กระบวนการหมักบิวทานอลจากน้ำอ้อยโดย *Clostridium acetobutylicum*
ที่ถูกตรึงบนรังไหมบาง

นางสาวณัฐชีรา กิตติธเนศวร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรดุษฎีบัณฑิต
สาขาวิชาวิศวกรรมเคมี ภาควิชาวิศวกรรมเคมี
คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

BUTANOL FERMENTATION PROCESS FROM SUGARCANE JUICE BY CLOSTRIDIUM ACETOBUTYLICUM IMMOBILIZED ON SILK COCOONS

Miss Nutshera Kittithanesuan

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Engineering Program in Chemical Engineering
Department of Chemical Engineering
Faculty of Engineering
Chulalongkorn University
Academic Year 2014

Thesis Title	BUTANOL FERMENTATION PROCESS FROM SUGARCANE JUICE BY CLOSTRIDIUM ACETOBUTYLICUM IMMOBILIZED ON SILK COCOONS
By	Miss Nutshera Kittithanesuan
Field of Study	Chemical Engineering
Thesis Advisor	Associate Professor Muenduen Phisalaphong, Ph.D.

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

.....Dean of the Faculty of Engineering
(Professor Bundhit Eua-arporn, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Bunjerd Jongsomjit, Ph.D.)
.....Thesis Advisor
(Associate Professor Muenduen Phisalaphong, Ph.D.)
.....Examiner
(Associate Professor Artiwan Shotipruk, Ph.D.)
.....Examiner
(Associate Professor Joongjai Panpranot, Ph.D.)
.....External Examiner
(Associate Professor Sombat Teekasap, Ph.D.)

ณัฐชีรา กิตติธเนศวร : กระบวนการหมักบิวทานอลจากน้ำอ้อยโดย *Clostridium acetobutylicum* ที่ถูกตรึงบนรังไหมบาง (BUTANOL FERMENTATION PROCESS FROM SUGARCANE JUICE BY CLOSTRIDIUM ACETOBUTYLICUM IMMOBILIZED ON SILK COCOONS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. เหมือนเดือน พิศาลพงศ์, 104 หน้า.

บิวทานอลเป็นแอลกอฮอล์ชนิดหนึ่งที่สามารถนำมาใช้เป็นน้ำมันเชื้อเพลิงร่วมกับน้ำมันเบนซิน (แก๊สโซฮอล์) ให้กับรถยนต์และยานพาหนะอื่นๆ โดยแก๊สโซฮอล์จะช่วยลดการใช้น้ำมัน แต่เพิ่มประสิทธิภาพการทำงานของเครื่องยนต์ได้อีกด้วย นอกจากการแยกบิวทานอลออกมาจากน้ำมันปิโตรเลียมแล้ว การผลิตบิวทานอลยังทำได้โดยการใช้กระบวนการหมักด้วยจุลินทรีย์คลอสติเดียมซึ่งใช้น้ำตาลหรือแป้งเป็นแหล่งคาร์บอนทำให้เกิดผลิตภัณฑ์ผสมของอะซีโตน-บิวทานอล-เอทานอล อย่างไรก็ตามในปัจจุบันกระบวนการหมักดังกล่าวยังมีกำลังการผลิตต่ำ ดังนั้นในงานวิจัยนี้จึงพยายามปรับปรุงกระบวนการผลิตเพื่อให้สามารถผลิตบิวทานอลได้ในเชิงพาณิชย์ในกำลังการผลิตที่สูงขึ้น รวมไปถึงการใช้วัตถุดิบที่มีราคาถูกแต่มีปริมาณน้ำตาลสูง เช่น น้ำอ้อย เป็นแหล่งคาร์บอนของจุลินทรีย์ เพื่อลดต้นทุนในกระบวนการหมัก งานวิจัยนี้มีการใช้กระบวนการหมักแบบกะด้วยจุลินทรีย์*คลอสติเดียม อะซีโตบิวทีลิคัม* (ATCC 824) นอกจากนี้ยังนำรังไหมบางซึ่งเป็นวัสดุเหลือใช้จากโรงงานไหมมาใช้เป็นแหล่งไนโตรเจนและใช้เป็นวัสดุตรึงเซลล์ในกระบวนการหมักอีกด้วย โดยในแต่ละรอบของกระบวนการหมักได้ดำเนินการภายใต้สภาวะไร้ออกซิเจนและควบคุมอุณหภูมิที่ 35 องศาเซลเซียส ความเป็นกรด-ด่าง 4.5-5.0 และความเร็วในการปั่นกวน 200 รอบต่อนาที จากการศึกษาผลของแหล่งไนโตรเจนโดยใช้รังไหมบางร่วมกับสารสกัดยีสต์ในกระบวนการหมัก พบว่าการใช้รังไหมบางร่วมกับสารสกัดยีสต์สามารถเป็นแหล่งไนโตรเจนที่ดีให้กับจุลินทรีย์ได้ ซึ่งให้ผลิตภัณฑ์อะซิโตน บิวทานอล และ เอทานอลที่มีความเข้มข้นสูงสุดประมาณ 8.03, 12.72 และ 1.50 กรัมต่อลิตร ตามลำดับ เมื่อศึกษาผลของความเข้มข้นของน้ำตาลเริ่มต้น (80, 90 และ 100 กรัมต่อลิตร) ที่มีผลต่อความเข้มข้นของผลิตภัณฑ์ผสมที่ได้ พบว่า สภาวะที่เหมาะสมที่สุดคือการใช้น้ำอ้อยที่มีความเข้มข้นของน้ำตาลเริ่มต้นเท่ากับ 80 กรัมต่อลิตรโดยใช้เวลาในการหมัก 132 ชั่วโมง ทำให้เกิดผลิตภัณฑ์อะซิโตน บิวทานอล และ เอทานอลที่มีความเข้มข้นสูงสุดประมาณ 4.2, 15 และ 1.6 กรัมต่อลิตร ตามลำดับ โดยมีอัตราการผลิต 0.15 กรัมต่อลิตรต่อชั่วโมง เมื่อเปรียบเทียบกับกระบวนการในระบบเซลล์อิสระที่สภาวะเดียวกัน พบว่า ระบบเซลล์อิสระสามารถผลิตอะซิโตนและบิวทานอลได้น้อยกว่าคือ 3.46 และ 14.03 กรัมต่อลิตร ตามลำดับ ในขณะที่ทั้งสองระบบสามารถผลิตเอทานอลได้ใกล้เคียงกันคือ 1.4-1.6 กรัมต่อลิตร และอัตราการผลิตโดยรวมสามารถเพิ่มขึ้นได้โดยการหมักแบบกะที่มีการทำซ้ำโดยเซลล์ถูกตรึงบนรังไหมบาง พบว่าสามารถผลิตบิวทานอลได้ความเข้มข้น 5.8 กรัมต่อลิตรสำหรับการหมักรอบที่ 1 ที่เวลาในการหมัก 72 ชั่วโมง แต่การหมักรอบที่ 2, 3 และ 4 จะได้ความเข้มข้นของบิวทานอลเท่ากับ 13.0, 12.7 และ 12.8 กรัมต่อลิตร ตามลำดับ ที่เวลาในการหมักแต่ละรอบเท่ากับ 48 ชั่วโมง ซึ่งคิดเป็นอัตราการผลิตเท่ากับ 0.43 กรัมต่อลิตรต่อชั่วโมง นอกจากนี้ได้ศึกษาการประเมินผลกระทบต่อสิ่งแวดล้อมในแง่ของการปล่อย CO_2 ที่เกี่ยวข้องกับสามกระบวนการได้แก่ การเตรียมวัตถุดิบ กระบวนการหมัก และ สาธารณูปโภคไฟฟ้า ใช้เครื่องมือการประเมินวัฏจักรชีวิต (LCA) ตามฐานข้อมูล Ecoinvent จากข้อมูลการทดลอง กำหนดให้ 1000 กรัมของผลิตภัณฑ์หลัก (บิวทานอล) ภายใต้การดำเนินการในกระบวนการหมักแบบกะและกระบวนการหมักแบบกะที่มีการทำซ้ำถูกใช้เป็นพื้นฐานในการประเมินผลกระทบต่อสิ่งแวดล้อม เนื่องจากผลผลิตบิวทานอลและอะซีโตนจะเพิ่มขึ้นอย่างมากเมื่อใช้ตรึงเซลล์บนรังไหมบาง ในกระบวนการหมักแบบกะที่มีการทำซ้ำปริมาณการปลดปล่อย CO_2 รวมต่อการผลิตบิวทานอล 1 กิโลกรัมลดลงอย่างมาก การประเมินผลแสดงให้เห็นว่าการปล่อย CO_2 ทั้งหมดจากกระบวนการหมักแบบกะและกระบวนการหมักแบบกะที่มีการทำซ้ำเป็น 32.07 และ 3.36 กิโลกรัม CO_2 ต่อกิโลกรัมบิวทานอลตามลำดับ

ภาควิชา วิศวกรรมเคมี ลายมือชื่อนิสิต .....

สาขาวิชา วิศวกรรมเคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557

5471405021 : MAJOR CHEMICAL ENGINEERING

KEYWORDS: BUTANOL / CLOSTRIDIUM ACETOBUTYLICUM / SUGAR CANE JUICE / IMMOBILIZED CELLS / THIN SHELL SILK COCOON / ABE FERMENTATION / ACETONE / BUTANOL / REPEATED BATCH

NUTSHERA KITTITHANESUAN: BUTANOL FERMENTATION PROCESS FROM SUGARCANE JUICE BY CLOSTRIDIUM ACETOBUTYLICUM IMMOBILIZED ON SILK COCOONS. ADVISOR: ASSOC. PROF. MUENDUEN PHISALAPHONG, Ph.D., 104 pp.

Butanol, a kind of alcohol, can be used as a fuel oil in forms of gasoline-alcohol blend (gasohol) for automobiles and other vehicles. The gasohol basically demotes gasoline usage, but promotes the efficiency of the combustion engine. Besides butanol is recently derived from petroleum, it is obtained from fermenting with Clostridium strain as butanol-producing bacteria in the presence of sugar or starch as a carbon source. Butanol produced from the fermentation generally presents in the mixture of acetone, butanol, and ethanol (ABE). However, currently, the particular ABE fermentation yields low butanol productivity. Therefore, this study aimed to improve the butanol production to achieve higher productivity in the industrial scale, and to reduce the production cost by using the sucrose-rich sugarcane juice as a cheaper carbon source. In the present study, the batch fermentation was performed using *Clostridium acetobutylicum* (ATCC824). In addition, thin shell silk cocoon (TSC), a residual from the silk industry, was used as a nitrogen source as well as a support material for the immobilization of *C. acetobutylicum*. The ABE fermentations were operated under an anaerobic condition at the controlled temperature of 35 °C, pH of 4.5-5.0, and the rotational speed of 200 rpm in all batches. In the study of effect of nitrogen sources which were yeast extract and thin-shell silk cocoon fragment on the fermentation, it was found that those nitrogen sources were a great combined nitrogen source providing ABE products at the highest concentrations of approximately 8.03, 12.72, and 1.50 g/L, respectively. Furthermore, in the immobilized culture (IC-TSC), the initial concentration of sugarcane juice (80, 90, 100 g/L) affected the ABE concentrations. The optimum initial sugar concentration and fermentation period were 80 g/L and 132 h yielding ABE at the greatest concentrations of 4.2, 15, and 1.6 g/L, respectively (productivity based on the total ABE concentration of 0.15 g/L/h). As compared the IC-TSC to the suspended culture (SC) under the identical condition, SC provided the lower contents of acetone and butanol (3.46 and 14.03 g/L, respectively). Whereas, the similar concentration (1.4-1.6 g/L) of ethanol in both culture systems was shown. In the repeated batch of IC-TSC, butanol at 5.8 g/L was appeared in the first cycle at 72 h of fermentation period, but 13.0, 12.7 and 12.8 g/L were obtained in the second, third and fourth cycle, respectively after 48 h of the fermentation (productivity based on the total ABE concentration of 0.43 g/L/h). In addition, the evaluation of the environmental impact, in terms of CO_2 emissions was performed associated with three processes: raw material preparation, fermentation process, and electric utility in the production. A Life Cycle Assessment (LCA) based on the Ecoinvent database was utilized as a tool for this study. According to the information from the experimental data, 1000 g of the main product (butanol) operated in batch and repeated batch systems were set as the basis of the LCA. Since butanol and acetone productivities are considerably enhanced by using immobilized culture (IC) on TSC in repeated batch fermentation, the total CO_2 emissions per butanol production significantly decreased. The evaluation reveals that the total CO_2 emissions from batch and repeated batch systems are 32.07 and 3.36 kg CO_2/kg butanol, respectively.

Department:	Chemical Engineering	Student's Signature	.....
Field of Study:	Chemical Engineering	Advisor's Signature	.....
Academic Year:	2014		

ACKNOWLEDGEMENTS

I would like to express my earnest gratitude to my advisor, Assoc. Prof. Muenduen Phisalaphong, Ph.D. for her encouragement, support, guidance, and unfailing faith all the way through my thesis work and study.

Thanks to all of my thesis committee, Assoc. Prof. Bunjerd Jongsomjit, Ph.D., Assoc. Prof. Artiwan Shotipruk, Ph.D., Assoc. Prof. Joongjai Panpranot, Ph.D. and Assoc. Prof. Sombat Teekasap, Ph.D. for their kind advices and recommendations which are invaluable for improving my work.

Many thanks are also addressed to Mrs. Sunee Pakprapan and Mrs. Rujiporn Prateepasin (Scientific and Technological Research Equipment Centre, Chulalongkorn University) for their kind assistance in commencing Gas Chromatography (GC) and Scanning Electron Microscopy (SEM).

I would like to acknowledge the support from the 90th anniversary of Chulalongkorn University (Ratchadaphiseksomphot Endowment Fund) and Chemical Engineering Research Unit for Value Adding of Bioresources, Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University.

I express my warm thanks to Ms. Sawittree Mulalee, Ms. Siriporn Taokeaw, Mr. Atthapon Srifa, Ms. Nadda Cheiwprakobkij and Mr.Teerawat Suratako for their aspiring guidance, invaluably constructive criticism and friendly advice during my thesis work. Sincere thanks are given to all members of the Biochemical Engineering Research Laboratory and staffs in the Department of Chemical Engineering, Chulalongkorn University for their assistance, support, and warm collaborations.

Finally, I would like to express my highest gratitude to my parents for their affectionate support, blessings, inspiration, and love which guide me all the way throughout my life and study.

CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I INTRODUCTION

1.1 Background and rationale

Biofuels are received increasing public and scientific attention, driven by factors such as uncertainties related to oil price, greenhouse gas emission, and the need for increased energy security and diversity. Biofuels are a wide range of fuels derived from biomass. Butanol is one of competitive renewable biofuels for use in internal combustion engines [1]. Announcements by a consortium of companies to produce fuel butanol by fermentation on the industrial scale have increased the interest. There are efforts by private companies for example Gevo Inc., CO, or Cobalt Biofuels, CA [2] and to develop fermentation process for butanol production.

1-Butanol (butyl alcohol or n-butanol) is a four carbon straight chained alcohol with a molecular formula of C_4H_9OH (MW 74.12 g/mol) and boiling point of 118 °C. 1-Butanol is an important chemical precursor for paints, polymers and plastics [3]. Consequently it is easier to blend with gasoline and other hydrocarbon products and also contains more heat energy than ethanol, which equates to a 25% increase in harvestable energy (Btu's). Butanol contains 110,000 BTUs per gallon, closer to gasoline's 115,000 BTUs, and is safer to handle with a Reid Value of 0.33 psi, which is a measure of a fluid's rate of evaporation when compared to gasoline at 4.5 and ethanol at 2.0 psi [4].

Butanol can be used either as neat fuel or blend fuel in SI engines without any engine modification, due to its fuel properties are much closer to gasoline. Butanol also has several advantages over ethanol. It has lower vapor pressure which makes it safer to store and handle. It has higher energy content, lower water absorption, better blending ability and less corrosive. It can be produced from biomass (biobutanol) as well as fossil fuels (petrobutanol). However, biofuels are attractive alternatives to prevent further increase of carbon dioxide emissions [5].

Butanol can be produced from sugars and other carbohydrates by a number of clostridia via fermentation. Clostridia are rod-shaped, spore-forming gram positive bacteria and typically strict anaerobes. In acetone-butanol-ethanol (ABE) fermentation,

the first phase is the acidogenic phase, during which the acids forming pathways are activated and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products. This acidogenic phase usually occurs during the exponential growth phase. The second phase is the solventogenic phase, during which acids are reassimilated and used in the production of acetone, butanol, and ethanol [1]. The most popular bacteria species used for fermentation is *Clostridium acetobutylicum*.

Immobilization of microorganisms can be used for improving fermentation processes and allow easy recovery and reuse of the bacteria. Bacteria can be immobilized in a wide range of different configurations either entrapped in a polymeric matrix or attached to a solid support. The different methods for immobilization will affect the performance of the bacteria; for example entrapment in a polymeric matrix will introduce a diffusion barrier through which nutrients and (waste) products need to be transported [6]. Previously, a thin-shell silk cocoon (TSSC), a residual from the silk industry, was successfully used as a support material for yeast immobilization by adsorption because of its advantages, which include its biocompatibility, light weight, high strength, low cost, chemical stability, high porosity and high surface area [7]. Moreover, TSSC contains a lot of nitrogen sources, which might help increase productivity of many fermentation processes [8].

This research study aims to increase production rate and efficiency of ABE fermentation process by immobilization technique. The ABE fermentations were carried out by *C. acetobutylicum* immobilized on a thin-shell silk cocoons (TSSC) under batch and repeated batch fermentation using sugarcane juice as the main substrate. The fermentation performances were then examined and compared with those using suspended cells. The fermentations were carried out in anaerobic condition under controlled temperature, rotational speed and pH at 35 °C, 200 rpm and pH 4.5-5.0, respectively.

1.2 Objectives

1. To develop cell immobilization technology for improved ABE fermentation by *C. acetobutylicum* ATCC 824.

2. To develop a batch and repeated batch process for enhanced butanol production rate using *C. acetobutylicum* ATCC 824 immobilized on thin-shell silk cocoons (TSSC).

3. To reduce production costs of ABE fermentation using TSSC substitute for yeast extract as a nitrogen source.

4. To evaluate the environmental impact, in terms of CO_2 emissions, associated with three processes: raw material preparation, fermentation process, and electric utility in the production.

1.3 Research scopes

1. ABE fermentation by *C. acetobutylicum* immobilized on thin-shell silk cocoons (TSSC) in batch operation is studied. The fermentation in this work is carried out in 700 mL stirred tank fermenter. Sugarcane juice is used as the main substrate at the initial sugar concentrations of 80, 90 and 100 g/L. For the performance evaluation, the results are compared to those of the system using the suspended culture. The comparison is included the concentrations of products and cell, the productivities and the yields of products.

2. ABE fermentation by *C. acetobutylicum* immobilized on TSSC is further carried out in repeated batch operations in 700 mL fermenter. Sugarcane juice is used as the main substrate at the initial sugar concentrations of 80, 90 and 100 g/L. The effects of the initial sugar concentration is examined. The productivities are compared to the batch system.

3. The effect of using TSSC substitute for yeast extract as a nitrogen source in ABE fermentation is investigated. The fermentation in this work are carried out in 700 mL stirred tank fermenter in batch operation. Sugarcane juice is used as the main substrate at the initial sugar concentrations of 80 g/L. Yeast extract, TSSC fragment and a combination of yeast extract and TSSC fragment are used as a nitrogen sources.

4. A Life Cycle Assessment (LCA) based on the Ecoinvent database was utilized as a tool for this study. According to the information from the experimental data, 1000 g of the main product (butanol) operated in batch and repeated batch systems were set as the basis of the LCA. The life cycles of biobutanol, including the raw materials, fuels, and energy used in fermentation and separation are analyzed.

5. Parameters were analyzed, including:

- Morphology of the cells and support material for immobilization by SEM
- Concentrations of butanol, ethanol, acetone, butyric acid and acetic acid by Gas chromatography (GC)
- Cells concentration by UV spectrophotometer
- Reducing sugar concentrations by DNS method by UV spectrophotometer
- Production rates of cells and their products
- Yield of cells and their products
- Specific production rates of cell and products
- Sugar consumption rate

CHAPTER II THEORY AND LITERATURE REVIEWS

2.1 History of ABE fermentation

The production of butanol in a microbial fermentation was first reported by Pasteur in 1861; in 1905 the first fermentative production of acetone was reported. In 1911 Fernbach isolated a culture, which was able to ferment potatoes to butanol. In 1914 Weizmann discovered *C. acetobutylicum*. This organism had a number of unique properties, including the ability to use variety of starchy substances and to produce much better yields of acetone and butanol than did Fernbach's original culture. During the First World War great amounts of acetone were needed for the manufacture of munitions. As Great Britain's supplies of acetone were almost cut off in 1916, six distilleries in Great Britain were adapted for the production of acetone by the Weizmann process because of the promising results at pilot-scale plants [9].

Between 1950 and 1960 the microbial production of acetone and butanol decreased rapidly. The reason therefore was twofold. First molasses, so far used as substrate for ABE fermentation, began to be used in substantial amounts as cattle feed. Second the competition between the fermentation and chemical process had become very acute [10].

Butanol is a very competitive renewable biofuel for use in internal combustion engines given its many advantages. It is a superior fuel to ethanol and an industrial solvent that can be produced from renewable resources employing a number of organisms including *C. acetobutylicum* or *C. beijerinckii*. Historically, acetone–butanol–ethanol (ABE): in the fermentation broth, the typical ratio of ABE is 3:6:1, where butanol is a major product fermentation is second to ethanol and there were plants that operated during WW I and WW II [1, 11]. Figure 1 shows a scheme of the respective production pathway through ABE [12].

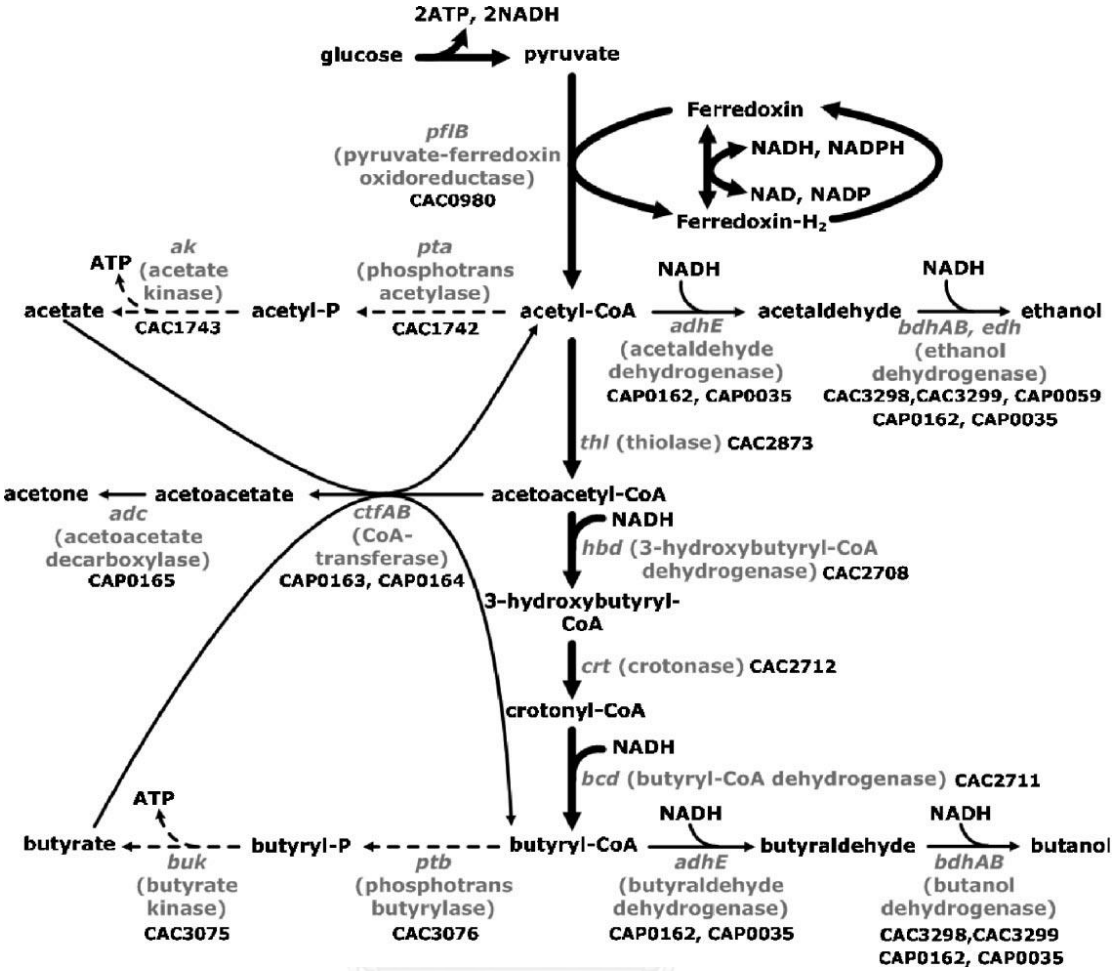


Figure 1 Metabolic pathways in *C. acetobutylicum* [13].

It is well known that during the growth phase of a batch culture of *C. acetobutylicum* the main end products are acetic acid, butyric acid, hydrogen, and carbon dioxide. The pH of the medium is very important to the biphasic ABE fermentation [1].

The major challenges that lie ahead in ABE fermentation using the current strains of Clostridia include the following [14]:

1. attaining higher biomass on cheaper substrates
2. improving solvent tolerance to achieve higher butanol titers
3. overcoming strain disintegration

4. delaying the initiation of sporulation to have a longer solventogenesis window

5. improving the selectivity of ABE process to produce butanol preferentially

6. improving aero tolerance

7. developing/improving fermentation, in situ solvent removal, and recovery strategies [15].

2.2 Microorganisms used in fermentation

Traditionally, butanol is produced in batch reactors using the anaerobic bacteria *C. acetobutylicum* or *C. beijerinckii* [16]. The clostridial acetone–butanol–ethanol (ABE) fermentation represents one of the oldest industrial fermentation processes known. In the early 1920s, Chaim Weizmann, who later became Israel's first president, discovered the anaerobic bacterium *C. acetobutylicum* which naturally produces acetone, butanol, and ethanol in a ratio of approximately 3:6:1 during the stationary growth phase, whereas acetic and butyric acid are the main products during exponential growth [17-19]. Anaerobic bacteria such as the solventogenic clostridia are capable of converting a wide range of carbon sources (e.g. glucose, galactose, cellobiose, mannose, xylose, and arabinose) to fuels and chemicals such as butanol, acetone, and ethanol [20]. The growth of cells on a large scale is called industrial fermentation. Industrial fermentation is normally performed in a bioreactor, which controls aeration, pH, and temperature [21].

Microorganism, culture maintenance and inoculum preparation *C. acetobutylicum* ATCC 824 was obtained from America Type Culture Collection, Manassas, VA, USA [22, 23]. The organism used was *C. acetobutylicum* ATCC 824. Spores of the culture were stored at 4°C in RCM medium (reinforced clostridial medium).

2.3 Approaches for improving fermentation process

2.3.1 Batch fermentation

Under batch conditions the fermentation process of solvent-producing *clostridium* strains proceeds with the production of cells, hydrogen, carbon dioxide, acetic acid, and butyric acid during the initial growth phase (acidogenesis). As the acid concentrations increase (pH decrease), the metabolism of cells shifts to solvent production (solventogenesis) and acidogenic cells - able to reproduce themselves - shift to the solventogenesis state with a morphological change. During solventogenesis the active cells become endospores unable to reproduce themselves. Two different physiological states must be taken into account for Clostridia: one for the acidonegenic phase, and one for the solventogenic phase [24].

Initial studies employing *C. beijerinckii* BA101 were performed in batch reactors ranging in size from 1 to 10 L using cracked corn, cornstarch, maltodextrin, and glucose. Under batch operation conditions, the culture was able to produce 18–33 g/L ABE in 72 h of fermentation using glucose or starch as the substrate, the productivity was 0.25–0.46 g/(L·h). Productivity in batch reactors is often low due to long lag phase, and product inhibition. This problem can be eliminated using fed-batch techniques or continuous culture with the application of novel product removal techniques [25].

Conventional batch operation of ABE fermentation seldom yields more than 12-20 g/l solvents and the fermentation time is about 2-6 days due to solvent toxicity, and therefore the amount of sugars that can be fermented is also limited [26, 27]

Another reason for low reactor productivity is the low concentration of cells in the bioreactor. In a batch reactor, cell concentration over 3 g/L is rarely achieved. Therefore, reactor productivity can be improved by increasing the cell concentration in the reactor. An increased cell concentration can be achieved by fixing cells onto supports or gel particles. Another option is the application of a membrane that returns cells to the reactor while the aqueous solution containing the product permeates the membrane [1].

Inexpensive and readily available biomass feedstock have been investigated for ABE fermentation. It was demonstrated that *C. acetobutylicum* can ferment mannitol, a potentially major constituent in seaweed, either as a sole carbon source or in combination with glucose. The butanol yields of 0.12 g/g and total solvent yields of 0.16 g/g, respectively were obtained from the ABE fermentation of extracts from *Saccharina spp.* by *C. acetobutylicum* ATCC 824. The result indicated that significant improvements are still needed to make industrial-scale acetone-butanol fermentations of seaweed economically feasible [28].

Cassava as a substrate for bio-butanol production was deficient in nitrogen source, when fermenting on cassava (15–25%w/v) with *C. acetobutylicum* ATCC 824, a severe delay (18–40 h) was observed in the phase shift from acidogenesis to solventogenesis, compared to the cases of fermenting on corn. By adding yeast extract (2.5 g/L-broth) into cassava meal medium when the delay appeared, the phase shift was triggered and fermentation performances were consequently improved. Total butanol concentrations and butanol productivities, compared to those with cassava substrate alone, increased about 15% and 80%, respectively in traditional fermentation while 86% and 79%, respectively in extractive fermentation using oleyl alcohol as the extractant, and reached the equivalent levels of those using corn substrate [29].

2.3.2 Repeated batch fermentation

The repeated batch operation is performed by many cycle batch fermentation with the reuse of cells. The product is removed at the end of each cycle. After the removal all of the supernatant, the fresh fermentation medium is poured into the fermenter for the next cycle. For ABE fermentation each repeated batch is cultivated under anaerobic conditions [30].

The long-term n-butanol production from sucrose by *C. acetobutylicum* JB200 was evaluated with cells immobilized in a fibrous-bed bioreactor (FBB). This study showed stable performance with 16.2–18.5 g/L butanol and 24.7–28.7 g/L ABE, yield (~0.21 g/g sucrose) and productivity (~0.32 g/L h) for 16 consecutive batches over 800 h [31].

2.3.3 The Immobilization Technique

Butanol production in ABE fermentation can be improved by cell recycling and immobilization to increase cell density and reactor productivity. Immobilized cell cultures have many advantages over suspension cultures for large-scale fermentation. They provide high cell density, eliminate expensive cell recovery, alleviate cell washout at high dilution rates, and protect against shear damage. Thus, cell immobilization enhances productivity in continuous processes. The adsorption of cells onto inert support surfaces has been widely used for cell immobilization.

High cell concentrations result in high reactor productivity. Such systems are continuous where feed is introduced into a tubular reactor at the bottom with product escaping at the top. These systems are often non-mixing reactors where product inhibition is significantly reduced. To improve the reactor productivity, *C. beijerinckii* cells were immobilized onto clay brick particles by adsorption and achieved reactor productivity on the order of 15.8 g/(L·h) [32]. In these scaled-up reactors, productivity of 40–50 times greater than that obtained in batch reactors was achieved. This result suggests that immobilized cell continuous reactors could be used to achieve higher reactor productivity which leads to economic advantage [1].

The technique is based on the pulsed addition of nutrients to the reactor which otherwise is only fed with a non-growth production medium, lacking nitrogen and growth factors. Using this technique, continuous production of solvents could be obtained from cells of *C. acetobutylicum* ATCC 824 immobilized in calcium alginate in a sheet reactor or adsorbed onto beech wood shavings [6].

The study was investigated to determine an optimal carrier for the immobilization of *C. acetobutylicum* in ABE fermentation and was also investigated the optimal size and number of carriers used for cell immobilization in butanol production. Three materials investigated in this study are bricks, synthetic sponge and nonwoven fabric. Among the three materials investigated in this study, bricks were determined to be the most suitable carrier for ABE fermentation. An immobilization ratio of 20% (the proportion of brick to the working volume) and a brick size of 0.15–2.4 mm were established as the most appropriate immobilization condition. The

continuous operation with cell immobilization resulted in a steady butanol production of 8.71 g/L with an average productivity of 0.48 g/(L·h) for over 300 h, with a dilution rate of 0.054/h. However, when the dilution rate was increased to 0.108/h, butanol production decreased to 6.95 g/L while productivity increased to 0.71 g/(L·h) [6].

Butanol production by cell immobilization onto porous materials (brick and zeolite 13X) was investigated using *C. beijerinckii* TISTR 1461. After immobilization, both materials can enhance butanol titers from 5.29 to 5.80 g/L and 8.58 g/L using brick and zeolite, respectively. Butanol to glucose yield also improved from 0.14 to 0.16 g/g after immobilization. In addition, The repeated batch fermentation was performed to ensure its stability for long-term use (384 h) [33].

2.4 Silk cocoons

The silkworm cocoon silk fiber is composed of two cores of fibroin surrounded by a cementing layer of sericin in a structure known as a bave (each individual fibroin core is known as a brin). Fibroin and sericin account for about 75 and 25 wt%, respectively [31].

Silk fibers have evolved for a biological function, often within complex engineering structures such as silkworm cocoons, which have them evolved as tough composites to protect developing larva. Interest in silk cocoons in recent years stems from their combination of porous structure, mechanical strength, and potential for control of the gaseous environment inside the cocoon [32].

Cocoon shells were multilayered and porous structures constructed from highly cross-linked fibers that are densely packed within the sericin/gum. Fibers had fibrillar sub-structures running along the fiber axis and with greater number and size of voids [34].

A thin-shell silk cocoons (TSSC) was used as a support material for the immobilization of *Saccharomyces cerevisiae* M30 in ethanol fermentation. In batch fermentation with blackstrap molasses as the main fermentation substrate, an optimal ethanol concentration of 98.6 g/L was obtained using a TSSC-immobilized cell system

at an initial reducing sugar concentration of 240 g/L. The ethanol concentration produced by the immobilized cells was 11.5% higher than that produced by the free cells. Ethanol production in five-cycle repeated batch fermentation demonstrated the enhanced stability of the immobilized yeast cells. Under continuous fermentation in a packed-bed reactor, a maximum ethanol productivity of 19.0 g/(L·h) with an ethanol concentration of 52.8 g/L was observed at a 0.36/h dilution rate [8].

2.5 Factors affecting ABE fermentation

Bacterial growth requires a variety of nutrients, including carbon, nitrogen sources, vitamins, minerals, and trace elements. Most bacteria use organic compounds for their energy and carbon sources. These chemoheterotrophs usually use glucose and other sugars as the substrate for their growth, but methylotrophs can use simple one-carbon compounds, such as methanol and methane.

2.5.1 Carbon source

Economic analysis demonstrated that the fermentation substrate was one of the most important factors that influenced the price of butanol [35]. Since the cost of the fermentation substrate has the greatest influence on the price of butanol, some renewable and economically feasible substrates were investigated in the butanol fermentation production. As *Clostridium sp.* possesses strong amylase activities, they can effectively utilize starchy substrates without the need for hydrolysis. Molasses, whey permeate, cassava and Jerusalem artichokes have also been used as fermentation substrates for butanol production [35].

It has been reported for ABE fermentation of dilute sulfuric acid barley straw hydrolysates by *C. beijerinckii* P260, resulting in the production of 7.09 g/L acetone butanol ethanol (ABE), an ABE yield of 0.33, and productivity of 0.10 g/(L·h). BSH is toxic to the culture in order to reduce this potential toxicity effect, barley straw hydrolysates should be treated with lime [$Ca(OH)_2$] prior to ABE fermentation. The treated BSH resulted in a successful fermentation and ABE concentration of 26.64 g/L

was achieved, in which an ABE yield of 0.43 and productivity of 0.39 g/(L·h) was obtained. It should be noted that using lime treated BSH, a specific productivity of 0.55/h was obtained as compared to 0.12/h in the control fermentation suggesting that more carbon was directed to product formation [35].

Fermentation-based production of n-butanol and ethanol from corn or switchgrass through the liquid fuel yield in terms of the lower heating value (LHV) were compared. The result showed that the energy yield of n-butanol is about half that of ethanol from corn or switchgrass using current ABE technology. Low yield increases n-butanol's life-cycle greenhouses gas emission for the same amount of LHV compared to ethanol. A given fermenter volume can produce only about one quarter of the LHV as n-butanol per unit time compared to ethanol [2].

A hyperamylolytic *Clostridium beijerinckii* BA101 has an enhanced capability to utilize starch and accumulate higher concentrations of butanol (17–21 g/L) in the fermentation medium [36]. In addition to the use of corn, liquefied corn meal, and corn steep liquor (a byproduct of corn wet milling process that contains nutrients leached out of corn during soaking) were also tested for ABE production. In the batch process with recovery, 60 g/L of liquefied corn meal and corn steep liquor yielded of 26 g/L of solvent. Since the cost of the fermentation substrate has the greatest influence on the price of butanol, some other renewable and economically feasible substrates such as starch-based packaging materials, corn fiber hydrolysate, soy molasses, fruit processing industry waste, and whey permeate were investigated for butanol fermentation. The total solvents produced from these alternative renewable resources ranged from 14.8 to 30.1 g/L [37]. Among the total polysaccharides, researchers focus on celluloses and hemicelluloses which are the most abundantly renewable and available resources on the planet. A number of cabohydrates have been used for butanol production when using *Clostridium beijerinckii* BA101 in batch fermentation, the detail data is shown in Table 1 [1].

Table 1 Production of butanol from different substrates using *C. beijerinckii* [1].

Fermentation parameters	Fermentation substrates								
	Glucose	Corn starch	Maltodextrins	Soy molasses	Agricultural waste	Packaging peanuts	Wheat bran	Corn fiber	DDGC
Acetone (g/L)	4.30	7.70	6.80	4.20	4.80	5.70	2.20	NC	2.70
Butanol (g/L)	19.60	15.80	18.60	18.30	9.80	15.70	8.80	NC	8.30
Ethanol (g/L)	0.30	1.20	0.70	0.30	0.20	0.30	0.80	NC	0.50
Total ABE (g/L)	24.20	24.70	26.10	22.80	14.80	21.70	11.80	0.00	11.50
ABE productivity (g/L h)	0.30	0.34	0.37	0.19	0.22	0.20	0.16	0.20	0.16

2.5.2 Vitamins and minerals

Some mineral salts, ammonium acetate, and glucose affected acetone and butanol fermentation by *C. acetobutylicum*. The vitamin requirements of *C. acetobutylicum*, *p*-Aminobenzoic acid and biotin were required for normal cell growth and for a good production of solvents at the respective concentrations of 1 and 0.01 mg/L. The vitamin is present in the yeast extract [38]. It can be replacing both vitamins B and folic acid with yeast extract.

2.5.2.1 Yeast extract: Yeast extracts (YEs) are frequently used as fermentation nutrient ingredients as nitrogen and vitamin source [38].

2.5.2.2 Magnesium: Cell growth was dependent on the presence of Mg^{2+} in the medium. When no $MgSO_4$ was added, growth was limited, but butanol and acetone were released in the medium [37].

2.5.2.3 Iron: Cell growth was dependent on the presence of Fe^{2+} in the medium. When only 1 mg/L of $FeSO_4$ was supplied to the bacteria, growth was good, the conversion of glucose was complete, and the production of solvents was normal. No differences were observed with concentrations of $FeSO_4$ between 1 and 50 mg/L [37].

2.5.2.4 Potassium: To investigate the influence of the K^+ concentration on fermentation without affecting the buffering actions of KH_2PO_4 and $K_2HP0_4{\cdot}3H_20$, these phosphate salts were substituted for by $NaH_2PO_4{\cdot}2H_20$ and $Na_2HP0_3{\cdot}12H_20$ to obtain an equivalent buffering effect. K^+ was supplied as KCl. The concentration of 600 mg/L of KCl was equivalent (for K^+ requirements) to 1 g/L of potassium phosphates. Cell growth increased with concentrations from 0 to 60 mg/L and then stayed at about the same level. The degradation of glucose regularly increased with increasing concentrations of KCl up to 15 mg/L, where the utilization of sugar was complete. At higher concentrations, no variation of the residual sugar concentrations was found (about 20 g/L utilized). Solvent yields became higher and higher with concentrations from 0 to 9 mg/L, reaching a maximum and then becoming constant. The production of solvents was not changed with concentrations from 0.6 to 8 g/L (6 g/L of solvents), but was slightly inhibited at 12 g/L of KCl (5.16 g/L of solvents) [37].

2.5.2.5 Ammonium acetate: the addition of ammonium acetate at concentrations of >2.2 g/L had deleterious effects on the production of solvents. Upon increasing the ammonium acetate concentration, an accumulation of acids (butyric) was observed. Thus, there was a good parallelism between the decreasing production of solvents and the increasing production of acids. No growth occurred in the absence of ammonium acetate [37].

2.5.2.6 Glucose: At concentrations of 5 and 10 g/L, only acetic and butyric acids were produced. At 20 g/L, two phases could be distinguished: (i) the formation of acids, and (ii) the disappearance of these acids concurrent with the formation of solvents. At 40 and 80 g/L, the two stages were more pronounced since the conversion of acids to solvents was almost complete and final levels of acetic and butyric acids were very low (traces of butyrate). A total of 2% of solvents were present in the medium at the glucose concentration of 80 g/L (the conversion of glucose to solvents was 31%) [37].

In summary, a medium of the following composition was suggested: $MgSO_4$, 50 to 200 mg/L; KCl (K is to be supplied as KH_2PO_4 and K_2HPO_4), 0.015 to 8 g/L; $FeSO_4$, 1 to 50 mg/L; CH_3COONH_4, 1.1 to 2.2 g/L; glucose, 20 to 60 g/L [37].

2.5.3 Temperature in the fermentation

The effect of temperature in ABE fermentation production by *Clostridium acetobutylicum* ATCC 824 has been reported. The optimum temperature for microbial growth and microbial solvent production in range of 30-37 ^{o}C [39, 40].

2.5.4 pH in the fermentation

Studies in batch and continuous culture do enable us to compile a number of observations regarding the physiological response to various environmental conditions. It is generally recognized that pH influences the outcome of a batch culture. At high pH, mainly acids are produced; whereas at low pH, solvents dominate. The optimum pH for solvent production varies with strain and culture conditions. *C. acetobutylicum* ATCC 824 strains rarely produce solvents above pH 6, but good solvent production can be achieved in the pH range of 4.3-5.5 (Isar, J. and V. Rangaswamy, Huesemann, M.H., et al.) [18, 25].

2.5.5 Heat shock

For efficient ABE fermentation, it is imperative to heat shock (to induce expression of heat shock proteins that enhance butanol tolerance and production) the solventogenic clostridia in media containing the representative carbohydrates that are present in the fermentation medium [19]. *C. acetobutylicum* ATCC 824 was heat shocked for 10 min at 70-80^{o}C before cooling on ice [20, 25, 41].

2.5.6 Influence of agitation

The effects of agitation on ABE production was investigated. With the increase of agitation from 0 to 150 rpm. The ABE yield increased gradually from 0.35 to 0.39 g/g-sugar, and then increased slightly more with a further increase of agitation rate. However the butanol/acetone ratio decreased dramatically from 2.8 to around 1.5 with the increase of agitation. The decreased fraction of butanol in the total ABE mixture led

to a decline in butanol yield (from a peak at 48 rpm) with the increase of agitation rate [40].

2.5.7 Anaerobic fermentation

Anaerobic fermentation occurs in the fermentation vessel once the oxygen is discharged and replaced with N_2, CO_2, or another by-product of the fermentation process. Anaerobic fermentation is usually a slower process. In the mid-1850s, the French chemist Louis Pasteur produced anaerobiosis by boiling the medium to drive out oxygen and then introducing inert gas for cultivation. He showed that a microorganism, probably *C. butyricum*, was responsible for butyric acid fermentation. In the 1960s and 1970s, anaerobic chambers were invented that allowed the cultivation of numerous anaerobic cultures for certain strictly anaerobic organisms, including *C. botulinum*. During World War I, industrial anaerobic fermentation was further demonstrated by Perkins and Weizmann, who worked on ABE fermentation with *C. acetobutylicum* [42].

2.5.8 Sugar consumption

Glucose and fructose started to be degraded right after inoculation of the culture, while sucrose utilization began 14 h after the start of fermentation. Previously, it was reported that during the fermentation process, approximately 70% of the glucose and fructose were utilized, while only about 50% of the sucrose was consumed. The results suggested that the monosaccharide glucose and fructose are more preferable for ABE production by *C. beijerinckii*, but the microorganism also has a good capability to degrade sucrose [40].

2.5.9 Nitrogen source

The source and content of nitrogen in the medium are very important in the development of alcoholic fermentations [43]. Although some amino acids can be used as sole carbon/nitrogen sources, Clostridia mainly prefer to ferment amino acids in pairs

within the so-called Stickland reaction [44]. Often the end- or by-product of the decomposition process is NH_3/NH_4^+, which can be used by various microorganisms directly as a nitrogen source and which additionally affects the buffer system of surrounding media [45].

2.5.10 Osmotic pressure

Osmotic pressure has been known to exert profound influence on cell growth and its metabolism [46]. It is the pressure which needs to be applied to a solution to prevent the inward flow of water across a semipermeable membrane. It is also defined as the minimum pressure needed to nullify osmosis.

The osmotic pressure π of an ideal solution with low concentration can be approximated using the Morse equation [27].

$$\pi = iMRT$$

Where, i = the dimensionless Van't Hoff factor

M = the molarity

R = 0.08205746 L atm K-1 mol-1 is the gas constant

T = the thermodynamic (absolute) temperature

The osmotic pressure of a solution increases with solute concentration (M) and with solution temperature (T). This equation gives the pressure on one side of the membrane; the total pressure on the membrane is given by the difference between the pressures on the two sides. The similarity of the above formula to the ideal gas law and also that osmotic pressure is not dependent on particle charge. This equation was derived by Van't Hoff.

CHAPTER III EXPERIMENTAL DETAILS

3.1 Equipment

3.1.1 Autoclave SS-325, TOMY, Japan

3.1.2 Centrifuger Kubota 5100 apan, Kubota Corporation, Japan

3.1.3 Controlled Environment Incubator Shaker New Brunswich Scientific Co., U.S.A.

3.1.4 Elemental Analyzer (EA) (CHNS/O Analyzer), Perkin Elmer PE2400 Series II

3.1.5 Fermentor 1 liter Biostat ®, B Braun Biotech International, Germany

3.1.6 Gas chromatography (GC-7AG, Shimadzu, Japan)

3.1.7 Hot Air Oven ULM 500, Memmert, Germany

3.1.8 Industrial Analyzer YSI Model 27 Yellow Springs Instrument Co., U.S.A.

3.1.9 pH Meter MP220, Mettler Toledo, Switzerland

3.1.10 Scanning electron microscope, JOEL (Tokyo, Japan), JSM-5410LV

3.1.11 Spectrophotometer UV-2450, Shimadzu, Japan

3.1.12 Stirrer RW 20 ZM.n. lka labortechnik, Germany

3.1.13 Vertical Laminar flow VS-124, ISSCO, U.S.A.

3.2 Chemical

3.2.1 Acetic acid (May & Baker, British)

3.2.2 Acetone (May & Baker, British)

3.2.3 Ammonium acetate (CH_3COONH_4) (Ajax Finechem, Australia)

3.2.4 Ammonium hydroxide (NH_4OH) (May & Baker, Britis)

3.2.5 Butyric acid (Merck)

3.2.5 Dipotassium phosphate (K_2HPO_4) (Ajax Finechem, Australia)

3.2.6 Ethanol (May & Baker, British)

3.2.7 Hydrochloric (HCl) (BDH laboratory, England)

3.2.8 Iron (II) sulfate ($FeSO_4 \cdot H_2O$) (Ajax Finechem, Australia)

3.2.9 Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$) (Ajax Finechem, Australia)

3.2.10 Manganese (II) sulfate ($MnSO_4 \cdot 7H_2O$) (Ajax Finechem, Australia)

3.2.11 Sugarcane juice without preservatives from Bon Marche market

3.2.12 Monopotassium phosphate (KH_2PO_4) (Ajax Finechem, Australia)

3.2.13 n-Butanol (May & Baker, British)

3.2.14 Potassium Sodium tartrate (K-Na tartrate)

3.2.15 Reinforced Clostridial Medium (Himedia, India)

3.2.16 Sodium Chloride (NaCl) (Ajax Finechem, Australia)

3.2.17 Sodium Hydroxide (NaOH) (Ajax Finechem, Australia)

3.2.18 Sucrose (Ajax Finechem, Australia)

3.2.19 Sulfuric acid (H_2SO_4) (Fisher Chemical)

3.2.20 Yeast extracts (Himedia, India)

3.2.21 3,5-dinitrosalicylic acid powder

3.3 Microorganisms used in fermentation

Clostridium acetobutylicum ATCC 824

3.4 The Fermentation Process

3.4.1 Preparation of strain and medium

Step 1 Preparation of strain

The bacterial strain used in this study was *C. acetobutylicum* ATCC 824. The stock culture was stored as frozen spore suspension. In order to prepare the seed culture, the 2 ml of the stock culture was aseptically transferred to 18 ml of reinforced clostridial medium (RCM) in a glass tubes (diameter, 3 cm; length, 15 cm). The culture medium was kept anaerobic by bubbling sterile filtered nitrogen for 15 min and then the culture was heat-shocked at 70-80^{o}C for 5-10 min, followed by cooling in the inoculation chamber for 5 min. After that it was incubated at 35°C for 48 hours. It was then applied as the seed culture. The sub-culture of 2 ml was inoculated into a glass tube (3 cm OD x 15 cm high) containing 18 ml of reinforced clostridial medium (RCM) and incubated for 115 rpm, 48 h at 35 ^{o}C.

Step 2 Preparation of medium in flask

The pre-culture medium of 60 ml Erlenmeyer flask was then added with the seed culture at a ratio of 10% (v/v) and incubated in an incubation shaker (Controlled Environment Incubator Shaker New Brunswich Scientific Co., U.S.A.) at 35°C, 115 rpm for 48 hours. The pre-culture medium contained 40 g/L glucose, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KH_2PO_4, 3 g/L CH_3COONH_4 and 8.4 g/L Yeast extract. The pH of the culture medium was adjusted to 4.5-5.0 prior to sterilization in an autoclave. All chemicals used in this work were analytical grade and were purchased from local suppliers in Thailand.

Step 3 Preparation of medium in reactor

The fermentation medium was then inoculated with seed culture at a ratio of 10% (v/v) [47]. The fermentation medium contained the following components (per

600 ml): carbon source, glucose or sugarcane juice 80 g/L; the following components 0.006 g $FeSO_4 \cdot 7H_2O$, 0.18 g $MgSO_4 \cdot 7H_2O$, 0.3 g KH_2PO_4, 1.8 g CH_3COONH_4, and 5.046 g yeast extract ($FeSO_4 \cdot 7H_2O$ 0.01 g/L, $MgSO_4 \cdot 7H_2O$ 0.3 g/L, KH_2PO_4 0.5 g/L, CH_3COONH_4 3 g/L, yeast extract 8.4 g/L) [27]. The pH and temperature were adjusted controlled to 4.5-5 and 35°C prior to sterilization.

3.4.2 Screening of carriers for cell immobilization

Carriers was thin-shell silk cocoons (TSSCs), it was evaluated for ABE production and compared to that of free cells. The fermentation and cell immobilization processes were performed simultaneously. In the batch of cells immobilized on TSSCs, 3 g of TSSCs was placed into a reactor.

3.4.3 Cell immobilization on thin-shell silk cocoons and the repeated batch fermentation process

Thin-shell silk cocoons (TSSCs) were obtained from the Queen Sirikit Department of Sericulture (Saraburi). Before use, each TSSC was cleaned and placed into a reactor containing 600 ml of fermentation medium. The repeated batch operation was carried out in a glass bottles containing 600 ml of fermentation medium for each batch. The product sample was removed via sterilized silicone tube at the end of each cycle. After the removal all of the supernatant, 600 ml of fresh fermentation medium was poured into the glass tubes in order to commence the next cycle. Each repeated batch received 48 h of cultivation under anaerobic conditions.

3.5 Analytical methods

Concentrations of acetone, butanol, and ethanol were determined using gas chromatography (GC-7AG, Shimadzu, Japan) equipped with a flame-ionization detector. Residual sugar content was measured from the absorbance at 590 nm using the 3,5-dinitrosalicylic acid (DNS) method [8] with an Spectrophotometer UV-2450 Shimadzu, Japan. Free cell dry weight was determined from the absorbance at 650 nm

measured by a UV-2450 UV–Visible spectrophotometer (Shimadzu Scientific Instruments, Inc., Japan) and converted to dry cell concentration using a corresponding standard curve. For cell dry weight determination, a 5 ml sample of the fermentation broth was centrifuged at 2000 rpm for 20 min. The cell pellet was resuspended in distilled water and washed twice with distilled water, dried at 85 ^{o}C for 24 hours and then weighed. A series of SEM images was taken to provide a visual characterization of the cell carrier. The samples were frozen in liquid nitrogen, immediately snapped, vacuum-dried, sputtered with gold and then photographed. Images were collected on a JOEL (Tokyo, Japan) JSM-5410LV scanning electron microscope. Nitrogen content was determined on an Elemental Analyzer (EA) (CHNS/O Analyzer), Perkin Elmer PE2400 Series II. Sucrose, fructose and glucose were measured by the high-performance liquid chromatography (HPLC) instrument equipped with an UV, Fluorescence, Refractive Index, Conductivity, and Evaporative Light Scattering Detectors, Shimadzu, LC-3A, LDC 4100, 2000 ES, (Japan), using Prevail carbohydrate Column size 250 mm x 4.6 mm (ID) with column temperature of 25 ^{o}C, 5 μL sample injection volume. The mixture of acetonitrile (69%) and water (31%) was used as mobile phase at flow rate of 0.9 mL/min.

CHAPTER IV RESULTS AND DISCUSSION

4.1 Thin-shell silk cocoon (TSSC) as a nitrogen source of ABE fermentation by *Clostridium acetobutylicum*

Cost of the substrate is an important part of the overall cost of bio-butanol production. Inexpensive and easy-degradable feedstock is desirable for the ABE fermentation [48]. Thailand is a tropical country and sugar cane is cultivated in various parts of the country. Sugar cane (*Saccharum officinarum*) is a high biomass tropical crop. It contains 12–17% total sugars, of which 90% is sucrose and 10% is glucose and/or fructose [48]. In addition, the nitrogen sources play an important role in the fermentative production of acetone, butanol and ethanol by *C. acetobutylicum*. The organism prefers utilization of natural organic nitrogen sources for the fermentative production of acetone, butanol and ethanol [48]. In the present study, a thin-shell silk cocoon (TSSC), a residual from the silk industry was investigated for the use as a cheap substitute of yeast extract in the ABE fermentation.

4.1.1 Screening of different nitrogen sources

Yeast extracts (YEs) are frequently used as fermentation nutrient ingredients [38]. It is commonly used as a source of amino acids and vitamins, and as a food additive [49]. Moehn *et al*. [50] reported that YE comprises of a high content of the main amino acids, such as lysine, alanine, leucine and glutamic acid is shown in Table 3.

Silk cocoon is composed of two cores of fibroin surrounded by a cementing layer of sericin in a structure known as a bave (each individual fibroin core is known as a brin). Fibroin and sericin account for about 75 and 25 wt%, respectively [31]. Fibroin and sericin comprises of a high content of the main amino acids containing Alanine, Glysine and Serine [51] are shown in Table 3. Silk fibers have evolved for a biological

function, often within complex engineering structures such as silkworm cocoons, which have them evolved as tough composites to protect developing larva. Interest in silk fiber in recent years is due to its combination of porous structure, mechanical strength, and potential for control of the gaseous environment inside the cocoon [52]. A thin-shell silk cocoon (TSSC), a residual from the silk industry was used as a N-source for ABE fermentation in this study. TSSC was obtained from the Queen Sirikit Department of Sericulture (Saraburi, Thailand). Before using, TSSC was cut into very small pieces, which herein referred to as "$TSSC_f$".

Nitrogen, carbon and hydrogen content in $TSSC_f$ compared to those in yeast extract are shown in Table 2. $TSSC_f$ has a significantly higher content in the nitrogen as compared to YE. Since TSSC is a residual cocoon left over after removing silk fibers, $TSSC_f$ is cheap. $TSSC_f$ is also simple to prepare and use, non-toxic and high biocompatibility.

Table 2 Nitrogen, carbon and hydrogen content in yeast extract (YE) and thin shell silk cocoon fragment ($TSSC_f$) determined by CHN analysis.

N-source	Carbon [%]	Hydrogen [%]	Nitrogen [%]
Yeast extract	33.53	6.06	10.04
$TSSC_f$	43.64	6.44	16.13

Table 3 Composition of the amino acids (%) in yeast extract (YE) and thin shell silk cocoon (TSSC) from previous reports.

Amino acid	% Amino acid	
	YE [*1]	TSSC [*2]
Lysine	8.3	0.2
Alanine	9.1	29.4
Glycine	5.1	44.6
Serine	4.9	12.1
Tyrosine	2.4	5.2
Leucine	7.9	0.7
Aspartic acid	10.1	1.3
Arginine	5.2	0.5
Glutamic acid	16.8	1.0
Phenylalanine	4.0	0.1
Threonine	4.6	0.9
Valine	6.0	2.4
Cysteine	0.8	0.1
Histidine	2.1	0.1
Proline	4.2	0.8
Isoleucine	5.8	0.8
Methioine	1.4	0.2
Tryptophan	1.3	-

[*1] LABORATORIOS CONDA, S.A.CAT No:1702, www.condalab.com

[*2] Takasu et al., 2002

4.1.2 The batch fermentation process

The ABE fermentations were carried out by *C. acetobutylicum* ATCC 824 under batch fermentation using sugar cane juice as the main substrate at the total sugar concentration of 80 g/L. The composition of the fermentation medium was similar to the pre-culture medium except for the C- and N-sources. The experiment was initiated by transferring the precultures at a ratio of 10% (v/v) into a 1-L glass fermenter (Biostat Q®, B Braun Biotech International, Germany) containing 600 ml fermentation medium and the system was purged under nitrogen at a rate of 0.1 vvm for 15 min in order to promote anaerobic condition. The agitation speed was controlled at 200 rpm and the pH was adjusted to 4.5-5.0 by automatic addition of 4 N NaOH solution. The incubation temperature was 35°C. The study was conducted with different nitrogen sources as follows. YE at the concentration of 8.41 g/L was used as a nitrogen source in the control. To investigate the use of TSSC as an alternative nitrogen substitute, instead of using YE as a sole N-source, $TSSC_f$ at 5.27 g/L and the mixture of YE (4.21 g/L) and $TSSC_f$ (2.64 g/L) were examined as N-sources. Therefore, the N-source in each system was prepared by having the same total amount of nitrogen content at 0.84 g/L as shown in Table 4.

Table 4 The different organic nitrogen sources: YE, $TSSC_f$ and combine source (YE+$TSSC_f$) supplemented in the ABE fermentation system. The same total nitrogen content in each batch was resulted.

System	Organic N-source		Total N-content (g/L)
	YE (g/L)	TSCf (g/L)	
Control	8.41	-	0.84
Experiment 1	-	5.27	0.84
Experiment 2	4.21	2.64	0.84

Acetone, butanol and ethanol (ABE) fermentations were performed by *C. acetobutylicum* ATCC 824 with different of nitrogen sources in anaerobic condition under controlled temperature at 33-37 °C and controlled pH at 4.5-5.0. Sugarcane juice was used as the main carbon source for the ABE fermentation. Sugarcane juice had on average 12-14% total sugar content, which had to be diluted to the solutions with total reducing sugars of 80 g/L for the ABE fermentation. Previously, the optimum initial sugar concentration for ABE fermentation by the suspended culture of *C. acetobutylicum* ATCC 824 using sugarcane juice medium was 80 g/L [53]. To investigate the use of $TSSC_f$ as an alternative N-source for the ABE fermentation, the N-source in each system was prepared by using the same total amount of nitrogen content, but it was provided by different sources as follows: (1) YE, (2) $TSSC_f$ and (3) the mixture of YE and $TSSC_f$. The fermentations were carried out in 600 mL stirred tank fermenter in batch operation.

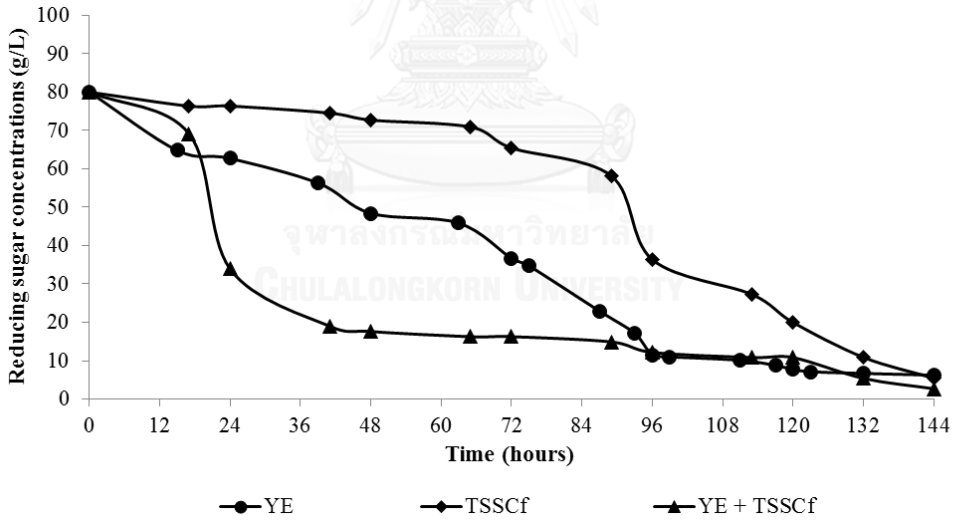


Figure 2 Sugar consumptions of *C. acetobutylicum* ATCC 824 in the presence of different organic nitrogen sources: YE (●), $TSSC_f$ (◆), and combine source ($YE+TSSC_f$) (▲) throughout 144 h of ABE fermentation.

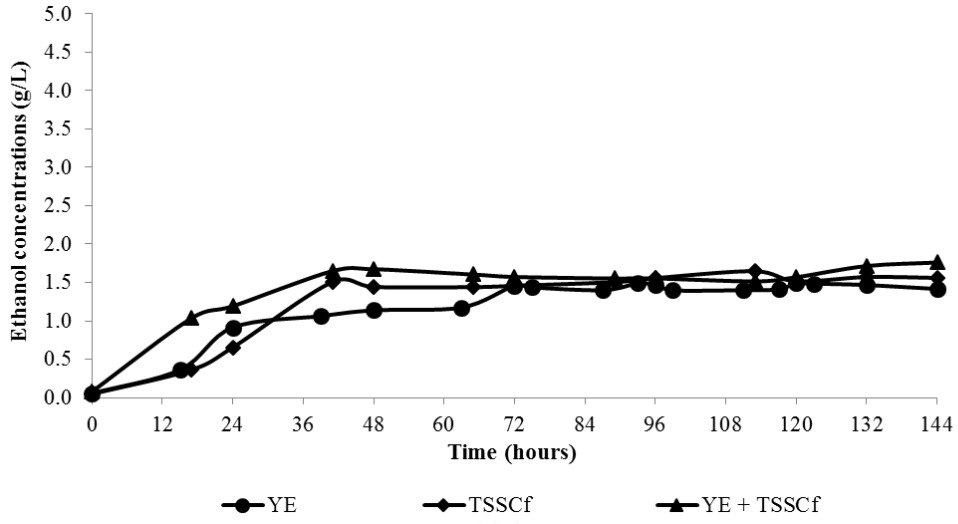


Figure 3 Ethanol productions of *C. acetobutylicum* ATCC 824 in the presence of different organic nitrogen sources: YE (●), $TSSC_f$ (◆), and combine source ($YE+TSSC_f$) (▲) throughout 144 h of ABE fermentation.

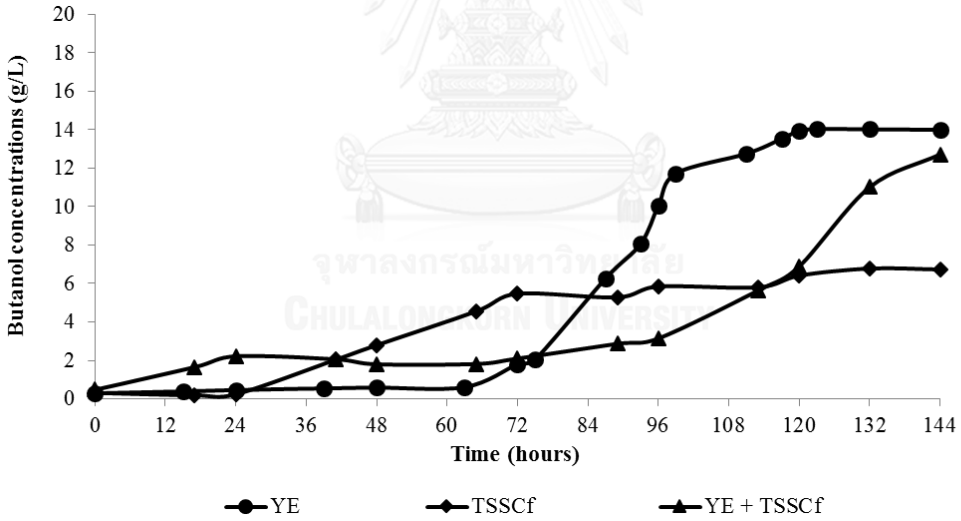


Figure 4 Butanol productions of *C. acetobutylicum* ATCC 824 in the presence of different organic nitrogen sources: YE (●), $TSSC_f$ (◆), and combine source ($YE+TSSC_f$) (▲) throughout 144 h of ABE fermentation.

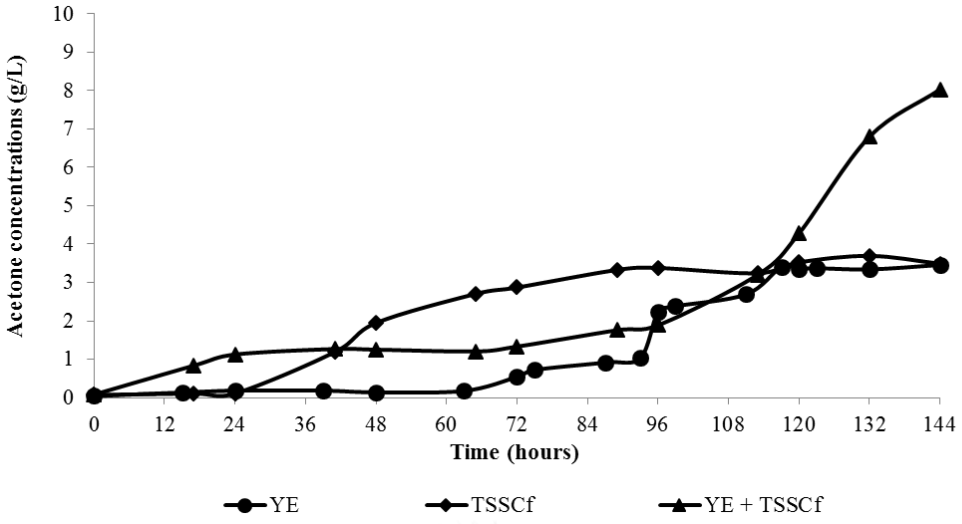


Figure 5 Acetone production of *C. acetobutylicum* ATCC 824 in the presence of different organic nitrogen sources: YE (●), $TSSC_f$ (◆), and combine source (YE+$TSSC_f$) (▲) throughout 144 h of ABE fermentation.

The results showed that the *C. acetobutylicum* ATCC 824 could use $TSSC_f$ and the mixture of YE and $TSSC_f$ as N-sources. However, the cell metabolisms in the system with different N-sources were quite different. From the comparison of the sugar consumption rate as shown in Figure 2, the rate of utilization of sugar was much faster in the system using the mixture of YE and $TSSC_f$, which was greater than that from the systems using YE and $TSSC_f$, respectively. However, after 144 h of the fermentation, almost all of the sugars in all systems were utilized (with residual sugar concentration of about 5-6 g/L). The result indicated that the use of the mixture of YE and $TSSC_f$ might promote the cell metabolism.

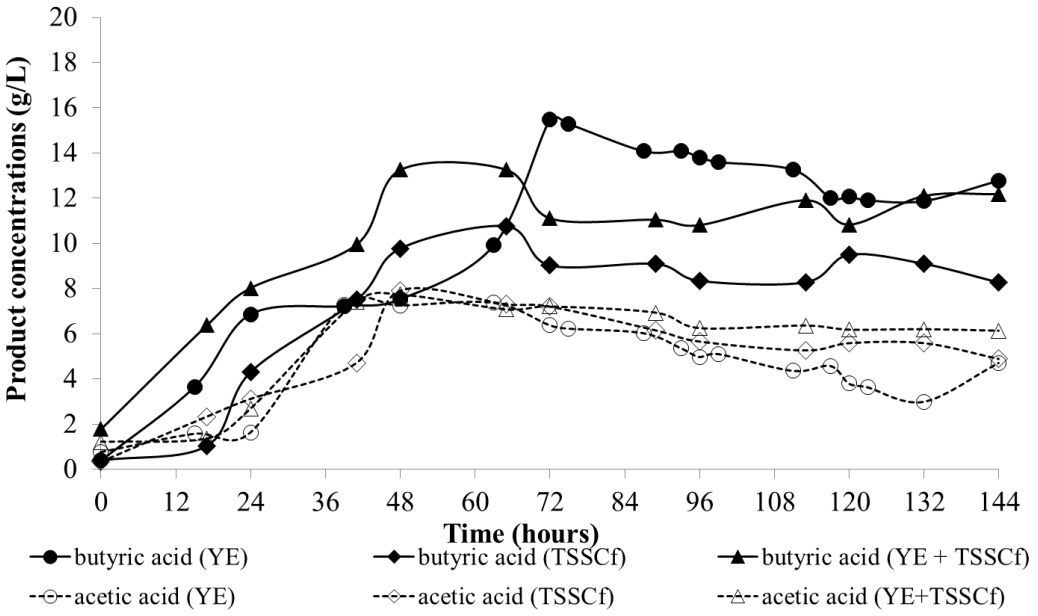


Figure 6 Butyric acid (—) and acetic acid (---) productions of *C. acetobutylicum* ATCC 824 in the presence of different organic nitrogen sources: YE (●), $TSSC_f$ (◆), and combine source (YE+$TSSC_f$) (▲) throughout 144 h of ABE fermentation.

According to Figure 6, acid production was dramatically increased in the first 48 hours of the fermentation. After that, the production yield will be gradually decreased, which is similar to the results of butanol and acetone production (from Figure 4 and Figure 5).

At the end of the fermentations, *C. acetobutylicum* ATCC 824 in all systems were mostly in the form of rod-shaped cells as shown in Figure 7. The relatively larger cells were observed in the system using $TSSC_f$ as N-source.

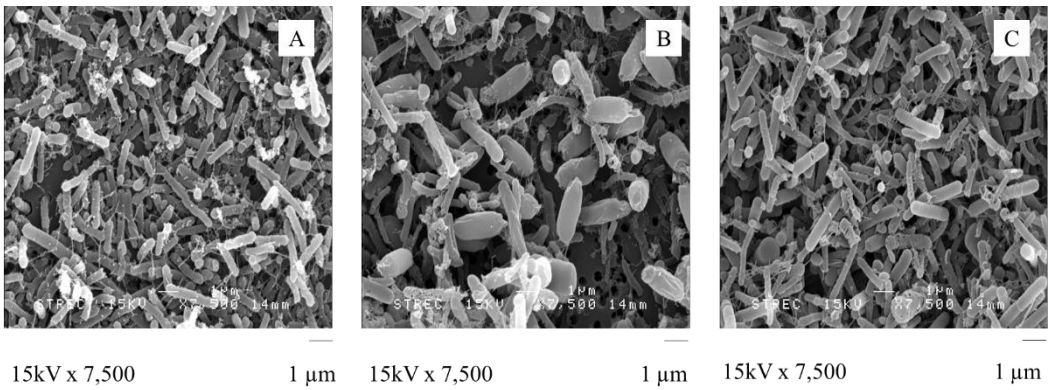


Figure 7 SEM image of *C. acetobutylicum* ATCC 824 in the systems using: (A) YE, (B) $TSSC_f$ and (C) the mixture of $TSSC_f$ and YE.

Previously, it was reported that YE could promote phase shift of bio-butanol fermentation by *C. acetobutylicum* ATCC 824 using cassava as substrate [26]. In this study, although the total amount of the initial nitrogen content and the other controlled conditions were the same, the butanol and acetone productions in each system were significantly different. The maximum butanol concentration was obtained at 14.0 g/L in the system using YE as a sole N-source, whereas the final butanol concentration in the system using the mixture of YE and $TSSC_f$ was 12.7 g/L, and only 6.7 g/L of butanol was obtained in the system using $TSSC_f$ without YE. However, after 144 h of the fermentation, the maximum acetone production and the maximum total solvent of 22.30 g/L (composed of 12.72 g/L butanol, 8.03 g/L acetone and 1.50 g/L ethanol) were obtained in the system using the mixture of YE and $TSSC_f$ as N-sources, in which the sugar consumption, ABE productivity and conversion yield were 74.58 g/L, 0.15 g/L/h and 0.30 g ABE/g sugar consumption, respectively. Therefore, it was found in this study that TSSC might help promote cell growth and phase shift of acetone in the ABE fermentation by *C. acetobutylicum* ATCC 824. Effect of different nitrogen source on the ABE production, productivity, yield and conversion from this work by *C. acetobutylicum* ATCC 824 was summarized in Table 5.

Table 5 The ethanol, acetone, butanol production, productivity, yield and conversion of *C. acetobutylicum* ATCC824 in ABE fermentation with different organic N-source. The initial sugar concentrations of 80 g/L.

Organic N-source	Time (h)	Concentrations (g/L)			
		Ethanol	Acetone	Butanol	Total ABE
YE	144	1.41	3.45	14.00	18.86
$TSSC_f$	144	1.47	3.49	6.73	11.69
$YE+TSSC_f$	144	1.50	8.03	12.72	22.30

Organic N-source	Sugar consumption (g/L)	Productivity (g/L·h)	Yield	Conversion
YE	73.74	0.13	0.26	0.24
$TSSC_f$	74.55	0.08	0.16	0.15
$YE+TSSC_f$	74.58	0.15	0.30	0.28

4.2 Enhanced acetone-butanol production from sugarcane juice by immobilized *Clostridium acetobutylicum* ATCC 824 on thin-shell silk cocoons

This research aims to increase the productivity and efficiency of ABE fermentation by an immobilization technique. In practice, the success of immobilized cell (IC) cultures depends upon the supporting materials and immobilization methods. A suitable cell support will ensure a simple, economical and energy-saving physical adsorption process. Previously, we immobilized *Saccharomyces cerevisiae* M30 on a thin-shell silk cocoon (TSC), a residual from the silk industry, and improved the activity and stability of ethanol production during the fermentation process [8]. TSC has a special microstructure with high porosity and high surface area, is mechanically strong and chemically stable. Additional advantages of TSC are its biocompatibility, light weight and low cost. The silk filament of TSC is composed of two proteins: fibroin and sericin. Fibroin is rich in the amino acids glycine and alanine, whereas sericin contains

no less than 18 amino acids, including essential amino acids, and is especially rich in serine [8, 54, 55]. On account of these advantageous properties, TSCs are promising cell carriers for enhancing the productivity of many fermentation processes. Therefore, in this work, we immobilized the ABE fermenter *C. acetobutylicum* (ATCC 824) on TSCs during batch and repeated batch fermentation, using sugarcane juice as the main substrate, and determined the optimal initial sugar concentration for ABE production. We discuss the ABE production performance and the process stability of cyclic batch operation using IC on a TSC (IC-TSC). To our knowledge, we report the first use of TSCs as an immobilized cell support for improved ABE fermentation by Clostridium cells.

4.2.1 Cell immobilization and fermentations

Thin-shell silk cocoons obtained from the Queen Sirikit Department of Sericulture (Saraburi, Thailand) were used as the immobilization material. Prior to use, impurities on the outer shell of the TSCs were removed by hand. The carbon source was sugarcane juice, provided at total reducing sugar concentrations of 80, 90, and 100 g/L. Apart from the additional carbon source, the fermentation medium contained the same ingredients as the pre-culture medium. To prepare the ABE fermentation by the immobilized cells on TSCs, 3 g of TSCs were added to 600 mL of fermentation medium in a 1 L glass fermenter (Biostat®, B Braun Biotech International, Germany) prior to sterilization. Fermenter flasks containing 600 mL fermentation medium with and without TSCs were then sterilized by autoclaving for 15 min at 121°C and were cooled to room temperature (30°C) before use. The experiment was initiated by transferring 10% (v/v) of the pre-cultures into the fermenter and purging the fermentation medium with nitrogen (0.1 vvm) for 15 min to establish anaerobic conditions. The fermentations were carried out in duplicate under 200 rpm agitation at 35°C, and the pH was controlled to 4.5 ~ 5.0 by automatic addition of 4 N NaOH solution.

The repeated 4-cycle IC-TSC batch fermentation was set up in a 1-L fermenter flask containing 600 mL fermentation medium. The main ABE fermentation substrate was sugarcane juice, supplied at an initial reducing sugar concentration of 80 g/L. The

first batch was continued for 72 h; subsequent batches were each conducted for 48 h. At the end of each batch, the fermentation broth was removed from the fermenter and replaced with the same volume of fresh medium, followed by purging with nitrogen. All fermentations were performed at 35°C and 200 rpm, with the pH controlled at 4.5 ~ 5.0.

In a previous section (4.1), we also cultivated *C. acetobutyricum* ATCC 824 in medium containing small fragment TSC (TSCf), to assess whether TSC is an effective nitrogen supplement in ABE fermentation. This study was conducted in batch mode for 132 h at 35°C and pH 5.0. The medium was sugarcane juice with a total reducing sugar concentration of 80 g/L. Control cultures containing 8.4 g/L yeast extract (YE) as the sole organic N-source were also prepared. The experimental cultures were reared in (i) 5.3 g/L TSCf and (ii) a mixture of YE (4.2 g/L) and TSCf (2.6 g/L).

4.2.2 Effects of thin-shell silk cocoons as a nitrogen source

The nitrogen, carbon and hydrogen contents of TSC and yeast extract (YE) are compared in Table 2. Besides being significantly richer in nitrogen than YE, TSC is a cheap N source because it constitutes the residual cocoon with the silk fibers removed. It is also simple to prepare, non-toxic and biocompatible. Table 6 compares the results of the preliminary experiment in terms of the ABE productivity. After 132 h fermentation, almost all of the sugars in the YE-only and the mixed YE–TSCf systems were utilized. These systems yielded comparable ABE concentrations (about 19 g/L) and ABE productivities (0.14 ~ 0.15 g/L/h).

However, in the TSCf system (lacking YE), the sugar consumption and total ABE production were relatively low. From the ratio of acetone to butanol in the broth, it appears that during ABE fermentation by *C. acetobutylicum* ATCC 824, YE and TSC promote a phase shift to biobutanol and acetone, respectively. This result demonstrates the utility of TSC as a partial substitute for organic N sources in ABE fermentation.

Table 6 Preliminary results of ABE fermentation supplemented with fragments of thin-shell silk cocoon (TSCf) as an alternative N source to yeast extract (YE).

N-source	Residual sugar (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Total solvent (g/L)	Productivity (g/L/h)
YE	6.5	3.3	14.0	1.4	3.0	11.9	18.7	0.14
$TSSC_f$	16.4	3.7	6.8	1.6	5.6	9.1	12.0	0.09
$YE+TSSC_f$	5.4	6.8	11.0	1.5	6.2	12.1	19.3	0.15

4.2.3 Suspended cells vs. immobilized cells on thin-shell silk cocoons

Encouraged by the above result, we further investigated ABE batch fermentation using immobilized *C. acetobutylicum* (ATCC 824) on TSC (IC-TSC). The ABE production and reducing sugar consumption in the IC-TSC system were compared with those of suspended cells (SC). In both systems, the main carbon source was sugarcane juice (initial sugar concentration = 80 g/L). In our previous studies, the optimum initial sugar concentration for ABE fermentation by *C. acetobutylicum* (ATCC 824) suspended in glucose and sugarcane juice media was 60 and 80 g/L, respectively [53]. ABE fermentation is inhibited at initial glucose concentrations exceeding 60 g/L [53, 56]. At the same mass concentration (g/L), glucose solution exerts greater osmotic inhibition than sucrose solution. The sugarcane juice used in this study comprised 91.2% sucrose, approximately 4.5% glucose and 4.4% fructose. Therefore, ABE production could be enhanced by cultivation in sugarcane juice medium, which exerts no inhibitory effect at 80 g/L [53].

Conventional ABE batch fermentation seldom yields more than 12 ~ 20 g/L of solvents and the fermentation time is limited to approximately 2 ~ 6 days by the solvent toxicity. Therefore, the quantity of sugar that can be fermented is limited [6, 25, 53]. Low productivity is also contributed by the low concentration of cells in the bioreactor. In typical ABE batch fermentations, the cell concentration rarely exceeds 3 g/L. Therefore, suitable cell immobilization improves the productivity by increasing the cell

concentration in the reactor. In this work, *C. acetobutylicum* (ATCC 824) cells were immobilized by adsorbing them to TSC surfaces (Figure 8(A)). During the 6-day batch fermentation, the TSCs were partially degraded (Figure 8(B)), indicating that *C. acetobutylicum* was capable of digesting the TSC constituents. The degradation yielded a complex mixture of amino acids and small peptides, providing C and N sources for the Clostridium cells. However, the roles of TSCs on the metabolic pathway of ABE should be further investigated.

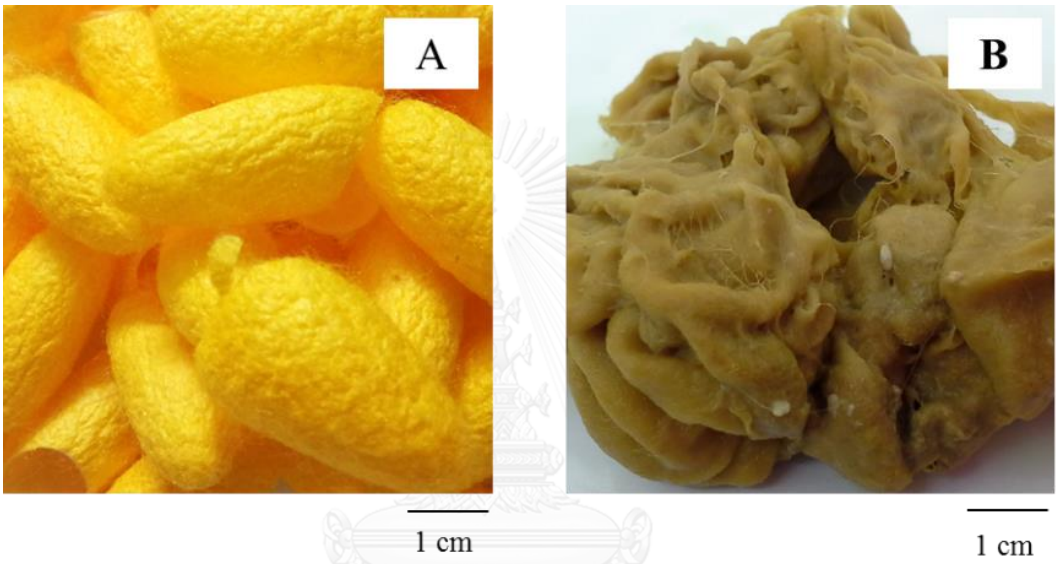


Figure 8 Thin-shell silk cocoons (TSSCs) of the mulberry silkworm: at the initial (A) and after being used in the ABE fermentation for 132 hours (B).

Figure 9 shows the morphology of free cells in the SC system (Figure 9 (A)) and free cells and ICs in the IC-TSC system (Figure 9(B) and 9 (D)). The general morphology of *C. acetobutylicum* was unchanged by the immobilization process. The free cell concentration in the SC system rapidly increased after 24 h, reaching its maximum (1.6 g/L) after 87 h. In the IC-TSC culture, the free cell concentration was initially very low; most of the cells were adsorbed to the TSC surfaces. The free cell concentration increased after 24 h and climbed to its maximum of 1.3 g/L after 78 h. At the end of the fermentation (132 h), *C. acetobutylicum* existed predominantly as rod-shaped cells in both systems, as shown in Figure 9 (A) and 9 (B). Small fragments of degraded TSCs were observed on the surfaces of Clostridium cells in the IC-TSC

system. The free cells in the effluent of this system may have descended from free cells in the broth or may have desorbed from the immobilization supports. Desorption of biomass from immobilization supports has been previously observed [23, 57]. Technical difficulties precluded the separation of the ICs from the TSCs. Additionally, the total mass of the TSC was not constant, but reduced as the materials were degraded by Clostridium cells. For these reasons, the concentration of IC-TSC was not determined in the study.

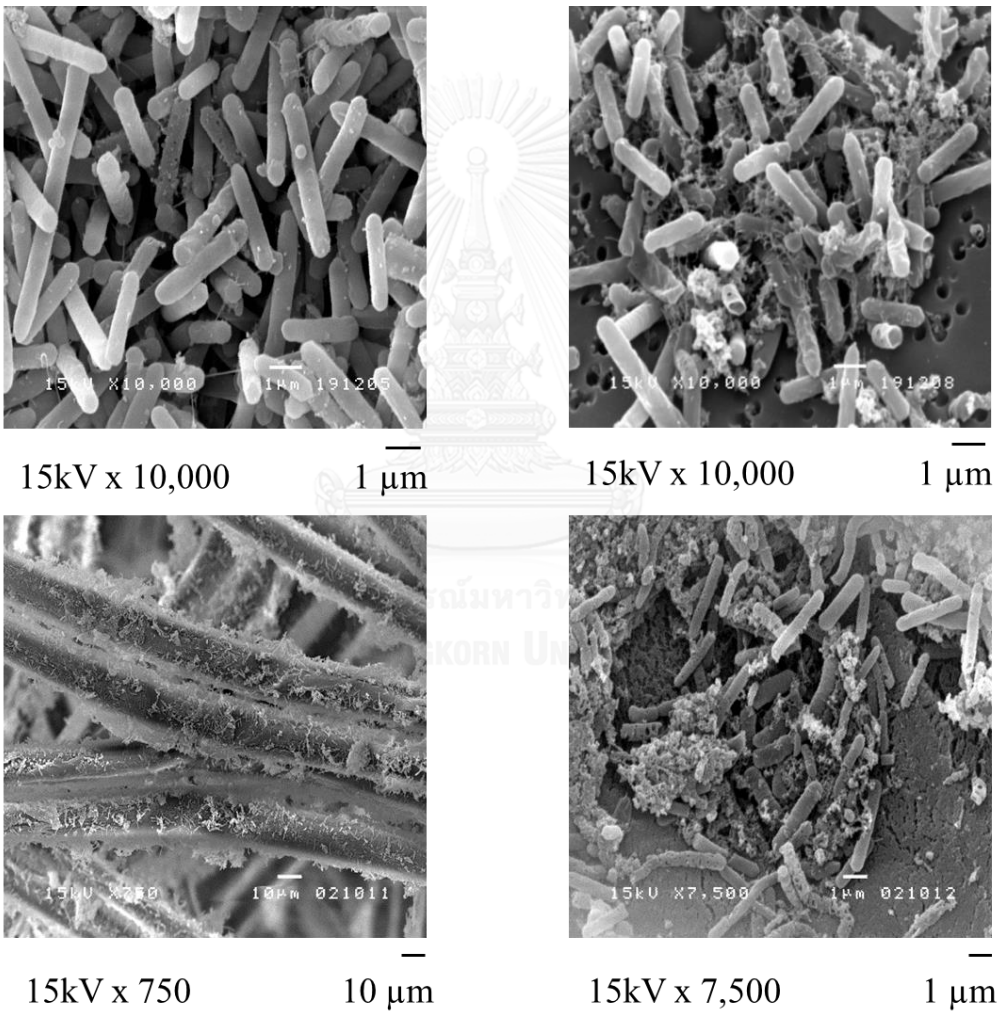


Figure 9 SEM images of *C. acetobutylicum* (ATCC 824) in the systems using the suspended cells (SC) and the immobilized cells on thin-shell silk cocoons (IC-TSSC): (A) free cells in the system using SC, (B) free cells in the system using IC-TSSC, (C) IC-TSSC and (D) a closer look of IC-TSSC.

Figure 10 and Figure 11 compares the residual sugar and ethanol concentrations between the systems using either SC or IC-TSSC cultures. The results show that the sugar consumption and ethanol production during the fermentation of the IC-TSSC system were similar to those of the SC system. The glucose concentration decreased from the initial concentration at 80 g/L to 6.3 g/L and 8.3 g/L in the systems using SC and IC-TSSC cultures, respectively. Therefore, the addition of TSSCs in the system would not interfere with the sugar uptake rate. The results were similar to those previously reported [57, 58]. The maximal ethanol concentrations in both SC and IC systems were approximately 1.4-1.6 g/L.

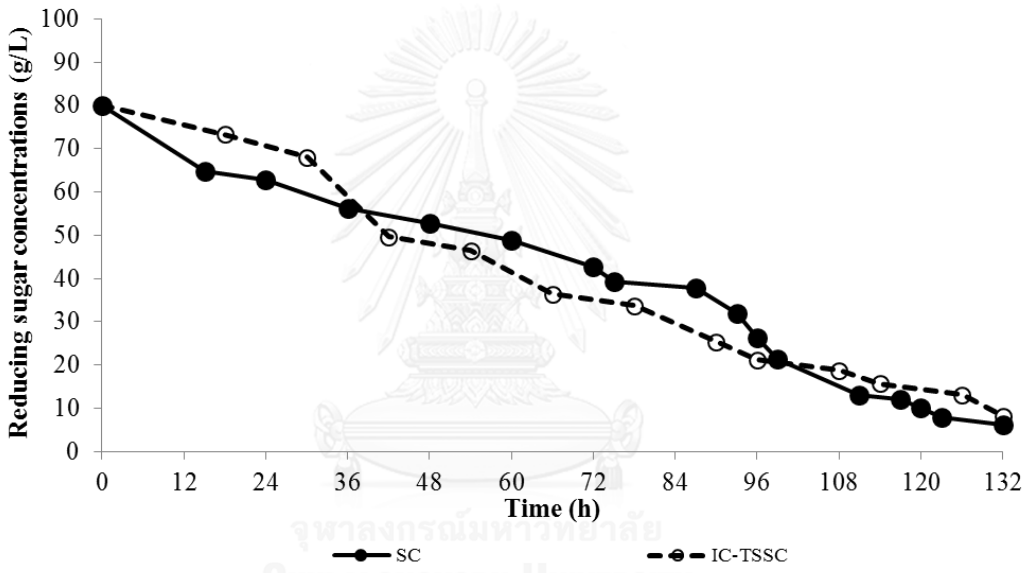


Figure 10 Effect of different culture systems: the suspended cells (SC) (—) and the immobilized cells on thin-shell silk cocoons (IC-TSSC) (---) on sugar consumption of *C. acetobutylicum* in ABE fermentation throughout 132 h. The initial sugar concentration, presented in sugarcane juice, of 80 g/L was utilized.

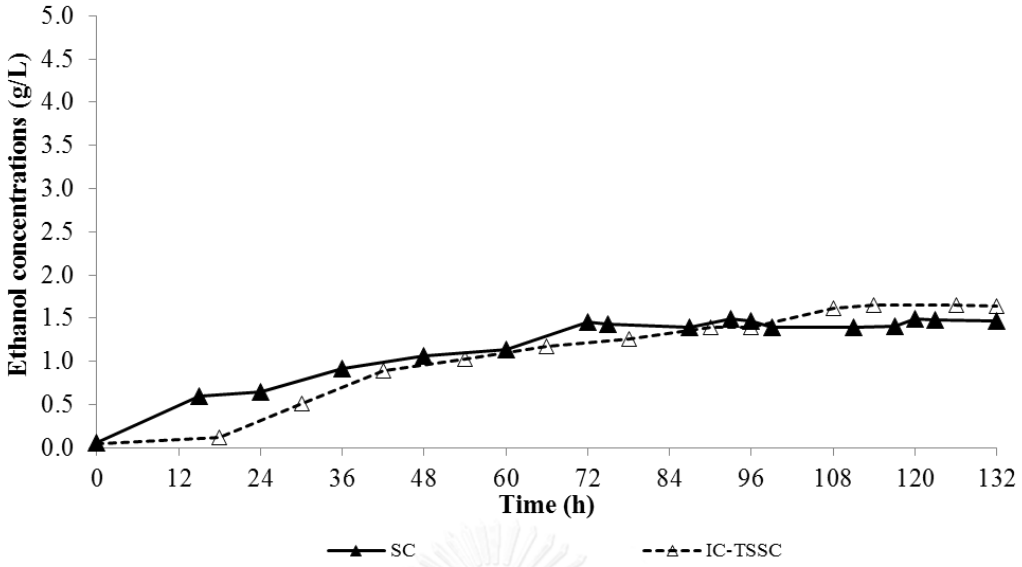


Figure 11 Effect of different culture systems: the suspended cells (SC) (—) and the immobilized cells on thin-shell silk cocoons (IC-TSSC) (---) on ethanol production of *C. acetobutylicum* in ABE fermentation throughout 132 h. The initial sugar concentration of 80 g/L was utilized.

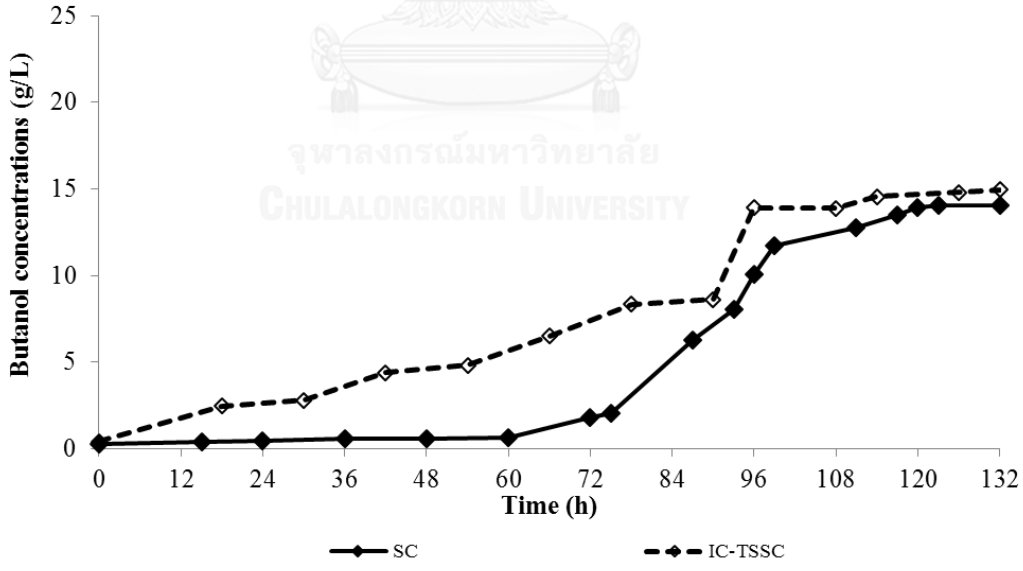


Figure 12 Effect of different culture systems: the suspended cells (SC) (—) and the immobilized cells on thin-shell silk cocoons (IC-TSSC) (---) on butanol production of *C. acetobutylicum* in ABE fermentation throughout 132 h. The initial sugar concentration of 80 g/L was utilized.

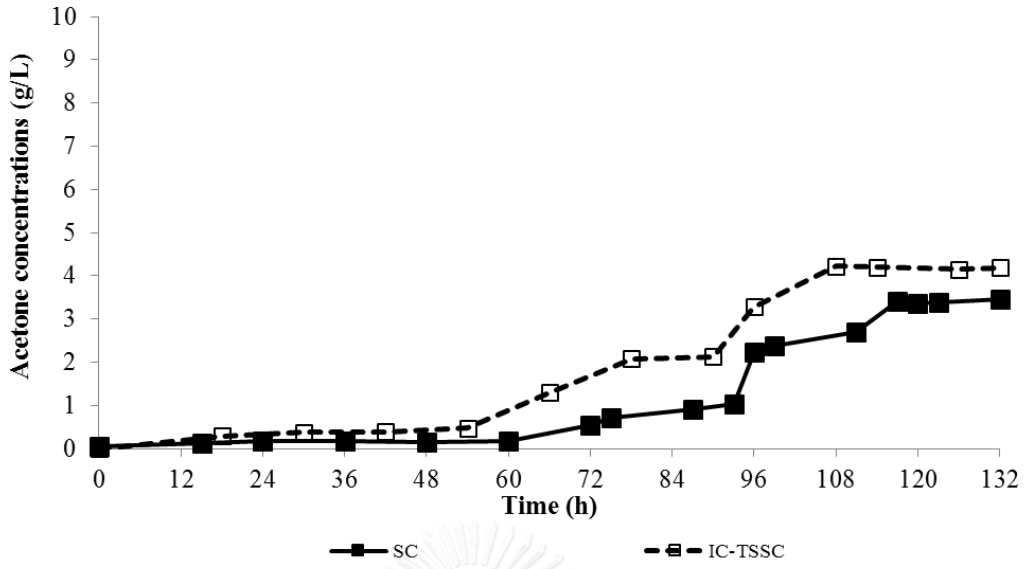


Figure 13 Effect of different culture systems: the suspended cells (SC) (—) and the immobilized cells on (---) thin-shell silk cocoons (IC-TSSC) on acetone production of *C. acetobutylicum* in ABE fermentation throughout 132 h. The initial sugar concentration of 80 g/L was utilized.

Figure 12 and Figure 13 compares the butanol and acetone between the systems using either SC or IC-TSSC cultures. The results show that the relatively higher acetone and butanol production in the system using the IC-TSSC culture compared to the system using SC were observed. Commonly, in ABE fermentation, during the initial growth phase the acid concentrations increase (acidogenesis) and then the metabolism of cells shifts to solvent production (solventogenesis). In the system using the SC culture, the conversion of organic acids (acetate and butyrate) into solvents (acetone and butanol) gradually increased during 0-72 h; more acetone and butanol productions were observed after 72 h. The results show that with the supplement of TSSCs, butanol and acetone could be produced from the earlier period and the rates of acetone and ethanol production were enhanced. After 132 h of IC-TSC fermentation, the total solvent accumulation was maximized at approximately 21.2 g/L, and comprised 4.2 g/L acetone, 15.0 g/L butanol and 1.6 g/L ethanol. The production rate of total solvent was 0.16 g/L/h. Meanwhile, the maximum acetone, butanol and ethanol concentrations in the SC culture were 3.5, 14.0, and 1.4 g/L, respectively.

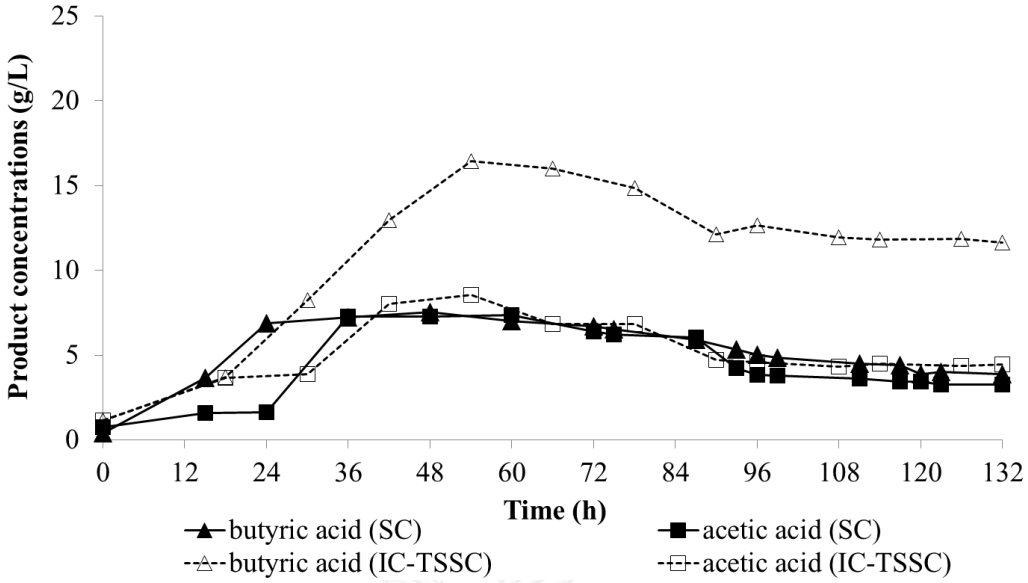


Figure 14 Effect of different culture systems: the suspended cells (SC) (—) and the immobilized cells on thin-shell silk cocoons (IC-TSSC) (---) on butyric acid and acetic acid production of *C. acetobutylicum* in ABE fermentation throughout 132 h.

Figure 14 showed the effect of different culture system on butyric and acetic acid productions. The results obtained during fermentation could be considered that immobilized-cell culture system could produce higher butyric acid which is in a good agreement with the results of butanol production (from Figure 12).

Although the fermentation required a relatively long time, the acetone and butanol concentrations in the IC-TSC system were higher than in previous reports of *C. acetobutylicum* (ATCC 824) immobilized on various support materials, such as activated carbon, vermiculite, coke, kaolinite and silica [20]. The source and content of nitrogen in the medium is very important in ABE production. Although Clostridia can utilize some amino acids as sole carbon/nitrogen sources, they preferentially ferment paired amino acids [44]. In 1934, Stickland importantly discovered that Clostridium sporogenes is a poor utilizer of single amino acids, but readily degrades certain amino acid pairs in coupled oxidation–reduction reactions (today, such reactions are known as Stickland reactions) [59]. Cocoon shells are packed within two natural macromolecular proteins: sericin and fibroin [33]. Sericin comprises 18 amino acids, most of which possess hydroxyl, carboxyl and amino groups. The dominant amino acids in sericin are

serine (33.4%) and aspartic acid (16.7%) [48]. Here, we demonstrated that *C. acetobutylicum* uses the degraded products of TSCs to promote ABE production.

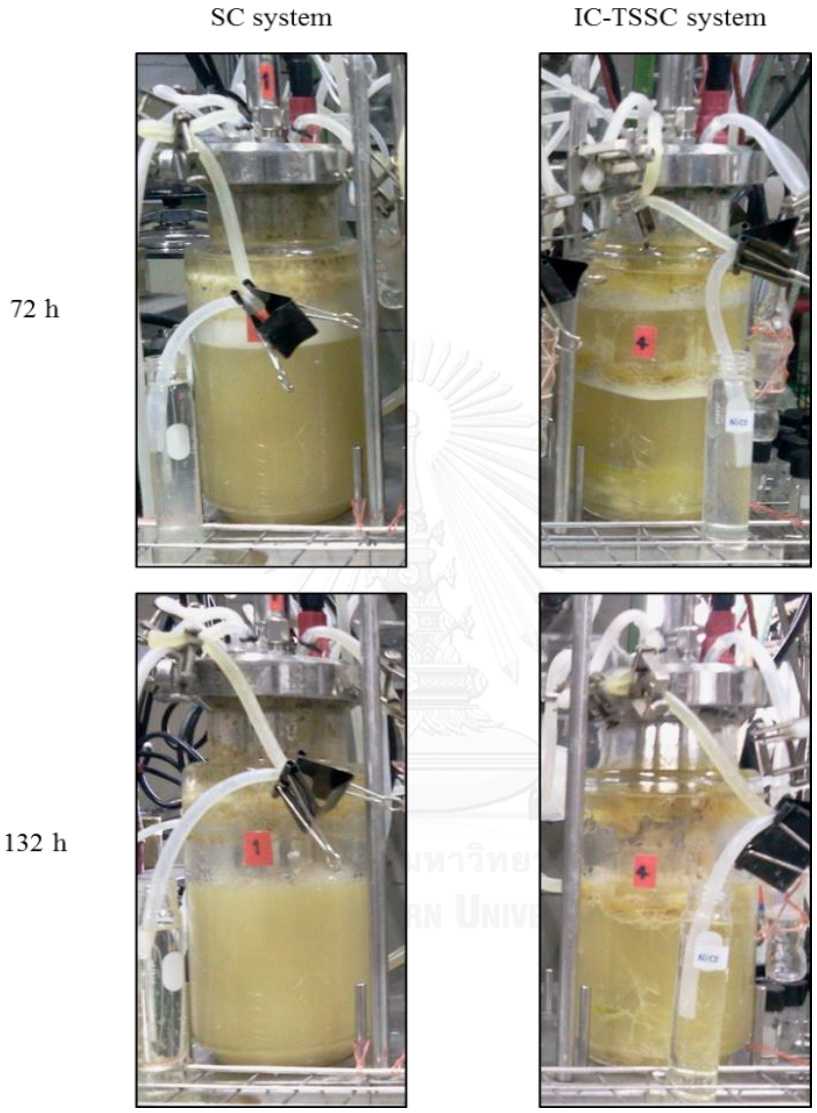


Figure 15 ABE productions in different culture systems: the suspended cells (SC) (left) and the immobilized cells on thin-shell silk cocoons (IC-TSSC) (right) at 72 h (top) and 132 h (bottom). The initial sugar concentration of 80 g/L was utilized.

4.2.4 Effects of initial sugar concentrations on ABE production by IC-TSSC culture

Next, we examined the optimal initial sugar concentration for ABE production by *C. acetobutylicum* immobilized on TSCs, using sugarcane juice as the carbon source. Figure 14 to Figure 17 shows the performance of ABE production in a 1 L batch reactor containing IC-TSC culture initially supplemented with 80, 90, and 100 g/L sugar. The sugar consumption and ethanol production was slightly higher at an initial sugar concentration of 90 g/L than at 80 g/L, and relatively higher acetone and butanol concentrations (6.1 and 15.9 g/L, respectively) were obtained at the end of the fermentation (132 h). Nevertheless, at such high initial sugar content (90 g/L), the ABE fermentation required an extended time (90 h) to shift from the acidogenic phase to the solventogenic phase. In contrast, the lower initial sugar concentration (80 g/L) yielded earlier butanol and acetone production, with final acetone and butanol concentrations of 4.2 and 15.0 g/L, respectively. An initial sugar concentration of 100 g/L exerted an inhibitory effect; the final acetone, butanol and ethanol concentrations were approximately 31% lower than at 90 g/L initial sugar concentration. Such high sugar concentration may have increased the osmotic strength, inhibiting the cell metabolism. However, the effects of excessive initial sugar concentration were mitigated in the IC-TSC system, which proved more efficient than the SC system. Immobilized cells were also more tolerant to butanol toxicity than their free counterparts [60]. In previous reports of ABE fermentation, solventogenesis was shown to be favored at high cell densities, whereas acidogenesis was favored at low cell densities [61]. Consistent with these findings, we found that immobilization on TSCs improved the ability of *C. acetobutylicum* to metabolize glucose at high initial sugar concentrations (80 ~ 90 g/L). The total solvent concentration yielded by the IC-TSC culture under optimal conditions was higher than obtained in a previous study [6], which evaluated ABE fermentation by *C. acetobutylicum* immobilized on bricks and initially supplemented with 60 g/L glucose [6].

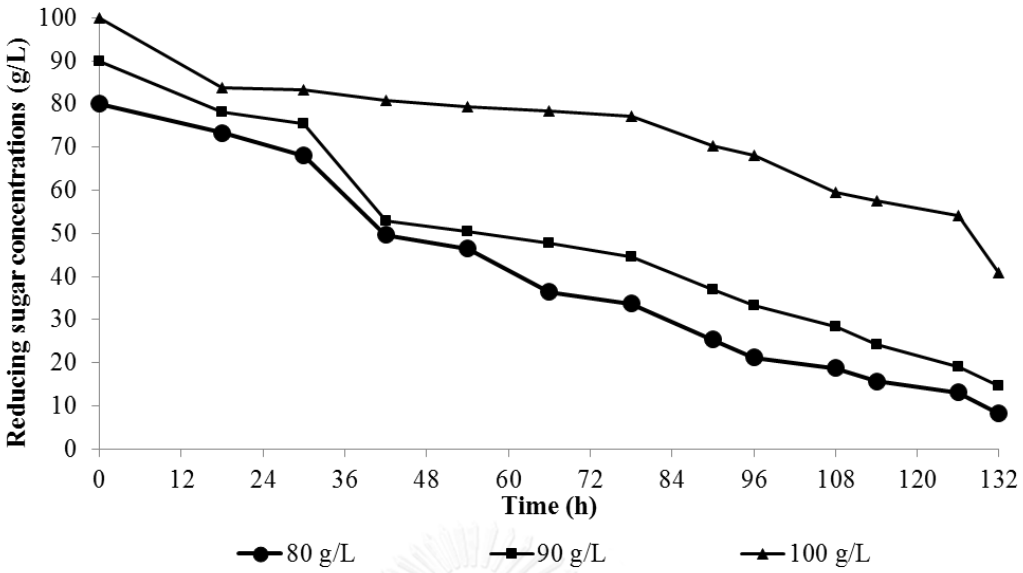


Figure 16 Effect of initial sugarcane juice contents as sugar concentrations (80 (●), 90 (■) and 100 (▲) g/L) on sugar consumption of immobilized *C. acetobutylicum* in the ABE fermentation throughout 132 h.

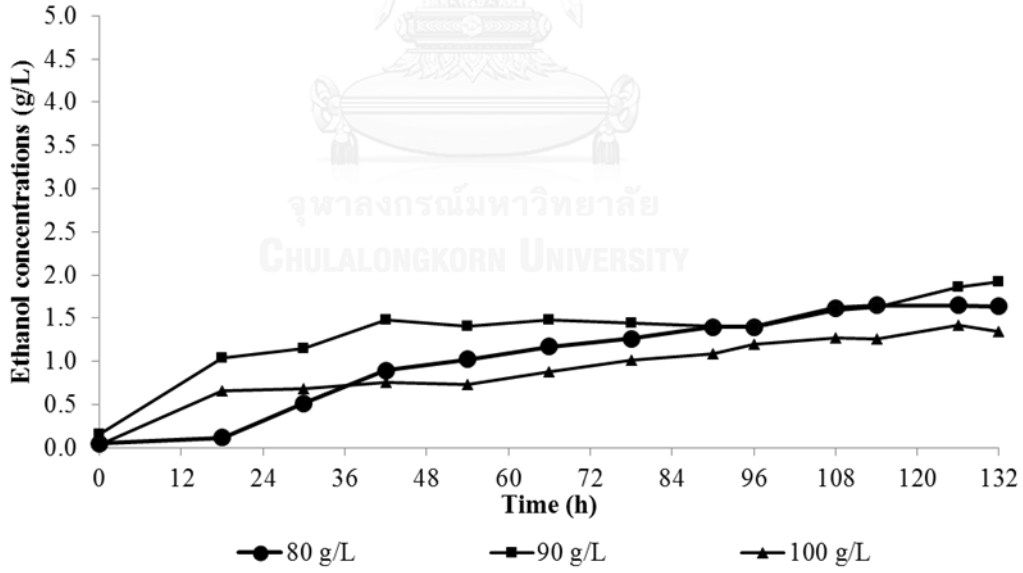


Figure 17 Effect of initial sugarcane juice contents as sugar concentrations (80 (●), 90 (■) and 100 (▲) g/L) on ethanol production of immobilized *C. acetobutylicum* in the ABE fermentation throughout 132 h.

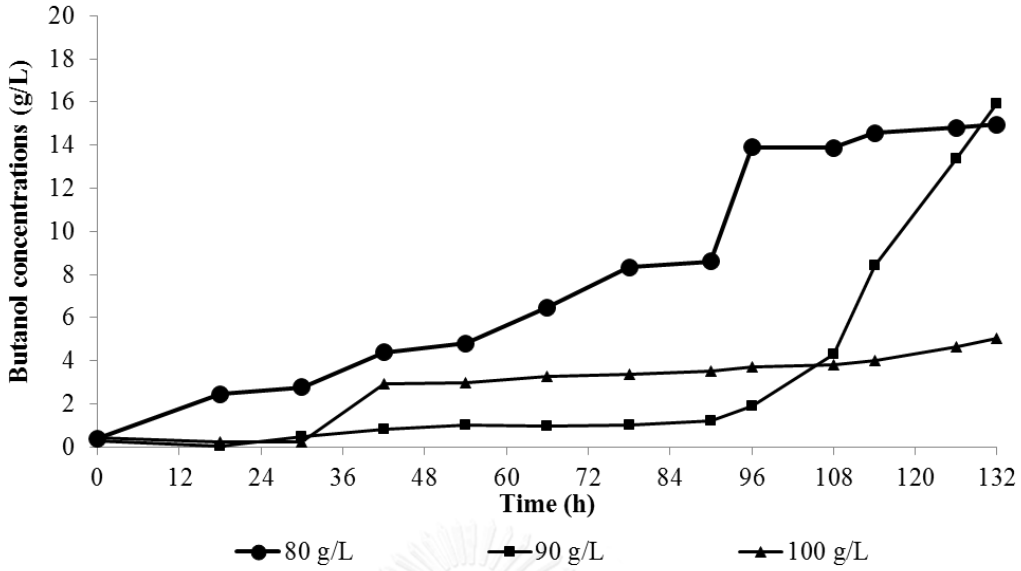


Figure 18 Effect of initial sugarcane juice contents as sugar concentrations (80 (●), 90 (■) and 100 (▲) g/L) on butanol production of immobilized *C. acetobutylicum* in the ABE fermentation throughout 132 h.

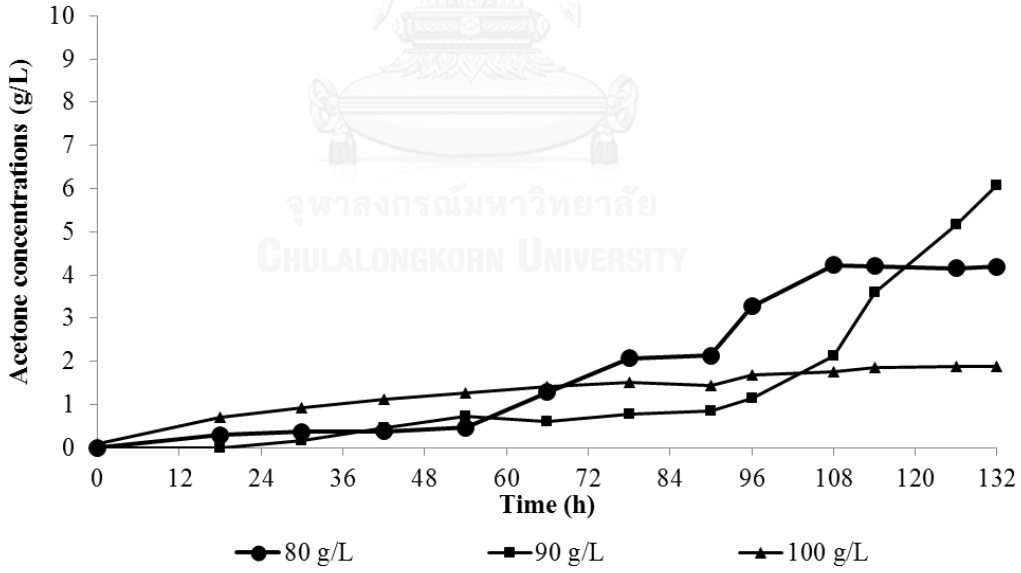


Figure 19 Effect of initial sugarcane juice contents as sugar concentrations (80 (●), 90 (■) and 100 (▲) g/L) on acetone production of immobilized *C. acetobutylicum* in the ABE fermentation throughout 132 h.

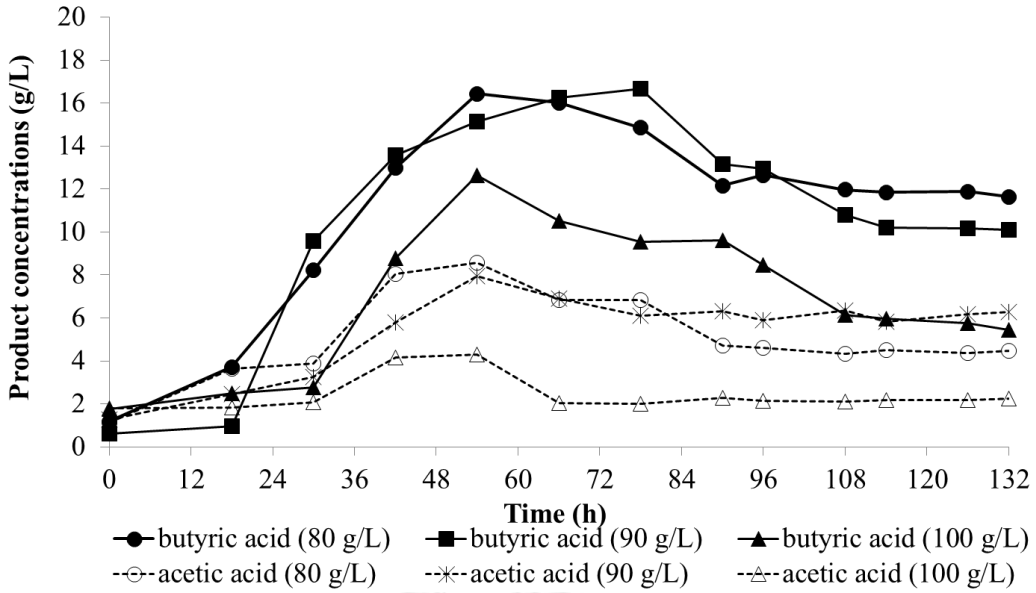


Figure 20 Effect of initial sugarcane juice contents as sugar concentrations (80 (●), 90 (■) and 100 (▲) g/L) on butyric acid (―) and acetic acid (---) production of immobilized *C. acetobutylicum* in the ABE fermentation throughout 132 h.

It could be observed in Figure 20. That the system with both initial sugar concentration 80 and 90 g/L could produce high butyric and acetic acid. However, the main drawback of this batch fermentation with the initial sugar concentration 90 g/L was it took long times to convert acid into acetone and butanol. Hence, the initial sugar concentration at 80 g/L was optimal to be chosen for the further study of fermentation development.

4.2.5 Repeated batch fermentation of IC-TSSC

In the previous subsection (4.2.3), we showed that the adsorbed *C. acetobutylicum* extract nutrients from the TSCs to promote ABE fermentation. In this subsection, ABE production by *C. acetobutylicum* immobilized on TSCs was further examined in 4-cycle repeated batch fermentation. The experimental duration was 72 h for the first batch (Batch 1) and 48 for consecutive batches (Batches 2, 3 and 4). The main carbon source was sugarcane juice, supplied at an initial concentration of 80 g/L. The concentration profiles of residual sugar, ethanol, acetone and butanol are plotted in Figure 21 to Figure 26.

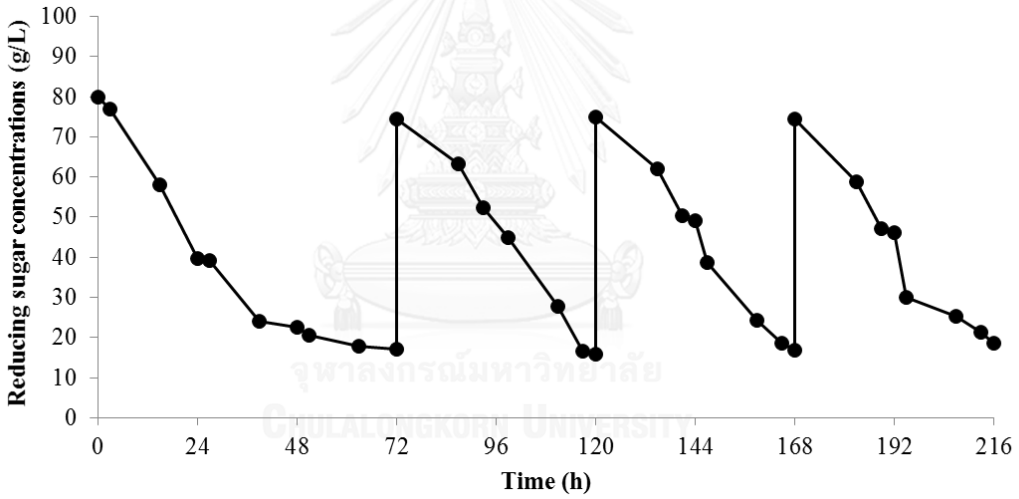


Figure 21 Sugar consumption rate of immobilzed *C. acetobutylicum* on TSSCs in the repeated batch fermentation throughout 216 h. After 72, 120 and 168 h, fermentation broths were removed and immediately replace by fresh medium with sugarcane juice at the sugar concentration of 80 g/L.

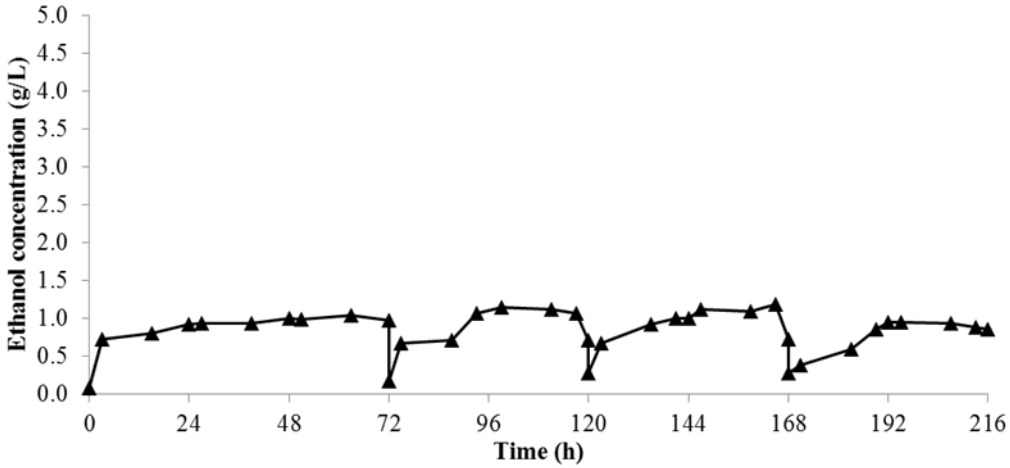


Figure 22 Ethanol production of immobilzed *C. acetobutylicum* on TSSCs in the repeated batch fermentation throughout 216 h. After 72, 120 and 168 h, fermentation broths were removed and immediately replace by fresh medium with sugarcane juice at the sugar concentration of 80 g/L.

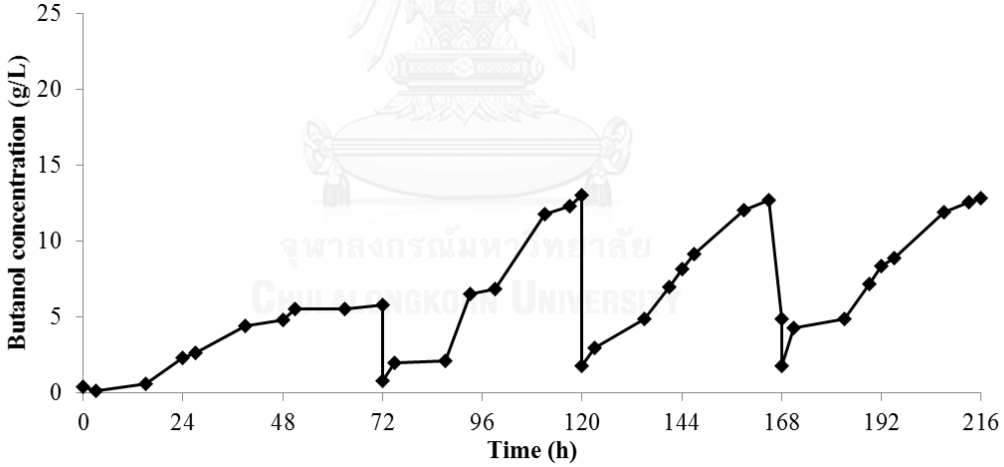


Figure 23 Butanol production of immobilzed *C. acetobutylicum* on TSSCs in the repeated batch fermentation throughout 216 h. After 72, 120 and 168 h, fermentation broths were removed and immediately replace by fresh medium with sugarcane juice at the sugar concentration of 80 g/L.

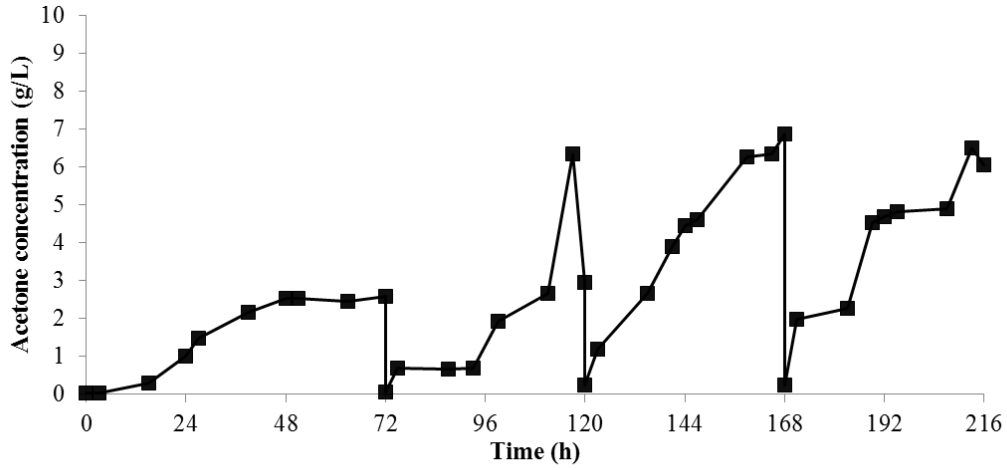


Figure 24 Acetone production of immobilzed *C. acetobutylicum* on TSSCs in the repeated batch fermentation throughout 216 h. After 72, 120 and 168 h, fermentation broths were removed and immediately replace by fresh medium with sugarcane juice at the sugar concentration of 80 g/L.

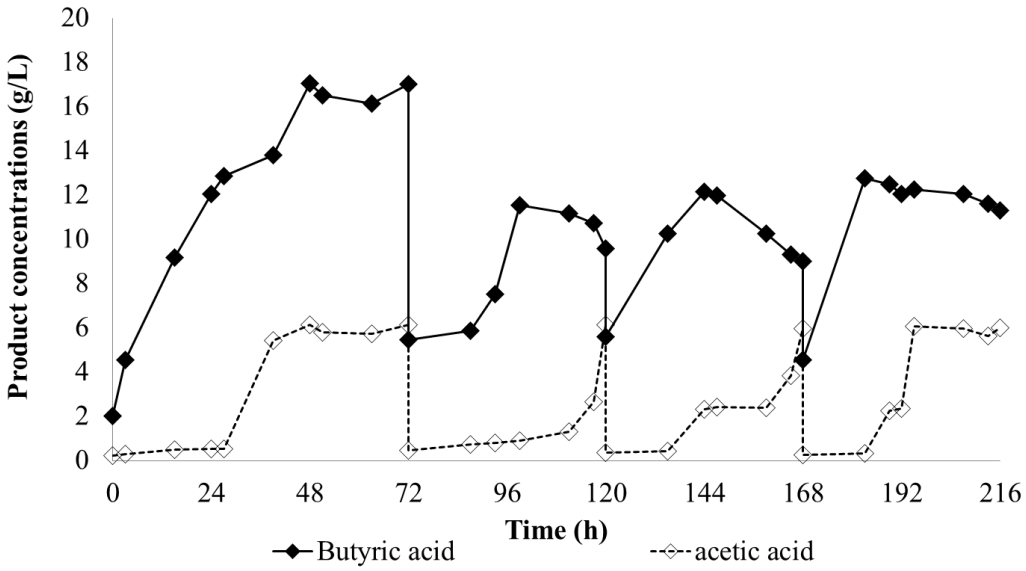


Figure 25 Butyric acid (—) and acetic acid (---) productions of immobilzed *C. acetobutylicum* on TSSCs in the repeated batch fermentation throughout 216 h.

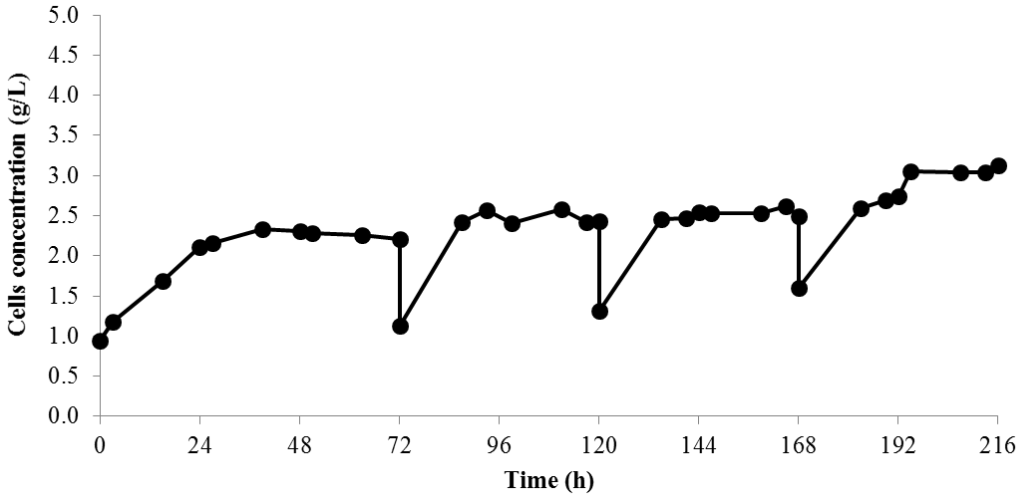


Figure 26 Cell concentrations (g/L) in culture broth in the repeated batch fermentation throughout 216 h. After 72, 120 and 168 h, fermentation broths were removed and immediately replace by fresh medium with sugarcane juice at the sugar concentration of 80 g/L.

The initial rates of ABE production and sugar consumption were lower in Batch 1 than in subsequent batches. The final ethanol and residual sugar concentrations in Batch 1 after 72 h were almost identical to those in Batches 2 ~ 4 after 48 h. Additionally, Batches 2 ~ 4 yielded significantly higher productivities and final concentrations of acetone and butanol than Batch 1.

The average free cell concentration at the end of Batch 1 was approximately 2.0 g/L; in subsequent batches, it increased to approximately 3.5 g/L. Because Batch 1 was the initial batch, fewer cells were immobilized on the TSCs than in subsequent batches. The consequent lower digestion of TSCs likely explains the lower acetone and butanol production in Batch 1 than in Batches 2 ~ 4. At an initial sugar concentration of 80 g/L, the IC-TSC culture completed the fermentation in approximately 100 ~ 120 h. In the repeat batch fermentations, more of the cells became immobilized on the TSCs. As the number of cells digesting the TSC materials increased, butanol and acetone was produced at a higher rate. After 48 h, the average acetone and butanol concentrations in the repeat batches (Batches 2 ~ 4) were 6.6 and 12.9 g/L, respectively. The average ABE productivity was 0.43 g/L/h, almost three times that of the one-batch process. It should be noted that, unlike acetone and butanol, the final ethanol concentration did not benefit from the repeated batch fermentation (its value of 1.1 g/L nearly matches that

of SC and IC-TSC batch fermentations during the same time interval). We infer that TSC does not promote ethanol productivity.

The IC-TSC system increases the cell density, cell reutilization and metabolic activities during the fermentation, and thereby enhances acetone and butanol production. The IC-TSC culture is catalytically stable, implying that the TSCs protect the cells from toxic conditions accumulating during the fermentation process. Previously, we demonstrated the potential utility of yeast cells immobilized on TSCs in repeated batch ethanol fermentation. Similar to the present study, we found that cyclic fermentation increased the number of yeast cells adsorbed to the inner/outer surfaces of the TSCs and embedded in the micro-porous structure of the fibril networks. Moreover, the TSCs conferred a protective effect against toxic metabolic products [8]. The average butanol and acetone productivities and concentrations obtained in the repeated IC-TSC batch fermentations exceeded those of ABE fermenter cells immobilized on other substrates under continuous or cyclic batch operation [6]. Unlike the inert immobilization matrices adopted in previous studies, we adopted a substrate that was digestible by the Clostridium cells, thus providing a nitrogen source during the ABE fermentation. In the experiments, 3 g of TSC (the immobilization matrix) was packed into the 1 L fermenter (600 mL fermentation medium). Although the total amount of TSC gradually decreased during the 216 h of repeated 4-cycle fermentation, a sufficient amount remained to maintain high ABE productivity. However, long-term repeated batch or continuous ABE fermentation would require a TSC feeding strategy. Table 7 summarizes the concentrations of residual sugar, end products and productivities of the batch and repeated batch fermentations.

Table 7 ABE fermentation of sugarcane juice at various initial sugar concentrations by *C. acetobutylicum* ATCC 824.

Operation mode	Culture	Initial sugar (g/L)	Residual sugar (g/L)	Acetone (g/L)	Butanol (g/L)	Ethanol (g/L)
Batch (132 h)	SC	80	6.5	3.5	14.0	1.4
Batch (132 h)	IC-TSSC	80	8.3	4.2	15.0	1.6
Batch (132 h)	IC-TSSC	90	14.6	6.1	15.9	1.9
Batch (132 h)	IC-TSSC	100	40.9	1.9	5.0	1.4
Repeated batch 1 (72 h)	IC-TSSC	80	17.1	2.6	5.8	1.0
Repeated batch 2 (48 h)	IC-TSSC	80	15.8	6.4	13.0	1.1
Repeated batch 3 (48 h)	IC-TSSC	80	18.7	6.9	12.7	1.2
Repeated batch 4 (48 h)	IC-TSSC	80	18.5	6.5	12.8	1.0

Operation mode	Culture	Initial sugar (g/L)	Acetic acid (g/L)	Butyric acid (g/L)	Total solvent (g/L)	Productivity (g/L·h)
Batch (132 h)	SC	80	3.0	11.9	18.7	0.14
Batch (132 h)	IC-TSSC	80	4.5	11.6	21.2	0.16
Batch (132 h)	IC-TSSC	90	6.3	10.1	23.9	0.18
Batch (132 h)	IC-TSSC	100	2.3	5.5	8.3	0.06
Repeated batch 1 (72 h)	IC-TSSC	80	6.2	17.0	9.4	0.13
Repeated batch 2 (48 h)	IC-TSSC	80	6.1	9.6	20.5	0.43
Repeated batch 3 (48 h)	IC-TSSC	80	6.0	9.0	20.7	0.43
Repeated batch 4 (48 h)	IC-TSSC	80	6.0	11.3	20.3	0.42

In addition, the ABE productions in repeated batch systems using immobilzed *C. acetobutylicum* on TSSCs at the initial sugar concentrations of 90 and 100 g/L were investigated. The concentration profiles of residual sugar, ethanol, acetone, and butanol are shown in Figure 27. The results show that the glucose concentration profiles during the fermentations using the initial sugar concentration of 90 g/L was quite similar to that using the initial sugar concentration of 100 g/L, however lower sugar consumptions were observed in the latter one. After the end of the ABE fermentations in the batch 1 and batch 2, the sugar consumptions were about 49.48, 30.60 g/L and 30.31, 11.66 g/L in the systems using the initial sugar concentrations at 90 g/L and 100 g/L, respectively, which were considerably lower than that in the system of 80 g/L. However, slight change in ethanol production was observed with the increase of the initial sugar concentration from 80 to 90 and 100 g/L.

On batch 1 (72 hours), the total solvent concentrations in the systems with the initial sugar concentrations of 90 and 100 g/L were approximately 10.06 g/L and 7.12 g/L, whereas the final concentrations of acetone were 1.48 g/L and 1.83 g/L and the final concentrations of butanol were 6.98 g/L and 4.06 g/L, respectively. On batch 2 (48 hours), the total solvent concentrations in the systems with the initial sugar concentrations of 90 and 100 g/L were approximately 6.44 g/L and 6.07 g/L, whereas the final concentrations of acetone were 2.28 g/L and 1.44 g/L and the final concentrations of butanol were 3.25 g/L and 3.54 g/L, respectively. It was shown that in the repeated batch fermentation with the use of the initial sugar at concentrations higher than 80 g/L, the acetone and butanol production tended to decrease with the increase of the batch cycle. These results were different from those with the initial sugar concentration at 80 g/L. It was suggested that the increase of osmotic strength, affected by a high sugar concentration in the medium, could cause serious inhibitory effect on cell metabolisms for acetone and butanol productions, resulting in lower productivities and concentrations of acetone and butanol. Therefore, the optimal initial sugar concentration in repeated batch systems by immobilized *C. acetobutylicum* on TSSCs using sugar cane juice medium was as same as that of the batch system, which was 80 g/L.

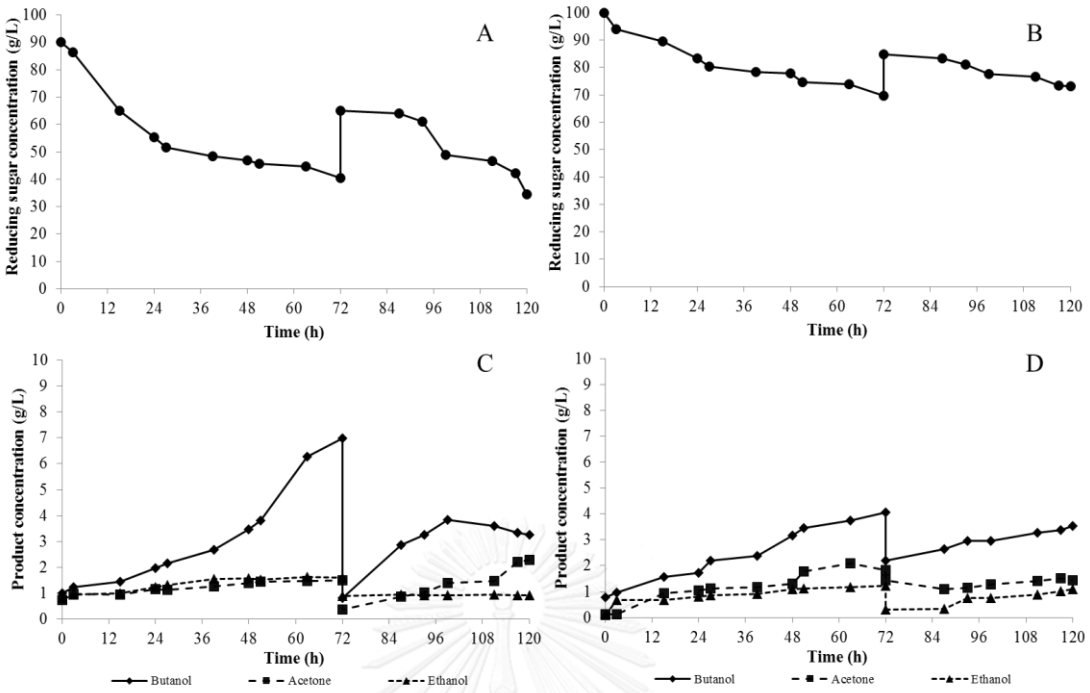


Figure 27 Sugar consumptions (top) and ABE productions (bottom) of immobilized *C. acetobutylicum* on TSSCs in repeated batch fermentation throughout 120 h. After 72 h, fermentation broths were removed and immediately replaced by fresh medium with sugarcane juice at the sugar concentration of 90 (A), (C) and 100 g/L (B), (D).

4.2.6 Comparison of repeated batch in this study to other reports

The fermentation conditions, which are different, such as *Clostridium* strains, fermentation periods, and carbon sources could impact the obtained products in the ABE fermentation. The comparison of repeated batch in this study to other reports was summarized in Table 8.

In the previous study on the repeated batch fermentation using *C. acetobutylicum* ATCC 824 immobilized on bricks, initial glucose substrate of 60 g/l and fermentation period of 60 h for each batch were utilized [6]. Average concentrations of 7, 10, and 1.8 g/L of acetone, butanol, and ethanol (ABE), respectively were yielded. In this study, the repeated batch fermentation using *C. acetobutylicum* ATCC 824 immobilized on TSSC was operated using sugarcane juice

at the initial sugar concentration of 80 g/L as a main substrate and produced butanol at concentration of 12.8 g/L (with 6.6 and 1.1 g/L of acetone and ethanol, respectively). This might be because the higher initial sugar concentration could be used when cane juice was used at the C-source in ABE fermentation. Moreover, the higher concentration of produced butanol, in this study, probably caused by the benefits of TSSC immobilized support giving rise to the increasing activity of butanol pathway.

Aragão Börner (2014) was reported that the 3-cycle repeated batch of ABE fermentation (127, 134, and 120 h respectively) using *C. acetobutylicum* DSM792 immobilized on cryogel and 60 g/L of the initial glucose produced ABE at the total concentrations of 12.2 g/L, which contained 8.5 g/L of butanol [7]. The lower product concentration of the previous study was confirmed the improvement in ABE production of this recent study. With the several-times reuse, easy separation from the medium, and achievement of high yield butanol production, Börner also indicated that cryogel was a great support material in the cross-linked-cells system due to cryogel possessed a special microstructure with high porosity and surface area which were suitable for applying as a cell carrier [7]. Therefore, in this study, utilization of TSSC which belongs to those surface properties as well might play a vital role to improve the productivity in ABE fermentation.

The stability of the ABE production performed by 16-cycle repeated batch throughout 800 h of fermentation using *C. acetobutylicum* JB200 and sucrose substrate at the initial concentration of 80 g/L was reported by Jiang (2014). Except for the first batch, the narrow concentration ranges of butanol, acetone, and ethanol (16–21, 8–10, and ~ 1.4 g/L, respectively) were obtained. Additionally, for the 3-cycle repeated batch fermentation in the presence of sugarcane juice (the initial sugar concentration of 83 g/L) in the same study, the slightly lower product concentrations were noticed (16.6, 7.4 g/L, and 1.4 g/L for butanol, acetone, and ethanol, respectively) as compared to the use of 16 cycles and sucrose substrate. However, those results from two conditions were not significantly different [28]. It was also reported that the concentrations of ABE in the repeated batch fermentation for 4 cycles using sugarcane juice measured as 80 g/L of initial sugar concentration as a substrate were similar to the previous report of Jiang (2014). Since both sucrose and sugarcane juice in those repeated batch systems

provided the similar fermentation kinetics of ABE fermentation, this suggested that sugarcane juice is a promising low-cost feedstock for butanol production.

Table 8 The average productions of *C. acetobutylicum* in repeated batch fermentation from this study and other reports.

Organism	Batch time (h)	Carbon source	Average product concentration				Author
			Butanol	Acetone	Ethanol	ABE	
C. acetobutylicum ATCC824	60	Glucose 60 g/L	10	7	1.8	18.8	Yen et al. 2011
C. acetobutylicum DSM792	(≥120)	Glucose 60 g/L	8.5	ND	ND	12.2	Aragão et al. 2014
C. acetobutylicum JB200	50	Sucrose 80 g/L	18.5	9.0	1.4	28.9	Jiang et al. 2014
C. acetobutylicum JB200	48	Sugarcane juice 80 g/L	16.6	7.4	1.4	25.4	Jiang et al. 2014
C. acetobutylicum ATCC824	48	Sugarcane juice 80 g/L	12.8	6.6	1.1	20.5	This study

4.3 Life Cycle Assessment of Acetone-Butanol Production from Sugarcane Juice by Immobilized *Clostridium acetobutylicum* (ATCC 824) on Thin-Shell Silk Cocoons

In our previous work [62], a method of immobilized cells (IC) on thin shell silk cocoon (TSC) was successfully applied to improve the productivity of ABE fermentation. It is well recognized that a high density of immobilized cells can improve the product yield and the volumetric productivity of bioreactors [63]. Regarding the environmental issues, the assessment for generation of CO_2 of butanol production in

batch and repeated batch fermentation by IC-TSC using sugarcane as feedstock was evaluated in this study.

4.3.1 Life cycle assessment

Life cycle assessment (LCA) represents an important tool to determine the positive or negative environmental impacts of products, processes, or services through interlinked production, usage, and disposal stages [64]. With the systematic inventory, an environmental profile can be set up and identify the weak points in the life cycle of system studied. It was noted that the results of interpretation of LCA must be consistent with the defined goal and scope [65, 66]. The life cycles of biobutanol, including the raw materials, fuels, and energy used in fermentation and separation are analyzed. Butanol production data based on the conditions in our previous studies [62] are used. The goal of this research is to carry out an evaluation of the environmental impact in terms of CO_2 emissions of the improved ABE fermentation process biosynthesized by immobilized cells on TSC in batch and repeated batch operations using sugarcane juice as a feedstock. In this study, Gate-to-Gate ABE production (Figure 28) was used to evaluate CO_2 emissions using the LCA methodology with Ecoinvent database. The CO_2 emissions from three stages of processing life cycle which are raw material, fermentation, and product separation were assessed. The results are compared to other studies of biobutanol productions. Furthermore, LCA of biobutanol production was compared to that of bioethanol production.

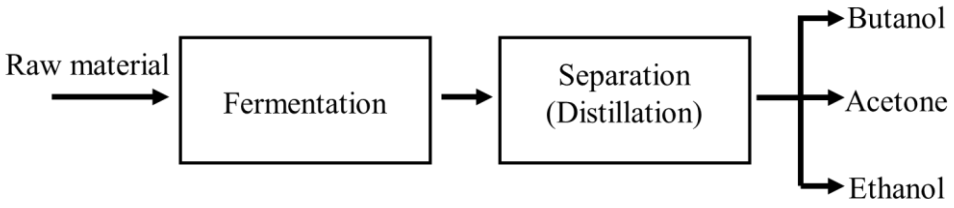


Figure 28 Block flow diagram of Gate-to-Gate ABE production process in this study.

4.3.2 Inventory analysis

The inventory analysis was preliminarily employed to indicate factors relevant to the environmental concerns as shown in Table 9. In this study, ABE fermentation from sugarcane juice was improved by using immobilized *Clostridium acetobutylicum* ATCC 824 on TSC. Compared to other works, different bacterial species might affect the fermentation performance resulting in alterations in the production rates and product concentrations.

Table 9 Information for raw material, ABE producing bacteria, fermentation time and product concentrations of this study in comparison to those of previously published works.

	References				
	Li et al., 2013	**Jiang et al., 2014**	**Vichuviwat et al., 2014**	**In this study**	
Process	batch	batch	batch	batch	repeated batch
Raw material	Glucose 60 g/L	Glucose 60 g/L	Cane molasses 60 g/L	Sugarcane juice 80 g/L	Sugarcane juice 80 g/L
Microorganism	*Clostridium Beijerinckii* L 175	*Clostridium Beijerinckii* IB4	*Clostridium Beijerinckii* TISTR 1461	*Clostridium Acetobutylicum* ATCC 824	*Clostridium Acetobutylicum* ATCC 824
Fermentation time (h)	84	40	120	132	48
Product concentrations (g/L)					
-Butanol	14	15.68	8.58	15	12.83
-Acetone	3.7	8.63	5.43	4.2	6.6
-Ethanol	2.1	0.32	0.62	1.6	1.1

4.3.3 Functional unit (FU)

In LCA, the functional unit (FU) provides a reference to which inputs and outputs are related. If the environmental impacts of the raw material under the study are compared, then a common base for the comparison must be identified before defining the functional unit, ensuring that the choice of FU stands in close relation to the goal and scope of the study.

4.3.4. Cell immobilization and fermentations

Thin-shell silk cocoons obtained from the Queen Sirikit Department of Sericulture (Saraburi, Thailand) were used as the immobilization material. Prior to use, impurities on the outer shell of the TSCs were removed by hand. The carbon source was sugarcane juice, provided at total reducing sugar concentrations of 80 g/L. Apart from the additional carbon source, the fermentation medium contained the same ingredients as the pre-culture medium. To prepare the ABE fermentation by the immobilized cells on TSCs, 3 g of TSCs were added to 600 mL of fermentation medium in a 1-L glass fermenter (Biostat®, B Braun Biotech International, Germany) prior to sterilization. TSCs was packed between stainless screen plates, and a magnetic stierrer was placed near the bottom of the reactor as shown in Figure 29. The reactor containing 600 ml fermentation medium with TSCs were then sterilized by autoclaving for 15 minutes at 121 °C and were cooled to room temperature (30 °C) before use. The experiment was initiated by transferring 10% (v/v) of the pre-cultures into the fermenter and purging the fermentation medium with nitrogen (0.1 vvm) for 15 min to establish anaerobic conditions. The fermentations were carried out in duplicate at 200 rpm agitation and 35° C. The pH was controlled to 4.5–5.0 by the automatic addition of 4 N NaOH solution. The repeated 4-cycle IC-TSC batch fermentation was also set up in the 1-L reactor containing 600 ml fermentation medium. The first batch was continued for 72 h; subsequent batches were each conducted for 48 h. At the end of each batch, the fermentation broth was removed from the fermenter and replaced with the same volume of fresh medium, followed by purging with nitrogen. All fermentations were performed at 35 °C and 200 rpm, with the pH controlled at 4.5–5.0.

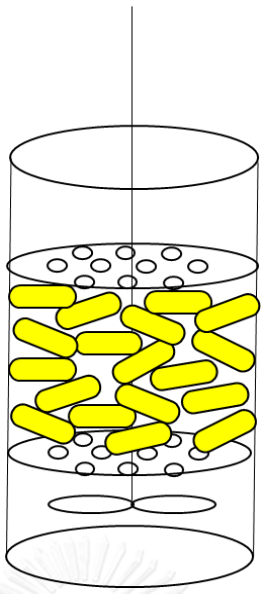


Figure 29 Schematic diagram represents a reactor for ABE production in this study.

4.3.5 Results and discussion

Sugarcane juice was used as the feedstock for the ABE fermentation. Sugarcane juice had 13.4% total sugar content (91.2% sucrose, 4.5% glucose and 4.4% fructose), which had to be diluted to the solutions with total reducing sugars of 80 g/L for the ABE fermentation. The optimum initial sugar concentration for ABE fermentation by the suspended culture (SC) of *C. acetobutylicum* ATCC 824 using sugarcane juice medium was 80 g/L [62, 67]. Generally, ABE fermentation is inhibited at initial glucose concentrations exceeding 60 g/L [53, 56]. At the same mass concentration (g/L), sugarcane juice exerts less osmotic inhibition than glucose or cane molasses media, thus the optimum initial sugar concentration in sugarcane juice medium was higher than those of glucose or cane molasses media. Conventional ABE batch fermentation takes approximately 3–6 days and seldom yields more than 12–20 g/L of solvents

limited by the solvent toxicity. Table 9 demonstrates ABE productions from this study as compared to those from other published works.

4.3.6 LCA characterization

The environmental impacts of ABE fermentation in this study were compared to those from other works in terms of CO_2 emissions. Butanol production of 1000 g was set as the basis of LCA and the contents of acetone, ethanol, sugar and inorganic chemicals were calculated according to the ABE ratio of the individual work. Due to the similar characteristics of fermenters and operating conditions used in ABE fermentations in batch mode, the electrical power consumed in the fermentation process was calculated from the information of the fermenter (Biostat®, B Braun Biotech International, Germany) used in this study and the electrical power consumed in the distillation process was estimated based on the information from the prior study of M. Wu et al., 2007 [68]. The electricity of each process was subsequently estimated based on the required batch fermentation time. After assessing the life cycle, equivalent CO_2 emissions to the air were yielded as shown in Table 10.

Table 10 The LCA characterizations of this study in comparison to those of previously published works.

	References					Unit
	Li et al.,2013	Jiang et al., 2014	Vichuviwat et al.,2014	This study		
System	batch	batch	batch	batch	repeated batch	
Product						
Butanol	1000	1000	1000	1000	1000	g
Acetone	264.286	550.383	634.033	280.268	514.419	g
Ethanol	150.000	20.408	72.261	109.699	85.737	g
Inputs						
Sugar	8571.429	1913.265	12587.413	8918.618	10392.310	g
Inorganic chemicals	85.714	109.375	799.534	424.749	494.934	g
Electricity						
- Fermentor	129.360	61.600	184.800	203.280	73.920	kWh
- Distillation[a]	0.430	0.430	0.430	0.430	0.430	kWh
Emissions to the air						
Carbon dioxide biogenic (fermentation)						
- Raw material process	-0.307	1.020	6.010	-10.300	-10.500	kg CO_2 eq
- Fermentation process	0.115	0.132	0.889	0.580	0.587	kg CO_2 eq
- Electricity						
- Fermentor	26.000	11.200	30.800	41.700	13.200	kg CO_2 eq
- Distillation[a]	0.087	0.078	0.072	0.088	0.077	kg CO_2 eq
total	25.895	12.430	37.771	32.068	3.364	kg CO_2 eq

[a] Electricity consumed in the distillation process was estimated based on the information from M. Wu, et al. (2007).

Under the batch process of this study method, the estimated total CO_2 emissions to the air were 32.068 kg CO_2/kg butanol. The net CO_2 emissions to the air from using sugarcane juice as a raw material are -10.30 kg CO_2/kg butanol, which is the lowest

one in comparison to those using the other raw materials, whereas the net CO_2 emission by using cane molasses as a raw material is relatively high due to a low production rate and low product concentrations of this process [30]. Cane molasses is a low cost by-product from the sugar industry, which can be used as a raw material in many fermentation processes, including ethanol fermentation. However, cane molasses might contain some toxic components, which have inhibitory effects on ABE fermentation as compared to glucose or cane sugar juice, resulting in lower product concentrations. The CO_2 emissions from ABE fermentation processes are 0.12 - 0.89 kg CO_2/kg butanol depending on the types of microorganism and the process conditions; however, this term slightly affects the total CO_2 emissions. The CO_2 emissions from electric generation for the distillation are 0.07-0.09 kg CO_2/kg butanol depending on the final product concentration. Thus, this term also causes only minor effects on the total CO_2 emissions. It was shown that the CO_2 emitted from electric generation for the fermenter has been the dominant factor affecting the total CO_2 emissions. During ABE fermentation, it is necessary to carefully control the operating pH and temperature in the appropriate ranges. Therefore, sufficient mixing to homogenize the culture broth and to promote sufficient mass and heat transfer rate during the fermentation process is required. For ABE production in a standard stirred tank fermenter, a power input of about 13-25 w/L is required for the fermenter unit [69]. A power input of 23.02 w/L is required for the fermenter unit in this work. Generally it would take 3–6 days for one batch of ABE fermentation; therefore, the CO_2 emission from electric generation for the fermenter unit is considerbly high compared to those of the other parts. In the case of the batch fermentation of this study, the CO_2 emission of this part is 32.068 kg CO_2/kg butanol, which appeared to be relatively high compared to those from the other

cases, due to time consumed in the fermentation process (132 h). On the other hand, the use of the productive strain as the mutant *C. beijerinckii IB4* and using glucose as a carbon source could reduce the fermentation time to only 40 h [70]. Consequently, the CO_2 emission of this part was reduced to 12.43 kg CO_2/kg butanol. However, the production cost of butanol from glucose was estimated to be about 4 fold higher than that of sugarcane because of its (glucose) high cost [71]. It was shown that the positive way to effectively reduce the CO_2 emissions of biobutanol production is to improve productivity in the fermentation process.

As compared to the batch system, a considerably higher productivity of acetone and butanol at approximately 0.43 g/L/h could be achieved by using the repeated batch system of the IC-TSC culture, which was increased to almost threefold of that of the batch process [62]. It was demonstrated that in ABE fermentation, *C. acetobutylicum* adsorbed on the TSCs and then digested TSCs into amino acids, which later were used as an N-source to stimulate solvent production [62, 67]. With a significant improvement in ABE productivity, the fermentation time in the repeated batch fermentation using IC-TSC could be reduced to 48 h [62]. The comparison of CO_2 emissions of the repeated batch system as compared to the batch system is shown in the last two columns of Table 10. With the shorter fermentation time, the energy consumed in the repeated batch fermentation is considerably less than that in the batch system. Consequently, the CO_2 emission from electric generation for fermenter in the repeated batch was only 0.31 of that of the typical batch system. It was estimated that the total CO_2 emission from the modified process could be reduced to 3.36 kg CO_2/kg butanol or only ≈ 1/10 of that from the typical batch fermentation. Therefore, the modified repeated batch ABE

fermentation with IC-TSC not only has contributed to substantial benefits in term of high productivity, but their environmental impact is also considerably decreased.

4.3.7 The LCA characterization in batch and repeated batch system of ABE fermentation vs ethanol fermentation

Currently, ethanol is the most widely used as an alternative fuel source from biomass. Production of bio-butanol could not at this time be compared to bio-ethanol. The major disadvantages of ABE fermentation are its low product concentrations, low productivity, and low conversion yield. However, butanol has superior fuel properties when compared to ethanol. Butanol has higher energy content than ethanol [3] and it is far less corrosive. Butanol-gasoline blends do not separate in the presence of water, and butanol can be blended with gasoline in any percentage up to 100%. Therefore, fermentation-derived butanol is considered a possible alternative to ethanol as a biomass-based liquid transportation fuel [2]. In this work, we compare the impact on environment in terms of total CO_2 emission of the fermentation-based production of butanol from sugarcane juice vs. ethanol from blackstrap molasses [8].

In our previous work [8], ethanol fermentation process was successfully improved by using TSC as a support material for the immobilization of *Saccharomyces cerevisiae* M30 in batch and repeated batch with blackstrap molasses as the main substrate. At the initial sugar concentration of 240 g/L, the maximum ethanol concentration of 98.6 g/L was obtained after 72 h in batch system; and at the initial sugar concentration of 220 g/L, an average ethanol concentration of 81.9 g/L was obtained after 48 h in repeated batch system. The environmental impacts of ethanol

fermentation in our previous study [8] and ABE fermentation in this study were compared in terms of CO_2 emission. Initially, 1000 g of initial sugar was set as the basis of the LCA. The contents of butanol, acetone, ethanol, and inorganic chemicals were then calculated according to the experimental data of the individual work. The electrical power consumed in the fermentation process was estimated from the information of the fermenter (Biostat®, B Braun Biotech International, Germany) used in this study and the electrical power consumed in the distillation process was estimated based on the information from the prior study of M. Wu et al., 2007 [68]. The electrical power consumed in ethanol fermentation and distillation process was estimated based on the information from the prior study of S. Soam, et al. (2015) [72]. This report focused on the environmental impacts and used the LCA to consider the gate-to-gate cycle by considering the process of ethanol and ABE fermentation, in terms of energy consumption, raw materials usage and productions.

LCA characterizations are shown in Table 11. It is demonstrated that the batch ABE fermentation produces the total CO_2 emission at 40.605 kg CO_2/kg initial sugar, which is almost two times higher than that of the batch ethanol fermentation process (20.790 kg CO_2/kg initial sugar). The major CO_2 emission is from electric generation for the fermenter unit during the fermentation process. In batch mode, bioethanol production requires a shorter fermentation time and a lower fermenter size, thus the energy consumed in the ethanol process was less than that in the ABE process. However, it is shown that by using the IC-TSC repeated batch process, the environmental impact of the ABE process becomes quite comparable to that of the ethanol process, in which the CO_2 emissions of ABE and ethanol processes decrease to 12.247 and 11.190 kg CO_2/kg initial sugar, respectively. Although the conversion

yields and concentrations of products in the ABE fermentation process are relatively low compared to those of the ethanol fermentation process, the market prices of butanol and acetone are relatively higher than the ethanol price. Hence, through developments of effective microbial strains and ABE fermentation processes, biobutanol production should be able to compete with bioethanol production in the near future.

Table 11 The LCA characterizations in batch and repeated batch processes of biobutanol vs ethanol fermentations.

	Ethanol [20]		**Butanol (in this study)**		**Unit**
Inputs					
Sugar	1000	1000	1000	1000	g
Inorganic chemicals	2.083	2.083	47.625	47.625	g
Electricity	75.78[a]	41.97[a]	203.71[b]	74.35[b]	kWh
Product					
Butanol	-	-	186.875	160.375	g
Acetone	-	-	52.375	82.500	g
Ethanol	410.833	372.273	20.500	13.750	g
Emissions to the air					
Carbon dioxide biogenic (fermentation)					
- Raw material process	-0.814	-0.814	-1.160	-1.010	kg CO_2 eq
- Fermentation process	0.004	0.004	0.065	0.057	kg CO_2 eq
- Electric generation	21.600	12.000	41.700	13.200	kg CO_2 eq
total	20.790	11.190	40.605	12.247	kg CO_2 eq

[a] Electricity consumed in the fermenter and distillation process was estimated based on the information from S. Soam, et al. (2015);

[b] Electricity consumed in the distillation process was estimated based on the information from M. Wu, et al. (2007).

CHAPTER V CONCLUSION

5.1 Thin-shell silk cocoon (TSSC) as a nitrogen source of ABE fermentation by *Clostridium acetobutylicum*

A thin-shell silk cocoon (TSSC), a residual from the silk industry, was used as an alternative N-source in ABE fermentation by *C. acetobutyricum* ATCC 824. The experimental studies were performed in batch fermentation at 35 °C and pH 5.0. Effects of dfferent N-sources: (1) YE, (2) $TSSC_f$ and (3) the mixture of YE and $TSSC_f$ for cell charateristics and ABE production were examined. It was found that TSSC might help promote cell growth and phase shift of acetone in the ABE fermentation by *C. acetobutylicum* ATCC 824, whereas YE could promote phase shift of biobutanol. Under optimum conditions, total solvent of 22.30 g/L (12.72 g/L butanol, 8.03 g/L acetone and 1.50 g/L ethanol) was obtained from the ABE fermentation using the mixture of YE and $TSSC_f$ as N-sources. The productivity and conversion yield were 0.15 g/L/h and 0.30 g ABE/g sugar consumption, respectively. The results showed that TSSC could be used as a cheap substitute for YE for a certain amount to promote ABE fermentation.

5.2 Enhanced acetone-butanol production from sugarcane juice by immobilized *Clostridium acetobutylicum* (ATCC 824) on thin-shell silk cocoons

To increase the production and efficiency of ABE fermentation, we immobilized *C. acetobutylicum* (ATCC 824) by adsorbing the cells to TSC surfaces. In a preliminary experiment, the nitrogen sourced from TSC fragments seemed to promote a phase shift to acetone in the ABE fermentation. Therefore, the adsorbed *C. acetobutylicum* ATCC 824 cells extract nutrients from the TSCs to promote ABE fermentation. Next, we conducted a comparative study of batch fermentation using

sugarcane juice as the major C-source. Acetone and butanol production was higher in the IC-TSC system than in the SC system, in which cells were freely suspended. The effect of initial sugar concentration on batch ABE fermentation by the IC-TSC culture was also investigated. At an initial sugar concentration of 90 g/L, the ABE fermentation required more time to shift from the acidogenic to the solventogenic phase than at 80 g/L, but the final acetone and butanol concentrations were higher (6.1 and 15.9 g/L, respectively, versus 4.3 and 15.0 g/L, respectively, at 80 g/L). The ABE productivities were further enhanced in repeated batch fermentations using IC-TSC culture. At an initial sugar concentration of 80 g/L, the repeated 4-cycle batch fermentation yielded average acetone and butanol concentrations of 6.6 and 12.9 g/L, respectively, with productivities of 0.43 g/L/h. These results demonstrate that the IC-TSC system enhances ABE fermentation by virtue of its high biocatalytic stability, reusability, cell regeneration and enhanced productivity of acetone and butanol.

5.3 Life Cycle Assessment of Acetone-Butanol Production from Sugarcane Juice by Immobilized *Clostridium acetobutylicum* (ATCC 824) on Thin-Shell Silk Cocoons

LCA analysis was used to evaluate the CO_2 emissions of ABE fermentations by using IC-TSC in batch and repeated batch operations. Butanol at 1000 g production was set as the basis of the LCA. The life cycles of biobutanol, including raw material preparation, fermentation process, and electric utility in this production were analyzed. From the evaluation, the total CO_2 emission at 29.87 and 2.67 kg CO_2/kg butanol are produced from the batch and repeated batch operations, respectively. The modified repeated batch ABE fermentation not only has contributed to a substantial benefit in terms of high productivity, but also significantly reduces the environmental impact. The result of LCA characterization in the repeated batch ABE fermentation using IC-TSC in terms of CO_2 emissions per kg of sugar was comparable to that of the ethanol

fermentation. In this study, we demonstrate that by using the modified process, biobutanol production could compete with bioethanol production in the near future**.**

5.4 Recommendation for further study

1. Study on the use of agricultural or industrial wastes for ABE fermentation in order to lower the production cost.

2. Study on the improvement of microbial strain in order to improve the productivities and concentrations of acetone and butanol.

3. Study on the improvement of process, such as integration of solvent separation unit with the fermentation unit in order to improve ABE productivities and yields.

4. Study on the effects of bio-butanol obtained from ABE fermentation in comparison with other biofuels, on the environmental concerns such as life-cycle energy usage and greenhouse gas (GHG) emission by using a life-cycle assessment (LCA).

REFERENCES

1. Jin, C., et al., *Progress in the production and application of n-butanol as a biofuel.* Renewable and Sustainable Energy Reviews, 2011. **15**(8): p. 4080-4106.
2. Pfromm, P.H., et al., *Bio-butanol vs. bio-ethanol: A technical and economic assessment for corn and switchgrass fermented by yeast or Clostridium acetobutylicum.* Biomass and Bioenergy, 2010. **34**(4): p. 515-524.
3. Green, E.M., *Fermentative production of butanol—the industrial perspective.* Current Opinion in Biotechnology, 2011. **22**(3): p. 337-343.
4. Nigam, P.S. and A. Singh, *Production of liquid biofuels from renewable resources.* Progress in Energy and Combustion Science, 2011. **37**(1): p. 52-68.
5. Zhang, Z., et al., *Combustion and particle number emissions of a direct injection spark ignition engine operating on ethanol/gasoline and n-butanol/gasoline blends with exhaust gas recirculation.* Fuel, 2014. **130**(0): p. 177-188.
6. Yen, H.-W., R.-J. Li, and T.-W. Ma, *The development process for a continuous acetone–butanol–ethanol (ABE) fermentation by immobilized Clostridium acetobutylicum.* Journal of the Taiwan Institute of Chemical Engineers, 2011. **42**(6): p. 902-907.
7. Aragão Börner, R., et al., *Immobilization of Clostridium acetobutylicum DSM 792 as macroporous aggregates through cryogelation for butanol production.* Process Biochemistry, 2014. **49**(1): p. 10-18.
8. Rattanapan, A., S. Limtong, and M. Phisalaphong, *Ethanol production by repeated batch and continuous fermentations of blackstrap molasses using immobilized yeast cells on thin-shell silk cocoons.* Applied Energy, 2011. **88**(12): p. 4400-4404.
9. Li, H.-g., et al., *Acetone, butanol, and ethanol production from cane molasses using Clostridium beijerinckii mutant obtained by combined low-energy ion beam implantation and N-methyl-N-nitro-N-nitrosoguanidine induction.* Bioresource Technology, 2013. **137**(0): p. 254-260.
10. Stoeberl, M., et al., *Biobutanol from food wastes – fermentative production, use as biofuel an the influence on the emissions.* Procedia Food Science, 2011. **1**(0): p. 1867-1874.
11. Qureshi, N., B. Saha, and M. Cotta, *Butanol production from wheat straw hydrolysate using Clostridium beijerinckii.* Bioprocess and Biosystems Engineering, 2007. **30**(6): p. 419-427.
12. Dürre, P., *Fermentative production of butanol — the academic perspective.* Current Opinion in Biotechnology, 2011. **22**(3): p. 331-336.
13. Sukumaran, R.K., et al., *Chapter 25 - Butanol Fuel from Biomass: Revisiting ABE Fermentation*, in *Biofuels*, A.P.L.C.R.-G.D. Gnansounou, Editor. 2011, Academic Press: Amsterdam. p. 571-586.
14. Papoutsakis, E.T., *Engineering solventogenic clostridia.* Current Opinion in Biotechnology, 2008. **19**(5): p. 420-429.
15. Qureshi, N. and I.S. Maddox, *Reduction in Butanol Inhibition by Perstraction: Utilization of Concentrated Lactose/Whey Permeate by Clostridium*

acetobutylicum to Enhance Butanol Fermentation Economics. Food and Bioproducts Processing, 2005. **83**(1): p. 43-52.
16. Lütke-Eversloh, T. and H. Bahl, *Metabolic engineering of Clostridium acetobutylicum: recent advances to improve butanol production.* Current Opinion in Biotechnology, 2011. **22**(5): p. 634-647.
17. Hönicke, D., et al., *Global transcriptional changes of Clostridium acetobutylicum cultures with increased butanol:acetone ratios.* New Biotechnology, 2012. **29**(4): p. 485-493.
18. Isar, J. and V. Rangaswamy, *Improved n-butanol production by solvent tolerant Clostridium beijerinckii.* Biomass and Bioenergy, 2012. **37**(0): p. 9-15.
19. Ezeji, T.C., N. Qureshi, and H.P. Blaschek, *Bioproduction of butanol from biomass: from genes to bioreactors.* Current Opinion in Biotechnology, 2007. **18**(3): p. 220-227.
20. Zhang, Y., B. Han, and T.C. Ezeji, *Biotransformation of furfural and 5-hydroxymethyl furfural (HMF) by Clostridium acetobutylicum ATCC 824 during butanol fermentation.* New Biotechnology, 2012. **29**(3): p. 345-351.
21. Sun, Z. and S. Liu, *Production of n-butanol from concentrated sugar maple hemicellulosic hydrolysate by Clostridia acetobutylicum ATCC824.* Biomass and Bioenergy, 2012. **39**(0): p. 39-47.
22. Dolejš, I., et al., *Butanol production by immobilised Clostridium acetobutylicum in repeated batch, fed-batch, and continuous modes of fermentation.* Bioresource Technology, 2014. **169**(0): p. 723-730.
23. Ezeji, T.C., N. Qureshi, and H.P. Blaschek, *Butanol fermentation research: upstream and downstream manipulations.* The Chemical Record, 2004. **4**(5): p. 305-314.
24. Lee, S.Y., et al., *Fermentative butanol production by clostridia.* Biotechnology and Bioengineering, 2008. **101**(2): p. 209-228.
25. Huesemann, M.H., et al., *Acetone-butanol fermentation of marine macroalgae.* Bioresource Technology, 2012. **108**(0): p. 305-309.
26. Li, X., et al., *Yeast extract promotes phase shift of bio-butanol fermentation by Clostridium acetobutylicum ATCC824 using cassava as substrate.* Bioresource Technology, 2012. **125**(0): p. 43-51.
27. Leewattana, N., *Biobutanol Production from Sugar Cane Molasses by Immobilized Clostridium acetobutylicum on Thin Shell Silk Cocoons.* Chemical engineering, Chulalongkorn University, 2013.
28. Jiang, W., et al., *Stable high-titer n-butanol production from sucrose and sugarcane juice by Clostridium acetobutylicum JB200 in repeated batch fermentations.* Bioresource Technology, 2014. **163**(0): p. 172-179.
29. Qureshi, N. and I.S. Maddox, *Continuous solvent production from whey permeate using cells of Clostridium acetobutylicum immobilized by adsorption onto bonechar.* Enzyme and Microbial Technology, 1987. **9**(11): p. 668-671.
30. Vichuviwat, R., et al., *Enhanced butanol production by immobilized Clostridium beijerinckii TISTR 1461 using zeolite 13X as a carrier.* Bioresource Technology, 2014. **172**(0): p. 76-82.

31. Jiang, P., et al., *Tensile behavior and morphology of differently degummed silkworm (Bombyx mori) cocoon silk fibres.* Materials Letters, 2006. **60**(7): p. 919-925.
32. Chen, F., D. Porter, and F. Vollrath, *Morphology and structure of silkworm cocoons.* Materials Science and Engineering: C, 2012. **32**(4): p. 772-778.
33. Teshome, A., et al., *Study on the microstructure of African wild silk cocoon shells and fibers.* International Journal of Biological Macromolecules, 2012. **50**(1): p. 63-68.
34. Jang, Y.-S., et al., *Butanol production from renewable biomass by clostridia.* Bioresource Technology, 2012. **123**(0): p. 653-663.
35. Qureshi, N., et al., *Production of butanol (a biofuel) from agricultural residues: Part I – Use of barley straw hydrolysate.* Biomass and Bioenergy, 2010. **34**(4): p. 559-565.
36. Ni, Y. and Z. Sun, *Recent progress on industrial fermentative production of acetone–butanol–ethanol by Clostridium acetobutylicum in China.* Applied Microbiology and Biotechnology, 2009. **83**(3): p. 415-423.
37. Monot, F., et al., *Acetone and Butanol Production by Clostridium acetobutylicum in a Synthetic Medium.* Applied and Environmental Microbiology, 1982. **44**(6): p. 1318-1324.
38. Potvin, J., et al., *An automatic turbidimetric method to screen yeast extracts as fermentation nutrient ingredients.* Journal of Microbiological Methods, 1997. **29**(3): p. 153-160.
39. Afschar, A.S. and K. Schaller, *Production of acetone and butanol from starch by continuous bioprocess.* Journal of Biotechnology, 1991. **18**(3): p. 255-264.
40. Wang, Y. and H.P. Blaschek, *Optimization of butanol production from tropical maize stalk juice by fermentation with Clostridium beijerinckii NCIMB 8052.* Bioresource Technology, 2011. **102**(21): p. 9985-9990.
41. Bankar, S.B., et al., *Continuous two stage acetone–butanol–ethanol fermentation with integrated solvent removal using Clostridium acetobutylicum B 5313.* Bioresource Technology, 2012. **106**(0): p. 110-116.
42. Huang, W.-C. and I.C. Tang, *Chapter 8 - Bacterial and Yeast Cultures—Process Characteristics, Products, and Applications*, in *Bioprocessing for Value-Added Products from Renewable Resources*, S.-T. Yang, Editor. 2007, Elsevier: Amsterdam. p. 185-223.
43. Torija, M.a.J., et al., *Effect of the nitrogen source on the fatty acid composition of Saccharomyces cerevisiae.* Food Microbiology, 2003. **20**(2): p. 255-258.
44. Wagner, A.O., et al., *Effects of different nitrogen sources on the biogas production – a lab-scale investigation.* Microbiological Research, 2012. **167**(10): p. 630-636.
45. Chen, Y., J.J. Cheng, and K.S. Creamer, *Inhibition of anaerobic digestion process: A review.* Bioresource Technology, 2008. **99**(10): p. 4044-4064.
46. Yang, L.-B., et al., *A novel osmotic pressure control fed-batch fermentation strategy for improvement of erythritol production by Yarrowia lipolytica from glycerol.* Bioresource Technology, 2014. **151**(0): p. 120-127.
47. Al-Shorgani, N., et al., *Bioconversion of Butyric Acid to Butanol by Clostridium saccharoperbutylacetonicum N1-4 (ATCC 13564) in a Limited Nutrient Medium.* BioEnergy Research, 2012. **5**(2): p. 287-293.

48. Zhang, Y.-Q., *Applications of natural silk protein sericin in biomaterials.* Biotechnology Advances, 2002. **20**(2): p. 91-100.
49. Pozo-Dengra, J., et al., *Screening of autolytic yeast strains for production of l-amino acids.* Enzyme and Microbial Technology, 2006. **40**(1): p. 46-50.
50. Moehn, S., P. Groenewegen, and R.O. Ball, *Amino acid digestibility of a yeast-derived protein source and gut endogenous losses in newly weaned pigs.* Livestock Science, 2010. **134**(1–3): p. 221-224.
51. Takasu, Y., H. Yamada, and K. Tsubouchi, *Isolation of Three Main Sericin Components from the Cocoon of the Silkworm, Bombyx mori.* Bioscience, Biotechnology, and Biochemistry, 2002. **66**(12): p. 2715-2718.
52. Chen, F., D. Porter, and F. Vollrath, *Silk cocoon (Bombyx mori): Multi-layer structure and mechanical properties.* Acta Biomaterialia, 2012. **8**(7): p. 2620-2627.
53. A. Sutcharit, C.S., C. Muangnapoh, V. Burapatana, and M. Phisalaphong, *Butanol production from sugarcane juice by Clostridium spp. in batch fermentation.* KKU Engineering journal, 2010. **37**: p. 339-347.
54. Tsujimoto, K., et al., *Cryoprotective Effect of the Serine-Rich Repetitive Sequence in Silk Protein Sericin.* Journal of Biochemistry, 2001. **129**(6): p. 979-986.
55. Aramwit, P., et al., *The Effect of Sericin with Variable Amino-Acid Content from Different Silk Strains on the Production of Collagen and Nitric Oxide.* Journal of Biomaterials Science, Polymer Edition, 2009. **20**(9): p. 1295-1306.
56. R. Shaheen, M.S., and D.T. Jones, *Comparative fermentation studies of industrial strains belonging to four species of solvent-producing clostridia.* Journal of Molecular Microbiology and Biotechnology, 2000. **2**: p. 115-124.
57. Yen, H.-W. and R.-J. Li, *The effects of dilution rate and glucose concentration on continuous acetone–butanol–ethanol fermentation by Clostridium acetobutylicum immobilized on bricks.* Journal of Chemical Technology & Biotechnology, 2011. **86**(11): p. 1399-1404.
58. Li, L., et al., *Enhanced butanol production by coculture of Clostridium beijerinckii and Clostridium tyrobutyricum.* Bioresource Technology, 2013. **143**(0): p. 397-404.
59. Barker, H.A., *Amino Acid Degradation by Anaerobic Bacteria.* Annual Review of Biochemistry, 1981. **50**(1): p. 23-40.
60. Chen, Y., et al., *Production of butanol from glucose and xylose with immobilized cells of Clostridium acetobutylicum.* Biotechnology and Bioprocess Engineering, 2013. **18**(2): p. 234-241.
61. Maddox, I.S., *The Acetone-Butanol-Ethanol Fermentation: Recent Progress in Technology.* Biotechnology and Genetic Engineering Reviews, 1989. **7**(1): p. 189-220.
62. Kittithanesuan N. , M.P., *Enhanced acetone-butanol production from sugarcane juice by immobilized Clostridium acetobutylicum (ATCC 824) on thin-shell silk cocoons.* Biotechnology and Bioprocess Engineering, 2015.
63. Phisalaphong, M., et al., *Alginate-loofa as carrier matrix for ethanol production.* Journal of Bioscience and Bioengineering, 2007. **104**(3): p. 214-217.

64. Rocha, M.H., et al., *Life cycle assessment (LCA) for biofuels in Brazilian conditions: A meta-analysis.* Renewable and Sustainable Energy Reviews, 2014. **37**(0): p. 435-459.
65. Menten, F., et al., *A review of LCA greenhouse gas emissions results for advanced biofuels: The use of meta-regression analysis.* Renewable and Sustainable Energy Reviews, 2013. **26**(0): p. 108-134.
66. Rebitzer, G., et al., *Life cycle assessment: Part 1: Framework, goal and scope definition, inventory analysis, and applications.* Environment International, 2004. **30**(5): p. 701-720.
67. Kittithanesuan N. , M.P., *Thin-shell Silk Cocoon (TSC) as a Nitrogen Source of ABE Fermentation by Clostridium acetobutylicum.* Applied Mechanics and Materials, 2015. **705**: p. 14-18.
68. Wu, M., et al., *Life-cycle assessment of corn-based butanol as a potential transportation fuel*. 2007. p. Medium: ED.
69. Pereira, L.G., et al., *Life cycle assessment of butanol production in sugarcane biorefineries in Brazil.* Journal of Cleaner Production, 2015. **96**(0): p. 557-568.
70. Jiang, M., et al., *Enhanced acetone/butanol/ethanol production by Clostridium beijerinckii IB4 using pH control strategy.* Process Biochemistry, 2014. **49**(8): p. 1238-1244.
71. Kumar, M., et al., *Comparative economic assessment of ABE fermentation based on cellulosic and non-cellulosic feedstocks.* Applied Energy, 2012. **93**(0): p. 193-204.
72. Soam, S., et al., *Life cycle assessment of fuel ethanol from sugarcane molasses in northern and western India and its impact on Indian biofuel programme.* Energy, 2015. **83**(0): p. 307-315.

APPENDIX

APPENDIX A EXPERIMENTAL METHODS

A-1 Preparation of selection strong strain

Clostridium acetobutylicum ATCC 824 was obtained from the American Type Culture Collection. The stock culture of cells was stored as spore suspensions frozen at -20 ◦C. Reinforced Clostridia Medium (RCM) was used as medium for stock cultures. The selection done heat shock by the weak cells is died by heat at 75-80 ºC. For sterilization, TOMY SS-325 autoclave was used. Preparation steps of selection strong strain in details are:

1. Mix 7.6 g RCM powder with 200 ml de-ionized (DI) water in 500 ml glass beaker.
2. Stir the solution with magnetic stirrer until all powder is dissolved as indicated by the formation of clear yellowish solution.
3. Transfer 18 ml solution into 25 x 200 mm screw cap culture tube.
4. Sterilize all RCM containing tubes, line for purge N_2 and tip 1 ml at 121ºC for 15 minutes in autoclave. (Set the tube's cap to be rather loose before autoclaving to facilitate gas expansion inside the tube during sterilization)
5. Check item in UV laminar flow follows: micropipette 1 ml, tissues paper, alcohol lamp and lighters.
6. After sterilization, tighten the tube's cap and let to cool down the tubes, line for purge N_2 and tip 1 ml in UV laminar flow 30-60 min. And leave stock culture out from freezer and let to dissolve.
7. After 30-60 min in UV laminar flow, burn RCM cap tube and purge N_2 about 5-10 min. After that, leave 2 ml stock culture into RCM tube and close tube.
8. After add stock culture, heat shock the RCM tubes at 80 ºC, 10 min.
9. Finally, incubate in shaker at115 rpm, 35°C for 48 hours.

A-2 Preparation to propagate cells

The strong cells of *Clostridium acetobutylicum* ATCC 824 propagate among cells in Reinforced Clostridia Medium (RCM). Preparation steps of propagate among cells in details are:

1. Precipitate the cells in RCM tube in part selection strong stain with gravity.
2. Preparation of new RCM with mix 7.6 g RCM powder with 200 ml de-ionized (DI) water in 500 ml glass beaker. After that stir the solution with magnetic stirrer until all powder is dissolved as indicated by the formation of clear yellowish solution. And then transfer 18 ml solution into 25 x 200 mm screw cap culture tube.
3. Sterilize all RCM containing tubes, line for purge N_2 and tip 1 ml at 121ºC for 15 minutes in autoclave. (Set the tube's cap to be rather loose before autoclaving to facilitate gas expansion inside the tube during sterilization.) And check item in UV laminar flow follows: micropipette 1 ml, tissues paper, alcohol lamp and lighters
4. After sterilization, tighten the tube's cap and let to cool down the tubes, line for purge N_2 and tip 1 ml in UV laminar flow 30-60 min.
5. After 30-60 min in UV laminar flow, burn RCM cap tube and purge N_2 about

5-10 min.

6. The cells in RCM tube in part selection strong stain were precipitated. Leave out supernatant until the volume of sediment about 2 ml. After that, leave 2 ml of strong cells into RCM tube and close tube.
7. Finally, incubate in shaker at115 rpm, 35°C for 24 hours.

A-3 Preparation of medium in flask

1. Weigh chemical follows:

Chemical	Weigh (g)
$FeSO_4.7H_2O$	0.005
$MgSO_4.7H_2O$	0.15
KH_2PO_4	0.25
CH_3COONH_4	1.5
Yeast extract	4.2
Sucrose	20

2. Mix chemical with 500 ml de-ionized (DI) water in 1 L beaker.
3. Stir the solution with magnetic stirrer until all powder is dissolved as indicated by the formation of clear yellowish solution.
4. Transfer 60 ml solution into 600 ml flask.
5. Sterilize all flasks, line for purge N_2 and tip 1 ml at 121ºC for 15 minutes in autoclave.
6. Check item in UV laminar flow follows: micropipette 1 ml, tissues paper, alcohol lamp and lighters
7. After sterilization, let to cool down the flasks, line for purge N_2 and tip 1 ml in UV laminar flow 30-60 min.
8. After 30-60 min in UV laminar flow, purge N_2 about 10-15 min burn cap flask and. After that, leave 2 ml sediment culture from RCM tube to medium in flask.
9. Finally, incubate in shaker at115 rpm, 35°C for 48 hours.

A-4 Preparation of medium in fermenter

1. Weigh chemical follows:

Chemical	Weigh (g)
$FeSO_4.7H_2O$	0.006
$MgSO_4.7H_2O$	0.18
KH_2PO_4	0.3
CH_3COONH_4	1.8
Yeast extract	5.046
Carbon source	-

2. Mix chemical with 600 ml de-ionized (DI) water in 1L beaker.
3. Stir the solution with magnetic stirrer until all powder is dissolved as indicated by the formation of clear solution.
4. Transfer medium into fermenter. And add silk cocoon for immobilized support and N-source.
5. And then sterilize fermenters and line for purge N_2 at 121ºC for 15 minutes in autoclave.
6. Check item in UV laminar flow follows: tissues paper, alcohol lamp and lighters
7. After sterilization, let to cool down the fermenters and line for purge N_2 in UV laminar flow 30-60 min.
8. After 30-60 min in UV laminar flow purge N_2 about 30 min. After that, leave culture from flask into reactor.
9. Finally, incubate at 200 rpm, 35°C.

A-5 Cell immobilization

Thin-shell silk cocoon (TSSC) were immobilized support. Cells immobilized on surface of silk cocoon with adsorption technic. Process immobilization carries out in fermenter. *Clostridium acetobutylicun* ATCC 824 adsorb on surface of TSSC with them.

A-6 ABE fermentation

A-6.1 Batch fermentation

The cell culture was mixed with 600 mL molasses medium, which contained sugar at 80-100 g/L, $FeSO_4 \cdot 7H_2O$ 0.006 g, $MgSO_4 \cdot 7H_2O$ 0.18 g, KH_2PO_4 0.3 g, CH_3COONH_4 1.8 g, and Yeast extract 5.046 g in fermenter 1 L Biostat®, B Braun Biotech International, Germany. ABE fermentation were purged with N_2 in order to ensure 100% anaerobic conditions. The pH and temperature were controlled to pH 4.5-5 and 35°C and fermentations were carried out for 132-144 hr.

A-6.2 Repeated batch fermentation

The repeated batch operation was carried out in a glass bottles containing 600 ml of fermentation medium for each batch. The product sample was removed via sterilized silicone tube at the end of each cycle. After the removal all of the supernatant, 600 ml of fresh fermentation medium was poured into the glass tubes in order to commence the next cycle. Each repeated batch received of cultivation under anaerobic conditions.

A-7 Sugar analysis

Sugar (sucrose) concentration was determined using a modified DNS reagent method. All disaccharides in the samples and standard sucrose solutions were first hydrolyzed to their monomers by using acid solution at elevated temperature. The acid residue was then neutralized using a basic solution and the resulting precipitates were settled by centrifugation. After centrifugation, the supernatant was reacted with DNS reagent at high temperature resulting in the formation of brown colored solution. The solution was then diluted before being analyzed by using spectrophotometer. The absorbance of the sample was compared with standard sucrose solutions to obtain the corresponding sucrose concentration. Complete step by step procedures are provided in the following sections.

A-7.1 NaOH and HCl solution preparation

NaOH 20% w/v was prepared by dissolving 200 g of NaOH pellets in 100 mL of water. The reaction is highly exothermic so that the preparation should be done in water bath in order to avoid excess heat generation. Weighing time of NaOH pellets should be minimized because of the hygroscopic nature of NaOH. Solution of 37% w/v HCl was obtained by diluting concentrated HCl solution with DI water. Beware of the acid vapor and wear protective gloves when preparing the solutions. Commercially available HCl 37% can be also be used directly.

A-7.2 DNS reagent preparation

DNS powder is toxic and easy to airborne so that it should be handled with caution. This powder should be added slowly in the mixing process because it is not easy to dissolve. After preparation, the resulting yellow colored reagent is best used in fresh condition so that it is not suggested to keep unused for long time (more than 1

month). The reagent is usually kept in brown bottle to protect it from degradation originated from light for example sun light. The complete preparation steps are:

1. Dissolve 1.633 g NaOH 98% w/w in 20 ml of water. Mix the solution with magnetic stirrer.
2. Under stirring, slowly add 1 g of 3,5-dinitrosalicylic acid powder into the solution.
3. Dilute by adding 50 ml of water. Stir until it is homogeneous.
4. Add 30 g Na-K tartrate & mix it thoroughly.
5. Adjust the volume to 100 ml.
6. Keep the reagent for 3 days before use.

A-7.3 Standard sucrose solution preparation

Standard sucrose solutions were prepared first by making the source solution which was the solution with the highest sucrose concentration as the upper limit. The source solution was then diluted with water so that a set of standard solution with increasing sucrose concentration (for instance 0, 6.25, 12.5, 18.75, and 25% w/v) was obtained. The detailed procedures are as follows:

1. Dry 3.0 g sucrose at 100-105°C in hot air oven for 2 hours.
2. Put the dried sucrose in desiccator for cooling.
3. Dissolve 2.5 g of the sucrose in 10 ml of water to obtain the source solution.
4. Prepare each 2 ml standard solution in small labeled bottle by serial dilution of suitable amount of source solution and diluting it with water as shown in detail in Table A-7.3. Use auto pipette for the transfer purpose.

Table A-7.3 Standard sucrose solution preparation

Sugar concentration (% w/v)	Sucrose solution (ml)	Water (ml)
0	0	2.0
6.25	0.5	1.5
12.50	1.0	1.0
18.75	1.5	0.5
25.00	2.0	0

A-7.4 Sample treatment I

In the first treatment, sample was hydrolyzed using HCl 37% in boiled water bath. After the hydrolysis reaction was stopped, NaOH was added into the solution. The sample was then centrifuged for removing suspended solids. Procedures of the first treatment are:

1. Mix 0.2 ml of sample with 0.8 ml DI water in screw cap tube.
2. Blend the sample with 0.5 ml HCl 37%.
3. Put the tubes in boiling water bath for 10 minutes.
4. Stop the reaction by placing the tubes in ice bath.
5. Add 0.5 ml NaOH 20% w/v and then mix with vortex mixer.
6. Add 10 ml DI water and then mix with vortex mixer.
7. Centrifuge the sample at 2000 rpm for 20 minutes.
8. Precautions and notes:
   a) Use vortex mixer for mixing the fluid in the tubes.
   b) Be cautious when handling the hot apparatus.
   c) The level of boiled water and ice bath must be sufficiently higher than the liquid level in the tubes to ensure good heating and cooling of the sample.

A-7.5 Sample treatment II

In treatment II, supernatant obtained from treatment I was reacted with DNS reagent in boiled water bath. The solution's color transformed from yellow to reddish brown in the course of reaction. The color intensity represents the corresponding sugar concentration. Solution with higher sugar content will have darker color. After the reaction was ended, the solution was diluted with sufficient amount of water until its absorbance spectrum obtained by spectrophotometer was well distributed along the range of concentration being considered (the absorbance measured was not more 0.7). Shimadzu UV-2450 UV-Visible spectrophotometer was used for absorbance measurement. Sample containing only water (0% sugar) which had been treated in the same manner as the other samples was used as blank. At every absorbance

measurement, fresh standard solution should be used. Complete procedures are described in the following paragraph.

1. Mix 0.2 ml of supernatant obtained from treatment I with 1.0 ml DNS reagent in screw cap tube.
2. Boil the solution for 10 minutes using water bath.
3. Put the tubes in ice bath to stop the reaction.
4. Add 10 ml DI water and then mix with vortex mixer.
5. Measure the absorbance at 520 nm. Use sample with 0% sugar as blank.
6. Obtain the standard curve by plotting absorbance versus sucrose concentration of standard sucrose solution.
7. Use the standard curve to gain sugar concentration of the samples.

A-8 Specimen preparation for SEM

1. Fix specimens in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 2 h/overnight at 4^{o}C.
2. Rinse specimens twice in phosphate buffer for 10 min/each and once in distilled water for 10 min.
3. Dehydrate specimens with a graded series of ethanol. (30%, 50%, 70%, 95% 10 min/each and absolute ethanol 3 times, 10 min/time).
4. Critical point dry (Critical point dryer, Balzers model CPD 020), mount and coat with gold (Sputter coater, Balzers model SCD 040).
5. Observe under a SEM (JEOL, model JSM-5410LV).

APPENDIX B EXPERIMENTAL DATA

B-1 Experimental data of batch fermentation with different organic nitrogen sources

Table B-1.1 Data of ethanol, acetic acid, acetone, butanol, butyric acid, reducing sugar and cell concentrations of ABE fermentation using yeast extract (YE) as N-source. The initial sugar concentration, presented sugarcane juice, of 80 g/L was utilized.

Time	Concentrations (g/L)						
(h)	Ethanol	Acetone	Acetic acid	Butanol	Butyric acid	Reducing sugar	Cells
0	0.05	0.04	0.78	0.29	0.40	80.00	0.22
15	0.36	0.13	1.59	0.39	3.66	64.80	0.22
24	0.91	0.18	1.64	0.46	6.87	62.72	0.19
39	1.06	0.18	7.29	0.55	7.24	56.31	0.30
48	1.14	0.14	7.27	0.59	7.54	48.42	0.43
63	1.17	0.18	7.39	0.61	9.92	45.88	0.89
72	1.46	0.54	6.39	1.81	15.48	36.65	1.42
75	1.44	0.72	6.24	2.05	15.28	34.71	1.49
87	1.40	0.92	6.04	6.27	14.09	22.94	1.64
93	1.49	1.03	5.38	8.08	14.09	17.13	1.61
96	1.47	2.23	4.99	10.06	13.80	11.47	1.61
99	1.40	2.38	5.12	11.73	13.60	11.02	1.45
111	1.40	2.69	4.37	12.76	13.26	10.13	1.43
117	1.41	3.40	4.59	13.53	12.02	8.79	1.45
120	1.49	3.35	3.80	13.95	12.07	7.75	1.37
123	1.48	3.37	3.64	14.04	11.91	7.15	1.39
132	1.47	3.46	3.01	14.03	11.88	6.70	1.36
144	1.41	3.45	4.72	14.00	12.78	6.53	1.34

Table B-1.2 Data of ethanol, acetic acid, acetone, butanol, butyric acid, reducing sugar and cell concentrations of ABE fermentation using thin-shell silk cocoon fragments ($TSSC_f$) as N-source. The initial sugar concentration, presented sugarcane juice, of 80 g/L was utilized.

Time	Concentrations (g/L)						
(h)	Ethanol	Acetone	Acetic acid	Butanol	Butyric acid	Reducing sugar	Cells
0	0.05	0.07	0.33	0.31	0.41	80.00	0.23
17	0.36	0.11	2.34	0.19	1.05	76.36	0.44
24	0.66	0.11	3.12	0.24	4.32	76.36	0.54
41	1.51	1.19	4.71	2.04	7.51	74.55	1.48
48	1.44	1.95	7.92	2.80	9.78	72.73	1.50
65	1.44	2.70	7.33	4.56	10.76	70.91	1.60
72	1.46	2.87	7.22	5.47	9.03	65.45	1.79
89	1.51	3.33	6.17	5.28	9.10	58.18	1.84
96	1.56	3.38	5.67	5.84	8.35	36.36	1.86
113	1.65	3.24	5.28	5.80	8.29	27.27	1.93
120	1.52	3.53	5.60	6.41	9.51	20.00	2.13
132	1.57	3.70	5.60	6.78	9.10	16.36	2.08
144	1.47	3.49	4.90	6.73	8.27	5.45	2.04

Table B-1.3 Data of ethanol, acetic acid, acetone, butanol, butyric acid, reducing sugar and cell concentrations of ABE fermentation using the mixture of yeast extract (YE) and thin-shell silk cocoon fragments ($TSSC_f$) as N-source. The initial sugar concentration, presented sugarcane juice, of 80 g/L was utilized.

Time	Concentrations (g/L)						
(h)	Ethanol	Acetone	Acetic acid	Butanol	Butyric acid	Reducing sugar	Cells
0	0.08	0.08	1.21	0.47	1.78	80.00	0.34
17	1.04	0.84	1.37	1.65	6.38	69.15	0.70
24	1.19	1.12	2.68	2.22	8.03	33.90	0.82
41	1.65	1.27	7.41	2.06	9.96	18.98	1.07
48	1.67	1.25	7.74	1.80	13.26	17.63	1.24
65	1.60	1.20	7.08	1.82	13.26	16.27	1.49
72	1.57	1.33	7.21	2.10	11.11	16.27	1.90
89	1.55	1.76	6.93	2.86	11.05	14.92	2.17
96	1.55	1.89	6.25	3.15	10.81	12.20	2.10
113	1.51	3.20	6.36	5.64	11.92	10.85	2.16
120	1.57	4.28	6.18	6.90	10.84	10.85	2.13
132	1.54	6.80	6.20	11.01	12.10	5.42	2.08
144	1.50	8.03	6.14	12.72	12.18	5.42	2.04

B-2 Experimental data of batch fermentation with different initial sugar concentration (80, 90 and 100 g/L) using immobilized cells on thin-shell silk cocoon.

Table B-2.1 Data of ethanol, acetic acid, acetone, butanol, butyric acid, reducing sugar and cell concentrations of ABE fermentation using the initial sugar concentration, presented sugarcane juice, of 80 g/L was utilized.

Time	Concentrations (g/L)						
(h)	Ethanol	Acetone	Acetic acid	Butanol	Butyric acid	Reducing sugar	Cells
0	0.05	0.00	1.15	0.40	1.17	80.00	0.29
18	0.12	0.30	3.65	2.47	3.71	73.30	0.15
30	0.52	0.37	3.89	2.78	8.24	68.07	0.24
42	0.90	0.39	8.04	4.38	12.97	49.66	0.25
54	1.03	0.47	8.58	4.81	16.43	46.48	1.06
66	1.17	1.30	6.83	6.49	16.02	36.36	1.28
78	1.26	2.08	6.85	8.34	14.85	33.75	1.32
90	1.40	2.13	4.72	8.61	12.15	25.34	1.27
96	1.40	3.28	4.61	13.90	12.65	21.25	1.11
108	1.62	4.23	4.35	13.87	11.97	18.75	1.12
114	1.65	4.20	4.50	14.55	11.84	15.68	1.11
126	1.65	4.15	4.36	14.80	11.87	13.18	1.10
132	1.64	4.19	4.46	15	11.64	8.30	1.09

Table B-2.2 Data of ethanol, acetic acid, acetone, butanol, butyric acid, reducing sugar and cell concentrations of ABE fermentation using the initial sugar concentration, presented sugarcane juice, of 90 g/L was utilized.

Time	Concentrations (g/L)						
(h)	Ethanol	Acetone	Acetic acid	Butanol	Butyric acid	Reducing sugar	Cells
0	0.16	0.00	1.27	0.30	0.60	90.00	0.20
18	1.04	0.00	2.45	0.06	0.98	78.09	0.21
30	1.15	0.17	3.26	0.49	9.56	75.55	0.27
42	1.49	0.46	5.78	0.82	13.59	52.91	1.17
54	1.41	0.73	7.96	1.00	15.15	50.37	1.55
66	1.48	0.62	6.90	0.99	16.23	47.83	1.77
78	1.44	0.78	6.12	1.01	16.68	44.51	2.08
90	1.41	0.85	6.30	1.20	13.15	36.90	2.05
96	1.40	1.15	5.89	1.91	12.94	33.19	2.07
108	1.59	2.12	6.34	4.32	10.79	28.50	2.09
114	1.63	3.61	5.84	8.44	10.21	24.21	1.96
126	1.86	5.17	6.16	13.36	10.18	19.13	1.94
132	1.92	6.06	6.28	15.92	10.09	14.64	1.93

Table B-2.3 Data of ethanol, acetic acid, acetone, butanol, butyric acid, reducing sugar and cell concentrations of ABE fermentation using the initial sugar concentration, presented sugarcane juice, of 100 g/L was utilized.

Time	Concentrations (g/L)						
(h)	Ethanol	Acetone	Acetic acid	Butanol	Butyric acid	Reducing sugar	Cells
0	0.04	0.10	1.79	0.42	1.77	100.00	0.17
18	0.66	0.70	1.83	0.22	2.49	83.84	0.17
30	0.68	0.92	2.08	0.26	2.77	83.33	0.22
42	0.76	1.14	4.15	2.95	8.78	80.81	0.96
54	0.74	1.28	4.28	2.96	12.64	79.29	1.26
66	0.88	1.42	2.03	3.28	10.52	78.28	1.45
78	1.01	1.51	2.00	3.38	9.55	77.27	1.70
90	1.09	1.44	2.29	3.54	9.61	70.20	1.67
96	1.20	1.70	2.16	3.70	8.47	68.18	1.69
108	1.27	1.75	2.10	3.80	6.12	59.60	1.71
114	1.27	1.86	2.17	4.03	5.95	57.58	1.60
126	1.41	1.89	2.17	4.65	5.75	54.04	1.58
132	1.35	1.88	2.25	5.04	5.45	40.91	1.57

B-4 Experimental data of repeated batch fermentation

Table B-4.1 Data of ethanol, acetic acid, acetone, butanol, butyric acid, reducing sugar and cell concentrations of 4 cycles repeated batch of ABE fermentation using the initial sugar concentration, presented sugarcane juice, of 80 g/L was utilized.

Batch	Time (h)	Concentrations (g/L)						
		Ethanol	Acetone	Acetic acid	Butanol	Butyric acid	Reducing sugar	Cells
I	0	0.09	0.03	0.22	0.41	2.01	80.00	0.94
	3	0.72	0.03	0.29	0.13	4.54	76.87	1.18
	15	0.81	0.30	0.50	0.60	9.18	57.91	1.69
	24	0.93	0.99	0.52	2.30	12.06	39.62	2.11
	27	0.93	1.48	0.54	2.64	12.87	39.24	2.15
	39	0.93	2.16	5.42	4.38	13.81	24.08	2.33
	48	1.01	2.53	6.13	4.81	17.03	22.65	2.30
	51	0.99	2.53	5.78	5.55	16.49	20.66	2.28
	63	1.04	2.46	5.73	5.52	16.13	17.91	2.26
	72	0.98	2.59	6.14	5.77	17.01	17.06	2.21
II	0	0.67	0.70	0.47	2.00	5.47	74.50	1.13
	3	0.71	0.66	0.74	2.08	5.87	63.13	2.42
	15	1.06	0.70	0.79	6.50	7.51	52.42	2.57
	24	1.14	1.92	0.89	6.82	11.54	44.83	2.40
	27	1.12	2.67	1.30	11.77	11.16	27.77	2.58
	39	1.07	6.35	2.67	12.29	10.74	16.68	2.42
	48	1.09	6.41	6.12	13.02	9.57	15.83	2.43

Table B-4.1 (continue) Data of ethanol, acetic acid, acetone, butanol, butyric acid, reducing sugar and cell concentrations of 4 cycles repeated batch of ABE fermentation using the initial sugar concentration, presented sugarcane juice, of 80 g/L was utilized.

Batch	Time	Concentrations (g/L)						
	(h)	Ethanol	Acetone	Acetic acid	Butanol	Butyric acid	Reducing sugar	Cells
III	0	0.67	1.18	0.35	2.98	5.60	74.98	1.31
	3	0.93	2.65	0.43	4.88	10.24	61.99	2.47
	15	1.00	3.90	2.32	6.96	12.15	50.33	2.55
	24	1.00	4.45	2.40	8.18	11.97	49.19	2.53
	27	1.12	4.60	2.37	9.17	10.24	38.77	2.53
	39	1.09	6.25	3.84	12.06	9.31	24.27	2.62
	48	1.18	6.87	5.97	12.67	9.01	18.67	2.50
IV	0	0.72	1.97	0.25	4.90	4.56	74.31	1.60
	3	0.59	2.26	0.33	4.84	12.76	58.77	2.59
	15	0.86	4.52	2.24	7.19	12.47	47.01	2.70
	24	0.94	4.68	2.36	8.35	12.04	46.16	2.74
	27	0.95	4.82	6.07	8.85	12.24	30.05	3.05
	33	0.93	4.90	5.97	11.90	12.05	25.31	3.04
	39	0.98	6.04	5.63	12.57	11.61	21.42	3.04
	48	1.00	6.50	5.99	12.79	11.29	18.48	3.12

Table B-4.2 Data of ethanol, acetic acid, acetone, butanol, butyric acid, reducing sugar and cell concentrations of 2 cycles repeated batch of ABE fermentation using the initial sugar concentration, presented sugarcane juice, of 90 g/L was utilized.

Batch	Time	Concentrations (g/L)						
	(h)	Ethanol	Acetone	Acetic acid	Butanol	Butyric acid	Reducing sugar	Cells
I	0	0.80	0.73	2.12	0.98	3.88	90.00	0.12
	3	0.95	0.96	2.25	1.22	5.35	86.48	0.23
	15	1.00	0.94	3.43	1.45	6.77	65.15	0.70
	24	1.23	1.14	3.60	1.97	12.10	55.24	0.70
	27	1.30	1.13	4.01	2.15	12.21	51.61	0.71
	39	1.53	1.26	4.71	2.66	13.65	48.41	1.02
	48	1.58	1.38	5.06	3.47	13.77	46.81	1.04
	51	1.53	1.44	4.69	3.82	11.19	45.64	1.41
	63	1.61	1.47	4.43	6.28	9.49	44.68	1.39
	72	1.60	1.48	4.27	6.98	9.04	40.52	1.30
II	0	0.90	0.36	2.31	0.81	8.39	65.05	0.18
	3	0.95	0.87	2.12	2.87	8.11	63.98	0.23
	15	0.90	1.01	2.70	3.25	10.57	61.10	0.36
	24	0.92	1.40	2.56	3.84	9.38	48.84	0.46
	27	0.93	1.47	2.33	3.60	8.22	46.71	1.00
	39	0.92	2.21	2.36	3.32	7.21	42.12	0.71
	48	0.90	2.28	4.58	3.25	7.65	34.44	0.72

Table B-4.3 Data of ethanol, acetic acid, acetone, butanol, butyric acid, reducing sugar and cell concentrations of 2 cycles repeated batch of ABE fermentation using the initial sugar concentration, presented sugarcane juice, of 100 g/L was utilized.

Batch	Time	Concentrations (g/L)						
	(h)	Ethanol	Acetone	Acetic acid	Butanol	Butyric acid	Reducing sugar	Cells
I	0	0.09	0.11	0.10	0.77	1.70	100.00	0.11
	3	0.66	0.12	0.14	0.97	2.49	94.06	0.24
	15	0.68	0.92	0.20	1.57	12.11	89.57	0.59
	24	0.80	1.04	0.40	1.73	12.19	83.37	0.48
	27	0.85	1.13	0.72	2.19	12.37	80.40	0.46
	39	0.91	1.18	0.72	2.38	12.86	78.49	0.45
	48	1.08	1.31	0.68	3.16	11.65	77.86	0.35
	51	1.12	1.77	0.60	3.45	10.46	74.64	0.18
	63	1.17	2.09	0.47	3.75	9.55	73.87	0.15
	72	1.23	1.83	0.38	4.06	7.15	69.69	0.15
II	0	0.29	1.44	0.37	2.19	5.87	84.83	0.14
	3	0.33	1.10	0.31	2.66	5.50	83.28	0.16
	15	0.76	1.14	4.15	2.95	5.87	81.14	0.22
	24	0.74	1.28	4.28	2.96	6.80	77.64	0.22
	27	0.88	1.42	2.03	3.28	10.52	76.71	0.18
	39	1.01	1.51	2.00	3.38	9.55	73.51	0.18
	48	1.09	1.44	2.29	3.54	9.61	73.16	0.17

APPENDIX C CALCULATION

C-1 Productivity

$$\text{Productivity}\quad (g/L.h) = \frac{ABE\quad Concentration\quad (g/L)}{Fermentation\quad Time\quad (h)}$$

C-2 Yield

$$Yield = \frac{ABE\quad Concentration\quad (g/L)}{Sugar\quad Consumption\quad Concentration\quad (g/L)}$$

C-3 Conversion

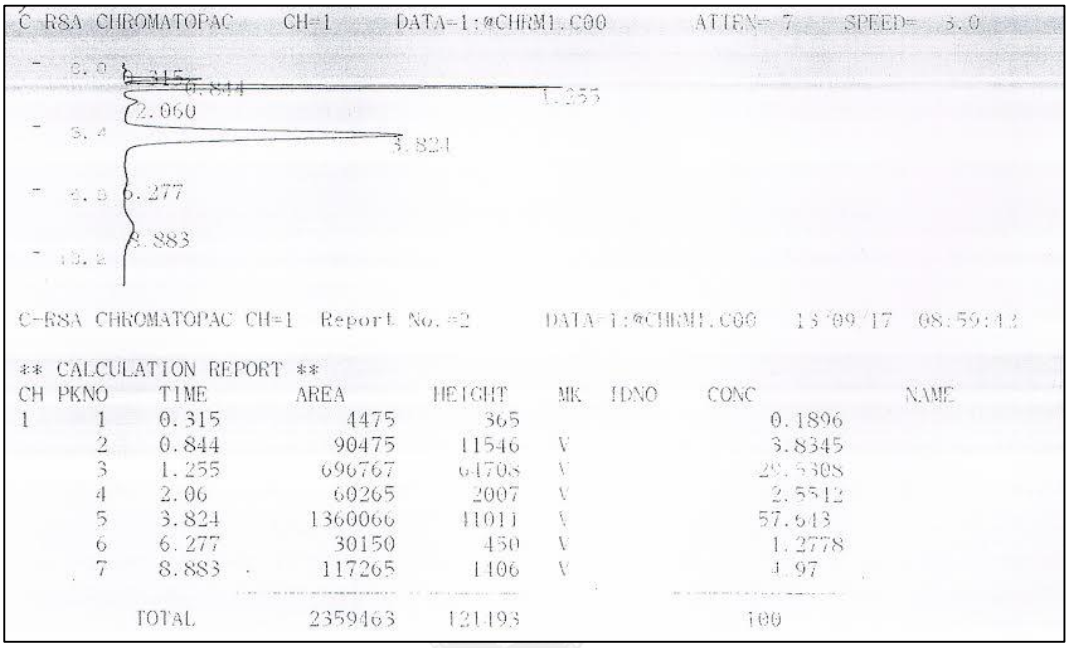
$$Conversion = \frac{ABE\quad Concentration\quad (g/L)}{Initail\quad Sugar\quad Concentration\quad (g/L)}$$

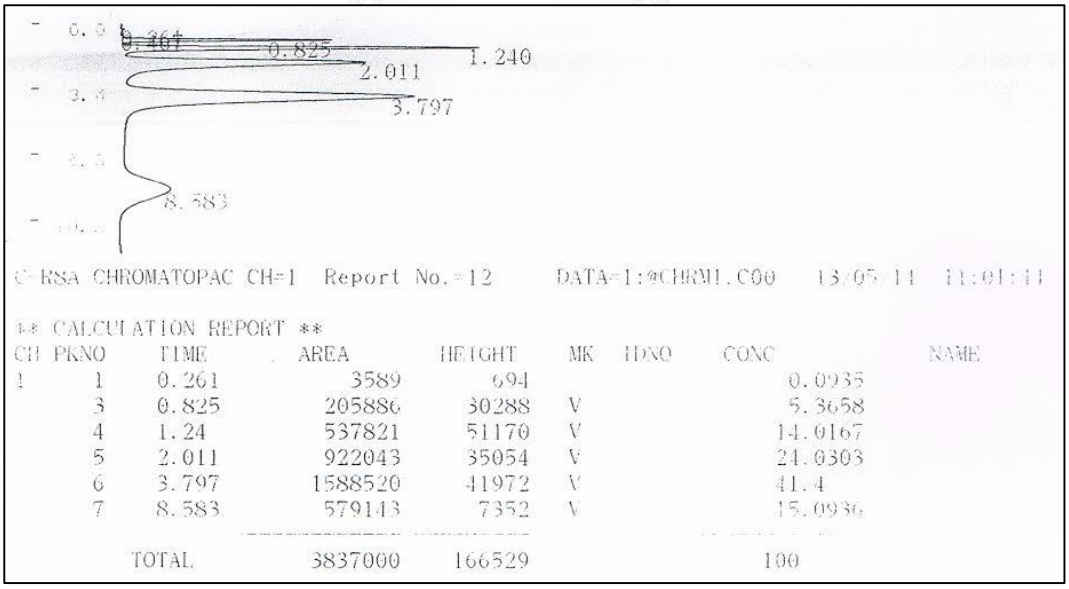
C-4 Percent remaining sugar

$$Percent\quad \text{Remaining}\quad Sugar\quad (\%) = \frac{\text{Remaining}\quad Sugar\quad Concentration\quad (g/L)}{Initail\quad Sugar\quad Concentration\quad (g/L)} \times 100$$

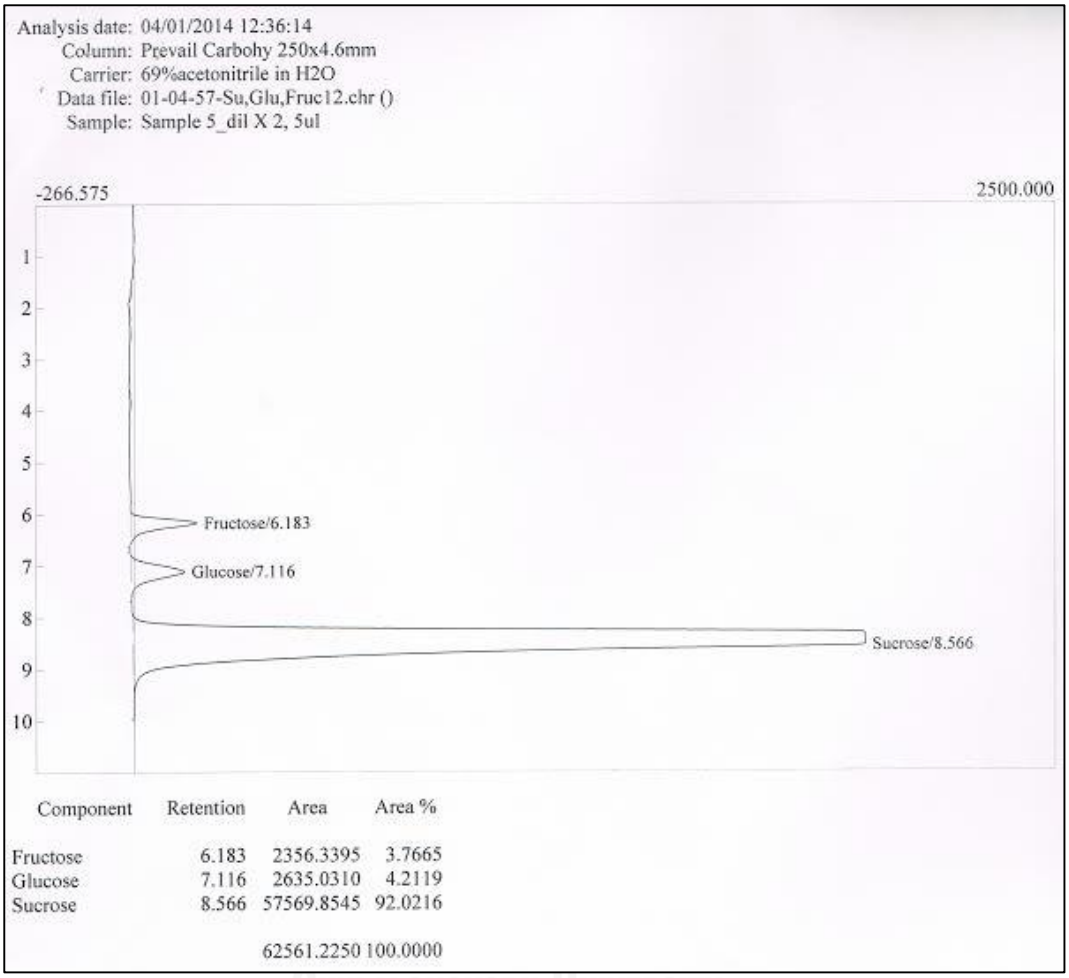
APPENDIX D PICTURE

D-1 Example of result from GC





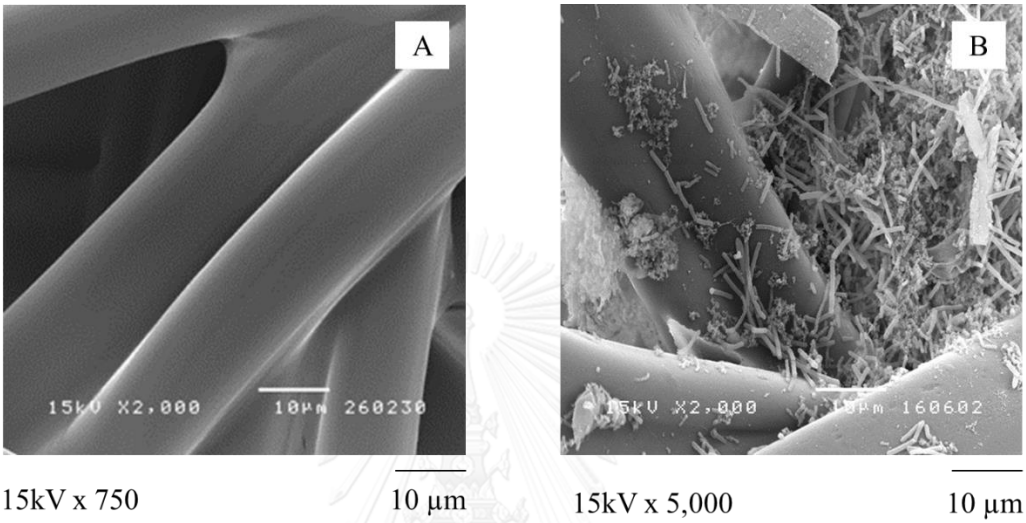
D-2 Example of result from HPLC



Component	Retention	Area	Area %
Fructose	6.183	2356.3395	3.7665
Glucose	7.116	2635.0310	4.2119
Sucrose	8.566	57569.8545	92.0216
		62561.2250	100.0000

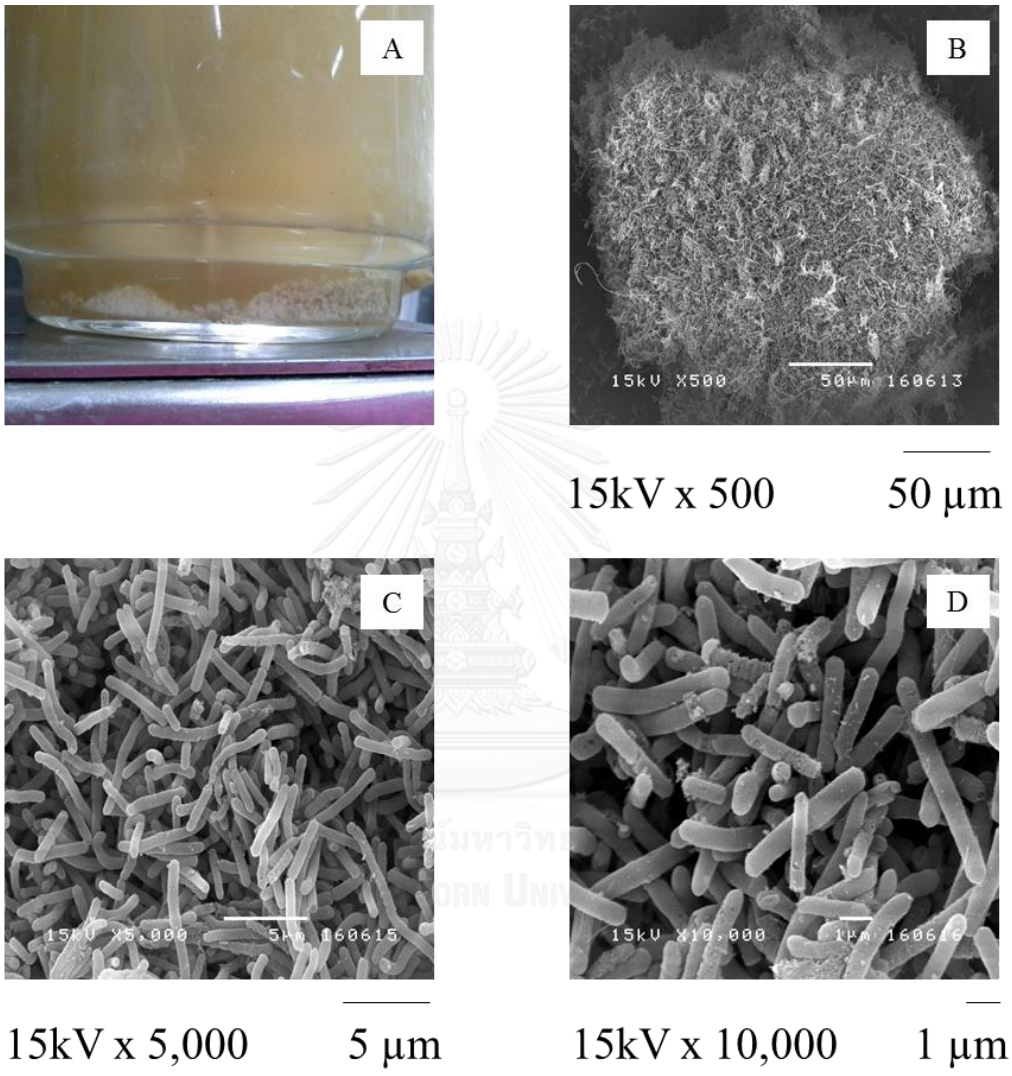
D-3 SEM images

D-3.1 SEM images of thin-shell silk cocoons before the ABE fermentation (A) and *C. acetobutylicum* (ATCC 824) in the systems using the immobilized cells on thin-shell silk cocoons (IC-TSSC) after the ABE fermentation (B)



15kV x 750 10 µm 15kV x 5,000 10 µm

D-3.2 The sediment of *C. acetobutylicum* (ATCC 824) (A) and SEM images of *C. acetobutylicum* in the systems using the immobilized cells on thin-shell silk cocoons (IC-TSSC) (B), (C) and (D)



VITA

Miss Nutshera Kittithanesuan was born on November 8th, 1983 in Bangkok, Thailand. She received her Bachelor's Degree of Chemical Engineering at Thammasat University in Apirl 2007 and Master Degree in Chemical Engineering, Kasetsart University in Apilr 2009. She continued Ph.D. degree in Chemical Engineering at Chulalongkorn University in June, 2012.

Research contributions

-Analysis of Combustion and Emission Characteristics of Cylindrical, Honey-comb Cross-section Coal Briquettes using an Experimental Design Technique, Proceeding for the conference of TiChe, 2007.

-On the Size and g-factor of Uniform Star Polymers in a Dilute Solution: A Monte Carlo Simulation, Kasetsart Journal, Natural Science, Vol. 43, pp. 198, 2009.

-Conformation and Size of Semi-flexible, Multi-block Copolymer Chains, 2nd International Conference on Polyolefin Characterization, Valencia, Spain, 2008.

-Characteristics and Conformation of Linear Homopolymers with Flory Distribution, Meeting of Modelling and Simulation Society of Thailand, 2008.

-Characteristics and Conformation of Semi-flexible Diblock Copolymers, The 17th National Chemical Engineering and Applied Chemistry Conference, 2007.

-Thin-shell silk cocoon (TSC) as a nitrogen source of ABE fermentation by Clostridium acetobutylicum, Applied mechanics and Materials, Vol. 705, pp 14-18, 2015.

-Enhanced acetone-butanol production from sugarcane juice by immobilized Clostridium acetobutylicum (ATCC 824) on thin-shell silk cocoons, Biotechnology and Bioprocess Engineering, 2015. (in press)

-กระบวนการหมักอะซิโตน-บิวทานอล-เอทานอล โดยใช้เทคนิคการตรึงเซลล์แบคทีเรียสายพันธุ์ในกลุ่มคลอสตริเดียม (Clostridium ap.) ด้วยรังไหมหรือรังไหมบาง (สิทธิบัตรเลขที่คำขอ 1301007002)