การแสดงออกและลักษณะสมบัติของรีคอมบิแนนต์เอนโดกลูคาเนสจาก *Trichoderma reesei* เพื่อการผลิตเซลลูโลสิกเอทานอล

นางสาว มณัลยา หมาดหล้า

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EXPRESSION AND CHARACTERIZATION OF RECOMBINANT ENDOGLUCANASE FROM *Trichoderma reesei* FOR THE PRODUCTION OF CELLULOSIC ETHANOL

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biochemistry

Department of Biochemistry

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Thesis Title	EXPRESSION AND CHARACTERIZATION OF RECOMBINANT ENDOGLUCANASE FROM *Trichoderma reesei* FOR THE PRODUCTION OF CELLULOSIC ETHANOL
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Field of Study	Program in Biochemistry
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ไบโอเอทานอลต่างจากเชื้อเพลิงปิโตรเลียม เนื่องจากเป็นรูปหนึ่งของพลังงานหมุนเวียนที่สามารถผลิตได้จากวัตถุดิบทางการเกษตร มักถูกใช้เป็นเชื้อเพลิงในการขนส่ง ส่วนใหญ่ใช้เป็นสารเติมแต่งเชื้อเพลิงชีวภาพสำหรับน้ำมันเชื้อเพลิง การผลิตเอทานอลจากลิกโนเซลลูโลสมีความได้เปรียบในด้านของวัตถุดิบที่มีปริมาณมากและหลากหลายเมื่อเทียบกับแหล่งอื่น เช่น ข้าวโพดและอ้อย อย่างไรก็ตามการผลิตเอทานอลจากวัตถุดิบเหล่านี้ต้องการเพิ่มขั้นตอนในการผลิต คือ แซคคาริฟิเคชัน โดยมวลชีวภาพของลิกโนเซลลูโลสจะถูกย่อยสลายเป็นกลูโคสก่อนที่จะถูกเปลี่ยนเป็นเอทานอลโดยวิธีการหมัก ปัจจุบันการย่อยสลายเซลลูโลสโดยใช้ตัวเร่งปฏิกิริยาทางชีวภาพเซลลูเลสได้รับความสนใจเพิ่มขึ้น อย่างไรก็ตามเซลลูเลสจากจุลินทรีย์ธรรมชาติไม่สามารถผลิตได้เพียงพอสำหรับใช้ในอุตสาหกรรม ดังนั้นงานวิจัยนี้จึงมีเป้าหมายในการโคลนและแสดงออกยีนเอนโดกลูคาเนส คือ ยีน *eg1* และ ยีน *eg2* จากเชื้อ *Trichoderma reesei* ซึ่งเป็นเชื้อที่เซลลูเลสมีประสิทธิภาพสูง จากผลการทดลองพบว่ายีน *eg1* ประกอบด้วย 713 คู่เบส แปลรหัสเป็นกรดอะมิโนได้ 234 ตัว และมีน้ำหนักโมเลกุลประมาณ 25 กิโลดาลตัน ในขณะที่ยีน *eg2* ประกอบด้วย 1257 คู่เบส แปลรหัสเป็นกรดอะมิโนได้ 418 ตัว และมีน้ำหนักโมเลกุลประมาณ 44 กิโลดาลตัน โดยพลาสมิดลูกผสม pPICαA-*eg1* และ pPICαA-*eg2* ถูกถ่ายเข้าสู่ *Pichia pastoris* โดยวิธีอิเล็กโทรพอเรชัน จากนั้นทำการคัดเลือกเซลล์ที่มีเวคเตอร์ลูกผสมที่แสดงออกเป็น เอนโดกลูคาเนส1 (EG1) และ เอนโดกลูคาเนส2 (EG2) เพื่อหาภาวะที่เหมาะสมในการแสดงออก พบว่า การแสดงออกของ EG1 และ EG2 สูงที่สุด เมื่อถูกชักนำด้วยเมทานอลปริมาณ 4 เปอร์เซ็นต์และ 5 เปอร์เซ็นต์ เป็นเวลา 5 วัน ตามลำดับ EG1 แสดงแอกทิวิตีสูงที่สุดที่ความเป็นกรดด่างเท่ากับ 5.0 ที่อุณหภูมิ 60 องศาเซลเซียสและมีความเสถียรในช่วงความเป็นกรดด่าง 4.0 ถึง 5.5 ที่อุณหภูมิต่ำกว่า 75 องศาเซลเซียส เอนโดกลูคาเนส2 แสดงแอกทิวิตีสูงที่สุดที่ความเป็นกรดด่างเท่ากับ 5.0 ที่อุณหภูมิ 70 องศาเซลเซียส และมีความเสถียรในช่วงความเป็นกรดด่าง 3.5 ถึง 5.5 ที่อุณหภูมิต่ำกว่า 70 องศาเซลเซียส เมื่อทำการศึกษาจลนศาสตร์ของปฏิกิริยาของเอนโดกลูคาเนสที่มีต่อ CMC พบว่า EG2 มีประสิทธิภาพในการเร่งปฏิกิริยาได้ดีกว่า EG1 ประมาณ 6 เท่า อย่างไรก็ตามเอนไซม์ทั้งสองชนิดสามารถย่อยสารตั้งต้นเซลลูโลสิกหลายชนิดรวมทั้งลิกโนเซลลูโลสด้วย ดังนั้นจากการศึกษานี้สามารถสรุปได้ว่าเอนโดกลูคาเนสที่ผลิตจาก *Pichia pastoris* สายพันธุ์ลูกผสมสามารถนำไปประยุกต์ใช้สำหรับขั้นตอนแซคคาริฟิเคชันในการผลิตไบโอเอทานอลได้

ภาควิชา ชีวเคมี

สาขาวิชา ชีวเคมี ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS: CLONING, ENDOGLUCANASE, *Trichoderma reesei*, BIOETHANOL

MANANYA MARTLA: EXPRESSION AND CHARACTERIZATION OF RECOMBINANT ENDOGLUCANASE FROM *Trichoderma reesei* FOR THE PRODUCTION OF CELLULOSIC ETHANOL. ADVISOR: PAKORN WINAYANUWUTTIKUN, Ph.D., 78 pp.

Bioethanol, unlike petroleum, is a form of renewable energy that can be produced from agricultural feedstocks. It can be used as a transport fuel, mainly as a biofuel additive for gasoline. The production of ethanol from lignocellulose has the advantage of abundant and diverse raw material compared to other sources like corn and cane sugars. However, it requires one more additional step; saccharification which the lignocellulosic biomass is hydrolyzed to glucose before converted to ethanol by fermentation. Recently, the hydrolysis of cellulose using biocatalysts, cellulase has been interestingly increased. Nevertheless, natural microbial cellulase cannot be sufficiently produced for the industrial application. Therefore, this research attempted to clone and express the genes of endoglucanase; *eg1* and *eg2* from *Trichoderma reesei*, one of the most effective producers of cellulase. From the results, the *eg1* gene was composed of 713 bps, encoding 234 amino acids, molecular weight approximately 25 kD while *eg2* gene was composed of 1257 bps, encoding 418 amino acids, molecular weight approximately 44 kD. The recombinant plasmid, pPICαA-*eg1* and pPICαA-*eg2*, were transformed into *Pichia pastoris* X33 by electroporation. The transformants expressing the endoglucanase1 (EG1) and endoglucanase2 (EG2) were selected for optimizing expression conditions. The EG1 and EG2 were highly expressed by 4% and 5% methanol induction for 5 days, respectively. EG1 showed the highest activity at pH 5.0 and 60°C and it was stable at pH range of 4.0–5.5 and temperature below 75°C. EG2 showed the highest activity at pH 5.0 and 70°C and it was stable at pH range of 3.5–5.5 and temperature below 70°C. The steady-state kinetic study indicated that the EG2 was six folds greater catalytic efficiency toward carboxymethyl cellulose substrate than EG1. However, both of them could hydrolyze varieties of cellulosic substrate including lignocellulosic materials. From this study, it could be concluded that endoglucanase produced from recombinant *Pichia pastoris* can be applied for saccharification process in bioethanol production.

Department : Biochemistry

Field of study : Biochemistry Student's Signature .....

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ABBREVIATIONS

bp	basepair	µl	microliter
°C	degree Celcius	µM	micromolar
et al.	et alia (latin)	mg	milligram
mM	millimolar	ml	milliliter
Fig.	figure	min	minute
g	gram	M	Molar
hr	hour	mol	mole
kb	kilobase	nm	nanometer
kD	kilodalton	psi	pound (force) per square inch
kg	kilogram	s	second
L	liter	v/v	volume by volume
m	meter	w/w, wt	weight by weight
µg	microgram		

CHAPTER I

INTRODUCTION

1.1 Statement of purpose

As a consequence of industrial development and population growth, there is an increase of energy consumption in the world. The worldwide energy consumption has increased 17-folds in the last century (Demirbas, 2007). However, conventional energy resources, like fossil fuels, cannot meet the increasing energy demand. The quantities of conventional non-renewable energy resources are limited and they have a considerable negative environment impact e.g. increased greenhouse gas emission. Therefore, the use of biofuels as alternative energy sources has many advantages, such as contribution to the reduction of CO_2 emission, lower dependency on the import of oil for non-oil producing countries, new employment opportunities and development of rural communities. They are easily available from common biomass sources, biodegradable and contribute to the sustainability (Baras *et. al.,* 2002). Bioethanol is one of the most promising biofuel from renewable resources. Fermentation derived ethanol can be produced from sugar, starch or lignocellulosic biomass. Sugar and starch based feedstocks are currently predominant at the industrial level and they are so far economically favorable (Nikolic *et. al.,* 2009). However, lignocellulosic biomass, like weeds such as grasses, or agricultural waste such as rice straw, seems to be suitable low-cost feedstocks for the production of fuel ethanol and other chemicals in the future since it is the most abundant organic material on earth. On the other hand, the application of cellulosic biomass as feedstock requires one additional step for bioethanol production. It must primarily be hydrolyzed to fermentable sugars using cellulolytic enzymes or acids. More attractive hydrolysis by the enzymes is environment-friendly, yielding high sugar for ethanol fermentation, mild condition, low energy requirement, high specific reaction, non toxic, simultaneous saccharification and fermentation and non corrosion making it amenable to industrial production. However, the

high cost of cellulases remains the most significant barrier to the economical production of bioethanol from cellulosic biomass (Fang *et.al.,* 2009)

Cellulase enzyme complex consists of 3 types of enzymes that acts synergistically in cellulose hydrolysis. Cellobiohydrolases (CBH) (EC 3.2.9.11) or exoglucanase cleave at the end of cellulose chains liberating cellobiose as a main product, endoglucanase (EG) (EC 3.2.1.4) randomly attacks cellulose chains and produces the new attack site of CBH, cello-oligasacharides. β-glucosidase (BGL) (EC 3.2.1.21) completes process through hydrolysis of cellobiose and other short oligosaccharides to glucose. Cellulase is an enzyme complex found in some fungi and bacteria. Filamentous fungi, typically *Trichoderma reesei*, are wood-degrading fungi, secrete one of the most successful cellulose systems (Johnvesly *et al.*, 2002). The *Trichoderma reesei* cellulase is mostly targetted for industrial purposes. Although *Trichoderma reesei* is widely used for this purpose due to its high production of secretion, disadvantages and limitations still remain from the low growth rate and difficult manipulation (Nikolic *et. al.,* 2009). Therefore, this research was interested in cloning the endoglucanase from *Trichoderma reesei* for the industrial applications of bioethanol production.

1.2 Objectives of this research

The aim of the study was to clone, express and characterize endoglucanase from *Trichoderma reesei* in *Pichia pastoris* for the production of cellulosic ethanol.

1.3 Scopes of the investigation

1.3.1 To clone endoglucanase gene from *Trichoderma reesei*

1.3.2 To express endoglucanase in *Pichia pastoris*

1.3.3 To characterize recombinant endoglucanase

1.4 Expected result

Endoglucanase can be produced from recombinant *Pichia pastoris* in order to apply for saccharification process in bioethanol production.

1.5 Thesis organization

This thesis consists of five chapters as follows: Chapter 1 is the introduction. Chapter 2 gives the theoretical and literature reviews. Chapter 3 comprises materials and methods. The results can be found in Chapter 4 and the final chapter contains the discussion and conclusion.

CHAPTER II
THEORETICAL BACKGROUND AND LITERATURE REVIEWS

The background information about bioethanol, cellulase, endoglucanase gene cloning and properties of recombinant endoglucanase are reviewed in this chapter.

2.1 Bioethanol

Alternatives to petroleum-derived fuels have been searched for in order to reduce the world's dependence on non-renewable resources. The most common renewable fuel today is bioethanol, an alternative fuel to both petrol and diesel. Usually, it can be used as a gasoline additive to increase octane and improve vehicle emissions. Bioethanol is a renewable energy source which can be produced through fermentation of sugars. The raw materials of bioethanol production are primarily from sugarcane, corn and sugar beets. It is a sustainable energy resource that may offer environmental and long-term economic advantages over fossil fuels.

Bioethanol use closes the carbon balance between the production of glucose by photosynthesis during the biomass growth and its combustion in a mechanical engine (Herna´ndez and Kafarov, 2009).

As follows, glucose is fermented to carbon dioxide and ethanol for use as fuel.

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

During combustion, ethanol reacts with oxygen to produce energy, water and carbon dioxide.

$$2C_2H_5OH + 6O_2 \longrightarrow 4CO_2 + 6H_2O + \text{heat}$$

Carbondioxide is utilized to produce glucose by combining with water in the presence of light in photosynthesis.

$$6CO_2 + 6H_2O \longrightarrow C_6H_{12}O_6 + 6O_2$$

The production of fuels from food crops may place upward pressure on the price and availability of food. The likelihood of such a ‘‘food versus fuel” conflict may increase

as the world's population grows. As an alternative, lignocellulosic biomass shows promise as a feedstock for bioethanol production because of its abundance and low cost. It is seen as an attractive feedstock for future supplies of ethanol. However, there are technical and economical impediments to the development of a commercial processes utilizing biomass. Technologies are being developed that will allow cost-effective conversion of biomass into fuels and other chemicals. These technologies include low-cost thermochemical pretreatment, highly effective conversion of cellulose to glucose and efficient, robust fermentative microorganisms. Many advances have been made over the past few years that making commercialization more promising (Gray *et. al.,* 2006).

2.2 Lignocellulose

A potential source for low-cost ethanol production is to utilize lignocellulosic materials such as crop residues, grasses, sawdust, wood chips, and solid animal waste. Lignocelluloses are often a major or sometimes the sole components of different waste streams from various industries, forestry, agriculture and municipalities. They are mainly composed of cellulose fibrils which are attached together by hemicelluloses, amorphous polymers of different sugars as well as other polymers such as pectin, and covered by lignin.

Cellulose or β-1-4-glucan is a linear polysaccharide polymer of glucose made of cellobiose units. The crystalline and strong cellulose chains are packed by hydrogen bonds. The microfibrils are often associated in the form of bundles or macrofibrils. This special and complicated structure makes cellulose resistant to both biological and chemical treatments (Figure 1).

Hemicellulose, in contrast to cellulose, is heteropolymers that contain mannose, xylose, galactose, arabinose, and small amounts of rhamnose, glucuronic acid, methyl glucuronic acid, and galacturonic acid. They have a random, amorphous, and branched structure with little resistance to hydrolysis but are more easily hydrolyzed by acids to their monomeric components.

Lignin is a very complex molecule constructed of phenylpropane units linked in a three dimensional structure. Lignin is the most recalcitrant component of the plant cell

wall, making it resistant to chemical and enzymatic degradation. There are chemical bonds between lignin and hemicellulose and even cellulose. Lignin is one of the drawbacks of using lignocellulosic materials in fermentation. Therefore, the removal of lignin including hemicelluloses can reduce the cellulose crystallinity and increase porosity to improve hydrolysis efficiency (Taherzadeh and Karimi, 2008).

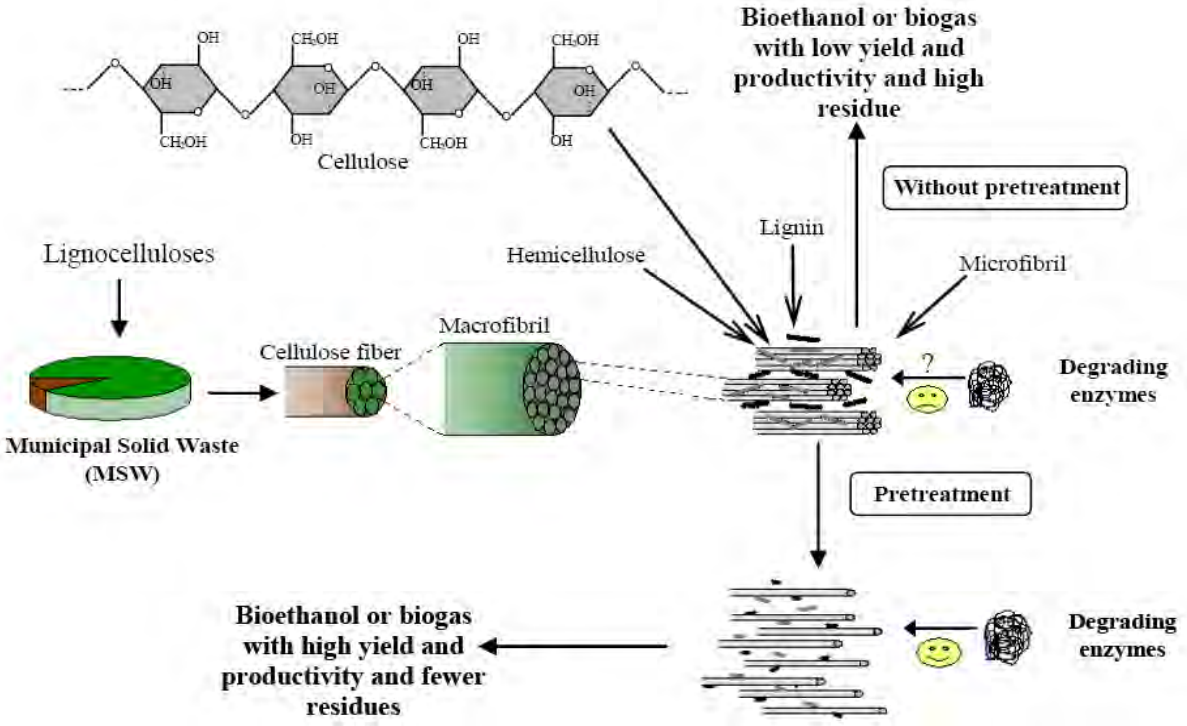


Figure 2-1 The effect of pretreatment on accessibility of degrading enzymes

2.3 Process of production

The completed conversion of lignocellulosic materials to ethanol includes three mainly processes; 1) pretreatment to remove lignin and hemicelluloses from lignocellulosic materials, 2) hydrolysis of cellulose to fermentable reducing sugars and 3) fermentation of the sugars to ethanol (Sun and Cheng, 2002).

2.3.1 Cellulose pretreatment

The properties of native lignocellulosic materials make them resistant to chemical or enzymatic attack. The purpose of pretreatment is to change these

properties in order to prepare the materials for degradation. The best methodology for pretreatment lignocellulosic materials depends greatly on the type of lignocelluloses, the crystallinity of cellulose, degree of cellulose polymerization, and degree of acetylation of hemicelluloses. Several methods have been introduced prior to cellulose hydrolysis. These methods have been classified into 4 types.

2.3.1.1 Physical pretreatment

Physical pretreatment can increase surface area and size of pores, and decrease the crystallinity and degrees of polymerization of cellulose. Different types of physical processes such as grinding, chipping, milling by ball, two-roll, hammer, colloid, or vibro energy and irradiation by gamma rays, electron beam or microwaves, can be used to improve the enzymatic hydrolysis or biodegradability of lignocellulosic waste materials. This method is simple and easy. However, it is not effective and requires high energy.

2.3.1.2 Chemical pretreatment

Chemical pretreatment uses varieties of chemicals such as acid, alkaline, organic solvent for degradation of lignin and hemicellulose. This method is quite effective. However, some of chemicals are expensive, toxic or can produce corrosive waste water.

2.3.1.3 Physicochemical pretreatment

Physicochemical processes are combination of chemical and physical processes, such as steam explosion, ammonia fiber explosion (AFEX), CO_2 explosion,and liquid hot-water pretreatment. This method is more effective than physical and chemical pretreatment alone. However, the disadvantages are similar to physical and chemical method.

2.3.1.4 Biological pretreatment

Microorganisms can also be used to treat the lignocelluloses by enzymatic hydrolysis. The applied microorganisms usually degrade lignin and hemicellulose. Several fungi, e.g. brown-, white- and soft-rot fungi, have been used for this purpose. White-rot fungi are among the most effective microorganisms for biological pretreatment of lignocelluloses. This method requires low energy, mild environmental condition, no chemical and more specific than the others. The examples of lignocellulose pretreatment were shown in Table2-1.

Table 2-1 Pretreatment processes of lignocellulosic materials (Taherzadeh and Karimi, 2008)

Pretreatment method	Processes	Studied application	Possible changes in biomass	Notable remarks
Physical pretreatments	**Milling:** - Ball milling - Two-roll milling - Hammer milling - Colloid milling - Vibro energy milling	Ethanol	- Increase in accessible surface area and pore size - Decrease in cellulose crystallinity - Decrease in degrees of polymerization	- Most of the methods are highly energy demanding - Most of them cannot remove the lignin - It is preferable not to use these methods for industrial applications
	Irradiation: - Gamma-ray irradiation - Electron-beam irradiation - Microwave Irradiation	Ethanol and biogas		

Pretreatment method	Processes	Studied application	Possible changes in biomass	Notable remarks
	Others: - Hydrothermal - High pressure steaming - Expansion - Extrusion - Pyrolysis	Ethanol and Biogas		- No chemicals are generally required for these methods
Chemical and physicochemical pretreatments	**Explosion:** - Steam explosion - Ammonia fiber explosion (AFEX) - CO_2 explosion - SO_2	Ethanol and biogas	- Increase in accessible surface area - Partial or nearly complete delignification - Decrease in cellulose crystallinity - Decrease in degrees of polymerization - Partial or complete hydrolysis of hemicelluloses	- These methods are among the most effective and include the most promising processes for industrial applications - Usually rapid treatment rate - Typically need harsh conditions - There are chemical Requirements
	Alkali: - Sodium hydroxide - Ammonia - Ammonium Sulfite	Ethanol and biogas		
	Acid: - Sulfuric acid - Hydrochloric acid - Phosphoric acid	Ethanol and biogas		

Pretreatment method	Processes	Studied application	Possible changes in biomass	Notable remarks
	Solvent extraction of lignin: - Ethanol-water extraction - Benzene-water extraction - Ethylene glycol extraction - Butanol-water extraction - Swelling agents	Ethanol and biogas		
Biological pretreatments	Fungi and actinomycetes		- Delignification - Reduction in degree of polymerization of cellulose - Partial hydrolysis of hemicellulose	- Low energy requirement - No chemical requirement - Mild environmental conditions - Very low treatment rate - Did not consider for commercial application

2.3.2 Hydrolysis of cellulose

The cellulose polymers in lignocellulosic materials need to be converted to simple sugars before fermentation through a process called hydrolysis. Various methods for the hydrolysis have been classified into two groups.

2.3.2.1 Chemical hydrolysis

Chemical hydrolysis involves exposure of lignocellulosic materials to a chemical agent for a period of time at a specific temperature. The chemical pretreatment and the hydrolysis process may be carried out in a single step. Acids are predominantly applied in chemical hydrolysis. Both dilute acid and concentrated acid such as hydrochloric acid, hydrogen fluoride, sulphuric acid have been used in the process. Sugar degradation and consequently low process yields limit the economic feasibility of dilute acid hydrolysis. In contrast, concentrated acid hydrolysis processes affect little sugar degradation and give yields approaching 100%. However, acid hydrolysis has a number of disadvantages that severely limit their commercial application such as the corrosion problems, the high cost of acid consumption and recovery. Particularly with the present requirements for increasingly stringent environmental controls, the waste water treatment problems posed by byproduct formation and strongly acidic off-streams conspire to limit the likelihood of future implementation of acid hydrolysis technologies.

2.3.2.2 Enzymatic hydrolysis

Enzymatic hydrolysis of natural lignocellulosic materials is a very slow process because cellulose hydrolysis is hindered by structural parameters of the substrate, such as lignin and hemicellulose content, surface area, and cellulose crystallinity. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (both pH and temperature), and does not have a corrosion problem.

The enzymatic hydrolysis has currently high yields (75–85%) and improvements are still projected (85– 95%), as the research field is only a decade young. Moreover, enzymatic hydrolysis is an environmental friendly alternative that involves using carbohydrate degrading enzymes to hydrolyze lignocelluloses into fermentable sugars. Comparison of process conditions and performance of three methods in cellulose hydrolysis is given in Table2-2

Table2-2 Comparison of process conditions and performance of three hydrolysis processes (Balat, 2011)

	Consumables	Temperature (^{0}C)	Time	Glucose yield
Dilute acid	<1% acid	215	3 min	50–70 %
Concentrated acid	30–70% acid	40	2–6 h	90%
Enzymatic	Cellulase	50	1.5 days	75-95%

2.3.3 Fermentation

The supernatant from enzymatic hydrolysis of lignocelluloses contain both hexoses and pentoses sugars, depending on the lignocellulose source. The hydrolysate typically consists of glucose, xylose, arabinose, galactose, mannose, fucose, and rhamnose. Microorganisms such as yeasts and bacteria have been used to ferment lignocellulose-derived sugar to bioethanol. Microorganisms for bioethanol fermentation can best be described in terms of their performance parameters and other requirements such as compatibility with existing products, processes and equipment. The performance parameters of fermentation are temperature, pH, alcohol tolerance, growth rate, productivity, osmotic tolerance, specificity, yield, genetic stability, and inhibitor tolerance. The most frequently used microorganism for fermenting bioethanol in industrial processes is *Saccharomyces cerevisiae*. It has proved to be very robust and well suited to the fermentation of

lignocellulosic hydrolysates. Fermentation can be performed as a batch, fed-batch or continuous process.

2.3.3.1 Batch process

Batch culture can be considered as a closed culture system which contains an initial, limited amount of nutrient, which is inoculated with microorganisms to allow the fermentation. It is very simple method since during the fermentation, nothing is required after inoculation except possibly acid or alkali for pH control or air for aerobic fermentation.

2.3.3.2 Continuous process

In the continuous process, feed, which contains substrate, culture medium and other required nutrients, is pumped continuously into an agitated vessel where the microorganisms are active. The product, which is taken from the top of the bioreactor, contains bioethanol, cells, and residual sugar (Balat, 2011, Sun and Cheng, 2002).

2.3.3.3 Fed-batch process

Fed-batch reactors are widely used in industrial applications because they combine the advantages from both batch and continuous processes. The major advantage of fed-batch, comparing to batch, is the ability to increase maximum viable cell concentration, prolong culture lifetime, and allow product accumulation to a higher concentration. This process allows for the maintenance of critical process variables (e.g. temperature, pH, and dissolved oxygen) at specific levels through feedback control.

2.4 Enzymatic hydrolysis

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific. The products of the hydrolysis are usually reducing sugars including glucose.

Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endocellullase or endoglucanase (EG, endo-1,4-D-glucanohydrolase; EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91.) which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose to produce glucose. The enzymatic mechanism of cellulose hydrolysis was shown in figure 2. In addition, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase. During the enzymatic hydrolysis, cellulose is degraded to reducing sugars that can be directly further fermented by yeasts or bacteria to ethanol (Sun and Cheng, 2002).

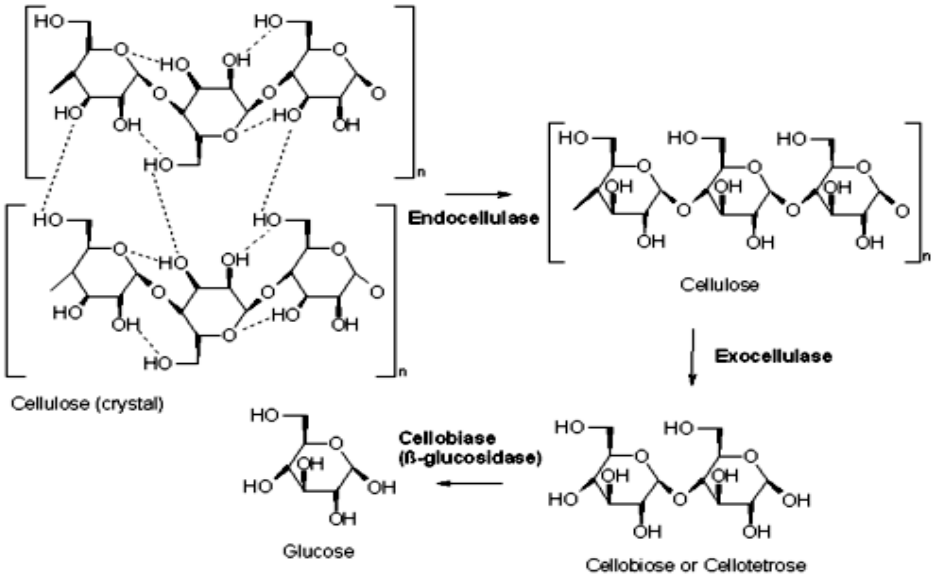


Figure 2-2 The enzymatic hydrolysis mechanism of cellulose

2.5 *Trichoderma reesei*

T. reesei is filamentous fungus that has been widely used in the fermentation industry. It becomes the principal source of cellulase enzymes and other metabolites.

2.5.1 Morphology

Trichoderma species are fungi that are present in nearly all soils and other diverse habitats. In soil, they frequently are the most prevalent culturable fungi. They are favored by the presence of high levels of plant roots. These fungi appear in the form of colonies of mold which start out transparent, turning white or yellowish over time. When the mold matures and begins producing spores, it darkens and turns green to gray. Many *Trichoderma* species have a distinctive sweet scent which is often compared to coconuts. *Trichoderma* molds tend to prefer moderate temperature which allows them to thrive in a variety of climates.

T. reesei (also known as *Hypocrea jecorina*) is a mesophilic and filamentous fungus which is one of the most efficient xylanase and cellulase producers. Industrial strains of *T. reesei* can achieve protein production level of up to 100 g/l (Cherry & Fidantsef, 2003). The efficient secretory ability together with the cheap cultivation of *T. reesei* make it be a useful organism for a variety of industrial applications and the large-scale production of enzymes. Moreover, *T. reesei* can also be used for this purpose (Hui et al., 2001).

2.5.2 Cellulase in *T. reesei*

T. reesei produces one of the most effective cellulase system for hydrolysis of cellulosic materials. This cellulase system consists of at least six genetically different cellulases: two cellobiohydrolases (CBH I and II; E.C.3.2.1.91) and four endoglucanases (EG I, II, III and V; E.C.3.2.1.4). All *Trichoderma* cellulases, except EG III, have a characteristic two domain structures with a catalytic core and a cellulose binding domain, CBD. The two domains are connected with a flexible and glycosylated hinge region. The enzymes appear in multiple isoforms in culture

filtrates of *T. reesei* and act in synergism with each other during hydrolysis of cellulosic substrates. The adsorption of the cellulases on the solid substrate is an important initial step in the hydrolysis (Medve, Lee, and Tjerneld, 1997).

2.5.3 Application

T. reesei are widely employed in the production of proteins at the industrial scale. The laundry and the pulp and paper industries use cellulases secreted by this microorganism for the hydrolysis of cellulosic substrates. For example, cellulases from these fungi are used in "biostoning" of denim fabrics to give rise to the soft, whitened fabric--stone-washed denim. The enzymes are also used in poultry feed to increase the digestibility of hemicelluloses from barley or other crops. Another application of these enzymes is the transformation of agricultural wastes into fermentable sugars for the production of bioethanol. However, the scale-up leads to many engineering challenges, and submerged cultures are still predominantly used in industrial processes partly because of the pressure of increasing industrial rationalization and standardization. Fungal fermentations are complex systems into which the operating conditions, the broth rheology, the enzyme production, the morphology of the microorganisms, and their physiological state are all interrelated. It is necessary to understand the relationship between these parameters in order to improve the enzyme production (Lecault, Patel, and Thibault, 2007).

2.6 DNA technology

The utilization of gene technology has made industrial enzymes with improved properties or better cost performance available. The benefits to the customers are considerable in cost savings of the application process, improved product quality and a significantly reduced negative impact on the environment. This technology enables the use of safe, well documented host organisms easy to cultivate, the microbial production of enzymes of animal and plant origin, the realization of enhanced efficiency and high

product purity, and also the production of enzymes with improved stability and activity (Falch, 1991). *T. reesei*, wood-degrading fungi, secretes one of the most active cellulase making it a target for industrial purposes (Johnvesly *et al*., 2002). Although *Trichoderma reesei* itself is widely used for this purpose due to its high secretion, it has some disadvantages and limitations as in low growth rate and difficult manipulation (Nikolic *et. al.,* 2009). Therefore, this research was interested in cloning of endoglucanase from *Trichoderma reesei* for industrial application in bioethanol production.

CHAPTER III
MATERIALS AND METHODS

3.1 Equipments

Agarose gel electrophoresis	(Biorad, USA)
Autoclave	(Ta Chang Medicalinstrument, Taiwan)
Balance	(Sartorius, Germany)
Blender	(Moulinex, france)
Centrifugal Filter Devices	(Millipore, USA)
Digital Balance	(Mettler Teledo, USA)
Digital Dry Bath	(Labnet international, Inc., USA)
Gel Documentation	(UVP, UK)
Incubator	(Gallenkamp, UK)
Incubator Shaker	(New Brunswick Scientific Co., Inc., USA)
Laminar flow	(Thermo electron corporation, USA)
Magnetic stirrer	(KIKA Labotechnik, Malaysia)
Magnetic bar	(Lio Lab Limted Partnership)
Microcentrifuge	(Satorious, Germany)
Microplate spectrophotometer	(ASYS Hitech GMBH, Australia)
Micropulser	(Bio-Rad, USA)
Microrefrigerated centrifuge	(Hettich, USA)
Microwave	(Sharp, Thailand)
Milling machine	(Ngawhaudyu, Thailand)
pH meter	(Metler Toledo, USA)
Refrigerated incubator shaker	(New Brunswick Scientific Co., Inc., USA)
Slab gel electrophoresis equipment	(BioRad, USA)
Test sieve	(Ngawhaudyu, Thailand)
Thermal cycler	(BioRad, USA)
ThermoE	(Bioer technology Co., Ltd, China)
UV-VIS spectrophotometer	(Thermo scientific, UK)
Vortex	(Scientific industries, USA)
Water bath	(T.S. Instrument, Thailand)

3.2 Chemicals and reagent

All chemicals and reagents used throughout this study were analytical grade, and/or molecular biological grade and were purchased from various manufacturers (Sigma (USA), Merk (Germany), Fluka (Switzerland), Carlo Erba (Italy), Ajax Finecchem (Australia), Difco (USA), Usb (USA), Biorad (USA) and Invitrogen (Canada)

3.3 Microorganism strains

Trichoderma reesei was used as the source of chromosomal DNA *Escherichia coli strain* DH5α (F-$\varnothing$80*lacZ*ΔM15Δ(*lacZYA*-argF)U169 *deoR recA*1 *endA1hsdR*17(*rk*,*mk*+)*phoA supE*44 *thi*-1 *gyrA*96*relA*1λ) was used as host for plasmid propagation.

Pichia pastoris strain X33 was used as host for expression of the gene constructed in pPICZαA vector.

3.4 Enzymes

*Eco*R I	New England Biolabs (USA)
Not I	New England Biolabs (USA)
RBC *Taq* DNA polymerase	RBC Bioscience (Italy)
T4 DNA ligase	New England Biolabs (USA)
Sac I	New England Biolabs (USA)
SuperScript III reverse transcriptase	Invitrogen (USA)

3.5 Commercial plasmids

pPICZαA vector (Figure 3-1)	Invitrogen (USA)

3.6 Commercial kits

RNA easy kit	Stratagene (USA)
Nucleospin extraction kit	Macherey-Nagel (Germany)

3.7 Synthetic oligonucleotides

All synthetic oligonucleotides used as primers, were purchased from Bio Basic Inc. (Canada). The primers information was shown in Table 3-1.

Table 3-1 List of primers

Primers	Sequences	T_m (°C)
EG1_F	5'-GG▼AATTCATGAAGTTCCTT CAAGTCCTCCC-3'(*Eco*RI)	61.6
EG1_R	5'-ATAAGAATGC▼GGCCGCGTTGATAGATGCGGTCCAGG-3'(*Not*I)	69
EG2_F	5'-CG▼AATTCATGAACAAGTCCGTGGCTCC-3'(*Eco*RI)	61.3
EG2_R	5'-ATCATATTGC▼GGCCGCCTTTCTTGCGAGACACGAGC-3'(*Not*I)	69

*The restriction sites were shown as the underlines

3.8 Miscellaneous

TriDye ™ 1 kb DNA Ladder	New England Biolabs (USA)
Prestained Protein Ladder, Broad Range	Fermentas (Canada)
6X DNA loading dye	Fermentas (Canada)

3.9 Data analysis

Sequences analysis program	(BioEdit, Chromas Lite and Blast program)
Statistical analysis program	(Graph Pad InStat3)
Graph analysis program	(Graph Pad Prism4)

3.10 Research methodology

3.10.1 Cloning of *eg1* and *eg2* genes from *Trichoderma reesei*

3.10.1.1 Preparation of endoglucanase genes

Trichoderma reesei was cultured in Mandel medium at 30 °C, 250 rpm for 7 days. The medium was initially checked for all 3 types of cellulase activities; CBH, EG and BGL were measured by using filter paper, CMC and salicin as substrates (Ghose, 1987). Total cellular RNA was isolated from cells using

RNAeasy kit. For cDNA synthesis, 1 µl of 10 pmol oligodT primer was added to 2 µg RNA in 20 µl reaction medium and incubated at 70 ^{o}C for 10 minutes. Then 200 units of SuperScript III reverse transcriptase was added to the mixture, followed by incubation first at 42 ^{o}C for 50 min and then for an additional at 70 ^{o}C 15 min. The obtained cDNA was amplified with two sets of primers, designed on basis of the *eg1* and *eg2* gene from *Trichoderma reesei*. The 50 µl of PCR mixture contained 1 µl of each primer, 1 µl dNTP, 2 µl cDNA as template, 10X reaction buffer and 0.5 µl RBC Taq DNA polymerase. The PCR was performed with a cycle at 94 °C for 3 min; 32 cycles at 94 °C, 3 min; 45-60 °C, 1 min, 72 °C, 1 min and a cycle at 72 °C for 7 min. Then, the PCR product was analyzed by 1% agarose gel electrophoresis in 1X TAE buffer. The PCR product mixed with 6X DNA loading dye was loaded onto agarose gel and run at constant voltage of 100 volts for 1 hr. The gel was stained with 10 µg/ml ethidium bromide (EtBr) solution for 5 min and destained in water for 15-20 min. The stained gel was visualized under UV light and photographed. The PCR products were extracted and purified by 1% agarose gel with Nucleospin extraction kit.

3.10.1.2 Preparation of pPICZαA

A single colony of *E.coli* strain DH5α carrying pPICZαA was inoculated to LB low salt medium containing 25 µg/ml zeocin and incubated at 250 rpm, 37 °C overnight. The cells were collected by centrifugation at 4 °C, 4,000 rpm for 10 min. The pPICZαA was extracted using CTAB method. The obtained pellets were resuspended in 400 µl of STET buffer (Appendix A) before transferring to 1.5 ml microcentrifuge tube. Next, 10 µl of 50 mg/ml lysozyme was added and then incubated at 37 °C for 10 min. After incubation, these mixtures were boiled for 45 sec, followed by centrifugation at 12,000 rpm for 15 min. and the pellets were removed by sterile toothpick, respectively. Subsequently, 5% CTAB was added and centrifuged at 13,000 rpm for 15 min. The pellets were resuspended in 300 µl of 1.2 M NaCl and mixed well. 3 µl of 10 mg/ml RNAse A was then

added and incubated at 37 °C for 3 hr. Before centrifugation at 12,000 rpm for 5 min, the mixture was added with 300 µl of chloroform-isoamyl alcohol mixture (24:1 v/v). The obtained solution was separated into two phases. The upper phase was then transferred to the new tubes before precipitating with two volumes of cold absolute ethanol and kept frozen at -20 °C for 30 min. After freezing, this mixture was later centrifuged at 13,000 rpm for 15 min. The pellet was twice washed with 100 µl of 70% ethanol followed by drying at 55 °C for 10 min. and resuspended in 20-30 µl of distilled water. Finally, the plasmid was analyzed by agarose gel electrophoresis, visualized under UV light and photographed.

3.10.1.3 Double digestion of endoglucanase gene and pPICZαA plasmid

The *eg1* and *eg2* genes and pPICZαA were double digested with 8 units of *Not* I and *Eco*R I in 30 µl of appropriate reaction buffer and incubated at 37 °C for 3 hr. Digested pPICZαA, *eg1* and *eg2* genes were analyzed by agarose gel electrophoresis and visualized under UV light and photographed.

3.10.1.4 Ligation of digested endoglucanase genes into plasmid

The digested *eg1* and *eg2* gene were ligated into digested pPICZαA with 3:1 molar ratio of insert: plasmid. A 10X T4 DNA ligase buffer and steriled distilled water was added to make final concentration of 1X before addition of 3 units of T4 DNA ligase and incubated at 16 °C overnight.

3.10.1.5 Preparation of competent cells by Simple and efficient method (SEM)

A single colony of *E.coli* strain DH5α was inoculated to 25 ml of SOB media and then incubated at 18 °C, 250 rpm overnight. Twenty five ml of incubated medium were further inoculated to new 250 ml SOB media and incubated at 18 °C, 250 rpm until the absorbance at 600 nm was approximately

0.6. The *E.coli* culture was chilled on ice for 10 min and centrifuged at 4 °C, 2,500 rpm for 10 min. The cell pellets were resuspended in 80 ml of ice-cold TB buffer and further incubated on ice for 10 min and centrifuged at 2,500 at 4 °C for 10 min. The cell pellets were collected and resuspended in 20 ml of ice-cold TB buffer. Then, dimethyl sulfoxide (DMSO) was slowly added and gently mixed to give the final concentration of 7% and the mixture was incubated on ice for 10 min. The competent cells were divided into 200 µl each in microcentrifuge tubes, frozen immediately with liquid nitrogen and stored in -80 °C until use.

3.10.1.6 Transformation into *E.coli* DH5α competent cells

The ligation products of pPICZαA-eg1 and pPICZαA-eg2 were transformed into *E.coli* DH5α by heat shock method. The 10 µl of reaction was mixed with 200 µl of *E.coli* DH5α competent cells. The mixture was incubated on ice for 30 min and immediately heat shocked at 42 °C for 90 sec. The reaction was quickly chilled on ice for 5 min. Then, 1 ml LB broth was added and incubated at 37 °C, 250 rpm for 1 hr. The transformed cells were spread on LB agar plate containing 25 µg/ml zeocin. The plates were further incubated at 37 °C overnight.

3.10.1.7 Screening for recombinant clones

Both recombinant clones of pPICZαA-eg1 and pPICZαA-eg2 were primarily checked by rapid size screening method. A single colony of each recombinant clone was picked and lysed in 30 µl of prewarmed lysis buffer (Appendix A). The reaction was incubated at 37 °C for 5 min, followed by chilling on ice for 5 min and centrifuged at 13,000 rpm for 5 min. The aqueous phase was loaded on agarose gel electrophoresis. The clones, containing plasmid DNA with larger size than control plasmid, were selected for plasmid extraction. Finally, recombinant clones were verified by restriction analysis with *Not* I and *Eco*R I.

3.10.1.8 DNA sequencing and sequence analysis

The plasmid of expected recombinant clones which contained *eg1* and *eg2* fragment were verified by nucleotide sequencing using automate DNA sequencer (1st BASE, Malaysia). Nucleotide sequences were checked by using Chromas Lite and BioEdit program and compared to the data from the BLAST (http://blast.ncbi.nlm.nih.gov).

3.10.2 Expression of the EG1 and EG2 in *Pichia pastoris*

3.10.2.1 Preparation of competent cell *P. pastoris*

Single colony of *P. pastoris* strain X33 was grown in 10 mL of yeast peptone dextrose (YPD) overnight at 30 °C, 250 rpm. Then, the cells were collected by centrifugation at 2,000 rpm, 4 °C for 5 min. Cell pellet was pretreated by incubating in YPD HEPES (200 mM, pH 8.0) containing 20 mM dithiothreitol and 100 mM lithium acetate at 30 °C for 15 min. The 150 ml of cold water was then added and centrifuged at 2,000 rpm, 4 °C for 10 min. The obtained pellets were washed by 100 ml of cold water and then centrifuged at 2,000 rpm, 4 °C for 10 min. The cell pellets were resuspended twice, firstly in 8 ml of cold 1M sorbitol and later in 0.4 ml cold 1M sorbitol. The competent cells were divided into 80 µl each and keep on ice until use.

3.10.2.2 Transformation of *Pichia pastoris* and selection of transformants

The plasmids containing *eg* gene were linearized with *Sac* I and transformed into competent *P. pastoris* strain X33 by electroporation using a Micropulser. The competent cell was mixed with 10 µg of linearized plasmid and 4 µl of DNA carriers and then chilled on ice for 1 min. The mixture was then transferred to cold electroporation cuvette. After pulsation, 1 ml of 1M ice-cold sorbitol was immediately added into the cuvette. Then, the mixture was transferred to 15 ml tube and incubated at 30 °C for 1 hr followed by the addition of 1 ml YPD and shaken at 30 °C, 250 rpm for 3 hr. The colonies with the

recombinant plasmids were preliminary selected on YPD plate with 100-200 µg/ml zeocin. Colonies appeared after 2–3 days of incubation at 30 °C. Subsequently, recombinant *P. pastoris* X33 were grown in YPD medium by shaking at 250 rpm at 30 °C until the culture reached the absorbance value of 5–6 at 600 nm. They were later transferred to buffered glycerol-complex medium, BMGY and incubated at 250 rpm at 30 °C until the culture reached the absorbance value of 5–6 at 600 nm. The cells were then harvested by centrifugation at 6,000 rpm for 10 min and the cell pellet was resuspended in buffered methanol-complex medium, BMMY. The culture was further cultivated at 30 °C, 250 rpm for 5 days. Methanol was added to a final concentration of 3% every 24 hr. The culture was collected and the expression level was determined by measuring endoglucanase activity and SDS-PAGE.

3.10.2.3 Optimized expression of endoglucanase in *Pichia pastoris*

In order to achieve high expression level of endoglucanase, different culture parameters including the optimal inducing time (1, 2, 3, 4, 5, 6, 7 days) and methanol daily addition to final concentration (1%, 2%, 3%, 4%, 5% (v/v)) were varied and evaluated for the endoglucanase expression level as described above.

3.10.3 Characterization of recombinant EG1and EG2

3.10.3.1 Endoglucanase activity assay

Enzymatic assays were performed in triplicate in the reaction mixture containing an appropriate volume of enzyme with 2% CMC (w/v) in 50 mM sodium citrate buffer, pH 4.8. Reactions were performed at 50 °C for 30 min and then terminated by adding dinitrosalicylic acid (DNS). The mixture was boiled in a boiling bath for 5 min and finally measured for absorbance at 540 nm. One unit of enzyme is defined as the amount of enzyme that catalyzes the formation of

1 μmol glucose/min under the assay conditions. The enzyme activity was calculated as shown in Appendix.

3.10.3.2 Protein determination

The protein concentration was determined by Bradford's method. The mixture consisting of 5 μl of sample and 300 μl of Bradford reagent was incubated at room temperature for 5 min, and later measured for the absorbance at 595 nm. Standard curve was prepared to determine concentration of protein using bovine serum albumin (BSA) at the concentration of 0.1-0.6 mg/ml. The calculation method was shown in Appendix E.

3.10.3.3 SDS-PAGE

The sample was prepared by mixing with 5X sample buffer to final concentration of 1X sample buffer. The reaction was heated at 95 °C for 5 min and centrifuged at 13,000 rpm for 5 min. The protein sample was analyzed by 12% SDS-PAGE.

3.10.3.4 Optimization condition of endoglucanase

The effect of pH and temperature on the endoglucanase activity was spectrophotometrically determined using carboxymethyl cellulose (CMC) as the substrate. The optimum pH for enzyme activity was determined at 50 °C from pH 3.5 to 6.0 in sodium citrate buffer. The optimum temperature for endoglucanase activity was determined by measuring the rate of reaction at temperatures ranging from 30 to 80 °C at optimum pH.

3.10.3.5 Stability of endoglucanase

The effect of pH on endoglucanase stability was determined by incubating the 0.5 mg/ml endoglucanase in the buffers at different pH values (pH 3.0-6.0) for 4 hr at optimum temperature and residual activity was spectrophotometrically

assayed at the optimum condition towards each endoglucanase. To determine the influence of temperature on the enzyme stability, the 0.5 mg/ml endoglucanase was preincubated for 2 hr at temperature range of 60–90 °C in 50mM sodium citrate buffer at optimum pH. Subsequently, the residual activity was analyzed at optimum condition towards each endoglucanase.

3.10.3.6 Substrate specificity

The 4 different cellulosic substrates; carboxymethyl cellulose (CMC), Avicel PH101, Sigmacell 101 and α-cellulose were spectrophotometrically analysed for specificity. Cellulase activity was performed with a final concentration of 2% cellulose (w/v) of each substrate in 50 mM sodium citrate buffer. The mixtures were incubated as triplicates on a thermomixer at 50°C, 500 rpm. Depending on the substrate, the incubation time was 10 min for CMC, 120 min for Avicel PH101, 30 min for Sigmacell 101 and 60 min for α-cellulose. After centrifugation at 9,000 rpm for 30 min, 100 µl of the supernatant was used to determine the reducing sugar by the DNS method. Dinitrosalicylic acid (DNS) was added and the mixture was boiled in a boiling bath for 10 min and finally the absorbance was measured at 540 nm.

3.10.3.7 Kinetic parameters

The kinetic parameters of endoglucanase were determined in 50mM citrate buffer at optimum conditions, and the initial reaction rates were assayed with 10 min incubation for EG1 and 5 min incubation for EG2. The concentrations of CMC ranged from 0.25 % to 5% (w/v). The amount of reducing sugar was measured using the dinitrosalicylic acid reagent method. Apparent kinetic constants, V_{max} and K_m were determined by fitting the collected data to a Michaelis-Menten equation by non linear regression analysis using GraphPad Prism 5 (GraphPad soflware).

3.10.3.8 Thermal stability

To observe the physical property of the enzyme, thermal stability assay was performed. In practical terms, an enzyme molecule is a very delicate and fragile structure. If the molecule absorbs too much energy, the tertiary structure will be disrupted and the enzyme will be denatured resulting in loss of the catalytic activity. The 0.5 mg/ml EG1 and EG2 were incubated at 75 °C in 50 mM citrate buffer. The inactivation time courses were determined by taking suitable aliquots of samples at the different time points for the assay of remaining activity to calculate half-life of the enzyme using the Equation 1-2 (Winayanuwattikun *et.al.,* 2008).

$$\text{Slope} = k / 2.3 \quad \text{(Equation 1)}$$

$$k = 0.693 / t_{1/2} \quad \text{(Equation 2)}$$

The slope was obtained from the linear plot between log percentage of original activity and the time point of preincubation.

3.10.4 Hydrolysis for production of cellulosic ethanol

3.10.4.1 Physical pretreatment

Grasses (Dwarf Napier, Ruzi, Pangola) were dried at 60 °C for 3 days. Then, they were milled in a blender and milling machine. Finally, it was passed through a 0.4 mm test sieve. The milled grasses were stored at room temperature.

3.10.4.2 Alkaline Peroxide Pretreatment

60 mg milled grasses was slurried in 0.4 ml 7.5% H_2O_2 pH 11.5. The mixtures were incubated in a thermomixer at 50°C, 500 rpm for 24 hr. The pretreated grasses were adjusted to pH 4.8 by concentrated HCl and then further added with 0.5 ml 50 mM sodium citrate buffer pH 4.8 before enzymatic saccharification.

3.10.4.3 Enzymatic saccharification

The enzymatic saccharification of the alkaline peroxide pretreated grasses was performed in thermomixer at 50°C, 500 rpm for 72 hr after adding 100 µl of enzyme for endoglucanase 1 and 50 µl for endoglucanase 2. Finally, reducing sugar was quantitated by the DNS method

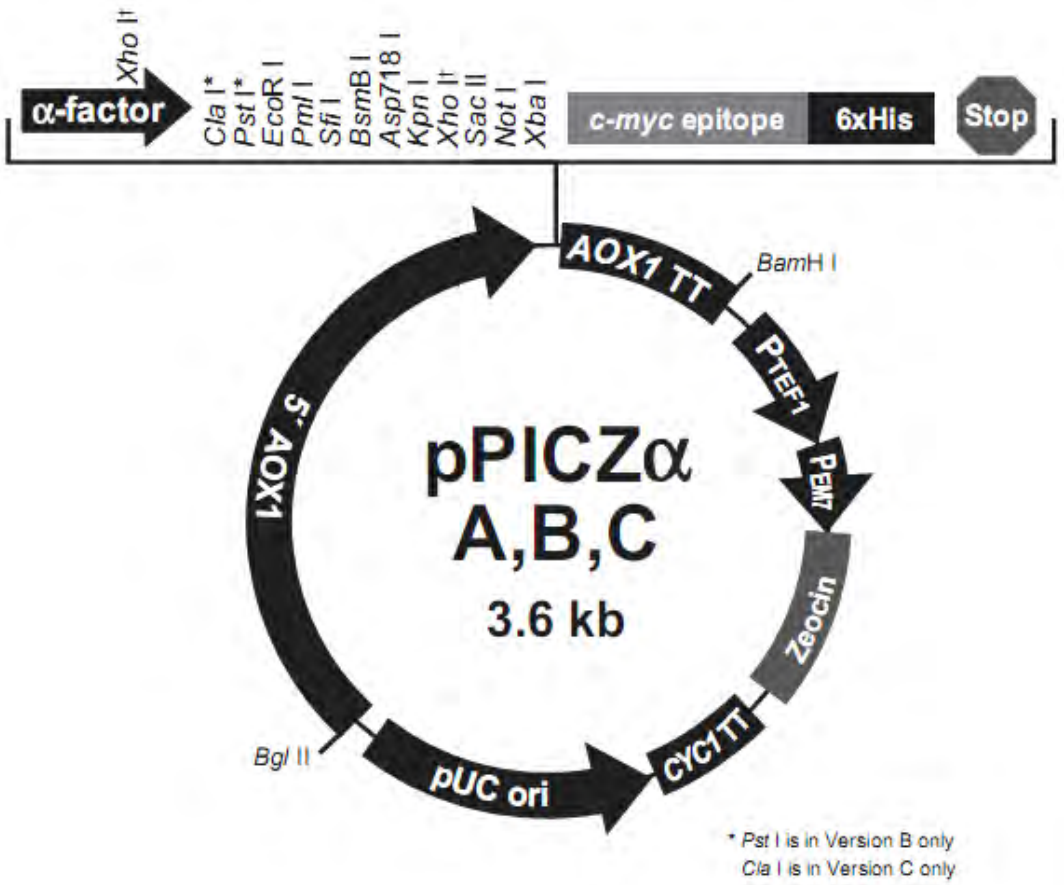


Figure 3-1 Physical map of pPICZαA vector

5' *AOX1* promoter region	1-941
5' *AOX1* priming site	855-875
α- factor signal sequence	941-1207
Multiple cloning site	1208-1276
c-myc epitope	1275-1304
Polyhistidine (6xHis) tag	1320-1337
3' *AOX*1 priming site	1423-1443
*AOX*1 transcription termination region	1341-1682
*TEF*1 promoter	1683-2093
EM 7 promoter	2095-2162
Sh ble ORF	2163-2537
CYC 1 transcription termination region	2538-2855
pUC origin	2866-3539

CHAPTER IV
RESULTS

4.1 Cloning of *eg1* and *eg2* genes from *Trichoderma reesei*

T. reesei was cultured in cellulose production medium. The determination of the enzyme activities confirmed that all 3 types of cellulases; cellobiohydrolases, endoglucanase and β-glucosidase were expressed under culture condition (Table 4-1).

Table 4-1 Enzyme activities of cellulases, cellobiohydrolases, endoglucanase and β-glucosidase from *T. reesei*

Organism	Activity (U/ml)		
	Exocellulase	Endocellulase	β-glucosidase
T.reesei	0.93 ± 0.01	18.56 ± 0.32	0.22 ± 0.01

Total RNA was isolated from the collected hyphae followed by qualitative analysis with formaldehyde gel electrophoresis. cDNA was synthesized using reverse transcriptase and poly-T oligonucleotide primers. Two endoglucanase genes, *eg1* and *eg2*, from *T. reesei* were further amplified by PCR using cDNA as template. Two endoglucanase genes; *eg1* and *eg2* were amplified by PCR technique. The sizes of PCR products were approximately 700 bp and 1200 bp as shown in Figure 4-1.

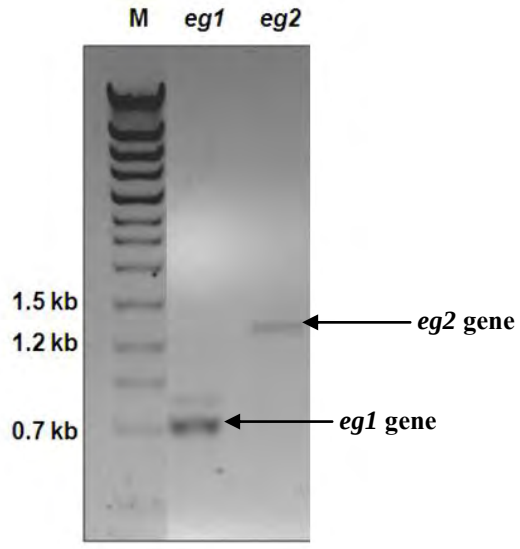


Figure 4-1 PCR products of *eg1* and *eg2* gene

Lane M: DNA markers

Lane 1: PCR products of *eg1* gene

Lane 2: PCR products of *eg2* gene

Purified PCR products were ligated into pPICZαA vector and the recombinant plasmid pPICZαA-*eg1* and pPICZαA-*eg2* were preliminarily checked by rapid size screening and further verified by restriction analysis. Two bands from the gel were observed; 700 bps of *eg1*, 1200 bps of *eg2* and 3100 bps of vector (Figure 4-2). Recombinant plasmids were subjected to DNA sequencing. Nucleotide sequences showed 100% identity with *eg1* gene (EU149644) and 99% identity with *eg2* gene (DQ178347). The *eg1* gene was composed of 713 bps, encoding 234 amino acids with molecular weight approximately 25 kDa whereas *eg2* gene was composed of 1257 bps, encoding 418 amino acids with molecular weight approximately 44 kDa. Amino acid sequence alignment showed 100% and 99% identity with *eg1* and *eg2* from *T. reesei* (Figure 4-3, 4-4). Two amino acid variations in *eg2* were observed from valine-20 to alanine and from glutamic acid-144 to aspartic acid.

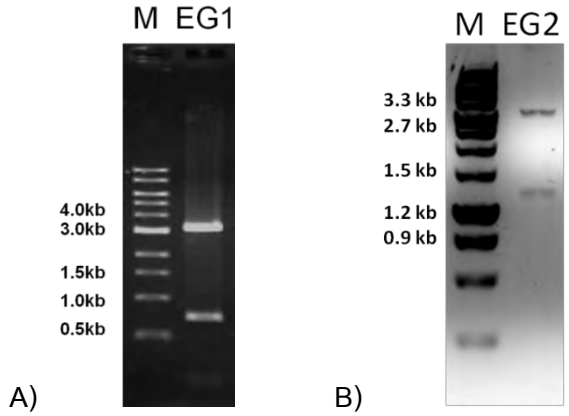


Figure 4-2 Restriction analysis of recombinants of *eg1* gene and *eg2* gene by double digestion with *Not* I and *Eco*R I; A) pPICZαA-*eg1* and B) pPICZαA-*eg2*

```
EG1         : MKFLQVLPALIPAALAQTSCDQWATFTGNGYTVSNNLWGASAGSGFGCVTAVSLSGGASWHADWQWSGGQNNVKSYQNSQ :  80
Wild type   : MKFLQVLPALIPAALAQTSCDQWATFTGNGYTVSNNLWGASAGSGFGCVTAVSLSGGASWHADWQWSGGQNNVKSYQNSQ :  80
              MKFLQVLPALIPAALAQTSCDQWATFTGNGYTVSNNLWGASAGSGFGCVTAVSLSGGASWHADWQWSGGQNNVKSYQNSQ

                      *       100         *       120         *       140         *       160
EG1         : IAIPQKRTVNSISSMPTTASWSYSGSNIRANVAYDLFTAANPNHVTYSGDYELMIWLGKYGDIGPIGSSQGTVNVGGQSW : 160
Wild type   : IAIPQKRTVNSISSMPTTASWSYSGSNIRANVAYDLFTAANPNHVTYSGDYELMIWLGKYGDIGPIGSSQGTVNVGGQSW : 160
              IAIPQKRTVNSISSMPTTASWSYSGSNIRANVAYDLFTAANPNHVTYSGDYELMIWLGKYGDIGPIGSSQGTVNVGGQSW

                      *       180         *       200         *       220         *
EG1         : TLYYGYNGAMQVYSFVAQTNTTNYSGDVKNFFNYLRDNKGYNAAGQYVLSYQFGTEPFTGSGTLNVASWTASIN : 234
Wild type   : TLYYGYNGAMQVYSFVAQTNTTNYSGDVKNFFNYLRDNKGYNAAGQYVLSYQFGTEPFTGSGTLNVASWTASIN : 234
              TLYYGYNGAMQVYSFVAQTNTTNYSGDVKNFFNYLRDNKGYNAAGQYVLSYQFGTEPFTGSGTLNVASWTASIN
```

Figure 4-3 The alignment of amino acid sequence of EG1 gene to database by using ClustalX program

```
EG2         : MNKSVAPLLLAASILYGGAAQQTVWGQCGGIGWSGPTNCAPGSACSTLNPYYAQCIPGATTITTSTRPPSGPTTTTRAT :  80
Wild type   : MNKSVAPLLLAASILYGGAVAQQTVWGQCGGIGWSGPTNCAPGSACSTLNPYYAQCIPGATTITTSTRPPSGPTTTTRAT :  80
              MNKSVAPLLLAASILYGGA AQQTVWGQCGGIGWSGPTNCAPGSACSTLNPYYAQCIPGATTITTSTRPPSGPTTTTRAT

                      *       100         *       120         *       140         *       160
EG2         : STSSSTPPTSSGVRFAGVNIAGFDFGCTTDGTCVTSKVYPPLKNFTGSNNYPDGIGQMQHFVNDDGMTIFRLPVGWQYLV : 160
Wild type   : STSSSTPPTSSGVRFAGVNIAGFDFGCTTDGTCVTSKVYPPLKNFTGSNNYPDGIGQMQHFVNEDGMTIFRLPVGWQYLV : 160
              STSSSTPPTSSGVRFAGVNIAGFDFGCTTDGTCVTSKVYPPLKNFTGSNNYPDGIGQMQHFVN DGMTIFRLPVGWQYLV

                      *       180         *       200         *       220         *       240
EG2         : NNNLGGNLDSTSISKYDQLVQGCLSLGAYCIVDIHNYARWNGGIIGQGGPTNAQFTSLWSQLASKYASQSRVWFGIMNEP : 240
Wild type   : NNNLGGNLDSTSISKYDQLVQGCLSLGAYCIVDIHNYARWNGGIIGQGGPTNAQFTSLWSQLASKYASQSRVWFGIMNEP : 240
              NNNLGGNLDSTSISKYDQLVQGCLSLGAYCIVDIHNYARWNGGIIGQGGPTNAQFTSLWSQLASKYASQSRVWFGIMNEP

                      *       260         *       280         *       300         *       320
EG2         : HDVNINTWAATVQEVVTAIRNAGATSQFISLPGNDWQSAGAFISDGSAAALSQVTNPDGSTTNLIFDVHKYLDSDNSGTH : 320
Wild type   : HDVNINTWAATVQEVVTAIRNAGATSQFISLPGNDWQSAGAFISDGSAAALSQVTNPDGSTTNLIFDVHKYLDSDNSGTH : 320
              HDVNINTWAATVQEVVTAIRNAGATSQFISLPGNDWQSAGAFISDGSAAALSQVTNPDGSTTNLIFDVHKYLDSDNSGTH

                      *       340         *       360         *       380         *       400
EG2         : AECTTNNIDGAFSPLATWLRQNNRQAILTETGGGNVQSCIQDMCQQIQYLNQNSDVYLGYVGWGAGSFDSTYVLTETPTG : 400
Wild type   : AECTTNNIDGAFSPLATWLRQNNRQAILTETGGGNVQSCIQDMCQQIQYLNQNSDVYLGYVGWGAGSFDSTYVLTETPTG : 400
              AECTTNNIDGAFSPLATWLRQNNRQAILTETGGGNVQSCIQDMCQQIQYLNQNSDVYLGYVGWGAGSFDSTYVLTETPTG

                      *
EG2         : SGNSWTDTSLVSSCLARK : 418
Wild type   : SGNSWTDTSLVSSCLARK : 418
              SGNSWTDTSLVSSCLARK
```

Figure 4-4 The alignment of amino acid sequence of EG2 gene to database by using ClustalX program

*The glycosilation sites were shown as the blue and amino acid variations were shown as the red.

4.2 Expression of the *eg1* and *eg2* genes in *Pichia pastoris*

In order to select the highest expression clone, the recombinant *P. pastoris* were cultured and induced by 3% methanol for 5 days. Endoglucanas=e activities were detected in culture medium of both pPICZαA-*eg1* and pPICZαA-*eg2* transformants. The recombinant clones with the highest enzyme activity were 4.69 U/ml and 5.86 U/ml for pPICZαA-*eg1* and pPICZαA-*eg2*, respectively. The supernatants of both *eg1* and *eg2* clones were further analyzed by SDS-PAGE (Figure 4-5). The molecular weight of EG1 and EG2 were found to be 30 and 48 kDa, respectively which were higher than the calculated molecular mass.

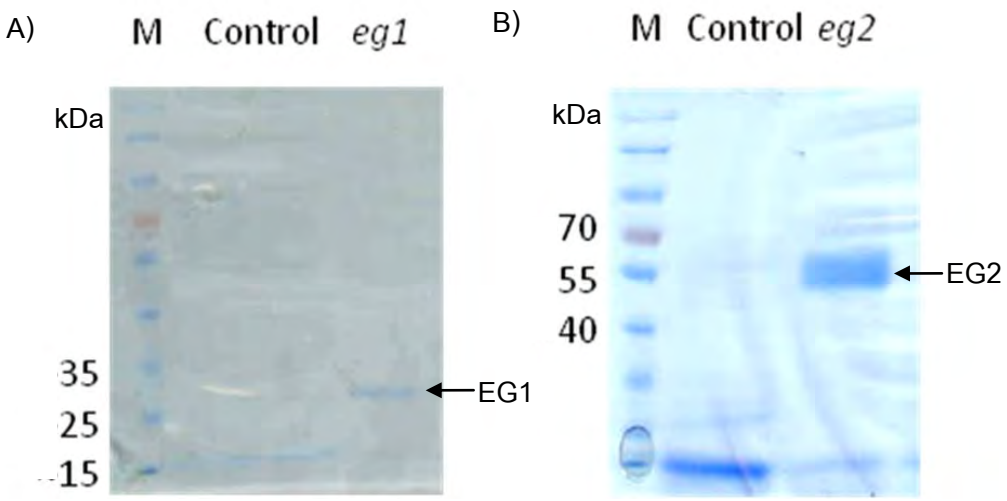


Figure 4-5 SDS-PAGE analysis revealed the expression of (A) EG1 and (B) EG2 in *P. pastoris* strain X33 induced with 3% methanol for 5 days.

4.2.1 Optimization of expression conditions

4.2.1.1 Effect of the methanol induction

To optimize expression conditions for the production of endoglucanase, the recombinant clone which produced the highest level of enzyme activity was grown under a variety of conditions. The effect of methanol concentration was examined by measuring total endoglucanase activity after 5 days of induction. Various concentrations of methanol ranging from 1 to 5 % v/v were added once a day. The results of EG1 were shown in Table 4-2 and Figure 4-6, EG1 expression

and the hydrolysis activity increased with the increase of the amount of methanol added. It can be seen that the maximum hydrolysis activity was reached, 6.68 ± 0.31 U/ml , with 4% methanol induction. The results of EG2 were shown in Table 4-3 and Figure 4-7. Similar to EG1, the expression level and the hydrolysis activity increased when increasing added methanol. It can be seen that the maximum hydrolysis activity was reached, 9.81 ± 0.16 U/ml, with 5% of methanol induction.

Table 4-2 The effect of methanol induction toward the expression of recombinant EG1

% MeOH	Hydrolysis activity (U/ml)
1	5.02 ± 0.14
2	5.20 ± 0.07
3	6.05 ± 0.20
4	6.68 ± 0.31
5	5.76 ± 0.08

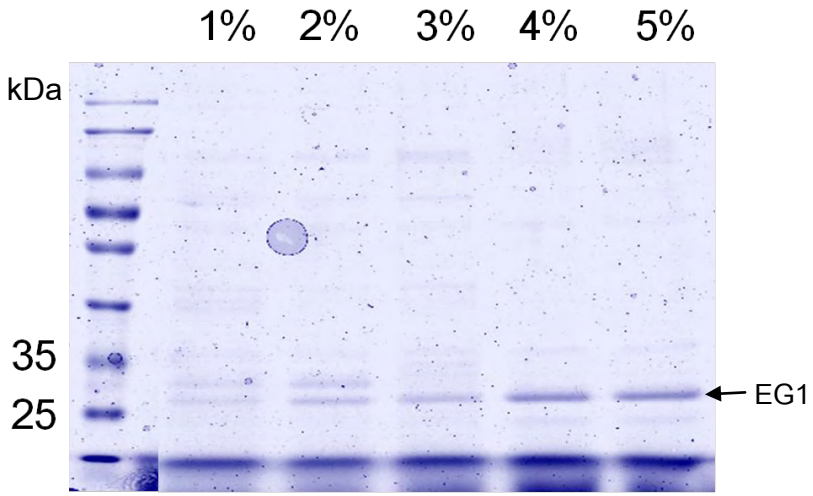


Figure 4-6 SDS-PAGE analysis of %methanol induction on the expression of EG1 after 5 days

Table 4-3 The effect of methanol induction toward the expression of recombinant EG2

% MeOH	Hydrolysis activity (U/ml)
1	7.95 ± 0.11
2	7.98 ± 0.75
3	8.42 ± 0.40
4	9.36 ± 0.49
5	9.81 ± 0.16

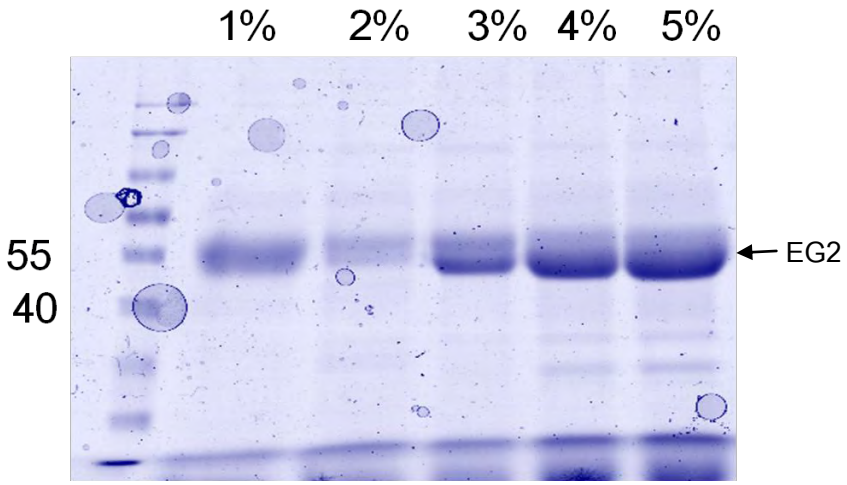


Figure 4-7 SDS-PAGE analysis of % methanol induction on EG2 expression after 5 days

4.2.1.2 Effect of the induction time

To determine the optimum time for methanol induction, cells were harvested during 7 days after induction and endoglucanase activity was measured. Both EG1 and EG2 recombinant clones had maximum activity at 5 days of induction. The total activity was decreased from days 6 to 7 (Table 4-4 and Figure 4-8 for EG1 and Table 4-5 and Figure 4-9 for EG2). The induction by 4% methanol for 5 days was therefore employed as optimal expression conditions for EG1 and 5% methanol and 5 days for EG2, respectively.

Table 4-4 The effect of induction time toward the expression of recombinant EG1

Days	Hydrolysis activity (U/ml)
0	2.54 ± 0.68
1	7.07 ± 0.09
2	6.62 ± 0. 09
3	6.47 ± 0.64
4	6.72 ± 0.64
5	7.17 ± 0.59
6	5.68 ± 0.00
7	5.74 ± 0.48

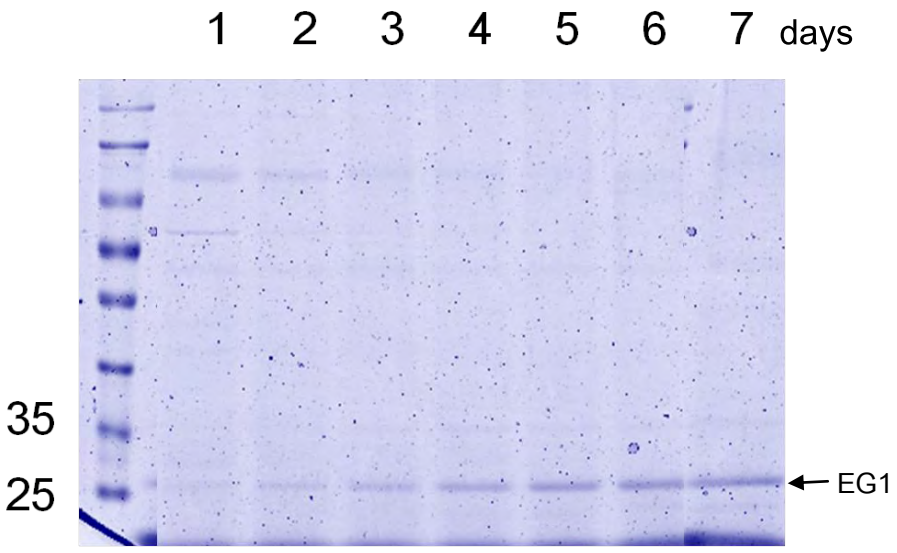


Figure 4-8 SDS-PAGE analysis for optimization of induction time for EG1

Table 4-5 The effect of induction time toward the expression of recombinant EG2

Days	Hydrolysis activity (U/ml)
0	2.60 ± 0.20
1	8.77 ± 0.28
2	9.11 ± 0.28
3	8.88 ± 0.33
4	9.73 ± 0.10
5	9.99 ± 0.19
6	8.78 ± 0.52
7	8.52 ± 0.37

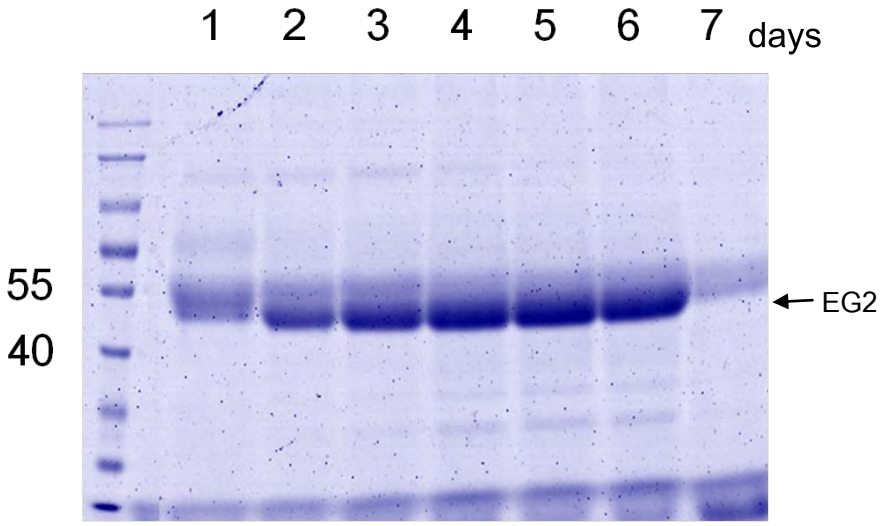


Figure 4-9 SDS-PAGE analysis for optimization of induction time for EG2

4.3 Characterization of recombinant EG1and EG2

The large scale production of EG1 and EG2 was performed. The concentrated enzymes were verified by SDS-PAGE. The results revealed only one major band of EG1 and EG2 (Figure 4-10). Thus, the purification step was not required. Both EG1 and EG2 were further characterized for the catalytic properties.

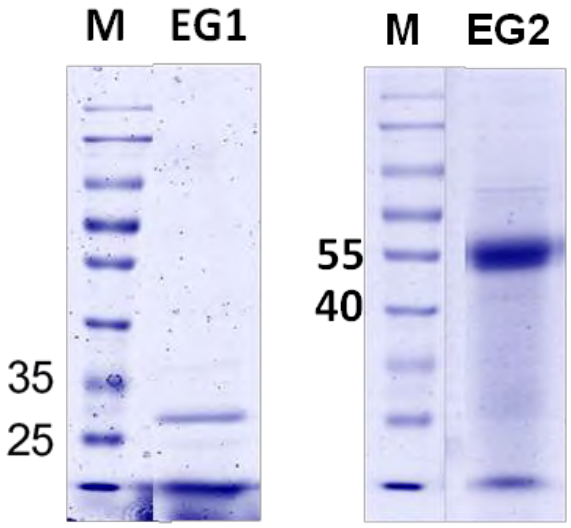


Figure 4-10 SDS-PAGE analysis of concentrated media from recombinant EG1 and EG2 after induction with 4% methanol for 5 days

4.3.1 Optimization condition of endoglucanase

4.3.1.1 Optimum pH

In order to determine the optimum pH of endoglucanase curves, the CMC-substrates were dissolved and enzyme activity was measured in 50 mM sodium citrate buffer, pH ranging between 3.5 and 6.0. The results showed that the optimal pH of both EG1 and EG2 were 5 (Figure 4-11).

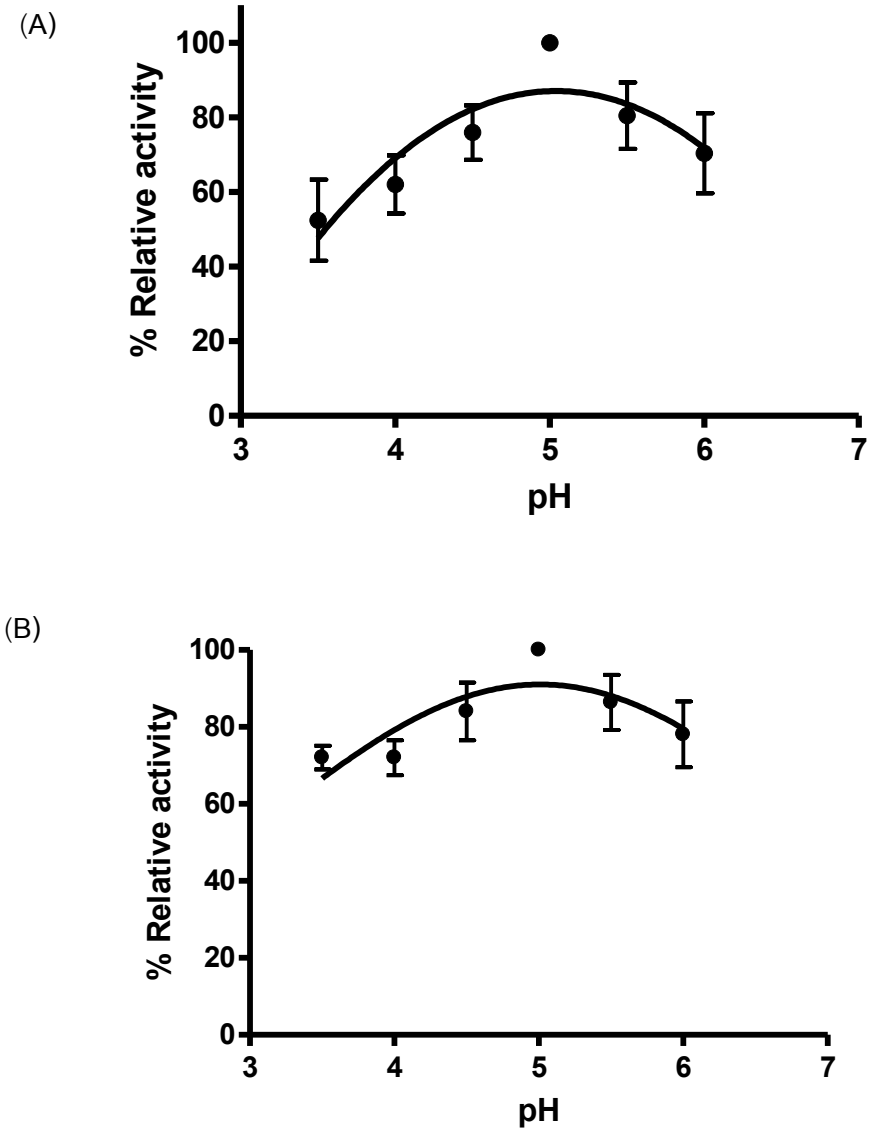


Figure 4-11 Effect of pH on the recombinant (A) EG1 and (B) EG2 at 50 ^{O}C

4.3.1.2 Optimum temperature

To determine the optimal temperature of endoglucanase, the enzyme-catalyzed reaction was performed at various temperatures ranging from 30 to 80 ^{O}C. The result showed that the optimal temperature EG1 was 60 ^{O}C and EG2 was 70 ^{O}C at pH 4.8 (Figure 4-12).

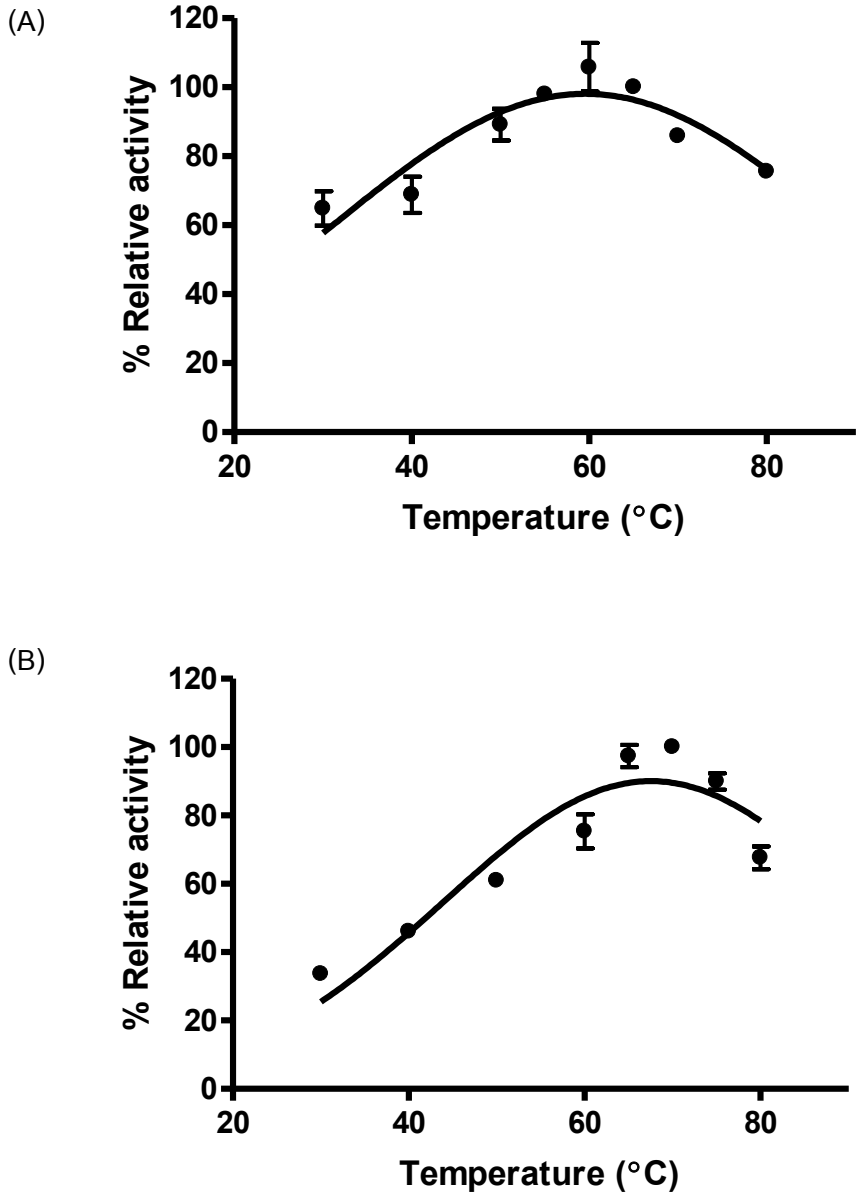


Figure 4-12 Effect of temperature on the recombinant (A) EG1 and (B) EG2 at pH 4.8

4.3.2 Stability of endoglucanase

4.3.2.1 pH stability

The pH stability was determined by incubating the enzyme in different pH of 50mM citrate buffer ranging of 3.0–6.0 for 4 hr and then measured the enzyme activity at optimum temperature. As shown in Figure 4-13, EG1 was stable between pH 4.0 and 5.5, retaining the activity approximately 70% of its original activity while EG2 was stable between pH 3.5 and 5.5.

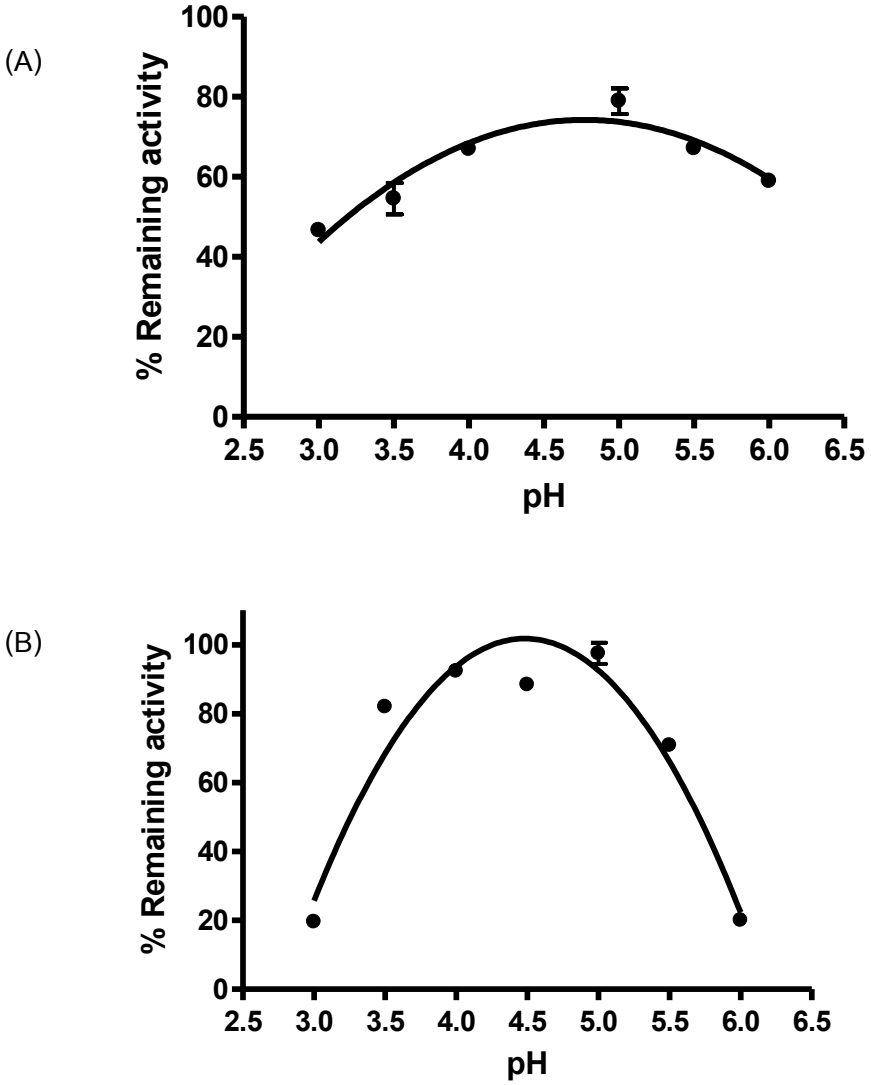


Figure 4-13 pH stability of (A) EG1 at 60 ^{O}C and (B) EG2 at 70 ^{O}C

4.3.2.2 Thermal stability

The effect of temperature on stability was investigated at various temperatures for 2 hr at optimum pH. Thermal inactivation was primarily examined by incubating the enzyme at temperatures from 60 to 90 ^{O}C. Aliquots were withdrawn to test the remaining activity at standard condition. Non-incubated reaction was considered as control and assumed to have 100% activity. It was observed that the endoglucanase activity of both EG1 and EG2 began to decrease at 75 ^{O}C (Figure 4-14). This temperature was used to determine half-life stability for recombinant EG1 and EG2.

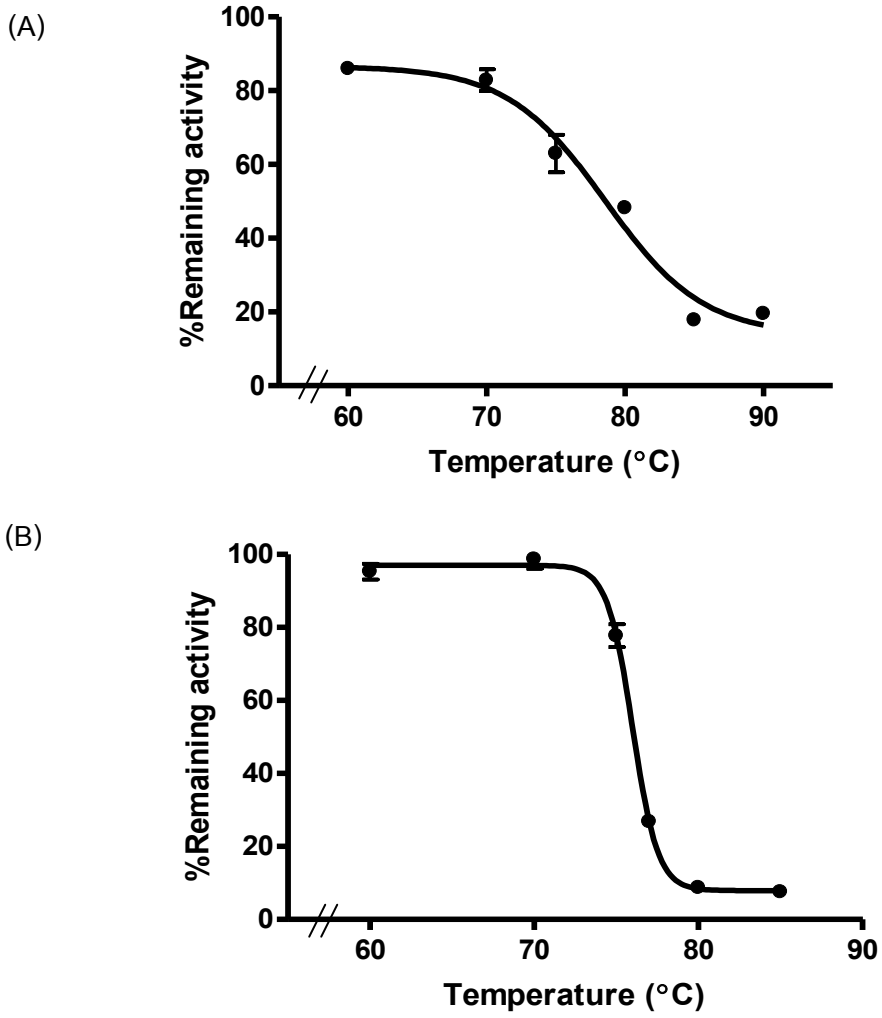


Figure 4-14 Thermal inactivation on (A) EG1 and (B) EG2

The inactivation time courses were determined by withdrawing suitable aliquots at the different time points for the assay of remaining activity to calculate half-life of the enzyme (Figure 4-15). The half-life corresponds to the time of preincubation when there is 50% remaining activity. The activity of EG2 was more stable than EG1 by ~68 folds (Table 4-6).

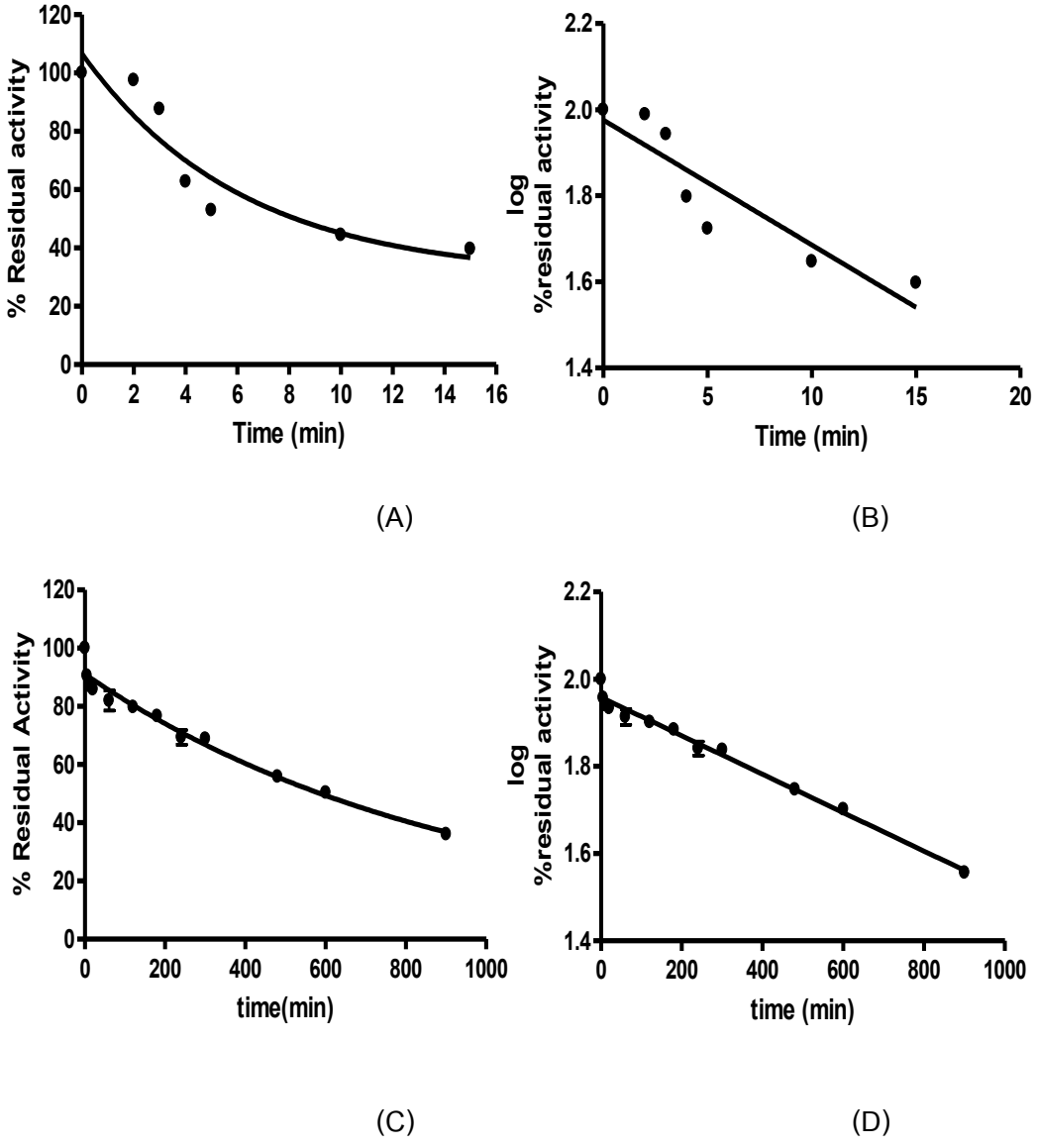


Figure 4-15 Time course inactivation of (A-B) EG1 and (C-D) EG2

Table 4-6 Thermal stability of EG1 and EG2

	Half-life at 75 °C (min)
EG1	10.39 ± 0.07
EG2	684.2 ± 26.8

4.3.2.3 Kinetic study

To determine the appropriate reaction time for steady state kinetic study of endoglucanase, the reaction was carried out at various time at optimum conditions. The slope of the initial rate period is the initial rate of reaction. The result showed that the proper reaction time for steady- state kinetic study of EG1 and EG2 was 10 and 5 minutes, respectively (Figure4-16).

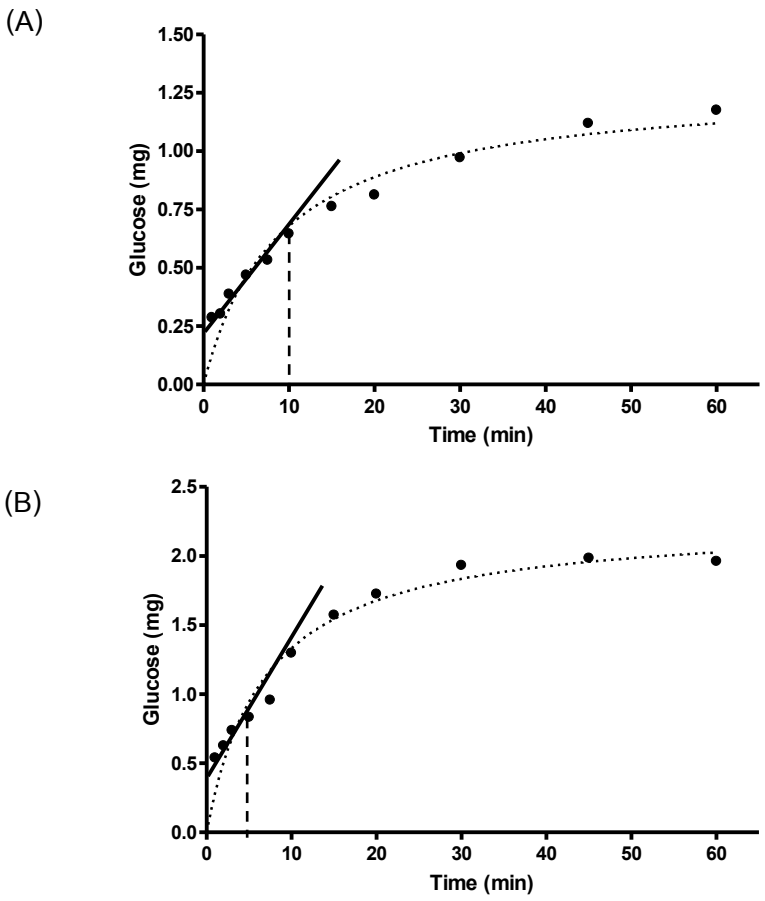


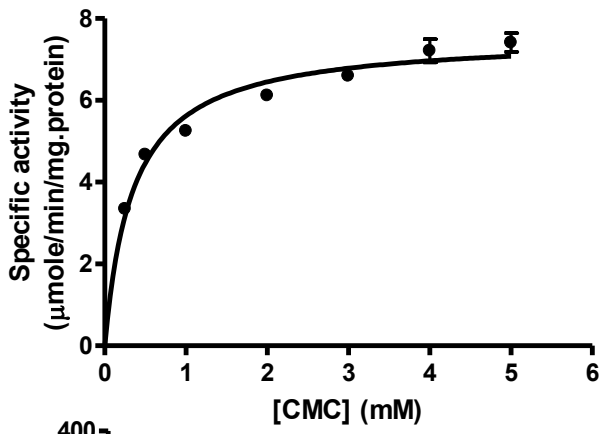
Figure 4-16 Reaction progress curve of CMC hydrolysis catalyzed by (A) EG1 and (B) EG2

Steady-state kinetics was performed with various concentration of CMC as substrate. The result shown in Figure 4-17 suggested that the enzyme followed Michaelis-Menten kinetics. The kinetic parameter k_{cat} and K_m were determined by non-linear regression analysis (Table 4-7). The k_{cat} value of EG2 in CMC hydrolytic reaction was significantly higher than EG1 by approximately 35 folds while the K_m values for CMC of EG1 was 6-fold lower than EG2. The k_{cat},/K_m values of EG2 show that it possesses about 6–fold greater catalytic efficiency for CMC than EG1.

Table 4-7 Steady-state kinetic parameters of EG1 and EG2 for the CMC hydrolysis

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/ K_m ($mM \cdot s^{-1}$)
EG1	181.68	0.35 ± 0.05	519.09
EG2	6,244.2	2.20 ± 0.16	2,838.27

(A)



(B)

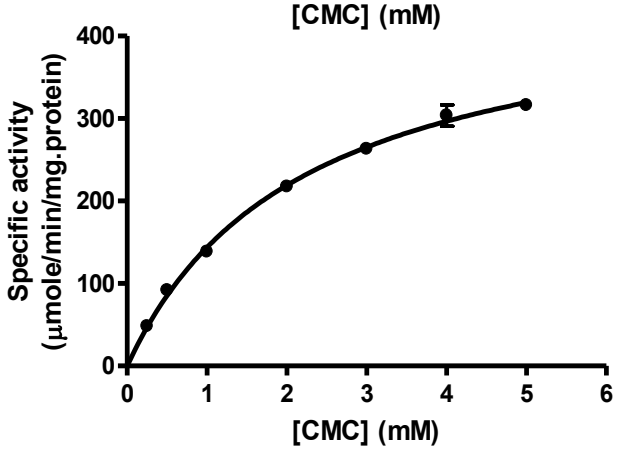


Figure 4-17 Saturation curve of CMC hydrolysis catalyzed by (A) EG1 and (B) EG2

4.3.3 Substrate specificity

Substrate specificity determination revealed differences in the specificity or the interaction of enzymes with several substrates (Table 4-8). EG1 and EG2 showed similar trend of activity toward different substrates; high specific activities to CMC and low activities to Avicel.

Table 4-8 Substrate specific activity toward different cellulosic substrates.

substrate	time (min)	specific activity (μmol/min/mg protein)	
		EG1	EG2
1% CMC	10	4.96 ± 0.38	14.86 ± 0.06
1% Sigmacell	30	0.60 ± 0.03	1.32 ± 0.03
1% α-cellulose	60	0.26 ± 0.00	0.47 ± 0.00
1% avicel	120	0.15 ± 0.00	0.33 ± 0.00

4.4 Hydrolysis cellulosic material for bioethanol production

The main composition of raw grass is taken as 33% cellulose, 34% hemicellulose and 4.5% lignin. Lignin hinders carbohydrate molecules from reacting with chemical agents such as acids, bases or cellulose solvents. The criteria for successful lignocellulose pretreatment have been defined from the high contents of cellulose and hemicellulose recovery but lower lignin content in pretreated biomass. The main objective is to increase the accessibility of cellulose to cellulase for the production of fermentable sugar (Nguyen *et. al.,* 2010).

The biomass was initially pretreated with 7.5% H_2O_2, pH 11.5 for 24 hr and later hydrolyzed at 50 ˚C, pH 5.0 for 72 hr. Three types of biomass; Dwarf Napier, Ruzi and Pangola were hydrolyzed by EG1 and EG2. Total sugars were determined by DNS assay. The percent conversion of different grasses was presented in Table 4-9. EG1 showed equal % conversion towards Pangola and Russy and low %conversion towards Dwarf Napier similar to EG2. However, % conversion obtained from EG2 was higher than EG1 by 2-folds.

Table 4-9 Hydrolysis of lignocellulosic materials by EG1 and EG2

substrate	Alkaline pretreatment (mg glucose)		Total (mg glucose)		Enzymatic hydrolysis (mg glucose)		% Conversion	
	EG1	EG2	EG1	EG2	EG1	EG2	EG1	EG2
Pangola	1.52 ± 0.06	1.59 ± 0.06	1.65 ± 0.05	3.46 ± 0.11	0.13 ± 0.05	1.87 ± 0.11	4.02 ± 0.13	8.42 ± 0.26
Dwarf Napia	0.47 ± 0.11	0.55 ± 0.11	0.73 ± 0.00	1.79 ± 0.20	0.26 ± 0.00	1.24 ± 0.20	1.74 ± 0.01	4.27 ± 0.48
Rusy	1.09 ± 0.01	1.16 ± 0.01	1.72 ± 0.07	2.49 ± 0.12	0.63 ± 0.07	1.33 ± 0.12	4.24 ± 0.17	6.12 ± 0.30

CHAPTER V
DISCUSSION

5.1 Cloning of *eg1* and *eg2* genes from *Trichoderma reesei*

The cDNA obtained from reverse transcription was amplified by using two pairs of primers; namely, EG1_R, EG1_F, EG2_R and EG2_F. The PCR products of *eg1* and *eg2* were 0.7kb and 1.3kb DNA, respectively (as shown in Figure 4-1). Furthermore, purified PCR products were successfully ligated into pPICZαA vector and were then subjected to DNA sequencing. The nucleotide sequences showed 100% identity with *eg1* gene and 99% identity with *eg2* gene. As regard the former, the *eg1* gene was composed of 713 bps, encoding 234 amino acids and molecular weight approximately 25 kDa. Concerning *eg2* gene, it was composed of 1257 bps, encoding 418 amino acids and molecular weight approximately 44 kDa. When the amino acid sequence were aligned, the homologue of such sequence were 100% and 99% identity with *eg1* and *eg2* from *T. reesei* (EU149644 and DQ178347), respectively. From the results shown in Figure 4-3 and 4-4, it was clearly seen that there were two amino acid variations observed at valine-20 to alanine and glutamic acid-144 to aspartic acid. The amino acid sequence showed that EG2 was larger than EG1 probably from the presence of the cellulose-binding domain (CBD) normally found in both hydrolytic and nonhydrolytic proteins such as cellulases and xylanases.

5.2 Expression of the *eg1* and *eg2* genes in *Pichia pastoris*

Expression of recombinant cellulases has previously been reported in many microoraganisms be that of bacteria, yeast, or filamentous fungi. However, when the recombinant cellulase was overexpressed in *E.coli,* the produced enzyme was not extracellularly secreted and it remained in the inactive form, inclusion bodies. In contrast, the cellulase secreted by *S. cerevisiae* was hyper-glycosylated resulting in reducing substrate-binding capacity and catalytic activity and the expression level was quite low (Shao *et. al.,* 2002). Previous studies showed that a wide variety of cellulose and hemicellulose-degrading enzymes could be produced in *Pichia pastoris*, including

exoglucanases, endoglucanase, xylanases, and xyloglucanases. The growth mode of such yeast was well constructed. It has a methanol strong inducible promoter, because of capability to metabolite methanol as a sole carbon source (Lindenmuth and McDonald, 2011). Thus, it can be used to promote the high expression level of heterologous protein. Moreover, the post-translational modifications make it more favourable than *S. cerevisiae*. Owing to the fact that this species does not secrete a lot of extracellular protein, thus it is easier to isolate heterologous protein. However, the methanol concentration and incubation time have to be optimized for higher yield of expression. At lower methanol concentration, it may be insufficient for expression but at too high concentration, it may lead to reduce the expression of protein or even death. Similarly, the shorter induction time may not be suitable for expression of yeast cell to obtain the maximum yield whereas the longer time may attribute to cell damage. In this study, the recombinant plasmid containing endoglucanase gene was transformed and expressed in *Pichia pastoris* strain X33. The recombinant protein was secreted into the culture medium. The level of protein expression was verified induction with different methanol concentrations and induction time. The optimal conditions of expression were selected for the further studies which were 4% methanol for 5 days for EG1 and 5% methanol for 5 days for EG2.

Regarding the large scale production of both EG1 and EG2, the concentrated enzymes were verified by SDS-PAGE and measuring enzymatic activity. The result distinctly showed that a major sharp band of EG1 and EG2 were found. Therefore, such proteins did not require the further additional step of purification.

The molecular weight of EG1 and EG2 were found to be 30 kDa and 48 kDa, respectively. Their molecular weights were significant larger than the calculated from amino acid sequence, 25 kDa and 44 kDa. The molecular weight of recombinant EG2 was consistent with the native EG2 from *Trichoderma reesei* (Qin *et. al.,* 2008). This result may be attributed from the post-translation modification, glycosylation, which is ubiquitous modification process in eukaryotic cells. It is a dynamic enzymatic process in which saccharides are usually attached to proteins or lipoproteins at the unique amino acid residues namely; serine (S), threonine (T) or tyrosine (Y) for O-link and asparagines (N) or

arginine (R) for N-link (Caragea *et. al.,* 2007). From the result, the site of N-glycosylation (Asn-Tyr-Ser) was found at position-183 in EG1 while two N-glycosylation sites (Asn-Lys-Ser and Asn-Phe-Thr) were found at position-2 and 124 in EG2.

5.3 Characterization of recombinant EG1 and EG2

5.3.1 Optimum pH

According to the result in 4.3.2.1, the optimal pH of both EG1 and EG2 were 5.0. It indicated that EG1 and EG2 are acidophilic endoglucanase enzyme. The result was similar to native EG2 from *Trichoderma reesei*, which showed the optimal pH is 4.8. Likewise, the recombinant EG2 from *S. cerevisiae* and from *Trichoderma reesei* also showed the optimal pH of 4.8 (Qin *et. al.,* 2008). Moreover, the other cellulases from different sources such as *Streptomyces* spp., *Bacillus* spp., *Anaerocellum thermophilum*, *Ulockadium chartarum* have similar optimum pH ranging between 4.5 -6.0 (Busto *et. al.,* 1996).

5.3.2 Optimum temperature

From the profile of enzymatic hydrolysis of CMC in Figure 4-12, the optimal temperature of EG1 and EG2 were 60 °C and 70 °C, respectively. These results were similar to endoglucanase obtained from wild type *Trichoderma reesei.* The activity of cellulase was detected in a wide range of temperature, from 50 °C to 70°C (Busto *et. al.,* 1996). Nevertheless, the highest one from *Trichoderma* spp. and other mesophilic cellulolytic fungi were observed when the activity was assayed at about 50°C.

5.3.3 pH stability

As the result of cellulose being by nature relatively recalcitrant to enzymatic hydrolysis even after pretreatment, thus the hydrolysis to glucose takes several days. To achieve the efficient hydrolysis, the high stability of cellulolytic enzymes is required for bioethanol producing conditions. Moreover, the optimal conditions for cellulose hydrolysis using a fungal cellulolytic enzyme has been frequently reported as pH 5.0 and 50 ˚C (Krogh *et. al.* 2009). From this study, the pH stability of recombinant endoglucanase was tested by using various pH of citrate buffer ranging of 3.0–6.0 for 4 hr at 60 ˚C for EG1 and 70 ˚C for EG2 as shown in Figure 4-13. It was found that the recombinant EG1 was stable between pH 4.0 and 5.5 with the retaining the activity more than 70% of its original activity while the recombinant EG2 was slightly more stable at the lower pH between pH 3.5 and 5.5.

5.3.4 Thermal stability

From the study of thermal stability, the recombinants EG1 and EG2 were likely to tolerate the intensive heat since their activities were retained more than 80% when incubated at 70 °C for 2 hr (Figure 4-14). Nonetheless, recombinants EG1 was more stable than that of EG2 from *Trichoderma reesei* expressed in *S. cerevisia* because it could retain activity only 50% of the initial activity when incubated for 1 hr. In addition, the total activity of native EG2 from *Trichoderma reesei* was 50% decreased within 30 min of incubation (Qin *et. al.,* 2008). The half-life of EG1 and EG2 at 75°C were found to be 10 minutes and 11.5 hr, respectively. This result indicated that EG2 is 68-folds more stable than EG1. However, both enzymes showed no evidence of the denaturation since the incubation temperature used was lower than their optimal temperature. These enzymes are suitable for application in cellulose hydrolysis.

5.3.5 Kinetic study

The kinetic parameters; k_{cat} and K_m of the EG1 and EG2 were determined with various concentrations of substrate, CMC. From the result in Figure 4-17, it indicated that enzyme velocity, as the function of substrate concentration, often follows the Michaelis-Menten equation. The steady-state kinetic parameters were calculated by non-linear regression analysis (Table 4-7). The k_{cat} value of EG2 was 35-fold higher than EG1. This result indicated that EG2 had significantly higher activity than EG1. In contrast, the K_m value of EG1 toward CMC substrates was six folds lower than EG2. The results showed that EG1 required small amount of substrate to achieve the maximum velocity of the reaction. The result did not seem to be that EG1 had higher affinity toward CMC substrate than EG2 because the K_m is not the true dissociation constant and it may cause its low activity. When the k_{cat}/K_m values were calculated, the catalytic efficiency of EG2 was six folds greater than EG1. The results suggested that EG2 was more effective catalyst than EG1. It is possible that the EG2 contains cellulose- binding domain (CBD). The CBD is a discrete domain that concentrates the catalytic domains on the surface of the insoluble cellulose substrate. Moreover, it is present in proteins with no hydrolytic activity as part of a scaffolding subunit which is part of the catalytic subunits organized into a cohesive multienzyme complex, cellulosome. This complex was found to function more efficiently in the degradation of cellulosic substrates. Hence, the removal of such domain from the cellulose molecule or from the scaffolding in cellulosomes dramatically decreased enzymatic activity (Levy *et. al.,* 2002).

The comparison of kinetic parameter between recombinant EG2 and native EG2 of *Trichoderma reesei* showed that the k_{cat} of recombinant EG2 was six folds higher than native whereas the K_m values of recombinant and native EG2 was roughly the same. The result indicated that the recombinant EG2 was more efficient than native EG2 from *Trichoderma reesei*.

5.3.6 Substrate specificity

The influences of artificial model substrates and α-cellulose on activities of endoglucanase were compared. The substrates for cellulase activity assays can be divided into two categories, based on their solubility (Table 5-1). CMC is long degree of polymerization cellulose derivatives which can be dissolved in water because of their ionic substitutions. It is often used for determining endoglucanase activity. The other substrates; α-cellulose, avicel and sigmacell, are insoluble cellulose. Although, they are good substrates for exoglucanase which degrade the molecule further to cellobiose from the free chain-ends but some endoglucanases can release considerable reducing sugars from them (Zhang *et. al.,* 2006). α-cellulose is an insoluble fibrous residues obtained by extraction of wood, straw pulps and holocelluloses with strong alkali under carefully controlled conditions. It contains major cellulose and a small amount of hemicellulose. Avicel or microcrystalline cellulose contains a significant fraction of amorphous cellulose. It is partially hydrolyzed cellulose prepared by heat-treating α-cellulose from wood with strong mineral acids, vigorously agitating the slurry, and spray drying. Sigmacell or cellulose powder is unsubstituted cellulose. It is an artificial substrate processed from α-cellulose obtained by mechanically disintegrating purified α-cellulose (dry or wet grinding and drying) generally from wood containing both broken fibers and irregularly shaped particles. The mechanical treatment results in partial amorphous character and limited depolymerization of cellulose. From the result shown in Table 4-8, both EG1 and EG2 showed high specific activities towards CMC whereas low activities towards avicel, sigmacell and α-cellulose. This is similar to the results from native *Trichoderma reesei* (Jäger *et. al.,* 2010). Thus, the capability of hydrolysis by both enzymes was different. Sigmacell could be hydrolyzed by endoglucanase more than avicel and α-cellulose from the high degree of amorphous structure. Nevertheless, the activities of EG2 were solely two times higher than the activities of EG1.

Table 5-1 Physical properties and product information of applied cellulosic substrates (from Jäger *et. al.,* 2010)

Substrate	Solubility in water	Impurities	CrI [%]	DPw [AGU]	dp [μm]
CMC	Soluble	Pure	-	64	-
Avicel PH101	Insoluble	Pure	82	200 - 240	43.82
Sigmacell 101	Insoluble	Pure	Amorphous	1590 - 1960	15.86
α-cellulose	Insoluble	impure: Xylan	64	2140 - 2420	68.77

*CrI, the crystallinity index of cellulose; DPw, the weight-average degree of polymerization; dP, the geometric mean particle size.

5.4 Hydrolysis for production of cellulosic ethanol

Cellulases have been used to hydrolyze pretreated cellulosic materials to sugars which can be fermented to commodities such as bioethanol and biobased. The effective conversion of recalcitrant lignocelluloses to fermentable sugars requires three sequential steps: (1) size reduction, (2) pretreatment, and (3) enzymatic hydrolysis (Zhang *et. al.,* 2006). However, this study focused only on enzymatic hydrolysis for production of cellulosic ethanol. Three species of grass; namely, Dwarf Napier, Ruzi and Pangola were pretreated by alkaline peroxide and only hydrolyzed by recombinant EG1 and EG2 and the results were shown in Table 4-9. The uses of recombinant EG1 showed an equal percentage of conversion of glucose towards Pangola and Russy and low conversion towards Dwarf Napier similar to EG2 which showed higher capability of hydrolysis than EG1 by two folds. However, % conversion of glucose obtained from enzyme-catalyzed reaction was quite low. It may be from the fact that the hydrolysis of lignocellulosic materials is very complicated and the type of lignocellulose is also the crucial barrier since it requires all 3 groups of cellulases to complete the reaction. Furthermore, the crystallinity of cellulose may be considered as the main factor that affects the rate of biological

degradation of lignocelluloses by the enzymes (Taherzadeh and Karimi, 2008). Neverless, both recombinant EG1 and EG2 could be applied for saccharification of raw materials.

CHAPTER VI
CONCLUSION

The genes of endoglucanase; *eg1* and *eg2* from *Trichoderma reesei* were cloned into the plasmid pPICαA to produce recombinant plasmid, pPICαA-*eg1* and pPICαA-*eg2*. After sequencing and analyzing the molecular properties of those genes, the *eg1* gene was composed of 713 bps, encoding 234 amino acids while *eg2* gene was composed of 1257 bps, encoding 418 amino acids. Furthermore, the recombinant plasmids; pPICαA-*eg1* and pPICαA-*eg2*, were transformed and both endoglucanase1 (EG1) and endoglucanase2 (EG2) were successfully expressed in *Pichia pastoris* X33. The EG1 and EG2 were highly expressed by 4% and 5% methanol induction for 5 days, respectively. Moreover, large scale expression showed that only single band was found with their molecular weights approximately 30 kD and 48 kD for EG1 and EG2, respectively. Thus, both endoglucanases were further characterized without additional purification step. It was found that EG1 showed the highest activity at pH 5.0 and 60°C, stable at pH range of 4.0–5.5 at the temperature below 75°C while EG 2 showed the highest activity at pH 5 and 70°C, stable at pH range of 3.5–5.5 at temperature below 70°C. Besides, the steady-state kinetic towards CMC substrate was also investigated. The catalytic efficiency of EG2 was six folds greater than EG1. The assay of substrate specificity showed that both EG could hydrolyze a variety of substrates; CMC, Sigmacell, α-cellulose and avicel in different extents. Recombinant endoglucanases were later studied for their capability of sacharification towards three species of grasses, Pangola, Russy and Dwarf Napier. EG1 showed an equal percentage of conversion of glucose toward Pangola and Russy, and low conversion towards Dwarf Napier as similar as EG2. However, % conversion of glucose obtained from catalyzing of recombinant EG2 was higher than EG1 by two folds. From this study, it indicated that the recombinant endoglucanase produced from *Pichia pastoris* could be applied for saccharification process in bioethanol production.

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APPENDICES

APPENDIX A

Reagent preparation

1. Preparation for media

1.1 Mandel medium

$(NH_4)_2SO_4$	1.4	g
KH_2PO_4	2.0	g
Urea	0.3	g
$CaCl_2.2H_2O$	0.4	g
$MgSO_4 \cdot 7H_2O$	0.3	g
$FeSO_4 \cdot 7H_2O$	5.0	mg
$MnSO_4 \cdot H_2O$	1.6	mg
$ZnSO_4 \cdot H_2O$	1.4	mg
$CoCl_2$	2.0	mg
Yeast extract	0.25	g
Peptone	0.75	g
α-cellulose	10	g

All components were dissolved in 950 ml distilled water. The pH of solution was adjusted to 5.0 and then brough the volume up to 1,000 ml by distilled water. Subsequently, the mixture was autoclaved at 121° C, 15 psi for 15 min.

1.2 Luria-Bertini (LB) broth

Bactotryptone	10	g
Yeast extract	5	g
NaCl	10	g

Dissolve all components up to 1,000 ml distilled water, autoclave at 121°C, 15 psi for 15 min

1.3 LB agar

Bactotryptone	10	g
Yeast extract	5	g
NaCl	10	g
agar	15	g

Dissolve all components with 1,000 ml distilled water, autoclave at 121°C,15 psi for 15 min

1.4 Low Salt Luria-Bertini broth (LB low salt)

Tryptone	10	g
Yeast extract	5	g
NaCl	5	g

All components were dissolved in 950 ml distilled water. The pH of solution was adjusted to 7.5 with NaOH and then brough the volume up to 1,000 ml by distilled water. Subsequently, the mixture was autoclaved at 121° C, 15 psi for 15 min.

1.5 Yeast peptone dextrose medium (YPD)

yeast extract	10	g
peptone	20	g

Dissolve all components with 900 ml distilled water, autoclave at 121°C, 15 psi for 15 min. Next, the mixture was cooled down at room temperature and added 20% D-glucose 100 ml

1.6 YPD agar

yeast extract	10	g
peptone	20	g
agar	20	g

Dissolve all components with 900 ml distilled water, autoclave at 121°C, 15 psi for 15 min. Next, the mixture was cooled down at room temperature and added 20% D-glucose 100 ml

1.7 Buffered glycerol-complex medium (BMGY)

yeast extract	10	g
peptone	20	g

Dissolve all components with 700 ml distilled water, autoclave at 121°C, 15 psi for 15 min. Next, the mixture was cooled down at room temperature and added the following and mix well:

1 M potassium phosphate buffer, pH 6.0	100	ml
10X YNB	100	ml
500X B ($4x10^{-5}$ biotin)	2	ml
10X Glycerol	100	ml

Stored media at 4° C

1.8 Buffered methanol-complex medium (BMMY)

yeast extract	10	g
peptone	20	g

Dissolve all components with 700 ml distilled water, autoclave at 121°C, 15 psi for 15 min. Next, the mixture was cooled down at room temperature and added the following and mix well:

1 M potassium phosphate buffer, pH 6.0	100	ml
10X YNB	100	ml
500X B ($4x10^{-5}$ biotin)	2	ml
10X M (0.5% methanol)	100	ml

Stored media at 4° C

2. Preparation for DNA extraction

2.1 Washing buffer

PVP (polyvinylpyrrolidone)	2	g
Ascorbic acid	1.76	g
1M Tris buffer pH 8.0	20	ml
2- mercaptoethanol	4	ml

The steriled water was added for adjusting volume to 200 ml and stored at 4° C

2.2 2X CTAB lysis buffer

CTAB	4	g
1M Tris buffer pH 8.0	20	ml
1M EDTA pH 8.0	8	ml
NaCl	16.36	g
2- mercaptoethanol	1	ml

The steriled water was added for adjusting volume to 200 ml and stored at 4° C

2.3 20 % Polyethylene glycol 6000 (PEG)

PEG	20	g
NaCl	14.61	g

The steriled water was added for adjusting volume to 200 ml and stored at 4° C

2.4 50X Tris-Acetate-EDTA buffer (TAE buffer)

Tris base	242	g
Acetic Acid	57.1	ml
0.5 M EDTA	100	ml

The distilled water was added for making up volume to 1 liter and its pH was adjusted to 8.5 by using KOH. Then, it was autoclaved at 121 °C, 15 psi

for 15 min. and stored at room temperature.

2.5 50X Tris-EDTA buffer (TE buffer)

1M Tris buffer pH 8.0	500	ml
0.5M EDTA pH 8.0	100	ml

The distilled water was added for adjust volume to 1 liter and autoclaved at 121° C, 15 psi for 15 min, followed by storing at room temperature.

2.6 1% Agarose gel

Agarose	1	g
1X TAE buffer	100	ml

2.7 STET buffer

8% sucrose	16	g
50mM Tris-HCl pH 8.0	10	ml
50mM EDTA pH 8.0	20	ml
0.1% Triton X-100	0.2	ml

The distilled water was added for adjust volume to 200 µl and autoclaved at 121 °C, 15 psi for 15 min, followed by storing at room temperature.

3. Reagent for heat shock transformation

3.1 Super Optimal Broth (SOB)

Bacto tryptone	20	g
Yeast extract	5	g
NaCl	0.5	g
250 mM KCl	10	ml

Adjust the pH to 7.0 with 5 N NaOH and make to 1 liter with distilled water then autoclave. After autoclaving, cool down, add sterile 100 mM $MgCl_2$ 100 ml and store at 4°C.

3.2 Transformation buffer (TB) 500 ml

10 ml Pipes	1.512	g
15 mM $CaCl_2$	1.103	g
250 mM KCl	7.320	g

Adjust the pH to 6.7 with 5 N KOH and add 56 mM $MnCl_2$ (~5.443 g), filter-sterile with 0.45 μm filter membrane and store at 4° C

4. Preparation for polyacrylamide gel electrophoresis

4.1 Stock reagent

4.1.1 30 % Acrylamide, 0.8 % bis-acrylamide 100 ml

Acrylamide	29.2	mg
N,N–methylene-bis-acrylamide	0.8	g

The distilled water was added for adjust volume to 100 ml.

4.1.2 1.5 M Tris-HCl, pH 8.8

Tris (hydroxymethyl)-aminomethane	18.17	g

The pH of solution was adjusted to 8.8 by HCl and the volume was brought up to 100 ml distilled water.

4.1.3. 1 M Tris-HCl, pH 6.8

Tris (hydroxymethyl)-aminomethane	12.1	g

The pH of solution was adjusted to 6.8 by HCl and the volume was brought up to 100 ml distilled water.

4.1.4 10 % Ammonium persulfate

Ammonium persulfate	0.1	mg
Distilled water	1	ml

4.1.5 10 % SDS

SDS	0.1	mg
Distilled water	1	ml

4.2 5X Sample buffer for SDS-PAGE

1M Tris-HCl, pH 6.8	0.6	ml
Glycerol	2.5	ml
10 % SDS	2	ml
2-mercaptoethanol	0.5	ml
1 % bromophenol blue	1	ml
Distilled water	3.4	ml

4.3 SDS-PAGE

4.3.1 12% separating gel

Distilled water	3.3	ml
1.5 M Tris-HCl, pH 8.8	2.5	ml
30 % acrylamide solution	4	ml
10 % SDS	0.1	ml
10 % Ammonium persulfate	0.1	ml
TEMED	0.004	ml

The distilled water was used for adjusting volume to 1 liter.

4.3.2 5.0% stacking gel

Distilled water	1.4	ml
1. M Tris-HCl, pH 6.8	0.25	ml
30 % acrylamide solution	0.33	ml
10 % SDS	0.02	ml
10 % Ammonium persulfate	0.02	ml
TEMED	0.002	ml

The distilled water was used for adjusting volume to 1 liter.

4.4 10X Electrophoresis buffer for SDS-PAGE 1 L

Tris (hydroxymethyl)-aminomethane	30.3	g
Glycine	144	g
SDS	10	g

The distilled water was used for adjusting volume to 1 liter.

4.5 Staining solution

Coomassie brilliant blue R-250	0.5	g
Methanol	250	ml
Glacial acetic acid	50	ml

The distilled water was used for adjusting volume to 500 ml and mixed well.

4.6 Destaining solution

Methanol	100	ml
Glacial acetic acid	100	ml

Add distilled water to 1000 ml and mix

5. Preparation of solutions for hydrolysis assays

5.1 1M sodium citrate buffer, pH 4.8

Sodium citrate dehydrate	29.44	g
Distilled water	80	ml

The pH of solution was adjusted to 4.8 by HCl and brought the volume up to 1,000 ml distilled water. Stored at 4° C and diluted to 50 mM sodium citrate buffer before use.

5.2 DNS reagent

Distilled Water	1416	ml
3,5-Dinitrosalicylic acid	10.6	g
NaOH	19.8	g

Dissolve above, then add:

Rochelle salts (Na-K tartarate)	306	g
Phenol (melt at 50°C)	7.6	ml
Na metabisulfite	8.3	g

Stored at 4°C in amber glass bottle

APPENDIX B

Methods

1. Phenol/Chloroform Extraction

1) Add an equal volume of phenol: chloroform (1:1) (at least 200 µl) to digestion reaction
2) Mix well, spin at 13000 rpm for 5 min.
3) Carefully remove the aqueous layer (upper phase) to a new tube, avoid the interface
4) Repeat step 1-3 until an interface is no longer visible
5) To remove traces of phenol, add an equal volume of chloroform to the aqueous layer
6) Spin at 13000 rpm for 2 min.
7) Remove aqueous layer (upper phase) to new tube
8) Clean sample by ethanol precipitation

2. Ethanol Precipitation

1) Add 1/10 volume of 3M NaOAc pH 4.6
2) Add 3.5 volume of 95% ethanol
3) Spin at 13000 rpm for 20 min
4) Wash with 200 µl of 70% ethanol
5) Air dry
6) Resuspend with 10 µl sterile distilled water

APPENDIX C

Protein determination

1. Preparation of solutions for protein assays

The assay reagent is prepared by diluting 1 volume of the bradford reagent with 4 volumes of distilled H_2O. Then solution was filtered by filter paper, Whatman No. 1. The solution should appear brown, and have a pH of 1.1. It is stable for 4 weeks in a brown bottle at 4°C.

2. Standard curve of BSA

Protein standards should be prepared in the same buffer as the samples to be assayed. A convenient standard curve can be made using bovine serum albumin (BSA) with concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/ml. The method is as follows;

2.1 Prepare stock bovine serum albumin with concentration 20 mg/ml.

2.2 20 mg/ml BSA was diluted with distilled water as 0.1-0.6 mg/ml (Table C-1)

Table C-1 Composition for standard BSA

BSA (mg)	Reagent volume (μl)	
	stock of BSA	dH_2O
0	-	1000
0.1	5	995
0.2	10	990
0.3	15	985
0.4	20	980
0.5	25	975
0.6	30	970

2.3. Pipet 5 μl of each standard from stock solution was into 96 wells microplate. Protein solutions are normally assayed in duplicate.

2.4. Add 300 μl of diluted dye reagent to each well and incubated at room temperature for 5 minutes.

2.5. The product was measured by an increase in the absorbance at 595 nm with micro plate spectrophotometer.

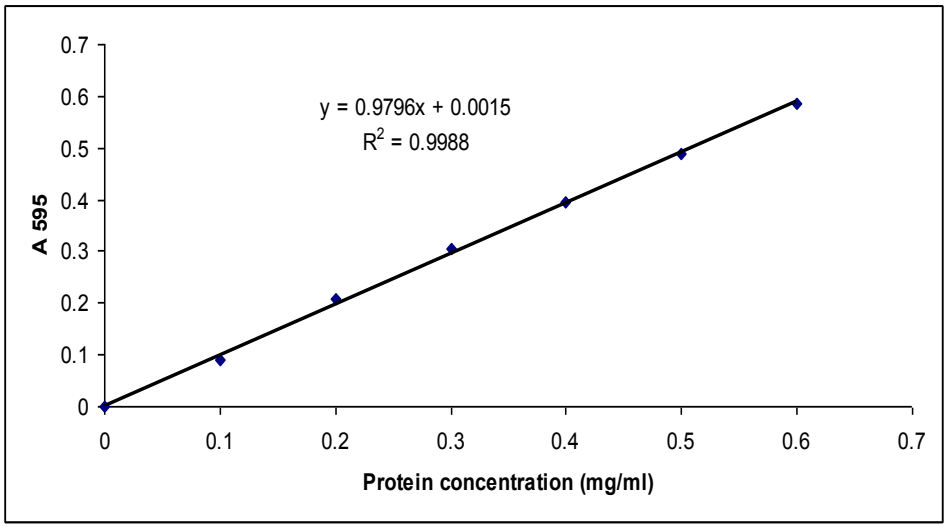

Figure C-1 Calibration curve for protein determination by Bradford's method

3. Calculation of total protein

The absorbance value at 595 nm was calculated by:

$$Y = aX + b$$

Where

Value X axis = Standard protein concentration (mg/ml)

Value Y axis = Absorbance at 595 nm

APPENDIX D

Calculation of the cellulase activity

1. Standard curve of glucose

Glucose standard solutions should be prepared in 50 mM citrate buffer, pH 4.8. A convenient standard curve can be made using glucose with concentrations of 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1 and 2.4 mg/ml. The method is as follows;

1.1. Prepare stock glucose with concentration 10 mg/ml.

1.2. 10 mg/ml glucose was diluted with distilled water as 0.1-0.6 mg/ml (Table D-1)

Table D-1 Composition for standard glucose

Glucose (mg)	Reagent volume (μl)	
	stock of glucose	50 mM Citrate buffer, pH 4.8
0	0	1000
0.3	30	970
0.6	60	940
0.9	90	910
1.2	120	880
1.5	150	850
1.8	180	820
2.1	210	790
2.4	240	760
2.7	270	730

1.3. Pipet 50 μl of each standard from stock solution into eppendorf tube. Incubated at 50°C for 10 min and then terminated by adding 250 μl

dinitrosalicylic acid (DNS). The mixture was boiled in a boiling bath for 5 min

1.4. The product was measured by an increase of the absorbance at 540 nm with micro plate spectrophotometer.

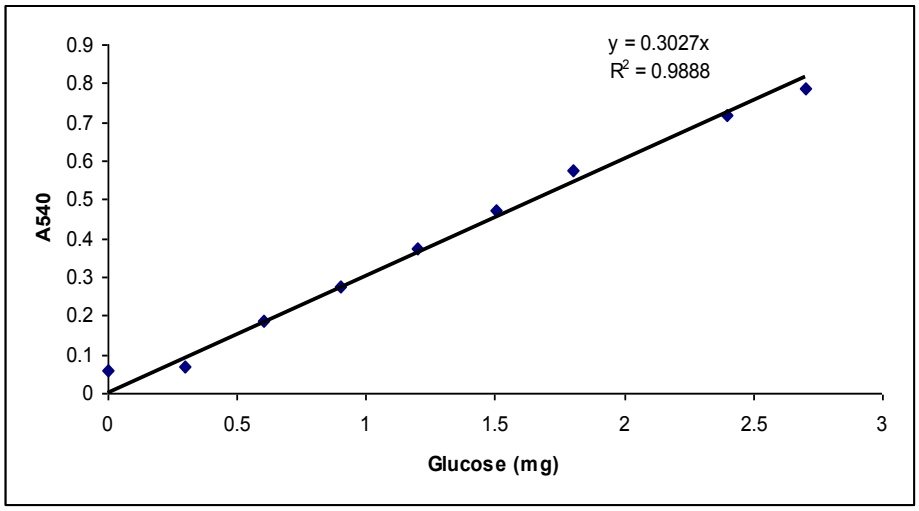

Figure D-1 Calibration curve for glucose determination by DNS method

2. Calculation of total glucose

The absorbance value at 540 nm was calculated by:

$$Y = aX + b$$

Where

Value X axis = Standard Glucose (mg)

Value Y axis = Absorbance at 540 nm

3. Calculation of the cellulase activity

1 IU = 1 mol min^{-1} of liberated hydrolysis product

= 0.18 mg mm^{-1} when the product is glucose

The critical amount of glucose in the CMC assay is X mg:

X mg glucose = X/0.18 µmol

Activity (µmol /min) = mg glucose× (1/ (0.18 × volume of enzyme (ml) × time(min)))

Specific activity = Activity / protein concentration

4. **Calculation of % conversion**

Table D-2 Components of biomass (Wongwatanapaiboon, 2008)

Biomass	Components of biomass (%)				
	Cellulose	Hemicellulose	Lignin	Ash	Other
Pangola	33.07	35.46	4.47	0.28	26.72
Dwarf Napia	35.64	34.19	3.66	0.13	26.38
Rusy	33.64	34.01	4.56	0.27	27.52

Initial substrate = (% cellulose + % hemicelluloses) x mass of sample

i.e., initial substrate in pangola

Initial substrate = (33.07 + 35.46)/100 x total lignocelluloses

Calculate percent conversion of each substrate as

% Conversion = (E/D) x 100

E = Total glucose released

D = Initial substrate

BIOGRAPHY

Miss Manunya Martla was born on Febuary 25, 1985 in Nakhon Si Thammarat, Thailand. She graduated with the Bachelor Degree of Science in chemistry from Department of chemistry, Faculty of Science, Mahidol University in 2007 and furthered her Master's Degree of Science in Biochemistry, Department of Biochemistry, Faculty of Science, Chlalongkorn University. During her postgraduate studies, she has got the international proceeding by presenting part of her work in this thesis as poster presentation at The 22nd Annual Meeting of the Thai Society for Biotechnology. The theme of symposium was "International Conference on Biotechnology for Healthy Living" on the topic of "Expression and characterization of recombinant endoglucanase from *Trichoderma reesei* for the production of cellulosic ethanol" at Prince of Songkla University, Trang Campus, Thailand on 20-22 October, 2010. As for the national level, she also presented her work as the poster presentation at The 1st Joint Symposium CU-NUT on the same topic at Chulalongkorn University, Bangkok, Thailand on 23-24 December, 2010.