Screening of *Clostridium* sp. for butanol production from Sato (Thai rice wine) factory wastewater

Miss Julalak Buranaprasopchai

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
FACULTY OF SCIENCE
Chulalongkorn University
Academic Year 2022

การคัดกรอง *Clostridium* sp. เพื่อการผลิตบิวทานอลจากน้ำเสียโรงงานผลิตสาโท (ไวน์ข้าว)

น.ส.จุฬาลักษณ์ บูรณะประสพชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ ไม่สังกัดภาควิชา/เทียบเท่า
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2565

Thesis Title	Screening of *Clostridium* sp. for butanol production from Sato (Thai rice wine) factory wastewater
By	Miss Julalak Buranaprasopchai
Field of Study	Biotechnology
Thesis Advisor	Professor WARAWUT CHULALAKSANANUKUL, Ph.D.
Thesis Co Advisor	Professor Isabelle Meynial-Salles, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the FACULTY OF SCIENCE
(Professor POLKIT SANGVANICH, Ph.D.)

DISSERTATION COMMITTEE

..... Chairman
(Associate Professor PATTAMAWADEE YANATATSANEEJIT, Ph.D.)

..... Thesis Advisor
(Professor WARAWUT CHULALAKSANANUKUL, Ph.D.)

..... Thesis Co-Advisor
(Professor Isabelle Meynial-Salles, Ph.D.)

..... Examiner
(Associate Professor SUCHADA CHANPRATEEP NAPATHORN, Ph.D.)

..... Examiner
(Associate Professor SIRILUX POOMPRADUB, Ph.D.)

..... Examiner
(Assistant Professor CHOMPUNUCH GLINWONG, Ph.D.)

..... External Examiner
(Nassapat Boonvitthya, Ph.D.)

จุฬาลักษณ์ บูรณะประสพชัย : การคัดกรอง *Clostridium* sp. เพื่อการผลิตบิวทานอลจากน้ำเสียโรงงานผลิตสาโท (ไวน์ข้าว). (Screening of *Clostridium* sp. for butanol production from Sato (Thai rice wine) factory wastewater) อ.ที่ปรึกษาหลัก : ศ. ดร.วรวุฒิ จุฬาลักษณานุกูล, อ.ที่ปรึกษาร่วม : ศ. ดร.อิซาเบล เมย์เนียล แซลล์

สาโทจัดเป็นเครื่องดื่มแอลกอฮอล์พื้นบ้านของไทยชนิดหนึ่ง และกระบวนการผลิตสาโทมักก่อให้เกิดน้ำเสียเป็นจำนวนมาก โดยส่วนประกอบของน้ำเสียมักประกอบไปด้วยเศษข้าวที่หลงเหลือจากกระบวนการหมัก จึงส่งผลให้ปริมาณของบีโอดี และซีโอดีในน้ำสูง ก่อให้เกิดปัญหาสิ่งแวดล้อม โดยน้ำเสียจะส่งกลิ่นเหม็นคล้ายไข่เน่าที่เกิดจากแก๊สไฮโดรเจนซัลไฟด์ เพื่อแก้ปัญหาดังกล่าวในงานวิจัยนี้จึงทำการศึกษาองค์ประกอบของน้ำเสียทั้งทางกายภาพและเคมี เพื่อประเมินศักยภาพในการนำน้ำเสียมาใช้เป็นสารตั้งต้นสำหรับกระบวนการหมักแอซีโทน-บิวทานอล-เอทานอล (ABE) ด้วยแบคทีเรียสกุลคลอสทริเดียม ซึ่งยังไม่เคยมีรายงานถึงการนำน้ำเสียจากโรงงานผลิตสาโทมาใช้ในการผลิต ABE มาก่อน แบคทีเรียสกุลคลอสทริเดียมที่คัดแยกได้จากแหล่งธรรมชาติในประเทศไทย และสามารถผลิตบิวทานอลได้ในปริมาณสูงสุดคือ ไอโซเลท CUEA02 โดยสามารถผลิตบิวทานอลได้ 8.32 ± 0.08 กรัมต่อลิตร ในการหมักระดับฟลาสก์ที่มีการควบคุมความเป็นกรด-ด่างเริ่มต้นเท่ากับ 6.5 อุณหภูมิ 35 องศาเซลเซียส หัวเชื้อร้อยละ 10 และน้ำตาลกลูโคส 50 กรัมต่อลิตร จากนั้นทำการวิเคราะห์ลำดับจีโนมของแบคทีเรียที่คัดแยกได้ เพื่อใช้ระบุสายพันธุ์และประเมินความสามารถในการใช้แหล่งคาร์บอนต่าง ๆ ซึ่งพบว่าไอโซเลท CUEA02 มีความใกล้เคียงกับ *Clostridium beijerinckii* โดยมีค่าเฉลี่ยความเหมือนของนิวคลีโอไทด์ (%ANI) ร้อยละ 95.14 และสามารถเจริญเติบโตและผลิตบิวทานอลในแหล่งคาร์บอนต่าง ๆ ได้ดี โดยเฉพาะแหล่งคาร์บอนที่เป็นแป้งและคาร์บอกซีเมทิลเซลลูโลส จากนั้นทำการประเมินความสามารถในการใช้น้ำเสียเพื่อผลิต ABE ด้วยการนำน้ำเสียมาเจือจาง 10, 20, 30 และ 40 เท่า ตามลำดับ โดยไอโซเลท CUEA02 สามารถผลิตบิวทานอลได้ 0.23 กรัมต่อกรัม ภายใต้การเจริญเติบโตในสภาวะไร้อากาศในน้ำเสียที่ถูกเจือจาง 10 เท่า และเมื่อทำการปรับค่าความเป็นกรด-ด่างจาก 4.75 เป็น 6.5 ส่งผลให้ผลผลิตบิวทานอลเพิ่มขึ้น 2.13 เท่า (0.49 กรัมต่อกรัม) นอกจากนี้ยังพบว่าการเติมสารสกัดจากยีสต์ที่ 2 กรัมต่อลิตร สามารถช่วยเพิ่มการผลิตบิวทานอลในน้ำเสียได้ถึงร้อยละ 29

สาขาวิชา	เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต .....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5772808723 : MAJOR BIOTECHNOLOGY

KEYWORD: ABE fermentation, Butanol, Clostridium beijerinckii CUEA02, Sato wastewater

Julalak Buranaprasopchai : Screening of *Clostridium* sp. for butanol production from Sato (Thai rice wine) factory wastewater. Advisor: Prof. WARAWUT CHULALAKSANANUKUL, Ph.D. Co-advisor: Prof. Isabelle Meynial-Salles, Ph.D.

The production of Sato, a Thai traditional beverage, yields Sato wastewater (STW), which contains residual rice constituents with high chemical and biological oxygen demands and so forms an environmental problem. In addition, STW often smells rotten from the formation of hydrogen sulfide. To solve this problem, the composition of STW was analyzed for its physiological and chemical characteristics to evaluate its potential as a substrate for ABE fermentation using *Clostridium* spp., which has never been reported for use in acetone-butanol-ethanol (ABE) fermentation before. Solvent-producing *Clostridium* isolates were obtained from environmental sources in Thailand, and that producing the highest butanol level (isolate CUEA02 from the sludge of a biodiesel plant) was selected. The butanol production was 8.32 ± 0.08 g/L in flask fermentation under an initial pH of 6.5, 35 °C, 10% (v/v) inoculum size, and 50 g/L of glucose. Whole-genome sequencing analysis was used to identify this strain and its ability to use different carbon sources for ABE fermentation was evaluated. Isolate CUEA02 was closely related to *Clostridium beijerinckii* with an average nucleotide identity of 95.14% and could grow and produce butanol in various carbon sources, especially starch and carboxymethyl cellulose. The STW was diluted 1:10, 1:20, 1:30, and 1:40 for ABE production by CUEA02, where the obtained butanol yield (0.23 g/g) under an anaerobic condition was highest with the 1:10 diluted STW. To improve the butanol production, the initial pH was adjusted from 4.75 to 6.5, resulting in a 2.13-fold enhanced butanol yield (0.49 g/g). Furthermore, adding yeast extract at 2 g/L enhanced butanol production in STW utilizing *C. beijerinckii* CUEA02 by up to 29%.

Field of Study:	Biotechnology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

Firstly, I would like to express my gratitude to my advisor, Professor Warawut Chulalaksananukul, Ph.D., for accepting me as a Ph.D. student and supporting my educational opportunities. Moreover, I would like to thank him for his invaluable advice, guidance, support, and encouragement throughout my studies and thesis. I would like to thank my thesis co-advisor, Professor Isabelle Meynial-Salles, Ph.D., for great opportunities, suggestions, and assistance in facilitating my experimental work at the laboratory of the Toulouse Biotechnology Institute, Institut National des Sciences Appliquées (INSA), Toulouse, France.

I would like to thank members of my thesis committee, including Associate Professor Pattamawadee Yanatatsaneejit, Ph.D., Associate Professor Suchada Chanprateep Napathorn, Ph.D., Associate Professor Sirilux Poompradub, Ph.D., Assistant Professor Chompunuch Glinwong, Ph.D., and Nassapat Boonvitthya, Ph.D., for their kind assistance and helpful comments.

My sincere gratitude also goes to all the professors and staff in the Biotechnology Program and Department of Botany, Faculty of Science, Chulalongkorn University, for offering laboratory facilities for this research. Furthermore, I would like to express my gratitude to all members of the EAD3, INSA, Toulouse, and Biofuels by Biocatalyst Research Unit, Chulalongkorn University, for their friendship, warm support, assistance, and sharing of the best memories throughout my studies.

I would like to acknowledge the Chulalongkorn University Dussadeepipat Scholarship and the PTT Public Company Limited for their financial support with my doctoral scholarship.

Finally, I would like to express my heartfelt gratitude and deepest appreciation to my family for their unending love and strong encouragement, which offer me limitless willpower to solve problems and overcome obstacles throughout my Ph.D. life.

Julalak Buranaprasopchai

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

STW	Sato wastewater
ABE	Acetone-butanol-ethanol
BOD	Biochemical Oxygen demand
COD	Chemical Oxygen demand
VFAs	Volatile fatty acids
OM	Organic matter

CHAPTER I

INTRODUCTION

Recently, increasing worldwide attention on the energy crisis and global climate change has led to the further development of alternative energy as a substitute for fossil fuel (Cook, 2021). Biofuels are environmentally friendly energy sources to fulfill the global energy demand. Especially, butanol has attracted attention. Butanol has been produced biologically via anaerobic acetone-butanol-ethanol (ABE) fermentation using many solvent-producing clostridia. The advantages of *Clostridium* species are producing a high level of ABE production and producing endospore for resistance to the extreme environment. Moreover, it can utilize a large variety of substrates from monosaccharides and polysaccharides such as cassava, corn, and sugar cane (Kanno et al., 2013; Lee et al., 2012). In butanol fermentation, the cost of feedstocks can account for up to 75% of the total cost. Therefore, food waste, lignocellulosic biomass, and agricultural residues are non-food crops and considered the ideal substrate for butanol production (Wayne Chew et al., 2018). However, sugar extraction from these substrates is significantly more challenging.

Consequently, the main bottleneck with these substrate's fermentation is the high cost of pretreatment. To discover the sustainable feedstocks, alcoholic beverage wastewater was considered. Due to the use of wheat, rice, corn, molasses, and barley as a feedstock to produce alcoholic beverage. As a result, a large volume of rich carbohydrate wastewater is produced throughout the alcoholic beverage manufacturing process, especially Sato wastewater. Sato is a unique Thai traditional alcoholic beverage that made from glutinous rice and look pang (Sato yeast ball) (Dung et al., 2007). In addition to that, this wastewater still contains relatively high levels of carbohydrates. However, the BOD and COD in this wastewater has still a high concentration, and it always produce bad odor from hydrogen sulfide that severe to environment and human health (Athanasopoulos, 1987; Vijayaraghavan & Ramanujam, 2000). To solve this problem, this wastewater will be used to produce a value-added substance like butanol. Moreover, it has not been a previous report to use Sato wastewater as a substrate for ABE production. This research will focus on the screening and identification of *Clostridium* spp. from natural sources in Thailand. The potential of Sato wastewater as feedstock using a solventogenic *Clostridium* sp.

for butanol production will be investigated. Moreover, this research will estimate bacterial growth, ABE production, and optimization condition of solventogenic *Clostridium* sp.

Objectives of this study

1. To isolate and identify a solvent producing *Clostridium* sp. which produce butanol from natural sources in Thailand

2. To evaluate butanol production from the isolated *Clostridium* sp. using Sato wastewater as feedstock

CHAPTER II

LITERATURE REVIEWS

1. Biofuels – renewable energy for substitute petroleum product in the future

Fossil fuels are classified as a fuel that the world still requires and are insatiable, which supply 80% of our global energy requirements (Nigam & Singh, 2011) Over the following decade, oil consumption is expected to climb further, with the United States accounting for 85% of global output (Cook, 2021). Besides that, globalization, economic expansion, and population growth affected the demand for fossil fuels. Concerns about the security of oil supplies, along with issues regarding global warming caused by greenhouse gas emissions, have prompted a surge in research into alternate, renewable fuel sources. More sustainable feedstocks for fuel generation, such as biomass, natural oils, and waste gases, are being researched. These formerly regarded waste feedstocks can be turned into fuel using a number of techniques such as fractionation, liquefaction, transesterification, pyrolysis, hydrolysis, fermentation, and gasification (Demirbas, 2009). For example, hydrogen (H_2), carbon

monoxide, and carbon dioxide were derived from biomass, municipal waste, and plastic by the gasification process. However, these processes used the high temperature and pressure, including high costs of production. Therefore, the focus has been shifted toward the use of microbial catalysts. Due to these catalysts have many advantages over chemical catalysts. It used milder temperature, pressure, and low costs for operation.

The use of ammonia (NH_3)-, H_2-, and butanol-derived energy to replace coal and achieve zero emissions seems appealing. Although NH_3 has numerous benefits over H_2, including a higher volumetric energy density and a low cost, it also has the drawbacks of sluggish burning velocity and significant nitrogen oxide (NO_x) emissions. H_2 is a carbon-free fuel and a clean energy source. Nonetheless, the cost of transportation and equipment for liquid H_2 remains significant. As can be shown, both NH_3 and H_2 have drawbacks that make them unsuitable for widespread application (Chai, Chew, et al., 2021). Butanol is one of the bio-alcohols that has attracted great interest. It can be produced by the biological process using

Clostridium sp. In addition, butanol can also be employed in industry as a chemical precursor and solvent as well as a biofuel(Qureshi & Blaschek, 2001).

2. The genus *Clostridium* – the traditional species that produce acetone-butanol-ethanol (ABE) production via ABE fermentation

There are approximately 231 species in the genus *Clostridium. Clostridium* sp. is a gram-positive and obligate anaerobic bacteria. The morphology of *Clostridium* sp. is rod shaped and colony can be divided into several categories such as flat, raised, convex, pulvinate, and umbonate (Figure 1). The highlight of *Clostridium* sp. is producing an endospore to help them to survive in the harsh environment. In general, *Clostridium* sp. can grow from 3.3 to 80 °C depending on the species. However, the suitable of temperature for their growth is approximately 25 to 40 °C (Brasca et al., 2022). *Clostridium* sp. can produce industrially relevant products such as solvents and toxins, among other products. The most reported *Clostridium* strains to butanol production are *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* (Qureshi & Ezeji, 2008). On the other hand, *C. perfringens* produces potent toxins such as enterotoxin. In addition, *C. botulinum* and

C. tetani produce neurotoxins (Rupnik et al., 2005). Moreover, *Clostridium* sp. has potential to utilize various carbon sources including pentoses and hexoses. For example, sugar cane, corn, cassava, agro-industrial waste, and wastewater.

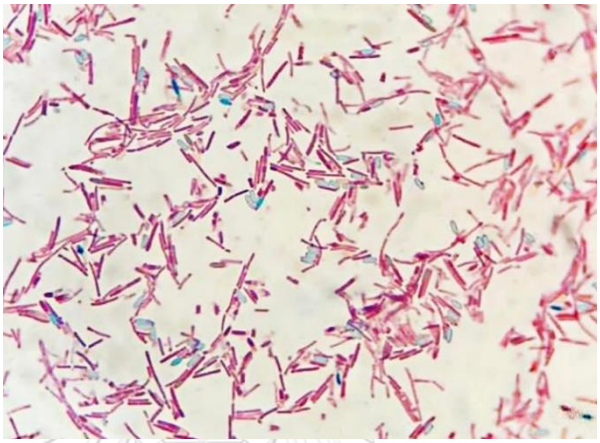


Figure 1 The morphology of *Clostridium* sp. – rod shaped cell and endospore forming (Buranaprasopchai et al., 2022).

The life cycle and growth of *Clostridium* sp. begin with the introduction of a carbohydrate source into the cell. When a carbohydrate source reaches the clostridial cells, it begins with the vegetative cell, which is formed like a rod. Clostridial cells produced organic acids such as acetate and butyrate during this stage, and the cells accumulated granulose. Following that, clostridial cells began to

sporulate, and organic solvents were produced. Furthermore, the cells became fatter and cigar-shaped. Finally, the cells sporulated (Figure 2).

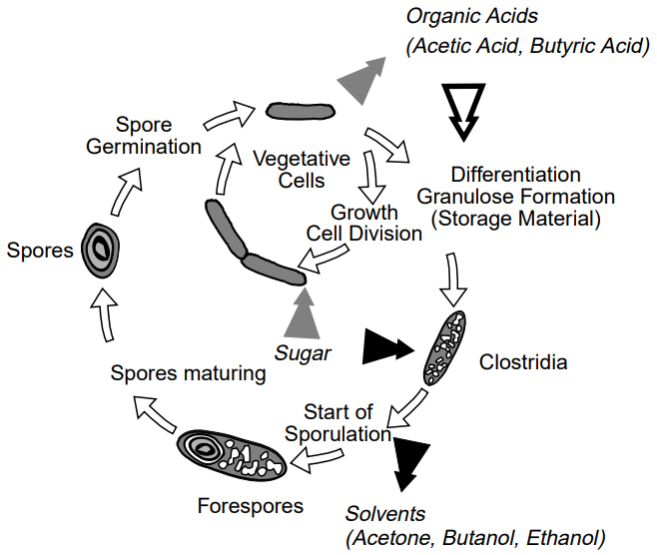


Figure 2 Cell cycle in each step of organic acids and solvents from *C. acetobutylicum* ATCC 824 (Schuster et al., 1998).

3. Acetone-butanol-ethanol fermentation – the history of ABE production for green energy in the future

In the early 20th century, acetone production was popular. Due to World War I, the high demand for acetone was rising. Acetone can be used as a precursor to produce explosive cordite (Jones & Woods, 1986; Killeffer, 1927). The first industrial scale of ABE fermentation was discovered in 1916 by Chaim Weizmann using *C.*

acetobutylicum. This bacteria strain was used and developed in ABE fermentation, which is widespread in many countries, such as the United States, England, and Russia. After that, various fermentation processes were developed, and the demand for butanol also increased. In 1936, Beesch (1952) stated that a more cost-effective process was developed that used molasses or other industrial sugars as carbon sources and reduced the fermentation temperature to 31 °C. It makes the ABE fermentation process economically viable. However, the increasing price of carbon sources and the rapid development of the petrochemical industry led to a decline in research on ABE fermentation and the closing of many ABE fermentation factories in several countries except South Africa, Russia, and China (Dürre, 2007; Jiang et al., 2015; Zverlov et al., 2006). In addition, the ABE production process has many problems, including the low yield of butanol, the toxicity of solvents, and the high cost of operation (Moon et al., 2016). In the 1970s, the oil crisis happened. Therefore, butanol has become attractive as a substitution for petrochemical fuel. After that, the study of molecular genetics was widespread and well known, and metabolic engineering to improve butanol production and reduce the toxicity of other solvents

from *Clostridium* sp. was evaluated. The timeline of ABE fermentation is shown in Figure 3

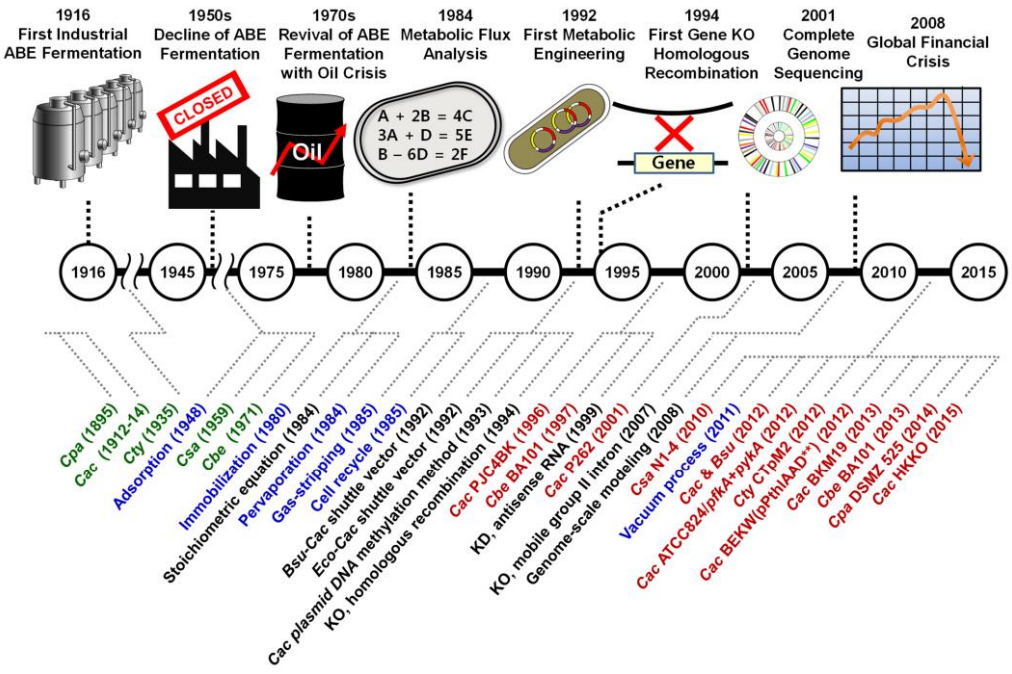


Figure 3 Timeline of the ABE production history of *Clostridium* sp. from 1916 to 2016 (Moon et al., 2016).

3.1 ABE fermentation factors

3.1.1 Type and concentration of substrate

The majority of the raw materials used in ABE fermentation are sugar, simple carbohydrates, and a few polymers (cassava, maize, sugarcane, and other agricultural residues). The initial sugar concentration is an important

factor in ABE fermentation. The initial sugar concentration was high (more than 20 g/L), which contributed to the low ABE production yield (Lee et al., 2008). If the initial sugar concentration was greater than 60 g/L, it could improve ABE production (Madihah et al., 2001). However, ABE production was reduced because the initial sugar concentration was greater than 80 g/L. ABE production is extremely low at an initial sugar concentration of 120 g/L. (Ezeji et al., 2003; Qadeer et al., 1980).

3.1.2 Temperature

Temperature is one of the factors that influences the fermentation of anaerobic bacteria. It affects the components and structure of the cell membrane, which are consistent with the stress response from the environment and other solvents (Zhang et al., 2016). For example, *C. ragsdalei* was cultured at three different temperatures (32, 37, and 42 °C). The highest solvent production and cell growth occurred at 37 °C. Furthermore, temperatures above 37 °C had a direct impact on cell growth and solvent production (Kundiyana et al., 2011). McNeil and Kristiahsen (1985)

investigated the effect of temperature on *C. acetobutylicum* solvent production from 25 to 40 °C. The increasing temperature reduced the yield of solvent production, particularly acetone. Interestingly, butanol was unaffected by temperature increases. The optimal temperature for solvent production was determined in this study to be 35 °C.

3.1.3 pH

The pH of ABE fermentation is critical to butanol production. The optimal pH range was 5.0 to 6.5. (Jones and Woods, 1986). Al-Shorgani et al. (2014) investigated the optimal pH of *C. acetobutylicum* YM1 for butanol production. When cultured with glucose as a carbon source, the optimal initial pH of 6.2 resulted in the highest yield of butanol. Moreover, the increase in pH from 4.9 - 5.2 to 5.5 and 6.0 caused dramatic increases in butanol production by *C. beijerinckii* IB4 (Jiang et al., 2014).

4. Butanol – the ecologically friendly energy source to fulfill the global energy demand

Recently, butanol has become interested in running out of fossil fuels, climate change, environmental pollution, and global warming. Besides that, awareness of environmental sustainability has made renewable energy more popular. The utilization of butanol has been predicted to increase from 90 million USD in 2020 to around 106 billion USD by 2025, with a compound annual growth rate of 10.6% (Choi et al., 2021). At present, butanol has received more attention than other biofuels because it can replace gasoline. Butanol refers to a four-carbon alcohol. The properties of butanol are colorless, low boiling and melting points, volatile, and flammable (Table 1) (Ndaba et al., 2015). For the application of butanol, it can be used for chemical feedstock and product formulations such as paints and lubricating oils. In addition, the properties of butanol are similar to those of gasoline. Therefore, butanol has been a leading biofuel in transportation. Butanol has several advantages when compared to ethanol. For example, butanol has a higher energy density, lower vapor pressure, and higher-octane number than ethanol (Berezina et

al., 2012; Birgen et al., 2019; Dürre, 2007; Lee et al., 2008). The comparison of ethanol and butanol properties was shown in Table 2 (Lee et al., 2008).

Table 1 The properties of n-butanol (Ndaba et al., 2015).

Properties	**Butanol**
Formula	C_4H_9OH
Boiling point; °C	118
Energy density; MJ Kg^{-1}	33.1
Air fuel ratio	11.2
Heat of vaporization; MJ Kg^{-1}	0.43
Research octane number	96
Motor octane number	78

Table 2 The properties of ethanol and butanol (Lee et al., 2008).

Properties	Ethanol	Butanol
Energy density (MJ/L)	19.6	29.2
Air-fuel ratio	9	11.2
Heat of vaporization (MJ/kg)	0.92	0.43
Energy content/value (BTU/gal)	84,000	110,000
Solubility	Soluble	Insoluble
Research octane number	129	96
Motor octane number	102	78

Butanol can be classified into 4 isomers that are comprised of *n*-butanol, *sec*-butanol, *iso*-butanol, and *tert*-butanol (Figure 4) (Sahoo et al., 2019). When considering butanol, it was found that butanol is a toxic metabolite that *Clostridium* sp. can tolerate better than other bacteria species. However, each isomer of butanol has different properties. Moreover, the isomers of butanol, it was found that *n*-butanol has the largest share of the global butanol market, and it can be produced by only *Clostridium* sp. (Russmayer et al., 2019).

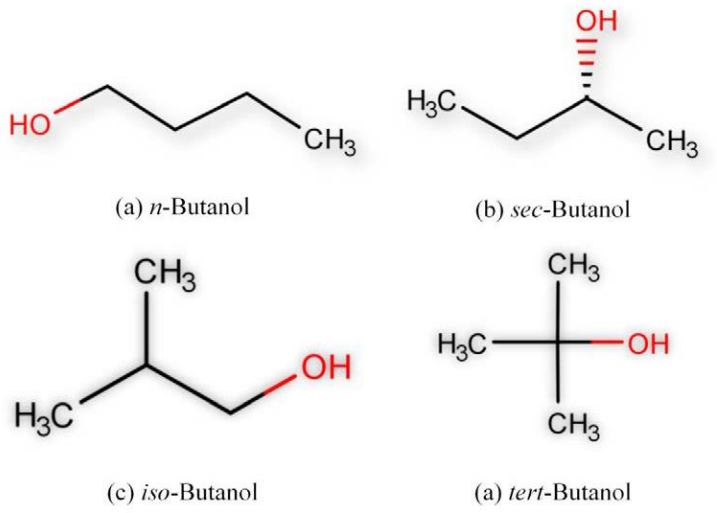


Figure 4 The isomers of butanol and their chemical structures (Sahoo et al., 2019).

Butanol can be produced via petrochemical and biological processes. In the petrochemical process, butanol can be produced via either the crotonaldehyde hydrogenation (aldol) process from acetaldehyde or the oxo process from propylene. The first chemical process to produce butanol is crotonaldehyde hydrogenation or the aldol process. The raw material in this process is acetaldehyde, and it consists of aldol condensation, dehydration, and hydrogenation. This process was to start with acetaldehyde produced from the dehydrogenation of ethanol (Figure 5). In this oxo process, propylene undergoes hydroformylation to form aldehydes. After that, aldehydes were further hydrogenated to *n*-butanol. The first step is to start with the

reaction of propylene with carbon monoxide and hydrogen using cobalt or rhodium as a catalyst. In the second step, the mixture of *n*- and isobutyraldehyde is hydrogenated to *n*- and isobutyl alcohols for distillation to recover butanol (Figure 5). However, the price of propylene in this process is highly sensitive to the price of crude oil on the market, and it regulates the cost of synthetic butanol production.

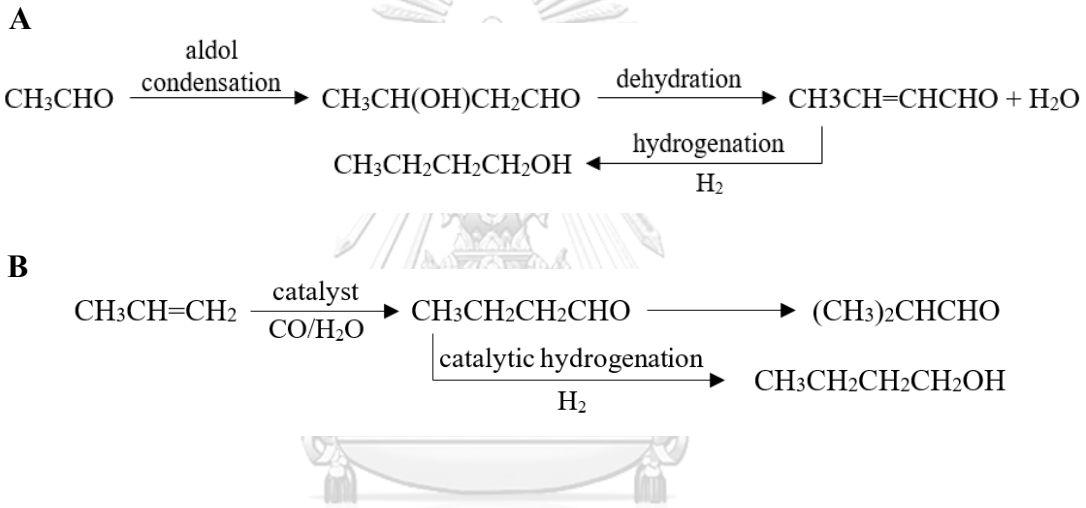


Figure 5 (A) n-butanol synthesis from the crotonaldehyde hydrogenation (aldol) process and (B) the propylene oxo process.

Because of the aforementioned issue with unpredictable global petroleum costs, attention has switched to biological processes. In the biological process, butanol can be produced by numerous microbes such as *Clostridium* sp., genetically engineered *Escherichia coli*, and *Saccharomyces cerevisiae* via acetone-butanol-

ethanol (ABE) fermentation, of which butanol production by *Clostridium* sp. has gained the most attention. This is due to the fact that these bacteria are a kind of solventogenic bacteria that can produce endospores, which help in their survival in harsh environments. They can also metabolize a wide range of substrates, including sugar cane, crops, and cassava (Kanno et al., 2013; Lee et al., 2012).

The ABE fermentation is characterized by its biphasic nature. The pH of the fermentation is reduced during the acidogenesis stage due to the quick synthesis of acetic and butyric acids, which is followed by solventogenesis, in which acids are reassimilated and butanol and acetone are formed (Lee et al., 2008). After that, the growth of bacteria was in a steady state (Huang et al., 2010). Buffering of the medium can cause the beginning of solventogenesis to be delayed, resulting in higher butanol yields (Figure 6) (Bryant & Blaschek, 1988; Jang et al., 2012; Yu et al., 2015). *C. acetobutylicum* and *C. beijerinckii* are the most commonly reported *Clostridium* strains for butanol synthesis (Qureshi & Ezeji, 2008).

However, ABE fermentation often results in poor butanol yield and productivity due to other by-products such as acetone and butyric acid being

created during the butanol synthesis route (Dadgar & Foutch, 1988; Gu et al., 2011). Furthermore, production costs are an important element influencing the economic feasibility of butanol production.

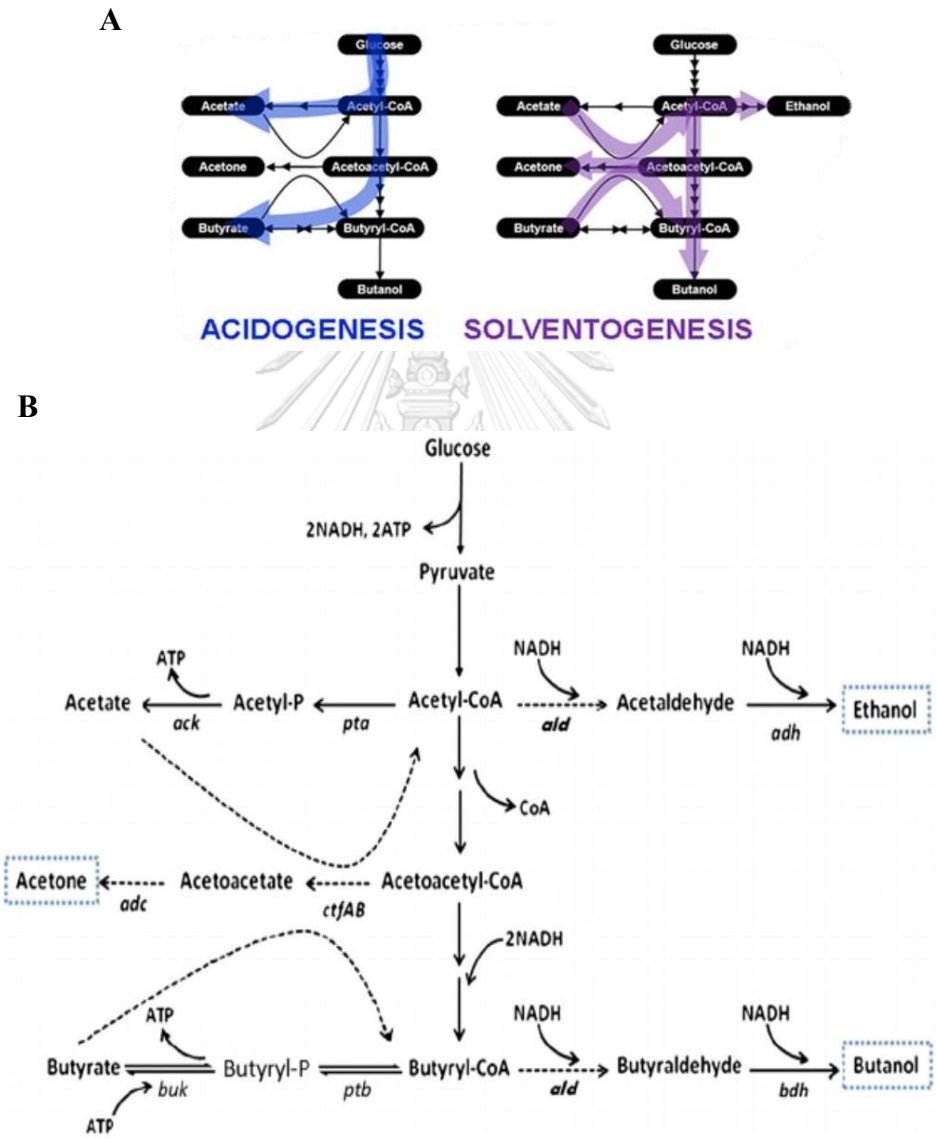


Figure 6 The ABE fermentation in *Clostridium* sp.: acidogenesis and solventogenesis phases (A) metabolic pathway to produce butanol by solvent producing Clostridium sp. (B) (Jang et al., 2012; Yu et al., 2015).

When considering the enzymes involved in the butanol synthetic pathway, there are six enzymes required to convert acetyl-CoA to butanol: thiolase (THL), β-hydroxybutyryl-CoA dehydrogenase (BHBD), 3-hydroxy-butyryl-CoA dehydratase (CRT, also termed crotonase), butyryl-CoA dehydrogenase (BCD), bifunctional acetaldehyde-CoA/alcohol dehydrogenase (ADHE, also known as ALD), and butanol dehydrogenase (BDH). ADHE and BDH play a key role in the production of butanol.

Many scientists have attempted to enhance butanol production by inserting, knocking out, or deleting key genes that code for corresponding enzymes. *C. acetobutylicum* is a well-known *Clostridium* species that has been studied for the butanol production process. When the *adhE2* gene in *C. acetobutylicum* ATCC 824 was overexpressed, butanol production increased (Yu et al., 2011). When the *adhE1* gene was inactivated, butanol production in *C. acetobutylicum* ATCC 824 decreased (Lehmann et al., 2012).

5. Typical feedstock for butanol production – the generation of biological processes for butanol production

In the first generation of butanol production, butanol can be produced by using a simple sugar, e.g., hexose sugar, that is derived from starchy crops such as sugarcane, corn, rice, wheat, and cassava. Table 3 summarizes the literature on various types of raw materials for first-generation butanol, including the microorganisms used and their product yields. Glucose is a simple sugar that is easy to use by bacteria. *C. beijerinckii* NCIMB 8052 produced butanol production of 11.2 g/L using glucose 60 g/L as a substrate (Lee et al., 2008). In accordance with *Clostridium* sp. strain G117, 6.45 g/L of butanol was produced using 30 g/L of glucose as a substrate. Butanol production was increased to 13.5 g/L when 0.4% yeast extract was supplemented and 60 g/L of glucose was used (Chua et al., 2013). Besides that, *C. saccharoperbutylacetonicum* N1-4 produced a high yield of butanol using cassava starch and cassava chips as substrates (21.0 and 19.4 g/L, respectively) when compared to glucose (24.2 g/L). Furthermore, solvent production from cassava starch was 42% to 63% higher than from corn or sago starch (Thang et al., 2010). In

addition, *C. acetobutylicum* GX01 can be used for various carbon sources, especially cassava flour. The yield of butanol was 17.1 g/L using 100 g/L of cassava flour mixed with 3 g/L of soybean meal. To improve production, GX01 was cultured in 10 and 30-L bioreactors. The result showed that butanol production was improved to 18.3 and 18.8 g/L, respectively (Li et al., 2015). Maize stalk juice is also very interesting. Due to the fact that the juice contained a high level of sugar, both fructose and sucrose, solvent production from *C. beijerinckii* NCIMB 8052 was also high. The initial sugar concentration of 42.2 g/L can produce 11.5 g/L of butanol (Wang & Blaschek, 2011). For the other raw materials, sago, corn, potato, and tapioca starch were also used in the first generation of butanol production (Madihah et al., 2001).

Table 3 The raw materials used in the production of second-generation butanol from *Clostridium* sp.

Type of feedstocks	Yield of butanol (g/L)	Pretreatment	Fermentation strategy	Strains	References
Glucose		No pretreatment		*C. beijerinckii* NCIMB 8052	(Lee et al.,
Supplemented					2008)
with	11.2		Batch (250 mL serum		
- 36 mM butyrate	13.4		bottle)		
- 36 mM butyrate			Continuous batch (5 L		
+ immobilize cell			bioreactor)		
reactor					
Glucose	6.45	No pretreatment	Batch (160 mL serum	*Clostridium* sp. strain G117	(Chua et al.,
Supplemented			bottle)		2013)

with 0.4% yeast extract	13.5				
Glucose	24.2	-	Batch (1 L bioreactor)	*C. saccharoperbutylacetonicum* N1-4	(Thang et al., 2010)
Cassava starch	21.0	-			
Cassava chips	19.4	Enzymatic hydrolysis			
Corn starch	16.2	-			
Glucose	7.39	No pretreatment	Batch (250 mL serum bottle)	*C. acetobutylicum* P262	(Madihah et al., 2001)
Sago starch	8.38				
Corn starch	8.61				
Potato starch	3.34				
Tapioca starch	4.89				

Glucose	19.12	No pretreatment	Fed-batch (5 L bioreactor)	*C. acetobutylicum* ATCC 824 */pfkA+pykA*	(Ventura et al., 2013)
Cassava **Corn meal**	11.85 15.5	Enzymatic hydrolysis	Batch (7 L bioreactor)	*C. acetobutylicum* ATCC 824	(Li et al., 2012)
Cassava starch + soybean meal	17.1 18.3 18.8	Enzymatic hydrolysis	Batch (250 mL serum bottle) Batch (10 L bioreactor) Batch (30 L bioreactor)	*C. acetobutylicum* GX01	(Li et al., 2015)
Cassava starch	4.37	No pretreatment	Batch (500 mL flask)	*C. acetobutylicum* DSMZ 792	(da Silva et al., 2022)
Sugarcane juice; SJ	4.1 6.4	No pretreatment	Batch without gas stripping	*C. beijerinckii* DSM 6423	(Rochon et al., 2019)

SJ + sweet sorghum	7.8		Batch without gas stripping Batch with *in-situ* gas stripping (5 L bioreactor)		
Maize stalk juice	11.5	No pretreatment	Batch (sixfors bioreactor system)	*C. beijerinckii* NCIMB 8052	(Wang & Blaschek, 2011)

However, these crops are also food for humans, and they were grown at an increasing cost of raw materials. As a result, many researchers attempted to produce butanol using non-edible food crops and plants. This is the beginning of the second generation of butanol. Lignocellulosic biomass, including agricultural waste, crop residues, and sustainably collected wood and forest residues, was used as a raw material to produce butanol. This second generation of butanol does not compete with human food crops and decreases the carbon source's price. Cotton stalk, soybean hull, sugarcane bagasse, and corn fiber were lignocellulosic residues. To utilize these wastes for butanol production, these substrates were treated with acid pretreatment and enzymatic hydrolysis before being used by *C. tyrobutyricum* Ct**Δ**ack-adhE2. The results showed that *C. tyrobutyricum* Ct**Δ**ack-adhE2 produced 15 g/L of butanol (Li et al., 2019). In addition, rice straw is one of the most plentiful sources of lignocellulosic biomass. Many researchers tried to use rice straw as a carbon source for ABE production. Gottumukkala et al. (2013) evaluated rice straws treated with enzymes and acids using *C. sporogenes* BE01. The results indicated that *C. sporogenes* BE01 produced 3.43 g/L of butanol. Furthermore, this result is

consistent with the findings of Moradi et al. (2013), who discovered that using alkali and acid pretreatment with rice straw resulted in yields of 163.5 and 192.3 g/kg of untreated rice straw, respectively. *C. acetobutylicum* NRRL B-591 produced 1.4 g/L butanol from alkali-treated rice straw, while it produced 2.0 g/L butanol from acid-treated rice straw. Additionally, *C. acetobutylicum* NRRL B-591 produced 7.1 g/L of butanol when pretreated with organosolv and hydrolyzed with rice straw (Amiri et al., 2014). It can be seen that rice straw has the potential to use as a substrate in ABE fermentation. Besides that, wood chips, sawdust, palm oil fiber, and corn cobs are abundant agricultural wastes with the potential for ABE production using *Clostridium* sp., as summarized in Table 4. However, the raw material in this generation has a disadvantage because it still requires enzyme, acid, or alkali pretreatment before being utilized. For this reason, it will have a high cost of production.

Table 4 The raw materials used in the production of second-generation butanol from *Clostridium* sp.

Type of feedstocks	**Yield of butanol (g/L)**	**Pretreatment**	**Fermentation strategy**	**Strains**	**References**
Corn cob	3.37	Alkali hydrolysis	Batch (2 L bioreactor)	*C. cellulovorans-adhE2*	(Ou et al., 2017)
Corn cob and corn steep liquor	11.65	Wet disk milling + enzymatic hydrolysis	Batch (100 L bioreactor)	*C. beijerinckii* SE-2	(Zhang & Jia, 2018)
Corn straw supplemented with		Thermochemically pretreated + enzymatic hydrolysis	Batch (2 L bioreactor + SSF and SHF)	*C. acetobutylicum* DSM 1731	(Dziemianowicz et al., 2022)
- mineral salts	24.03				
- mineral	28.64				

compounds					
Palm oil			Batch (1 L bioreactor)		(Komonkiat &
- Sap	14.4	No pretreatment		*C. acetobutylicum* DSM	Cheirsilp, 2013)
- Trunk fiber	10.0	Acid hydrolysis		1731	
				C. beijerinckii TISTR 1461	
Cotton stalk	15.6	Acid and enzymatic	Batch (fibrous-bed	*C. tyrobutyricum Ct*Δ*ack-*	(Li et al., 2019)
Sugarcane	12	hydrolysis	bioreactor)	*adhE2*	
bagasse					
Soybean hull	11.7				
Corn fiber	13				
Rice straw	3.43	Enzymatic hydrolysis	Batch (100 mL serum	*C. sporogenes* BE01	(Gottumukkala
			bottle)		et al., 2013)

Rice straw	1.4 2.0	Alkali hydrolysis Acid hydrolysis	Batch (118 mL serum bottle)	*C. acetobutylicum* NRRL B-591	(Moradi et al., 2013)
Rice straw	7.1	Organosolv pretreatment	Batch (118 mL serum bottle)	*C. acetobutylicum* NRRL B-591	(Amiri et al., 2014)
Saw dust	4.2 3.1 2.5	Autohydrolysis pretreatment + enzymatic hydrolysis	Batch (100 mL serum bottle + SHF, SSF, and PSSF process)	*C. beijerinckii* DSM 6423	(Cebreiros et al., 2019)
Soybean hull supplemented with methyl viologen	8.1 15.7	Acid + enzymatic hydrolysis	Batch (1 L bioreactor)	*C. tyrobutyricum* ATCC 25755 Ct(Δack)-pTBA	(Yu et al., 2015)

Sugarcane bagasse	5.85	Hydrothermally pretreatment + acid hydrolysis	Batch (300 mL bioreactor)	*C. sacharoperbutylacetonicum* DSM 14923	(Zetty-Arenas et al., 2019)
Wheat bran	8.8	Acid hydrolysis	Batch (100 mL serum bottle)	*C. beijerinckii* ATCC 55025	(Liu et al., 2010)
Wheat straw	7.05 5.05	Enzymatic hydrolysis	Batch - SHF process SSF process	*C. acetobutylicum* ATCC 824	(Wang et al., 2013)

* SSF Simultaneous saccharification and fermentation, SHF separate hydrolysis and fermentation, PSSF pre-saccharification and simultaneous saccharification and fermentation.

For the third generation of butanol production, the focus was shifted to wastewater and algae, including industrial wastewater, microalgae, and macroalgae. Due to the properties of algae, which contain a high level of oil (more than 50%), it is suitable for biodiesel production. After oil extraction from the algae, there is a lot of waste. Therefore, to reduce and make the value-added from that waste. To evaluate the potential of algae as a substrate for butanol production, Castro et al. (2015) investigated the sugar release of microalgae treated with acid hydrolysis. With 166.1 g/kg of dry algae, the amount of sugar was high, and it can be converted into 3.74 g/L of butanol production using *C. saccharoperbutylacetonicum* N1-4. Moreover, *C. acetobutylicum* ATCC 824 produced 3.86 g/L of butanol when using the sugar release of microalgae at 60 g/L (Table 5) (Cheng et al., 2015). In addition, wastewater algae are an interesting carbon source. *C. saccharoperbutylacetonicum* N1–4 produced butanol 2.26 g/L when pretreated with 10% wastewater algae with acid and base. Besides that, acid/base pretreatment with a 1% glucose supplement helped to improve the butanol production to 5.61 g/L (Ellis et al., 2012). Besides that, red and brown seaweeds were also employed as a carbon source for ABE

fermentation. *Clostridium* sp. strain NJ4 produced 12.56 g/L butanol using red seaweed hydrolysate, which contained 43.18 g/L of glucose after being pretreated with acid and concentration (Jiang et al., 2022), while *C. beijerinckii* DSM-6422 produced 7.16 g/L using brown seaweed that was pretreated by an enzyme (Hou et al., 2017). In addition, other techniques were used for improved butanol production from seaweed. The immobilized *C. acetobutylicum* B-1787 cells were studied on ABE production using microalgae biomass, it produced 10.91 g/L of butanol (Efremenko et al., 2012). Moreover, industrial wastewater is the other carbon source that has the potential for ABE production of *Clostridium* sp. The suspended brewery liquid waste, starch industrial wastewater, and apple pomace ultra-filtration sludge were pretreated with acid before being used as a substrate for ABE production. The result showed that *C. beijerinckii* NRRL B-466, produced butanol at 1.8, 4.68, and 1.4 g/L, respectively (Maiti et al., 2016). Furthermore, *C. butyricum* TISTR1032 produced 0.85 g/L when cultured with cassava wastewater (Virunanon et al., 2013).

Table 5 The raw materials used in the production of third-generation butanol from *Clostridium* sp.

Type of feedstocks	Yield of butanol (g/L)	Pretreatment	Fermentation strategy	Strains	References
Dry algae	3.74	Acid hydrolysis	Batch (125 mL serum bottle)	*C. saccharoperbutylacetonicum* N1-4	(Castro et al., 2015)
Microalgae residues	3.86	Acid and alkali hydrolysis	Batch (100 mL serum bottle)	*C. acetobutylicum* ATCC 824	(Cheng et al., 2015)
Wastewater algae supplemented with - 1% glucose	2.26 5.61	Acid and alkali hydrolysis	Batch (125 mL serum bottle)	*C. saccharoperbutylacetonicum* N1–4	(Ellis et al., 2012)

Red seaweed	12.56	Acid hydrolysis	Batch	*Clostridium sp.* strain NJ4	(Jiang et al., 2022)
Arthrospira (Spirulina) platensis	10.91	Thermal treatment + immobilized cells	Batch (1 L fermentor)	*C. acetobutylicum* strain B-1787	(Efremenko et al., 2012)
Apple pomace sludge	1.4	Acid hydrolysis	Batch	*C. beijerinckii* NRRL B-466	(Maiti et al., 2016)
Brewery liquid waste	1.8				
Starch industry wastewater	4.68				
Cassava wastewater	0.85	No pretreatment	Batch (60 mL serum bottle)	*C. butyricum* TISTR1032	(Virunanon et al., 2013)

6. Agro-industrial wastes and wastewater – the new carbon sources to replace food crops and human food

In terms of wastewater, it is one of the most important environmental problems in the world. Besides that, as the world's population grows, massive amounts of agro-industrial waste are continuously thrown into nature, resulting in pollution. Furthermore, the volume of these wastes tends to grow over time. The expected increase in water demand will reach 4.35 trillion m^3 by 2040, leading to increased wastewater generation (Lahlou et al., 2022). Generally, wastewater treatment was comprised of four successive steps: (1) preliminary pretreatment, also known as pre-treatment: the first step is removing coarse and large suspended substances from raw sewage. (2) primary treatment: the process of reducing the Biochemical Oxygen Demand (BOD) and Total Suspended Solids (TSS) in wastewater. In this step, the materials in the wastewater were settled by gravity. Then, removing floatable objects and reducing the pollution will make secondary treatment easier. (3) secondary treatment: the biological treatment takes place in this step, which is known as activated sludge. When the water reaches the secondary treatment

process, microorganisms are added to it. Microorganisms consumed some pollutants in the water, such as ammonia, nitrogen, nitrate, and phosphate. Therefore, the BOD was decreased. (4) tertiary or final treatment: this step removes contaminants that secondary treatment could not remove. This step frequently employs a combination of physical and chemical processes to remove potentially harmful microbiological contaminants from wastewater (Crini & Lichtfouse, 2019). Following that, the treated wastewater was either reused or discharged into the environment. The wastewater treatment processes were shown in Figure 7. It can be seen that the treatment of industrial wastewater is a crucial step. However, the cost of the treatment system is quite high, so the majority of wastewater is discharged into natural water sources without being treated (Dutta et al., 2021).

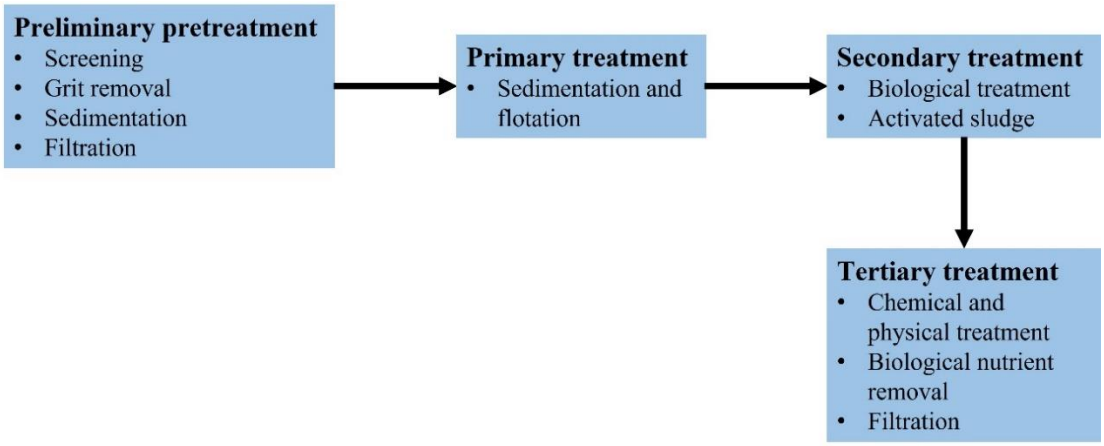


Figure 7 Wastewater treatment processes (Akbar et al., 2023; FAYSSAL et al.).

To address the issue of high wastewater treatment system costs, biological methods of treatment will be considered. The majority of wastewater is comprised of organic matter (OM), raw material residue, NH_3, and phosphate. When considering the wastewater treatment process, several treatment processes are being studied to remove the OM and nutrients, and this has been the focus of current research. Chai, Tan, et al. (2021) presented that microalgae can be used as a biotreatment to remove NH_3 from water. Since NH_3 is essential for the growth of microalgae, they are among the identified biological methods for removing NH_3 from wastewater.

In addition, microalgae can eliminate many of the pesticides that pollute wastewater. As a result, the biological and chemical demand (BOD and COD) in the

water also decreased (Chai, Chew, et al., 2021). Furthermore, microalgae are versatile and can be used as a sustainable source for a variety of applications around the world, including biomass feedstock, conversion into green biofuels, and integration into the human food chain (Ong et al., 2019). Moreover, modified techniques and materials are used to increase the efficiency of treatment processes and produce value-added substances. For example, Rambabu et al. (2021) used nanomaterials to disperse pollutants and produce biohydrogen from rice mill wastewater. To improve value-added substances from wastewater, alternative methods have been considered. Lin et al. (2021) investigated methane production from traditional pig manure wastewater, but the methane production was low. As a result, two-stage anaerobic systems were used to enhance methane production. However, there is still a scarcity of research on the use of Sato wastewater (STW).

7. Thai traditional beverage (Sato) wastewater

Thailand is mostly an agricultural country, with rice being the most important crop. Rice is produced and exported in massive amounts all over the world each year. Jasmine rice (*Oryza sativa L.*) accounts for the majority of rice exported for

distribution and has a unique aroma (Watchararuji et al., 2008; Zhou et al., 2020). However, there are many types of traditional Thai rice, such as glutinous rice and rice berry, that are still cultivated for human use. Each type of rice has its distinct aroma; other species have a similar aroma to jasmine rice, but farmers are less popular with jasmine rice, so those rice types are at risk of extinction. Therefore, increasing the value of these native rice varieties will help preserve them by processing them into brown rice or alcoholic beverages like Sato.

Sato is a distinct Thai traditional alcoholic beverage that was extensively made in the past based on the wisdom of previous generations. Glutinous rice and look pang (a sato yeast ball) are the essential elements in the Sato-making process. Sato yeast balls are made up of fungus and yeast that work together as an inoculum to accomplish the fermentation process, which involves breaking down starch and turning sugar into alcohol (Dung et al., 2007). The majority of the wastewater generated by the Sato manufacturing process comes from two sources: slop from alcohol distillation and cleaning fermenters and bottles. The wastewater from both sources is treated in aeration ponds before being utilized for agriculture and farming.

The activated sludge technique, a biological treatment, is used for the majority of wastewater treatment in the alcoholic beverage industry. However, this treatment procedure has several drawbacks and may not be suited for all applications. The complex structure of the substrate, in particular, takes a long time to remove. As a result, residual slop wastewater frequently turns from cloudy white to black due to sulfate-reducing bacteria producing hydrogen sulfide, which is difficult to remove, causes a bad odor, and can lead to serious environmental and health problems, as presented in Figure 8 (Athanasopoulos, 1987; Vijayaraghavan & Ramanujam, 2000). To address this issue, wastewater might be utilized as a fermentation substrate, which should help reduce production costs, maximize waste recovery, and finally eliminate waste.

Figure 8 The Sato manufacturing process and its byproducts, including wastewater effluent; STW.

In general, the substrates of the (Sato) factory (e.g., white glutinous rice) are converted to Sato, and the rest is discharged as Sato wastewater (STW). The STWs still contain relatively high levels of carbohydrates, such as starch, glucose, mannose, xylose, and arabinose. Consequently, using STW as a substrate in the ABE

fermentation process is an interesting new alternative way to produce butanol. However, although the STW is quite acidic and contains high BOD and COD levels (Kida et al., 1995; Satyawali & Balakrishnan, 2008), it has sufficient nutrients and minerals to support the growth of microorganisms. Starch and sugar are the main components in the STW because they are the major carbohydrates in Sato at approximately 42–49% by dry weight (Phantuwong, 2017). This is an interesting point to consider because carbohydrates can be used as a precursor to produce butanol by *Clostridium* sp.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Alcoholic beverage wastewater (Sato wastewater)

The Sato wastewater was collected from a Sato factory in Nakhon Ratchasima province, Thailand.

1.2 Chemical and reagents

All the chemicals and reagents used in this study were analytical and HPLC grades manufactured by various companies, as listed below.

- Acetone (Qrec, New Zealand)
- Ammonia (Panreac, Spain)
- Ammonium acetate (CH_3COONH_4) (Univar, USA)
- Arabinose (Sigma-Aldrich, USA)
- Biotin (Sigma-Aldrich, USA)
- Butanol (Qrec, New Zealand)

- Butyric acid (Sigma-Aldrich, USA)

- Carboxymethyl cellulose (CMC) (Sigma-Aldrich, USA)

- Casein hydrolysate (Glentham, United Kingdom)

- Crystal violet (Lobachemie, India)

- 3,5-Dinitrosalicylic acid (DNS) (Sigma-Aldrich, USA)

- Di-potassium hydrogen phosphate (K_2HPO_4) (Univar, USA)

- Di-sodium hydrogen orthophosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) (Univar,USA)

- Di-potassium hydrogen phosphate (K_2HPO_4) (Univar, USA)

- Ethanol (Qrec, New Zealand)

- Fructose (Univar, USA)

- Galactose (Univar, USA)

- Glacial acetic acid (CH_3COOH) (RCI Labscan, Thailand)

- Glucose (Kemaus, Australia)

- Glycerol (Kemaus, Australia)

- Hydrochloric acid (HCl) (Lobachemie, India)

- Iodine crystal (Lobachemie, India)

- Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) (Univar, USA)

- Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (Univar, USA)

- Malachite green (Sigma-Aldrich, USA)

- Manganese (II) sulfate monohydrate ($MnSO_4 \cdot H_2O$) (Univar, USA)

- Mannose (Himedia, India)

- p-aminobenzoic acid (PABA) (Fluka, India)

- Potassium dihydrogen phosphate (KH_2PO_4) (Univar, USA)

- Potassium iodide (KI) (Univar, USA)

- Potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6 \cdot 4H_2O$) (Kemaus, Australia)

- Safranin O dye (Sigma-Aldrich, USA)

- Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) (Univar, USA)

- Sodium hydroxide (NaOH) (Lobachemie, India)

- Soluble starch (Univar, USA)

- Sucrose (Kemaus, Australia)

- Thiamine HCl (Fluka, Germany)

- Tryptone (Himedia, India)

- Xylose (Himedia, India)

- Yeast extract (Himedia, India)

1.3 Equipment, consumables, and supplies

- Gas chromatography; GC-2010A (Shimadzu, Japan)

- Shaking incubator (Wiggens, Germany)

- Incubator (Shel Lab, USA)

- Vortex mixer; KMC-1300V (Vision Scientific, Korea)

- Analytical Balance 2 digits; BJ 1000C (Precisa, Switzerland)

- Analytical Balance 4 digit; PX224 Pioneer (Ohaus, USA)

- pH meter (Eutech instruments, USA)

- Microplate reader (BioTek, USA)

- Microscope; CH30RF200 (Olympus, Japan)

- Laminar flow cabinet (Biobase, USA)

- Fume hood (Biobase, USA)

- Autoclave (Daihan Scientific, Korea)

- Water bath (Memmert, Germany)

- Centrifuge; Combi 514R (Hanil science industrial, Korea)

- Anaerobic chamber; Bactron (Shel Lab, USA)

- Microtubes (Axygen, USA)

- 96-well plates (Thermo Scientific, USA)

- 10 µL loops (Thermo Scientific, USA)

- Plastic petri dishes (Thermo Scientific, USA)

- Pipette tips (Axygen, USA)

2. Method

2.1 Part I Isolation and identification of solventogenic *Clostridium* sp. from environmental sources in Thailand

2.1.1 Sample collection of *Clostridium* sp.

The samples were included the soil, sediment, sludge, and wastewater from two different sources: a biodiesel production plant and a Sato factory in Prachinburi and Nakhon Ratchasima, respectively, were collected. The samples used

in this study were listed in Table 6. These samples were collected in 50 mL sterile tubes and stored in an ice box before being transported to the laboratory.

Table 6 The list of samples from two different sources that were used in this study.

Samples	Habitat	Coordinated
- Soil, - Sludge (under the aeration pond)	Energy Absolute Public Company Limited, Prachinburi province, Thailand	14.073951466714558, 101.82477325415034
- Soil, - Rice waste residues - Wastewater	Sumrit Mankong Limited Partnership, Nakhon Ratchasima province	14.494278, 101.641389

2.1.2 Isolation of solventogenic *Clostridium* sp.

To isolate solventogenic *Clostridium* sp., the Clostridium Basal Medium (CBM) was used as an enrichment medium. The CBM medium contained (1L): 200 mg $MgSO_4 \cdot 7H_2O$, 7.58 mg $MnSO_4 \cdot H_2O$, 1 mg p-aminobenzoic acid, 0.002 mg biotin, 1 mg thiamine HCl, and 4 g casein hydrolysate, and the pH was adjusted to 7.0. Then, 15

g/L of filter sterilized K_2HPO_4 and KH_2PO_4 were added after autoclaving. One milliliter of the sample was diluted in 9 mL of CBM medium. Then, 3 mL of the dilution was inoculated into 27 mL of modified CBM medium containing 5 g/L of butanol. The serum bottles were heat-shocked at 80 °C for 10 min to eliminate non-spore forming bacteria and incubated at 37 °C for 5 days. Solvent-producing bacteria were isolated by plating on CBM agar containing 5 g/L of butanol and incubated the plates at 37 °C for 5 days. Bacterial colonies with different morphologies were selected and streaked on fresh modified CBM agar containing 5 g/L butanol. To obtain pure colonies, all colonies growing on these plates were re-streaked three times on modified CBM agar containing 5 g/L butanol.

2.1.3 Identification of solventogenic *Clostridium* sp.

2.1.3.1 Morphology based identification

The isolated solventogenic *Clostridium* sp. was identified by using morphological and 16S rDNA gene sequencing. The cell morphology of isolated strains was examined under a light microscope after Gram-staining.

2.1.3.2 16S rDNA gene sequencing

The chromosomal DNA of the 11 isolated *Clostridium* sp. were extracted using a bacterial DNA kit (OMEGA Bio-Tek, USA). 16S rDNA gene was amplified by PCR technique using universal primer, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). After that, the PCR was carried out in a DNA thermal cycler and the PCR procedure is as follows: initial denaturation 94 °C for 2 min, 30 amplification cycles of denaturation at 94 °C for 20 s, annealing at 54 °C for 20 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR products was purified and sequenced by Pacific Science Co., Ltd (Bangkok, Thailand). The identity of the 11 isolated *Clostridium* sp. were characterized by sequencing of their 16S subunit ribosomal RNA genes. The BLASTN web program was used to compare each bacterial strain's 16S rDNA sequence to other 16S rDNA sequences in GenBank. The phylogenetic tree of CUEA02 nucleotide sequences was built on the RaxML platform using Randomized Axelerated Maximum (RaxML) with fast bootstrapping.

2.1.4 The ability of starch hydrolysis

The experiment was carried out following the methodology of Su et al. (2015). Amylase activity: the isolated solventogenic *Clostridium* sp. was cultured on TYA agar medium including (g/L): 15 soluble starch, 0.01 trypan blue, 1 L-cysteine, 15 agar, and the pH was adjusted to 6.5. The 11 isolated *Clostridium* sp. were incubated at 37 °C for 24 h. The clear zone appearance is indicating amylase activity.

2.2 Part II Determination of ABE production of isolated solventogenic *Clostridium* sp.

Tryptone-yeast extract-ammonium acetate (TYA) medium was used as a growth medium for butanol production enhancement (Al-Shorgani et al., 2018). The TYA medium contained (g/L): 50 glucose, 6 tryptone, 2 yeast extract, 3 ammonium acetate, 0.001 $FeSO_4 \cdot 7H_2O$, and 0.3 $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 6.5 with acetic acid and flushed with N_2 (99.99%) to create an anaerobic condition. The isolated solventogenic *Clostridium* sp. was cultured on TYA medium for 144 h, and controlled temperature at 37 °C. The samples were collected at the appropriate

times, centrifuged at 6000 x *g* for 10 min and then analyzed by GC (GC-2010A Shimazu, Kyoto, Japan) equipped with a flame ionization detector and a 30 m DB-wax capillary column of 0.25 μM film thickness (inner diameter 0.530 mm) (Agilent, Santa Clara, CA, USA) to measure the concentration of ABE, acetic acid, and butyric acid. The temperature of the injector and detector were adjusted to 240 °C, while the column temperature was initially set to 45 °C and then gradually increased from 45 to 240 °C. The residual reducing sugar concentration was analyzed by the modified dinitrosalicylic acid method.

2.3 Part III Whole genome sequencing (WGS)

The whole genome of *C. beijerinckii* CUEA02 was sequenced using the Illumina MiSeq sequencer at the Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand). Genomic DNA was extracted from strain CUEA02 using a bacterial DNA kit (OMEGA Bio-Tek, USA), verified by 0.8% (w/v) agarose gel electrophoresis, and identified with visible UV light. A 100-ng portion of the genomic DNA was subjected to DNA sequencing library preparation using the QIAGEN FX kit

(Qiagen, USA), while the FASTQC software was used to evaluate the raw read quality (Andrews, 2010). The assembled genome was annotated using RASTtk (Brettin et al., 2015), and the average nucleotide identity (ANI) was also calculated and compared using JSpeciesWS, a web server tool (Richter et al., 2016). To perform the functional annotation, the OmicsBox program was employed (Götz et al., 2008). Gene ontology (GO) annotation was used to analyze the main sources of functional analysis, which were comprised of cell components, molecular functions, and biological processes (Conesa et al., 2005). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number JAJSOL000000000.

2.4 Part IV Enzymatic activity measurement

2.4.1 Enzyme assay

Cell pellets were washed and resuspended in 0.1M Tris/HCl pH 7.2 with 2 mM of reducing agent dithiothreitol (DTT). Then, break cells by using sonication (for 30 sec, 4 cycles), the cell lysate was centrifuged at 11,000 x g for 10 min. Cell pellet was discarded and the supernatant was collected to measure the butanol

dehydrogenase (BDH) activity. To measure the BDH activity, 44 mM butyraldehyde was used as a substrate and 2 mM NADH/NADPH in Tris buffer (0.1M, pH 8) was used as a cofactor. Each reaction mixture is 1 mL, and it contains cell lysate or enzyme solution except blank control. The BDH enzyme activity was measured at absorbance 340 nm by using a UV-Vis spectrophotometer. One unit (U) of activity is equivalent amount of enzyme required for oxidation of 1 μmol of NAD(P)H/min. The specific activity was determined by the amount of total protein that was measured by the Bradford assay. The specific activity can be calculated from ΔOD as follows:

Specific activity (U/mg) = (ΔOD/min)/(6.22 x cell extract(mL)) x amount of protein

* 6.22 is an extinction coefficient of NAD(P)H.

2.4.2 Protein assay

The Bradford protein assay was used to determine the amount of protein. The total volume of reaction is 1 mL, containing 0.8 mL of cell extract and 0.2 mL of Bradford reagent. The mixture was mixed and sat for 10 min at room temperature. The protein was measured at an absorbance of 595 nm.

2.5 Part V Optimization condition and biochemical characterization of newly isolated *C. beijerinckii* CUEA02 for butanol production

According to the initial pH, temperature, inoculum size, and substrate concentration are important factors in the fermentation of solvents in *Clostridium* species (Khamaiseh et al., 2012). The inoculum was cultured in a 60-mL serum bottle containing 27 mL of TYA medium at 37 °C for 48 h, and then the inoculum was transferred to fresh TYA medium 10% (v/v) and incubated at 37 °C in a shaking incubator at a rotation speed of 130 rpm.

2.5.1 Effect of initial pH

The newly isolated *C. beijerinckii* CUEA02 was cultured in TYA medium at 37 °C with different initial pH values (4.5, 5.5, 6.5, 7.5, and 8.5) and the OD600 measurements were taken after 24 h. The sample was analyzed by GC-FID.

2.5.2 Effect of temperature

To investigate the optimum fermentation temperature of *C. beijerinckii* CUEA02, the temperature was tested at 30, 35, and 37 °C for 24 h with an initial

ferment pH from the previous step. Then, OD600 measurements were taken after 24 h. The sample was analyzed by GC-FID.

2.5.3 Effect of inoculum size

To determine the effect of inoculum size of *C. beijerinckii* CUEA02 on its butanol production, the inoculum size was tested at 5%, 10%, 20%, 30%, and 40% under the optimal initial pH and temperature. After that, OD600 measurements were taken after 24 h. and the sample was analyzed by GC-FID.

2.5.4 Effect of substrate concentration

The initial substrate concentration was evaluated by varying the glucose concentration in the TYA medium at 40, 50, and 60 g/L at optimum an initial pH, temperature, and inoculum size for 24 h. The OD600 was measured and analyzed ABE production by GC-FID after 24 h.

2.5.5 Physiological and biochemical characterization

2.5.5.1 Carbon source utilization

To evaluate the capability of *C. beijerinckii* CUEA02 to utilize various carbon sources, xylose, arabinose, glucose, galactose, fructose, mannose, sucrose, starch, carboxymethyl cellulose (CMC), and glycerol were used as sole carbon source at 2% (w/v) medium supplements in the TYA to investigate the capability of butanol production. The temperature and rotation speed were controlled at 37 °C and 130 rpm, respectively, and samples were taken every 24 h and analyzed for ABE production by GC-FID.

2.5.5.2 Butanol and ethanol tolerance

To examine the butanol and ethanol tolerance, the butanol and ethanol were varied at different levels ranging from 1 to 2.5 % (v/v). The OD600 measurement was taken after 24 h.

2.6 Part VI Determination of Sato wastewater for butanol production

2.6.1 Characteristic of the Sato wastewater

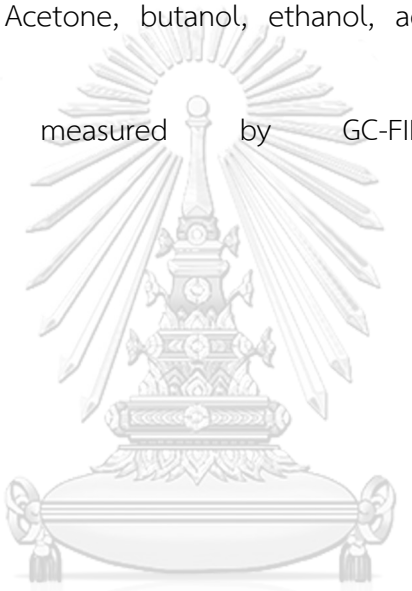
The Sato wastewater used in this study was collected from a Sato factory in Nakhon Ratchasima province, Thailand. The sample was stored at -20 °C until used. The pH, BOD (mg/L), COD (mg/L), total solids (TS; mg/L), total suspended solids (TSS; mg/L), total Kjeldahl nitrogen (TKN; mg/L), and volatile solids (VS; mg/L) of the sample was measured by the Environmental Research Institute, Chulalongkorn University, Thailand. Moreover, the Sato wastewater was investigated the levels of Invert sugar (AOAC 968.28 procedures) and starch (South African sugar factory laboratory handbook method) by Oversea Merchandise Inspection Co., Ltd., Thailand.

2.6.2 Preparation of the Sato wastewater

The STW was diluted 1:10, 1:20, 1:30, and 1:40 and then aliquoted at 45 mL per 160-mL serum bottle. The bottles were then flushed with N_2 (99.999%) for 15 min, sealed with a Neoprene stopper, and sterilized in an autoclave (121 °C, 1.5 mPa for 15 min).

2.6.3 Measurement of butanol production from Sato wastewater

Cell density was analyzed by UV–vis spectrophotometer at 600 nm. *C. beijerinckii* CUEA02 was cultured in TYA medium at 35 °C for 144 h under anaerobic condition and taken sample every 24 h. Then, the mixture was centrifuged to separate supernatant. Acetone, butanol, ethanol, acetic acid, and butyric acid production were measured by GC-FID (Shimadzu, Japan).



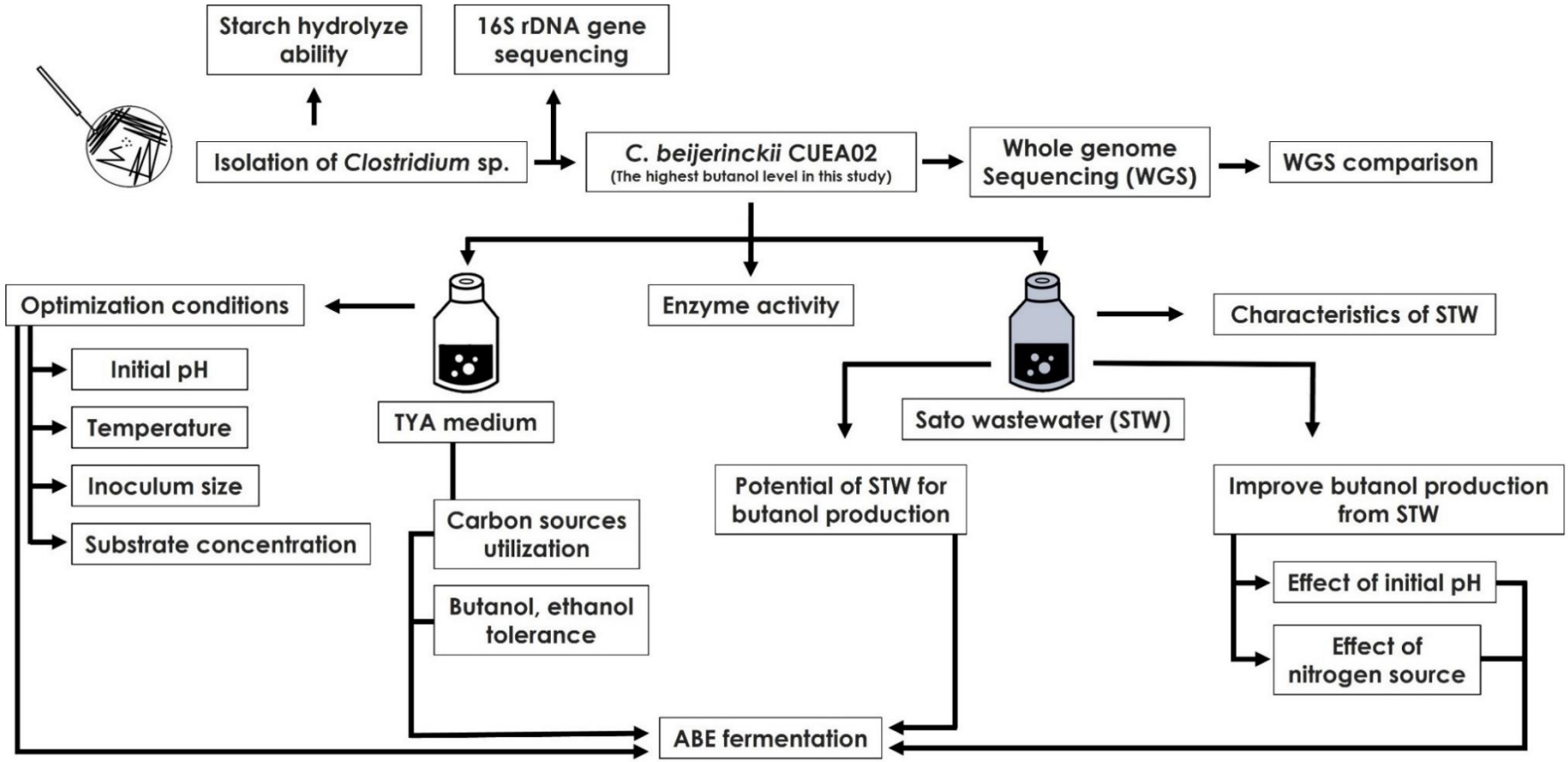


Figure 9 A summary of the experimental methods for producing butanol from *C. beijerinckii* CUEA02 utilizing STW.

CHAPTER IV

RESULTS AND DISCUSSION

1. Part I: Isolation and identification of solventogenic *Clostridium* sp. from environmental sources in Thailand

1.1 Isolation, identification, and production of solventogenic *Clostridium* sp.

Clostridial bacteria are naturally butanol producers. The model of ABE research has been *C. acetobutylicum, C. beijerinckii, C. saccharolyticum,* and *C. saccharoperbutylacetonicum* (Tirado-Acevedo et al., 2010). The isolated *Clostridium* sp. was identified and evaluated the ABE production in this study. A total of ten environmental samples were collected from two separate locations: a biodiesel plant in Prachinburi and a Sato factory in Nakhon Ratchasima, respectively. Soil, sludge, and wastewater were among the samples collected. All samples were cultured in a CBM medium containing 5 g/L butanol before being heat-shocked at 80 °C for 10 min. Following that, 36 isolated were streaked three times on CBM agar containing 5 g/L of butanol. Within 24 hours of incubation after the third re-streak,

approximately 30% of the samples treated had formed colonies. All isolates were rod shaped, gram positive, and endospore-forming bacteria. The 11 isolated strains were identified by sequencing their 16S subunit ribosomal RNA genes (Kolbert & Persing, 1999). Genomic DNA was extracted and 16S rRNA amplified by PCR using the primers 27F and 1492R. Each bacterial strain's 16S rDNA sequence was compared to other 16S rDNA sequences in GenBank using the BLASTN web program (Wang & Zhang, 2000). Table 7 shows the organism with the highest similarity score for each sequence. Moreover, the isolated strains were gram stained and observed under the microscope for sporulation, as shown in Figure 10.

Table 7 BlastN results for 16S sequences.

Isolate	Top BLASTN Hit	Identity
GW-1	*C. beijerinckii* strain B17 16S ribosomal RNA gene, partial sequence	1174/1198 (98%)
GW-2	*Clostridium sp.* strain WANY51 16S ribosomal RNA gene, partial sequence	1193/1228 (97%)
GW-3	*Clostridium* sp. strain WANY51 16S ribosomal RNA gene, partial sequence	1188/1228 (97%)
GW-4	*C. beijerinckii* DSM 6423 genome assembly, chromosome: I	1181/1218 (97%)
AW-1	*C. beijerinckii* strain B17 16S ribosomal RNA gene, partial sequence	1187/1222 (97%)
CUEA02	*C. beijerinckii* strain L8 16S ribosomal RNA gene, partial sequence	1162/1170 (99%)
FGR-1	*Clostridium* sp. strain WANY51 16S ribosomal RNA gene, partial sequence	1191/1225 (97%)
FGR-2	*Clostridium* sp. strain DSM 105335 16S ribosomal RNA gene, partial sequence	1190/1213 (98%)
FGR-3	*C. beijerinckii* strain OM 16S ribosomal RNA gene, partial sequence	1195/1221 (98%)
FGR-4	*C. beijerinckii* strain OM 16S ribosomal RNA gene, partial sequence	1197/1233 (97%)
FGR-5	*C. beijerinckii* strain OM 16S ribosomal RNA gene, partial sequence	1195/1233 (97%)

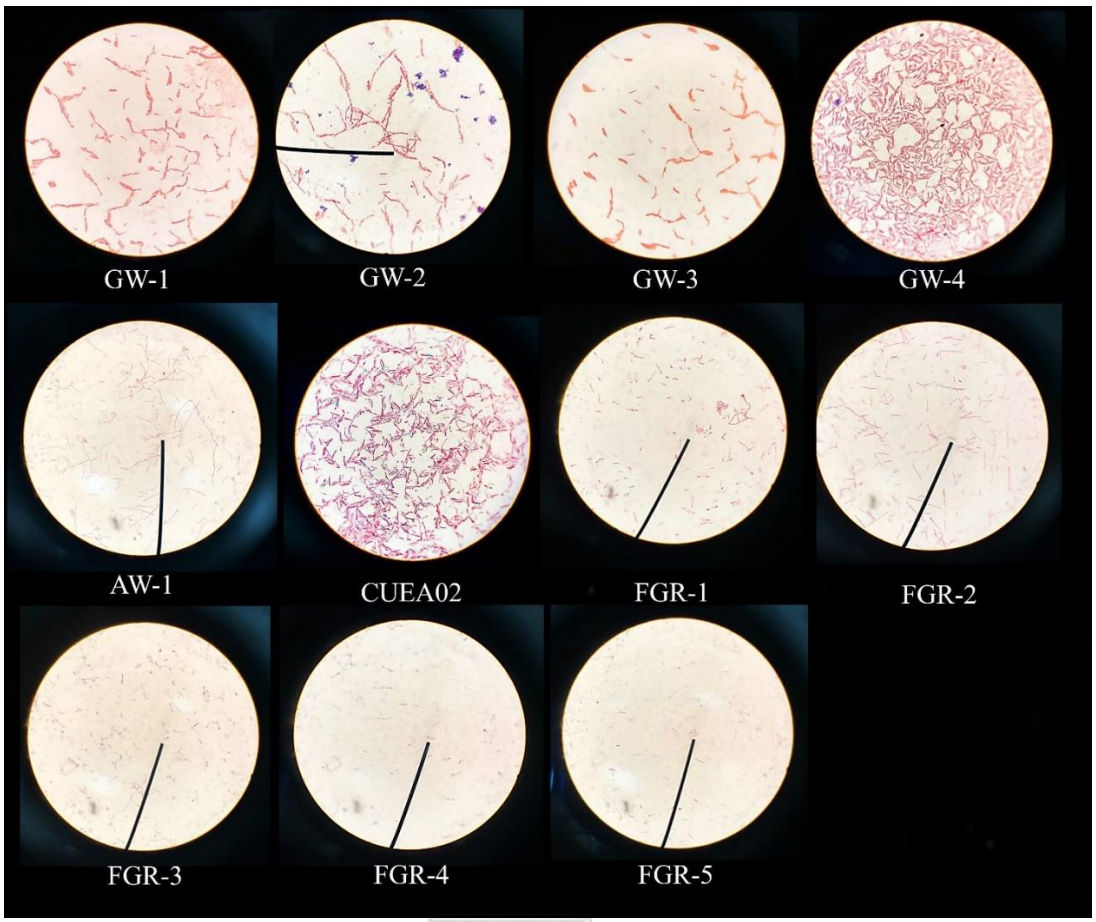


Figure 10 *Clostridium*'s cell stained with safranin o and malachite green of the 11 isolated strains from various sources located in Thailand.

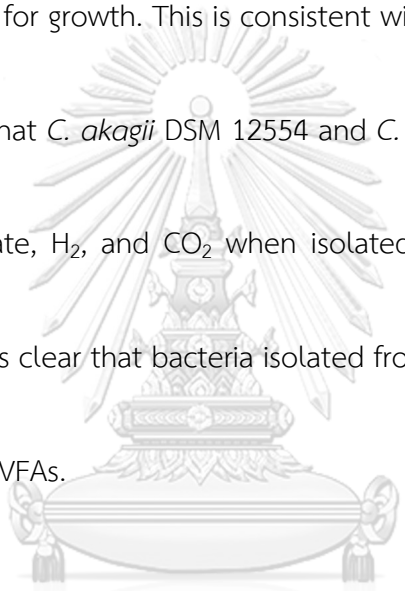
Identification of the isolated *Clostridium* sp. were performed by sequencing of partial the 16S rDNA gene. The 11 isolates were identified as 2 different strains, including *Clostridium* sp. (isolates GW-2, GW-3, FGR-1), *C. beijerinckii* (isolates GW-1, GW-4, AW-1, CUEA02, FGR-2, FGR-3, FGR-4, FGR-5). To evaluate the potential of ABE

and volatile fatty acids (VFAs) production from 11 isolated strains, all isolated strains were cultured in TYA medium for 144 h.

Batch fermentation was performed in a 60 mL serum bottle containing glucose as a carbon source. Bacteria were divided into two groups after ABE production was determined by GC analysis. Isolated bacteria, which were isolated from biodiesel plant, produced both VFAs and solvents (isolates GW-1, GW-2, GW-3, GW-4, AW-1, and CUEA02). On the other hand, isolated bacteria from the Sato factory produced only VFAs (isolates FGR-1, FGR-2, FGR-3, FGR-4, and FGR-5). Figure 11 presents the amount of ABE production by all 11 isolated *Clostridium* strains after 72 h. It can be seen that *C. beijerinckii* CUEA02 produced the highest level of butanol production. Therefore, this strain was selected for further study in Sato wastewater utilization.

According to the result from the 16S rDNA gene, the result showed that the majority (8/11) of the isolated bacteria were *C. beijerinckii*. It is well known that *C. beijerinckii* is a solvent-producing bacteria. It is frequently employed in the

manufacture of ABE as well as *C. acetobutylicum*. Surprisingly, none of the *C. beijerinckii* isolates from the Sato factory produced ABE solvents. Due to the initial hypothesis, it may be because the natural source where bacteria grow was extremely acidic (pH 3.5). As a result, they must adapt to surviving in acidic conditions and/or be chosen to use acids for growth. This is consistent with the findings of Kuhner et al. (2000), who observed that *C. akagii* DSM 12554 and *C. acidisoli* DSM 12555 produced acetate, butyrate, lactate, H_2, and CO_2 when isolated from acidic beech litter and peat-bog soil (pH 3). It is clear that bacteria isolated from acidic environments tended to produce exclusively VFAs.



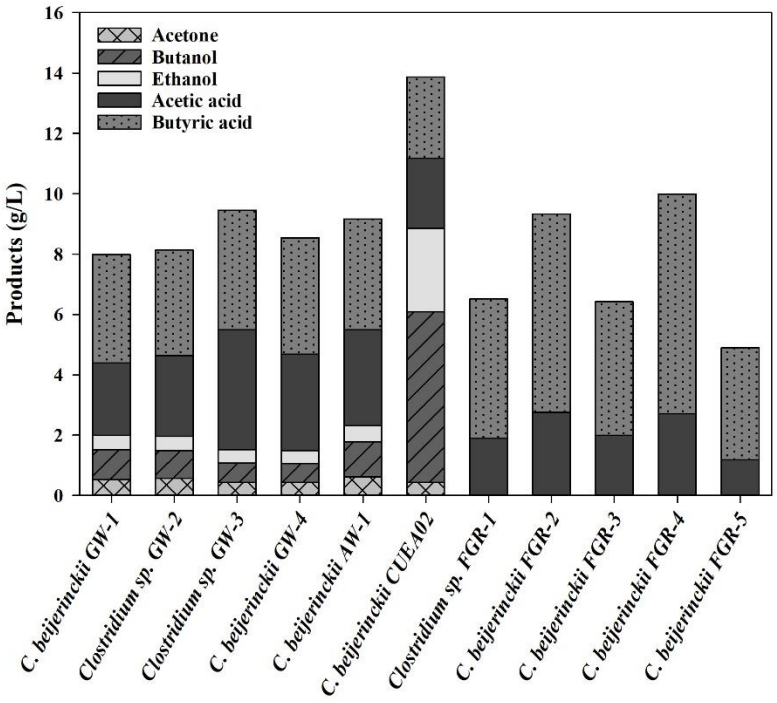


Figure 11 The level of ABE fermentation (72 h) by isolated solvent-producing *Clostridium* sp. in a 60 mL serum bottle at 37 ˚C using 2% (w/v) glucose as a carbon source in TYA medium. The data are presented as the mean from triplicate trials (Buranaprasopchai et al., 2022).

In addition, when considering the pH of the culture medium, it was found that the pH was a substantial component and has an influence on the formation of VFAs and solvents. However, the research related to solvent producing *Clostridium* sp. and the impact of pH on VFAs production has seldom been studied. However, it

has recently been reported that *C. beijerinckii* NRRL B-598 can be used to produce butyric acid, with the highest butyric acid concentration obtained being 9.69 and 11.5 g/L when cultured at pH 6.5 and 7.0, respectively. These studies discovered that a neutral pH improves acid production whereas a mildly acidic pH stimulates solvent production (Drahokoupil & Patáková, 2020). Furthermore, Katagiri et al. (1961) supported the study on the influence of pH on VFAs production. When *C. acetobutylicum* was cultivated at pH 7.0 or higher, it produced more lactic acid. As a result, the initial ferment pH is essential in determining acids and solvents formation, with an alkaline pH being optimal for acidogenic fermentation. In this study, all isolated strains were cultured in a TYA medium which adjusted the initial pH to 6.5. Therefore, this may be one reason that *C. beijerinckii* isolates from the Sato factory may only be capable of producing acids.

1.2 Selection of solventogenic *Clostridium* sp.

In this study, a potentially isolated *Clostridium* strain belonging to *C. beijerinckii* CUEA02 was selected to optimize and determine the ABE production

including VFAs. Strain CUEA02 was cultured in TYA medium for 144 h and controlled temperature at 37 °C. The amounts of solvents produced by the CUEA02 strain with the highest ABE and butanol production at 72 h (8.86 ± 0.12 g/L and 5.65 ± 0.09 g/L, respectively) were determined by GC analysis. The ABE and VFAs' production profile of CUEA02 is shown in Figure 12.

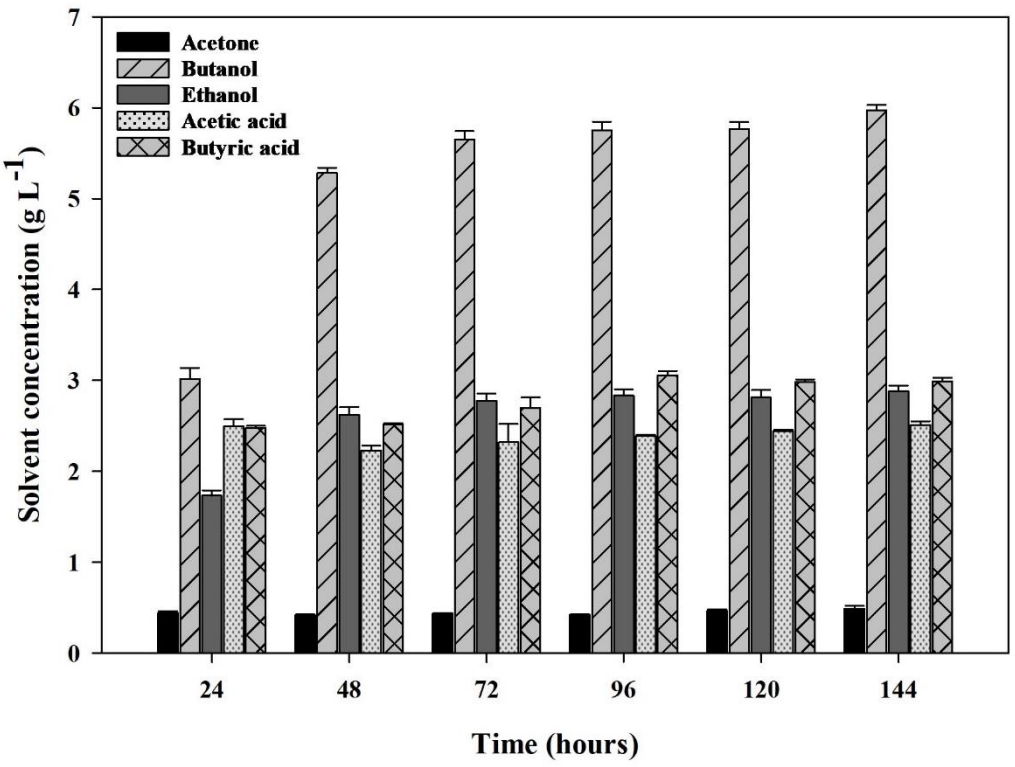


Figure 12 CUEA02 production profile in a 60 mL serum bottle at 37 °C with 50 g/L glucose as a carbon source in TYA medium. The data are provided as the mean of triplicate trials.

The cell morphology of CUEA02 was rod-shaped and spore-forming cells (Figure 13A), whereas the colonies were cream, irregular, convex, and with an undulate margin (Figure 13B). The phylogenetic trees of 16S rRNA sequences were constructed for distinct species of *Clostridium* (Figure 14) and closely related solventogenic species. According to these trees, it is apparent that isolated CUEA02 is 100% identical to *C. beijerinckii* NCIMB 8052. In addition, the ANI of the whole genome was used to establish the species identification of the CUEA02 strain based on genomic information. The result indicated that the genome of the CUEA02 strain is consistent with *C. beijerinckii* BGS1 with 95.14% ANI. As previously stated, the phylogenetic tree and ANI findings differed because the 16S rRNA gene phylogenetic tree can only identify microorganisms up to the genus level (Figueras et al., 2014).

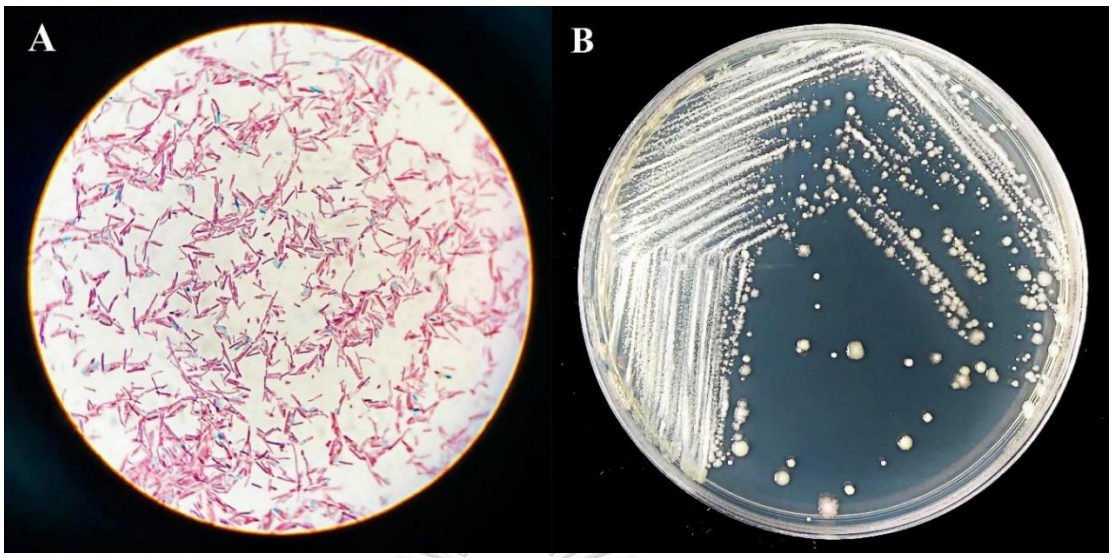


Figure 13 Representative images of *C. beijerinckii* CUEA02 morphology cultured in TYA at 37 °C. (A) Rod-shaped and spore-forming cells; (B) cream, uneven, convex, and undulate colonies (Buranaprasopchai et al., 2022).

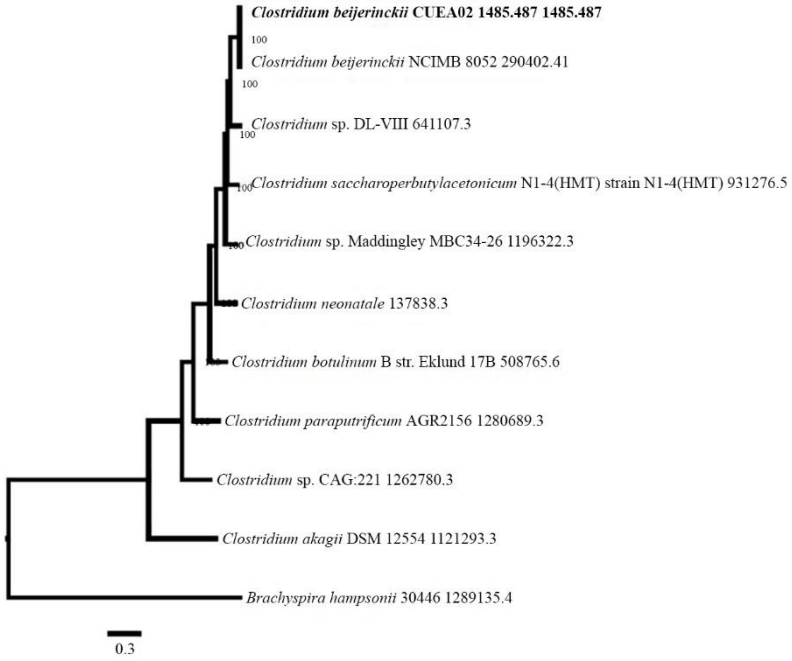


Figure 14 *C. beijerinckii* CUEA02 evolutionary analysis using the Randomized Axelerated Maximum Likelihood method. The accession codes for GenBank are displayed after the name. The bar represents evolutionary distance (Buranaprasopchai et al., 2022).

1.3 The ability of starch hydrolysis from 11 isolated *Clostridium* sp.

According to the 11 isolates of *Clostridium* sp., isolated from biodiesel plant, it has the ability to produce both solvents and acids production. Conversely, isolated from the Sato factory, it solely produced acids production. Due to the isolated *Clostridium* sp. being selected and applied to starchy wastewater, it would be good if

that isolated could produce an amylase enzyme to hydrolyze starch in that wastewater. To investigate the amylase enzyme, all isolated *Clostridium* sp. were cultured on TYA medium agar, which contains trypan blue to observe the clear zone, and controlled at 37 °C for 24 h. Figure 15 demonstrates the clear zone from all isolated *Clostridium* sp.

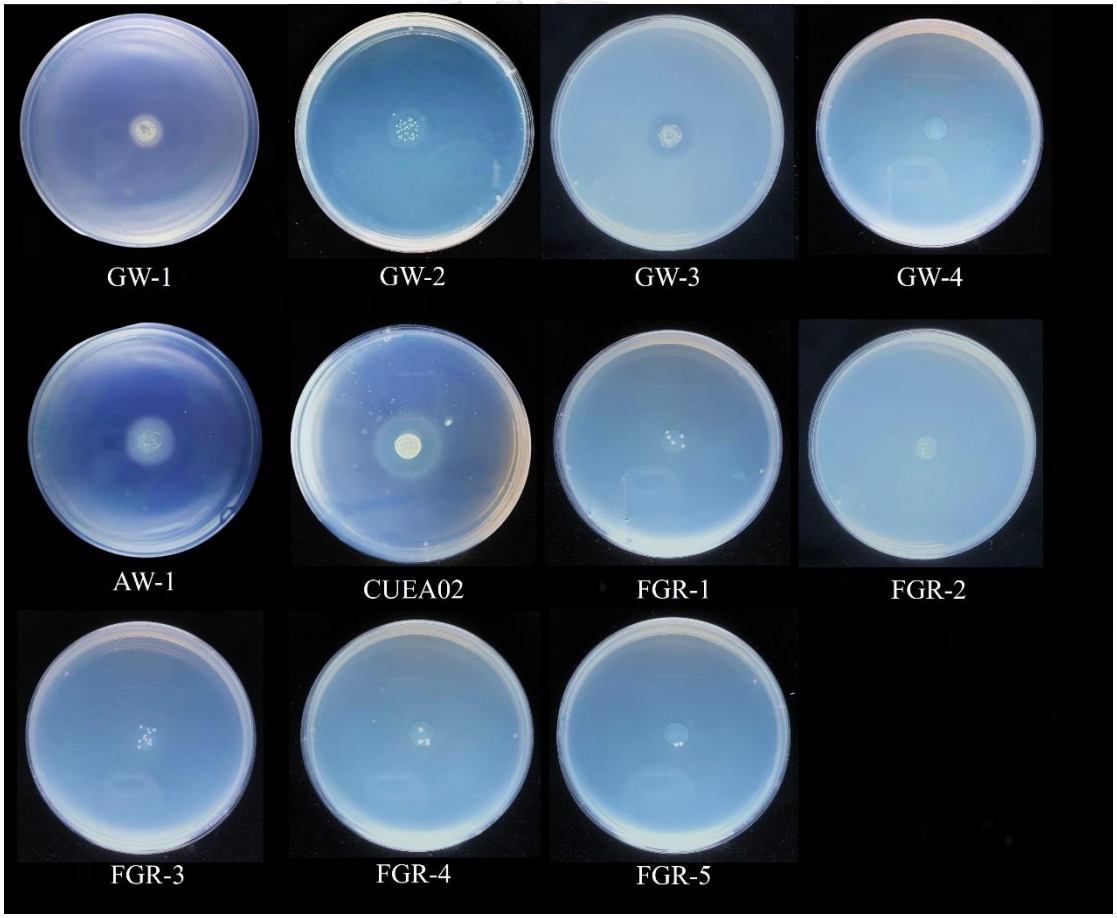


Figure 15 the clear zone from all isolated *Clostridium* sp. after 24 h.

2. Part II: Whole genome sequencing of *C. beijerinckii* CUEA02

2.1 The whole genome profile of *C. beijerinckii* CUEA02

The *C. beijerinckii* CUEA02's genome is a single circular genome of 5,768,209 bp with an average GC content of 29.54%. This assembled genome had 134 large contigs ranging in length from 16 to 125,868 bases. No plasmids could be identified. The taxonomy of this genome is cellular organisms > Bacteria > Terrabacteria group > Firmicutes > Clostridia > Clostridiales > Clostridiaceae > *Clostridium*. The draft genome and functional annotation of *C. beijerinckii* CUEA02 are demonstrated in Figure 16. The genome of CUEA02 has 5371 protein coding sequences (CDS), 83 transfer RNA (tRNA) genes, and six ribosomal RNA (rRNA) genes, as revealed by using the RAST tool kit (RASTtk) to annotate and evaluate the sequence data. The annotation revealed 1,450 hypothetical proteins and 3,921 functional proteins. The proteins with functional assignments comprised 3,036 with Enzyme Commission (EC) numbers (Schomburg et al., 2004), 4110 with GO assignments (Ashburner et al., 2000), and 772 with KEGG pathway mapping (Kanehisa et al., 2016), as shown in Table 8.

The DDBJ/ENA/GenBank accession number for this Whole Genome Shotgun project is JAJSOL000000000.

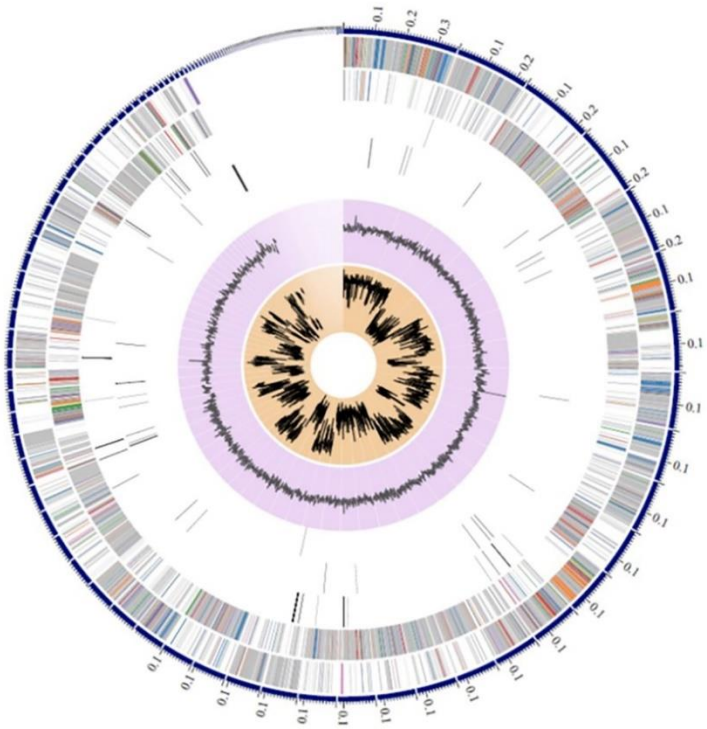


Figure 16 A circular graphical representation of the *C. beijerinckii* CUEA02 genome, including contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, and the GC content and GC skew, from outer to inner rings (Buranaprasopchai et al., 2022).

Table 8 Summary statistics for *C. beijerinckii* CUEA02 transcriptome assembly (Buranaprasopchai et al., 2022).

Statistics	CUEA02 Transcriptome
Genome length	5,768,209 bp
GC content	29.54
Contigs	134
Contig L50	16
Contig N50	125,868
Chromosome	0
Plasmids	0
CDS	5,371
tRNA	83
rRNA	42
No. of hypothetical proteins	1,450
No. of functional proteins	3,921
No. of transcripts with GO annotations	4,110
No. of transcripts with EC annotations	3,036
No. of transcripts with KEGG annotations	772

The functional study of *C. beijerinckii* CUEA02 genes was performed using several functional databases. The annotated gene functions were integrated into the

transcriptome with GO terms using the OmicsBox program. The CUEA02 transcriptome had a total of 12,651 annotations, with a mean GO level of 2.982 (Figure 17A). A total of 4110 genes were discovered and assigned to the three functional GO terms of biological processes (18 sub-categories), molecular function (12 sub-categories), and cellular component (two sub-categories) in the GO distribution of functional annotations (Figure 17B). The primary roles of biological processes were the cellular process (32.4%) and metabolic process (31.8%), whereas catalytic activity (62.1%) was the most common molecular function, and cellular anatomical entity (99.2%) was the largest cellular component. All 3036 proteins with EC numbers were divided into seven enzyme groups, as shown in Figure 17C, with the predicted enzyme code distribution. The most abundant EC class in CUEA02 was transferases (32.11%) and hydrolases (29.15%), with roughly 13.97% as oxidoreductases and 10.44% as translocases. Lyases, isomerases, and ligases comprised the remaining three enzyme classes, accounting for 14.33% of the reported enzyme assignments.

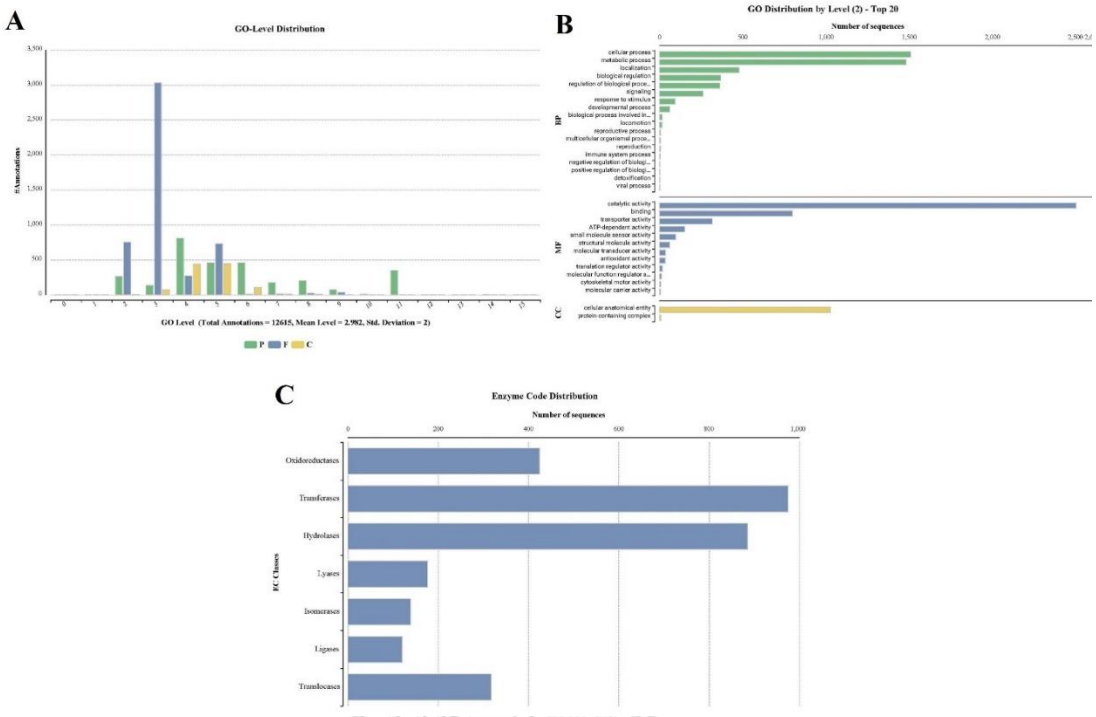


Figure 17 (A) GO annotation distribution in *C. beijerinckii* CUEA02. There was a total of 12,615 annotations with a 2.982 median GO level (P: biological process, F: molecular function, and C: cellular component). (B) Distribution of *C. beijerinckii* CUEA02 Level 2 GO terms. The three types of GO terms are biological process (BP), molecular function (MF), and cellular component (CC). (C) Distributions of enzyme code (EC) classes in *C. beijerinckii* CUEA02 (Buranaprasopchai et al., 2022).

2.2 The comparison of whole genome sequencing of *C. beijerinckii* CUEA02 with the other *Clostridium* sp.

From the data of the gene annotation, it was found that the CUEA02 strain contained three types of butanol dehydrogenase (BDH) genes, including NADH-dependent butanol dehydrogenase A; BDH I (EC 1.1.1-), NADH-dependent butanol dehydrogenase, and NADPH-dependent butanol dehydrogenase, and one type of bifunctional alcohol and aldehyde dehydrogenase (ADHE) gene. Butanol dehydrogenases and bifunctional alcohol and aldehyde dehydrogenase are essential enzymes that catalyze the final step in the production of butanol. Normally, these enzymes are found in solventogenic *Clostridium* sp. To investigate the differences between the genes involved in butanol production in CUEA02 and the top 5 %ANI, the IGV software was performed. The top 5 %ANI was presented in Table 9. This study focused on ADHE and BDH gene of *C. beijerinckii* BGS1, *C. beijerinckii* NCIMB 14988, *C. beijerinckii* DSM 791, and *C. beijerinckii* NCIMB 8052 compared with CUEA02 strain.

Table 9 the top 5 %ANI with the CUEA02 strain.

Genome	ANI (%)
C. beijerinckii BGS1	95.14
C. beijerinckii NCIMB 14988	95.11
C. beijerinckii DSM 791	94.95
C. beijerinckii ATCC 35702 SA-1	94.93
C. beijerinckii NCIMB 8052	94.93

The CUEA02 strain contains one copy of the ADHE gene and four copies of the BDH gene. When considering the nucleotide sequences of the ADHE and BDH genes of CUEA02 and other strains, a large number of mutations were observed. In particular, more than 100 mutation points were observed in the ADHE gene and less than 30 mutation points in the BDH gene. However, when examining mutations in protein sequences, it was shown that DSM 791 had the largest mutations at 18 positions in the ADHE gene, followed by 12 positions of NCIMB 8052, 10 positions of NCIMB 14988, and seven positions of BGS1. The BDH gene has fewer mutations than the ADHE gene, both at the nucleotide and protein sequences. The most protein sequence mutation points in the BDH A gene were found in seven positions of NCIMB

14988, followed by DSM 791 and BGS1 at six positions, and the least positions were detected in four positions of NCIMB 8052. Tables 10 and 11 present an overview of point mutation data in nucleotide and protein sequences.

Table 10 comparison of point mutations in nucleotide sequences between CUEA02 and the other strains in the top 5% ANI.

Strain	**%ANI**	**BLASTN (point mutations)**				
		ADHE (2,583 bp)	BDH (1,167 bp)	BDH (1,164 bp)	BDH A (1,179 bp)	BDH (1,167 bp)
1. BGS1	95.14	97	8	22	25	13
2. NCIMB 14988	95.11	106	6	24	19	11
3. DSM 791	94.95	128	11	20	21	7
4. NCIMB 8052	94.93	148	30	21	16	15

Table 11 comparison of point mutations in protein sequences between CUEA02 and the other strains in the top 5% ANI.

Strain	**%ANI**	**BLASTP (point mutations)**				
		ADHE (860 aa)	BDH (388 aa)	BDH (387 aa)	BDH A (392 aa)	BDH (388 aa)
1. BGS1	95.14	7	1	3	6	4
2. NCIMB 14988	95.11	10	0	3	7	3
3. DSM 791	94.95	18	1	3	6	0
4. NCIMB 8052	94.93	12	4	2	4	3

In addition, the CUEA02 genome was examined to determine its potential capacity for utilizing carbon sources. Several copies of genes involved in carbon sources being utilized were found, including alpha-xylosidase, L-arabinose isomerase, 6-phospho-beta-glucosidase, galactokinase, fructokinase, alpha-amylase, and others. Remarkably CUEA02 also had a high copy number of enzymes involved in the glycolytic pathway, that include glycerol dehydrogenase (EC 1.1.1.6; three copies)

and dihydroxyacetone kinase (EC 2.7.1.29; one copy). The CUEA02 enzyme involved in carbon utilization is summarized in Table 12. Glycerol dehydrogenases are widely identified in *C. pasteurianum*, an effective hyper-glycerol-utilizing bacteria (Malaviya et al., 2012). For example, *C. pasteurianum* ATCC 6013 contains glycerol dehydrogenase (dhaD1) and dihydroxyacetone kinase (dhaK), which is another gene in the glycolytic pathway. Furthermore, this strain was shown to possess three additional glycerol dehydrogenase paralogs (gldA1, gldA2, and dhaD2) in its genome (Sandoval et al., 2015).

In comparison with other *Clostridium* strains, *C. beijerinckii* NCIMB 8052 possesses just one glycerol dehydrogenase gene. Both CUEA02 and NCIMB 8052 have a single copy of the dihydroxyacetone kinase (dhaK) gene (Agu et al., 2019). When compared to *C. pasteurianum* ATCC 6013, CUEA02 possesses three copies of glycerol dehydrogenase and a single copy of dihydroxyacetone kinase. As a result, CUEA02 may be an effective glycerol utilizer.

Table 12 Enzymes involved the carbon utilization of *C. beijerinckii* CUEA02 (Buranaprasopchai et al., 2022).

EC code	Enzymes	Number encoded in genome
EC 5.3.1.5	Xylose isomerase	3
EC 3.2.1.177	alpha-xylosidase	2
EC 5.3.1.4	L-arabinose isomerase	1
EC 3.2.1.86	6-phospho-beta-glucosidase	13
EC 2.7.1.199	PTS system, glucose-specific IIA component	5
EC 2.7.1.-	Sugar kinase and transcription regulator	3
EC 2.7.1.6	Galactokinase	1
EC 2.7.7.10	Galactose-1-phosphate uridylyltransferase	2
EC 5.4.2.2	Phosphoglucomutase	2
EC 2.7.1.4	Fructokinase	2
EC 2.7.1.56	1-phosphofructokinase	1
EC 2.7.1.11	6-phosphofructokinase	2
EC 3.2.1.26	beta-fructofuranosidase	1
EC 5.3.1.8	Mannose-6-phosphate isomerase	3
EC 3.2.1.24	alpha-mannosidase	1
EC 2.7.1.211	PTS system, sucrose-specific IIB component	2
EC 3.2.1.26	Sucrose-6-phosphate hydrolase	3
EC 3.2.1.20	alpha-glucosidase	3
EC 3.2.1.21	beta-glucosidase	3
EC 3.2.1.1	alpha-amylase	1
EC 2.4.1.25	4-alpha-glucanotransferase (amylomaltase)	2
EC 3.2.1.135	Neopullulanase	2
EC 3.2.1.41	Pullulanase	1
EC 3.2.1.73	Endo-beta-1,3-1,4 glucanase (licheninase)	1

	Glucan 1,4-beta-glucosidase	2
EC 3.2.1.21	beta-glucosidase	3
EC 1.1.1.6	Glycerol dehydrogenase	3
EC 2.7.1.30	Glycerol kinase	1
EC 2.7.1.107	Diacylglycerol kinase	1
EC 2.7.1.29	Dihydroxyacetone kinase	1
EC 1.1.5.3	Glycerol-3-phosphate dehydrogenase	2
EC 1.1.1.94	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	2

3. Part III: The activities of key solventogenic enzymes during growth on glucose

According to whole genome sequencing data of CUEA02, it was revealed that CUEA02 contains four copies of BDH genes, including two copies of NADPH-dependent butanol dehydrogenase, one copy of NADH-dependent butanol dehydrogenase, and one copy of NADH-dependent butanol dehydrogenase A. These enzymes are an essential key enzyme in butanol synthesis. To examine the BDH activity, CUEA02 was cultured under anaerobic conditions for 120 h. The cells were extracted by sonicate and 2 mL of supernatant was used to measure the BDH activity. The result showed that CUEA02 has 0.0005 and 0.012 U/mg of NADH-BDH and NADPH-BDH specific activity, respectively. It seems that the NADH-BDH and

NADPH-BDH activity of CUEA02 was not quite high. When considering the other reports, it was revealed that the time for collecting cells affected BDH activity. Normally, the appropriate time to collect cells for BDH enzyme measurement ranges from 12 to 36 hours, depending on the strain. For instance, *C. beijerinckii* NCIMB 8052 had the highest BDH activity at 24 h, whereas *C. beijerinckii* IB4 had the greatest BDH activity at 16 h (Kong et al., 2016). Additionally, *Clostridium* sp. BOH3 had the largest BDH activity at 24 h (Rajagopalan et al., 2013). Therefore, the BDH activity of CUEA02 was reconsidered at 24 h. The BDH activity of CUEA02 at 24 h was increased to 0.005 and 0.041 U/mg in BDH-NADH and BDH-NADPH, respectively. In comparison, both NADH and NADPH-BDH activity at 24 and 120 h, was shown that the BDH activity at 24 was considerably greater than BDH activity at 120h. Consequently, this result is consistent with the findings of Jiang et al. (2014), who revealed that BDH activity was higher at the beginning of the solventogenesis phase. Additionally, the results were consistent with Rajagopalan et al. (2013), who observed that BDH activity reached a high level when the cells began the early phase of solventogenesis.

4. Part IV: Optimization condition and biochemical characterization of newly isolated *C. beijerinckii* CUEA02 for butanol production

4.1 Effect of initial pH

The crucial factors of butanol synthesis in *Clostridium* species are initial pH, temperature, inoculum size, and substrate concentration (Khamaiseh et al., 2012; Xin et al., 2017). The ideal pH range for butanol synthesis is typically between 5.0 and 6.5 (Jones & Woods, 1986; Maiti et al., 2016). To determine the initial pH that is suitable for the CUEA02 strain, the initial pH was varied in different values (4.5, 5.5, 6.5, 7.5, and 8.5) in the TYA medium and controlled temperature at 37 °C. The OD_{600} measurement was taken every 24 h for their growth. The result indicated that CUEA02 did not grow at pH 4.5 but performed well at pH 5.5 - 8.5, with the maximum OD_{600} at pH 6.5 (data not shown). CUEA02 produced butanol at 0.57 ± 0.01 and 0.87 ± 0.02 g/L after 24 hours at initial pH of 5.5 and 6.5, respectively (Figure 18A). However, raising the initial pH to 7.5 and 8.5 reduced butanol production to 0.75 ± 0.03 and 0.57 ± 0.03 g/L, respectively. According to these data, the initial pH had a substantial influence on butanol production. As a result, the optimal initial pH

of CUEA02 was determined to be 6.5, and this pH was employed to evaluate other CUEA02 culture conditions. The result of this study was consistent with the findings of Hijosa-Valsero et al. (2018), who discovered that the optimal pH range for solvent formation by *C. acetobutylicum* P262 was 5.0 - 6.5. Furthermore, when the initial pH was 6.5, *C. beijerinckii* ATCC 10132 produced higher butanol (Isar & Rangaswamy, 2012).

4.2 Effect of temperature

The temperature of the fermentation is also an essential component in butanol production. *Clostridium* species can grow at temperatures ranging from 25 to 40 °C (Brasca et al., 2022; da Silva et al., 2022; Ranjan et al., 2013); however, the optimal temperature for *Clostridium* species is between 30 and 37 °C (Cheng et al., 2019; Johnravindar et al., 2021). Nevertheless, the ideal pH and temperature for a given organism might vary depending on the strain and medium composition (Khamaiseh et al., 2013). CUEA02 was evaluated at 30, 35, and 37 °C for 24 h with an initial ferment pH of 6.5 to determine the most optimal fermentation temperature in

terms of butanol production. The result showed that CUEA02 produced the maximum butanol and ABE levels at 0.55 ± 0.09 and 1.39 ± 0.15 g/L, respectively, when cultivated at 35 °C (Figure 18B). On the other hand, other research has indicated a lower temperature. Yao et al. (2017) reported that *C. acetobutylicum* NRRL B527 produced the highest butanol level using glucose as a substrate at 30 °C, while *C. saccharoperbutylacetonicum* N1-4 produced 15.1 g/L butanol when cultured with 80 g/L glucose at 30 °C. Moreover, Shukor et al. (2014) also found that *C. saccharoperbutylacetonicum* N1-4 produced the greatest butanol production when the temperature was controlled at 28 °C. However, some reports revealed that the higher temperature can improve butanol production. *C. acetobutylicum* CICC 8008 produced the maximum butanol level at 35 °C (Lin et al., 2011), but some publications claimed that butanol production decreased with increasing temperature (Nakayama et al., 2011). It can be seen that the temperature might be different in each *Clostridium* sp.

4.3 Effect of inoculum size

Microbial concentration is an important element in every biological system. The appropriate concentration is crucial for initiating a production process (Montville & Schaffner, 2003). *C. beijerinckii* CUEA02 inoculum size was examined at 5%, 10%, 20%, 30%, and 40% under ideal initial pH and temperature to investigate the influence on butanol production. Figure 18C demonstrates the results of butanol production using various inoculum sizes in a TYA medium. Increasing the inoculum size from 5% to 10% resulted in enhanced butanol production up to 3.01 ± 0.12 g/L, which was 75% greater at a 10% (v/v) inoculum size than a 5% (v/v) inoculum size. This result was consistent with the findings of Nasrah et al. (2017), who discovered that increasing the inoculum size by 10% raised the butanol yield to 0.3054 g/g. Moreover, the inoculum size that was suitable for *C. acetobutylicum* using an oil palm decanter cake hydrolysate for ABE fermentation was 16.2% (Razak et al., 2013), while a 15% inoculum size was optimal for *C. saccharoperbutylacetonicum* N1–4 in butanol production (Al-Shorgani et al., 2015). In contrast, a 5% inoculum size was ideal for butanol production by *C. acetobutylicum* MTCC 481 from rice straw

hydrolysate (Ranjan et al., 2013). It is clear that the appropriate inoculum size depended on the microorganism and substrate.

Regarding the inoculum size had an effect on butanol synthesis. Increased inoculum size enhances butanol production due to increased cell concentration, and the lag phase of microbial development is shortened. Conversely, in this study, the inoculum size of more than 10% did not improve butanol production. This finding was supported by Ranjan et al. (2013), who found that the inoculum size level had no significant influence on the lag phase of microbial growth and cell activity, which resulted in butanol production.

4.4 Effect of substrate concentration

The last factor to be determined in this study is substrate concentration. To improve ABE solvent production, the initial glucose concentration in the TYA medium was varied at 40, 50, and 60 g/L (at 35 °C, 10% inoculum size, and an initial pH of 6.5 for 24 h). The OD600 was taken to measure their growth, and it was found that there was no significant difference (Data not shown). Therefore, ABE production was

considered to find the optimal substrate concentration. The result indicated that increasing the glucose concentration from 40 to 50 g/L enhanced the production of butanol from 6.32 ± 0.51 g/L to 8.32 ± 0.08 g/L. When the glucose concentration increased to 60 g/L, it decreased the butanol production to 5.59 ± 0.30 g/L (Figure 18D). Based on this finding, it is consistent with Al-Shorgani et al. (2018), who reported that butanol production at 50 g/L glucose was higher than butanol production at 20 g/L glucose. However, other reports have stated that *Clostridium* sp. could produce significant amounts of butanol in the presence of 40 - 60 g/L glucose in the medium (Chua et al., 2013; Formanek et al., 1997; Monot et al., 1982).

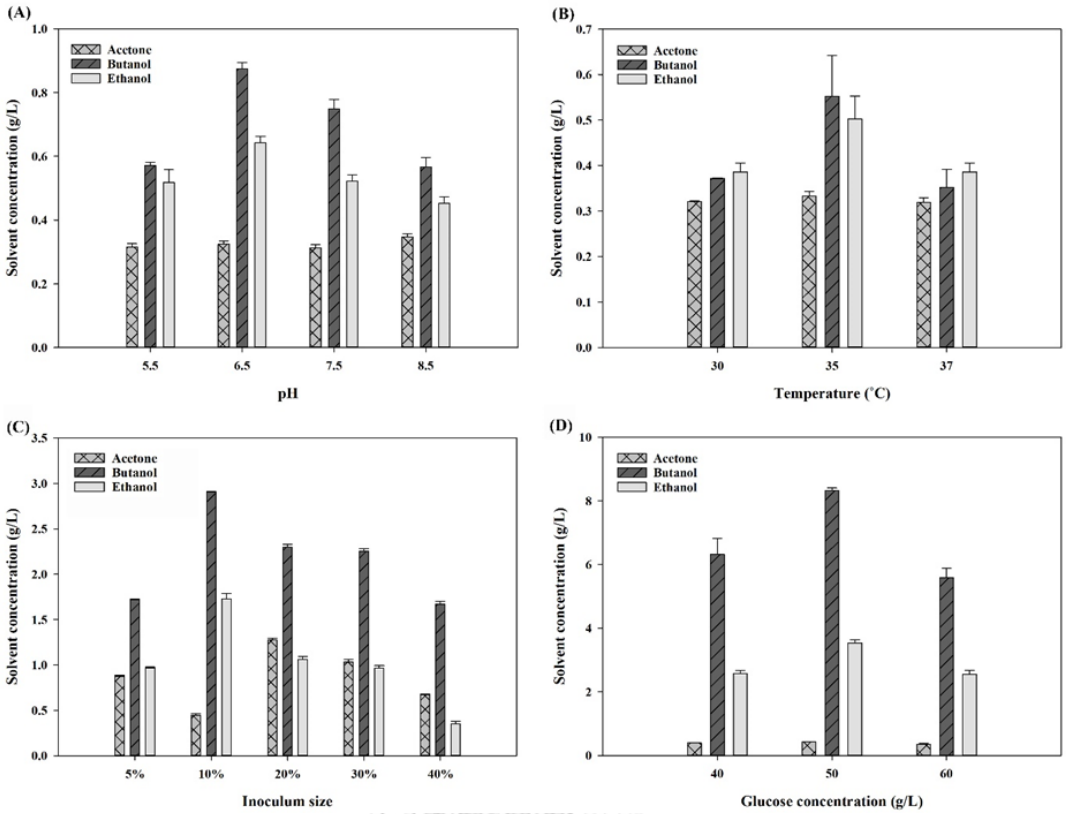


Figure 18 The effect of (A) initial pH (at 37 °C and 50 g/L glucose), (B) temperature (at pH 6.5 and 50 g/L glucose), (C) inoculum size (at pH 6.5, 35 °C, and 50 g/L glucose) and (D) glucose concentration (at pH 6.5, 35 °C, and 10% (v/v) inoculum size) on the ABE solvent production by *C. beijerinckii* CUEA02 in TYA medium after 24 h (Buranaprasopchai et al., 2022).

From aforementioned data, the optimal conditions on ABE production of CUEA02 are adjusted initial pH to 6.5, controlled temperature at 35 °C, 10% (v/v) inoculum size, and 50 g/L glucose as a substrate. To evaluate the ABE production

under optimal conditions, CUEA02 was cultured in a TYA medium for 144 h. The sample was taken every 24 h for analyzed ABE production. The result indicated that CUEA02 produced 13.48 ± 0.25 g/L and 8.32 ± 0.08 g/L of ABE and butanol production, respectively, as presented in Figure 19.

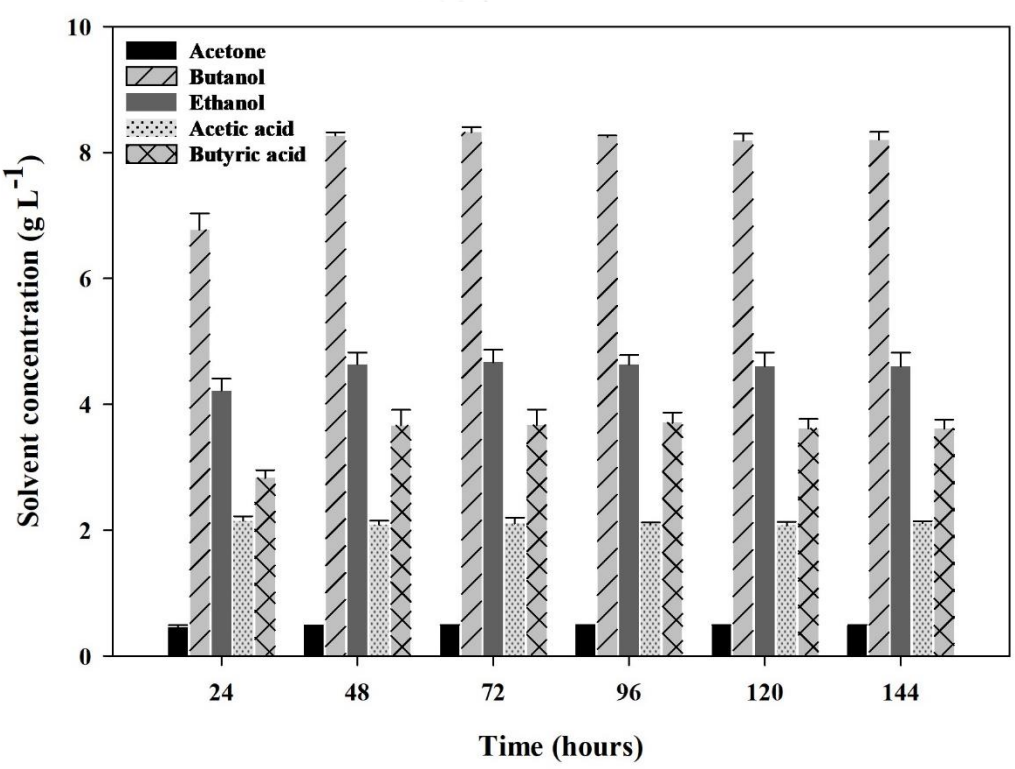


Figure 19 The levels of ABE production of *C. beijerinckii* CUEA02 cultured in TYA medium under optimal conditions (initial pH of 6.5, 35 °C, 10% (v/v) inoculum size, 50 g/L of glucose). The data are provided as the mean of triplicate trials.

4.5 Physiological and biochemical characterization

4.5.1 Carbon source utilization

The carbon utilization of *C. beijerinckii* CUEA02 was investigated by using various carbon sources, including pentoses, hexoses, disaccharides, and polysaccharides. The batch culture was employed using 2% (w/v) concentrations of xylose, arabinose, glucose, galactose, fructose, mannose, sucrose, starch, CMC, and glycerol in a TYA medium. The CUEA02 strain was cultured in a 60 mL serum bottle that contained 30 mL of TYA medium at pH 6.5 and 37 °C using 10% (v/v) inoculum. The growth was measured at OD600 every 24 h, and ABE production was measured at 72 h. In this study, it was found that *C. beijerinckii* CUEA02 grew in all of the carbon sources indicated above and produced ABE solvents as well as two VFAs: acetic and butyric acids, as presented in Figure 20.

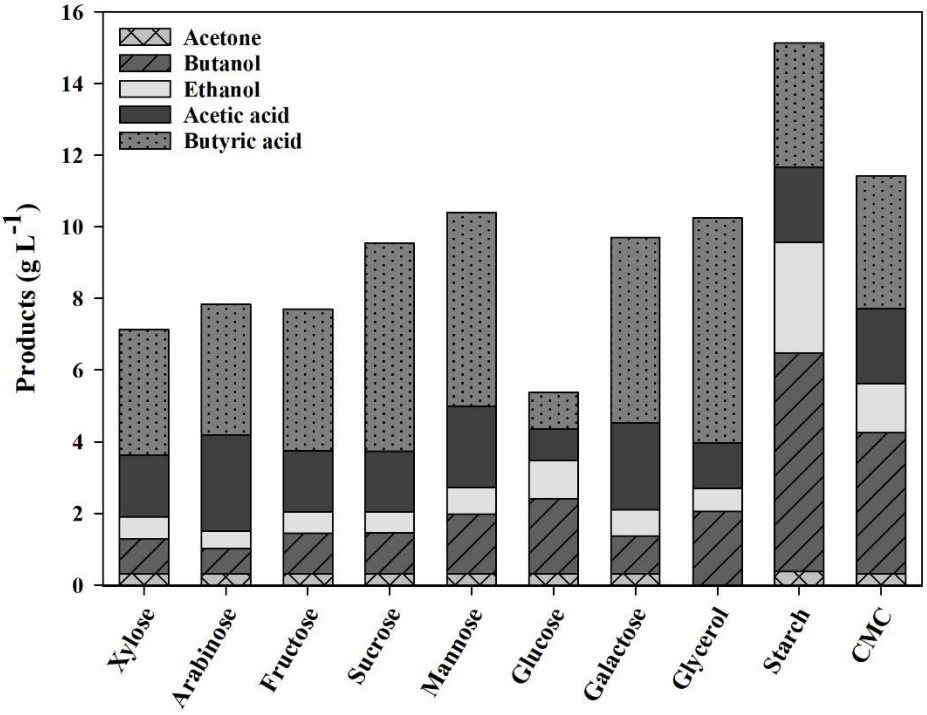


Figure 20 *C. beijerinckii* CUEA02 produced ABE solvents and volatile fatty acids at 2% (w/v) in TYA medium after 72 h at 37 °C. The data are presented as the mean of triplicate trials (Buranaprasopchai et al., 2022).

Considering each carbon source, *C. beijerinckii* CUEA02 produced a high level of butanol when cultured with starch, CMC, glucose, and glycerol (6.11 ± 0.10, 3.92 ± 0.05, 2.10 ± 0.08, and 2.05 ± 0.04 g/L, respectively). On the other hand, this strain produced butanol at a low level when cultured with xylose and arabinose (0.98 ± 0.02 and 0.70 ± 0.01 g/L, respectively). This is consistent with its growth,

which is lower than that of other carbon sources. These findings correspond with a previous study that found solvent-producing *Clostridium* sp. can completely consume some carbohydrates, including glucose, fructose, sucrose, mannose, and starch, while xylose, arabinose, raffinose, galactose, inulin, and mannitol were only partially utilized (Al-Shorgani et al., 2011; Jones & Woods, 1986).

Regarding the total acid production of the CUEA02 strain, it seems that CUEA02 produced large amounts of total acids from a variety of carbon sources. However, the acid production was at the lowest level when cultured CUEA02 with glucose (1.91 ± 0.03 g/L). Based on the aforementioned experimental data, it is possible to deduce that *C. beijerinckii* CUEA02 might be a good butanol producer from various carbon sources utilization, particularly starch, CMC, glucose, and glycerol, it is potentially appropriate for application in the fermentation of agricultural waste, industrial waste, and wastewater substrates. Therefore, this would assist in minimizing the cost of ABE production and waste recycling.

4.5.2 Butanol and ethanol tolerance

The *Clostridium* genus is known for producing solvents. However, the high concentration of solvents can affect the growth of *Clostridium* species. Basically, butanol is a toxic solvent that has an effect on cells and limits the production of ABE fermentation (Vasylkivska & Patakova, 2020). It has microorganisms that can tolerate butanol, such as *Clostridium* sp., *Pseudomonas* sp., *Zymomonas* sp., *Bacillus* sp., *Lactobacillus* sp., and *Enterococcus* sp. However, only a few butanol-tolerant bacterial species can survive in more than 2.0% (v/v) butanol (Kanno et al., 2013; Li et al., 2010). To evaluate the solvent tolerance of the CUEA02 strain, butanol was added to the TYA medium at different values (1.0%, 1.5%, 2.0%, and 2,5% v/v). The OD600 measurement was taken every 24 h for 3 days. The result showed that CUEA02 grew when cultured in TYA medium supplemented with butanol at 1.0% and 1.5% (v/v), as presented in Figure 21. When considering the growth rate, it was found that the CUEA02 growth decreased by approximately 50% and 70% at 1.0% and 1.5% (v/v), respectively. Although CUEA02 tolerated butanol concentrations up to 1.5% (v/v), this strain was tolerated better than *C. acetobutylicum* ATCC 824

(pGROE1), which overexpressed the *groESL* operon, which helps to improve butanol production and tolerance, at 0.75% (v/v) (Tomas et al., 2004). In addition, *C. tyrobutyricum* mutants can tolerate butanol up to 1.5% (v/v) with a 30% to 50% growth decrease (Yu et al., 2011), and *C. acetobutylicum* NT642 mutants can tolerate butanol at 3.0% (v/v) (Liu et al., 2012). Although CUEA02 is tolerant to butanol at 1.5% (v/v), it is still a wild-type strain. Therefore, this strain tends to be more tolerant to butanol than 1.5% (v/v) when improved by using genetic modification, as mentioned in the previous publication.

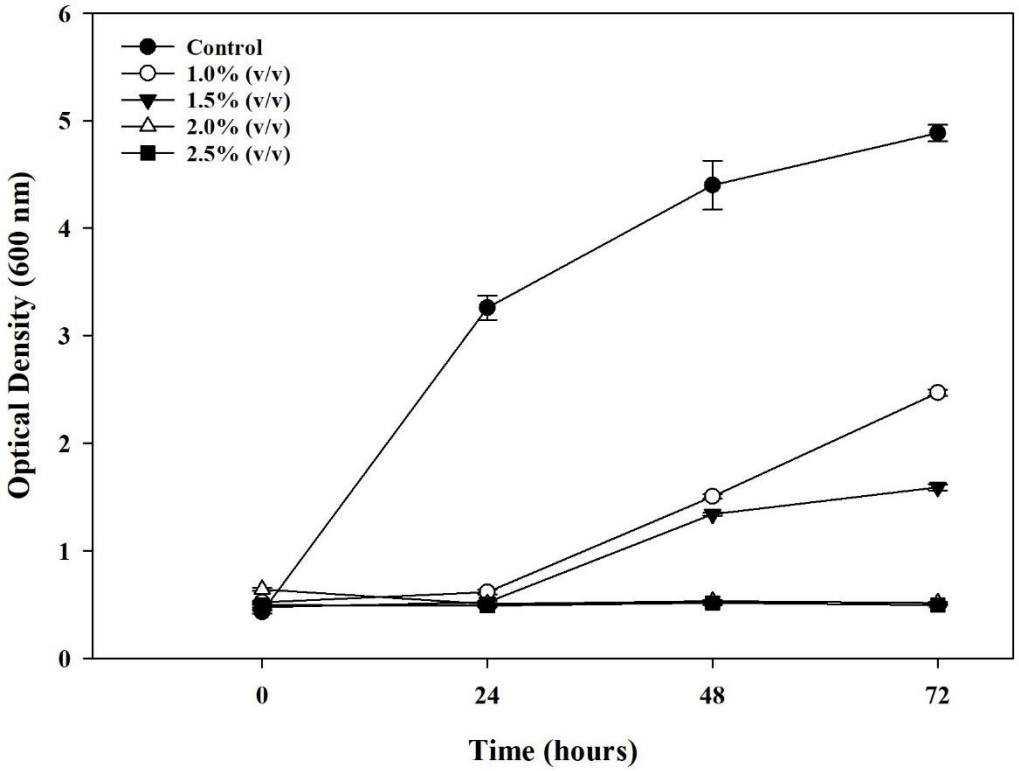


Figure 21 CUEA02 culture growth profile for butanol tolerance. Solid circle, control; open circle, 1.0% (v/v); solid triangle, 1.5% (v/v); open triangle, 2.0% (v/v); and solid square, 2.5% (v/v).

In addition, one of the remaining barriers to the industry is this microorganism's low ethanol tolerance. The ethanol toxicity causes denaturation of these macromolecules (proteins, DNA, RNA, and lipids), enhanced membrane fluidization, and altered nutrition transport. It has an effect on ATP production, resulting in cellular stress and probable cell death (Casey & Ingledew, 1986; Cray et

al., 2015). Normally, *C. thermocellum* is a thermophilic bacterium that can utilize biomass and produce high levels of ethanol. This means that this strain has a strong ethanol tolerance and can survive up to 20 g/L of butanol. To examine the ethanol tolerance in the CUEA02 strain, the ethanol was varied at 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, and 5.0% (v/v). After 72 h, it was found that CUEA02 grew when the ethanol concentration reached 4.0% (v/v), as presented in Figure 22. The ethanol concentration of 4.0% (v/v) decreased CUEA02 growth by approximately 65% as compared to the control. In comparison to *C. thermocellum*, an ethanol-tolerant strain, CUEA02 was shown to tolerate ethanol up to 31 g/L, which was greater than that strain. There is much research that has tried to enhance the ethanol tolerance of *C. thermocellum*. For example, a *C. thermocellum* mutant strain that can survive and grow at 40 g/L of ethanol production (Kuil et al., 2022) and *C. thermocellum* ATCC 27405, which is an ethanol-adapted strain, can tolerate ethanol at 50 g/L. However, it can be shown that CUEA02 has a relatively high level of ethanol resistance, making it appropriate for use in wastewater from alcoholic factories, which is likely to include ethanol.

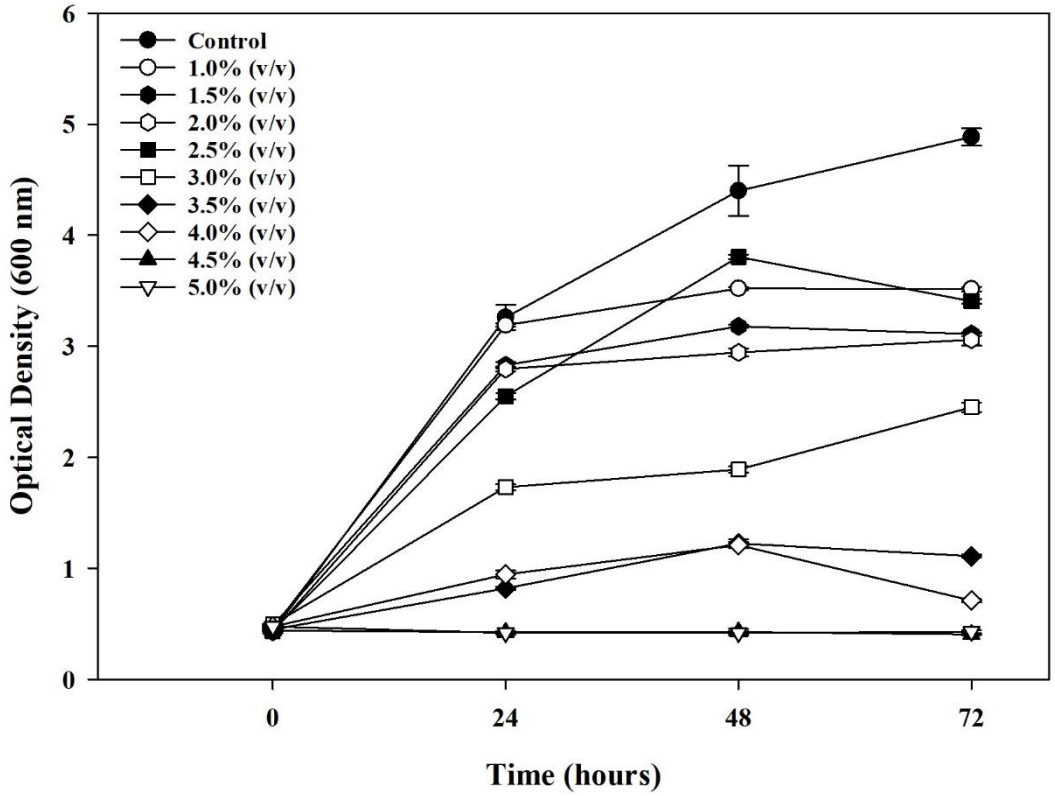


Figure 22 CUEA02 ethanol tolerance culture growth profile. Solid circle, control; open circle, 1.0% (v/v); solid hexagon, 1.5% (v/v); open hexagon, 2.0% (v/v); solid square, 2.5% (v/v); open square, 3.0% (v/v); solid diamond, 3.5% (v/v); open diamond, 4.0% (v/v); solid triangle, 4.5% (v/v); and open triangle, 5.0% (v/v).

5. Part V: Determination of Sato wastewater for butanol production

5.1 Characteristic of the Sato wastewater

The physiological and chemical properties of the STW were examined in order to determine its potential as a substrate for ABE fermentation. Table 13 shows the

results of an analysis of the pH, BOD, COD, TSS, TS, VS, TKN, reducing sugar, and starch levels. The STW had a milky white color with a pH of 3.5 (at 25 °C), which is highly acidic. The BOD and COD levels were excessive in comparison to the typical water pollution and limits enforced by Thailand's Pollution Control Department. The STW contained OMs and sugars, the most important of which were starch and reducing sugar. STW has the potential to be used as a carbon source by microorganisms as well as a substrate for solvent synthesis via ABE fermentation due to its starch and reducing sugar concentrations. Considering the starch and reducing sugar concentrations, STW has the potential to be used as a carbon source by microorganisms as well as a substrate for solvent production via ABE fermentation.

Table 13 Physical and chemical properties of Thai traditional beverage (Sato) factory effluent; STW (Buranaprasopchai et al., 2022).

Parameters[a]	**Unit**	**Sato wastewater; STW**
pH		3.5
BOD_5	mg/L	68,400
COD	mg/L	142,714
TSS	mg/L	573
TS	mg/L	10,192
VS	mg/L	8,852
TKN	mg/L	399
Starch content	mg/100g	26.58
Reducing sugar	mg/100g	60

[a] BOD: Biochemical oxygen demand, COD: Chemical oxygen demand, TSS: Total suspended solids, TS: Total solids, VS: Volatile solids, TKN: Total Kjeldahl nitrogen.

5.2 ABE production from *C. beijerinckii* CUEA02 by using Sato wastewater.

To reduce the price of the ABE fermentation process, the use of wastewater as a substrate is one possible method because this waste is plentiful and inexpensive. As a result, STW was chosen as a typical wastewater substrate for

butanol production in this study. In general, starch, reducing sugar, TKN, and other components are present in STW, making it potentially viable as a substrate for ABE production. However, among the other remaining components in STW was a large (inhibitory) level of ethanol (55 g/L). Therefore, the potential of STW as a substrate for ABE fermentation was examined using various dilutions (1:10, 1:20, 1:30, and 1:40). The ABE production was then measured by GC analysis.

The amount of ABE solvents and acids produced by *C. beijerinckii* CUEA02 from various dilutions of STW is shown in Figure 23. It can be seen that every STW dilution can be used as a substrate to produce butanol, with yields of 0.59 ± 0.03, 0.46 ± 0.03, 0.47 ± 0.05, and 0.43 ± 0.02 g/L for the 1:10, 1:20, 1:30, and 1:40 dilutions, respectively. While the neat STW, *C. beijerinckii* CUEA02 cannot grow and produce ABE production. Due to the composition of STW containing high levels of ethanol (55 g/L), CUEA02 can tolerate ethanol up to 31 g/L. This is a reason why CUEA02 cannot grow in neat STW. According to the data, the optimal concentration

for *C. beijerinckii* CUEA02 to produce butanol was 1:10 STW dilution, with total ABE and butanol production of 6.40 ± 0.66 and 0.59 ± 0.03 g/L, respectively.

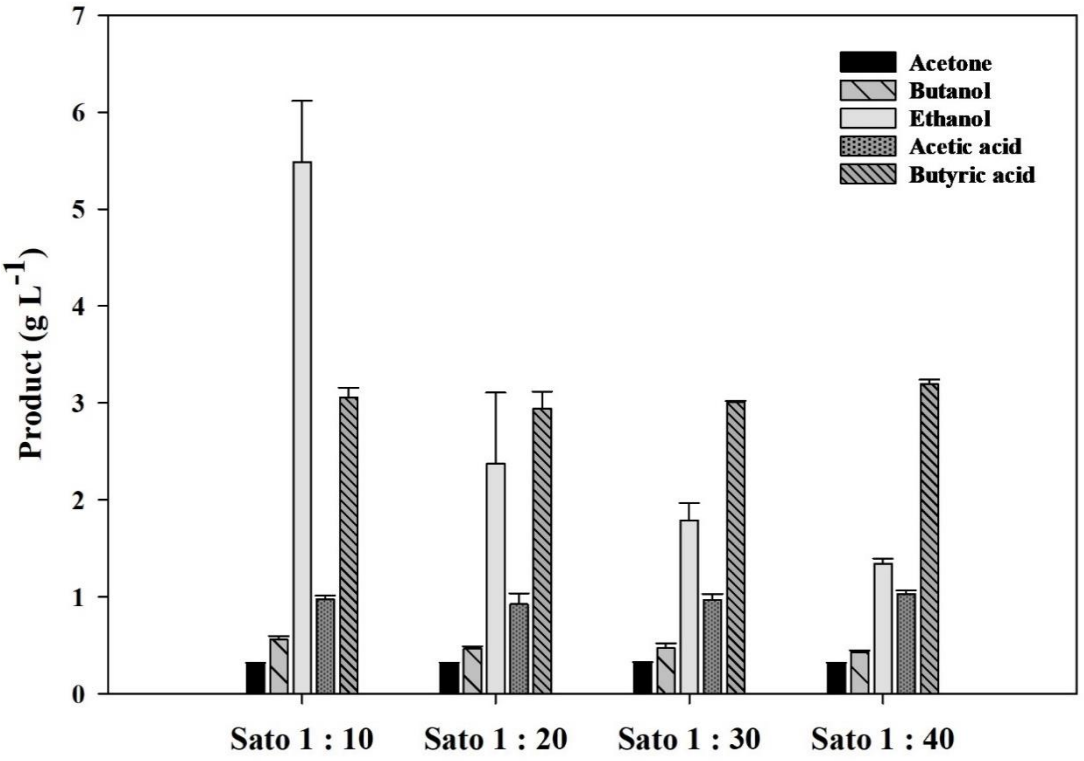


Figure 23 the level of ABE solvents and acids produced by *C. beijerinckii* CUEA02 from various dilutions of STW at 72 h, controlled temperature at 35 °C.

Although the fact that 1:10 STW dilution is the optimum concentration suited for use as a substrate for ABE fermentation, the production of butanol remained at a low level. From the mentioned above, there are many factors that can help to improve the levels of ABE production. The pH is a key factor that has a huge effect

on production. Therefore, to enhance butanol production, the initial pH of STW was considered. Due to the initial pH of the 1:10 diluted STW being 4.75, which is quite acidic. Whereas the optimal initial pH for CUEA02 in this study is 6.5. The 1:10 diluted STW was adjusted to the initial pH of 6.5 and used for the next experiment. Considering the growth of CUEA02, the OD600 was 0.67 when cultured in the 1:10 STW at pH 4.75, but it increased 1.7-fold when the initial pH was adjusted to 6.5. In addition, the production of the solvent increases in accordance with cell growth, with butanol production 2.67-fold greater at pH 6.5 than at pH 4.75 (Figure 24). Besides that, the levels of reducing sugar in 1:10 diluted STW with an initial pH of 6.5 was completely consumed in 48 h as opposed to 144 h at an initial pH of 4.75, leading to a 2.13-fold increase in butanol yield to 0.49 g/g. As a result, it is clear that the initial pH influences cell growth, sugar consumption, and butanol production in this *Clostridium* species. This finding was supported by Al-Shorgani et al. (2014), who reported that the initial pH has an important influence on butanol production. Furthermore, *C. acetobutylicum* NCIMB 13357 was observed to enhance ABE production when the pH was initially 5.5 - 5.8, but this reduced when the pH was

initially 6.0 (Kalil et al., 2003). This conclusion is also similar to the findings of Ouephanit et al. (2011), who discovered that increasing the initial pH from 4.5 to 5.5 improved the butanol production from *C. butyricum* and *C. acetobutylicum*, but reducing it to an initial pH of 6.5. As a result, the ideal pH for butanol production is determined by the *Clostridium* species.

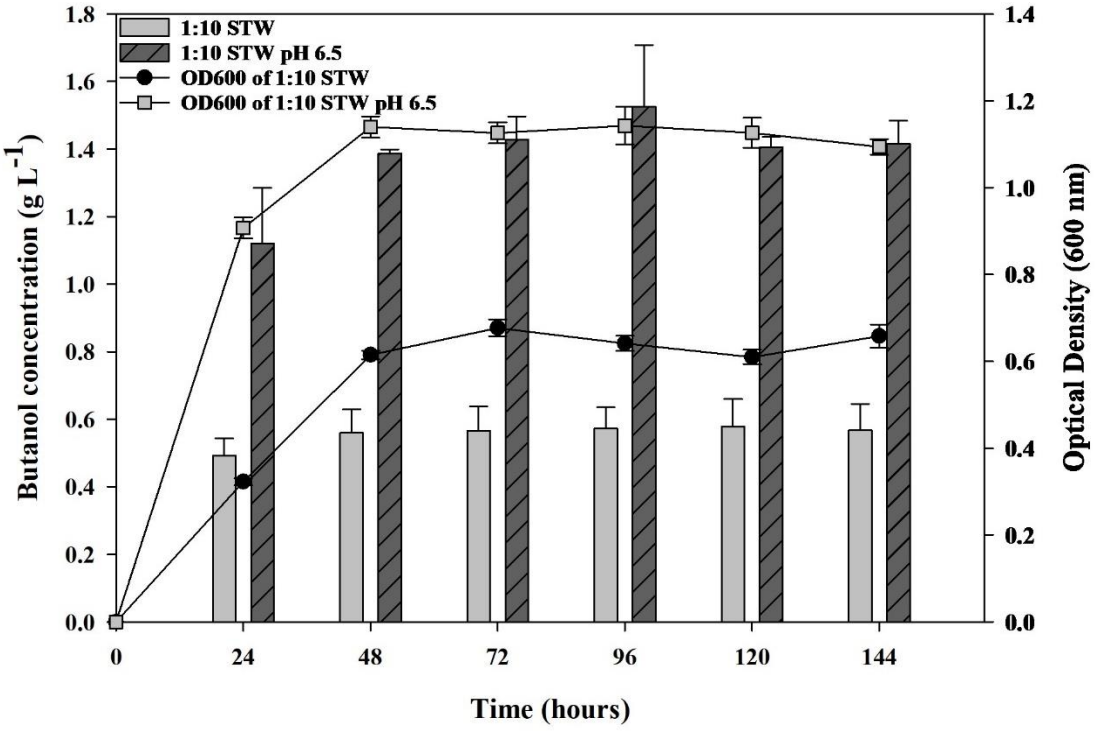


Figure 24 butanol production and cell growth characteristics of *C. beijerinckii* CUEA02 cultured in a 1:10 STW dilution (with and without modified initial pH to 6.5) at 35 °C.

5.3 The effect of nitrogen content supplementation in Sato wastewater on ABE production in *C. beijerinckii* CUEA02

The change in medium composition throughout the fermentation process influences not only the growth of microbes but also the synthesis of metabolites. It has been reported to improve metabolite production by adding some components to the medium, such as nitrogen sources, vitamins, and trace elements (Im et al., 2021; Papizadeh et al., 2020). To examine the effect of nitrogen sources on ABE production, yeast extract was supplemented in 1:10 diluted STW at different concentrations (2, 4, 6, and 8 g/L) with an initial pH of 6.5. The result indicated that butanol production in 1:10 diluted STW was enhanced to 1.96 ± 0.04, 1.86 ± 0.01, 1.83 ± 0.01, and 1.82 ± 0.04 for the 2, 4, 6, and 8 g/L of yeast extract, respectively. The production of butanol when supplemented with yeast extract in 1:10 dilute STW is shown in Figure 25. From Figure 25, butanol production was slightly different at each yeast extract concentration. In this study, yeast extract 2 g/L improved butanol production from 1.52 to 1.96 g/L, increasing by more than 29%. Earlier research found that adding yeast extract to the medium containing cassava as a substrate

improves at the beginning of the solventogenesis phase in *C. acetobutylicum*. When compared to employing cassava as a sole substrate, the butanol concentration increased by 15% (Li et al., 2012). Furthermore, yeast extract was determined to be outstanding for enhancing the butanol production level from starch wastewater among various nitrogen sources (Luo et al., 2018). In addition, Mao et al. (2019) confirmed these findings by evaluating the butanol production of *C. acetobutylicum* CGMCC1.0134 using fern root as a substrate. When yeast extract was added to the medium, the butanol production increased from 3.74 to 11.8 g/L. It is obvious that yeast extract can enhance butanol production when used at the appropriate concentration.

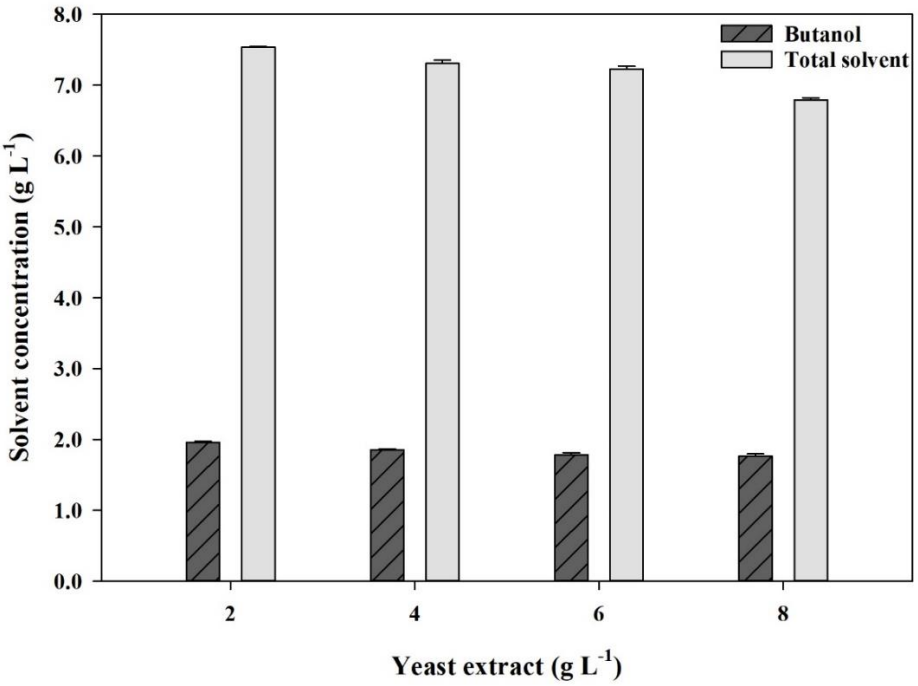


Figure 25 the level of butanol and total solvents of *C. beijerinckii* CUEA02 in 1:10 diluted STW with an initial pH of 6.5 and yeast extract supplementation (Buranaprasopchai et al., 2022).

CHAPTER V

CONCLUSION

This is the first study of the production of ABE in *Clostridium* sp. using Sato wastewater as a substrate. CUEA02, a new solventogenic *C. beijerinckii* isolated from a biodiesel plant, was discovered. This strain is highly capable of producing ABE and can consume a wide range of carbohydrates, particularly starch, to effectively generate butanol. The starch hydrolyzes confirmed this strain can consume starch by clear zone around the colony which indicated to amylase enzyme. The CUEA02 strain produced 13.48 ± 0.25 g/L and 8.32 ± 0.08 g/L of ABE and butanol production, respectively, when culture under optimal conditions with initial pH of 6.5, a temperature of 35 °C, 10% (v/v) of inoculum, and 50 g/L of glucose. This strain also produces NADH-BDH and NADPH-BDH activity at 24 h (0.005 U/mg and 0.041 U/mg, respectively) but it is not quite high. When considering the comparison of WGS with other *Clostridium* sp., it was found that the CUEA02 strain has a large number of point mutations in nucleotide sequences of ADHE and BDH genes. This study intended to evaluate the potential of CUEA02 on ABE production from consolidated

bioprocess using Sato wastewater. Because of the CUEA02's ability to consume starch, which is a main component in STW. CUEA02 could produce butanol from 1:10 diluted STW (0.23 g/g) at pH 4.75 but increasing the initial pH to 6.5 increased the production of butanol to 0.49 g/g, which is 2.13-fold compared to the not adjusted initial pH. Furthermore, the supplement of yeast extract to 1:10 diluted STW also enhanced butanol production by 29%. It can be concluded that CUEA02 has an effective potential to utilize STW as a low-cost substrate for butanol synthesis. In addition, it can also help to solve the wastewater, which frequently unpleasant smell and is damaging to the environment and humans. Furthermore, it is one possible way that contributes to the increased value-added chemical substances from wastewater.

REFERENCES

Agu, C. V., Ujor, V., & Ezeji, T. C. (2019). Metabolic engineering of *Clostridium beijerinckii* to improve glycerol metabolism and furfural tolerance. *Biotechnology for biofuels*, *12*, 1-19.

Akbar, M., Yaqoob, A., Ahmad, I., & Ahmad, A. (2023). Wastewater treatment: an overview. *Sodium Alginate-Based Nanomaterials for Wastewater Treatment*, 19-34.

Al-Shorgani, N. K., Tibin, E. M., Kalil, M. S., Yusoff, W. M. W., & Hamid, A. A. (2014). Biobutanol production by a new local isolate of *Clostridium acetobutylicum* YM1. AIP Conference Proceedings,

Al-Shorgani, N. K. N., Kalil, M. S., Yusoff, W. M. W., & Hamid, A. A. (2018). Impact of pH and butyric acid on butanol production during batch fermentation using a new local isolate of *Clostridium acetobutylicum* YM1. *Saudi journal of biological sciences*, *25*(2), 339-348.

Al-Shorgani, N. K. N., Mohd, S. K., & Wan, M. (2011). The effect of different carbon sources on biobutanol production using *Clostridium saccharoperbutylacetonicum* N1-4. *Biotechnology*, *10*(3), 280-285.

Al-Shorgani, N. K. N., Shukor, H., Abdeshahian, P., Nazir, M. Y. M., Kalil, M. S., Hamid, A. A., & Yusoff, W. M. W. (2015). Process optimization of butanol production by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) using palm oil mill effluent in acetone–butanol–ethanol fermentation. *Biocatalysis and Agricultural Biotechnology*, *4*(2), 244-249.

Amiri, H., Karimi, K., & Zilouei, H. (2014). Organosolv pretreatment of rice straw for efficient acetone, butanol, and ethanol production. *Bioresource technology*, *152*, 450-456.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., & Eppig, J. T. (2000). Gene ontology: tool for the unification of biology. *Nature genetics*, *25*(1), 25-29.

Athanasopoulos, N. (1987). Anaerobic treatment of beet molasses alcoholic

fermentation wastewater in a downflow filter. *Resources and conservation*, *15*(1-2), 147-150.

Beesch, S. C. (1952). Acetone-butanol fermentation of sugars. *Industrial & Engineering Chemistry*, *44*(7), 1677-1682.

Berezina, O., Zakharova, N., Yarotsky, C., & Zverlov, V. (2012). Microbial producers of butanol. *Applied biochemistry and microbiology*, *48*, 625-638.

Birgen, C., Dürre, P., Preisig, H. A., & Wentzel, A. (2019). Butanol production from lignocellulosic biomass: revisiting fermentation performance indicators with exploratory data analysis. *Biotechnology for biofuels*, *12*(1), 1-15.

Brasca, M., Morandi, S., & Silvetti, T. (2022). *Clostridium* spp. In P. L. H. McSweeney & J. P. McNamara (Eds.), *Encyclopedia of Dairy Sciences (Third Edition)* (pp. 431-438). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-08-100596-5.22989-2

Brettin, T., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Olsen, G. J., Olson, R., Overbeek, R., Parrello, B., & Pusch, G. D. (2015). RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Scientific reports*, *5*(1), 8365.

Bryant, D. L., & Blaschek, H. P. (1988). Buffering as a means for increasing growth and butanol production by *Clostridium acetobutylicum*. *Journal of industrial microbiology*, *3*, 49-55.

Buranaprasopchai, J., Boonvitthya, N., Glinwong, C., & Chulalaksananukul, W. (2022). Butanol production from Thai traditional beverage (Sato) factory wastewater using newly isolated *Clostridium beijerinckii* CUEA02. *Biochemical engineering journal*, *187*, 108648.

Casey, G. P., & Ingledew, W. M. (1986). Ethanol tolerance in yeasts. *CRC Critical Reviews in Microbiology*, *13*(3), 219-280.

Castro, Y. A., Ellis, J. T., Miller, C. D., & Sims, R. C. (2015). Optimization of wastewater microalgae saccharification using dilute acid hydrolysis for acetone, butanol, and ethanol fermentation. *Applied Energy*, *140*, 14-19.

Cebreiros, F., Ferrari, M. D., & Lareo, C. (2019). Cellulose hydrolysis and IBE fermentation

of eucalyptus sawdust for enhanced biobutanol production by *Clostridium beijerinckii* DSM 6423. *Industrial Crops and Products*, *134*, 50-61.

Chai, W. S., Chew, C. H., Munawaroh, H. S. H., Ashokkumar, V., Cheng, C. K., Park, Y.-K., & Show, P. L. (2021). Microalgae and ammonia: a review on inter-relationship. *Fuel*, *303*, 121303.

Chai, W. S., Tan, W. G., Munawaroh, H. S. H., Gupta, V. K., Ho, S.-H., & Show, P. L. (2021). Multifaceted roles of microalgae in the application of wastewater biotreatment: a review. *Environmental Pollution*, *269*, 116236.

Cheng, C., Bao, T., & Yang, S.-T. (2019). Engineering *Clostridium* for improved solvent production: recent progress and perspective. *Applied Microbiology and Biotechnology*, *103*, 5549-5566.

Cheng, H.-H., Whang, L.-M., Chan, K.-C., Chung, M.-C., Wu, S.-H., Liu, C.-P., Tien, S.-Y., Chen, S.-Y., Chang, J.-S., & Lee, W.-J. (2015). Biological butanol production from microalgae-based biodiesel residues by *Clostridium acetobutylicum*. *Bioresource technology*, *184*, 379-385.

Choi, H., Han, J., & Lee, J. (2021). Renewable butanol production via catalytic routes. *International Journal of Environmental Research and Public Health*, *18*(22), 11749.

Chua, T. K., Liang, D.-W., Qi, C., Yang, K.-L., & He, J. (2013). Characterization of a butanol–acetone-producing *Clostridium* strain and identification of its solventogenic genes. *Bioresource technology*, *135*, 372-378.

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., & Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, *21*(18), 3674-3676.

Cook, M. (2021). Trends in global energy supply and demand. In *Developments in Petroleum Science* (Vol. 71, pp. 15-42). Elsevier.

Cray, J. A., Stevenson, A., Ball, P., Bankar, S. B., Eleutherio, E. C., Ezeji, T. C., Singhal, R. S., Thevelein, J. M., Timson, D. J., & Hallsworth, J. E. (2015). Chaotropicity: a key factor in product tolerance of biofuel-producing microorganisms. *Current opinion in biotechnology*, *33*, 228-259.

Crini, G., & Lichtfouse, E. (2019). Advantages and disadvantages of techniques used for

wastewater treatment. *Environmental Chemistry Letters*, *17*, 145-155.

da Silva, D. B., Fernandes, B. S., & da Silva, A. J. (2022). Butanol production by *Clostridium acetobutylicum* DSMZ 792 from cassava starch. *Environmental Sustainability*, *5*(1), 91-102.

Dadgar, A. M., & Foutch, G. L. (1988). Improving the acetone-butanol fermentation process with liquid-liquid extraction. *Biotechnology progress*, *4*(1), 36-39.

Demirbas, M. F. (2009). Biorefineries for biofuel upgrading: a critical review. *Applied Energy*, *86*, S151-S161.

Drahokoupil, M., & Patáková, P. (2020). Production of butyric acid at constant pH by a solventogenic strain of *Clostridium beijerinckii*. *Czech Journal of Food Sciences*, *38*(3), 185-191.

Dung, N., Rombouts, F., & Nout, M. (2007). Characteristics of some traditional Vietnamese starch-based rice wine fermentation starters (men). *LWT-Food Science and Technology*, *40*(1), 130-135.

Dürre, P. (2007). Biobutanol: an attractive biofuel. *Biotechnology Journal: Healthcare Nutrition Technology*, *2*(12), 1525-1534.

Dutta, D., Arya, S., & Kumar, S. (2021). Industrial wastewater treatment: Current trends, bottlenecks, and best practices. *Chemosphere*, *285*, 131245.

Dziemianowicz, W., Kotarska, K., & Świerczyńska, A. (2022). Increase Butanol Production from Corn Straw by Mineral Compounds Supplementation. *Energies*, *15*(19), 6899.

Efremenko, E., Nikolskaya, A., Lyagin, I., Senko, O., Makhlis, T., Stepanov, N., Maslova, O., Mamedova, F., & Varfolomeev, S. (2012). Production of biofuels from pretreated microalgae biomass by anaerobic fermentation with immobilized *Clostridium acetobutylicum* cells. *Bioresource technology*, *114*, 342-348.

Ellis, J. T., Hengge, N. N., Sims, R. C., & Miller, C. D. (2012). Acetone, butanol, and ethanol production from wastewater algae. *Bioresource technology*, *111*, 491-495.

Ezeji, T. C., Groberg, M., Qureshi, N., & Blaschek, H. P. (2003). Continuous production of butanol from starch-based packing peanuts. Biotechnology for Fuels and

Chemicals: The Twenty-Fourth Symposium,

FAYSSAL, F., MORTADA, A., & GHANDOUR, M. Wastewater Treatment: Overview, Types, Energy Consumption & Actual State in Lebanon with Proposal of Using Renewable Energy. *Acta Materialia Turcica*, *4*(2), 1-9.

Figueras, M. J., Beaz-Hidalgo, R., Hossain, M. J., & Liles, M. R. (2014). Taxonomic affiliation of new genomes should be verified using average nucleotide identity and multilocus phylogenetic analysis. *Genome announcements*, *2*(6), e00927-00914.

Formanek, J., Mackie, R., & Blaschek, H. P. (1997). Enhanced butanol production by *Clostridium beijerinckii* BA101 grown in semidefined P2 medium containing 6 percent maltodextrin or glucose. *Applied and environmental microbiology*, *63*(6), 2306-2310.

Gottumukkala, L. D., Parameswaran, B., Valappil, S. K., Mathiyazhakan, K., Pandey, A., & Sukumaran, R. K. (2013). Biobutanol production from rice straw by a non acetone producing *Clostridium sporogenes* BE01. *Bioresource technology*, *145*, 182-187.

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., Robles, M., Talón, M., Dopazo, J., & Conesa, A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic acids research*, *36*(10), 3420-3435.

Gu, Y., Jiang, Y., Wu, H., Liu, X., Li, Z., Li, J., Xiao, H., Shen, Z., Dong, H., & Yang, Y. (2011). Economical challenges to microbial producers of butanol: feedstock, butanol ratio and titer. *Biotechnology journal*, *6*(11), 1348-1357.

Hijosa-Valsero, M., Garita-Cambronero, J., Paniagua-García, A. I., & Díez-Antolínez, R. (2018). Biobutanol production from coffee silverskin. *Microbial Cell Factories*, *17*(1), 1-9.

Hou, X., From, N., Angelidaki, I., Huijgen, W. J., & Bjerre, A.-B. (2017). Butanol fermentation of the brown seaweed *Laminaria digitata* by *Clostridium beijerinckii* DSM-6422. *Bioresource technology*, *238*, 16-21.

Huang, H., Liu, H., & Gan, Y.-R. (2010). Genetic modification of critical enzymes and involved genes in butanol biosynthesis from biomass. *Biotechnology advances*, *28*(5), 651-657.

Im, H., An, T., Kwon, R., Park, S., & Kim, Y.-K. (2021). Effect of organic nitrogen supplements on syngas fermentation using *Clostridium autoethanogenum*. *Biotechnology and Bioprocess Engineering*, *26*, 476-482.

Isar, J., & Rangaswamy, V. (2012). Improved n-butanol production by solvent tolerant *Clostridium beijerinckii*. *Biomass and bioenergy*, *37*, 9-15.

Jang, Y.-S., Lee, J. Y., Lee, J., Park, J. H., Im, J. A., Eom, M.-H., Lee, J., Lee, S.-H., Song, H., & Cho, J.-H. (2012). Enhanced butanol production obtained by reinforcing the direct butanol-forming route in *Clostridium acetobutylicum*. *MBio*, *3*(5), e00314-00312.

Jiang, M., Chen, J.-n., He, A.-y., Wu, H., Kong, X.-p., Liu, J.-l., Yin, C.-y., Chen, W.-f., & Chen, P. (2014). Enhanced acetone/butanol/ethanol production by *Clostridium beijerinckii* IB4 using pH control strategy. *Process Biochemistry*, *49*(8), 1238-1244.

Jiang, Y., Li, J., Ni, Y., Tang, Y., Wu, M., Zhang, W., Xin, F., & Jiang, M. (2022). High butanol production from galactose riched red seaweed *Gracilaria lemaneiformis* by solventogenic *Clostridium* sp. strain NJ4. *Fuel*, *307*, 121867.

Jiang, Y., Liu, J., Jiang, W., Yang, Y., & Yang, S. (2015). Current status and prospects of industrial bio-production of n-butanol in China. *Biotechnology advances*, *33*(7), 1493-1501.

Johnravindar, D., Elangovan, N., Gopal, N. O., Muthaiyan, A., & Fei, Q. (2021). Biobutanol production from cassava waste residue using *Clostridium* sp. AS3 in batch culture fermentation. *Biofuels*, *12*(10), 1259-1266.

Jones, D. T., & Woods, D. (1986). Acetone-butanol fermentation revisited. *Microbiological reviews*, *50*(4), 484-524.

Kalil, M. S., Kit, P., Yusoff, W. W., Sadazo, Y., & Rahman, R. A. (2003). Direct fermentation of palm oil mill effluent to acetone-butanol-ethanol by solvent producing Clostridia. *Pakistan Journal of Biological Sciences (Pakistan)*.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic acids research*, *44*(D1), D457-D462.

Kanno, M., Katayama, T., Tamaki, H., Mitani, Y., Meng, X.-Y., Hori, T., Narihiro, T., Morita, N., Hoshino, T., & Yumoto, I. (2013). Isolation of butanol-and isobutanol-tolerant

bacteria and physiological characterization of their butanol tolerance. *Applied and environmental microbiology*, *79*(22), 6998-7005.

Katagiri, H., Sugimori, T., Imai, K., Komaki, S., Okuzumi, M., & Anyoji, H. (1961). On the Metabolism of Organic Acids by *Clostridium acetobutylicum*: Part III. Purification and Properties of Racemiase Part IV. Mechanism of Racemiase Action Part V. Further Studies on the Formation of Racemiase Part VI. Metabolism of Crotonic Acid Part VII. Reduction of Crotonic Acid. *Agricultural and biological chemistry*, *25*(4), 281-313.

Khamaiseh, E., Hamid, A., Yusoff, W., & Kalil, M. S. (2013). Effect of some environmental parameters on biobutanol production by *Clostridium acetobutylicum* NCIMB 13357 in date fruit medium. *Pakistan journal of biological sciences: PJBS*, *16*(20), 1145-1151.

Khamaiseh, E. I., Mohd, S. K., Olujimi, D., Ibrahim, E.-S., & Wan, M. (2012). Date fruit as carbon source in RCM-modified medium to produce biobutanol by *Clostridium acetobutylicum* NCIMB 13357. *Journal of Applied Sciences*, *12*(11), 1160-1165.

Kida, K., Morimura, S., Abe, N., & Sonoda, Y. (1995). Biological treatment of Schochu distillery wastewater. *Process Biochemistry*, *30*(2), 125-132.

Killeffer, D. (1927). Butanol and acetone from corn1: a description of the fermentation process. *Industrial & Engineering Chemistry*, *19*(1), 46-50.

Kolbert, C. P., & Persing, D. H. (1999). Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Current opinion in microbiology*, *2*(3), 299-305.

Komonkiat, I., & Cheirsilp, B. (2013). Felled oil palm trunk as a renewable source for biobutanol production by *Clostridium* spp. *Bioresource technology*, *146*, 200-207.

Kong, X., He, A., Zhao, J., Wu, H., Ma, J., Wei, C., Jin, W., & Jiang, M. (2016). Efficient acetone–butanol–ethanol (ABE) production by a butanol-tolerant mutant of *Clostridium beijerinckii* in a fermentation–pervaporation coupled process. *Biochemical engineering journal*, *105*, 90-96.

Kuhner, C. H., Matthies, C., Acker, G., Schmittroth, M., Göβner, A. S., & Drake, H. L. (2000). *Clostridium akagii* sp. nov. and *Clostridium acidisoli* sp. nov.: acid-tolerant, N2-

fixing clostridia isolated from acidic forest soil and litter. *International journal of systematic and evolutionary microbiology*, *50*(2), 873-881.

Kuil, T., Yayo, J., Pechan, J., Küchler, J., & van Maris, A. J. (2022). Ethanol tolerance of *Clostridium thermocellum*: the role of chaotropicity, temperature and pathway thermodynamics on growth and fermentative capacity. *Microbial Cell Factories*, *21*(1), 273.

Kundiyana, D. K., Wilkins, M. R., Maddipati, P., & Huhnke, R. L. (2011). Effect of temperature, pH and buffer presence on ethanol production from synthesis gas by "*Clostridium ragsdalei*". *Bioresource technology*, *102*(10), 5794-5799.

Lahlou, F.-Z., Mackey, H. R., & Al-Ansari, T. (2022). Role of wastewater in achieving carbon and water neutral agricultural production. *Journal of Cleaner Production*, 130706.

Lee, J., Jang, Y.-S., Choi, S. J., Im, J. A., Song, H., Cho, J. H., Seung, D. Y., Papoutsakis, E. T., Bennett, G. N., & Lee, S. Y. (2012). Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol-butanol-ethanol fermentation. *Applied and environmental microbiology*, *78*(5), 1416-1423.

Lee, S.-M., Cho, M. O., Park, C. H., Chung, Y.-C., Kim, J. H., Sang, B.-I., & Um, Y. (2008). Continuous butanol production using suspended and immobilized *Clostridium beijerinckii* NCIMB 8052 with supplementary butyrate. *Energy & fuels*, *22*(5), 3459-3464.

Lehmann, D., Hönicke, D., Ehrenreich, A., Schmidt, M., Weuster-Botz, D., Bahl, H., & Lütke-Eversloh, T. (2012). Modifying the product pattern of *Clostridium acetobutylicum*: physiological effects of disrupting the acetate and acetone formation pathways. *Applied Microbiology and Biotechnology*, *94*, 743-754.

Li, J., Du, Y., Bao, T., Dong, J., Lin, M., Shim, H., & Yang, S.-T. (2019). n-Butanol production from lignocellulosic biomass hydrolysates without detoxification by *Clostridium tyrobutyricum* Δack-adhE2 in a fibrous-bed bioreactor. *Bioresource technology*, *289*, 121749.

Li, J., Zhao, J., Zhao, M., Yang, Y., Jiang, W., & Yang, S. (2010). Screening and characterization of butanol-tolerant micro-organisms. *Letters in applied*

microbiology, *50*(4), 373-379.

Li, S., Guo, Y., Lu, F., Huang, J., & Pang, Z. (2015). High-level butanol production from cassava starch by a newly isolated *Clostridium acetobutylicum*. *Applied biochemistry and biotechnology*, *177*, 831-841.

Li, X., Li, Z., Zheng, J., Shi, Z., & Li, L. (2012). Yeast extract promotes phase shift of bio-butanol fermentation by *Clostridium acetobutylicum* ATCC824 using cassava as substrate. *Bioresource technology*, *125*, 43-51.

Lin, C.-Y., Chai, W. S., Lay, C.-H., Chen, C.-C., Lee, C.-Y., & Show, P. L. (2021). Optimization of hydrolysis-acidogenesis phase of swine manure for biogas production using two-stage anaerobic fermentation. *Processes*, *9*(8), 1324.

Lin, Y., Wang, J., Wang, X., & Sun, X. (2011). Optimization of butanol production from corn straw hydrolysate by *Clostridium acetobutylicum* using response surface method. *Chinese Science Bulletin*, *56*, 1422-1428.

Liu, X.-B., Gu, Q.-Y., Yu, X.-B., & Luo, W. (2012). Enhancement of butanol tolerance and butanol yield in *Clostridium acetobutylicum* mutant NT642 obtained by nitrogen ion beam implantation. *The Journal of Microbiology*, *50*(6), 1024.

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

Luo, W., Zhao, Z., Pan, H., Zhao, L., Xu, C., & Yu, X. (2018). Feasibility of butanol production from wheat starch wastewater by *Clostridium acetobutylicum*. *Energy*, *154*, 240-248.

Madihah, M., Ariff, A., Sahaid, K., Suraini, A., & Karim, M. (2001). Direct fermentation of gelatinized sago starch to acetone–butanol–ethanol by *Clostridium acetobutylicum*. *World Journal of Microbiology and Biotechnology*, *17*, 567-576.

Maiti, S., Sarma, S. J., Brar, S. K., Le Bihan, Y., Drogui, P., Buelna, G., & Verma, M. (2016). Agro-industrial wastes as feedstock for sustainable bio-production of butanol by *Clostridium beijerinckii*. *Food and Bioproducts Processing*, *98*, 217-226.

Malaviya, A., Jang, Y.-S., & Lee, S. Y. (2012). Continuous butanol production with reduced byproducts formation from glycerol by a hyper producing mutant of *Clostridium pasteurianum*. *Applied Microbiology and Biotechnology*, *93*, 1485-

1494.

Mao, B., Liu, W., Chen, X., Yuan, L., & Yao, J. (2019). Enhanced biobutanol production from fern root using *Clostridium acetobutylicum* CGMCC1. 0134 with yeast extract addition. *BioResources*, *14*(2), 4575-4589.

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

Monot, F., Martin, J.-R., Petitdemange, H., & Gay, R. (1982). Acetone and butanol production by *Clostridium acetobutylicum* in a synthetic medium. *Applied and environmental microbiology*, *44*(6), 1318-1324.

Montville, R., & Schaffner, D. (2003). Inoculum size influences bacterial cross contamination between surfaces. *Applied and environmental microbiology*, *69*(12), 7188-7193.

Moon, H. G., Jang, Y.-S., Cho, C., Lee, J., Binkley, R., & Lee, S. Y. (2016). One hundred years of clostridial butanol fermentation. *FEMS microbiology letters*, *363*(3).

Moradi, F., Amiri, H., Soleimanian-Zad, S., Ehsani, M. R., & Karimi, K. (2013). Improvement of acetone, butanol and ethanol production from rice straw by acid and alkaline pretreatments. *Fuel*, *112*, 8-13.

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

Nasrah, N. S. M., Zahari, M. A. K. M., Masngut, N., & Ariffin, H. (2017). Statistical optimization for biobutanol production by *Clostridium acetobutylicum* ATCC 824 from Oil Palm Frond (OPF) juice using response surface methodology. MATEC Web of Conferences,

Ndaba, B., Chiyanzu, I., & Marx, S. (2015). n-Butanol derived from biochemical and chemical routes: A review. *Biotechnology Reports*, *8*, 1-9.

Nigam, P. S., & Singh, A. (2011). Production of liquid biofuels from renewable resources. *Progress in energy and combustion science*, *37*(1), 52-68.

Ong, M. Y., Syahira Abdul Latif, N.-I., Leong, H. Y., Salman, B., Show, P. L., & Nomanbhay,

S. (2019). Characterization and analysis of Malaysian macroalgae biomass as potential feedstock for bio-oil production. *Energies*, *12*(18), 3509.

Ou, J., Xu, N., Ernst, P., Ma, C., Bush, M., Goh, K., Zhao, J., Zhou, L., Yang, S.-T., & Liu, X. M. (2017). Process engineering of cellulosic n-butanol production from corn-based biomass using *Clostridium cellulovorans*. *Process Biochemistry*, *62*, 144-150.

Ouephanit, C., Virunanon, C., Burapatana, V., & Chulalaksananukul, W. (2011). Butanol and ethanol production from tapioca starch wastewater by *Clostridium* spp. *Water Science and Technology*, *64*(9), 1774-1780.

Papizadeh, M., Rohani, M., Hosseini, S. N., Shojaosadati, S. A., Nahrevanian, H., Talebi, M., & Pourshafie, M. R. (2020). Screening for efficient nitrogen sources for overproduction of the biomass of the functionally probiotic *L. plantarum* strain RPR42 in a cane molasses-based medium. *AMB Express*, *10*, 1-14.

Phantuwong, N. (2017). Functional and biological properties of Sang Yod rice bran hydrolysate prepared by enzymatic hydrolysis and its application in rice pudding product. Prince of Songkla University.

Qadeer, M., Choudhry, F. M., Ahmad, S., Rashid, S., & Akhtar, M. (1980). Acetone-butanol fermentation of cane molasses by *Clostridium acetobutylicum*. *Pakistan Journal of Scientific Research*, *32*(3/4), 157-163.

Qureshi, N., & Blaschek, H. (2001). Recent advances in ABE fermentation: hyper-butanol producing *Clostridium beijerinckii* BA101. *Journal of Industrial Microbiology and Biotechnology*, *27*, 287-291.

Qureshi, N., & Ezeji, T. C. (2008). Butanol,'a superior biofuel'production from agricultural residues (renewable biomass): recent progress in technology. *Biofuels, Bioproducts and Biorefining: Innovation for a sustainable economy*, *2*(4), 319-330.

Rajagopalan, G., He, J., & Yang, K.-L. (2013). A highly efficient NADH-dependent butanol dehydrogenase from high-butanol-producing *Clostridium* sp. BOH3. *BioEnergy Research*, *6*, 240-251.

Rambabu, K., Bharath, G., Thanigaivelan, A., Das, D., Show, P. L., & Banat, F. (2021). Augmented biohydrogen production from rice mill wastewater through nano-

metal oxides assisted dark fermentation. *Bioresource technology*, *319*, 124243.

Ranjan, A., Mayank, R., & Moholkar, V. S. (2013). Process optimization for butanol production from developed rice straw hydrolysate using *Clostridium acetobutylicum* MTCC 481 strain. *Biomass Conversion and Biorefinery*, *3*, 143-155.

Razak, M. N. A., Ibrahim, M. F., Yee, P. L., Hassan, M. A., & Abd-Aziz, S. (2013). Statistical optimization of biobutanol production from oil palm decanter cake hydrolysate by *Clostridium acetobutylicum* ATCC 824. *BioResources*, *8*(2), 1758-1770.

Richter, M., Rosselló-Móra, R., Oliver Glöckner, F., & Peplies, J. (2016). JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics*, *32*(6), 929-931.

Rochon, E., Cebreiros, F., Ferrari, M. D., & Lareo, C. (2019). Isopropanol-butanol production from sugarcane and sugarcane-sweet sorghum juices by *Clostridium beijerinckii* DSM 6423. *Biomass and bioenergy*, *128*, 105331.

Rupnik, M., Pabst, S., Rupnik, M., von Eichel-Streiber, C., Urlaub, H., & Soling, H.-D. (2005). Characterization of the cleavage site and function of resulting cleavage fragments after limited proteolysis of *Clostridium difficile* toxin B (TcdB) by host cells. *Microbiology*, *151*(1), 199-208.

Russmayer, H., Marx, H., & Sauer, M. (2019). Microbial 2-butanol production with *Lactobacillus diolivorans*. *Biotechnology for biofuels*, *12*(1), 1-11.

Sahoo, R. K., Das, A., Gaur, M., Dey, S., Sahoo, S., & Subudhi, E. (2019). Bacteria for Butanol Production: Bottlenecks, Achievements and Prospects. *Journal of Pure and Applied Microbiology*, *13*(3), 1429-1440.

Sandoval, N. R., Venkataramanan, K. P., Groth, T. S., & Papoutsakis, E. T. (2015). Whole-genome sequence of an evolved *Clostridium pasteurianum* strain reveals Spo0A deficiency responsible for increased butanol production and superior growth. *Biotechnology for biofuels*, *8*, 1-18.

Satyawali, Y., & Balakrishnan, M. (2008). Wastewater treatment in molasses-based alcohol distilleries for COD and color removal: a review. *Journal of environmental management*, *86*(3), 481-497.

Schomburg, I., Chang, A., Ebeling, C., Gremse, M., Heldt, C., Huhn, G., & Schomburg, D.

(2004). BRENDA, the enzyme database: updates and major new developments. *Nucleic acids research*, *32*(suppl_1), D431-D433.

Schuster, K. C., Van Den Heuvel, R., Gutierrez, N., & Maddox, I. (1998). Development of markers for product formation and cell cycle in batch cultivation of *Clostridium acetobutylicum* ATCC 824. *Applied Microbiology and Biotechnology*, *49*, 669-676.

Shukor, H., Al-Shorgani, N. K. N., Abdeshahian, P., Hamid, A. A., Anuar, N., Abd Rahman, N., & Kalil, M. S. (2014). Production of butanol by *Clostridium saccharoperbutylacetonicum* N1-4 from palm kernel cake in acetone–butanol–ethanol fermentation using an empirical model. *Bioresource technology*, *170*, 565-573.

Su, H., Zhao, Y., Wang, M., & Xu, Y. (2015). Development and application of a novel screening method and experimental use of the mutant bacterial strain *Clostridium beijerinckii* NCIMB 8052 for production of butanol via fermentation of fresh cassava. *RSC Advances*, *5*(17), 12624-12637.

Thang, V. H., Kanda, K., & Kobayashi, G. (2010). Production of acetone–butanol–ethanol (ABE) in direct fermentation of cassava by *Clostridium saccharoperbutylacetonicum* N1-4. *Applied biochemistry and biotechnology*, *161*, 157-170.

Tirado-Acevedo, O., Chinn, M. S., & Grunden, A. M. (2010). Production of biofuels from synthesis gas using microbial catalysts. *Advances in applied microbiology*, *70*, 57-92.

Tomas, C. A., Beamish, J., & Papoutsakis, E. T. (2004). Transcriptional analysis of butanol stress and tolerance in *Clostridium acetobutylicum*. *Journal of bacteriology*, *186*(7), 2006-2018.

Vasylkivska, M., & Patakova, P. (2020). Role of efflux in enhancing butanol tolerance of bacteria. *Journal of Biotechnology*, *320*, 17-27.

Ventura, J.-R. S., Hu, H., & Jahng, D. (2013). Enhanced butanol production in *Clostridium acetobutylicum* ATCC 824 by double overexpression of 6-phosphofructokinase and pyruvate kinase genes. *Applied Microbiology and Biotechnology*, *97*, 7505-7516.

Vijayaraghavan, K., & Ramanujam, T. (2000). Performance of anaerobic contact filter in series for treating distillery spentwash. *Bioprocess Engineering*, *22*, 109-114.

Virunanon, C., Ouephanit, C., Burapatana, V., & Chulalaksananukul, W. (2013). Cassava pulp enzymatic hydrolysis process as a preliminary step in bio-alcohols production from waste starchy resources. *Journal of Cleaner Production*, *39*, 273-279.

Wang, Y., & Blaschek, H. P. (2011). Optimization of butanol production from tropical maize stalk juice by fermentation with *Clostridium beijerinckii* NCIMB 8052. *Bioresource technology*, *102*(21), 9985-9990.

Wang, Y., & Zhang, Z. (2000). Comparative sequence analyses reveal frequent occurrence of short segments containing an abnormally high number of non-random base variations in bacterial rRNA genes. *Microbiology*, *146*(11), 2845-2854.

Wang, Z., Cao, G., Jiang, C., Song, J., Zheng, J., & Yang, Q. (2013). Butanol production from wheat straw by combining crude enzymatic hydrolysis and anaerobic fermentation using *Clostridium acetobutylicum* ATCC824. *Energy & fuels*, *27*(10), 5900-5906.

Watchararuji, K., Goto, M., Sasaki, M., & Shotipruk, A. (2008). Value-added subcritical water hydrolysate from rice bran and soybean meal. *Bioresource technology*, *99*(14), 6207-6213.

Wayne Chew, K., Ying Yap, J., Sabariah Din, S., Chuan Ling, T., Monash, P., & Loke Show, P. (2018). Developments in fermentative butanol production as an alternative biofuel source. *Journal of Energy Resources Technology*, *140*(4), 040801.

Xin, F., Chen, T., Jiang, Y., Lu, J., Dong, W., Zhang, W., Ma, J., Zhang, M., & Jiang, M. (2017). Enhanced biobutanol production with high yield from crude glycerol by acetone uncoupled *Clostridium* sp. strain CT7. *Bioresource technology*, *244*, 575-581.

Yao, D., Dong, S., Wang, P., Chen, T., Wang, J., Yue, Z.-B., & Wang, Y. (2017). Robustness of *Clostridium saccharoperbutylacetonicum* for acetone-butanol-ethanol production: Effects of lignocellulosic sugars and inhibitors. *Fuel*, *208*, 549-557.

Yu, L., Xu, M., Tang, I. C., & Yang, S. T. (2015). Metabolic engineering of *Clostridium*

tyrobutyricum for n-butanol production through co-utilization of glucose and xylose. *Biotechnology and bioengineering*, *112*(10), 2134-2141.

Yu, M., Zhang, Y., Tang, I.-C., & Yang, S.-T. (2011). Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production. *Metabolic engineering*, *13*(4), 373-382.

Zetty-Arenas, A. M., Alves, R. F., Portela, C. A. F., Mariano, A. P., Basso, T. O., Tovar, L. P., Maciel Filho, R., & Freitas, S. (2019). Towards enhanced n-butanol production from sugarcane bagasse hemicellulosic hydrolysate: Strain screening, and the effects of sugar concentration and butanol tolerance. *Biomass and bioenergy*, *126*, 190-198.

Zhang, J., & Jia, B. (2018). Enhanced butanol production using *Clostridium beijerinckii* SE-2 from the waste of corn processing. *Biomass and bioenergy*, *115*, 260-266.

Zhang, J., Wang, S., & Wang, Y. (2016). Biobutanol production from renewable resources: recent advances. *Advances in Bioenergy*, *1*, 1-68.

Zhou, H., Xia, D., & He, Y. (2020). Rice grain quality—traditional traits for high quality rice and health-plus substances. *Molecular Breeding*, *40*, 1-17.

Zverlov, V., Berezina, O., Velikodvorskaya, G., & Schwarz, W. (2006). Bacterial acetone and butanol production by industrial fermentation in the Soviet Union: use of hydrolyzed agricultural waste for biorefinery. *Applied Microbiology and Biotechnology*, *71*, 587-597.

APPENDIX

Appendix A: Calibration curves

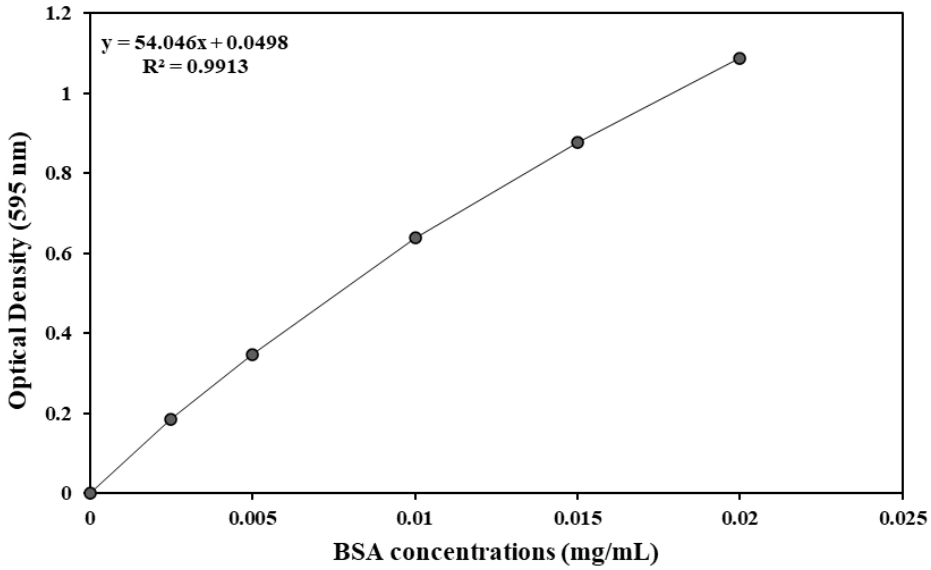


Figure A.1 BSA standard calibration curve

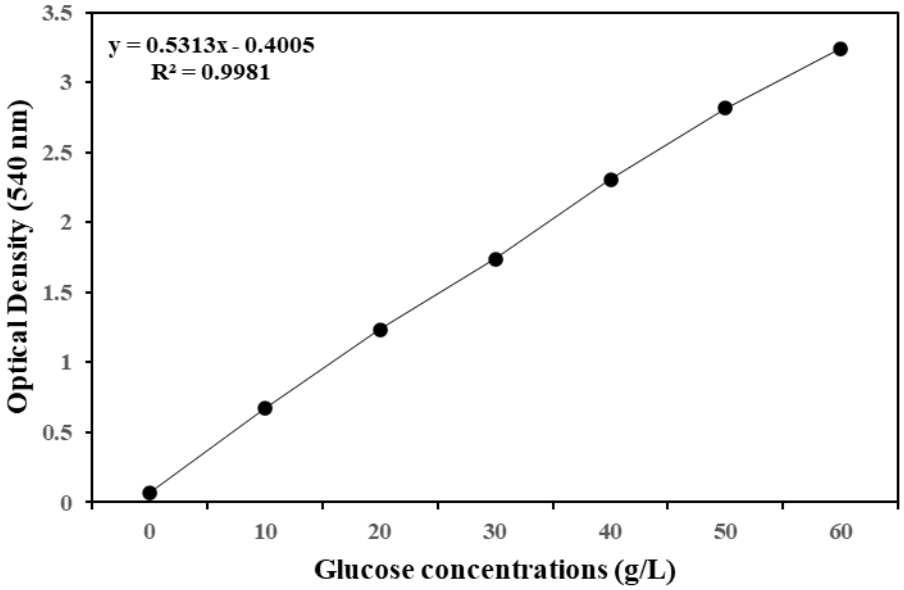
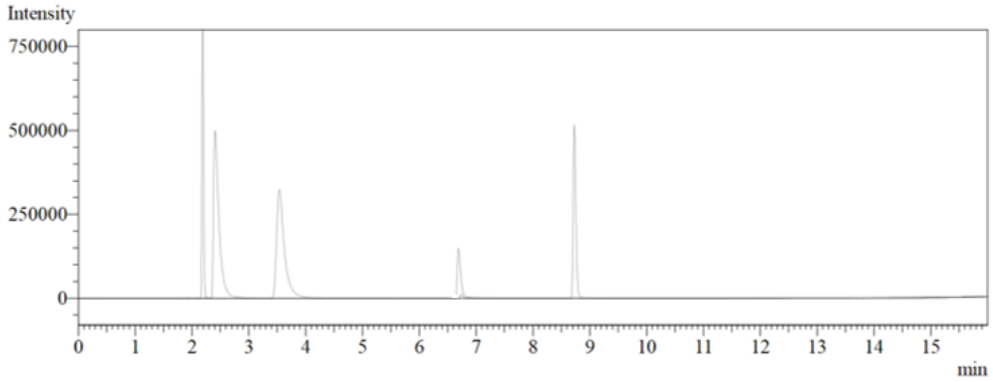


Figure A.2 Glucose standard calibration curve

Appendix B: Standard of ABE production profile using GC-FID

Analysis Date & Time : 10/03/2565 15:46:45
User Name : Admin
Vial# : 1
Sample Name : STD
Sample ID :
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : D:\Keng\STD Old Column 26.02.64\STD.gcd
Method Name : D:\Keng\STD new method 16.06.2023.gcm

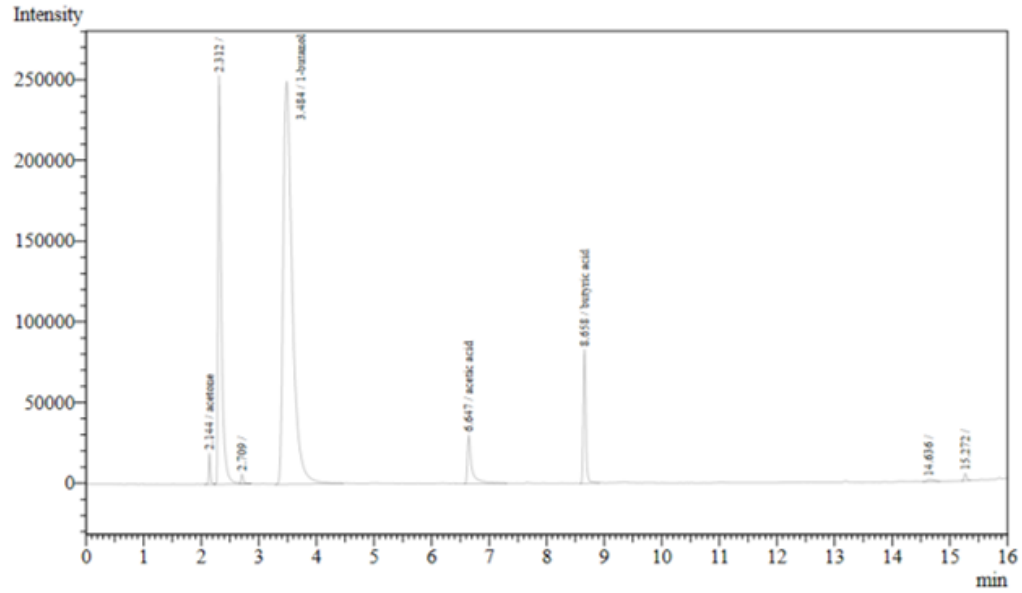


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	2.189	1904559	1104002	9.528	g/L	SV	1	acetone
2	2.410	3364188	498702	15.188	g/L	SV	2	ethanol
3	3.540	3108397	323896	9.544	g/L		3	1-butanol
4	6.690	677958	149906	7.079	g/L	S	4	acetic acid
5	8.729	1722205	516022	13.678	g/L		7	butyric acid
6	9.369	13627	1830	0.000		T		
7	14.427	15106	1988	0.000		V		
8	14.594	77975	10509	0.000		V		
9	15.281	69374	22161	0.000		V		
Total		10953389	2629016					

Figure B.1 GC-FID chromatogram of standard acetone, ethanol, butanol, acetic acid, and butyric acid.

Analysis Date & Time : 30/06/2564 13:27:38
User Name : Admin
Vial# : 11
Sample Name : YTA50 Air2 72h 2
Sample ID :
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : D:\Keng\YTA50mL Air2 30.06.64\YTA50 Air2 72h 2.gcd
Method Name : D:\Keng\STD new method 16.06.2023.gcm

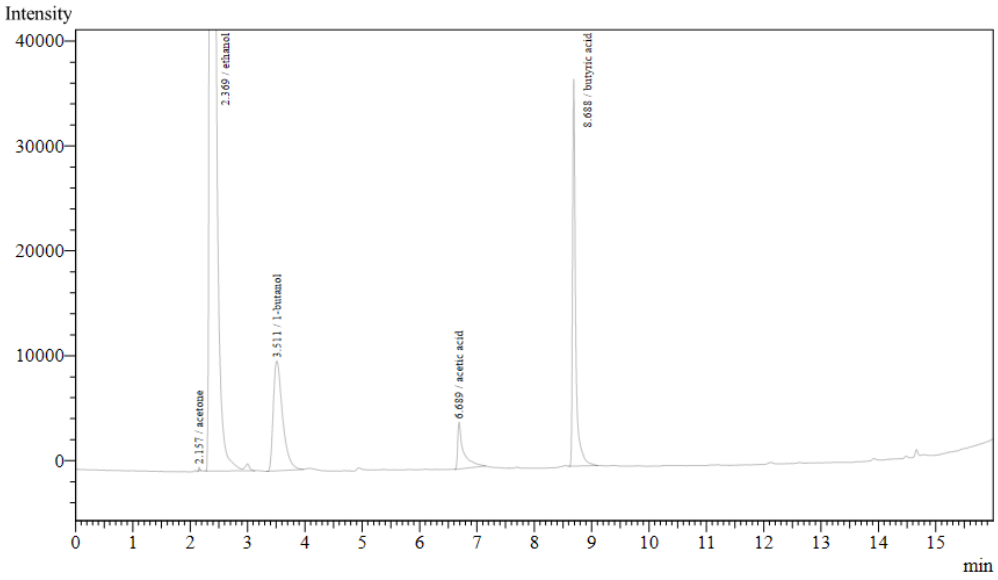


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	2.144	36625	19047	0.493	g/L		1	acetone
2	2.410	1029444	251818	4.672		SV	2	ethanol
3	2.709	15903	5684	0.000		T		
4	3.484	2733016	249479	8.417	g/L	S	3	1-butanol
5	6.647	162166	30073	2.177	g/L	S	4	acetic acid
6	8.658	256667	82293	3.756	g/L	S	7	butyric acid
7	14.636	10759	1337	0.000				
8	15.272	13182	4400	0.000				
Total		4257762	644131					

Figure B.2 GC-FID chromatogram of ABE production from *C. beijerinckii* CUEA02 cultured under optimal conditions and using glucose as a carbon source.

Analysis Date & Time : 25/04/2564 19:07:27
User Name : Admin
Vial# : 28
Sample Name : Sato10x 72h 2
Sample ID :
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : D:\Keng\Eth20x Sam3 Eth30x Sam2 3 Sato10x Sam2 3 25.04.64\Sato10x 72h 2.gcd
Method Name : D:\Keng\STD new method 16.06.2023.gcm

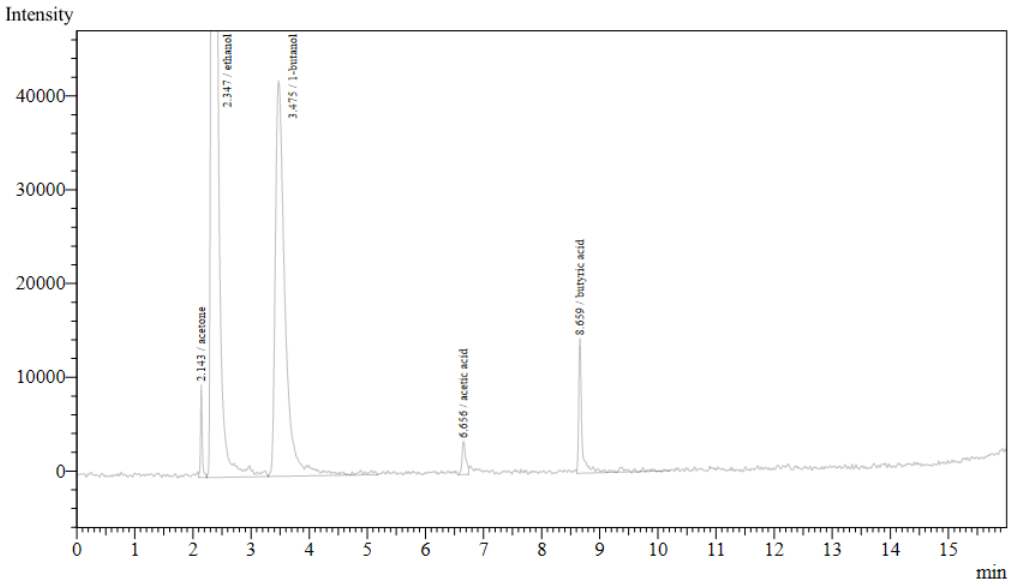


Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	2.157	446	313	0.318	g/L		1	acetone
2	2.369	1110618	199179	5.184	g/L	S	2	ethanol
3	3.511	118057	10483	0.569	g/L		3	1-butanol
4	6.689	31927	4461	0.939	g/L		4	acetic acid
5	8.688	137472	36817	2.949	g/L		7	butyric acid
Total		1398520	251253					

Figure B.3 GC-FID chromatogram of ABE production from *C. beijerinckii* CUEA02 cultured on 1:10 STW without adjusted initial pH.

Analysis Date & Time : 02/07/2564 15:00:19
User Name : Admin
Vial# : 14
Sample Name : Sato10x pH6.5 96h 2
Sample ID :
Sample Type : Unknown
Injection Volume : 1.00
ISTD Amount :

Data Name : D:\Keng\Sato30x pH6.5 120-144h Sato10x pH6.5 Sam 2 3 02.07.64\Sato10x pH6.5 96h 2.gcd
Method Name : D:\Keng\STD new method 16.06.2023.gcm



Peak#	Ret.Time	Area	Height	Conc.	Unit	Mark	ID#	Cmpd Name
1	2.143	19802	9777	0.412	g/L	V	1	acetone
2	2.347	1274728	229436	5.913	g/L	SV	2	ethanol
3	3.475	479771	42179	1.654	g/L	SV	3	1-butanol
4	6.656	16130	3545	0.789	g/L		4	acetic acid
5	8.659	48472	14309	2.346	g/L	SV	7	butyric acid
Total		1838903	299246					

Figure B.4 GC-FID chromatogram of ABE production from *C. beijerinckii* CUEA02 cultured on 1:10 STW and adjusted initial pH to 6.5.

Appendix C: The diameter of clear zone from 11 isolated *Clostridium* sp.

Table C.1 Clear zone area of 11 isolated *Clostridium* sp. in the TYA agar containing starch after 24 h

Strain	Clear zone (cm^2)
GW-1	1.3
GW-2	1.6
GW-3	1.7
GW-4	1.1
AW-1	1.9
CUEA02	2.7
FGR-1	0
FGR-2	0
FGR-3	0
FGR-4	0
FGR-5	0

Appendix D: Calculation of butanol dehydrogenase activity

Table D.1 The ΔOD/min of NADH-BDH with cell extract of CUEA02

NADH-BDH	Cell extract (μL)	ΔOD/min
	100	0.0040888
		0.011949
	200	0.002627
		0.017261

Cell extract: 100 μL ΔOD/min = 0.011949 - 0.0040888

= 0.0078602

(ΔOD/min)/cell extract (mL) = 0.0078602/0.1

= 0.0786

Cell extract: 200 μL ΔOD/min = 0.017261 - 0.002627

= 0.00732

(ΔOD/min)/cell extract (mL) = 0.00732/0.1

= 0.0732

Average of ΔOD/min = (0.0786 + 0.0732)/2

= 0.0759

Specific activity = (ΔOD/min)/6.22/protein concentration

= 0.0759/6.22 = 0.0122

= 0.0122/2.435 = 0.005 U/mg

Table D.2 The ΔOD/min of NADPH-BDH with cell extract of CUEA02

NADPH-BDH	Cell extract (μL)	ΔOD/min
	100	0.001246
		0.063233

Cell extract: 100 μL ΔOD/min = 0.063233 - 0.001246

= 0.061987

(ΔOD/min)/cell extract (mL) = 0. 061987/0.1

= 0.61987

Specific activity = (ΔOD/min)/6.22/protein concentration

= 0.61987/6.22 = 0.0997

= 0.0997/2.435 = 0.041 U/mg

Appendix E: Calculation of protein concentration for calculated BDH activity

Table E.1 The protein concentration is measured using the Bradford protein assay (595 nm).

Dilution of protein sample	A595 nm	Protein (mg/mL)	Protein*dilution factor
10x	1.907	0.03436	0.3436
100x	1.126	0.01991	1.9912
200x	0.688	0.01180	2.3616
500x	0.321	0.00501	2.5089
1000x	0.171	0.00224	2.2425

The appropriate protein concentration values based on the BSA standard are 200x and 500x.

The protein concentration = (2.3616 + 2.5089)/2

= 2.435 mg/mL

VITA

NAME	Julalak Buranaprasopchai
DATE OF BIRTH	29 May 1992
PLACE OF BIRTH	Kanchanaburi, THAILAND
INSTITUTIONS ATTENDED	Bachelor of Science (BIOLOGY) (2nd. Class Honors, GPA 3.40) from the Department of Biology, Faculty of Science, Silpakorn University, THAILAND (2014)
HOME ADDRESS	75/15 South Sangchuto Rd, Tha Lo, Tha Muang, Kanchanaburi, 71000
PUBLICATION	Julalak Buranaprasopchai, Nassapat Boonvitthya, Chompunuch Glinwong, Warawut Chulalaksananukul, 2022. Butanol production from Thai traditional beverage (Sato) factory wastewater using newly isolated Clostridium beijerinckii CUEA02. Biochemical Engineering Journal. 187, 108648.
AWARD RECEIVED	The Chulalongkorn University Dussadeepipat Scholarship to study for the degree of Doctor of Philosophy in Biotechnology Program, Faculty of Science, Chulalongkorn University.