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Monitoring dark hydrogen fermentation performance of indigenous *Clostridium butyricum* by hydrogenase gene expression using RT-PCR and qPCR

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ABSTRACT

Hydrogenase is the key enzyme responsible for H_2 production in dark fermentation. Therefore, the expression of hydrogenase gene may be a good indicator for the performance of a dark H_2 fermentation culture. In this study, we investigated the correlation between expression of the functional gene (*hydA* encoding for hydrogenase in *Clostridium butyricum*) and bioH_2 production activity during batch growth of an indigenous H_2-producing isolate *C. butyricum* CGS5 using sucrose as the sole carbon source. The copy number of *hydA* mRNA was determined by using reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR). The results show that the specific hydrogen production rate of *C. butyricum* CGS5 was essentially linearly proportional to the level of *hydA* expression (represented by the copy umber of *hydA* cDNA), whereas the profiles of microbial growth and volumetric H_2 production rate followed a similar trend to that of the *hydA* DNA copies.

1. Introduction

Today the global energy requirements are mostly dependent on fossil fuels. In addition to the rapid decline in global reserve of fossil fuels, the pollution arising from the utilization of fossil fuels (such as formation of CO_2, NO_x, SO_x, C_xH_x, and other organic pollutants) has become a great environmental threat and resulted in severe climate change [1]. The current trend of environmental goals tends to develop alternative energy resources that are cleaner and more sustainable [2–4]. In recent years, hydrogen has been regarded as one of the most promising energy carriers of the future because it is clean, efficient, and recyclable [1,5]. Producing hydrogen by microorganisms seems to be cost-effective, pollution-free, and energy-saving. Biological hydrogen production systems can be classified as biophotolysis of water using algae and cyanobacteria [1,6,7], photodecomposition of organic compounds by photosynthetic bacteria (e.g., *Rhodobacter* spp.) [8–11], light-independent dark hydrogen fermentation from organic compounds by obligate anaerobes (e.g., acidogenic bacteria) or facultative anaerobes (e.g., *Enterobacter* spp.) [12–16]. Moreover, hybrid H_2-producing systems combining

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dark and photofermentation processes are of particular interest due to the potential of attaining the highest possible theoretical yield of 12 mol H_2/mol hexose [1].

Among the aforementioned biohydrogen systems, using anaerobic acidogenic bacteria to produce H_2 from a wide variety of organic compounds (or organic wastes) by dark fermentation is considered the most economically feasible way of bioH_2 production [2]. Dark H_2 fermentation is able to achieve a much higher hydrogen production rate than that obtained from the other biological means [2]. In addition, it also has the advantage of simultaneous waste reduction and clean energy (i.e., H_2) generation [17]. *Clostridia* species are the most often used bacterial population for dark fermentative hydrogen production [18–22].

Hydrogenases are the key enzymes in biohydrogen production by catalyzing the interaction of proton and electron to form H_2 gas or the reverse reaction [23]. Hydrogenases can be categorized into two major families, i.e., nickel–iron (NiFe) hydrogenases and iron only (FeFe) hydrogenases [24,25], on the basis of the metal content of their respective dinuclear catalytic centers. Some NiFe hydrogenases also contain selenium at their catalytic center [24,25]. The hydrogenase contains Ni and Se facilitates the uptake of hydrogen, whereas those containing Fe alone often catalyze the production of hydrogen [24,26]. Many hydrogen-producing microorganisms in dark fermentation belong to the genus *Clostridium*. However, only a few hydrogenase gene sequences of *Clostridium* species are sequenced and characterized [27].

In our recent work [22], an indigenous strain, *Clostridium butyricum* CGS5, was isolated from a highly efficient H_2-producing anaerobic bacterial microflora [28]. The *C. butyricum* strain exhibited excellent H_2-producing activity in batch and continuous culture [22]. In this study, a nucleic acid probe targeting the *hyd*A gene of Fe–Fe hydrogenase from *C. butyricum* was designed and used to monitor H_2 production behavior of the specific strain via quantitative detection of mRNA of hydrogenase using reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR). As most of Fe–Fe hydrogenases predominantly catalyze the production of H_2 [26,29], using *hyd*A gene as a biomarker of hydrogen production bacteria or an indicator of hydrogen production performance seems to be reasonable [29,30,45]. This work aimed to demonstrate the feasibility of using molecular biological tools to correlate H_2 production activity with functional gene (i.e., hydrogenase) expression. Based upon that information, strategies leading to effective monitoring, control, and improvement of the dark H_2 fermentation could be developed.

2. Materials and methods

2.1. Strain, media, and cultivation

C. butyricum CGS5 is an anaerobic hydrogen-producing bacterial strain isolated from effluent sludge of an anaerobic bioreactor as described in our resent study [22]. The seed sludge used in the H_2-producing bioreactor was collected from a municipal wastewater treatment plant in Taiwan. The CGS5 strain was isolated anaerobically at 37 °C on CH agar medium (pH 7.5) consisting of (per liter) 15 g sucrose, 1 g yeast extract, 5 g Na_2HPO_4, 1 g KH_2PO_4, 1 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.025 g $FeSO_4$, 20 g agar and 2.0 ml trace element solution containing (g/l) H_3BO_3, 2.86; $MnSO_4 \cdot 4H_2O$, 2.03; $ZnSO_4 \cdot 7H_2O$, 0.22; $Na_2MoO_4 \cdot 2H_2O$, 0.14; $CuSO_4 \cdot 5H_2O$, 0.08; $FeCl_3$, 0.1. In addition to morphological and biochemical identification, the isolated pure strain of *C. butyricum* CGS5 was confirmed by 16S rDNA sequencing and phylogenetic analysis [22]. The NCBI GenBank accession number for the 16S rDNA sequence of this strain is AY540109.

C. butyricum CGS5 was grown in 50 ml PM medium at 37 °C for 3 days and subsequently 5 ml of the fully grown culture was injected into 150 ml serum vial containing 100 ml oxygen free fresh PM medium for the hydrogen fermentation experiments. The ingredients of PM medium (pH 7.2) per liter were sucrose, 15 g; Na_2HPO_4, 5 g; KH_2PO_4, 1 g; NaCl, 2 g; $MgSO_4$, 0.1 g; $(NH_4)_2SO_4$, 3 g; $Na_2S \cdot 9H_2O$, 0.5 g; resazurin, 0.001 g.

2.2. Total DNA and RNA extraction

One milliliter of grown *C. butyricum* was centrifuged at 13 000 rpm at 4 °C for 5 min. The cell pellets were collected and used for nucleic acids' extraction as described by Yu and Mohn [31]. For DNA extraction, a mixture of 1.5 g of zirconia/silica beads (1 mm in diameter) and 1.0 ml of extraction buffer [50 mM Tris–HCl (pH 8.0), 5 mM EDTA, and 3% sodium dodecyl sulfate (SDS)] was added to the cell pellet and agitated using a bead beater (Vortex-Genie 2, VWR scientific, and Vortex Adapter, Mo bio Laboratories Inc.) at 5000 rpm for 10 min. The supernatant was collected in a fresh tube after centrifugation at 13 000 rpm for 3 min. The supernatant was treated with 2 M (final concentration) ammonium acetate (10 M stock solution) and kept on ice for 10 min. After ammonium acetate precipitation, the supernatant was collected by centrifugation at 13 000 rpm for 10 min. Total nucleic acid in the supernatant was extracted by the phenol/chloroform/isopropanol extraction procedures [32]. The resulting nucleic acid pellet was washed with 70% ethanol, air dried and re-suspended in 50 μl deionized (DI) water.

For RNA extraction, a mixture of 2.0 g of zirconia/silica beads (1 mm in diameter), 1.0 ml of extraction buffer, and 30 μl of 100% diethylpyrocarbonate (DEPC) was added to the cell pellet and agitated using a bead beater at 5000 rpm for 10 min at 4 °C. The resulting cell homogenate was cool on ice for 1 min, and the supernatant was collected after centrifugation at 13 000 rpm for 3 min at 4 °C. The supernatant was treated with 2 M (final concentration) ammonium acetate (10 M stock solution) and kept on ice for 5 min. After ammonium acetate precipitation, the supernatant was collected by centrifugation at 13 000 rpm for 10 min at 4 °C. Total nucleic acid in the supernatant was extracted by the phenol/chloroform/isopropanol extraction procedures [32]. The resulting nucleic acid pellet was washed with 70% ethanol, air-dried and re-suspended in 50 μl DI water. To obtain total RNA, 20 μl of crude nucleic acid were incubated with 2 μl of RQ1 RNase-free DNase (Promega Corporation, Madison, WI) and 2 μl of RQ1 RNase-free DNase 10× reaction buffer at 37 °C for 30 min. The reaction was terminated by the addition of 2 μl of RQ1 DNase stop solution and the DNase was further inactivated at 65 °C for 10 min.