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SUPPRESSION OF HYDROGEN CONSUMING BACTERIA IN ANAEROBIC HYDROGEN FERMENTATION

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Abstract : Severe loss of hydrogen occurred in most anaerobic hydrogen fermentation reactors. Several selected methods were applied to suppress the consumption of hydrogen and increase the potential of production. As the first trial, pH shock was applied. The pH of reactor was dropped nearly to 3.0 by stopping alkalinity supply and only feeding glucose (5 g/L-d). As the pH was increase to 4.8 ± 0.2, the degradation pathway was derived to solventogenesis resulting in disappearance of hydrogen in the headspace. In the aspect of bacterial community, methanogens weren't detected after 22 and 35 day, respectively. Even though, however, there was no methanogenic bacterium detected with fluorescence in-situ hybridization (FISH) method, hydrogen loss still occurred in the reactor showing a continuous increase of acetate when the pH was increased to 5.5 ± 0.2. This result was suggesting the possibility of the survival of spore forming acetogenic bacteria enduring the severely acidic pH. As an alternative and additive method, nitrate was added in a batch experiment. It resulted in the increase of maximum hydrogen fraction from 29 (blank) to 61 % (500 mg NO_3/L). However, unfortunately, the loss of hydrogen occurred right after the depletion of nitrate by denitrification. In order to prevent the loss entangled with acetate formation, CO_2 scavenging in the headspace was applied to the hydrogen fermentation with heat-treated sludge since it was the primer of acetogenesis. As the CO_2 scavenging was applied, the maximum fraction of hydrogen was enhanced from 68 % to 87 %. And the loss of hydrogen could be protected effectively.

Key Words : Hydrogen, anaerobic fermentation, inhibitor, suppression of hydrogen consumption, CO_2 scavenging

INTRODUCTION

Hydrogen has been suggested as a promising alternative to fossil fuels as an energy carrier for transportation needs. Hydrogen gas has a high energy capacity per unit mass (118.2 kJ/g) and its reaction with oxygen does not produce green house gases such as CO_2. In contrast, methane has a relatively lower energy capacity (36.3 kJ/g) and CO_2 is released during its combustion in conventional engines. However, most hydrogen is usually produced from stored methane reserves and other non-renewable materials resulting in net increases of CO_2 in the environment. Shifting a fossil fuel economy

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to a hydrogen economy offers few environmental advantages if both are based on the net consumption fossil fuels.[1] Thus, it is essential for reducing CO_2 emissions that hydrogen production not release a net amount of CO_2 into the atmosphere, and that the technologies and materials used to produce hydrogen are sustainable.

Anaerobic fermentation is a promising method of sustainable hydrogen production since organic matter, including waste products, can be used as feed-stocks for the process.[2,3] However, the yield of hydrogen is still low as much as 2 mol-H_2/mol-glucose because hydrogen is lost during fermentation. The highest yields of hydrogen have been reported with strains of *Clostridia* in pure cultures or mixed cultures where *Clostridia* are predominant. For example, Collet *et al.* obtained 2.1-3.0 mol-H_2/mol-lactose with *Clostridium thermolacticum* and Yokoi *et al.* achieved 2.6 mol-H_2/mol-glucose with a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes* in continuous culture tests.[4,5] Batch tests often produce relatively lower yields than continuous culture tests. For example, Mizuno *et al.* obtained a yield of 0.88 mol-H_2/mol-glucose using a mixed anaerobic culture in batch tests.[6] A yield of 0.99 mol-H_2/mol-glucose of hydrogen was obtained in batch tests with a heat-treated inoculum[7] but the same inoculum produced a yield of 0.91 to 1.8 mol-H_2/mol-glucose in continuous culture.[8] Continuous culture experiments with mixed cultures have produced yields in other studies of 1.7 to 2.54 mol-H_2/mol-glucose.[6,7,9-11]

The reasons for lower yields in anaerobic hydrogen fermentation are not fully understood, although differences in the microbial community are certainly important. Using pure cultures helps to minimize hydrogen losses by excluding methanogens and certain homoacetogens. However, the use of pure cultures is not feasible for waste materials due to the different bacteria needed to break down the various components of the organic matter in the waste, and the prohibitive cost of sterilizing wastewater streams. Mixed cultures may contain a diverse array of bacteria, but *Clostridia* spp. and other hydrogen producing, spore-forming bacteria can easily be obtained by heat-treatment of a soil or digested sludge sample by boiling or oven drying.[7,12] Heat-treatment kills non-spore forming methanogens so that hydrogen losses due to methanogenesis can be prevented.[13] Even with a heat-treated inoculum, however, the efficiency of hydrogen production is low.

Reducing the partial pressure of hydrogen in reactor vessels can increase hydrogen production. In batch tests, continuous versus intermittent release of gas pressure increased hydrogen production by 43%.[7] Reducing the hydrogen partial pressure using a vacuum or by sparging the reactor vessel with nitrogen gas has also increased hydrogen yields.[7,14] It was assumed in these tests that this increase in hydrogen production was related to reduction in hydrogen partial pressure. However, hydrogen can also be consumed via acetogenesis, according to:

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (1)$$

In this study, we tried to reduce hydrogen consumption by suppressing acetogenesis. Basically, most hydrogen consumers such as methanogens, homoacetogens, sulphate reducers, etc. use acetyl-CoA pathway where CODH (carbon monoxide dehydrogenase) and CO_2 make important roles.[15] The CODH is a strictly anaerobic enzyme which is highly sensitive to oxygen and cyanide and suppressed in low pH below 5.0.[16,17] Thus we applied pH shock, nitrate and cyanide ions and CO_2 removal. Nitrate ion was employed instead of oxygen in order to form anoxic condition. The CO_2 removal was examined since CO_2 was the essential component for acetogenesis with hydrogen. In the CO_2 removal test, a heat-treated sludge was used as seed in order to get rid of the possibility of methanogenesis and examine only the effect on acetogenesis whereas anaerobic mixed culture was used in other tests. It has been discussed that hydrogen loss in

hydrogen fermentation with heat-treated sludge was caused by homoacetogenesis.[18]

MATERIALS AND METHODS

Master Culture Reactor (MCR) Operation

The MCR had been seeded with digested sludge from Gwangju Sewage Treatment Plant in Korea and operated for 6 months to secure bacterial consortia enriched to glucose in a constant temperature room at 35±1°C. The total volume of reactor was 15 L and the working volume was 10 L. Hydraulic retention time (HRT) was kept at 10 days with the organic loading rate of 1 g glucose/L-d. The alkalinity was supplied as much as 0.8 g $NaHCO_3$/L-d. The substrate was fed once a day with nutrient/mineral (N/M) solution.[11] Table 1 shows the characteristics of the MCR before applied to the specific tests.

Table 1. Characteristics of MCR used in the experiment

Parameters		Concentration
pH		7.0±0.1
Alkalinity, mg/L as $CaCO_3$		580
SCOD, mg/L		3,200
MLSS, mg/L		2,800
VFAs, mg/L	Acetate	630
	Propionate	150
	n-Butyrate	1,200
	i-Butyrate	5
	n-Valerate	40

Experiment with Anaerobic Mixed Culture

pH shock. All the experiments with anaerobic mixed culture were basically carried out using pH-shocked culture. Enriched culture of 3 L was transferred from the MCR to a 5 L-pyrex bottle in order to examine the effect of pH drop at first. The reactor was operated in semi-continuous mode. The pH was dropped by stopping alkalinity supply and increasing organic loading rate with 3 days of HRT. It was dropped near 3.0 after 10 days of operation. The loading rate was increased up to 5 g glucose/L-d. After 12 days of operation, $NaHCO_3$ began to be added as much as 2 g $NaHCO_3$/L-d. The ratio of glucose and $NaHCO_3$, 5:2 was determined by pre-experiment to maintain the pH at 5.0±0.2 with 3 days of HRT. Then, the reactor was operated for 50 days with periodical monitoring. Beside the total gas production, the contents of monitoring were headspace gas composition, volatile fatty acids, solvents, and microbial community. Microbial community was analyzed with fluorescence in-situ hybridization (FISH) method and epi-fluorescent microscopy.

Effects of nitrate and cyanide ions. Severe loss of hydrogen still occurred in the pH shocked reactor. Then the pH of the reactor was increased up to 5.5±0.2 by increasing the concentration of $NaHCO_3$ in the feed to 3 g $NaHCO_3$/L and operated for 17 days before nitrate and cyanide ion tests. Before the increase of alkalinity source, culture in the pH-shocked reactor had been fed with only N/M solution for 6 days in order to dilute the accumulated by-products. During the operation, volatile fatty acids (VFAs), several solvents, and headspace gas composition were monitored once a day. In fact, nitrate and cyanide ions were selected as inhibitors to suppress the activity of hydrogen consumers using acetyl-CoA pathway because some evidence of autotrophic hydrogen consumption was obtained during the reactor operation. The batch experiments with nitrate and cyanide were carried out in duplicate.

In order to examine the effect of nitrate ion, a part of the pH-shocked culture was transferred to four 250-mL medium bottles, respectively. The experiment was carried out in batch in duplicate. The concentrations of glucose and $NaHCO_3$ were 5 and 3 g/L, respectively. The volume of liquid in each bottle was 200 mL (134 mL of culture + 66 mL of feed solution). Nitrate ion was added to each bottle in different concentrations, 0, 100, 200, and 500 mg NO_3/L using $NaNO_3$. Then nitrate ion and headspace gas composition were monitored to the end of the batch test.

Another part of the pH-shocked culture was

transferred to five 250-mL medium bottles, respectively for testing the effect of cyanide. The experimental conditions were the same as the nitrate test. The concentration of cyanide ion was adjusted 0, 6.5, 32.5, 65, and 130 mg CN^-/L with KCN, respectively.

CO_2 Scavenging from Headspace

Culture and medium. Dewatered and thickened sludge was obtained from at the Pennsylvania State University Wastewater Treatment Plant in State College, PA, and heat-treated as previously described.[7] The sample was either used within one day or stored in a sealed container at room temperature (24°C). Glucose (2 g/L) and sludge (1 g/L) were added to a mineral salt medium[11] buffered to a pH of 6.2 using 0.07 M of 2-(N-mopholino) ethanesulfonic acid monohydrate (MES) (Mallinckrodt Baker Inc., Phillipsburg, New Jersey, U.S.A.).

Experimental apparatus. Batch tests were conducted in duplicate using 250 mL of media in 300 mL bottles stirred at 300 rpm in a constant temperature room (30±1 °C). All bottles were initially flushed with nitrogen gas and sealed. CO_2 was removed from the headspace using 6 mL of KOH (30 wt %) placed in a small tube open only to the headspace. Biotic controls were prepared in the same manner using distilled water instead of KOH. Gas production was measured using a respirometer (Challenge Environmental Systems AER-200, Fayetteville, Arkansas, U.S.A.) with bio-hydrogen production calculated from headspace gas measurements and the total volume of biogas produced at each sampling interval as previously described.[18] The difference in gas production between the treatment and control was used to calculate total CO_2 gas production. Four bottles prepared in the same manner (with and without CO_2 scavenging) were connected to 1 L-polypropylene gas packs (SPELCO) open to the headspace of the bottles instead of the respirometer for analysis of liquid products. Liquid analysis was conducted to evaluate the effect of CO_2 scavenging on the change in by-product composition.

Bacterial Community Analysis with FISH

Sample preparation and cell fixation for FISH. Samples were taken periodically with 20-mL syringe and transferred to 50-mL centrifuge tubes for the quantitative analysis of bacterial community under severe pH shock. Each sample was spun down by centrifuge at 4,500 rpm for 17 min in a 50-mL centrifuge tube. Then the supernatant was transferred to another 50-mL tube and stored at -20°C after 7 mL of 10 mM phosphate-buffered saline (PBS) and 3 mL of 50 % glycerol were added.

One mL of each sample was transferred to a 1.5-mL centrifuge tube and spun down at 12,000 rpm for 5 min. Then it was washed with 10 mM PBS by vortex for 5 min. this procedure was repeated three times. After that, cells were fixed with 1 mL of 4% paraformaldehyde in PBS and left for 5 hr at 4°C. Finally, the sample was washed with PBS twice and stored at -20°C with 50% EtOH and 50% PBS.

Probes for FISH and hybridization. The 16S rRNA targeted oligonucleotide probes and their FISH conditions were demonstrated in Table 2. Probes were 5'-end labelled with TAMRA (tetramethyl rhodamine) and purified by reverse-phase high-pressure liquid chromatography were obtained from ThermoHybaid GmbH (Ulm, Germany). In situ hybridization was performed at 46°C for 3 h with hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.1% sodium dodecyl sulfate [SDS]) containing 2.5 ng of each probe per μL of hybridization buffer. Formamide was added to the hybridization buffer to control stringency in the hybridization steps. Unhybridized probes were washed at 46°C for 30 min with respective washing buffers (Tris-HCl [pH 7.2], 0.1% SDS) containing different concentrations of NaCl (Table 2). FISH was followed by 4',6-diamidino-2-phenylindole (DAPI) staining to stain total cells. DAPI stained cells were washed with distilled water at room temperature and air-dried. The cells were visualized using a Zeiss Axiolab,

Table 2. Probes used in the study and their FISH conditions

Probe name	% FA[a]	NaCl (mM) in washing buffer	Target group	Reference
ARC915	35	80	Archaea	20)
EUB 338	30	150	Bacteria	21)
MB1174	35	80	*Methanobacteriaceae*	22)
MT757	20	225	*Methanosaeta*	23)
MS821	20	225	*Methanosarcina*	22)

[a] % (v/v) formamide in hybridization buffer.

with a 50W-mercury lamp. The cell counting was achieved with at least 10 random microscopic fields in each well.

Analysis

Liquid samples (triplicate) were prepared for analysis by adding 20 μL of $HgCl_2$ (16 g/L) into 1 mL of sample in a 1.5-mL centrifuge tube, centrifuging the tubes at 6610 × g for 15 min, and then removing the supernatant for analysis. Samples were stored at 4°C prior to analysis. Then the liquid samples were analyzed using gas chromatography (GC, Hewlett Packard 5890 series II) equipped with an FID detector and a glass column packed with carbopack B-DA. Fifty μL of the gas in the headspace of the reactor was sampled with a constant rate syringe (CR-700-200, HAMILTON Co.) and analyzed in order to determine the fraction of H_2, CO_2, and CH_4 with gas chromatography (GC, Hewlett Packard 5890 series II), which was equipped with a TCD detector and a CROMPACK column. The pH was measured with a pH meter (ISE/pH/mV/ORP/Temp. meter, Orion, Model 290A).

RESULTS AND DISCUSSION

Effect of pH Shock

During the operation of pH shocked culture, obvious change in gas composition, by-products of glucose degradation and also bacterial community(Figure 1). The pH of reactor was 4.8± 0.2. According to the headspace gas analysis, methane production was perfectly controlled after 33 days of operation but severe loss of hydrogen was observed between 30 and 40 day. The loss of hydrogen was not contributed to methanogenesis since there's no methane production during the period. Otherwise, sudden increase of butanol was detected along with decrease of butyrate when the loss occurred. After the ratio of butanol and butyrate ran over 0.75, no hydrogen was observed in the headspace (Figure 1(a)). It could be suggested that the loss of

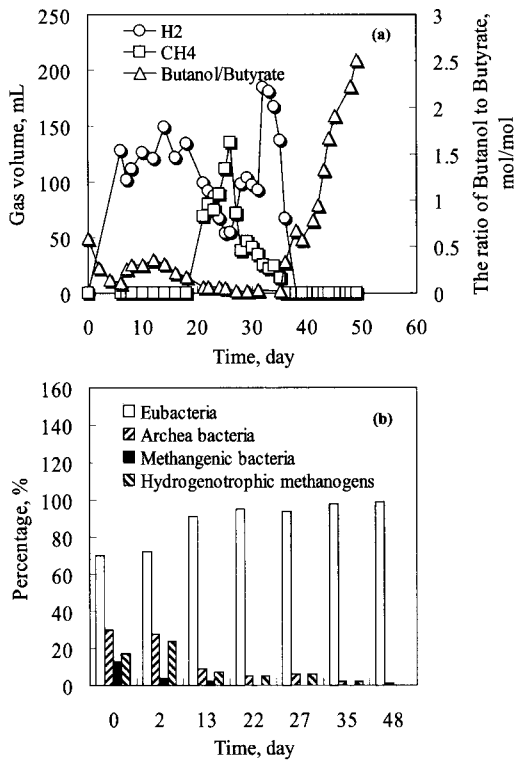

Figure 1. Effect of pH drop to the anaerobic fermentation and microbial community. ((a) Gas production and composition and the ration of butanol and butyrate. (b) Change in microbial community by pH drop.)

hydrogen was caused by the change in glucose degradation pathway to solventogenesis. Solventogenesis contributing to the production of ethanol, acetone, butanol, etc. has been known to cause hydrogen consumption.[11] This result could be supported by the bacterial communication analysis. Archaea were mostly hydrogenotrophic methanogens and it was less than 7% when the severe loss occurred (Figure 1(b)). Therefore, methanogens couldn't be suggested as the origin of loss. The only possibility was the very bacteria contributing to solvent production. Here, it could be suggested from this operation that the pH shock was effective for the suppression of methanogens but reactor operation at this low pH (4.8 ± 0.2) would cause the pathway change to solventogenesis accompanied with the severe loss of hydrogen. A similar result on the pathway change has been reported in a research about bio-hydrogen production.[24]

Effect of pH Increase in pH shocked Culture

The effect of pH increase on the pH shocked culture was examined because hydrogen was still lost even after the shock, which meant some hydrogen consuming bacteria still active. The pH of reactor was adjusted higher by increasing the concentration of $NaHCO_3$ to 3 g/L. After that, it was increased up to 5.5 ± 0.2. However, there's still occurring the consumption of hydrogen apparently by some biological reaction suspected as acetogenesis (Figure 2). Continuous increase of acetate was observed along with the decrease of the hydrogen concentration, whereas other by-products were maintained at nearly constant concentrations. Butyrate accumulation was also observed from the beginning of operation but stopped after 12 day at $2{,}100 \pm 50$ mg/L of concentration (Figure 2(a)). The increase of acetate, probably, could be suggested the cause of hydrogen loss accompanied with acetogenesis like the equation (1). The consumption of hydrogen by acetogenesis has been discussed before.[18] When CO_2 and H_2 coexist, acetogenesis always occur in a mixed anaerobic culture. The result supporting the possibility was suggested in Figure 2(b). It was sure that the fraction of carbon dioxide decreased with the decrease of hydrogen in the headspace. Other parameters such as pH, alkalinity source, and organic concentration were fixed at the designed values. Carbon dioxide was probably consumed by the acetogenesis with hydrogen resulting in the increase of acetate. From this result, it could be suggested that the acetogenic bacteria survived from the pH shock even after 50 days of operation and thus contributed to the hydrogen consumption when the pH was increased to 5.5.

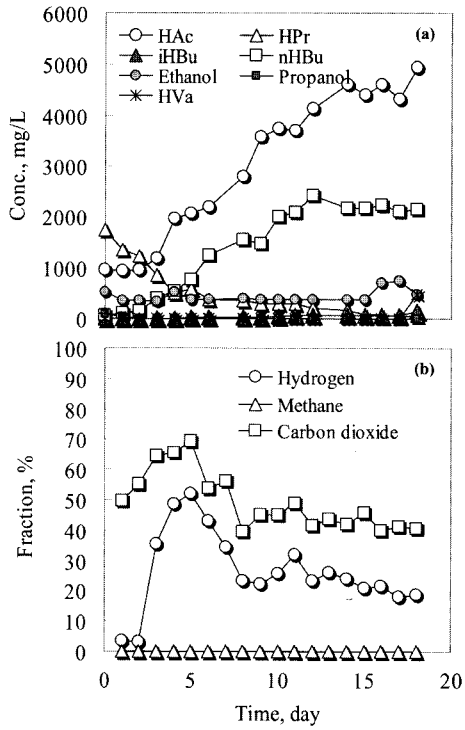

Figure 2. Change in by-products and gas composition as the pH increased to 5.5. (The pH was increased from 4.8 to 5.5.)

The survival of acetogenic bacteria was an interesting phenomenon. The bacteria contributing to acetogenesis have been reported to be inactivated at pH 5.0 because the CODH loses its activity.[17] However, amazingly, the acetogenic activity was observed again as the pH increased to 5.5. The survival of acetogenic bacteria might be contributed to the spore-forming nature of some acetogenic bacteria such as

Clostridium aceticum.[19] Therefore, it could be inferred, not affirmative, that the spore-forming bacteria weren't washed out even under the extreme condition. After that, they geminated as the pH increased and then contributed to the acetogenesis.

Effects of Nitrate and Cyanide Ions

The loss of hydrogen was suggested due to acetogenesis. A sequent batch experiment using nitrate ion as the inhibitor showed that the loss of hydrogen could be retarded by anoxic condition. However, unfortunately, the positive effect of nitrate ion was just temporary. The concentration of hydrogen was increased up to 61% at 500 mg NO_3/L by applying nitrate injection whereas 29% in the blank test, where hydrogen was totally consumed within 29 hr. In other bottles applied nitrate addition, the consumption could be prevented for a while but occurred after the depletion of nitrate (Figure 3). Consequently, it could be suggested that the nitrate would be effective for the suppression of hydrogen consumption but the effect not persist. If a wastewater contains, uniquely, both nitrate and organics, the result could be useful for hydrogen production from the wastewater but if not, it couldn't. As an alternative way, an annexed treatment of a wastewater with organics and a wastewater with nitrate will be recommendable.

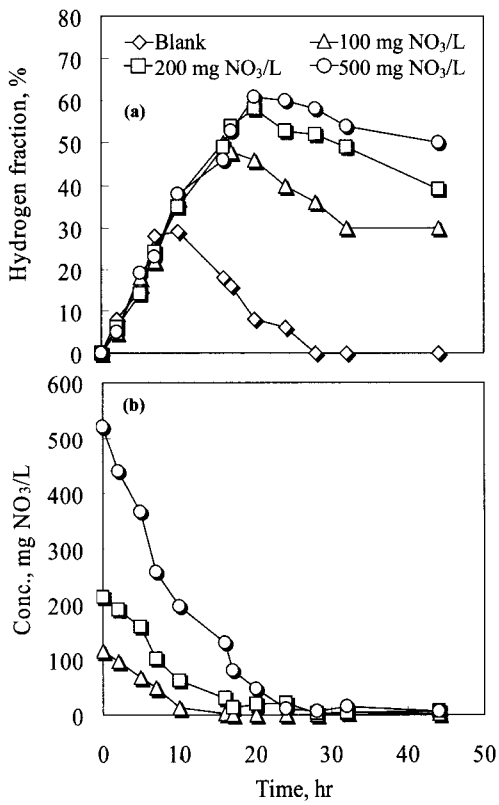

Figure 3. Effect of nitrate ion to the gas composition in the headspace. ((a) Change in headspace gas composition. (b) Change in nitrate concentration in liquid. Nitrate concentration was measured to verify the occurrence of denitrification.)

Cyanide ion didn't show any effectiveness. When the ion was added, the total gas production was decreased to 10% of the blank. It has been reported that the ion inhibits the activity of CODH[17] but other anaerobic bacteria were not exceptional.

CO_2 Scavenging from Headspace

In this test, heat-treatment was employed in order to get rid of the possibility of the hydrogen loss by methanogens. CO_2 removal was purposed to suppress acetogenesis. The removal of CO_2 provided a reasonable and simple solution for the purpose. Normally, the maximum concentration of hydrogen was ranged between 60 and 70% with heat-treated sludge.[7,18] In this experiment, the maximum concentration of hydrogen was 68% without CO_2 removal. Then the hydrogen was consumed fast to be zero at 52 hr accompanied with the reduction of CO_2 to be less than 5% (Figure 4(a) and (b)). During the decrease of H_2 and CO_2, a dramatic increase of acetate was observed from 474 to 1,137 mg /L. This type of hydrogen loss (acetogenesis) has been discussed previously by Oh *et al.*.[18] On the other hand, it was up to 87 % as CO_2 was removed. Then it was deceased slightly to 79% at 52 hr (Figure 4(a)). The loss of hydrogen was well protected by scavenging CO_2. Acetogenesis can be processed only when both CO_2 and H_2 exist. Basically, all the acetogens use the acetyl-CoA pathway for the synthesis of

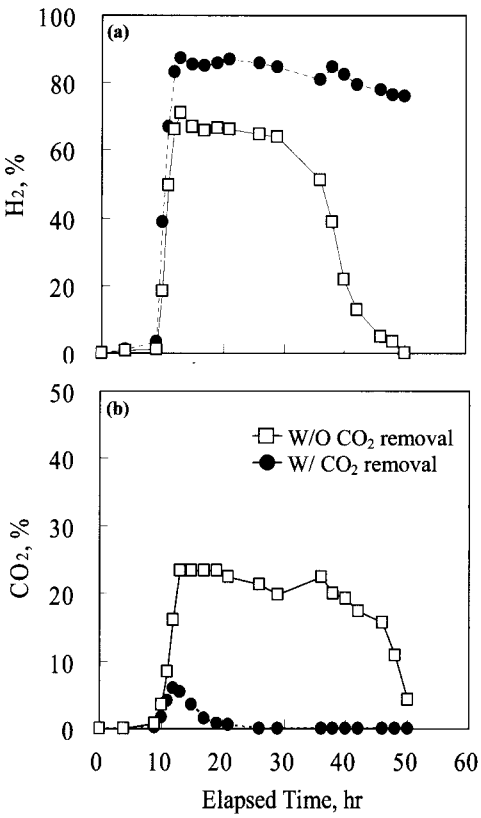

Figure 4. Effect of CO_2 scavenging from the headspace. ((a) Change in hydrogen fraction. (b) Change in CO_2 fraction. CO_2 fraction was shown to suggest the evidence of hydrogen loss by acetogenesis.)

cell materials accompanied with acetate production. Therefore, it might be suggested that they would not synthesize cell materials resulting in disappearance in a long term operation. But it can not be affirmative without communication analysis.

CONCLUSIONS

Several methods for the suppression of hydrogen consumption have been employed in order to reduce the loss and increase the production potential of hydrogen. The following are the intensive results of the research.

1. The pH control could be effective for the suppression of methanogens but not enough to prevent the hydrogen loss. The low pH below 5.0 caused solventogenesis and deteriorated the condition for hydrogen evolution. After the solventogenesis occurred, no hydrogen was detected.
2. Even though the severe drop of pH was applied, the bacteria responsible for acetate production could not be separated from the culture. As the pH was increased to 5.5, they restarted to show a huge activity resulting in fast consumption of hydrogen.
3. Nitrate ion could be suggested as an effective inhibitor for the suppression of hydrogen consumption. It resulted in the increase of maximum fraction of hydrogen from 29 (blank) to 61% (500 mg NO_3/L). However, the loss of hydrogen occurred right after the depletion of nitrate. Consequently, therefore, nitrate could be suggested as an outstanding inhibitor but temporary.
4. Cyanide ion didn't show any effectiveness. It caused a serious effect in biogas production itself resulting in 90% decrease in total gas production.
5. The removal of CO_2 contributed to the outstanding protection of hydrogen loss. The maximum fraction of hydrogen was enhanced even to 87% whereas 68% in the comparative test. CO_2 scavenging may provide a more direct and cost effective method than other application.

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