J. Microbiol. Biotechnol. (2012), **22**(5), 668–673
http://dx.doi.org/10.4014/jmb.1110.10076
First published online March 19, 2012
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Metabolic Pathways of Hydrogen Production in Fermentative Acidogenic Microflora

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Received: October 25, 2011 / Revised: January 9, 2012 / Accepted: January 12, 2012

Biohydrogen production from organic wastewater by anaerobically activated sludge fermentation has already been extensively investigated, and it is known that hydrogen can be produced by glucose fermentation through three metabolic pathways, including the oxidative decarboxylation of pyruvic acid to acetyl-CoA, oxidation of NADH to NAD^+, and acetogenesis by hydrogen-producing acetogens. However, the exact or dominant pathways of hydrogen production in the anaerobically activated sludge fermentation process have not yet been identified. Thus, a continuous stirred-tank reactor (CSTR) was introduced and a specifically acclimated acidogenic fermentative microflora obtained under certain operation conditions. The hydrogen production activity and potential hydrogen-producing pathways in the acidogenic fermentative microflora were then investigated using batch cultures in Erlenmeyer flasks with a working volume of 500 ml. Based on an initial glucose concentration of 10 g/l, pH 6.0, and a biomass of 1.01 g/l of a mixed liquid volatile suspended solid (MLVSS), 247.7 ml of hydrogen was obtained after a 68 h cultivation period at 35 ± 1°C. Further tests indicated that 69% of the hydrogen was produced from the oxidative decarboxylation of pyruvic acid, whereas the remaining 31% was from the oxidation of NADH to NAD^+. There were no hydrogen-producing acetogens or they were unable to work effectively in the anaerobically activated sludge with a hydraulic retention time (HRT) of less than 8 h.

Keywords: Biohydrogen production, metabolic pathways, fermentation, NADH/NAD^+, Acetogenesis

Hydrogen is regarded as a prospective fuel owing to its high energy value and conversion efficiency, recyclability, and nonpolluting nature [3, 4]. Biological hydrogen production processes have also been found to be more environment-friendly and less energy intensive than thermochemical and electrochemical processes [6, 12], where hydrogen production by fermentation has attracted particular interest, as the hydrogen production rate is generally faster than photohydrogen evolution by photosynthetic microorganisms [26]. Consequently, several breakthroughs have recently been reported in relation to biohydrogen production from organic wastewater by anaerobically activated sludge fermentation [5, 6, 8, 16, 22, 23, 31]. However, when a pilot-scale study of biohydrogen production was performed in a continuous flow anaerobic fermentation reactor with an available volume of 1.48 m^3 [21], the evolution rate of hydrogen was not perfect owing to the occurrence of several by-products, including organic acids and alcohols, during the metabolism. In this case, metabolic pathway control is perhaps the most effective approach to improve the yield of hydrogen. For an anaerobically acidogenic microbial community, there are three potential pathways for H_2 production [1, 17, 19, 24, 26, 27]: (i) oxidative decarboxylation of pyruvic acid, *via* the Embden–Meyerhof Pathway (EMP), to acetyl-CoA, (ii) oxidation of NADH to NAD^+, and (iii) acetogenesis by hydrogen-producing acetogens. However, the exact or dominant pathway for hydrogen production *via* anaerobically activated sludge fermentation has not yet been identified.

Accordingly, this study used a continuous stirred-tank reactor (CSTR) and obtained a specifically acclimated acidogenic fermentative microflora under certain operation conditions. The hydrogen production activity of the acidogenic microflora was then determined. Furthermore, to investigate the potential pathways of hydrogen evolution by the acidogenic microflora, the conversions of ethanol, propionic acid, and butyric acid were examined in batch cultures inoculated with the acidogenic microflora from the CSTR.

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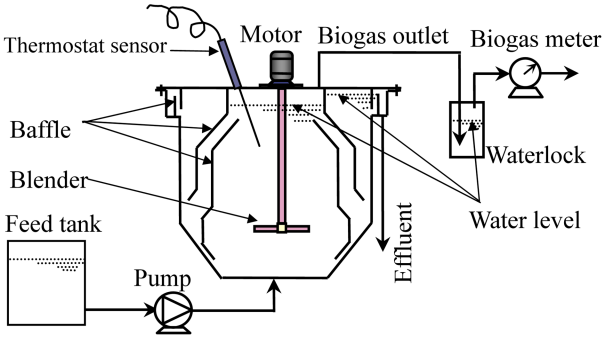

Fig. 1. Schematic diagram of the CSTR for acclimatization of anaerobic activated sludge.

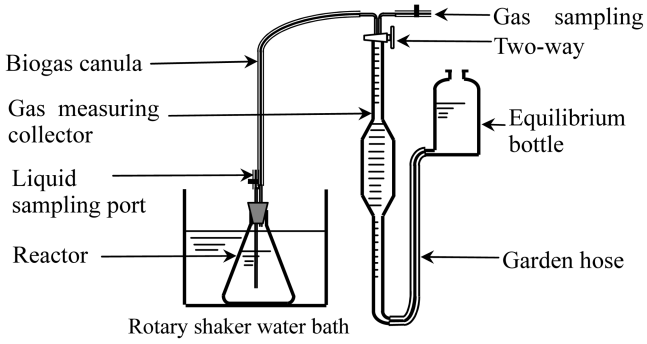

Fig. 2. Schematic diagram of batch culture.

Materials and Methods

Acclimatization of Anaerobically Acidogenic Microbial Community in Continuous Stirred-Tank Reactor

A 17 L CSTR with an effective volume of 9.6 L was used to acclimatize the activated sludge and obtain a stable mixed fermentation community for the batch culture investigation (Fig. 1). The temperature was maintained at 35 ± 1°C. Based on previous experimental results, $NaHCO_3$ was added to the feeding solution to maintain a pH of 6.5–7.5 in the influent and pH of 4.5 in the reactor [18, 20]. A feeding solution containing diluted molasses (5,000 mg COD/l) was continuously fed into the CSTR. The hydraulic retention time (HRT) of the CSTR was kept at 8 h. The COD:N:P in the feeding solution was maintained at a ratio of 200–300:5:1 by adding a synthetic fertilizer to supply microorganisms with adequate nitrogen and phosphorus. The resulting biogas was collected in a waterlock and the volume measured daily using a wet gas meter (Model LML-1, Changchun Filter Co., Ltd.). The waterlock and wet gas meter were both filled with water at pH 3.0 to prevent the biogas from dissolving.

The reactor was inoculated with excess sludge from a secondary settling tank at a local brewing wastewater treatment plant and started up with a biomass of 13 g MLVSS/l. After 40 days, the pH, biogas yield, hydrogen content, and liquid fermentation products became stable in the CSTR, and a mixed microbial community of anaerobically activated sludge was developed [13, 14]. The total amount of liquid fermentation products was 2,291.13 mg/l, where the concentrations of ethanol, acetic acid, propionic acid, butyric acid, and valeric acid were 333.63, 922.80, 308.35, 641.81, and 84.44 mg/l, respectively. The characteristics of the mixed acid fermentation indicated the presence of various microbes and metabolic pathways in the acidogenic microflora. At the end of the acclimatization period, the biomass in the reactor was approximately 13.5 g MLVSS/l. Some of the acclimatized mixed microbial community was then used for batch cultures.

Batch Cultures

Batch cultures (Fig. 2) were used to investigate the hydrogen productivity of different substrates with the anaerobically activated sludge microbe community. The working volume of the Erlenmeyer flask was 500 ml, and the culture was maintained at 35 ± 1°C in a rotary shaker water bath. The initial concentrations of glucose, propionic acid, butyric acid, and ethanol were 10,000, 801.54, 618.54, and 573.46 mg/l, respectively.

The batch cultures were inoculated with the anaerobically activated sludge (acclimatized acidogenic microflora in CSTR) at a concentration of 1.01 g MLVSS/l. Several minerals were also added to the batch cultures to fulfill the requirements for microbial growth (1.5 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.1 g $CaCl_2 2H_2O$, 0.1 g $MgCl_2 6H_2O$, and 3 mg $FeSO_4 \cdot 7H_2O$ in 1.0 L solution). The total volume of each batch culture was 500 ml, and the pH was adjusted to 6.0 by 0.1 mol/l NaOH or 0.1 mol/l HCl solution. For each tested substrate, the experiment was conducted with duplicate batch cultures under identical experimental conditions. In one of the duplicate cultures, the equilibrium bottle and gas measurement collector were filled with a 20% NaOH solution to absorb the CO_2 in the biogas, thereby allowing the hydrogen content to be measured during the fermentation process. In the other duplicate culture, the equilibrium bottle and gas measurement collector were filled with water (pH 3.0) to measure the total volume of the biogas during the fermentation process. To ensure anaerobic conditions for the batch culture, the equilibrium bottle was sparged with nitrogen gas for 20 min.

Analytical Methods

The MLVSS and pH analysis were conducted according to standard methods [2]. The concentration of glucose was measured using the sulfuric acid–phenol method [9]. During the batch culture experiments, about 5 ml was sampled every 5 or 2 h from the liquid sampling port of each Erlenmeyer flask. The samples were then filtered and immediately injected into the analyzer. The concentrations of volatile fatty acids (VFAs) and ethanol in the fermentation solution were analyzed using a gas chromatograph (Shanghai Anal. Inst. Co., GC112) equipped with a hydrogen flame ionization detector and stainless steel column (2 m × 5 mm) packed with GDX-103 (60–80 meshes). The operation of the stainless steel column was amenable to a temperature programming process within 100–200°C. N_2 was used as the carrier gas at a flow rate of 50 ml/min, hydrogen was the combustion gas at 50 ml/min, and O_2 was the combustion-supporting gas at 500 ml/min. The biogas was analyzed using another gas chromatograph (Qingdao, Shandong Lunan Instrument Factory, Model SC-7) equipped with a thermal conductivity detector and stainless steel column (2 m × 5 mm) filled with Porapak Q (50–80 meshes). Nitrogen was used as the carrier gas at a flow rate of 40 ml/min. The injected sample dose was 0.5 ml each time.

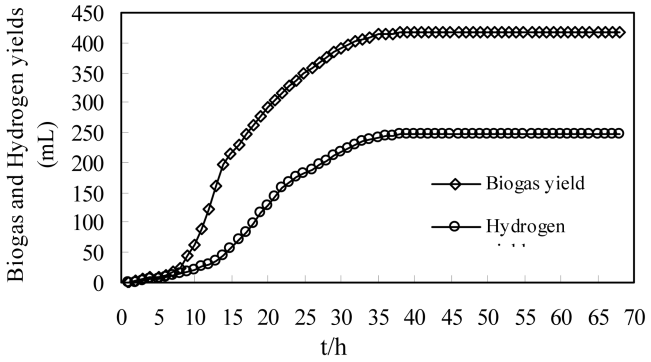

Fig. 3. Biogas and H_2 yields from glucose by fermentation.

RESULTS AND DISCUSSION

Hydrogen Production from Glucose in Batch Cultures

The batch tests for the production of biohydrogen from glucose by the acidogenic microflora were carried out for 68 h (Fig. 3). After a lag stage of 8 h, a biogas with hydrogen content began to evolve gradually, and the stabilization stage was achieved after 36 h. Finally, 417.95 ml of biogas with a hydrogen content of 247.7 ml was collected over the 68-h cultivation period. By the end of the batch culture, the medium pH decreased to 3.2 from 6.0 owing to the accumulation of VFAs produced from glucose during the fermentation process, plus there was still 4.45 g/l of glucose left in the culture. According to a previous study [18], the lowest pH value that acidogenesis can endure is 3.8. Thus, the low pH (3.2) restrained the metabolism of the bacteria in the acidogenic microflora, thereby inhibiting the biogas production after 36 h of cultivation. By the end of the cultivation, the liquid fermentation products (2,266.67 mg/l in total) were found to include ethanol 635.82 mg/l, acetic acid 764.12 mg/l, propionic acid 129.48 mg/l, and butyric acid 737.25 mg/l (Fig. 4). As such, this variety of terminal metabolites indicated diversity in the metabolic pathways for the acidogenic microflora.

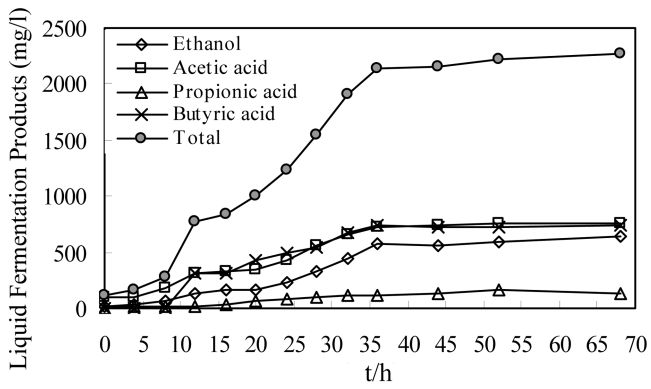

Fig. 4. Liquid products from glucose fermentation.

Pyruvic acid produced by EMP can be converted into acetic acid, propionic acid, butyric acid, ethanol, and lactic acid, and also catalyzed by special enzymes. These metabolic approaches can also take place spontaneously according to Gibbs free energy change ($\Delta G_0'$) (Reactions (1)–(5)) [11, 15, 20, 25, 28].

$$C_6H_{12}O_6 + 4H_2O + 2NAD^+ \rightarrow 2CH_3COO^- + 2HCO_3^- + 2H_2 + 2NADH + 6H^+$$
$$\Delta G_0' = -215.67 \text{ kJ/mol} \quad (1)$$

$$C_6H_{12}O_6 + 2NADH \rightarrow 2CH_3CH_2COO^- + 2H_2O + 2NAD^+$$
$$\Delta G_0' = -357.87 \text{ kJ/mol} \quad (2)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + 2HCO_3^- + 2H_2 + 3H^+$$
$$\Delta G_0' = -261.46 \text{ kJ/mol} \quad (3)$$

$$C_6H_{12}O_6 + 2H_2O + 2NADH \rightarrow 2CH_3CH_2OH + 2HCO_3^- + 2NAD^+ + 2H_2$$
$$\Delta G_0' = -234.83 \text{ kJ/mol} \quad (4)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOO^- + 2H^+$$
$$\Delta G_0' = -217.7 \text{ kJ/mol} \quad (5)$$

According to Reactions (1) to (5), the proportion of hydrogen and carbon dioxide in a biogas produced by glucose fermentation should be 1. However, the actual percentage of hydrogen in the biogas produced by glucose fermentation in the batch culture was 59% (247.7 ml), whereas the percentage of carbon dioxide was 41% (170.25 ml) (Fig. 3). Thus, the proportion of hydrogen and carbon dioxide was higher than 1, which also means that 77.45 ml of hydrogen (up to 31% of the hydrogen yield) was not produced from the oxidative decarboxylation of pyruvic acid. In addition, the experimental results indicated the existence of other pathways of hydrogen evolution in the anaerobic activated sludge.

Hydrogen Production by Hydrogen-Producing Acetogens

Hydrogen-producing acetogens can convert propionic acid, butyric acid, and ethanol to acetic acid while evolving hydrogen under anaerobic condition (Reactions (6)–(8)) [7, 10, 29, 30].

$$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + 3H_2 + CO_2$$
$$\Delta G_0' = +76.1 \text{ kJ/mol} \quad (6)$$

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2$$
$$\Delta G_0' = +48.1 \text{ kJ/mol} \quad (7)$$

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + 2H_2 + H^+$$
$$\Delta G_0' = +19.2 \text{ kJ/mol} \quad (8)$$

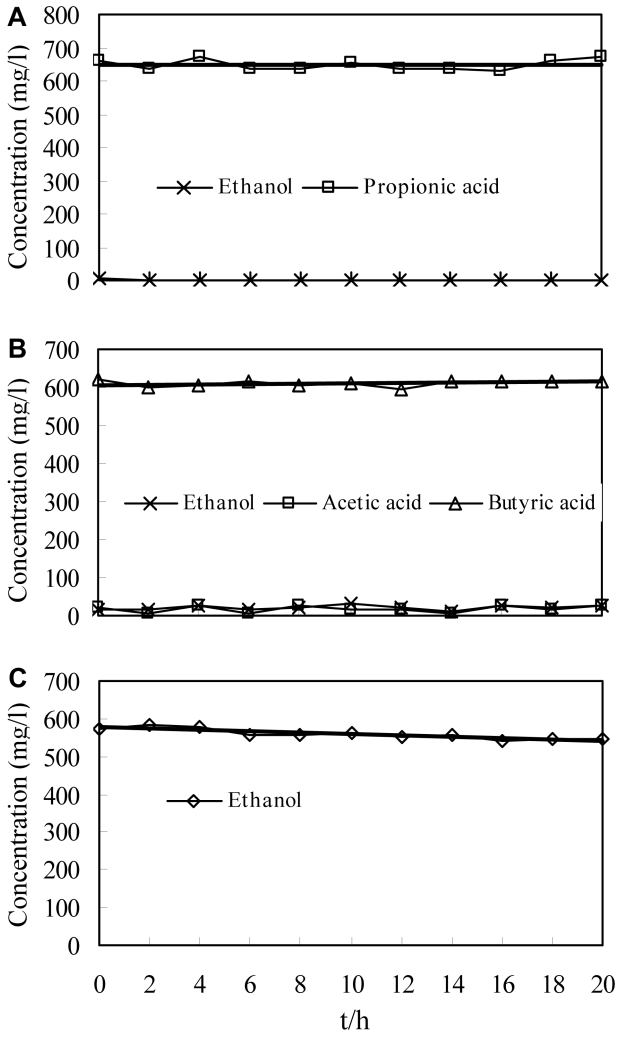

Fig. 5. Conversion of propionate, butyrate, and ethanol by acidogenic microflora.
(**A**) Concentrations of ethanol and VFAs in propionate medium; (**B**) Concentrations of ethanol and VFAs in butyrate medium; (**C**) Concentrations of ethanol and VFAs in ethanol medium.

Besides acetic acid, large amounts of ethanol, propionic acid, and butyric acid were also produced by the anaerobically activated sludge in the CSTR, as described in above. If there are hydrogen-producing acetogens in the CSTR, the acidogenic microflora will produce hydrogen while converting ethanol and VFAs to acetic acid. Therefore, the characteristics of the hydrogen production from ethanol, propionic acid, and butyric acid by the acidogenic microflora from the CSTR were investigated using batch cultures. The results showed no noticeable changes in the concentrations of propionic acid and butyric acid. Furthermore, no biogas or additional acetic acid was detected in any of the batch cultures during the 20 h cultivation process (Fig. 5A and 5B). Small quantities of ethanol and acetic acid were detected in the propionic acid medium (only residual ethanol was detected at a concentration of 0.62 ± 0.4 mg/l) (Fig. 5A) and butyric acid medium (the residual ethanol and acetic acid were 21.46 ± 5.42 and 17.40 ± 9.05 mg/l, respectively) (Fig. 5B), yet there were no changes in the concentrations during the cultivation process. Although no acetic acid or biogas yield was detected from the ethanol during the fermentation process, its concentration in the medium decreased gently (Fig. 5C), possibly as a result of volatilization of the ethanol.

It has previously been reported [19, 29, 30] that hydrogen production from ethanol and VFAs by hydrogen-producing acetogens can only occur under the condition of a low hydrogen partial pressure. As such, ethanol can only be converted to acetic acid and hydrogen when the hydrogen partial pressure is as low as 0.1 atm. Meanwhile, the maximal hydrogen partial pressure for the conversion of butyric acid and propionic acid to acetic acid is 2×10^{-3} atm and 1×10^{-4} atm, respectively. However, the hydrogen partial pressure in the CSTR was about 0.1 atm at least [21], thereby inhibiting the biochemical conversion of ethanol and VFAs to acetic acid and hydrogen, regardless of the existence of hydrogen-producing acetogens in the anaerobically activated sludge.

Hydrogen Production from NADH

As described above, the proportion of hydrogen and carbon dioxide in the biogas produced by the EMP should be theoretically equal during the glucose fermentation process. Yet, the batch tests for the production of hydrogen from glucose in the mixed microbial community fermentation (Fig. 3) showed that the hydrogen yield was much higher than the carbon dioxide in the accumulated biogas. However, the observation that ethanol, propionic acid, and butyric acid could not be converted into acetic acid by the acidogenic fermentative microflora from the CSTR indicated the absence or ineffectiveness of hydrogen-producing acetogens in the anaerobically activated sludge at a HRT of less than 8 h. Thus, the remaining hydrogen yield of 77.45 ml should logistically be produced through the NADH pathway [26]. The oxidation of organic material by fermentation is normally accomplished by dehydrogenation, which produces a large amount of NADH owing to the lack of an electron transport chain. For the fermentation to proceed continuously and maintain a proper ratio of $NADH/NAD^+$, the residual NADH is reoxidized to NAD^+. Part of the NADH can be rapidly utilized by cellular synthesis at a higher pH or converted to hydrogen and NAD^+ by dehydrogenation at a lower pH in the presence of acetyl-CoA [19]. The evolution of hydrogen through the NADH pathway is driven by the necessity of reoxidizing the residual NADH of the metabolic reaction as follows [26]:

$$NADH + H^+ \rightarrow NAD^+ + H_2 \quad (9)$$

Tanisho *et al*. [24] analyzed the mechanism of hydrogen evolution from NADH *via* membrane-bound hydrogenase. Thus, if metabolic reactions can be controlled so as to increase the amount of residual NADH, the yield of hydrogen will be improved remarkably.

In summary, the conclusions from the above results are as follows: (i) the oxidative decarboxylation of pyruvic acid to acetyl-CoA was the dominant pathway for hydrogen evolution in the anaerobic microflora with a contribution of 69% to the total hydrogen yield; (ii) the NADH pathway was another important pathway for hydrogen evolution, where its potential contribution to the total hydrogen yield from the anaerobic activated sludge was 31%; and (iii) there were no hydrogen-producing acetogens or they were unable to work effectively in the anaerobic activated sludge with a HRT of less than 8 h. However, to obtain more evidence, the structure and species composition of the anaerobic activated sludge should be investigated in depth.

Acknowledgments

This work was supported by the State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology (No. 2010DX06) and the National Natural Science Foundation of China (No. 51178136).

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