CHAPTER III
BIO-HYDROGEN PRODUCTION FROM SUGARCANE JUICE BY IMMOBILIZED *Clostridium butyricum* ON SUGARCANE BAGASSE

3.1 Introduction

As a sustainable energy source, hydrogen is a promising alternative to fossil fuels. It is a clean and environmental friendly fuel, which produces water instead of greenhouse gases after combusted. 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 environmental friendly and less energy intensive compared to thermo-chemical and electro-chemical processes (Kapdan, Kargi, 2006). Fermentative hydrogen production can be generated by various types of microorganisms. *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 which is higher than that of the other fermentative bacteria (Yokoi et al., 1998; Chen et al., 2005; Lin et al., 2004).

Sugarcane is one of the important industrial crops in Thailand. It can be cultivated in all parts of Thailand, except in the Southern of Thailand, with the cultivation area of more than 960,000 ha. Approximately 48 million tons of sugarcane is produced per year (Office of the Cane and Sugar Board, 2006). Sugarcane juice is mainly used to produce sugar. However, from the report of Office of the Cane and Sugar Board (Thailand), sugar production from sugarcane every year is greater than sugar consumption (Office of the Cane and Sugar Board, 2006). Therefore, this research was designed to investigate an alternative way to value-added sugarcane by producing a clean and renewable energy i.e. hydrogen. The main sugar found in sugarcane juice is sucrose with approximately concentration of 200 g/L. Sucrose has been reported as a substrate for producing hydrogen by various types of microorganisms such as *C. butyricum* CGS5 (Chen et al., 2005) and *C. pasteurianum* (Lin et al., 2004) with the yield of 2.78 and 4.8 mol hydrogen/mol sucrose,

respectively. Therefore, sugarcane juice has a potential to be used as substrate for hydrogen production.

In the present study, a bio-hydrogen production from sugarcane juice using the immobilized *C. butyricum* on sugarcane bagasse was investigated in batch system with an attempt to optimize environmental conditions for hydrogen production. The two most important environmental factors affecting hydrogen production including initial pH and initial total sugar concentration were investigated. The optimum conditions obtained from batch system were further used in repeated batch hydrogen fermentation with the varying end-of-the batch-harvested volume. The hydrogen produced from repeated batch fermentation at the optimum end-of-the batch-harvested volume was purified and combined to the fuel cell system to examine the possibility for electricity generation.

3.2 Materials and Methods

3.2.1 Sugarcane juice 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 by squeezer and filtrating through a cheesecloth and kept at -20 ^{O}C. Frozen sugarcane juice was thawed by placing at room temperature prior the usage. Compositions of the sugarcane juice were presented in Table 19.

Table 19 Compositions of sugarcane juice

Composition	Concentration (g/L)
Fructose	3.2
Glucose	3.4
Sucrose	198.9
Total Sugar	209.48
Total soluble solid (Brix)	19.6
pH	5.07

3.2.2 Inocula

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 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/ 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 of the cell culture, 10^6 cells/mL, into 60 mL fresh TSY medium and incubated at the given conditions before using as inoculum (Sneath et al., 1986). Each liter of TSY contains 5.0 g of tryptone; 3.0 g of sucrose; 5.0 g of yeast extract; 1.0 g of K_2HPO_4 (Holdman et al., 1977).

3.2.3 Support material

Sugarcane bagasse was used as a support material for cell immobilization. It was cut by knife into small pieces (approximately 0.5 X 0.5 X 0.5 cm) and passed through 0.5-1 cm sieve. After that, they were boiled in 1% NaOH for 3 h with a mass ratio of solid (g dry weight) to liquid (mL) at 1:24 to remove lignin which might be toxic to microorganisms and then thoroughly washed with tap water 3 times and soaked in distilled water overnight (Iconomou et al., 1995). The delignified sugarcane bagasse was kept at -20 ^{O}C until the usage.

3.2.4 Immobilization of *C. butyricum* on sugarcane bagasse

Seven grams dry weight of delignified sugarcane bagasse was added into 63 mL TSY medium in serum bottle capped with rubber stopper and aluminum cap and then autoclaved at 121 ^{O}C for 15 min before inoculating with 10% (v/v) of *C. butyricum* (final cell density of 10^6 cell/mL). The bottle was flushed with argon to create the anaerobic condition and incubated at 37°C for 10 h at 150 rpm on the orbital shaker. After the incubation, the culture medium was drained and the immobilized cell was washed three times by sterile 0.85% NaCl. The final cell number in support materials were approximately 10^7 cells/g dry wt of support materials. The photographs of sugarcane bagasse and immobilized cell were determined by scanning electron microscopy (SEM, JSM-5410LV, JEOL, Japan) (Rachman et al., 1998).

3.2.5 Experimental procedure

The batch experiments for hydrogen production by free and immobilized *C. butyricum* were conducted with the varying initial pH and sucrose concentration in the substrate. A 100-mL serum bottle with a working volume of 70 mL comprised of 60 mL of sugarcane juice, 7 mL of *C. butyricum* (10^6 cells/mL) for free cell or 7 g dry wt of immobilized cell (10^7 cell/g dry wt of support materials), 1.5 mL of 3.75% (w/v) L-cysteine as a reducing agent and 1.5 mL of nutrient stock solution. After replacement of the gas phase with argon to create the anaerobic condition, the serum bottle was incubated at 37°C and 150 rpm for 24 h on the orbital shaker. All treatments were carried out in duplicates. Nutrient solution contains (in g/L) NH_4HCO_3, 80; KH_2PO_4, 1.24; $MgSO_4.7H_2O$, 0.1; NaCl, 0.01; $Na_2MoO_4.2H_2O$ 0.01; $CaCl_2.2H_2O$, 0.01; $MnSO_4.7H_2O$, 0.015; and $FeCl_2$, 0.0278 (Lay et al., 1999). The effect of the initial pH was conducted at the initial sucrose in culture medium of 20 g-COD/L with the varying initial pH in the range of 4.5-7.0 in order to obtain the optimum pH for hydrogen production. The effect of initial sucrose concentration as substrate in the range 20-40 g-COD/L was further investigated at the optimum pH in order to determine the optimum initial sucrose concentration for hydrogen production. During incubation, the volume of biogas was measured by plunger displacement method (Owen et al., 1978), the content of biogas and volatile fatty acids (VFAs) concentrations in culture medium were determined by GC and sugar concentration in culture medium was analyzed by HPLC.

3.2.6 Reusability of the immobilized cell

At the end of the batch experiment, the culture medium in the serum bottle was replaced by 63 mL fresh medium (60 mL of sugarcane juice, 1.5 mL of 3.75% (w/v) L-cysteine as a reducing agent and 1.5 mL of nutrient stock solution) with the optimum initial pH and sucrose concentration, and incubated at a given condition for 24 h. The volume and content of biogas and VFAs and sucrose concentration in culture medium were determined during incubation. This process was repeated 5 times to determine the reusability of the immobilized *C. butyricum* for hydrogen production.

3.2.7 Repeated-batch operation

The repeated-batch experiment was conducted in 2 L glass bioreactor (Biostat B, B.Braun Biotech International, Germany) with a working volume of 1.5 L at the optimum conditions obtained from the batch experiment. The immobilization was conducted by adding 150 g dry wt of delignified sugarcane bagasse into 1.5 L TSY medium in the reactor and then autoclaved at 121 ^{O}C for 15 min. After the sterilization, the reactor was inoculated with 10% (v/v) of *C. butyricum* (10^6 cell/mL). The reactor was flushed with argon through a 0.2 μm cellulose acetate air filter to create the anaerobic condition. The reactor was operated at a control temperature of 37 ^{O}C using a temperature controller and a stirring speed of 50 rpm. A pH of 6.5 was maintained using the pH controller and 1N NaOH or 1N HCl. After the incubation time of 24 h, the culture medium was drained and the immobilized cell was washed three times by sterile 0.85% NaCl. The final cell number in support materials were approximately 10^7 cell/g dry wt of support materials. The immobilized cells were used as the inoculums.

The repeated batch experiment was firstly operated as a batch fermentation by adding 1.5 L of substrate (1,440 mL of sugarcane juice, 30 mL of 3.75% (w/v) L-cysteine as a reducing agent and 30 mL of nutrient stock solution) into the bioreactor containing the immobilized *C. butyricum* and operating condition was conducted as abovementioned. The end-of-the batch harvested volumes were varied at 25%, 50% and 75%. At each the end-of-the batch harvested volume, the repeated batch fermentation was conducted by replacement of culture broth in the bioreactor with substrate at designated the end-of-the batch harvested volume every 24 h of reactor operation. The biogas production and liquid samples were investigated every 6 h until the cumulative hydrogen production and substrate consumed was stable.

3.2.8 Electricity generation by a biohydrogen- fuel cell system

The hydrogen producing system with the optimum end-of-the batch harvested volume was connected to a fuel cell unit (Item 1919, h-tec, Germany) (Figure 9) after the constant hydrogen production between batches was achieved. The produced biogas from the repeated batch system was purified by three bottles in series of 4N NaOH before connected to fuel cell. The fuel cell specifications were proton exchange membrane type, electrode area; 16 cm^2, power; 600 mW H_2/O_2, 200 mW

(H_2/air) and generated voltage (short-cicrcuit-proof); 0.40-0.96 V DC. The condition for electricity generation was examined using 10 Ω load resistors connected with the fuel cell. Electric power (voltage) was measured by a digital multi-meter (Kewtech-KT200, Kyoritsu, Japan). Generated electricity was applied to rotate a DC motor fan (power consumption; 20mW, Item 1914, h-tec, Germany).

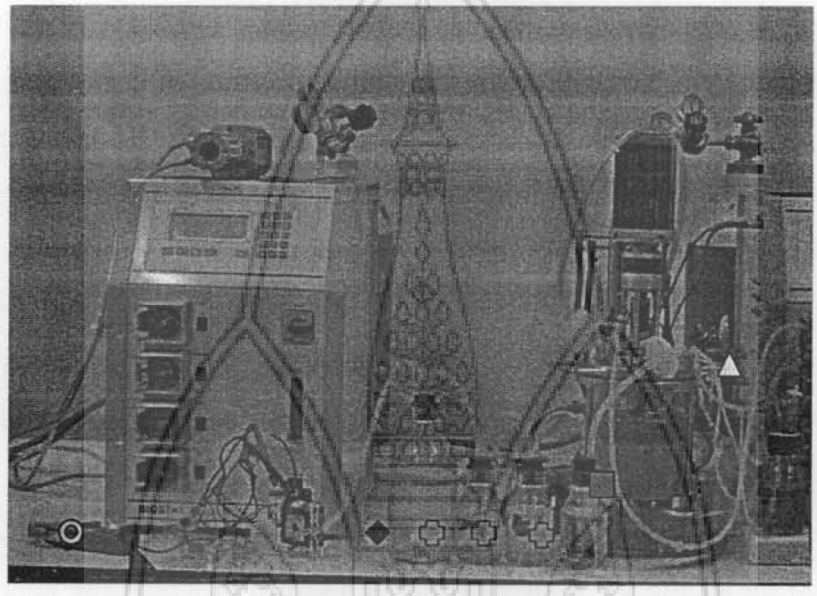

Figure 9 Photograph of biohydrogen producing system connected to fuel cell unit: △ = Fermenter controller, ■ = Bio-reactor for fermentative hydrogen production, ✤ = CO_2 absorber, ◆ = Hydrogen storage, ● = fuel cell, ◣ = Fan and ○ = Digital multi-meter.

3.2.9 Analytical method

3.2.9.1 Determination of cell concentration

Free cell concentration was defined as the amount (dry weight) of cells per mL of culture broth. It was measured by centrifuging five mL of culture broth at 12,000 rpm for 15 min. Cell pellets were washed twice with distilled water, dried to constant weight at 105 ^{o}C, before final weighing. The amount of immobilized cell was conducted by measuring the difference in dry weights between the biomass-associated matrix and the matrix alone (Chang et al., 2002).

3.2.9.2 Sugar analysis by HPLC

Sugarcane juice was filtered through a 0.45 μm cellulose acetate membrane and analyzed by HPLC for glucose, fructose and sucrose. The HPLC analysis was carried out using a LC-10AD (Shimadzu, Japan) with an Pinnacle II Amino column (oven temperature 40 ^{O}C), 80% acetonitrile as mobile phase at the flow rate of 1.0 mL/min and a refraction index detector (RID). Total sugar concentration in the sugarcane juice is a summation of glucose, fructose and sucrose concentrations obtained from HPLC analysis.

3.2.9.3 Biogas analysis by GC

The component of biogas in the headspace including hydrogen, nitrogen, methane and carbon dioxide was determined using a gas chromatography (GC, Shimadzu 2014, Japan) equipped with a thermal conductivity detector (TCD) and 2 m x 2.5 mm diameter stainless-steel column packed with Unibead C (60/80 mesh) (GL Science Inc., Japan). The temperatures of injector port, detector and column oven were 150, 145 and 150 °C, respectively. Argon was used as the carrier gas at a flow rate of 25 mL/min. The concentrations of Volatile Fatty Acids (VFAs) including acetic (HAc), propionic (HPr), normal butyric (HBu) acids and solvents including ethanol (EtOH) and butanol (BuOH) were determined using GC (Shimadzu 17A, Japan) equipped with a flame ionization detector (FID) and a 3 m x 3.2 mm diameter glass column packed with Unisole F-200 (30/60 mesh) (GL Science Inc., Japan). The operational temperatures of the injector port, detector and column oven were 150, 140 and 150 °C, respectively. Nitrogen, hydrogen and pressured air were used as carrier gases with flow rate of 50, 60 and 500 mL/min, respectively.

3.2.9.4 Kinetic analysis

Volume of biogas produced was calculated by a mass balance equation previously described by Zheng, Yu (2005). The cumulative hydrogen production followed the modified Gompert equation (Equation 1) (Zwietering et al., 1990)

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

Where H is the cumulative volume of hydrogen produced (L), P is the hydrogen production potential (L), Rm is the maximum hydrogen production rate (L

H_2/h), λ is the lag phase time (h), t is the incubation time (h) and e is the exp (1) = 2.718. The hydrogen production ability of the biomass in the reactor was calculated as a specific hydrogen production rate (SHPR) (mmol H_2/g cell.day) obtained by dividing R_m by the cell dry weight (g cell). Hydrogen production (HP, L H_2/L substrate) and hydrogen production rate (HPR, L H_2/L substrate.day) were calculated by dividing the hydrogen production potential (P) and maximum hydrogen production rate (Rm), respectively, obtained from the modified Gompertz equation by the volume of substrate (0.07 L) which was converted to the unit of liter before taking into the calculation.

3.3 Results and Discussion

3.3.1 Effect of initial pH on hydrogen and VFAs production from sugarcane juice by free and immobilized *C. butyricum*

The effect of initial pH of sugarcane juice on hydrogen production by free and immobilized cell of *C. butyricum* was investigated. Results indicated that the initial pH values significantly affected the hydrogen production by free and immobilized cell in which the highest volume of hydrogen was obtained at the initial pH of 6.5 while the initial pH of 4.5 gave the lowest volume of hydrogen (Table 20). Increase in the initial pH led to an increase in HP and hydrogen yield (HY) (Table 20). For hydrogen fermentation by free cell, the results indicated that an initial pH of 6.5 gave the highest HP and HY of approximately 2.96 L H_2/L substrate and 2.66 mol H_2/mol sucrose consumed, respectively (Table 20).

The effect of initial pH of sugarcane juice on HP and HY by the immobilized *C. butyricum* were similar to those obtained from free cell. The highest HP and HY of approximately 2.84 L H_2/L substrate and 2.67 mol H_2/mol sucrose consumed were achieved at the initial pH of 6.5 the initial sucrose concentration of 25 g-COD /L (Table 20). Greater HPR and SHPR values could be obtained from the immobilized cell, which suggested the immobilized cell was more efficient in producing hydrogen than free cell. The immobilization was reported to lead a high productivity rate and it is the best choice for feasibility of continuous processing

Table 20 Hydrogen production performance from sugarcane juice by free and immobilized cells of *C. butyricum* under different initial pH and initial sucrose concentration of sugarcane juice

Types	Conditions		Final pH	H_2 content (%)	HP (L H_2/L substrate)	HPR (L H_2/L substrate.day)	HY (mol H_2/mol sucrose consumed)	SHPR (mmol H_2/g dry cell. day)
	Initial pH	Initial sucrose concentration(g-COD/L)						
Free cell of *C. butyricum*	4.5	25	3.63	44	1.19	1.41	1.50	112.67
	5.0		4.10	44	1.69	1.43	1.97	87.38
	5.5		4.13	44	2.23	1.68	2.03	106.44
	6.0		4.33	45	2.56	2.95	2.28	280.41
	6.5		4.23	45	2.96	3.00	2.66	283.49
	7.0		4.41	45	2.26	2.33	2.42	157.84
	6.5	20	4.21	45	2.44	2.15	2.36	198.47
		25	4.23	45	2.96	3.00	2.66	283.49
		30	4.17	44	2.67	1.98	2.22	194.46
		35	4.06	44	2.64	1.82	2.09	172.68
		40	4.00	44	2.59	1.79	1.84	170.41
Immobilized *C. butyricum* on sugarcane bagasse	4.5	25	3.58	44	1.25	2.06	1.59	169.24
	5.0		4.09	44	1.61	2.16	2.05	132.93
	5.5		4.18	44	1.78	2.49	2.26	162.55
	6.0		4.27	45	1.96	1.60	2.50	153.56
	6.5		4.13	45	2.84	3.11	2.67	438.16
	7.0		4.38	44	2.15	2.72	2.32	298.22
	6.5	20	4.11	45	2.47	2.42	2.19	228.03
		25	4.13	45	2.84	3.11	2.67	438.16
		30	4.03	44	2.51	2.22	1.64	214.06
		35	3.98	45	2.56	2.26	1.65	223.12
		40	3.86	45	2.52	3.06	1.61	302.04

HP: Hydrogen production (L H_2/L substrate); HPR: Hydrogen production rate (L H_2/L substrate.day); HY: Hydrogen yield (mol H_2/mol sucrose); SHPR: specific hydrogen production rate (mmol/g dry cell.day)

(Kourkputas et al., 2004). This is because the immobilized-cell system has a feature of creating a local anaerobic environment, which is well suited to oxygen-sensitive fermentative hydrogen production (Wu et al., 2005; Chang et al., 2001).

At a weak acid condition (initial pH of 5.5-6.5), *Clostridia* species can be activated to extrude the express proton from cytoplasm to facilitate the resumption of the cell growth (Ferchichi et al., 2005) as well as producing hydrogen which normally exhibit at the exponential growth phase of this species. Our results were consistent with the report of Jo et al. (2008) who found that pH of 6.3 was the optimum pH for hydrogen production from glucose by *C. tyrobutyricum* JM1. In addition, the pH range of 5.5-6.5 was reported as the optimal pH range for the optimal hydrogen evolution efficiency of anaerobic fermentative bacteria without the solventogenic phase (Valdez-Vazquez, Poggi-Varaldo, 2009).

The lowest hydrogen production from sugarcane juice was observed at the initial pH of 4.5 (Table 20). The results implied that the adjustment of the initial pH in fermentative broth by adding an acid to obtain the pH of 4.5 in substrate might result in a protonation of undissociate weak acids in the medium which can pass through the cell membrane into cytoplasms. This can cause the loss of glycolytic enzyme and structural damaging of cell membrane, DNA and protein which could slow down the growth of the microorganisms (Ferchichi et al., 2005). Our results indicated that the pH control has a significant impact on hydrogen production. Therefore too high or too low pH can result in a low hydrogen production in which the activity of hydrogenase could be inhibited (Kim et al., 2004).

The low sucrose degradation efficiency of approximately 50% could be observed in all batch experiments (Table 21). This might due to the fact that hydrogen production was accompanied by VFAs production coupled with solvent production. The VFAs produced led to the decrease in pH of the fermentation broth to be less than 4.5 (Table 20) which could inhibit the growth and metabolism of the cell as described above. Our results were similar to the report of Wang et al. (2008) who found that substrate degradation efficiency trended to decrease with increasing ethanol, acetic, propionic and butyric acid concentrations of more than 600 mg/L during fermentative hydrogen production from glucose by mix cultures.

Table 21 Soluble metabolites distribution by free and immobilized cells of *C. butyricum* at each initial pH and initial sucrose concentration in sugarcane juice

Type	Condition		Sucrose concentration (g-COD/L)		Soluble metabolites (mg-COD/L)							HBu/ TVFAs (%)	HBu/ HAc ratio
	Initial pH	Initial sucrose concentration (g-COD/L)	Remaining	Consumed	HAc	HPr	HBu	EtOH	BuOH	TVFAs	SMP		
Free cell of *C. butyricum*	4.5		13.48±0.50	11.52±0.54	418±0.01	68±0.02	777±0.06	1,783±0.15	1,912±0.20	1,263±0.09	4,957±0.26	62	1.09
	5.0		12.71±1.16	12.29±1.10	419±0.00	50±0.01	1,438±0.21	1,577±0.03	1,571±0.09	1,906±0.22	5,054±0.16	75	2.01
	5.5		8.87±1.55	16.13±1.63	726±0.08	49±0.02	3,178±0.34	1,611±0.04	836±0.18	3,954±0.28	6,401±0.51	80	2.60
	6.0	25	8.87±1.01	16.13±1.10	780±0.07	55±0.01	3,704±0.13	1,333±0.15	485±0.04	4,539±0.05	6,357±0.13	82	2.80
	6.5		9.06±0.89	15.94±0.81	845±0.08	78±0.00	4,592±0.10	296±0.00	511±0.01	5,514±0.02	6,321±0.01	83	3.21
	7.0		11.75±1.82	13.25±1.90	668±0.12	04±0.02	3,647±0.26	273±0.02	470±0.22	4,419±0.16	5,163±0.39	82	3.27
		20	5.22±0.50	14.78±0.81	791±0.09	204±0.04	4,174±0.13	310±0.01	343±0.02	5,169±0.26	5,822±0.28	81	3.11
		25	9.06±0.89	15.94±0.81	845±0.08	78±0.00	4,592±0.10	296±0.00	511±0.01	5,514±0.02	6,321±0.01	83	3.21
	6.5	30	12.33±1.95	17.66±2.17	876±0.09	248±0.01	4,559±0.46	447±0.11	594±0.13	5,684±0.54	6,724±0.77	80	3.05
		35	16.76±1.27	18.24±1.36	897±0.06	337.0.06	4,572±0.14	693±0.11	630±0.18	5,806±0.03	7,130±0.26	79	2.99
		40	19.85±1.23	20.16±1.36	962±0.13	440±0.02	4,571±0.15	846±0.10	1,093±0.14	5,973±0.00	7,913±0.24	77	2.82
Immobilized *C. butyricum* on sugarcane bagasse	4.5		13.67±1.82	11.31±1.90	488±0.03	92±0.01	840±0.21	1,500±0.45	1,888±0.15	1,421±0.20	4,809±0.79	59	1.02
	5.0		13.48±2.25	11.52±2.17	445±0.06	36±0.00	1,303±0.39	1,412±0.01	1,726±0.37	1,785±0.44	4,922±0.82	73	1.70
	5.5		13.48±0.47	11.52±0.54	546±0.07	35±0.00	2,759±0.16	534±0.07	791±0.25	3,342±0.08	4,667±0.39	83	3.00
	6.0	25	13.67±1.82	11.33±1.90	577±0.15	61±0.32	2,956±0.37	317±0.05	538±0.05	3,593±0.55	4,448±0.64	82	3.06
	6.5		9.83±0.35	15.17±0.27	667±0.08	42±0.01	4,520±0.16	341±0.07	445±0.01	5,229±0.24	6,015±0.31	86	3.99
	7.0		11.75±1.28	13.25±1.36	578±0.01	84±0.04	3,644±0.53	344±0.06	469±0.06	4,307±0.58	5,120±0.59	85	3.67
		20	3.68±1.72	16.32±1.36	952±0.14	239±0.01	4,448±0.26	355±0.06	435±0.11	5,639±0.41	6,429±1.11	79	2.76
		25	9.83±0.35	15.17±0.27	667±0.08	42±0.01	4,520±0.16	341±0.07	445±0.01	5,229±0.24	6,015±0.31	86	3.99
	6.5	30	12.53±1.14	17.47±1.36	1,292±0.22	225±0.05	4,456±0.31	386±0.02	509±0.01	5,974±0.49	6,868±0.52	74	2.04
		35	16.19±0.46	18.82±0.54	1,316±0.07	372±0.01	4,424±0.16	739±0.04	682±0.11	6,113±0.10	7,534±0.25	72	1.98
		40	19.46±0.69	20.54±0.81	1,322±0.01	516±0.12	4,371±0.14	907±0.01	1,222±0.02	6,209±0.26	8,338±0.29	70	1.94

Total sucrose concentration (g/L) = sucrose (g/L) +0.95(Glucose (g/L) + Fructose (g/L)).

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

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Anaerobic hydrogen production is always accompanied with VFAs production. Production of soluble products by free and immobilized cells including HBu, HAc, ethanol and butanol during hydrogen fermentation was summarized in Table 21. The main metabolite in the fermentative broth was HBu in which its highest concentrations in free and immobilized cells experiment of 83% and 86%, respectively, (Table 21) were found in fermentative broth with the initial pH of 6.5. Results suggested that hydrogen production by *C. butyricum* was butyrate-type fermentation as described in Equation (2) (Khanal et al., 2004).

$$C_{12}H_{22}O_{11} + H_2O \longrightarrow 2CH_3CH_2CH_2COOH + 4CO_2 + 4H_2 \qquad (2)$$

Ethanol and butanol could be observed in the fermented broth at the end of incubation (Table 21). This result indicated the occurrence of solvent phase which could take place when VFAs were accumulated in the hydrogen production system of *Clostridia* species (Lin, Lay, 2004). A drop of pH to approximately 4 (Table 21) can inhibit the growth of microorganisms. In order to be able to survive at a low pH, the new enzyme system of *Clostridia* species would be activated leading to the formation of solvents such as the ethanol and butanol in metabolic pathway (Lenz, Moreira, 1980).

3.3.2 Effect of initial sucrose concentration

Hydrogen and carbon dioxide were the main biogas products in fermentative broth throughout the experiment (Table 20). There was no methane produced. Cumulative hydrogen volume increased with an increase in concentration of sucrose (Table 20). Effect of an initial sucrose concentration on HP and HY of the immobilized *C butyricum* were similar to those obtained from free cell (Table 2). The highest HP and HY of free and the immobilized cells were obtained at the initial sucrose concentration of 25 g-COD/L (Table 20). A slightly decreased in HP and HY when the initial sucrose concentration was increased to be greater than 25 g-COD/L might be a result from a substrate inhibition. An inhibitory effect of high substrate concentration generally occurs in the fermentation process depending on types of substrates and microorganisms. Chen et al. (2005) reported that hydrogen production from sucrose by *C. butyricum* CGS5 showed the best performance at the initial

sucrose concentration of 20-g COD/L, while the fermentation process was inhibited at the initial sucrose of 30-g COD/L.

Our results indicated that substrate concentration apparently influenced the hydrogen production. Increase in substrate concentration could increase in hydrogen production up to a certain level. However, an excessive substrate concentration can cause a build-up of VFAs in the system leading to a decline of pH in the reactor and could inhibit the growth of hydrogen producer (Wu, Chang, 2007). Moreover, an increase in substrate concentration could lead to a partial pressure in the fermentation system. When partial pressure was accumulated in a headspace of reactor enough to a certain level, the hydrogen production will be switched to solvent production, thus inhibit the hydrogen production (Fan et al., 2004).

Soluble products in fermentative broth of free and immobilized cells were presented in Table 3. A butyrate type of hydrogen fermentation was found in all experiments. High HBu/HAc ratio of 3.21 and 3.99 were obtained from free and immobilized cells, respectively, at pH 6.5 (Table 3). The high HBu/HAc ratio indicated an efficient hydrogen production since HAc and HBu productions are positively correlated to hydrogen production. Positive correlation between hydrogen production and VFAs was also observed in which the maximum % HBu/TVFAs ratio in culture broth was obtained at the initial sucrose concentration of 25 g-COD/L by both free and immobilized experiments.

3.3.3 Reusability of the immobilized cell

Since the results of batch fermentation indicated that hydrogen was optimally produced at an initial pH 6.5 and an initial sucrose concentration in sugarcane juice of 25 g-COD/L, therefore, further investigation on the reusability of immobilized cell at this optimum condition for 5 runs was conducted. The results indicated that the HPR and HY values obtained from 2^{nd} to 5^{th} runs were similar to that from the first run (Fig. 2). At the 5^{th} repeated run, *C. butyricum* could still survive in a sugancane bagasse as shown in Fig. 3c. This result implied that the immobilized *C. butyricum* on sugarcane bagasse can be reused up to 5 times in batch fermentation without a reduction in HY and HPR. Result indicated the potential of using the immobilized cell of *C. butyricum* on sugarcane bagasse for practical applications in continuous hydrogen production processes. Though the previous report showed that

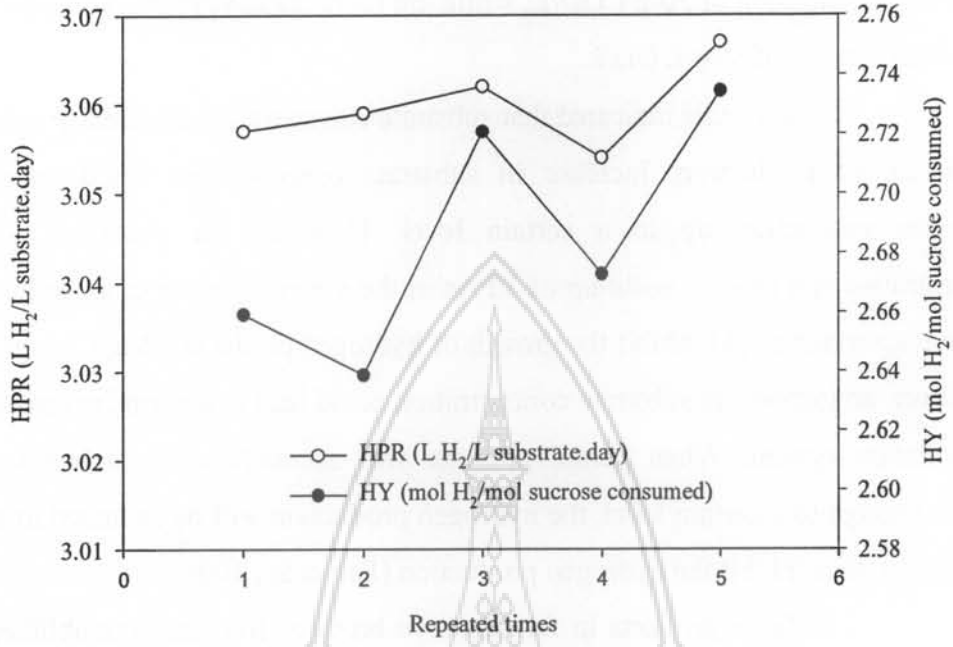

Figure 10 Hydrogen production rate (HPR, mL H_2/L substrate.day) and hydrogen yield (HY, mol H_2/mol sucrose consumed) by the immobilized *C. butyricum* at each reusability runs.

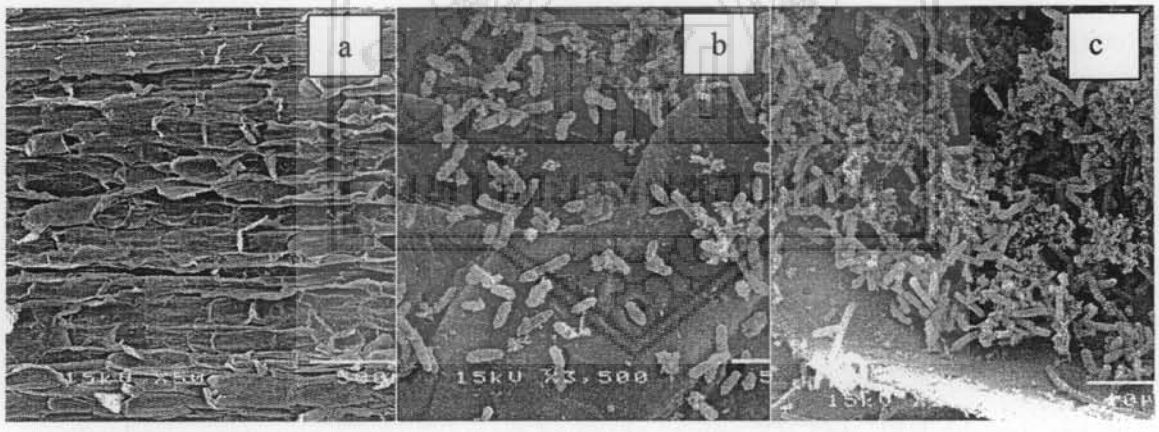

Figure 11 Scanning electron microscope studies of sugarcane bagasse (SCB) as a support material, 50X, (a); *C. butyricum* immobilized on SCB, 3,500X, (b); and immobilized *C. butyricum* on SCB after 5 repeated runs of continuous hydrogen fermentation processes, 3,500X, (c).

the immobilized of anaerobic sludge on synthetic polymer i.e., ethylene-vinyl acetate copolymer using sucrose as a carbon source could maintain stable and efficient hydrogen production up to 15 runs of repeated experiment (Wu et al., 2005) but sugarcane bagasse has the advantage over synthetic polymer in the aspect of biodegradable and environmental friendly (Kaumar, Das, 2001).

3.3.4 Repeated-batch hydrogen production

Repeated batch hydrogen production was conducted with the varying end-of-the-batch harvested volume of 25%, 50% and 75%. The hydrogen production and sucrose remaining time courses profile were shown in Figs. 4, 5 and 6. Results indicated that sucrose concentration depleted during the fermentation process until 24 h (Figure 12, 13 and 14). This result coincided with the cumulative HP which was observed to be stable at the volume of approximately 1.35, 2.29 and 3.35 L H_2/L substrate at 24 h for the end-of-the-batch harvested volume of 25%, 50% and 75%, respectively (Figure 12, 13 and 14). The repeated batch system achieved the relatively high HY ranged between 2.68 and 3.07 mol H_2/mol sucrose in which the maximum HY

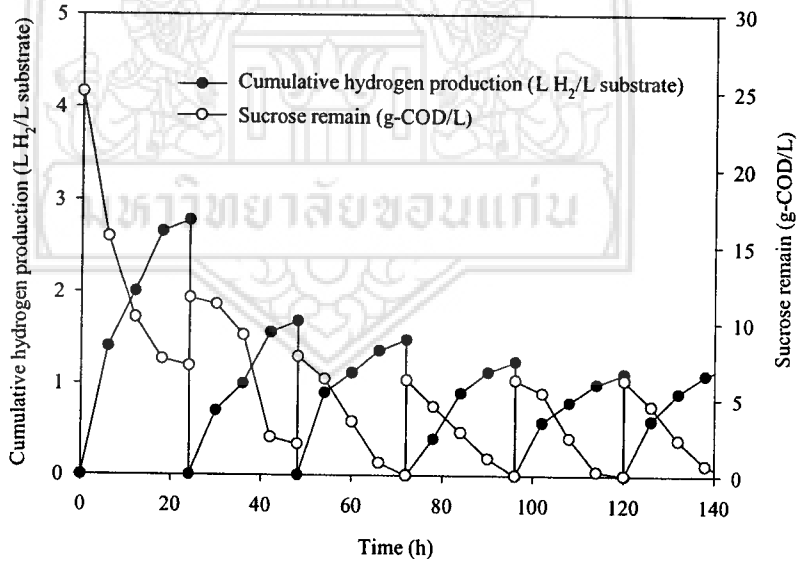

Figure 12 Time course profile of cumulative hydrogen production and sucrose concentration remaining at 25% end-of–the-batch harvested volume.

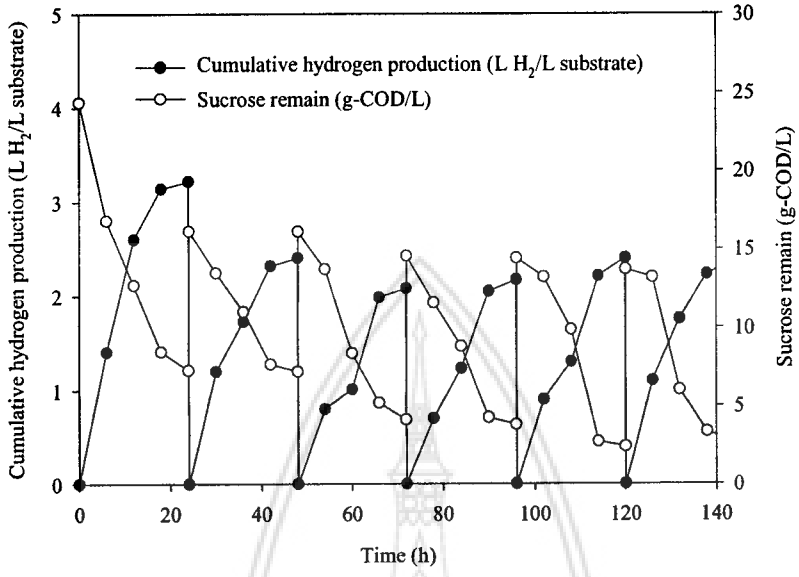

Figure 13 Time course profile of cumulative hydrogen production and sucrose concentration remaining at 50% end-of–the-batch harvested volume.

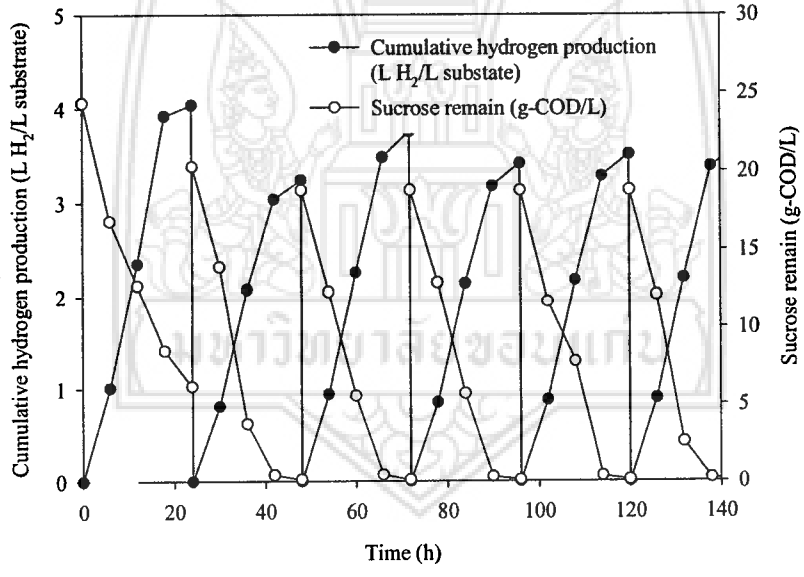

Figure 14 Time course profile of cumulative hydrogen production and sucrose concentration remaining at 75% end-of–the-batch harvested volume.

could be obtained at the end-of-the-batch harvested volume of 50% (Table 4). The maximum HPR of 3.50 L H_2/L substrate.day was obtained at the end-of-the-batch harvested volume of 75% following by 50% and 25% of 2.29 and 1.35 L H_2/L substrate.day (Table 22). According to results, if the HY was considered as the most important parameter for hydrogen production, the optimum the end-of-the-batch harvested volume of would be 50%. However, if the HPR was considered as the most important parameter, the end-of-the-batch harvested volume of 75% would be the most suitable for repeated-batch hydrogen production from sugarcane juice by the immobilized *C. butyricum*.

In comparison to batch fermentation, the results indicated that repeated batch hydrogen production was more effective than batch in which the higher HY and HPR could be achieved (Table 22). This result was similar to the finding of Yokoi et al. (2002) who reported that HY from sweet potato starch waste by mixed culture of *C. butyricum* and *Enterobacter aerogenes* in repeated batch fermentation was higher than batch fermentation in which HY of repeated batch was 2.7 mol H_2/mol glucose while HY of batch was 1.7 mol H_2/mol glucose. In addition, Sangyoka et al. (2007) reported that HP from cassava starch wastewater by heat-treated granules anaerobic sludge in repeated batch fermentation was higher than batch fermentation. This phenomena might due to the fact that a substrate in repeated batch was periodically supplied during the fermentation resulting in a continuously hydrogen production without the shortage of substrate (Sangyoka et al., 2007).

Major VFAs production in repeated batch experiment were HAc and HBu (Table 4). HBu was observed as the main product throughout every end-of–the-batch harvested volume of repeated batch experiment (Table 4).

HY of 3.04 mol H_2/mol sucrose consumed at 50% end-of–the-batch harvested volume in repeated batch fermentation was compared very favorably to those reported in the literature (Table 5). It's worth noting that, most of the studies were conducted using sugar as substrate. Results from this study revealed that hydrogen production from sugarcane juice was feasible.

Table 22 Hydrogen and soluble metabolites production from sugarcane juice by the immobilized *C. butyricum* in repeated batch operation

End-of-the batch harvested volume	H_2 content (%)	HP (L H_2/L substrate)	HPR (L H_2/L. substrate day)	HY (mol H_2/mol sucrose)	Soluble metabolites (mg-COD/L)							HBu/ TVFAs (%)	HBu/HAc ratio
					HAc	HPr	HBu	EtOH	BuOH	TVFAs	SMP		
Batch	45	3.35	3.35	2.75	686±0.12	268±0.08	4,631±0.42	725±0.22	886±0.26	5,585±0.24	7,197±0.21	83	4.07
25%	45	1.35	1.35	2.74	309±0.11	92±0.04	2,276±0.27	298±0.07	667±0.22	2,677±0.38	3,642±0.64	85	4.42
50%	45	2.29	2.29	3.04	436±0.06	49±0.03	2,904±0.41	277±0.03	425±0.12	3,389±0.43	4,091±0.55	86	3.89
75%	45	3.50	3.50	2.68	905±0.14	343±0.07	5,268±0.54	505±0.06	699±0.12	6,516±0.62	7,721±0.53	81	3.41

HP: Hydrogen production (L H_2/L substrate); HPR: Hydrogen production rate (L H_2/L. substrate day); HY: Hydrogen yield (mol H_2/mol sucrose) HAc: acetic acid; HBu: normal butyric acid; HPr: propionic acid; EtOH: ethanol; BuOH: butanol; TVFAs (total volatile fatty acids) = HAc+HBu+HPr; SMP (soluble microbial products) = TVFAs+EtOH+BuOH.

Table 23 Comparison of hydrogen yield from sugar by various types of microorganisms

Microorganism	Substrate	Types	Fermentation process	H_2 yield (mol H_2/mol substrate)	Reference
C. butyricum	Sucrose	Free cell	Batch	2.78	Chen et al. (2005)
C. saccharoperbutylacetonicum	Disaccharide	Free cell	Batch	2.69	Ferchichi et al. (2005)
C. butyricum	Sugarcane juice (sucrose)	Free cell	Batch	2.67	This study
C. butyricum	Sugarcane juice (sucrose)	Immobilized cell/sugarcane bagasse	Batch	2.66	This study
Anaerobicsludge (*Clostridium*species dominate)	Glucose	Immobilized cell/activated carbon	Continuous fermentation	0.87	Wu et al. (2005)
Anaerobicsludge (*Clostridium*species dominate)	Glucose	Immobilized cell/granular sludge	Continuous fermentation	1.57	Wu et al. (2005)
C. tyrobutyricum JM1	Glucose	Immobilized cell/polyurethane foam	Continuous fermentation	1.79	Wu, Chang (2007)
C. butyricum TISTR 1032	Sugarcane juice (sucrose)	Immobilized cell/sugarcane bagasse	Semi-Continuous fermentation (Repeated Batch)	3.04	This study

3.3.5 Preliminary results of electricity generation using a biohydrogen-fuel cell system

The hydrogen producing system with a 50% (v/v) end-of-the-batch harvested volume was connected to a fuel cell unit after the constant hydrogen production between batches was achieved. Results showed the ability to generate the

maximum and constant electrical power of 49 mW during 8–20 hours of the fermentation. However, the electric power decreased after 20 h of reaction time, because the hydrogen production supplied by hydrogen fermentation decreased after 20 h of incubation time (Figure 15). Fuel cell was connected to motor fan (electric requirement; 20 mW) and showed a rotating of the fan during 12 h. Previous research reported that hydrogen production by the immobilized *E. coli* MC13-4 immobilized in alginate gel beads using glucose as a carbon source could generate electric power of 20 mW (using 1 Ω load-resistors) during 40-60 min (Ishikawa et al., 2006).

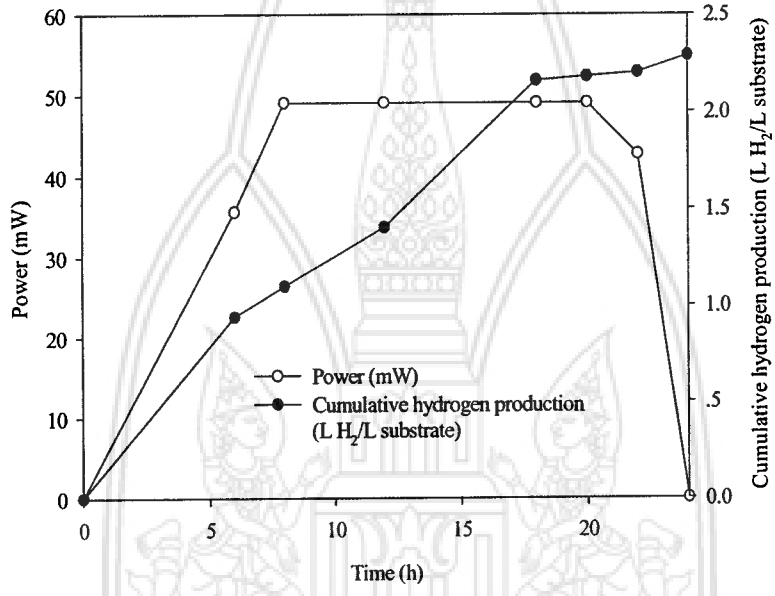

Figure 15 Time course profile of electricity generation and hydrogen production using fuel cell system.

3.3.6 Feasibility analysis of hydrogen production from sugarcane juice for electricity generation

The feasibility of hydrogen production from sugarcane juice for electricity generation was evaluated and summarized in Figure 16. The information on hydrogen fermentation from sugarcane juice by *C. butyricum* in repeated batch reactor was taken in to the calculation with the yield of 3.04 mol H_2/mol sucrose which equivalent to 75% of the maximum yield (4 mol H_2/mol sucrose). According to the data of sugarcane crop in Thailand (Office of the Cane and Sugar Board, 2006), sugarcane can be cultivated one crop per year yielding 103 ton/ha.crop. The sucrose

content of sugarcane is approximately 15% (Office of the Cane and Sugar Board, 2006). Therefore, harvested sugarcane from one ha could be use to produce 0.206 ton of hydrogen. Assuming that the heating value of hydrogen is 286 KJ/mol hydrogen (DOE, 2001), thus, in terms of combustion enthalpy, the production of 0.206 ton hydrogen yielded the heating value of 29.5 GJ. In order to demonstrate the use of hydrogen produced by supplying the electricity to 100 households, the 15000 KW-h/month (648 GJ/year) would be needed (PEA, 2009). Therefore, a 22 ha per year of sugarcane was needed to sufficiently supply the substrate i.e., sugarcane juice for the hydrogen- generation system (Figure15).

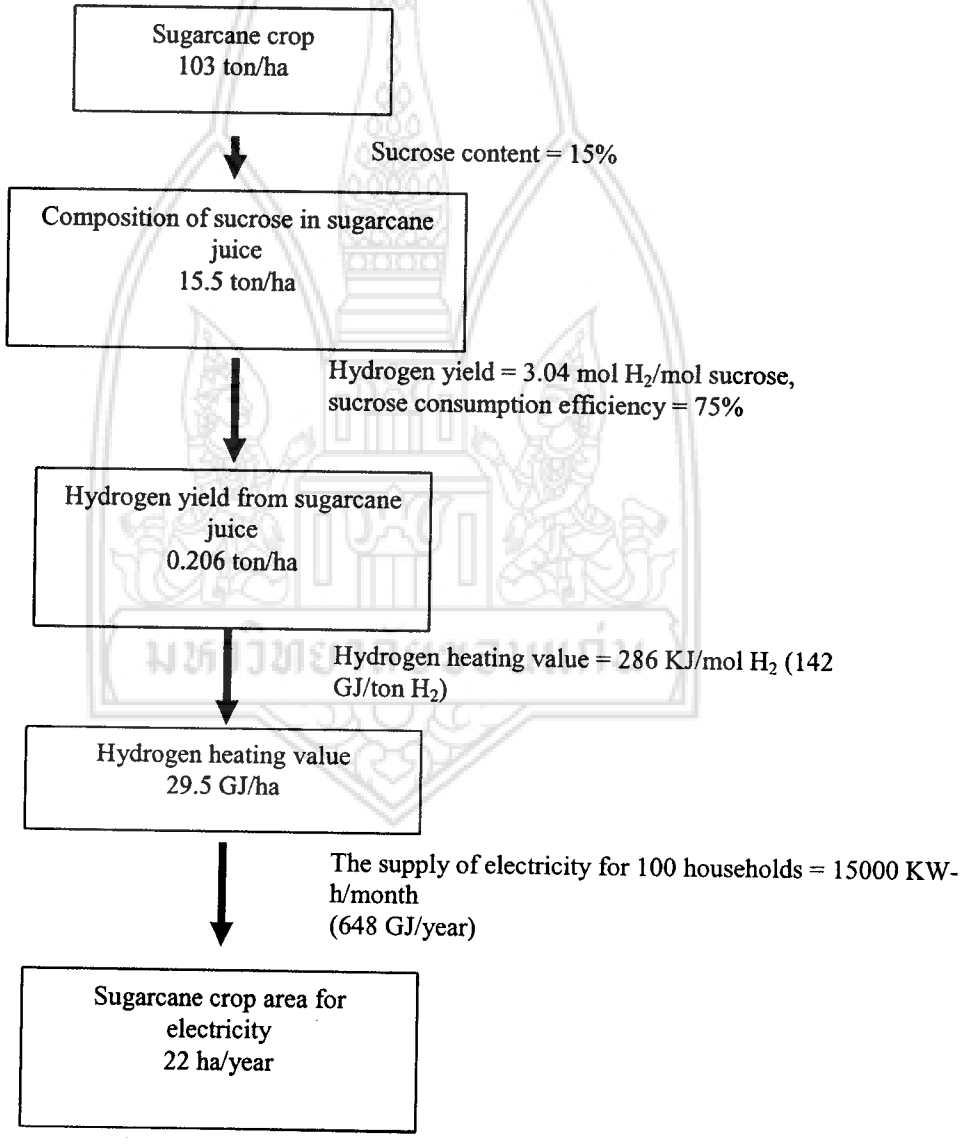

Figure 16 Feasibility analysis of hydrogen production from sugarcane juice for electricity generation.

3.4 Conclusion

Sugarcane juice has a potential to be used as the substrate for hydrogen production. Hydrogen was optimally produced from sugarcane juice at an initial pH of 6.5 and initial sucrose concentration of 25 g-COD/L by free and immobilized *C. butyricum*. The maximum hydrogen production at the optimum condition for free and immobilized cell experiment were 2.96 L H_2/L substrate and 2.84 L H_2/L substrate, respectively, and the maximum HY were 2.66 and 2.67 mol H_2/ mol sucrose consumed, respectively. The hydrogen content in the biogas was 44-45% and no methane was detected. The hydrogen production by *C. butyricum* was essentially butyrate-type fermentation. Main by-product was HBu in which accounted for 45-66% of VFAs. Solvents i.e. ethanol and butanol were detected in this study. Immobilized *C. butyricum* in batch fermentation can be reused up to 5 times without a reduction in HY and HPR. The optimal condition of repeated batch fermentations by the immobilized cell was 50% end-of–the-batch harvested volume which resulted in the highest HY and HPR of 3.04 mol H_2/mol sucrose and 2.29 L H_2/L substrate.day, respectively. The biohydrogen-fuel cell system showed the ability to generate the maximum and constant electrical power of 49 mW during 8–20 hours of the fermentation. In order to supply the electricity for 100 households, the 15000 KW-h/month (648 GJ/year) would be needed. Thus, a 22 ha/year of sugarcane is needed to sufficiently supply the substrate i.e., sugarcane juice for the hydrogen-electricity generation system of 100 households.

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References

Chang JS, Chou C, Chen SY. Decolorization of azo dye with immobilized cells of *Pseudomonas luteola*. **Process Biochem** 2001; 36: 757-763.

Chang JS, Lee KS, Lin PJ. Biohydrogen production with fixed-bed bioreactors. **Int J Hydrogen Energy** 2002; 27: 1167–1174.

Chen WM, Tseng ZJ, Lee KS, Chang JS. Fermentative hydrogen production with *Clostridium butyricum* GCS5 isolated from anaerobic sewage sludge. **Int J Hydrogen Energy** 2005; 30: 1063-1070.

Department of Energy (DOE). **Properties of hydrogen**. [online] 2008 [cite 2008 Feb 10] Available from: <http://www.eere.energy.gov/hydrogenandfuelcells/fuelcells/pdfs/fcm01r0.pdf.

Fan YT, Li CL, Lay JJ, Hou HW, Zhang GS. Optimization of initial substarte and pH levels for germination of sporing hydrogen-producing anaerobes in cow dung compost. **Biores Technol** 2004; 91: 189-193.

Ferchichi M, Crabbe E, Gil GH, Hintz W, Almadidy A. Influence of initial pH on hydrogen production from cheese whey. **J Biotechnol** 2005; 120: 402-409.

Han SK, Shin HS. Biohydrogen production by anaerobic fermentation of food waste. **Int J Hydrogen Energy** 2004; 29: 569-577.

Holdeman LV, Cato EP, Moore WEC. **Anaerobe laboratory manual.** 4thed. Blacksburg, Virginia USA: Southern printing Co.; 1977.

Iconomou L, Psariannos C, Koutinas A. Ethanol fermentation promoted by delighified cellulosic material. **J Ferment Bioeng** 1995; 79: 294-296.

Ishikawa M, Yamamura S, Takamura Y, Sode K, Tamiya E, Tomiyama M. Development of a compact high-density microbial hydrogen reactor for portable bio-fuel cell system. **Int J Hydrogen Energy** 2006; 31: 1484-1489.

Jo JH, Lee DS, Park JM. The effects of pH on carbon material and energy balances in hydrogen-producing *Clostridium tyrobutyricum* JM1. **Biores Technol** 2008; 99: 8485-8491.

Kapdan LK, Kargi F. Bio-hydrogen production from waste materials. **Enzyme Microbial Technol** 2006; 38: 569–582.

Khanal SK, Chen WH, Li L, Sung S. Biohydrogen production : effect of pH and intermediate products. **Int J Hydrogen Energy** 2004; 29: 1123-1134.

Kim SH, Han SK, Shin HS. Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. **Int J Hydrogen Energy** 2004; 29: 1607-1616.

Kourkputas Y, Bekatorou A, Banat IM, Marchant R, Koutinas AA. Immobilization technologies and support material suitable in alcohol beverages production: a review. **Food Microbiol** 2004; 21: 377-397.

Kumar N, Das D. Electron microscopy of hydrogen producing immobilized *E. cloacae* IIT-BT 08 on natural polymers. **Int J Hydrogen Energy** 2001; 26: 1155-1163.

Lay JJ, Lee YJ, Noike T. Feasibility of biological hydrogen production from organic fraction of municipal solid waste. **Water Res** 1999; 33: 2579-2586.

Lenz TO, Moreira AR. Economic evaluation of the acetone butanol fermentation. **Ind Eng Chem Prod Res Dev** 1980; 19: 478-4783.

Lin CY, Lay CH. Carbon/nitrogen-ratio effect on fermentative hydrogen production by mixed microflora. **Int J Hydrogen Energy** 2004; 29: 41-45.

Kim InS, Hwang MH, Jang NJ, Hyun SH, Lee ST. Effect of low pH on the activity of hydrogen utilizing methanogen in bio-hydrogen process. Int J Hydrogen Energy 2004; 29: 1133-40.

Office of the Cane and Sugar Board. **Summarize of the situation on sugar consumption, export and import in Thailand 2006.** [online] 2006 [cite 2006 Feb 10]. Available from: http://www.ocsb.go.th/uploads/contents/14/attachfiles/F5397.df.

Owen W, Stuckey C, Healy J, Young L, McCarty P. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. **Water Res** 1978; 13: 485-493.

Provincial Electricity Authority (PEA). **Electricity Tariffs.** [online] 2008 [cite 2008 Jan 10]. Available from: http://www.pea.co.th/rates/Rate2006.pdf.

Rachman MA, Nkashimada Y, Kakizono T, Nishio N. Hydrogen production with high yield and high evolution rate by self-flocculated cell of *Enterobacter aerogenes* in a packed-bed reactor. **Appl Microbiol Biotechnol** 1998; 49: 450-454.

Sangyoka S, Reungsang A, Moonamart S. Repeated-batch fermentation for bio-hydrogen production from cassava starch manufacturing wastewater. **Pak J Biol Sci** 2007; 10: 1782-1789.

Sneath PHA, Mair NS, Sharpe ME, Holt JG. **Bergey's manual of systematic bacteriology volume 2.** Baltimore: Williams and Wilkins; 1986.

Valdez-Vazquez I, Poggi-Varaldo HM. Hydrogen production by fermentative consortia. **Renew Sustain Energy Rev** 2009; 13: 1000-1013.

Wang B, Wan W, Wang J. Inhibitory effect of ethanol, acetic acid, propionic acid and butyric acid on fermentative production. **Int J Hydrogen Energy** 2008; 33: 7013-7019.

Wu SY, Lin CN, Chang JS, Chang JSh. Biohydrogen production with anaerobic sludge immobilized by ethylene-vinyl acetate copolymer. **Int J Hydrogen Energy** 2005; 30: 1375-1381.

Wu KJ, Chang JS. Batch and continuous fermentative production of hydrogen with anaerobic sludge entrapped in a composite polymeric matrix. **Process Biochem** 2007; 42: 279-284.

Yokoi H, Maki R, Hirose J, Hayashi S. Microbial production of hydrogen from starch-manufacturing wastes. **Biomass Bioenergy** 2002; 22: 389-395.

Yokoi H, Mori S, Hirose J, Hayashi S, Takasaki Y. Hydrogen production from starch by a mixed culture of *Clostridium butyricum* and *Rhodobacter* sp. M-19. **Biotechnol Lett** 1998; 2: 895-899.

Zheng XJ, Yu HQ. Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures. **J Environ Management** 2005; 74: 66-70.

Zwietering MH, Jongenburger L, Rombouts FM, Van't RK. Modeling the bacterial growth curve. **Appl Environ Microbiol** 1990; 56: 1875-1881.


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