PRODUCTION OF ACETIC AND BUTYRIC ACID FROM TREATED LEACHATE BY *CLOSTRIDIUM BUTYRICUM* NCIMB 7423 IN STIRRED TANK AND MEMBRANE REACTOR

by

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science

August 2016

ACKNOWLEDGEMENT

Alhamdulillah.

I would like to thank my mother, Azizah bt Daud and my late father, Othman bin Awang for their constant encouragement and support. I also would like to express gratitude to my supervisor Dr. Husnul Azan bin Tajarudin for giving me an opportunity and guidance to complete this research. Not forgetting both my co-supervisors, Assoc. Professor Wan Nadiah bt. Wan Abdullah and Dr. Noor Aziah bt. Serri for their constructive ideas and advice.

One of the best part during my postgraduate journey is having a very supportive colleague, Muhammad Redzwan bin Tamat who continuously supported me in facing all obstacles throughout one and half years of lab work. This gratitude also goes for members of lab 407 and all postgraduate students of Bioprocess Technology Division.

I also would like to express my gratitude to all staff and Lab Assistants of Bioprocess Technology, and Environmental Technology Divisions especially Mr. Azmaizan and Mdm. Najmah. Besides that, a big thanks to School of Civil Engineering and Solid Waste Management Cluster for the use of various facilities in their school.

Last but not least, I would like to acknowledge the Government of Malaysia (203.PTEKIND.6711373) and Universiti Sains Malaysia (1001.PTEKIND.811262) for the research grants that financed this study.

Mohd Firdaus bin Othman

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LIST OF ABBREVIATIONS

µm	Micrometre
AF	Anaerobic filter
APHA	American Public Health Association
ASBR	Anaerobic sequencing STR reactor
BOD	Biological oxygen demand
C. butyricum	*Clostridium butyricum*
cm	Centimetre
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
CW	Constructed wetland
EPA	Environmental Protection Agency
g	Gram
GC	Gas chromatography
h	Hour
HR	High range
HRT	Hydraulic retention time
kg	Kilogram
L	Litre
LPI	Leachate pollution index
m	Meter
MBR	Membrane bioreactor
MF	Microfiltration
Min	Minutes
ml	Millilitre
mm	Millimetre
mmol	Millimoles
MSW	Municipal solid waste
N.D.	Not determined
NCIMB	National Collection of Industrial, Food and Marine Bacteria

OD	Optical density
OFN	Oxygen free nitrogen
PBLS	Pulau Burung landfill site
PFR	Plug-flow reactor
RBC	Rotating biological contactor
rpm	Revolution per minute
RTBS	Reed bed treatment systems
SBR	Sequencing STR reactor
SRT	Sludge retention time
SSF	Solid state fermentation
STR	Stirred tank reactor
t	Time
TKN	Total Kjeldahl nitrogen
UASB	Upflow anaerobic sludge blanket
UF	Ultrafiltration
v	Volume
v/v	Volume per volume
VFA	Volatile fatty acid
VSS	Volatile suspended solid
vvm	Volume per volume per minute

LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage
±	Plus minus
-	Minus
+	Plus
x	Multiply
US$	United States dollar
C	Carbon
Ca	Calcium
CO_2	Carbon dioxide
H_2	Hydrogen
H_2SO_4	Sulfuric acid
Mg	Magnesium
N	Nitrogen
N_2	Nitrogen gas
Ni	Nickel
P	Phosphorus
Sr	Strontium
V	Vanadium

PENGHASILAN ASID ASETIK DAN ASID BUTARIK DARIPADA BAHAN LARUT LESAP TERAWAT OLEH *CLOSTRIDIUM BUTYRICUM* NCIMB 7432 DALAM BIOREAKTOR TANGKI TERADUK DAN BIOREAKTOR MEMBRAN

ABSTRAK

Bahan larut lesap merupakan cecair tercemar yang terhasil daripada tapak pelupusan, yang meninggalkan kesan yang buruk kepada alam sekitar. Kajian ini tertumpu kepada biopenukaran bahan larut lesap terawat kepada asid asetik dan asid butarik oleh *Clostridium butyricum* NCIMB 7423 dan bertujuan untuk menyelesaikan masalah rawatan bahan larut lesap, pada masa yang sama menghasilkan produk bernilai tambah daripada rawatan tersebut. Bahan larut lesap telah diambil dari tapak pelupusan Pulau Burung (PBLS) dan diprarawat dengan menggunakan batu kapur untuk membuang asid lemah meruap. Dua jenis pelarasan dibuat terhadap bahan larut lesap sebelum penapaian, iaitu diubah berdasarkan keperluan oksigen biologi (BOD) dan diubah berdasarkan jumlah karbohidrat. Bioreaktor tangki teraduk (STR) dan bioreaktor membran digunakan dalam penapaian oleh *C. butyricum* NCIMB 7423 dengan pH 6.5 dan suhu 37°C. Media sintetik dengan kondisi yang sama digunakan sebagai tanda aras, dan bahan larut lesap yang tidak dilaraskan digunakan sebagai kawalan. Penapaian STR media sintetik menunjukkan produktiviti asid asetik, asid butarik, CO_2 dan H_2 yang masing-masing 0.46, 0.67, 8.24, dan 0.001 g/L/j dengan 91.3% pemulihan karbon. Bagi penapaian STR bahan

larut lesap tanpa penyelarasan, produktiviti asid asetik, asid butarik, CO_2 dan H_2 adalah masing-masing 0.00, 0.004, 0.39 dan 0.0009 g/L/j dengan 38.9% pemulihan karbon. Manakala produktiviti asid asetik, asid butarik, CO_2 dan H_2 untuk bahan larut lesap diubah berdasarkan BOD masing-masing mencapai 0.26, 0.36, 1.94 dan 0.0002 g/L/j dengan pemulihan karbon sebanyak 78.9%. Produktiviti asid asetik, asid butarik, CO_2 dan H_2 bagi bahan larup lesap yang diubah berdasarkan jumlah karbohidrat adalah masing-masing 0.04, 0.03, 0.32 and 0.00005 g/L/j dengan 24.9% pemulihan karbon. Di samping itu, penapaian membran media sintetik mencapai produktiviti asid asetik, asid butarik, CO_2 dan H_2 masing-masing pada 0.02, 0.01, 3.39 dan 0.0002 g/L/j dengan 65.6% pemulihan karbon. Penapaian bahan larut lesap menggunakan reaktor membran mencapai produktiviti asid asetik, asid butarik, CO_2 dan H_2 masing-masing pada 0.04, 0.08, 10.17, dan 0.002 g/L/j dengan 72.0% pemulihan karbon. Penapaian bahan larut lesap dalam tangki teraduk menghasilkan produktiviti yang sedikit rendah dibandingkan dengan media sintetik manakala penapaian bahan larut lesap dalam bioreaktor membran menunjukkan produktiviti dan pemulihan karbon yang lebih tinggi daripada media sintetik dalam bioreaktor membran. Bahan larut lesap yang diubah berdasarkan BOD dipilih sebagai pelarasan terbaik untuk penapaian berdasarkan nilai pemulihan karbon.

PRODUCTION OF ACETIC AND BUTYRIC ACID FROM TREATED LEACHATE BY *CLOSTRIDIUM BUTYRICUM* NCIMB 7423 IN STIRRED TANK AND MEMBRANE REACTOR

ABSTRACT

Leachate is a contaminated liquid generated from landfill, imposing a devastating effect to the environment. This study focuses on bioconversion of treated leachate to acetic and butyric acid by *Clostridium butyricum* NCIMB 7423 which aims to solve the leachate treatment problem and at the same time produce value added product from the treatment. Leachate was taken from Pulau Burung Landfill Site (PBLS) and pretreated with limestone to remove volatile fatty acids (VFA). Two types of adjustments were carried out on leachate prior to fermentation namely altered leachate according to Biological Oxygen Demand (BOD) and altered leachate according to total carbohydrate. Stirred tank (STR) and membrane reactors were used in the fermentation of pretreated leachate by *C. butyricum* NCIMB 7423 of pH 6.5 and 37°C. Synthetic medium fermentation with the exact condition was carried out as a benchmark for leachate fermentation, and non-altered leachate fermentation as the control. Synthetic medium in STR fermentation showed productivity of acetic acid, butyric acid, CO_2 and H_2 of 0.46, 0.67, 8.24, 0.001 g/L/h respectively, with 91.3% of carbon recovery. For leachate STR fermentation, the productivity of non-altered leachate for acetic acid, butyric acid, CO_2 and H_2 were 0.00, 0.004, 0.39 and 0.0009 g/L/h respectively with 38.9% of carbon recovery. While the

productivity of altered leachate according to BOD for acetic acid, butyric acid, CO_2 and H_2 reached 0.09, 0.11, 1.94 and 0.0002 g/L/h respectively, and carbon recovery of 78.9%. The productivity of altered leachate according to the total carbohydrate for acetic acid, butyric acid, CO_2 and H_2 were 0.04, 0.03, 0.32 and 0.00005 g/L/h respectively with 24.9% of carbon recovery. Besides, synthetic medium membrane fermentation achieved productivity of acetic acid, butyric acid, CO_2 and H_2 at 0.02, 0.01, 3.39 and 0.0002 g/L/h respectively, with 65.6% of carbon recovery. Leachate fermentation in a membrane reactor achieved productivity of acetic acid, butyric acid, CO_2 and H_2 at 0.04, 0.08, 10.17, and 0.002 g/L/h respectively, with 72.0% of carbon recovery. Leachate fermentation in STR resulted in slightly lower productivity compared to similar fermentation using synthetic medium, whereas leachate fermentation in a membrane reactor showed relatively high production and carbon recovery compared to synthetic medium fermentation in membrane reactor. Leachate altered to BOD was selected as the best alteration for fermentation based on the carbon recovery.

CHAPTER 1. INTRODUCTION

1.1. Solid Waste Problem in Malaysia

As one of the aggressively developing countries in Asia, Malaysia faces the problem of solid waste disposal. A study by the Malaysian government in 2013 showed that each day, a person produces 800 g of solid waste. On the other hand, those who live in the urban area produce 1.25 kg of waste per person every day. The government estimated that in 2013, Malaysian would be producing 30 000 to 33 000 tonnes of solid wastes a day. This figure was alarming because in the current situation, these had already exceeded the governments projection of 30 000 tonnes of solid waste daily for 2020 (Ismail, 2014).

Municipal Solid Waste (MSW) consists of solid items we use and then discard. It is commonly known as trash or garbage (Agency, 2014). In a developing country such as Malaysia, problems in handling of MSW which includes collection coverage, irregular collection services, crude open dumping and burning without control cause air and water pollution, and the breeding of vermin and flies (Samsudina & Dona, 2013). MSW composition differ between countries. In Malaysia, the main composition of MSW are foods, papers and plastics and this reflects on the Malaysian lifestyle (Samsudina & Dona, 2013). The composition of MSW for 2005 is tabulated in Table 1.1 below.

Table 1.1: The composition of MSW in Malaysia for 2005 (Samsudina & Dona, 2013).

Waste Composition	Percentage of Wet Weight
Organic	44.8
Paper	16.0
Plastic	15.0
Glass	3.0
Metal	3.3
Textiles	2.8
Wood	6.7
Others	8.4

1.1.1. Solid Waste Management in Malaysia

In Malaysia, MSW is managed by the Ministry of Urban Wellbeing, Housing and Local Government with the support from the private sector. Studies show that MSW management in this country is still highly underdeveloped (Badgie *et al.*, 2012). However, the Malaysian government shows a very high commitment in the management of solid waste. The government has plenty of plans and strategies, and has ratified its commitment in the Agenda 21, United Nations Framework Convention on Climate Change, and Kyoto Protocol, which required that sustainability be considered in its development (Abas & Wee, 2014). Management of MSW in Malaysia is a big issue because it costs local authorities up to 60 percent of their annual budget, which comes with a price of RM 110 to RM 130 to collect and dispose one tonne of MSW. This corresponds to RM 1.98 million to RM 2.34 million a day (Masirin *et al.*, 2008). The cost to manage MSW in states that under Solid Waste Management and Public Cleansing Corporation supervision in 2013

had reached RM 1.6 billion (Astro Awani, 2014) while, a landfill in Johor which located at Tanjung Langsat Industrial Area charged RM 45 per tons of waste dumped to maintain the landfill which cost around RM 1.5 million a year (Sabeen *et al*., 2016).

Land filling is the main method of disposal of MSW in Malaysia (Abas & Wee, 2014). However, proper sanitary landfills are not fully implemented due to financial and technology constraints and Malaysia keeps on practicing open dumping or controlled dumping (Theng *et al.,* 2005). Sanitary landfill gives an advantage such as a simple disposal method, low cost and landscape restoring effect from mineral workings (Aziz *et al.,* 2010). The consequence from landfills is the generation of contaminated leachate which is an environmental concern (Wiszniowski *et al.*, 2007).

In Japan, the researchers solved open dumping of MSW by introducing the use of aerobic and anaerobic landfill. Japanese government struggle to find a best way to minimize the pollution from landfill sites. From their findings (Figure 1.1), the aerobic condition showed effectiveness in rapidly reducing Biological Oxygen Demand (BOD) concentration of leachate and continued highly removed under anaerobic condition (Yamamoto, 2002). According to Theng *et al.* (2005), Fukuoka method is a method where the leachate and gas is continuously removed by leachate collection and gas venting systems, in which the ambient air will flow into the waste through leachate pipe hence improve microorganism activities in the waste body thus will also improve waste stabilization process and leachate quality. Malaysia is one of few countries in the world that tested and proved the effectiveness of the Fukuoka method semi-aerobic system to

manage solid waste (Theng *et al.*, 2005). From the study, Theng *et al.* (2005) also suggested that Fukuoka method is a cost effective method to be implemented in Malaysia.

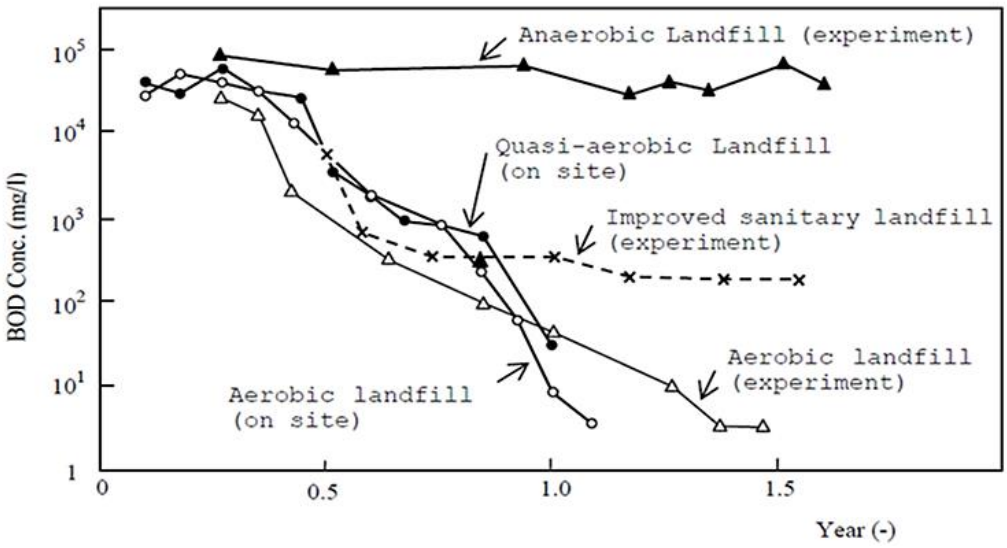

Figure 1.1: Changes of BOD concentration by various landfill method (Yamamoto, 2002).

1.2. Leachate

What is leachate? Leachate is produced when moisture enters MSW in a landfill and extracts out contaminants from MSW into liquid phase. This moisture is high enough to produce a liquid flow (Hamidi *et al.*, 2004). Even after closure of the landfill, leachate is still being produced as the decomposition of refuse continues thus understanding of landfill leachate is critical to counter the problem (Kjeldsen *et al.*, 2002). Leachate may contain various contaminants which include organic contaminants, ammonia, halogenated hydrocarbons, suspended solids, heavy metals, inorganic salts, phenol, nitrogen and phosphorus (Aziz *et al.*, 2010), and can impose a devastating effect to the environment, especially related to groundwater and surface water pollution (Kjeldsen *et al.*, 2002).

A team of researchers from the School of Civil Engineering, Universiti Sains Malaysia (USM) had investigated the significance effect of leachate pollution index (LPI) to the immediate attention of leachate treatment. The index is very important because characterization of leachate is difficult to carry out. This is because the composition of leachate is highly influenced by various factors which include waste composition and site hydrology (Umar *et al.*, 2010). At early stage of leachate production, aerobic decomposition occurs nearly at neutral pH. As the decomposition of leachate develops, the anaerobic decomposition takes place which involves acetogenic phase. After several months or years of decomposition, leachate enters the methanogenic phase which indicates that biodegradation of leachate is near completion (Salem *et al.*, 2008). Each and every phase of leachate development must be fully understood in order to design the suitable treatment for leachate before it meets the requirement of discharge regulation and can be discharged to the river or surface water. The main purposes of this research is to study the conversion of leachate to acetic and butyric acids by biological process.

1.3. Research Scope and Objectives

In this study, batch fermentation in stirred tank and membrane reactor was used to investigate the production of acetic and butyric acids by *C. butyricum* NCIMB 7423 using leachate. The leachate was collected from Pulau Burung Landfill Site (PBLS). Before the fermentation was started, leachate has first undergone a pretreatment process (adsorption) to reduce volatile fatty acids. During fermentation, the production of hydrogen (H_2), carbon dioxide (CO_2), acetic and butyric acid were monitored. Besides that, the changes

in biological oxygen demand (BOD), chemical oxygen demand (COD), nitrogen, phosphorus, carbohydrates and volatile fatty acid (VFA) in the leachate were checked.

The objectives of the research were as follows:

1. To reduce inhibitors of *C. butyricum* NCIMB 7423 such as acetic and butyric acid from raw leachate.
2. To identify the kinetic profile and carbon recovery during synthetic and leachate medium fermentation in stirred tank reactor systems.
3. To determine the best leachate alteration method based on carbon balance for leachate fermentation:
    a. BOD alteration
    b. Total carbohydrate alteration
4. To study the capability of membrane bioreactor to produce acetic and butyric acid by *C. butyricum* NCIMB 7423.

1.4. Description of the Study

This research attempted not only to treat leachate but at the same time produce valuable chemicals (acetic and butyric acid) using *C. butyricum* NCIMB 7423 fermentation. Leachate treatment is a high cost treatment and give high burden to the government. Many problems can be solved by producing chemical from leachate through fermentation. An attempt of growing *C. butyricum* NCIMB 7423 in a solely leachate medium was not successful because leachate contain inhibitor and must undergo pretreatment first to

remove it. This study does not gave detailed attention to the pretreatment as the objective is just to remove inhibitor.

C. butyricum NCIMB 7423 was selected as suitable candidate for leachate fermentation simply because of *Clostridia* is the absolute choice for producing valuable product from complex organic matter (Ljungdahl *et al.*, 2013). The novelty of this research lies in the fact, to date no research has been documented on the treatment of leachate by using *C. butyricum* and at the same time producing valuable product. The result of this research enables the understanding in leachate treatment as well as open more possibilities for green and sustainable practices.

1.5. Thesis Outline

This thesis consists of five chapters which explained in detail as follows:

Chapter 1. Introduction

This chapter presents the background of the study. It also includes problem statements, objectives and scope of the research, as well as a description of the study.

Chapter 2. Literature Review

This chapter discusses in detail regarding *C. butyricum* which is the bacteria used in the study to treat the leachate and also about the products from *C. butyricum*. This chapter also discusses about the type and method to treat leachate.

Chapter 3. Methodology

In chapter 3, the detail of all procedures for the experiment was described. The chapter also includes materials and instruments used in the research.

Chapter 4. Result and Discussion

The findings and results from the research conducted was compiled in this chapter. The research findings are compared and discuss in detail.

Chapter 5. Conclusion and Recommendations

The findings from the research was summarized and appropriate recommendations for future research was suggested.

CHAPTER 2. LITERATURE REVIEW

2.1. *Clostridium butyricum*

In the Kingdom Procaryota one of the largest genus is *Clostridium. Clostridium* is a strictly anaerobic and gram-positive bacteria. Their morphology is cylindrical-shape (Szymanowska-Powalowska *et al.*, 2014). *Clostridium* is a spore forming bacteria and strains can be isolated from soil, waste water, animal digestive systems and contaminated dairy products (Zigov & TurdK, 2000). *Clostridium* is well known for producing bad smell. Mankind also label *Clostridium* as biological threat and foe to them even though not all of *Clostridium* produce toxins (Bahl & Dürre, 2001). Optimal cultivation conditions for *Clostridium* are at temperature ranging from 35-37°C, pH range of 4.5-7.0 and an atmosphere of pure carbon dioxide (CO_2) or nitrogen (N_2) or N_2 and CO_2 mixture in the ratio of 1:9 (Zigová *et al.*, 1999). The product of *Clostridium* metabolism is CO_2, H_2, organic compounds (butyric, lactic, acetic and succinic acids) and solvents (butanol, acetone and isopropanol) (Szymanowska-Powalowska *et al.*, 2014).

In 1861, Louis Pasteur found the bacteria that was capable of growth without air. This finding was a sensation at that time. Later Louis Pasteur named this bacteria *Vibrion butyrique*. This event is a brief discovery of anaerobiosis (Porter, 1972; Bahl & Dürre, 2001) . There are reports which said similar *Clostridia* include *Bacillus amylobacter* and *Bacillus butylicus*. Later Van Tieghem concluded that his *Bacillus amylobacter* is the same bacteria as *Vibrion butyrique* found by Louis Pasteur but all the credit went to Adam Prazmowki who suggested that *Vibrion butyrique, Bacillus amylobacter, Bacillus*

butylicus were all the same bacteria and should be named *Clostridium butyricum* (Bahl & Dürre, 2001).

Clostridium butyricum can be found in the soil, healthy humans and animal's intestine (Takahashi *et al.*, 2000). The metabolism of *C. butyricum* involved acetic acid (main byproduct from butyrate production) been taken up by the bacteria and converted into butyrate. This pathway is inhibited by its end product which is butyrate. The fermentation of *C. butyricum* results in production of acetic acid, butyric acid, H_2 and CO_2. The first three products which caught a great deal of attention nowadays is discussed in detail in Section 2.3, 2.4 and 2.5 respectively. The fermentation of *C. butyricum* follows the equation 2.1 below (Zhang *et al.*, 2009):

$$\text{Glucose} \rightarrow 0.8\ \text{butyrate} + 0.4\ \text{acetate} + 2\ CO_2 + 2.4\ H_2 \qquad (2.1)$$

2.1.1. Metabolisms of *C. butyricum*

Clostridium butyricum can utilize a variety of carbohydrate from mono to disaccharides and complex polysaccharides which include glucose, lactose from whey, sucrose from molasses, starch, potato wastes, wheat flour, cellulose or dextrose (Bahl & Dürre, 2001; Kong *et al.*, 2006; Tracy *et al.*, 2012; Azan *et al.*, 2013). The carbohydrate intake from surrounding by *Clostridia* follows phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) which is associated with uptake and phosphorylation of substrate. After carbohydrate intake into cytoplasm by *C. butyricum*, carbohydrate is metabolized to pyruvate *via* Embden-Meyerhof-Parnas (EMP) pathway (Bahl & Dürre,

2001; Azan *et al.*, 2013; Ljungdahl *et al.*, 2013). Figure 2.l depicts the EMP pathway and general metabolite pathway of *Clostridia*.

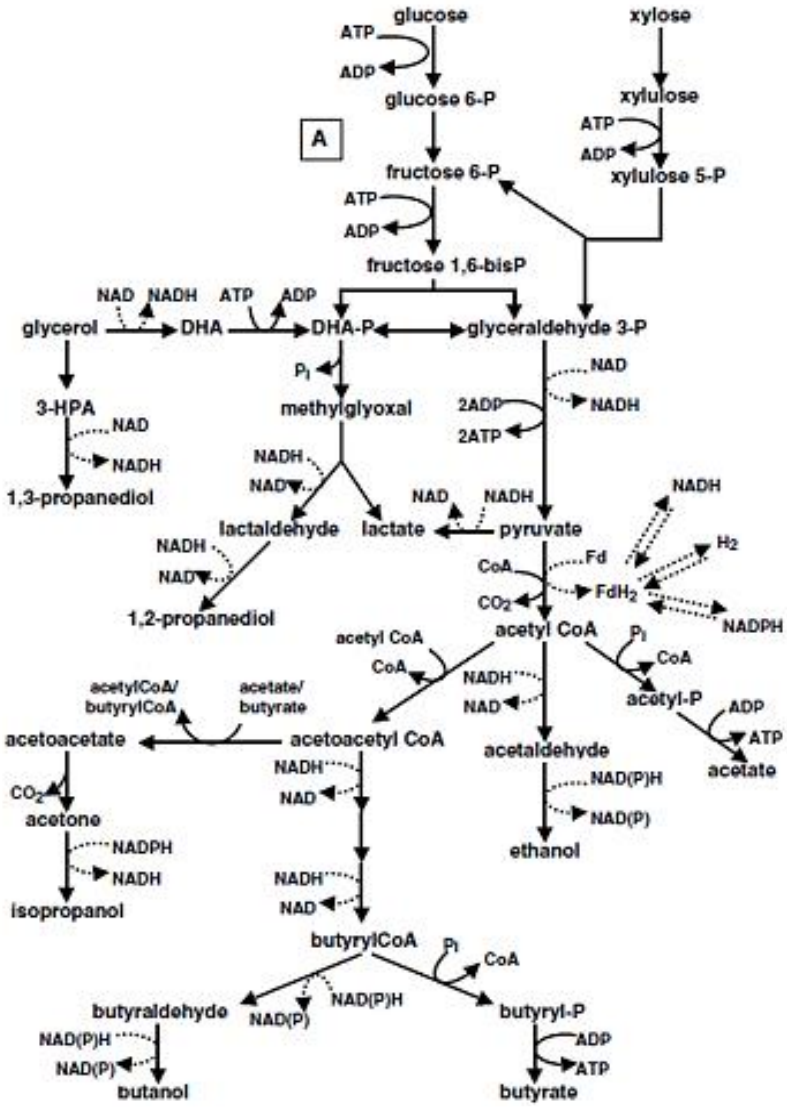

Figure 2.1: EMP pathway (A) of carbohydrate intake by *C. butyricum* and general *Clostridia* metabolite pathway (Bahl & Dürre, 2001).

From Figure 2.2, at first *C. butyricum* transports glucose into cytoplasm *via* the PTS and the compound is metabolized to pyruvate *via* EMP. Two mol of ATP and two mol of NADH result from the metabolism of the glucose from each mol of hexose (Bahl & Dürre, 2001). Then, pyruvate is decarboxylated and oxidized simultaneously by pyruvate-ferredoxin oxidoreductase to yield CO_2, acetyl-CoA, and reduced ferredoxin (Reaction (2)). The reduced ferredoxin form Reaction (2) is then catalyse by hydrogenase to produce H_2 (Reaction (3)). Acetyl-CoA is a product intermediate. Acetyl-CoA being converted to acetate by phosphotransacetylase (Reaction (4)) and acetate kinase (Reaction (5)). Two acetyl-CoA combine together into acetoacetyl-CoA (Reaction (6)) in which this molecules further reduce in three step resulting in butyryl-CoA, with ß-hydroxybutyryl-CoA and crotonyl-CoA intermediate (Reaction (7), (8) and (9)). Throughout Reaction (7)-(9), two NADH is generated and used as an electron donor. Butyryl-CoA is transformed into Butyryl-P and lastly butyrate by butyryl-CoA dehydrogenase, phosphotransbutyrylase and butyrate kinase (Reaction (9), (10) and (11)) (Ljungdahl *et al.*, 2013). Ferredoxin and hydrogenase are both easily poisoned by oxygen, resulting in disruption of metabolic pathway of *C. butyricum* (Azan *et al.*, 2013). Pyruvate is an important intermediate in *Clostridia* metabolism, and can be converted to form acetyl-CoA and CO_2 by pyruvate-ferredoxin oxidoreductase. Acetyl CoA produced can be further converted to oxidized product (acetate, CO_2) and reduced product (butyrate) (Gheshlaghi *et al.*, 2009).

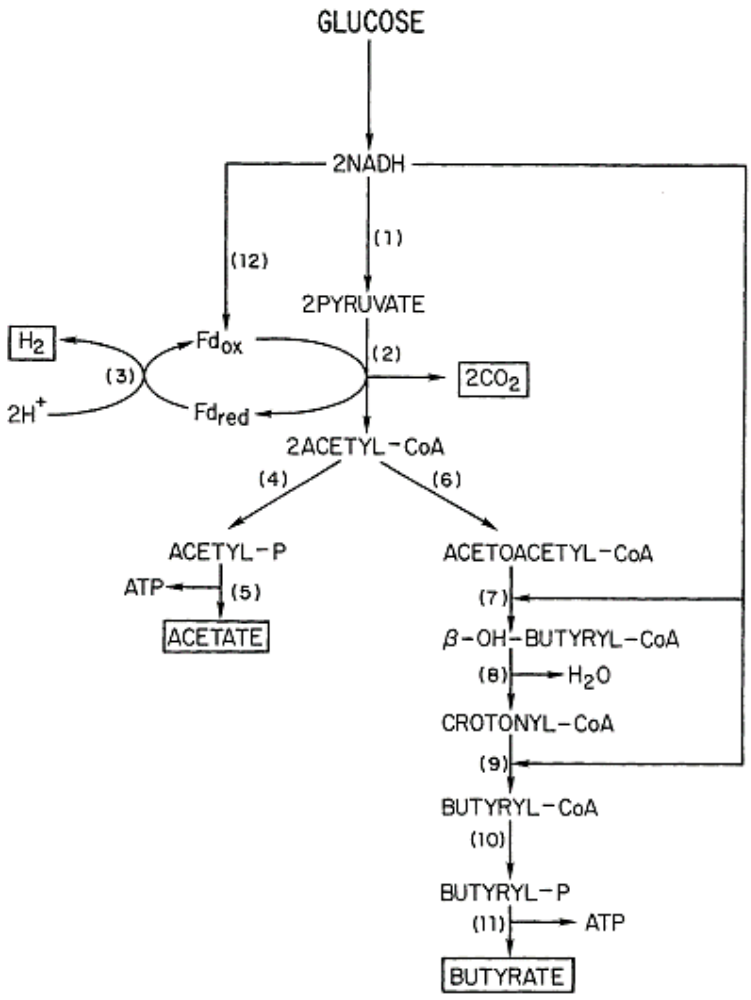

Figure 2.2: Metabolite pathway of glucose fermentation in *C. butyricum* (Ljungdahl *et al.*, 2013)

2.2. Application of *C. butyricum* in Industry

2.2.1. Probiotics

Probiotic is a live microbial supplement which will benefit the host by improving intestinal floral balance (Fuller, 1991). There are many reports regarding probiotic properties of *C. butyricum* which have been intensively studied (Takahashi *et al.*, 2000; Bahl & Dürre, 2001; Seki *et al.*, 2003; Kong *et al.*, 2011; Yang *et al.*, 2012;

Szymanowska-Powalowska *et al.*, 2014; Tanasupawat *et al.*, 2014; Isa *et al.*, 2015). *Clostridium butyricum* can withstand low pH and high bile concentrations making it a suitable candidate for probiotics compared to *Lactobacillus casei subsp. casei*, *Lactobacillus delbrueckii subsp. Lactis*, *Lactobacillus acidophilus* BFC, *Lactobacillus acidophilus* ATCC 4356, *Bifidobacterium bifidum* 1.1852 and *Bifidobacterium adolescentis* LCL (Kong *et al.*, 2011). According to the author and co-workers, this high tolerance is associated with ability of *C. butyricum* to produce endospores.

Clostridium butyricum MIYAIRI 5881® (CBM 5881®) is the most popular strain that is used as a probiotic. CBM 5881® was isolated in 1960's from soil in Nagano, Japan. Currently CBM 5881® is widely used as probiotics in human and animal (Isa *et al.*, 2015). MIYAIRI strain is used to treat and prevent the non-antimicrobial-induced diarrhoea and antimicrobial-associated diarrhoea in human and animal (Takahashi *et al.*, 2000).

2.2.2. Green Energy (Hydrogen and Biobutanol)

Clostridium butyricum produces hydrogen and butyric acid during fermentation. Both hydrogen (H_2) and butyric acid are an important alternative to biofuel. The interest in finding a new way to produce H_2 with almost no carbon emission have been an issue from past few years. The most promising procedure is through bacteria fermentation (Beckers *et al.*, 2010). Beckers and co-workers work showed that *C. butyricum* produce hydrogen more efficiently compared to *Citrobacter freundii*, and theoretically *C. butyricum* can produce maximum 4 mol of H_2 per mol of carbon source (Table 2.1).

Table 2.1: Comparison of substrate conversion ratio, hydrogen production and hydrogen yield of *Citrobacter freundii* and *C. butyricum* with 5 different substrate investigated by Beckers and co-workers (Beckers *et al.*, 2010).

		Substrate conversion ratio	Hydrogen production (ml)	Hydrogen yield ($mol_{H2}.mol_{hexose}^{-1}$)
Citrobacter	Glucose	98.2 %	46.1 ± 5.7	0.24 ± 0.03
freundii	Maltose	N.D.	N.D.	N.D.
CWBI952	Sucrose	92.5%	19.0 ± 2.8	0.10 ±0.02
	Lactose	99.4%	35.3 ± 7.2	0.18 ±0.04
	Starch	0	0	N.D. (0)
C. butyricum	Glucose	89.3%	95.9 ± 2.0	0.58 ± 0.01
CWBI1009	Maltose	97.2%	100.8 ± 2.0	0.51 ± 0.01
	Sucrose	99.1%	98.3 ± 0.5	0.52 ± 0.00
	Lactose	93.3%	123.9 ± 2.0	0.69 ± 0.00
	Starch	85.6%	79.1 ± 2.1	0.49 ± 0.02

N.D.: Not determined

Biobutanol production can be achieved by fermentation of *C. butyricum.* This new type of fuel can solve many problem faced by bioethanol. Due to the increase world fuel prices and awareness on environmental pollution, biobutanol is one of the solution for clean energy source (Dwidar *et al.*, 2012).

2.2.3. Food Industry

Flavour market worldwide represents almost 7 billion US$ a year and currently the demand on microbial-derived flavour is staggering. Butyric acid can be used as pure acid in dairy industry or in form of ester in food additive (He *et al.*, 2005). Butyric acid that is produced by *C. butyricum* is used as natural cheese aroma and by converting butyric acid into ethyl butanol, produce an important fruity flavour (Dubal *et al.*, 2008). Recently, a

Japanese company, Miyarisan Pharmaceutical Co., plan to market *C. butyricum* as a novel food ingredient in European Union (EU) (Media, 2013). Novel food referred to a food that has been not significantly consume by human in EU prior to 1997 (Commission, 2016).

2.2.4. Environment

The role of *C. butyricum* in environment is not well recognized. Szymanowska-Powalowska *et al.* (2014) addressed an important role of *C. butyricum* in environment in which *C. butyricum* is involved in soil mineralization and conversion of organic matter. A study in 1977 shows that *C. butyricum* capable of dechlorinated and also degraded y-hexachlorocyclohexane (y-HCH) in soil (Jagnow *et al*., 1977). Besides that, *C. butyricum* also capable of fixing nitrogen in soil thus play a vital role in soil fertility (Parker, 1954; Ross, 1960).

2.3. Acetic Acid

Acetic acid is a colourless liquid, have strong vinegar-like odour and is considered as volatile organic compound (Inventory, 2014). The structural formula of acetic acid is shown in Figure 2.3. Global acetic acid market in 2014 was valued at 12,124.30 kilo metric tons and expected to increase to 16,155.09 kilo metric tons by 2020 while Asia Pacific becomes the largest consumer of acetic acid in the world (LLP, 2016). Acetic acid is an important chemical in industry which represent as key block in manufacturing Vinyl Acetate Monomer (VAM), Purified Terephthalic Acid (PTA), Acetate Esters, Acetic Anhydride and Calcium Magnesium Acetate (CMA) (Jin *et al.*, 2005; LLP, 2016). Figure 2.4 summarizes the application of acetic acid.

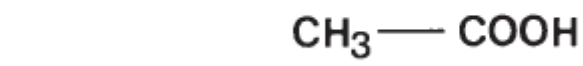

Figure 2.3: Structural formula for acetic acid (Thurman, 1985).

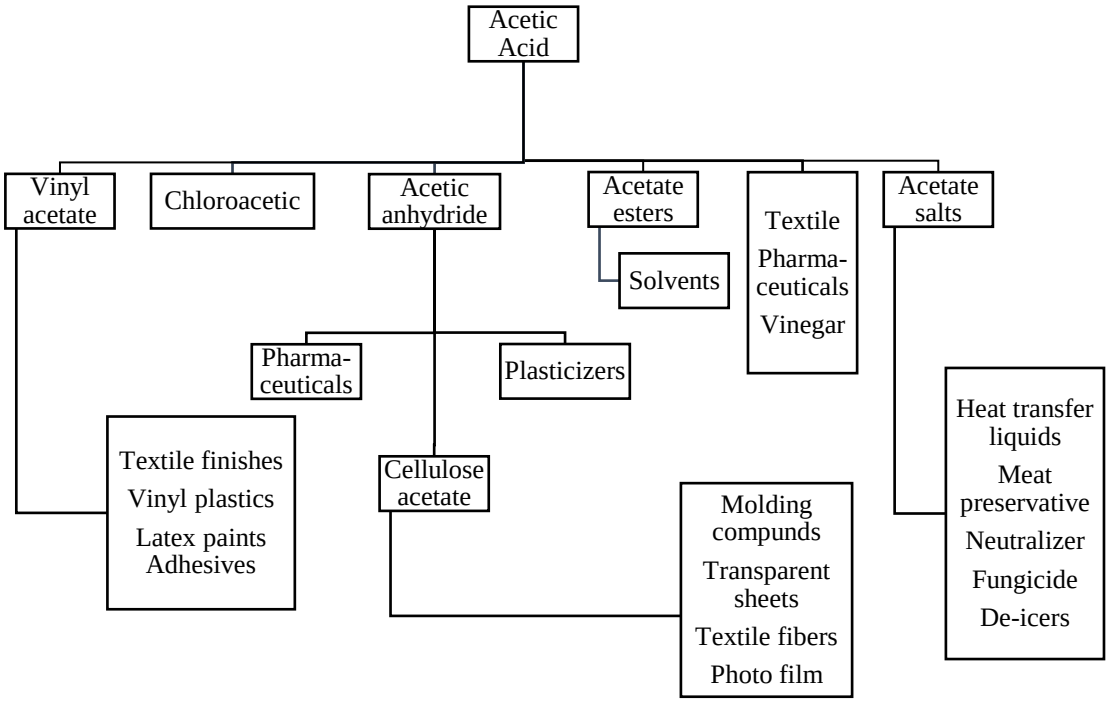

Figure 2.4: Summary of application of acetic acid (Rogers *et al.*, 2006).

Acetic acid is the main component in vinegar, which was also reported to possess the antimicrobial property. Due to this property, acetic acid help in preventing spoilage in fermented food, however, it is not suitable for wine and beverages (Gullo *et al.*, 2014). The earliest vinegar manufacturing was a slow process known as Orleans Process. In this process, wooden casks were filled with fresh vinegar that act as inoculum then a wine was added on a weekly basis. At around five weeks, some of the vinegar was replaced by wine

and a thick bacterial layer can be observed. An air was sparged into the wooden casks then created a continuous production of vinegar (Rogers *et al.*, 2006)

2.3.1. Production of Acetic Acid

Before Christ, vinegar (diluted acetic acid) was produced by alcohol fermentation but was replaced by dry distillation wood when the demand of acetic acid increased in various fields. Later this process was replaced by a synthetic process. The process that can be used to produce acetic acid include (Sano *et al.*, 1999):

i. Methanol carbonylation process
ii. Acetaldehyde oxidation process
iii. Hydrocarbon (butane, naphtha) oxidation process
iv. Direct oxidation of ethylene

Despite all the chemical process described above, acetic acid can also be produced by fermentation using renewable biomass. Acetic acid is a low-value commodity. So, the conversion yield is important in term of commercialization which was demonstrated well by *Clostridium thermoaceticum* when it manages to convert 1 mol of glucose to 3 mol of acetic acid (Klemps *et al.*, 1987). The production of acetic acid by fermentation can be done by using solid state fermentation (SSF) or liquid fermentation (submerged). Submerged system has advantages over SSF, a submerged system has high yield and faster process (Gullo *et al.*, 2014).

The current technique used in production of acetic acid which is by using chemical synthesis might not be favourable in future due to petroleum depletion. This will open a

wide opportunity to the fermentation technology in replacing current method. The major bacteria use in acetic acid production is *Acetobacter* species (aerobic) and *Clostridium* species. *Clostridium* fermentation gives more advantages because it only requires one-step vinegar process, has higher yield, low cost (because no aeration needed) and ability to consume CO_2 and other one-carbon precursor permits their use in conversion of synthesis gases from waste. Thus, the anaerobic fermentation to produce acetic acid is more favourable compared to aerobic fermentation (Rogers *et al.*, 2006).

2.4. Butyric Acid

Butyric acid, or also known as butanoic acid is a colourless oily liquid that has an extremely pungent smell. This chemical belongs to a group of short-chain fatty acid (Załęski *et al.*, 2013; Health, 2015) and contains 4 carbon (Figure 2.5). The main application of butyric acid is in the manufacture of cellulose acetate butyrate plastics, an important material in textile fibre production. Besides that, butyric acid is also an important chemical that is used directly in fibre as an additive for heat and sunlight resistance enhancement. Butyric acid also use in chemical, food and pharmaceutical industry (Zhang *et al.*, 2009).

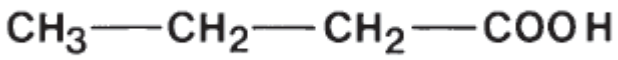

Figure 2.5: Structural formula of butyric acid (Thurman, 1985).

Esterification of butyric acid with ethanol produce ethyl butyrate which has an important fruity flavour (Dubal *et al.*, 2008). According to Dubal *et al.* (2008), the cost of

production of ethyl butyrate from fermentation is 180 US$/kg. Recently, it is reported that butyric acid is capable of treating irritable bowel syndrome (IBS), a functional bowel disorder without any possible side effect (Załęski *et al.*, 2013). Butyric acid acts as anti-proliferative agent which directly inhibits DNA synthesis and cell growth. This mechanism suggests for relaxing DNA and giving time to repair an enzyme and ultraviolet damage allowing for greater survival time (Bingham, 1990). Bingham (1990) also suggested that butyric acid is capable of preventing colon and rectum cancer.

The most interesting application of butyric acid to date is as a precursor of biofuel. Because of environmental concerns and high fuel price, biofuel is the solution to the alternative energy source to the world. Biobutanol is the most promising biofuel to replace gasoline (Dwidar *et al.*, 2012). Biobutanol offers many advantages over bioethanol and gives solutions to most of bioethanol problems. The advantages of biobutanol are: 1) Three carbon-carbon bond in butanol provides more energy compared to double bond in two molecules of ethanol. 2) Biobutanol can be used directly into gasoline without any modification. 3) Biobutanol allows drivers to travel further on a single full tank compared to ethanol blend fuel. 4) Biobutanol potentially can be mixed in higher ratio with gasoline. 5) Reported more friendly compared to bioethanol as it captures more biomass carbon as fuel. 6) Biobutanol is less attracted to water and can be transported in an existing pipeline and if spilled, will spread less in ground water (Nexant and Chemical Strategies, 2009; Dwidar *et al.*, 2012).

2.4.1. Production of Butyric Acid

To keep up with global demand, butyric acid production has evolved from time to time. Butyric acid is produced from a chemical process which involves petroleum-based chemical synthesis. Chemical synthesis is more preferable because of its lower cost and large production scale (Zhu & Yang, 2004; Zhang *et al.*, 2009; Dwidar *et al.*, 2012). The other ways of producing butyric acid are by extraction from butter, which has butyric acid concentration ranging from 2% - 4%. The downside of this method is, that this method is difficult and expensive in extracting butyric acid compared to the chemical synthesis (Zigov & TurdK, 2000).

The production of butyric acid by means of bioprocessing is increasing in demand because of public respond to the pollution cause by petrochemical industry and consumer preference on bio-based product (Liu *et al.*, 2006). Currently, butyric acid obtained from microbial fermentation is more favourable even though the cost of production is higher compared to chemically synthesised butyric acid (Zigová *et al.*, 1999; Zigov & TurdK, 2000; Zhu & Yang, 2004; Liu *et al.*, 2006; Zhang *et al.*, 2009; Dwidar *et al.*, 2012). There are several bacteria that can produce butyric acid and this bacteria is strictly anaerobic. This bacteria belong to genera *Clostridium*, *Butyrivibrio*, *Butyribacterium*, *Sarcina*, *Eubacterium*, *Fusobacterium* and *Megasphera*. According to Zigov & TurdK (2000), genera *Clostridium, Butyrivibrio and Butyribacterium* are commonly use bacteria to produce butyric acid but *Butyribacterium* is widely use microorganism and *Clostridium* is mostly use for commercial production of butyric acid because of its stability and high productivity.

2.5. Hydrogen

Hydrogen is known as fuel of the future. Experts list the advantages of hydrogen fuel in its purest form, which include zero emission, endless supply and production of hydrogen may use a variety of sources including renewable resource (Johnston *et al.*, 2005). Hydrogen is a main component/compound found in water or hydrocarbon with combinations of oxygen and carbon respectively. Once it is extracted, hydrogen which is a colourless, odourless and tasteless gas becomes a useful 'feedstock' or input to a variety of industrial activities (Dunn, 2003).

Hydrogen-based energy system is intensively developed by the United States (US) government. The key driver to this situation is concern for long term energy security, environmental quality and economic vitality (US Department of Energy, 2007). Governments around the world seem interested in hydrogen fuel, which leads to various researches in searching for the most affordable way in manufacturing hydrogen. Iceland, Canada, the US, Japan and Germany have taken the lead in exploring the advantages and benefits offered by hydrogen as an energy source (Johnston *et al.*, 2005). Hydrogen can be produced from domestic energy resources around the world (Turner *et al.*, 2008). Some of the main resources for the production of hydrogen include coal, natural gas, biomass, wind, solar, nuclear and nuclear energy (US Department of Energy, 2007). This topic will focus only on hydrogen production by a biological process. The pathway of hydrogen production by biological process depends on the substrates and microorganism. To produce hydrogen *via* biological process, various waste materials can be used (Kapdan & Kargi, 2006):

i. Starch and Cellulose Containing Agricultural or Food Industry Wastes

Annually, the yield of lignocellulosic biomass worldwide was estimated to exceed 220 billion tons (Ren *et al.*, 2009). From this figure, it can be concluded that the lignocellulosic biomass offer attractive and low cost feedstock for hydrogen production. Lignocellulosic biomass cannot be utilized directly; hence, further pretreatment is required.

ii. Carbohydrate Rich Industrial Wastewaters

Some industries discharge wastes that are rich in carbohydrate content. Such industries are dairy industry, olive mill, baker's yeast and brewery (Kapdan & Kargi, 2006). Since industrial wastewaters are in use, further pretreatment may be required to remove undesirable component and for balancing the nutrient content. Wastewater may contain varying concentrations of detergent, surfactants and saline, which have different influence on hydrogen production (Karadag *et al.*, 2014).

iii. Waste Sludge from Wastewater Treatment Plants

Waste sludge is rich in polysaccharide and protein, thus is a very suitable substrate for hydrogen production (Guo *et al.*, 2010).

2.5.1. Production of Hydrogen

Up to date, many car manufacturers is taking an initiative to develop a car that can run on hydrogen completely. BMW, Honda, Toyota, Mercedes-Benz and Nissan are among the manufacturers that promotes in developing hydrogen technology as a source of energy for their cars. The most promising car came from Toyota with their Mirai hydrogen car. Mirai has a range of 483 km and refuelled in just five minutes. Toyota claimed Mirai only emits water vapour (Tan, 2015).

Massachusetts Institute of Technology (MIT) researcher announced that they have successfully modified a virus to split water into hydrogen. This virus, called M13, acts as 'scaffolding'. This lead to non-intensive and efficient way to produce hydrogen (Chino, 2010). This renewable energy also gives hope to the world in searching for renewable energy that is zero carbon emission. Billions of dollars are already spent on the research and development in production of hydrogen as a new energy source replacing the fuels.

There are a lot of research done in the production and utilization of hydrogen energy. Hydrogen can be extracted from water by mechanical or chemical process. Unfortunately, in order to get hydrogen gas by extracting the water molecule, we need to use more energy (Shinnar, 2003). Microbial activities shows a promising future to produce hydrogen. Table 2.2 shows the research done for producing hydrogen using microorganism.