# Butanol Production from Micro-algae by Combining Dark Fermentation and ABE Fermentation Equipped with Gas Sparging

**Nikannapas Usmanbaha**

**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Energy Technology**

**Prince of Songkla University**

**2023**

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| **Major Program** | Energy Technology |

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**ชื่อวิทยานิพนธ์** การผลิตบิวทานอลจากจุลสาหร่ายด้วยกระบวนการหมักสองขั้นตอนที่มีการแยกบิวทานอลโดยระบบเป่าไล่ด้วยแก๊ส
**ผู้เขียน** นางสาวนิกานต์ณภัส อุสมันบาฮา
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## บทคัดย่อ

จุลสาหร่าย *Chlorella* sp. ได้ถูกนำมาใช้สำหรับการผลิตบิวทานอลด้วยกระบวนการสองขั้นตอนของการหมักไร้แสงและการผลิตตัวทำละลาย ทั้งนี้ได้ศึกษาปัจจัยของการหมักไร้แสงในแบทช์ที่ปัจจัยต่างๆได้แก่ ความเข้มข้นของจุลสาหร่าย (*Chlorella* sp.) เริ่มต้น (20, 40, 60, 80, 100, และ 120 g-VS/L), pH เริ่มต้น (5 และ 7), อุณหภูมิ (35 ºC และ 55 ºC), และนำมาใช้ของแหล่งกล้าเชื้อ (กล้าเชื้อผสมที่สภาวะเมโซฟิลิก และกล้าเชื้อผสมที่สภาวะเทอร์โมฟิลิก) ต่อการผลิตกรดไขมันระเหยได้โดยเฉพาะกรดบิวทิริกซึ่งเป็นสารตั้งต้นสำหรับการผลิตบิวทานอลในขั้นตอนการผลิตตัวทำละลาย ปัจจัยที่เหมาะสมคือสภาวะที่ pH เริ่มต้น 7 ด้วยกล้าเชื้อผสมจากแหล่งอุณหภูมิเทอร์โมฟิลิก (55 ºC) โดยผลผลิตกรดบิวทิริกสูงสุดจากจุลสสาหร่ายคือ 10.67 g/L ที่ความเข้มข้นเริ่มต้นของจุลสาหร่าย 80 g-VS/L โดยผลการทดลองเมื่อทำการปรับสภาพจุลสาหร่าย 60 g-VS/L ด้วยวิธี thermolysis พบว่าผลผลิตกรดได้ 11.42 g/L จากการหมักร่วมกับการเติมสารอาหาร BA medium โดยให้ผลผลิตแก๊สไฮโดรเจน 224.43 mL-$H_2$ (46.76 mL-$H_2$/g-VS)

นอกจากนี้ศึกษาสภาวะการผลิตกรด ในถังปฏิกรณ์ CSTR 2 L ที่ปริมาตรการทำงาน 1.35 L มีการศึกษาการทดลอง 9 สภาวะ โดยสภาวะที่เหมาะสมในการผลิตกรดระเหยง่ายจากการหมักจุลสาหร่าย 50 g/L *Chlorella* sp. ร่วมกับน้ำตาลซูโครส 10 g/L ที่ระยะเวลาการกักเก็บ HRT 6 วันให้ผลผลิตกรดบิวทิริก 5.23-5.49 g/L และผลผลิตไฮโดรเจน 119-124 mL-$H_2$/g-VS หรือ อัตราการผลผลิตต่อวัน 1190-1288 mL-$H_2$/L.d โดยนำสภาวะที่ได้จากการทดลองมายกระดับขนาดถังหมักที่ 10 L ที่ปริมาตรการทำงาน 5 L ได้ทำการเพิ่มขนาดการหมัก พบว่าให้ผลผลิตไฮโดรเจน 161-189 mL-$H_2$/g-$VS_{add}$ หรืออัตราการผลิตต่อวัน 1,938-2,269 mL-$H_2$/$L_{reactor}$ และมีแก๊สชีวภาพเกิดขึ้น 23.4 L/d สามารถใช้เป็นแก๊สสำหรับการเป่าไล่ในขั้นต่อไป แม้ว่าจะมีปริมาณที่ต้องใช้น้อยกว่าเนื่องจากการเป่าใล่ใช้ 3 L/min อย่างไรก็ตามหากมีการใช้แก๊สแบบหมุนเวียนก็มีความสามรถที่เป็นไปได้ในการนำไปใช้ในอนาคต โดยมีผลิตกรดอะซิติก กรดโพรพิออนิก และกรดบิวทิริกที่เกิดขึ้นระหว่างการทดลองอยู่ในช่วง 5.25±0.14 g/L, 0.76±0.02 g/L และ 7.50±0.15 g/L ตามลำดับ โดยพบว่ามีแบคทีเรียที่ตรวจพบทั้งหมด

280 สายพันธ์ จากการหมักเชื้อผสมนี้ โดยมีกลุ่มแบคทีเรียหลักๆ ได้แก่ *Comamonas* spp. (33%), *Lysinibacillus macrolides* (23%), *Advenella* sp. (13%), *Dysgonomonas mossii* (9%), *Proteiniphilum* spp. (4%), *Acinetobacter* sp. (3%), *Clostridiales* spp. (3%), *Acetobacter pasteurianus* (2%) และ *Caproicproducens* spp. (2%) เป็นต้น

จากการศึกษาผลผลิตบิวทานอลในชุดการทดลองแบบแบทช์จากเชื้อสองชนิดของ *Clostridium butylicum* TISTR1032 และ *Clostridium beijerinckii* ATCC10132 ที่ใช้กลูโคสความเข้มข้นเริ่มต้น 20, 40, 60 และ 80 g-VS/L พบว่าการใช้กลูโคส 40 g-VS/L จากเชื้อ *Clostridium beijerinckii* ATCC10132 ให้ผลผลิตบิวทานอลสูงสุด 10.17 g/L และเมื่อทำการทดสอบการหมักโดยใช้จุลลสาหร่ายที่มีการปรับ และมีการเติมสารอาหารพบว่าได้ผลผลิตบิวทานอล 2.30 g/L ดีกว่าไม่ปรับสภาพ นอกจากนี้การศึกษา B/G ratio จากการออกแบบการทดลองด้วยทากูชิ พบว่าที่การใช้สัดส่วนกรดบิวทิริก 10 g/L ร่วมกับน้ำตาลกลูโคส 20 g/L ให้ผลผลิตบิวทานอลสูงสุด 3.67 g/L ด้วยการใช้เชื้อร้อยละ 15 โดยปริมาตร ใขขณะที่ความเข้มข้นบิวทิริกที่ 14 g/L ก่อให้เกิดการยับยั้ง

นอกจากนี้การศึกษาผลผลิตบิวทานอลในถัง CSTR ด้วยการป้อนแบบกึ่งต่อเนื่องด้วยอาหาร TYA ที่มีน้ำตาลกลูโคส 20 g-VS/L ที่ระยะเวลาการกักเก็บของของเหลว (HRT) 2 วัน ให้ผลผลิตบิวทานอลคงที่อยู่ในช่วง 5.51 g/L พบไฮโดรเจนที่ร้อยละ 38 ซึ่งมีผลผลิตไฮโดรเจนเฉลี่ย 74 mL-$H_2$/g-VS โดยมีอัตราการผลิตคงที่เฉลี่ย 740 mL-$H_2$/L/d เมื่อศึกษาการเป่าไล่ด้วยแก๊ส 70% $CO_2$ ร่วมกับ 30% $H_2$ ที่อัตราการเป่าไล่ 3 L/min เป่าใล่ในระบบหมักตลอดการทดลองต่อเนื่อง 48 ชั่วโมง ได้ร้อยละการเก็บเกี่ยวของบิวทานอลเท่ากับ 43.86 มีค่าประสิทธิภาพการควบแน่นร้อยละ 75.69 โดยระหว่างการเป่าไล่ในช่วงแรกจากน้ำหมัก ที่มีความเข้มข้นบิวทานอล 5.51 g/L เมื่อเป่าไล่ในช่วง 4 ชั่วโมงแรกพบว่าได้บิวทานอลเข้มข้นสูงสุดในชุดควบแน่นแรกที่ 45.17g/L ทั้งนี้จากการทดสอบแสดงให้เห็นว่าระบบสามารถใช้งานได้เหมาะสมหากมีการผลผลิตตัวทำละลายจากกระบวนการหมักที่สูงขึ้นได้ นอกจากนี้ผลการทดสอบการผลิตบิวทานอลจากการหมักจากน้ำหมักที่อุดมไปด้วยกรดบิวทิริก ร่วมกับน้ำตาล 20 g-VS/L พบว่าในช่วงแรกสามารถให้ผลผลิตบิวทานอล โดยได้ผลผลิตบิวทานอล 5.54 g/L ผลผลิตแก๊ส 117.68 mL-$H_2$/g-VS แต่เมื่อมีการป้อนเข้าออกพบว่า ปริมาณกรดต่างๆมีการสะสมเพิ่มขึ้นในระบบและส่งผลต่อการเจริญและกระบวนการทำงานของเชื้อ *Clostridium beijerinckii*. การคำนวณสมดุล COD ของสารตั้งต้นจากการใช้จุลสาหร่ายผ่านระบบการหมักสอง ขั้นตอน สามารถให้ผลผลิตสุดท้ายในรูปของแก๊ส และสารเคมีภัณฑ์ในส่วนของของเหลว จากการใช้ g-COD เริ่มต้นไปร้อยละ 45.79 ตั้งแต่การป้อนสารตั้งต้นของจุลสาหร่ายในระบบหมักทั้งสองขั้นตอนจนกระทั้งได้ผลผลิตสุดท้าย

คำสำคัญ: บิวทานอล, กรดบิวทิริก, ไฮโดรเจน, *Chlorella* sp., *Clostridium beijerinckii*, การเป่าไล่ด้วยแก๊ส

**Thesis Title** Butanol Production from Micro-algae by Combining Dark Fermentation and ABE Fermentation Equipped with Gas Sparging
**Author** Miss Nikannapas Usmanbaha
**Major Program** Energy Technology
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## ABSTRACT

Micro-algae *Chlorella* sp. as the third-generation biomass for biofuel production was used to evaluate the production of butanol via two-stage dark fermentation and solventogenesis process. Batch dark fermentation was carried out to investigate the effects of initial algae concentration (20, 40, 60, 80, 100, 120 g-VS/L), initial pH (5 and 7) , Temperature (35 ºC and 55 ºC), and inoculums (mesophilic mixed-cultures and thermophilic mixed-cultures) on formation of volatile fatty acids (VFAs). The optimal effect of thermophilic mixed-cultures fermentation for 72 h at 55 ºC, initial pH 7 was investigated. The highest butyric acid production from initial algae concentration at 80 g-VS/L was produced of 10.67 g/L which is a precursor for butanol generation in the solventogenesis stage. Thermolysis pretreated microalgae 60 g-VS/L with BA medium can produce butyric acid 11.42 g/L higher than without pretreatment, which produced hydrogen production of 224.43 mL-$H_2$ (46.76 mL-$H_2$/g-VS).

Production of volatile acid in 2 L CSTR was operated at 9 conditions. The condition of the highest product is both hydrogen production (119-124 mL-$H_2$/ g-VS or 1,190-1288 mL-$H_2$/L-reactor.d) and butyric acid of 5.23-5.49 was investigated on 1.35 L working volume at operation of 6-day HRT from microalgae pretreated 50 g-VS/L along with 10 g-sucrose/L by thermophile mixed culture source at 55 °C. The optimal condition from 2 L CSTR reactor was operated under scale up to 10 L CSTR reactor at 5 L working volume. The production of hydrogen yield of 161-189 mL-$H_2$/g-$VS_{add}$ and hydrogen production rate of 1,938-2,269 mL-$H_2$/L-reactor with the production of acetic acid propionic acid and butyric acid produced during the experiment were in the range of 5.25±0.14 g/L, 0.76±0.02 g/L and 7.50±0.15 g/L, respectively was investigated. biogas production 23.4 L-biogas/d of this stage can be used as a concept for gas stripping of butanol product recovery in second CSTR fermentation. Although, flow rate 3 L/min or 4,320 L/day was used as stripping gas recovery. However, gas stripping recovery can used as circulation system of gas flow with flow rate control to get the desired flow rate value and this process can reduce the cost of operating the system. The total tags data of this sample is 172,245 with 280 observed species. The main bacterial in the fermentation of mixed culture were *Comamonas* spp. (33%), *Lysinibacillus macrolides* (23%), *Advenella* sp. (13%),

*Dysgonomonas mossii* (9%), *Proteiniphilum* spp. (4%), *Acinetobacter* sp. (3%), *Clostridiales* spp. (3%), *Acetobacter pasteurianus* (2%), and *Caproicproducens* spp. (2%).

Two ABE fermentation bacteria of *Clostridium butylicum* TISTR1032 and *Clostridium beijerinckii* ATCC10132 was investigated at difference initial concentration of 20, 40, 60, and 80 g-VS/L. Highest butanol concentration of 10.17 g/L was obtained from *Clostridium beijerinckii* ATCC10132 at 40 g-VS/L initial glucose concentrations. The initial concentration 40 g-VS/L of microalgae pretreatment with TYA nutrient addition could provide butanol 2.30 g/L which is higher than untreated microalgae. In addition, by studying the B/G ratio from the Taguchi experimental design, the combination of 10 g/L butyric acid with 20 g/L glucose yielded the highest butanol yield at 3.67 g/L from the 15% inoculation concentration, which butyric acid concentrations up to 14 g/L can inhibit for this fermentation system.

In addition, Butanol production yield from CSTR with semi-continuously fed 20 g-VS/L glucose on TYA medium at HRT 2 day was constant in the range of 5.51 g/L, 38% hydrogen concentration with yield of 74 mL-$H_2$/g-VS (740 mL-$H_2$/L/d). Using stripping gas having 70% $CO_2$ and 30% $H_2$ at 3 L/min and using sparging time for 48 hours, the stripping gas efficiency 43.86% can calculated after removal of butanol from fermenter with gas stripping while 75.69% efficiency of condensation. Recovered butanol was obtained highest concentration of 45.17g/L during the first 4-hr gas stripping from the fermented of 5.51 g/L butanol concentration. This stripping process is suitable when fermentation process provides high solvent production yield. In addition, using rich butyric acid dark fermentation effluent with 20 g-VS/L glucose could be capable of covering 5.54 g/L butanol with hydrogen production 117.68 mL-$H_2$/g-VS in the early stage of ABE fermentation. Acid content was increased and consequently inhibited growth and activity of *Clostridium beijerinckii*. COD balance of substrate from microalgae in 10 L CSTR system from two-stage fermentation was investigated. The last production in the form of solution and hydrogen at the final stage can be produced from g-COD of the substrate in the system as 45.79%.

Keywords: Butanol, Butyric acid, Hydrogen, *Chlorella* sp., *Clostridium beijerinckii*, Gas stripping.

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Nikannapas Usmanbaha

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# CHAPTER 1
# INTRODUCTION

## 1.1 Background and problem statement

Butanol is produced commercially from fossil fuels while the utilization of energy from petroleum fuels with drilling from underground results in reduced resources and causes crisis pollution in the environment (Moon *et al*., 2018 and Walsh *et al*., 2018). Butanol utilization is used as a solvent for industrial of textile production processes, a paint thinner and in organic synthesis as well as energy fuel. However, biobutanol can be produced from biomass by biodegradation process as one of alternative way which can greatly reduce the consumption and dependence on petroleum resources. The performance of butanol has been higher than ethanol in terms of utilization as biofuel and the butanol combustion heating value of 29.2 MJ/dm$^3$ while ethanol combustion heating value of 19.6 MJ/dm$^3$ (Kaminski *et al*., 2011). Butanol has octane number 96 similar to gasoline for internal combustion engines. The properties of the butanol better than ethanol have been reported, such as vapor pressures and corrosion capacity are less than ethanol. While the use of ethanol mixed with gasoline higher than 15% caused corrosion problems in various parts of the engine. Thus, biobutanol is expected potentially to replace bioethanol in the near future (Green, 2011). Butanol is suitable for transporting through pipelines and can be produced from biomass as the substrate in the fermentation process.

At present, traditional chassis hosts for biorefineries are mainly *Escherichia coli*, yeasts, and *Corynebacterium glutamicum* (Yang *et al*.2022). Among these hosts, E. coli is commonly used for the production of biofuels and other alcohol fuels such as n-butanol, and n-propanol. However, E. coli is less robust against changes in temperature, salts, and pH, and exhibits lower alcohol tolerance than yeast and *Clostridia*, which is limited in industrial utilization. While yeasts and *C. glutamicum* cannot produce butanol (Weber *et al*., 2010). The production of butanol from Acetone Butanol Ethanol (ABE) fermentation process can be used from biomass. *Clostridium* bacterium was used as inoculum for anaerobic ABE fermentation. The main products of this ABE fermentation are acetone, butanol, and ethanol (3:6:1 molar ratio). While The products of hydrocarbons, carbon dioxide, and Hydrogen are also consequently produced in the fermentation process (Ezeji *et al*., 2007). At the exponential phase of growth curve, carbohydrates are majorly converted to butyric acid and acetic acids along with hydrogen and carbon dioxide from ABE fermentation process. Thus, the first stage of ABE fermentation is acidogenesis stage to produce the main product as volatile fatty acid, which the pH of the fermentation system starts to drop because of the accumulation of organic acids in the fermentation system. Follow with the second stage as a solventogenesis stage producing during stationary phase.

The acetone butanol and ethanol as composition of solvent are converted from butyric acid and acetic acids from the first stage. The research of Lee *et al*. (2008), found that the identity characteristics of butanol producing bacteria are the rapid conversion of butyric acid to butanol. While butanol producing bacteria can use butyric acid as the precursor for butanol generation by solventogenic activity.

Anyhow, the butanol production process from fermentation is feasibly separated into two sub-processes including acidogenesis or dark fermentation and solventogenesis, which two stage process can be used with different types of bacteria. (Angenent *et al*., 2004). The two stage process is efficient for increasing butanol production yield with producing high butyric acid along with hydrogen from dark fermentation. Acetic acid and butyric acid production as two main acids by acidogenic bacteria in dark fermentation stage are important precursors to produce solvent from ABE production process (Angenent *et al*., 2004 and Agler *et al*., 2011 ). So that, hydrogen was produced via both butyric acid and acetic pathways of dark fermentation which thermodynamically controlled by hydrogen partial pressure. At hydrogen partial pressure less than 60 Pa, 1 mole of glucose will be directed to 2 moles of acetic acid and 4 moles of hydrogen. In contrast, a dark fermentation process at hydrogen partial pressure greater than 60 Pa, the direction of production is in the form of 1 mole of butyric acid and 2 moles of hydrogen. Thus, the fermenter of dark fermentation contained majorly with butyrate is favored to be used for enhancing butanol production in the second stage of solventogenesis. Substrate fermentation can be cellulose and starch without hydrolysis by enzyme (Lee *et al*., 2008).

Butanol, acetone, and ethanol are toxic to microorganisms. Especially, the more concentrated butanol (>2% v/v) leads to cause product inhibition resulting in decreasing solvent products of ABE fermentation process (Abdehagh *et al*., 2014). Furthermore, butanol can be extremely toxic to microorganisms when concentrated while it may stop fermentation and substrate may not be consumed, thus increasing waste treatment. The gas stripping separating technique of butanol involves spraying gas through the fermentation tank to remove the solvent as a product, The solvent product was separated from the fermenter and condensed from the condenser and the gas can be recycled back into the process. The gas blowing process can use gas generated by the fermentation process. This technique can increase the contact between the substrate and the enzyme. According to Lu *et al*., (2013) and de Vrije *et al*., (2013) reported that the use of gas stripping during fermentation can increase production rate by nearly two times and result in increased yields.

*Chlorella* sp., *Scenedesmus* sp., *Chlorococum* sp., *Tetraselmis* sp. and *Chlamydomonas* sp. are unicellular microscopic algae that can grow easily in various water sources. They are mostly carbohydrates (starch and cellulose), the main substrate for biofuel production when using microbial fermentation, up to about 55% of dry weight (Kin

*et al.*, 2011). Classified as the third-generation biomass for bio-fuel production, microalgae have advantages over the first-generation biomasses such as flour and sugar derived from food crops and the second-generation biomasses such as lignocelluloses in terms of biofuel production. Microalgae do not compete for arable land, can be grown in saline or freshwater environments, and can absorb $CO_2$. Furthermore, microalgae can be practically cultivated in open raceway ponds cost effectively (Banerjee *et al*., 2017). Microalgae do not contain lignin and have fast growth potential (Demirbas 2011 and Davis *et al*., 2011). Especially, *Chlorella* sp. are considered promising feedstock for ABE production because they have cellulose and hemicellulose of cell walls and accumulated starch as main carbohydrate sources (37–55%) (Chen *et al*., 2013). Most of the cell wall and starch can be converted to sugars for acid and ABE production (Phanduang *et al*., 2017)

In this PhD research, optimization of butanol production from microalgae *Chlorella* sp. via two-stage process of dark fermentation and solventogenesis is first determined in both batch and continuous mode of operation. The first stage dark fermentation is employed enriched mixed cultures to produce butyric acid. *Clostridium beijerinckii* ATCC10132 is later to convert butyric acid rich solution to ABE from the second stage ABE reactor. The second reactor was equipped with a gas sparging system to investigate enhancement of ABE fermentation and butanol separation.

### 1.2 Objective of research

1. To investigate butyric acid from microalgae *Chlorella* sp. in batch mode with first stage of mixed cultures and produce ABE from *Clostridium beijerinckii* ATCC10132 at second stage.
2. To produce ABE from two-stage fermentation of continuous system of Continuous Stirred Tank Reactor (CSTR).
3. To separate butanol via two-stage fermentation process equipped with gas sparging system.

### 1.3 Scope of Study

1. The optimization of butanol production from two-stage with microalgae *Chlorella* sp. as a substrate in batch mode.
2. Butyric acid and ABE fermentation were produced from two-stage fermentation
3. Continuous Stirred Tank Reactor (CSTR) working volume 5 liter will used to produce butyric acid from *Chlorella* sp. as substrate using mixed culture at 55 °C.
4. Second CSTR series working volume 3 liter will used to produce ABE from fermented of first CSTR series with *Clostridium beijerinckii* ATCC10132 at 37 °C.
5. Separation of butanol via two-stage fermentation process equipped with gas sparging system

### 1.4 Expected Outcome

1. The optimization of butanol production from microalgae *Chlorella* sp. in batch mode will be obtained.
2. The ABE production from two-stage fermentation of continuous system of Continuous Stirred Tank Reactor (CSTR) will be obtained.
3. Simultaneous enhancement of ABE fermentation and efficient butanol separation could be achieved via two-stage fermentation process equipped with gas sparging system.

# CHAPTER 2

# LITERATURE REVIEWS

## 2.1 Background of butanol

Butanol ($C_4H_9OH$) is four-carbon alcohol which butanol isomers boiling points and melting points have different about 83-117.7 ºC and 25-115 ºC, respectively. While butanol solubility (g/L) of n-butanol, iso-butanol, and sec-butanol has different are 77, 80 and 120, respectively. Butanol has 4-isomer structure such as straight-chain primary alcohol as n-butanol, primary alcohols as Iso-butanol, secondary alcohol as sec-butanol, and branched-chain tertiary alcohol (Atsumi *et. al*., 2008). However, tert-butanol is fully miscible with water above tert-butanol's melting point. The hydroxyl group could enhance the molecule polar, and solubility in water. Meanwhile, the longer hydrocarbon chain could mitigate the polarity and reduce solubility (Karabektas and Hosoz, 2009).

Commercial butanol is produced from fossil fuels in the form of Petro butanol. The propene was used as starts common process become to form butanal through a hydroformylation reaction and then butanal is reduced of hydrogen to butanol. Butanol is capable of use as an intermediate in chemical synthesis, as a chemical solvent including as a fuel. Butanol has properties more similar to gasoline when compared with ethanol. Especially, biofuel–gasoline blending, butanol was offering several advantages such as first butanol has a lower vapor pressure the second there is higher energy content and the last it is offers better fuel economy than ethanol. Butanol gasoline blends are less susceptible to separation, which facilitates their use in existing gasoline supplies and distribution channels. Specifically, butanol can be shipped through existing pipelines and is far less corrosive than ethanol (Dürre, 2007). Butanol has been demonstrated to work in vehicles designed for use with gasoline without modification. However, biobutanol was produced from a biological fermentation process of biomass by microbial degradation to convert the carbon source to butanol. The energy at a given volume of butanol contains have more than ethanol and almost as much as gasoline while butanol can be added to diesel fuel to reduce soot emissions (Antoni *et al.*, 2007). Moreover, biobutanol can be produced from ABE fermentation process. Acetone butanol ethanol as a main solvent product was produced from ABE fermentation process in industry by *Clostridia* for more than 100 years. The first industrial-scale ABE fermentation using *C. acetobutylicum* based on the Weizmann process was operated in 1916, within two years, the production of acetone and butanol as 3000 tons and 6000 tons in the fermentation process (Moon *et al*., 2016). Furthermore, in industrial level, butanol has been successfully promoted and produced from the fermentation process such as South Africa, Russia, and China countries (Zverlov *et al*., 2006; Ni and Sun, 2009).

## 2.2 Butanol production of Clostridium

Butanol can be produced from ABE fermentation process, which the productions of ABE fermentation process are produced acetone and ethanol together with butanol as a solvent product from *Clostridiam* species. Clostridia, a diverse group of strictly anaerobic bacteria, include known pathogenic and toxinogenic bacteria such as *Clostridium difcile* or *Clostridium botulinum* but also non-pathogenic industrially important species such as *Clostridium acetobutylicum*, *Clostridium beijerinckii* or *Clostridium ljungdahlii*. Among them, butanol-producers, *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum* and others offer a wide range of options related to substrate choice and utilization because they can produce a spectrum of hydrolytic enzymes and can utilize different, often unusual, and insufficiently described metabolic pathways to produce valuable chemical compounds that are currently produced from oil or its derivatives (Patakova *et.al.* 2019). The *Clostridia* such as *C. acetobutylicum, C. butylicum, C. beijerinckii* and other species are gram positive bacteria with rod-shaped, endospore-forming and typically strict anaerobes (Yang *et. al.* 2022) Figure 1. Batch ABE fermentation from *Clostridium* sp. can be divided into two phase: acidogenic and solventogenic phase (Patakova *et.al.* 2019).

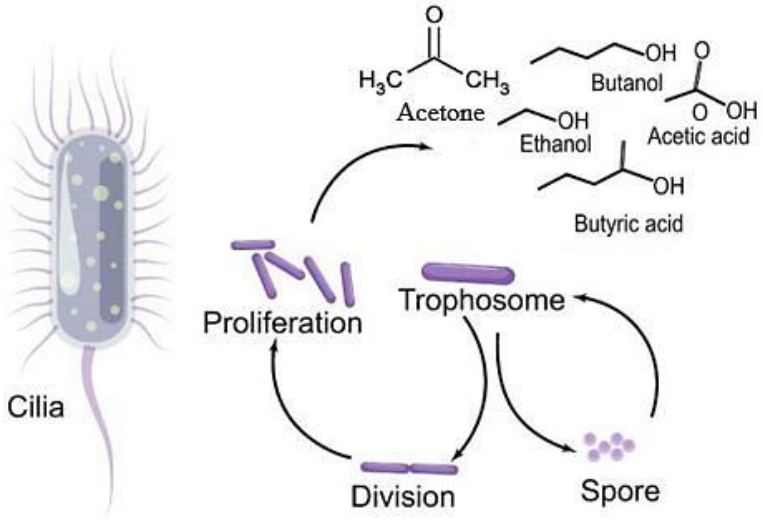



**Figure 1.** Schematic diagram of morphological and physiological characteristics of *Clostridium*. (Adapted from Yang *et al*., 2022).

The utilization of *Clostridial* species via ABE fermentation is the most used process for butanol production with acetone, butanol, and ethanol production in the ratio of 3:6:1 (Ezeji *et al*., 2007). The bacteria that have been used for the study are *Clostridium*

*beijerinckii*, *Clostridium acetobutylicum*, *Clostridium butylicum* and *Clostridium saccharoperbutylacetonicum* (Lee *et al*., 2008).

The biochemical pathways (Figure 2) utilized for the conversion of carbohydrates to fatty acids, hydrogen, carbon dioxide, and solvents with *C. acetobutylicum* have been established. The solvent producing of clostridia from sugars in form hexose and pentose is difference metabolized pathway, the Embden-meyerhof pathway from hexose sugars and the Pentose phosphate pathway from pentose sugars. The 1 mol sugars in form of hexose can be converted to 2 mols of pyruvate, which this reaction can occur the net product of NADH (reduced nicotinamide adenine dinucleotide) 2 mols and ATP (adenosine triphosphate) 2 mols. However the pentose sugars form also can be shift to pentose 5-phosphate which dissimilated through the transketolase-transaldolase sequence. fructose 6-phosphate and glyceraldehyde 3-phosphate are resultant of production after pass through pentose phosphase pathway while ATP 5 mols and NADH 5 mols was occurred at a final of the glycolytic pathway (Tashiro and Sonomoto, 2010). The glycolysis pathway, pyruvate can occur to carbon dioxide, acetyl-CoA, and reduced ferredoxin from pyruvate ferredoxin oxidoreductase enzyme with the presence of Coenzyme A (CoA). While Acetyl-CoA as a central intermediate in the branched pathways of ABE fermentation can convert to acid and solvent production as a last product. Under correct conditions, the reduced ferredoxin is transferred electrons to an iron-containing hydrogenase, which could use protons as a final electron acceptor and produce molecular hydrogen (Chen and Liao, 2016). Pyruvate is a key intermediate in *clostridial* metabolism. Under certain conditions, most saccharolytic *clostridia* are able to convert pyruvate to lactate by lactate dehydrogenase. Lactate is generated additionally when the activity of hydrogenase enzyme is inhibited from carbon monoxide or iron in cells depleted, leading to levels of ferredoxin and hydrogenase reduced. Pyruvate is mainly cleaved by pyruvate-ferredoxin oxidoreductase to form acetyl- CoA and $CO_2$ with concurrent reduction of ferredoxin (Jones and Woods, 1986).

The carbon flow from acetyl-CoA through the main branches of the pathway leading to the formation of acids and solvents is shown in Figure 2. The cells grow rapidly and form carboxylic acids (such as acetate and butyrate) occurred during the acid-producing phase, which the excretion of these acids can affect to decrease the external pH. Phosphotransacetylase and acetate kinaseand as enzyme is active acetyl-CoA become to acetate. Meanwhile, butyrate production can be produced from butyryl-CoA by enzyme of phosphotransbutyrylase and butyrate kinase. Acetate and butyrate are generated to produce ATP. Four enzymes involving in the metabolic pathway responsible for the formation of butyryl-CoA from acetyl- CoA are thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase (Chen and Liao, 2016).

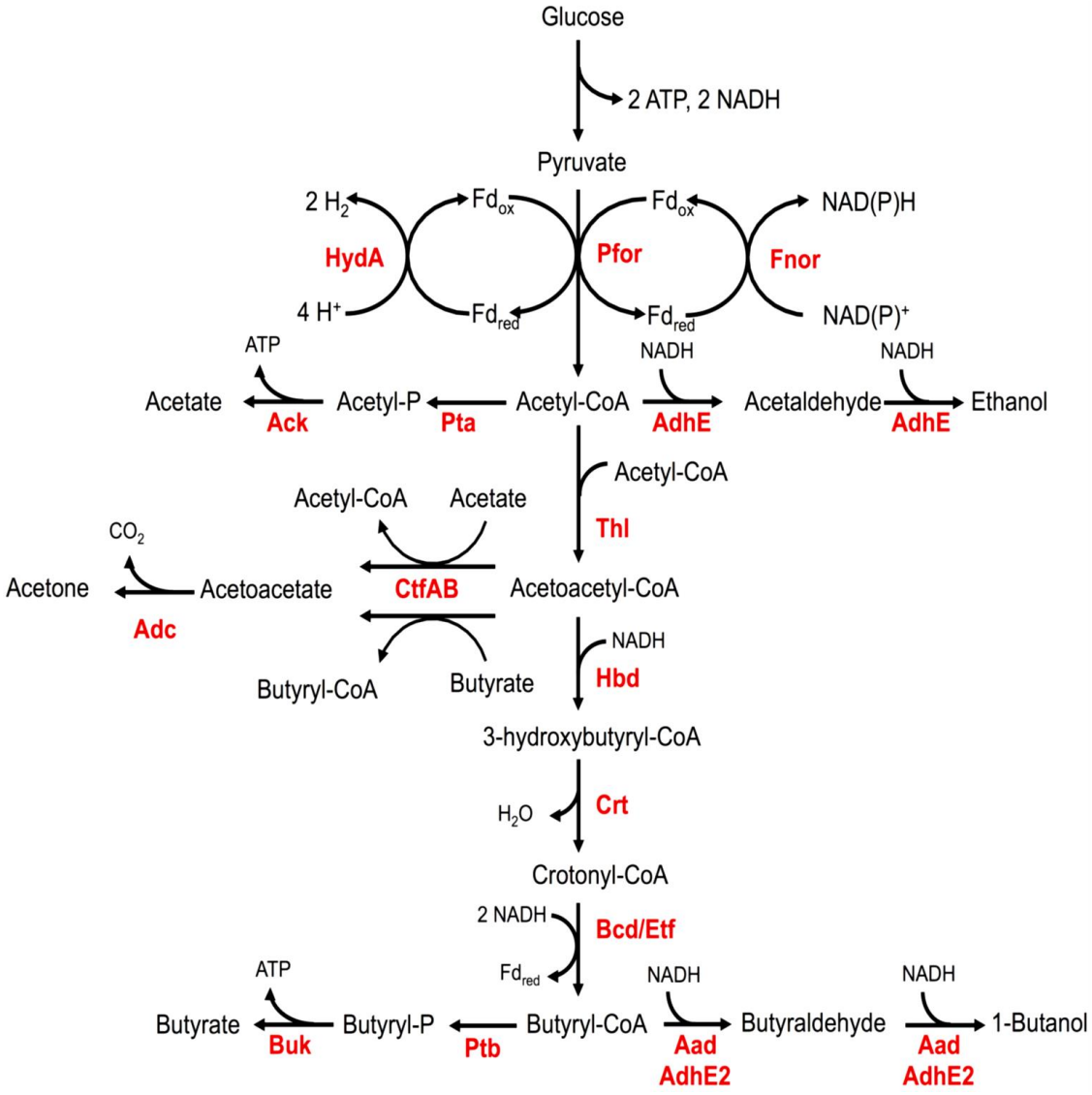



**Figure** 2. Biochemical pathways in *C. acetobutylicum* (Chen and Liao, 2016).

Pfor, pyruvate: ferredoxin oxidoreductase; HydA, hydrogenase; Fnor, ferredoxin: $NAD(P)^+$ oxidoreductase; Pta, phosphotransacetylase; Ack, acetate kinase; Ptb, phosphotransbutyrylase; Buk, butyrate kinase; CtfAB, acetoacetyl-CoA: acyl-CoA transferase; Adc, acetoacetyl-CoA decarboxylase; Thl, thiolase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd, butyryl-CoA dehydrogenase; Etf, electron transfer flavoprotein; Fd, ferredoxin; Aad and AdhE2 are both bifunctional alcohol/aldehyde dehydrogenase.

The major products of acetone ethanol and butanol were produced as a final of the ABE fermentation process. However, acetyl-CoA and butyryl-CoA functions are an important production intermediates for solvent production (ethanol and butanol). Acetylaldehyde and butyraldehyde can be produced from acetyl-CoA and butyryl-CoA with enzyme acetyldehyde dehydrogenase and butyraldehyde dehydrogenase, respectively. Furthermore, acetylaldehyde and butyraldehyde can be produced ethanol by ethanol dehydrogenase and butanol by butanol dehydrogenase. At the beginning of the solvent production process, the acid production was stopped in the process which increased the pH because acid was uptaken and convert to solvent. (Gheshlaghi *et al*., 2009). Acetoacetyl-CoA:acetate/butyrate:CoA transferase is an important role of enzyme to uptake acids during acetone production. Acetoacetate is produced from Acetoacetyl-CoA transferase. During the conversion of acetoacetyl-CoA to acetoacetate by enzyme acetoacetyl-CoA transferase, the CoA from Acetoacetyl-CoA was combined with acetate or butyrate as a CoA acceptor to be Acetyl-CoA and butyryl-CoA. The acetoacetate is then decarboxylated to produce acetone by acetoacetyl-CoA decarboxylase enzyme as the irreversible step (Tashiro and Sonomoto, 2010). When entering the solventogenic phase, the activity of acid production including phosphate acetyltransferase, phosphate butyryl-transferase, and acetate kinase rapidly decreases. As a result, the uptake of acids does not occur through a reversal of the acid-forming pathways (Gheshlaghi *et al.,* 2009). Understanding the pathway of ABE production could be beneficial to improve the butanol production process. The production of Acetic acid and butyric acid has been impacted to butanol and ethanol production. reported the utilization of butyric acid as the precursor could enhance the production of butanol by Clostridia (Al-Shorgani *et al*., 2012 and Tashiro *et al*., 2004).

## 2.3 Factor effect of butanol production

### 2.3.1 Temperature

Cultivation temperature is one of major factors impacting on ABE production process for butanol production. The research of Kundiyana *et al*., (2011) studied fermentation of *C. ragsdalei* at different temperatures 32 °C, 37 °C, and 42 °C. It was found that at 37 °C, the highest acid production and 32 °C gave the highest yield of bacteria cells and solvents. Furthermore, temperature at 40 °C found that all yields of acid, solvent and bacteria cell volume was decreased and very low volume. Jones and Woods, (1986) review the butanol ratio was increased by decreasing the temperature of the fermentation from 30- 24°C after 16 h. In addition, genetic engineering can improve butanol production of bacteria such as using plasmid of *E. coli* (Inui *et al*., 2008). Interestingly, mixed culture as many of bacteria communities that can be fermented at high temperatures and high acid yields at 37-55 °C which the research of Stein *et al*., (2017), the fermentation can produce butyric acid 8-10 g/L from batch dark fermentation. While butanol production can be

produced from butyric acid at lower temperatures. Corresponding with the research of Al-shargani *et al*. (2012), the butyric acid and sugar fermentation from various ratios can increase butanol production at temperature of 30 °C.

### 2.3.2 pH

The pH is a very important effect of the fermentation system to butanol production for ABE fermentation. The acidogenesis phase of acetic acid and butyric acids was rapid formation which pH is decreased in the fermenter. Until the process starts for solventogenesis stage, the pH on this stage is increased because the rich acid is reassimilated to be solvent product of butanol and acetone. Therefore, low pH is necessary for solvent production (Kim *et al*., 1984). The crisis pH decreases in the stage of acid formation lower than 4.5 before change to solventogenesis stage, The acid cannot convert to solvent which solventogenesis stage will be unproductive. So, the buffering capacity is important for ABE fermentation process, which the increasing buffer of medium is simple way to control the pH of the fermentation system. in addition, the utilization of buffer in ABE fermentation process can promote microorganism cell growth and carbohydrate degradation (Lee *et al*., 2008). However, the report of Gheshlaghi *et al*. (2009), at initial pH, the appropriate pH should not be less than 6 for beginning of ABE fermentation which that pH is the most of enzymes required in the mechanism. Corresponding with the research of Al-Shorgani *et al.* (2012) The initial medium pH was adjusted to 6.2 for ABE fermentation of glucose to the butyric acid ratio with *C. saccharoperbutylacetonicum* N1-4 which the rang pH of 4.5 to 5.7 at the final of fermentation was decreased.

### 2.3.3 Initial concentration

The utilizing of substrate for acetone, butanol, and ethanol production from ABE fermentation is important especially initial concentration of substrate. Initial sugar concentration less than 20 g/L can be produced low productivity with a small amount of organic acid for ABE fermentation (Lee *et al*., 2008). However, high sugar concentration (higher than 60 g/L) can produce more organic solvents (Madihah *et al*., 2001). However, at high concentrations above 80 g/L resulting product inhibition from high acid occurs acidogenesis phase. While concentrations of sugar up to 120 g/L, fermentation activity occurs only slightly (Qadeer *et al*., 1980). Also, at high concentration of sugar (160 g/L) was substrate inhibition which toxic to *C. beijernckii* BA101 (Ezeji *et al*., 2003).

### 2.3.4 Concentration of solvent

The concentration of product as solvent toxicity can be inhibited in the fermentation process which is an essential problem associated. During solventogenesis phase, cell metabolism usually continues until the concentration of the solvent reaches inhibitory levels as around 20 g/L, at butanol concentration 13 g/L, after which further cell

metabolism ceases. However, The product of butanol from concentrated sugar degradation can be toxic to microorganisms for ABE fermentation which can cause low yield of acetone butanol ethanol and concentration as well as low reactor productivity (Jones and Woods, 1986; Ezeji *et al*., 2004). The introduction of acetone and ethanol was reduced growth by approximately 50% at a concentration of 40 g/L, and total growth inhibition arise at a concentration of 70 g/L of acetone and 50-60 g/L of ethanol (Costa and Moreira, 1983). To resolve solvent toxicity problem butanol removal has various alternative methods, including membrane-based systems, adsorption, liquid-liquid extraction, and gas stripping. Those methods to be the most achievable path to follow have been reviewed from Xue *et al*., (2017).

### 2.3.5 Carbon and nitrogen ratio

The condition of carbon and nitrogen ratios (C/N ratios) was recognized as one of the important factors in biological fermentation processes for attaining the maximum production of butanol. Which nitrogen supplementation is an important source for the cultural growth of microorganisms in fermentation. However, butanol production using *Clostridium* sp as a solvent-producing bacteria has C/N ratio as a key role factor in the ABE fermentation process. The varied C/N ratio of from 5-120 for fermentation medium was researched with three nitrogen sources of tryptone, yeast extract, and ammonium acetate in a ratio of 6:2:3, respectively (Al-Shorgani *et al*., 2016). Comparing tryptone and yeast extract, tryptone was important than yeast extract. Because high concentration of butanol production from ABE fermentation process was provided at higher tryptone concentrations than yeast extract, while the using of only tryptone or yeast extract alone was increased only the cell growth. In addition, the ammonium acetate was an essential component of cell growth in the medium for ABE production. So, these three types of nitrogen sources were optimized to increase butanol production. Thus, the optimization of C/N ratio for ABE producing of *Clostridium* strains is important to increasing butanol production (Al-Shorgani *et al*., 2012; Tashiro *et al*., 2007). The research of Al-Shorgani *et al*. (2016) found that the glucose concentration of 50 g/L with three nitrogen sources under the ratio of 6:2:3 was optimal for the butanol production. While the optimal C/N ratio of 12.8 for butanol production was operated, butanol of 9.21 g/L was produced in fermentation process. However, the butanol and total solvent production have gradual decrease at the increasing C/N ratio. While butanol and ABE production in fermentation was low but high acetic acid concentration at the lower C/N ratios.

### 2.3.6 Effect of oxygen

The research of O'Brien and Mortis, (1971) found that the oxygen has effect of on *C. acetobutylicum* growth and metabolism Low oxygen exposures of cells were not lethal and often eliminate oxygen or reactive derivatives via NAD(P)H-dependent reduction to

survival in this system. However, it is not insufficient to establish long-term tolerance of aerobic conditions because depleted NAD(P)H (Hillmann *et al*., 2008). cell growth was decreased when exposed to high oxygen concentration result in the glucose consumption rate. Due to the unit of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) including with protein syntheses were halted. The effect of oxygen using the oxidation reduction potential (Eh) on the culture were measured with Radiometer pH meter. In addition, a review of Jones and Woods, (1986) showed that effects of oxygen exposure to cultures cause an increase in spore formation. From that review the exposure of cultures to short bursts of aeration of between 2 and 3 min every 1 to 2 h has been reported to increase the output of butanol by 3.1 to 9.1%.

**2.4 Butanol production from two stage anaerobic digestion**

*Clostridia*l ABE fermentation possesses sequential acidogenic and solventogenic biphasic fermentation. Under the exponential phase of acidogenic bacteria cell growth, the main product of acid, $H_2$, and $CO_2$ was converted from the substrate which is the acidogenic phase. During the solventogenic phase, the final products of acetone, butanol, and ethanol were produced from acetic acid, and butyric acid usually occurs in the stationary phase. (Wieczorek *et al*., 2014). ABE fermentation has two main phases: acidogenesis and acetogenesis which each phase is different condition due to a lot of acid at the first phase lead to low pH in system. The review condition of first stage was shown at Table 1. Three important conditions of acidogenesis stage can be used to optimize hydrogen production in the research from Table 1. This mentions the important condition is the initial concentration of substrate, pH, and temperature. While acetogenesis is second phase higher pH than acidogenesis phase while solvent can inhibit the acid production pathway of ABE fermentation. Therefore, if the fermentation process is split into two steps may be one way to reduce the inhibition of acid production in the first step and to increase the solvent yield in the second stage. According to Bankar *et al*. (2012), report that during continuous two-stage ABE fermentation with 60 g/L glucose of *Clostridium acetobutylicum* B 5313 and sugarcane bagasse was used for immobilized cells. An overall ABE production of 25.32 g/L (consisted of acetone butanol and ethanol of 5.93, 16.90 and 2.48 g/L, respectively) was observed as compared to 15.98 g/L in the single stage chemostat with highest solvent productivity and solvent yield of 2.5 g/L h and of 0.35 g/g, respectively. The second stage has different conditions for produce the product with the first stage. Especially, the type and initial concentration of substrate, pH, temperature, and inoculum of *Clostridium* sp. were reviewed at Table 2.

In addition, ABE production from two stage continuous culture was spatial separation in two reactors for acidogenesis and solventogenesis. Separation with two stage fermentation was used with difference of cell growth and product from ABE fermentation

process. Two stages of continuous fermentation have many benefits over than a single stage from the review of Richter *et al*. (2013),: (1) The temperature and pH of fermentation system can be optimized individually in each stage. (2) Dilution and cell growth rates of two stage fermentation can be adjusted for promote of acid production with fast growth in first stage and solvent production in low dilution rate of last stage. (3) The first stage has produced acid convert to solvent. Acid from the first stage can be used to substrate for solvent production during solventogenesis in the second stage. (4) Dilution rate was low in second stage, result to accumulation of biocatalyst. Demonstrate reactor productivity is high and can keep the cells by filtering the effluent to enhance the cells in the reactor. (5) The second stage nutrients may alter to adjust sufficient balance for solventogenic bacteria viability and limitation of nutrients to maintaining the solventogenic stage. Different condition of two stage fermentation was used for ABE production. In a lot of research was use difference maintaining for both stage of acidogenesis stage and solventogenesis stage depend on type of substrate reactor and microorganism. The research of Richter *et al*., (2013) operate two stage continuous fermentation for produce solvent with difference of pH (optimal pH was use for first stage and second stage of 5.5-5.7 and 4.4-4.8, respectively) with *Clostridium ljungdahlii*.

The acid production in the first stage has been enhanced for being converted to solvent in the second stage. There are different means such as selection of bacteria efficient to degradation of substrates (Maron *et al*., 2014) and using genetic engineering for improving the performance of bacteria to increase products (Berezina *et al*., 2010) or be tolerant to oxygen (Hillmann *et al*., 2008). The selection of mixed culture has been another way used to increase butanol production. The report of Angenent *et al*. (2004), during the first acidogenesis stage of acetic acid and butyric acid production is linked to hydrogen production. Ferredoxin oxidoreductase is the main enzyme used in metabolism to produce acid. The hydrogen production via butyric acid and acetic acid pathways is thermodynamically controlled by hydrogen partial pressure in dark fermentation process. At hydrogen partial pressure less than 60 Pa, 1 mole of glucose is directed to 2 moles of acetic acid and 4 moles of hydrogen, but most of the NADH will may be oxidized trough other fermentation, such as butyric acid fermentation. Hydrogen partial pressures above 60 Pa, the direction of production is in the form of 1 mole of butyric acid and 2 moles of Hydrogen (Figure 3). Major acid of effluent that consists of rich butyric acid can be utilized to enhance butanol product in the second stage of solventogenesis in the fermentation process. However, the utilization of mixed culture is possibly operated in the first stage to encourage rich butyric acid production. Bioprocess of mixed cultures for conversion complex mixture of carbohydrate (such as lignocellulosic hydrolysates) to butyric acid with acidogenesis in first stage bioprocess may be a superior alternative than the pure culture (Wang and Yin, 2018).

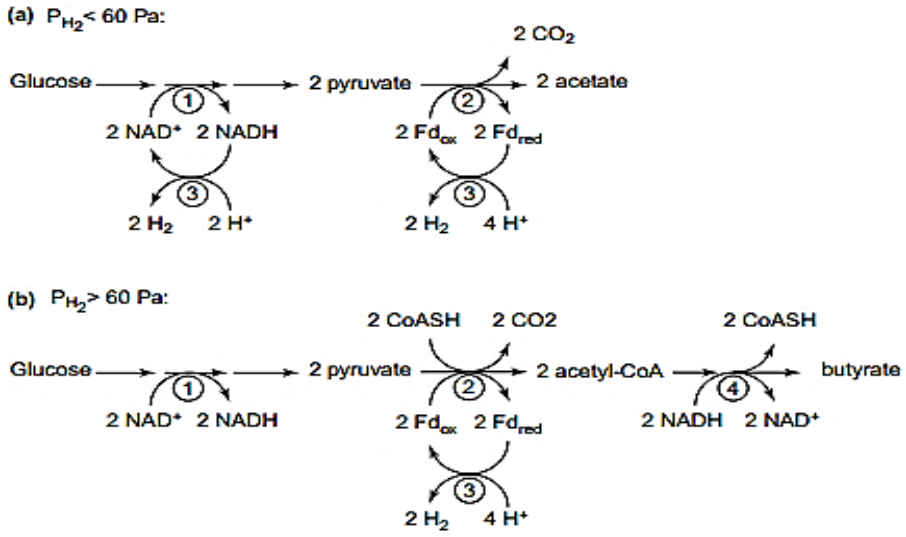



**Figure 3.** Schematic of biological butanol production (Angenent *et al*., 2004).
(a) Hydrogen partial pressure less than 60 Pa
(b) Hydrogen partial pressures above 60 Pa

Stein *et al*., (2017) studied the production of acid from mixed cultures in the anaerobic digestion of food waste with varied pH and temperatures. The maximum yields of butyric and acetic acids were 9.01 and 2.05, respectively. Which optimized condition of pH and temperature giving the maximum butyric acid production were 7 and 55°C respectively. While the acid production was obtained from fermenter will be used as precursor for ABE fermentation in the next step. According to research by Al-shorgani *et al*. (2012), and Tashiro *et al*. (2004) butanol production using butyric acid as precursor was performed by the pure culture of *Clostridium saccharoperbutylacetonicum* N1-4(ATCC13564). While butyric acid can be used for butanol production with a high yield of 0.95 C-$mol_{butanol}$/C-$mol_{substrate}$ but at low of butanol concentration, due to low pH and lack of electron donors from NADH. So, the organic carbon source (Glucose) is carbon source for microbial growth. Especially it's still needed as a source of ATP and electrons for conversion butyric acid to butanol from ABE fermentation process. Thus, the studied of Al-shorgani *et al*. (2012), glucose and butyric acid ratio of 20g/L and 10 g/L can be produced high butanol production and yield of 12.99 g/L and 0.99 C-$mol_{butanol}$/C-$mol_{substratev}$, respectively. Therefore, production of the acid in the first step was produced from the mixed cultures. After that acid on effluent of first stage was used to precursor for solvent production at the second stage by pure culture for the ABE fermentation process, is one option to increase the yield of butanol.

**Table 1** Condition of dark fermentation in batch

| References | Substrate | C/N ratio | Inoculum | pH | Rpm. | T.(℃) | $H_2$ P. | BA. P. |
|---|---|---|---|---|---|---|---|---|
| Yossan *et al*., (2012) | POME (35g-VS/L) | 42 | Anaerobic seed sludge | 6 | NA. | 35 | 27 [a] | 44.94mM |
| | | | | 6 | NA. | 55 | 28 [a] | 40.05mM |
| Marone *et al*., (2014) | Glucose (10g-VS/L) | NA. | Selected isolate from vegetable waste | 6.8-7 | 120 | 28 | 80-107 [a] | NA. |
| Mamimin *et al*., (2012) | POME (11g-VS/L) | 40 | Thermoanaerobacterium-rich | 5.5 | 150 | 60 | 243.9 [a] | 6.3 g/L |
| Li *et al*. (2018) | Rice straw (20g-TS/L) | NA. | Consortium DCB17 | 6.5 | 140 | 35 | 33 [b] | 6.87 g/L |
| Jehlee *et al*., (2017) | *Chlorella* sp. (16%TS) | 5.7 | Anaerobic sludge | 5.5 | NA. | 55 | 124.9 [a] | NA. |
| Phanduang *et al*., (2017) | *Chlorella* sp. (39%TS) | NA. | Anaerobic sludge | 6 | NA. | 30 | 18.58 [a] | 3.94 g/Kg sample |
| Roy *et al*., (2014) | *C. Sorokiniana* (14 g-TS/L) | NA. | Anaerobic sludge | 6.5 | NA. | 60 | 338 [b] | NA. |
| Wieczorek *et al*., (2014) | *C. valgaris* (10 g-TS/L) | NA. | Anaerobic sludge | 7.5 | 130 | 60 | 39 [b] | NA. |
| Sun *et al*., (2011) | *Chlorella* sp. (4-40 g-TS/L) | NA. | Anaerobic sludge | 6.5 | NA. | 35 | 0.37-7.13[b] | NA. |
| Ortigueira *et al*., (2015) | *Scenedesmus obliquus* (10-50 g-TS/L) | NA. | Anaerobic sludge | 7 | 150 | 58 | 0.7-15.3 [b] | NA. |
| Chen *et al*., (2012) | Rice straw (90 g-TS/L) | NA. | Anaerobic sludge | 6.5 | 150 | 55 | 24.8 [b] | NA. |
| He *et al*., (2014) | Rice straw (200 g-TS/L) | NA. | Anaerobic sludge | 7 | NA. | 35 | 28 [b] | NA. |

a = g-$H_2$/g-VS, b = g-$H_2$/g-TS, BA. P. = Butyric acid, $H_2$ P. = Hydrogen Production, Rpm = revolutions per minute, T. = Temperature , NA. = not available

**Table 2** Condition of butanol production in batch fermentation

| Reference | Inoculum | Substrate | Initial Conc. | pH | T. (°C) | Rpm. | ABE P. (g/L) | Bu.P. (g/L) |
|---|---|---|---|---|---|---|---|---|
| Al Shorgani *et al.*, (2012) | *C. saccharo-perbutylacetoncum* N1-4 (ATCC13564) | G/BA. | 20/10 (g/L) | 6.2 | 30 | NA. | 15.41 | 13 |
| Li *et al.*, (2018) | *C. beijerinckii* 8052 | G | 30 (g/L) | 6.8 | 37 | 140 | NA. | 6.7 |
| | | G/BA. | 30/5 (g/L) | 5 | 37 | 140 | NA. | 11.5 |
| | | rice straw hydrolysis/SFC | 40/60% | 5 | 37 | 140 | NA. | 13.8 |
| Maiti *et al.* (2016) | *C. beijerinckii* NRRL B-466 | Agro-industry waste water hydrolysate | 30 (g/L) | NA. | 37 | 150 | NA. | 4.68 |
| Wang *et al.* (2016) | *C. acetobutylicum* ATCC824 | *C. vagaris* STSC-6 | 150 (g/L) | 5 | 37 | NA. | NA. | 13.1 |
| Baba *et al*. (2012) | *C. saccharoper-butylacetoncum* N1-4 | G/BA. | 20/10 (g/L) | 6.5 | 37 | NA. | NA. | 8 |
| Al-Shorgani *et al*.(2018) | *C. acetobutylicum* YM1 | G | 30 (g/L) | 6 | 30 | NA. | NA. | 13.5 |
| | | G/BA. | 30/4 (g/L) | 6 | 37 | NA. | NA. | 16.5 |
| Sirisantimethakom *et al.* (2016) | *C. beijerinckii* TISTR1461 | Sweet sorghum juice | 27-30 (g/L) | 6.5 | 37 | 100 | NA. | 15.46 |
| Boonsombuti *et al*.(2015) | *C. beijerinckii* TISTR1461 | G | 40 (g/L) | 6.6 | 37 | NA. | 11.64 | NA. |
| Loyarkat *et al*. (2013) | *C. beijerinckii* TISTR1461 | G/BA. | 15/10 (g/L) | 6.5 | 37 | NA. | NA. | 12 |
| Vichuviwat *et al*. (2014) | Immobilized *C. beijerinckii* TISTR1461 | G | 60 (g/L) | 6.5 | 37 | 150 | NA. | 8.58 |
| This study | *C. beijerinckii* ATCC10132 | G | 40 (g/L) | 6 | 37 | 150 | 13.44 | 10.17 |
| | | Pretreated *Chlorella* sp. | 40 (g/L) | 6 | 37 | 150 | 2.67 | 2.30 |
| | | G/BA. | 20/10 (g/L) | 6 | 37 | 150 | 4.63 | 3.67 |

ABE P. = ABE production, BA = butyric acid, Bu. P. = Butanol production, G = Glucose, NA. = not available, Rpm = revolutions per minute., SFC = Supernatant of fermentation culture and T. = Temperatur

**2.5 Microalgae as major substrate**

The compositions of carbohydrate or oil rich substrate from microalgae can be produced for biofuels (Bellido *et al*., 2014; Milledge and Heaven, 2014). Biomass in the form of microalgal becomes a promising feedstock for biofuel production. It can be used for direct energy production from lipid extraction, or with the fermentation process for production of other biofuels (Lakaniemi *et al*., 2013). Microalgae can grow very fast and has high efficiency of photosynthetic when compared with terrestrial plants. It consists of many autotrophic organisms and can grow through photosynthesis, the same with plants. The cultivation of micro-algae can be cultivated in seawater and wastewater with no need land area, lead to not compete for resources of conventional agriculture at terrestrial (Harun *et al*., 2010). In addition, microalgae can decrease carbon dioxide emissions, and microalgae especially have valuable components to microorganism for fermentation such as carbohydrates and glycerol. Although microalgae for biobutanol production have potential to replace fuel from petroleum. But butanol production from microalgae has no commercial technologies that could overcome the related technical and economic barriers (Zhou *et al*., 2014)

Microalgae are the simplest photosynthetic plants and oldest on earth. There is diversity of physiologically and genetically with both single cellular and simple multicellular organisms, as well as it can be growth with autotrophic, heterotrophic, and mixing of autotrophic and heterotrophic. The report of Zue (2015), microalgae could be used its main components including carbohydrates, lipids, and proteins for commercially nutritional supplement. In addition, some microalgal types are rich in lipids as storage products in cell can be used for biodiesel production (Chen *et al*., 2011). In contrast, microalgal rich of carbohydrates can be used as feedstock for biofuel production via fermentation to producing biofuel in form butanol and ethanol (Ho *et al*., 2013). The main compositions (carbohydrates, lipids, and proteins) of microalgae are varied by the species of microalgal and conditions of cultivation (Vitova *et al*., 2015). Microalgae could increasingly produce protein during exponential phase of the cell growth. This phase can be improved to increase protein with optimizing cell growth. On the other hand, both of carbohydrates and lipids accumulation of cellular in microalgae has been under stress conditions. The most of strategy of nutrient depletion employment condition was used to promote accumulation of lipids and starch in cell of microalgae (Breuer *et al*., 2012; Li *et al*., 2008 and Ho *et al*., 2012).

The genera *Scenedesmus*, *Chlorella*, *Chlorococcum*, and *Tetraselmis* of the *Clorophyta* division and *Synechococcus* among other *Cyanobacteria* have been extensively studied as feedstock for this type of bioethanol production. In general, the cultivation in a high light intensity ranged from 150 to 450 $m^{-2}$ $s^{-1}$ using a mix of $CO_2$ in air between 2% and 5% and mesophilic temperatures (20–30 °C) achieves around 50% of carbohydrate

content under nutrient starvation, mainly nitrogen (Silva and Bertucco, 2016). Especially *Chlorella* sp. as micro-algae have contains cellulose and hemicellulose in cell wall and main storage product in form starch and lipid. The composition of carbohydrates varied at 37-55% (Chen *et al*., 2013) and oil content at 28-32% (percentage of dry weight) (Demirbas and Demirbas, 2011). According to a report by Sunja *et al*. (2011), the C/N ratio of *Chlorella* sp. is in the range of 5-10, which is close to Wang *et al*. (2010), at 11.5. Al-Shorgani *et al*. (2016), found that the optimum C/N ratio for the production of butanol was 12.8. In the research of Bhatnagar *et al*. (2010), they used growth conditions of *Chlorella* sp. with BG-11 medium cultivation ($NaNO_3$ 1.5 g $K_2HPO_4$ 0.04 g, $MgSO_4 \cdot 7H_2O$ 0.075 g, $CaCl_2 \cdot 2H_2O$ 0.036 g, Citric acid 0.006 g, Ferric ammonium citrate 0.006 g, EDTA (disodium salt) 0.001 g, $Na_2CO_3$ 0.02 g, Trace metal mix A5 1.0 ml ($H_3BO_3$ 2.86 g/L, $MnCl_2 \cdot 4H_2O$ 1.81 g/L, $ZnSO_4 \cdot 7H_2O$ 0.222 g/L, $NaMoO_4 \cdot 2H_2O$ 0.39 g, $CuSO_4 \cdot 5H_2O$ 0.079 g and $Co(NO_3)_2 \cdot 6H_2O$ 49.4 mg)), gathering with Light throughout the 10 days. Biomass production was found to be 73.03 mg/L with protein, carbohydrate, and lipid of 41.1, 9.6 and 3.94 mg/L respectively. The composition of cell may be different by various conditions of lighting or sugar added. In addition, the composition of *Chlorella* sp. MP-1 with also used BG-11 medium culture has been moisture 6.8% volatile matter 72.19% fixed carbon 15.08% and ash 5.93%, there are relatively high volatile matter and low residual ash indicate the amount of organic material that can be used as substrate for fermentation with microorganism (Phukan *et al*., 2011).

Microalgal cells needed pretreatment to break down the cell wall of microalgal biomass for hydrolysis micro-algae as feedstock to sugars with fermentative bacteria. Technologies of pretreatment for microalgal has four technologies widely used (Passos *et al*., 2014), thermal pretreatment, mechanical pretreatment, chemical pretreatment, and enzymatic pretreatment methods. The ABE production of *Chlorella vulgaris* JSC-6 was 19.9 g/L with an A:B:E ratio of 2:6:1. The butanol concentration, butanol yield, and butanol productivity were 13.1 g/L, 0.58 mol/mol sugar (0.24 g/g sugar), and 0.66 g/L.h, respectively. Glucose consumption was nearly completed (97.5%), demonstrating that there was no inhibitor in the hydrolysate resulting from sequential alkali-acid pretreatment using NaOH (1%) and $H_2SO_4$ (3%) (Wang *et al*., 2016). In addition, at the same of micro-algae and pretreatment with sulfuric acid followed by enzymatic process (cellulase) but difference of microorganism could affect to butanol production (Van der Wal *et al*., 2013). Butanol production is not only depending on type of microorganism but also type and composition of microalgae, which have been used for fermentation. Both *Nannochloropsis* sp. and *Dunaliella tertiolecta* were used 0.1 M Sulfuric acid pretreatment and produced closely of sugar concentration, but *Dunaliella tertiolecta* produced lower of butanol than *Nannochloropsis* sp. obviously. Because of *Dunaliella tertiolecta* was cultured in brine conditions. Therefore, high salt content may affect the inhibition of microorganisms in the

butanol fermentation process (Efremenko *et al*., 2012). The review of Chen *et al*., (2013) reported much lower ethanol production yield of microalgae without pretreatment. On the other hand, using mixed cultures for fermentation microalgae may improve production of butanol. Because some of microorganism in mixed cultures such as *bacillus* sp. can be produced amylase enzyme to hydrolysis starch (Silva and Bertucco, 2016) and from *Clostridium thermocellum* and *bacillus* sp. also can produce cellulase enzyme to hydrolysis cellulose of microalgae (Sadhu and Maiti, 2013). And temperature used is suitable at thermophilic temperature of 55 °C for producing more butyric acid as precursor for butanol production (Stein *et al*., 2017). Thus, there is an alternative for no pretreatment prior anaerobic digestion with mixed cultures fermentation for butanol production.

**2.6 Reactor Configuration**

There are several types of reactors, but the Continuous Stirred Tank Reactor (CSTR) reactors are used for fermentation in high suspended solids. stirred reactors are more efficient because their employment may reduce product inhibition over cellulose (Nguyenhuynh *et al*. 2017). CSTR is widely used in research studies by Richter *et al.* (2013) and Lay *et al*. (2010). Two reactors of the first and second stages of this research used CSTR for acid production and butanol production. The first stage for acidogenesis have pH and temperature suitable range of 5 to 9 and 35 to 70 °C, respectively, which depended on microbial. The report of Richter *et al*. (2013), study for acid production begins with a series of continuous reactor. While the temperature of acid fermentation from mixed culture was suitable at 55 °C (Stein *et al*., 2017). For HRT and Speed of rotation can be monitored from suitable for CSTR follow with the Table 3. Under process of acid fermentation. The acid product from the first series was continued for second series CSTR tank to produce butanol production by *Clostridia*.

However, ABE production if more than 20 g/L will inhibit the microorganism in system, so that the recovery system can used to integrate ABE from fermenter. Especially *in situ* recovery with gas stripping to remove of ABE production can help to increase vapor–liquid contact than without gas stripping (Kumar *et al*., 2011). From Table 4 were show the condition of ABE and butanol production with gas stripping. The research almost used glucose for the substrate to produce ABE and butanol from *Clostridium* sp. in varies reactor type at pH and temperature range of 4.5 to 6 and 35 to 37 °C, respectively. Which the flow rate range of gas stripping was used 4 to 6 L/min with $H_2$-$CO_2$ at condensate temperature range -10 to 2 °C all show at table 4.

**Table 3**. Conditions for hydrogen and butyric acid production in CSTR.

| Reffrence | Inoculum | Substrate | pH | T. (ºC) | HRT | RS. | $H_2$ P. | BA.P. |
|---|---|---|---|---|---|---|---|---|
| Kongian *et al*. (2010) | Mixed culture | Hemicellulose-rich hydrolysate | 5.2-5.5 | 70 | 3 day | NA. | 184 ml/L.d | NA. |
| Stein *et al*., (2017) | Mixed microbial | Food waste | 9 | 37 | 2 day | NA. | N.A. | 8.5 g/L |
| | Mixed microbial | Food waste | 7 | 55 | 2 day | NA. | N.A. | 10.5 g/L |
| Ding *et al*., (2014) | Anaerobic-activated sludge | 53% Sugar | 6.5-7.5 | 35 | 8 h | 50-70 rev/min | 11.8 L/d | NA. |
| Lay *et al*., (2010) | Seed sludge | Soluble condensed molasses | 5.5 | 35 | 3-24 h | 250 rpm | 390 mmol/L.d | NA. |
| This study | Mixed culture | Pretreated *Chlorella* sp. | 7 | 55 | 6 day | NA. | 2,103.5 mL /L.d | 7.50g/L |

BA.P. = Butyric acid production, $H_2$ P. = Hydrogen production, NA. = Not available and RS. = Rotation speed T. = Temperature.

**Table 4.** Condition of ABE recovery with gas stripping.

| Ref. | Reactor type | Sub. (g/L) | Inoculum | pH | Ferment T. (ºC) | Gas type | Flow rate (L/min) | Cond. T. (ºC) | ABE Production (g/L) | Butanol Production (g/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| Xue *et al*. (2012) | Fed batch FBB $_{TV=1L\ WV=7.5L}$ | G 475 | *C. acetobutylicum* JB200 | 5 | 37 | $H_2$-$CO_2$ | 1.5 | ~2 | 172 | 113 |
| Xue *et al*. (2013) | batch FBB $_{WV=1\ L}$ | G 80 | *C. acetobutylicum* JB200 | 5 | 37 | $H_2$-$CO_2$ | 1.5 | ~2 | 227 | 175 |
| Chen*et al*. (2014) | Fed batch FBB $_{WV=1\ L}$ | G 290 | *C. acetobutylicum* B3 | 4.5 | 37 at 6h after that 70 | $H_2$-$CO_2$ | 1.5 | -5 to -10 | 106 | 66 |
| Ezeji *et al*. (2013) | Cont.$_{TV=2L\ WV=1L}$ | S 1,125 | *C. beijerinckii* BA101 | 5 | 35 | $H_2$-$CO_2$ | 4-6 | 1 | 461 | NA. |
| Lin *et al*. (2017) | Fed batch $_{TV=1L\ WV=0.5L}$ | G 840 | TSH06 | 6 | 37 | $H_2$-$CO_2$ | NA. | -5 | 267 | 185 |
| This study | Cont.$_{TV=10L\ WV=3L}$ | G20 | *C. beijerinckii* ATCC10135 | 5-5.5 | 37 | $H_2$-$CO_2$ | 3 | -4 | 45* | 52* |

Cont. = Continuous, FBB = Fibrous bed bioreactor, NA. = Not available, G = Glucose, S = Sugar, Sub. = Substrate, Ref = Reference, TV = Total volume and WV = Working volume. Ferment T. = Fermentation temperature. Cond. T. = Condensate temperature and *= at highest product concentration of removal in a condensate.

**2.7 Butanol separation with gas stripping**

Distillation is currently applied for product purification in the butanol fermentation industry. While Solvent of ABE fermentation process usually produced lower concentration of butanol than 2% (w/v), Due to the limitation of the bacteria's tolerance to butanol. Butanol is more toxic than ethanol to cells, which inhibits cell growth and hinders solvent accumulation. The research of Oudshoorn *et al*., 2009, butanol recovery will need to integrate with ABE fermentation, which butanol recovery was required high energy especially with distillation recovery technique.

Due to, the removal of inhibitors from the fermentation process was popularly investigated by an integrated recovery process during fermentation (Kujawska *et al*., 2015; Staggs and Nielsen, 2015). However, the several techniques of *in situ* product recovery processes of ABE fermentation were studied in many research such as, liquid liquid extraction, pervaporation, adsorption, and gas stripping. The effect of high ABE production in the fermenter from the fermentation process can be maintained by the fermentation with continuously removing solvent product during fermentation, which is one of the main advantages for mitigating inhibition by product and enhancing fermentation productivity.

Gas stripping is a process of solvents production removal from fermentation broth by sparging gas through the bioreactor or column with condenser to separate the solvents. This kind of process is attempting to make the ABE recovery from the fermentation broth simpler and more economical. Fermented gases can be used to recover butanol during ABE fermentation by gas stripping recovery (Ezeji *et al*., 2013). Thus, *in situ* recovery by gas stripping, ABE fermentation process could be fed with high sugar concentration and could reduce butanol inhibition impact (Kumar *et al*., 2011). The gases can be captured ABE in the fermenter with a bubble gas passed through the ABE fermentation broth, and then cooled in a condenser with a receiver vessel to collect the product (Vrije *et al*., 2013). In some research, after the solvents are condensed, the gas is recycled back to capture more ABE in the fermenter. This process continues until all the sugar in the fermenter is utilized by the culture in fermentation process is depleted. Consistent in research Ezeji *et al*. (2003), and Ezeji *et al*. (2004), the stripper effluent is then recycled back to the reactor. While the productivity of research of Xue *et al*. (2012), The butanol and total solvent ABE concentrations were increase of 113.3 g/L and 172 g/L respectively from the intermittent strategy of fed-batch ABE fermentation with gas stripping. Gas stripping was used to recovery of butanol more than 8 g/L in fermentation broth which final product after spontaneous phase separation of butanol was more than 60% (w/v).

Furthermore, the biogas ($H_2$ and $CO_2$) from anaerobic fermentation was used instead of additional $N_2$ in the fermentation with off-gas for ABE recovery which circulated in a closed circuit of reactor and condenser to prevent any loss (Chen *et al*., 2014 and Ezeji *et al*., 2013). Also study of Lin *et al*., (2017) using fed batch

fermentation from *C. acetobutylicum* TSH1 and *B. cereus* TSH2 to produce butanol with *in situ* recovery by gas stripping ($H_2$ and $CO_2$) and obtain butanol recovery ratio of 97.36%. The several advantages of gas stripping process have such as simplicity, no fouling to the culture in fermented and easy operation. Furthermore, only volatile products can be removed from aqueous fermented. While organic acids and other nutrients were remained in fermentation broth for metabolic activity of bacteria culture (Xue *et al*., 2017). The Table 5 review of ABE production of fermentation recovery by gas stripping was compared of non-integrate with product recovery in fermentation systems.

The significant disadvantage is that excessive amounts of foam produced by gas stripping may result in the necessity of antifoam agent addition. Most fermentation problems are foaming. Normally, it is caused by the natural of culture medium is changes and foam causes some microorganisms to exit the culture medium and then release the protein from autolysis cell. The resulting foam is more stable and the gas stripping into the fermentation system may even result in increased foaming. Foaming can be solved by adding antifoam as suggested by Richter *et al*., (2013) for using antifoam in the second stage, and Ezeji *et al.*, (2005) and Vrije *et al*., (2013) using antifoam for ABE fermenter with gas stripping. In addition, butanol integrates with gas stripping have limited by equilibrium with water and butanol while interfacial of gas bubbles have the limit performance of gas stripping. However, gas stripping was operated under the best simulation still is a competitive process for recovery of butanol integrated with fermentation.

**Table 5.** A comparisons of ABE production systems with non-integrate and integrate gas stripping.

| Reactor | Culture | Substrate | Sugar used (%) | Total ABE (g/L) | Butanol (g/L) | Butanol Yield (g/g) | ABE P. (g/L.h) | Ref. |
|---|---|---|---|---|---|---|---|---|
| Batch | *C. beijerinckii* CC101. | Wood pulping hydrolysate | 64/78 | 7.85/13.0 | 5.58/9.38 | 0.23/0.29 | 0.11/0.17 | Lu *et al.*, (2013) |
| Batch | *C.acetobutylicum* JB200 | Glucose | 100/100 | 26/32 | 16.2/19.8 | 0.20/0.25 | 0.48/0.66 | Xue *et al.*, (2013) |
| Batch | *C.beijerinckii* BA101 | Sugar | 78/100 | 18/76 | 11/8.5 | 0.39/0.47 | 0.28/0.60 | Ezeji *et al.*, (2013) |
| FB. | | | -/95 | -/232 | -/3.7 | -/0.46 | -/1.16 | |
| | | | -/93 | -/461 | -/4.3 | -/0.41 | -/0.92 | |

FB. = Fed-batch continuous, Cont. = Continuous, ABE P. = ABE production and

**Theory of gas stripping**

Gas stripping is the transfer of mass from the liquid phase to the gas phase (liquid to gas mass transfer). The mass transfer has occurred between both surface of parts liquid and gas. This theory assumes that the parts of the gas and liquid are completely mixed, causing the surface of the gas and liquid to contact and transfer the mass of the substance in the gas or liquid with diffusion of the molecule through the membrane between the gas and liquid, until two phase were balance. Important variables for gas stripping depend on the physical properties of the gas such as diffusion, flow rate, concentration, density, and solvents condition such as boiling point, solubility in water and vaper pressure, et cetera (Table 6) (Plaza *et al*., 2013). Moreover, can adjust the gas flow conditions to improve the efficiency of the stripping better, such as the experiment of Lodi and Pellegrini, 2016 was found that from different flow rate the ratio of nitrogen and broth flow rates lower than 0.84 was suable to used, because of high of gas flow result to the increasing water removal from the fermentation broth.

**Table 6.** Properties of the acetone butanol ethanol components (Plaza *et al*., 2013).

| Compound | Molecular weight (g $mol^{-1}$) | Boiling point (°C) | Solubility in water 25 °C (g $L^{-1}$) | Vapor pressure at 25 °C (mmHg) |
|---|---|---|---|---|
| Acetone | 58.08 | 56.50 | Miscible | 228.86 |
| Ethanol | 46.07 | 78.39 | Miscible | 58.71 |
| Butanol | 74.12 | 117.70 | 73.90 | 6.70 |

**Henry's law**

Water in a closed vessel consists of dissolved gases. The volatile compound concentration in both of the gas phase and the water phase will be equilibrium, according to Henry's law. Henry's law can be used to calculate equilibrium concentration.

$$C_w = K_H \cdot C_g$$

$C_w$ represent the equilibrium concentration of a gas dissolved in the water with the unit of [$g/m^3$] and Henry's constant is $k_H$ as distribution coefficient while $C_g$ represents the concentration of the gas in the air with the unit of [$g/m^3$]

The differences of gas type and temperature affect to the distribution of coefficient $k_H$. As well as the water phase, pollution and impurities of water can affect equilibrium. The Henry's law constant can be found in many forms of calculation. The partial pressure in air is often used with the gas concentration, while in water often used

molar concentration as weight concentration. Therefore, this results unit will be different for the distribution coefficient, or Henry's law constant such as [mol/($m^3$ Pa)] or [mol/l/atm]. For using with gas stripping, the volatility is often given instead of the gas solubility. In this case, the distribution coefficient is inverted (gas/water, instead of water/gas) Seader and Henley, (2006).

**Table 7.** Distribution coefficient and molecular weight of gases.

| Gas | Distribution coefficient ($k_H$) | | | |
|---|---|---|---|---|
| | T = 10 °C | T = 20 °C | T = 25 °C | Molecular weight (MW) [g/mol] |
| Nitrogen ($N_2$ ) | 0.019 | 0.016 | 0.015 | 28 |
| Carbon dioxide ($CO_2$ ) | 1.23 | 0.942 | 0.830 | 44 |
| Hydrogen ($H_2$ ) | - | - | 0.019 | 2 |

**Table 8** Mole fractions of acetone butanol ethanol and water in the liquid phases of condensation after gas stripping. (Lodi and Pellegrini, 2016)

| Component | Organic phase | Liquid phase |
|---|---|---|
| Acetone | 0.016 | 0.005 |
| Butanol | 0.398 | 0.028 |
| Ethanol | 0.013 | 0.004 |
| Water | 0.572 | 0.962 |

The distribution coefficient of gas value depends on water temperatures, the gas value will change at different water temperatures and the value of gas can be achieved from linear interpolation (Table 7). Show that nitrogen and hydrogen have low values of $k_H$. It means that nitrogen and hydrogen gases hardly dissolve in water, and they can be easily out of stripper chamber. The removal of gas from the water phase was difficult under the gas value of high $k_H$ and easy to dissolve in the water phase.. Moreover, the liquid and gas mass transfer properties of solvent from ABE fermentation are different. The henry's law constant of solvent from Plaza *et al*. (2013).

$$K_H=K_H'\bullet RT$$

$K_H$ is Henry's low constant (atm•$m^3$/mol), $K_H$'is Henry's low constant (dimensionless) of ($C_g/C_{aq}$ is concentration of solute in gas or liquid phase), R is ideal gas constant (atm•$m^3$/mol•K) and T is equilibrium temperature (K). The henry's law constant of acetone ($4.25\bullet E^{-5}$ atm•$m^3$/mol) was higher than butanol ($0.56\bullet E^{-5}$ atm•$m^3$/mol) and ethanol ($0.50\bullet E^{-5}$ atm•$m^3$/mol) at 25 °C. So that the acetone was easily dissolved in the gas phase follow by butanol and ethanol at the same initial

concentration of solvent. But the ABE fermentation from *Clostridium* sp. was produced with the ratio of ABE 3:6:1. The butanol is higher concentration than the acetone resulted that the butanol was easy to transfer to gas phase with gas stripping. The research of Lin *et al*. (2017), used hydrogen and carbon dioxide for stripping solvent ABE fermentation process with a butanol recovery ratio of 97.36%. In addition, the research of Lodi and Pellegrini (2016), was study gas stripping of synergetic medium of acetone butanol and ethanol with nitrogen gas, result at condensation of ABE solution has been two phase of organic phase and liquid phase. Butanol is more dissolving in organic phase (Table 8), So that the solvent can separate and remove of liquid phase for increase ABE concentration and to decrease costs for separate with distillation.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Substrate and Inoculum preparation

### 3.1.1 *Chlorella* sp. from Taiwan. and Inoculum from mesophilic source

Microalgae (*Chlorella* sp.) purchased from Cheng Yang Instrument Corp, Taiwan as a dry, green powder. They were stored in a desiccator at room temperature prior to further use.

The mixed cultures used as inoculum for fermentation were taken from APEC Research Center for Advanced Biohydrogen Technology (ACABT), Feng Chia University, Taichung, Taiwan. The inoculum was originally cultivated in the CSTR dark fermentation tank at the mesophilic temperature (35 °C) for hydrogen and acid production by feeding using sucrose at a concentration of 80 g-VS/L supplemented with Endo nutrients ($MnSO_4.H_2O$ 9.79 g/L, $FeSO_4.7H_2O$ 25 g/L, $CuSO_4.5H_2O$ 5 g/L and $CoCl_2.6H_2O$ 0.125 g/L) (Endo *et al*., 1982). The inoculum was later adapted to two different temperatures (mesophilic and thermophilic conditions), as have researchers demonstrated that mesophilic cultures can serve as sources for cultivation under thermophilic as well as hyperthermophilic conditions (De la Rubia *et al*., 2013). The obtained inoculum was acclimatized by adding 50 g-VS/L sucrose as a substrate without Endo nutrients and then leaving for three days at 150 rpm in an incubator shaker at mesophilic (35 °C) or thermophilic temperature (55 °C).

### 3.1.2 *Chlorella* sp. from China and Inoculum from thermophilic source

Microalgae (*Chlorella* sp.) Yantai Hearol Biotechnology Co. Ltd., Shangdong, China as a dry, green powder. and before used stored at temperature 4 °C.

The mixed cultures used as inoculum for fermentation were taken from laboratory scale 10 L CSTR reactor of Bio-mass Conversion to Energy and Chemicals (Bio-MEC Research Unit), Faculty of Science and Technology, Prince of Songkla University, Pattani Campus, Thailand. The inoculum was originally cultivated in the CSTR dark fermentation tank at the Thermophilic temperature (55 °C) for hydrogen and acid production by feeding using sucrose at a concentration of 20 g-VS/L supplemented with Basic Anaerobic (BA) medium (Solution A 10 mL: $NH_4Cl$ 100 g/L; NaCl 10 g/L; $MgC_{l2}•6H_2O$, 10 g/L, $CaCl_2•2H_2O$ 5 g/L ; Solution B 2 mL: $K_2HPO_4•3H_2O$ 200 g/L; Solution C 1 mL: $C_{12}H_6NO_4Na$ 0.5 g/L ; Solution D 1 mL: $H_3BO_3$ 0.05 g/L, $ZnCl_2$ 0.05 g/L, $CuCl_2•2H_2O$ 0.038 g/L, $MnCl_2•4H_2O$ 0.05 g/L, $(NH_4)_6Mo_7O_{24}•4H_2O$ 0.05 g/L, $AlCl_3$ 0.05 g/L, $CoCl_2•6H_2O$ 0.05 g/L, $NiCl_2•6H_2O$ 0.092 g/L, EDTA 0.5 g/L, $Na_2SeO_3•5H_2O$ 0.066 g/L ; $NaHCO_3$ 50 mL of 52 g/L ;Yeast extract, 1.07 g/L adjust with DI water for 1 L (Angelidaki *et al*., 2009). The inoculum

also was later adapted to two different temperatures (mesophilic and thermophilic conditions).

### 3.1.3 Strain for ABE fermentation

*Clostridium butylicum* TISTR 1032 and *Clostidium beijerinckii* ATCC 10132 taken from dry cell tube into serum bottle with Tryptone-yeast extract-acetate (TYA) (Glucose 20 g/L, Yeast extract 2 g/L, Tryptone 6 g/L, $CH_3COONH_4$ 3 g/L, $KH_2PO_4$ 0.5 g/L, $MgSO_4.7H_2O$ 0.3 g/L and $FeSO_4 \cdot 7H_2O$ 10 mg/L) (Al-Shorgani *et al*., 2012) incubate at 37 °C 48 h with 150 rpm under anaerobic condition for active bacteria. 10% of activated bacteria was used to enhance bacteria for butanol fermentation in serum bottle with working volume 300 ml, incubate at 37 °C 24 h at 150 rpm anaerobic condition. The enhanced bacteria was used as inoculum for butanol fermentation process and some parts were taken into 15 % glycerol and stored at -40 °C to be stock bacteria.

*Clostridia* are anaerobic bacteria which inoculum preparation without oxygen inhibition are necessary for fermentation process. Laminar air flow was used to protect for contamination when preparation bacteria (Figure 4). *Clostridium* sp. live under -40 °C with 15% glycerol for more than 20 years. However, *Clostridium* sp. can be spore form under lac medium condition so bacteria that prepared on serum bottle with TYA also can be used after -4 °C stored which need to shock with 80 °C for 5 min before incubator at temperature of 37 °C (Ezeji *et al*., 2003).

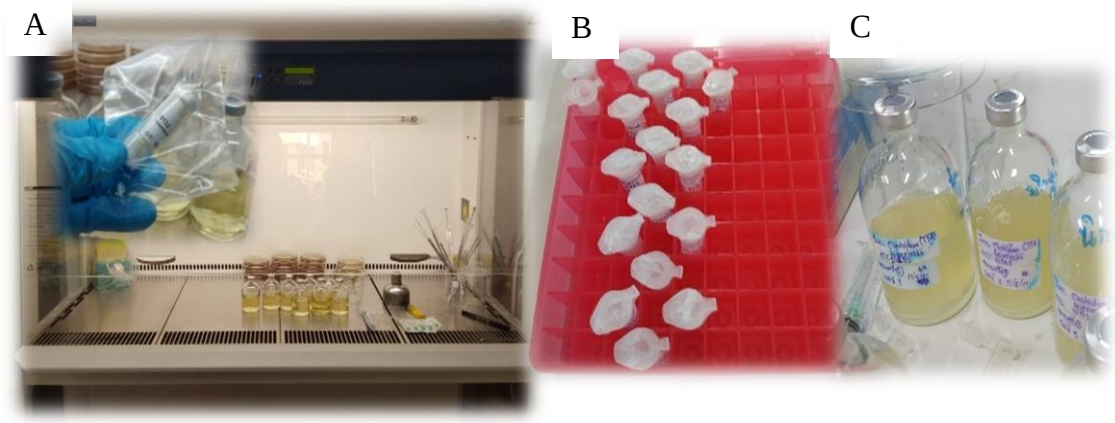



**Figure 4.** *Clostridium* sp. preparation.
(A) *Clostridium* sp. preparation with TYA medium under Laminar air flow
(B) *Clostridium beijerinckii* stored in 15% glycerol
(C) Active *Clostridium beijerinckii* in serum bottle with TYA medium

## 3.2 Batch experiment for acidogenesis stage

### 3.2.1 Mesophilic source mixed culture for batch acidogenesis fermentation

3.2.1.1 Batch Fermentation of *Chlorella* sp. with and without Endo Nutrients

The batch experimental assay was first carried out to investigate the effect of either adding or not Endo nutrients on hydrogen production from *Chlorella* sp. in batch fermentation at the initial concentration of 80 g-VS/L and pH 7 with 15 mL total volume of serum bottle. The serum bottle contained 80 g-VS/L of *Chlorella* sp. and 4 mL of mixed cultures with or without Endo nutrient (the concentration of Endo nutrients: 9.79 g $MnSO_4 \cdot H_2O$, 25 g $FeSO_4 \cdot 7H_2O$, 5 g $CuSO_4 \cdot 5H_2O$ and 0.125 g $CoCl_2 \cdot 6H_2O$ in 1 L). The amount of nutrients used depended on the working volume (g-nutrient/L-working volume). The initial pH 7 ± 0.1 was adjusted with 0.5 M phosphate buffer (1.5 mL) followed by distilled water to 10 mL working volume. The serum bottles were capped with tight rubber stoppers and aluminum, and the headspace was flushed with 5 min of nitrogen gas to ensure anaerobic conditions. They were then placed in a batch incubator shaker at 150 rpm and 35 °C. Gas production from anaerobic dark fermentation was measured using a 50 mL glass syringe drawing from the headspace of the batch serum bottles for cumulative gas values and, for the composition gas hydrogen production, 1 mL plastic syringes were drawn daily. Both were analyzed with gas chromatography.

3.2.1.2 Optimization of hydrogen and butyric acid production from *Chlorella* sp.

For optimization of hydrogen and butyric acid production from *Chlorella* sp. using mixed cultures as a substrate, it was carried out at temperatures of 35 °C and 55 °C (two levels), the initial pHs of 5 and 7 (two levels) and the initial substrate concentrations of 40, 60, 80, and 100 g-VS/L (four levels) to determine the optimum conditions in batch dark fermentation of *Chlorella* sp. by using Taguchi method to aid experimental design.

The Taguchi method as a statistical tool for biotechnological applications is an easy and popular method used for experimental design. As reviewed by Rao *et al*. (2008), comparative studies between response surface methodology (RSM) and the Taguchi techniques revealed that both techniques have similar results, however Taguchi technique requires half the time as RSM technique. Thus, Taguchi technique was selected for this investigation. Table 9 shows a set of experimental assays created using Taguchi design method. Eight batch test sets were used for the Taguchi design. However, in the experiment, there was one additional set of experiments (Batch Set 7), with the intensity of the substrate with 80 g-VS/L, at pH 7 and temperature 35 °C to compare the same initial substrate concentration and temperature with different pH.

Thus, nine total batch test sets were performed. The batch fermentation was conducted in 235 mL serum bottles by adding 60 mL of enriched inoculum of the mixed culture. The substrate was added at different initial concentrations of

*Chlorella* sp. (40, 60, 80, and 100 g-VS/L) with 63 mL distilled water. Sucrose was used as a positive control assay, representing microalgae and the blank assay was added with only distilled water, i.e., no substrate. 3N Hydrochloric acid and 3N sodium hydroxide were added to adjust to the initial of pH 5 ± 0.1 and 7 ± 0.1, respectively. Then, 22 mL of 0.5 M phosphate buffer was used in each bottle, followed by distilled water to 150 mL working volume.

**Table 9.** Experiment from apply of Taguchi design.

| Batch Run | Conc. (g-VS/L) A | pH B | Temp. (°C ) C |
|---|---|---|---|
| 1 | 40 | 5 | 35 |
| 2 | 60 | 5 | 35 |
| 3 | 80 | 7 | 35 |
| 4 | 100 | 7 | 35 |
| 5 | 40 | 7 | 55 |
| 6 | 60 | 7 | 55 |
| 7 | 80 | 7 | 55 |
| 8 | 80 | 5 | 55 |
| 9 | 100 | 5 | 55 |

The bottles and microalgae were not sterilized before use and the fermentation was followed above. The fermentation broth was taken from each bottle after batch fermentation finished to measure pH and volatile fatty acids (VFAs).

### 3.2.2 Thermophilic source mixed culture for batch acidogenesis fermentation

3.2.2.1 Optimization butyric acid from microalgae

The production of butyric acid from *Chlorella* sp. using thermophilic mixed culture was operated for two temperature 35 °C and 55 °C with the concentration of substrate (20, 40, 60, 80, 100, and 120 g-VS/L). In batch acid production was conducted in a 120 ml total volume and 70 working volume of serum bottle with mixed culture 21 mL of the working volume, bottle was placed in incubator shaker 150 rpm at 35 °C and 55 °C. The serum bottles were capped with tight rubber stoppers and aluminum, and the headspace was flushed with 5 min of nitrogen gas to ensure anaerobic conditions. Hydrogen gas was taken every day of each batch for analyst concentration and cumulative hydrogen volume as well as concentration of volatile fatty acid (VFA).

3.2.2.2 Hydrogen and acid production from pretreated microalgae

Microalgae was investigated capacity of degradation by mixed culture under pretreatment and adding BA medium for acidogenesis fermentation. Microalgae thermolysis pretreatment was operated under temperature of 108 °C for 30 min (Efremenko *et al*., 2012). Initial concentration *Chlorella* sp. 60 g-VS/L was prepared for four set batch (A) unpretreated *Chlorella* sp. with added BA medium (B) pretreated *Chlorella* sp. with added BA medium (C) unpretreated *Chlorella* sp. without BA medium and (D) pretreated *Chlorella* sp. without BA medium. This batch dark fermentation was conducted in a 120 ml total volume and 70 working volume of serum bottle with mixed culture 21 mL of the working volume, bottle was placed in incubator shaker 150 rpm at temperature of 55 °C. The serum bottles were capped with tight rubber stoppers and aluminum, and the headspace was flushed with 5 min of nitrogen gas to ensure anaerobic conditions. Hydrogen gas was taken every 3, 6, 12 or 24 h of each batch until out off gas production about 48 h or 72 h for analyst concentration and cumulative hydrogen volume as well as concentration of volatile fatty acid (VFA).

## 3.3 Batch fermentation for Butanol production

### 3.3.1 Optimizing butanol production from glucose

Comparison conditions of shaking, *clostridium* sp. types and initial glucose concentration on butanol production were operated under an anaerobic digestion temperature 37 °C in batch mode. Shaking condition at 150 rpm was compared with without shaking of initial glucose concentration 20 g-vs/L for butanol production by *clostridium beijerinckii*, which was operated in serum bottle of 500 ml total volume and 300 ml working volume. Meanwhile both *Clostridium butylicum* and *Clostridium beijerinckii* for butanol production were operated from glucose with various initial concentrations of 20, 40, 60, and 80 g-VS/L. Which was conducted on a 120 ml total volume and 70 ml working volume of serum bottle with inoculum 10% (v/v) . The serum bottles were capped with tight rubber stoppers and aluminum, and the headspace was flushed with 5 min of nitrogen gas to ensure anaerobic conditions and placed in an incubator shaker at 150 rpm.

### 3.3.2 Butanol production from microalgae

Microalgae was investigated for butanol production by *Clostridium beijerinckii*. Pretreatment of 40 g-VS/L microalgae was carried out under 108 °C autoclaved for 30 min. While the fermentation was compared from pretreated microalgae and without together with adding TYA medium and without for butanol production. Which was conducted on a 500 ml total volume and 250 ml working volume of serum bottle with inoculum 10% (v/v) placed in an incubator shaker at 150 rpm. The serum bottles were capped with tight rubber stoppers and aluminum, and the headspace was flushed with 5 min of nitrogen gas to ensure anaerobic conditions. The sample of fermentation broth

and gas was taken from each batch for analyzing the composition of gas and concentration of acetone butanol ethanol and VFAs.

### 3.3.3 Butanol production from butyric acid and glucose ratios

The various concentrations of butyric acid and glucose ratios were designed with Taguchi experimental design (Table 10). Three concentration factors of Glucose (0, 2, 4, 8, 10, and 14 g/L), butyric acid (0, 20, and 40 g/L), and inoculum (5, 10, and 15% (v/v) of *Clostridium beijerinckii*) were investigated on a 120 ml total volume and 70 ml working volume of a serum bottle with adding TYA-N (nitrogen-free medium) and placed in an incubator shaker at 150 rpm.

**Table 10.** Experiment from apply of Taguchi design.

| Batch Run | Butyric acid (g/L) | Glucose (g/L) | % *C. beijerinckii* |
|---|---|---|---|
| 1 | 0 | 0 | 5 |
| 2 | 0 | 20 | 10 |
| 3 | 0 | 40 | 15 |
| 4 | 2 | 0 | 5 |
| 5 | 2 | 20 | 10 |
| 6 | 2 | 40 | 15 |
| 7 | 4 | 0 | 10 |
| 8 | 4 | 20 | 15 |
| 9 | 4 | 40 | 5 |
| 10 | 8 | 0 | 15 |
| 11 | 8 | 20 | 5 |
| 12 | 8 | 40 | 10 |
| 13 | 10 | 0 | 10 |
| 14 | 10 | 20 | 15 |
| 15 | 10 | 40 | 5 |
| 16 | 14 | 0 | 15 |
| 17 | 14 | 20 | 5 |
| 18 | 14 | 40 | 10 |

## 3.4 Butyric acid production from first CSTR series

At first continuous fermentation was carried out CSTR at total volume 2 L (working volume 1.35 L) will be used for butyric acid production from continuous dark fermentation. The inoculum will be used mixed culture acidogenic microorganism for dark fermentation with the suable ratio from 3.1.2 and control temperature at 55 °C using water jacket. The varies concentration substrate will load into system from hydraulic retention time (HRT) 6 day and 3 day and take gas production and solution

to analyses of composition of gas and VFA and also characterization of COD pH, Alkalinity. After follow condition at maximum hydrogen and butyric acid production. The scale up to CSTR 10 L with 5 L WV for achieved more effluent with rich butyric acid. Microbial community analysis of fermentation serum after process was investigated.

### 3.5 Butanol production from second CSTR series

CSTR at total volume 10 L (working volume 3 L) will be used for butyric acid production. Pure inoculum *Clostidium beijerinckii* will be used 10, 15% at 35 °C in anaerobic condition. The fermentation process was operated up to optimal from butyric acid and glucose ratio (B/G) from topic (3.3.3) and use TYA medium. The system was operated HRT 2 day. Gas production and solution were analyzed composition of gas, VFA, ABE, OD and pH.

### 3.6 Gas stripping test of $H_2$-$CO_2$ in CSTR

Install sequential distillation column ID size 30 mm. x high 300 mm. connected to the reactor CSTR of working volume 3 L (Figure 5). The system will operate from the condition of 3.4 for butanol production of ABE fermentation process. When the system was steady will flow mixed of 30 % hydrogen and 70 % carbon dioxide from anaerobic digestion of CSTR fermentation system and sprayed through the bottom of the reactor with flow rate of 3.0 L/min. Flow rate of gas stripping will use to study for butanol production and take the sample of butanol from condensed to analyze of VFA, ABE and sugar.

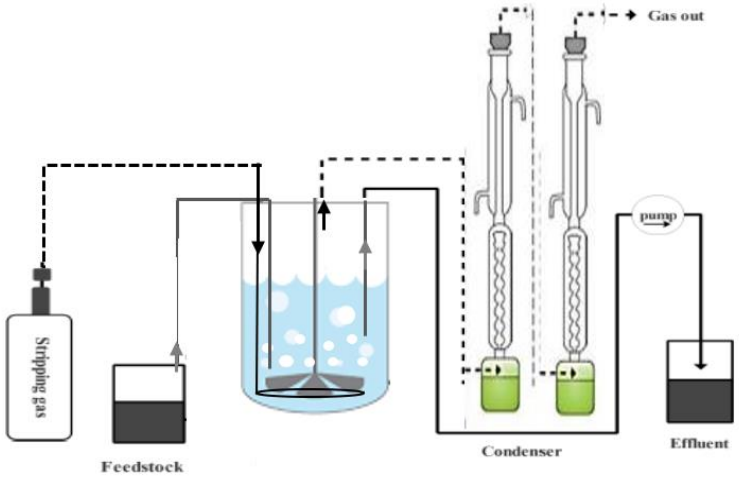



**Figure 5.** Schematic diagram of two stage fermentation by CSTR *in situ* with gas stripping recovery for butanol production.

### 3.7 Microbial community analysis

Microbial community analysis acidogenic anaerobic digestion samples were taken during fermentation of Pretreated microalgae 50 with sucrose 10 at 6-day HRT from First 10 L CSTR series system. The microbial communities were analyzed by the Next Generation Sequencing (NGS) technology. The CTAB/SDS technique was used to extract total genomic DNA from the samples. On 1% agarose gels, the concentration and purity of DNA were measured. DNA was diluted to 1ng/L using sterile water according to the concentration. The barcode was utilized to amplify 16S rRNA/18S rRNA/ITS genes from separate locations. Phusion® High-Fidelity PCR Master Mix was used in all PCR experiments (New England Biolabs). Quantification and qualifying of PCR products by mixing the same volume of 1X loading buffer (containing SYB green) with the PCR products and electrophoresis on a 2% agarose gel for detection. For the next experiments, samples having a bright main strip 79 between 400 and 450 bp were used. Qiagen Gel Extraction Kit was used to purify the mixed PCR products (Qiagen, Germany). The Illumina platform would analyze the libraries created with the NEBNext® UltraTM DNA Library Prep Kit for Illumina and measured by Qubit and Q-PCR.

### 3.8 Analysis methods

The composition of micro-algae, gas production and liquid in batch fermentation system were analyzed. The analysis of total sugar used Anthrone Sulfuric Acid Method. While total solid, volatile solid and Ash were determined in follow by standard methods the procedures described in APHA 1998. Chemical oxygen demand (COD), oil and grease, and total Kjeldahl nitrogen (TKN) were determined in accordance with the procedures described in the Standard Methods (APHA, 1999). The liquid phase of samples were centrifuged at 10,000 rpm at 10 min and filtered through nylon membrane with hole size 0.45 µm. Volatile fatty acid (VFA) was analyzed by GC-FID. Gas volume under operating CSTR fermentation was measured by water displacement method while gas volume from batch fermentation was operated by 5-50 mL wetted glass syringe. Meanwhile composition of hydrogen content was analyzed by a gas chromatography (GC) Shimazu GC-14A model equipped with a thermal conductivity detector with column as stainless steel Shin-carbon (80/100 mesh). The temperatures of 100°C was operated for injection port, oven and detector with argon gas as a carrier gas. Analysis CHONS were analyzed by analyzer CHNS/O analyzer (Thermo Quest Flash EA 1112). However some tests were measured in Taiwan with difference condition as follows. Components of microalgae were analyzed using Ion Chromatography Plasma (ICP) (main minerals analyzed: Na, K, Fe, Mg, Ca, As, Cr, Cd, Cu, Pb, Zn, and Mn). The suspensions were centrifuged at 10,000 rpm for 10 min with control temperature of 4 °C and the supernatants were filtered through nylon membrane with hole size of 0.45 µm into 2 mL vial bottle. Volatile fatty acids (VFAs)

including acetic acid, propionic acid and butyric acid were analyzed using high performance liquid chromatography equipped with SphereClone$^{TM}$ 5 µm ODS (2) 80 Å, LC Column 50 × 4.6 mm, Ea, with stationary phase: C18, UV-detector WL 210 nm Hitachi L7400. The operating temperature was 25 °C with Solution A 90% of 0.5 mM $H_2SO_4$ and Solution B 10% of 99.9% methanol as mobile phase at a flow rate of 0.6 mL/min, and the injection sample volume was 20 µL. The concentration of acetic acid, propionic acid and butyric acid were calculated using the linear equation obtained from various concentrations (0.05, 0.1, 1, 5 and 10 g/L) of standard mixed acid solutions. The concentration of hydrogen gas, nitrogen gas and carbon dioxide gas were analyzed by gas chromatography (GC) equipped with thermal conductivity detector (China Chromatograph Personal GC 1000). Argon gas was used as a carrier gas flow into 1/8 mm ID × 4 m Steel column (Porapak Q 10%). The temperatures of injection, oven and detector were 40, 28, and 40 °C, respectively.

The cumulative hydrogen was calculated from the linear equation of standard gas (hydrogen gas, nitrogen gas, and carbon dioxide). Then, the gas concentration obtained was used to calculate the hydrogen volume from total gas. The hydrogen gas on each day was combined to calculate the cumulative hydrogen (unit: mL-$H_2$). Hydrogen production yield was calculated from cumulative hydrogen by dividing it by the volatile solid of initial substrate concentration (unit: mL-$H_2$/g-VS).

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Acidogenesis stage in batch fermentation

### 4.1.1 Production from mesophilic mixed culture

4.1.1.1 *Chlorella* sp. from Taiwan characterization and batch fermentation of *Chlorella* sp. with and without Endo nutrients

The characterization of *Chlorella* sp. is shown in Table 11. TS of 97.7% consisted of VS of 91% and ash of 6.7% (w/w). TKN, total sugar, oil and grease of 4.6%, 6.1%, and 2.6% (w/w), respectively, were recorded. *Chlorella* sp. microalgae in powder form had high volatile solids and low ash, indicating high organic matter suitable for biodegradation (Lee *et al*., 2015 and Sun *et al*., 2011). Essential elements for bacteria growth such as Mg, Fe, Cu, K, and Co (Bhatnagar *et al*., 2010 and Phukan *et al*., 2011) was also found in *Chlorella* sp. Although Co was not analyzed in this study, it was previously found as one of the main elements in *Chlorella* sp.(Wieczorek *et al*., 2014)

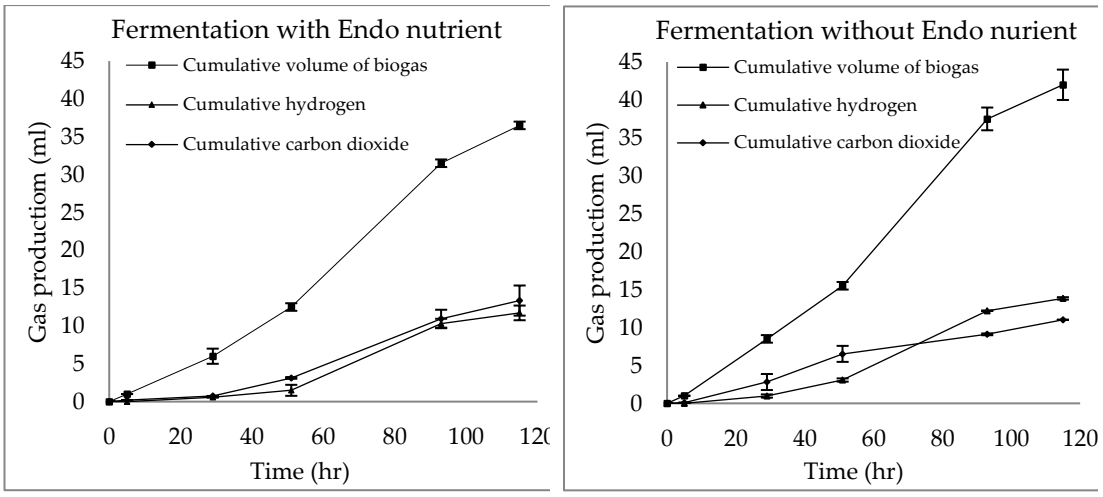
**Table 11.** Characterizations of the *Chlorella* sp. from Taiwan.

| **Character of *Chlorella* sp.** | **Value** | **Character of *Chlorella* sp.** | **Value** |
|---|---|---|---|
| TS % (w/w) | 97.7 | Mg (mg/kg) | 2458.66 |
| VS % (w/w TS) | 91.0 | Ca (mg/kg) | 1919.06 |
| Ash % (w/w TS) | 6.7 | As (mg/kg) | ND |
| Total Sugar % (w/w) | 6.1 | Cr (mg/kg) | 5.22 |
| COD (g-COD/g-VS) | 1.43 | Cd (mg/kg) | ND |
| Oil and grease % (w/w) | 2.6 | Cu (mg/kg) | 7.4 |
| TKN % (w/w) | 4.6 | Pb (mg/kg) | 5.22 |
| Na (mg/kg) | 248.04 | Zn (mg/kg) | 110.1 |
| K (mg/kg) | 7267.19 | Mn (mg/kg) | 48.74 |
| Fe (mg/kg) | 574.41 | | |

ND, not detected.

Cumulative hydrogen generated from batch fermentation with and without Endo nutrients was 12 ± 1.0 and 14 ± 0.2 mL-$H_2$, respectively, under mesophilic temperature (35 °C) and initial pH 7. The compositions of gas production in this study were shown in Figure 6. Hydrogen represented around 46% of total gas generated from batch without Endo nutrients. Meanwhile, the batch fermentation with Endo nutrient had hydrogen content of 40% of total gas generated, i.e., slightly lower than that from

the batch fermentation adding Endo nutrient, demonstrating that *Chlorella* sp. itself contained sufficient nutrients for bacteria growth (Abu-Ruwaida *et al*., 1991). In the fermentation process, microorganisms degrade complex biomass prior to taking up nutrients and trace elements for microbial metabolism activities ( Ghimire *et al*., 2015). The hydrogen production yields of anaerobic fermentation from *Chlorella* sp. with and without nutrient were 14.66 ± 1.2 and 17.29 ± 0.2 mL-$H_2$/g-VS, respectively. The effect of nutrients ($FeSO_4$.$7H_2O$, Urea, and $Na_2HPO_4$) was previously studied by Yossan *et al*. (2012). They found $FeSO_4$.$7H_2O$ Urea and $Na_2HPO_4$ were important nutrients affecting hydrogen production at a proper amount, which were sufficiently contained in the substrate used. Batch dark fermentation of *Chlorella* sp. with Endo nutrients provided lower hydrogen yield than batch fermentation without Endo nutrients, indicating too high amount of important minerals achieved by adding Endo nutrients could be toxic instead of enhancing the microorganisms. Hydrogen yield obtained from the batch fermentation without adding nutrient is in accordance with hydrogen yield result reported by Wieczorek *et al*. (2014), The batch dark fermentation of *Chlorella vulgaris* at different initial concentrations of 5, 10, 20, and 30 g-VS/L at 60 °C without adding nutrients could provide highest hydrogen yield of 19 mL-$H_2$/g-VS at initial concentration of 10 g-VS/L. Therefore, the fermentation of hydrogen production from *Chlorella* sp. without Endo nutrient was selected to further optimize the conditions of pH, temperature and initial concentration for batch anaerobic dark fermentation.




(A) (B)

**Figure 6.** Cumulative gas production (hydrogen and carbon dioxide) from *Chlorella* sp. in batch fermentation with (A) and without (B) Endo nutrient.

4.1.1.2 Hydrogen Production

In dark fermentative system, hydrogen and carbon dioxide are simultaneously generated with volatile fatty acids (VFAs). Butyric acid, a major VFAs generated from dark fermentation, can be converted to butanol by pure culture-based ABE fermentation (Li *et al*., 2007). In this research, *Chlorella* sp. was used as the main carbon source under various conditions during batch fermentation using mixed acidogenic bacteria, resulting in different amounts of gases. Figure 7 shows the cumulative total gas and hydrogen production as well as the concentration of hydrogen in the bio syngas for the nine batch sets using *Chlorella* sp. as a substrate and sucrose for control batch fermentation set. The different types of substrate affected the batch anaerobic dark fermentation (Sangyaga *et al*., 2016). Only Batch Sets 3 and 4 of sucrose fermentation could produce hydrogen of 199 ± 17 mL-$H_2$ (17 mL-$H_2$/g-VS) and 170 ± 14 mL-$H_2$ (10 mL-$H_2$/g-VS), respectively, which were lower than Batch Sets 3 and 4 of *Chlorella* sp. fermentation. As synthetic nutrients were not added for this optimization, higher hydrogen production obtained from *Chlorella* sp. fermentation could be strong evident that nutrients contained in *Chlorella* sp. could enhance hydrogen production. Therefore, not adding synthetic nutrients to the sucrose fermentation led improper nutrient concentrations for bacteria growth metabolism. Nutrients are necessary for enzymatic activities and cell growth in anaerobic digestion process (Ghimire *et al*., 2015). Although nutrients were not adequate, batch sucrose fermentation under condition of 35 °C and initial pH 7 might be suitable for cell growth of microorganisms for hydrogen production (Yun *et al*., 2012). The low initial pH 5 inhibited hydrogen production (Chen *et al*., 2002). The initial concentration of substrate is an important parameter affecting microbial fermentation process (Fabiano *et al*., 2002). The initial concentrations used in this study were 40, 60, 80, and 100 g-VS/L and did not differ significantly ($P_{value} > 0.05$) for hydrogen production under initial pH 7 and operating temperature of 35 °C or 55 °C. Cumulative gas production from *Chlorella* sp. was highest under Batch Set 4 (initial concentration 100 g-VS/L, pH 7 and 35 °C) with total cumulative gas of 646 ± 48 mL and cumulative hydrogen of 330 ± 32 mL-$H_2$. The productions of cumulative gas and cumulative hydrogen at Batch Set 3 were 592 ± 42 mL and 266 ± 25 mL-$H_2$, respectively, obtained under conditions of initial concentration 80 g-VS/L, pH 7 and 35 °C were insignificantly lower than those of Batch Set 4 ($P_{value} > 0.05$). However, higher initial concentration of substrate was more likely to result in more hydrogen production. Yun *et al.* (2012), reported the optimum conditions of initial pH 7.4 at 35 °C with high initial concentration of *Chlorella vulgaris* 76 g/L could produce hydrogen yield of 31.2 mL-$H_2$/g of dry cell weight. Meanwhile, under thermophilic temperature (55 °C) and initial pH 7, the cumulative gas production (hydrogen production) obtained from *Chlorella* sp. at different initial concentration of 40, 60, and 80 g-VS/L (Batch Sets 5–7) were 349 ± 17 mL (107 ± 10 mL-$H_2$), 347 ± 19 mL (141 ± 13 mL-$H_2$) and 352 ± 21 mL (118 ± 12 mL-$H_2$), respectively. At the lower

substrate concentrations, anaerobic dark fermentation caused a lower hydrogen productivity (Jo *et al*., 2008). Increasing the initial concentration to a certain level can increase hydrogen productivity. Because the excess substrate concentration caused accumulation of cell and VFAs concentration, low pH could inhibit acidogenic bacteria in the fermentation process (Yun *et al.*, 2012). Higher hydrogen concentration of 64-73 % than theory (not more than 50-66% as a followed of equation (1) and (2)) was obtained from sucrose fermentation may due to high pressure in the fermenter (Wiebe and Gaddy, 1940), the biogas composition of carbon dioxide was soluble in to the medium which lead the concentration of hydrogen in the fermentation was increased.

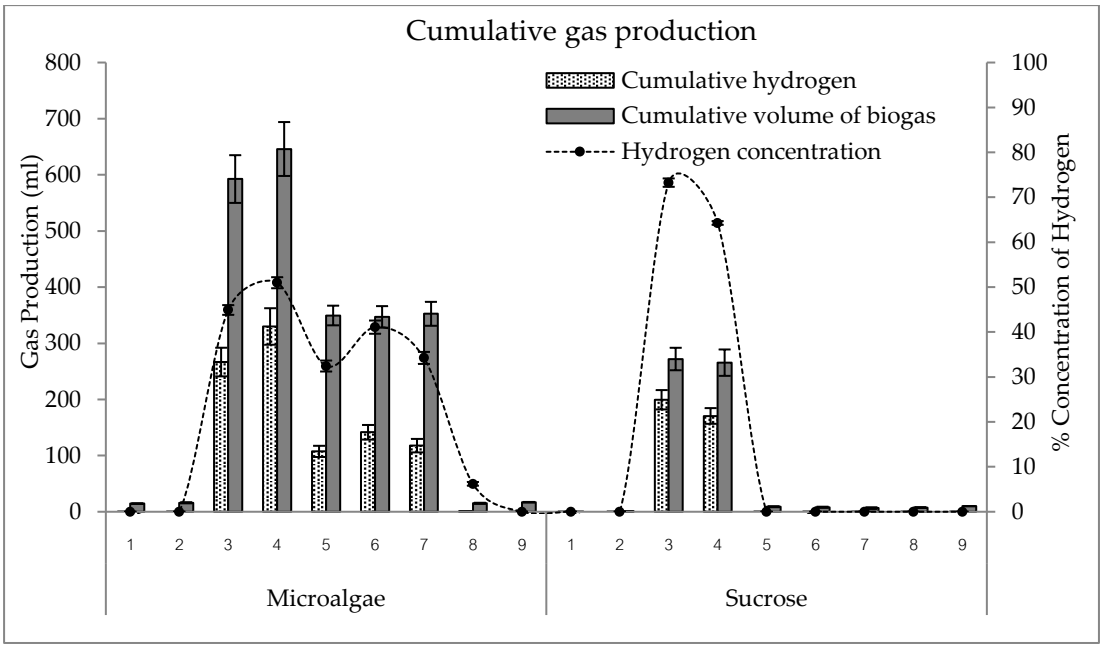



**Figure 7.** Hydrogen production of batch fermentation from microalgae (*Chlorella* sp.) with sucrose as a control set under different conditions for initial concentration, pH, and temperature (Batch Sets 1–9) based on Taguchi design.

Two operating temperatures, mesophilic temperature (35 °C) and thermophilic temperature (55 °C), were employed for fermentative hydrogen production under acidogenic anaerobic dark fermentation in batch mode. Temperature is an essential factor for microbial biodegradation to produce hydrogen and VFAs (Qiu *et al*., 2017 and Valdez-Vazquez *et al*., 2005). As demonstrated in Figure 8, at initial pH 7, hydrogen production yields obtained from *Chlorella* sp. fermentation at initial concentrations of 80 g-VS/L (Batch assay No. 3) and 100 g-VS/L (Batch assay No. 4) at 35 °C were 22.2 ± 2.1 mL-$H_2$/g-VS and 22.0 ± 2.2 mL-$H_2$/g-VS, which are higher than these obtained at 55 °C of 18.9 ± 1.6 mL-$H_2$/g-VS (initial concentration: of 40 g-VS/L of Batch Set 5), 15.9 ± 1.4 mL-$H_2$/g-VS (60 g-VS/L of run 6) and 10.1 ± 1.0 mL-$H_2$/g-VS (initial concentration: 80 g-VS/L of Batch Set 7). This results demonstrate

that the hydrogen production yields obtained from fermentation conducted at mesophilic (35 °C) conditions were higher than those at thermophilic (55 °C) conditions. Although the inoculum was brought from the mesophilic condition, in a mixed culture, some bacteria can grow at high temperatures, in accordance with the research of Stein *et al*. (2017), and De la Rubia *et al.* (2013), who used inoculum from a mesophilic condition for fermentation at thermophilic or even hyperthermophilic temperature. However, Qui *et al.* (2017) studied the effect of temperature, finding that the mesophilic range of 35-40 °C yielded higher hydrogen production. They used active sludge from sewage treatment plant and the mesophilic range was familiar for microbes. In addition, the mesophilic condition (35 °C) was used by Yun *et al.* (2012), for fermentation with *Chlorella vulgaris* at 31.2 mL-$H_2$/g-VS of dry cell weight. Yokoyama *et al*. (2007), presented lower hydrogen production at the temperature of 55 °C than at 35 °C, and the highest hydrogen production yield (392 mL-$H_2$/L of slurry) at 60 °C using cow waste slurry with anaerobic microflora. Thus, the production of hydrogen depends on the microbes used as inoculum. In our research, as the anaerobic sludge used as inoculum was taken from mesophilic operation conditions, operating at higher thermophilic temperature could lead to some of the microorganisms being inhibited, resulting in decreased microbial community diversity and consequently reduced hydrogen productivity (Qui *et al*., 2017 and Yokoyama *et al*., 2007).

The initial pH is another crucial parameter affecting fermentation systems for hydrogen production. It can affect the metabolism in anaerobic acidogenic fermentation pathway of microorganisms (Khanal *et al*., 2004 and Hu *et al*., 2005). The effect of initial of pH 5 and 7 on hydrogen production yield of *Chorella* sp. was tested in various conditions, as shown in Figure 9. The hydrogen production yield obtained when using pH 7 was obviously different from when using pH 5. Hydrogen production from *Chlorella* sp. was successfully obtained by fermentation at pH 7 only for both temperatures and all initial substrate concentrations. The research of Yun *et al*. (2012), obtained the maximum hydrogen production of 30.74 mL-$H_2$/g-dry cell at pH 7 and 35 °C. In addition, the optimum conditions in several studies on anaerobic digestion were different for mixed culture including the pH range. For example, Yun *et al*. (2012) found the optimum condition to be pH 7.4 (after testing pH 4.2–9) at 35 °C. While the optimum condition was pH 6 (after testing pH range 5.5–7.7) at 39 °C in the study by De Gioannis *et al*. (2014). Therefore, the optimal pH for dark fermentation depends on inoculum sources, enrichment of inoculum and type of substrate (Ghimire *et al*., 2015 and Wang *et al*., 2008). However, using initial pH 5 in this research could not produce hydrogen at either 35 °C or 55 °C in mixed-culture fermentation. Although the research of Fang and Liu (2002), studied the pH in the range of 4–7 to produce hydrogen from glucose with a mixed culture, it is found that the pH range 4.5–5.5 can provide good hydrogen yield.

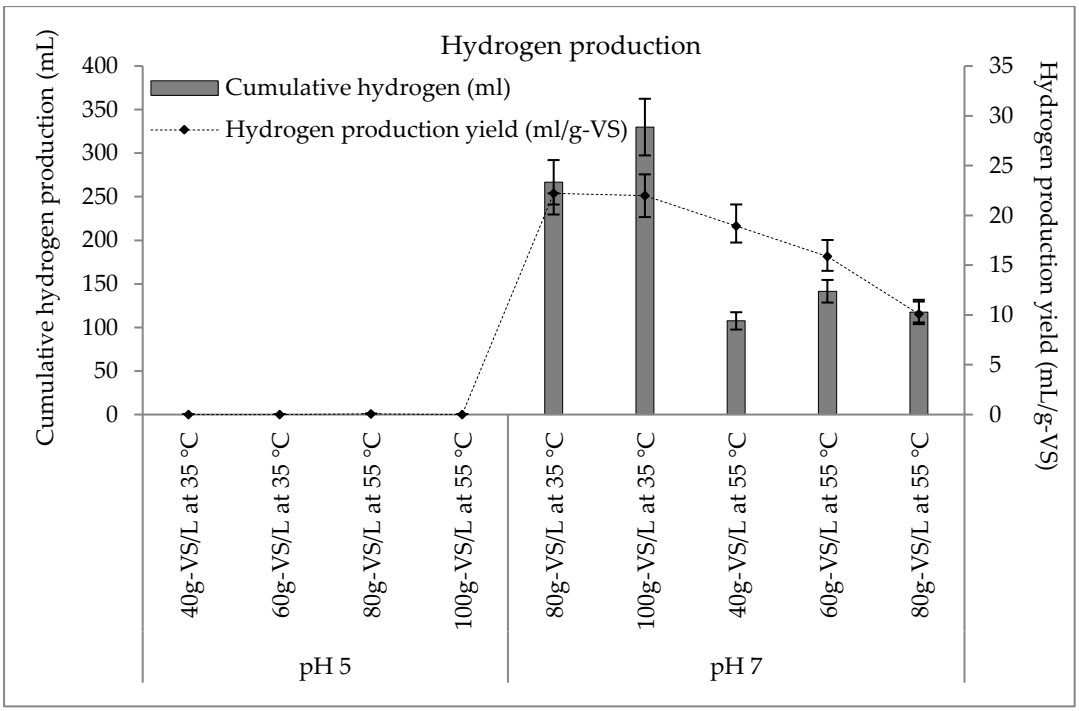
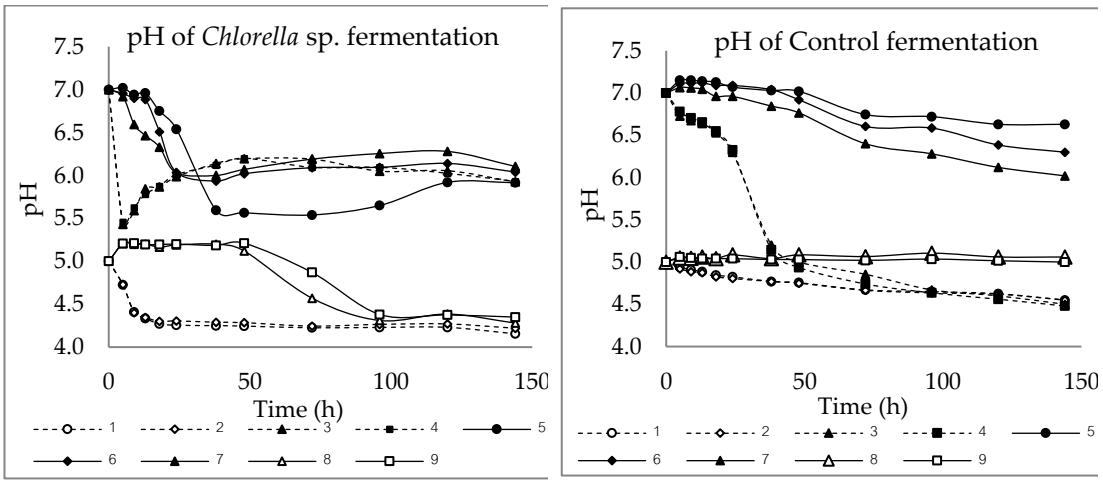



**Figure 8.** Cumulative hydrogen and hydrogen production yields from initial pH of 7 and 5 at different initial concentrations (40-100 g-VS/L) and temperature (35 °C and 55 °C).

On the other hand, this research shows low hydrogen yields when using initial pH 5, possibly due to enzyme deactivation in the metabolic pathway favoring fermentative hydrogen production (Lay, 2000). Initial pH 5 inhibited hydrogen production, while high initial pH 9 declined the lag phase but still yielded low hydrogen production (Chen *et al*., 2002 and Khanal *et al*., 2004). During fermentation, the initial pH 7 at 35 °C was rapidly decreased to pH 5.4 in one day and then slightly increased to pH 5.6–6.2 by day 6 of microalgae fermentation. The initial pH 7 at 35 °C of sucrose rapidly decreased in the first two days to nearly pH 5 and then dropped to pH 4.5 on day 6. Inconstant, low pH 4.1–4.3 on day 6 from the initial pH 5 was found for anaerobic fermentation of microalgae (Figure 9). The results demonstrate that pH control is important for hydrogen production (Lay *et al*., 2000). Furthermore, the pH from initial pH 5 and 7 at 35 °C was more rapidly decrease than fermentation at 55 °C. The acid accumulation was formed of decomposition process by acid-producing bacteria to produce acid which led to the pH reduction in the fermentation system (Stein *et al*., 2017). Especially, clostridia, a kind of acidogenic bacteria, can produce acid along with the production of hydrogen (Yossan *et al*., 2012). The reduction of pH demonstrates that hydrogen production occurred in the fermentation process. However, the decrease in low pH would greatly inhibit the metabolism of bacterial growth and cause a decrease in hydrogen production (Al-Shorgani *et al*., 2018).




(A) (B)

**Figure 9.** pH during fermentation in different batches: (A) *Chlorella* sp., and (B) control.

4.1.1.3 Butyric acid Production

Butyric acid can be used as a precursor for butanol production (Li *et al*.,2018 and Stein *et al*., 2017). The results indicate that organic acid production from *Chlorella* sp. fermentation varied with the different initial concentrations, pH and temperatures by acidogenic mixed cultures. Figure 10 shows the production of butyric acid, propionic, and acetic acids at 35 °C and 55 °C.

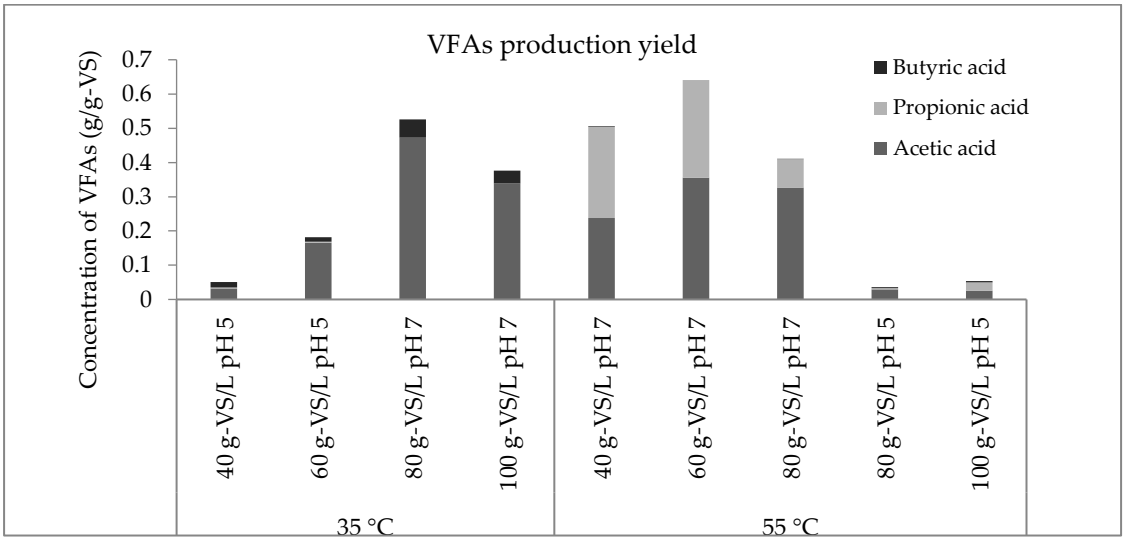



**Figure 10**. Acetic acid, propionic acid, and butyric acid production from batch fermentation.

The fermentation process at the initial pH 5 showed that VFAs production had relatively low amounts, corresponding to very low hydrogen yield for both operating temperatures. On the other hand, fermentation at initial pH 7 and any concentration or temperature can produce VFAs with higher yield. Acetic acid and butyric acid are the main products of the fermentation of microalgae at 35 °C, similar to the main products in the study by Giang *et al.* (2019), at the same temperature, while at operating temperature 55 °C, the main VFAs generated were acetic acid and propionic acid. High butyric acid production in batch fermentation was obtained at initial concentration of 80 and 100 g-VS/L at pH 7 with concentration of butyric acid production of 4.27 g/L (0.05 g/g-VS) and 3.81 g/L (0.04 g/g-VS), respectively (Table 12)

**Table 12.** The hydrogen and VFAs product of *Chlorella* sp. fermentation by the Taguchi method.

| Run | Conc. (g-VS/L) | pH | T (ºC) | Acetic â (g/g-VS) | Propionic â (g/g-VS) | Butyric â (g/g-VS) | Total â (g/g-VS) | $H_2$ Yield (mL/g-VS) | % COD Removal |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 40 | 5 | 35 | 0.03 | 0.00 | 0.016 | 0.05 | 0.0 | 0.0 |
| 2 | 60 | 5 | 35 | 0.17 | 0.00 | 0.013 | 0.18 | 0.0 | 0.0 |
| 3 | 80 | 7 | 35 | 0.47 | 0.00 | 0.053 | 0.56 | 22.2 | 1.0 |
| 4 | 100 | 7 | 35 | 0.34 | 0.00 | 0.038 | 0.38 | 22.0 | 1.0 |
| 5 | 40 | 7 | 55 | 0.24 | 0.27 | 0.002 | 0.80 | 17.9 | 0.7 |
| 6 | 60 | 7 | 55 | 0.36 | 0.28 | 0.000 | 0.81 | 15.7 | 0.7 |
| 7 | 80 | 7 | 55 | 0.33 | 0.08 | 0.001 | 0.46 | 9.8 | 0.4 |
| 8 | 80 | 5 | 55 | 0.03 | 0.00 | 0.004 | 0.03 | 0.1 | 0.0 |
| 9 | 100 | 5 | 55 | 0.03 | 0.02 | 0.003 | 0.07 | 0.0 | 0.0 |

Butyric and acetic acids are obligatory produced along with hydrogen production. Carbohydrate monomer in the form hexose was directed to volatile fatty acids along with carbon dioxide and/or hydrogen, as shown in Reactions (1)–(3) Maaroff *et al*. (2019),:

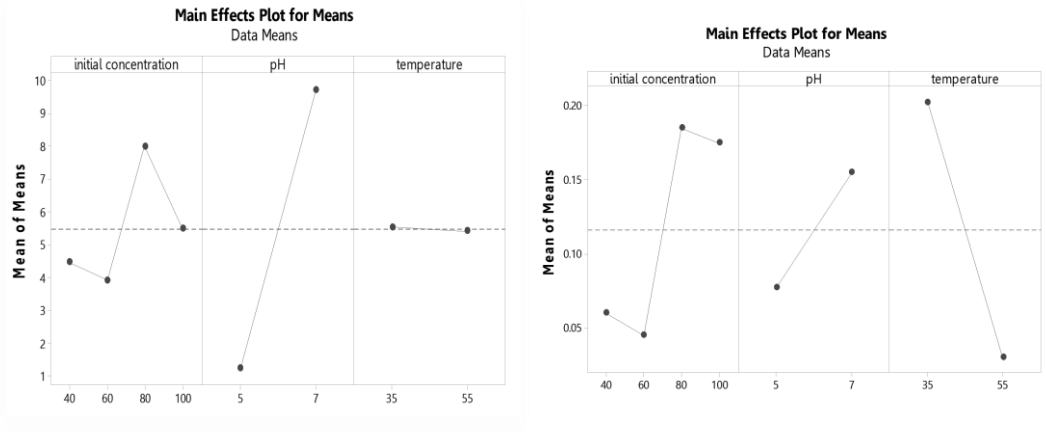
$$C_6H_{12}O_6 + 2H_2O \longrightarrow 4H_2 + 2CO_2 + 2C_2H_4O_2 \quad (1)$$

$$C_6H_{12}O_6 \longrightarrow 2H_2 + 2CO_2 + 2C_4H_8O_2 \quad (2)$$

$$C_6H_{12}O_6 + 2H_2 \longrightarrow 2C_3H_6O_2 + 2H_2O \quad (3)$$

Reaction (1): One mole of glucose can be converted to two moles of acetic acid concurrently with four moles of hydrogen production. Reaction (2): One mole of glucose can produce butyric acid along with only two moles of hydrogen. Reactions (1) and (2) can simultaneously produce butyric and acetic acids along with hydrogen. Reaction (3): Propionic acid production cannot occur simultaneously but two moles of hydrogen with one mole of glucose can further produce two moles of propionic acid. The temperature of 55 °C produced high propionic acid yield in contrast with low hydrogen yield. The reaction for propionic acid does not produce hydrogen. The temperature of 35 °C produced lower total VFAs than 55 °C at initial pH 7, while yielding acetic acid and butyric acid as the main products. According to Giang *et al.* (2019), when the total acid production is increased, the hydrogen production yield will decrease, as the hydrogen synthesis byproduct found in fermented solution contains mostly acetic acid and butyric acid (Ghimire *et al*., 2015). Thus, the temperature of 55 °C yielded not only the highest acetic acid production but also propionic acid as a product, resulting in low hydrogen gas production because propionic acid is produced through a hydrogen consuming reaction (Cappai *et al*., 2015).

In addition, the production of hydrogen and butyric acid under different conditions was analyzed by ANOVA in Minitab. The most significant ($P_{value} < 0.05$) factors for hydrogen and butyric acid production are pH ($P_{value} = 0.001$) followed by temperature ($P_{value} = 0.024$). Therefore, the main factors affecting butyric acid and hydrogen production in *Chlorella* sp. fermentation using mixed cultures were operating temperature and initial pH (Figure 11). In accordance with the review of Ghimire *et al*., (2015), pH and temperature are the most crucial parameters for acidogenesis fermentation. In addition, the biomass used in the fermentation process is decomposed by microorganisms, transforming into volatile fatty acids, mixed carboxylate and cell mass, which remain in the system (Agler *et al.*, 2011), but only COD contributed to hydrogen production is removed from the fermentation system (Kongjan *et al.*, 2010 and Kleerebesem and Van Loosdrecht, 2007). Therefore, the hydrogen yield obtained (Table 12) shows that there was a slight decrease in COD in the system. The hydrogen production yield from microalgae of Batch Sets 3 and 4 could be eliminated, being only about 1%, which represents that, after degradation, the decomposed matter remained in the system, especially in a volatile fatty acid form that can be used as a potential substrate to produce butanol in pure-culture ABE fermentation. Li *et al.* (2018), used rice straw in the fermentation process to produce acids, which were subsequently used to produce butanol in ABE fermentation of *Clostridium beijerinckii* NCIMB 8052.




(A) (B)

**Figure 11.** Response table from three factors of initial concentration of substrate, pH and temperature for means of: (A) hydrogen production; and (B) butyric acid production.

### 4.1.2 Production from Thermophilic mixed culture

4.1.2.1 *Chlorella* sp. from China characterization

The characterization of *Chlorella* sp. is shown in Table 14. TS of 91.7% consisted of VS of 80.91% and ash of 10.75% (w/w). Crude protein Crude fat Crude fiber Carbohydrates of 55.52%, 4.67%, 2.74% and 31.41% (w/w of TS), respectively, were recorded. This *Chlorella* sp. microalgae in powder had high crude protein follow with carbohydrates composition demonstrate that it was suitable for biodegradation (Lee *et al*., 2015; Sun *et al*., 2011). Corresponding with Phanduang *et al*. (2017), The major component of biomass is protein followed by carbohydrate. In addition, *Chlorella* sp. was reported that many of amino acid with glutamic acid to be a major. However the main organic bast composition of carbon (45.260±0.020 %(W/W)) hydrogen (6.829±0.174 %%(W/W)) oxygen (31.353±0.346 %%(W/W)) nitrogen (7.896±0.153 %%(W/W)) and sulfur (0.614±0.002 %%(W/W)) were analyzed by analyzer CHNS/O analyzer.

4.1.2.2 Hydrogen and acid production from microalgae

The fermentation was produced from glucose to be a control (Control, C) and microalgae as a substrate (Sample, S) by mixed culture form thermophilic source, it was used as inoculum for incubator under mesophilic (M) 35 °C and thermophilic (T) 55 °C for batch fermentation. The initial substrate concentration 20, 40, 60, 80, 100, and 120 g-VS/L (1-6) were used for fermentation. The results showed that hydrogen yield was the first yield obtained from fermentation that could be analyzed during the experiment.

**Table 13.** Characterizations of the *Chlorella* sp. from China.

| **Parameters** | ***Chlorella* sp.** | **unit** |
|---|---|---|
| Total solid | 91.7±0.004 | %(W/W)[a] |
| Volatile solid | 80.91±0.08 | %(W/W)[b] |
| Ash | 10.75±0.08 | %(W/W)[b] |
| Crude protein | 55.52 | %(W/W)[b] |
| Crude fat | 4.67 | %(W/W)[b] |
| Crude fiber | 2.74 | %(W/W)[b] |
| Carbohydrates | 31.41 | %(W/W)[b] |
| Total sugar | 9.12±0.194 | %(W/W)[a] |
| COD | 24.3±4.06 | g/L[a] |
| Nitrogen | 7.896±0.153 | %(W/W) [a] |
| Carbon | 45.260±0.020 | %(W/W) [a] |
| Hydrogen | 6.829±0.174 | %(W/W) [a] |
| Sulfur | 0.614±0.002 | %(W/W) [a] |
| Oxygen | 31.353±0.346 | %(W/W) [a] |

a Micro-algae substrate
b Dry micro-algae

It was found that, highest hydrogen production at 35 °C was achieved from sugar control (CM) set at a concentration 60 g-VS/L with the highest yield 341.8±9.8 mL-$H_2$ (81.4±2.3 mL-$H_2$/g-VS). Meanwhile, the maximum hydrogen production from microalgae (SM) fermentation at 35 °C of 195.5±8.2 mL-$H_2$ (46.5±1.9 mL-$H_2$/g-VS) was observed at the same concentration of highest sugar control (Figure 12). The lowest concentration 20 g-VS/L was produced low hydrogen 159±5.8 mL-$H_2$ (113.5±4.1 mL-$H_2$/g-VS). The hydrogen production may probably be produced under non-pretreatment of microalgae. As reported of Wieczorek *et al*. (2014), using *Chlorella* sp. without pretreatment for hydrogen fermentation, it was produced 19 mL-$H_2$/g-VS. In opposite of the investigated hydrogen production for fermentation was compared under temperature 55 °C. Both hydrogen productions of sugar as a control (CT) and microalgae as a substrate (ST) were produce lower production gas than temperature of 35 °C. The concentration ranges 20-120 g-VS/L of microalgae can be produced hydrogen production ranges of 49.2±3-129.4±12.9 mL-$H_2$. Which highest initial

microalgae concentration of 120 g-VS/L (ST6) was promoted a maximum hydrogen production of 129.4±12.9 mL-$H_2$ (15.6±1.5 mL-$H_2$/g-VS). while 14.9±1.4 and 16.2±1.6 mL-$H_2$/g-VS at high concentrations of 80 g-VS/L (ST4) and 100 g-VS/L (ST5), respectively were investigated.

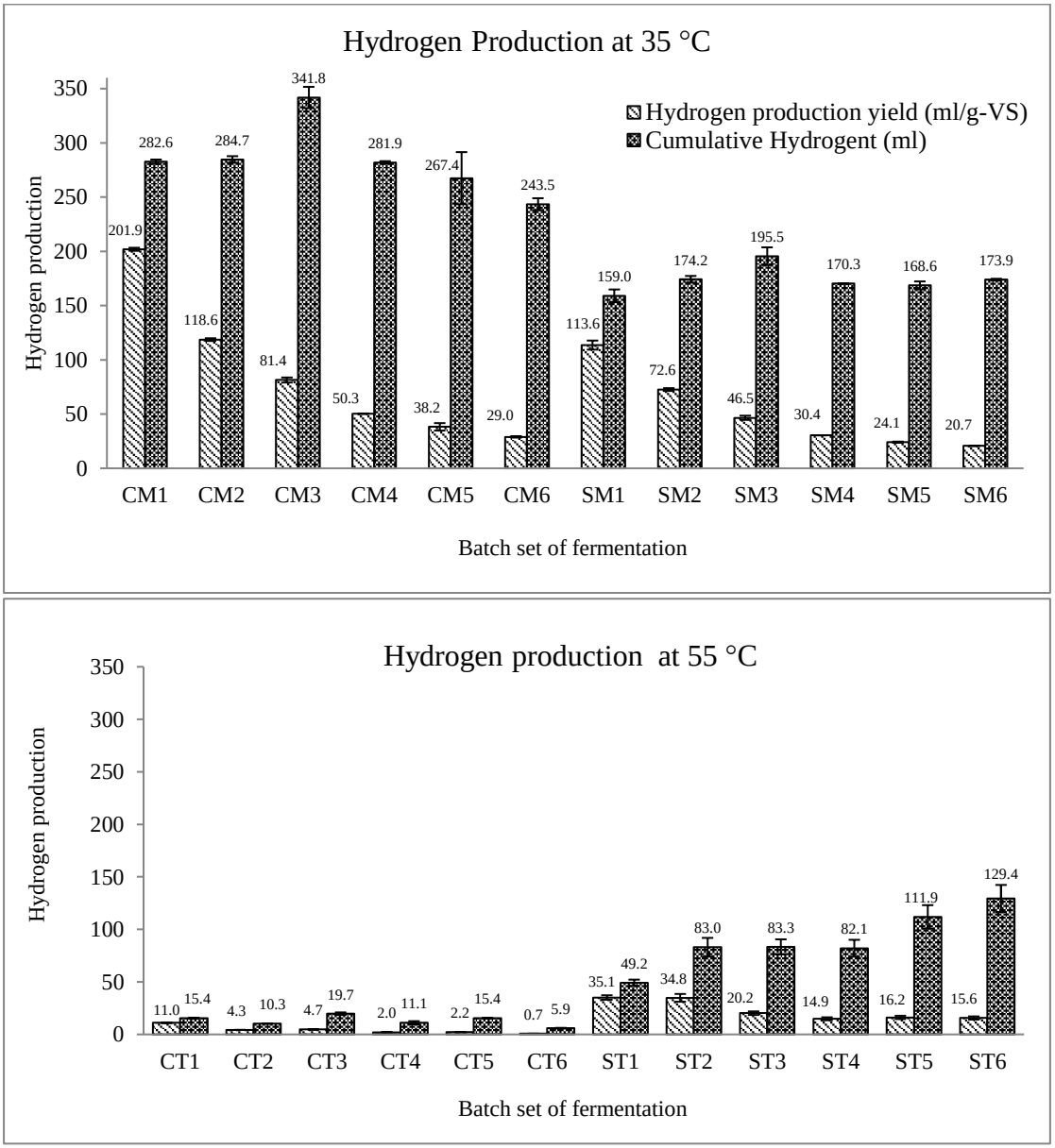



**Figure 12.** Hydrogen production at varies initial concentration (g-VS/L) 20, 40, 60, 80, 100, and 120 of *Chlorella* sp. on operated temperature (A) 35 °C and (B) 55 °C by mixed culture.

In contrast, at low microalgae concentrations at 20 g-VS/L can be produced lowest hydrogen of 49.2±3.0 mL-$H_2$, but the hydrogen production yield was 35.1±2.1 mL-$H_2$/g-VS higher than high substrate concentration at thermophilic condition.

Although high hydrogen yield was obtained at temperature of 35 °C fermentation condition. However, the acid production in form of acetic acid may have higher among of hydrogen production than when produced butyric acid form. Because the reaction of hydrogen production from fermentation that was produced together with acetic acid, the product of hydrogen can be achieved higher than reaction of butyric acid production as a followed of equation (1) and (2). Furthermore the fermentation at 55 °C still tends to yield hydrogen in substrate concentrations as high as 120 g-VS/L. Demonstrate that, the fermentation of 55 °C using inoculum mixtures was able to promote biomass degradation in the biodegradation process and increase the product from acidogenic fermentation process (Mamimin *et al*., 2012) (Kongjan *et al*., 2010 and Yossan *et al*., 2012) However, Inoculum obtained from different sources can affect hydrogen yield and volatile fatty acid type (Wang and Wan, 2009; Ghimire *et al*., 2015). Moreover, acidogenesis fermentation needs to be investigated butyric acid before indicating optimum conditions.

Analysis results of volatile fatty acids (acetic acid, propionic acid, and butyric acid) were analyzed by GC-FID from the fermenter, samples were collected at 72 hours after fermentation. Both temperature conditions gave the highest yield of butyric acid, when compared with acetic acid and propionic acid (Figure 13). It also provides the lowest concentration of propionic acid, which is an advantage. High concentrations of propionic acid can inhibit microbial growth in dark fermentation and not promote hydrogen production or even conversion to butanol production (Cappai *et al*., 2015). Most of the acid yield was in the form of acetic and butyric acid (Giang *et al*., 2019). However, at 35 °C the acetic acid yield was relatively high. Which, at the same time, produces high butyric yields as well, the maximum butyric acid production of 9.13±0.94 g/L was obtained 60 g-VS/L, which may have caused a higher hydrogen yield than 55 °C fermentation, with the high yield in form of butyric acid only. These results at 55 °C tended to produce higher butyric acid production, highest at 10.67±1.35 g/L at 80 g-VS/L substrate concentration. The highest butyric acid productions of temperature of 35 °C and 55 °C were 49% and 45% of butyric acid production increase from butyric acid that produced from 20 g-VS/L substrate. Microalgae fermentation at concentrations of 20, 40, 60, 80, 100, and 120 g-VS/L were produced butyric acid production (g/L) of 4.51±0.16 (0.226±0.008 g/g-VS), 5.74±0.76 (0.144±0.019 g/g-VS), 9.13±0.94 (0.152±0.016 g/g-VS) 8.01±0.73 (0.100±0.009 g/g-VS), 6.10±0.17 (0.061±0.002 g/g-VS) and 6.88±0.93(0.057±0.008 g/g-VS), respectively, temperature condition of 35 °C. While, temperature condition of 55 °C for fermentation, butyric acid production (g/L) were obtained of 4.85±0.19 (0.243±0.009 g/g-VS), 4.54±0.08 (0.113±0.002 g/g-VS), 4.93±1.06 (0.082±0.018 g/g-VS), 10.67±1.35 (0.133±0.017

g/g-VS), 8.31±1.58 (0.083±0.016 g/g-VS), and 10.44±1.46 (0.087±0.012 g/g-VS), respectively. While butyric acid production yield of theory of 0.98 g/g-glucose was calculated from equation (2).

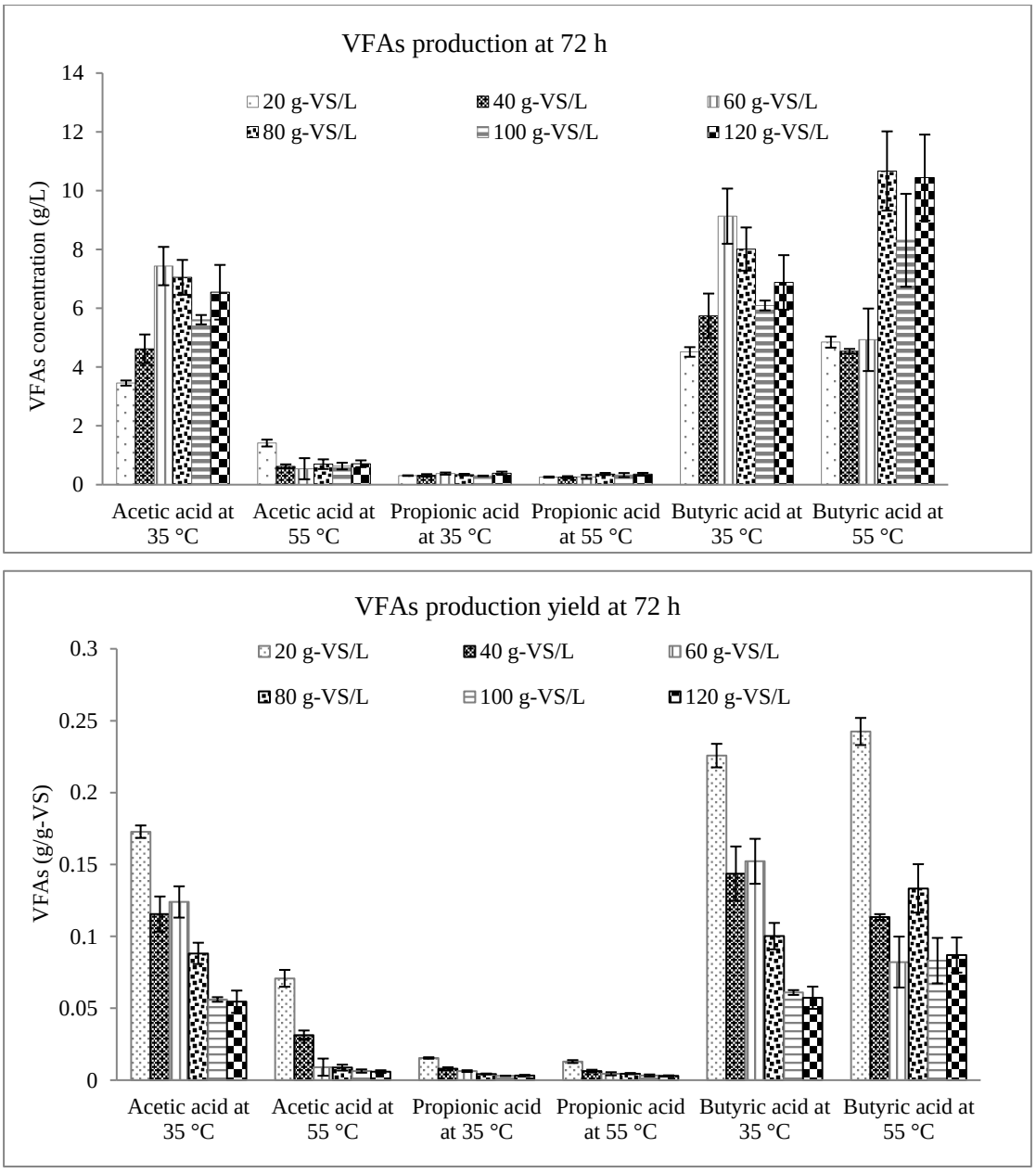



**Figure 13.** VFAs at varies initial concentration (g-VS/L) 20, 40, 60, 80, 100, and 120 of *Chlorella* sp. of on 35 °C and 55 °C was demonstrated of (A) VFA production and (B) VFA production yield by mixed culture.

The results showed that 60 g-VS/L of substrate produced butyric acid production yield with the same range when compared to 80-120 g-VS/L of substrate concentration. However, from the above experimental results of using *Chlorella* sp, the

high concentration of 120 g-VS/L microalgae can be used as substrate in the anaerobic fermentation process with mixed cultures. However, too high an initial concentration may result in the inhibition of microbial biodegradation (Wu and Shi, 2008).

4.1.2.3 Hydrogen and acid production from pretreated microalgae

Microalgae at initial concentration *Chlorella* sp. 60 g-VS/L was prepared for acidogenesis fermentation. Batch fermentation sets were operated for four set batch fermentation with (A) unpretreated *Chlorella* sp. and added BA medium (B) pretreated *Chlorella* sp. and added BA medium (C) unpretreated *Chlorella* sp. without BA medium and (D) pretreated *Chlorella* sp. without BA medium. Figure 14 show the amount of hydrogen gas production during each period, Hydrogen yield concentration and cumulative hydrogen content. Microalgae pretreated with and without BA medium can be produced 224.43±0.42 mL-$H_2$ and 193.48±11.65 mL-$H_2$ (46.76±0.09 mL-$H_2$/g-VS and 40.31±2.45 mL-$H_2$/g-VS respectively). Comparing non pretreated microalgae with and without BA medium were produced 175.09±7.76 mL-$H_2$ (36.48±1.62 mL-$H_2$/g-VS) and 138.12±6.29 mL-$H_2$ (28.78±1.31 mL-$H_2$/g-VS) in the fermentation process.

The fermentation process of non-added BA medium shows the importance of nutrients, if insufficient or suitable for the microbial population, can affect the microbial degradation process (Yossan *et al*., 2012). The cumulative hydrogen production was gradually decreased on without nutrient adding in batch fermentation, with the final cumulative hydrogen production was obtained at 48 h. Cumulative hydrogen production time of without BA medium was used shorter time for hydrogen production than the fermentation of BA medium solution adds, at 72 h.

In addition, the yield of butyric acid as shown in Figure 15, The resulting of butyric acid production was corresponding to the hydrogen production. The condition with added BA medium and pretreated microalgae can produce a maximum butyric acid concentration of 11.42±0.66 g/L, While without BA medium yielded 8.22±0.55 g/L of butyric acid. As opposed to the unpretreated microalgae condition, under using BA medium and without BA medium added condition were produced less butyric acid production of 5.29±0.09 g/L and 5.14±0.25 g/L, respectively. From the experimental results, it was clearly seen that the microalgae pretreatment *Chlorella* sp. before fermentation can promote both hydrogen and butyric acid production (Efremenko *et al*., 2012; Phanduang *et al*., 2017 and Lunprom *et al*., 2019).

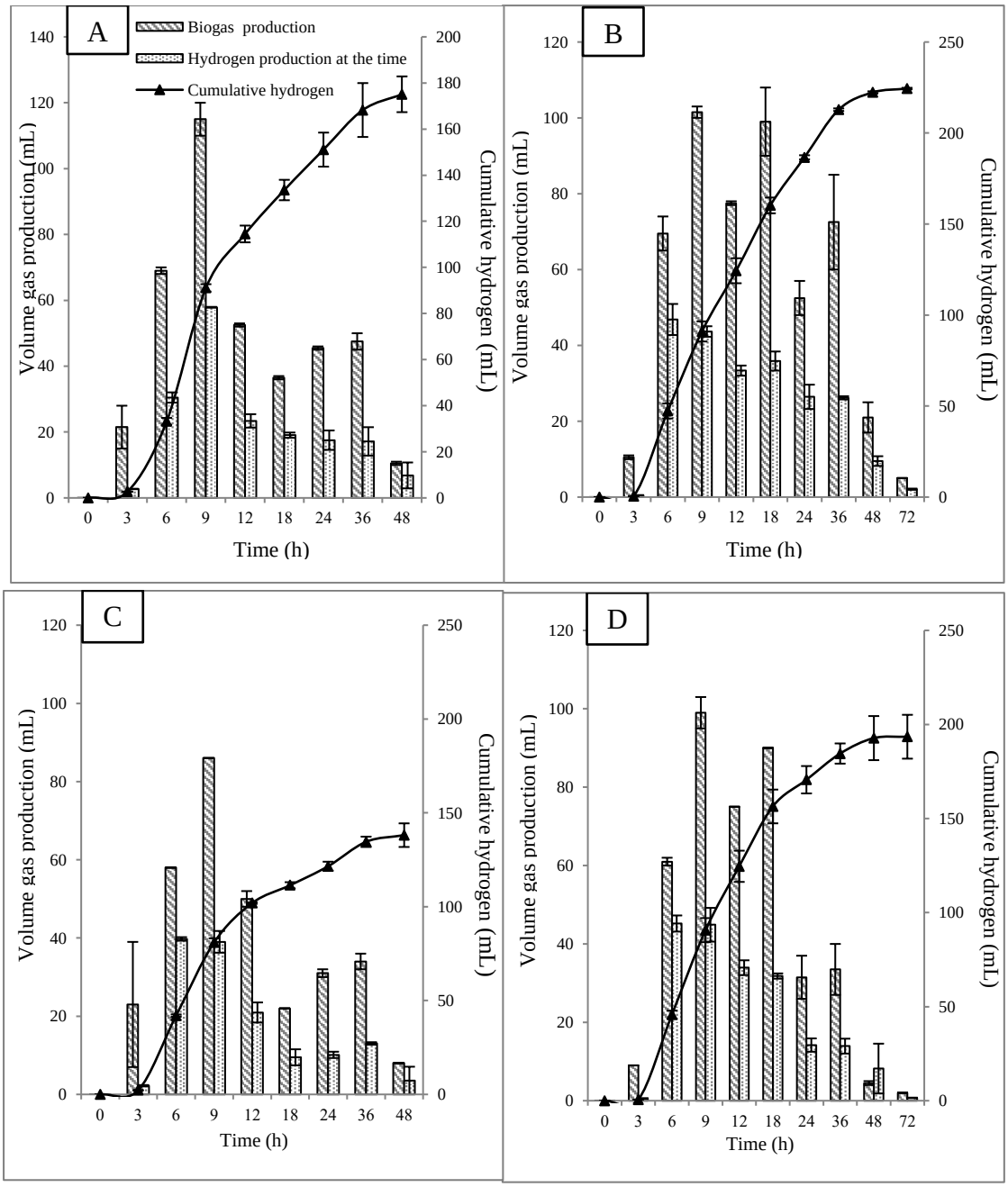



**Figure 14.** Biogas production hydrogen production and cumulative hydrogen from initial concentration 60 g-VS/L of (A) unpretreated *Chlorella* sp. with added BA medium (B) pretreated *Chlorella* sp. with added BA medium (C) unpretreated *Chlorella* sp. without BA medium (D) pretreated C*hlorella* sp. without BA medium at 55 °C by mixed culture.

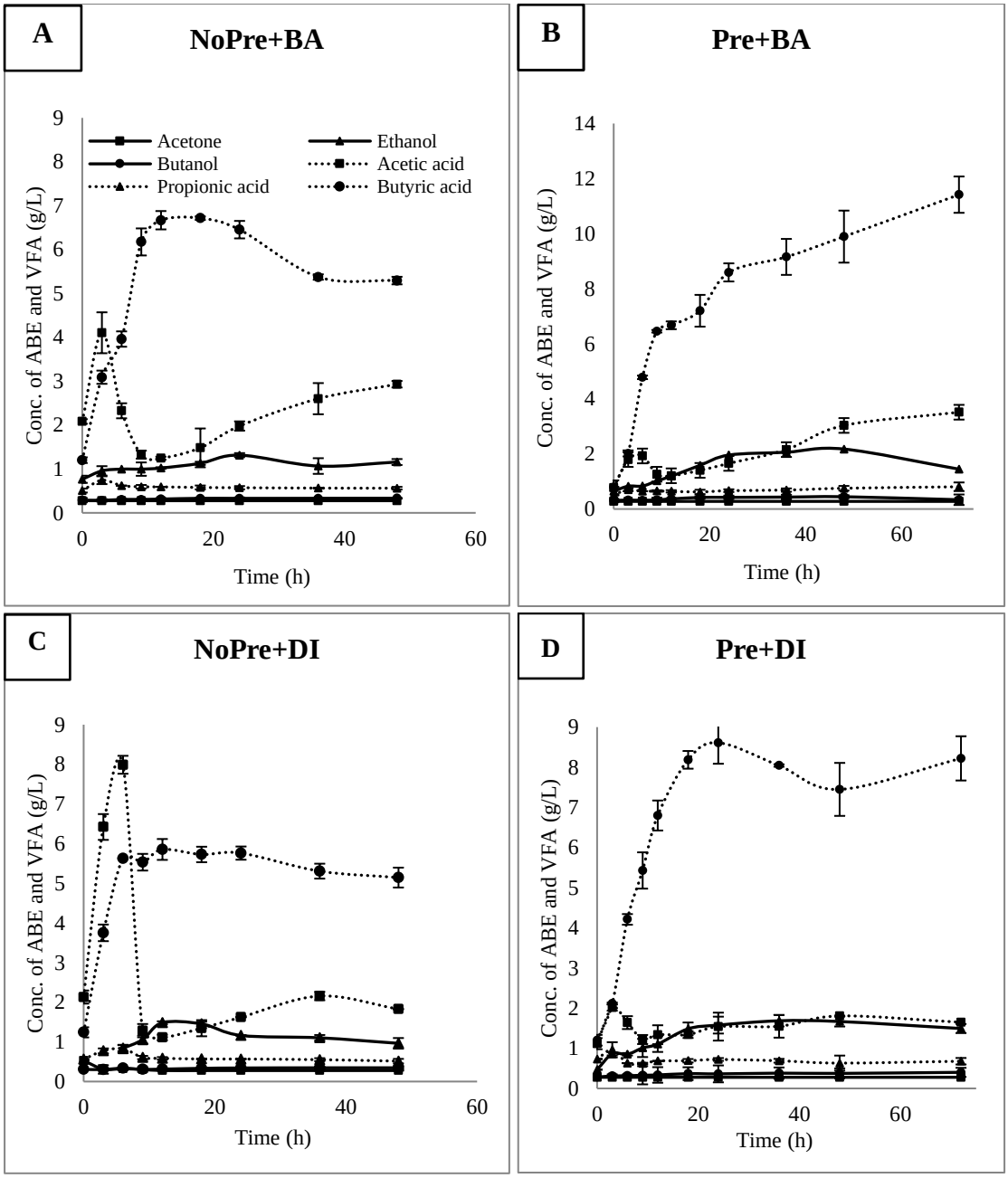



**Figure 15.** VFA production from initial concentration 60 g-VS/L of (A) unpretreated *Chlorella* sp. with added BA medium (B) pretreated *Chlorella* sp. with added BA medium (C) unpretreated *Chlorella* sp. without BA medium (D) pretreated *Chlorella* sp. without BA medium at 55 °C by mixed culture.

Therefore, the conclusions from the study of butyric acid production factors by mixed cultures from different sources. It was found that pH control was an important factor for butyric acid production. The main bacteria of mixed culture for acidogenesis fermentation have pH control in fermentation as a necessary factor for acid production

type. Lactic acid and acetic acid occurred from the pH fermentation control range below 6, while if the pH was higher but not more than 7.2, the yield had a higher concentration in form of butyric acid (Dudek *et al*., 2022). Meanwhile, the temperature factor of fermentation depends on the type of inoculum. Therefore, groups of microorganisms coming from different temperature sources had better metabolism capacity and proliferating abilities in different optimum temperature ranges (Chi *et al*., 2018 and Jiang and Xu, 2016). So, the condition of the acidogenesis fermentation step experiment with the mixed culture at thermophilic temperature of 55 °C from the thermolysis pretreated microalgae of 108 °C with the addition of BA medium will be used as a condition for continuous fermentation.

## 4.2 Butanol production on batch fermentation

### 4.2.1 Optimizing butanol production from glucose

4.2.1.1 Characteristics of *Clostridium beijericnkii* and butanol production effect of with and without shaking on batch fermentation

*Clostridium beijerinckii* enriched fermentation in TYA medium after incubated for 24 h was characterized. *Clostridium* sp. characterizations can be operated by a light microscope from 1000x (Figure 16). This bacterium was tested with gram strain which it can be absorbed color of crystal violet as gram-positive, rod-shaped, this bacteria is endospore forming bacteria It was found that the bacteria that appeared were characteristic of pure bacteria. There were no other cellular characteristics, so it could mean there was no contamination of other microorganisms as well as can be extended and used in further experiments.

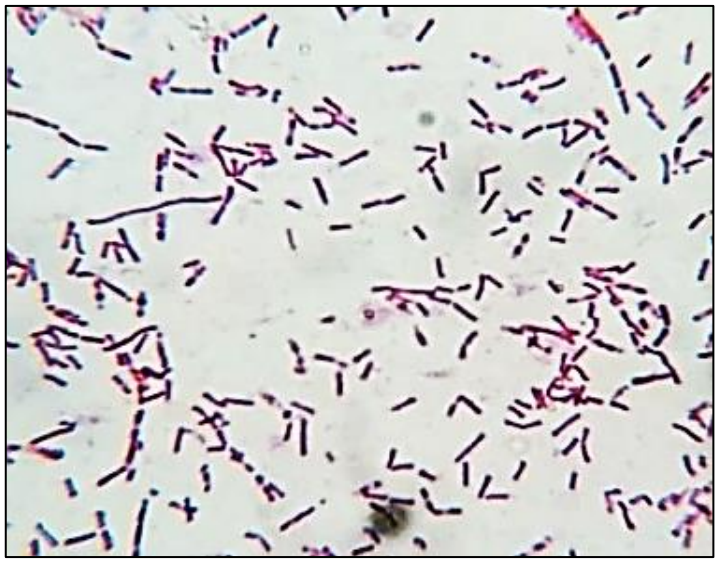


**Figure 16.** *Clostridium beijerinckii* gram stained.

Comparison of butanol yields obtained from the experimental set shaken at 150 rpm and unshaken under temperature of 35 °C by *Clostridium beijerinckii*. From tracking the yield butanol from shaking set, found that productivity increased into a steady state at 24 h. The butanol production 5.59 g/L was produced at 72 h (Figure 17). While the butanol yield in the unshaken experimental set showed a gradually increase of butanol production at the end of the 96-hour experiment, which butanol was produced lower than shaking of 4.1 g/l.

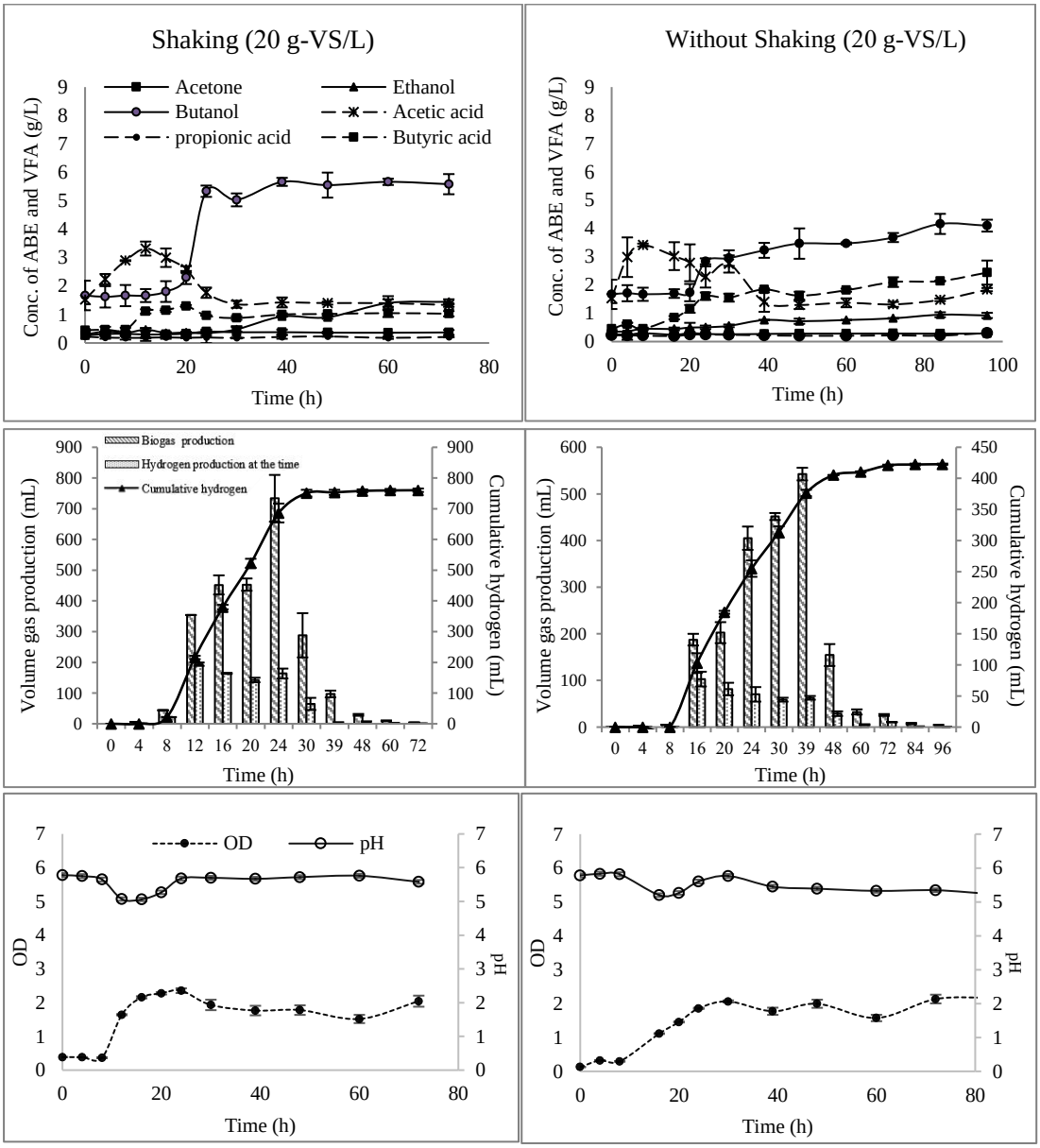



**Figure 17.** Follow-up of (A) VFA-ABE production (B) gas production and (C) OD and pH of with and without shaking comparison on batch fermentation of glucose 20 g-VS/L by *Clostridium beijerinckii*.

4.2.1.2 Butanol production from varies concentration of glucose and inoculum

*Clostridium butylicum and Clostridium beijerinckii* cells were prepared from 3.1.3 batch fermentation serum bottle to examine cell characteristics (Figure 17) and cell growth characteristics of both types on 20 g/L initial glucose concentration. The growth cells of both *Clostridium butylicum and Clostridium beijerinckii* was showed at Figure 18, which was demonstrated that both of cell growth were enter to steady state of *Clostridium beijerinckii* at 24 h with higher optical density than *Clostridium butylicum* while longer time to steady stage about 36 h and lower optical density of *Clostridium butylicum* than *Clostridium beijerinckii*. The optimum OD is in the range of 1.3-1.5. (Maiti *et al.*, 2016) which at OD around 1.5 of *Clostridium beijerinckii* was growth faster at 12 (OD=1.593 h; $3.0*10^7$ cells) than *Clostridium butylicum* at 16 h (OD=1.625; $8.1*10^7$cells).

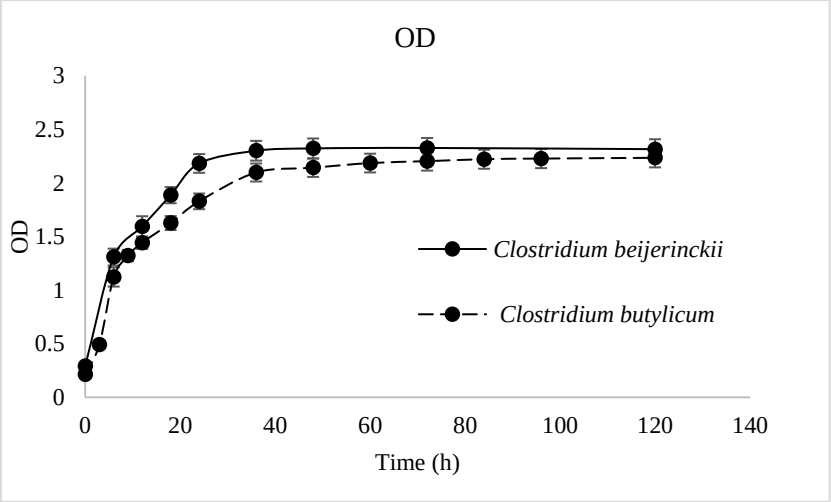



**Figure 18.** Follow-up of OD from glucose 20 g-VS/L by *Clostridium butylicum* TISTR 1032 and *Clostridium beijerinckii* ATCC10132.

The production of acetone butanol and ethanol on varies initial glucose concentration was operated by *Clostridium butylicum and Clostridium beijerinckii* as inoculum. The results are shown in Table 15. The highest butanol production of 2.95 g/L of *Clostridium butylicum* was operated under initial concentration of 20 g-VS/L. While *Clostridium beijerinckii* produced the highest of 10.17 g/L at 40 g-VS/L, however initial glucose concentration 10.16 g/l butanol production was produced from 60 g-VS/L initial glucose concentration nearby with concentration before.

**Table 14.** ABE production *from Clostridium butylicum* and *Clostridium beijerinckii* with difference initial concentration of glucose.

| | *Clostridium butylicum* TISTR 1032 | | | | *Clostridium beijerinckii* ATCC10132 | | | |
|---|---|---|---|---|---|---|---|---|
| Glucose initial concentration (g/L) | 20 | 40 | 60 | 80 | 20 | 40 | 60 | 80 |
| Acetone production (g/L) | 0.056 ±0.002 | 0.055 ±0.007 | 0.061 ±0.003 | 0.058 ±0.002 | 2.17 ±0.05 | 3.11 ±0.06 | 3.31 ±0.14 | 3.43 ±0.06 |
| Ethanol production (g/L) | 0.886 ±0.056 | 0.754 ±0.041 | 0.581 ±0.031 | 0.509 ±0.028 | 0.09 ±0.00 | 0.16 ±0.02 | 0.15 ±0.01 | 0.14 ±0.03 |
| Butanol production (g/L) | 2.957 ±0.095 | 2.563 ±0.085 | 2.454 ±0.080 | 2.157 ±0.048 | 6.02 ±0.04 | 10.17 ±0.22 | 10.16 ±0.19 | 9.88 ±0.08 |
| ABE production (g/L) | 3.899 | 3.372 | 3.096 | 2.724 | 8.28 | 13.44 | 13.62 | 13.45 |
| Butanol yield (g-butanol/g-VS) | 0.148 ±0.005 | 0.064 ±0.002 | 0.041 ±0.001 | 0.027 ±0.001 | 0.30 ±0.002 | 0.254 ±0.006 | 0.169 ±0.003 | 0.124 ±0.001 |
| Initial pH | 6.02 | 5.95 | 5.92 | 5.93 | 5.94 | 5.9 | 5.8 | 5.81 |
| Final pH | 4.82 | 4.7 | 4.59 | 4.59 | 6.07 | 5.18 | 5.07 | 5.06 |

Demonstrated that *Clostridium beijerinckii* has efficiency for glucose using at 40 g-VS/L to produce butanol production yield of 0.254 g/g-glucose. If used glucose more than 40 g, the butanol production yield may likely decrease. According to the trend from the results, butanol production of both bacteria was decreased at initial substrate concentration more than 80 g-vs/L (Figure 19). pH and OD of all initial substrate concentration from *clostridium beijerinckii* were followed up until 120 h (Figure 20). However, the butanol production of *Clostridium beijerinckii* produced higher butanol than production of *Clostridium butylicum* as well as acetone and ethanol of solvent phase. Due to *Clostridium butylicum* is the main bacteria for acid production which high yields and acid concentration tolerance (Dudek *et al*., 2022). While *Clostridium beijerinckii* as a solventogenic strain can produce solvent better than acid when compare with *Clostridium butyricum* and *Clostridium tyrobutyricum* strains (Drahokoupil and Patáková, 2020).

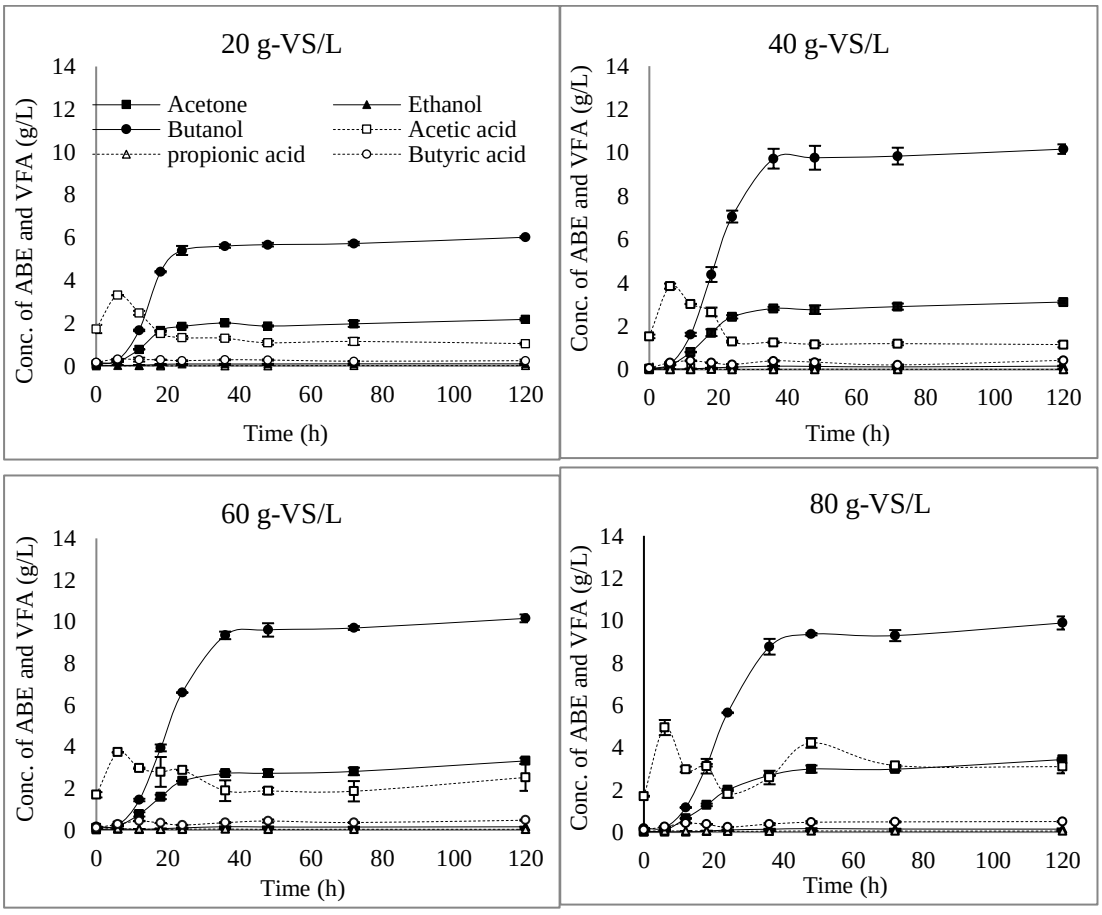



**Figure 19.** VFA and ABE production from difference initial concentration of glucose 20 g-VS/L, 40 g-VS/L, 60 g-VS/L and 80 g-VS/L by *Clostridium beijerinckii.*

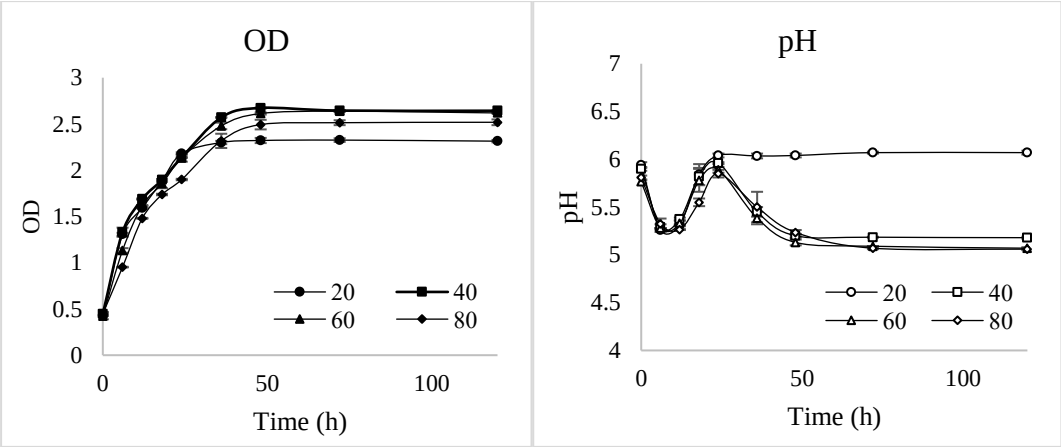



**Figure 20.** Follow-up of (A) OD and (B) pH from glucose 20 g-VS/L 40 g-VS/L 60 g-VS/L and 80 g-VS/L by *Clostridium beijerinckii*.

### 4.2.2 Butanol production from microalgae

Microalgae (*Chlorella* sp.) was investigated for butanol production at optimum concentration 40 g-VS/L by *Clostridium beijerinckii* . The initial concentration was chosen from high butanol production on previous initial glucose concentration experiment. The microalgae were pretreated by autoclave at temperature of 108 °C 30 min. Including with TYA medium was compared on batch fermentation for butanol production (Figure 21).

From each set of experiments, it was found that the pretreated microalgae produced significantly better yields than those without the pretreatment compared to both the nutrient and without nutrient added sets. The highest butanol production was carried out under microalgae pretreatment condition included TYA medium of 2.30 g/L butanol production at final pH 5.13 by *Clostridium beijerinckii* (Figure 22-23). While microalgae pretreatment without TYA medium produced butanol of 1.80 g/L at final pH 4.68. Under the condition non-pretreatment, the butanol production of 1.07 g/L with TYA and 1.08 g/L with DI water were produced. The butanol production yield also higher at pretreatment microalgae than non-pretreatment (Figure 22). However, using DI water represented TYA of fermentation was effect to pH, final pH of butanol fermentation was lower than used TYA medium, 4.61-4.68 of non-adding TYA medium, 5.13-5.29 of adding TYA medium (Figure 23).

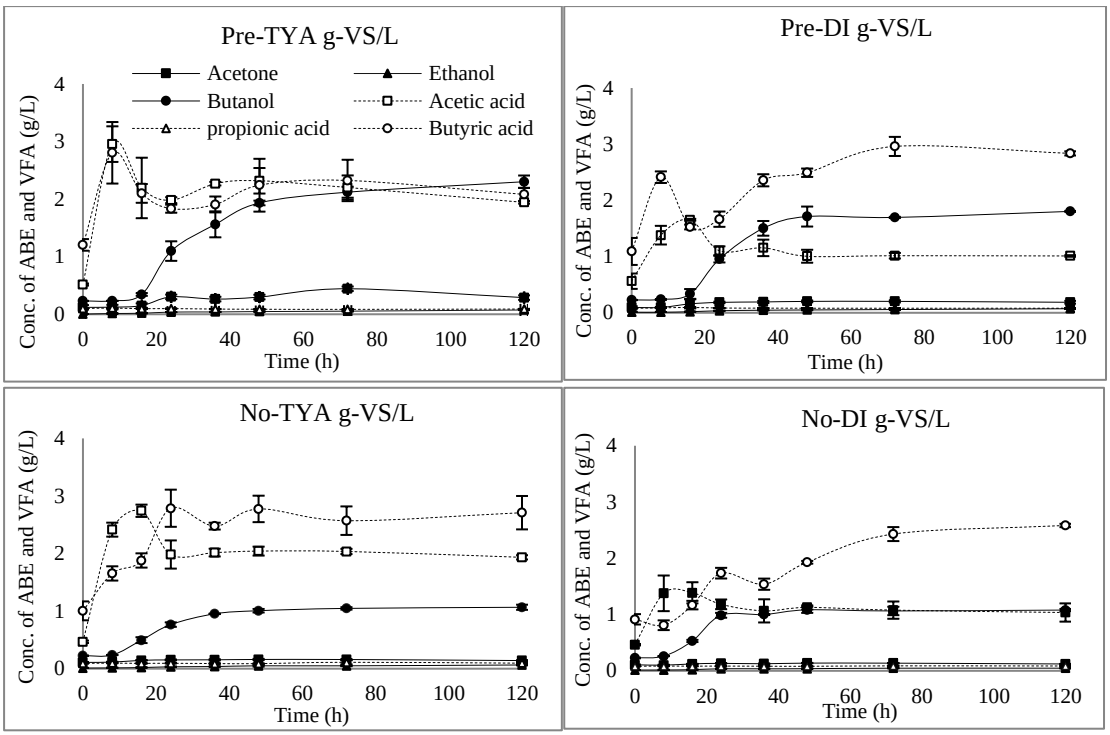



**Figure 21.** VFA and ABE production from 40 g-VS/L microalgae by *Clostridium beijerinckii.*

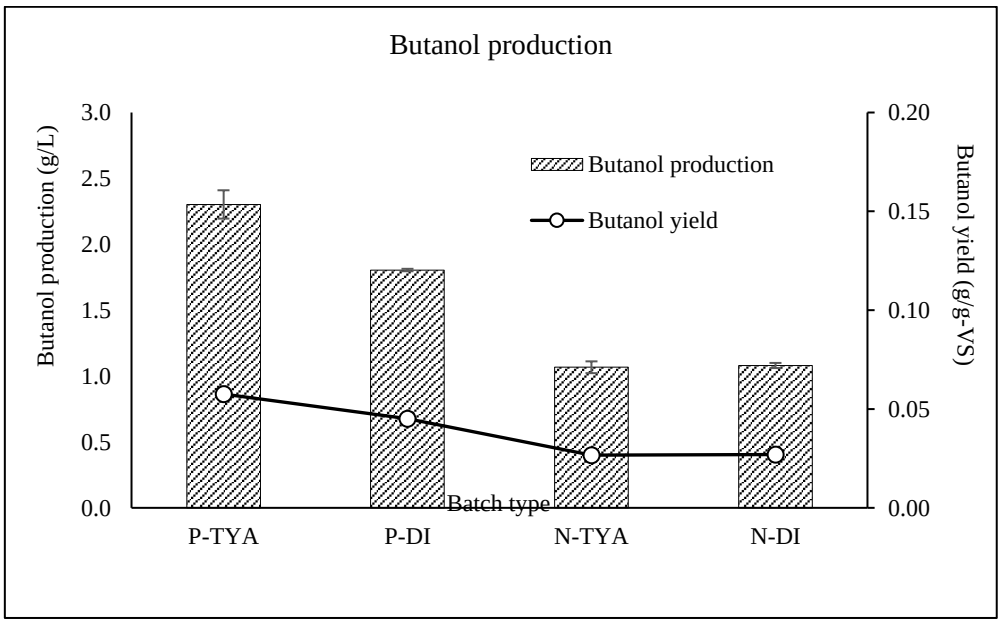



**Figure 22.** Butanol production from 40 g-VS/L microalgae by *Clostridium beijerinckii*.

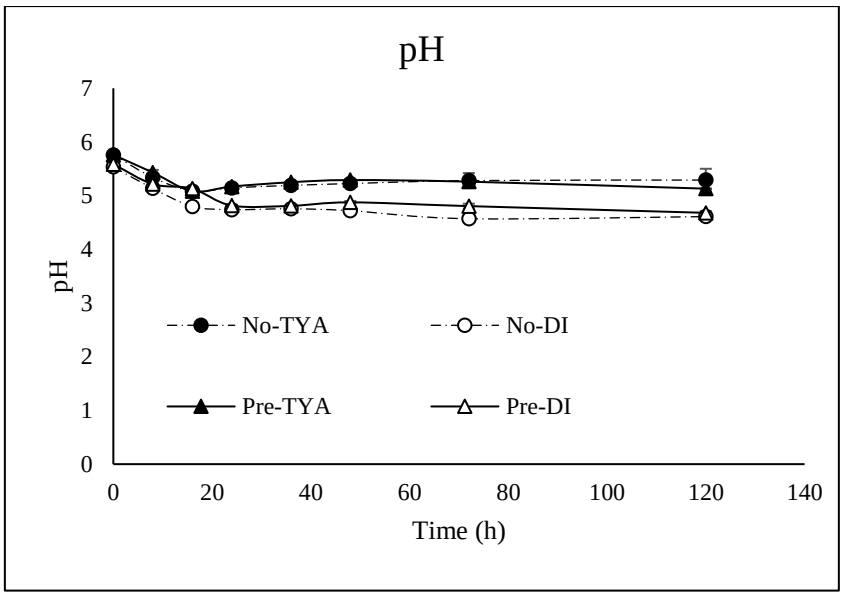



**Figure 23.** Following of pH from 40 g-VS/L microalgae fermentation by *Clostridium beijerinckii*.

### 4.2.3 Butanol production from butyric acid and glucose ratios

The butanol production from various butyric acid and glucose (B/G) ratios was operated with difference concentration of inoculum *Clostridium beijerinckii* 5, 10 and 15 %(v/v). Three main factor concentration of butyric acid (6 level of 0, 2, 4, 8, 10, and 14 g/L) glucose (3 level of 0, 20, and 40 g/L) and inoculum ( 3 level of 5, 10, and 15 %(v/v)) was optimized for butanol production from Taguchi design. The results of three concentration factors demonstrated that butyric acid around 2-10 g/L can produce butanol although more butyric acid than 14 g/L was inhibit for fermentation system together with glucose. The curves of solvent and acid production are seen in supplementary Appendix A1 in accordance with the research of Zhou *et al.* (2019), studying the butyric acid ratio ranging from 8-15 g/L added to the process. Butanol production was decreased at butyric acid of 12 g/L as well as no production at all at butyric acid concentration of 15 g/L onwards. It can be concluded that when the acid content is higher than the bacteria's tolerability, the activity can be inhibited (Yang *et al.*, 2013).While inoculum concentration at 15% can produce more butanol production than 5 and 10% (v/v). The highest butanol production was produced 3.67 g/L from B/G ratio as 10/20 with 15% (v/v) inoculum (Figure 24).

Although the result of butanol production of this test was shown the set was high production. However, after analyzed one way ANOVA in Minitab (Figure 25-26), the data of butanol production yield of three factor was non-significant difference ($P_{value}$ >0.05) (Figure 25-26). $P_{value}$ of three factors (butyric acid, glucose, and inoculum) for butanol production were investigated as 0.324, 0.061 and 0.218, respectively.

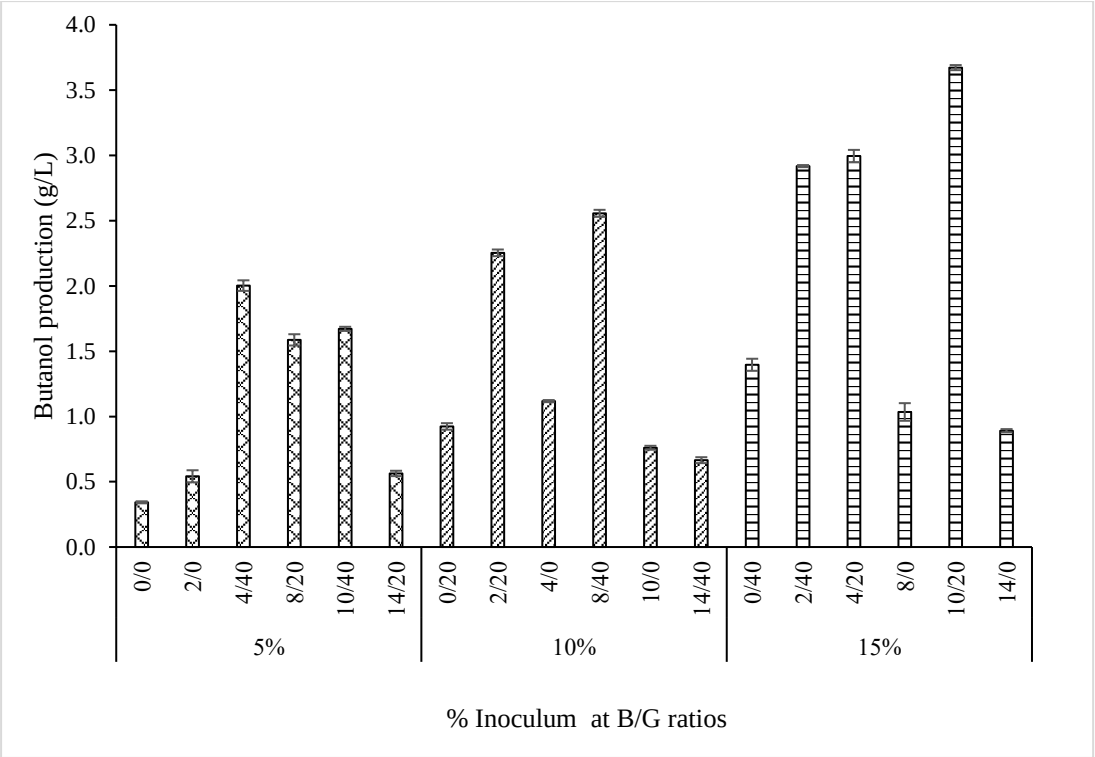
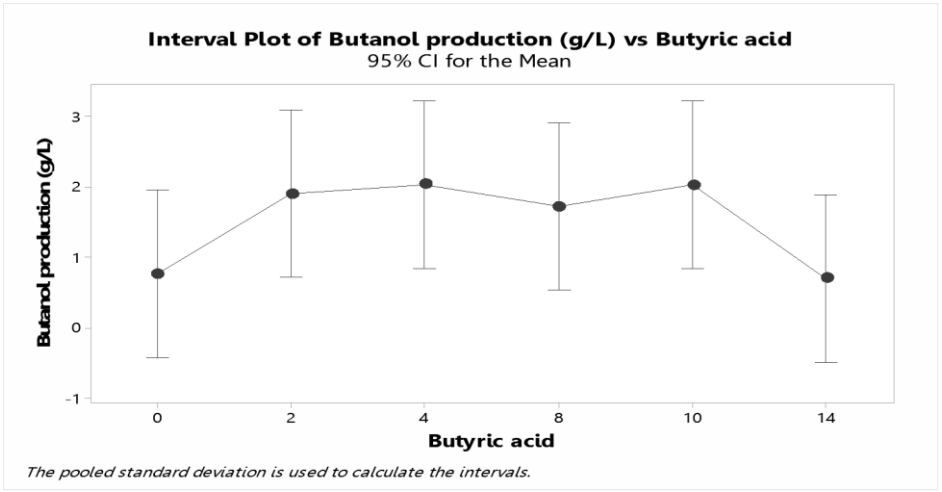
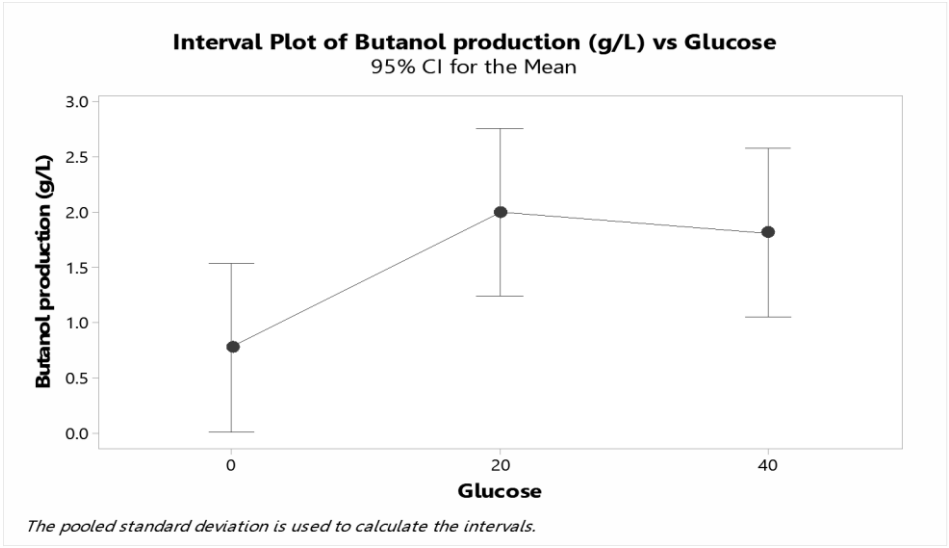



**Figure 24.** Butanol yield from B/G ratio at 5, 10 and 15 % (V/V) of *Clostridium beijerinckii*.



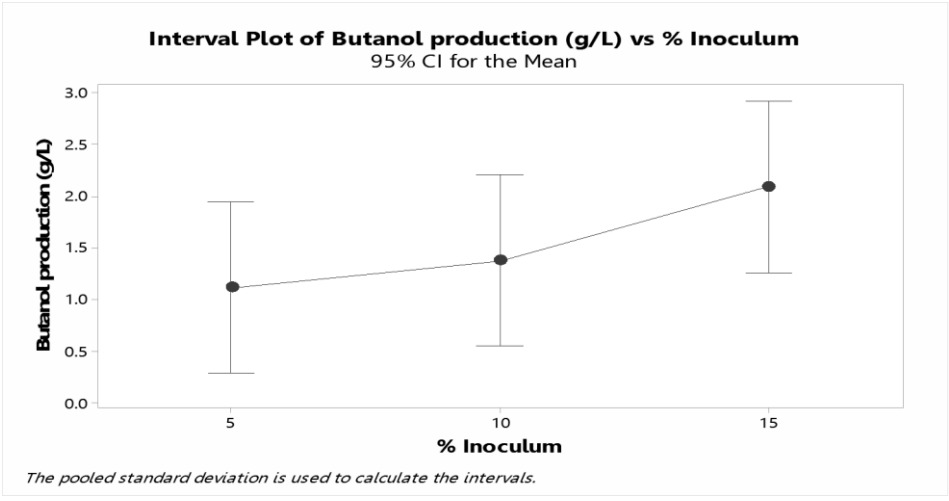




**Figure 25.** Interval plot of butanol production with (A) Butyric acid, (B) Glucose, and (C) % inoculum.

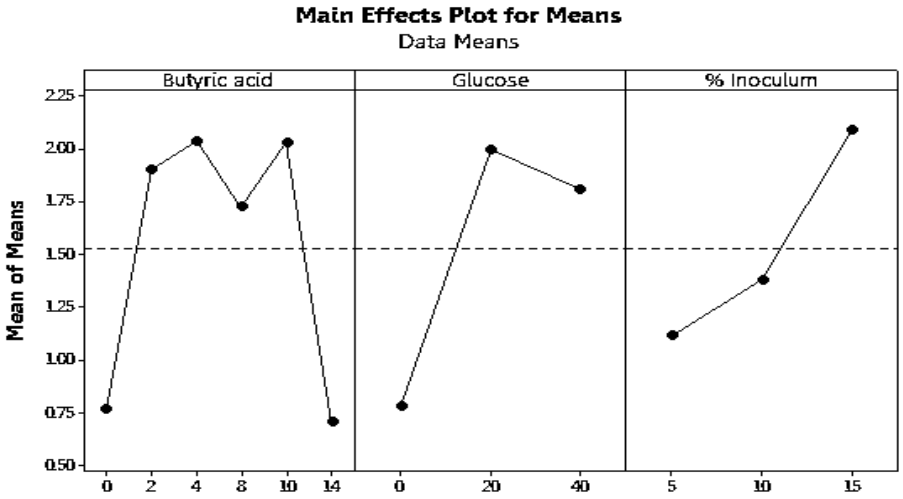



**Figure 26.** Response table from three factors of butyric acid concentration, glucose and concentration of inoculum for means of: butanol production.

## 4.3 Butyric acid production in CSTR

### 4.3.1 2 L CSTR

This Experiment was operated 2 L CSTR with 1.35 working volume to study condition that can be produce butyric acid and gas production, 3-day HRT and 6 day-HRT with substrate concentration 20 g-VS/L and 60 g-VS/L. The substrate concentration was operated between microalgae and sucrose. 9 conditions were studied for the substrate feeding in the form of sucrose and microalgae pretreated and untreated on anaerobic acidogenesis fermentation (Table 16). The follow-up results of Hydrogen production yield, hydrogen production rate (Figure 27), Influent pH, Effluent pH (Figure 28), solvent and VFAs (Figure 29) were reported.

The resulting hydrogen gas production is shown in Figure 27. The graph shows the daily hydrogen production rate and pH The fermentation results from the different conditions were performed in the CSTR reactor by starting the system at condition 1 with the addition of the inoculant taken from the lab-scale CSTR reactor used in the dark anaerobic fermentation process with palm oil mill effluent and brown sugar as a substrate.

Condition 2 was operated with non-pretreatment microalgae 20 g-VS/L without BA medium at 6 day-HRT on 3-12 day of experimentation. After fermentation the hydrogen production gradually decreases. Alkalinity control was necessary to control pH of fermentation system by $NaHCO_3$. pH range was controlled for suitable within the cell acidogenic growth bacteria range, which are the main groups in the production of hydrogen and volatile acids (Kuribayashi *et al*., 2017), because pH has a strong effect on microbial growth (Yossan *et al*., 2012) and acid processing enzymes (Ghimire *et al*., 2015).

**Table 15.** Operated condition for dark fermentation.

| Conditions | periods | Details of the conditions |
|---|---|---|
| 1 | 1-2 | Start up |
| 2 | 3-12 | Microalgae 20 g-VS/L at 6-day HRT |
| 3 | 13-18 | Microalgae 20 g-VS/L at 3-day HRT |
| 4 | 19-27 | Microalgae 10 + sucrose 10 = 20 g-VS/L at 6-day HRT |
| 5 | 28-32 | Microalgae 50 + sucrose 10 = 60 g-VS/L at 6-day HRT |
| 6 | 33-40 | Pretreated microalgae 10 + sucrose = 20g-VS/L at 6-day HRT |
| 7 | 41-44 | Pretreated microalgae 20 g-VS/L at at 6-day HRT |
| 8 | 45-52 | Pretreated microalgae 60 g-VS/L at 6-day HRT |
| 9 | 53-62 | Pretreated microalgae 50 + sucrose 10 = 60 g-VS/L at 6-day HRT (augmentation) |

HRT reduction for 3-day HRT, with the sample's feeding characteristics being the same concentration as that of the experimental condition 2, it was found that the hydrogen yield gradually increased but slightly. Despite the gradual increase in hydrogen production, the pH in the system remains in the same range as the first state. is in the range of 6.40-6.38, including the resulting butyric acid gradually decreasing from the original the resulting hydrogen yield was relatively low. Although the amount of initial feed into the system increased. Therefore, it must be resumed at reduced feed conditions 6-day HRT to prevent the loss of microorganisms from the system (Wash out).

Condition 4, in which BA medium solution was added to promote yield. Minerals are an important factor in promoting enzyme activity in the bacterial degradation process. As a result, the degradation process cannot continue if there is not enough (Yossan *et al*., 2012). It will continue to be used in all conditions. This condition was performed at a microalgae concentration of 10 g-VS/L with 10 g-VS/L of brown sugar with a total feed initial concentration of 20 g-VS/L. Adding sucrose to promoted easily digestion stimulate and increase microorganisms in the system. Due to, a small carbon source is easy to use in microbial degradation processes (Jang *et al*., 2012). The resulting hydrogen yield tended to increase and remained stable in the range of 363-349 mL-$H_2$ /$L_{reactor}$.d or 109-105 mL-$H_2$/g-VS as well as the pH in the system was down

to 5.81-5.74 as the optimum condition with constant butyric acid in the range of 2.18-2.28 g/L. Corresponding with the experiment of wheat straw hydrolysate, the pH 5.2 and 5.3.of fermenter serum was obtained more acid production form on system than when final pH fermenter of 6.6 and 6.9. which high acetic acid and butyric acid can be promoted hydrogen production (Kongjan *et al.* 2010).

Condition 5 was performed using thermolysis pretreated microalgae at 108 °C at a concentration of 50 g-VS/L with 10 g-VS/L of sucrose. A total feed of 60 g-VS/L at 6-day HRT operation. The high hydrogen production was obtained in the range of 653-566 mL-$H_2$/$L_{reactor}$ d. while pH in the range of 5.69-. 5.73 and butyric acid in the range of 3.36-3.46 g/L were investigated. Thermolysis pretreatment of microalgae can convert carbohydrates into sugar form, making it easier to use by microorganisms. Microalgae pretreatment promoted a greater increase in hydrogen production compared to no microalgae pretreatment (Efremenko *et al.*, 2012 and Wieczorek *et al*., 2014).

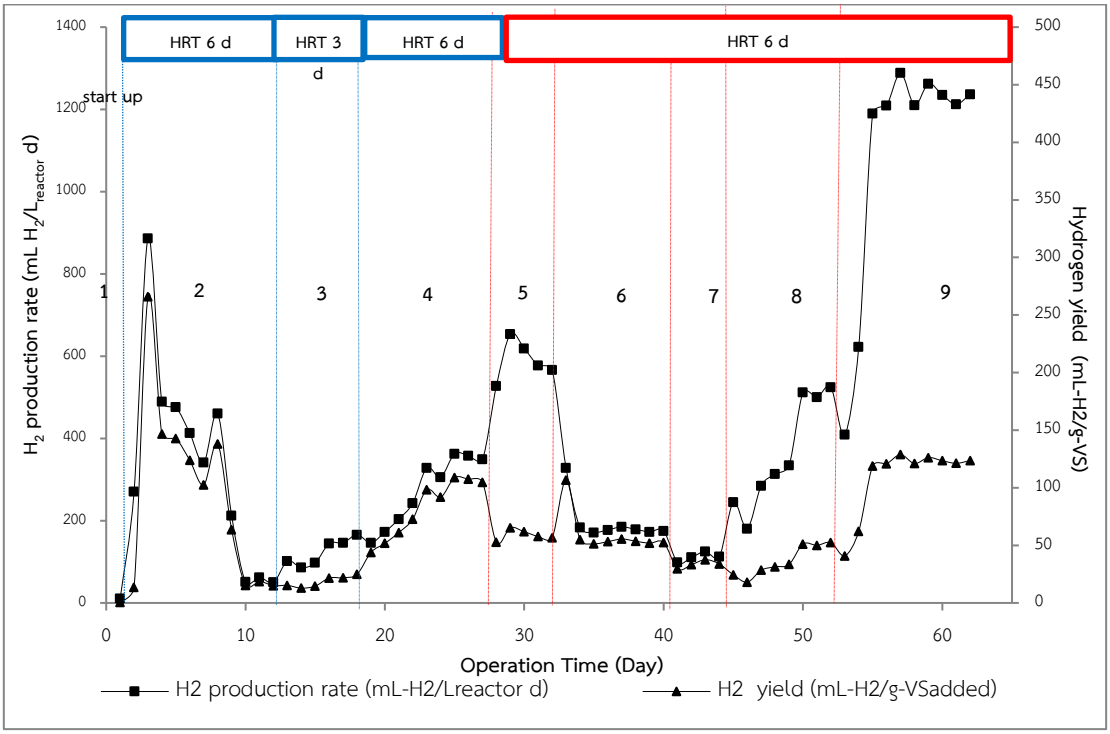



**Figure 27.** Follow-up of hydrogen production rate and hydrogen production yield on CSTR fermentation from *Chlorella* sp. by mixed culture.

Condition 6: The total initial concentration was reduced back to 20 g-VS/L by using 10 g-VS/L of algae conditioning microalgae and 10 g-VS/L sucrose to compared with untreated microalgae in condition 4. Although hydrogen yield was produced in the range of 183-175 mL-$H_2$/$L_{reactor}$ d. But the production of hydrogen yield in the range of 55-52 mL-$H_2$/g-VS, as well as the butyric acid in the range of 2.45-2.64 g/L (Figure 29) were increased.

Condition 7, Initial concentration reduction test of 20 g-VS/L from conditioned microalgae only. The reduction in hydrogen yield per day was found to be in the range of 99-113 mL-$H_2$/$L_{reactor}$ d as well as 30-38 mL-$H_2$/g-VS.

Condition 8, Initial concentration using 60 g-VS/L of algal microalgae alone cab be produced the daily hydrogen yield rang increased to 512-524 mL-$H_2$/$L_{reactor}$. d (51-. 52 mL-$H_2$/g-VS) and butyric acid yield 2.49-2.73 g/L.

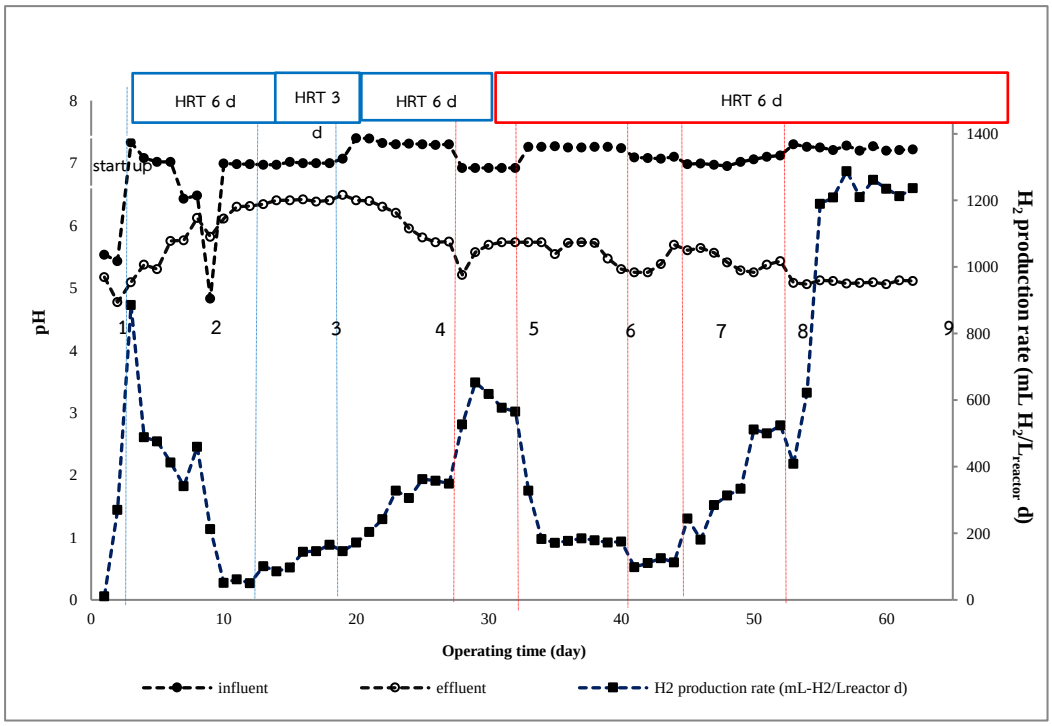



**Figure 28.** Follow-up of hydrogen production rate and pH of influent and effluent on CSTR fermentation from *Chlorella* sp. by mixed culture.

The condition 9, pretreated microalgae was used 50 g-VS/L together with sugar 10 g-VS/L total organic is 60 g-VS/L at HRT 6 day. This is the same condition as condition 5, but differing from this condition, 10% mixed culture augmentation was added to the system prior to operation. It was found that the daily hydrogen yield increased to a range of 1,190-1,288 mL-$H_2$/$L_{reactor}$ d as well as 119-124 mL-$H_2$/g-VS hydrogen yield. In addition the production of butyric acid was increase about 5.23-5.49 g/L. Corresponding to Wieczorek *et al.* (2014) research, Hydrogen production form *Chlorella vulgaris* with enzyme pretreatment, fermentation under 60 °C which produce Hydrogen 130 mL-$H_2$/g-VS more than non-pretreated 19 mL-$H_2$/g-VS (Wieczorek *et al.*, 2014)

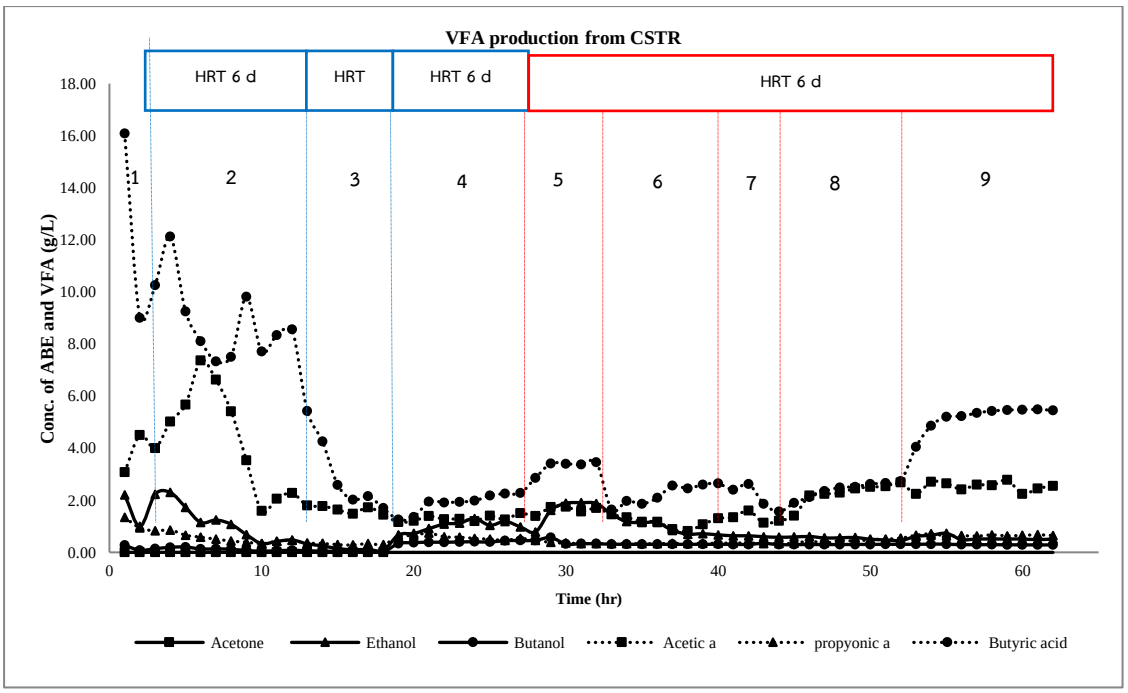



**Figure 29.** Follow-up of VFA and pH of influent and on CSTR fermentation from *Chlorella* sp. by mixed culture.

Therefore, from different operated conditions from the CSTR tank found that microalgae pretreatment with constant addition of BA medium along with system pH control can be promote better production yield of fermentation system. Although the 60 g-VS/L pretreated microalgae only from 6-days HRT can be produce high hydrogen and butyric acid production. However, the optimum operating conditions were obtained using 50 g-VS/L of conditioned microalgae combined with 10 g-VS/L of sucrose for a total concentration of 60 g-VS/L with Augmentation inoculum. Due to mixed culture are the diverse population of microbial (Kleerebezem and van Loosdrecht, 2007). When the growth of the main cultivars was reduced under certain conditions, the fermentation process resulted in a decrease of hydrogen and acetic and butyric acid production even under optimum conditions.

### 4.3.2 10 L CSTR

Butyric acid production from the anaerobic fermentation process with mixed microorganisms for acid production was performed in a 1.35 L working volume in CSTR tank containing microalgae substrate and sucrose. At the total initial concentration of 60 g-VS/L, the highest butyric acid yield conditions were 5.23-5.49 g/L and hydrogen gas yields were 119-124 mL-$H_2$/g-VS. The hydrogen yield per day increased to a range of 1190-1288 mL-$H_2$/$L_{reactor}$. The effluent from the anaerobic fermentation process was prepared from the algae microalgae obtained under the

studied conditions above. 10 L CSTR was operated at a working volume of 5 L (Figure 31) and HRT for 6 days. The butyric acid yield and the pH of incoming and outgoing nutrients were monitored (Figure 30). The pH of the system was changed after entering the five days of the experiment. Biogas production rate 5,042±367mL-biogas/L.d was produced along with 45% hydrogen production at pH stabilized in the range of 5.5-5.2 and 4,981 mg-$CaCo_3$/L of alkalinity. Meanwhile hydrogen production yield of 178±11 mL-$H_2$/g-$VS_{added}$ and production rate per day 2,103.5±165.5 mL-$H_2$/$L_{reactor}$ were investigated. Producing acetic acid propionic acid and butyric acid were produced 5.25±0.14 g/L, 0.76±0.02 g/L and 7.50±0.15 g/L, respectively during days 10-26 of the experiment, which butyric acid production yield 0.13 g/g-VS with a COD value of 118.8. g-COD/L, total sugar 2.45 g/L, total solids (TS) 51.1 g/L, volatile solids (VS) 45.99 g/L and ash 5.11 g/L were investigated. To be used as a substrate rich in butyric acid for the production of butanol in combination with glucose by *Clostridium beijerinckii* in the next step. In addition, biogas production of this stage can be used as a concept for gas stripping of butanol product recovery in second CSTR fermentation. Biogas production of the first CSTR system at 5 L reactor was produced of 23.4 L-biogas/day. While flow rate 3 L/min or 4,320 L/day was used as stripping gas recovery. So that, even lower the biogas production of this study than utilizing flow rate per day, however gas stripping recovery can used as circulation system of gas flow with flow rate control to get the desired flow rate value and this process can reduce the cost of operating the system (Ezeji *et al*., 2013).

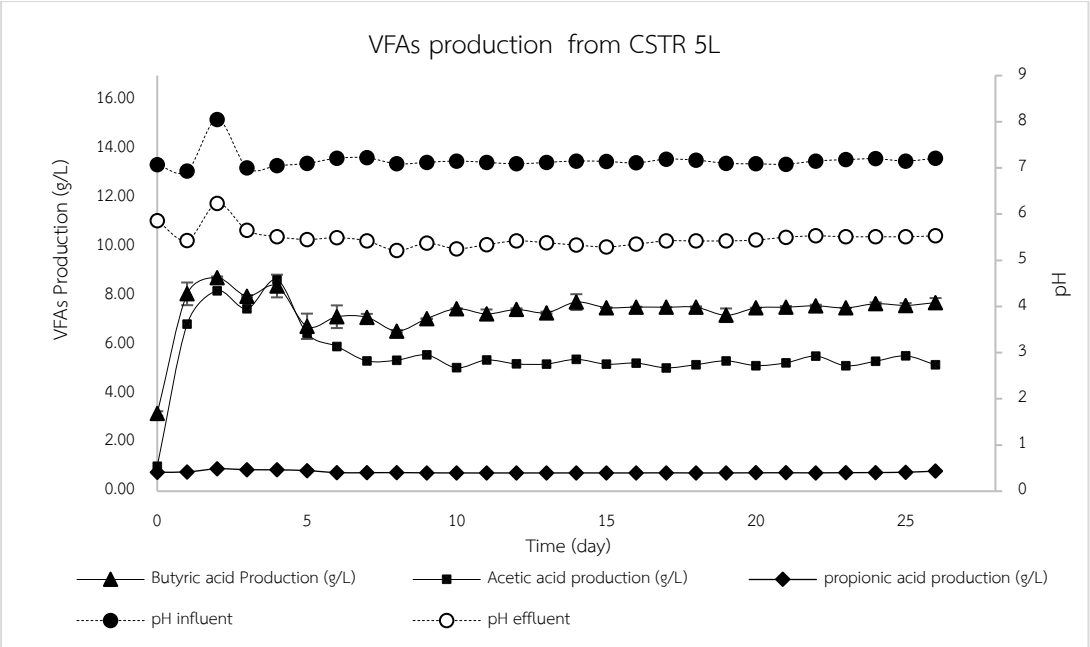



**Figure 30.** Acid and Butyric acid from microalgal fermentation by mixed culture in CSTR at 6-day HRT.


**Figure 31.** First CSTR series for acidogenic fermentation process.

4.3.3 Microbial community structure in CSTR dark fermentation from mixed culture

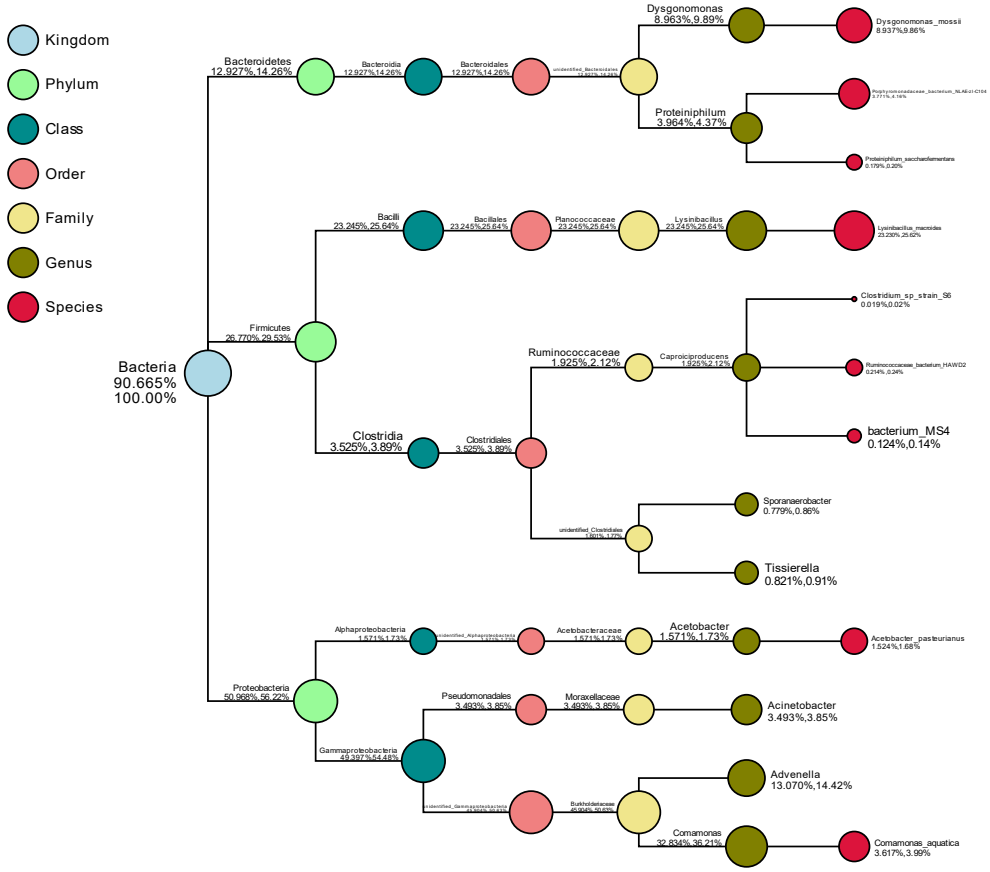



**Figure 32.** Taxonomy tree from the first CSTR system.

The Bacteria communities of fermenter from pretreated microalgae for acidogenesis stage fermentation process at 6-day HRT of first CSTR series with mixed culture as inoculum were investigated by the Next Generation Sequencing (NGS) technology. The rich butyric acid of volatile fatty acid was produced from an acidogenic anaerobic fermentation process by mixed cultures. Volatile fatty acid consisting of acetic acid butyric acid and propionic acid was produced from this stage, while the productions of hydrogen gas and carbon dioxide were produced at the same time. In order to study the microbial community composition in each sample, Operational Taxonomic Units (OTUs) were obtained by clustering with 97% identity on the Effective Tags of sample, and then identified. The total tags data of this sample is 172,245 with 280 observed species. The main bacteria in the fermentation of *Comamonas* spp. (33%), *Lysinibacillus macrolides* (23%), *Advenella* sp. (13%), *Dysgonomonas mossii* (9%), *Proteiniphilum* spp. (4%), *Acinetobacter* sp. (3%), *Clostridiales* spp. (3%), *Acetobacter pasteurianus* (2%), and *Caproicproducens* spp. (2%) were later identified from the anaerobic fermentation process by mixed culture.

It can be seen that some bacteria have the potential to produce rich butyric acid (Dudek *et al*., 2022) and hydrogen gas (Baeyens *et al*., 2020)with the capability to degrade the organic composition of the substrate in the form of proteins carbohydrates and oils.

**4.4 Butanol production on CSTR fermentation**

The experiment was carried out in a volume 10 L (working volume 3 L) will be used for butyric acid production CSTR reactor equiped with igas stripping system installed (Figure 33). The fermentation CSTR tank was connected the heating system to control the temperature of cell growth in the fermentation tank. Hydrogen and carbon dioxide gas were passed through the gas entered the fermentation system and then entered the condenser. Two condensate units were connected to the cooling system for condense the gaseous into a solvent solution. The product of the fermentation system was recovering into the sampling container. The concentration and type of substance were analyzed.

At the beginning of the system, butanol fermentation system was operated from TYA medium (pH 5.93±0.1) containing 20 g-VS/L glucose with and 10% *Clostridium beijerickii* until 3 L working volume at first of experiment.

**Figure 33.** Second CSTR system for butanol production equip with gas stripping process.

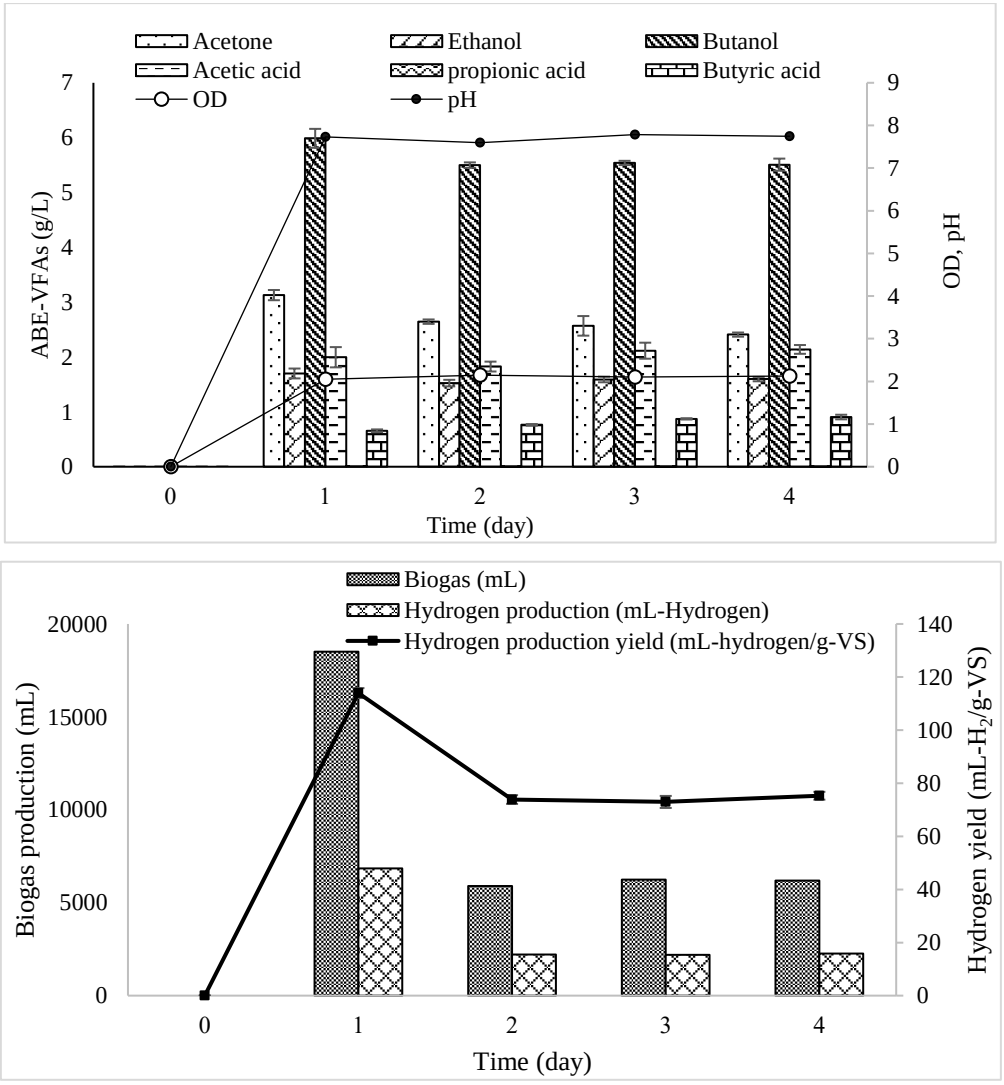



**Figure 34.** ABE-VFAs , OD, pH and gas production of CSTR effluent using glucose 20 g-VS/L with 2-day HRT by *Clostridium beijerinckii.*

After 24 hours, 1,500 mL of substrate was fed in and discharged from the system each day at 2-day HRT. Feeding was divided into two periods per day. The produce was collected in the outgoing fermentation daily to monitor the concentration of solvents and acids production in the fermentation process. It was found that after continuous feeding until the 4th day of the experiment. Butanol yield was stable in the range of 5.51 g/L.

However, the anaerobic process with *Clostridium beijerinckii* can produce hydrogen gas during fermentation. The resulting biogas contained 38% hydrogen concentration with gas production as shown at Figure 34, an average yield of 74 mL-$H_2$/g-VS with an average hydrogen production rate of 740 mL- $H_2$/L/d.

**4.5 Butanol production and gas sparging recovery on CSTR fermentation**

After the production of the second CSTR fermentation was steady. The substrate was added separately 6 times a day into the fermenter. The fermentation system was combined with gas stripping (70% $CO_2$ and 30% $H_2$) recovery solvent on CSTR fermentation system. The installing condensation was operated for two sets of condensation 1 and condensation 2. The results of monitoring the changes in solvent and acid concentrations in the system are shown in Figure 35.

The fermentation system in the CSTR fermenter and the monitoring of bacterial turbidity and pH characteristics Figure 36. Over time, the turbidity or cell sediment decreases due to the operation under 2-day HRT as a steady state period of bacteria cell grow. The result of solvent on CSTR was decreased after combining with gas stripping recovery with flow gas of 3 L/min until concentration of butanol left to 1.55 g/L (4.64 g).

The ability to gas strip was compared to the butanol production that should be obtained from 1 day nutrient feeding at 2 day-HRT. At steady half of the yield should be obtained from the fermentation at the same conditions but without the gas strip. The product concentration is 5.51 g/L (in 3 L there is 16.52 g of butanol). Therefore, the expected productivity in 1 day obtained from HRT feeding for 2 days is 2.755 g/L (in 3 L, there is 8.26 g of butanol). Butanol remaining in the fermentation system can be calculated from grams of solvent (Table 17). After butanol lower than 1.55 g/L (in 3 L there is 4.65 g) concentration, this gas stripping condition cannot remove butanol out of the butanol fermentation system. The butanol removal percentage of 43.86 % as stripping gas efficiency was calculated from gram butanol before striping (8.26 g) minus gram butanol left in the fermenter (4.65 g) and divided by gram butanol before striping (8.26 g) into percentage of removal. However, this test revealed that the blow-drying technique was only suitable for fermentation conditions with high butanol yields, which can increase the removal efficiency. While butanol concentration on condensates 1 and 2 were achieved from gas stripping with high concentration of butanol (Figure 37). However, the volume of solvent was small amount from gas stripping on CSTR (Table 16).

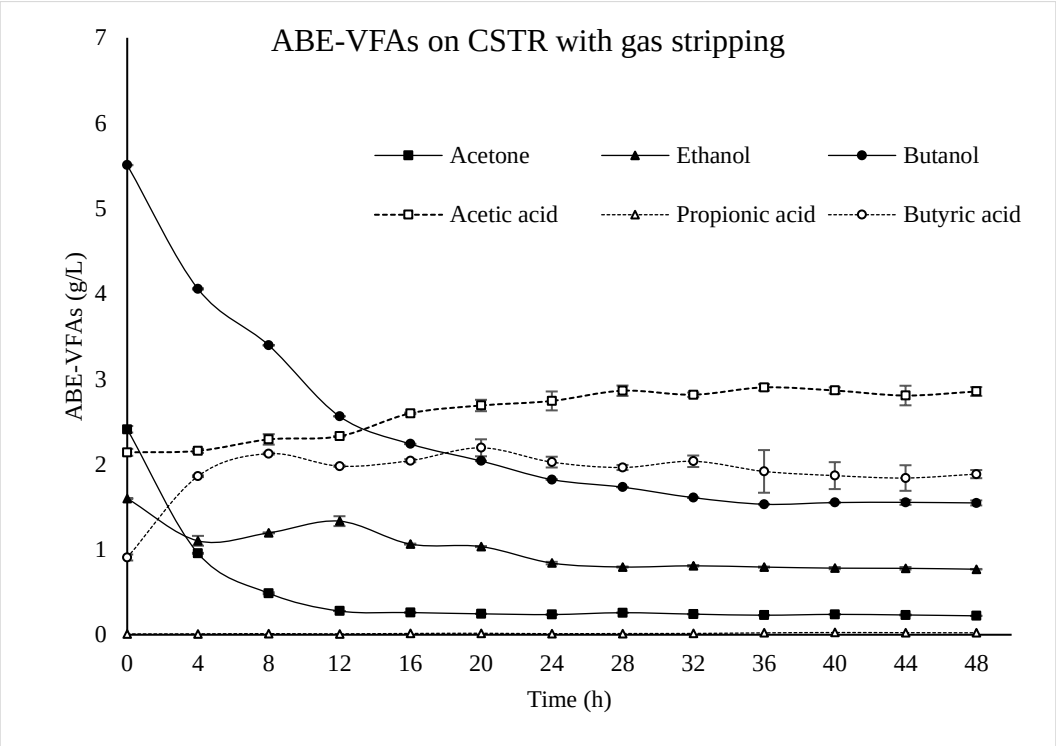



**Figure 35.** ABE-VFAs and pH of CSTR from glucose 20 g-VS/L fermentation equipped with gas sparging at 2-day HRT by *Clostridium beijerinckii*.

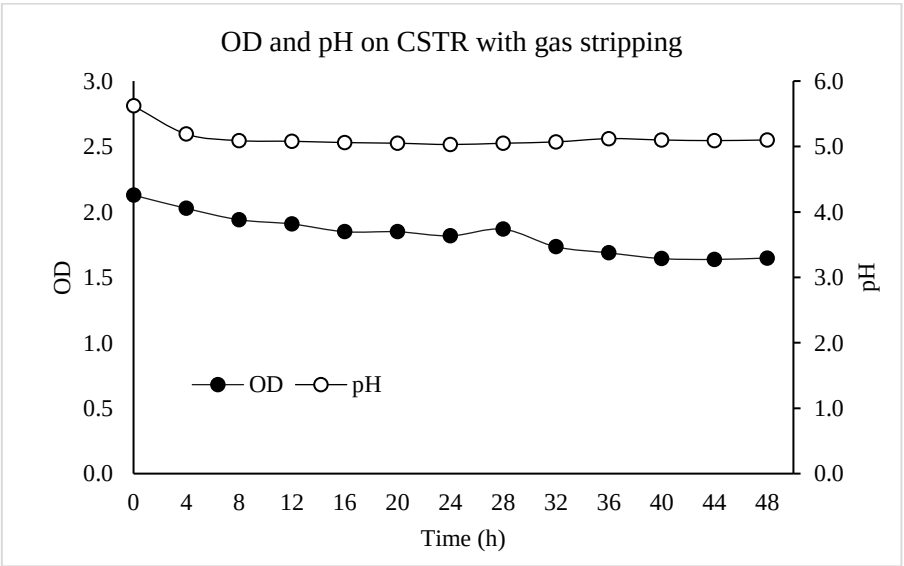



**Figure 36.** OD and pH of CSTR from glucose 20 g-VS/L fermentation equipped with gas sparging at 2-day HRT by *Clostridium beijerinckii*.

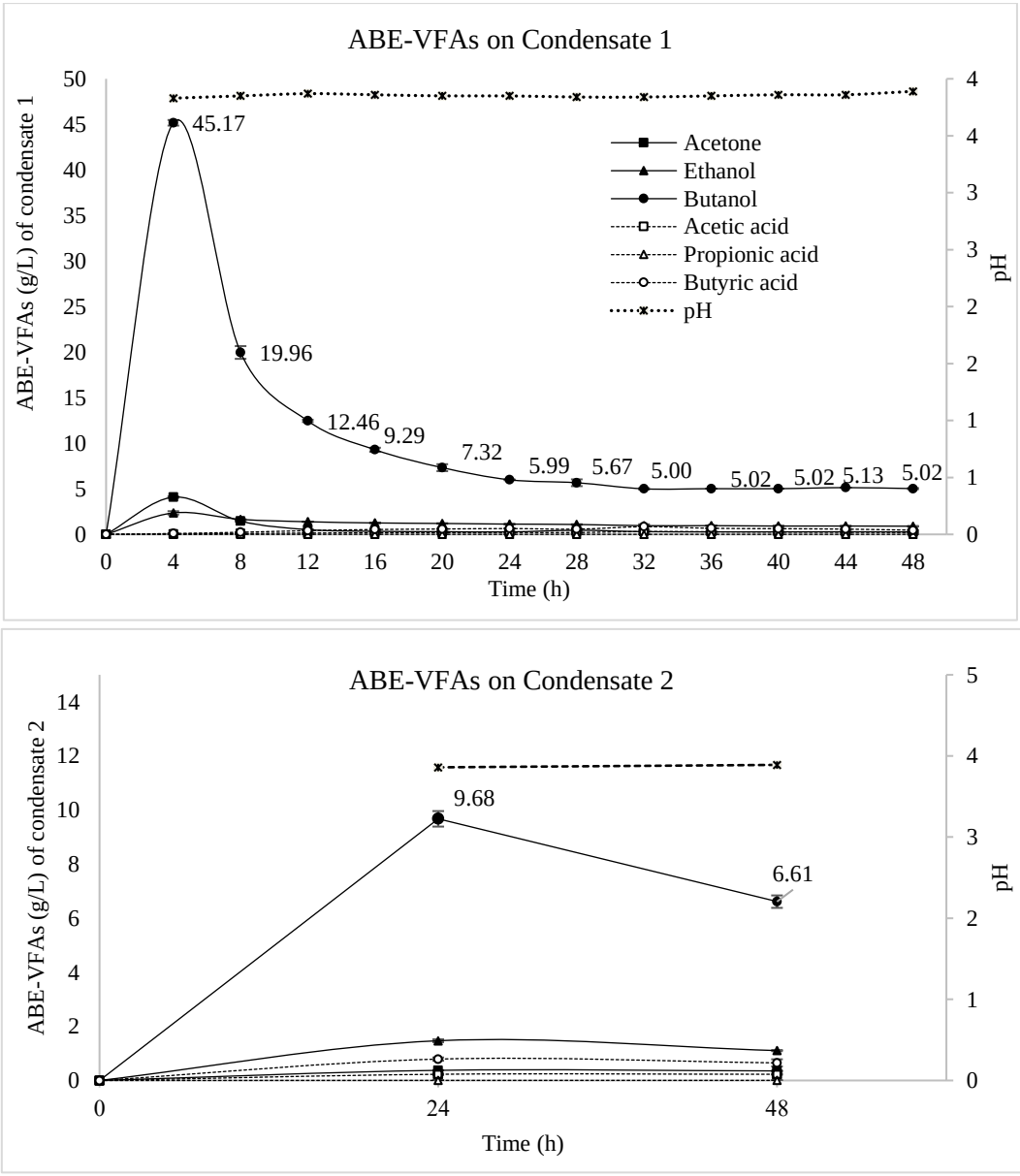



**Figure 37.** pH and ABE-VFAs recovery of condensate 1 and 2 by gas sparging.

Gas striping can be removing the organics pass through installed condensing unit with the cooling water from 50% ethylene glycol at -4 °C to be condensed and harvested of organics. It was harvested every 4 h. At the beginning butanol production of the harvest was found that the solution concentration was 45.17 g/L butanol, Figure 37. which when gas flow continuously the concentration and volume of solvent were obtained with relatively stable. It represents the harvest potential produced by a stable system. The condensation was done in two sets of experiments to increase the efficiency of condensing organic matter obtained from the gas stripping process. In addition, the pH of the extract was found to be relatively acidic. Demonstrated that, some acid can

be removed out of fermenter, but in low concentrations. However, this experiment revealed the ability to condense including substances that have been caught before leaking out with impinger trap in distilled water. This gas stripping had a condensation efficiency of butanol 75.69% which was calculated from the weight of the butanol in grams unit at the recovery system ( 1.30 g butanol of condensate 1 + 0.04 g butanol of condensate 2 + 1.40 g butanol of impinger) and divided by gram of butanol removal from fermenter (8.26 g of butanol at start – 4.65 g butanol left in the fermenter) into percentage of efficiency (Table 17).

**Table 16.** Solution volume of condensate 1 and 2 from gas stripping at different time points.

| Time (h) | Condensed volume (mL) | | pH | |
|---|---|---|---|---|
| | Condensate 1 | Condensate 2 | Condensate 1 | Condensate 2 |
| 0 | 0.00 | | 0 | |
| 4 | 35.00 | | 3.83 | |
| 8 | 50.00 | | 3.85 | |
| 12 | 50.00 | | 3.87 | |
| 16 | 42.00 | | 3.86 | |
| 20 | 40.00 | | 3.85 | |
| 24 | 41.00 | 5.00 | 3.85 | 3.86 |
| 28 | 42.00 | | 3.84 | |
| 32 | 40.00 | | 3.84 | |
| 36 | 42.00 | | 3.85 | |
| 40 | 42.00 | | 3.86 | |
| 44 | 42.00 | | 3.86 | |
| 48 | 44.00 | 5.60 | 3.89 | 3.89 |
| Total | 510.00 | 10.60 | | |
| Total of 2 condensate | 520.60 | | | |

**Table 17.** ABE-VFAs of fermentation system with gas stripping.

| ABE-VFAs | In CSTR 0 h (g) | In CSTR 48 h (g) | Organic (g) | | | |
|---|---|---|---|---|---|---|
| | | | Condensate 1 | Condensate 2 | Impinger | Total organic solution |
| Acetone | 7.23 | 0.666 | 0.357 | 0.004 | 0.661 | 1.687 |
| Ethanol | 4.79 | 2.305 | 0.621 | 0.014 | 1.159 | 4.099 |
| Butanol | 16.52 | 4.637 | 5.427 | 0.085 | 3.935 | 14.084 |
| Acetic acid | 6.42 | 8.557 | 0.100 | 0.002 | 0.006 | 8.665 |
| Propionic acid | 0.03 | 0.068 | 0.002 | 0.000 | 0.004 | 0.074 |
| Butyric acid | 2.72 | 5.651 | 0.264 | 0.008 | 0.035 | 5.958 |

Rich butyric acid from acidogenesis fermentation stage was used as a substrate with glucose to produce butanol with acetic acid butyric acid and propionic acid as 5.17, 7.71 and 0.82 g/L respectively. However, butanol can be produced of 6 g/L butanol in the beginning at 24 h, after 2 days of fermentation, the production was stopped at 60 h pH 4.92, while the hydrogen and butanol production at pH 5.54 at 24 h (Figure 38). Demonstrate that, the substrate can be used for butanol production which should be used at lower of this substrate initial concentrations to decrease the inhabitation from acid. The highest production of butanol and hydrogen before inhibiting were produced of 5.54 g/L and 117.68 mL-$H_2$/g-VS, respectively ( Figure 38). Although expelling is a technique that involves some investment in condensing and heating refrigeration energy as well as impeller agitation. But energy consumption in this integrated harvesting system can be reduced compared to conventional distillation. It was found that the combined use of gas stripping and pervaporation studies (Pervaporation process) is a process to recover the solvent from fermentation. using only 29.2% of the energy used in conventional distillation processes (Cai *et al*., 2016).

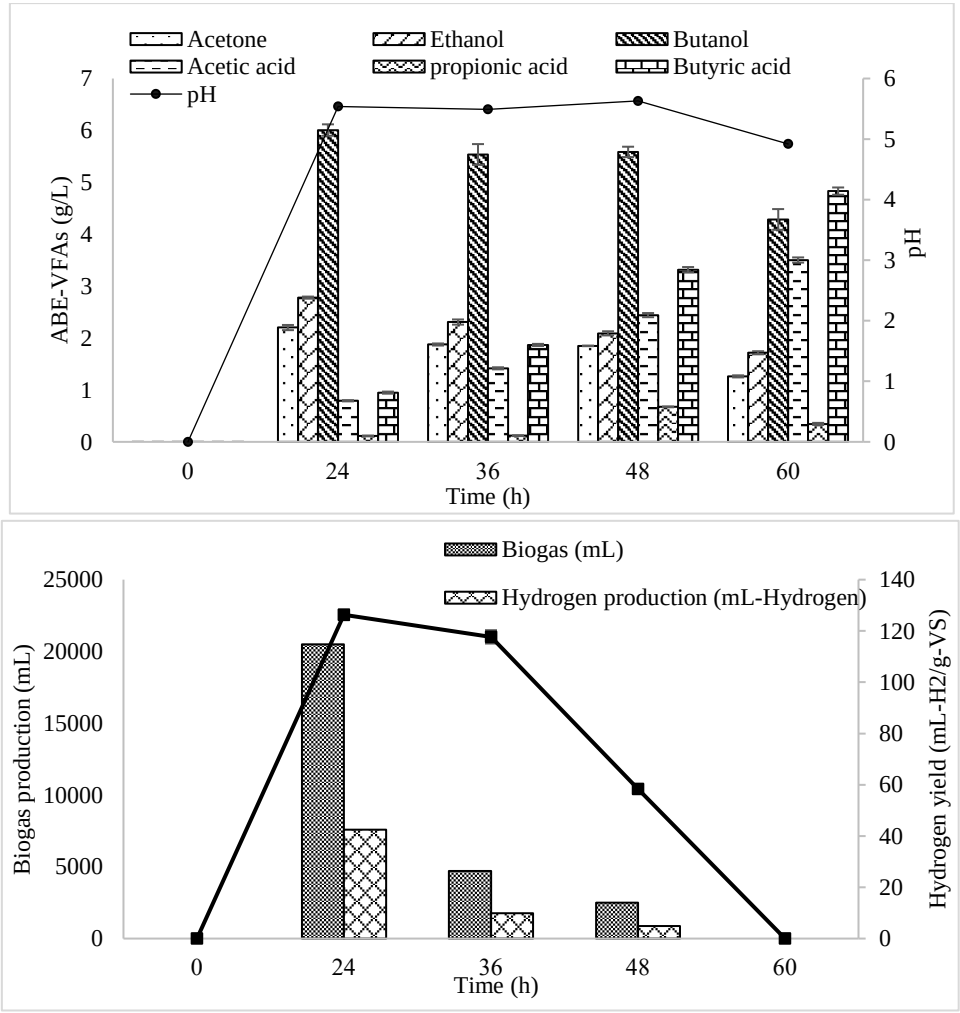



**Figure 38.** ABE-VFAs and gas production of CSTR using glucose 20 g-VS/L mixed with rich butyric acid from acidogenesis fermentation for 2-day HRT by *Clostridium beijerinckii.*

COD balance was investigated under the continuous system of the first CSTR system for acidogenesis fermentation by mixed culture and the second CSTR system for solventogenesis stage by *Clostridium beigerinckii* ATCC10132. The first CSTR fermentation was obtained *Chlorella* sp. 41.66 g-VS with 8.33 g-VS in 833.3 mL/day (136.4 g-COD/g-VS and 8.91 g-COD/g-Sucrose; Total COD of initial substrate of 145.31 g-COD) under operated condition of 5 L working volume, 6-day HRT at 55 °C. The production of this stage was produced in form of solution along with hydrogen gas (10.52 L-$H_2$) (Figure 39). Therefore, solution as a rich of butyric acid was converted to the second CSTR series for 3 L working volume of solventogenesis fermentation by *Clostridium beigerinckii* ATCC10132. The process was operated under 2-day HRT, 1,500 mL/d with rich of butyric acid 50% (v/v) or 750 mL and 750 mL TYA-N with 30 g-glucose at a temperature of 37 °C. acetone ethanol butanol acetic acid propionic

acid and butyric acid as well as hydrogen production as a last product was investigated and calculated for g-COD. The COD balance of substrate was shown as a figure 39.

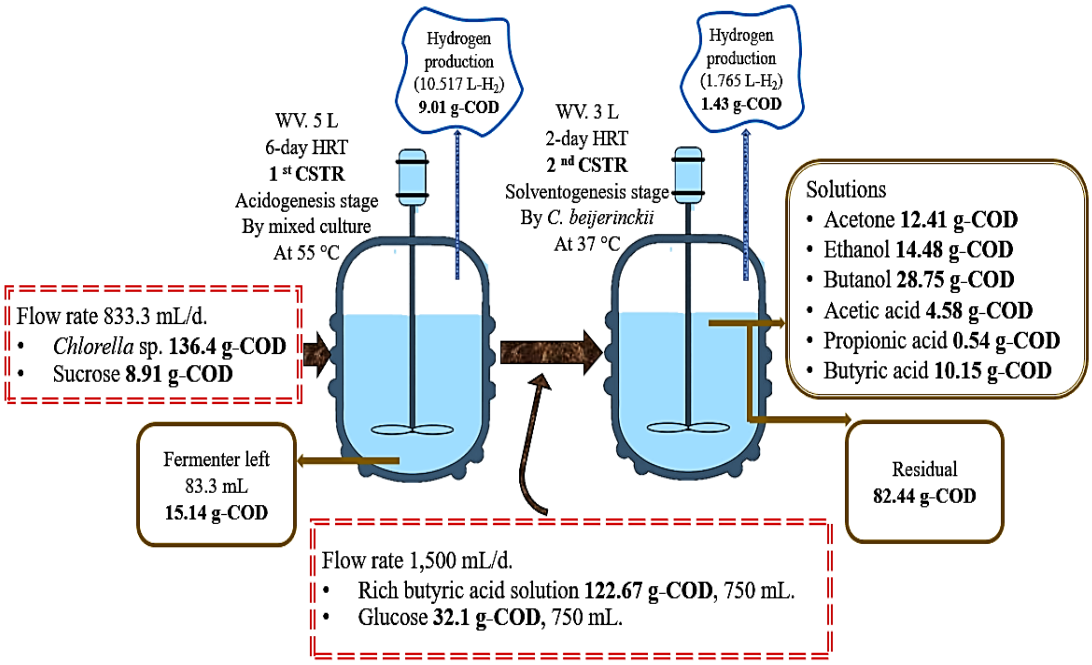



**Figure 39.** COD balance of substrate from microalgae (*Chlorella* sp. ) for two-stage fermentation in 10 L CSTR

# CHAPTER 5
# CONCLUSION AND RECOMMENDATIONS

## 5.1 Conclusion

The optimal effect of acidogenesis batch fermentation of initial pH 7 with a wide range of initial microalgal concentration as a substrate of 20-120 g-VS/L with the thermolysis pretreatment of microalgae at 108 °C with the addition of BA medium to produce butyric acid and hydrogen in batch dark fermentation by mixed culture at thermophilic temperature source of 55 °C was investigated. pH control was an important factor for butyric acid production. The main bacteria of mixed culture for acidogenesis fermentation have pH control in fermentation as a necessary factor for acid production type. Meanwhile, the temperature factor of fermentation depends on the type of inoculum. Therefore, groups of microorganisms coming from different temperature sources had better metabolism capacity and proliferating abilities in different optimum temperature ranges.

However, the condition of highest product both hydrogen production (119-124 mL-$H_2$/g-VS or 1,190-1288 mL-$H_2$/L-reactor.d mL) and butyric acid of 5.23-5.49 in CSTR reactor was investigated on 1.35 L working volume at operation of 6-day HRT from microalgae pretreated 50 g-VS/L along with 10 g-sucrose/L by thermophile mixed culture source at 55 °C. The optimal condition from 2 L CSTR reactor was operated under scale up to 10 L CSTR reactor at 5 L working volume. The production of hydrogen yield of 161-189 mL-$H_2$/g-$VS_{add}$ and hydrogen production rate of 1,938-2,269 mL-$H_2$/Lreactor with the production of acetic acid propionic acid and butyric acid produced during the experiment were in the range of 5.25±0.14 g/L, 0.76±0.02 g/L and 7.50±0.15 g/L, respectively was investigated. biogas production 23.4 L-biogas/day of this stage can be used as a concept for gas stripping of butanol product recovery in second CSTR fermentation. Although, flow rate 3 L/min or 4,320 L/day was used as stripping gas recovery. However, gas stripping recovery can used as circulation system of gas flow with flow rate control to get the desired flow rate value and this process can reduce the cost of operating the system. the microbial community composition in each sample, Operational Taxonomic Units (OTUs) were obtained by clustering with 97% identity on the Effective Tags of sample, and then identified. The total tags data of this sample is 172,245 with 280 observed species. The main bacterial in the fermentation of mixed culture were *Comamonas* spp. (33%), *Lysinibacillus macrolides* (23%), *Advenella* sp. (13%), *Dysgonomonas mossii* (9%), *Proteiniphilum* spp. (4%), *Acinetobacter* sp. (3%), *Clostridiales* spp. (3%), *Acetobacter pasteurianus* (2%), and *Caproicproducens* spp. (2%).

Higher butanol concentration of 10.17 g/L in batch fermentation was produced from *Clostridium beijerinckii* ATCC10132 at 40 g-VS/L with incubator shaker initial

glucose concentrations than 20 60 and 80 g-VS/L. The initial concentration 40 g-VS/L of microalgae pretreatment with 108 °C autoclave for 30 min with nutrient addition was produced butanol 2.30 g/L which higher than untreated. In addition, the highest butanol yield of 3.67 g/L at the B/G ratio (10 g/L butyric acid with 20 g/L glucose) from the Taguchi experimental design was investigated. Which initial butyric acid concentration of 2-10 g/L had better yield when compared with high butyric acid concentrations up to 14 g/L.

Butanol production yield from CSTR with continuously fed 20 g-VS/L glucose on TYA medium at HRT 2 day was constant in the range of 5.51 g/L, 38% hydrogen concentration with yield of 74 mL-$H_2$/g-VS (740 mL-$H_2$/L/d). When Stripping gas (70% $CO_2$ and 30% $H_2$ ) 3 L/min, blowing in the fermentation system for 48 hours continuously, it was found that the harvest butanol was 43.86% of stripping gas efficiency with 75.69% efficiency of condensation. In the first 4 hours, the highest butanol concentration was obtained in the first condensing unit at 45.17g/L. This stripping process is suitable to recovery with high solvent yield of CSTR. In addition, the results of butanol production from the effluent of rich butyric acid together with 20 g-VS/L-glucose were found that in the early period butanol production 5.54 g/L with hydrogen production 117.68 mL-$H_2$/g-VS. The acid content is increased in the system and affects the growth and activity of *Clostridium beijerinckii*. A reduction in the acidic substrate concentration is necessary since the acid in the fermentation solution is not only butyric acid but also contains other types of volatile acids. COD balance of substrate from microalgae in 10 L CSTR system from two-stage fermentation was investigated. The last production in the form of solution and hydrogen at the final stage can be produced from g-COD of the substrate in the system as 45.79% with 8.59% left from the solution of the first stage and 48.68% as a residual of the final fermenter system.

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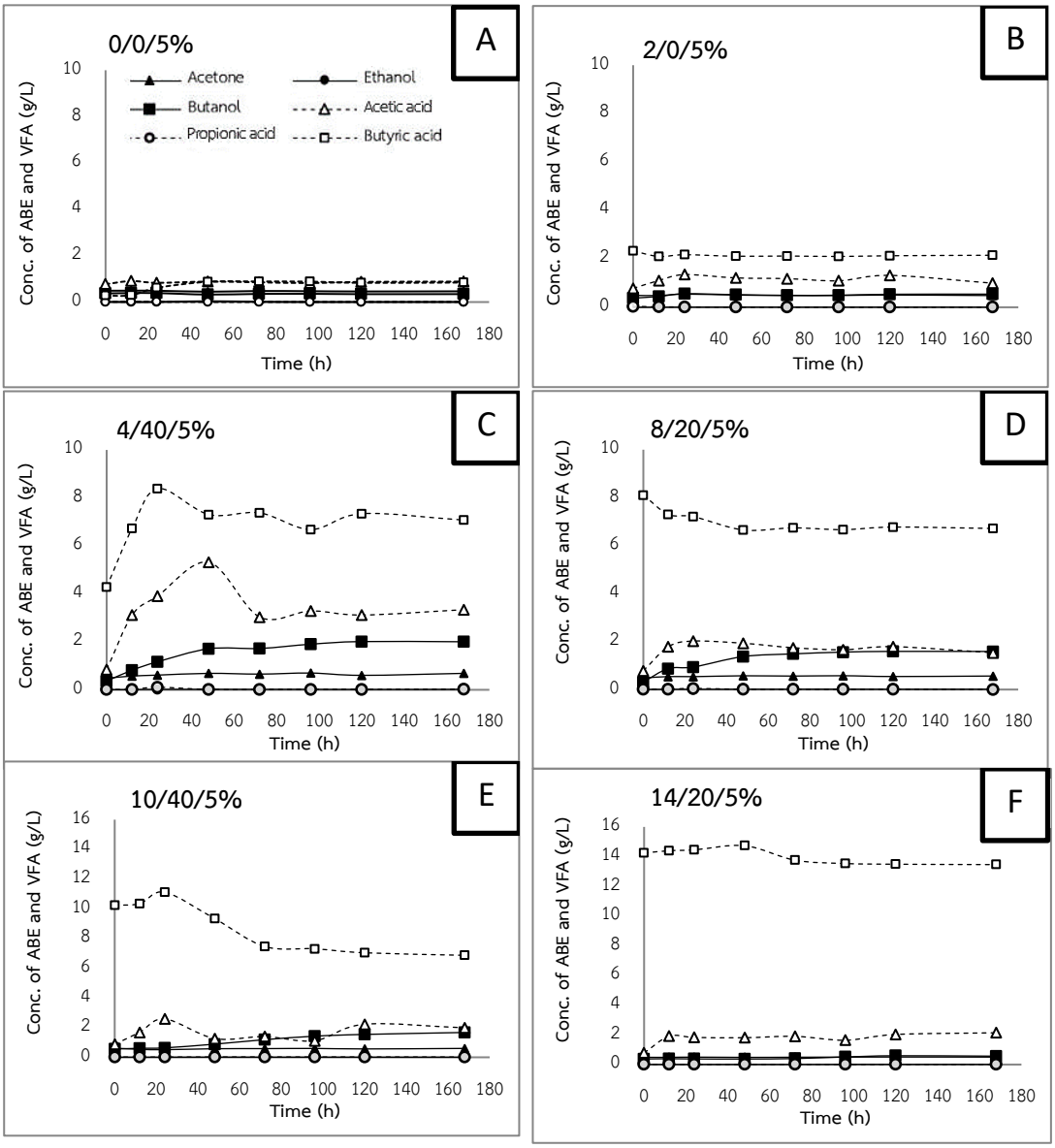
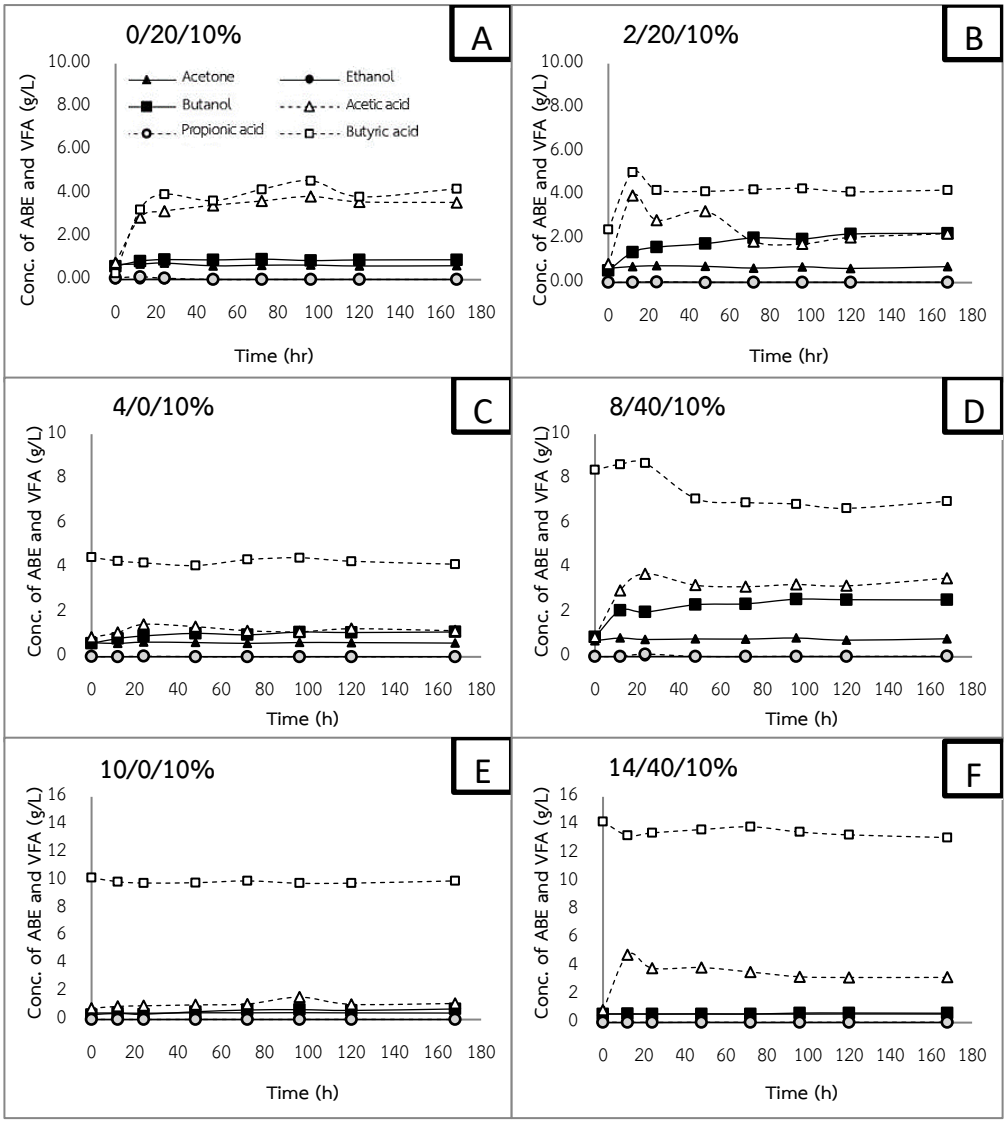
# APPENDICES

## Appendix A

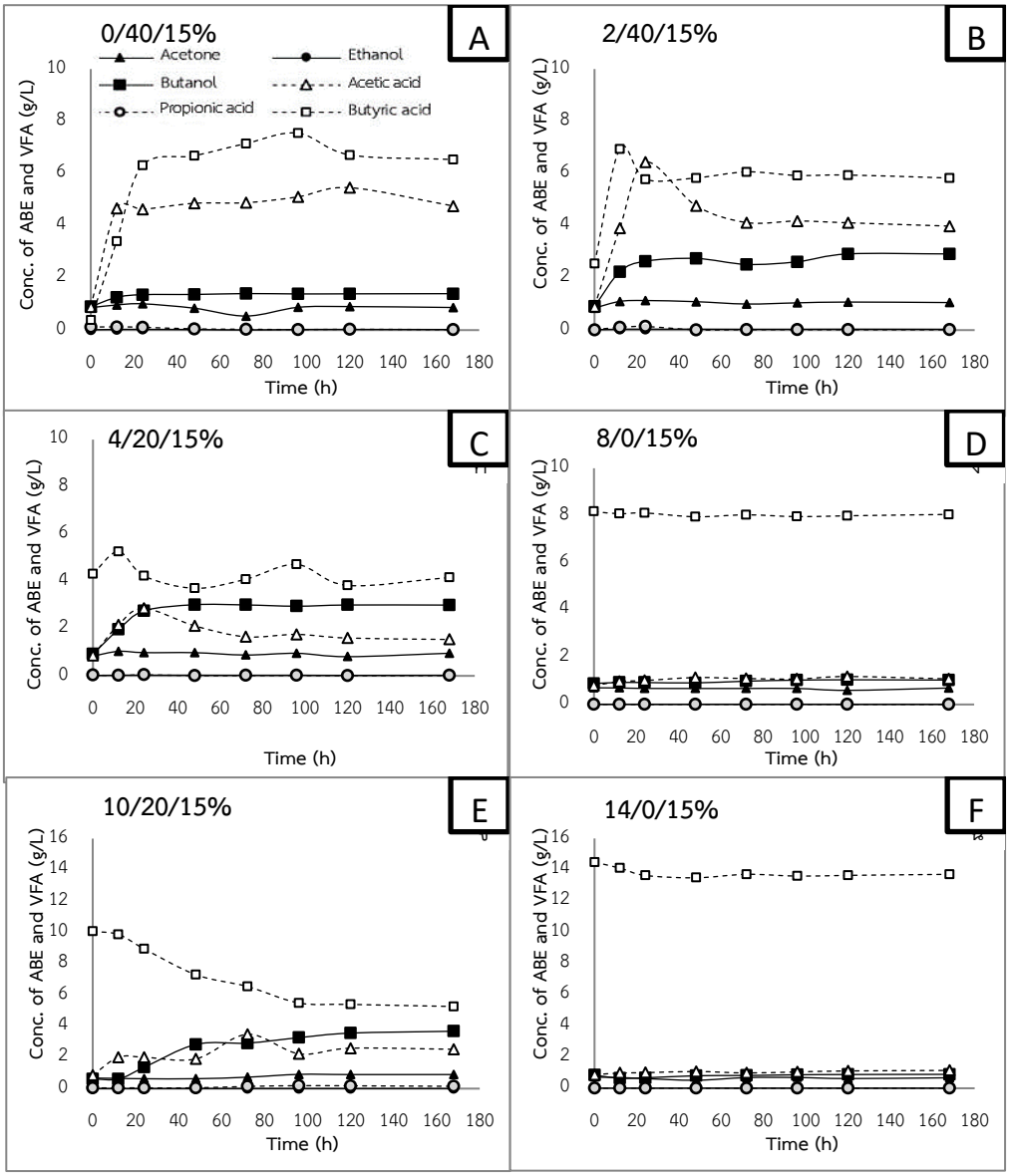
A1 Follow-up of acetone, butanol, ethanol, acetic acid, butyric acid and propionic acid yields obtained from fermentation at butyric acid/glucose ratios (B/G ratios). at an inoculum (*Clostridium beijerinckii*) concentration of 5% by volume



A2 Follow-up of acetone, butanol, ethanol, acetic acid, butyric acid, and propionic acid yields obtained from fermentation at butyric acid/glucose ratios (B/G ratios). at an inoculum (*Clostridium beijerinckii*) concentration of 10% by volume



A3 Follow-up of acetone, butanol, ethanol, acetic acid, butyric acid, and propionic acid yields obtained from fermentation at butyric acid/glucose ratios (B/G ratios). at an inoculum (*Clostridium beijerinckii*) concentration of 15% by volume



**Appendix B**

Grams of solvent and volatile acid obtained from condensers 1 and 2 of stripping system at each time point. and the total in 48 hours from second CSTR fermentation glucose

| Time (h) | Acetone (g) | | Ethanol (g) | | Butanol (g) | | Acetic acid (g) | | Propionic acid (g) | | Butyric acid (g) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | condensate | | | | | | | | | | | |
| | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| 0 | 0.000 | | 0.000 | | 0.000 | | 0.000 | | 0.000 | | 0.000 | |
| 4 | 0.143 | | 0.083 | | 1.581 | | 0.001 | | 0.000 | | 0.003 | |
| 8 | 0.073 | | 0.082 | | 0.998 | | 0.005 | | 0.000 | | 0.012 | |
| 12 | 0.026 | | 0.069 | | 0.623 | | 0.007 | | 0.000 | | 0.021 | |
| 16 | 0.014 | | 0.053 | | 0.390 | | 0.007 | | 0.000 | | 0.022 | |
| 20 | 0.012 | | 0.048 | | 0.293 | | 0.008 | | 0.000 | | 0.024 | |
| 24 | 0.011 | 0.002 | 0.046 | 0.007 | 0.246 | 0.048 | 0.009 | 0.001 | 0.000 | 0.000 | 0.026 | 0.004 |
| 28 | 0.019 | | 0.046 | | 0.238 | | 0.008 | | 0.000 | | 0.024 | |
| 32 | 0.012 | | 0.038 | | 0.200 | | 0.013 | | 0.000 | | 0.034 | |
| 36 | 0.012 | | 0.040 | | 0.211 | | 0.012 | | 0.000 | | 0.028 | |
| 40 | 0.012 | | 0.038 | | 0.211 | | 0.011 | | 0.000 | | 0.026 | |
| 44 | 0.012 | | 0.038 | | 0.216 | | 0.010 | | 0.000 | | 0.024 | |
| 48 | 0.012 | 0.002 | 0.039 | 0.006 | 0.221 | 0.037 | 0.009 | 0.001 | 0.000 | 0.000 | 0.020 | 0.004 |
| Total | 0.357 | 0.004 | 0.621 | 0.014 | 5.427 | 0.085 | 0.100 | 0.002 | 0.002 | 0.000 | 0.264 | 0.008 |
| Total of two condensate | 0.360 | | 0.635 | | 5.512 | | 0.102 | | 0.002 | | 0.272 | |

# VITAE

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**Educational Attainment**

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|---|---|---|
| Master of Science (Applied Chemistry) | Prince of Songkla University | 2015 |
| Bachelor of Science (Chemistry-Biology) | Prince of Songkla University | 2013 |

**Scholarship Awards during Enrolment**

Prince of Songkla University -Ph.D. Scholarship 2016.
The Ministry of Science and Technology, Taiwan (MOST 106-2221-E-035 -086 -MY2) and Taiwan Experience Education Program (TEEP), MOE.
National Research Council of China (NRCT) for grants to the Prince of Songkla University in fiscal year 2018 (SAT610100S)
Thailand Research Fund for Senior Research Scholar (Grant No. RTA 5980004).

**List of Publication and Proceeding**

**Publications**

Usmanbaha N., Jariyaboon R., Reungsang A., Kongjan P. and Chu C.-Y. (2019) Optimization of batch dark fermentation of *Chlorella* sp. using mixed-cultures for simultaneous hydrogen and butyric acid production. *Energies* **12**, 2529.

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Kongjan P., Usmanbaha N., Khaonuan S., Jariyaboon R., Sompong O. and Reungsang A. (2021) Butanol production from algal biomass by acetone-butanol-ethanol fermentation process. In: Clean Energy and Resources Recovery (pp. 421- 46). Elsevier.

**Proceeding**

Usmanbaha N., Jariyaboon R., O-Thong S. and Kongjan P. Effect of Different Mixing Ratios of Palm Oil Mill Effluent (POME) and *Cerathophyllum demersum* on

Two-Stage Anaerobic Co-Digestion for Hydrogen and Methane Production. The 39 th National Graduate Research Conference and AU International Graduate Research Conference 2016. Assumption University, Smutprakar, Thailand, on 30 June–1 July, 2016.

Usmanbaha N., Jariyaboon R., Kongjan P. and Chu C.-Y. Optimizing Batch Dark Fermentation of *Chlorella sp*. Using Mixed-cultures to Butyric Acid Production for Further Acetone Butanol Ethanol (ABE) Fermentation. The 6th Asian Conference on Innovative Energy and Environmental Chemical Engineering (ASCON-IEEChE). Fleur de Chine, Sun Moon Lake,TAIWAN, on 4-7 November 2018.