# CHAPTER 1
# INTRODUCTION

## 1.1 Rational/Problem Statement

Hydrogen is one of the most promising alternatives to fossil fuels due to its clean, renewable, and high energy yield (122 kJ $g^{-1}$) (Pisutpaisal *et al*., 2010). Hydrogen can be either directly combusted or used to produce electricity through fuel cells. Many processes, thermochemical, electrochemical, and biological can be used to generate hydrogen (Chen *et al*., 2006). Thermochemical and electrochemical processes are not renewable due to their dependence on fossil fuels. Biological hydrogen production more environmentally friendly since it is less energy intensive than non-biological processes. Biohydrogen can be generated from different metabolic pathways including direct water biophotolysis by green algae, indirect water bio-photolysis by cyanobacteria, photofermentation by photosynthetic purple non-sulfur bacteria, hydrogen synthesis via water-gas shift reaction by microalgae in Rhodospirillaceae family, and dark fermentation by heterotrophic anaerobic bacteria. Higher production rate, wider spectrum of substrates, and lower energy requirement make dark fermentation more advantageous than other processes (Hallenbeck and Benemann, 2002; Levin *et al*., 2004; Wakayama and Miyake, 2001).

Various dark fermentative bacteria such as *Enterobacter, Bacillus,* and *Clostridium* are capable of producing hydrogen by breaking down organic substrates under mesophilic, thermophilic, or extreme thermophilic conditions. Broad types of organic feedstocks such as glucose, starch, municipal waste, livestock manure, crop residues, food waste, and wastewater have been utilized as substrates in the dark fermentation (Cakır *et al*., 2010; Datar *et al*., 2007; Noike and Mizuno, 2000; Xing *et al*., 2010). Food waste is an important waste material that constitutes a major fraction of municipal solid wastes. High carbohydrate content and low cost feedstock make food waste a potential feedstock for biohydrogen production. Over the last decade, $H_2$ production from dark fermentation of food waste has been reported using lab-scale bioreactors under mesophilic and thermophilic conditions (Ismail *et al*., 2009; Li *et al*., 2008). Up-scaling production of $H_2$ to commercial scale is now on progress.

Efficient hydrogen production by dark fermentation depends highly on process conditions, substrate compositions, and microbial community. The key microbes in

hydrogen production may originate from substrates, microbial seed or both. Previous studies mainly used pure bacteria strains for pure substrates and mixed cultures derived from natural sources for non-sterile feedstock in the hydrogen fermentation. A range of inocula have been used for $H_2$ production, including sewage sludge, soils (Hawkes *et al*., 2002), cattle dung compost (Fan *et al*., 2006), and river sediment (Zuo *et al*., 2005). *Clostridium* spp. is the most common bacteria present in the hydrogen fermentation process. *Bacillus, Enterobacter*, and *Thermoanaerobacterium* spp. have also been found during the fermentation, but less frequently than *Clostridium* spp. (Kraemer and Bagley 2005; Shizas and Bagley, 2005)

Many researchers have attempted to improve $H_2$ production to reach the maximum theoretical yield of 12 mol $H_2$ per mol glucose from complete glucose conversion (Eq.1), but this phenomenon has never been exploited in known biological systems up to now (Fang *et al*., 2006; Westermann *et al*., 2007; Das *et al*., 2009). In practice, combined volatile fatty acids and/or alcohol were allowed to produce through dark $H_2$ fermentation. $H_2$ synthesis can reach to a maximum theoretical yield of 4 mol per mol glucose if acetic acid is the sole end product (Eq.2) while a maximum theoretical yield of 2 mol per mol glucose can be obtained if butyric acid as the end product (Eq.3) (Liu *et al*., 2008; Das *et al*., 2009). An enormous amount of research has concentrated upon the optimization of environmental conditions in the fermentation process together with the selection of $H_2$ producers. However, $H_2$ yield lower than 2 mol/ mol glucose has been still reported from many research studies. The low conversion yield might be involved with some crucial metabolic activity preferentially developed for cell growth instead of $H_2$ including some competitive reaction such as $H_2$ consumption or diversion to other products that the currently proposed metabolic reactions and equations might be ignored. (Hallenbeck and Benemann, 2002; Cai *et al*., 2010). Therefore, total metabolic networks correlated with $H_2$ fermentation could be understood.

$$C_6H_{12}O_6 + 6H_2O \text{ ----> } 6CO_2+12H_2 \quad (1.1)$$

$$C_6H_{12}O_6 + 2H_2O \text{ ----> } 2CH_3COOH + 2CO_2 +4H_2 \quad (1.2)$$

$$C_6H_{12}O_6 \text{ ----> } CH_3CH_2CH_2COOH + 2CO_2 +2H_2 \quad (1.3)$$

In the present study, the performance of the hydrogen production of non-sterile food waste by the best candidate of hydrogen producing pure bacteria, *C. butyricum* TISTR1032 and heat-shocked anaerobic sludge was evaluated based on kinetic parameters such as $H_2$ productivity, $H_2$ production rate and yield. The best condition achieved the

highest yield of $H_2$ production was chosen to scale up in 5L semi-batch reactor. Carbon mass balance, metabolic profiles and population community in long term fermentation were examined. Moreover, to understand total metabolic networks, MFA for the fermentative $H_2$ production from rice starch, a representative of carbohydrate rich substrate, by anaerobic sludge was used to analyze the intracellular flux involved with the activity of dominant microorganism in the reactor, including maximize metabolites production correlated with $H_2$ flux pathway.

## 1.2 Literature Review

### 1.2.1. Factors affecting biohydrogen production

Dark fermentative hydrogen production is a very complex process and depends on many factors, such as type of microbial culture, ingredient of substrate, reactor type, supplementary nutrient for bacterial growth, metal ion, media composition, temperature and pH. The effects of these factors on fermentative hydrogen production have been briefly introduced and discussed. However, the main factors affecting biohydrogen production in this study could be summarized as:

#### 1.2.1.1 Ecological factors for fermentation of hydrogen producer

To optimize and control the ecological factors suitable for hydrogen producer is a key to improve $H_2$ yield in the fermentation process while methanogens and acetogens, hydrogen consumer, would be inhibited. Environmental factors such as pH and HRT affect the metabolic balance especially for the growth phase in batch culture. Temperature results in the adaptation of microorganism to reach steady state for hydrogen production rate. When temperature changes frequently, it directly effects with the growth of microorganism which greatly responses fluctuation of hydrogen producing rate during temperature rises and drops. Lay *et al*. (2000) reported that *Clostridia spp*. produced VFA and $H_2$ in the exponential growth phase and rapid alcohol production would be obtained in late growth phase. That an optimal HRT of 0.5 days gave maximum hydrogen production (14 mmol/g carbohydrate) from wastewater by anaerobic consortia in the presence of a chemostat culture was revealed by Ueno *et al*. (1996). When the HRT was increased from 0.5 days to 3 days, the hydrogen production rate was reduced from 198 to 34 mmol/L.day, while the carbohydrates in the wastewater were decomposed at an increasing efficiency from 70 to 97%.

#### 1.2.1.2 Undissociated acid inhibition and pH changes

In acidogenesis, hydrogen plus VFAs and/or alcohol are produced as the end products (Lee *et al*., 2002). When a high concentration of sugar was fed into these cultures, high concentrations of acids produced and finally inhibited hydrogen production. A study on effect of undissociated acetic and butyric acid on hydrogen production had been conducted by Van Ginkel and Logan (2005). It revealed that adding these acids into the cultures (external supplementation) or increasing glucose concentrations to synthesize these acids by normal metabolism of bacteria itself caused the inhibitory effect of hydrogen production. Experimental results apparently illustrated that both conditions could reduced hydrogen production up to 93%. When glucose concentration was above 40 g/L, solventogenesis was induced and hydrogen yields were inhibited more by self-produced acids.

Zheng and Yu (2005) supported this result of inhibited factor by conducting a comparative study. The experiment was operated by adding butyrate as external supplementation. A noncompetitive and non-linear inhibition model was developed with maximum hydrogen production rate at 59.3 ml $H_2$/g-SS/ hr, critical added butyrate concentration of 25.08 g/L and inhibition degree of 0.323, respectively. Ionic strength of solution would be increased at high concentration of dissociated acids condition resulting in the solvent production was induced and hydrogen production was stopped. The inhibition effect occurred when nonpolar undissociated acids could pass through intracellular cell at low pH condition (pH<pKa), and then released proton at higher internal pH (Van *et al*., 2001). This caused the increase of energy requirement that involved the coenzyme A and phosphate pools for maintaining neutrality. As the immediate effect, the flux of glucose through glycolysis was reduced.

To determine the parameters during the shift from acidogenesis to solventogenesis, Grupe and Gottschalk. (1992) reported that. an intracellular acid concentration up to 440 mM started to induce solventogenesis. The key parameter to maintain the intracellular acid concentration was the pH of extracellular environment. The pH was related with three important facts: (1) methanogen growth limitation; (2) $H_2$ production performance and (3) regulation of shift to solventogenesis (Valdez-Vazquez and Poggi-Varaldo., 2009). The optimum pH for hydrogen production was observed in the range of 5–7 (Fang and Liu, 2002; Kawagoshi *et al*., 2005), with the optimum at pH 5.5 (Van *et al*., 2001 ; Khanal *et al*., 2004). However, some investigators reported the optimum

pH range between 6.8 and 8 (Collet *et al*., 2004; Liu and Shen, 2004; Zhang *et al*., 2003; Kanai *et al*., 2005; Lay, 2001). Gradual decrease in pH was able to inhibit hydrogen production as pH affectted the activity of iron containing hydrogenase enzyme (Dabrock *et al*., 1992). Therefore, controlling of pH at the optimum level was required. For microbial growth, initial pH was an important factor since it obviously influenced the extent of lag phase in batch hydrogen production. Liu and Shen (2004) reported that the initial pH of 4.0-4.5 resulted in longer lag phase while high initial pH of 9.0 could reduce lag time along with lower yield of hydrogen production was reported by Zhang *et al*. (2003).

**1.2.1.3 Hydrogen partial pressure**

Hydrogen partial pressure in the headspace of the reactor was a key factor affecting hydrogen production. Hydrogen synthesis pathways were sensitive to hydrogen concentrations and were subjected to the inhibition of the end product. Since $H_2$ concentration was increased, it apparently affected the synthesis of $H_2$ gas that the metabolic pathway would be shifted to produce more reduced substance such as lactate, ethanol, acetone or butanol. Hydrogenase, key enzyme involved in hydrogen production reversibly oxidized and reduced ferrodoxin (Nicolet *et al*., 2002). The oxidation of reduced ferrodoxin was less favorable when hydrogen concentration in liquid phase was increased that reduction of oxidized ferrodoxin would be took place. In short, hydrogen in liquid phase would oxidize to proton, therefore the yield of hydrogen was significantly reduced.

The most common solution method for decreasing dissolved gas concentrations in fermentative $H_2$-producing reactors has been chosen gas sparging. . In pure cultures, sparging has altered the relative amounts of metabolic products. Tanisho *et al*. (1998) revealed that utilizing of argon and $H_2$ sparging in a culture of *Enterobacter aerogenes* could decrease the production of succinate while acetate was produced in high concentration. Crabbendam *et al*. (1985) observed in a culture of *Clostridium butyricum*, a lower acetate:butyrate ratio (A/B ratio) was occuredwhen $N_2$ was passed over the fermentation liquid. These studies implied that more $H_2$ gas was produced with sparging. Similarly, the $H_2$ yield increased to 47% when *Rhodopseudomonas palustris* P4 was intermittently purged with argon, reported by Oh *et al*. (2002).

In mixed cultures, the $H_2$ yield was increased when gas sparging passed through the culture that noticeably in comparison to unsparged conditions (Mizuno *et al*., 2000; Hussy *et al*.,2003,2005; Kyazze *et al*., 2006; Kim *et al*., 2006a ). For $N_2$ sparging, 80–120% of $H_2$ yield has been improved, 20–70% for $CO_2$ sparging, 88% for

methane sparging, and 0–12% for $H_2/CO_2$ (biogas) sparging (Kramer *et al*., 2006). Mizuno *et al*. (2000) showed that sparging nitrogen to the liquid phase lowered the dissolved hydrogen concentration resulting in an increase of hydrogen yield at 68%. On the other hand, the dissolved hydrogen in liquid phase could also be reduced by increasing agitation speed where the increase of hydrogen production was also doubled (Lay, 2000).

#### 1.2.1.4 Metal ions

Many researches had been studied about metal ions at cellular level affecting on cell growth due to the fact that their characteristic related with enzyme cofactor, transport processes and dehydrogenases. Lin and Lay (2005) summarized the research studies that had been carried out on nutrient supplementation for hydrogen production. The Taguchi orthogonal array was applied to find out the most significant nutrients supporting the growth and the activity of the $H_2$ producer. Magnesium, sodium, zinc and iron were the most important trace metals that affected hydrogen production. Of these, Magnesium was the most significant. Hydrogen production reached to the maximum yield at 3.52 mol $H_2$/ mol sucrose when $MgCl_2$ and NaCl concentrations were added 120 and 1000 mg/L, respectively.

Voet *et al*. (1999) reported that magnesium ion was an important cofactor that could activate almost 10 enzymes including hexokinase, phosphofructokinase and phosphoglycerate kinase during glycolysis process. Iron was also reported as an essential element that resulted in formation of ferredoxin and it was required for hydrogenase, being the key enzyme for hydrogen production (Nicolet *et al*., 2002). Zhang *et al*. (2004) illustrated that higher yield of hydrogen production was observed at the iron concentration of 1600 mg $FeSO_4$/L. Lee *et al*. (2001) studied the effect of iron concentration on hydrogen production using seed sludge from digester as inoculum. The maximum hydrogen production rate was found to be 24 mL/gVSS/hr at 4000 mg/L $FeCl_2$ (1760 mg$Fe^{2+}$/L). On the other hand, Lay *et al*. (2005) illustrated the lowest optimal concentration of $Fe^2$,132 mg$Fe^{2+}$/L) in food waste fermentation gave the highest yield of hydrogen production. Dabrock *et al*. (1992) demonstrated that the growth of *Clostridium pasteurianum* was limited when iron concentration of the medium was lower than 10mmol/L and iron limitation had changed the product pattern during glucose fermentation.

Significant amounts of lactate were produced as the major product instead of butyrate at an iron concentration of 5.7 mmol/L, and the reduction of hydrogen production in continuous fermentation was not observed. For non-limiting iron concentration (iron concentration up to 25 mmol/L), metabolism of *C. acetobutyricum* was fluxed to acidogenesis and hydrogen production (Peguin and Soucaille, 1995). Zhang and Shen. (2006) conducted the research study with a concerted effect of temperature and iron concentration. The effects of iron for hydrogen production decreased with increasing reactor temperature. The researchers suggested that when the ambient temperature was relatively low, bacteria need more ferrous ion to activate the hydrogenase so that it could oxidize reduced ferredoxin to produce more molecular hydrogen. Even though external ions were not significant factors to enhance hydrogen production, iron was needed for the long term preservation of bacteria.

### 1.2.2 Microbial community involving biohydrogen production

Biohydrogen fermentation starts forming the degradation of organic matter by microbial consortia corresponded with the cooperation of a microbial population that could be divided into 4 main groups involving the pathway of biohydrogen production. Firstly hydrolysis reaction, hydrolytic bacteria hydrolyze polymeric carbohydrates and proteins to monomeric substance. Acidogenesis will be reacted afterwards. Facultative bacteria convert monomeric substance such as glucose, amino acid or fatty acid to simple organic acid (acetic acid, propionic acid, butyric acid) and/or alcoholplus $H_2$ and $CO_2$ (Fig. 1.1). At this point, $H_2$ and acetate can be utilized and/or produced by several microbial groups. Therefore, acetate is generated during acetogenesis from $CO_2$ reduction and $H_2$ by autotrophic acetogens via the Wood–Ljungdahl pathway, a process named homoacetogenesis (Muller, 2003). However, synthophic bacteria can also generate acetate along with additional $H_2$ from short-chain organic acids (except acetate). Eventually, to complete anaerobic degradation of organic matter acetoclastic/hydrogenotrophic methanogens can utilize organic acids and $H_2$ for producing $CH_4$ and $CO_2$ as the end product (Garcia *et al*., 2000). In addition, when sulfates or nitrates are present, sulfate-reducing bacteria (SRB) and nitrate-reducing bacteria (NRB) are capable to use $H_2$ as electron donors generating sulfides and ammonia, respectively (Fig. 1.1). Thus, $H_2$ is a key intermediate consumed mainly by methanogens, NRB, SRB and homoacetongens. Due to the $H_2$ consumer, methanogen can grow in the short range of pH and it cannot resist in high temperature, so the pre-treatment by thermal stress is essential for limiting this organism.

However, the microbial population correlated with biohydrogen fermatation can be classified into two main types:

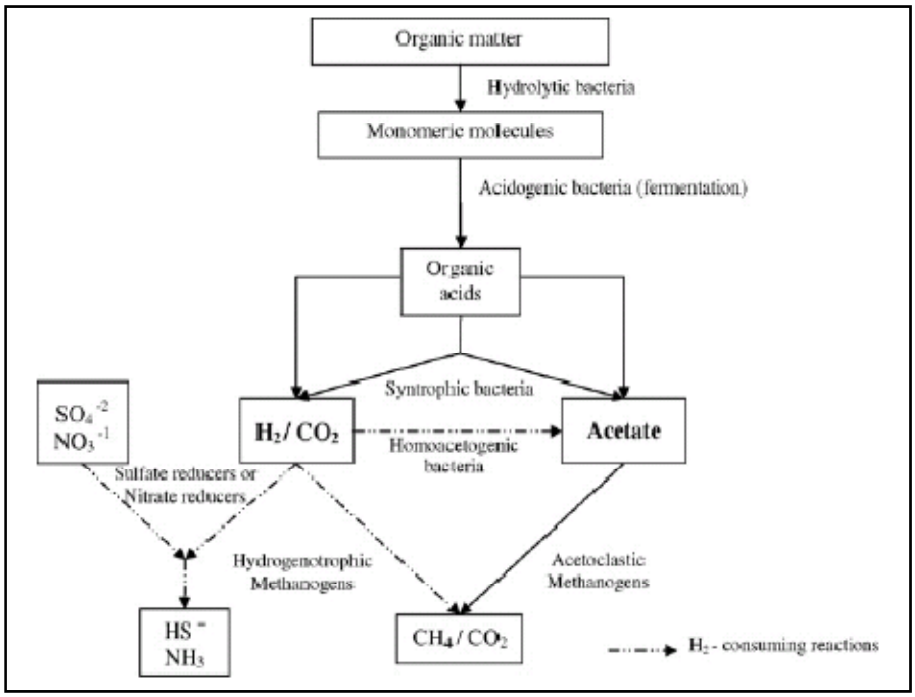


**Figure 1.1:** Hydrogen role in the anaerobic degradation of organic matter by microbial consortia (Valdez-Vazquez and Poggi-Varaldo, 2009)

#### 1.2.2.1 Pure culture

Several types of bacteria could be used to produce hydrogen from various substrates. Table 1.1 summarizes the studies on each strain of pure cultures for hydrogen fermentative production. *Clostridium* and *Enterobacter* were the most widely type that used as inoculum for fermentative hydrogen production. Species of genus *Clostridium* are gram-positive, rod shaped, strict anaerobes and endospore formers, whereas *Enterobacter* are gram-negative, rod-shaped, and facultative anaerobes (Li and Fang 2007). *Clostridium spp*., the spore forming anaerobic bacteria, was the most attractive $H_2$ producer. It could convert sucrose to hydrogen with the yield ranged between 2.0 and4.8 mol of hydrogen/mol sucrose (Yokoi *et al*., 1998; Chen *et al*., 2005; Lin and Lay, 2004). These were higher than the yields observed from the other fermentative bacteria such as *Enterobacter spp*. achieved the yield of 1mol hydrogen/mol hexose (Kapdan and Kargi, 2006). Hence, it is advantageous to select for clostridial species as the inoculums due to the maximum yield achieved in $H_2$ fermentation. However, the disadvantages of

clostridia were their limitations due to the absence of $O_2$ in the system, the specific nutrient and other environmental requirements for spore germination which response in unfavourable environmental conditions such as lack of nutrients.

Most studies using pure cultures of bacteria for fermentative hydrogen production were operated in batch mode and used glucose as a substrate. However, it was more desirable to produce hydrogen from organic wastes using pure cultures in continuous mode because continuous fermentative hydrogen production from organic wastes was more feasible for industrialization to realize the goal of waste management and alternative clean energy generation. Thus, many researchers attract the pure cultures for continuous fermentative hydrogen production from organic wastes. Various studies reported the efficiencies of $H_2$ production by using continuous flow reactor that glucose was used as substrate were 1.9-2.4 mol $H_2$/mol glucose production (Lay, 2001; Ueno *et al*., 2001a; Fang and Liu. 2002). Up-scaling continuous fermentative production of $H_2$ to commercial scale by pure culture from organic waste is now on progress.

**1.2.2.2 Mixed culture or co-culture**

Many previous studies have revealed that biohydrogen production, by pure strain $H_2$ producers, is about 2 mol $H_2$/mol glucose by *Clostridium spp*. and 1 mol $H_2$/mol glucose by *Enterobacter spp,* facultative bacteria. (Lin and Lay, 2004; Chen *et al*., 2005; Kapdan and Kargi, 2006). However, the cultivation of strict anaerobic bacteria was rather difficult since it sensitized with trace amounts of oxygen inhibited their growth that finally decreased ATP yield and reducing power (NADH). Supplementation of reducing agent such as L-cysteine HCl was an alternative way to maintain the anaerobic environment including $H_2$ yield.

Yuan *et al*. (2008) conducted the experiment to confirm this way that the maximum hydrogen production rates observed in the study increased to 1.5-2.9 times. However, using chemical substances increase in the cost of the biohydrogen production process. Yokoi *et al*. (1998) suggested biological way to reduce the present of $O_2$ in the fermentative reactor by using a co-culture of *C. butyricum* and *E. aerogenes*. *E. aerogenes* will first consume dissolved oxygen in the liquid fraction creating anaerobic condition that was desirable to *C. butyricum*. Typically, under the condition without addition of the reducing agent, hydrogen yield was achieved at 2 mol $H_2$/mol glucose while co-immobilization of both strains on porous glass beads obtain a high yield at 2.6 mol $H_2$/mol glucose.

Experimental results have supported the hypothesis that co-culturing reaches a higher yield than does pure culturing. Yokoi *et al*. (2002) reported the more efficient $H_2$ production operated in continuous flow by using mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes* achieved yield of 2.7 mol $H_2$ / mol glucose. Isolation of $H_2$ producer from natural source or biogas fermentative plant had been an attractive way to select the specific organism groups. Wang *et al*. (2008) reported that *C.acetobutyricum* X9, $H_2$ producer isolated from pilot-scale fermentor, exhibited the highest capability of hydrogen producing from microcrystalline cellulose compared to the other strains. However, co-culture of *C. acetobutyricum* X9 with *E. harbinense* B49 produced hydrogen more efficiently from microcrystalline cellulose, yielding hydrogen at the early stage of fermentation, with maximum hydrogen production at 1810 ml $H_2$/ml medium.

It was illustrated that *E. harbinense* B49 hydrolyzed cellulose to reducing sugars and these sugars were immediately utilized by *C.acetobutyricum* X9 and then biohydrogen was produced during this metabolic pathway. Co-culture of *C. thermocellum* and *T. thermosaccharolyticum* indicated the same effect that hydrogen production increased about 2-fold and hydrogen yield increased to 1.8 mol $H_2$/mol glucose (Liu *et al*, 2008). Consortia from various sources have been used as seed inocula for hydrogen production. However, the selection of consortia might be difficult due to the co-existence of hydrogen consuming bacteria such as hydrogenotrophic metanogens and homoacetogen. Thermal stress or chemical stress can kill, suppress or limit hydrogen consumer since most of the hydrogen producer can produce spore at severe environment and then it can be activated when the environmental conditions were appropiate for hydrogen production (Cheng *et al*., 2002; Zhang *et al*., 2005). Denaturing Gradient Gel Electrophoresis (DGGE) method was a molecular tool that was capable of analyzing and determining the microbial communities during $H_2$ fermentation by anaerobic sludge (Ueno *et al*., 2001; Fang *et al*., 2006).

**Table1.1** The pure bacterial cultures for fermentative hydrogen production (Wang *et al*., 2008)

| Organism | Substrate | Reactor type | Maximum yield of $H_2$ (mol $H_2$/mol substrate) | Reference |
|---|---|---|---|---|
| *Clostridium acetobutylicum* | Glucose | Batch | 2.0 mol $H_2$/mol glucose | Chin *et al*., 2003 |
| *Clostridium acetobutylicum* ATCC 824 | Glucose | Continuous | 1.08 mol $H_2$/mol glucose | Zhang *et al*., 2006 |
| *Clostridium butyricum* CGS 5 | Xylose | Batch | 0.73 mol/mol xylose | Lo *et al*., 2008 |
| *Clostridium butyricum* CGS 2 | Starch | Batch | 9.95 mmol/g COD | Chen *et al*., 2007 |
| *Clostridium pasteurianum* $CH_4$ | Sucrose | Batch | 2.07 mol/mol hexose | Lo *et al*., 2008 |
| *Clostridium paraputrificum* M-21 | Chitinous waste | Batch | 2.2 mol/mol substrate | Evvyernie *et al*., 2001 |
| *Clostridium thermocellum* 27405 | Cellulosic biomass | Batch | 2.3 mol/mol glucose | Levin *et al*., 2006 |
| *Clostridium thermolacticum* | Lactose | Continuous | 3.0 mol/mol lactose | Collet *et al*., 2004 |
| *Clostridium sp.* Strain no.2 | Cellulose | Continuous | 0.3 mol/mol glucose | Taguichi *et al*., 1996 |
| *Clostridium sp.* Fanp2 | Glucose | Batch | 0.2 mol/L medium | Pan *et al*., 2008 |
| *Enterobacter aerogenes* HO-39 | Glucose | Batch | 1.0 mol/mol glucose | Yokoi *et al*., 1995 |
| *Enterobacter aerogenes* NBRC 13534 | Glucose | Batch | 0.05 mol/L medium | Ogino *et al*., 2005 |
| *Enterobacter aerogenes* | Glucose | Batch | - | Jo *et al*., 2008 |
| *Enterobacter aerogenes* HU-101 | Glycerol | Batch | 0.6 mol/mol glycerol | Nakashimada., 2002 |
| *Enterobacter aerogenes* | Starch | Batch | 1.09 mol/mol starch | Fabiano and Perego, 2002 |
| *Enterobacter aerogenes* E82005 | Molasses | Continuous | 3.5 mol.mol sugar | Tanisho and Ishiwata, 1995 |

**Table1.1** The pure bacterial cultures for fermentative hydrogen production (cont')

| Organism | Substrate | Reactor type | Maximum yield of $H_2$ (mol $H_2$/mol substrate) | Reference |
|---|---|---|---|---|
| *Enterobacter cloacae* II-BT08 | Glucose | Continuous | 3.5 mol/mol sugar | Kumar and Das, 2001 |
| *Enterobacter cloacae* II-BT08 | Sucrose | Batch | - | Kumar and Das, 2000 |
| *Enterobacter cloacae* II-BT08 | Cellobiose | Batch | 6 mol/mol sucrose | Kumar and Das, 2000 |
| *Escherichia coli* MC 13-4 | Glucose | Batch | 1.2 mol/mol glucose | Ishikawa *et al*., 2006 |
| *Escherichia coli* | Glucose | Batch | 2.0 mol/mol glucose | Bisaillon *et al*., 2006 |
| *Escherichia coli* | Glucose | Continuous | 2.0 mol/mol glucose | Turcot *et al*., 2008 |
| *Pseudomonas* sp.GZ1 | Waste sludge | Batch | 0.007 mol/g TCOD | Guo *et al*., 2008 |
| *Thermoanaerobacterium thermosaccharolyticum* KU001 | Glucose | Batch | 24 mol/mol glucose | Ueno *et al*., 2001 |
| *Thermotoga kodakaraensis* KOD1 | Starch | Continuous | - | Kanai *et al*., 2005 |
| *Thermotoga elfii* | Glucose | Batch | 84.9 mol/L medium | Van *et al*., 2002 |
| Hydrogen-producing bacteria B49 | Glucose | Batch | 0.1 mol/L culture | Wang *et al*., 2007 |
| *Ruminococcus albus* | Glucose | Batch | 2.52 mol/mol glucose | Ntaikou *et al*., 2008 |
| *Hafnia alvei* | Glucose | Batch | - | Podesta *et al*., 2007 |
| *Citrobacter amalonaticus* Y19 | Glucose | Batch | 8.7 mol/mol glucose | Oh *et al*., 2008 |
| *Ethanoligenens harbinense* Yuan-3 | Glucose | Continuous | 1.93 mol/mol glucose | Xing *et al*., 2008 |

### 1.2.3 Metabolic profile in biohydrogen production

Generally, the typical metabolic pathways of glucose fermentation processing by *Clostridium spp*. are well known. Figure 1.2 shows that glucose is fermented via glycolysis and pyruvate is the end product. Electrons are transferred to reducing power, nicotinamide-adenine dinucreotide (NADH). Pyruvates are oxidized by puruvate: ferredoxin oxidoreductase (PFOR) to acetyl-CoA and $CO_2$, with electrons being transferred to ferredoxin (Fd). Acetate, butyrate, ethanol and butanol are the end products produced from acetyl-CoA under different conditions.

Production of acetate or butyrate allows for ATP generation whereas ethanol and butanol do not. Even though the electrons from NADH are utilized by the more reduced products, thereby maintaining the redox balance. NADH can alternatively be re-oxidized by electron transfer to ferridoxin by NADH:Fd oxidoreductase (NFOR). While hydrogenase enzymes play important roles in fermentative hydrogen production, which catalyzes proton reduction using electrons from ferredoxin (the reduced Fd). Therefore, a high yield of hydrogen production depends on the quantities of the reduced Fd, which can be formed from two sources: (1) Pyruvate oxidation by PFOR and (2) NADH oxidation by NFOR.

According to this pathway, a part of the electrons is transferred to proton to form molecule of hydrogen and the other to $NAD^+$ to generate $NADH_2$. $NADH_2$ is then used to produce hydrogen in the second pathway which relate hydrogenase enzyme where electron will be transferred to ferredoxin and then to $H^+$. During the batch fermentation two main phases of the process can be distinguished as (1) the acid production phase and (2) the solvent production phase. Firstly, during the via Embden-Meyerhof pathway, 1 mol of hexose is metabolized to 2 mol of pyruvate with the production of 2 moles of reduced nicotinamide adenine dinucleotide (NADH) and 2 mol of adenosine triphosphate (ATP). Meanwhile Clostridia can also utilize the pentose phosphate pathway for the conversion of 3 mol of pentose to 5 mol of ATP and 5 mol of NADH (Roger 1984).

Pentose sugars are fermented to pentose 5-phosphate, then fructose 6-phosphate and glyceraldehyde 3-phosphate are produced and can enter to glycolytic pathway. Subsequently, pyruvate generated from fermented hexose/pentose sugars is cleaved by pyruvate ferredoxin oxidoreductase in the presence of coenzyme A (CoA) to generated acetyl- CoA, reduced ferredoxin and carbon dioxide. The acetyl-CoA produced is the essential intermediate in both acid-producing and solvent-producing pathways. Then

acetate or butyrate and ATP can be produced from phosphorylation processing of Acetyl-CoA, which involves the phosphotransacetylase-kinase or phosphotransbutylase-kinase enzyme.

When organic acids are generated, there are not any reductions and reduced ferredoxin is able to transfer electrons to a hydrogenase that allows the use of protons as a final electron acceptor. Thus, ferredoxin is re-oxidized and molecular $H_2$ is released from the cell. The proton reduction ($H_2$ evolution) is essential in pyruvate fermentation or in the disposal of excess electrons. Under certain conditions (e.g. high $H_2$ partial pressure), the law of mass action limits the formation of $H_2$ and the cell is forced to channel electrons through NADH:ferredoxin oxidoreductase to reduce acetyl-CoA and butyryl-CoA to ethanol and butanol, respectively. Thus, solvent production involves a switch in the carbon flow from the acid production pathway to the solvent production pathway (Fig 1.3).

These reactions require two sets of dehydrogenases to achieve the necessary reductions to produce ethanol and butanol. Since solvent production involved reductions ferredoxin is unable to transfer electrons to a hydrogenase for $H_2$ evolution. For this reason, it is necessary to avoid the conditions that force the cell to switch the acidogenic to solventogenic fermentation. From the evolutionary perspective, the low yield of hydrogen is caused from the ability of hydrogen producing from microorganisms, which have developed their metabolic pathways preferentially for cell growth rather than $H_2$ synthesis. Therefore, the maximum theoretical yield of 12 mol $H_2$ / mol glucose from the complete conversion of glucose to $H_2$ and $CO_2$ is never obtained in biological in vivo system (Das, 2009). The maximum $H_2$ yield from fermentative $H_2$ production is 4 mol $H_2$/mol glucose (HP = 33%), which occurs if acetic acid is the only VFA produced and no electrons are used for growth (Angenent *et al.*, 2004). The $H_2$ yield is lower if other metabolites are produced, such as butyric acid (2 mol $H_2$/mol glucose, HP = 17%) or ethanol (0 mol $H_2$/mol glucose, HP = 0%). Thus, production of VFAs is preferred over alcohols and acetate is preferred over butyrate. However, the accumulation of acetate in the medium does not necessarily imply higher biohydrogen production since several microbial species can convert hydrogen and carbon dioxide to acetate:

$$2CO_2 + 4H_2 \rightarrow \ CH_3COOH + 2H_2O \qquad (1.4)$$

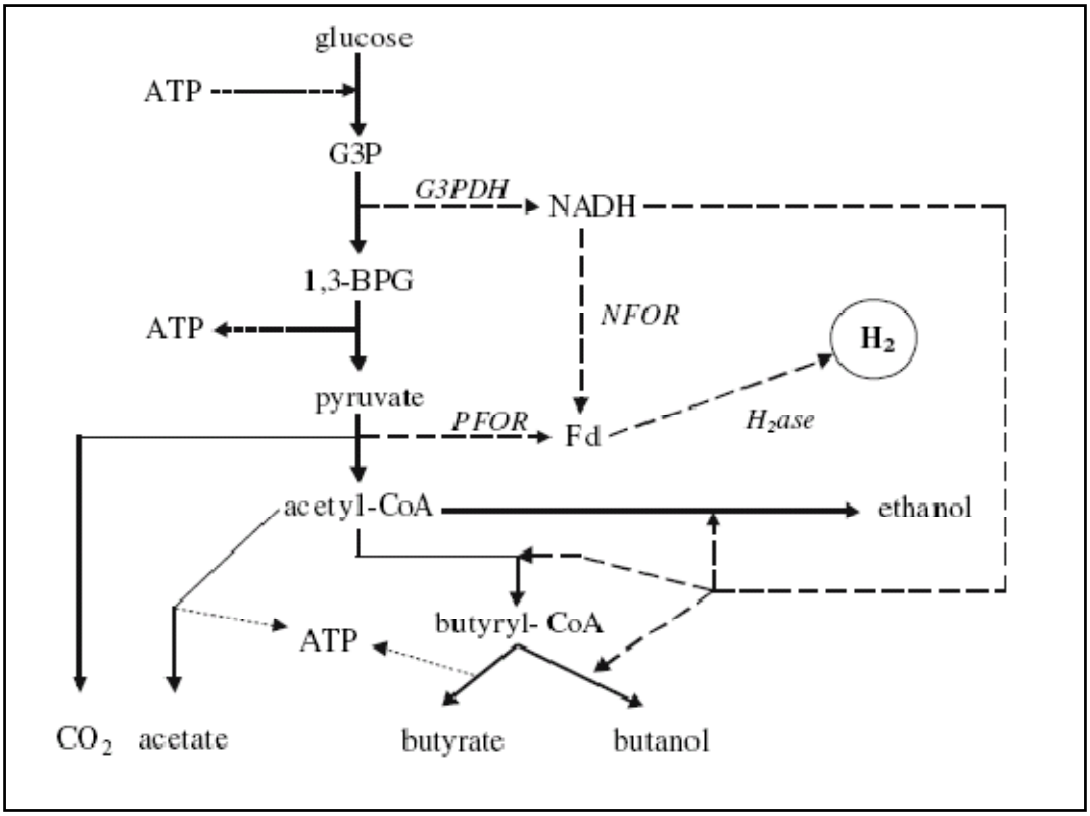


**Figure 1.2**: Metabolic pathways in *Clostridium spp*. fermenting glucose. Solid lines indicate substrate transformations, dotted lines indicate ATP creation or utilization, and dashed lines indicate electron flow. ATP = adenosine triphosphate; G3P = glyceraldehyde-3-phosphate; G3PDH = G3P dehydrogenase; 1,3-BPG = 1,3-bisphosphoglycerate; NADH = nicotinamide-adenine dinucleotide; Fd = ferredoxin; PFOR = pyruvate: Fd oxidoreductase; NFOR = NADH:Fd oxidoreductase; $H_2$ase = hydrogenase; CoA = coenzyme A. (Kraemer and Bagley. 2006)

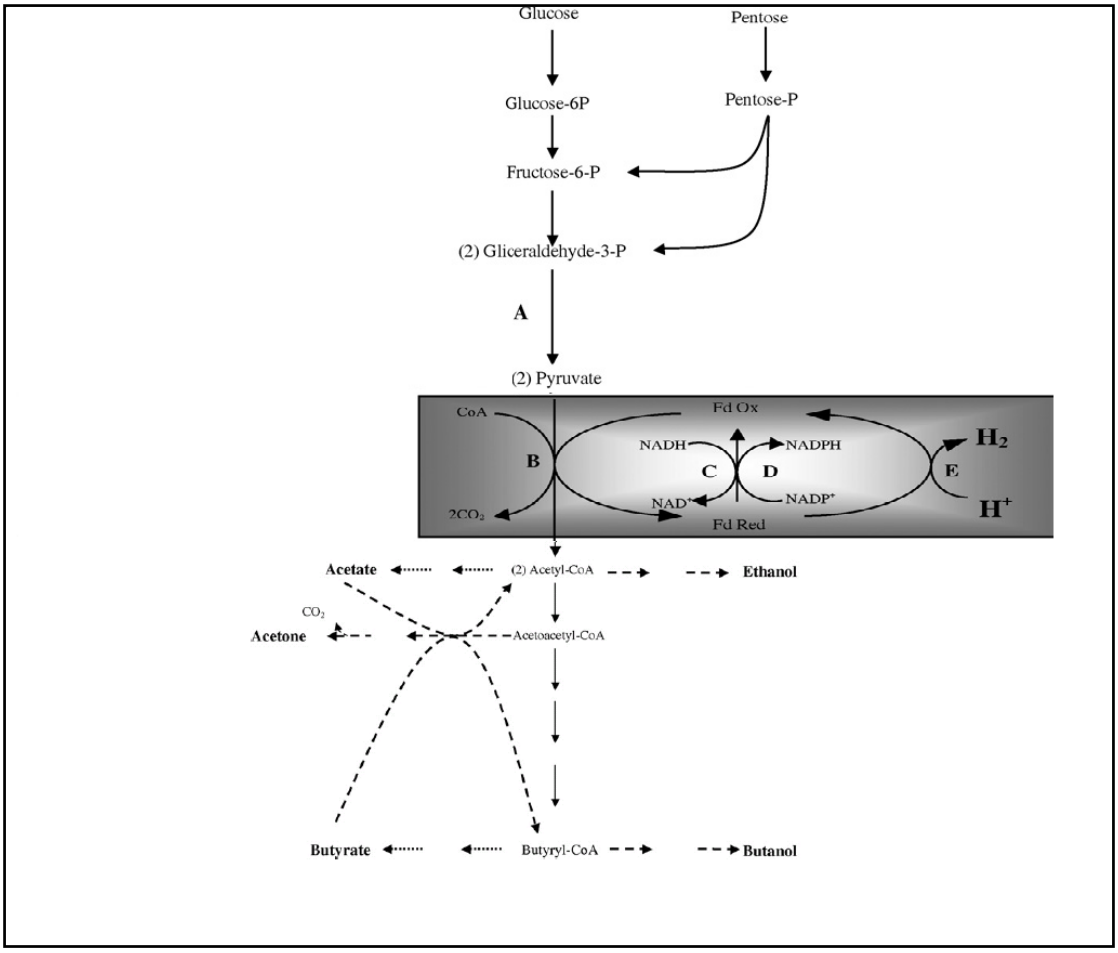


**Figure 1.3**: Biochemical pathways utilized by *Clostridium spp*. for the conversion of carbohydrates to hydrogen, carbon dioxide, organic acids and solvents. Solid lines indicate reaction during both acidogenic and solventogenic phases. Dotted lines indicate reactions that predominate during the acidogenic phase and dashed lines indicate reactions during the solventogenic phase. (Valdez-Vazquez and Poggi-Varaldo. 2009)

### 1.2.4 Metabolic flux analysis

Available $H_2$ fermentation pathways have been mostly observed from *Clostridia spp*. fed glucose in several previous reports (Zhu and Yang, 2004; Cai *et al*., 2010; Liu *et al*., 2010). Various reports revealed that multiple metabolic pathways were constructed in the network reaction in accordance with different environmental conditions of fermentation and/or type of microorganisms. Metabolic flux analysis (MFA) is the powerful tool to elucidate and analyze the complex metabolic network from different microorganisms to produce specific metabolites and to assist in optimizing process (Cai *et al*., 2010). According to previous literatures, MFA could be used for analyzing electron or

carbon flux distribution patterns and maximizing the yield of valuable end products such as organic acid, amino acids, polysaccharide and antibiotics (Stephanopoulos *et al*., 1998; Edwards *et al*., 2002; Kim *et al*., 2004).

Base on the basis of stoichiometric analysis and mass balances, MFA can be used to quantify intracellular fluxes to understand the overwhelming complexity of metabolic network of cellular responses (Stephanopoulos *et al*., 1998). However, MFA is intensively used for analyzing intracellular flux correlated with maximizing $H_2$ production and specific growth rate is selected for objective function (Table 1.2). Most MFA studies pointed out pure strain cultures and simple forms of substrate to analyze the capability of $H_2$ production and other valuable metabolites under different conditions by considering biomass formation as an objective function in optimizing process. It indicated that biomass formation seemed to be a significant metabolic flux that directly corresponded with the overall process of $H_2$ synthesis from the pure strain.

Only some of the literature has used the MFA for $H_2$ production by mixed cultures with simple substrates and/or complex substrates. A considerable issue of research study for mixed cultures in batch fermentation was conducted to understand the activity of organisms which could resist for stress condition in fermentation reactor such as acidic condition and to optimize conditions corresponding with $H_2$ synthesis. The end-products of anaerobic fermentation such as VFAs and/or alcohol could be used as an indicator to understand microbial activity of hydrogen producing bacteria in a reactor (Fan *et al*., 2004).

Manish *et al*. (2007) applied MFA to biohydrogen production with *Escherichia coli* and concluded that the mutant strain without ldh had enhanced hydrogen production. Oh *et al*. (2008) illustrated that based on metabolic constructed model for *Citrobacter amalonticus Y9*, the yield of hydrogen reached to 8.7 mol/mol glucose was possibly observed when glucose metabolism flowed to PP pathway and NAD(P)- linked hydrogenase was used to produce hydrogen. A metabolic network model for *Clostridium butyricum W5* was developed by Cai *et al*. (2010). Influence of pH condition on hydrogen yield was evidently observed rather than that of initial glucose concentration based on MFA.

In batch operation, Jiang *et al*., (2013) showed that MFA results obtained from fed batch experiments and reported that pH condition was critical to $H_2$ yield. While Cheng *et al*. (2013) constructed the metabolic network based on MFA to evaluated results

of hydrogen fermentation by continuous mode operation and expressed that HRT presented significant impact on hydrogen production from glucose. For mixed cultures, based on the metabolic pathway involved the activity of various microorganisms in the reactor, Chaganti *et al*. (2011) developed and utilized MFA to explain the impact of pH and LA on $H_2$ yield. However, by applying MFA to mixed cultures, it was lack of study published until now.

**Table 1.2** Summary of MFA applied to $H_2$ dark fermentation

| Microorganism | Substrate | Study condition | Objective function | Reference |
|---|---|---|---|---|
| *Clostridium butyricum W5* | glucose | pH and organic loading | specific growth rate | Cai *et al*., 2010 |
| *Clostridium tyrobutyricum* | glucose/ lactate/acetate | HRT | - | Cheng *et al*., 2013 |
| *Escherichia coli* | glucose | metabolically engineered | specific growth rate | Kim *et al*., 2008 |
| *Citrobacter amalonaticusY19* | glucose | organic loading | specific growth rate, $H_2$ production rate | Oh *et al*., 2007 |
| *Klebsiella pneumonia ECU-* | glucose | temperature, pH, organic loading | - | Niu *et al*., 2011 |
| Anaerobic sludge | glucose | pH and linoleic acid | acetic acid and acetone production | Chaqanti *et al*., 2011 |
| Anaerobic sludge | starch | organic loading | acetic acid production | This study |

## 1.3 Objectives

1.3.1 Compare the performance of biohydrogen production from *C.butyricum* and heat shocked anaerobic sludge under the various organic loadings of food wastes in 0.5 batch reactor.

1.3.2 Monitor the stability of *C.butyricum* and identify the co-dominant bacterial strains responsible for the peak biohydrogen production and long time fermentation in 5 L semi-batch reactor.

1.3.3 Analyze carbon mass balance and metabolic profiles during the fermentative hydrogen production process in order to determine the direction of metabolic pathway and inhibition factors of biohydrogen processes in 5 L semi-batch reactor.

1.3.4 Analyze metabolic flux network and constructa metabolic flux model in order to predict and maximize the end target products that directly correspond to biohydrogen production pathways.

# CHAPTER 2
# THEORIES

## 2.1.Biohydrogen production method

Biological hydrogen production processes can be classified into 4 main methods as follows:

### 2.1.1 Biophotolysis

With 178,000 TW hitting the Earth per year, solar energy is the most abundant renewable resource (Rupprecht *et al*., 2006). This widely available resource has attracted many studies into the photobiological production of hydrogen. However, many recent reviews have not interested this method of production since the efficiency of hydrogen conversion by solar energy is low and this method has expensive cost and complicated photo-bioreactors (Hallenbeck and Beneman. 2002; Levin *et al*., 2004; Das and Veziroglu. 2001). It is generally agreed that a stable and cost-effective solar conversion efficiency of at least 10% needs to be achieved so that this form of hydrogen production can compete with photoelectrical systems (Hallenbeck and Beneman. 2002; Prince and Kheshgi. 2005; Kruse et al., 2005 and Esper et al., 2006). Fortunately, several advances in direct photolysis, indirect photolysis, and photo-fermentation have shown that metabolic engineering can be used to increase conversion efficiencies and improve productivity (Kruse et al., 2005; Mussgnug *et al*., 2007). In this process, green algae and blue-green algae split water molecules into hydrogen ion and oxygen via direct and indirect biophotolysis.

#### 2.1.1.1 Direct biophotolysis

The conversion of water to hydrogen by green algae may be represented by the following general reaction:

$$2H_2O + \text{light energy} \rightarrow 2H_2 + O_2 \qquad (2.1)$$

The well-known $H_2$-producing green algae, *Chlamydomonas reinhardtii*, under anaerobic conditions, can either generate $H_2$ or use $H_2$ as an electron donor (Winkler *et al*., 2002). The generated hydrogen ions are converted into hydrogen gas in the medium with electrons (donated by reduced ferredoxin) by hydrogenase enzyme present in the cells. Light energy absorbed by photosystem II (PSII) generates electrons that are transferred to ferredoxin using light energy absorbed by photosystem I (PSI). A

reversible hydrogenase accepts electrons directly from the reduced ferredoxin to generate $H_2$ in the presence of hydrogenase. This enzyme is very sensitive to $O_2$. Hydrogenase activity has also been observed in other green algae, such as *Scenedesmus obliquus* (Winkler *et al*., 2002), Chlorococcum littorale (Nandi and Sengupta. 1998), Platymonas subcordiformis (Nandi and Sengupta. 1998) and Chlorella fusca (Winkler *et al*., 2002). On the other hand, there are several green algae types that do not have hydrogenase activity such as Dunaliella salina and Chlorella vulgaris (Nandi and Sengupta, 1998).

#### 2.1.1.2 Indirect biophotolysis

The general reaction for hydrogen formation from water by cyanobacteria can be represented by the following reactions:

$$12H_2O + 6CO2 + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2 \quad (2.2)$$

$$C_6H_{12}O_6 + 12H_2O + \text{light energy} \rightarrow 12H_2 + 6CO_2 \quad (2.3)$$

Cyanobacteria are also known as blue-green algae, cyanophyceae or cyanophytes. They are a large and diverse group of photoautotrophic microorganism. Cyanobacteria contain photosynthetic pigments, such as chl a, carotenoids and phycobiliproteins, and can perform oxygenic photosynthesis. Morphologically these organisms fall into a diverse group that includes unicellular, filamentous and colonial species. Hydrogen is produced both by hydrogenase and nitrogenase enzymes. Within the filamentous cyanobacteria, vegetative cells may develop into structurally modified and functionally specialized cells. The nutritional requirements of cyanobacteria are simple air ($N_2$ and $O_2$), water, mineral salts and light.

Hydrogen producing cyanobacteria may be either nitrogen fixing or non-nitrogen fixing. The examples of nitrogen fixing organisms are non-marine Anabaena sp., marine cyanobacteria *Calothrix sp*., *Oscillatoria sp*. Non-nitrogen fixing organisms are Synechococcus sp., *Gloebacter sp*. and *Anabaena sp*. They are found suitable for higher hydrogen evolution as compared to other cyanobacteria species (Nandi and Sengupta. 1998; Pinto et al., 2001; Winkler *et al*., 2002; Levin *et al*., 2004).

Heterocystous Filamentous, *Anabaena cylindrical* is a well-known hydrogen producing cyanobacterium (Nandi and Sengupta, 1998). But Anabaena variabilis has received more attention in recent years, because of higher hydrogen yield (Liu *et al*., 2006). The growth conditions for *Anabaena* are simple and include nitrogen free media, illumination, $CO_2$ and $N_2$. Nitrogenase plays an important role in hydrogen generation. The

activity of the nitrogenase is inhibited by oxygen. Hydrogen production takes place under anaerobic conditions. Some cultures require $CO_2$ during hydrogen evolution phase, although $CO_2$ is reported to give some inhibition effects on photo-production of $H_2$. Lower $CO_2$ concentrations (4–18% w/v) have been reported to increase cell density during growth phase, resulting in higher hydrogen evolution in the later stage. Simple sugars have been found suitable for hydrogen production. Recently more emphasis has been given to increase hydrogenase activity and bidirectional hydrogenase deficient mutants of *Anabaena sp*. to increase the rate of hydrogen production. However, at the present time the rate of hydrogen production by *Anabaena sp*. is considerably lower than that obtained by dark or photo-fermentations (Nandi and Sengupta. 1998; Pinto et al., 2001; Winkler *et al*., 2002; Levin *et al*., 2004; Liu *et al*., 2006).

With dinitrogen

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (2.4)$$

or, without dinitrogen

$$8H^+ + 8e^- + 16ATP \rightarrow 4H_2 + 16ADP + 16Pi \quad (2.5)$$

### 2.1.2 Photo fermentation

One of the other main sunlight-dependant hydrogen production methods is called photo-fermentation (Levin *et al*., 2004), which is carried out by nonoxygenic photosynthetic bacteria that use sunlight and biomass to produce the hydrogen (Zaborsky. 1998). Purple non-sulfur (PNS) and green sulfur (GS) bacteria such as *Rhodobacter spheroids* and *Chlorobium vibrioforme*, respectively, are capable of producing hydrogen gas by using solar energy and reduced compounds (Rupprecht *et al*., 2006). Their photosynthetic systems differ from oxygenic photosynthesis due to their requirement for reduced substrates and their inability to oxidize water (Rupprecht *et al*., 2006).

Nitrogenases are the main enzymes utilized by these bacteria and require nitrogen-deficient conditions in order to produce hydrogen (Levin *et al*., 2004). The PNS bacteria are capable of using a range of organic acids, while the GS bacteria prefer $H_2S$, S, or thiosulfate as a substrate (Rupprecht *et al*., 2006). The substrate is oxidized using the tricarboxylic acid cycle (TCA) and the produced electrons are shuttled through an electron transport chain that uses NAD/NADH and ferredoxin before a nitrogenase combines the derived electrons with a proton to produce hydrogen gas and restore redox balance to the cell (Rupprecht *et al*., 2006; Vignais *et al*., 2006). This production method has high conversion efficiencies from organic substrates but is still plagued by the reliance on ATP-

consuming nitrogenases, expensive photo-bioreactors and inefficient light harvesting antennae (Hallenbeck and Benemann, 2002; Rupprecht *et al*., 2006).

### 2.1.3 Dark fermentation

Dark hydrogen fermentation is a ubiquitous phenomenon under anoxic conditions (i.e. no oxygen present as an electron acceptor). When bacteria grow on organic substrates (heterotrophic growth), these substrates are degraded by oxidation to provide building blocks and metabolic energy for growth. This oxidation generates electrons which need to be disposed of to maintain electrical neutrality. In oxic environments, oxygen is reduced and water is the product. In anoxic environments, other compounds, e.g., protons, which are reduced to molecular hydrogen ($H_2$), need to act as electron acceptor (Das and Verziroglu, 2001; Levin *et al*., 2004). In the hydrogen fermentation process, glucose is initially converted to pyruvate by the glycolytic pathways. This is oxidized to acetyl-CoA, which can be converted to acetyl phosphate and results in the generation of ATP and the excretion of acetate. Pyruvate oxidation to acetyl-CoA requires ferredoxin (Fd) reduction. Reduced Fd is oxidized by hydrogenase which generates Fd (ox) and releases electrons to produce molecular hydrogen (Nath and Das. 2005). The overall reaction of the process can be described as follows:

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd (ox)} \rightarrow \text{acetyl - CoA} + 2\text{Fd (red)} + \text{CO2} \quad (2.6)$$

$$2H^+ + \text{Fd (red)} \rightarrow H_2 + \text{Fd (ox)} \quad (2.7)$$

Anaerobic fermentation enables the mass production of hydrogen via relatively simple processes from a wide spectrum of potentially utilizable substrates, including refuse and waste products. Moreover, fermentative hydrogen production generally proceeds at a higher rate and does not rely on the availability of light sources. Carbohydrates, mainly glucose, are the preferred carbon sources for fermentation processes, which predominantly give rise to acetic and butyric acids together with hydrogen gas, as follows:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2.8)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2COOH + 2CO_2 + 2H_2 \quad (2.9)$$

The end-products of glucose fermentation by anaerobic and facultative anaerobic chemoheterotrophs, e.g., clostridia and enteric bacteria, are produced through pyruvate. Facultative anaerobic bacteria give 2 mol of hydrogen per mol of glucose, whereas strictly anaerobic bacteria give four moles. Facultative anaerobes are less sensitive to oxygen, and are sometimes able to recover hydrogen production activity after accidental

oxygen damage to them by rapidly depleting oxygen present in the broth. As a consequence, a facultative anaerobe is considered a better microorganism than a strict anaerobe to carry out fermentative hydrogen production process (Oh *et al*., 2003).

One of the main constraints of fermentative biohydrogenation process is the lower yield of hydrogen, maximally 4 mol/mol glucose, compared with other processes. A yield of 2 mol $H_2$/mol glucose was reported for butyrate fermentation (Hawkes *et al*., 2002). However, by a modification of fermentation pathways only a maximum of 4 mol $H_2$/mol glucose can be expected from ideal acetate fermentation. This yield is too low to be economically viable as an alternative to existing chemical or electrochemical processes of hydrogen generation (Hallenbeck and Benemann. 2002). Therefore, the ultimate goal, and challenge, for fermentative hydrogen research and development focuses essentially on attaining higher yields of hydrogen. The present review provides a critical discussion of the various practical and theoretical approaches towards improvement of overall yield of hydrogen in fermentative process.

### 2.1.4 Two-stage process (integration of dark and photo fermentation)

In fermentation, complete oxidation of 1 mole of glucose yields 12 moles of hydrogen. However, complete oxidation of glucose into hydrogen and carbon dioxide is not possible as the corresponding reaction is not feasible thermodynamically

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO2,\ \Delta G_o = +3.2\ kJ. \quad (2.10)$$

With an external energy supply (photon-energy in photofermentation), theoretically 12 moles of hydrogen per mole of glucose can be produced. However, this process cannot be operated in the absence of light. On the other hand, in the absence of external energy (in the case of dark-fermentation), oxidation of glucose by fermentative bacteria results in other by-products also and maximum 4 moles of hydrogen are produced per mole of glucose consumption with acetate as the sole by-product:

$$C_6H_{12}O_6 + 2H_2O \rightarrow\ 4H_2 + 2CO_2 + 2CH_3COOH,\ \Delta G_o = -206\ kJ. \quad (2.11)$$

Acetate produced in the dark-fermentation stage can be oxidized by photosynthetic bacteria to produce hydrogen:

$$CH_3COOH + 2H_2O + \text{‘light energy’} \rightarrow 4H_2 + 2CO_2,\ \Delta G_o = +104\ kJ. \quad (2.12)$$

Hence, continuous production of hydrogen at maximum yield can be achieved by integrating dark- and photo-fermentation methods.

## 2.2 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a particular type of gel electrophoresis in which constant heat (about 60ºC) and an increasing concentration of denaturing chemicals are used to force DNA molecules to unwind. This molecular biology approach is a fingerprinting methodology that has led to revolutionary changes in many of the traditional routines used in assessing microbial populations. Denaturing gradient gel electrophoresis (DGGE) is a technique used for separating DNA fragments according to their mobilities under increasingly denaturing conditions (usually increasing formamide/ urea concentrations).

In practice, the DNA fragments are usually produced via PCR amplification. The DGGE technique exploits (among other factors) the difference in the stability of G-C pairing (3 hydrogen bonds per pairing) as opposed to A-T pairing (2 hydrogen bonds). A mixture of DNA fragments of different sequence is separated by electrophoresis on an acrylamide gel containing a linearly increasing gradient of DNA denaturants (usually urea and formamide). In general, DNA fragments richer in GC will be more stable and remain double-stranded until reaching higher denaturant concentrations. Double-stranded DNA fragments migrate better in the acrylamide gel, while denatured DNA molecules slow down or stop in the gel. In this manner, DNA fragments of differing sequence can be separated in an acrylamide gel.

DGGE is commonly performed for partial 16S rRNA gene, but also functional genes may be used. A GC (guanine plus cytosine) rich sequence can be incorporated into one of the primers used in the PCR to modify the melting behaviour of the fragment of interest and to improve the separation of the fragments. The DGGE gels can be stained with DNA binding fluorescent dyes, such as SYBR Green and visualized under UV light. Known standards may be used for comparing the samples on different gels. Ideally one band on the gel corresponds to one species, and therefore the number of bands gives an idea of the diversity of the sample. The gene fragments can be excised from the gel, eluted e.g. into sterile water and amplified for sequencing. The relative abundance of various microorganisms can be estimated by measuring the intensity of their bands relative to the intensity of all bands in the corresponding sample. Figure 2.1 show an example of microbial community analysis by using the DGGE technique.

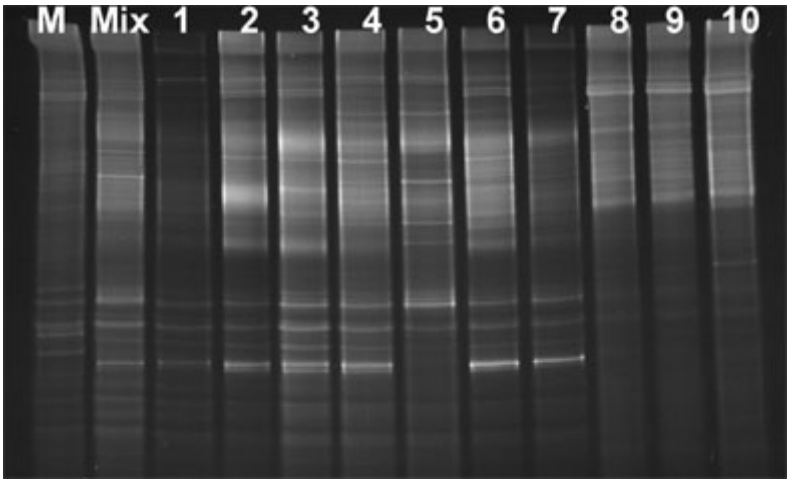


**Figure 2.1**: An example of DGGE gel showing band compositions of various population samples that are representative of complex microbial ecosystems. Each band in each lane represents a 16S amplified product migrating to a unique position in the gel, which melts in a sequence dependent manner.

**Table 2.1** Advantages and disadvantages of different hydrogen production processes (Das *et al*., 2009)

| Process | Advantage | Disadvantage |
|---|---|---|
| **Direct biophotolysis** | - can produce $H_2$ directly from water and sunlight<br>- Solar conversion energy increase by tenfold as compared to trees, crops | - require high intensity of light<br>- $O_2$ can be dangerous for the system<br>- lower photochemical efficiency |
| **Direct biophotolysis** | - can produce $H_2$ from water<br>- has the ability to fix $N_2$ from atmosphere | - uptake hydrogenase enzymes need to be removed to stop degradation of $H_2$<br>- about 30% $O_2$ present in gas mixture |
| **Photo fermentation** | - A wide spectral light energy can be used by these bacteria<br>- can use different waste materials such as distillery effluents, waste, etc. | -low light conversion efficiencies, high energy demand by nitrogenase, expensive hydrogen impermeable photobio-reactor required |
| **Dark fermentation** | - can produce $H_2$ all day long without light<br>- a variety of carbon sources can be used as substrates<br>- produce valuable metabolites such as butyric, lactic and acetic acids as by products<br>- no $O_2$ limitation problem | - $O_2$ is a strong inhibitor of hydrogenase<br>- relatively lower achievable yields of $H_2$<br>- as yields increase $H_2$ fermentation becomes thermodynamically unfavorable<br>- product gas mixture contains $CO_2$, which has to be separated |
| **Two-stage fermentation (dark fermentation)** | - stoichiometric yield of 12 mol $H_2$ per mol hexose represents the ultimate target for biohydrogen | |

# CHAPTER 3
# METHODOLOGY

## 3. Materials and Methods

### 3.1. Materials

#### 3.1.1 Food waste

Synthetic food waste was prepared from typical locally-produced food waste to mimic the food waste compositions collected in the canteen of King Mongkut's University of Technology North Bangkok, Thailand. The food waste, containing 65% carbohydrate (rice), 17% vegetable and 18% meat (w/w), was ground in a blender to particles of diameter approx. 0.5 mm and used as the feedstock with no prior sterilization. The ground food waste contains total solid (TS) and total volatile solid (VS) of 45,520 and 27,578 mg $L^{-1}$. Minimal amount (< 1 mM) of volatile fatty acids (VFAs) and ethanol was detected in the ground food waste. Prior to use, the food waste was adjusted to the desire concentration with distilled water.

#### 3.1.2 Inocula

##### 3.1.2.1 Mixed consortia

Anaerobic granules were obtained from a full-scale upflow anaerobic sludge blanket reactor treating fruit juice processing wastewater (Malee Industry Co. Ltd., Nakornpathom, Thailand and Eiamburapa Industry Co. Ltd., Sa Kaeo, Thailand). Prior to use, the granules were sieved to the size <0.5 mm to remove coarse matters and then washed twice with tap water and subsequently heat-shocked by boiling at 100$^{o}$C for 1 hr. The heat-treated granules were re-cultivated in 0.5% (w/v) glucose solution for 24 hr and washed with the distilled water twice before use as a seed microbial inoculum for the hydrogen fermentation.

##### 3.1.2.2 Pure culture

*C. butyricum* (TISTR 1032) was purchased from the Thailand Institute of Scientific and Technological Research (TISTR). Prior to cultivation, the *C. butyricum* was reactivated by transferring 2 mL of the stock culture into 20 mL of Reinforced Clostridial Medium (RCM). The cultural serum bottle was flushed with nitrogen gas for 2 min to create anaerobic condition and incubated at 37 $^{o}$C for 10 hr in 150 rpm incubator shaker. The culture was further enriched by inoculating 10% v/ v of previous culture into

60 mL Tryptone Sucrose Yeast Extract (TSY) and incubated under the same conditions. Three re-cultures of inocula were grown before use in the fermentation process. The optical density (600 nm) of the inoculum was determined and controlled about 0.8 before using. Composition of TSY (per liter) contains 5.0 g of tryptone; 3.0 g of sucrose; 5.0 g of yeast extract; 1.0 g of $K_2HPO_4$ (Pattra et al., 2008).

## 3.2 Method

### 3.2.1 Hydrogen production in 0.5 L batch reactor

A batch fermentation system was set up in 500 mL screw-cap bottles with a working volume of 500 mL (375 mL of food waste and 125 mL of inoculum). The microbial seeds were fixed at 4.75 g total volatile solids (VS) and 0.15 g per batch for anaerobic sludge and *C. butyricum* inocula, respectively. The food waste was added at varying concentrations between 2.5-12.5 g VS/L for pure culture batch test and F/M ratio of 1-10 for mixed culture batch test. The initial pH was adjusted to 6.0 with 6 N NaOH or concentrated $H_3PO_4$. The system was flushed with nitrogen gas to generate anaerobic conditions. $H_2$ fermentation was conducted at 37°C with rotary shaking at 150 rpm. All experiments were setup in duplicate. During the fermentation experiment, total gas volume and composition were periodically monitored by gas counters and gas chromatography, respectively. The liquid samples were analyzed for pH and VFAs every 4-6 hr.

### 3.2.2 Hydrogen production by *C. butyricum* in 5 L semi-batch reactor

The 5 L reactor was set up with 4 L of working volume. The initial pH was adjusted to 6.0. The culture was then purged with nitrogen gas to create anaerobic conditions and incubated at 37 $^{o}$C with constant shaking at 100 rpm. The controlled pH condition was performed using 1 M NaOH and concentrated acid solution of orthophosphoric acid throughout the experiment. In semi-batch operation, fresh substrate was added to maintain the same concentration (2.5% TVS) when the production of hydrogen was decreased to zero. This point is recorded as the new feed starter while the mixed liquor (both culture medium and inoculum) in the reactor was discharged for the same volume. The ratio of new substrate replacement was approximately at 1:4 (1L: 4L) of the total working volume. During the experiment, total gas volume and gas composition were periodically monitored by gas counter and gas chromatography. The mixed liquor samples were analyzed for pH, chemical oxygen demand (COD), total solid (TS), TVS,

total organic carbon (TOC) and volatile fatty acids (VFA) in every 6-12 hr.; the analytical methods used in the experiment were followed the standard methods.

### 3.2.3 Batch fermentation for MFA

A batch fermentation system from rice starch was set up in 500 mL screw-cap bottles with a working volume of 500 mL. The seed microbial inocula were fixed at 5.45 g total volatile solids (VS). The rice starch solution with distill water was adjusted for the final concentration varied in a range 2.5-12.5 g/L. The initial pH was adjusted to 6.0 with 6 N NaOH or concentrated $H_3PO_4$. The system was flushed with nitrogen gas to generate anaerobic conditions. $H_2$ fermentation was conducted at 37°C with rotary shaking at 150 rpm. All experiments were setup in duplicate. During the fermentation experiment, total gas volume and composition were periodically monitored by gas counters and gas chromatography, respectively. The liquid samples were analyzed for pH, VFAs, residual starch and glucose every 5 hr.

## 3.3 Analytical techniques

Gas volume and composition were periodically monitored by water displacement method and a liquid sample from each bottle was analyzed for pH, COD, TS, TVS and volatile fatty acids (VFA) every 4 hrs. The analytical methods used were in accordance with standard methods 2540 G and 5220 B, respectively (APPA, 2005).

The initial concentrations of the chemical substances in the synthetic food waste and the remaining chemical substances, such as carbohydrate and protein in the culture medium, were determined. Carbohydrate was examined using the phenol sulfuric method (Dubois *et al*., 1956). TOC was examined by high temperature combustion using heat (680°C or higher) in a stream pure oxygen and cobalt oxide as a catalyst. In the process, the oxygen-rich atmosphere converts all carbon to carbon dioxide to be measured. Non Dispersive Infrared Analyzer (NDIR) was used as the modern analyzer to detect the carbon dioxide produced from combustion. Total carbon in solid biomass was analyzed by high temperature combustion using heat (680°C). Thermal conductivity detector (TCD) was used to detect the carbon dioxide produced from combustion.

For starch analysis, samples were acidified and boiled with concentrated acid (2.5 N HCl) for 3 hr to completely hydrolyze starch into sugar and the total sugar concentration was determined by using the phenol acid spectrometric method (Dubois *et al*., 1956).

Starch concentration was determined by dividing sugar concentrations by 1.10, since the starch-to-glucose ratio is 1.10 in the hydrolysis reaction of starch. Therefore, total glucose concentration at given time was equal to free glucose plus glucose from hydrolyzed starch. For biomass measurement, 10 mL fermentation broth samples were centrifuged at 8000 rpm for 20 min (TOMY MX-301 High Speed Refrigerated Micro Centrifuge, Japan). Pellets were wash twice with distill water and weighted after drying at 70$^{o}$C overnight.

### 3.3.1 Gas chromatography

The amount of generated biogas was recorded using liquid displacement gasometers. Biogas content ($H_2$, $CH_4$, and $CO_2$) was measured periodically every 4-6 hr using a gas chromatograph (Shimadzu GC-8A, Kyoto, Japan) equipped with a thermal conductivity detector (TCD) with a Unibeads C 60/80 column (GL Sciences, Inc., Tokyo, Japan). Helium was used as a carrier gas. The temperatures of the injection port and the detector were 150 and 80°C, respectively. VFAs were analyzed by gas chromatography (Shimadzu GC-7A system equipped with a flame ionization detector and a Stabilwax DA capillary column (Restek Corporation, PA, USA). The temperatures of the injection port and detector were maintained at 240°C (Nathao *et al*., 2013).

### 3.3.2 High performance liquid chromatography

Free glucose was analyzed by High Performance Liquid Chromatography (Agilent Technologies 1200 series, Germany equipped with reflextive index detector, UV detector and an Aminex HPX-87H column, 300 x 7.8 mm, Biorad, USA). The mobile phase was 5mM $H_2SO_4$ at flow rate of 0.7 mL/min and the column temperature was 64$^{o}$C.

### 3.3.3 Denatured gradient gel electrophoresis (DGGE) analysis

The total genomic DNA of the bacteria samples from the 5L reactor was collected at the initial time, at the peak of $H_2$ production on each feed and the final time of the experiment. DNA extraction was applied in accordance with Zhou *et al* (1996). Firstly, samples were homogenized and extracted with extraction buffer pH 8 (100 mM Tris-HCl, 100 mM EDTA, 1.5 M NaCl). Lysozyme was added for the final concentration of 10 mg/ml and incubate at 37 ºC for 60 min. Then 20% (w/v) SDS was added and incubated at 70 ºC for 30 min. The lysate was then centrifuged and the supernatant (solution dissolved with extracted DNA) was kept for the next step in the cleaning and the removal of non-specific substance. An equal volume of isoamyl:chloroform (1:24) was added to the supernatant to precipitate DNA from the solution together with 0.6 volume of isopropanol and incubated at 4 $^{o}$C overnight. After precipitation, DNAs were washed with absolute

ethanol before centrifugation. The DNAs were dried and dissolved with TE buffer pH8 (1 M Tris-HCl, 0.5 M EDTA).

A primer set of 338F (5'-ACTCCTACGGGAGGCAGCAG-3') plus GC-clamp (5'-CGCCCGGGGCGCGCCCCG GGCGGGGCGGGGGCACGGGGGG-3') and 518R (5'-ATTACC GCG GCT GCT GG-3') were used for amplifying the approximate 200–bp target product of 16S rDNA-V3 regions (Nielson et al., 1999). 50 µL of Polymerase Chain Reaction (PCR) mixtures is generally contained 0.2 mM of dNTPs, 0.5 µM of each primers, 1.5 mM of $MgCl_2$, 1 U of Taq DNA polymerase (Kapa Biosystems, US), and the 1X PCR buffer. The condition used for PCR amplification is as followed: DNA denaturing step at 95$^{o}$C for 5 min followed by 30 cycles at 95$^{o}$C for 30 sec (denaturation), 60$^{o}$C for 30 sec (annealing), and 72$^{o}$C for 50 sec (extension) and then by a final elongation step at 72 $^{o}$C for 7 min to ensure full extension of the product. All PCR reactions were performed on an Eppendorf® Mastercycler Gradient (Eppendorf, Germany). PCR products were analyzed by electrophoresis at 100 V for 25 min through 1.5% (w/v) agarose gel before DGGE. The amplified products were visualized under UV light after ethidium bromide staining. Variation of DNA sequence of PCR amplifying fragments were assessed by DGGE.

The PCR-DGGE profile of the PCR-amplified DNA was obtained by using a DGGE-2000 system apparatus (CBS Scientific Company, Del Mar, CA.). The 8% (w/v) acrylamide solution was used to cast a gel with denaturant gradients ranging from 40 to 60% denaturant. Electrophoresis was conducted in a 1X TAE buffer solution at 80 V and 60$^{o}$C for 14 hr. The gels were stained for 10 min with SYBR Gold nucleic acid gel strain (Invitrogen, USA) and visualized with UV illuminator and take a photo under UV light. The target DGGE bands were excised and then dissolved in 20 µL $dH_2O$ at 4$^{o}$C overnight. The eluted DNA was used as DNA template in a following PCR amplification with primers 338GC-F-M13R(5'-*CAGGAAACAGCTATGAC*<u>GGGCGGGGCGGGGGCACGGGG GG</u>ACTCCTACGGGAGGCA-3') and 518R, the primer 338GC-F-M13R binding sequences described by O'Sullivan *et al* (2008). (M13R primer binding sites are in italics; GC clamp are underlined). PCR products were purified using Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan) before sequencing by 1$^{st}$ BASE, Malaysia. The DNA sequences were analysed with ChromasPro Program version 1.49 beta and compared to the available databases using the BLASTn tool of the NCBI for the phylogenetic affiliations.

### 3.3.4 In silico model construction and metabolic flux analysis

The anaerobic starch metabolic network for the mixed cultures that had been constructed in the *in silico* model was developed from the concept of universal bacterium under the anaerobic degradation of organic matter. The network was constructed with 26 reactions, 14 intracellular metabolites and 12 extracellular compounds (Appendix A and B). In order to avoid redundancy, the values for starch and residual starch were ignored from the flux calculation. Biomass synthesis was not included into the model since it hardly observed in this study. For living systems, the objective functions of prokaryotic metabolism would be allowed to include energy production and biomass growth that it was presented in term of the network variables (Chaganti et al., 2011).Nevertheless, the particular objective function would be selected under the rationale that the organism can maximize its target product under stress condition (Lee et al., 2006). Maximizing acetic acid production was chosen as the objective function and the criterion to estimate an accordance of the results from the experiments and the *in silico* model. Starch hydrolysis, glucose utilization, metabolites such as acetic, butyric, lactic acids and ethanol production defined in stoichiometric coefficients comparing to 1 mol of initial starch concentration were used as constraints in the *in silico* model. Carbon balance was evaluated to check the validity of all external fluxes before using in the model. Undetermined fluxes could be solved by linear optimization using the program package MetaFluxNet (Lee *et al*., 2003).

### 3.3.5 Carbon mass balance

Carbon mass balance was used to determine the carbon fraction in each phase (solid, liquid and air) during the fermentative period that resulted in monitoring the metabolic direction of the biohydrogen production process. Carbon fraction could be in gas phase in the form of $CO_2$, liquid phase in the form of volatile fatty acid (VFA), alcohol and chemical substance remained from food waste (eg. carbohydrate and protein) including solid phase in the form of insoluble chemical substance remained from food waste and biomass (bacterial cells).

Therefore, the carbon mass balance can be calculated from:

Total carbon = carbon in gas phase + carbon in solid phase + carbon in liquid phase

The calculation of each fractional carbon is shown as:

1. Soluble carbohydrate digested with strong acid by the phenol sulfuric method was reported as the concentration of glucose (g/ L).

2. VFA and ethanol was calculated by using the results from GC analysis in the unit of molar of product per volume of the culture medium (mol/ L).

3. Carbon dioxide was calculated by using the results from GC analysis in the unit of volume and then converted into molar units using the ideal gas law.

4. Solid phase was calculated by using the results from CHNO analysis in the unit of gram carbon per gram solid biomass (g/ g).

### 3.4 Kinetic analysis

The modified Gompertz Equation (Eq. 1) was used to fit the cumulative hydrogen/methane production data obtained from each batch experiment (Lay *et al*., 1999). This model has long been used for describing hydrogen production in batch fermentation experiments.

$$H(t) = P \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\} \qquad (1)$$

where $H(t)$ is cumulative hydrogen production (mL) during the incubation time, $t$ (hr), $P$ is the hydrogen production potential (mL), $R_m$ is the maximum production rate (mL/ hr), $\lambda$ is the lag phase duration (hr), and e is the exp(1) = 2.718.

Hydrogen yield (Y) is calculated by dividing the hydrogen production potential by the amount of VS substrate, working volume of reactor and volatile solids of inocula.

# CHAPTER 4

# Comparative Performance between Heat-Shocked Anaerobic Sludge and *Clostridium butyricum TISTR 1032* Inocula in Biohydrogen Production from Food Waste

## 4.1 Hydrogen fermentation by *C. butyricum* in 0.5 L reactor

Hydrogen was produced after 12 hr incubation for all cases (Fig. 4.1). The highest hydrogen content (32%) and volume (80 mL) was observed at 2.5 % after 12 hr incubation. Hydrogen content and the volume of hydrogen were slightly increased until 72 hr that the maximum hydrogen content occurred about 67% was observed at 10% VS while the maximum volume 276 mL of 2.5% VS was observed at 96 hr. The highest cumulative hydrogen production was obtained at lower concentration of food waste, 2.5 and 5%, about 1,069 and 854 mL whereas at high concentration of food waste, 10 and 12.5%, the results showed that the cumulative hydrogen production was significantly lower than that in the low substrate concentrations (293 and 196 mL). Results indicated that the concentration of the food waste (organic loading) influenced the ability of biohydrogen production. Under the over-loading substrate condition, high concentration of VFA was quickly produced in acidogenic phase. At acidic pH, the VFA was favourably in undissociated form inhibiting biohydrogen production. This condition disturbed the equilibrium state inside the cell of organism that eventually the reaction was switched to produce more reduced substance to reach equilibrium state and to reduce toxicity for cell together with to maintain regular metabolic inside the cell.

Fig. 4.2 showed that at high food waste concentration (10 and 12.5 %), acetic and propionic acids were the main final products in the liquid phase. The accumulation of propionic acid in anaerobic fermentation process is often observed in the condition of high yield of $NADH_2$ (Ren. 1994). The ratio of $NADH_2$ ($NADH+H^+$) and $NAD^+$, the medium metabolism product, also affect directly in biohydrogen production process since to reach equilibrium state inside the cell of organism, the proper ratio between ($NADH+H^+$) and $NAD^+$ ($NADH+H^+/NAD^+$) during oxidation reduction reaction should be maintained. If the concentration of hydrogen gas in the reactor headspace was too high, it can dissolve back into the liquid phase. In this condition, molecules of hydrogen around the cells were not released in time and affected hydrogen formation from the $NADH+H^+$ channel such that the electron flow was stopped and superfluous $NADH+H^+$ had accumulated. To

regenerate superfluous $NADH+H^+$, more reduced substance such as propionic acid, lactic acid and alchohol was produced to maintain general metabolism of organism. Generally, propionic acid conversion rate is often increased to exit the oxidation reduction reaction at or close to equilibrium state that also adjust the metabolism of the microorganism (Li *et al*., 2007).

On the contrary, at low concentration of substrate, volatile acids produced in this case were less compared with in high concentration of substrate that acetic and butyric acid were the main final product. In this condition, hydrogen formation from $NADH+H^+$ was allowed that firstly the reduced ferredoxin was oxidized to produce molecule of hydrogen. At the same time, the resulting oxidized ferredoxin was recycled back to the reduced form by NADH-ferredoxin oxidoreductase (NFOR) that oxidized $NADH+H^+$ into $NAD^+$ essential for driving glycolysis process. Therefore, the concentrated volatile acid produced during anaerobic fermentation process was a factor inhibited hydrogen formation from $NADH+H^+$ channel. The high accumulated undissociated acid above 19 mM was found to be the inhibition factor which was a threshold concentration for significant decreasing hydrogen yield and beginning solventogenesis (Ginkel and Logan. 2005).

The results revealed that the total undissociated acids had accumulated in the broth tend to increase when the concentrations of food waste were increased (Fig. 4.2). High concentration of substrate was not only resulted in metabolites accumulated in the broth, but it also affected directly in the total concentration of undissociated acids that finally reduced the efficiency of biohydrogen production. On the contrary, at high concentration of substrate (10 and 12.5%), the efficiency of COD removal was higher than that at low concentration of substrate (2, 5 and 7.5%) (Fig.4.3).

The value of $H_{max}$ was observed to be highest at 2.5% VS and slightly decreasing at 5, 10, 12.5 and 7.5 % VS respectively. Similarly, the value of $R_{max}$, was the highest at 2, 5, 10, 12.5 and 7.5 % VS respectively (Fig. 4.4 and Table 4.2). The maximum values of 3.7 mol $H_2$/mol $hexose_{added}$ and 134 mL $H_2$/ g $VS_{added}$ from this study obtained from 2.5% VS food waste (Fig. 4.5 and Table 4.2) under the condition of initial pH 6, 37$^oC$ in batch operation mode were capable to compare with previous studies (Table 4.1). Results from this study showed that the efficiency of degradation of food waste and biohydrogen production by *C. butyricum* were feasible and practical to scale up and develop to industrial portion in the future.

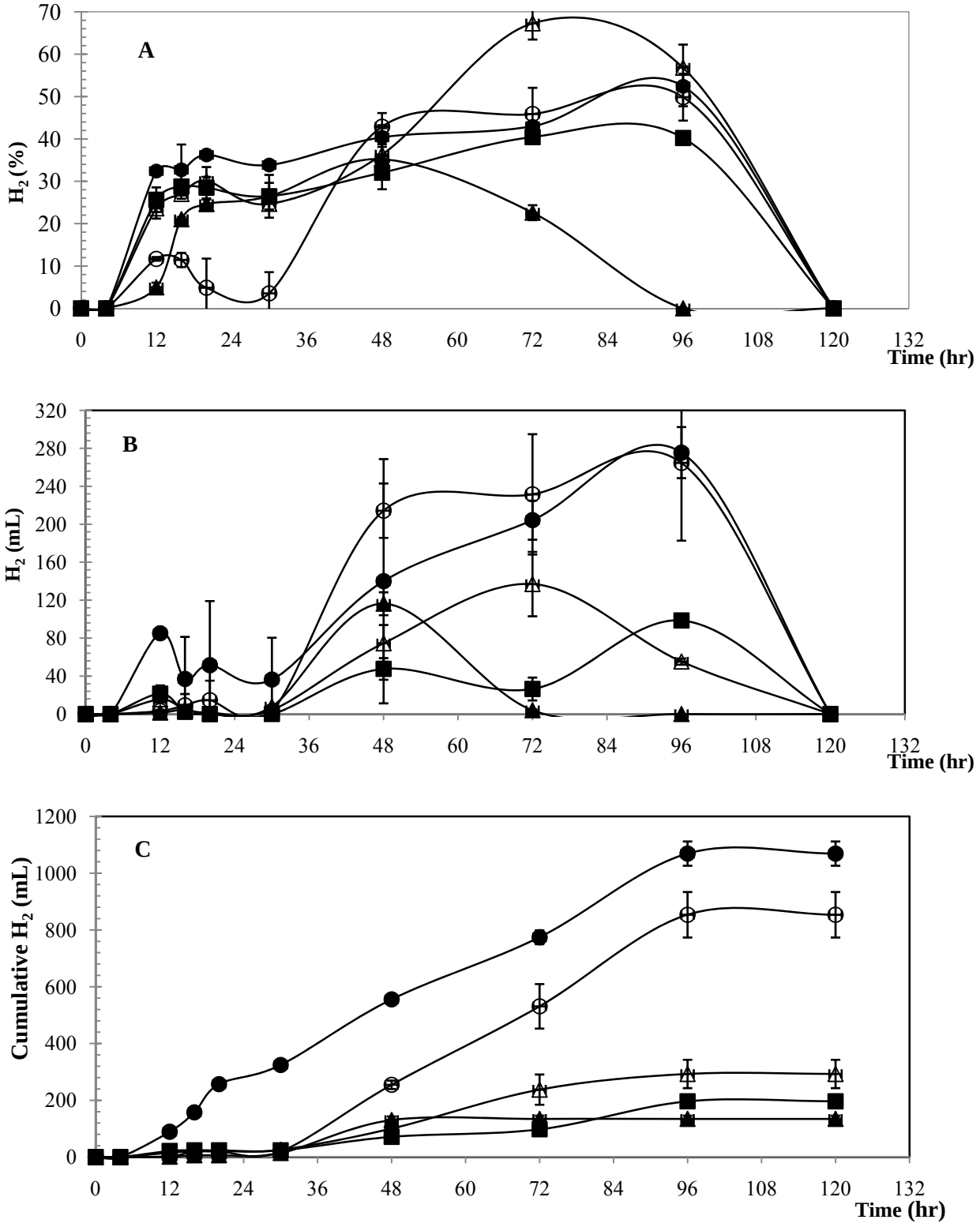


**Figure 4.1:** (A) Hydrogen content, (B) hydrogen volume, and (C) cumulative hydrogen volume produced from varying food waste concentrations, 2.5 (•), 5 (○), 7.5 (▲), 10 (△), 12 (■) % VS. Symbols represent mean values of duplicate measurements. Error bars represent one standard deviation.

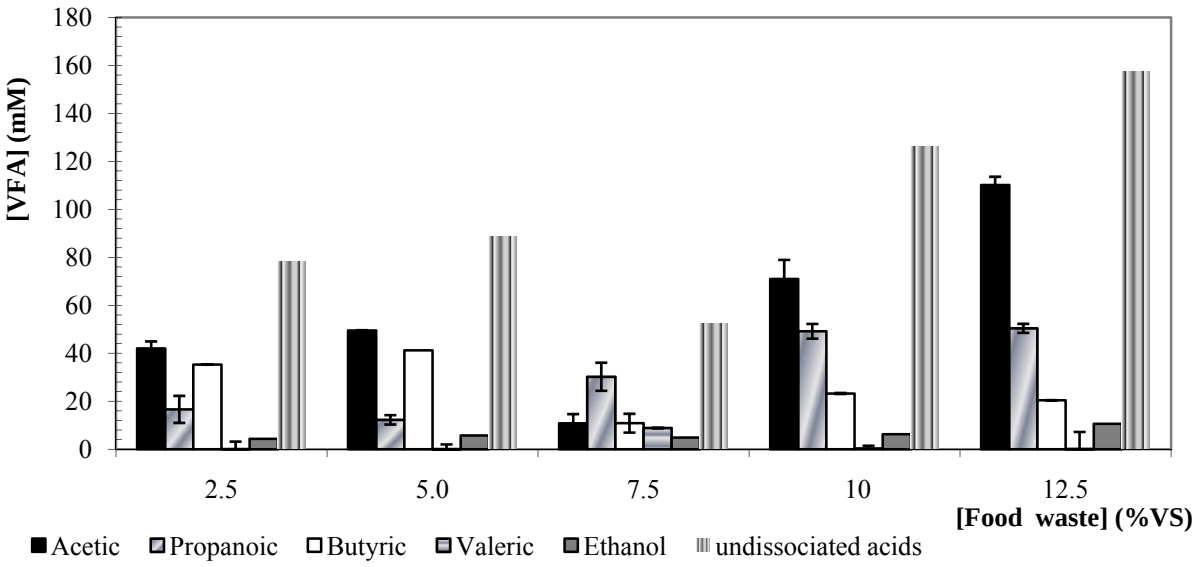


**Figure 4.2:** VFA accumulation in the reactor content after 96 hr fermentation. Histograms represent mean values of duplicate measurements. Error bars represent one standard deviation.

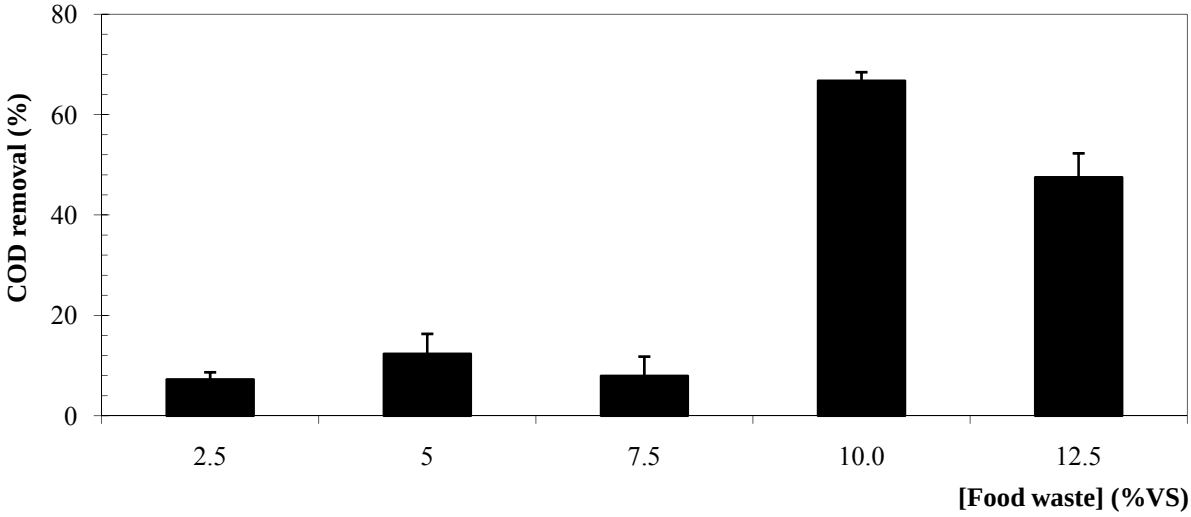


**Figure 4.3:** COD removal after 96 hr fermentation. Histograms represent mean values of duplicate measurements. Error bars represent one standard deviation.

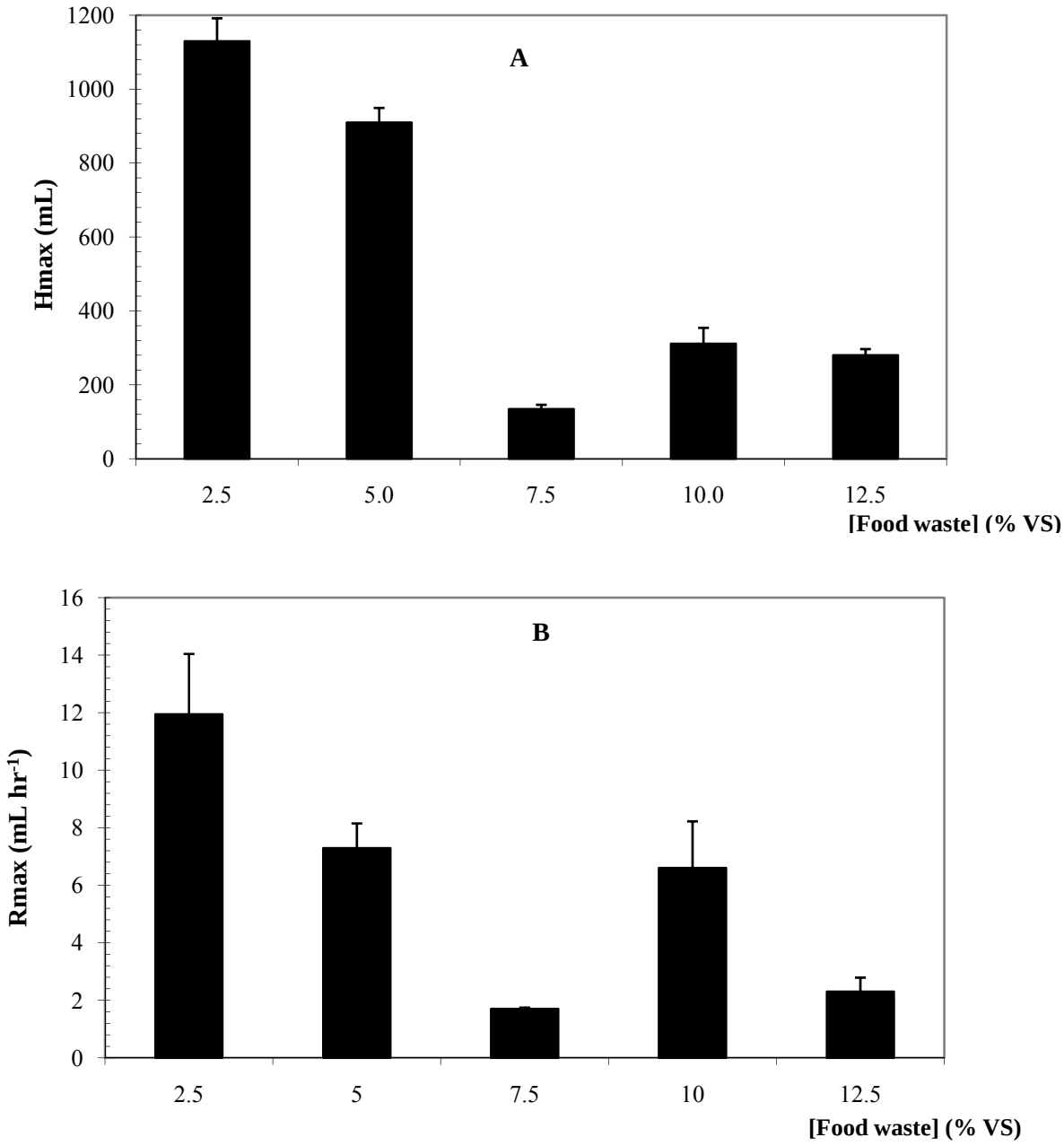


**Figure 4.4**: Cumulative hydrogen ($H_{max}$) (A) and maximum hydrogen production rate ($R_{max}$) (B). Histograms represent mean values of duplicate measurements. Error bars represent one standard deviation.

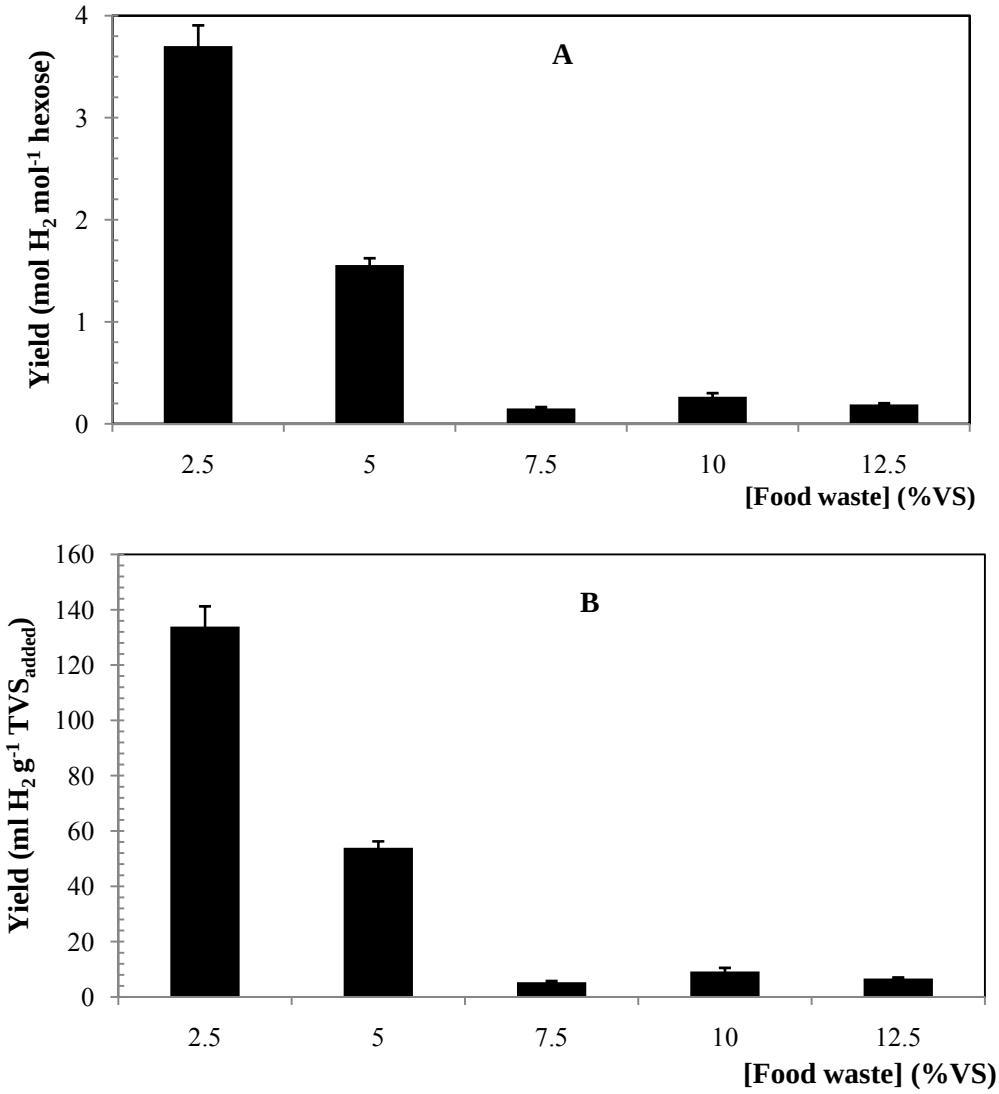


**Figure 4.5:** Hydrogen production yield based on mole of hexose (A), and gram of food waste addition (B). Histograms represent mean values of duplicate measurements. Error bars represent one standard deviation.

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**Table 4.1** Yield of biohydrogen production by pure culture from various substrates

| Inoculum | Substrate | Maximum hydrogen yield | Reference |
|---|---|---|---|
| *Clostridium butyricum* | sucrose | 2.8 mol$H_2$/ mol sucrose | Chen *et al*., 2005 |
| *Clostridium butyricum CGS5* | xylose | 0.68-0.73 mol$H_2$/ mol xylose | Lo *et al*., 2008 |
| *Clostridium butyricum CWBI1009* | glucose<br>strach | 1.7 mol$H_2$ /mol glucose<br>2.0 mol$H_2$/ mol hexose | Masset *et al*., 2010 |
| *Clostridium butyricum* | sugarcane juice | 2.0 mol$H_2$ /mol hexose | Pattra *et al*., 2010 |
| *Clostridium acetobutylicum ATCC824* | glucose | 1.8 mol$H_2$ /mol glucose | Oh *et al*., 2009 |
| *Clostridium butyricum TISTR1032* | food waste | 3.7 mol$H_2$ /mol hexose | This study |

**Table 4.2** Summarized yield and rate of biohydrogen production by *C. butyricum*

| % VS | Yield | | Rate | |
|---|---|---|---|---|
| | **mL$H_2$/gVS** | **mL$H_2$/gVS.L** | **mL$H_2$/gVS.hr** | **mL$H_2$/hr.L** |
| 2.5 | 133.896±7.34 | 267.792±14.67 | 1.395±0.08 | 23.886±4.20 |
| 5 | 53.919±2.33 | 107.839±4.65 | 0.562±0.02 | 14.578±1.72 |
| 7.5 | 5.31±0.45 | 10.622±0.89 | 0.055±0.005 | 3.408±0.08 |
| 10 | 9.228±1.27 | 18.435±2.53 | 0.096±0.01 | 13.203±3.24 |
| 12.5 | 6.642±0.39 | 13.283±0.77 | 0.069±0.004 | 4.605±0.97 |

### 4.2 Hydrogen fermentation by mixed culture in 0.5 L reactor

Fig. 4.6 showed that F/M ratio affects on hydrogen content and volume of hydrogen produced. Hydrogen evolution was occurred at first eight hours all cases. At this period the maximum hydrogen content about 68% was observed at F/M of 2 and 63 % at F/M of 1, while the little volume of hydrogen was observed in all cases. The maximum volume of hydrogen (530 mL) and hydrogen content (50%) were observed at F/M of 5 after 35 hrs fermentation. However, throughout the fermentation course, maximum cumulative hydrogen (1,306 mL) was present at F/M ratio of 5. Acetic and butyric acids were the main final products in liquid phase for all cases except at the F/M 10, in which ethanol was the main metabolite (Fig. 4.7).

Hydrogen was not observed under F/M ratio of 10. Two main metabolites produced during the batch processing of all cases indicated that this fermentative process was butyric acid fermentation type so that acetic and butyric acids were the main liquid products. However, the concentrated volatile acid produced during anaerobic fermentation process was one factor inhibited hydrogen formation from $NADH+H^+$ channel. The high accumulated undissociated acid above 19 mM was found to be the inhibition factor which was a threshold concentration for significant decreasing hydrogen yield and beginning solventogenesis (Ginkel and Logan. 2005). Therefore, the direction of fermentation process might depended on ecology circumstance that pH played a main role in affecting fermentation process especially in equilibrium state.

When the pH in the culture was low (less than 4.5), acidic terminal products accumulated in cells were affected the equilibrium state and toxic for bacterial cell, so the reaction would be switched to produce more reduced substance, such as ethanol and butanol, that easily reaches to equilibrium state and maintain the normal metabolism. Ethanol has been one of the more reduced substances produced in the solventogenesis reaction. It was produced in the unbalanced condition of protons, including the detoxicity condition in the bacterial cell. In the acidity condition, undissociated form can be infiltrated through the cells that in this time proton will be released inside the cells under the neutral condition (higher internal pH) resulting in toxicity condition. Therefore, solventogenesis will be generated instead of acidogenesis for maintaining the neutral condition of normal organism metabolism process.

As for the results, the pH declined to lower than 5 after 10 hr incubation for all cases and solventogenesis was apparently observed at F/M of 7 rather than that observed at the lower F/M ratio (1, 2 and 5) conditions. The highest value of $H_{max}$ was observed at F/M ratio of 5 about 1029.68 mL similar as the highest values of $R_{max}$ (59 mL/ hr) (Fig. 4.8 and Table 4.3). While the maximum biohydrogen molar yield was observed at F/M of 2 about 2.68 mol $H_2$ /mol hexose and 84 mL $H_2$ /g VS under the condition of initial pH 6, 37$^{o}$C in batch operation mode. However this yield can also be compared with the previous reports in the literature (Table 4.4). The results from this study indicated that the efficiency of degradation of food waste and biohydrogen production by mixed culture were feasible and practical for scaling up to improve and enhance the yield of hydrogen using in industrial scale in the future. Moreover, this research can use to integrate the waste management and alternative energy generation together in the same time.

### 4.3 Comparative performance of anaerobic sludge and *C. butyricum* in biohydrogen fermentation from food waste

Organic loading (i.e. food waste concentration) and types of inocula have strong effects on $H_2$ production. Combined microflora in the non-sterile food waste and the anaerobic sludge inoculum, in the anaerobic sludge reactor, appeared to facilitate hydrolysis of food waste resulting shorter lag time of $H_2$ production. Higher cell concentration of $H_2$ producing bacteria (*C. butyricum*) in the *C. butyricum* reactor led to higher capacity of hydrogen fermentation from food waste, regarding to the $H_2$ production yield, than the anaerobic sludge reactor, which contains only spore forming $H_2$-producing bacteria in the heat-shocked sludge inoculum. The increase of food waste concentration can cause overloading substrate, results high accumulation of VFAs and abrupt pH drop in the reactor content.

This phenomenon causes the inhibiting effect on the hydrogen producing bacteria in both reactors. However, previous studies revealed that metabolites of anaerobic fermentation such as VFAs could be used as an indicator to understand microbial activity of hydrogen producing bacteria in a reactor (Fan *et al*., 2006, Nathao *et al*., 2013). The main metabolites in the anaerobic sludge reactor were normal butyrate (a concentration ranging from 18.2-46.1 mM), acetate (a concentration ranging from 17.9-31.8 mM), and ethanol (a concentration ranging from 4.5-22.2 mM); however, propionate was

insignificant. Little fermentation end-product with main ethanol was detected in F/M of 10 (Fig. 4.7). On the other hand, acetate (a concentration ranging from 42.1-49.5 mM) and butyrate (a concentration ranging from 35.2-41.2 mM) were the main end-product in *C. butyricum* reactor with 2.5-5.0% food waste.

Propionate appeared to increase as the food waste concentrations were increased. The type of main fermentation end-products appeared to be consistently corresponding to the hydrogen production yield. Dominant end-products as acetate achieved higher hydrogen yield in the *C. butyricum* reactor than that in the anaerobic sludge reactor, which containing butyrate as the main end-products. The presence of propionate and ethanol involved the low hydrogen yield at high organic loading. Therefore, *C. butyricum* and the optimal organic loading of 2.5% VS food waste condition achieved the highest $H_2$ yield were chosen to scale up into 5 L semi-batch reactor for studying in more details involved in $H_2$ fermentation by using several techniques such as DGGE for monitoring microbial community in long term fermentation, Carbon mass balance for evaluating the direction of fermentation and MFA for analyzing the internal flux and optimizing some products resulted in $H_2$ fermentation pathway.

## 4.4 Conclusion

Food waste loading not only governed the metabolite accumulation in the fermentative process, but also directly affected the total concentration of undissociated acids, and the efficiency of biohydrogen production. The end products of fermentative processes were significant factors controlling the fermentative direction in biohydrogen production process.

This study successfully demonstrated the increased yield of hydrogen fermentation from the 2.5% food waste by *C. butyricum* TITSR1032 over the anaerobic sludge inocula by 59% in 0.5 L batch reactor. Better performance of $H_2$ fermentation by *C. butyricum* over the anaerobic sludge regarding the hydrogen yields and specific $H_2$ production rate for most food waste content. Acetate and butyrate are normal end-products for both inocula. Addition of a pure $H_2$-producing bacterium such as *Clostridium* spp into the $H_2$ fermentation process of non-sterile food waste could be a strategic technique to enhance the performance of the process.

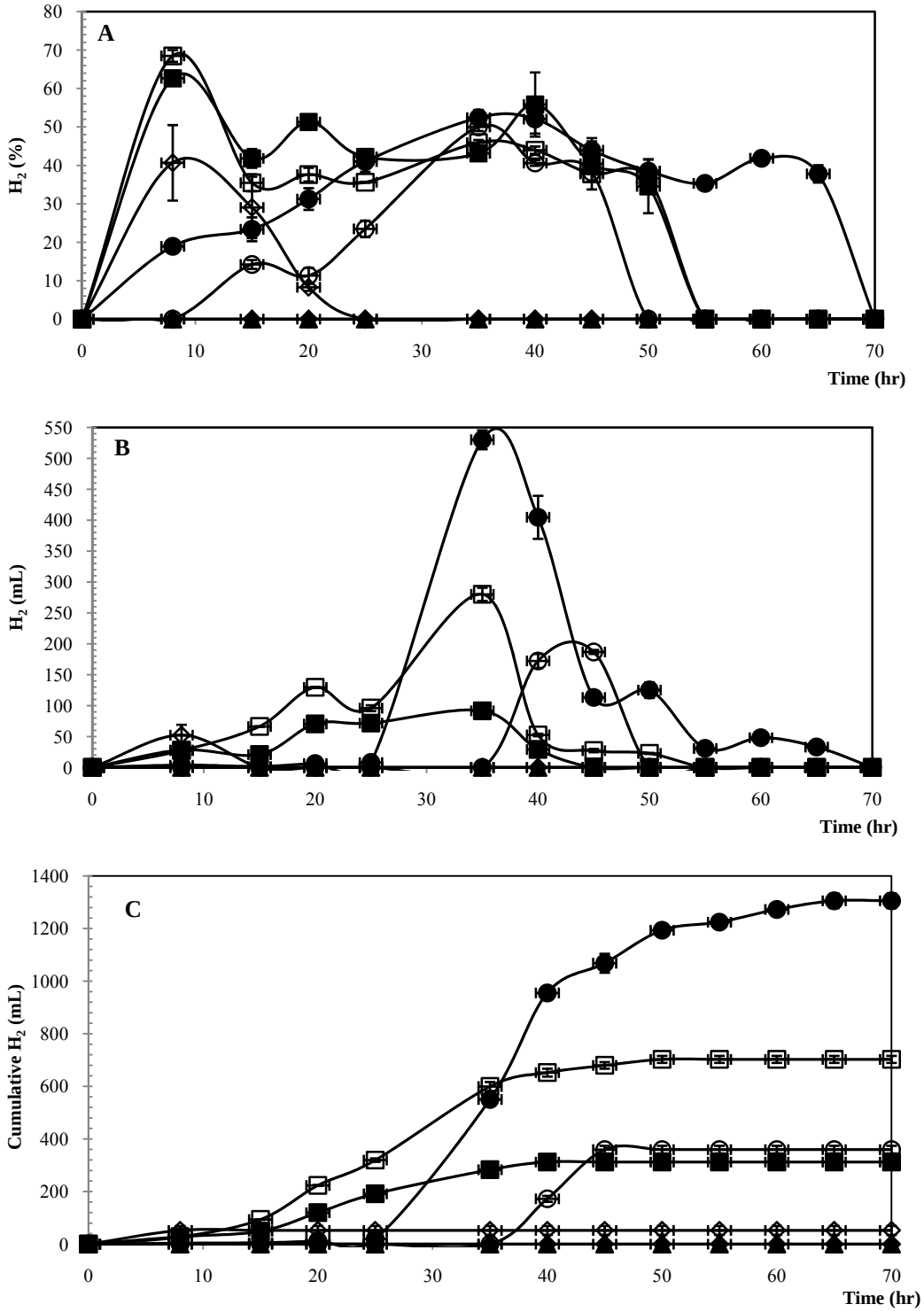


**Figure 4.6**: (A) Hydrogen content, (B) volume of hydrogen and (C) cumulative hydrogen produced by anaerobic sludge in various F/M ratio (1:■, 2:□, 5:●, 7: ○,10:◆, seed control:◇ and food control waste:▲). Each data point represents average value of duplicate experiments (n=2). Error bar represents standard deviation.

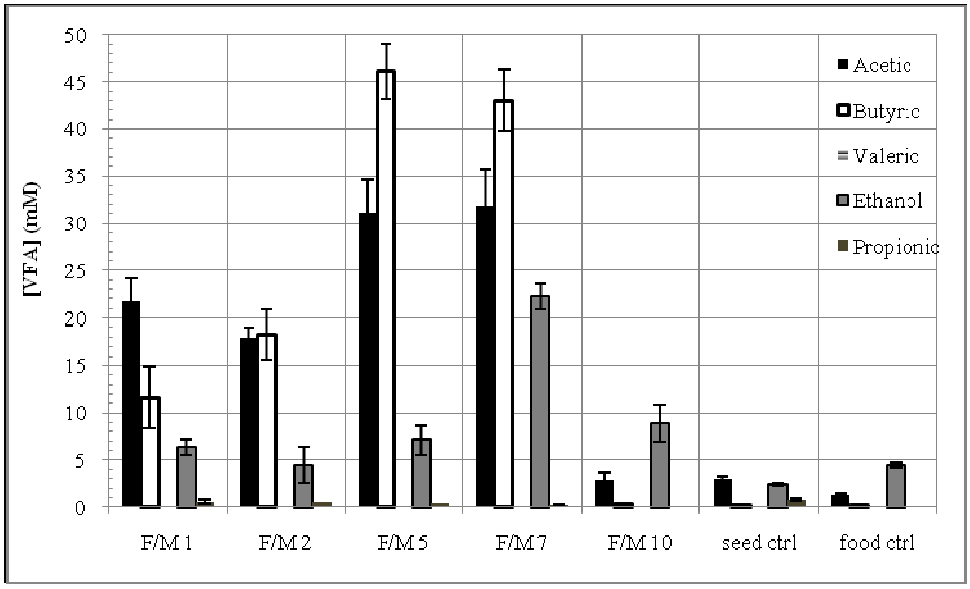


**Figure 4.7**:VFA concentration after 70 hr fermentation. Histogram bar represents represents average value of duplicate experiments (n=2). Error bar represents standard deviation.

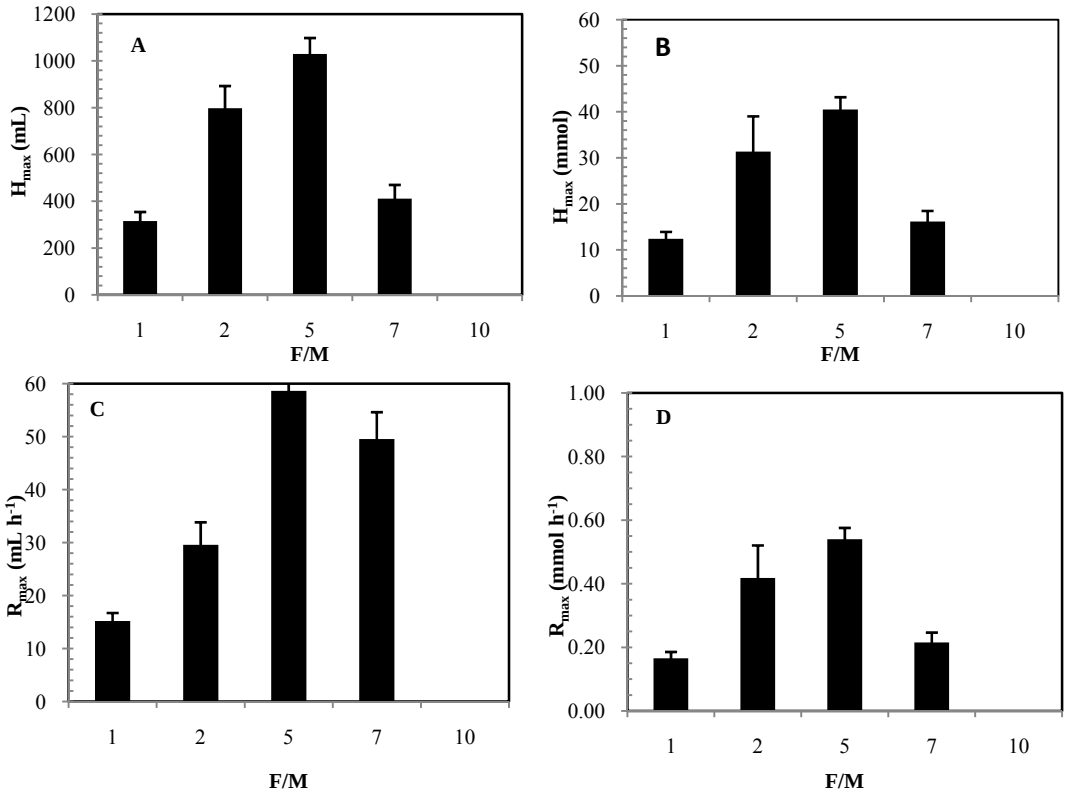


**Figure 4.8:** Maximum hydrogen cumulative value in (A) mL, (B) mmol; and maximum hydrogen production rate in unit mL/ hr (C), mmol/ hr (D). Histogram bar represents represents average value of duplicate experiments (n=2). Error bar represents standard deviation.

**Table 4.3** Yield of bio-hydrogen production by mixed cultures from various substrates.

| Substrate | Operation mode | Yield | | Reference |
|---|---|---|---|---|
| | | mol $H_2$ /mol substrate | mL $H_2$/ g VS | |
| glucose | batch | 0.9 | NA[a] | Logan and Ginkel. 2002 |
| sucrose | batch | 1.8 | NA[a] | |
| sucrose | batch | 3.7 | NA[a] | Wang *et al*., 2005 |
| Garbage slurry+paper | continuous | 2.4 | 46 | Ueno *et al*., 2007 |
| food waste | batch | 1.05 | NA[a] | Kim *et al*., 2004 |
| food waste | batch | 1.80 | 92 | Shin *et al*., 2004 |
| food waste | continuous | 1.22 | 81 | Kim and Shin. 2008 |
| food waste | batch | 2.68 | 84 | This study |

**[a] NA= not available.**

**Table 4.4** Summarized yield of biohydrogen production by mixed cultures

| F/M ratio | Yield | | | Rate | | |
|---|---|---|---|---|---|---|
| | **$mLH_2$/gVS** | **$mLH_2$/gVS.hr** | **$mLH_2$/gVS.L** | **$mLH_2$/hr.L** | **$mLH_2$/hr.CDW** | **$mLH_2$/hr.L.gCDW** |
| 1 | 66.457±8.04 | 0.886±0.11 | 132.915±16.08 | 30.39±3.03 | 3.2±0.32 | 6.40±0.64 |
| 2 | 83.993±20.49 | 1.120±0.27 | 167.986±40.97 | 59.13±8.49 | 6.22±0.89 | 12.45±1.79 |
| 5 | 43.355±2.88 | 0.058±0.01 | 86.710±5.75 | 117.29±15.18 | 12.35±1.60 | 24.69±3.20 |
| 7 | 12.365±1.76 | 0.165±0.02 | 24.73±3.52 | 95.10±13.01 | 10.43±3.17 | 20.86±6.34 |

# CHAPTER 5

# Investigation of Metabolic Profiles and Microbial Community on Biohydrogen Production by *Clostridium butyricum TISTR1032* from Food Waste

## 5.1 Hydrogen production and cumulative hydrogen production in 5 L semi-batch reactor

Hydrogen production and cumulative hydrogen production in both controlled and uncontrolled pH conditions were observed after 12 hr incubation (Fig. 5.1A) while the maximum $H_2$ volume of 5.1 and 3.9 L was obtained after 18 and 24 hr incubation under controlled and uncontrolled pH conditions, respectively. After the end of each feed, the volume of hydrogen was decreased until zero and the fresh waste was added to mark as new feed starter cycle. Hydrogen production in the second feed was immediately observed in both conditions. The results indicated that organisms in both conditions did not take the lag time for acclimation themselves. On the other hand, these organisms can continue fermentative process and produce some products such as organic acid, $H_2$ and $CO_2$. Stability of *C.butyricum* and the other organisms were monitored in every feeds to explain the performance of organisms in the reactor.

After the second feed, Hydrogen was not generated in the controlled pH condition while it was still observed in uncontrolled pH condition throughout the sixth feed. However, the volume of hydrogen slightly decreased after the second feed and then it was not observed after 180 hr incubation at the last feed (the sixth feed). The fermentation period in the controlled pH condition was shorter than that in the uncontrolled pH condition for all feeding cycles. The highest cumulative hydrogen productions in the first feed was observed about 7 and 11.5 L after 30 and 48 hr incubation in controlled and uncontrolled pH condition respectively while 2.2 and 3.9 L were observed in the second feed after 48 and 72 hr incubation (Fig. 5.1B and 5.2B).

Subsequently, the cumulative hydrogen production in the next feed under uncontrolled pH condition was also observed about 4.3, 4.3, 3 and 0.6 L after 100, 126, 156 and 180 hr incubation respectively (Table 4.1). Stability based on the fermentation periods and the cumulative $H_2$ after the first feed was observed about 24.8±3.0 hr and 3.9±0.6 mL under uncontrolled pH condition. While the fermentation period in the first

and second feed of controlled pH condition was shorter than that of uncontrolled pH condition. Results indicated that under optimal pH condition (pH 6) of *C. butyricum* was suitable for growth condition while other groups of bacteria involved anaerobic digestion may be suppressed the normal activity, especially, methanogen groups. No $CH_4$ was detected throughout the fermentative process.

Generally, organisms related with anaerobic fermentation process consist of hydrolytic bacteria, fermentative bacteria, acetogenic bacteria, syntrophic bacteria, metanogenic bacteria, sulfate reducing bacteria (SRB) and nitrate reducing bacteria (NRB). However, firstly, hydrolytic groups also degrade complex substrates or polymeric substrates to simple forms and then fermentative groups can utilize these substrates and convert to various products depended on some controlled conditions and types of active organisms groups in that time. Under the controlled pH condition, performance of the activity of organisms in the reactor was significantly different from that under the uncontrolled pH condition. Although pH 6 was the optimal condition for growth condition of *C.butyricum* and it was in the range of suitable condition for hydrogen production (5.5-6.5) (Fang and Liu. 2002).

According to the preliminary experiment from the previous study, under sterile food waste conditions, the results showed that hydrogen production from waste substrate by only the pure strain, *C. buturicum*, was less than that in non-sterile food waste conditions that allowed the indigenous organisms from food waste to work together with *C. butyricum*. Reaction direction under the controlled pH condition proceeded to enhance the number of cells rather than produce hydrogen molecule. Chen *et al* (2005) reported that the operation with pH 6 and 6.5 in biohydrogen production by *C. butyricum* CGS5 obtained higher cell growth rate and cell yield but it resulted in lower hydrogen production and hydrogen yield compared with the operation with pH 5.5.

This condition promoted the rapid conversion of the carbon source into cell biomass instead of formation of hydrogen molecule. Moreover, the competition of other organism groups to *C.butyricum* had to be determined by considering some metabolite profiles together with microbial community to check the feasibility of the involved hydrogen consuming direction and inhibition pathway which respected to the stability of hydrogen production in long term operation comparing between two different pH

conditions. Hydrogen production obtained from the first feed and the second feed in the controlled pH condition was lower about 1.6 - 1.7 times than that in the uncontrolled pH condition.

Better performance of hydrogen production from the non-sterile food waste by *C. butyricum* from the previous study in 0.5 L batch reactor suggested that addition of pure strain of Clostridia into reactor during hydrogen fermentation resulted in the increase of the $H_2$ producer can enhance hydrogen concentration and yield. However, after scaling up to 5L semi- batch reactors and using *C. butyricum* as started seed inocula, the number of *C. butyrium* after discharging was an important factor to maintain continuously of $H_2$ fermentative reaction in both different pH condition reactors. The period and the ratio of circulation for new substrate and cultured broth must be kept at the proper length of time and the sufficient quantities to dilute the acidic condition and to maintain the availability of organisms in the reactor. However, they have to be carefully examined.

**Table 5.1** Fermentative periods and cumulative hydrogen production under uncontrolled pH condition

| Feeding cycle | fermentative period (hr) | cumulative $H_2$ production (L) |
|---|---|---|
| 1 | 48 | 11.5 |
| 2 | 24 | 3.9 |
| 3 | 30 | 4.3 |
| 4 | 24 | 4.3 |
| 5 | 22 | 3 |
| 6 | 24 | 0.6 |

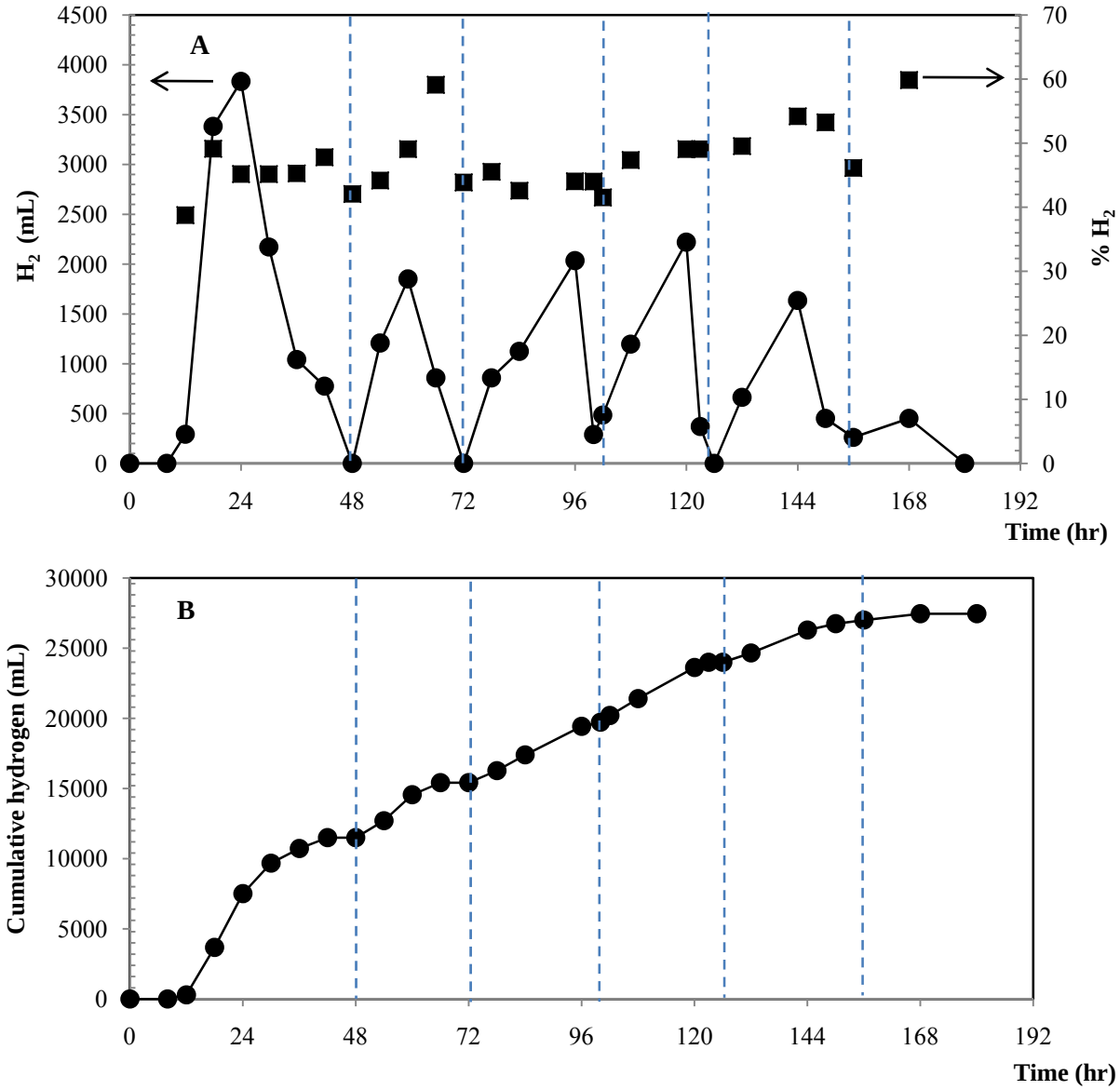


**Figure 5.1:** (A) Hydrogen production and (B) cumulative hydrogen by *C.butyricum* under uncontrolled pH condition.

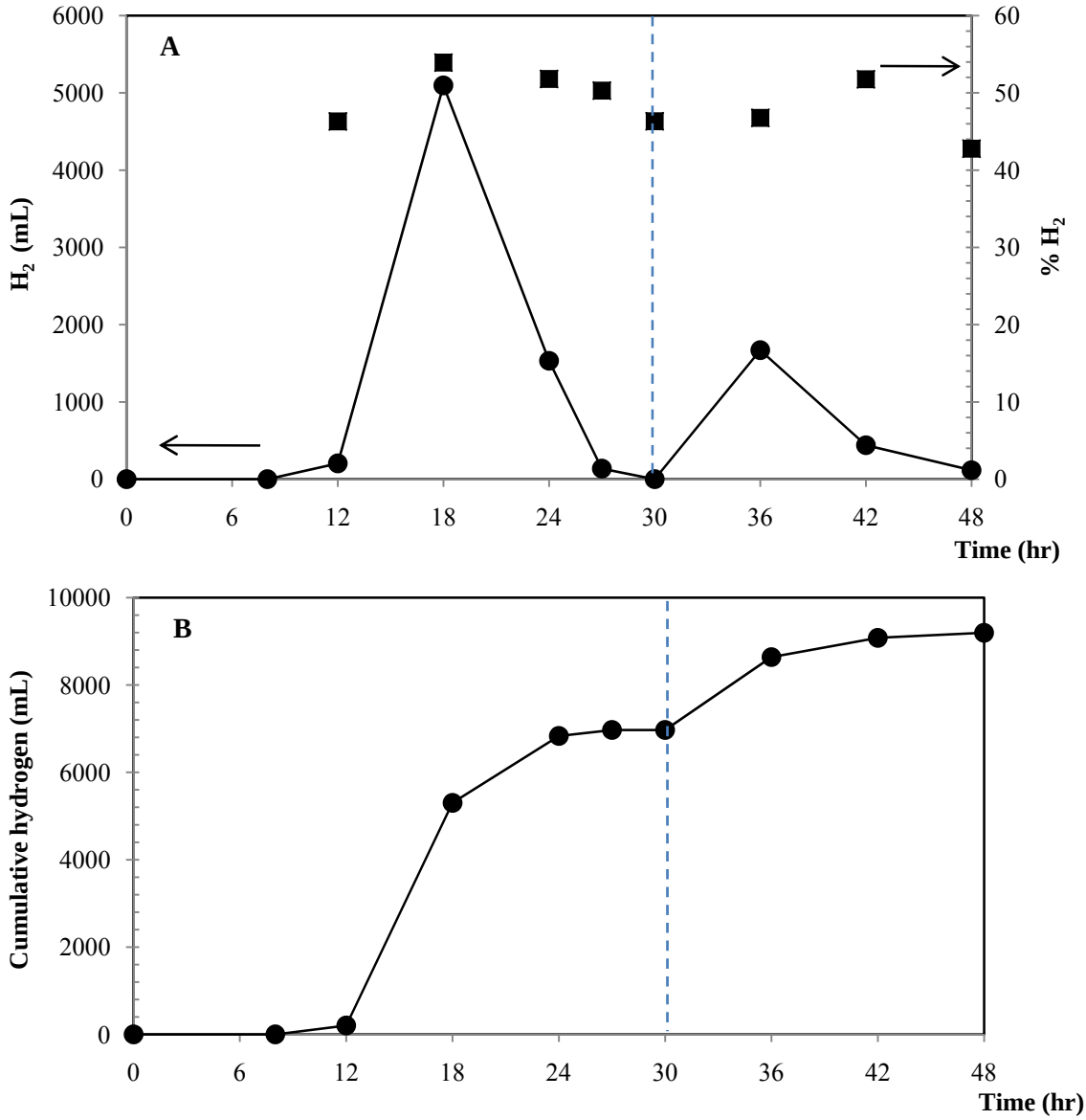


**Figure 5.2:** (A) Hydrogen production and (B) cumulative hydrogen by *C.butyricum* under controlled pH condition.

## 5.2 Metabolites accumulated during fermentative process

Fig. 5.3A showed that the initial pH 6 under uncontrolled pH condition quickly decreased to lower than 4.5 at 24 hr incubation. Subsequently, it deviated in the narrow pH range of 4.1-4.3 until the end of first feed. After starting the next feed cycle, pH was still deviated in narrow pH range of 3.9-4.3 throughout the fermentation process. The results showed that this condition was not optimal pH for growth condition of *Clostridium spp* and other organisms in the reactor. However, many types of hydrolytic bacteria along with fermentative bacteria can survive in acidic condition although the growth rate may be low compared to the neutral condition (O'Sullivan *et al*., 2008).

Increase of pH resulted from dilution of new substrate to the rector can reduce total undissociated acids accumulated in the broth. Hydrogen production was continuously obtained after feeding new substrate. The volume of hydrogen production was the same trend in the second feed until the fifth feed. The metabolites illustrated that the pattern of fermentative process was butyric acid fermentation type (i.e., butyric acid and acetic acid was the dominant metabolites) throughout fermentation process.

Drop of pH during fermentation causes an unbalance of $NADH_2/NAD^+$ ratio and lower hydrogen production yield (Li *et al*., 2007). At low pH, $NADH_2$ can be regenerated through $NAD^+$ and allowed proton as electron acceptor to form hydrogen molecule to reach equilibrium state. Because high concentration of protons around cell were increased as high accumulation of total undissociated acids in the broth that it was observed more than 50 mM at first 24 hr and still remained at high concentration between 49-72 mM throughout the end of experiment (Fig. 5.3B).

Hydrogen can be not released throughout bacterial cell in time. Hydrogen production would be stopped and produced more reduced substances. The high accumulated undissociated acid above 19 mM was found to be the inhibition factor which was a threshold concentration for significant decreasing hydrogen yield and beginning solventogenesis (Ginkel and Logan. 2005). Results showed that total concentration of undissociated acid had been higher than 19 mM after first 18 hr incubation until the end of experiment. Not only butyric acid and acetic acid were accumulated in same pattern and high concentration, ethanol also accumulated in the high level compared with the other metabolites (propionic acid and valeric acid).

Ethanol is one of more reduced substance produced in the solventogenesis reaction. It is produced in the unbalance condition of proton including the detoxicity condition in the bacterial cell. In the acidity condition, undissociated form can be infiltrated through the cells that proton will be released inside the cells under the neutral condition (higher internal pH) resulting in toxicity condition. Therefore, solventogenesis will be generated instead of acidogenesis for maintaining the neutral condition for the normal metabolism process. Under this condition, type of metabolites and total concentrations of undissociated acid were the controlled factors of the fermentative direction.

Under controlled pH condition, the pattern of metabolites was obviously different from that under uncontrolled pH condition. Butyric acid and acetic acid were still the main metabolites whereas ethanol was hardly different from the initial period until the end of experiment (Fig. 5.4A). The results indicated that solventogenesis had not been induced. Neutral condition was suitable for growth condition of most organisms to maintain their normal metabolisms. In this study, pH 6 was maintained throughout the fermentative process that organisms can be increasingly proliferated. To generate new cells, more energy in form of ATP was greatly required in order to assure normal anabolism process. Butyric acid and acetic acid pathways were preferable for obtaining higher ATP yield rather than the others organic acid or alcohol pathways.

An intermediate, $NADH+H^+$, can be throughly utilized by regenerated organisms and then released in the form of $NAD^+$ returned to drive glycolysis process. Under this condition, total concentration of undissociated acids was not an inhibition factor in fermentative process (Fig. 5.4B). However, a considerable issue observed under this condition was the pattern and quantity of butyric and acetic acid production. Although total undissociated acids did not influence to overall $H_2$ production process, an enormous of butyric plus acetic acid was not significantly different from those observed under uncontrolled pH condition. While acetic acid trended to increase before the end of experiment, $H_2$ production was reversed.

The results implied that this acid formation might be constructed from other processes, such as acetogenesis or butyric acid oxidation (Eq. 5.1 and 5.2). $H_2$ was consumed as electron donor to produce acetic acid under acetogenesis process. It resulted in the net value of $H_2$ production. Therefore, acetic acid production was not only considered for supporting $H_2$ production but it also corresponded with $H_2$ consuming pathway via acetogenesis. However, the activity of organisms in the reactor had to be more

understand not only in metabolite profiles. Metabolic flux analysis can be used as a powerful tool for evaluating the intracellular flux and extracellular flux involved with overall $H_2$ fermentation process from non sterile food waste.

$4H_2+2CO_2$ ----> $CH_3COOH+2H_2O$ (5.1)

$CH_3CH_2CH_2COOH$ ----> 2 $CH_3COOH+2\ H_2$ (5.2)

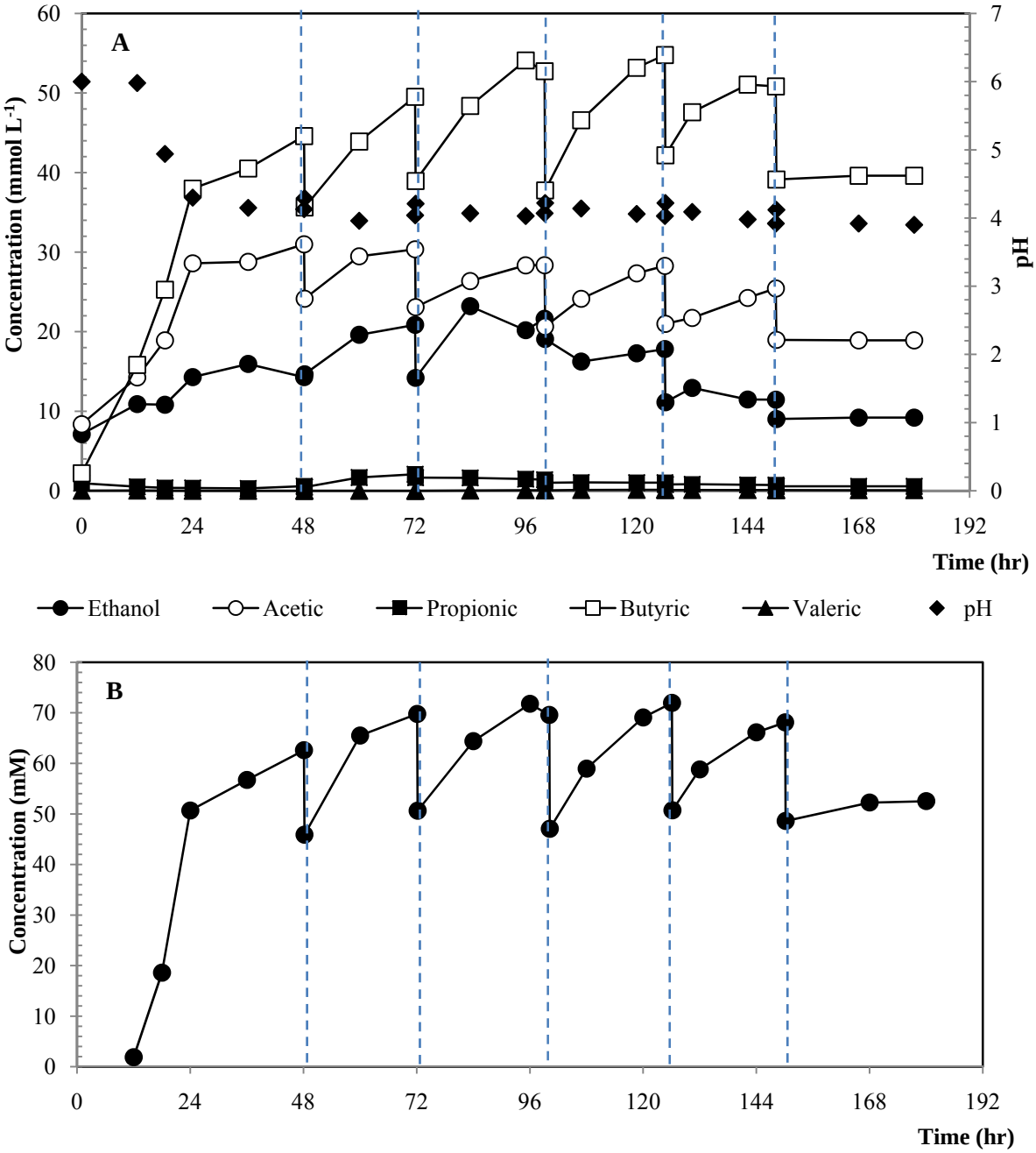


**Figure 5.3:** (A) Variation of pH (◆), metabolites (●: ethanol, ○: acetic, ■: propionic, □: butyric, ▲: valeric acids) and (B) total concentration of undissociated acid during the hydrogen fermentation under uncontrolled pH condition.

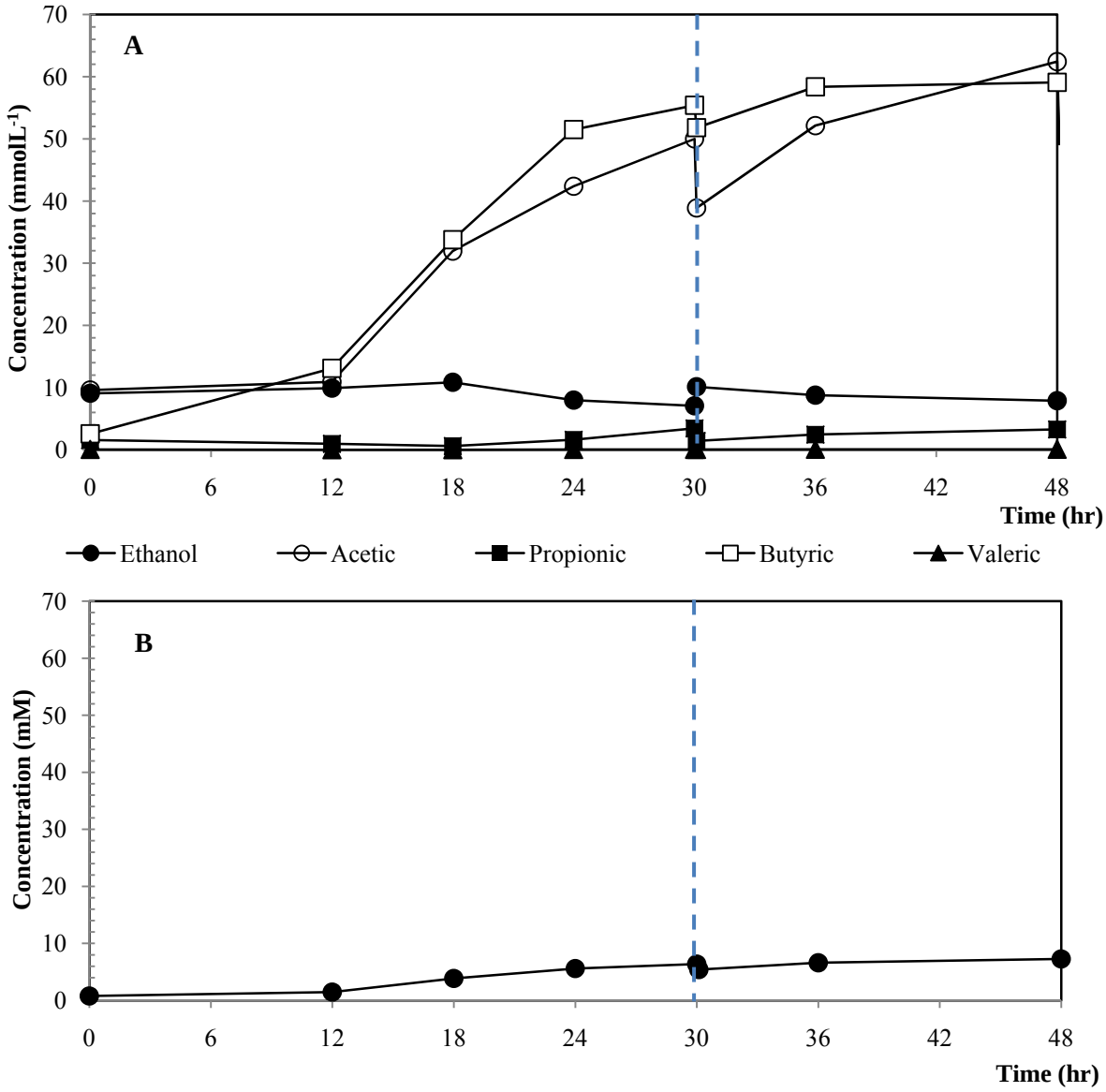


**Figure 5.4:** (A) Variation of metabolites (●: ethanol, ○: acetic, ■: propionic, □: butyric, ▲: valeric acids) and (B) total concentration of undissociated acid during the hydrogen fermentation under controlled pH condition.

### 5.3 Substrate degradation and utilization during fermentative process.

TS, VS and soluble carbohydrate showed a declining pattern after 18 hr incubation under the uncontrolled pH condition while in the controlled pH condition, these processes occurred within 12 hr after incubation (Fig. 5.5 and 5.6). The total solid substrate was degraded through soluble substances in the liquid phase, and then were easily used and uptaken by bacterial cells to produce more products under different conditions of fermentation process. The results indicated that under growth condition (controlled pH), degradation of solid substrate was rapid in the first period (18 hr) similar as utilization of soluble carbohydrate since in this time, it was in the growth period which need high energy (ATP) from carbon source in catabolism process to regenerate new cells in anabolism process. While under the uncontrolled pH condition, organisms took more times to degrade and uptake carbon source into their cells for normal metabolism that under this condition, the growth rate of organisms was lower than that in controlled pH condition. However, more than 50% of solid substrates were not hydrolyzed for both cases. It implied that the hydrolysis step may have caused an inhibition factor in the overall fermentation. However, carbon mass balance and carbon distribution would be used to confirm this assumption.

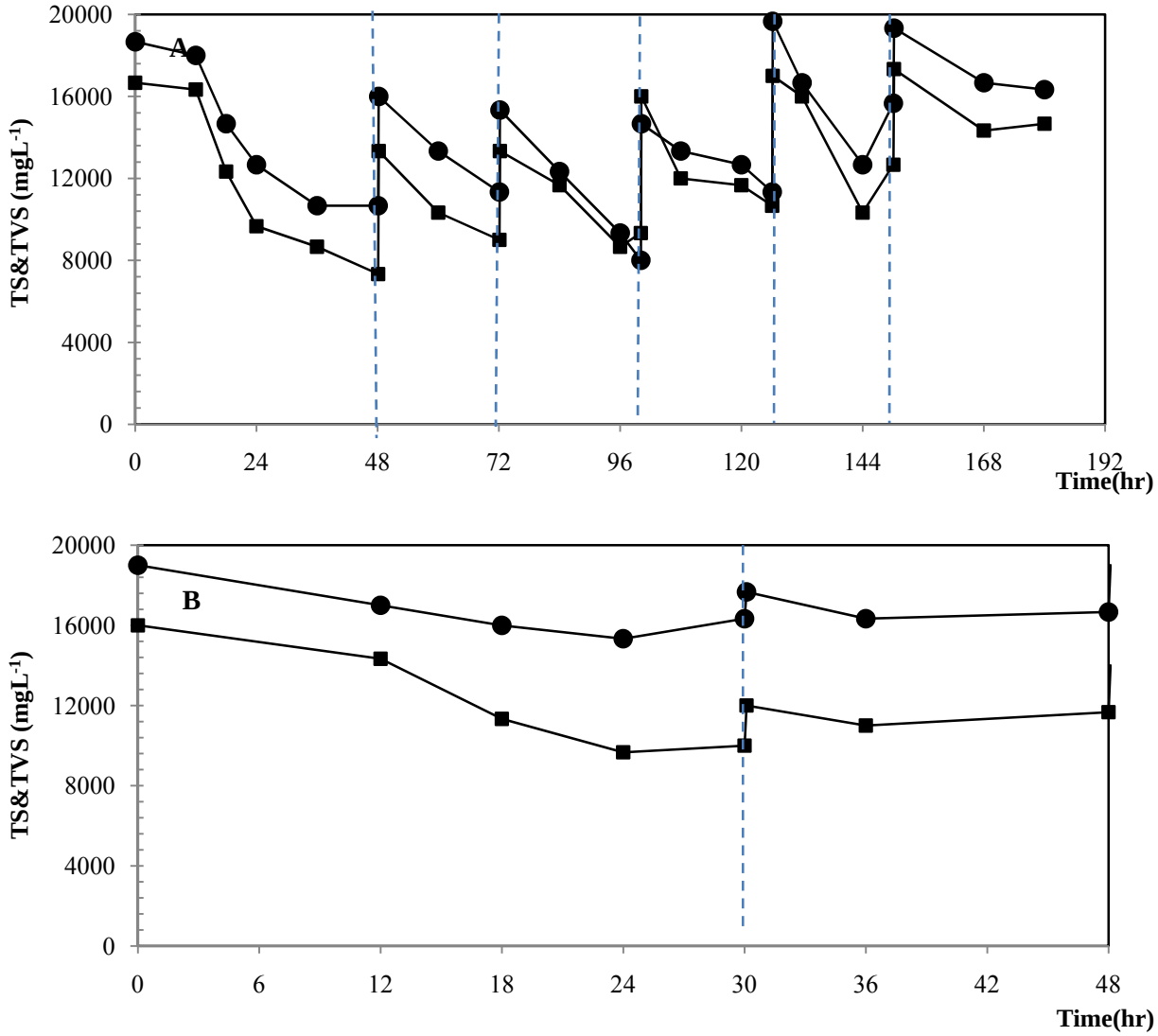


**Figure 5.5:** Variation of TS (●) and VS (■) during the hydrogen fermentation under (A) uncontrolled pH condition and (B) controlled pH condition.

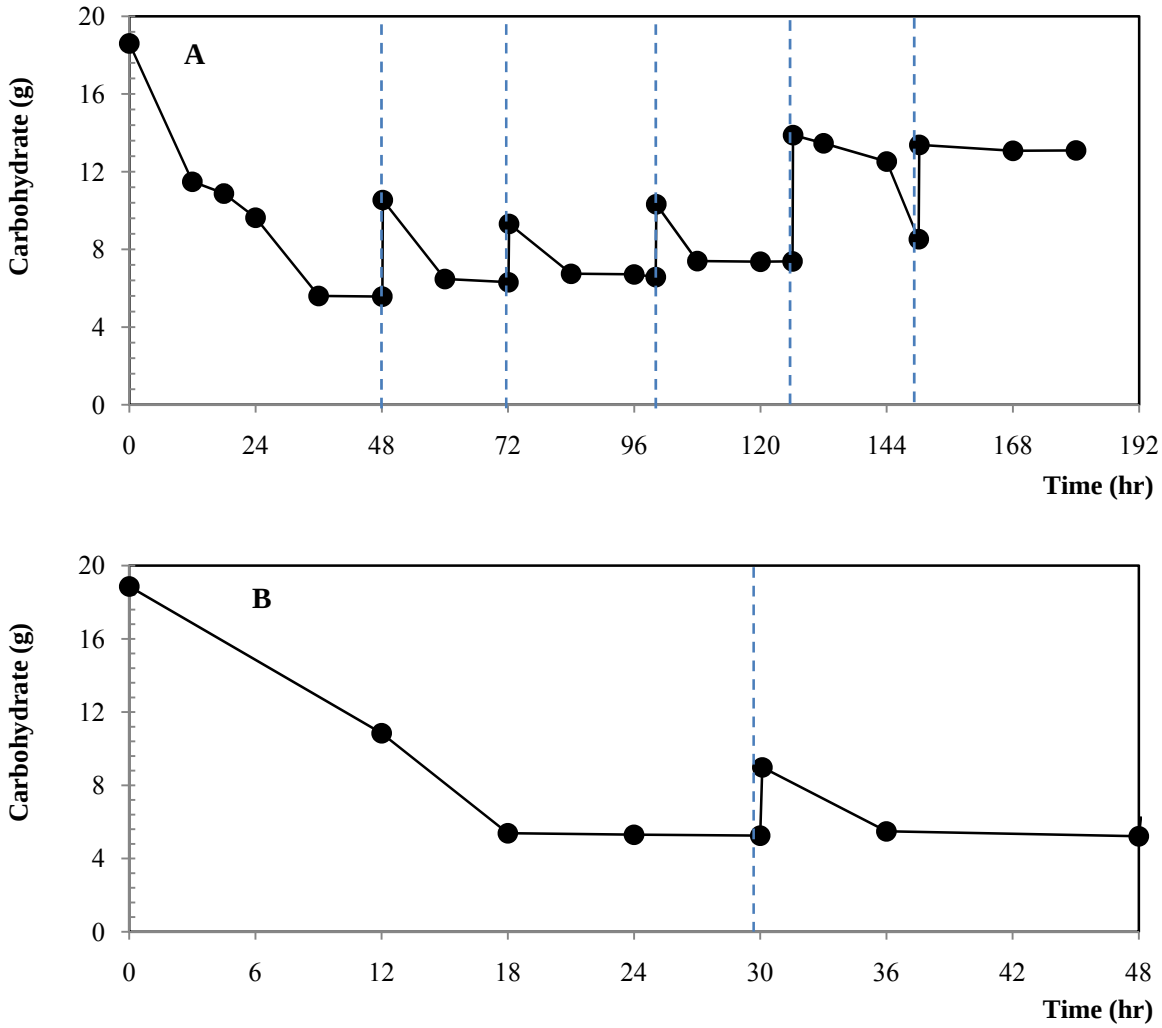


**Figure 5.6:** Variation of soluble carbohydrate during the hydrogen fermentation under (A) uncontrolled pH condition and (B) controlled pH condition.

## 5.4 Carbon distribution under uncontrolled pH condition

In this study, carbon distribution was examined under uncontrolled pH condition at the initial time and the peak time of hydrogen production observed from the volume of hydrogen of each feed. Carbon distributions were quite same pattern and the percentage of total carbon distributions were more than 90% in every time. In this case, carbon fraction in solid phase was degraded and converted in the form of butyric acid and acetic acid which directly related with hydrogen production pathway. The results could be elucidated by the carbon distribution analysis to determine the direction of fermentation process.

Carbon distributions can be separated into three major phases (solid, liquid and gas) in order to identify the metabolic pathway that resulted in fermentative type and metabolite products (Figs. 5.7 and 5.8). In the first feed, most carbon fractions of the initial cultivation period were in the solid phase in the form of insoluble substrate and inocula (63.4%) and the remained carbon fraction was observed in the liquid phase (36.6 %) in the form of soluble carbohydrate (91%). After 24 hr incubation, insoluble substrate was degraded by microorganism about 14.2% and eventually converted and accumulated into liquid phase in the form of butyric acid (35.67%) and still remained in soluble carbohydrate (47.3%) as the main final liquid products. In the second feed, only 4.37% of solid substrate was degraded and accumulated in liquid phase in the form of butyric (44%) while utilization of soluble substrate was observed 17.38 % which decreased from the first feed (43.7%).

The results revealed that carbon utilization in soluble substrates was maiximized in the first feed and then declined in the next feeding cycle. The results also indicated a similar pattern of solid substrate degradation. Both processes were slightly decreased from the first feeding cycle and hardly changed in the last feed. Efficiency of hydrolysis process under acidic condition was a problem under this condition since the remained solid substrates were still the main carbon proportion of total carbon distribution in every collection time. Especially, after the second feed, carbon distribution in solid fraction was about 50% and it was hardly changed in the next feed (the $4^{th}$ feed to the $6^{th}$ feed). Therefore not only acidic condition was the controlled factor for $H_2$ fermentative process, but hydrolysis was a limitation step which affected the stability of $H_2$ production in long term fermentation.

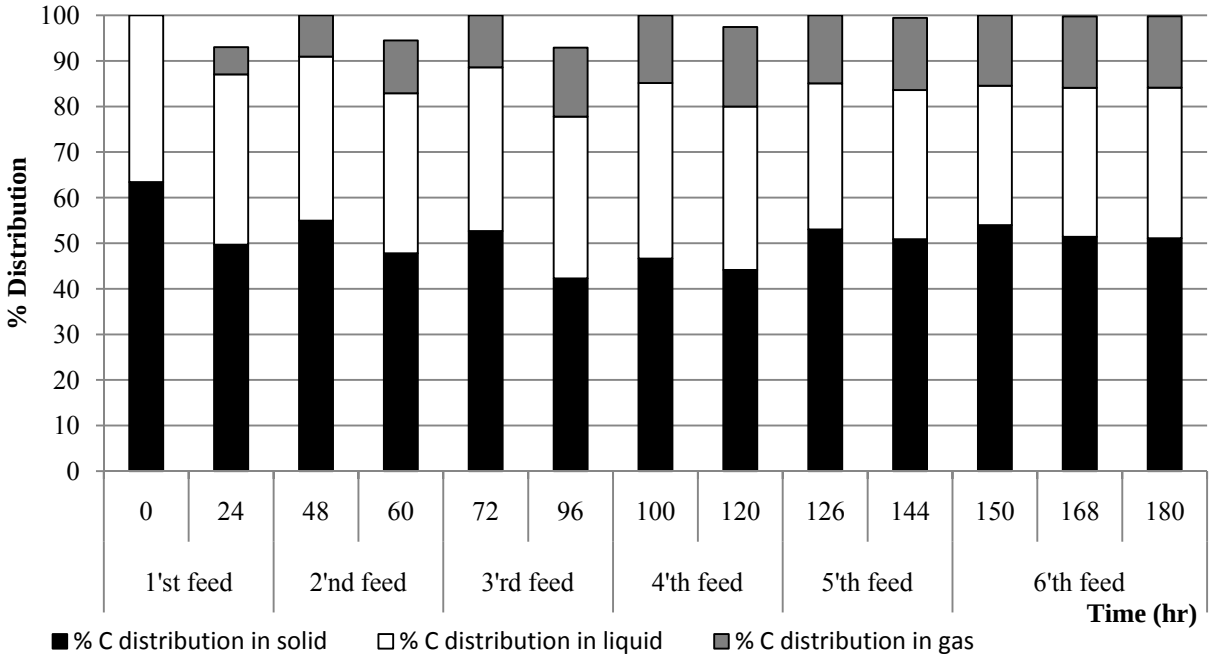


**Figure 5.7:** Carbon distribution during the hydrogen fermentation under uncontrolled pH condition.

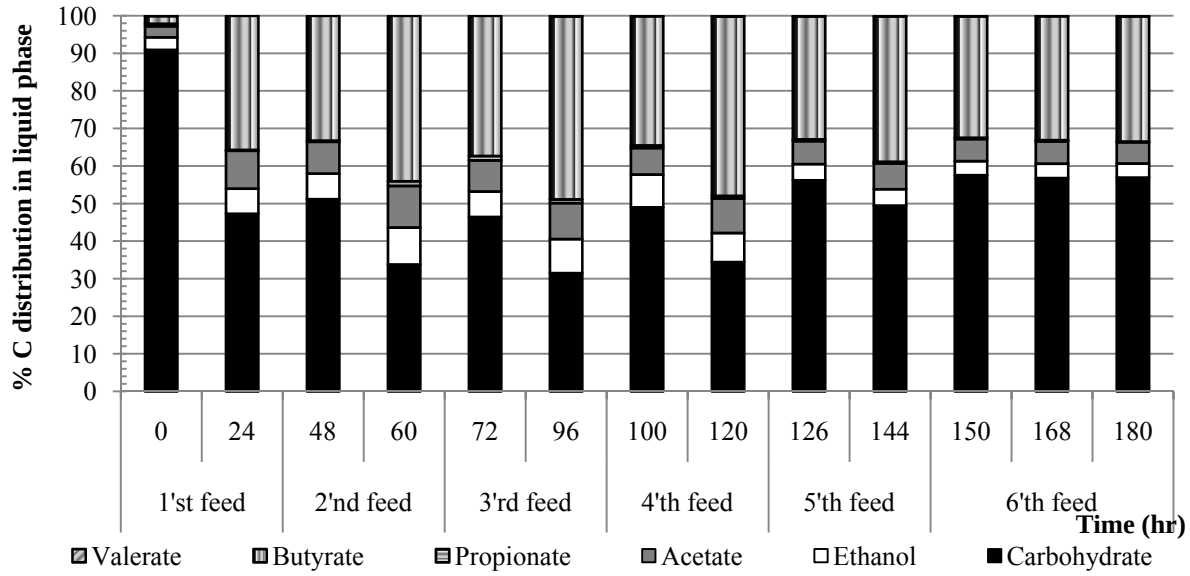


**Figure 5.8:** Carbon distribution in liquid phase during the hydrogen fermentation under uncontrolled pH condition.

## 5.5 Microbial dynamics analysis

DGGE profiles generated from the universal bacteria primer (338F and 518R) illustrated the structural pattern of the bacterial community in the long term fermentation under uncontrolled pH condition using *C. butyricum* on hydrogen fermentation from non sterile food waste. PCR-DGGE products were checked by 1.5% agrarose gel to confirm the quality of amplified PCR products and the results were shown as Fig. 5.9. However, in this study, the comparison of microbial community in the first feed between the controlled pH reactor and uncontrolled pH reactor was evaluated. Results showed that *C. butyricum, Klebsiella oxytoca, Straphylococcus spp, Enterobacter spp, Lactococcus spp* and *Acinetobacter spp.* were dominant species under uncontrolled pH condition (Fig. 5.10) throughout the experimental period. The presence of DGGE band responsible for *C. butyricum*, started seed and confirmed its stability in the non-sterile culture condition.

The Clostridia species has been reported in the previous literatures to be responsible for hydrogen production via butyric acid-type fermentation (Fang *et al*., 2002a; Oh *et al*., 2009). Hydrogen fermentation by Clostridia species was accompanied and corresponded with VFAs and/or solvent production under different culture conditions (Kramer and Bagley. 2006). Various non- Clostridia species were not $H_2$ producer, but they can contribute to break down complex organic substrates maintain a strict anaerobic environment in hydrogen fermentation (Table 5.2). *Klebsiella spp*. and *Enterobacter spp*, the gram negative facultative bacteria, could promote biohydrogen production by maintaining a strictly anaerobic condition and break down of complex substrates as reported previously (Hung *et al*., 2011; Lee *et al*., 2011). *Klebsiella spp*. was also known as the solvent production strains which can utilize various kind of substrates and produce alcohol such as 2,3 butanediol, isopropanol and ethanol along with hydrogen and carbon dioxide as soluble and gases metabolites (Wu *et al*., 2008; Minnan *et al*., 2008).

According to previous studies, some species of *Acinetobacter spp*. were also involved in biohydrogen production, such as *Acinetobacter baumannii*, the predominant species responsible for biohydrogen production from press mud (Saravanane and Radjaram. 2011). Lo *et al*., (2010) reported that *Acinetobacter junii* F6-02 was used as hydrolytic organism to produce cellulolytic enzymes to hydrolyze and pretreat rice straw before entering hydrogen production. *Lactococcus spp*., with small amount presented in this group, was believed to have an inhibiting effect on hydrogen production by ethanol

and butyric type fermentation (Ren *et al*., 2007a). Fig. 5.11 showed the microbial community in the first feed under both conditions.

The results showed that *Clostridium butyricum, Klebsiella oxytoca, Straphylococcus spp, Enterobacter spp, Lactococcus spp* and *Acinetobacter spp.* were still the dominant species in both conditions. Stability of *C. butyricum* in long term fermentation was consistency based on the intensity of DGGE bands for both cases. It indicated that the efficiency of hydrogen fermentation depend upon the performance of other co-organisms in the reactor resulting in the pattern of metabolites during the fermentative process. The relative DGGE bands of *Lactococcus spp.* observed under controlled pH condition were stronger intensity than that under uncontrolled pH condition while the stronger intensity of the relative DGGE bands for *Klebsiella oxytoca* were observed under uncontrolled pH condition.

The results supported hydrogen production and the direction of fermentative process that under controlled pH condition, total cumulative hydrogen production in the first feed was lower than that under uncontrolled pH condition due to the existence of *Lactococcus spp*., while solventogenesis pathway was obviously observed under uncontrolled pH condition. Therefore, the performance of dominant organisms in the reactor was an important factor controlled the direction of fermentative pathway, yield of hydrogen production and long term fermentation process.

**Table 5.2** Function of co-existing organisms in fermentative reactors involved with hydrogen production

| DGGE band | Organisms | Influence on $H_2$ fermentation | Reference |
|---|---|---|---|
| 1-4, 20 | *C. butyricum* | $H_2$ producer | Pattra *et al*., 2008 |
| 5-8 | *K. oxytoca* | Maintain an anaerobic environment and breakdown of complex organic substrates | Hung *et al*., 2011; Lo *et al*., 2008 |
| 9,15 | *Acinetobacter spp.* | Hydrolytic organisms | Saravanane and Radjaram. 2011; Lo *et al*., 2010 |
| 10 | *Straphylococcus spp* | - | - |
| 11-13 | *Lactococcus spp* | $H_2$ consumer | Ren *et al*., 2007 |
| 14 | *Enterobacter spp* | Maintain an anaerobic environment | Yokoi *et al*., 1998 |
| 16-19 | *Clostridium spp* | -$H_2$ producer | Chin *et al*., 2003 Zhang *et al*., 2006 |
| | | - Acetogenic bacteria, such as *C. aceticum*, *C. thermoautotrophicum* | Oh *et al*., 2003 |

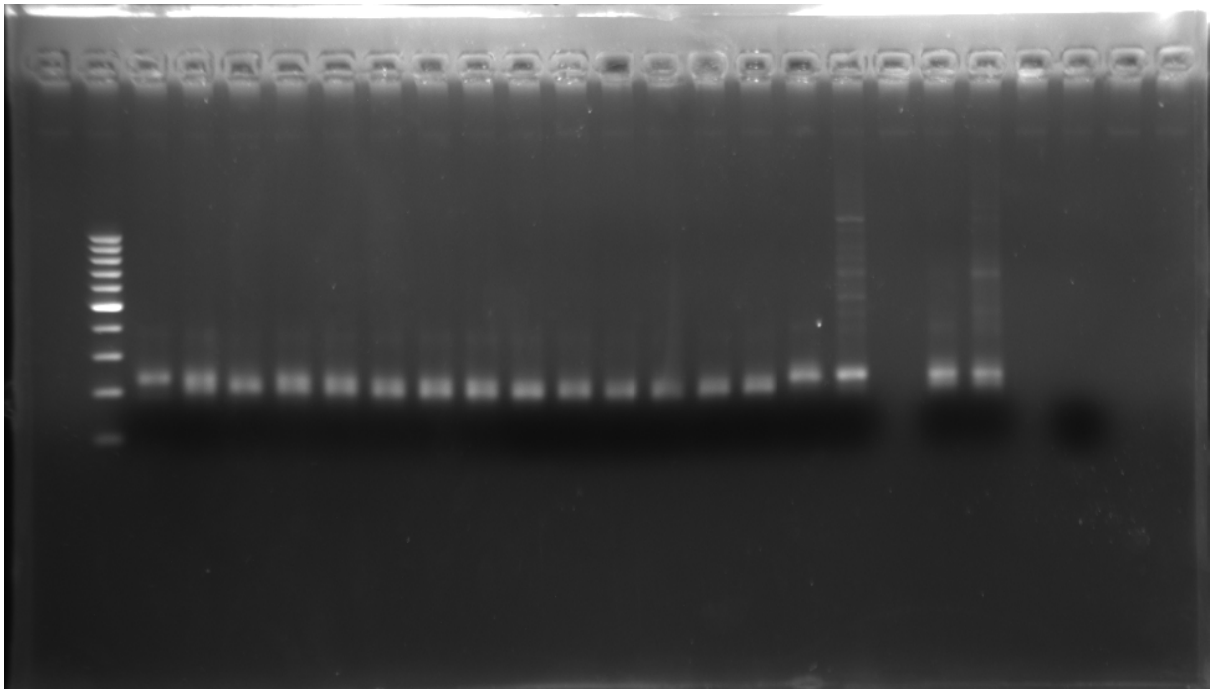

**Figure 5.9:** Amplification of partial 16S rRNA genes sequence with GC-clamp for DGGE analysis.

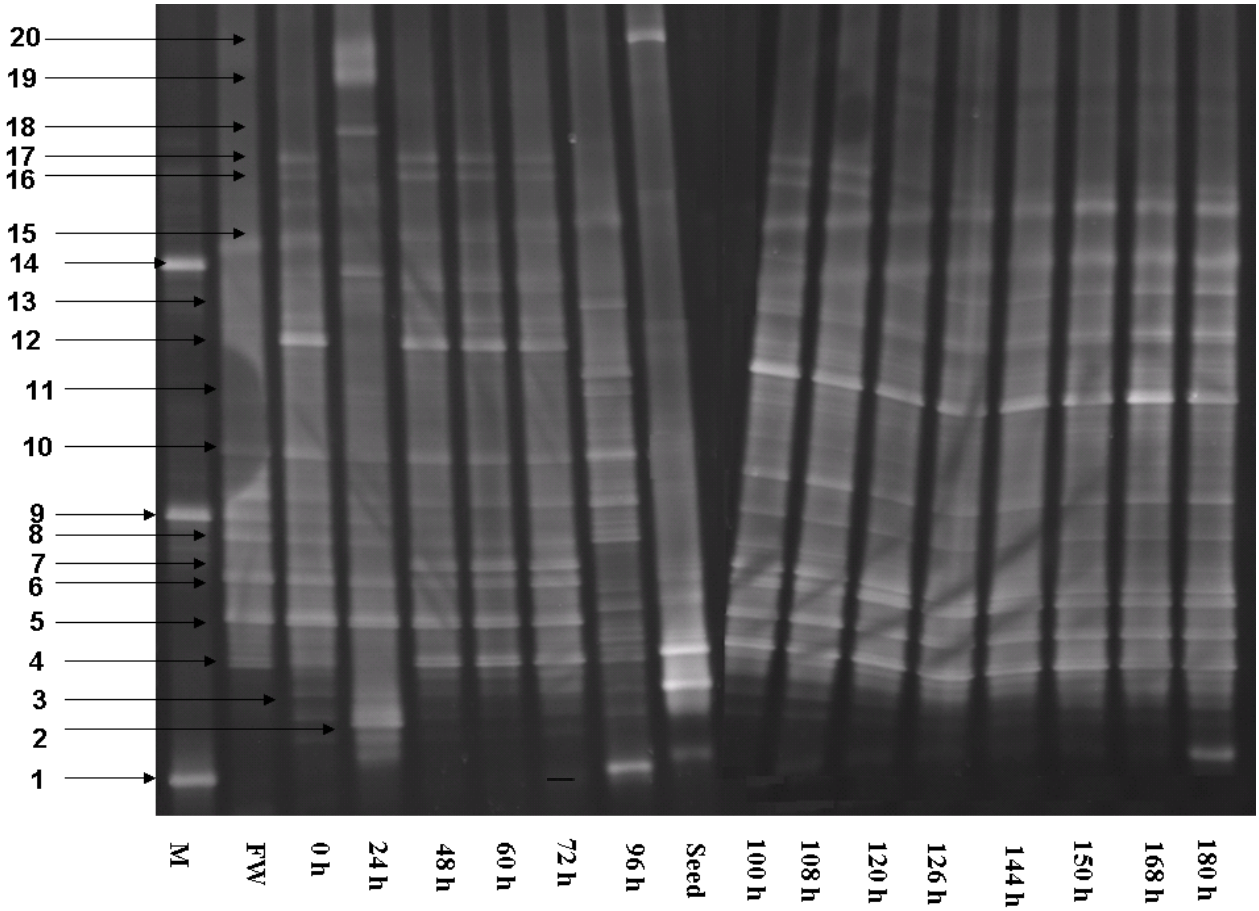


**Figure 5.10**: Dynamics of microbial community structure in long-term uncontrolled pH condition. (1-4, 20: *Clostridium butyricum*, 5-8: *Klebsiella oxytoca*, 9,15: *Acinetobacter spp,* 10: *Straphylococcus spp*, 11-13: *Lactococcus spp,* 14: *Enterobacter spp* 16-19: *Clostridium spp*. )

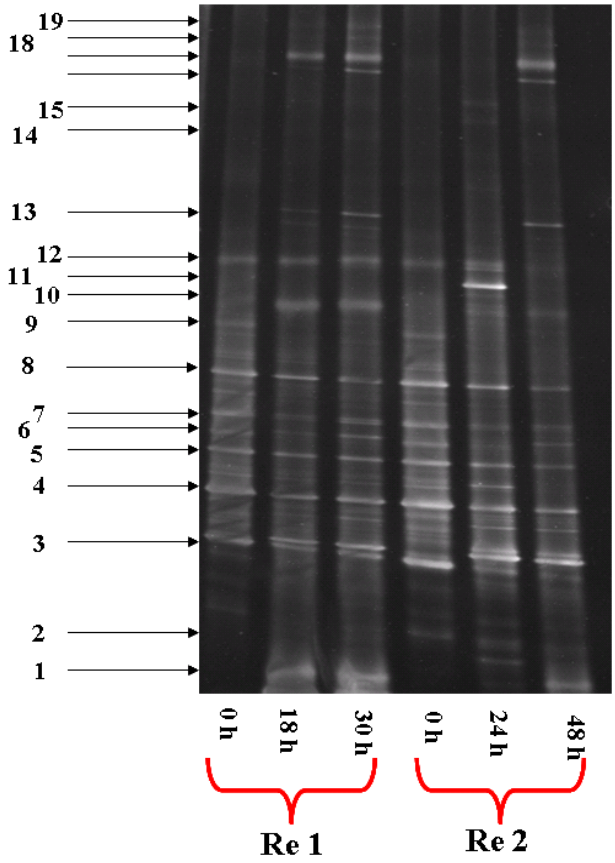


**Figure 5.11**: Structure of microbial dynamics in first feed compared between that in controlled pH condition (Re1) and that in uncontrolled pH condition (Re2). (1-3 : *Clostridium butyricum*, 4-6: *Klebsiella oxytoca*, 7,13: *Acinetobacter sp,* 8: *Straphylococcus sp*, 9-10: *Lactococcus sp,* 11-12: *Enterobacter sp* 14-19: *Clostridium spp*. )

## 5.6 Kinetic parameters of hydrogen production

The kinetic parameters of this study are summarized in Table 5.3 compared between under uncontrolled and controlled pH condition in the first feed of 5 L semi-batch reactor. Under controlled pH condition, maximum hydrogen production rate and specific hydrogen production rate were significantly higher than those under uncontrolled pH conditions whereas maximum hydrogen production, specific hydrogen production and yield of hydrogen under uncontrolled pH condition were higher than those under controlled pH condition. Under uncontrolled pH condition, the obtained cumulative hydrogen production was highest in the first feed (11. 5 L) and obviously decreased in the next feed throughout the end of experiment (3.9, 4.3, 4.3, 3.0 and 0.4 L). Similarly, under controlled pH condition, the highest cumulative hydrogen production was evidently observed in the first feed. A comparison of kinetic parameters obtained in this study with other studies was exhibited in Table 5.4. It successfully demonstrated the efficiency of hydrogen fermentation by adding *C.butyricum* in the semi-batch reactor and using non sterile food waste.

## 5.7 Conclusion

Under uncontrolled pH condition, maximum hydrogen yield, maximum hydrogen production rate and specific hydrogen production rate were 362 mL $H_2$/g $VS_{removed}$, 695 ml/hrand 174 ml/hr.L respectively while 350 mL $H_2$/g $VS_{removed}$, 1,092 ml/hrand 273 ml/hr.L were observed under controlled pH condition. Butyric acid fermentation type was the fermentation type in both conditions that butyric acid and acetic acid were the main metabolites in liquid phase. Acidic condition resulted from total concentration of undissociated acids was the main factor which controlled the route of fermentative process under uncontrolled pH condition. DGGE profiles revealed that *Clostridium butyricum, Klebsiella oxytoca, Straphylococcus spp, Enterobacter spp, Lactococcus spp* and *Acinetobacter spp.* were the dominant species observed in both conditions that *K. oxytoca* was involved in solventoganesis process under uncontrolled pH condition while *Lactococcus spp* related with the low yield of hydrogen production under controlled pH condition.

The stability of *C. butyricum* in the long-term fermentation of the semi-batch reactor appeared to be significant in both of the different pH conditions. The circulation period and ratio have been carefully evaluated to maintain environmental condition and a number of active organisms in the reactor. Acetogenesis was a $H_2$ consuming process observed under the controlled pH condition that affected the net $H_2$ production. Therefore, not only metabolite profiles and carbon mass balance used to understand the activity of organisms in the reactor, but more details of intracellular flux involved with the net $H_2$ production have to be intensively considered along with other profiles to improve and enhance the efficiency of fermentation.

**Table 5.3** Summarized kinetic parameters in 5 L semi-batch reactor

| Kinetic parameters | Unit | Condition | |
|---|---|---|---|
| | | Controlled pH | Uncontrolled pH |
| $R_{max}$ | mL $hr^{-1}$ | 1,091.08 | 695.45 |
| | mmole $hr^{-1}$ | 43.72 | 24.87 |
| $H_{max}$ | mL | 6,994.47 | 11,577.85 |
| | Mmole | 280.28 | 463.95 |
| Y | mL $H_2$ $g^{-1}$ $VS_{removed}$ | 349.72 | 361.81 |
| | mmole $H_2$ $g^{-1}$ $VS_{removed}$ | 14.01 | 14.50 |
| Y’ | mL $H_2$ $hr^{-1}$ $g^{-1}$ $VS_{removed}$ | 11.66 | 10.05 |
| | mmole $H_2$ $hr^{-1}$ $g^{-1}VS_{removed}$ | 0.47 | 0.40 |
| R | mL $hr^{-1}$ $L^{-1}$ | 272.77 | 173.86 |

**Table 5.4** Comparison of kinetic parameters in biohydrogen production

| Substrate | inocula | Maximum yield ($mLH_2$/g substrate) | Volumetric $H_2$ production rate ($mLH_2$/hr) | Molar $H_2$ production rate (mmol $H_2$/hr) | Normalized $H_2$ production rate (mmol $H_2$/L.hr) | Reference |
|---|---|---|---|---|---|---|
| Glucose | *C acetobutylicum* | - | 27.20 | 1.09 | 8.90 | Zhang *et al*., 2006 |
| Sugarcane hydrolysate | *C. butyricum TISTR1032* | - | 75.91 | 3.042 | 3.38 | Pattra *et al*., 2010 |
| Food waste | sludge digester | 120 mL/g $VSS_{removed}$ | - | - | - | Zhu *et al*., 2011 |
| Food waste | anaerobic mixed culture | 102.63mL/g $VSS_{removed}$ | | | | Sreela *et al*., 2011 |
| Palm oil mill effluent | *C. butyricum* | 306 mL/g carbohydrate | 914.00 | | | Chong *et al*., 2009 |
| Food waste | Anaerobic digester | 80.9 mL/g VS | | | | Kim *et al*., 2008 |
| Food waste | *C. butyricum TISTR1032* | 362mL/g $VS_{removed}$ | 695.45 | 24.87 | 6.22 | this study (uncontrolled pH) |
| Food waste | *C. butyricum TISTR1032* | 350mL/g $VS_{removed}$ | 1,092.45 | 43.70 | 10.90 | this study (controlled pH) |

# CHAPTER 6

# Metabolic Flux Analysis of Hydrogen Production from Rice Starch by Anaerobic Sludge under Varying Organic Loading

Metabolic Flux Analysis is a powerful tool to analyze intracellular flux corresponding with hydrogen fermentation pathway. To better understand, the intracellular flux analysis based on the *in silico* model which constructed of totally possible reactions involved in hydrogen fermentation was examined. Due to the limitation factor of MFA for complex substrate such as measurement of initial molar of substrate and concentration of biomass (seed inocula) at given time, experimental plan under this study was designed to analysis the performance of anaerobic sludge for hydrogen fermentation by conducting batch fermentation under varying organic loading conditions from 2.5 to 12.5 g/L rice starch. Nevertheless, only *C. butyricum* was still limited in hydrolysis process and took more time in the lag phase (12 hr fermentation) than the lag time which observed of heat shocked anaerobic sludge (8 hr fermentation) in 0.5 L batch reactors.

For long-term fermentation, it may be difficult to use a pure culture for hydrogen production from organic wastes since it is easily out competed by $H_2$ consumer groups such as methanogen and acetogen groups. Moreover, the extraction of hydrogen molecules from short chain organic acids, metabolites of acidogenesis, such as butyric and propionic acid to produce acetic acid, acetogenesis, by some syntrophic bacteria in anaerobic sludge is an interesting strategy to enhance the net $H_2$ production from organic rich wastes. Therefore, in this study, rice starch fermentation by anaerobic sludge was conducted to evaluate the intracellular flux of organisms in the reactor involving in hydrogen fermentation since the structure of rice starch was similar as rice, a major fraction of food waste (65% w/w).

## 6.1 Carbon balance and recovery for starch fermentation

To check the validity of the data before using the metabolic construction model, the carbon balance of starch fermentation was examined. Table 6.1 showed that carbon recoveries were higher than 96% in most cases (5-12.5 g/L) at the $20^{th}$ hr fermentation. At this time, cumulative $H_2$ production reached saturated condition for all cases (Fig. 6.1). According to previous study, biomass yield from anaerobic fermentation fed by glucose

was presumed to be relatively low and observed in the range 11-22% (Chaganti *et al*., 2011). However, in this study, biomass formation was hardly observed (less than 1%) for all cases, so it was not included in carbon balance analysis and MFA model. Hydrolysis was decreased as the concentration of starch was increased (Fig. 6.2A). On the other hand, utilization of glucose appeared to increase in higher concentration while $H_2$ production reached to maximum at 5g/L starch and continuously reduced in the higher concentration cases (from 7.5 to 12.5 g/L).

The capability of hydrolysis and utilization process by organism was the first step of anaerobic degradation of organic substance to drive normal metabolism and finally to produce the combined volatile fatty acid and/or alcohol. Under this study, $H_2$ production at 5 g/L condition gave the maximum production and the highest efficiency of hydrolysis process along with utilization process. The main carbon fraction of fermentation end-product in the starch fermentation reactor at the $20^{th}$ hr fermentation was acetic acid for all cases (Fig. 6.2B). Normally, the fermentation end-products of anaerobic fermentation, such as VFAs and/or alcohol, could be used as an indicator to understand microbial activity of hydrogen-producing bacteria in a reactor (Fan *et al*., 2004, Nathao *et al*., 2013).

Acetic acid production is not only correlated with hydrogen-producing pathway (acidogenesis), but it can be produced via homoacetogenesis which is hydrogen consuming pathway. According to Reaction 6.1, the presence of homoacetogen (acitogenic hydrogen consumers) is the main factor resulting in the low yield of hydrogen production that it cannot be suppressed by thermal stress ($104^oC$ for 2 hr) (Oh *et al*., 2003). Therefore, considering the pathway of acetic acid production together with hydrogen production could be examined to evaluate the feasibility of the net $H_2$ production and $H_2$ consumption through starch fermentation.

$$4H_2+2CO_2 \text{ ----> } CH_3COOH+2H_2O \qquad (6.1)$$

## 6.2 Metabolic Flux Analysis

The metabolic reaction for $H_2$ production constructed *in silico* model and the optimum flux for different starch fermentation (2.5-12.5 g/L) showed in Fig. 6.3 and Appendix A. For flux analysis, 14 intra-cellular and 12 extracellular compounds were constructed in configuring the network (Appendix B). Starch is hydrolyzed to glucose as the simple form by hydrolytic bacteria and then converts to pyruvate via glycolysis

pathway and finally to hydrogen plus many end- products such as VFAs and alcohol. No $CH_4$ was detected for all cases under this study. For sequence of reaction involved hydrogen production, acetic acid production pathway is defined as hydrogen producing pathway that can achieve the highest theoretical yield 4 mol $H_2$ per mol glucose if acetic acid is the sole end product (Eq. 6.2) (Das *et al*.,2009). However, acetic acid production can be produced from acetogenesis pathway (Eq. 6.1) and short chain fatty acid degradation such as propionic acid (Eq.6.3), and butyric acid (Eq. 6.4) (Muller *et al*., 2003; Veldez-Vazquez *et al*., 2005) under anaerobic conditions.

Normally, the anaerobic oxidation of propionic and butyric acid to acetic acid, $CO_2$ and $H_2$ are highly endergonic for standard conditions at 25$^oC$ ($\Delta G^0_{propionate}$ = +76.1 kJ/mol and $\Delta G^0_{butyrate}$ = +48.1 kJ/mol). However, under the maintained condition of low $H_2$ partial pressure, these reactions can be achieved (Schink and Stams, 2005).

$CH_3CH_2CH_2COOH$ ----> 2 $CH_3COOH$+2 $H_2$ (6.3)

$CH_3CH_2COOH + 3H_2O$ ----> $CH_3COOH + 3H_2 + CO_2$ (6.4)

According to Gibbs free energy equation related with standard conditions follow as:

$\Delta G = \Delta G^0 + RT \ln Q$

where $\Delta G$ = free energy at any moment, $\Delta G^0$ = standard state free energy, R= ideal gas constant, T= temperature ($^oK$) and Q = reaction quotient ([product]/[substrate])

at equilibrium $\Delta G = 0$, thus $\Delta G^0 = - RT \ln K$

In this study, under anaerobic conditions with maintaining the mesophilic condition (310$^oK$), results showed that acetic acid was the main metabolites, and it was significantly higher than other end-products (butyric acid, lactic acid and ethanol) (Fig. 6.4). Therefore, acetic acid formation via Reaction 6.3 was feasible to occur thermodynamically under this condition.

### 6.3 Maximizing $H_2$ production via acetic acid production pathway

The net production of acetic acid was obtained from acetyl coA conversion (R15), homoacetogenesis (R17) and butyric acid oxidation (R26*2). However, the results calculated from the *in silico* model and measured in the experiment had very good agreement with respect to acetic acid production used as the objective function in this study (Fig. 6.5A). Although acetic acid was defined as the correlative pathway of hydrogen

production in the acidogenesis process, the pathway of production could be intensively considered to understand the activity of the involved microorganism in a fermentation reactor. The maximum flux of acetic acid production was observed from R26, R17 and R15 respectively for all cases (Fig. 6.5B).

The results indicated that butyric acid oxidation reaction was the major pathway to generate acetic acid and $H_2$ as the final products. However, the acetogenesis process was the $H_2$ consuming pathway in which 4 mol of hydrogen was consumed to produce 1 mol of acetic acid. Therefore, the flux of acidogenesis (R17) affected directly the low yield of $H_2$ production whereas the increased $H_2$ yield resulted from flux of butyric acid oxidation (R24). Normally, as a R15, acetyl coA would be converted to acetic acid that allows to produce $H_2$ in normal condition (Kramer *et al*.,2006). The highest flux of R26 (1.2 mol) was observed at 5 g/L condition corresponding with the maximum net $H_2$ production (0.98 mol) from R14 (Fig. 6.6A). However, re-oxidizing ferredoxin (R13) was the main flux for hydrogen production observed under low starch fermentation (2.5 and 5 g/L) and it was significantly higher than $H_2$ flux from butyric acid oxidation (R26). On the other hand, trends in both R13 and R26 flux observed under high starch fermentation (7.5, 10 and 12.5 g/L) were not significantly different (less than 10%).

The results illustrated that the initial starch concentration resulted in the major flux of $H_2$ production. The net $H_2$ production (R14) trends to increase from 0.57 to 0.98 mol when starch concentration was increased (2.5 to 5 g/L) and reduce to 0.43, 0.33 and 0.27 mol in 7.5, 10 and 12.5 g/L starch fermentation respectively. Besides, homoacetogenesis was the major flux correlated with $H_2$ consumption in the anaerobic fermentation. Moreover, $H_2$ consuming acetogenic activity was not suppressed by thermal stress and pH adjustment (Chaganti *et al*.,2011). $H_2$ consuming flux (R17*4) observed in this study was higher than other hydrogen fluxes for all cases. This flux appeared to be a major reaction resulted in the low $H_2$ production that it was strongly affected in the high starch fermentation (10 and 12.5 g/L). $H_2$ productivity tended to increase with increased starch concentration from 2.5 to 5 g/L and decrease with the higher starch concentration (Fig. 6.6B). The highest productivity was 18.15 mL $H_2$ /hr at 5 g/L starch condition.

The results illustrated that acetic acid production pathway was significantly correlated with $H_2$ synthesis. Therefore, not only $H_2$ production from normal flux, ferredoxin re-oxidation could be maintained in the optimal condition of fermentation, but

others correlated with $H_2$ flux, such as acetogenesis (R17) and butyric acid oxidation (R26) could be used as the major issues for enhancement the efficiency of hydrogen production.

## 6.4 Conclusion

In this study, metabolic flux analysis constructed in the *in silico* model successfully agreed with the results from the experiment with respect to acetic acid production used as the objective function. Maximizing acetic acid is not only correlated in $H_2$ production but it's also resulted in $H_2$ consumption through acetogenesis. Butyric acid oxidation (R26) corresponded with $H_2$ production under anaerobic fermentation. Acetic acid formation from R26 was the maximum flux for all cases of starch fermentation. The initial starch concentration resulted in the major flux of $H_2$ production. R13 was the major $H_2$ formation flux observed in the low starch fermentation (2.5 and 5 g/L). The maximum $H_2$ productivity obtained at 5 g/L starch fermentation. To achieve high yield of hydrogen production from starch fermentation, besides normal acetic acid fermentation pathway, butyric acid oxidation could be a strategic reaction target to promote the performance of the involved microorganism in fermentation reactor.

**Table 6.1** Carbon balance for starch fermentation after 20 hrs incubation by mixed culture

| [starch] | Time (hr) | Carbon (mol) | | | | | | | Total (mol) | % recovery |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Starch | Glucose | Acetic | Butyric | Lactic | Ethanol | $CO_2$ | | |
| **2.5** | 0 | 0.0458 | 0.0000 | 0.0157 | 0.0000 | 0.0012 | 0.0003 | 0.0000 | 0.0630 | |
| | 20 | 0.0002 | 0.0204 | 0.0217 | 0.0009 | 0.0025 | 0.0010 | 0.0073 | 0.0541 | 85.78 |
| **5.0** | 0 | 0.0917 | 0.0000 | 0.0166 | 0.0000 | 0.0013 | 0.0004 | 0.0000 | 0.1099 | |
| | 20 | 0.0002 | 0.0205 | 0.0598 | 0.0073 | 0.0011 | 0.0013 | 0.0164 | 0.1066 | 96.96 |
| **7.5** | 0 | 0.1375 | 0.0000 | 0.0124 | 0.0000 | 0.0014 | 0.0003 | 0.0000 | 0.1516 | |
| | 20 | 0.0827 | 0.0172 | 0.0340 | 0.0005 | 0.0027 | 0.0009 | 0.0130 | 0.1509 | 99.56 |
| **10.0** | 0 | 0.1833 | 0.0000 | 0.0165 | 0.0000 | 0.0015 | 0.0003 | 0.0000 | 0.2016 | |
| | 20 | 0.0907 | 0.0192 | 0.0657 | 0.0002 | 0.0042 | 0.0013 | 0.0157 | 0.1969 | 97.68 |
| **12.5** | 0 | 0.2292 | 0.0000 | 0.0166 | 0.0000 | 0.0014 | 0.0003 | 0.0000 | 0.2475 | |
| | 20 | 0.0799 | 0.0207 | 0.1174 | 0.0052 | 0.0048 | 0.0011 | 0.0132 | 0.2422 | 97.85 |

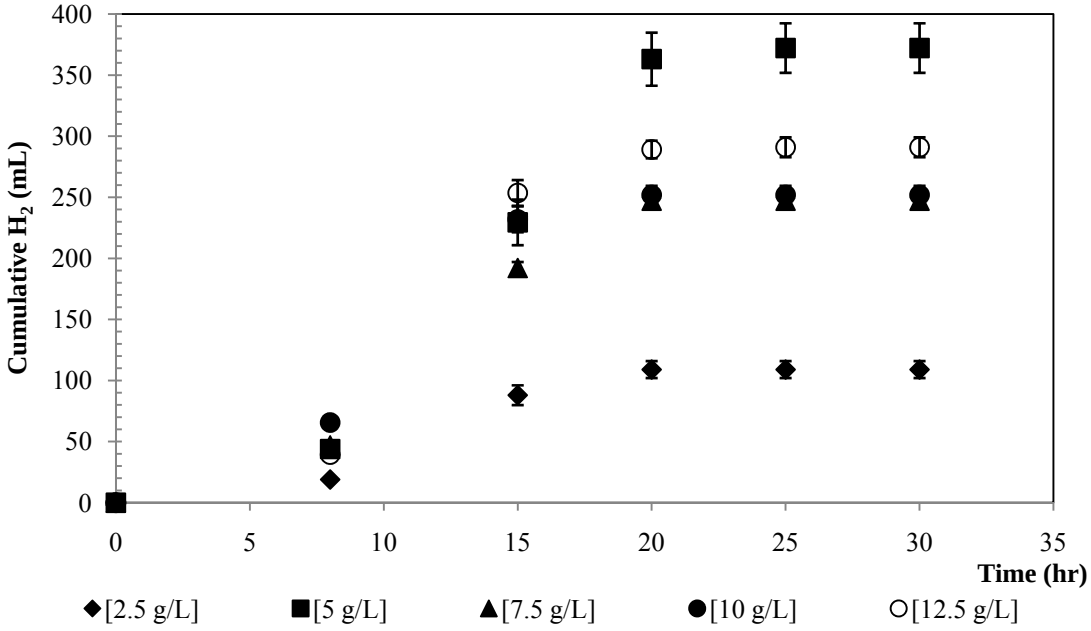


**Figure 6.1:** Cumulative hydrogen production by anaerobic sludge at different starch concentrations. Each data point represents average values of duplicate experiments (n=2). Error bar represents standard deviation.

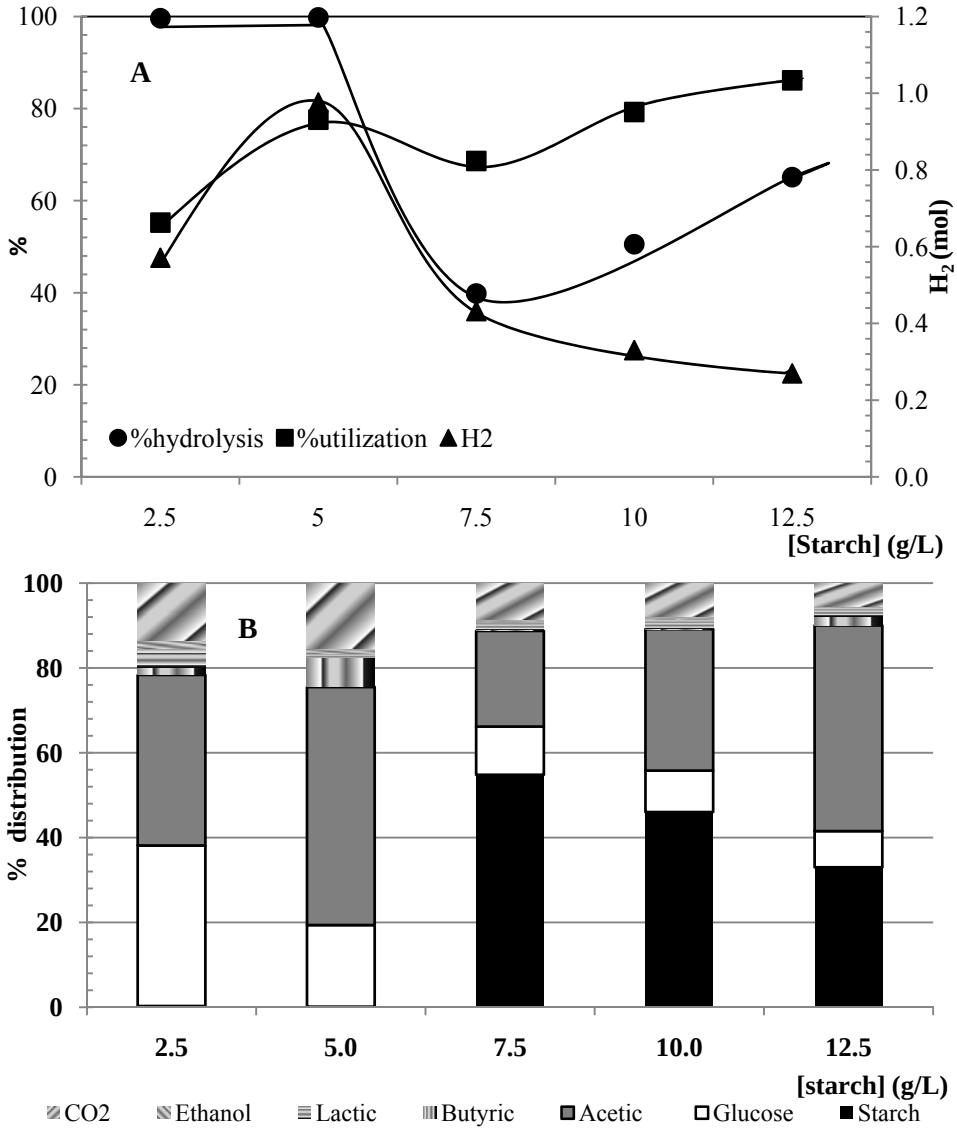


**Figure 6.2:** (A) Hydrolysis, Utilization and $H_2$ production and (B) Carbon balance analysis at 20 hr starch fermentation at different concentrations.

**Figure 6.3:** Molar flux distributions of dark fermentation using starch as substrates at different concentrations (2.5, 5, 7.5, 10 and 12.5 g/L). Rn (1 to 26) represents the reaction number. The flux is normalized to make 1 mol of substrate.

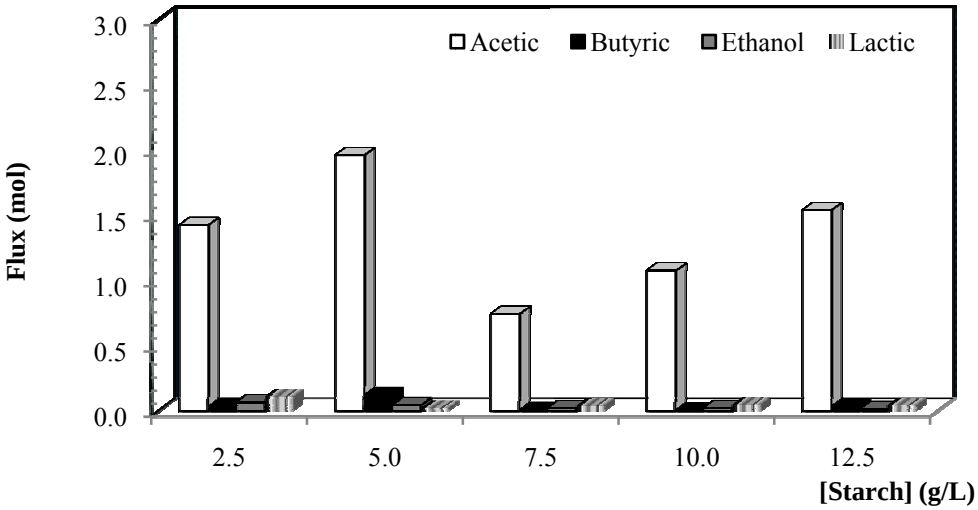


**Figure 6.4:** Metabolite fluxes on starch fermentation at different concentrations.

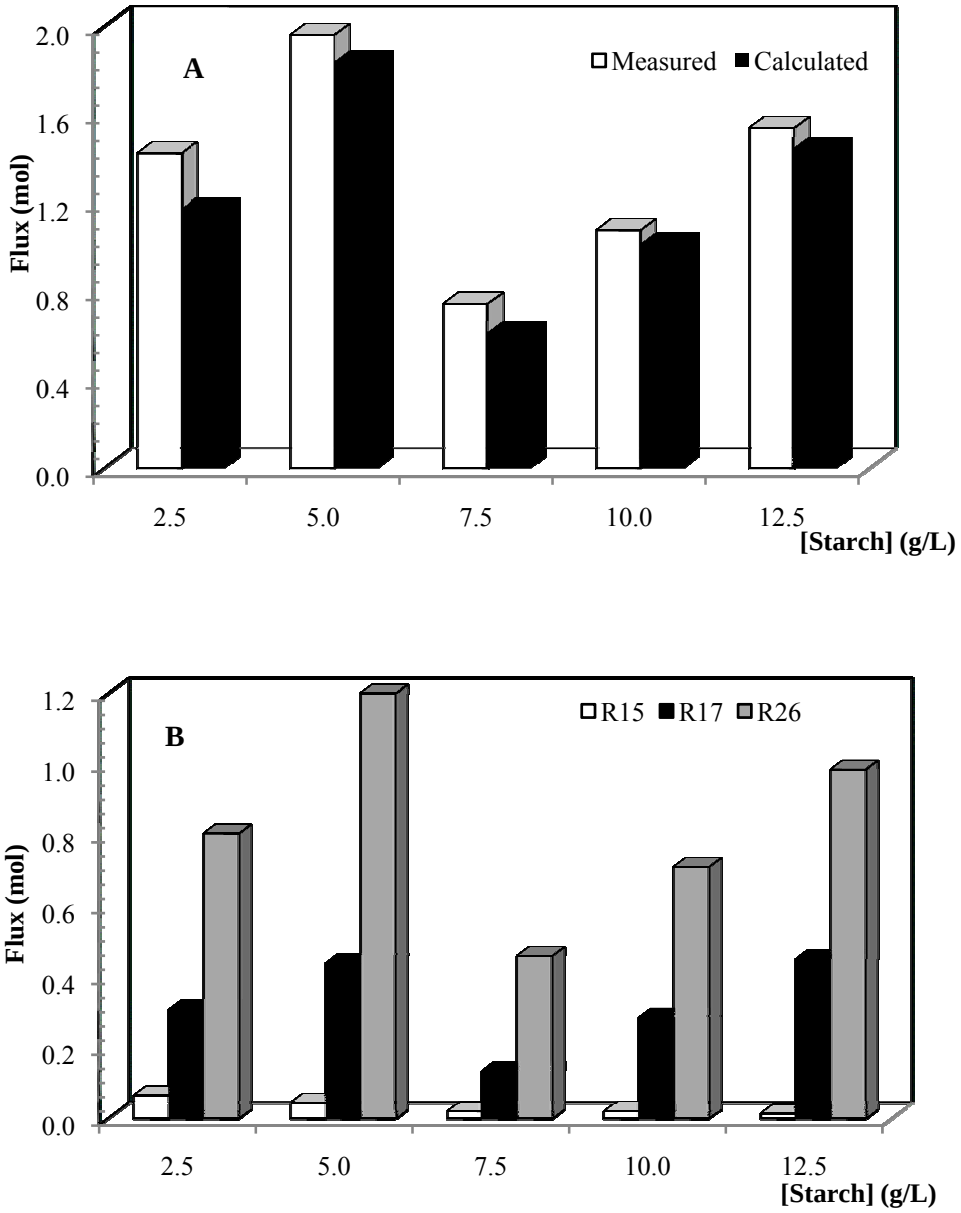


**Figure 6.5:** (A) Acetic acid production measured from experiment and calculated from flux distribution and (B) Acetic acid production by ACCOA intermediate pathway ( R15), acetogenesis (R 17) and butyric acid oxidation {(R26)*2} on starch fermentation at different concentrations.

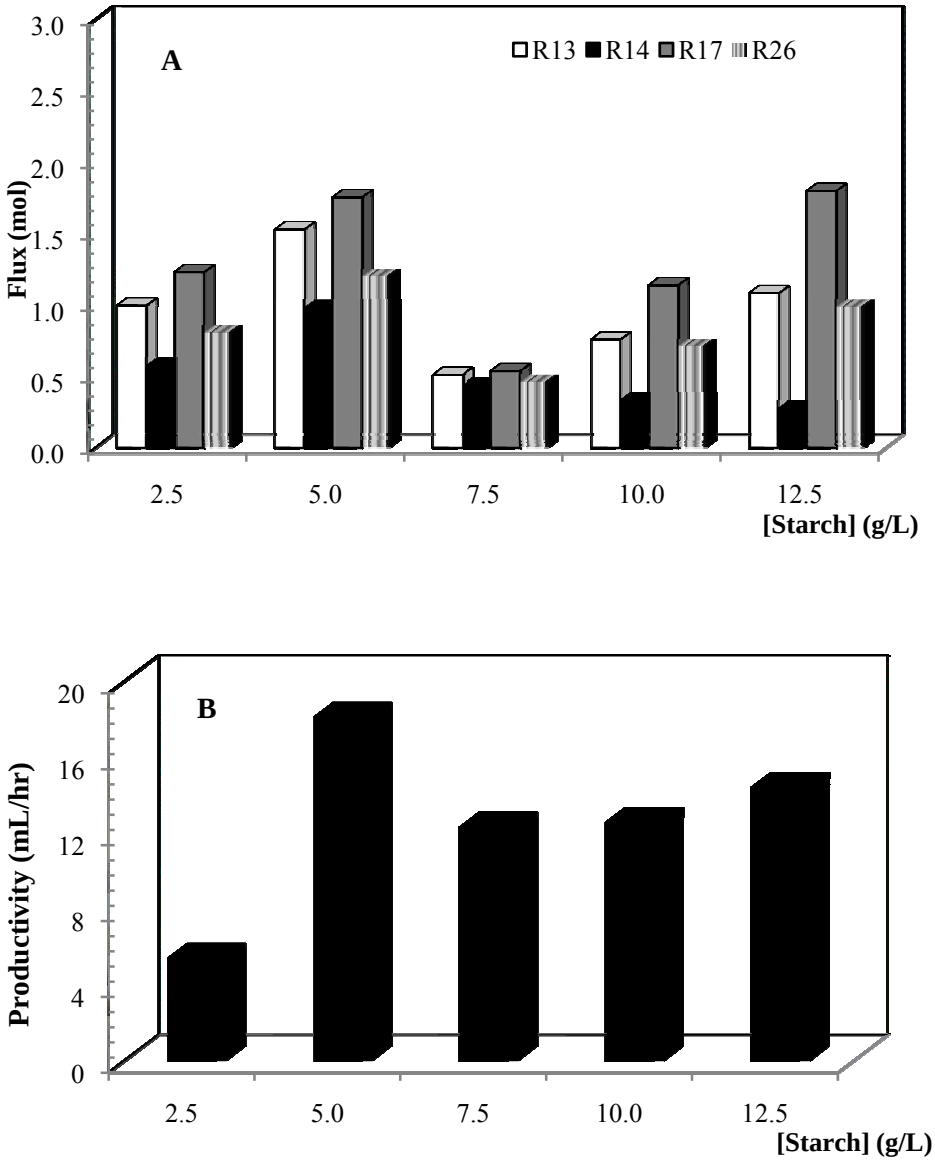


**Figure 6.6:** (A) $H_2$ production (R15) from ferredoxin pathway, butyric acid degradation (R26*2) and hydrogen consumption (R 17*4) fluxes and (B) $H_2$ productivity on starch fermentation at different concentrations.

# CHAPTER 7
# CONCLUSION

Biohydrogen production from organic rich waste has been improved and developed for the industrial sector to be used as an alternative renewable energy source in the future. However, the results of this study can be used as a basic guideline for full scale hydrogen production. The summarized issues can be listed as given below:

1. Very good performance of *C.butyricum* for hydrogen fermentation by food waste was constructed in batch mode operation (0.5 L batch reactor). The maximum yield 134 $mLH_2$/g VS food waste was observed at 2.5% VS food waste.
2. Organic loading causes in the pattern of metabolites, particularly, under high organic loading condition (7.5-12.5% VS food waste) for both inocula fermentations. Results showed that propionic acid was the major limitation factor of hydrogen production for *C.butyricum* batch fermentations while ethanol was the limitation factor for anaerobic sludge batch fermentations.
3. Hydrogen fermentation in 5L semi-batch reactor performed the efficiency of hydrogen production. The maximum hydrogen yield, maximum hydrogen production rate and specific hydrogen production rate observed under uncontrolled pH condition were 362 mL $H_2$ $g^{-1}$ $VS_{removed}$, 695 ml $hr^{-1}$and 174 ml $hr^{-1}L^{-1}$ respectivelywhile 350 mL $H_2$ $g^{-1}$ $VS_{removed}$, 1,092 ml $hr^{-1}$and 273 ml $hr^{-1}L^{-1}$ were observed under controlled pH condition.
4. Microbial community was monitored for long-term fermentation in 5 L semi-batch reactor to check the stability of *C.butyricum* used as started seed inocula included to determine the dominant organisms involved in $H_2$ production under the different pH condition. *C. butyricum* can be dominant group together with the other indigenous groups from food waste in both different pH fermentors. The results illustrate that *Klebsiella spp*. was the major group affecting $H_2$ production process in the uncontrolled pH fermentor, while *Lactococcus spp*. and some Clostridia played significant roles for hydrogen production in the controlled pH fermentor.

5. Carbon mass balance can be used as a technique to monitor carbon distribution in 3 phases (solid, liquid, gaseous) for evaluating the ability of hydrolysis, utilization and fermentation direction. The results show that hydrolysis was a limitation factor for dark fermentation by *C.butyricum* from waste substrate.
6. Metabolite dynamics could be used to evaluate the direction of fermentation along with the inhibition factor resulting in $H_2$ production.
7. Solventogenesis and acidity conditions were limiting factors in $H_2$ production under uncontrolled pH condition in 5L semi-batch reactor regarding the pattern of alcohol and the total concentration of undissociated acid. While acitogenesis was presumed as an inhibition factor under controlled pH condition that metabolite profiles showed the reverse trend of acetic acid and $H_2$ production
8. MFA for the $H_2$ production by mixed culture with rice starch successfully agreed with the results from the experiment.
9. Acetic acid production could be used as an objective function for MFA in an *in vivo* system.
10. Acetic acid production via acetogenesis and butyric acid oxidation can be observed in MFA
11. Maximizing of acetic acid is not only correlated in $H_2$ production but also resulted in $H_2$ consumption through acetogenesis.
12. Butyric acid oxidation was a strategic reaction target to enhance the $H_2$ yield by mixed culture.
13. Organic loading resulted in the major flux of $H_2$ production.
14. Maximum hydrolysis and utilization was observed at 5 g/L starch fermentation related to maximum $H_2$ productivity while the re-oxidation of ferredoxin was the major flux of $H_2$ synthesis pathway.

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## APPENDIXES

**Table A.** Reactions used in metabolic reaction network.

| Reaction No. | Reaction |
|---|---|
| 1 | Starch ----> GLC (ext) |
| 2 | Starch ----> Starch (Res) |
| 3 | GLC (ext) ----> GLC |
| 4 | GLC ----> Biomass |
| 5 | GLC ----> Res GLC |
| 6 | GLC + 2 $NAD^+$ ----> 2 PYR+2 NADH |
| 7 | PYR+NADH ----> HLa+$NAD^+$ |
| 8 | HLa ----> HLa (ext) |
| 9 | HLa+NADH ----> HPr+$NAD^+$ |
| 10 | HPr ----> HPr(ext) |
| 11 | PYR+CoA+2$Fd^{2+}$ ----> ACCOA+$CO_2$+2$Fd^+$ |
| 12 | NADH+2$Fd^{2+}$ ----> $NAD^+$+2$Fd^+$ |
| 13 | 2$Fd^+$+2$H^+$ ----> 2$Fd^{2+}$+$H_2$ |
| 14 | $H_2$ ----> $H_2$ (ext) |
| 15 | ACCOA ----> HAc+CoA |
| 16 | HAc ----> HAc (ext) |
| 17 | 4$H_2$+$CO_2$ ----> HAc |
| 18 | ACCOA+2NADH ----> EtOH+2$NAD^+$+CoA |
| 19 | 2ACCOA ----> ACACCOA+CoA |
| 20 | ACACCOA+2NADH ----> BTCOA+2$NAD^+$ |
| 21 | BTCOA ----> HBu+CoA |
| 22 | HBu ----> HBu (ext) |
| 23 | HAc ----> $CO_2$+$CH_4$ |
| 24 | $CO_2$+4$H_2$ ----> $CH_4$+2$H_2O$ |
| 25 | $CH_4$ ----> $CH_4$ (ext) |
| 26 | HBu ----> 2HAc+2$H_2$ |

**Table B** Metabolites used in MFA.

| **Abbreviation** | **Compound** |
|---|---|
| HAc | Acetic acid |
| HAc (ext) | Acetic acid (external) |
| ACACCOA | Acetoacetyl-CoA |
| ACCOA | Acetyl- coA |
| HBu | Butyric acid |
| HBu (ext) | Butyric acid (external) |
| BTCOA | Butyryl-CoA |
| Biomass | Biomass |
| EtOH | Ethanol |
| $Fd^+$ | Ferridoxin (oxidized) |
| GLC | Glucose |
| GLC (ext) | Glucose (external) |
| $H_2$ | Hydrogen |
| $H_2$ (ext) | Hydrogen (external) |
| HLa | Lactic acid |
| HLa (ext) | Lactic acid (external) |
| $CH_4$ | Methane |
| $CH_4$ (ext) | Methane (external) |
| NADH | Nicotinamide adenine dinucleotide (reduced) |
| HPr | Propionic acid |
| HPr (ext) | Propionic acid (external) |
| PYR | Pyruvate |
| Res GLC | Residual glucose |
| Starch | Starch |
| Starch (Res) | Residual starch |

ext.= external to the cell.