MICROBIAL CONVERSION OF CELLULOSE TO SUGARS BY BACTERIAL ENZYME

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A Dissertation Submitted in Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy
The Petroleum and Petrochemical College, Chulalongkorn University
in Academic Partnership with
The University of Michigan, The University of Oklahoma,
and Case Western Reserve University
2017

Thesis Title:	Microbial Conversion of Cellulose to Sugars by Bacterial Enzyme
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ABSTRACT

5281003063: Petrochemical Technology Program
Kessara Seneesrisakul: Microbial Conversion of Cellulose to Sugars by Bacterial Enzyme
Thesis Advisors: Prof. Sumaeth Chavadej and Prof. Erdogan Gulari
118 pp.
Keywords: Cellulase/ Cellulose/ Cloning/ Continuous bubble reactor/ Corncob/ Endoglucanase/ Microbial hydrolysis/ Pretreatment/ Sugars

This work studied the conversion of lignocellulosic materials to sugars, the intermediates for bioethanol production, by a bacterial enzyme. Due to the complex structure of lignocellulose, a cellulase enzyme cannot effectively access and hydrolyse cellulose fibers. Various pretreatment processes of lignocellulose have been studied to enhance enzymatic hydrolysis to date. In this work, corncob was firstly studied for microbial pretreatment using three microorganisms including two bacterial strains of *Bacillus subtilis* A 002 and *Cellulomonas* sp. TISTR 784 and a fungal strain of *Phanerochaete sordida* SK7. The results showed that the microbial pretreatment with *P. sordida* SK7 was the most effective for enhancing enzymatic hydrolysis with approximately 40% improvement, compared to no treatment. Whereas, *Bacillus subtilis* A 002 was able to hydrolyse some cellulose fraction in lignocellulose but the reducing sugar concentration was very low due to its simultaneously consumption. In the following part, the native cellulase-producing bacteria *B. subtilis* was developed to produce enzyme at high level using PCR-based cloning technique. Endoglucanse gene encoding from *B. subtilis* M015 was cloned into plasmid (pFLAG-CTS) and transferred into *E. coli* JE5505. The clone, named *E. coli* Glu5, could greatly produce endoglucanase with higher enzymatic activity than that of the native strain, approximately 17 times. Finally, *E. coli* Glu5 was used for a microbial hydrolysis of soluble cellulose (carboxymethyl cellulose) in a continuous bubble reactor. The results demonstrate the feasibility to consolidate the steps of enzyme production and enzymatic hydrolysis together in continuous system at a low organic loading rate of 5 kg/m^3d.

บทคัดย่อ

เกศรา เสนีย์ศรีสกุล : การเปลี่ยนแปลงวัสดุเซลลูโลสเป็นน้ำตาลโดยอาศัยการย่อยสลายด้วยเอนไซม์จากเชื้อแบคทีเรีย (Microbial Conversion of Cellulose to Sugars by Bacterial Enzyme) อาจารย์ที่ปรึกษา : ศ.ดร. สุเมธ ชวเดช และ ศ.ดร. เออดอร์แกน กูลารี 118 หน้า

งานวิจัยนี้มีจุดประสงค์เพื่อศึกษาการเปลี่ยนแปลงเศษวัสดุลิกโนเซลลูโลสที่เหลือใช้จากการเกษตรเป็นน้ำตาลโดยอาศัยการย่อยสลายด้วยเอนไซม์จากเชื้อแบคทีเรีย เพื่อใช้น้ำตาลเป็นสารตั้งต้นในกระบวนการผลิตไบโอเอทานอล เนื่องจากโครงสร้างของลิกโนเซลลูโลสมีความซับซ้อนจึงยากต่อการถูกย่อยสลายด้วยเอนไซม์ ดังนั้นกระบวนการบำบัดขั้นต้นของลิกโนเซลลูโลสเพื่อช่วยเพิ่มประสิทธิภาพการทำงานของเอนไซม์ให้ดียิ่งขึ้นจึงได้รับความสนใจศึกษาอย่างต่อเนื่องตั้งแต่อดีตจนถึงปัจจุบัน ในงานวิจัยนี้ ส่วนแรกซังข้าวโพดถูกนำมาใช้ในกระบวนการบำบัดขั้นต้นด้วยวิธีทางชีวภาพ (Biological Pretreatment) โดยใช้เชื้อจุลินทรีย์ 3 ชนิด ได้แก่ แบคทีเรีย 2 สายพันธุ์ (*Bacillus subtilis* A 002 และ *Cellulomonas* sp. TISTR 784) และเชื้อราสายพันธุ์ 1 สายพันธุ์(*Phanerochaete sordida* SK7) จากผลการทดลองพบว่าการบำบัดขั้นต้นด้วยเชื้อราให้ประสิทธิภาพสูงสุดในการช่วยส่งเสริมการย่อยสลายซังข้าวโพดด้วยเอนไซม์ไปเป็นน้ำตาล ในขณะเดียวกันพบว่า *Bacillus subtilis* A 002 มีความสามารถในการย่อยเซลลูโลสได้ดีแต่ได้ผลผลิตน้ำตาลต่ำเพราะแบคทีเรียใช้น้ำตาลเพื่อการเจริญเติบโตด้วย ในส่วนงานวิจัยต่อมาจึงมุ่งเน้นการพัฒนาสายพันธุ์แบคทีเรียเพื่อให้มีความสามารถสูงในการผลิตเอนไซม์เซลลูเลสโดยใช้เทคนิคพีซีอาร์ (PCR-based cloning technique) ในการโคลนยีนเอนโดกลูคาเนส (Endoglucanase) จากแบคทีเรียต้นแบบ *Bacillus subtilis* M015 เพื่อเชื่อมต่อเข้ากับดีเอ็นเอพาหะ (pFLAG-CTS) และนำเข้าสู่เซลล์ *E. coli* JE5505 แบคทีเรียสายพันธุ์ผสมใหม่นี้ถูกตั้งชื่อว่า *E. coli* Glu5 สามารถผลิตเอนโดกลูคาเนสที่มีความสามารถสูงขึ้นมากกว่าเซลล์ต้นแบบถึง 17 เท่า ในขณะเดียวกันพบว่ามีการใช้น้ำตาลน้อยกว่าเซลล์ต้นแบบ นอกจากนี้ยังพบว่า *E. coli* Glu5 มีความสามารถในการย่อยวัสดุเซลลูโลสในระบบถังปฏิกรณ์แบบต่อเนื่องชนิดเป่าฟองอากาศ (Continuous Bubble Reactor) โดยในการศึกษาเบื้องต้นพบว่า ค่าความสามารถสูงสุดในการเจริญเติบโตของ *E. coli* Glu5 และการย่อยวัสดุเซลลูโลสที่ละลายน้ำได้ (Carboxymethy cellulose, CMC) อยู่ที่อัตราการเติมสารออร์แกนิก (Organic loading rate, OLR) ที่ 5 กิโลกรัมต่อลูกบาศก์เมตรต่อวัน

ACKNOWLEDGEMENTS

It is my pleasure to thank all of the following individuals and organizations, who made this thesis successful.

First of all, I would like to express my deep and sincere gratitude to my advisor, Prof. Sumaeth Chavadej for giving me an opportunity to continue my research interest on this Ph.D. program. I have greatly received support and allowing me a freedom to carry out a research. At the same time, his guidance make me move forward through every problem that I have met.

My great honours and appreciations also go to my co-advisor Prof. Erdogan Gulari and Dr. Saadet Guralp Albarak for taking care of me and also giving me great advice and support during work at University of Michigan, USA.

My sincere thanks go to Prof. Ratana Jiraratananon, Prof. Adrian Flood, Prof. Suwabun Chirachanchai, Prof. Pitt Supaphol, Assoc. Prof. Boonyarach Kitiyanan and Dr. Ampira Charoensaeng for being my dissertation committees and giving useful suggestions on this work.

I am grateful for the scholarship and funding from Royal Golden Jubilee Ph.D Program; the Petroleum and Petrochemical College; the Center of Excellence on Petrochemical and Materials Technology, Thailand; and the Thai Oil Public Company Limited.

I am grateful for PPC faculties, staffs and all SC group members for their helpful during working on this research. I also offer my regards and blessings to my Ph.D. friends, Ms. Paweena Kanokkarn and Ms. Achiraya Jiraprasertwong, who continual encouraged and supported me at any respect from the initial to the final of the project.

Special thanks to Mr. Sahaporn Singtothong, who always provides taking care of me and helping me get through the difficult times.

Finally, all of the success would have been impossible without my family who are always beside me. This thesis is dedicated to my parents for their endless love, support and encouragement.

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CHAPTER I
INTRODUCTION

World-wide use of bioethanol as an alternative fuel has been steadily increasing because of main problems of the petroleum shortage and the environmental concern. Biomass conversion to ethanol generally consists of two steps including a hydrolysis of biomass into reducing sugars and a fermentation of reducing sugars to ethanol (Badger, 2002). Different kinds of biomass have been used as a feedstock which can be classified into three categories; simple sugar, starch and lignocellulosic materials (Balat and Balat, 2009). Present bioethanol production processes use edible plant materials like sugars and starches which are easily to be hydrolyzed. However, the edible feedstock is in a human food chain, therefore, it affects food supply and the price of the ethanol production (Scharf and Tartar, 2008; Alvira *et al.*, 2010). To overcome the feed conflict problems, lignocellulose biomass like agricultural residues is considered to be a promising alternative feedstock for sugar production.

The complex structure of cellulose, resulted from a lignin coverage and a rigid crystallinity of cellulose, is responsible for low sugar yield (Mosier *et al.*, 2005). Therefore, a pretreatment process is needed to reduce the biomass recalcitrance by breaking lignin seals and disrupting the crystalline structures of cellulose (Wan and Li, 2010). Compared with the other pretreatment methods (e.g. diluted acid, alkali extraction, steam explosion, and hydrothermolysis), biological pretreatment of lignocellulosic materials is an environmentally friendly process since there is no severe chemicals, less energy input, no requirement for pressurized and corrosion-resistant reactors, and less inhibition to fermentation (Keller *et al.*, 2003). There are two mechanisms of the biological pretreatment that enhances enzymatic hydrolysis: (1) lignin removal and (2) cellulose breaking down. White rot fungi have been receiving extensive attention for biodelignification of lignocellulosic biomass but several weeks to months are generally needed to obtain a high degree of lignin degradation (Vicentim and Ferraz, 2007; Wan and Li, 2010; Liew *et al.*, 2011). Bacterial pretreatment of office paper using *Sphingomonas paucimobilis* MK1 and *Bacillus circulans* MK2 showed a high sugar recovery (94%) within a short time (4-d pretreatment). The

bacterial strains acted as an endoglucanase to hydrolyze the amorphous areas of cellulose randomly (Kurakake *et al.*, 2007).

The enzymatic hydrolysis of cellulose requires effective cellulase enzymes. Due to the high cost of enzyme production, biofuel production from cellulose is not feasible at the present time (Maki *et al.*, 2009; Klein-Marcuschamer *et al.*, 2012). A consolidated bioprocess, combining cellulase production and conversion of cellulose into desired products as one step, has been the subject of increased research efforts in recent years (Olson *et al.*, 2012). The absence of cellulase-whole cells separation step can lowered overall process cost (Wu *et al.*, 2013). The term "microbial hydrolysis" referred to growing cultures of cellulolytic bacteria in the presence of cellulose substrate, which is to be hydrolyzed, without added enzyme (Lu *et al.*, 2006). Currently, there is little research available on cellulose hydrolysis with whole cells (Lu *et al.*, 2006; Wu *et al.*, 2013). Hence, a study on this issue is of great interest since it will lead to potential applications for a large scale biofuel production from lignocellulosic materials.

A number of cellulolytic bacteria from termite gut and other sources have been studied and continually developed by genetic engineering techniques to achieve desired properties and high productivity of endoglucanase for industrial applications (Rastogi *et al.*, 2010; Taechapoempol *et al.*, 2011; Zhang *et al.*, 2011; Akaracharanya *et al.*, 2014; Chuan Wei *et al.*, 2015; Cheng *et al.*, 2016). Biologically meditated processes are promising for energy conversion, especially the conversion of lignocellulosic biomass into bioethanol. Although, mostly batch or fed-batch bioprocesses have been studied, due to less complexity of these systems, continuous bioprocess is promising for capital cost reduction. For the process dealing with cell culture like microbial hydrolysis of cellulose, bubble reactors are of great interest. A bubble reactor works as a three phase reactor where microorganisms are utilized as solid suspensions in order to convert cellulose into sugars in liquid media and aeration is provided for cell growth and mixing (Bredwell and Worden, 1998).

In this research, there were 3 separated parts: (i) A study of biological pretreatment of corncob using cellulase producing bacteria, *Bacillus subtilis* A 002 and *Cellulomonas sp*. TISTR 784, comparing with the use of ligninase-producing fungus, *Phanerochaete sordida* SK7, for enhancement of enzymatic hydrolysis by commercial

cellulase enzyme, (ii) *Escherichia coli* expressing endoglucanase gene of *Bacillus subtilis* M 015 for enzymatic and microbial hydrolysis of different cellulosic materials, and (iii) the recombinant *E. coli* strain was used for microbial hydrolysis of carboxymethyl cellulose in an aseptic 3-L continuous bubble reactor (CBR) with a working volume of 1.5 L to determine the feasibility of consolidated process of enzyme production and enzymatic hydrolysis as a continuous system.

CHAPTER II
LITERATURE REVIEW

2.1 Bioethanol in Thailand

Bioethanol is widely used as a partial gasoline replacement for transportation in many countries. Using bioethanol can significantly reduce a limited fossil fuel use and greenhouse gas emission. Bioethanol derived from biomasses is a promising renewable energy because biomass is relatively clean (Sajjakulnukit *et al.*, 2005). Additionally, many researchers have reported the achievement of zero net carbon dioxide emission from use of bioenergy (Hoffert *et al.*, 2002; Demain, 2004; Demain *et al.*, 2005; Percival Zhang *et al.*, 2006). In order to convert biomass to ethanol, mainly two processes are involved including hydrolysis of cellulose in biomass to produce reducing sugars and fermentation of the sugars to ethanol (Sun and Cheng, 2002). The Thai government has continually supported production and promoted use of bioethanol in order to reduce import demand of crude oil, mitigate global warming impact and activate the grass root economy by stabilizing the income of farmers and generating employment in the local community (Silalertruksa and Gheewala, 2009). The manufacturing of vehicles compatible with E20 and E85 gasohol was also supported by the excise tax rate reduction. The ethanol consumption was aimed to increase from 1.2 billion liters in 2015 to 3.3 billion liters by 2021 and up to 4.1 billion liters by 2036 through gasohol intake (Preechajarn and Prasertsri, 2016). In 2014, there were 22 factories producing ethanol with a total capacity of 5.31 million liters per day and the demand of ethanol was at 3.25 million liters per day (Alternative Energy Development Plan (AEDP), 2015). Currently, bioethanol in Thailand is in first generation, produced from sugar-based and starch-based technology, which will be not practical in the future because sugar and starch supplies for ethanol production can directly affect food production became of the limited agricultural land (Sun and Cheng, 2002; Nguyen *et al.*, 2007; Nguyen *et al.*, 2008). Lignocellulosic biomass such as agricultural residues, grasses, sawdust, and wood chips are cheap, renewable, abundantly available resources for low-cost ethanol production, as second generation. Thailand is an agricultural based country with abundant crop products, such as

sugarcane, rice, corn, etc. Consequently, a large amount of agricultural wastes associated with the crop production can be a potential source for producing energy and also other more valuable chemicals. In 2010, the total amount of agricultural residues from 10 species of high productive crops, including sugarcane, paddy, oil palm, coconut, cassava, maize, groundnut, cotton, soybean, and sorghum was estimated to be around 72 million tons per year, only a small portion of 18 million tons was utilized for energy purposes, as shown in Table 2.1 (Sajjakulnukit *et al.*, 2005).

Although lignocellulose is potential resource for bioethanol production, its conversion process is more difficult than that of sugar and starch due to its complex structure. Thus, intensive research and development of conversion of lignocellulose into ethanol has been performed over the last few decades (Lange, 2007).

2.2 Lignocellulosic Materials

The chemical structure and the major organic components in biomass are important in the development of processes for producing derived fuel and chemicals. Lignocellulosic materials contain a mixture of carbohydrate polymers (cellulose and hemicellulose), lignin and a small amount of extractives. The basic structure of all woody biomass consists of three basic polymers: cellulose $(C_6H_{10}O_5)_n$, hemicellulose such as xylan $(C_5H_8O_4)_m$, and lignin $(C_9H_{10}O_3(OCH_3)_{0.9–1.7})_n$ in trunk, foliage, and bark. The carbohydrate polymers are tightly bound to lignin mainly through hydrogen bonding, but also through some covalent bonding (Saxena *et al.*, 2009). The structure of lignocellulosic materials is shown in Figure 2.1. Some common agricultural lignocellulosic materials investigated for the contents of cellulose, hemicellulose, and lignin, as listed in Table 2.2 (Sun and Cheng, 2002).

Table 2.1 Estimated amount of agricultural residues of Thailand in year 2010 (Sajjakulnukit *et al.*, 2005).

Product	Annual Production (Mt/Y)	Residue Part	Residue Available for Energy		Energy Use		Remaining Amount (Mt/Y)
			Residue Product Ratio	Amount (Mt/Y)	Factor	Amount (Mt/Y)	
Sugarcane	68.58	Bagasse	0.250	17.15	0.793	13.60	3.55
		Top and trash	0.298	20.42	0.000	0.00	20.42
Paddy	24.66	Husk	0.230	5.67	0.531	3.01	2.66
		Straw (top)	0.306	7.54	0.000	0.00	7.54
Oil palm	5.20	Empty bunches	0.263	1.37	0.030	0.04	1.33
		Fber	0.146	0.76	0.858	0.65	0.11
		Shell	0.031	0.16	0.588	0.09	0.07
		Frond	2.602	13.53	0.000	0.00	13.53
		Male bunches	0.233	1.21	0.000	0.00	1.21
Coconut	1.42	Husk	0.317	0.45	0.289	0.13	0.32
		Shell	0.127	0.18	0.413	0.07	0.11
		Empty bunches	0.049	0.07	0.144	0.01	0.06
		Frond	0.218	0.31	0.159	0.05	0.26
Cassava	14.59	Stalk	0.036	0.52	0.000	0.00	0.52
Maize	6.07	Corn cob	0.216	1.31	0.193	0.25	1.06
Groundnut	0.17	Shell	0.294	0.05	0.000	0.00	0.05
Cotton	0.08	Stalk	3.000	0.24	0.000	0.00	0.24
Soybean	0.36	Stalk, leaves, Shell	2.028	0.73	0.007	0.01	0.72
Sorghum	0.29	Leaves and stem	0.966	0.28	0.118	0.03	0.25
Total	121.40			71.95		17.95	54.00

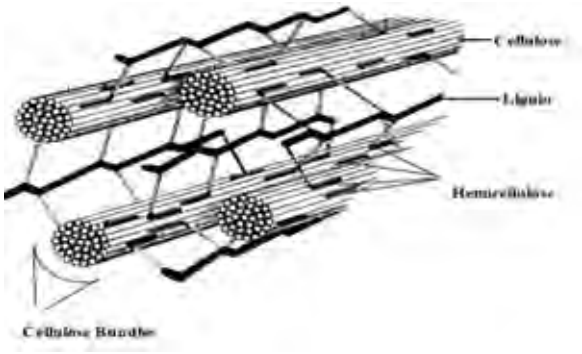


Figure 2.1 Structure of lignocellulosic materials (Scott *et al.*, 2002).

Table 2.2 Composition of some agricultural lignocellulosic materials (Sun and Cheng, 2002)

Lignocellulosic materials	Composition (%, dry basis)		
	Cellulose	Hemicellulose	Lignin
Hardwoods stems	40–55	24-40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Paper	85–99	0	0–15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15–20	80–85	0
Cotton seed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Waste papers from chemical pulps	60–70	10–20	5–10
Primary wastewater solids	8-15	No data	24–29
Swine waste	6	28	No data
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12

2.2.1 Cellulose

Cellulose fibers provide wood's strength and comprise 40–50% of dry wood. Cellulose is a high molecular weight (10^6 or more) of glucose polymer

containing linear chains of β-(1,4)-D-glucopyranose units in the alpha configuration and the substituents HO-2, HO-3, and CH_2OH are oriented equatorially (Figure 2.2). Cellulose $(C_6H_{10}O_5)_n$ consists of around 2,500–5,000 basic repeating unit of cellobiose unit—two glucose anhydride units. Crystal forming of cellulose by extensive inter- and intra-molecular hydrogen bond makes it completely insoluble in normal aqueous solutions; however, it is soluble in more exotic solvents such as ionic liquids or near supercritical water. The crystalline structure resists thermal decomposition better than hemicelluloses. Amorphous regions in cellulose contain waters of hydration, and free water is present within the wood. When this water is rapidly heated such as by steam explosion, the structure disrupts (Mohan *et al.*, 2006).

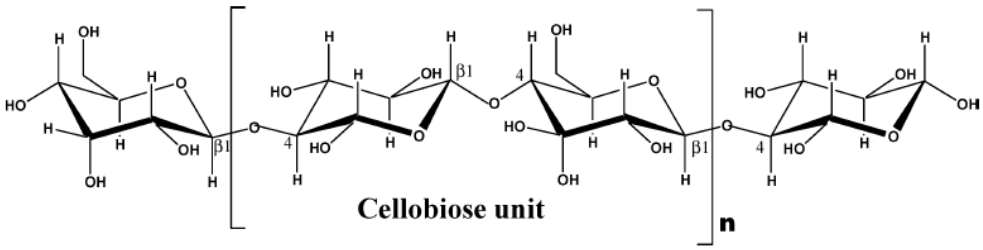


Figure 2.2 Chemical structure of cellulose (Mohan *et al.*, 2006).

2.2.2 Hemicellulose

Hemicelllulose is a second major constituent in lignocellulosic materials and also known as polyose because it contains a mixture of various polymerized monosaccharides such as glucose, mannose, galactose, xylose, arabinose, 4-O-methyl glucuronic acid and galacturonic acid residues, as shown in Figure 2.3 (Mohan *et al.*, 2006). Xylose is the predominant pentose sugar derived from the hemicellulose of most hardwood feedstocks while arabinose is the dominant amount of pentose sugar derived from various agricultural residues and other herbaceous crops which are being considered as dedicated energy crops. Arabinose makes only 2–4% of the total pentoses in hardwoods whereas it represents 10–20% of the total pentoses in many herbaceous crops. In addition, arabinose contents can be as high as 30–40% of

the total pentoses in corn fiber, a by-product of corn processing (Mohagheghi *et al.*, 2002).

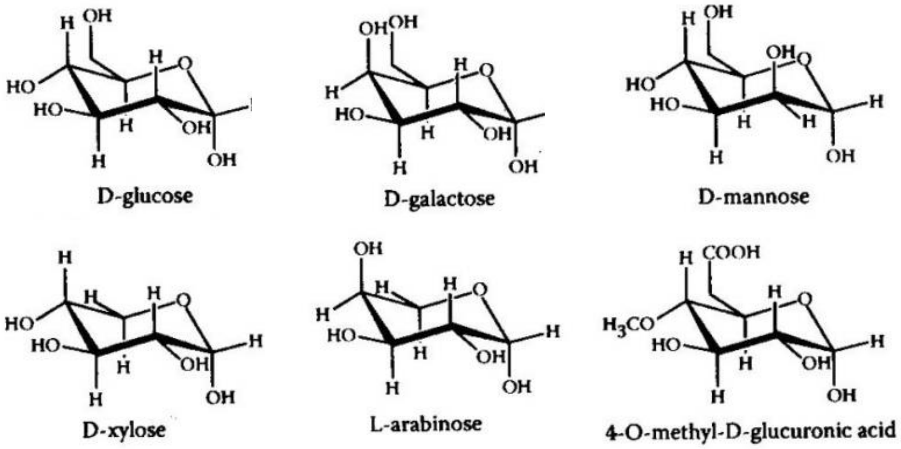
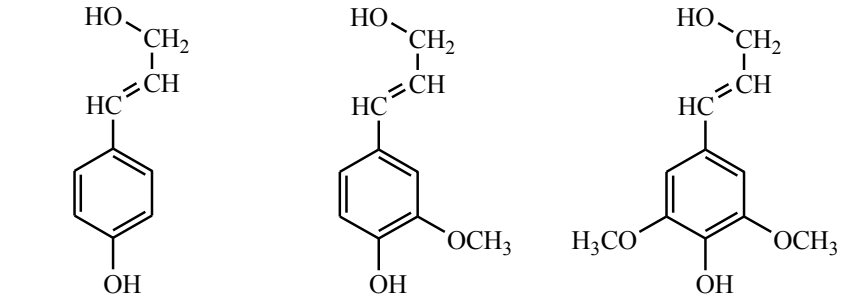


Figure 2.3 Basic component of hemicellulose (Mousdale, 2008).

2.2.3 Lignin

Lignin (15–25% of total feedstock dry matter) is highly branched, substituted, mononuclear aromatic polymers with the substituents connected by both ether and carbon-carbon linkages in the cell walls of the certain biomass, especially woody species, and are often adjacent to cellulose fibers to form a lignocellulosic complex (Saxena *et al.*, 2009). The principal building blocks of lignin composed of p-coumaryl alcohol (p-hydroxyphenyl propanol), coniferyl alcohol (guaiacyl propanol), and sinapyl alcohol (syringyl propanol) (Figure 2.4). This matrix comprises a variety of functional groups, such as hydroxyl, methoxyl and carbonyl, which impart a high polarity to the lignin macromolecule. Softwood lignin and hardwood lignin belong to the first and second category, respectively. Softwoods generally contain more lignin than hardwoods (Demirbas, 2007). Lignin is one of the drawbacks of using lignocellulosic materials in fermentation, as it makes lignocellulose resistant to chemical and biological transformation (Taherzadeh and Karimi, 2008).



p-Coumaryl alcohol Coniferyl alcohol Sinapyl alcohol

Figure 2.4 Monolignol building blocks of lignin (Sannigrahi *et al.*, 2010).

2.3 Conversion of Cellulose to Ethanol

Ethanol can be produced from lignocellulosic materials in various ways. The main features of the different ethanol processes are outlined in Figure 2.6. All processes comprise the main steps: hydrolysis of cellulose and hemicellulose to fermentable reducing sugars, fermentation of the sugars to ethanol, and product recovery and concentration by distillation. The main difference between the process alternatives is the hydrolysis steps, which can be performed by a dilute acid, concentrated acid or enzyme. Some of the process steps are more or less the same, independent on the hydrolysis method. For example, enzyme production will be omitted in an acid hydrolysis process; likewise, the recovery of acid is not necessary in an enzymatic hydrolysis process (Galbe and Zacchi, 2002).

Dealing with the environmental concern, the enzymatic hydrolysis seems to be the alternative process as it can be carried out under mild conditions with less chemical use. The enzymatic hydrolysis is usually catalyzed by cellulase enzymes, and the fermentation is carried out by yeasts or bacteria. However, the presence of lignin and hemicellulose in lignocellulosic materials makes the access of cellulase enzymes to cellulose difficult, as a result of reducing of hydrolysis efficiency. Aside from lignin and hemicellulose content, the factors that have been identified to affect the hydrolysis of cellulose include porosity (accessible surface area) of the waste materials and cellulose fiber crystallinity. Removal of lignin and hemicellulose, increase of porosity, and reduction of cellulose crystallinity in pretreatment processes can significantly

improve the hydrolysis (McMillan, 1994). The overall reaction in the ethanol production process is shown below:

$$(C_6H_{12}O_5)_n \rightarrow C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \qquad (2.1)$$

Theoretically, 1 kg of glucose will produce 0.51 kg of bioethanol and 0.49 kg of carbon dioxide. Practically, the actual yield is less than 100% because part of the glucose is used by microorganisms for growth (Balat and Balat, 2009).

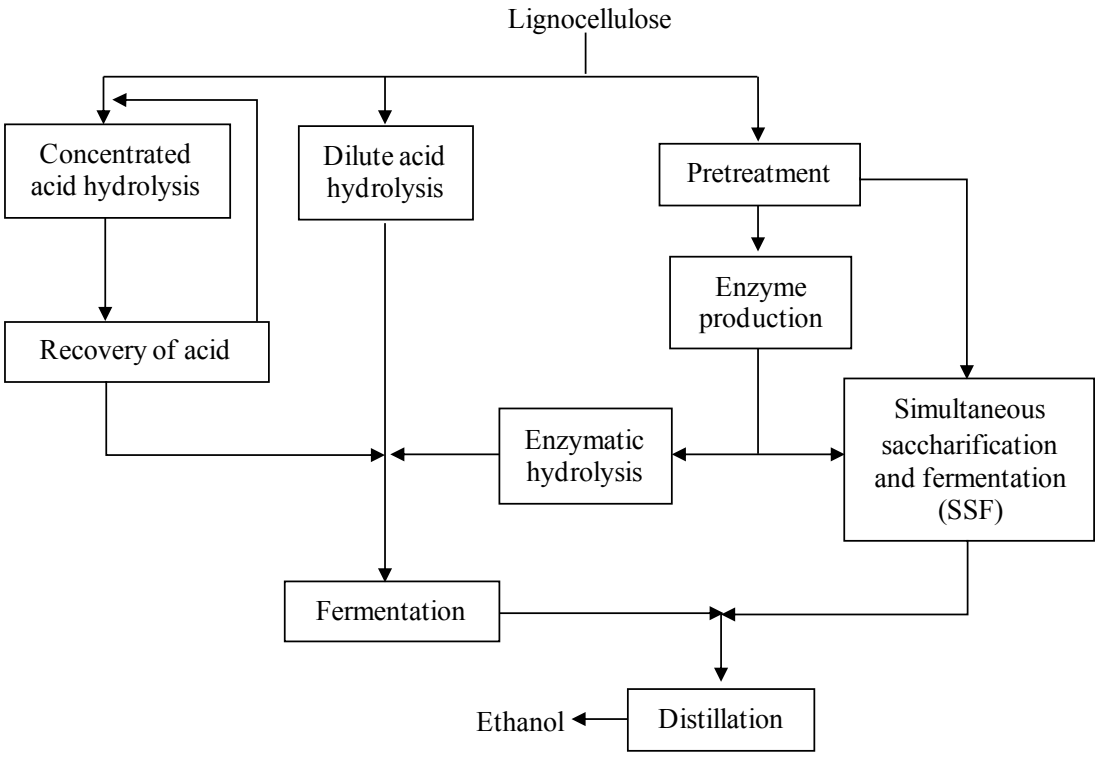


Figure 2.5 Production of ethanol from lignocellulosic materials (Galbe and Zacchi, 2002)

2.3.1 Pretreatment Step

The purpose of the pretreatment is to improve the efficiency of enzymatic hydrolysis which includes removing of lignin and hemicellulose, reducing of cellulose crystallinity, and increasing in the porosity of the materials, as shown in

Figure 2.6. Various techniques have been used for pretreatment of lignocellulosic materials and can be classified into 5 categories: (1) mechanical pretreatment, (2) physical pretreatment, (3) physicochemical pretreatment, (4) chemical pretreatment, and (5) biological pretreatment.

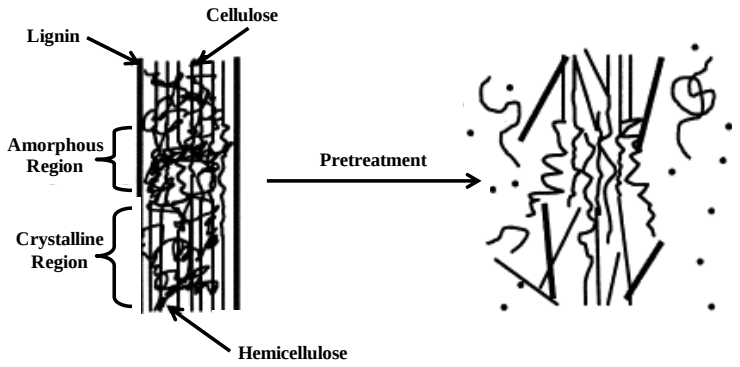


Figure 2.6 Schematic role of pretreatment of lignocellulosic biomass.

2.3.1.1 Mechanical Pretreatment

Mechanical pretreatment is normally a primary step when lignocellulosic materials are used as raw materials for conversion process. Lignocellulosic materials is basically comminuted by a combination of chipping, grinding and milling in order to reduce the size and crystallinity of lignocellulosic materials to be convenient for loading. Ball milling is a effective and low cost technique in breaking down the cellulose crystallinity and improving the digestibility of the biomass (Mtui, 2009).

2.3.1.2 Physical Pretreatment

Thermogravimetric treatment is one of the effective physical pretreatment methods based on elevated temperature and radiation. The temperature will be increased to 827 °C under either inert or oxidative atmosphere in order to improve the decomposition of cellulose, hemicellulose, and lignin (Mtui, 2009). On the other hand, pyrolysis is a thermochemical decomposition of organic materials at the temperature range of 327–927 °C without the participation of oxygen. It involves

the simultaneous change of chemical composition and physical phase. Cellulose rapidly decomposes to produce gaseous products and residual char (Kilzer and Broido, 1965; Shafizadeh and Bradbury, 1979). Irradiation can cause significant breakdown of liognocellulose. Microwave irradiation pretreatment at 700 W at various exposure times caused of weight loss due to the decomposition of cellulose, hemicellulose and lignin. The rate of decomposition was found to be considerably enhanced by an addition of alkali (Zhu *et al.*, 2006; Mtui, 2009).

2.3.1.3 Physicochemical Pretreatment

Physicochemical pretreatment is the combination of chemical and physical treatment systems, leading to dissolving hemicellulose and alteration of lignin structure and providing an improved accessibility of the cellulose for hydrolytic enzymes (Hendriks and Zeeman, 2009). Various successful physico-chemical pretreatments include thermochemical treatments such as steam explosion (autohydrolysis), liquid hot water (LHW), ammonia fiber explosion (AFEX) and CO_2 explosion (Sun and Cheng, 2002). These techniques are similar in concept to each other; chipped biomass is treated with high-pressure saturated steam, liquid ammonia or CO_2 and then the pressure is swiftly reduced, making the materials to undergo an explosive decompression.

Steam explosion is typically initiated at a temperature of 160–260 °C (corresponding pressure of 0.69–4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicellulose degradation and lignin transformation due to the high temperature, thus increasing potential of cellulose hydrolysis. A variety in addition of H_2SO_4, SO_2, and CO_2 in steam explosion can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds, and lead to more complete removal of hemicellulose. The advantages of steam explosion pretreatment include low energy requirement, as compared to mechanical comminution and no recycling or environmental costs. However, there have limitations in aspect of destruction of a portion of the xylan fraction, incomplete disruption of the lignin-carbohydrate matrix, and generation of compounds that may be inhibitory to microorganisms used in downstream processes (Mackie *et al.*, 1985; Sun and Cheng, 2002). On the contrary,

wet oxidation pretreatment at 200–210 °C in the presence of alkali or Na_2CO_3 led to lignocellulose solubilization and better enzymatic convertibility to value-added products (Mtui, 2009).

LHW pretreatment utilizes pressurized hot water at a pressure less than 5 MPa and in the temperature range of 170–230 °C for several minutes followed by decompression up to atmospheric pressure. Bagasses, corn stalk and straws of wheat, rice and barley pretreated by LHW could achive 80–100 % conversion of hemicellulose to produce 45–65 % xylose (Sun and Cheng, 2002; Cardona and Sánchez, 2007).

On the other hand, in AFEX pretreatment, the dosage of liquid ammonia ranging from 1–2 kg ammonia/kg dry biomass at a temperature of 90 °C, and a residence time of 30 min could significantly improve the saccharification rates (Chundawat *et al*., 2006; Martín and Thomsen, 2007). In CO_2 explosion process, 75 % of the theoretical glucose released during 24 h of the enzymatic hydrolysis was reported (Sun and Cheng, 2002). Ethanol yield of up to 83 % of the theoretical value was achieved for lignocellulosic materials subjected to physicochemical treatment (Jeoh and Agblevor, 2001; Mtui, 2009).

2.3.1.4 Chemical Pretreatment

Chemicals ranging from oxidizing agents, acids, alkalis and organic solvents can be used to decompose lignin, hemicellulose and cellulose in lignocellulosic materials. Powerful oxidizing agents such as ozone and H_2O_2 effectively degrade lignin, leading to the enhancement of the susceptibility to enzymatic hydrolysis. Ozonolysis does not produce toxic residues for the downstream processes and the reactions are carried out at room temperature and pressure. However, a large amount of ozone is required, making the process expensive (Sun and Cheng, 2002).

In acid pretreatment processes, a concentrated acid or dilute acid such as H_2SO_4 and HCl have been used to treat lignocellulosic materials. A concentrated acid is powerful but toxic, corrosive and hazardous. In addition, a recovery step is required to make the process economically feasible (Von Sivers and Zacchi, 1995). The dilute acid hydrolysis was successfully developed for pretreatment

at both moderate and high temperatures. It could achieve a high xylan-to-xylose conversion yield which is necessary for overall process economics because xylan accounts for up to a third of lignocellulose (Hinman *et al.*, 1992). Although the dilute acid pretreatment can significantly improve the cellulose hydrolysis, its cost is usually higher than other physicochemical pretreatment processes such as steam explosion or AFEX. A pH neutralization step is necessary for the downstream enzymatic hydrolysis or fermentation processes (Sun and Cheng, 2002).

On the other hand, some bases can also be used for pretreatment of lignocellulosic materials such as NaOH, $Ca(OH)_2$, NaOH-urea, Na_2CO_3. The mechanism of alkali hydrolysis is supposed to be saponification of intermolecular ester bonds crosslink hemicelluloses and other components, for example, lignin and other hemicellulose, When these pretreatments are performed by using 0.5–2 M alkali at 120–200 °C, they substantially facilitate saccharification, resulting in separation of structural linkages between lignin and carbohydrates, disruption of the lignin structure, an increase in internal surface area, a decrease in the degree of polymerization and a decrease in crystallinity (Tarkow and Feist, 1969; Sun and Cheng, 2002). The digestibility of NaOH-treated hardwood increased from 14 % to 55 % with the decrease in lignin content from 24–55 % to 20 %. However`, no effect of the dilute NaOH-pretreatment was observed for softwoods with lignin content greater than 26% (Millett *et al.*, 1976). Ammonia was also used for the pretreatment to remove lignin. Iyer and coworker described an ammonia recycled percolation process (temperature, 170 °C; ammonia concentration, 2.5–20 %; reaction time, 1 h) for the pretreatment of corncobs/stover mixture and switchgrass. The efficiency of delignification was 60–80 % for corncobs and 65–85 % for switchgrass (Iyer *et al.*, 1996).

Recent studies have shown that when acids are combined with alkali, they play a more effective role in lignocellulosic material pretreatment than acids and alkalis alone (Damisa *et al.*, 2008).

Organic acids such as oxalic, acetylsalicylic and salicylic acid can be used as catalysts in the organosolv process whereby an organic or aqueous organic solvent mixture with inorganic acids (HCl or H_2SO_4) are used to break the internal lignin and hemicellulose bonds. The organic solvents used in the process

include methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol (Sun and Cheng, 2002). The use of a dicarboxylic acid catalyst, maleic acid, for hemicellulose hydrolysis in corn stover overcame the technical and economic hurdle of hemicellulose hydrolysis (Lu and Mosier, 2007; Mtui, 2009).

Recently, a new interesting pretreatment method, which can enhance the sequential cellulose hydrolysis rate (and is more environmentally friendly), is solvent pretreatment using ionic liquids (ILs) to reduce the crystallinity of the cellulose (Liu and Chen, 2006; Zhao *et al*., 2009). It was reported that 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), one type of effective ILs, could dissolve up to 25 wt % of the cellulose without any degradation products. The precipitation of [BMIM]Cl-dissolved cellulose could be achieved by adding water or alcohol. After the IL-treatment step, the cellulose structure was found to change from crystalline to amorphous, which resulted in a significant increase in the enzymatic hydrolysis rate and cellulose conversion (Swatloki *et al.*, 2002).

2.3.1.5 Biological Pretreatment

Biological treatment involves the use of whole organisms or enzymes in pretreatment of lignocellulosic materials. Both fungi and bacteria have been used for biotreatment of lignocellulose materials; however, commercial preparations of fungal and bacterial hydrolytic and oxidative enzymes are also widely used instead of these microorganisms. The microscopic filamentous structures of fungi generally are much larger than bacteria and usually are 5-10 μm in width, whilst, most of bacteria fall into a size range of 0.5-3.0 μm (Speight, 2000). Fungal pretreatment of agricultural residues is a new method for improvement of digestibility by degradation of lignin and hemicellulose in waste materials (Mtui, 2009). Brown-rot fungi mainly attack cellulose, while white- and soft-rot fungi attack both cellulose and lignin. White rot fungi *Irpex lacteus* CD2 were used to destroy hemicellulose and lignin which enhanced saccharification of corn stover (Xu *et al*., 2010). Interestingly, recombinant strains of *Saccharomyces cerevisiae* have been genetically engineered to carry out simultaneous saccharification and fermentation (SSF) to produce extracellular endoglucanase and β-glucosidase that are able to ferment cellulose and hemicellulose

to 6-carbon and 5-carbon sugars and subsequent fermentation to ethanol (Wisselink *et al*., 2007). Bacterial pretreatment of lignocellulosic materials involves both anaerobic and aerobic systems. Anaerobic degradation utilizes usually mesophillic, rumen derived bacteria (Yue *et al*., 2008). Aerobic-anaerobic systems have an upper hand when it comes to degradation of lignocellulosic materials richer in lignin content (Mshandete *et al*., 2008) while in aerobic system alone. *Actinomycete Streptomyces Griseus* is able to produce high levels of extracellular hydrolytic enzyme that degrade lignocellulose (Arora *et al*., 2005). *Escherichia coli* and *Klebsiella oxytoca* strains have been genetically engineered to produce microbial biocatalysts that produce bioethanol from lignocellulosic materials (Peterson and Ingram, 2008). Biological pretreatment with two bacterial strain *Sphingomonas paucimobilis* MK1 and *Bacillus circulans* MK2 was studied to improve enzymatic hydrolysis of office paper. The strains act as endoglucanase, which hydrolyzes amorphous areas randomly and gave high sugar recovery after hydrolysis (Kurakake *et al*., 2007). Enzymatic pretreatment of lignocellulose materials utilizes hydrolytic and oxidative enzymes which are mainly derived from fungi and bacteria. The advantages and disadvantages of all the pretreatment methods discussed above are summarized in Table 2.3.

2.3.2 Enzymatic Hydrolysis Step

2.3.2.1 Cellulase and Hydrolysis Mechanism

Cellulase refers to a group of enzymes that contribute to the degradation of cellulose to glucose. Several cellulase components can form a cellulase complex which synergistically hydrolyses cellulosic substrates. Studies of the cellulolytic enzyme system include the mode of action of cellulase, activity of each enzyme component, synergistic action among the enzyme components, and the inhibitory effect on the enzyme action by the reaction intermediates and products (Gan *et al.*, 2003). The widely accepted mechanism for enzymatic cellulose hydrolysis involves synergistic actions by three different subenzymes: endoglucanases (1,4-β-D-glucan-4-glucanohydrolase, E.C. 3.2.1.4) randomly hydrolyze cellulose chain at intramolecule β-1,4-glucosidic bonds to produce new chain ends; exoglucanases (1,4-β-D-glucan cellobiohydrolase, E.C. 3.2.1.91) processively cleave chain ends of cellulose to release cellobiose or glucose; and β-glucosidases (E.C. 3.2.1.21) hydrolyze

cellobiose to glucose. The three hydrolysis processes occur simultaneously, as shown in Figure 2.7 (Percival Zhang *et al.*, 2006). Primary hydrolysis occurring on the surface

Table 2.3 Advantages and disadvantages of pretreatment

Pretreatment	Advantages	Disadvantages	Reference
Mechanical comminution	Improve the digestibility of biomass	Require exorbitant amount of energy	(Kumar *et al.*, 2009)
Steam explosion (autohydrolysis)	- Low energy requirement compared to mechanical comminution - No recycling or environmental costs	- Formation of inhibitory compounds - Destruction of xylan fraction - Incomplete disruption of the lignin-carbohydrate matrix	(Dimian and Bildea, 2008)
Ammonia fiber explosionb (AFEX)	- Significantly improve saccharification rates of various herbaceous crops - Not produce inhibitors for downstream biological process - Not require small particle size for efficacy	Not very effective for biomass with high lignin content	(Kumar *et al.*, 2009)
CO2 explosion	- More cost effective than ammonia fiber explosion - No formation of inhibitory compounds	Low yield compared to steam or ammonia explosion	(Kumar *et al.*, 2009)
Ozonolysis	- Effectively remove lignin - Not produce toxic residues for the downstream process - Carry out at room temperature and pressure	Large amount of ozone required, making the process expensive	(Kumar *et al.*, 2009)
Acid hydrolysis	- Achieve high xylan-to-xylose conversion yields (less severe conditions) - Significant improve cellulose hydrolysis	- Higher cost than some physico-chemical pretreatment - Need neutralization of pH	(Dimian and Bildea, 2008)
Alkaline hydrolysis	- Decrease degree of polymerization and crystallinity - Separation of structural linkages between lignin and carbohydrates - Disruption of lignin structure	No effect for soft woods with lignin content greater than 26%	(Dimian and Bildea, 2008)
Organosolv	Hydrolyzes lignin and hemicelluloses	- Solvents need to be drained from the reactor, evaporated, condensed, and recycled - High cost	(Dimian and Bildea, 2008)
Biological pretreatment	- Mild environmental conditions - Low energy requirement	Very low hydrolysis rate	(Kumar *et al.*, 2009)

of substrates releases soluble sugars with a degree of polymerization (DP) up to 6 into the liquid phase by endoglucanases and exoglucaases. The primary step is considered

to be the rate-limiting step of hydrolysis mechanism. Secondary hydrolysis takes place in the liquid phase by β-glucosidase. This enzyme hydrolyzes cellobiose from primary hydrolysis into glucose. During cellulose hydrolysis, the changing of solid substrate characteristics is varied with time, including changes in a number of cellulose chain ends, resulting from endoglucanase and exoglucanase, and changes in cellulose accessibility, resulting from substrate consumption and cellulose fragmentation. Synergistic effects between endoglucanase and exoglucanase causes changes at the substrate's surface along the time, resulting in rapid changes in hydrolysis rates (Percival Zhang *et al.*, 2006).

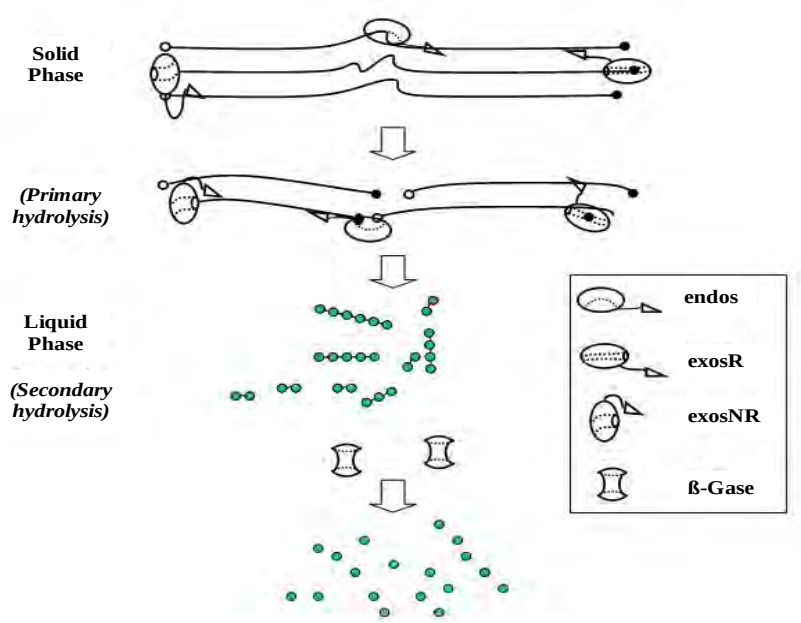


Figure 2.7 Mechanism of hydrolysis processes involve synergistic actions by three different enzymes: endoglucanases, exoglucanases, and β-glucosidases (Percival Zhang *et al.*, 2006).

2.3.2.2 Cellulase Sources

Cellulolytic enzymes have been continually searched, screened, and isolated from several sources from environment (pool, soil, and mud) or animal species like cattle, crayfish, and insect along the time to obtain the novel one (Lynd *et al.*, 2002). Cellulase are induced by a wide type of microorganisms in nature.

They are generally produced by saprophytic microorganisms growing on dead and decaying organic matters and some are produced by plant pathogens. Most cellulase-producing microbes are isolated from soil obtained from forest and nature reservoirs, hot spring, compost, sewage, animal manure, and bovine rumen. Insects, such as termites, have been of interest to be a source of cellulase-producing microbe because they require symbiotic relationships with bacteria and fungi to break down cellulose (Wenzel *et al.*, 2002; Taechapoempol *et al.*, 2011). However, some insects have now been shown to produce their own cellulases in the midgut or salivary glands.(Fischer *et al.*, 2013). Some examples of native cellulase-producing microbes that have been isolated are listed in Table 2.4.

2.3.2.3 Cellulase-Producing Bacteria

Cellulase-producing bacteria can be classified by using oxygen as growth factor and different in cellulolytic strategy into three main groups : (1) fermentative anaerobes, typically gram-positive (*Clostridium, Ruminococcus* and *Caldicellulosiruptor*) but containing a few gram-negative species, (2) aerobic gram-positive bacteria (*Cellulomonas* and *Thermobifida*), and (3) aerobic gliding bacteria (*Cytophaga* and *Sporocytophaga*), as shown in Table 2.5 (Lynd *et al.*, 2002).

Anaerobes degrade cellulose primarily via complex cellulase systems Cellulolytic enzymes in *Clostridium thermocellum* cultures are typically distributed both in the liquid phase and on the surface of the cells. Several anaerobic species that utilize cellulose do not release measurable amounts of extracellular cellulase, and instead have localized their complexed cellulases directly on the surface of the cell or the cell-glycocalyx matrix. Most anaerobic cellulolytic species grow optimally on cellulose when attached to the substrate, and in at least a few species this adhesion appears to be obligate. Cellulolytic anaerobes resemble other fermentative anaerobes in that their cell yields are low, with the bulk of substrate being converted to various fermentation end products, including ethanol, organic acids, CO_2, and H_2.

Aerobic bacteria utilize the cellulose through the production of substantial amounts of extracellular cellulase enzymes that are freely recoverable from culture supernatants, although enzymes are occasionally present in complexes at the cell surface. The individual enzymes often display strong synergy in the hydrolysis of

Table 2.4 Native microbial isolates that express cellulases

Source of microorganisms	Isolated microorganism (s)	Enzyme (s)	Country of Origin	Reference
Termite gut	*Bacillus subtilis* A 002, M 015, F 018	Endoglucanase, Exoglucanase,	Thailand	(Taechapoempol *et al.*, 2011)
	Clostridium sp. CT1112	β-glucosidase Cellulosome	Congo	(Hethener *et al.*, 1992)
Agriculture soil	*Cellulomonas* sp. TSU-03	Cellulosome	Thailand	(Sangkharak *et al.*, 2011)
Organic fertilizers	*Paenibacillus* sp. E2	Cellulosomes	Canada	(Maki *et al.*, 2011)
Persimmon vinegar	*Gluconacetobacter* sp. RKY5, *Gluconacetobacter intermedius* TF2	Cellulosome	Korea	(Wee *et al.*, 2011)
Cow dung	*Bacillus* sp.	Endoglucanase	India	(Das *et al.*, 2010)
Droppings of elephant	*Clostridium thermocellum* CT2	Cellulosome	India	(Harish *et al.*, 2010)
Gut of silk worm	*Bacillus circulans, Proteus vulgaris, Klebsiella Ineumonia, Escherichia coli, Citrobacter freundii, Serratia liquefaciens, Enterobacter* sp. *Pseudomonas fluorescens, P. aeruginosa, Aeromonas* sp. *Erwinia* sp.	Exoglucanase, Endoglucanase	India	(Anand *et al.*, 2010)
Empty fruit bunch and palm oil mil effluent compost	*Geobacillus pallidus*	Cellulosome	Malaysia	(Baharuddin *et al.*, 2010)
Deep subsurface of the Homestake gold mine	*Brevibacillus, Paenibacillus, Bacillus, Geobacillus*	Endoglucanase	USA	(Rastogi *et al.*, 2009)
Hot water spring	*Anoxybacillus flavithermus, Geobacillus thermodenitrificans, Geobacillus stearothermophilus*	Cellulosome	Egypt	(Ibrahim and El-diwany, 2007)
Anaerobic digester fed with pineapple peels	*Bacteroides* sp. P1	Cellulosome	Thailand	(Ponpium *et al.*, 2000)
Ripe olives	*Cellulomonas flavigena*	Cellulosome	USA	(Patel and Vaughn, 1973)

Table 2.5 Some cellulase-producing bacteria which are classified by oxygen relationship (Lynd et al., 2002)

Conditions	Bacteria	Representative Species	Gram Reaction
Aerobic	*Acidothermus*	*A. cellulolyticus*	+
	Bacillus	*B. pumilis*	+
	Caldibacillus	*C. cellovorans*	+
	Cellulomonasc	*C. flavigena, C. uda*	+
	Cellvibrio	*C. fulvus, C. gilvus*	-
	Cytophaga	*C. hutchinsonii*	-
	Erwinia	*C. carotovora*	-
	Micromonospora	*M. chalcae*	+
	Pseudomonas	*P. fluorescens var. cellulosa*	-
	Sporocytophaga	*S. myxococcoides*	-
	Streptomyces	*S. reticuli*	+
	Thermobifida	*T. fusca*	+
Anaerobic	*Acetivibrio*	*D. cellulolyticus*	-
	Anaerocellum	*D. thermophilum*	+
	Butyrivibrio	*B. fibrisolvens*	+
	Caldicellulosiruptor	*C. saccharolyticum*	-
	Clostridium	*C. thermocellum, C.cellulolyticum*	+
	Eubacterium	*E. cellulosolvens*	+
	Fervidobacterium	*F. islandicum*	-
	Fibrobacter	*F. succinogenes*	-
	Halocella	*H. cellulolytica*	-
	Ruminococcus	*R. albus, R. flavefaciens*	+

cellulose. Many aerobic bacteria adhere to cellulose, physical contact between cells and cellulose does not appear to be necessary for cellulose hydrolysis.

Many studies showed that cellulolytic aerobes produce higher cell yields as compared with anaerobes. On the other hand, cellulose-utilizing bacteria, which can live either aerobic or anaerobic conditions, are called facultative bacteria. These aerobes are still required some investigation about genus of bacteria and mechanisms that use for cellulose degradation (Lynd *et al.*, 2002).

2.3.2.4 Cellulase-Producing Bacteria from Termite

Termites, the small insect, but powerful wood destroyer, have drawn many researcher attraction for being cellulase producer (Wenzel *et al.*, 2002; Ohkuma, 2003; Taechapoempol *et al.*, 2011; Brune, 2014; Pramono *et al.*, 2017). Termites digest lignocellulose by using endogenous and symbiont-produced digestive enzymes. Termite gut symbionts consist of diverse microorganisms such as protozoa, bacteria, spirochetes, fungi and yeast (Scharf and Boucias, 2010). From phylogenetic identification, termites can be classified into two subgroups: lower termites and higher termites. Lower termites are termites from families *Mastotermitidae*, *Hodotermitidae*, *Kalotermitidae*, *Rhinotermitidae*, and *Serritermitidae*, which use symbiotic protozoans in the hind intestine for cellulose digestion. Higher termites are termites from family *Termitidae*, which use cellulolytic bacteria in their hindgut for cellulose digestion (Mo *et al.*, 2004).Several reports show the possibility to isolate cellulolytic bacteria from termites. Wenzel *et al*. (2002) isolated 119 cellulolytic strains from the gut of *Z. angusticollis*, which were assigned to 23 groups of aerobic, facultative anaerobic or microaerophillic cellulolytic bacteria (Wenzel *et al.*, 2002). They concluded that higher termites did not have cellulolytic fagellates, and the cellulolytic bacteria partly took over the role of the fagellates in cellulose degradation. Bakalidou *et al*. (2002) isolated novel cellulolytic and xylanolytic bacterium, *Cellulosimicrobium* strain MX5T from the hindgut of the Australian termite, *Mastotermes darwiniensis*. The isolate was a facultative anaerobe, which had a gram-positive cell wall profile (Bakalidou *et al.*, 2002). Taechapoempol *et al*. (2011) isolated cellulase-producing bacteria, *Bacillus subtilis* strain A 002, M 015 and F 018) from Thai higher termites, *Microceroterms* sp. which provided the high endoglucanase, FPase and β-glucosidase activities (Taechapoempol *et al.*, 2011).

2.3.2.5 Over-Expression of Cellulases in Bacteria and Activies

In cellulose hydrolysis bioprocesses, a large volume of enzymes is one of the important prerequisites for industry, resulting to high production cost. Over-expression of individual cellulases using recombinant DNA technology is the routinely used method (Juturu and Wu, 2014).

E. coli and *B. subtilis are* the most commonly used platform bacteria for expressing the recombinant proteins. The use of these systems for large scale production of enzyme have increased because of their advantages including short doubling times, ease of cultivation at high cell densities using inexpensive media (Westers *et al.*, 2004), simple techniques required for transformation, and easy isolation and purification of expressed proteins. Although *E. coli* is well known to be an ideal host for recombinant protein production, extracellulary secreting proteins into the medium has been a difficult task in *E. coli* (Zafar *et al.*, 2014). If *E. coli* could efficiently secrete recombinant proteins, such as cellulases, a consolidated bioprocessing approach could be applied where the same organism could hydrolyze the biomass and produce biofuel (Lynd *et al.*, 2005). In addition to these two bacteria, a few other expression platforms including *Zymomonas mobilis*, *Pseudomonas cellulosa* and *Streptomyces lividans* are also considering for expressing cellulases (Juturu and Wu, 2014). Details of various heterologously expressed microbial cellulase are listed in Table 2.6.

2.3.2.6 End-Product and Inhibitor of Enzymatic Hydrolysis

Cellulase enzymes are inhibited by cellobiose, glucose, or both products. Glucose inhibites cellobiase, Cellobiose inhibites endo-glucanase and/or cellobiohydrolase whereas exo-glucosidase is not inhibited by the end products (Kastel'yanos *et al*.,1995). Ghose and Das (1971) studied a enzymatic hydrolysis using *T.viride* and found that the nature of cellobiose inhibition was represented by competitive inhibition. However, in most cases, the inhibition was either competitive or non-competitive. The key factors relating to the inhibition patterns are supposed to be a function of cellulase binding constant, enzyme concentration, enzyme/substrate concentration ratio, available surface area of cellulose and β-glucosidase activity (Gan, 2003).

Several methods have been developed to reduce the inhibition, including the use of high concentrations of enzymes, the supplementation of β-glucosidases during hydrolysis, and the removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and fermentation (SSF).

Table 2.6 Heterologous expression of cellulases in microbial hosts

Source of enzyme gene	Expression host	Enzyme type	Enzyme activity	Reference
Bacillus sp.Z-16	*E. coli* BL21 (DE3)	Endoglucanase	0.5584 U/mL	(Gao *et al.*, 2015)
B. subtilis UMC7	*E. coli* BL21 (DE3)	Endoglucanase	0.73 ± 0.002 U/mL	(Chuan Wei *et al.*, 2015)
B. animalis subsp. lactis AD011	*B. bifidum* BGN4	β-Glucosidase	1.9 U/mL	(You *et al.*, 2015)
B. subtilis I15	*E. coli* BL21 (DE3)	Endoglucanase	2.82 U/ml	(Yang *et al.*, 2010)
B. subtilis DR	*E. coli* BL21 (DE3)	Endoglucanase	0.82 U/mL	(Li *et al.*, 2008)
X. fastidiosa	*E. coli* DH5α	Endoglucanase	2.39 μKat	(Wulff *et al.*, 2006)
Bacillus sp.KSM-64	*B. subtilis* ISW1214	Endoglucanase	21,700 U/L	(Sumitomo *et al.*, 1995)
Azoarcus sp. BH72	*E. coli* DH5α	Exoglucanase	30 U/mg of protein	(Reinhold-Hurek *et al.*, 1993)
R. flavefaciens 186	*E. coli* POP-13	Endoglucanase	0.455 U/mg protein	(Huang *et al.*, 1989)
R. flavefaciens	*E. coli* HB101	Endoglucanase	19.4 μg/min/mg	(Barros and Thomson, 1987)
T. fusca	*S. lividans* TK24(Str-6)	Endoglucanase	10 U/ml	(Ghangas and Wilson, 1987)
Thermonospora YX	*E. coli* SK2284	Endoglucanase	58,000 μmol/min/mg	(Collmer and Wilson, 1983)

The most significant breakthrough, simultaneous saccharification and fermentation (SSF) is a notable method employed to produce ethanol from lignocellulosic materials. The glucose and other fermentable sugars are first produced in the saccharification step and then fermented in the next step. This is very promising for the SSF for ethanol production from lignocellulose due to its possibility to improve hydrolysis rates, yields, and product concentrations compared to the two-stage hydrolysis–fermentation systems. The SSF has the various advantages such as a higher hydrolysis rate by conversion of sugars that inhibit the cellulase activity, a lower enzyme requirement, a higher product yield, lower requirements for sterile conditions since glucose is removed immediately to yield ethanol, a shorter process time, and a smaller reactor volume because a single reactor is used. However, some drawbacks of the SSF are needed to be considered, including the incompatible temperature of hydrolysis and fermentation, ethanol tolerant level of microbes, and inhibition of enzymes by ethanol (Sun and Cheng, 2002). The microorganisms used in the SSF are usually fungus (*T. reesei*) and yeast (*S. cerevisiae*). The optimal temperature for the SSF is around 38 °C, which is a compromise between the optimal temperatures for hydrolysis (45–50 °C) and fermentation (30 °C) (Philippidis, 1996). The hydrolysis step is usually the rate-limiting process in the SSF (Philippidis and Smith, 1995). Thermotolerant yeasts and bacteria have been used in the SSF to raise the temperature close to the optimal hydrolysis temperature**.**

2.3.2.7 Enhancement of Enzymatic Hydrolysis by Additives

Applying additives has shown to be a promising way for improving cellulase effectiveness. Most studies added small amount of surfactant which non-ionic surfactants or polymers containing ethylene oxide (EO) are the most effective on lignocellulose substrates (Börjesson *et al.*, 2007). The non-ionic surfactant Tween 80 was found to enhance the enzymatic hydrolysis rate of newspaper cellulose by 33% (Castanon and Wilke, 1981) and improve enzymatic hydrolysis yields for steam exploded poplar wood by 20% in the simultaneous saccharification and fermentation (SSF) process (Ballesteros *et al.*, 1998). Another research found that several cationic surfactants improved performance with tissue paper, while anionic surfactants did not (Ooshima *et al.*, 1986). Tween 20 improved the enzymatic hydrolysis of dilute sulfuric-

acid-pretreated saline Creeping Wild Ryegrass by 14% (Zheng *et al.*, 2008). Addition of Tween 20 reduced cellulase adsorption on substrate and allowed a 50% reduction in cellulase loadings to obtain the same conversion for steam pretreated spruce (SPS) while having little effect on yields for delignified SPS (Eriksson *et al.*, 2002). The addition of surfactant decreased cellulase adsorption on cellulose substrate due to hydrophobic interaction of surfactant with lignin on the lignocellulose surface, which releases unspecifically bound enzyme (Figure 2.8). Others showed that Tween 20 reduced thermal deactivation of cellulase (Kaar and Holtzapple, 1998). Addition of poly(ethylene glycol) PEG) to enzyme hydrolysis of lignocellulose increased the conversion from 42% without addition to 78% (Börjesson *et al.*, 2007).

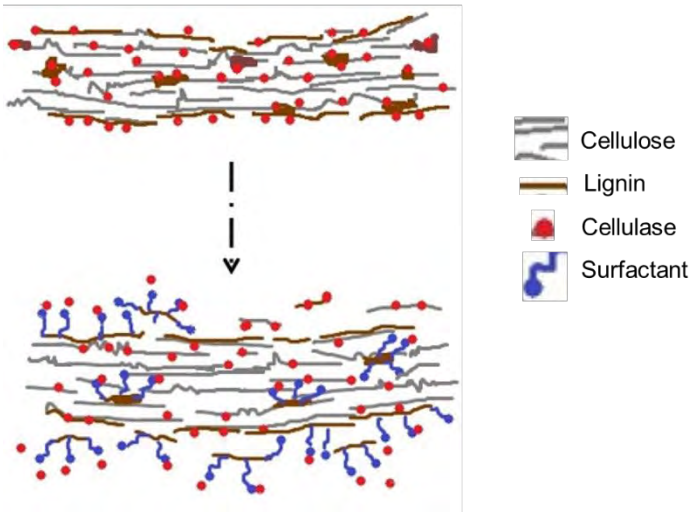


Figure 2.8 A schematic of enhanced enzymatic hydrolysis of lignocellulosic biomass by surfactant (Eckard *et al.*, 2013).

Other additives, besides surfactant, were conducted to enhance enzymatic hydrolysis on the same hypothesis of ligin covering. Bovine serum albumin (BSA) and other proteins were studied to adsorb competitively and irreversibly on lignin and it could be added prior to enzymatic hydrolysis to improve the effectiveness of cellulase (Yang and Wyman, 2006).

2.3.3 Fermentation Step

Fermentation is a biological process in which enzymes produced by microorganisms catalyze chemical reactions that break large molecule organic compounds (starch, oil, and protein) to simple sugars and amino acids and further convert into lower molecular weight materials such as organic acids and neutral solvents such as ethanol. Although organisms (bacteria, yeasts and fungi) can break down virtually any organic material, five- and six- carbon sugars are widely available in a vast variety of crops. These microorganisms digest simple one and two molecule sugars to produce the energy and chemicals they need to live and reproduce, and give off byproducts such as carbon dioxide, organic acids, hydrogen, ethanol, and other products.

Fermentation of glucose sugar derived from enzymatic hydrolysis of lignocellulosic hydrolysate generally does not pose special difficulties, as the inhibitor concentration should be very low. However, compared to starch and sugar fermentations, a sugar concentration after hydrolysis step is often low with values approaching typically not more than 70 g/L due to challenges in feeding a solution with a solid concentration higher than about 10% by weight to a fermentor and end-product inhibition of cellulase enzymes by the sugars released. Thus, a concentration step, e.g., vacuum evaporation, might be needed to achieve higher concentrations, with additional extra costs possibly counterbalanced by savings in the final distillation step (Maiorella *et al*., 1984; Brethauer and Wyman, 2010).

2.4 Cellulose Hydrolysis Bioreactor

Despite the fact that cellulose hydrolysis in large scale of bioprocess is not feasible at present due to the difficulty of conversion on complex structure of cellulose. Many studies have been reported an attempt in operation or modeling the hydrolysis of cellulose in bioreactors. The process of enzymatic hydrolysis of cellulose can be carried out in various type of reactors: a batch stirred reactor (Herr, 1980; South *et al.*), a continuous plug-flow column reactor (Gusakov *et al.*, 1985; Gusakov *et al.*, 1987), and a flowed stirred reactor with ultrafiltration (Henley *et al.*, 1980; Bélafi-Bakó *et al.*,

2006). Some advantages and disadvantages of such reactors due to specificity in its operation have been reported.

For batch stirred reactors, it showed an advantage of providing a higher concentration of products. While, the most negative effect was produced by cellobiose inhibition and by inactivation of the cellobiose-producing enzymes. The efficiency of the process was also confined by substrate crystallinity (Gusakov *et al.*, 1985). For plugflow column reactor, it gives a higher productivity and a higher degree of substrate conversion and its application for enzymatic cellulose hydrolysis offers good prospects and is highly promising because the negative effects of both product inhibition and enzyme inactivation were partially eliminated. However, cellulase adsorption ability, desorption and flowtransfer of the enzymes between the layers of the reactor, and the flow rate were of particular importance in the column reactor (Gusakov *et al.*, 1987). For membrane reactor, it has been developed from various membrane modules where different kinds of reactions were carried out, using catalysts in most cases (Mulder, 2012). One of the main advantages of the membrane bioreactors is that they make recovery and reuse of biocatalysts possible. Usage of membrane bioreactors is especially beneficial for polymer degradation processes, where the small size product may have inhibitory effect, but can be separated easily by a proper porous membrane, like enzymatic hydrolysis of cellulose. The long polysaccharide chain as well as the biocatalyst (enzyme or cell) are rejected by the membrane, while the product (glucose) passes through the membrane into the other phase. In such a system, continuous uptake of substrate and release of product without loss of biocatalysts can be achieved (Bélafi-Bakó *et al.*, 2006). Ohlson et al. reported that the enzymatic cellulose hydrolysis rate increased four times in a membrane bioreactor compared with that obtained in a conventional batch reactor (Ohlson *et al.*, 1984).

CHAPTER III
STUDY ON MICROBIAL PRETREATMENT FOR ENHANCING ENZYMATIC HYDROLYSIS OF CORNCOB

3.1 Abstract

The complex structure of lignocellulose leads to great difficulties in converting it to fermentable sugars for the ethanol production. The major hydrolysis impediments are the crystallinity of cellulose and the lignin content. To improve the efficiency of enzymatic hydrolysis, microbial pretreatment of corncob was investigated using two bacterial strains, *Bacillus subtilis* A 002 and *Cellulomonas* sp. TISTR 784, expected to break open the crystalline part of cellulose, and a lignin-degrading fungus strain, *Phanerochaete sordida* SK7, expected to remove lignin from lignocellulose. The microbial pretreatment was carried out with each strain under its optimum conditions. The pretreated corncob samples were further hydrolyzed to produce reducing glucose with low amounts of commercial cellulase (25 U/g corncob) from *Aspergillus niger*. The corncob samples were determined for composition change by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscope (SEM). According to the results, the microbial pretreatment with fungus, *P. sordida* SK7 was the most effective for enhancing enzymatic hydrolysis with approximately 40% improvement. And, this would lead to better ethanol production.

3.2 Introduction

Worldwide use of ethanol as an alternative fuel has been steadily increasing because of the main problems of petroleum shortage and environmental concern. The biomass conversion to ethanol generally consists of the hydrolysis of biomass into reducing sugars and the fermentation of reducing sugars to ethanol (Badger, 2002). Different kinds of biomass have been used as a feedstock which can be classified into three categories; simple sugar, starch, and lignocellulosic materials (Balat and Balat, 2009). Present processes are likely to use edible plant materials like sugar and starch

which are easily hydrolyzed; however, they are in human food chain, affecting food supply and price (Scharf and Tartar, 2008; Alvira *et al.*, 2010). To overcome the food conflict problem, lignocellulose biomass like agricultural residues is considered to be a promising alternative feedstock for sugar production.

Thailand is an agricultural country with abundant crop products, such as corn, sugarcane, rice, etc. Consequently, large amounts of agricultural residues associated with the crop production can be a potential resource for producing energy and other more valuable chemicals. Corncob residue around 1.13 million ton is produced each year (Meehom *et al.*, 2012). Some portion is used as animal feed, but the excess remaining residues still leads to an environmental problem. Hence, a use of agricultural residues for sugar production is of great interest. The contents of cellulose and hemicelluloses in corncob are very high and vary with species, growing area, season, and so on. Commonly, the main composition of corncob is consisted about 45% cellulose, 35% hemicellulose, and 15% lignin (Sun and Cheng, 2002).

The complex structure of cellulose, resulted from lignin coverage and rigid crystallinity of cellulose, is responsible for low sugar yield (Mosier *et al.*, 2005). Therefore, a pretreatment is needed to reduce the biomass recalcitrance by breaking lignin seals and disrupting the crystalline structures of cellulose (Wan and Li, 2010). Compared with the other pretreatment methods (e.g. diluted acid, alkali extraction, steam explosion, and hydrothermolysis), microbial pretreatment of lignocellulosic materials is an environmentally friendly process in aspects of no severe chemicals, less energy input, no requirement for pressurized and corrosion-resistant reactors, and less inhibition to fermentation (Keller *et al.*, 2003). Microbes used for pretreatment of lignocellulosic materials can be both fungal and bacterial species. White rot fungi have been receiving extensive attention for biodelignification of lignocellulosic biomass but several weeks to months are generally needed to obtain a high degree of lignin degradation (Vicentim and Ferraz, 2007; Wan and Li, 2010; Liew *et al.*, 2011). A white rot fungus *Irpex lacteus* CD2 was found to have high efficiency for pretreatment of corn stover with a saccharification ratio up to 66.4% of (Xu *et al.*, 2010). In 2007, Kurakake and coworkers (Kurakake *et al.*, 2007) studied bacterial pretreatments of office paper, mainly consists of cellulose, and found that the sugar recovery up to 94% was achieved after 4 d. They explained that the strains acted as an endoglucanase, to

hydrolyze the amorphous cellulose randomly. Normally, the microscopic filamentous structures of fungi generally are much larger than bacteria and usually are 5-10 μm in width, whilst, most of bacteria fall into a size range of 0.5-3.0 μm. In comparison, a growth rate of bacteria is much higher than that of fungi. The life cycle of fungi is dependent on the ambient conditions and it take several months or years to get a stationary phase. Whilst, bacteria are generally given the proper conditions and grow at a very high rate of binary fission (Speight, 2000).

As mentioned previously, Thailand annually produces a large amount of corncob residue which can be explored as an alternative source for producing valuable products e.g. bioethanol. However, pretreatment of corncob needs to be done to reduce the impact of complex structure on the hydrolysis process. Therefore, microbial pretreatment using either fungi or bacteria is an interesting environmental-friendly process and has not been intensively studied on corncob. In this study, the enhancement of enzymatic hydrolysis by microbial pretreatments using both fungus and bacteria were investigated . The corncob samples were characterized by X-ray diffraction (XRD) analysis, Fourier transform infrared (FTIR) spectroscope, and scanning electron microscopy (SEM) in order to correlate the enzymatic hydrolysis efficiency to the structural changes as a result from the pretreatment.

3.3 Experimental

3.3.1 Materials and Microbial Strains

Corncob collected from Kanchanaburi province, Thailand, was dried overnight at 105 °C. Dried corncob was milled and sieved to the size ranges of 40–60 mesh (0.25–0.42 mm) and stored in an air tight container at room temperature before use. Bacterial pretreatment of corncob was performed by two cellulose-hydrolyzing bacteria including *Bacillus subtilis* A002 isolated from Thai higher termites, *Microceroterms* sp., by our research group (Taechapoempol *et al.*, 2011) and *Cellulomonas* sp. TISTR 784 obtained from sugarcane field provided by Thailand Institute of Scientific and Technological Research. Both studied bacteria were activated in a 65 modified DSMZ broth medium and were then maintained on a 65 modified DSMZ agar medium at 4 °C. The white-rot fungi, *Phanerochaete sordida*

SK7 used for the pretreatment study was kindly provided by Plant Biomass Utilization Research Unit, Chulalongkorn University, Thailand. The medium and all equipments were sterilized in an autoclave at 121 °C for 15 min. Distilled water was used for preparing all solutions.

3.3.2 Bacterial Pretreatment

For bacterial inoculation, a 65 modified DSMZ nutrient broth (5 g/L Carboxymethyl cellulose (CMC), 4 g/L yeast extract, and 10 g/L malt extract), pH 7.2 was prepared for culturing *B. subtilis* A 002, and a medium containing 5 g/L CMC, 0.5 g/L yeast extract, 1 g/L $NaNO_3$, 1 g/L K_2HPO_4, 1 g/L KCl, 0.5 g/L $MgSO_4$, and 1 g/L glucose at pH 7.2 was prepared for *Cellulomonas* sp. TISTR 784. Each pure colony was cultured in a 250 mL Erlenmeyer flask containing 50 mL of each medium. The culturing flask was incubated at 37 °C in a shaking incubator at 180 rpm for 12 h. Then, an inoculum (10^7 colony forming units (CFU)/mL) was transferred into a 1 L bottle containing 450 mL of each medium with 5 g of the 40–60 mesh corncobs for pretreatment. The bacterial pretreatment was carried out in the medium without CMC or glucose and incubated at 37 °C in a shaking incubator at 180 rpm for 3 d. After that, the pretreated samples were washed with 200 mL DI water and dried until constant weight for weight loss determination and composition analysis.

3.3.3 Fungal Pretreatment

The tropical white-rot fungus *P. sordida* SK7 was cultured on a malt extract (ME) agar plate, containing 30 g/L malt extract, 5 g/L mycological peptone, 10 g/L glucose, and 16 g/L agar, at 30ºC for 3-5 d until the mycelium fully grew inside the petri dish. Twenty mycelium blocks (10 mm in Diameter) from the pre-cultured fungus were inoculated in a 250 mL Erlenmeyer flask containing 200 mL of ME broth at room temperature with static conditions for 10 d. The grown mycelium mat was aseptically filtered and washed with sterile distilled water and homogenized by a homogenizer (Omni TH) for three 20-s cycles and adjusted the mycelium suspension to be 3.5 mg/L on dry basis. The fungal pretreatment was carried out in the solid state, following the method published with some modification (Vicentim and Ferraz, 2007; Liew *et al.*, 2011). A quantity of 5 gram of the dried corncob was placed in a 250 ml

Erlenmayer flask and supplemented with 0.5% corn steep liquor then conditioned with distilled water. The mixture was subjected to sterilization at 121ºC for 15 min and cooled prior to inoculation. Each flask was loaded with mycelium suspension on the top of the substrate corresponding to a fungal mycelium (mg): corncob (g) ratio of 1:2. The pretreatment was carried out at 30 ºC with static conditions for 20 d. After that, the pretreated samples were washed with 200 ml DI water and dried until constant weight for weight loss determination and composition analysis (Sripat, 2013).

3.3.4 Composition Analysis

For chemical composition analysis, corncob samples were analyzed for cellulose, hemicelluloses, lignin, and extractives. The amount of extractives was determined from the weight loss from solvent extraction using 60 mL acetone per gram of a dried corncob sample at 90 °C for 2 h and drying at 105 °C. The hemicellulose content was represented by the weight loss from the alkaline dissolution step. A 10 mL of a 0.5 M of sodium hydroxide (NaOH) solution was added to 1 g of extractive-free dried biomass, and the mixture was held at 80 °C for 3.5 h. After that, the mixture was washed several times by DI water and dried to a constant weight. To determine the lignin fraction, 30 mL of a 98% sulfuric acid (H_2SO_4) was added to the residue after the alkaline dissolution step, and the mixture was held at ambient temperature for 24 h and boiled at 100 °C for 1 h. The mixture was filtered, and then the residue was washed with distilled water until the sulfate ion in the filtrate was undetectable (via titration of a 10% barium chloride solution). Finally, the remaining residue was dried at 105 °C until constant weight. The weight of the dried residue was recorded as the lignin content. The cellulose content was calculated by the weight difference of the remains residues by the NaOH and H_2SO_4 extractions (Di Blasi *et al.*, 1999; Lin *et al.*, 2010). The elemental compositions of samples were also determined by a CHNS/O analyzer (Leco, TruSpec® Elemental Determinator). All samples were assayed in triplicate.

3.3.5 Enzymatic Hydrolysis Experiments

The cellulase from *Aspergillus niger* (Sigma Chemical Co.) with the CMCase activities of 0.35 U/mg (following Ghose's method) was used to hydrolyze

both untreated and pretreated corncob samples (GHOSE, 1987). The enzymatic hydrolysis was carried out with 1% corncob loading and 25 U of cellulase in 100 ml of a 50 mM sodium acetate buffer solution, pH 4.8 and then the hydrolytic mixture was incubated in a shaking incubator at 180 rpm and 50 °C for 3 d with periodically sample collecting. A 0.01% sodium azide was added to prevent the microorganism contamination (Chang *et al.*, 2011).

3.3.6 Sugar Analysis

The supernatant of each sample taken during the hydrolysis experiments after centrifugation at 8000 rpm for 3 min was filtrated through a 0.22 μm nylon filter and used for sugar analysis by high-performance liquid chromatography (HPLC) equipped with a refractive index detector (Model 6040 XR, Spectra-Physics, USA) and Aminex HPX- 87H column, (Bio-Rad Lab, USA). A 0.005 M H_2SO_4 solution was used as a mobile phase at a flow rate of 0.6 $mL \cdot min^{-1}$ and a column temperature of 65 °C.

3.3.7 Measurement of the Degree of Crystallinity of Concob

The crystalline structures of the untreated and pretreated corncob samples were determined by X-ray diffraction (XRD). The dried samples were scanned and recorded by using a Rigaku X-Ray Diffractometer (RINT-2200) with a Ni filter and Cu K_α radiation (1.5406 Å) generated at 30 mA and 40 kV. A scan speed of 5° (2θ) $\cdot min^{-1}$ with a scan step of 0.02 (2θ) was used for the continuous run in 5 to 50° (2θ) range. Crystallinity indices of corncob samples were calculated from the X-ray diffraction patterns by the following equation (Cao and Tan, 2005):

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100\% \qquad (3.1)$$

where I_{002} is the maximum intensity from the (002) lattice plane (2θ = 22.5°) referring to crystalline peak and I_{am} is the minimum intensity of amorphous phases (2θ =19°).

3.3.8 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) analysis was used for analyzing the differences of crystallinity and chemical structure of corncob sample before and after microbial pretreatment. The FTIR spectra of untreated corncob and pretreated corncobs were measured by direct transmittance using the KBr pellet technique. Spectra were recorded using a Nicolet Nexus 670. All the spectra are measured at 100 scans per sample with spectral resolution of 4 cm^{-1}, in the frequency range of 4000–400 cm^{-1} (Pandey and Pitman, 2003).

3.3.9 Morphology Examination

The morphological changes of corncob entities due to the hydrolysis process were observed using a scanning electron microscope (SEM), Hitachi/S-4800.

3.4 Results and Discussion

3.4.1 Change of Corncob Composition Results

The chemical composition of corncob is generally different, depending on breeds, growing area, season, and also analysis method (Silverstein *et al.*, 2007). In this study, corncob was analyzed on dried weight basis. The original corncob sample used in this study consists of 43.64 ±1.94% cellulose, 33.59 ± 0.97% hemicellulose, 12.20 ± 0.94% lignin, and 10.55 ± 0.68% extractives, as shown in Table 3.1. It had a quite high portion of cellulose with lower contents of hemicellulose and lignin inhibitors of the cellulolytic hydrolytic reaction, compared with other agricultural residues (Sun and Cheng, 2002). After the microbial pretreatments with different strains, the weight losses of corncob samples during 3-d pretreatment with *B. subtilis* A 002 and *Cellulomonas* sp. TISTR 784 were 10.73 ± 0.28% and 10.93 ± 0.14%, respectively. And the weight losses of samples during 25-d pretreatment with *P. sordida* SK 7 was 6.84 ± 2.35%. The results showed that both bacterial pretreatments with *B. subtilis* A 002 and *Cellulomonas* sp. TISTR 784 which were carried out in a liquid state caused higher extraction of corncob particles (responding well to the lower extractive content of the washed corncob samples) and then higher weight loss than

the fungal pretreatment with *P. sordida* SK 7 which was carried out in a semi-solid state.

For the chemical composition change, after the microbial pretreatment with *P. sordida* SK 7, the lignin content (10.10 ±0.55%) was significantly lower than that of the untreated corncob (12.20 ± 0.94%) whereas the extractive fraction also increased remarkably from 10.55 ± 0.68% to 18.31 ± 1.33%. The results imply that the lignin was substantially degraded to low molecular phenolic compounds by the ligninase enzyme of the white rot fungi, *P. sordida* SK 7. This is consistent with the result of Lee and coworkers who found that the content of extractives increased after fungal pretreatment because of degradation products of carbohydrates and lignin (Lee *et al.*, 2010). At the same time, the cellulose content of corncob sample with *P. sordida* SK 7 pretreatment for 25 d decreased by 12%, comparing to that of the untreated corncob sample indicating that cellulose was decomposed, consistent with previous report (Highley *et al.*, 1984). The white rot fungi growing on cellulose fibers can mainly decompose cellulose by two processes: (i) fungi release cellulose-degradation enzyme system to act on the immediate vicinity of the hyphae and exposed surface of the cell wall (Cowling, 1961), (ii) whit-rot fungi produce cavity-shaped fissures in the S_2 layer of the secondary wall at some distance from hyphae and then the cellulose-degradation enzymes of fungi diffuse into the cell wall to create cavities, causing completely cell wall disruption (Liese, 1970; Wilcox, 1970). Normally, the hemicellulose attaching to lignin after lignin removal can be simultaneously removed. However, this hemicellulose fraction was found to be insignificantly different from the untreated corncob after all pretreatment methods, implying that the removal of lignin from this corncob samples might mostly occur on the outer surface of covered lignin. For the microbial pretreatment with both *B. subtilis* A 002 and *Cellulomonas* sp. TISTR 784, the cellulose content slightly decreased, correlating with the increase of crystallinity index (see Table 3.2). The results indicating that the amorphous phase of cellulose in the corncob samples was preferentially degraded during the pretreatment. There are consistent with the study of Kurakake and coworker who reported that bacterial pretreatment have an influence on the amorphous area of cellulosic materials by acted as endoglucanase to hydrolyze the amorphous cellulose randomly (Kurakake *et al.*, 2007). However, the contents of hemicellulose and lignin seem to be higher than that

of the untreated corncob sample due to they are relative value to the decrease of cellulose an extractive contents.

Table 3.1 Chemical compositions of untreated corncob and pretreated corncob by different microbes after 2 d at 37 ºC for both *B. subtilis* A 002 and *Cellulomonas* sp. TISTR 784 and 20 d at 25 ºC for *P. sordida* SK 7

Microbial pretreatment strains	Weight loss (%)	Corncob composition (%)			
		Cellulose	Hemicellulose	Lignin	Extractives
None	-	43.64 ± 1.94	33.59 ± 0.97	12.20 ± 0.94	10.55 ± 0.68
B. subtilis A 002	10.73 ± 0.28	43.03 ± 2.97	34.28 ± 0.70	14.90 ±2.20	7.76 ± 0.29
Cellulomonas sp. TISTR 784	10.93 ± 0.14	42.20 ± 4.98	35.00 ± 2.36	17.00 ±4.29	5.77 ± 5.49
P. sordida SK 7	6.84 ± 2.35	38.47 ± 3.52	33.14 ± 0.61	10.10 ±0.55	18.31 ± 1.33

3.4.2 Crystallinity Measurement by XRD

The structure of corncob contains crystalline portion of cellulose and amorphous portion of mostly hemicellulose and lignin. The former portion makes the access of cellulase enzymes to cellulose difficult, whilst the latter portion is inhibitor. Thus, the transformation from crystalline to amorphous cellulose during a pretreatment and the removal of inhibitor in amorphous phase can improve the sub-sequential step of enzymatic hydrolysis efficiency (Lee *et al.*, 2007; Qing and Wyman, 2011). Therefore, XRD was used to investigate the degree of crystallinity after microbial pretreatment and enzymatic hydrolysis, comparing to that of the untreated corncob sample in order to predict the circumstance of the change of corncob structure. Figure 3.1 shows the X-ray diffractograms of untreated and microbial pretreated corncob samples. The peak for the (002) plane at $2\theta = 22.5°$ in the diffractograms, referring to crystalline peak, of the corncob samples with *B. subtilis* A 002 and *Cellulomonas* sp. TISTR 784 pretreatment become slightly sharper than that of the untreated corncob samples. Corresponding to the Cr.I. values (Table 3.2), they increased up to 68.01 and 58.04% for *B. subtilis* A 002 and *Cellulomonas* sp. TISTR 784 pretreatment, respectively, comparing to the untreated corncob (57.14%). These results can be explained by the fact that *B. subtilis* A 002 and *Cellulomonas* sp. TISTR 784 are cellulase-producing bacteri (Paice et al., 1986; Wolf et al., 1995; Saleem et al., 2002; Lo et al., 2009; Taechapoempol et al., 2011). Therefore, the produced cellulase in the

system can preferentially act on amorphous region, causing the increase in crystallite size regarding to the (002) plane (Cowling, 1961). These results confirmed the decrease in cellulose content after bacterial pretreatments. For corncob sample with *P. sordida* SK 7 pretreatment, the diffractogram exhibited that the intensity of (002) plane was significantly decreased compared with that of other samples, indicating that the crystal lattice was distracted by fungi. The Cr.I. value was found to be 54.35%, lower than that of untreated corncob about 5%. Sripat reported that *P. sordida*, SK 7 had the ability to produce ligninase enzyme—Ligninase peroxidase and Laccase (Sripat, 2013). Therefore, ligninase can degrade lignin, causing the increase of pore sizes and accessible surface area to cellulase (Wan and Li, 2010) and then being less crystallinity. In addition, the corncob structure disruption also results in the decrease in Cr.I. value.

A peak intensity of the $(10\bar{1})$ plane ($2\theta = 16.3°$) of any given pretreated corncob samples insignificantly change, except *P. sordida* SK 7 pretreated corncob samples. This result consistent with previous work reported that the cellulase enzymes preferentially attack the (002) plane in native cellulose (Cao and Tan, 2005).

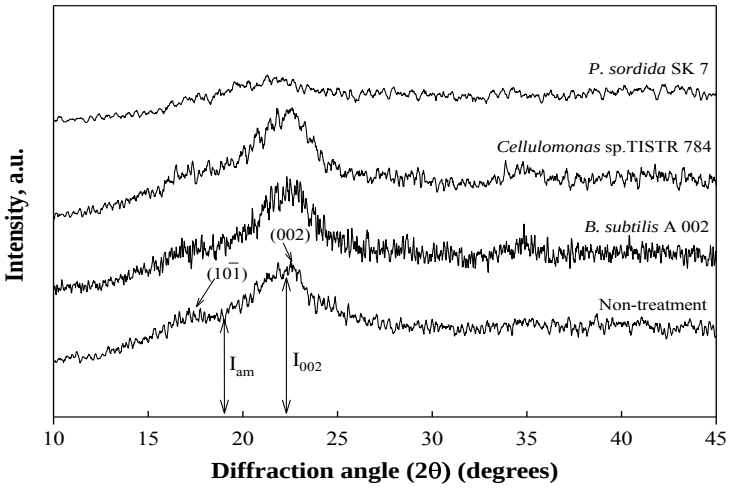


Figure. 3.1 X-ray diffractograms of untreated and pretreated corncob samples.

Table 3.2 Crystallinity index (Cr.I.) of corncob after microbial pretreatment (3 d for *B. subtilis* A002 and *Cellulomonas* TISTR 784 pretreatments, and 25 d for *Phanerochaete sordida* SK 7 pretreatment) and enzymatic hydrolysis for 3 d

Microbial pretreatment strains	Cr.I. (%) of corncob		Cr.I. reduction (%)
	Post-pretreatment	Post- hydrolysis	
None	57.14	66.02	-
Bacillus subtilis, A 002	68.01	64.07	2.96
Cellulomonas sp., TISTR 784	58.04	55.74	15.57
Phanerochaete sordida, SK 7	54.35	53.22	19.38

After the enzymatic hydrolysis, the Cr.I. values decreased in any given pretreated corncob samples, suggesting that the commercial cellulase subsequentially hydrolyzed on the crystalline cellulose. On the other hand, the untreated corncob shows the increase in Cr.I. value since the hydrolysis occurred on the amorphous region. A value of Cr.I. reduction after hydrolysis, compared with the Cr.I. of untreated corncob can be used to refer how much the pretreatment can improve enzymatic hydrolysis, the higher value means the enzyme can be more accessible to cellulose. The highest value of Cr.I. reduction was found with the microbial pretreatment with *Phanerochaete sordida*, SK 7, indicating that this pretreatment could enhance enzyme-substrate accession.

3.4.3 Change in Chemistry of Pretreated Corncob by FTIR

The Foureir transform infrared (FTIR) spectroscopic results of microbial pretreated corncob samples are shown in Figure 3.2. The analysis of corncob is based on spectra band absorptions for lignocelluloses (Pandey and Pitman, 2003), as shown in Table 3.3. The stronger bands of hydrogen bonded (O-H) stretching absorption at 3400 cm^{-1} (1) and C–H stretching absorption at 2912 cm^{-1} (2) was found in the pretreated corncob samples compared with untreated corncob. When extractives or lignin were removed after pretreatment, the portion of cellulose and hemicellulose increased, resulting in these stronger bands. The existence of band at 1735 cm^{-1} (3) in all pretreated corncobs represented the unconjugated C=O in xylan or hemicellulose which still remained after the microbial pretreatment, suggesting that the microbial pretreatment step could not break down the hemicellulose in the studied corncob. The intensities of absorbance bands at 1113 cm^{-1} (13) of aromatic skeletal and C–O stretch

and 1601 cm^{-1} (5) of aromatic skeletal in lignin obviously decreased after the fungal pretreatment by *P. sordid* SK 7, indicating that the selective removal of the lignin occurred by this fungal strain.

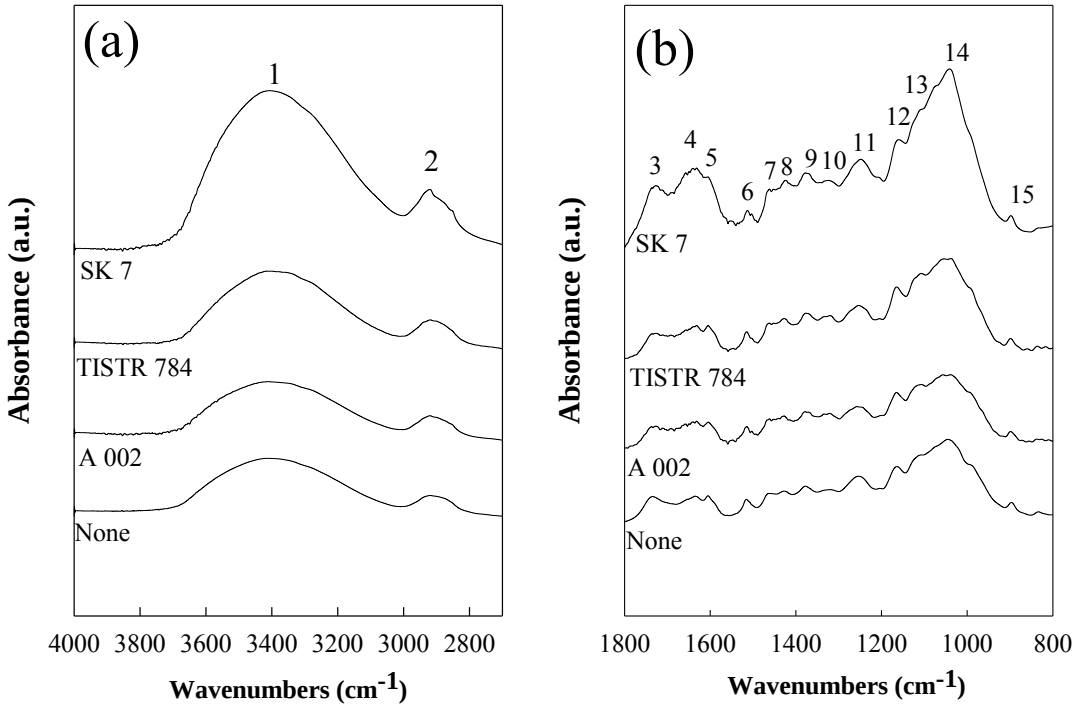


Figure 3.2 FTIR spectra of untreated and pretreated corncob samples. (a) the OH and CH stretching vibrations in the 4000-2700 cm^{-1} region and (b) the fingerprint region with different stretching vibrations of different groups in the 1800-800 cm^{-1} region

Table 3.3 Key FTIR absorptions for lignocellulose (Pandey and Pitman, 2003)

Peak number	Key spectra	Wavenumbers (cm^{-1})
1	A strong hydrogen bonded (O–H) stretching absorption	3400
2	A prominent C–H stretching absorption	2912
3	Unconjugated C = O in xylans (hemicellulose)	1735
4	Absorbed O–H and conjugated C–O,	1634
5	Aromatic skeletal in lignin	1601
6	Aromatic skeletal in lignin	1516
7	C–H deformation in lignin and carbohydrates	1463
8	C–H deformation in lignin and carbohydrates	1429
9	C–H deformation in cellulose and hemicellulose	1378
10	C–H vibration in cellulose and Cl–O vibration in syringyl derivatives	1325
11	Guaiacyl ring breathing, C–O stretch in lignin and C–O linkage in guiacyl aromatic methoxyl groups	1257
12	C–O–C vibration in cellulose and hemicellulose	1166
13	Aromatic skeletal and C–O stretch	1113
14	C–O stretch in cellulose and hemicellulose	1050
15	C–H deformation in cellulose	895

3.4.4 Surface Morphology by SEM

The smooth surface was observed at the untreated corncob, as shown in Figure 3.3 (a). The surface morphology was obviously changed to be porous after the all microbial pretreatments of corncobs, as shown in Figure 3.3 (b-d). Interestingly, the fungal pretreatment shows the decompose surface with fungal fibers remaining on surface. The change in surface morphology confirms microbial action on corncob as mentioned previously.

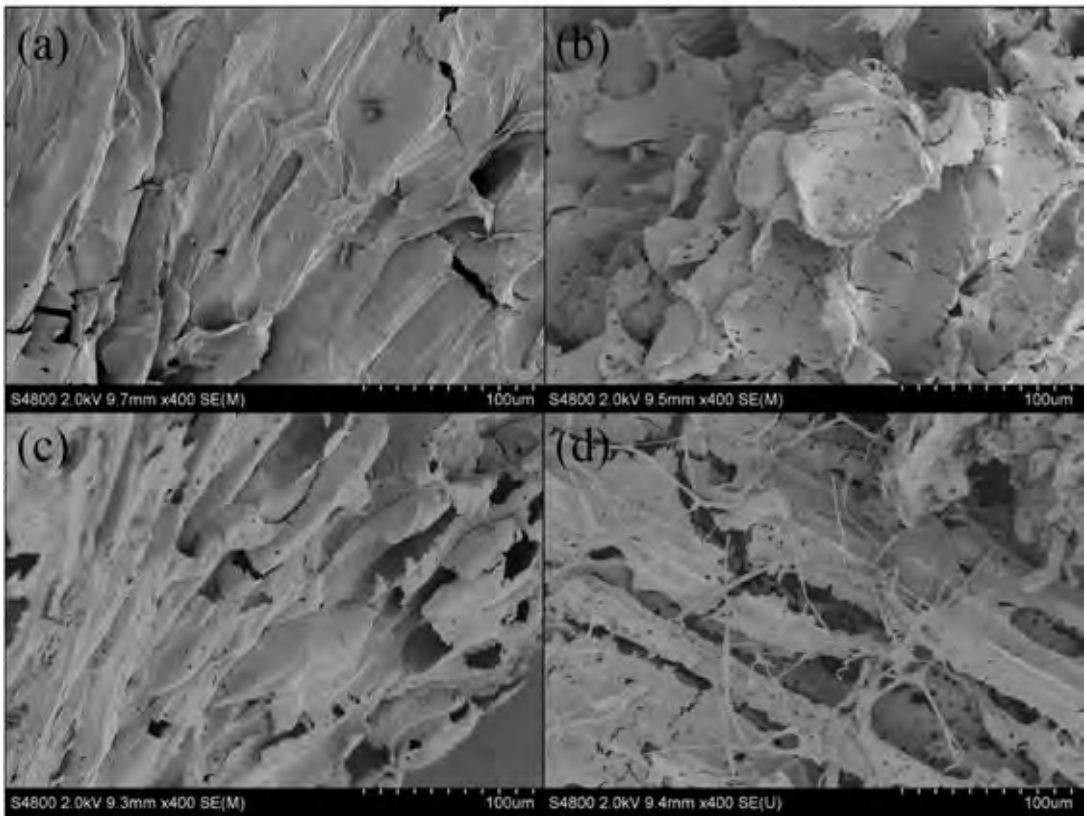


Figure 3.3 Scanning electron micrographs of the 40–60 mesh corncob surfaces (a) untreated corncob; (b) pretreated with *B. subtilis* A 002; (c) pretreated with *Cellulomonas* sp. TISTR 784; and (d) pretreated with *P. sordida* SK 7.

3.4.5 Enzymatic Glucose Production Abilities

The excess cellulase (100 U/g corncob) from *Aspergillus niger* (Sigma Chemical Co.) was used to hydrolyze untreated and pretreated corncob samples in 100 mL of a 50 mM acetate buffer solution at pH 4.8 in order to compare the abilities of glucose production. The efficiencies of microbial pretreatments by different strains were evaluated by the amount of produced glucose obtained from the enzymatic hydrolysis step. No other soluble sugar, except glucose, was observed forming during the hydrolysis. The profiles of glucose concentrations over a 72 h period are shown in Figure 3.4.

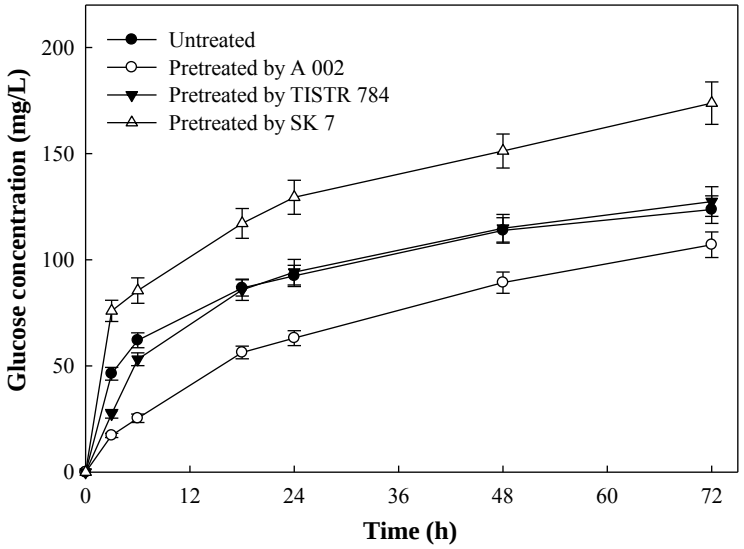


Figure 3.4 Glucose concentrations (g/L) from enzymatic hydrolysis of untreated and pretreated corncob.

For any given microbial pretreatment, the glucose concentration from the enzymatic hydrolysis of corncob increased with a fast rate within the first 6 h and continually increased with a decreasing rate until 24 h. After that, it still increased with a constant rate. The results can be explained by the fact that the enzymatic hydrolysis of corncob is a heterogonous catalytic reaction, which depends directly on the available interfacial surface area. At the beginning of the reaction, there is more possibility of the soluble enzyme to adsorb on the more vacant site of corncob surface to undergo the reaction, leading to the decrease in available surface. Afterwards, the reaction takes place with the decreasing available surface, resulting in a slower glucose production rate. This result is in a good agreement with a report of Highina and coworker (Highina *et al.*, 2012).

At 72-h-pretreatment, the enzymatic hydrolysis of the untreated corncob provided 124 mg/L of glucose. Greatly, the enzymatic hydrolysis of corncob with the fungal pretreatment using *P. sordida* SK 7 provided the highest glucose concentration (174 mg/L). Therefore, the enzymatic hydrolysis of corncob with fungal pretreatment was improved up to 40%, compared with the enzymatic hydrolysis of untreated corncob. The increase in glucose yield indicates the decrease in the recalcitrance of the enzymatic hydrolysis, which results from the altering of the

corncob characteristics during the pretreatment with the white rot fungi. These characteristics might include an increase in adsorption capacity to cellulase and a decrease in lignin content (Mooney *et al.*, 1998; Boussaid and Saddler, 1999; Gardner *et al.*, 1999; Sun and Cheng, 2002; Zhang *et al.*, 2007). For the purpose of using *B. subtilis* A 002 for the bacterial pretreatment of corncob in order to hydrolyze partially the crystalline cellulose fraction into amorphous form; however, it provided the negative result of glucose production after the enzymatic hydrolysis. The glucose produced from pretreated corncob by *B. subtilis* A002 was lower than the untreated corncob (107 mg/L). It can be explained by the XRD result which showed the increase of crystallinity index of corncob after pretreatment (more details on the following section). The result imply that the pretreatment using *B. subtilis* A 002 major acted on amorphous cellulose and left the crystalline portion which was difficult to be hydrolyzed. This is the reason why the pretreated corncob provided glucose less than the untreated corncob, which possessed more amorphous cellulose, did. This situation also occurred with pretreated corncob using *Cellulomonas* sp. TISTR 784. However, it was possible that less activity of *Cellulomona*s sp. TISTR 784 made glucose insignificantly differ from the untreated one (127 mg/L).

3.5 Conclusions

Microbial pretreatment is the environmentally friendly process for lignocellulose pretreatment before enzymatic hydrolysis because it can be carried out at mild conditions and no hazardous chemical requirement. The results showed that the microbial pretreatment using lignin degrading fungus, *P. sordid* SK7 on corncob was an effective method in improving enzymatic hydrolysis accessibility (approximately, 40% improvement of no pretreatment. Whilst, the use of cellulase producing bacteria, *B. subtilis* A002 and *Cellulomonas* sp. TISTR 784 show no improvement of enzymatic hydrolysis but they are potential to be employed as enzyme source for hydrolysis of lingo cellulose. Due to *P. sordid* SK7 can produce ligninase for degrading the lignin which is the major impediment in enzymatic hydrolysis. However, fungal pretreatment is time consuming process compared with bacterial

pretreatment, because of slow growth rate of fungus. More extensive study needs to be performed to compare in aspect of economic feasibility.

3.6 Acknowledgments

The authors acknowledge the following organizations: the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0244/2552); the Center of Excellence on Petrochemical and Materials Technology, Chulalongkorn University. The authors also sincerely thankful to Plant Biomass Utilization Research Unit, Chulalongkorn University, Thailand for providing fungal pretreated corncob. Moreover, the authors gratefully acknowledged the Thailand Institute of Scientific and Technological Research (TISTR) for the contribution to this work is highly appreciated.

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CHAPTER IV
ESCHERICHIA COLI EXPRESSING ENDOGLUCANASE GENE FROM THAI HIGHER TERMITE BACTERIA FOR ENZYMATIC AND MICROBIAL HYDROLYSIS OF CELLULOSIC MATERIALS

4.1 Abstract

Endoglucanase plays a major role in initiating cellulose hydrolysis. Various wild-type strains were searched to produce this enzyme but mostly low extracellular enzyme activities were obtained. To improve extracellular enzyme production for potential industrial applications, the endoglucanase gene of *Bacillus subtilis* M015, isolated from Thai higher termite, was expressed in a periplasmic-leaky *Escherichia coli*. Then, the use of crude recombinant endoglucanase (EglS) with a commercial cellulase (Cel) for hydrolyzing celluloses and microbial hydrolysis using whole bacterial cells was evaluated. *E. coli* Glu5 expressing endoglucanase at high levels was successfully constructed. It produced EglS (55 kDa) with extracellular activity of 18.56 U/mg total protein at optimal hydrolytic conditions (pH 4.8 and 50ºC). EglS was highly stable (over 80% activity retained) at 40-50ºC after 100 h. The addition of EglS significantly improved the initial sugar production rates of Cel on the hydrolysis of carboxymethyl cellulose (CMC), microcrystalline cellulose and corncob about 5.2, 1.7, and 4.0 folds, respectively, as compared to those with Cel alone. *E. coli* Glu5 could secrete EglS with high activity in the presence of glucose (1% w/v) and Tween 80 (5% w/v) with low glucose consumption. Microbial hydrolysis of CMC using *E. coli* Glu5 yielded 26 mg reducing sugar/g CMC at pH 7.0 and 37ºC after 48 h. In conclusion, the recombinant endoglucanase activity was improved by 17 times compared with that of the native strain, and could greatly enhance the enzymatic hydrolysis of all studied celluloses when combined with a commercial cellulase.

4.2 Introduction

Cellulosic biomass, obtained from agricultural residues, offers a higher potential than grains and edible feedstocks to serve as a sustainable resource with

lower production costs for alternative liquid fuel production in the future (Prasad *et al.*, 2007). The conversion of cellulose to ethanol is typically carried out via a first step of enzymatic hydrolysis of cellulosic materials to reducing sugars and a second step of fermentation of the produced sugars to ethanol (Nigam and Singh, 2011). The enzymatic hydrolysis of cellulose involves synergistic actions of endoglucanases, exoglucanases, and β-glucosidases. Endoglucanases play a major role in initiating cellulose hydrolysis by randomly cleaving internal glucosidic bonds in cellulose fibers and creating free chain ends; exoglucanases release cellobiose either from reducing or non-reducing chain ends; and β-glucosidases finally hydrolyze the cellobiose to glucose (Gupta and Verma, 2015).

The use of cellulolytic bacteria for large scale production of enzyme has increased in recent years because of their short doubling times and ease of cultivation at high cell densities using inexpensive carbon and nitrogen sources (Westers *et al.*, 2004). A number of cellulolytic bacteria from termite gut and other sources have been studied and continually developed by genetic engineering techniques to achieve desired properties and high productivity of endoglucanase for industrial applications (Akaracharanya *et al.*, 2014; Cheng *et al.*, 2016; Chuan Wei *et al.*, 2015; Rastogi *et al.*, 2010; Taechapoempol *et al.*, 2011; Zhang *et al.*, 2011).

Due to the high cost of enzyme production, biofuel production from cellulose is not feasible at the present time (Klein-Marcuschamer *et al.*, 2012; Maki *et al.*, 2009). A consolidated bioprocess, combining cellulase production and conversion of cellulose into desired products as one step, has been the subject of increased research efforts in recent years (Olson *et al.*, 2012). The absence of cellulase-whole cells separation step can lowered overall process cost (Wu *et al.*, 2013). The term "microbial hydrolysis" referred to growing cultures of cellulolytic bacteria in the presence of cellulose substrate, which is to be hydrolyzed, without added enzyme (Lu *et al.*, 2006). Currently, there is little research available on cellulose hydrolysis with whole cells (Lu *et al.*, 2006; Wu *et al.*, 2013). Hence, a study on this issue is of great interest since it will lead to potential applications for a large scale biofuel production from lignocellulosic materials.

In this study, an endoglucanase gene from *Bacillus subtilis* M015, isolated from Thai higher termites, *Microcerotermes* sp. (Taechapoempol *et al.*, 2011) was

cloned into a periplasmic-leaky *Escherichia coli* strain. The crude recombinant endoglucanase produced by *E. coli* expressing endoglucanase gene was characterized. A mixture of the recombinant endoglucanase and the commercial cellulase enzyme was tested the hydrolytic activity of different cellulosic materials of soluble cellulose, insoluble microcrystalline cellulose, and corncob, as compared with the commercial enzyme alone. The recombinant bacteria were also studied for glucose consumption and microbial hydrolysis. Additionally, the effects of Tween 80 on bacterial growth and endoglucanase production were determined.

4.3 Experimental

4.3.1 Bacterial Strains, Vectors, and Materials

B. subtilis M015 (GenBank accession number **KP192484**), isolated from Thai higher termites (*Microcerotermes* sp.) by our research group (Taechapoempol *et al.*, 2011), was preserved by lyophilization and grown in a 65 modified Deutsche Sammlung von Mikroor Ganismen und Zellkulturen (DSMZ) medium, containing 5 g/L of carboxymethyl cellulose (CMC, Sigma-Aldrich), 4 g/L of yeast extract (Hi-Media), and 10 g/L of malt extract (Hi-Media) at pH 7.0. *E. coli* strain JE5505 (carrying an *lpp*-deletion), obtained from The Coli Genetic Stock Center (CGSC), Yale University, was maintained in a lysogeny broth (LB; 10 g/L of tryptone (Hi-Media), 5 g/L of yeast extract, and 5 g/L of sodium chloride (RCI labscan) at pH 7.0) to be used as an expression host in this study. The expression plasmid, pFLAG-CTS, carrying an OmpA secretion signal for the periplasmic expression of recombinant proteins, and ampicillin sodium salt (100 μg/mL) used as the selection agent, were purchased from Sigma-Aldrich. One mM of isopropyl β-D-thiogalactopyranoside (IPTG, Invitrogen Life Technologies) was added to the culture to induce protein expression.

4.3.2 Amplification, Cloning and Expression of Endoglucanase Gene

To obtain the DNA template for gene amplification, genomic DNA was extracted from *B. subtilis* M015 cell pellets, harvested in the mid-exponential growth phase and separated by centrifugation, using the DNeasy Blood and Tissue Kit

(QiaGen, USA). For the amplification of the gene coding for endoglucanase by polymerase chain reaction (PCR), two primers were designed, based on the reference sequence of endoglucanase (*egl*S) gene of *B. subtilis* subsp. *subtilis* 168 (GenBank accession number **BSU18130**); forward primer: 5'-GATCCAAGCTTCTATGAAACGGTCAATCTCTAT-3', reverse primer: 5'-CTCACTCGAGTTACTAATTTGGTTCTGTTCCC-3'. Two restriction sites were incorporated, *Hin*dIII and *Xho*I, respectively, to allow the directional cloning of PCR products into the expression vector pFLAG-CTS. The *egl*S gene was amplified using Phusion Hot Start II polymerase (Thermo Scientific, USA) under the following PCR conditions: 2 min initial denaturation at 98°C, 30 cycles of 30 s denaturation at 98°C, 30 s annealing at 55°C and 65°C, and 30 s extension at 72°C, and 5 min final extension at 72°C.

PCR products were double-digested with *Hin*dIII and *Xho*I and cloned into plasmid, pFLAG-CTS, also digested with the same restriction enzymes. The ligation products (pCTS-*egl*S) were transformed into *E. coli* JE5505 by electroporation. The positive clone was confirmed by recovering plasmid DNA using the QIAprep Spin Miniprep Kit (QiaGen, USA) and running on a 0.8% agarose gel.

The presence of the endoglucanase gene was confirmed by DNA sequencing. Then, the transformed *E. coli* containing pCTS-*egl*S was named *E. coli* Glu5. The *E. coli* carrying the empty plasmid was included as a control in the study. All molecular biology studies were performed by following the standard techniques (Sambrook and Russell, 2001).

4.3.3 Preparation of Secreted Recombinant Endoglucanase and Size Verification

E. coli Glu5 was cultured overnight in 2 mL LB-ampicillin as an inoculum in an orbital shaker at 180 rpm and 37°C under aerobic conditions, and then diluted at a ratio of 1:400 into a 250 mL Erlenmeyer flask containing 100 mL of the fresh LB-ampicillin medium. The flask was incubated with constant shaking. At mid-exponential phase of the culture when the optical density (OD_{600}) reached 0.5, protein expression was induced with a 1 mM IPTG. The optimal culturing time of *E. coli* Glu5 for endoglucanase (EglS) production was determined during 40 h post-induction by

DNS assay (explained below) and further used for producing enzyme stock. The secreted recombinant EglS used as crude form was obtained by centrifugation of the cell culture at 11000 × g for 10 min at 4°C and filtered via a 0.22 µm membrane.

To verify the molecular size of the secreted mature protein, the secreted EglS enzyme was concentrated ten-fold by ultrafiltration (10 kDa MW membrane cut-off, Amicon, Beverly, MA, USA). The protein profile of the concentrated enzyme was determined by resolution through SDS-PAGE (12% (w/v) acrylamide resolving gel) and native-PAGE (containing 1% (w/v) CMC) for zymogram analysis (Laemmli, 1970). The cell-free supernatant of *E. coli* carrying the empty plasmid was prepared the same way and used as a control. The theoretical moleculer weight of EglS was calculated using the online tool available at Georgetown University Protein Information Resources Website (http://pir.georgetown.edu/cgi-bin/comp_mw.pl).

4.3.4 Hydrolytic Ability Assay

E. coli Glu5 was tested for hydrolytic ability by the Congo red assay (Li *et al.*, 2008). The inoculum (5 µL) was spotted on LB-ampicillin agar plates containing 1% (w/v) CMC and 1 mM IPTG. In comparison with the cloned strain (*E. coli* Glu5), *B. subtilis* M015, was assayed on 65 modified DSMZ agar plates at 37°C for 24 h, and then hydrolysis zones were visualized by a 1% (w/v) Congo red reagent staining for 10 min and destaining with a 1 M sodium chloride solution. The presence of a clear zone around the colonies indicated the hydrolysis ability on cellulose by the secretion of active endoglucanase. The hydrolysis capacity (HC) value was calculated from the clear zone diameter divided by the colony diameter.

4.3.5 Enzymatic Activity and Protein Determination

The endoglucanase activity was determined from crude enzyme by the standard DNS method using glucose as the standard reference (Miller, 1959). The assays were conducted in triplicates in 96-well-microplates. Each diluted crude enzyme sample was incubated with 2% (w/v) CMC solubilized in a 50 mM sodium citrate buffer (pH 4.8) at an equivalent ratio (total volume of 60 µL). The mixture was incubated at 50°C for 30 min and the reaction was stopped by adding an equal volume of a DNS solution (1.4% (w/v) 3,5-dinitrosalicylic acid, 0.28% phenol, 1.4% sodium

hydroxide, 28% sodium potassium tartrate, and 0.07% sodium sulfite), heated at 95°C for 5 min, and finally quickly cooled to room temperature. The degree of enzymatic hydrolysis of the soluble cellulose (CMC) was determined spectrophotometrically by measuring the absorbance at 540 nm in a VersaMax Microplate Reader (Molecular Probes, USA). One Unit (U) of endoglucanase activity is defined as the amount of enzyme producing 1 μmol of reducing sugars from CMC per min under the assay conditions. The total protein concentration was determined by the Bradford method (Bio-Rad) using bovine serum albumin standards. The specific activity was calculated from the number of units of enzyme activity per mg of total proteins.

The hydrolytic mixture of added EglS enzyme at a volume ratio of 1:40 in 50 mM sodium citrate buffer containing 1% (w/v) CMC, (9.4 U/g CMC), was incubated at the optimum pH (4.8) and temperature (50°C) for 30 min, and then analyzed for sugar products using a high performance liquid chromatograph (HPLC) equipped with a refractive index detector (6040 XR, Spectra-Physics, USA) and an Aminex HPX-87H column (Bio-Rad Lab, USA). A 5 mM sulfuric acid (H_2SO_4) solution was used as a mobile phase at a flow rate of 0.6 mL/min and the column temperature was controlled at 60°C. The supernatant of cultured *E. coli* carrying the empty plasmid was used as control.

4.3.6 Characterization of Crude Endoglucanase Enzyme

To determine optimum pH for maximum endoglucanase activity of the crude EglS enzyme, samples were diluted with citrate and phosphate buffers of various pH (50 mM sodium citrate buffer for pH 3.0–6.0 and 50 mM sodium phosphate buffer for pH 7.0–8.0), containing 2% (w/v) CMC, at an equivalent volumetric ratio and incubated at 50ºC. The effect of temperature on the endoglucanase activity was determined by incubating the diluted samples with 2% (w/v) CMC in a 50 mM sodium citrate buffer (pH 4.8) at an equivalent volumetric ratio under different temperatures of 25–80ºC. The enzymatic hydrolytic reactions were stopped after 30 min by adding the DNS solution and the endoglucanase activities were assessed as previously described. Thermal stability was determined by pre-incubating the diluted EglS in a 50 mM sodium citrate buffer (pH 4.8) at different temperatures of 40, 50 and 60ºC.

The residual endoglucanase activity of samples was determined as percentages of the initial activity during the incubation period of 100 h.

The kinetic parameter values of the fresh crude EglS on CMC hydrolysis in terms of Michaelis-Menten constants (K_m) and maximum velocity (V_{max}) were determined using the Lineweaver-Burk plots. The reaction was started by addition and mixing of enzyme (0.715 U/mL of reaction mixture) to CMC solutions of various initial CMC concentrations (2.5-47 mg/mL). The initial reaction velocity of the enzyme that produced reducing sugars was determined under the optimal conditions (pH 4.8 and 50ºC) within 10 min reaction time.

4.3.7 Enzymatic Hydrolysis Studies with Various Cellulosic Substrates

The crude EglS, the commercial cellulase (Cel), and a mixture of the two enzymes were tested for the hydrolytic abilities on various cellulosic substrates including a soluble cellulose (CMC), an insoluble microcrystalline cellulose (Avicel PH-101, Sigma-Aldrich), and a milled corncob (CC) as lignocellulose. The Cel exoglucanase activity from *Trichoderma reesie* ATCC 26921 (Sigma Aldrich) was determined to be 62 FPU/mL by DNS method. Both CMC and Avicel substrates were used as received without treatment. A corncob sample, obtained from Kanchanaburi province, Thailand, was dried overnight at 105°C, milled by a hammer mill (Fitzmill Fitzpatrick DAS06 5HP), and sieved to obtain the particle size range of 60-80 mesh. The CC, composed of 44 ± 2% cellulose, 34 ± 1% hemicellulose, 12 ± 1% lignin, and 10 ± 1% extractives on dry weight basis, was used in the experiment without any treatment (Seneesrisakul *et al.*, 2014). The properties of all test cellulosic substrates are summarized in Table 4.1 (Mittal *et al.*, 2011; Percival Zhang *et al.*, 2006; Seneesrisakul *et al.*, 2014; Zhang and Zhao, 2009). The enzymatic hydrolysis tests were carried out with a 100 ml of total reaction volume in Erlenmeyer flasks. The enzyme solutions of EglS (50 U), Cel (50 FPU), and the mixture of EglS and Cel (50 U and 50 FPU, respectively) were loaded into each flask containing 0.5 g of each dried substrate in a 50 mM sodium citrate buffer (pH 4.8). A 0.01% sodium azide reagent was added to prevent microbial contamination. For controls, all studied substrates were suspended in the buffer solution without added enzymes. All flasks were incubated at 50°C in a shaking incubator (180 rpm). The samples were collected every 20 h for 100

h and boiled for 5 min to stop the hydrolytic reaction. Subsequently, the samples were centrifuged and filtered via a 0.22 μm filter for sugar analysis using HPLC. The initial and overall sugar production rates of each hydrolytic reaction were calculated from the total concentration of produced glucose, cellobiose, and xylose from the first 20 h and the entire range of 100 h, respectively.

Table 4.1 Properties of cellulosic substrates as crystallinity index (CrI), the fraction of β-glucosidic bond accessible to cellulase (F_a), the number average of degree of polymerization (DP_n), the degree of substitution (DS),and the fraction of reducing ends (F_{Re}) (Mittal *et al.*, 2011; Percival Zhang *et al.*, 2006; Seneesrisakul *et al.*, 2014; Zhang and Zhao, 2009)

Substrate	Size (μm)	CrI	F_a (%)	DP_n	DS	F_{Re} (%)
Soluble						
Carboxymethyl cellulose (CMC)	-	-	100	400	0.65-0.9	0.05-1
Insoluble						
Microcrystalline cellulose (Avicel)	50	0.7-0.9	0.6	90-220	-	0.2-0.67
Corncob (CC)	300	0.57	-	-	-	-

4.3.8 Study on Effect of Glucose on Bacterial Growth and Endoglucanase Production

To determine the effects of glucose on the growth profile and endoglucanase production of *E. coli* Glu5 compared with *B. subtilis* M015, glucose (1% w/v) was supplemented in the medium (pH 7.0). *E. coli* Glu5 was inoculated (1:400) in the LB-ampicillin (100 mL) with glucose and without glucose as a control. One mM of IPTG was used to induce protein expression at 4 h post-inoculation. *B. subtilis* M015 was diluted at the same ratio (1:400) in LB with glucose and without glucose. CMC (1% w/v) was used as the enzyme-induced substrate. All flasks were incubated at 37°C and 180 rpm for 48 h. The absorbance of samples taken from the cultures were measured at OD_{600} to determine cell concentration. The endoglucanase activity of cell-free supernatant samples were determined by DNS assay. The un-

metabolized glucose was determined by HPLC. All experiments were performed in triplicate.

4.3.9 Microbial Hydrolysis Experiments of CMC using *E. coli* Glu5

Microbial hydrolysis of CMC using *E. coli* Glu5 was investigated by growing *E. coli* Glu5 in the medium containing CMC as the substrate to determine if the secreted EglS from bacterial cells could directly hydrolyze the substrate presented in the medium and produce sugar products. Various concentrations of CMC (10-50 g/L) were added to the LB-ampicillin medium (pH 7.0). *E. coli* Glu5 inoculum was then added into each medium at a volumetric ratio of 1:400, and the entire experiment was performed at 37°C and 180 rpm for 48 h. The reducing sugar amount in the culture solution was determined using the DNS reagent.

4.3.10 Study of Toxicity of Tween 80 on *E. coli* Glu5 Growth and Secreted Enzyme Activity

Polyoxyethylenesorbitan monooleate (Tween 80, Sigma-Aldrich), a nonionic surfactant, was added to the LB-ampicillin at different concentrations (0.1-10% (w/v)). The OD_{600} of cultures and EglS activities of the supernatants taken from the stationary phase were measured to determine toxicity of the added surfactant.

4.4 Results and Discussion

4.4.1 Cloning and Sequencing Results

The amplification of the endoglucanase (*egl*S) gene of *Bacillus subtilis* M015 yielded a 1,520 bp fragment, as shown in Figure 4.1(a). The intensity of PCR product at annealing temperature of 55°C was higher than that of 65°C. Thus, the annealing temperature was fixed at 55°C for further amplification. The fragment was subsequently cloned into plasmid, pFLAG-CTS, and sequenced. Figure 4.1(b) showed the successfully transformation of plasmid containing *egl*S (pCTS-*egl*S) into *E. coli.* Two bands of positive clone (colonies 1 and 5) at 1.5 kbp and 5.3 kbp refers to *egl*S and pFLAG-CTS, respectively. The DNA sequencing of the cloned fragment yielded a 1,497 bp long open reading frame (ORF) encoding for a 499 amino-acid long protein

with a calculated molecular weight of 55,294 Da. Further investigation of the sequence across GenBank database revealed that the amino acid sequence of *B. subtilis* M015 endoglucanase (accession number **KP223322**) is 99% identical to that of *B. subtilis* EGD-AK10 (**WP021479778.1**) and E1 (**CCU58432.1**), 98% identical to that of *B. subtilis* subsp. *subtilis* 168 (**CAB13696**) and *B. sp*. HY2-3 (**AAV34758.1**), and 93% identical to that of *B. sp*. NK2 (**ADO85705.1**). The alignment of the *B. subtilis* M015 endoglucanase with the two reference sequences is shown in Figure 4.2. These findings confirm that the endoglucanase belongs to the glycoside hydrolase (GH) family 5.

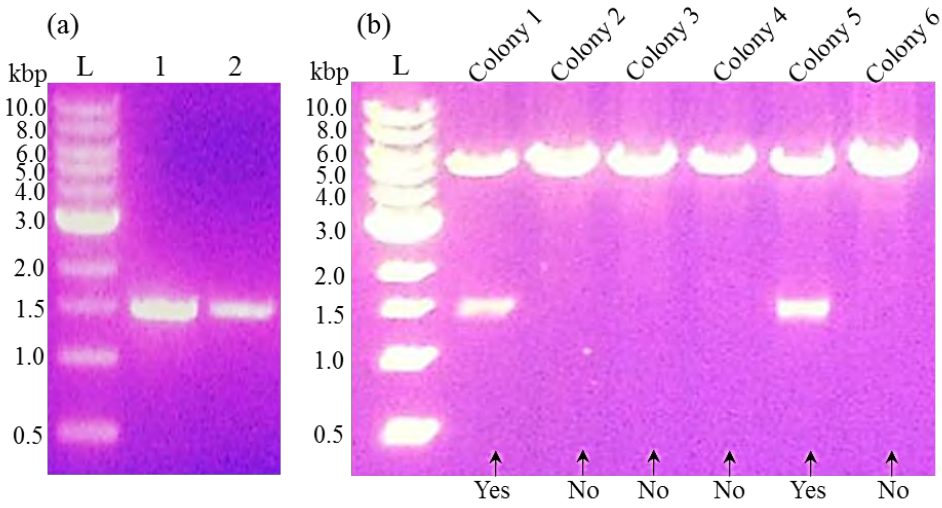

Figure 4.1 (a) Amplification of *B. subtilis* M015 endoglucanase gene. Lane L – 1-kb DNA ladder (New England Biolabs); lane 1 and 2 – PCR product of *B. subtilis* M015 endoglucanase gene with annealing temperature of 55°C and 65°C, respectively. (b) The transformation of pCTS-*egl*S into *E.coli* was successful in colonies 1 and 5. These two colonies were selected for further study.

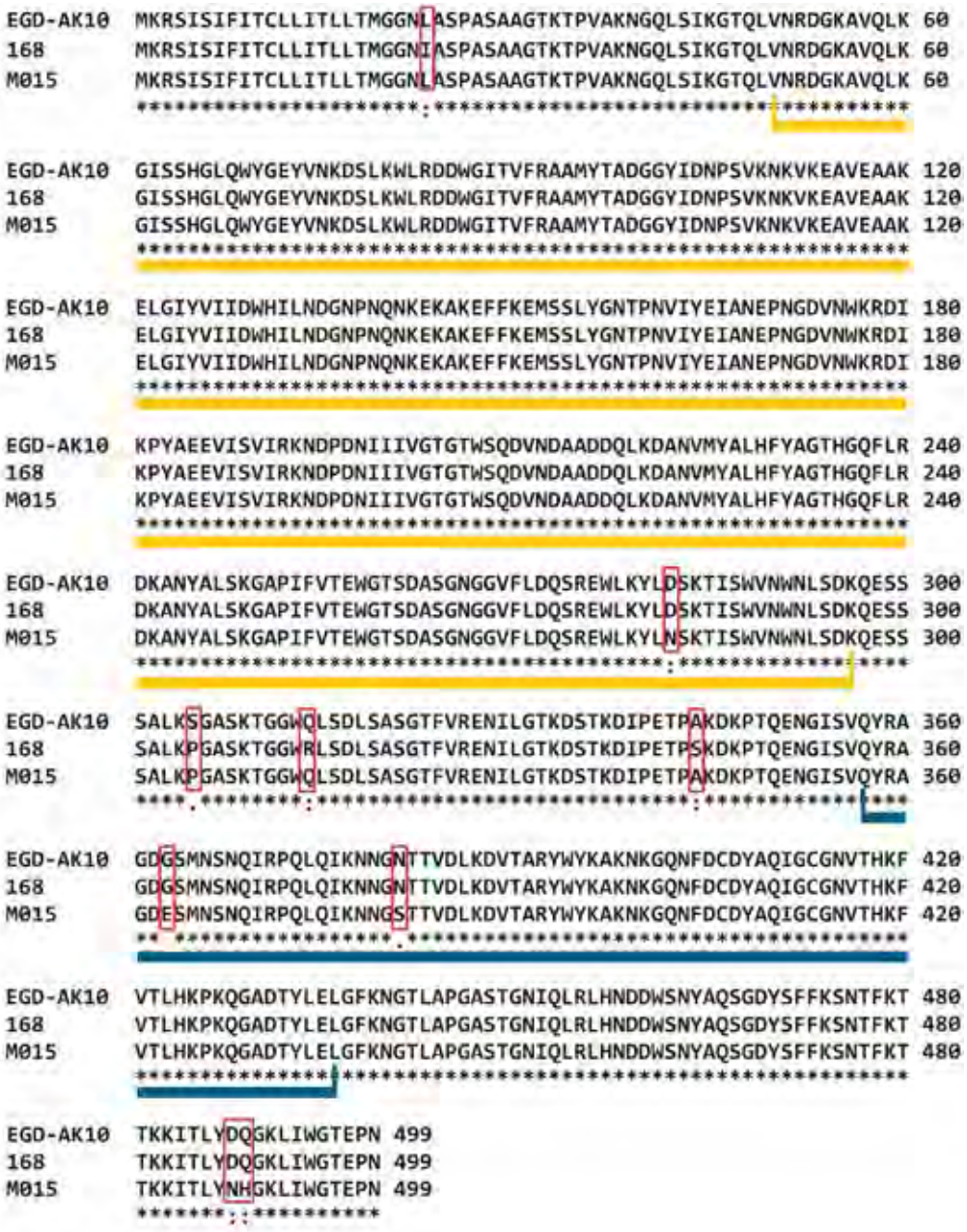

Figure 4.2 Sequence alignment of *B. subtilis* M015 endoglucanase (**KP223322**) to that of *B. subtilis* subps. *subtilis* 168 and EGD-AK10 (**CAB13696**, **WP021479778.1**). The differences between sequences are framed in red. Conserved domains are shown by straight colored bars; yellow bar shows residues 50 to 296 corresponding to cellulase encoding sequence (glycosyl hydrolase family 5) and blue bar shows the residues 357 to 436 corresponding to cellulose binding domain (CBM3) (determined by NCBI Protein Blast).

4.4.2 Secretory expression and high activity of recombinant endoglucanase (EglS)

To verify the expression and secretion of the recombinant endoglucanase (EglS) from periplasmic-leaky *E. coli*, the supernatant samples were visualized on a 12% SDS-PAGE gel stained with Coomassie Blue (Biorad) (Figure 4.3(a)). The band corresponding to recombinant endoglucanase was observed at 55 kDa (lane 2). Two additional proteins around 41 kDa and 37 kDa were also observed on the gel which are most likely the truncated products of EglS due to proteolysis. A barely visible band around 37 kDa was also present in the concentrated supernatant samples of induced *E.coli* carrying empty plasmid (lane 4) which possesses no detectable cellulase activity. The CMC-zymogram of EglS (lane 5) showed two bands consistent with lane 2 which confirms that the smaller band does correspond to truncated EglS with some preserved cellulase activity.

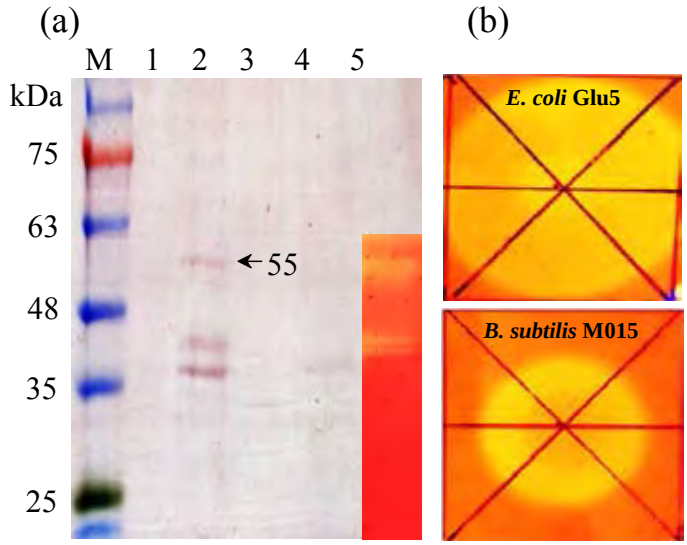

Figure 4.3 (a) SDS-PAGE analysis of endoglucanase (EglS) secreted into the culture medium by the recombinant *E. coli*. Lane M – Protein marker, lane 1 – uninduced *E. coli* Glu5 (before adding IPTG), lane 2 – induced *E. coli* Glu5 (after adding IPTG for 16 h), lane 3 – uninduced *E. coli* carrying empty plasmid, lane 4 – induced *E. coli* carrying empty plasmid, and lane 5 – CMC-zymogram of EglS. (b) Congo red plate assay of *E. coli* Glu5 and native strain *B. subtilis* M015.

The secretion of the recombinant EglS was further verified by the Congo red assay. The presence of a clear zone around the colony of *E. coli* Glu5 indicated the hydrolytic activity on the added CMC by the secreted extracellular enzyme under aerobic incubation whereas no clear zone was found around the control colonies. The clear zone of *E. coli* Glu5 was significantly larger than that of native strain *B. subtilis* M015 (Figure 4.3 (b)) with hydrolysis capacity (HC) values of 2.66 ± 0.34 and 1.63 ± 0.02, respectively, corresponding to a 63% increase due to enhanced expression and secretion yield of the recombinant EglS enzyme by *E. coli*.

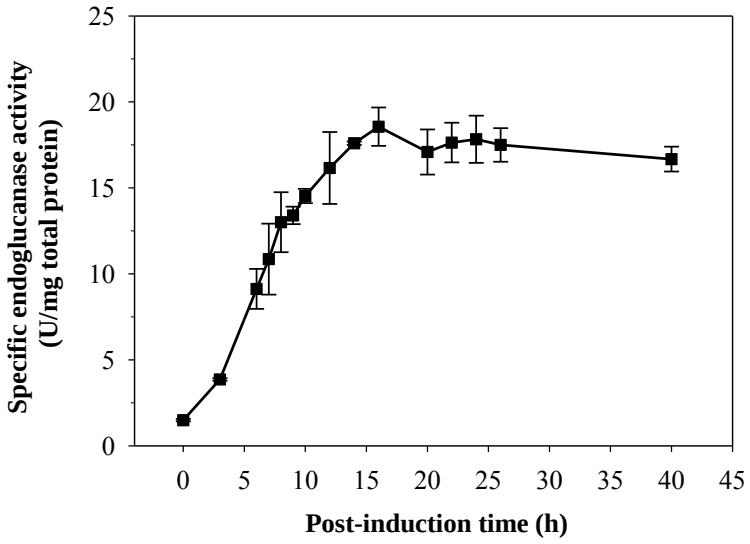

Figure 4.4 Crude EglS production rate of *E. coli* Glu5 cultured at 37°C and 180 rpm.

From the crude enzyme production profile (Figure 4.4), *E. coli* Glu5 secreted crude EglS with a maximum specific endoglucanase activity of 18.56 U/mg total protein (an endoglucanase activity of 3.68 U/mL and a total protein of 0.198 mg/mL) at 16 h of post-induction cultivation. This level is approximately 17 times higher than that of the native strain *B. subtilis* M015 (1.098 U/mg total protein) (Taechapoempol *et al.*, 2011). This high activity of crude EglS towards CMC, achieved by *E. coli* JE5505-based expression system, also appears to be superior to other crude endoglucanases from *Bacillus velesensis* P3-1 and P4-6 (0.015 U/mL) (Akaracharanya *et al.*, 2014) and other purified recombinant endoglucanases produced by other *E. coli* strains: *E. coli* harboring endoglucanase gene from *Ruminococcus*

flavefaciens strain 186 (0.183-0.455 U/mg protein under the studied conditions) (Huang *et al.*, 1989), *E. coli* BL21 (DE3) harboring cellulase *Cel*DR of *B. subtilis* DR (0.82 U/mL) (Li *et al.*, 2008), and *E. coli* harboring endoglucanase gene of *B. subtilis* UMC7 (0.73 ± 0.002 U/mL) (Chuan Wei *et al.*, 2015). In 2015, Gao and coworkers (Gao *et al.*, 2015) reported an effort to produce extracellular cellulase by using *E. coli* expressing heterologous cellulase with N20 from Cel-CD. It produced higher extracellular protein of 514 mg/L with a lower CMCase activity of 558.4 U/L, compared to our results. The results reveal that *E. coli* Glu5 has great ability to produce highly active extracellular endoglucanase without a need for downstream purification steps which would be very cost effective for cellulosic biofuel production industry.

Figure 4.5(a) shows the sugar end products of the enzymatic hydrolysis of CMC using the crude EglS, as compared to the control. The broad CMC peak (peak 4) of the control (RT 6.34 min) represents the distribution of degree of polymerization ($DP_n \geq 3$) of CMC, indicating that it was not hydrolyzed. In contrast, the narrower CMC peak (peak 4) with a much higher height of the *E. coli* Glu5 system shifted to RT 6.20 min, confirming that CMC was partially hydrolyzed by EglS and still retained gluco-oligomer portion (peak 4 and 5). Figure 4.5(b), the enlarged version of Figure 4.5(a), shows the presence of three small peaks of glucose (peak 1), cellobiose (peak 3) and remaining gluco-oligomers (peak 5) since endoglucanases randomly cleave the internal bonds of the polymer and not necessarily resulting in direct soluble sugars (Engel *et al.*, 2012). The results suggest that a combination of EglS with an exoglucanase and β-glucosidase will be needed to complete the hydrolysis of CMC into glucose. A further attempt will be carried out to insert both exoglucanase and β-glucosidase genes in to *E. coli* Glu5 for complete hydrolysis of cellulosic materials by using a mixed solution of these three enzymes.

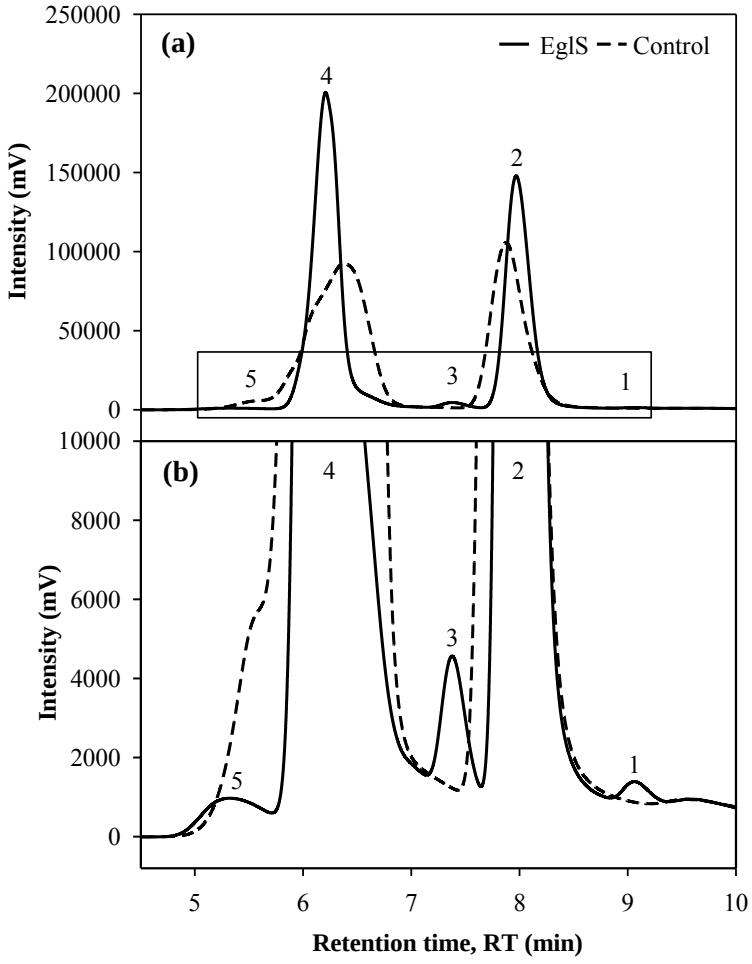

Figure 4.5 HPLC chromatograms of sugar end products from hydrolysis of CMC (1% w/v) by recombinant endoglucanase (EglS) and control in the 50 mM sodium citrate buffer (pH 4.8) at 50°C for 30 min, peak 1 – glucose, 2 – sodium citrate, 3 – cellobiose, and 4-5 – gluco-oligomers of CMC. The rectangular area in chromatogram (a) is enlarged in (b).

4.4.3 EglS Enzyme Characterization Results

The crude EglS enzyme showed a maximum activity at pH 4.8 in the sodium citrate buffer, and was able to retain over 70% of its maximum activity across the broad pH range of 4.0–8.0 in sodium citrate/phosphate buffer, as shown in Figure 4.6(a). Similarly, the EglS activity at pH 5.0 was insignificantly different (only 0.2%) from it at optimum pH 4.8. The maximum endoglucanase activity at pH 5.0 was also found for other cellulolytic enzymes such as CMCase from *Bacillus* sp. DUSELR13

(Rastogi *et al.*, 2010), and cel28a from *E. coli* DH5α, expressing *cel28a* with high activity retained in a narrower pH range than the crude EglS (Cheng *et al.*, 2016).

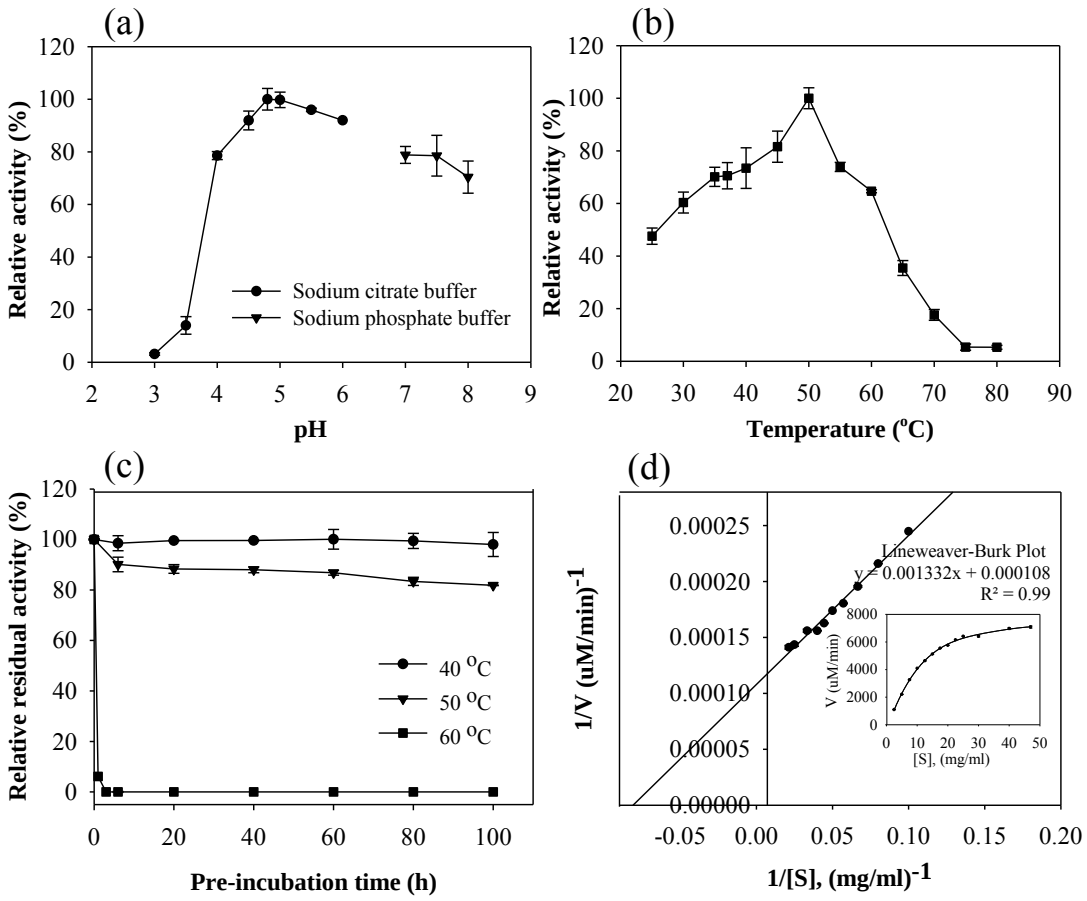

Figure 4.6 (a) Effect of pH on endoglucanase activity measured at 50°C for 30 min using various pH buffers; 50 mM sodium citrate buffer for pH 3.0–6.0; 50 mM sodium phosphate buffer for pH 7.0–8.0, (b) Effect of temperature on endoglucanase activity measured in 50 mM sodium citrate buffer (pH 4.8) for 30 min, (c) Thermal stability of the recombinant EglS at different temperatures in 50 mM sodium citrate buffer (pH 4.8) for 100 h measured from residual endoglucanase activity, and (d) Kinetic analysis of crude EglS toward various concentration of CMC using Lineweaver-Burk plot.

As shown in Figure 4.6(b), the maximum endoglucanase activity is at 50°C with 50% of its maximum activity in the temperature range of 30–60°C. This is consistent with other endoglucanases from *B. subtilis* DR, *E. coli* BL21 (DE3) (Li *et*

al., 2008), and *Bacillus velesensis* (Akaracharanya *et al.*, 2014), tested at slightly different pHs (6.5, 6.0 and 7.0, respectively). When the temperature increased to 75°C, the EglS activity approached zero, which is most likely due to denaturation of the enzyme.

The thermal stability of the crude EglS sample in the 50 mM sodium citrate buffer (pH 4.8) was determined at different temperatures of 40, 50 and 60°C for 100 h. The crude enzyme showed high thermal stability at 40°C and 50°C with residual activities of 98 and 82%, respectively, after 100 h of incubation, as shown in Figure 4.6(c), similar to non-specific endoglucanase (cel28a) tested at a much shorter incubation time (1 h) (Cheng *et al.*, 2016). The EglS lost almost all of its total enzymatic activity (94%) after 1 h of incubation at 60°C. Highly stability of EglS during extended incubations up to 100 h will be beneficial to enzymatic processes which are generally carried out at 50°C for 3 d or longer. For industrial applications, material costs can be reduced significantly by using more stable enzymes like EglS (Buchholz *et al.*, 2012).

The kinetic parameter values of the crude EglS toward CMC were calculated using Lineweaver-Burk plots, as shown in Figure 4.6(d). The K_m and V_{max} values were 12 mg/mL and 9259 μM/min (0.83 mg/min), respectively. In principle, Km is independent of the enzyme concentration (Fogler, 2006). Lower K_m values refer to higher affinity between enzyme and substrate, indicating that the crude EglS had higher affinity for CMC compared to the purified endoglucanase (Thcel9A) from *E. coli* BL 21(DE) expressing endoglucanase gene from *Thermobifida halotolerans* YIM 90462 (K_m of 37 mg/mL) (Zhang *et al.*, 2011) and purified endoglucanase from *Aspergillus niger* B03 (K_m of 21.01 mg/mL) (Dobrev and Zhekova, 2012), but lower than the purified cellulase from *Bacillu*s sp. MSL2 (K_m of 0.8 mg/mL) (Sriariyanun *et al.*, 2016). In addition, Km value of crude enzyme is normally higher than purified enzyme due to the kinetic efficiency of enzyme that might be retarded by the complex mixture of crude enzyme in medium and other available proteins (Cavalcante Braga *et al.*, 2013; Rastogi *et al.*, 2010).

4.4.4 Enzymatic Hydrolysis of Various Cellulosic Substrates

Figure 4.7 shows all hydrolysis products of three different cellulosic materials over time (soluble cellulose, CMC; insoluble microcrystalline cellulose, Avicel; and 60-80 mesh milled corncob, CC, as a representative of agricultural residue) using crude EglS and the commercial enzyme (Cel) with and without EglS addition. Both EglS and Cel were tested to ensure the enzyme stability after 100 h of incubation at 50°C and found that they were highly stable with 18% and 4% loss of their initial activity, respectively. The hydrolysis results of the three substrates showed that glucose was the main component of sugar products followed by cellobiose. A small concentration of xylose was found in CC which contains hemicellulose but not in CMC and Avicel. EglS displayed much lower hydrolytic ability with much lower sugar production, due to the fact that EglS has only the endoglucanase activity while Cel has multifunctional cellulolytic ability of endoglucanase, exoglucanase, and β-glucosidase. Interestingly, the use of Cel with added crude EglS showed a great enhancement on the initial sugar production rate at the first 20 h for any studied substrate (Table 4.2). The initial sugar production rates from CMC, Avicel, and CC using the mixed enzymes of Cel and EglS were higher than those of the sole Cel by 5.23, 1.67, and 5.77 times, respectively. This can be reasonably explained as follows; EglS possessing endoglucanase activity cleaves cellulose at random sites to shorter cellulose chains and increases active sites or reducing ends for subsequent enzymes, specifically exoglucanase to act on (Gupta and Verma, 2015). The enzyme mixture could hydrolyze CMC, Avicel and CC to produce maximum total sugar concentrations of 196, 749 and 197 mg/g substrate, respectively, at 100 h. The sugar level from Avicel hydrolysis was much higher than those from CMC and CC hydrolysis, resulted from exoglucanase activity and specificity of Cel towards Avicel. Conversely, CMC, which is a modified cellulose with degree of substitution of 0.65-0.9, has a quite low accessibility (only non-substituted glucose units are accessible for the enzyme) with a high DP_n (Percival Zhang *et al.*, 2006). For CC, it is a lignocellulosic material which contains not only cellulose but also hemicellulose and lignin as inhibitors to enzymatic hydrolysis. Moreover, an average particle size of corncob was relatively high, and consequently, its available surface area was much lower than that of Avicel.

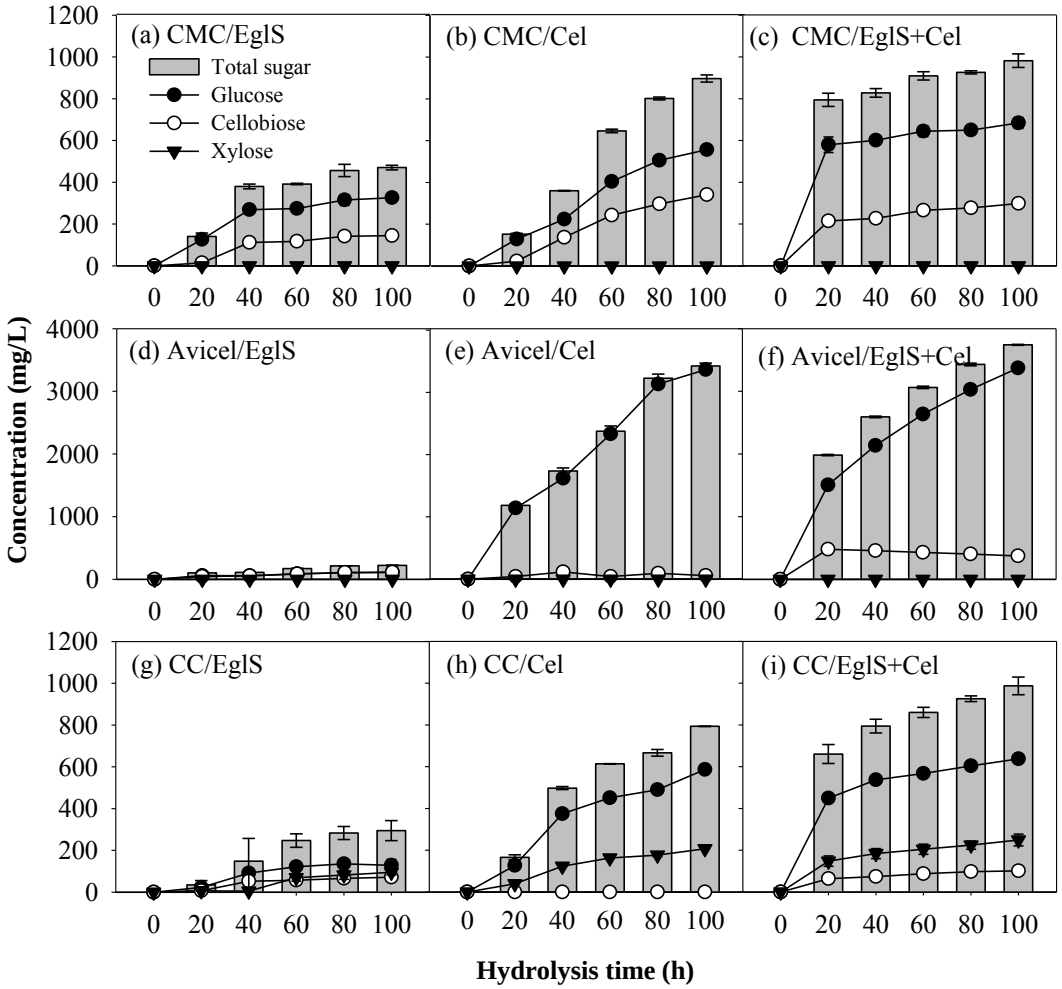

Figure 4.7 Sugar products from enzymatic hydrolysis of different cellulosic substrates (5 g/L); (a-c) carboxymethyl cellulose (CMC), (d-f) microcrystalline cellulose (Avicel), and (g-i) corncob (CC) using crude recombinant EglS with 25 U/g substrate, commercial cellulase (Cel) with 25 FPU/g substrate, and enzyme mixture of EglS and Cel with 25 U/g substrate and 25 FPU/g substrate, respectively. The reactions were carried out at 50°C and 180 rpm for 100 h. Bar charts represent total sugar concentration based on glucose, cellobiose, and xylose.

Table 4.2 Summary of initial and overall sugar production rate from the enzymatic hydrolysis at 50°C and pH 4.8

Substrate	Enzyme	Total Sugar Production (mg/L)	Sugar Production Rate (mg/L/h)	
			Inintial Rate (20 h)	Overall Rate (100 h)
CMC	EglS	470 ± 11	7.02 ± 0.80	4.70 ± 0.11
	Cel	897 ± 17	7.59 ± 0.20	8.97 ± 0.17
	EglS+Cel	982 ± 32	39.71 ±1.58	9.82 ± 0.32
Avicel	EglS	222 ± 03	5.19 ±0.55	2.22 ± 0.03
	Cel	3424 ±46	59.20 ± 0.84	34.24 ± 0.46
	EglS+Cel	3743 ± 07	99.10 ± 0.49	37.43 ± 0.07
CC	EglS	294 ± 47	1.75 ± 1.00	2.94 ± 0.48
	Cel	793 ± 01	5.72 ± 0.40	7.93 ± 0.01
	EglS+Cel	987 ± 42	33.02 ± 2.26	9.87 ± 0.42

Comparisons between this work and other previous reports elsewhere are not straightforward due to the differences in studied conditions such as substrate type and enzyme concentration, reaction volume, pH, temperature, reaction time and purity of enzyme used. However, in comparison with other published works done under relatively similar conditions, the use of enzyme mixture in this study could produce relatively higher reducing sugar content: a purified enzyme mixture (CelZ and CelY) from *Erwinia chrysanthemi* produced 105.12 mg reducing sugar/g CMC (Zhou and Ingram, 2000), and a purified cellulase mixture (EG II, CBH I, CBH II, and β-glucosidase) produced approximately 430 mg glucose/g Avicel (Woodward *et al.*, 1988). The hydrolysis of Avicel using an enzyme mixture of commercial cellulase and β-glucosidase (Novozymes) provided a lower carbohydrate conversion of 69.6% at 96 h (Yu *et al.*, 2012), as compared to that of our enzyme mixture (75% conversion at 100 h). For the hydrolysis of milled corncob using the purified enzyme mixture of endoglucanase and endoxylanase from *Aspergillus niger* B03, it gave a reducing

sugar of 540 mg/L (Dobrev and Zhekova, 2012), which is lower than that in the present work (987 mg/L). These results reveal that crude EglS has great potential to be utilized for the enhancement of commercial cellulase, especially for the enzymatic hydrolysis of agricultural residues.

4.4.5 Effects of Glucose on Bacterial Growth and Endoglucanase Production

In the microbial hydrolysis of cellulose, bacterial cells have a role on cellulolytic enzyme production for hydrolyzing cellulose mixed in the liquid medium. Simultaneously, the hydrolysis products i.e. reducing sugars, mainly glucose, can be further utilized as a carbon source for bacterial growth, leading to the reduction of sugar yield. A lower sugar level can directly decrease the ethanol production rate of downstream fermentation (Sofer and Zaborsky, 2012). Besides, glucose at high concentrations can inhibit bacterial growth and enzyme activity (Epps and Gale, 1942). Therefore, the effects of glucose present in the culture media of *E. coli* Glu5 and *B. subtilis* M015 on bacterial growth and endoglucanase activity were investigated by adding glucose (1% w/v) in both systems of *E. coli* Glu5 and *B. subtilis* M015.

Figure 4.8(a) shows that the addition of 1% glucose can inhibit the growth of *E. coli* Glu5 up to 40%, as compared to the growth without added glucose at 48 h. The specific growth rate (μ) obtained from its exponential phase decreased from 0.177 h^{-1} in the absence of glucose to 0.087 h^{-1} in the presence of glucose. The pH of 48-h culture with added glucose was found to dramatically decrease (from the initial value of 7.0 to 5.0) whereas the pH of 48-h culture without added glucose slightly increased (7.5). The results imply that the presence of glucose in the medium causes acid formation, resulting in lower pH, and consequently, both lower cell density and growth rate were observed (Epps and Gale, 1942). For *B. subtilis* M015, glucose supplemented in LB medium with CMC (1% w/v) as endoglucanase inducer caused a biphasic growth, resulting from the two carbon sources (glucose and CMC) available in the medium. Glucose was preferentially utilized as a carbon source for *B. subtilis* M015 and rapidly consumed during the 8–20 h (Figure 4.8(b)), corresponding to the first exponential phase observed in Figure 4.8(a). After 20 h of incubation, the growth of *B. subtilis* M015 slightly dropped with a leveling off of glucose.

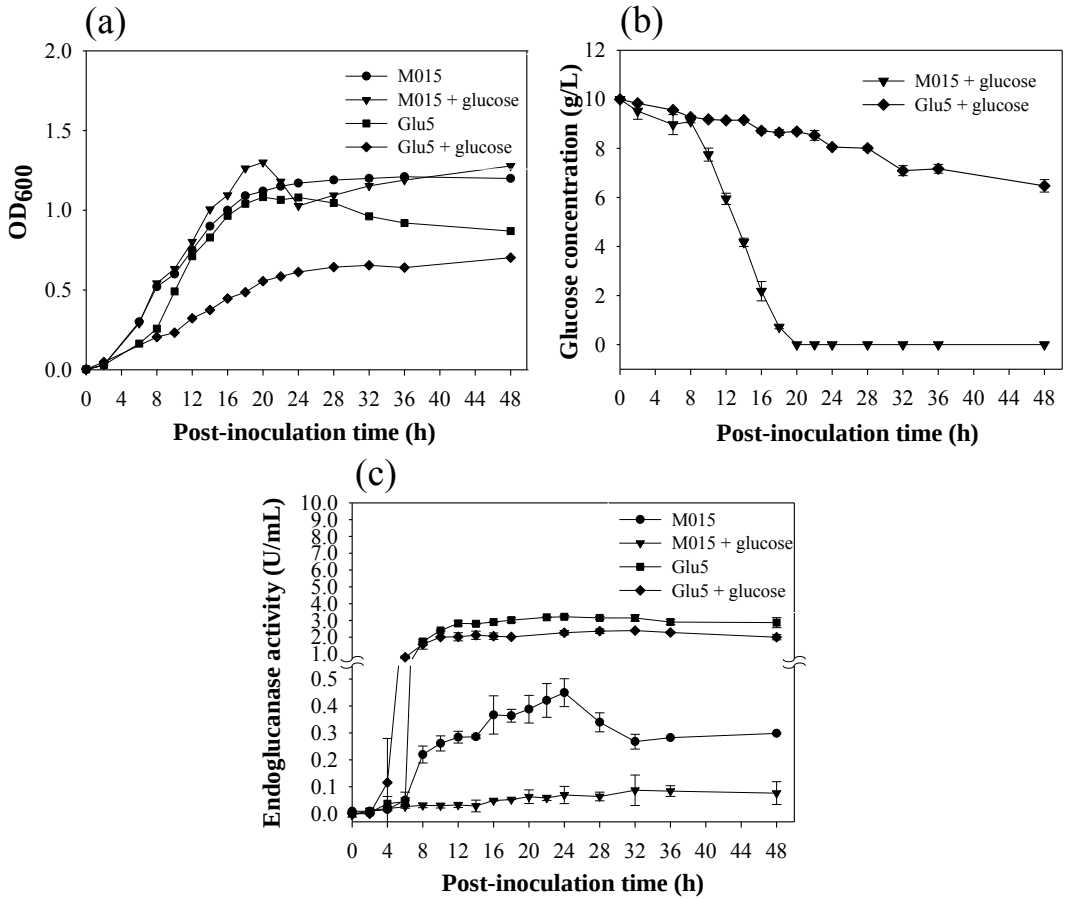

Figure 4.8 Effects of glucose (1% w/v) on bacterial growth and endoglucanase production. (a) Growth, (b) glucose consumption, and (c) endoglucanase activity of *B. subtilis* M015 in LB (*circle*) and LB with glucose (*triangle*), and *E. coli* Glu5 in LB-ampicillin (*square*) and LB-ampicillin with glucose (*diamond*). One mM of IPTG and 1% (w/v) of CMC were used to induce enzyme of *E. coli* Glu5 and *B. subtlis* M015, respectively, at 37°C and 180 rpm for 48 h.

The new exponential phase started at 24 h, suggesting that *B. subtilis* M015 started to utilize CMC as a carbon source. Interestingly, glucose was found to be slowly consumed by *E. coli* Glu5 with almost 65% of the initial glucose remaining unutilized after 2 days of culturing. This is most likely because LB medium contains plentiful of catabolizable amino acids as a carbon source for *E. coli* (Sezonov *et al.*, 2007).

The presence of glucose in the culture medium resulted in a lower endoglucanase production (activity) in both strains, compared with the absence of

glucose (Figure 4.8(c)). For *E. coli* Glu5, the lower endoglucanase production (26% relative activity) was consistent with the lower cell density due to acid formation from glucose. This was confirmed by measuring endoglucanase activities of *E. coli* Glu5 grown at pH 5.0 medium which was approximately 25% relative activity, as compared to that at pH 7.0. Whereas the lower endoglucanase production in *B. subtilis* M015 might be caused by catabolite repression, preventing expression of catabolic systems (endoglucanase activity) by preferred carbon sources (glucose) that enable the use of secondary substrates (CMC) (Deutscher, 2008).

Based on our findings, *E. coli* Glu5 produced endoglucanase with higher activity than that of *B. subtilis* M015 and consumed less glucose. The effect of glucose on lowering endoglucanase production can be reduced by controlling the pH of the culture.

4.4.6 Microbial Hydrolysis Activity of CMC by *E. coli* Glu5

An investigation of microbial hydrolysis using whole cells of *E. coli* Glu5 was conducted at different initial CMC concentrations for 48 h. As shown in Figure 4.9, an increase in CMC concentration increases the reducing sugar concentration whereas the sugar yield slightly decreases. A compromise of the two was found to be at a CMC concentration of 40 g/L, resulting in a reducing sugar concentration of 1,060 mg/L or a sugar yield of 27 mg reducing sugar/g CMC. The reducing sugar concentration produced in this study is comparable to that of the hydrolysis of CMC (10 g/L) with an excess of purified endoglucanase Hi Cel5A at room temperature for 48 h (reducing sugar concentration of 5.9 mM or 1,062 mg/L); however, sugar yield in this study was lower than that of Hi Cel5A about 4 times (Karlsson *et al.*, 2002). Zhou and coworkers (2000) reported that enzymatic hydrolysis of CMC (20 g/L) with two endoglucanases of CelY and CelZ provided a reducing sugar concentration of 3.83 mM (689 mg/L) and 3.98 mM (716 mg/L), respectively, at 35°C for 4 h (Zhou and Ingram, 2000). The report showed that the sugar product concentration obtained from purified enzyme (CelY and CelZ) is similar to that obtained from the microbial hydrolysis of the present study with a shorter time. The use of whole cells of *E. coli* Glu5 for microbial hydrolysis is technically feasible for cellulosic conversion processes. The direct use of *E. coli* Glu5 will cut down the cost

of enzyme production from both separation and purification steps. However, reducing sugars produced can be further consumed by bacteria as mentioned in section 4.4.5, resulting to the lower sugar product. Hence, a use of two-step process (enzyme production at a lower temperature and enzymatic hydrolysis step at a higher temperature) can improve the sugar concentration (Wu *et al.*, 2013).

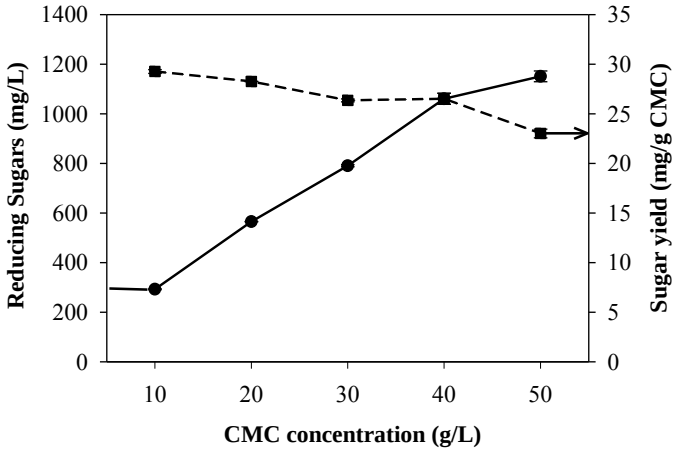

Figure 4.9 Reducing sugar profile from microbial hydrolysis of various concentrations of CMC (10-50 g/L) by *E. coli* Glu5 culturing in LB-ampicillin (pH 7.0) at 37°C and 180 rpm for 48 h.

4.4.7 Toxicity Results of Tween 80

Tween 80, a nonionic surfactant, has received great interest to be utilized in the enhancement of enzymatic hydrolysis of lignocellulosic materials because the hydrophobic interaction of surfactant with lignin can prevent the enzyme loss by being adsorbed onto the lignin surface, leading to higher availability of enzyme (Zheng *et al.*, 2008). It can also stimulate microbial enzyme production by increasing cell permeability (Akaracharanya *et al.*, 2014). However, a high concentration of Tween 80 can inhibit bacterial growth (Rose *et al.*, 1966). Therefore, the effect of Tween 80 concentration on cell growth rate and secreted enzyme activity of *E. coli* Glu5 was investigated. The results indicated that Tween 80 insignificantly affected the cell growth rate at the concentration ≥0.5% (w/v) and could enhance the EglS secretion of *E. coli* Glu5 approximately 10% at 0.2% (w/v) (Figure 4.10). Another

report showed that CMCase activity of enzyme secreted from *Isoptericola variabilis* sp. IDAH9 was increased approximately 36% by the addition of 0.6% Tween 80 (Azizi *et al.*, 2015). The effect of Tween 80 on enhancing enzyme secretion of *E. coli* Glu5 was smaller than that of the wild-type strain *I. variabilis* sp. IDAH9 which is most likely due to the fact that the cell wall of *E. coli* strain used in this study was already permeable for recombinant proteins to exit. After 16 h of cultivation, *E. coli* Glu5 could grow in 5% (w/v) Tween 80 with 35% reduction in cell density and 18% reduction in enzymatic activity. Compared to another report, *E. coli* (ATCC 11303) growth was inhibited at 4% of Tween 80 with a half of viable cells remaining in nutrient broth (Rose *et al.*, 1966). The results reveal that *E. coli* Glu5 has a robust ability to both grow and secrete enzymes under the presence of a high concentration of Tween 80 which will be useful for microbial hydrolysis process of lignocellulose with Tween 80 treatment.

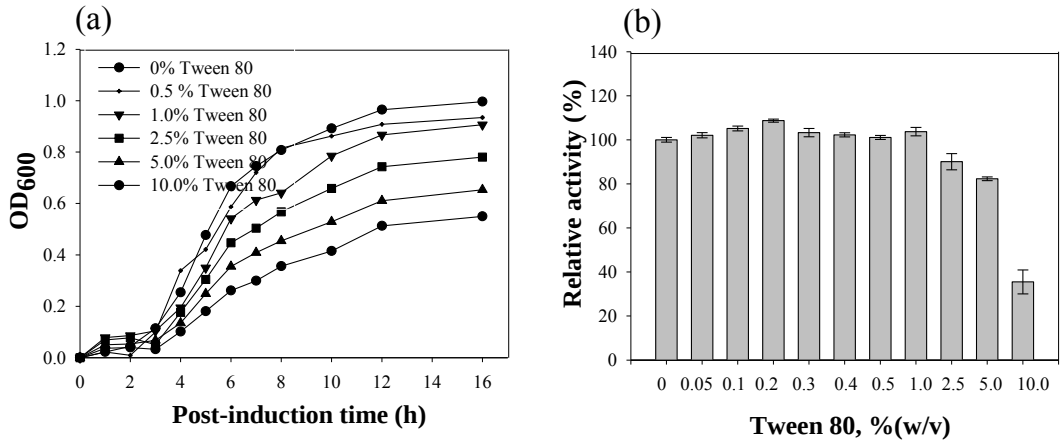

Figure 4.10 Effects of Tween 80 addition on (a) bacterial growth and (b) endoglucanase production of *E. coli* Glu5 culturing in LB-ampicillin (pH 7.0) at 37°C and 180 rpm for 16 h post-induction time.

4.5 Conclusions

In this study, crude recombinant endoglucanase (EglS) was successfully produced by *E. coli* Glu5 with high activity. The addition of EglS could greatly

enhance the initial sugar production rate of the commercial cellulase by hydrolysis of soluble cellulose (CMC), microcrystalline cellulose (Avilcel), and corncob (CC). Remarkably, *E. coli* Glu5 showed lower glucose consumption than the native strain (*B. subtilis* M015) and retained high enzyme activity at high sugar concentrations, making it a potential candidate for the microbial hydrolysis process. Furthermore, *E. coli* Glu5 also showed a high tolerance to Tween 80. These findings can help in developing an economical biofuel production process in the future.

4.6 Acknowledgements

Financial support from the Thailand Research Fund (TRF) through The Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0244/2552) to the first author, TRF Senior Scholar Research (Grant No. RTA5780008) to the corresponding author, and Thai Oil Group Company are greatly appreciated. We would like to acknowledge the help of all members of the Gulari Research Group, especially Alison L. Banka, at the Department of Chemical Engineering, University of Michigan. Protein profile analysis was performed by Dr. Ruethairat Boonsombat at the Institute of Biotechnology and Genetic Engineering, and Assoc. Prof. Dr. Sehanat Prasongsuk at the Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University. The authors also thank The Center of Excellence on Petrochemical and Materials Technology, Chulalongkorn University for providing some of the equipment for this research.

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CHAPTER V
MICROBIAL HYDROLYSIS OF CARBOXYMETHYL CELLULOSE BY ENDOGLUCANASE FROM *ESCHERICIA COLI* GLU5 USING BATCH AND CONTINUOUS BUBBLE REACTORS (CBR)

5.1 Abstract

Biologically meditated processes are promising for energy conversion, especially the conversion of lignocellulosic biomass into bioethanol. Although, most batch or fed-batch bioprocesses have been studied due to less complexity of system, continuous bioprocess is promising for capital cost reduction. For the process dealing with cell culture like microbial hydrolysis of cellulose, bubble reactors are of great interest. A bubble reactor works as three phase reactor where microorganisms are utilized as solid suspensions in order to convert cellulose into sugars in liquid media and aeration is provided for cell growth and mixing. In this work, a microbial hydrolysis of soluble cellulose, carboxymethyl cellulose (CMC), was carried out in continuous bubble reactor at different organic loading rates (5-50 kg/m^3d). The optimum organic loading rate (OLR) was 5 kg/m^3d. The reducing sugar concentration at optimum OLR was 1473±5 mg/L (7.35%yield). The results from this experiment demonstrate the feasibility of consolidated step of enzyme production with the step of enzymatic hydrolysis in term microbial hydrolysis process using continuous bubble reactor (CBR) at low organic loading rate.

5.2 Introduction

Biologically meditated processes seem promising for energy conversion, especially the conversion of lignocellulosic biomass into fuels (Lynd *et al.*, 2005). A continuous culture bioprocess has been more advantageous than a batch culture because biocatalysts, generally microorganisms once grown, remain viable for a long time (theoretically indefinitely), while a batch culture has to be re-grown after each fermentation run to become productive again (Richter *et al.*, 2013). Although processes featuring a step dedicated to the production of cellulase enzymes have been

the focus of most research efforts to date, consolidated bioprocessing, including cellulase production, cellulose hydrolysis, and fermentation in one step, is alternative approach with outstanding potential to reduce costs for capital, raw materials, and utilities associated with cellulase production (Lynd *et al.*, 2005).

Bubble columns are used as multiphase contactors and reactors in chemical, biochemical and petrochemical industries. Several advantages achieve during operation and maintenance such as high heat and mass transfer rates, compactness and low operating and maintenance costs (Kantarci *et al.*, 2005). In biochemical applications, bubble column reactors are three phase where microorganisms are utilized as solid suspensions in order to manufacture industrial valuable bioproducts in liquid media and aeration is provided for cell growth and mixing. Moreover, bubble columns with micro bubble generators have been reported to be one of the most efficient reactor types for gas distribution while require a lower volumetric energy input (Bredwell and Worden, 1998).

In previous work, we successfully developed an endoglucanase-producer, *Eschericia coli,* Glu5 (Seneesrisakul *et al.*, 2017). In this study, we continually studied on microbial hydrolysis of cellulose to sugar as a batch and continuous culture in a bubble reactor using *E. coli* Glu5 in order to determine the feasibility of consolidation of enzyme production and cellulose hydrolysis in one step. Due to *E. coli* Glu5 produces only endoglucanase, soluble carboxymethyl cellulose (CMC), specific substrate of endoglucanase, was used in this experiment. Effect of pH values on cell growth and microbial hydrolysis efficiency was determined. Then, effect of substrate loading rates on microbial hydrolysis of CMC in batch and continuous bubble reactor was studied. Finally, the flocculant were preliminary tested in order to recovery microbial cells in reactors.

5.3 Experimental

5.3.1 Materials and Microbial Strains

E. coli Glu5 obtained from the previous work (Seneesrisakul *et al.*, 2017) was preserved by lyophilization and cultured in a lysogeny broth (LB; 10 g/L of tryptone (Hi-Media), 5 g/L of yeast extract, and 5 g/L of sodium chloride (RCI labscan) at pH

7.0). An ampicillin sodium salt (100 μg/mL) used as the selection agent, were purchased from Sigma-Aldrich. An isopropyl β-D-thiogalactopyranoside (IPTG) used as a protein inducer was purchased from Life Technologies CO., Ltd. Carboxymethyl cellulose (CMC, Sigma-Aldrich) was used as substrate in this experiment. Three types of flocculant; nonionic polyacrylamide (MAXFLOC 912), anionic polyacrylamide (MAXFLOC 917), and cationic polyacrylamide (MAXFLOC 415) were kindly obtained from Thai Chemical & Engineering Co.,Ltd., Thailand.

5.3.2 Preparation of *E. coli* Glu5 Inoculum

E. coli Glu5 was kept on Lysogeny broth (LB; 10 g/L of Tryptone, 5 g/L of yeast extract, and 5 g/L of sodium chloride) agar plate at 4°C. A loop of cell was cultured overnight in 2 mL LB-ampicillin as an inoculum in an orbital shaker at 180 rpm and 37°C under aseptically aerobic conditions overnight until saturation. The nutrient broth were sterilized in the autoclave at 121°C for 15 min and cooled to room temperature in the laminar flow hood before use. A 100 μg/mL of ampicillin sodium salt (Sigma-Aldrich) was used as selection agent and a 1 mM of isopropyl β-D-thiogalactopyranoside (IPTG, Invitrogen Life Technologies) was added to the culture to induce endoglucanase enzyme. Carboxymethyl cellulose (CMC) used as substrate was purchased from Sigma-Aldrich, Singapore. The chemicals were used as received.

5.3.3 Effect of Initial pH of Medium on Cell Growth and Microbial Hydrolysis

LB containing 1% of CMC with various initial pH values (4–7) were prepared in a 250 mL Erlenmayer flask with working volume of 100 mL and adjusted pH with a 1 M hydrochloric acid (HCl) and a 1 M sodium hydroxide (NaOH). *E. coli* Glu5 inoculum was diluted at a ratio of 1:400 into each pH medium. The culturing was performed at 37 °C and 180 rpm for 24 h with continuous shaking. At each interval time, a 200 μL culture of each sample were pipetted into a 96-well-microplate and measured an optical density at 600 nm (OD_{600}) using a microplate absorbance reader (Infinite F200, Tecan, Switzerland) for cell growth determination. The effect of culture medium pH on the microbial growth rate was determined by calculating the specific growth rate (μ, h^{-1}) from the exponential phase of the growth curve. The supernatant

of each culture sample also taken for reducing sugar production by DNS assay, described following. The tests were carried out in triplicate.

5.3.4 Effect of IPTG Concentration on Endoglucanase Production

An *E. coli* Glu5 inoculum culture was added into an Erlenmayer flask containing 150 mL LB ampicillin and shaking-incubated until mid-log phase in an orbital shaker at 180 rpm and 37°C. Ten millilitre of culture was separated into 13 culture tubes. Various concentration of Isopropyl β-D-thiogalactopyranoside (IPTG, Invitrogen Life Technologies) in the range of 0-1.5 mM was added to each culture tube and continued shaking for 16 h. Each collected sample was centrifuged at 8000 rpm for 5 min and filtered via 0.2 um nylon-filter. Supernatant was used for determination of endoglucanase activity, described following.

5.3.5 Microbial Hydrolysis of CMC in Bubble Reactor

5.3.5.1 Configuration of a Continuous Bubble Reactor (CBR)

Figure 5.1 illustrates the configuration of a continuous bubble reactor (CBR) for microbial hydrolysis. The CBR unit was consisted of a round-bottom vessel with a total volume of 3,000 mL and a working volume of 1,500 mL. The CBR was operated under aseptic conditions. The CBR temperature was controlled at 37°C by circulating water from water bath through the jacket of the reactor. A constant flow rate of feed and drain liquid was controlled by peristaltic pump. A level probe was used to control the liquid level inside the CBR along the experiment. Compressed air was filtered through a 0.2 μm-pore size filter before entering the reactor via a connected spreader to achieve the aerobic condition and complete mixing inside the reactor.

5.3.5.2 Reactor Start-Up and Operating Conditions in CBR

The CBR column was sterilized by soaking with 70% Ethanol 2 h and rinsing. After that, the CBR was heat-up to 90 °C with filtered air blowing to remove the residual ethanol, then, cooled to 37 °C for microbial hydrolysis. The *E. coli* Glu5 inoculum was loaded into CBR containing 1,500 mL of fresh LB-ampicillin

containing 2% CMC at volumetric ratio of 1:400. Filtered air was pumped into the reactor at a flow rate of 0.5 L/min. When the OD_{600} of culture reached mid-exponential phase, continuous mode was started by continuous feeding substrate solution (2% CMC in LB-ampicilline) and continuously draining products. The operating conditions of carboxymethyl cellulose hydrolysis in CBR are shown in Table. 5.1. Batch hydrolysis was also carried out in the same bubble reactor without coninuous feed and drain. An IPTG at a minimal concentration was mixed in the feed tank to induce endoglucanase enzyme. Samples were collected along the experiment in order to investigate the effect of organic loading rate (OLR, kg/m^3d) on the reducing sugar production (mg/L) and enzyme activity (U/mL). OD_{600}, bacterial cell dried weight (CDW, g/L), and pH of each sample was also measured.

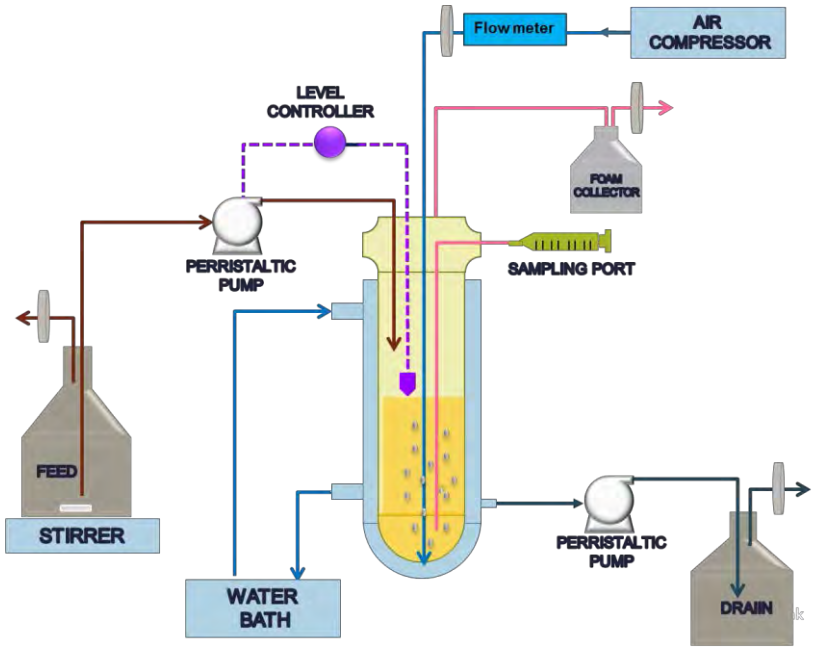

Figure 5.1 Configuration of a single-stage continuous bubble reactor (CBR).

Table 5.1 Operating conditions at different organic loading rate in CBR

Organic Loading Rate (kg/m^3d)	Space Time (h)	Feed Flow Rate (mL/min)	Dilution Rate (h^{-1})
5	96	0.26	0.01
10	48	0.52	0.02
20	24	1.04	0.04
50	10	2.6	0.1

5.3.6 Determination of Flocculant Type and Concentration for *E. coli* Glu5

E. coli Glu5 was grown in LB-ampicillin (100 mL) until saturated (OD_{600} ~1.5). Flocculants were prepared in distilled water from viscous liquid to give a 1% (w/v) stock solution. Experiments were performed in vials with stirred magnetic bars (40 rpm). Flocculant types were tested to flocculate *E. coli* Glu5 cells by dosing 40 ppm of each type into 20 mL of cell culture and observed turbidity change after 5 min and 2 days. The suitable type of flocculant was determined the optimum dosing for flocculating *E. coli* Glu5 cells at various concentration of 0-35 ppm. Cell density was followed turbidimetrically as OD_{600}.

5.3.7 Analytical Method

5.3.7.1 Bacterial Cell Growth Determination

The OD_{600} of each cultured was periodically monitored using DR3800 spectrophotometer (Hach, USA). Cell dried weight was obtained from a 1 mL of samples in triplicate. The samples were centrifuged at 11000 × g for 10 min. The cell pellet samples were washed twice with distilled water and dried at 80 ˚C overnight until constant weight obtained.

5.3.7.2 Enzymatic Activity Determination

The endoglucanase activity was determined by the standard DNS method. The amount of reducing sugars produced was measured using a dinitrosalicylic acid (DNS) reagent with glucose as the standard reference (Miller, 1959). The assays were conducted in round bottom test tube (14 mL). Each diluted sample (0.25 mL) was incubated with 0.25 mL of CMC solution (2% CMC in a 50

mM sodium citrate buffer, pH 4.8) at 50 ˚C for 30 min. A 0.5 mL of DNS solution was added to the mixed solution and incubated at 90 ˚C for 5 min. Next, the samples were quickly cooled in ice bath and added with 5 mL of distilled water. The degree of enzymatic hydrolysis of the soluble cellulose (CMC) was determined by measuring the absorbance at 540 nm in a DR3800 spectrophotometer (Hach, USA). One Unit (U) of endoglucanase activity is defined as the amount of enzyme producing 1 μmol of reducing sugars per min under the assay conditions. Glucose was used as a standard sugar.

5.3.7.3 Reducing Sugar Determination

A mixture of sample (0.25 mL), distilled water (0.25 mL), and DNS solution (0.5 mL) in round bottom test tube (14 mL) was heat at 90 ˚C for 5 min, then, cooled in ice bath, and added with 5 mL of distilled water. The amount of reducing sugar was determined by measuring the absorbance at 540 nm as mentioned previously.

5.3.7.4 Acetic Concentration Determination

The acetic acid was analyzed by a high performance liquid chromatograph (HPLC) equipped with a refractive index detector (6040 XR, Spectra-Physics, USA) and an Aminex HPX-87H column (Bio-Rad Lab, USA). A 5 mM sulfuric acid (H_2SO_4) solution was used as a mobile phase at a flow rate of 0.6 mL/min and the column temperature was controlled at 60 °C.

5.3.7.5 pH and Dissolved Oxygen Determination

Dissolved oxygen was measured by dissolved oxygen meter (Model: DO5512SD). pH of the hydrolysis medium was measured by pH meter (Mettler: S20 SevenEasy).

5.4 Results and Discussion

5.4.1 Effect of Initial Medium pH on Cell Growth and Microbial Hydrolysis Efficiency

Most cellulolytic enzymes including endoglucanse from *E. coli* Glu5, prefer slightly acidic conditions (pH ~5) while bacterial culture has been carried out at neutral pH values (pH 7) (Bajaj *et al.*, 2009; Rastogi *et al.*, 2010; Liu *et al.*, 2011). For effective utilization of *E. coli* Glu5 cells on microbial hydrolysis, combining steps of enzyme production from bacterial cells and enzymatic hydrolysis should be carried out under an appropriate pH.

The effect of initial pH on the bacterial growth profile is shown in Figure 5.2(a). The specific growth rate, obtained from the growth phase, showed that *E. coli* Glu5 could develop at pH 6 (μ of 0.49 h^{-1}) with about 13% inhibition as compared to that at pH 7 (μ of 0.56 h^{-1}). At pH 5, the growth of *E. coli* Glu5 was considerably inhibited (μ of 0.14 h^{-1}) and it took about 18 h to reach the stationary phase. The growth of *E. coli* Glu5 was found to be totally inhibited at pH 4. The results suggest that *E. coli* Glu5 is sensitive at acid conditions.

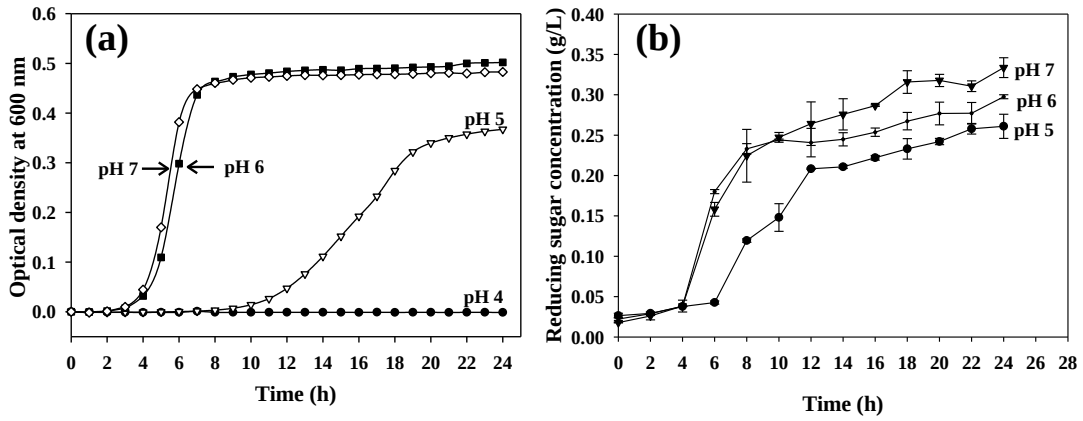

Figure 5.2 Growth curves of *E. coli* Glu5 and reducing sugar concentration of microbial hydrolysis of carboxymethyl cellulose (CMC) at differemnt medium pH values.

Figure 5.2(b) shows reducing sugar concentration produced from microbial hydrolysis of CMC at different initial pH values using *E. coli* Glu5. At 24-h culturing, the reducing sugar concentration decreased from 0.33 g/L at pH 7 to 0.30 g/L and 0.26 g/L at pH 6 and 5, respectively. The growth inhibition at a low pH can be explained by the detrimental effect of H^+ ions on cellular components, resulting in a lower bacterial population and lower extracellular enzyme level secreted into the medium orderly (Russell and Dombrowski, 1980). In conclusion, even *E. coli* Glu5 could grow at pH 5, however, both growth rate and reducing sugar production were considerably lower. The decrease of reducing sugar concentration, corresponding to cell growth, when decreasing culture pH from 7 to 5 was similar to the decrease of endoglucanase activity when increasing enzyme function pH from optimum pH of 5 to neutral pH of 7 reported previously (Seneesrisakul *et al.*, 2017), approximately 22-23% relative activity. Hence, the microbial hydrolysis of cellulose utilized *E. coli* Glu5 should be carried out at pH 7 to maximize growth rate with a reasonably high enzymatic activity.

5.4.2 Effect of IPTG Concentration on Endoglucanase Production

Due to IPTG is very expensive, the use in large scale should be minimized to control production cost. Higher concentration of IPTG may become toxic to the cell and lower concentration may not be enough to titrate the repressor molecule completely. In the previous work, IPTG was carried out at 1 mM constantly. In this work, the effect of various concentration of IPTG (0-1.5 mM) on the endoglucanase production was studied and shown in Figure 5.3. A maximum endoglucanase activity of 3.13 U/mL was found at the addition of IPTG concentration of 0.6 mM. However, a 0.1 mM of IPTG can provide the endoglucanase activity of 2.5 U/mL, corresponding to 80% relative activity of the maximum. Therefore, 0.1 mM of IPTG was chosen for endoglucanase induction as standard procedure for large scale CBR experiment in order to minimize an operation cost. Without the IPTG addition, the endoglucanase activity was found to be 1.52 U/mL (50% relative activity), resulting from the unexpected leaky of promoter.

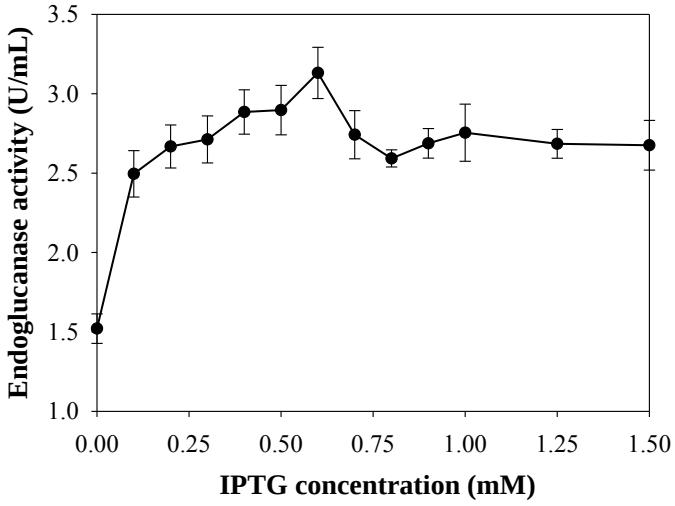

Figure 5.3 Effect of IPTG concentration on endoglucanase productivity of *E. coli* Glu5.

5.4.3 Results of Microbial Hydrolysis of CMC in CBR

5.4.3.1 OD600 and Cell Dried Weight (CDW) Results

Figure 5.4(a) shows that OLR of 5 kg/m^{3}d is chosen to be an optimum for microbial hydrolysis of CMC using CBR in this experiment. Cell density could maintain constant at higher value than that of the other conditions as observed from the values of OD_{600}, corresponding to CDW values (Figure 5.4(b)). The over OLR resulted in higher cell washed-out as obviously shown by low values of OD_{600} and CDW of OLR of 50 kg/m^{3}d at 120 h. The OD_{600} of OLR of 50 kg/m^{3}d reached the maximum value at only ~1.5 whereas the OD_{600} of ~2.25 could be reached by the lower OLR of 5 to 20 kg/m^{3}d and by batch reactor. At the same time, the steady CDW of OLR of 50 kg/m^{3}d was only 0.9 g/L whereas CDW of OLR of 5, 10, 20 kg/m^{3}d could reach at 1.3-1.7 g/L.

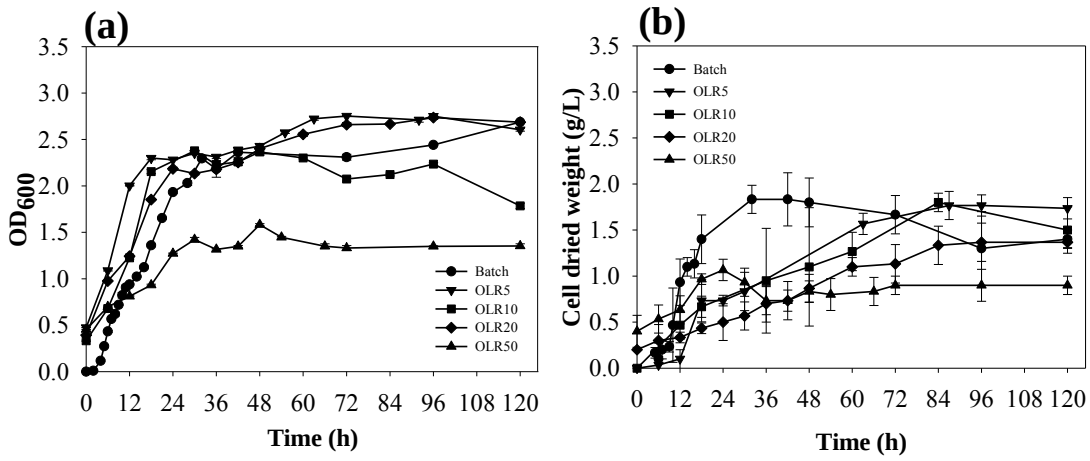

Figure 5.4 Microbial hydrolysis of CMC in terms of (a) the optical density at 600 nm (b) the cell dried weight at organic loading rates of 5 kg/m^{3}d (triangle down), 10 kg/m^{3}d (square), 20 kg/m^{3}d (diamond), 50 kg/m^{3}d (triangle up), and batch mode (circle).

5.4.3.2 Endoglucanase Activity Results

From Figure 5.5 , the endoglucanase activity profile of OLR of 5 kg/m^{3}d was gradually increased until constant at 84 h (3.2 U/mL). The OLR of 5 kg/m^{3}d in CBR could reach steady-state of enzyme production while the higher OLR could not. The higher OLR of 5, 10, and 20 kg/m^{3}d was operated, the shorter space time in CBR was achieved, that resulted in cell washed-out and lower enzyme produced. The enzyme activity of OLR of 10, 20 and 50 kg/m^{3}d continually decreased and seem to go to zero level. The other explanation of the decrease of enzyme activity at higher OLR might be due to *E. coli* Glu5 lose plasmid carrying endoglucanase gene when long operating time was carried out, espescially the higher OLR resulting in higher dilution rate of feed to CBR make the endoglucanase losing faster as the profiles shown. For batch hydrolysis, endoglucanase activity was maintained at high value because of enzyme accumulation in the system from the initial operation. However, it might be because the nutrient in reactor ran out and by-products accumulated in the system resulting in lower endoglucanase achieved comparing to continuous hydrolysis at OLR of 5 kg/m^{3}d.

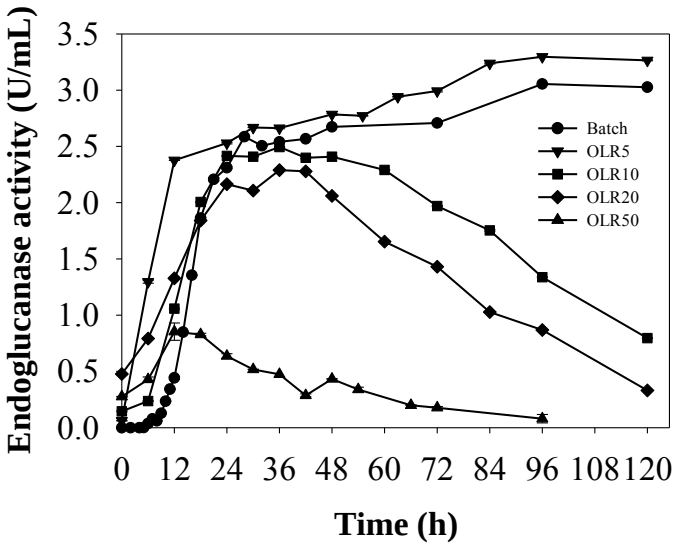

Figure 5.5 Microbial hydrolysis of CMC in terms of the endoglucanase activity at organic loading rates of 5 kg/m^{3}d (triangle down), 10 kg/m^{3}d (square), 20 kg/m^{3}d (diamond), 50 kg/m^{3}d (triangle up), and batch mode (circle).

5.4.3.3 Acetate and pH Evolution

Acetate have known to be inhibitor of bacterial growth and recombinant protein production (Kleman and Strohl, 1994). At neutral pH (7.0-7.5), acetate is presented in equilibrium with undissociated acetic acid. The latter, unlike charged acetate ions, can migrate uncontrolledly through bacterial membranes, disrupting the transmembrane ΔpH and impairing cells viability (Axe and Bailey, 1995). However, acetate was found that can be a carbon source for *E. coli* BL21 for recombinant sweet protein production in defined medium (Leone *et al.*, 2015).

In this experiment, acetate and pH evolution profiles are demonstrated in Figure 5.6 (a) and (b), respectively. The acetate evolution profiles of all experiments were similar. At earlier profile (0-36 h), acetate concentration gradually increased, then, decreased after reach the plateau. The OLR 5 kg/m^{3}d produced higher level of acetate concentration than the other OLR which might be the result of a high cell density and a lower dilution rate of feed allow the accumulation of acetate in the system. From Figure 5.6 (b), the environmental pH inside the CBR at OLR of 5, 10 kg/m^{3}d and batch hydrolysis without pH control was likely to be an alkalinity shift to pH of 8.5, resulting from the amino acid catabolism of LB medium

leading to an excretion of the excess ammonium (Sezonov *et al.*, 2007). For the OLR of 50 kg/m^{3}d, low bacterial density was in the system resulting in the excess carbon source from CMC, thus *E. coli* produce acidic by-products, in particular acetate . Thus, the acetate accumulation making the system acidic. Wang and coworker reported that the alkaline shift of the medium pH (pH 7.5-8.5) helps in reducing acetate stress on cell growth and protein production in rich medium culture (Wang *et al.*, 2014). This suggested that pH control might not be required in this system at low OLR.

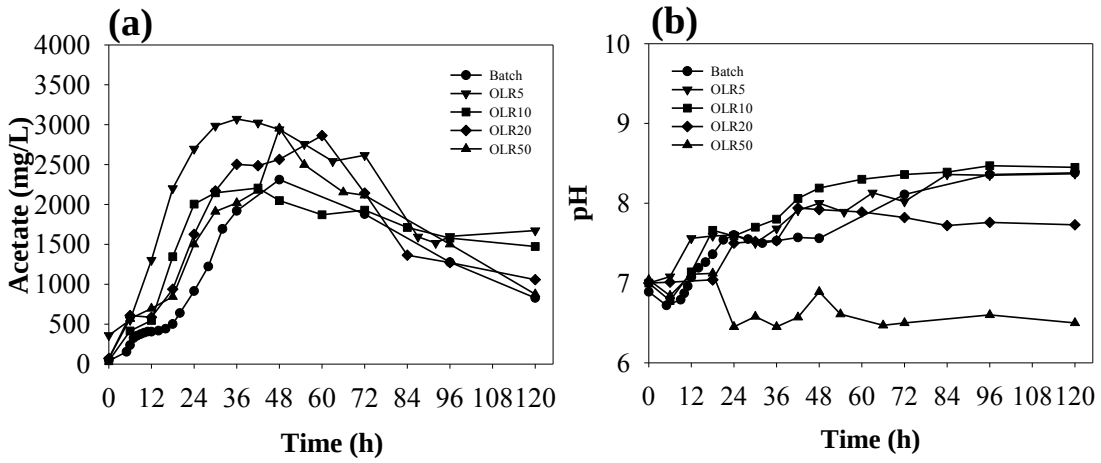

Figure 5.6 Microbial hydrolysis of CMC in terms of (a) the acetate concentration (b) the pH of the system at organic loading rates of 5 kg/m^{3}d (triangle down), 10 kg/m^{3}d (square), 20 kg/m^{3}d (diamond), 50 kg/m^{3}d (triangle up), and batch mode (circle).

5.4.3.4 Reducing Sugar Production Results

The profiles of reducing sugar over the experimental period at all OLR for continuous hydrolysis and batch hydrolysis were similar (Figure 5.7). At the initial production (initial 24 h for continuous mode (CBR) and initial 32 h for batch mode), reducing sugar concentration drastically increased to the maximum concentration, then gradually decreased and maintain slightly constant level. The final reducing sugar concentration at 120 h obtained from batch hydrolysis was the highest value due to product accumulation and decreased with increasing of OLR in continuous hydrolysis (Table 5.2). For CBR, optimum OLR of 5 kg/m^{3}d yielded the highest reducing sugar of 7.35 % reducing sugar/CMC applied at 120 h. The reducing

sugar productivity of the CBR is inverse to the final reducing sugar concentration. However, at higher OLR than 5 kg/m^{3}d, the endoglucanse activity ran out and could not be operated longer than 120 h.

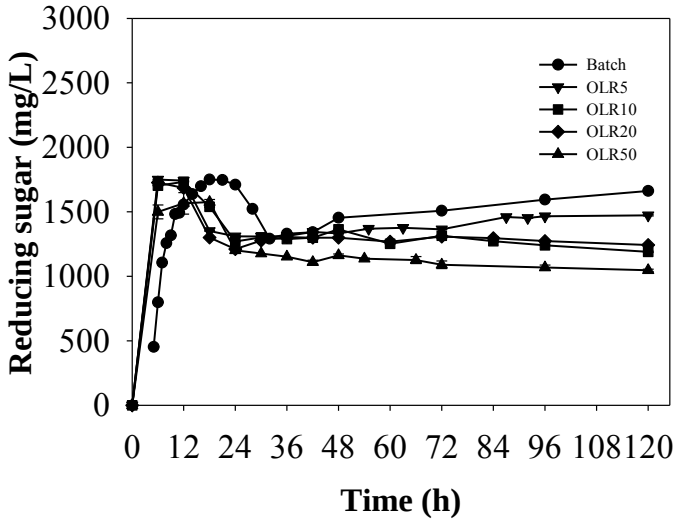

Figure 5.7 Microbial hydrolysis of CMC in terms of (a) the reducing sugar concentration at organic loading rates of 5 kg/m^{3}d (triangle down), 10 kg/m^{3}d (square), 20 kg/m^{3}d (diamond), 50 kg/m^{3}d (triangle up), and batch mode (circle).

Table 5.2 Summary of reducing sugar production from each system.

System	Organic Loading Rate (kg/m^{3}d)	Final Reducing Sugar concentration (mg/L)	Reducing Sugar Productivity (kg/m^{3}d)	Yield (% reducing sugar/CMC applied)
Batch	-	1,661 (±1)	-	
CBR	5	1,473 (±5)	0.368	7.35
CBR	10	1,190 (±4)	0.594	5.94
CBR	20	1,243 (±2)	1.241	6.20
CBR	50	1,046 (±9)	2.611	5.22

5.4.4 Results of Flocculant Type Test and Determination of Optimum Dosing

A cationic polyacrylamide flocculant provided a positive result for flocculating *E. coli* Glu5 cells and maintaining flocculation stability over 2-d as seen in Figure. 5.5. This results can be explained that *E. coli* is gram-negative bacteria,

which is negative charge cell wall, thus cationic charges of flocculant can perform charge attraction to cell wall and bridge bacterial cells together as flocculates. The mechanism of flocculation can be demonstrated as Yang' report, Figure 5.6 (Yang *et al.*, 2014).

In Figure 5.7, the optimum concentration of cationic polyacrylamide were determined and found that 20 ppm is the optimum dose for floculating *E. coli* Glu5 in LB-ampicillin medium. Hence, cationic flocculant can be potential way for microbial cell recovery from the liquid medium. However, there are many factors involved to efficiency of cell flocculation such as fluid shear, pH, bridge stability, etc., needed to be further study. Moreover, cell activities and non-degradation of products are also the important for the purpose of microbial cell recovery in continuous system.

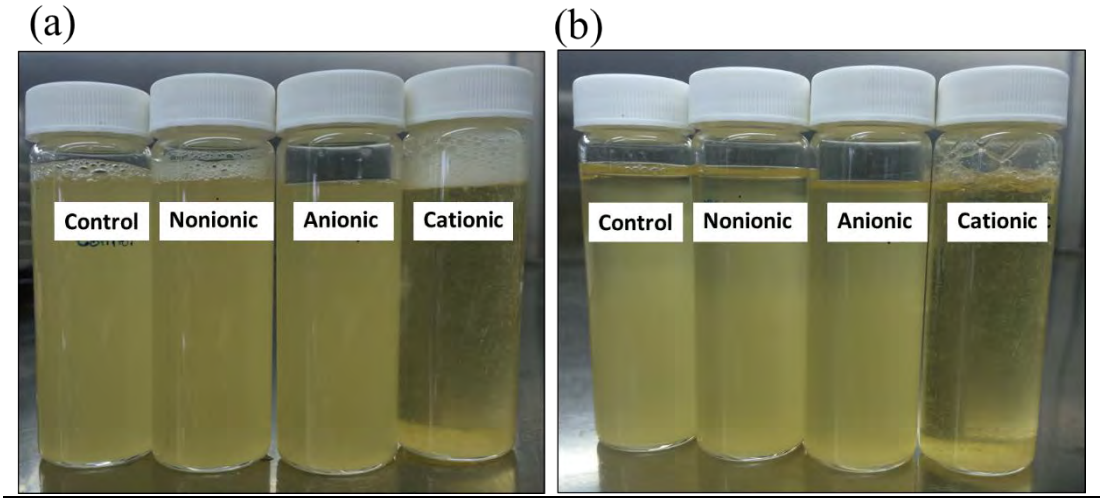

Figure 5.8 Tests of flocculant (40 ppm) types; nonionic, anionic, and cationic polyacrylamide, to flocculate *E. coli* Glu5 cells in LB-ampicilin medium: (a) the photograph of 5-min post flocculant addition, (b) the photograph of 2-d post flocculant addition.

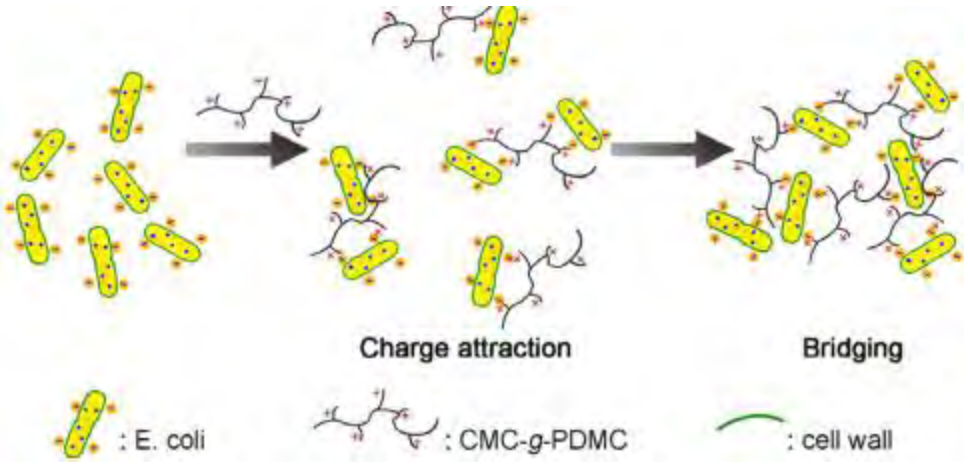

Figure 5.9 Model of flocculation of *E. coli* glu5 cells in LB-ampicilin medium by cationic polyacrylamide (Yang *et al.*, 2014).

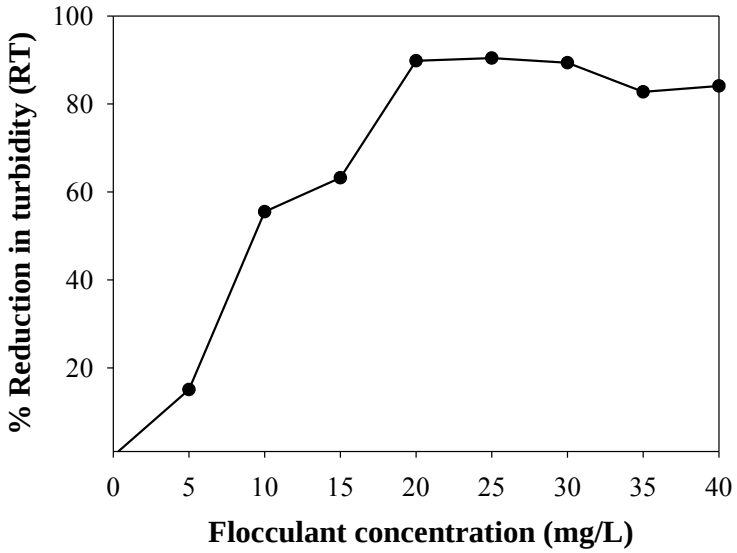

Figure 5.10 Cationic polyacrylamide flocculant dose optimization curve for *E. coli* Glu5 in LB-ampicillin medium.

5.5 Conclusion

Microbial hydrolysis of soluble substrate cellulose, carboxymethyl cellulose, by *E. coli* Glu5 using a continuous bubble column reactor in this experiment

demonstrates the feasible way to consolidate the step of cellulose conversion to sugars for biofuel production. This process will be benefit for biofuel industry because it can reduce operation cost of enzyme production step. However, this study tested on one sub-cellulase, i.e. endoglucanase, it cannot use for completing hydrolysis of natural cellulose. Hence, further intensive study are need to be tested on other cellulase-producer and cellulose materials next.

5.6 Acknowledgements

Financial support from the Thailand Research Fund (TRF) through The Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0244/2552) to the first author, TRF Senior Scholar Research (Grant No. RTA5780008) to the corresponding author, and Thai Oil Group Company are greatly appreciated. We would like to acknowledge The Center of Excellence on Petrochemical and Materials Technology, Chulalongkorn University for providing some of the equipment for this research.

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CHAPTER VI
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

We have studied the microbial pretreatment on lignocellulosic biomass, corncob by using fungus (*Phanorachae sordida)* and bacteria (*Bacillus subtilis* M 015 and *Cellulomonas* sp.). The results showed that fungal pretreatment is the best for lignocellulose pretreatment by removing lignin recalcitrant from the substrate allowing enzyme adsorption on cellulose. Moreover, we found that *B. subtilis* M 015 gave the interesting results on producing cellulase enzyme for microbial hydrolysis of lignocellulose. Hence, it sparked an interesting to move to next chapter of research, cloning endoglucanase gene from *B. subtilis* M 015 into *Eschericia coli.*in order to increase enzyme production and use in microbial hydrolysis.

We have successfully constructed an *E. coli* Glu5 expressing endoglucanase gene from native strain *B. subtilis* M015 at high level. Remarkably, there was a smaller glucose consumption of *E. coli* Glu5 than the native strain, making it a potential candidate for the microbial hydrolysis process. Since only endoglucanase cannot complete the cellulose hydrolysis, thus, a combination of recombinant endoglucanase (EglS) with cellulase is required. EglS shows a great enhancement of commercial cellulase enzymes for the hydrolysis of corncob to produce sugar. These findings can help in developing an economical biofuel production process in the future.

Finally, we scaled up the experiment of microbial hydrolysis from flask scale into 3-L reactor. Microbial hydrolysis of soluble substrate cellulose, carboxymethyl cellulose, by *E. coli* Glu5 using a continuous bubble column reactor was carried out. The results demonstrates the feasible way to consolidate the step of cellulose conversion to sugars for biofuel production. This process will be benefit for biofuel industry because it can reduce operation cost of enzyme production step. However, this study tested on one sub-cellulase, i.e. endoglucanase, it cannot use for completing hydrolysis of natural cellulose. Hence, further intensive study are need to be tested on other cellulase-producer and cellulose materials next.

6.2 Recommendations

From the experiment of microbial hydrolysis using a single stage continuous reactor (CBR), we suggested to further study on two-stage CBR for separation the step of enzyme production and downstream of cellulose hydrolysis.

To optimize the sugar production in 2S-CBR system on the effect of organic loading rate of stage two, many experiments are required to be done. The medium type for *E.coli* Glu5 will be changed from LB rich medium to be minimal medium in order to obtain high enzymatic activity with the low cost medium. Moreover, LB is easy to be contaminated when long process is operated. A screening of flocculant type and optimization of flocculant concentration for cell removal from the crude enzyme from stage one will be tested with the test of effect of flocculant addition on enzymatic activity. Then, the organic loading rate of cellulose will be varied and the optimization of sugar productivity will be investigated.

Because *E. coli* Glu5 can produce only endoglucanase which cannot complete the cellulose hydrolyzing. Therefore, new cellulase producer or supplement commercial enzyme are required to supply in the system. To optimization of sugar productivity, the two-stage CBR system operating at ambient pressure: (1) an enzyme production unit operated at pH 7 and 37 °C, allowing the optimal growth and enzyme secretion of *E. coli* Glu5 (2) an enzymatic hydrolysis of unit operated at pH 4.8 and 50 °C, allowing the optimal enzymatic activity of cellulase. Stage one was equipped with a cell removal unit remove cells out of the crude enzyme from stage one before loading to stage two using flocculant addition. Foam will be controlled with an antifoam injection system.

We have proposed to establish a two-stage continuous bubble reactor (2S-CBR) process for sugar production from lignocellulosic residue such as corncob with endoglucanase producer, *E. coli* Glu5, supplemented with commercial enzyme (Figure 5.2). The system consists of a 3-L continuously bubble reactor (1.5-L working volume) as an enzyme production stage by *E. coli* Glu5 equipped with 1-L of cell removal unit and a 3-L bubble column reactor (1.5-L working volume) as a sugar production stage. Operating conditions in both stages will be optimized for the respective purpose (enzyme secretion in stage one and sugar production in stage two). The stage one will be fed with an optimal minimal medium for *E. coli* Glu5grow, then, the culture will be transferred via cell removal unit and crude enzyme will be provided to next stage. In

stage two, the maximum enzyme activity will be maintained. Sugar production will be continuously monitored for maximum production rate. Foam control will be essential to maintain reactor stability.

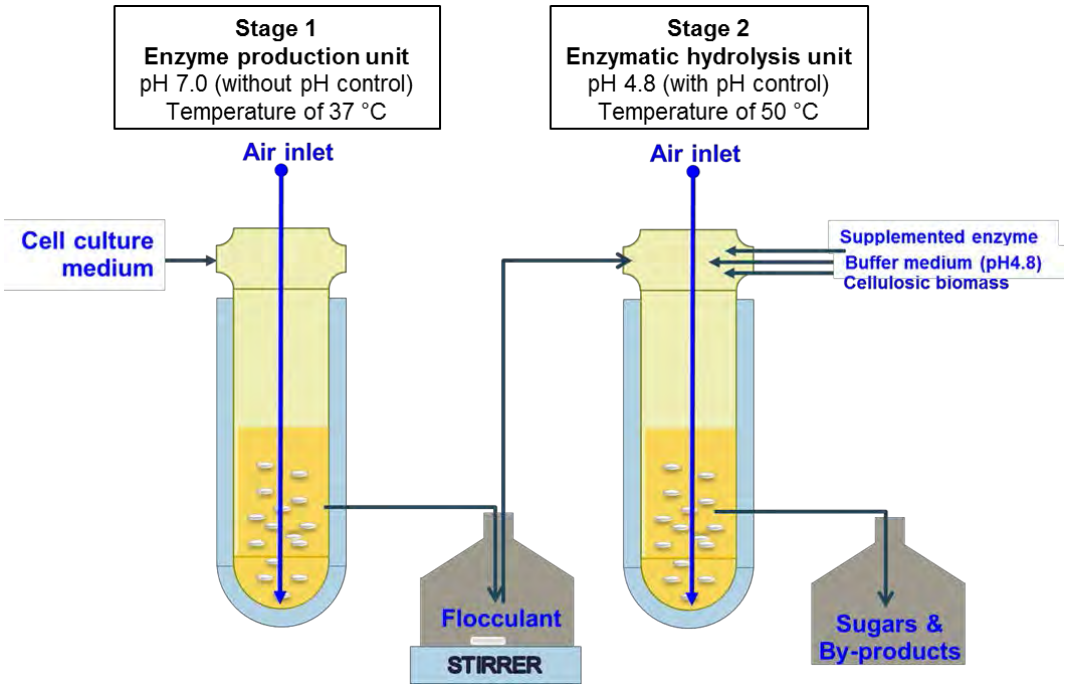

Figure 6.1 Configuration of two-stage continuous bubble reactor (CBR)

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APPENDICES

Appendix A Lineweaver-Burk plots of the crude EglS toward CMC

The kinetic parameter values of the crude EglS on CMC hydrolysis in terms of Michaelis-Menten constants (K_m) and maximum velocity (V_{max}) were determined using the Lineweaver-Burk plots, as shown in Figure 4.6 (d).

Table A1 Data of Lineweaver-Burk plots of the crude EglS toward CMC

[S], mg/mL	1/[S], $(mg/mL)^{-1}$	V_o, (uM/min)		$1/V_o$, $(uM/min)^{-1}$	
		Mean	SD	Mean	SD
2.5	0.400	1095	2	0.0009132	0.0000016
5.0	0.200	2196	2	0.0004554	0.0000004
7.5	0.133	3252	2	0.0003075	0.0000002
10.0	0.100	4084	0	0.0002448	0.0000000
12.5	0.080	4630	6	0.0002160	0.0000003
15.0	0.067	5115	20	0.0001955	0.0000008
17.5	0.057	5543	7	0.0001804	0.0000002
20.0	0.050	5751	0	0.0001739	0.0000000
22.5	0.044	6145	14	0.0001627	0.0000004
25.0	0.040	6409	3	0.0001560	0.0000001
30.0	0.033	6411	56	0.0001560	0.0000014
40.0	0.025	6970	70	0.0001435	0.0000014
47.0	0.021	7084	84	0.0001412	0.0000017

Appendix B Sugar concentration of the enzymatic hydrolysis of different cellulosic substrates

Sugars were the hydrolysis products of the three different cellulosic materials (carboxymethyl cellulose, Avicel PH-101, and 60-80 mesh milled corncob) using crude recombinant EglS with 25 U/g substrate, commercial cellulase (Cel) with 25 FPU/g substrate, and enzyme mixture of EglS and Cel with 25 U/g substrate and 25 FPU/g substrate, respectively. The reactions were carried out at 50°C and 180 rpm for 100 h.

Table B1 Concentration of sugar products from enzymatic hydrolysis of carboxymethyl cellulose (CMC)

Enzyme	Time (h)	Glucose (mg/L)		Cellobiose (mg/L)		Xylose (mg/L)		Total (mg/L)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
EglS	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	20	126.4	15.6	14.0	0.3	0.0	0.0	140.4	15.9
	40	268.4	6.3	111.7	5.1	0.0	0.0	380.1	11.4
	60	274.2	1.9	117.2	5.3	0.0	0.0	391.4	3.4
	80	314.8	25.5	141.5	4.0	0.0	0.0	456.3	29.5
	100	326.0	9.2	144.2	2.1	0.0	0.0	470.2	11.2
Cel	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	20	128.6	0.4	23.3	3.6	0.0	0.0	151.8	4.0
	40	223.3	4.1	136.7	2.8	0.0	0.0	360.0	1.4
	60	404.1	6.2	242.4	2.1	0.0	0.0	646.5	8.4
	80	505.4	0.9	296.4	6.0	0.0	0.0	801.8	7.0
	100	556.3	7.6	341.1	9.8	0.0	0.0	897.5	17.4
EglS+Cel	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	20	579.7	37.4	214.6	5.8	0.0	0.0	794.3	31.6
	40	601.0	1.1	226.7	21.5	0.0	0.0	827.8	20.3
	60	644.2	25.8	265.2	5.9	0.0	0.0	909.4	19.9
	80	649.4	19.8	276.7	12.1	0.0	0.0	926.1	7.7
	100	683.6	25.9	298.3	6.4	0.0	0.0	982.0	32.3

Table B2 Concentration of sugar products from enzymatic hydrolysis of Avicel PH-101

Enzyme	Time (h)	Glucose (mg/L)		Cellobiose (mg/L)		Xylose (mg/L)		Total (mg/L)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
EglS	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	20	59.4	11.3	44.4	0.4	0.0	0.0	103.8	10.9
	40	57.4	0.9	53.5	0.5	0.0	0.0	111.0	1.4
	60	86.5	4.1	86.0	3.7	0.0	0.0	172.4	0.5
	80	105.2	0.0	108.3	1.0	0.0	0.0	213.5	1.0
	100	109.9	1.8	111.9	2.5	0.0	0.0	221.8	2.5
Cel	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	20	1141.8	14.3	42.2	2.6	0.0	0.0	1184.0	16.9
	40	1619.1	25.1	116.9	24.2	0.0	0.0	1736.0	49.3
	60	2332.3	37.1	43.4	46.3	0.0	0.0	2375.6	83.5
	80	3133.4	33.4	93.4	32.1	0.0	0.0	3226.7	65.5
	100	3365.5	5.7	58.4	5.1	0.0	0.0	3423.9	45.7
EglS+Cel	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	20	1503.6	8.5	478.3	1.4	0.0	0.0	1981.9	9.9
	40	2135.2	14.0	456.3	1.0	0.0	0.0	2591.5	12.9
	60	2635.5	8.4	427.1	10.5	0.0	0.0	3062.6	18.9
	80	3027.7	3.5	401.9	18.2	0.0	0.0	3429.6	21.7
	100	3371.3	3.6	371.6	10.7	0.0	0.0	3742.9	7.1

Table B3 Concentration of sugar products from enzymatic hydrolysis of 60-80 mesh-milled corncob (CC)

Enzyme	Time (h)	Glucose (mg/L)		Cellobiose (mg/L)		Xylose (mg/L)		Total (mg/L)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
EglS	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	20	22.2	1.0	6.3	22.7	6.5	1.7	34.9	20.0
	40	90.7	14.7	52.5	0.4	5.2	93.8	148.3	109.0
	60	121.6	13.5	56.6	2.7	68.6	16.5	246.9	32.7
	80	135.5	10.1	65.8	0.2	82.0	20.9	283.2	31.2
	100	128.3	28.0	71.1	6.6	95.1	13.2	294.5	47.8
Cel	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	20	126.6	9.0	0.0	0.0	39.4	2.7	166.0	11.7
	40	374.6	8.2	0.0	0.0	122.9	0.9	497.5	7.3
	60	450.5	5.1	0.0	0.0	162.9	4.0	613.4	1.1
	80	489.9	9.0	0.0	0.0	176.4	7.2	666.2	16.2
	100	586.1	1.8	0.0	0.0	207.2	0.7	793.3	1.0
EglS+Cel	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	20	449.4	15.6	63.3	5.1	147.7	24.5	660.4	45.1
	40	537.0	9.7	73.2	1.2	183.9	24.1	794.1	32.6
	60	567.5	0.9	87.6	1.2	204.5	24.5	859.6	24.2
	80	604.1	5.1	97.4	0.6	224.0	19.2	925.5	13.5
	100	637.3	14.9	100.9	0.4	248.7	27.9	986.9	42.4

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Publications:

1. Seneesrisakul, K., Sutabutr, T. and Chavadej, S. (2018) The effect of temperature on the methanogenic activity in relation to micronutrient availability. Energies 11(5), 1057.
2. Seneesrisakul, K., Guralp S.A., Gulari E., and Chavadej S. (2017) *Escherichia coli* expressing endoglucanase gene from Thai higher termite bacteria for enzymatic and microbial hydrolysis of cellulosic materials. Electronic Journal of Biotechnology, 27, 70-79.
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