CHAPTER IV
TWO STAGES FERMENTATION OF BIO-HYDROGEN AND ETHANOL BY *Clostridium butyricum* FROM SUGARCANE JUICE

4.1 Introduction

Being a sustainable energy source, hydrogen is a promising alternative to fossil fuels, as it is a clean and environmental friendly fuel, which produces water instead of greenhouse gases after combustion. Hydrogen has a high energy yield of 122 KJ/g which is 2.75 times greater than that of hydrocarbon fuel. Hydrogen can be generated mainly from fossil fuels, biomass and water by chemical or biological process (Han, Shin, 2004; Kim et al., 2004). Biologically, hydrogen can be produced by the photosynthetic and fermentative routes which are more environmentally friendly and less energy intensive compared to thermo-chemical and electro-chemical processes (Kapdar, Kargi, 2006). In comparison to photosynthetic microorganisms, fermentative hydrogen-producing microorganisms are advantageous in that hydrogen can be evolved in a reactor continuously without light (Koku et al., 2002).

Among the fermentative hydrogen producers, the organisms of genus *Clostridium* such as *C. butyricum* (Yokoi et al., 1998; Chen et al., 2005), *C. acetobutylicum* (Chin et al., 2003) *C. saccharoperbutylacetonicum* (Ferchichi et al., 2005), *C. pasteurianum* (Liu, Shen, 2004; Lin, Lay, 2004) are often used to produce hydrogen (Hawkes et al., 2007). *Clostridium* spp., the spore forming anaerobic bacteria, is one of those organisms capable of converting sucrose to hydrogen with the yield ranged between 2.0 and 4.8 mol of hydrogen/mol sucrose (Yokoi et al., 1998; Chen et al., 2005; Lin et al., 2004) which is higher than that of the other fermentative bacteria such as *Enterobacter* sp. (1 mol hydrogen/mol hexose) (Kapdan, Kargi, 2006). When pure culture is used for hydrogen fermentation, the reactors are mostly started up and operated under sterile condition which requires high cost for hydrogen production in industrial scale. To overcome this problem, the addition of pure culture to the reactor under non-sterile condition has drawn our attention.

During hydrogen production under non-sterile condition, the relationship of competitive and cooperative of microbial populations could be found. For the

competitive relationship, some isolates augmented into the reactors can not become dominant due to their inability to grow in the communities depending on the environmental condition (Xing et al., 2008). In contrast, for the cooperative relationship, the mixed indigenous microorganisms in the substrate could consume volatile fatty acids (VFAs) produced during hydrogen production, which in turn facilitate hydrogen production of bioaugmented producer by reducing the product inhibitory effect (Xing et al., 2008). According to these previous reports, the microbial community in the hydrogen production system under non-sterile condition should be investigated for the dominate species responsible for hydrogen production. Therefore, in this study the microbial community in the hydrogen fermentation system after adding *C. butyricum* under non-sterile condition was explored in order to verify the relationship between the augmented microorganism and normal flora in the substrate i.e. sugarcane juice.

Throughout the successful process of hydrogen production from sugarcane juice, large amounts of organic wastewater containing residual organic matter such as butyric and acetic acids and residual sugar were generated. In the recent reports, obligate and facultative anaerobes such as *Clostridia* and *Enterobacter* species are capable of producing solvent by using VFAs as substrate and/or simultaneously producing hydrogen and solvent from sugar (Wu et al., 2007; Steinbusch et al., 2008; Li, Rosazza, 2000). These published data drawn our interest for using the effluent from hydrogen production process, hydrogenogenic effluent, as a substrate for ethanol production.

In the present study, sugarcane juice was used as a substrate to produce hydrogen continuously by *C. butyricum* in the Continuous Stirred Tank Reactor (CSTR). The hydrogenogenic effluent from hydrogen production process was further used for ethanol production by carried over microorganisms consortium from previous hydrogen production. The relationship of the augmented microorganism i.e. *C. butyricum* and normal flora in the fermentation system under non-sterile condition were analyzed by the Denatured Gradient Gel Electrophoresis (DGGE) method.

4.2 Material and Methods

4.2.1 Hydrogen production from sugarcane juice in the CSTR

4.2.1.1 Fermentative medium preparation

Sugarcane (*Saccharum officinarum* Linn.) used in this study was harvested from sugarcane filed, Lopburi Province, Thailand. Sugarcane juice was prepared by crushing the sugarcane stalk using squeezer and filtrating through a thin layer cloth, then boiled to concentrate in order to obtain the sugarcane syrup. The sugarcane syrup has a final concentration of 2,000 g/L (2,245 g-COD sucrose/L) of total sugar and was kept at -20 °C until the usage. Frozen sugarcane syrup was thawed by placing at room temperature prior to use as substrate for hydrogen production. For medium preparation, the sugarcane syrup was diluted with distilled water to a concentration of 25 g-COD sucrose/L and supplemented with sufficient inorganic nutrients for bacterial growth including (mg/L); NH_4HCO_3 5240, K_2HPO_4 125, $MgCl_2.6H_2O$ 15, $FeSO_4.7H_2O$ 25, $CuSO_4.5H_2O$ 5, $CoCl_2.5H_2O$ 0.125, $NaHCO_3$ 6720 (Wu et al., 2008).

4.2.1.2 Seed inoculum preparation

Clostridium butyricum TISTR 1032 was obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand. It was grown in cooked meat medium (CMM) (Himedia, India) at 37 ^{O}C under the anaerobic condition for 10 h and kept at 4 ^{O}C as a stock culture. Prior to cultivation, *C. butyricum* was activated by transferring 1 mL of the stock culture at a cell concentration of 10^7 cells/mL into 10 mL of fresh Tryptone Sucrose Yeast Extract (TSY) medium, incubated at 37°C for 10 h at 150 rpm using an orbital shaker under the anaerobic condition. The culture was further enriched by inoculating 10% v/v, cell concentration of 10^6 cells/mL, of the culture into 60 mL fresh TSY medium and incubated at the given conditions before using as inoculum (Sneath et al., 1986). Each liter of TSY containing 5.0 g of tryptone; 3.0 g of sucrose; 5.0 g of yeast extract; 1.0 g of K_2HPO_4 (Holdeman et al., 1977).

4.2.1.3 CSTR operation and monitoring

A 1-L CSTR with a working volume of 900 mL was used to produce hydrogen from sugarcane juice. The schematic diagram of the bioreactor was depicted in Figure 1. The reactor was fed with 810 mL of fermentative medium and

90 mL of *C. butyricum* (10% (v/v), final cell density of 10^6 cell/mL) as seed inoculum under anaerobic condition and operated at a controlled temperature of 37 °C with a constant stirring at 150 rpm using impeller. A pH of fermented broth was maintained at 6 using a pH controller and 1N NaOH. The oxidation-reduction potential (ORP) was measured using ORP meter (MV7615, B&C Electronic, Italy) to ensure the anaerobic condition. After 12 h of reactor operation, the fermentative medium was continuously fed from medium tank to the feed-in port at the bottom of the reactor using peristaltic pump. The effluent overflowed from the reactor at the feed-out port. The starting hydraulic retention time (HRT) was 36 h and the HRT was further shortened to 24, 12, 6 and 4 after a steady state was reached. Steady-state conditions reached when the product concentration such as hydrogen gas content, biogas volume and metabolite concentration (i.e. VFAs and ethanol) were stable (less than 10% variation) for 5-7 days.

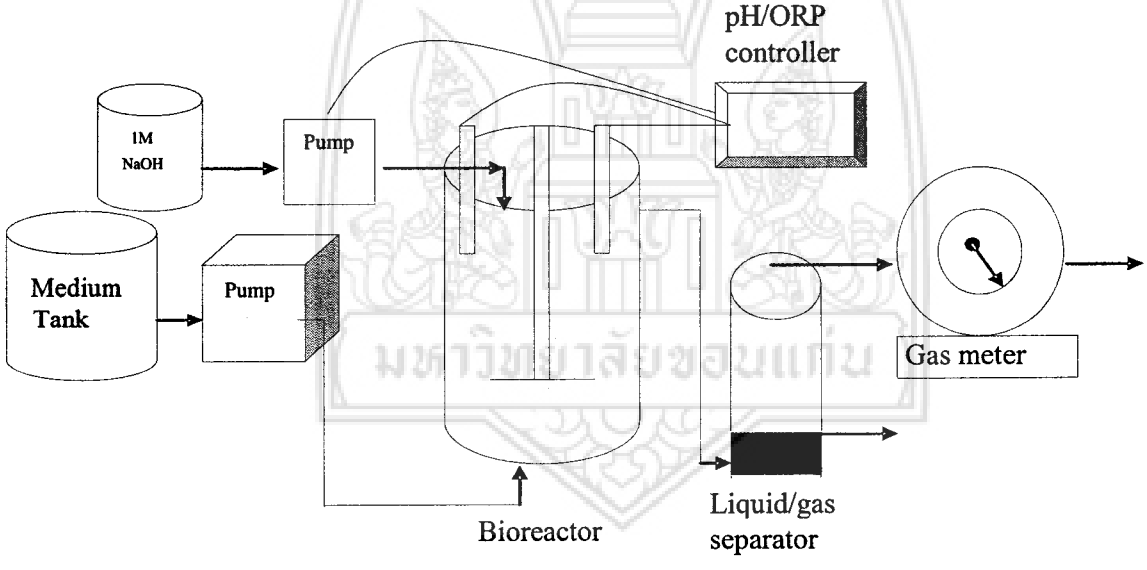

Figure 17 Schematic diagram of the CSTR (not subject to scale)

During reactor operation, the amount of biogas produced was recorded daily using a wet-gas meter (Ritter Inc, Germany, Type: TG 1/5). The biogas contents were analyzed by using Gas Chromatography (GC). The measured hydrogen volume was adjusted to the volume at standard temperature (0 °C) and pressure (760

mm Hg, STP). The effluent was sampled every day to determine the sucrose residues and the concentrations of biomass, VFAs and ethanol produced. The optimum HRT was justified by the greatest hydrogen production rate and yield. The hydrogenogenic effluent, the effluent from hydrogen fermentation system, from the CSTR operated at the optimum condition was collected and used as substrate for ethanol production.

4.2.2 Ethanol production from hydrogenogenic effluent in the batch experiment

4.2.2.1 Hydrogenogenic effluent

Effluent from the bio-hydrogen production using the CSTR at the optimum HRT was used as a substrate for ethanol (EtOH) production. The compositions of hydrogenogenic effluent were shown in Table 24. VFAs were the main metabolites detected in the hydrogenogenic effluent with the residual sucrose of 8.63 g-COD/L.

Table 24 Characteristics of hydrogenogenic effluent

Compositions	Concentration
Volatile suspended solids (VSS, mg/L) as biomass	1,301±10
HAc (mg-COD/L)	1,580±30
HBu (mg-COD/L)	1,552±6
HPr (mg- COD/L)	318±14
EtOH (mg-COD/L)	2,857±11
Sucrose concentration (g-COD/L)	8.63±0.10
pH	6.00±0.05

4.2.2.2 Ethanol production

The EtOH production by carried over microorganisms consortium from previous hydrogen fermentation was conducted in this study. The experiments were conducted in 200 mL serum bottles capped by rubber stopper and aluminum cap with the working volume of 100 mL. The effect of initial pH on ethanol production was firstly investigated. The pH of the hydrgenogenic effluent was adjusted to be 5.0, 6.0, 7.0 and 8.0 before adding to the serum bottle. After replacement of the gas phase with argon to create the anaerobic condition, the serum

bottle was incubated at 37°C and 150 rpm. During the batch experiments, the volume and contents of biogas produced and culture broth compositions were analyzed at the interval time for 48 h. Each run was duplicated and averaged results were reported. The optimum pH was justified by the greatest molar yield of EtOH produced per molar sucrose consumed.

The effect of sugar concentration on ethanol production was studied in batch experiment at the optimum pH obtained. The initial sucrose concentration of the hydrogenogenic effluent was varied by adding sugarcane syrup to the concentration of 25, 50, 100, 200 and 300 g-COD/L. The experiment was conducted in a similar manner as explained in the pH variation experiment.

4.2.3 Analytical methods

The composition of biogas was measured by using a GC (China 8700T, Taiwan) equipped with a thermal conductivity detector (TCD) and the stainless steel column packed with Porapak Q (80/100 mesh, Water crop., USA). Oven, injector and detector temperatures were 50, 50 and 50°C, respectively, and argon was used as the carrier gas.

The concentrations of VFAs and EtOH were determined by gas chromatography (Shimadzu GC-14A, Japan) equipped with a flame ionization detector (FID) and a glass column packed with FON (Shimadzu, Japan). The oven, injector and detector temperatures were 145, 175 and 175°C, respectively, and nitrogen was used as the carrier gas. Volatile suspended solids (VSS) represented as the biomass concentration and Chemical Oxygen Demand (COD) were measured according to Standard Methods (APHA, 1995). The sugar concentration in fermentative medium was determined according to the phenol-sulfuric acid method using sucrose as a standard (Saha, Brewer, 1994).

Hydrogen yield (HY) (mol H_2/mol hexose) and hydrogen production rate (HPR) (mmol H_2/L substrate.h) were calculated by using equation (1) and (2), respectively.

$$HY = \frac{\text{volume hydrogen produced per day (L) /22.4 (L/mol)}}{\text{hexose consumed per day (g) /342 (g/mol)}} \quad (1)$$

$$HPR = \left[\frac{\text{volume hydrogen produced per day (L)/22.4 (L/mol)}}{\text{volume of substrate consumed per day}}\right] X \frac{\text{day}}{24\ \text{h}} \qquad (2)$$

4.2.4 DNA isolation and PCR-DGGE analysis

Total genomic DNA of samples was extracted and purified by using the Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene, Taiwan) following the manufacturer's instructions. Primer set of EUB968F (5'-AACGCGAAGAACCTTAC-3') plus GC-clamp (5'-CGCCCGGGGCGCGCCCCG GGCGGGGCGGGGGCACGGGGGG-3') and UNIV1392R (5'-ACGGGCGGTGTG TRC-3') were used for amplifying the target universal-consensus of 16S rDNA fragment (Nielsen et al., 1999). Polymerase Chain Reaction (PCR) mixtures (50 μL) contained 200 μM of each deoxynucleoside triphosphate, 1.5 mM of primers, 0.2 μM of $MgCl_2$, 1.25 U of Taq DNA polymerase (Promega, USA), and the PCR buffer supplied with the enzyme. The amplification consisted of a DNA denaturing step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 2 min. The cycling included a final extension step at 72°C for 3 min to ensure full extension of the product. All PCR operations were performed with an automatic thermal cycler iCyclerTM (Bio-Rad, U.S.A.). PCR products were analyzed by electrophoresis at 100 V for 30 min through 1.5% (w/v) agarose gel. The amplified products were visualized under UV light after being stained with ethidium bromide. Before PCR-amplifying the extracted rDNA fragments, the sequence variations were assessed by DGGE. The DGGE profile of the PCR-amplified DNA was obtained following the method of Muyzer et al. (Muyzer, 1993) using a DCodeTM Universal Mutation Detection System (BioRad, U.S.A.). The 6% (w/v) acrylamide solution was used to cast a gel with denaturant gradients ranging from 30 to 65%. Electrophoresis was conducted in a 1X TAE (Tris/acetic acid/EDTA) buffer solution at 180 V and 60°C for 5 h. The gels were stained for 10 min with ethidium bromide and visualized with UV radiation. The number of operational taxonomic units in each sample was defined as the number of DGGE bands.

4.3 Results and Discussion

4.3.1 Hydrogen production from sugarcane juice in the CSTR

4.3.1.1 Effect of HRT on hydrogen production and microbial community in the CSTR

The effect of HRT on continuous hydrogen production from sugarcane juice under non-sterile condition in the CSTR augmented with *C. butyricum* was investigated. The time courses profile of continuous biogas and hydrogen production in the CSTR are shown in Figure 2. The summarized operating parameters at steady state for each HRT are shown in Table 2. Results indicated that variation of HRT led to the variation in operating parameters such as total sugar consumption, biogas production, hydrogen content, HPR and HY (Figure 18). The biogas produced in this study consisted of mainly carbondioxide and hydrogen without the detection of methane. The result implied that a slightly acidic condition at pH 6.0, which was maintained by controlling pH of the fermented broth, was favorable for hydrogen production.

At the long HRT of 36-12 h, the hydrogen production was observed to be stable with the HY and HPR of 0.05-0.10 mol H_2/mol hexose and 0.25-0.49 mmol H_2/L substrate.h, respectively. The HY and HPR values increased when the HRT was shortened from 12 h to 6 and 4 h, respectively. The maximum HY and HPR of 1.00 mol H_2/mol hexose and 3.38 mmol H_2/L substrate.h, respectively, were obtained at the HRT of 4 h which indicated that the HRT of 4 h was suitable for hydrogen production from sugarcane juice in the CSTR augmented with *C. butyricum*. The HY of 1.00 mol H_2/mol hexose is in the average range of previous reports (Table 26). Our findings were similar to Wu et al. (2008) who reported that HPR from glucose by anaerobic sludge increased from 0.19 to 0.6 L H_2/L substrate.h when the HRT was shortened from 12 to 6 h. Lin et al. (2008a) reported that a decrease in HRT led to an increase in organic loading rate and long HRT favored the degradation of substrate. In our study, as the sucrose concentration in sugarcane juice was fixed at 25 g-COD/L, a decrease in HRT represented an increase in substrate loading rate. Therefore, if H_2-producing bacterial population can be stably maintained in the bioreactor against an increase in hydraulic dilution arising from a decrease in HRT, the HPR would increase with decreasing HRT. Approximately 24.5 g-COD/L

of sugar consumed at steady state was observed at the HRT of 36-16 h. The total sugar consumed decreased to 18.15 and 16.37 g-COD/L when the HRTs were shortened to 6 and 4 h, respectively. Hydrogen content was considered as HRT-dependent parameter. At the start up, the hydrogen content increased continuously and peaked at 45% after 3 d of reactor operation then decreased continuously to be stable at 6.3% at 36-h HRT. The further decrease in HRT to 24 h resulted in an increase in hydrogen content of 24.2%. At HRT in the range of 12-4 h, the hydrogen content in the biogas produced was observed to be relatively low at approximately 17% (Figure17, Table 24). The volume of produced biogas peaked at the HRT of 4 with the relatively low H_2 content of 19.1%.

The microbial determination in a DGGE analysis for the biomass sample at steady state at each HRT indicated that microbial population was HRT-dependent with *C. butyricum, Klebsiella pneumoniae* and *Lactobacillus harbinensis* (Figure 19, Table 26) are dominant species. *Clostridia* species have been reported to be responsible for hydrogen production via butyrate type fermentation (Lin et al., 2006; Dinopoulou et al., 1988; Yokoi et al., 1997). Hydrogen fermentation by *Clostridia* species is accompanied with VFAs and/or solvent production (Lin et al., 2006). *Klebsiella* species is known as the solvent production strains. They can utilize various kind of substrates and produce alcohol such as 2,3 butanediol, isopropanol and ethanol and hydrogen and carbondioxide as soluble and gases metabolites (Wu et al., 2008; Rosenberg, 1980). Lactic acid bacteria such as *Lactobacillus* sp. had been reported to decrease hydrogen content in biogas production due to its inhibitory effect caused by the excreted bateriocins which have an adverse effect on hydrogen producing bacteria (Noike et al., 2002).

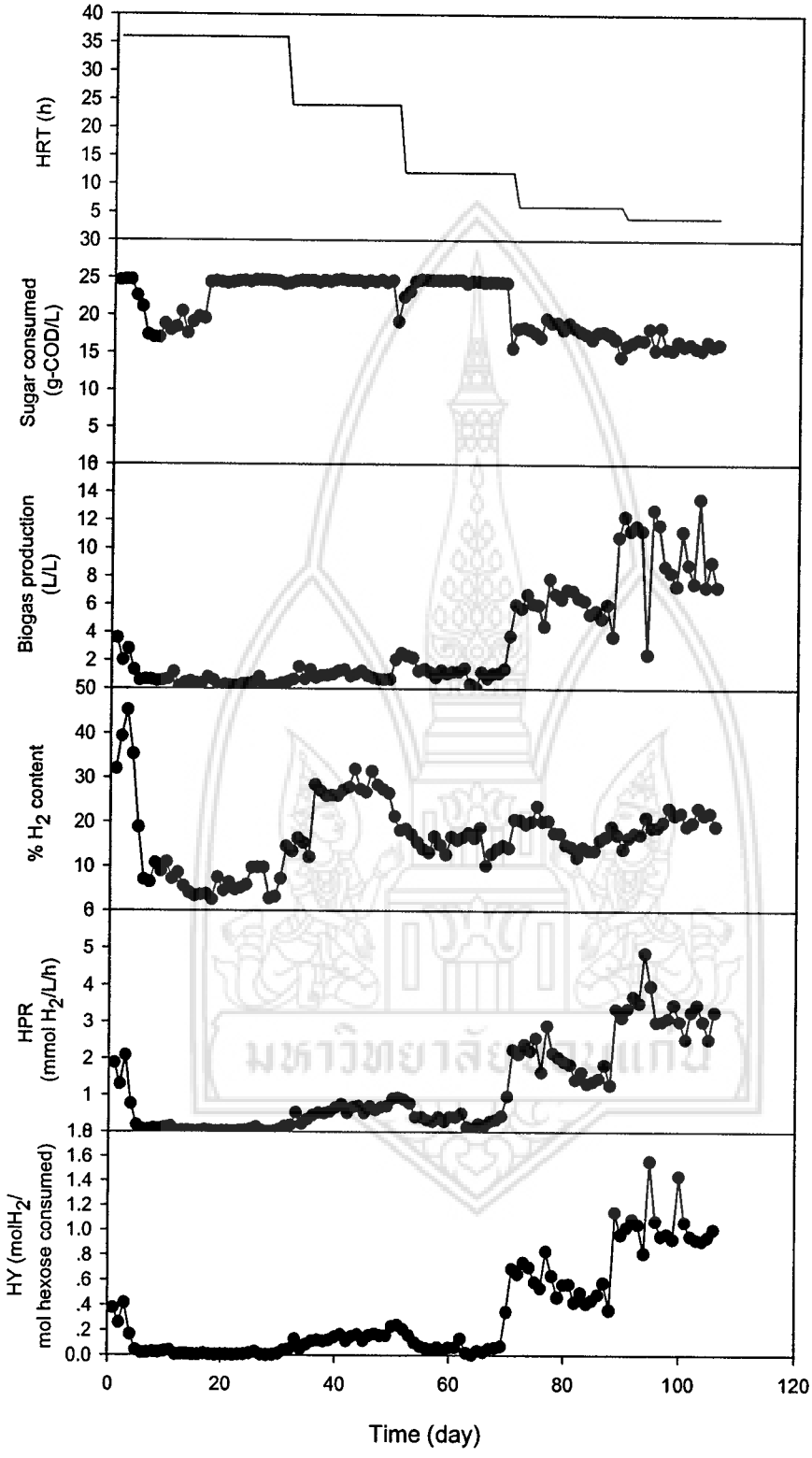

Figure 18 Operating parameters for hydrogen production from sugarcane juice in the CSTR augmented with *C. butyricum* under non-sterile condition.

Table 25 Experiment data under steady-state condition in continuous hydrogen fermentation at each HRT

HRT (h)	%H_2 content	HPR (mmol H_2/L substate.h)	HY (mol H_2/mol hexose)	Total sugar consumed (g-COD/L)	mg-COD/L							HBu /HAc ratio	Biomass (mg- VSS/L)
					EtOH	HAc	HPr	HBu	HLa	VFA	SMP		
36	6.3	0.25	0.05	24.50	2,583	1,407	1,741	4,799	3,566	12,226	14,809	3.41	1,074
24	24.2	0.49	0.10	24.53	2,059	2,430	2,499	1,694	2,443	9,159	11,217	0.70	1,371
12	15.8	0.42	0.08	24.55	4,096	2,280	2,914	1,586	1,961	9,338	13,434	0.70	2,019
6	17.4	1.85	0.40	18.15	3,002	1,728	655	1,157	4,438	7,296	10,298	0.67	1,547
4	19.1	3.38	1.00	16.37	2,857	1,580	318	1,552	3,379	6,895	9,752	0.98	1,301

HAc: acetic acid; HBu: normal butyric acid; HPr: propionic acid; HLa= Lactic acid; EtOH: ethanol; TVFAs (total volatile fatty acids) = HAc+HBu+HPr+HLa; SMP (soluble microbial products) = TVFAs+EtOH.

Table 26 Hydrogen yield (HY) values in the literature search

Feedstock	Optimal HRT	Reactor type	Inoculum starter	HY(mol H_2/mol substrate)	Reference
Sucrose	12	CSTR	Municipal sewage sludge	1.6 mol H_2/mol hexose	Lee et al., 2003
Starch	12	CSTR	Anaerobic digester sludge	0.92 mol H_2/mol glucose	Arooj et al., 2008
glucose	2.5	CSTR	*Ethanoligenens harbinense* YUAN-3	1.93 mol H_2/mol glucose	Xing et al., 2008
Wheat	15	CSTR	Anaerobically digested sludge	1.88 mol H_2/mol substrate	Hussy et al., 2003
Starch	4	CSTR	Sewage sludge	1.5 mol H_2/mol hexose	Lin et al., 2008a
Sugarcane juice	4	CSTR	*C. butyricum*	1.00 mol H_2/mol hexose	This study

The hydrogen content seemed to be directly related with the type of dominant species. In this study, *L. harbinensis* was dominant at every HRT (Figure 18, Table 25). This could be responsible for a reduction of hydrogen production efficiency resulting in low a hydrogen content at steady state at every HRT (lesser than 25%) as compared to the published report in which the hydrogen content was higher than 40% (Lin et al., 2008a; Xing et al., 2008; Lee et al., 2003). Noike et al. (2002) suggested that the heat treatment of substrate should be conducted as a pretreatment step to suppress the effect of lactic bacteria and increase the efficiency of hydrogen production.

K. pneumoniae was dominant microorganism at the HRT of 36 h and 4, while *C. butyricum* could be detected at every HRT except at a 36-h HRT (Figure 19, Table 26). When there was only the presence of *K. pneumoniae* a very low hydrogen content was observed at the HRT of 36 h (Figure 19, Table 26). At the HRT of 24 h, *C. butyricum* and *K. pneumoniae* were dominant and the hydrogen content was approximately 3.8 times greater than that of 36-h HRT (Figure 19, Table 26). At the HRT of 12 and 6 h, *C. butyricum* was dominant without the detection of *K. pneumoniae,* the hydrogen content was found to decrease slightly (1.5 times) as compared to the 24-h HRT (Table 26). When *K. pneumoniae* reappeared as a dominant species together with *C. butyricum* at the HRT of 4 h, a slightly increase in hydrogen content of 1.2 times in comparison to that at the HRT of 12 and 6 could be observed (Figure 19, Table 26). According to these results, *C. butyricum* was the major microorganisms responsible for hydrogen production, while *K. pneumoniae* also demonstrated the capability to produce hydrogen but it did not play a significant role.

In addition, it should be noted that there have some unknown species presence in the DGGE analysis results (Figure 20). These unknown species might be dark fermentative bacteria which can grow well at mesothermal condition such as the other *Clostridia* sp., *Pseudomonas* sp. and *Enterobacter* sp (Wang, Wan, 2009).

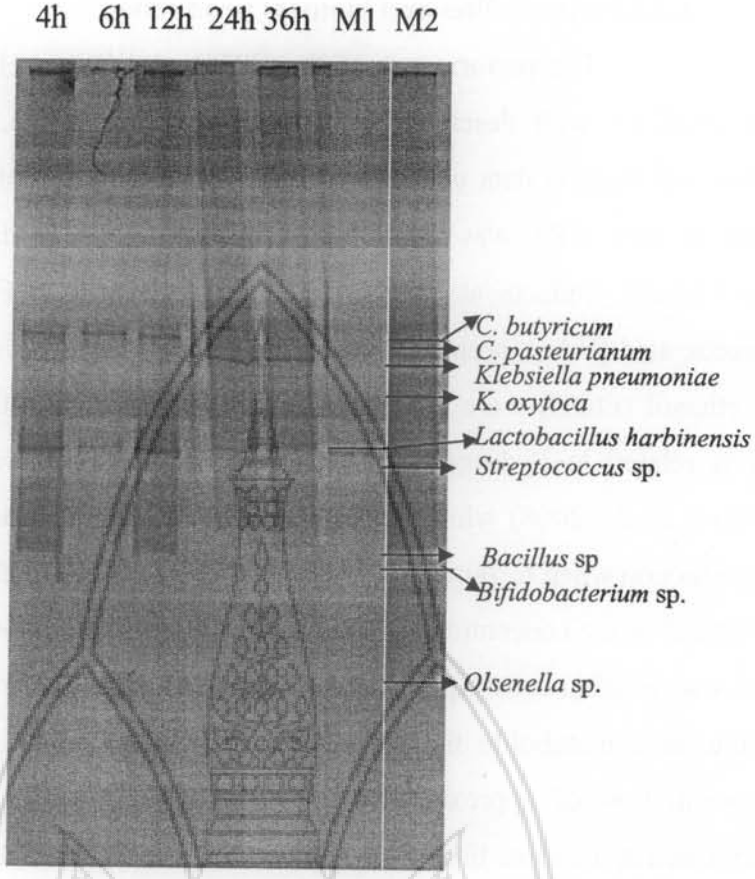

Figure 19 DGGE analysis of microbial community in hydrogen production at different HRT. M1, M2 = Markers

Table 27 Summary of DGGE analysis results for microbial community from hydrogen production system at different HRT

HRT (h)	*C. butyricum*	*K. pneumoniae*	*L. harbinensis*
36	-	+	+
24	+	+	+
12	+	-	+
6	+	-	+
4	+	+	+

4.3.2.2 Metabolites and biomass production

The performance of the CSTR at different HRT on hydrogen production could be well described by the metabolic products. The metabolite concentration and fraction data under steady-state condition in continuous hydrogen fermentation at each HRT was summarized in Table 25. The daily variation of biomass and liquid products at different HRT were shown in Figure 20. VFAs including acetic acid (HAc), propionic acid (HPr), butyric acid (HBu) and lactic acid (HLa) and ethanol (EtOH) were the main metabolites detected in the effluent. HBu production is related to hydrogen production when mixed-cultures were used as inoculum (Kim et al., 2008) while HAc, HBu and EtOH production are related to hydrogen production when *C. butyricum* was used (Saint-Amans et al., 2001). Ethanol could be detected at the concentration ranged from 2,059-4,096 mg-COD/L indicated that ethanol was simultaneously produced at the same time of hydrogen production. HBu was the main metabolite from hydrogen production at the start-up with the highest concentration of approximately 9,500 mg-COD/L at 3 days of reactor operation in which at the same time, hydrogen content peaked at 45%.

The concentration of HPr at steady state increased from 1,741 to 2,914 mg-COD/L with the increase in HRT from 36 h to 12 h, but a drastically decrease in HPr concentration to 318 mg-COD/L was observed when the HRT was decreased to 4 h (Figure 21 and Table 25). The decrease in HPr production was correlated to the increase in HY in which the maximum HY and minimum HPr could be obtained at the HRT of 4 h (Table 25). Propionate production involves consumption of both organic substrate and hydrogen which is produced according to the equation (3):

$$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3CH_2COOH + 2H_2O \quad (3)$$

A reduction in retention time could prevent the combination of organic matter and hydrogen leading to a reduction in HPr production in anaerobic hydrogen production system (Lin, Chang, 2004; Hussy et al., 2003). Hussy et al. (2003) reported that immediate reduction of HPr production was observed in continuous hydrogen fermentation from wheat starch when HRT was shortened to 12

h. Lin, Chang (2004) also reported that the HPr level decreased significantly when the HRT was shortened to 6 h.

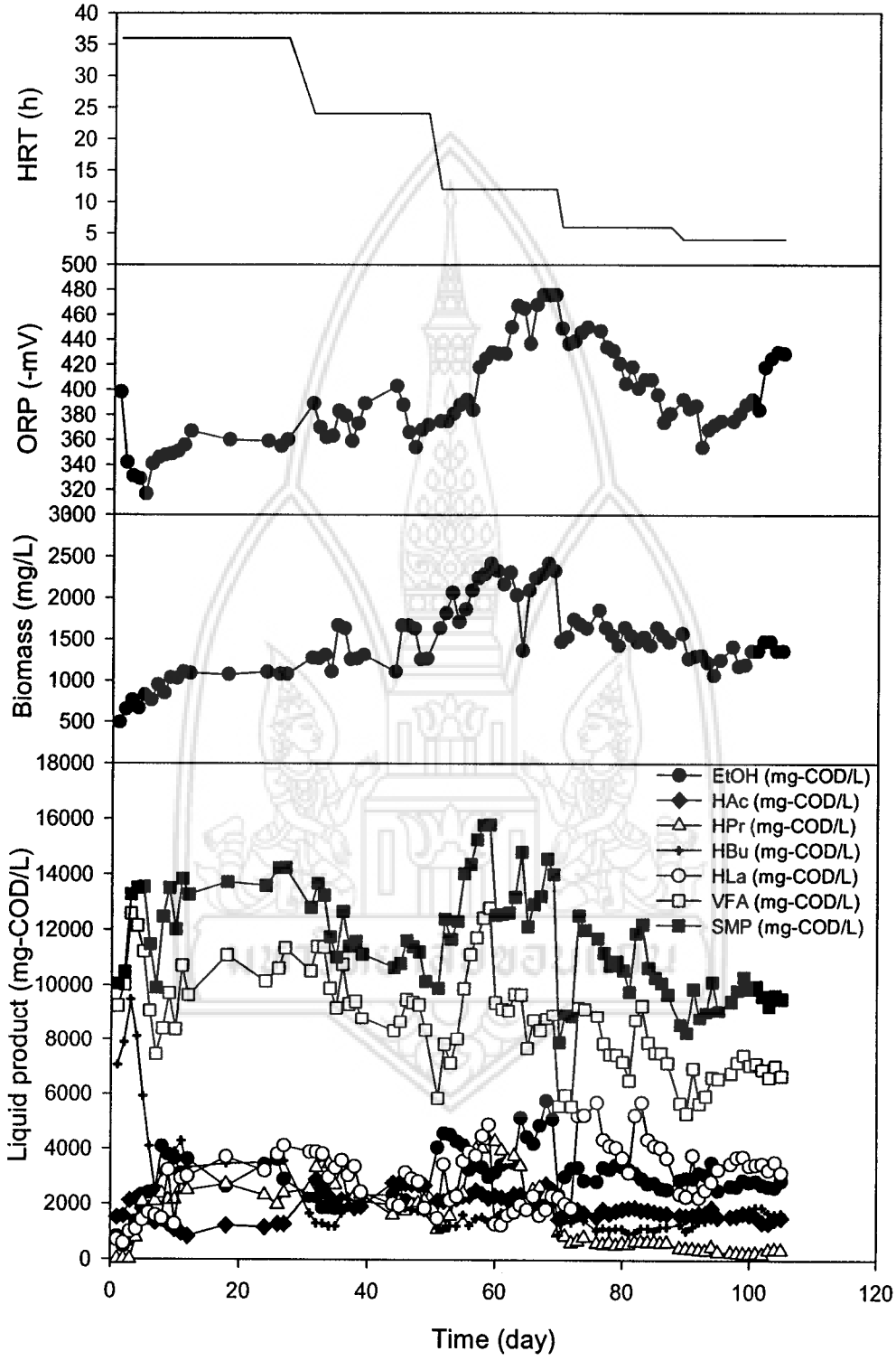

Figure 20 ORP variation, biomass and metabolic production during the CSTR operation.

A greater concentration of HLa than normally found in biohydrogen production was detected at every HRT operation (Figure 21, Table 25). This might be a result from the lactic acid producing bacteria, *L. harbinensis,* presence in the hydrogen fermentation system (Figure 20).

The production of biomass at steady state obviously increased from 1,074 to 2,019 mg VSS/L with the decrease in HRT from 36 to 12 h (Figure 21 and Table 25). This might due the fact that a shorter HRT attributed to a higher organic loading rate could provide a greater amount of substrate for cell growth. Further decrease of HRT to 6 and 4 h resulted in a decrease in biomass concentration to 1,574 and 1,301 mg VSS/L, respectively (Figure 21 and Table 25). This implied that the biomass growth in the CSTR operated at this HRT apparently cannot compete with the dilution effect due to the faster volumetric feeding rate, resulting in the washout of cells (Lin et al., 2008a). The hydrogen production at the HRT of shorter than 4 h was not operated in order to maintain the biomass concentration in the system and to avoid the worst wash-out condition. The average ORP data range was -300 to -490 mV ensured that the CSTR system was maintained under the anaerobic condition.

As the results indicated that the optimum HRT for hydrogen production from sugarcane juice using the CSTR augmented with *C. butyricum*, the hydrogenogenic effluent at the steady state of 4 h-HRT was further used as the substrate for ethanol fermentation.

4.3.2 Ethanol production from hydrogenogic effluent by carried over microorganism consortium from hydrogen fermentation

4.3.3.1 Effect of initial pH on ethanol production

The effect of an initial pH on ethanol fermentation from hydrogenogenic effluent by carried over microorganism consortia from previous hydrogen fermentation was investigated. Figure 5 depicted EtOH production, EtOH yield, specific EtOH production and biomass concentration over initial pH of hydrogenogenic effluent. Results indicated that EtOH production and EtOH yield increased from 383 to 696 mg COD/L and 0.08 to 0.17 mol EtOH/mol hexose with the increase in initial pH in the effluent from 5 to 7, respectively (Figure 5). The further increase in pH value to 8 did not markedly affect the EtOH production and

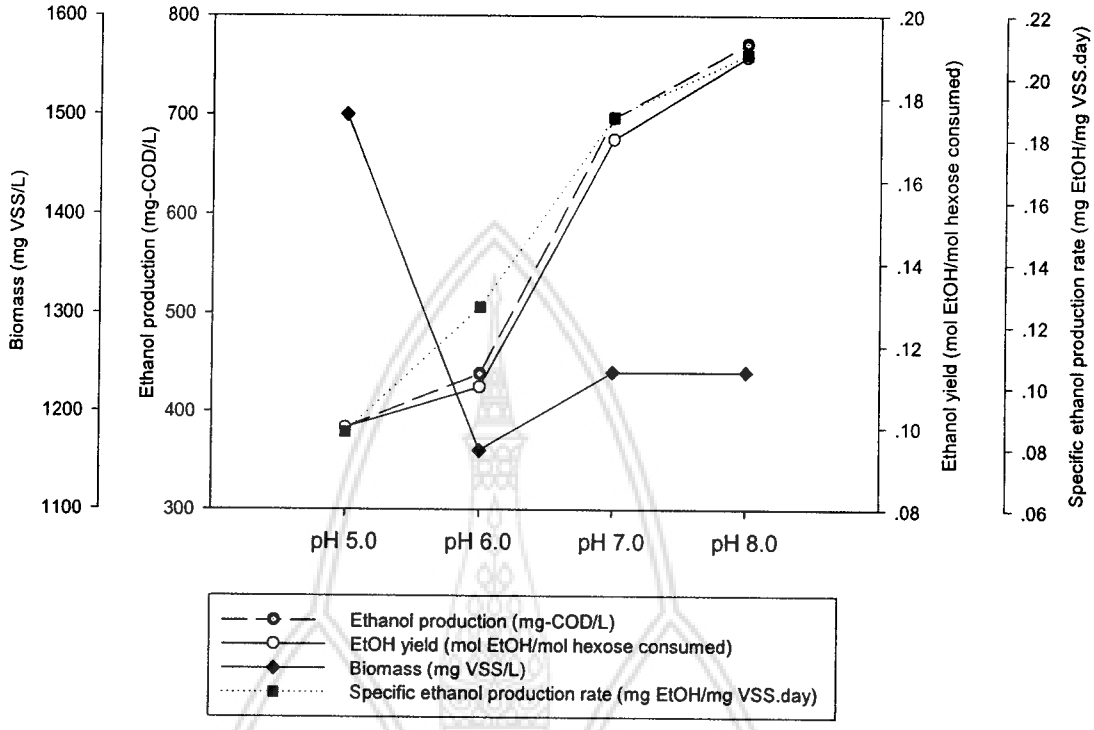

Figure 21 Effect of initial pH on ethanol production, ethanol yield, specific ethanol Production rate and biomass concentration from hydrogenogenic effluent by carried over microorganism at the end of ethanol fermentation.

EtOH yield which suggested that pH 7 was the optimum pH for ethanol fermentation from hydrogenogenic effluent. These results were consistent with the report of Lin, Hung (2008) who found that pH range of 7.0-8.0 was an optimum pH for hydrogen/ethanol production from cellulose by mixed anaerobic cultures. At the end of EtOH production, the metabolites including HAc, HPr, and HBu were produced with the concentration of approximately 1,831, 2,985 and 3,201 mg-COD/L, respectively (Table 25, 28). Valeric acid (HVa) was the additional metabolite found in this system (Table 28). The remained sugar concentration from hydrogen production was almost completely consumed to produce EtOH at every initial pH (Table 285) while both EtOH and VFAs were produced as the by products. Results indicated that the metabolic pathway in the EtOH production system from hydrogenogenic effluent did not completely enter the solventogenesis which microorganisms would utilize

VFAs for solvent production (Steinbusch et al., 2008), but they utilized sugar to produce sugar and solvent simultaneously (Wu et al., 2007). The pathway of simultaneous hydrogen and alcohol production can be found in some fermentative bacteria such as *Clostridia* and *Klebsiella* species (Wu et al., 2007; 2008).

Table 28 Metabolic products in fermentative broth from ethanol production at different initial pH

Initial pH	Final pH	Sugar consumed (g-COD/L)	mg-COD/L			
			HAc	HPr	HBu	HVa
5.0	5.22	8.34	1,944	3,664	3,044	1,031
6.0	6.30	8.52	1,527	2,634	3,187	1,801
7.0	6.58	8.52	1,894	2,916	3,404	1,676
8.0	6.62	8.50	1,958	2,724	3,170	1,627

4.3.3.2 Effect of initial sucrose concentration on ethanol production

The effect of sucrose concentrations on ethanol production from hydrogenogenic effluent was studied at the fixed pH value of 7. Sugarcane syrup was used to adjust the concentration of sugar in hydrogenogenic effluent to 25-300 g-COD/L. Figure 6 depicted the effect of initial sucrose concentration on EtOH production, EtOH yield, specific EtOH production and biomass. Results demonstrated that when the sucrose concentration was increased from 8.63 to 25 g-COD/L, EtOH production and EtOH yield increased markedly from 696 to 1418 mg-COD/L and 0.17 to 0.31 mol EtOH/mol hexose, respectively (Figure 22). HAc and HPr concentrations decreased while HBu and HVa increased with the increase in EtOH production (Table 29). Further increase in sugar concentration to 50 and 100 mg-COD/L markedly reduced EtOH production to 995 mg-COD/L and the EtOH yield was obviously decreased to 0.16 and 0.03 mol EtOH/mol hexose, respectively, (Table 6). In addition, the biomass concentration was found to be greater than 2,000 mg VSS/L. These results implied that the reduction of EtOH yield was not the result of substrate inhibition. It can be seen that a concentration of HBu increased approximately 5.8 and 8.3 times at the initial sugar concentration of 50 and 100 g-COD/L, respectively, in comparison to the initial sucrose concentration of 25 g-

COD/L (Table 29). A shift of metabolic pathway from alcohol production to butyrate-type hydrogen fermentation can explain the phenomena which was confirmed by the hydrogen production (HP) values that increase from 68 to 166 mL H_2/L substrate when the sugar concentration was increased from 25 to 100 g-COD/L (data not shown).

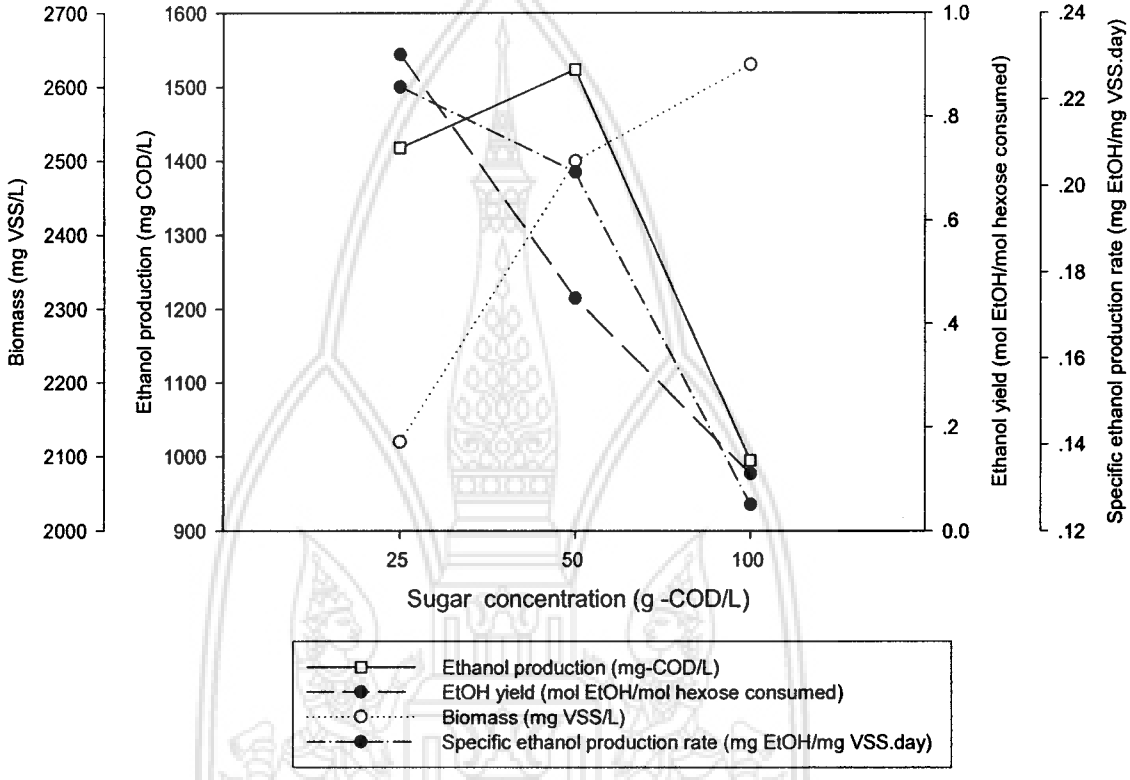

Figure 22 Effect of initial sucrose concentration on ethanol production, ethanol yield, specific ethanol production and biomass from hydrogenogenic effluent by carried over microorganisms at the end of ethanol fermentation.

When sugar concentration was increased to 200 and 300 g-COD/L, the fermentation process could not be observed indicating the occurrence of a substrate inhibition. An inhibitory effect of high substrate concentration on growth of microorganisms might result in a decrease in the efficiency of ethanol production. The study of Crabbendam et al. (1985) found that the growth of *C. butyricum* could be worsen at the input glucose concentration above 40 g/L and could be completely

inhibited at the glucose concentration above 55 g/L with concomitant to the accumulation of residual (unused) glucose in the culture medium.

The maximum ethanol yield of 0.31 mol EtOH/mol hexose consumed could be obtained at the optimum condition which is in the average range of previous reports (Table 30). Therefore, the optimum condition for EtOH fermentation from hydrogenogenic effluent by carried over microorganisms was at pH 7 and initial sucrose concentration of 25 g-COD/L.

Table 29 Metabolic products in the fermentative broth of ethanol production at different initial sugar concentrations

Initial sucrose concentration (g-COD/L)	Sugar consumed (g-COD/L)	Final pH	mg-COD/L			
			HAc	HPr	HBu	HVa
25	9.50	4.41	5,821	8,746	1,120	328
50	19.89	4.39	5,431	7,040	6,533	1,608
100	75.43	4.38	5,508	4,266	9,307	4,211
200	NF	NF	NF	NF	NF	NF
300	NF	NF	NF	NF	NF	NF

NF=Non Fermentation

Table 30 Comparison of ethanol production by various types of microorganism with sugar-type substrate.

Microorganism	Substrate	Fermentation process	Optimal production value	Reference
Klebsiella sp. HE1	Sucrose (10 g-COD/L)	Batch	0.41 mol ethanol/mol hexose	Wu et al. (2008)
Anaerobic sludge	Sucrose (20 g-COD/L)	Continuous	0.49 mol ethanol/mol hexose	Wu et al. (2007)
Anaerobic sludge	Glucos (20 g-COD/L)	Continuous	0.53 mol ethanol/mol hexose	Wu et al. (2007)
Carried over microorganisms consortium	Sugarcane juice (25 g-COD/L)	Batch	0.31 mol ethanol/mol hexose	This study

The microbial community responsible for ethanol production as the results of variation in initial pH and sucrose concentration is depicted in Figure 24. The results indicated that *C. butyricum* was dominant in all treatments (Figure 247) and responsible for every metabolic pathways in this experiment. The unknown band in DGGE result (Figure 24) indicated that there might be some other microorganisms that work together with *C. butyricum* on ethanol fermentation.

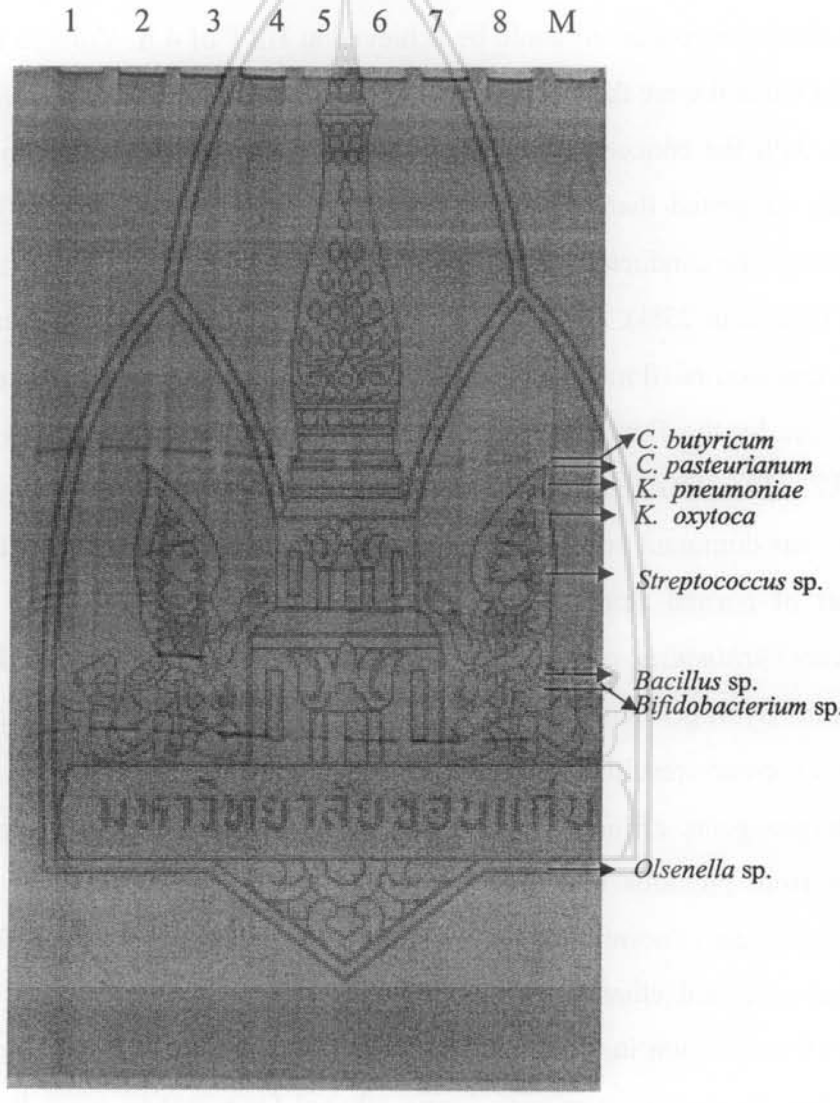

Figure 23 DGGE analysis for microbial community responsible for ethanol production at different initial pH; 1=pH 5, 2= pH 6, 3=pH 7 and 4= pH 8 and at different sucrose concentration; 5 = 25 g-COD/L, 6= 50 g-COD/L, 7 = 100 g-COD/L and M = Marker.

4.4 Conclusion

The research aims to explore the possibility of using sugarcane juice to produce bio-hydrogen and subsequently ethanol by *Clostridium butyricum* under non-sterile condition. Bio-augmentation of *C. butyricum* for continuous hydrogen production from sugarcane juice in the Continuous Stirred Tank Reactor (CSTR) at various hydraulic retention time (HRT) was examined. Results indicated that the maximum hydrogen production rate and yield of 3.38 mmol H_2/L substrate.h and 1.00 mol H_2/mol hexose consumed could be achieved at HRT of 4 h. Volatile Fatty Acids (VFAs) and ethanol were the main metabolites detected in the effluent from hydrogen production with the concentrations of 6,895 and 2,857 mg-COD/L at the optimum HRT which suggested that hydrogen production under non-sterile condition by *C. butyricum* could be conducted. However, the hydrogen content obtained was observed to be low (less than 25%). The relationship of the augmented microorganism i.e. *C. butyricum* and normal flora in the fermentation system under non-sterile condition were analyzed by the Denaturing Gradient Gel Electrophoresis (DGGE) method at every HRT. The DGGE results revealed that augmented microorganism, *C. butyricum*, was dominant and played an important role on hydrogen production with the support of normal flora, *Klebsiella pneumoniae*, under non-sterile condition. *Lactobacillus harbinensis*, one of the dominant normal flora, was observed to be able to grow in the hydrogen fermentation system but played a role in reducing hydrogen production efficiency resulting in a low hydrogen content obtained. The possibility of using hydrogenogenic effluent to produce ethanol by carried over microorganisms consortium from previous hydrogen fermentation was further explored at various initial pH and sugar concentration. The highest ethanol yield of 0.31 mol EtOH/ mol hexose consumed and ethanol production of 1,418 mg-COD/L, respectively, were achieved at the optimum initial sugar concentration of 25 g-COD/L and pH of 7. *C. butyricum* was the dominant species during ethanol fermentation of hydrogenogenic effluent at every pH values and sugar concentrations.

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