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Characterization and potential of three temperature ranges for hydrogen fermentation of cellulose by means of activity test and 16s rRNA sequence analysis

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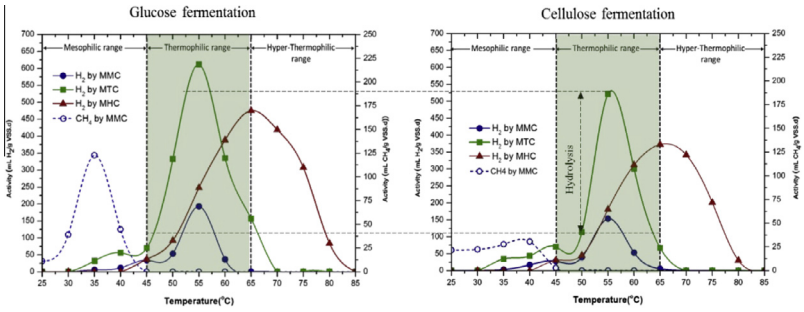
HIGHLIGHTS

- The characterization of three temperature ranges of H_2 fermentation was evaluated.
- The H_2 gas produced effectively at 55 and 65 °C, while the CH_4 produced at 35 °C.
- The highest activity of 521 mL H_2/g VSS d was obtained by TMC at 55 °C.
- The H_2 producing bacteria were close to *Enterobacter* genus in the MMC.
- The H_2 producing bacteria were close to *Thermoanaerobacterium* in the TMC & HMC.

GRAPHICAL ABSTRACT



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ABSTRACT

A series of standardized activity experiments were performed to characterize three different temperature ranges of hydrogen fermentation from different carbon sources. 16S rRNA sequences analysis showed that the bacteria were close to *Enterobacter* genus in the mesophilic mixed culture (MMC) and *Thermoanaerobacterium* genus in the thermophilic and hyper-thermophilic mixed cultures (TMC and HMC). The MMC was able to utilize the glucose and cellulose to produce methane gas within a temperature range between 25 and 45 °C and hydrogen gas from 35 to 60 °C. While, the TMC and HMC produced only hydrogen gas at all temperature ranges and the highest activity of 521.4 ml H_2/g VSS d was obtained by TMC. The thermodynamic analysis showed that more energy is consumed by hydrogen production from cellulose than from glucose. The experimental results could help to improve the economic feasibility of cellulosic biomass energy using three-phase technology to produce hythane.

1. Introduction

The fossil fuels burning as the main source of our energy causes some serious environmental problems such as greenhouse effect, ozone layer depletion and acid rains (Lay et al., 1999). A clear and sustainable solution is to replace the fossil fuels with renewable resources such as wind, solar, and biomass energy etc. Currently, biomass energy utilization has gained particular interest in recent years due to the progressive depletion of conventional fossil fuels and environmental security. The dark hydrogen fermentation is currently one of the most promising and environmental technology to generate clean hydrogen energy with no energy input required. Additionally dark-fermentation can use various

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types of organic wastes. In particular, the cellulosic-dark hydrogen fermentation is getting more importance because around 9.2×10^{11} tones cellulose are produced at the annual rate of 0.85×10^{11} tones per year (Lieth and Whittaker, 2012), which consider an important source for biomass energy technology. A range of factors are important in the hydrogen fermentation reactions from organic substrates. These factors include the substrate concentration, type of inoculants, pH and temperature, etc. Among these factors, the temperature is the most significant key factor for bacterial growth. Based on our previous study, the reaction rates are temperature-dependent. However, this can be fitted to microbiological processes in limited temperature ranges. Temperature affects not only the rate of biochemical processes, but also the composition of the microbial community (Gadow et al., 2012; Hernon et al., 2006). At the thermophilic temperature (55 °C), stable hydrogen production was obtained for over 6 months; on the other hand, hydrogen was not produced continuously but intermittently at mesophilic temperature (35 °C) (Gadow et al., 2012). This is thought to be mainly due to the presence of hydrogenotrophic homoacetogens, which were more active at mesophilic than at thermophilic temperature (Hernon et al., 2006). At thermophilic temperatures, the cellulose to hydrogen biodegradation is more efficient, while the microbial structure is more genetically diverse at mesophilic temperatures (Hernon et al., 2006). Therefore, the most common temperatures are ranged from 35 to 55 °C for cellulosic-hydrogen fermentation in the literature. In order for cellulosic-hydrogen production to be practical at a commercial-scale, non-sterile feedstock and readily available inoculants should be available. Therefore, the purpose of this study was to characterize three different temperature ranges for hydrogen fermentation based on activity test using glucose and cellulose as sole of different carbon source as well the activation energies were studied. Another aim of the study was to explore the microbial community structure and its functional implications.

2. Methods

2.1. Anaerobic mixed culture source

The inoculum used in the activity tests were taken from the three different continuous reactors, operated at mesophilic (37 ± 1 °C), thermophilic (55 ± 1 °C) and hyper-thermophilic (70 ± 1 °C) temperatures, under their steady-state conditions. These reactors were operated up to 260 days for cellulosic-hydrogen fermentation using anaerobic mixed cultures (digested sludge).

2.2. Activity test experiments

One-hundred ninety-five activity experiments were conducted in serum bottles (120 ml) to compare efficiency of three different anaerobic mixed cultures to the temperature variations. In each bottle, with a working volume of 80 ml, was added 16 ml of inoculum from three different mixed cultures to 64 ml of synthetic medium containing 0.1% of cellulose (Cellulose powder E, Toyo Roshi Kaisha, Ltd. Japan) or glucose as sole of carbon source, 2 g NH_4HCO_3, 3 g $NaHCO_3$, 0.1 g K_2HPO_4, 0.1 g KH_2PO_4, 0.01 g NaCl, 0.015 g $CaCl_2{\cdot}2H_2O$, 0.01 g $MgCl_2{\cdot}6H_2O$, 0.025 g $FeSO_4{\cdot}7H_2O$, 0.001 g $MnSO_4{\cdot}6H_2O$, 0.0005 g $CuSO_4{\cdot}5H_2O$, 0.002 g $CoCl_2{\cdot}5H_2O$, 0.0005 g $Na_2MoO_4{\cdot}2H_2O$, 0.001 g $ZnCl_2$, 0.001 g $NiCl_2{\cdot}6H_2O$, 1 mg resazurin and 40 ml reducing solution (200 mL NaOH (0.2 M); 2.5 g $Na_2S{\cdot}9H_2O$; 2.5 g cysteine $HCl{\cdot}H_2O$). The pH was adjusted at 5.5 by NaOH (2 M) and HCl (2 M) solutions. After inoculation, all bottles were flushed with pure nitrogen gas for 5 min to confirm anaerobic condition. Beside the test bottles, in which both inoculum and synthetic medium were contained, a control bottle of inoculum employed. The bottles were incubated in the shaking water bath with temperature control. The gas production and composition were measured by glass syringe and gas chromatography, respectively. The metabolic by-products such as volatile fatty acids (VFAs), lactic acid and alcohols were analyzed.

2.3. Analytical methods

The gases percentages in the biogas mixture were analyzed by a gas chromatograph [Shimadzu 8A] equipped with a thermal conductivity detector [TCD] and a stainless steel column packed with a molecular sieve 5A [60/80 3 mmØ]. The temperatures of the detector and the column were maintained at 100 °C and 60 °C, respectively. The VFAs and other by-products concentrations were determined by a gas chromatograph (Agilent-6890) equipped with a DB-WAXetr capillary column and a flame ionization detector (FID). The temperature of the injector/detector and oven were 250 °C and 125 °C, respectively. The volatile solid (VS), volatile suspended solid (VSS), protein and chemical oxygen demand (COD) were analyzed according to the procedures described in the Standard Methods (Federation and Association, 2005).

2.4. Microbial community structure analysis

2.4.1. Sample collection and DNA extraction

The bacterial cells were collected by centrifugation at 10,000 rpm for 2 min. The precipitated cells were washed with phosphate buffer saline (PBS) three times. According to the manufacturer's instructions of Ultra Clean Soil DNA Isolation Kit (MO-BIO), the DNA extraction was performed from samples.

2.4.2. 16S rRNA gene cloning analysis

The microbial communities were analyzed by 16S rRNA gene cloning and sequencing. To perform 16S rRNA amplification, EUB8F and UNIV1500R primers were used (Uemura et al., 2010). Twenty-five thermal cycles of the polymerase chain reaction (PCR) were applied, which contained of 30 s of denaturing at 94 °C, 40 s of annealing at 50 °C and 1 min of extraction at 72 °C. The PCR products were purified by Micro Spin™ S-400 HR (Amersham Pharmacia GE, USA). The purified DNA was cloned with the TOPO TA Cloning® Kit (Invitrogen, USA) and transformed into *Escherichia coli* DH5a competent cells. The white ones were randomly picked out, which incubated at 37 °C for 24 h, and transferred to LB medium with another 6 h of continuous incubation. The cell suspensions were used in a PCR reaction containing the M13F/M13R universal primers. Sequence reactions were carried out at TAKARA BIO dragon genomics center (Yokkaichi, Japan). Clone sequences were compared with the GenBank nucleotide database using BLAST microbial genomes searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine their closest phylogenetic neighbours.

3. Results and discussion

3.1. Microbial community structure

3.1.1. 16S rRNA analysis of mesophilic mixed culture

The cloning results show that a total of 37 OTUs were detected from a total of 100 clones. According to analysis results (see Table 1), the 52% of the microbial community were able to hydrolyze the cellulose effectively such as *Enterobacter cloacae*, *Pedobacter* sp. and *Thermoanaerobacterium thermosaccharolyticum* (Soares et al., 2012). Several bacteria such as *Clostridium*, *Bacillus*, *Enterobacter* and *Pseudomonas* produce cellulases such as