การแสดงออกของไซแลเนสยีน

จาก *Penicillium citrinum* ใน *Pichia pastoris* และ *Yarrowia lipolytica*

นางสาวชนิกา อื้อพานิช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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EXPRESSION OF XYLANASE GENE

FROM *Penicillium citrinum* IN *Pichia pastoris* AND *Yarrowia lipolytica*

Miss Chanika Ouephanit

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By	Miss Chanika Ouephanit
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Warawut Chulalaksananukul, Ph.D.
Thesis Co-Advisor	Sophie Bozonnet, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

....Chairman
(Assistant Professor Tosak Seelanan, Ph.D.)

....Thesis Advisor
(Associate Professor Warawut Chulalaksananukul, Ph.D.)

....Thesis Co-Advisor
(Sophie Bozonnet, Ph.D.)

....Examiner
(Assistant Professor Kittinan Komolpis, Ph.D.)

....Examiner
(Assistant Professor Chompunuch Glinwong, Ph.D.)

....External Examiner
(Nassapat Boonvitthya, Ph.D.)

ชนิกา อื้อพานิช : การแสดงออกของไซแลเนสยีนจาก *Penicillium citrinum* ใน *Pichia pastoris* และ *Yarrowia lipolytica* (EXPRESSION OF XYLANASE GENE FROM *Penicillium citrinum* IN *Pichia pastoris* AND *Yarrowia lipolytica*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.วรวุฒิ จุฬาลักษณานุกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.โซฟี โบซอนเนท, 121 หน้า.

ไซแลนคือองค์ประกอบหลักของเฮมิเซลลูโลสซึ่งเป็นพอลิแซคคาไรด์ที่มีจำนวนมากเป็นอันดับที่ 2 บนโลกและมีไซโลสเป็นน้ำตาลองค์ประกอบหลัก ไซแลเนสเป็นเอนไซม์หลักที่ใช้ในการย่อยสลายไซแลนเป็นไซโลสและไซโลโอลิโกแซคคาไรค์สายสั้น ไซแลเนสมีศักยภาพในการประยุกต์ใช้ในหลากหลายอุตสาหกรรม ไซแลเนสที่ใช้ในอุตสาหกรรมส่วนมากถูกผลิตจากราเส้นใยที่มีความสามารถผลิตเอนไซม์ในกลุ่มไซแลเนสหลากหลายชนิดซึ่งยากต่อการทำให้บริสุทธิ์ วัตถุประสงค์ของงานวิจัยนี้คือ เพื่อตรวจสอบการแสดงออกของยีนไซแลเนสจาก *Penicillium citrinum* ในระบบการแสดงออกของยีสต์ เพื่อเพิ่มการการแสดงออกของไซแลเนสทำให้บริสุทธิ์ และตรวจสอบคุณสมบัติของไซแลเนส ยีนไซแลเนสสังเคราะห์จาก *P. citrinum* ถูกโคลนและแสดงออกสำเร็จใน *Pichia pastoris* โดยใช้ระบบการแสดงออกแบบตลอดเวลาภายใต้การควบคุมของโพรโมเตอร์กลีเซอร์รอลดีไฮด์-3-ฟอสเฟต ดีไฮโรจีเนส (p*GAP*) และระบบการแสดงออกแบบเหนี่ยวนำภายใต้การควบคุมของโพรโมเตอร์แอลกอฮอล์ออกซิเดส1 (p*AOX1*) การแสดงออกของไซแลเนสภายใต้การควบคุมของ p*GAP* มีค่าแอคทิวิตี 119.5 ยูนิตต่อมิลลิลิตร ที่เวลา 48 ชั่วโมง ซึ่งมากกว่าไซแลเนสดั้งเดิม 34 เท่า ไซแลเนสที่ผลิตภายใต้การควบคุมของ p*AOX1* มีแอคทิวิตีสูงสุดเท่ากับ 676 ยูนิตต่อมิลลิลิตร เมื่อถูกเหนี่ยวนำด้วยเมทานอล 1 เปอร์เซ็นต์โดยปริมาตร ทุก 24 ชั่วโมง เป็นเวลา 5 วัน ซึ่งมากกว่าไซแลเนสดั้งเดิม 193 เท่า ค่าแอคทิวิตีที่เหมาะสม คือ ที่อุณหภูมิ 55 องศาเซลเซียส และ พีเอช 5 ตามลำดับ ไซแลเนสมีความเสถียรที่พีเอช 3-10 หลังจากบ่มที่อุณหภูมิ 40 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง เอนไซม์มีแอคทิวิตีคงเหลือ 80 เปอร์เซ็นต์ ค่า K_m และ k_{cat} คือ 2.8 มิลลิกรัมต่อมิลลิลิตร และ 243 ต่อวินาที ตามลำดับ นอกจากนี้ ยีนไซลาเนสสังเคราะห์จาก *P. citrinum* ถูกโคลนและแสดงออกสำเร็จใน *Yarrowia lipolytica* โดยใช้ระบบการแสดงออกแบบตลอดเวลาภายใต้การควบคุมของโพรโมเตอร์ทรานสโลเคชัน อีลองเกชัน แฟคเตอร์ 1 แอลฟา (p*TEF*) ใช้สัญญาณการหลั่งของยีนดั้งเดิมและยีน prepro*Lip2* ไซลาเนสที่ผลิตได้มีค่าแอคทิวิตีมากกว่าไซแลเนสดั้งเดิม 11 และ 52 เท่า ตามลำดับ ไซแลเนสที่ใช้สัญญาณการหลั่งของยีน prepro*Lip2* มีค่าแอคทิวิตี 180 ยูนิตต่อมิลลิลิตร ที่ 48 ชั่วโมง ไกลโคซิเลชันส่งผลต่อมวลโมเลกุลของไซแลเนสซึ่งเพิ่มขึ้นมากกว่า 60 กิโลดาลตัน หลังจากดีไกลโคซิเลชันไซแลเนสมีมวลโมเลกุล 20 กิโลดาลตัน ค่าแอคทิวิตีที่เหมาะสม คือ ที่อุณหภูมิ 55 องศาเซลเซียส และ พีเอช 5 ตามลำดับ ไซแลเนสมีความเสถียรที่พีเอช 3-9 หลังจากบ่มที่อุณหภูมิ 40 องศาเซลเซียส เป็นเวลา 6 ชั่วโมง เอนไซม์มีแอคทิวิตีคงเหลือ 80 เปอร์เซ็นต์ ค่า K_m และ k_{cat} คือ 5.2 มิลลิกรัมต่อมิลลิลิตร และ 245 ต่อวินาที ตามลำดับ

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ปีการศึกษา 2558	ลายมือชื่อ อ.ที่ปรึกษาหลัก _____
	ลายมือชื่อ อ.ที่ปรึกษาร่วม _____

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CHANIKA OUEPHANIT: EXPRESSION OF XYLANASE GENE FROM *Penicillium citrinum* IN *Pichia pastoris* AND *Yarrowia lipolytica*. ADVISOR: ASSOC. PROF. WARAWUT CHULALAKSANANUKUL, Ph.D., CO-ADVISOR: SOPHIE BOZONNET, Ph.D., 121 pp.

Xylan is the major component of hemicelluloses, the second most abundant polysaccharide on earth, and contains xylose as the major component sugar. Xylanases are the main xylan degrading enzymes which hydrolyze the xylan to produce xylose and short-chain xylooligosaccharides. Xylanases have potential applications in a wide range of industries. The most of common industrial xylanases are produced from filamentous fungi which have ability to produce many xylanolytic enzymes that are difficult to purify. The purpose of this study was to investigate expression of xylanase gene from *Penicillium citrinum* in yeast expression platform for increasing xylanase expression, purification and determine the properties of xylanase. A synthetic xylanase gene from *P. citrinum* was successfully cloned and expressed in *Pichia pastoris* using either constitutive expression system under the control of glyceraldehyde-3-phosphate dehydrogenase promoter (p*GAP*) or inducible expression system under the control of alcohol oxidase 1 promoter (p*AOX1*). Expression of xylanase under control of the p*GAP* showed the activity 119.5 U/ml after 48 h which was higher than the native xylanase activity for 34 folds. The recombinant xylanase under the control of the p*AOX1* showed the maximum activity of 676 U/ml when induced with 1% v/v methanol every 24 h for 5 days which was higher than the native xylanase activity for 193 folds. Optimal xylanase activity was observed at 55°C and pH 5, respectively. The recombinant xylanase was stable at a pH of 3 to 10. After pre-incubation at 40°C for 1 hour, the enzyme retained around 80% of the original activity. The K_m and k_{cat} were 2.8 mg/ml and 243 per s, respectively. Moreover, a synthetic xylanase gene from *P. citrinum* was successfully cloned and expressed in *Yarrowia lipolytica* using constitutive expression system under the control of translation elongation factor-1α (p*TEF*) using either native or prepro*LIP2* secretion signals. The recombinant xylanase was showed the activity higher than native xylanase for 11 and 52 folds, respectively. The recombinant xylanase using the prepro*LIP2* secretion signal showed the activity 180 U/ml after 48 h. Glycosylation affected the molecular mass of recombinant xylanase, which increased to larger than 60 kDa. After deglycosylation, the recombinant xylanase displayed molecular mass of 20 kDa. Optimal xylanase activity was observed at 55°C and pH 5, respectively. The recombinant xylanase was stable at a pH of 3 to 9. After pre-incubation at 40°C for 6 hour, the enzyme retained around 80% of the original activity. The K_m and k_{cat} were 5.2 mg/ml and 245 per s, respectively.

Field of Study: Biotechnology   Student's Signature ....

Academic Year: 2015   Advisor's Signature ....

                                                      Co-Advisor's Signature ....

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LIST OF ABBREVIATIONS

AZCL-Arabinoxylan	Azurine-cross-linked-Arabinoxylan
BSA	Bovine Serum Albumine
DNA	Deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid
dNTP	Deoxyribonucleotide
GH	Glycoside hydrolase
Ka	Kilodalton
LB	Luria-Bertani culture media
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulfate
Tris	Trishydroxymethylaminomethane
XYN	Xylanase

CHAPTER I

INTRODUCTION

Plant cell walls are the main polysaccharide-containing renewable resource on earth which are generated by photosynthesis. Cellulose, hemicellulose and lignin are three major components of plant cell walls, with cellulose being the most abundant carbohydrate polymeric component followed by hemicelluloses. Structurally, hemicelluloses are much more complex than cellulose. Hemicellulose is composed of xylan as a major component and accounts for approximately one third of all renewable organic carbon on earth. Xylan is a complex heteropolysaccharide having a backbone made up of β-1,4-linked D-xylopyranose residues with substitutions of L-arabinofuranose and 4-O-methyl-D-glucuronic acid at 2' and 3' positions. Complete hydrolysis of xylan requires the action of several main-chain- and side-chain-cleaving enzymes. Xylanases (EC 3.2.1.8) play a key role in the xylan hydrolysis to xylooligosaccharides, while β-xylosidases act on these xylooligosaccharides releasing xylose.

Xylanases are widely used for various biotechnological applications. The forest products industry is well suited to play a significant role in biomass conversion to alleviate the use of fossil fuels and food crops for energy and chemicals production. They are widely distributed. They can be found in both prokaryotes and eukaryotes including bacteria, actinomycetes, fungi, and yeasts. Filamentous fungi are the most common industrial xylanase producers. *Penicillium citrinum* is an interesting xylanase producing fungi because their xylanases were stable over a wide pH range (pH 2-10).

However, filamentous fungi have a wide spectrum of genes encoding xylanolytic enzymes, so it is difficult to obtain a pure form of a particular enzyme from a fungal preparation. Therefore, recombinant DNA technology, that enables to analyze a single gene product, can be applied with more success for these purposes.

Expression of proteins in yeast systems provides additional benefits over bacterial expression systems such as ability to perform eukaryotic post-translational modifications, ability to grow to very high cell densities and ability to secrete proteins into fermentation media. Moreover, many types of yeast are considered as non-toxigenic, non-pathogenic and several processes based on this organism were calssified as Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA, USA). Heterologous gene expression by *Pichia pastoris* is a useful alternative to produce genetically engineered enzymes for research and even for the industrial purposes.

In addition to *P. pastoris*, oleaginous yeast *Yarrowia lipolytica* is an upcoming protein expression host. Application of this organism for heterologous protein expression has multiple advantages such as growth capacity, ability of this organism to metabolize glucose, alcohols, acetate and hydrophobic substrates such as alkanes, fatty acids and oils, well-characterized secretory system that yields high levels of recombinant proteins, glycosylation resembling mammalian system, easy screening for multi-copy strains using defective selection marker and usage of a single integration site.

Objective

To investigate expression of xylanase gene from *Penicillium citrinum* in yeast expression platform

Scope of study

Gene encoding xylanase A from *P. citrinum* was cloned and expressed in heterologous host *P. pastoris* using either constitutive expression system or inducible expression system. Moreover, gene encoding xylanase A from *P. citrinum* was also cloned and expressed in *Y. lipolytica* using either native or prepro*LIP2* secretion signals with the objectives of overproducing and characterization the enzyme.

Expected results

To construct the high-level expression of xylanase in yeast expression platform and characterization of recombinant enzyme

CHAPTER II

LITERATURE REVIEW

Part I Lignocellulosic biomass

Plant cell walls are the main polysaccharide-containing renewable resource on earth which generated by photosynthesis and are the major stock of fixed carbon sources in nature (Yang *et al.*, 2007). The three major components of plant cell walls are cellulose (35-50%), followed by hemicellulose (20-35%) and lignin (10-25%), respectively (Sá-Pereira *et al.*, 2003; Saha, 2003). The exact composition of lignocellulose depends on the type, species and even source of the biomass (Table II-1). The lignocelluloses contained in the cell walls of plants are responsible for the specific mechanical properties. During cell formation, the middle lamella and primary wall are formed first. The subsequent cell-thickening is onset when the bulk of the cellulose and hemicellulose are deposited in the secondary wall (Timell, 1964). Celluloses are deposited to form microfibrils, which are key to the mechanical strength of the cell. The hemicelluloses are linked to the celluloses in such a manner as to orientate the microfibrils. The orientation of the fibrils gives the cell flexibility and directional strength (Lima *et al.*, 2001). Lignin forms in the middle lamella and secondary cell wall and serves to bind the fibres in the wood. In the simplest form, therefore, celluloses form the skeletal structure, which is surrounded by hemicelluloses and bound by lignin (Sjöström, 1993).

Table II-1 Composition of some lignocellulosic materials (Sánchez, 2009)

Lignocellulosic residues	Lignin (%)	Hemicellulose (%)	Cellulose (%)	Ash (%)
Hardwood stems	18–25	24–40	40–55	NA
Softwood stems	25–35	25–35	45–50	NA
Nut shells	30–40	25–30	25–30	NA
Corn cobs	15	35	45	1.36
Paper	0–15	0	85–99	1.1–3.9
Rice straw	18	24	32.1	NA
Sorted refuse	20	20	60	NA
Leaves	0	80–85	15–20	NA
Cotton seeds hairs	0	5–20	80–95	NA
Newspaper	18–30	25–40	40–55	8.8–1.8
Waste paper from chemical pulps	5–10	10–20	60–70	NA
Primary wastewater solids	24–29	NA	8–15	NA
Swine waste	NA	28	6	NA
Solid cattle manure	2.7–5.7	1.4–3.3	1.6–4.7	NA
Coastal Bermuda grass	6.4	35.7	25	NA
Switch grass	12.0	31.4	45	NA
S32 rye grass (early leaf)	2.7	15.8	21.3	NA
S32 rye grass (seed setting)	7.3	25.7	26.7	NA
Orchard grass (medium maturity)	4.7	40	32	NA
Grasses (average values for grasses)	10–30	25–50	25–40	1.5
Sugar cane bagasse	19–24	27–32	32–44	4.5–9
Wheat straw	16–21	26–32	29–35	NA
Barley straw	14–15	24–29	31–34	5–7
Oat straw	16–19	27–38	31–37	6–8
Rye straw	16–19	27–30	33–35	2–5
Bamboo	21–31	15–26	26–43	1.7–5
Grass Esparto	17–19	27–32	33–38	6–8
Grass Sabai	22.0	23.9	NA	6.0
Grass Elephant	23.9	24	22	6
Bast fiber Seed flax	23	25	47	5
Bast fiber Kenaf	15–19	22–23	31–39	2–5
Bast fiber Jute	21–26	18–21	45–53	0.5–2
Leaf Fiber Abaca (Manila)	8.8	17.3	60.8	1.1
Leaf Fiber Sisal (agave)	7–9	21–24	43–56	0.6–1.1
Leaf Fiber Henequen	13.1	4–8	77.6	0.6–1
Coffee pulp	18.8	46.3	35	8.2
Banana waste	14	14.8	13.2	11.4
Yuca waste	NA	NA	NA	4.2

NA = Not available.

The plant cell wall structure is shown in Figure II-1. It also contains smaller amounts of pectins, inorganic compounds, proteins and extractives, such as waxes and lipids, which also have potential value. The exact composition of lignocellulose

depends on the species, the plant tissue and the growth conditions (Rose and Bennett, 1999).
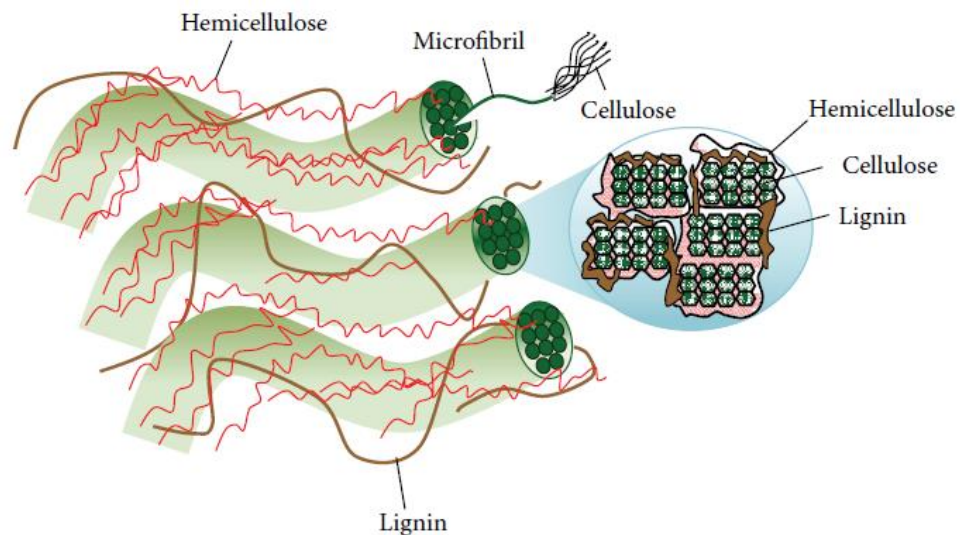
Figure II-1 Plant cell wall structure and microfibril cross-section (strands of cellulose molecules embedded in a matrix of hemicellulose and lignin) (Lee *et al.*, 2014)

1. Hemicellulose

Hemicelluloses are the world's second most abundant renewable polymers after cellulose in lignocellulosic materials. They were originally considered to be intermediates in the biosynthesis of cellulose. It is now known that hemicelluloses belong to a group of heterogeneous polysaccharides, which are formed by biosynthetic routes different from that of cellulose (Sjöström, 1993). They represent a type of hetero-polysaccharide with complex structure containing glucose, xylose, mannose, galactose, arabinose, rhamnose, glucuronic acid and galacturonic acid in various amounts, depending on the source (Table II-2). Pentoses comprise about 75% of the monomer of hemicellulose.

Table II-2 Hemicelluloses composition of various lignocellulosic materials (Gírio *et al.*, 2010)

Raw material	Xyl	Ara	Man	Gal	Rha	UA	AcG
Softwoods							
Douglas fir	6.0	3.0	–	3.7	–	–	–
Pine	5.3–10.6	2.0–4.2	5.6–13.3	1.9–3.8	–	2.5–6.0	1.2–1.9
Spruce	5.3–10.2	1.0–1.2	9.4–15.0	1.9–4.3	0.3	1.8–5.8	1.2–2.4
Hardwoods							
Aspen	18–27.3	0.7–4.0	0.9–2.4	0.6–1.5	0.5	4.8–5.9	4.3
Birch	18.5–24.9	0.3–0.5	1.8–3.2	0.7–1.3	0.6	3.6–6.3	3.7–3.9
Black locust	16.7–18.4	0.4–0.5	1.1–2.2	0.8	–	4.7	2.7–3.8
Eucalypt	14–19.1	0.6–1	1–2.0	1–1.9	0.3–1	2	3–3.6
Maple	18.1–19.4	0.8–1	1.3–3.3	1.0	–	4.9	3.6–3.9
Oak	21.7	1.0	2.3	1.9	–	3	3.5
Poplar	17.7–21.2	0.9–1.4	3.3–3.5	1.1	–	2.3–3.7	0.5–3.9
Sweet gum	19.9	0.5	0.4	0.3	–	2.6	2.3
Sycamore	18.5	0.7	1.0	–	–	–	3.6
Willow	11.7–17.0	2.1	1.8–3.3	1.6–2.3	–	–	–
Agricultural and agro-industrial materials							
Almond shells	34.3	2.5	1.9	0.6	–	–	–
Barley straw	15	4.0	–	–	–	–	–
Brewery's spent grain	15	8	0	1	0	2	0.8
Cardoon	26.0	2.5	3.7	1.4	0.9	–	–
Corn cobs	28–35.3	3.2–5.0	–	1–1.2	1	3	1.9–3.8
Corn fibre	21.6	11.4	–	4.4	–	–	–
Corn stalks	25.7	4.1	<3.0	<2.5	–	–	–
Corn stover	14.8–25.2	2–3.6	0.3–0.4	0.8–2.2	–	–	1.7–1.9
Olive stones	2.0–3.7	1.1–1.2	0.2–0.3	0.5–0.7	0.3–0.5	1.2–2.2	–
Rice husks	17.7	1.9	–	–	–	–	1.62
Rice straw	14.8–23	2.7–4.5	1.8	0.4	–	–	–
Sugar cane bagasse	20.5–25.6	2.3–6.3	0.5–0.6	1.6	–	–	–
Wheat bran	16	9	0	1	0	2	0.4
Wheat straw	19.2–21.0	2.4–3.8	0–0.8	1.7–2.4	–	–	–

The average degree of polymerization (DP) of hemicelluloses is in the range of 80–200. They are usually bonded to other cell-wall components such as cellulose, cell-wall proteins, lignin and phenolic compounds by covalent and hydrogen bonds, and by ionic and hydrophobic interactions (Peng *et al.*, 2012; Sun *et al.*, 2000). They are insoluble in water but soluble in alkaline solutions. Association with cellulose and lignin plays an important structurally supportive role on building up of plant cell walls (Dobrev *et al.*, 2007; Nakamura *et al.*, 2003). The major component of hemicelluloses is different depending on its sources (Table II-3). Hemicelluloses are usually named according to the main sugar residues in the backbone, e.g., xylans, glucomannans, galactans and glucans (Viikari *et al.*, 1993).

Table II-3 Main types of polysaccharides present in hemicelluloses (Peng *et al.*, 2012)

Polysaccharide	Biological origin	Amount[a]	Units			DP[b]
			Backbone	Side chains	linkages	
Arabinogalactan	Softwoods	5–35	β-D-Galp	β-D-Galp	β-(1→6)	100–600
				α-L-Araf	α-(1→3)	
				β-L-Arap	β-(1→3)	
Xyloglucan	Hardwoods, softwoods, and grasses	2–25	β-D-Glcp	β-D-Xylp	β-(1→4)	
			β-D-Xylp	β-D-Galp	α-(1→3)	
				α-L-Araf	β-(1→2)	
				α-L-Fucp	α-(1→2)	
				Acetyl	α-(1→2)	
Galactoglucomannan	Softwoods	10–25	β-D-Manp	β-D-Galp	α-(1→6)	40–100
			β-D-Glcp	Acetyl		
Glucomannan	Hardwoods	2–5	β-D-Manp			40–70
			β-D-Glcp			
Glucuronoxylan	Hardwoods	15–30	β-D-Xylp	4-O-Me-α-D-GlcpA	α-(1→2)	100–200
				Acetyl		
Arabinoglucuronoxylan	Grasses and softwoods	5–10	β-D-Xylp	4-O-Me-α-D-GlcpA	α-(1→2)	50–185
				α-L-Araf	α-(1→3)	
Glucuronoarabinoxylan	Grasses	15–30	β-D-Xylp	α-L-Araf	α-(1→2)	
				4-O-Me-α-D-GlcpA	α-(1→3)	
				Acetyl		
Homoxylan	Algae		β-D-Xylp[c]			
β-(1→3, 1→4)-glucan	Grasses	2–15	β-D-Glcp		β-(1→3)	
					β-(1→4)	

[a] %, Dry biomass.
[b] Degree of polymerization.
[c] May also present β-(1→3) linkages on the backbone.

2. Xylan

Xylan is the major component of hemicellulose and among the most abundant biopolymers, after cellulose, comprising approximately one third of all renewable organic carbon on earth (Nair *et al.*, 2008; Prade, 1996). Xylan is a complex heteropolysaccharide having a backbone made up of β-1,4-linked D-xylopyranose residues with substitutions of L-arabinofuranose and 4-O-methyl-D-glucuronic acid at 2' and 3' positions (Subramaniyan and Prema, 2002; Wakiyama *et al.*, 2008). Xylan does not form tightly packed structures hence is more accessible to hydrolytic enzyme. Consequently, the specific activity of xylanase is 2-3 times greater than the hydrolases of other polymers like crystalline cellulose (Gilbert and Hazlewood, 1993).

The xylan from hardwood is O-acetyl-4–O-methylglucuronoxylan. This polysaccharide consists of at least 70 β-xylopyranose residues (average degree of polymerization (DP) between 150 and 200), linked by β-1,4-glycosidic bonds (Puls, 1993). Every tenth xylose residue carries a 4-0-methylglucuronic acid attached to the 2 position of xylose (Figure II-2A). Hardwood xylans are highly acetylated (e.g., birchwood xylan contains more than 1 mol of acetic acid per 2 mol of xylose). The presence of these acetyl groups is responsible for the partial solubility of xylan in water. These acetyl groups are readily removed when xylan is subjected to alkali extraction (Beg *et al.*, 2001; Sunna and Antranikian, 1997).

In the case of soft wood plants xylan is mainly arabino-4-O-methylglucuronoxylan, which is not acetylated, contains β-D-xylopyranose, 4-O-methyl-α-D-glucuronic acid and L-arabinofuranose in a ratio of 100:20:13 (Puls, 1993). Softwood xylans are shorter than hardwood xylans, with a DP between 70 and 130. They are also less branched (Beg *et al.*, 2001; Sunna and Antranikian, 1997).

The xylan contained in grasses is also arabino-4-O-methylglucuronoxylan with a DP of 70. It has less 1,2-linked 4-O-methyl-α-D-glucuronic acid than does hardwood xylan but does have a large content of L-arabinofuranosyl side chains (Puls, 1993) (Figure II-2B). These are linked to C-2 or C-3, or both, of the β-D-xylopyranosyl backbone units. In addition, such xylans contain 2-5% by weight of O-acetyl groups linked to C-2 or C-3 of the β-D-xylopyranosyl backbone units (Bacon *et al.*, 1975). Morever, 6% of the arabinosyl side chains are themselves substituted at C-5 with feruloyl groups, while 3% are substituted with p-coumaroyl residues (Coughlan and Hazlewood, 1993).

A

B
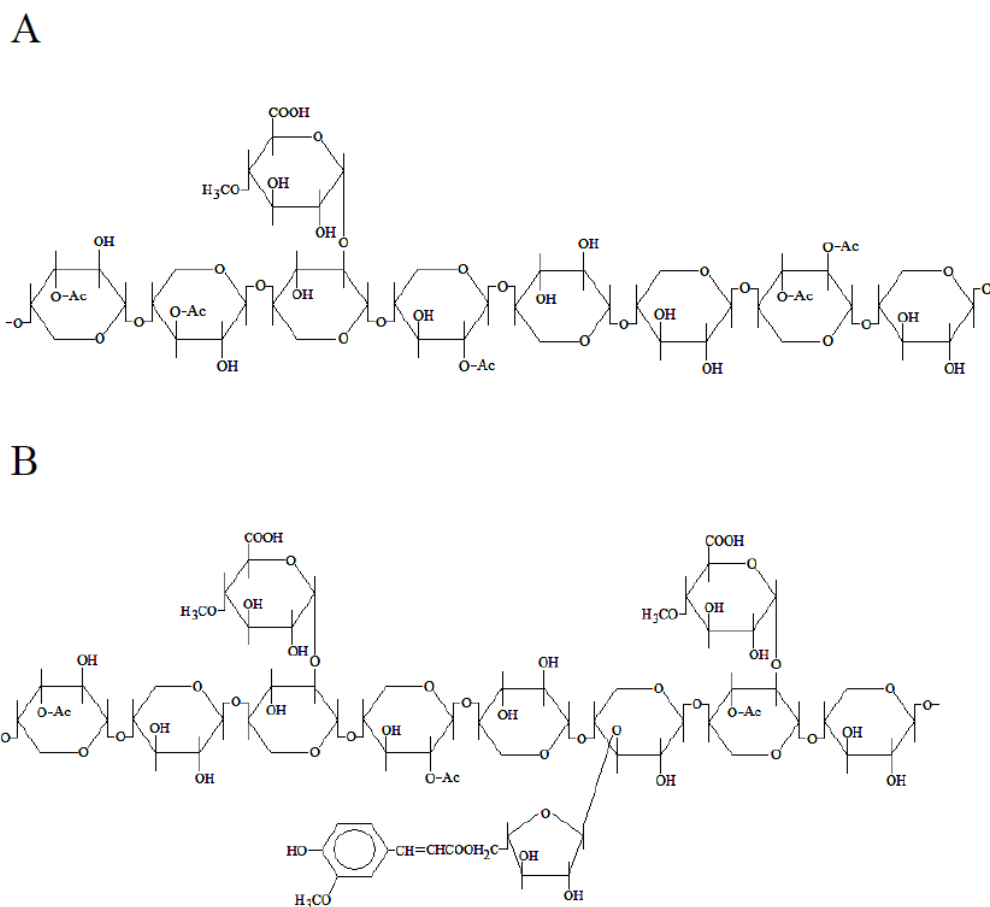
Figure II-2 Composition of a typical hardwood O-acetyl-4-O-methylglucuronoxylan (A) and a typical cereal arabino-4-O-methylglucuronoxylan (B). Ac, Acetyl group (Redrawn from Coughlan and Hazlewood, 1993)

3. Pretreatment of lignocellulosic biomass

The purpose of a pretreatment is to efficiently separate and provide easier access to the main biomass components (cellulose, hemicellulose and lignin), eventually removing lignin, preserving the hemicellulose, reducing the cellulose crystallinity and increasing the porosity of the material in order to improve the rate of

enzymatic hydrolysis and increase yield of fermentable sugars from cellulose or hemicellulose (Balat *et al.*, 2008; Sun and Cheng, 2002).

Numerous pretreatment strategies have been developed to enhance the reactivity of cellulose and to increase the yield of fermentable sugars. Typical goals of pretreatment include (Brodeur *et al.*, 2011) :

(1) Production of highly digestible solids that enhances sugar yields during enzyme hydrolysis.

(2) Avoiding the degradation of sugars (mainly pentoses) including those derived from hemicellulose.

(3) Minimizing the formation of inhibitors for subsequent fermentation steps.

(4) Recovery of lignin for conversion into valuable coproducts.

(5) To be cost effective by operating in reactors of moderate size and by minimizing heat and power requirements.

Pretreatment technologies are usually classified into physical, chemical, physicochemical and biological.

Mechanical pretreatment

Physical pretreatment involves breakdown of biomass size and crystallinity by chipping, grinding and milling. Ultrasonic pretreatment employs ultrasonic radiation to breakdown the complex network of polymerization in biomass (Li *et al.*, 2015). Improved hydrolysis results due to the reduction in crystallinity and improved mass transfer characteristics from reduction in particle size (Chiaramonti *et al.*, 2012). Reduction of particle size is often needed to make material handling easier and to

increase surface/volume ratio (Harmsen *et al.*, 2010). Mechanical pretreatment is usually carried out before a following processing step.

Chemical pretreatment

This group belong the pretreatments that are purely initiated by chemical reactions for disruption of the biomass structure. Acid hydrolysis can be performed either at low temperature with concentrated acids or at high temperature with diluted acids (Chiaramonti *et al.*, 2012). In the alkaline treatment, biomass is treated with alkali such as sodium, potassium, calcium and ammonium hydroxides at normal temperature and pressure. The main advantage of the process is efficient removal of lignin from the biomass (Maurya *et al.*, 2015). Organosolv processes use an organic solvent or mixtures of organic solvents with water for removal of lignin (Jönsson and Martín, 2016).

Physicochemical pretreatment

These methods combine mechanical and chemical action. Steam explosion (uncatalysed or catalysed) is one of the most applied pretreatment processes owing to its low use of chemicals and limited energy consumption (Harmsen *et al.*, 2010). Liquid hot water (LHW) processes are biomass pretreatments with water at high temperature and pressure (Mosier *et al.*, 2005). Ammonia fibre explosion (AFEX) process, biomass is treated with liquid ammonia at high temperature and pressure (Teymouri *et al.*, 2005). CO_2 explosion is similar to steam and ammonia fibre explosion; high pressure CO_2 is injected into the batch reactor and then liberated by

an explosive decompression (Sun and Cheng, 2002). The wet oxidation pretreatment catalyzes the formation of acids (Maurya *et al.*, 2015).

Biological pretreatment

Most pretreatment technologies require expensive instruments or equipment that has high energy requirements, depending on the process. Especially, physical and thermochemical processes require abundant energy for biomass conversion. Biological pretreatment is based on the use of microorganisms able to degrade lignin, hemicellulose and cellulose. Cellulose resulted to be the most resistant component to biological attack. Brown-, white and soft-rot fungi are used to treat biomass and enhancing enzymatic hydrolysis (Palmqvist and Hahn-Hägerdal, 2000). Advantages and disadvantages of different pretreatment methods of lignocellulosic biomass are shown in Table II-4.

Table II-4 Advantages and disadvantages of different pretreatment methods of lignocellulosic biomass (Maurya *et al.*, 2015)

Pretreatment method	Advantages	Disadvantages
Milling	-Decrease of cellulose crystallinity and degree of polymerization -Reduction of particle size to increase specific surface area and pore size	-High power and energy consumption
Steam explosion	-Causes lignin transformation and hemicellulose solubilization -Lower cost -Higher yield of glucose and hemicellulose in the two-step method	-Generation of toxic compounds -Partial hemicellulose degradation
Liquid hot water	-Size reduction of the biomass is not needed -No chemicals are generally required -No requirement of corrosion-resistant materials	-High energy and high water requirement -Formation of toxic compounds
Ammonia fiber expansion (AFEX)	-Increases accessible surface area -Less inhibitors formation -Does not require small particle size of biomass	-Not very effective for the biomass with high lignin content -High cost of large amount of ammonia
CO_2 explosion	-Increase accessible surface area -Availability at relatively low cost -Do not form inhibitory compounds -Non-flammability -Easy recovery after extraction and environmental acceptability	-Very high pressure requirements
Wet oxidation	-High degree of solubilization of hemicellulose and lignin -Avoid formation of degradation compounds	-High cost of oxygen and alkaline catalyst
Concentrated acid	-High glucose yield -Ambient temperatures	-High cost of acid and need to be recovered -Corrosion-resistant equipments are required -Concentrated acids are toxic and hazardous
Diluted acid	-High recovery of sugars at the end of the process -Low formation of toxic products	-Concentration of reducing sugars is relatively low -Generation of degradation products
Alkali	-Decrease in the degree of polymerization and crystallinity of cellulose -Disruption of lignin structure	-High cost -Not used for large-scale plant
Ozonolysis	-Effectively removes lignin content -Does not produce toxic residues -Reaction is carried out at room temperature and pressure	-High cost of large amount of ozone
Organosolv	-Causes lignin and hemicellulose hydrolysis	-Solvents need to be drained and recycled -High cost
Biological	-Low energy requirements -Delignification -Reduction in degree of polymerization of cellulose -Partial hydrolysis of hemicelluloses -No chemical requirements -Mild environmental conditions	-Slow process rate -Very low treatment rate -Not very effective for commercial application

Part II Microbial xylanolytic enzymes

   1. Source of xylanase

      Xylanases are widespread in nature. They can be found both in prokaryotes and eukaryotes and have been reported from some marine algae, marine and terrestrial bacteria, rumen bacteria, yeast, fungi, seed of terrestrial plants, insects, snails and crustaceans (Basaran *et al.*, 2001; Dekker and Richards, 1976; Morosoli *et al.*, 1992; Prade, 1996; Subramaniyan and Prema, 2002; Sunna and Antranikian, 1997; Wang *et al.*, 2007; Wong *et al.*, 1988).

      Xylanases are reported to be produced mainly by microorganisms that can produce cell wall-degrading enzymes to solubilize the complex components to simple molecules for completing the carbon cycle. Most of the microorganisms isolated from soil/waste/composting waste material are capable of producing a spectrum of cell wall-degrading enzymes (Badhan *et al.*, 2007). The presence of xylanolytic microorganisms was reported over 100 years ago by Hoppe-Seyler (1889), who described a gas production process using wood xylan suspension and rivermud microbes (Bastawde, 1992). There are many reports on the purification and characterization of xylanases from microbial sources (Table II-5).

Table II-5 Characteristics of xylanases from different microorganisms (Beg *et al.*, 2001)

Microorganism	Molecular weight (kDa)	Optimum		Stability		pI	K_m (mg/ml)	V_{max} (μM/mine per mg)
		pH	Tempera-ture (°C)	pH	Tempera-ture (°C)			
Bacteria								
Acidobacterium capsulatum	41	5	65	3–8	20–50	7.3	3.5	403
Bacillus sp. W–1	21.5	6	65	4–10	40	8.5	4.5	–
Bacillus circulans WL–12	15	5.5–7	–	–	–	9.1	4	–
Bacillus stearothermophilus T–6	43	6.5	55	6.5–10	70	7, 9	1.63	288
Bacillus sp. strain BP-23	32	5.5	50	9.5–11	55	9.3	–	–
Bacillus sp. strain BP-7	22–120	6	55	8–9	65	7–9	–	–
Bacillus polymyxa CECT 153	61	6.5	50	–	–	4.7	17.1	112
Bacillus sp. strain K-1	23	5.5	60	5–12	50–60	–	–	–
Bacillus sp. NG-27	–	7, 8.4	70	6–11	40–90	–	–	–
Bacillus sp. SPS-0	–	6	75	6–9	85	–	–	–
Bacillus sp. strain AR-009	23, 48	9–10	60–75	8–9	60–65	–	–	–
Bacillus sp. NCIM 59	15.8, 35	6	50–60	7	50	4, 8	1.58, 3.50	0.017, 0.742
Cellulomonas fimi	14–150	5–6.5	40–45	–	–	4.5–8.5	1.25–1.72	–
Cellulomonas sp. N.C.I.M. 2353	22, 33, 53	6.5	55	–	–	8	1.7, 1.5	380, 690
Micrococcus sp. AR-135	56	7.5–9	55	6.5–10	40	–	–	–
Staphylococcus sp. SG-13	60	7.5, 9.2	50	7.5–9.5	50	–	4	90
Thermoanaerobacterium sp. JW/SL–YS 485	24–180	6.2	80	–	–	4.37	3	–
Thermotoga maritima MSB8	40, 120	5.4, 6.2	92–105	–	–	5.6	1.1, 0.29	374, 4760
Fungi								
Acrophialophora nainiana	17	6	50	5	50	–	0.731, 0.343	–
Aspergillus niger	13.5–14.0	5.5	45	5–6	60	9	–	–
Aspergillus kawachii IFO 4308	26–35	2–5.5	50–60	1–10	30–60	3.5–6.7	–	–
Aspergillus nidulans	22–34	5.4	55	5.4	24–40	–	–	–
Aspergillus fischeri Fxn1	31	6	60	5–9.5	55	–	4.88	5.88
Aspergillus sojae	32.7, 35.5	5, 5.5	60, 50	5–8, 5–9	50, 35	3.5, 3.75	–	–

Table II-5 Characteristics of xylanases from different microorganisms (Continued)

Microorganism	Molecular weight (kDa)	Optimum		Stability		pI	K_m (mg/ml)	V_{max} (µM/mine per mg)
		pH	Tempera-ture (°C)	pH	Tempera-ture (°C)			
Aspergillus sydowii MG 49	30	5.5	60	–	–	–	–	–
Cephalosporium sp.	30, 70	8	40	8–10	–	–	0.15	–
Fusarium oxysporum	20.8, 23.5	6	60, 55	7–10	30	–	9.5; 8.45, 8.7	0.41, 0.37
Geotrichum candidum	60–67	4	50	3–4.5	45	3.4	–	–
Paecilomyces varioti	20	4	50	–	–	5.2	49.5	–
Penicillium purpurogenum	33, 23	7, 3.5	60, 50	6–7.5, 4.5–7.5	40	8.6, 5.9	–	–
Thermomyces lanuginosus DSM 5826	25.5	7	60–70	5–9	60	4.1	7.3	–
Thermomyces lanuginosus–SSBP	23.6	6.5	70–75	5–12	60	3.8	3.26	6300
Trichoderma harzianum	20	5	50	–	40	–	0.58	0.106
Trichoderma reesei	20, 19	5–5.5, 4–4.5	45, 40	3–8.5, 2.5–8.5	–	9, 5.5	3–6.8, 14.8–22.3	–
Yeast								
Aureobasidium pullulans Y-2311–1	25	4.4	54	4.5	55	9.4	7.6	2650
Cryptococcus albidus	48	5	25	–	–	–	5.7, 5.3	–
Trichosporon cutaneum SL409	–	6.5	50	4.5–8.5	50	–	–	–
Actinomycete								
Streptomyces sp. EC 10	32	7–8	60	–	–	6.8	3	–
Streptomyces sp. B–12–2	23.8–40.5	6–7	55–60	–	–	4.8–8.3	0.8–5.8	162–470
Streptomyces T7	20	4.5–5.5	60	5	37–50	7.8	10	7610
Streptomyces thermoviolaceus OPC–520	33, 54	7	60–70	–	–	4.2, 8	–	–
Streptomyces chattanoogensis CECT 3336	48	6	50	5–8	40–60	9	4, 0.3	78.2, 19.1
Streptomyces viridisporus T7A	59	7–8	65–70	5–9	70	10.2–10.5	–	–
Streptomyces sp. QG-11-3	–	8.6	60	5.4–9.2	50–75	–	1.2	158.85
Thermomonospora curvata	15–36	6.8–7.8	75	–	–	4.2–8.4	1.4–2.5	–

There are lots of reports on microbial xylanases starting from 1960 (Gascoigne and Gascoigne, 1960). Filamentous fungi have been used for more than 50 years in the production of industrial enzymes (Dalbøge, 1997). They are widely used as xylanase producers and are generally considered as more potent xylanase producers than bacteria and yeasts (Pedersen *et al.*, 2007; Polizeli *et al.*, 2005). They produce xylanases extracellularly into the medium, eliminating the need for cell disruption prior to purification. Furthermore, xylanase levels from fungal cultures are typically much higher than those from yeast or bacteria (Polizeli *et al.*, 2005; Sunna and Antranikian, 1997). Species of fungi genera that are known to produce xylanases include *Aspergillus, Disporotrichum, Penicillium, Neurospora, Fusarium, Chaetomium* and *Trichoderma* (Kulkarni *et al.*, 1999; Mach and Zeilinger, 2003; Saleem *et al.*, 2008). However, xylanases are produced mainly by *Aspergillus* and *Trichoderma* on the industrial scale (Haltrich *et al.*, 1996; Lu *et al.*, 2008).

2. Classification of hemicellulases

Although glycoside hydrolases are categorized by the Enzyme Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) based on the type of reaction that enzyme catalyze and on their substrate specificity, the category does not reflect the structural features of these enzymes. In 1991 Henrissat classified these glycoside hydrolases (GH) based on their amino acid sequence similarities which there is direct relationship between sequence and folding similarities (Henrissat, 1991).

Hemicellulases can be grouped based on their catalytic properties. The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyse glycosidic bonds or carbohydrate esterases (CEs), which hydrolyze ester bonds (Table II-6).

Table II-6 Enzymes associated with hemicellulose degradation and their specific mode of action (Lima *et al.*, 2001)

Enzyme	Mode of action	Catalytic classification
Exo–xylanase	Hydrolyses β–1,4–xylose linkages releasing xylobiose	GH
Endo–xylanase	Hydrolyses mainly interior β–1,4–xylose linkages of the xylan backbone	GH
β–Xylosidase	Releases xylose from xylobiose and short chain xylooligosaccharides	GH
α–Arabinofuransidase	Hydrolyses terminal non-reducing α–Arabinofuranose from arabinoxylans	GH
α–Glucuronidase	Releases glucuronic acid from glucuronoxylans	GH
Acetyl xylan esterase	Hydrolyses acetylester bonds in acetyl xylans	CE
Ferulic acid esterase	Hydrolyses feruloyester bonds in xylans	GH
p–Coumaric acid esterase	Hydrolyses p–Coumaryl ester bonds in xylans	GH
Endo–β–1,4–mannanase	Hydrolyse mannan-based hemicelluloses, liberate β–1,4–mannooligomers	GH
Exo–β–1,4–mannosidase	Hydrolyse β–1,4–mannooligomers to mannose	GH
Endo–galactanase	Hydrolyses β–1,4–galactan	GH
Acetyl mannan esterase	2– or 3–O–acetyl xylan	CE

GH: glycoside hydrolysis
CE: carbohydrate esterases

These enzymes are classified into families. In some families protein folds are more conserved than their amino acid sequences and these families are grouped into clans (Juturu and Wu, 2012). According to amino acid sequence homologies and hydrophobic cluster analysis, xylanases have been grouped mainly into two families

of glycosyl hydrolases (GH): family 10 (formerly known as family F) and family 11 (formerly known as family G) (Jeffries, 1996).

However, other glycoside hydrolase families, 5, 7, 8 and 43, have also been found to contain distinct catalytic domains with a demonstrated endo-1,4-β-xylanase activity. According to the continually updated Carbohydrate Active enzymes (CAZy) database, members of families 5, 7, 8, 10, 11 and 43 differ in their physico-chemical properties, structure, mode of action and substrate specificities (Cantarel *et al.*, 2009; Collins *et al.*, 2005). Representative structures of enzymes from various glycoside hydrolase families are shown in Figure II-3.

GH family 10 consists of xylanases with a high M_r of > 30 kDa, a high pI value and $(\alpha/\beta)_8$ barrel fold structure likened to a 'salad bowl' (Pell *et al.*, 2004) whereas GH family 11 consists of xylanases with relatively low M_r ranging from 19 to 25 kDa, a low pI value and β-jelly roll fold structure likened to a 'left hand' (Henrissat and Bairoch, 1993; Henrissat and Bairoch, 1996). GH family 11 consists solely of xylanases. Moreover, these xylanases are true xylanases as they are exclusively active on D-xylose containing substrates (Collins *et al.*, 2005). It is considered that due to their relatively small sizes these endo-xylanases can pass through the pores of hemicellulose network bringing efficient hydrolysis (Juturu and Wu, 2012). Characteristics of some recent (GHs 10 and 11) xylanases produced from different sources are shown in Table II-7.
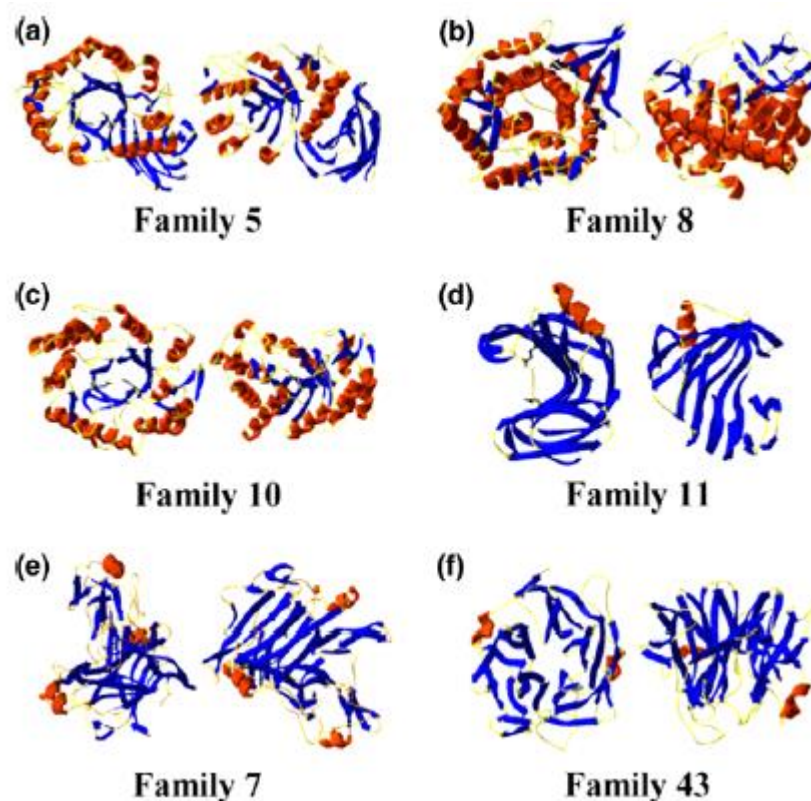
Figure II-3 Representative structures of enzymes from various glycoside hydrolase families. (a) Structure of the family 5 enzyme, XynA, from *Erwinia chrysanthemi*. The $(\beta/\alpha)_8$ barrel structure of the catalytic domain and the β_9-barrel of the small domain are shown. (b) Structure of the family 8 xylanase, pXyl, from *Pseudoalteromonas haloplanktis* TAH3a. The $(\alpha/\alpha)_6$ barrel structure of the catalytic domain is shown in two perpendicular views. (c) Structure of the *Streptomyces lividans* xylanase showing the typical family 10-fold. (d) Structure of the *Trichoderma reesei* family 11 xylanase showing the typical family 11-fold. (e) Structure of the *T. reesei* family 7 non-specific EGI. (f) Structure of the *Cellvibrio japonicas* family 43 α-L-arabinanase. Figures were prepared with Swiss-Pdb Viewer v3.7b2 (Collins *et al.*, 2005).

Table II-7 Characteristics of some recent (GHs 10 and 11) xylanases produced from different sources (Topakas *et al.*, 2013)

Microorganism	Name	Accession Number	MW (kDa)	pI	T_{opt}	pH$_{opt}$
GH 11						
Bispora sp. MEY-1	XYL11B	FJ212324	23	n.d.	65	2.6
Fusarium graminearum	XylA	AY289919	27	n.d.	35	8.0
F. graminearum	XylB	XM_383800	26	n.d.	35	7.0
Fusarium oxysporum f. sp. *ciceris*	–	–	21.6	8.2–9.0	55	5.5
Lentinula edodes	Xyl11A	AF411252	29.5	4.6	50	4.5
Paecilomyces thermophila	PtxynA	FJ593504	28	n.d.	75	7.0
Penicillium citrinum	XynA	AB198065	20	<3.5	55	5.0
Penicillium funiculosum	XYNB	AJ489605	22	5.0	n.d.	3.7–4.7
Sporotrichum thermophile ATCC 34628	StXyn1	–	24	8.7	60	5.0
Streptomyces olivaceoviridis A1	XYNB	CAC19491	26	n.d.	60	5.2
GH 10						
[b]*Sphingobacterium* sp. TN19	XynA19	FJ436401	42	n.d.	45	6.5
[c]*Cellulosimicrobium* sp. HY-13	XylK	FJ375341	36	4.7	55	6.0
[d]*Cellulosimicrobium* sp. HY-12	XynA	EU179736	39	n.d.	60	6.0
[e]*Flavobacterium* sp.	Xyn10	DQ059337	42	n.d.	30	6–7
[e]*Penicillium* strain FS010	XYL	AY583585	64[a]	n.d.	25	5.5
Penicillium funiculosum	XYND	AJ634957	36	4.6	n.d.	4.2–5.2
Aspergillus terreus BCC129	–	DQ087436	33	n.d.	60	5.0
Aureobasidium pullulans ATCC 20524	XynII	AB201542	39	8.9	70	6.0
Clostridium acetobutylicum ATCC 824	Xyn10A	Q97TP5	34	n.d.	60	5.0
Geobacillus sp. MT-1	–	DQ143882	62[a]	n.d.	70	7.0
Glaciecola mesophila KMM241	XynA	FJ715293	43	n.d.	30	7.0
Metagenomic library (soil sample)	XynH	AY860981	39	n.d.	40	7.8
Schizophyllum commune	XynB	–	30.5	2.8	50	5.5
Sporotrichum thermophile ATCC 34628	StXyn2	–	48	8.0	60	5.0

3. Mode of action

Hemicellulases are the enzymes that break down hemicellulose (Shallom and Shoham, 2003). Since xylan is a complex heteropolysaccharide, complete conversion of the xylan requires the interaction of several main-chain- and side-chain-cleaving enzymes activities, which are collectively called xylanolytic enzymes. Xylanolytic enzymes involved in the complete enzymatic hydrolysis of xylan are endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan 1,4-β-xylosidase, E.C.3.2.1.37), α-D-glucuronidase (α-glucuronidase, E.C.3.2.1.139), α-L-arabinofuranosidase (non-reducing end alpha-L-arabinofuranosidase, E.C.3.2.1.55),

acetylxylan esterase (E.C. 3.1.1.72)　 and feruloyl esterases (E.C. 3.1.1.73). Endoxylanases randomly hydrolyze the main chain of xylan to produce a mixture of xylooligosaccharides. β-xylosidase liberate xylose from short oligosaccharides. α-D-glucuronidase hydrolyze the methyl glucuronate residues. α-L-arabinofuranosidase remove l-arabinofuranose side chains. Acetylxylan esterase hydrolyze acetate groups from the main chain. Feruloyl esterases hydrolyze the respective aromatic acids linked to the arabinofuranoside residues (Figure II-4) (Chávez *et al.*, 2006; Choi *et al.*, 2000).
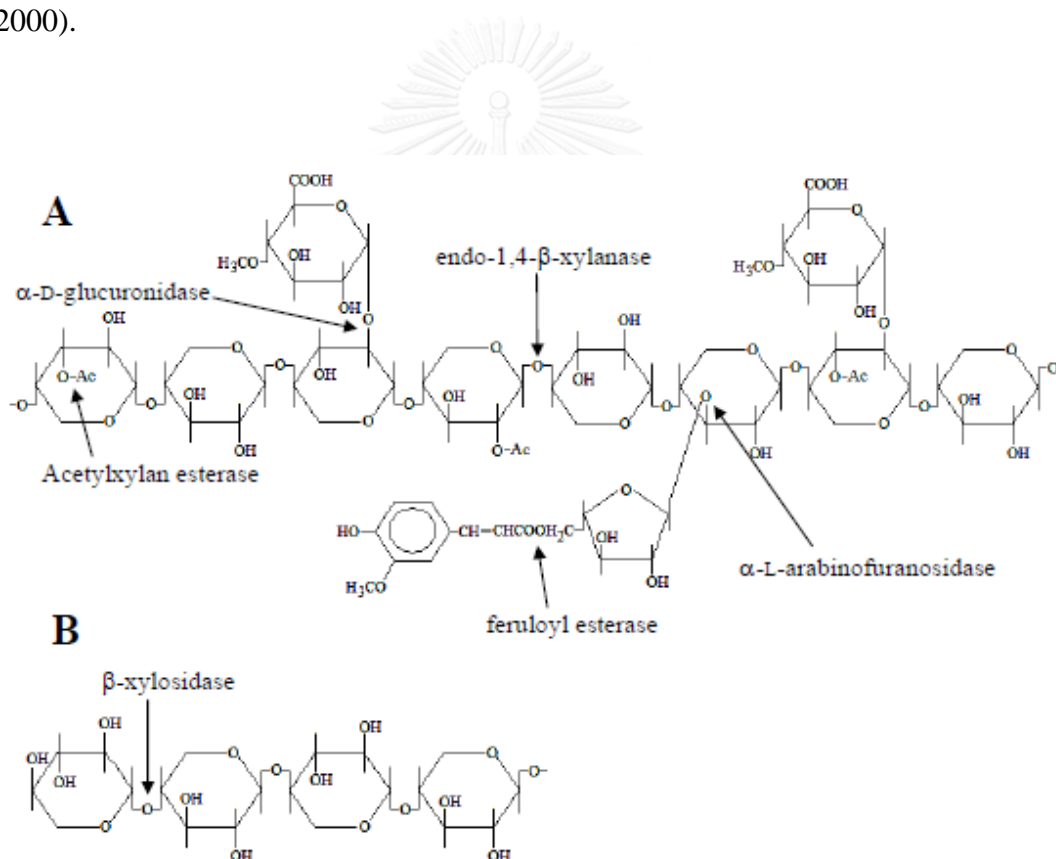
Figure II-4 The sites of attack by xylanolytic enzymes on xylan (A) and hydrolysis of xylo-oligosaccharides by β-xylosidase (B). Ac, Acetyl group (Coughlan and Hazlewood, 1993; Sunna and Antranikian, 1997)

4. Regulation of xylanase biosynthesis

Overall regulation can be considered as a combination of induction and catabolic repression. The general view of regulation of synthesis of xylanase is that low levels of constitutively produced hydrolytic enzymes produce small cell wall fragments that can enter the microbial cells and induce the synthesis of the corresponding enzyme for depolymerization of the cell wall substrate (Peltonen *et al.*, 1994; Thomson, 1993). In some cases, readily metabolizable sugars, such as glucose and/or xylose, are suppressors of xylanase biosynthesis (Bataillon *et al.*, 1998; Beg *et al.*, 2000; Liu *et al.*, 1999).

In 1999, Kulkarni and co-workers have proposed regulation model of xylanases. The initial release of low molecular mass substances (xylose and oligosaccharides) from xylan plays a key role in full induction of xylanases. The inducers need transferase enzymes for their translocation into the cytoplasm (Kulkarni *et al.*, 1999). Hence the level of inducers and/or the required enzymes in the culture filtrate also affect the xylanase synthesis (Figure II-5). The products of xylan hydrolysis are small molecular weight (xylose, xylobiose, xylotriose and other oligosaccharides) and easily enter the microbial cells and sustain the growth by acting as energy and carbon source (Biely, 1985; Haltrich *et al.*, 1995).
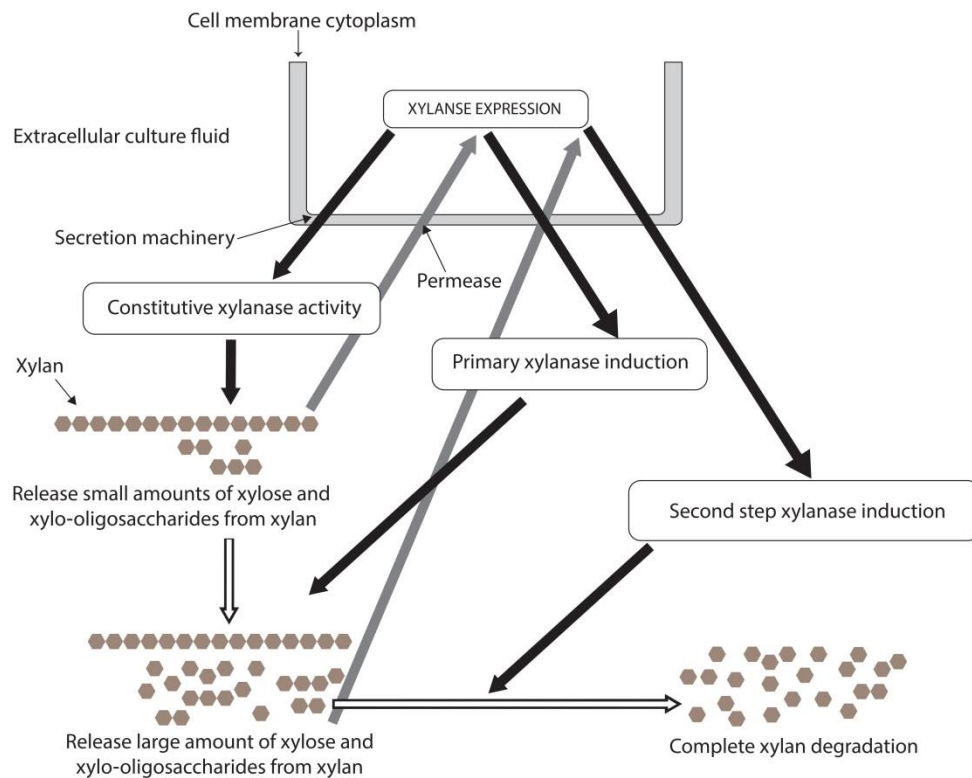
Figure II-5 Regulation of xylanase biosynthesis (hypothetical's model). Constitutive xylanases degrade xylan to xylo-oligosaccharides and xylobiose, which are taken up by the cell and induce other xylanase genes. The inducible xylanases degrade xylan further to xylo-oligosaccharides and xylobiose. The β-xylosidases, which may be produced constitutively and/or inducibly, convert xylobiose to xylose. These compounds are uptaken into the cell and act as additional inducers of genes encoding xylanolytic enzymes (Adapted from Kulkarni *et al.*, 1999).

5. *Penicillium* **species xylanolytic enzyme**

Penicillium is well known and one of the most common fungi occurring in a diverse range of habitats, from soil to vegetation to air, indoor environments, various food products textiles and other materials undergoing biodegradation. All common species grow and sporulate well on synthetic or semi-synthetic media (Pitt, 1979). *Penicillium* species play important roles as decomposers of organic materials and cause destructive rots in the food industry where they produce a wide range of mycotoxins (Visagie *et al.*, 2014). Penicillin discovery by Fleming's legendary observations on the inhibition of bacterial growth by *Penicillium notatum* is one of the greatest advances in medicine. This is due to the importance that this antibiotic appreciated during the Second World War and even today as it continues to be a source of many β-lactam semisynthetic derivatives of clinical use (Rodríguez-Sáiz *et al.*, 2005). Citrinin is also the mycotoxin, first isolated from filamentous fungus *Penicillium citrinum* (Hetherington and Raistrick, 1931).

A great number of *Penicillia* are active producers of xylanolytic enzymes and the use of xylanases from these species has growing importance in biotechnological applications (Chávez *et al.*, 2006). The presence of both family-10 and family-11 xylanases has been reported for *Penicillium purpurogenum* (Chávez *et al.*, 2002). The production of xylanolytic enzymes by *Penicillia* has been explored in a number of species (Table II-8). *Penicillium citrinum* is an interesting xylanase producing fungi because their xylanases were stable over a wide range of pH 2-10 (Dutta *et al.*, 2007; Tanaka *et al.*, 2005; Wakiyama *et al.*, 2008).

Table II-8 *Penicillium* species, which have been examined for the production of xylanolytic enzymes (Chávez *et al.*, 2006).

Species	Enzymes detected	Best carbon source tried
P. brasilianum	Endoxylanase	Wet-oxidized wheat straw
	β-Xylosidase	Wet-oxidized wheat straw
P. canescens	Endoxylanase	Soya meal + wheat straw
	Endoxylanase	Wheat straw + xylan
P. capsulatum	Endoxylanase	Beet pulp + wheat bran
P. chermisinum	β-Xylosidase	Xylan
P. chrysogenum	Endoxylanase	Xylan (oat)
	Endoxylanase	Xylan
P. commune	Endoxylanase	Cellulose
P. corylophilum	Endoxylanase	Xylan (oat)
	Endoxylanase	Xylan
P. duclauxi	Endoxylanase	Xylan
P. expansum	Endoxylanase	Cellulose
P. funiculosum	Endoxylanase	Cellulose + wheat bran
	Endoxylanase	Xylan (oat)
	β-Xylosidase	Xylan (oat)
	Arabinofuranosidase	Xylan (oat)
	Endoxylanase	Xylan
P. herquei	Endoxylanase	Xylan
	β-Xylosidase	Xylan (oat)
P. hirsutum	Endoxylanase	Cellulose
P. islandicum	β-Xylosidase	Xylan
P. janthinellum	Endoxylanase	Xylan (oat)
	β-Xylosidase	Xylan (oat)
P. kloeckeri	Endoxylanase	Xylan (birchwood)
P. oxalicum	Endoxylanase	Xylan
P. persicinum	Endoxylanase	Xylan (oat)
	β-Xylosidase	Xylan (oat)
	Arabinofuranosidase	Xylan (oat)
P. pinophilum	Endoxylanase	Cellulose + barley straw
P. purpurogenum	Endoxylanase	Wheat straw
	Acetyl xylan esterase	Acetylated xylan

6. Industrial application of xylanase

Xylanases are widely used for various biotechnological applications. Several commercial products have been launched successfully worldwide in the past few years (Table II-9).

Table II-9 Commercial xylanases and their industrial suppliers (Beg *et al.*, 2001).

Supplier	Product trade name	Application
Alko Rajamaki, Finland	Ecopulp	Pulp bleaching
Sandoz, Charlotte, N.C. and Basle, Switzerland	Cartazyme	Pulp bleaching
Clarient, UK	Cartazyme HS 10, Cartazyme HT, Cartazyme SR 10 Cartazyme PS10, Cartazyme 9407 E, Cartazyme NS 10, Cartazyme MP	Pulp bleaching
Genercor, Finland; Ciba Giegy, Switzerland	Irgazyme 40–4X/Albazyme 40–4X, Irgazyme-10A, Albazyme-10A	Pulp bleaching
	Multifect xylanase	Baking, food
Voest Alpine, Austria	VAI Xylanase	Pulp bleaching
Novo Nordisk, Denmark	Pulpzyme HA, Pulpzyme HB, Pulpzyme HC	Pulp bleaching
	Biofeed Beta, Biofeed Plus	Feed
	Ceremix	Brewing
Bicon India, Bangalore	Bleachzyme F	Pulp bleaching
Rohn Enzyme 0Y; Primalco, Finland	Ecopulp X-100, Ecopulp X-200, Ecopulp X-200/4, Ecopulp TX-100, Ecopulp TX-200, Ecopulp XM	Pulp bleaching
Meito Sankyo, Nogaya Japan	Xylanase	Research
Rohm, Germany	Rholase 7118	Food
Solvay Interox, USA	Optipulp L-8000	Pulp bleaching
Thomas Swan, UK	Ecozyme	Pulp bleaching
Iogen, Canada	GS-35, HS70	Pulp bleaching
Sankyo, Japan	Sanzyme PX, Alpelase F	Feed
	Sanzyme X	Food
Enzyme Development, USA	Enzeko xylanase	Baking, food, feed

Pulp and paper industry

The most common use of xylanase is as a bleaching agent in the pulp and paper industry. The chemical bleaching of paper pulp is traditionally achieved by the addition of chlorine and chlorine dioxide (Subramaniyan and Prema, 2000; Viikari *et al.*, 1994). The expulsion of hazardous chemicals during conventional pulp production to the environment cause serious public concern. Figure II-6 shows stages of the bleaching of cellulose pulp for paper production.
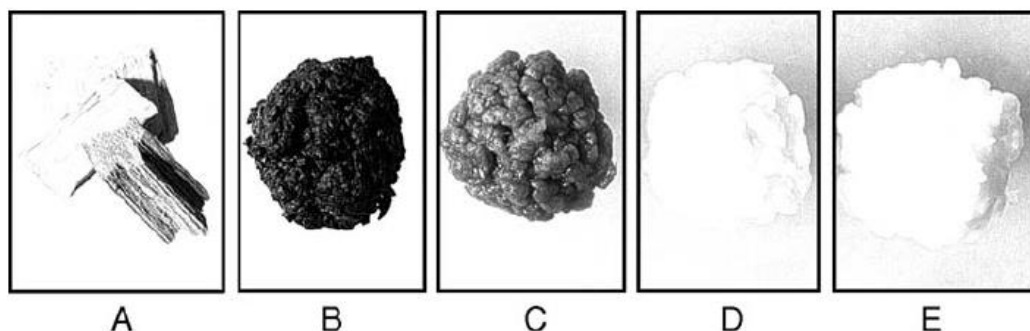
Figure II-6 Stages of the bleaching of cellulose pulp for paper production. Wood shavings (A), aspect of the cellulose pulp after the Kraft process (B), stage of pre-bleaching through the use of oxygen (C), cellulose pulp bleached with ozone and chlorine dioxide (D), stage using chlorine dioxide only, after the period of treatment with sodium hydroxide, hydrogen peroxide and oxygen (E) (Polizeli *et al.*, 2005).

Studies with fungal xylanases have resulted in the reduction of chlorine consumption. However, there was also an unacceptable drop in viscosity due to cellulase contamination of the xylanase preparations. Use of cellulase-free xylanases in pulp and paper industries would permit the production of superior quality dissolving pulp, as cellulase-free xylanases selectively remove hemicellulose components with minimal damage to cellulose (Jurasek and Paice, 1986; Polizeli *et al.*, 2005).

The xylanases preferred for bio-bleaching applications are usually family 11 xylanases (Bajpai, 1999; Buchert *et al.*, 1994). This is because the family 11 xylanases are relatively small, which makes it easy for this enzyme to pass through the pulp fibre, so that the enzyme has contact with relatively large areas. The other reason is that family 11 lacks cellulose activity, which can affect the pulp yield and strength negatively (Gomes *et al.*, 1993; Juturu and Wu, 2012).

Animal feed

The use of enzymes in the production of feed is an important sector of agribusiness (Polizeli *et al.*, 2005). The major component of cereal grains that is used for animal feed, especially for chicken and pigs, contains high amounts of xylan. This is not of any nutritional value to these animals. When such components are present in soluble form, they may raise the viscosity of the ingested feed, interfering with the mobility and absorption of other components resulting in difficulties in penetration of digestive enzymes and absorption of nutrients (Bedford and Classen, 1992). The effect is that the feed is not efficiently utilised resulting in poor growth of animals, especially chickens. Xylanases are used in animal feed along with glucanases, pectinases, cellulases, proteases, amylases, phytase, galactosidases and lipases. These enzymes break down arabinoxylans in the ingredients of the feed and reducing the viscosity of the raw material, thus improving nutrient availability (Twomey *et al.*, 2003).

Food industry

In the baking industry, xylanases may be employed in bread-making, together with α-amylase, malting amylase, glucose oxidase and proteases (Polizeli *et al.*, 2005). Xylanases break down the xylan component of wheat-flour, helping in the redistribution of water and leaving the dough softer and easier to knead. With the use of xylanases, there has been an increase in bread volumes, greater absorption of water and improved resistance to fermentation (Camacho and Aguilar, 2003; Jiang *et al.*, 2005). It also achieves desirable texture and mouth feel and increases bread volume and shelf life of baked products (Courtin *et al.*, 2001).

The fruit juice, beer and wine industries make up a good part of the enzyme market. The production of fruit and vegetable juices requires methods of extraction, clearing and stabilization (Debyser *et al.*, 1997). The addition of xylanases helped in the degradation of water-soluble xylan. These water soluble xylans cause cloud formation and high viscosity during fermentation which in turn makes filtering of the beer difficult. The use of xylanase decreased viscosity, thus making the filtration step much easier and faster and reduced haze formation resulting in a clarified product which is more acceptable to the consumers. This enzyme is also used in coffee-bean mucilage (Wong *et al.*, 1988). The main desirable properties for xylanases for use in the food industry are high stability and optimum activity at an acid pH (Polizeli *et al.*, 2005).

Pharmaceutical and chemical applications

Xylitol is used as an alternative food sweetener for diabetics. Slow adsorption of xylitol, artificial low-calorie sweeteners, into metabolic pathways is independent of insulin and does not cause rapid fluctuations in blood sugar levels. It is also used in orthodontical applications because of its beneficial properties such as teeth hardening, remineralisation of tooth enamel and antimicrobial properties (Saha, 2003; Winkelhausen and Kuzmanova, 1998). It is a non-cariogenic sweetener, suitable for diabetic and obese individuals and recommended for the prevention of osteoporosis and respiratory infections, lipid metabolism disorder, kidney and parenteral lesions (Polizeli *et al.*, 2005).

Several other applications of xylitol are also receiving renewed interest, such as its use as sweetener in sweets, chewing gums and chocolates, baked

goods and food marinades and sauces. It can also be used as sweetener in liquid pharmaceutical applications as it will not cause damage to teeth (Emodi, 1978). The steps for xylitol production are the delignification of hemicellulose material rich in xylan, followed by hydrolysis by xylanases and hemicellulases, to produce sugars such as β-D-xylopyranosyl units. Next, the products are fermented, mainly by yeasts (*Candida shehatae* and *Pichia stipitis*) to produce xylitol (Figure II-7) (Jin *et al.*, 2005; Kastner *et al.*, 1996).

Biofuel

The rise in global energy usage, together with the disappearance of fossil fuel reserves, has highlighted the importance of developing technologies to harness new and renewable energy sources (Chang, 2007). Limited risk of biofuels significantly affecting the price of food crops, the use of organic waste and agricultural/forestry residues and of lignocellulosic crops can reduce competition with food.

For cost-effective production of bioethanol from lignocellulosic biomass, the high efficiency utilization of both carbohydrate fractions (cellulose and hemicellulose) is required. Bioethanol production from lignocellulosic biomass involves several steps such as pretreatment, hydrolysis of complex carbohydrates, fermentation and distillation for product recovery (Gray *et al.*, 2006; Gupta *et al.*, 2009). The fermentation process would be economically viable only if both hexose and pentose sugars present in the hydrolysates are converted to ethanol. Xylose can be fermented by *Pachysolen tannophilus, Candida shehatae, Pichia stipitis* and *Kluveromyces marxianus* to produce ethanol (Abbi *et al.*, 1996; Hahn-Hägerdal *et al.*,

2007; Kuhad *et al.*, 1997). Figure II-7 shows the simplified scheme of xylitol and ethanol production by bacteria and yeasts from lignocellulosic materials.
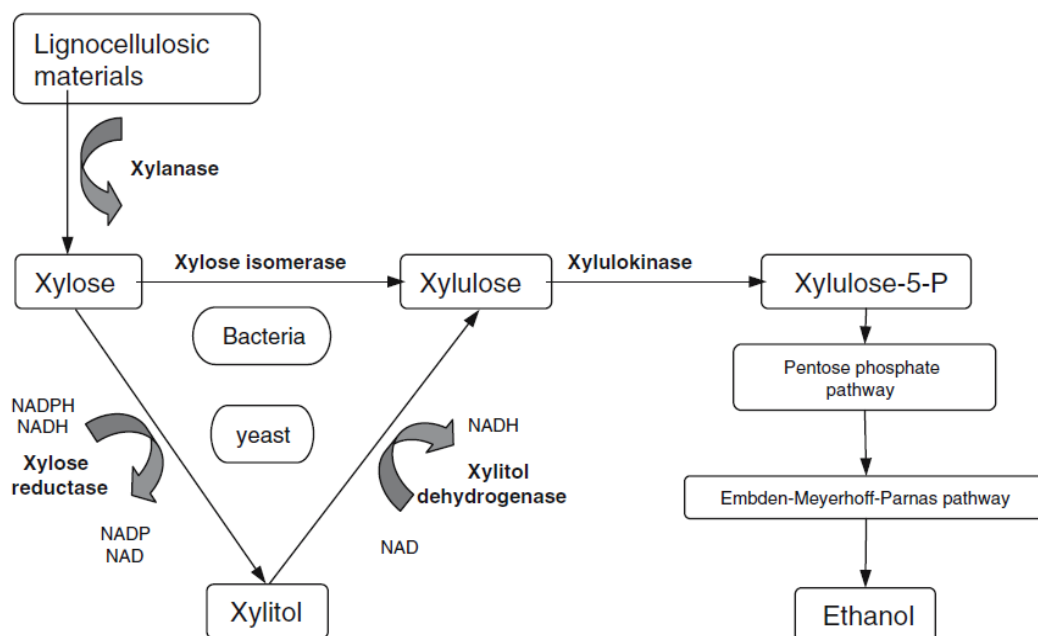
Figure II-7 Simplified scheme of xylitol and ethanol production by bacteria and yeasts from lignocellulosic materials (Polizeli *et al.*, 2005).

Part III Heterologous expression of xylanases

Recent advances in molecular biology and genetic engineering have opened up the areas of application of gene cloning and recombinant DNA technology. Cultivation of filamentous fungi for large scale protein production is very complicated often ending up in many interfering enzymes. Purification of target enzymes from a pool of proteins requires many purification steps thereby increasing their costs (Juturu and Wu, 2012; Kormelink *et al.*, 1993). Recombinant DNA techniques offer new opportunities for construction of genetically modified microbial strains with selected enzyme machinery. A number of genes of industrially important enzymes

have been cloned and expressed for enhanced production of enzymes and other commercially useful properties. Genes encoding xylanases have been isolated, cloned and expressed in homologous and heterologous hosts with the objectives of overproducing the enzyme and altering its properties to suit commercial applications (Baba *et al.*, 1994; Beg *et al.*, 2001; Jamil *et al.*, 2005). The main objectives for recombinant DNA technology in xylan bioconversion are: construction of producers of xylanolytic systems free of cellulolytic enzymes and the improvement of fermentation characteristics of the industrially important organisms by introducing genes for xylanase (Biely, 1985).

1. Bacterial expression system

Among the many systems available for heterologous expression, the gram-negative bacterium *Escherichia coli* is usually the first choice for cloning and expression of heterologous proteins. Due to its long history as a model system and because *E. coli* genetics are very well characterized, many tools have been developed for chromosome engineering and to facilitate gene cloning and expression (Mergulhao *et al.*, 2005; Sørensen and Mortensen, 2005; Terpe, 2006). Success of this host is attributed to multiple factors such as rapid growth on inexpensive media and simple techniques required for transformation and relatively simple process scale-up (Lee, 1996). Cloning of different fungal xylanase genes in *E. coli* is shown in Table II-10.

Table II-10 Cloning of different fungal xylanase genes in *E. coli* (Ahmed *et al.*, 2009)

Source organism	Gene	Vector	Host
Aspergillus cf. *niger* BCC14405	*xyn*	pGEM-T Easy	*E. coli* DH5α
Aspergillus oryzae KBN 616	*xynF3*	pNAN-d	*E. coli* DH5α
Aspergillus usamii E001	*xynII*	pET-28a(+)	*E. coli* BL21-CodonPlus (DE3)-RIL
Aureobasidium pullulans Y-2311-1	*xynA*	pCRII λ ZapII pBluescript	*E. coli* INVαF′, SURE, XL-Blue
Claviceps purpurea	*cpxyl1* *cpxl2*	pUC19 and pBluescriptII SK(−)	*E. coli*
Cochliobolus sativus	xyl2	Lambda ZAP	*E. coli* SOLR
Cochliobolus sativus	xyl1	Lambda ZAP	*E. coli*
Helminthosporium turcicum H-2	*htxyl2*	pBluescript SK(+)	*E. coli* DH5α
Neocallimastix frontails	*xyn11A* *xyn11B*	pET-21a	*E. coli*
Neocallimastix patriciarum	*xynA*	pBTac2	*E. coli*
Neocallimastix patriciarum	*xyns20*	pTriptx2-S20	*E. coli*
Penicillium purpurogenum	*xynA*	pATH-3	*E. coli*
Penicillium purpurogenum	*xynB*	pATH3	*E. coli* RR1
Penicillium sp. 40	*xynA*	pUC119	*E. coli* DH5α
Pichia stipitis NRRL Y-11543	*xynA*	pUC19	*E. coli* DH5αF′
Thermomyces lanuginosus DSM 5826	*xynA*	pBluescript II SK(−)	*E. coli* SURE
Trichoderma harzianum E-58	*xyn2*	pUC18, FLAG	*E. coli* DH10B
Trichoderma reesei Rut C-30	*xyn2*	pET-28a	*E. coli* BL21 (DE3)

The main limitation of using *E. coli* as expression host is that not every protein is secreted efficiently. RT-PCR amplification *xynIII* gene from *T. reesei* was expressed in *E. coli* JM109 transformed with pAG9-3xyn3 (Ogasawara *et al.*, 2006).

An activity was observed in the cell free extract and higher activity (13.2 U/ml medium) was recovered from inclusion bodies in cell debris. cDNA clones of the fungal xylanases are expressed in *E. coli* as intron processing is absent in the host. In many cases, the recombinant xylanases expressed in *E. coli* accumulate in the cytoplasm or periplasm (Schlacher *et al.*, 1996).

Although *E. coli* has also been used as an expression host successfully by some researchers for xylanase genes of fungal origin, eukaryotic genes are usually not expressed in *E. coli.* In 2001, Basaren and co-workers expressed a β-xylanase from *P. stipitis* under its own promoter in *E. coli*, the enzyme activity was significantly lower (4 U/mg) compared to the activity from the parent strain (30 U/mg) (Basaran *et al.*, 2001). Absent of post-translational modifications may also be a reason for the low levels of activity of the recombinant enzymes (Wong *et al.*, 1988). Lack of glycosylation in *E. coli* is an important factor that accounts for lower affinity of the enzyme with substrate and decrease in stability of enzyme. For these reasons, yeasts are attractive hosts for expressing heterologous protein from fungus.

2. Yeast expression system

Production procedures had to be developed that employ platforms which meet both the demand for efficient mass production and criteria of safety and authenticity of the produced compounds (Gellissen, Strasser*, et al.*, 2005). Although *E. coli* is still the first choice for genetic manipulation and expression of heterologous proteins, yeasts also have some attractive features. Expression of proteins in yeast systems provides additional benefits over bacterial expression systems such as ability to perform eukaryotic post-translational modifications, ability to grow to very high cell

densities and ability to secrete proteins into fermentatation media (Juturu and Wu, 2012). Moreover, many types of yeast are considered as non-toxigenic, non-pathogenic and several processes based on this organism were calssified as Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA, USA). In this respect, yeasts offer considerable advantages over alternative microbial and eukaryotic cellular systems in providing low-cost screening and production systems for authentically processed and modified compounds (Gellissen, Kunze, *et al.*, 2005). *Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis* and *Yarrowia lipolytica* are commonly used for heterologous protein expressions (Buckholz and Gleeson, 1991).

Saccharomyces cerevisiae

The initial yeast system developed for heterologous gene expression was based on the bakers yeast *Saccharomyces cerevisiae. S. cerevisiae* has been used for thousands of years by mankind in brewing and baking. This yeast is an ideal organism accessible to various genetic manipulations due to a well defined life cycle, development of transformation protocols, availability of suitable auxotrophic strains and availability of vector systems. All these properties have been put together for the production of foreign proteins for medical, research and industrial use. Recombinant protein expression was first described in 1981 for human α –interferon (Hitzeman *et al.*, 1981). This platform has been successfully applied to the production of various FDA-approved pharmaceuticals, including insulin (Melmer, 2005) and HBsAg (Harford *et al.*, 1986). A large number of xylanases from filamentous fungi

Aspergillus niger, A. nidulans, A. kawachii, Crptococcus flavus and *Penicillium purpurogenum* were successfully expressed in this yeast (Table II-11).

Table II-11 Cloning of different fungal xylanase genes in *S. cerevisiae* (Ahmed *et al.*, 2009)

Source organism	Gene	Vector	Host
Aspergillus kawachii IFO4308	*xynA*	pG3	*Saccharomyces cerevisiae* DBY747
Aspergillus kawachii IFO4308	*xyn3*	Multicopy episomal plasmid	*Saccharomyces cerevisiae*
Aspergillus nidulans G191	*xlnA* *xlnB*	Yeplac181	*Saccharomyces cerevisiae* OL1
Aspergillus niger ATCC 90196	*xyn4* *xyn5*	pDLG1	*Saccharomyces cerevisiae* Y294
Aureobasidium pullulans	*xynA*	pYES2	*Saccharomyces cerevisiae* INSC1
Aureobasidium pullulans var. *melanigenum* ATCC 20524	*xyn1*	pYES2	*Saccharomyces cerevisiae* INVSc1
Cryptococcus flavus I-11	*Cfxyn1*	Yep351PGK	*Saccharomyces cerevisiae* MFL
Penicillium purpurogenum ATCC MYA-38	*xynA*	pYEplac181	*Saccharomyces cerevisiae* YM335:: RY171

However, when using this system, certain limitations that have diminished its applications as a host for heterologous expression. These are instability of recombinant plasmid DNA, need for fed-batch fermentation for attaining high cell densities, hyperglycosylation of secreted proteins and retention of proteins within periplasmic space decreasing yields of protein expression (Buckholz and Gleeson, 1991). Hyperglycosylate recombinant proteins; N-linked carbohydrate chains are

terminated by mannose attached to the chain via an α-1,3 bond, which is considered to be allergenic (Gellissen, Kunze, *et al.*, 2005). Therefore, other yeast expression systems have been developed (Mishra and Baranwal, 2009).

Pichia pastoris

The use of the methylotrophic yeast, *Pichia pastoris*, as a cellular host for the expression of recombinant proteins has become increasing popular in recent times. The Phillips Petroleum Company was the first to develop media and protocols for growing *P. pastoris* on methanol in continuous culture at high cell densities (>130 g/ L dry cell weight (Cereghino and Cregg, 2000). During the 1970s, *P. pastoris* was evaluated as a potential source of single-cell protein due to the ability of this yeast to utilize methanol as sole carbon source. In the following decade, Phillips Petroleum, together with the Salk Institute Biotechnology/ Industrial Associates Inc. (SIBIA, La Jolla, CA, USA), studied *P. pastoris* as a system for heterologous protein expression. The gene and promoter for alcohol oxidase were isolated by SIBIA, who also generated vectors, strains and corresponding protocols for the molecular manipulation of *P. pastoris*. In 1993, *P. pastoris* expression system is available in kit form from Invitrogen Corporation (Carlsbad, CA, USA), the consequence of which has been an explosion in the knowledge base on the system as described in numerous recent publications (Macauley-Patrick *et al.*, 2005). Heterologous gene expression by *P. pastoris* is a useful alternative to produce genetically engineered enzymes for research and even for the industrial purposes (Berrin *et al.*, 2000).

Heterologous gene expression by *P. pastoris* has several advantages over *S. cerevisiae* as a host for heterologous expression because of high secretion

efficiency, high cell densities attained in inexpensive culture media, the relative ease of scale up of industrial process and *Pichia* also secrete very low levels of native proteins, thus making it easier to recover the foreign secreted protein from the fermentation fluid and serves as the first step in purification of the protein (Barr *et al.*, 1992; Macauley-Patrick *et al.*, 2005). It is a particularly attractive expression host due to the availability of strong and regulatory promoters that are involved in methanol metabolism (Gellissen, 2000; Tsai and Huang, 2008).

A. niger xylanase (*XylA*) was the first microbial xylanase efficiently secreted and correctly processed using the methylotrophic *P. pastoris* by Berrin and co-workers (Berrin *et al.*, 2000). Recombinant XylA *P. pastoris* showed 30% higher activity compared to native XylA *A. niger*. In 2012, Fan and co-workers expressed a xylanase gene from the thermophilic fungus *Paecilomyces thermophila* in the methylotrophic *P. pastoris*, the XynA activity was higher (300 U/ml) than expression in *E. coli* (8.3 U/ml) by Zhang and co-workers (Fan *et al.*, 2012; Zhang *et al.*, 2010). Expression of *P. citrinum xynB* cDNA in *P. pastoris*, the tranformant showed a xylanase activity of 5.74 U/ml in the culture supernatant after 5 days induction by methanol which higher than the native xynB *P. citrinum* for 1.4 folds (4.15 U/ml) (Wakiyama *et al.*, 2008). Cloning of different fungal xylanase genes in *P. pastoris* is shown in Table II-12.

Table II-12 Cloning of different fungal xylanase genes in *P. pastoris* (Ahmed *et al.*, 2009)

Source organism	Gene	Vector	Host
Aspergillus niger	*anxA*	pPIC9K	*P. pastoris* GS115
Aspergillus niger	*xylA*	pHIL-D2	*P. pastoris* GS115
Aspergillus niger BCC14405	*xylB*	pPICZαA	*P. pastoris* KM71
Aspergillus niger CGMCC1067	*xynB*	pGAPZαA	*P. pastoris* X33
Aspergillus niger IBT-90	*xyn6*	pPICZB	*P. pastoris* GS115
	xynB	pPICZαA	
Aspergillus sulphureus	Endo-β-1, 4-xylanase	pGAPZαA	*P. pastoris* X33
Aspergillus terreus BCC129	*xyn10*	pPICZαA	*P. pastoris* KM71
Aureobasidium pullulans ATCC20524	*xynII*	pPIC3.5	*P. pastoris* GS115
Aureobasidium pullulans	*xyl6*	pPIC3.5 pHILS1 pPIC9	*P. pastoris* GS115
Cryptovalsa mangrovei BCC7197	Xylanase 10	pPICZα A (Invitrogen)	*P. pastoris* KM71
Lentinula edodes CS-2	*xyn11A*	pGAPZαA	*P. pastoris* GS115
Magnaporthe grisea CP987	*xyl6*	pPicH	*P. pastoris*
Neocallimastix frontalis	*Xyn11B*	pPK9K	*P. pastoris* KM71
Penicillium citrinum	*xynA*	pPIC3.5	*P. pastoris* GS115

Yarrowia lipolytica

In addition to *P. pastoris*, oleaginous yeast *Yarrowia lipolytica* is an upcoming protein expression host. Application of this organism for heterologous protein expression has multiple advantages such as growth capacity, ability of this organism to metabolize glucose, alcohols, acetate and hydrophobic substrates such as alkanes, fatty acids and oils, well-characterized secretory system that yields high levels of recombinant proteins, glycosylation resembling mammalian system, easy screening for multi-copy strains using defective selection marker and usage of a single integration site (Juturu and Wu, 2012; Madzak *et al.*, 2004; Müller *et al.*, 1998). Expression of foreign proteins is achieved through the use of shuttle vectors (Gellissen, Kunze*, et al.*, 2005). Integration of a linearized plasmid into the *Y. lipolytica* genome generally occurs by homologous recombination, which results in a high transformation efficiency and the accurate targeting of the monocopy integration into the genome (Gellissen, Strasser*, et al.*, 2005). Comparison of expression systems in *Yarrowia lipolytica* and four other yeasts (*Saccharomyces cerevisiae, Hansenula polymorpha, Klyveromyces lactis and Schizosaccharomyces pombe*) the comparison was carried out on six fungal genes encoding the enzymes galactanase, lipase, polygalacturonase, xylanase and two cellulases by Müller and coworkers in 1998. They also developed novel promoter *TEF* and *RPS7* instead of *XPR2* promoter. They concluded that with the using novel *TEF* promoter, *Y. lipolytica* was shown to be the most effective protein expression system (Müller *et al.*, 1998). General characteristics and genomic information of *S. cereviciae, P. pastoris* and *Y. lipolytica* developed as hosts for recombinant protein production are shown in Table II-13.

Table II-13 General characteristics and genomic information of various yeasts developed as hosts for recombinant protein production (Kim *et al.*, 2015)

Yeast species	Attributes
Saccharomyces cerevisiae	Favorable public acceptance GRAS status The most well studied of simple eukaryotes Amenable to both classical genetics and modern recombinant DNA techniques Versatile vector systems (episomal, integrative, copy-number regulated) are available (Invitrogen) A wide range of mutant strains Well-established fermentation and downstream processing Hypermannosylation with immunogenic terminal α-1,3-linked mannose residues Genome sequencing: Reference strain S288C; 12 157 Kb (6273 ORFs); Accession number PRJNA128
Pichia pastoris	GRAS status Tightly regulated, methanol-inducible AOX promoters A Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes Can grow rapidly on inexpensive media at high cell densities (up to 150 g DCW L^{-1}) Integrated vectors developed that help genetic stability of the recombinant elements, even in continuous and large-scale fermentation processes Well-established commercial vector systems and host strains (Invitrogen) A lesser extent of hypermannosylation compared to *S. cerevisiae*; No terminal α-1,3-linked mannose residues Genome sequencing: Reference strain GS115; 9216 Kb (5040 ORFs); Accession number PRJNA39439, PRJEA37871
Yarrowia lipolytica	An oleaginous yeast, based on its ability to accumulate large amounts of lipids GRAS status Can grow in hydrophobic environments, that is able to metabolize triglycerides, fatty acids, *n*-alkanes, and *n*-paraffins as carbon sources for the bioremediation of environments contaminated with oil spills Can secrete a variety of proteins via cotranslational translocation and efficient secretion signal recognition similar to higher eukaryotes Availability of a commercial expression kit (YEASTERN BIOTECH CO., LTD.) Salt tolerance A lesser extent of hypermannosylation compared to *S. cerevisiae*; a lack of the immunogenic terminal α-1,3-mannose linkages Genome sequencing: Reference strain CLIB122; 20 503 Kb (7042 ORFs); Accession number PRJNA12414

3. *Pichia pastoris* expression platform

3.1 Characteristics

The use of the methylotrophic yeast, *P. pastoris*, as an excellent host for the expression of recombinant proteins provides additional benefits include (Li *et al.*, 2007; Macauley-Patrick *et al.*, 2005) :

1) Rapid growth rate to high cell density in inexpensive and non-complex culture medium

2) High level of expression

3) Ease of genetic manipulation

4) The availability of an efficient host and vector system

5) The existence of tightly controlled (repressed by glucose and depressed by glycerol) and highly inducible promoters

6) The capability of eukaryotic post-translation modification processes such as polypeptide folding, glycosylation, methylation, acylation, sulphation, phosphorylation, lipidation, proteolytic adjustment, and targeting to subcellular compartments

7) The absence of known proteases concomitant to a very low level of endogenous proteins, allowing easy purification.

3.2 Expression vectors

The insertion of a foreign coding sequence into the expression vector is usually carried out in *E. coli*. The expression vectors have been designed as *E. coli/P. pastoris* shuttle vectors having origin of replication and selection markers for propagation in both of the hosts (Cereghino and Cregg, 2000). They contain at the same time an origin of replication for plasmid maintenance in *E. coli*, multi-cloning sites, a promoter to drive the expression of the gene of interest in the yeast and selectable markers functional in one or both organisms (antibiotic resistance genes, auxotrophy markers). The list of expression vectors for *P. pastoris* heterologous expression is presented in table II-14.

Table II-14 *P. pastoris* expression vectors (Higgins and Cregg, 1998)

Vector name	Selectable markers	Features
Intracellular		
pHIL-D2	*HIS4*	*Not*I sites for *AOX1* gene replacement
pAO815	*HIS4*	Expression cassette bounded by *Bam*HI and *Bgl*II sites for generation of multicopy expression vector
pPIC3K	*HIS4* and *kan*r	Multiple cloning sites for insertion of foreign genes; G418 selection for multicopy strains
pPICZ	*ble*r	Multiple cloning sites for insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags
pHWO10	*HIS4*	Expression controlled by constitutive *GAPp*
pGAPZ	*ble*r	Expression controlled by constitutive *GAPp*; multiple cloning site or insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags
Secretion		
pHIL-S1	*HIS4*	*AOX1p* fused to *PHO1* secretion signal; *Xho*I, *Eco*RI, and *Bam*HI sites available for insertion of foreign genes
pPIC9K	*HIS4* and *kan*r	*AOX1p* fused to α-MF prepro signal sequence; *Xho*I (not unique), *Eco*RI, *Not*I, *Sna*BI and *Avr*II sites available for insertion of foreign genes; G418 selection for multicopy strains
pPICZα	*ble*r	*AOX1p* fused to α-MF prepro signal sequence; multiple cloning site for insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags
pGAPZα	*ble*r	Expression controlled by constitutive *GAPp*; *GAPp* fused to α-MF prepro signal sequence; multiple cloning site for insertion of foreign genes; Zeocin selection for multicopy strains; potential for fusion of foreign protein to His_6 and *myc* epitope tags

A shuttle vector pPICZαA is the vector of choice by most of the workers and contains tightly regulated *AOX1* promoter (Ruanglek *et al.*, 2007). They integrate at a specific site in either single or multi-copy. Gene insertion events at the *AOX1* loci arise from a single crossover event between the loci and either of the two *AOX1* regions on the pPICZα vectors: the *AOX1* promoter or the *AOX1* transcription termination region (TT) (Figure II-8). A shuttle vector pGAPZαA contains constitutive *GAP* promoter. Gene insertion events at the *GAP* promoter locus arise from a single crossover event between the locus and the P_{GAP} region on the pGAPZα vectors (Figure II-8). Both vectors contain sequences encoding a secretion signal that are in frame with the foreign gene for secretion of foreign proteins. This secretion signal derives from *S. cerevisiae* α-mating factor (α-MF) (Li *et al.*, 2007).
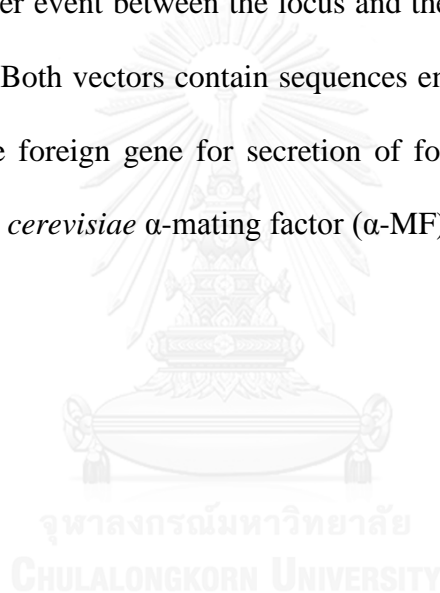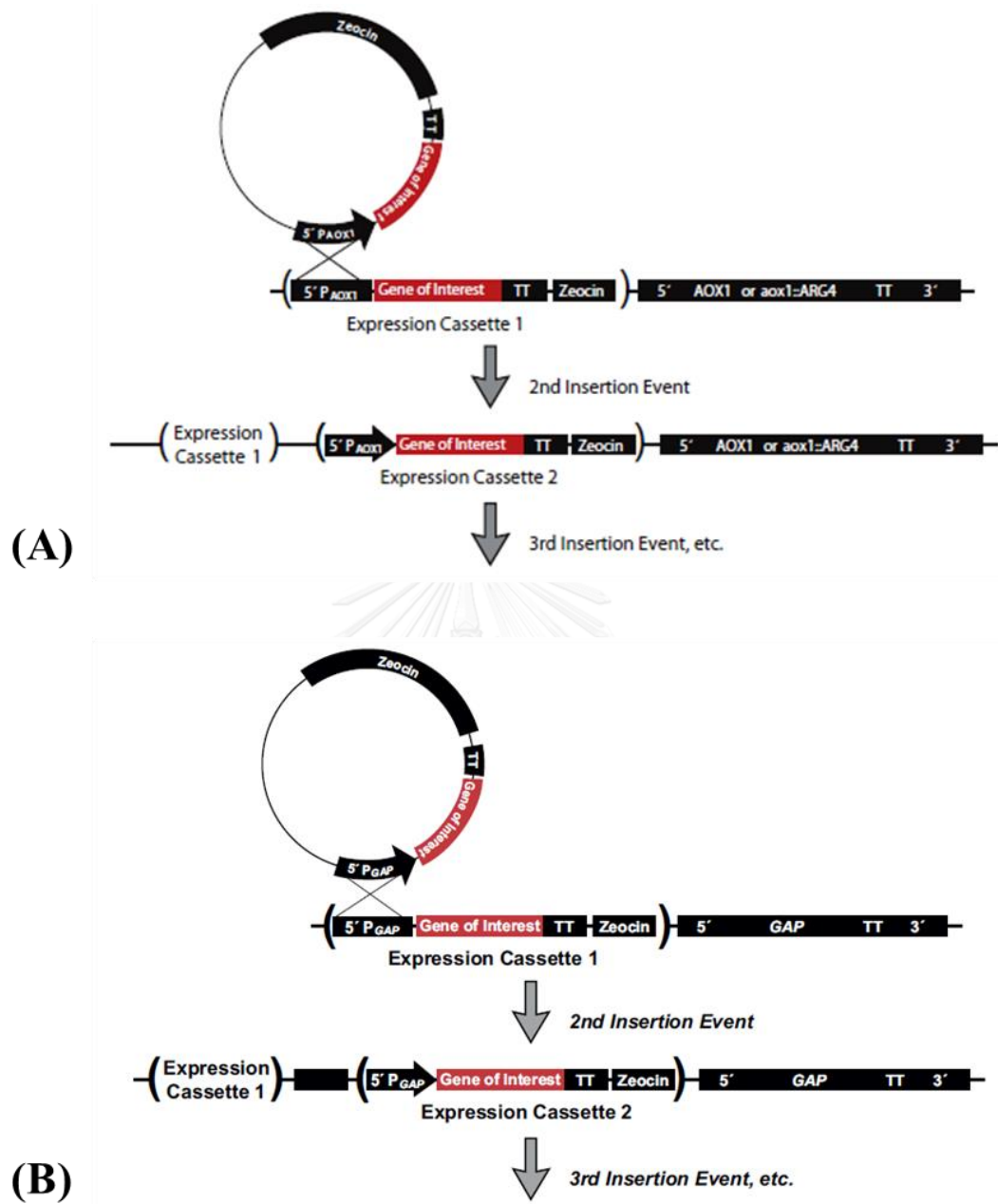
Figure II-8 Mechanisms of integration of expression cassettes in *P. pastoris* genome.

(A) Insertion of gene at *AOX1* locus. (B) Insertion of gene at *GAP* locus. (Invitrogen)

3.3 Promoters

Alcohol oxidase (AOX) is the enzyme that catalyzes the first step in the methanol utilization pathway (Egli *et al.*, 1980). It is encoded by two genes in *P. pastoris*: *AOX1* and *AOX2*. The *AOX1* gene accounts for the majority of *AOX* activity in the cell (Cregg *et al.*, 1989). It is synthesized in large amounts (up to 30% of total protein). Therefore, *AOX1* is a promoter of choice by most researchers for the expression of xylanase genes in *P. pastoris* as it gives high levels of xylanase expression under methanol induction.

The alcohol oxidase I (*AOX1*) promoter is one of the strongest and tightly regulated promoters known to man. This promoter is repressed by glucose, derepressed by glycerol and induced by methanol which can serve to regulate induction of expression of foreign proteins (Gellissen, Kunze*, et al.*, 2005). Aerobic growth with methanol induction ends up in high levels of recombinant proteins with *AOX1* promoter system (Müller *et al.*, 1998). Multi-copy strains with the recombinant gene under the *AOX1* promoter control could be the choice for industrial scale protein expression (Juturu and Wu, 2012).

Cheng *et al.*, (2005) and Chantasingh *et al.,* (2006) attained high xylanase activity (342.2 U/ml and 238.5 mg/ml, respectively) under this promoter (67-fold and 4-fold higher recombinant xylanase activity, respectively, compared to the native fungal xylanases) (Chantasingh *et al.*, 2006; Cheng *et al.*, 2005). However, this promoter may not be suitable under certain industrial applications. For instance, it is not appropriate to use methanol for the induction of genes in the food industry due to its toxicity. Moreover, it may be a potential fire hazard in large-scale fermentations (Ahmed *et al.*, 2009).

The *P. pastoris* glyceraldehyde 3-phosphate dehydrogenase (*GAP*) gene promoter provides strong constitutive expression on glucose at a level comparable to that seen with the *AOX1* promoter (Waterham *et al.*, 1997). *GAP* promoter activity levels in glycerol- and methanol-grown cells are approximately two-thirds and one-third of the level observed for glucose, respectively. The advantage of using the *GAP* promoter is that methanol is not required for induction, nor is it necessary to shift cultures from one carbon source to another, making strain growth more straightforward and this promoter is more suitable for large-scale production of heterologous recombinant proteins (Cereghino and Cregg, 2000; Zhang *et al.*, 2009).

A constitutive *GAP* gene promoter has been employed successfully for the xylanase gene expression in *P. pastoris* by some workers (Cao *et al.*, 2007; Deng *et al.*, 2006; Lee *et al.*, 2005). Deng *et al.*, (2006) obtained 50-fold increase in *XynB* expression under this promoter compared to the expression of the enzyme by the native species *A. niger* (Deng *et al.*, 2006).

3.4 Secretion signals

Heterologous expression in *Pichia pastoris* can be either intracellular or secreted. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. While several different secretion signal sequences have been used successfully, including the native secretion signal present on some heterologous proteins, success has been variable. In general, yeast signal sequences are more likely to be successful and yield 2-3 fold higher extracellular production than native signal peptide. Alpha mating factor (α-MF) signal peptide from *S. cerevisiae* is the most widely used and successful secretion signal with the

most success (Cregg *et al.*, 1993; Scorer *et al.*, 1993). It consists of a pre-sequence of 19-amino acid followed by a pro-region of 66 residues. Three N-linked glycosylation sites and a Kex2 endopeptidase processing site are present on the (pro) sequence (Cereghino and Cregg, 2000; Kurjan and Herskowitz, 1982). Alternative signal sequences exist such as those from *H. Polymorpha* acid phosphatase *PHO1*, *S. cerevisiae* invertase *SUC2*, and *Phaseolus vulgaris* phytohaemagglutinin *PHA-E* (Macauley-Patrick *et al.*, 2005). The amount of recombinant Xyn1 secreted into the culture medium of *P. pastoris* under the native signal was found comparable to that secreted with α-MF signal peptide and two-fold higher than that secreted by *PHO1* signal peptide (Tanaka *et al.*, 2004).

3.5 Selection markers

A number of selectable marker genes are known for the molecular genetic manipulation of *P. pastoris*, as represented in Table II-15. There are two groups of selectable markers used in *P. pastoris* and other yeast expression systems including biosynthetic pathway genes and drug resistance gene. Initially, markers were limited to biosynthetic pathway genes including *HIS4* (histidinol dehydrogenase gene) from either *P. pastoris* or *S. cerevisiae*, *ARG4* (argininosuccinate lyase gene) from *S. cerevisiae* and the *Shble* gene from *Streptoalloteichus hindustanus* which confers resistance to the bleomycin-related drug zeocin (Cregg *et al.*, 1985; Higgins and Cregg, 1998).

After that, a number of biosynthetic pathway genes have been developed such as *URA3* (orotidine 5'- phosphate decarboxylase) and *ADE1* (PR-amidoimidazolesuccinocarboximide synthase). Alternatively, drug resistance gene

such as *Aspergillus tererus* blasticidin S deaminase gene or *Sorangium cellulosum* acetyl-CoA carboxylase gene are also used to select recombinant clones. Among these markers, the zeocin resistant gene is the most widely used because of its small size, and its easy use in both *E. coli* and *P. pastoris* (Li *et al.*, 2007; Macauley-Patrick *et al.*, 2005; Nel *et al.*, 2009).

Table II-15 Selectable marker genes for use with the *Pichia pastoris* expression system (Macauley-Patrick *et al.*, 2005)

Gene	Origin	Function
HIS4 (histidinol dehydrogenase gene)	P. pastoris or S. cerevisiae	Biosynthetic pathway gene
ARG4 (argininosuccinate lyase gene)	S. cerevisiae	Biosynthetic pathway gene
Zeo^R (zeocin resistance gene)	Streptoalloteichus hindustanus	Confers resistance to the bleomycin-related drug
Blasticidin S deaminase gene	Aspergillus tererus	Confers resistance to the drug blasticidin
ADE1 –PR-amidoimidazolesuccinocarboximide synthase	P. pastoris	Biosynthetic pathway gene
URA3–orotidine 5′-phosphate decarboxylase	P. pastoris	Biosynthetic pathway gene
Sor^R–acetyl-CoA carboxylase	Sorangium cellulosum	Confers resistance to the macrocyclic polyketide soraphen A

4. *Yarrowia lipolytica* expression platform

4.1 Characteristics

The hemiascomycetous yeast *Y. lipolytica* has been formerly known as *Candida, Endomycopsis* or *Saccharomycopsis lipolytica*. *Y. lipolytica* is a non-pathogenic yeast and classified as generally regarded as safe (GRAS) by the Food and Drug Administration (FDA, USA). It is naturally found in high lipid containing products such as cheeses, dairy products, plant oils and sewages (Madzak *et al.*, 2004). It is a dimorphic that grows as budding cells, hyphae or pseudohyphae depending on growth conditions. *Y. lipolytica* can use glucose (but not sucrose

because it does not have invertase), alcohols, acetate and hydrophobic substrates (such as alkanes, fatty acids and oils) as carbon sources. It is strictly aerobic and grows at temperatures ranging from 5°C to 30°C (Barth and Gaillardin, 1997). The genome of *Y. lipolytica* is organized in 6 chromosomes, representing a total of 20.5 Mb (much more than *S. cerevisiae* and *S. pombe*), with size varying between 2.6 and 4.9 Mb. No plasmidic DNA was identified (Casaregola *et al.*, 2000; Dujon *et al.*, 2004). Proteins from viruses and different organisms, ranging in size from 6 to 116 kDa, have been expressed successfully in this yeast (Mishra and Baranwal, 2009). *Y. lipolytica* has become a reliable and popular system for heterologous protein expression in both academic purposes and commercial applications because of high transformation efficiency, plasmid stability, high secretion efficiency, good product yield, performance reproducibility and facilitated high-throughput screening (Bordes *et al.*, 2007; Müller *et al.*, 1998).

4.2 Expression vectors

The transforming vectors for expression in *Y. lipolytica* are shuttles vectors which allowing at the same time replication in bacterial hosts and expression in yeast. Integrative vectors used to transport foreign genes function by simple crossing-over at homologous site such as *LEU2, URA3* in the ribosomal DNA or the platform of chromosomal integration. Random integration in the genome can also be carried out by non-homologous insertion (Hamsa and Chattoo, 1994), due zeta zones (the long terminal repeat zeta of the *Y. lipolytica* retrotransposon Ylt1) flanking the expression cassette (Nicaud *et al.*, 2002; Pignède *et al.*, 2000). A technique of transformation by lithium acetate method is possible to obtain a good effectiveness of

transformation (Xuan *et al.*, 1988). Transformation of competent *Y. lipolytica* with linearized integrative expression cassettes based on a single crossover recombination event (using the lithium acetate method) resulted in transformation efficiencies of up to 10^6 transformants/μg DNA where more than 80 % of the transformants will harbor a single copy of the expression cassette integrated at the correct site into the genome and results in a stable integration as the gene is retained in the genome after more than 100 generations without needing a pressure of selection (Barth and Gaillardin, 1997; Madzak *et al.*, 2004; Xuan *et al.*, 1988). The list of these elements is showed in Table II-16.

Table II-16 Components available for *Y. lipolytica* expression/secretion vectors (most commonly used and most interesting items are underlined) (Madzak *et al.*, 2004).

Component[a]	Characteristics
Marker genes	
LEU2, URA3, LYS5, ADE1	Auxotrophy complementation
ura3d4	Item + copy number amplification (cf. text)
Phleo[R], hph (E. coli)	Antibiotic resistance (respectively, to phleomycin and hygromycin B)
SUC2 (S. cerevisiae)	Sugar utilization
Promoters (source)	
pLEU2 (β-isopropylmalate dehydrogenase)	Inducible by leucine precursor
pXPR2 (alkaline extracellular protease)	Inducible by peptones
pPOX2, pPOT1 (respectively, acyl-CoA oxidase 2, 3-oxo-acyl-CoA thiolase)	Inducible by fatty acids and derivatives, and alkanes
pICL1 (isocitrate lyase)	Inducible by fatty acids and derivatives, alkanes, ethanol and acetate
pPOX1, pPOX5 (acyl-CoA oxidases 1 and 5)	Weakly inducible by alkanes
pG3P (glycerol-3-phosphate dehydrogenase)	Inducible by glycerol
pMTP (bidirectional: metallothioneins 1 and 2)	Inducible by metallic salts
hp4d (hybrid promoter derived from pXPR2)	Growth-phase-dependent
pTEF, pRPS7 (respectively, translation elongation factor-1α, ribosomal protein S7)	Constitutive
Secretion signals[b]	
Native	Frequently efficient in Y. lipolytica
XPR2 prepro	13 aa pre/10 XA or XP dipeptides/122 aa pro/KR cleavage site
XPR2 pre + dipeptide	13 aa pre/5–10 XA or XP dipeptides
XPR2 pre	13 aa pre/LA cleavage site
LIP2 prepro	13 aa pre/4 XA or XP dipeptides/10 aa pro/KR cleavage site
Terminators	
XPR2t, LIP2t, PHO5t	Respectively, 430, 150 and 320 bp fragments
Minimal XPR2t	PCR-synthesized 100 bp fragment with added restriction sites
Elements for maintenance in yeast cells	
ARS18, ARS68	Autonomously replicative vectors can maintain only 1–3 copies/cell[c]
Homology to genome	Homologous integration (in LEU2, URA3, XPR2 terminator, rDNA, or, when present, in zeta or pBR322 docking platform)
Zeta (Ylt1 LTR)	Non-homologous integration in Ylt1-devoid strains (cf. text)

4.3 Promoters

The native form of the *XPR2* promoter (p*XPR2*) was initially applied to the expression of heterologous proteins. However, its complex regulation hindered its industrial use. This strong promoter is active only at pH above 6, on media lacking preferred carbon and nitrogen sources, and its full induction requires high levels of peptones in the culture medium. The functional dissection of the p*XPR2* showed that one of its upstream activating sequences, UAS1, was poorly affected by environmental conditions (Blanchin-Roland *et al.*, 1994; Madzak *et al.*, 2004). Muller et al. (1998) described two novel promoters isolated from *Y. lipolytica*. These promoters, from *TEF* (translation elongation Factor-1 alpha) and *RPS7* (ribosomal protein S7) genes, are strong constitutive promoters, leading to increased numbers of positive clones after transformation and producing sufficient amounts of protein for characterization (Müller *et al.*, 1998). Inducible and strong promoters were also described such as the promoter from isocitrate lyase (*ICL*), acyl-CoA oxidase (*POX*) and 3-oxo-acyl-CoA thiolase (*POT*). These promoters activate the expression of enzymes involved in the metabolism of hydrophobic substrates and are thus inducible by alkanes and fatty-acids (Madzak *et al.*, 2004).

4.4 Secretion signals

Secretion signal is needed to target the heterologous protein to the secretion pathway. The most commonly used secretion signal peptide are the prepro sequence of the *XPR2* gene, which encodes for the extracellular alkaline protease and the prepro sequence the *LIP2* gene (Madzak *et al.*, 2004; Müller *et al.*, 1998).

4.5 Selection markers

Y. lipolytica is resistant to most commonly used drugs with the exception of a few antibiotics such as bleomycin/phleomycin (Gaillardin and Ribet, 1987) and hygromycin B (Otero and Gaillardin, 1996). Thus, the selection by complementation of auxotroph strains (mainly for *LEU2* and *URA3*) remains the mostly used (Barth and Gaillardin, 1997).

CHAPTER III

MATERIALS AND METHODS

PART I Cloning, expression, purification and characterization of endo-1,4-β-xylanase from *Penicillium citrinum* in *Pichia pastoris*

1.1 Organism and culture conditions

The bacterial strain Escherichia coli DH5α (Gibco) was used for vector propagation. *E. coli* was grown in a Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37°C. Kanamycin (50 µg/ml) was added as a selective agent for pUC57-Kan selection. Zeocin (Invitrogen) (25 µg/ml) was added as a selective agent for pGAPZα A and pPICZα A selection.

P. pastoris X-33[(mut+)] (Invitrogen) was cultured in yeast extract-peptone-dextrose (YPD) liquid medium (1% yeast extract, 2% bacteriological peptone and 2% dextrose) at 30°C and shaken at 200 rpm. The transformants were selected on YPDS plates (YPD with 1 M sorbitol) containing 100-2,000 µg/ml Zeocin.

1.2 DNA manipulations

A codon-optimized *Penicillium citrinum xynA* gene was synthesized and introduced into a pUC57-Kan vector by GenScript based on the nucleotide database (GenBank: accession no. AB198065.1) (Tanaka *et al.*, 2005). Constitutive expression vector (pGAPZα A), methanol-inducible expression vector (pPICZα A) were all provided by Invitrogen (Figure III-1). The synthetic *xynA* gene was amplified without

a signal peptide or stop codon from pUC57-Kan containing optimized codon *xynA* gene by PCR using the following specific primers: *xynA*_Pc-F (5´-GCGGTACCGAGTCATATACTTCTTCCTCAACC-3´) and *xynA*_Pc-R (5´-GAGCGGCCGCGGAAACGGTAATGTCAGCG-3´). *Kpn*I-HF and *Not*I-HF restriction sites (underlined) were added to the forward and reverse primers, respectively, which allowed the *xynA* gene to be ligated in frame with the α-factor secretion signal sequence and the C-terminal peptide in expression vectors. Reaction mixtures contained 1x Phusion HF buffer, 200 μM each dNTPs, 0.5 μM each of primers , 10 ng of template and 1 U of Phusion High-Fideelity DNA polymerase (New England BioLabs) in a total volume of 50 μl. The PCR conditions were as follows: at 98°C for 30 s, 30 cycles of 98°C for 10 s, 54.1 °C for 30 s and 72°C for 20 s and followed by one cycle of 72°C for 10 min. The amplified fragment was purified and then digested with *Kpn*I-HF and *Not*I-HF before cloning into expression vectors predigested with the same restriction enzymes and transformation into *E. coli* DH5α competent cells. The constructed plasmid designated as pGAPZαA-*xynAPc* and pPICZαA-*xynAPc* were screened on low-salt LB agar plate (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar) containing 25 μg/ml Zeocin to detect the presence of the recombinant plasmid. The insertion was verified by double restriction enzyme digestion and sequencing using the α-factor and 3´AOX sequencing primers (Invitrogen) by Pacific Science (Bangkok, Thailand).
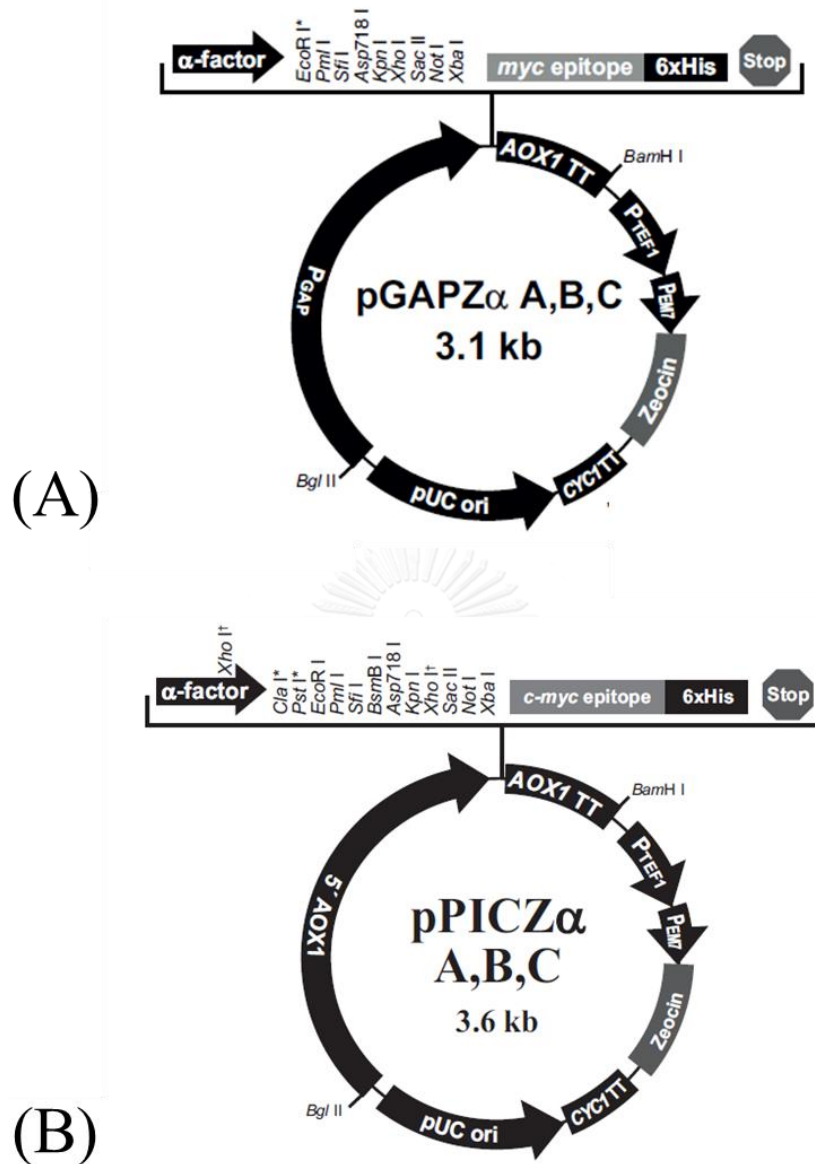
Figure III-1 The features of the pGAPZα A vector (constitutive expression vector) (A) and the pPICZα A vector (methanol-inducible expression vector) (B). Both vectors contain α-factor secretion signal for efficient secretion of most proteins from *Pichia* and Zeocin resistance gene for selection (Invitrogen).

1.3 Yeast transformation and selection

The pGAPZαA-*xynAPc* and pPICZαA-*xynAPc* plasmid were linearlized with *Avr*II and *Pme*I, respectively, for integration into *P. pastoris* genome. Each linearized DNA was transformed into *P. pastoris* x-33 strain using the lithium acetate and dithiothreitol method by electroporation (Wu and Letchworth, 2004). About 10 µg linearized plasmid combined with 25 µg salmon sperm DNA (Invitrogen) were used to transform 90 µl competent *P. pastoris* x-33$^{(mut+)}$ cells using Gene Pulser Xcell (Bio-Rad), and immediately recovered in 1 ml of 1 M sorbitol for 2 h at 30°C. The transformants were selected on YPDS agar plate containing 100-2,000 µg/ml Zeocin. The transformants under the control of p*GAP* were chosen for preliminary selection on YP with 0.2% Azo-xylan agar plate.

1.4 Heterologous expression of *xynA* gene in *P. pastoris*

The expression of *xynA* gene in *P. patoris* was achieved according to the supplier's instructions (Invitrogen). For the constitutive expression (p*GAP*), *P. pastoris* transformants were cultured in YPD medium for 4 days, whereas for the methanol-inducible expression (pPIC), *P. pastoris* transformants were employed by using buffered glycerol-complex medium (BMGY: 1% yeast extract, 2% peptone, 100 mM KH_2PO_4/KOH (pH 6.0), 1.34% Yeast Nitrogen Base, 4 x 10^{-5}% biotin and 1% glycerol) and buffered minimal-methanol medium (BMMY: 1% yeast extract, 2% peptone, 100 mM KH_2PO_4/KOH (pH 6.0), 1.34% Yeast Nitrogen Base, 4 x 10^{-5}% biotin and 0.5% methanol). Absolute methanol was added every 24 h to maintain induction for 5 days. The final concentration of methanol was varied from 0.5 to 1% in BMMY medium and 0.5 to 3% for daily induction to optimize the methanol

concentration upon the obtained recombinant protein expression level. All enzyme expressions were operated in baffled shake flask at 30°C with constant shaking at 200 rpm. The culture supernatant was collected at periodic intervals to analyze the expression levels and determine the optimal time post-induction to harvest. The maximum xylanase activity was selected for purification of recombinant Pp-xynA and characterization.

1.5 Xylanase activity assay and protein determination

The xylanase activity was assayed by following the release of reduced sugars from xylan. The reaction mixture consisted of 0.5 ml of 4% (w/v) birchwood xylan in deionized water, 0.4 ml of 0.15 M acetate buffer (pH 5) and 0.1 ml of suitably diluted enzyme. The reactions were incubated at 50°C for 5 min. Aliquots (75 μl) were taken every minute and stopped by adding an equal amount of DNS (3, 5-dinitrosalicylic acid) before boiling for 5 min (Miller, 1959). The reactions were cooled before adding 0.75 ml deionized water and the absorption was measured at 540 nm. Each absorbance was plotted on a linear graph. The equations of the reaction were used to calculate the xylanase activity. One unit of xylanase activity (U) was defined as the amount of enzyme that liberates 1 μmol of xylose equivalents per min per ml under the assay condition. Protein concentration was measured at 595 nm following the *DC* Protein Assay (Bio-Rad) using bovine serum albumin (BSA) as a standard.

1.6 Enzyme purification

All purification steps were performed at 4°C. Yeast cells from liquid cultures were sedimented by centrifugation at 4,500 x g for 10 min. The supernatant was

concentrated and exchanged for 20 mM acetate buffer (pH 5.5) by ultrafiltration through ultra centrifugal filter devices with 10 kDa cut-offs (Amicon) and loaded onto a HiLoad^{TM} 16/10 Q Sepharose High Performance column (GE Healthcare Life Sciences) previously equilibrated with 20 mM acetate buffer (pH 5.5). The adsorbed proteins were eluted at a flow rate of 2.0 ml/min with a linear gradient of 0 to 0.6 M NaCl in the same buffer.

1.7 SDS-PAGE and zymographic analysis

The purified recombinant Pp-xynA was subjected to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-Protean$^{®}$ TGX Stain-Free^{TM} gels to determine the molecular weight and confirm the purity of the existing enzymes. Precision Plus Protein Standards (Bio-Rad) ranging from 10 to 250 kDa were used as the reference protein.

The zymographic analysis for xylanase was performed on 10% polyacrylamide gel containing 1% Azo-xylan as substrate running at the same time with 10% polyacrylamide gel without Azo-xylan. The sample was mixed with the loading buffer without 2-mercaptoethanol and boiled at 70°C for 5 min. The electrophoresed gel containing 1% Azo-xylan was soaked in 25% isopropanol to remove SDS and renature protein and then washed with 60 mM acetate buffer pH 5 with gentle shaking at 4°C for 30 min, 4 times before incubating in the same buffer at room temperature overnight. The activity bands were observed as clear colorless areas.

1.8 Enzyme characterization

The optimum pH of the purified recombinant Pp-xynA activity was evaluated at 50°C over a pH range of 3.0 to 10.0 using appropriate buffer (60 mM):citric acid – sodium citrate buffer (pH 3.0-6.0), sodium phosphate buffer (pH 6.0-8.0) and sodium carbonate (pH 9.0-10.0). A pH profile was produced with the enzyme activity at the optimum pH set at 100%. The pH stability of the purified recombinant Pp-xynA was investigated by incubating in various pH buffers at room temperature for 24 h and the residual activity was measured under standard assay condition. The optimum temperature of the purified recombinant Pp-xynA activity was studied by incubating the purified enzyme at pH 5.0 and temperatures ranging from 24°C (room temperature) to 60°C. A temperature profile was produced with the enzyme activity at the optimum temperature set a 100%. Thermostability of the purified recombinant *xyn*Asyn was evaluated at temperatures of 40-60°C for different time periods before measuring the residual activity. The kinetic parameters (K_m, V_{max} and k_{cat}) of the purified recombinant Pp-xynA enzyme were determined using different substrate concentrations (0.1-2% xylan) with the same method described previously at pH 5.0, 40°C for 10 min. All assays were performed in triplicate and the mean values with their standard deviation (SD). Kinetic analyses were performed using Mechaelis-Menten and Lineweaver-Berk plots.

PART II Cloning, expression, purification and characterization of endo-1,4-β-xylanase from *Penicillium citrinum* in *Yarrowia lipolytica*

1.1 Organism and culture conditions

The bacterial strain Escherichia coli DH5α (Gibco) was used for vector propagation. *E. coli* was grown in a Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37°C. Kanamycin (50 µg/ml) was added as a selective agent for pUC57-Kan, pTEF_nat*XynAPc* and pTEF_fus*XynAPc* selection.

Y. lipolytica was cultured in YPD medium (1% yeast extract, 1% bactopeptone and 1% glucose) at 30°C and shaken at 130 rpm. Solid YPD medium contains 1.5% agar. To select for complementation (Ura$^+$) of auxotrophic mutant strains, transformants were grown on solid YNB medium (0.17% w/v YNB, 1% glucose w/v, 0.5% w/v ammonium chloride, 0.2% w/v casamino acids and 50 mM sodium–potassium phosphate buffer, pH 6.8). For heterologous protein expression, *Y. lipolytica* transformants were cultured in YTD medium (1% w/v yeast extract, 2% w/v tryptone, 5% w/v glucose and 100 mM phosphate buffer, pH 6.8).

1.2 DNA manipulations

A *Penicillium citrinum xynA* gene was synthesized and introduced into a pUC57-Kan vector by GenScript based on the nucleotide database (GenBank: accession no. AB198065.1) (Tanaka *et al.*, 2005). Plasmid JMP62_TEF_Ura3Ex was used for the production of recombinant enzymes (Figure III-2) (Meunchan *et al.*, 2015).

For native secretion signal, the synthetic *xynA* gene was amplified from pUC57-Kan containing *xynA* gene by PCR using the following specific primers: *xynA*_Yl_nat-F (5´-CTTCTT<u>GGATCC</u>CACAATGCCATCCCTTACTTCATTGTTT TCA-3´) and *xynA*_Yl_-R (5´-GAGGAG<u>CCTAGG</u>TTAGGAAACGGTAATGTCAG CGG-3´). *BamH*I and *Avr*II restriction sites (underlined) were added to the forward and reverse primers, respectively. For fusion with prepro*LIP2* secretion signal using the following specific primers: *xynA*_Yl_fus-F (5´-AAGCGAGTGTACAATACTTC TTCCTCAACCGGTACTAGTAACGG-3´) and *xynA*_Yl_-R (5´-GAGGAG <u>CCTAGG</u>TTAGGAAACGGTAATGTCAGCGG-3´) *BsrG*I and *Avr*II restriction sites (underlined) were added to the forward and reverse primers, respectively, which allowed the *xynA* gene to be ligated in frame with the prepro*LIP2* secretion signal sequence in JMP62_TEF_Ura3Ex. The PCR conditions were as follows: at 98°C for 30 s, 30 cycles of 98°C for 15 s, 60 °C for 20 s and 72°C for 30 s, followed by one cycle of 72°C for 5 min. The amplified fragments were purified and then digested with *Bam*HI/*Avr*II or *Bsr*GI/*Avr*II before cloning into corresponding expression vectors predigested with the same restriction enzymes and transformation into *E. coli* DH5α competent cells. The constructed plasmid designated as pTEF_nat*XynAPc* and pTEF_fus*XynAPc* were screened on a LB agar plate containing 50 µg/ml Kanamycin to detect the presence of the recombinant plasmid. The insertion was verified by double restriction enzyme digestion and DNA sequencing (GATC Biotech, Konstanz, Germany).
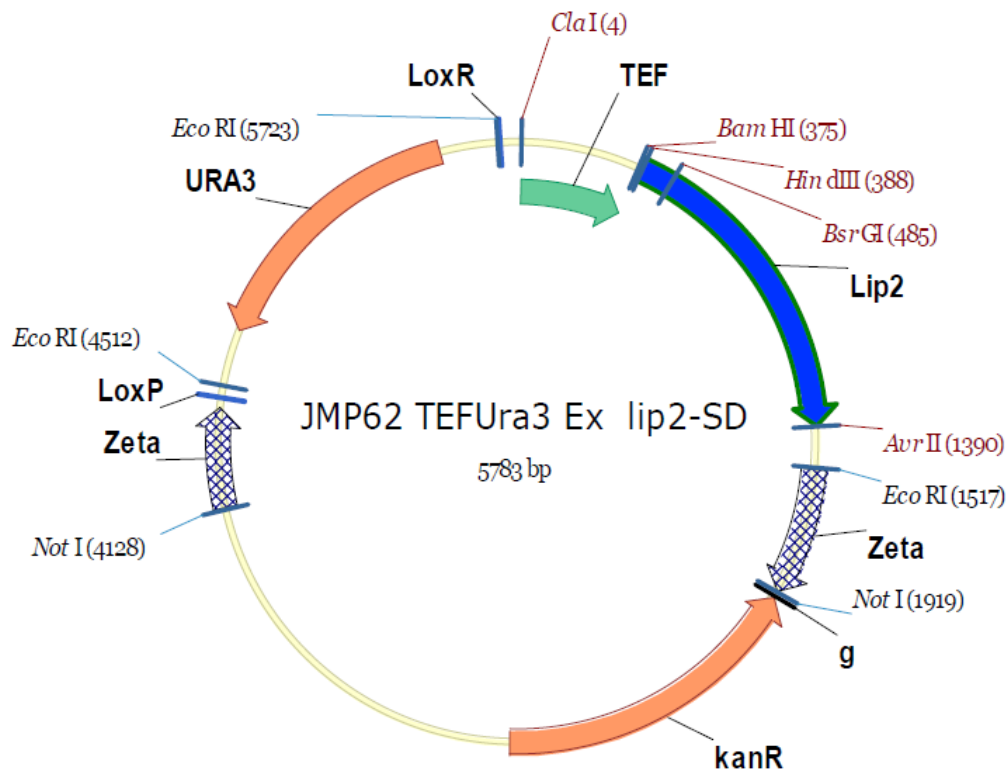
Figure III-2 The features of the JMP62_TEF_Ura3EX vector (constitutive expression vector). For native secretion signal, the *xyn*A gene with native secretion signal sequence is positioned between *Bam*HI and *Avr*II sites. For fusion with prepro*LIP2*, the mature *xyn*A gene is positioned between *Bsr*GI and *Avr*II sites.

1.3 Yeast transformation and selection

pTEF_nat*XynAPc* and pTEF_fus*XynAPc* plasmid were linearlized with *Not*I for integration into *Y. lipolytica* genome. Each linearized DNA was transformed into *Y. lipolytica* using the lithium acetate method (Duquesne *et al.*, 2012). Briefly, the cells were grown at 28°C in 25 ml of YPD containing 50 mM citrate buffer (pH 4) until a cell density of between 8×10^7 to 1.5×10^8 cells/ml was attained. Cells were collected by centrifugation at 3000 rpm for 5 min and washed using 20 ml of TE (50

mM Tris HCl, 5 mM EDTA, pH 8.0). After centrifugation, the cells were treated with 25 ml of 100 mM lithium acetate (pH 6.0) at 28°C for 1 h without shaking. They were then centrifuged at 2000 rpm, 4°C for 3 min and resuspended delicately in 2 ml of lithium acetate. Plasmid DNA linearized by *Not*I (~100-300 ng combined with 25 µg of carrier DNA) was incubated in 100 µl of *Y. lipolytica* competent cells at 28°C for 15 min without shaking, and then 700 µl of 40 % (w/v) PEG4000 in lithium acetate was delicately added and incubated at 250 rpm and 28°C for 1 h. After that, the cells were transformed by heat shock at 39°C for 10 min and immediately recovered in 1.2 ml of 100 mM lithium acetate. Transformants were selected on YNB agar plate. The Ura-positive colonies were randomly chosen for preliminary selection on YNB with 0.2% AZCL-Arabinoxylan agar plate.

1.4 Heterologous expression of *xynA* gene in *Y. lipolytica*

Y. lipolytica transformants were cultured in YTD medium for 16 h. Then, the cultures were used to inoculate a fresh YTD medium with various initial cell density OD_{600} from 0.1 to 0.5. All enzyme expressions were operated in baffled shake flask at 30°C with constant shaking at 130 rpm. After completion of glucose utilization, the culture supernatant was collected to analyze production levels and to allow purification of recombinant xylanase and characterization.

1.5 Xylanase activity assay and protein determination

The xylanase activity was assayed by following the release of reduced sugars from xylan. The reaction mixture consisted of 0.5 ml of 4% (w/v) birchwood xylan in deionized water, 0.4 ml of 0.15 M acetate buffer (pH 5) and 0.1 ml of suitably diluted

enzyme. Reactions were incubated at 50°C for 5 min and aliquots (75 µl) were taken every minute and stopped by transferring into an equal amount of DNS (3, 5-dinitrosalicylic acid) before boiling for 5 min (Miller, 1959). After cooling, 0.75 ml deionized water were added and the absorption was measured at 540 nm. Each absorbance was plotted on a linear graph. The equations of the reaction were used to calculate the xylanase activity. One unit of xylanase activity (U) was defined as the amount of enzyme that liberates 1 µmol of xylose equivalents per min per ml under the assay condition. Protein concentration was measured at 595 nm following the *DC* Protein Assay (Bio-Rad) using bovine serum albumin (BSA) as a standard.

1.6 Protein deglycosylation

Yeast cells from liquid cultures of Yl-fus*XynAPc* were sedimented by centrifugation at 10,000 rpm, 4°C for 20 min. The supernatant was concentrated by ultrafiltration through ultra centrifugal filter devices with 10 kDa cut-offs (Amicon). The enzyme solution was treated with EndoH according to the manufacturer's instruction at room temperature overnight.

1.7 Enzyme purification

The native deglycosylated xylanase was exchanged for 20 mM acetate buffer (pH 5.5) by ultrafiltration through ultra centrifugal filter devices with 10 kDa cut-offs (Amicon) and loaded onto a HiLoad^TM 16/10 Q Sepharose High Performance column (GE Healthcare Life Sciences) previously equilibrated with 20 mM acetate buffer (pH 5.5). The adsorbed proteins were eluted at a flow rate of 2.0 ml/min with a linear gradient of 0 to 1 M NaCl in the same buffer.

1.8 SDS-PAGE and zymographic analysis

The sample were subjected to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-Protean® TGX Stain-Free™ gels to determine the molecular weight and confirm the purity of the existing enzymes. Precision Plus Protein Standards (Bio-Rad) ranging from 10 to 250 kDa were used as the reference protein.

The zymography analysis for xylanase was performed on 10% polyacrylamide gel containing 1% Azo-xylan as substrate running at the same time with 10% polyacrylamide gel without Azo-xylan. The sample was mixed with the loading buffer without 2-mercaptoethanol and boiled at 70°C for 5 min. The electrophoresed gel containing 1% Azo-xylan was soaked in 25% isopropanol to remove SDS and renature protein and then washed with 60 mM acetate buffer pH 5 with gentle shaking at 4°C for 30 min, 4 times, before incubating in the same buffer at room temperature overnight. The activity bands were observed as clear colorless areas.

1.9 Enzyme characterization

The optimum pH of the purified recombinant Yl-xynA activity was evaluated at 50°C over a pH range of 3.0 to 6.0. A pH profile was produced with the enzyme activity at the optimum pH set at 100%. The pH stability of the purified recombinant xynA was investigated by incubating in various pH buffers using appropriate buffer (60 mM): citric acid – sodium citrate buffer (pH 3.0-6.0), sodium phosphate buffer (pH 6.0-8.0) and sodium carbonate (pH 9.0-10.0) at room temperature for 24 h and the residual activity was measured under standard assay condition.

The optimum temperature of the purified recombinant Yl-xynA activity was studied by incubating the purified enzyme at pH 5.0 and temperatures ranging from 40°C to 60°C. A temperature profile was produced with the enzyme activity at the optimum temperature set at 100%. Thermostability of the purified recombinant Yl-xynA was evaluated at 40°C and 45°C for different time periods before measuring the residual activity.

The kinetic parameters (K_m, V_{max} and k_{cat}) of the purified recombinant Yl-xynA enzyme were determined using 9 different substrate concentrations (0.1-2% xylan) with the same method described previously at pH 5.0, 40°C for 10 min. All experiments were repeated three times. Kinetic analyses were performed using Michaelis-Menten and Lineweaver-Berk plots. Each data represents the mean of at least three independent experiments.
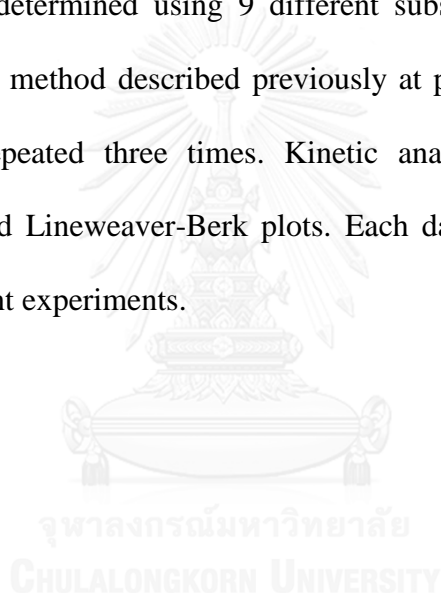
CHAPTER IV

RESULTS AND DISCUSSION

PART I Cloning, expression, purification and characterization of endo-1,4-β-xylanase from *Penicillium citrinum* in *Pichia pastoris*

1.1 Construction of expression vectors, transformation and selection

In order to overexpress and study the biochemical properties of the xylanase A from *P. citrinum* FERM P-15944, the *xynA* gene was codon optimized (*xynAPc*) to enhance transcription levels in *P. pastoris*. The gene encoding mature xynA was cloned in frame with the native *Saccharomyces cerevisiae* α-factor secretion signal and C-terminal sequence under the control of *AOX1* or *GAP* promoter by introducing an artificial *Kpn*I restriction site at the 5′-end and *Not*I restriction site at the 3′-end of *xynAPc*. These constructions were ligated with the *P. pastoris* expression vectors pGAPZαA and pPICZαA at the multiple cloning site to produce the recombinant expression plasmids pGAPZαA-*xynAPc* and pPICZαA-*xynAPc*, respectively. After transformation into *E.* coli, the plasmids were found to be positive by *Kpn*I-HF/*Not*I-HF restriction enzyme digestion and sequencing while pGAPZαA-*xynAPc* and pPICZαA-*xynAPc* were transformed and integrated into the *P. pastoris* X-33[(mut+)] genome. Integration of multiple gene copies, a quick and direct way to potentially allow higher protein production, was allowed by plating the transformation mix on increasing concentrations of Zeocin[TM]. In this study, the transformants were selected on YPDS agar plate containing 100-2,000 µg/ml Zeocin[TM].

Concerning pGAPZαA-*xynAPc*, YPDS agar plate containing 100 μg/ml Zeocin[TM] showed the highest amount of transformants, and this number decreased as the concentration of Zeocin[TM] increased, though none grew on YPDS agar plate containing 2,000 μg/ml Zeocin[TM]. Since the *GAP* promoter is a constitutively expressed promoter, the transformants were preliminary selected on YP with 0.2% Azo-xylan agar plate. The results showed that all selected transformants from each plate were detected the xylanase activity as the substrate was hydrolysed. It would diffuse more rapidly than the native substrate, giving zones of clearing, whereas the negative control, *P. pastoris* X-33[(mut+)] without transformation, did not show any clearing zone (Figure IV-1).

For pPICZαA-*xynAPc*, the transformants could grow on all YPDS agar plates containing 100-2,000 μg/ml Zeocin[TM], though the number of transformants decreased when the concentration of Zeocin[TM] was increased.
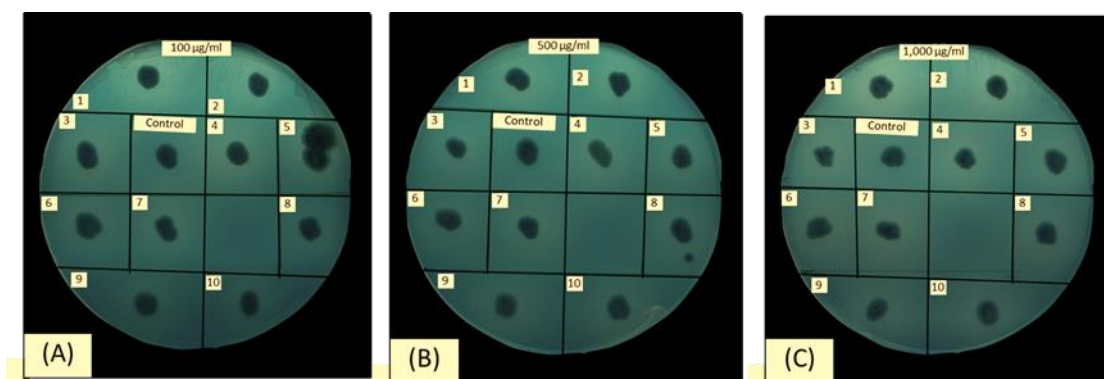
Figure IV-1 Prelimary selection of the pGAPZαA-*xynAPc* transformants on YP with 0.2% Azo-xylan agar plate. (A) Selected transformants from YPDS agar plate containing 100 µg/ml Zeocin, (B) 500 µg/ml Zeocin and (C) 1,000 µg/ml Zeocin. Control is *P. pastoris* without transformation.

1.2 Expression of *xynAPc* in *P. pastoris*

All transformants were cultured at 30°C and monitored every 24 h for growth and recombinant xynAPc activity in the supernatant. We first checked that the wild type *P. pastoris* X-33[(mut+)] had no detectable extracellular xylanase activity. For constitutive expression (p*GAP*), the growth of all transformants reached a steady state after 48 h, whereas for the methanol-inducible expression (pPIC), all transformants were still in the growing phase after induction with methanol every 24 h during 5 days. The maximum xylanase activity of the pGAPZαA-*xynAPc* transformants was from a clone picked on a YPDS agar plate containing 100 µg/ml Zeocin[TM], after 48 h of culture. Activity reached 119.5 µmol/ml/min which was 34 folds higher than the native xylanase activity from *P. citrinum* at 72 h (3.5 µmol/ml/min) (Tanaka *et al.*, 2005).

Concerning pPICZαA-*xynAPc* containing clones, in order to maximize recombinant protein production level, methanol concentration was varied in BMMY medium during induction (Boonvitthya *et al.*, 2012). The results showed that the maximum xylanase activity of the pPICZαA-*xynAPc* transformants was from a clone selected on a YPDS agar plate containing 500 µg/ml Zeocin™, cultured in 1% methanol in BMMY and induced with 1% methanol every 24 h for 5 days (676 µmol/ml/min) which was 193-fold higher than that of the native strain.

Recombinant expression of *xynA* from *P. citrinum* FERM P-15944 had already been investigated in the *P. pastoris* strain GS115 using the integrative yeast expression vector pPIC3.5 (Tanaka *et al.*, 2005), but our results clearly demonstrate a significant higher production (39 fold) of xylanase activity by pPICZαA-*xynAPc* (17 µmol/ml/min at 120 h). Besides the use of different strains of *P. pastoris,* our results benefit from the fusion to a secretion signal and from the use of a codon optimized sequence the combination of which resulted in an improved level of xylanase expression as shown in Table IV- 1.

1.3 Purification, SDS-PAGE and zymographic analysis

Recombinant Pp-xynA produced from pPICZαA-*xynAPc* transformants was chosen for purification and characterization. Culture supernatant was concentrated with 10 kDa molecular weight cut-offs and subjected to anion exchange chromatography to obtain the purified Pp-xynA enzyme. After the first step, the concentrate still retained 95.3% of total activity but protein yield dramatically decreased to 26.4% after anion exchange chromatography (Table IV-2).

Table IV-1 Xylanase activity of xynA from *P. citrinum* in culture supernatant

Microorganisms	Signal sequence	Promoters	Activity (μmol/ml/min)	Comparison with the native stain (fold)	References
P.citrinum					
the native stain	native	native	3.5	-	Tanaka *et al.*, 2005
P. pastoris GS115					
†pPIC3.5	native	AOX1/methanol inducible	17	5	Tanaka *et al.*, 2005
E.coli BL21					
†pEXP401	Ap-xynI[1]	T7/IPTG inducible	16.8	5	Ohta *et al.*, 2011
P.pastoris X-33					
†pPICZαA-*xynAPc*	Sc-aSP[2]	AOX1/methanol inducible	676	193	This study
P.pastoris X-33					
†pGAPZαA-*xynAPc*	Sc-aSP	GAP/constitutive	119.5	34	This study

[1] Ap-xynI = *Aureobasidium pullulans xynI* signal sequence

[2] Sc-aSP = *S. cerevisiae* alpha factor signal sequence

This protocol afforded 33.8-fold purification of the Pp-xynA enzyme from the culture supernatant with a final specific activity of 1258.96 U/mg. The molecular weight of the Pp-xynA enzyme was determined by running the SDS-PAGE (without Azo-xylan) and confirmed by running the zymogram (containing 1% Azo-xylan) at the same time. It was estimated to be 20 kDa, the same as that of the native fungal *P. citrinum* xylanase. Moreover, these results show that Pp-xynA does not undergo post-translational glycosylation. The Pp-xynA represented the major protein in culture supernatant, and it was resolved as a single band after anion exchange chromatography (Figure IV-2). These results support the reports of Barr and *et al.* and Lafond *et al.* showing that *P. pastoris* secretes only very low levels of native proteins and the secreted heterologous protein constitutes the majority of the total protein in the medium (Barr *et al.*, 1992; Lafond *et al.*, 2011).

Table IV-2 Purification of extracellular recombinant xynAPc expressed in *P. pastoris* X-33

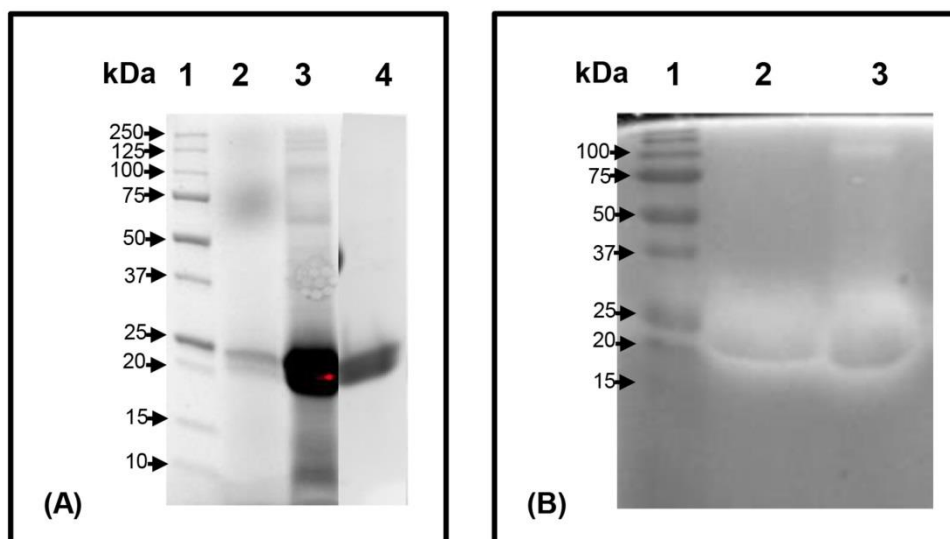| Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	81118	2175.57	37.29	1.00	100
Concentrate	77305	533.25	144.97	3.89	95.3
Q Sepharose	21425	17.02	1258.96	33.77	26.4

Figure IV-2 SDS-PAGE analysis and zymogram of recombinant xynAPc expressed in *P. pastoris* X-33. (A: SDS-PAGE), (B: Zymogram) Lane 1: Size markers, Lane 2: Supernatant, Lane 3: Concentrated and changed buffer with 10 kDa MWCO, Lane 4: Purified Pp-xynA.

1.4 Characterization of xylanase A protein produced in *P. pastoris*

The effect of pH on the enzymatic activity and stability of the purified Pp-xynA enzyme was also determined. As shown in Figure IV-3 and IV-4, the purified enzyme showed an optimum activity at pH 5.0. Concerning its stability to pH, after 24 h, the enzyme retained more than 80% of its original activity when incubated in buffers going from pH 3.0 to pH 9.0. At pH 10, the enzyme conserved around 80% of the original activity after 2 h and gradually declined to 70% and 50% after 6 and 27 h, respectively. These results show that the purified Pp-xynA enzyme was stable for a long time over a wide pH range and, thus, can be applied to many industries.

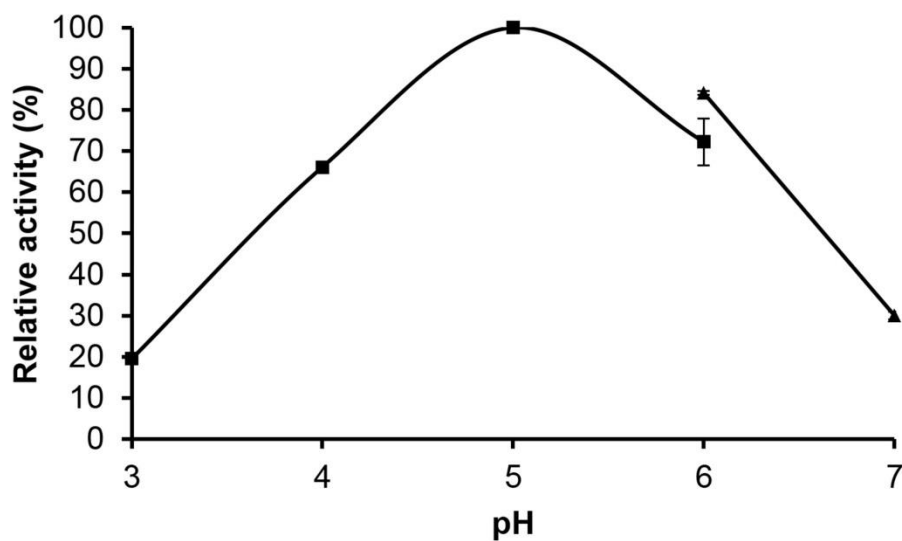**Figure IV-3** Effect of pH on the activity of purified Pp-xynA with reaction mixture incubated at 50°C over a pH range of 3.0 to 7.0. The buffers used were 60 mM citric acid – sodium citrate buffer (pH 3-6) (*solid square*) and 60 mM sodium phosphate buffer (pH 6-7) (*solid triangle*). Each data point represents the mean of at least three independent experiments and the error bars indicate the standard deviation.
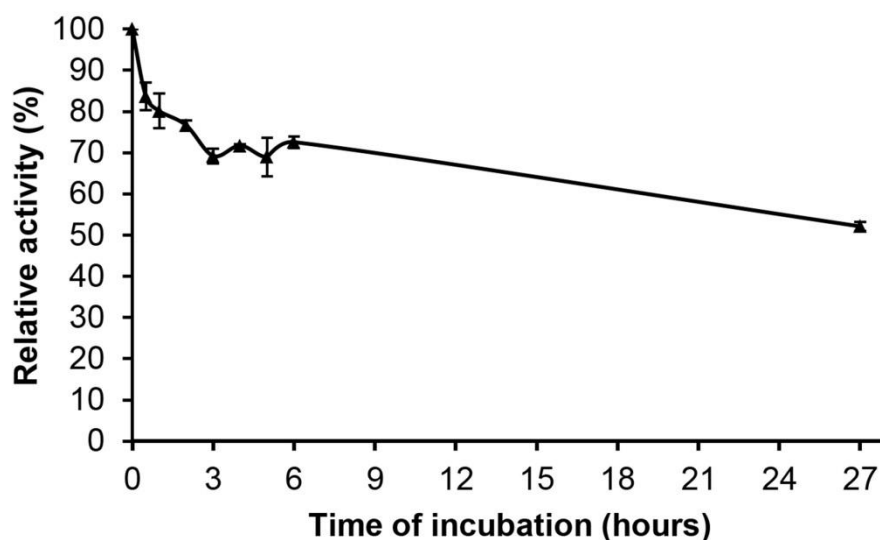
Figure IV-4 pH stability profile of the Pp-xynA. The purified recombinant Pp-xynA was pre-incubated in sodium carbonate buffer, pH 10 at room temperature for 27 h and then the residual activity was measured under optimal condition. Each data point represents the mean of at least three independent experiments and the error bars indicate the standard deviation.

The effect of temperature on the enzymatic activity and thermostability of Pp-xynA was investigated. At pH 5.0, the optimum temperature for the enzyme activity was 55°C as shown in Figure IV-5. After incubation at 40°C for 1 h, the enzyme retained around 80% of the original activity and slightly dropped to 20% of the original after pre-incubation for 6 h as shown in Figure IV-6.

The kinetics of catalytic reactions were determined at 40°C, pH 5.0 using 9 different substrate concentrations (0.1-2% birchwood xylan). The K_m, V_{max} and k_{cat} were 2.8 mg/ml, 310.7 μmol/min and 243 per s, respectively. In comparison to other family 11 β-1,4 xylanases using biechwood xylan as substrate, the K_m of Pp-xynA

was lower than that for Nf Xyn11A, native XylA and reXylA as shown in table IV-3 (Berrin *et al.*, 2000; Zhang *et al.*, 2011). The lower K_m of Pp-xynA indicated that it had a higher affinity for birchwood xylan. The Pp-xynA also had a higher turnover number k_{cat} and catalytic efficiency k_{cat}/K_m, indicating high catalytic activity.
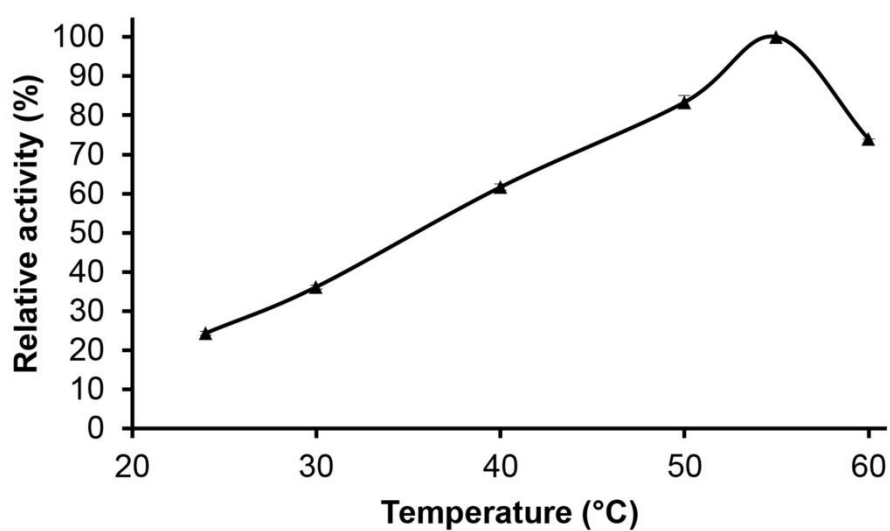
Figure IV-5 Effect of temperature on activity of purified Pp-xynA. Each data point represents the mean of at least three independent experiments and the error bars indicate the standard deviation.

Figure IV-6 Thermal stability profile of Pp-xynA. The purified Pp-xynA was incubated in acetate buffer, pH 5 at 40°C, for 6 h and then the residual activity was meas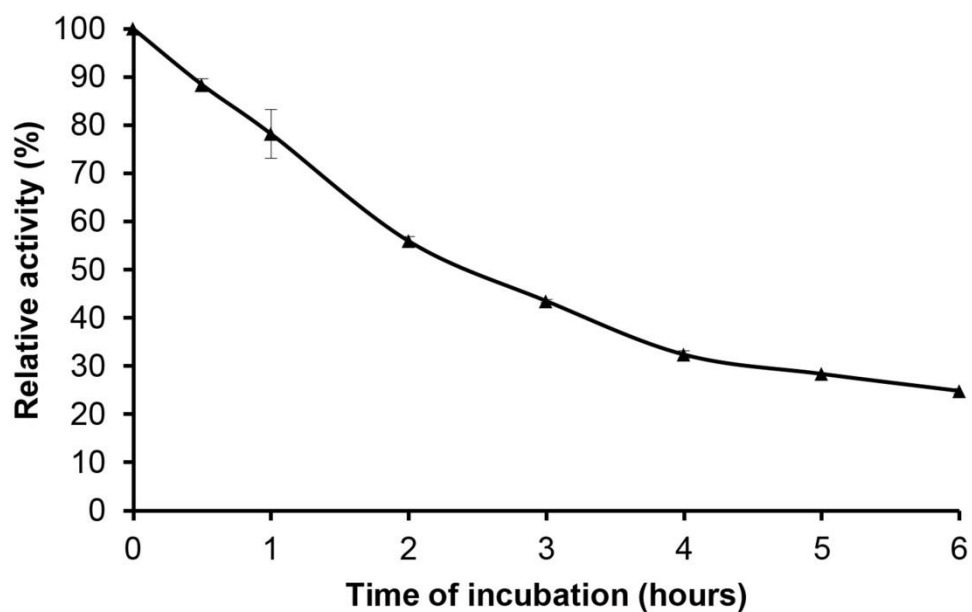ured under optimal condition. Each data point represents the mean of at least three independent experiments and the error bars indicate the standard deviation.

Table IV-3 Comparison of enzymatic properties between recombinant Pp-xynAPc and those of other β-1,4 xylanases[a] from family 11

Enzyme	K_m (mg/ml)	k_{cat} (S^{-1})	k_{cat}/K_m $(mg^{-1}S^{-1}ml)$	References
Native XylA[b]	6.8	85	12.5	Berrin et al., 2000
reXylA[c]	12.6	150	11.9	Berrin et al., 2000
Nf Xyn11A[d]	6	136.9	22.8	Zhang et al., 2011
Pp-xynAPc	2.8	243	86.8	This study

[a]Kinetic values were analyzed using birchwood xylan as substrate.

[b]Native xylanase produced in *Aspergillus niger*

[c]Recombinant xylanase produced in *Pichia pastoris*

[d]Xylanase produced in *Nonomuraea flexuosa*

PART II Cloning, expression, purification and characterization of endo-1,4-β-xylanase from *Penicillium citrinum* in *Yarrowia lipolytica*

2.1 Construction of expression vectors, transformation and selection

To overexpress and study xylanase A from *P. citrinum* FERM P-15944, the *xynA* gene was synthesized and expressed in *Y. lipolytica*. Plasmid JMP62_TEF_Ura3Ex contains the *Y. lipolytica* constitutive *TEF* promoter and *URA3ex* excisable selection marker, which is flanked by a Zeta fragment that serves as the homologous integration site (Bordes *et al.*, 2007). To evaluate the efficiency of *xynA* signal peptide, the expression and secretion level, the gene encoding mature *xynA* was cloned in frame with the prepro*LIP2* secretion signal and compared with the native secretion signal. The constructed plasmid was designated as pTEF_fus*XynAPc* and pTEF_nat*XynAPc*, respectively. Since the *TEF* promoter is a constitutively expressed promoter, transformants producing an active enzyme were directly selected for activity on YNB with 0.2% AZCL-Arabinoxylan agar plate, as the substrate was hydrolysed and released soluble dye-labelled fragments around the colony, whereas the negative control, *Y. lipolytica* without transformation, did not show any coloured halo (Figure IV-7).
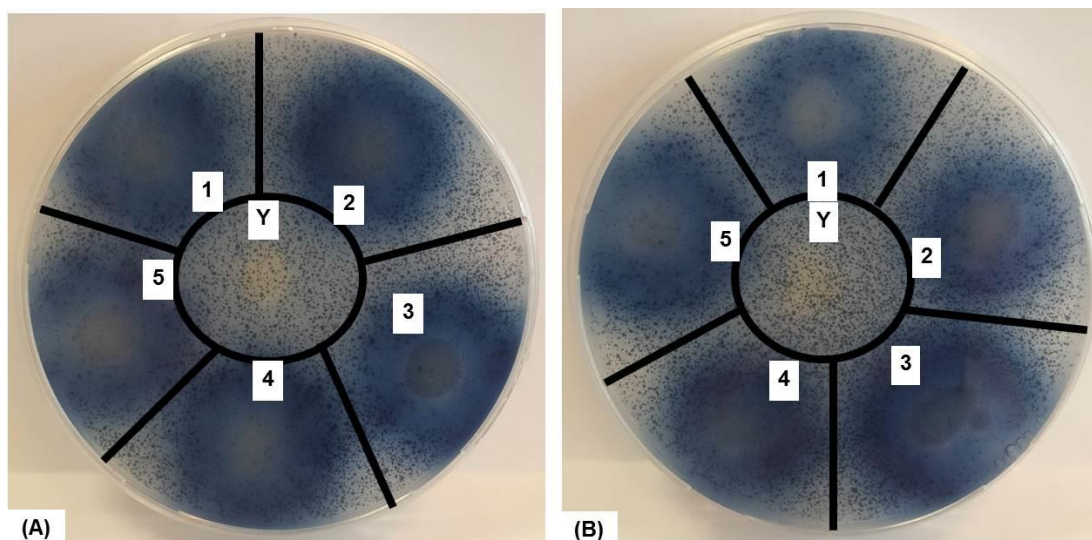
Figure IV-7 Prelimary selection of the transformants on YNB with 0.2% AZCL-Arabinoxylan agar plate. (A) *Y. lipolytica*-natXynA, (B) *Y. lipolytica*-fusXynA; 1-5: selected transformants, Y: *Y. lipolytica* without transformation.

2.2 Expression of *xynA* in *Y. lipolytica*

All selected transformants were cultured and monitored for utilization of glucose. Since the xylanase activity was assayed by following the release of reduced sugars from xylan and YTD medium contained glucose as carbon source, the culture were harvested after complete utilization of glucose. The maximum xylanase activity of the Yl-natXynAPc at 48 h (38.01 µmol/mL/min) was higher by 10.9 folds, than that of the native strain *P. citrinum* at 72 h (Tanaka *et al.*, 2005). Maximum xylanase activity of Yl-fusXynAPc was reached at 48 h (180.26 µmol/ml/min) and was 51.5-fold higher than that of the native culture. *Y. lipolytica* without transformation had no detectable extracellular xylanase activity. The various initial cell density at the OD_{600} values of 0.1, 0.3 and 0.5 were approximately similar xylanase activity. To compare with expression of *xynA* in *P. pastoris* containing constitutive *GAP* promoter fusion

with *S. cerevisiae* α-factor signal peptide (in the result part 1.2), expression in *Y. lipolytica* using constitutive *TEF* promoter fusion with prepro*LIP2* signal peptide showed the higher xylanase activity than that for 1.5 folds. Besides expression of the *xynA* from *P. citrinum* FERM P-15944 in *Y. lipolytica* and *P. pastoris* in this study, it was already investigated in the *P. pastoris* strain GS115 using the integrative yeast expression vector pPIC3.5. In comparison, xylanase activity of Yl-natXynAPc and Yl-fusXynAPc was 2.2 and 10.6 folds higher, respectively. Fusion of the signal peptide of *Aureobasidium pullulans XynI* in place of the native one of *xynA* from *P. citrinum* was tested to improve protein secretion in *E. coli* BL21(DE3) (Ohta *et al.*, 2011). Xylanase activity of the Yl-natXynAPc and Yl-fusXynAPc strains were again higher than that of this heterologous *E. coli*, for 2.3 and 10.7 folds, respectively. Expression of *xynA* from *P. citrinum* using different promoters, secretion signal peptides and host strains affect expression level as shown in Table IV-4. In this study, recombinant Yl-xynA enzyme from Yl-fusXynAPc transformant was used to purify for characterization.

Table IV-4 Xylanase activity of *xynA* from *P. citrinum* in culture supernatant

Microorganism	Signal sequence	Promoter	Activity (μmol/ml/min)	Comparison with the native stain (fold)	References
P.citrinum the native stain	native	native	3.5	-	Tanaka *et al.*, 2005
P.pastoris GS115 +pPIC3.5	native	AOX1/methanol inducible	17	5	Tanaka *et al.*, 2005
E.coli BL21 +pEXP401	Ap-xynI[1]	T7/IPTG inducible	16.8	5	Ohta *et al.*, 2011
P.pastoris X-33 +pPICZαA-*xynAPc*	Sc-aSP[2]	AOX1/methanol inducible	676	193	This study
P.pastoris X-33 +pGAPZαA-*xynAPc*	Sc-aSP	GAP/constitutive	119.5	34	This study
Y.lipolytica zeta +pTEF_nat*XynAPc*	native	TEF/constitutive	38	11	This study
Y.lipolytica zeta + pTEF_fus*XynAPc*	ppLIP2[3]	TEF/constitutive	180.3	52	This study

[1]Ap-xynI = *Aureobasidium pullulans xynI* signal sequence

[2] Sc-aSP = *S. cerevisiae* alpha factor signal sequence

[3] ppLIP2 = preproLIP2 signal sequence

2.3 Native protein deglycosylation

The molecular weight of the Yl-xynA enzyme was determined by running the SDS-PAGE (without 1% Azo-xylan) and confirmed by running the zymogram (with 1% Azo-xylan in the polyacrylamide gel) at the same time. Electrophoretic and zymography analysis of recombinant xylanase from *Y. lipolytica* revealed that molecular weight of the recombinant protein was higher than that of the native xynA (20 kDa), as a large smear reaching 60kD could be detected. This was similarly observed in the expression of several cellulases, *YlBGL2, TrEGII and TrCBHII* with their apparent molecular mass larger than the native protein since glycosylation (Boonvitthya *et al.*, 2012; Guo *et al.*, 2015). Therefore, it was suspected that the molecular weight differences might have arisen from protein hyperglycosylation mediated by the yeast production hosts. Hyperglycosylation was confirmed by treating the Yl-xynA with endoglycosidase H to remove asparagine-linked *N*-glycosyl motifs from Yl-xynA enzyme. After deglycosylation, smear of xylanase at high molecular mass were decreased and displaying a molecular mass closest to the one of native xynA native protein (Figure IV-8).

2.4 Purification, SDS-PAGE and zymographic analysis

The native deglycosylated xylanase was exchanged against 20 mM acetate buffer (pH 5.5) and subjected to anion exchange chromatography to obtain the purified Yl-xynA enzyme. Table IV-5 summarizes the procedure for the purification of Yl-xynA. After concentration, the concentrate still retained 81.8% of the initial activity but recovery dramatically decreased to 3.53% after anion exchange chromatography. This protocol afforded 185-fold purification of the Yl-xynA enzyme

from the culture supernatant with a specific activity of 695 U/mg. Figure IV-8 shows SDS-PAGE and the zymogram of protein in each step of purification. The purified Yl-xynA enzyme was resolved as a single band after anion exchange chromatography. SDS-PAGE and zymographic analysis indicated that the molecular mass of the xylanase A produced from *Y. lipolytica* was 20 kDa, which is similar to that expressed in *P. pastoris* and reported for the native xylanase from *P. citrinum.*

Table IV-5 Purification of extracellular Yl-xynA enzyme

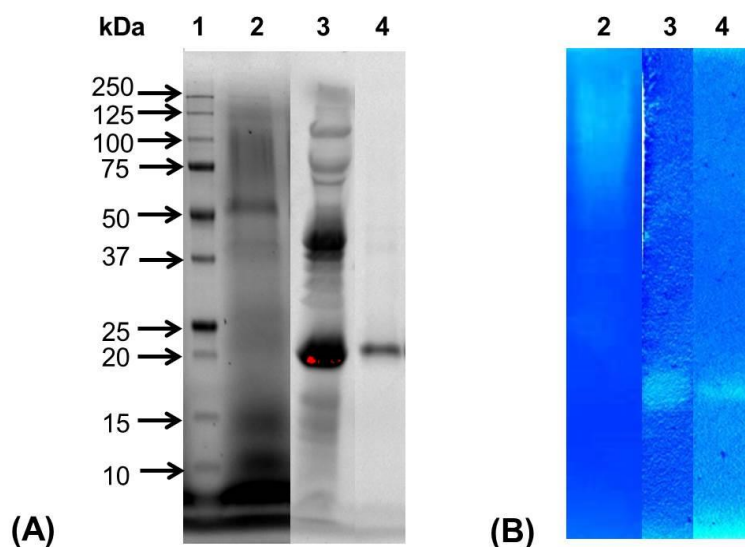| Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	27038	7214.63	3.75	1.00	100
Concentrate	22126	2195.79	10.08	2.69	81.83
Q Sepharose	954	1.37	695.23	185.51	3.53

Figure IV-8 SDS-PAGE analysis and zymography of recombinant xynAPc expressed in *Y. lipolitica* zeta. (A: SDS-PAGE), (B: Zymography) Lane 1: Size markers, Lane 2: Supernatant, Lane 3: Concentrated Yl-xynAPc treated with EndoH, Lane 4: Purified Yl-xynA.

2.5 Characterization of xylanase A protein produced in *Y. lipolytica*

The effect of pH on the enzymatic activity and stability of the purified Yl-xynA enzyme was also determined. As shown in Figure IV-9 and IV-10, the purified enzyme showed an optimum activity at pH 5.0. The pH stability of the purified enzyme was investigated in a pH range 3.0 to 10.0. After 24 h, the enzyme retained more than 80% of the original activity between pH 3.0 to 9.0. At pH 10 however, the enzyme retained around 80% of the original activity after 30 min and gradually declined to 40% and 27% after 5 and 24 h, respectively. These results show that the purified Yl-xynA enzyme was stable for a long time over a wide pH range and, thus, can be applied to many industries.
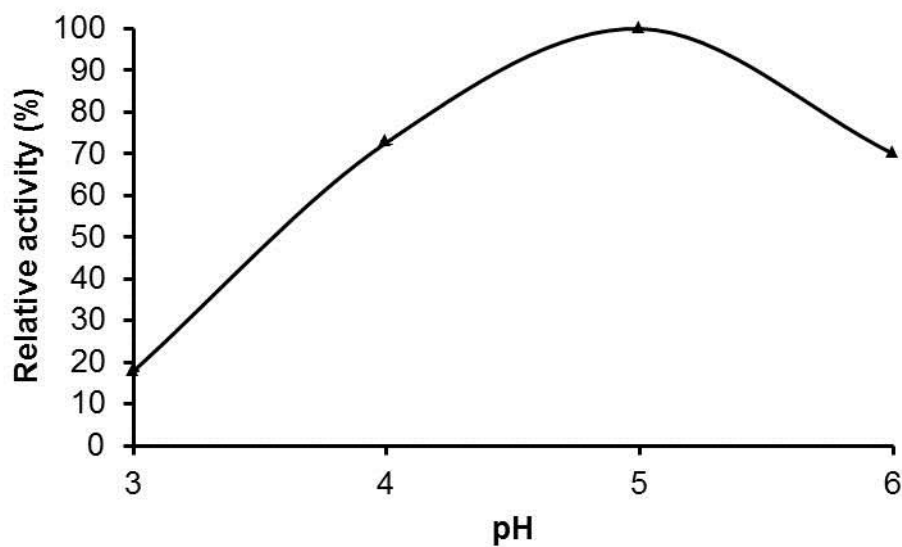
Figure IV-9 Effects of pH on the activity of purified Yl-xynA with reaction mixture incubated 24 h at 50°C over a pH range of 3.0 to 6.0 under standard enzyme assay condition. Each data point represents the mean of at least three independent experiments and the error bars indicate the standard deviation.

Figure IV-10 Stability of the purified Yl-xynA at pH 10 at room temperature measured under standard enzyme assay condition. Each data point represents the mean of at least three independent experiments and the error bars indicate the standard deviation.

The effect of temperature on the enzymatic activity and thermostability was investigated on the purified Yl-xynA enzyme. At pH 5.0, the optimum temperature for the enzyme activity was 55°C as shown in Figure IV-11. The enzyme exhibited good stability at temperatures up to 40°C. After a 6 h incubation, the enzyme remained around 80% of the original activity. At 45°C, the enzyme retained around 70% of the original activity after 30 min and gradually declined to 17% and 6% after 3 and 5 h, respectively (Figure IV-12), whereas expression of *xynA* in *P. pastoris* (in the result part 1.4) exhibited stability at temperatures up to 40°C. After pre-incubation

for 1 h, the Pp-xynA remained around 80% of the original activity and slightly

dropped to 20% of the original after a for 6 h incubation.
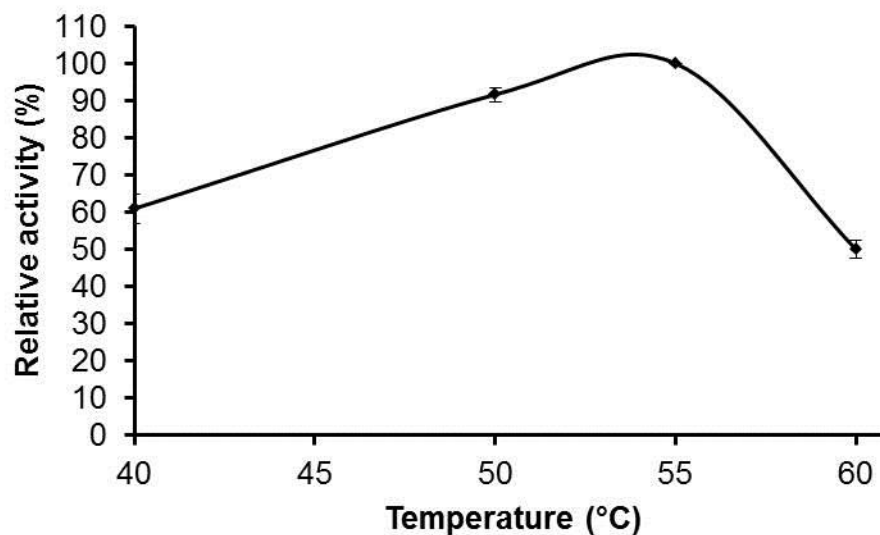
Figure IV-11 Effects of temperature on activity of purified Yl-xynA under standard

enzyme assay condition. Each data point represents the mean of at least three

independent experiments and the error bars indicate the standard deviation.
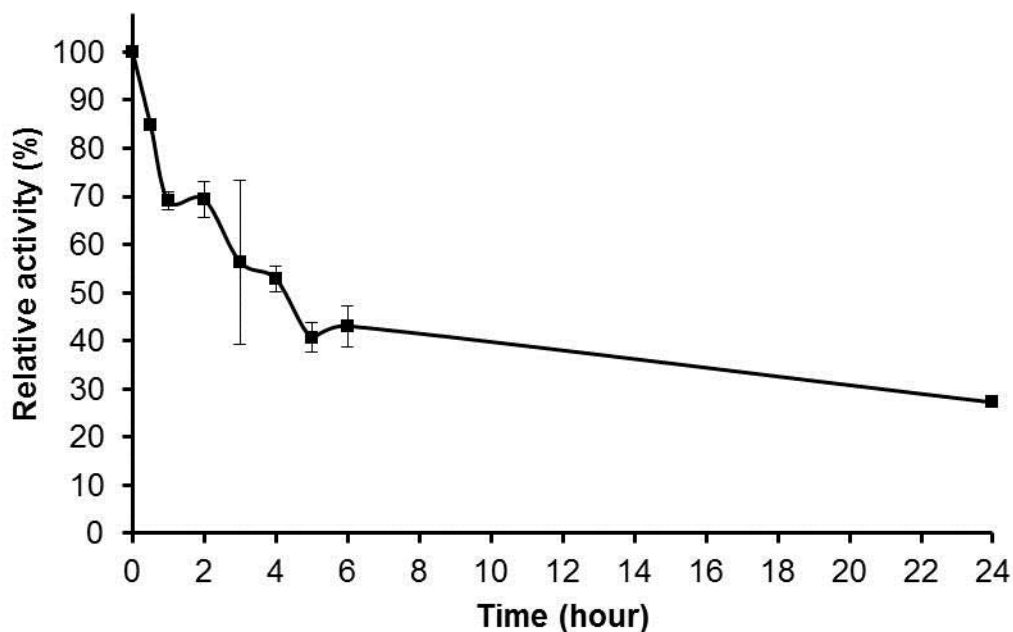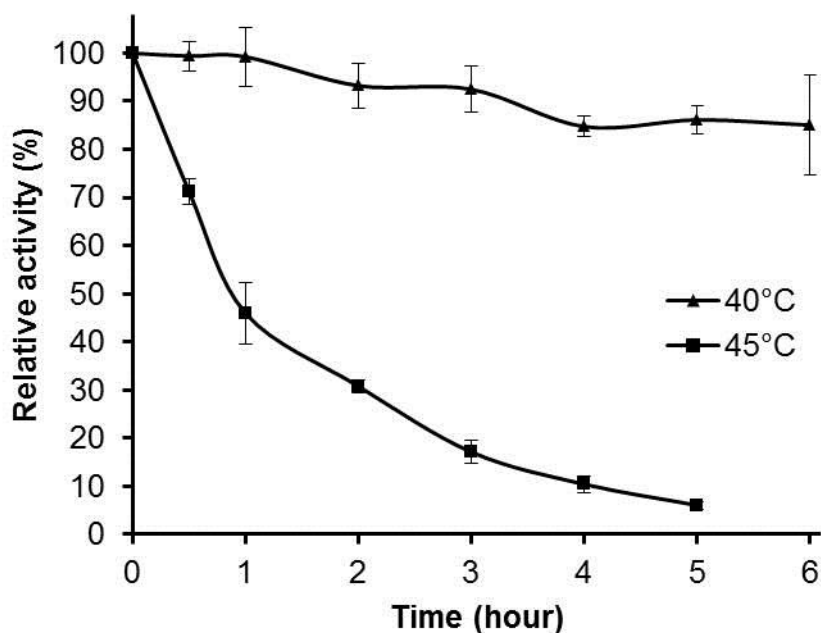
Figure IV-12 Thermal stability of purified re-xynA at 40 and 45°C measured under standard enzyme assay condition. Each data point represents the mean of at least three independent experiments and the error bars indicate the standard deviation.

Yl-XynA kinetics were performed at 40°C, pH 5.0 using 9 different substrate concentrations (0.1-2% birchwood xylan). The K_m, V_{max} and k_{cat} were calculated to be 5.2 mg/ml, 560.5 µmol/min and 245 per s, respectively. In comparison to other family 11 β-1,4 xylanases, the Yl-xynA K_m was lower than that for Nf Xyn11A, native XylA and reXylA as shown in Table IV-6 (Berrin *et al.*, 2000; Lafond *et al.*, 2011). The lower K_m of Yl-xynA indicated that it had a higher affinity for birchwood xylan. The Yl-xynA also had a higher turnover number k_{cat} and catalytic efficiency k_{cat}/K_m, indicating high catalytic activity.

Table IV-6 Comparison of enzymatic properties between Yl-xynA and those of other

family 11 β-1,4 xylanase[a]

Enzyme	K_m (mg/ml)	k_{cat} (S^{-1})	k_{cat}/K_m (mg^{-1}S^{-1}ml)	References
Native XylA[b]	6.8	85	12.5	Berrin *et al.*, 2000
reXylA[c]	12.6	150	11.9	Berrin *et al.*, 2000
Nf Xyn11A[d]	6	136.9	22.8	Lafond *et al.*, 2011
Pp-xynA	2.8	243	86.8	This study
Yl-xynA	5.2	245	47.1	This study

[a]Kinetic values were analyzed using birchwood xylan as substrate.

[b]Native xylanase produced in *Aspergillus niger*

[c]Recombinant xylanase produced in *Pichia pastoris*

[d]Xylanase produced in *Nonomuraea flexuosa*

CHAPTER V

CONCLUSION AND FUTURE PERSPECTIVES

For sustainable development, xylan, one of the major components of lignocellulosic biomass, presents an attractive source for biorefinery applications because it is the non-edible portion of the plant and therefore, they do not interfere with food supplies. Xylanases play a key role in the xylan hydrolysis to xylooligosaccharides and have potential applications in a wide range of industries as well as the reduction of environmental impact of large-scale. Filamentous fungi, such as *Penicillia*, *Trichoderma* and *Aspergilli*, are known to be efficient producers of xylanolytic enzymes. Cultivation of filamentous fungi for large scale protein production is very complicated often ending up in many interfering enzymes. Therefore, recombinant DNA technology, that enables to analyze a single gene product, can be applied with more success.

Major findings in this dissertation can be concluded as follows:

1. Cloning, expression, purification and characterization of endo-1,4-β-xylanase from *Penicillium citrinum* in *Pichia pastoris*

A codon optimized *xynA* gene encoding xylanase A from *Penicilium citrinum* was successfully cloned and expressed in *Pichia pastoris* under the control of either *GAP* or *AOX1* promoter. The recombinant xylanase was expressed as a soluble protein. The effect of culture conditions on recombinant xylanase production was studied. The recombinant xylanase under the control of the *GAP* promoter was

expressed as a total activity production reached 34 times the level of activity produced by the native strain. The recombinant xylanase under the control of the *AOX1* promoter was produced at its maximum activity when cultured in BMMY medium (pH 6.0, 1% methanol and induced with 1% methanol every 24 h). After 5 days, activity of recombinant xylanase reached a peak at 676 U/ml, which was 193 times as high as that of the native xylanase (3.5 U/ml). Characterization of the purified recombinant xylanase showed an optimum temperature and pH of 55°C and pH 5, respectively. The recombinant xylanase was stable at a pH of 3 to 10 and retained around 80% of the original activity after pre-incubation at 40°C for 1 h. With birchwood xylan as substrate, the enzyme showed a K_m of 2.8 mg/ml and k_{cat} of 243 per s.

2. Overexpression, purification and characterization of endo-1,4-β-xylanase from *Penicillium citrinum* in *Yarrowia lipolytica*

A *xynA* gene encoding xylanase A from *Penicilium citrinum* was successfully cloned and expressed in *Yarrowia lipolytica* under the control of a *TEF* promoter. Native and prepro*LIP2* secretion signals were used for comparison of the expression and secretion level. The recombinant xylanase was expressed as a soluble protein, and total activity production reached 11 and 52 times the level of activity produced by the native strain, respectively. Maximum activity was observed with the prepro*LIP2* secretion signal when cultured in YTD medium (180 U/ml). Post translational glycosylation affected the molecular mass of the recombinant xylanase, resulting in an apparent molecular weight larger than 60 kDa, whereas after deglycosylation, the recombinant xylanase displayed a molecular mass of 20 kDa. The native

deglycosylated xylanase was purified by ion exchange chromatographic method. It was optimally active at 55°C and pH 5. It was stable at pH of 3 to 9. After 24 h, the enzyme retained more than 80% of the original activity. It conserved around 80% of the original activity after pre-incubation at 40°C for 6 h. With birchwood xylan as substrate, the enzyme showed a K_m of 5.2 mg/ml and k_{cat} of 245 per s.

Unlike *P. pastoris* expression platform, recombinant xylanase produced by *Y. lipolytica* was glycosylated. However, both yeast expression platforms showed the high level of secretion and interesting stability over a wide range of pH and at moderate temperatures lead to the recombinant xynA and might be useful for biotechnological applications. For further studies, copy number of xylanase gene could be determined to study the association with the xylanase activity.
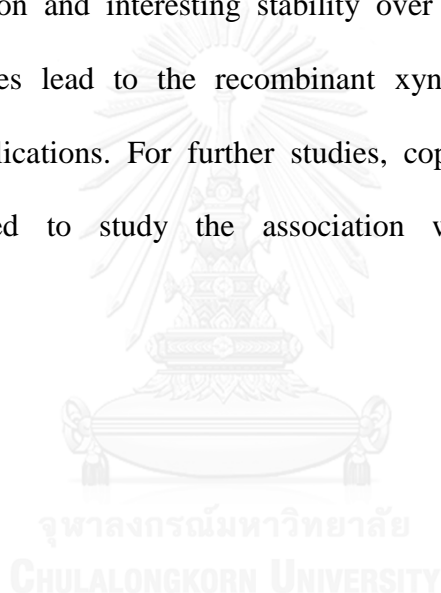
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APPENDIX

APPENDIX A

A codon-optimized *Penicillium citrinum xynA* gene was synthesized and introduced into a pUC57-Kan vector by GenScript based on the nucleotide database (GenBank: accession no. AB198065.1). DNA alignment of optimized sequence of xylanase A from *P. citrinum* FERM P-15944 is shown in Figure A-1.

```
Optimized    1    ATGCCATCCCTTACTTCATTGTTTTCATTTTTCGCCCTTGCTTCAGGAGCCTTTTCAGCC
Original     1    ATGCCGTCTCTGACTTCGCTATTCTCGTTCTTCGCTCTCGCTTCAGGCGCTTTCAGTGCT

Optimized   61    ACAGCCGATTTGTCCAAGAGAGAGTCATATACTTCTTCCTCAACCGGTACTAGTAACGGA
Original    61    ACTGCTGATCTCTCGAAAAGAGAATCTTATACTTCCAGCTCGACTGGAACTAGCAATGGC

Optimized  121    TACTACTACTCTTTCTGGACTGATGGTCAAGGAGACATTACATATTCCAACGGTGCTGCC
Original   121    TATTACTACTCCTTCTGGACGGATGGCCAGGGCGATATCACCTACTCCAACGGTGCCGCT

Optimized  181    GGAGAATACTCCGTTACTTGGTCAGGAGATGGAAATTTTGTCGCCGGTAAAGGATGGAAC
Original   181    GGCGAGTACAGCGTGACCTGGTCTGGTGATGGAAACTTCGTTGCTGGAAAGGGTTGGAAC

Optimized  241    CCAGGTGGATCAAGAGAGGTTACATTCAAAGGTTCTTACAACCCTAATGGAAACTCCTAT
Original   241    CCTGGTGGTAGCCGGGAGGTTACCTTCAAGGGCTCCTACAACCCCAACGGAAACAGCTAC

Optimized  301    TTGTCAGTTTACGGTTGGACCCAAAACCCACTTATCGAATTCTACATCGTCGAGGACTTC
Original   301    CTCTCCGTCTACGGCTGGACTCAAAACCCCCTGATCGAATTCTACATCGTCGAAGACTTC

Optimized  361    GGAACCTACAACCCTAGTTCTGGTGCTACTAAGAAAGGAACAGTTACCAGTGATGGTTCT
Original   361    GGCACTTACAACCCCTCCAGCGGCGCAACCAAGAAGGGCACTGTTACCAGTGATGGAAGT

Optimized  421    GTCTATGACATCTACACTTCCGAAAGAGTTAATCAGCCATCAATCGAGGGTACTGCAACA
Original   421    GTCTACGACATTTACACCAGTGAGCGAGTCAATCAGCCTTCTATTGAGGGCACTGCGACT

Optimized  481    TTCACCCAATATTGGAGTGTTAGACAGAACAAGAGATCCGAAGGTACAGTCACTACAGGA
Original   481    TTCACCCAGTACTGGTCCGTTCGTCAGAACAAGCGTTCTGAGGGAACTGTTACTACTGGA

Optimized  541    AACCATTTCAACGCTTGGAAGAATTTGGGTATGGATCTTGGATCTTTCAACTACATGATT
Original   541    AATCACTTTAATGCATGGAAAAACCTGGGCATGGACCTTGGTTCGTTCAACTATATGATT

Optimized  601    GTTGCCACTGAAGGTTATTACTCCTCTGGTTCCGCTGACATTACCGTTTCCTAA
Original   601    GTTGCTACAGAGGGATATTATAGCAGTGGTTCTGCTGATATTACTGTTAGCTAG
```

Figure A-1 DNA alignment of optimized sequence of xylanase A from *P. citrinum* FERM P-15944

Michaelis–Menten kinetics of xylanse from *P. pastoris* contains pPICZαA-*xynAPc* for birchwood xylan was shown as Figure A-2.
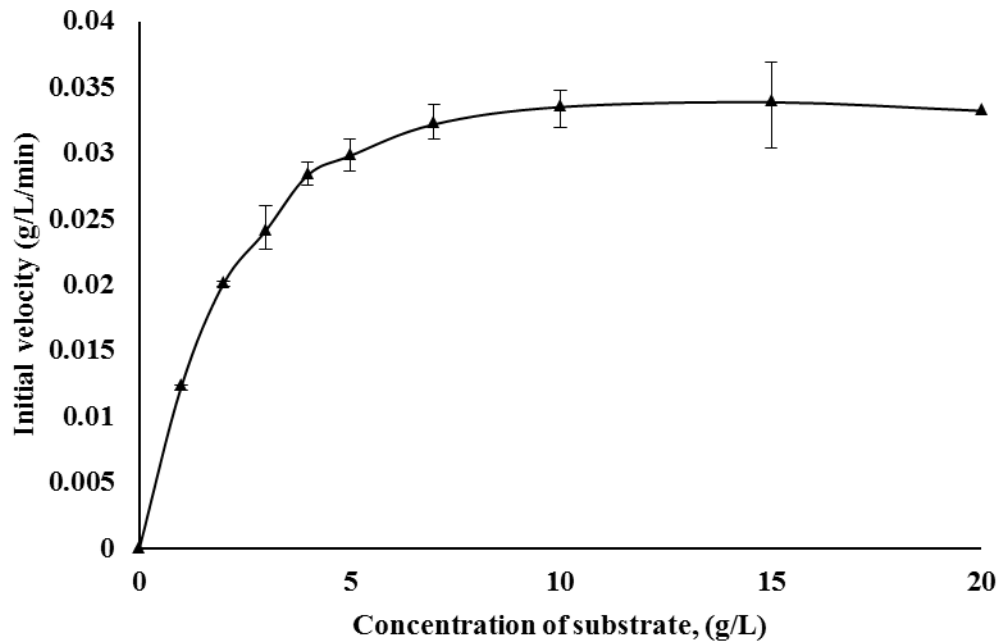

Figure A-2 Michaelis–Menten saturation curve for xylanase reaction showing the relation between nine different concentrations of birchwood xylan and reaction velocity

VITA

Miss Chanika Ouephanit was born on December 28th, 1984 in Bangkok, Thailand. She got a Bacherlor's Degree of Science in Genetics, Faculty of Science, Chulalongkorn University in 2006. Later in 2007, she furthered her Master's Degree of Science from Program in Biotechnology Faculty of Science, Chulalongkorn University, at which she finished in 2008 with "very good" merit from her defense examination. Then, she continue studied in Doctoral Degree in Program in Biotechnology, the same place, with received a 3 years-grant from the Royal Golden Jubilee Ph.D. Program (RGJ), Thailand Research Fund and including financial support from France Embassy in Thailand for 1 year during corroboration under supervisor of Dr. Michael J. O'Donohue and Dr. Sophie Bozonnet, in the Laboratory of Biological system engineering and Bioprocess(LISBP), Institut National des Sciences Appliquées (INSA), Toulouse, France.

Outcome from this study:

2 Publications

Ouephanit, C., Bozonnet, S., Boonvitthya, N., and Chulalaksananukul, W. 2016. Overexpression, Purification and Characterization of Endo-1,4-β-Xylanase from Penicillium citrinum in Pichia pastoris. (submitted in Enzyme and Microbial Technology)

Ouephanit, O., Boonvitthya, N., Chulalaksananukul, W., and Bozonnet, S. 2016. Overexpression, Purification and Characterization of Endo-1,4-β-Xylanase from Penicillium citrinum in Yarrowia lipolytica. (submitted in FEMS Microbiology Letters)

2 Patents

Patent no. 1501001990 (Thailand)

Patent no. 1501003653 (Thailand)