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Failure of biohydrogen production by low levels of substrate and lactic acid accumulation

Jeong-Hoon Park [a, b], Sang-Hoon Lee [a], Hyun-Jun Ju [c], Sang-Hyoun Kim [c], Jeong-Jun Yoon [b], Hee-Deung Park [a, *]

[a] School of Civil, Environmental and Architectural Engineering, Korea University, Seoul 136-714, South Korea
[b] IT Convergence Materials R&D Group, Korea Institute of Industrial Technology, Chungnam 330-825, South Korea
[c] Department of Environmental Engineering, Daegu University, Gyeongbuk 712-714, South Korea

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ABSTRACT

To identify causes affecting the stability of biohydrogen production from the microbial community perspective, a fermentor was operated using galactose as a substrate for 30 days. The fermentor demonstrated that hydrogen production yield gradually increased with concomitant decrease of galactose concentration until day 27. Meanwhile, hydrogen producing clostridia gradually increased their proportion to 75% of bacteria analyzed until day 11, and then gradually decreased to 23% with increases in other groups of bacteria such as *Bacilli*. After day 27, hydrogen production abruptly failed without deterioration of galactose utilization. At that time, lactic acid accumulated to 920 mg COD/L and *Ruminococcaceae* increased its proportion to 30%. In conclusion, low substrate levels, beginning on day 11, provided a selective disadvantage to the hydrogen producing clostridia and proliferated other groups of bacteria, which resulted in accumulation of lactic acid and, ultimately, failure of hydrogen production.

1. Introduction

Hydrogen is one of the most promising clean energy resources that can replace conventional fossil fuels [1]. Biological hydrogen production from organic waste or non-food biomass using mixed microbial populations has also received attention as an environmentally friendly process [2]. Over the last three decades many studies have been conducted to optimize and increase hydrogen production by modulating hydraulic retention time (HRT) [3], substrate loading rate [4], pH [5], and other variables [6]. Nevertheless, biohydrogen production still has limitations, including slow reaction times and low production yields [6]. Considering that both the rate and the production yields are directly dependent on activity and quantity of biohydrogen producing microorganisms [7], characterizing the microbial community structure during hydrogen production is needed for further optimizing hydrogen fermentors.

Introduction of culture-independent molecular techniques have significantly enhanced the understanding of key microorganisms involved in biohydrogen production [8], of population dynamics within continuous-flow hydrogen fermentors [9], and of operational parameters affecting the stability of the microbial community structure [10]. It is reported that bacteria within the *Clostridium* and *Enterobacter* genera are frequently detected when hydrogen is stably produced [11], while other groups of bacteria (e.g., *Lactobacillus*) outcompete biohydrogen fermenting bacteria when hydrogen production starts to deteriorate [12]. Although some operational parameters such as pH and substrate loading rate appear to be important in maintaining hydrogen producing bacteria [13,14], ecological processes influencing the stability of the hydrogen-producing microbial community structure are still unclear.

The aim of this study was to identify causes affecting the stability of biohydrogen production from the microbial community perspective. To this end, a continuous flow fermentor was operated using galactose, the main monomeric sugar of marine red algae [15], as a feedstock. Bacterial communities, reactor performance, and biochemical data were all collected from the fermentor operation.

* Corresponding author.
E-mail address: heedeung@korea.ac.kr (H.-D. Park).

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2. Materials and methods

2.1. Inocbulum

Seed sludge was obtained from an anaerobic digester at the Gyeongsan wastewater treatment plant (Gyeongsan, Korea). The digester treats waste activated sludge and food waste together in mesophilic conditions (37 ± 1.2 °C). At the time of collection, the total suspended solids (TSS) and volatile suspended solids (VSS) of the seed sludge were 33.5 ± 0.7 g TSS/L and 22.5 ± 0.6 g VSS/L, respectively. Before seeding, the seed sludge was heated to 90 °C for 10 min to inactive all microorganisms except for hydrogen producing spore-forming bacteria.

2.2. Set-up and operation of hydrogen fermentor

Initially, pre-heated seed sludge and deionized water were filled into a 5 L acrylic fermentor (3 L working volume) to generate 15 g VSS/L. Galactose and nutrients were then dissolved into the mixture to generate 15 g/L galactose, 0.96 g/L NH_4Cl, 0.22 g/L KH_2PO_4, 0.12 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2$, 0.01 g/L $MnSO_4 \cdot H_2O$, and 0.043 g/L $FeCl_2$. The initial pH was 7.5. The composition of the medium was adapted based on a medium for producing biohydrogen production [16], and the initial pH was determined by the previous reports evaluating optimum pH for hydrogen fermentation [17,18]. The fermentor was purged with high purity N_2 gas for 5 min with 5 L/min, and then incubated at 35 ± 0.1 °C at 150 rpm for 21 h in batch mode. During the fermentation operation, pH was maintained above 5.5 ± 0.1 using 3 M NaOH automatically. After 21 h, galactose medium with nutrients was continuously fed into the fermentor at 3 L/d (1.0 day HRT) for 23 days. After day 23, nitrogen source (10 g/L peptone, 10 g/L beef extract, and 3 g/L yeast extract) was supplemented to the medium to evaluate whether the addition of nitrogen source is effective in increasing hydrogen production.

2.3. Analyses of biogas, organic acids, and solids

Biogas composition was analyzed using gas chromatography (series 580, Gow-Mac Instrument Co., Bound Brook, NJ, USA) with a previously described protocol [15]. Organic acids and galactose were analyzed using high performance liquid chromatography (HPLC) (YL9100 series, Young Lin Co., Inchon, South Korea) using a refractive index detector and an ultraviolet detector at 210 nm with a previously described protocol [15]. The liquid samples were filtered through a 0.45 μm membrane filter before injection into the HPLC. TSS and VSS were measured according to the Standard Methods.

2.4. DNA extraction, clone library construction, and sequencing

Total DNA was extracted from sludge samples using a MoBio PowerSoil DNA extraction kit (Solana Beach, CA, USA) following the manufacturer's protocol. Bacterial 16S rRNA genes were PCR-amplified using 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′) universal primers. Detailed information about the reaction mixture and thermal profile is described in a previous study [19]. After reaction, purification and cloning of the PCR amplicon were conducted using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA), respectively. Detailed information was described in a previous study [20]. From the clones, 16S rRNA gene fragments were sequenced using BigDye terminator chemistry with an automated capillary sequencer (Applied Biosystems, Foster City, CA, USA). Mothur utility [21] was used to remove potential chimeric sequences, to define operational taxonomic units (OTUs), and to estimate diversity indices. Each OTU was taxonomically identified using RDP's Classifier (http://rdp.cme.msu.edu).

2.5. Phylogenetic analysis

A phylogenetic tree was constructed with major OTUs (OTUs constituting >2% in each sample) and their closest relative references by the neighbor-joining algorithm using MEGA 5 software [22], and the evolutionary distances were calculated according to Kimura's 2-parameter model [23]. The detailed information for the phylogenetic analysis is described in a previous study [19].

3. Results and discussions

3.1. Galactose utilization and hydrogen production

Fig. 1(a) shows galactose concentrations and hydrogen production yields in the fermentor during the 30 days of fermentor operation. Galactose rapidly decreased from 15 g/L to 2 g/L within 21 h of batch mode hydrogen fermentation. After shifting to continuous-flow mode, galactose gradually increased up to 11.3 g/L on day 8, while hydrogen production yield decreased to 0.08 mol H_2/mole galactose. From day 8 to day 21 galactose gradually decreased to 0.95 g/L and was maintained at the same concentration until day 25. On day 21 the hydrogen production yield was 1.0 mol H_2/mole galactose. After supplementing nitrogen sources to the reactor on day 23, galactose decreased further to almost undetectable levels at day 30 and the hydrogen yield reached 1.3 mol H_2/mole galactose on day 27. This yield falls well within the values (0.6–1.8 mol H_2/mole sugar) observed in hydrogen fermentors with hexose-based feedstock [24]. Then, hydrogen production abruptly ceased on day 28 and was not recovered until end of fermentor operation (e.g., day 30), although the galactose concentration was maintained at almost undetectable levels. This result suggests that certain conditions lead to complete inhibition of hydrogen fermentation without deterioration of galactose utilization.

3.2. Organic acids production

Organic acid production was also monitored to elucidate the galactose utilization pattern and the hydrogen production yield during the fermentor operation. Acetic acid, butyric acid, and lactic acid were analyzed, and the sum of these acids was assumed to be the total organic acids (TOA) produced. Fig. 1(b) shows TOA and the relative proportion of each organic acid produced. TOA sharply increased to 6710 mg COD/L at 39 h following batch mode operation. After shifting to continuous-flow mode, TOA concentration gradually decreased to 1720 mg COD/L at day 7, and then increased to 7640 mg COD/L at day 21 and maintained a similar concentration until the end of fermentor operation. The pattern of TOA fluctuation was similar to that of hydrogen production yield, except for the early continuous-flow mode and the fermentor closure period. The TOA mostly consisted of acetic acid and butyric acid (95–99% of total organic acids), except for the 11 h and 28 day samples in which lactic acid was observed at 15% (140 mg COD/L) and 13% (920 mg COD/L), respectively. Interestingly, the proportion of acetic acid appears to be inversely related to TOA production during the continuous-flow mode. When the proportion of acetic acid concentration was near maximum (86% of TOA) at day 6, TOA concentration was minimal at 1940 mg COD/L (Fig. 1(a and b)). Several researchers who studied biohydrogen production also reported similar results [2]. The addition of nitrogen sources did not