

# EFFECTS OF PH AND REPEATED HEAT-SHOCK TREATMENT ON HYDROGEN FERMENTATION OF SUCROSE BY A MIXED CULTURE

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**Abstract** : This study was conducted to investigate the effects of pH and repeated heat-shock treatment on the production of hydrogen and metabolites in hydrogen fermentation of sucrose by a mixed culture. The batch experiments were repeatedly performed three times using the same sludge as a seed microorganism in each serum bottle. Initial pH 6.5 led to the maximum hydrogen production from sucrose with only initial heat-shock treatment. The sucrose degradation efficiency increased up to 98.0%. The maximum values of the hydrogen yield and the specific hydrogen production rate were 2.0 mol H<sub>2</sub> (mol hexose)<sup>-1</sup> and 103.2 ml H<sub>2</sub> (g VSS · day)<sup>-1</sup>, respectively. However, at initial pH 7.0 or higher, methane was detected so that repeated heat-shock treatment was needed. Butyrate and acetate were the two most abundant species in hydrogen fermentation. At initial pH 6.5, butyrate production reached the maximum while acetate production reached the minimum, resulting in the highest butyrate/acetate (B/A) ratio of 5.0. Compared with the value of stoichiometric reaction, the hydrogen yield of 2.0 mol H<sub>2</sub> (mol hexose)<sup>-1</sup> meant that most of the sucrose was converted to butyrate, instead of acetate. Thus, *Clostridium* species are considered to be the dominant microorganisms because these bacteria are responsible for butyrate fermentation. The high concentrations of butyrate indicate that the hydrogen- and acid-producing pathway dominated the metabolic flow. In this study using sucrose as a substrate by a mixed culture, the optimum initial pH of 6.5 was a dividing line between acid and alcohol production for an undefined inoculum.

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**Key Words** : initial pH, heat-shock treatment, hydrogen yield, butyrate, *Clostridium* species

## INTRODUCTION

When fossil fuels are burned in coal-fired power plants or automobiles, carbon dioxide and other pollutants are generated. The greenhouse effect caused by excess carbon dioxide in the atmosphere is a serious global environmental problem. As a clean and sustainable source of energy in the future, hydrogen is a promising

alternative to fossil fuels.<sup>1)</sup> It produces water instead of greenhouse gases when combusted. It has a high energy yield (122 kJ g<sup>-1</sup>), which is about 2.75 times greater than that of hydrocarbon fuels, and can be directly used to produce electricity through fuel cells.<sup>2,3)</sup>

Hydrogen can be generated in a number of ways, for example through fossil fuel processing or by electrolysis using solar energy.<sup>4,5)</sup> However, these processes are energy intensive and therefore expensive. Biohydrogen production is potentially attractive, especially if organic waste

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could be used as a raw material.<sup>6,7)</sup> Microorganisms are capable of producing hydrogen via either photosynthesis or fermentation.<sup>1,8)</sup> Fermentation is generally preferred, because it does not rely on the availability of light sources and the transparency of the mixed liquor.<sup>9)</sup> Thus, biohydrogen production from organic wastes by a fermentative H<sub>2</sub> producer (i.e., *Clostridium* sp.) represents an important area of bioenergy production for global environmental considerations.<sup>1,10-12)</sup>

Clostridia are Gram-positive, spore-forming and rod-shaped bacteria. Endospores might be very resistant to heat or harmful chemicals, and cannot be destroyed easily.<sup>13)</sup> The clostridial bacteria could be enriched from soil, compost, sludge and comparable sources using an inhibition technique to exclude non-spore formers.<sup>14)</sup> The inhibition of hydrogen consumers is essential for net hydrogen production and for further scale-up and industrial application. The inhibition technique should be cost-effective and compatible with bacterial characteristics. There are three major inhibitors including oxygen, 2-bromoethanesulfonate (BES) and acetylene.<sup>15)</sup> However, simple heat-shock treatment can be quickly performed in the laboratory or field. If a liquid medium containing bacteria is brought to a 15 min boil, many of the bacteria will not survive. Those that do survive are likely to be spore formers.<sup>2,16,17)</sup> Once the liquid medium is subjected to natural bacterial growing conditions, these spores can germinate and give rise to active vegetative cells.<sup>13)</sup> However, if pH increases from acidic to neutral conditions, the activity of hydrogenotrophic methanogens could be recovered. In this case, repeated heat-shock treatment is required to produce hydrogen continuously.

The understanding of the properties of hydrogen fermentation lies in an appreciation of the response to environmental changes that influence hydrogen metabolism. Hydrogen fermentation is affected by several factors such as pH, carbon source, F/M ratio and phosphate levels as well as the nature of the microbial flora.<sup>18-20)</sup> Proper

pH control is a key factor to improve the germination of the clostridia, as well as to initiate and operate a hydrogen-producing bioprocess.<sup>21)</sup> If the pH cannot be maintained in a desired range, it could be inhibitory to hydrogen production or cause a metabolic shift, resulting in a cessation of hydrogen evolution. Thus, it is important to monitor and maintain the pH at the optimum level in the biohydrogen production process. Some experiments were conducted to investigate hydrogen fermentation depending on pH. It was reported that the control of pH plays an important role to hydrogen fermentation, due to the effect of pH on hydrogenase activity<sup>20)</sup> and on metabolic pathways.<sup>16)</sup> However, the optimal initial pH values for hydrogen production from sucrose in literature are conflicting, varying from pH 9.0 for batch fermentation of sucrose<sup>22)</sup> to pH 4.0-4.5 and pH 4.7-5.7, respectively, for the continuous fermentation of sucrose<sup>23)</sup> and starch.<sup>16)</sup>

This study was, therefore, performed to find the optimum pH in hydrogen production from sucrose by a mixed culture, and also to investigate the effects of pH and repeated heat-shock treatment on the production of hydrogen and metabolites in hydrogen fermentation.

## MATERIALS AND METHODS

### Seed Microorganisms

The seed sludge was taken from an anaerobic digester in the Daejeon Wastewater Treatment Plant (Korea). The digester was operated at a temperature of 35°C and a hydraulic retention time (HRT) of 25 days by feeding a mixture (1.5~2.0% VS) of primary sludge and waste activated sludge. The pH and volatile suspended solids (VSS) concentration of the sludge were 7.5 and 14,600 mg L<sup>-1</sup>, respectively.

### Experimental Set-up

The batch experiments were conducted under mesophilic conditions (35±1°C) using 160 ml serum bottles. Sucrose was used as a sole carbon source at a concentration of 7,500 mg L<sup>-1</sup>.

Rapidly metabolized carbohydrate, glucose, is generally avoided in germination media because it is vigorously fermented by certain saccharolytic species of *Clostridium*, resulting in considerable acid production.<sup>24)</sup> Seed sludge was boiled at 93°C in an open beaker for 15 min to inhibit hydrogenotrophic bacteria and to harvest anaerobic spore-forming bacteria (i.e., *Clostridium* sp.).<sup>2,16)</sup> After cooling in the air, 10 ml of seed sludge and 1.0 ml of nutrient stock solution were added into individual serum bottles. Each liter of nutrient stock solution contained 200 g of NH<sub>4</sub>HCO<sub>3</sub>, 100 g of KH<sub>2</sub>PO<sub>4</sub>, 4 g of K<sub>2</sub>HPO<sub>4</sub>, 10 g of Na<sub>2</sub>HPO<sub>4</sub>, 10 g of MgSO<sub>4</sub>(7H<sub>2</sub>O), 1.0 g of NaCl, 1.0 g of Na<sub>2</sub>MoO<sub>4</sub>(2H<sub>2</sub>O), 1.0 g of CaCl<sub>2</sub>(2H<sub>2</sub>O), 1.5 g of MnSO<sub>4</sub>(7H<sub>2</sub>O), and 0.278 g of FeCl<sub>2</sub>, which was slightly modified from Bahl et al.<sup>25)</sup> The bottles were filled to 100 ml using deionized water. The initial pH of the bottles was adjusted from 5.0 to 9.0 by using either 2 M HCl or 2 M KOH. The bottles were then flushed with nitrogen gas and capped tightly with rubber septum stoppers before being placed in an orbital shaker running at 180 rpm. The volume of biogas was determined using glass syringes of 5–50 ml according to Owen et al.<sup>26)</sup> The batch experiments were repeatedly performed three times using the same sludge as a seed microorganism in each serum bottle. Second heat-shock treatment was employed at initial pH 7.0–9.0 in the third experiment, because methane was produced in the second experiment.

### Analytical Methods

Biogas composition was analyzed by a gas chromatograph (GC, GowMac series 580) equipped with a thermal conductivity detector (TCD) and two columns. The contents of methane and carbon dioxide were determined using a 1.83 m × 3.18 mm (inside diameter) stainless-steel column packed with Porapak Q (80/100 mesh). Hydrogen content was measured with a 1.83 m × 3.18 mm (inside diameter) stainless-steel column packed with molecular sieve 5A. The operational temperatures of injector, detector and

column were kept at 80, 90 and 50°C, respectively. Helium was used as a carrier gas at a flowrate of 40 ml min<sup>-1</sup>. The concentrations of individual volatile fatty acids (VFA) were analyzed by a high-performance liquid chromatograph (LC, Spectra SYSTEM P2000) equipped with an ultraviolet (210 nm) detector and a 300 m × 7.8 mm Aminex HPX-87H column after pretreatment with a 0.45 μm membrane filter. H<sub>2</sub>SO<sub>4</sub> of 0.005 M was used as a mobile phase at a flowrate of 0.6 ml min<sup>-1</sup>. Alcohols were determined by a high-performance liquid chromatograph (LC, DX-600 Bio-LC system) equipped with an ED50A electrochemical detector and a 250 m × 4 mm CarboPac PA10 column after pretreatment with a 0.45 μm membrane filter. Deionized water was used as the mobile phase at a flowrate of 0.6 ml min<sup>-1</sup>. Carbohydrate concentration was measured by the phenol-sulfuric acid method using glucose as a standard.<sup>27)</sup> The parameters such as chemical oxygen demand (COD), VSS and pH of the samples were measured according to Standard Methods.<sup>28)</sup>

### Data Analysis

Once cumulative hydrogen production data were obtained over the course of an entire batch experiment, a curve was modeled to the data using the following modified Gompertz Eq. (i).<sup>2,16,17)</sup>

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

where

H(t) ; cumulative methane production during the incubation time t (mL hr<sup>-1</sup>)

λ ; lag phase time (hr)

P ; hydrogen production potential (mL)

R<sub>m</sub> ; hydrogen production rate (mL hr<sup>-1</sup>)

e ; 2.718281828

The hydrogen yield was obtained by dividing P by an amount of sucrose consumed, and then

converted to the unit of  $\text{mol H}_2 (\text{mol hexose})^{-1}$ . On the other hand, the specific hydrogen production rate ( $\text{ml H}_2 (\text{g VSS} \cdot \text{day})^{-1}$ ) was obtained by dividing  $R_m$  by the dry biomass weight.

## RESULTS

### Performance of Repeated Batch Fermentation for Hydrogen Production

Figure 1 illustrates the cumulative biogas production and pH profiles of the fermentation. Figure 1(a) shows that the cumulative hydrogen production increased from 102.7 ml at initial pH 5.0 to 199.7 ml at initial pH 6.5 in the first batch experiment. However, further increase of pH drastically lowered the cumulative hydrogen production. Methane was not detected in the biogas. As the operation time increased, the pH decreased due to VFA generated during hydrogen production. Initial pH 6.5 reduced to 5.3-5.4 after 33 hr of incubation, resulting in the largest hydrogen production. Therefore, the activity of hydrogen-consuming methanogens was effectively inhibited by initial heat-shock treatment and then suppressed under acidic conditions caused by initial pH 6.5.

Figure 1(b) illustrates that the same hydrogen production was obtained at initial pH 5.0-6.5 in the second batch experiment without repeated heat-shock treatment. However, the hydrogen production at initial pH 7.0-9.0 was lower than that of the first experiment. This is due to the fact that the biogas was free of methane at initial pH 6.5 or lower, while considerable quantities of methane were produced at initial pH 7.0 or higher. The methane production increased from 3.6 ml at initial pH 7.0 to 14.4 ml at initial pH 9.0, accompanied by the increased pH of 0.5-0.6. It indicated that, without repeated heat-shock treatment, the activity of hydrogenotrophic methanogens was recovered over initial pH 7.0.

Figure 1(c) shows that the hydrogen production at initial pH 7.0-9.0 increased to the level of the first experiment due to repeated heat-shock treatment. The seed sludge in the serum

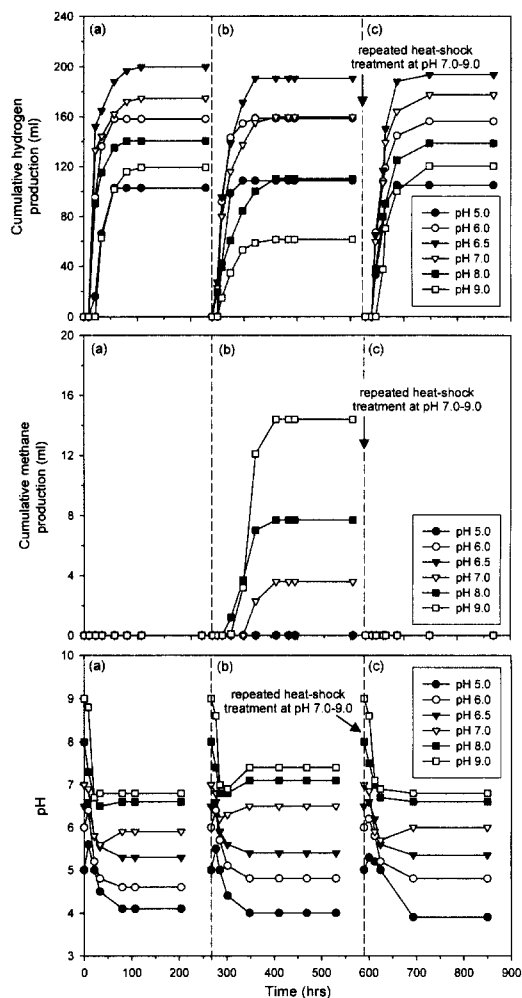


Figure 1. Performance of batch fermentation for hydrogen production: (a) the first experiment (with initial heat-shock treatment); (b) the second experiment (using the same sludge as an inoculum without repeated heat-shock treatment); (c) the third experiment (using the same sludge as an inoculum with repeated heat-shock treatment at pH 7.0-9.0).

bottles (initial pH 7.0-9.0) was boiled at  $93^{\circ}\text{C}$  in an open beaker for 15 min again and then added into the serum bottles after cooling in the air. Methane was not detected in the biogas. The pH range after 33 hr was similar to that of the first experiment. Thus, only initial heat-shock treatment was needed below initial pH 6.5, whereas repeated heat-shock treatment was needed over initial pH 7.0. It was concluded

that initial pH 6.5 was an optimum value for hydrogen fermentation of sucrose, leading to the maximum hydrogen production with only initial heat-shock treatment.

### Hydrogen Production at Various Initial pH Values

Figure 2 illustrates hydrogen production at various initial pH values in the experiments. Figure 2(a) shows that sucrose degradation efficiency increased from 65.5% at initial pH 5.0 to 98.0% at initial pH 6.5 and then decreased to 84.6% at initial pH 9.0. When methane was

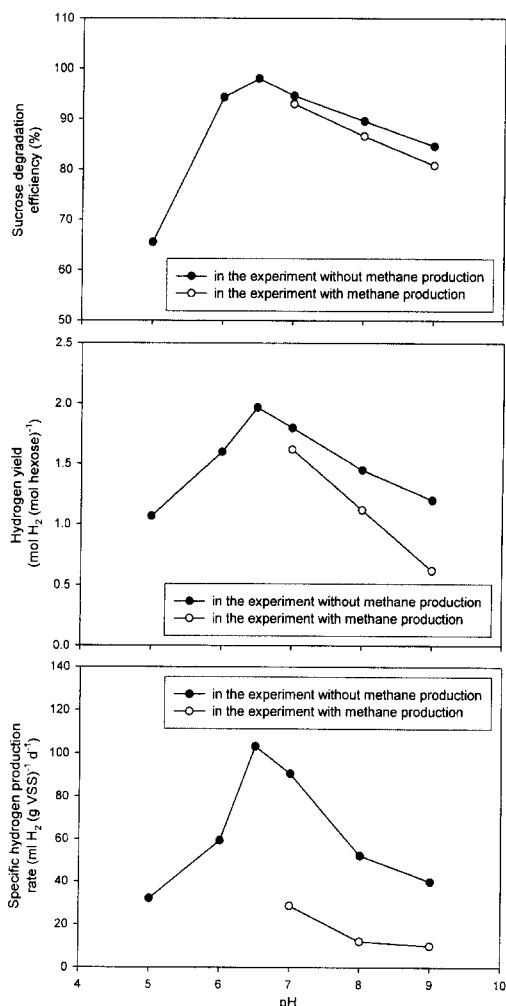


Figure 2. Hydrogen production at various initial pH values: (a) sucrose degradation; (b) hydrogen yield; (c) specific hydrogen production rate.

produced, the efficiency decreased to 80.8% at initial pH 9.0. The maximum sucrose degradation efficiency of 98.0% was observed at initial pH 6.5.

Figure 2(b) and (c) show that the hydrogen yield and the specific hydrogen production rate reached the optimum at initial pH 6.5. Hydrogen evolution by *Clostridium* sp. was inhibited in the pH range of 4.0-5.0,<sup>25,29)</sup> but, in this study, initial pH 6.5 prevented pH from dropping below 5.4. Thus, the high yield of 2.0 mol H<sub>2</sub> (mol hexose)<sup>-1</sup> was obtained. The specific hydrogen production rate ranged 32.0-103.2 ml H<sub>2</sub> (g VSS · day)<sup>-1</sup> at initial pH 5.0-9.0. The highest specific hydrogen production rate was 103.2 ml H<sub>2</sub> (g VSS · day)<sup>-1</sup> at initial pH 6.5.

### COD balance and Metabolite Production

Table 1 and 2 summarize the overall COD balance. The COD of sucrose was converted to biomass, VFA, alcohol, hydrogen and methane in the biogas. Butyrate and acetate were the two most abundant species in the products. Increase of initial pH from 5.0 to 6.5 resulted in the increase of butyrate but in the decrease of acetate. At initial pH 6.5, the production of butyrate reached the maximum (53.7% of COD<sub>in</sub>) but the production of acetate reached the minimum (10.8% of COD<sub>in</sub>). If hydrogen builds up in the system, a higher molecular weight acid such as butyrate is produced instead of acetate.<sup>30)</sup> On the other hand, propionate production was suppressed at low pH as observed by others,<sup>31)</sup> but it increased remarkably at initial pH 7.0-9.0, accompanied by the increased production of methane.

Butyrate/acetate (B/A) ratio has been studied to determine available hydrogen production from sucrose.<sup>8,32)</sup> The optimal B/A ratio for *Clostridium butyricum* was 2.0 using glucose as substrate.<sup>33)</sup> However, the optimal B/A ratio in converting disaccharide to hydrogen has not been reported. In this work, the optimal B/A ratio was 5.0 at initial pH 6.5 for hydrogen production from sucrose by a mixed culture, suggesting that the optimal B/A ratio is rather

Table 1. COD balance of hydrogen fermentation without methane production

pH	COD <sub>in</sub> (mg)	HAc <sup>a</sup> (%)	HPr <sup>a</sup> (%)	HBu <sup>a</sup> (%)	HVa <sup>a</sup> (%)	HCa <sup>a</sup> (%)	HFO <sup>a</sup> (%)	HLA <sup>a</sup> (%)	EtOH <sup>a</sup> (%)	Sucrose (%)	H <sub>2</sub> (%)	CH <sub>4</sub> (%)	Biomass(%)	B/A*
5.0	842.1	18.6	1.7	7.3	1.2	0.1	4.6	8.9	11.5	34.5	8.9	-	2.7	0.4
6.0	842.1	13.8	1.8	48.8	1.0	0.0	1.0	5.3	5.7	5.7	13.4	-	3.5	3.5
6.5	842.1	10.8	1.2	53.7	1.0	0.1	0.7	5.4	5.4	2.0	16.5	-	3.1	5.0
7.0	842.1	12.8	2.6	46.2	2.7	0.2	2.3	3.2	6.0	5.4	14.9	-	3.6	3.6
8.0	842.1	18.3	3.3	33.8	2.5	0.0	2.7	6.4	6.6	10.4	12.1	-	3.9	1.8
9.0	842.1	18.4	15.2	20.7	6.4	0.4	0.2	2.5	7.2	15.4	10.2	-	3.3	1.1

<sup>a</sup>HAc=acetic acid; HPr=propionic acid; HBu=butyric acid; HVa=valeric acid; HCa=caproic acid; HFO=formic acid; HLA=lactic acid; EtOH=ethanol.

\*B/A=HBu/HAc

Table 2. COD balance of hydrogen fermentation with methane production

pH	COD <sub>in</sub> (mg)	HAc <sup>a</sup> (%)	HPr <sup>a</sup> (%)	HBu <sup>a</sup> (%)	HVa <sup>a</sup> (%)	HCa <sup>a</sup> (%)	HFO <sup>a</sup> (%)	HLA <sup>a</sup> (%)	EtOH <sup>a</sup> (%)	Sucrose (%)	H <sub>2</sub> (%)	CH <sub>4</sub> (%)	Biomass(%)	B/A*
7.0	842.1	18.5	8.0	37.0	2.3	0.5	0.1	6.4	2.9	6.0	13.5	1.2	3.6	2.0
8.0	842.1	24.1	11.0	25.2	1.3	0.0	0.8	5.2	3.2	13.4	9.3	2.6	3.9	1.0
9.0	842.1	25.9	18.2	12.3	1.4	0.0	0.2	5.7	2.7	19.2	5.2	4.9	4.3	0.5

<sup>a</sup>HAc=acetic acid; HPr=propionic acid; HBu=butyric acid; HVa=valeric acid; HCa=caproic acid; HFO=formic acid; HLA=lactic acid; EtOH=ethanol.

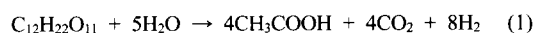
\*B/A=HBu/HAc

substrate dependent.

Ethanol was the third most abundant species in the products. Increase of initial pH from 5.0 to 6.5 resulted in the decrease of ethanol. The production of ethanol was the lowest at initial pH 6.5. Production of alcohol is considered to cause negative effects on hydrogen production, whereas acid formation is favorable to hydrogen production.<sup>34)</sup> This is consistent with our observation that hydrogen production was the largest when ethanol concentration was the lowest.

## DISCUSSION

The formation of hydrogen is accompanied by VFA or alcohol production during an anaerobic digestion process. The distribution of metabolites formed during hydrogen fermentation is a useful indicator in evaluating the efficiency of hydrogen fermentation.<sup>35,36)</sup> Sucrose may be converted to either acetate or butyrate in the production of hydrogen as the following:



It produces eight moles of hydrogen when one mole of sucrose is converted to acetate, but only four moles of hydrogen when sucrose is converted to butyrate. The yield of 2.0 mol H<sub>2</sub> (mol hexose)<sup>-1</sup> observed in this study reflects the observation that most of the sucrose was converted to butyrate, instead of acetate. *Clostridium* species are, therefore, considered to be the dominant microorganisms because these bacteria are responsible for butyrate fermentation.<sup>35,37)</sup> The high concentrations of butyrate indicate that the hydrogen- and acid-forming pathway dominated the metabolic flow. B/A ratio has frequently been used as the indicator for monitoring hydrogen production.<sup>33,38)</sup> During hydrogen fermentation, it may vary with growth conditions within thermodynamically determined limits.<sup>39)</sup> In this study, the B/A ratios were 0.3-5.0 at initial pH 5.0-9.0. As the B/A ratio increased, higher hydrogen yields and specific hydrogen production rates were obtained. The changes in B/A ratio imply a metabolic alteration due to environmental changes such as pH, partial pressure of hydrogen, and the accumulated amounts of intermediate products in the

reaction.

The control of pH is fundamental to the maintenance of optimal bacterial growth and/or conversion processes in anaerobic microbial systems.<sup>2,12,22,40</sup> In this study, initial pH 6.5 was the most favorable to trigger hydrogen production at a higher production rate. However, rapid hydrogen production was accompanied by VFA accumulation, which in turn depleted the pH very quickly to inhibitory levels. The hydrogen producers could not adapt to the fast change in environment, thereby halting the reaction. On the other hand, at acidic conditions, the starting environment might not be suitable for hydrogen producers, however, with their adaptation and limited self-adjustment of environmental conditions such as pH,<sup>41</sup> they started to produce hydrogen gradually at a moderate production rate.

Some researchers have obtained hydrogen production with pure cultures of clostridia at pH 6.0-7.0.<sup>42-44</sup> In this study using a mixed culture, the optimum initial pH of 6.5 was a dividing line between acid and alcohol production for an undefined inoculum. When the hydrogen partial pressure increases to a certain level in the serum bottle headspace, the metabolism shifts to rapid alcohol production. Clostridia produce VFA during the exponential growth phase and alcohol during the stationary growth phase.<sup>2,34</sup> The shift in metabolic activity is accompanied by a corresponding shift in the cellular content of enzymes involved in the hydrogen-, acid- and alcohol-producing pathways.<sup>45,46</sup> Apparently, the build up of VFA and hydrogen during the exponential growth phase would potentially induce this shift. Kim and Zeikus<sup>47</sup> reported that initiation of solventogenesis by *Clostridium acetobutylicum* is directly related to a decrease in hydrogen production caused by the regulation of hydrogenase activity. An acidic pH below 5.0 was found to be optimal for acetone and butanol production (solventogenesis) and at higher pH values only acids are generally excreted.<sup>25,48,49</sup> In this study, initial pH 6.5 was able to delay the onset of the

pH inhibition to the hydrogen production caused by the metabolic shift from acidogenesis to solventogenesis.

## CONCLUSIONS

1. Initial pH 6.5 was an optimum value for hydrogen production from sucrose, leading to the maximum hydrogen production with only initial heat-shock treatment. However, at initial pH 7.0 or higher, considerable quantities of methane were produced so that repeated heat-shock treatment was needed.
2. The highest efficiency of sucrose degradation was 98.0% at initial pH 6.5. The maximum values of the hydrogen yield and the specific hydrogen production rate were 2.0 mol H<sub>2</sub> (mol hexose)<sup>-1</sup> and 103.2 ml H<sub>2</sub> (g VSS · day)<sup>-1</sup>, respectively, at initial pH 6.5. The results of this study and those reported for hydrogen fermentation seem to suggest that the maximum hydrogen yield by sucrose fermentation was about 2.0-2.3 mol H<sub>2</sub> (mol hexose)<sup>-1</sup>.
3. Butyrate and acetate were the two most abundant species in the products. At initial pH 6.5, the production of butyrate reached the maximum while the production of acetate reached the minimum. The highest B/A ratio of 5.0 was obtained at initial pH 6.5. As the B/A ratio increased, higher hydrogen yields and specific hydrogen production rates were obtained. Ethanol was the third most abundant species in the metabolites.
4. Compared to the value of stoichiometric reaction, the hydrogen yield of 2.0 mol H<sub>2</sub> (mol hexose)<sup>-1</sup> indicated that most of the sucrose was converted to butyrate, instead of acetate. Thus, *Clostridium* species are considered to be the dominant microorganisms because these bacteria are responsible for butyrate fermentation. The high concentrations of butyrate indicate that the hydrogen- and acid-producing pathway dominated the metabolic flow. In this study using sucrose as a substrate by a mixed culture, the optimum initial pH of 6.5 was a dividing line between

acid and alcohol production for an undefined inoculum.

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## REFERENCES

1. Benemann, J., "Hydrogen biotechnology: progress and prospects," *Nature Biotechnol.*, **14**, 1101~1103 (1996).
2. Lay, J. J., Lee, Y. J. and Noike, T., "Feasibility of biological hydrogen production from organic fraction of municipal solid waste," *Water Res.*, **33**, 2579~2586 (1999).
3. Mizuno, O., Dinsdale, R., Hawkes, F. R., Hawkes, D. L. and Noike, T., "Enhancement of hydrogen production from glucose by nitrogen gas sparging," *Bioresource Technol.*, **73**, 59~65 (2000).
4. Momirlan, M. and Veziroglu, T., "Recent directions of world hydrogen production," *Renewable and Sustainable Energy Reviews*, **3**, 219~231 (1999).
5. Momirlan, M. and Veziroglu, T., "Current status of hydrogen energy," *Renewable and Sustainable Energy Reviews*, **6**, 141~179 (2002).
6. Gray, C. T. and Gest, H., "Biological formation of molecular hydrogen," *Science*, **148**, 186~191 (1965).
7. Borkris, J. O. M., A hydrogen economy, *Science*, **178**, 1323 (1973).
8. Nandi, R. and Sengupta, S., "Microbial production of hydrogen: an overview," *Critical Reviews in Microbiology*, **24**, 61~84 (1998).
9. Hart, D., "Hydrogen power: the commercial future of the ultimate fuel," Financial times energy publishing, London (1997).
10. Brosseau, J. D., Yan, J.-Y. and Lo, K. V., "The relationship between hydrogen gas and butanol production by *Clostridium saccharo-perbutylaceticum*," *Biotechnol. Bioeng.*, **28**, 305~310 (1986).
11. Noike, T. and Mizuno, O., "Hydrogen fermentation of organic municipal wastes," *Water Sci. Technol.*, **42**, 155~162 (2000).
12. Van Ginkel, S., Sung, S. and Lay, J.-J., "Biohydrogen production as a function of pH and substrate concentration," *Environ. Sci. Technol.*, **35**, 4726~4730 (2001).
13. Brock, T. D., Madigan, M. T., Martinoko, J. M. and Parker, J., *Biology of microorganisms*. Prentice-Hall, New York USA (1994).
14. Biebl, H., *Clostridium acetobutylicum*. In: *Encyclopedia of Food Microbiology*, Robinson R., Batt C. and Patel P. (ed.), Academic Press (1999).
15. Sparling, R., Risbey, D. and Poggi-Varaldo, H. M., "Hydrogen production from inhibited anaerobic composters," *Int. J. Hydrogen Energy*, **22**, 563~566 (1997).
16. Lay, J. J., "Modeling and optimization of anaerobic digested sludge converting starch to hydrogen," *Biotechnol. Bioeng.*, **68**, 269~278 (2000).
17. Lay, J. J., "Biohydrogen generation by mesophilic anaerobic fermentation of microcrystalline cellulose," *Biotechnol. Bioeng.*, **74**, 280-287 (2001).
18. Kondratieva, E. N. and Gogotov, I., "Production of molecular hydrogen in microorganisms," *Adv. Biochem. Eng.*, **28**, 139~191 (1983).
19. Bowles, L. K. and Ellefson, W., "Effects of butanol on *Clostridium acetobutylicum*," *Appl. Environ. Microbiol.*, **50**, 1165~1170 (1985).
20. Dabrock, B., Bahl, H. and Gottschalk, G., "Parameters affecting solvent production by *Clostridium pasteurianum*," *Appl. Environ. Microbiol.*, **58**, 1233~1239 (1992).
21. Craven, S. E., "Increased sporulation of *Clostridium perfringens* in a medium prepared with the pre-reduced anaerobically sterilized technique or with carbon dioxide or carbonate," *J. Food Prot.*, **51**, 700~706 (1988).



22. Lee, Y. J., Miyahara, T. and Noike, T., "Effect of pH on microbial hydrogen fermentation," *J. Chem. Technol. Biotechnol.*, **77**, 694~698, (2002).
23. Ren, N., Wang, B. and Ma, F., Hydrogen bio-production of carbohydrate fermentation by anaerobic sludge process. In: Proceedings 68<sup>th</sup> Annual Water Environmental Federal Conference. Miami, USA, pp. 145~152 (1995).
24. Labbé, R. G. and Rey, D. K., "Raffinose increases sporulation and enterotoxin production by *Clostridium perfringens* Type A," *Environ. Microbiol.*, **37**, 1196~1200 (1979).
25. Bahl, H., Andersch, W., Braun, K. and Gottschalk, G., "Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture," *Eur. J. Appl. Microbiol. Biotechnol.*, **14**, 17~20 (1986).
26. Owen, W. F., Stuckey, D. C., Healy, J. B. Jr., Young, L. Y. and McCarty, P.L., "Bio-assay for monitoring biochemical methane potential and anaerobic toxicity," *Water Res.*, **13**, 485~493 (1979).
27. Dubios, M., Gilles, K. L., Hamilton, J. K., Rebers, P. A. and Smith, F., "Colorimetric method for determination of sugars and related substances," *Anal. Chem.*, **28**, 350~356 (1956).
28. APHA, *Standard methods for the examination of water and wastewater*, 20th Ed. Amer. Public Health Assoc., Washington, D. C., USA (1998).
29. Roychowdhury, S., Cox, D. and Levandowsky, M., "Production of hydrogen by microbial fermentation," *Int. J. Hydrogen Energy*, **13**, 407~410 (1988).
30. Grady, C. P. L. Jr., Daigger, G. T. and Lim, H. C., *Biological wastewater treatment*. Second Ed. Chapter 13.9. (1999).
31. Inanc, B., Matsui, S. and Ide, S., "Propionic acid accumulation in anaerobic digestion of carbohydrates: an investigation on the role of hydrogen gas," *Water Sci. Technol.*, **40**, 93~100 (1999).
32. Ueno, Y., Kawai, T., Sato, S., Otsuka, S. and Morimoto, M., "Biological production of hydrogen from cellulose by natural anaerobic microflora," *J. Ferment. Bioeng.*, **79**, 395~397 (1995).
33. White, D., *The physiology and biochemistry of prokaryotes*. Oxford University Press, New York, USA (1995).
34. Yan, R. T., Zhu, C. X., Golembosik, C. and Chen, J. S., "Expression of solvent-forming enzymes and onset of solvent production in batch culture of *Clostridium butyricum*," *Appl. Environ. Microbiol.*, **54**, 642~648 (1988).
35. Dinopoulou, G., Rudd, T. and Lester, J. N., "Anaerobic acidogenesis of a complex wastewater. I. The influence of operational parameters on reactor performance," *Biotechnol. Bioeng.*, **31**, 958~968 (1988).
36. Cha, G. C. and Noike, T., "Effect of rapid temperature change and HRT on anaerobic acidogenesis," *Water Sci. Technol.*, **36**, 247~253 (1997).
37. Yokoi, H., Maeda, Y., Hirose, J., Hayashi, S. and Takasaki, Y., "H<sub>2</sub> production by immobilized cells of *Clostridium butyricum* on porous glass beads," *Biotechnol. Tech.*, **11**, 431~433 (1997).
38. Annous, B. A., Shieh, J. S., Shen, G. J., Jain, M. K. and Zeikus, J. G., "Regulation of hydrogen metabolism in *Butyribacterium-Methylotrophicum* by substrate and pH," *Appl. Microbiol. Biotechnol.*, **45**, 804~810 (1996).
39. Thauer, R. K., Jungermann, K. and Decker, K., "Energy conservation in chemotrophic anaerobic bacteria," *Bacterial Rev.*, **1**, 100~180 (1977).
40. Stronach, S. M., Lester, J. N. and Rudd, T., "The influence of variable organic loading on the start-up of an anaerobic fluidized bed reactor," *Biotechnol. Lett.*, **8**, 521~524 (1986).
41. Gottwald, M. and Gottschalk, G., "The internal pH of *Clostridium acetobutylicum* and its effect on the shift from acid to solvent formation," *Arch. Microbiol.*, **143**, 42~

- 46 (1985).
42. Kataoka, N., Miya, A. and Kiriya, K., "Studies on hydrogen production by continuous culture system of hydrogen producing anaerobic bacteria," *Water Sci. Technol.*, **36**, 41~47 (1997).
43. Reimann, A., Biebl, H. and Deckwer, W. D., "Influence of iron, phosphate and methyl viologen on glycerol fermentation of *Clostridium butyricum*," *Appl. Microbiol. Biotechnol.*, **45**, 47~50 (1996).
44. Taguchi, F., Mizukami, N., Taki, T. S. and Hasegawa, K., "Hydrogen production from continuous fermentation of xylose during growth of *Clostridium* sp. strain No-2," *Can. J. Microbiol.* **41**, 536~540 (1995).
45. Jones, D. T., van der Westhuizen, A., Long, S., Allcock, E. R., Reid, S. J. and Woods, D. R., "Solvent production and morphological changes in *Clostridium acetobutylicum*," *Appl. Environ. Microbiol.*, **43**, 1434~1439 (1982).
46. Afschar, A. S., Schaller, K. and Schugerl, K., "Continuous production of acetone and butanol with shear-activated *Clostridium acetobutylicum*," *Appl. Microbiol. Biotechnol.*, **23**, 315~322 (1986).
47. Kim, B. H. and Zeikus, J. G., "Importance of hydrogen metabolism in regulation of solventogenesis by *Clostridium acetobutylicum*," *Dev. Ind. Microbiol.*, **26**, 549~556 (1985).
48. Monot, F., Engasser, J. M. and Petitdemange, H., "Influence of pH and undissociated butyric and on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*," *Appl. Microbiol. Biotechnol.*, **19**, 422-426 (1984).
49. Li, H., Gibbins, L. N. and Forsberg, C. W., "Transmembrane pH gradient and membrane potential in *Clostridium acetobutylicum* during growth under acetogenic and solventogenic conditions," *Appl. Environ. Microbiol.*, **50**, 1043~1047 (1985).