Effect of Nitrogen-Load Condition on Hydrogen Production and Bacterial Community in Continuous Anaerobic Hydrogen Fermentation Process

Yasunori Kawagoshi*+ / Masaharu Nakao* / Naoe Hino* / Tomonori Iwasa* / Kenji Furukawa*

Abstract : Effect of nitrogen-load condition on hydrogen (H_2) production and bacterial community in a continuous anaerobic hydrogen fermentation were investigated. The slight H_2 production on extremely low nitrogen-load condition (C/N ratio: 180) at the start-up period. The highest H_2 production was obtained when the C/N ratio was 36, the H_2 production yield (mol-H_2 / mol-glucose) reached to 1.7, and it was indicated that Clostridium pasteurianum mainly contributed to the H_2 production. The H_2 production was decreased on both the lower (C/N: 72) and higher (C/N: 18) nitrogen-load conditions. The excess nitrogen-load was not always suitable for the hydrogen production. The fluctuation of H_2 production seemed to be caused by a change in the bacterial community according to the nitrogen-load condition, while a recovery of H_2 productivity was possible by a control of nitrogen-load condition through the bacterial community change. When the nitrogen-load condition was not suitable for hydrogen production, the lactic acid concentration was increased and also lactic acid bacteria were definitely detected, which suggested that the competition between hydrogen fermentator and lactic acid producer was occurred. These results demonstrated that the nitrogen-load condition affect on the H_2 productivity through the change of bacterial community in anaerobic hydrogen fermentation.

Keyword : Hydrogen fermentation, Nitrogen load, Bacterial community, DGGE

Introduction

In recent years, energy issues has been more serious because the depletion of a finite resources are feared. On the other hand, waste problem has been considerable concerned worldwide. These problems are especially serious for the country like Japan, which is poor in natural resources and has insufficient waste-disposal site. The one of the key to these problems is the establishment of recycling-based society supported by renewable resources. There are a variety of renewable energy sources altanative to the fossil energy, e.g., solar thermal energy, hydraulic energy, wind power and biomass energy. The biomass energy has a great advantage in the aspect of waste-recycle if a biomass waste wii be efficiently utilized to produce an energy. Currently, liquid fuels such as the ethanol and the biogases like methane or hydrogen gas (H_2) are commonly produced from the biomass materials. Hydrogen gas is expected as one of the promising future energy source because it is consideredto be the most valuable material for power fuel cell and has a higher energy content per unit weight than any other fuel energy source (Boyles, D., 1984). Hydrogen gas commonly has been produced by steam reforming of natural gas and electrolysis of water so far, however, these process are high-cost and environmentally- unfriendly. Therefore, biological H_2 production processes, which can convert an organic waste to H_2, have received considerable attention as a promising technology from the perspective of recycling-based society.

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Anaerobic hydrogen fermentation is the most developed process from various organic materials including biomass, compared with other biological H_2 produciton methods (Levin, B.D. *et al.*, 2004). There have been studies about anaerobic hydrogen fermentation process from the pure substrates such as glucose and real mixed source such as a kitchen waste in recent years (Kim, S.H. *et al.*, 2005; Kotsopouls, T.A. *et al.*, 2006; Lay, J.J. *et al.*, 1999; Liu, H. *et al.*, 2003; Liu, D. *et al.*, 2006; Ueno, Y. *et al.*, 2006; Ueno, Y. *et al.*, 2006; Yokoi, H. *et al.*, 2002; Yu, H. *et al.*, 2002; Zhu, H.G. *et al.*, 2002). These studies demonstrated the possibility of H_2 production using the bacterial inoculums such as anaerobic digested sludge on strictly anaerobic condition, and the effects of culture conditions of pH, temperature, hydraulic retention time, etc., on H_2 production (Kim, S.H. *et al.*, 2005; Kotsopouls, T.A. *et al.*, 2006; Lay, J.J. *et al.*, 1999; Liu, H. *et al.*, 2003; Liu, D. *et al.*, 2006; Ueno, Y. *et al.*, 2006; Ueno, Y. *et al.*, 2006; Yokoi, H. *et al.*, 2002; Yu, H. *et al.*, 2002; Zhu, H.G. *et al.*, 2002; Fang, H.H.p. *et al.*, 2002; Kawano, T. *et al.*, 2004; Lee, Y.J. *et al.*, 2001; Lee, Y.J. *et al.*, 2002; Mu,Y. e*et al.*, 2006). However, there are only a few study on the effect of nutrient component like a nitrogen on the hydrogen fermentation (Lin, Y. *et al.*, 2004), and there is no study on the interaction between hydrogen fermentation condition and the related bacterial community. This study therefore aimed to clarify the effect of nitrogen-load on the H_2 production activity and related bacterial community in the continuous hydrogen fermentation.

Materials and Methods

Inoculum and medium

Anaerobic digestion sludge from sewage waste water treatment plant was used as an inoculum. The synthetic medium was prepared with 20 g/*l* or 40 g/*l* glucose as a carbon source and other inorganic supplements: 125 mg/*l* K_2HPO_4, 2 g/*l* Na_2CO_3, 5 mg/*l* $CuSO_4 \cdot 5H_2O$, 100 mg/*l* MgCl $\cdot 6H_2O$, 15 mg/*l* MnSO4 $\cdot 4H_2O$, 0.125 mg/*l* $CoCl_2 \cdot 6H_2O$, 25 mg/*l* $FeSO_4 \cdot 7H_2O$, and the prescribed amount of NH_4HCO_3 was added. The medium was adjusted pH of 6.0 and sterilized by autoclave before use.

Continuous H_2 fermentation on various nitrogen-load conditions

The synthetic medium of 1.6 *l* was put into a continuous stirred tank reactor (CSTR) of 2 *l* and deaerated by argon gas purge. The temperature and pH were maintained constant at 35 ºC and 6.0, respectively, by the CSTR controller. The medium was continuously supplied into the reactor and drained by peristaritic pumps as shown in Figure 1. The ammonium concentration in the medium was accordingly changed from the range of 44 mg-nitrogen (N)/*l* to 880 mg-N/*l* during experimental period.

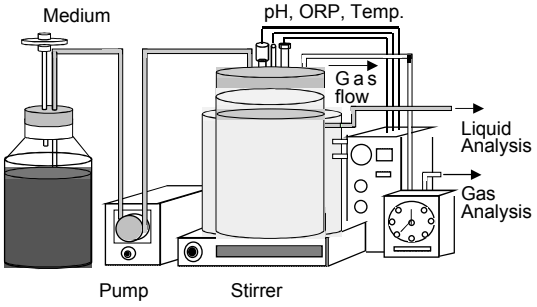

Fig. 1. Schematic diagram of lab-scale continuous anaerobic hydrogen fermentation reactor

Analysis

The gas composition (H_2, CO_2, CH_4, N_2, O_2)

and percentage were analyzed by a gas chromatograph equipped with a thermal conductivity detector (TCD) using SHINCARBON ST column (50/80 mesh, 4 m x 3 mm I.D.) (Shimadzu, Kyoto, Japan), and argon gas was used as a carrier. The volatile fatty acids (VFAs; acetic acid:HAc, propionic acid: HPr, n-butyric acid: n-HBu, isobutyric acid: i-HBu, n-valeric acid: n-HVa, isovaleric acid: i-HVa and lactic acid: HLa) were analyzed by a high-performance liquid chromatograph (HPLC) with a UV-detector (at 210 nm) using Shim-pack SCR-120H column (300 x 8 mm I.D.) (Shimadzu) for acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and lactic acid. The glucose (hexose) concentration was measured by phenol-sulfuric acid method (APHA., 1995) and the concentration of volatile suspended solids (VSS) was determined as a biomass amount according to Standard methods (APHA., 1995).

PCR-DGGE

A culture of 1.0 ml was accordingly taken from the reactor and centrifuged at 12,000 x g for 5 min. The harvested cells were immediately extracted or stored at −80 °C until analysis. DNA extraction and purification were performed by UltraClean Soil DNA Isolation Kit (Mo Bio, USA). Polymerase chain reaction (PCR) was performed using the extracted DNA samples as templates according to our previous study (Kawagoshi, Y. *et al.*, 2005). The bacterial universal primer set was used, i.e., forward primer attached with a GC-clamp: U341f-GC(5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGAGCAG-3') and reverse primer: U907r: (5'-CCCCGTCAATTCCTTTGAGTTT-3') (Weisburg, W.G et al., 1991). The amplified DNA was verified by an agarose gel electrophoresis and supplied for denaturing gradient gel electrophoresis (DGGE). The DGGE gel was made using SJ-1060GF gradient gel maker (ATTO, Japan) and electrophoresis was carried out using AE-6290E system (ATTO) according to our previous study (Kawagoshi, Y. et al., 2005). The gel was stained with SYBR GREEN I and DNA bands were verified by UV radiation. The visible DNA bands were taken from the gel and reamplified by PCR according to the same procedure as above. The DNA sequencing was performed according to the manufacturer's protocol using the same forward primer as above. The DNA sequence alignment and phylogenetic analysis were performed using BioEdit (Hall, T.A., 1999) and Clustal W softwares (Thompson, J.D. et al., 1994). The homology search of the obtained DNA sequences were determined by comparing with FASTA database (Person, W.R. et al., 1988).

Results and Discussion

Start up of continuous hydrogen fermentation on low nitrogen-load condition

Figure 2 shows the time course of H_2 production yield (mol of H_2 / mol of glucose), glucose consumption rate (%), VSS concentration (g/*l*) and VFAs cocentrations during the experimental period. Figure 3 shows the DGGE result at each experimental condition.

Continuous culture was started on a very low nitrogen-load condition (C-I in Figure 2) of 20 g/*l* glucose and 44 mg/*l* of nitrogen (C/N ratio: 180), which is the same condition as that of anaerobic digested sludge used as a source of inoculum, and continued for 10 days. As shown in Figure 2, both H_2 production yield and glucose consumption rate increased at the beginning of culture, but the glucose consumption rate rapidly decreased from 80 %

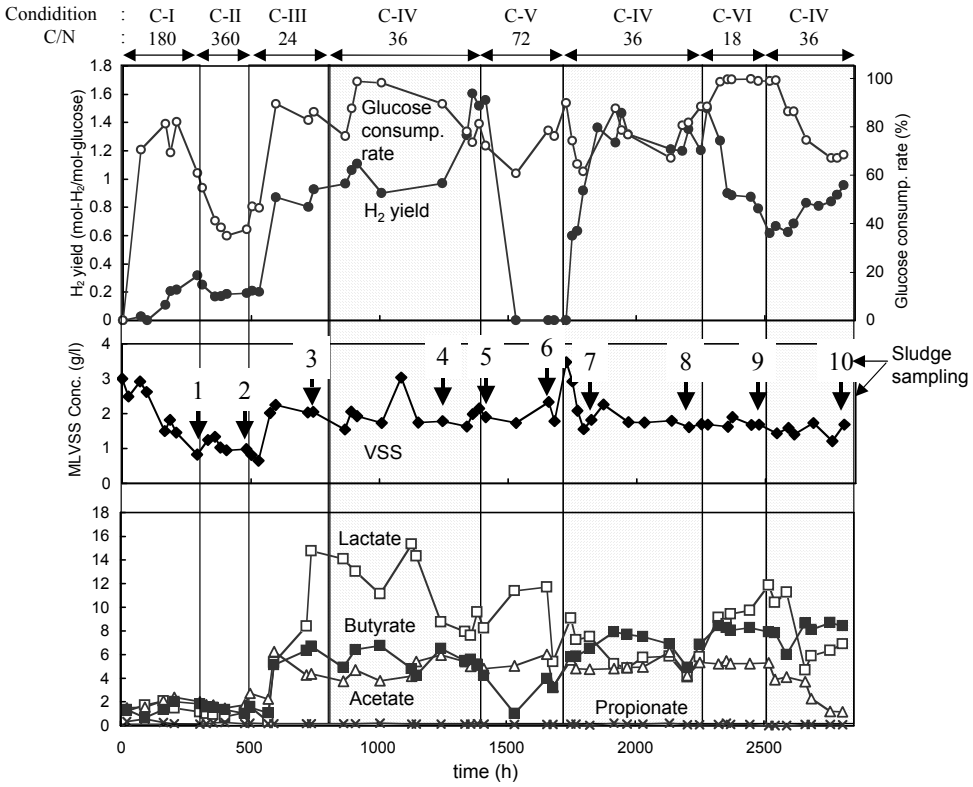

Fig. 2. Time course of H_2 production yield (mol of H_2 / mol of glucose), glucose consumption rate (%), VSS concentration (g/l) and VFAs cocentrations.

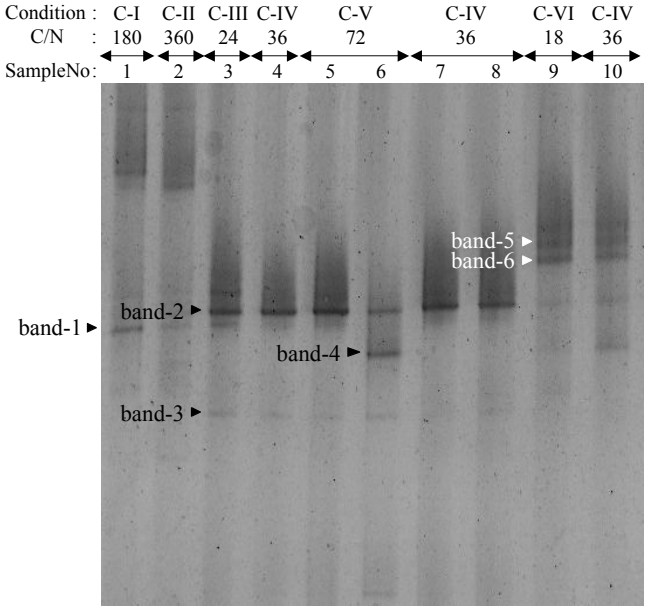

Fig. 3. DGGE result on each nitrogen-load condition

Table 1. Phylogenetic identification of major DGGE bands FASTA database

Band number*	Closest relative	Identity(%)	Accession number
1	*Clostridium* sp. FRB1	98.3	AY925092
2	*Clostridium pasteurianum*	99.0	M23930
3	*Lactobacillus paracasei* isolate 4C	92.4	AY773954
4	*Sporolactobacillus terrae*	99.8	AJ634662
5	*Clostridium tyrobutyricum*	99.1	M59113
6	*Clostridium tyrobutyricum*	98.9	M59113

* The band numbers are corresponding to them on DGGE (Fig. 3)

to 50 % after 250 hours. The VSS concentration decreased from around 3 g/*l* at the beginning to 0.8 g/*l* at the end of this condition, which indicated biomass reduction. The slight VFAs were produced, i.e., butyrate and acetate, which are common metabolites in hydrogen fermentation, were only less than 2g/l and other VFAs were not detected. On the other hand, the H_2 production yield showed the trend of slight increase, and additionally one relatively clear DNA band (band-1) was detected on DGGE gel (sample 1 in Figure 3). The sequence of this DNA band showed 98 % similarity to Clostridium sp. known as a candidate of hydrogen fermentating bacteria (Table 1), therefore it was suggested that this bacteria contributed to H_2 producing activity although it was quite low on this condition (Ueno, Y. *et al.*, 2006; Kawano, T. *et al.*, 2004; Wu, S.Y. *et al.*, 2006).

It was suggested that low production of the biomass and H_2 were due to the lack of nitrogen, therefore, in order to confirm this hypothesis the glucose concentration was further increased to 40 g/*l*(C-II in Figure 2). On an extremely low nitrogen-load condition, the glucose consumption rate further decreased to around 40 % and the H_2 production yield continued to be 0.2. VSS and VFAs concentration also continued to be flat in the low levels of 1 g/*l* and less than 2g/*l*, respectively. Additionally, there is no distinct DNA band on DGGE gel at the end of this phase as shown in Figure 3 (sample 2). Finally, no nitrogen was detected in effluent at the end of this phase. From these results, it was concluded that the quite low nitrogen-load (C/N Ratio: 360~180) caused a reduction of the biomass including hydrogen fermentating bacteria and the H_2 hydrogen productivity.

Suitable nigrogen-load condition for continuous hydrogen fermentation and related bacterial community

Since the above results obviously indicated a lack of nitrogen source for biomass, the nitrogen concentration was set at 680 mg/*l* (C-III, C/N: Thompson, J.D. *et al.*, 1994) referred to previous studies (Kawagoshi, Y. *et al.*, 2005; Nokie, T. *et al.*, 2002). As shown in Figure 2, the glucose consumption rate and the H_2 production yield drastically increased to 90% and 1.0, respectively, and the VSS concentration also increased from 1g/*l* to 2g/*l*, which suggested that the nitrogen-load condition significantly affect not only biomass but also H_2 producing activity. The concentrations of HLa, n-HBu and HAc rapidly increased along with the other items, particularly the HLa concentration was remarkable, however, HPr, which were considered unfavorable metabolites for H_2 production, was not detected.

As shown in Figure 3, the distinct DNA band

(band-2) and the another thin band (band-3) were observed on DGGE gel (sample 3). The band-2 showed 99 % sequence similarity to Clostridium *pasteurianum* which was reported as a main hydrogen producer in high rate bioreactor (Wu, S.Y. *et al.*, 2006) operated with sucrose of 35.6 g/l (71.2 g/l hexose) at HRT of 0.5 h. In this study, the glucose concentration was also higher (40 g/l hexose) than other previous studies (Lee, Y. J. *et al.*, 2002; Lin C.Y. *et al.*, 2004; Chen, C.C. *et al.*, 2002; Logan, B.E. *et al.*, 2002), therefore high carbon-load condition might be suitable for the growth of *C. pasteurianum*. On the other hand, the band-3 was presumed a lactic acid bacteria *Lactobaccilus* sp. from the result of sequence similarity although the similarity percentage was only 90 %, it was suggested that the HLa production on this condition was due to the band-3 bacteria.

A little excess nitrogen-load was presumed on the C-III condition because that the effluent ammonium concentration was 200 mg/*l*, thus the nitrogen concentration was set at 440mg/*l* (C-IV, C/N:36), next. The H_2 production yield increased to 1.7 and more than 90% of glucose was stably consumed. In addition, the effluent nitrogen concentration was decreased to less than 10 mg/*l*, which suggested that the nitrogen concentration of 440mg/*l* was adequate amount for 40g/*l* glucose utilization by biomass in the reactor. The main DNA band on DGGE gel was still the band-2, thus it was concluded that the bacterium possessing the band-2 contributed mainly to H_2 production in the reactor, and this nitrogen condition was optimal for hydrogen fermentation in this experiment. The concentrations of butyrate and acetate were constant, and the HLa concentration remarkably increased at the beginning of this condition, and then gradually decreased with the increase of H_2 production yield, while the density of band−3 was attenuated (sample-3 to sample-4 in Figure 3). These results indicated that the reduction of lactic acid bacteria resulted in the decrease of lactate production and the improvement of H_2 production which was the same result as that in our previous study (Kawagoshi, Y. *et al.*, 2005).

Effect of low nitrogen-load on continuous hydrogen fermentation and bacterial community

In order to examine the effect of low nitrogen load on H_2 production and bacterial community, nitrogen concentration was decreased to 220 mg/*l* (C-V, C/N:72), a rapid decrease of H_2 production yield was observed in spite of slight decrease of the glucose consumption rate and no change of VSS amount, while nitrogen in the effluent was not detected on this condition. The concentration of HLa increased again, while of HBu rapidly dropped. The band-2 was attenuated, and new DNA band (band-4), which showed 99.5 % sequence similarity to lactic acid bacterium *Sporolactobacillus terrae*, was observed on DGGE gel (sample 6 in Figure 3). These results indicated that the nitrogen condition affected on not only a metabolism in hydrogen fermentation but also a bacterial community without remarkable change of biomass volume.

Since the recovery of H_2 production was not expected on this condition, the nitrogen concentration was reset at 440mg/*l* (C-IV, C/N:36). Both the H_2 production yield and the HBu concentration rapidly increased, while the HLa showed slight decreasing trend. In addition, the band-2 became predominant and the band-4 was remarkably attenuated on DGGE (samples 7, 8 in Figure 3). These results indicated that it was possible to recover the H_2 producion due to the reconstruction of bacterial community

according to the condition.

Effect of high nitrogen-load on continuous hydrogen fermentation and bacterial community

The nitrogen concentration was next increased from 440 mg/*l* to 880 mg/*l* (C-VI, C/N:18). The H_2 production yield decreased although the glucose consumption rate was maintained more than 90%. On this condition, the effluent nitrogen concentration was over 400 mg/l. The HBu and HAc concentrations were almost constant, but the HLa concentration showed increasing trend. The bacterial community became quite different from those on other conditions, i.e., the band-2 was attenuated and the other new bands (band-5, band-6) showing 98% similarity to Clostridium tyrobuty-ricumappeared, in addition, the band-4 was slightly detected again. Therefore, in order to recover H_2 productivity, the nitrogen con-centration was returned to 440 mg/lagain (C-IV, C/N:36), the HLa concentration rapidly decreased, however, the recovery of H_2 production yield was slowly and the glucose consumption rate once decreased. The change of bacterial community was also gradual, i.e., the band-1 only became littlebit thick. From these results, it was suggested that the excess nitrogen-load condition was not always suitable for H_2 production, in addition, the nitrogen-load affect on the bacterial community within genus level and the H_2 productivity was likely changed depending on the type of Clostridiumspecies. Furthermore, if the bacterial community was changed among the competitive hydrogen producers, the rapid change of bacterial community and the H_2 productivity seemed to be difficult.

Conclusions

Anaerobic hydrogen fermentation process has received attention as one of the most promising technology to produce H_2 from the perspective of recycling-based society. We investigated that the effect of the nitrogen-load condition on H_2 production and bacterial community in the continuous anaerobic hydrogen fermentation process, and obtained interesting results. The highest H_2 production was obtained when the C/N ratio was 36, and it showed declining trend either when the C/N ratio was more or less than 36. The H_2 production was changed through the bacterial community by the nitrogen-load condition, while the recovery of H_2 productivity was possible by a control of nitrogen-load condition. When the condition was not suitable for hydrogen production, the distinct HLa production and lactic acid bacteria were observed. These results demonstrated that the nitrogen-load condition likely affect on the H_2 productivity through the change of bacterial community in anaerobic hydrogen fermentation process.

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