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# Effect of hydraulic retention time on the hydrogen yield and population of *Clostridium* in hydrogen fermentation of glucose

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**Abstract**

The conversion of glucose to hydrogen was evaluated using continuous stirred tank reactor at pH 5.5 with various hydraulic retention times (HRT) at 30°C. Furthermore, the population dynamics of hydrogen-producing bacteria was surveyed by fluorescence *in-situ* hybridization using probe Clost IV targeting the genus *Clostridium* based on 16S rRNA. It was clear that positive correlation was observed between the cells quantified with probe Clost IV and hydrogen yield of the respective sludge. The numbers of hydrogen-producing bacteria were decreased gradually with increasing HRT, were $9.2 \times 10^8$, $8.2 \times 10^8$, $2.8 \times 10^8$, and $6.2 \times 10^7$ cell/mL at HRT 6, 8, 12, and 14 h, respectively. The hydrogen yield was 1.4–1.5 mol $H_2$/mol glucose at the optimum HRT range 6–8 h. It is considered that the percentage of the hydrogen-producing bacteria to total bacteria is useful parameter for evaluation of hydrogen production process.

**Key words**: bio-hydrogen; fluorescence *in-situ* hybridization; genus *Clostridium*
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## Introduction

Hydrogen is a promising candidate as a clean energy carrier. The energy content per unit mass of liquid hydrogen is about 2.75 times higher than that of hydrocarbon fuels (Chang and Lin, 2003). Biological hydrogen production has been investigated in two groups of bacteria: photosynthetic anaerobic bacteria, and anaerobic fermentative bacteria (Das and Veziroglu, 2001; Lee *et al*., 2002). The latter possess the ability to generate hydrogen without photoenergy, making them useful for treating large quantity of high strength organic wastes. Although the members of genus *Clostridium* are known for evolving hydrogen during anaerobic fermentation (Karube *et al*., 1982; Esteso *et al*., 1996; Kataoka *et al*., 1997; McTavish, 1998; Singh *et al*., 1999) and their existence in hydrogen production processes had been shown (Lay, 2001; Ueno *et al*., 2001a; Fang *et al*., 2002a), limited information about detection and quantification in hydrogen production is available.

Traditionally, microbes are identified by isolating individual cultures and examining their physiological, biochemical, and morphological characteristics. However, such identification is often unreliable. First, microbes may not be properly isolated from the artificial growth medium. Second, many microbes grow syntrophically with others and thus cannot be cultured individually (Pike and Curds, 1971; Wagner *et al*., 1993). Third, many microbes share similar physiological, biochemical, and morphological characteristics and thus cannot be distinguished.

More recently, a number of molecular techniques have been developed for the quantitative analysis of microbial communities. Among them, 16S rRNA-based fluorescence *in-situ* hybridization (FISH) have been extensively applied in the study of activated sludge, biofilms, ocean mats, sediments (Amann *et al*., 1990; Raskin *et al*., 1994; Sekiguchi1 *et al*., 1999; Syutsubo *et al*., 2001), etc.

In this study, a mesophilic microbial community converting carbohydrate into hydrogen in wastewater was developed. Glucose was chosen as the model carbohydrate. The quantitative analysis of the hydrogen-producing bacteria was analyzed using 16S rRNA-based FISH technique. Furthermore, the quantity result of FISH was compared with the hydrogen generation.

## 1 Materials and methods

### 1.1 Experiments of hydrogen production

As shown in Fig. 1, four series of hydrogen production experiment were conducted by a 0.5-L continuous stirred tank reactor (CSTR). The reactors were operated at (30 ± 1)°C and pH was adjusted to 5.5 using 3 mol/L NaOH.

The composition of the hydrogen growth medium

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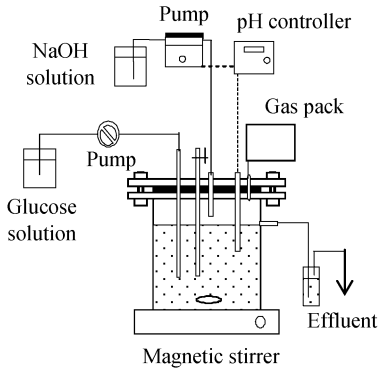


**Fig. 1** Schematic of a chemostat reactor.

was as follows (mg/L) (Mizuno *et al*., 2000): glucose 10000, $NH_4Cl$ 2600, $K_2HPO_4$ 250, $MgCl{\cdot}6H_2O$ 125, $MnCl_2{\cdot}4H_2O$ 2.5, $FeSO_4{\cdot}7H_2O$ 5, $CoCl_2{\cdot}6H_2O$ 2.5, KI 2.5, $Na_2MoO_4{\cdot}2H_2O$ 0.5, $H_3BO_4$ 0.5, $NiCl_2{\cdot}6H_2O$ 0.5, $ZnCl_2$ 0.5.

The inoculum obtained from anaerobic digestion sludge was heated at 70°C for 30 min to inhibit the bioactivity of hydrogen consumers and to develop its hydrogen producing capabilities. Two hundreds microliter inoculum was acclimated with hydrogen growth medium in a CSTR for 1 d. Four series of CSTR were then operated with different hydraulic retention times (HRTs) (6, 8, 12, and 14 h).

### 1.2 Analyses

The amount of biogas produced in the reactor was collected daily using a gas pack. The proportions of $H_2$, $CH_4$, $N_2$, and $CO_2$ in the biogas were determined using a gas chromatogram (GC-8A, Shimadzu, Japan) equipped with a thermal conductivity detector (TCD). The concentrations of the volatile fatty acids (VFAs) and alcohols were determined using a gas chromatogram (GC14B, Shimadzu, Japan) equipped with a flame ionization detector (FID). The concentration of lactate was determined using a liquid chromatograph (LC-10AD, Shimadzu, Japan) equipped with ultraviolet detector. The pH was determined by a pH meter (TOA, Japan) equipped with a GST-5721C probe. The concentrations of volatile suspended solids (VSS), and $COD_{Cr}$ were determined according to the standard methods (APHA, 1995). Carbohydrate concentration was measured by the phenol-sulfuric acid method using glucose as the standard (Dubios *et al*., 1956).

### 1.3 Quantification of hydrogen-producing bacteria

Sludge samples collected from reactors during the steady state condition at various HRTs were fixed with 3% paraformaldehyde for 2 h at 4°C, afterwards the fixed samples were centrifuged (15000 r/min, 10 min) and washed three times with PBS. Then it was stored in an equal volume of PBS-ethanol solution at –20°C.

Fixed samples (3 μL) were spotted on gelatin-coated fourteen-field glass slides and air dried, and subsequently dehydrated in 50%, 80%, and 95% ethanol (3 min each). The 16S rRNA-targeted oligonucleotide probe ClostIV (5'-GCA CCC TTT ACA CCC-3') was used to target the genus *Clostridium* (Weber *et al*., 2001). The probe was synthesized and fluorescently labelled with the hydrophilic sulfoindocyanine dye CY3 (TaKaRa, Shiga, Japan). Hybridization was carried out in a water-saturated equilibration chamber at 46°C for 2 h. The hybridization buffer contained 0.9 mol/L NaCl, 30% formamide, 20 mmol/L Tris-HCl (pH 7.2), 0.01% SDS (sodium dodecyl sulphate) and 0.44 pmol/L probe. The washing step was done at 48°C for 20 min with washing buffer containing 20 mmol/L Tris-HCl (pH 7.2), 112.4 mmol/L NaCl, 0.01% SDS. The washing buffer was removed by rinsing the slides with $ddH_2O$, then the slides were air dried. The cells were counter-stained with SYBR Green I. Before examination, the slides were covered with vectashield mounting medium for fluorescence H-1000 (Vector Laboratories, Japan). Fluorescence was detected with a confocal laser scanning microscope (TCS 4D, Leica, Germany). For counting of bacteria, at least 4 slides for each sample, and at least 10 fields were counted for each slide. The average number of bacteria was taken.

## 2 Results and discussion

### 2.1 Hydrogen productivity

Wastewater containing 10000 mg/L of glucose was treated at 30°C in all experiments, and HRT varied from 6 to 14 h. Four sets of extensive analysis were conducted at each HRT under steady-state condition. Figure 2 shows the HRT effects on biogas content, hydrogen yield, and specific hydrogen production rate. Figure 2a illustrates that biogas comprised mostly hydrogen and carbon dioxide. The hydrogen keeps constant in the range of 50%–53% at HRT 6–8 h, and decrease immediately along with HRT further increasing. No hydrogen production was detected at 14 h. The carbon dioxide content in biogas followed an opposite trend of hydrogen. The biogas was free of methane before 8 h, but considerable quantities of methane were produced with increasing HRT, due to the bioactivity of hydrogenotrophic methanogens. The methane content increased from 15% at 12 h to 22% at 14 h, which was accompanied with the decrease of hydrogen content.

Figure 2b illustrates that the hydrogen yield reached the optimum during 6–8 h, and the maximum hydrogen yield of 1.4–1.5 mol $H_2$/mol glucose was observed. Table 1 lists the hydrogen yields, reported in publications, from glucose at continuous process for comparison. The characteristic of seed organisms is likely a main factor on hydrogen yield.

Figure 2c illustrates that the specific hydrogen production rate were decreased accompanied as HRT increased. The specific hydrogen production rate varied in the range of 2.7–2.9 L/(g VSS·d) during 6–8 h.

### 2.2 COD mass balance in the process

The products in the hydrogen production process could be divided into a biomass, soluble, and biogas, and the COD mass balance can be calculated according to the COD conversion coefficient of each product (Li and Noike, 1987). The conversion of VSS was calculated assuming the composition of $C_5H_9O_3N$ (Speece and McCarty, 1964). Table 2 summarizes the overall COD mass balance.

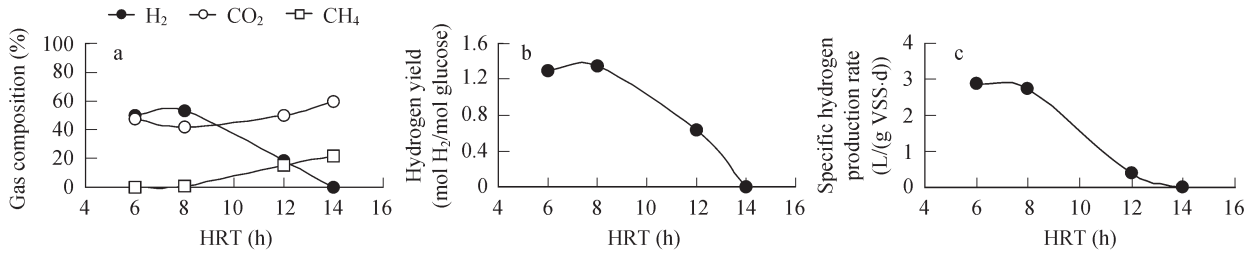


**Fig. 2** Effect of HRT on biogas composition (a), hydrogen yield (b), and specific hydrogen production rate (c).

**Table 1** Comparison of hydrogen yield from glucose at continuous process

| Microorganism | HRT (h) | pH | *T* (°C) | *Y* (mol $H_2$/mol glucose) | Reference |
|---|---|---|---|---|---|
| Digested sludge | 6 | 5.7 | 35 | 1.71 | Lin and Chang, 1999 |
| Soybean-meal | 8.5 | 6 | 35 | 1.43 | Mizuno *et al*., 2000 |
| Mixed culture | 6 | 5.5 | 36 | 2.1 | Fang and Liu, 2002b |
| Compost sludge | 12 | 6.6 | 60 | 1.19 | Ueno *et al*., 2001b |
| River sediments | 6.5 | 5.0–5.2 | 37 | 1.98 | Zhang *et al*., 2006 |
| Digested sludge | 6–8 | 5.5 | 30 | 1.45 | This study |

HRT: hydraulic retention time; *Y*: hydrogen yield.

As shown in Table 2, butyrate was the most abundant VFA in the effluent with a influent COD range 19.5%–21.6%, followed by acetate with a range of 10.7%–12.5%. According to the metabolic pathway of glucose, converting 1 mol of glucose into acetate would produce 4 mol of hydrogen. Whereas converting 1 mol of glucose into butyrate would produce 2 mol of hydrogen. The hydrogen production in this study reflects the observation that most of the glucose was converted to butyrate, instead of acetate.

Table 2 also shows that ethanol and lactate have an equal concentration range, and ethanol was increased, but lactate was decreased along with increasing HRT. Propionate was the lowest in the metabolic products at 4.5% or less. Glucose decomposition and biomass yield were increased with increasing HRT, degrading 1 g of glucose produced 0.17 g VSS at the optimal HRT 8 h.

### 2.3 Quantification of hydrogen-producing bacteria

For the quantification of hydrogen-producing bacteria, FISH was performed with sludge samples during the steady state condition at various HRTs. Probe Clost IV targeting the genus *Clostridium* based on 16S rRNA sequences were used. Figure 3 illustrates fluorescence image of sludge samples after FISH with CY3-labelled probe Clost IV. It was found that the hydrogen-producing bacteria were mostly composed of bacilli of various lengths, plus some diplobacilli and streptobacilli. The numbers of hydrogen-producing bacteria were $9.2\times10^8$, $8.2\times10^8$, $2.8\times10^8$, and $6.2\times10^7$ cell/mL, at HRT 6, 8, 12, and 14 h, respectively.

Bacterial cells stained with SYBR Green I were counted as total bacteria (bacteria and archaea). As a result, the numbers of total bacteria were $9.4 \times 10^8$, $9.2 \times 10^8$, $6.5 \times 10^8$ and $6.2 \times 10^8$ cell/mL, at HRT 6, 8, 12, and 14 h, respectively. It was clarified that HRT seemed to have little impact on the number of total bacteria, in contrast to the number of hydrogen-producing bacteria.

The percentage of the hydrogen-producing bacteria *clostridium* to total bacteria was determined. As shown in Fig. 4, there was a significant relationship between

**Table 2** Products distribution based on the COD mass balance at various HRTs (%)

| HRT (h) | Influent | Effluent | | | | | | Biogas | | Biomass VSS | Recovery (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Glucose | Lactate | Acetate | Propionate | Butyrate | Ethanol | $H_2$ | $CH_4$ | | |
| 6 | 100 | 9.9 | 10.8 | 10.7 | 2.1 | 20.5 | 8.8 | 13.1 | 0 | 19.2 | 95.2 |
| 8 | 100 | 8.6 | 7.7 | 11.6 | 4.5 | 21.6 | 9.7 | 13.4 | 0 | 20.6 | 97.7 |
| 12 | 100 | 8.1 | 5.9 | 12.5 | 2.8 | 20.8 | 12.8 | 5.2 | 6.5 | 22.5 | 97.0 |
| 14 | 100 | 5.4 | 6.4 | 10.8 | 0 | 19.5 | 15.7 | 0 | 10.2 | 27.3 | 95.3 |

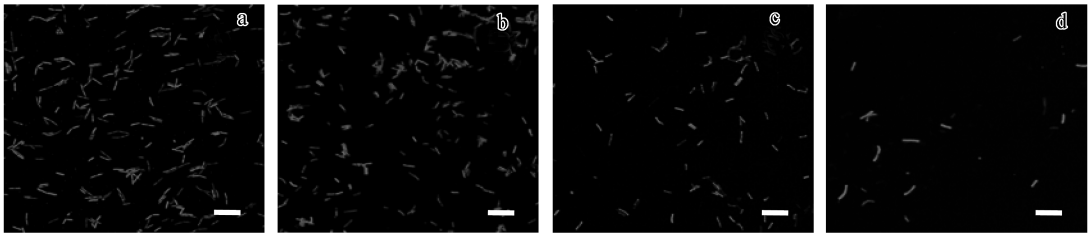


**Fig. 3** Fluorescence images of sludge samples from continuous fermentative reactors after FISH with CY3-labelled probe Clost IV. Sludge samples from the reactor with HRT 6 (a), 8 (b), 12 (c) and 14 h (d). Bar equals 10 μm.

the percentage of hydrogen-producing bacteria and HRT. The percentage of the hydrogen-producing bacteria was decreased from 97% at 6 h to 10% at 14 h. The decrease of the hydrogen-producing bacteria at high HRT was likely due to the increase of hydrogenotrophic methanogens, as evidenced by the increased methane production in the biogas. The results indicated that quantification of hydrogen-producing bacteria by FISH with probe Clost IV was in consensus with the hydrogen production. In other words, with probe Clost IV, it should be offered a base to develop an activity index for evaluating hydrogen-producing efficiency.

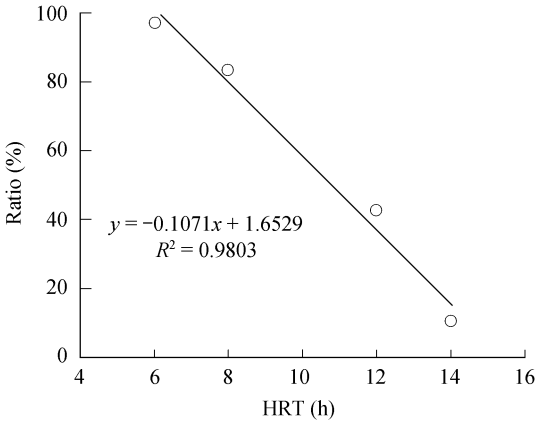


**Fig. 4** Effect of HRT on the ratio of *Clostridium* to total bacteria.

## 3 Conclusions

For quantification of hydrogen-producing bacteria by FISH, to our knowledge this is first attempted using probe Clost IV targeting the genus *Clostridium* based on 16S rRNA sequences. Furthermore, we focused on the relationship between hydrogen-producing bacterial populations and hydrogen yield at various HRTs. At the optimum HRT range 6–8 h, the numbers of hydrogen-producing bacteria and hydrogen yield were in the range of $(8.2–9.2)\times10^8$ cell/mL and 1.4–1.5 mol $H_2$/mol glucose, respectively. It is considered that the percentage of the hydrogen-producing bacteria to total bacteria is useful parameter for evaluation of hydrogen production process.

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