CLONING AND EXPRESSION OF A *CRYPTOVALSA* SP. (BCC7197) XYLANASE GENE IN *PICHIA PASTORIS*

KATEWADEE BOONYAPAKRON

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CLONING AND EXPRESSION OF A *CRYPTOVALSA* SP. (BCC7197) XYLANASE GENE IN *PICHIA PASTORIS*

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ABSTRACT

Cryptovalsa sp. (BCC7197), a filamentous fungus, has been previously collected from Phuket, Thailand. A preliminary testing for the potential enzyme activities demonstrated that BCC7197 produces xylanases at high level when compared to over 1,000 other fungi. Since xylanases are one of the industrially valuable enzymes, this work aims to isolate gene(s) that encode xylanase from BCC7197 and express it in *Pichia pastoris*. First, the culturing condition used to induce xylanase production in BCC7197 was optimized. The result demonstrated that maximal xylanase activities were detected after directly growing BCC7197 in the medium containing a supernatant of 5% wheat bran with continuous shaking at 30°C, 250 rpm for 8 days. This condition was used for culturing the fungus for total RNA isolation. RT-PCR and RACE methods were performed to identify the full-length xylanase cDNA from BCC7197. The result showed that the xylanase cDNA has an open reading frame of 978 bp encoding 325 amino acid residues. The BLAST search in GenBank database revealed that the BCC7197 xylanase gene belonged to glycosyl hydrolase family 10 and showed high sequence similarity to other fungal xylanases. Expression of the full-length xylanase gene, with or without its native leader sequence in *P. pastoris* expression vector, pPICZαA, was performed. The results showed that the active BCC7197 xylanase protein was successfully produced and secreted from *P. pastoris* with or without its own leader sequence.

KEY WORDS: *CRYPTOVALSA* SP./ XYLANASE/ GLYCOSYL HYDROLASE FAMILY 10/ *PICHIA PASTORIS* EXPRESSION/ XYLAN

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การโคลนและการแสดงออกของยีนที่สร้างเอนไซม์ไซแลนเนสจากเชื้อ *Cryptovalsa* sp. (BCC7197) ในยีสต์ *Pichia pastoris* (CLONING AND EXPRESSION OF A *CRYPTOVALSA* SP. (BCC7197) XYLANASE GENE IN *PICHIA PASTORIS*)

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บทคัดย่อ

Cryptovalsa sp. (BCC7197) จัดอยู่ในกลุ่มของเชื้อราเส้นใย ซึ่งเก็บตัวอย่างได้ในประเทศไทยที่จังหวัดภูเก็ต จากการศึกษาความสามารถในการผลิตเอนไซม์ เบื้องต้นพบว่า BCC7197 สามารถผลิตเอนไซม์ไซแลนเนสได้ในปริมาณสูงอย่างมีนัยสำคัญเมื่อเทียบกับเชื้อราอื่นๆกว่าพันชนิด ซึ่งไซแลนเนสเป็นเอนไซม์ที่มีความสำคัญอย่างยิ่งในอุตสาหกรรม งานวิจัยนี้ได้ทำการโคลน cDNA ของเอนไซม์ไซแลนเนสจากเชื้อ BCC7197 และศึกษาการแสดงออกในยีสต์ *Pichia pastoris* ในขั้นแรกเชื้อ BCC7197 ได้ถูกเหนี่ยวนำให้เกิดการสร้างเอนไซม์ไซแลนเนสในสูตรอาหารต่างๆ ผลการทดลองพบว่าสูตรอาหารที่ประกอบด้วยรำข้าวสาลี 5% ที่ผ่านการกรองสามารถเหนี่ยวนำให้เกิดการสร้างเอนไซม์ไซแลนเนสได้สูงสุด เมื่อทำการเลี้ยงที่อุณหภูมิ 30 องศาเซลเซียสเป็นระยะเวลา 8 วัน จากนั้นทำการสกัด total RNA จากเชื้อที่ผ่านการเหนี่ยวนำและนำ total RNA ที่สกัดได้มาใช้สังเคราะห์ cDNA ด้วยวิธีการ RT-PCR และ Rapid Amplification of cDNA Ends (RACE) ผลการศึกษาพบว่า cDNA ของเอนไซม์ไซแลนเนสให้ open reading frame มีความยาว 978 นิวคลีโอไทด์ ซึ่งถอดรหัสให้โปรตีนประกอบด้วยกรดอะมิโนจำนวน 325 ตัว เมื่อนำลำดับอะมิโนไปเปรียบเทียบกับฐานข้อมูลใน GenBank พบว่า cDNA ของเอนไซม์ไซแลนเนสของเชื้อ BCC7197 จัดอยู่ใน glycosyl hydrolase กลุ่มที่ 10 ลำดับอะมิโนมีความเหมือนกับเอนไซม์ไซแลนเนสกลุ่มที่ 10 ของเชื้อราชนิดอื่นๆ ผลการศึกษาการแสดงออกของ cDNA ของเอนไซม์ไซแลนเนสในยีสต์ *Pichia pastoris* พบว่า cDNA ของเอนไซม์ไซแลนเนสของเชื้อ BCC7197 สามารถผลิตและหลั่งออกนอกเซลล์ได้ ทั้งในแบบที่มี leader sequence และไม่มี leader sequence

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LIST OF ABBREVIATIONS

A_{260}	=	absorbance at 260 nm
A_{280}	=	absorbance at 280 nm
AOX	=	alcohol oxidase gene
APS	=	ammonium persulfate
ATP	=	adenosine triphosphate
BCC7197	=	*Cryptovalsa* sp. (BCC7197)
BLAST	=	Basic Local Alignment Search Tool
BSA	=	bovine serum albumin
BMGY	=	buffered minimal glycerol complex medium
BMMY	=	buffered minimal methanol complex medium
bp	=	base pair (s)
CTAB	=	cetyl trimethyl ammonium bromide
°C	=	degree Celcius
cDNA	=	complementary DNA
dATP	=	deoxyadenosine-5'-triphosphate
dCTP	=	deoxycytosine-5'-triphosphate
dGTP	=	deoxyguanosine-5'-triphosphate
dTTP	=	deoxythymidine-5'-triphosphate
dNTP	=	dATP, dCTP, dGTP, dTTP
DMSO	=	dimethyl sulfoxide
DNA	=	deoxyribonucleic acid
DTT	=	dithiothreitol
EtBr	=	ethidium bromide
E. coli	=	*Escherichia coli*
EDTA	=	ethylenediamine tetraacetic acid
g	=	gram
xg	=	centrifugal force
h	=	hour (s)

LIST OF ABBREVIATIONS (CONT.)

IPTG	=	isopropyl-β-D-thiogalactopyranoside
kb	=	kilobase (s)
kDa	=	kilodalton (s)
KEX2	=	killer expression 2 protein
LB	=	Luria-Bertani (medium)
M	=	molar
mM	=	millimolar
mg	=	milligram
min	=	minute (s)
ml	=	milliliter
µg	=	microgram
µl	=	microliter
mRNA	=	messenger RNA
ng	=	nanogram
nt	=	nucleotide (s)
OD	=	optical density
PAGE	=	polyacrylamide gel electrophoresis
PCR	=	polymerase chain reaction
pmol	=	picomole
P. pastoris	=	*Pichia pastoris*
RACE	=	rapid amplification of cDNA ends
RNA	=	ribonucleic acid
RNase A	=	ribonuclease A
rpm	=	revolution per minute
RT-PCR	=	reverse transcription-polymerase chain reaction
SDS	=	sodium dodecyl sulfate
sec	=	second (s)
T_m	=	melting temperature

LIST OF ABBREVIATIONS (CONT.)

TAE	=	tris-acetate EDTA
TEMED	=	N,N,N',N'-tetramethyl-ethylenediamine
Tris-HCl	=	tris-(hydroxylmethyl)-aminoethane hydrochloric acid
UV	=	ultraviolet
V	=	volt
v/v	=	volume/volume
w/v	=	weight/volume
X-gal	=	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YEPD	=	yeast extract peptone dextrose

CHAPTER I
INTRODUCTION

1.1 *Cryptovalsa* sp. (BCC7197)

Cryptovalsa sp. (BCC7197) is a filamentous fungus belonging in the phylum *Ascomycota*, class *Ascomycetes*, subclass *Sordariomycetes*, order *Incertae sedis*, and family *Incertae sedis* (Figure 1), which was collected from Panwa Beach, Phuket, Thailand. It was characterized to the genus level by E. B. G. Jones (BIOTEC, Thailand). *Cryptovalsa* contains 19 species (1) which grow in terrestrial habitats and are widely distributed geographically. They can be found from mangrove or coastal habitats, such as salt marsh or intertidal zone, in both temperate and tropical regions (2). The characteristics of the fungi in this genus include the formation of ascomata and the production of asci and ascospores. BCC7197 was isolated from a piece of wood and after detailed examination was found to contain multispores per ascus. When tested for the activities of potential enzymes, it was shown that BCC7197 produces xylanase at high level when compared to over 1,000 other fungi (personal communication, BIOTEC).

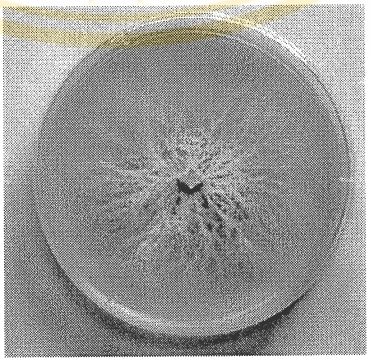


Figure 1. Filamentous fungus, *Cryptovalsa* sp. (BCC7197)

1.2 Structure and occurrence of xylan

Hemicelluloses are noncellulosic polysaccharides that are found in plant tissues (3). In the cell wall of land plants, xylan is the most common hemicellulosic polysaccharide, representing up to 30%-35% of the total dry weight (4). Xylans are composed of 1,4-linked β-D-xylopyranosyl residues (5, 6). Most xylans occur as heteropolysaccharides, containing different substituent groups in the backbone chain and in the side chain (7). The common substituents found on the backbone of xylan are acetyl, arabinosyl, and glucuronysyl residues (8).

1.2.1 Hemicellulose from hardwood

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 (9). Every tenth xylose residue carries a 4-*O*-methylglucuronic acid attached to the 2 position of xylose. Hardwood xylans are highly acetylated (e.g., birchwood xylan contains more than 1 mol of acetic acid per 2 mol of xylose). Acetylation is more frequent at the C-3 than at the C-2 position, however, acetylation at both positions has been reported (10, 11). The presence of these acetyl groups is responsible for the partial solubility of xylan in water (9). These acetyl groups are readily removed when xylan is subjected to alkali extraction (12).

1.2.2 Hemicellulose from softwood

The xylan from softwood is composed of arabino-4-*O*-methylglucuroxylans. They have higher 4-*O*-methylglucuronic acid content than do hardwood xylans. The 4-*O*-methylglucuronic acid residues are attached to the C-2 position. Softwood xylans are not acetylated, and instead of an acetyl group they have α-L-arabinofuranose units linked by α-1,3-glycosidic bonds at the C-3 position of the xylose (13). The arabinosyl substituents occur on almost 12% of the xylosyl residues (14).

1.3 Xylanolytic enzymes system

Xylanolytic enzymes are the glycosyl hydrolase which degrades the xylan. Xylanases are usually composed of repertoire of hydrolytic enzymes: β-1,4-

endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and ρ-coumaric acid) esterase (Figure 2) (5, 12).

(1) β-1,4-Endoxylanase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.8) cleaves the internal glycosidic linkages of the heteroxylan backbone, resulting in the production of xylooligosaccharides.

(2) β-D-Xylosidases (β-D-xyloside xylohydrolase; EC 3.2.1.37) are exoglycosidases that hydrolyze short xylooligosaccharides and xylobiose from the nonreducing end to liberate xylose.

(3) α-L-Arabinofuranosidases; there are two types of arabinases, the exo-acting α-L-Arabinofuranosidases (EC 3.2.1.55), which is active against ρ-nitrophenyl-α-L-Arabinofuranoside and on branched arabinans, and the endo-1,5-α-L-arabinase (EC 3.2.1.99), which is active only toward linear arabinans.

(4) α-D-Glucuronidases (3.2.1-) hydrolyze the α-1,2 linkages between glucuronic acid and xylose residues in glucuronoxylan.

(5) Acetylxylan Esterases (EC 3.1.1.6) remove the *O*-acetyl substituents at the C-2 and C-3 positions of xylose residues in acetylxylan.

(6) Ferulic and ρ-coumaric acids are linked to xylan by esterase bonds. Ferulic acid esterase (EC 3.1.1-) cleaves the ester linkages between arabinose side chains and ferulic acids in xylan. Similarly, ρ-coumaric acid esterase (EC 3.1.1-) cleaves the ester linkage between arabinose and ρ-coumaric acid.

All these enzymes act cooperatively to convert xylan into its constituent sugars. The presence of such multifunctional xylanolytic enzymes system is quite widespread among bacteria and fungi (15, 16). Heteroxylans contain different substituent groups in the backbone and side chain. Thus, the degradation of such a complex polysaccharide may involve synergistic action between the different components of the xylanolytic enzymes system (17, 18, 19, 20).

1.4 Classification of endo-β-1,4-xylanases families

In 1988, Wong *et al.* have divided xylanases into two categories: (a) the low molecular weight, basic xylanases and (b) high molecular weight, acid xylanases (14). The low molecular weight basic xylanases are usually endo-1,4-β-xylanases with

specific activity on xylan only, whereas the other group seems to contain endo-xylanases with cellulase activity. These groups roughly correspond to β-glycanase families G and F, respectively.

In 1991, a classification of glycosyl hydrolases was proposed on the basis of amino acid sequence similarities (21). The relatedness of enzymes can be demonstrated either by pairwise alignments of the protein sequences or by the basic local alignment search tool (BLAST) to discern sequence similarity (22).

In 1997, hydrophobic cluster analysis (HCA) have been used to classify the glycosyl hydrolase (23). The hydrophobic cluster analysis is a comparative method designed to predict protein folding based on the hydrophobic/hydrophilic patterns and is used to correlate members of a protein group of similar catalytic function (24). HCA is a very sensitive tool to uncover distant relatedness between proteins for which standard alignments methods are unreliable (25). Based on the amino acid sequence similarities and hydrophobic cluster analysis, endo-β-1,4-xylanases (EXs, EC 3.2.1.8) can be divided into two major families. Acidic high-molecular mass EXs (> 30 kDa) were found to belong to one family, assigned as glycanase family 10 (formerly family F), and basic low-molecular-mass EXs, were found to belong to another family, designated as glycanase family 11 (formerly family G) (26, 27, 28).

At past few years, the tertiary structures of EXs have been established by crystallography. EXs of family 11 appear to be smaller and well-packed molecules, formed mainly of β-sheets (29, 30, 31, 32) (Figure 3B). EXs of family 10 is a typical 8-fold α/β barrel $(\alpha/\beta)_8$ resulting in 'salad bowl' shape of the molecule (33, 34, 35) (Figure 3A).

1.5 Xylanases induction

Xylanases are widespread in nature. They occur both in prokaryotes and eukaryotes and have been reported from marine and terrestrial bacteria, rumen bacteria, fungi, marine algae, snail, crustaceans, insects and seeds of terrestrial plants (36). Most of the xylanases known to date are from bacterial and fungal sources (12). Xylanase are usually inducible enzymes secreted in media containing pure xylan or xylan-rich residues (37). However, constitutive production of xylanase has also been

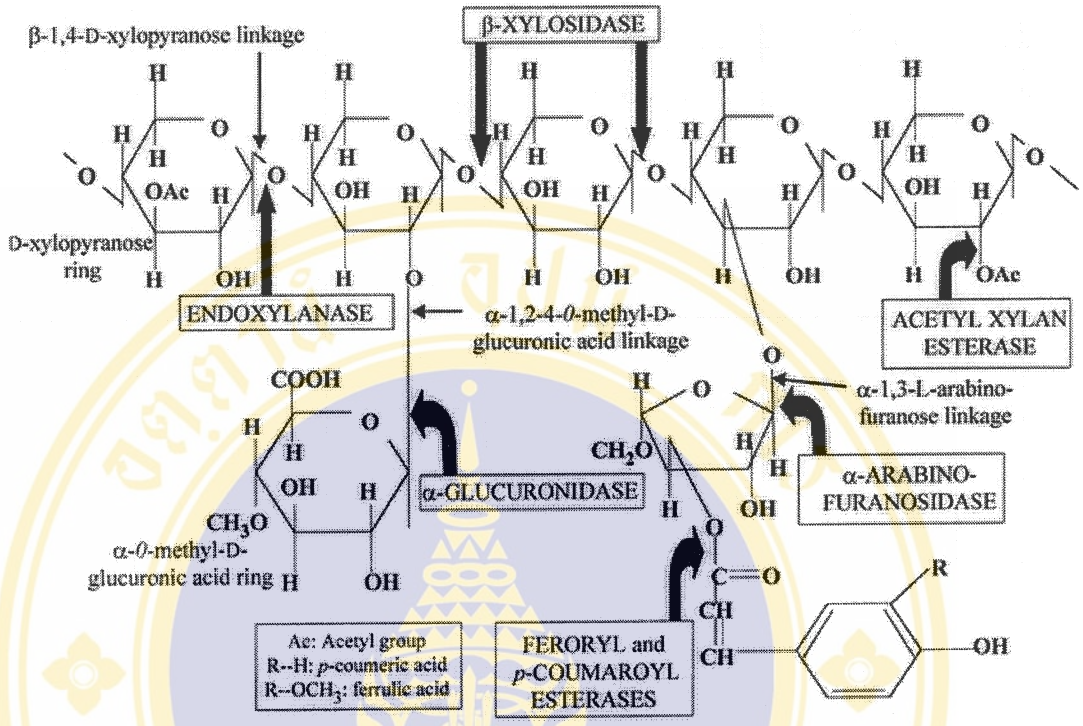
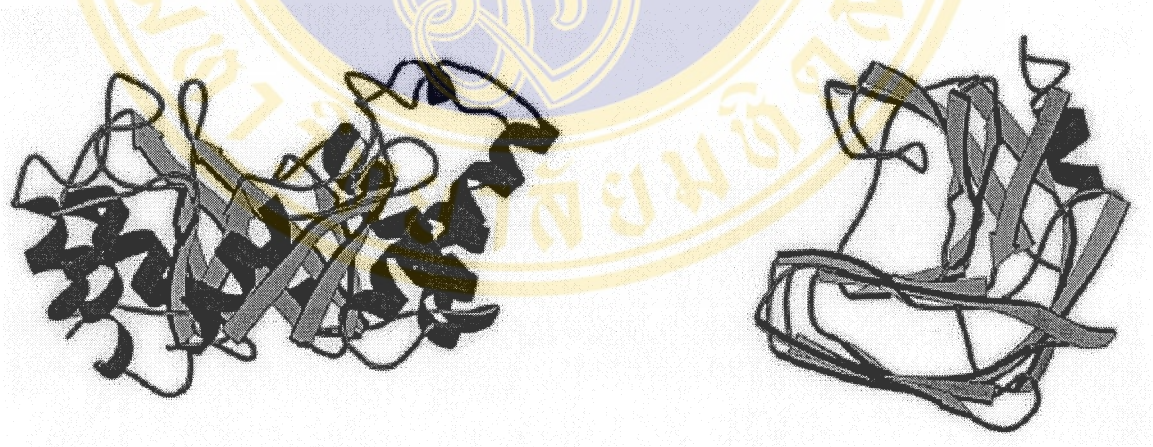


Figure 2. Xylanolytic enzymes system (Taken from: Hoondal, 2001) (5)



(A) EX of family 10 **(B) EX of family 11**

Figure 3. Ribbon representation of the main fold of the catalytic domains of EXs of family 10 and 11 (Adapted from: Klurpfel, 1997) (28)

reported (38, 39, 40). Induction is mostly by xylan in *Trametes trogii* (41), *Aspergillus awamori* (42), and *Streptomyces* sp QG-11-3 (43). However, in *Cellulomonas flavigena*, xylan is a poor inducer (44). Induction of xylanase by several other compounds, such as L-sorbose, various xylooligosaccharides, and xylose has been reported. L-sorbose in medium induces the xylanase production in *Sclerotium rolfsii* (45) and *Trichoderma reesei* PC-3-7 (46). In *Bacillus* sp. BP-7 (47) and *Trichosporon cutaneum* SL409 (48), xylanase is induced by xylose, but is repressed in the presence of glucose. Several reports have shown xylanase induction by hemicellulosic substrates like corn cob, wheat bran, rice bran, rice straw, corn stalk and bagasse (49, 50). These substrates have also been found to be most suitable for the production of xylanses in the case of certain microorganisms such as *Aspergillus awamori*, *Penicillium purpurogenum* (51) and alkaliphilic thermophilic *Bacillus* sp. NCIM 59 (52). In 1997, Penttila *et al.* studied the expression of the hemicellulase gene of *Trichoderma reesei*. They found that the expression patterns of ten hemicellulase-encoding genes are different in various culture conditions including polymeric and di- and monomeric sugars (53). The enhancement of xylanase production in the presence of amino acids has also been shown in *Bacillus* sp. No. C-125 (54), *Trametes trogii* (41), and *Streptomyces* sp. QG-11-3 (55). However, some reports have been shown that growing the cells under a lower nitrogen/carbon ratio in the medium may be one strategy for producing xylanolytic enzymes (56).

1.6 Applications of xylanolytic enzymes

Xylanolytic enzymes from microorganisms have attracted a great deal of attention in the last decade, particularly because of their biotechnology potential in various industrial processes (57), such as food, feed, and pulp and paper industries. Xylanases have shown an immense potential for increasing the production of several useful product in a most economical way. The main possibilities are the production of single cell proteins (SCPs), enzymes, liquid or gaseous fuel, and solvents and sugar syrups, which can be used as such or as feed stock for other microbiological processes (58).

(1) Currently, the most promising application of xylanases is in the probleaching of kraft pulps. Enzyme application improves pulp fibrillation and water retention,

reduction of beating times in virgin pulps, restoration of bonding and increased freeness in recycled fibers, and selective removal of xylans from dissolving pulps.

(2) Depression in weight gain and feed conversion efficiency in rye-fed broiler chicks has been associated with intestinal viscosity. Incorporation of xylanase into a rye-based diet of broiler chickens results in reduced intestinal viscosity, thus improving both the weight gain of chicks and their feed conversion efficiency (59).

(3) The efficiency of xylanases in improving the quality of bread has been seen with an increase in specific bread volume (60).

(4) Xylan is present in large amounts in wastes form and food industries. Hence, xylanase are used for conversion of xylan into xylose in waste water. The development of an efficient process of enzymatic hydrolysis offers new prospects for treating hemicellulosic wastes (61).

(5) Xylanase are used concurrently with cellulase and pectinase for clarifying must and juices, and for liquefying fruits and vegetables (62).

(6) Xylanase in synergism with several other enzymes, such as mannanase, ligninase, xylosidase, glucanase, etc., can be used for the generation of biological fuels, such as ethanol and xylitol, from lignocellulosic biomass (58).

1.7 Methods for assay xylanases activity

1.7.1 Quantitative assays

(1) Reductometric method

Xylanase (EC 3.2.1.8) activity is generally determined by measuring the reducing sugars released from xylan by the Somogyi-Nelson or the dinitrosalicylic acid (DNS) method (63, 64). Based on a round-robin test that involved 20 laboratories, Bailey *et al.* (1992) proposed a suitable procedure for preparing xylan and measuring xylanase activity (65). There are some limitations these methods may lead to false interpretations in the presence of oxidizing or reducing agents and are not suitable for the detection of low levels of xylanase activity.

(2) Viscometric method

A viscometric method using a soluble xylan (arabinoxylan or carboxymethyl xylan) has been used for the determination of xylanase activity in sample containing high background sugar values (66). Although this method detects the ability of an

enzyme sample to hydrolyse xylan, it does not reveal the type of products released, which may be important when evaluating the ability of an enzyme to degrade xylan.

(3) Dyed substrate method

Another specific assay, which can be used to determine xylanase activity in the present of either large amounts of reducing sugars or viable cell utilizing xylan fragments, involves the use of soluble and covalently dyed xylan (Remazol Brilliant Blue) (RBB-xylan). The assays are based on photometric measurements of the enzyme-released dyed fragments soluble in the presence of organic solvents which precipitate the original substrates and their high-molecular weight fractions. The assays are advantageous for rapid analyses of large amount of samples and also permit evaluation at a high level of reducing compounds (67).

A nephelometric (turbidometric) assay using insoluble xylan (Azurine blue dyed and cross-linked xylan: AZCL-xylan) has also been found to be suitable for determining xylanase activity (68). Xylan molecules were dyed and made insoluble by chemical cross-linking, which makes the partly soluble xylan into insoluble particles form. Xylanase activity is determined by measuring the dye-linked soluble fraction released from the insoluble substrate (69).

1.7.2 Qualitative assays

Qualitative assays have been developed either to select microbial strains producing high levels of xylanases, or to identify and/or characterize these enzymes in a given sample. Insoluble xylan and RBB-xylan are ideal substrates to select microorganisms producing xylanase on solid agar medium (70). Similarly, methods using xylan stained with Congo Red can be used to select microorganisms producing xylanase activity.

1.8 Cloning and expression of xylanase gene(s)

For the commercial realization and economic viability of xylanase production, it is necessary to identify organisms which can hyperproduce the enzymes. Recombinant DNA techniques offer the means to enhance protein production. Xylanase gene have already been cloned and characterized from different microbial genera.

The heterologous expression of genes from various organisms, for example in *E. coli* have been reported, *Butyrivibrio fibrosolvens* (71), *Bacillus polymyxa* (72), and

Bacillus subtilis (73). However, the expression in *E. coli* is generally found to be lower than parent organisms and confined to the cytoplasmic or the periplasmic fractions. The absence of post-translational modifications such as glycosylation in *E. coli* and the intracellular accumulation of the recombinant xylanases have been suggested to be the key reasons for the low levels of activity (74). Extracellular activity has been reported in recombinant *E. coli* for the xylanase from alkaliphilic, *Aeromonas* (75), thermophilic *Bacillus* sp. (76), and *Cellulomonas* sp. (77).

The heterologous expression has also been reported in yeast, such as *Saccharomyces cerevisiae* and *Pichia pastoris*. These organisms have several advantages over bacteria in that the yeast has the potential to perform many of the post-translational modifications typically associated with higher eukaryotes, such as processing of signal sequences, folding, disulfide bridge formation, and glycosylation. The successful expression of xylanase genes in yeast have been reported from various microorganisms, for example *Aspergillus nidulans* (78) *Cryptococus albidus* (79), *Trichoderma reesei* (80), *Aureobasidium pullulans* (81), and *Aspergillus niger* (82).

1.9 *Pichia pastoris* expression system

Pichia pastoris is one of the eukaryotic expression systems that is widely used as it is capable of many of the post-translational modifications performed by higher eukaryotic cells, such as proteolytic processing, folding, disulfide bond formation, and glycosylation. Thus, many proteins that end up as inactive inclusion bodies in the baterial system are produced as biologically active molecules in *P. pastoris*. The *P. pastoris* system is also generally regarded as being faster, easier, and less expensive to use than expression systems derived from higher eukaryotes, such as insect and mammalian tissue culture cell systems, and usually gives higher expression levels and the downstream processes of rapid purification. Therefore, it has become highly successful for the expression of heterogous genes (83).

1.10 General characteristics of *P. pastoris*

P. pastoris is classified in Genera *Ascomycetous*, Family *Saccharomycestaceae* and Order *Endomycetales* (84). *P. pastoris* is a methylotrophic yeast, capable of

metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. This enzyme has a poor affinity for O_2, and therefore *Pichia pastoris* compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous protein expression in *Pichia*.

There are two genes in *P. pastoris* that encode for alcohol oxidase *AOX1* and *AOX2*. The *AOX1* gene is responsible for the vast majority of alcohol oxidase activity in the cell. Expression of the *AOX1* gene is tightly regulated and induced by methanol to very high level, typically ≥ 30% of the total soluble protein in cells grown with methanol as the carbon source. The *AOX1* gene has been isolated and a plasmid-borne version of the *AOX1* promoter is used to drive expression of the gene of interest encoding the desired heterologous protein.

1.11 Advantages of *P. pastoris* as an expression system

P. pastoris expression system offers several advantages over other expression systems.

1.11.1 Genetics and growth

P. pastoris has many of the genetic characteristics that found in *S. cerevisiae*. Its genome can be complemented, disrupted and replaced with a wide variety of genes from a number of sources.

The ability to integrate an expression cassette into the genome of *P. pastoris* at precisely defined locations, compared to the plasmid driven expression that is found with *S. cerevisiae*, enables greater stability of recombinants in both the shaked flasks and fermented cultures.

The growth kinetics of *P. pastoris* allows extremely high cell densities in comparison with other expression systems. The cell density could approach 150 grams of dye weight per liter within a generation time of approximately three hours (85). Under these conditions, the expression levels can reach up to 30% of total cellular proteins for intracellular expression (86) or up to 80% for expression with secreted recombinant proteins (87, 88). This is more than that found in *S. cerevisiae*, where the

maximal recombinant protein concentration is approximately 5% of cellular proteins (89).

1.11.2 Post-translational modifications and targeting

The ability to secrete the heterologous protein product through the secretory pathway of the yeast expression system permits post-translational events similar to those in higher eukaryotes such as proteolytic maturation, glycosylation and disulfide bond formation (90). The expression of disufide-bonded recombinant proteins in *E. coli* system is not suitable, as the *E. coli* cytoplasm is reducing environment, which prevents disulfide bond formation. In contrast, *P. pastoris* has been successfully used to express disulfide-bonded proteins with high yield, suggesting that all of the necessary folding to the eukaryotic secretory pathway is glycosylated prior to secretion. This has been a problem for heterologous proteins containing N-linked oligosaccharides when they are expressed in *S. cerevisiae*, where hyperglycosylation occur frequently (91, 92, 93, 94). This hyperglycosylation of *S. cerevisiae* may cause differences in immunogeneicity and diminish activity of foreign protein. In contrast, *P. pastoris* has been used to express a number of heterologous proteins without extensive hyperglycosylation (95, 96)

1.11.3 Expression levels

P. pastoris has the ability to express up to gram/liter amounts of recombinat protein such as human serum albumin (4.0 g/l) (97), α-amylase (2.5 g/l) (98) and α-galactosidase (0.4 g/l) (99). These levels far exceed many other expression systems. The differences between the *S. cerevisiae* and *P. pastoris* systems are illustrated by the expression of murine epidermal growth factor. In *S. cerevisiae* expression levels of up to 0.6 mg/l were recorded. In constrast, *P. pastoris* expressed up to 22.4 mg/l (100). This represents an approximately 37-fold increase in overall expression levels.

1.12 Computer program for sequence analysis

1.12.1 NCBI-BLAST

BLAST (Basic Local Alignment Search Tool) is a set of programs designed for sequence database similarity searching. The query sequences can be both DNA and protein. The BLAST programs have been designed for a less sensitivity to distantly

related sequences. In BLAST program, sequence alignment is performed in pair-wise fashion. Results of searches may be compared directly by using alignment score (S). Alignment score (in bits) is calculated from the number of identity residues, mismatch residues and gap penalties. This score represents the degree of similarity between the query sequence and the compared sequence in the database. The higher the score, the greater the degree similarity. Moreover, the blast results also show the significance of each sequence alignment in the value of the statistical expectation value (E value). The E value is the number of different alignments with scores equivalent to or better than S that are expected to occur in a database search by chance. Lower E value indicates more significance of the alignment score (101).

There are many different types of BLAST. Choosing the right one depends on the type of query sequence and the NCBI nucleotide sequences.

(1) Standard nucleotide-nucleotide BLAST (blastn) compares query nucleotide sequence against the NCBI nucleotide sequences.

(2) Standard protein-protein BlAST (blastp) compares query protein sequence against the protein sequences in protein sequence database.

(3) Nucleotide query-protein db (blastx) compares a nucleotide query sequence translated in all reading frames against a protein sequence database.

(4) Protein query-translated db (tblastn) compares a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames.

(5) Nucleotide query-translated db (tblastx) compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

1.12.2 Clustal X (Sequence alignment program)

Clustal X is a new window interface of program Clustal W. It can be used to perform multiple alignment of any group of protein or nucleic acid sequences that are related to each other over their entire lengths. A set of short fragment will not work in Clustal X. Clustal X will align the most closely related sequences first in order to build a representative profile of the family. Divergent sequences are delayed by default until this profile is available. Clustal X plays the sequence alignment in a window on the screen. New features include the ability to cut and paste sequences to change the order of the alignment, selection of a subset of the sequences to be realigned, and selection

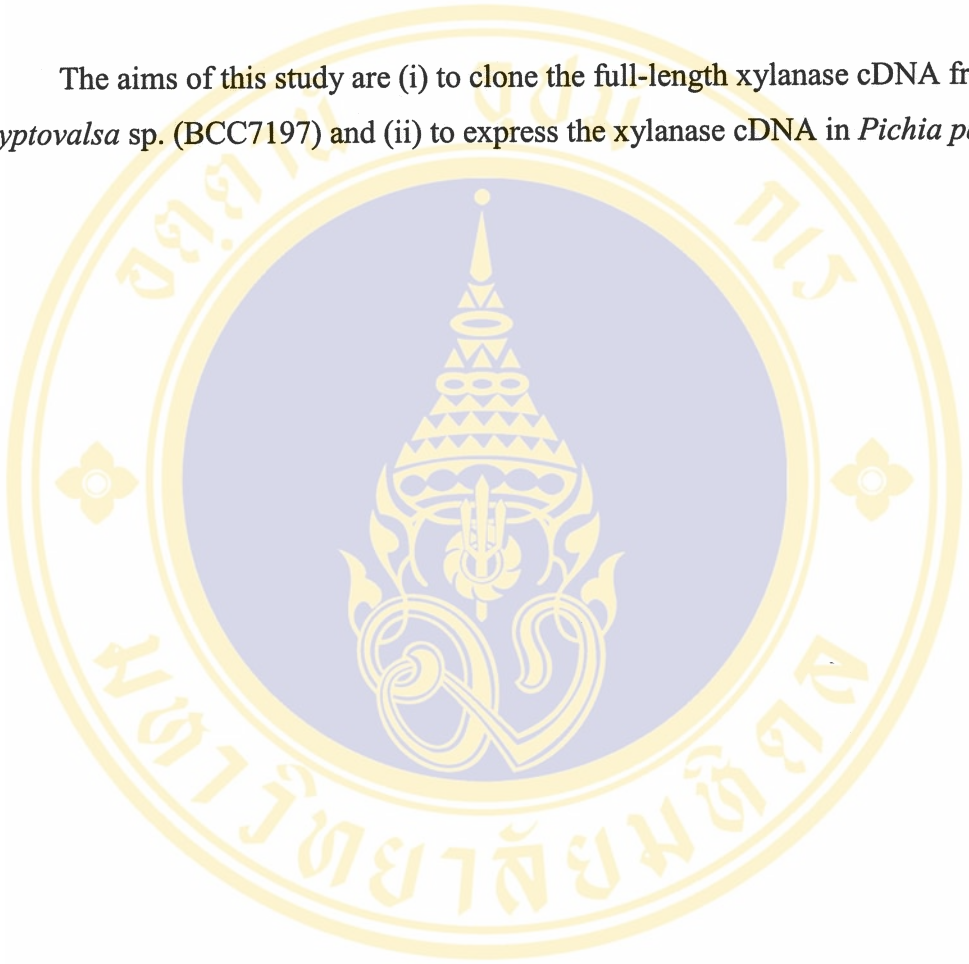
of a sub-range of the alignment to be realigned and inserted back into the original alignment. Quality analysis and realignment of selected residue ranges provide the user with a powerful tool to improve and refine difficult alignments and to trap errors in input sequences. Moreover, Clustal X can calculate phylogenetic trees by using the Neighbour-Joining method, a widely used and relatively fast algorithm that clusters sequences by minimizing the sum of branch lengths. However, Clustal X does not display trees. A simple tree-display program, NJplot is included in the Clustal X distribution package. Like Clustal X, NJplot is available for all computer platforms. NJplot reads the phylip-format tree output of Clustal X and displays trees as dendrograms. Basic manipulations of the text labels, branch flips and rerooting of the tree can be performed. However, the underlying tree topology cannot be changed (102, 103).

1.12.3 SWISS-MODEL

SWISS-MODEL is a server for automated comparative modeling of three-dimensional (3D) protein structures. MODEL provides several levels of user interaction through its World Wide Web interface: in the 'first approach mode' only an amino acid sequence of a protein is submitted to build a 3D model. Template selection, alignment and model are done completely automated by the server. In the 'alingment mode', the modeling process is based on a user-defined target-template alignment. Complex modeling tasks can be handled with the 'project mode' using Deep View (Swiss-PdbViewer), an integrated sequence-to-structure workbench (104).

CHAPTER II
OBJECTIVES

The aims of this study are (i) to clone the full-length xylanase cDNA from *Cryptovalsa* sp. (BCC7197) and (ii) to express the xylanase cDNA in *Pichia pastoris*.



CHAPTER III
MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 *Cryptovalsa* sp. (BCC7197)

The filamentous *Cryptovalsa* sp. (BCC7197) was obtained from the BIOTEC Culture Collection, BIOTEC, Thailand.

3.1.2 Bacterial strain

Escherichia coli, DH5α [*sup*E44 Δ*lac*U169 (Φ80 *lac*Z ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1] was used as a host for plasmid propagation.

3.1.3 Yeast strain

Pichia pastoris KM71 (*arg4, his4, aox1::Arg4*) was used as a host cell for expression of xylanase.

3.1.4 Plasmid vector

3.1.4.1 pGEM®-T Easy vector (Promega) was used in all cloning steps. A physical map of pGEM®-T Easy vector was shown in Figure 4.

3.1.4.2 pPICZαA vector (Invitrogen) was used in *P. pastoris* expression. A physical map of pPICZαA vector was shown in Figure 5.

3.1.5 Synthetic oligonucleotides

All synthetic oligonucleotides (primers) were custom synthesized by Bioscience Unit, BIOTEC. Information of primers is shown in Table 1.

3.1.6 Culture media

3.1.6.1 Fungal culture media.

BCC7197 was grown in PDB [(Potato Dextrose Broth); 0.4% (w/v) potato extract, and 2% (w/v) dextrose] supplemented with 17 ppt marine salt. For agar plates, 1.5% (w/v) of bacteriology agar was added.

3.1.6.2 Bacterial culture medium (for transformants containing pGEM®T Easy vector)

E. coli strain DH5α was grown in LB [1% (w/v) peptone (Difco), 0.5% (w/v) NaCl, and 0.5% (w/v) yeast extract (Difco)]. The *E. coli* transformants were grown in LB containing 100 μg/ml ampicillin (Sigma, USA). For agar plates, 2% (w/v) of bacteriology agar was added. For X-gal/IPTG agar plates, 40 μl of a stock solution 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; 20 mg/ml of X-gal in dimethyl-formamide) and 4 μl of a solution of isopropylthio-β- D-galactoside (IPTG) were added in agar media.

3.1.6.3 Bacterial culture medium (for transformants containing pPICZαA vector)

E. coli (strain DH5∝) was cultured in low salt LB medium containing 1% (w/v) tryptone or peptone (Gibco BRL, USA), 0.5% (w/v) NaCl and 5% (w/v) yeast extract (Gibco BRL, USA), The pH of the medium was adjusted to 7.5 with NaOH. The *E. coli* transformants were grown in low salt LB medium containing 25 μg/ml Zeocin™ (Invitrogen, Canada) as a selectable marker.

3.1.6.4 Yeast culture medium

3.1.6.4.1 Culture and selective media

P. pastoris (strain KM71) was grown in a rich medium YEPD [2% (w/v) peptone, 2% (w/v) glucose (Sigma, USA) and 1% (w/v) yeast extract]. The *P. pastoris* transformants were cultured under selective condition in YEPD containing 100 μg/ml Zeocin™.

3.1.6.4.2 Expression medium

Buffered minimal glycerol complex medium (BMGY) is composed of 0.67% (w/v) yeast nitrogen base, 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 0.00004% biotin (w/v), and 1% (w/v) glycerol.

Buffered minimal methanol medium (BMMY) is composed of 0.67% (w/v) yeast nitrogen base, 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 0.00004% biotin, and 3% methanol.

3.1.7 Standard xylanase and xylan

Standard xylanase from *Trichoderma viride* was purchased from Fluka, Switzerland. Xylan from birchwood was purchased from Sigma, USA and Merck, Germany.

3.1.8 Chemicals

Zeocin™ was purchased from either Invitrogen, Canada or CAYLA, France. Other chemicals and solvents (analytical grade) were purchased from Gibco BRL, USA; Fluka, Switzerland; Sigma, USA and Merck, Germany.

3.1.9 Enzymes

Restriction enzymes and other modification enzymes were purchased from New England Biolabs, USA; Boehringer Mannheim, Germany; Promega, USA; Gibco BRL, USA; Stratagene, USA and Sigma, USA.

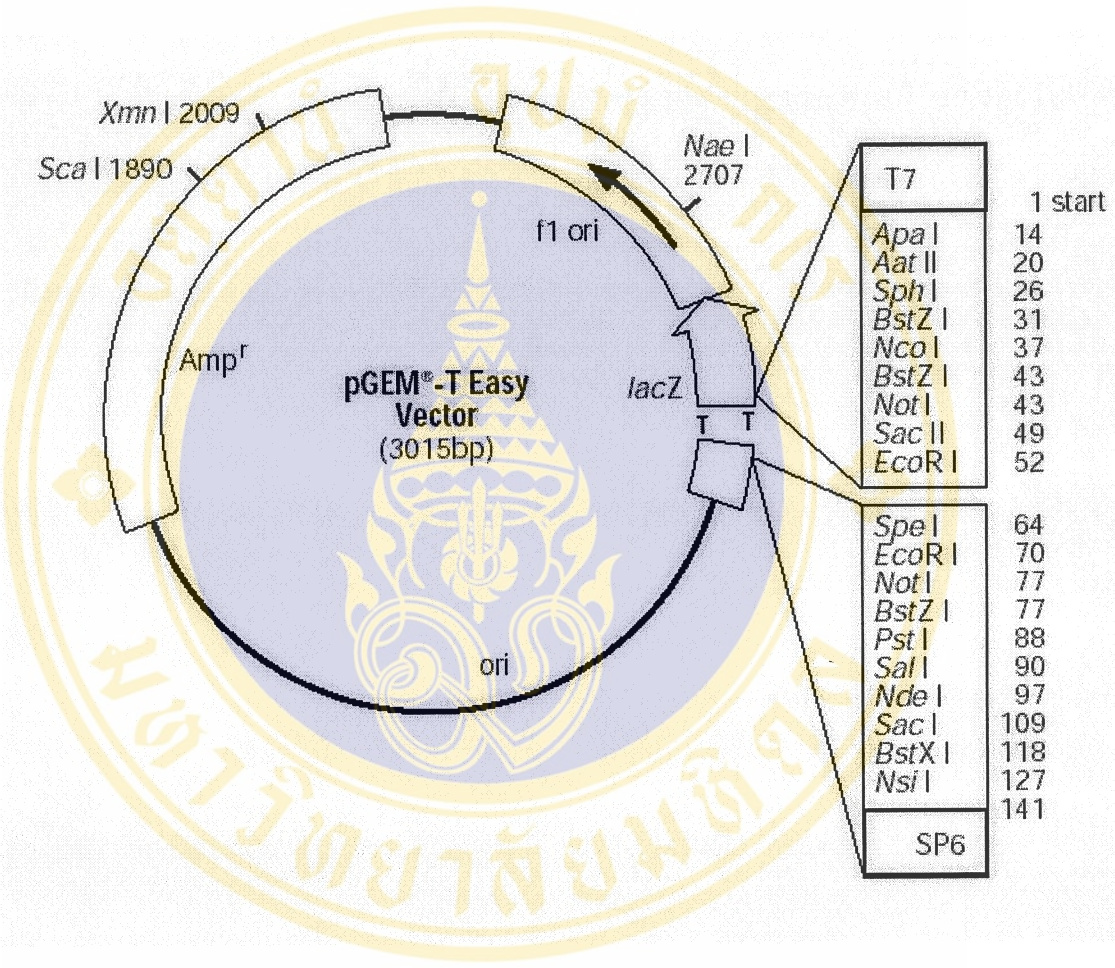


Figure 4. A physical map of pGEM®-T Easy vector (Taken from: pGEM®-T Easy vector manual book)

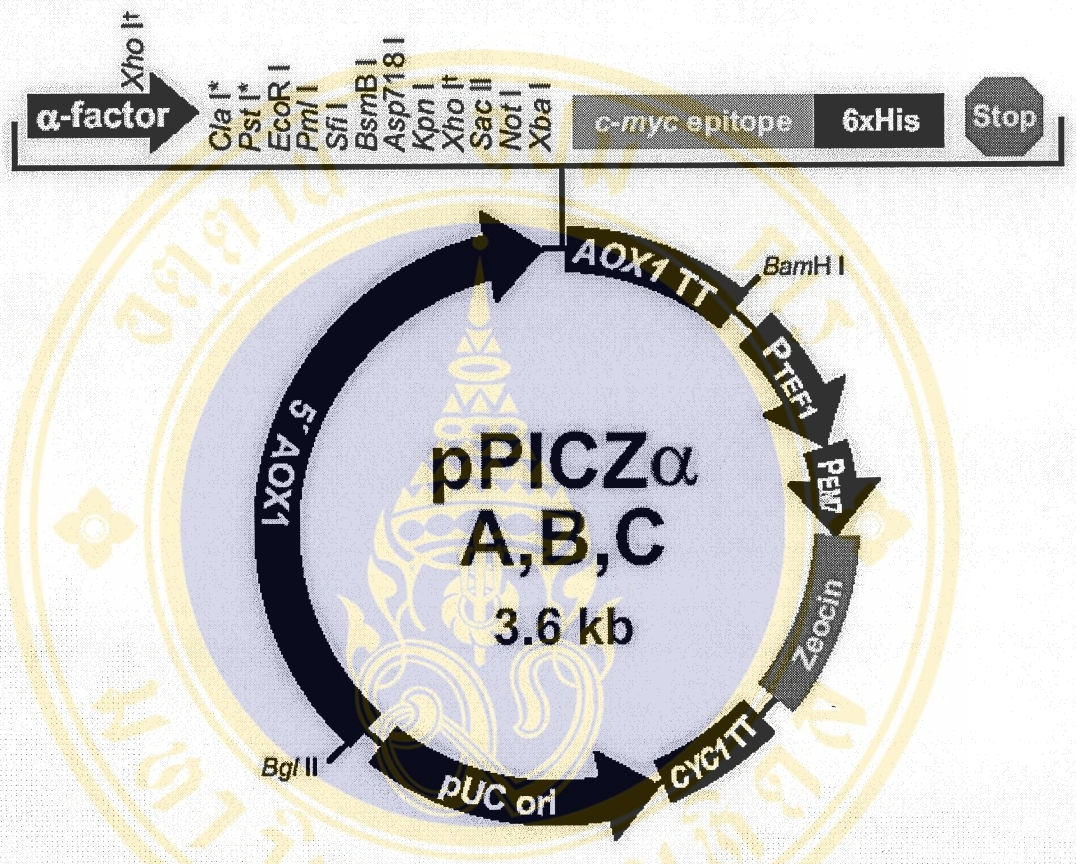


Figure 5. A physical map of pPICZαA vector (From Invitrogen's instruction manual No. V195-20)

Table 1. Primers used in each experiment. For the degenerate oligonucleotides, following abbreviations are used (H= A, T, G; N= A, T, C, G; Y= C, T; M= A, C; S= C, G; R= A, G)

Experiment and Primer name	Sequence (5'→ 3')	Size (bp)	T_m (°C)
Reverse transcription			
PM1 oligo(dT)	CCGGAATTCAAGCTTCTAGAGGATCCTTT TTTTTTTTTTTT	42	70
Adapter primer			
PM2	CCGGAATTCAAGCTTCTAGAGGATCC	26	60.5
Partial cDNA amplification			
Xyl2-F1	ACNCCNGARAAYTCNATGAAR	21	51.1
Xyl2-B1	RTCRTTHATRTANAGYTTNGC	21	50.7
Xyl2-B2	HATYTCRTTNACNACRTCCCA	21	43.3
3'-end cDNA amplification			
3'RaceXyn	ACGCGATACAAGGGTAAGATCAC	23	54.1
5'-end cDNA amplification			
5'RaceXyn1	TCGCGAGTTCTCGGTTTGATCC	22	60
5'RaceXyn2	TCAGCTTGGGCGAGGTTGAAC	21	58.8
Full-length cDNA amplification			
XynWF1	CAACTCCGTGCACGCAATCC	20	59.5
XynR1	TCACCTGATCCGAGGCGATC	20	59.5
***P. pastoris* expression**			
PP-XynF1	CGGAATTCCTCGAGAAAAGAAAGTCATC CCTCGCCCTCCT	40	71.4
PP-XynF2	CCGCTCGAGAAAAGACAGGCCGCTGAGA GC	30	74
PP-XynR1	GGAAGCTTCTAGACTAGGAGCTGAG	25	62.2

3.2 METHODS

3.2.1 Optimization of cell growth

3.2.1.1 Xylanase activity test using congo red staining

0.2% of birchwood xylan (Sigma, USA) was resuspended in 0.1 M phosphate buffer pH 5.8, then 1.2% of bacteriology agar was added. The 20 ml of xylan agar was poured into a sterile petri dish plate. Once, the agar has solidified the wells for applying sample were punctured using a sterile 5 mm diameter tube. A volume of 20 µl of supernatant was applied in each well. The plate was incubated at 30 °C overnight. The xylanase activity was detected by staining with a congo red solution for 30 min then washing twice with 1 M NaCl for 10 min. The blue zone was developed on the red background after an hour at room temperature. The level of xylanase activity was determined by measuring the length of halo from edge of the well to edge of the blue zone in mm.

3.2.1.2 Xylanase activity test using AZCL-xylan

0.1% of AZCL-xylan (Megazyme, Australia) was immersed in 96% ethanol for 1 h. then mixed with the prewarm 1.2% of bacteriology agar in 0.1 M phosphate buffer pH 5.8. The mixture was poured into a sterile plate using 20 ml of mixture per plate. Once, the agar has solidified the wells for applying sample were punctured using a sterile 5 mm diameter tube. A volume of 20 µl of supernatant was applied in the well. The plate was incubated at 30 °C. The blue zone was developed after overnight incubation. The xylanase activity was determined by measuring the length of halo from edge of the well to the edge of the blue zone in mm.

3.2.1.3 Xylanase optimization

The stock culture BCC7197 was prepared by growing on the potato dextrose agar supplemented with 17 ppt marine salt at 30°C for 7 days.

The enrichment method was carried out by growing the stock culture in 100 ml of potato dextrose broth supplemented with 17 ppt marine salt at 250 rpm, 30°C for 7 days. The enriched culture was collected by filtration. One gram of enriched culture was transferred into 100 ml of the induction medium containing either 5% wheat bran or 0.2% birchwood xylan. One ml of the supernatant was collected at different days of growth. The xylanase activities were detected using the method modified from

Molecular and Enzyme Screening Laboratory of BIOTEC based on agar plate diffusion procedure.

The culture was directly inoculated into 100 ml of either the supernatant of 5%wheat bran or the supernatant of 5% wheat bran and 1% yeast extract. The culture was growing at 250 rpm, 30°C. One ml of the supernatant was collected after culturing for 5 days. The xylanase activities were detected as mentioned in 3.2.1.1.

3.2.2 RNA isolation

3.2.2.1 Total RNA isolation by TRI REAGENT

Total RNA was isolated from the BCC7197 mycelium after culturing in the supernatant of 5% wheat bran for 8 days. The mycelia were frozen in liquid nitrogen and ground in mortar until a floury consistency was reached. The RNA was extracted by homogenizing the sample in TRI-REAGENT® (Molecular Research Center, USA) as described by the manufacture. Specifically, 10 ml of TRI-REAGENT was used per gram of mycelium. After homogenization, the homogenate was incubated at room temperature for 5 min, then 0.2 volumes of chlorofrom was added, followed by vigorously shaking for 15 s and incubation at room temperature for 15 min. The mixture was centrifuged at 10,000 rpm, 4 °C for 20 min. Then the aqueous phase was transferred to a new tube. To precipitate RNA, 0.5 volumes of isopropanol were added, followed by incubation at room temperature for 10 min. The precipitated RNA was collected by centrifugation at 10,000 rpm, 4 °C for 20 min. The RNA pellet was washed with 1 volume of 75% ethanol. Then it was centrifuged at 7,500 rpm, 4 °C for 5 min and shortly air dried (approximately 10 min). The dried RNA pellet was dissolved in DEPC-treated water. The isolated total RNA was either stored at -80° C or used immediately.

3.2.2.2 RNA concentration and purity determination

The RNA quantity and quality were determined by using a spectrophotometer (Hitachi, Japan) at the absorbance of 260 nm and 280 nm. The RNA concentration was calculated from absorbance value measured at 260 nm by the formula.

$$\text{RNA concentration } (\mu g/\mu l) = \frac{A_{260} \times (\text{dilution factor}) \times 40}{1000}$$

The RNA purity was determined by the absorbance ratio of A_{260}/A_{280}. A ratio should be between 1.8-2.0 which represents a high purity of RNA.

3.2.2.3 RNA electrophoresis

Quality and integrity of RNA was determined by using gel electrophoresis in 1% agarose gel containing 3.5% formaldehyde. RNA agarose gel was prepared by boiling 0.4 g of agarose in 34 ml of sterile distilled water and cooling down to 60 °C. After that, 4 ml of 10X MOPS buffer [200 mM of morpholinopropanesulfonic acid (MOPS), 50 mM sodium acetate, and 10 mM Na_2EDTA] and 2.2 ml of 37% formaldehyde were added, mixed and poured quickly. The gel was cast in a fume hood for approximately 1 h.

RNA sample was prepared by mixing 2 µg of RNA with 2 µl of RNA loading buffer (50% glycerol, 0.4% bromophenol blue, 1 mM EDTA, 1 mg/ml EtBr) and 6 µl of RNA sample buffer (10 ml formamide, 3.5 ml of 37% formaldehyde, 2 ml of 10X MOPS). Then the RNA mixture was heated at 65°C for 10 min and quickly cooled on ice for 5 min.

RNA agarose gel electrophoresis was performed by immersing the gel in 1X MOPS buffer. The RNA mixture were loaded into the gel, run at 120 volt for 5 min to allow the RNA entered into the gel then run at 80 volt for 1 hr. or until the dye front is about the 2/3 of the gel length. The integrity of RNA was analyzed and photographed under UV light.

3.2.3 cDNA amplification of BCC7197 xylanase partial sequences by RT-PCR

3.2.3.1 First stranded cDNA synthesis

To synthesize the first strand cDNA from BCC7197, total RNA isolated from BCC7197 mycelium at the time of maximal xylanase activity was used as template. To generate the first strand cDNA RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas, Lithuania) was used. The reaction conditions were as recommended by the supplier. Specifically, first strand cDNA was synthesized in a 20 µl volume, containing 1x RT-buffer, 10 mM each of dNTPs (dATP, dCTP, dGTP and dTTP), 200 ng of random hexamer, 1 µg of total RNA, 20 U RNase inhibitor, and 200 U MMLV-H minus reverse transcriptase. The reaction mixture was incubated at 42 °C for 1 hr. The reaction was then terminated by heating at 70 °C for 5 min. After the first strand cDNAs were successfully synthesize, it was kept at –20 °C until used.

3.2.3.2 Degenerate primer design

The degenerate oligonucleotide primers were designed so that they corresponded to the highly conserved amino acid sequence across published Sordariomycetes' xylanases. They are:

(1) Xyl2-F1 (TPENSM): 5'-ACNCCNGARAAYTCNATGAAR-3'

(2) Xyl2-B1 (AKLYIND): 5'-RTCRTTHATRTANAGYTTNGC-3'

(3) Xyl2-B2 (WDVVNET): 5'-HATYTCRTTNACNACRTCCCA-3'

All primers were obtained from BioService Unit (BIOTEC).

3.2.3.3 PCR amplification

After reverse transcription, the first strand cDNAs were used as a template for PCR amplification. Two rounds of PCR amplification (Semi-nested PCR) were performed using the three degenerate primers. The first round of PCR reaction was performed in a total volume of 50 µl that contained 20 µM of Xyl2-F1 and Xyl2-B1, 1x DynaZyme buffer, 2.5 mM each of dNTPs, 2 mM $MgCl_2$, 4 µl of cDNA and 1 U DyNAzyme EXT™ DNA polymerase (Finnzyme, Finland). Amplification cycles consist of one cycle of 1 min at 94 °C, 35 cycles with 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C, then 10 min at 72 °C. The next round of PCR was carried out using 4 µl of the previously PCR products using Xyl2-F1 and Xyl2-B2 as primers under the same PCR condition as previous. The PCR products were analyzed on 1% agarose gel electrophoresis.

3.2.3.4 DNA electrophoresis

The agarose gel was prepared by melting 1% (w/v) of agarose gel in 1X TBE (Tris borate buffer; 89 mM boric acid 89 mM Tris-HCl, 2 mM EDTA, pH 8.0). The gel was poured into an electrophoretic tray and allowed to set at room temperature for 20-30 min. 1X TBE was used as an electrophoretic buffer. The digested DNA or PCR products were mixed with 30% (v/v) of loading dye (25% glycerol, 60 mM EDTA and 0.25% bromophenol blue) and loaded into the prepared gel and run at a constant voltage at 110 volts for 1 h. The gel was then stained in 2.5 µg/ml of ethidium bromide solution for 5 min. and destained in water for 15-30 min. The DNA was visualized under the UV light (Gel Doc model 1000, Bio-Rad, USA) and photographed.

3.2.4 Xylanase cDNA cloning

3.2.4.1 Competent cell preparation using DMSO

E. coli strain DH5α was inoculated into 250 ml SOB [2% peptone (Difco), 0.5% yeast extract (Difco), 0.05% NaCl] broth with shaking at 250 rpm at 18 °C until OD_{600} reached 0.6. The cell culture was transferred into five steriled 50 ml polypropylene centrifuge tubes and the tubes were chilled on ice for 10 min. After centrifugation at 3,000 rpm for 7 min at 4 °C, the supernatant was discarded. The pellet was resuspended in 80 ml of ice cold TB buffer (10 mM pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl). The tubes were chilled on ice for 10 min and cells were harvested at 3,000 rpm for 7 min at 4 °C. The cell pellet was resuspended in 20 ml of ice cold TB buffer. Dimethyl sulfoxide (DMSO) was added to the final concentration of 7% and competent cells were stored at –80 °C (105).

3.2.4.2 DNA purification by QIAquick gel extraction kit (QIAGEN kit)

A band containing DNA of interest was cut from the gel under UV light. QIAGEN gel extraction kit was used to purify the PCR product. Specifically, three volumes of buffer QX1 were added to the excised gel containing the PCR product. The mixture was incubated at 50 °C for 10 min or until the gel was completely dissolved. The color of the solution should be yellow to ensure that pH was not less than 7.5 which give high efficiency of DNA binding to QIAquick column. Then, one volume of isopropanol was added to the mixture to increase the yield of DNA fragments that was smaller than 500 bp or larger than 4 kb. The mixture was, then, applied to the QIAquick column which was placed in a 2 ml collection tube, centrifuged at 14,000 rpm for 1 min. The flow through was discarded. Then 0.5 ml of buffer QX1 was added to QIAquick column to remove all traces of agarose, the column was then centrifuged as previous. Next, 0.75 ml of buffer PE was added to the QIAquick column for washing. The column was left at room temperature for 2-5 min then, centrifuged twice. Then, the column was placed in a fresh 1.5 ml tube. A volume of 30 µl of elution buffer was added to the QIAquick column and the column was left at room temperature for 10 min. to dissolve DNA. The tube was then centrifuged as above. The step of DNA elution with the elution buffer was repeated to obtain the maximal amount of DNA. Then, 1-2 µl of concentrated DNA was analyzed by gel electrophoresis in order to determine purity and concentration.

3.2.4.3 DNA ligation

The purified DNA was directly ligated to the pGEM-T Easy vector (Promega, USA) in a molar ratio of 1:3 (vector: insert). The appropriate amount of DNA used in ligation reaction was calculated from the following formula.

$$\text{ng of in insert} = \frac{\text{ng of vector x kb size of insert x (insert : vector molar ratio)}}{\text{kb size of vector}}$$

In this study, 25 ng of pGEM-T Easy vector was used in a total volume of 10 µl. Ligation mixture contained 1X rapid ligation buffer of T4 DNA ligase [30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM EDTA, 1 mM ATP and 5% polyethylene glycol] and 3 units of T4 DNA ligase (Promega, USA). The ligation mixture was mixed and incubated overnight at 4 °C.

3.2.4.4 Transformation of competent *E. coli* DH5α cells

An amount of 10 ng of ligated product was mixed with 100 µl of competent cells. The mixture was placed on ice for 30 min, and immediately heat shocked at 42 °C for 90 sec, then placed back on ice for 5 min. A volume of 900 µl of SOC [2% peptone (Difco), 0.5% yeast extract (Difco), 0.05% NaCl, 20 mM glucose] medium was added to the mixture and further incubated at 37 °C for 1 h with constant shaking. The cells were then spread on LB agar plate containing 100 µg/ml of ampicillin, 100 µl of 100 mM IPTG (isopropylthio-β-D-galactoside) and 20 µl of 50 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (IPTG and X-gal were spread over the surface of an LB plate). The agar plate was incubated at 37 °C for 12-16 hr.

3.2.4.5 Master plate preparation and recombinant clone screening

After incubation for 12-16 hr, blue and white colonies were observed. Approximately 20 white clones were picked and spotted (8-12 points per clone) on a LB agar plate containing ampicillin so called, master plate. The master plate was incubated at 37 °C for 12-16 hr. To screen recombinant clones, simplified rapid size screening was performed. One colony per clone was picked by toothpick from the master plate and lysed in 30 µl of pre-warm lysis buffer [5mM EDTA, 10% (w/v) sucrose, 0.25% (w/v) SDS, 100 mM NaOH, 60 mm KCL and 0.05% (w/v) bromophenol blue]. The lysed cells were incubated at 37 °C for 5 min, placed on ice for 5 min and centrifuged at 13,000 rpm for 5 min. Then 20 µl of supernatant was

analyzed on 0.8% agarose gel electrophoresis. Those clones containing the plasmid DNA with the larger size than that of the vector alone were selected for further plasmid isolation.

3.2.4.6 Extraction of plasmid DNA using CTAB method

A selected colony was inoculated into 3 ml of LB broth containing 100 µg/ml ampicillin and incubated with vigorous shaking at 37 °C for 12-16 h. To collect the cell pellet, the cell suspension in a microcentrifuge tube was centrifuged at 10,000 rpm for 10 s. The cells pellet was resuspended in 200 µl of STET buffer (8% (w/v) sucrose, 1% Triton X-100, 50 mM EDTA and 50 mM Tris pH 8.0) by vigorously vortexing. A volume of 10 µl of 100 mg/ml lysozyme was then added. The tube was left at room temperature for 5 min and then placed in a boiling water bath for 45 s and left on ice for 30 s. Cell debris was removed by using a sterile toothpick. A volume of 10 µl of 10 mg/ml Ribonuclease A was added and incubated at 37 °C for 30 min. Then, 20 µl of 5% of cetyl trimethyl ammonium bromide (CTAB) was added, then the solution was mixed by inverting the tube. The mixture was incubated at room temperature for 30 min and centrifuged at 12,000 rpm for 5 min. Then the supernatant was removed and the DNA pellet was resuspended in 300 µl of 1.2 M NaCl. A volume of 300 µl (an equal volume of aqueous phase) of chloroform was added and the mixture was vortexed for 30 s, then centrifuged as before. The aqueous phase was transferred to a fresh microtube. To precipitate plasmid DNA, approximately 600 µl (2 volumes of aqueous phase) of absolute ethanol was added and the mixture was incubated at room temperature for 5 min. The precipitated plasmid DNA was centrifuge at 12,000 rpm for 15 min. The supernatant was discarded, then the DNA pellet was washed twice in 200 µl of 70% (v/v) ethanol and air dried. A completely dried plasmid DNA pellet was resuspended in 20-30 µl of sterile distilled water. The quality and concentration of plasmid DNA was analyzed on 1% agarose gel electrophoresis.

3.2.4.7 Extraction of plasmid DNA using QIAprep Spin Miniprep kit (QIAGEN)

Specifically, the overnight cultured cells were collected by centrifugation at 5,000 rpm for 10 min at 4 °C, and the supernatant was discarded. The pellet was resuspended in 250 µl of Buffer P1 and transferred to a new microcentrifuge tube. A 250 µl of

Buffer P2 was added and mixed by gently inverting the tube 4-6 times. A volume of 350 µl of Buffer N3 was added to the mixture and the tube was inverted gently 4-6 times, then centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to the QIAprep spin column inserted in a collection tube. The QIAprep spin column was then centrifuged at 10,000 rpm for 1 min. The flow-through was discarded and the QIAprep spin column was washed with 500 µl of Buffer PB. After centrifugation at 10,000 rpm for 1 min, the flow-through was discarded. The QIAprep spin column was then washed with 750 µl of Buffer PE and centrifuged at 10,000 rpm for 1 min. The flow-through was discarded, and the column was centrifuged for an additional 1 min to remove residual wash buffer. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5), left standing for 1 min and followed by centrifugation at 10,000 rpm for 1 min.

3.2.4.8 Insert size screening by restriction enzyme digestion

After recombinant plasmid was purified, the size of insert was checked by *Eco*R I digestion. Plasmid DNA (1µl) was added into the reaction mixture containing 1X buffer H [90 mM Tris-HCL (pH 7.5), 50 mM NaCl and 10 mM $MgCl_2$] and 2-4 units of *Eco*R I restriction endonuclease enzyme (Promega, USA) in a final volume of 10 µl. The reaction mixture was incubated at 37 °C for 3 hr. The size of insert was analyzed using an appropriate percentage of agarose gel.

3.2.5 DNA sequencing

After screening with restriction enzyme analysis, the recombinant clones harbouring interested cDNA were further analyzed by DNA sequencing (Macrogen, Korea).

3.2.6 Sequence analysis

DNA sequences obtained were translated into a deduced amino acid sequence by using the Bioedit Sequence Alignment Editor program. The DNA sequences from different clones were aligned together by Clustal X program. The nucleotide and the deduced amino acid sequences were blasted in GenBank database in NCBI website (http://www.ncbi.nlm.nih.gov) and compared with other related sequences by Clustal X program.

3.2.7 Amplification of the 3' end cDNA by 3' RACE method

The 3' end cDNA of a BCC7197 xylanase was elucidated by 3' rapid amplification of cDNA end (3' RACE) method (Figure 6A). A forward gene specific primer, 3'RaceXyn was designed from previously identified sequence of a partial xylanase sequences. At first, the first stranded cDNA was generated from 1 μg of total RNA from BCC7197 mycelium with PM1, oligo-dT-adaptor primer, according to the manufacturer's instruction of MMLV-H minus reverse transcriptase (Fermentas, Lithuania) as described in method 3.2.3.1. The PCR amplification was then performed using 3'RaceXyn, a gene specific primer and PM2, an adaptor primer in the same condition as described in method 3.2.3.3 (Figure 6B). The PCR product was analyzed on 1% agarose gel electrophoresis. Then it was purified, cloned and sequenced as described in protocol 3.2.4.2-3.2.4.7 and 3.2.5. The 3' end cDNA sequence was analyzed as described in 3.2.6.

3.2.8 Amplification of the 5' end cDNA by 5' RACE method

3.2.8.1 First stranded cDNA synthesis by partial heat denaturation method

In this study 5' end cDNA of a partial xylanase sequence was investigated by 5' RACE method. The first strand cDNA was constructed using partial heat denaturation reverse transcription method (106), in order to overcome a strong RNA secondary structure problem. Five μg of total RNA from BCC7197 mycelium was reverse transcribed by using 10 μM of 5'RaceXyn2, a gene specific primer. The first stranded cDNA was generated by MMLV-H minus reverse transcriptase (Fermentas, Lithuania) as described in method 3.2.3.1. The partial heat denaturation method was then performed. The mixture was heated at 85 °C for 3 min and immediately put on ice-water slurry for 2 min. The reverse transcription was continued by adding 1 μl (200 unit) of MMLV-H minus reverse transcriptase, followed by another incubation at 42 °C for 60 min. Reverse transcriptase was then heat-inactivated at 70 °C for 15 min. The RNA template was removed by adding 2.5 μl of 5 M NaOH. The mixture was then incubated at 55 °C for 30 min and neutralized with 72 μl of 1% acetic acid.

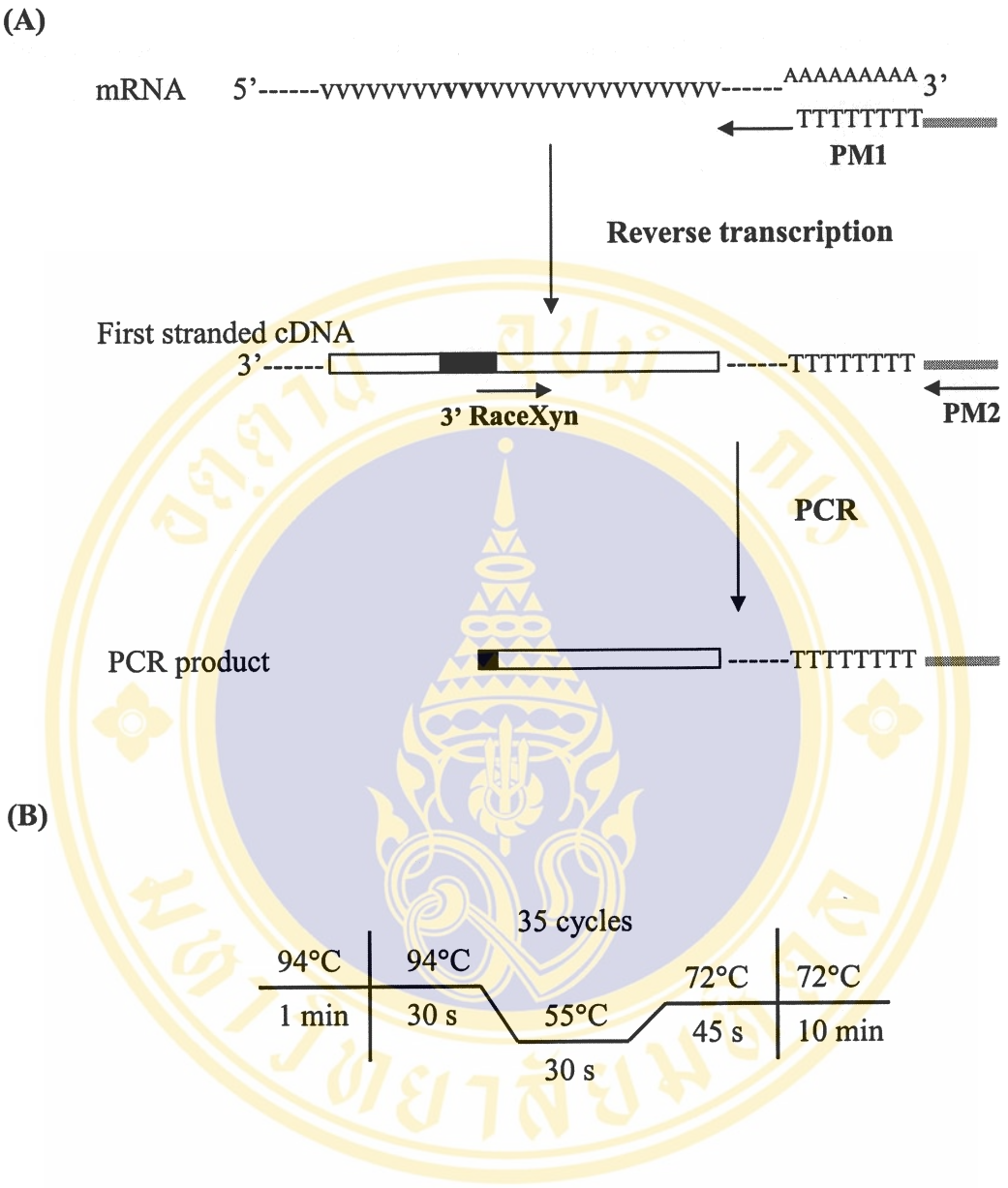


Figure 6. A schematic diagram and PCR profile of the 3' end cDNA amplification by 3' RACE method

(A) A schematic diagram of the 3' end cDNA amplification by 3' RACE method. A black box indicates an internal sequence of xylanase cDNA. Dotted lines represent the untranslated regions (UTR). Arrows indicate position and direction of primers. (B) PCR profile of the 3' end cDNA amplification of BCC7197 xylanase.

3.2.8.2 QIAquick PCR purification

Before poly-A tailing, it is necessary to purify the first strand cDNAs. In this study, QIAquick PCR purification kit (QIAGEN) was used to separate the first stranded cDNA from dNTPs, ATP, salts and primer. The purification process was performed according to manufacturer's instruction. Five volumes of PB buffer was added to the cDNA solution and the mixture was then applied to a QIAquick spin column placed on a 2 ml collection tube and centrifuged at 13,000 rpm at room temperature for 1 min. After the flow-through was discarded, 0.75 ml of PE buffer was added following with centrifugation at 13,000 rpm for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min at 13,000 rpm. Then the column was placed into a new microcentrifuge tube. To elute the first stranded cDNA, at least 30 µl of sterile distilled water was added into the center of membrane. After incubation at room temperature for 1 min, the column was centrifuged at 13,000 rpm for 1 min. The eluted cDNA was collected in a microcentrifuge tube.

3.2.8.3 First stranded cDNA poly-A tailling

In order to allow oligo(dT) primer to bind, poly-A tail was added to the 3' end of the first-strand cDNAs (which corresponds to the 5'-end of the mRNA). In a final volume of 30 µl reaction, 21 µl of purified first stranded cDNA was added into the reaction mixture containing 2 µl of 25 mM dATP, 1X TdT buffer [100 mM cacodylate buffer (pH 6.8), 1 mM $CoCl_2$, and 0.1 mM DTT], and 1 µl of 50 unit/µl terminal deoxynucleotidyl transferase (TdT) (Promega, USA). The mixture was then incubated at 37 °C for 10 min. The TdT was inactivated by incubation at 65 °C for 10 min. The poly-A tailed first stranded cDNA was stored at –20 °C until used.

3.2.8.4 Synthesis of the 5' end cDNA by PCR amplification

To amplify the 5' end cDNA of a putative xylanase, two reverse gene specific primers, 5'RaceXyn2 and 5'RaceXyn1 were designed form previously identified sequence of a putative xylanase. Two rounds of PCR amplification were performed using two different primer pairs (Figure 7A). The first PCR was performed with 5'RaceXyn2, a gene specific primer and PM1, an oligo-dT-adaptor primer. Then the first PCR product was subjected to the nested PCR performed with 5'RaceXyn1, a gene specific primer and PM2, an adaptor primer. Both PCR reactions were performed in the presence of 4 µl cDNA template, 20 µM of each primer, 1x DynaZyme buffer,

2.5 mM each of dNTP's, 2 mM $MgCl_2$ and 1 U DyNAzyme EXT™ DNA polymerase (Finnzyme, Finland) in a final volume of 50 µl. PCR amplification was carried out by incubating at 94 °C for 5 min, followed by touch down PCR in which annealing temperature was decreased 1 °C per cycle from 58-54 °C at first five cycles. Then PCR amplification for 18 cycles of denaturation at 94 °C for 30 sec, annealing at 53 °C for 15 sec and extension at 74 °C for 2 min was performed, followed by a final extension at 74 °C for 7 min (Figure 7B). The nested PCR was performed in the same condition but in the touch down step, annealing temperature was decreased from 60-55 °C of 6 cycles and annealing temperature was changed to 54 °C in the PCR amplification of the next 25 cycles (Figure 7B). The PCR product was analyzed on 1% agarose gel electrophoresis then, it was purified, cloned and sequenced as described in protocol 3.2.4.2-3.2.4.7 and 3.2.5. The 5' end cDNA sequence was combined with the previously identified sequence of a putative xylanase and analyzed as described in protocol 3.2.6.

3.2.9 Amplification of the full-length xylanase cDNA

In order to amplify the full-length xylanase cDNA from BCC7197, two gene specific primers, XynWF1 and XynR1 corresponding to the 5' and 3' ends, respectively were designed from a combined full-length xylanase sequence (Figure 8A). At first the first strand cDNA was synthesized from a total RNA isolated from the BCC7197 mycelium using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Lithuania). The reaction conditions were as recommended by the supplier as described in protocol 3.2.3.1. The PCR reaction was performed in 50 µl that contained 10 µM of XynWF1 and XynR1, 1x DynaZyme buffer, 2.5 mM each of dNTP's, 2 mM $MgCl_2$, 3µl of cDNA and 1 U DyNAzyme EXT™ DNA polymerase (Finnzyme, Finland). Amplification cycles were one cycle of 1 min at 94 °C, 35 cycles with 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C, then 10 min at 72 °C (Figure 8B). The PCR product was analyzed on 1% agarose gel electrophoresis. Then it was purified, cloned and sequenced as described in protocol 3.2.4.2-3.2.4.7 and 3.2.5. The full-length xylanase cDNA sequence was analyzed as described in protocol 3.2.6.

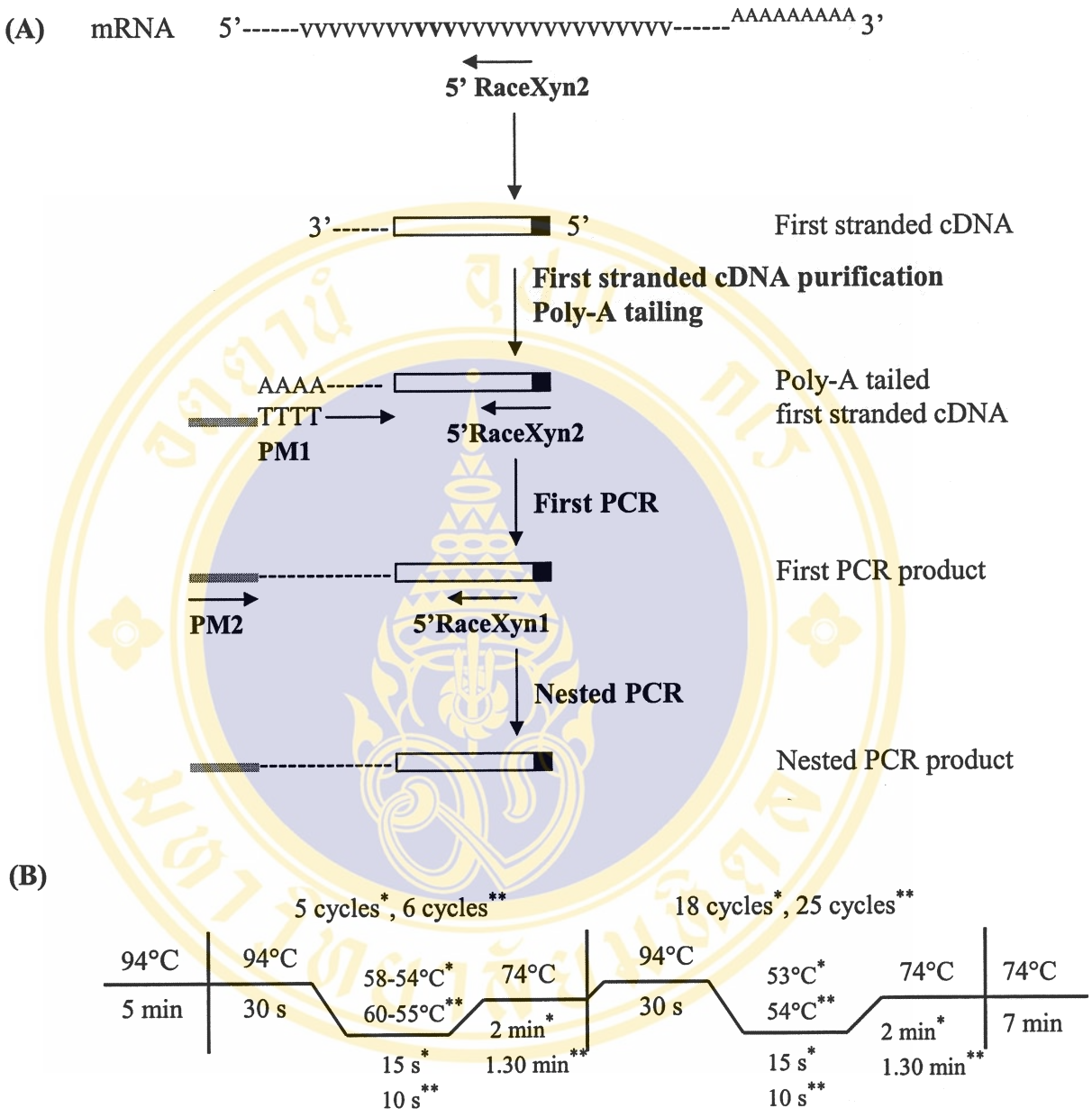


Figure 7. A schematic diagram and PCR profile of the 5' end cDNA amplification by 5' RACE method

(A) A schematic diagram of the 5' end cDNA amplification by 5' RACE method. A black box indicates an internal sequence of xylanase cDNA. Dotted lines represent the untranslated regions (UTR). Arrows indicate position and direction of primers. (B) PCR profile of the 5' end cDNA amplification of BCC7197 xylanase. Asterisks represent the different condition of the first (*) and nested (**) PCR reactions.

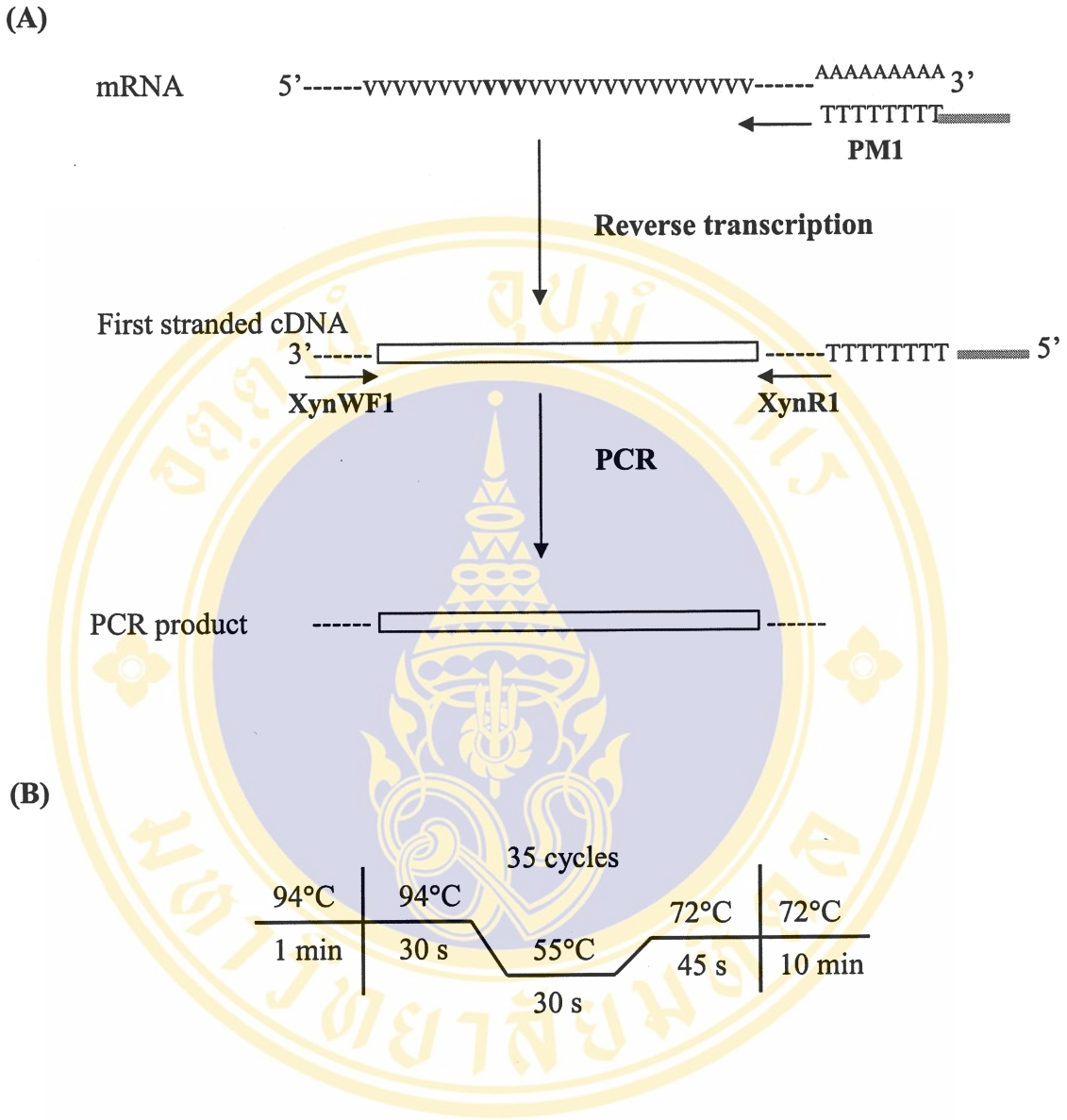


Figure 8. A schematic diagram and PCR profile of the full-length cDNA amplification

(A) A schematic diagram of the full-length cDNA amplification. Dotted lines represent the untranslated regions (UTR). Arrows indicate position and direction of primers. (B) PCR profile of the full-length cDNA synthesis of BCC7197 xylanase.

3.2.10 Construction of xylanase in *P. pastoris* expression vector

3.2.10.1 Amplification of xylanase cDNA

The full-length xylanase gene, with or without a leader sequence were constructed. The PCR was performed using PP-XynF1 and PP-XynR1 for amplifying xylanase gene with a leader sequence, while PP-XynF2 and PP-XynR1 for amplifying xylanase gene without a leader sequence. The PCR reaction was performed in a 50 µl reaction that contained 10 µM of each PP-XynF1 or PP-XynF2 and 10 µM of PP-XynR1, 1x DynaZyme buffer, 2.5 mM each of dNTP's, 2 mM $MgCl_2$, 10 ng of full-length xylanase in pGEM-T Easy vector and 1 U of DyNAzyme EXT™ DNA polymerase (Finnzyme, Finland). Amplification cycles were one cycle of 1 min at 94 °C, 35 cycles with 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C, then 10 min at 72 °C. The PCR product was analyzed on 1% agarose gel electrophoresis and purified using QIAGEN gel extraction kit.

3.2.10.2 Preparation of linearized pPICZαA

In order to subclone the full-length xylanase gene with or without a leader sequence into the *P. pastoris* vector, pPICZαA. The linearized pPICZαA vector was prepared by double digestion with *Xho* I and *Xba* I restriction enzymes (Promega, USA). A reaction contained 5 µg of pPICZαA vector, 20 unit of restriction enzyme, 1X reaction buffer and sterile distilled water to give a total volume of 100 µl. After the digestion had been completed, the digested products were purified using QIAGEN gel extraction kit. DNA ligation and transformation were then performed as described in 4.3 and 4.4, respectively. The transformants were screened on low salt LB medium [1% peptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl] containing 25 µg/ml Zeocin™ (Invitrogen).

3.2.11 Transformation of *P. pastoris*

3.2.11.1 Preparation of *P. pastoris* competent cells

A single colony of *P. pastoris* or a small amount of liquid culture grown from a single colony was grown in 100 ml of YEPD at 30°C with shaking until OD_{600} reached 1.3-1.5. The cells were harvested and washed twice with 30 ml cold steriled water and centrifuged at 4,000 rpm for 5 min at 4 °C. The cells were resuspended in 5 ml of ice-cold 1 M sorbitol and centrifuged at 4,000 rpm for 5 min at 4°C. The cells were finally

resuspended in 800 µl of ice-cold 1 M sorbitol. 50 µl of the competent cells were used for a transformation reaction.

3.2.11.2 Preparation of linearized recombinant plasmids

Recombinant plasmids were linearized by digesting with *Dra* I. The linearized plasmids were then precipitated with 60 µl of 95% (v/v) ethanol in the presence of 3 M sodium acetate and 1.5 µl of yeast tRNA. After incubation at –80°C for 15 min, the mixture was centrifuged at 10,000 rpm at 4°C for 10 min. The pellet was wash with 70% ethanol, and air-dried. The dried pellet was resuspended in 10 µl steriled distilled water.

3.2.11.3Transformation of *P. pastoris* competent cells by electroporation

The reaction mixture was composed of 50 µl of *P. pastoris* cells and 10 µl of linearized recombinant plasmid. The mixture was transferred to and ice-cold 0.2 cm electro-cuvette and left on ice for 5 min. The cells were pulsed by using Bio-Rad Gene Pulser with the following conditions: 1.5 kV, 25 µF, and 200 Ω (time constant should be 4.5). Then, 900 µl of YEPD was added to the cuvette and the solution was transferred to a steriled microcentrifuge tube. The tube was incubated at 30°C without shaking for 1 h, then the culture was centrifuged at 3,000 rpm at 4°C for 5 min and the supernatant was discarded. The cell pellets were resuspended in 100 µl sterile distilled water. The cell suspension was spread on YEPD plate containing 100 µg/ml Zeocin™, and was incubated at 30°C for 2-3 days until colonies formed.

3.2.11.4 Total DNA isolation from *P. pastoris*

Both the recombinant and the wild-type *P. pastoris* strains were grown at 30°C until OD_{600} reached 5-10 in YEPD containing 100 µg/ml Zeocin™. The cells were collected by centrifugation at 3,000 rpm for 5 min at room temperature. The pellet was washed with 1 ml steriled water, then resuspended in 200 µl of SCED buffer, pH 7.5 (1 M sorbitol, 10 mM sodium citrate, pH 7.5, 10 mM EDTA, 10 mM DTT). A volume of 5 µl of lyticase (25 U/µl) was then added. The mixture was incubated at 30°C for 3-4 h, then 100 µl of 2% (w/v) SDS was added, mixed gently and chilled on ice for 5 min. Next, 150 µl of 5 M potassium acetate pH 8.9 was added, and mixed gently. The mixture was centrifuged at 10,000 rpm for 5-10 min at 4 °C. The supernatant was collected and transferred into a new microcentrifuge tube. To the supernatant, 1 ml of

absolute ethanol was added, and incubated at 4°C overnight. After centrifugation at 10,000 rpm for 20 min at 4°C, the supernatant was removed, and the pellet was resuspended gently in 500 µl of TE buffer (10 mM Tris HCl pH 7.4, 1 mM EDTA pH 8.0). The DNA solution was incubated with 10 µl of 5 mg/ml RNase A at 37°C for 1 h. An equal volume of phenol, pH 8 was added, mixed gently, then centrifuged at 14,000 rpm for 5 min. The nucleic acid in an aqueous phase was transferred into a new microcentrifuge tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed gently then centrifuged at 14,000 rpm for 5 min. The nucleic acid in an aqueous phase was transferred to a new microcentrifuge tube. This step was repeated once then, a half volume of 7.5 M ammonium acetate, pH 7.5 and two volumes of absolute ethanol were added to the tube, which was then placed at -80°C for 10 min. The tube was centrifuged at 10,000 rpm for 20 min at 4°C. The cell pellet was washed with 70% (v/v) ethanol. The DNA was resuspended in 40 µl and stored at –20°C until use.

3.2.11.5 PCR analysis of *P. pastoris* integrants

For screening of *P. pastoris* integrants containing integrated recombinant plasmid. The PCR was set up as follows. A PCR reaction consisting of 1x Reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2 mM $MgCl_2$, 2.5 mM of dNTP, 10 pmole of each primer (*5'AOX1* forward primer and *3'AOX1* reverse primer), 50-100 ng of genomic DNA and 0.25 µl of Amplitaq polymerase (5U/µl from Stratagene, USA). The reaction was adjusted to a total volume of 50 µl with sterile distilled water. The sample reactions were operated in an automated thermal cycler GeneAmp PCR system model 2400 (Perkin Elmer Cetus, USA). Amplification cycles were one cycle of 2 min at 94 °C, 30 cycles with 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, then 7 min at 72 °C. A 5 µl aliquot of the PCR products were analyzed by running on 1.0% (w/v) agarose gel electrophoresis.

3.2.12 Expression of recombinant xylanase in *P. pastoris*

A single colony of *P. pastoris* KM71 recombinants was inoculated into 2 ml of YEPD and incubated at 30°C with shaking at 250 rpm for 48 h. Then, the cell culture was transferred to 5 ml of fresh BMGY medium and grown using the condition as mentioned above until the culture reached an OD_{600} of 5-6. In the induction step, the

cell pellet was harvested by centrifugation at 4,000 rpm for 5 min at room temperature. The supernatant was decanted and the cell pellet was resuspended in BMMY using 1/5 the volume of original culture (1 ml). The cell suspension was placed in a 20 ml of a glass tube. Absolute methanol was added every 24 h to a final concentration of 3% (v/v) to maintain induction. The expression culture was collected at 0, 24, 48, 72 and 96 h. The collected cells were centrifuged at the maximal speed in a bench-top microcentrifuge for 2-3 min at room temperature. The supernatant was transferred to a separate tube. The secreted protein were analyzed by using SDS-PAGE and also analyzed for the xylanase activity.

3.2.13 Proteins electrophoresis

3.2.13.1 Sample preparation

Protein samples were mixed with 4X protein sample buffer [60 mM Tris-HCL (pH 7.5), 2% (w/v) SDS, 10% glycerol, 0.025% (w/v) Bromophenol blue, 100 mM DTT] at the ratio of 3:1, then boiled for 10 min. The heated samples were kept on ice, then centrifuge at 13,000 rpm for 5 min. The protein samples were loaded into SDS-polyacrylamide gel.

3.2.13.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Stacking and separating gel for SDS-PAGE were prepared by mixing various solution described in Table 2. The proteins were eletrophorised in protein running buffer (25 mM Tris-HCl, pH 8.2, 192 mM glycine and 0.1% (w/v) SDS) using a constant voltage of 150 volts for 60 min or until the dye front reached the bottom of the gel. The gels were stained in staining buffer (45% (v/v) methanol, 10% (v/v) acetic acid and 0.1% (v/v) coomassie blue R-25) for 1-2 h. The excess stain was washed in destaining buffer (10% (v/v) methanol and 10% (v/v) acetic acid) overnight with a mild shaking. Destaining buffer was removed by rinsing several times in distilled water. The gel was dried by placing between two sheets of wet cellophane paper, which later were clamped together. Air bubbles were removed. The gel was air-dried at room temperature overnight.

Table 2. Components of SDS-PAGE for protein determination

Preparation of 5% of stacking SDS-PAGE	
Solution	Volume (ml)
30% Acrylamide[a]	0.83
H_20	3.40
1 M Tris-HCl, pH 6.8	0.63
10% SDS[b]	0.05
10% APS[c]	0.05
TEMED[d]	0.005
Total volume	3.965
Preparation of 12% of separating SDS-PAGE	
Solution	Volume (ml)
30% Acrylamide[a]	4.0
H_20	3.3
1.5 M Tris-HCl, pH 8.8	2.5
10% SDS[b]	0.1
10% APS[c]	0.1
TEMED[d]	0.004
Total volume	10.004

[a] acrylamide: N,N'-methylene-bis-acrylamide 29:1

[b] sodium dodecyl sulfate

[c] ammonium persulfate

[d] N,N,N',N'-tetramethyl-ethylenediamine

CHAPTER IV
RESULTS

4.1 Optimal condition for xylanase induction in BCC7197

In order to isolate gene(s) that encode xylanase from BCC7197, optimal culturing condition for xylanase production was determined. The enrichment method was performed by growing the stock culture in PDB supplemented with 17 ppt marine salt for 7 days then transferring into the induction medium containing either 5% wheat bran or 0.2% birchwood xylan. The supernatant was collected for detection of the xylanase activity at various days. The result demonstrated that the maximal xylanase activity was detected at day 9 and 13 in 5% wheat bran and 0.2% birchwood xylan, respectively, with a maximal halo length of xylanase at 5 mm. (Table 3). Although, the xylanase can be induced using this condition, it was impossible to separate the growing BCC7197 from the suspended wheat bran or birchwood xylan. Thus, a modified method is needed. In this method, the stock culture of BCC7197 was directly inoculated into two types of modified media which were either the supernatant of 5%wheat bran or the supernatant of 5% wheat bran plus 1% yeast extract. The cultures were grown at 250 rpm at 30°C. The supernatant was collected to detect for xylanase activity after culturing for 5 days and continue each day until day 10. The result demonstrated that the maximal xylanase activity was at day 8 in both types of media, with a maximal halo length of xylanase at 7 mm. (Table 4). This suggested that 5% wheat bran by itself is sufficient to induce xylanase at a significant level. The 1% yeast extract may not be important for xylanase production. In short, the optimal condition for inducing xylanase production was directly growing BCC7197 in the medium containing the supernatant of 5% wheat bran with continuously shaking at 30°C, 250 rpm for 8 days. This condition was used for culturing the fungus for total RNA isolation.

Induction medium	Induction time (day)								
	1-7	8	9	10	11	12	13	14	15
5% wheat bran	PDB+17ppt marine salt	0	5	5	5	5	5	5	5
0.2% birchwood xylan		0	0	0	0	4	5	5	5

Table 3. Induction of xylanase production by using enrichment method

BCC7197 was enriched in PDB supplemented with 17 ppt marine salt. The enriched culture was transferred into the induction medium containing either 5% wheat bran or 0.2% birchwood xylan. At various days (8 to 15 days), 20 µl of supernatant was loaded onto the 0.2% birchwood xylan plate. The radius of the clear zone or halo length (in mm) shown in the table indicates the xylanase activity.

Induction medium	Induction time (day)					
	5	6	7	8	9	10
5% filtrated wheat bran	5	5.5	6	7	7	7
5% filtrated wheat bran + 1% yeast extract	4	5	6	7	7	7

Table 4. Induction of xylanase production by using modified method

BCC7197 was directly introduced into the induction medium containing either 5% filtrated wheat bran or 5% filtrated wheat bran supplemented with 1% yeast extract. At various days (5 to 10 days), 20 µl of supernatant was removed and added onto 0.2% birchwood xylan plate. The radius of the clear zone or halo length (in mm) shown in the table indicates the xylanase activity.

4.2 Isolation of total RNA from BCC7197 using TRI REAGENT

Total RNA was isolated from the BCC7197 mycelium at the time of maximal xylanase activity using TRI REAGENT. RNA concentration and purity were determined by spectrophotometry at 260 and 280 nm. The result showed that RNA sample presented an absorbance ratio (A_{260}/A_{280}) in the range of 1.8-2.0, indicating that RNA sample was relatively pure. Moreover, the quality of the RNA was determined by formaldehyde agarose gel electrophoresis. The result showed that RNA sample presented a predominant band in size of about 3.6 and 1.9 kb corresponding to 28S and 18S rRNA, respectively. However, an unknown band of approximately 9.4 kb was observed (Figure 9).

4.3 Isolation of partial sequences of BCC7197 xylanase by using RT-PCR

The degenerate primers (Xyl2-F1, Xyl2-B1, and Xyl2-B2) were designed based on the conserved amino acid sequence of endoxylanase from various Sordariomycetes (Figure 10). These primers were used in a PCR reaction to amplify a xylanase gene fragment from BCC7197 cDNA. A semi-nested PCR result showed approximately 300 bp and 200 bp products (Figure 11). These PCR products were purified and cloned into pGEM-T Easy vector. DNA sequence analysis using WU-BLAST revealed that the 300 bp PCR product showed the sequence identity with various fungal xylanases such as *Magnaporthe grisea* endo-β-1,4 xylanase and *Hypocrea jecorina* xylanase 3. The length of partial xylanase cDNA are 225 nucleotides and encode deduce amino acid sequences of 75 amino acids (Figure 12). However, the 200 bp PCR product is not related to any of the fungal xylanases.

The alignment of the deduced amino acid sequence of the BCC7197 xylanase with other fungal xylanases using Clustal X program revealed a significant similarity. The results revealed a 63% amino acid identify to *Thermoascus aurantiacus* endo-1,4-beta-D-xylanase A, a 59% identity to *Aspergillus oryzae* xylanse F3, *Penicillium purpurogenum* endo-1,4-beta-D-xylanase A and *Aspergillus sojae* endo-1,4-beta-D-xylanase A, and a 57% identity to both *Magnaporthe grisea* endo-beta-1,4-D-xylanase and *Hypocrea jecorina* xylanase III (Figure 13).

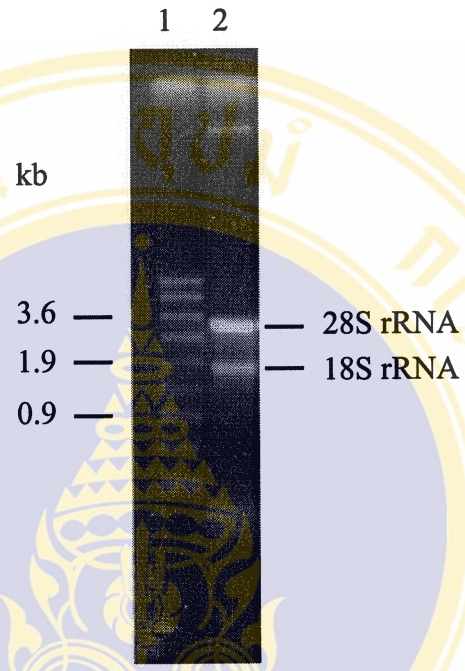


Figure 9. Total RNA isolated from BCC7197 mycelium

Total RNA was isolated from BCC7197 mycelium using TRI REAGENT. RNA was size-fractionated on 1% formaldehyde agarose gel electrophoresis. Lane 1 is 2 μg RNA molecular weight marker. Lane 2 is RNA from BCC7197. The size of intact bands are about 3.6 and 1.9 kb corresponding to 28S and 18S rRNA, respectively.

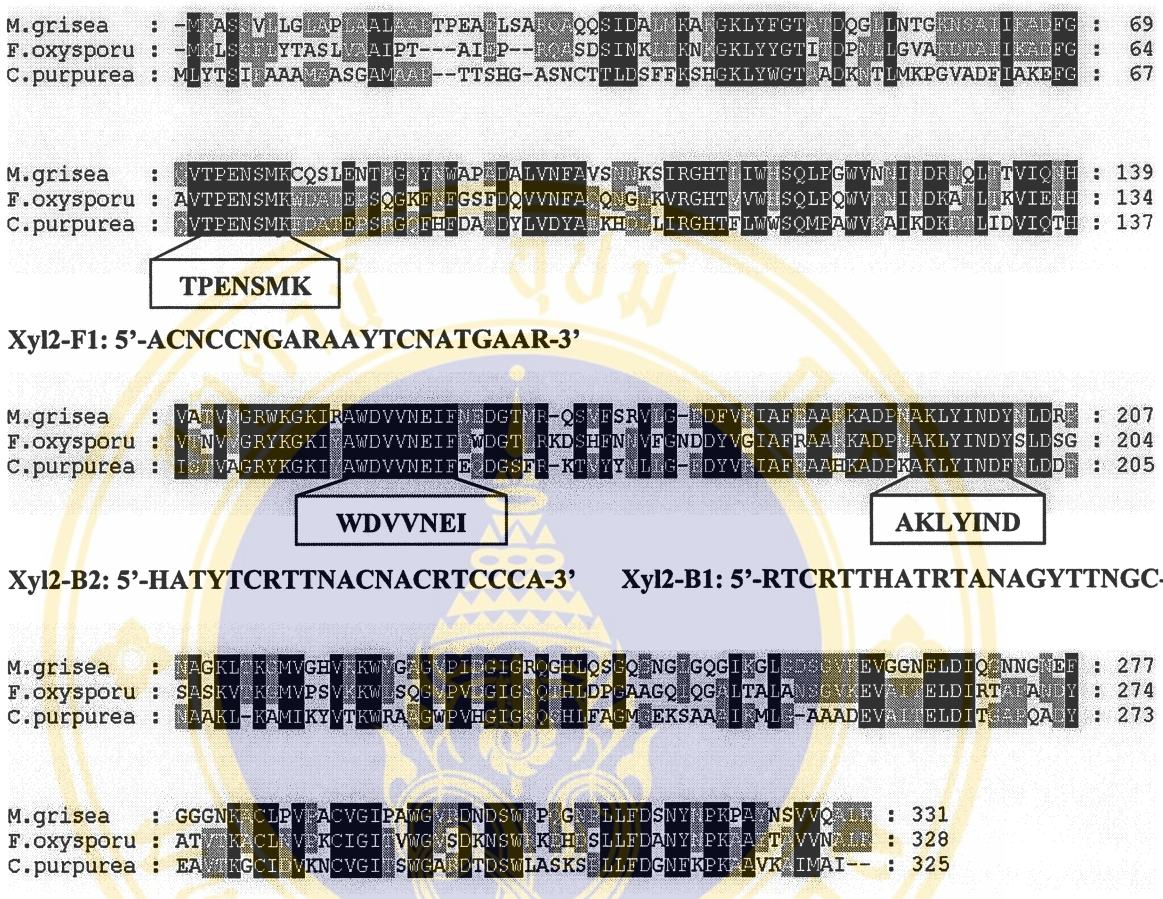


Figure 10. Degenerate primer design

Three degenerate primers were designed from amino acid sequences of xylanase from Sordariomycetes (*Magnaporthe grisea*, accession number L37530; *Fusarium oxysporum*, accession number AF052583 and *Claviceps purpurea*, accession number Y16970). In the degenerate oligonucleotides, abbreviations of mix nucleotides are used (H = A, T, G; N = A, T, C, G; Y = C, T; M = A, C; S = C, G; R = A, G).

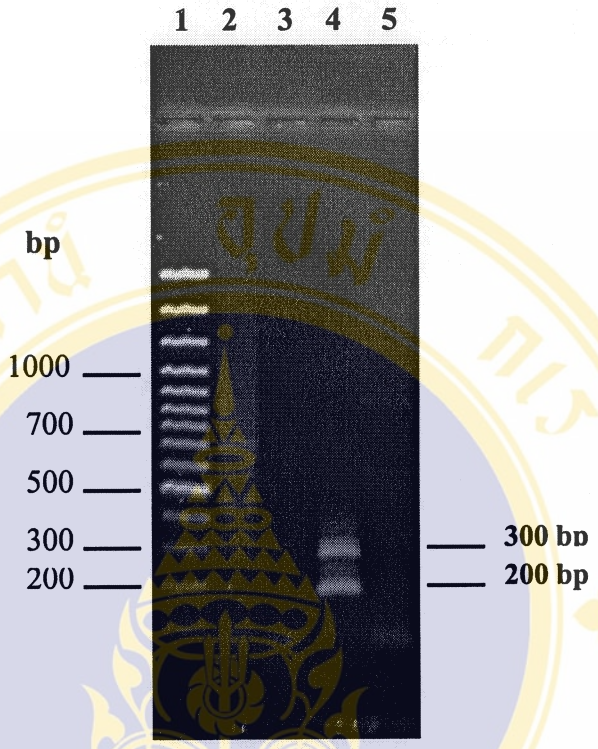


Figure 11. The RT-PCR products of BCC7197 xylanase partial sequences

RT-PCR showed the expression of xylanase gene from BCC7197. Lane 1 is a 100 bp ladder DNA marker. Lane 2 is the PCR product using Xyl2-F1 and Xyl2-B1 primers. Lane 3 is negative control of 1st round PCR performed with distilled water. Lane 4 is the semi-nested PCR product using Xyl2-F1 and Xyl2-B2 primers. Lane 5 is negative control of semi-nested PCR. PCR products were size fractionated on 1% agarose gel electrophoresis.

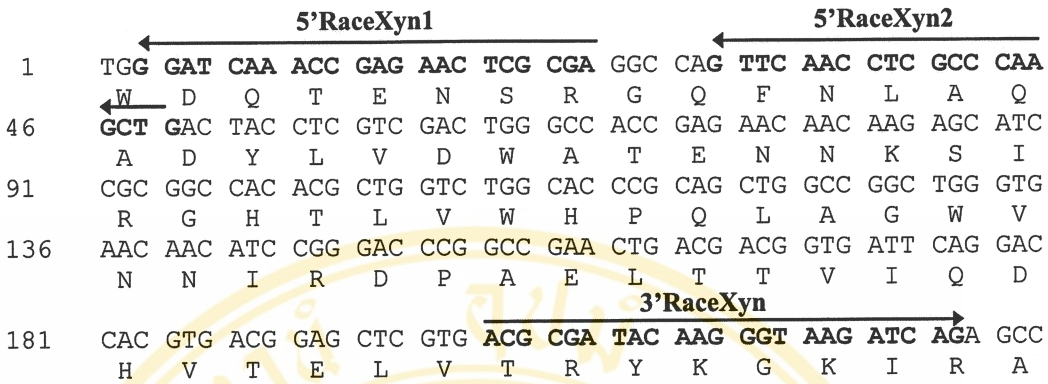


Figure 12. Nucleotide and deduced amino acid sequences of partial xylanase cDNA from BCC7197

DNA sequencing result showed 255 nucleotides of the partial xylanase cDNA. Primers for 3' RACE (3'RaceXyn) and 5' RACE (5'RaceXyn1 and 5'RaceXyn2) used to amplify 3' and 5' end cDNA, were designed and indicated by arrows.

Figure 13. An alignment of the deduced amino acid sequence of partial xylanase cDNA from BCC7197

The deduced amino acid sequence of xylanase from BCC7197 was aligned by Clustal X program to those of other fungal xylanases: *Magnaporthe grisea* endo-beta-1,4-D-xylanase (AY124591), *Hypocrea jecorina* xylanase III (AB036796), *Aspergillus oryzae* xylanse F3 (Q96VB6), *Penicillium purpurogenum* endo-1,4-beta-D-xylanase A (Q9P8J1), *Aspergillus sojae* endo-1,4-beta-D-xylanase A (Q9P955), and *Thermoascus aurantiacus* endo-1,4-beta-D-xylanase A (P23360). Shading of the alignment represents the degree of conservation. The darkest shading shows the most conserved region among all sequences compared.

4.4 Amplification of the 3' end cDNA by 3' RACE method

3' RACE was performed in order to obtain the 3' end cDNA of BCC7197 xylanase gene. Positions of gene specific primers used to amplify 3' end cDNA are indicated in Figure 12. The 3' RACE result showed approximately 750 bp product (Figure 14). This fragment was purified and cloned into pGEM-T Easy vector. The result of DNA sequencing revealed that the length of 3' end cDNA is 716 nucleotides (Figure 15). Translation of amino acid sequence found the stop codon at the 189th position. Therefore, only 564 nucleotides of the 3' end cDNA encode a deduced amino acid sequence of 188 amino acids (Figure 15). DNA sequence analysis using NCBI-BLAST revealed sequence identity with various fungal xylanases for example *Aspergillus nidulans* xlnC, *Magnaporthe grisea* endo-β-1,4 xylanase, and *Thermoascus aurantiacus* endo-β-1,4 xylanase. The alignment result using Clustal X program revealed 85% nucleotide sequence identity to *Aspergillus nidulans* xlnC gene. Although no AATAAA consensus polyadenylation signal was observed from the 3'-noncoding region of BCC7197 xylanase, the sequence ACTAAA was found at the position 72 downstream from the stop codon (Figure 15). This sequence may represent an alternative polyadenylation signal which is also found in the 3'-UTR of xylanase family 10 xylanase from *Penicillium chrysogenum* (107). Polyadenylation site and poly-A tail suggested that 3' end cDNA of BCC7197 xylanase was completely identified.

4.5 Amplification of the 5' end cDNA by 5' RACE with partial heat denaturation method

The 5' end cDNA of BCC7197 xylanase gene was identified using 5' RACE method. Two gene specific primers, 5' RaceXyn1 and 5' RaceXyn2 located upstream of the internal sequence of BCC7197 xylanase gene were employed (Figure 12). The nested PCR amplification result revealed approximately 400 bp PCR product (Figure 16). This fragment was purified and cloned into pGEM-T Easy vector. Three clones harbouring 5' end cDNA were sequenced. The result of sequencing reaction showed the length of cDNA clone to be 340 bp. There are 237 nucleotide sequences from the start codon (ATG) that encode a deduced amino acid sequences of 79 amino acids

(Figure 17). Although a perfect Kozak translation initiation sequence (ANNATGG) was not observed (108), A found at the position -3 of the start codon of BCC7197 xylanase. This context may represent the variation of Kozak sequence (Figure 17). Sequence analysis using NCBI-BLAST showed the sequence identity with various fungal xylanases belong in the same group of 3' RACE result. The alignment of nucleotide sequence revealed 89% identity to *Magnaporthe grisea* endo-β-1,4 xylanase 88% identity to *Thermoascus aurantiacus* xynA.

4.6 Amplification of the full-length xylanase cDNA

After the 3' end and 5' end cDNA of BCC7197 xylanase were identified. These sequences were combined with the partial xylanase sequences in order to obtain the predicted full-length of xylanase cDNA. XynWF1 and XynR1, gene specific primers were designed to cover the whole sequence of xylanase gene. The PCR amplification showed approximately 1,100 bp product (Figure 18). This fragment was purified and cloned into pGEM-T Easy vector. Restriction enzyme analysis was performed to screen for the clones containing the full-length cDNA. The expected clones gave a band of 1,121 bp when digested with *Eco*R I. Two bands of 274 bp and 847 bp when digested with *Eco*R I and *Ava* I, two bands of 378 bp and 743 bp when digested with *Eco*R I and *Cla* I (Figure 19, 20). DNA sequencing was performed and the result revealed that the nucleotide sequence of the cDNA fragment contained 978-bp open reading frame that encodes a 325-amino acid sequence with a calculated mass of 35 kDa. and a pI of 4.67 (Figure 21). Analysis of the sequences obtained showed that the BCC7197 xylanase gene belonged to glycosyl hydrolase family 10 which high sequence similarity with other fungal xylanases (Figure 22). The alignment of the deduced amino acid sequence of the BCC7197 xylanase with other fungal xylanases revealed 52%-63% amino acid identity (Figure 23).

In order to determine the mature xylanase sequence, the deduced amino acid sequences of BCC7197 xylanase were aligned with the mature fungal xylanase family 10. The result indicated that the mature xylanase of BCC7197 may start at the position 22 (Figure 24).

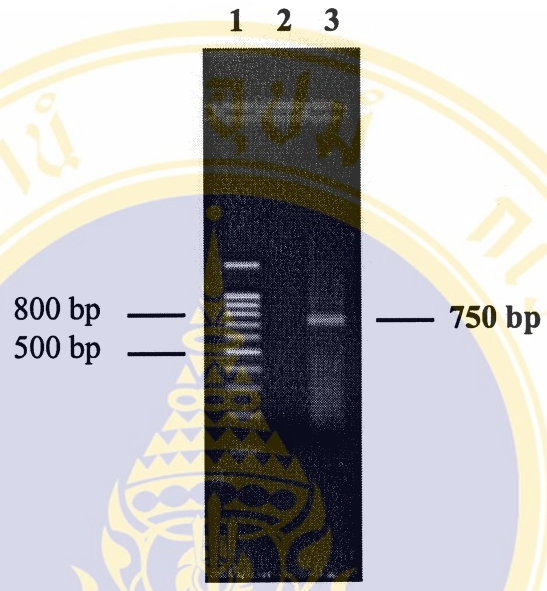


Figure 14. The 3' RACE product of BCC7197 xylanase

The 3' RACE was performed with 3'RaceXyn, a gene specific primer and PM2, an adapter primer. Lane 1 is a 100 bp ladder DNA marker. Lane 2 is negative control using distilled water. Lane 3 is a 3' RACE product. The size of cDNA fragments is about 750 bp.

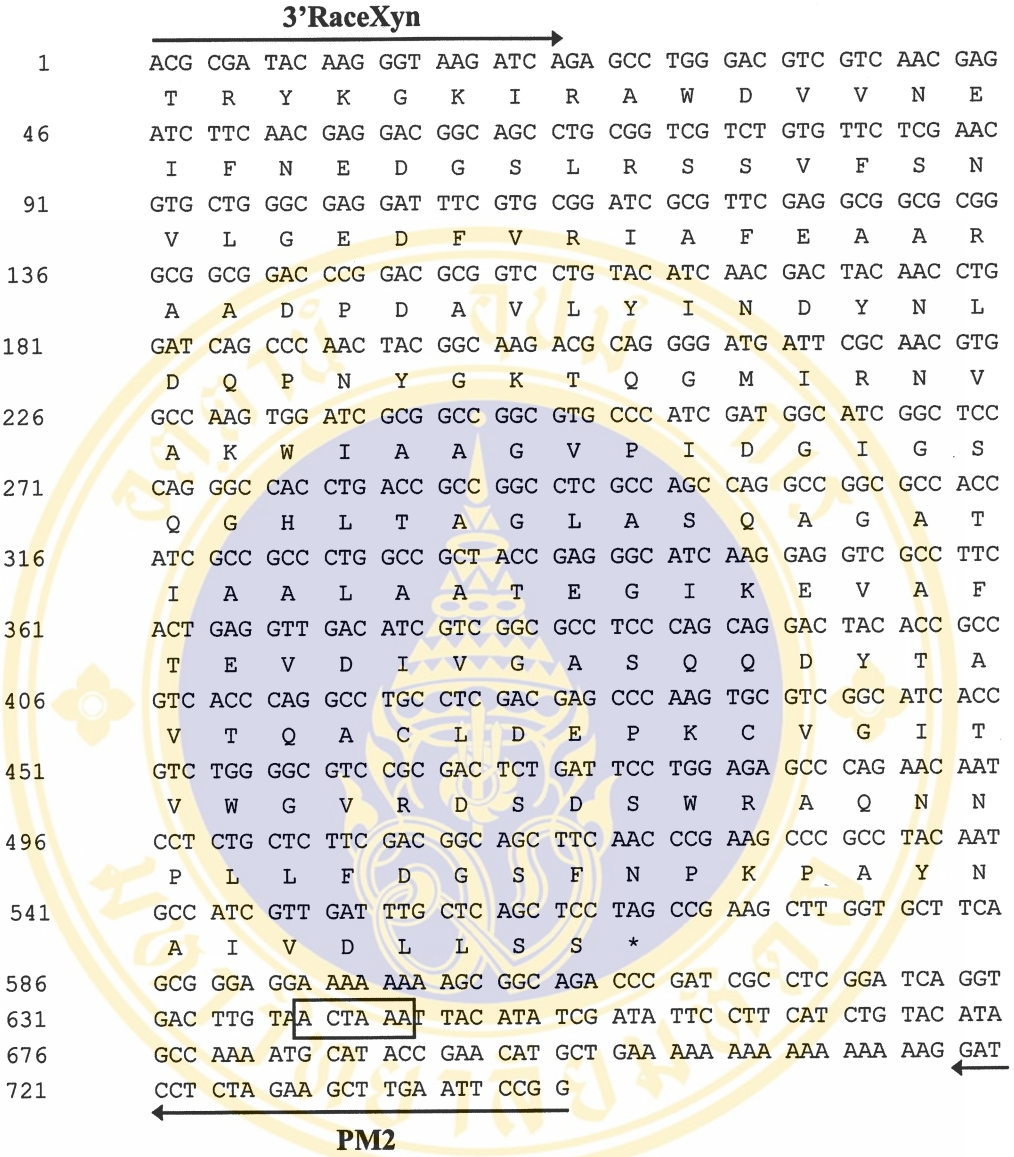


Figure 15. Nucleotide and deduced amino acid sequences of 3' end cDNA of BCC7197 xylanase

3' end cDNA was obtained from BCC7197 xylanase using 3' RACE method. DNA sequencing revealed 716 nucleotides that encoded 188 amino acids. Star represents a stop codon at the 189th position. 3'-UTR sequence is presented after the stop codon and without the translated amino acids. Sequence within the black box represents the predicted polyadenylation site. Arrows indicate positions of primers.

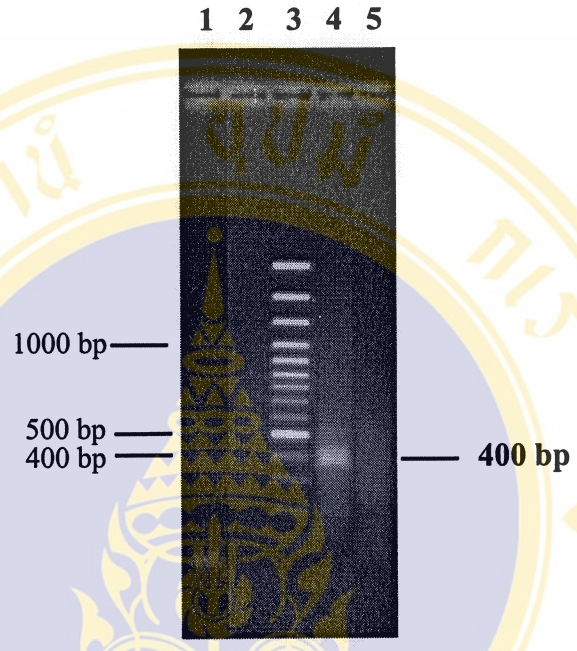


Figure 16. The 5' RACE product of BCC7197 xylanase

The 5' RACE was performed to identify the 5' end cDNA. Lane 1 is the PCR product using 5'RaceXyn2 and PM1 primers. Lane 2 is a negative control of the 1st round PCR which was performed using distilled water. Lane 3 is a 100 bp ladder DNA marker. Lane 4 is the semi-nested PCR product using 5'RaceXyn1 and PM2 primers. Lane 5 is a negative control of semi-nested PCR. PCR products were analyzed on 1% agarose gel.

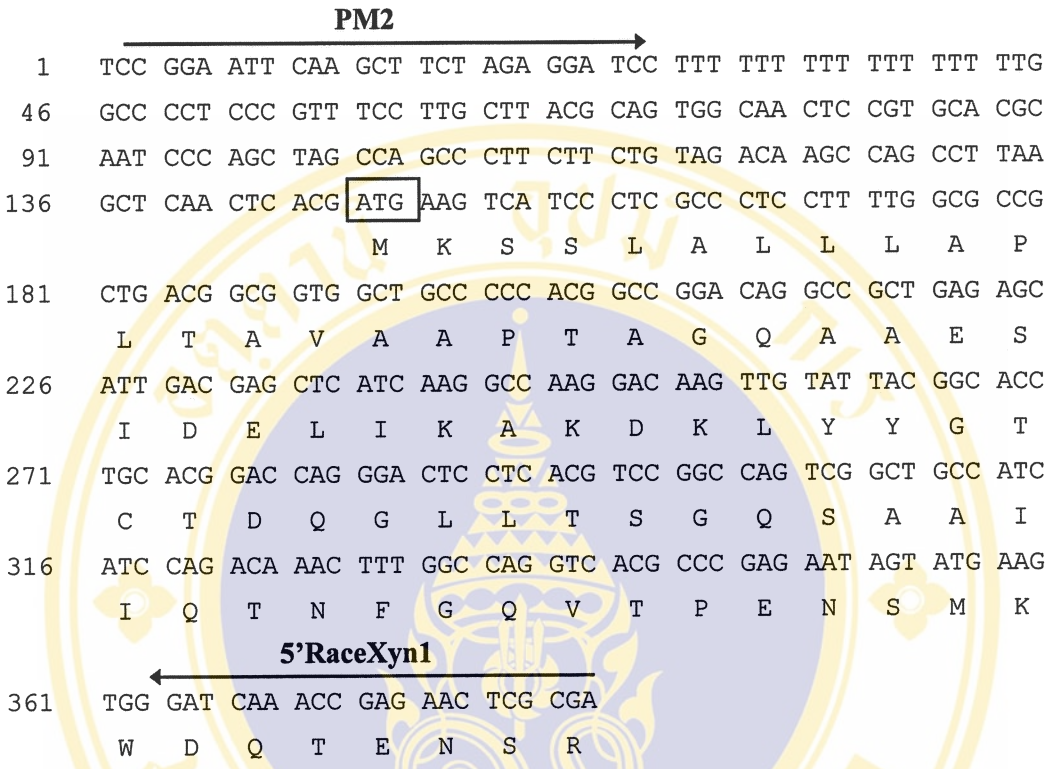


Figure 17. Nucleotide and deduced amino acid sequences of 5' end cDNA of BCC7197 xylanase

5' end cDNA was obtained from BCC7197 xylanase using 5' RACE method. DNA sequencing revealed 237 nucleotides that encode 79 amino acids. Arrows indicate positions of primers. Sequence within the black box represents the translation initiation sequence.

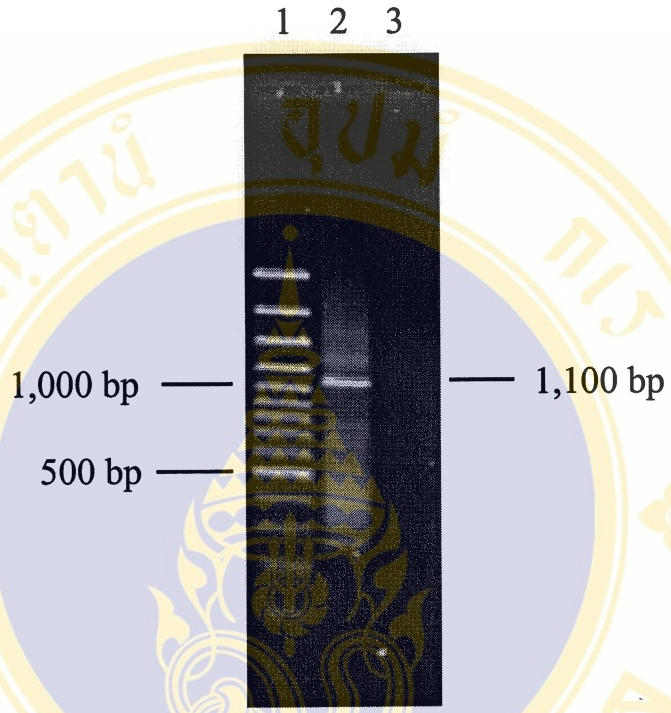


Figure 18. PCR product of the full-length BCC7197 xylanase

The amplification of full-length xylanase cDNA using XynWF1 and XynR1 primers showed approximately 1,100 bp product. Lane 1 is a 100 bp ladder DNA marker. Lane 2 is the PCR product. Lane 3 is water control.

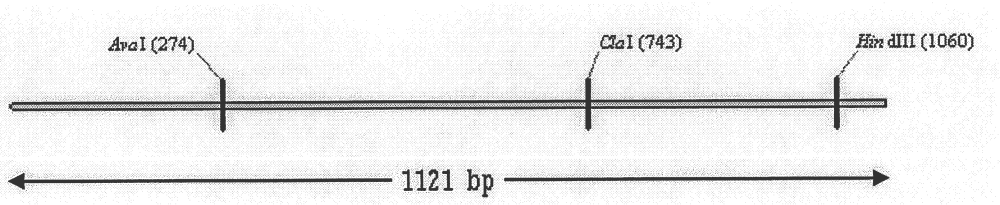


Figure 19. Predicted restriction site of the full-length xylanase cDNA

Restriction site of the full-length xylanase cDNA was predicted using Vector NTI program suites. The result showed the full-length xylanase cDNA amplified by XynWF1 and XynR1 primers contain *Ava* I, *Cla* I, and *Hin*d III restriction site at the position 274, 743, and 1,060 nucleotide, respectively.

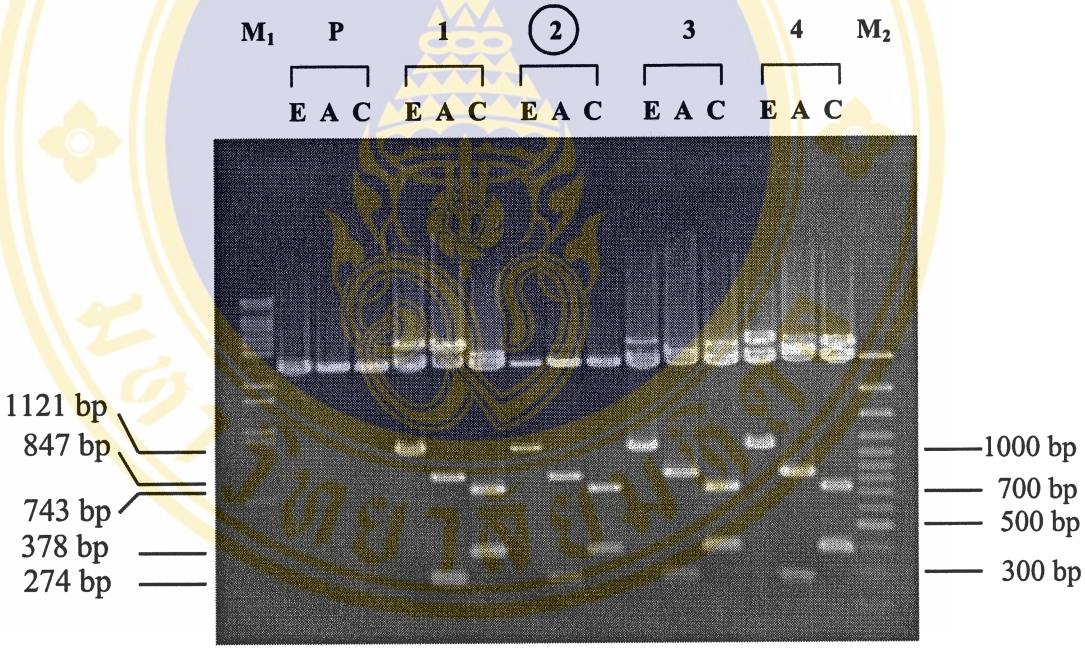
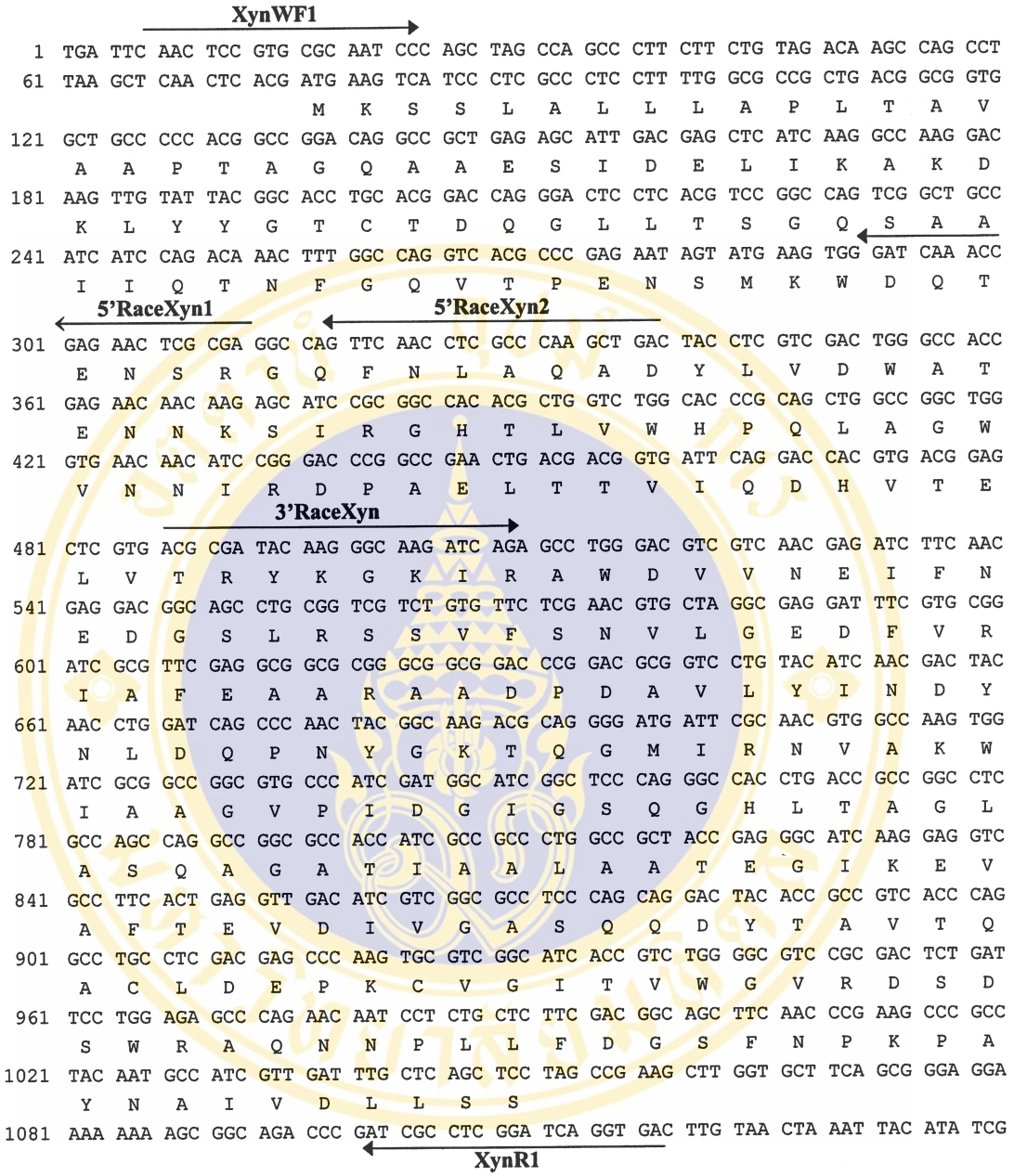


Figure 20. Screening of the full-length xylanase cDNA clones by restriction enzyme analysis

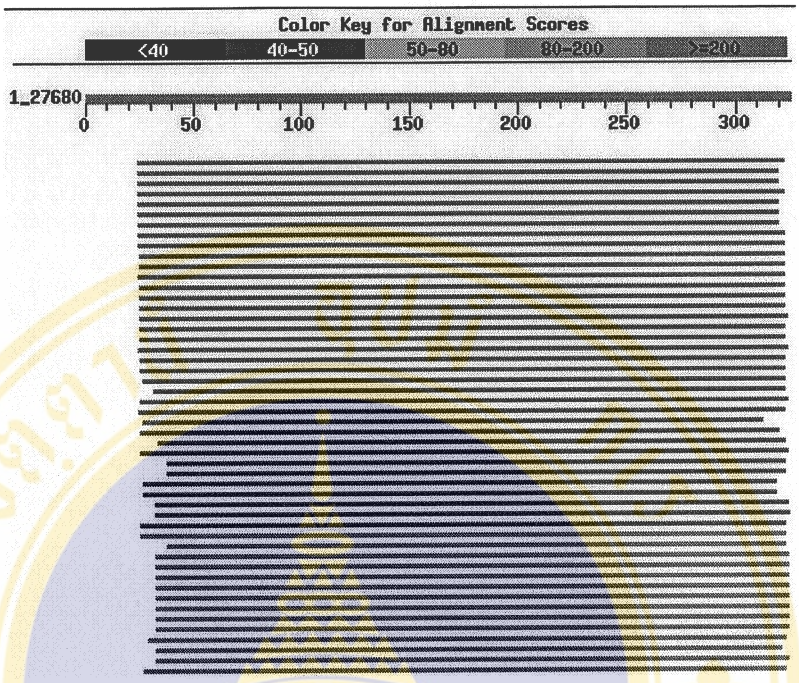
Recombinant plasmid DNAs were digested with *Eco*R I (E), *Eco*R I & *Ava* I (A), and *Eco*R I & *Cla* I (C) then analyzed with gel electrophoresis using 1.2 % agarose gel. Lane M_1 is λ/*Bst*E II DNA marker. Lane M_2 is a 100 bp ladder DNA marker. Lane P is pGEM-T Easy vector. Numbers represent clone numbers. Clone number with circle represents clone selected for DNA sequencing.




```
                                                                  M   K   S   S   L   A   L   L   L   A   P   L   T   A   V
  1 TGA TTC AAC TCC GTG CGC AAT CCC AGC TAG CCA GCC CTT CTT CTG TAG ACA AGC CAG CCT
 61 TAA GCT CAA CTC ACG ATG AAG TCA TCC CTC GCC CTC CTT TTG GCG CCG CTG ACG GCG GTG
121 GCT GCC CCC ACG GCC GGA CAG GCC GCT GAG AGC ATT GAC GAG CTC ATC AAG GCC AAG GAC
      A   A   P   T   A   G   Q   A   A   E   S   I   D   E   L   I   K   A   K   D
181 AAG TTG TAT TAC GGC ACC TGC ACG GAC CAG GGA CTC CTC ACG TCC GGC CAG TCG GCT GCC
      K   L   Y   Y   G   T   C   T   D   Q   G   L   L   T   S   G   Q   S   A   A
241 ATC ATC CAG ACA AAC TTT GGC CAG GTC ACG CCC GAG AAT AGT ATG AAG TGG GAT CAA ACC
      I   I   Q   T   N   F   G   Q   V   T   P   E   N   S   M   K   W   D   Q   T
301 GAG AAC TCG CGA GGC CAG TTC AAC CTC GCC CAA GCT GAC TAC CTC GTC GAC TGG GCC ACC
      E   N   S   R   G   Q   F   N   L   A   Q   A   D   Y   L   V   D   W   A   T
361 GAG AAC AAC AAG AGC ATC CGC GGC CAC ACG CTG GTC TGG CAC CCG CAG CTG GCC GGC TGG
      E   N   N   K   S   I   R   G   H   T   L   V   W   H   P   Q   L   A   G   W
421 GTG AAC AAC ATC CGG GAC CCG GCC GAA CTG ACG ACG GTG ATT CAG GAC CAC GTG ACG GAG
      V   N   N   I   R   D   P   A   E   L   T   T   V   I   Q   D   H   V   T   E
481 CTC GTG ACG CGA TAC AAG GGC AAG ATC AGA GCC TGG GAC GTC GTC AAC GAG ATC TTC AAC
      L   V   T   R   Y   K   G   K   I   R   A   W   D   V   V   N   E   I   F   N
541 GAG GAC GGC AGC CTG CGG TCG TCT GTG TTC TCG AAC GTG CTA GGC GAG GAT TTC GTG CGG
      E   D   G   S   L   R   S   S   V   F   S   N   V   L   G   E   D   F   V   R
601 ATC GCG TTC GAG GCG GCG CGG GCG GCG GAC CCG GAC GCG GTC CTG TAC ATC AAC GAC TAC
      I   A   F   E   A   A   R   A   A   D   P   D   A   V   L   Y   I   N   D   Y
661 AAC CTG GAT CAG CCC AAC TAC GGC AAG ACG CAG GGG ATG ATT CGC AAC GTG GCC AAG TGG
      N   L   D   Q   P   N   Y   G   K   T   Q   G   M   I   R   N   V   A   K   W
721 ATC GCG GCC GGC GTG CCC ATC GAT GGC ATC GGC TCC CAG GGC CAC CTG ACC GCC GGC CTC
      I   A   A   G   V   P   I   D   G   I   G   S   Q   G   H   L   T   A   G   L
781 GCC AGC CAG GCC GGC GCC ACC ATC GCC GCC CTG GCC GCT ACC GAG GGC ATC AAG GAG GTC
      A   S   Q   A   G   A   T   I   A   A   L   A   A   T   E   G   I   K   E   V
841 GCC TTC ACT GAG GTT GAC ATC GTC GGC GCC TCC CAG CAG GAC TAC ACC GCC GTC ACC CAG
      A   F   T   E   V   D   I   V   G   A   S   Q   Q   D   Y   T   A   V   T   Q
901 GCC TGC CTC GAC GAG CCC AAG TGC GTC GGC ATC ACC GTC TGG GGC GTC CGC GAC TCT GAT
      A   C   L   D   E   P   K   C   V   G   I   T   V   W   G   V   R   D   S   D
961 TCC TGG AGA GCC CAG AAC AAT CCT CTG CTC TTC GAC GGC AGC TTC AAC CCG AAG CCC GCC
      S   W   R   A   Q   N   N   P   L   L   F   D   G   S   F   N   P   K   P   A
1021 TAC AAT GCC ATC GTT GAT TTG CTC AGC TCC TAG CCG AAG CTT GGT GCT TCA GCG GGA GGA
      Y   N   A   I   V   D   L   L   S   S
1081 AAA AAA AGC GGC AGA CCC GAT CGC CTC GGA TCA GGT GAC TTG TAA CTA AAT TAC ATA TCG
1141 ATA TTC CTT CAT CTG TAC ATA GCC AAA ATG CAT ACC GAA CAT GCT GAA AAA AAA AAA AAA
1201 AA
```

Figure 21. Nucleotide and deduced amino acid sequence of xylanase from BCC7197

The nucleotide sequence of the xylanase cDNA contained 978-bp open reading frame. The deduced amino acid sequence is shown in a single letter under the respective codon. Primers for 3' RACE (3'RaceXyn), 5' RACE (5'RaceXyn1 and 5'RaceXyn2) and full-length xylanase (XynWF1 and XynR1) are indicated by arrows.



Sequences producing significant alignments:		Species	Score(bits)	E Value
gi|13432255|sp|P23360|	endo-1,4-beta-xylanase	*Thermoascus aurantiacus*	356	3e-97
gi|576483|gb|AAC41684.1|	endo-1,4-beta-xylanase	*Magnaporthe grisea*	348	5e-95
gi|15823785|dbj|BAB69073.1|	xylanaseF3	*Aspergillus oryzae*	343	3e-93
gi|7328942|dbj|BAA92882.1|	endo-1,4-beta-xylanase	*Aspergillus sojae*	340	3e-92
gi|7328936|dbj|BAA89465.2|	xylanaseIII	*Hypocrea jecorina*	339	4e-92
gi|3660011|pdb|1BG4|	xylanase	*Penicillium simplicissium*	330	2e-89
gi|7960269|gb|AAF71268.1|	endo-1,4-beta-xylanase	*Penicillium purpurogenum*	325	9e-88
gi|2981135|gb|AAC06239.1|	family F xylanase	*Fusarium oxysporum*	312	4e-84
gi|3660499|emb|CAA76571.1|	endo-1,4-beta-xylanase	*Claviceps purpurea*	291	8e-78
gi|9796014|emb|CAC03463.1|	putative xylanase	*Agaricus bisporus*	285	1e-75
gi|32402371|gb|AAP81158.1|	xylanase	*Volvariella volvacea*	247	2e-64

Figure 22. BLAST search results for xylanase from BCC7197 from GenBank database

A red line with scale numbers represents the xylanase sequence. Colors according to score are indicated. Analysis was performed using a blastp (protein-protein blast) in http://www.ncbi.nlm.nih.gov/blastp

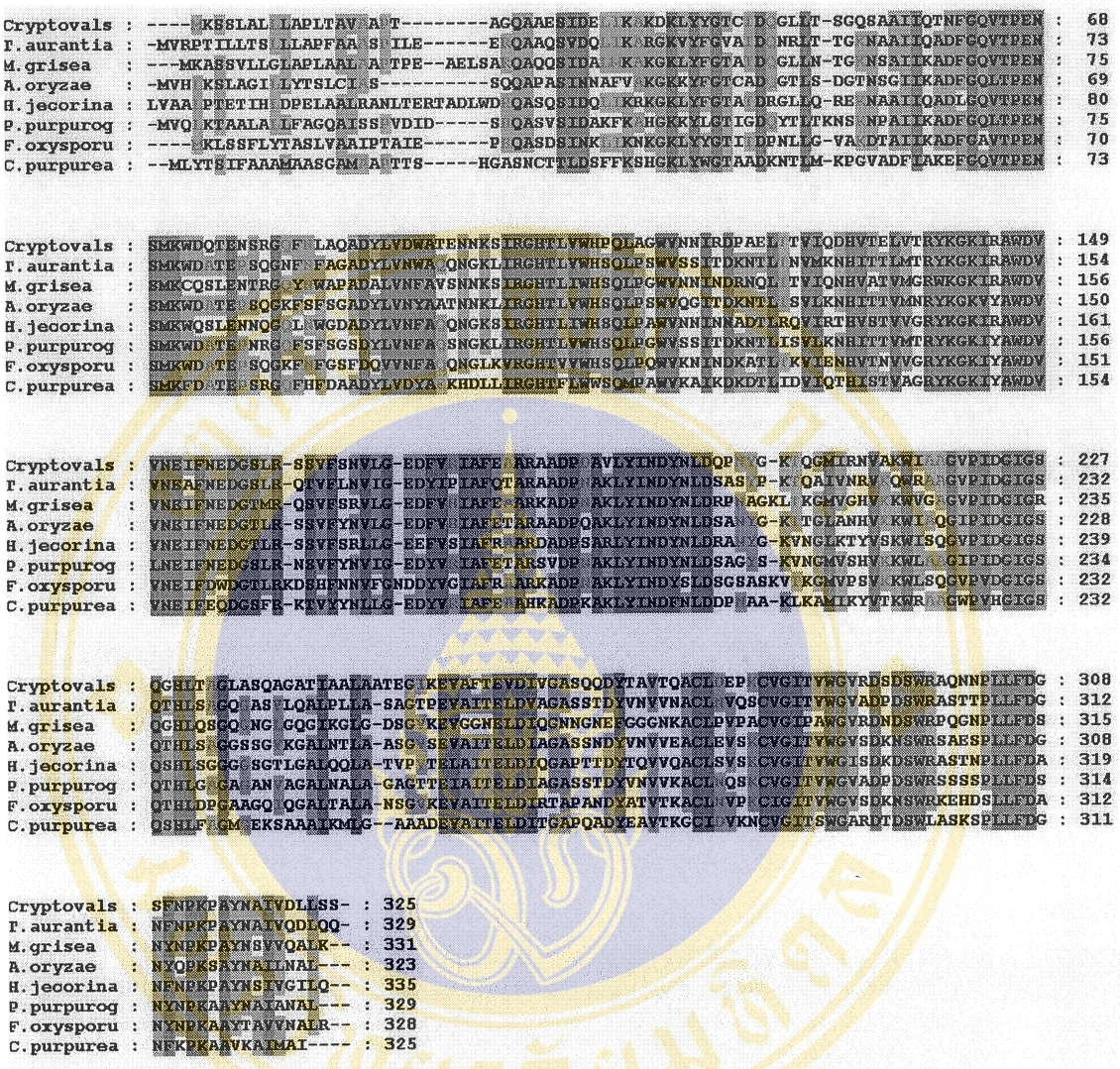


Figure 23. An alignment of deduced amino acid sequence of BCC7197 xylanase with other fungal xylanases

Sequence alignment of the deduced amino acid sequence of xylanase from BCC7197 with those of other fungal xylanases: *Thermoascus aurantiacus* xylanase A (AJ132635.1), *Magnaporthe grisea* putative endo-beta-1,4-D-xylanase (AAC41684), *Aspergillus oryzae* xylanse F3 (BAB69073), *Hypocrea jecorina* xylanase III (BAA89465), *Penicillium purpurogenum* endo-1,4-beta-D-xylanase A (AAF71268), *Fusarium oxysporum* family F xylanase (AAC06240), *Claviceps purpurea* endo-1,4-beta-xylanase (CAA76571). Shading of the alignment represents the degree of conservation.

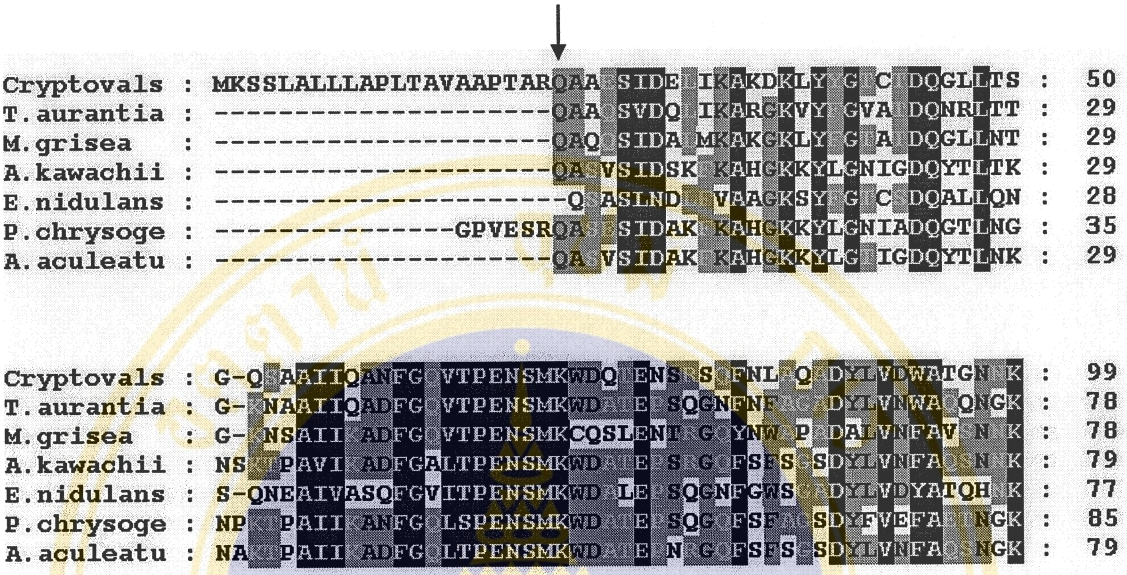


Figure 24. Alignment of partial deduced amino acid sequence of BCC7197 xylanase with mature xylanase from other fungal

Sequence alignment of the partial deduced amino acid sequence of xylanase from BCC7197 with mature xylanase from other fungal: *Thermoascus aurantiacus* xylanase A (AJ132635.1), *Magnaporthe grisea* putative endo-beta-1,4-D-xylanase (AAC41684), *Aspergillus kawachii* endo-1,4-beta-xylanase (P33559), *Emericella nidulans* endo-1,4-beta-xylanase (Q00177), *Penicillium chrysogenum* endo-1,4-beta-xylanase (P29417), *Aspergillus aculeatus* endo-1,4-beta-xylanase (O59859). Shading of the alignment represents the degree of conservation. An arrow indicates where the start position of the mature xylanase of BCC7197 might be.

The 3D structure of BCC7197 xylanase was preliminary predicted from the deduced amino acid sequences using the SWISS-MODEL program. The result showed that the 3D structure of the BCC7197 xylanase depicts the $(\alpha/\beta)_8$ fold (Figure 25B) like other xylanases in the glycosyl hydrolase familly 10 such as thermostable xylanase form *Thermoascus aurantiacus* (Figure 25A). This result confirmed that BCC7197 xylanase gene belonged to glycosyl hydrolase family 10.

4.7. Construction of xylanase in *P. pastoris* expression vector

In order to express the secreted xylanase in *P. pastoris*, PP-XynF1, PP-XynF2 and PP-XynR1 primers were designed in-frame with *S. cerevisiae* α-factor secretion signal and cloned into pPICZαA vector. The PCR amplification was performed using PP-XynF1 and PP-XynR1 for full-length xylanase with leader sequence whereas PP-XynF2 and PP-XynR1 for mature xylanase without leader sequence (Figure 26). The PCR result showed approximately 950 bp (with leader sequence) and 900 bp (without leader sequence) products (Figure 27). The PCR products were double digested with *Xho* I and *Xba* I, purified and then cloned into the pPICZαA between *Xho* I and *Xba* I sites. Rapid size screening and restriction enzyme analysis were performed to screen for the clones containing the xylanase insert fragment. The expected clones gave a band of 950 bp (with leader sequence) and 900 bp (without leader sequence) when double digested with *Xho* I and *Xba* I (Figure 28). DNA sequencing was then performed to analyze the inserted sequence. The sequencing result revealed that the fragments were inserted at the correct site and fused in-frame with the α-factor secretion signal (Figure 29). Moreover, no mutation was observed in the sequences of xylanase with or without leader sequence.

4.8 Determination of xylanase cDNA integration into *P. pastoris* genome

The recombinant plasmids, pPICZαA-xylanase with and without leader sequence were linearized with *Dra* I, which is a unique site in the 5' AOX promoter. The linearized plasmids were transformation into the *P. pastoris* host strain KM71 by electroporation. *P. pastoris* transformants were firstly screened for Zeocin™-resistant

transformants using the rich medium (YEPD) containing 100 ug/ml Zeocin™. Genomic DNA was extracted from Zeocin™-resistant transformants and further identified for integration by PCR amplification using 5' AOX and 3' AOX primers. PCR screening of *P. pastoris* transformants with leader sequence (WO) and without leader sequence (MWO) showed the products with the approximately size of 1,600 and 1,500 bp, respectively. While a band of 600 bp was observed from *P. pastoris* transformant containing only pPICZαA vector (Figure 30). These results indicated that the linearized plasmids containing xylanase fragments (WO, MWO) integrated into the *P. pastoris* genome.

4.9 Expression of BCC7197 xylanase in *P. pastoris*

P. pastoris integrants that contained the xylanase cDNA with leader sequence (WO 5-2, WO 5-4) and without leader sequence (MWO1-1, MWO1-3) were chosen for small-scale expression. These clones were grown in BMGY until OD_{600} reached 5-6 units. To induce the expression, cells were pelleted and resuspended in BMMY containing 3% (v/v) of methanol. The methanol was added to a final concentration of 3% (v/v) into the culture every 24 h to maintain the induction state. The culture supernatant was collected every 24 h of induction from 0-4 days; the secreted xylanase expression was detected by AZCL-xylan and SDS-PAGE.

4.9.1 Xylanase activity test by AZCL-xylan

20 µl of culture supernatant was applied in each well of AZCL-Xylan, then incubated at 30°C for 18 h. The result showed that the blue zone developed from all four clones after a day and lasted at least 4 days of induction. Interestingly, the blue zone from the clone MWO (mature xylanase) gave the largest zone after 4 days of incubation while that from the clone WO (with its own leader sequence) gave the largest zone after 1 day and 4 days of incubation. The blue zone was not observed from the transformant containing only pPICZαA vector (Figure 31). The result suggested the active xylanase protein was successfully produced and secreted from *P. pastoris* containing xylanase cDNA with or without its own leader sequence.

4.9.2 Protein analysis by SDS-PAGE

In order to determine the secreted protein, 40 µl of culture supernatant was loaded into 12% SDS-PAGE then stained with coomassie brilliant blue. The result showed that the protein of the expected size (33-35 kDa) was observed after 1 day until 4 days of induction from the *P. pastoris* integrants containing xylanase with leader sequence (Figure 32A) and without leader sequence (Figure 32B). While this protein was not observed form the *P. pastoris* integrant containing only pPICZαA vector. The result has suggested that the protein of 33-35 kDa is BCC7197's xylanase, although the xylanase activity assay or the N-terminal sequencing of this 33-35 kDa should be performed to confirm the result.

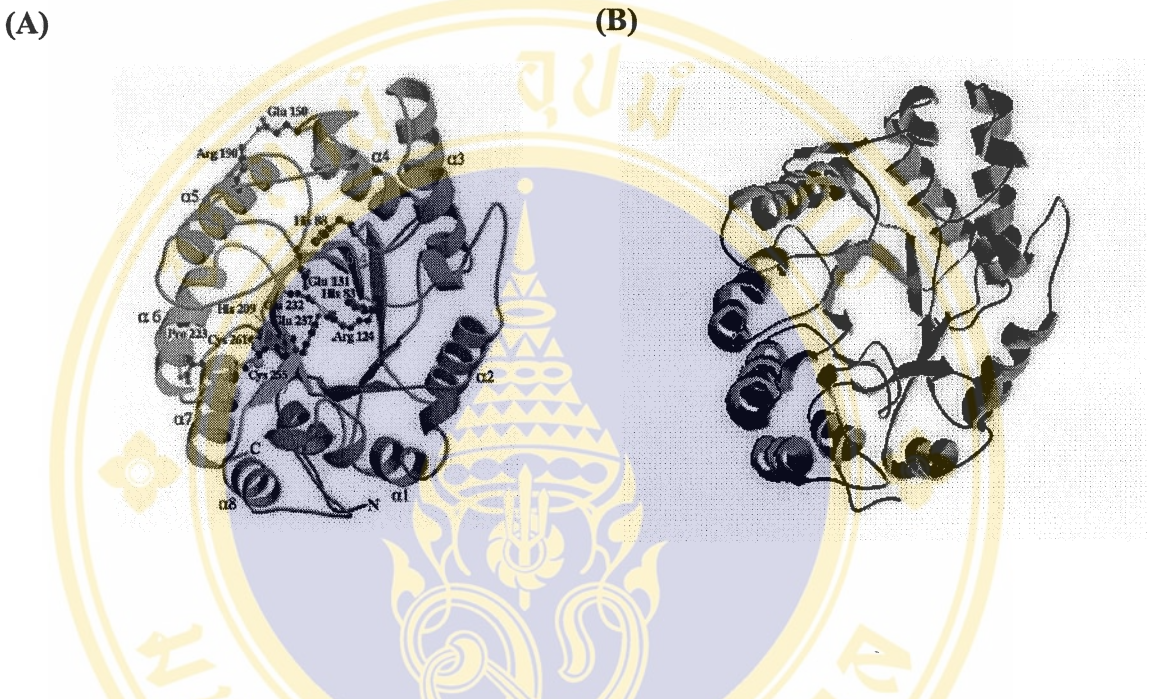


Figure 25. Prediction of 3D structure of BCC7197 xylanase

(A) Representation of *T. aurantiacus* xylanase, depicting the $(\alpha/\beta)_8$ fold. (B) Prediction of the tertiary structure of BCC7197 xylanase was performed using An Automated Comparative Protein Modelling Server, SWISS-MODEL in www.expasy.org/swissmod/SWISS-MODEL.html.

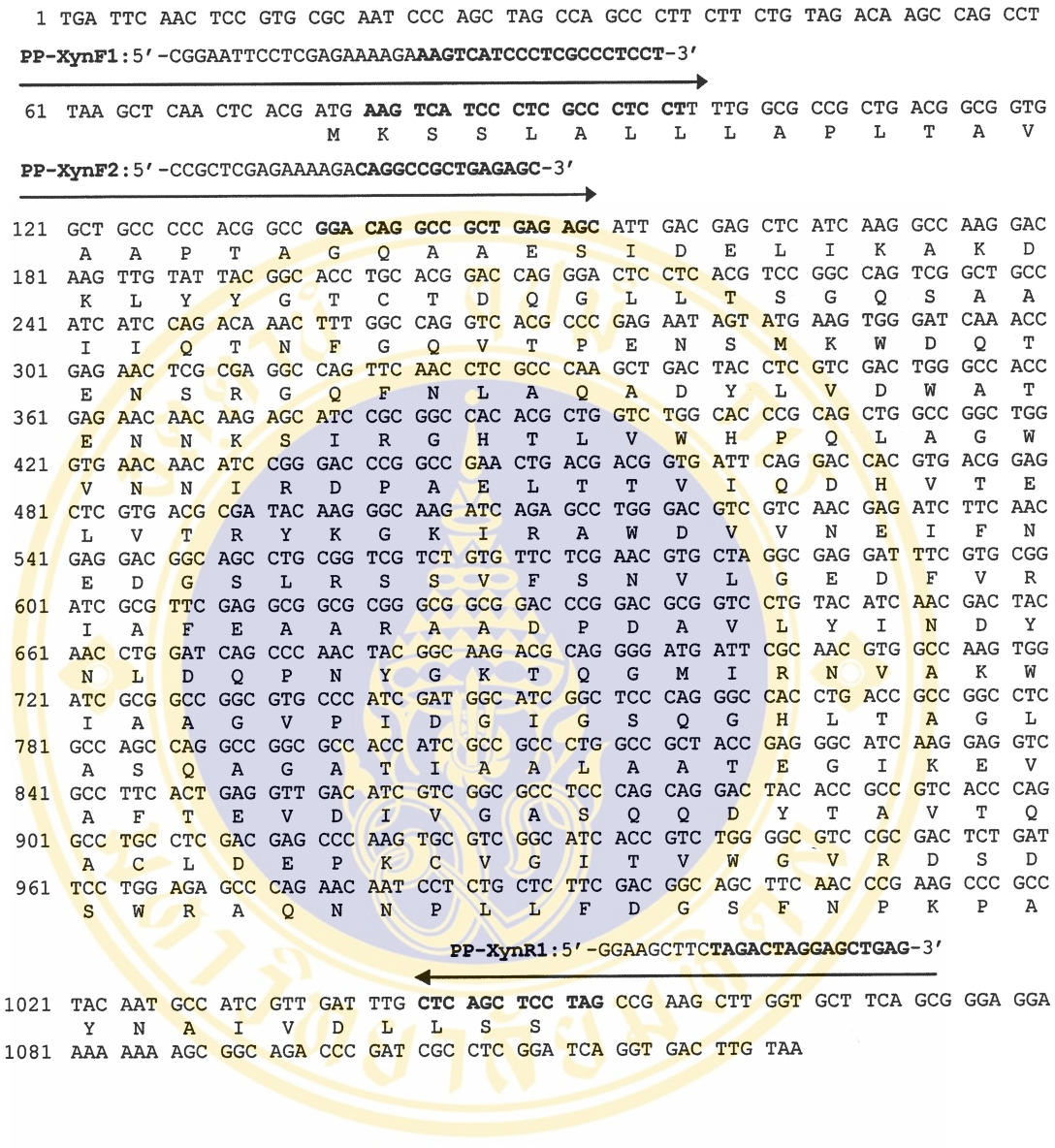


Figure 26. *P. pastoris* expression primers

P. pastoris expression primers were designed in-frame with *S. cerevisiae* α-factor secretion signal containing Kex2 cleavage site. Forward primers for amplification of full-length xylanase with leader sequence (PP-XynF1), mature xylanase without leader sequence (PP-XynF2), and reverse primer (PP-XynR1) are indicated by arrows.

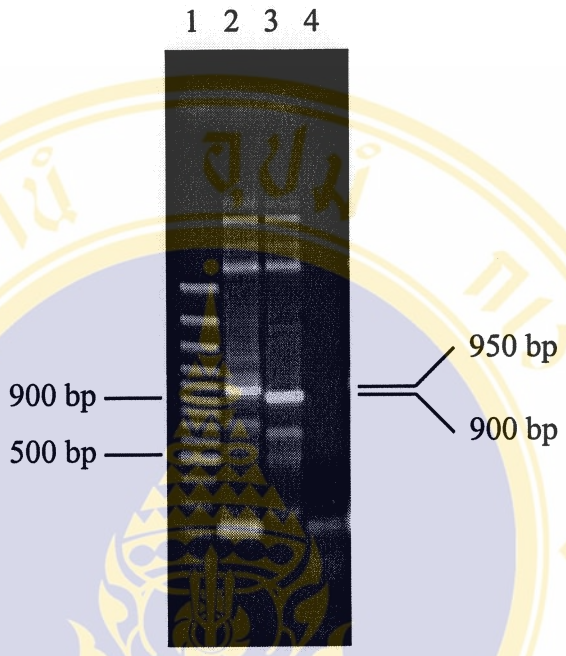


Figure 27. PCR product of xylanase for *P. pastoris* expression

The PCR result showed approximately 950 bp (with leader sequence) and 900 bp (without leader sequence) products. The PCR products were double digested with *Xho* I and *Xba* I, then cloned into the pPICZαA. Lane 1 is a 100 bp marker. Lane 2 is the PCR product using PP-XynF1 and PP-XynR1. Lane 3 is the PCR product using PP-XynF2 and PP-XynR1. Lane 4 is negative control which performed using distilled water.

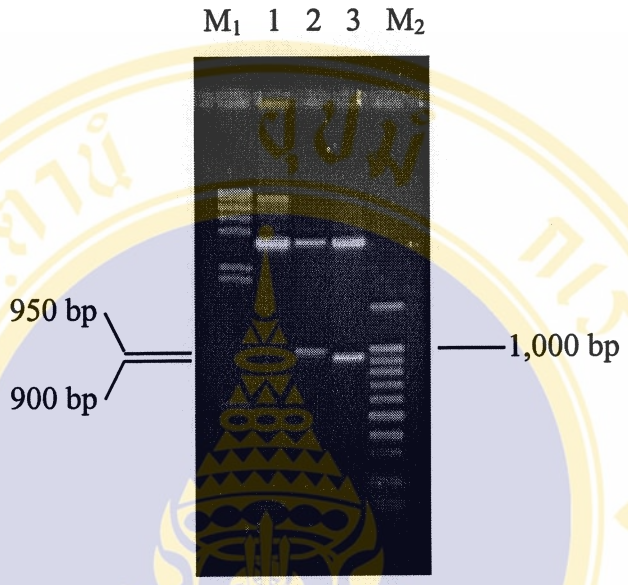


Figure 28. Restriction endonuclease analysis of plasmid pPICZαA-xylanase

Recombinant plasmids containing full-length xylanase with leader sequence (Lane2) and mature xylanase without leader sequence (Lane 3) were digested with *Eco*R I. Lane 1 is pPICZαA vector. Lane M_1 is λ/*Hin*d III DNA marker. Lane M_2 is a 100 bp ladder DNA marker.

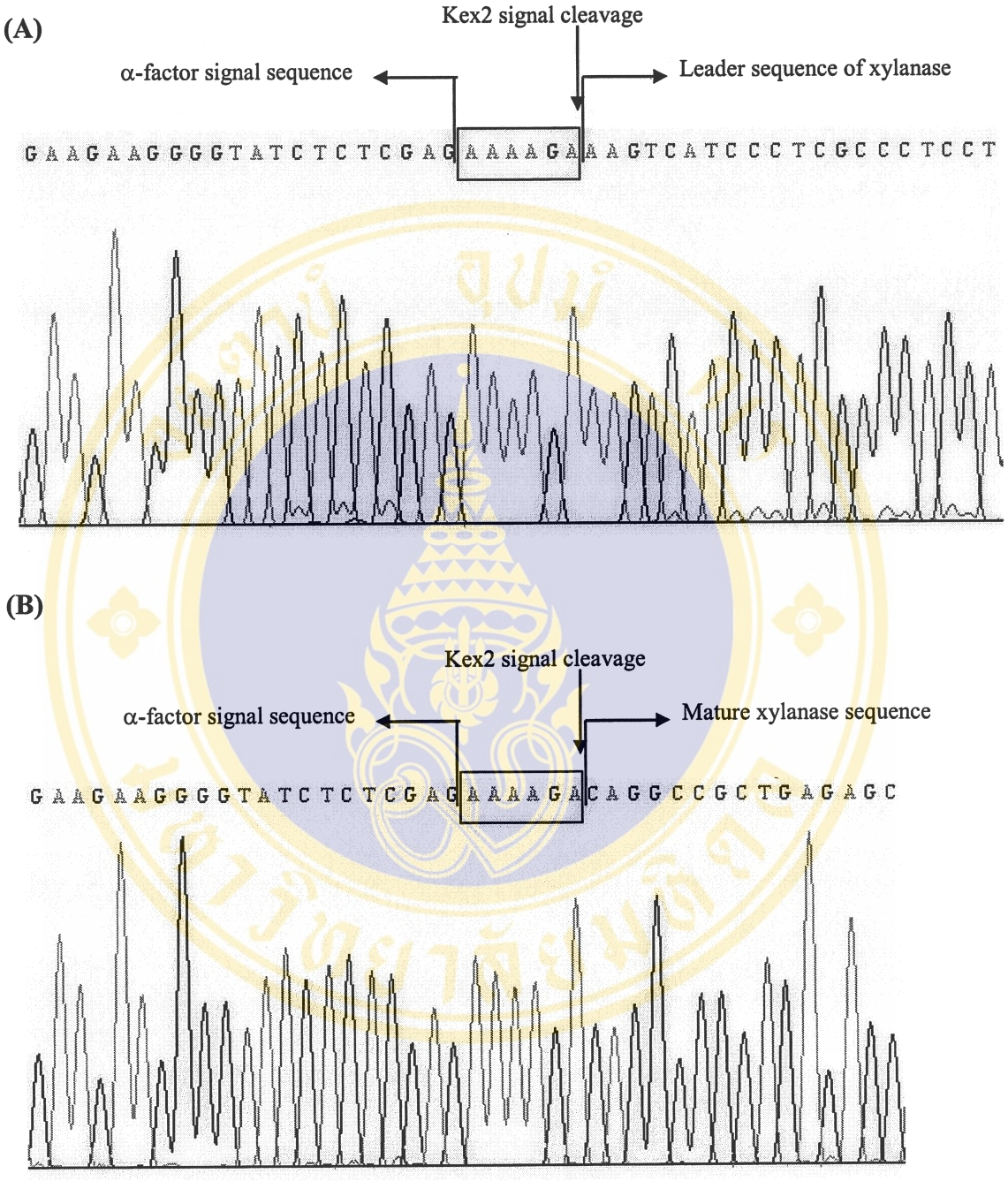


Figure 29. Chromatogram showing a part of nucleotide sequence analysis of xylanase with or without leader sequence recombinant plasmid

The sequences show that either xylanase with or without leader sequence fragment was inserted and fused in-frame to the *P. pastoris* expression vector at a correct position in panel A and B, respectively.

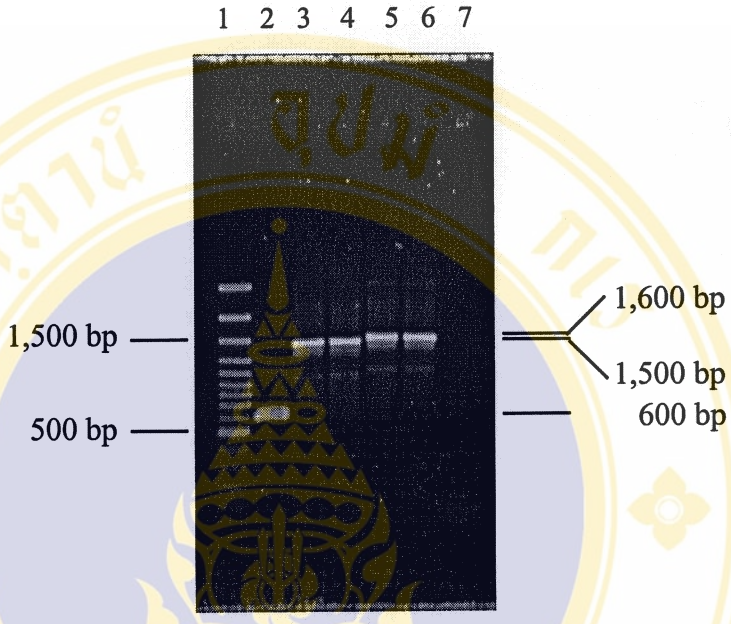


Figure 30. PCR analysis of *P. pastoris* integrants

PCR screening for genome integration using 5' AOX and 3' AOX primers showed that the xylanase gene insert into the *P. pastoris* genome. Lane 1 is a 100 bp ladder DNA marker. Lane 2 is the PCR product of *P. pastoris* integrant containing pPICZαA. Lane 3, 4 are the PCR product of *P. pastoris* integrants containing xylanase without leader sequence clone 1 (WO5-2) and 2 (WO5-4), respectively. Lane 5, 6 are the PCR product of *P. pastoris* integrants with leader sequence clone 1 (MWO1-1) and 2 (MWO1-3), respectively. Lane 7 is a negative control was performed with distilled water.

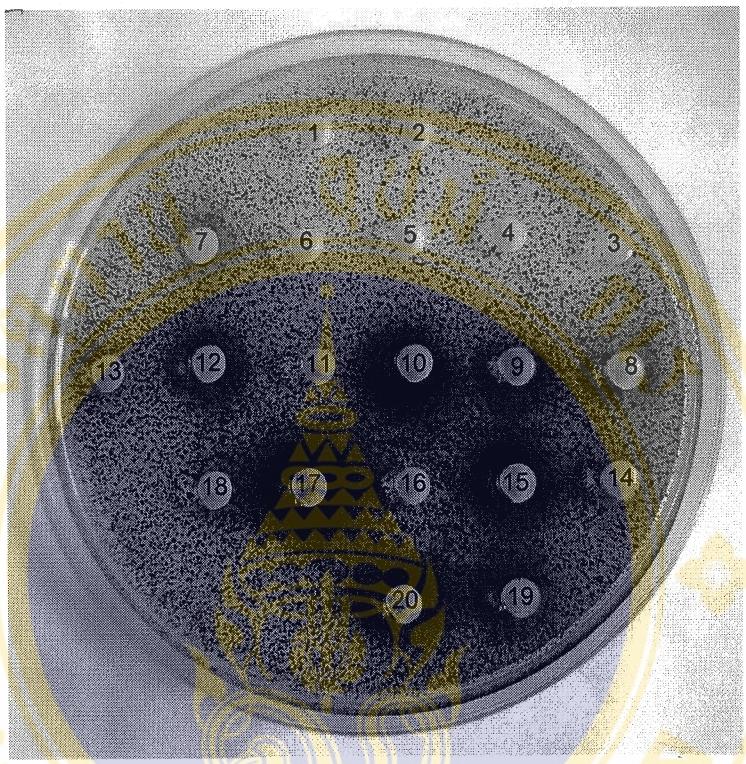


Figure 31. Xylanase activity test by AZCL-xylan

P. pastoris integrants containing the xylanase cDNA and only pPICZαA vector were induced in BMMY supplemented with 3% methanol (v/v). The culture supernatant was collected every 24 h of induction from 0-4 days. A 20 µl of culture supernatant was used to assay the xylanase activity on AZCL-xylan plate. The blue zone about 2-3 mm was observed after incubating at 30° C for 18 h. Well 1-5 is *P. pastoris* integrant containing pPICZαA after induction for 0-4 days, respectively. Well 6-10 is MWO1-1 after induction for 0-4 days, respectively. Well 11-15 is WO5-2 after induction for 0-4 days, respectively. Well 16-20 is WO5-4 after induction for 0-4 days, respectively. MWO1-3 result is the same as MWO1-1 (data not show).

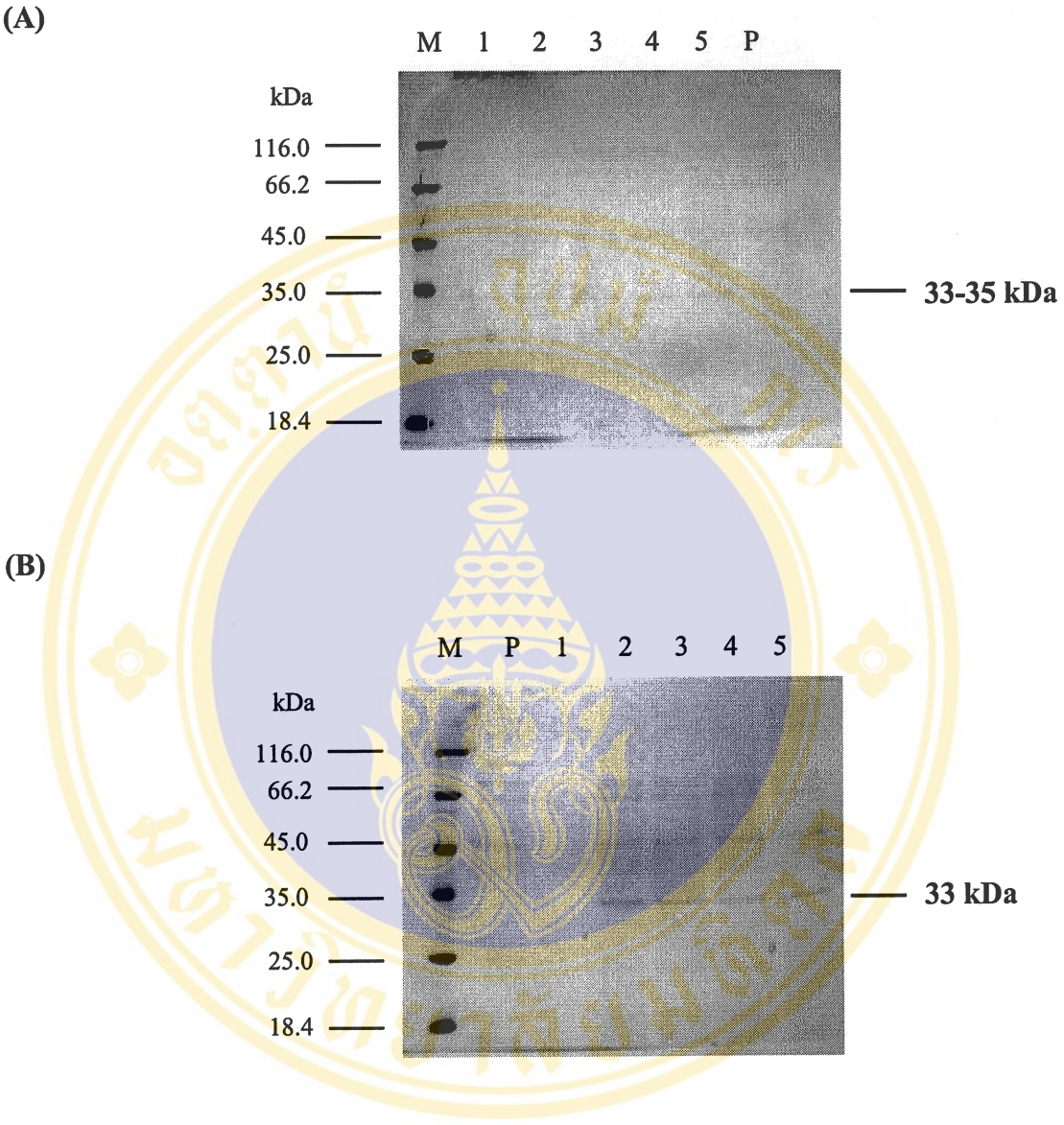


Figure 32. SDS-PAGE protein profile of the *P. pastoris* integrants

(A) SDS-PAGE protein profile of *P. pastoris* clone WO5-2 (with leader sequence). (B) SDS-PAGE protein profile of *P. pastoris* clone MWO1-1 (without leader sequence). These were grown in BMGY and were induced in BMMY supplemented with 3% methanol (v/v). The culture supernatant was collected every 24 h of induction from 0-4 days. 40 µl of culture supernatant was loaded into each lane (Lane 1-5). Lane M represents protein molecular weight marker (Fermentas). Lane P is *P. pastoris* containing only pPICZαA vector after induction for 4 days.

CHAPTER V

DISCUSSION

5.1 Efficient xylanase production using medium containing 5 % wheat bran

The choice of an appropriate substrate is of great importance for the successful production of xylanases. The substrate not only serves as a carbon and energy source, but also provides the necessary inducing compounds for the organism.

In order to identify the xylanase gene BCC7197, appropriate inducing substrate and optimal culturing condition for xylanase production are desired. In this study, hemicellulosic substrates, birchwood xylan and wheat bran, were used as inducers. These substrates have also been found to be most suitable for the production of xylanses in other certain microorganisms such as *Penicillium purpurogenum* (51) and an alkaliphilic thermophilic *Bacillus* sp. NCIM 59 (52).

In this study, a modified method was developed for xylanase induction. The successful xylanase production was performed by directly growing BCC7197 in the supernatant of 5% wheat bran with continuously shaking at 30°C, 250 rpm for 8 days. This might be because wheat bran which is a complex polysaccharide, may up regulate a broad spectrum of genes encoding the xylanolytic enzymes. Using this procedure, not only higher xylanase activity was obtained, the growing BCC7197 can also be easily separated from the insoluble, fine particulate inducers present in the culture medium.

5.2 Molecular cloning of the full-length gene encoding xylanase from BCC7197

RT-PCR and RACE were used to identify the full-length xylanase cDNA from BCC7197. The partial xylanase sequence was achieved by RT-PCR with three degenerate primers, which were designed based on the conserved amino acid sequence

of endoxylanase from various Sordariomycetes. The 3' end of cDNA was obtained by 3' RACE. For the 5' RACE, in order to avoid RNA secondary structures such as hairpins and stem-loop formation which often compromise the synthesis of cDNAs, partial heat denaturation method during reverse transcription (106) was used to construct the first stranded cDNA. Using this method, the 5' end of cDNA was amplified and subsequently, the full-length xylanase cDNA was obtained. The xylanase cDNA had an open reading frame of 978 bp encoding a polypeptide of 325 amino acid residues with a calculated mass of 35 kDa. and a pI of 4.67. Thus, this BCC7197 xylanase would fit into the acidic high-molecular-mass category of xylanase described by Wong *et al.* (14). This result agreed with the classification of glycanase based on the amino acid sequence similarity, which revealed that the BCC7197 xylanase gene belong to glycosyl hydrolase family 10 (21). Similarity of the deduced amino acid sequence of the BCC7197 xylanase with other fungal xylanases revealed 52%-63% identity. In contrast to the family 11 which has only endo-β-1,4-xylanase (EXs, EC 3.2.1.8) activity, family 10 possesses several catalytic activities which are: endo-β-1,4-xylanase (EC 3.2.1.8), endo-β-1,3-xylanase (EC 3.2.1.32) and cellobiohydrolase (EC 3.2.1.91).

In recent years, 3D crystal structure of glycanase family 10 such as *Cellulomonas fimi* (108), *Streptomyces lividans* (33) and *Thermoascus aurantiacus* (35) have been published. They revealed that EXs of family 11 appear to be small and well-packed molecules, and formed mainly β-sheets, while EXs of family 10 is a typical 8-fold α/β barrel $(\alpha/\beta)_8$ resulting in 'salad bowl' shape of the molecule. In this study, the 3D structure of BCC7197 xylanase was preliminary predicted using its deduced amino acid sequence via SWISS-MODEL program. The result showed that the 3D structure of the BCC7197 xylanase depicting the $(\alpha/\beta)_8$ fold. This observation is in agreement with 3D structure of EXs of family 10.

5.3 Expression of BCC7197 xylanase cDNA in *P. pastoris*

Expression of fungal xylanase genes have been reported in various host organisms such as *Escherichia coli*, *Saccharomyces cerevisiae, Pichia pastoris* and *Aspergillus oryzae* (74). In this study, *P. pastoris* was also used as the expression host.

P. pastoris is capable of many posttranslational modifications, such as glycosylation, disulfide bond formation and proteolytic processing. Therefore, expression in *P. pastoris* may provide soluble, secreted products.

The BCC7197 xylanase was subcloned into *P. pastoris* expression vector, pPICZαA, which contained the MFα-1 signal sequence that directed the secretion of the heterologous protein. However the N-terminal sequences of the BCC7197 mature xylanase was not yet been determined. To obtain the secreted active protein, two different recombinant plasmids were therefore constructed. (1) the full-length xylanase sequence including its own leader peptides, and (2) the mature xylanase sequence determined from the alignment of xylanases from several fungal family 10 xylanase. The predicted mature xylanase had a polypeptide of 304 amino acid residues and a calculated mass of 33 kDa.

Prior to *P. pastoris* transformation, the insert gene in the pPICZαA-xyn was analyzed by DNA sequencing. The result showed that the xylanase fragments were inserted at the correct site and fused in-frame with the prepro-α-factor secretion signal. Moreover, no mutation was observed in the sequences of xylanase with or without its own leader sequence. The recombinant plasmids were linearized, and then transformed into *P. pastoris* by electroporation. *P. pastoris* integrants were first screened for zeocin-resistant and identified for integration by PCR amplification using 5' AOX and 3' AOX primers.

P. pastoris integrants containing either the full-length xylanase sequence or the mature xylanase sequence were expressed in a small-scale (1 ml). The expression was performed in the BMMY containing 3% methanol at vary times of induction (1-4 days). The culture supernatant was collected every 24 h of induction. The secreted proteins were analysed for the xylanase activity by using AZCL-xylan. The result showed that the blue zone developed from all clones after a day and lasted at least 4 days of induction. Interestingly, the blue zone from the clone containing the mature xylanase gave the largest zone after 4 days of incubation while that from the clone containing the xylanase with its own leader sequence gave the largest zone after 1 day and 4 days of incubation. The reason for this is not clear at present. The activity shown by this AZCL-xylan assay of the recombinant xylanase indicated that the xylanase protein was successfully produced and secreted from *P. pastoris*.

Analysis of the secreted protein by SDS-PAGE showed that the protein of expected size (33 kDa) was observed from *P. pastoris* integrants containing the mature xylanase cDNA without its own leader sequence during the time of induction. This result was found to be in concert with the analysis of xylanase activity by AZCL-xylan, suggesting that the xylanase fusion protein was successfully synthesized in *P. pastoris*. The MFα-1 pre-signal sequence was removed by signal peptidase and then Kex2 endopeptidase cleaved after Lys-Arg of the MFα-1 pro-leader sequence allowing the mature xylanase to be secreted into the culture medium. Optimization such as vary concentration of methanol may give the higher yield of recombinant protein. In order to further confirm that the protein of 33 kDa is xylanase, zymogram analysis (109) and/or the N-terminal sequencing should also be performed.

For the *P. pastoris* integrants containing the full-length xylanase sequence, it gave the protein band of approximately 33-35 kDa as analyzed by SDS-PAGE. However, at this stage, it was not possible to determine if the xylanase leader sequence of the BCC7197 was completely cleaved form the xylanase, as the size of the band between the full-length and the native xylanase is not significantly different. In order to determine this, N-terminal sequencing of the protein need to be performed or the higher percentage of gel should be used to allow the more efficient protein separation.

CHAPTER VI
CONCLUSION

1. A modified method was developed in this study for xylanase induction (BCC7197 was culturing in the supernatant of 5% wheat bran with continuously shaking at 30°C, 250 rpm for 8 days).
2. The full-length of BCC7197 xylanase cDNA contained 978 bp open reading frame encoding 325 amino acid residues with a calculated mass of 35 kDa and a pI of 4.67.
3. The BCC7197 xylanase cDNA was classified into the glycosyl hydrolase family 10 based on the amino acid sequence similarity.
4. The predicted 3D structure of the BCC7197 xylanase cDNA depicted the $(\alpha/\beta)_8$ fold like other xylanases in the glycosyl hydrolase familly 10.
5. The active BCC7197 xylanase protein was successfully expressed in *P. pastoris* and secreted into the culture medium using α-factor signal sequence with or without its own leader sequence.

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