Butanol Production from Two-Stage ABE Fermentation of Food Waste

Prince of Songkla University
Pattani Campus

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Applied Chemistry

Prince of Songkla University

2018

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ชื่อวิทยานิพนธ์ การผลิตบิวทานอลจากการหมักเอบีอีสองขั้นตอนของเศษอาหาร

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บทคัดย่อ

ในงานวิจัยนี้ใช้เศษอาหารเป็นซับสเตรทสำหรับการผลิตไฮโดรเจนและกรดบิวทิริกจากการหมักไร้แสงโดยใช้เชื้อผสมในระดับห้องปฏิบัติการแบบแบทช์และแบบถังปฏิกรณ์ชนิดถังกวนแบบต่อเนื่อง (continuous stirred tank reactor: CSTR) ภายใต้สภาวะเทอร์โมฟิลิก (55 °C) จากการทดลองในระบบแบบแบทช์ให้ศักยภาพการผลิตไฮโดรเจนที่ 135.2 mL H_2/g-VS_{added} ที่ความเข้มข้นเริ่มต้นของเศษอาหารที่ 5.8 g-VS/L กรดบิวทิริกที่ความเข้มข้น 34.57 mM ซึ่งเป็นกรดหลักที่ผลิตได้ นอกจากนี้ยังพบกรดแลกติก กรดอะซิติก และกรดโพรพิโอนิก ความเข้มข้นน้อยกว่า 5 เท่า ที่ 7.62, 5.76 และ 2.30 mM ตามลำดับ การหมักกรดบิวทิริกในถังปฏิกรณ์ชนิดถังกวนแบบต่อเนื่องใช้เศษอาหารเป็นซับสเตรทดำเนินระยะเวลาการกักเก็บของเหลวในระบบเท่ากับ 4 วัน และอัตราการป้อนที่ 10 g-VS/$L_{reactor}$ สามารถผลิตแก๊สไฮโดรเจนดีที่สุดคือ 100 mL H_2/g-V_{Sadded} และสามารถผลิตกรดบิวทิริกได้ที่ 6.14 g/L

สารละลายที่มีกรดบิวทิริกมากมีศักยภาพในการเปลี่ยนไปเป็นบิวทานอลโดยใช้เชื้อ *Clostridium* sp. ในขั้นที่สอง (การผลิตเอบีอี) ทดลองแบบแบทช์ควบคุมอุณหภูมิ 37 °C ศึกษาผลของเชื้อแบคทีเรียต่างชนิดกัน (*Clostridium beijerinckii* ATCC 55025 และ *Clostridium butylicum* TISTR 1032) ศึกษาผลความเข้มข้นเชื้อเริ่มต้น (5%, 10% และ15% เชื้อ) ศึกษาผลของอาหารต่างกัน (อาหาร Tryptone Yeast extract Acetate (TYA) และอาหาร Free-N) ศึกษาผลความเข้มข้นน้ำตาลเริ่มต้น (20-60 g/L) และศึกษาผลของอัตราส่วนกรดบิวทิริกต่อกลูโคส (0/20. 1/20, 5/20, 10/20 และ 15/20 g/L) ความเข้มข้นบิวทานอลที่ผลิตได้สูงสุด 7.93 g/L จากการหมักของความเข้มข้นน้ำตาลเริ่มต้น 30 g/L เชื้อ 10% ของ *Clostridium beijerinckii* ATCC 55025 ที่เสริมด้วยอาหาร TYA

ศึกษาการผลิตเอบีอีแบบป้อนต่อเนื่องภายใต้อุณหภูมิ 37 °C โดยใช้ถังปฏิกรณ์แพคเบด (Packed Bed Reactor: PBR) ปริมาตรการทำงาน 2.5 L ใช้กลูโคส (30 g/L) เสริมด้วยอาหาร

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TYA โดยมีอัตราการป้อน 500 mL/d ค่า pH ในระบบควบคุมอยู่ในช่วง 4.0-6.5 ผลการทดลองพบ ผลิตภัณฑ์หลักคือกรดบิวทิริกที่ความเข้มข้น 3-5 g/L

คำสำคัญ: การหมักแบบไร้แสง, เศษอาหาร, บิวทานอล, การผลิตเอบีอี

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Thesis Title	Butanol Production from Two-Stage ABE Fermentation of Food Waste
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Major Program	Applied Chemistry
Academic Year	2017

ABSTRACT

In this study, food waste was used as substrate to feasibly investigate simultaneous hydrogen and butyric acid production from dark fermentation by using enriched mixed-cultures in laboratory scale batch reactor and continuous stirred tank reactor (CSTR) under thermophilic conditions (55 °C). The batch experimental results show that satisfied hydrogen production yield of 135.2 mL H_2/g-VS_{added} was achieved by using initial food waste concentration 5.8 g-VS/L. Butyric acid with concentration of 34.57 mM is dominant metabolic product, while other metabolic products of lactic acid, acetic acid and propionic acid were found at more than 5 times lower concentrations of 7.62, 5.76 and 2.30 mM, respectively. The CSTR fed with food waste was operated at 4-day HRT, corresponding to organic loading rate (OLR) of 10 g-VS/$L_{reactor}$ could produce highest hydrogen production yield of 100 mL H_2/g-VS_{added}, gathering with 6.14 g/L butyric acid.

Butyric acid rich solution could be potentially converted to butanol by using *Clostridium* sp. In the second stage (ABE production), batch experiments under mesophilic conditions (37 °C) were set up to investigate the effect on ABE fermentation by using different bacteria strains (*Clostridium beijerinckii* ATCC 55025 and *Clostridium butylicum* TISTR 1032), various initial inoculums concentration (5%, 10% and 15% by volume), different ABE fermentation mediums (Tryptone Yeast extract Acetate, TYA medium and Free-N medium), various initial sugar concentration (20-60 g/L), and different butyric acid to glucose (B/G) ratios (0/20. 1/20, 5/20, 10/20 and 15/20, g/L basis). The highest butanol concentration of 7.93 g/L was achieved from batch fermentation at initial 30 g/L sugar, 10% *Clostridium beijerinckii* ATCC 55025, and supplemented with TYA medium.

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The continuous ABE production under temperature 37 ^{o}C was studied by using Packed Bed Reactor (PBR) having 2.5- L working volume. Glucose (30 g/L) supplemented with TYA at flow rate of 500 mL/d. Meanwhile, pH in the reactor was maintained in range of 4.0-6.5. The results show that the main products were butyric acid with concentration of 3-5 g/L and butanol was not detected.

Keywords: Dark fermentation, Food waste, Butanol, ABE fermentation

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ACKNOWLEDGEMENT

I would like to express my deepest and sincere gratitude to my advisor, Associate Professor Dr. Prawit Kongjan for his kindness, valuable supervision, valuable suggestion, guidance and support encouragement all the time during this work.

I would like to thank my co-advisor, Associate Professor Dr. Rattana Jariyaboon and Dr. Shahrul Ismail for their helpful and valuable suggestion, support, guidance and criticism.

I would like to thank my committee, Dr. Somrak Panphon and Dr. Tussanee Srimachai for their helpful suggestion, constructive comment and criticism.

I would like to thank the Science and Technology Asian (SAT-Asian), Faculty of Science and Technology, and Graduate School, Prince of Songkla University (PSU), Pattani Campus for financial support of this work.

Appreciation is also expressed to all their practical help, my friends and my juniors for their support and help and the members of Universiti Malaysia Terengganu (UMT) for their help and friendship during in Malaysia.

I am also grateful to lecturer and staffs under Chemistry Division, Department of Science, Faculty of Science and Technology and School of Ocean Engineering, Universiti Malaysia Terengganu, Kuala Terengganu, Terengganu Darul Iman, for the valuable knowledge, encouragement, practical and technical assistance.

Last but not least, I would like to express my very special thanks to my family for their love, cheerfulness, understanding and encouragement throughout my life.

Thank you all.

Surananee Hayeeyunu

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LIST OF ABBREVIATIONS AND SYMBOLS

TS	Total solid
VS	Volatile solid
GC	Gas chromatography
CSTR	Continuous Stirrer Tank Reactor
PBR	Packed-Bed Reactor
TYA	Tryptone-yeast extract-acetate medium
HRT	Hydraulic retention time
OLR	Organic loading rate
VFA	Volatile Fatty Acid
PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis

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CHAPTER 1
INTRODUCTION

1.1 Research background

The increase in population, the demand for energy increases as well. The problem is that the continuing decline of oil and petroleum prices rise, so finding new energy sources. Bio-gas, bio-diesel and bio-alcohol are interesting in developing renewable energy. Ethanol is currently used to fill or replace petroleum-based oil. Bio-ethanol is an alcohol that has been widely studied and applied. But nowadays, with the attention butanol increased dramatically. Butanol is a liquid fuel that offers numerous advantages over ethanol, butanol has an energy value (29 MJ/L) similar to gasoline (32 MJ/L) and much higher than ethanol (16 MJ/L) (Lee *et al*., 2008b). In addition butanol has higher energy content, lower volatility, and lower miscibility with water, less hygroscopic and less corrosive, a higher flash point and can be transported in existing pipelines. Moreover, it can reduce hydrocarbon emissions by 95% and oxides of nitrogen by 37% (Bellido *et al*., 2014). Butyrate as the precursor for butanol generation from solventogenesis of butanol producing bacteria can be separately produced in conjunction with hydrogen production via dark fermentation. An enriched group of bacteria having multi-enzyme systems involved in two-step dark fermentation; hydrolysis and acidogenesis can produce hydrogen from carbohydrates along with the pathways of butyrate and acetate with the theoretical yields of 249 and 498 mL-H_2/g-sugars, respectively (Kongjan *et al*., 2011). Hydrogen is preferable to be produced through butyrate pathway, when the hydrogen partial pressures are above 60 Pa. During acidogenesis, hydrogen partial pressures higher than 60 Pa can inhibit proton reduction by NADH, shifting the fermentation towards butyrate or other reduced products instead of toward acetate (Angenent *et al*., 2004). And hydrogen gas is a clean, recyclable and high energy fuel (122 kJ/g) with lower air pollution and reduced greenhouse effects (Ozmihci, and Kargi, 2010).

In Thailand, food wastes generated is around 600,000 kg/day, approximately 50% of total waste in wet-weight ratio. It's one of the critically problems in environmental concerns. Alternatively, food wastes containing high

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amount of carbohydrates (starch, celluloses and hemicelluloses) have attracted attention in recent years as the potential substrates for the microbiological conversion of bio-fuels (biogas, ethanol, butanol and bio-hydrogen) (Lee *et al*., 2010). Total solids (TS) content and volatile solids (VS) content were 18.9% and 90.1% for food waste, respectively. Carbon (C) and Nitrogen (N) were 41.1 and 3.4%, respectively. (Wan *et al*., 2013).

Molasses, a viscous by-product of the refining of sugarcane is produced in large amount every year. Besides, containing high sugars content approximate 50-60% by weight, molasses also contains significant amount of protein, mineral salts and vitamins, which are suitable nutrient for microbial fermentation. Therefore it is considered as the potential and inexpensive substrate for producing value-added fuels such as ethanol, butanol and hydrogen (Wang and Jin, 2009).

Two-stage anaerobic digestion, two-phase digestion process is based on two physiologically different groups of microorganism. One group of acidogenic bacteria that converts organic matter into hydrogen, carbon dioxide, soluble organic acid (acetic acid, propionic acid and butyric acid) and alcohol. Soluble organic acid (butyric acid) from the first stage are fed into second stage, this phase for further anaerobic butanol production by sequential solventogenesis. There are literatures reported that two-stage fermentation was better than single-stage. By obtaining the optimum environmental conditions for each group of organisms, the two-stage anaerobic process provides several advantages over the conventional single stage.

In this research project, a new approach applying to producing of butanol from food waste and molasses is proposed by coupling dark fermentation using thermophilic mixed cultures and solventogenesis using *clostridias* strain of *Clostridium beijerikii* ATCC 55025. Biostat A plus is proposed to be used for fermentation of food waste in the first stage due to its capability of handling with substrates having a high suspended solid (SS) content, typically with a volatile solid (VS) content greater than 2% (Liu *et al*., 2008). Furthermore, it is simple to construct and is easy to regulate both acidity and temperature. Complete homogeneous mixing for direct contact between the substrate and active biomass can be achieved efficiently within continuous stirrer tank reactor (CSTR) (Hallenbeck and Ghosh, 2009). In solventogenic stage, cells of *Clostridium beijerikii* ATCC 55025 was immobilized on

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plastic carriers packed inside a packed-bed reactor (PBR). This type of immobilized reactor could effectively increase cell concentration in the reactor and consequently improve reactor productivity (Ezeji*et al*., 2007).

1.2 Research objectives

This research has its general objective it has three objectives as follow:

1.2.1 To optimize food waste concentration for butyrate production in first stage batch fermentation.

1.2.2 To optimize the ratio of butyrate and sugar used for butanol production in batch fermentation.

1.2.3 To study feasibility continuous for butanol production from glucose by using Pack Bed Reactor (PBR).

1.3 Scope of the research

1.3.1 First stage

Food waste concentration of 5%, 8%, 10% and 13%TS in single fermentation was optimized to obtain optimum butyric acids along with hydrogen and carbon dioxide using a 5-L jacketed fermentor of the model Biostat A plus (Sartorius, Melsungen, Germany) with working volume of 3.5 L fed with 10 %TS (W/V) of food waste.

Enriched mixed thermophiles originally obtained from digested POME are used as inoculums in the fermentor of the model Biostat A plus (Sartorius, Melsungen, Germany).

Fermentation at different butyric acid to glucose (B/G) ratios of 0/20. 1/20, 5/20, 10/20 and 15/20 was studied to butanol production in batch fermentation with working volume of 300 mL.

1.3.2 Second stage

2500 mL-PBR with working volume of 2200 mL was set up for butanol production from glucose.

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Clostridia strain used in solventogenic stage is *Clostridium beijerinckii* ATCC 55025 for butanol production was purchased from the American Type Culture Collection (ATCC). While, *Clostridium butyricum* TISTR 1032 was obtained from Thailand Institute of Scientific and Technological Research (TISTR).

1.4 Expected benefits from this study are:

a) Optimum food waste concentration to get highest butyrate in first stage batch fermentation was obtained.

b) Optimum ratio of butyrate and sugar (content in molasses) used for butanol production in batch fermentation was investigated.

c) Continuous butanol production in Pack Bed Reactor (PBR) was determined.

1.5 Conceptual framework

Concept of this research work was divided into 2 phase as shown in Figure 1.1

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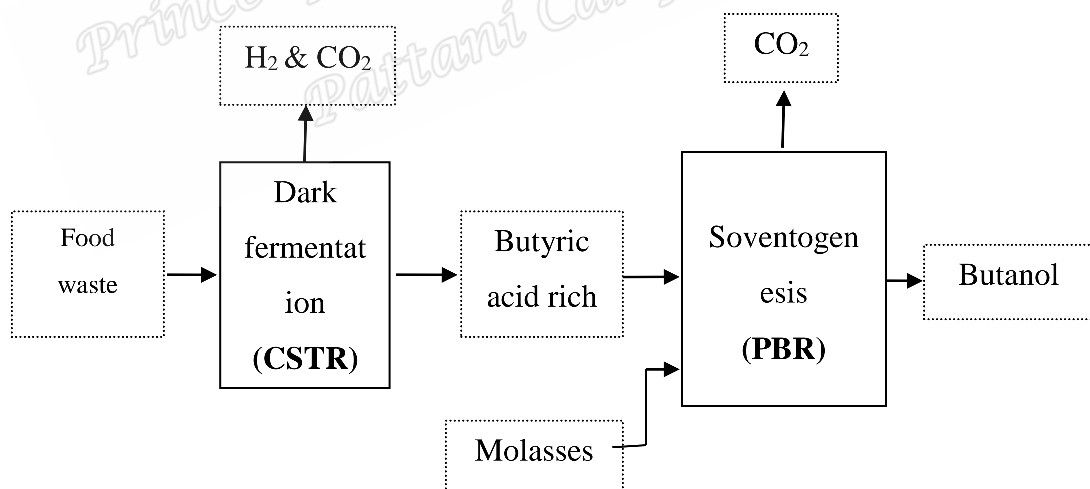

Figure 2.1 Proposed schematic of the two-stage ABE fermentation for production of rich butanol, CSTR: Continuous Stirred Tank Reactor, PBR: Packed Bed Reactor.

CHAPTER 2
LITERATURE REVIEW

2.1 Butanol

2.1.1 Butanol properties

Butanol (IUPAC nomenclature, 1-butanol, CAS no. 71-36-3), a four carbon (Primary alcohol) with a molecular formula of C_4H_9OH (MW 74.12 $g \cdot mol^{-1}$) (Figure 2.1), commonly known as n-butanol, butyl alcohol or methylolpropane. Butanol is igneous, slightly hydrophobic, colorless, obvious banana-like and strong alcohol odor. However butanol can cause irritation of the nasal mucosa. May cause sensitization by inhalation and skin contact. Butanol can dissolve early in the solution with other solvents but has less solubility in water. The physicochemical properties of butanol are shown in Table 2.1

Butanol can be produced in three different ways: (1). From the chemical process of the oil or hydrocarbon. (2). The fermentation process from some biomass. (3). From Advanced Processes it has a variety of substrates. The most popular way to do this is by fermentation using the appropriate bacteria and yeast. And the bacteria in *Clostridia* family often of butanol production.

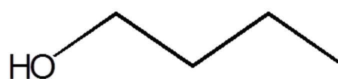

Figure 2.1 Structure of butanol

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Table 2.1 Physicochemical properties of butanol

Properties	Values
Atomic weight(g/mol)	74.12
Density (g/L)	0.0810
Boiling point (°C)	118
Melting point (°C)	-89.3
Flash point (°C)	25-29
Specific gravity	0.810-0.812
Igniting temperature (°C)	35-37
Auto-igniting temperature (°C)	343-345
Critical temperature (°C)	287
Critical pressure (hPa)	48.4
Explosive limits (vol% in air)	1.4-11.3
Water solubility 9.0 mL/100 mL	7.7 g/100 mL at 20 °C
Relative vapor density (air:1.0)	2.6
Vapor pressure (kPa at 20 °C)	0.58

Source: Wikipedia, 2018

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2.1.2 Application of butanol

Nowadays there are some serious environmental problems and global climate change, which affect life on earth from burning of problem. The problem is that the continuing decline of oil and petroleum prices rise, so finding new energy sources. Renewable energy sources such as solar, wind, water and biomass are attracting in developing renewable energy, which could be applied, worthily and effectively. Butanol production from the fermentation process with the attention increased dramatically, it can use the same type of biomass used to produce bioethanol. Butanol produced from the biological process can be used as a liquid fuel in the engine in the near future. Butanol can be produced from biomass by biochemical processes and can be mixed with gasoline at any concentration. Compared with ethanol, butanol has higher energy content and lower volatility and is less hygroscopic and less corrosive (Lee *et al.*, 2008) and energy content as compare to ethanol (Table 2.2). Beside,

butanol has been demonstrated to work in vehicles designed for use with gasoline without any modification, while ethanol can only be blended up to 85% with gasoline. Butanol, in contrast, can be blended at the refinery and storage infrastructure (pipelines, tanks, pumps, filling stations, etc.). Due to lower vapor pressure and safer to handle. Other physical properties of butanol are shown in Table 2.1.

Table 2.2 Specification of alcohol and conventional fossil fuel

Properties	Butanol	Gasoline	Ethanol	Methanol
Boiling point (°C)	117-118	27-221	78	64.7
Density at 20°C (g/L)	0.8098	0.7-0.8	0.7851	0.7866
Solubility in 100 g of water	immiscible	immiscible	immiscible	immiscible
Energy density (MJ/1)	27.29.2	32	19.6	16
Energy content/value (BTU/gal)	110000	115000	84000	76000
Air-fuel ratio	11.2	14.6	9	6.5
Heat of vaporization (MJ/kg)	0.43	0.36	0.92	1.2
Liquid Heat capacity (Cp) at STP (kJ/k-mol.°K)	178	160-300	112.3	81.14
Research octane number	96	91-99	129	136
Motor octane number	78	81-89	102	104
Octano/Water Partition Coefficient (as $\log P_{O/W}$)	0.88	3.52±0.62	-0.31	-0.77
Dipole moment (Polarity)	1.66	n.a.	1.7	1.6
Viscosity (10^{-3}Pa.s)	2.593	0.24-0.32	1.078	0.5445

Source: Loyarkat, 2014

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2.2 Metabolism of butanol production from *Clostridium* sp.

Biobutanol production can be produced from many microorganism species. The most of microorganisms used are genus *Clostridium* including, *Clostridium acetobutylicum*, *Clostridium beijerinkii*, *Clostridium saccharoperbutylacetonicum* and *Clostridium saccharobutylicum* are strictly anaerobe, spore-forming and heterofermentative bacteria. Other *Clostridium* species are able to form minor amounts of butanol as well such as *Butyribacterium methylotrophicum, Hyperthermus butylicus* and *Thermoanaerobacterium thermosaccharoyticum*.

ABE (acetone/butanol/ethanol) fermentation is process for butanol production via *Clostridium* species can be divided into two production phase, acidogenic phase (acid production phase) and solventogenic phase (solvent production phase). During exponential growth (first phase), the cells grow rapidly and producing acetic acid, butyric acid, CO_2 and H_2. Accumulation of the acids causes a rapid decrease in pH of the system.

Figure 2.2 show catabolic pathways of acid and solvent formation in *Clostridium acetobutylicum*, degrades carbohydrate to product. Inside the cell, mono- and disaccharides are then taken up by phosphoenolpyruvate-dependent phosphotransferase systems, which are already well described in *C. acetobutylicum* and *C. beijerinckii* (Kopke and Durre, 2011). Hexose sugars are metabolized to pyruvate via the Embden-Meyerhof-Parnas (EMP) pathway. The convert of 1 mol of a hexose yields 2 mol of pyruvate 2 mol of adenosine triphosphate (ATP) and 2 mol of reduced nicotinamide adenine dinucleotide (NADH). While, the pentose sugars via pentose phosphate pathway, which enter the glycolytic pathway. The uptake of 3 mol pentose to pyruvate yields 5 mol ATP and 5 mol NADH.

Pyruvate is converted to acetyl-CoA by enzyme a pyruvate: ferredoxin-oxidoreductase, lactate and acetoin are produced from pyruvate (Figure 2.1), catalyzed bylactate dehydrogenaseand acetolactate synthase plusacetolactate decarboxylase, respectively.

Acetyl-CoA is the central intermediate leading to the formation of acetate, butyrate, ethanol, acetone and butanol by CO_2 and H_2 as byproduct. Acetate is produced via acetyl phosphate by successive action of phosphotransacetylase (Pta)

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and acetate kinase (Ack). Meanwhile, butyrate is produce via butyryl-CoA and butyryl phosphate by successive action of enzymes phosphotransbutyrylase (Ptb) and butyrate kinase (Buk), respectively. Butyryl-CoA itself is produced from two molecules of acetyl-CoA by successive action of four enzymes are thiolase (ThlA), 3-hydroxybutyryl-CoA dehydrogenase (Hbd), crotonase (Crt) and butyryl-CoA dehydrogenase (Bcd) (Chen and Liao, 2016).

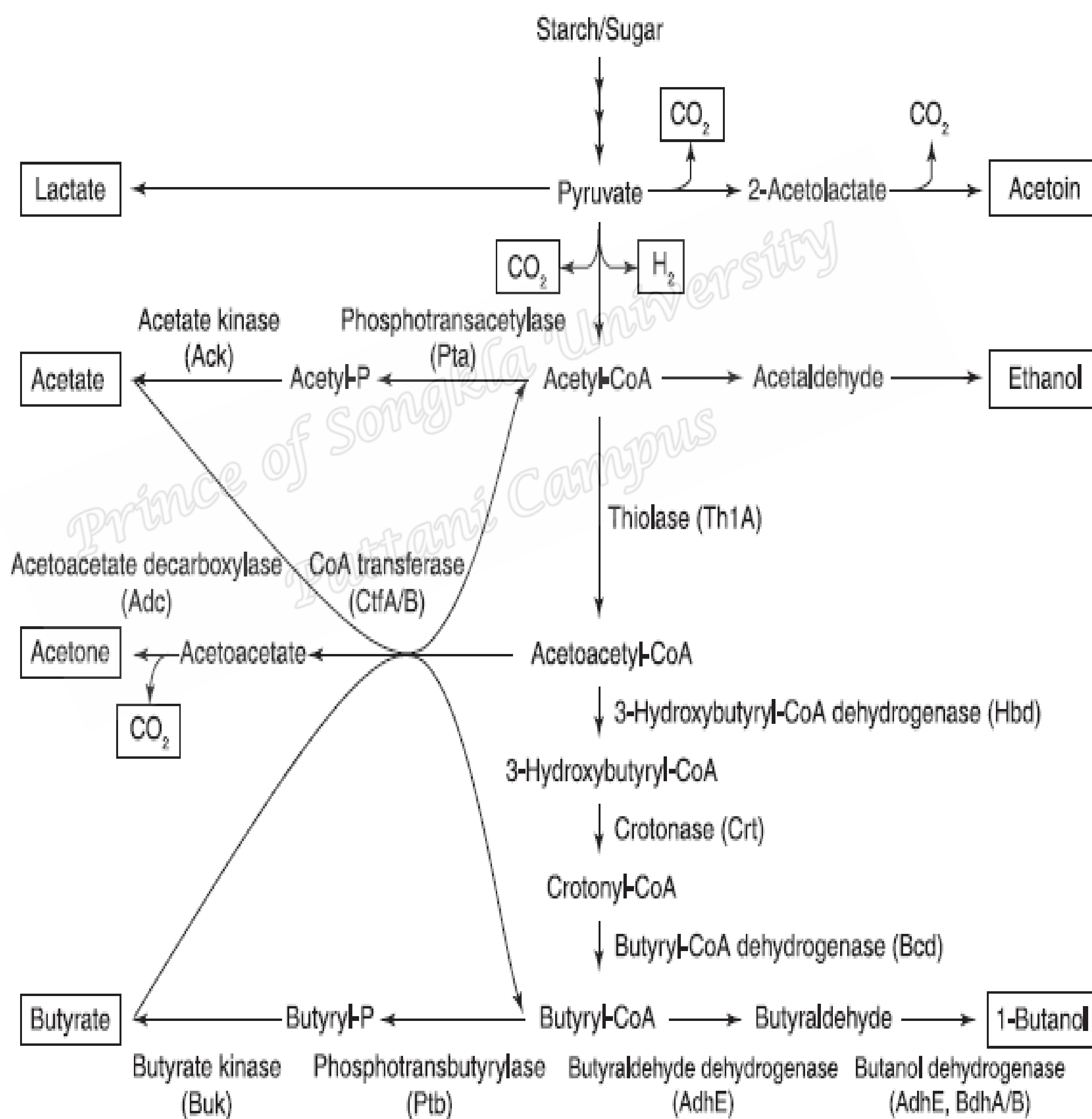

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Figure 2.2 Catabolic pathways of acid and solvent formation in *Clostridium acetobutylicum.*

Source: Kopke and Durre, 2011

Formation of solvents starts with an acetoacetyl-CoA:acetate/butyrate-CoAtransferase (CtfA/B), which converts the previously produced acids acetate and butyrate into the respective acetyl-CoA derivatives and acetoacetate, while the recycled acetyl-CoA and butyryl-CoA is used for the production of alcohols such as ethanol and butanol. Ethanol and butanol are produced from acetylaldehyde and butyraldehyde by successive action of ethanol dehydrogenase and butanol dehydrogenase, respectively. Ceases of acid production when solvent production begins in the solvent production step, solvent result were uptake to acid the pH increase (Gheshlaghi *et al*., 2009).

2.3 Factors effecting butanol fermentation

2.3.1 pH

The pH value has very important for fermentation system, which affects on enzyme activity in microorganism because each group of microorganism has different optimal pH range. Furthermore, enzymes also active within the specific pH range and also have maximum activity under optimum pH. In first phase is the acetogenesis phase, accumulation of the excreted acetic acids and butyric acid causes a rapid decrease in pH of the system. This serious threat to *C. acetobutylicum*, due to anaerobe bacterium is unable to maintain an internal pH, which is generally 1 unit higher than the external pH (Kopke and Durre, 2011). In second phase is the solventogenesis phase, when pH reaches a critical point, while high acids are assimilated butanol and acetone are produced. If the pH decreases less than 4.5 before sufficient acids are produced, solventogenesis will be quick and unproductive.

2.3.2 Temperature

A range of temperature is optimal for ABE fermentation by *Clostridium* spp., mesophilic and between 30 and 37 ^{o}C. From report of Al-shargani *et al*., (2012) the fermentation of 10 g/L butyric acid and 20 g/L glucose at various ratios with 30 ^{o}C can produce high butanol at 13 g/L by *Clostridium saccharoperbutylacetonicum*culture. Loyarkar *et al*., (2013) also reported high butanol 12 g/L at 37 ^{o}C by *Clostridium beijerinckii* culture.

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2.3.3 Sugar concentration

The initial sugar concentration is an important operational parameter for acetone-butanol-ethanol production. High sugar concentration (160 g/L) was found inhibit to *C. beijernckii* BA101 (Ezeji*et al*., 2003), it is toxic lead to decrease in biodegradability and process failure. However, at low sugar concentration (less than 20 g/L) was found small amount of acid in ABE production (Lee *et al*., 2008a).

.3.4 Solvent concentration

The product inhibition proved to be the most severe limitation during the ABE fermentation process. Acetone and ethanol are only moderately toxic, even at low concentrations of butanol has disastrous effects on bacterial cells. Butanol concentration at 1.1% (120 mM), the growth rate of *C. acetobutylicum* is decreased by 50% and butanol concentration at 1.5% (165 mM), growth is almost completely inhibited (Baer *et al*., 1987) this effect is caused by an increase in membrane fluidity and inhibition of membrane proteins such as transporters and ATPases

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2.3.5 Effect of oxygen

Butanol producing organisms require anaerobic conditions. A low redox potential (below -250 mV) is crucial for ABE fermentation. The research of O'Brien and Mortis (1971) found that *C. acetobutylicum* can detoxify molecular oxygen by NADH without forming H_2O_2. If in system was high oxygen concentration the rate of glucose consumption decreased and cell growth, RNA, DNA and protein syntheses were paused. In addition, summarize biochemical and physical consequences of oxygenation in organism for ABE fermentation are

a) NADH oxidase activity is increase.

b) The intracellular ATP level is fall.

c).Break of butyric acid establishes.

d) Starvation of energy and draining of reducing power.

2.4 Butanol production from two-stage

Two-stage ABE fermentation process divided into two phase, first stage (acedogenesis phase) produced via dark fermentation from mixed-culture for hydrogen production, the crucial stage to producing rich butyric acid. Second stage (solventogenesis phase) produced butanol from *Clostridium* species. In the first phase occurs during cells growth, substrate transform into primary metabolite (organic acid) by H_2 and CO_2 as byproduct. In second phase is stationary phase from organic acid converted to solvent (acetone and butanol) (Zverlov*et al*., 2006). Each stage of ABE fermentation different conditions due to in first phase produces acid cause to low pH in system while second phase high pH than first phase and which solvent can inhibit acid production of fermentation. Therefore thus one way to reduce the inhibition of acid production in the first stage if the fermentation process is divided into two stages and to increase the solvent yield in the second stage. According to Li *et al*., (2018) report that two-stage ABE fermentation in first with 20 g/L pretreated rice straw of *Clostridium beijerinckii* NCIMB 8052 the acidogenic fermentation produced 6.87 g/L butyric acid, with a total VFAs of 9.52 g/L in the supernatant of fermentation culture (SFC). With 40% rice straw hydrolysate and 60% SFC as co-substrate, butanol productions of and 13.8 g/L, were obtained in the ABE fermentation. The ABE-producing *clostridia* sp. as a good hydrogen production. Actually, in addition to ABE fermentation, hydrogen can also be collected during acidogenic fermentation.

two-stage continuous culture advantages over than a single-stage continuous fermentation setup because from report of Richter *et al*., (2013): (1) the pH and temperature can be optimized for each stage; (2) each stage can be adjusted to set different dilution and growth rates by the working volume, because of nutrient consumption in first stage and low dilution rates in second stage; (3) the acid produced in first stage can be converted into solvent in second stage during solventogenesis; (4) result to the accumulation of biocatalyst in second stage for high reactor productivity and can be further enhanced; and (5) the second stage nutrients to adjust sufficient balance for solventogenic bacteria viability and limitation of nutrients to maintaining the solventogenic state.

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2.5 Characteristic of food waste and its applications

Food wastes containing high amount of carbohydrates (starch, celluloses and hemicelluloses) and also high of protein and nutrient due to come from food, have attracted attention in recent years as the potential substrates for the microbiological conversion of bio-fuels (biogas, ethanol, butanol and bio-hydrogen) (Lee *et al*., 2010). Food waste not only production for energy, but also treats waste for environmental and social benefits (Zhang *et al*., 2013). The characteristic of food waste show in the Table 2.3.

Table 2.3 Characteristic of food waste

Parameter	Wang *et al*. (2014)	Agyeman and Tao (2014)	Wan *et al*. (2013)
pH	6.1	4.4	-
Total solid (%)	24.0	29.3	18.9
Volatile solid (%)	-	-	90.1
SCOD (g/L)	25.2	-	-
Total carbohydrate (%)	39.5	-	-
Total nitrogen (%)	1.8	3.8 (%TS)	3.4
Total protein (%)	11.0	-	-
Total carbon (%TS)	-	48.4	41.1
Carbon/Nitrogen	-	12.74	12.09
Sulfur (g/kg TS)	-	3.4	0.92(%)
Total calcium (g/kg TS)	-	1.7	-
Total magnesium (g/kg TS)	-	0.7	-
Total potassium (g/kg TS)	-	9.6	-
Total sodium (g/kg TS)	-	10.1	-
Total iron (mg/kg TS)	-	41	-
Total zinc (mg/kg TS)	-	32	-
Total copper (mg/kg TS)	-	5	-
Manganese (mg/kg TS)	-	8	-
Molybdenum (mg/kg TS)	-	0.3	-

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CHAPTER 3
METERIALS AND METHODS

3.1 Materials

3.1.1 Microorganisms

Mixed-culture butyrate and hydrogen production was collected from biogas production system from palm oil mill effluent, Pattani province. The sludge used through the removal of microorganisms produces methane and enrich for hydrogen producing bacteria by shock load method with sucrose 50 g/L.

Clostridium beijerinckii ATCC 55025 for butanol production was purchased from the American Type Culture Collection (ATCC). *Clostridium butyricum* TISTR 1032 were obtained from Thailand Institute of Scientific and Technological Research (TISTR) Bangkok.

3.1.2 Media

Tryptone yeast extract acetate (TYA) medium was used preculture and main culture for ABE fermentation by used pure culture *Clostridium* spp. and Free-N medium are variance for fermentation. The compositions of TYA and Free-N medium were summarized in Table 3.1. The pH was adjusted to 6.2 (Al-Shorgani *et al*., 2011).

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Table 3.1 The compositions of Tryptone-Yeast extract-Acetate (TYA) and Free-N media

TYA medium		Free-N medium	
Component	(g/L)	Component	(g/L)
Glucose	20-80	Glucose	0-20
Yeast extract	2	KH_2PO_4	0.5
Tryptone	6	$FeSO_4 \cdot 7H_2O$	0.01
CH_3COONH_4	3	Distilled water	1 L
KH_2PO_4	0.5		
$MgSO_4 \cdot 7H_2O$	0.3		
$FeSO_4 \cdot 7H_2O$	0.01		
Distilled water	1 L		

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Basic anaerobic (BA) medium was used for butyrate production by used mixed-culture are summarized in Table 3.2.

Table 3.2 The compositions of basic anaerobic (BA) medium

Component	Chemical, Concentration (g/L)	Volume (mL)
A	NH_4Cl, 100; NaCl, 10; $MgCl_2.6H_2O$, 10; $CaCl_2.2H_2O$, 5	10
B	$K_2HPO_4.3H_2O$, 200	2
C	$C1_2H_6NO_4Na$, 0.5	1
D	H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2.2H_2O$, 0.038;$MnCl_2.4H_2O$, 0.05; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2.6H_2O$, 0.05; $NiCl_2.6H_2O$, 0.092; EDTA, 0.5; $Na_2SeO_3.5H_2O$, 0.066	1
$NaHCO_3$	$NaHCO_3$, 52	50
Vitamin	Yeast extract, 1	-

Volume (mL): Solution volume (mL) for 1 L BA-medium preparation

Source: Angelidaki & Sanders, 2004

3.1.3 Substrate

Food waste was collected from the main canteen (Lan-It) of Prince of Songkla University, Pattani campus. After collection the waste was undergo preparation and characterization processes.

Molasses was purchased by local shop in Pattani province. The chemical composition was characteristic after purchased.

3.1.4 Chemicals

The following chemicals were used in this study

Chemical	Grade	Suppliers
n-Butanol	AR	Ajax Finechem, Australia
Ethanol	AR	VWR, France
Acetone	AR	Fisher Scientific, UK
Sodium Hydroxide	AR	LOBAChemie, India
Yeast extract powder	-	HiMedia, India
Sodium hydrogen carbonate	AR	Ajax Finechem, Australia
D-Glucose	AR	Ajax Finechem, Australia
Tryptone	-	HiMedia, India
Phenol	AR	Carlo Erba
Sulfuric acid	AR	ANaPURE
Acetic acid	AR	J.T.Baker, China
Butyric acid	AR	Panreac, Spain
Propionic acid	AR	LOBAChemie, India
Potassium Dihydrogen Orthophosphate	AR	Ajax Finechem, Australia
Boric acid	AR	Ajax Finechem, Australia
Magnesium Sulfate	AR	Panreac, Spain
Sodium Thiosulfate Pentahydrate	AR	Ajax Finechem, Australia
Ammonium Acetate	AR	LOBAChemie, India

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3.1.4 Apparatus

The apparatus was used in this study:

3.1.2.1 Erlenmeyer flask 125 and 250 mL

3.1.2.2 Beaker 50 and100 mL

3.1.2.3 Cylinder10 and100 mL

3.1.2.4 Volumetric flask 10, 50 and100 mL

3.1.2.5 Volumetric pipette 1 and2 mL

3.1.2.6 Micropipette 100 and1000 µL

3.1.2.7 Crucible

3.1.2.8 Buchner filter

3.1.2.9 Test tube

3.1.2.10 Syringe filter

3.1.2.11 Cuvette

3.1.2.12 Serum bottle

3.1.2.13 Syringe

3.2 Instruments

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The following instruments were used for analysis in this study

Instruments	Series	Suppliers
Autoclave	HVE-50	HIRAYAMA, Japan
Balance 4 digit	PA413	OHAUS, USA
Centrifuge	TT-3K-10K	Hercuvam
Incubator	30-1060	Memmert, Germany
Gas Chromatography (FID)	G1530A	Hewlett Packard, USA
Gas Chromatography (TCD)	GC-14A	SHIMAZU, Japan
pH meter	MP220	Mettler Toledo, UK
Spectrophotometer	LIBRA-S22	Biochrom, England
Vortex	G-560E	SCIENTIFIC, USA
Water bath	SC-100	Thermofisher, USA
Hotplate Stirrer	HTS-1003	LMS, Japan
Vacuum pump	DOA- V130-BN	Waters, USA
Peristaltic pump	WT600-2J-A	Longerpump, China

3.3 Experiment method

3.3.1 Food waste preparation

Food waste was collected from the main canteen (Lan-It) of Prince of Songkla University, Pattani campus. Bones in collected food waste was separated out before crushed with electric blender and stored at temperature -4 ^{o}C until further use.

3.3.2 Food waste and molasses characterization

Food waste and molasses will be characterized the following parameters: Total Solis (TS), Volatile Solid (VS), Total carbohydrates, Total protein, Total nitrogen, Total ammonia nitrogen, Volatile fatty Acids (VFA) and pH, as show in Table 4.1.

Table 3.3 Analytical methods for food waste and molasses characteristics.

Characteristics	Analysis Methods
Total Solid (TS)	AOAC Official Method 920.193 (1995)
Volatile Solid (VS)	AOAC Official Method 920.193 (1995)
Ash content	AOAC Official Method 920.193 (1995)
Total Kjeldhl Nitrogen (TKN)	AOAC Official Method 973.48 (2000)
Total carbohydrates	Phenol-Sulfuric acid method
C H N O and S	CHNS-O Analyzer

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3.3.3 Dark fermentation in first stage

3.3.3.1 Batch fermentation

Thermophilic mixed culture originally obtained from hydrogen production reactor using Palm Oil Mill Effluent (POME) as substrate (Department of Science, Faculty of Science and Technology, Prince of Songkla University, Pattani Campus) will be used inoculums. 125 mL-bottle with a working volume of 60 mL with consist of 10% TS (W/V) of food waste in 15 mL basic anaerobic (BA) medium and 15 mL thermophilic mixed culture will be used for mixed culture stimulation. The bottle will be subsequently sealed with butyl stoppers, secured with aluminum crimps purged with and purged with N_2 for 3–5 min to ensure anaerobically conditions. It will be then incubated at 55 ºC for 7 days. Gas samples will be taken daily for

measurement of volume composition biogas. All experiment will be performed in triplicate.

A 5-L jacketed fermentor of the model Biostat A plus (Sartorius, Melsungen, Germany) with 3.5 L working volume will be first started up as batch reactor adding with 150 mL of the inoculums and 1200 mL at 5, 8, 10, 13% TS (W/V) food waste in basic anaerobic (BA) medium. The temperature will be controlled at 55 ºC by hot water circulating in a water jacket. The soluble starch was used as positive control. Liquid samples will be also taken periodically (2-3 days/times) for analysis of VFAs, alcohols, total sugars and microbial community. The effluents generated from the optimum conditions will be collected and kept at -4 ºC for further use in the solventogenic stage.

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3.3.3.2 Bacteria Community Analysis in mixed culture

Total gDNA was extracted from TIANamp Soil DNA Kit (TIANGEN Biotech (Beijing) Co., Ltd.). Amplification mixtures were used 5x PCR Master Mix II (GMbiolab Co., Ltd., Taiwan) with a final volume of 25 µl. The mixtures contained 25 pmol of each primer, The region 16S rRNA genes of bacteria was PCR-amplified by the first polymerase chain reaction (PCR) with universal primer 1525r (5' AAGGAGGTGWTCCARCC 3') and 27f 5' GAGTTTGATCCTTGGCTCAG 3') under conditions of an initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 40 sec, extension at 72 °C for 1 min, with final extension at 72 °C for 10 mins. The PCR product was analyzed on 1.0% agarose gels electrophoresis. In the second PCR, primer 518r (5' GTATTACCGCGGCTGCTGG 3') and 357f (5' CTCCTACGGGAGGCAGCAG3') with CG clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGG GG) were used to amplify the fragment of V3 region of 16S rDNA product from first PCR for bacteria. The PCR program were corresponded to an initial denaturation 95 °C for 3 min follow by 30 cycles of three steps: 95 °C for 1 min, 55 °C for 30 sec, and 72 °C for 1 min and final extension at 72 °C for 10 min. PCR products were stored at 4 °C and analyzed on 1.0% agarose before DGGE. The DGGE analysis of the PCR products was performed by electrophoresis for 20 min at 20 V and 16 hrs. at 70 V through 8% polyacrylamide gel containing a linear gradient of denaturant (100%

denaturant corresponds to 7 M urea and 40% (v/v) formamide deionised with AG501-X8 (mixed bed resin) ranging from 40% to 70% in 0.5 x TAE buffer at a constant temperature of 60 ℃ (DGGE unit, V20-HCDC, Scie-Plas Limited, UK). The gel was stained with Sybr-Gold (1000x concentration) for 1 h. and visualized on a UV transilluminator. Most of the bands were excised from the gel and re-amplified with the forward primer without a GC clamp and the reverse primer. After re-amplification, PCR products were purified using E.Z.N.A cycle pure kit (Omega Bio-tek, USA) and sequenced using reverse primer 518r andsequenced by the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). The closest matches for partial 16S rRNA gene sequences were identified by the ribosomal database project (http://rdp.cme.msu.edu/). This used the SeqMatch program and basic local alignment search tool (BLAST) with nucleotide database in the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3.3.3 Dark fermentation in Continuously Stirred Tank Reactor (CSTR)

The 10 L-CSTR reactor (Figure 3.1) was operate for butyric acid production 7 L working volume was maintain at thermophilic condition (55 °C). The enriched hydrogen producing bacteria from serum bottle. Start up with 800 mL of molasses in 4200 mL basic (BA) medium and 2000 mL of mixed culture. Subsequently, operate with food waste as substrate at the HRTs a 4 days, organic loading rates (OLRs) of 10 g-VS/$L_{reactor}$ d (1,019 g/d).

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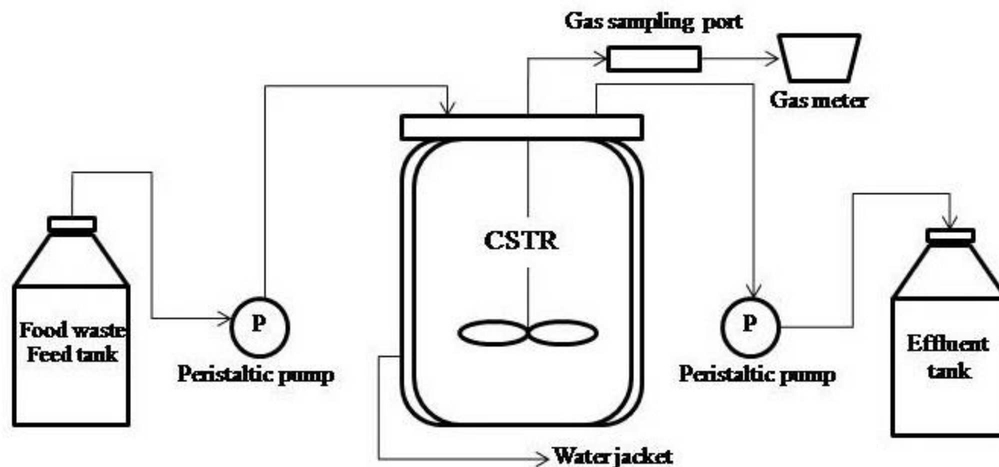

Figure 3.1 Schematic of Continuously Stirred Tank Reactor (CSTR) system

3.3.4 Second stage reactor set up and operation (Solventogenesis)

3.3.4.1 Batch fermentation

A spore of *Clostridium* strains stock culture was transferred into 270 mL of sterilized Tryptone-yeast extract-acetate medium (TYA), incubated anaerobically for 12-16 h, OD_{600} about 0.5 was used to fermentation. The ingredients of TYA media are summarized in Table 3.1.

Batch fermentation will be set up to determine the effect of different strains (*Clostridium beijerinckii* ATCC 55025 and *Clostridium butylicum* TISTR 1032), effect of initial inoculums concentration (5,.10 and 15% inoculums), effect of medium (Tryptone Yeast extract Acetate (TYA) medium and Free-N medium) and effect of different butyrate to glucose (B/G) ratios of 0/20. 1/20, 5/20, 10/20 and 15/20 (Al-Shorgani *et al.*, 2012) on butanol production. 500 mL bottles with a working volume of 300 mL were used for co-fermentation of first stage at. Triplicate batch fermentation will be carried out using 10% (V/V) seed culture, which will be *Clostridium* sp. previously enriched in a capped serum bottle containing tryptone-yeast extract-acetone (TYA) medium (Tashiro *et al.*, 2004). Will be set-up as the control. Blank bottles will be also prepared by using only pure TYA medium and inoculums in order to account for possible background butanol production which will be to be subtracted from butanol produced in the bottles with various mixing B/G ratios. Initial pH of fermentation broth in all bottles will be adjusted to 6.2 by using 10

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M NaOH (Al-Shorgani *et al*., 2012). The bottles will be subsequently sealed with butyl stoppers, secured with aluminum crimps and purged with N_2 for 3–5 min to ensure anaerobic conditions. All bottles will be then put into a 37 ºC incubator for 48 hours. Periodically, Liquid samples will be taken periodically for analysis of VFAs, acetone, butanol, ethanol and total sugars.

3.3.4.2 Acetone-Butanol-Ethanol production in Packed Bed Reactor (PBR)

A 2500-mL packed bed reactor (PBR) (Figure 3.2) with 2000 mL liquid volume, medium, plastic carriers will be sterilized for 20 min. at 121 ºC. The PBR packed randomly with plastic carriers will be then inoculated with 200 mL of seed culture, which will be *Clostridium beijerinckii* ATCC 55025 previously enriched in a capped serum bottle containing tryptone-yeast extract-acetone (TYA) medium (Tashiro *et al*., 2004). The reactor will be to flush with nitrogen gas for 10 min to ensure an anaerobic atmosphere. An optimum mixture of acidogenic effluent and molasses obtained from batch fermentation is to be added until the liquid volume in the reactors reach 200 mL. A recirculating flow rate of 18 mL/h is applied for 3 days in the PBR reactor to allow the seed culture to be attached on plastic carriers. The reactors will be switched to continuous feed mode with solution containing acidogenic effluents and glucose at the B/S ratio of 0.5 in TYA medium at 20 hr-HRT, corresponding to dilution rate 1 h (Napoli *et al*., 2010). The temperature was maintained at 37 ºC by circulating hot water inside the water jacket of the PBR. The optimum B/S ratio will be used to determine the optimum dilution rate of PBR by varying HRT (20, 10, 5, 2.5 and 1 h). Liquid samples will be taken periodically (2-3 days/time) for analysis of VFAs, acetone, butanol, ethanol and total sugars.

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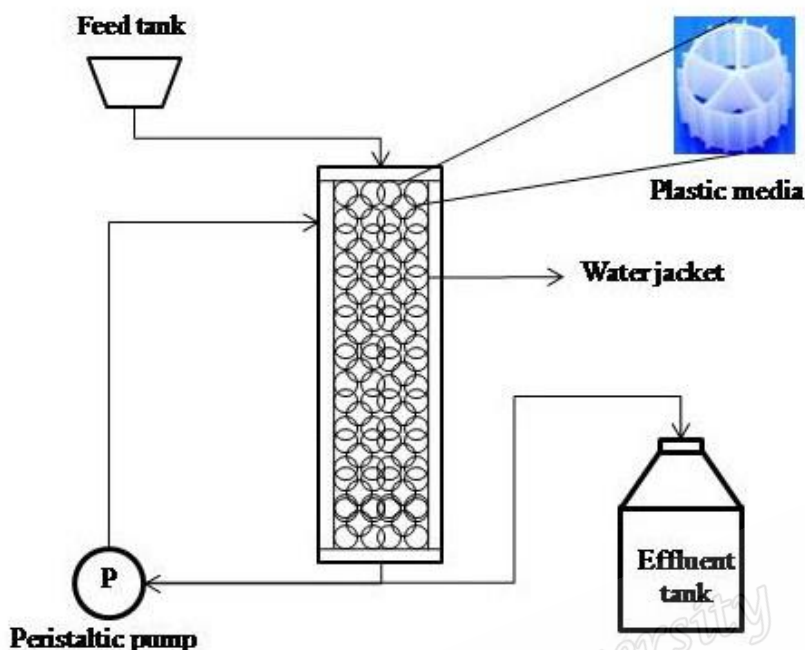

Figure 3.2 Schematic of Packed Bed Reactor (PBR) system

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3.4 Analytical methods effluent

3.4.1 Growth measurement

The concentration of inoculums (*Clostridium beijerinckii* ATCC 55025 and *Clostridium butyricum* TISTR 1032) in the fermentation broth was determined by ultraviolet-visible spectrophotometer (Libra S22, Biochrom, UK) at 600 nm (OD_{600}).

3.4.2 Determination of products

Acetone, butanol, ethanol, acetic acid propionic acid and butyric acid was determined by gas chromatography (Hewlett Packard, G1530A, USA) equipped with a flame ionization detector (FID) and a HP-INNOWax (19091N-113, 30 m × 0.32 mm × 0.25 µm, Agilent technologies) is a polyethylene glycol (PEG) stationary phase. The oven temperature was run to increase from 70 to 230 °C with ramping of 25 °C/min. The injector and detector temperature was set to 240 °C. Helium was used as a carrier gas at a flow rate of 1 mL/min.

3.4.3 Determination of gas

The hydrogen content was analyzed with gas chromatography (Shimadzu, GC-14A, Japan) equipped with a 1.5 m stainless column paced with molecular sieve 58 (80/100 mesh) and thermal conductivity detector (TCD). Argon was used as a carrier gas with a flow rate of 15 mL/min. The injector, oven and detector temperature was set 100, 50 and 100 ^{o}C, respectively.

3.4.4 Determination of total sugar

Total sugar concentrations were determined according to Dubois et al. (1956) by adding 1 mL of sample to test tubes and then mixed by vortexing with 5% phenol solution. 5 mL of conc. sulfuric acid was added to each test tube and left at room temperature for 10 minutes. The samples were analyzed on an ultraviolet-visible spectrophotometer (Libra S22, Biochrom, UK) against the blank at a wavelength of 490 nm. Total sugar concentration was determined from the calibration curve obtain from with glucose standard.

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3.4.5 Calculation of C/N ratio

The equation for calculating C/N was as follows:

C/N ratio = Carbon concentration (%TS)/nitrogen concentration (%TS)

Carbon concentration and nitrogen concentration was determined by CHNS-O Analyzer

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Characteristics of food waste and molasses

Food waste's main substrate in this research collected from the canteen Aquacalture Tropical Institute (Akuatrap) of Universiti Malaysia Terengganu, the canteen Lan-It and Lan-lay of Prince of Songkla University, Pattani campus. Primary characteristics are shown in Table 4.1. The high quantity of organic (VS), usually organic matter are main compound in food waste such as main biochemical type carbohydrate protein and lipid such biomolecules can be degraded by anaerobic microorganisms fermentative bacteria group to fatty acids along with carbon dioxide and hydrogen. C/N ratios, which are the ratio parameters of organic carbon to nitrogen. C/N ratios of food waste is 14, shows that the quantity of carbon and nitrogen are suitable that is used a substrate for fermentation consistent with Kongjan *et al*., 2016 found that C/N ratios in the range13-15 co-substrate of palm oil mill effluent and skim latex serum can produce a satisfactory hydrogen yield (69-85 ml-H_2/g-VS) by batch fermentation at thermophilic temperature without nutrients and buffer supplement.

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Table 4.1 The characteristics of food waste and molasses

Parameter	**Food waste**	**Molasses**
TS (%)	17.44-24.42*	68-74.03*
VS (%TS)	97.15 -99.59*	90.32-92.58.*
Ash(%TS)	1.39-2.85*	7.42.-9.68*
Total sugar (g/g)	0.035 ±3.55	845
Protein (g/g)	0.043 $\pm 9.0\times10^{-2}$	-
C/N ratio	14.08	33.65
Oil&grease (g/$g_{fresh\ FW}$)	0.105$\pm 1.0\times10^{-3}$	-

* n=3 sample

4.2 Dark fermentation

4.2.1 Dark fermentation in batch experiment

4.2.1.1 Hydrogen production from inoculums preparation

Initial mixed-culture use food waste as substrate for hydrogen production along with the production of butyric acid are the anaerobic seed sludge was collected from biogas production system from palm oil mill effluent. The sludge used through the removal of microorganisms produce methane and enrich for hydrogen producing bacteria by load shock method (O-Thong *et al*., 2009) with sucrose 50 g/L. When used mixed-culture after treated ferment with initial loading 10 g-VS_{added}/L of food waste and glucose without nutrients and buffer supplement were 135.2 mL H_2/ VS_{added} and 80.6 mL H_2/ VS_{added}, respectively as showed in Figure 4.1 when compare with glucose as positive control hydrogen yield of food waste higher than that control about 40.38%. Hydrogen was produced simultaneously throughout the fermentation. Gas production was initially low, but increased rapidly in the exponential and stationary phases. Hydrogen production from food waste higher than glucose without nutrients and buffer supplement, show that food waste had appropriate ratio of carbon and nitrogen source so can produce hydrogen higher. Anaerobic fermentation of carbohydrate for hydrogen production to be butyric acid and acetic acid, bacteria produced butyric acid higher than when pressure of hydrogen higher than 60 Pa (Angenent *et al*., 2004) so when uncontrolled pressure of hydrogen lower than 60 Pa in fermentation system can produce hydrogen along with butyric acid production, can be as substrate for butanol production by solventogenic bacteria group such as *Clostridium acetobutylicum* and *Clostridium beijerinckii*.

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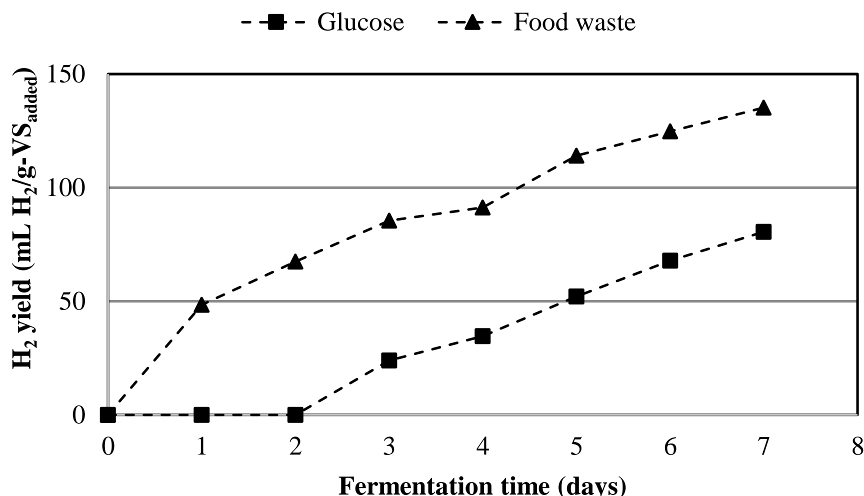

Figure 4.1 Hydrogen production yields of inoculums without medium addition.

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4.2.1.2 Butyric acid production from different initial concentration of food waste in batch fermentation

Food waste with different initial concentration of 5, 8, 10 and 13%TS were fermented in comparison in 5-L jacketed fermenters of the model Biostat A Plus (Sartorius, Melsungen, Germany) with working volumes of 2 L. Process temperature was maintained at 55 ± 3 °C by using a heating jacket. Concentration of carbohydrate during fermentation at different initial concentration (Figure 4.3) decrease rapidly between fermentation show that the ability of carbohydrates to transform organic acids of bacterial fermentative groups.

Only the process of manufacture of butyric acid obtains the theoretical hydrogen production of 249 mL-H_2/g-sugar (Reaction(1)) and only the process of manufacture of acetic acid obtains the theoretical hydrogen production of 498 mL-H_2/g-sugar (Reaction(2)), while propionic acid and ethanol production without hydrogen production. Moreover Homo acetogens bacteria group can produce acetic acid by using hydrogen and carbondioxide as substrates.

$$C_6H_{12}O_6 + 2H_2O \longrightarrow C_3H_6COOH + 2H_2 + 2CO_2 \quad \Delta G^0=-251.7 \text{ kJ/mol} \quad (1)$$

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad \Delta G^0=-2641.0 \text{ kJ/mol} \quad (2)$$

Figure 4.2 shows the volatile fatty acid (butyric acid, acetic acid and propionic acid) and ethanol production during fermentation using various concentrations of food waste such as 5, 8, 10 and 13%TS. The highest concentration of butyrate 6.76 g/L was obtained at 13%TS, follow by 4.53 g/L in 5%TS, 1.71 g/L in 8%TS and 2.55 g/L in 10%TS of food waste.

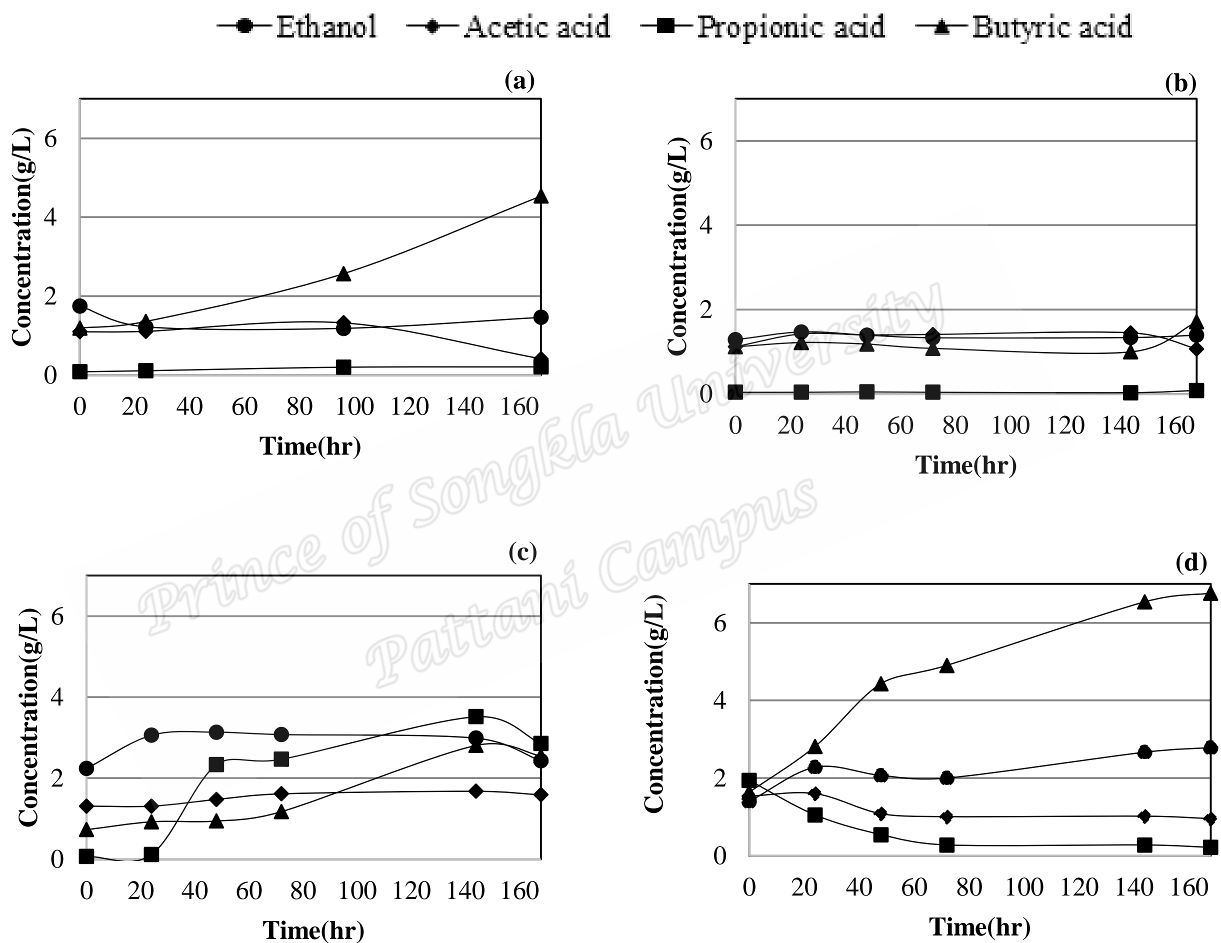

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Figure 4.2 Volatile fatty acid (VFA) and ethanol at different concentration of food waste, (a) 5%TS (b) 8%TS (c) 10%TS and (d) 13%TS.

The butyrate concentrations generated from batch having 5%TS and 13%TS of food waste are higher thanthat having 8%TS and 10%TS of food waste due to the main dominant strain in the reactor of 13%TS and 5%TS of food waste is *Clostridium* sp. (Figure 4.4 and Table 4.2). This strain is capable to convert sugar to butyrate. As evidently shown in Figure 4.3, Total sugars from batch having 13%TS of food waste was rapidly degraded and converted to butyrate.

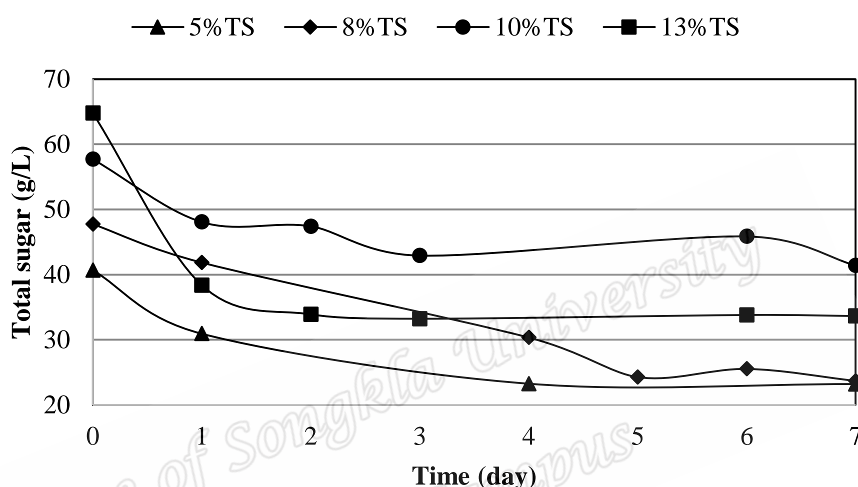

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Figure 4.3 Total carbohydrates at different concentration of food waste.

4.2.1.3 Bacteria Community Analysis

Fermentation broth from hydrogen and butyrate production under anaerobic conditions at various 5%, 8%, 10% and 13%TS of food waste were taken to analyze micro-communities by using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) assay. This analysis method could provide structures and quantities of microorganisms containing in mixed- cultures group from DNA band intensities (Stamper *et al*., 2003). Result of PCR-DGGE assay are shown in Figure 4.4.

Microorganism populations found are the main structure of those produce hydrogen and butyrate. During dark fermentation process at different concentrations of food waste (5%, 8% 10% and 13%TS of food waste), the main microorganisms of *Clostridium* sp. *Lactobacillus* sp., *Weissella* sp., *Ruminococcus* sp. and *Leuconostoc* sp. (Figure 4.4 and Table 4.2). *Clostridium* sp. has been previously reported as

hydrogen producing bacterium due to these bacteria can degrade carbohydrate into acetic acid/butyric acid coupled with hydrogen in anaerobic process in thermophilic condition (Jariyaboon *et al*., 2015). Therefore, *Lactobacillus* sp., *Weissella* sp. and *Leuconostoc* sp. are lactic acid producing bacteria and inhibit in *Clostridium* sp. bacteria group, if the main of this bacteria group to decrease in hydrogen production process (Adams and Nicolaides, 1997), this is confirmed by the accumulation of lactic acid in effluent of the hydrogen production process. *Ruminococcus* sp. is a genus of bacteria in the class Clostridia, they obtain nutrients by breaking down cellulose that comes through the digestive system of the host organism, these organisms are also capable of fermenting glucose and xylose, and do not produce spores. Corresponding with 13%TS of food waste in this experiment obtain butyric acid was the highest due to there are bacteria here. In addition, also found *Pediococus* sp. *Bacillus* sp. *Shewanella* sp. and *Sulfitobacter* sp.

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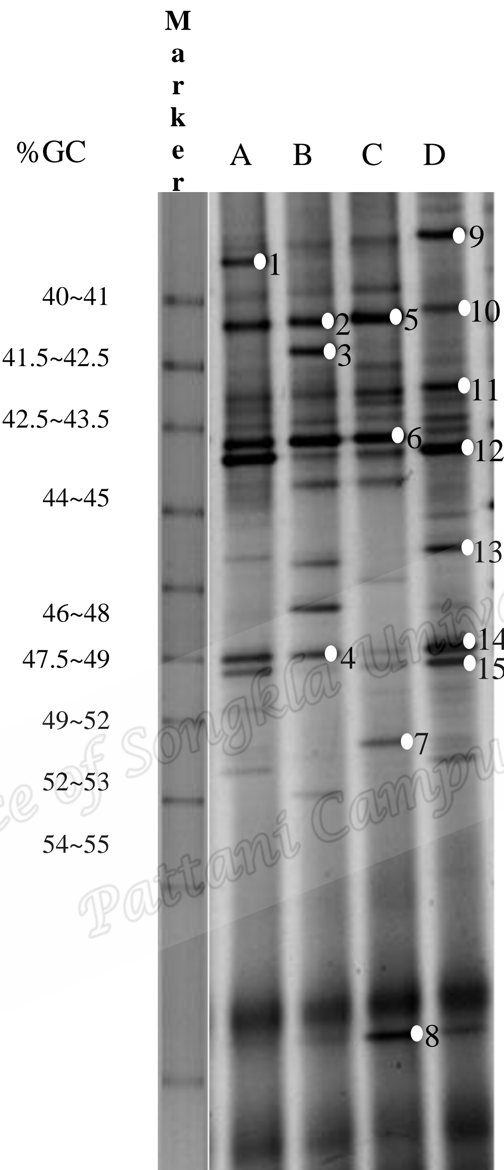

Figure 4.4 PCR-DGGE profiles from sludge with different reactor. A) 8%TS at 144 hr, B) 5%TS at 168 hr, C) 10%TS at 168 hr and D) 13%TS at 168 hr.

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Table 4.2 Result of DGGE bands (Figure 4.4) with GenBank database.

Band	strains	Access No.	Sequence
1	*Lactobacillus* sp.	EU715408	TCTCCTACGGGAGGCAGCAGTAGGGAATCTTC CACAATGGACGCAAGTCTGATGGAGCAACGCC GCGTGAGTGAAGAAGGTTTTCGGCTCGTAAAG CTCTGTTGTTGGTGAAGAAGGATAGAGGCAGT AACTGGTCTTATATTGACGGAATCAACCAGAA CGCGGAATT
2	*Lactobacillus* sp.	HE858543	TCTCCTACGGGAGGCAGCAGTAGGGAATCTTC CACAATGGACGCAAGCCTGATGGAGCAACACC GCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAG CTCTGTTGTTAAAGAAGAACAAGTACGAGAGT AACTGTTCATACGTGACGGTCCAACCAGAACT CCGGAGC
3	*Leuconostoc* sp.	LC097081	CTCCTACGGGAGGCAGCAGTAGGGAATCTTCC AAATGGGGGAAAGGGTGATGGAGCAAATGGG GTGAGTGATGAAGGTTTTCGGGTGGTAAAGCT ATGTTGTATTGAAGAAGAAAAAGTATAGTAAA TGGTTTTATTTGACCGTACTAACGGTATGCGAC GCAGGCC
4	*Clostridium* sp.	EF639852	TCTCCTACGGGAGGCAGCAGTAGGGTCTCCTA CGGGAGGCAGCAGTGGGGAATATTGCGCAAT GGGGGAAACCCTGACGCAGCAACGCCGCGTG AGCGATGAAGGTCTTCGGATTGTAAAGCTCTG TCTTTAGGGACGATAATGACGGTACCTAAGAG ACGCACGATGG
5	*Lactobacillus* sp.	HE858543	TCTCCTACGGGAGGCAGCAGTAGGGAATCTTC CACAATGGGCGCAAGCCTGATGGAGCAACACC GCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAG CTCTGTTGTTAAAGAAGAACACGTATGAGAGT AACTGTTCATACGTGACGGTATAACCAGAACT CACGACCC
6	*Lactobacillus* sp.	HE858547	CATTGGGAGGGTACCGTTTTGTTTTTTCCTACG GGAGGCAGCAGTAGGGAATCTTCCACAATGGA CGAAAGTGTGATGGAGCAACACCGCGGGAGT GAAGAAGGTTTTCGGTTCGTAAAGCTCTGTTG TTGGAGAAGAAGGATAGAGAGAGTAACTGTTC TTAATTGACGTAATTACAGAAAGCAGGACTGT CGCC

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Table 4.2 Result of DGGE bands (Figure 4.4) with GenBank database (cont.)

Band	strains	Access No.	Sequence
7	*Pediococcus* sp.	EU85194	CTCCTACGGGAGGCAGCAGTAGGGAATCTTCC ACAATGGACGCAAGTCTGATGGAGCAACGCCG CGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGC TCTGTTGTTGGGAGAAGACACGTGCCGGAGAG TCACTGCGCCGACGTGACGGTACCGACCAGAA TTCACGGAGT
8	*Lactobacillus* sp.	AJ508724	ATTATACAGGGGGTCATAAATGGCCATGGAAA TAAAATTTTTTTTCTCCTACGGGAGGAGCAGTA GGGAATCTTCCACAATGGGCGAAAGCCTGATG GAGCAACACCGCGTGAGTGAAGAAGGGTTTCG GCTCGTAAAGCTCTGTTGTTAGAGAAGAAAAC GTAGTGAGTAACGTTCGCAGGACGTGACCGTA TTCAACCAGAAGCACGCAG
9	*Weissella* sp.	HQ897604	TCTCCTACGGGAGGCAGCAGTAGGGAATCTTC CACAATGGGCGAAAGCCTGATGGAGCAACGC CGCGTGTGTGATGAAGGGTTTCGGCTCGTAAA ACACTGTTGTAAGAGAAGAATGACATTGAGAG TAACTGTTCAATGTGTGACGGTATCTACCAGA ACGGAGCGG
10	*Ruminococcus* sp.	FJ371845	AGGGGAGGAAGAAGTGGGGTATCTTACGGAA GGGGGGAAAGGGGGATGGAGCAAAACGGAGG GAGTGAGGAAGGGTTTGGGCTGGTAAAGCGAT GATGGTGGTGAGGAAGTAAAGGGGTGGTATA GGGTCGGGAGAGTGACCGTAGGGCAAGAGAA GCGGTATGGCC
11	*Bacillus* sp.	DQ779013	TACGGGAGGCAGCAGTAGGGTTTCTTCCGGAA GGGAGGAATGGGGGATGGAGCAAAGTGGGGT GAGTGATGAAGGTTTTAGGGTGGTAAAGCTAT GTTGTTGGTGAAGAAGTAAAGGTGTTGTTTTGT TGTGGATTTTGACGGTATGGTAAAGGGGAAGG GGGTCTGGA
12	*Lactobacillus* sp.	AF095563	TTCTCCTACGGGAGGCAGCAGTAGGGACTCTT CCACAAGGGACCAAAGTCTGATGGAGCACCGC CGCGTGAGTGAAGAAGGTCTTCGGCTCCTAAA GCTCTGTTGTTGGTGAAGAAGTATAGCTGCAG TAACTGGTCCGATAATGACGGTAACAACCAGA

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Table 4.2 Result of DGGE bands (Figure 4.4) with GenBank database (cont.)

Band	strains	Access No.	Sequence
13	*Shewanella* sp.	EU268274	AAGGGAGGGAGCAGTAGGGATTTTTAGGAA AGGAAGAAAGGGGGAATATTGAACAATGGGG GAAAGAAGGAGGTATTAAGGTCGTAAAAGTG ATGAAGGTTTAAGATTGTAAAGATAGTGTTAA GTAAATGAATAGATGACGTAATAAAGGAAAA AGATTCTTGAC
14	*Clostridium* sp.	NR_044849	TTCTCCTACGGGAGGCAGCAGTAGGGTCTCCT ACGGGAGGCAGCAGTGGGGAATATTGCGCAA TGGGGGAAACCCTGACGCAGCAACGCCGCGTG AGCGATGAAGGTCTTCGGATTGTAAAGCTCTG TCTTTAGGGACGATAATGACGGACCTAAGGAG GAAACGCAGATATG
15	*Sulfitobacter* sp.	JQ349481	TTTCTTTTCCTACGGGAGGCAGCAGTGGGGTA TCTTACGGGAGGGAGGAGTGGGGAATATAGG AAAATGGGGGAAATGATGAAGGAGTAAGGCT GGGAAAGCGATGAAGGTCTTAGGATAGTAAA GCTGTGTCTTTAGGAGGGATGAATGACCGTAG GGAAGAGAGCGTGATGTGGCC

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4.2.2 Dark fermentation in Continuously Stirred Tank Reactor (CSTR)

The butyric acid-CSTR reactor was operating at the HRTs a 4 days, organic loading rates (OLRs) of 10 g-VS/$L_{reactor}$d (1,019 g/d) under thermophilic condition (55 ^{o}C) and maintained pH in the reactor. Figure 4.5 shows hydrogen production rate and hydrogen production yield after 51 days, operation at 0-2 day hydrogen production rate is highest because exposed it to high load (shock load) of molasses (100 g/L). During fermentation at these high loads, appropriate for hydrogen production, high concentrations of volatile fatty acid (VFA) are accumulated resulting in acidification and inhibition of methanogen (Kongjan *et al.*, 2011). For 51 days of operation, no methane was detected, indicating that methanogenic archaea were completely repressed by the operating conditions, due to the low pH between 4.82-

6.25 (Figure 4.6) that was established in the reactor, which was self-regulated by bicarbonate buffer added the substrate generate in the process.

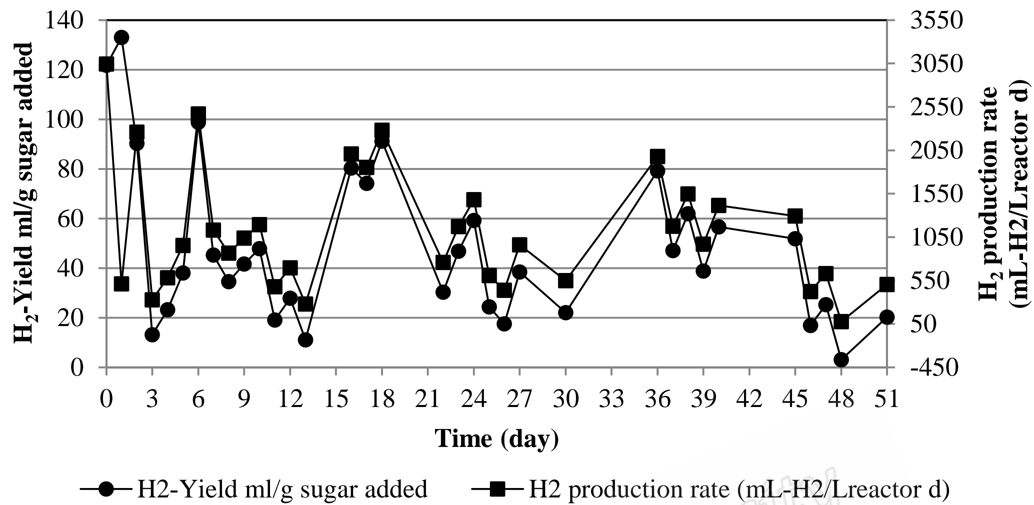

Figure 4.5 H_2 production yield and H_2 production rate under thermophilic temperature from CSTR reactor by H_2 Yield (mL/g $_{\text{sugar added}}$) and H_2 production rate (mL-H_2/$L_{reactor}$ d).

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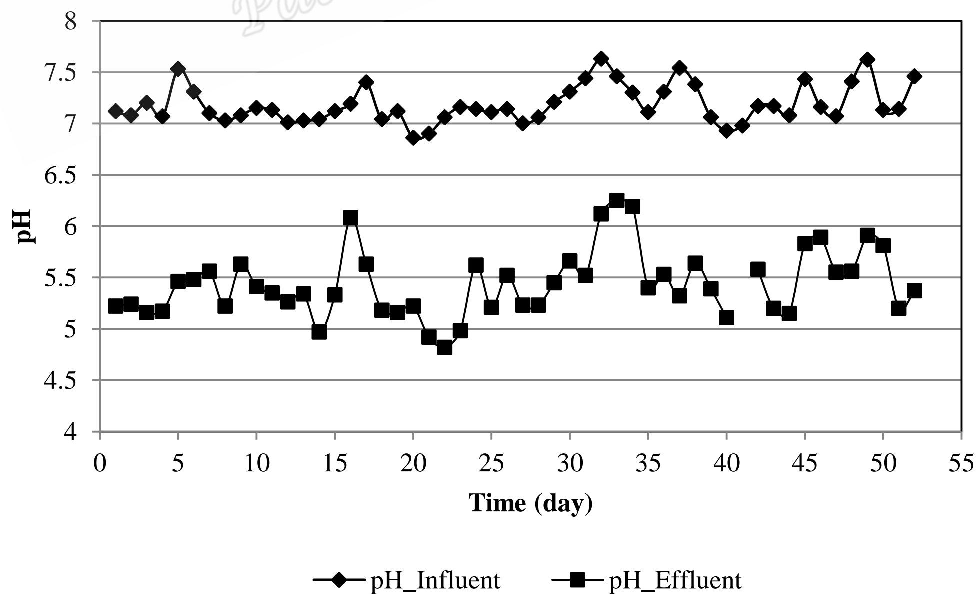

Figure 4.6 pH influent and effluent under thermophilic temperature from CSTR reactor.

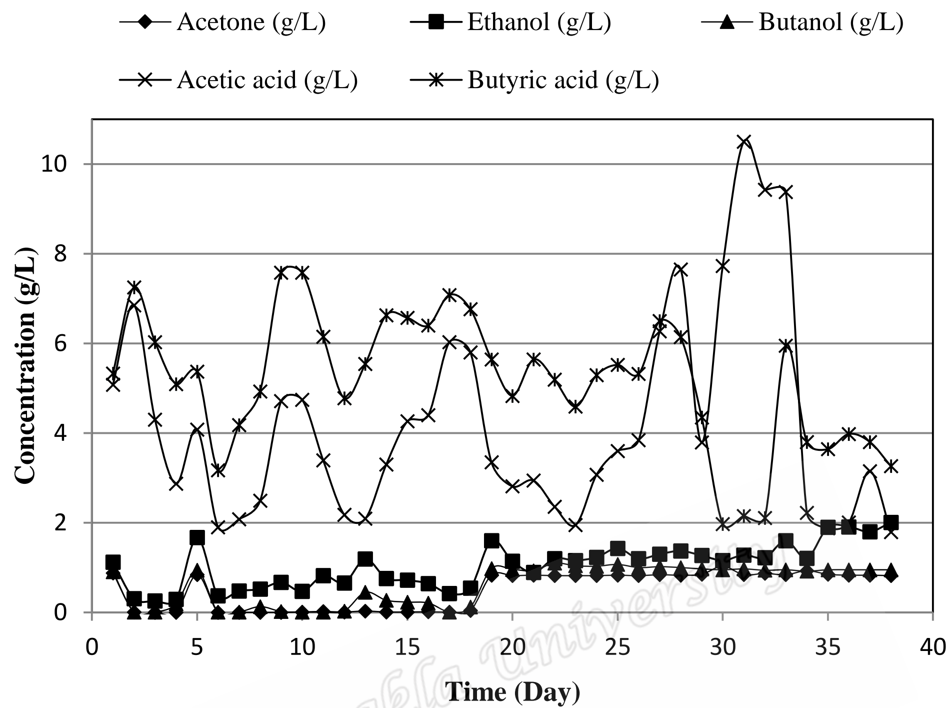

Figure 4.7 VFA and alcohol production thermophilic temperature from CSTR.

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Figure 4.7 shows the result of fermented food waste by CSTR under anaerobic conditions at 55 °C resulted in the production of organic acids (Acidogenesis), which transformed the organic matter into organic acids include acetic acid and butyric acid. Analysis of volatile fatty acids after fermentation at 0-29 day butyric acid has a higher concentration than acetic acid, an average of 6.14 g/L, it shows that the amount of organic material entering the system has an appropriate amount for the bacteria to decompose these organic substances. During the 30-33 days, the acetic acid is higher than the butyric acid because that is appropriate on producing acetic acid and between 34-38 days, the concentration of both acids decreased steadily, but the concentration of the butyric acid tended to increase and higher than acetic acid.

Between day 30-33, acetic acid is higher than butyric acid due todark fermentation via both butyric and acetic pathways for hydrogen production controlled by hydrogen partial pressure. When hydrogen partial pressure less than 60 Pa, 1 mole of glucose are directed to 2 moles of acetic acid and 4 moles of hydrogen. Hydrogen

partial pressures above 60 Pa, the direction of production is in the form of 1 mole of butyric acid and 2 mole of hydrogen (Figure 4.8). Major of effluent with butyric acid is favor to be used to enhancing butanol production in the second stage of solventogenesis (Angenent *et al.*, 2004).

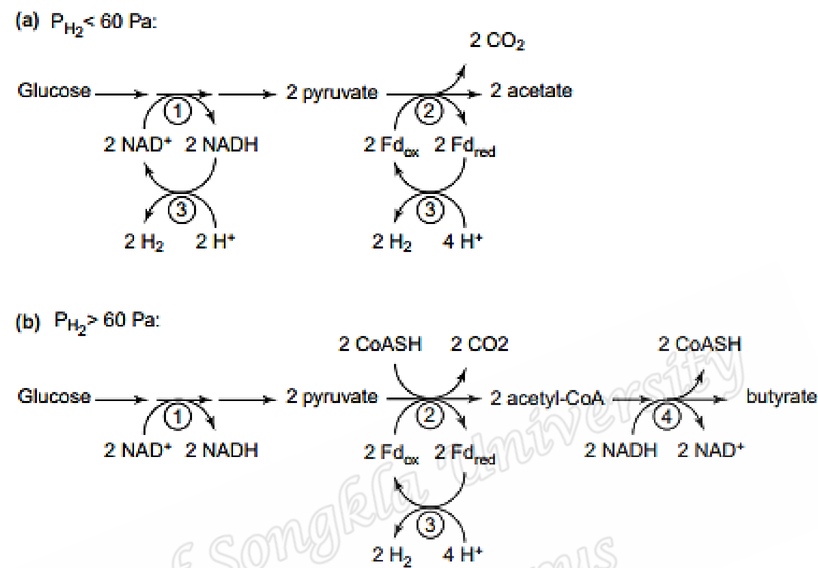

Figure 4.8 Schematic of biological butanol production

(a) hydrogen partial pressure less than 60 Pa.

(b) Hydrogen partial pressures above 60 Pa.

Source : Angenent *et al.*, 2004

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4.3 Acetone-Butanol-Ethanol (ABE) production

4.3.1 Effect of different strain

The acetone-Butanol-Ethanol (ABE) fermentation was conducted at a controlled temperature of 37 °C. The batch cultivation of *Clostridium beijerinckii* ATCC 55025 and *Clostridium butyricum* TISTR 1032. Glucose was used as the carbon source for butanol fermentation. The concentration of 20, 40, 60 and 80 g/L were examined for the batch fermentation. Figure 4.9 shown cumulative H_2 in batch different of initial of glucose concentration and strains.

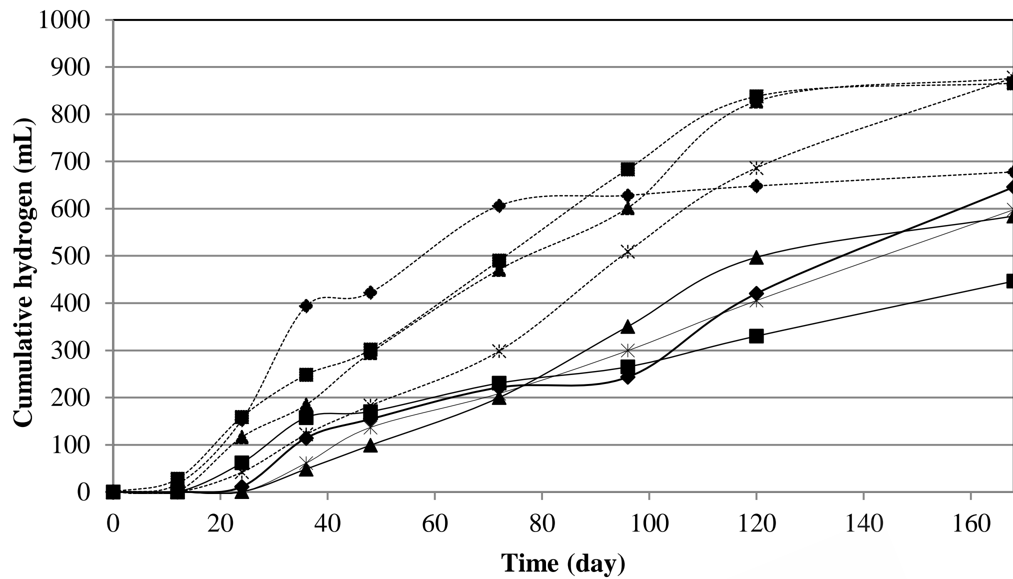

Figure 4.9 Cumulative H_2 in batch different of initial of glucose concentration and Strains(■ 20 g/L, ◆ 40 g/L, ▲ 60 g/L and ✳ 80 g/L of glucose by used *C. beijerinckii* ATCC 55025 and ■ 20 g/L, ◆ 40 g/L, ▲ 60 g/L and ✳ 80 g/L of glucose by used *C. butyricum* TISTR 1032 (10% inoculums, TYA medium).

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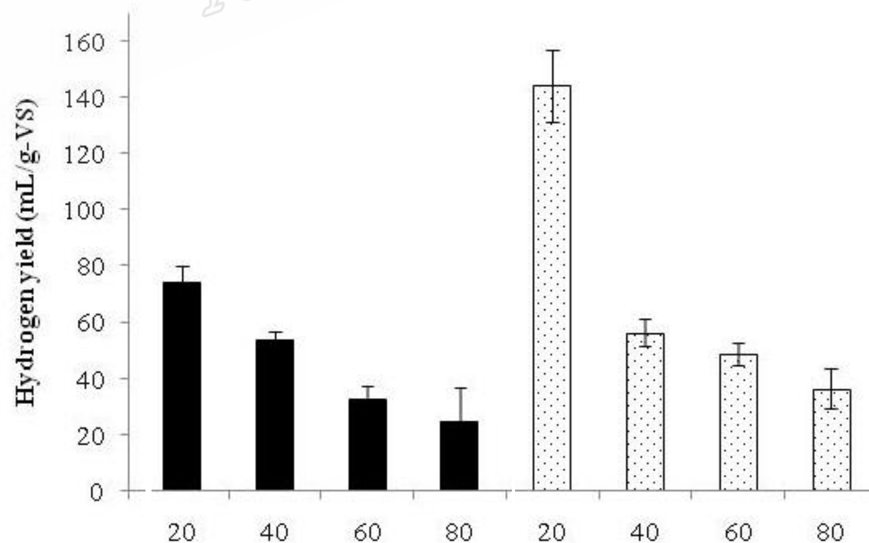

Figure 4.10 Hydrogen yield in batch fermentation, ■*Clostridium beijerinckii* ATCC 55025 and □*Clostridium butyricum* TISTR 1032 (10% inoculums, TYA medium).

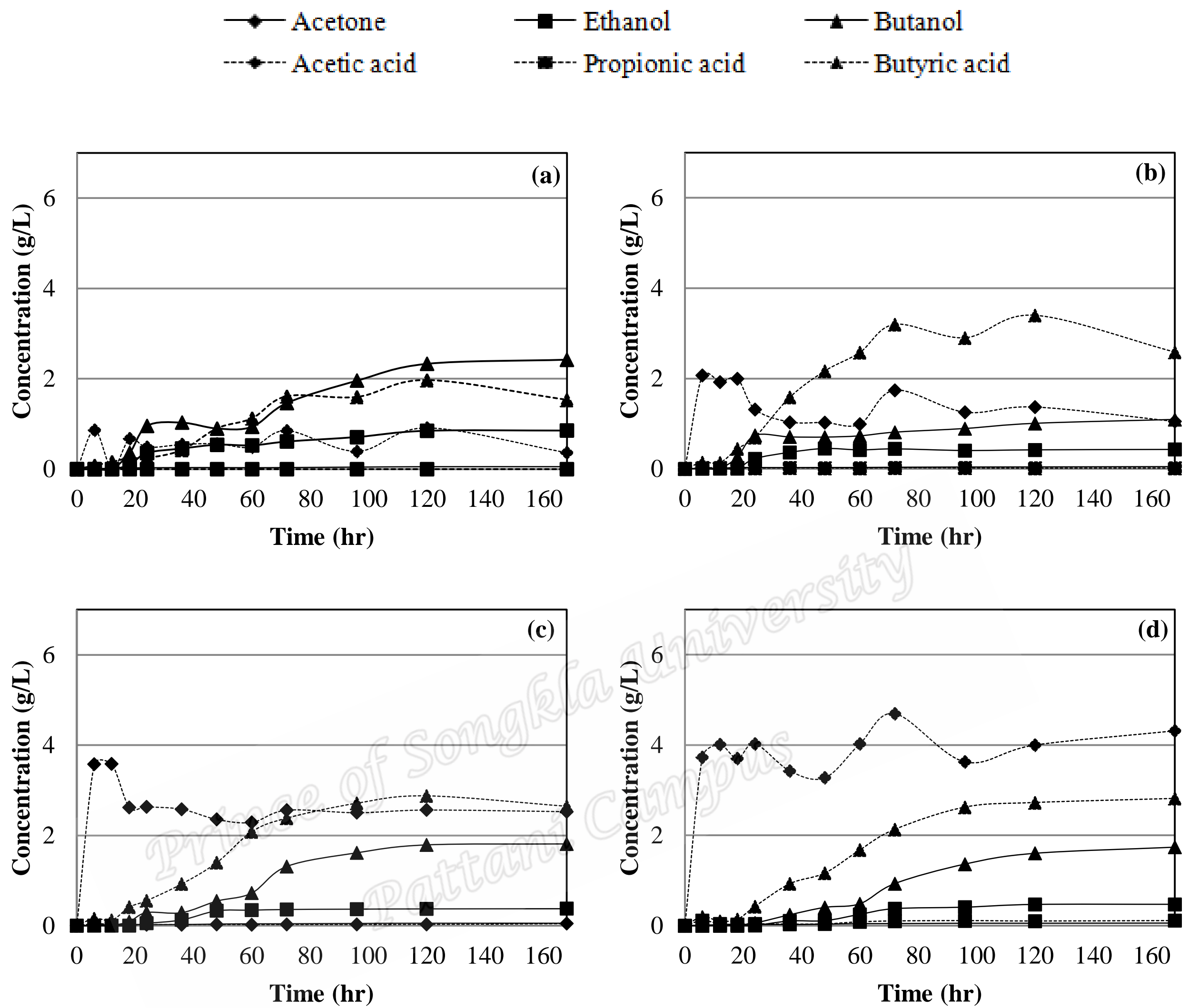

Figure 4.11 ABE productions in batch fermentation by culture *Clostridium butyricum* TISTR 1032 at concentration (a) 20 g/L, (b) 40 g/L, (c) 60 g/L and (d) 80 g/L of glucose (10% inoculums, TYA medium).

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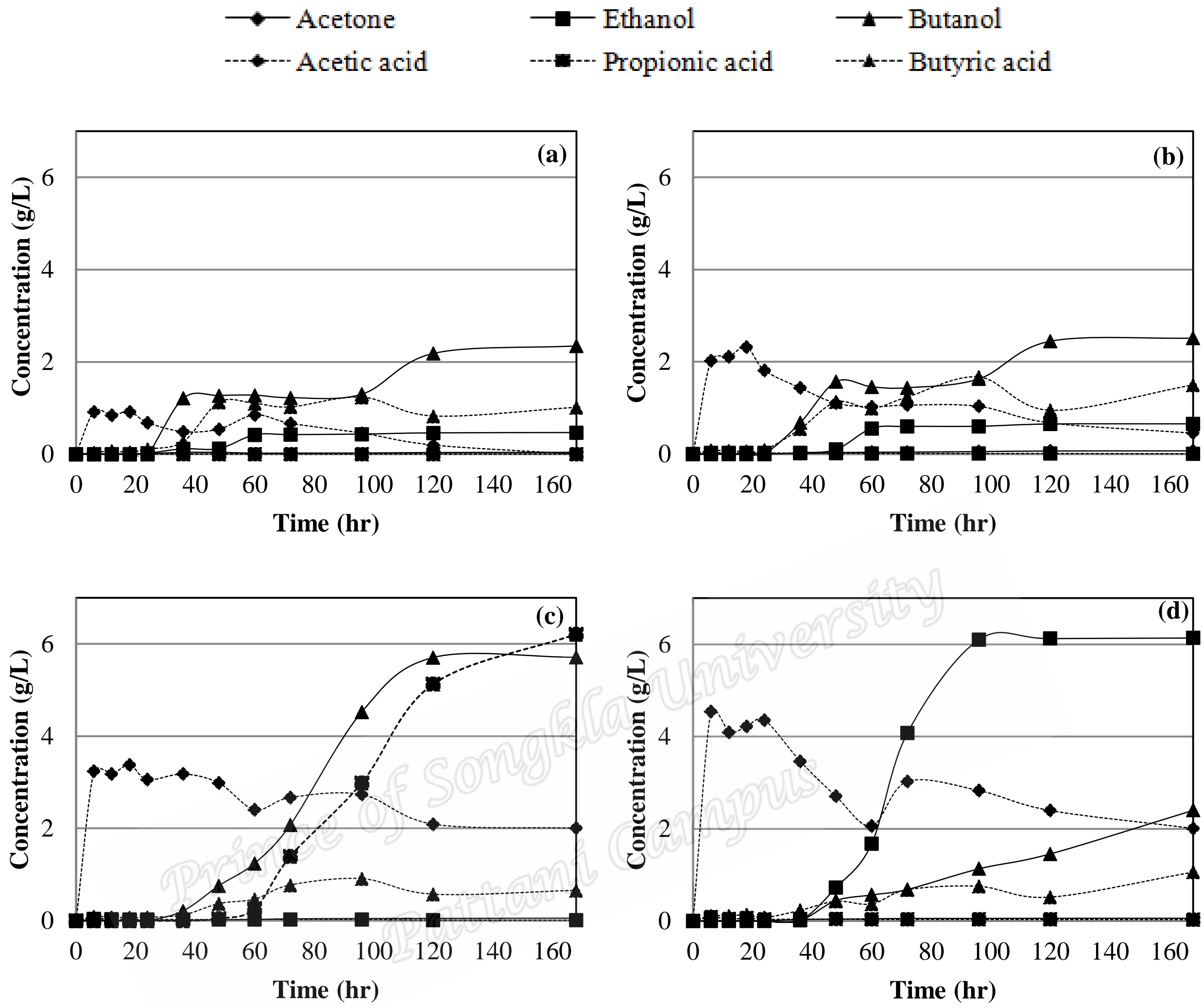

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Figure 4.12 ABE productions in batch fermentation by culture *Clostridium beijerinckii* ATCC 55025 at concentration (a) 20 g/L, (b) 40 g/L, (c) 60 g/L and (d) 80 g/L of glucose (10% inoculums, TYA medium).

Butanol fermentation was also carried out using different concentration of glucose as the carbon source, and different of microorganism strain, the result shown in Figure 4.11 and Figure 4.12. Figure 4.12 can be see that butanol was successfully produced using *Clostridium beijerinckii* ATCC 55025 at 60 g/L glucose, but long lag phase (36-60 h), after 60 h butanol increased rapidly. Provide highest butanol about 5.71 g/L time at 168 hour.

In the Figure 4.11 from *Clostridium butylicum* TISTR 1032 of every concentration lower solvent concentration were detected. Total sugar of *Clostridium beijerinckii* ATCC 55025 at 60 g/L glucose (concentration of produce highest butanol). For the first 24 hr, total sugars were decreased rapidly. After that, it was gradually decreased to about 28.59 g/L as show in the Figure 4.13. Microorganism *Clostridium beijerinckii* ATCC 55025 consume sugar (glucose) in acedogenesis phase for primary metabolites production such as acetic acid butyric acid and ethanol. Then primary matabolites were then converted to secondary metabolites of ethanol, acetone and butanol during stationary phase (Tashiro *et al*., 2004).

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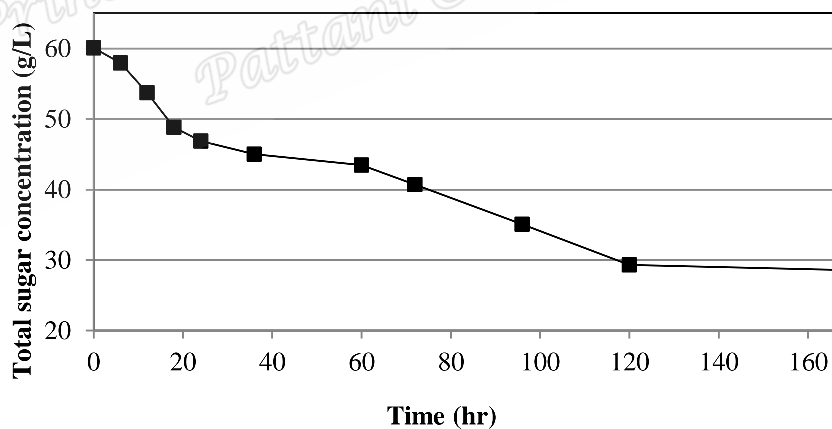

Figure 4.13 Total sugar of ABE production by culture *Clostridium beijerinckii* ATCC 55025 at concentration 60 g/L (10% inoculums, TYA medium).

4.3.2 Effect of initial inoculums concentration

There were batch fermentations of butanol production between 30 g/L glucose and 30 g/L_{sugar}molasses as substrate in TYA medium. Various initial inoculums concentrations contained with *C. beijerinckii* ATCC 55025 were used at 5%, 10% and 15% by incubate at 37 ^{o}C. Figure 4.14 shows that at 30 g/L_{sugar}molasses having 10% and 15% inoculums could generate solvents about 6.7-9.7 g/L, mainly containing with ethanol at168 hr. Ethanol tends to be increased in the fermentation system. The acetic acid was gradually increased in the first phase of fermentation and it is gradually decreased to produce solvents. However, butanol is not detected in this experiment.

This experiment found that the optimum inoculums size is 10%inoculums by volume. Microorganism *C. beijerinckii* ATCC 55025 could have growth from sugar (glucose) via the Embden-Meyerhof-Parnas (EMP) pathway then change to volatile fatty acid and solvent. Previously, the optimum sugar concentration for butanol production was 60 g/L, in this investigation, 30 g-sugar/L (2 times dilution) was deployed to investigate ABE fermentation. However, butanol is not still detected.

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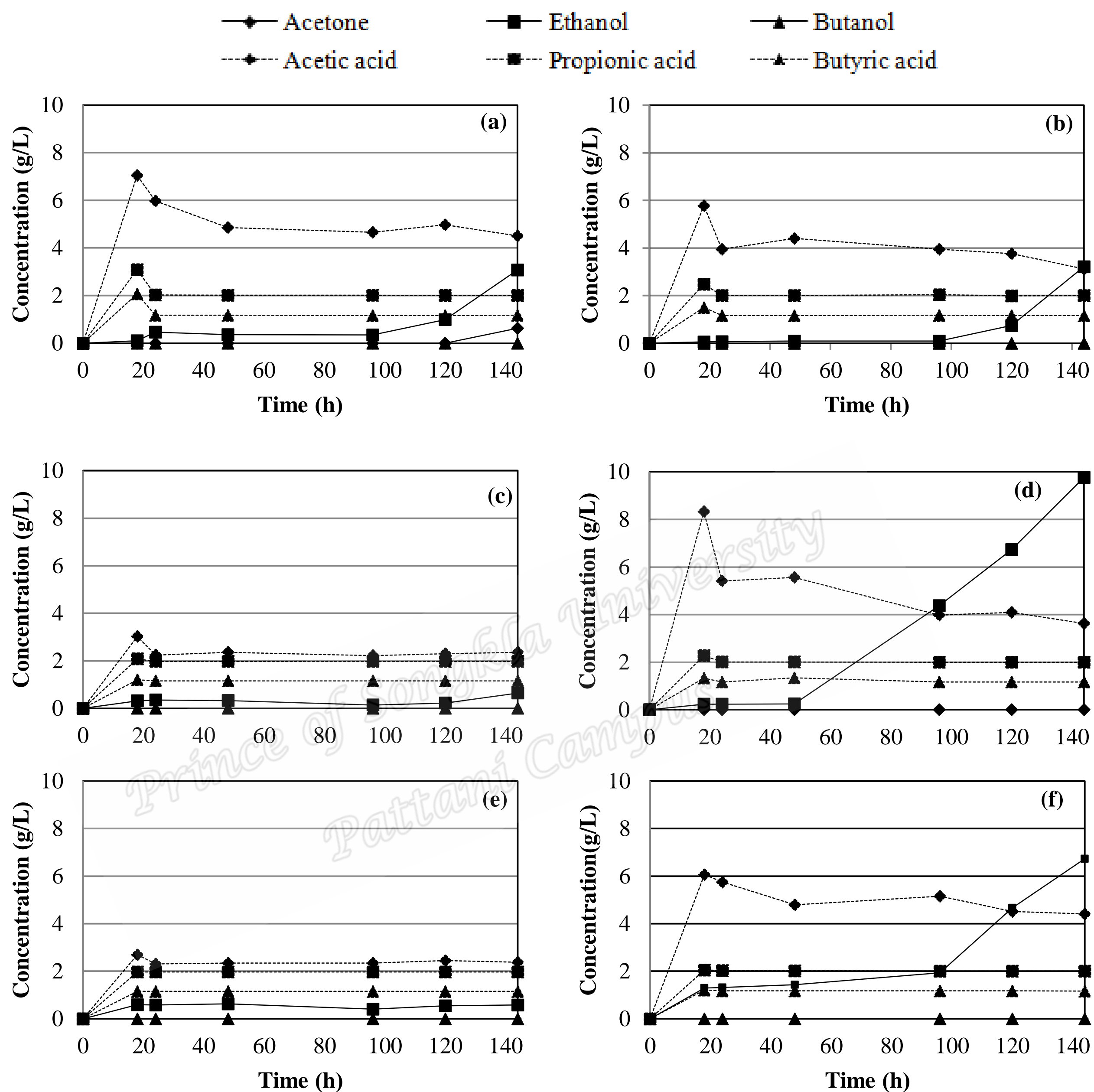

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Figure 4.14 ABE productions in batch fermentation by culture *C. beijerinckii* ATCC 55025 at (a) 5% inoculum in 30g/L glucose, (b) 5% inoculum in 30 g/L_{sugar} molasses, (c) 10% inoculum in 30 g/L glucose (d) 10% inoculum in 30 g/L_{sugar} molasses (e) 15% inoculum in 30 g/L glucose (f) 15% inoculum in 30 g/L_{sugar} molasses (TYA medium).

4.3.3 Effect of medium

4.3.3.1 TYA medium and Free-N medium

Clostridium sp.ABE fermentation of glucose is biphasic fermentation, in the first phase is the acedogenesis phase as primary metabolite production such as butyric acid together with growth of cells generated and in second phase is the solventogenesis phase as secondary metabolite production such as butanol and acetone produced. In this study the cells in stationary phase (solventogenesis phase) could produce butanol from butyric acid with Free-N medium, indicating that non growth microorganism in the stationary phase could have its ability of butanol generation without nitrogen requirement.

Batch fermentation of butanol production by adding TYA medium or Free-N medium at initial glucose concentration of 30 g/L and 10% of *C. beijerinckii* ATCC 55025 was incubated at 37 °C. Figure 4.15, at 30 g/L glucose in TYA medium obtains highest butanol production about 7.9 g/L at168 hr. The butyric acid is gradually increased in the first phase of fermentation and it is gradually decreased to produce a solvent (butanol) in 48 hr, corresponding to gradually increasing of butanol concentration till hour 12and increasing rapidly later on till hour 48. The OD_{600} value (Figure 4.16) demonstrates that microorganisms showed an increasing in growth rate, gathering with pH in the appropriate range of not less than 5.2, which is the trigger for butanol producing bacteria.

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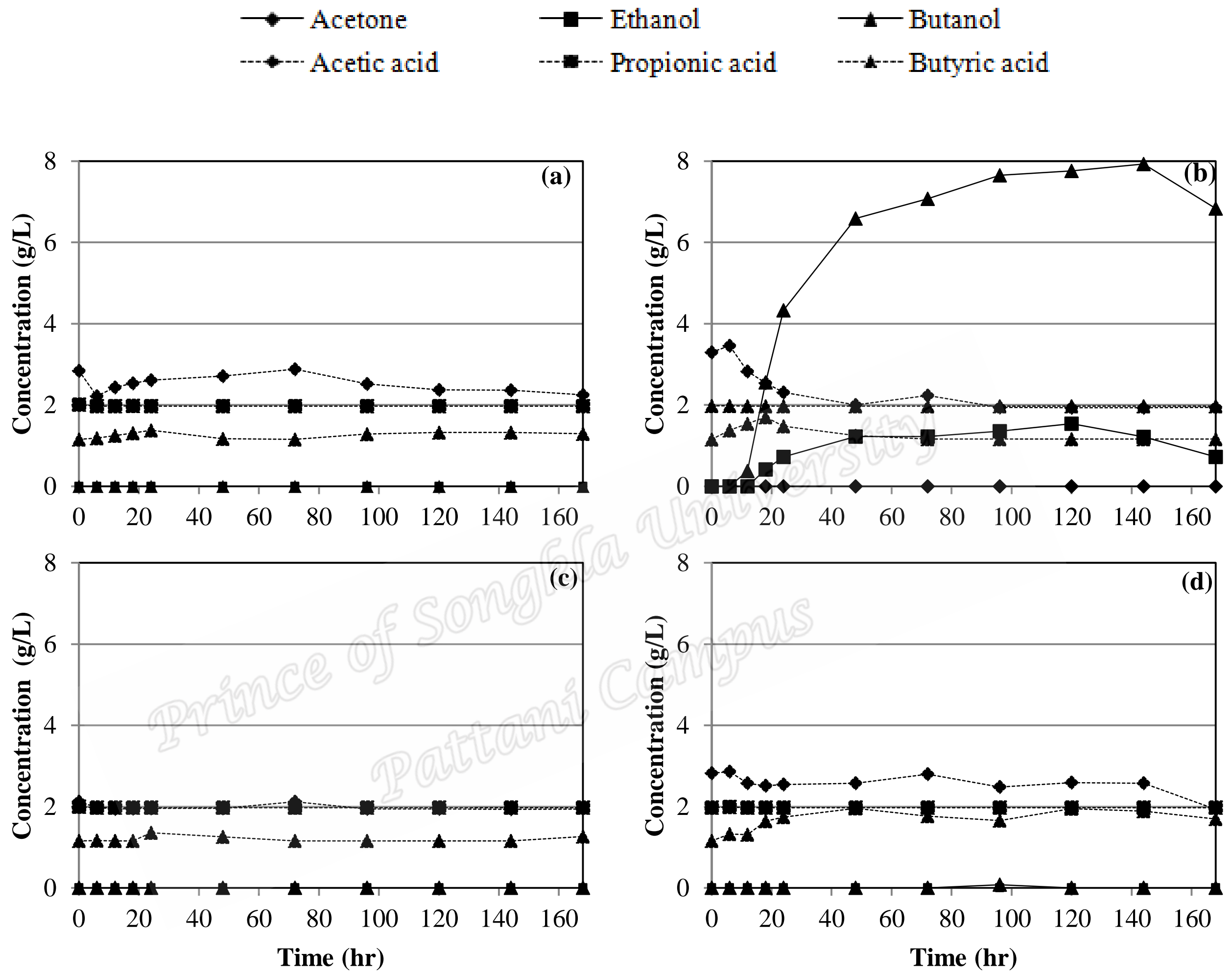

Figure 4.15 VFA and alcohol concentration in batch fermentation by *C. beijerinckii* ATCC 55025 (a) blank, (b) 30 g/L glucose in TYA medium and (c) blank, (d) 30 g/L glucose in Free-N medium (10% inoculums).

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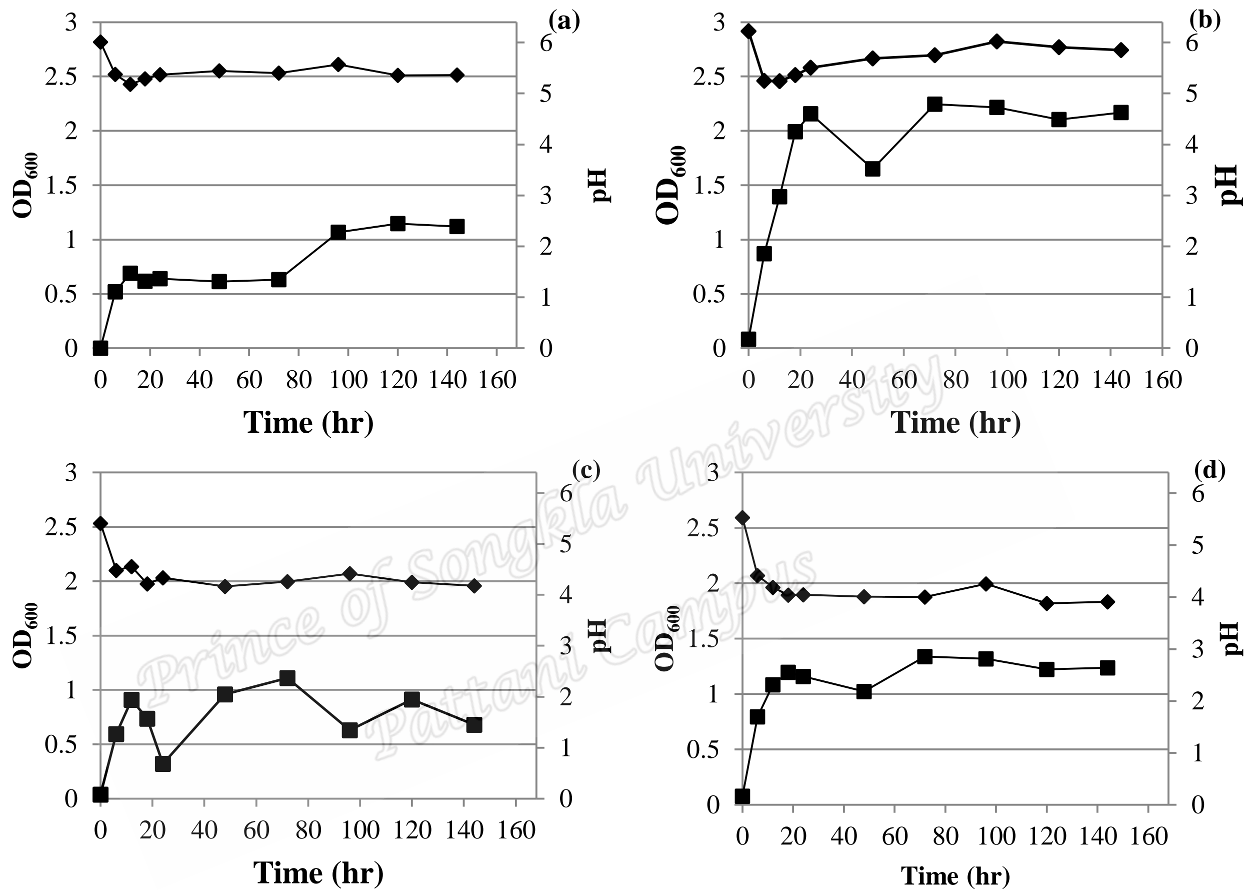

Figure 4.16 OD_{600} and pH in batch fermentation by culture *C. beijerinckii* ATCC 55025 (a) blank, (b) 30 g/L glucose in TYA medium and (c) blank, (d) 30 g/L glucose in Free-N medium (-■-OD_{600},-◆- pH) (10%inoculums).

In other experiments (Figure 4.15), batch adding TYA medium without glucose or adding Free-N medium and 30 g/L glucose as substrate or adding Free-N medium without glucose could not produce butanol.

4.3.4 Effect of butyric acid to glucose (B/G) ratio

Effect of butyric acid to glucose (B/G) at various ratios on direct conversion to acetone-butanol-ethanol by *Clostridium beijerinckii* ATCC 55025 was investigated. Butyric acid (AR grade) was used in this study. *C. beijerinckii* ATCC 55025 cells under stationary phase could convert butyric acid to butanol via their sole solventogenic activity. This activity is further enhanced butanol by the presence of glucose as a co-substrate (Loyarkat, 2014). Glucose is used as electron donor in butyric acid reduction for butanol conversion (Loyarkat *et al*., 2013).

Batch fermentation of butanol production from butyric acid (B) and glucose (G) in TYA mediumby controlling temperature at 37 ^{o}C at various of (B/G) ratios of 0/20, 1/20, 5/20, 10/20 and 15/20 g/L was carried out by using *C. beijerinckii* ATCC 55025 at 10% by volume of inoculums. It was found that (Figure 4.17) for all experiment assays, no butanol is detected but ethanol with low concentration is generated and high concentration of acids (mainly butyric acid) is still remained.

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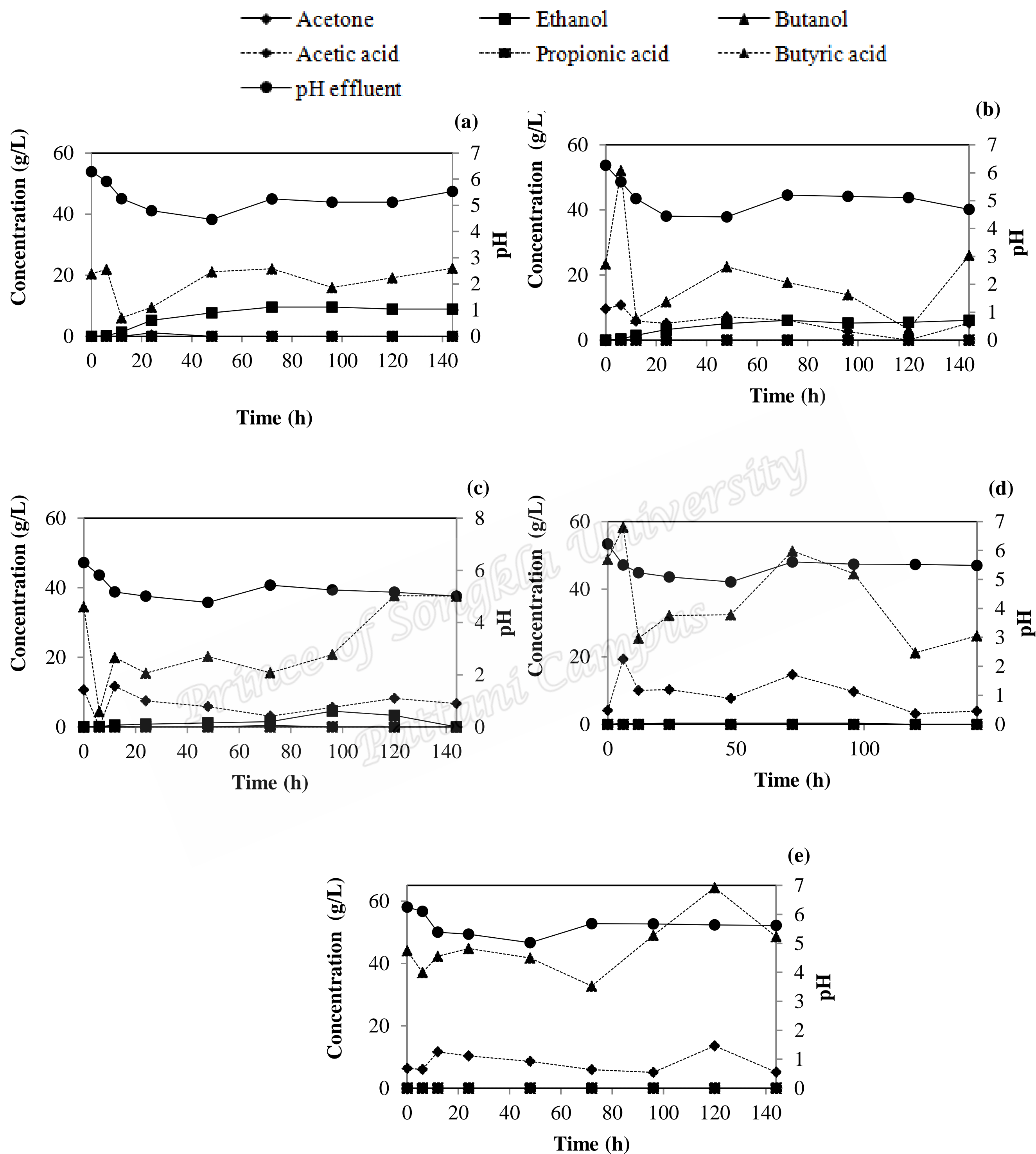

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Figure 4.17 VFA and alcohol concentration of B/G (a) 0/20, (b) 1/20, (c) 5/20, (d) 10/20 and (e) 15/20 g/L by used *Clostridium beijerinckii* ACT 55025 (10% inoculums, TYA medium).

4.3.5 Glucose fermentation in Packed Bed Reactor (PBR) by using *Clostridium beijerinckii* ACTT 55025

Packed Bed Reactor (PBR) with 2.5-L working volume is packed with plastic media for *Clostridium beijerinckii* ACT 55025 cells immobilized. To facilitate comparative fermentation studies, TYA medium having glucose as a carbon source (Hango and Murata, 1965) was designated as an adaptable fermentation medium that support a good solventogenic fermentation.

The ABE-PBR reactor was operated at 30 g/L of glucose and maintained pH in the reactor in a range of 4-6.5. Figure 4.23 shows the VFA, alcohol concentration and pH for 55 days operation. On day 0-8, butyric acid with high concentration in a range of 1-5 g/L is generated. After day 10, acids are decreased to day 44. Acids could be produced again on day 45 onwards but low concentration (0.9-3.1g/L). During fermentation hydrogen was detected about 30%. pH in a range of 3.91-6.55 (Figure 4.18) was established in the reactor, which was regulated by bicarbonate buffer added the substrate generate in the process.

The 1 g/L of butyric acid is not only stimulous the solventogenesis pathway but also consumed as a substrate to butanol production (Al-Shorgani *et al*. 2012). One possibility would be energy-rich substances such as ATP or NADH to support the assimilation of acid during the solventogenesis phase (Shinto *et al*., 2007). Therefore, in the study of Al-Shorgani *et al*. (2012) there found that when glucose was added the cells in stationary phase produced more butanol from butyric acid.

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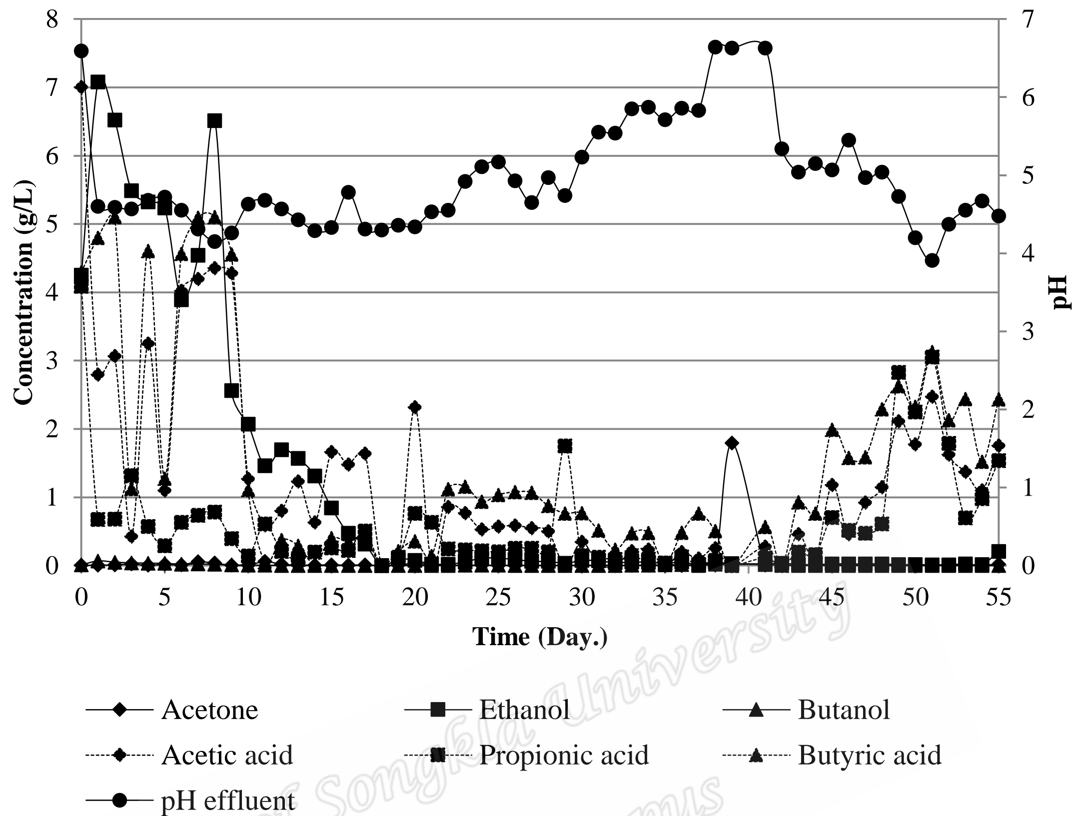

Figure 4.18 VFA and alcohol production by culture *C. beijerinckii* ATCC 55025 under 37 ^{o}C temperature from PBR.

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CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

For the first stage of acidigenesis, the hydrogen yield was obtained at concentration of food waste 5.8 gVS/L with 135.2 mL H_2/gVS$_{added}$. The main metabolites dominated mainly butyrate. The highest hydrogen production yield of 100 mL H_2/g-VS$_{added}$ and 6.14 g/L butyric acid achieved from CSTR mode.

In the second stage of ABE production, the highest butanol concentration of 7.93 g/L achieved from the batch fermentation of initial 60 g/L sugar by 10% *Clostridium beijerinckii* ATCC 55025 supplemented with TYA. For the continuous mode was not success in butanol production. The continuous ABE production was preformed in Packed Bed Reactor (PBR).The results show that the main products were butyric acid with concentration of 3-5 g/L.

5.2 Recommendations

5.2.1 The effect of the initial inoculums concentration on butanol production should studied in continuous.

5.2.2The process of B/G should be further investigated in a continuous system for better understanding in its sustainability.

5.2.3The condition on PBR operation should be adjusted to produce ABE such as nutrient type and pH control.

5.2.4 The immobilized cells should be further applied in a continuous.

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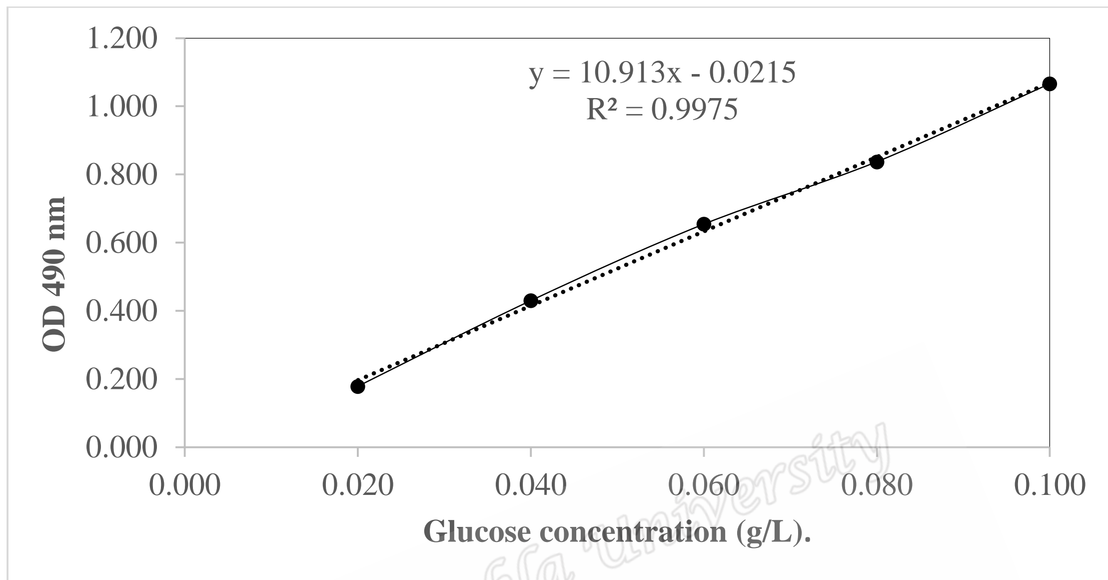
APPENDICES

Appendix A

1. **Glucose standard curve for total sugar**



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Financial support from Science and Technology Asian (SAT-Asian), Faculty of Science and Technology.

List of Publication and Proceeding

Surananee Hayeeyunu, Rattana Jariyaboon, Shahrul Ismail and Prawit Kongjan. 2016. Dark fermentation of food waste for hydrogen production: the crucial stage to producing butyrate for butanol production. 39th National Graduate Research Conference & 4th International Graduate Research Conference, June 30th - July 1st, 2016. Assumption University, Suvarnabhumi Campus, Samut Prakan, Thailand.

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