnesium sulfate heptahydrate: Scharlau, Spain
- Manganese sulfate heptahydrate: Scharlau, Spain
- Methanol: Merck, Germany
- Sodium carbonate: Scharlau, Spain
- Sodium chloride: Scharlau, Spain
- Sodium dodecyl sulfate: Scharlau, Spain
- Sodium thiosulfate: Merck, Germany

- Sodium hydrosulfite: Carlo Erba, Italy
- Sodium hydroxide: Ajex Finechem, New Zealand
- Zinc sulfate heptahydrate: Scharlau, Spain

3.3 Procedures

3.3.1 *Aureobasidium* isolates

Twenty five isolates of *Aureobasidium pullulans* were obtained from the fungal culture collection of the Plant Biomass Utilization Research Unit (PBURU), Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand (Manitchotpisit et al., 2009; Rotjanagusol, 2010). The reference strain, *A. pullulans* Y-2311-1, was obtained from the ARS Culture Collection, USDA, Peoria, IL, USA. The yeast was grown in yeast malt (YM) agar medium (Atlas, 1993) at room temperature (28±2°C) for 72 hours and the short-term stock culture was stored at 4°C. For long-term storage, the strain was stored at –20°C in YM broth containing 20% (v/v) glycerol.

3.3.2 Screening for xylanase producing isolates

For quantitative assay, inocula of *Aureobasidium* isolates were grown in basal medium (Leathers et al., 1984) containing 1% (w/v) glucose at room temperature with 150-rpm agitation for 48 hours. The inoculum was transferred to the basal medium containing 1% (w/v) beechwood xylan (Fluka, Switzerland) instead of glucose. The cultures were incubated at the same condition up to 48 hours. After this, cells were removed by centrifugation at 18,000 x *g* for 10 min and the supernatant was used as the crude enzyme for xylanase assay. The assay method involved mixing crude enzyme (100 µl) with 1% (w/v) beechwood xylan in 50 mM acetate buffer pH 6.0 (900 µl) and incubated for 30 min at a selected temperature. Two incubation temperatures, 50 and 70°C, were chosen to detect the thermophilic property of the crude enzyme. The amount of released reducing sugars was determined by adding 3 mL of DNS solution (Appendix B) and heated in boiling water bath for 5 minutes. The mixture was then added with 10 mL of distilled water, cooled to room temperature and determined for its optical density at 540 nm (Miller, 1959). A standard curve was performed using xylose solutions at different concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0

mg/mL). One unit (U) of xylanase was defined as the amount of enzyme required to release 1 µmole xylose equivalent per minute under the optimum conditions. The experiments were performed in triplicate.

3.3.3 Xylanase production from agricultural residues

Locally available agricultural products (wheat bran and germ), agricultural wastes (rice straw and corncob) and weed biomass (water hyacinth) were collected and cleaned. These materials were initially cut to small sizes, air dried, grinded and sieved through 80-mesh screen. The chemical compositions of these ground biomasses were analyzed according to method of Goering & Van Soest (1970) (Appendix C). Seed culture was prepared by growing one colony of the selected *Aureobasidium* isolate in basal medium containing 1% (w/v) glucose at room temperature with 150-rpm agitation for 72 hours. The inoculum was adjusted to 2.5×10^7 cells/mL using sterile distilled water and 100 µl was transferred into a 250-mL Erlenmeyer flask containing 100 mL basal medium supplemented with 1% (w/v) ground biomass as the sole carbon source. The cultures were incubated at room temperature with 150-rpm agitation for 48 hours. Cells were separated from the culture broth by centrifugation (18,000 × *g*, 10 min) at 4°C. The supernatant was used as the crude enzyme solution for xylanase assay as described above. Biomass that yielded the highest xylanase activity was selected for further medium optimization for xylanase production.

3.3.4. Medium optimization for xylanase production

3.3.4.1 Screening of nutrient compositions using a Plackett–Burman design

In order to assess the effects of medium compositions on the xylanase production, the experiment was designed based on Plackett & Burman (1946) using the Design Expert software (Version 8.0.2.0, Stat-Ease Inc., Minneapolis, USA). A total of 11 variable components were analyzed including a selected biomass, xylose, glucose, urea, $(NH_4)_2SO_4$, yeast extract, peptone, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, KH_2PO_4 and tween 80. All variables were investigated at two spaced intervals designated as -1 (low level) and +1 (high level) and listed in Table 3.1. The experimental design for the screening of the

variables is described in Table 3.2. All experiments were performed in three replicates and the mean values are given. Nutrient compositions that significantly enhanced the xylanase production were selected for further concentration optimization.

Table 3.1 Nutrient screening using a Plackett-Burman design

Variables		Level (g/L)	
Nutrient code	Nutrients	Low (-1)	High (+1)
X_1	Glucose	0.5	1.5
X_2	Xylose	0.5	1.5
X_3	Selected biomass	5.0	10.0
X_4	$(NH_4)_2SO_4$	0.5	1.5
X_5	Urea	0.5	1.5
X_6	KH_2PO_4	0.5	1.5
X_7	Yeast extract	0.5	1.5
X_8	Peptone	0.5	1.5
X_9	$MgSO_4 \cdot 7H_2O$	0.2	1.0
X_{10}	$CaCl_2 \cdot 2H_2O$	0.2	1.0
X_{11}	Tween 80	0.5	1.5

Table 3.2 Plackett-Burman experimental design matrix for screening of important variables for xylanase production

Trials	Coded value										
	X_1*	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

* X_1: glucose, X_2: xylose, X_3: selected biomass, X_4: $(NH_4)_2SO_4$, X_5: urea, X_6: KH_2PO_4, X_7: yeast extract, X_8: peptone, X_9: $MgSO_4 \cdot 7H_2O$, X_{10}: $CaCl_2 \cdot 2H_2O$ and X_{11}: tween 80

3.3.4.2 Optimization of nutrient concentration using Response Surface Methodology

Based on the results from Plackett–Burman experiments, factors that significantly enhanced the xylanase production were further optimized for their optimal concentration using Response Surface Methodology (RSM). The Box-Behnken experimental design with five factors and three levels including three replicates at the center point was used to fit the second order response surface (Box & Behnken, 1960). All variables were taken at a central coded value of zero. The minimum and maximum ranges of variables investigated are listed in Table 3.3 and 3.4. Upon the completion of experiments, the average of xylanase activities was taken as the response and analyzed in a statistical manner using regression. A multiple regression analysis of the data was

carried out for obtaining an empirical model that related the response measured to the independent variables. Xylanase production was analyzed by multiple regressions through the least squares method to fit the following equation:

$$B = \beta_0 + \Sigma\beta_i A_i + \Sigma\beta_{ij} A_i A_j + \Sigma\Sigma\beta_{ii} A_i^2 \quad (1)$$

Where B was the measured response, β_0 was the intercept term, β_i was linear coefficients, β_{ii} was quadratic coefficient, β_{ij} was interaction coefficient and A_i and A_j were coded independent variables. For statistical calculation, the experimental variable A_i has been coded as a_i according to the following transformation equation:

$$a_i = \frac{A_i - A_0}{\delta A} \quad (2)$$

Where a_i was the dimensionless coded value of the independent variable, A_i was the real value of an independent varisble, A_0 was the value of A_i at the center point and δA was the step change. Statistical analysis of the data was performed by design package Design-Expert software (version 8.0.7.1, Stat-Ease, Inc., Minneapolis, USA) to evaluate the analysis of variance (ANOVA) and to determine the significance of each term in the equations fit. The fitted polynomial equation was then expressed in the form of three-dimensional response surface plots to illustrate the main and interactive effects of the independent variables on the dependent ones. In addition, the optimal concentrations of the critical variables were also obtained by these plots. The combination of different optimized variables, which yielded the maximum response, was determined to verify the validity of the model. In order to verify the accuracy of the predicted model, the repeated experiment was carried out using the optimized medium. Growth pattern and xylanase production of the selected *A. pullulans* strain were determined every 12 hours up to 120 hours at room temperature with 150-rpm agitation.

Table 3.3 Experimental range and levels of independent test variables used in Box-Behnken experimental design.

Independent variables (g/L)	Variable codes	Level		
		-1	0	+1
Selected biomass	A_1	30.0	35.0	40.0
$(NH_4)_2SO_4$	A_2	2.0	3.0	4.0
Xylose	A_3	1.0	1.5	2.0
KH_2PO_4	A_4	1.0	1.5	2.0
Tween 80	A_5	1.0	1.5	2.0

Table 3.4 Box-Behnken experimental design matrix of xylanase production by a selected *A. pullulans* strain

Trials	Variables (g/L)				
	Selected biomass	$(NH_4)_2SO_4$	Xylose	KH_2PO_4	Tween 80
1	+1	+1	0	0	0
2	0	0	0	0	0
3	0	0	0	+1	+1
4	0	0	0	0	0
5	0	0	-1	-1	0
6	0	+1	0	0	+1
7	0	0	+1	+1	0
8	+1	0	+1	+1	0
9	0	+1	+1	0	0
10	-1	+1	0	-1	0
11	0	0	0	0	-1
12	0	-1	0	0	-1
13	0	+1	0	0	0
14	0	0	-1	+1	0
15	0	0	+1	0	-1
16	0	-1	0	-1	+1

Table 3.4 Box-Behnken experimental design matrix of xylanase production by a selected *A. pullulans* strain (continued)

Trials	Variables (g/L)				
	Selected biomass	$(NH_4)_2SO_4$	Xylose	KH_2PO_4	Tween 80
17	-1	0	0	0	+1
18	0	0	-1	+1	-1
19	-1	0	-1	+1	0
20	0	-1	0	0	0
21	+1	0	0	-1	+1
22	+1	-1	0	0	0
23	0	-1	+1	0	0
24	0	0	0	0	0
25	-1	-1	0	+1	0
26	0	+1	0	0	-1
27	0	+1	0	-1	0
28	-1	0	+1	0	0
29	0	0	0	+1	-1
30	0	0	0	+1	+1
31	+1	0	-1	0	0
32	0	0	-1	-1	+1
33	0	-1	0	0	0
34	0	+1	-1	0	0
35	-1	0	0	0	0
36	0	-1	-1	+1	0
37	-1	0	0	0	0
38	-1	0	0	-1	-1
39	+1	0	0	0	-1
40	+1	0	0	+1	0
41	0	0	+1	+1	+1
42	0	0	+1	0	0
43	+1	0	0	-1	0

3.3.5 Purification of endoxylanase

3.3.5.1 Preparation of crude enzyme

Crude xylanase was prepared by culturing the selected *A. pullulans* strain in 500 mL of the optimized production medium. After 72-hours incubation at room temperature (28 ± 2°C), culture supernatant was recovered by centrifugation (18,000 × *g*, 10 min) at 4°C. The culture supernatant (445 mL) was fivefold concentrated by ultrafiltration (Vivaflow 50, 10 kDa MW membrane cut-off, Sartorius AG, Goettingen, Germany). The enzyme activity was determined as described above. Protein concentration was determined using the Lowry method (Lowry et al., 1951). A standard curve (appendix C) was performed using bovine serum albumin (George et al.) solution at different concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/mL). The optical density was measured at 750 nm. This crude enzyme was used for further purification step.

3.3.5.2 Ammonium sulfate precipitation

The crude xylanase was purified at 4°C throughout the process. The required amount of ammonium sulfate was calculated to give the sequential fractionations of saturations at 0-20%, 20-40%, 40-60% and 60-80% (Appendix E). The amount of ammonium sulfate was slowly added to crude enzyme from the previously section (50 mL) until the next saturation was achieved with mild stirring. After incubation for 4 hours, the precipitate was recovered by centrifugation (18,000 × *g*, 30 min) and totally dissolved in 50 mM acetate buffer (pH 6.0) in total volume of 50 mL. The suspension was dialyzed against the same buffer. This procedure was repeated for the other fractions by adding ammonium sulfate to the supernatant until the desired saturation was obtained and the precipitate was recovered as described above. Xylanase activity and protein concentration in each fraction were determined as described in 3.3.2 and 3.3.5.1, respectively. The fractions given the high xylanase activity were pooled, concentrated by ultrafiltration and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) for the subsequent column chromatography.

3.3.5.3 Anion exchanger chromatography

The enzyme solution was continuously purified using anion exchange chromatography. The HiTrap DEAE-sepharose Fast Flow (FF) column (1.6×2.5 cm, 5 mL; GE Healthcare Bio-Sciences, Uppsala, Sweden) was pre-equilibrated with 20 mM Tris-HCl buffer, pH 8.0 (50 mL). The concentrated enzyme (30 mL) was applied to this column by using a flow rate of 1.0 mL/minute. After the application of protein, the column was washed with the same buffer at least 5 column volumes (25 mL) until no protein appeared in the effluent. Purified enzyme was then eluted with a linear NaCl gradient (0 to 1.0 M, over 50 mL) in the same buffer at a flow rate of 1.0 mL/min. Five-mL of each fractions were collected. The enzyme solution was measured for protein concentration and xylanase activity as described above. Fractions with xylanase activity were pooled, dialyzed against an excess volume of 50 mM acetate buffer (pH 6.0) for overnight, and then concentrated by ultrafiltration.

3.3.5.4 Gel filtration chromatrography

The concentrated enzyme (5 mL; 4 mg total protein/mL) obtained from 3.3.5.3 was applied to Sephacryl S-200 High Resolution Column (30x5 cm,120 mL; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) that was pre-equilibrated with 50 mM acetate buffer, pH 6.0 (240 mL). The protein was eluted with the same buffer at a flow rate of 0.5 mL/minute. The fractions (3 mL) were collected and monitored for protein and enzyme activity as described above. The fractions with xylanase activity were pooled, dialyzed against an excess volume of 50 mM acetate buffer (pH 6.0) and then concentrated by ultrafiltration. Xylanase activity and protein concentration of the purified enzyme were then determined as described in 3.3.2 and 3.3.5.1.

3.3.5.5 Protein profiles by SDS-PAGE and zymogram analysis

The protein profiles of the purified xylanase were determined by separation through an SDS-PAGE with 5% stacking gel in 0.5 M Tris-HCl buffer (pH 6.8) and 12% (w/v) resolving gel in 1.5 M Tris-HCl, pH 8.8 (LaemmLi, 1970). The purified xylanase fraction (20 µg proteins, 10 µl) were mixed with the same volume of sample buffer (Appendix F) and boiled for 5 min. The samples were loaded into an SDS-PAGE

and the electric current at 150 V was applied for 55 min. The gel was stained in Coomassie Brilliant Blue R-250 solution (appendix E) for 1 h and repeatedly destained in a solution of 40% (v/v) methanol and 10% (v/v) acetic acid in water to visualize the protein bands. The molecular weights of the proteins were estimated using protein marker of 10-250 kDa range (Precision Plus Protein™, Bio-Rad Laboratories, USA).

For zymogram analysis, the purified xylanase (10 µg proteins, 10 µl) was mixed with sample buffer without SDS and β-mercaptoethanol. The mixture was not denaturated by heating. The samples were loaded into 12% (w/v) native-polyacrylamide gel containing 0.5% (w/v) beechwood xylan. After electrophoresis, the gel was rinsed in the assay buffer (50 mM acetate buffer, pH 6.0) containing 1% (v/v) Triton X-100 for 1 hour at room temperature (Her et al., 1999). The gel was further washed twice with the assay buffer for 15 minutes and incubated for 1 hour and a half at room temperature to allow the enzymatic digestion of xylan. After incubation, the gel was stained with 0.1% (w/v) Congo red for 30 minutes and destained in 1 M NaCl to reveal a clear zone resulting from the hydrolysis of xylan.

3.3.6 Characterization of the purified xylanase

3.3.6.1 Optimal condition for xylanase activity

The optimal condition for xylanase activity was assayed as a combination of two factors (temperature and pH) at the same time. The reaction mixtures containing the purified xylanase (4.5 U/mL, 50 µl) and 1% (w/v) beechwood xylan were incubated at various temperatures, ranging from 50–90°C, and at different pHs. Two different buffers, 50 mM sodium acetate buffers (pH 3.0–6.0) and 50 mM phosphate buffers (pH 6.0–11.0), were used to adjust the pH of reaction mixtures. The relative activity was calculated as the percentage of the maximum xylanase activity detected. The results presented were mean ± one standard deviation derived from three replications.

3.3.6.2 Thermostability of the purified xylanase

To determine its thermostability, the purified xylanase (4.5 U/mL) was incubated at different temperatures ranging from 40-70°C, in the absence of xylan. Aliquots of the enzyme were removed every 30 minutes up to 180 minutes and immediately chilled at 4°C for 10 minutes. The residual xylanase activity was assayed at the optimum condition. The purified enzyme kept at 4°C was used as a control and defined as 100% relative activity. The results presented were mean ± one standard deviation derived from three replications.

3.3.6.3 Thermodynamic analysis

The data obtained from the thermal stability profile were used to analyze the thermodynamic parameters related to the xylanase activity. The half-life of the xylanase ($t_{1/2}$, 1/min) was determined from equation (3) (Polakovič & Vrábel, 1996).

$$t_{1/2} = \ln 2/k_d \quad (3)$$

The D_t values, decimal reduction time or time required to inactivate 90% of the original enzyme activity at a constant temperature, were calculated from equation (4).

$$D_t = \ln 10/k_d \quad (4)$$

The activation energy for xylanase denaturation (E_d) was determined by an Arrhenius plot of the log denaturation rate constants (ln k_d) versus the reciprocal of the absolute temperature in Kelvin (T) using equation (5) (Arrhenius, 1889).

$$\text{Slope} = -E_d/R \quad (5)$$

Where R is the gas constant (8.314 J/mol. K). The changes in enthalpy (ΔH_o, kJ/mol), free energy (ΔG_o, kJ/mol) and entropy (ΔS_o, J/mol. K) for the thermal denaturation of xylanase were determined using equation (6)-(8) (Gummadi, 2003)

$$\Delta H_o = E_d - RT \quad (6)$$

$$\Delta G_o = -RT \ln (k_d h/ k_B T) \quad (7)$$

$$\Delta S_o = (\Delta H_o - \Delta G_o)/T \quad (8)$$

Where h is the Planck constant (11.04×10^{-36} J. min) and k_B is the Boltzman constant (1.38×10^{-23} J/K).

3.3.6.4 Effect of polyols on xylanase thermostability

In order to improve the thermal stability of the xylanase, polyols including ethylene glycol (2C), glycerol (3C), xylitol (5C), sorbitol (6C) and mannitol (6C) were separately added to the enzyme solutions at 0.5 M final concentration prior to incubation at 70°C. Aliquots were withdrawn every 30 minutes, ice-cooled and then the residual xylanase enzyme activity was assayed under the optimal condition. The stability of the enzyme was expressed as a percentage of residual activity (% RA) compared with the initial enzyme activity (before incubation and no polyol added). The polyol that most improved the thermostability was selected for further study on optimal concentration (0.25-1.00 M) at 70°C. Thermostability and thermodynamic of purified xylanase in the present of optimum polyol at optimum concentration was then determined as described in 3.3.6.2 and 3.3.6.3.

3.3.6.5 Effects of ions

To investigate the effect of ions on the enzyme activity, $CaCl_2$, $CuCl_2$, $MgCl_2$, $FeSO_4$, $CoCl_2$, $ZnCl_2$ and EDTA were separately added to the reaction mixtures at two different final concentrations of 1 and 10 mM, respectively, prior to performing the enzyme assay under the optimum condition. The relative activity was calculated as a percentage of enzyme activity without the addition of ion.

3.3.6.6 Substrate specificity

The substrate specificity of the purified xylanase was tested with each of the followings; viz. beech wood xylan, oat spelt xylan, rice straw xylan, cellulose and carboxymethyl cellulose (CMC) final concentration at 1%. Oat spelt and rice straw xylans were prepared according to the method of (Höije et al., 2005). The xylanase activity was assayed under the optimum condition. β-Xylosidase was also assayed as described by Bachmann and McCarthy (1989) by using 5 mM p-nitrophenyl-3-D-xylopyranoside (PNP-xyloside) in 50 mM acetate buffer (pH 6.0).

3.3.6.7 Kinetic parameters

Kinetic parameters of the purified xylanase were determined by measuring the enzymatic activity toward xylan from beechwood, oat spelt and rice straw at various concentrations (0.1-4 mg/mL in 50 mM sodium acetate buffers, pH 6.0). The activity assay of the enzyme was conducted at the optimal condition for 30 minutes and the reducing sugars were determined by the DNS method. The kinetic parameters including Michaelis-Menten constant (*K*m) and maximal reaction velocity (*V*max) were then calculated by fitting the initial velocity data to the Michaelis–Menten equation and linear regression of the Lineweaver and Burk double-reciprocal plot.

$$\frac{1}{V} = \frac{1}{V\text{max}} + \frac{K\text{m}}{V\text{max}} \left(\frac{1}{[S]} \right) \qquad (9)$$

3.3.7 Application of the crude xylanase for prebleaching of rice straw pulp

3.3.7.1 Pulping of rice straw

Rice (*Oryza sativa*) straw was collected from a local rice field in Suphanburi province, Thailand. The material was cut into segments of approximately 3 centimeters and air dried before use. The moisture content of the dried material was analyzed by the moisture balance (FD-600, Kett Electric Laboratory, Japan). The pulping of rice straw was carried out with 15% (w/w) NaOH based on weight of the oven-dried material and charged into autoclave digester, (UEC-2017A, Universal Engineering, India) with the required amount of chemical solution at liquor to solid ratio of 10:1. The digester was gradually heated to operating temperature at 120 ℃ for 2 hours (Chaiarrekij et al., 2011; Thongmeethip, 2013). After the reaction, the resulting pulp was filtered, extensively washed with tap water to remove the alkali and then kept in a zipper-lock bag at 4 ℃ until used in the next experiment. The qualities of pulp and process were characterized in the term of chemical components, pulp yield, rejects of pulps and Kappa number.

3.3.7.2 Bleaching process

For prebleaching of the rice straw pulp, the xylanase pretreatment (18.6 U crude xylanase/g dry pulp, with or without a selected polyol at an optimized concentration) was performed in transparent plastic bags with 10% (w/v) rice straw pulp suspended in 50 mM sodium acetate buffer (pH 6.0). The reaction was performed at 70°C for 2 hours (Viikari et al., 2007). Reducing sugars in the hydrolysates were determined by the DNS method. The enzyme-treated pulp and the enzyme untreated pulp, as the control, were transferred to the H_2O_2 solution at 10% (v/v) final concentration and incubated for 1 hour at the same temperature as in the prebleaching step. The resultant bleached pulps were made into 60 g/m hand sheets on a Rapid-Köthen sheet former (RK-2A KWT, PTI, Austria) according to the ISO Standard Method 5269–2.

3.3.7.3 Property determination of hand sheets

The brightness and opacity of the hand sheets were measured using an optical tester (Color Touch PC, Technidyne, U.S.A.), based on the ISO Standard Methods 2470 and 2471, respectively. The tensile and tear indexes were determined after tensile strength and tear resistance were measured using a tensile strength tester (Strograph E-S, Toyo Seiki, Japan) and a tear strength tester (Protear, Thwing-Albert, U.S.A.) according to TAPPI Standard Method T 494 om-01 and T414 om-04, respectively. Fiber morphology was also analyzed using a fiber quality analyzer (FQA LDA02, OpTest Equipment, Canada) according to the TAPPI Standard Method T271 om-12. Hand sheets made from untreated pulp was used as the control.

3.3.8 Data analysis

Statistical differences among the means of data were calculated using one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) or Student's t-test (2 tailed) with the SPSS 17.0 software package (SPSS Inc., Chicago, U.S.A.). Differences at $P<0.05$ were considered significant.

CHAPTER IV

RESULTS

4.1 Screening for thermophilic xylanase producing isolates

All isolates of *A. pullulans* cultivated in the production medium containing 1% (w/v) beechwood xylan exhibited the xylanase activity in a range between 1.49 ± 0.08 to 7.44 ± 0.37 U/mL and 0.23 ± 0.09 to 4.87 ± 0.18 U/mL when assayed at 50°C and 70 °C, respectively (Fig.4.1). Although six *A. pullulans* isolates showed the best xylanase activity (> 6.0 U/mL) at 50 °C, the crude enzymes from these isolates were thermosensitive as nearly or more than half of their activities were lost at 70°C. On the contrary, a thermophilic xylanase was produced by the isolate PBU-109 with the higher xylanase activity at 70°C (1.2-fold) than that at 50°C (Fig. 4.1). Therefore, the isolate PBU-109 was selected for further study. This isolate was deposited at the Centraalbureau voor Schimmelcultures, The Netherlands, received CBS number 135684.

4.2 Xylanase production from agricultural wastes

The chemical composition on a dry matter in each xylan-containing biomasses used in this study were presented in Table 4.1. Corn cob contained a significantly high proportion of hemicellulose (42.50%), compared to other biomasses such as wheat germ (28.40%), wheat bran (35.54%), rice straw (23.50%) and water hyacinth (23.70%). In lignin content, the highest value was observed in rice straw (29.10%), followed by wheat bran (22.33%), wheat germ (18.9%), water hyacinth (15.67%) and corncob (12.80%), respectively. High content of cellulose was found in all these biomass. The highest value was found in rice straw (39.20%) and it was not significantly different with wheat bran (38.59%). Effects of various kinds of xylan-containing biomasses, including rice straw, corncob, wheat germ, wheat bran and water hyacinth biomass, on the PBU-109 xylanase production were investigated in liquid cultures. The maximum xylanase production (4.10 ± 0.10 U/mL) was observed in the basal medium containing 1% (w/v) corncob at room temperature (28 ± 2°C) for 48 hours (Table 4.1). Consequently, corncob was selected for further medium optimization for the xylanase production by PBU-109.

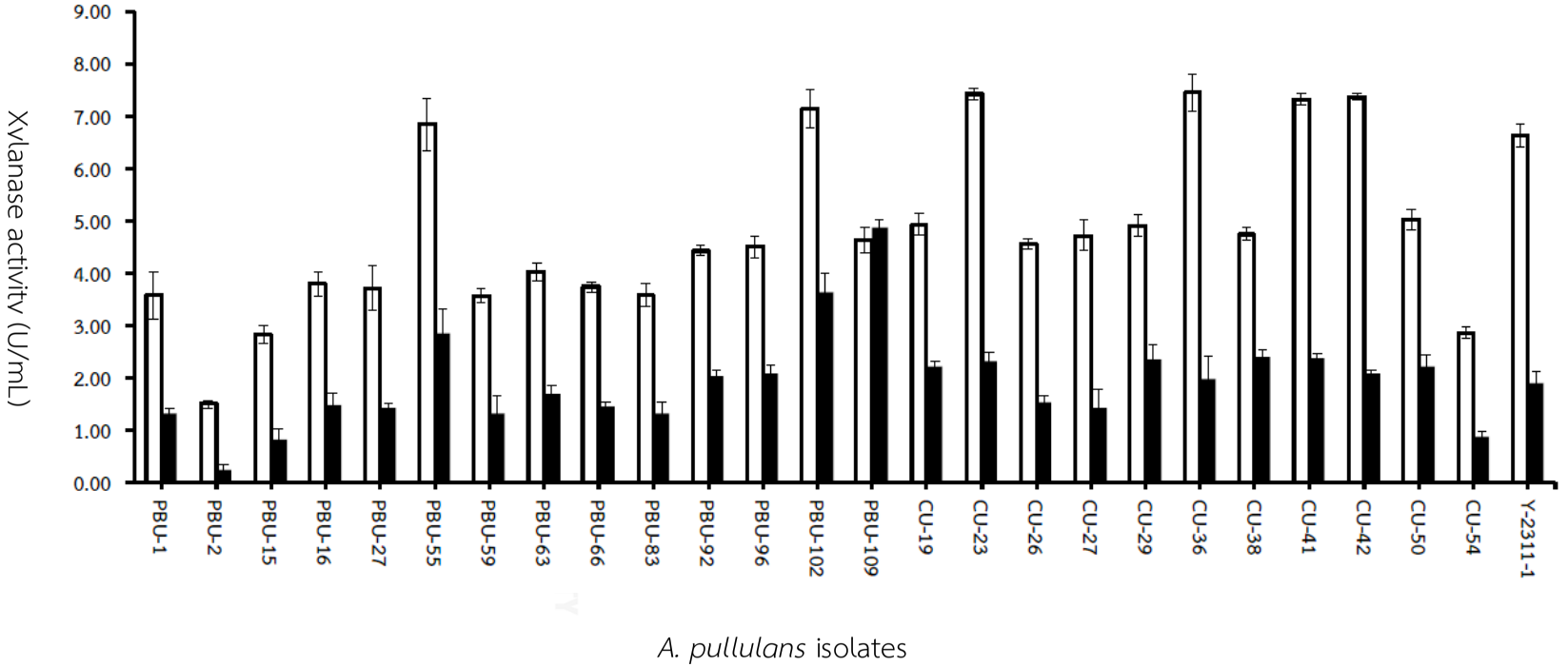

Figure 4.1 Xylanase activity produced by *A. pullulans* isolated in Thailand. White bars indicated activity assayed at 50°C and black bars indicated activity assayed 70°C. Results are the mean values with one standard deviation derived from three replicates. Y-2311-1 was the reference strain obtained from the ARS Culture Collection, USDA, Peoria, IL, USA.

Table 4.1 Xylanase activity produced by *A. pullulans* PBU-109 cultivated in the basal medium containing 1% (w/v) biomass as the carbon source at room temperature (28±3°C) with 150-rpm agitation for 3 days. Composition of each biomass was determined by Goering and Van Soest method (1970) and presented in percentage of dry weight.

Biomass	Xylanase activity (U/mL)	Component (% dry weight)		
		Cellulose	Hemicellulose	Lignin
Wheat germ	3.25 ± 0.13^{d}	30.50 ± 0.95^{A}	28.40 ± 1.21^{b}	18.90 ± 0.89^{C}
Wheat bran	2.59 ± 0.08^{c}	38.59 ± 0.90^{CD}	35.54 ± 0.68^{c}	22.33 ± 1.41^{D}
Corncob	4.10 ± 0.10^{e}	34.10 ± 1.03^{B}	42.50 ± 1.18^{d}	12.80 ± 0.73^{A}
Rice straw	1.64 ± 0.13^{a}	39.20 ± 2.20^{D}	23.50 ± 1.20^{a}	29.10 ± 1.76^{E}
Water hyacinth biomass	1.92 ± 0.13^{b}	36.58 ± 1.34^{BC}	23.70 ± 0.92^{a}	15.67 ± 1.58^{B}
Beechwood xylan	7.23 ± 0.15^{e}	-	-	-

The values in this table were presented as mean ± one standard deviation derived from three replicates (N = 3). Different superscript letter in the same column indicated the values were significantly different (ANOVA and DMRT, $P < 0.05$).

4.3 Medium optimization for xylanase production

4.3.1 Screening of the medium composition for xylanase production using the Plackett–Burman design

To evaluate the medium composition affecting xylanase production, 11 components were tested using the Plackett–Burman design. The independent variables examined in the Plackett–Burman design and their settings are shown in Table 4.2. The experimental data of xylanase production in this experiment showed a wide variation in xylanase activity from 2.27 U/mL (trial number 8) to 6.51 U/mL (trial number 11), which reflected the importance of certain medium components that enhance the enzyme production. The main effect was estimated based on the differences between the sum of responses obtained at the high level (+1) and at the low level (−1) of each component.

Table 4.2 The Plackett–Burman experimental design matrix for medium composition screening of xylanase production by *A. pullulans* PBU-109.

Trials	Variable factors* (g/L)											Xylanase Activity (U/mL)	Predicted values (U/mL)
	X_1*	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}		
1	1.0	0.5	5.0	0.5	1.5	0.5	1.5	1.5	0.2	1.0	1.5	2.48	2.17
2	1.0	1.0	10.0	0.5	0.5	0.5	1.5	0.5	1.0	1.0	0.5	3.26	3.27
3	1.0	0.5	10.0	1.5	0.5	1.5	1.5	1.5	0.2	0.2	0.5	4.73	4.38
4	1.0	0.5	10.0	1.5	1.5	0.5	0.5	0.5	1.0	0.2	1.5	3.95	4.43
5	0.5	1.0	10.0	0.5	1.5	1.5	1.5	0.5	0.2	0.2	1.5	6.08	6.20
6	0.5	1.0	5.0	1.5	1.5	0.5	1.5	1.5	1.0	0.2	0.5	3.86	3.87
7	1.0	1.0	5.0	0.5	0.5	1.5	0.5	1.5	1.0	0.2	1.5	3.64	3.65
8	0.5	0.5	5.0	0.5	0.5	0.5	0.5	0.5	0.2	0.2	0.5	2.27	2.39
9	0.5	0.5	5.0	1.5	0.5	1.5	1.5	0.5	1.0	1.0	1.5	5.04	5.05
10	1.0	1.0	5.0	1.5	1.5	1.5	0.5	0.5	0.2	1.0	0.5	3.44	3.60
11	**0.5**	**1.0**	**10.0**	**1.5**	**0.5**	**0.5**	**0.5**	**1.5**	**0.2**	**1.0**	**1.5**	**6.51**	**6.20**
12	0.5	0.5	10.0	0.5	1.5	1.5	0.5	1.5	1.0	1.0	0.5	4.62	4.67

* X_1: glucose, X_2: xylose, X_3: corncob, X_4: $(NH_4)_2SO_4$, X_5: urea, X_6: KH_2PO_4, X_7: yeast extract, X_8: peptone, X_9: $MgSO_4{\cdot}7H_2O$, X_{10}: $CaCl_2{\cdot}2H_2O$ and X_{11}: tween 80

The effect of each medium component on xylanase production was shown in Figure 4.2. It was found that eight components, including xylose, corncob, $(NH_4)_2SO_4$, KH_2PO_4, yeast extract, peptone, $CaCl_2$ and tween 80, could enhance xylanase production while the remaining three components (glucose, urea and $MgSO_4$) gave the negative influence on the xylanase yield. The model *F*-value of 24.74 and *P* at < 0.05 indicated that the model was significant. Regression analysis was performed on the results and a first order polynomial equation was derived representing xylanase production as a function of the independent variables as follows:

$$Y = 4.15\text{-}0.57X_1+0.31X_2+0.70X_3+0.43\ X_4\ +0.44X_6+0.46X_{11} \quad (1)$$

Where, *Y* was the response value or xylanase activity (U/mL). X_1, X_2, X_3, X_4, X_6 and X_{11} were glucose (g/L), xylose (g/L), corncob (g/L), $(NH_4)_2SO_4$(g/L), KH_2PO_4 (g/L) and tween 80

(mL/L), respectively. Statistical analysis (*F*-value and *P*-value) demonstrated that, among the positive main effects, xylose, corncob, $(NH_4)_2SO_4$, KH_2PO_4 and tween 80 had effects above 95% level and hence were considered to significantly influence to xylanase production (Table 4.3). The optimal concentration of these five components was determined by the subsequent Box-Behnken design.

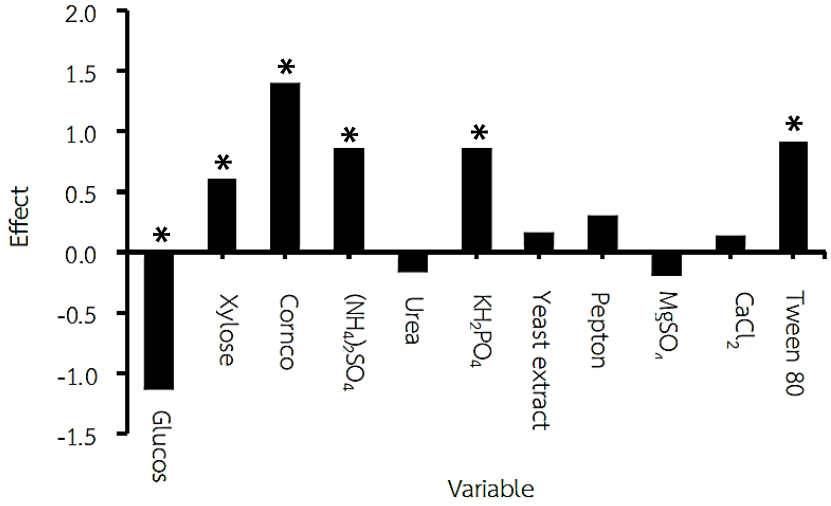

Figure 4.2 Pareto chart showing the effects of medium components on xylanase activity by *A. pullulans* PBU-109.

Table 4.3 Analysis of variance (ANOVA) of the Plackett–Burman experimental model developed for xylanase production by *A. pullulans* PBU-109.

Source	Sum of Squares	df	Mean square	*F*-value	*P*-value (Prob > *F*)
Model	18.0393	6	3.0065	24.7358	0.001441*
X_1-Glucose	3.9445	1	3.9445	32.4528	0.002325*
X_2-Xylose	1.1408	1	1.1408	9.3860	0.027985*
X_3-Corncob	5.9080	1	5.9080	48.6071	0.000934*
X_4-$(NH_4)_2SO_4$	2.2360	1	2.2360	18.3965	0.007796*
X_7- KH_2PO_4	2.2707	1	2.2707	18.6817	0.007555*
X_{11}-tween 80	2.5392	1	2.5392	20.8907	0.005998*
Residual	0.6077	5	0.1215		
Cor Total	18.6471	11			

R^2 = 0.967; Adj R^2= 0.928; Coefficient of variance = 8.39%; Significant at P<0.05

4.3.2. Optimization of the medium compositions for xylanase production using response surface methodology

Five medium components, xylose, corncob, $(NH_4)_2SO_4$, KH_2PO_4 and tween 80, were chosen to determine the optimal concentration based on Box-Behnken design. The observed and predicted responses from 43 experimental trials of which contained different combinations of the five variables at three different concentrations were shown in Table 4.4. The maximum xylanase production was found to be 8.94±0.84 U/mL in the trial number 8 and the minimum 1.80 ±0.15 U/mL was found in the trial number 25. For estimation of the RSM model errors, the center point in the design was repeated three times. The second-order regression equation provided the levels of xylanase activity as the function of variables was presented in terms of coded factors as in the following equation:

$$\begin{aligned} Y = {} & 5.64A_1+10.39A_2+12.86A_3+18.37A_4+18.54A_5-0.14A_1A_2-0.03A_1A_3-0.22A_1A_4 \\ & -0.20A_1A_5-0.05A_2A_3+0.39A_2A_4+0.09A_2A_5+0.13\ A_3A_4-0.10A_3A_5+0.02A_4A_5 \\ & -0.06A_1^2-0.95A_2^2-3.33A_3^2-3.89A_4^2-3.61A_5^2-155.36 \end{aligned}$$

Where, Y was the response value or xylanase activity (U/mL). A_1, A_2, A_3, A_4 and A_5 were corncob (g/L), $(NH_4)_2SO_4$ (g/L), xylose (g/L), KH_2PO_4 (g/L) and tween 80 (mL/L), respectively.

The analyzed results by ANOVA were shown in Table 4.5. ANOVA for xylanase production indicated that the model was significant due to the *F*-value (151.97) and model terms values of *P* less than 0.0001. The coefficient of determination (R^2) was calculated to be 0.9928, which indicated that the model could explain up to 99% of the variability and only less than 1% of the total variance could not be explained by the model. The adjusted determination coefficient (adjusted R^2 = 0.9863) and predicted determination coefficient (R^2 = 0.9730) also confirmed the significance of the model. The lack of fit indicated the unfit probability between the experimental and the predicted values. In this case, the model showed a statistical insignificance in lack of fit (*P* =0.7724) which indicated that the second-order model equation was adequate for the prediction of xylanase production across the specified range of variables employed. The plot of observed versus predicted xylanase activities under optimum production

conditions showed that the actual xylanase activity values were similar to the predicted response values (Fig 4.3). A lower value of coefficient of variation (CV = 3.63%) indicated a greater preciseness and reliability of the experiment performed. Thus, the estimated model fitted the experimental data adequately. The plot of observed xylanase activity versus the predicted xylanase activity under optimum production conditions (Figure 4.3) showed that the actual xylanase activity values were not significantly different with the predicted response values. It was confirmed by the coefficient of determination (R^2 = 0.9905).

Table 4.4 Box-Behnken design matrix with experimental and predicted values of xylanase production by *A. pullulans* PBU-109.

Trials	Variables (g/L)					Xylanase Activity (U/mL)	Predicted values (U/mL)
	Corncob (A_1)	$(NH_4)_2SO_4$ (A_2)	Xylose (A_3)	KH_2PO_4 (A_4)	Tween 80 (A_5)		
1	15.0	40.0	1.5	3.5	3.5	7.24	7.50
2	12.5	35.0	1.5	3.5	3.5	7.42	7.66
3	12.5	35.0	1.5	5.0	5.0	6.50	6.34
4	12.5	35.0	1.5	3.5	3.5	7.62	7.66
5	12.5	35.0	1.0	2.0	3.5	4.74	4.76
6	12.5	40.0	1.5	3.5	5.0	6.68	6.73
7	12.5	35.0	2.0	5.0	3.5	7.10	7.01
8	**15.0**	**35.0**	**2.0**	**5.0**	**3.5**	**8.74**	**8.65**
9	12.5	40.0	2.0	3.5	3.5	7.35	7.31
10	10.0	40.0	1.5	2.0	3.5	4.08	4.06
11	12.5	35.0	1.5	3.5	2.0	4.87	5.24
12	12.5	30.0	1.5	3.5	2.0	5.04	4.97
13	12.5	40.0	1.5	3.5	3.5	5.94	5.87
14	12.5	35.0	1.0	5.0	3.5	4.97	5.08
15	12.5	35.0	2.0	3.5	2.0	6.52	6.53

Table 4.4 (continue) Box-Behnken design matrix with experimental and predicted values of xylanase production by *A. pullulans* PBU-109.

Trials	Variables (g/L)					Xylanase activity (U/mL)	Predicted values (U/mL)
	Corncob (A_1)	$(NH_4)_2SO_4$ (A_2)	Xylose (A_3)	KH_2PO_4 (A_4)	Tween 80 (A_5)		
16	12.5	30.0	1.5	2.0	5.0	5.52	5.60
17	10.0	35.0	1.5	3.5	5.0	3.74	3.77
18	12.5	35.0	1.0	5.0	2.0	4.57	4.61
21	15.0	35.0	1.5	2.0	5.0	7.44	7.56
22	15.0	30.0	1.5	3.5	3.5	7.72	7.86
23	12.5	30.0	2.0	3.5	3.5	6.40	6.32
24	12.5	35.0	1.5	3.5	3.5	7.93	7.66
25	10.0	30.0	1.5	5.0	3.5	1.80	1.64
26	12.5	40.0	1.5	3.5	2.0	6.02	5.93
27	12.5	40.0	1.5	2.0	3.5	6.78	6.65
28	10.0	35.0	2.0	3.5	3.5	3.70	3.96
29	12.5	35.0	1.5	5.0	2.0	5.40	5.62
31	15.0	35.0	1.0	3.5	3.5	7.03	6.91
32	12.5	35.0	1.0	2.0	5.0	5.42	5.38
33	12.5	30.0	1.5	3.5	3.5	5.14	5.22
34	12.5	40.0	1.0	3.5	3.5	5.42	5.49
35	10.0	35.0	1.5	3.5	3.5	2.02	2.08
36	12.5	30.0	1.0	5.0	3.5	4.38	4.40
37	10.0	35.0	1.5	3.5	3.5	3.38	3.57
38	10.0	35.0	1.5	2.0	2.0	2.30	2.03
39	15.0	35.0	1.5	3.5	2.0	8.04	7.86
40	15.0	35.0	1.5	5.0	3.5	8.26	8.00
41	12.5	35.0	2.0	5.0	5.0	7.27	7.19
42	12.5	35.0	2.0	3.5	3.5	6.74	6.56
43	15.0	35.0	1.5	2.0	3.5	7.42	7.29

Table 4.5 Analysis of variance (ANOVA) of the Box-Behnken experimental model developed for xylanase production by *A. pullulans* PBU-109.

Source	Coefficient factor	Sum of Squares	Df	Mean square	*F*-value	*P*-value (Prob > *F*)
Model	7.66	132.60	20	6.63	151.97	< 0.0001*
A_1- Corncob	2.41	92.73	1	92.73	2125.43	< 0.0001*
A_2-$(NH_4)_2SO_4$	0.52	4.33	1	4.33	99.27	< 0.0001*
A_3- Xylose	0.93	13.95	1	13.95	319.77	< 0.0001*
A_4- KH_2PO_4	0.19	0.60	1	0.60	13.84	0.0012*
A_5-tween 80	0.36	2.06	1	2.06	47.3	< 0.0001*
A_1A_2	-0.69	1.90	1	1.90	43.65	< 0.0001*
A_1A_3	-0.16	0.20	1	0.20	10.37	0.0054*
A_1A_4	-0.55	1.21	1	1.21	27.73	< 0.0001*
A_1A_5	-0.51	1.04	1	1.04	23.85	< 0.0001*
A_2A_3	-0.02	2.10E-003	1	2.10E-003	0.048	0.8285
A_2A_4	0.20	0.15	1	0.15	3.49	0.0753
A_2A_5	0.04	7.74E-003	1	7.74E-003	0.18	0.6776
A_3A_4	0.03	4.23E-003	1	4.23E-003	0.097	0.7586
A_3A_5	-0.03	2.70E-003	1	2.70E-003	0.062	0.8057
A_4A_5	5.50E-003	1.21E-004	1	1.21E-004	2.77E-03	0.9585
A_1^2	-1.45	13.51	1	13.51	309.73	< 0.0001*
A_2^2	-0.95	5.76	1	5.76	131.98	< 0.0001*
A_3^2	-0.83	4.45	1	4.45	101.92	< 0.0001*
A_4^2	-0.97	6.06	1	6.06	138.85	< 0.0001*
A_5^2	-0.90	5.21	1	5.21	119.37	< 0.0001*
Residual		0.96	22	0.04		
Lack of Fit		0.83	20	0.04	0.63	0.7724
Residual		0.13	2	0.07		
Cor Total		133.56	42			

R^2 = 0.993; Adj R^2= 0.986; Coefficient of variance = 3.63%; Significant at P<0.05

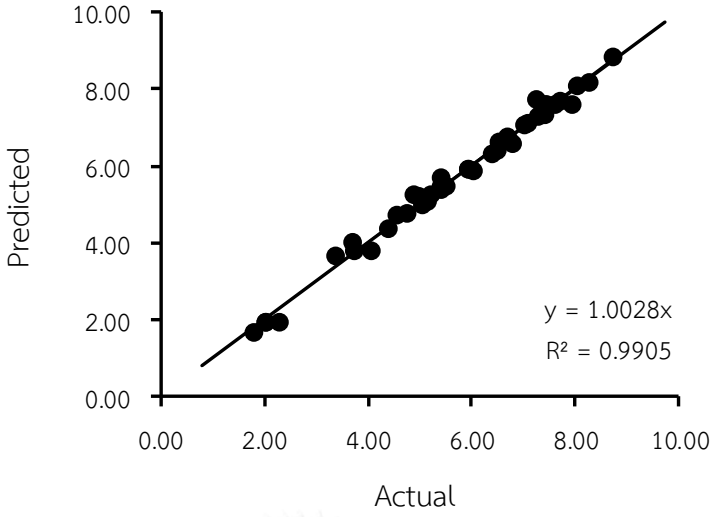

Figure 4.3 Observed xylanase activity versus the predicted xylanase activity under the optimum conditions.

Three-dimensional response plots and their corresponding contour plots were drawn on the basis of the model equation to investigate the interaction among the variables and to determine the optimum concentration of each factor for maximum xylanase production. Figure 4.4-4.9 presented the effect of two variables on the production of xylanase, while the other variables were held at central (0) level. The significant interactions were found in A_1A_2 (corncob and $(NH_4)_2SO_4$), A_1A_3 (corncob and xylose), A_1A_4 (corncob and KH_2PO_4) and A_1A_5 (corncob and tween 80) which is evident from *P*-value less than 0.05 (Table 4.5). It can be seen from Figure 4.4-4.7 that xylanase production tends to increase with gradually increasing corncob concentration. However, no interactions between A_2A_3 ($(NH_4)_2SO_4$ and KH_2PO_4), A_2A_4 ($(NH_4)_2SO_4$ and KH_2PO_4), A_2A_5 ($(NH_4)_2SO_4$ and tween 80), A_3A_4 (xylose and KH_2PO_4), A_3A_5 (xylose and tween 80) and A_4A_5 (KH_2PO_4 and tween 80) were found to contribute to the response at a significant level, due to the high *P*-value ($P>0.05$) (Table 4.5). Figure 4.8 described the effects of A_2 ($(NH_4)_2SO_4$) and A_3 (xylose) in term of nitrogen sources and inducer on xylanase production, respectively. It indicated that when A_2 ($(NH_4)_2SO_4$) and A_3 (xylose) were in the ranges of 2.4 to 3.4 and 1.5 to 2.0, respectively, the xylanase activity was higher than 8.5 U/mL. Figure 4.9 shows the effects of A_3 (xylose) and A_4 (KH_2PO_4) on xylanase production.

Evidently, the middle level of the KH_2PO_4 in the medium ensured more xylanase production. Similar result was found in Figure 4.10 that the middle level of tween-80 affected the higher xylanase production.

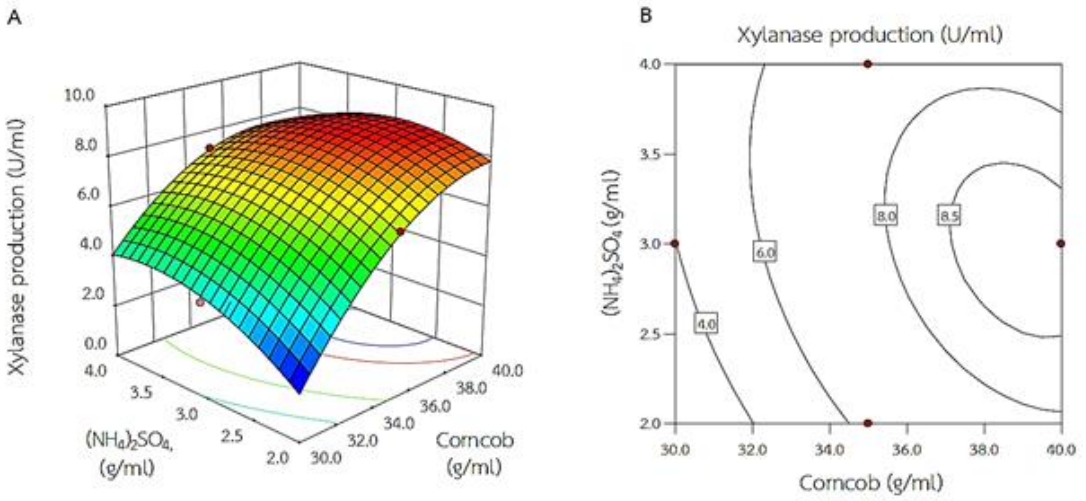

Figure 4.4 Response surface plot (A) and contour plot (B) of the combined effects of corncob (A_1) and $(NH_4)_2SO_4$ (A_2) on xylanase production by *A. pullulans* PBU-109.

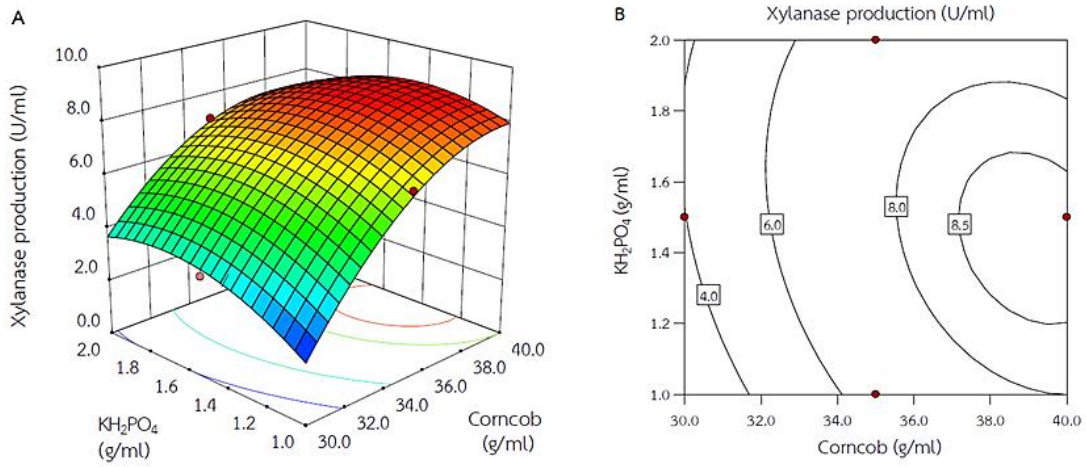

Figure 4.5 Response surface plot (A) and contour plot (B) of the combined effects of corncob (A_1) and KH_2PO_4 (A_4) on xylanase production by *A. pullulans* PBU-109.

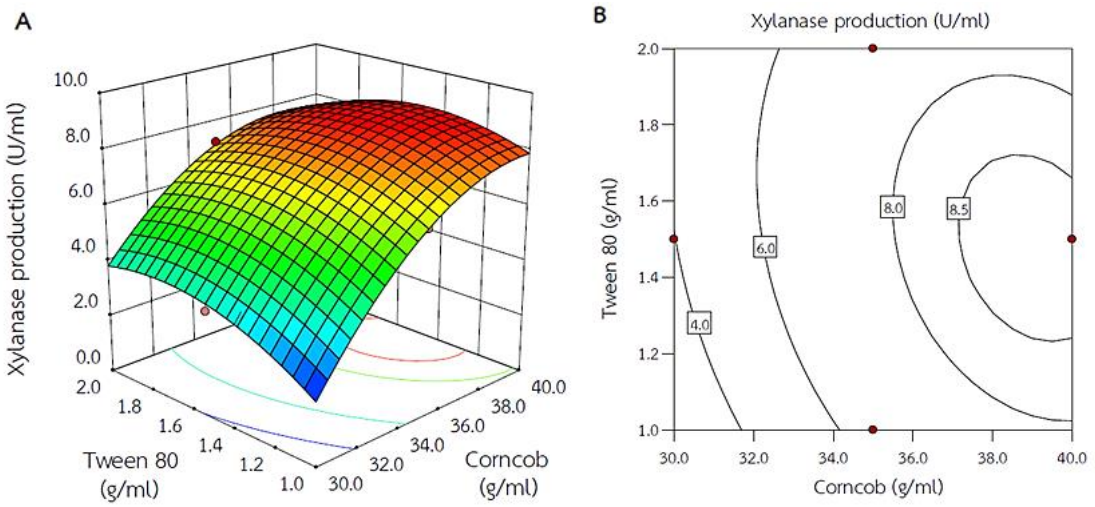

Figure 4.6 Response surface plot (A) and contour plot (B) of the combined effects of corncob (A_1) and tween 80 (A_5) on xylanase production by *A. pullulans* PBU-109.

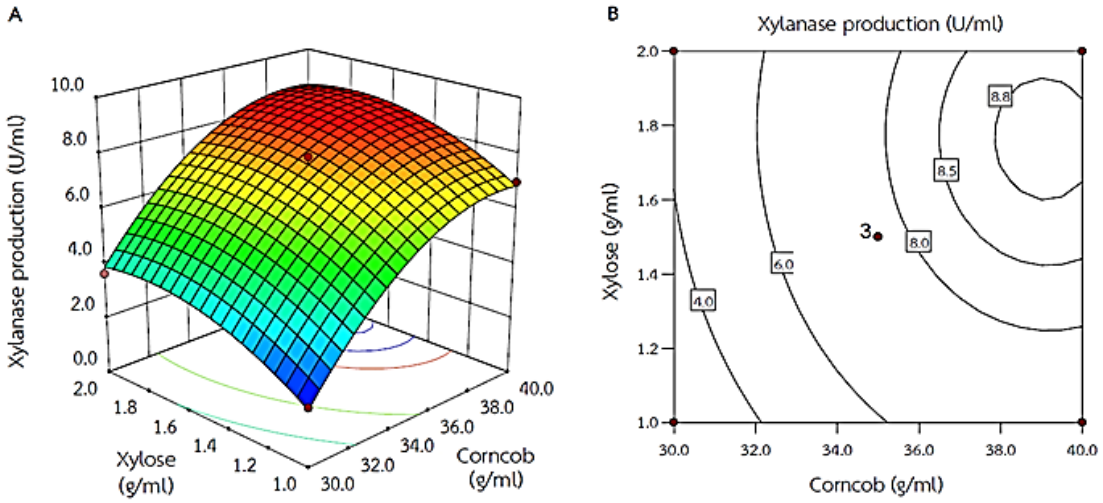

Figure 4.7 Response surface plot (A) and contour plot (B) of the combined effects of corncob (A_1) and xylose (A_3) on xylanase production by *A. pullulans* PBU-109.

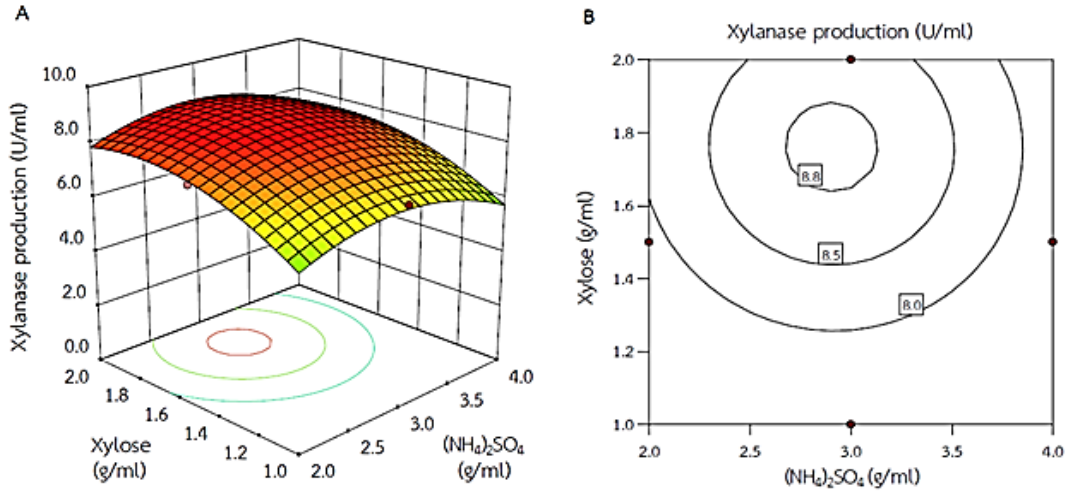

Figure 4.8 Response surface plot (A) and contour plot (B) of the combined effects of $(NH_4)_2SO_4$ (A_2) and xylose (A_3) on xylanase production by *A. pullulans* PBU-109.

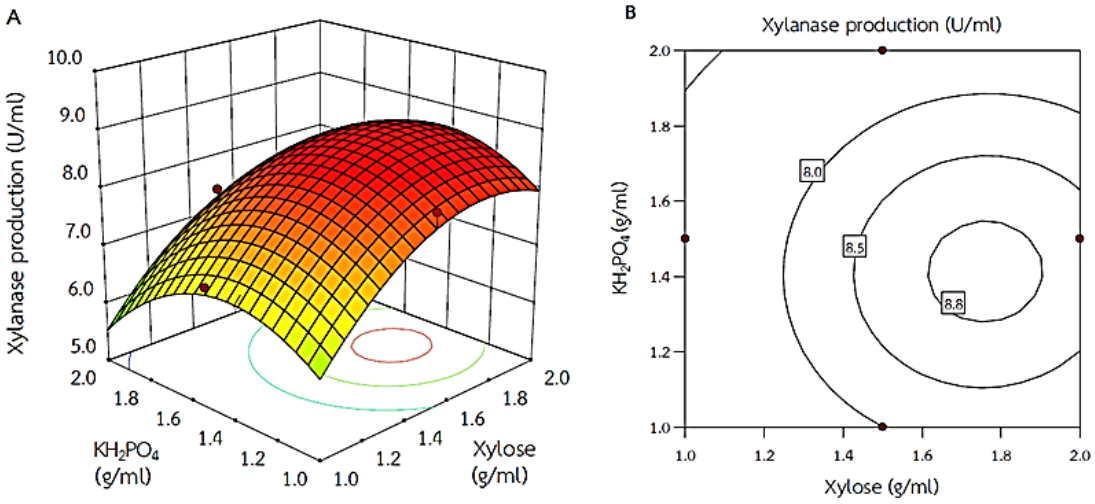

Figure 4.9 Response surface plot (A) and contour plot (B) of the combined effects of xylose (A_3) and KH_2PO_4 (A_4) on xylanase production by *A. pullulans* PBU-109.

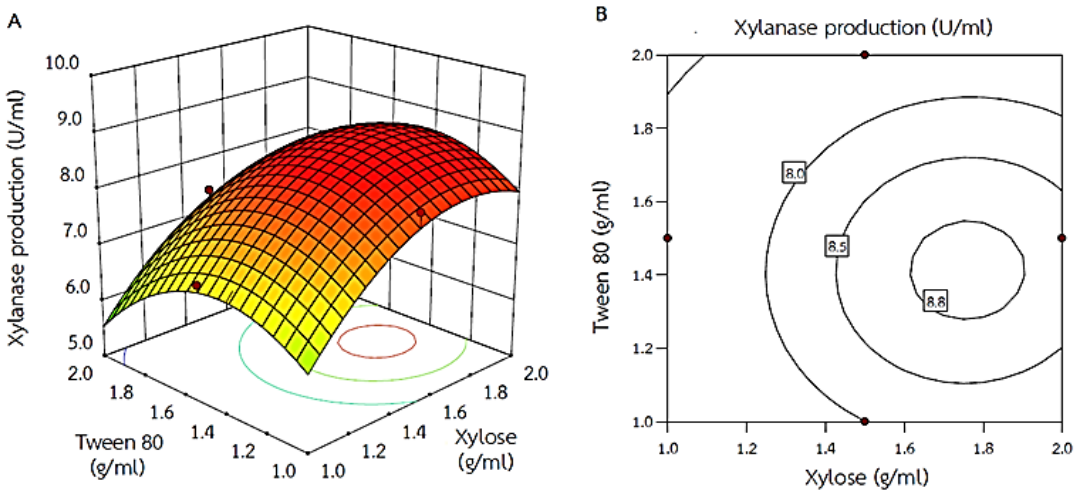

Figure 4.10 Response surface plot (A) and contour plot (B) of the combined effects of xylose (A_3) and tween 80 (A_5) on xylanase production by *A. pullulans* PBU-109.

The optimum composition for the maximum xylanase production was determined by regression equation and response surface analysis. As the results of Box-Behnken design experiments, the optimal concentrations of corncob, $(NH_4)_2SO_4$, xylose, KH_2PO_4 and tween 80 were 39.0 g/L, 3.0 g/L, 1.8 g/L,1.4 g/L and 1.4 g/L, respectively. The model predicted that the xylanase activity from *A. pullulans* PBU-109 would reach up to 8.82±0.21 U/mL when produced in the medium containing these five components at the optimized concentrations. Synergistic effect of the selected variables corresponded to a 2.15-fold increase in xylanase activity compared to their individual effects. The validity of the results predicted by the regression model was confirmed by repeated experiments under optimal conditions. The result obtained from three replications showed that the maximum xylanase production obtained (8.74±0.84 U/mL) was close to the predicted value (8.82±0.21 U/mL). This result indicates that there was an excellent correlation between experimental and predicted values and in turn proved the validity of the model.

The profiles of the growth and xylanase production by *A. pullulans* PBU-109 in the basal medium with 1% (w/v) corncob and the optimized production medium were showed in Figure 4.10. Xylanase production reaches a peak value of 9.48±0.87

U/mL after 72 hours of cultivation in the optimized medium, while a maximum level of 4.14±0.17 U/mL was observed in the basal medium after 48 hours. Although xylanase production was peaked in different period, the patterns of cell growth in both media were similar. The cell density increased exponentially with no apparent lag phase for 48 hours of cultivation. After this period, the cell density increased at a slower rate and entered the stationary phase after 72 hours of cultivation. However, the cell density of *A. pullulans* PBU-109 in the optimized medium was noticeably lower than that of the basal medium.

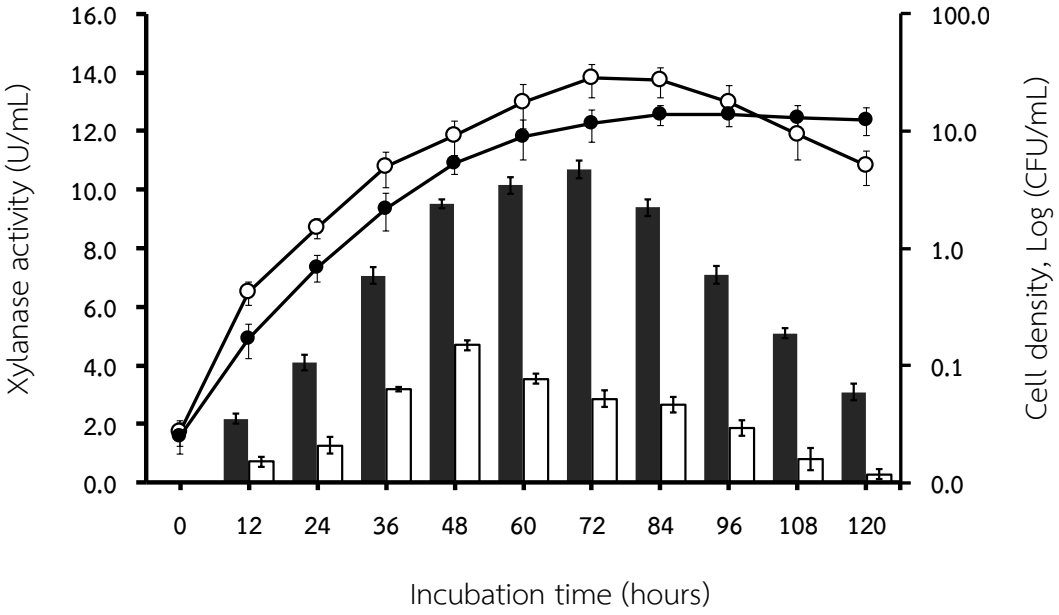

Figure 4.11 Time course of growth and xylanase production of *A. pullulans* PBU-109 at room temperature in the basal medium containing 1% (w/v) corncob and the optimized production medium. Open circles (○) and solid circles (●) indicated cell density in the basal medium and the optimized production medium, respectively. White bars and black bars indicated xylanase activity in the basal medium and the optimized production medium, respectively. Results were showed as a mean with one standard deviation derived from three replicates

4.4 Purification of xylanase

4.4.1 Ammonium sulfate precipitation

Crude xylanase was prepared from 1 liters of the optimized production medium as described in 3.3.3. After cell removal, the culture supernatant (465 mL) was concentrated by ultrafiltration to yield 100 mL of the crude enzyme with 4,431.65 ± 27.31 units of xylanase and 1,376.00 ± 26.18 mg protein. Thus, the specific activity of the enzyme in the crude preparation was 3.22 ± 0.06 U/mg proteins (Table 4.6). The crude enzyme solution was further purified by ammonium sulfate precipitation. To determine the suitable ammonium sulfate concentration for precipitation of xylanase, the experiment was performed by a stepwise increase at 20% increment from 0-80%. The enzyme activity was mainly detected in the 40-60% and 60-80% fractions with the most activity in 60-80% fractions (Table 4.6). Therefore, to harvest the enzyme, protein fractionation was carried out in the range of 40-60% and 60-80% saturated ammonium sulfate precipitation. Total protein content was obtained at 1,376.00 ± 26.18 mg with total enzyme activity at 2,640.00 ± 14.24 units (about 56.77% recovery from the filtrated crude enzyme). The specific activity of the enzyme from this step was 5.48 ± 0.02 U/mg proteins (Table 4.7).

4.4.2 HiTrap DEAE-sepharose column (Anion exchanger chromatography)

The enzyme obtained from ammonium sulfate precipitation was applied onto HiTrap DEAE-sepharose FF column as described in 3.3.4.3. The chromatographic profile was shown in Figure 4.11. The unbound proteins were eluted from the column with 20 mM Tris-HCl buffer (pH 8.0). The bound proteins were eluted with a linear NaCl gradient from 0 to 1 M in the same buffer. The fractions with xylanase activity were pooled, concentrated by ultrafiltration and dialyzed against 50 mM acetate buffer (pH 6.0). Two peaks of proteins with xylanase activity were detected. The first peak was unbound to the column whereas the second peak was bound to the DEAE-sepharose and was eluted by NaCl at the concentration between 0.3 and 0.6 M. The second peak, which represented the major portion of xylanase activity, was further fractionated using Sephacryl S-100 gel filtration chromatography. This step yielded the enzyme with 21±1.04

mg protein, 834±9.84 activity units and the specific activity of 39.70±1.76 U/mg proteins. The enzyme was purified to 16.61-fold with about 17.2% recovery (Table 4.7).

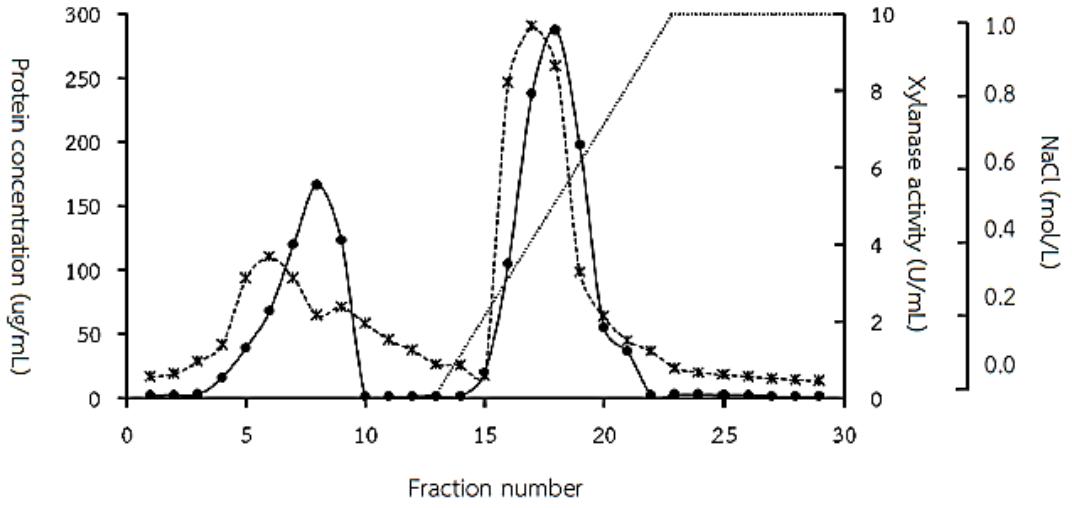

Figure 4.12 Chromatographic profiles of xylanase activity and protein content fractionated through a HiTrap DEAE-sepharose Fast Flow (FF) column. The chromatography was carried out in 20 mM Tris-HCl buffer (pH 8.0) at 4ºC with a flow rate of 1.0 mL/min.

Table 4.6 Ammonium sulfate precipitation of the xylanase from *A. pullulans* PBU-109.

Sample	Total volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)
Crude extract	465	2,030.53 ± 32.24	4,650.23 ± 36.42	2.39 ± 0.04	1.00
Ultrafiltration	100	1,376.63 ± 26.18	4,431.65 ± 27.31	3.22 ± 0.06	1.35
0-20 % $(NH_4)_2SO_4$	15	233.26 ± 2.12	4.56 ± 1.31	0.02 ± 0.00	0.01
20-40 % $(NH_4)_2SO_4$	15	220.19 ± 6.24	15.93 ± 0.98	0.07 ± 0.00	0.03
40-60 % $(NH_4)_2SO_4$	15	402.38 ± 5.84	1,044.59 ± 2.40	2.60 ± 0.02	1.09
60-80 % $(NH_4)_2SO_4$	15	178.35 ± 1.92	2,132.27 ± 4.28	12.68 ± 0.04	5.31
80% $(NH_4)_2SO_4$ (supernatant)	110	134.65 ± 1.12	68.34 ± 0.88	0.50 ± 0.00	0.21

Table 4.7 Purification step summary of the xylanase from *A. pullulans* PBU-109.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
Culture supernatant	2,030.86 ± 32.24	4,650.23 ± 36.42	2.39 ± 0.04	1.00	100.00
Ultrafiltration	1,376.64 ± 26.18	4,431.65 ± 27.31	3.22 ± 0.06	1.35	95.30
$(NH_4)_2SO_4$ precipitation (40–80% saturation)	560.95 ± 1.24	2,640.26 ± 14.24	5.48 ± 0.02	2.29	56.78
DEAE-Sepharose	21.15 ± 1.04	834.38 ± 9.84	39.70 ± 1.76	16.61	17.94
Sephacryl S-100	16.22 ± 1.24	662.19 ± 10.46	41.40 ± 2.85	17.32	14.24

4.4.3 Sephacryl S-100 High Resolution Column (Gel filtration chromatography)

The concentrated enzyme (5 ml; 4 mg total protein/ml) obtained from the anion-exchanger chromatography was subsequently purified using a Sephacryl S-200 High Resolution Column. The chromatographic profile is shown in Figure 4.12. The protein was eluted from the column with 50 mM acetate buffer (pH 6.0). The fractions with xylanase activity were pooled, concentrated by ultrafiltration and dialyzed against 50 mM acetate buffer (pH 6.0). The protein content remained in this step was approximately 16.00 ± 1.24 mg with 662.00 ± 10.46 activity units and specific activity of 41.40 ± 2.85 U/mg. The enzyme was purified 17.32-fold with 13.6% recovery. The purified enzyme was kept as aliquots at 4ºC for further experiments.

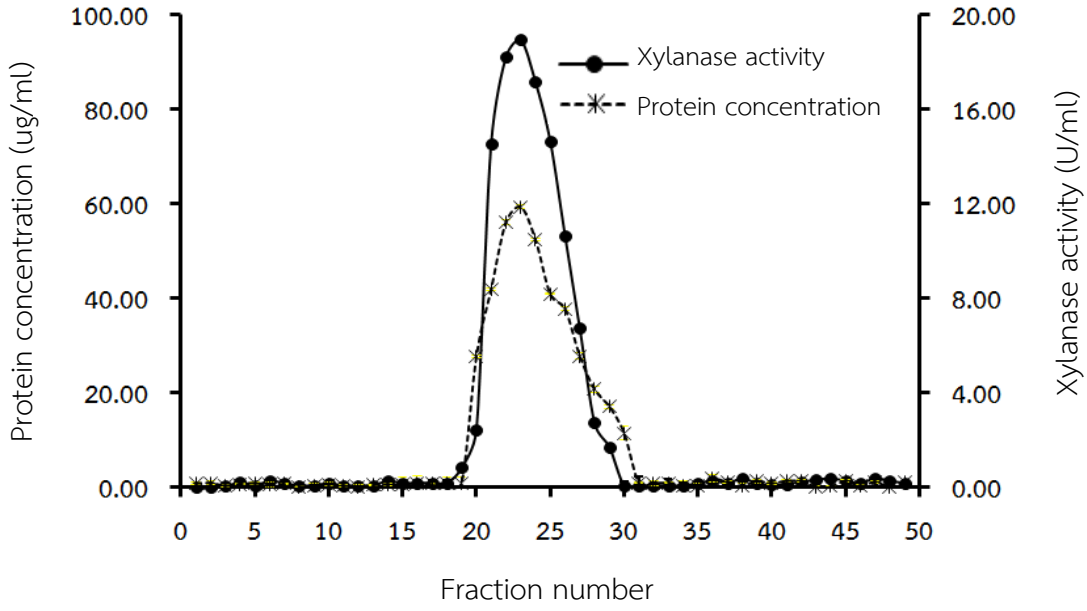

Figure 4.13 Chromatographic profiles of xylanase activity and protein content fractionated through a Sephacryl S-200 High Resolution column. The chromatography was carried out in 20 mM Tris-HCl buffer (pH 8.0) at 4ºC with a flow rate of 0.5 ml/min.

4.4.4 Protein analysis by SDS-PAGE and zymogram analysis

The purified xylanase was analyzed for purity and mass by SDS-PAGE as described in section 3.3.4.5. A single band of protein was observed on the denatured gel indicating the purity of the enzyme, and this band corresponded with the xylanase activity detected in a native gel after activity staining (Fig. 4.13). By comparison to a standard protein marker, the mass of the protein was calculatedly to be 73 kDa and suggestively consisted of a single polypeptide.

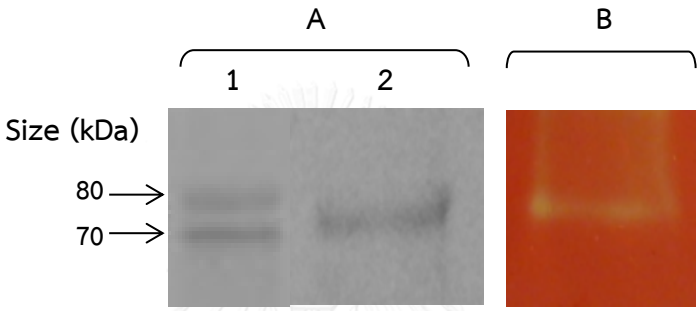

Figure 4.14 SDS-PAGE (A) and zymogram (B) analysis of the purified xylanase from *A. pullulans* PBU-109. Protein mass and purity were determined by SDS-PAGE on a 12% acrylamide gel in 1.5 M Tris-HCl (pH 8.8) of which the gel was stained by Coomassie Blue (lane 1, 2). Xylanase activity of the protein was determined in a native PAGE containing 0.1% (w/v) beechwood xylan stained with 0.5% (w/v) Congo Red (lane 3).

Lane 1: Precision Plus Protein Standards, 23.4 µg (SDS-PAGE)

Lane 2: Purified xylanase from gel filtration chromatography, 10 µg protein (native PAGE)

Lane 3: Purified xylanase from gel filtration chromatography, 10 µg protein (native PAGE)

4.5 Characterization of the purified xylanase

4.5.1 Optimal conditions for xylanase activity

The optimal condition for xylanase activity was determined as described in 3.3.5.1. The effects of pH and temperature on the activity of the purified xylanase were summarized in Figure 4.14. The purified xylanase was active over a broad pH range with

the maximum activity at pH 6, and more than 70% of the maximal activity was observed between pH 4 and10. The enzyme activity dramatically decreased at pH lowers than 4. When the effect of temperature on the xylanase activity was observed, the enzyme activity was found to be significantly increased (at every pH) as the incubation temperature was raised from 50℃ to the optimal at 70℃. A significantly lower xylanase activity was noted at 90℃ (60% of the optimum activity at pH 6.0). At pH 11.0, the xylanase activity was not detectable. The optimal pH (pH 6.0) and temperature (70℃) of the purified xylanase were similar to those of the crude enzyme.

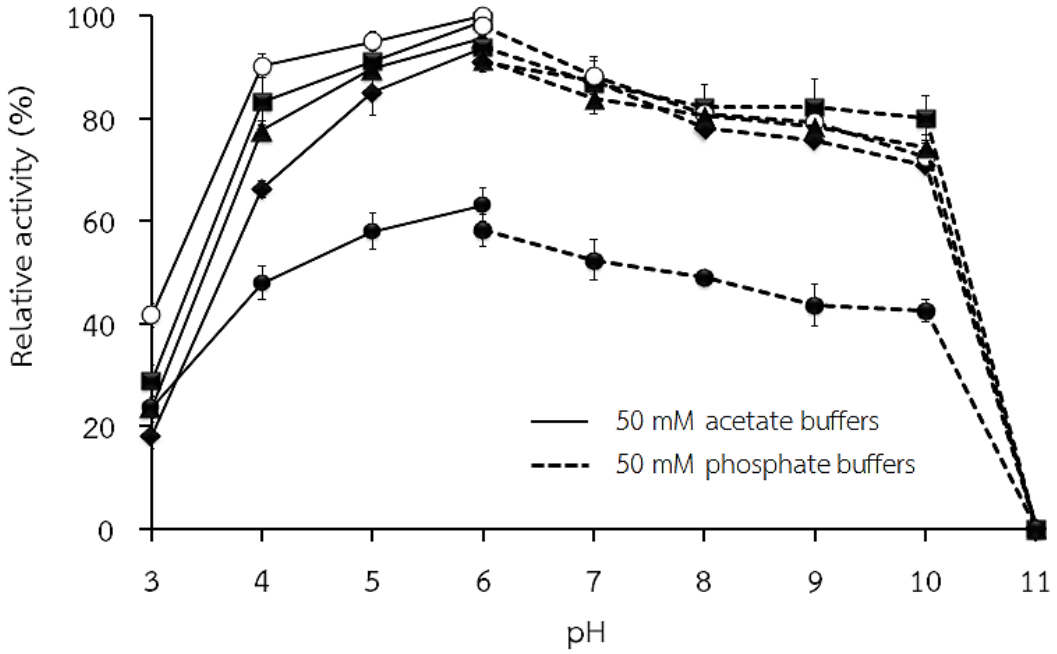

Figure 4.15 Effects of pH and temperature on activity of the purified xylanase from *A. pullulans* PBU-109. Reaction mixtures containing 1% (w/v) beech wood xylan as the substrate were incubated for 30 mins at various temperatures including 50℃ (♦), 60℃ (■), 70℃ (○), 80℃ (▲) and 90℃ (●) (100% = 8.42 ± 0.24 U/ml).

4.5.2 Thermostability and thermodynamic analysis of purified enzyme

In this experiment, the thermostability of the purified xylanase from *A. pullulans* PBU-109 was assessed at 40-70℃ and pH 6.0. The purified enzyme was found to be relatively stable at 40℃ and 45℃ with more than 80% of its original activity

remaining after 3 h incubation (Fig. 4.15). After incubation for 60 min at 50°C, 55°C, 60°C, 65°C or 70°C, some 95%, 84%, 60%, 40% or 23% of the initial enzyme activity remained, respectively. The enzyme quickly lost its activity at 70°C with less than 50% residual activity after 30 min. The plots of the residual activity of the purified xylanase versus incubation time were linear, with R^2 >95%, indicating that the inactivation could be expressed as a first order kinetics (Fig 4.16). The activation energy for irreversible inactivation (E_d) of the xylanase was determined to be 86.1 kJ/mol from the Arrhenius plot (Fig. 4.17). Thermodynamic parameters of the purified enzyme were summarized in Table 4.8. The rate of enzyme deactivation (k_d) was found to increase at higher temperatures while the half-life ($t_{1/2}$) of xylanase and the enthalpy of activation of the thermal denaturation (ΔH) tended to decrease. The Gibbs free energy (ΔG) for the thermal unfolding increased from 106.0 to 107.6 kJ/mol with increasing temperature from 50 to70°C (Table 4.8).

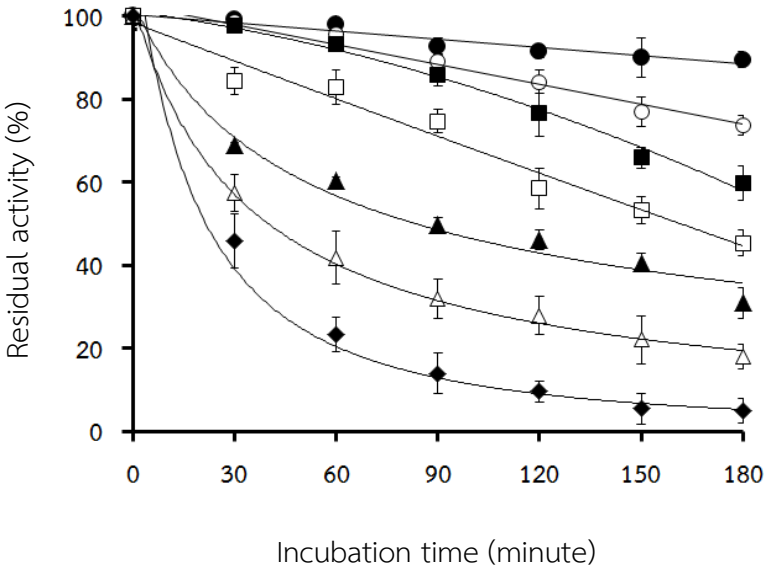

Figure 4.16 Thermostability profile of the purified xylanase from *A. pullulans* PBU-109. The purified enzyme was solubilized in 50 mM of sodium acetate buffers (pH 6.0) and separately incubated at 40°C (●), 45°C (○), 50°C (■), 55°C (□), 60°C (▲), 65°C (Δ) and 70°C (♦) prior to enzyme assay at optimal pH and temperature.

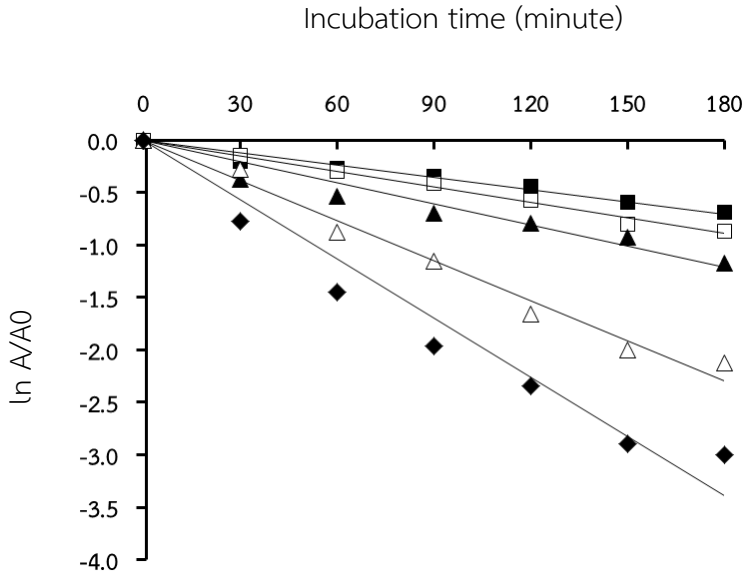

Figure 4.17 First order thermal deactivation of the purified xylanase from *A. pullulans* PBU-109 at 50°C (■), 55°C (□), 60°C (▲), 65°C (Δ) and 70°C (♦).

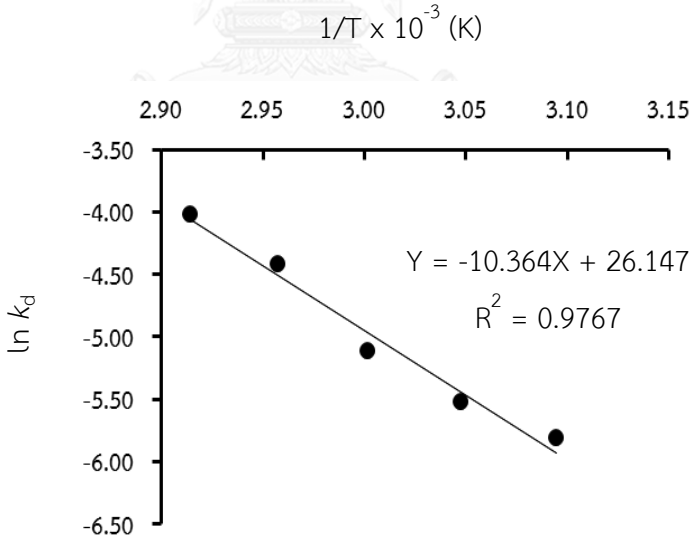

Figure 4.18 The Arrhenius plot for the calculation of activation energy (E_d) for thermal denaturation of the purified xylanase from *A. pullulans* PBU-109.

Table 4.8 Thermodynamic parameters for the irreversible thermal inactivation of the purified xylanase from *A. pullulans* PBU-109 at different temperatures.

Temperature (°C)	k_d* (1/min)	$t_{1/2}$ (min)	D_t (min)	ΔH° (kJ/mol)	ΔG° (kJ/mol)	ΔS° (J/mol.K)
50	0.003	231	768	83.45	105.96	-0.070
55	0.004	173	576	83.40	106.85	-0.071
60	0.006	116	384	83.36	107.40	-0.072
65	0.012	58	192	83.32	107.10	-0.070
70	0.018	39	128	83.28	107.57	-0.071

k_d = denaturation rate constants, $t_{1/2}$ = half-life of the xylanase, D_t = decimal reduction time, E_d = 86.13 kJ/mol, ΔH° = Variations in enthalpy; ΔG° = Variations in free energy; ΔS° = Variations in entropy in free energy; ΔS° = Variations in entropy.

4.5.3 Effect of polyols on xylanase thermostability

To improve the thermostability of the purified xylanase from *A. pullulans* PBU-109, five different polyols were tested for their ability to prolong the enzyme activity at 70°C. When each polyol was separately added to the enzyme solution, a significant ($P<0.05$) protection against thermal denaturation was observed in comparison to that of the control (Figure 4.16). However, differences in the degree of protection were also evident. The highest protective effect was provided by sorbitol which resulted in 64.2% retention of the initial enzyme activity after 180 min at 70°C, some 14.5-fold higher than that of the enzyme without polyol addition. Xylitol and ethylene glycol gave the next highest protection, but respectively some 1.2- and 1.3- fold less effective than sorbitol. Mannitol and glycerol were far less effective protectants (2.3- and 5.1-fold less effective than sorbitol, respectively). Sorbitol was clearly the best thermoprotectant at 0.5 M, therefore the effect of sorbitol concentration on the thermostability of the purified xylanase was further evaluated. Increasing the sorbitol concentration up to 0.75 M further improved the thermostability of the purified xylanase with ~80% of the original

activity remaining after 180 min at 70°C (Fig. 4.17). No further significant change in the xylanase activity was observed at 1.0 M sorbitol.

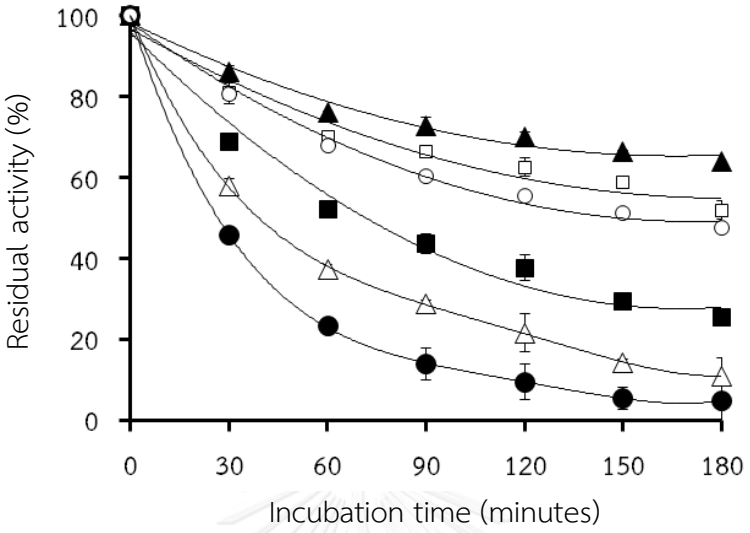

Figure 4.19 The effect of polyols on thermostability of the purified xylanase from *A. pullulans* PBU-109. The enzyme solutions were preincubated at 70°C without the substrate for various periods with the presence of glycerol (Δ), sorbitol (▲), mannitol (■), xylitol (□) and ethylene glycol (○) at a concentration of 0.5 M prior to enzyme assay at the optimal condition. No polyol was added in the control experiment (●).

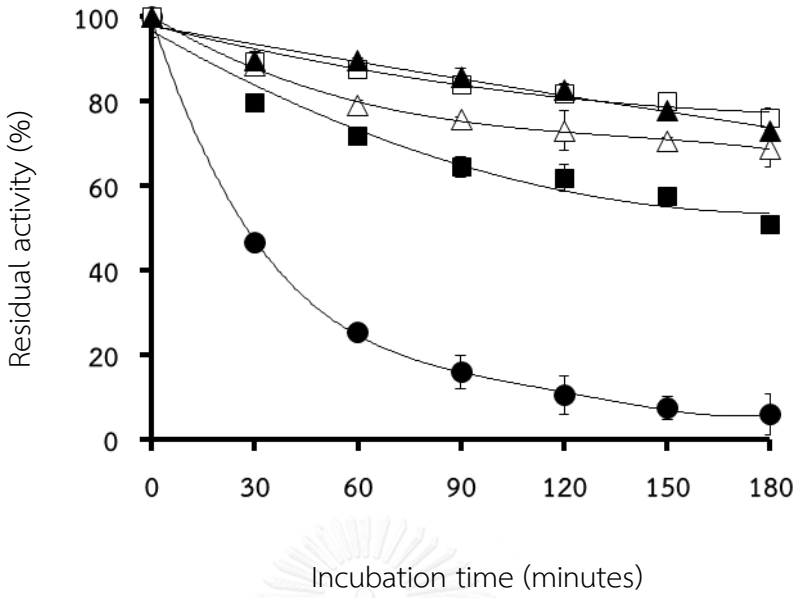

Figure 4.20 The effect of sorbitol concentration on thermostability of the purified xylanase from *A. pullulans* PBU-109. The enzyme solutions were preincubated at 70°C without the substrate for various periods with the presence of sorbitol at concentrations of 0.25 M (■), 0.50 M (Δ), 0.75 M (▲) and 1.00 M (□) prior to enzyme assay at the optimal conditions. No sorbitol was added in the control experiment (●).

The thermostability and thermodynamic profiles of the purified xylanase in the presence of 0.75 M sorbitol were also assessed at 70-90°C and pH 6.0. The purified enzyme plus 0.75 M sorbitol was found to be relatively stable at 70°C with more than 80% of its original activity remaining after 3 h incubation compared to less than 20 % of the control (without sorbital) (Fig. 4.19-4.20). However, the enzyme quickly lost more than half of its activity at 90°C within the first 30 min. The plots of the residual activity of purified xylanase with 0.75 M sorbitol versus incubation time were linear, with R^2 >0.95, indicating that the inactivation could be expressed as first order kinetics (Fig 4.21). Thermodynamic parameters of the purified enzyme in the presence of 0.75 M sorbitol were summarized in Table 4.9. The activation energy for irreversible inactivation (E_d) of the xylanase was determined to be 121.8 kJ/mol from the Arrhenius plot (Fig. 4.22). It was found to increase in 1.4-fold higher than that of the enzyme without sorbitol addition. At 70°C, the value of k_d was found to decrease (9-fold) while the half-life of

xylanase, ΔH and D_t values tended to increase in 10-fold when compared with the purified xylanase alone. The Gibbs free energy (ΔG) for the thermal unfolding decreased from 114.25 to 113.82 kJ/mol with increasing temperature from 70 to 90°C (Table 4.9). The value of ΔG was observed in positive value indicating the absence of enzyme aggregation.

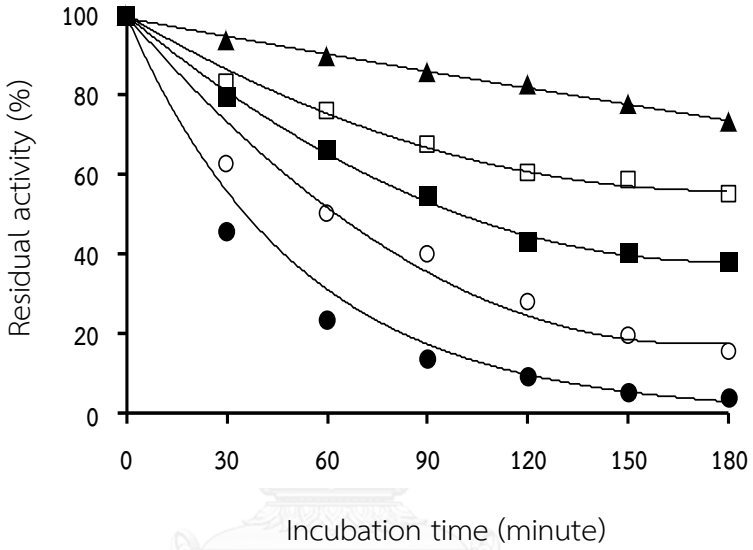

Figure 4.21 Thermostability profile of the purified xylanase in the presence of 0.75 M sorbitol. The purified enzyme was solubilized in 50 mM of sodium acetate buffers (pH 6.0) and separately incubated at 70°C (▲), 75°C (□), 80°C (■), 85°C (○) and 90°C (●) prior to enzyme assay at optimal pH and temperature.

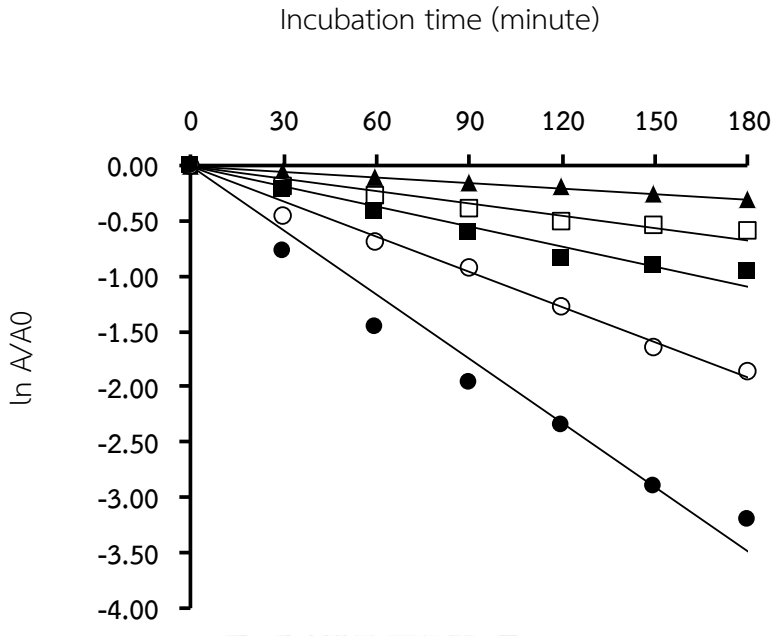

Figure 4.22 First order thermal deactivation of the purified xylanase in the presence of 0.75 M sorbitol at 70°C (▲), 75°C (□), 80°C (■), 85°C (○) and 90°C (●).

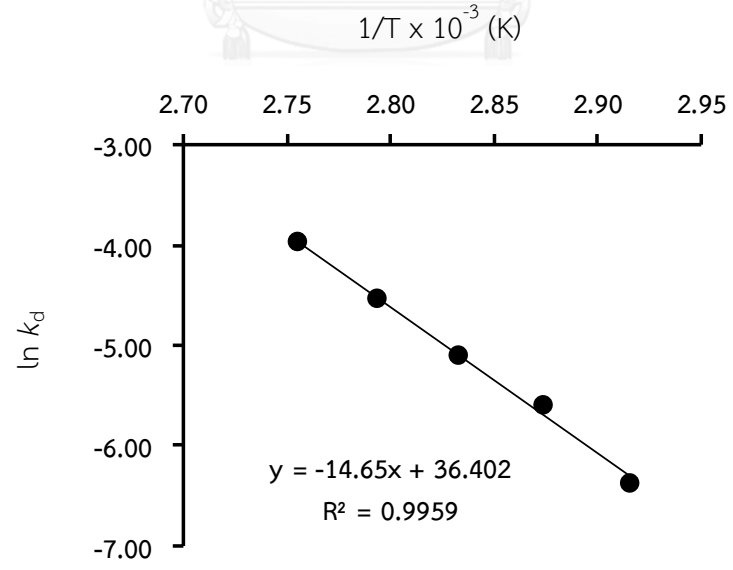

Figure 4.23 The Arrhenius plot for the calculation of activation energy (E_d) for thermal denaturation of the purified xylanase in the presence of 0.75 M sorbitol.

Table 4.9 Thermodynamic parameters for the irreversible thermal inactivation of the purified xylanase in the presence of 0.75 M sorbitol at different temperatures.

Temperature (℃)	k_d* (1/min)	$t_{1/2}$ (min)	D_t (min)	ΔH° (kJ/mol)	ΔG° (kJ/mol)	ΔS° (J/mol.K)
70	0.002	408	1,355	119.75	114.25	0.0160
75	0.004	187	622	119.71	113.71	0.0162
80	0.006	114	378	119.67	113.92	0.0163
85	0.011	65	215	119.62	113.90	0.0160
90	0.019	37	122	119.58	113.82	0.0159

k_d = denaturation rate constants, $t_{1/2}$ = half-life of the xylanase, D_t = decimal reduction time, E_d = 121.80 kJ/mol, ΔH° = Variations in enthalpy; ΔG° = Variations in free energy; ΔS° = Variations in entropy in free energy; ΔS° = Variations in entropy.

4.5.4 Effects of ions

The effects of EDTA and six cations commonly found in pulp on the xylanase activity are summarized in Table 4.10. At 1 mM, none of the metal ions significantly affected the enzyme, except Ca^{2+}. The presence of Ca^{2+} was equally stimulating at 1 and 10 mM, whilst Co^{2+} and Mg^{2+} significantly enhanced the xylanase activity only at 10 mM. The treatment of the purified xylanase with EDTA and Zn^{2+} did not significantly decrease the xylanase activity. The present of Fe^{2+} and Cu^{2+} showed a dose-dependent inhibitory effect against the enzyme, which was significant at 10 mM.

Table 4.10 Effects of different cations with the purified xylanase from *A. pullulans* PBU-109.

Additives	Relative enzyme activity (%)* at an additive concentration of:	
	1 mM	10 mM
Untreated	100.00 ± 2.01^{abc,NS}	100.00 ± 1.04^{C,NS}
$FeSO_4$	99.93 ± 4.47^{abc,2}	74.07 ± 1.19^{B,1}
$MgCl_2$,	105.86 ± 3.03^{c,1}	111.67 ± 5.07^{E,2}
$CaCl_2$	112.43 ± 1.45^{d,NS}	112.37 ± 2.84^{E,NS}
$CuCl_2$	95.48 ± 2.16^{a,2}	29.85 ± 2.68^{A,1}
$CoCl_2$	103.41 ± 4.10^{bc,1}	106.10 ± 3.54^{D,2}
$ZnCl_2$	99.20 ± 2.50^{ab,NS}	98.54 ± 4.23^{C,NS}
EDTA	100.93 ± 5.32^{abc,NS}	99.60 ± 4.23^{C,NS}

* Different superscript letter in the same column indicated that the values were significantly different (ANOVA and DMRT, $P < 0.05$) and different superscript number in the same row indicated that the values were significantly different (Student's t-test, $P < 0.05$). NS = not significantly different.

4.5.5 Substrate specificity and kinetic parameters

The purified xylanase showed different degree of specificity towards xylans from different sources (Table 4.11). In contrast, no activity was observed when α-cellulose, filter paper, CMC or p-Nitrophenyl-β-D-xylopyranoside was used as substrate. Among them, the highest activity was observed with 1% (w/v) beech wood xylan (4.10 ± 0.1 U/ml), followed by oat spelt xylan (3.87 ± 0.23 U/ml) and rice straw xylan (3.48 ± 0.12 U/ml), respectively (Table 4.11).

Table 4.11 Effect of substrates on activity of purified xylanase from *A. pullulans* PBU-109.

Substrate	Xylanase activity (U/ml)
Beechwood xylan*	7.10 ± 0.12[a]
Oat spelt xylan	6.87 ± 0.23[a]
Rice straw xylan	6.48 ± 0.12[b]
p-nitrophenyl-β-D-xylopyranoside	Not detectable
α-cellulose	Not detectable
Filter paper	Not detectable
Carboxymethylcellulose	Not detectable

*From Sigma-Aldrich Inc., USA. Different superscript letter in the same column indicated that the values were significantly different (ANOVA and DMRT, $P < 0.05$).

The effect of concentration of different substrates on xylanase activity was found to be similar with slightly different rate of reaction under optimal pH and temperature (Fig. 4.23). The xylanase activity exponentially increased until reaching the limiting rate at 1 mg/ml of substrate concentration in all xylans. At this point, the purified xylanase was saturated with the substrate and further increase in the substrate concentration resulted in a minimal change in the reaction rate. The determination coefficients (R^2) of the linear plots (Fig 4.24) were calculated to be in a range of 0.98 to 0.99 for substrate concentrations ranging from 0.1 to 1.0 mg/ml which indicated that the model could be expressed as first order kinetics. The derived *K*m and *V*max values of the purified *A. pullulans* PBU-109 were determined by the slope and y intercept of Lineweaver-Burk plot and shown in Table 4.12. The highest value of the maximum rate of reaction (Vmax) was found with beechwood xylan following with oat spelt xylan and rice straw xylan. The affinity of enzyme was not different.

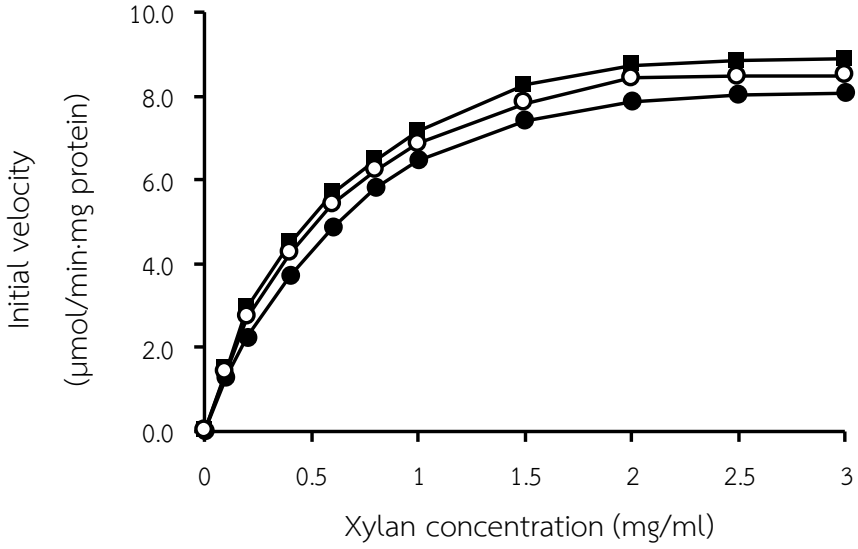

Figure 4.24 Effect of xylan from beechwood (■), oat spelt (○) and rice straw (●) at various concentrations on enzyme activity of purified xylanase from *A. pullulans* PBU-109.

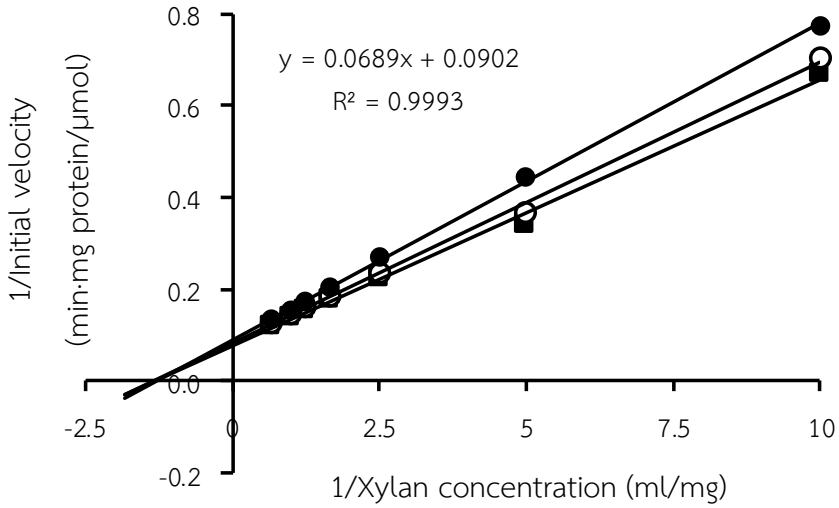

Figure 4.25 Lineweaver-Burk plot for kinetic parameters estimation in xylan from beechwood (■), oat spelt (○) and rice straw (●) of purified xylanase from *A. pullulans* PBU-109.

Table 4.12 The kinetic parameters of the purified xylanase of from *A. pullulans* PBU-109.

Substrate	*V*max (μmol/min/mg)	*K*m (mg/ml)	Linear equation	R^2
Beech wood xylan	12.82	0.741	Y = 0.0578X + 0.0780	0.9956
Oat spelt xylan	12.25	0.751	Y = 0.0816X + 0.0816	0.9977
Rice straw xylan	11.09	0.763	Y = 0.0689X + 0.0902	0.9993

4.6 Application of *A. pullulans* PBU109 xylanase for prebleaching of rice straw pulp

4.6.1 Pulping of rice straw

Before carrying out the pulping experiment, chemical analysis of the rice straw was performed in accordance with Goering and Van Soest methods (1970). Composition of the rice straw was found to be 39.20 ± 2.20% cellulose, 31.10 ± 1.76% hemicellulose and 12.50 ± 1.20% lignin. After soda pulping, the components of pulps were found to be 68.20 ± 1.68% cellulose, 27.35 ± 0.96% hemicellulose and 8.27 ± 1.04% lignin. The residual alkali charge was 0.16 ± 0.04 g/L in black liquor. The pulp yield, rejects of pulps and Kappa number were 40.52 ± 0.49%, 0.07 ± 0.00% and 3.56 ± 0.17, respectively. The appearance of rice straw after pulping was more fibrillate, soft, flexible and darker in color than the original material (Fig. 4.25).

A B

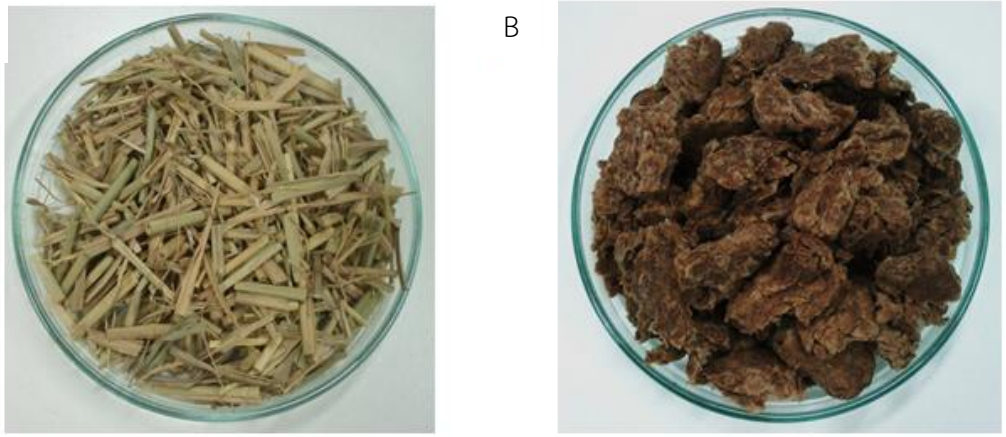

Figure 4.26 Appearance of rice straw before (A) and after (B) 15% NaOH pulping at 120°C for 2 hr.

4.6.2 Property of hand sheets after bleaching process

Paper sheets made from the bleached pulp with and without pretreatment by the crude xylanase enzyme were presented in Figure 4.26. Reducing sugar contents in the hydrolysates after xylanase pretreatment of pulp with and without 0.75 M sorbitol were 41.1 ± 1.3 μmole and 46.6 ± 3.05 μmole, respectively. A significant increase in pulp brightness was observed with the rice straw pulp pretreated with either xylanase or xylanase with 0.75 M sorbitol before bleaching, compared to those of the pulp bleached with H_2O_2 alone and unbleached pulp (Table 4.13). The paper strength, in terms of the tear indexes, was found to be significantly increased along with the curl and kink indexes of the fibers after xylanase pretreatment (Table 4.14). Rice straw pulp treated with xylanase and 0.75 M sorbitol presented higher values of kink and curl indexes, compared with pulp treated with xylanase alone while the tensile and tear indexes were not significant. From the observation of fiber morphology, no significant changes were noticed in the fiber length while the fines content was significantly decreased by the xylanase pretreatment of the pulp.

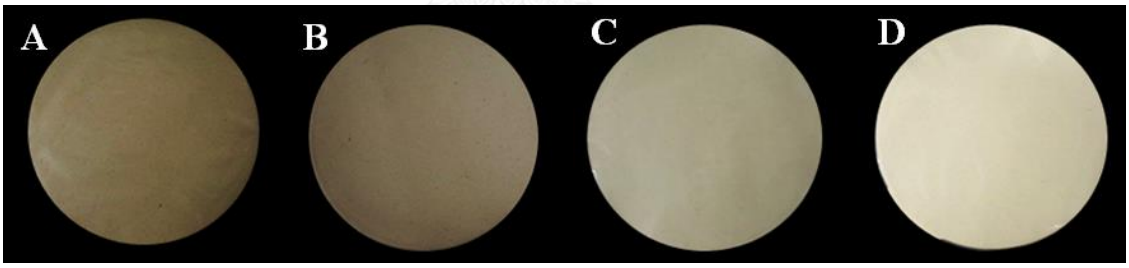

Figure 4.27 Paper sheets made from rice straw pulp from different treatments. A: control without xylanase pretreatment and chemical bleaching, B: no xylanase pretreatment before H_2O_2 bleaching, C: xylanase pretreatment before H_2O_2 bleaching, D: xylanase with 0.75 M sorbitol pretreatment before H_2O_2 bleaching.

Table 4.13 Effects of xylanase pretreatment on optical properties of the paper sheets made from rice straw pulps.

Treatment	Brightness (ISO Units)*
Untreated	39.5 ± 0.10^{a}
Xylanase	40.3 ± 1.00^{a}
Sorbitol	38.7 ± 0.67^{a}
H_2O_2	55.5 ± 1.00^{b}
Xylanase + H_2O_2	62.8 ± 0.40^{c}
Xylanase + Sorbitol + H_2O_2	63.0 ± 0.58^{c}

* Different superscript letter in the same column indicated that the values were significantly different (ANOVA and DMRT, $P < 0.05$).

Table 4.14 Effects of xylanase pretreatment on the strength of the paper sheets made from rice straw pulps.

Treatment	Tensile index ($N.m\ g^{-1}$)	Tear index* ($mN.m^2\ g^{-1}$)
Untreated	39.8 ± 5.90^{A}	7.68 ± 0.50^{a}
Xylanase	40.2 ± 3.76^{A}	7.36 ± 0.37^{a}
Sorbitol	39.6 ± 2.87^{A}	7.20 ± 0.59^{a}
H_2O_2	35.8 ± 7.00^{A}	6.56 ± 0.61^{a}
Xylanase + H_2O_2	42.4 ± 3.90^{A}	12.2 ± 0.75^{b}
Xylanase + Sorbitol + H_2O_2	46.3 ± 4.90^{A}	13.1 ± 1.30^{b}

* Different superscript letter in the same column indicated that the values were significantly different (ANOVA and DMRT, $P < 0.05$).

Table 4.15 Effects of xylanase pretreatment on fiber morphology of the rice straw pulps.

Treatment	Fiber length (mm)	Fines content (%)	Fiber curl index	Fiber kink index
Untreated	0.93 ± 0.05^{A}	49.3 ± 0.34^{a}	0.07 ± 0.002^{A}	1.48 ± 0.04^{b}
Xylanase	0.89 ± 0.04^{A}	48.4 ± 0.36^{a}	0.07 ± 0.004^{A}	1.49 ± 0.03^{b}
Sorbitol	0.90 ± 0.09^{A}	49.5 ± 0.74^{a}	0.06 ± 0.008^{A}	1.47 ± 0.07^{b}
H_2O_2	1.01 ± 0.05^{A}	45.2 ± 0.14^{b}	0.07 ± 0.003^{A}	1.42 ± 0.03^{a}
Xylanase + H_2O_2	0.93 ± 0.05^{A}	40.6 ± 0.90^{c}	0.09 ± 0.002^{B}	1.59 ± 0.02^{c}
Xylanase + Sorbitol + H_2O_2	1.00 ± 0.16^{A}	39.7 ± 0.30^{c}	0.10 ± 0.008^{C}	1.65 ± 0.02^{d}

* Different superscript letter in the same column indicated that the values were significantly different (ANOVA and DMRT, $P < 0.05$).

CHAPTER V
DISCUSSION AND CONCLUSION

5.1 Screening for thermophilic xylanase producing isolates

Microbial xylanases have shown their potential in various industrial applications, especially the pulp and paper production (Viikari et al., 2007). Xylanases are often used in bleaching process to reduce the consumption of chlorine chemicals and contamination of toxic derivatives in effluent (Suurnakki et al., 1997). In a continuous pulp production process, xylanases active at high temperature (≥ 70°C) are particularly desired for the thermal pulping and bleaching steps (Beg et al., 2001). Consequently, a search for potential microbial thermophilic xylanases has been conducted extensively around the world. Although bacterial xylanases have been shown to possess diverse properties, but most of them are intracellular enzymes and produced in a lower yield compared to fungal xylanases (Polizeli et al., 2005). Among the xylanase-producing fungi, *Aureobasidium pullulans* has been studied by many researchers due to its cellulase-free property suitable for the pulp and paper industry as the enzyme will not cause any damage on the cellulose fibers. However, only a few strains have been reported to produce thermophilic xylanase (Leathers, 1986; Leathers et al., 1984). Moreover, even though *A. pullulans* is a ubiquitous species, the cellulase-free xylanase was produced mostly in color variant strains that have been isolated only from tropical or subtropical latitudes (Leathers et al., 1984; Wickerham & Kurtzman, 1975). From the report of Manitchotpisit et al. (2009) Thailand is an apparent source of color variant strains of *A. pullulans*. Therefore, 25 Thai isolates with high xylanase production of *A. pullulans* (Manitchotpisit et al., 2009; Rotjanagusol, 2010) from the Plant Biomass Utilization Research Unit, Department of Botany, Chulalongkorn University, were selected for screening of the thermophilic xylanase activity in this study. *A. pullulans* NRRL Y-2311-1 was included as a reference strain since it has been used for commercial xylanase production although its enzyme was unstable at high temperature (Leathers, 1986). Since thermophilic enzymes are defined as those catalyze at an optimum temperature between 60 and 80 °C (Vieille et al., 2001), the screening experiment was

conducted at two temperatures, the standard assay temperature (50 ºC) and the thermophilic assay temperature (70 ºC). Based on the xylanase assay at 50ºC, notably high xylanase producers were the strains (6.83 ± 0.50 U/ml), PBU 102 (7.13 ± 0.36 U/ml), CU 23 (7.42 ± 0.13 U/ml), CU 36 (7.44 ± 0.37 U/ml), CU 41 (7.33 ± 0.10 U/ml) and CU 42 (7.37 ± 0.08 U/ml) when compared with the reference strain NRRL Y-2311-1 (6.63 ± 0.23 U/ml). However, when assay at 70ºC, a significant drop in xylanase activity was found in almost all strains except one, PBU 109, that its xylanase activity was slightly increase. It could be concluded that the crude xylanase from *A. pullulans* PBU 109 was thermophilic and thus this strain was selected for further study.

5.2 Xylanase production from xylan-containing biomasses

For a production of an industrial enzyme, the cost of substrate and product yield are crucial in a commercial perspective. In general lab-scale studies, pure xylans from beech wood or birch wood have been widely used for xylanase production (Gübitz & Walter, 1996; Raj et al., 2013). However, such purified xylans are expensive and therefore not suitable for a large-scale commercial production. Since xylan is present in all plant biomass as a part of major cell wall components (i.e. hemicellulose), any cellulosic biomass can theoretically be used as an inducer for xylanase production. In this study, five xylan-containing biomasses were screened to find an appropriated substrate for the xylanase production by *A. pullulans* PBU 109. In addition to nutrient- and xylan-rich wheat germ and wheat bran (Vieille et al., 2001), two most abundant agricultural wastes in Thailand such as corncob and rice straw were selected in this study (Boonmee, 2012). These agricultural residues are produced up to more than 2-20 million tons per year while most of them are not used for any commercial purpose and become problematic in disposal (Binod et al., 2010). Similarly, the uncontrolled dispersal of water hyacinth is a serious ecological problem in Thailand in term of invasive plant species, aquatic biodiversity threat and water pollution (Chu et al., 2006). This rapidly growing plant species can double its biomass within a few weeks (Agarwal, 2005) and therefore makes it an endless source of xylan. Utilization of these biomasses not only provides an alternative disposal method but also adds a value to them.

According to the component analysis, all selected biomasses contain hemicellulose more than 20 % of their dry weight suggesting that they could be used to induce xylanase production. The highest xylanase production was found in the medium containing 1% (w/v) corncob, following by those contain wheat germ, wheat bran, rice straw and water hyacinth, respectively. The enhancing effect of corn cob on xylanases production was also reported in another yeast-like fungus, *Thermomyces lanuginosus* (Singh et al., 2000a). The differences in xylanase production when using different biomasses could be caused by their varying contents of hemicellulose and lignin. The highest amount of hemicellulose was found in corncob of which lignin content was the lowest. Although the hemicellulose content was higher in wheat bran than that of wheat germ, the xylanase activity is higher in the medium containing wheat germ. It was possibly due to the high lignin content in wheat bran that adversely affected the degradation and utilization of hemicellulose hence the enzyme production was less enhanced (Rakotoarivonina et al., 2012). Similar situation was found in case of rice straw (1.64±0.13 U/ml) and water hyacinth (1.92±0.13 U/ml) that the hemicellulose contents in theses biomasses were not significantly different, but the lignin content was higher in rice straw. Even though the xylanase production from corncob (4.10 ±0.10 U/ml) was 1.76-fold lower than that obtained from commercial beech wood xylan (7.23±0.15 U/ml), corncob was practically more attractive due to its abundance, extremely low cost, and availability of other organic nutrients such as amino acids and reducing sugars (Benedetti et al., 2013). To improve the yield of xylanase production from corncob, optimization of the production medium employing statistical analysis was conducted in the following experiments.

5.3 Medium optimization for xylanase production

5.3.1 Screening of nutrient compositions using a Plackett–Burman design

Plackett-Burman design was used in this study to statistically select medium components positively affecting the xylanase production by *A. pullulans* PBU 109 when corncob was used as the main carbon substrate and inducer (Goyal et al., 2008; Zhang et al., 2013). As xylanases are inducible enzymes, the study on their

inducers and repressors was necessary to develop an optimal xylanase production medium. The generally known inducers of xylanase production are xylan and its short fragments, such as xylooligo-saccharides (Kulkarni et al., 1999; Li et al., 2006). Such long and short chains of xylan are present in corncob (Dumitriu, 2012) However, the effect of other inducers, especially monosaccharides such as glucose and xylose, on xylanase production is still not clear. Glucose reportedly acted as an inducer for xylanase production in *Penicillium purpurogenum* (Ravanal et al., 2012), or *Trichoderma reesei* (Kurzatkowski et al., 1996), as well as a weak inducer in *Aspergillus nidulans* biA1 (Orejas et al., 2001) and *A. pullulans* Y-2311-1 (Li & Ljungdahl, 1994). For xylose, it served as a strong inducer for xylanase production in *Aureobasidium pullulans* CBS 58475 (Dobberstein & Emeis, 1989) and *A. pullulans* NCIM 1050 (Karni et al., 1993). Therefore, both glucose and xylose were included in the experimental design to investigate their effects on the xylanase production of *A. pullulans* PBU 109.

The effect of nitrogen sources on the xylanase production was observed with the different organic (yeast extract, peptone and urea) and inorganic nutrients ($(NH_4)_2SO_4$). Peptone and yeast extract are complex organic nitrogen containing various growth factors which have been reported to enhance the growth and enzyme production in several fungi including *Penicillium canescens* 10-10c (Bakri et al., 2003), *Cochliobolus sativus* Cs6 (Bakri et al., 2008), *Trichoderma viride*-IR05 (Irfan et al., 2014), and *Candida xylanilytica* (Boonmak et al., 2011). The addition of urea to production medium was reportedly boosted xylanase production in *Melanocarpus albomyces* comparing with other nitrogen sources including peptone, $(NH_4)_2SO_4$, or $NaNO_3$ (Gupta et al., 2013). On the other hand, inorganic nitrogen such as $(NH_4)_2SO_4$ have also reported to play an important role in enhancing the xylanase production. Ammonium sulfate was also incorporated in the medium to enhance the xylanase production in *Geobacillus stearothermophilus* KIBGE-IB29 (Bibi et al., 2014). In addition, the highest level of xylanase has been obtained when *A. pullulans* SN090 was cultivated in submerged medium using wheat bran and $(NH_4)_2SO_4$ as carbon and nitrogen sources, respectively (Nasr et al., 2013).

Mineral salts are well known as essential elements in cell growth and enzyme synthesis that play an important role in maintaining the cellular osmotic

pressure and as co-factors of metabolite enzymes (Bibi et al., 2014) The influence of different salts on xylanase production was examined in several studies. The presence of $MgSO_4$, $CaCl_2$ and KH_2PO_4 have been reported to enhance biocompound production in certain strains of *A. pullulans*, (Gostinčar et al., 2014; Lotrakul et al., 2009) but the effect of these salts on xylanase production was still not investigated.

In addition to carbon, nitrogen and minerals, certain surfactants were reported to enhance xylanase production. The supplementation of tween 80 in the production medium of *A. pullulans* NCIM 1050 resulted in 1.2-fold increase of xylanase production. Therefore, a total of 11 variable components, including a corncob, xylose, glucose, urea, $(NH_4)_2SO_4$, yeast extract, peptone, $MgSO_4$, $CaCl_2$, KH_2PO_4 and tween 80, were analyzed in this experiment.

Out of the 11 medium components tested, five factors comprising of xylose, corncob, $(NH_4)_2SO_4$, KH_2PO_4, and tween 80 were found to significantly enhance the overall xylanase production with a maximum yield of 6.51 U/ml. Almost all organic and inorganic nitrogen sources, except urea, could induce xylanase production although in different rate. Although urea was considered as an utilizable nitrogen source in fungi, but it might be affected with the xylanase activity in medium by cleavage hydrogen bond and lead to the change of tertiary structure of enzyme (Rajagopalan et al., 1961). The low rate of xylanase production was found in organic sources because high concentration of yeast extract or peptone acted as a good inducer for proteases and leading to the increased degradation of other enzymes (Subramaniyan, 2002). The strong inhibition of xylanase production by *A. pullulans* PBU 109 was found in the presence of glucose. Similar result was observed in *T. reesei* and it was suggested that glucose caused the repression of xylanase encoding gene due to the cross talk between cellulase/xylanase regulatory pathways (Aro et al., 2003). The shared-domain of carbon catabolic repressor make it be able to regulate both cellulase and xylanase gene cassettes, therefore, glucose acted as a strong repressor for xylanase in this case (Aro et al., 2003; Margolles-clark et al., 1997). According to the report, it might be conclude that xylanase production from *A. pullulans* PBU 109 was inhibited by glucose due to the catabolic repression. In the case of xylose, the induction of xylanase synthesis was depended on its concentration. Xylanase production from *T. reesei* was found to be

induced by xylose at an optimal concentration ranging from 0.5 to 1 mM while the repression of xylanase gene was observed at a xylose concentration higher than 10 mM (Mach-Aigner et al., 2010). In this experiment, xylose was added to the medium at concentrations less than 10 mM; therefore the enhancement of xylanase production was observed as expected. The positive effect was also found in the presence of tween 80, a non-ionic surfactant, which was probably due to the increase in permeability of cell membrane that caused a rapid secretion of the enzymes (Ahlawat et al., 2007). The mechanism that influences to increase the fungal cell permeability by tween 80 is still not clear. However, it has been purposed that the changes of cell membrane permeability cause by the degradation and conversion of tween 80 into polyoxyethylenic acids at cellular compartment called the periplasmic space in fungal cell. This intermediate was used to synthesis novel series of glycolipids at cell wall and leaded to increase the permeability of cell wall (Urek & Pazarlioglu, 2007; Zheng & Obbard, 2001). The presence of KH_2PO_4 was found to significantly increased xylanase production from *A. pullulans* PBU-109 while MgSO4 and $CaCl_2$ were not. It was probably due to the effect of phosphate in the culture medium that controlled the synthesis of metabolites including deoxyribonucleic acid (DNA), adenosine triphosphate (ATP) and ribonucleic acids (RNA) (Lounes et al., 1996; Singh & Chhatpar). These metabolites play an important role in cellular respiration, control of ATP levels, carbohydrate metabolism and the synthesis of protein including xylanase enzyme.

With this experimental design, the role of each medium component on *A. pullulans* PB109 xylanase production was obtained and it was confirmed by the coefficient of determination (R^2 = 0.967) for the Plackett–Burman model and the significant probability value for regression model [(prob > F) <0.05]. Xylanase activity from the composition-adjusted medium in this experiment was found to increase about 1.6-fold (6.51 ± 0.23 U/ml) comparing to the unmodified medium containing 1% (w/v) corncob (4.10 ± 0.10 U/ml). However, the xylanase activity obtained from this new medium was still slightly lower than that from the original production medium containing commercial beech wood xylan (7.23 ± 0.15 U/ml). Therefore, the appropriated concentrations of all five positive factors were optimized in the following experiments.

5.3.2 Optimization of nutrient concentration using Response Surface Methodology

After the critical medium components have been identified via Plackett–Burman model, the optimal concentration of these five components including xylose, corncob, $(NH_4)_2SO_4$, KH_2PO_4 and tween 80 were determined by employing the Box-Behnken design experiment with a proposed three-level design for fitting response surfaces in the 43-run experiments. The maximum xylanase production was found to be 8.74±0.84 U/ml which was very close to the predicted value (8.82±0.21 U/ml). The enzyme yield increased approximately 2-fold when compared to the unmodified medium (4.10±0.10 U/ml). The model for xylanase production in this study was significant based on the *F*-value (151.97) and the model terms value of Prob > *F* less than 0.0001. The goodness of fit of the model was also checked by determination coefficient (R^2) that was calculated to be 0.9928. The adjusted determination coefficient (adjusted R^2 = 0.9863) and predicted determination coefficient (R^2 = 0.9730) also confirmed the significance of the model. A lower value of coefficient of variation (CV = 3.63%) indicated a greater preciseness and reliability of experiment performed. Comparison of predicted and experimental values revealed good correspondence between them. In this case, the model showed insignificance in lack of fit (p =0.7724). It indicated second-order model equation was adequate for the prediction of xylanase production across the specified range of variables employed. Thus, the estimated model fits the experimental data adequately.

In this experiment, the linear effects (A_1, A_2, A_3, A_4 and A_5), the interactive effects (A_1A_2, A_1A_3, A_1A_4 and A_1A_5) and square effects (A_1^2, A_2^2, A_3^2, A_4^2 and A_5^2) were significant model terms ($P< 0.05$) for xylanase production (B). In order to get a better understanding of the effects of the variables on xylanase production by *A. pullulans* PBU-109, the predicted model was further assessed using RSM. In this study, the contour plots affirmed that the objective function was unimodal which show an optimum at the boundaries. The canonical analysis revealed a maximum xylanase activity of 8.74±0.84 U/ml under the optimal conditions of corncob, 39.0 g/L; $(NH_4)_2SO_4$, 3.0 g/L; xylose, 1.8 g/L; KH_2PO_4 1.4 g/L and tween 80, 1.4 g/L. The synergistic effect of selected variables corresponded to 1.34 and 2.13-fold increase in xylanase activity compared to their individual effects from Plackett–Burman model and original medium, respectively. From counterplots, it showed that xylanase production tended to increase with gradually increasing value in corncob concentration. The optimal concentration of corncob (3.9 %

(w/v)) obtained in this study fell in the same range (1% to 4% (w/v)) reported for other strains of *A. pullulans* (Chen et al., 2014; Nasr et al., 2013), *Aspergillus terricola* (Michelin et al., 2012) and *A.niger* (Jin et al., 1997). It was important to note that xylanase production from this experiment was 1.2-fold higher than that from the unmodified medium containing commercial beechwood xylan. Validation experiment was also carried out with three replications to verify the accuracy of the models, and the results showed that the predicted values agreed well with the experimental values (8.70±0.66 U/ml). It implied that empirical models derived from RSM can be used to adequately describe the relationship between the factors and response in xylanase production by *A. pullulans* PBU-109.

Two parallel experiments were conducted to study the pattern of cell growth and xylanase production in the optimized and original media (basal medium containing 1% (w/v) corncob as in 3.3.3 under the same condition. The results indicated that, during the first 72 hours, the growth patterns and growth rates were similar in both cultures. The cell density in the optimized medium was slightly lower than original medium throughout the study period. Although the cell density of *A. pullulans* PBU-109 was lower in optimized medium, the xylanase production was strongly increased. The maximal activity (10.09±0.27 U/ml) was found in the optimized medium after 72 hours of incubation, during the late log phase of the culture. In the original medium, the maximal activity was 4.10±0.10 U/ml after 48 hours of incubation which was 2.5-fold lower than that of the optimized medium. The subsequent decrease in enzyme activity during the stationary phase was generally common for the primary metabolites and has also been noted in xylanase production by other fungi (Shah & Madamwar, 2005). It was probably due to proteolysis of the enzyme and/or the decline of actively growing cells as a result of nutrient depletion (Pal & Khanum, 2011; Shah & Madamwar, 2005). The results also suggested that the appropriate time for harvesting the xylanase enzyme produced by *A. pullulans* PB109 grown in the optimized medium was at 72 hours after inoculation. The xylanase production cost of optimized medium (0.50 THB/10 units) was found to lower than basal medium (18.78 THB/10 units) in 37–fold (Appendix G).

5.4 Purification of endoxylanase

In general, most of the xylanolytic fungi produce multiple xylanases, therefore, several protein purification methods were required. For instance, the 20 kDa xylanase

from *A. pullulans* NRRL Y-2311-1 was purified using ultrafiltration at 10 kDa cut-off, ion exchange (CM-Sephadex) and gel filtration (Sephadex G-75) column chromatography, respectively, to a recovery of 45% of initial (Leathers & Timothy, 1989). Tanaka et al. (2006) observed a novel family 10 xylanase (XynII) from *A. pullulans* var. *melanigenum* ATCC 20524. Ultrafiltration, cation exchange chromatography (CM-Cellulofine C-500) and gel permeation chromatography on a Superdex 75 pg were used to purify. In this experiment, three purification strategies were adopted including ammonium sulfate precipitation, anion exchange chromatography using DEAE-Sepharose followed by gel filtration using Sephacryl S-100. Ammonium sulfate precipitation was employed in the first step to partially eliminate some unwanted proteins and concentrate the enzyme in the crude extract based on salting out property of the protein. In general, proteins of higher molecular weight precipitate in a lower concentration of ammonium sulfate (Cohn et al., 1940). Xylanases from *A. pullulans* PBU-109 in this experiment were mainly precipitated at 40 to 60% saturation of ammonium sulfate while xylanases with the mass around 20-25 kDa from other strains of *A. pullulans* were precipitated in a range of 50 to 80 % saturation of ammonium sulfate (Li & Ljungdahl, 1994). This suggested that the molecular weight of xylanases from *A. pullulans* PBU-109 might be higher than those of the other *A. pullulans.* Salts in the solution are then removed by dialysis through porous cellulose tubing of Vivaflow (10 kDa membrane cut-off). Total protein content in this step was 56.77 % recovery from the crude enzyme and specific activity of the enzyme was 5.48 ± 0.02 U/mg proteins.

To separate a specific protein based on its overall charges, an ion exchange chromatography is commonly used. In this study, an anion exchange column composing of positively charges of the weak anion exchanger (diethyl-aminoethyl groups; DEAE), with dextran-based sepharose as ion exchange matrix was used. The elution of bound proteins was performed by increasing the ionic strength of 20 mM tris-HCl buffer (pH 8.0) with linear gradient of NaCl from 0 to 1 M. Two peaks of proteins with xylanase activity were detected in fraction number 6-9 and 16-20, respectively. Such two or more xylanases have been previously reported in the color variant *A. pullulans* NRRL Y-2311-1 (Li et al. 1993) and *A. pullulans* ATCC 20504 (Tanaka et al., 2006). The first peak of xylanase (xylanase I) was not bound to the column suggesting its positive or neutral-charged nature. The second peak (xylanase II), which represented the major portion of the xylanase activity, was bound to the column and eluted by NaCl at the concentration between 0.3 and 0.6 M suggesting that the enzyme was negatively charge in 20 mM

tris-HCl buffer (pH 8.0). The high amount of protein and xylanase activity was concurred in the same fractions, so it was implied that xylanase enzyme was the major protein in these fractions. Proteins from both peaks were separately ultra-filtrated in 50 mM acetate buffer (pH 6.0) to eliminate the salts and assayed for their xylanase activity at 50 and 70°C. Both of them were more active at high temperature while the relative xylanase activity of xylanase II (142.14 ± 1.45 %) was higher than xylanase I (133.76 ± 2.50 %) at 70°C. It could be concluded that xylanase II was more active than xylanase I at high temperature; therefore xylanase II was selected to further purify in following experiment due to the higher activity at high temperature. The enzyme was purified to 16.61 fold with about 17.2% recovery in this step.

The other column using in this experiment was gel filtration chromatography or size-exclusion chromatography. In this step, protein molecules are separated according to their hydrodynamic diameter (Andrews, 1965). The column was packed with chromatographic resin, Sephacryl S-200, which are inert and porous spheres with pore size in range of 50-250 kDa. Proteins larger than the diameter of the pores will flow through the column whereas proteins smaller than the pores will be retained and thus elute later ((de Lima Leite)). Gel filtration of xynII from anion-exchange chromatography was done by elution with 50 mM acetate buffer (pH 6.0) that resulted in a single and only peak of major protein with detectable xylanase activity. Although the single peak of protein from gel filtration column may conceal different proteins of the same size, result from SDS-PAGE analysis confirmed the presence of a single protein with a molecular mass of 73 kDa. Result from a native PAGE with activity staining also supported result of the denature PAGE that it was a monomeric protein with xylanase activity.

The majority of xylanases fall into two glycoside hydrolase families: family-10 xylanases with high molecular masses of greater than 30 kDa and family-11 xylanases with a relatively low molecular masses ranging from 19 to 25 kDa (Henrissat and Bairoch, 1996). Most strains of *A. pullulans* have been reported to produce a family-11xylanase with different molecular masses in the range of 20-30 kDa (Dobberstein & Emeis, 1991; Li & Ljungdahl, 1994) while the molecular masses of the purified xylanase from *A. pullulans* PBU-109 in this study was higher than 30 kDa. Therefore, xylanase in this study might be a novel enzyme that related with the family-10 xylanases. However, amino acid sequence of the purified xylanase from *A. pullulans* PBU-109 must be studied before its GH family can be classified. The overall level of recovery was

approximately 13.6% with a 17.32-fold enrichment and a specific activity of 41.40 ± 2.85 U/mg protein.

5.5 Characterization of the purified xylanase

5.5.1 Optimal conditions for xylanase activity

To determine the optimum condition, the purified xylanase from *A. pullulans* PBU-109 was assayed with 1% (w/v) beechwood xylan over various temperatures and different pHs. The purified xylanase was found to catalyze well in a wide range of pH with more than 70% of the maximal activities in the pH range of 4.0-10.0 and exhibited maximum activity in 50 mM sodium acetate buffers at pH 6.0. In additions, the xylanase activity gradually increased with increasing temperature reached to peak at 70°C similar response was observed at every pH tested. Therefore, the optimum condition for xylanase activity from *A. pullulans* PBU-109 was at 70°C, pH 6.0 which was not different in both purified and crude enzymes. Xylanases that are active in an alkaline environment and at high temperature are uncommon in yeasts (Techapun et al., 2003). Most of yeast and fungal xylanases have been reported to catalyze well in a mildly acid to neutral condition (pH 4.0-7.0) (Georis et al., 2000). Similarly, xylanases from *A. pullulans* in different strains have been reported to active at acidic environment, pH 4.5-4.8 (Leathers & Timothy, 1989; Li et al., 1993), therefore, this study is the first report of alkalo-tolerant xylanase from *A. pullulans*.

Most of xylanases produced by thermophilic fungi are usually more active at high temperature than those of mesophilic fungi (Singh et al., 2000a). In contrast, the results of this study showed that the xylanase from *A. pullulans* PBU-109, mesophilic fungi, was active in the wide range of high temperature from 50°C to 80°C and retained more than 50% of the maximal activities at 90°C at the optimum pH. Depending on the optimal temperature, enzymes can be classified as mesophilic (40-60°C), thermophilic (50-80°C) and hyper-thermophilic (>80°C) (Polizeli et al., 2005), therefore, the *A. pullulans* PBU-109 xylanase was suggested to be a thermophilic enzyme. Alkalo-tolerant and thermophilic xylanases were rarely found to produce from fungi while have been reported in a number of bacteria, including *Bacillus* sp. (Zheng et al., 2000) and *Clostridium absonum* CFR-702 (Rani & Nand, 2000). With its broad optimum pH and thermophilic properties, the xylanase from *A. pullulans* PBU-109 may be a strong candidate for several industrial applications for example pulp bleaching and the bioconversion of lignocellulosic materials.

5.5.2 Thermostability and thermodynamic analysis of purified enzyme

Stability of enzymes, especially during thermal processes, remains an important concern in modern biotechnology. The loss of enzyme activity during exposure to elevated temperatures is related to the significant changes in the enzyme (Cui et al., 2008) that can be estimated according to the Arrhenius equation and thermodynamic parameters (Marangoni, 2003)The purified xylanase was found to be relatively stable up to 45℃ while the half-life of enzyme decreased sharply and were 231, 173, 116, 58 and 39 minutes after incubation at 50℃, 55℃, 60℃, 65℃ and 70℃, respectively. Similarly, the time needed to reduce the xylanase activity by 90%, *D*-value, decrease at higher temperature. At 70℃, half-life of purified xylanase from many thermophilic organisms including *Thermoascus aurantiacus* C436 (Tan et al., 1987), *Thermomyces lanuginosus* DSM 5826 (Lin et al., 1999) and *Bacillus thermantarcticus* (Lama et al., 2004)were longer than 120 minutes. It could be concluded that purified xylanase from *A. pullulans* PBU-109 was the thermophilic enzyme while its activity was unstable at high temperature.

Thermostability corresponds to the capability of an enzyme molecule to resist against thermal unfolding in the absence of substrate, while thermophilicity represents the ability of an enzyme to work at high temperatures in the presence of substrate (Bhatti et al., 2006). Thermal denaturation of enzymes could be occurred in two steps (Iyer & Ananthanarayan, 2008) as shown below:

$$N \leftrightarrow U \longrightarrow D$$

Where, *N* is the native enzyme, *U* is the unfolded inactive enzyme that could be reversibly refolded upon cooling and *D* is the denatured enzyme form after prolonged exposure to heat and therefore cannot be recovered upon cooling. The irreversible refolding of enzyme at high temperature was directly related with the energy of denaturation (E_d). As the temperature of system is increased, some of heat could be converted into chemical potential energy. Increasing of chemical potential energy in greater amount than E_d is accompanied by the breakage of weak interactions, including ionic effects, hydrogen bonds, and hydrophobic interactions, which are prime determinants of protein tertiary structures (Wang et al., 2010). The intermolecular aggregation among unfolded proteins or the incorrect structure formation was occurred upon the exposure of the hydrophobic surfaces of enzyme, and this phenomenon

became the major problem because of the irreversible inactivation (Lumry & Eyring, 1954). E_d was determined by applying the Arrhenius plots and was found to be 86.1kJ/mole in this study. This value is in a range estimated for many microbial enzymes, 67–293 kJ/mole (Tari et al., 2008) that was higher than a number of other reported xylanase, such as a purified xylanase from *Aspergillus niger* DFR-5 with E_d of 63.76 kJ/mole (Pal & Khanum, 2010) and *Penicillium occitanis* with E_d of 79.27 kJ/mole (Driss et al., 2011). The higher value of E_d referred to lower trend of enzyme denaturation at the same condition (Saqib et al., 2010); therefore xylanase from this experiment might be more stable than *Aspergillus niger* DFR-5 and *Penicillium occitanis*. The stability of xylanase in this study was also higher than the immobilized xylanase from *Armillaria gemina* (Dhiman et al., 2013b). It was indicated from 1.35-folds higher E_d value of xylanase in this study (86.1 kJ/mole) compared to the immobilized xylanase (49.3 kJ/mole).

The disruption of non-covalent linkages was reflected with the enthalpy of deactivation (ΔH) (Marín et al., 2003). The ΔH values decreased with rise in temperature revealing that lesser energy is required to denature enzyme at high temperature. In contrary, the Gibbs free energy (ΔG) for the thermal unfolding increased with higher temperature indicating that the resistance of system was declined at a particular temperature. The opening up of the enzyme structure is also accompanied to the disorder or entropy of deactivation (ΔS) (Nosoh & Sekiguchi, 1990). The negative values of ΔS were found at all temperatures. It indicated the aggregation of the xylanase in which a few inter and/or intra-molecular bonds were formed (Anema & McKenna, 1996). The negative values of ΔS have been observed during deactivation of xylanases from different organisms including *Armillaria gemina* (Dhiman et al., 2013a), *Thermotoga petrophila* (ul Haq et al., 2012) and *Scopulariopsis* sp. (Afzal et al., 2005). All these results confirmed that the purified xylanase of *A. pullulans* PBU-109 was unstable at temperature higher than 50°C.

5.5.3 Effect of polyols on xylanase thermostability

In order to avoid thermal inactivation, several approaches were carried out to improve stabilization of an enzyme in soluble form including chemical modification, cross-linking, immobilization, protein engineering and treatment with additive. The addition of small compounds to an enzyme solution can provide a simple but practical means of increasing the enzyme stability by changing its microenvironment

(Pal & Khanum, 2010). Polyols, also known as sugar alcohols, are small carbohydrates that were found to preserve the enzymatic activity under the heat treatment (Kumar et al., 2012). The addition of polyols in aqueous solutions has been found to improve the thermostability of enzymes from fungi (Georis et al., 2000) including xylanases from *Trichoderma reesei* QM 9414 (Cobos & Estrada, 2003) and *Aspergillus awamori* (at 52°C) (Lemos et al., 2000). The selection of the appropriate polyol depends on the nature of the enzyme while (Lemos et al., 2000) suggested that the molecular size and number of hydroxyl groups per polyol molecule are play a crucial role in mediating the protection against thermal inactivation. Therefore, the different hydroxyl groups or molecular size of polyol including ethylene glycol (C2), glycerol (C3), xylitol (C5), sorbitol (C6) and mannitol (C6) were investigated for the thermal protective property of purified xylanase from *A. pullulans* PBU-109 in this experiment.

The stability of xylanase improved with addition of all polyol additives while the highest retention of original activity was found in the presence of sorbitol at the same condition. It was a good indication that the sorbitol treated xylanase could withstand a higher temperature without losing its functional structure and activity. The effects of sorbitol on the thermostability of xylanase have been recognized in several filamentous fungi and bacteria. The use of 2M sorbitol for xylanase from *Aspergillus niger* DFR-5 enhanced the thermo-stability of the enzyme at 70°C by 2.27-fold increasing in half-life (Pal & Khanum, 2010). This positive effect of sorbitol on enzyme thermostability was also reported by (George et al., 2001), they found that the addition of 4M sorbitol into purified xylanase from *Thermomonospora* sp. resulted in an increase in the half-life from 8 to 42 minutes at 80°C. A similar improvement in the thermostability in the present of 400 mg/ml sorbitol was observed for bacterial xylanase from *Bacillus amyloliquefaciens* (Breccia et al., 1998) that 63-fold increased the half-life of the enzyme from 29 minutes to about 30 hours at 65°C. However, the effect on the thermostability of xylanase from yeast has not yet been reported.

Polyols was classified in terms of their protective effect on xylanase heat stability as follows: sorbitol (C6) > xylitol (C5) > ethylene glycol (C2) > mannitol (C6) > glycerol (C3). It indicated that hydroxyl content and molecular size were not sufficient to predict the protective effect of polyol in this experiment. It has further been suggested that the protective role of polyols is due to their capability to form hydrogen bonds with native enzyme to support and stabilize the native conformation of the enzyme and make it more resistant to thermal unfolding. Most of the polyols acted in a dose-

dependent manner. The efficiency of sorbitol in thermal protection increased with the increasing concentration. However, its protective effect at 0.75 M was not significantly different from that at a higher concentration.

The role of polyols in stabilizing the enzyme can be explained in two ways. The first possibility is that these additives restrict conformational changes by forming hydrogen bonds with functional groups on enzyme surface (Cobos & Estrada, 2003). Therefore the efficiency of thermal protection depends on the nature of the enzyme including hydrophilic and hydrophobic properties, and on the degree of interaction with the polyols (George et al., 2001). The second possibility is thereby increasing the free energy of the system (Kendrick et al., 1997). To clarify this, thermostability profile and thermodynamic parameters of the purified xylanase treated with 0.75 M sorbitol was evaluated in a temperature range of 70-90°C for 0-180 min compared with that of the xylanase without sorbitol at optimum condition.

The 1.77-fold increasing in half-life clearly indicated that sorbitol treated xylanase was more stable at 70°C, comparing with xylanase without sorbitol. It was also observed that sorbitol affected to retain the activity of the xylanase considerably and a good correlation with decreasing in rate of enzyme deactivation (k_d). Similarly, *D*-value increased in the presence of sorbitol, further proving its thermoprotecting effect on the purified xylanase from *A. pullulans* PBU-109. At 70°C, the addition of sorbitol also increased E_d of xylanase (121.80 kJ/mole) 1.41-fold in comparison to the xylanase without sorbitol (86.13 kJ/mole) which clearly indicated that more energy was required to denature the treated enzyme, resulting in more resistant to thermal denaturation (Tayefi-Nasrabadi et al., 2011). The calculation of ΔH was based on the value of E_d and, therefore, these values presented in the same trend. In this study, ΔH value of xylanase without sorbitol was 83.45 kJ/mole while that of sorbitol treated xylanase was 114.25 kJ/mole. The conformational change was also observed due to the decreasing of ΔH values at higher temperatures.

The value of ΔG for thermal unfolding at 70°C was calculated to be 114.25 and 107.57 kJ/mole for xylanase with and without sorbitol, respectively. These values suggested that treated xylanase offered more resistance to unfolding, once transition phase is achieved. ΔG values in sorbitol treated xylanase were also higher than native enzyme, whereas it was incubated at higher temperatures. The positive value of ΔS found for sorbitol treated xylanase indicating the lesser compaction of treated enzyme which further revealed more resistance to inactivation than the negative ΔS of

xylanase without sorbitol. Furthermore there was a slight change in the entropy of treated xylanase with the increase in temperature. It demonstrated the increasing of stability of enzyme.

As all results, it could be concluded that the protective role of sorbitol to stabilize enzyme at high temperature were due to the property to resist enzyme conformation and also to increase free energy in the system. The increasing of E_d, ΔH and ΔG values in the presence of sorbitol was refer to the enhancement in free energy while a change in the ΔS indicated that stabilization of xylanase was from conformation resistance. Accordingly, the addition of sorbitol in xylanase from *A. pullulans* PBU-109 provided useful information on improvement of enzyme stability for use in several biotechnological processes including pulp and paper.

5.5.4 Effect of ions

The effects of metal ions on activities of the purified xylanase were examined under the optimum conditions in the presence of a metal chelator and different metal ions that were often found in non-wood pulp (Doherty & Rainey, 2006; Li et al., 1997), at two different final concentrations (1 and 10 mM, respectively). Several publications have been reported the inhibitory activity of EDTA against fungal xylanases (Heine et al., 1997; Yang et al., 2010). However, the addition of EDTA did not significantly affect the purified xylanase from *A. pullulans* PBU-109 in both concentrations, suggesting that a divalent cation was not essential for xylanase activity or the xylanase in this study might be not a metalloenzyme. The metalloenzyme referred to enzyme that strongly bind with the metal ion or required the metal ions to maintain its native form, while the enzyme weakly bind with metal ion only during catalytic reaction referring to metal activated enzyme (Sudha, 2012). This result was similar to bacterial xylanases including those from *Bacillus* sp. N16-5 (Zhang et al., 2010) and *Anoxybacillus* sp. Ip-C (Hauli et al., 2013). The presence of Zn^{2+} had no effect on xylanase activity which was different from previous report that a purified xylanase from *A. pullulans* ATCC 42023 was inhibited by Zn^{2+} and Co^{2+} (Seo et al., 2004). In contrast, Fe^{2+} and Cu^{2+} showed a dose-dependent inhibitory effect against the enzyme, which was significant at high concentration (10 mM) normally unfound in non-wood pulp. Inactivation of xylanases by Fe^{2+} and Cu^{2+} has been reported among other fungi including *Aspergillus usamii* (Wang et al., 2011)and *Aspergillus awamori* VTCC-F312 (Do et al., 2012). These ions were suggested to react

with the thiol groups, carboxyl groups and histidine residues in the enzymes and therefore disrupt its active conformation (Lama et al., 2004).

5.5.5 Substrate specificity and kinetic parameters

A number of xylanase has been reported as bifunctional enzyme that significantly contained with cellulolytic activity, therefore the application of these enzymes was not suitable for pulp and paper industry. However, cellulase-free xylanases also have been reported including in tropical isolate of *A. pullulans* (Leathers, 1986). Therefore, substrate specificity of the xylanase from *A. pullulans* PBU 109 was also observed in this experiment using various polysaccharides as the substrates including different types of xylan, PNP-xyloside, α-cellulose, CMC and filter paper that were the specific substrate for estimation of endoxylanase, β-xylosidase, exoglucanase, endoglucanase and total cellulase, respectively. The purified and crude xylanases of *A. pullulans* PBU 109 were able to hydrolyze xylan from beech wood, oat spelt and rice straw while these was no activity toward α-cellulose, filter paper, CMC or PNP-xyloside. These results indicated that xylanase from *A. pullulans* PBU-109 was cellulase-free and it catalyzed only long chain xylan or it was a strictly endoxylanase which has been suggested to be typical for extracellular xylanase from *A. pullulans* (Leathers & Timothy, 1989; Nasr et al., 2013; Ohta et al., 2010). In general, many fungal xylanase often exhibited cellulolytic activities (Bischof et al., 2013; Lee & Forsberg, 1987). Therefore, the xylanase in this study provided a great potential in pulp and paper industry as it can selectively remove xylan in pulp with minimal damage to cellulose fibers (Shrinivas et al., 2010). The highest xylanase activity was observed with beech wood xylan and oat spelt xylans while it exhibited the lowest activity on rice straw xylan. It might be due to the fact that the side chains substitution in the rice straw xylan (arabino-glucuronoxylan) was higher than that of the beech wood xylan (glucuronoxylan) and oat spelt xylan (arabinoxylans) (Yoshida et al., 1990). Furthermore, the activity of the xylanase might be affected by side chain substituents on the xylan main chain which made the enzyme unable to cleave linkages next to the substituted residues (Collins et al., 2005). To better understand the action of the xylanase, kinetic parameters of the purified enzyme from *A. pullulans* PBU-109 were determined by measuring the enzymatic activity toward different xylan at various concentrations, 0.1-3 mg/ml, under optimum condition. Michaelis-Menten constant (*K*m) and maximal reaction velocity (*V*max) were calculated using linear regression of the Lineweaver and Burk double-reciprocal plot. The *K*m values from beech wood xylan, oat spelt xylan and

rice straw xylan were 0.741, 0.751 and 0.763 mg/ml, respectively that was fall within the generally range of fungal xylanase from 0.1-14 mg/ml (Kuhar et al., 2007). The lowest *K*m of purified xylanase with beech wood xylan referred to lower amount of substrate to saturate the enzyme at half of the maximal velocity for the reaction, indicating a high affinity for beech wood xylan. This result was related with the highest value of *V*max (12.82 μmol/min/mg) with beech wood xylan, comparing with *V*max values of oat spelt xylan (12.25 μmol/min/mg) and rice straw xylan (11.09 μmol/min/mg). It indicated that the purified xylanase was capable to hydrolyze this substrate faster than other. However, the values of *K*m and *V*max were slight different within substrates that revealed the wide substrate specificity in comparable rate.

5.6 Application of *A. pullulans* PBU109 xylanase for prebleaching of rice straw pulp

5.6.1 Pulping of rice straw

Rice straw is an abundant agricultural waste that was produced more than 20 million tons annually in Thailand (Kanokkanjana & Garivait, 2013). Although rice straw was partly used in livestock and agricultural facility, a large quantity of the remaining straw were burnt in the field without further utilization (Rodríguez et al., 2008). Therefore, the use of rice straw as an alternative raw material in pulp and papermaking may provide more economic value and disposal alternative in this waste. The benefit of rice straw as a non-wood material was its high content of cellulose (39.20 ± 2.20%) and hemi-cellulose (31.10 ± 1.76%) while small amount of lignin (21.50 ± 1.20%) in fibers that indicated the effective pulping capability. The pulping process of non-wood material was often done using sodium hydroxide solution as cooking liquor, known as soda pulping, to obtain the high quality pulp with low environmental problems (Chaiarrekij et al., 2011). Alkalinity was an important factor for soda pulping that directly related with the amount of residual lignin and pulp yield. Although the delignification was more effective at high alkali charges, but carbohydrate polymers might be damaged and lead to considerable losses in pulp yield (Silva et al., 2010). The strong alkalinity may also lead to the formation of chromophore structure in lignin resulting in the alkali-darkening of pulp, despite a high degree of lignin separation (Karlsson & Agnemo, 2010). Therefore, it was important to optimize the alkali content that it must be sufficient until the end of the pulping process to avoid precipitation and/or recondensation of higher molecular weight lignin into the fibers (Hergert, 1998). In this experiment, pulping of rice straw was carried out with 15% (w/w) sodium

hydroxide at 120°C for 2 hours (Chaiarrekij et al., 2011) resulting in 40.52 ± 0.49% of pulp yield with the small amount of pulp reject. The yield obtained in this study was selectively high compare to those reported from other non-woods (29.24 % to 48.53%) (Ogunsile & Quintana, 2010; Rodríguez et al., 2008). The residual lignin in pulp was presented in the term of Kappa number that is the volume of 0.1N potassium permanganate solution consumed by one gram of the oven-dried pulp and calculatedly corrected to 50% consumption of the amount of permanganate (Mohammadi-Rovshandeh et al., 2005). The low value of Kappa number from rice straw pulp (3.56 ± 0.17) referred to the high rate of lignin elimination in pulp that required less potassium permanganate for analysis. It also indicated that rice straw pulp should be easy to bleach by less sequent processes due to the low content in residual lignin. The residual alkali charge was observed in black liquor that exhibited the available amount of alkali charges in the system. The cellulosic portion of the fibers was minimal damaged from soda pulping while the amount of hemicellulose was found to decrease. The degradation of carbohydrate was by the oxidation at reducing end (peeling reaction) and alkaline hydrolysis at glycosidic linkage during pulping (Dumitriu, 2012). Cellulose was degraded in lesser extent than the hemicelluloses according to the protection by its crystalline structure as well as the high degree of polymerization. The hemicelluloses on the other hand have a higher amount of reducing end-groups as a result of their lower degree of polymerization and are thus more susceptible to the peeling reaction (Sjostrom, 1993).

5.6.2 Bleaching process and property determination of hand sheets

The dark color of pulp was a common phenomenon occurring in soda pulping due to the presence of residual and recondensed lignin in fibers. In order to achieve a higher quality pulp, lignin and its chromophores must be degraded and dissolved during bleaching. Chlorine based reagents were usually used as selective chemicals in bleaching to avoid the damage of cellulose fibers and pulp yield. However, by-products from this reaction were toxic and highly resistant to biodegradation that became the major source of environmental pollution (Beg et al., 2001). Enzymatic has been focused as an alternative method in order to reduce the use of these toxic chemicals. Among several enzymes, a combination of xylanase prebleacing with oxygen-based chemical bleaching sequence has gained much attention due to its delignification efficiency, increased the final pulp brightness in pulp, low costs,

and less ecological impact (Abrantes et al., 2007). Hydrogen peroxide bleaching processes are normally carried out at high temperature in the range of 60-80°C to maintain the pulp yield and efficient work (Sundara, 1998). Most of commercial xylanase was active at lower temperature (Viikari et al., 2007). Therefore, the application of xylanase in bleaching at an industrial scale remains limited.

Cellulase-free xylanase from *A. pullulans* PBU 109 presented as an appropriated enzyme in the continuous bleaching sequence of rice straw pulps according to its thermophilic property and wide substrate specific (Viikari et al., 2007)The crude xylanase was used in this experiment to reduce the cost of purification process while its properties were similar with purified enzyme. The condition of rice straw pulp prebleaching and chemical bleaching in this study was set at optimum temperature of xylanase from *A. pullulans* PBU 109 at 70°C. The reaction of xylanase prebleaching was performed for 2 hours while the half-life of this enzyme was 39 minutes, therefore the addition of sorbitol to prolong the half-life (into 6 hours) was also investigated. The addition of sorbitol to the crude enzyme solution significantly increased the amount of reducing sugars released from the pulp compared to that pretreated with the crude xylanase alone. The release of reducing sugars indirectly indicated the efficiency of xylan degradation. Moreover, the amount of reducing sugars released from untreated pulp was not detected.

The significant increase in pulp brightness was observed with pretreatment of the rice straw pulp with either xylanase or xylanase plus sorbitol, compared to those of the pulp treated with hydrogen peroxide alone (1.13- and 1.15-fold, respectively) and the unbleached pulp (1.60- and 1.62-fold, respectively). The positive effect of the xylanase pretreatment on pulp bleaching was generally attributed to the bleaching of covalent linkages between the lignin-hemicellulose complexes that leads to the releasing of chromophores and lignin from the pulp (Pérez et al., 2002). In addition, after alkaline pulping, certain amounts of xylan were re-precipitated on the fiber surface. The xylanase pretreatment could hydrolyze such precipitated xylan from the fiber surface, which would facilitate the penetration of subsequent bleaching agents (Suurnakki et al., 1997). At the same time, xylanase pretreatment enhances the removal of transition metal ions, such as iron, manganese, magnesium and calcium, which cause the formation of colored metallic complexes in the carbohydrates (Kirk & Jeffries, 1996). Increasing pulp brightness after xylanase prebleaching was previously reported in non-wood pulps. The brightness of the bagasse pulp after treated with *Arthrobacter* sp.

xylanase at 70°C for 2 hours enhanced by 9.6% ISO compared to the untreated pulp (Kulkarni et al., 1999). The boosting effect of commercial xylanase (Ecopulp TX-200) pretreatment at 70°C for 1 hour was also examined in giant reed pulp that led to increasing in pulp brightness by 0.6-1.0% ISO (Shatalov & Pereira, 2007). *Bacillus halodurans* xylanase treatment on wheat straw pulp at 70°C for 1.5 hours showed 5.2% ISO increasing in brightness (Lin et al., 1999) while increasing in the brightness of rice straw pulp in this study was enhanced by 58% ISO compared to the chemical bleached pulp.

From the observation of fiber morphology, no significant change was noticed in the average fiber length that could be referred to the selective digestion of xylanase in this prebleaching process and thus the cellulose component of the fiber remained relatively unchanged (Saake et al., 1995). The fines content was significantly decreased in the xylanase pretreated pulp which might be due to the high specific surface area to weight ratio of fines that the enzyme preferentially attacked this fiber fraction (Mansfield et al., 1996). The lower fines content led to the increase of contacting area between fiber-to-fiber and thus the paper strength was enhanced as observed in tear index (Liu et al., 2012b; Pan, 2004). In general, the mechanical properties of a paper sheet are influenced by the fiber morphology which can be adversely affected by the kink and curl of fibers (Pan, 2004). Fiber deformations can be occurred during all processes in different types including smooth, angular folds, twist and sharp bends. All of these will give a high value of fiber curl index that leads to low tensile strength and high tear index in paper sheet (Moraleda et al., 2009). The kink of fiber refers to the fiber deformation by changing of axial direction in sharp bend that can become weak site of fibers, therefore high value of kink index will lead to low value of tensile strength (Mark et al., 2002). Although the significant increase in kink index was observed with xylanase treated pulp, but values of the tensile strength were not significantly different in all treatments. It indicated that the kink of fiber was still in the low rate that could not affect with the tensile strength of paper. The highest value of kink index was found in pulp treated with xylanase plus sorbitol, referring to the enhancement of the xylanase stability during pretreatment at high temperature and high rate of degradation. The paper strength in terms of the tear indexes increased along with the curl indexes of the fibers after xylanase pretreatment. This may be due to the digested fibers becoming more flexible, thus the moderate curl of the fibers could enhance the interweaving between them which led to the strengthened bonding between the pulp fibers (Liu et

al., 2012a). These results clearly indicated that the crude xylanase from *A. pullulans* had a high potential for treatment of rice straw before bleaching for paper manufacture since it significantly improved the paper brightness without compromising fiber quality, especially when enhanced its stability by sorbitol. Therefore, it was possible to be used in other kind of pulp.

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APPENDIX A

CULTURE MEDIA

1. Yeast Malt Agar (YMA), (Atlas, 1993)

Yeast extract	5.0 g
Malt extract	5.0 g
Bacto-peptone	5.0 g
Dextrose	20.0 g
Agar	15.0 g

Dissolved in distilled water to final volume 1 liter.

2. Yeast Malt Agar (YMXA), (Christov and Prior, 1993)

Yeast extract	5.0 g
Malt extract	5.0 g
Bacto-peptone	5.0 g
Commercial xylan	20.0 g
Agar	15.0 g

Dissolved in distilled water to final volume 1 liter.

3. Basal Medium (Leathers *et al.*, 1984)

Glucose	3.0 g
Yeast-nitrogenous base	3.0 g
Bacto-peptone	5.0 g
Dextrose	10.0 g
Agar	20.0 g

Dissolved in distilled water to final volume 1 liter.

APPENDIX B

XYLANASE ASSAY

1. Chemical reagent

1.1 Dinitrosalycyclic acid solution (DNS) (Miller 1959)

Distilled water	1,416	ml
3,5-Dinitrosalycyclic Acid	10.6	g
NaOH	19.5	g
Rochelle salt (sodium potassium tartrate)	300	g
Phenol solution (pH 7.0)	7.5	ml
Sodium metabisulphate	8.3	g

After dissolving the above ingredients, the solution was stored at room temperature in an amber colored bottle to avoid photo oxidation.

1.2 Phosphate buffer (50 mM), pH 6-10

Make up the following solutions:

Solution A: 14.2 g Na_2HPO_4 per liter (0.1 M)

Solution B: 0.1 M HCl

Solution C: 0.1 M NaOH

Table 1B Preparation of 50 mM sodium phosphate buffers

Desired pH	Solution A (ml)	Solution B (ml)	Solution C (ml)
6.0	687.7	312.3	-
7.0	756.0	244.0	-
8.0	955.1	44.9	-
9.0	955.0	45.0	-
10.0	966.4	-	33.6

1.3 Acetate buffer (50 mM), pH 3-6

Make up the following solutions:

Solution A: 13.6 g sodium acetate per liter (0.1M)

Solution B: 0.1 M acetic acid

Table 2B Preparation of 50 mM sodium acetate buffers

pH	Solution A (ml)	Solution B (ml)
3.0	982.3 ml	17.7 ml
4.0	847.0 ml	153.0 ml
5.0	357.0 ml	643.0 ml
6.0	52.2 ml	947.8 ml

1.4 Standard solution

The standard solution was prepared by dissolve xylose in distilled water at seven different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mg/mL).

2. Method

The standard method involved mixing crude enzyme (100 µl) with 1% (w/v) beechwood xylan in 50 mM acetate buffer pH 6 (900 µl) as substrate and was incubated at 70°C for 30 min. Control tube was made by adding 1 ml of 1% substrate. Then 3 ml of DNS solution was added in each reaction and heated in boiling water bath for 5 min. After heating, the mixture was added with 10 ml of distilled water, allowed to cool at room temperature and optical density read at 540 nm (Miller 1959). The amount of reducing sugar was calculated according to a standard xylose graph.

3. Standard graph

One ml of the each concentration was added in a test tube along with 3 ml DNS solution. The test tubes were kept in boiling water for 5 minutes and cooled. A blank was also prepared (1.0 ml of distilled water and DNS solution). Absorbance was determined at 540 nm and a graph was drawn by plotting absorbance against concentration (Fig 1).

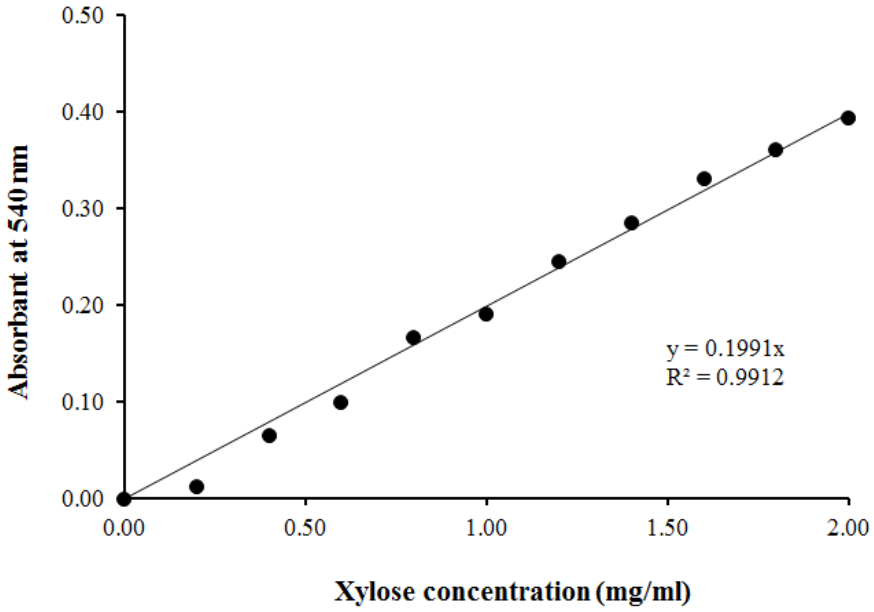

Fig. 1A Xylose standard curve

4. Activity calculation

According to the International Union of Biochemistry, one international unit of xylanase

(1 IU) corresponds to the amount of enzyme required to release 1 micromole of reducing sugar (xylose) in 1 minute.

$$\text{Xylanase activity (U/ml)} = \frac{\text{mg xylose from reaction/ (0.15013 mg xylose/}\mu\text{mol)}}{\text{0.1 ml x enzyme dilution x 30 min}}$$

APPENDIX C

PROTEIN ASSAY BY LOWRY'S METHOD

1. Chemical reagent

1.1 Solution A

NaOH 2.86 g

Na_2CO_3 14.31 g

Dissolved in distilled water to final volume 500 ml.

1.2 Solution B

$CuSO_4 \cdot 5(H_2O)$ 1.42 g

Dissolved in distilled water to final volume 100 ml.

1.3 Solution C

$Na_2Tartrate \cdot 2(H_2O)$ 2.85 g

Dissolved in distilled water to final volume 100 ml.

1.4 Lowry solution

The mixture of solution A, B and C with a ratio 100:1:1

1.5 Folin reagent

2N Folin Ciocalteu's phenol reagent 5 mL

Distilled water 6 mL

This reagent should be freshly prepared and kept in an amber container.

1.6 Standard protein

Five different concentrations (0.02, 0.04, 0.06, 0.08 and 0.10 mg/mL) of bovine serum alubumine (BSA) were prepared in distilled water.

2. Method

The standard method involved mixing sample or standard protein (500 µl) with Lowry solution (700 µl). The mixture was incubated in dark place at room temperature for 20 min. Control tube was made by adding 500 µl of distilled water. Then 100 µl of Folin reagent was added in each reaction and incubated once more for 30 min in the same condition. The mixture was optical density read at 750 nm (Lowry et al., 1951). The amount of protein was calculated according to a standard protein graph.

3. Standard graph

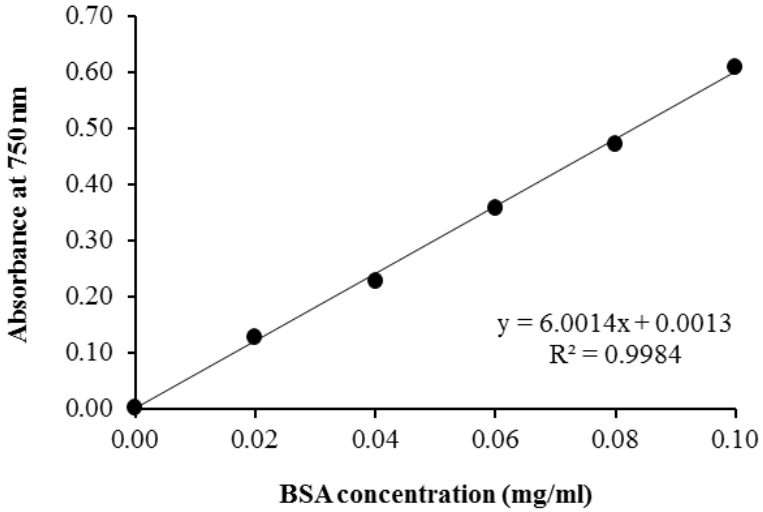

Fig. 1C Protein standard curve

APPENDIX D

FORAGE FIBER ANALYSES

1. Chemical reagent

1.1 Neutral-detergent solution

Sodium lauryl sulfate	30.00	g
Disodium ethylenediaminetetraacetate (EDTA)	18.61	g
Sodium borate decahydrate	6.81	g
Disodium hydrogen phosphate	4.56	g
2-ethoxyethanol (ethylene glycol monoethyl ether)	10.00	ml

Dissolved EDTA and sodium borate decahydrate in distilled water and add sodium lauryl sulfate and 2-ethoxyethanol (ethylene glycol monoethyl ether) to solution. Dissolved disodium hydrogen phosphate in distilled water and add to solution containing other ingredients. Check pH to range 6.9 to 7.1.

1.2 Acid-detergent solution

Concentrated sulfuric acid	26.20	ml
Cetyl trimethylammonium bromide (CTAB)	20.00	g

Dissolved CTAB in sulfuric acid and make up to final volume 1 liter with distilled water

1.3 Saturated potassium permanganate

$KMn0_4$	50.0	g
Ag_2S0_4	0.05	g

Dissolve $KMn0_4$ and Ag_2S0_4 in distilled water 1 liter. Keep out of direct sunlight.

1.4 Lignin buffer solution

Ferric nitrate	6.00	g
Silver nitrate	0.15	g
Acetic acid	500	ml
Potassium acetate	5.00	g
Tertiary butyl alcohol	400	ml
Distilled water	100	ml

Dissolve ferric nitrate and silver nitrate in distilled water. Combine with acetic acid and potassium acetate. Add tertiary butyl alcohol and mix.

1.5 Demineralizing solution

Oxalic acid dihydrate	50	g
95% ethanol	700	ml
12 N hydrochloric acid	50	ml
Distilled water	250	ml

Dissolve oxalic acid dihydrate in 95% ethanol and add concentrated hydrochloric acid. Adjust volume with distilled water.

2. Method (Goering and Van Soest, 1970)

2.1 Neutral detergent

Dry crucibles at 100° C for 12 h and weigh. Weigh 1.0 g air-dry sample into a beaker of the refluxing apparatus. Add in order, 100 ml neutral-detergent solution, 2 ml decahydronaphthalene and 0.5 g sodium sulfite. Adjust boiling to 80°C and reflux for 60 minutes. Remove the suspend solids into crucible. Rinse sample in crucible with hot water (90-100°C) and wash twice with acetone in same manner. Dry crucibles at 100° C for 12 h and weigh.

2.2 Acid-detergent fiber

Remove residual sample into beaker of the refluxing apparatus and add 100 ml acid detergent solution and 2 ml decahydronaphthalene. Reflux 60 minutes from onset of boiling at 80°C. After that, filter the suspend solids on a previously crucible and wash twice with hot water (90-100°C). Rinse sides of the

crucible in the same manner. Repeat wash with 80% ethanol until it removes no more color. Dry crucibles at 100° C for 12 h and weigh.

2.3 Permanganate lignin, cellulose, insoluble ash and silica

Add 25 ml of combined saturated potassium permanganate and lignin buffer solution (2:1 by volume) to the crucibles containing the acid-detergent fiber and incubate on ice for 90 min. Remove crucibles to filtering apparatus and suck to dry. Fill crucibles no more than half full with demineralizing solution to avoid spillage by foaming. After 5 minutes, suck dry on filter and refill half full with demineralizing solution. Repeat after second interval if solution is very brown and treat until fiber is white. Total time required is not over 20 min. Fill and thoroughly wash crucible with 80% ethanol. Wash twice in similar manner with acetone. Dry at 100° C for 16 h and weigh.

2.4 Ash content

Allow crucibles with residual sample to ash in furnace at 500 °C. After that, cool and weigh crucible to calculate lignin content as loss in weight from the original tare.

3. Activity calculation

3. Activity calculation

$$\%\ \text{NDF} = \frac{(W_{NDF}-W_T)}{S} \times 100 \quad (1)$$

$$\%\ \text{ADF} = \frac{(W_{ADF}-Wt)}{S} \times 100 \quad (2)$$

$$\%\ \text{Hemicellulose} = \%\ \text{NDF} - \%\text{ADF} \quad (3)$$

$$\%\ \text{Lignin} = \frac{(W_{ADF}-W_L)}{S} \times 100 \quad (4)$$

$$\%\ \text{Cellulose} = \frac{(W_L-W_A)}{S} \times 100 \quad (5)$$

$$\%\ \text{Ash} = \frac{(W_A- W_T)}{S} \times 100 \quad (6)$$

where: W_{NDF} = weight of oven-dry crucible including natural-detergent fiber;

W_{ADF} = weight of oven-dry crucible including acid-detergent fiber;

W_L = weight of oven-dry crucible including lignin extracted fiber;

W_A = weight of oven-dry crucible including ash;

W_T = tared weight of oven-dry crucible;

S = oven-dry sample weight

Table1E The weight (g) of ammonium sulfate to be added to one liter of solution to produce a desired change in the concentration (% saturation) of ammonium sulfate based on the values at 25°C.

%	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
10		28	57	86	118	137	150	183	216	251	288	326	365	406	449	494	540	592	640	694
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	69	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	110	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39

APPENDIX F

SDS-PAGE ANALYSIS

1. Chemical reagents

1.1 Acrylamide-bis Stock, 100 ml:

30% acrylamide

0.8% N,N'-methylene bis acrylamide

1.2 2x Resolving Buffer, 100 ml:

0.75 M Tris-HCl, pH 8.8

0.2% SDS

1.3. 2x Stacking Buffer, 100 ml:

0.25 M Tris-HCl, pH 6.8

0.2% SDS

1.4. 5x Electrode Buffer, 100 ml:

0.125 M Tris-HCl, pH 8.3

0.96 M glycine

0.5% SDS

1.5. TEMED full strength

1.6. 2x Sample buffer 0.125 M Tris-HCl, pH 6.8

4% SDS

20% glycerol

0.002% Bromphenol blue

10% mercaptoethanol (add just before use)

1.7 Stain: 450 ml water

500 ml methanol

75 ml acetic acid

5 g Coomassie brilliant blue

1.8 Destain I: 1.0 litre water

1.0 litre methanol

200 ml acetic acid

1.9 Destain II: 150 ml methanol

225 ml acetic acid

bring to 1.0 litre with water

APPENDIX G

XYLANASE PRODUCTION COST

1. The basal medium (Leather et al., 1984)

Table 1G Xylanase production cost of basal medium

Components	Concentrations	Price*
Yeast nitrogenous base	6.7 g	19.11 THB/g
Asparagine	2.0 g	22.26 THB/g
KH_2PO_4	5.0 g	6.94 THB/g
Beechwood xylan	10.0 g	11,492 THB / 100 g

*Reference price from Sigma-Aldrich Inc., USA

1,358 THB/ Liter (7.23 U/mL)

1,358 THB/ 7,230 Units

10 Unit = 18.78 THB

2. The optimized medium (in this study)

Table 2G Xylanase production cost of optimized medium

Components	Concentrations	Price*
Corncob	39.0 g	5.00 THB/g
$(NH_4)2SO_4$	3.0 g	4.80 THB/g
Xylose	1.8 g	11.05 THB/g
KH_2PO_4	1.4 g	6.94 THB/g
Tween-80	1.4 g	2.46 THB/g

*Reference price from Sigma-Aldrich Inc., USA

48.04 THB/ Liter (9.48 U/mL)

48.04 THB/ 9,480 Units

10 Unit = 0.50 THB

APPENDIX H

PROPERTIES OF CRUDE AND PURIFIED XYLANASE

Table 1H Properties of crude and purified xylanase from *A. pullulans* PBU109

Properties	Crude xylanase	Purified xylanase
Optimal condition	70°C and pH 6.0	70°C and pH 6.0
Half-life at 70°C	45 min	39 min
Half-life at 70°C + 0.75 M sorbitol	432 min	408 min
Activator(1 mM)	Ca^{2+}	Ca^{2+}
Inhibitor (1mM)	Not detectable	Not detectable
Activator (10 mM)	Mg^{2+}, Ca^{2+}, Co^{2+}	Mg^{2+}, Ca^{2+}, Co^{2+}
Inhibitor (10 mM)	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}
Cellulolytic activity	Not detectable	Not detectable
Specified substrate	Beechwood xylan > Oat spelt xylan > Rice straw xylan	

VITA

Miss Wichanee Bankeeree was born on October 11th, 1983 in Bangkok, Thailand. She graduated from Depsirinromklao School in March 1990 and received the Bachelor of Science degree with a major in Biotechnology from King Mongkut's Institute of Technology Ladkrabang in April 2005. She continued to study for the Master degree of Science in Biotechnology at the same institute and graduated in October 2008. Since June 2009, she has studied the degree of Philosophy of Science in Biological Sciences Program, at the Faculty of Science, Chulalongkorn University.

Visiting researchs:

1. Korea University, Seoul, Republic of Korea, June-August, 2011

   "Molecular characterization of cellulolytic and xylanolytic genes from tropical fungi"

2. Mulawarman University, Samarinda, Indonesia, May, 2014

   "Effect of lignosulfonate in enhancing enzymatic saccharification of palm-oil mill fibers using crude cellulase produced from fungi isolated in Indonesia

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