METINEE WASOONTHARAWAT : TREATMENT OF RICE STRAW FOR BUTANOL PRODUCTION BY *CLOSTRIDIUM ACETOBUTYLICUM*

Matinee Wasoontharawat

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2013

การปรับสภาพของฟางข้าวต่อการผลิตบิวทานอลโดยเชื้อ *CLOSTRIDIUM ACETOBUTYLICUM*

นางเมทินี วสุนธราวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2556

TREATMENT OF RICE STRAW FOR BUTANOL PRODUCTION BY *CLOSTRIDIUM ACETOBUTYLICUM*

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

(Assoc. Prof. Dr. Montarop Yamabhai)
Chairperson

(Asst. Prof. Dr. Sunthorn Kanchanatawee)
Member (Thesis Advisor)

(Asst. Prof. Dr. Kaemwich Jantama)
Member

(Assoc. Prof. Dr. Isabelle Meynial-Salles)
Member

(Assoc. Prof. Dr. Pattana Laopaiboon)
Member

(Prof. Dr. Sukit Limpijumnong)
Vice Rector for Academic Affairs and Innovation

(Asst. Prof. Dr. Suwayd Ningsanond)
Dean of Institute of Agricultural Technology

เมทินี วสุนธราวัฒน์ : การปรับสภาพของฟางข้าวต่อการผลิตบิวทานอลโดยเชื้อ *CLOSTRIDIUM ACETOBUTYLICUM* (TREATMENT OF RICE STRAW FOR BUTANOL PRODUCTION BY *CLOSTRIDIUM ACETOBUTYLICUM*)
อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุนทร กาญจนทวี, 258 หน้า.

โครโมโซมของ *Clostridium acetobutylicum* ATCC 824 ซึ่งเป็นสายพันธุ์ที่ผลิตอะซิโตน บิวทานอล และเอทานอล (ABE) มียีนที่สร้างเอนไซม์เซลลูเลสที่เรียกว่า *cel48A* ซึ่งอยู่ในรูปที่ไม่สามารถแสดงกิจกรรมของเอนไซม์เซลลูเลสในการย่อยคริสตัลไลน์เซลลูโลสได้ ในการศึกษานี้ได้พยายามแทนที่ยีน *cel48A* ด้วยยีนพันธุ์ลูกผสมที่สร้างเอนไซม์เซลลูเลสซึ่งสามารถแสดงแอคติวิตีของเอนไซม์และย่อยคริสตัลไลน์เซลลูโลสได้ที่เรียกว่า *SAFA* hybrid gene ในโครโมโซมของ *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* mutant โดยคาดหวังที่จะได้สายพันธุ์ลูกผสมใหม่ที่มีความสามารถทั้งย่อยเซลลูโลสและใช้น้ำตาลจากการย่อยเซลลูโลสเป็นแหล่งคาร์บอนในการผลิตบิวทานอลได้ อย่างไรก็ตามโคลนที่ได้ไม่แสดงการสอดแทรกของ *SAFA* hybrid gene บนโครโมโซม ความเป็นไปได้ของปัญหานี้น่าจะเกี่ยวข้องกับโครงสร้างที่ไม่เหมาะสมของ integration plasmid ที่ได้สร้างขึ้นในส่วนของ strong promoter และ reporter system การขาด scaffolding หรือ cohesion ที่ทำงานร่วมกันแบบเสริมสร้างซึ่งกันและกันระหว่าง cellulosome gene ในโครโมโซมและยีนพันธุ์ลูกผสม *SAFA* รวมถึงการขัดขวางระบบการขับเอนไซม์ภายในเซลล์โดย precursor ของยีนพันธุ์ลูกผสม *SAFA*

ในส่วนของแหล่งคาร์บอนสำหรับ *Cl. acetobutylicum* เพื่อใช้ในกระบวนการหมักสำหรับการสร้าง ABE นั้น เซลลูโลสและเฮมิเซลลูโลสในฟางข้าวสามารถใช้เป็นสารตั้งต้น ทางเลือกใหม่สำหรับเป็นแหล่งคาร์บอนและแหล่งพลังงานให้กับ *Cl. acetobutylicum* ได้เมื่อฟางข้าวได้ถูกเปลี่ยนให้อยู่ในรูปน้ำตาลที่ใช้ในกระบวนการหมักดังนั้นจึงทำการศึกษาถึงประสิทธิภาพของ Pretreatment โดยวิธีที่ต่างกันต่อคุณภาพของเซลลูโลสจากฟางข้าวที่ผลิตได้และสภาวะที่เหมาะสมของการเปลี่ยนเซลลูโลสจากฟางข้าวเป็นน้ำตาลที่ใช้ในกระบวนการหมักโดยวิธีการย่อยด้วยเอนไซม์หรือจุลินทรีย์หรือสารเคมี ผลการทดลองทำให้ทราบว่า วิธี A2WB1 pretreatment มีประสิทธิภาพสูงสุดในการปรับโครงสร้างของเซลลูโลสของฟางข้าวเมื่อเทียบกับวิธีอื่น ปริมาณที่เหมาะสมของเอนไซม์เซลลูเลสอย่างหยาบเพื่อให้ได้น้ำตาลรวมทั้งหมดที่มากที่สุด (96.83±1.25 g/L, เรียกน้ำตาลนี้ว่า GE) จากกระบวนการ saccharification คือ 0.7 มิลลิลิตรต่อกรัมเซลลูโลสแห้ง A2WB1 ส่วนการย่อยเซลลูโลสโดยจุลินทรีย์ พารามิเตอร์ที่สำคัญ ได้แก่ ความเข้มข้นของเซลลูโลส ความเข้มข้นของไนโตรเจนจากกากยีสต์แห้งจากโรงงานเบียร์ อุณหภูมิในการเลี้ยง และการเขย่าของผสมมีผลอย่างมีนัยสำคัญต่อการเพิ่มความเข้มข้นของน้ำตาลที่ผลิตได้หลังการเลี้ยง *Cl. cellulolyticum* DSM 5812

สำหรับการผลิตน้ำตาลไซโลสโดยกระบวนการย่อยฟางข้าวด้วยกรดสองครั้งทำให้ได้สารละลายไฮโดรไลเสท (ที่เรียกว่า ARSH) ซึ่งมีน้ำตาลทั้งหมด 44.67 กรัมต่อลิตร

ลำดับต่อมาได้ทำการศึกษาการผลิต ABE จากน้ำตาลไซโลสที่อยู่ใน ARSH ซึ่งได้ผ่านกระบวนการกำจัดสารพิษด้วยวิธีการต่างกัน ผลการทดลองสามารถสรุปได้คือ *Cl. acetobutylicum* ATCC 824 สามารถการเจริญและผลิตสารละลาย ABE ได้มากที่สุดใน ARSH ที่ผ่านการกำจัดสารพิษด้วยวิธี over-titration plus activated charcoal absorption สุดท้ายทำการศึกษาการใช้ *Cl. acetobutylicum* ร่วมสองสายพันธุ์คือ *Cl. acetobutylicum* ATCC 824 และ *Cl. acetobutylicum* TISTR 1462 และใช้น้ำตาล GE เพื่อปรับปรุงการผลิต ABE ผลการทดลองแสดงให้เห็นว่าความเข้มข้นของ ABE ที่ผลิตได้จากการกระบวนการหมักแบบกะที่ใช้เชื้อสองสายพันธุ์ร่วมกันมีค่าต่ำกว่ากระบวนการหมักเดียวกันแต่ใช้เชื้อเพียงชนิดเดียวอย่างไรก็ตามผลผลิตและความเข้มข้นของ ABE มากที่สุดที่ผลิตได้โดยเชื้อชนิดเดียวที่ใช้น้ำตาล GE มีค่าสูงกว่าวิธีการหมักเดียวกันที่ใช้น้ำตาลกลูโคสในเชิงการค้า สรุปภาพโดยรวมทั้งหมดของการศึกษากล่าวได้ว่า ฟางข้าวมีศักยภาพที่จะใช้เป็นสารตั้งต้นจำพวกลิกโนเซลลูโลสในการผลิตบิวทานอลในกระบวนการหมักแบบกะด้วย *Cl. acetobutylicum* ATCC 824 และ TISTR 1462 ได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

METINEE WASOONTHARAWAT : TREATMENT OF RICE STRAW FOR BUTANOL PRODUCTION BY *CLOSTRIDIUM ACETOBUTYLICUM*. THESIS ADVISOR : ASST. PROF. SUNTHORN KANCHANATAWEE, Ph.D., 258 PP.

BUTANOL PRODUCTION/*CLOSTRIDIUM ACETOBUTYLICUM*/RICE STRAW/ PRETREATMENT/DETOXIFICATION/CO-CULTURE

The chromosome of *Clostridium acetobutylicum* ATCC 824 is an acetone-butanol-ethanol (ABE) producing strain which consists of the inactive cellulase gene *cel48A* which results in a lack of cellulase activity towards crystalline cellulose. In this study, the replacement of the *cel48A* with a *SAFA* hybrid gene as an active cellulase gene in the chromosome of *Cl. acetobutylicum* ATCC 824 *Δcac1502ΔuppΔcel48A* mutant was used to attempt the construction of a new recombinant strain as a cellulolytic-solvent producing strain. However, the clones obtained did not show the insertion of the hybrid gene on the chromosome. This could probably be attributed to the unsuitable gene structure of the integration plasmid such as the strong promoter and reporter system; lacking the complementary scaffolding or cohesion in the *Clostridium* chromosomal cellulosome gene to the hybrid gene employed; and blocking from the secretory system of the *Clostridium* cell from the precursor of the *SAFA* gene.

Regarding the carbon source for ABE fermentation by *Cl. acetobutylicum*, cellulose and hemicelluloses of rice straw can be an alternative substrate as a carbon source and energy for fermentation when they are converted to fermentable sugars. Thus, the effectiveness of various pretreatments of rice straw on the quality of rice straw cellulose (RSC) and the optimum condition of the RSC to fermentable sugars conversion

by enzymatic, microbial and chemical hydrolyses were investigated. The results revealed that the A2WB1 pretreatment method had the highest effective modification of the RSC compared with other methods. The optimum dose of crude cellulase enzymes to achieve maximal total sugars (96.83±1.25 g/L, this sugar called GE) from the saccharification was 0.7 mL/g of dry A2WB1 cellulose. For the cellulose hydrolysis of the microorganism, major parameters including cellulose concentration, spent's brewer yeast concentration, growth temperature and agitation had a significant effect on the increase of glucose concentration after fermentation by *Clostridium cellulolyticum* DSM 5812. With xylose production by the two-stage acid pretreatment of rice straw, acid rice straw hydrolysate (ARSH) was obtained. It consisted of 44.67 g/L total sugars.

The investigation of ABE production from xylose in ARSH was carried out by different detoxification methods. The results showed that *Cl. acetobutylicum* ATCC 824 could grow and produce ABE in the detoxified ARSH with over-titration plus activated charcoal carbon absorption. Finally, the use of co-culture between *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462 and GE sugar was investigated with the aim of improving ABE production. The results demonstrated that the amount of ABE obtained from batch fermentation by the co-culture was lower than the single culture. However, the maximum ABE yield and ABE concentration by using GE were higher than those of commercial glucose. Overall, the results of this study demonstrate that rice straw is a potential lignocellulosic substrate for ABE production in batch fermentation by both *Cl. acetobutylicum* ATCC 824 and TISTR 1462 strains.

School of Biotechnology Student's Signature_____

Academic Year 2013 Advisor's Signature _____

Co-Advisor's Signature _____

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGTURES

Figure **Page**

LIST OF FIGTURES (Continued)

Figure **Page**

LIST OF FIGTURES (Continued)

Figure **Page**

LIST OF FIGTURES (Continued)

Figure **Page**

LIST OF FIGTURES (Continued)

Figure **Page**

LIST OF FIGTURES (Continued)

Figure **Page**

LIST OF FIGTURES (Continued)

Figure **Page**

LIST OF FIGTURES (Continued)

Figure **Page**

LIST OF ABBREVIATONS

NASA	=	Aeronautics and Space Administration
BP	=	British Petroleum
ATCC	=	American Type Culture Collection
ABE	=	Acetone-butanol-ethanol
INSA	=	Institute National des Sciences Appliquées
NCIMB	=	National Collection of Industrial Food and Marine Bacteria
TISTR	=	Thailand Institute of Scientific and Technological Research
USA	=	The United States of America
DSM	=	Deutsche Sammiung von Mikroorgansimen
NRRL	=	Agricultural Research Service Culture Collection
CPC patent	=	The Cooperative Patent Classification
IFP patent	=	Industrial production of intermediate food product patent
RVP	=	Reid vapor pressure
ºC	=	Degree Celsius
g	=	Gram (s)
M	=	Molar
mg	=	Milligram (s)
min	=	Minute (s)
h	=	Hour (s)
mL	=	Millitre (s)
μL	=	Microlitre (s)

LIST OF ABBREVIATONS (continued)

mm	=	Millimetre (s)
μm	=	Micrometre (s)
OD600	=	Optical density at 600 nm
Pa	=	Pascal
rpm	=	Revolutions per minute
% (w/v)	=	Percentage weight by volume
% (v/v)	=	Percentage volume by volume
kg/L	=	Kilogram (s) per Liter
mg/L	=	Milligram (s) per Liter
g/l.h	=	Gram per liter and hour
mm^2/sec	=	Square millimeter/second
MJ/Kg	=	Megajoules per Kilogram
MJ/L	=	Megajoules per Litre
BTU	=	British thermal unit
C_4H_9OH	=	Butanol
CO_2	=	Carbondioxide
CO	=	Carbon monoxide
ATP	=	Adenosine triphosphate
NAD	=	Nicotinamide adenine dinucleotide (Oxidized form)
NADH	=	Reduced form of Nicotinamide adenine dinucleotide
EMP	=	Embden-Meyerhof-Parnas pathway
PP	=	Pentose Phosphate pathway

LIST OF ABBREVIATONS (continued)

CoA	=	Coenzyme
PFOR	=	Pyruvate-ferrodoxin oxidoreductase
Acetyl-CoA	=	Acetyl-coenzyme A
F_{red}	=	Reduced ferredoxin
PTA	=	Phosphotransacetylase
AK	=	Acetate kinase
DH	=	Acetaldehyde dehydrogenase
AAD	=	Ethanol dehydrogenase
THL	=	Acetoacetyl-CoA thiolase or thiolase
CoAT	=	CoA-transferase
AADC	=	Acetoacetate decarboxylase
BK	=	Butyrate kinase
BHBD	=	β-hydroxybutyryl-(CoA) dehydrogenase
CRD	=	Crotonase
BCD	=	Butyryl-CoA dehydrogenase
PTB	=	Phosphotransbutyrylase
BDHA	=	Butyraldehyde dehydrogenase
BDHB	=	Butanol dehydrogenase
LDH	=	Lactate dehydrogenase
HYD	=	Hydrogenase
NFO	=	NADH-ferredoxin oxidoreductase or ferridoxin-NAD-reductase

ACKNOWLEDGEMENTS

First, my success in this Ph.D. program would not have been possible without the scholarship under the program of development of the lecturer potentiality in the Udon Thani Rajabhat University, as one of the oldest community universities in the northeast of Thailand.

I wish to express my sincere appreciation to my advisors, Assistant Professor Dr. Sunthorn Kanchanatawee, Professor Dr. Philippe Soucaille, and Professor Dr. Isabelle Meynial-Salles for providing me good opportunity of collaboration between the Thai laboratory at Suranaree University of Technology (SUT) and the French laboratory at Institut National des Sciences Appliquees (INSA), Toulouse, France. Moreover, I am grateful to the French Embassy in Bangkok, Thailand in providing me with portial scholarship.

My extend sincere appreciation to my co-advisor, Assistant Professor Dr. Kaemwich Jantama, for all advising, adding my graduated experience, and being the idol of scientists who has deeply knowledge in the field of the metabolic engineering.

I would like to special thank to Dr. Christian Croux for providing me the molecular biology knowledge. He has answered countless questions and has always guided me in the right direction.

I would like to thank my French friends, especially, Céline Foulquier and Mathieu Cesari, my mentors in the laboratory. They instructed me on laboratory procedures, protocols, laboratory techniques and how to use the analytical equipments.

My special thanks are also exteded to the following person, Phattaraporn Yudthachit who gave me the strength during the last time of my study, Wallapha Wongsirichan for improving my English Grammar, and all my friends in the team of metabolic engineering group for helpful assistance during my Ph.D study as well as also being various idols of life styles in the graduate school.

Last, I would like to deeply thank to Sitha Chan, my Cambodian friend, to endure reading and proving careful my first draft of the thesis document in the first period of writing. Subsequently, her kindness made my English writing successful until getting this whole of the thesis document. I was impressed by her word that "You have to read and write it with your heart."

I would like to dedicate this Degree of Doctor of Philosophy to my mother, my daughter and son, my husband, and every member in my big family, especially to my late father for all their love and encouragement and for always been supportive of my choices.

Metinee Wasoontharawat

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Fossil fuels, such as coal, petroleum, and natural gas, have a significant impact upon the world's population. Fossil fuels are needed to create a wide range of natural goods and provide many of the services. For instances, fossil fuels were used for heat, electricity (industrial, commercial and residential), transportation fuels and chemicals including pharmaceuticals, detergents, synthetic fiber, plastics, pesticides, fertilizers, lubricants, solvent, waxes, coke, and asphalt (Scott *et al.*, 2010). Eighty five percent of the commercial energy resources used in the world came from fossil fuels (Hinrich and Kleinbach, 2006). Hence, the fluctuations in fuel resources and prices affect industrialization including the global social and economic development.

Currently, the increasing worldwide fossil oil price appears as a result of depletion of fossil fuels, the limited nature of the source of petroleum and fossil, awareness in greenhouse gas emissions during combustion of fossil fuels and potential impacts of this activity on global warming. In the past two centuries, cheap supply of fossil fuels drove the age of industrialization. Coal and gas are used to make electricity and oil is used for transportation. However, burning fossil fuels to makeenergy causes seriously problems (Lange, 2007). Burning a fuel containing sulfur or sulfur dioxide causes acid rain. In addition, carbon-fuels burning released about 40% of carbon dioxide into the atmosphere (United State environmental

protection agency, 2013). Large amounts of carbon dioxide act as insulator and cause greenhouse effect which contributed to climate change. Nowadays, more evidence of this shows earth's climate changes (The Earth Science Communication Team-National Aeronautics and Space Administration (NASA), 2013).

The increase of crude oil prices have resulted in energy sustainable biorefinery. Biofuels are environmentally friendly fuels, due to their clean-burning unless CO_2 emission to the atmosphere (Kumar *et al.*, 2009). In fact, biofuels preceeded petroleum-based fuels in the late 1800's (Cordos *et al.*, 2011). For example, corn derived ethanol and peanut oil were alternative power sources for vehicles and diesel engine, respecitvely. In the past decades, the biofuel majority of effort is bioethanol and biodiesel (fatty acid methyl ester). Recently, biobutanol, one of the biofuel, has been renewed interest in both a potential fuel and a chemical feedstock. A turn point after the World War II (1950s-1960s), industrial butanol production stopped due to the development of petrochemical industries including shortage of feedstock for the fermentation process and the high cost of recovery.

Butanol has several advantages over ethanol including a direct replacement of gasoline, a fuel additive, a higher energy content, lower volatility, less hydroscopic, and less corrosive (Dürre, 2007; Tashiro and Sonomoto, 2010). Butanol used as a fuel additives has not only similar characteristics to gasohol, but it can also be used in some vehicles without the need for any modifications in their engines or fuel system. Thus, butanol has become an important industrial product (Kahl *et al.*, 2003; Kharkwal *et al.*, 2009). The processes of commercial butanol production by fermentation have also been developed by some companies. British Petroleum (BP) and DuPont, announced to develop a process of butanol fermentation from sugar beet in June 2006

(Mariano *et al.*, 2009). Butamax™Advanced Biofuels demonstrated the purpose of commercialized biobutanol by 2013 (Michael *et al.*, 2013).

Early industrial butanol production was based on fermentation using bacterium *Clostridium acetobutylicum* ATCC 824. This strain can ferment various carbohydrates to generate acetone, butanol and ethanol. The major products are butanol and acetone (Lee *et al.*, 2008). Starchy materials, used as substrates for butanol production, are corn, sugarcane molasses, wheat, rice, rye, and cassava starches (Campos *et al.*, 2002; Syed *et al.*, 2008). Availability of these starchy substrates is also used as food for human and/or animal (Kumar and Gayen, 2011). The cost of fermentation substrate, is one of the important factors influence the butanol production. Therefore, use of these substrates, as human food and animal feed, affects the economics of biofuel production (Dürre, 2007).

Among the total carbohydrate materials, lignocellulosic biomass is considered to be inexpensive material and has great potential as a substrate for butanol fermentation (Esteghlalian *et al.*, 1997; Qureshi and Blascheck, 2005). In fact, it is the extremely abundant renewable materials on the planet. It is derived from rapidly growing non-food plants such as agricultural and foresty residues, municipal and industrial solid wastes, as well as herbaceous and woody bioenergy plants. Moreover, lignocellulosic material is natural composed of cellulose (glucose polymer) and hemicelluloses (sugar polymer) as a main component. They contain the high sugar content. This is indicated that one single crop residue or biomass would address the biofuel demand.

Rice straw is one of the largely abundant lignocellulosic agricultural wastes around the world. It is distributed in Africa, Asia, Europe, and America (Singh and Bishnoi, 2012). Rice straw as a renewable energy source has a huge potential in Thailand (Sajjakulnukit *et al.*, 2005; Prasertsan and Sajjakulnukit, 2006). The amount

of rice straw available for energy has been estimated around 26 million tons or 43% of total agricultural wastes available (Ministry of energy, Thailand, 2013). Therefore, this study emphasized on the use of rice straw as a lignocellulosic substrate for the butanol production.

Necessary steps in the process of lignocellulosic biomass conversion to butanol are pretreatment, enzymatic hydrolysis, fermentation, and butanol purification (Satimanont *et al.*, 2012). Typically, lignocellulosic biomass mainly consists of celluloses, hemicelluloses, and lignin. The complex structure of these components is difficult to break down by enzymatic hydrolysis or microorganism to utilize it for butanol production. Pretreatment is an essential prerequisite to modify lignocellulose's structural features by removing lignin and separating hemicelluloses, or disrupting the crystalline structure of cellulose prior to the enzymatic hydrolysis. Numerous pretreatment strategies have been suggested or constructed to enhance the reactivity of cellulose and to facilitate the enzymatic hydrolysis of cellulose (Laser *et al.*, 2002; Rosgaard *et al.*, 2007; Silverstein *et al.*, 2007; Alhasan and Kuang, 2010). However, the best pretreatment methods and conditions depend greatly on the types and properties of lignocellulosic biomass (Fang *et al.*, 2010). No single method has also been found suitable to all biomasses. Therefore, the first objective of this study was to compare the influence of different pretreatment strategies on the accessibility of pretreated rice straw cellulose and then to select the suitable pretreatment method in order to prepare the rice straw for sugar production by microbial or enzymatic hydrolysis.

After pretreatment process, pretreated cellulose is less crystalline, allowing enzyme to break down it into fermentable sugars. Enzymes are very specific for cellulose digestion. Cellulose hydrolysis using enzymes does not release toxic

derivatives to a culture medium. Application of enzymes for cellulose hydrolysis depends on process variables such as temperature, pH, reaction time, enzyme concentration, substrate concentration, intensity of agitation, and the presence of other chemical species that may inhibit or accelerate their rates of reaction (Kaya *et al.* 2000). In order to improve the efficiency of cellulose hydrolysis, the optimum condition for the enzymatic hydrolysis is required. Therefore, the second objective of the present study was to evaluate the effect of enzyme loading on cellulose hydrolysis.

Clostridia are a group of Gram-positives, spore-forming, motile, rod-shape and obligate anaerobes. Solvent producing clostridia have been assigned to 4 species such as *Clostridium saccharoperbutylacetonicum, Cl. acetobutylicum, Cl. beijerinckii,* and *Cl. aurantibutyricum* (Tashiro and Sonomoto, 2010; Raganati *et al.*, 2012). They naturally possess pathways that allow the conversion of sugar into solvent. These pathways are well known as acetone-butanol-ethanol (ABE) fermentation. They can utilize a wide range of carbohydrates, including mono- and di-saccharides (i.e. glucose, cellobiose, fructose, maltose, or xylose), starches (i.e. corn, cassava, sago), and other substrates (i.e. glycerol, pectin, inulin, whey, and xylan) (Jones and Woods, 1986; Madihah *et al.*, 2001; Thang *et al.*, 2010; Cappelletti *et al.*, 2011). Industrial clostridium strains have been recognized based on ratio of the solvent produced (Beesch *et al.*, 1952; Dürre and Bahl, 1996). The most extensively species for the study of the butanol production is *Cl. acetobutylicum,* which typically produces acetone, butanol, and ethanol at the mass ratio of 3:6:1 (Jones and Woods, 1986; Lee *et al.*, 2008; Lee *et al.*, 2009). Another species, *Cl. beijerinckii* produces butanol almost in the same ratio as *Cl. acetobutylicum*, but it produces isopropanol instead of acetone (George and Chen, 1983). *Cl. aurantibutyricum* produces acetone and isopropanol along with butanol (George *et al.*, 1983). For *Cl. tetanomorphum*, it produces

only butanol and ethanol because of only having butyric acid production and conversion of butyric acid into butanol by other strains (Kharkwal *et al.*, 2009). Typically, *Cl. acetobutylicum* cannot naturally utilize crystalline cellulose as a substrate for butanol production (Lee *et al.*, 1985; Lee *et al.*, 1987). Surprisingly, *Cl. acetobutylicum* ATCC 824 genome has indicated the presence of a number of genes encoding cellulolytic enzymes. Some of these genes (i.e. *Cel48F*) appear to be grouped into a gene cluster similar to the cluster found in true cellulolytic clostridia, such as *Cl. cellulovorans* and *Cl. cellulolyticum*. Moreover, *Cl. acetobutylicum* grows very well on cellobiose, devided sugar from cellulose (López-Contreras *et al.*, 2004). However, some study reported that the Cel48A endocellulase protein, which is much more abundant than the other catalytic subunits, of cellulosome of *Cl. acetobutylicum* ATCC 824 does not present cellulase activity (unpublished data, INSA). In the last decade, advanced knowledge in the metabolism, physiology and genetics of solventogenic clostridia have been studied. Besides, the ability of clostridia to produce butanol can be achieved using genetic tool for transformation through metabolic engineering (Reysset and Sebald, 1993). For instance, *Cl. beijerinckii* NCIMB 8052 was transformed with desired genes coding for active extracellular hydrolytic enzymes. It would be able to increase substrate utilization range and enable to degrade cellulose and hemicellulose more efficiently (Bergstrom and Foutch, 1985). Hence, it may be possible to engineer solvent producing *Cl. acetobutylicum* ATCC 824 in order to grow on cellulose by introducing cellulases gene from another cellulolytic bacterium. Hence, the third objective of this study was to develop cellulolytic solvent-producing *Cl. acetobutylicum* ATCC 824.

Besides improving solvent producing clostridial strain with genetic engineering, Berstrom and Foutch (1985) reported butanol fermentation process

development using a coculture of *Cl. pateurianum* and *Cl. butylicum.* The coculture of both species produced a 20% higher yield which was equivalent to 70 weight per volume butanol. Therefore, the last objective of this study also aimed to develop butanol production using co-culture of *Cl. acetobutylicum* ATCC 824 and *Cl. acetobu-tylicum* TISTR 1462.

1.2 Scope and limitations of the study

As shown in Figure 1, the purpose of this study was to construct the cellulolytic solvent-producing *Cl. acetobutylicum* ATCC 824 strain and butanol production from rice straw. First, the recombinant *Cl. acetobutylicum* ATCC 824 was constructed by induction of a hybrid cellulase gene encoding an active cellulase instead of an inactive cellulase gene in clostridia genome. Expected output in the research was that the recombinant *Cl. acetobutylicum* would be able to produce butanol from rice straw in a single process of fermentation. Second, the comparison of different pretreatment method strategies on the accessibility of pretreated rice straw cellulose was performed in order to prepare the rice straw for sugar production by microorganism or enzymatic hydrolysis. Finally, the comparison of different detoxification methods of toxins which generated from acid hydrolysis of rice straw, the optimization of butanol production, and the investigation of potential microbe synergy of *Cl. acetobutylicum* ATCC 824 in a co-culture with *Cl. acetobutylicum* TISTR 1462 for butanol production were investigated. The results would reveal whether that rice straw fermentable sugars would be employed as a potential substrate for butanol production by *Cl. acetobutylicum*.

1.3 Research objectives

1.3.1 To construct the recombinant *Cl. acetobutylicum* ATCC 824 strain which was expected to be able to actually degrade the cellulose and produce ABE all in single step.

1.3.2 To produce fermentable sugars from rice straw by physicochemical and enzymatic hydrolysis.

1.3.3 To produce butanol from rice straw in batch fermentation by *Cl. Acetobutylicum*.

1.3.4 To produce butanol from rice straw by co-culturing of *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462.

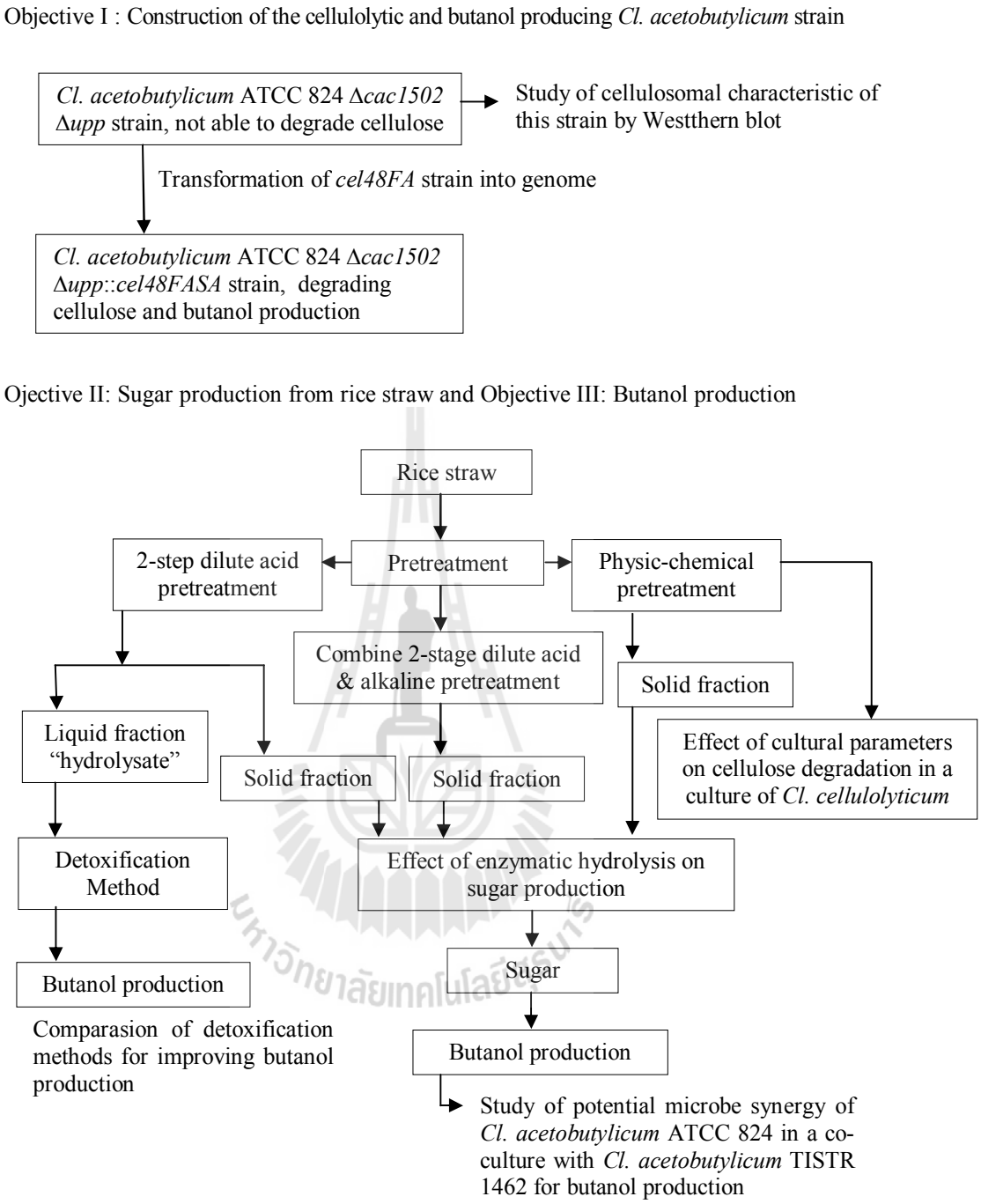


Figure 1.1 A schematic diagram for the scope of the study of butanol production using a *Cl. acetobutylicum* from rice straw. There were 3 objectives: objective I construction of cellulolytic and butanol producing *Cl. acetobutylicum* strain; objective II, sugar production from rice straw; and objective III: butanol production.

CHAPTER II

LITERATURE REVIEW

2.1 Butanol

Butanol is commonly known as butyl alcohol, n-butanol or methylolpropane (Figure 2.1). It is a four carbon linear and aliphatic primary alcohol, existing the molecular formula of C_4H_9OH and has a molecular weight of 74.12 g/mol. It is a colorless hydrophobic liquid with a distinct banana-like aroma, flammable, slightly and strong alcoholic odor. Direct contact causes severe eye irritation and moderate skin irritation. Butanol's vapor has an irritant effect on mucous membranes and a narcotic effect when inhaled in high concentrations. It is completely miscible with most organic solvents, but partly miscible with water (Lee *et al.*, 2008). Other members in the same alcohol family include methanol (1-carbon), ethanol (2-carbon), and propanol (3-carbon) (Brekke, 2007).

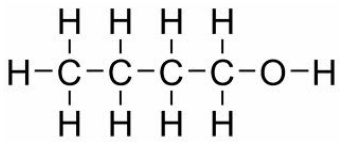


Figure 2.1 Structural molecule of butanol.

Butanol is widely used as an industrial solvent and chemical feedstock precursors for the production of other organic chemicals such as paints, lacquer finishes, thinners, plastics, butyl rubber, resins, adhesives, elastomers, emulsifiers, flocculants, absorbents, brake and hydraulic fluids, dicing fluids, and cleaning fluids (Dürre, 2008; Jones and Woods, 1986). Interestingly, butanol has highly potential application as a biofuel, which is a superior fuel compared with ethanol, and an environmentally friendly fuel. Table 2.1 shows the several advantages of butanol over ethanol including: 1) butanol offers a number of attractive features as a liquid transportation fuel which is used in conventional internal combustion engines without any modifications; 2) it contains 22% oxygen, so this composition makes butanol a beneficiary fuel extender that is cleaner burning than ethanol; 3) it is a higher octane rating, which is less likely to knock; 4) it has lower hydroscopicity (not absorb moisture), thus being less affected by weather changes; 5) it is an alternative fuel having complete miscibility with gasoline and diesel fuel without phase separation in the presence of water; 6) it is lower volatility (less explosive). Butanol's Reid vapor pressure (RVP) is 7.5 times lower than ethanol, 7) it is high flash point and lower vapor pressure resulting in its safety more than ethanol, and 8) it is less corrosive. These properties promote butanol to be a much safe alternative fuel that can be dispersed through existing pipelines and filling station and storage infrastructure (pipeline, storage tanks, filling station, etc) (Dürre, 2007; Shapovalov and Ashkinazi, 2008).

Table 2.1 Comparison of physical and chemical properties of butanol with other biofuels (Davis and Morton III, 2008; Lee *et al.*, 2008b).

Property	Butanol	Gasoline	Ethanol	Biodiesel	Diesel
Reid Vapour Pressure	23	62	15	-	<3
Lower Flamibility limit					
Concentration (Volume%)	1.4	1.4	3.3	-	0.6
Temperature (ºC)	36	-45	13	-	64
Upper Flamibility Limit					
Concentration (Volume%)	11.2	7.6	19	-	5.6
Temperature (ºC)	-	-20	42	-	150
Flash Point	36	-43	13	>120	64
Autoignition Temperature	343	300	366	-	230
Cloud Point	-89	NA	NA	0	-26
Density kg/L	0.81	0.791	0.785	0.86	0.863
Vapour Specific Gravity	2.6	3.5	1.6	-	5.5
Kinematic Viscosity mm^2/sec	3.7	NA	NA	3.5-5	2-Aug.
Lower Heating Value					
Mass (MJ/Kg)	33.22	43.9	27	37.8	42.6
Volume (MJ/L)	26.9	32.7	21.2	32.5	36.7
BTU per Gallon	84,000	115,000	110,000	120,000	13,000
Research Octane Number	96	90-100	108	NA	NA
Cetane Number	17	NA	2-Dec.	>51	40-47.5

2.2 History of butanol production

The production of butanol by using a microbial fermentation was first reported by Pasteur in 1861. Until the beginning of the 20th century, a shortage of natural rubber caused an increasing rubber prices. This led to the development of synthetic rubber production. Considering on n-butanol as the best feedstock chemical for the synthesis of butadiene, butanol is the raw material for synthetic rubber. This event initiated the microbial fermentation and further intensive research on the isolation of butanol producing microorganisms (Killeffer, 1927).

In 1911, Fernbach isolated an acetone butanol-producing bacterium. Unfortunately, the strain had limited substrate range and low solvent yield (Gabriel and Crawford, 1930). In addition, Fernbach and Strange's patent was issued for solvent fermentation using Fernbach strain and potato as a substrate. By the middle of 1913, British company, Strange and Graham Ltd., initiated acetone production, additional to butanol, from potatoes in industrial scale. Between 1912 and 1914, Weizmann had succeeded in the isolation of an organism capeble of ferment various substances including starchy materials to solvent. Importantly, the isolated strain produces many better yields of n-butanol and acetone than does Fernbach's culture (Gabriel and Crawford, 1930). This organism was named "*Bacillus granulobacter pectinovorum*" and given the nickname BY (B for bacteria or bacillus; Y for Weizmann). It was renamed as *Clostridium acetobutylicum* by McCoy and his coworkers (McCoy *et al.*, 1926). In 1915, Weizman's patent was issued for solvent process using *Cl. acetobutylicum* as biocatalyst and maize mash in addition to potato.

During World War I, demand for acetone was rising to produce gunpowder for the British navy (Jones and Woods, 1986). Acetone was used as the colloidal solvent

for nitrocellulose, which used to cordite in the manufacture of explosives. Butanol fermentation opened a way of producing large quantities of acetone for England at the time. After the war, the plants were closed down because there was no further a high demand for acetone. Next, the automobile industry developed rapidly to optional produce more cars. n-Butanol was in demand again, being as a precursor for synthesized butyl acetate as a suitable solvent for lacquer as automobile paints (Dürre and Bahl, 1996). In 1927, industrial butanol fermentation reached its peak capacity. For example, in Peoria, Illinois, the United States, 96 production fermentors with a volume of 50,000 gallons each were operated (Gabriel and Crawford, 1930).

The industrial fermentation of butanol was carried on until the 1950's, after World War II. It then started to decline due to growing petrochemical industry and increasing prices of fermentative substrates as food for human and animal. All acetone and n-butanol are currently produced from petroleum derived propylene. Then, in the 1960's, the industrial butanol production in Europe and North America ceased because of the unfavorable economics. Until in 1981, the ABE fermentation plant in South Africa was closed (Jones and Woods, 1986).

However, based on increasing oil prices about two to four times higher than in July–August 2000 and declining oil reserves (Middle East), William Jefferson Clinton issued Executive Order 13134, titled "Developing and Promoting Biobased Products and Bioenergy" (64 FR 44639, August 16, 1999) to hasten research in order to get rid off the crisis. Therefore, biobutanol have been introduced and attracted renewed interests because of the goals for these initiatives. It is an alternative liquid transportation fuel which its property is more similar to gasoline than ethanol and provides a number of advantages over ethanol. Moreover, the recent studies indicated that butanol fermentation is becoming more economical based on process technology

and availability of low cost substrate from renewable resources such as corn, sugar beets, sorghum, cassava, sugarcane, corn stalks, and other biomass (Dürre, 2008). Many researchs demonstrate that cellulosic materials were substrate for butanol fermentation by using clostridial strain, are shown in Table 2.2.

Table 2.2 Strains of butanol producing bacteria from wood or agricultural residue in batch fermentation.

Substrate	Microorganism	ABE productivity (g/l.h)	Butanol productivity (g/l.h)	Reference
Wheat straw	*Cl. beijerinckii* P260	0.63	0.36	Qureshi *et al.*., 2007.
	Cl. acetobutylicum IFP926	0.48	0.28	Marchal *et al.,* 1984.
Wheat bran	*Cl. beijerinckii* ATCC 55025	0.16	0.12	Liu *et al.*, 2010.
Corn stover	*Cl. acetobutylicum* P262	1.08	-	Parekh, 1988.
Corn fiber	*Cl. beijerinckii* BA101	0.13	-	Qureshi *ei al.*., 2008
Bagasse	*Cl. saccharoperbutaylacetonicum* ATCC 27022	0.30	-	Soni, 1982.
Rice straw	Above	0.15	-	
Packing peanut	*Cl. beijerinckii* BA101	0.20	-	Jesse *et.al.,* 2002
Agricultural waste (packing peanut, cracked corn apple and pears	Above	0.22	0.15	
Pine Wood	*Cl. acetobutylicum* P262	0.73	-	Parekh, 1988.
Aspen Wood	Above	0.84-1.03	-	
Solka floc or mixture of solka floc and xylan	*Cl. thermocellum* and *Cl. acetobutylicum* ATCC 824	0.04	0.003	Yu *et al.,* 1985

Note: (-): no data.

2.3 Naturally butanol-producing clostridia

Cl. acetobutylicum ATCC 824 bacterium was isolated from Connecticut garden soil in 1924 (Wey and Rettger, 1927). It is a species of strictly anaerobic, spore-forming, Gram-positive, and motile bacillus with peritrichous flagella. *Cl. acetobutylicum*

requires anaerobic condition for vegetative cell growth. The vegetative cell can survive for several hours in aerobic condition, whereas the endospore form can lay dormant for long periods and remain viable even in aerobic conditions. Endospore germinates into vegetative cell again when anaerobic growth conditions return. Actively growing cells are straight rod shaped bacillus ranging in size of 0.5-1.5x1.5-6 μm (Robinson, 2000). *Cl. acetobutylicum* can exist as single cells, in pairs or chains, depending on the growth phase and medium used. *Cl. acetobutylicum* is a mesophilic bacterium which has optimum growth temperature at 37°C, but the optimum temperature for solvent production is 35°C (McNeil and Kristiahsen, 1985). The optimal pH for growth and acid production is at 6.5, while pH of lower than 5.3 is required for production of solvent (Bahl *et al.*, 1982a; Monot *et al.*, 1984).

There are several wild types of solvent-producing microorganisms, dominated by clostridia (Kharkwal *et al.* 2009), as tabulated in Table 2.3. The solventogenic clostridia comprise of 4 species such as *Cl. acetobutylicum, Cl.beijerinckii* (synonym. *Cl. butylicum*), *Cl. saccharobutylicum* (syn. *Cl. beijerinckii* P262), and *Cl. saccharo-perbutylacetonicum* (George *et al.*, 1983; Gottwald *et al.*, 1984; Annous and Blaschek, 1991). Table 2.4 demonstrates strains of solvent-producing clostridia surving in culture collections with significant potential for renewable biobutanol and chemical production. It was noticed that only the *Cl. acetobutylicum* and *Cl. beijerinckii* have been developed for the industrial solvent production due to their type and ratio of the solvent (Beech, 1952; Dürre and Bahl, 1996). *Cl. acetobutylicum* ferments starch, hexoses, or pentoses to acetone, butanol, and ethanol in the ratio of 3:6:1, respectively (Blaschek, 2008; Lee *et al.*, 2008; Qureshi, 2001). Another species *Cl. beijerinckii* produces largely butanol and a smaller quantity of isopropanol (in place of acetone) and ethanol by using hexose or starch as fermentative substrates (George and Chen,

1983). Unlike *Cl. acetobutylicum*, *Cl. beijerinckii* specie can produce solvents from sugars without pH control in a culture. Remarkably, the most commonly studied strain is *Cl. acetobutylicum* ATCC 824 strain.

Cl. acetobutylicum ATCC 824 strain is closely related to the historical Wiezman strain for the early industrial acetone production. It is studied as model for endospore formation in bacteria and solvent-producing strain important in terms of fundamental research on strictly anaerobic bacteria. The strain has been utilized by the majority of investigators working in the USA, Canada, Sweden, France, and Germany. Several studies on the characterization of physiology, molecular biology and genetic engineering to use alter specific substrate were investigated for the efficiency of solvent production (Nölling *et al.,* 2001; Sabathé, 2002).

Table 2.3 Potential microbial strains for butanol production (Jones and Keis, 1995).

Microoraganism	**Comments**
Cl. acetobutylicum	The most potential *Clostridium* sp. for butanol production, in terms of yield. Mutants for degeneration resistance, butanol tolerance, improved butanol production, and regulated sporulation have been made.
Cl. beijerinskii	In ABE process, it produces isopropanol in place of acetone. Mutant for higher butanol production has been made.
Cl. carboxidivorus	Butanol was produced, using CO as a substrate
Cl. tetanomorphum	Butanol was produced by using D-glucose as a substrate
Cl. aurantibutyricum	Produces both acetone and isopropanol along with butanol.
Lactobacillus sp.	Reported as a capable butanol producing strain
Pseudomonas putida	Reported as capable butanol producing strain
Kluyveromyces lactis	Butanol production reported
Pichia sp.	Good for ethanol production, Reported as a capable candidate for butanol production

Table 2.4 Groups of culture collection strains of solvent producing clostridia according to species and substrate utilization (Jones and Keis, 1995).

ATCC	DSM	NCIMB	NRRL	Strain	Origin
Group 1. *Cl. acetobutylicum* (starch utilization)					
824	792	-	B527	McCoy W	Weyer/Rettger (type strain)
[862]	-	-	B-528	McCoy D	Donker/Kluyer
4259	1731	619	B-530	McCoy T	Thaysen (Weizmann
-	1732	2951	-	Thaysen	Weizman patent
3265	1737	-	B-529	McCoy H	Hall (Weizmann strain?)
8529	1738	-	-	Castell	McCoy 48
-	1733	6441	-	Wisconsin B-3	
-	-	6442	-	Wisconsin B-5	
-	-	6443	-	Wisconsin B-10	
39236	-	-	-	B-11-3	CPC patent (ATCC 4259 mutant)
43084	-	-	-	3003	Benassi (Cassava strain)
Group 2. *Cl. acetobutylicum* (molasses utilization)					
-	-	-	B-591	Wisconsin A-8	(from CSC)
-	-	6444	-	Wisconsin A-13	
-	-	6445	-	Wisconsin A-14	
10132	1739	8049	B-594	Wisconsin A-14	
X	x	8052	X		Listed as type strain ATCC 824
-	-	-	B-597	Wisconsin A-72	
-	-	-	B-643		CSC
39057	-	-	-	IFP-903	IFP patent
39058	-	-	-	IFP-904	IFP patent (IFP-903
Group 3 *Cl. Beijerinckii*					
14823	-	-	-	79	
-	-	-	B-593	Wisconsin A-21	Fernbach
-	-	-	B592	Wisconsin A-39	Fernbach
-	-	-	B-596	Wisconsin A-38	
			B-466	Reid	Wisconsin 39-90
Group 4 *Cl. saccharoperbutylacetonicum*					
27021	-	-	-	N1-4(HMT)	Hongo
27022	-	-	-	N1-504	Hongo

2.4 Morphological and physiological changes of *Clostridium acetobutylicum* during solvent fermentation

Most *Clostridium* cultures contain different cell forms including young vegetative cells, cells in different sporulation stages, free endospores, and germinated endospores (Dürre and Hollergschwandner, 2004; Perkins, 1965). Morphological and physiological changes, which occurred in *Cl. acetobutylicum* during growth, associate with solvent production in a batch ABE fermentation process (Jones and Keis, 1995; Tracy *et al.*, 2008), as shown in Figure 2.2.

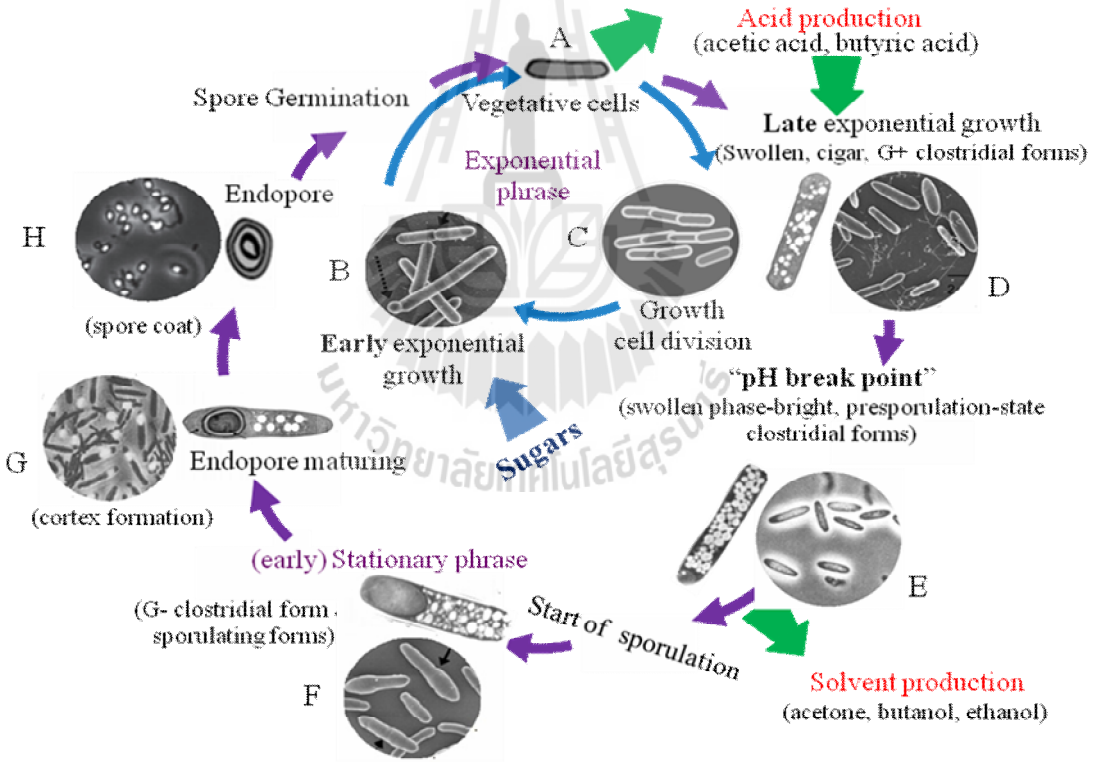


Figure 2.2 The morphological and cytological changes of *Cl. acetobutylicum* during the process of ABE batch fermentation. (A-E) exponential growth state, and (F-H) stationary phrase and sporulation. (Adapted from Long *et al.*, 1983; Reysenbach *et al.*, 1986; Schuster *et al.*, 1998).

The morphological and cytological changes of *Cl. acetobutylicum* begins after cell inoculation, the *Cl. acetobutylicum* cells appear as elongated rods which develop septa before cell separation (Figure 2.2B) and result in the formation of long chains, strongly Gram-positive cells with sporadic and sluggish motility. Biotin and 4-aminobenzoate are usually required as growth factors, because deficiency of biotin leads to interruption in lipid synthesis and cytoplasmic membrane synthesis, and suppress the sporulation on glucose limitation (Al-Sum and White, 1977).

The earliest exponential growth, the *Cl. acetobutylicum* cell chains begin to break up and release individual actively growing rods which are highly motile. The actively motile cell is regarded as a prerequisite for good solvent production (Gutierrez and Maddox, 1987). This stage, the growth rate of *Cl. acetobutylicum* is at its maximum. The period between the early (active-growth) and late (zero-growth) stage is associated with the production of titratable acids and a decrease in the pH from 6.8 to 5.1 The end of late exponential growth is named pH breakpoint the changeover point between the early (active-growth) and late (zero-growth) stages in the fermentation process.

Before the pH breakpoint, the presence of granulose is firstly detected in the *Cl. acetobutylicum* cells only at the late end exponential growth (Reysenbach *et al.*, 1986). Granulose, a reserve polyglucan, can be comsumed as an energy and carbon source for the spore formation and their maturation (Strasdine, 1972). *Cl. acetobutylicum* cells are the formation in Gram positive, and swollen, cigar-shaped clostridial stages (Figure 2.2D). The clostridial cell form is generally supposed to be the solvent-producing cell type in solventogenic clostridia (Tracy *et al.*, 2008). Reysenbach *et al.* (1986) suggested that the accumulation of granulose occurs concomitantly with the

shift from an acid-producing metabolism to a solvent-producing metabolism at the end of the exponential growth phase.

At the pH breakpoint, stress in acidic pH can induce solventogenesis, shifting from acid production to solvent production. The maximum granulose deposition within cell is observed to show as a swollen phase-bright clostridial stage, capsule production, and the initiation of a forespore septum. Reysenbach *et al.* (1986) indicated that the clostridia strains, producing less or no granulose, exhibited lower levels of sporulation, because the reserved granulose usually is degraded during spore formation. At the latest growth, most of the cells are arrested and sluggishly motile or non-motile in the early stationary state.

In the stationary state, solvent is produced and most of the vegetative rods are converted to negatively stained clostridial forms which cannot motile. In case of *Cl. acetobutylicum* growth in glucose minimal medium, solvents and clostridial forms were not produced (Long *et al.*, 1983). During the solvent production, the titratable acids decrease due to conversion of acid to solvent, and the pH increase to about 5.6. The event of premature endospores occurs at the terminal ends of the vegetative cells. Next, endospores can be observed as round phase bright bodies which allow endospores to mature and the majority is released from the mother cells, as shown in Figure 2.2F, G and H.

2.5 Acetone-butanol-ethanol (ABE) fermentation

The *Cl. actobutylicum* ATCC 824 strain is a promising biofuel producer, which can convert a great variety of carbon resources to solvents (Girbal and Soucaille, 1995). The process of acetone-butanol-ethanol (ABE) fermentation by *Cl. acetobutylicum* in batch culture is divided into two distinctive phases namely acidogenesis and solventogenesis (Shinto *et al.*, 2008). The acidogenesis is an acid production phase occurred in the exponential growth phase, while the solventogenesis is a solvent production phase occurring in the stationary phase.

During the first acidogenic phase in exponential growth phase, the cells grow rapidly, and produce carboxylic acetic and butyric acids with adenosine triphosphate (ATP) formation. The excretion of these acids lowers the external pH. Ballongue *et al.* (1985) suggested that the acetic and butyric acids act as inducers for the biosynthesis of the solventogenic enzymes during a second fermentative phase.

During the second solvetogenic phrase, cell growth slows down and enters the stationary phase. Then, the acetic and butyric acids are formed earlier reenter the cells. These acids act as co-substrates for the production of neutral solvents. The partial of acids are reutilized to produce acetone, butanol and ethanol. Additonary, cells stop producing acid and cease growth. The medium pH increases slightly due to the acid uptake, indicating the acidogenic-solventogenic transition (Spivey, 1978; Terracciano and Kashket, 1986). The switch from acid production to acid re-assimilation is an adaptive response of the cells to the low medium pH, resulting from high yield production of acid (Bahl *et al.*, 1982). A typical ABE fermentation, butanol is a major end product, while acetone and ethanol are minor products. At the end of the fermentation, the solvent concentrations reach an inhibitory level leading to halt the

metabolism (Bowles and Ellefson, 1985). Toxicity of the high accumulated solvents alters both of membrane function and membrane fluidity of *Cl. acetobutylicum* (Moreira *et al.*, 1981; Vollherbst-Schneck *et al.*, 1984).

2.6 Metabolic pathway and gene of *Clostridium acetobutylicum* for ABE production

Carbohydrate metabolism of the *Cl. acetobutylicum* strain for ABE fermentation in batch cultures depends on the nature of the carbon source and the culture conditions (Abou-Zeid *et al.*, 1976; Compere and Griffith, 1979). Figure 2.3 illustrates the metabolic pathways of the carbohydrates for the acid/solvent formation by *Cl. acetobutylicum*. Carbon flow proceeds through hexose sugars that are metabolized via the Embden-Meyerhof-Parnas (EMP) pathway or glycolysis. One molecule of hexose is converted to 2 pyruvate molecules, with net production of 2 ATP and 2 NADH molecules. For pentose sugars, they are metabolized via the pentose phosphate (PP) pathway. The pentose is converted to pentose 5-phosphate and dissimilated by the transketolase-transaldolase sequence. Fructose 6-phosphate and glyceraldehyde 3-phosphate are generated and enter the glycolytic pathway. The fermentation of 3 pentose molecules provides 5 pyruvate, 5 ATP and 5 NADH molecules.

In the presence of coenzyme (CoA), puruvate is oxidized by pyruvate-ferrodoxin oxidoreductase (PFOR), which is transcribed from pyruvate:ferredoxin oxidoreductase (*pfor*) gene in decarboxylation to carbon dioxide (CO_2), acetyl-coenzyme A (acetyl-CoA), and reduced ferredoxin (F_{red}). Acetyl-CoA is the branch-point intermediate leading to the production of organic acids (acetate and butyrate),

solvents (acetone, butanol and ethanol), and gases (carbondioxide and hydrogen). Acetyl-CoA is converted to acetate in two steps by phosphotransacetylase (PTA, encoded by *pta*) and acetate kinase (AK, encoded by *ack*), respectively, in an operon. Acetyl-CoA is also converted to ethanol by acetaldehyde dehydrogenase (DH, encoded by *aad*) and ethanol dehydrogenase (AAD, encoded by *aad*).

Condensation of two acetyl-CoA molecules by acetoacetyl-CoA thiolase or thiolase (THL, encoded by *thl*) produces one molecule of acetoacetyl-CoA. This reaction is the central pathway, because it plays an important role in determining the ratio between the two-carbon (acetate, ethanol) and the three-carbon (acetone) and the four-carbon (butyrate, butanol) products. The *in vivo*, the thiolase activity is regulated by the coenzyme-A:acetyl-CoA ratio. Acetoacetyl-CoA is the precursor of formation of acetone and butyryl-CoA, which is the precursor of butyrate and butanol. Acetoacetyl-CoA is converted to acetone by CoA-transferase (CoAT, encoded by *ctf*A and *ctf*B) and acetoacetate decarboxylase (AADC, encoded by *adc*). The steps for acetone formation involve the transfer of the CoA moiety to either acetate or butyrate, which catalyzed by acetate kinase and butyrate kinase (BK). Besides these stepes are primary routes of ATP generation for *Cl. acetobutylicum*. Acetoacetyl-CoA is also reduced to butyryl-CoA by *β*-hydroxybutyryl-(CoA) dehydrogenase (BHBD, encoded by *hbd*), crotonase (CRD, encoded by *crt*), and butyryl-CoA dehydrogenase (BCD, encoded by *bcd*) in a single operon. Butyryl-CoA creates butyrate via two steps catalyzed by phosphotransbutyrylase (PTB) and butyrate kinase, respectively. It is suggested that two CoA derivatives, including acetoacetyl-CoA and butyryl-CoA from conversion of acetyl-CoA, are the central intermediates leading to both acid and ABE production. An *in vitro* CoA-transferase activity is inhibited by butanol and acetone concentrations which feedback inhibit the reaction (Hiisemann and Papoutsakis,

1989). Butanol is formed from butyryl-CoA by two sequential reductions catalyzed by two dehydrogenase enzymes including butyraldehyde dehydrogenase (BDHA, encoded by *bdhA*) and butanol dehydrogenase (BDHB, encoded by *bdhB*).

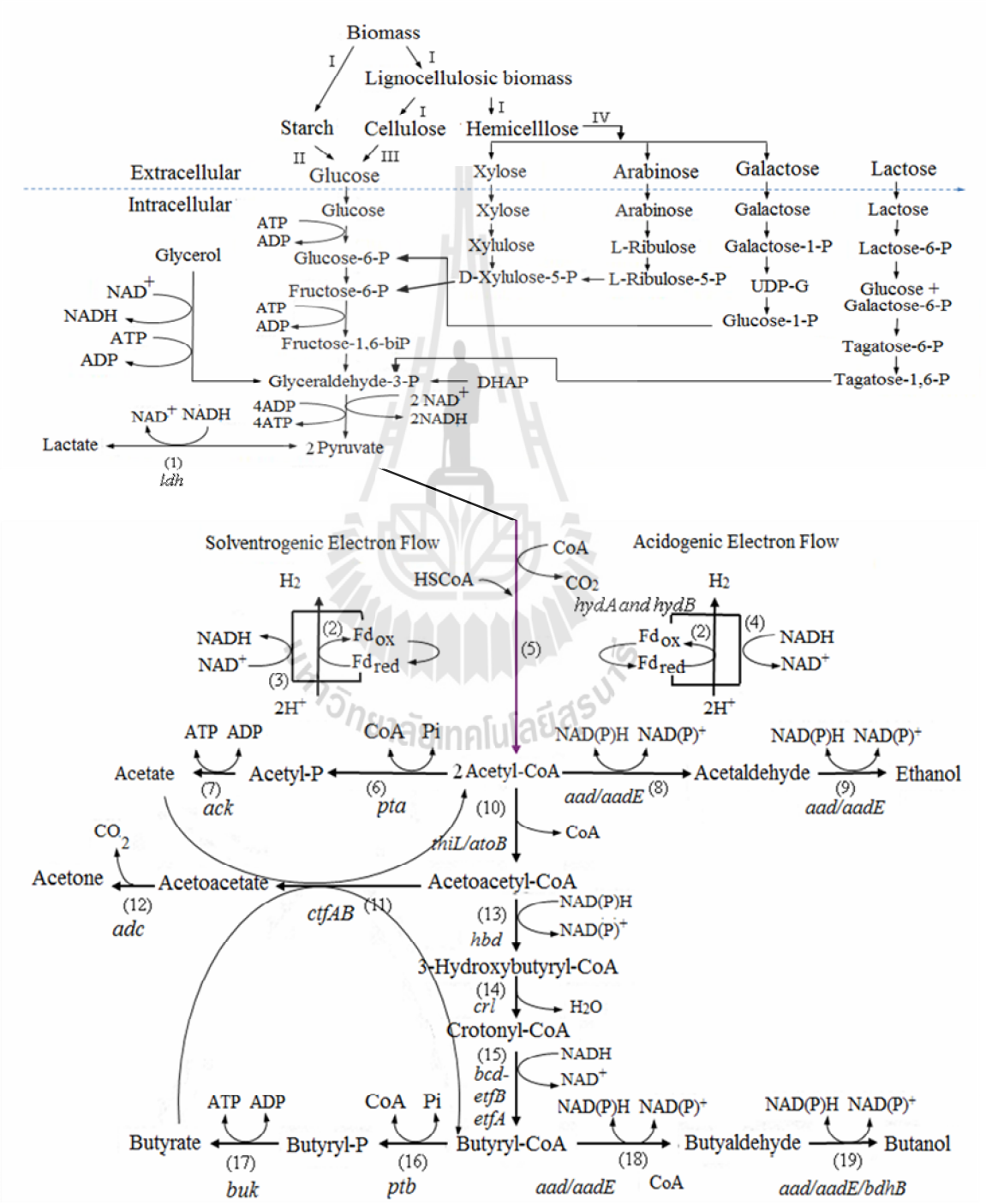


Figure 2.3 Metabolic pathways of carbohydroate metabolism in *Cl. acetobutylicum* for ABE production. (Adapted from Jones and Woods,1986; Girbal and Soucaille, 1998; Servinsky *et al.*, 2010) Process and enzymes are indicated by letters as follows: (I), Pretreatment of corn and lignocellulose; (II), starch hydrolysis (α-amylase, β-amylase, pullulanase, glucoamylase, α-glucosidase); (III), cellulose hydrolysis (cellulases,b-glucosidase); (IV), hemicellulose hydrolysis; (1) LDH, lactate dehydrogenase; (2) HYD, hydrogenase; (3) NFO, NADH-ferredoxin oxidoreductase or ferridoxin-NAD-reductase; (4) FNR, NADPH-ferredoxin reductase or NADPH-ferredoxin oxidoreductase; (5) PFOR, pyruvate-ferredoxin oxidoreduc-tase; (6) PTA, phosphate acetyltransferase (phosphotransacetylase); (7) AK, acetate kinase; (8) DH, acetaldehyde dehydrogenase; (9) AAD, ethanol dehydrogenase; (10) THL, thiolase (acetyl-CoA acetyltrans-ferase); (11) CoAT, acetoacetyl-CoA;acetate/butyrate:CoA transferase; (12) AADC, acetoacetate decarboxylase; (13) BHBD, 3-hydroxylbutyryl-CoA dehydrogenase; (14) CRD, crotonase; (15) BCD, butyryl-CoA dehydrogenase; (16) PTB, phosphate butyltransferase or phosphobutyry-lase; (17) BK, butyrate kinase; (18) BDHA, butryraldehyde dehydro-genase; (19) BDHB, butanol dehydrogenase. The several genes encoding each metabolic enzyme are also indicated with abbreviations in the metabolic map. Note: AAD is believed to be the primary enzyme for butanol and ethanol formation but additional genes that code for alcohol forming enzymes (*adh E2, bdhA, bdhB*).

During solventogenesis, butyrate kinase (BK) is important in solvent production as compared with other enzymes such as acetaldehyde dehydrogenase and ethanol dehydrogenase (AAD). BK linked two crucial intermediate butyrate (But) and butyryl phosphate (BuP). Besides, BK coupled phosphate butyltransferase (PTB) to generate butyrate as well as catalyzing its reassimilation. Figure 2.4 demonstrates the BK, CoAT and AK's influences on butanol production. Butyrate reassimilation has more influence on butanol production than that of acetate reassimilation. BK activity's impact is greater than that of acetate kinase (AK). Unlike the positive correlation of BK for butanol production, CoAT has negative correlation with butanol production, because both BK and CoAT accept butyrate as substrate. However, BK has more efficiency than CoAT during solventogenesis and butyrate reassimilation relies on BK rather than CoAT. Therefore, BK plays the role in acid (butyrate) reassimilation efficiency, which enhances butanol production.

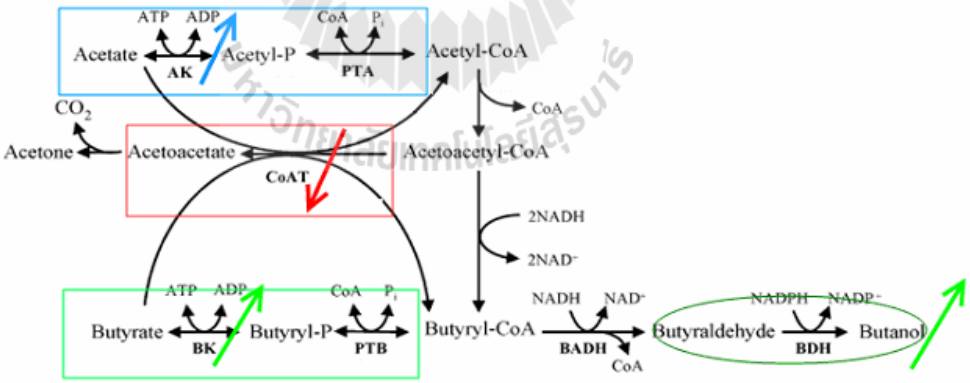


Figure 2.4 Illustration of BK, CoAT and AK's influences on butanol production. BK's influence is positive correlation with butanol production shown by the green arrow. CoAT's influence is negative correlation shown by the red arrow. AK's influence is positive correlation shown by the blue arrow, indicating smaller magnitude than BK (Source: Li *et al.*, 2010).

Some study reported that butyryl phosphate (BuP) is a key metabolite for butanol production, even the detailed mechanism of how BuP acts to regulate ABE process has not been very clear yet (Li *et al.*, 2010). BuP is the intermediate between butyryl-CoA (BCoA) and butyrate (But). Many important genes involving solventogenic enzymes include *adhE1, adhE2, ctfA, ctfB, adc, bdhA, bdhB*, etc. These gene are strictly correlated with BuP kinetics (Zhao *et al.*, 2005). The enzymes are regulated by complex factors (e.g. transcription control) to exhibit different activities to fulfill conditional requirements of different periods. Enzyme activity levels are constant by default and they are only regulated by substrates/products. In solventogenic phase, the acidogenic enzymes shut-down results in the glucose sufficiency and inactivation of solventogenic enzymes at the beginning of acidogenesis. The regulatory effects of complex factors can be described by using a time division pattern, according to enzyme activity variations. The 2-phase mode of ABE process is: 1) acids are vigorously generated during the earlier phase, and 2) solvents are produced later. Therefore, BK-PTB path generates ATP for bacteria growth during acidogenesis, while the bacteria do not need to grow in solventogenesis where there is surplus ATP. These surplus ATPs are utilized to proceed butyrate reassimilation. It is noteworthy that acids are severely poisonous to bacteria cells and it is a priority for the bacteria to convert acids to other forms. In addition, enhanced butanol production means more acids are converted. Therefore acid reassimilation is the reason why the bacteria prefers path BK-PTB at the expense of ATP, therefore, BK is efficient for responding to severe poison stress and the energetic basis for this process is the ATP surplus generated during acidogenesis.

The enzymes of the solventogenesis pathway are regulated by genes locating in both genome and plasmid of *Cl. acetobutylicum*. The genome of *Cl. acetobutylicum* ATCC 824 consists of a 3.94-Mb chromosome with 11 ribosomal operons and a 210 Kb megaplasmid, named pSOL1 (Nölling *et al.,* 2001). Figure 2.5 shows the *sol* operon locus on the pSOL1 megaplasmid of *Cl. acetobutylicum*. The megaplasmid carries the *sol* operon containing *adh E/aad, aad, ctf, adc* genes but *bdh* gene locating on chromosome. Loss of the megaplasmid leads to the total abolition of solvent production. Furthermore, the continuous culture being operated for a long time leads to the loss of the solvent formation capability of strain.

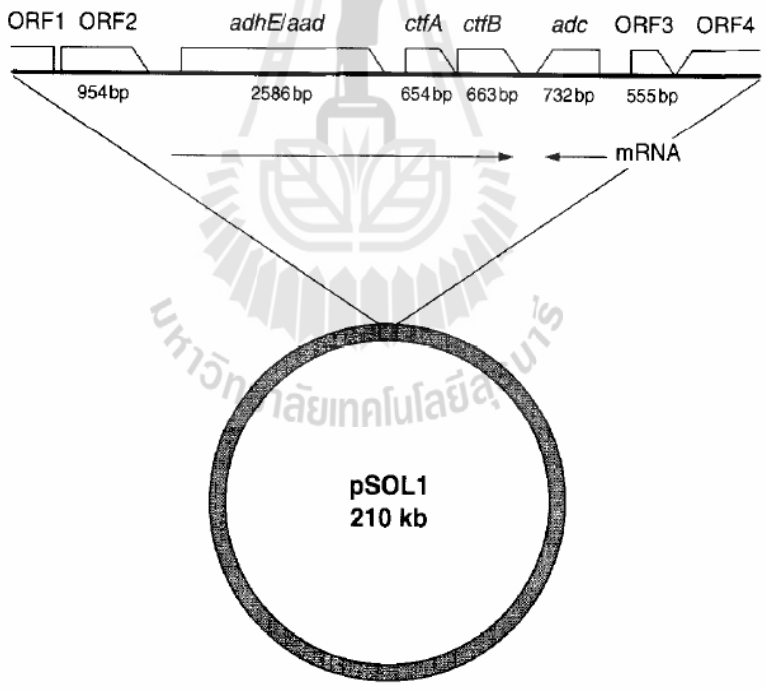


Figure 2.5 The *sol* operon locus on the pSOL1 megaplasmid. The functions of ORF1 and ORF3 are unknown; ORF2 regulates *adhE/aad, ctf*A and B, and *adc*; and ORF4 codes for a putative α-amylase (Girbal and Soucaille, 1998).

For alcohol formation (butanol and ethanol), acetyl-CoA and butyryl-CoA are converted to ethanol and butanol by aldehyde dehydrogenase and alcohol dehydrogenase, respectively. The *aad* gene in the megaplasmid of *Cl. acetobutylicum* ATCC 824 is part of the *sol* operon and encodes a bifunctional alcohol/aldehyde dehydrogenase, such as ethanol dehydrogenase, and butanol dehydrogenase. These enzymes catalyzes the two-step conversion of butyryl-CoA to butanol or of acetyl-CoA to ethanol (Nair *et al.*, 1994). The alcohol/aldehyde dehydrogenase is critical in alcohol production (ethanol and butanol) (Girbal and Soucaille, 1998). The inactivation of *aad* gene decreases butanol formation. Acetyl-CoA is converted to ethanol by acetaldehyde dehydrogenase and ethanol dehydrogenase. For butanol production, acetyl-CoA is converted to butanol by butyraldehyde dehydrogenase and butanol dehydrogenase. The two NADH-dependent butanol dehydrogenases are BDH I (*bdh*A gene) and BDH II (*bdh*A gene) are located the *Cl. acetobutylicum* chromosome.

2.7 Regulation of electron flow in ABE fermentation

In glycolysis, *Cl. acetobutylicum* generates less ATP and more NAD(P)H, which are required for biosynthesis and growth in batch culture. At the optimal condition (pH 6.8), a more efficient consumption of glucose occurred resulting in an increase in the acetate production during acidogenesis during exponential phase. The production of acetic acid generates the additional ATP, but not consumes the NAD(P)H. On the other hand, the production of butyric acid consumed the NADH, which is produced during glycolysis. Besides the net amount of ATP generated during the formation of acetate and butyrate differs. The net yield of ATP obtained from acetic acid production is double that these of obtained from butyric acid production (Thauer *et al.*,

1977). The production of acetate by the cell results in a net excess generation of NADH. Importantly, growing cells must act to keep the continued operation of glycolysis to metabolite substrate for the generation of energy. Additionally, the performance of glycolysis process does require a sufficient supply of NAD+ to accept electrons which obtain from glycolysis. Thus, only a portion of excess NADH must be oxidized to regenerate the NAD+ by hydrogen production. The process of hydrogen production is achieved by the action of NADH ferredoxin oxidoreductase, which transfers electrons or reducing equivalents from NADH to generate reduced ferredoxin. Then, the reduced ferredoxin in turn is reoxidized by the reversible hydrogenase using protons as terminal electron acceptors for hydrogen production. Thus, the increase in the production of acetate is accompanied by the increase in the production of hydrogen and also in the activity of NADH ferredoxin oxidoreductase. It concludes that during the acidogenic phase, a major proportion of the electron flow is directed to hydrogen production, while the carbon flow is mainly directed to acid production, resulting in the maximum generation of energy. The cells of solvent producing clostridria would also dispose only a portion of the excess reducing equivalents by production of hydrogen gas. The reason is that the concentration of hydrogen is apparently kept sufficiently low to allow the redox potential of the H^+/H_2 couple to be raised under the growth conditions.

In regulation of direction of electron and carbon flow through the branched acid-producing pathways during acidogenesis, NADH oxidoreductase and hydrogenase play a key important role. The ratio of acetate/butyrate produced is directly regulated by the activity of these complex enzymes. In case an increase of high hydrogen pressure, the complex enzymes are inhibited, excess NADH must be

utilized in the production of butyrate to maintain the correct redox balance (Crabbendam *et al.*, 1985).

In the solventogenesis, the shift from acid-producing metabolism to solvent-producing metabolism occurs at midway in the ABE fermentation. The shift is accompanied by a change in the ratios of hydrogen and CO_2, which generated during acidogenesis. A decrease in hydrogen production and an increase in CO_2 production control the direction of the carbon and electron flow in the cell from the acid production to the solvent production. Commonly during acidogenesis, hydrogen activity and hydrogen production are high in parallel with increasing growth rate and increasing glucose consumption during the initial growth stage. Then, when the decrease of the growth rate of culture and the reduction of metabolic activity result in the decrease in the rate of hydrogen product, however, the specific hydrogenase activity associated with the whole cell did not alter. The hydrogen production decreases continually when the shift to solvent production causing the decrease of hydrogen production rate and hydrogenase activity. Thus, the decrease in hydrogen production in the solventogenic phase was due to the regulation of hydrogenase production in the stationary phase of cell growth.

2.8 Composition of lignocellulosic material

Lignocellulosic or cellulosic biomass is typically plant materials. Lignocellulose materials can obtained from various resources such as agricultural residues (e.g. corn stove and wheat straw), agricultural wastes (e.g. stover, sugarcane baggasse), wood residues from many wood industries (e.g. sawmill and paper mill discards), woody (e.g. poplar trees, eucalyptus tree) dedicated energy crops (e.g. switchgrass), and municipal paper waste (e.g. newspaper) (Bayer, 2007). The

lignocellulosic biomass composes of cellulose (35-50%), hemicelluloses (25-30%), lignin (20-25%), pectin, extractives (i.e. proteins, terpenic oils, fatty acids/esters and inorganic materials, mainly based on N, P and K), and ashes (Mabee *et al.*, 2006). However the ratio between various constituents in biomass depends on the plant species, age, stage of growth and growth conditions (Table 2.5). A mixture of cellulose and hemicelluose, as called holocellulose, is the total carbohydrate polymers. Holocellulose can be used as a carbon substrate for cellulolytic microorganisms, which convert insoluble cellulosic substrates to soluble sugars. These sugars can be subsequently converted to valuable industrial chemicals and biofuels by the microbial fermentation. The chemical compositions of the various lignocellulosic materials are given in Table 2.6. Hence, lignocellulosic substrates are attractive because of the high glucose content, foreseeable source of fuel, low cost, and plentiful supply.

2.8.1 Cellulose

Cellulose is the structural component of the primary cell wall of green plants, many forms of algae, oomycetes, some animals (e.g., tunicates) and a few bacteria (Lynd *et al.*, 1999). However, the cell wall structure of plant taxa contains high cellulose content comparing with that of anothers. Cellulose $(C_6H_{10}O_5)_n$ is a polysaccharide comprising of linear chain polymers of D-glucopyranose units. The glucose units are joined together by $\beta(1\rightarrow4)$ glycosidic bond. The average degree of polymerization (DP) of plant cellulose varies between 7,000 and 15,000 glucose units, depending on the plant species or cellulose sources. The repeating unit of the cellulose chain is the cellobiose unit.

Table 2.5 Main compositions in some lignocellulosic biomass (percent of dry weight) (Sun and Cheng, 2002; Nigam *et al.*, 2009).

Lignocellulose materials	Cellulose	Hemicellulose	Lignin
Barley straw	33.8	21.9	13.8
Corn cobs	33.7	31.9	6.1
Corn stalks	35.0	16.8	7.0
Cotton stalks	58.5	14.4	21.5
Oat straw	39.4	27.1	17.5
Rice straw	36.2	19.0	9.9
Rye straw	37.6	30.5	19.0
Soya stalks	34.5	24.8	19.8
Sugarcane bagasse	40.0	27.0	10.0
Sunflower stalks	42.1	29.7	13.4
Wheat straw	32.9	24.0	8.9
Coastal bermudagrass	25	35.7	6.4
Corn cobs	45	35.0	15
Cotton seed hairs	80-90	5-20	0
Grasses	25-40	35-50	10-30
Hardwoods steam	40-55	24-40	18-25
Leaves	15-20	80-85	0
Newspaper	40-55	25-40	18-30
Nutshells	25-30	25-30	30-40
Paper	85-99	0	0-15
Primary wastewater solids	8-15	NA	24-29
Softwoods stems	45-50	25-35	25-35
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Sorted refuse	60	20	20
Swine waste	6.0	28	NA
Switchgrass	45	31.4	12
Waste papers from chemical pulps	60-70	10-20	5-10

NA, not available.

Table 2.6 Chemical compositions of different lignocellulosic biomass (percent of dry weight) (Bak *et al.,* 2009; Mussanto *et al.,* 2010).

Lignocellulos	Cellulos	Xyla	Manna	Galacta	Arabina	Ligni	Extracti
Spruce wood	41.9	6.1	14.3	NA	1.2	27.1	9.6
Pine wood	37.7	4.6	7.0	NA	NA	27.5	10.8
Birch wood	38.2	18.5	1.2	NA	NA	22.8	4.8
Poplar wood	49.9	17.4	4.7	1.2	1.8	18.1	NA
Corn stover	36.4	18.0	0.6	1.0	3.0	16.6	7.3
Wheat straw	38.2	21.2	0.3	0.7	2.5	23.4	13.0
Swichgrass	31.0	20.4	0.3	0.9	2.8	17.6	17.0
Rice straw	35.7	10.8	3.7	2.5	3.3	19.7	NA

NA, not available.

The cellulose chain is stablilized by strong hydrogen bonds along the direction of the chain due to the linearity and the existing hydrogroup at the ends of the cellulose chains. These hydroxyl groups are able to interact with each other intrachain and interchain by forming hydrogen bonds (Figure 2.6). In fact, H-bonds do not only exist between OH-groups of cellulose, but they also are in water molecules. So, these hydroxyl groups make the surface of cellulose largely hydrophobic. In addition, the formation of the hydrogen bonds in β-sheet in plant cell wall causes the cellulose chains arrange in parallel to form a crystalline structure that is far more difficult to depolymerize than the α-bonds in amorphous starch. The multiple hydroxyl groups on the glucose residues from one cellulose chain of hydrogen bonds with the oxygen molecules on the same or on a neighbor chain holds the chains firmly together side-by-side with weak intersheet van der Waals forces and forming microfibrils that makes a recalcitrant compact structure. The microfibrils are group of (about 30) individual cellulose chains, and approximately 100 microfibrils are packed

to form fibrils and these fibrils are further packed to form the cellulose fiber (Brown and Saxena, 2000) (Figure 2.7). The cellulose microfibrils have both highly crystalline regions (around 2/3 of the total cellulose) and less-ordered amorphous regions. More ordered or crystalline cellulose is less soluble and less degradable (Zhang and Lynd, 2004; Taherzadeh and Karimi, 2008). The crystalline nature of cellulose implies a structural order in which all of the atoms are fixed in discrete positions with respect to one another. An important feature of the crystalline array is that the component molecules of individual microfibrils are packed tightly to prevent penetration not only by enzymes but even by small molecules such as water (Zhang *et al.*, 2006; Kumar *et al.*, 2008).

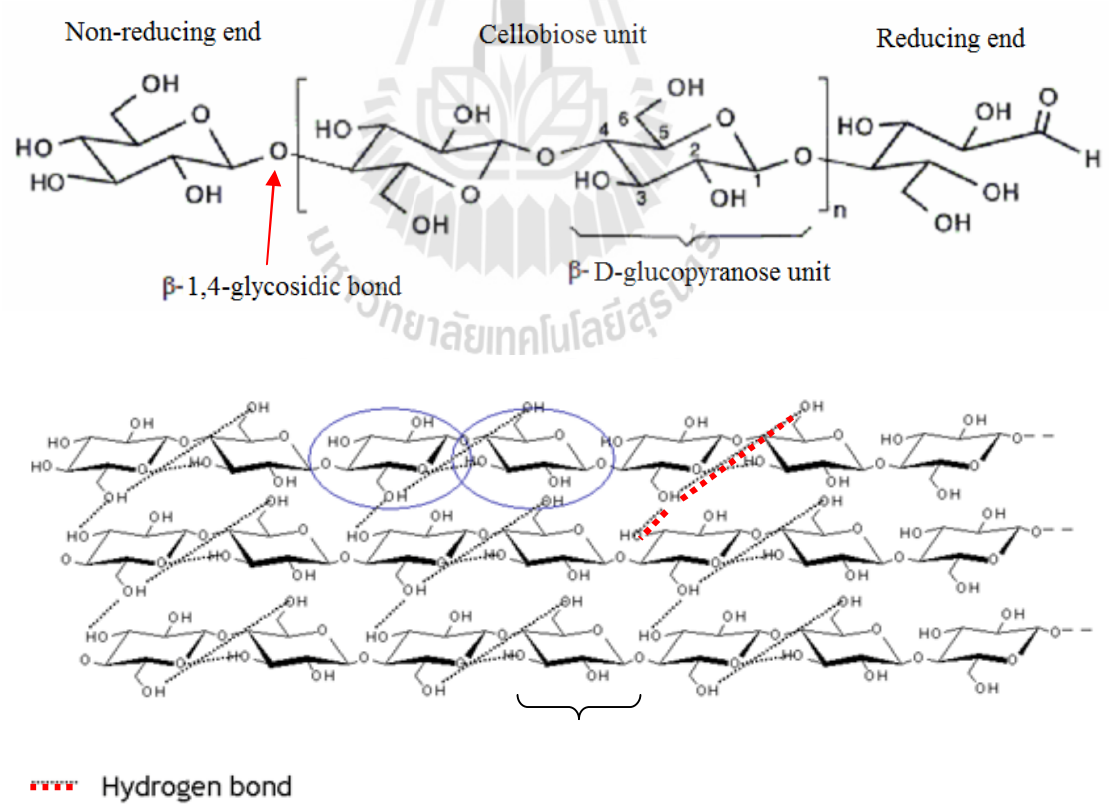


Figure 2.6 Chemical structure of cellulose chains.

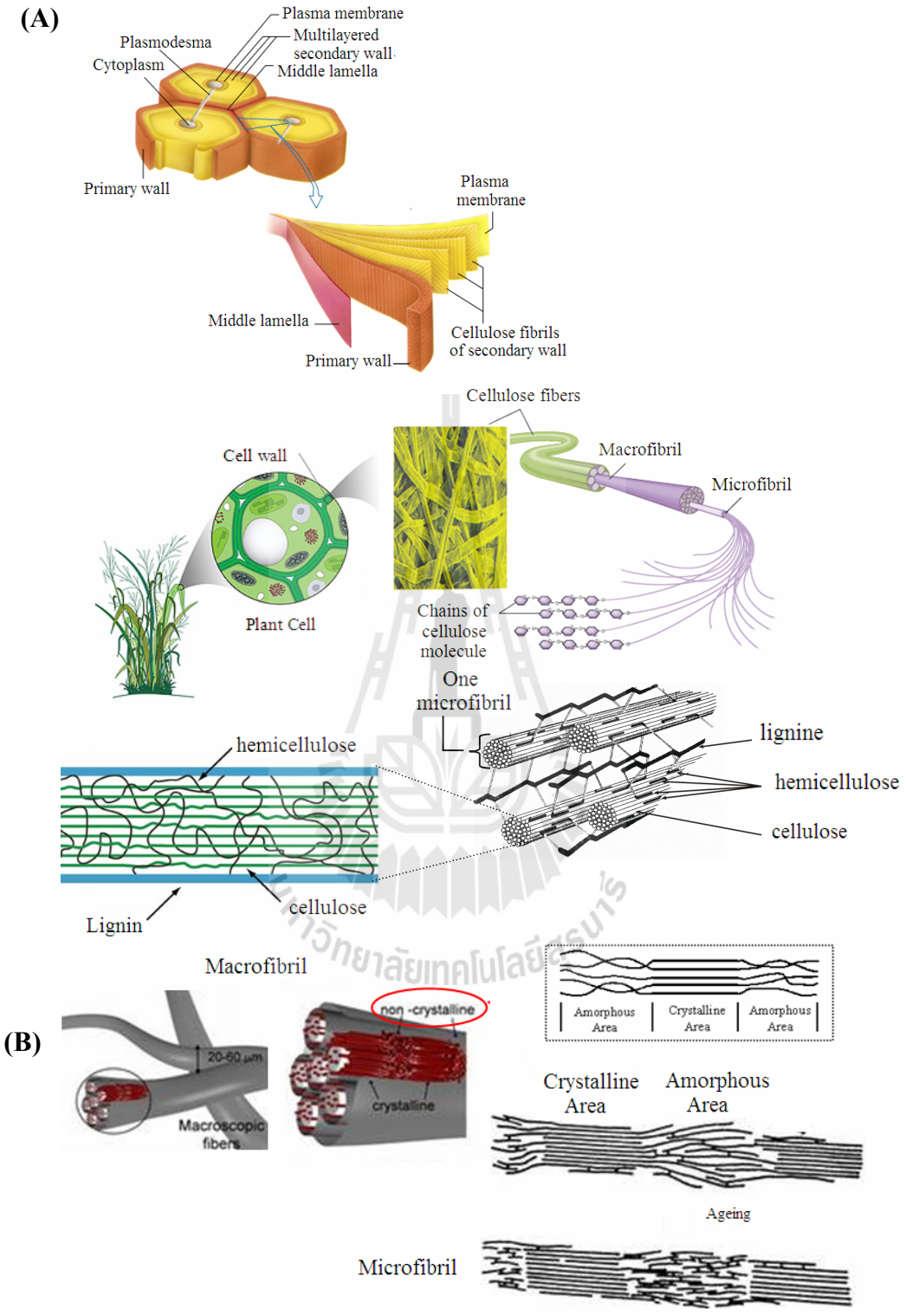


Figure 2.7 Cellulose structure (A) components of plant biomass; and (B) crystalline and amorphous structures of cellulose (Adapted from Fengel and Wegner, 1989; Hsu *et al.,* 1980).

In addition to the crystalline and amorphous areas, cellulose fibers compose with various types of irregularities, such as twists of the microfibrils, surface micropores, large pits, and capillaries (Blouin *et al.*, 1970; Cowling, 1975; Fan *et al.*, 1980). Therefore, the total surface area of a cellulose fiber is much greater than the surface area of an ideally smooth fiber of the same dimension. The net effect of structural heterogeneity within the fiber is that the fibers are at least partially hydrated by water when immersed in aqueous media, and some micropores and capillaries are sufficiently spacious to permit penetration by relatively large molecules including, in some cases, cellulolytic enzymes (Stone and Scallan, 1968; Stone *et al.*, 1969).

Purified celluloses used for studies of hydrolysis and microbial utilization . The commercial microcrystalline cellulose shows in Table 2.7. Crystalline of Avicel and Sigmacel are nearly pure cellulose due to removal of hemicellulose with the dilution acid treatment. These commercial microcrystalline celluloses are different in particle size distribution, which was significant for the rate of hydrolysis and utilization. Bacteria also certain cellulose called bacterial cellulose. It is different from plant cellulose for the arrangement of glucosyl units within the cellulose unit (Atalla *et al.*, 1993).

Table 2.7 The commercial cellulose.

Name	% Crystalline cellulose	Property	Source
BC (Bacterial cellulose)	76-92%	Highly crystalline cellulose	Bacterial
BMCC (Bacterial Micro-crystalline Cellulose)			
Avicel (Sigma)	47%	Microcrystalline cellulose	Plant cell
CMC (Carboxymethyl cellulose)		High soluble cellulose	

2.8.2 Hemicellulose

Hemicellulose is consisted of both linear and branched heteropolymers of D-xylose, L-arabinose, D-mannose, D-glucose, and D-galactose as well as other components such as acetic, glucuronic, and ferulic acids. The several different polymers in hemicelluloses include xylans, mannans, galactans, glucans, glucuronoxylans, arabinoxylans, glucomannans, galactomannans, galactogluco-mannans, β-glucan, and xyloglucans. Typically, hemicellulose in hardwoods and annual plants are mainly xylan, whereas hemicellulose in softwood is composed of galactoglucomannans and xylan. The largest amount of xylose is always the sugar monomer present in hemicellulose, but mannose and galacturonic acids also tend to be present. In addition to hemicellulose structure, hemicellulose consists of shorter chains about 500-3,000 sugar units as opposed to 7,000-15,000 glucose molecules per polymer seen in cellulose. Hemicelluloses are embedded in the cell walls of plants, sometimes in chains that form a ground. They bind with pectin to cellulose to form a network of cross-linked fibres. Unlike cellulose, hemicellulose is amorphous, which makes it more easily hydrolyzed than cellulose (Fengel *et al.*, 1989). Compared with cellulose, hemicelluloses differ in composition of sugar units, presence of shorter chains, and a branching of main chain molecules in amorphous form.

2.8.3 Lignin

Lignin is a complex, hydrophobic, and cross-linked aromatic polymer. In nature, lignin is mostly found as an integral part of the plant cell wall, especially in tracheids, xylem fibres and sclereids. Lignin is embedded in a polymer matrix of cellulose, hemicelluloses, and pectin components. Lignins are polymers of phenylpropene units: guaiacy units, syringyl units, and p-hydroxypheny units. In addition, lignins are divided into two major categories: guaiacyl lignins and guaiacyl-

syringyl lingnin in hardwood and grass lignins (Gibbs, 1958). The softwood is more resistant to lignin removal by alkaline extraction than hardwood (Ramos *et al.*, 1992). It has been suggested that guaiacyl lignin restricts fiber swelling and thus the enzymatic accessibility is more than that in syringyl lignin. Lignin consists of amorphous regions and structured forms such as oblong particles and globules (Novikova *et al.,* 2002). The hydroxyl and methoxyl groups in lignin precursors and oligomers may interact with cellulose microfibrils despite hydrophobicity of lignin (Houtmanand and Atalla, 1995). The functional groups affecting the reactivity of lignin include free phenolic hydroxyl, methoxyl, benzylic hydroxyl, benzyl alcohol, noncyclic benzyl ether and carbonyl groups. Lignin can be regarded as a group of amorphous, high molecular-weight, chemically related compounds. Function of lignin is to provide rigidity and cohesion to the material cell wall, to confer water impermeability to xylem vessels, and to form a physical–chemical barrier against fungal or pathogen attack (Fengel and Wegener, 1989). Due to its molecular configuration, lignins are extremely resistant to chemical and enzymatic degradation by nonspecific adsorption of lignin-degrading and cellulolytic enzymes and enzyme inaccessibility (Palmqvist and Hahn-Hagerdal, 2000a, b). Thus increasing removal of lignins greatly enhances the hydrolysis of cellulolose.

2.8.4 Pectin

In plant cells, pectin consists of a complex set of polysaccharides. It presents in the most primary cell walls, middle lamella and particularly abundant in the non-woody parts of terrestrial plants. Pectin penetrates throughout primary cell walls and the middle lamella between plant cells. Thus it helps to bind cells together. The amount, structure and chemical composition of the pectin differs between plants, although the different parts of a plant. In the ripen fruit, pectin is easily to be broken

down by the pectinase and pectinesterase enzymes. It is also found in the abscission zone of the petioles of plant causing leaf fall. The sugars are mainly D-galactose, L-arabinose, and D-xylose, depending on the types and proportions of neutral sugars varying with the origin of pectin.

2.9 Pretreatment methods

Approximately 90% of the dry weight of most plant materials are stored in the form of cellulose, hemicelluloses, lignin, and pectin. A lignin layer is a protective barrier. The cellulose and hemicellulose are also densely packed and embedded in the lignin layer, illustrated in Figure 2.8. This structural robustness of lignocellulosic feedstocks is resistant to degradation by fermenting microbes and enzymatic hydrolysis. Thus, pretreatment is a necessary upstream process in order to render the recalcitrant cellulose fraction of the biomass more accessible to hydrolytic enzymes by altering its structure. Goal of pretreatment is to remove lignin and hemicellulose, to reduce cellulose crystallinity and to increase porosity of biomass prior to the enzymatic process (Kumar *et al.*, 2009). The pretreatment is a critical step in the cellulosic fuels and chemical technology affecting the quality and the cost of the carbohydrates in form of cellulose (Balat *et al.*, 2008).

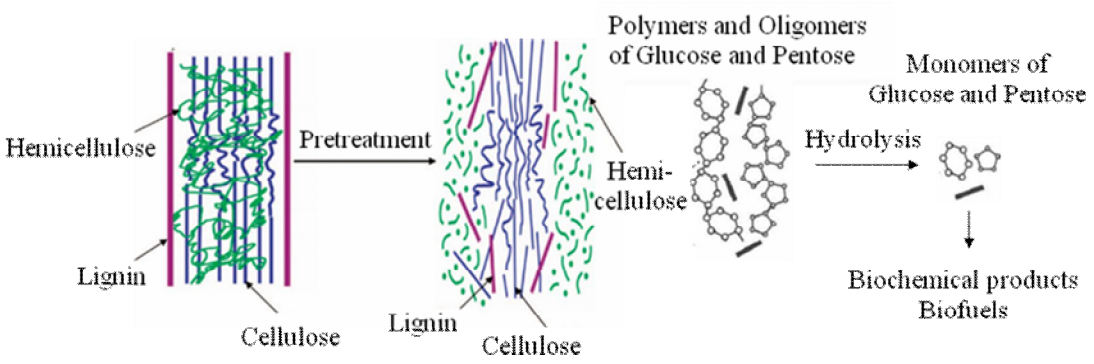


Figure 2.8 A schematic of the overall goals of the pretreatment on the lignocellulosic materials and the conversion of the lignocellulosic biomass to high-value industrial products (Adapted from Hsu *et al.*, 1980).

A wide range of pretreatment methods have been developed for the various biomass feedstocks. The pretreatment methods can be categorized into physical, chemical, biological properties (Sun and Cheng, 2002; Chandra *et al.*, 2007; Kumar *et al.*, 2009). Table 2.8 classifies the pretreatment methods for structural alteration of lignocellulosic materials. The main physical pretreatment methods are mechanically comminution or reducing the particle size, pyrolysis and steam explosion in order to increase the surface area of cellulose and break down the cellulose crystallinity (Shafizadeh and Stevenson, 1982; Bernado *et al.*, 2011). The physical pretreatment commonly combined with heating and adding chemicals to enhance the effective enzymatic degradation of cellulose into glucose. Some reports proposed irrigation by microwaves, gamma rays, and electron beam to cleave the β-1,4-glycosidic bonds in cellulose chain. These methods provided the large surface area and the lower crystallinity (Hetal, 2006; Li *et al.*, 2012).

Chemical pretreatments are adding solvents or chemicals to remove lignin and hemicelluloses for the increased digestibility of pretreated materials. The main chemical pretreatment methods include ozonolysis; acid hydrolysis; alkaline hydrolysis; oxidative delignification; solvent extraction (Hsu *et al.*, 2010; Sarkar and Aikat, 2012; Sathitsuksanoh *et al.*, 2012; Ndukwe *et al.*, 2013; Travaini *et al.*, 2013). Although chemical method is effective pretreatment procedure, it requires more energy and chemicals than biological process and may cause secondary pollution problems (Sivers and Zacchi, 1995). The effect of chemical procedures also depends upon the lignin content of the materials (Fan *et al.*, 1987; McMillan, 1994).

Table 2.8 The overview effects of pretreatment methods on alteration of chemical and/or physical structure of lignocellulosic materials (Kumar *et al.*, 2009).

		Operating condition	Advantages	Disadvantages
Physical	Chipping Grinding Milling	Room temperature Energy input < 30Kw per ton biomass	Reduces cellulose critallinity	Power consumption higher than inherent biomass energy
Physio-chemical	Steam pretreatment	160-260ºC (0.69-4.83 MPa) for 5-15 min.	Causes hemicelluloses auto hydrolysis and lignin transformation; cost-effective for hardwoods and agricultureal residues	Destruction of a portion of the xylan fraction; incomplete distruption of the lignin-carbohydrate matrix; generation of inhibitory compounds; less effective for softwoods

Table 2.8 The overview effects of pretreatment methods on alteration of chemical and/or physical structure of lignocellulosic materials (Kumar *et al.*, 2009) (continued).

	Operating condition	Advantages	Disadvantages
AFEX (Ammonia fiber explosion method)	90ºC for 30 min. 1-2 kg ammonia/kg dry biomass	Increases accessible surface area, removes lignin and hemicelluloses	Do not modify lignin neither hydrolyzes hemicelluloses
ARP (Ammonia recycle percolation method)	150-170ºC for 14 min Fluid velocity 1 cm/min.	Increases accessible surface area, removes lignin and hemicelluloses	Do not modify lignin neither hydrolyzes hemicelluloses
CO2 explosion	4 kg CO2/kg fiber at 5.62 Mpa 160 bar for 90 min at 50ºC under supercritical carbon dioxide	Not produce inhibitors for downstream process,. Increases accessible surface area, does not cause formation fo inhibotry compounds.	It is not suitable for biomass with high lignin content (such as woods and nut shells). Does not modify lignin neither hydrolyze hemicellulolse.
Ozonolysis	Room temperature	Reduce lignin content; does not produce toxic residues.	Expensive for the ozone required.
Wet oxidation	148-200ºC for 30 min.	Efficient removal of lignin; low formation of inhibitor of inhibitors; low energy	High cost of oxygen and alkaline catalyst.

Table 2.8 The overview effects of pretreatment methods on alteration of chemical and/or physical structure of lignocellulosic materials (Kumar *et al.*, 2009) (continued).

		Operating condition	Advantages	Disadvantages
	Acid hydrolysis: dilute-acid pretreatment	Type I: T>160ºC, continuous-flow process for low solid loading 5-10%) Type II: T<º, batch process for high solid loadings (10-40%).	Hydrolyzes hemicellulose to xylose and other sugar; alters lignin structure.	Equipment corrosion; formation of toxic substances.
	Alkaline hydrolysis	Low temperature; Long time high, concentration of the base; for soybean straw; ammonia liquor (10%) for 24 h at room temperature.	Removes hemicelluloses and lignin; increases accessible surface area.	Residual salts in biomass.
	Organosolv	150-200ºC with or without addition of catalysts (oxalic, salicylic, acetylsalicylic acid).	Hydrolyzes lignin and hemicelluloses.	High costs due to the solvent recovery.
Biological		Several fungi (brown-, white-, and soft-rot fungi)	Degrades lignin and hemicelluloses; low energy requirements.	Slow hydrolysis rate

In particular, the physical and chemical processes require expensive instruments or equipments and require high energy for biomass conversion. On the other hand, process of biological pretreatment is capable of not only degrading the extensive lignin but also environmentally friendly and energy saving due to performance at low temperature and no need to use any chemicals. The main biological pretreatment involves utilizing microorganisms to attack lignin, hemicelluloses, and cellulose through the action of lignin-degrading enzymes (i.e. peroxidases and laccase) and cellulolytic enzymes (Boominathan and Reddy, 1992; Sun and Cheng, 2002; Taniguchi *et al.*, 2005). Many effective microorganisms are used for the biological pretreatment including fungi (white- and soft-rot fungi: *Pleurotus ostreatus*, *Phanerochaete sordida*, *Pycnoporus cinnabarinus*, *Trichroderma reesei*, *Aspergillus niger* etc.) and bacteria (*Bacillus macerans*, *Cellulomonas cartae*, *C. uda* etc.) (Hatakka, 1983; Okano *et al.*, 2005; Ballesteros *et al.*, 2006; Singh *et al.*, 2008; Zhang and Cai, 2008; Mohan *et al.*, 2012). These microbes are able to rapidly adapt their metabolism to varying carbon and nitrogen sources through the production of a large set of intra- and extra-cellular enzymes that are able to degrade the complex lignocellulosic materials (Saratale *et al.*, 2007). Although the biological pretreatment possess many advantages, as described, it has a drawback including consumption of the hydrolyzed products (such as reducing sugars) during cell growth. To overcome these problems, some studies suggested a two-stage process using mixed or pure microbial culture for hydrolysis and the subsequent fermentative bioenergy production (Lo *et al.*, 2009; Saratale *et al.*, 2010).

Pretreatment of biomass both prior operation (e.g., lignocellulose particle size reduction) and subsequent operations (e.g., enzymatic hydrolysis and fermentation) currently represents a critical and expensive step for production of sugars from

biomass. In addition, several pretreatment processes for lignocellulosic biomass have their advantages and disadvantages. Importantly, it must be emphasized that it is not always possible to use just one type of pretreatment method for biomass conversion with all types of feedstock. Thus, handling of the feedstock for the biochemical conversion of lignocellulosic materials must consider the features of an effective pre-treatment strategy including 1) breaking the lignocellulosic complex; 2) producing highly digestible solids that enhances sugar yields during enzymatic hydrolysis; 3) avoiding the degradation of hemicellulose sugars; 4) minimizing the formation of fermentation inhibitory compounds released from degradation of products during hydrolysis method; 5) minimizing both energy inputs and use of extraneous chemicals; 6) requiring a more specificity of substrate; 7) generating high value lignin co-products; 8) minimizing the production of toxic and hazardous wastes; and 9) generating minimum amount of waste water and cost effective treatment (Kumar *et al.*, 2008; Saratale *et al.*, 2008; Zhang *et al.*, 2009).

2.10 Fermentation inhibitors

One commonly observed obstacle for acid pretreatment of lignocellulosic materials is the generation of toxic inhibitory compounds as byproducts, as shown in Figure 2.9. The inhibitors are a major factor limiting the bioconversion of lignocellulosic biomass to industrial chemical products including biofuels. Toxicity of inhibitors causes low rate of sugar uptake with simultaneous decay in product formation, low productivity of desired product, and killing of fermenting microorganisms.

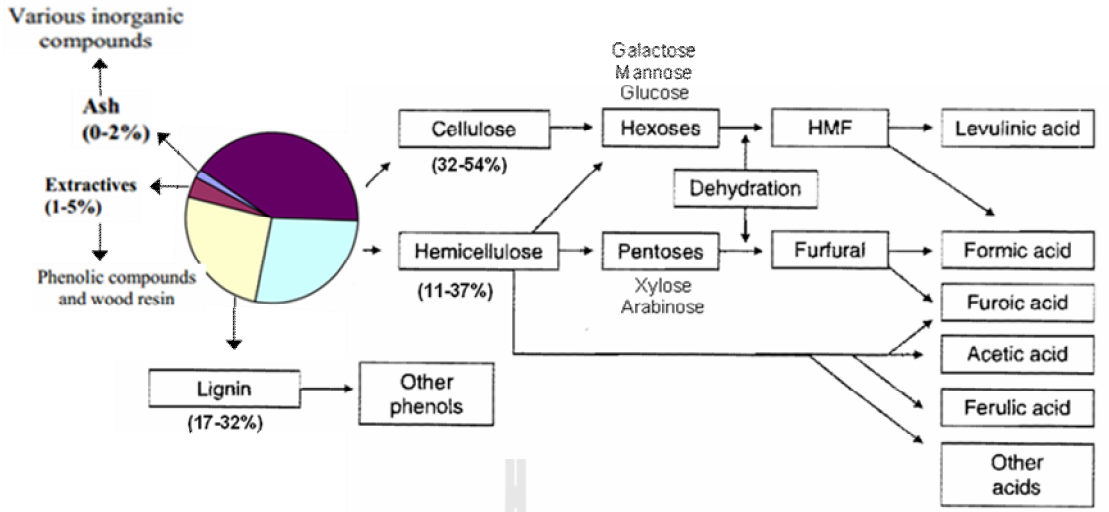


Figure 2.9 Composition of lignocellulosic biomass and their potential degraded products (Taherzadeh and Karimi,2007; Liu and Blaschek, 2010).

Type, toxicity, and concentration of the inhibitors presented in the hydrolysates depend upon types of biomass and conditions of acid hydrolysis. Table 2.9 displayes inhibitor profiles from different lignocellulosic materials. The toxic inhibitors are produced mainly by dehydration of sugars and degradation of lignin fractions during lignocellulosic hydrolysis. Several inhibitors are divided into four groups: (1) sugar degradation products, (2) substances from the hemicellulose hydrolysis, (3) lignin degradation products, and (4) metals from corrosion of equipment (Olsson and Hahn-Hägerdal 1996).

Table 2.9 Inhibitor profiles from different lignocellulosic materials (Chandel *et al.*, 2007).

Linocellulosic material	Inhibitor profile (g/L)				
	Furans	**HMF**	**Phenolics**	**Acetic acid**	**The others**
Sugarcane bagasse	1.89	-	2.75	5.45	-
Wheat straw	0.15	-	-	2.7	-
Rice straw	0.25	0.15	-	1.43	-
Corn stover	0.56	-	0.08	1.48	-
Spruce	1	3.3	0.44	5	Levulinic acid, 0.2 Formic acid 0.7
Eucalyptus globulus	0.26	0.07	2.23	3.41	-
Saccharum spontaneum	1.54	-	2.01	-	-
Poplar	-	-	-	-	2-furoic acid, 0.3 mg/g; 3,4-HBA, 2.5; Salicylic acid, 56; Syringaldehyde, 6.0; Fureulic acid, 4.7
Soft wood	2.2	-	-	5.3	-

Pentoses and some hexoses liberated from structure of hemicelluloses. Under high temperature and pressure as well as acidic conditions, the dehydration of these sugars leads to the formation of 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural; HMF) (Larsson *et al.*, 1999; Lewkowski, 2001). Furfural and HMF are considered to be the representative inhibitors of microbial growth and fermentation (Taherzadeh *et al.*, 2000; Ezeji *et al.*, 2007). Furfural and HMF can be further broken down to levulinic acid, formic acid, and furoic acid. Many reports also revealed that these inhibitory compounds reduced

enzymatic biological activities, damaged DNA, and inhibited protein and RNA synthesis (Sanchez and Bautista, 1988; Khan and Hadi, 1994; Modig *et al.*, 2002).

Apart from the sugars derived from hemicelluose fraction, there are other organic acids as inhibitors including acetic acid, formic acid, and levulinic acid. These weak acids are the most common carboxylic acids, which have been detected in the hydrolysates. Among these acids, acetic acid has been known to be the most prevalent organic acid. Acetic acid was also produced by hydrolysis method and fermentative process. Some report suggested that the effect of the undissociated acetic acid is larger than the effect of the dissociated butyric acid for solventogenic fermentation (Assobhei *et al.*, 1998; Thomas *et al.*, 2002). This was explained that the undissociated weak acids are liposoluble and can diffuse through the cell membrane resulting in a low level of intracellular pH (Huang *et al.*, 1986). A decrease in intracellular pH causes the different pH value (ΔpH) between inner and outer membrane and the collapse of the proton gradient across the membrane and the impairment of the nutrient transport (Russell, 1992; Axe and Bailey, 1995). Cho *et al.* (2012) reported that the solvent production of *Cl. beijerinckii* was affected by the acetic acid concentration of up to 11.7 g/L, however, *Cl. acetobutylicum* was more susceptible to formic acid than *Cl. beijerinckii*.

Lignin is essential for the structural integrity of plant cell walls and considered a first line defense against successful penetration of invasive pathogens. However, phenolic compounds, which are released from lignin dehydration, have negative impacts on biofuel production due to inhibition of microbial growth (McMillan, 1994). The main phenol structure building blocks in lignin are p-hydroxyphenyl, guaiacyl, and syringyl. These phenolic compounds have low water solubility, so the presence of phenolic compounds is a limited degradation of lignin during the

hydrolysis process. The phenolic inhibitors in the hydrolysates have been divided into three forms such as acid, ketone and aldehyde. (Palmqvist and Hahn-Hagerdal, 2000a,b; Mussatto and Roberto, 2004; Parawira and Tekere, 2011). The aldehyde compounds from lignin degradation are more potent inhibitor than those derived from sugar dehydration (Lee *et al.*, 1999). The phenolic compounds disturb the membranes in turn affecting their membrane fluidity and membrane permeability to serve as selective barriers and enzyme matrices, thus, affecting the cell growth and sugar assimilation (Heipieper *et al*., 1994; Palmqvist and Hahn-Hagerdaal, 2000b). Their toxicity is thought to be proportional to the molecular weights. The lower molecular weight of phenolic compounds is generally lethal to the microorganisms than those of higher molecular weight compounds (Clarck and Mackie, 1984).

In general, furans and phenolic aldehydes are more toxic than organic acids (Leonard and Hajny, 1945; McMillan, 1994). In reality, toxic compounds in a hydrolysate have synergistic inhibitive effects over that of the sum of individual toxic effects. However, removal of only the major inhibitors often results in significantly improved microbial growth and fermentation (Tran and Chambers, 1986; Buchert *et at*., 1990; Larsson *et al.*, 1999; Klinke *et at*., 2003).

2.11 Detoxification methods to remove fermentation inhibitors

For economic reasons, dilute acid hydrolysis is commonly used in biomass degradation for hydrolysis of the hemicellulose fraction and increase fiber porosity to allow enzymatic saccharification and fermentation of the cellulose solid fraction (Bothast and Saha, 1997; Larsson *et al.*, 1999; Saha, 2003). In addition, this method generates a mixture of fermentable sugars and inhibitory compounds in liquid fraction, which is referred to hydrolysate. The inhibitory compounds include furan

derivatives (furfural, 5-hydroxymethylfurfural (5-HMF)), lignin derivatives (vanillin, 4-hydroxybenzal-dehyde, guaiacol, and phenol), and aliphatic organic acids (acetic acid, levulinic acid, formic acid, and ferulic acid) (Palmqvist and Hahn-Hagerdal, 2000a, b). These compounds are toxic to the various fermentative microorganisms, to severely inhibit enzymatic hydrolysis, and to affect butanol fermentation performance (Ranatunga *et al.*, 2000; Meinita *et al.*, 2012). It suggested that the inhibitors decrease the butanol productivity and consequently make the butanol production expensive. Therefore, the inhibitors in hydrolysate must be kept at low concentration or eliminated through a detoxification process prior to fermentation (Baek and Kwon, 2007; Ge *et al.*, 2011).

Various detoxification methods such as physical methods (evaporation, membrane separation), chemical methods (neutralization, ammonium hydroxide over-liming, calcium hydroxide over-liming, activated charcoal treatment, ion exchange resins, and extraction with ethyl acetate), and biological methods (enzymatic mediated using laccase, lignin peroxidase; microbial detoxification) are used to remove the inhibitors (Zhang *et al.*, 2010; Gyalai-Korpos *et al.*, 2011; Ge *et al.*, 2011). The method of non-biological detoxification is often employed comparing with another method (Table 2.10). The biological detoxification by microorganisms and/or the enzymes possess many advantages including 1) the potential for alternation of chemical nature of inhibitors in hydrolysates, and 2) the more feasible and environmental friendly method due to fewer side-reactions and less energy requirements (Zhang *et al.*, 2010; Parawira and Tekere, 2011). On the other hand, the biological method has the following disadvantages: 1) slow reaction rate of microbial/ enzymatic detoxification, and 2) loss of fermentable sugars make them less attractive (Yang and Wyman, 2008).

Several researches attempted to improve the methods of non-biological detoxification (Table 2.10). Indeed, it is difficult to compare non-biological detoxifications based on the selection of lignocellulosic hydrolysates and types of specific strain of the fermentative microorganism, which is inherently able to resist the toxicity of inhibitors. Moreover, the type and level of inhibitory compounds presenting in a hydrolysate depend upon the type and condition of pretreatments and biomass materials utilized in the process (Mussatto and Roberto, 2004). However, several researchers have investigated methods to detoxify hydrolysate to improve fermentation efficiency. Each method represents its specificity to eliminate particular inhibitor from the lignocellulosic hydrolysate.

Table 2.10 Different strategies of non-biological detoxification for the removal of inhibitors in lignocellulosic hydrolysates.

Lignocellulose Hydrolysate	Detoxification method	Detoxification condition	Changes in hydrolysate composition	Reference
Corn stove	vacuum evaporation	vacuum evaporation, acid hydrolysate condensation times, BÜCHI Rotavapor R-200, 70ºC and 170 mbar.	Removal of formic acid (59.89%), acetic acid (77.72%)	Zhu *et al.*, 2009
Corn stove	Membrane based organic phase alamine	using two different LiquiCel Membrane Contactors (Membrana, Charlotte, NC); acid hydrolysate; the organic phase containing octanol and Alamine 336; flow the prganic phase rate in the system, 0.07 bar 520).	Removal of acetic acid (60%)	Grzenia *et al.*, 2008
Sugarcane bagasse	anion exchange resin	The hydrolysate, weak anion-exchange resins (Dowex-MWA1, 1 meq ml/L, 16–50 mesh); ratio of the hydrolysates and the resins , 5:1 (v/w); stirring, 1 h, room temperature; adjusting pH to to 5.5 with $Ca(OH)_2$ or H_2SO_4.	Removal of furans (63.4 %), and total phenolics (75.8%).	Chandel *et al.*, 2007

Table 2.10 Different strategies of non-biological detoxification for the removal of inhibitors in lignocellulosic hydrolysates (continued).

Lignocellulose Hydrolysate	Detoxification method	Detoxification condition	Changes in hydrolysate composition	Reference
Corn stove	steam stripping	Steam stripping, acid hydrolysate, heating 100 ºC, 120 min.	Removal of formic acid (58.79%) and acetic acid (80.83%)	Zhu *et al.*, 2009
Sugarcane bagasse	Activated charcoal	The acid hydrolysate, granular activated charcoal ca. 2.5 mm (Merck, Darm-stadt, Germany), ratio of charcoal and hydrolysate, 1:10 (w/v), stirring, 1 h,oom temperature, adjusting pH to 5.5.	Removal of furans and (38.7%), and total phenol-lics (57.5%).	Chandel *et al.*, 2007
Oak wood	Activated charcoal	Hydrolysate, ration of powdered charcoal to hydrolysate, 1:10 (g/g), room temperature, 1 hour, filtration, retreating.	Removal of phenolics (95.40%)	Converti and Dominguez , 1999
Yello poplar	Over-liming, calcium hydroxide ($Ca(OH)_2$)	the hydrolysate; adjusting pH to 10.0-10.5, solid calcium hydroxide; heating, 50°C for 30 min; adjusting pH to 7, 96% H_2SO_4; filtration, storing, 4°C, 5 days.	Removal of acetic acid (99%), HMF (34%), furfural (30%), and pheno-lic compounds (88%)	Ranatunga *et al.*, 2000

Table 2.10 Different strategies of non-biological detoxification for the removal of inhibitors in lignocellulosic hydrolysates (continued).

Lignocellulose Hydrolysate	Detoxification method	Detoxification condition	Changes in hydrolysate composition	Reference
Norway spruce (*Picea abies*)	Over-liming, ammonium hydroxide (NH_4OH)	The dilute-acid hydrolysate; adjusting a increased pH, pH 10, 28% NH_4OH; adjusting a decreased pH, pH 5.5, 37% HCl.	Avoid of gypsum formation, addition of a nitrogen source	Alriksson *et al.*, 2005
Spruce wood	Dithionite and sulfite	Twenty-six milliliter hydrolysate; adjusting pH to 5.5,5 M NaOH;. Adding sodium dithionite, final concentration 10 mM; adding sodium sulfite, final concentration 10 mM, stirring, room temperature, 10	No major change in composition of hydrolysates	Alriksson *et al.*, 2011
Wheat straw	Overliming, $Ca(OH)_2$ +Ion-exchange-D 311	Overlimed hydrolysate, using D311 ion-exchange resin	Remaval of furfurals (90.36%), phenolics (77.44%), and acetic acid (96.29%)	Zhuang *et al.*, 2009
Corn stove	A combination of vacuum evaporation and over-liming	vacuum evaporation, acid hydrolysate condensation times, BÜCHI Rotavapor R-200, 70ºC, 170 mbar; over-liming, solid calcium hydroxide, adjusting pH 11, 60ºC, 120 min,	NA	Zhu et al., 2009

Table 2.10 Different strategies of non-biological detoxification for the removal of inhibitors in lignocellulosic hydrolysates (continued).

Lignocellulose Hydrolysate	Detoxification method	Detoxification condition	Changes in hydrolysate composition	Reference
Corn stove	A combination of steam stripping and overliming	vacuum evaporation, acid hydrolysate condensation times, BÜCHI Rotavapor R-200, 70ºC and 170 mbar. Steam stripping, acid hydrolysate, heating 100 ºC, 120 min.	NA	Zhu *et al.*, 2009

NA, not applicable.

2.12 Enzymatic hydrolysis

The polymersaccharides in the lignocellulosic materials are broken down into simple sugars before fermentation, through a process called hydrolysis. The most commonly applicable method is enzymatic hydrolysis due to several advantages of enzymatic hydrolysis such as high substrate specificity and high hydrolysis efficiency, less energy requirement, performance under mild reaction conditions (pH around 5 and temperature lower than 50ºC), high yield of sugars, and less environmental impact, as well as avoiding any corrosion problems from hydrolysis process (Martin *et al.*, 2002; Wen *et al.*, 2004; Liao *et al.*, 2005). In the bioconversion of lignocelluloses into value chemical feedstock and biofuels, the enzymatic hydrolysis is considered as an economically feasible process (Taherzadeh and Karimi, 2007).

Enzymatic hydrolysis of cellulose is a conversion of lignocellulosic biomass to fermentable sugars by cellulases. The cellulases are typically a mixture of several enzymes that hydrolyze crystalline/amophorous cellulose to fermentable sugars (Duff and Murray, 1996). The individual enzymes in cellulase enzymes involving formation of soluble sugars from holocellulose in agricultural residues include β-endoglucanase (EC3.2.1.4), β–exoglucanase or cellobiohydrolase (EC3.2.1.91.), and β-D-glucosidase (EC3.2.1.21). Figure 2.10 illustrates the function of the three celluase groups. The endoglucanases attacks regions of low crystallinity in the cellulose fiber creating free chain ends. And then, it cleaves intramolecular β-1,4-glucosidic linkages randomly and releases reducing sugars in the reaction mixture. The exoglucanases acts on the accessible ends of cellulose free chains from the endogluconases's action to liberate glucoses, cellobioses, and cellodextrins. The β-D-glucosidases hydrolyze soluble cellobiose and other cellodextrins to produce glucose in the aqueous phase. In addition

to the three main cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996). Table 2.11 exhibits the cellulolytic enzyme involving in the hydrolysis of lignocelluloses materials.

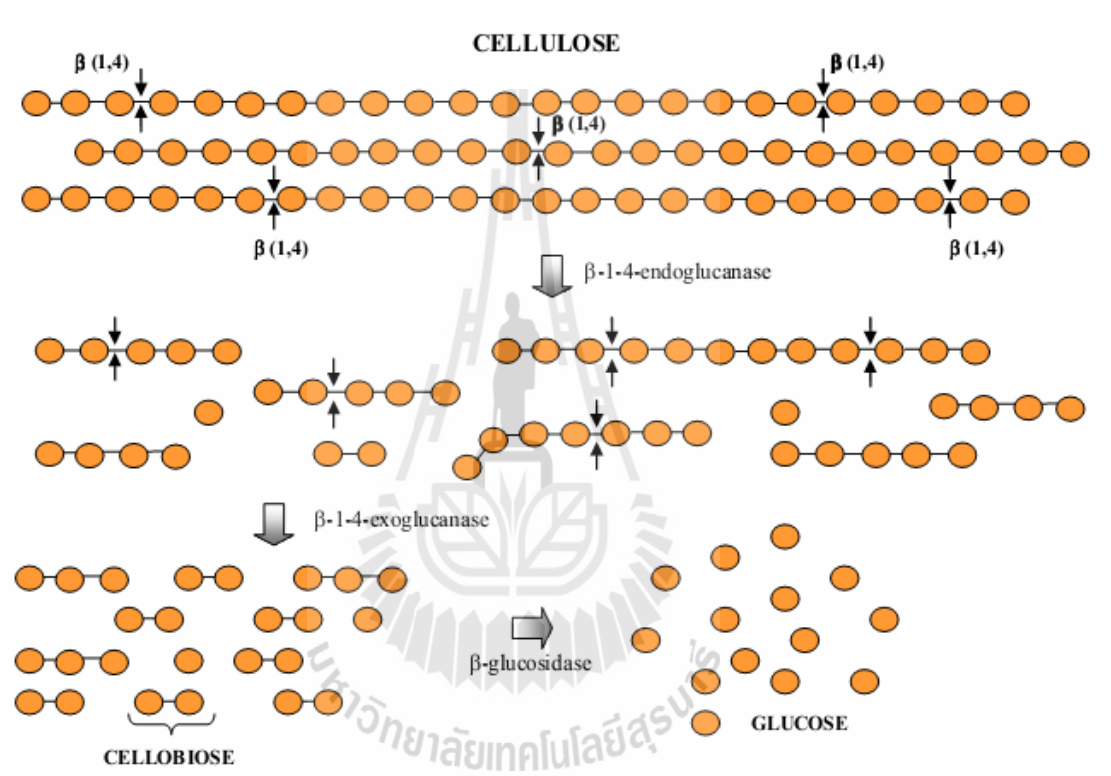


Figure 2.10 Schematic cellulose degradation by the cellulase enzymes (Mussatto *et al.*, 2010).

The interaction between hydrolytic enzymes and cellulosic substrates is complex. Enzymatic hydrolysis of cellulosic biomass consists of following three steps: 1) adsorption of cellulase enzymes onto the surface of the cellulose, 2) biodegradation of cellulose to fermentable sugars, and 3) desorption of cellulase (Saratale *et al.*, 2010). In hydrolysis, retardation of cellulase activity might be caused

by the irreversible adsorption of cellulase on cellulose (Zhang *et al.*, 2006). High sugar, released from the enzymatic hydrolysis process, depends on hydrolysis condition and cellulolytic enzyme concentration (Sun and Cheng, 2002).

Table 2.11 Enzymes involved in the hydrolysis of lignocelluloses materials consisting of cellulose and hemicellulose (Adapted from Saha and Bothast, 1999).

Enzyme	Mode of action
β-endoglucanase	Hydrolysis of intramolecular β-1,4-glucosidic linkages randomly of low crystalline regions and releases reducing sugars in the reaction mixture
β–exoglucanase or cellobiohydrolase	Hydrolysis of oligosaccharide short chain from the endogluconases's action to cellodextrins, cellobiose, and glucose
β-D-glucosidase	Hydrolysis of soluble cellobiose and other cellodextrins to produce glucose
Endo-xylanase	Hydrolysis of mainly interior *β*-1,4-xylose linkages of the xylan backbone
Exo-xylanase	Hydrolysis of *β*-1,4-xylose linkages releasing xylobiose
β-xylosidase	Releasing of xylose from xylobiose and short chain xylooligosaccharides
α-arabinofuranosidase	Hydrolysis of terminal nonreducing *α*-arabinofuranose from arabinoxylans
α-glucoronidase	Releasing glucoronic acid from glucoronoxylans
Acetylxylan esterase	Hydrolysis of acetylester bonds in acetyl xylans
Ferulic acid esterase	Hydrolysis of feruloyester bonds in xylans
p-coumaric acid esterase	Hydrolysis of *p*-coumaryl ester bonds in xylans

2.13 Cellulosome

Cellulosome is a multienzyme complex having a high activity for hydrolysis of crystalline cellulose, hemicellulose, pectin, and chitin to microbial cell mass and products (e.g. ethanol and butanol). The cellulase activity of cellulosome depends on the source of the cellulosomes (Bayer, 1998; Doi *et al.*, 2003). In nature, cellulosome is supposed to be the largest extracellular enzyme complex since polycellulosomes,

which is as large as 100 MDa, although the individual cellulosomes range are in the form 650 kDa to 2.5 MDa (Doi *et al.*, 2003).

2.13.1 Characterization of cellulosome

Generallly, a feature of the clostridial cellulosome consists of catalytic or cellulolytic components as called cellulosomal enzymes and non-catalytic (or non-cellulolytic components as called scaffolding proteins or scaffoldins (Figure 2.11).

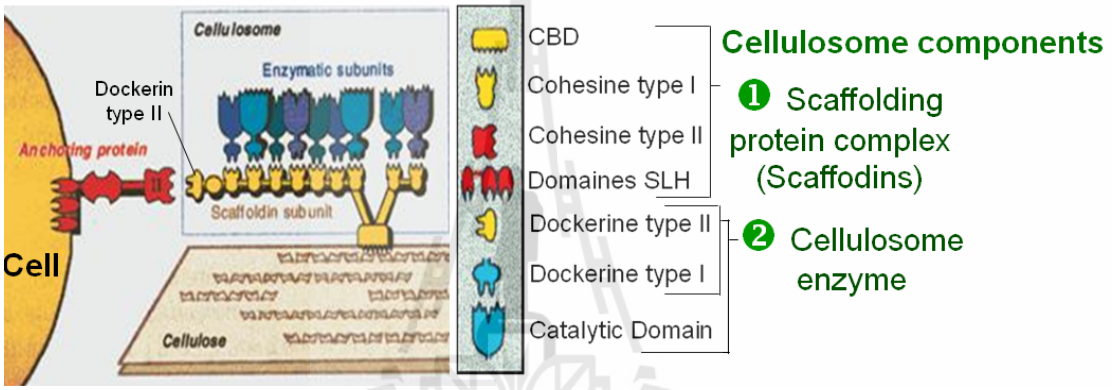


Figure 2.11 Schematic representation of a mini-cellulosome (Adapted from Shoham *et al.,* 1999).

The cellulosome consists of a scaffolding protein named CbpA, CipA, or CipC (Doi *et al.*, 2003). Scaffoldins are large nonenzyme proteins containing a number of cohesion domain (Coh), cellulose binding domains (CBDs), and hydrophilic domain (HLDs). The cohesins bind to the dockerin domains of catalytic domain as cellulosomal enzymes. The number of cohesions presenting in various scaffoldins is shown in Figure 2.12. The cohesin-dockerin interaction plays a key role in the assembly of the cellulosome (Doi *et al.*, 2003). The cohesins recognize very high specificity with their dockerin. For example, the cohesins presenting in CipA of *Cl. thermocellum* did not interact with the dockerins presenting in *Cl. cellulolyticum*

(Pages *et al.*, 1997). The cohesion-dockerin is high sequence homology among analog via components. The cohesion-dockerin specifically interactions are in all cognate cellusomal enzymes (Morag and Lamed, 1990).

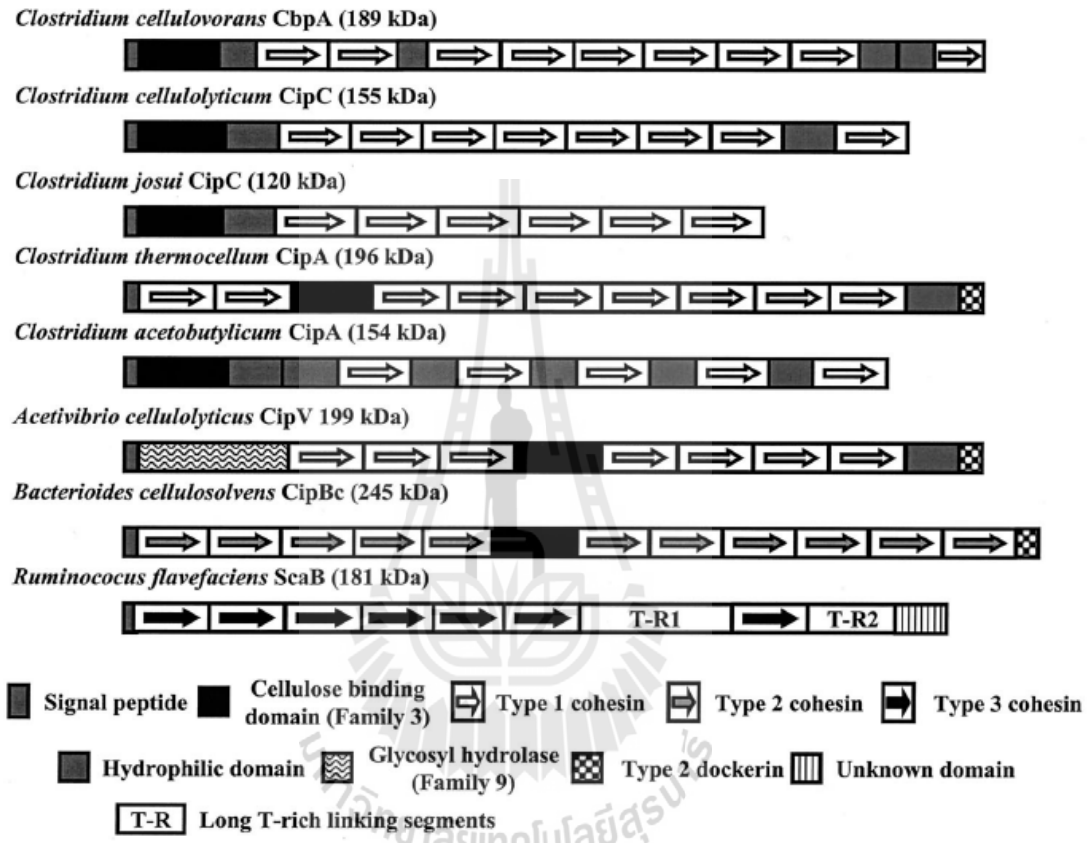


Figure 2.12 The number of cohesions present in various scaffoldins from various bacterial species (Doi *et al.,* 2003).

The CBD or the carbohydrate binding module (CBM) binds tightly the cellulosome to the cellulose molecule. The binding of CBD helps to increase the efficiency of cellulose hydrolysis. The CBD is the ability of recognizing and attaching itself to the surface of cellulosomes. Additionally, it has the ability to recognize and hold onto enzyme and the surface substrate (Kataewa *et al.*, 1997). The CBD binding

the cellulosome to the crystalline form of cellulose is more efficiently than that to amorphous form of cellulose (Goldstein *et al.*, 1993). The CBDs are divided into a number of families based on their amino acid sequences such as family 1, 2, 3, and 4. The family 3 CBDs are able to bind with crystalline cellulose.

The hydrophilic domain plays roles in binding the cellulosome to the cell surface (Tamaru and Doi, 1999; Kosugi *et al.*, 2002). It is a tight association with both the substrate and cell surface. The domain involves the facility of cellulosome binding both the substrate and the cell surface for degradation of cellulosic substrates. However, it has been reported that the binding of the cellulosome of *Cl. thermocellum* to the cell surface does not depend on SLH.

The catalytic component of *Clostridium* sp. composes of non-catalytic domain and catalytic domain. The non-catalytic domain is called dockerin. The cohesin-dockerin interaction is a species specific. The dockerin plays a key role for assembly of cellulosomal catalytic domain by indicating a celluosomal enzyme complex of cellulosome. In general, the major groups of enzyme activity, which found in catalytic domains of cellulosme, were endoglucanase, exoglucanase, and β-glucosidase. The enzymes system show synergistic activity (Figure 2.10): these enzymes are able to hydrolyze β-1,4-glucosidic bonds between glucosyl residues. Exoglucanases act in a processive manner on the reducing or non-reducing ends of cellulose polysaccharide chains. The exoglucanase, also known as exo-1, 4-β-glucanase, liberates a major product either glucose or cellulobiose by enzyme activity of glucanohydrolases and cellobiohydrolase, respectively. Endoglucanase randomly cut at internal amorphous sites in cellulose chain, resulting in various lengths of oligosaccharides. The oligosaccharide from a major product of endoglucanase are completely hydrolysed by exogluconase and β-glucidase. The

other name of endoglucanase was endo-1,4-β-glucanase or 1,4-β-D-glucan-4-glucanohydrolase. β-Glucosidases hydrolyze soluble cellodextrins or cellobiose from end product of exoglucanase and endoglucanase to glucose.

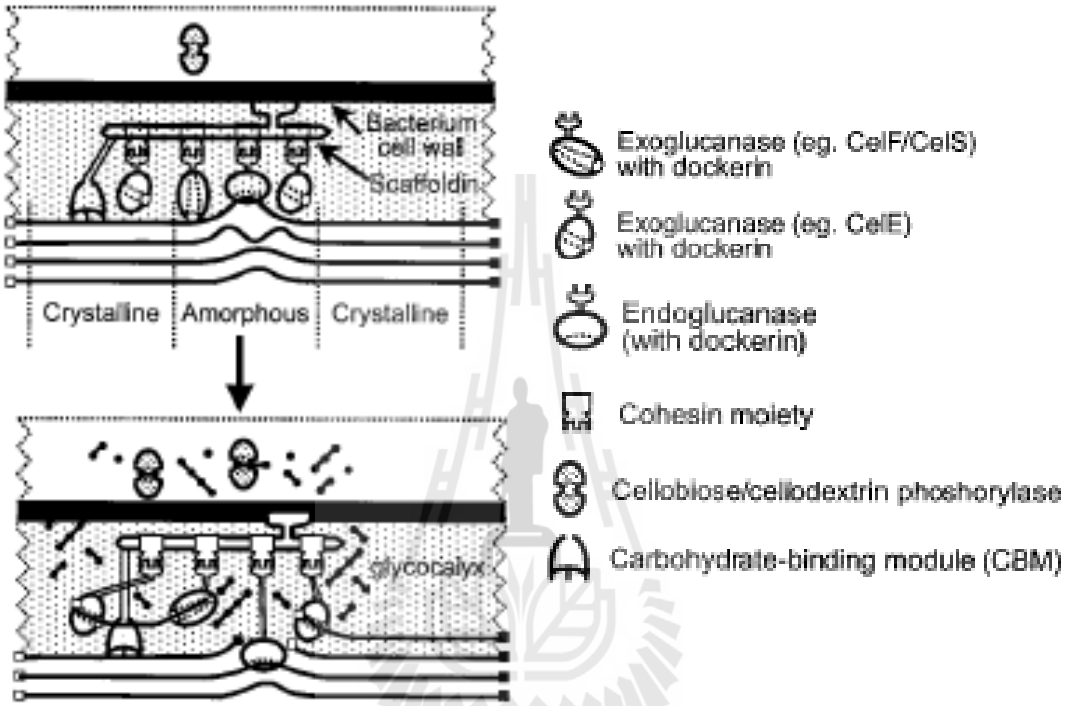


Figure 2.13 The hydrolysis of amorphous and microcrystalline cellulose by cellulase complex of cellulosome .

The cellulosomal enzymes show four forms for the synergistic interactions following (i) endo-exo synergy between endoglucanases and exoglucanases, (ii) exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains, (iii) synergy between exoglucanases and β-glucosidases to remove cellobiose as end products of the first two enzymes, and (iv) intramolecular synergy between catalytic domains and CBMs (Teeri, 1997; Teeri *et al.*, 1998).

The cellulosome-cell associated mechanism may not completely consist of scaffolding protein, cohesion, CMB, and HLD. Some scaffoldin can bind to cellulosome, although it has no HLD, for example of the *Cl. thermocellum* scaffoldin. In addition, the cellulosome-cellulose association is not necessary depending on CMB, cellulosome has yet been able to bind to cellulose for example scaffoldin of *Ruminococcus flavefaciens* (Doi *et al.*, 2003).

2.13.2 Designer cellulosome

The combination of designer cellulosomes concentrates on to produce the high value product. It is necessary for economical conversion of cellulosic materials to biofuels.

Cl. acetobutylicum ATCC 824 posses both endoglucanase and cellobiase activties for the hydrolysis of amorphous cellulose, but it lacks of exogluconase activity, being necessary for the hydrolysis of crystalline cellulose (Lee *et al.*, 1985). It is thus not able to grow on cellulosic materials as the sole source of carbon (Sabathé *et al.*, 2002). However, a large cellulosomal gene cluster encoding a complex of 665 kDa, was in sequenced genome of *Cl. acetobutyliucm* ATCC 824 by sequence analysis. In addition, by applying biochemical and western blot analysis, the cellulosomal component of the *Cl. acetobutylicum* ATCC 824 was structured with the least four major cellulosomal proteins including the scaffolding CipA, the endoglucanases Cel48A, Cel9X, Cel9C or Cel9E protiens. However, cellulolytic activity has not yet expressed. Cel48A protein is much more abundant than the other catalytic subunits a most role in the degradation of cellulose. Cel9X and Cel9C protein are active (unpublished data, INSA, Toulouse, France), whereas Cel48A protein sequence has no activity when they were expressed in *E.coli.* By analysis of amino acid sequencing, it found that Cel9X and Cel9C belong to the family 9 of

glycosyl hydrolases, a family 3c CBM, and a family 4 CBM (Sabathé *et. al*., 2002). The recombinant Cel9C and Cel9X expressed the cellulolytic activity on carboxymethylcellulose (CMC), amorphous cellulose, and crystalline cellulose due to forming a complex with the CipA scaffolding protein. CipA is an important receptor for the assembly of a minicellulosome in *Cl. acetobutylicum* ATCC 824. CipA contains an N-terminal signal peptide, a family 3a cellulose-binding domain (CBD), five type I cohesion domains. A mini-CipA scaffolding protein only consists of a CBD3a and two cohesion domains can be overexpressed in *Cl. acetobutylicum* ATCC 824. Thus the deficiency of cellulase activity of Cel48A could lead to an inactive cellulosome of *Cl. acetobutylicum* ATCC 824 for hydrolysis of crystalline cellulose (Lee *et. al.*, 1985; Sabathé and Soucaille,, 2003).

Cellulosome composition varies from species to species, but it has similarity among the same species. Sabathé and co-workers (2002) have reported that the cellulosomal gene clusters of *Cl. acetobutylicum* and *Cl. cellulolyticum* are similar in the high amino acid sequence level. Cel48A of *C. acetobutylicum* ATCC 824 has homology with Cel48F of *Cl. cellulolyticum* at 52%. The Cel48A of *Cl. acetobutylicum* ATCC 824 is an inactive cellulase gene, but the one of the Cel48F of *Cl. cellulolyticum* is an active cellulase gene. Therefore, this similarity of homology means that the opportunity is high for species specific dockerin domain with catalytic domain. This is well defined to construct plasmid containing the hybrid molecular between Cel48F as one of two major cellulases of *Cl. cellulolyticum*, with Cel48A as a deficiency of cellulase activity of *Cl. acetobutylicum*. The recombinant hybrid Cel48A dockerin-Cel48F catalytic domain protein has been constructed into *E.coli* DH5 cell to be expressed. This strain can express hybrid protein or hybrid enzyme activity by

culturing in crystalline cellulose (Avicel-cellulose) as good as effective native Cel48F (unpublished data, INSA, Toulouse, France).

2.14 Coculture

A promising strategy to overcome this impediment involves the production of cellulolytic enzymes, hydrolysis of biomass, and fermentation of resulting sugars to desired products in a single process step via a cellulolytic microorganism. Such "con-solidated bioprocessing" (CBP) offers very large cost reductions if microorganisms can be developed that possess the required combination of substrate utilization and product formation properties (Lynd *et al.*, 1996)

Mixed culture biotechnology (MCB) could be an alternatively one to traditional pure culture based biotechnology for the sustainable production of chemicals, bioplastics and bioenergy by fermentation of mixed organic substrate using a sequencing batch process (Kleerebezem and Loosdrecht, 2007). In nature, many microorganisms coexist by interacting with each other while many microorganisms are most effective only when they are present in association with other groups of organisms (Kato *et al.*, 2004). Hence, process development in MCB is only based on natural and ecological selection by manipulating the operation of the bioprocess or by varying the source of the natural inoculums to enrich the desired metabolic capacities and the corresponding microbial population. MCB has several advantages when compared with pure culture based industrial biotechnology including: adaptive capacity owing to microbial diversity, the capacity to use mixed substrates, and the possibility of a continuous process (Kleerebezem and Loosdrecht, 2007). For example, Bergstrom and Foutch (1983) patented the invention of relation between *Cl. pasteurianum* to *Cl. butylicum* in order to the butanol production by glucose

fermentation. They found that the use of a co-culture at least two different *Clostridium* species substantially increases a 20% enhancement in butanol yield over that those obtained using a culture employing only one microorganism.

Some research determined the optimal conditions for maximal butanol production on batch by response surface methodology (RSM) and using mixed bacterial microflora strains such as *Cl. saccharoperbutylacetonicum, Cl. butylicum, Cl. beijernckii,* and *Cl. acetobutylicum*, which were isolated from H_2-producing sludge taken from a sewage treatment plant (Cheng *et al.*, 2012). They demonstrated the feasibility of isolated bacterial microflora for butanol production performance combining butyric acid addition and an innovative pressurized fermentation strategy. This fermentation strategy enhanced the butanol fermentation, and provided product yield, productivity and a maximum butanol concentration of 0.8 mol butanol/mol glucose, 1.25 g/L.h, and, 21.1 g/L, respectively. Therefore, a mixed difference clostridia strain has the potential to produce butanol from glucose fermentation in coculture.

CHAPTER III

CONSTRUCTION OF SUITABLE INTEGRATION PLASMIDS FOR REPLACING *CEL48A* WITH HYBRID CELLULASE GENE IN *CLOSTRIDIUM ACETOBUTYLICUM*

3.1 Introduction

Cl. acetobutylicum ATCC 824 converts sugars (i.e. glucose, mannose, xylose, and arabinose) that obtain from plant cell degradation to solvents (Vasconcelos *et al*., 1994; Thormann *et al.*, 2002). However, it is unable to utilize cellulosic substrates due to secretion of inactive cellulosomes lacking activity towards crystalline cellulose (Sabathé *et al.,* 2002). Considering an economically competitive ABE process in the generation of biobutanol, it is largely attributable to the prohibitive cost of the feedstocks and the capabilities of the *Clostridium* strains employing in terms of both butanol productivity and extension of the substrate utilization (Ni and Sun, 2009). As mentioned, the combination of both cellulolytic and solventogenic phenotypes is an attractive challenge.

In this chapter, a synthetic gene called a hybrid gene *cel48SAFA* previously constructed was used. This hybrid gene encodes a hybrid *cel48SAFA* functional cellulase was expressed in *E. coli*. The hybrid *cel48SAFA* was constructed by species specific combination of *Cel48A* signal sequence and dockerin domain from

Cl. acetobutylicum ATCC 824 with *Cel48F* catalytic domain from *Cl. cellulolyticum*. Therefore, the aim of this study was to introduce the cellulase hybride gene into the chromosome of recombinant ATCC 824 strain. The process was followed by deleting *cel48A* gene from *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp* chromosome. Next, the growth and cellulase activity of strain ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* was tested on different cellulose sources. Additionally, the extracellular cellulosomal components secreted by *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* were purified and characterized. Subsequently, three artificial plasmids containing the *cel48SAFA* hybrid gene were constructed and introduced the hybrid gene into the *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp* Δ*cel48A* genome. The transformed clones should express the functional recombinant cellulase gene from the 48 family.

3.2 Materials and Methods

3.2.1 Strains and cultivation conditions

The microorganisms used are listed in Table 3.1. *Cl. acetobutylicum* ATCC 824 (wild type) and *Cl. acetobutylicum* mutant culture stocks were stored as spore suspensions at −80°C in mineral salts (MS) medium containing 60 g/L of glucose. The MS medium consists of (g/liter): 0.22, $MgSO_4.7H_2O$; 0.55, KH_2PO_4; 0.55, K_2HPO_4: 0.011, $FeSO_4.7H_2O$; 0.1, PABA; 0.001, Biotin; 0.0075, Resazurin; and 7, acetic acid (100%w/v). The pH was adjusted to 6.5 with 28% ammonium (NH_4) solution. The Clostridial growth medium (CGM) was used as culture and preculture media for the Clostridia wild type and the mutant. The CGM medium composed of the following components (g/L): 50, glucose; 6.25, yeast extract; 0.95, KH_2PO_4; 0.95, K_2HPO_4; 0.5, $MgSO_4.7H_2SO_4$; 0.013, $MnSO_4.7H_2O$; 0.013, $FeSO_4.7H_2O$; 1.25, NaCl;

2.5, asparagines; 2.5, $(NH_4)_2SO_4$; and 0.63, cystein. The medium pH was adjusted to 6.8 by 28% ammonium solution. The medium was made anaerobically by sparging with nitrogen gas before autoclaving. Medium for transformation was 2YTG. It is a nutrient-rich bacterial growth medium. The 2YTG medium consisted of (g/L): 16, tryptone; 10, yeast extract; 4, sodium chloride; and 5, glucose.

Table 3.1 Bacterial strains and plasmids used in this study.

Strains or Plasmids	Relevant characteristics
Bacterial strains	
E. coli TOP10	Invitrogen
E. coli Codon Plus (DE3) RIPL	Invitrogen
Cl. acetobutylicum ATCC 824	Wild type
Cl. acetobutylicum ATCC824 Δ*cac1502*Δ*upp*	*cel48A*, deletion of *cac1502*, and *upp*
Cl. Acetobutylicum ATCC 824 Δ*cac1502*Δ*upp*Δ *cel48A*	Deletion of *cac1502*, *upp*, and *cel48A*
Plasmids	
pSOS95-*Flp*	*repL, amp*^{R}*, colE1, , MLSr, flp1*
p ET-FH3	*SAFA*
pSCB-*FRT-Pptb-catP AZI*	*Lac* promoter, *catP, kan, amp, pUC*
pREP *cel48A::upp2*	*repL catP, upp, ori colE1, cipA, cel5B, MLSr*
pREP *cel48A::upp2ΔMLSr*	*repL catP, upp, ori colE1, cipA, cel5B,* deletion of *MLSr*
pREP-*cel48A*::*upp-catP* -11	*repl*, *upp*, *ori colE1, cipA, catP, pptb, cel5B*
pGR48A-ES5	Thiam^{r} , *cipA*, *ori*, *upp*, *catP*, *cel5B*, *cel48SAFA*, *repL*
pGR48A-ES5Δ*repL*	Thiam^{r} , *cipA*, *ori*, *upp*, *catP*, *cel5B*, *cel48SAFA* deletion of *repL*
pGR48A-ES5Δ*repL::Pptb-catP*	Thiam^{r} , *cipA*, *ori*, *upp*, *catP*, *cel5B*, *cel48SAFA*, *Pptb* promoter, deletion of *repL*
pGR48A-ES5::*Pptb-catP*	Thiam^{r} , *Pptb- catP* promoter

Note: *cac1502*, a putative gene encoding restriction endonulcease; *upp*, gene encoding uracil phosphoribosyltransferase (UPRTase); *cel48A*, gene encoding inactive cellulase protein; thiam^{r}, thiamphenicol resistance gene; *repL*, replication protein gene as an origin of replication for gram-positive organism; *celSAFA*, active hybrid cellulase gene; *Pptb*, phosphotransbutyrylase gene; *catP*, chloramphenicol acetyltrans-ferase gene; *ori colE1,* gene for a functional replication origin. Its replication is controlled independently of the replication of the host chromosome, *cipA,* encoding the *Cl. acetobutylicum* scaffolding protein*; cel5B*, encoding a *β*-1,4-endoglucanase

and a member of family 5 of glycoside hydrolases (GH5); MLSr, encoding macrolide-lincosamide-streptogramin B resistance.

For the cellulolytic activity, the *Clostridium* strains were grown in MS medium with xylose (20 g/L) and Avicel microcrystalline cellulose (10 g/L) as carbon sources. For *E. coli*, it was grown in the Luria-Bereani (LB) liquid medium with ampicillin (50 μg/mL) and streptomycin (75 μg/mL). The LB medium composed of (g/L): 10, tryptone; 5, yeast extract; and 10, sodium chloroide (NaCl). In the experiments, cellulose subtrates were used as follow: carboxymethyl cellulose (CMC), phosphoric acid swollen cellulose (PASC), Whatman no. 1 filter paper, and Avicel-PH101. The CMC is non-crystalline cellulose, whereas the PASC, Whatman no. 1 paper, and Avicel are microcrystalline cellulose.

3.2.2 DNA isolation and manipulation

The genomic DNA of *Cl. acetobutylicum* ATCC 824 was isolated using the GeneJET™Genomic DNA Purification Kit (Fermentas K0721, EU) and the purified DNA was dissolved in double distilled water. The kits for plasmid isolation were purchased from Fermentas (GenJET™Plasmid Miniprep Kit K502, EU), and the isolated plasmid was dissolved in TE buffer (pH 8.0). All genes and plasmid were stored at −20 °C prior to use. Restriction enzymes were purchased from Fermentas (Fermentas FastDigest®, EU) and the restriction enzyme mapping was performed using either single- or double-enzyme digestions. The agarose gel electrophoresis was run with 1 % agarose and 1×TAE as running buffer (pH 8.0) at 100 V for 15 to 30 min. The gene or plasmid DNA on the agarose gel was stained with trace amount of ethidium bromide for analysis. For purification, desired DNA bands in agarose gel were stained with SYBR® Safe DNA gel stain (Invitrogen). Standard DNA ladder (10

kb) (Fermentas O'GeneRuler™Express DNA Ladder, EU) was used to compare the isolated DNA after illuminating the electrophorized gel under UV light (312 nm). A desired DNA fragment was purified from a 0.8% low melting point (LMP) agarose gel (Seplaque GTG; FMC, Rockland, Maine). For ligation, linear fragments were ligated by quick ligation kit (M2200S, Biolab, New England). For preparation of plasmid, an amount of plasmids was prepared by the plasmid minipreparation (Qiagen).

3.2.3 PCR

DNA fragments were amplified by PCR with genomic DNA from the strains of ATCC 824, ATCC 824 mutant, and transformants as template DNA. PCR mixture per reaction comprised of DNA, 1μL; buffer, 10 μL; dNTPs 2 mM, 2μL; ddH2O, 28.5 μL; Phusion polymerase, 0.5 μL. Corresponding primers were listed in Table 3.2. A total of 35 cycles were performed in a DNA thermal cycler. Each cycle consisted of initiation denaturation at 95°C for 5 min, denaturing at 95°C for 15 sec, annealing at 52°C for 30 sec, elongation at 72°C for 5 min, and final extension at 72°C for 2-2.5 min depending on the lengths of the DNA fragments.

3.2.4 Plasmid construction and isolation

Table 3.1 summarizes all the plasmid vectors used in this study. Four designed plasmids including pGR48A-ES5Δ*repL*, pGR48A-ES5Δ*repL::Pptb-catP*, pGR48A-ES5*::Pptb-catP*, and pGR48A-ES5 were constructed. These vectors harbored the *SAFA* gene, encoding active a Cel48A hybrid cellulase. This active hybrid cellulase could digest cellulose to glucose. Each plasmid was transferred to host cells by electroporation.

Table 3.2 Primers used in this study.

Primer	Nucleotide no.	Sequence
Chromosome		
celA-OB	30	5'TAAAGATATACAGGATGCAGCTTCTAACGG3'
celA-5	28	5'GCCTACATTATGGAAAGACGATGCTAGT3'
Plasmid		
ccf3 bis	18	5'TATAAGACTGGCGATGCC3'
ccf1	21	5'GACCTAGGTTGTGCTTCTTCA3'
catP 5'-R	27	5'GTACAAGGTACACTTGCAAAGTAGTGG3'
catP 3'-D	23	5'GGAAGGAAAGCCAAATGCTCCGG3'
dock2	33	5'GATATTATGGAACTTACTAAATTAATTGCAAAA3'
UPP-R 5	28	5'GGTCTTGGAGATGCTGGAGATAGATTAT3'
cac3	50	5'CCCCCTTTTTAGGCCTCCCCTCGAACTTATTAGAAT GATTAAGATTCCGG3'
cac4	30	5'TCATTAAATTTCCTCCATTTTAAGCCTGTC3'

3.2.5 Preparation of clostridia competent cell and transformation

Cl. acetobutylicum ATCC 824 and *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* were used as competent cells for transformation by electroporation. *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* was an ATCC 824 strain being deleted genes of *cac1502, upp,* and *cel48A*. Each 1 mL of spore suspension was transferred into 60 mL of CGM medium in a 100 mL serum bottle covering with aluminium cap. The spores were heat shocked at 80ºC for 10 min. The activated spore culture was allowed to grow at 37ºC for overnight (16-18 h). When turbidity of broth culture at 600 nm reached 2.0, the culture was diluted to OD 600 nm = ~ 0.2-0.3 by calculating volume of original cell suspension, which needed to add to a new CGM medium in order to produce dormant clostridia cells. The diluted culture

was incubated until an initial cell-density reached the OD600 nm of approximately 0.8-1.2. This level of turbidity was the best result obtained at early- to mid-log phase. The suspension of electrocompetent cells was kept in ice bath prior to transformation.

The fresh electrocompetent cell culture was splited into two of 50 mL falcon tubes. Each centrifuge tube contained 25 mL working volume. The culture was harvested by centrifugation in cold rotor at 3000 x relative central force (rcf) maximum for 15 min. Much of supernatant as possible was removed by pouring off a few cells. Then, remaining liquid, which was in cell pellet, was absorbed by sterile cotton butt until dry. The cell pellet was resuspended gently in 4 mL of chilled electroporation buffer. Centrifugation to harvest cells and drying the tube were performed again. Two cell pellets in each tube were resuspended gently and pooled together in 500 μL of chilled buffer. The suspension was kept as close to 0°C as possible in an ice bath before the transformation process. The cell suspension was transferred into the cold electro-cuvette. DNA vector was added in the cell suspension with well mixed. Mixture was sat on ice for~0.5-1 min.

Pulser apparatus was set at 1.80 kV, 50 μF, and 600 Ω. The cuvette was placed in a chilled safety chamber slide and pulsed once. The cuvette from the chamber was removed and immediately transferred into 10 ml of cold 2YTG medium. The transformants were incubated at 37°C for 6 h. The transformant culture was concentrated by centrifugation to harvest cell, pouring off the medium and then resuspending the cells in the same culture medium to the final volume of 300 μL. the concentrated transformant culture (250 μL) was spreaded on reinforced clostridial medium (RCA) supplemented with thiamphenicol 10 μg/mL. Culture plate was incubated at 37°C until appearance of the colony.

3.2.6 Cellulolytic activity assay and growth measurement

The strains of *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48* were grown on agar plate to test for the cellulase activities. Five types of the solid cellulose media were used. The cellulose media were different in the medium composition between the top layer (TL) and the bottom layer (BL) as follow: 1) TL, MS medium plus xylose and PASC/ BL, MS medium plus xylose; 2) TL, MS plus PASC/ BL, MS; 3) TL, MS plus PASC and yeast extract/ BL, MS plus PASC; 4) MS plus CMC; and 5) MS plus PASC and xylose. The following carbon and nitrogen substrates were used at the indicated final concentrations (g/L): 10 g PASC, 10 g CMC, 20 g xylose, and 0.5 g yeast extract in medium (pH 6.5). Cellulolysic activities were determined by observing the clear zone around the colony. The culture growth was monitored by measuring the optical density at 600 nm (OD_{600}).

3.2.7 Preparation of phosphoric acid-swollen cellulose

Avicel PH-101 power (2 g) was mixed with 100 ml of ice-cold 85% phosphoric acid by vigorous stirring until the cellulose suspension solution became white cloudy and colloid solution. Next, the solution was kept at 4°C overnight to allow complete solubilization of the cellulose, until it was clear and no lumps remained in the reaction mixture. The mixture was then poured into 500 ml of ice-cold distilled water with occasional stirring. The white cloudy and colliod solution were appeared. The swollen cellulose solid was harvested by centrifugation at 5,000 x g at 4°C for 20 min. The cellulose solid was washed with cold 1% (w/v) $NaHCO_3$ solution to neutralize the residual phosphoric acid in the cellulose fraction. The swollen cellulose was suspended with cold distilled water until final pH about 7. Finally, the cellulose was resuspended in 100 ml of ice-cold water. The final concentration of the amorphous (homogeneous) cellulose slurry was 2%. It was stored at 4°C.

3.2.8 Cellulosomal Purification of *Clostridium*

The *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48* strains were grown anaerobically at 37ºC with shaking at low shaking speed (100 rpm) in 250 mL blue top bottle of the MS medium with xylose and Avicel. Before total consumption of xylose, cellulose was harvested by decantation of the liquid fraction and washed two times, first in 50 ml of 100 mM potassium phosphate buffer (PBB), pH 7.0, to remove cells. Then the cellulose pellet was washed two times with 50 ml of 100 mM PBB, and one time with 50 ml of 25 mM PBB by centrifugation at 15,000 x g for 15 min. The cellulosome was then eluted with 10 ml 1% triethylamine. The eluant fraction was centrifuged to remove all of the insoluble substances, and the supernatant was concentrated to the final volume of 1 mL by centrifugation using the Amicon concentrator (Millipore, cut-off 10 kDa).

3.2.9 Cellulase Purification of *E. coli*

For the cultivation of the *E. coli*, it was grown aerobically in 80 mL of the Luria-Bereani (LB) liquid medium with ampicillin (50 μg/mL) and streptomycin (75 μg/mL) at 37ºC, 150 rpm, until the OD of the culture medium at 600 nm (OD_{600}) reached 1.2. The culture was then cooled to 25ºC and isopropyl-L-D-thiogalactopyranoside (IPTG) was added to the final concentration of 0.2 mM. The culture was further shaken at 16ºC, 120 rpm until the OD 600 nm of the culture reached 2.5-3.0. The cells were cooled down to 4ºC prior to harvesting by centrifugation. Then, the harvested cell pellet was resuspended in cold 30 mM Tris-HCl (pH 8.0) buffer (THB), and lysed in a French pressure cell. The crude extract was centrifuged at 26,000 x g for 15 min, and the supernatant was loaded onto a 3 ml Ni-nitrilotriacetic acid column (NTA) resin (Qiagen) equilibrated with RB. After washing the column with THB supplemented with 5, 10 and 20 mM imidazole, the protein was

eluted with THB by adding 50 mM imidazole. The eluant fraction was immediately dialyzed against THB and concentrated by the Amicon concentrator (Millipore, cut-off 10 kDa).

3.2.10 SDS-PAGE analysis

The concentrated proteins from the *Clostridium* strains and *E. coli* were determined for the apparent size of the protein by performing with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (14%). Samples were heated at 100°C for 5 min in 2.5% SDS and 5% 2-mercaptoethanol. Protein bands were stained with Coomassie blue solution. Calibration proteins ranging in molecular weight from 15,000 to 170,000 Da (molecular-weight calibration kit; Pharmacia) were used for the estimation of the molecular weight of the concentrated cellulosomal proteins.

3.2.11 estern blot analysis

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech) by standard procedures. The membrane was incubated overnight at 4ºC with shaking in blocking buffer (10% milk powder, 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 0.3% Tween 20). The dilution used for the primary antibody was 1/1000. The Coh5 cohesin domain and anti-rabbit immunoglobulins, including anti CelE, anti CelC, and anti Cel5A, were used as the purified primary antibodies. The anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Sigma) was used as the secondary antibody, according to the manufacturer's instructions. The membrane was then incubated with the primary antibody for 1 h at room temperature in blocking buffer and washed three times with Wash Buffer (50 mM Tris-HCl buffer, pH 7.5 containing 150 mM NaCl and 0.3% Tween 20). The membrane was incubated at least for 1 h at room temperature with alkaline phosphatase-conjugated antibody in blocking buffer and washed three times

with Washing Buffer. The bands were visualized using 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT) liquid substrate system (Sigma).

3.3 Results

3.3.1 Deletion of the *cel48A* gene of the *Clostridium* mutant

The *cel48A* gene located on chromosome of the *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp* cell encodes an inactive cellulase. Thus, to remove the *cel48A* gene of this mutant, the gene deletion strategy was developed and the the pREP-*cel48A*::*upp-catP* replicative plasmid was constructed.

Then this designed plasmid was transformed into the mutant cells. Clone was selected on RCA medium supplemented with thiamphenicol and 5-fluorouracil (5-FU) substrate to remove pREP-*cel48A*::*upp-catP*. With regarding to the presence of the *upp* and *catP* genes on the pREP-*cel48A*::*upp-catP*, the *upp* gene encoding uracil phosphoribosyl transferase causes the cell containing the pREP-*cel48A*::*upp-catP* plasmid to be sensitive to 5-fluorouracil (5-FU) substrate. In addition, the *catP* gene is a chloramphenicol resistant gene in case of *E. coli* cell and a thiamphenical resistant gene in case of clostridium cell. Therefore, this was a reason to use thiamphenical and 5-FU substance for screening clone without the pREP-*cel48A*::*upp-catP*. The *FRT-catP-FRT* cassette on the *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A ::catP* chromosome was deleted with the *FRT/Flp* system of pSOS95-*Flp* plasmid. Finally, pSOS95-*Flp* plasmid was removed by growing culture on RCA medium without antibiotic.

3.3.1.1 Construction of the pREP-*cel48A*::*upp-catP*

The pREP-*cel48A*::*upp-catP*-11 plasmid was constructed by ligation the *StuI* digested genes between the 1,230 bp of the *FRT-catP-FRT* gene cassette from pSCB-*FRT-Pptb-catP AZI* plasmid and the 5,815 bp of the *catP-UPP* gene cassette from pREP *cel48A::upp2ΔMLSr* plasmid (Figure 3.1). This plasmid was transformed into the TOP10 chemically *E. coli* competent cell. The clone carrying this plasmid was screened on LB medium supplemented with chloramphenicol. The results from restriction analysis by using *StuI* demonstrated and confirmed that the plasmid configuration was correct as expected (Figure 3.2).

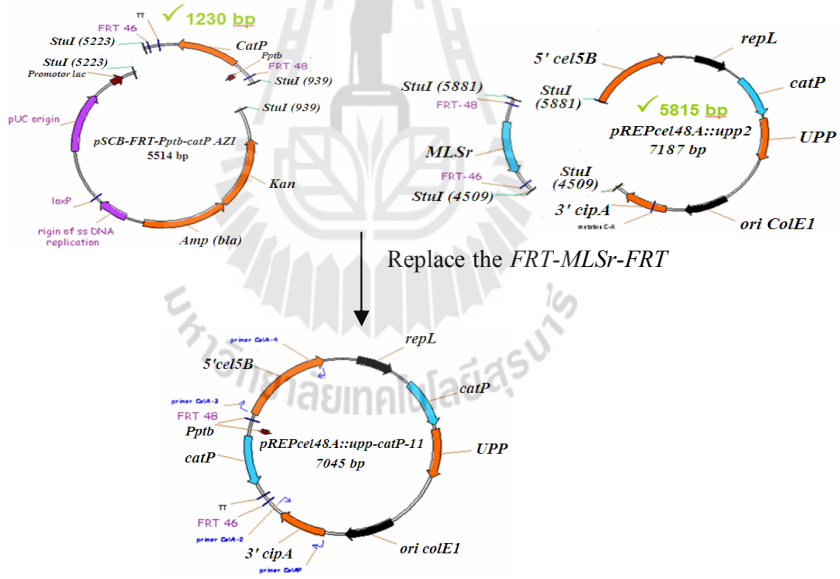


Figure 3.1 The construction of the pREP-*cel48A:: upp-catP-11* plasmid.

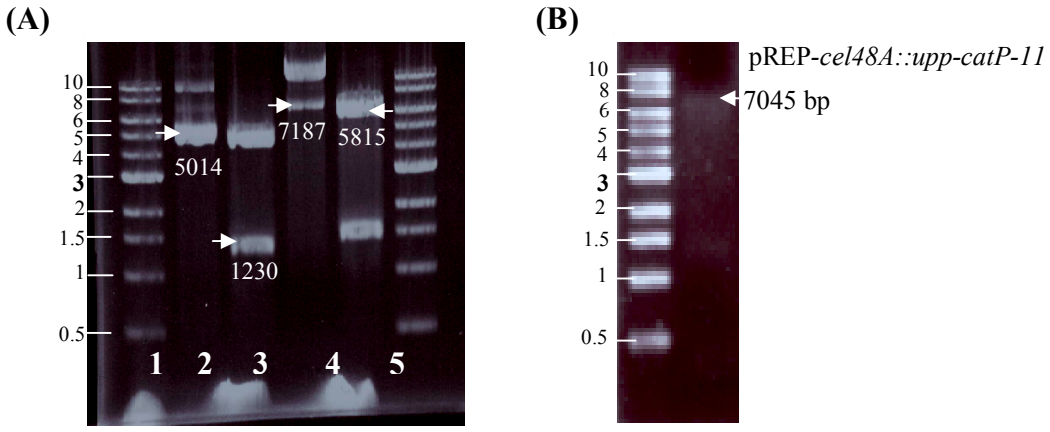


Figure 3.2 (A) Electrophoresis analysis of the digested plasmid with *StuI*. Lanes: 1, 6, molecular mass marker (1 kb DNA ladder for the mass calibration kit from Biolabs, New England); 2, the undigested DNA plasmid of the pSCB-*FRT-Pptb-catP AZI* plasmid (5,014 bp); 3, the 1,230 bp of the pSCB-*FRT-Pptb-catP AZI* plasmid which was digested by *StuI* and purified; 4, the undigested DNA plsmid of the pREP*cel48A::upp2* plasmid (7,187 bp); and 5, the 5,815 bp of the pREP*cel48A::upp2* plasmid which was digested by *StuI* and purified; and (B) the 7,045 bp of the artificaial pREP-*cel48A::upp-catP-11* plasmid.

3.3.1.2 Transformation of the pREP-*cel48A::upp-catP*-11 into *Clostridium acetobutylicum* ATCC824 Δ*cac1502*Δ*upp* cell

The pREP-*cel48A*::*upp-cat*P-11 plasmid was employed to remove the chromosomal located *cel48A* gene within the ATCC 824 mutant cell by double crossing over process after transformation (Figure 3.3). The transformation results showed that a number of transformed colonies could be formed on RCA medium supplemented with thiamphenicol (5 μg/mL), since the presence of the thiamphenicol resistant *catP* gene carrying by the plasmid. These transformants were selected again

on RCA with thiamphenicol and followed by PCR analysis to verify the presence of the plasmid. The PCR results demonstrated that these clones displayed the presence of the plasmid.

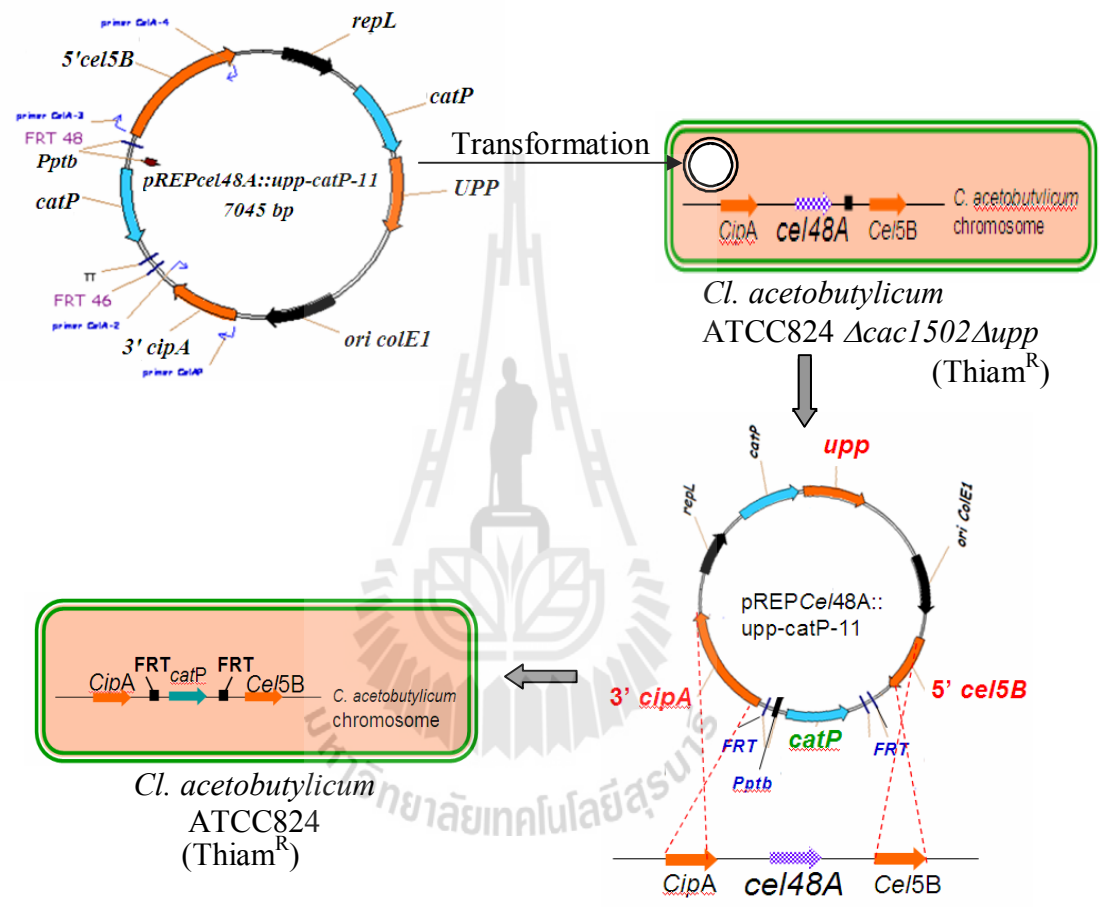


Figure 3.3 Schematic of the deletion of *cel48A* gene by the double crossing over phenomenon.

3.3.1.3 Selection of clone for the double crossing over

To delete the *cel48A* gene on chromosome, the ATCC 824 Δ*cac1502*Δ*upp* cells were induced with the pREP-*cel48A*::*upp-cat*P-11 plasmids and plated on RCA medium supplemented with thiamphenicol (5 μg/mL) and 5-FU (100 μM). Five clones were visible after 2–5 days of incubation. Mixture of these clones was cultured on 2YTG liquid medium for the cell dilution. Then the diluted culture was spreaded on RCA supplemented with thiamphenicol and 5-FU. Five clones were screened by PCR, and all were shown to be double-cross over clones (Figure 3.3). These results could explainethe double crossing over between the plasmid and the chromosome was pressured by 5-FU substrate (Figure 3.4). It resulted that the *cel48A* gene position on chromosome was replaced by the *FRT-catP-FRT* gene cassette. The transformant was designed as the ATCC 824 Δ*cac 1502*Δ*upp*Δ*cel48A::catP* strains. It was resistant to thiamphenicol and sensitive to 5-FU substrate (Thiam^{R}5-FU^S).

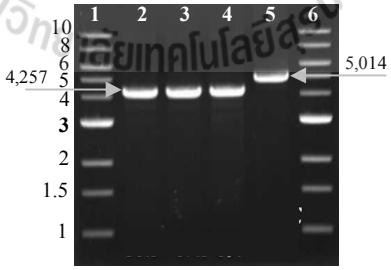


Figure 3.4 The PCR products of clones from the transformation of the pREP-*cel48A*::*upp-cat*P-11 plasmid into *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp* cell to analysis the integration and the presence of plasmid. Lanes; 1 and 6; 10 kb ladder; and 2-5; clones from transformation.

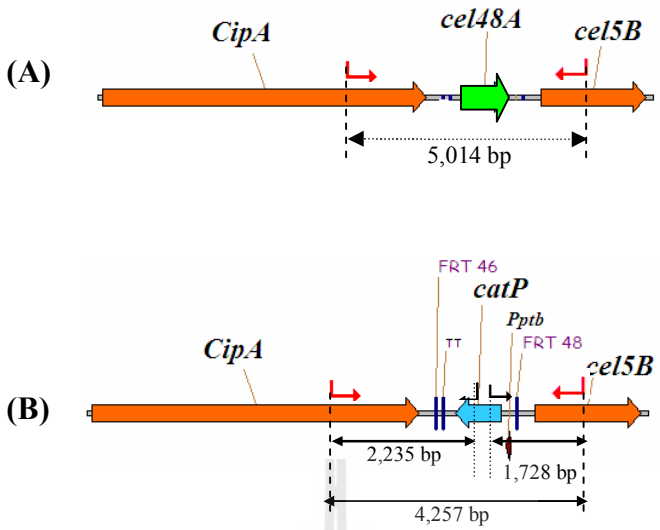


Figure 3.5 (A) The structure of chromosome for *Cl. acetobutylicum* ATCC 824 *Δcac 1502Δupp*; and (B) the integration of the *FRT-catP-FRT* gene cassette on chromosome for *Cl. acetobutylicum* ATCC 824 *Δcac1502ΔuppΔcel48A:: catP* strain.

3.3.1.4 Removal of the *FRT-catP-FRT* cassette on the *Clostridium acetobutylicum* ATCC 824 *Δcac1502ΔuppΔcel48A::catP* chromosome with the Flp/FRT system

The removal of the *FRT-catP-FRT* cassette on the chromosome of the strain ATCC 824 *Δcac1502ΔuppΔcel48A::catP* based on the *Flp* gene of pSOS95-*Flp* plasmids to produce Flp recombinase, which cut this cassette gene at FRT sites. The pSOS95-*Flp* contained a *MLS* gene as a gene marker. This gene marker conferred resistance to erythromycin. The gene arrangement of the pSOS95-*Flp* plasmid was showed in Figure 3.6(A). Thus, pSOS95-*Flp* plasmids were transformed into the mutant *Cl. acetobutylicum* ATCC 824 *Δcac1502ΔuppΔcel48A ::catP* cells. The result displayed that there were more than 200 clones appeared on RCA medium

supplementeded with erythromycin (40 μg/mL) and thiamphenicol (5 μg/mL). It indicated that these clones, as the thiamphenicol resistant clones, contained the plasmid. Subsequently, to remove the *FRT-catP-FRT* cassette on the mutant chromosome, these transformants were recultured on 2YTG liquid medium without antibiotic following plating on RCA medium supplemented with erythromycin (40μg/mL). The result exhibited that only two clones could grow on medium. However, it implied that the *FRT-catP-FRT* cassette had been removed. Later, to remove the pSOS95-*Flp* plasmid, the clones was resuspended in 2YTG liquid medium without erythromycin and then plated on RCA medium without erythromycin. The results showed a lot of clones on medium. Some clones were chosen to be restreaked on RCA medium with erythromycin. The result displayed that 90% of clones were sensitive to both thiamphenicol and erythromycin, indicating the absence of plasmid and the gene cassette on chromosome. It can conclude that the construction of mutant *Cl. acetobutylicum* ATCC 824 lacking the *cel48A* was successful. This $Ery^{S}Thiam^{S}$ strain was called as *Cl. acetobutylicum* ATCC 824 *Δcac1502ΔuppΔcel48A* strain. This strain was employed to be a host cell for introduction of hybrid cellulase gene on chromosome by using other designed plasmids harboring hybrid gene.

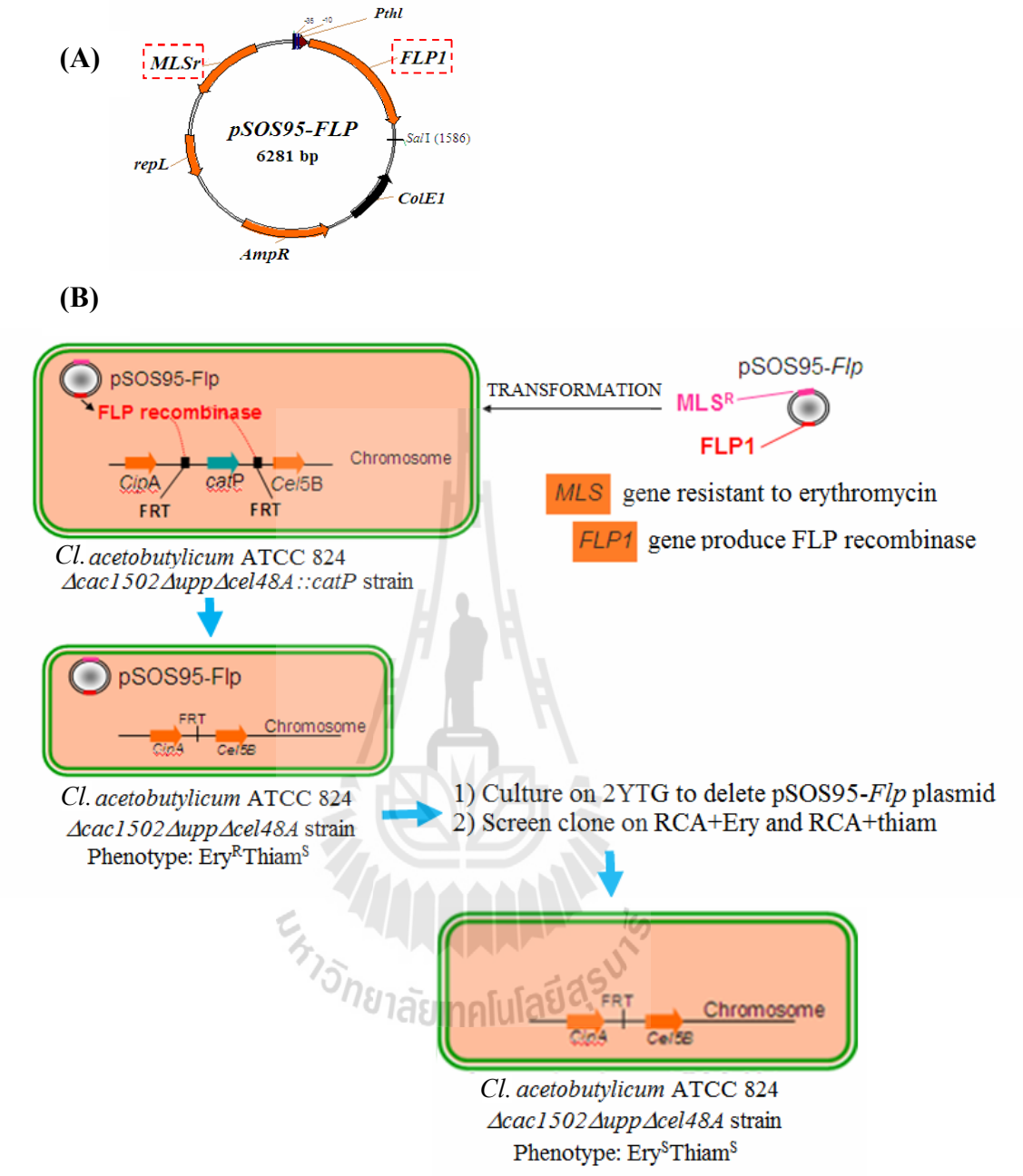


Figure 3.6 (A) The map of the pSOS95-*Flp* plasmid; and (B) the modification of the chromosome structure of *Cl. acetobutylicum* ATCC 824 *Δcac1502Δupp Δcel48A::catP* strain after the transformation of the pSOS95-*Flp* plasmid.

3.3.2 Growth and cellulolytic activity of the recombinant *Clostridium* strains on different cellulose substrates

Two recombinant *Cl. acetobutylicum* strains, including the ATCC 824 Δ*cac1502*Δ*upp* and ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48* strains, were detected for the cellulolytic activity on different cellulosic substrates. Each recombinant strain was cultivated on solid media containing carboxymethyl cellulose (CMC) and phosphoric acid swollen cellulose (PASC) supplemented with xylose or yeast extract. The ability to degrade the cellulose was observed from the clear zone around the colony. The results showed that both strains did not show the colony formation on the surface of the solid cellulose media even cellulose media contained xylose or yeast extract as a carbon source or nitrogen source, respectively, (Table 3.3). It may be due to agar media were not suitable for culturing the *Clostridium* strains. Subsequently, the growth and cellulose digestion of these strains were verified by culturing in liquid cellulose medium supplemented with and without xylose. The cellulose substrates used were Avicel, Whatman No.1 paper, and CMC. The growth was determined by measuring the optical density at 620 nm. Cellulolytic activity was observed from the solubility of celluloses in Avicel and Whatman No.1, and the viscosity of the soluble CMC. The result exhibited that both recombinant strains grew in the liquid cellulose media supplemented with xylose, but failed to grow in media without xylose (Table 3.4). It indicated that these celluloses were not degraded by both of the recombinant *Clostridium* strains. They also were capable of utilizing xylose as a carbon source for growth. This is supported by the fact that the strain ATCC 824 possesses xylose catabolic enzyme genes in the genome. Furthermore, it would secrete the active xylose isomerase and xylulokinase, which convert xylose to xylulose-5-P through pentose phosphate and phosphoketolase pathways (Liu *et al.*, 2012; Gu *et al.*, 2010).

Notably, the growth of the strain ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48* was faster than that of other strain on all of the cellulose subtrates in the liquid medium supplemented with xylose (Figure 3.7). It is not clearly whether the deletion of *cel48A* would contribute to the capacity of growth.

Table 3.3 Cellulolytic activities of the cultures of mutant *Cl. acetobutylicum* ATCC 824 strains grown on solid medium with different cellulosic substrates.

	Agar medium (pH 6.5)		***Cl. acetobutylicum* ATCC 824**	
			Δ*cac1502*Δ*upp*	**Δ*cac1502*Δ*upp*Δ*cel48A***
1	Top layer	MS+X 0.5 g/L+1%PASC	No growth	No growth
	Bottom layer	MS+X 0.5 g/L		
2	Top layer	MS +1%PASC	No growth	No growth
	Bottom layer	MS		
3	Top layer	MS+ 1%PASC+Yeast extract 0.5 g/L	No growth	No growth
	Bottom layer	MS+ 1%PASC		
4	Whole	MS+CMC20 g/L	No growth	No growth
5	Whole	MS+CMC20 g/L+X20g/L	No growth	No growth

Table 3.4 Growth and cellulolytic activities of the cultures of mutant *Cl. acetobutylicum* ATCC 824 strains grown in liquid medium with different cellulosic substrates.

Liquid cellulose media	***Cl. acetobutylicum* ATCC 824**	
	Δ*cac1502*Δ*upp*	**Δ*cac1502*Δ*upp*Δ*cel48***
MS+Avicel-PH101 10 g/L	No growth	No growth
MS+Avicel-PH101 10 g/L+xylose 20 g/L	Growth	Growth
MS+Whatman No.1	No growth	No growth
MS+Whatman No.1+xylose 20 g/L	Growth	Growth
MS+CMC 20 g/L	No growth	No growth
MS+CMC 20 g/L+xylose 20 g/L	Growth	Growth

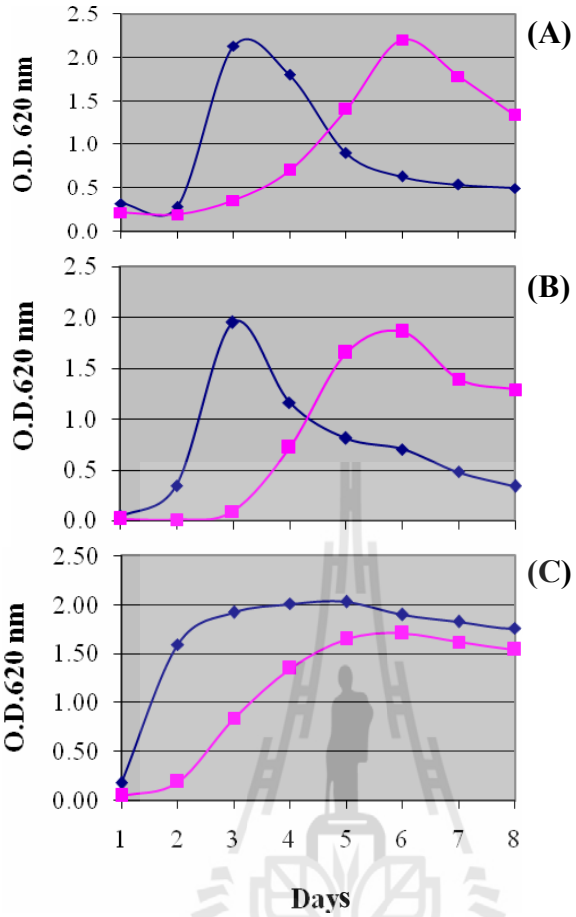


Figure 3.7 The growth of the *Cl. acetobutylicum* ATCC 824 Δ*cac1502* Δ*upp*Δ*cel48* strains (blue) and *Cl. acetobutylicum* ATCC 824 wild type strain (pink) in different medium. (A), MS medium with Avicel-PH101 and xylose; (B), MS with Whatman No.1 and xylose; (C) , MS with CMC and xylose.

3.3.3 Characterization of extracellular cellulosomal cellulase

Cl. acetobutylicum ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48* strain was studied for the cellulosome components by determination of the apparent size of the purified recombinant protein with SDS-PAGE analysis. Its cellulosomal components were also identified by Western blot anaylysis. The *Cl. acetobutylicum* ATCC 824 as a wild type strain, and the recombinant *Escherichia coli* harboring pET-FH3::*SAFA* plasmid

were used as control. These strains were cultured and then the cellulosomes from each culture were purified and concentrated for further analysis for celllulosomal composition. Figure 3.8 showed the patterns of difference molecular weight of each concentrated cellulases fraction under SDS-PAGE gel. The gel revealed that the minicellulosome of protein fractions composed of Cel48A (80.9 kDa) was produced by the *Clostridium* wild type, whereas SAFA (70 kDa) was overexpressed by recombinant *E. coli* as a control strain. In addition, there was no appearance of a Cel48A band for the recombinant *Clostridium* strain, where the *cel48A* gene was deleted. The molecular masses of Cel48A and SAFA were in a good agreement with the theoretical molecular sizes.

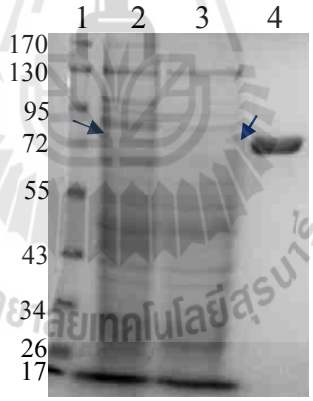


Figure 3.8 SDS-PAGE of the purified cellulosomal cellulases from three recombinant strains. Lane 1: low-molecular-weight Bio-Rad standard markers. The patterns of cellulase fraction obtained from the different organisms: Lane 2, wild type *Cl. acetobutylcium*; Lane 3, the *Cl. acetobutylcium* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48* strain; Lane 4, the recombinant *E. coli* strain containing the *SAFA* hybrid cellulase gene.

Subsequently, the minicomplexes were identified as a type of cellulosomal components by Western blotting (Figure 3.9). The proteins including Coh5 cohesin domain, anti CelE, anti CelC, and anti Cel5A, the purified primary antibodies from rabbit were used as anti-antibody to the cellulase proteins. The results exhibited that the *Clostridium* wild type strain possessed CipA, Cel48A, Cel9C, and Cel5A. Concerning the ATCC 284 Δ*cac1502*Δ*upp*Δ*cel48* strain, their proteins corresponded with the CipA, Cel9C and Cel5A, but no protein corresponded with the Cel48. Furthermore, the SAFA molecule was specifically recognized by CelF antibody. This suggested that SAFA protein was the active cellulase. These results were in parallel with those observations in the SDS-PAGE analysis. According to these results, it was evident that ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48* strain could not produce the Cel48A.

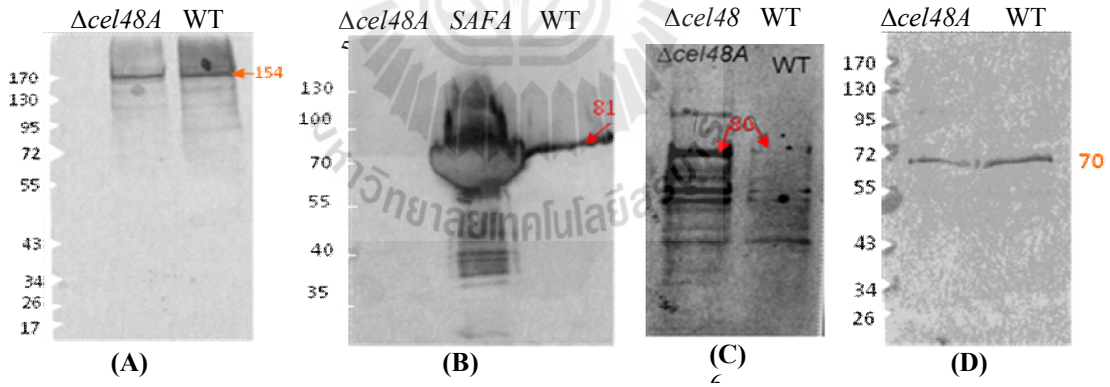


Figure 3.9 Immunological detection of the cellulosomal complex from the recombinant strain including *Cl. acetobutolicum* ATCC 824 strain (WT), *Cl. acetobutolicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48* strain (Δ*cel48*), and *E. coli* Δ*celSAFA* strain (*SAFA*) by Western blot analysis. Western blots obtained with *Cl. cellulolyticum* Coh5 (A), Cel48F (B), Cel9C (C), and CBD-Cel9G (D) specific antibodies.

3.3.4 Introduction of a *cel48SAFA* hybrid cellulase gene into the Δ*cac1502* Δ*upp*Δ*cel48A chromosome* of *Clostridium acetobutylicum* ATCC 824

Cl. acetobutylicum ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* was used as a host cell for introducing a *cel48SAFA* hybrid gene. The hybrid gene coded for the active cellulase enzymes. Four plasmids, haboring the *SAFA* active cellulase gene, were designed as follow: 1) pGR48A-*ES5ΔrepL*, 2) pGR48A-ES5*ΔrepL::Pptb-catP*, and 3) pGR48A-*ES::Pptb-catP*. Each designed plasmid was transformed into the mutant strain by electroporation. The hybrid gene was introduced into chromosome of the mutant cells by a single crossing over through non-replicative plasmids including pGR48A-*ES5ΔrepL* and pGR48A-*ES5ΔrepL::Pptb-catP*. As to replicative plasmids including pGR48A-ES5*::Pptb-catP* and pGR48A-ES5, the desirable gene was inserted by double crossing over.

3.3.4.1 Construction and transformation of pGR48A-*ES5 repL* plasmid into *Clostridium acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp* Δ*cel48A*

Figure 3.10 shows the construction of pGR48A-*ES5*Δ*repL* plasmid. The 8068-bp plasmid pGR48A-*ES5* was digested with *ZraI* and *SmaI* to remove a *repL* gene encoding origin of replication. The 7291-bp pGR48A-*ES5* linear fragments without *repL* gene were generated. The 7291-bp linear fragment was ligated. Then, the plasmid was stored by transformation into *E. coli* TOP10. Transformed clones containing plasmid were selected on Luria-Bertani (LB) agar medium supplemented with 30 μg/mL of chloramphenicol. The result showed a number of clones on medium, indicating these clones contained the plasmid. Four clones were chosen to prove for the correct size of plasmid by the digestion with *ZraI*

and *SmaI* (Figure 3.11). The PCR result exhibited the correct size, indicating the correction of plasmid configuration was constructed.

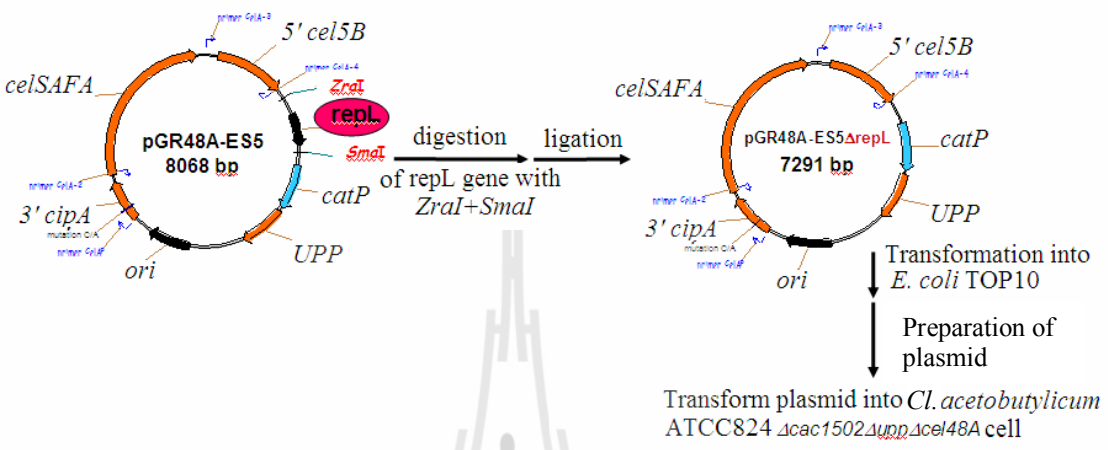


Figure 3.10 Construction of pGR48A-*ES5*Δ*repL* plasmid and transformation of plasmid into *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* cell to introduce a hybrid *SAFA* gene on chromosome.

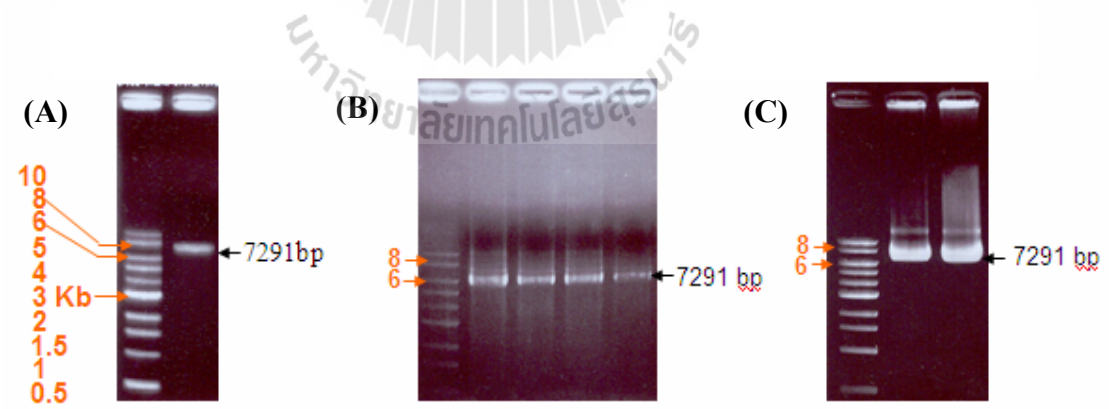


Figure 3.11 (A) the 7291 bp of pGR48A-*ES5*Δ*repL* plasmid from digestion with *ZraI*+*SmaI*; (B) the 7291 bp fragment of *E. coli* TOP 10 clone no. 1, 2, 3 and 4 from miniplasmid preparation; and (C) the 7291 bp fragment of *E. coli* TOP 10 clone no. 2 from miniplasmid preparation.

To introduce the hybrid *cel48SAFA* gene from the pGR48A-*ES5*Δ*repL* into the mutant *Clostridium* chromosome, the clostridial cells were transformed with this plasmid by electroporation. Some clones were able to grow on RCA medium supplemented with thiamphenicol. These obtained clones were proved for the integration between the hybrid gene and chromosome by PCR analysis. The hybrid *cel48SAFA* gene from the pGR48A-*ES5*Δ*repL* plasmid was expected to be introduced into the chromosome of the *Cl. acetobutylicum* ATCC 824 Δ*cac1502* Δ*upp*Δ*cel48A* cell by single crossing over twice after the transformation (Figure 3.12). The result exhibited only the presence of plasmids in the cells by the appearance of 914 bp PCR fragement (Figure 3.13), but did not show the PCR size of the integration (Figure 3.14). Many attempts of transformation were performed by increasing the amount of plasmid DNA and varying different amount of thiamphenicol added to the selective RCA medium (Table 3.5). The result exhibited a few of the obtained clones, indicating the low transformation efficiency of obtained clones. Additionally, the obtained clones did not display the integration. Thus, a new plasmid would be constructed to improve the introduction of the hybrid celluase gene.

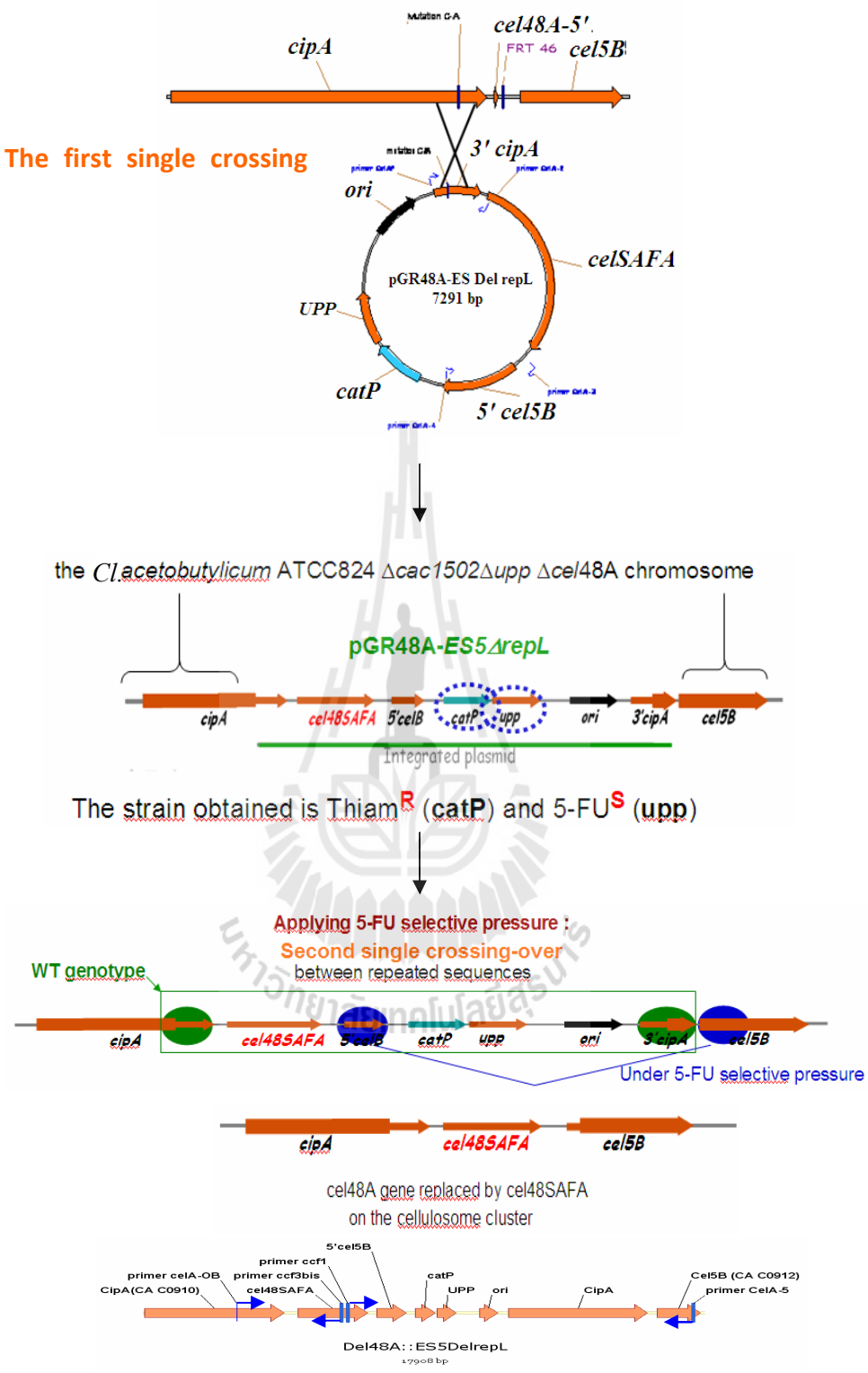


Figure 3.12 The introduction of *celSAFA* hybrid gene from the pGR48A-*ESΔrepL* plasmid into the chromosome structure of *Cl. acetobutylicum* ATCC824 *Δcac1502ΔuppΔcel48A* strain by single crossing over after the transformation.

Table 3.5 Results and conditions of electrophoration for transformation of pGR48A-*ES5ΔrepL* plasmid into *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp* Δ*cel48A* strain.

Item	Time no.				
	1	**2**	**3**	**4**	**5**
Preparing competent cell					
OD620 nm = 2.0	1.63	1.96	1.99	1.98	1.99
OD620 nm = 0.8-1.2	0.798	0.96	0.87	1.29	0.94
Transformation					
Time constant (mSec)	12.61	29.8	7.66	16.6	11.8
Amount of DNA plasmid (μg)	32	32	83	78	78
RCA medium +Thiam (μg/mL), transformant number	Thiam10, 0	Thiam10,0	Thiam5, 12	Thiam5, 9 Thiam10, 16	Thiam5,0

Abbreviations: Thiam, thiamphenicol antibiotic.

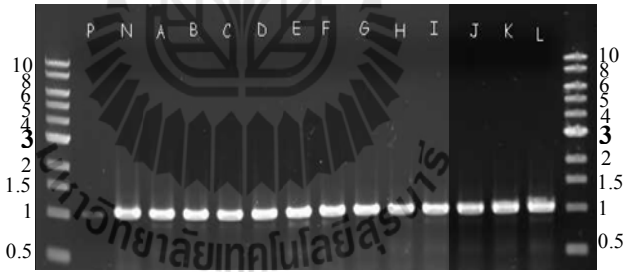


Figure 3.13 The presence of plasmid. The PCR products of pooled clones from the transformation of the pGR48A-*ESΔrepL* plasmid into *Cl. acetobutylicum* ATCC 824 *Δcac1502 Δupp* cell to analyze for the integation by PCR analysis by using *cac3* and *cac4* primers. Lanes; 1 and 16, 1 kb ladder (cat. no. Biolab, New England); Lane 2, pGR48A-*ESΔrepL* DNA plasmid as a negative control; Lane 3, genomic DNA of *Cl. acetobutylicum* ATCC 824 *Δcac1502ΔuppΔcel48* strain as a negative control; Lane 4-15, cell of pooled clones from transformantion.

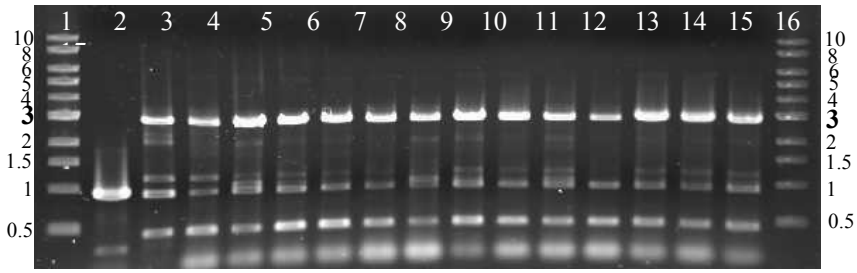


Figure 3.14 The presence of integration PCR confirmation of single-cross over hybrid gene inserted transformants with primers of oligo UPP-R and catP3'-D. No clones contained the single crossover recombination. Lane no1, 17, 10 kb DNA maker; Lane 2, positive control, pGR48A-*ES5ΔrepL* plasmid DNA; Lane 3, negative control, genomic DNA of *Cl. acetobutylicum* ATCC 824 Δ*cel48A* strain; Lane 4, negative control, genomic DNA of transformanted cells; Lane 5-16, clones obtaining from transformation.

3.3.4.2 Construction and transformation of pGR48A-*ES5ΔrepL::Pptb-catP* non-replicative plasmid into *Clostridium acetobutylicum* ATCC 824 *Δcac1502ΔuppΔcel48A* cell.

Based on the low transformation efficiency of pGR48A-*ES5 ΔrepL* plasmid, pGR48A-*ES5ΔrepL::Pptb-catP* plasmid was designed and constructed by insertion of *Pptb-catP* gene from pSCB-*Pptb-catP*-AZI_*EcoRVSacI* plasmid into pGR48A-*ES5ΔrepL* plasmid. Generally, the *Pptb-catP* gene as a strong promoter was used in our laboratory. This promoter was expected to be able to control the expression of *Pptb-catP* gene for screening clone. It could also enhance expression of cellulase hybrid gene.

The p*GR48A-ES5ΔrepL::Pptb-catP* plasmid was constructed by ligation of the *Pptb-catP* gene from pSCB-*Pptb-catP*-AZI plasmid and p*GR48-ES5 ΔrepL* plasmid (Figure 3.15). Both plasmids were digested by *EcoRV* and *Sac*. Following the pGR48A-*ES5ΔrepL::Pptb-catP* plasmids were transformed into *E. coli* TOP10 and spreaded onto the LB agar medium supplemented with chloramphenicol (20 μg/mL) as the selective medium. The result displayed a number of transformants appeared on the medium, indicating the presence of the plasmid in the cells. Four clones were chosen and digested with *EcoRV* and *SacI* for checking the correct size. The PCR result showed that the correct size of pGR48A-*ES5ΔrepL::Pptb-catP* plasmid appeared on all of the clones (Figure 3.15).

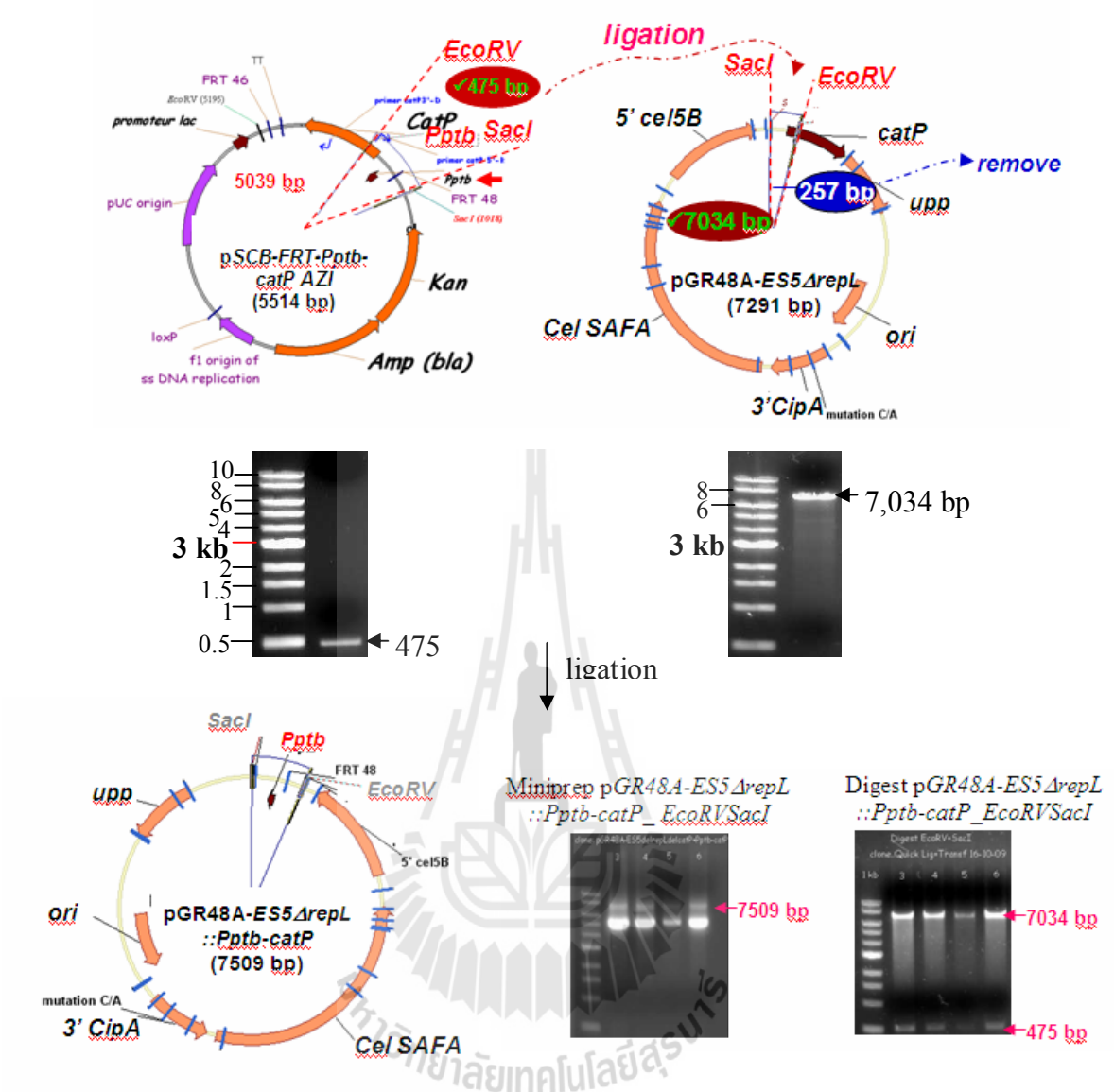


Figure 3.15 Construction of pGR48A-*ES5ΔrepL::Pptb-catP* plasmid.

For transformation, the amount of plasmid pGR48A-*ES5ΔrepL::Pptb-catP* was prepared by the plasmid minipreparation. The transformation of this plasmid was expected that the hybrid gene cassette would be inserted into the chromosome by double crossing over (Figure 3.16). After transformation, the result exhibited only one clone on RCA medium supplemented with thiamphenicol (5 μg/mL) and 5-FU (100 μM). Consequently, the clone was analysed for the integration and insertion by PCR. The results exhibited that clone did not show the insertion of the desired hybrid gene in the Δ*cac1502*Δ*upp*Δ*cel48A* chromosome (Figure 3.17). Besides, many transformations were carried out by vary the amount of DNA plasmid and the antibiotic concentration on the selective RCA medium, but none of the clones was able to grow on the medium (Table 3.6).

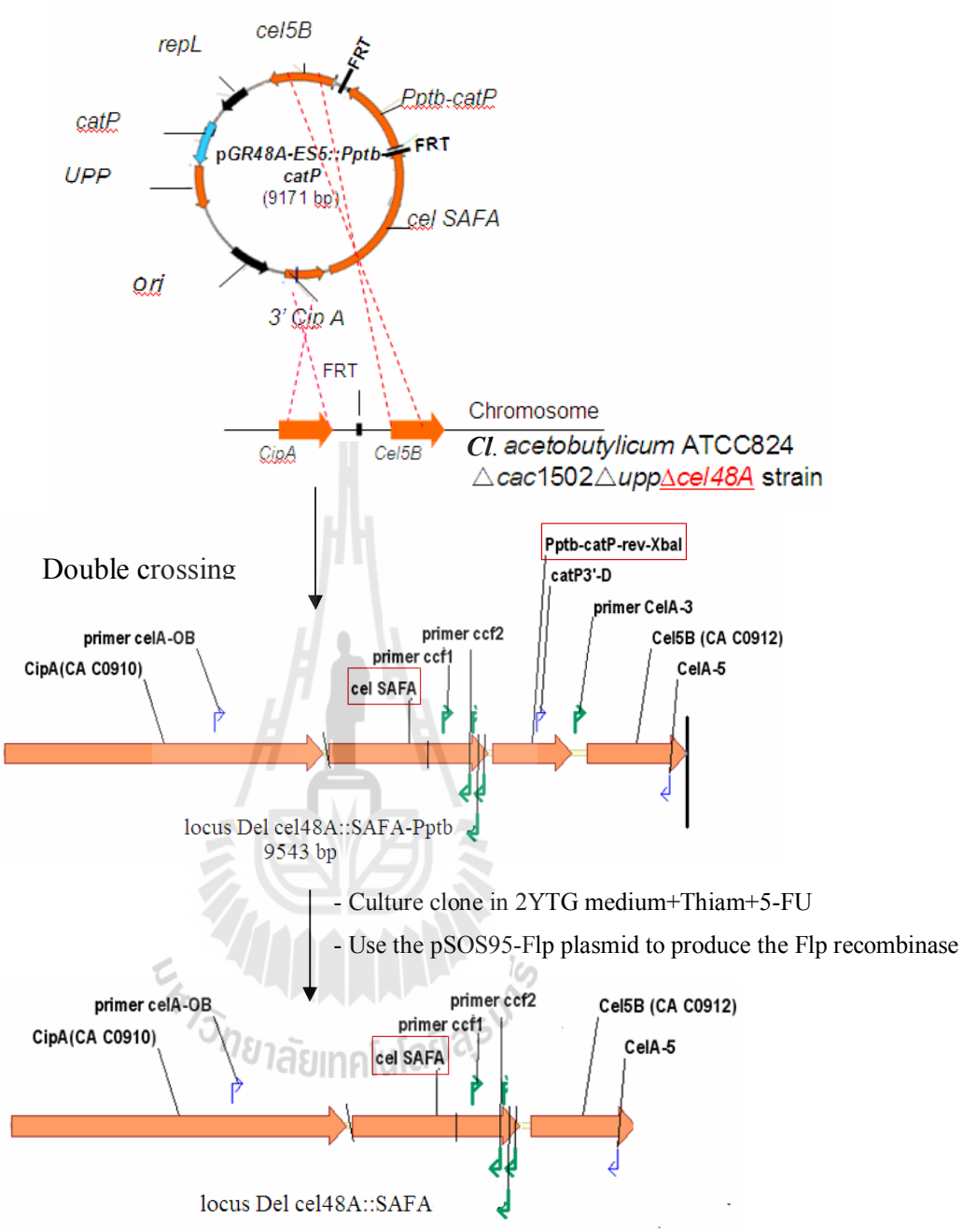


Figure 3.16 Model for the introduction of the *cel48SAFA* gene on the Δ*cac 1502*Δ*upp*Δ*cel48A* chromosome by double crossing over using pGR48A-*ES5ΔrepL::Pptb-catP* non-replicative plasmid.

Table 3.6 Conditions of electroporation for transformation of pGR48A-*ES5ΔrepL* plasmid into *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp* Δ*cel48A* strain.

Item	Time no.			
	1	**2**	**3**	**4**
Preparing competent cell				
OD620 nm = 2.0	2.17	2.09	1.96	2
OD620 nm = 0.8-1.2	0.83	0.95	0.85	0.93
Transformation				
Time constant (mSec)	16.3	15.8	29.7	16.7
Amount of DNA plasmid (μg)	157	157	100	100
RCA medium +Thiam (μg/mL), transformant number	Thiam5, NC Thiam10, NC	Thiam10,1	Thiam10, NC	Thiam10, NC
RCA medium +Thiam 10 μg/mL +5-FU 100 mM	-	Grow	-	-

NC, no clone appeared; -, no data.

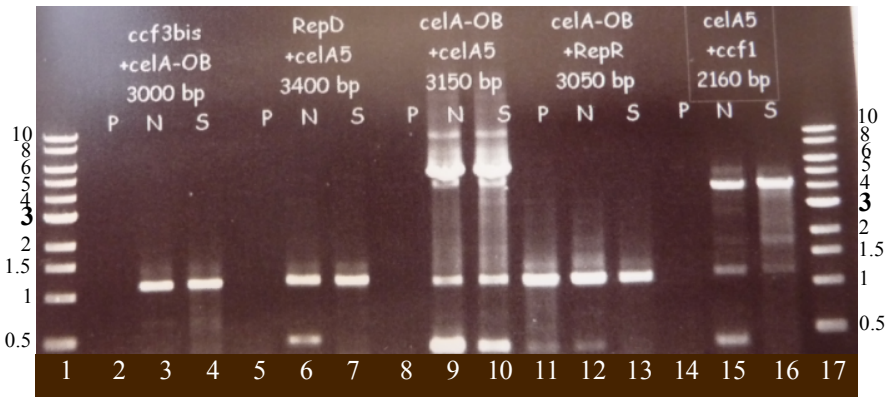


Figure 3.17 PCR confirmation of single-crossover hybrid gene inserted transformants with 5 pairs of primers 1) ccf3bis+celA-OB, 3 kb PCR product; 2) RcpD+celA5, 3.4 kb PCR product; 3) celA-OB+celA5, 3.15 kb PCR product; 4) celA-OB+RepR, 3.05 kb PCR product; 5) celA5+ccf1, 2.16 kb PCR product]. One clone did not show the PCR product indicating the single cross over recombination. Lane no.1 and 17, 10 kb DNA maker. Set of PCR products with ccf3bis+celA-OB: lane no. 2, P, positive control, pGR48A-*ES5ΔrepL::cel48SAFA::Pptb-catP* plasmid DNA; 3, N, negative control, genomic DNA of *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp* strain; 4, S, genomic DNA of transformant Set of PCR products with RcpD+celA5: lane no. 5, P; 6, N; 7, genomic DNA of transformant. Set of PCR products with celA-OB+celA5: lane no. 8, P; 9, N; 10, genomic DNA of transformant. Set of PCR products with celA-OB+RepR : lane no. 11, P; 12, N; 13, genomic DNA of transformant. Set of PCR products with celA5+ccf1: lane no. 14, P; 15, N; 16, genomic DNA of transformant.

To evaluate transformation efficiency of pGR48A-*ES5ΔrepL* plasmid and *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* competent cell, this plasmid was transformed into the competent cell (Table 3.7). The pGR48A-*ES5* plasmid and the ATCC 824 *Δcac3535Δcac1502Δupp* strain were used as control. The results demonstrated that no growth of the transformed cell on RCA medium supplemented with thiamphenicol, indicating the low efficiency of the plasmid. For further work, a new plasmid was designed by construction of pGR48A-*ES5::Pptb-catP* as replicative plasmid.

Table 3.7 The efficiency of pGR48A-*ES5ΔrepL* plasmid for transformation of *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A*.

Item	**Transformation with *Cl. acetobutylicum* competent cell**			
	ATCC 824 *Δcac3535Δcac1502Δupp* strain		**ATCC 824 *Δcac1502ΔuppΔcel48A* strain**	
Plasmid	pGR48A-*ES5 ΔrepL*	pGR48A-*ES5*	pGR48A-*ES5 ΔrepL*	pGR48A-*ES5*
Amount of DNA (*μ*g)	100, 100	100, 100	100, 100	100, 20
Time constant (mSec)	29.7, 29.5	10.2, 13.4	29.7, 16.7	10.2, 16.9
No. of clone on RCA+Thiam 5 μg/mL	NC, NC	>300, >300	NC, NC	>300, 22-27

NC, no clone appeared.

3.3.4.3 Construction and transformation of pGR48A-*ES5::Pptb-catP* replicative plasmid into *Clostridium acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* cell.

The p*GR48A-ES5::Pptb-catP* plasmid was constructed by ligation of the *Pptb-catP* gene from pSCB-*Pptb-catP*-AZI plasmid and p*GR48-ES5* plasmid (Figure 3.18). Both plasmids were digested by *NarI* and *ZraI*. The pGR48A-*ES5::Pptb-catP* plasmids were transformed into *E. coli* TOP10 and then spreaded on the LB agar medium supplemented with chloramphenicol (20 μg/mL). The result showed that a number of transformants appeared on the medium, indicating the presence of the plasmid in the cells. Some clones were chosen and digested with *NarI* and *ZraI*. The PCR result showed that the correct size of pGR48A-*ES5::Pptb-catP* plasmid appeared on all of the clones.

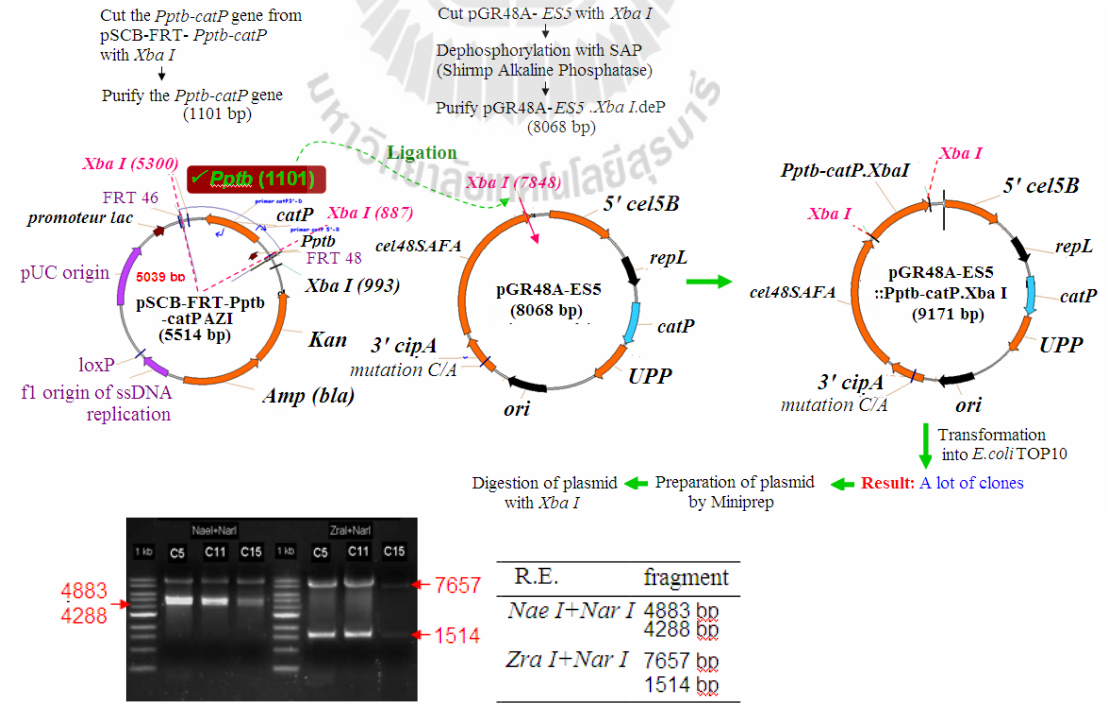


Figure 3.18 Construction of pGR48A-*ES5::Pptb-catP* plasmid.

The pGR48A-ES5*::Pptb-catP* replicative plasmid was introduced into *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* competent cell. The results displayed more 300 clones on RCA medium with 10 μg/mL thiamphenicol supplementation. These clones resisted to thiamphenicol ($Thiam^R$). Figure 3.19 illustrates model for introducing a hybrid gene *cel48SAFA* into the chromosome of *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* by double crossing over. Twenty transformants ($Thiam^R$) were chosen and restreaked on RCA medium supplemented with 10 μg/mL thiamphenicol as an original plate. Twelve of twenty transformed clones appeared on medium. In order to determine the desired hybrid gene integration on both right and left sides of the *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* chromosome of these putative clones, the polymerase chain reaction (PCR) technique with two of primer pairs was used. The primer pairs used were pairs of cat3'D+celA-5 and celA-OB+ccf2. Specific positions of primer binding were showed in Figure 3.5B. The expected sizes of the integrant right and left side loci were 1.9 and 3.5 kb, respectively. Two of the twelve screened clones (i.e. clone no. 7 and 17) exhibited two PCR product size of the adjacent gene, while none of the control strains did (Figure 3.20).

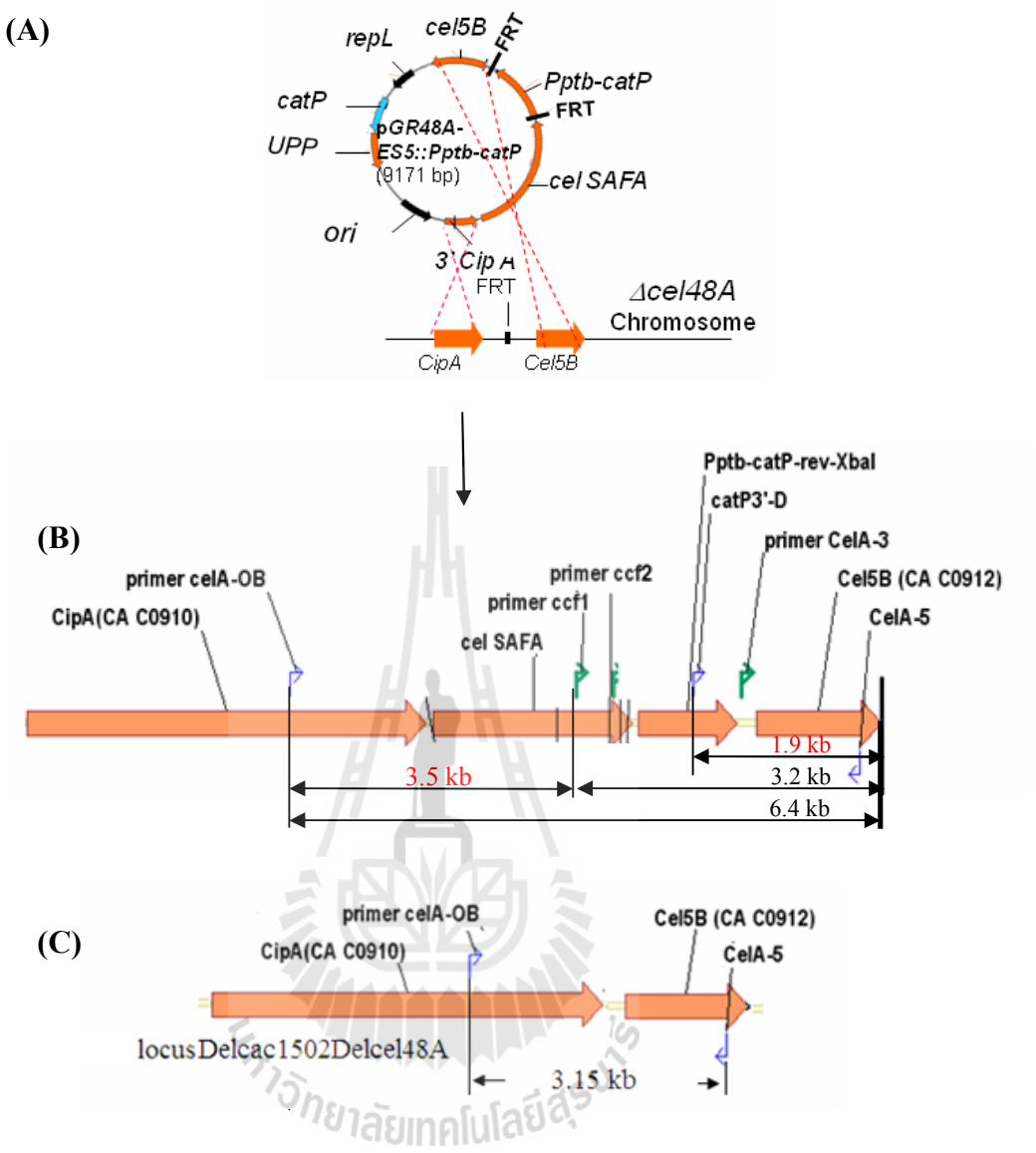


Figure 3.19 (A) Model for introduce a hybrid gene *cel48SAFA on the* Δ*cac1502* Δ*upp*Δ*cel48A chromosome* of *Cl. acetobutylicum* ATCC824 by double crossing over using pGR48A-ES5*::repL::Pptb-catP:: cel48SAFA* replicative plasmid. (B) The insertion of the desired hybrid gene on the *Cl. acetobutylicum* mutant chromosome. (C) Gene structure of the ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* as a negative control.

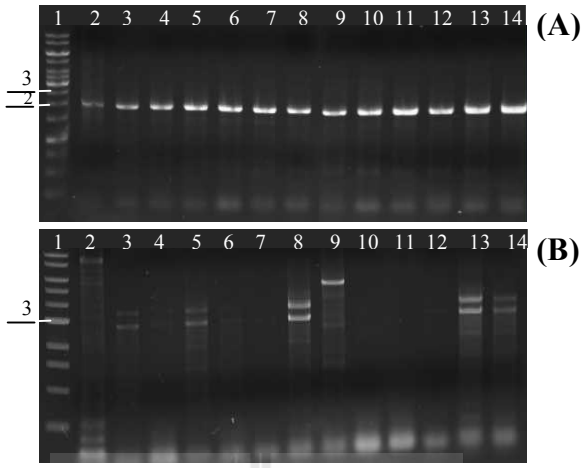


Figure 3.20 PCR screening and confirmation of integration transformants with primers indicated in panel (Figure 3.6.B and 3.6C). The expected sizes of the integrant loci were 1.9 and 3.5 kb. The expected size of the inserted locus was 6.4 kb. (A) the left integration (1.9 kb). (B) the right integration (3.5 kb). Lane no. 1, 10 kb DNA ladder. Lane2, negative control, the pGR4A-ES5*:: repL.::Pptb-catP::cel48*SAFA plasmid. Lane no./clone no.: 3/1, 4/2, 5/3, 6/4, 7/5, 8/6, 9/7, 10/8, 11/9, 12/10, 13/11, 14/12. Clone 7 and 17 was chosen according to the size of PCR products showing the integration size of PCR products (A) 1.9 kb (B) 3.5 kb.

To further reexamine for the integration of putative integrant, the chosen clone 7 was isolated and screened again by reculturing in liquid 2YTG medium without antibiotic and spreading onto the solid RCA medium with 10 μg/mL thiamphenicol and 100 μM 5-FU. Then, twenty three picked clones were screened for the integration by PCR. One screened clone (code 7.9.3) showed the expected three size bands of integration as 1.9, 3.2, and 3.5 kb (Figure 3.21).

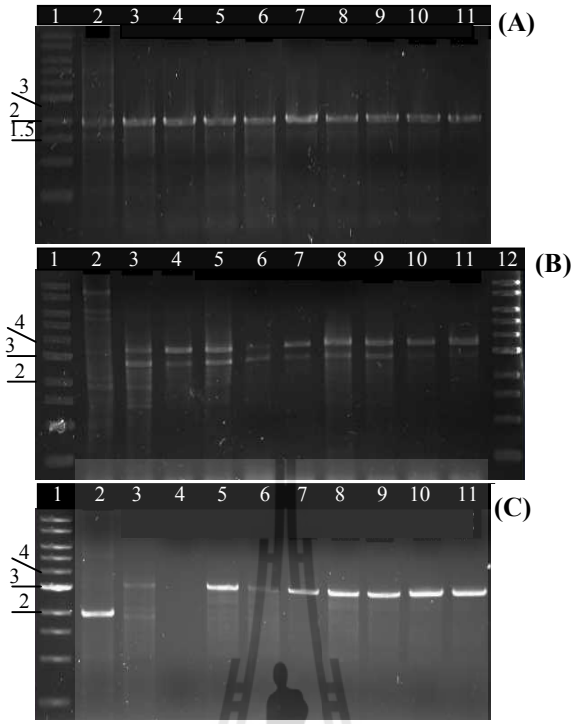


Figure 3.21 Clone 7.9.3 (A) 1.9 kb integration of the right side of the hybrid gene. (B) 3.2 kb integration of the right side of the hybrid gene. (C) 3.5 kb. integration of the left side of the hybrid gene. Lane no.1, 10 kb DNA marker. Lane no. 2, negative control, pGR4A ES5*::repL::Pptb-catP::cel48*SAFA. Lane no./clone no.: 3/7.1.3, 4/7.2.3, 5/7.3.3, 6/7.4.3, 7/7.5.3, 8/7.6.3, 9/7.7.3, 10/7.8.3, 11/7.9.3.

In order to confirm the desired recombinant strain again, the clone no. 7.9.3 was retested for the resistance of thiamphenicol and 5-FU. Two picked clones [i.e. code 7.9.3(1) and 7.9.3(2)] were examined for morphology by microscope, and analysed for the integration and the insertion of the desired active cellulase hybrid gene by PCR. Under the microscope, the cell of both clones showed rod shape. The only clone no. 7.9.3(1) displayed three sizes of the expected PCR products indicating

the integration and the insertion of the hybrid gene. However, the inserted PCR band was very pale. Therefore, the integration and insertion of the desired gene with the clone no. 7.9.3(1) was rechecked by picking the clone no. 7 from the original plate and the clone no. 7.9.3 from the master plate. Many attemps were performed to verify the integration and insertion of the hybrid gene, however, the PCR results displayed the unclear band of the 6.4 kb inserted fragment. This implied that none of the clones was the desired recombinant strain. Consequently, the pGR48A-*ES5* replicative plasmid was used for introduction.

3.4 Discussion

Cl. acetobutylicum ATCC 824 have been employed for the commercial production of acetone and butanol. It could also be an appropriate candidate for genetic engineering the solventogenic-cellulolytic bacterium, which could utilize cellulosic material to produce solvents. Taking this into account, some reports was successful to introduce the genes encoding miniscaffoldins or a mannanase-miniscaffoldin complex from the designed introducing plasmid into *Cl. acetobutylicum* (Perret *et al.*, 2004; Mingardon *et al.*, 2005). The obtained recombinant strain could secrete the cellulase enzymes in the supernatant. In this respect, the ATCC 824 strain was expected to be a suitable solventogenic host for introducing the *SAFA* hybrid gene encoding the active cellulase enzymes by electrotransformation in the present study.

In this work, four designed artificial plasmids containing the *SAFA* hybrid gene were designed and to integrate the hybrid SAFA gen into the chromosome of *Cl. acetobutylicum* ATCC 824 strain. The chromosome of this recombinant strain was methylated. It was also deleted the *cac1502*, the *upp*, and *cel48A* genes (encoding the inactive cellulose). These plasmids were pGR48A-*ES5ΔrepL*, pGR48A-ES5*ΔrepL::*

Pptb-catP, pGR48A-*ES::Pptb-catP*, and pGR48A-*ES5*. The SAFA hybrid gene was cloned in the plasmid downstream of the strong and constitutive promoter *Pptb* gene encoding phosphotransbutyrylase. The *catP* gene encoding thiamphenicol resistance was used as a reporter system for mornitoring the expression of *Pptb* promoter. In experiment, each artificial introducing plasmid was transformed into recombinant ATCC 824 cells. After transformation, only a few clones obtained from transformation of the recombinant ATCC 824 strain with pGR48A-ES5*ΔrepL::Pptb-catP*, whereas the transformation of the recombinant strain with other plasmids did not generate any thiamphenicol-resistant clone. The obtained clones can grow on medium containing thiamphenicol and 5-FU at 37ºC. The PCR results displayed that these clones contain the pGR48A-ES5*ΔrepL::Pptb-catP*, but they did not show the integration and insertion of hybrid cellulase gene in the recombinant ATCC 824 chromosome after several attempts of transformation. Therefore, the obtained results from transformation with pGR48A-ES5*ΔrepL::Pptb-catP* was selected to clarify the causes of the failure for the integration and insertion.

First, possible explanations are efficient integrational plasmids and frequency of transfer of plasmid to the *Cl. acetobutylicum* host cell for electrotransformation. The integrational plasmids are extremely powerful tools to detect homologous recombination involving Gram-positive bacterial chromosomes (Wilkinson and Young, 1994). Generally, an integrational plasmid contains a desired gene together with gene marker being used to select the desired recombinant clones after transformation. The integrational plasmids have to be non-replicative plasmids to prevent the occurrence of the plasmid loss in the host cells. Moreover, plasmid sequences at the target site have to be flanked by two directly homologous region of

the host replicon. One importance is the effect of frequency of plasmid transfer on the occurrence of genetic exchange. The high recovery of transformants, containing non-replicative plasmids inserted into the bacterial chromosome, is based on the frequency of sufficiently transfer of plasmid into host cell (Wikinson and Young, 1994). For example, the frequency of plasmid mobilization for electrotransformation of the *Cl. acetobutylicum* NCIMB 8052 strain was 10^3 cells per μg plasmid DNA. Commonly, *Cl. acetobutylicum* ATCC 824 is closely related to the NCIMB 8052 strain. The used pGR48A-ES5*ΔrepL::Pptb-catP* in this experiment has a property according to the detail above. Additionally, 80-100 μg of the plasmid DNA was used for electrotransformation. The frequency of pGR48A-ES5*ΔrepL::Pptb-catP* plasmid transformation was investigated. It showed low transformation frequency compared with the pGR48A-ES5 plasmid control which is a replicative plasmid. The PCR results exhibited the presence of this plasmid in the recombinant *Cl. acetobutylicum* cells, but they did not show integration. Some studies suggested that recombination frequency was affected by the length of the region of homology between the inserted DNA fragment from the integrational plasmid and the chromosomal DNA from host cell (Vagner and Ehrlich, 1988; Biswas *et al.*, 1992; Wilkinson *et al.*, 1994). As the results for pGR48A-ES5*ΔrepL::Pptb-catP* plasmid transformation, it implied that some of the pGR48A-ES5*ΔrepL::Pptb-catP* gene structure may not be suitable for the integration.

Second, the potential of the promoter contributed to low level of gene expression in *Cl. acetobutylicum*. In fact, for the development of artificial genetic systems, a limiting factor is the availability of promoter elements with potent activity in transformed cells. Process of transcription in DNA-based RNA systems can be

driven by stronger promoters. In addition to introducing plasmid DNA into *Cl. aceetobutylicum*, a gene expression reporter system is one of the critical tools for the metabolic engineering (Tummala *et al.*, 1999). The reporter system has been used to monitor the strong activity of promoter by observing from the transcriptional level (Quixley and Reid *et al.*, 2000). An understanding of promoter strength and regulation results in more effective clostridial expression vector. In the present work, the reporter system of pGR48A-ES5*ΔrepL::Pptb-catP* plasmid contained strong *Pptb* promoter and *catP* reporter gene controlling gene expression in *Cl. acetobutylicum* ATCC 824. A chloramphenicol acetyltransferase gene (*catP*) from *Cl. perfringens* was a good candidate for potential use as a reporter gene for *Cl. acetobutylicum*. The *catP* gene used in this study was reported by many studies as an effective reporter gene of *Clostridium.* For example, the *catP* gene from *Staphylococcus* plasmid pC194 has been used to estimate the activity of the *Cl. pasterurianum* ferrodoxin promoter (Minton *et. al.,* 1993). The *catP* has been used to monitor the *Cl. perfringens* alpha-toxin-encoding *plc* gene (Matsushita *et al.*, 1994; Bullifent *et. al.,* 1995) Notwithstanding, *Cl. acetobutylicum* contains high levels of nonspecific coenzyme A transferases which might interfere with the chloramphenicol acetyltransferase assay (Tummala *et al.* 1999). It was considered that effective regulatory mechanisms controlling gene expression differ extremely from one bacterium to another (Girbal *et al.*, 2003). Additionally, a method for integrating DNA into the chromosome depends on coupling the expression of a promoterless antibiotic resistance marker to that of the constitutively expressed gene promoter (Al-Hinai *et al.*, 2012). In this study, since SAFA cellulase gene did not express, it may be possible that the coupling of these gene did not fit for introducing SAFA gene from pGR48A-ES5*ΔrepL::Pptb-catP*

plasmid into the recombinant *Cl. acetobutylicum* chromosome. Nevertheless, some studied reported the available strong promoters and gene expression reporter systems for *Clostridium*, which were successfully used. For instance, the couple of the gene reporter and the strong gene promoter used in the gene expression reporter systems for *Cl. acetobutylicum* ATCC 824 was 1) the *Escherichia coli* reporter *gusA* expression under the control of *Clostridium* promoter *thlA* encoding thiolase (Girbal *et al.*, 2003), 2) the reporter *adc* gene coding for acetoacetate-decarboxylase and the promoter *thlA* gene (Mingardon *et al.*, 2005), and 3) the *E. coli* reporter *lacZ* gene coding for acetoacetate-decarboxylase and the *Cl. pasteurianum* promoter *recA* gene (Heap *et al.*, 2012).

Third, the evidence of no clones exhibited the insertion of the target gene in *Cl. acetobutylicum* chromosome may result from the lack of complementary scaffolding or cohesion in *Cl. acetobutylicum* (Mingardon *et al.*, 2011). For example the cohesion-containing protein OrfXp gene plays the role for the cellulosome assembly (Pagés *et al.*, 1999). This gene is presented in the central part of the large *cip-cel* cluster of *Cl. cellulolyticum*.

Forth, a possible explain is that the precursor of the *SAFA* hybrid gene may induce a blockage of the secretory system. This led to the effect of the toxicity of its Cel48F cellulase on the transformant's growth. Mingardon *et al.* (2011) were successful to construct a genuine celluloytic *Cl. acetobutylicum* secreting two miniscaffoldins after introducing the genes coding for the cellulosomal cellulase from *Cl. cellulolyticum* into *Cl. acetobutylicum* chromosome. They demonstrated the toxicity of its Cel48F cellulase to the transformant's growth. They had verified the impact of these by introducing pSOS952-*cel48F* deleting the native Cel48F signal

sequence to *Clostridium* cells. The pSOS952-cel48F plasmid containing Cel48F signal was used to be a plasmid control. The electrotransformation of *Cl. acetobutylicum* with control plasmid provided a number of clones, while no clones obtained from introduction of plasmid lacking the cellulase signal.

Finally, the fact is that *Cl. acetobutylicum* lacks the chaperone gene in the translocation system. This chaperone protein involves in the prevention of protein folding and targeting the proteins to the Sec translocon. Thus, the overexpression of cellulase gene was harmful to the cells. The lack of the secretory system results in the loss of the cellulotytic phenotype of *Cl. acetobutylicum*. It may be possible for the formation of clone if the corresponding chaperone gene will be coexpressed afterwards with the *SAFA* hybrid cellulase gene in the *Cl. acetobutylicum* cell.

3.5 Conclusion

The *SAFA* hybrid gene coding for the active cellulases could not express in the *Cl. acetobutylicum* due to several reasons including the genetic tools and the unique feature of this strain. The failure may be due to the effectiveness of integrational or replicative plasmids involving the homologous DNA sequence of the designed hybrid gene structure for crossing over with the partial gene on chromosome, the type of promoter, the gene expression reporter system, or the secretion machinery system of *Cl. acetobutylicum*. Further works should be carried out to prove for the above mentioned reasons in order to develop a new cellulolytic-solventogenesis *Cl. acetobutylicum* strain.

CHAPTER IV

EFFECT OF PHYSICOCHEMICAL PRETREATMENT OF RICE STRAW ON ENZYMATIC HYDROLYSIS BY *CLOSTRIDIUM CELLULOLYTICUM* FERMENTATION

4.1 Introduction

Rice straw is a biopolymer consisting of cellulose (38.3%), hemicellulose (28%), lignin (14.9%), and other minor components (18.80%) (Zhang and Cai, 2008). It is converted to sugars which can subsequently be fermented to desired products by suitable microorganisms as biocatalysts. A variety of pretreatment methods have been developed to remove hemicellulose and/or lignin. It is the key factor to increase the accessible surface area of cellulose for microbial and enzymatic attacks. A chemical pretreatment (i.e. acid dilution and alkaline pretreatment) is a promising method. This pretreatment can break down lignin and hemicellulose that surround cellulose fraction in lignocelluloses composite material. Nevertheless, these methods cause problems in a slow cellulose digestion by the enzyme or microbial attack and low sugar yields (Moiser *et al*., 2005). It could cause the lignin to condense on the cellulose surface. It can potentially block enzyme accessibility to cellulose for sugar production (Zhu *et al*., 2009). It is well known that the cost of enzymes is the most important factor in the production cost of industrial chemical production based on the lignocellulosic materials.

The aims of this chapter were thus (1) to investigate the synergistic effects of combined sodium hydroxide (NaOH) and liquid hot water (LHW) pretreatments or sulfuric acid (H_2SO_4) and liquid hot water pretreatments with reducing particle size (S) of rice straw under the pressurized steam on the modification of the cellulose microstructure, and (2) to investigate the effect of heat retention time in the process of LHW pretreatment on the digestibility of the alkaline pretreated rice straw of cellulose fermentation by *Clostridium cellulolyticum* DSM 5812.

4.2 Materials and Methods

4.2.1 Raw materials

Rice straw obtained from the Suranaree University of Technology Farm in Nakhon Ratchasima province, Thailand, was dried at 65°C until constant weight. The dried rice stalks were chopped into 20-25 mm lengths prior to grinding to average 10 mm size. The rice straw was then milled to pass through a 0.5 mm sieve screen. The rice straw samples were kept at room temperature until use.

4.2.2 Microorganism and inoculum preparation

Cl. cellulolyticum DSM 5812 was purchased from Leibniz Institute Deutsche Sammlung von. Mikroorganismen und Zellkulturen GmbH (DSMZ), the German collection of microorganisms and cell cultures, Germany. Spores were heat shocked at 80℃ for 10 min in CM3 medium containing 3 g/L of cellobiose. The heat-shocked spores were incubated at 35℃ for a few days (4-5 days). Ten percentages of actively motile cell were transferred to 80 mL of CM3 medium supplemented with 3 g/L of cellobiose. The CM3 medium consisted of $(NH_4)_2SO_4$ 1.3 g/L, KH_2PO_4 1.5 g/L, K_2HPO_4 2.9 g/L, $MgCl_2.6H_2O$ 0.2 g/L, $CaCl_2.2H_2O$ 0.075 g/L, $FeSO_4.7H_2O$ 1.25

mg/L, yeast extract 2 g/L and resazurin 0.001 g/L and 48 mM MOPS with trace elements. Medium pH was adjusted to 7.2. The culture medium was heated and then sparged with oxygen free nitrogen gas for 15 min to ensure anaerobiosis. The medium was sterilized at 121 ℃ for 15 min. Cellobiose was filtered prior to adding to 80 mL of CM3 medium.

4.2.3 Alkaline pretreatment method

Sodium hydroxide (NaOH) at concentration of 2% (w/v) was used to pretreat 50 g of each milled, grinded and chopped rice straw at a solid to liquid ratio of 1:6. Each reaction mixture was performed under the mild operating condition at 121°C or 15 psi for 60 min. Thereafter, the NaOH treated rice straw solid fraction was collected by filtering through cotton cloth. The treated solid fraction was washed with tap water until the pH of the washing-off water was neutral. The pretreated solid fraction was then dried at 65°C until constant weight. After pretreatment, the reduced dried weight of rice straw was calculated for the final cellulose yield. The dried pretreated rice straw was stored at room temperature until further use. These rice straw solids were used as cellulose substrates for cellulose fermentation by cellulolytic *Cl. cellulolyticum* DSM5812.

4.2.4 Acid pretreatment method

Fifty grams of grinded rice straw were pretreated with 0.5, 1, 3, and 5% v/v sulfuric acid (H_2SO_4) at a solid to liquid ratio of 1:8 under at 121°C or 15 psi for 30 min.

4.2.5 Liquid hot water pretreatment method

Liquid hot water (LHW) pretreatment was performed in a 100 mL serum bottle. Each bottle contained a mixture of chemical pretreated rice straw and water at a

solid to liquid ratio of 1:7. The slurry mixture was heated at 121ºC for 30-120 min. After LHW pretreatment, the pretreated solid slurry was used to prepare CM3 medium.

4.2.6 Fermentation

Batch fermentation experiments were performed in 100 mL serum bottle under anaerobic conditions. Anaerobic conditions in the serum bottle were generated by sparging with an oxygen-free nitrogen gas**.** The 10% inoculum of exponentially active motile culture was added to the medium. The pretreated rice straw was fermented by *Cl. cellulolyticum* DSM 5812 in 80 mL of CM3 medium with 7 g/L of pretreated rice straw as substrate at 34°C and agitation at 150 rpm for 14 days. Fermentation broth was sampled every 24 hr. The broth was centrifuged at 13,400 rpm for 5 min. The supernatant was kept at -80°C for further analysis.

4.2.7 Experimental design

The purpose of this work was to investigate the pretreatment methods that enable the cellulose structure accessible for further hydrolysis to fermentable products by the fermentation of *Cl. cellulolyticum* DSM 5812. The following different pretreatment conditions were investigated: the chopped rice straw particle size (0.5, 10, 25 mm) with 2% NaOH pretreatment, and the diluted sulfuric acid concentration (0.5%, 3%, 5%). Next, the heating residence time (0, 30, 60, 90, 120 min) for LHW pretreatment was examined. Schematic of the experimental design illustrated in Figure 4.1. These pretreated rice straw solid fraction were used as cellulose substrate for subsequent fermentation. The sugars yield and the end products obtained from the fermentation of these solid fractions were compared.

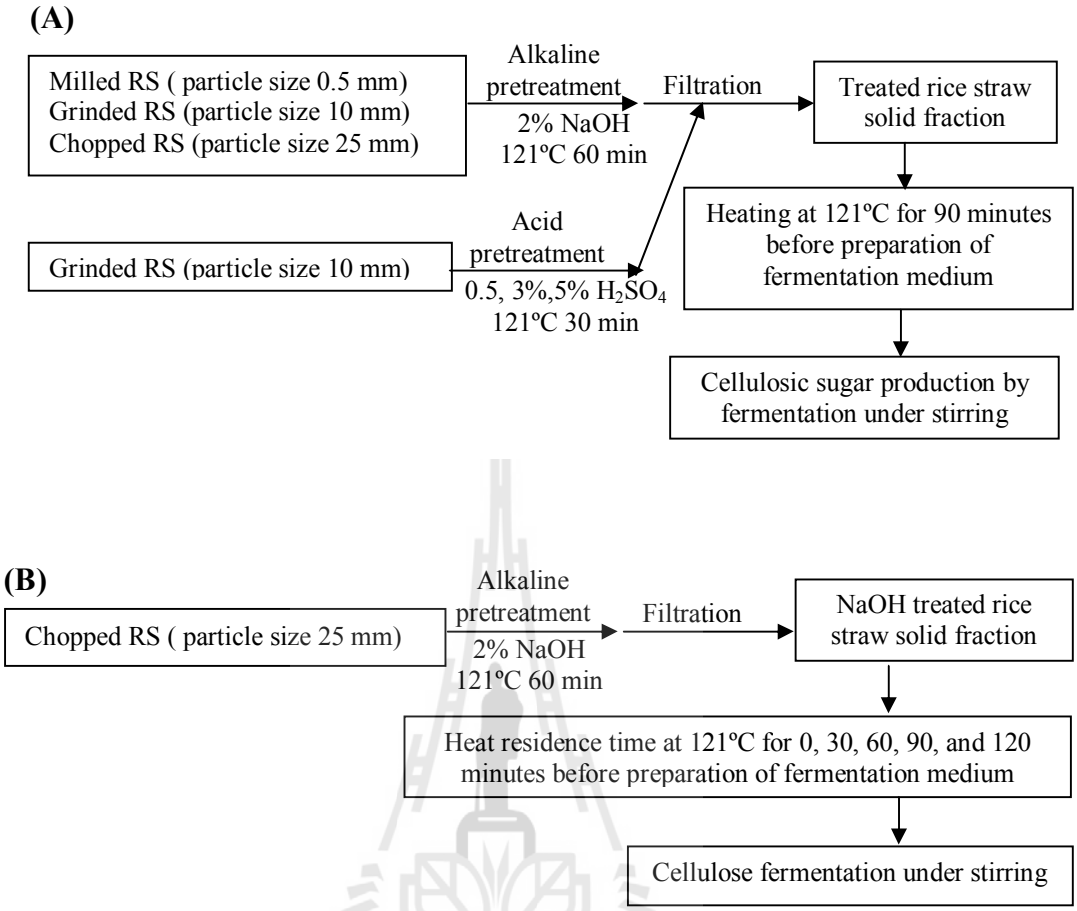


Figure 4.1 Schematic diagram of the experimental design for cellulosic sugar production by *Cl. cellulolyticum* DSM 5812. (A) the effect of the particle size and the different chemical treatments on the quality of treated RS for cellulose fermentation and (B) the effect of the heat residence time in NaOH-LHW-S pretreatment.

4.2.8 Chemical analysis

All the fermentation broth samples were centrifuged at 13,400 rpm and filtered through 0.2 μm filter membrane. The concentration of sugars and fermentation products were quantitatively analyzed by HPLC (Algilent 1200 series) equipped with refractive index detector. The supernatant was analysed by using an Aminex HPX-

87H ion exchange column (Bio-Rad, Richmond, USA) maintained at 45°C with 4 mM H_2SO_4 as the mobile phase.

The residual insoluble cellulose was harvested by centrifugation at 10,000 rpm for 20 min. The supernatant was drawn off gently. Solid fraction was dried at 65°C until constant weight. The reduced dried weight of rice straw was noted and calculated for the final cellulose degradation.

The morphology of the untreated and pretreated rice straw structures under different alkaline or dilute acid pretreatments was visualized by the scanning electron microscope (SEM) model JSM-6400 SEM (Japan).

4.3 Results and Discussions

4.3.1 Effect of acid pretreatment on cellulose conversion

The objective of this experiment was to investigate the effects of sulfuric acid (H_2SO_4) concentration on rice straw pretreatment and end products from the microbial digestibility of different dilute acid pretreated rice straws by the fermentation of *Cl. cellulolyticum* DSM 5812. The grinded rice straw (10 mm) was hydrolyzed in different H_2SO_4 concentrations (0.5%, 3% and 5%v/v) under the saturated steam at 121ºC or 15 psi for 30 min. The sugar accumulation from the digestion of the pretreated rice straws was recorded from the fermentation of *Cl. cellulolyticum* during 14 days.

The results revealed that increasing in the H_2SO_4 concentration resulted in low levels of total residual sugars yield, total organic compounds and low percentage of rice straw degradation (Table 4.1). This would imply that the acid pretreatment of rice straw may not be suitable for modifying the complex structure of cellulose. This

result is obviously shown that acid pretreatment was only able to disrupt the lignocellulosic structure by mainly dissolving its hemicelluloses and partially delignification, whereas the structure of cell wall was not disrupted (Silverstein *et al*. 2007; Li *et al*., 2010). In addition, the cause of the presence of lignin, condensed lignin-carbohydrate complex and acetyl groups remained on the crystalline cellulose surface after acid pretreatment may inhibit the cellulase attack on cellulose (Zhu *et al*. 2009).

Moreover, the nonproductive binding of cellulases to the lignin component has been attributed to depress cellulose hydrolysis rate by the whole cellulase complex (Berin *et al*., 2005). Therefore, the acid pretreated rice straw was not suitable for subsequent enzymatic hydrolysis. In addition, the results were clearly shown that the glucose concentration were higher than xylose concentration in all of the treatments (Table 4.1). This suggested that the hemicellulose fraction was only depolymerized by the acid hydrolysis and the structure of major microfibrous cellulose still remained.

For liquid hot water (LHW) pretreatment of rice straw, the digestibility of LHW pretreated rice straw was slightly higher than that of diluted acid RS during the fermentation by *Cl. cellulolyticum* DSM 5812. It may be possible that water and organic acids, which were generated from acetyl groups within hemicelluloses during the LHW hydrolysis, act together as a weak acid. Thus it makes this process effects the process of the dilute acid hydrolysis (Wan and Li, 2011; Abril *et al.,* 2012). Typically, the released acetyl groups are the form of acetic acid as an acid catalyst in the hydrolysate for the disruption of the pretreated lignocellulosic materials (Kim *et al*., 2009; Yu *et al*., 2010). It is also reported that the LHW pretreatment could attribute to the liberation of low molecular weight soluble materials and the partial

depolymerization of hemicellulose fraction at lower temperature than the cellulose fraction (Ibbett *et al*., 2011). In addition to this, the presence of acetyl groups and lignin in the acid pretreated solid residue after washing step results in cellulase inhibition. Moreover, some reports have suggested that most of the lignin was not removed from rice straw by dilute acid pretreatment (Hsu *et al*., 2010). Therefore, it suggested that the cell wall structure of rice straw was modified by steam pressured pretreatment, and the dilute acid pretreatment could result in decreasing in its recalcitrance for the fermentation of *Cl. cellulolyticum* DSM 5812.

Table 4.1 The combined effect of physicochemical pretreatments of rice straw on the end products and the percentage of degradation of pretreated rice straw in cellulose fermentation by *Cl. cellulolyticum* DSM 5812.

Pretreatment	Size of rice straw (mm)	Residual sugar concentration (g/L)					Fermentable Products concentration (g/L)					% Degradation
		Cellobiose	Glucose	Xylose	Arabinose	Total residual sugars	Succinic acid	Lactic acid	Formic acid	Acetate	Ethanol	Total products	
2% NaOH +LHW	0.5	0.17±0.02	0.07±0.01	0.213±0.02	0.030	0.47±0.06	0.03	1.35±.0.02	0.96±0.09	1.14±0.08	0.78±0.03	4.26±0.14^{a}	77.35±0.38$^{\alpha}$
2% NaOH +LHW	10	0.34±0.02	0.26±0.02	0.276±0.06	0.030	0.90±0.01	0.03	1.52±0.07	0.930	1.15±0.08	0.81±0.03	4.44±0.18^{b}	85.53±0.49$^{\beta}$
2% NaOH +LHW	25	0.42±0.02	0.34±0.01	0.322±0.02	0.030	1.12±0.06	0.02	1.66±0.04	1.10±0.01	1.18±0.07	0.84±0.01	4.79±0.12^{c}	82.34±0.59$^{\gamma}$
0.5% H_2SO_4 +LHW	10	0.00	0.13±0.01	0.01	0.00	0.14±0.02	0.05	0.27±0.01	0.21±0.01	1.01±0.02	0.30±0.02	1.85±0.06^{d}	48.17±0.67$^{\varepsilon}$
3.0% H_2SO_4 +LHW	10	0.00	0.13±0.01	0.00	0.00	0.12±0.01	0.05	0.23±0.02	0.16±0.01	0.59±0.07	0.13±0.01	1.16±0.11^{e}	38.24±0.54$^{\delta}$
5.0% H_2SO_4 +LHW	10	0.00	0.12±0.01	0.00	0.00	0.12±0.01	0.04	0.16±0.02	0.160	0.66±0.05	0.020	1.04±0.07^{e}	39.21±0.45$^{\eta}$
H_2O	10	0.00	0.13±0.01	0.00	0.00	0.14±0.01	0.04	0.14	0.25±0.02	1.14±0.08	0.31±0.01	1.87±0.09^{d}	47.14±0.35$^{\kappa}$

- All data represent the averages of two fermentations with standard deviation.
- The value of the total fermentable products bearing different font symbol are significant difference (p<0.05).
- The value of the percentage of degradation bearing different Greek symbol are significant difference (p<0.05).

4.3.2 Effect of alkaline pretreatment on cellulose conversion

In order to obtain the physicochemical pretreatment which have been used to render rice straw to be more susceptible for cellulolytic microbial or enzymatic hydrolysis, experiments were carried out to investigate the combined effect of physical size reduction and alkaline pretreatment on microbial conversion to sugars. The milled, ground and chopped rice straws were pretreated by heating with 2% NaOH at 121ºC for 60 min before it was used as cellulose substrates for the fermentation of *Cl. cellulolyticum* DSM 5812.

As shown in Table 4.1, the alkaline pretreated chopped rice straw was more suitable substrate than that of fine rice straw in enhancing cellulose fermentation by *Cl. cellulolyticum* DSM 5812 to sugars production and cellulose degradation. Possible explanation for this could be that the good aeration between the stalks of chopped rice straw absorbed culture medium by capillary action during fermentation more than that of the fine rice straw saturated with culture medium (He *et al*., 2006). On the other hand, the pretreated fine rice straw had little effect on cellulose degradation compared with the milled and chopped RS. It was possible that the physicochemical pretreatment may break the open parts in the rice straw structure. These parts of the rice straw may be non-lignified yet. Therefore, *Cl. cellulolyticum* DSM 5812 and cellulase produced was unable to penetrate. Cellulases were also adsorbed to the lignified areas of cellulose resulting in unable to hydrolyze the cellulose. Moreover, opening up pores by the hydrolysis of the xylan resulted in an increased in xylose released, whereas it caused agglomeration and the exposed surface area of cellulose to vary in a complex manner affecting cellulase's adsorption and the rate of hydrolysis (Coverse *et al*., 1990).

Total sugars yield and percentage of cellulose degradation resulted from the fermentation of all different pretreated particle size rice straws with NaOH were typically higher those of the hydrothermal and acid pretreated rice straw. These results were not surprising because sodium hydroxide have commonly been used to pretreat lignocellulosic materials for delignification by breaking the ester bonds cross-linking lignin and xylan and cleavage of glycosidic linkages in the lignocellulosic cell wall matrix. This led to the increasing in the porosity of biomass due to the alteration of the structure of lignin, the reduction of the lignin-hemicellulose complex, cellulose swelling and partial decrystallization of cellulose (He *et al*., 2008; Cheng *et al*., 2010). These results were supported by other studies reported on NaOH pretreatment of other lignocellulosic materials on the removal of lignin and hemicelluloses. For example, Verma *et al*. (2011) reported 95% reduction in lignin content as a result of pretreatment of corn stover with 10% NaOH for 1 h in the autoclave. The high reduction level may be attributed to a higher NaOH concentration of 10%, which in thier study was limited only to 2%. The maximum reduction in lignin was 65.63% with 2% NaOH treatment for 90 min at 121ºC (15 psi). Sodium hydroxide pretreatment resulted in xylan solubilization in the range of 13.90% (0.5%, 90 min, 90ºC) to 40.02% (2%, 90 min, 90ºC). Koullas *et al*. (1992) showed that alkaline delignification of wheat straw was a very strong pretreatment method, since it can increase by four times the maximum enzymatic conversion. Overall, NaOH pretreated RS enhanced microbial hydrolysis or cellulose fermentation efficiency when compared with the dilute acid pretreated RS as previously discussed.

An interesting observation in this study was that xylose was the main component in the fermented broth from the alkaline pretreated ground rice straw, whereas cellobiose was the major component in the alkaline pretreated milled and

chopped rice straw (Table 4.1). This might be due to the fact that the removal of lignin and at the same time the partial degradation of hemicelluloses during NaOH pretreatment as well as the different absorption of culture medium between the large and the fine rice straw size, which led to the change in the different component of RS structure after NaOH pretreatment. Thereby, it may be possible that the difference in the particle sizes of rice straw which were further treated with alkaline showed the different major types of sugars end products from the fermentation of *Cl. cellulolyticum* DSM 5812.

Also, it was noteworthy that all NaOH-pretreated rice straws were relatively difficult to wash after pretreated due to the presence of higher concentrations of non-degradated fractions, non-soluble small fragments and silica (Cheng *et al*., 2010). These substances were not removed from the rice straw mixture during washing step. Furthermore, it was observed that the use of chopped RS appeared to offer several advantages when compared with the fine particle rice straw. This includes less energy requirement and the loss of solid during recovery process, which was expressed as solids remaining after NaOH pretreatment. Although the washing step causes an additional cost for the low-value lignocellulosic material, however, it was essential step for neutralization of the chemical pretreated lignocellulosic materials before being used as substrate for cellulolytic microorganism or enzymatic hydrolysis. The washing step also removed non-cellulose compounds including lignin, phenol derivatives, furfural hydroxymethyfurfual and other compound as inhibitors in the fermentation process.

In Figure 4.2, the percentage of solid recovery of chopped rice straw was higher than that of the other pretreated rice straw. It showed that the solid recovery was decreased when compared with the reduced particle size rice straw under the

same condition of pretreatment. Although the percentages of solid recovery from LHW pretreatment and all of the dilute acid pretreatments were higher than that of alkaline pretreatment, these recovered solids were digested in low level, as discussed earlier.

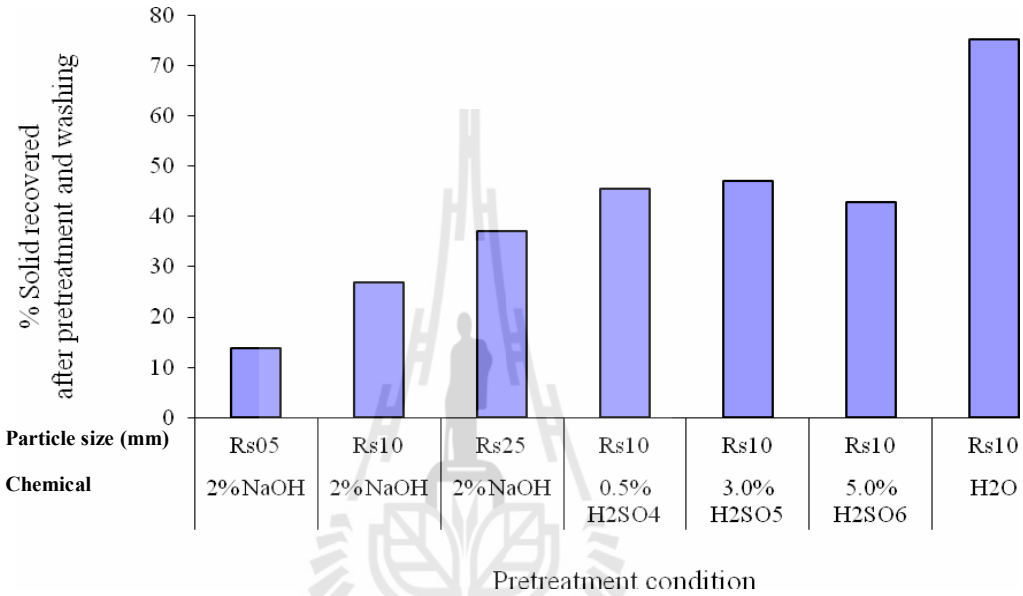


Figure 4.2 Effect of alkaline, acid and LHW pretreatment on solid recovery.

4.3.3 Effect of heating residence time on the susceptibility of NaOH pretreated rice straw for microbial hydrolysis

The combined effect of liquid hot water (LHW) and alkaline pretreatments (NaOH) with size reduction (S), as called NaOH-LHW-S pretreatment, on the yield of total end products during cellulose fermentation by *Cl. cellulolyticum* DSM 5812 was investigated. In Table 4.2, the result of NaOH- LHW-S pretreated rice straw showed the highest total end products (i.e. sugars, organic acids and ethanol) at 121ºC (15 psi) for 120 min residence time compared with those of the other residence times (0, 60, and 90 min). Without LHW pretreatment, the NaOH-S pretreated showed low

fermentable products. This implied that increased in cellulose digestibility depended on LHW pretreatment temperature and time. Our results were in good agreement with those observed by Cara *et al*. (2007) and Yu *et al*. (2010). Negro *et al*. (2003) who also reported that the higher the temperatures applied for liquid hot water and steam explosion, the higher enzymatic hydrolysis yield for LWH pretreatment was obtained in comparison with that of microbial hydrolysis. The effect of LHW pretreatment on the susceptibility of enzymatic hydrolysis may due to an increasing in the removal of particular hemicelluloses and lignin which remained after pretreatment and then modification of cell wall structure of the pretreated material (Wan and Li, 2011). Therefore, LHW pretreatment significantly improved the efficiency of enzymatic hydrolysis of NaOH pretreated rice straw in term of sugars accumulation in the fermentation by *Cl*. *cellulolyticum* DSM 5812.

Table 4.2 Effect of heating residence time of NaOH-S pretreated rice straw with LHW pretreatment under agitation at different retention time on the end product formation in batch cellulose fermentation by *Cl. cellulolyticum* DSM 5812.

End Products after cellulose fermentation	LHW pretreatment conditions (min, rpm) for NaOH pretreated rice straw				
	0,0	**0, 200**	**60, 200**	**90, 200**	**120, 200**
Cellobiose (g/L)	0.71±0.06	1.55±0.17	1.86±0.11	2.15±0.04	1.30±0.12
Glucose (g/L)	0.48±0.08	0.52±0.15	0.66±0.13	0.70±0.07	0.58±0.15
Xylose (g/L)	0.29±0.03	0.13±0.03	0.16±0.03	0.18±0.04	0.13±0.03
Arabinose (g/L)	0.06±0.02	0.05±0.02	0.06±0.01	0.06±0.01	0.05±0.00
Succinate (g/L)	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00
Lactate (g/L)	3.03±0.14	3.73±0.21	3.86±0.15	4.42±0.07	6.61±0.12
Formate (g/L)	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
Acetate (g/L)	0.80±0.04	0.86±0.11	0.94±0.03	0.97±0.04	1.06±0.04
Ethanol (g/L)	0.16±0.03	0.12±0.01	0.13±0.03	0.14±0.03	0.13±0.03
Total residual sugars (g/L)	1.53±0.20	2.25±0.26	2.73±0.28	3.10±0.16	2.05±0.31
Total fermentable products (g/L)	4.03±0.26[a]	4.74±0.25[b]	4.96±0.25[c]	5.58±0.15[d]	7.84±0.21[e]

- All data represent the averages of two fermentations with standard deviation.
- The value of the total fermentable products bearing different font symbol are significant different (p<0.05).

4.3.4 Morphological alterations in pretreated rice straw

Scanning electron microscope (SEM) was used to visually observe the physical and microstructure alterations of the rice straw. Micrographs of microfibril surfaces and vascular bundle structures of unpretreated, pretreated NaOH-LHW-S, and pretreated H_2SO_4-LHW-S rice straw under different conditions were examined (Figure 4.3). The morphological changes could be clearly observed in fiber structure of all pretreated NaOH-LHW-S, LHW-S and pretreated H_2SO_4-LHW-S, compared with that of the untreated rice straw which displayed rigid and highly ordered fibril as lignin.

Considering the appearance of vascular bundle and microfibril surface structure of the treated RS samples under various dilute acid conditions (Figure 4.3 g-h, k-l, and o-p), the structure of vascular bundle was irregular and shrank. In addition, part of the outer surface displayed the wire of cell wall structure. The concavity on the outer surface was increased with increasing the dilute acid concentration during pretreatment process. These results were also similar to the study of Li *et al*. (2012) who reported the disruption of the cell walls of the rice straw using microwave-assisted dilute lye pretreatment.

For the changes of cell wall of the different particle size of NaOH-LHW pretreated rice straws (Figure 4.3 e-f, i-j, and m-n), the results of SEM showed that the vascular bundle structure of NaOH-LHW pretreated chopped rice straw was more severely shrank when compared with that of the other particle size pretreated rice straws. The wire and concavity of the outer surface disappeared resulted from the removal of lignin and hemicelluloses from the rice straw (Wongiewboot *et al*., 2010). This suggested that the NaOH pretreatment of rice straw could enhance more of the lignin removal than the H_2SO_4 pretreatment leading to the increasing in the surface area of cellulose to be more accessible to enzymatic and microbial attack.

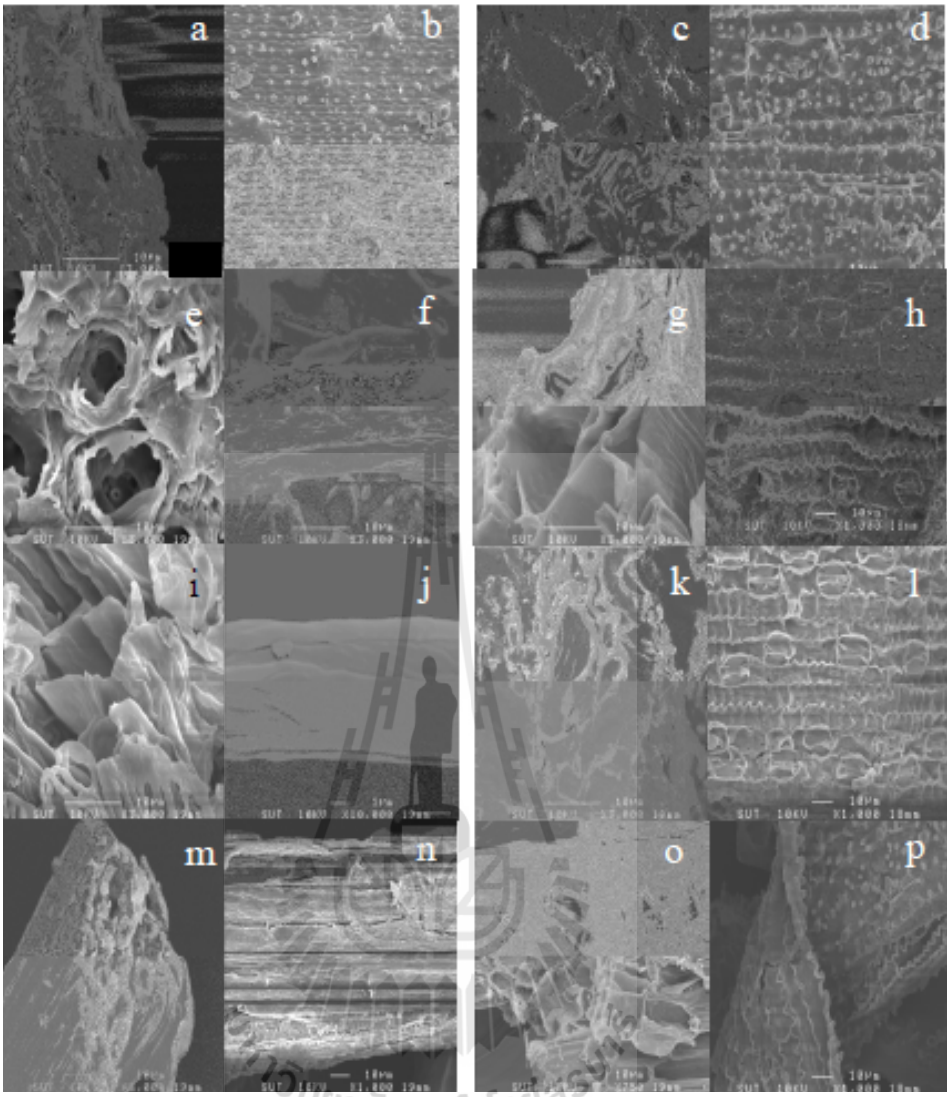


Figure 4.3 Scanning electron microscope micrographs of unpretreated and pretreated rice straw structure (RS) in transverse section (row 1 and 3) and longitudinal section (row 2 and 4). (a, b) unpretreated RS as raw material. (c, d) LHW pretreated RS. (e, f) NaOH-LHW pretreated milled RS, 2% NaOH. (i, j) NaOH-LHW pretreated grinded RS, 2% NaOH. (m, n) NaOH-LHW pretreated chopped RS, 2% NaOH. (g, h) H_2SO_4-LHW pretreated grinded RS, 0.5% H_2SO_4. (k, l) H_2SO_4-LHW pretreated grinded RS, 3% H_2SO_4. (o, p) H_2SO_4-LHW grinded RS, 5% H_2SO_4.

4.4 Conclusion

Rice straw contains a substantial amount of cellulose and is a potential lignocellulosic material for biofuel production. The combinatorial NaOH and LHW pretreatment with the size reduction of the rice straw is an effective method to enhance the rice straw to generate fermentable products. However, one disadvantage of this combined LHW-NaOH-S pretreatment method under the pressurized steam is that it required excess water to posttreat excess alkaline and alkaline waste. This water requirement plays significant roles in the economic viability of the industrial process. The results of this study will provide useful information for further study of butanol production by fermentation using sugars obtained from the hydrolysis of pretreated rice straw.

CHAPTER V

EFFECTS OF CULTURE PARAMETERS ON CELLULOSE DEGRADATION AND SUGAR ACCUMULATION FROM RICE STRAW CELLULOSE IN A CULTURE OF *CLOSTRIDIUM CELLULOLYTICUM*

5.1 Introduction

Rice straw is the potential of lignocellulosic materials as the sole feedstock due to the presence of fermentable sugars in the form of carbohydrate polymers (cellulose and hemicelluloses). Rice straw structure through pretreatment process is easy to hydrolyze to fermentable sugars by actions of cellulase enzymes and cellulose-utilizing microorganisms. *Clostridium cellulolyticum* could be able to converse lignocellulosic materials to glucose because of their cellulosome complexes as multi-cellulolytic enzyme machines (Gal *et al.,* 1997; Bayer *et al.,* 1998). In the past, fundamental studies of *Cl. cellulolyticum* were devoted essentially to the cellulase system (Gal *et al.,* 1997; Mickael *et al.,* 2004). In addition, its cellulolytic system has been studied by molecular genetics approaches (Reverbel-Leroy *et al.,* 1996; Maamar *et. al.,* 2004; Blouzard *et. al.,* 2010). However, the literature review on effect of culture parameters on enhancement of cellulose to sugars conversion by *Cl. cellulolyticum* is very limited. Therefore, the aim of this work was to investigate in more details on the effect of various culture parameters on the fermentability of alkaline pretreated rice straw cellulose to soluble sugar by *Cl. cellulolyticum* DSM 5812.

5.2 Materials and Methods

5.2.1 Microorganism and media

Cl. cellulolyticum DSM 5812, was obtained from the Leibniz Institut DSMZ-German Culture Collection of Microorganisms and Cell Cultures (DSM), was employed for all experiments. CM3 medium supplemented with 7 g/L of pretreated rice straw was used as a fermentation medium. The pretreated rice straw obtained from the alkaline pretreatment, see chapter 3. This medium consisted of compositions as follows (per liter): 1.3 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 2.9 g $K_2HPO_4.3H_2O$, 0.2 g $MgCl_2.6H_2O$, 0.075 g $CaCl_2.2H_2O$, 1.25 mg $FeSO_4.7H_2O$, 1 mL trace element, 1 mg resazurin, 0.5 g cysteine-HCl, and 2g yeast extract. The trace elements contained (per liter): 10 mL HCl (25%; 7.7 M), 1.5 g $FeCl_2$ $.4H_2O$, 70 mg 100 mg $ZnCl_2$, $MnCl_2$ $.4H_2O$, 6 mg H_3BO_3, 190 mg $CoCl_2$ $.6H_2O$, 2 mg $CuCl_2$ $.2H_2O$, 24 mg $NiCl_2$ $.6H_2O$, and 36 mg Na_2MoO_4 $.2H_2O$. The initial pH of culture medium was adjusted to 7.2 by 3 N NaOH. Anaerobic condition was maintained by passing into the culture broth with oxygen-free nitrogen gas before autoclaving at 121ºC for 15 min.

CM3 medium supplemented with 3 g/L cellobiose was used as media for induction of spore germination. Steriled CM3 medium (80 mL) was added 2.4 mL of 100 g/L of filter-sterile cellobiose solution.

5.2.2 Cellulose fermentation

Cl. cellulolyticum spores (1 mL) was added in CM3 medium supplemented with 3 g/L cellobiose, and then were heated to 80ºC for 10 min and incubated at 34ºC for 2-3 days. Later, the active motile cell was transferred to CM3 medium supplemented with 7 g/L pretreated rice straw. The culture was incubated at 34ºC for 3 days. The two-day old culture was used as seed culture for all of the experiments, except the study effect of seeding culture age on improvement of cellulose to sugar

conversion by *Cl. cellulolyticum*. Inoculum size used was 10% (v/v). Batch fermentation was performed under unregulated pH and anaerobic condition with mixing thoroughly (about 50 rpm) for 3 weeks.

5.2.3 Experimental design

All experiments were performed in a 80 mL working volumes of 120 mL septum bottle. The culture parameters tested were as follows: inoculum size (5%, 10% and 15% v/v); inoculum age (1, 2 and 3 days old); phosphate sources KH_2PO_4 (0, 11, 15, and 20 mM) and K_2HPO_4 (0, 17, 30, 50, 70 and 100 mg/L), $MgCl_2$ (0, 0.2, 0.5, and 0.7 g/L), $NaHCO_3$ (0, 15, 20, and 25 g/L); nitrogen sources using spent brewer's yeast (1, 2, 3, and 5 g/L); concentrations of cellulose from alkaline pretreated rice straw (4, 7, 10, 20, and 30 g/l); temperature of 34 and 37°C; and culture conditions with or without agitation.

5.2.4 Determination of fermentation end products

Cellobiose, glucose, xylose, arabinose and fermentation end product were quantified by a high-performance liquid chromatography (HPLC) with a Refractive Index (RI) detector (Lab Alliance Essence System). One milliliter of a three-week old culture was centrifuged at 13,400 rpm for 5 min and the supernatant was filtered through a 0.45 μm pore size filter membrane. The filtrate was analysed by using an anion exchange column (Aminex 87H; BioRad) operated at 45°C with 4 mM sulphuric acid as running buffer, 0.4 mL/min flow rate and RI detector.

5.2.5 Statistical analysis

The results were statistically analyzed by Duncan's multiple range test (DMRT) at 95% significance confidence ($p<0.05$) using SPSS statistical software (SPSS for Windows, version 16). Each data was presented as the average value of triplicate determinations.

5.3 Results and Discussion

5.3.1 Effect of inoculum size on sugars accumulation

Amount of inoculum play a role in enhancing capacity of cellulose to sugar conversion by *Cl. cellulolyticum* DSM 5812. Inoculum sizes of 5%, 10% and 15% were inoculated under culture condition at 34ºC, mixing, unregulated pH and 7 g/L of cellulose from alkaline pretreated rice straw in CM3 medium. Cellulose degradation in all samples was measured by analysis of accumulated sugar concentration (Giallo *et al.,* 1985; Samaniuk *et al.,* 2011). Sugar accumulation in *Cl. cellulolyticum* DSM 5812 culture was monitored for 21 days. The result showed that sugar accumulation was affected significantly by the inoculum size ($p \leq 0.05$). The highest level of sugar accumulation was obtained when inoculum size was 15% (v/v) (Figure 5.1A). The result indicated that increasing the inoculums size resulted in increasing sugar accumulation. This result was in agreement with the previous reports (Mourino *et al.,* 2001; O'Sullivan *et al.*, 2008). They demonstrated that amount of initial cell density, which used in rumen and rumen inoculated systems, were a controlling factor on the rate of cellulose degradation. Furthermore, some reports suggested that the increase of the inoculum size caused the rapid cell growth and enzyme production because of the fast degradation of the substrate in the fermentation process (Sarao *et al*., 2010). Moreover, Jensen *et al.* (2009) proposed that the rate of cellulose solubilization was only affected by the effective initial inoculum size and was independent of cell growth. From the result presented here, it could be concluded that the inoculum size was able to improve the cellulose degradation and sugar accumulation.

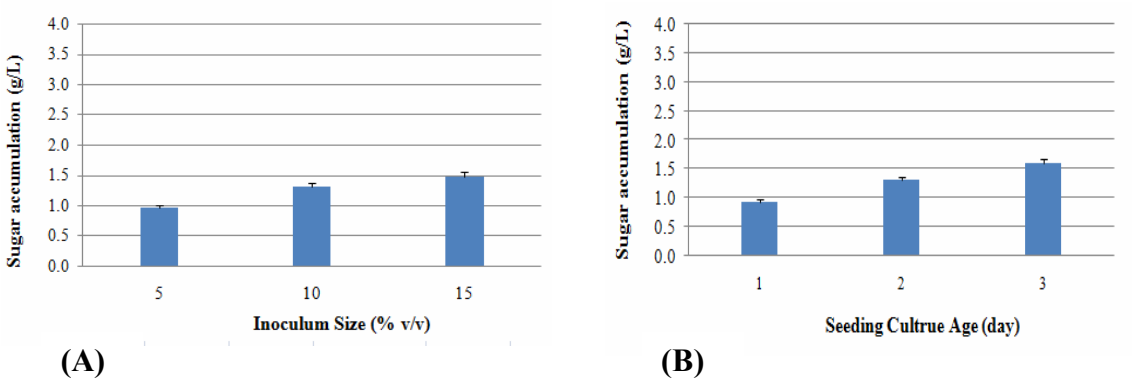


Figure 5.1 Effect of inoculum size (A) and seeding culture age (B) on sugar accumulation in *Cl. cellulolyticum* DSM 5812 culture at the end of three weeks of fermentation under culture condition at 34ºC with cellulose 7 g/L in CM3 medium.

5.3.2 Effect of culture seeding age on sugar accumulation

In order to investigate the effect of culture seeding age on cellulose degradation and sugar accumulation during fermentation of alkaline pretreated rice straw cellulose by *Cl. cellulolyticum*, the culture seeding ages of 1 day, 2 days and 3 days were respectively transferred into CM3 medium containing 7 g/L of cellulose under the same culture condition as described above. The results displayed that the significance of each culture seeding age showed the following order: 3 > 2 > 1 days of seeding culture age ($p \leq 0.5$) (Figure 5.1B). This result indicated that culture seeding age affected the accumulation of sugar end product during cellulose fermentation. A possible explanation for these results might be due to bacteria cell density. The cells attached to cellulose surface until some adhesion sites were hydrolyzed, leading to the detuched of bacterial cells from cellulose to the liquid fraction. Then, these released cells could adhere again and colonize to another site. This suggested that the growth

of *Cl. cellulolyticum* on cellulose occurred according to the following process: adhesion, colonization, release and readhesion (Gelhaye *et al.,* 1993). The generation time of about 24 h for *Cl. cellulolyticum* on cellulose MN 300 as a crystalline celluose was also reported (Giallo *et al.,* 1985). The situation occurred according to the kinetics of cellulose degradation that could be divided into three periods, the maximum cellulose solubilization rate appeared on days 0 to 2 or 3 and after 10 days when the remaining cellulose solubilization continued slowly. The rate of cellulose degradation was also variable and decreased between these two extremes. Nevertheless, the use of older seeding cultures age resulted in the decrease of the cellulase production (Vu *et al.,* 2011).

5.3.3 Effect of phosphate concentration on sugar accumulation

The effect of different phosphate sources such as KH_2PO_4 and K_2HPO_4 on cellulose degradation by *Cl. cellulolyticum* was illustrated in Figure 5.2. In the experiment using KH_2PO_4 as a phosphate source, control media were CM3 medium supplemented with 1.5 g/L of KH_2PO_4 and 17 g/L of K_2HPO_4, and CM3 medium supplemented with only 17 g/L of K_2HPO_4. The KH_2PO_4 concentration in fermentation media was varied as follows (g/L): 1.5, 2.0, and 2.7. The result showed that sugar accumulation in culture broth was affected significantly by the presence of KH_2PO_4, which was noticed from the two control media (Figrue 5.2A). The most effective cellulose hydrolysis was found at 2.0 g/L of KH_2PO_4 as compared with other KH_2PO_4 concentration as well as control. This result implied that KH_2PO_4 as phosphate source was readily available for *Cl. cellulolyticum*. Nevertheless, cellulose utilization was so sensitive to K_2HPO_4 concentration as when the concentration was higher than 2.0 g/L causing cellulose degradation was decreased.

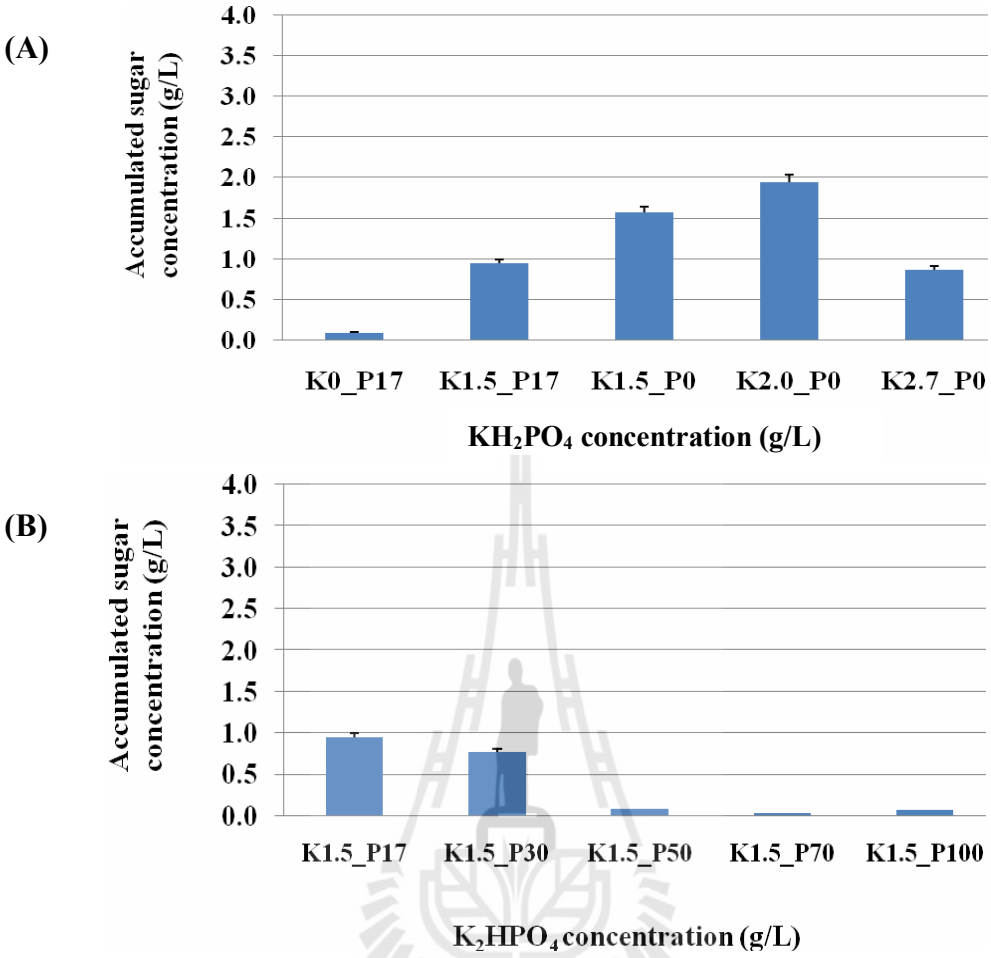


Figure 5. 2 Effect of phosphate source and concentration on sugar accumulation by *Cl. cellulolyticum* under culture condition at 34ºC, mixing and cellulose 7 g/L in CM3 medium. Phosphate source: (A) KH_2PO_4, (B) K_2HPO_4.

For the experiment using K_2HPO_4 as a phosphate source, the result showed that the highest concentration of accumulated sugar was attained when cells were culture in CM3 medium supplemented with 1.5 g/L of KH_2PO_4 and 17 g/L of K_2HPO_4. This medium was used as a control medium. Increasing K_2HPO_4 concentration in CM3 medium supplemented with 1.5 g/L KH_2PO_4 greatly influenced by decreasing sugar accumulation in cellulose fermentation by *Cl. cellulolyticum*

(Figure 5.3B). It implied that K_2HPO_4 greater 17 g/L in medium supplemented with KH_2PO_4 (1.5 g/L) had a negative effect on the ability of cells to degrade cellulose to sugar. Similar results had been obtained by Khan *et al.* (1979), who studied a cell system of a mixed anaerobic microbial population in the cellulose degradation process of converting cellulose to methane. In addition, similar observations in *Saccharomyces cerevisiae* to induce invertase synthesis demonstrated that the optimum phosphate concentration enhanced the rate of cell growth and sugar uptake which were related to the maximal secretion of extracellular enzyme as the cellulolytic enzymes in this study (Shafiq *et. al.,* 2002; Ikram-Ul-Haq *et al.,* 2004). Therefore, the optimum concentration and the source of phosphate salts were a crucial parameter to obtain maximal extracellular cellulase enzyme secretion. Moreover, the rate of cell growth and enzyme secretion were reduced in the fermentation medium supplemented with excess phosphate. This phenomenon could be described by the increasing in K_2HPO_4 concentration, which increased the alkalinity of the medium resulting in highly unfavorable condition for cell growth and enzyme stability of *Cl. cellulolyticum* (Desvaux *et al.,* 2001; Ikram-Ul-Haq *et al.,* 2004; Underkofler and Hickey, 1954).

5.3.4 Effect of $MgCl_2$ concentration on sugar accumulation

The effect of different $MgCl_2$ concentrations on the accumulation of sugar was examined. The maximum sugar accumulation (1.74 g/L) was obtained in the presence of 0.7 g/L $MgCl_2$ (Figure 5.3). The sugar accumulation was affect significantly by the amount of $MgCl_2$ ($p \leq 0.05$). It was observed that the production of sugar increased when $MgCl_2$ concentration was increased. Medium without $MgCl_2$ as control also showed low accumulation of sugar indicating that *Cl. cellulyticum* needed $MgCl_2$ for cellulose degradation. This could be explained by the fact that the cellulolytic enzyme required Mg^{2+} ions as cofactor for catalytic activity. This

observationwas supported by Vu *et al.*'s study (2011) with cellulase production by *Aspergillus* sp. They demonstrated that solid medium containing wheat bran supplemented with $MgCl_2$ could produce highest yield of cellulase compared with all tested metal salts such as $MnCl_2$, $CuSO_4$, $CoSO_4$, $FeSO_4$, $ZnSO_4$, KCl and $CaCl_2$. Therefore, it suggested that the activity of cellulolytic enzyme might depend on the concentration of heavy metal Mg^{2+} ions.

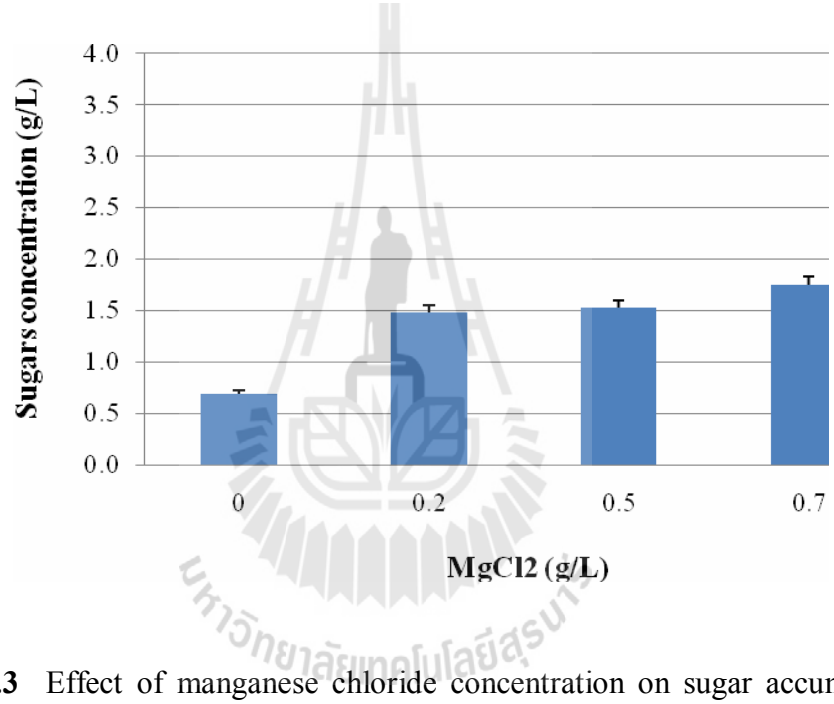


Figure 5.3 Effect of manganese chloride concentration on sugar accumulation by *Cl. cellulolyticum* under culture condition at 34ºC, mixing and cellulose 7 g/L in CM3 medium.

5.3.5 Effect of $NaHCO_3$ concentration on sugar accumulation

The optimum concentration of $NaHCO_3$ for cellulose conversion to the accumulation of sugar in cellulose fermentation was 15 g/L (Fig. 6.4). The amount of accumulated sugars decreased at concentration of $NaHCO_3$ higher than 15 g/L. There has been no direct evidence to support the effect of $NaHCO_3$ on cellulolytic system of cellulolytic bacteria in vitro. Nonetheless, in the previous study the addition of

$NaHCO_3$ in the animal diets could increase the number of total ruminal bacteria, cellulolytic and amylolytic bacteria as well as cellulolytic bacteria. Increasing cell biomasses have contributed to improve digestibility of cellulose (Koul *et al.*, 1998; Santra *et al.,* 2003) and microbial protein synthesis (Harrison and McAllan, 1980). Generally, it is known well that sodium bicarbonate is mineral salt acting as a buffer to prevent acidic conditions. Addition of a high sodium bicarbonate concentration in the diet would be expected to increase ruminal osmolality which led to effectively control the sudden fall in rumen pH being related to enhance the cellulolytic activity of ruminal microorganisms (Dehority and Tirabasso, 2012). For low rumen pH led to decrease the ruminal cellulolytic microbial activity resulting in poor digestibility of cellulose (William and Coleman, 1992). Although this study employed only monoculture of *Cl. cellulolyticum*, cellulose degradation was affected significantly by the increase of the $NaHCO_3$.

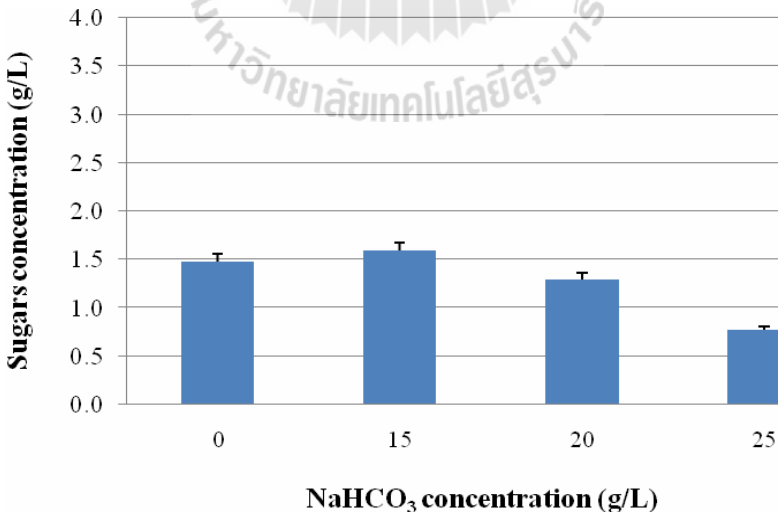


Figure 5.4 Effect of sodium bicarbonate on sugar accumulation by *Cl. cellulolyticum* under culture condition at 34ºC with 7 g/L of cellulose in CM3 medium.

5.3.6 Effect of nitrogen concentration on sugars accumulation

In order to define effect of spent brewer's yeast as an inexpensive nitrogen source instead of commercial yeast extract on cellulose degradation and sugar accumulation, different concentrations of spent brewer's yeast (1-5 g/L) were added to the fermentation medium compared with the medium with commercial yeast extract (Figure 5.5). The results demonstrated that the concentration of sugars accumulation obtained from fermentation medium containing 1 g/L of spent brewer's yeast was very close to that observed in the fermentation medium containing 2 g/L of commercial yeast extract. These results revealed that the spent brewer's yeast served as an alternative nitrogen source to replace the commercial yeast extract, which as expensive nitrogen source. This might be due to the presence of high level of protein, lipid, RNA, vitamin B complex, and minerals containing in the spent brewer's yeast (Jiang *et al.,* 2010). Typically, nitrogenous compounds defined as nitrogen sources play an important role in cell growth by building cellular materials for cell division. Therefore, an increase of spent brewer's yeast utilization by *Cl. cellulolyticum* resulted in increasing in high accumulated sugar. This could be explained by the utilization of nitrogen source led to high cell density which could boost the cellulolysis rate and amount of sugars released during cellulose fermentation (O'Sullivan *et al.,* 2008).

The maximum production of sugars from cellulose fermentation was obtained in the medium containing 3 g/L of spent brewer's yeast, whereas higher concentration of spent brewer's yeast (up to 5 g/L) had an affected on a decrease of sugars accumulation. Presumably, higher concentrations of nitrogen source caused distortion of C:N ratio leading to other pathways (Agarwal *et al.,* 2006).

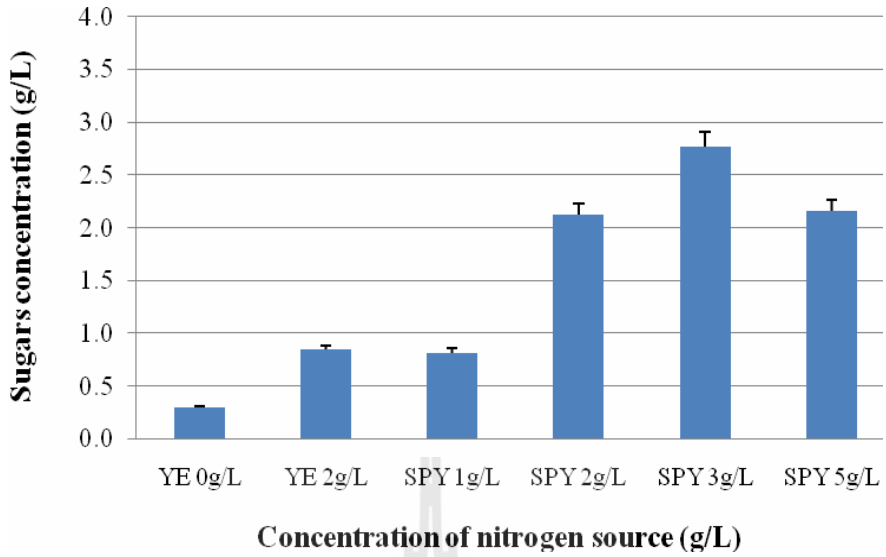


Figure 5.5 Effect of nitrogen source (YE, yeast extract; SBY, spent brewer's yeast) and concentration on sugar accumulation by *Cl. cellulolyticum* under culture condition at 34ºC with cellulose 7 g/L in CM3 medium.

5.3.7 Effect of cellulose concentration on sugar accumulation

Carbon sources play a crucial role in the cell metabolism and cellulase production. To examine the effect of cellulose concentration on the utilization of cellulose and sugars accumulation by *Cl. cellulolyticum,* experiments were conducted using different concentrations of rice straw cellulose (4-30 g/L) in the fermentation medium. Results showed the final accumulated sugars concentration depending on the initial cellulose concentration (Figure 5.6). An increase of initial cellulose concentration resulted in an increase in sugars production. These results were in agreement with previous study which reported a very low amount of soluble sugars was produced during the stationary phase and remained unchanged until the end of fermentation under the low-cellulose culture conditions (Isam *et al.*, 2009). For the high cellulose culture condition, the amount of accumulated sugars increased rapidly

in the late exponential growth phase and continued at a slower rate during the stationary phase until the end of fermentation (Islam *et al.,* 2009). This was supported by the fact that the maximum degradation rate was closely linked to the sugars accumulation and biomass production in parallel with the maximum cellulase activity and the adhesion of most of the bacteria on the cellulose particles at the end of fermentation under the culture condition with an excess of initial cellulose concentration (≥6.7 g/L) (Desvaux *et al.,* 2000).

At the concentration of 4 g/L initial cellulose, the low amount of accumulated sugars was observed. This could be the result of exhaustion of the cellulose observing from the residual cellulose at the end of fermentation (data not shown) as supported by another researcher (Desvaux *et al.,* 2000).

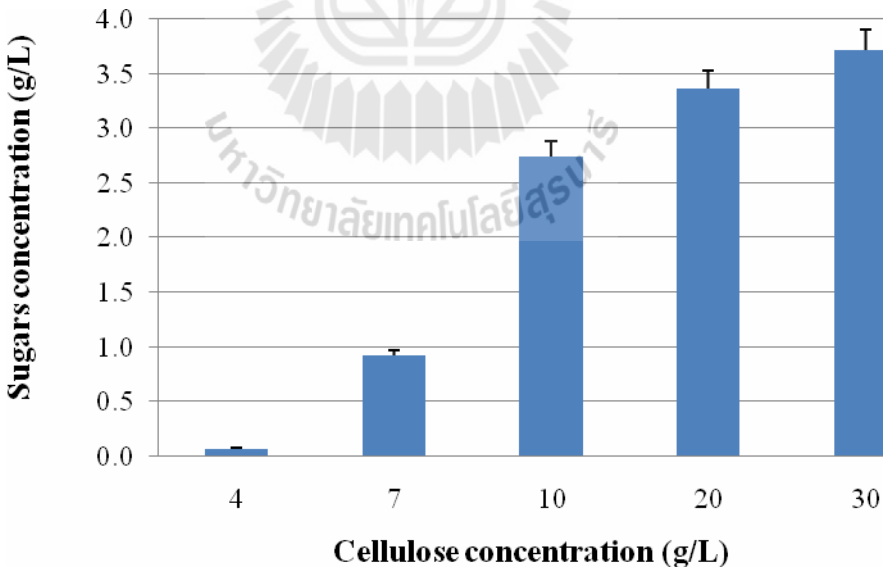


Figure 5.6 Effect of cellulose concentration on sugars accumulation by *Cl. cellulolyticum* under culture condition at 34ºC.

5.3.8 Effect of culture temperature and agitation on sugars accumulation

Culture temperature is one of the most vital parameters essential for the achievement of a cellulose conversion to fermentative sugars by enhancing enzymatic activity, depending on the microorganisms. Temperature is an important factor in the activity of enzymes. The activity of the enzymes could be measured by the formation of accumulated soluble sugars (Gautam *et al.,* 2011). The result showed that the cellulose fermentation by *Cl. cellulolyticum* was most effective at 37ºC as compared with 34ºC (Figure 5.7A). This result revealed that the slight change in culture temperature could have strong affect on cellulose conversion. Many studies reported different temperatures for maximum cellulose production which depend on the variation of the microorganism strain as biocatalyst used. For example, the optimal temperature for exoglucanase and endoglucanase activity produced by *A. niger* was between 40 and 50°C, whereas the optimum temperature for β-glucosidase activity was between 45 and 55°C (Gautam *et al.,* 2011). An optimum temperature range and the maximum temperature for cellulase production by *Streptomyces* sp. were 45 to 60°C and 60°C, respectively (Jaradat *et al.,* 2008). A high temperature at 50°C influenced the increase rate of cellulose utilization, sugars accumulation and ethanol production in *Cl. thermocellum* (Özpinar and Özkan, 2007).

According to the results, it clearly showed that the amount of sugars production was increased significantly under culture condition with well agitation and than that of non-agitated culture condition (Figure 6.7B). These results were corresponded with the data previously reported by Palmqvist *et al.* (2001), Ingesson *et al.* (2001), Mussatto *et al.* (2008), and Samaniuk *et al.* (2011). Roche *et al*. (2009) proposed that the rate of cellulose hydrolysis increased because the enzymes were more uniformly distributed initially when the samples were thoroughly mixed. In fact,

the enzyme adsorption onto cellulose caused an effective cellulose hydrolysis and was considered to be prerequisite condition. Therefore, this result indicated that an efficient initial agitation having an influenced on the increasing of enzyme distribution and the enzyme adsorption rate under short reaction time. This resulted in the cellulose conversion yield and accumulated sugars yield (Ingesson *et al.,* 2001).

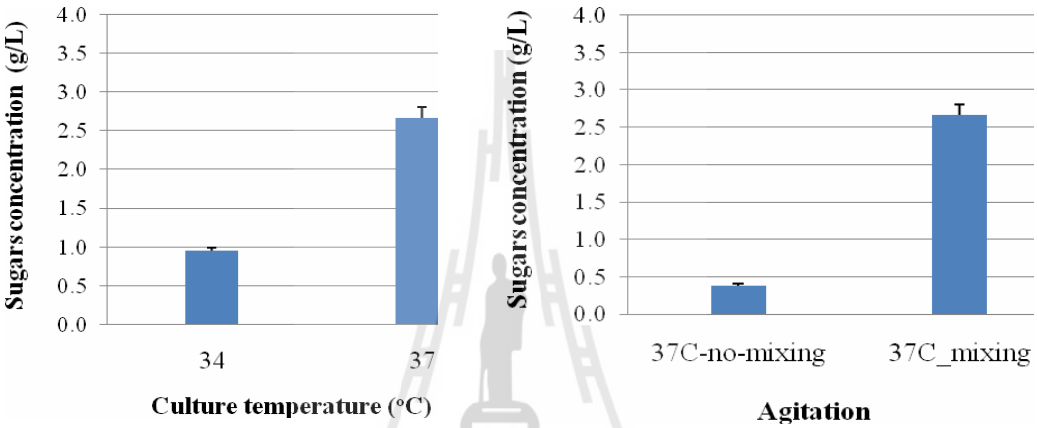


Figure 5.7 Effect of culture temperature (A) and agitation (B) on sugars accumulation by *Cl. cellulolyticum* under culture condition at 34ºC with cellulose 7 g/L in CM3 medium.

5.4 Conclusion

It could be concluded from the study that the nutritional and environmental parameters significantly affected on the cellulose degradation and sugars accumulation by *Cl. cellulolyticum* DSM 5812. The results also indicated the possibility of utilization of the rice straw as cheap carbon substrate for the fermentable sugars production. These data could further be of great important to industrial applications of anaerobic digestion technologies or guidance for the optimization of culture condition to enhance cellulose degradation by response surface methodology (RSM).

CHAPTER VI

EFFECT OF TWO-STAGE DILUTE ACID PRETREATMENT ON XYLOSE PRODUCTION FROM RICE STRAW HEMICELLULOSES

6.1 Introduction

Xylan is the largest portion of hemicelluloses in the rice straw, so the dominant sugar in hemicelluloses is xylose. It is the most important indicator of effectiveness of the dilute acid pretreatment (Chen *et al.*, 2011). Many studies have been demonstrated that the rich xylose in the acid hydrolyzate from biomass could serve as a carbon source in fermentation medium for industrial microorganisms. These microorganism are able to converse xylose to various commercial chemicals such as xylitol, butyric acid, ethanol, and butanol (Zhu and Yang, 2004; Baek and Kwon, 2007; Sumphanwanich *et al.*, 2008; Lui *et al.*, 2010).

In the present study, a two-step acid hydrolysis of rice straw pretreatment under mild condition was investigated in order to develop a simple technique for highly efficient pretreatment of rice straw for the maximum production of xylose and the minimum generation of sugar decomposition products. This work started to define the optimal conditions of the different two-step pretreatment processes of rice straw, and to estimate the pretreatment efficiency of the processes. The pretreatment process efficiency was evaluated by estimating the total xylose yield and the minimum amount of sugar decomposition products after the pretreatment. This investigation

aims to reveal whether the process of xylose liberation by the two-step acid saccharification pretreatment of the hemicellulose fraction of rice straw could produce significant amount of xylose which could be used as culture media for useful bioconversion.

6.2 Materials and Methods

6.2.1 Biomass material

Rice straw was used as raw material in the experiment. Rice straw was obtained from the Suranaree University of Technology Farm in Nakhon Ratchasima province of Thailand. It was dried at 65°C, cut into 20-25 mm average in size, and stored at room temperature for subsequent experiments.

6.2.2 Dilute acid pretreatment

The rice straw was cut to about 25 mm long, The raw material was oven-dried for 24 h at 65ºC. Then, it was used directly in acid saccharification studies. Ten grams of dry chopped rice straw were mixed with various H_2SO_4 solution (1, 1.5, 2, 3% w/v) with a solid to liquid ratio of 1:6, 1:8, and 1:10. The reaction mixtures were then autoclaved at 121ºC for reaction time depending on each experiment.

6.2.3 Experimental design

The flow process diagram of the two-stage dilute acid pretreatment process was shown in Figure 6.1 in order to improve the saccharification hemicellulose efficiency from rice straw. The operation variables in the two-step hydrolysis process examined in this study were (a) the solid to liquid ratio of 1:6, 1:8, and 1:10 at 121ºC, 30 min; (b) the sulfuric acid concentration, which was varied with 1, 1.5, 2, and 3% (w/v); and (c) hydrolysis retention time was varied for 30, 45, 60 or 90 min. In the first-stage dilute acid pretreatment, chopped rice straw (10 g) was

mixed with 1.5% H_2SO_4 solution to give a solid to liquid ratio of 1:6, 1:8, and 1:10 and then autoclaved at 121ºC for 30 min. In all the experiments, the first-stage liquor was drained. The optimum ratio of solid to liquid was determined based on the xylose concentration recovered. The best ratio of solid to liquid, provided high concentration of sugars, was chosen for further investigation in the second-stage hydrolysis. The residual solids were carried out with addition of the same acid concentration with the solid to liquid ratio of 1:6, 1:8, and 1:10. Then, they were autoclaved under the same condition mentioned above. The optimum combination ratio of solid to liquid in the first- and second-stage hydrolysis, provided the highest concentration of total sugars, was further investigated. Next, the optimum concentration of sulfuric acid for sugars production in the two-stage diluted acid pretreatement was performed at the same step as described above in the study of ratio between solid and liquid solution. Finally, the optimum reaction time for sugars production in the combined acid hydrolysis was determined.

6.2.4 Analysis methods

The composition of the hydrolyzates after pretreatment was analyzed by a high-performance liquid chromatography (HPLC) with a Refractive Index (RI) detector (Algilent 1200 series HPLC, USA) using an Aminex HPX-87H ion exchange column. The packed column was maintained at 45°C and the products containing in hydrolyzates were eluted with 4 mM H_2SO_4 at the flow rate of 0.4 mL/min.

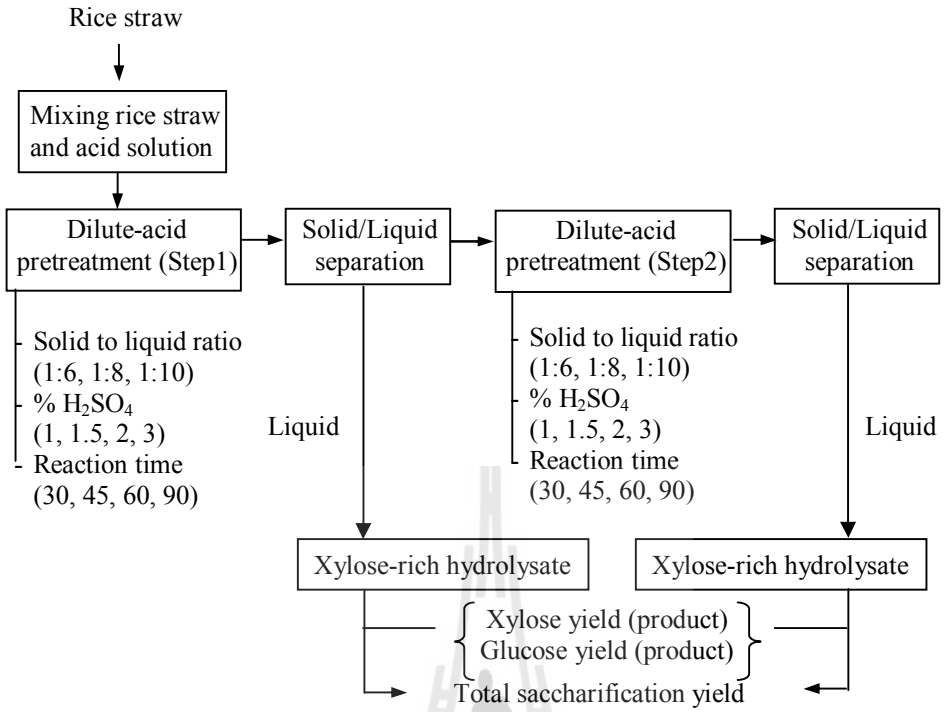


Figure 6.1 The flow process diagram of the 2-stage dilute-acid hydrolysis in this study.

6.2.5 The definition of amount and yield of products

The amount and yield of xylose, total sugars, and acetate are calculated by the following equations:

Xylose yield (g xylose/g rice straw) was expressed as amount of xylose (g) divided by 10 g rice straw provided.

Total sugar yield (g total sugars/g rice straw) was calculated as total sugar (g) divided by 10 g rice straw provided.

Acetate yield (g acetate/g rice straw) was calculated as amount of acetate (g) divided by 10 g rice straw provided.

The total saccharification yield formulated by combining yield of total xylose and glucose conversion for both process of hydrolysis.

6.2.6 Data analysis

The results obtained in the present study were statistically analyzed by Duncan's multiple range test (DMRT) at 95% significance level ($p<0.05$) using SPSS statistical software (SPSS for Windows, version 16). Each data was presented as the mean value of three determinations.

6.3 Results and Discussions

Two step processes of acid pretreatment under mild conditions were used to selectively obtain xylose-rich hydrolyzate from rice straw and to avoid the formation of sugar degradation products, as inhibitors for microbial fermentation. The effects of three operational variables, including solid to liquid ratio, sulfuric acid concentration, and reaction time, on the hydrolysis of the hemicellulose fraction of rice straw by the two-stage acid pretreatment were investigated.

6.3.1 Effect of solid to liquid ratio in two-stage acid pretreatment

Chopped rice straw was hydrolyzed in the first stage of acid hydrolysis at different solid to liquid ratio of 1:6, 1:8, and 1:10. These experiments were carried out at 121ºC for 30 min with 1.5% (v/v) sulfuric acid solution. The results of hydrolysis of rice straw hemicellulose are summarized in Table 6.1. The ratio of solid to liquid had significant effect on total sugars yield after the first-stage of the acid hydrolysis reaction ($p\leq0.05$). Increasing the ratio of solid to liquid from 1:6 to 1:10 caused a sharp positive effect on the amount of the total sugars recovery in the hydrolysis of chopped rice straw. These results coincided with the other researchers, who reported that reducing the solid to liquid ratio resulted in reducing rate of xylose decomposition as well as xylose production (Baek *et al.*, 2007; Lavarack *et al.*, 2002; Najafpour

et al., 2007;). This implied that lignocellulosic substrate could not be hydrolyzed by acid at high substrate concentration (Meinita *et al.,* 2012). A comparison of the highest yield of xylose and total sugars present in the hydrolyzate with different ratios of solid to liquid demonstrated the ability of the first-stage pretreatment to depolymerize hemicellullose at the ratio of 1:10. Thus, this ratio was selected for second step of the acid hydrolysis.

The second hydrolysis step was expected to provide an increased in xylose recovery resulting in high overall yield of xylose and total sugars. Hemicellulosic fraction was still present in the solid residue after the first-stage hydrolysis under non-severe condition. At the end of the first stage of the hydrolysis with the ratio of 1:10, the liquor containing soluble hemicellulosic materials was drained. Afterward, the solid residue was used in the second hydrolysis stage. In the set of experiments, the solid to liquid ratio was set to 1:6, 1:8, and 1:10 by adding 1.5% acid solution. These experiments were performed under the same condition as in the first-stage hydrolysis. Table 6.2 demonstrated the composition of end products in all of the hydrolysates after the second step of hydrolysis. The results revealed that the acid pretreated rice straw in the first pretreatment step still has significant amount of hemicelluloses. A decrease of the solid to liquid ratio in the second step resulted in an increase in yield of xylan to xylose conversion. In contrast, further increase in the ratio to 1:10 reduced the yield of recovered xylose. The maximum yield of xylose and total sugar was obtained from the ratio of 1:6 for the second stage of acid hydrolysis.

Table 6.1 Effect of solid to liquid ratio on the composition of hydrolyzates from 10 g. of dry rice straw in the first acid hydrolysis step.

Solid:liquid	**1:06**	**1:08**	**1:10**
Collected volume (mL)	10.33±0.58	25.33±1.53	45.33±1.53
Xylose (g/L)	14.67±0.07	16.99±0.02	14.18±0.06
Glucose (g/L)	1.06±0.02	1.11±0.02	1.04±0.03
Arabinose (g/L)	1.79±0.02	2.04±0.03	1.76±0.05
Acetate (g/L)	1.78±0.06	1.53±0.12	0.95±0.05
Total sugars (g/L)	17.52±0.05	20.14±0.04	16.98±0.14
Xylose yield (g xylose/g rice straw)	0.02	0.04	0.06
Total sugars yield (g total sugars/g rice straw)	0.02^{d}	0.05^{e}	0.08^{f}
Acetate yield (g acetate/g rice straw)	0.00	0.00	0.00

- All of the acid hydrolysis conditions were performed at 121ºC for 30 min with 1.5% (v/v) H_2SO_4.
- All data represent an average value of triplicates with standard deviation.
- The value of the total products from acid hydrolysis bearing different font symbols are significant difference ($p \leq 0.05$).

Table 6.2 Effect of the solid to liquid ratio on the composition of hydrolyzates from 10 g of dry rice straw in the second acid hydrolysis step.

Solid:liquid	1:06	1:08	1:10
Collected volume (mL)	35.33±0.58	35.67±0.58	35±0.58
Xylose (g/L)	16.40±0.08	11.81±0.03	9.15±0.07
Glucose (g/L)	1.46±0.03	1.10±0.03	0.96±0.05
Arabinose (g/L)	2.44±0.07	1.77±0.02	1.27±0.04
Acetate (g/L)	1.25±0.03	0.90±0.03	0.67±0.03
Total sugars (g/L)	20.30±0.12	14.68±0.06	11.38±0.09
Xylose yield (g xylose/g rice straw)	0.06	0.04	0.03
Total sugars yield (g total sugars/ g rice straw)	0.07^{d}	0.05^{e}	0.04^{f}
Acetate yield (g acetate/g rice straw)	0.00	0.00	0.00

- The residual solid from the first stage hydrolysis with the solid to liquid ratio of 1:10 were performed at 121ºC for 30 min with 1.5% (v/v) H_2SO_4.
- All data represent an average value of triplicates with standard deviation.
- The value of the total products from acid hydrolysis bearing different font symbols are significant difference ($p \leq 0.05$).

A summary of the effect of the solid to liquid ratio on the yield of xylan solubility by the combination of the first and the second hydrolysis step in the two-stage pretreatment were presented in Table 6.3. The suitable ratio of solid to liquid for the significant amount of liberated xylose was 1:10 and 1:6 in the first and the second stage of acid pretreatment, respectively.

Table 6.3 The effect of solid to liquid ratio on composition of all of the hydrolyzate from 10 g of dry rice straw in the two-stage acid pretreatment.

Solid:liquid	1:10&1:6	1:10&1:8	1:10&1:10
Collected volume (mL)	80.66±2.10	81.00±2.10	80.33±2.53
Xylose (g/L)	30.58±0.14	25.99±0.09	23.33±0.13
Glucose (g/L)	2.47±0.06	2.10±0.06	1.96±0.08
Arabinose (g/L)	4.23±0.12	3.56±0.07	3.06±0.09
Acetate (g/L)	2.19±0.08	1.85±0.08	1.62±0.08
Total sugars (g/L)	37.27±0.25	31.64±0.19	28.35±0.23
Xylose yield (g xylose/g rice straw)	0.25	0.21	0.19
Total sugars yield (g total sugars/g rice straw)	0.30^{d}	0.26^{e}	0.23^{f}
Acetate yield (g acetate/g rice straw)	0.02	0.01	0.01

- The residual solid from the first stage hydrolysis with solid to liquid ratio of 1:10 were performed at 121℃ for 30 min with 1.5% (v/v) H_2SO_4. Then the residual solid from the first stage hydrolysis was performed by adding 1.5% (v/v) H_2SO_4 with the ratio of 1:6, 1:8, and 1:10 under the same condition as mentioned above. Symbol of combined two-stage hydrolysis process was 1:10&1:6 (or 1:10&1:8 or 1:10&1:10) representing 1:10 as the first stage of hydrolysis reaction and 1:6 (1:8 or 1:10) as the second stage of hydrolysis reaction.
- All data represent on average value of triplicates with standard deviation.
- The value of the total products from the acid hydrolysis bearing different font symbols are significant difference ($p \leq 0.05$).

6.3.2 Effect of sulfuric acid concentration in two-stage acid pretreatment

In order to investigate effect of sulfuric acid in each stage of the two-stage acid pretreatment of rice straw hemicellulose on total sugars concentration, experiments were conducted by reacting hemicellulose with sulfuric acid at different concentrations of 1.0, 1.5, 2.0, and 3.0 % at the solid to liquid ratio of 1:10 (first

stage) at 121ºC for 30 min. Subsequently in the second stage of hydrolysis reaction, the remained solid from the solid to liquid ratio which provided the high amount of total sugars in the first-stage of hydrolysis, was preformed with different acid concentrations under the same condition. The optimum condition of the combined two-stage acid pretreatment was determined from the sum of the highest yield of total sugars from the first- and second-stage hydrolysis.

The results of different sulfuric acid concentrations on total sugars in the hydrolyzates from the first pretreatment step of rice straw are summarized in Figure 6.4. Increasing acid concentration from 1 to 1.5% (v/v) resulted in significant increase in xylose recovery ($p \leq 0.5$). This finding was also supported by the results of Mosier *et al.* (2002) that increasing acid concentrations led to the increase of catalyst activity, proportional to concentration of hydrogen ion in the solution. The more the hydrogen ions available, the more the rapidity of the hydrolysis proceed as well as increasing in breaking of glycosidic bonds which resulting in high conversion of hemicelluloses fraction into sugars. In this work, the maximum xylose and total sugar yield after pretreated was obtained when 2% sulfuric acid concentration was used. Furthermore, the results displayed that increasing in the sulfuric acid concentration up to 3% resulted in decreased in the yield of xylose and total sugars significantly. These situations were also experienced by Beak *et al.,* (2007). Some study reported that xylan content has generally been found to decline with increasing acid concentration and eventually plateau at approximately 3% sulfuric acid (Chen *et al.,* 2011). Besides, xylose is sensitive to degradation to furfural, especially when the concentration of acid used for the reaction is over 1% and the reaction temperature is greater than 120^{o}C (Lee *et al.*, 2001). Regarding with this issue, reduction in the yield of hemicellulose carbohydrate may have occurred as a result of degradation of pentose sugars (xylose

and arabinose) into decomposed products such as furfural, hydroxymethyfurfural (HMF), and acetate.

According to these results, it was obviously that the total sugars yield was dependent upon the sulfuric acid dose in this reaction condition of pretreatment. The result showed that the amount and yield of total sugars that obtained from 1.5% and 2% (v/v) acid concentration was not significantly different ($p \leq 0.05$). Thus, the solid to liquid ratio of 1:10 and 1.5% H_2SO_4 was the selected condition for the subsequent of the second-stage experiment.

Table 6.4 Effect of sulfuric acid concentration on the composition of hydrolyzates from 10 g of dry rice straw in the first hydrolysis step.

% H_2SO_4	**1**	**1.5**	**2**	**3**
Collected volume (mL)	35.33±0.58	35.33±0.58	35.67±0.58	35.33±0.58
Xylose (g/L)	16.20±0.03	16.48±0.09	16.76±0.07	14.17±0.12
Glucose (g/L)	1.05±0.06	1.61±0.05	1.75±0.03	1.40±0.03
Arabinose (g/L)	2.33±0.06	2.39±0.03	2.42±0.05	1.77±0.06
Acetate (g/L)	1.56±0.08	1.59±0.04	1.70±0.04	1.76±0.03
Total sugars (g/L)	19.58±0.07	20.48±0.13	20.92±0.12	17.35±0.07
Xylose yield (g xylose/g rice straw)	0-06	0.06	0.06	0.05
Total sugars yield (g total sugars/g rice straw)	0.07	0.07	0.07	0.06
Acetate yield (g acetate/g rice straw)	0.01	0.01	0.01	0.01

- All of the acid hydrolysis conditions with the solid to liquid ratio of 1:10 were performed at 121ºC for 30 min with 1.0, 1.5, 2.0, 3.0% (v/v) H_2SO_4.
- All data represent an average value of triplicates with standard deviation.
- The value of the total products from the acid hydrolysis bearing different font symbols are significant difference ($p \leq 0.05$).

The second pretreatment step was performed using rice straw residue that obtained from the first dilute-acid pretreatment. The effect of sulfuric acid concentrations on the hemicellulose saccharification of rice straw in the second step of pretreatment was shown in Table 6.5. Unlike the first hydrolysis step, increasing the acid dose from 1 to 2% (v/v) in the second hydrolysis step had no significant effect on the yield of xylose ($p \leq 0.05$), except at 3% (v/v) acid concentration. This indicated that the acid dose of 3% (v/v) was a high severity condition for xylose conversion, causing a decrease in xylose recovery. In addition, Himmel *et al.* (2007) proposed that the best model for xylose formation and degradation was a pair of parallel first-order reactions: one to be easy to hydrolyze and another to be difficult to hydrolyze. The easier hydrolysable fraction, xylan, has been reported to be consisted of 60–80% hemicelluloses in various lignocellulosic materials. The xylan residue is part of the difficult or uneasy hydrolysable fraction. Therefore, it could be concluded that most of the amount of xylose from the first hydrolysis step derived from the easier hydrolysable fraction of hemicelluloses xylan. After this, the content of stubborn xylan molecules did not change even under the same moderate condition. This suggested that the increase in acid concentration was sufficient to have an effect on the liberated xylose molecules by increasing xylose degradation.

Table 6.5 Effect of sulfuric acid concentration on the composition of hydrolyzates from 10 g of dry rice straw in the second hydrolysis step.

% (v/v) H_2SO_4	1	1.5	2	3
Collected volume (mL)	35.33±0.58	35.33±0.58	35.33±0.58	35.33±0.58
Xylose (g/L)	9.71±0.05	9.44±.08	9.16±0.04	8.28±0.08
Glucose (g/L)	0.84±0.05	1.08±0.03	1.12±0.03	1.20±0.03
Arabinose (g/L)	1.27±0.06	1.29±0.04	1.25±0.06	1.13±0.02
Acetate (g/L)	0.62±0.03	0.70±0.04	0.73±0.03	0.85±0.04
Total sugars (g/L)	11.82±0.03	11.81±0.11	11.53±0.11	10.61±0.05
Xylose yield (g xylose/g rice straw)	0.03	0.03	0.03	0.03
Total sugars yield (g total sugars/g rice straw)	0.04	0.04	0.04	0.04
Acetate yield (g acetate/g rice straw)	0.00	0.00	0.00	0.00

- The residual solid from the first stage hydrolysis with the solid to liquid ratio of 1:10 and 1.5% (v/v) acid were performed at 121ºC for 30 min with the solid to liquid ratio of 1:6 and vary of H_2SO_4 concentration at 1.0, 1.5, 2.0, and 3.0 % (v/v).
- All data represent an average value of triplicates with standard deviation.
- The value of the total products from the acid hydrolysis bearing different font symbols are significant difference ($p \leq 0.05$).

Notably, the results displayed that the released glucose was slightly increased under these pretreatment conditions despite the fact that cellulose fraction was not hydrolyzed. In contrast, hemicellulosic fraction was easier to be broken down under the same operating condition. Nonetheless, the greater glucose yield corresponded to the increase in acid concentration. Some studies reported that the main part of glucose generally originated from the cellulose fraction (Taherzadeh *et al.* 1997; Kadam *et al.*, 2000). However, glucose could originate from either the hemicellulose or cellulose fractions of lignocelluloses (Karami *et al.*, 2006).

Considering chemical reaction in the acid hydrolysis, the hydrolysis rate was gradually increased with respect to acid concentration, because hydrolysis reaction was generally controlled by hydrogen ion concentration (H^+). At the same time, the cellulose fragment dissolution performed as a function of hydroxyl ion concentration (Ladisch, 1989). Additionally, Xiang *et al.,* (2003) have proved that most of the cellulose was able to dissolve in 65% of H_2SO_4 or higher. Moreover, study of Karami *et al.* (2006) found that the yield of glucose was relatively low in the hydrolysis at lower pressure than 217ºC or 20 bar. Thus, the liberated glucose in this study might be probably originated from the hemicelluloses hydrolysis by acid.

In the second step of acid pretreatment, the acid concentration of 1% resulted in the highest yield of xylose formation. The xylose yield under this condition was not significantly different from that of acid concentration at 1.5% and 2.0% ($p \geq 0.05$). The result of the two-stage acid pretreatment for the recovery of rice straw hemicellulosic sugars was shown in Table 6.6. Under the combined two-step acid reaction conditions, no significant effect of the combination of acid concentration 1.5% (first step) and 1% (second step) as designated by 1.5&1, as well as 1.5&1.5 and 1.5&2.0, on yield of total sugars concentration was observed. Accordingly, the use of 1.5% and 1.0% sulfuric acid in the first and second step of acid hydrolysis, respectively, was deemed suitable for providing the best yield of xylose and total sugars in the two-stage acid pretreatment of rice straw.

Table 6.6 Effect of sulfuric acid concentration on the composition of hydrolyzates from 10 g of dry rice straw in the two-stage acid hydrolysis**.**

% H_2SO_4	1.5&1	1.5&1.5	1.5&2.0	1.5&3.0
Collected volume (mL)	71.00±1.15	70.66±1.15	71.00±1.15	70.66±1.15
Xylose (g/L)	25.91±0.12	25.92±0.15	25.91±0.11	22.45±0.15
Glucose (g/L)	1.89±0.09	2.68±0.06	2.87±0.06	2.61±0.06
Arabinose (g/L)	3.60±0.10	3.68±0.09	3.67±0.10	2.90±0.07
Acetate (g/L)	2.21±0.07	2.40±0.08	2.49±0.07	2.41±0.08
Total sugars (g/L)	31.40±0.15	32.29±0.24	32.45±0.24	27.95±0.18
Xylose yield (g xylose/kg rice straw)	0.18	0.09	0.09	0.08
Total sugars yield (g total sugars/g rice straw)	0.22	0.23	0.23	0.20
Acetate yield (g acetate/g rice straw)	0.02	0.02	0.02	0.02

- The residual solid from the first stage hydrolysis with the solid to liquid ratio of 1:10 were performed at 121ºC for 30 min with 1.5% (v/v) H_2SO_4. Then the residual solid from the first stage hydrolysis was performed by adding H_2SO_4 (v/v) of 1.0 or 1.5 or 2.0 or 3.0% (v/v) under the same condition as mentioned above. Symbol of combined two-stage hydrolysis process with different usage of acid dose was 1&1 (or 1.5&1.5 or 1.5&2.0 or 1.5&3.0) represent of the first stage of acid hydrolysis reaction using 1% acid and the second stage of acid hydrolysis reaction using 1% acid.
- All data represent an the average value of triplicates with standard deviation.
- The value of the total products from the acid hydrolysis bearing different font symbols are significant difference ($p \leq 0.05$).

6.3.3 Effect of reaction time in the two-stage acid pretreatment

Effect of various reaction time (30, 45, 60, and 90 min) on the formation of soluble hemicellulose sugars of rice straw using the solid to liquid ratio of 1:10 and 1.5% H_2SO_4 under hydrolysis condition at 121ºC in first-stage pretreatment was shown in Table 6.7. The results showed that when the reaction time increased from 30 to 60 min, the xylose concentration increased from 0.59 to 0.80 g/L, respectively. However, the concentration of xylose obtained decreased significantly, when the reaction time was increased to 90 min. This indicated that the first acid hydrolysis for 90 min was severe to hydrolyze the hemicellulose sugars to sugar degradation products. It was also indicated that the increase of acetate accumulation depended upon the increase of the time of reaction. This result was similar to the previous reports that a longer hydrolysis time resulted in increased in sugar decomposition (Saha *et al.*, 2005; Baek *et al.*, 2007; Meinita *et al.*, 2012). The highest total sugars (28.61±0.14 g/L), was achieved when the pretreatment reacting with 1.5% sulfuric acid was conducted at 121°C for 60 min in the first-step hydrolysis process.

Table 6.7 Effect of reaction time on the composition of hydrolyzates from 10 g of dry rice straw in the first acid hydrolysis step.

Reaction time (min.)	30	45	60	90
Collected volume (mL)	35.33±0.58	35.33±0.58	35.67±0.58	35.33±0.58
Xylose (g/L)	16.76±0.05	17.42±0.07	22.52±0.05	20.01±0.06
Glucose (g/L)	1.72±0.08	1.75±0.04	2.49±0.06	3.17±0.04
Arabinose (g/L)	2.37±0.06	2.42±0.05	3.60±0.04	3.22±0.05
Acetate (g/L)	1.48±0.07	1.76±0.03	1.84±0.05	1.97±0.04
Total sugars (g/L)	20.85±0.01	21.59±0.04	28.61±0.14	26.40±0.06
Xylose yield (g xylose/g rice straw)	0.06	0.06	0.08	0.07
Total sugars yield (g total sugars/g rice straw)	0.07	0.08	0.10	0.09
Acetate yield (g acetate/g rice straw)	0.01	0.01	0.01	0.01

- All of the acid hydrolysis conditions with the solid to liquid ratio of 1:10 and the 1.5% acid concentration were performed at 121ºC for 30, 45, 60, and 90 min.
- All data represent an average value of triplicates with standard deviation.
- The value of the total products from the acid hydrolysis bearing different font symbols are significant difference ($p \leq 0.05$).

Next, the residual solid from the first step of the hydrolysis, using the solid to liquid ratio of 1:10 and the reaction time of 60 min with 1.5% acid, was pretreated at the solid to liquid ratio of 1:6 with 1.0% sulfuric acid, and the reaction time of 30, 45, 60, and 90 min. The trend of hemicellulose solubilization was similar to the first step of acid hydrolysis (Table 6.8). When the reaction time was increased from 30 to 60 min, the xylose concentration was also increased. In contrast, the level of xylose concentration obtained decreased significantly when the hydrolysis time was increased to 90 min. At the reaction time of 60 min, the maximum amount of xylose

and total sugars presented in hydrolyzate were 11.81±0.05 g/L and 16.06±0.09 g/L, respectively. The amount of xylose dramatically dropped to 11.01±0.04 g/L when the reaction time of the pretreatment was increased to 90 min.

Table 6.8 Effect of reaction time on the composition of hydrolyzates of dry rice straw in the second pretreatment step.

Reaction time (min.)	**30**	**45**	**60**	**90**
Collected volume (mL)	35.33±0.58	35.67±0.58	35.33±0.58	35.33±0.58
Xylose (g/L)	9.16±0.03	10.19±0.02	11.81±0.05	11.01±0.04
Glucose (g/L)	1.13±0.03	2.13±0.06	2.33±0.07	2.38±0.04
Arabinose (g/L)	1.25±0.03	1.54±0.05	1.92±0.03	1.81±0.02
Acetate (g/L)	0.73±0.04	0.82±0.02	0.88±0.03	0.94±0.03
Total sugars (g/L)	11.53±0.02	13.86±0.08	16.06±0.09	15.20±0.07
Xylose yield (g xylose/g rice straw)	0.03	0.04	0.04	0.04
Total sugars yield (g total sugars/g rice straw)	0.04	0.05	0.06	0.05
Acetate yield (g acetate/g rice straw)	0.00	0.00	0.00	0.00

- The residual solid from the first stage hydrolysis (with the solid to liquid ratio of 1:10 and 1.5% (v/v) acid) were performed with the solid to liquid ratio of 1:6 and 1.0% acid concentration at 121ºC for 30, 45, 60, 90 min.
- All data represent an average value of triplicates with standard deviation.
- The value of the total products from the acid hydrolysis bearing different font symbols are significant difference ($p \leq 0.05$).

The composition of all the two-stage hydrozates were shown in Table 6.9. The results indicated that the optimum condition of the two-stage acid pretreatment of rice straw, providing the maximum total yield of hemicellulosic sugars, was achieved at the solid to liquid ratio of 1:10 with the sulfuric concentration of 1.5% at 121ºC for 60 min in the first pretreatment step. The liquid phase containing the soluble hemicellulosic sugars was separated. Then, the hemicelluloses reaction of the remaining solid residue from the second pretreatment step was further preceeded at the solid to liquid ratio of 1:6 with the H_2SO_4 concentration of 1.0%, at 121 ºC for 60 min. In this study, the maximum yield of xylose, total sugars, and acetate was 2.43, 3.16, and 0.19 g/g rice straw, respectively. Notably, the quantity of xylose recovery of rice straw under the mild acid hydrolysis condition in this work is closely related with other researchers using the severe acid hydrolysis condition. Baek and Kwon (2007) reported the use of solid to liquid ratio of 1:10 with 1.5% H_2SO_4 at 130ºC for 20 min to hydrolyze the chopped rice straw which resulting in the yield of xylose and acetate of 0.13 and 0.009 g/g rice straw, respectively. Furthermore, Hsu *et al.* (2010) investigated the pretreatment of milled rice straw under the hydrolysis reaction using a solid:liquid ratio of 1:10 with 1% H_2SO_4 (v/v) at 160ºC for 1 min. They obtained yields of xylose and acetate of 0.14 and 0.013 g/g rice straw, respectively.

Table 6.9 The composition of hydrolyzates obtaining from the optimal condition in the two-stage acid pretreatment for hemicelluloses carbohydrate recovery of 10 g dry rice straw.

Reaction time (min.)	60&30	60&45	60&60	60&90
Collected volume (mL)	70.67±1.15	70.67±1.15	70.67±1.15	70.67±1.15
Xylose (g/L)	31.68±0.07	32.71±0.07	34.33±0.10	33.53±0.05
Glucose (g/L)	3.62±0.09	4.62±0.12	4.82±0.13	4.87±0.06
Arabinose (g/L)	4.85±0.06	5.14±0.08	5.52±0.06	5.41±0.04
Acetate (g/L)	2.57±0.09	2.66±0.07	2.72±0.08	2.78±0.05
Total sugars (g/L)	40.14±0.16	42.47±0.22	44.67±0.23	43.81±0.14
Xylose yield (g xylose/g rice straw)	2.24^{a}	2.31^{b}	2.43^{c}	2.37^{d}
Total sugars yield (g total sugars/g rice straw)	2.84^{e}	3.00^{f}	3.16^{g}	3.10^{h}
Acetate yield (g acetate/g rice straw)	0.18	0.19	0.19	0.2

- The residual solid from the first stage hydrolysis with the solid to liquid ratio of 1:10 were performed at 121ºC for 60 min with 1.5% (v/v) H_2SO_4. Then the residual solid from the first stage hydrolysis was performed by adding 1.0% (v/v) H_2SO_4 for the different reaction time of 30, 45, 60 and 90 min under the same condition as mentioned above. Symbol of combined two-stage hydrolysis process with the different reaction time was 60&30 (or 60&45 or 60&60 or 60&90) representing the performance of the first stage at 121ºC for 60 min and the second stage at 121ºC for 30 min.
- All data represent on average value of triplicates with standard deviation.
- The value of the total products from the acid hydrolysis bearing different font symbols are significant difference ($p \leq 0.05$).

6.4 Conclusion

Significant enhancement on yield of fermentable sugar release and yield of hemicellulose saccharification could be achieved through the two-stage acid pretreatment under mild condition. The two-stage pretreatment with diluted acid for hydrolysis was the most suitable method to depolymerize xylan in hemicelluloses for maximum xylose production and reduction of liberation of inhibitory sugar degradation products. This study revealed that the three approximate combinations of the solid to liquid ratio, acid concentration and reaction time in the two-stage acid pretreatment could help increase the amount of xylan solubilization. Although the acid pretreatment in this study was performed under the mild acid condition, some inhibitory products were still generated in the significant amount. This could affect the growth and fermentation rate of microorganism. It is suggested to further investigation into the detoxification methods to reduce the inhibitory effect of the toxic compounds presented in the hydrolyzate and in order to improve the fermentability of rice straw by microorganisms.

CHAPTER VII

ENHANCED ENZYMATIC SACCHARIFICATION OF RICE STRAW TREATED WITH DOUBLE ACID PRETREATMENT AND ALKALINE PRETREATMENT

7.1 Introduction

The efficient process of enzymatic saccharification depends on quality of the treated cellulose from pretreatment method and enzyme loading in the enzymatic hydrolysis reaction (Fang *et al*., 2010; Singh and Bishnoi, 2012). Therefore, the purpose of this chapter was to investigate into the physical and microstructure of pretreated rice straw resulting from the different pretreatment methods, to investigate the effect of washing or non-washing the pretreated solid fraction between the different chemical pretreatment on the overall sugars yield after the enzymatic hydrolysis, and to investigate the suitable amount of enzyme loading on digestibility of the pretreated cellulose solids. For the investigation of the effect of washing, six different pretreatment experiments were set up as follows: 1) one-step dilute acid pretreatment (A), 2) one-step alkaline pretreatment (B), 3) two-step dilute acid pretreatment with separation and washing of the insoluble solid material and then the subsequently one-step alkaline pretreatment (A2WB1), 4) two-step acid pretreatment without washing followed by the one-step alkaline pretreatment (A2B1); 5) two-step acid pretreatment without washing followed by two-step alkaline pretreatment (A2B2), and 6) the one-step acid pretreatment without washing followed by the two-step alkaline pretreatment (A1B2). The characteristics of rice straw structure after

the synergistic acid-alkaline pretreatment were examined by scanning electron microscopy (SEM).

7.2 Materials and Methods

7.2.1 Biomass material and enzyme

Rice straw was cut to grinded (~1 cm) and chopped (~2.5 cm) rice straws, which was used for pretreatment. The cut rice straw was dried in oven at 65ºC until constant weight. Then, it was stored in plastic bags at room temperature till further use. For the experiments of enzymatic hydrolysis, crude enzyme (ACTIPRO VJ-52) was kindly provided from Siam Victory Chemicals Co. Ltd., Thailand. The commercial enzyme Cellulase VJ 52 consists of an endoglucanase acitivity of 2,200 - 2,800 carboxymethy-cellulase units (CMC U)/g and β-glucosidase activity of 450-775 pNP-glucosidase units (pNPG U)/g. The enzymes were produced from *Trichoderma reesei.*

7.2.2 Pretreatment of rice straw

7.2.2.1 Acid pretreatment

The acid pretreatment of rice straw was performed and modified according to the protocol described by Qureshi *et al.,* 2007. The grinded rice straw samples were soaked containing 1% (v/v) sulfuric acid solution at 10% (w/v) total solid loading in glass test tubes. The final total volume was 3 mL. The solutions were autoclaved at 121ºC for 60 min with double times. Prior to autoclaving the mixture, the tube was covered with aluminum foil. The tube containing the mixture was weighed before and after autoclaving to account for the loss of water. The lost water was replaced by adding distilled water to the mixture. Upon autoclaving, the mixture

was cooled to room temperature followed by adjusting pH to 4.8 with 5 M NaOH. The mixture subsequently used for enzyme hydrolysis experiments.

7.2.2.2 Alkaline pretreatment

Chopped rice straw at a solid to liquid ratio of 1:6 was treated with sodium hydroxide (NaOH) at concentration (2% w/v). The reaction mixture was performed under the mild pretreatment condition at 121°C or 15 psi for 60 min. Consequently, the treated solid fraction was collected by filtration through cotton cloth. The solid fraction was washed with tap water until the pH of washed water was neutral. The wet acid pretreated solid was stored at -20ºC until further use. To calculate percentage of dry treated cellulose, the samples were weighted before and after oven drying at 65ºC until constant weight.

7.2.2.3 Combined acid-alkaline pretreatment

Six different pretreatment strategies were investigated as schematically show in Figure 7.1. All of the pretreated rice straws were used as cellulose substrates for enzymatic hydrolysis in this study. In the beginning, procedure of the A2WB1 pretreatment was the combination of the two-stage dilute acid and one-stage alkaline pretreatments. At the first acid pretreatment step, the chopped rice straw to liquid ratio of 1:10 was mixed with 2% (v/v) sulfuric (H_2SO_4) solution at 121ºC for 60 min. The liquid phase containing the soluble hemicellulosic sugars was separated through cotton cloth. In the second acid pretreatment step, the remaining solid residue was consequently preceeded at the solid to liquid ratio of 1:6 with 1% (v/v) H_2SO_4, at the same reaction temperature and time as in the first hydrolysis step. The slurry was collected by filtration through cotton cloth. Next, the recovered solid was repeatedly washed with tap water until the pH of the washed water became neutral. Third, the washed residue was further pretreated with 2% (w/v) NaOH, the solid to liquid ratio

of 1:4, at 121ºC (15 psi) for 60 min. Finally, the solid fraction was washed with tap water until the pH of the washed water reached neutral pH. The wet solid residue was stored at -20ºC until further use for enzymatic hydrolysis. The percentage of dry treated cellulose was calculated as described in section 7.2.2.2.

The A2B1 pretreatment is the combined acid-alkaline pretreatment by sequential running the double acid pretreatment without washing solid, then the one-step NaOH pretreatment, and washing solid after alkaline pretreatment. The A2B2 pretreatment is the combined acid-alkaline pretreatment by sequential running the double-acid pretreatment with washing solid, and then the double-NaOH pretreatment with washing solid after alkaline pretreatment. The A1B2 pretreatment is the combined acid-alkaline pretreatment by sequential starting the single-step acid pretreatment without washing solid, and then the double-stage alkaline pretreatment with washing solid after alkaline pretreatment.

7.2.3 Enzymatic saccharification

The dosage of the enzyme cocktail was investigated to investigate the best hydrolysis conditions for rice straw and to compare the suitable pretreatment process for enzymatic saccharification. The experiments were carried out in glass test tubes with a working volume of 2 mL. The sacchrification was done by using crude cellulase enzymes (ACTIPRO VJ-52) from Siam Victory Chemicals Co. Ltd., Thailand. The pretreated cellulose sample was saccharified with different amount of enzyme mixture as follows: 0.3, 0.5, and 0.7 mL/g pretreated biomass. The chopped raw rice straw was used as control. In all cases, enzymatic hydrolysis experiments were conducted in duplicate. Enzymatic hydrolysis reaction was performed in a screw cap test tube containing 50 mM sodium citrate buffer (pH 4.8) and 10% (w/v) dry pretreated cellulose substrate at 50ºC water bath for 72 h. Samples were taken for

analysis every 24 h. The samples were centrifuged at 13,400 rpm for 5 min to remove solid residues. The hydrolysates were kept at -20ºC for analysis.

7.2.4 Analysis methods

After the completion of enzymatic hydrolysis, the sugar composition of the hydrolysates was analyzed by HPLC (Algilent 1200 series) equipped with refractive index detector. The separation of supernatant sample was run by using an Aminex HPX-87H ion exchange column (Bio-Rad, Richmond, USA) maintained at 45°C with 4 mM H_2SO_4 as the mobile phase. Total sugars yield was expressed as gram of total sugars per gram of dry biomass. The morphology of the untreated and pretreated rice straw structures under different alkaline or dilute acid pretreatments was visualized by the scanning electron microscope (SEM) model JSM-6400 SEM (Japan).

7.2.5 Data analysis

Two parallel samples were used in all analytical determination. The data represent the average value of duplicate analysis. The results obtained in the present study were statistically analyzed by Duncan's multiple range test (DMRT) at 95% significant confidence ($p<0.05$) using SPSS statistical software (SPSS for Windows, version 16).

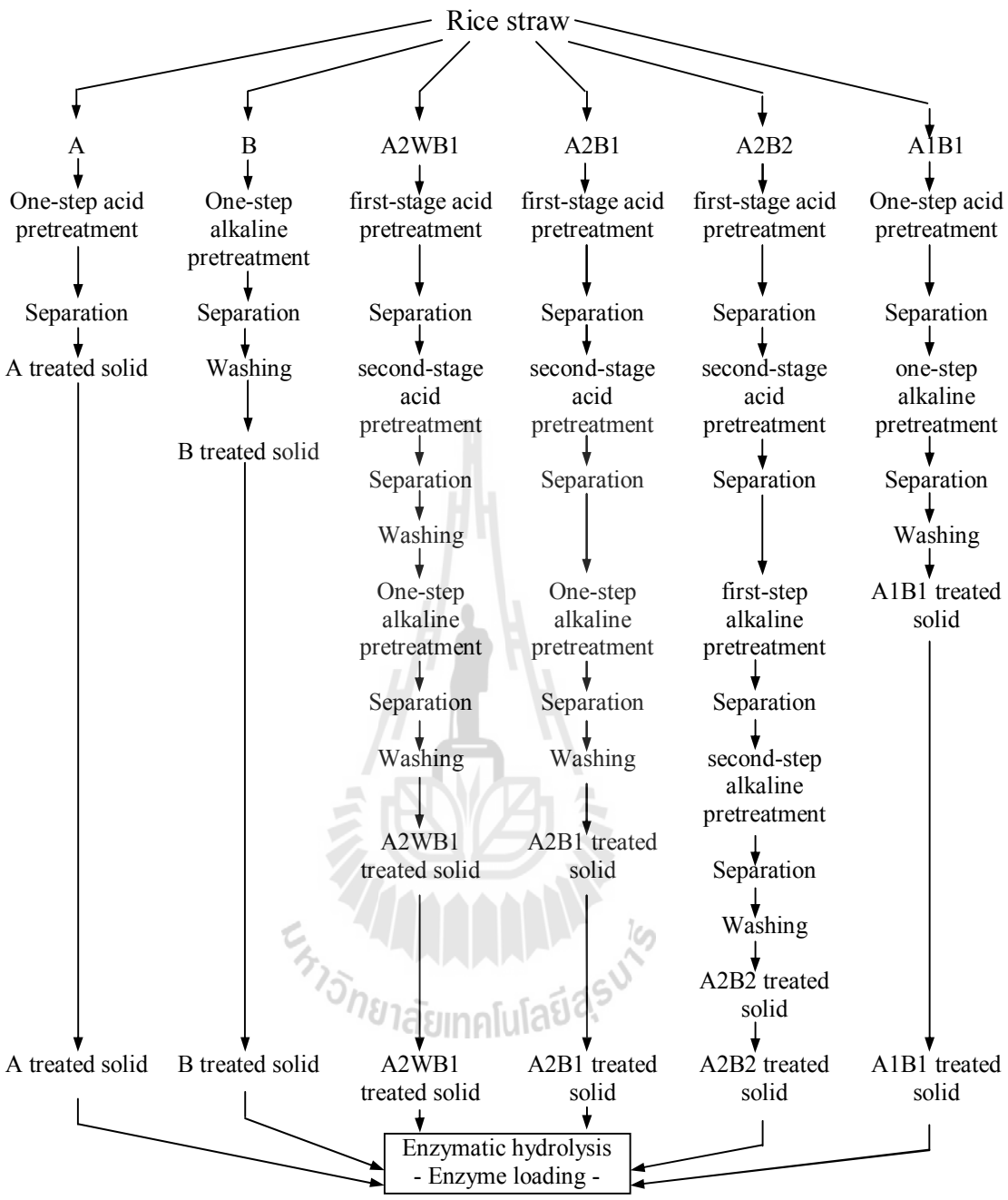


Figure 7.1 Schematic of experimental set up for evaluation of the different pretreatment procedures.

7.3 Results and Discussions

7.3.1 Microstructure changes of pretreated rice straw

Generally, fibers from cell wall structure of rice straw are composed of a bundle of single cells held together by cellulose, hemicelluloses, lignin and other binding materials. Figure 7.2 shows the physical texture of pretreated rice straws through the different pretreatment methods compared with those of the untreated rice straw. The basic texture of the tissue in the pretreated rice straw was severely shrank as well as became soft and loosened as compared with the untreated rice straw. By hand-touch of the material, the alkaline pretreated rice straw was much softer than the untreated and acid pretreated rice straw, which coincided with the results from the study of Zhang and Cai (2008) and Kim and Lee (2007).

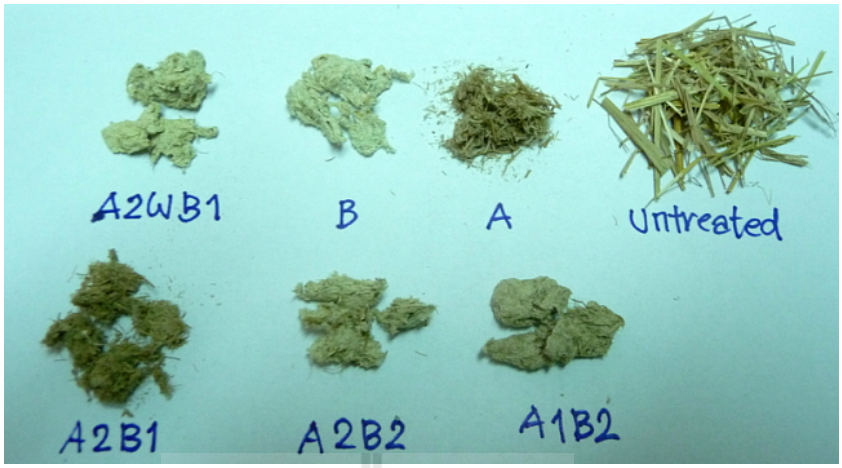


Figure 7.2 Physical texture of different combined pretreatment processes with untreated and pretreated rice straw. Untreated rice straw is raw material (U); one-step dilute acid pretreatment (A); the one-step alkaline pretreatment (B); the two-step dilute acid pretreatment with separation and washing of the water-insoluble solid material and then further alkaline pretreatment (A2WB1); two-step acid pretreatment, in which neither washing nor separation between steps was performed, and then the alkaline pretreatment (A2B1); two-step acid pretreatment, in which neither washing nor separation between steps was performed, following by supplementary double-step alkaline pretreatment (A2B2); one-step acid pretreatment, in which neither washing nor separation between steps was performed, following by supplementary double-step alkaline pretreatment (A1B2).

The characteristics of rice straw structure after the synergistic acid-alkaline pretreatment were studied by using SEM. Figure 7.3 shows SEM micrographs of rice straw before and after pretreatments. The results demonstrated the significant historical changes of the chemical pretreated rice straw. The untreated rice straw has mostly a layer of substances, which could be visualized by the rigid and highly ordered fibrils (Figure 7.3a, b). This feature of layer results from the presence of lignin, silica, and other non-cellulosic substances on the outer surface. Unlike the layer of untreated rice straw, the wire of the cellulose and the distorted cell wall structure of others acid pretreated material in a long section were appeared (Figure 7.3d). This results from the fact that, acid pretreatments usually remove hemicellulose, but it does not destroy the lignin fraction (Kumar *et al.,* 2009). In comparison, the fiber bundles of the alkaline pretreated rice straw had a smooth surface causing the removal of the large surface substances, including hemicelluloses and lignin (Zhang and Cai, 2008) (Figure 7.3e, f). It resulted from sodium hydroxide broken down the ether linkage of the lignin barrier (Lee, 1997; Varga *et al.*, 2002). It could be concluded that the alkaline pretreatment was better than the acid pretreatment for preparing the available cellulose to use as substrate in enzyme saccharification. Figure 7.3g-n demonstrated the microfibrils, which were exposed and separated from the initial connected structure. These indicated that the two-step pretreatment methods could disintegrate lignocellulosic structure of the rice straw significantly. Hence, the SEM results confirmed that the combined acid-alkaline pretreatment with washing step, especially A2WB1, had more powerful to induce historical changes in the rice straw structure. This leads to the increase in surface area and the accessibility of enzyme to convert cellulose into fermentable sugars.

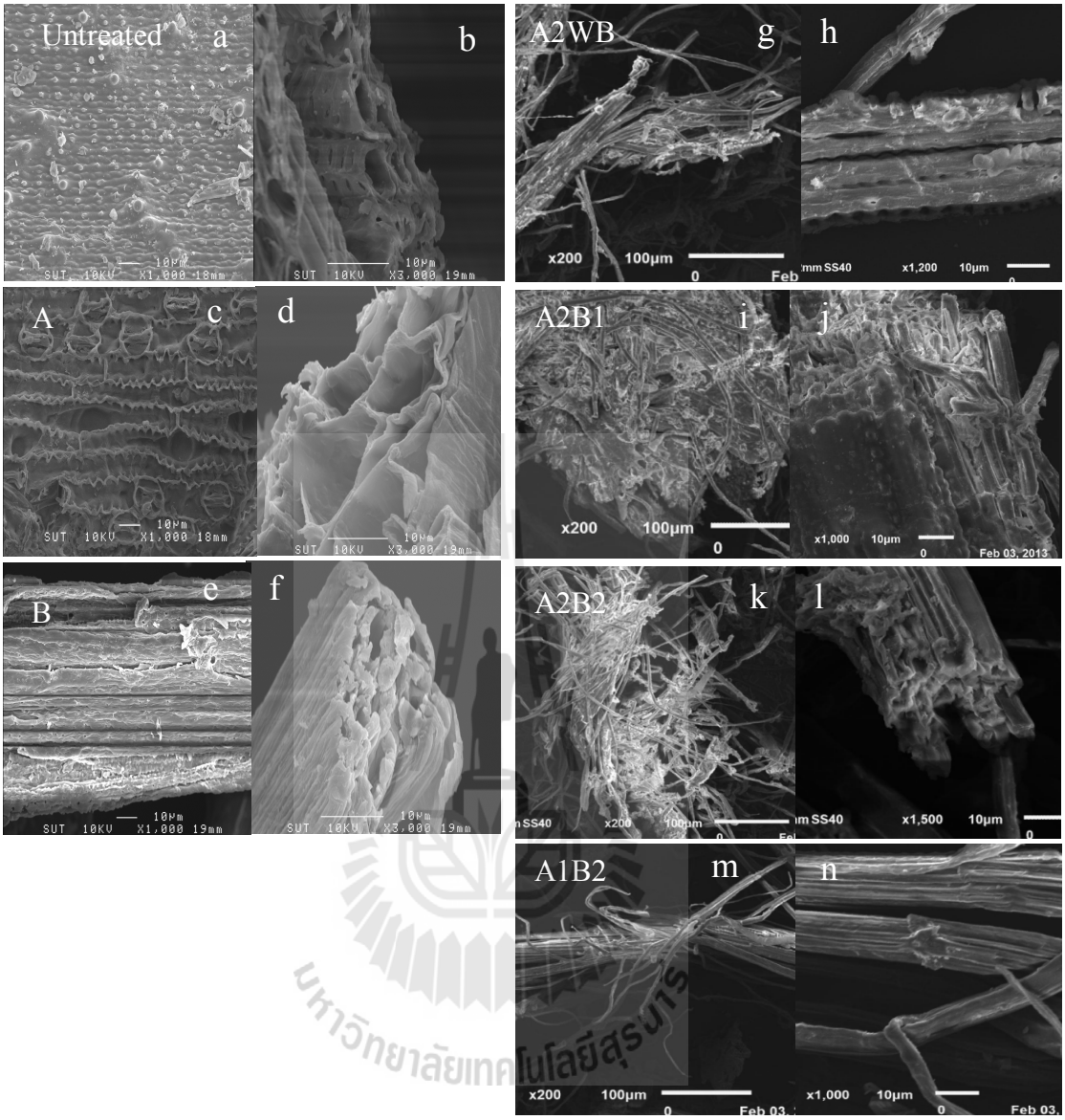


Figure 7.3 Scanning electron micrographs of rice straw structure through the different pretreatment processes. Transverse section of rice straw stem showed at the right of each pretreatment method, and longitudinal section of rice straw stem at the left of each pretreatment method. (a, b), untreated rice straw is raw material; (b, c), residual solid obtained from acid pretreatment; (e, f), residual solid from alkaline pretreatment; (g, h), A2WB1; (i, j), A2B; (k, l), A2B2; (m, n), A1B2.

7.3.2 Saccharification of rice straw cellulose with enzyme

Six pretreatment strategies were studied: one-step dilute acid pretreatment (A), one-step alkaline pretreatment (B), two-step dilute acid pretreatment with (A2WB1) or without (A2B1) separation and washing of the material between the steps following by one-step alkaline pretreatment, and one-step (A1B2) or two-step (A2B2) dilute acid pretreatment without separation and washing of the material and the subsequent two-step alkaline pretreatment. These pretreatment processes produced cellulose substrates for enzymatic saccharification. The chopped raw rice straw without the pretreatment was used as control. The cellulase enzyme complex containing endoglucanase and beta-glucosidase was employed as catalysts in the reaction of enzymatic saccharification. The various enzyme doses (0.3, 0.5, and 0.7 mL/g biomass) were optimized to achieve maximal total sugars from the saccharification of the treated rice straw. The pretreated materials were incubated with cellulase mixture at 50ºC for 72 h. The concentration of total sugar released was measured every 24 h.

One of the objectives in this study was to evaluate the effect of washing the pretreated material residues before treatment with other chemical pretreatment step to enhance the conversion of cellulose into sugar by the enzymatic hydrolysis. The results showed that the combine acid-alkaline pretreatment with washing between alternative pretreatment steps (A2WB1) resulted in the highest total sugars compared with the other combined two pretreatment cases without washing (i.e. A2B1, A1B2, A2B1) (Table 8.1). Notably, the lowest recovery of unhydrolyzed cellulose was found in the A2WB1 (data not shown). The major sugar in all the combined two pretreatment cases was glucose, and cellobiose as a minor sugar was found only in the A2WB1. As a result, the washing step was an effective step in enhancing enzymatic

hydrolysis reaction because it helped avoiding further degradation of sugars into inhibiting substrates (Boussaid *et al*., 2000; Nguyen *et al*., 2000; Söderström *et al*., 2003). For the two step pretreatments without washing, cellulose provided the lower liberated sugar after hydrolysis. It may be attributed to the condensed lignin and hemicelluloses, which may still remain on the surface of crystalline cellulosic material and could potentially block enzyme accessibility to the substrate for generation of monomeric sugars (Liu and Wyman, 2004; Zhu *et al*., 2009). In comparison, it was clearly demonstrated that the two-pretreatment with washing (A2WB1) provided more liberated sugar than the only one-step pretreatment methods (i.e. A and B pretreatment). This could reveal the double-step dilute acid pretreatment was effectively removed most of the hemicelluloses, causing an increasing in the pore volume and surface area of the solid residue, as well as increasing in cellulose fraction. Moreover, the washing step helped to protect sugar degradation to enzymatic inhibitors. Finally, alkaline acts mainly by dissolving lignin, as called delignification. The pretreatment of the cellulose also resulted in a swelling and disrupting of the crystalline structure of cellulose. It leads to an increase in internal surface area and a decrease in crystallinity, which makes cellulose fraction more accessible to enzymatic attack (Zhao *et al.,* 2008).

In comparison of the one-step pretreatment, the alkaline pretreatment was better than that of acid pretreatment without washing with following by enzymatic hydrolysis. It may be due to no removal of inhibitors. The hydrolysis of the acid pretreated and alkaline pretreated celluloses resulted in the generation of glucose as a major sugar followed by cellobiose, and arabinose, whereas the cellulose obtaining from the other two-step pretreatments resulted in only glucose. It indicated that the combined acid-alkaline pretreatment could remove hemicellulose completely. The

appearance of arabinose from the alkaline or acid pretreatment indicated the presence of hemicelluloses.

Table 7.1 The sugars from the different pretreated rice straw after enzymatic saccharification.

Pretreatments	End products after enzymatic saccharification (g/L)			
	Glucose	**Cellobiose**	**Arabinose**	**Total sugar**
Untreated	12.37±0.37	0	0.035±0.01	12.40±0.38
A	20.55±0.56	0.20±0.01	2.44±0.238	38.24±0.95
B	47.01±1.00	9.90±0.14	3.25±0.171	61.94±1.40
A2WB1	89.72±1.13	7.11±0.13	0	96.83±1.25
A2B1	47.74±0.85	0	0	47.74±0.85
A2B2	54.85±0.90	0	0	54.85±0.90
A1B2	24.43±0.99	0	0	24.43±0.99

Another objective of this study was to investigate a suitable amount of enzyme to digest the pretreated celluloses to available sugars. The results revealed that all of the pretreatment investigated, as the enzymatic hydrolysis dose was increased, the concentration of the released total sugar was also increased (Figure 7.4). The results also suggested that the cellulase hydrolysis reaction of rice straw was affected by the enzyme loading applied. This result was in accordance with Kaur *et al.* (1997). Based on reaction time, the maximal saccharification rate of all pretreated substrates was divided into 3 groups: 24 h (U, A, and A1B2), 48 h (B, A2WB1, and A1B2), and 72 h (A2b2). These implied that the different procedure of pretreatment influenced in the enhancement of the enzymatic activity. The effect of enzyme loading on the saccharification of A2WB1 pretreated rice straw was highest when compared with that of other pretreated substrates (Figure 7.4d). At this pretreatment method, the observed total sugars concentration was 96.83±1.25 g/L at 50ºC, pH 4.8 and 48 h of reaction time.

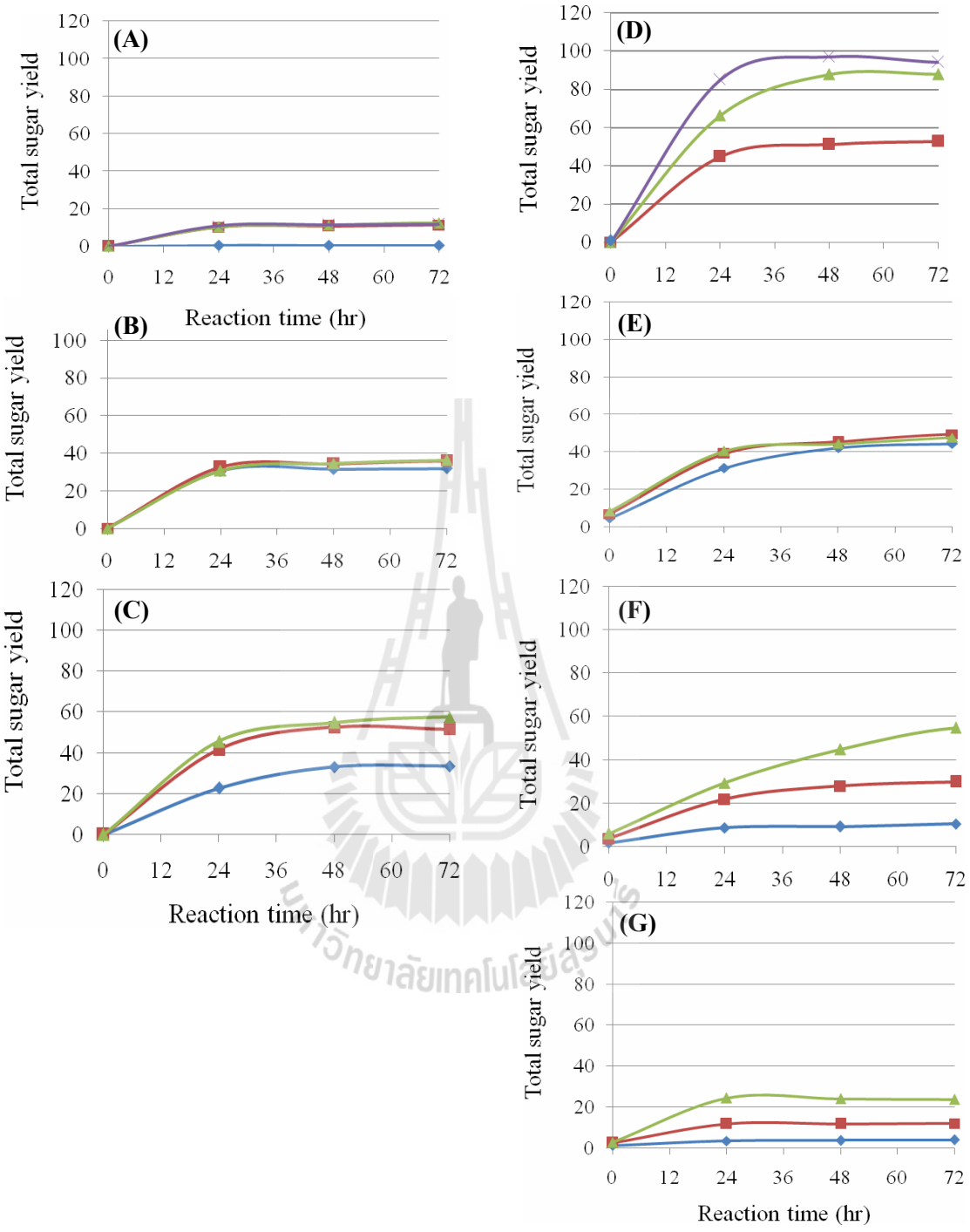


Figure 7.4 Profiles of total sugars yields at various enzyme loadings using the treated rice straw from different pretreatment processes. (A) untreated rice straw is raw material; (B), residual solid obtained from acid pretreatment; (C), residual solid from alkaline pretreatment; (D), A2WB1; (E), A2B1; (F), A2B2; (G), A1B2.

7.4 Conclusion

In this study, the SEM results revealed that the surface structure of the rice straw was significantly altered after A2WB1 pretreatment. In addition, the washing step between the A2WB1 pretreatment step was essential, and resulting in an increase in the amount of glucose released from the enzymatic reaction. The optimum enzyme loading condition in the cellulase-catalyzed process to hydrolyse the pretreated A2WB1 cellulose was 0.7 mL of cellulase mixture per gram of dry rice straw, and 10% (w/v) of dry pretreated substrate in 50 mM citrate buffer (pH 4.8) at 50°C, for 48 h of reaction. The A2WB1 pretreatment was an effective method of improving the enzymatic saccharification to enhance the liberation of glucose.

CHAPTER VIII

COMPARATIVE DETOXIFICATION OF RICE STRAW HYDROLYSATE FOR BUTANOL PRODUCTION

8.1 Introduction

Hemicellulose in rice straw can easily be liberated with process of dilute acid treatments under mild conditions to generate a mixture of sugars (D-glucose, D-xylose and L-arabinose) with xylose being the mainly sugar. Besides the xylose being produced, various inhibitory substances are formed during acid hydrolysis of rice straw. Toxicity of these inhibitors is the major factor limiting lignocellulosic bioconversion to industrial products from fermentation. These compounds inhibit growth of microorganisms which as biocatalysts. Several detoxification treatments were employed to remove these inhibitors.

The present chapter, the purposes of work were 1) to investigate efficiency of detoxification method to reduce toxicity of inhibitory compounds in rice straw hemicellulosic hydrolysate from 2-step acid hydrolysis, 2) to investigate the growth adaptation of *Cl. acetobutylicum* ATCC 824 in xylose hydrolysate and acetone butanol ethanol (ABE) production, and 3) to evaluate the production of ABE from concentrated detoxified hydrolysates of rice straw by this *Clostridium* strain.

8.2 Materials and Methods

8.2.1 Microorganism, medium and culture conditions

Cl. acetobutylicum ATCC 824 was used in this study. Spores suspension of was routinely maintained in mineral salt (MS) medium containing glucose (60 g/L) at -80ºC. The composition of this medium was previously described in Chapter 4.

The inoculum was prepared by transferring the spore suspension (1 mL) into 10 mL of the TYG medium. It was then heat shocked for 10 min at 80ºC followed by cooling in ice cold water. Cells were allowed to grow under anaerobic condition at 35ºC for 16-18 h. The active motile cell suspension was then transferred to 80 mL of P2 medium in 125 mL aluminium capped serum bottle and incubated at 35ºC for 5-7 h to be used as inoculums for fermentation of ABE. This would be used as an unadapted inoculum (A) for adaptation in hydrolysate based medium for ABE production.

For prepararation of an adapted inoculum (B), 10% (v/v) of the active culture of inoculum A was transferred to adaptation medium. This medium was prepared from P2 medium plus 2.5 fold diluted detoxified concentrated rice straw hydrolysate (DCRAH) by adding water and sugar to the final concentration of the solution mixture of 60 g/L. Then, cultures were incubated at 35ºC prior to moving into the fermentation media, similar to the media, which was used for adaptation.

Tryptone-glucose-yeast extract (TYG) medium was used as an enrich medium for spore germination. It consisted of (g/L): 8.0 tryptone; 4.0 yeast extract; and 1.0 glucose.

P2 medium was used for inoculum preparation and fermentation. It consisted of the following components (g/L): 0.5 KH_2PO_4; 0.5 K_2HPO_4; 0.4 $MgSO_4.7H_2O$; 0.01 $MnSO_4.4H_2O$; 0.01 $FeSO_4.5H_2O$; 1.0 yeast extract; 0.5 cysteine;

and 10 CaCO3. The final concentration of P2 medium composed 80 μg/L biotin and 1 mg/L 4-aminobenzoic acid. The P2 medium was boiled followed by flushing nitrogen gas to the medium containing carbon source and yeast extract (1 g/L) to get rid of oxygen in the medium. The pH was adjusted to 6.8 using 1 M NaOH prior to sterilizing the medium at 121ºC for 15 min. After cooling, the sterilized medium was added with 1 mL of each P2 stock solution of filter-sterilized buffer, vitamin, and mineral. The stock solution of buffer consisted of (g/L): 50 KH_2PO_4; 50 K_2HPO_4; and 220 ammonium acetate. The vitamin stock solution consisted of (g/L): 0.1 para-amino-benzoic acid; 0.1 thiamin; and 0.001 biotin. Finally, the mineral stock solution consisted of (g/L): 20 $MgSO_4.7H_2O$; 1 $MnSO_4.H_2O$; 1 $FeSO_47H_2O$; and 1 NaCl.

8.2.2 Preparation of concentrated rice straw hydrolysate

Procedure for preparing rice straw hydrolysate was previously mentioned in Chapter 6 using the two-stage dilute acid hydrolysis. The attained hydrolysate was concentrated by vacuum evaporation at 70ºC until a xylose concentration of 80 g/L (from initial sugars concentration 21 g/L in hydrolysate) was obtained. The concentrated hydrolysate, so called CRAH, was then kept at 4ºC for subsequent use in detoxification methods.

8.2.3 Detoxification treatment with over liming

Over liming was conducted by initially adjusting the pH of the CRAH from pH 10.0 to 10.5 using solid calcium hydroxide. The mixtures were then heated in a temperature-controlled water bath set at 50°C for 30 min. Finally, the pH was adjusted to 7.0 by using 96% H_2SO_4. The mixtures were filter steriled through a 0.2 μm filter membrane, and kept for a minimum of 5 days at 4°C. This over liming treated hydrolysate (O-CRAH) would be used for the substrate for fermentation.

8.2.4 Detoxification treatment with overtitration

The CRAH was overtitrated to pH 8 by adding NaOH pellets. The hydrolysate pH was then lowered to pH 6.5 by using 13 M H_2SO_4. After each pH shift, the treated hydrolysate was centrifuged at 2000 g for 20 min to get rid of precipitat and the supernatant of the over titration treated hydrolysate (T-CRAH) was finally obtained.

8.2.5 Detoxification treatment with charcoal absorption

One gram of activated charcoal was mixed with 40 mL of the CRAH. The mixture was incubated in the controlled temperature water bath shaker at 200 rpm, 30ºC for 1 h. The treated liquid fraction was then recovered by centrifugation. This charcoal treated hydrolysate was designated as C-CRAH.

8.2.6 Detoxification treatment with neutralization

The CRAH was neutralized by adjusting pH of the concentrated hydrolysate to 6.5 with 5M NaOH. This neutralization treated hydrolysate (N-CRAH) was used as a control substrate and further secondary detoxified with the activated charcoal carbon method.

8.2.7 Two-stage detoxification

The CRAH was firstly detoxified by overliming or overtitration or neutralization, and then the treated hydrolysate was secondly detoxified with the activated charcoal method, as previously described. These treated hydrolysates were designated as OC-CRAH, TC-CRAH, and NC-CRAH, respectively.

8.2.8 Fermentation

Fermentation was conducted in aluminium capped serum bottles (120 mL) containing 80 mL medium throughout the studies. In all experiments, the batch fermentation condition was maintained at 35ºC, without agitation or pH control.

During fermentation, samples were taken every 24 h for pH, ABE and sugars concentration. Prior to analysis, the samples were centrifuged at 13,400 rpm in a microcentrifuge for 10 min. Unless otherwise stated fermentation time was 7 days.

8.2.9 Experimental design

Figure 8.1 illustrates five different detoxification treatment methods for investigation of efficiency of approximately detoxification method to reduce the inhibitory toxicity of the CRAH. The detoxification methods included 1) one-step detoxification with overliming (O), overtitration (T), activated charcoal (C), or neutralization (N) and 2) two-step detoxification with overliming plus activated charcoal (OC), overtitration plus activated charcoal (TC), and neutralization plus activated charcoal (NC). The fermentation media with DCRAH plus glucose raised to 60 g/L were used to produce acetone, butanol, and acetone by *Cl. acetobutylicum* ATCC 824. The CRAH was used as untreated hydrolysate with pH 6.5. The P2 medium supplemented with glucose (G), or glucose obtaining from enzymatic hydrolysis of acid-alkaline pretreated rice straw (GE), or glucose plus xylose (GX) or GE plus xylose (GEX), which was used as a control medium. The initial sugar concentration of the solvent producing medium prior to fermentation was 60 g/L. Besides, the detoxified hydrolysates as well as fermentable end-products were analysed for composition by HPLC.

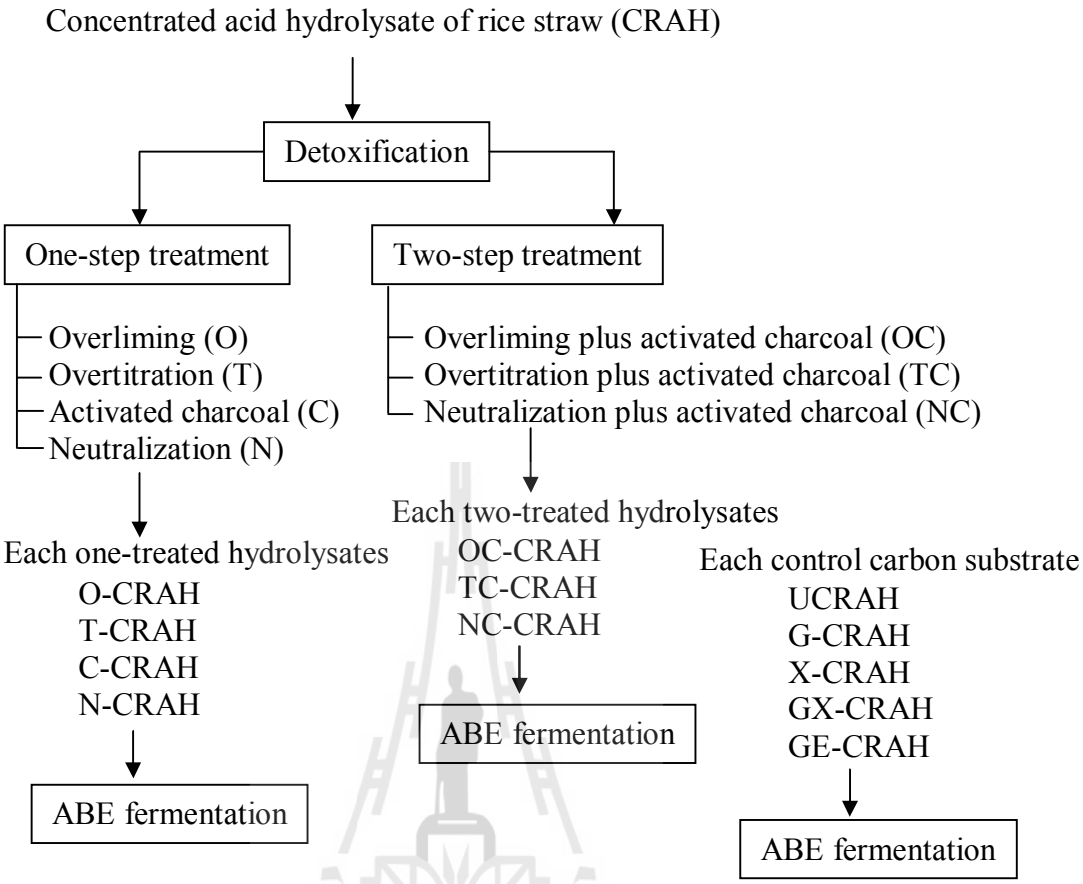


Figure 8.1 Experiment set up for the investigation of the different detoxification treatment methods of rice straw acid hydrolysate for ABE fermentation by *Cl. acetobutylicum* ATCC 824.

8.2.10 Analyses

Samples were analyzed for sugars, organic acids and solvents by HPLC as previously described. The results presented here were an average of duplicate experiments and have an error range of 8-12%. The concentration of solvents (acetone, butanol and ethanol) was determined by gas chromatography (GC) equipped with a flame ionization detector (FID). Separations of solvents were achieved by using a capillary column and H_2 as the carrier gas. The column temperature was held at 115^{o}C for 8 min and then programmed at 5^{o}C/min to 170^{o}C with a 10 min final hold.

Temperature of the detector and injector were set at 270 and 220^{o}C, respectively. Sample was injected as 2 μL aliquots. The results obtained in the present study were statistically analyzed by Duncan's multiple range test (DMRT) at 95% significance confidence ($p<0.05$) using SPSS statistical software (SPSS for Windows, version 16).

For the analysis of kinetic parameters, volumetic productivity was calculated as total ABE concentration (g/L) divided by the time (h) of fermentation. Fermentation time was expressed as the time period when the maximum ABE concentration was achieved. ABE yield (Yp/s, g ABE produced/g sugars consumed) was calculated as total ABE produced (g/L) divided by total sugars utilized (g/L).

8.3 Results and Discussions

8.3.1 Composition of different detoxified hydrolysates

In this study, the rice straw hydrolysate from the 2-step acid hydrolysis was concentrated by evaporation, because initial xylose concentration was lower than 60 g/L, which may resulted in the low production of acetone, butanol, and ethanol in the subsequent fermentation. Although some studies reported that 100% furfural or partial volatile compounds (i.e. acetic acid and formic acid) could be removed by evaporation process, in contrast, acetic acid and, hydroxymethylfurfural, and lignin degradation products increase (Mussatto *et al.*, 2004; Zhu *et al.*, 2009). Hence, the CRAH was detoxified with the one-step methods including overliming (O), overtitration (T), neutralization (N), and activated charcoal absorption (C), or the combination of two-step methods ,acitivated charcoal plus overliming (OC), plus overtitration (TC), and plus neutralization (NC) in order to improve ABE production by *Cl. acetobutylicum* ATCC 824. Table 8.1 illustrates the main components of raw (RAH), concentrated hydrolysates

(CRAH) and detoxified concentrated hydrolysates (DCRAHs). Xylose was the major sugar of the hydrolysates.

Table 8.1 Effect of detoxification procedures on sugar composition in the rice straw concentrated hydrolysate.

Treatments	Glucose	Xylose	Arabinose	Total sugars
RAH	2.81±0.26	19.47±0.36	2.23±0.17	24.51±0.49
CRAH	9.35±0.44	60.59±0.86	9.17±0.42	80.91±0.838
O	6.75±0.28	21.88±0.73	2.22±0.04	30.85±0.98
T	9.23±0.09	56.85±0.61	9.06±0.23	75.14±0.47
C	9.25±0.16	50.73±0.24	7.85±0.23	67.83±0.62
N	9.22±0.10	58.96±0.58	9.08±0.27	77.26±0.41
OC	6.51±0.06	20.73±0.58	1.98±0.07	29.22±0.71
TC	9.18±0.16	54.95±0.89	8.97±0.20	73.11±0.51
NC	8.98±0.62	49.83±0.67	8.17±0.06	66.97±0.11

The result showed the lost of sugar during the detoxification process. Percentage of sugar removal was different from each detoxification method (Figure 8.2). By comparing detoxification treatments, maximum removal of total sugars was observed from the results of the overliming and overliming plus activated charcoal procedures. Xylose as the main sugar in all of the DRAHs was gradually decreased. During overliming reaction, it was noteworthy that heat was highly released. This may be attributed to the heat effect on breaking down xylose. Further, it indicated that xylose molecule was more easily destroyed than glucose molecule due to its less stability (Zhu *et al.*, 2009). The results exhibited that the method of neutralization detoxification showed the lowest level of sugar removal relating to the lowest removal of inhibitory substances.

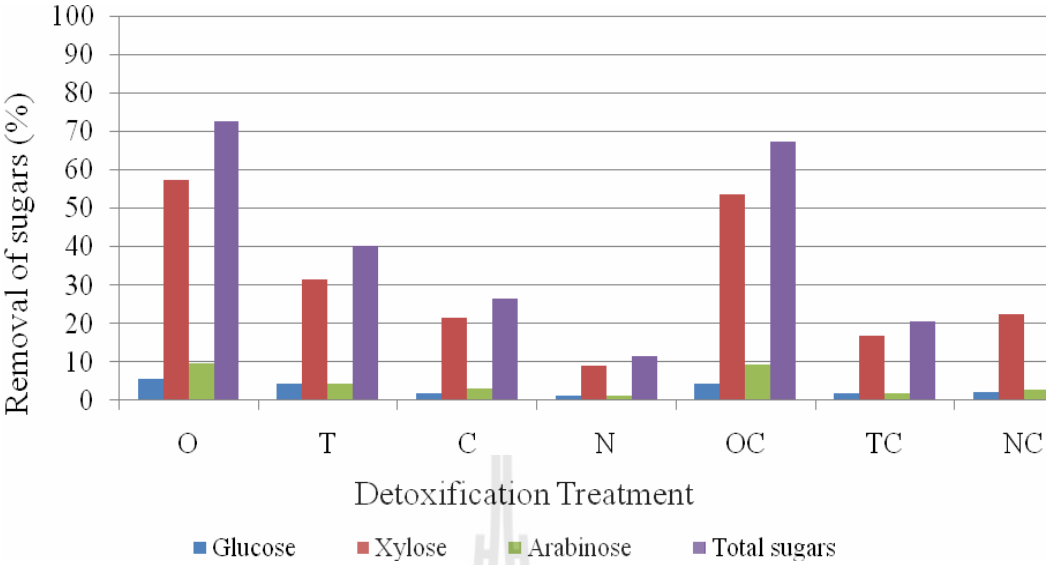


Figure 8.2 Removal of glucose, xylose, arabinose, and total sugars from concentrated rice straw hydrolysate obtained from seven detoxification treatments.

8.3.2 ABE fermentation using undiluted or diluted DCRAHs-based medium

In order to examine the use of DCRAHs as fermentation substrates for batch ABE fermentation by the *Cl. acetobutylicum* ATCC 824, the DCRAH based media supplemented with glucose to the final concentration of the solution mixture of 60 g/L were used as fermentation media. The untreated CRAH based medium was used as a control medium. Table 8.1 shows the compositions of sugars of the untreated CRAH and each of the undiluted DCRAH (i.e. O-, T-, C-, N-, OC-, TC-, NC-CRAH), which xylose was the main sugar. The fermentation results demonstrated that no cell growth and ABE fermentation were observed in all of the media (data not shown). It implied that amount of various toxic substances in the DCRAHs was still in high level that seriously interfered with the growth and fermentation process. Next, in order to overcome the problem associated with the presence of inhibitors in these hydrolysates, the overliming treated CRAH (O-CRAH) was chosen to examine for the effect of O-CRAH dilution on the

reduction of inhibitory toxicity for ABE fermentation. The O-CRAH was diluted with distilled water followed by supplementing with GE to the final concentration of the solution mixture of 60 g/L. The different ratio of the OCRAH to DW was 1:0, 1:1, 1:3, and 3:1. P2 media, composed of these diluted OCRAH plus GE, were used as fermentation media. The result showed that the growth and ABE fermentation of *Cl. acetobutylicum* were stimulated by the OCRAH to DW ratio of 3:1 (data not shown). This approximately DCRAH based medium consisted of about 12 g/L xylose and 48 g/L GE sugars. It suggested that this ratio was suitable to prepare DCRAH based medium as a fermentation medium.

8.3.3 Effect of microorganism adaptation on ABE fermentation from detoxified hydrolysates

The objective of the present investigation was to use the diluted DCRAHs to produce acetone-butanol-acetone solvents by using 5% (v/v) of an unadapted (A) or an adapted (B) inoculum. Fermentation broth used was P2 medium supplemented with DCRAH to water ratios of 3:1 and GE until 60 g/L final sugars concentration. The *Cl. acetobutylicum* was grown as inoculum A and B. Batch fermentations were conducted under anaerobic condition and static cultivation without pH control at 35ºC for 7 days. From Figure 8.3, it is evident that the use of adapted inoculums B significantly increased the production of ABE in most of the diluted DCRAH media, except in the diluted N-CRAH based medium. It might be explained that the mechanism of neutralization treatment, which was pH adjustment to induce precipitation and/or instability of the toxic substances, was not enough to eliminate the inhibitory toxicity compared with other detoxification treatments. Nevertheless, this could be concluded that the cell can adapt to the hydrolysate prior to fermentation provided an alternative in enhancing the ABE production.

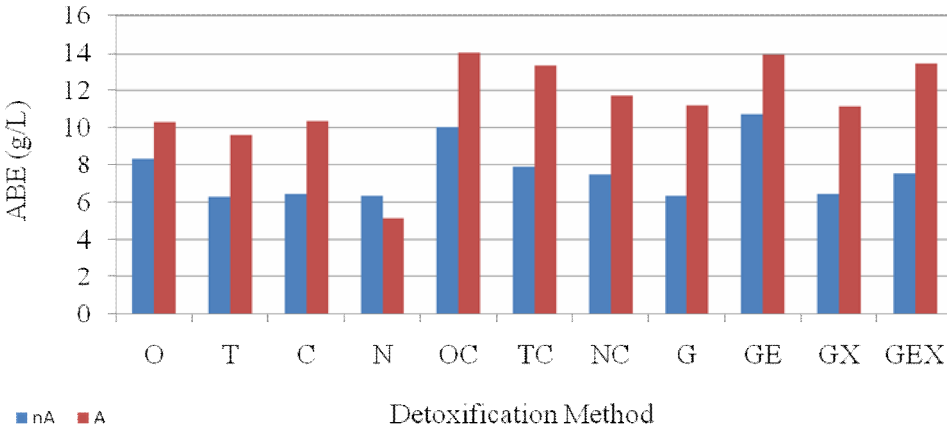


Figure 8.3 ABE production using rice straw concentrated hydrolysates from different detoxification methods for *Cl. acetobutylicum* ATCC 824 grown as unadapted inoculums A (blue bar) and adapted inoculums B (red bar), in comparison with the control media using pure glucose (G), rice straw enzymatic digested glucose (GE), mixture of pure glucose and xylose (GX), and mixture of GE and pure xylose.

8.3.4 Efficiency of the detoxification for ABE production from hydrolysates

The efficiency of the one-step or two-step detoxification method to prepare the CRAH as the fermentable substrate for ABE fermentation using *Cl. acetobutylicum* ATCC 824 was evaluated. The *Clostridium* was grown as the adapted inoculum followed by transferring into the medium containing different DCRAHs.

In the one-step detoxification, neutralization process of the acidic pH of CRAH is a pH adjustment to neutral value for microbial fermentation by using neutralizing agent (Parajo *et al.*, 1998). In this study, C-RAH was detoxified with one-step method (1S-DCRAH) including overliming (O), overtitration (T), neutralization (N) and absorption (C) using the neutralizing agent including $Ca(OH)_2$, $NaOH$-H_2SO_4,

NaOH, and activated charcoal, respectively. At the end of the fermentation, the ABE concentration with the detoxification procedure using $Ca(OH)_2$, $NaOH-H_2SO_4$, and activated charcoal was higher than that using NaOH (Figure 8.3). This suggested that the detoxification procedures by overliming, overtitration, and activated charcoal absorption were able to decrease more inhibitory toxicity than neutralization. These results coincided with the previous result,where the neutralization provided the lowest extent of sugar removal compared with others. It suggested that the lost of the low level of sugar related with eliminating the toxic inhibitors.

With regard to the efficiency of the detoxification methods, the result revealed that ABE production from the diluted DCRAHs from all of the two-step detoxification treatments (2S-DCRAHs) were significantly higher than those of the one-step detoxification treatments (1S-DCRAHs) (Figure 8.3). In addition, in comparison with the control media using pure glucose or xylose, or mixing both of them, the maximum ABE concentration in the 2S-DCRAH medium were near. This suggested that the activated charcoal treatment in the second detoxification step was an efficient and economic method in enhancement of the sugars-to-ABE conversion by *Cl. acetobutylicum* ATCC 824. This efficiency can be explained by the fact that the activated charcoal absorbs the phenolic compound and acetic acid resulting in the reduction of the inhibitory toxicity in hemicellulosic hydrolysates (Parajo *et al.*, 1996; Baek and Kwon, 2007). Furthermore, the results displayed that the concentration of ABE attained from fermentation of TC-CRAH was close to that of OC-CRAH. When considering the sugar losing from the aboved mentioned result, the loss of sugars in the overtitration was less than that of the overliming treatment. This could be concluded that the method of overtitration plus activated charcoal detoxification was an economical and effective method of reducing inhibitory toxicity of rice straw hydrolysate even the overliming method was widely used

due to easy separation of gypsum crystals (Larsson *et al.*, 1999: Nilverbrant *et al.*, 2003).

8.3.5 Evaluation of ABE fermentation from detoxified hydrolysates

From the above mentioned result (section 8.3.4), the 2-step detoxifications including OC, TC, and NC treatments illustrated good and effective method of hydrolysate detoxification. It had also the positive effect on the increased fermentability of rice straw detoxified hemicelluloses hydrolysate supplemented with GE sugar to the final concentration of the solution mixture of 60 g/L concentration to produce ABE employing *Cl. acetobutylicum* ATCC 824. Hence, the purpose of this experiment was to evaluate the ability of *Cl. acetobutylicum* ATCC 824 to produce solvents and organic acids and to metabolize sugars in the OC-, TC-, and NC-CRAH compared with the G, GE, GX, and GEX as control carbon substrates. The batch fermentations were performed for 168 h.

Prior to performing 2S-DCRAHs based fermentations, control batch fermentations were conducted using pure glucose or GE of 60 g/L, and mixture of glucose-xylose or GE-xylose in the ratio of 48:12 (w/w) at 60 g/L final sugar concentration in order to ascertain the ability of culture to ferment sugars present in the 2S-DCRAHs. Figure 8.4 G-J showed that *Cl. acetobutylicum* produced higher ABE concentration from GE than other sugars. ABE accumulation in the system employing G, GX, and GEX as carbon substrates was not different. These results indicated that G, GX, GE and GEX effectively supported the growth of *Cl. acetobutylicum*. Additionally, the culture was capable of utilized mixed sugars to produce solvents.

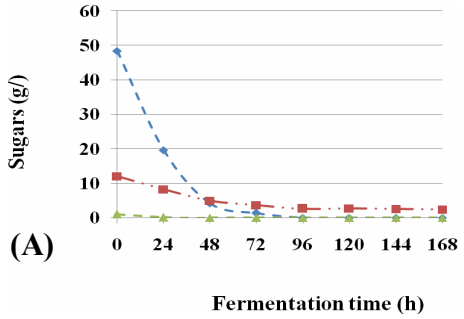
Subsequently, the ability of the culture to utilize sugars in the OC-, TC- and NC-DCRAHs for ABE production was evaluated. Each 2S-DCRAHs and GE was mixed in the ratio of 12:48 (w/w) in the solvent producing medium prior to being fermented. The results showed that total solvent in these media were slightly higher than that of the pure

sugar as a control, except GE. However, the fermentation times for the solvent producing microorganism were longer. The *Cl. acetobutylicum* produced maximum ABE concentration within 96 h for all of the 2S-DCRAHs, whereas 48 h for control (Figure 8.4). The fermentations resulted in the productivity of 0.14, 0.14, and 0.09 g/L.h in the fermentation broths containing OC-, TC-, and NC-CRAH, respectively, comparing with the productivity of the 0.23, 0.29, 0.21, and 0.28 g/L/h in fermentation broths containing G, GE, GX and GEX, respectively (Table 8.2). The results demonstrated that *Cl. acetobutylicum* could be able to metabolize sugars in the OC-, TC-, and NC-CRAH into solvents. It should be noted that high levels of total acids were present in the fermentation media (Table 8.2). Nevertheless, considering production of good level of ABE (13.32-13.54 g/L in OC- and TC-CRAH based medium), it would be hypothesized that the extent of produced acetic acid did not severely interfere with the switch from acids to solvents. In the previous studies, ABE concentration from other lignocellulosic hydrolysates such as distillers dried grains and soluble, corn stover and switchgrass, rice bran was similar (Ezeji and Blaschek, 2008; Jieun *et al.*, 2009; Qureshi *et al.*, 2010a). Further, the solvent production from the NC-CRAH based medium was lower than those of other media. It indicated that there was still the inhibitory toxicity presented in the hydrolysate, which was supported the above mentioned result.

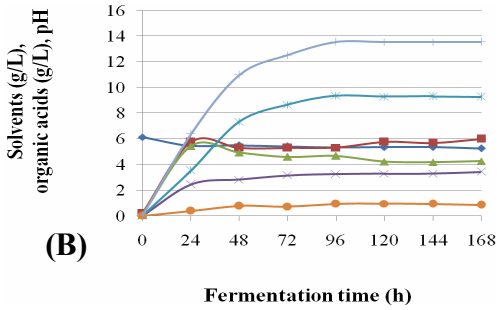
Table 8.2 ABE production and sugar utilization by *Cl. acetobutylicum* ATCC 824 with different substrates.

Kinetic parameters	OC	TC	NC	G	GE	GX	GEX
initial sugar conc. (g/L)	61.42	60.91	60.974	60	60	59.98	59.89
Sugars utillized (g/L)	58.78	56.137	53.358	60	60	59.98	59.89
Max. butyric acid conc. (g/L)	5.436	4.265	3.544	5.496	5.391	4.932	5.243
Max.acetic acid conc. (g/L)	5.761	5.753	4.897	3.764	5.279	4.425	5.737
Total organic acids conc. (g/L)	11.20	10.02	8.44	9.26	10.67	9.36	10.98
Max. acetone conc. (g/L)	3.24	3.56	1.23	1.63	3.25	1.84	2.84
Max. butanol conc. (g/L)	9.36	8.45	6.86	8.74	9.84	7.64	9.89
Max. ethanol conc. (g/L)	0.94	1.31	0.68	0.83	0.87	0.67	0.67
Total ABE conc. (g/L)	13.54	13.32	8.77	11.20	13.96	10.15	13.40
Fermentation time (day)	96	96	96	48	48	48	48
ABE productivity (g/L.h)	0.14	0.14	0.09	0.23	0.29	0.21	0.28
Yield (g ABE /g sugar)	0.23	0.24	0.16	0.19	0.23	0.17	0.22

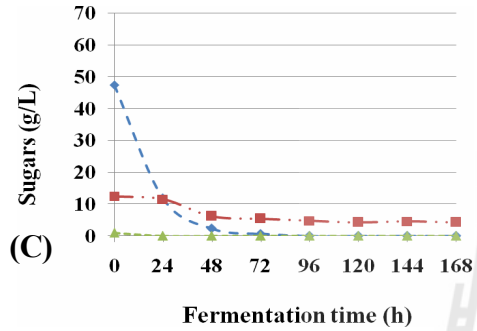
For the utilization of sugar in the fermentation broth, initial sugar concentrations in the 2S-DCRAH were (g/L) glucose 47, xylose 12, and arabinose 1 (Figure 8.4A, C, and E). The xylose concentration of 2.4, 4.5, and 7.5 g/L remained in the media containing OC-, TC-, and NC-CRAH, respectively. The reason for the residual sugar in fermentation system using these hydrolysates is not clear. However, many studies reported the residual sugar including glucose, xylose, and galactose in the bioreaction system for ABE production (Liu *et al.*, 2010; Qureshi *et al.*, 2010a; Qureshi *et al.*, 2010b), compared with controls, all of mixed sugars were completely consumed. This indicated the present of some interference compounds in hydrolysate.


Sugars (g/)
Fermentation time (h)

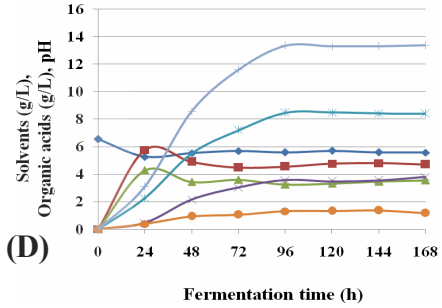
(A)


Solvents (g/L), organic acids (g/L), pH
Fermentation time (h)

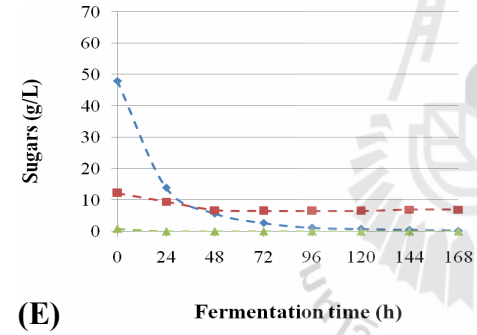
(B)


Sugars (g/L)
Fermentation time (h)

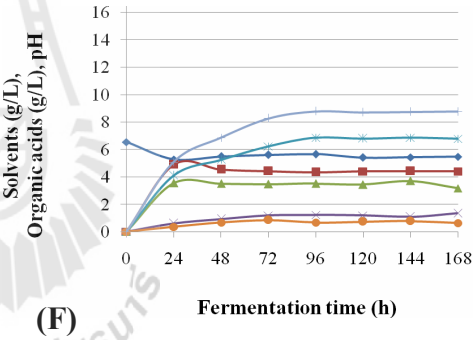
(C)


Solvents (g/L), Organic acids (g/L), pH
Fermentation time (h)

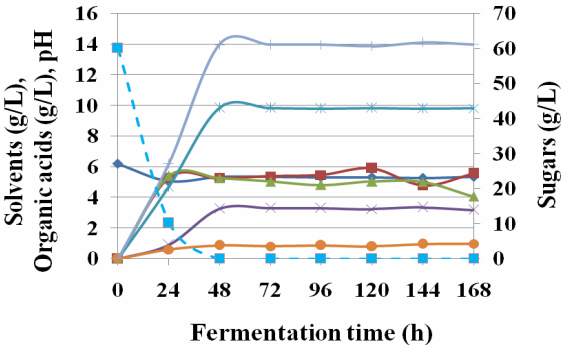
(D)


Sugars (g/L)
Fermentation time (h)

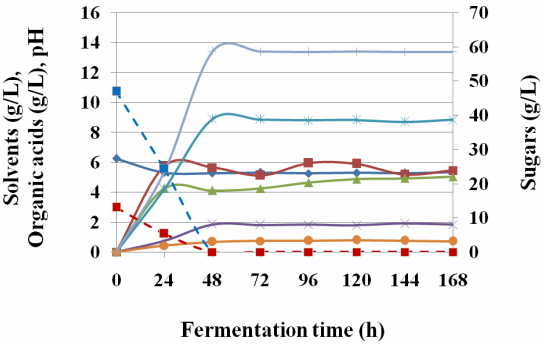
(E)


Solvents (g/L), Organic acids (g/L), pH
Fermentation time (h)

(F)


Solvents (g/L), Organic acids (g/L), pH
Sugars (g/L)
Fermentation time (h)

(G)


Solvents (g/L), Organic acids (g/L), pH
Sugars (g/L)
Fermentation time (h)

(H)

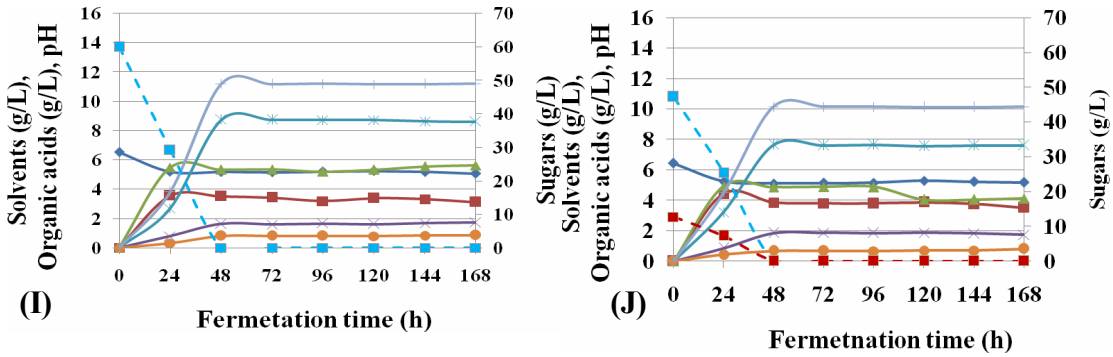


Figure 8.4 Production of ABE from individual detoxified concentrated rice straw hydrolysate containing 60 g/L sugars by *Cl. acetobutylicum* ATCC 824. (A) sugars consumption in OC-based medium, (B) pH and fermentable products using OC-based medium, (C) sugars consumption in TC-based medium, (D) pH and fermentable products using TC-based medium, (E) sugars consumption in NC-based medium, (F) pH and fermentable products using NC-based medium, (G) product profile in GE medium, (H) product profile in GEX medium, (I) product profile in G medium, (J) product profile in GX medium. Symbols represent:, butanol ( ), burytyric acid ( ), ethanol ( ), glucose ( ), arabinose ( ), pH ( ), xyolse ( ), acetone ( ), acetic acid ( ), ABE ( ).

8.4 Conclusion

Hemicellulose from rice straw was easily hydrolysed to monosaccharides by acid pretreatment. The sugars composition of hemicelluloses fraction included glucose, xylose, and arabinose,where xylose was the major sugar. Evaporation of acid hydrolysate increased the sugar concentration, which was enough to perform the ABE fermentation by the strain used. Even though evaporation contributed the reduced toxicity in the concentrated hydrolysate,the inhibitory compounds in the concentrated hydrolysate must be removed with the efficient detoxification methods. Over-titration plus adsorption with activated charcoal treatment enhanced the susceptibility of the concentrated hydrolysate to the ABE production by *Cl. acetobutylicum* ATCC 824. The total ABE concentration, productivity, and yield were 13.32 g/L, 0.14 g/L/h, and 0.24, respectively. The efficient method was close to the overliming plus activated charcoal absorption method, which would be the most economical and efficient method. The overliming plus activated charcoal absorption method resulted in provided total ABE concentration, productivity, and yield were were 13.54 g/L, 0.14 g/L/h, and 0.23, respectively.

CHAPTER IX

CO-CULTURE OF CLOSTRIDIA FOR BUTANOL PRODUCTION FROM RICE STRAW

9.1 Introduction

The main disadvantage of typical single solvent producing *Clostridium* strain to produce acetone-butanol-ethanol is its low efficiency of substrate to solvent conversion (Tashiro *et al.*, 2004). Coculture could be an attractive alternate to the typical single biocatalyst approaches leading to enhance solvent production by *Clostidium* strain (Nakayama *et al.*, 2011). Therefore, the object of this chapter is to investigate the potential use of two *Clostridium* strains including *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462 for ABE production from rice straw. Effect of the inoculums ratio *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462 on the co-culture system was investigated with the aim to improve the ABE production.

9.2 Materials and Methods

9.2.1 Microorganisms and media

Cl. acetobutylicum TISTR 1462 was purchased from the Culture Collection of the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. The stock spore was maintained in sterile water at 4ºC. Another strain was *Cl. acetobutylicum* ATCC 824. It was kindly provided by Prof. Dr. Philippe

Soucaille, INSA, Touluse, France. The stock culture was maintained in the form of spores in MSG medium at -80ºC.

TYG medium was used as an enrich medium for spore germinating.

MSG medium consisting of 60 g/L commercial glucose was used to pre-culture of both strains prior to transferring into fermentation medium.

P2 medium was used for fermentation medium throughout all experiments. It consisted of 60 g/L glucose deriving from enzymatic saccharification (GE) of combined acid-alkaline treated rice straw. P2 medium consisting of 60 g/L pure glucose was used as a control medium.

9.2.2 Inoculum preparation

To prepare each seed culture of *Cl. acetobutylicum* TISTR 1462 and *Cl. acetobutylicum* ATCC 824, 1 mL of spore suspension was aseptically transferred into 9 mL of TYG medium. Then, the mixture was subjected to heat shock by placing it in boiling water for 10 min and was subsequently incubated at 35C for 16-18 h. The actively motile cell culture (5%v/v) was inoculated to P2 medium and cultivated at the same condition as mentioned above for 5-7 h prior to transferring to a new fermentation P2 medium.

9.2.3 Co-culture fermentation

All batch experiments were carried out in 80 mL anaerobic serum bottles sealed with rubber stoppers and aluminium caps. The cultures were grown in P2 fermentation medium at 35°C for 7 days. The inoculation ratio of *Clostridium* strains either single or co-culture was fixed at 5% (v/v) inoculum size. Four milliters of 5-7 h-old pre-culture were inoculated into 80 mL of fermentation medium. The *Cl. acetobutylicum* TISTR 1462 to *Cl. acetobutylicum* ATCC 824 inoculation ratios was 1:1, 1:3, and 3:1 of the inoculums size. For proper controls, either single or

mixed- cultures of *Cl. acetobutylicum* TISTR 1462 and *Cl. acetobutylicum* ATCC 824 on the P2 medium containing pure glucose were used during incubation conditions identical to the mixed culture system.

9.2.4 Analysis methods

After complete enzymatic hydrolysis, the sugar composition of the hydrolysates was analyzed by HPLC (Algilent 1200 series) equipped with refractive index detector. The separation of supernatant sample was run by using an Aminex HPX-87H ion exchange column (Bio-Rad, Richmond, USA) maintained at 45°C with 4 mM H_2SO_4 as the mobile phase. Total sugar yield was expressed as gram of total sugar per gram of dry biomass. The concentration of solvents (acetone, butanol and ethanol) was determined by GC equipped with a flame ionization detector (FID). Separations of solvents were achieved by using a capillary column and H_2 as the carrier gas. The column temperature was held at 115^{o}C for 8 min and then programmed at 5^{o}C/min to 170^{o}C with a 10 min final hold.

9.2.5 Data analysis

The results presented here were an average of duplicate experiments and had an error in the range of 8-12%. The results obtained in the present study were statistically analyzed by Duncan's multiple range test (DMRT) at 95% significance level ($p<0.05$) using SPSS statistical software (SPSS for Windows, version 16).

9.3 Results and Discussions

Prior to performing ABE fermentation using sugar based on enzymatic saccharification of pretreated rice straw, control batch fermentations were performed with 60 g/L glucose as sole carbon source in order to determine the ability of different solvent producing *Clostridium* spp. to produce ABE. The fermentations were conducted under static anaerobic conditions without pH regulation at 35ºC for 5 days. In all experiments, the initial glucose level was 60 g/L. Cell density could not be measured because of the presence of calcium carbonate suspension in the fermentation mixture. Figure 9.1 exhibited a comparison of fermentation profile in above experiments. The results demonstrated that both *Clostridium* strains clearly showed the biphasic patterns of the fermentations. During the first 24 h, the cultures produced large amounts of acetic and butyric acids because of rapid glucose uptake resulted in rapid decrease of the media pH from 6.73 to 6.28 for *Cl. acetobutylicum* TISTR 1462 and from 6.69 to 5.95 for *Cl. acetobutylicum* ATCC 824. This period of glucose fermentations was an acidogenic phase. Thereafter, during 24 to 48 h of fermentations, the fermentations were solventogenic phase. The pH of culture media was very slightly increase. The accumulated acid concentrations decrease and remained constant, indicating acid reassimilation to solvents. The fermentations ceased after 72 h as a result of solvent toxicity. The toxicity of butanol disrupted both fluidity and function of cell membrane resulting in loss of ability of the cell to maintain internal pH, the low level of intracellular ATP, and inhibition of glucose uptake (Moreira *et al.*, 1981; Vollherbst-Schneck *et al.*, 1984; Bowles and Ellefson, 1985). In ABE fermentation, butanol concentration of 13 to 16 g/L was inhibitory to cell growth (Lin and Blaschec, 1983; Roos *et al.*, 1985).

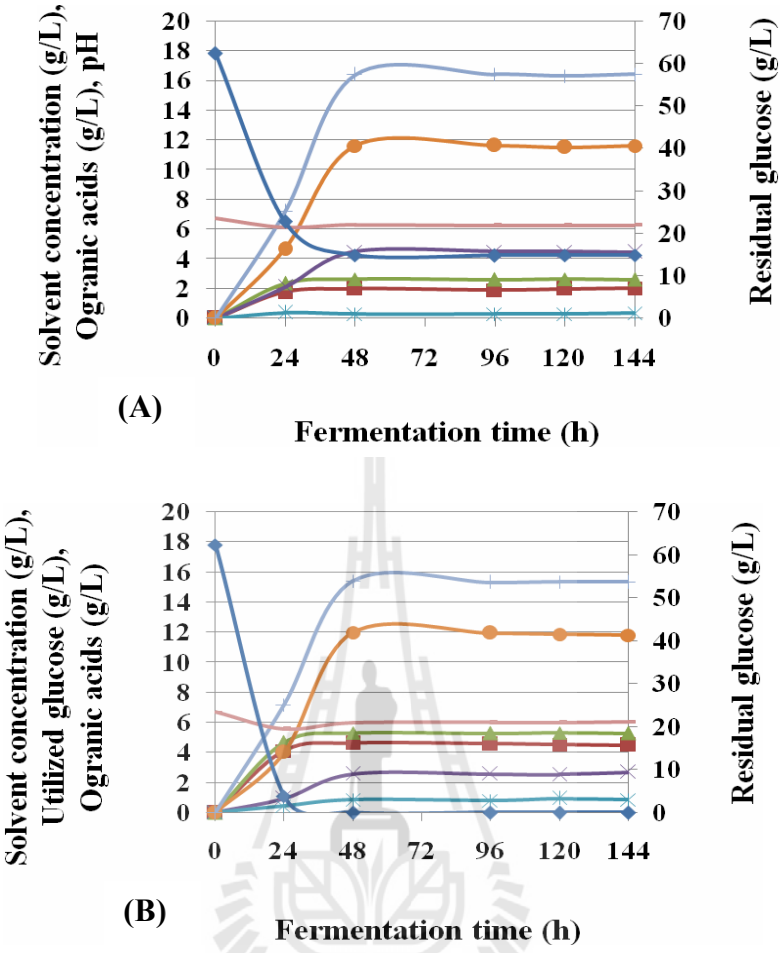


Figure 9.1 Production of ABE during the fermentation of glucose from different strain of *Clostridium* spp.: (A) *Cl. acetobutylicum* ATCC 824; (B) *Cl. acetobutylicum* TISTR 1462. Symbol represent: glucose ( ); acetic acid ( ); butyric acid ( ); acetone ( ); butanol ( ); ethanol ( ); total ( ); pH ( ).

With regard to glucose uptake, the result showed that at the end of fermentation *Cl. acetobutylicum* TISTR 1462 utilized 47.46 g/L glucose with 14.89 g/L residual, whereas the *Cl. acetobutylicum* ATCC 824 consumed almost all glucose. This result was in good agreement with previous studies (Ezeji and Blaschek, 2008; Sawisit,

2010). According to the Sawisit's study (2010) for ABE production by the *Cl. acetobutylicum* TISTR 1462 using 50 g/L initial sugar from cassava starch under batch fermentation without pH control, this strain used 47.98 g/L total sugar, leaving behind 2.22 g/L sugar in the system. Besides, Enzeji *et al.* (2008) showed that glucose was quickly and completely consumed during the fermentation of the *Cl. acetobutylicum* ATCC 824. The results could be concluded that glucose consumption by *Cl. acetobutylicum* ATCC 824 was better than *Cl. acetobutylicum* TISTR 1462.

Considering the production of ABE using glucose, the result displayed that the level of solvent produced by the two strains was not significant different ($p \leq 0.5$) (Table 9.1). The *Cl. acetobutylicum* TISTR 1462 produced maximum ABE concentration of 16.38 g/L or 11.61 g/L butanol, 4.48 g/L acetone, and 0.29 g/L ethanol. The level of ABE obtained from this strain was closely related to those reported by the previous study (Sawisit, 2010). For the fermentation the *Cl. acetobutylicum* ATCC 824, it produced 15.40 g/L total ABE or 11.97 g/L butanol, 2.57 g/L acetone, and 0.86 g/L ethanol. The fermentations resulted in the productivity and yield of 0.34 g/L.h and 0.35 g/g for the *Cl. acetobutylicum* TISTR 1462, and 0.32 g/L.h and 0.25 g/g for the *Cl. acetobutylicum* ATCC 824, respectively. This study demonstrated that the total solvent yield obtained from the *Cl. acetobutylicum* ATCC 824 was lower than that of the *Cl. acetobutylicum* TISTR 1462, even though the productivity of both strains was close. This may be result of the different glucose uptake between the two clostridial strains. The previous result of this study demonstrated that at the same time course of fermentation glucose consumption by the *Cl. acetobutylicum* ATCC 824 was better than that by the *Cl. acetobutylicum* TISTR 1462.

Table 9.1 Fermentation kinetic parameters of glucose and glucose obtaining from enzymatic hydrolysis of rice straw using single culture and co-culture of *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462.

Kinetic parameters	G_1462	G_0824	G_1:1	G_1:3	G_3:1	GE_1462	GE_0824	GE_1:1	GE_1:3	GE_3:1
Initial sugar conc. (g/L)	62.34	62.32	62.58	61.76	60.83	60.33	60.76	60.64	60.01	60.97
Sugars utillized (g/L)	47.46	62.32	62.58	61.76	60.83	48.84	60.76	60.64	60.01	60.97
Max. butyric acid conc. (g/L)	2.65	5.3	5.08	4.5	5.66	3	5.1	5.36	5.56	4.96
Max.acetic acid conc. (g/L)	1.99	4.61	4.83	4.12	5.46	2.07	5.06	5.27	5.35	4.76
Total organic acids conc. (g/L)	4.64	9.91	9.91	8.62	11.12	5.07	10.16	10.63	10.91	9.72
Max. acetone conc. (g/L)	4.48	2.57	3.52	3.89	3.12	5.25	2.42	3.39	3.73	3.23
Max. butanol conc. (g/L)	11.61	11.97	9.62	10.79	9.68	13.39	13.81	10.56	11.38	9.96
Max. ethanol conc. (g/L)	0.29	0.86	0.63	0.63	0.85	0.41	0.75	0.91	0.67	0.66
Total ABE conc. (g/L)	16.38	15.4	13.77	15.31	13.65	19.05	16.98	14.86	15.78	13.85
Fermentation time (day)	48	48	48	48	48	48	48	48	48	48
ABE productivity (g/L.h)	0.34	0.32	0.29	0.32	0.28	0.40	0.35	0.31	0.33	0.29
Yield (ABE g/sugar g)	0.35	0.25	0.22	0.26	0.22	0.39	0.28	0.25	0.26	0.23

Note: GE_1462 or 0824, fermentation by *Cl. acetobutylicum* TISTR 1462 and *Cl. acetobutylicum* ATCC 824 using glucose derived from enzymatic sacchrification of acid-alkaline pretreated rice straw; G_1462 or 0824, fermentation by *Cl. acetobutylicum* TISTR 1462 and *Cl. acetobutylicum* ATCC 824 using glucose; GE_1:1, 1:3, and 3:1, fermentation by co-culture of the *Cl. acetobutylicum* ATCC 824 to *CL. acetobutylicum* TISTR 1462 ratio of 1:1, 1:3, and 3:1 during fermentation using GE; G_1:1, 1:3, and 3:1, fermentation by co-culture of the *CL. acetobutylicum* ATCC 824 to *CL. acetobutylicum* TISTR 1462 ratio of 1:1, 1:3, and 3:1 during fermentation using G.

Next, the main object of this study was to investigate the possibility of using *Cl. acetobutylicum* TISTR 1462 for co-culturing with *Cl. acetobutylicum* ATCC 824 to improve the solvent production from available sugar present in the rice straw (GE). The co-cultures of *Cl. acetobutylicum* TISTR 1462 and *Cl. acetobutylicum* ATCC 824 were established under anaerobic condition without shaking at 35ºC for 5 days. The initial GE concentration was 60 g/L in all experiments. The culture ratio of *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462 were 1:0 (single culture of *Cl. acetobutylicum* ATCC 824), 0:1(single culture of *Cl. acetobutylicum* TISTR 1462), 1:1, 1:3, and 3:1 (v/v) of 5% (v/v) inoculum size.

According to the results in Figure 9.2, in all cases, total acids were produced rapidly during the first 24 h of fermentation. The accumulation of ABE production reached its highest value at 48 h of fermentation after inoculation. All of the fermentation stopped 72 h. Under the singe culture system, *Cl. acetobutylicum* TISTR 1462 and *Cl. acetobutylicum* ATCC 824 in the fermentation media containing GE as sole carbon substrates produced maximum ABE concentrations of 19.05 and 16.98 g/L, respectively (Table 9.1). The higher final titers of ABE from fermentation were found from using GE as substrate compared with that using glucose under the same fermentation conditions. It may be attributed to buffering capacity of the medium containing GE was higher than that of the medium containing glucose. This result was supported by that the amount of ABE production from all media containing GE was higher than that of all the media containing glucose (Figure 9.3). Based on process of enzymatic hydrolysis of pretreated rice straw for the cellulose to sugar conversion, the action of crude cellulase enzymes was implemented under 50 mM acetate buffer (pH 4.8) at 50ºC. This buffer composed of sodium acetate which contributed to support the activity CoA transferase responsible for reassimilation of acids generated during the solventrogenic phase (Haeng *et al.*, 2012).

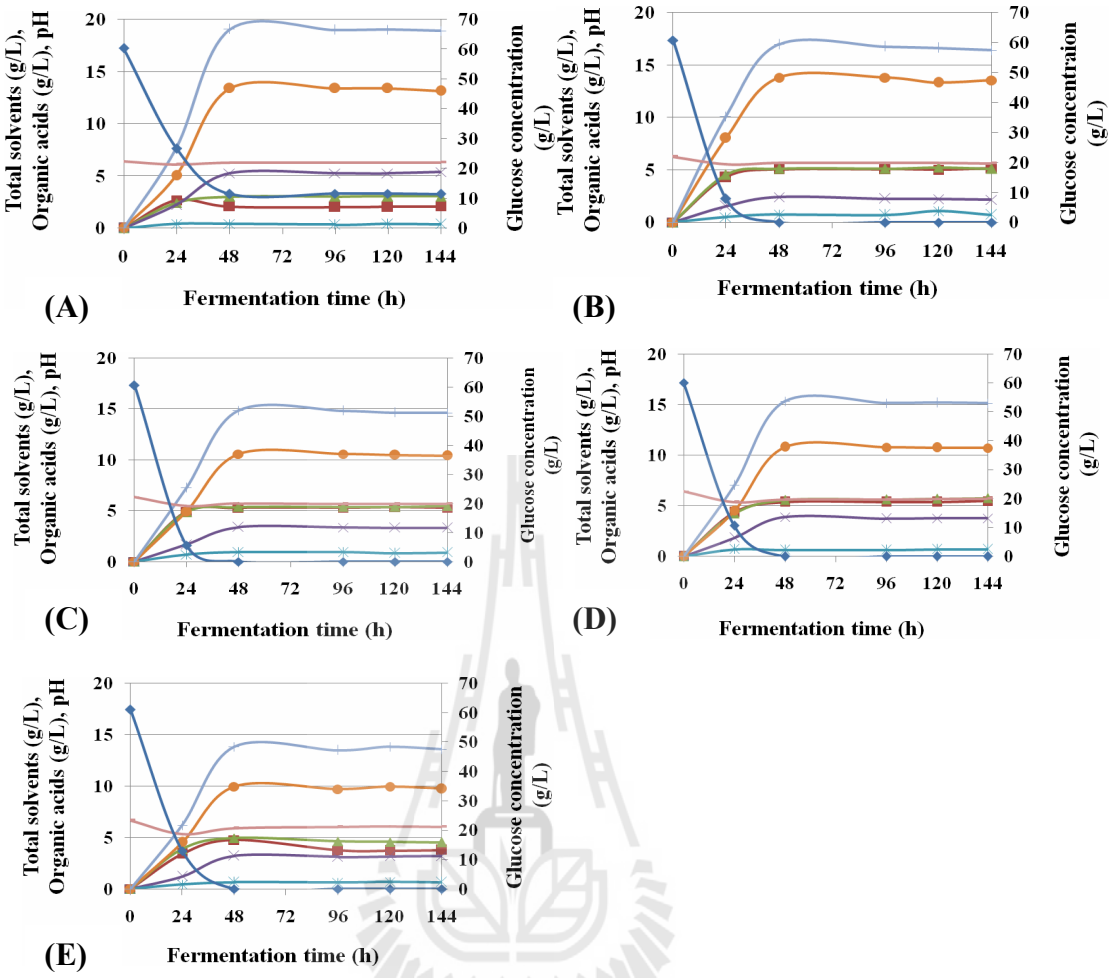


Figure 9.2 Production of ABE during the fermentation of glucose deriving from enzymatic saccharification (GE) of acid-alkaline pretreated rice straw by monoculture and co-culture between the strains of different *Clostridium* spp. The end product profile of co-culture of *Cl. acetobutylicum* TISTR 1462 (A); and *Cl. acetobutylicum* ATCC 824 (B). The end product profile of co-culture of the *Cl. acetobutylicum* ATCC 824 to *Cl. acetobutylicum* TISTR 1462 in the ratio of 1:1 (C); 1:3 (D); and 3:1(E) during fermentation. Symbol represent: glucose ( ); acetic acid ( ); butylic acid ( ); acetone ( ); butanol ( ); ethanol ( ); total ( ); pH ( ).

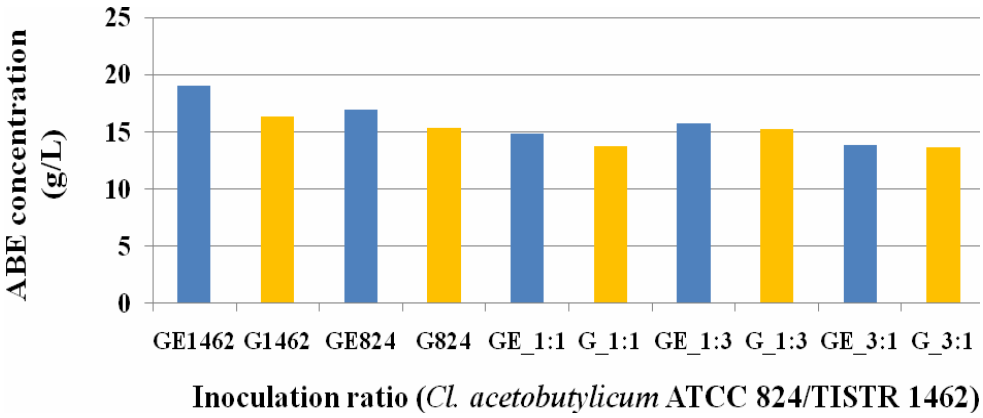


Figure 9.3 Production of ABE during the fermentation of sugar from rice straw (GE, blue bar) by monocultures and co-culture comparing with the fermentation of pure glucose (G, organge bar) as control. Monocultures include *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462. Co-culture at various inoculation ratios of *Cl. acetobutylicum* ATCC 824 to *Cl. acetobutylicum* TISTR 1462 was 1:1, 1:3, and 3:1 (v/v) of 5% (v/v) inoculum.

According to the results, the ABE production by *Cl. acetobutylicum* TISTR 1462 using GE was significantly different ($p \leq 0.05$) that by *Cl. acetobutylicum* ATCC 824, indicating the different glucose consumption. Under the co-culture, production of ABE was not significantly different ($p \leq 0.05$) in all of the co-cultures ratios (Table 9.1). This suggested that the enhancement of solvent production by co-culture of these *Clostridium* strains was not available. In addition, it were noticed that titer of total acids produced under the co-culture was higher compared with that of the single culture. The most probable reason for low ABE production could be the influence of the medium pH to solvent production (Bahl *et al.*, 1982; Monot *et al.*, 1984). The

medium pH is an essential regulatory factor on cellular metabolism of solvent producing clostridial cells to solvent production. Typically, with several strains of *Cl. acetobutylicum*, a pH of culture medium below 5.0 was found optimal to produce acetone and butanol (Bahl *et al.*, 1982). At higher pH, only acids are excreted. Decreasing the amount of undissociate butyric acid inside the cell cannot induce the shift from acidogenesis to solventogenesis (Grupe *et al.*, 1992). However, in this study, the medium pH ranges of single and co-cultures were 5.64 to 6.25 and 5.57 to 5.91, respectively. This showed that even though these medium pH were higher than 5.0, the productions of ABE were good. It suggested that high total acids produced did not negatively affect on ABE fermentation. This result here was consistent with other researcher reports using fermentable sugars from lignocelluloses for ABE production (Qureshi *et al.*, 2007; Jieun *et al.*, 2009).

9.4 Conclusion

This study demonstrated that there was no inhibitory effect of glucose obtained from enzymatic hydrolysis of rice straw on the growth and fermentation by *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462. The ABE fermentation employing the glucose of rice straw enhanced solvent production when compared with commercial glucose. The co-culture with *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462 could not significantly improve ABE production.

CHAPTER X

CONCLUSIONS

The first objective of this study was to delete *cel48A* encoding the inactive cellulaseand then introduce the *SAFA* gene encoding an active cellulase from the 48 family into the chromosome of *Clostridium acetobutylicum* ATCC 824 mutant. The deletion of the *cel48A* gene was done by gene knocking out facilitating by pREP-*cel48A*::*upp-catP*-11 plasmid and pSOS95-*Flp* plasmid. After the deletion of the *cel48A* gene, the *Cl. acetobutylicum* ATCC 824 Δ*cac1502*Δ*upp*Δ*cel48A* recombinant strain did not expressed cellulase Cel48A protein. Subsequent attempts were performed to insert the *SAFA* gene into the chromosome of this recombinant strain with four designed artificial plasmids. Nevertheless, the clones obtained, did not show the insertion of the *SAFA* gene in their chromosomes. This may be caused by a non-suited gene structure of the integration plasmid including a strong promoter and a reporter system, or lacking of the complementary scaffolding protein or cohesion in the *Clostridium* chromosomal cellulosome gene to the hybrid gene employed, or blocking of the secretory system of *Clostridium* cell from the precursor of the *SAFA* gene. In the previous reports, the conversion of *Cl. actobutylicum* into a celluloytic-biofuel producing microorganism was successfully performed by expression of hybrid cellulase genes in the artificial plasmids consisting of hybrid minicellulosomal genes in the *Cl. actobutylicum* cells (Fierobe *et al*., 2002; Perret *et al*., 2004; Mingardon *et al*., 2005; Mingardon *et al*., 2011). The hybrid genes of minicellulosome complexes were constructed by the combination of one or two *Cl. cellulolyticum* cellulases with divergent dockerins and a hybrid scaffoldin containing

the single family 3a CBM. Most of the cellulases expressed could degrade crystalline cellulose. However, the phenotype of these recombinant strains was not stable, although they were kept as spore suspension.

The second objective was to compare two different pretreatment methods on the accessibility of rice straw in order to prepare the rice straw cellulose for sugars production by *Clostridium cellulolyticum* DSM 5812 or by enzymatic hydrolysis. Rice straw was pretreated either by acid or by alkaline pretreatment combining with liquid hot water (LHW) and reduction in size of the rice straw (S) (H_2SO_4- or NaOH-LHW-S). The results showed that the NaOH-LHW-S pretreatment was a superior method to improve the accessibility of the rice straw as compared with the H_2SO_4-LHW-S pretreatment. However, this method required excess used of water to post-treat excess alkaline and its waste which considered disadvantage of the method. This disadvantage plays significant roles in the economic viability of the industrial process.

Regarding with the disadvantage of the method mentioned, another pretreatment method of the rice straw was investigated. These included four different series of the synergistic double acids and alkaline pretreatment including washing step on the improvement of enzymatic saccharification for sugar production in comparison to the NaOH-LHW-S pretreatment method. The results revealed that rice straw treated by the two-step dilute acid pretreatment (A2WB1) followed by post pretreatment washing enhanced the enzymatic hydrolysis leading to the maximum sugar liberation as compared with those of other synergistic double acids and alkaline pretreatments including the NaOH-LHW-S pretreatment. These results confirmed that the additional washing step to the post pretreatment resulted in significant affect on increasing the capacity of enzymatic hydrolysis of pretreated rice straw to sugar production. The sugars from degradation of pretreated rice straw by *Cl. cellulolyticum* DSM 5812 were glucose,

xylose, and arabinose, whereas by the enzymatic hydrolysis glucose was the only monosaccharides, indicating the effectiveness of the NaOH-LHW-S pretreatment to remove lignin and hemicellulose from rice straw structure. These results suggested that the A2WB1 pretreatment was the superior method for preparation of rice straw to be accessible prior to the enzymatic saccharification for sugar production. After the enzymatic saccharification using cellulase and *β*-glucosidase for 48 h, hydrolysate from the A2WB1 pretreated rice straw contained 96.83±1.25 g/L of glucose or 97 (g of sugar produced per 100 g of rice straw provideed) grass yield of glucose. The yield of glucose was closely related to those reported by with Kim *et al.* (2011)'s study. They reported 96.90 g of sugar per 100 g of rice straw with the a two-stage pretreatment process using aqueous ammonia and dilute sulfuric acid in a percolation mode. In addition, the amount and yield of glucose from our A2WB1 pretreated rice straw were higher than those of other reports using single-step pretreatments prior to enzymatic saccharification. For example, Yu *et al.* (2010) reported that about 90 g of sugars per 100 g of rice straw was produced by enzymatic hydrolysis of hot liquid water pretreated rice straw. Singh and Bishnoi (2012) had also reported 25 g/L of sugars obtained after 36 h of enzymatic hydrolysis of NaOH pretreated rice straw under optimum enzymatic conditions of cullulase, *β*-glucosidase, and tween 80. Hsu *et al.* (2010) reported that the highest yield of glucose from acid pretreated rice straw liberated during enzymatic saccharification was 25 g of sugars per 100 g of pretreated rice straw. The rice straw was pretreated at 160ºC with 1% of sulfuric acid and 5 min reaction time.

The last object was to investigate the different detoxification methods of toxins which were generated from the acid hydrolysis of rice straw. The production of butanol either from the detoxified acid hydrolysate sugars or from the rice straw enzyme hydrolysis sugar by single culture and co-culture of *Cl. acetobutylicum* ATCC

824 and *Cl. acetobutylicum* TISTR 1462 were also investigated. The results revealed that xylose and glucose were the major sugar from acid hydrolysis and from the enzymatic hydrolysis of rice straw, respectively. These sugars could be used as potential substrates for butanol production by *Cl. acetobutylicum*. The total ABE concentration and yield from rice straw sugar (60 g/L) were 19.05 g/L and 0.39 g ABE /g sugar for *Cl. acetobutylicum* TISTR 1462; 16.98 g/L and 0.28 g ABE /g sugar for *Cl. acetobutylicum* ATCC 824; and 15.78 g/L and 0.26 g ABE /g sugar for co-culture. In addition, the total acid concentration and yield from the ABE production were 5.07 g/L and 0.10 g total acid/g sugar for *Cl. acetobutylicum* TISTR 1462; 10.16 g/L and 0.17 g total acid/g sugar for *Cl. acetobutylicum* ATCC 824; and 9.72 g/L and 0.16 g total acid/g sugar for co-culture. Regarding with the results of total acid concentrations and yields, it implied that the lower amount and yield of ABE produced by *Cl. acetobutylicum* ATCC 824 and by the co-culture than those of *Cl. acetobutylicum* TISTR may result from the effect of high acid concentration which was only partially assimilated to solvent production. Comparing with ABE production with other studies using rice straw sugar as carbon substrate, Ranjan *et al.* (2012) obtained 16.91 g/L ABE from the fermentation of *Cl. acetobutylicum* MTCC 481. Cheng *et al.* (2012) obtained 2.92 g/L of ABE from 16.63 g/L initial concentration of sugar from rice straw by using mixed clostridial culture of *Cl. saccharoper-butylacetonicum, Cl. butylicum, Cl. beijernckii,* and *Cl. acetobutylicum*. The lower amount of ABE obtained resulted from the low initialted sugar concentration and the exhaustion of sugar during the fermentation. For ABE production from other lignocellulosic biomass by *Clostridium* spp. under batch fermentation, ABE concentration was in the range of 12.01-26.64 g/L (Jieun *et al.*, 2009; Qureshi *et al*., 2007; Qureshi *et al*., 2010; Ezeiji *et. al.*2007; Liu *et al.*, 2010). The highest amount of

total solvent (26.64 g/L) was obtained from the fermentation of treated barley straw hydrolysate (Qureshi *et al*., 2010). According to the results of this work, it could be concluded that the sugar from the rice straw hydrolysate by enzymatic saccharification was appearently adequate to support growth and solvent production by *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462.

According the PCR analysis, the insertion of the hybrid cellulase gene into the chromosome of the recombinant *Cl. acetobutylicum* strain was not found when using the designed artificial plasmids. It is recommended that a new plasmid should be constructed for more efficient promoter and signal sequence of hybrid cellulase gene to the secretary system of the recombinant *Cl. acetobutylicum*. For ABE production, *Cl. acetobutylicum* ATCC 824 and *Cl. acetobutylicum* TISTR 1462 were able to ferment sugar from the rice straw hydrolysate. In order to improve a high sugar utilization and solvent production, further works should be looking into the effect of culture parameters such as concentration of carbon and nitrogen as well as control pH on ABE production, and the effect of co-culture ratio between *Cl. acetobutylicum* ATCC 824 and TISTR 1462 under the fermentation with pH control. In addition, the study of ABE production using simultaneous saccharification and fermentation (SSF) comparing with separate hydrolysis and fermentation (SHF) should be further investigated to reduce steps in the separation of solid after enzymatic saccharification of pretreated rice straw. Furthermore, study of the feasibility of scaling up the ABE production using rice straw sugar should be evaluated to develop an efficient solvent production process.

REFERENCES

Abou-Zeid, A.A., Fouad, M., and Yassein, M. (1976): Fermentative production of acetone-butanol by *Clostridium acetobutylicum*. **Indian Journal of Technology** 14, 349-352.

Abril1, D., Medina, M., and Abril, A. (2012): Sugar cane bagasse prehydrolysis using hot water. **Brazilian Journal of Chemical Engineering** 29(1), 31-38.

Agarwal, L., Isar, J., Meghwanshi, G.K., and Saxena, R.K. (2006): A cost effective fermentative production of succinic acid from cane molasses and corn steep liquor by *Escherichia coli*. **Journal of Applied Microbiology** 100, 1348-1354.

Alhasan, M.A., and Kuang, D. (2010): Combined effect of nitric acid and sodium hydroxide pretreatments on enzymatic saccharification of rubber wood (*Heavea brasiliensis*). **International Journal of Chemical Technology** 2(1), 12-20.

Al-Hinai, M.A., Fast, A.G., and Popoutsakis, E.T. (2012): Novel system for efficient isolation of *Clostridium* double-crossover allelic exchange mutants enabling markerless chromosomal gene deletions and DNA integration. **Applied and Environmental Microbiology** 78(22), 8112-8121.

Alriksson B., Horváth, I.S., Sjöde, A., Nilvebrant, N.-O., and Jönsson, L.J. (2005): Ammonium hydroxide detoxification of spruce acid hydrolysates. **Applied Biochemistry and Biotechnology** 121-124, 912-922.

Alriksson, B., Cavka, A., and Jönsson, L.J. (2011): Improving the fermentability of enzymatic hydrolysates of lignocellulose through chemical in-situ detoxification with reducing agents. **Bioresource Technology** 102, 1254-1263.

Al-Sum, R.M, and White, P.J. (1977): Effects of biotin deficiency on growth, morphology and sporulatjion in *Bacillus megaterium* NCIB7581. **Journal of General Microbiology** 99, 343-351.

Annous, B.A., and Blaschek, H.P. (1991): Isolation and characterization of *Clostridium acetobutylicum*. **Applied and Environmental Microbiology** 57(9), 2544-2548.

Assobhei, O., Kanouni, A.E., Ismaili, M., Loutfi, M., and Petitdemange, H. (1998): Effect of acetic and butyric acids on the stability of solvent and spore formation by *Clostridium acetobutylicum* ATCC 824 during repeated subculturing. **Journal of Fermentation and Bioengineering** 85(2), 209-212.

Atalla, R.H., Hackney, J.M., Uhlin, I., and Thompson, N.S. (1993): Hemicelluloses as structure regulators in the aggregation of native cellulose. **International Journal of Biological Macromolecules** 15, 109-112.

Axe, D. D. and J. E. Bailey (1995): Transport of lactate and ace-tate through the energized cytoplasmic membrane of Escherichia coli. **Biotechnology and Bioengineering** 47: 8-19.

Baek, S.-C., and Kwon, Y.-J. (2007): Optimization of the pretreatment of rice straw hemicellulosic hydrolyzates for microbial production of xylitol. **Biotechnology and Bioprocess Engineering** 12, 404-409.

Baek, S.-C., and Kwon, Y.-J. (2007): Optimization of the pretreatment of rice straw hemicellulosic hydrolyzates for microbial production of xylitol. **Biotechnology and Bioprocess Engineering** 12, 404-409.

Bahl, H., Andersch, W., Braun, K., and Gottschalk, G. (1982): Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture. **European Journal of Applied Microbiology and Biotechnology** 14, 17-20.

Bak, J.S., Ko., J.K., Choi, I.-G., Park, Y.-C., Seo, J.-H., and Kim, K.H. (2009): Fungal pretreatment of lignocelluloses by *Phanerochaete chrysosporium* to produce ethanol from rice straw. **Biotechnology and Bioengineering** 104, 471-482.

Balat.,M., Balat, H., and Oz, C. (2008): Progress in bioethanol processing, **Progress and Energy Combustion science** 34(5), 551-573.

Ballesteros, I., Negro, M. J., and Oliva, J. M., Cabanas, A., Manzanares, P., Ballesteros, M. (2006): Ethanol production from steam explosion pretreated wheat straw. **Applied Biochemistry and Biotechnology** 129-132, 496-508.

Ballongue, J., Amine, J., Masion, E., Petitdemange, H., and Gay, R. (1985): Induction of acetoacetate decarboxylase in *Clostridium acetobutylicum*. **FEMS Microbiology Reviews** 29, 273-277.

Bayer, E. A., Shimon, L. J. W., Shoham, Y., and Lamed, R. (1998): Cellulosomes-structure and ultrastructure. **The Journal of Structural Biology** 124, 221-234.

Bayer, E.A., Chanzyt, H., Lamed, R., and Shoham, Y. (1998): Cellulose, cellulases and cellulosomes. **Current Opinion in Structural Biology** 8, 548-557.

Bayer, E.A., Lamed, R., and Himmel, M. (2007): The potential of cellulases and celulosomes for cellulosic waste management. **Current Opinion in Biotechnology** 18, 237-245.

Beesch, S.C. (1952): Acetone-butanol-fermentation of sugars. **Industrial and Engineering Chemistry** 39, 1677-1682.

Bergstrom, S.L., and Foutch, G.L.: US4539293 (1985).

Berin, A., Gilkes, N., Kurabi, A., Bura, R., Tu, M., Kilburn, D., and Saddler, J. (2005): Weak lignin-binding enzymes: a novel approach to improve activity of cellulases for hydrolysis of lignocellulosics. **Applied Biochemistry and Biotechnology** 121-124, 163-170.

Bernardo, C., Vidal, J.R., Bruee, S., Dien, S., and Ting, K.C. (2011): Influence of feedstock particle size on lignocelluloses conversion: a review. **Applied Biochemistry and Biotechnology** 164, 1405-1421.

Biswas, I., Vagner, V., and Ehrlich, S.D. (1992): Efficiency of homologous intermolecular recombination at different locations on the *Bacillus subtilis* chromosome. **Journal of Bacteriology** 174(17), 5593-5596.

Blaschek, H. P., Knott, G., Scheffran, J., Funk, T., and Overmyer, S. (2008): Overview for the Center for Advanced Bioenergy Research at the University of Illinois, Urbana-Champaign. ACS. **Chemical Biology** 3(1), 21-23.

Blouin, F. A., Martin, L. F. and Rowland, S. P.. (1970): Gel permeation properties of cellulose. III. Measurement of pore structure of unmodified and of mercerized cottons infibrous form. **Textile Research Journal** 40, 809-813.

Blouzard, J.-C., Valette, O., Tardif, C., and de Philip, P. (2010): Random mutagenesis of *Clostridium cellulolyticum* by using a Tn1545 Derivative. **Applied and Environmental Microbiology** 76(13), 4546-4549.

Boominathan, K., and Reddy, C.A. (1992). cAMP mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete *Phanerochaete chrysosporium*. **Proceedings of the National Academy of Sciences (USA)** 89 (12), 5586-5590.

Boussaid, A.-L., Esteghlalian, A.R., Gregg, D.J., Lee, K.H., and Saddler, J.N. (2000): Steam pretreatment of Douglas-Fir wood chilps. **Applied Biochemistry and Biotechnology** 84-86, 693-705.

Bowles, L., and Ellefson, W.L. (1985): Effects of butanol on *Clostridium acetobutylicum*. **Applied and Environmental Microbiology** 50(5), 1165-1170.

Brown, R.M., and Saxena, I.M. (2000) : Cellulose biosynthesis:a model for understanding the assembly of biopolymers. **Plant Physiology and Biochemistry,** 38: 57-67.

Buchert, J., NiemeJä, K., PuIs, J., and Poutanen, K. (1990): Improvement in fennentability ofsteamed hemicellulose hydrolysate by ion exclusion. **Process Biochemistry International** 176-180.

Bullifent, H. L., Moir, A. and Titball, R. W. (1995): The construction of a reporter system and use for the investigation of *Clostridium perfringens* gene expression. **FEMS Microbiology Letters** 131, 99-105.

Campos, E.J., Qureshi, N., and Blaschek, H.P. (2002): Production of acetone butanol ethanol from degermed corn using *Clostridium beijerinckii* BA101. **Applied Biochemistry and Biotechnology** 98-100, 553-561.

Cappelletti, B.M., Reginatto, V., Amante, E.R., and Antonio, R.V. (2011): Fermentative production of hydrogen from cassava processing wastewater by *Clostridium acetobutylicum*. **Renewable energy** 36, 3367-3372.

Cara, C., Romero, I., Oliva, J. M., Saez, F., and Castro, E. (2007): Liquid Hot Water Pretreatment of Olive Tree Pruning Residues. **Applied Biochemistry and Biotechnology** 136-140, 379-394.

Chandel, A.K., Kapoor, R.K., Singh, A., and Kuhad, R.C. (2007): Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. **Bioresource Technology** 98, 1947-1950.

Chandra, R. P., Bura, R., Mabee, W. E., Berlin, A., Pan, X., and Saddler, J. N. (2007): Substrate Pretreatment: The Key to Effective Enzymatic Hydrolysis of Lignocellulosics? **Biofuels Advances in Biochemical Engineering/ Biotechnology** 108, 67-93.

Chen, W.-H., Pen, B.-L., Yu, C.-T., and Hwang, W.-S. (2011): Pretreatment efficiency and structural characteristic of rice straw by and integrated process of dilute-acid and steam explosion for bioethanol production. **Bioresource Technology** 102, 2916-2924.

Cheng, C.-L., Che, P.-Y. Chen, B.-Y., Lee, W.-J., Chien, L.-J., and Chang, J.-S. (2012): High yield bio-butanol production by solvent-producing bacterial microflora. **Bioresource Technology** 113, 58-64.

Cheng, Y.-S., Zheng, Y., Yu, C.W., Dooley, T.M., Jenkins, B.M., and Gheynst, J.S. (2010): Evaluation of High Solids Alkaline Pretreatment of Rice Straw. **Applied biochemistry and biotechnology** 162, 1768-1784.

Cho, D.H., Shin, S.-J., and Kim, Y.H. (2012): Effects of acetic and formic acid on ABE Production by *Clostridium acetobutylicum* and *Clostridium beijerinckii*. **Biotechnology and Bioprocess Engineering** 17: 270-275 (2012).

Clark, T., and Mackie, K.L. (1984): Fermentation inhibitors in wood hydrolysates derived from the softwood Pinus radiata. **Journal of Chemical Technology and Biotechnology** 34, 101-110.

Compere, A.L., and Griffith, W.L. (1979): Evaluation of substrates for butanol production. **Developments in industrial microbiology** 20, 509-517.

Converti, A., Perego, P., and Dominguez, J.M. (1999): Xylitol production from hardwood hemicellulose hydrolysates by *P. tannophilus, D. hanseniiand C. guilliermondii.* **Applied Biochemistry and Biotechnology** 82, 141-151.

Coverse, A. O., Ooshima, H., Burns, D. S., Greenboum, E., and Wyman, C. E. (1990): Kinetics of enzymatic hydrolysis of ligno-cellulosic materials based on surface area of cellulose accessi-ble to enzyme and enzyme adsorption on lignin and cellulose. **Applied Biochemistry and Biotechnology** 24-25, 67-73.

Cowling, E. B. (1975): Physical and chemical constraints in the hydrolysis of cellulose and lignocellulosic materials. **Biotechnology and Bioengineering Symposium** 5, 163-181.

Crabbendam, P.M., Neijssel, 0.M. and Tempest, D.W. (1985): Metabolic and energetic aspects of the growth of *Clostridium butyricum* on glucose in chemostat culture. **Archives of Microbiology** 142, 375-382.

Davis, S. E., and Morton, S., A. III. (2008): Investigation of ionic liquids for the separation of butanol and water. **Separation Science and Technology** 43, 2460-2472.

Dehority, B. A., and Tirabasso, P. A. (2012): Effect of ruminal cellulolytic bacterial concentrations on in situ digestion of forage cellulose. **Journal of Animal Science** 76, 2905-2911.

Desvaux, M., and Petitdemange, H. (2001): Flux analysis of the metabolism of *Clostridium cellulolyticum* grown in cellulose-fed continuous culture on a chemically defined medium under ammonium-limited conditions. **Applied and Environmental Microbiology** 67(9), 3846-3851.

Desvaux, M.L., Guedon, E., and Petitdemange, H. (2000): Cellulose catabolism by *Clostridium cellulolyticum* growing in batch culture on defined medium. **Applied and Environmental Microbiology** 66(6), 2461-2470.

Doi,R.H., Kosugi, A., Murashima, K., Tamaru, Y., and Han, S.O. (2003): Cellulosomes from mesophilic bacteria. **Journal of Bacteriology** 185(20), 5907-5914.

Duff, S.J.B., and Murray, W.D. (1996): Bioconversion of forestproducts industry waste cellulosics to fuel ethanol - a review. **Bioresource Technology** 55, 1-33.

Dürre, P. (2007): Biobutanol: An attractive biofuel. **Biotechnology Journal** 2(12), 1525-1534.

Dürre, P. (2008): Fermentative butanol production bulk chemical and biofuel. **New York Academy of Sciences** 1125, 353-362.

Dürre, P., and Bahl, H. (1996): *Products of primary metabolism* (vol.6) (2nd). Germany**:** VCH Publisher.

Dürre, P., and Hollergschwandner, C. (2004): Initiation of endospore formation in *Clostridium acetobutylicum*. **Anaerobe** 10, 69-74.

Esteghlalian, A., Hashimoto, A.G., Fenske, J.J., and Penner, A.H. (1997): Modelling and optimization of dilute-sulfuric-acid pretreatment of corn stover, poplar, and switchgrass. **Bioresources technology** 59, 129-136.

Ezeji, T., and Blaschek, H.P. (2008): Fermentation of dried distillers' grains and soluble (DDGS) hydrolysates to solvents and value-added products by solventogenic clostridia. **Bioresource Technology** 99, 5232-5242.

Ezeji, T., Aureshi, N.M., and Blaschek, P. (2007): Butanol production from agricultural residues: Impact of degrasation products on *Clostridium beijerinckii.* **Biotechnology and Bioengineering** 97(6), 1460-1468.

Fan L.T., Gharpuray, M.M., and Lee, Y.H. (1987): Cellulose hydrolysis. **Berlin, Germany, Springer-Verlag** 3, 1-68.

Fan, L. T., Lee, Y.-H., and Beardmore, D. H. (1980): Mechanism of the enzymatic hydrolysis of cellulose: effects of major structural features of cellulose on enzymatic hydrolysis. **Biotechnology and Bioengineering** 22, 177-199.

Fang, H., Zhao, C., and Song, X.Y. (2010): Optimization of enzymatic hydrolysis of steam-exploded corn stover by two approaches: Response surface methodology or using cellulase from mixed cultures of *Trichoderma reesei* RUT-C30 and *Aspergillus niger* NL02. **Bioresource Technology** 101, 4111-4119.

Fang, X., Shen, Y., Zhao, J., Bao, x., and Qu, Y. (2010): Status and prospect fo lignocellulosic bioethanol production in Chian. **Bioresource Technology** 101(13), 4814-4819.

Fengel, D., and Wegener, G. (1989): *Wood: Chemistry, ultrastructure, reaction*. New York: Walter de Gruyter.

Fierobe, H.P., Bayer, E.a., Tardif, C., Czjzek, M., Mechaly, A., Bélaïch, A., Lamed, R., Shoham, Y., and Bélaïch, J.-P. (2002): Degradation of cellulose substrates by cellulosome chimeras: substrate targeting versus proximity of enzyme components. **The Journal of Biological Chemistry** 277(51), 49621-49630.

Gabriel, C.L., and Crawford, F.M. (1930): Development of the butyl-acetonic fermentation industry. **Journal of Industrial and Engineering Chemistry** 22, 1163-1165.

Gal, L., Pages, S., Gaudin, C., Belaich, A., Reverbel-leroy, C., Tardif, C., and Belaich, J.-P. (1997): Characterization of the Cellulolytic Complex (Cellulosome) Produced by *Clostridium cellulolyticum*. **Applied and Environmental Microbiology** 63(3), 903-909.

Gautam, S.P., Bundela, P.S., Pandey, A.K., Khan, J., Awasthi, M. K., and Sarsaiya, S. (2011): Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi. **Biotechnology Research International**, 1-8.

Ge, J.-P., Cai, B.-y., Liu, G.-M., Ling, H.-Z., Fang, B.-Z., Song, G., Yang, X.-F., and Ping, W.-X. (2011): Comparison of different detoxification methods for corn cob hemciellulose hydrolyzate to improve ethanol production by *Candida shehatae* ATCC 20335. **African Journal of Microbiology Research** 5(10), 1163-1168.

Gelhaye, E., Gehin, A. and Petitdemange, H. (1993): Colonization of crystalline cellulose by *Clostridium cellulolyticum* ATCC 35319. **Applied and Environmental Microbiology** 59(9), 3154-3156.

George, H.A., and Chen, J.S. (1983): Acidic conditions are not obligatory for onset of butanol formation by *Clostridium beijerinckii*. (synonym, *C. butylicum*). **Applied and Environmental Microbiology** 46, 321-327.

George, H.A., Johnson, J.L., Moore, W.E.C., Holdeman, L.V., and Chen, J.S. (1983): Acetone, isopropanol, and butanol production by *Clostridium beijerinkii* (*syn. Clostridium butylicum*) and *Clostridium aurantibutyricum.* **Applied and Environmental Microbiology** 45, 1160-1163.

Giallo, J., Gaudin, C., and Belaich, J.-P. (1985): Metabolism and solublization of cellulose by *Clostridium cellulolyticum* H10. **Applied and Environmental Microbiology** 49(5), 1216-1221.

Gibbs, R.D. (1958): *The physiology of forest trees*. New York: Ronald Press Company.

Girbal, L., and Soucaille, P. (1998): Regulation of solvent production in *Clostridium acetobutylicum.* **Trends in Biotechnol** 16, 11-16.

Girbal, L., Croux, C., Vasconcelos, I., and Soucaille, P. (1995): Regulation of metabolic shifts in *Clostridium acetobutylicum*. **FEMS Microbiology Reviews** 17, 287-297.

Girbal, L., Mortier-Barriére, I., Raynaud, F., Rouanet, C., Croux, C., and Soucaille, P. (2003): Development of a sensitive gene expression reporter system and an inducible promoter-repressor system for *Clostridium acetobutylicum*. **Applied and Environmental Microbiology** 69(8), 4985-4988.

Goldstein, M.A., Takagi, M., Hashida, S., Shoseyov, O., Doi, R.H., and Segel, I.H. (1993): Characterization of the cellulose-binding domain of the clostridium cellulovorans cellulose-binding protein A. **Journal of Bacteriology** 175(18), 5762-5768.

Gottwald, M., Hippe, H., and Gottschalk, G. (1984): Fomation of n-butanol from D-glucose by strains of "*Clostridium tetanomorphum"* group. **Applied and Environmental Microbiology** 48, 573-576.

Grupe, H., and Gottschalk, G. (1992): Physiological events in *Clostridium acetobutylicum* during the shift from acidogenesis to solventogenesis in Continuous culture and presentation of a model for shift induction. **Applied and Environmental Microbiology** 58(12), 3896-3902.

Grzenia, D.L., Schell, D.J., and Wickramasinghe, S.R. (2008): Membrane extraction for removal of acetic acid from biomass hydrolysates. **Journal of Membrane Science** 322, 189-195.

Gutierrez, N.A., and Maddox, I.S. (1987): Role of chemotaxis in solvent production by *Clostridium acetobutylicum.* **Applied and Environmental Microbiology** 53, 1924-1927.

Gyalai-Korpos, M., Mangel, R., Alvira, P., Dienes, D., Ballesteros, M., and Réczey, K. (2011): Cellulase production using different streams of wheat grain- and wheat straw-based ethanol process. **Industrial Microbiology and Biotechnology** 38, 791-802.

Harrison, D.G., and McAllan, A.B. (1980): *Factors affecting microbial growth yields in the retriculorumen*. Lancaster: MTP Press Limited.

Hatakka, A. I. (1983): Pretreatment of wheat straw by white-rot fungi for enzymatic saccharification of cellulose. **Applied and Environmental Microbiology** 18, 350-357.

He, Y., Pang, Y., Liu, Y., Li, X., and Wang, K. (2008): Physicochemical characterization of rice straw pretreated with sodium hydroxide in the solid state for enhancing biogas production. **Energy and Fuels** 22, 2775-2781.

Heap, J.T., Ehsaan, M., Cooksley, C.M., Ng, Y.-K., Cartman, S.T., Winzer, K., and Minton, N.P. (2012): Integration of DNA into bacterial chromosomes from plasmids without a counter-selection marker. **Nucleic Acids Research** 40(8), 2-10.

Heipieper, H.J., Weber, F.J., Sikkema, J., Keweloh, H., and Debont, J. (1994): Mechanisms of resistance of whole cells to toxic organic solvents. **Trends Biotechnology** 12, 409-415.

Hetal, G.A. (2006): Bioconversion of straw into improved fodder: Preliminary treatment of rice straw using mechanical, chemical and/or gamma irradiation. **Mycobiology** 34(1), 14-21.

Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., and Foust,T.D. (2007): Biomass recalcitrance: engineering plants and enzymes for biofuels production. **Science** 315, 804-807.

Houtman, C.J., and Atalla, R.H. (1995): Cellulose-lignin interactions. **Plant Physiology** 107, 977-984.

Hsu, T.-C., Guo, G.-L., Chen, W.-H., and Hwang, W.-S. (2010): Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. **Bioresource Technology** 101, 4907-4913.

Huang, C., Wu, H., Liu, Z.-J., and Cai, J. (2012): Wen-yong Lou and Min-hua Zong. Effect of organic acids on the growth and lipid accumulation of oleaginous yeast *Trichosporon fermentans*. **Biotechnology for Biofuels** 5, 4.

Ibbett, R., Gaddipati, S., Davies, S., Hill, S., and Tucker, G. (2011): The mechanisms of hydrothermal deconstruction of lignocellulose: new insights from thermal-analytical and complementary studies. **Bioresource Technology** 102, 9272-9278.

Ikram-Ul-Haq, Shafiq, K., and Ali, S. (2004): Kinetics of phosphate ions induced invertase synthesis by *Saccharomyces cerevisiae*. **International Journal of Agriculture and Biology** 6(2), 363-365.

Ingesson, H., Zacchi, G., Yang, B., Esteghlalian, A.R., and Saddler, J.N. (2001): The effect of shaking regime on the rate and extent of enzymatic hydrolysis of cellulose. **Journal of biotechnology** 88, 177-182.

Islam, R., Cicek, N., Sparling, R., and Levin, D. (2009): Influence of initial cellulose concentration on the carbon flow distribution during batch fermentation by *Clostridium thermocellum* ATCC 27405. **Applied and Environmental Microbiology** 82, 141-148.

Jaradat, Z., Dawagreh, A., Ababneh, Q., and Saadoun, I. (2008): Influence of Culture conditions on cellulase production by *Streptomyces* sp. (strain J2). **Jordan Journal of Biological Sciences** 1(4), 141-146.

Jensen, P.D., Hardin, M.T., and Clarke, W.P. (2009): Effect of biomass concentration and inoculum source on the rate of anaerobic cellulose solubilization. **Bioresource Technology** 100, 5219-5225.

Jesse, T.W., Zeji, T.C., Qureshi, N., and Blaschek, H.P. (2002): Production of butanol from starch-based waste packing peanuts and agricultural waste. **Journal of Industrial Microbiology and Biotechnology** 29, 117-223.

Jiang, M., Chen, K., Liu, Z., Wei, P., Ying, H., and Chang, H. (2010): Succinic acid production by *Actinobacillus succinogenes* using spent brewer's yeast hydrolysate as a nitrogen source. **Applied Biochemistry Biotechnology** 160, 244-254.

Jieun, L., Seo, E., Kweon, D.-H., Park, K., and Jin, Y.-S. (2009): Fermentation of rice bran and defatted rice bran for butanol production. **Journal of Microbiology and Biotechnology** 19(5), 482-490.

Jieun, L., Seo, E., Kweon, D.-H., Park, K., and Jin, Y.-S. (2009): Fermentation of rice bran and defatted rice bran for butanol production using *Clostridium beijerinkii* NCIMB 8052. **Journal of Microbiology and Biotechnology** 19(5), 482-490.

Jones, D.T., and Keis, S. (1995): Origins and relationships of industrial solvent-producing clostridial strains. **FEMS Microbiology Reviews** 17, 223-232.

Jones, D.T., and Woods, D.R. (1986): Acetone-butanol fermenatation revisited. **Microbiological reviews** 50, 484-524.

Kadam, K. L., Forrest, L.H., and Jacobson, W. (2000): A rice straw as a lingo-cellulosic resource collection, process-ing, transportation and environmental aspects. **Biomass Bioenergy** 8, 369-389.

Karami, K., Kheradmandinia, S., and Taherzadeh, M.J. (2006): Conversion of rice straw to sugars by dilute-acid hydrolysis. **Biomass and Bioenergy** 30, 247-253.

Kato, S., Haruta, S., Cui, Z.J., Ishii, M., and Igarash, Y. (2004): Effective cellulose degradation by a mixed-culture system composed of a cellulolytic *Clostridium* and aerobic non-cellulolytic bacteria. **FEMS Microbiology Ecology** 51, 133-142.

Kaur, P.P., Arneja, J.S., and Singh, J. (1997): Enzymatic hydrolysis of rice straw by crude cellulas from *Trichoderma reesei*. **Bioresource Technology**, 267-269.

Kaya, F., Heitmann, J.A., and Joye, T.W. (2000): Influence of lignin and its degradation products on enzymatic hydrolysis of xylan. **Journal of Biotechnology** 80, 241-247.

Khan, A.W., Trottier, T.M., Patel, G.B., and Martin, S.M. (1979): Nutrient requirement for the degradation of cellulose to methane by a mixed population of anaerobes. **Journal of General Microbiology** 112, 365-372.

Khan, Q., and Hadi, S. (1994): Inactivation and repair of bacteriophage lambda by furfural. **Biochemistry andMolecular Biology International** 32, 379-385.

Kharkwal, S., Karimi, I.A., Chang, M.W., and Lee, D.-Y. (2009): Strain improvement and process development for biobutanol production. **Recent Patents on Biotechnology** 3, 202-210.

Killeffer, D. H. (1927): Butanol and acetone from corn. A description of the fermentation process. **Journal of Industrial and Engineering Chemistry** 19, 46-50.

Kim, J.-W., Kim, K.S., Lee, J.-S., Park, S.M., Cho, H.-Y. Parkb, J.C., and Kim, J.S. (2011): Two-stage pretreatment of rice straw using aqueous ammonia and dilute acid. **Bioresource Technology** 102, 8992-8999.

Kim, P., and Mosier, N.S. (2009): Enzymatic digestion of liquid hot water pretreated hybrid poplar. **American Institute of Chemical Engineers** 25(2), 340-348.

Kim, T.H., and Lee, Y.Y. (2007): Pretreatment and fractionation of corn stover by ammonia recycle percolation process. **Bioresource Technology** 96, 2007-2013.

Kleerebezem, R., and van Loosdrecht, M.CM. (2007): Mixed culture biotechnology for bioenergy production. **Current Opinion in Biotechnology** 18, 207-212.

Klinke, H.B., Olsson, L., Thomsen, A.B., and Ahring, B.K. (2003): Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of Saccharomyces cerevisiae: wet oxidation and fermentation by yeast. **Biotechnology and Bioengineering** 81, 738-747.

Kosugi, A., Murashima, K., Tamaru, Y., and Doi, R.H. (2002): Cell surface anchoring role of N-terminal surface layer homology domains of *Clostridium cellulovorans* EngE. **Journal of Bacteriology** 184, 884-888.

Koul, V., Kumar, U., Sareen, V.K., and Singh, S., (1998): Effect of sodium bicarbonate supplementation on ruminal microbial populations and metabolism in buffalo calves. **The Indian Journal of Animal Sciences** 68, 629-631.

Koullas, D. P., Christakopoulos, P., Kekos, D., Macris, B. J., and Koukios, E. G. (1992): Correlating the effect of pretreatment on the enzymatic hydrolysis of straw. **Biotechnology and Bioengineering** 39, 113-116.

Kumar, P., Barrett, D.M., Deiwiche, M.J., and Stroeve, P. (2009): Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. **Industrial and Engineering Chemistry Research** 48, 3713-3729.

Kumar, R., Mago, G., Balan, V., and Wyman, C.E. (2009): Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. **Bioresource Technology** 100, 3948-3962.

Kumar, R., Singh, S., and Singh, O.V. (2008): Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. **The Journal of Industrial Microbiology and Biotechnology** 35, 377-391.

Ladish, M.R. (1989): *In Biomass Handbook*. New York: Science Publisher.

Lange, J.P. (2007): Lignocellulose conversion: an introduction to chemistry, process and economics. **Biofuels, Bioproducts and Biorefining** 1, 39-48.

Larsson, S., Palmqvist, E., Hahn-Hiigerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., and Nilvebrant, N. (1999): The generation of inhibitors during dilute acid hydrolysis of softwood. **Enzyme and Microbial Technology** 24, 151-159.

Larsson, S., Reimann, A., Nilvebrant, N.O., and Jönsson, I.J. (1999): Comparison of different methods for the detoxification of lignocelluloses hydrolyzates of spure. **Applied Biochemistry and Biotechnology** 77-79, 91-103.

Laser, M., Schulman, D., and Allen, S.G. (2002): A comparison of liquid hot water and steam pretreatment of sugar cane bagasse for bioconversion to ethanol. **Bioresource technology** 81, 33-44.

Lavarack, B. P., Griffin, G.J., and Rodman, D. (2002): The acid hydrolysis of sugarcane bagasse hemicelluloses to produce xylose, arabinose, glucose and other prouducts. **Biomass and Bioenergy** 23, 367-380.

Lee, J.Y., Jang, Y.S., Lee, J., Papoutsakis, E.T., and Lee, S.Y. (2009): Metabolic engineering of *Clostridium acetobutylicum* M5 for highly selective butanol production. **Biotechnology Journal** 4, 1432-1440.

Lee, S.F., Forsberg, C.W., and Gibbins, L.N. (1985): Cellulolytic activity of *Clostridium acetobutylicum*. **Applied and Environmental Microbiology** 50, 1068-1076.

Lee, S.F., Forsberg, C.W., and Rattray, J.B. (1987): Purification and characterization of two endoxylanases from *Clostridium acetobutylicum* ATCC 824. **Applied and Environmental Microbiology** 50, 644-650.

Lee, S.Y., Park, J.H., Jang, S.H., Nielsen, L.K., Kim, J., and Jung, K.S. (2008): Fermentative butanol production by Clostridia. **Biotechnology and Bioengineering** 101, 209-228.

Lee, W.G., Lee, J.S., Shin, C.S., Park, S.C., Chang, H.N., and Chang, Y.K. (1999): Ethanol production using concentrated oak wood hydrolysates and methods to detoxify. **Applied Biochemistry and Biotechnology** 77-79, 547-559.

Lee, Y.Y., Kim, J.-S., Xiang, D.Q., and Kim, T-H. (2001): *Enhancement of dilute-acid total-hydrolysis process for high-yield saacharification of cellulosic biomass*. Subcontract Final Report.

Leonard, RH., and Hajny, G.J. (1945): Fermentation of wood sugars to ethyl alcohol. **Industrial & Engineering Chemistry Research** 37, 390-395.

Lewkowski, I. (2001): Synthesis, chemistry and applications of 5-hydroxymethylfurfural and its derivatives. **Archive for Organic Chemistry I**, 17-54.

Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H.V., Auer, M, Vogel, K.P., Simmons, B.A., and Singh, S. (2010): Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. **Bioresource Technology** 101, 4900-4906.

Li, F.-H., Hu, H.-J., Yao, R.-Y., Wang, H., and Li, M.-M. (2012): Stucture and saccharification of rice straw pretreated with microwave-assisted dilute lye. **Industrial and Engineering Chemistry Research** 51, 6270-6274.

Li, R.-D., Li, Y.-Y., Lu, L.-Y., Ren, C., Li, Y.-X., and Liu, L. (2010): An improved kinetic model for acetone-butanol-ethanol pathway of *Clostridium acetobutylicum* and model-based perturbation analysis. **The Fourth Internationa Conference on Computational Systems Biology**, 62-71.

Liao, W., Wen, Z., Hurley, S., Liu, Y., Liu, C., and Chen, S. (2005): Effects of hemicellulose and lignin on enzymatic hydrolysis of cellulose from dairy manure. **Applied Biochemistry and Biotechnology** 121(124), 1017-1030.

Lin, Y.-L., and Blaschec, H.P. (1983): Butanol production by a butanol-tolerant strain of *Clostridium acetobutylicum* in extruded corn broth. **Applied and Environmental Microbiology** 45(3), 966-973.

Liu, Z., Ying, Y., Li, F., Ma, C., and Xu, P. (2010): Butanol production by *Clostridium beijerinckii* ATCC 55025 from wheat bran. **Journal of Industrial Microbiology and Biotechnology** 37(5), 495-501.

Lo, Y.C., Saratale, G.D., Chen, W.M., Bai, M.D., and Chang, J.S. (2009): Isolation of cellulose-utilizing bacteria for cellulosic biohydrogen production. **Enzyme and Microbial Technology** 44, 417-425.

Long, S., Jones, D.T., and Wood, D.R. (1983): Sporulation of *Clostridium acetobutylicum* P262 in a defined medium. **Applied and Environmental Microbiology** 45(4), 1389-1393.

Lui, C.G., and Wyman, C.E. (2004): Impact of fluid velocity on hot water only pretreatment of corn stove in flow through reactor. **Applied Biochemicstry and Biotechnology** 113-116, 977-987.

Lynd, L. R., Wyman, C. E. and Gerngross, T. U. (1999): Biocommodity engineering. **Biotechnology Progress** 15, 777-793.

Lynd, L. R., R. T. Elander, and C. E. Wyman. (1996): Likely features and costs of mature biomass ethanol technology. **Applied Biochemistry and Biotechnology** 57-58, 741-761.

Maamar, H., Valette, O., Fierobe, H.-P., Bélaich, A., Bélaich, J.-P., and Tardif, C. (2004): Cellulolysis is severely affected in *Clostridium cellulolyticum* strain cipC Mut1. **Molecular Microbiology** 51(2), 589-598.

Madihah, M.S., A Riff, A.B., Khalil, M.S., Suraini, A.A., and Karim, M.I.A. (2001): Anaerobic fermentation of gelatinized sago starch-derived sugars to aceton-1-butanol-ethanol solvent by *Clostridium acetobutylicum*. **Folia microbiologica** 46, 197-204.

Marchal, R., Rebeller, M., and Vandecasteele, J.P. (1984): Direct bioconversion of alkali-pretreated straw using simultaneous enzymatic hydrolysis and acetone-buanol fermentation. **Biotechnology Letters** 6(8), 523-528.

Martín C, Galbe M, Nilvebrant N-O, and Jönsson LJ. (2002): Comparison of the fermentability of enzymatic hydrolyzates of sugarcane bagasse pretreated by steam explosion using different impregnating agents. **Applied Biochemistry and Biotechnology** 98(100), 699-716.

Matsushita, C., Matsushita, O., Koyama, M. and Okabe, A. (1994): A *Clostridium perfringens* vector for the selection of promoters. **Plasmid** 31:317-319.

McCoy, E., Fred, E. B., Peterson, W. H., and Hastings, E. G. (1926): A cultural study of the acetone butyl alcohol organism. **The Journal of Infectious Diseases** 39, 457-484.

McMillan, J.D. (1994). *Enzymatic Conversion of Biomass for Fuels Production*. Washington, DC.: American Chemical Society.

McNeil, B., and Kristiahsen, B. (1985): Effect of temperature upon growth rate and solvent production in batch cultures of *Clostridium acetobutylicum*. **Biotechnology Letters** 7, 499-502.

Meinita, M.D.N., Hong, Y.-H., and Jeong, G.-T. (2012): Detoxification of acidic catalyzed hydrolyzate of *Kappaphhycus alvarezii* (cottonii). **Bioprocess and Biosystems Engineering** 35, 93-98.

Meinita, M.D.N., Hong, Y.-K., and Jeong, G.-T. (2012): Comparison of sulfuric and hydrochloric acids as catalysts in hydrolysis of *Kappaphycus alvarezii* (cottonii). **Bioprocess and Biosystems Engineering** 35, 123-128.

Mickael, D. (2004): Mapping of carbon flow distribution in the central metabolic pathways of *Clostridium cellulolyticum*: direct comparison of bacterial metabolism with a soluble versus an insoluble carbon source. **Journal of Microbiology and Biotechnology** 14(6), 1200-1210.

Mingardon, F., Chanal, A., Tardif, C., and Fierobe, H.-P. (2011): The issue of secretion in heterologous expression of *Clostridium cellulolyticum* cellulase-encoding genes in *Clostridium acetobutylicum* ATCC 824. **Applied and Environmental Microbiology** 77(9), 2831-2838.

Mingardon, F., Perret, S., Bélaïch, A., Tardif, C., Bélaïch, J.-P., and Fierobe, H.-P. (2005): Heterologous production, assembly, and secretion of a minicellulosome by *Clostridium acetobutylicum* ATCC 824. **Applied and Environmental Microbiology** 71(3), 1215-1222.

Ministry of Energy, Department of Alternative Energy Development and Efficiency, Bangkok. Retrived April 28, 2013, from: http://www.dede.go.th.

Minton, N. R., Berhm, J. K., Swinfield, T.-J., Whelm, S. M., Mauchline, M. L., Bodsworth, N., and Oultram, J. D. (1993): *The clostridia and biotechnology.* Baltimore: Butterworth-Heinemann.

Modig, T., Linden, G., and Taherzadeh, M.J. (2002): Inhibition effects offurfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. **Biochemical Journal** 363, 769-776.

Mohan, P.R., Ramesh, B., and Reddy, O.V.S. (2012): Biological pretreatment of rice straw by *Phenarocheate chrysosporium* for the production of cellulases and xylanases using *Asperigllus niger* isolate. **Research Journal of Microbiology** 7(1), 1-12.

Monot, F., Engasser, J.-M., and Peptidemange, H. (1984): Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobyticum*. **Applied and Environmental Microbiology** 19, 422-426.

Monot, F.., Engasser, J.M., and Petitdemange, H. (1984): Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*. **Environmental Microbiology** 19, 422-426.

Morag, E.A., and Lamed, R. (1990): Relationship of cellulosomal and noncellulosomal xylanases of *Clostridium thermocellum* to cellulose-degrading enzymes. **Journal of Bacteriology** 172, 6098-6150.

Moreira, A. R., Ulmer, D. C. and Linden, J. C. (1981): Butanol toxicity in the butylic fermentation. **Biotechnology and Bioengineering Symposium** 11, 567-579.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., and Ladisch, M. (2005): Features of promising technologies for pretreatment of lignocellulosic biomass. **Bioresource Technology** 96 (6), 673-686.

Mosier, N.S., Ladisch, C.M., and Ladisch, M.R. (2002): Characterization of acid catalytic domains for cellulose hydrolysis and glucose degradation. **Biotechnology and Bioengineering** 79(6), 610-618.

Mussatto, S.I., and Roberto, I.C. (2004): Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. **Bioresource Technology** 93, 1-10.

Mussatto, S.I., and Teixeira, J.A. (2010): *Current Research, Technology adn Education Topics in Applied Microbiology and Microbial Biotechnolog*. Spain: Formatex.

Najafpour, G., Ideris, A., Salmanpour, S., and Norouzi, M. (2007): Acid hydrolysis of pretreated palm oil lignocellulosic wastes. **International Journal of Engineering Transactions B: Applications** 20(2), 147-156.

Nakayama, S., Kiyoshi, K., Kadokura, T., and Nakazato, A. (2011): Butanol production from crystalline cellulose by cocultured *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum* N1-4. **Applied and Environmental Microbiology** 77(18), 6470–6475.

Ndukwe, N. A., Okiei, W. O., Alo, B.I., van Wyk, J.P.H., Mamabolo, T.M., and Igwe, C.C. (2013): Enzymatic bioconversion of kraft pulped and oxidative delignified sawdust from the Lagos Lagoon, Nigeria into fermentable sugars. **Scientific Research and Essays** 8(13), 493-500.

Negro, M. J., Manzanares, P., Ballesteros, I., Oliva, J.M., Cabañas, A., and Ballesteros, M. (2003): Hydrothermal pretreatment conditions to enhance ethanol production from Poplar biomass. **Applied Biochemistry and Biotechnology** 105-108, 87-100.

Nguyen, Q. A., Tucker, M.P., Keller, F.A., and Eddy, C.K. (2000): Two-stage dilute-acid pretreatment of softwoods. **Applied Biochemistray and Biotechnology** 84-46, 561-576.

Ni, Y., and Sun, Z. (2009): Recent progress on industrial fermentative production of acetone-butanol-ethanol by *Clostridium acetobutylicum* in China. **Applied Microbiology and Biotechnology** 83, 415-423.

Nigam, P.S., Gupta, N., and Anthwal, A. 2009): *Biotechnology for agro-industrial residues utilization* (1st ed). Netherlands: Springer.

Nilvebrant, N.-O., Persson, P., Reimann, A., de Sousa, F., Gorton, L., and Jönsson, L.J. (2003): Limits for alkaline detoxification of dilute-acid lignocelluloses hydrlysates. **Applied Biochemistry and Biotechnology** 105-108, 2003.

Nölling, J., Breton, G., Omelchenko, M.V., Makarova, K.S., Zeng, Q., Gibson, R., Lee, H.M., Dubois, J., Qiu, D., Hitti, J., and Sequencing, G. (2001): Genome Sequence and Comparative Analysis of the Solvent-Producing Bacterium *Clostridium acetobutylicum*. **Journal of Bacteriology** 183(16), 4823-4838.

Nölling, J., Breton, G., Omelchenko, M.V., Makarova, K. S. Zeng, Q., Gibson, R., Lee, H.M., Dubois, J., Qiu, D., Hitti, J., Wolf, Y. I., Tatusov, R. L., Sabathe, F., Doucette-Stamm, L., Soucaille, P., Daly, M. J., Bennett, G. N., Koonin, E. V., and Smith, D. R. (2001): Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. **Journal of Bacteriology** 183, 4823-4838.

Novikova, L.N., Medvedeva, S.A., Volchatova, I.V., and Bogatyreva, S.A. (2002): Changes in macromolecular characteristics and biological activity of hydrolytic lignin in the course of composting. **Applied Biochemistry and Microbiology** 38, 181-185.

O'Sullivan, C., Burrell, P.C., Clarke, W.P., and Blackall, L.L. (2008): The effect of biomass density on cellulo se solubilisation rates. **Bioresource Technology** 99, 4723-4731.

Okano, K., Kitagaw, M., Sasaki, Y., and Watanabe, T. (2005): Conversion of Japanese red cedar (*Cryptomeria japonica*) into a feed for ruminants by white-rot basidiomycetes. **Animal Feed Science and Technology** 120, 235-243.

Olsson, L., and Hahn-Hägerdal, B. (1996): Fermentation of lignocellulosic hydrolysates for ethanol production. **Enzyme and Microbial Technology** 18, 312-331.

Özpinar, Ö., and Özkan, M. (2007): Cellulose degradation and glucose accumulation by *Clostridium thermocellum* ATCC 27405 under different cultural conditions. **Annals of Microbiology** 57(3), 395-400.

Pages, S., Belaich, A., Belaich, J.-P., Morag, E., Lamed, R., Shoham, Y., and Bayer, E.A. (1997): Species-specificity of the cohesion-dockerin interaction between *Clostridium thermocellum* and *Clostridium cellulolyticum*: prediction of specificity determinants of the dockerin domain. **Proteins** 29, 517-527.

Pagés, S., Bélaïch, A., Fierobe, H.-P., Tardif, C., Gaudin, C., and Bélaïch, J.-P. (1999): Sequence analysis of scaffolding protein CipC and ORFXp, a new cohesin-containing protein in *Clostridium cellulolyticum*: comparison of various cohesin domains and subcellular localization of ORFXp. **Journal of Bacteriology** 181, 1801-1810.

Palmqvist, E., and Hahn-Hagerdal, B. (2000a): Fermentation of lignocellulosic hydrolyzates. I: inhibition and detoxification. **Bioresource Technology** 74, 17-24.

Palmqvist, B., Wiman, M. and Lidén, G. (2001). Effect of mixing on enzymatic hydrolysis of steam-pretreated spruce: a quantitative analysis of conversion and power consumption. **Biotechnology for Biofuels**, 4-10.

Palmqvist, E., and Hahn-Hagerdal, B. (2000b): Fermentation of lignocellulosic hydrolyzates.II: inhibitors and mechanism of inhibition: review. **Bioresource Technology** 74, 25-33.

Parajo, J. C., Dominguez, H., and Dominguez, J. M. (1998): Biotechnological production of xylitol. Part 3: operation in culture media made from lignocellulose hydrolysates. **Bioresource Technology** 66, 25-40.

Parawira, W., and Tekere, M. (2011): Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. **Critical Reviews in Biotechnology** 31, 20-31.

Parekh, S.R., Parekh, R.S., and Wayman, M. (1988) : Ethanol and butanol production by fermentation of enzymatically saccharified SO2-prehy-drolysed lignocellulosics. **Enzyme and Microbial Technology** 10, 660-668.

Perret, S., Casalot, L., Fierobe, H.-P., Tardif, C., Sabathé, F., Belaich, J.-P., and Belaich, A. (2004): Production of heterologous and chimeric scaffoldins by *Clostridium acetobutylicum* ATCC 824. **Journal of Bacteriology** 186(1), 253-257.

Prasertsan, S., and Sajjakulnukit, B. (2006): Biomass and biogas energy in Thailand: potential, opportunity and barriers. **Renewable Energy** 31, 599-610.

Quixley, K.W.M., and Reid, S.J. (2000): Construction of a reporter gene vector for *Clostridium beijerinckii* using a *Clostridium endoglucanase* gene. **Journal of Molecular Microbiology and Biotechnology** 2(1), 53-57

Qureshi, N., and Blaschek, H.P. (2005): *Food biotechnology* (2nd ed.) New York: Taylor and Francis.

Qureshi, N., Ezeji, T.C., Ebener, J., Dien, B.S., Cottaa, M.A., and Blaschek, H.P. (2008): Butanol production by *Clostridium beijerinckii*. Part I: Use of acid and enzyme hydrolyzed corn. **Bioresource Technology** 99, 5915-5922.

Qureshi, N., Lolas, A., and Blaschek, H.P. (2001): Soy molasses as fermentation substrate for production of butanol using *Clostridium beijerinckii* BA101. **Journal of Industrial Microbiology and Biotechnology** 26, 290-295.

Qureshi, N., Saha, B.C., Dien, B., Hector, R.E., and Cotta, M.A. (2010b): Production of butanol (a biofuel) from agricultural residues: Part I - Use of barley straw hydrolysate. **Biom ass and Bioenerg y** 34, 559-565.

Qureshi, N., Saha, B.C., and Cotta, M.A. (2007): Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*. **Bioprocess and Biosystems Engineering** 30, 419-427.

Qureshi, N., Saha, B.C., Hector, R.E., Dien, B., Hughes, S., Liu, S., Iten, L., Bowman, M.J., Sarath, G., and Cotta, M.A. (2010a): Production of butanol (a biofuel) from agricultural residues: Part II - Use of corn stover and switchgrass hydrolysates. **Biomass and Bioenergy** 34, 566-571.

Qureshi, N.T., and Blaschek, H. (2007): Butanol production from agricultural residues: Impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. **Biotechnology and Bioengineering** 97, 1460-1469.

Raganati, F., Curth, S., Götz, P., Olivieri, G., and Marzocchella, A. (2012): Butanol production from lignocellulosic-based hexoses and pentoses by fermentation of *Clostridium acetobutylicum.* **Chemical engineering transactions** 27, 91-96.

Ramos, L.P., Breuil, C., and Saddler, J.N. (1993): The use of enzyme recycling and the influence of sugar accumulation of cellulose hydrolysis byTrichoderma cellulases. **Enzyme and Microbial Technology** 15, 19-25.

Ramos, L.P., Breuil, C., and Saddler, J.N. (1991): Comparison of steam pretreatment of Eucalyptus, Aspen, and spruce wood chips and their enzymatic hydrolysis. **Applied Biochemistry and Biotechnology** 34(35), 37-47.

Ranatuanga, T.D., Jervis, J., Helm, R.F., McMillan, J.D., and Wooley, R.J. (2000): The effect of overliming on the toxicity of dilute acid pretreated lignocellulosics: te role of inorganics, uronic acids and ether-soluble organics. **Enzyme and Microbial Technology** 27, 240-247.

Reverbel-Leroy, C., Be1aich, A., Bernadac, A., Gaudin, C., Belaich, J.-P. and Tardif, C. (1996). Molecular study and overexpression of the *Clostridium cellulolyticum* celF cellulase gene in *Escherichia coli*. **Microbiology** 142, 1013-1023.

Reysenbach, A.L., Ravenscroft, N., Long, S., Jones, D.T., and Woods, D.R. (1986): Characterization, biosynthesis, and regulation of granulose in *Clostridium acetobutylicum*. **Applied and Environmental Microbiology** 52(1), 185-190.

Reysset, G., and Sebald, M. (1993): *The clostridia and biotechnology*. Butterworth: Boston.

Robinson, R. K. (2000): Encyclopedia of Food Microbiology. Retrieved Septemper 28, 2013, from http://www.elsevierdirect.com/companions/9780122270703/fmicauth.htm.

Roche, C. M., Dibble, C. J., Knutsen, J. S., Stickel, J. J., and Liberatore, M. W. (2009). Particle Concentration and Yield Stress of Biomass Slurries during Enzymatic Hydrolysis at High-Solids Loadings. **Biotechnology and Bioengineering** 104, 290-300.

Roos, J. W., McLaughlin, J. K., and Papoutsakis, E. T. (1985): The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentations of *Clostridium acetobutylicum*. **Biotechnology and Bioengineering** 27, 681-694.

Rosgaard, L., Pedersen, S., and Meyer, A.S. (2007): Comparison of different pretreatment strategies for enzymatic hydrolysis of wheat and barley straw. **Applied Biochemistry and Microbiology** 143,284-296.

Russell, J. B. (1992): Another explanation for the toxicity of fer-mentation acids at low pH: anioin accumulation versus uncoupling. **Journal of Applied Bacteriology** 73, 363-370.

Sabathé, F., and Soucaille, P. (2003): Characterization of the CipA Scaffolding protein and In vivo production of a minicellulosome in *Clostridium acetobutylicum*. **Journal of Bacteriology** 185(3), 1092-1096.

Sabathé, F., Belaïch, A., and Soucaille, P. (2002). Characterization of the cellulolytic complex (cellulosome) of *Clostridium acetobutylicum*. **FEMS Microbiology Letters** 217, 15-22.

Saha BC (2003). Hemicellulose bioconversion. **Journal of Industrial Microbiology and Biotechnology** 30, 279-291.

Saha, B.C., and Bothast, R.J. (1999): *Biopolymers: utilizing natures advanced materials*. Washington, D.C.: American Chemical Society.

Saha, B.C., Iten, L.B., Cotta, M.A., and Wu, Y.V. (2005): Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. **Process Biochemistry** 40, 3693-3700.

Sajjakulnukit, B., Rungrawee, Y., Virach, M., Veerawan, P., Bhattacharyab, S.C., and AbduSalam, P. (2005): Assessment of sustainable energy potential of non-plantation biomass resources in Thailand. **Biomass and Bioenergy** 29, 191-198.

Samaniuk, J.R., Scott, C.T., Root, T.W., and Klingenberg, D.J. (2011): The effect of high intensity mixing on the enzymatic hydrolysis of concentrated cellulose fiber suspensions. **Bioresource Technology** 102, 4489-4494.

Sanchez, B., and Bautista, J. (1988): Effects of furfural and 5-hydroxymethy1furfrual on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guilliermondii*. **Enzyme and Microbial Technology** 10, 315-318.

Santra, A., Chaturvedi, O.H., Tripathi, M.K., Kumar, R., and Karim, S.A. (2003): Effect of dietary sodium bicarbonate supplementation on fermentation characteristics and ciliate protozoal population in rumen of lambs. **Small Ruminant Research** 47, 203-212.

Sarao, L.K., Arora, M., and Sehgal, V.K. (2010): Use of *Scopulariopsis acremonium* for the production of cellulase and xylanase through submerged fermentation. **African Journal of Microbiology Research** 4(14), 1506-1509.

Saratale, G.D., Humnabadkar, R.P., and Govindwar, S.P. (2007): Presence of mixed function oxidase system in *Aspergillus ochraceus* (NCIM-1146). **Indian Journal of Microbiology** 47, 304-309.

Saratale, G.D., Saratale, R.G., Lo, Y.C., and Chang, J.S. (2010): Multicomponenet cellulase production by *Cellulomonas biazotea* NCIM-2550 and their applications for cellulosic biohydrogen production. **Biotechnology Progress** 26, 406-416.

Saratale, G.D., Chen, S.D., Lo, Y.C., Saratale, R.G., and Chang, J.S. (2008): Outlook of biohydrogen production from lignocellulosic feedstock using dark fermentation-a review. **Journal of Scientific and Industrial Research** 67, 962-979.

Sarkar, N., and Aikat, K. (2012): Alkali pretreatment of rice straw and enhanced cellulase production by a locally isolated fungus *Aspergillus fumigates* NITDGPKA3. **Journal of Microbiology and Biotechnology Research** 2 (5), 717-726.

Satimanont, S., Luengnaruemitchai, A., and Wongkasemjit, S. (2012): Effect of temperature and time on dilute acid pretreatment of corn cobs. **World Academy of Science, Engineering and Technology** 64, 531-535.

Sawisit, A. (2010): *Acetone-butanol-ethanol (ABE) production from cassava and glycerol by Clostridium acetobutylicum* (Master's Degree). Suranaree University of Technology. Nakhon Ratchasima. Thailand.

Schuster, K. C., van den Heuvel, R., Gutierrez, N. A., and Maddox, I. S. (1998): Development of markers for product formation and cell cycle in batch cultivation of *Clostridium acetobutylicum* ATCC 824. **Applied microbiology and biotechnology** 49, 669-676.

Scott, E.L., Kootstra, A.M.J., and Sanders, J.P.M. (2010): *Sustainable biotechnology.* The Netherland: Elsevier.

Shafiq, K., Ali, S., and Ikram-ul-Haq. (2002): Effect of different mineral nutrients on invertase production by *Saccharomyces cerevisiae* GCB-K5. **Biotechnology** 1(1), 40-44.

Shafizadeh, F., and Stevenson, T.T. (1982): Saccharification of douglas-firwood by a combination of prehydrolysis and pyrolysis. **Journal of Applied Polymer Science** 27, 4577-4585.

Shoham, Y., Lamed, R., and Bayer, E.A. (1999):The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends in Microbiology 275(7), 275-281.

Shapovalov, O.I., and Ashkinazi, L.A. (2008): Biobutanol: Biofuel of second generation. **Russian Journal of Applied Chemistry** 81(12), 2232-2236.

Shinto, H., Tashiro Y., Kobayashi G., Sekiguchi T., Hanai, T., Kuriya Y., Okamoto M., and Sonomoto K. (2008): Kinetic study of substrate dependency for higher butanol production in acetone-butanol-ethanol fermentation **Process Biochemistry** 43, 1452-1461.

Silverstein, R.A., Chen, Y., Sharma-Shivappa, R.R., Boyette, M.D., and Osborne, J. (2007): A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. **Bioresource technology** 98, 3000-3011.

Singh, A., and Bishno, N.R. (2012): Optimization of enzymatic hydrolysis of pretreated rice straw and ethanol production. **Applied Microbiology and Biotechnology** 93, 1785-1793.

Singh, P., Suman, A., Tiwari, P., Arya, N., Gaur, A., and Shrivastava, A. K. (2008): Biological pretreatment of sugarcane trash for its conversion to fermentable sugars. **World Journal of Microbiology and Biotechnology** 24, 667-673.

Sivers, M.V., and Zacchi, G. (1995): A techno-economical comparison of three processes for the production of ethanol from pine. **Bioresource Technology** 56, 43-52.

Söderström, J., Pilcher, L., Galbe, M., and Zacchi, G. (2003):Combined use of H_2SO_4 and SO_2 impregnation for steam pretreatment of spruce in ethanol production. **Applied Biochemistry and Biotechnology** 105-108, 127-140.

Soni, B., Das, K., and Ghose, T. (1982): Bioconversion of agro-wastes into acetone butanol. **Biotechnology Letters** 4, 19-22.

Spivey, M.J. (1978): The acetone/butanol/ethanol fermentation. **Process Biochemistry** 13, 2-5.

Stone, J. E., Scallan, A. M., Donefer, E., and Ahlgren, E. (1969): Digestibility as a simple function of a molecule of similar size to a cellulase enzyme. In **Advanced Chemistry Set** 95, 219.

Stone, J. E., and Scallan, A. M. (1968): A structural model for the cell wall of water-swollen wood pulpfibres based on their accessibility to macromole-cules. **Cellulose Chemistry and Technology** 3, 343-358.

Sun, Y., and Cheng, J. (2002): Hydrolysis of lignocellulosic materials for ethanol production: A review. **Bioresource Technology** 83(1), 1-11.

Syed, Q.A., Nadeem, M., and Nelofer, R. (2008): Enhanced butanol production by mutant strains of *Clostridium acetobutylicum* in molasses muedium. **Turkish Journal of Biochemistry** 33(1), 25-30.

Taherzadeh, M., and Karimi, K. (2007): Acid-based hydrolysis process for ethanol from lignocellulosic materials: A review. B**ioresources** 2(3), 472-499.

Taherzadeh, M.I., Gustafsson, L., Niklasson, E., and Linden, G. (2000): Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. **Applied Microbiology and Biotechnology** 53, 701-708.

Taherzadeh, M.J., and Karimi, K. (2008): Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. **International Journal of Molecular Sciences** 9, 1621-1651.

Taherzadeh, M.J., Eklund, R., Gustafsson, L., Niklasson, C., and Liden, G. (1997): Characterization and fermentation of dilute-acid hydrolyzates from wood. **Industrail and Engineering Chemistry Research** 35(11), 4659-4665.

Tamaru, Y., and Doi, R.H. (1999): Three surface layer homology domains at the N terminus of the *Clostridium cellulovorans* major cellulosomal subunit EngE. **Journal of Bacteriology** 181, 3270-3276.

Taniguchi, M., Suzuki, H., Watanabe, D., Sakai, K., Hoshino, K., and Tanaka, T. (2005): Evaluation of pretreatment with *Pleurotus ostreatus* for enzymatic hydrolysis of rice straw. **Journal of Bioscience and Bioengineering** 100, 637-643.

Tashiro, Y., and Sonomoto, K. (2010): *Advances in butanol production by clostridia. Current Research, Technology and Education Topic in Applied Microbiology and Microbial Biotechnology*. Retrived 1 Septemper, 2013, from http://www.formatex.org/ microbiology2/index.html.

Tashiro, Y., Takeda, K., Kobayashi, G., Sonomoto, K., Ishizaki, A., and Yoshino, S. (2004): High butanol production by *Clostridium saccharoperbutylacetonicum*N1-4 in fed-batchculturewithpH-stat continuous butyric acid and glucose feeding method. **Journal of Bioscience and Bioengineering** 98 (4), 263-268.

Teeri, T.T. (1997): Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. **Trends in Biotechnology** 15*,* 160-167.

Teeri, T.T., Koivula, T., Linder, M., Wohlfahrt, G., Divne, C., and Jones, T.A. (1998): *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? **Biochemical Society Transaction** 26, 173-178.

Terracciano, J.S., and Kashket, E.R. (1986): Intracellular conditions required for initiationof solvent production by *Clostridium acetobutylicum*. **Applied and Environmental Microbiology** 52, 86-91.

Thang, V.H., Kanda, K., and Kobayashi, G. (2010): Production of acetone-butanol-ethanol (ABE) in direct fermentation of cassava by *Clostridium saccharoperbutylacetonicum* N1-4. **Applied Biochemistry and Biotechnology** 161, 157-170.

Thauer, R.K., Jungermann, K., and Dekker, K. (1977): Energy conservation in chemotrophic anaerobic bacteria. **Bacteriological Reviews** 41, 100-180.

Thomas, K.C., Hynes, S.H., and Ingledew, W.M. (2002): Influence of medium buffering capacity on inhibition of *Saccharomyces cerevisiae* growth by acetic and lactic acids. **Applied and Environmental Microbiology** 68(4), 1616-1623.

Thormann, K., Feustel, L., Lorene, K., Nakojje, S and Dürre, P. (2002): Control of butanol formation in *Clostridium acetobutylicum* by transcriptional activation. **Journal of Bacteriology** 184, 1966-1973.

Tracy, B. P., Gaida, S.M., and Papoutsakis, E.T. (2008): Development and application of flow-cytometric techniques for analyzing and sorting endo-spore-forming clostridia. **Applied and Environmental Microbiology** 74:7497-7506.

Travaini, R., Otero, M.D.M., Coca, M., Da-Silva, R., and Bolado, S. (2013): Sugarcane bagasse ozonolysis pretreatment: Effect on enzymatic digestibility and inhibitory compound formation. **Bioresource Technology** 133, 332-339.

Tummala, S. B., Welker, N. E., and Papoutsakis, E. T. (1999): Development and characterization of a gene expression reporter system for *Clostridium acetobutylicum* ATCC 824. **Applied and Environmental Microbiology** 65, 3793-3799.

Underkofler, L.A., and Hickey, R.J. (1954): *Industrial Fermentations* (2^{nd}). Chemical NewYork: Publishing Company.

Vagner, V., and Ehrlich, S.D. (1988): Efficiency of homologous dna recombination varies along the *Bacillus subtilis* chromosome. **Journal of Bacteriology** 170(9), 3978-3982.

Vasconcelos, I., Girbal, L. and Soucaille, P. (1994): Regulation of carbon and electronflow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. **Journal of Bacteriology** 176, 1443-1450.

Verma, A., Kumar, S,. and Jain, P.K. (2011): Key pretreatment technologies on cellulosic ethanol production. **Journal of Scientific Research** 55, 57-63.

Vollherbst-Schneck, K., Sands, J.A., and Montencourt, B.S. (1984): Effect of butanol on lipid composition and fluidity of *Clostridiutm acetobutylicum* ATCC 824. **Applied and Environmental Microbiology** 47, 193-194.

Vollherbst-Schneck, K., Sands, J.A., and Montenecourt, B.S. (1984): Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. **Applied and Environmental Microbiology** 47(1), 193-194

Vu, V.H., Pham, T.A. and Kim, K. (2011): Improvement of fungal cellulase production by mutation and optimization of solid state fermentation. **Mycology** 39(1), 20-25.

Wan, C., and Li, Y. (2011): Effect of hot water extraction and liquid hot water pretreatment on the fungal degradation of biomass feedstocks. **Bioresource Technology** 102, 9788-9793.

Wan, C., Zhou, Y., and Li, Y. (2011): Liquid hot water and alkaline pretreatment of soybean straw for improving cellulose digestibility. **Bioresource Technology** 102, 6254-6259.

Wen Z, Liao W, and Chen S. (2004): Hydrolysis of animal manure lignocellulosics for reducing sugar production. **Bioresource Technology** 91, 31-39.

Weyer, E. R., and Rettger, L.F. (1927): A comparative study of six different strains of the organism commonly concerned in large-scale production of butyl alcohol and acetone by the biological process. **Journal of Bacteriology** 1, 399-424.

Wilkinson, S.R., and Young, M. (1994): Targeted integration of genes into the *Clostridiurn acetobutylicum* chromosorne. **Microbiology** 140, 89-95.

William, A.G., and Coleman, G.S., 1992. The rumen protozoa. Springer, New York.

Wongjewboot, I., Kangsadan, T., and Kongruang, S. (2010). Ethanol production rrom rice straw using ultrasonic pretreatment. International Conference on Chemistry and Chemical Engineering. Retrieved Septemper 28, 2010, from http://ieeexplore.ieee.org/xpls/abs_all.jsp?arnumber=5560351.

Xiang, Q., Lee, Y.Y., Pettersson, P.O., and Torget, R. W. (2003): Heterogeneous aspect of acid hydrolysis of α-cellulose. **Applied Biochemicistry and Biotechnology** 105 (108), 505-513.

Yang, B., and Wyman, C.E. (2008): Pretreatment: the key to unlocking low-cost cellulosic ethanol. **Biofuels, Bioproducts and Biorefining** 2, 26-40.

Yu, E.K.C., Chan, M.K.-H., and Saddler, J.N. (1985): Butanol production from cellulosic substrates by sequential co-culture of *Clostridium thermocellum* and *Clostridium acetobutylicum*. **Biotechnology Letters** 7(7), 509-514.

Yu, G., Yano, S., Inoue, H., Inoue, S., Endo, T., and Sawayama, S. (2010): Pretreatment of rice straw by a hot-compressed water process for enzymatic hydrolysis. **Applied Biochemistry and Biotechnology**, 160, 539-551.

Zhang, P.Y.H., Himmel, M.E., and Mielenz, J.R. (2006): Outlook for cellulase improvement: Screening and selection strategies. **Biotechnology Advances** 24, 452-481.

Zhang, Q.Z., and Cai, W.M. (2008): Enzymatic hydrolysis of alkali pretreated rice straw by Trichoderma reeseiZM4-F3. **Biomass Bioenergy** 32, 1130-1135.

Zhang, J., Zhu, Z., Wang, X., Wang, N., Wang, W., and Bao, J. (2010): Biodetoxification of toxins generated from lignocelluloses pretreatment using a newly isolated fungus, *Amorphoteca resinae* ZN1, and the consequent ethanol fermentation. **Biotechnology for Biofuels** 3, 26.

Zhang, P., Berson, Y.H., Sarkanen, E., and Dale, S. (2009): Pretreatment and biomass recalcitrance: Fundamentals and progress. **Applied Biochemistry and Biotechnology** 153, 80-83.

Zhang, Q., and Cai, W. (2008): Enzymatic hydrolysis of alkali-pretreated rice straw by *Trichoderma reesei* ZM4-F3. **Biomass and Bioenergy** 32, 1130-1135.

Zhang, Y-H.H., and Lynd, L.R. (2004): Kinetics and relative importance of phosphorolytic and hydrolytic cleavage of cellodextrins and cellobiose in cell extracts of clostridium thermocellum. **Applied and Environmental Microbiology** 70(3), 1563-1569.

Zhao, Y., Tomas, C.A., Rudolph, F.B., Papoutsakis, E.T., Bennett, G.N. (2005): Intracellular butyryl phosphate and acetyl phosphate concentrations in *Clostridium acetobutylicum* and their implications for solvent formation. **Applied and Environmental Microbiology** 71, 530-537.

Zhao, X.B., Wang, L., and Liu, D.H. (2008): Peracetic acid treatment of sugarcane bagasse for enzymatic hydrolysis; a continued work. **Journal of Chemical Technology and Biotechnology** 83, 950-960.

Zhu, J.-J., Yong, Q, Xu, Y., and Yu, S.-Y. (2009): Comparative detoxification of vacuum evaporation/stem stripping conbined with overliming on corn stover prehydrolyzate. **International Conference on Energy and Environment Technology**, 240-243.

Zhu, J.J., Yong, Q., Xu, Y., Chen, S.X., and Yu, S.Y. (2009): Adaptation fermentation of *Pichia stipitisand* combination detoxification on steam exploded lignocellulosic prehydrolyzate. **Natural Sciences** 1, 47-54.

Zhu, Y., and Yang, S.-T. (2004): Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*. **Journal of Biotechnology** 110, 143-157.

Zhu, Z., Sathitsuksanoh, N., Vinzant, T., Schell, D.J., McMillan, J.D., and Zhang, Y.H.P. (2009): Comparative study of corn stover pretreated by dilute acid and cellulose solvent-based lignocelluloses fractionation: enzymatic hydrolysis, supramolecular structure and substrate accessibility. **Biotechnology and Bioengineering** 103, 715-724.

BIOGRAPHY

Mrs. Metinee Wasoontharawat was born on February 22th, 1972 in Udon Thani province, Thailand. She attended and finished high school from the Udonpittayanukoon School in Udon Thani province. In 1990, she finished her Bachelor Degree in Biotechnology from Department of Biotechnolgy, Faculty of Technology, Khon Kaen University. In 2008, she received a scholarship for the program of development of the lecturer potentiality in the Udon Thani Rajabhat University to study the Degree of Doctor of Philosophy in Biotechnology Suranaree University of Technology. Later, she received a scholarship for the program of the sandwich Ph.D. program of the France-Thai cooperation in the Suranaree University of Technology in 2008 in order to do the partial part of her thesis research at Institut national des sciences appliquées (INSA), Toulouse in France for one year. Later, she received a scholarship under the Fronco-Thai scholarship program to do the thesis research for 4 months.