CHAPTER V

BIO-HYDROGEN PRODUCTION FROM THE FERMENTATION OF SUGARCANE BAGASSE HYDROLYSATE BY *Clostridium butyricum*

5.1 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. Its high energy yield of 122 KJ/g which is 2.75 times greater than that of hydrocarbon fuel. Hydrogen can be directly used to produce electricity through fuel cells [1]. Hydrogen can be generated mainly from fossil fuels, biomass and water by chemical or biological process [2]. 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 [3]. In comparison to photosynthetic microorganisms, fermentative hydrogen-producing microorganisms are advantageous in that hydrogen can be evolved in a reactor continuously without light [4]. Among the fermentative hydrogen producers, the organisms of genus *Clostridium* such as *C. butyricum* [5, 6], *C. acetobutylicum* [7] *C. saccharoperbutylacetonicum* [8], *C. pasteurianum* [9, 10] are often used to produce hydrogen [11]. *Clostridium* species are spore forming anaerobic organisms capable of converting hexose to hydrogen with the yield of 2 mol of hydrogen/mol hexose [3] which is higher than that of the other fermentative bacteria such as *Enterobacter* sp. (1 mol hydrogen/mol hexose) [3].

Sugarcane is one of important industrial crops in Thailand. It can be cultivated in all parts of Thailand, except in Southern Thailand, and with the cultivation area of more than 960,000 ha, approximately 48 million tons of sugarcane is produced per year [12]. Sugarcane bagasse (SCB) is a waste left after sugarcane extraction process. Since the bagasse accounted for approximately 25% of sugarcane mass, about 12 million tons of SCB is produced annually. The most common use for SCB is the energy production by combustion [13] which can cause environmental problem from the emissions of CO_2. In addition, SCB can be used also to produce chemical compounds such as furfural or hydroxymethylfurfural [14], paper paste [15] or ethanol [16].

SCB consists of three main fractions i.e., cellulose, hemicellulose and lignin. It contains ca. 30-35% of hemicellulose [17]. Dilute acid treatment of hemicellulose fraction in SCB yields a solution containing mainly glucose and xylose [18] with small amount of arabinose [17]. Since bonds in cellulose are stronger than in hemicellulose, therefore a solid waste formed by cellulose and lignin is obtained in the dilute acid hydrolysis of SCB [18]. Glucose and xylose were reported as a substrate for producing hydrogen by various types of microorganisms such as *C. acetobutylicum* [19] and *C. butyricum* [20] with the yield of 2.0 and 2.3 mol H_2/mol glucose, respectively. Due to its composition, hydrolysate of SCB is a very attractive raw material for the production of hydrogen.

Therefore, we are interested in using SCB hydrolysate to produce hydrogen by *C. butyricum*. In order to achieve this goal, we attempted to optimize the conditions for hydrolysis of SCB fraction with sulphuric acid in autoclave (121°C) in order to obtain the SCB hydrolysate that was suitable as a fermentation media (high sugar concentrations and low growth inhibitors) for producing hydrogen using *C. butyricum*. The two most important environmental factors affecting hydrogen production namely initial pH and initial total sugar concentration were also investigated. This approach will not only adding value to SCB, in a form of safe and clean energy, but also one of the solution approaches for making use of this abundant waste.

5.2 Materials and methods

5.2.1 Sugarcane bagasse

SCB used in this study was obtained from a local sugar juice shop (Khon Kaen, Thailand). SCB were air dried, milled and sieved through a 0.5 mm screen before storing at room temperature prior to usage. The composition (w/w) of the SCB was (values of three replicates, error lower than 0.1 % in all compounds): 37.56% cellulose; 24.88% hemicellulose; 4.31% lignin; 1.29% ash and 31.96% of other components. Low lignin content was due to the removal of the rind prior to content analysis.

5.2.2 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 anaerobic condition for 10 hrs 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/mL into 10 mL of fresh Tryptone Sucrose Yeast Extract (TSY) medium. Then, serum bottle was flushed with argon to create anaerobic condition and incubated at 37^{o}C for 10 hrs at 150 rpm using an orbital shaker. The culture was further enriched by inoculating 10% v/v of previous culture into 60 mL fresh TSY medium and incubated at the given conditions before using as inoculum [21]. 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 [22].

Cell concentration was defined as the amount (dry weight) of cells per mL of culture broth. This was conducted by centrifuging one 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 was conducted.

5.2.3 Acid hydrolysis of SCB

Acid hydrolysis of SCB hemicellulose fraction was conducted at 121 ^{O}C, 1.5 Kg/cm^2 for 60 min in autoclave with a mass ratio of solid (g dry weight) to liquid (mL) at 1:15 [13]. SCB was hydrolyzed by H_2SO_4 at various concentrations of 0.25, 0.5, 1, 2, 3, 4, 5, 6 and 7% (v/v). The hydrolysate from each condition was used as substrate for hydrogen production. The optimum concentration of H_2SO_4 that yield the hydrolysate which gave the highest cumulative hydrogen was further used to investigate an effect of reaction time on hydrolysis of SCB in a similar manner. Samples were collected at reaction time of 15, 30, 60, 90, 120, 180 and 240 min.

After hydrolysis, a solid residue was separated by filtration through a thin layer cloth. The pH of hydrolysate was adjusted to pH 10 with $Ca(OH)_2$, the resulting precipitate was removed by centrifugation (1,500 rpm, 15 min) and then reacidified to pH 7, followed by further centrifugation [23]. Supernatant was concentrated at 70 ^{O}C for 48 hrs in hot air oven.

The efficiency (*E*) of H_2SO_4 to hydrolyze hemicellulose fraction of SCB at various concentrations (volume %) was determined using Equation 1 [17]:

$$E = \sum S / \sum I \qquad (1)$$

Where $\sum S$ is the sum of total sugar concentrations (glucose, xylose and arabinose) and $\sum I$ is the sum of growth inhibitors concentrations (furfural, acetic acid) in the hydrolysate.

5.2.4 Experimental procedure

The batch experiments were set up in 100 mL serum bottle with a working volume of 70 mL comprised of 61 mL of SCB hemicellulose hydrolysate, 6 mL of *C. butyricum* at a cell concentration of 10^6 cells/mL, 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 anaerobic condition, the serum bottle was incubated at 37°C and 150 rpm in an orbital shaker. All treatments were carried out in triplicate. 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 [24]. The volume of biogas was measured by plunger displacement method [25].

5.2.5 Analytical method

Hydrolysate was filtered through 0.45 μm cellulose acetate membrane and analyzed by HPLC for glucose, xylose, arabinose, acetic acid and furfural. The HPLC analysis were carried out using a LC-10AD (Shimadzu, Japan) with an Aminex HPX-87H column (oven temperature 40 ^{O}C), 1 mM H_2SO_4 as mobile phase at the flow rate of 0.6 mL/min and a refraction index detector.

Total sugar concentration in the hydrolysate is a summation of glucose, xylose and arabinose concentrations obtained from HPLC analysis.

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 3 m x 3 mm diameter stainless-steel column packed with activated charcoal

(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 was used as carrier gases with flow rate of 50 mL/min.

5.2.6 Kinetic analysis

Volume of biogas produced was calculated by a mass balance equation previously described by Zheng and Yu (2005) [26]. The cumulative hydrogen production followed the modified Gompertz equation (Equation 2) [27]:

$$H(t) = P \exp\left\{-\exp\left[\frac{\underline{R_m}e}{P}(\lambda - t) + 1\right]\right\} \qquad (2)$$

Where H is the cumulative volume of hydrogen produced (mL), P is the H_2 production potential or HP (mL), *Rm* is the maximum hydrogen production rate or HPR (mL H_2/day), 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 specific hydrogen production rate or SHPR (mmol H_2/g cell.day) obtained from dividing R_m by the cell dry weight (g cell).

5.3 Results and discussion

5.3.1 Composition of SCB hemicellulose hydrolysate

Table 1 shows the composition of hemicellulose hydrolysate when hydrolyzed SCB at different H_2SO_4 concentrations. Glucose, and xylose were the main sugars found in the SCB hemicellulose hydrolysate. Arabinose was released in a small amount.

Glucose can be derived from the cellulosic fraction or from some heteropolymers of the hemicellulosic fraction [18]. Glucose is of important because it is the main carbon source for microorganisms. The highest glucose obtained was 10.99 g/L when 0.5% H_2SO_4 was used to hydrolyze SCB at a fixed reaction time of 60 min (Table 1). A distinct increase in glucose concentration was observed when H_2SO_4 concentration was increased from 0.25% (6.71 g/L) to 0.5% (10.99 g/L). However, an increase from 0.5% to 1.0% H_2SO_4 did not affect the glucose concentration in SCB hemicellulose hydrolysate. It was observed that glucose concentration in SCB hemicellulose hydrolysate decreased when the H_2SO_4 concentration was between 1.0-5% ($p \leq 0.05$) and then slightly increased at 6-7% H_2SO_4.

Xylose is found as the main sugar in SCB hemicellulose hydrolysate. Its concentration was highest when SCB was hydrolysed with 0.5% H_2SO_4 for 60 min (Table 1). The profile of its concentration was similar to those of glucose except that xylose concentration was lowest when SCB was hydrolysed by 7% H_2SO_4.

Arabinose is a sugar formed from arabinoxylans, hemicellulosic heteropolymers found in agricultural materials such as SCB [28]. In arabinoxylans, the arabinose is generally found in a low amount compared to xylose. Its concentration (2.22 g/L) was peak when 0.5% H_2SO_4 was used (Table 31) It was also observed that a profile concentration of arabinose derived from acid hydrolysis of SCB was similar to those of glucose and xylose.

The increase of acid concentration in the acid-hydrolyzing process could provide a strong or complete reaction for breaking down the chemical bonds inside cellulose materials yielding the hydrolyzed products mainly sugars in the hydrolysate [28]. However, further conversion of sugars to other substances such as furfural could potentially occur when the amount of acid added was in excess [18]. Furfural could be generated as a degradation product from pentose such as xylose in which it was found to increase with the concentration of the acid catalysts such as H_2SO_4 [18]. Our results indicated the same trend that furfural was significantly increased with an increase in H_2SO_4 concentration ($p \leq 0.05$) with the highest concentration of furfural (1.30 g/L) found at 7% H_2SO_4 (Table 31). Furfural has been reported to damage microorganisms by reducing enzymatic and biological activities, breaking down DNA and inhibiting protein and RNA synthesis [29]. Unfortunately, there is limited information in the literature in regard to the susceptible of *C. butyricum* to furfural. Hence, its effect on the bio-hydrogen fermentation of *C. butyricum* should be conducted in order to examine if it is a major limiting factor for *C. butyricum* to produce hydrogen. Therefore, apart from high sugar concentration obtained from SCB hemicellulose hydrolysis our selection criteria were low furfural concentration in order to prevent the damage to microorganisms.

Another inhibitory substances founded in SCB hemicellulose hydrolysate is acetic acid. Acetic acid can be generated when the hydrolysis reaction takes place at the acetyl group of hemicellulose [28]. Results showed that acetic acid concentration was not affected by the concentration of H_2SO_4 (Table 1) which was in contrast to those reported by Rodriguez-Chong et al. (2004) [28] that concentrations of acetic acid in SCB hydrolysate were increased with an increase in H_2SO_4 concentration. Acetic acid can be the inhibitors of microbial growth when its concentration is between 4-10 g/L because it can diffuse through the cell membrane and decreases intracellular pH causing the adverse effects to metabolism of the microorganisms [18, 28]. Our results revealed that the maximum concentration of acetic acid, 2.72 g/L when hydrolysed by 6% H_2SO_4 for 60 min, was lower than a toxic effect (Table 31).

In order to achieve the optimum condition for the acid hydrolysis of SCB hemicellulose which yielded high total sugar concentration and low concentration of growth inhibitors, we compared the efficiency (*E*) when hydrolyzed SCB with various concentrations of H_2SO_4 (volume %) (Table 31). Our results indicated that *E* increased from 6.7 to 9 when H_2SO_4 concentration increased from 0.25 to 0.5%. Then the value gradually decreased from 9 to 4.26 with an increase in H_2SO_4 concentration (Table 31). Therefore, results implied that high concentration of H_2SO_4 generated a limited positive effect on the hydrolysate due to higher growth inhibitors obtained. Economic

Table 31 Compositions of the SCB hydrolysate obtained at a fixed reaction time of 60 min under different percentage of H_2SO_4

H_2SO_4 (volume %)	Concentration (g/L) Glucose	Xylose	Arabinose	HAc	Furfural	Total sugar*	Efficiency (E) (dimensionless)
0.25	6.71^{a}	8.58^{cd}	1.86^{ab}	2.44^{a}	0.12^{a}	17.15	6.70
0.5	10.99^{c}	11.29^{e}	2.22^{b}	2.48^{a}	0.12^{a}	24.50	9.00
1.0	9.42^{bc}	9.65^{de}	2.16^{b}	2.41^{a}	0.25^{ab}	21.23	8.00
2.0	8.79^{abc}	8.48^{bcd}	2.09^{ab}	2.35^{a}	0.46^{bc}	19.35	6.89
3.0	7.90^{ab}	7.17^{abc}	1.91^{ab}	2.13^{a}	0.61^{c}	16.98	6.19
4.0	7.04^{ab}	5.99^{a}	1.76^{a}	1.90^{a}	0.66^{c}	14.79	5.78
5.0	8.54^{ab}	6.67^{ab}	1.94^{ab}	2.37^{a}	0.98^{d}	17.15	5.13
6.0	9.44^{bc}	6.77^{abc}	2.04^{ab}	2.72^{a}	1.11^{de}	18.24	4.76
7.0	9.21^{bc}	5.80^{a}	1.93ab	2.68^{a}	1.30^{e}	16.93	4.26

Different letters indicate significant differences among treatment by the Duncan test ($p<0.005$).

HAc: acetic acid.

*Total sugar = glucose + xylose + arabinose.

wise, we prefer to keep the acid concentration as low as possible. This would help to minimize a corrosion of equipments as well. Since the highest total sugar concentration of 24.50 g/L and the highest E ratio of 9 were obtained when 0.5% H_2SO_4 was used as a catalyst (Table 1) we, therefore, further investigated the effect of reaction time on SCB hydrolysis using 0.5% H_2SO_4 as a catalyst.

The results from Table 32 revealed that maximal concentrations of glucose (11 g/L) and xylose (13.64 g/L) were obtained when SCB was hydrolysed by H_2SO_4 for 60 min. However, arabinose concentration, 2.52 g/L, was highest at 180 min of reaction time. As reaction time increase, concentrations of glucose and xylose decrease which might be resulted from a continuously increased in furfural concentration in SCB hemicellulose hydrolysate (Table 32). Total sugar concentrations in SCB hemicellulose hydrolysate and E ratio increase with an increase in reaction time from 15 min to 60 min. The reaction time longer than 60 min did not result in an increase in total sugar concentration in SCB hemicellulose hydrolysate. E ratio decreased gradually with the increase in reaction time from 60 to

240 min which corresponded to high concentrations of inhibitors obtained at longer reaction time

Table 32 Compositions of SCB hydrolysate obtained at a fixed H_2SO_4 of 0.5% under different reaction time

Reaction time (min)	Concentration (g/L)						Efficiency (E)
	Glucose	Xylose	Arabinose	HAc	Furfural	Total sugar*	(dimensionless)
15	6.91^{a}	6.63^{a}	1.24^{a}	1.30^{a}	0.11^{a}	14.79	10
30	7.46^{ab}	8.15^{ab}	1.40^{a}	1.34^{a}	0.11^{a}	17.01	11
60	10.99^{c}	11.29^{c}	2.22^{b}	2.48^{b}	0.12^{a}	24.50	9
90	10.17^{bc}	12.26^{c}	2.20^{b}	2.46^{b}	0.13^{a}	24.63	10
120	10.97^{c}	12.82^{c}	2.37^{b}	2.73^{b}	0.16^{ab}	26.16	9
180	10.71^{c}	11.94^{c}	2.52^{b}	2.83^{b}	0.19^{bc}	27.17	9
240	10.79^{c}	10.89^{bc}	2.41^{b}	2.55^{b}	0.21^{c}	24.09	9

Different letters indicate significant differences among treatment by the Duncan test ($p<0.005$).

HAc: acetic acid.

*Total sugar = glucose + xylose + arabinose.

(Table 2). Therefore, 0.5% H_2SO_4, at 121 ^{O}C, 1.5 Kg/cm^2 and reaction time of 60 min were selected as hydrolysis condition because they resulted in solutions with 10.99 g glucose/L, 11.29 g xylose/L and low concentrations of furfural (0.12 g/L) and HAc (2.48 g/L). The SCB hydrolysate obtained by this condition was then used as a fermentation media for hydrogen production by *C. butyricum.*

The hydrolysis result from this study is comparable to other studies in term of total sugar and low furfural obtained (Table 33) considered that the liquid to solid ratio was higher in this study. A distinct result from this study was on glucose to xylose ratio obtained from hydrolysis that was shown to be higher when compared to other studies. This high glucose to xylose ratio indicated that more cellulosic fraction was degraded as the result of hydrolysis, which could be the result of the rind removal prior to hydrolysis process and small SCB particle (< 0.5 mm) used for hydrolysis. As the total sugar obtained from this study still appeared to fall in the range of industrial interest (more than 20 g-sugar/L), as well as low concentrations of inhibitors attained, the use of 0.5% H_2SO_4 was proved to be more attractive in term of cost and corrosion issues.

Apart from total sugar concentration obtained from hydrolysis, we further investigated the use of hydrolysate from all treatments in hydrogen production. The results (data not shown) suggested a strong correlation between cumulative hydrogen produced and *E* ratio which implied the importance of relative amount of inhibitor to sugar concentration in hydrogen production

3.2 Effect of initial pH on hydrogen production

The effect of an initial pH of SCB hydrolysate on hydrogen production by *C. butyricum* was investigated at a fixed initial total sugar concentration of 20 g-COD/L. Initial pH was varied from 5.5 to 8.0 with an increment of 0.5. Temperature used was 37°C. Regardless of initial pH value, pH of the culture dropped during the cultivation to the final value of around pH 4.2-4.5 (Table 34). The initial pH values affected the cumulative hydrogen production significantly in which the highest cumulative volume of hydrogen (180 mL) was obtained at the initial pH of 5.5 while the initial pH of 8.0 gave the lowest cumulative volume of hydrogen (75 mL) (Figure 24). An increase in initial pH led to a decrease in SHPR, HPR, HP and hydrogen yield (Figure 25) suggesting that low initial pH was a better condition for *C.*

Table 33 Comparison of sugar compositions and inhibitor concentration obtained from different acid hydrolysis conditions of sugarcane bagasse

Acid	Liquid to solid ratio (g/g)	Temp (°C), time (min)	Concentration (g/L)					Reference
			Glucose	Xylose	Arabinose	Acetic acid	Furfural	
6% HNO_3	10	122, 9.3	2.87	18.60	2.04	0.90	1.32	[28]
4% H_3PO_4	8	122, 300	3.00	17.60	2.60	4.00	1.20	[17]
2.5% HCl	10	140, 30	5.84	21.50	2.95	1.89	5.45	[30]
2% H_2SO_4	10	122, 24	3.00	21.60	NA	3.65	0.50	[18]
7% H_2SO_4	5	125, 120	4.50	47.2	6.2	10.7	0.83	[23]
0.5% H_2SO_4	15	121, 60	11.00	13.64	2.10	2.26	0.12	This study

NA: not applicable

butyricum to utilize SCB hydrolysate for hydrogen production than at high pH. It is also observed that an initial pH of 5.5 gave the highest SHPR, HP, HPR and hydrogen yield of approximately 303 mmol H_2/g cell.day, 180 mL, 1,611 mL H_2/L/day and 1.73 mol H_2/mol total sugar, respectively, (Figure 25). At a weak acid condition (an 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 as well as producing hydrogen which normally exhibited at the exponential growth phase of this species [8]. Our results were consistent with those reported by Chen et al. [6] that pH in the range of 5.5-6.0 was an optimum pH for hydrogen production by *C. butyricum.* In addition, the optimum pH range of 5.0-5.7 to produce hydrogen from glucose by anaerobic fermentative bacteria with the maximum hydrogen evolution efficiency was also reported [31].

Hydrogen could not be produced from SCB hydrolysate at the initial pH of 5 (data not shown). An adjustment of the initial pH in fermentative broth by adding an acid to obtain the pH of 5 in SCB 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 activities and structural damaging of cell membrane, DNA and protein which could slow down [8] or inhibit the growth of the microorganisms as well as inhibit the ability to produce hydrogen [6]. Too high or too low pH can result in a low hydrogen production in which the activity of hydrogenase could be inhibited [23]. Chen et al. [6] reported that at a higher pH cell growth was more efficient than hydrogen production.

Anaerobic hydrogen production is always accompanied with VFAs production. Results of production of soluble microbial products (SMP) including HBu, HAc, HPr, EtOH and BuOH during hydrogen fermentation were summarized in Table 34. The main soluble metabolite in the fermentative broth was butyrate in which the ratio of HBu/TVFAs was highest in fermentative broth at the initial pH of 5.5 and accounting for 55-66% of total SMP. Results from Table 4 also indicated that acetate is the second abundant soluble metabolite. Therefore, the hydrogen production by *C. butyricum* was essentially butyrate-type fermentation and can be described by Equation (3) [11, 32]. Our finding was in accordance with previous report [33, 34, 35].

$$3C_5H_{10}O_5 + 4C_6H_{12}O_6 + 3H_2O \longrightarrow 5C_4H_8O_2 + 3C_2H_4O_2 + 13CO_2 + 16H_2 \qquad (3)$$

Ethanol and butanol could be observed in the fermented broth at the end of incubation (Table 34) indicating the occurrence of solvent phase which take place when VFAs were accumulated in the hydrogen production system of *Clostridi*a species [10]. However, the TVFAs/SMP ratio of greater than 0.87 indicated that a predominant pathway of hydrogen production from SCB hemicellulose hydrolysate was acidogenic pathway over solventogenesis. Solventogenesis is unfavorable to bio-hydrogen production for the reason that additional free electrons from NADH was consumed [36]. The formation of solvent such as ethanol and butanol in metabolic pathway of *Clostridia* species was due to a development of new enzyme system at a drop of pH from 5.5-8.0 to approximately 4 in order to be able to survive at a low pH [37]. Hence, results implied that the pH control at an optimum value is needed in order to obtain high hydrogen produced. In our study, the optimal initial pH of

Table 34 Hydrogen production performance by *C. butyricum* for each initial pH at a fixed initial total sugar concentration of 20 g-COD/L of SCB hydrolysate

pH		Glucose (g-COD/L)		Xylose (g-COD/L)		Arabinose (g-COD/L)		H_2 (%)	SHPR (mmol H_2/ g cell .day)	H_2 yield (mol H_2/ mol sugar)
Initial	Final	Remaining	Consumed	Remaining	Consumed	Remaining	Consumed			
5.5	4.24	4.86	4.76	5.34	5.30	0.11	0.00	45	303.06	1.73
6.0	4.33	4.57	5.05	5.36	5.28	0.11	0.00	45	215.51	1.21
6.5	4.23	4.69	4.93	5.45	5.19	0.11	0.00	45	190.26	1.09
7.0	4.36	4.91	4.71	5.70	4.94	0.11	0.00	45	178.47	1.07
8.0	4.51	4.75	4.87	5.73	4.91	0.11	0.00	44	126.28	0.75

Table 35 Soluble metabolites distribute during hydrogen production by *C. butyricum* for each initial pH at a fixed initial total sugar concentration of 20 g-COD/L of SCB hydrolysate

Initial	Soluble metabolites (g-COD/L)							HBu/	TVFAs/	HBu/SMP
pH	HAc	HPr	HBu	EtOH	BuOH	TVFAs	SMP	TVFAs	SMP	(%)
5.5	1.46	0.02	6.77	0.55	0.31	8.25	9.11	0.82	0.91	66.36
6.0	1.65	0.06	5.68	0.59	0.10	7.39	8.08	0.77	0.91	63.82
6.5	1.73	0.09	5.70	0.38	0.72	7.52	8.61	0.76	0.87	54.83
7.0	1.83	0.06	5.85	0.33	0.42	7.74	8.49	0.76	0.91	58.04
8.0	2.02	0.07	4.68	0.32	0.11	6.77	7.20	0.69	0.94	55.11

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.

hydrogen production from SCB hydrolysate was 5.5, giving the highest SHPR of 303 mmol H_2/g cell.day and H_2 yield of 1.73 mol H_2/mol total sugar.

5.3.3 Effect of initial total sugar concentration on hydrogen production

To investigate the effect of initial total sugar concentration on hydrogen production, an initial total sugar concentration of SCB hydrolysate was adjusted to be 1.5, 2.5, 5.0, 10.0, 20.0 and 40.0 g-COD/L under a preferable initial pH, 5.5, obtained from earlier experiment and at 37°C. It was observed that hydrogen and carbon dioxide were the main biogas products in fermentative broth throughout the experiment (data not shown). The cumulative hydrogen production volume increased with an increase in concentration of SCB hydrolysate except at 40 g-COD/L that the cumulative hydrogen

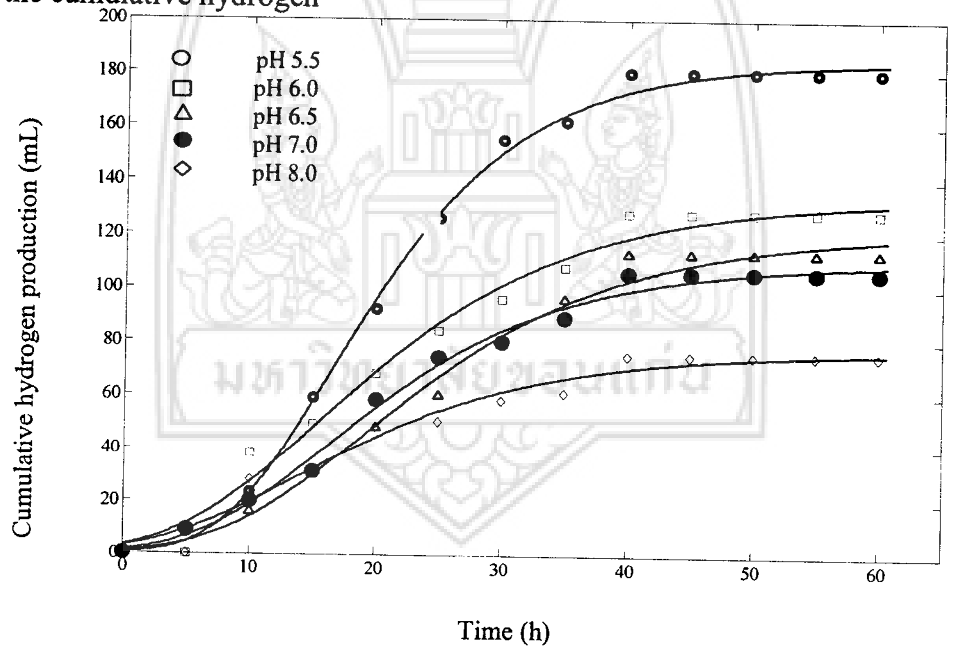

Figure 24 Cumulative hydrogen production profiles for *C. butyricum* at different initial pHs. (Temperature = 37°C, initial total sugar concentration = 20 g-COD/L; symbols: observed data, curves: prediction with Gompertz equation)

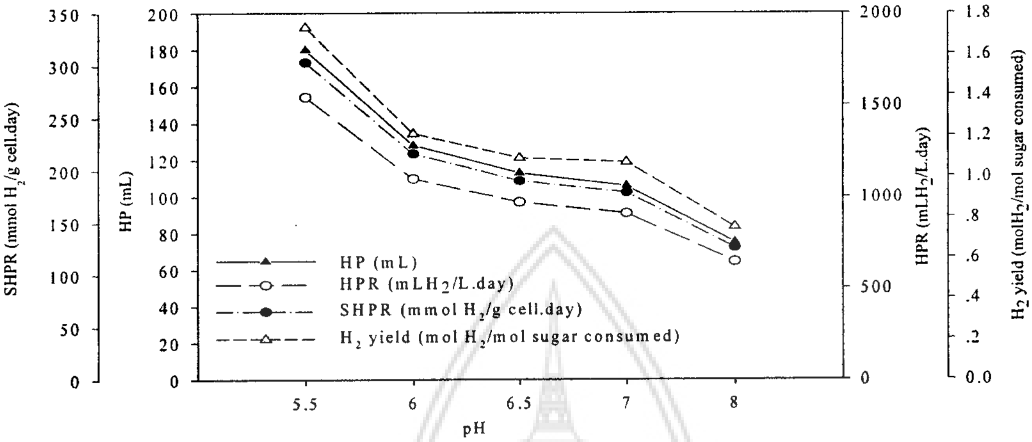

Figure 25 Relationships between specific hydrogen production rate (SHPR), hydrogen production (HP), hydrogen production rate (HPR), hydrogen yield (HY) and initial pH. (Temperature = 37°C, initial total sugar concentration = 20 g-COD/L)

was less than that obtained at 20 g-COD/L (Figure 26). Effect of an initial total sugar concentration on SHPR, HPR, HP and hydrogen (Figure 27) were similarly to those of cumulative hydrogen volume (Figure 27). The highest SHPR, HP, HPR and hydrogen yield of approximately 303 mmol H_2/g cell.day, 180 mL, 1,611 mL H_2/L/day and 1.73 mol H_2/mol total sugar were obtained at the initial total sugar concentration of 20 g/L (Figure 27). There was a decrease in SHPR, HPR, HP and hydrogen yield at 40 g-COD/L of SCB hydrolysate which might be the result of 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.* [6] 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 [6]. 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 [38]. 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 to some level, the hydrogen production will be switched to solvent production, thus inhibited the hydrogen production [39].

Similar to the results attained from the effect of initial pH, HBu (45-60% of SMP) was found to be the most abundant soluble metabolite followed by HAc (Table 5) suggesting a butyrate type of hydrogen fermentation. High TVFAs/SMP ratio (0.75-0.95) indicated an acidogenic pathway of hydrogen production from hydrolysate of SCB by *C. butyricum*.

Hydrogen molar yield of 1.73 mol H_2/mol total sugar obtained from our best hydrogen production performance condition i.e. initial pH 5.5., 37°C, 20 g COD/L initial total sugar concentration present in the SCB hydrolysate by *C. butyricum* was compared very favorably with those reported in literature (Table 36), though, most of the studies were conducted on glucose, xylose as the substrates. Results from this study revealed that hydrogen production from SCB hydrolysate was feasible.

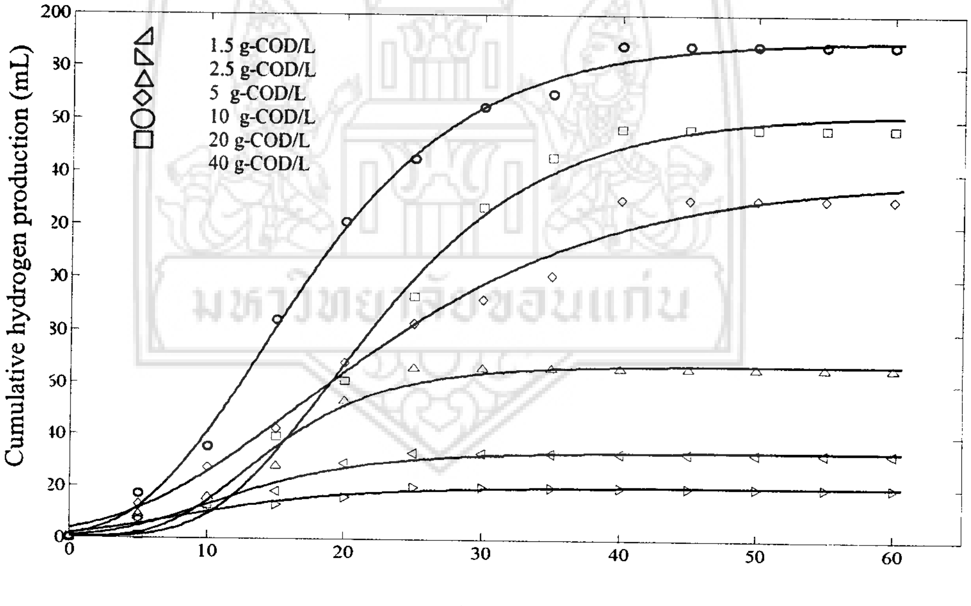

Figure 26 Cumulative hydrogen production profiles for *C. butyricum* at different initial total sugar concentrations. (Temperature = 37°C, initial pH = 5.5; symbols: observed data, curves: prediction with Gompertz equation)

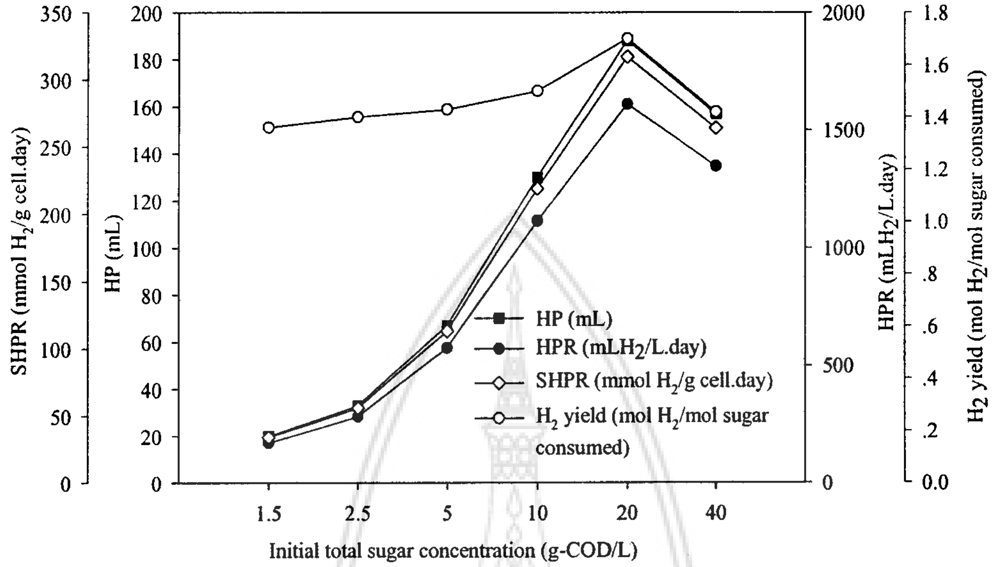

Figure 27 Relationships between specific hydrogen production rate (SHPR), hydrogen production (HP), hydrogen production rate (HPR), hydrogen yield (HY) and initial total sugar concentration. (Temperature = 37°C, initial pH = 5.5)

Table 36 Hydrogen production performance by *C. butyricum* for each initial total sugar concentration of SCB hydrolysate at a fixed initial pH of 5.5

Total sugar (g-COD/L)		Substrate consumption (%)	Glucose (g-COD/L)		Xylose (g-COD/L)		Arabinose (g-COD/L)		H_2 (%)	SHPR (mmol H_2/g cell day)	H_2 yields (mol H_2/ mol sugar)
initial	final		Remaining	Consumed	Remaining	Consumed	Remaining	Consumed			
1.49	0.23	84.56	0.22	0.25	0.00	1.01	0.01	0.00	43	33.67	1.46
2.59	0.29	88.80	0.23	0.99	0.04	1.30	0.01	0.00	45	55.56	1.39
5.33	0.87	83.18	0.69	1.65	0.15	2.81	0.03	0.00	45	112.81	1.43
10.55	2.36	77.13	1.97	2.89	0.33	5.31	0.05	0.00	46	218.88	1.51
20.66	10.01	51.41	4.86	4.76	5.34	5.30	0.11	0.00	45	303.06	1.73
40.74	30.13	26.04	14.98	4.26	14.93	6.35	0.22	0.00	44	264.34	1.41

Initial total sugar concentration = glucose + xylose + arabinose.

Table 37 Soluble metabolites distribution during hydrogen production by *C. butyricum* for each initial total sugar concentration of SCB hydrolysate at a fixed initial pH of 5.5

Soluble metabolites (g-COD/L)							HBu/	TVFA/	HBu/SMP
HAc	HPr	HBu	EtOH	BuOH	TVFAs	SMP	TVFA	SMP	(%)
0.14	0.003	0.47	0.16	0.047	0.61	0.82	0.77	0.75	53.57
0.34	0.05	1.21	0.13	0.044	1.61	1.79	0.75	0.90	45.24
0.50	0.07	2.29	0.32	0.063	2.86	3.24	0.80	0.88	60.90
1.08	0.06	4.13	0.28	0.322	5.27	5.88	0.78	0.90	60.90
1.46	0.02	6.77	0.55	0.31	8.25	9.11	0.82	0.91	66.36
1.64	0.09	5.45	0.32	0.041	7.18	7.54	0.76	0.95	58.03

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.

[a] Calculated by $(TS_i-TS_f)/TS_i$ x 100%, where TS_i and TS_f denote initial and final total sugar concentration, respectively

Table 38 Comparison of hydrogen yield in various types of sugar by different *Clostridium* species

Microorganism	Substrate	H_2 yield (mol H_2/mol substrate)	Reference
C. butyricum	glucose	1.40-2.30	[40]
C. beijerinckii	glucose & starch	1.20-20	[41]
C. acetobutylicum	glucose	1.97	[42]
C. paraputrificum M-21	glucose	1.40	[43]
C. beijerinckii AM21B	glucose	1.80-2.00	[44]
C. cellobioparm	glucose	2.73	[45]
C.pasteurianum	glucose	1.50	[44]
Clostridium sp.	glusoce	0.85	[46]
C. beijerinckii	glucose	2.00	[47]
Clostridium. sp. strain No. 2	glucose	2.36	[47]
C. acetolyticum	glucose	2.00	[19]
C. pasterium (dominant)	sucrose	4.80	[48]
Clostridium sp.	microcrystalline	2.18	[49]
Clostridium. sp. strain No. 2	xylose	1.80-2.10	[47]
Hydrogen producing sludge (dominated by *Clostridium* sp.)	xylose	1.30	[50]
C. uliginosum sp. nov.	xylose	2.59	[51]
C. butyricum CGS5	xylose	0.68-0.73	[34]
C. butyricum	SCB* hydrolysate (mixed sugars of glucose, xylose and arabinose)	1.73	This study

*SCB: sugarcane bagasse

5.4 Conclusion

We demonstrated that acid hydrolysate of SCB was suitable for producing hydrogen by *C. butyricum* due to its high sugar concentration (glucose, xylose, arabinose) and low growth inhibitors concentrations (HAc and furfural). The optimal condition for the hydrolysis of SCB was the use of 0.5% H_2SO_4 under 121 °C and 1.5 Kg/cm^2 in autoclave for 60 min. The highest HPR and hydrogen yield (1,611 mL H_2/L.day and 1.73 mol H_2/mol total sugar, respectively) were obtained at an optimal fermentation condition of initial pH 5.5, 37°C, and an initial total sugar in SCB hydrolysate of 20 g-COD/L. The hydrogen performance were compared favorably to those reported in the literature ensuring that SCB hydrolysate could be used as a fermentation media for hydrogen production by *C. butyricum*.

Acknowledgements

The authors are grateful to the Commission on Higher Education for financial support of this work. We greatly appreciate Sureewan Sittijunda for her valuable technical assistance.

References

[1] Han SK, Shin HS. Biohydrogen production by anaerobic fermentation of food waste. Int J Hydrogen Energy 2004;29:569-77.

[2] 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;29:1607-16.

[3] Kapdan LK, Kargi F. Bio-hydrogen production from waste materials. Enzyme Microbial Technol 2006;38:569–82.

[4] Koku H, Eroglu I, Gunduz U, Yucel M, Turker L. Aspects of the metabolism of hydrogen production by *Rhodobacter sphaerides*. Int J Hydrogen Energy 2002;27:1315-29.

[5] 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;2:895-9.

[6] 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;30:1063-70.

[7] Chin HL, Chen ZS, Chou CP. Fed-batch operation using *Clostriduim acetobutylicum* suspension culture as biocatalyst for enhancing hydrogen production. Biotechnol Progress 2003;19:383-8.

[8] Ferchichi M, Crabbe E, Gil GH, Hintz W, Almadidy A. Influence of initial pH on hydrogen production from cheese whey. J Biotechnol 2005;120:402-9.

[9] Liu G, Shen J. Effects of culture medium and medium conditions on hydrogen production from starch using anaerobic bacteria. J Biosci Bioeng 2004; 98:251–6.

[10] Lin CY, Lay CH. Carbon/nitrogen-ratio effect on fermentative hydrogen production by mixed microflora. Int J Hydrogen Energy 2004;29:41-5.

[11] Hawkes FR, Hussy I, Kyazze G, Dinadale R, Hawkes DL. Continuous dark fermentative hydrogen production by mesophilic microflora: principles and progress. Int J Hydrogen Energy 2007;32:172-84.

[12] 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.pdf.

[13] Neureiter M, Danner H, Thomasser C, Saidi B, Braum R. Dilute-acid hydrolysis of sugarcane bagasse at varying conditions. Appl Biochem Biotechnol 2002;49:98-100.

[14] Almazan O, Gonzalez L, Galvez L. The sugarcane, its by-products and co-products. Sugar Cane International 2001;7:3-8.

[15] Caraschi JC, Campana F, Curvelho AAS. Preparation and characterization of dissolving pulps obtained from sugar cane bagasse. Polimeros:Cienciae Technolgia 1996;6:24-9.

[16] Laser M, Schulman D, Allen SG, Lichwa J, Antal MJ, Lynd LR. A comparison of liquid hot water and steam pretreatments of sugarcane bagasse for bioconversion to ethanol. Bioresource Technol 2002;81:33-44.

[17] Gamez S, Gonzalez-Cabriales JJ, Ramirez JA, Garrote G, Vazquez M. Study of hydrolysis of sugarcane bagasse using phosphoric acid. J Food Eng 2005; 74: 78-88.

[18] Aguilar R, Ramirez JA, Garrote G, Vazquez M. Kinetic study of the acid hydrolysis of sugarcane bagasse. J Food Eng 2002;55:309-18.

[19] Chin HL, Chen ZS, Chou CP. Fed-batch operation using *Clostridium acetobutylicum* suspension cultures as biocatalyst for enhancing hydrogen production. Biotechnol Prog 2003;19:383-8.

[20] Yokoi H, Maeda Y, Hirose J, Hayashi S. Hydrogen production by immobilized cell of *Clostridium butyricum* on porous glass beads. Biotechnol Techniques 1997;11:431-3.

[21] Sneath PHA, Mair NS, Sharpe ME, Holt JG. Bergey's manual of systematic bacteriology volume 2. The Williams and Wilkins. Baltimore USA, 1986.

[22] Holdeman LV, Cato EP, Moore WEC. Anaerobe laboratory manual 4th. Southern printing Co. Blacksburg, Virginia USA, 1977.

[23] Nigam JN. Cultivation of *Candida langeronii* in sugarcane bagasse hemicellulosic hydrolysate for the production of single cell protein. World J Microbiol Biotechnol 2000;16:367-72.

[24] Lay JJ, Lee YJ, Noike T. Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Resources 1999;33:2579-86.

[25] Owen W, Stuckey C, Healy J, Young L, McCarty P. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Resources 1978;13:485-93.

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

[27] Zwietering MH, Jongenburger L, Rombouts FM, Van't RK. Modeling the bacterial growth curve. Appl Environ Microbiol 1990;56:1875-81.

[28] Rodriguez-Chong A, Ramirez JA, Garrote G, Vazquez M. Hydrolysis of sugarcane bagasse using nitric acid: a kinetic assessment. J Food Eng 2004;61:143-52.

[29] Liu ZL, Slininger PJ, Dien BS, Berhow MA, Kurtzman CP, Gorsich SW. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J Ind Microbiol Biotechnol 2004; 31:345-52.

[30] Chandel AK, Kapoor RK, Singh A, Kuhad RC. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. Bioresource Technol 2007; 98: 1947–50.

[31] Zoetemeyer RJ, Arnoldy P, Cohen A, Boelhouwer C. Influence of temperature on the anaerobic acidification of glucose in a mixed culture forming part of a two-stage digestion process. Water Resources 1982;16:313-21.

[32] Wu SY, Lin CY, Lee KS, Hung CH, Chang JS, Lin PJ, Chang FY. Dark fermentation hydrogen production from xylose in different bioreactors using sewage sludge microflora. Energy&Fuels 2008;22:113-119.

[33] Chen SD, Lee KS, Lo YC, Chen WM, Wu JF, Lin CY, Chang JS. Batch and continuous biohydrogen production from starch hydrolysate by *Clostridium* species. Int J Hydrogen Energy 2008;33:1803-12.

[34] Lo YC, Chen WM, Hung CH, Chen SD, Chang JS. Dark H_2 fermentation from sucrose and xylose using H_2-producing indigenous bacteria: feasibility and kinetic studies. Water Res 2008; 827-42.

[35] Wang CH, Chang JS. Continuous biohydrogen production from starch with granulated mixed bacterial microflora. Energy&Fuels 2008; 22:93-7.

[36] Das D, Veziroglu TN. Hydrogen production by biological processes: a survey of literature. Int J Hydrogen Energy 2001;26:13-28.

[37] Lenz TO, Moreira AR. Economic evaluation of the acetone butanol fermentation. Ind Eng Chem Prod Res Dev 1980;19:478-83.

[38] Fan YT, Zhang YH, Zhang SF, Hou HW, Ren BZ. Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. Bioresource Technol 2006;97:500-5.

[39] Fan YT, Li CL, Lay JJ, Hou HW, Zhang GS. Optimization of initial substrate and pH levels for germination of sporing hydrogen-producing anaerobes in cow dung compost. Bioresource Technol 2004;91:189-93.

[40] Kataoka N, Miya K, Kiriyama K. Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria. Water Sci Technol 1997; 36: 41-7.

[41] Taguchi F, Chang JD, Takiguchi S, Morimoto A. Efficient hydrogen production from starch by a bacterium isolate from termites. Ferment Bioeng 1992; 73: 244-5.

[42] Saint-Amans S, Girbal L, Andrade J, Ahrens K, Soucaille P. Regulation of carbon and electron flow in *Clostridium butyricum* VPI 3266 grown on glucose-glycerol mixtures J Bacteriol 2001; 183: 1748-54.

[43] Evvyerine D, Morimoto K, Karita S, Kimura T, Sakka K, Ohmiya K. Conversion of chitinous wastes to hydrogen gas by *Clostridium parputrificum* M-21. J Biosci Bioeng 2001; 91: 339-43.

[44] Nandi R, Sengupta S. Microbial production of hydrogen: an overview. Crit Rev Microbiol 1998; 24: 61-84.

[45] Chung KT. Inhibitory effects of H_2 on growth of *Clostridium cellobioparum.* Appl Environ Microbiol 1976;31:342-8.

[46] Mizuno O, Dinsdale R, Hawkes FR, Hawkes DL, Noike T. Enhancement of hydrogen production from glucose by nitrogen gas sparging. Biores Technol 2000; 73: 59-65.

[47] Taguchi F, Mizukami N, Saito-Taki T, Hasegawa K. Hydrogen production from continuous fermentation of xylose during growth of *Clostridium* sp. strain No. 2. Can J Microbiol 1995; 41: 536-40.

[48] Lin CY, Chang RC. Fermentative hydrogen production at ambient temperature. Int J Hydrogen Energy 2004;29:715-20.

[49] Lay JJ. Biohydrogen generation by mesophilic anaerobic fermentation of microcrystalline cellulose. Biotechnol Bioeng 2001;74:281-7.

[50] Lin CY, Wu CC, Hung CH. Temperature effects on fermentative hydrogen production from xylose using mixed anaerobic cultures. Int J Hydrogen Energy 2008b;33:43-50.

[51] Matthies C, Kuhner CH, Acker G, drake HL. *Clostridium uliginosum* sp. nov., a novel acid-tolerant, anaerobic bacterium with connecting filaments. Int J Syst Evol Microbiol 2001; 1119-25.