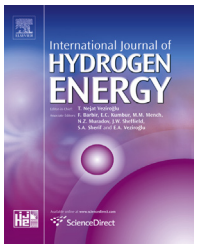
Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/he



Simultaneous saccharification and fermentation of cellulose for bio-hydrogen production by anaerobic mixed cultures in elephant dung

Arunsri Fangkum Saripan [a,b], *Alissara Reungsang* [b,c,*]

[a] *Environmental Science Program, Faculty of Science and Technology, Thepsatri Rajabhat University, Lopburi 15000, Thailand*

[b] *Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand*

[c] *Research Group for Development of Microbial Hydrogen Production Process from Biomass, Khon Kaen University, Khon Kaen 40002, Thailand*

ARTICLE INFO

Article history:
Received 30 January 2014
Received in revised form
1 April 2014
Accepted 7 April 2014
Available online 5 May 2014

Keywords:
Bio-hydrogen
Elephant dung
Thermophilic
Carboxymethyl cellulose
Sugarcane bagasse

ABSTRACT

The objective of this study was to optimize the culture conditions for simultaneous saccharification and fermentation (SSF) of cellulose for bio-hydrogen production by anaerobic mixed cultures in elephant dung under thermophilic temperature. Carboxymethyl cellulose (CMC) was used as the model substrate. The investigated parameters included initial pH, temperature and substrate concentration. The experimental results showed that maximum hydrogen yield (HY) and hydrogen production rate (HPR) of 7.22 ± 0.62 mmol H_2/g CMC_{added} and 73.4 ± 3.8 mL H_2/L h, respectively, were achieved at an initial pH of 7.0, temperature of 55 °C and CMC concentration of 0.25 g/L. The optimum conditions were then used to produce hydrogen from the cellulose fraction of sugarcane bagasse (SCB) at a concentration of 0.40 g/L (equivalent to 0.25 g/L cellulose) in which an HY of 7.10 ± 3.22 mmol H_2/g $cellulose_{added}$. The pre-dominant hydrogen producers analyzed by polymerase chain reaction-denaturing gel gradient electrophoresis (PCR-DGGE) were *Thermoanaerobacterium thermosaccharolyticum* and *Clostridium* sp. The lower HY obtained when the cellulose fraction of SCB was used as the substrate might be due to the presence of lignin in the SCB as well as the presence of *Lactobacillus parabuchneri* and *Lactobacillus rhamnosus* in the hydrogen fermentation broth.

Introduction

Hydrogen is a clean and sustainable energy carrier i.e. upon its combustion with oxygen, only water is obtained as a by-product. Hydrogen has a high energy content of 122 kJ/g which is 2.75 times higher than fossil fuel such as gasoline [1]. Biologically, hydrogen can be produced by photo production process and dark fermentation process. Dark fermentation process has the advantages over photo production process in terms of a higher rate of hydrogen production and more versatility of the substrates can be used [2–5].

* *Corresponding author*. Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand. Tel./fax: +66 43 362 121.
E-mail addresses: alissara@kku.ac.th, reungsang4@gmail.com (A. Reungsang).

http://dx.doi.org/10.1016/j.ijhydene.2014.04.066

Different types of feedstocks i.e. food crops (first generation feedstock), lignocellulosic materials (second generation feedstock) and microalgae (third generation feedstock) have been used to produce hydrogen by dark fermentation process [6]. However, the feedstock for hydrogen production should come from non-food crops in order to avoid a competition between food source and energy source. Hence, lignocellulosic materials are promising feedstock for producing hydrogen due to its compositions that are rich in polysaccharide that can subsequently be hydrolyzed to produce fermentable sugar. In addition, lignocellulosic materials are abundant with no/low cost. The compositions of lignocellulosic materials are 30–56% cellulose, 10–27% hemicellulose and 3–30% lignin [7]. Cellulose consists of linear and highly chains of glucose that is suitable as a carbon source to produce hydrogen by the biological method. However, due to its complex structure, cellulose is difficult to be used as a feedstock for hydrogen production. Therefore, only a few studies have been conducted using cellulose to directly produce hydrogen [5,8] while the research on using hydrolysate of cellulosic or lignocellulosic materials as substrate to produce hydrogen is well-documented [9–13]. Thus, a further investigation on finding efficient methods to directly produce hydrogen from cellulose is needed.

In this study, simultaneous saccharification and fermentation (SSF) process was selected as an efficient method to produce hydrogen due to its advantages including a higher hydrolysis rate, lower enzyme requirements, higher product yield, shorter process time (since glucose is removed immediately and hydrogen is produced) and a smaller reactor volume (using single reactor) [14]. The mixed cultures in elephant dung were chosen as a seed culture to produce hydrogen from cellulose with the hypothesis that the cellulolytic bacteria that can degrade cellulose to glucose and non-cellulolytic bacteria that can produce hydrogen from the resulting glucose would present in elephant dung due to the diet of the elephant which comprised of mainly plant materials.

In order to obtain a successful hydrogen production process, there is a need to optimize the important process parameters such as substrate concentration, initial pH and temperature. Substrate concentration affects metabolic pathways and microbial community structures [15,16], while the initial pH directly affects the activity of the iron-containing hydrogenase enzyme that is responsible for the production of hydrogen [17] as well as the extent of the lag phase in batch hydrogen production [18]. Temperature affects the rate of biochemical processes [19]. Hydrogen production at high temperature by mixed thermophilic bacteria favors reaction kinetics and has a positive effect on biocatalyst activity promoting hydrogen production [20].

Therefore, in order to maximize thermophilic hydrogen production from cellulose by microorganisms in elephant dung using the SSF process, the optimization of important process parameters including initial pH, temperature and cellulose concentration, was conducted. The possibility of producing hydrogen from natural cellulose i.e. the cellulose fraction of sugarcane bagasse (SCB) was explored under the optimum initial pH, CMC concentration, and temperature. The findings from this research would provide the important information toward the use of lignocellulosic materials for energy (hydrogen) production.

Materials and methods

Seed microorganisms

Elephant dung was obtained from the elephant village, Surin, Thailand. Before it was directly used as the seed cultures, 100 g of elephant dung was chopped into small pieces and heat-treated in an LDO-100E hot air oven (Lab Tech, Korea) for 2 h at 105 °C in order to inhibit methanogenic activity and to harvest hydrogen-producing spore forming anaerobes. The elephant dung comprised of 47.36% cellulose, 18.97% hemicellulose and 14.91% lignin. The volatile solid (VS) of heat-treated elephant dung was 827.34 g-VS/kg.

Cellulose fraction of SCB

SCB was obtained from a local chipboard industry (Panel Plus Ltd.), Chaiyaphum, Thailand. Compositions of SCB are 51.52% cellulose, 23.49% hemicellulose, and 8.33% lignin.

The cellulose fraction of SCB was the solid residue obtained after acid hydrolysis of SCB with 1% (v/v) H_2SO_4 at a mass ratio of SCB:H_2SO_4 of 1:15 [21]. Prior the usage, the cellulose fraction of SCB was washed and soaked in tap water for 5 min. This process was conducted several times until the pH of cellulose fraction of SCB was 7. Then, it was dried in an LDO-100E hot air oven (Lab Tech, Korea) at 105 °C for 3 h. The obtained cellulose fraction of SCB contained 62.50% cellulose, 8.80% hemicellulose and 16.41% lignin.

Batch hydrogen fermentation with carboxymethyl cellulose (CMC)

CMC was chosen as the representative of natural cellulose because of its amorphous cellulose structure and a high reducing sugar production rate. The optimization of initial pH, temperature and CMC concentrations for hydrogen production by thermophilic microorganisms in the elephant dung were conducted in a batch mode. The hydrogen production at different initial pH (pH 5.0, 6.0, 7.0 and 8.0) was first investigated at the initial CMC concentration of 2.5 g/L and 55 °C. The effects of temperature (45, 50, 55 and 60 °C) on hydrogen production were then observed under the obtained optimum initial pH and CMC concentration of 2.5 g/L. The effects of CMC concentrations (0.1, 0.25, 0.5 and 0.75 g/L) on hydrogen production were further investigated at the optimum initial pH and temperature.

All batch fermentations were conducted in 120 mL serum bottles with a working volume of 70 mL comprised 3.5 g of elephant dung (27.34 g-volatile suspended solid (VSS)/L) and 70 mL of CMC solution. The CMC solution was prepared in a buffer solution containing inorganic nutrients at the final concentration of (all in mg/L): 5240 NH_4HCO_3; 125 K_2HPO_4; 15 $MgCl_2{\cdot}6H_2O$; 25 $FeSO_4{\cdot}7H_2O$; 5 $CuSO_4{\cdot}5H_2O$ and 0.125 $CoCl_2{\cdot}5H_2O$ (modified from Endo et al., 1982 [22]). Citrate-phosphate buffer solution, phosphate buffer solution and sodium-phosphate buffer solution were used in the treatment

with an initial pH of 5.0, 6.0–7.0 and 8.0, respectively. The initial pH of the CMC solution inoculated with elephant dung was slightly adjusted to the designated values using 2 mol/L HCl or 2 mol/L NaOH. The serum bottles were purged with nitrogen gas for 5 min in order to create anaerobic condition. The serum bottles were closed with rubber stoppers and capped with aluminum caps. During the fermentation, biogas and soluble metabolite products (SMPs) were monitored until the end of hydrogen production. Control vial without CMC was conducted in order to account for background hydrogen production from self-fermentation of elephant dung. The background hydrogen production was subtracted from the hydrogen production in the vial with CMC for obtaining the actual hydrogen production. All treatments were conducted in triplicates.

Batch hydrogen fermentation with the cellulose fraction of SCB

The obtained optimum initial pH, temperature and CMC concentration were used to produce hydrogen from the cellulose fraction of SCB. Cellulose fraction of SCB was prepared in phosphate buffer solution. The experimental methods followed the protocols in section Batch hydrogen fermentation with carboxymethyl cellulose (CMC). Control vials without cellulose fraction of SCB and without elephant dung, respectively, were included in order to account for background hydrogen production from indigenous microorganisms in SCB and self-fermentation of elephant dung, respectively. The background hydrogen produced in the control vials was subtracted from the hydrogen produced in vial with the cellulose fraction of SCB and elephant dung in order to obtain the actual hydrogen production from cellulose fraction of SCB by mixed cultures in elephant dung. All treatments were conducted in triplicates.

Analytical method

During the incubation, volume of biogas was measured by the plunger displacement method [23]. Gas composition was measured by gas chromatography (GC) using a thermal conductivity detector (TCD) and Unibead C column. The GC conditions followed the method previously described by Pattra et al., 2008 [24]. Volatile fatty acids (VFAs), acetone and alcohols were detected by GC (model Shimadzu 14B, Japan) equipped with a flame ionization detector (FID) and Porapak Q column. The operational conditions of GC were similar to our previous report [21].

Prior to analysis of lactic acid concentration, the liquid sample was centrifuged at 10,000 rpm for 5 min, acidified by 0.2 N oxalic acid and filtered through a 0.2 μm nylon membrane [25]. The high performance liquid chromatography (HPLC) analysis was carried out to determine the concentration of lactic acid using an LC-10AD (Shimadzu, Japan) with an Aminex HPX-87H column. The operational conditions of HPLC followed the method of Fangkum and Reungsang [25].

Endoglucanase activity was measured using the modified method of Nitisinprasert and Temmes [26]. A reaction mixture contained 0.1 mL of crude enzyme and 0.9 mL of 0.1% CMC in acetate buffer (pH 4.5) and incubated at 55 °C for 30 min. The reaction was terminated by ice cool. The reducing sugar was estimated with dinitrosalicylic reagent method using glucose as standard [27].

The microbial community analysis in the hydrogen fermentation broth using CMC or cellulose fraction of SCB as the substrate under the optimum conditions was conducted by PCR-DGGE analysis according to the method described in our previous report [21].

Cumulative hydrogen production was calculated from the headspace measurement of the gas composition and total volume of biogas produced at each time interval using the mass balance equation [21,28]. The HPR was calculated from the cumulative hydrogen production divided by fermentation time (h) (mL-H_2/L h). The HY was calculated from total molaric amount of hydrogen divided by the amount of CMC added (mmol-H_2/g CMC_{added}). The total molaric amount of hydrogen was calculated using the ideal gas law [25,29].

Results and discussions

Hydrogen production from CMC by anaerobic mixed cultures in elephant dung

Effect of initial pH on hydrogen production

Results showed that the cumulative hydrogen production increased with an increase in the initial pH from 6.0 to 7.0 and sharply decreased with an increase in the initial pH from 7.0 to 8.0 (Table 1). A maximum cumulative hydrogen production of 761 mL-H_2/L was achieved at an initial pH of 7.0 (Table 1). At low pH (pH 5.0), hydrogen could not be produced which could possibly due to the formation of acidic metabolites that may destabilize the ability of the microbial cells to maintain internal pH, resulting in a decrease in the internal level of ATP and inhibiting substrate uptake [30]. At an initial pH of 7.0, the maximum HY and HPR were 11.32 ± 0.28 mmol-H_2/g-

Table 1 – Effect of initial pH on hydrogen yield (HY), hydrogen production rate (HPR), cumulative hydrogen production (CHP) and soluble metabolite product (SMP) concentrations from carboxymethyl cellulose at a concentration of 2.5 g/L and a temperature of 55 °C.

pH	HY (mmol-H_2/g CMC_{added})	HPR (mL-H_2/L h)	CHP (mL-H_2/L)	SMP (mM)				
				Ethanol	Acetic	Butyric	Butanol	Lactate
6	0.64 ± 0.12[a]	54.5 ± 10.3[a]	43	0.08	3.84	0.11	0.00	0.00
7	11.32 ± 0.28[c]	1074.5 ± 23.8[c]	761	0.81	16.21	0.02	0.17	0.01
8	5.50 ± 0.37[b]	467.0 ± 31.2[b]	320	0.27	18.18	0.03	0.06	0.04

Different letters indicate significant difference among treatment by the Duncan's test ($p < 0.05$).

Table 2 – Effect of temperature on hydrogen yield (HY), hydrogen production rate (HPR), cumulative hydrogen production (CHP) and soluble metabolite product (SMP) concentrations from carboxymethyl cellulose at a concentration of 2.5 g/L and initial pH of 7.

Temp. (°C)	HY (mmol-H_2/g CMC_{added})	HPR (mL-H_2/L h)	CHP (mL-H_2/L)	SMP (mM)				
				Ethanol	Acetate	Butanol	Butyric	Lactate
45	2.62 ± 0.38^a	314.9 ± 46.1^a	171	0.42	20.87	0.03	0.10	0.00
50	8.49 ± 1.51^b	642.7 ± 114.2^b	562	0.76	30.59	0.03	0.14	0.00
55	11.32 ± 0.28^c	1074.5 ± 26.4^c	761	0.32	29.74	0.03	0.07	1.10
60	8.90 ± 0.45^b	690.2 ± 35.3^b	604	0.12	17.57	0.10	0.05	0.00

Different letters indicate significant difference among treatment by the Duncan's test ($p < 0.05$).

CMC_{added} and 1074.5 ± 23.8 mL-H_2/L h, respectively. At high pH (pH 8), low hydrogen production was observed. Wang and Wan, 2009 [31] reported that the activity of hydrogenase is inhibited at high pH. Our optimal pH of 7 was in agreement with other studies investigating hydrogen production from cellulose under thermophilic conditions. For example, Lin and Hung, 2008 [1] reported an initial pH of 7.0 was optimal for thermophilic hydrogen production (55 °C) from cellulose by anaerobic cow dung microflora with an HY of 2.8 mmol-H_2/g-cellulose. At an optimal initial pH of 7.0, *Clostridium thermocellum* JN4 produced a maximum HY of 10 mmol-H_2/g-cellulose from cellulose at an incubation temperature of 60 °C [32].

Concentration of each VFAs and alcohol at the end of each batch test are presented in Table 1. The results demonstrate that a decrease in pH and an increase in VFAs occurred in parallel with hydrogen production. The most abundant SMPs was acetate, comprising greater than 94% of the total end products in all batch tests, followed by ethanol, butanol, butyrate and lactate, respectively. Results suggested that a hydrogen production from CMC by anaerobic mixed cultures in elephant dung was acetate type fermentation.

Effect of temperature on hydrogen production

The cumulative hydrogen production from CMC by anaerobic mixed cultures in the elephant dung was found to increase with an increase in the incubation temperature from 45 °C to 55 °C. A further increase in incubation temperature from 55 °C to 60 °C resulted in a decrease in the cumulative hydrogen production (Table 2). Temperature higher than the optimum temperature could induce a denaturation of hydrogenase resulting in a low microbial activity and a decrease in HY and HPR [33]. At a lower temperature, the inhibition of some essential enzyme such as hydrogenase and pyruvate-ferredoxin oxidoreductase occurred resulting in a reduction of hydrogen production and a shift of by-product composition [34,35]. Thus, the optimal temperature for hydrogen production from CMC by anaerobic mixed cultures in elephant dung was 55 °C. The trends of HY and HPR were similar to the cumulative hydrogen production in which the maximum HY and HPR of 11.32 ± 0.28 mmol-H_2/g-CMC_{added} and 1074.5 ± 26.4 mL-H_2/L h, respectively, were obtained at 55 °C. The optimum temperature in this study was different from the report of Lo et al., 2011 [36] that the optimum temperature for hydrogen production from filter paper by *Clostridium* sp. TCW1 was 60 °C. The different in the optimum temperature might be due to the different types of microorganisms.

The main SMPs at different incubation temperatures was acetate (Table 2). This result suggested that the fermentation type at the temperature in the ranges of 45–50 °C was acetate–ethanol type while at 55 °C was the acetate–lactate type and at 60 °C was acetate type. Under high temperature (60 °C), the hydrogen production by thermophilic anaerobic bacteria followed the Embden–Meyerhof pathway. In this pathway, glucose is converted to pyruvate and then pyruvate is converted to acetylcoenzyme A (acetyl-CoA), carbon dioxide, and hydrogen by pyruvate-ferredoxin oxidoreductase and hydrogenase [35]. Acetyl Co-A is finally converted to acetate, butyrate and ethanol, depending on the microorganisms and the environmental conditions [35,37].

Other SMPs i.e. butyrate was detected in the hydrogen fermentation broth at every temperature. The highest SMPs was observed at temperature of 55 °C corresponding to the highest cumulative hydrogen production, HY and HPR. The changes in acetate, ethanol and lactate concentration in the SMPs of each batch test with increasing temperature may be resulted from the metabolic pathway shift caused by different pre-dominant bacteria at each temperature.

Table 3 – Effect of carboxymethyl cellulose (CMC) concentration on hydrogen yield (HY), hydrogen production rate (HPR), cumulative hydrogen production (CHP) and soluble metabolite product (SMP) concentrations at 55 °C and initial pH of 7.

CMC concentration (g/L)	HY (mmol-H_2/g CMC_{added})	HPR (mL-H_2/L h)	CHP (mL-H_2/L)	SMP (mM)				
				Ethanol	Acetate	Butanol	Butyrate	Lactate
0.1	3.70 ± 1.01^b	17.1 ± 3.6^a	10	0.54	1.54	0.02	0.04	0.00
0.25	7.22 ± 0.62^c	73.4 ± 3.8^b	49	1.49	1.55	0.02	0.03	0.19
0.5	2.46 ± 0.26^b	56.6 ± 4.2^b	33	1.05	1.84	0.03	0.02	0.00
0.75	0.61 ± 0.06^a	18.0 ± 1.3^a	12	0.75	2.00	0.06	0.00	0.00

Different letters indicate significant difference among treatment by the Duncan's test ($p < 0.05$).

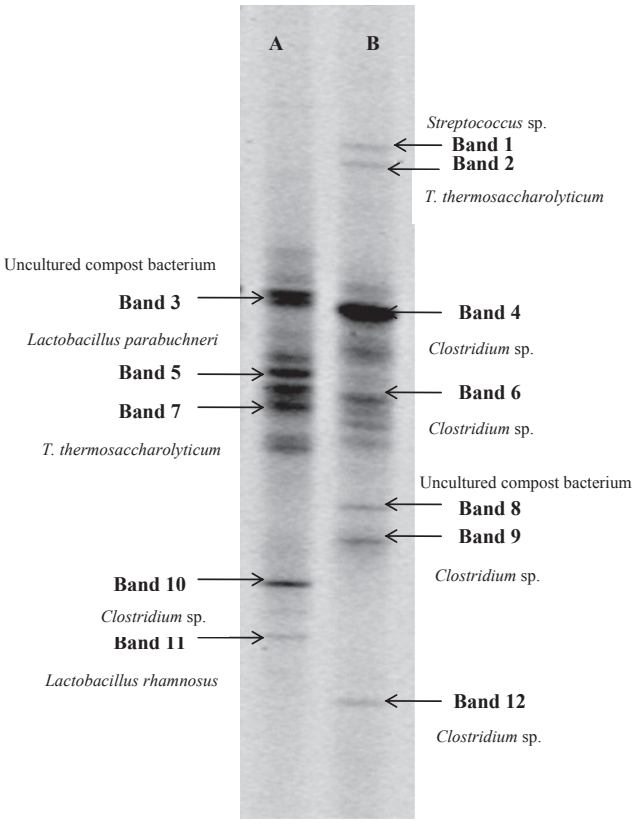


Fig. 1 – DGGE band profile for each substrate at the optimum conditions. Lanes: A, microorganism taken from hydrogen fermentation broth of cellulose fraction of sugarcane bagase (SCB); B, microorganism taken from hydrogen fermentation broth of carboxymethyl cellulose (CMC).

Effect of CMC concentration on hydrogen production

The effect of CMC concentration on hydrogen production was conducted under the optimal initial pH (7.0) and optimal temperature (55 °C). The cumulative hydrogen production increased with an increase in the CMC concentration from 0.1 to 0.25 g/L, but decreased from 49 mL-H_2/L to 12 mL-H_2/L with a further increase in the CMC concentration from 0.25 to 0.75 g/L (Table 3). The trends of HY and HPR were similar to cumulative hydrogen production. The HY and HPR were decreased when the CMC concentration was greater than 0.25 g/L which may be resulted from the substrate and by-product inhibition that occurred during hydrogen production [21,38,39].

During hydrogen fermentation processes, SMPs i.e. acetate, ethanol, lactate, butyrate and butanol were detected in the fermentation broth (Table 3). Among these, the content of acetate accounted for 90–96% of the total SMPs indicating that the hydrogen fermentation from CMC by anaerobic mixed cultures in the elephant dung is acetate type fermentation.

Therefore, the optimum conditions for hydrogen production from CMC by anaerobic mixed cultures in elephant dung were an initial pH of 7.0, temperature of 55 °C and CMC concentration of 0.25 g/L. Under the optimum conditions, the maximum cumulative hydrogen production, HY and HPR of 49 mL-H_2/L, 7.22 ± 0.62 mmol-H_2/g-CMC_{added} and 73.4 ± 3.8 mL-H_2/L h, respectively, were achieved. The cumulative hydrogen production of this study (49 mL-H_2/L) was a bit lower than that reported by Ren et al. [40] (1750 mL-H_2/L) which produced hydrogen from CMC by co-cultures of *Clostridium acetobutylicum* X_9 and *Ethanoigenens harbinense* B_{49}. Co-culture could rapidly hydrolyze CMC and produce hydrogen in which the strain X_9 is cellulose hydrolyzer while the strain B_{49} is hydrogen producer. In addition, the cumulative hydrogen production of this study was lower than that report by Nguyen et al. [41] (175.7 mL-H_2/L and 172.4 mL-H_2/L) which produced hydrogen from CMC by *Thermotoga maritina* and *Thermotoga neapolitana*. Such discrepancies depended on the different in inoculum used.

It is worth noting that the CMC concentration used in this study is quite low in comparison to the other potential substrate. Thus, we suggest that the pretreatment of CMC by dilute or strong acid, cellulase as well as a co-culture of cellulose hydrolyzer and hydrogen producer would improve the process performance at a high organic loading.

Despite the fact that we did not measure the amount of hexose and pentose sugars released during and at the end of the fermentative hydrogen production process, our findings together with the evidence of the endoglucanase activity of mixed cultures in the elephant dung in the ranges of 0.005–0.20 Unit/mL (data not shown) during the bio-hydrogen production process indicated that the mixed cultures in elephant dung were a potential cellulose hydrolyzer as well as hydrogen producer that can simultaneously hydrolyze cellulose and use the resulting sugar to produce hydrogen.

Hydrogen production from the cellulose fraction of SCB by the anaerobic mixed cultures in elephant dung

Previous researches focused on bio-hydrogen production from the hydrolysate of lignocellulosic materials such as beer less waste [9], agro-industrial materials [11], cellulolytic materials [12] and cellulose [13]. However, the report on bio-hydrogen production from cellulose fraction of SCB by SSF is still limited. Therefore, this experiment was designed to directly produce hydrogen from cellulose fraction of SCB by anaerobic mixed cultures in elephant dung, using SSF. The optimum conditions were used in practice to produce hydrogen from the cellulose fraction of SCB at a concentration of 0.4 g/L (equivalent to the optimal cellulose concentration of 0.25 g/L). Cumulative hydrogen production, HY and HPR of 47 mL-H_2/L, 7.10 ± 3.22 mmol-H_2/g-$cellulose_{added}$ and 115.2 ± 52.8 mL-H_2/L h, respectively, were achieved. Acetate (78.1%) was detected as the major SMPs, followed by lactate (14.4%), butyrate (5.6%), ethanol (1.5%) and butanol (0.3%), respectively. The results indicated that the anaerobic mixed cultures in elephant dung could simultaneously degrade cellulose fraction in SCB to sugar and use the resulting sugar for hydrogen production. Though, a low hydrogen production was obtained which may be due to the complex structure of the cellulose fraction of SCB. In addition, the presence of lactic acid bacteria might also contribute to a low HY and HPR (see section Microbial community structure analysis).

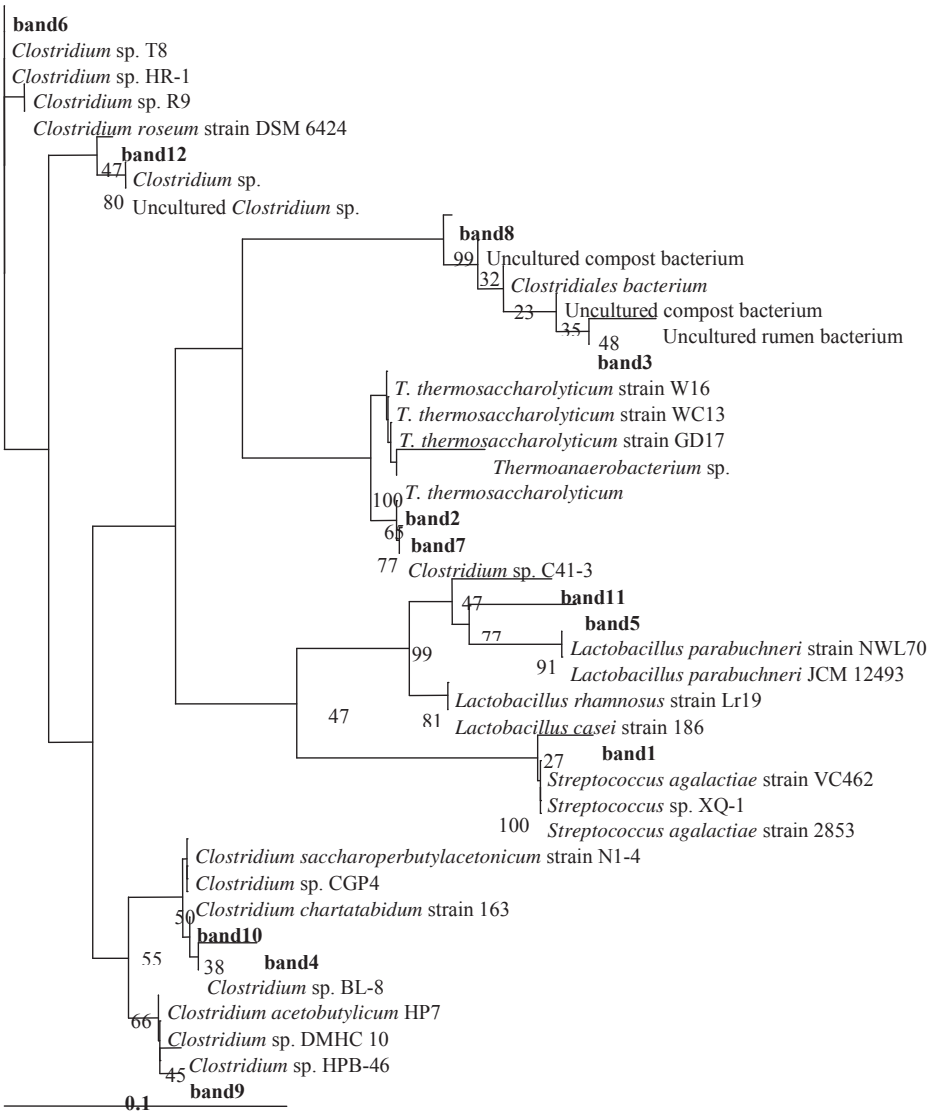


Fig. 2 – Phylogenetic tree showing the relationship between DGGE bands detected in this study and reference sequences based on a comparison of 16S rRNA sequences. The bar corresponds to a 10% difference in nucleotide sequence. The numbers shown next to the nodes indicate percent bootstrap values from 1000 iterations.

Microbial community structure analysis

The structures of microbial community in the fermentation broth using cellulose fraction of SCB as the substrate: Lane A and CMC as the substrate: Lane B are illustrated in Fig. 1. The phylogenetic tree of the dominant species and their close relatives based on partial 16S rRNA gene is shown in Fig. 2. Bands 3, 5, 7, 10 and 11 were found in the fermentation broth of cellulose fraction of SCB. Bands 3 and 5 were similar to uncultured bacteria and *Lactobacillus parabuchneri*, respectively. Bands 7 and 10 were similar to *Clostridium* sp. while Band 11 was similar to *Lactobacillus rhamnosus*. Bands 1, 2, 4, 6, 8, 9, 12 were found in the fermentation broth of CMC. Band 1 affiliated with *Streptococcus* sp. while band 2 affiliated with *Thermoanaerobacterium thermosaccharolyticum*. Bands 4, 5, 8, 9 and 12 affiliated with *Clostridium* sp. Band 6 was similar to uncultured bacteria. *T. thermosaccharolyticum* and *Clostridium* sp. are known hydrogen-producing bacteria [1,32,39]. *T. thermosaccharolyticum* could pre-dominant in the fermentation broth under thermophilic condition when using elephant dung as the source of inoculum. However, *T. thermosaccharolyticum* was not detected in the fermentation broth under mesophilic condition when elephant dung was used as the source of inoculum [21]. Therefore, the detection of thermophilic microorganisms i.e. *T. thermosaccharolyticum* in both hydrogen fermentation broth indicated a successful hydrogen fermentation under thermophilic condition [25,35].

The number of bands pre-dominant with hydrogen producer in fermentation broth of cellulose fraction of SCB (bands 7, 10) was lower than the number of bands dominant with hydrogen producer in the fermentation broth of CMC (bands 1, 2, 4, 6, 9 and 12). This could contribute to a low HY obtained when cellulose fraction of SCB was used as the substrate for hydrogen production. In addition, a low HY could be due to the

presence of lactic acid bacteria (LAB) i.e. *L. parabuchneri* and *L. rhamnosus*. These bacteria could produce bacteriocins that caused the adverse effect on hydrogen-producing bacteria [42]. In order to inhibit the activity of the LAB, the mandelic acid should be added to the fermentation broth [43].

Conclusions

This study demonstrated that anaerobic mixed cultures in elephant dung could simultaneously breakdown cellulose and utilized the resulting sugars to produce hydrogen. The optimum conditions for hydrogen production from CMC were an initial pH of 7.0, temperature of 55 °C and CMC concentration of 0.25 g/L. Under these optimum conditions, the cumulative hydrogen production, HY and HPR of 49 mL-H_2/L, 7.22 ± 0.62 mmol-H_2/g-CMC_{added} and 73.4 ± 3.8 mL-H_2/L h, respectively were obtained. The microorganisms in elephant dung could also use the cellulose fraction of SCB as the feedstock for hydrogen production with cumulative hydrogen production. The dominant hydrogen producers were *T. thermosaccharolyticum* and *Clostridium* sp. The detection of *L. parabuchneri* and *L. rhamnosus* in the hydrogen fermentation broth of the cellulose fraction of SCB may have been responsible for the low HY.

Acknowledgments

The authors appreciate the Ph.D. scholarship to AF from the Office of the Higher Education Commission, Thailand, under the Program Strategic Scholarships for Frontier Research Network for the Joint Thai Ph.D. Program. The authors are grateful for financial support received from Fermentation Research Center for Value Added Agricultural Products, Faculty of Technology, Khon Kaen University, and the National Research University Project through the Biofuels Research Cluster – Khon Kaen University, Office of the Higher Education Commission.

REFERENCES

[1] Lin CY, Hung WC. Enhancement of fermentative hydrogen/ethanol production from cellulose using mixed anaerobic cultures. Int J Hydrogen Energy 2008;33:3660–7.
[2] Kim MS, Lee DY. Fermentative hydrogen production from tofu-processing waste and anaerobic digester sludge using microbial consortium. Bioresour Technol 2010;101:548–52.
[3] Ren NG, Xu JF, Gao LF, Xin L, Qiu J, Su DX. Fermentative bio-hydrogen production from cellulose by cow dung compost enriched cultures. Int J Hydrogen Energy 2010;35:2742–6.
[4] Sittijunda S, Reungsang A, O-thong S. Biohydrogen production from dual digestion pretreatment of poultry slaughterhouse sludge by anaerobic self-fermentation. Int J Hydrogen Energy 2010;35:13427–34.
[5] Sreela-or C, Imai T, Plangklang P, Reungsang A. Optimization of key factors affecting hydrogen production from food waste by anaerobic mixed cultures. Int J Hydrogen Energy 2011;36:14120–33.
[6] Cheng CL, Lo YC, Lee KS, Lee DJ, Lin CY, Chang JS. Biohydrogen production from lignocellulosic feedstock. Bioresour Technol 2011;102:8514–23.
[7] Emtiazi G, Nahvi I. Multi-enzyme production by *Cellulomonas* sp. grown on wheat straw. Biomass Bioenerg 2000;19:31–7.
[8] Wang A, Gao L, Ren N, Xu J, Lin C. Bio-hydrogen production from cellulose by sequential co-culture of cellulosic hydrogen bacteria of *Enterococus gallinarum* G1 and *Ethanoigenens harbinense* B49. Biotechnol Lett 2009;31:1321–6.
[9] Fan YT, Zhang GS, Guo XY, Xing Y, Fan MH. Biohydrogen production from beer lees biomass by cow dung compost. Biomass Bioenerg 2006;30:493–6.
[10] Lo YC, Bai MD, Chen WM, Chang JJ. Cellulosic hydrogen production with a sequencing bacterial hydrolysis and dark fermentation strategy. Bioresour Technol 2008;99:8299–303.
[11] Prakasham RS, Sathish T, Brahmaiah P. Biohydrogen production process for use of agro-industrial material hydrolysate as substrate. Bioresour Technol 2010;101:5708–11.
[12] Taguchi F, Mizukami N, Yamada K, Hasegawa K, Saito-Taki T. Direct conversion of cellulolytic material to hydrogen by *Clostridium* sp. strain no 2. Enzyme Microbiol Technol 1995;17:147–50.
[13] Taguchi F, Yamada K, Hasegawa K, Taki-Saito T, Hara K. Continuous hydrogen production by *Clostridium* sp. strain no. 2 from cellulose hydrolysate in aqueous two-phase system. J Fermen Bioeng 1996;82:80–3.
[14] Sun Y, Cheng J. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 2002;83:1–11.
[15] Kim SH, Han SK, Shin HS. Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter. Process Biochem 2006;41:199–207.
[16] Kyazze G, Martinez-Perez N, Insdale R, Premeir GC, Hawkes FR, Guwy AJ. Influence of substrate concentration on the stability and yield of continuous biohydrogen production. Biotechnol Bioeng 2006;93:971–9.
[17] Dabrock B, Bahl H, Gottschal K. Parameters affecting solvent production by *Clostridium pasteurium*. Appl Environ Microbiol 1992;58:1233–9.
[18] Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzyme Microb Technol 2006;38:569–82.
[19] Fang HHP, Liu H. Effect of pH on hydrogen production from glucose by mixed culture. Bioresour Technol 2002;82:87–93.
[20] Chong ML, Sabaratnam V, Shirai Y, Ali Hassan M. Biohydrogen production from biomass and industrial wastes by dark fermentation. Int J Hydrogen Energy 2009;34:2377–87.
[21] Fangkum A, Reungsang A. Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: effects of initial pH and substrate concentration. Int J Hydrogen Energy 2011;36:8687–96.
[22] Endo G, Noike T, Matsumoto T. Characteristics of cellulose and glucose decomposition in acidogenic phase of anaerobic digestion. Proc Soc Civ Eng 1982;325:61–8.
[23] Owen WF, Stuckey DC, Healy Jr JB, Young LY, McCarty PL. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res 1979;13:485–93.
[24] Pattra S, Sangyoka S, Boonmee M, Reungsang A. Bio-hydrogen production from the fermentation of sugarcane bagasse hydrolysate by *Clostridium butyricum*. Int J Hydrogen energy 2008;33:6058–65.
[25] Fangkum A, Reungsang A. Biohydrogen production from mixed xylose/arabinose at thermophilic temperature by

anaerobic mixed culture in elephant dung. Int J Hydrogen Energy 2011;36:13928–38.
[26] Nitisinprasert S, Temmes A. The characteristics of a new non-spore-forming cellulolytic mesophilic anaerobe strain CMC126 isolated from municipal sewage sludge. J Appl Bacteriol 1991;71:154–61.
[27] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31:426–8.
[28] Zheng XJ, Yu HQ. Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures. J Environ Manage 2005;74:65–70.
[29] Zhang H, Bruns MA, Logan BE. Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor. Water Res 2006;40:728–34.
[30] Bowels LK, Ellefson WL. Effects of butanol on *Clostridium acetobutilicum*. Appl Environ Microbiol 1985;50:1165–70.
[31] Wang J, Wan W. Factor influencing fermentation hydrogen production: a review. Int J Hydrogen Energy 2009;34:759–811.
[32] Lin CY, Wu CC, Hung CH. Temperature effects on fermentative hydrogen from xylose using mixed anaerobic cultures. Int J Hydrogen Energy 2008;33:43–50.
[33] Hoster F, Daniel R, Gottsschalk G. Isolation of a new *Thermoanarobacterium thermosaccharolyticum* strain (FH1) producing a thermostable dextranase. J Gen Appl Microbiol 2001;41:187–92.
[34] O-Thong S, Prasertsan P, Karakashev D, Angelidaki I. Thermophilic fermentative hydrogen production by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* PSU-2. Int J Hydrogen Energy 2008;33:1204–14.
[35] Khamtib S, Reungsang A. Biohydrogen production from xylose by *Thermoanaerobacterium thermosaccharolyticum* KKU19 isolated from hot spring sediment. Int J Hydrogen Energy 2012;37:12219–28.
[36] Lo YC, Huang CY, Cheng CL, Lin CY, Chang JS. Characterization of cellulolytic enzymes and bioH_2 production from anaerobic thermophilic *Clostridium* sp. TCW1. Bioresour Technol 2011;102:8384–92.
[37] Lee DJ, Show KY, Su A. Dark fermentation on biohydrogen production: pure culture. Bioresour Technol 2011;102:8393–402.
[38] Wang J, Wan W. Effect of temperature on fermentative hydrogen production by mixed cultures. Int J Hydrogen Energy 2008;33:5392–7.
[39] Khamtib S, Plangklang P, Reungsang A. Optimization of fermentative hydrogen production from hydrolysate of microwave assisted sulfuric acid pretreated oil palm trunk by hot spring enriched culture. Int J Hydrogen Energy 2011;36:14204–16.
[40] Ren N, Wang A, Gao LF, Xin L, Lee DJ, Su A. Bioaugmented hydrogen production from carboxymethyl cellulose and partially delignified corn stalks using isolated cultures. Int J Hydrogen Energy 2008;33:5250–5.
[41] Nguyen TAD, Kim JP, Kim MS, Oh YK, Sim SJ. Optimization of hydrogen production by hyperthermophilic eubacteria, *Thermotoga maritime* and *Thermotoga neapolitana* in batch fermentation. Int J Hydrogen Energy 2008;33:1483–8.
[42] Noike T, Takabatake H, Mizuno O, Ohba M. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. Int J Hydrogen Energy 2002;27:1367–71.
[43] Jeon JM, Lee HI, Kim SG, Han SH, So JS. Differential inactivation of food poisoning bacteria and *Lactobacillus* sp. by mandelic acid. Food Sci Biotechnol 2010;9:583–7.