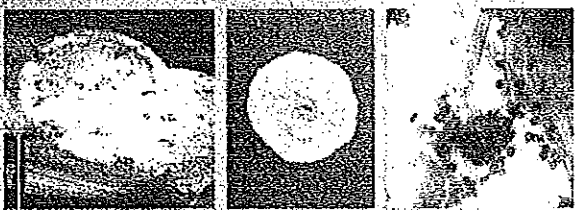


Microbes in Applied Research

Current Advances and Challenges



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A. Menéndez-Vilas

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Effect of initial pH on bio-hydrogen production from enzymatic hydrolysate of acid-pretreated sugarcane bagasse by elephant dung

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This research investigated the effect of initial pH on hydrogen production by elephant dung from enzymatic hydrolysate of acid-pretreated sugarcane bagasse (SCB). The acid-pretreated sugarcane bagasse was hydrolyzed with 20 U/g commercial cellulase derived from *Trichoderma reesei*. The resulting hydrolysate had the glucose concentration of 3.19 g/L which was subsequently used as the substrate to produce hydrogen by elephant dung in batch fermentation at various initial pH of 3-8 with an increment of 0.5. Elephant dung was heat-treated in boiling water for 2 h before used as the seed inoculum in order to inhibit methanogenic activity. The experiments were conducted in 10 mL serum bottles with a working volume of 7 mL. Culture of the hydrolysate at the initial pH 7 provided a maximum hydrogen production (HP) and hydrogen yield (HY) of 410.97 mL and 0.93 mol H₂/mol total sugars consumed, respectively. Main soluble products were acetate and butyrate suggesting that the hydrogen fermentation from the enzymatic hydrolysate of acid-pretreated SCB is the acetate-butyrate type.

Keywords acid pretreatment; enzymatic hydrolysis; hydrogen; sugarcane bagasse

1. Introduction

Hydrogen is a promising alternative energy source to conventional fossil fuels. It is a clean and environmental friendly fuel. It produces water after combustion instead of the greenhouse gases and can be directly used to generate electricity through fuel cells. Hydrogen has a high energy yield of 122 kJ/g, which is 2.75 times greater than fuel from hydrocarbon [1]. Generation of hydrogen can be achieved by a variety of technologies, among which biological hydrogen production is considered a promising route for hydrogen production [2]. Biological hydrogen production via dark fermentation has gained considerable interest in recent years due to its advantages of technical simplicity, no light is needed and the ability to utilize various kinds of substrates [3].

Lignocellulosic materials are recent considered as a potential feed stock for bioenergy production due to its large abundant and locally available as agricultural residues. Sugarcane bagasse (SCB) is one of the agricultural residues that showed a great potential to be used as the substrate for producing the renewable energy such as ethanol and hydrogen. It is a waste from sugar industry in which more than 400 million tons of SCB are annually generated from the sugar manufacturers around the world [4]. SCB consists of hemicelluloses (25-27%), cellulose (38-42%) and lignin (20-25%) [4,5]. Conversion of SCB to fermentable sugar is needed for the subsequent hydrogen fermentation. In our previous research, dilute acid hydrolysis have been applied to hydrolyze hemicelluloses fraction of SCB and the resulted hydrolysate was used to produce hydrogen by *Clostridium butyricum* TISTR 1032 with the yield of 0.84-1.73 mol H₂/mol total sugar [5,6]. After dilute acid pretreatment, the solid-particles consisting of cellulose and lignin fractions of SCB are remained. The cellulose fraction could be saccharified by hydrolysis method and yielded glucose which can be effectively used as substrate for hydrogen production [7]. In comparison to the other hydrolysis methods such as acid or alkaline hydrolysis, enzymatic hydrolysis using cellulase is typically carried out with the advantages of more specific and milder and it does not cause the sugar degradation [8]. Therefore, in this study the enzymatic hydrolysis was applied to hydrolyze cellulose fraction of dilute acid pretreated SCB to obtain the fermentable sugar for subsequent hydrogen production.

The bacterial mixed cultures from various natural environments have been used as the sources for hydrogen producing bacteria [9]. Elephant dung is expected to be abundant with cellulase producing microorganisms due to the elephant's diet which is mainly plant materials. Apart from cellulase-producing microorganisms, those that utilized sugar in the hydrolysate of cellulose are also expected. Our previous research reported the efficient

use of mixed cultures presence in elephant dung as a seed inoculum for biohydrogen production from pentose and hexose sugars as well as dilute acid hydrolysate of SCB [6,10]. In this research, the mixed culture in elephant dung was used as the seed inoculum to produce hydrogen from the enzymatic hydrolysate of acid pretreated SCB. The information obtained from this study would pave the way towards the utilization of the elephant dung for hydrogen fermentation from lignocellulosic materials.

Therefore, the goal of this research is to utilize the cellulose fraction in the SCB after dilute acid pretreatment as the substrate for hydrogen production. In order to achieve the main objective, the enzymatic hydrolysis was applied to hydrolyze acid pretreated SCB. The resulted hydrolysate containing fermentable sugar was used as substrate to produce hydrogen by microorganisms in the elephant dung in batch fermentation process. One of the most important environmental factors affecting hydrogen production i.e., initial pH was optimized in order to maximize hydrogen production from enzymatic hydrolysate of acid pretreated SCB.

2. Materials and Methods

2.1 Microorganism preparation

Elephant dung was collected from the elephant village of Surin province, Thailand. Before it is used, 30%(w/v) elephant dung was chopped by knife, placed into a beaker and heat treated in the boiling water at 100 °C for 2 h in order to inhibit the hydrogen consumer [6]. The inoculum was prepared by cultivating 210 g of elephant dung in 10 g/L glucose as a carbon source with the supplementation of 1 mL/L of each nutrient stock solution and 5.42 g/L NH_4HCO_3 . The stock solutions consist of (all in g/L) KH_2PO_4 , 125; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 25; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5; $\text{CoCl}_2 \cdot 5\text{H}_2\text{O}$, 0.125 [11]. The culture was incubated under anaerobic condition at 37 °C, shaken at 100 rpm for 24 h before used as the inoculum for hydrogen fermentation experiment. The volatile suspended solid of the inoculum is 17.5 g VSS/L.

2.2 Acid pretreatment of sugarcane bagasse (SCB) and enzymatic hydrolysis of acid pretreated SCB

SCB used in this study was obtained from a local sugar industry (Mitr-Phu-Wieng Industry, Khon Kaen, Thailand). SCB were air dried, milled and sieved through a 0.5 mm screen before storing at room temperature prior to usage.

Acid pretreatment of SCB by using 0.5% (w/v) H_2SO_4 was conducted at 121 °C, 1.5 kg/cm² for 60 min in autoclave with a mass ratio of solid (g dry weight) to liquid (mL) at 1:15 [5]. After the acid hydrolysis, the solid residue was recovered by filtration through a thin layer cloth, soaked in distilled water for 24 h and washed with distilled water 4 times to remove acid residues. The solid residue was further dried at 105°C for 4 h in hot air oven. The major compositions of the SCB before and after acid pretreatment including cellulose, hemicelluloses and lignin were analyzed using the method of Massatto and Roberto [12]. The acid pretreated SCB was subjected to the enzymatic hydrolysis in order to obtain the enzymatic hydrolysate of acid pretreated SCB.

Cellulase derived from *Trichoderma reesei* ATCC 26921 (Sigma Aldrich, USA) was used to hydrolyze the acid pretreated SCB. Enzymatic hydrolysis was conducted in a 250-mL Erlenmeyer flask with 7 g of dried acid pretreated SCB and 100 mL of acetate buffer (pH 4.8) containing cellulase (20 U/g SCB). The flask was incubated at 50°C and shaken at 160 rpm for 12 h. The solid particles were removed by filtration through a thin layer cloth and glass microfiber filter (70 mm), respectively. The resulting hydrolysate was analyzed for reducing sugar and glucose concentrations and further used as substrate for hydrogen fermentation.

2.3 Hydrogen production from enzymatic hydrolysate of acid pretreated SCB

The batch experiments were conducted in 10 mL serum bottles with a working volume of 7 mL comprised of 3.94 mL of enzymatic hydrolysate of acid pretreated SCB containing 5.42 g/L NH_4HCO_3 , 1 mL/L of each nutrient stock solution and 3 mL of seed inoculum. The effect of initial pH of fermentation substrate (pH of 3-8 with an increment of 0.5) was investigated. The serum bottles were sealed with rubber septa and aluminum cap. After replacement of the gas phase with nitrogen gas for 5 min to create anaerobic condition, the serum bottles were incubated at 37±2 °C and operated in an orbital shaker with a rotation speed of 150 rpm. At designed time, the total gas volume was measured by releasing the pressure in the bottles using wetted glass syringe [13] and then analyzed for gas content by gas chromatography (GC) equipped with a thermal conductivity detector (TCD). Effluent was collected by using a glass syringe and analyzed for volatile fatty acids (VFAs) and alcohol by gas chromatography with a flame ionization detector (FID). All treatments were conducted in three replications. The hydrogen production was continued until the biogas volume could not be measured.

2.4 Analytical method

The component of biogas in the headspace including hydrogen, nitrogen, methane and carbon dioxide was determined using GC (Shimadzu 2014, Japan) equipped with TCD and a 3 m × 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 ovens were 150, 145 and 150 °C, respectively. Argon was used as the carrier gas at a flow rate of 25 mL/min.

Sugar compositions and concentrations in the liquid samples were analyzed by HPLC. Before the HPLC analysis, the samples were centrifuged at 13,000 rpm for 10 min and filtered through 0.45 µm nylon membrane prior the analysis. HPLC analysis was carried out using a LC-10AD (Shimadzu, Japan) with an Aminex HPX-87H column (oven temperature 45 °C), 5 mM H₂SO₄ as mobile phase at the flow rate of 0.6 mL/min and a refraction index detector. For the analysis of VFAs including acetic (HAc), propionic (HPr), and butyric (HBu) acids and ethanol (EtOH) concentrations, liquid samples were centrifuged at 10,000 rpm for 5 min, acidified by 0.2 N oxalic acid and filtered through 0.45 µm nylon membrane. The GC (Shimadzu 2014, Japan) equipped with FID and a 2 m × 2 mm diameter packed column (Porapak Q 80/100 mesh) was used. The operational temperatures of the injector, detector and column ovens were 230, 250 and 175 °C, respectively. Nitrogen was used as carrier gases with pressure of 220 kpa.

Volume of biogas produced was calculated by a mass balance equation previously described by Zheng and Yu [14]. The volumetric hydrogen production rate (HPR) (mL H₂/L h) was calculated from cumulative hydrogen production (mL H₂/L) divided by fermentation time (h). Hydrogen yield (HY) (mol H₂/mol glucose) was calculated as total molaric amount of hydrogen divided by molaric amount of consumed glucose (as reducing sugar). The total molaric amount of hydrogen was calculated using the ideal gas law as: molar hydrogen production (mmol H₂/L) = cumulative hydrogen production (mL H₂/L) (RT), where R = 0.08205784 L atm/K mol, T = 310 K [15].

3. Results and Discussion

3.1 Enzymatic hydrolysate of acid pretreated SCB

Dilute acid pretreatment was applied to hydrolyze lignocellulosic material in SCB into hemicellulose and lignin fractions. After the pretreatment, the liquid fraction containing mainly sugar from hemicelluloses was used to produce hydrogen by *C. butyricum* TISTR 1032 and the result was reported by Pattra et al. [5]. The compositions of the remained solid fraction compared to non-acid pretreated SCB are determined. Results indicated that after the acid pretreatment, the content of cellulose was increased from 33.63 to 59.07% while the hemicelluloses content in SCB was decreased from 23.44 to 4.45%. Hemicelluloses can crosslink with each other, adsorb to cellulose, from the part of the matrix embedding cellulose and forms a physical barrier to enzymatic attack on cellulose [16]. Therefore, the decrease in hemicelluloses content would greatly facilitate the subsequent enzymatic hydrolysis of cellulose fraction in SCB. The content of lignin in SCB was increased from 4.31 to 22.55% after dilute acid pretreatment. The high lignin content can difficult the enzymatic hydrolysis of acid pretreated SCB such as by enzymatic sterical hindrance and prevention of fiber swelling. In addition, dissolved lignin is known to inhibit the activities of cellulases and xylanases which further decrease the hydrolysis efficiency [17].

The acid pretreated SCB was subjected to further enzymatic hydrolysis which yielded the hydrolysate containing 3.64±0.52 g/L of reducing sugar. Glucose is the main sugar detected in the hydrolysate with a concentration of 3.19±0.25 g/L. The results suggested that hydrolysis process took place at the β-1,4 glycosidic bond of cellulose in acid pretreated SCB. The sugar concentration in the hydrolysate was considered low which might be due to the inhibition of cellulases by lignin containing in acid pretreated. Acetic acid was detected in the hydrolysate at a low concentration of 0.22±0.01 g/L which would not affect the hydrogen fermentation process [5,11,18].

3.2 Hydrogen production from enzymatic hydrolysate of acid pretreated SCB

Effect of initial pH on hydrogen production from enzymatic hydrolysate of acid pretreated SCB by anaerobic mixed cultures from elephant dung was investigated at various initial pH values from 3.0 to 8.0. Parameters regarding hydrogen production are summarized in Table 1. Methane was not detected throughout the fermentation process. Results indicated that initial pH values markedly affected HP as well as HY. HP was increased from 34.37 to 410.79 mL H₂/L while HY was increased from 0.08 to 0.93 mol H₂/mol glucose when the initial pH was increased from 3.0 to 7.0 (Table 1). A low HP and HY at a strong acidic condition might be resulted from the inhibition of hydrogenase activity and structural damaging of cell membrane, DNA and

protein caused by the proton in the medium that pass through the cell membrane. These can decelerate the growth of microorganisms as well as their abilities to produce hydrogen [19]. In addition, the shift from acidogenesis to solventogenesis which is the route of microorganisms to prevent the obstructions in their metabolic flux could be occurred under acidic condition. With this kind of metabolic shift, the additional free electrons from NADH was consumed to produce neutral organic solvents such as alcohols which can cause the reduction in HP and HY. Therefore, an increase in the initial pH would delay the beginning of the inhibition caused by the metabolic shift hence improving the hydrogen production efficiency [20].

Table 1 Effect of pH on hydrogen production from enzymatic hydrolysate of acid pretreated SCB

Treatment	HP (mL H ₂ /L)	HPR (mL H ₂ /L h)	Lag time (h)	R ²	HY (mol H ₂ /mol glucose)
pH 3	34.37	1.05	39.16	0.988	0.08
pH 4	43.84	1.49	42.29	0.990	0.09
pH 4.5	63.54	2.40	44.23	0.994	0.14
pH 5	127.93	0.90	23.63	0.996	0.30
pH 5.5	148.03	0.73	35.52	0.994	0.33
pH 6	181.51	0.94	1.82	0.976	0.43
pH 6.5	277.91	1.50	33.50	0.997	0.64
pH 7	410.79	1.56	41.37	0.992	0.93
pH 7.5	194.16	0.60	40.17	0.993	0.44
pH 8	129.61	0.45	1.39	0.979	0.31

The HP and HY were decreased when the initial pH was greater than 7.0. The pH value that is higher than the optimum level was reported to cause negative effect on the hydrogenase activity and metabolic pathways of the hydrogen producing bacteria [20]. In addition, cell growth was more efficient than hydrogen production at a high pH level [19].

The values of HPR and lag time were varied regarding the initial pH values. The maximum HPR (2.4 mL H₂/L h) and the shortest lag period (1.39 h) were achieved at the initial pH of 4.5 and 8.0, respectively, while the lowest HPR (0.45 mL H₂/L h) and the longest lag time (44.23 h) were obtained at the initial pH of 8.0 and 4.5, respectively (Table 1). Considering the maximum HP and HY with the relatively high HPR of 1.57 mL H₂/L h obtained (Table 1), it could be concluded that the initial pH value of 7.0 was optimal for hydrogen production from enzymatic hydrolysate of acid pretreated SCB by anaerobic mixed cultures in the elephant dung.

3.3 Sugar consumption, pH variation and soluble metabolite products (SMPs)

Reducing sugar concentration in the medium was analyzed at 0 h and at the end of hydrogen fermentation (350 h) (Table 2). At day 0, the reducing sugar concentration in the fermentation medium was approximately 3.10 g/L. The reducing sugar was almost completely consumed during hydrogen fermentation in all treatments with the remained concentration of less than 0.18 g/L at the end of incubation.

Table 2 Hydrogen production performance and soluble metabolites distribution for hydrogen fermentation from enzymatic hydrolysate of acid pretreated SCB at different initial pH values

pH	Reducing sugar concentration (g/L)				SMPs (mg/L)								HAc/ TVFAs	HBu/ TVFAs
	Initial	Final	Initial	Final	Consumed	EtOH	HAc	HPr	HBu	TVFAs	TSMPS	TVFAs		
3.0	3.11	3.37±0.015	0.16±0.0016	3.21	3.3	50.31	2.02	7.26	59.59	62.89	0.84	0.12		
4.0	4.11	3.65±0.070	0.11±0.0008	3.54	6.79	60.16	2.31	8.84	71.31	78.1	0.84	0.12		
4.5	4.46	3.47±0.008	0.17±0.0008	3.30	8.13	69.91	1.08	10.22	81.21	89.34	0.86	0.13		
5.0	4.44	3.21±0.778	0.15±0.0008	3.05	36.48	84.19	1.48	26.14	111.81	148.29	0.75	0.23		
5.5	4.52	3.28±1.539	0.13±0.0008	3.15	49.21	82.39	1.97	17.75	102.11	151.32	0.81	0.17		
6.0	4.61	3.18±1.642	0.18±0.0008	3.00	30.87	110.17	1.65	21.22	133.04	163.91	0.83	0.16		
6.5	5.52	3.23±0.141	0.18±0.0008	3.05	20.18	114.21	2.18	60.95	177.34	197.52	0.64	0.34		
7.0	5.12	3.29±0.235	0.18±0.0008	3.11	13.49	126.58	2.05	72.31	200.94	214.43	0.63	0.36		
7.5	6.95	3.29±0.133	0.16±0.0008	3.13	16.38	84.07	4.65	11.95	100.67	117.05	0.84	0.12		
8.0	7.72	3.06±0.573	0.12±0.0008	2.94	13.15	59.6	1.82	9.06	70.48	83.63	0.85	0.13		

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

Results of final pH and production of SMPs i.e., EtOH, HAc, HBu, and HPr at the end of hydrogen fermentation were summarized in Table 4. Hydrogen is produced concomitantly with VFAs production causing the decrease in pH during hydrogen fermentation (Table 1 and 2). The decrease in pH at the initial pH of 3.0 to 4.5 was not observed and the amount of SMPs produced was very low which was coincided with the low amount of HP. At the initial pH of 5 to 8, the final pH was in the range of 4.52 and 7.72 and it increased

alongside the initial pH. The main SMPs in the fermentative broth was HAc in all treatments which accounted for 54-80% of total SMPs. The highest concentration (126.58 mg/L) was found in fermentative broth at the initial pH of 7.0. At the initial pH of 6.5-7.0, the second abundant SMPs was H₂Bu in which its highest concentration of 72.31 mg/L was found at the initial pH of 7.0. Since the production of acetic and butyric acids favors production of hydrogen [21], these results prove the result of hydrogen production which was found to be optimized at the initial pH of 7 (Table 1). Ethanol is the second abundant SMPs at the initial pH of 5.0-6.0 with the concentration of 30.87-49.21 mg/L which implied that the shift of acidogenesis to solventogenesis was occurred. This was caused by the accumulation of VFAs in the fermentation broth which led to the decrease in the pH to the inhibition level. These results were correlated with the low HP and HY obtained at this range of initial pH. SMPs productions decreased with an increase of initial pH from 7.5 to 8.0. This is in agreement with the tendency of hydrogen production which was found to decrease at the initial pH of greater than 7.0.

From these results, it could be concluded that the pH control at an optimum value is needed in order to effectively produce hydrogen from enzymatic hydrolysate of acid pretreated SCB by anaerobic mixed culture from elephant dung.

4. Conclusions

This study successfully used the enzymatic hydrolysate of acid pretreated SCB to produce hydrogen by anaerobic mixed cultures in elephant dung. The maximum HP and HY of 410.79 mL H₂/L and 0.93 mol H₂/mol glucose, respectively, with the relatively high HPR of 1.57 mL H₂/L h were achieved at the optimum initial pH of 7.0. The main SMPs detected in the fermented broth were acetic acid followed by ethanol and butyric acid. The highest concentrations of acetic and butyric acids were observed at the optimum initial pH of 7.0 while ethanol was observed at the initial pH of 5.0-6.0.

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