

Relationship among growth parameters for *Clostridium butyricum*, *hydA* gene expression, and biohydrogen production in a sucrose-supplemented batch reactor

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Abstract This study was undertaken to identify the relationship between the performance of dark H₂ fermentation and expression of the key functional gene (i.e., hydrogenase gene) involved in the bioH₂ production process. *Clostridium butyricum* CGS5 isolated from anaerobic sewage sludge was used as the model strain for this study. Copy number of the hydrogenase gene (*hydA*) and mRNA transcripts (cDNA *hydA*) (after amplification) and the total DNA and RNA (before amplification) were measured over the course of the growth of strain CGS5. Cell concentration was also determined by optical density and converted to dry weight. After amplification, the *hydA* gene increased 1,500-fold during late exponential growth phase after normalization to the copy number at time 0, and cDNA from mRNA transcripts of *hydA* also increased 500-fold after normalization. mRNA transcripts of *hydA* lagged behind the increase of total DNA and RNA, and increases in *hydA* more closely mimicked those of total DNA. Increases in both of these parameters corresponded with hydrogen production. Transcripts of 16s ribosomal RNA reached a maximum value earlier (38 h) than did those of *hydA* (47 h). All molecular characteristics matched those for sucrose utilization, growth,

and hydrogen production. These experiments indicated that transcription as measured by cDNA can be related to hydrogen production and possesses the potential to be used as tool for process control.

Keywords Biohydrogen · *Clostridium butyricum* · Expression of *hydA* gene · Hydrogenase · RT-PCR · qPCR

Introduction

Due to recent oil price increases and global warming arising from intensive utilization of fossil fuel, much research effort has been devoted to developing new energy alternatives. Hydrogen is considered a promising energy carrier of the future since it is clean, CO₂ neutral, renewable, and efficient (Das and Veziroglu 2001; Levin et al. 2004; Nath and Das 2004). Both abiotic and biotic pathways can be used to produce hydrogen. For instance, hydrogen gas can be produced from electrolysis of water (Rao and Hall 1996), thermocatalytic destruction of hydrogen-rich organic compounds such as natural gas and coal, as well as biomass conversion via fermentation or carbon fixation via photosynthesis using water as the terminal electron acceptor (Levin et al. 2004; Nandi and Sengupta 1998). Biological production of hydrogen (called biohydrogen), using microorganisms, a newer technological development offering usable hydrogen particularly as stationary energy sources, is more environmentally friendly and has been the subject of intensive studies. Biohydrogen systems are composed of direct biophotolysis, indirect biophotolysis, photofermentation, or dark fermentation (Das and Veziroglu 2001; Levin et al. 2004; Nandi and Sengupta 1998; Nath and Das 2004; Woodward et al. 1996). Recently, research has focused on the fermentative production of hydrogen

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from renewable substrates, such as mixed waste, animal, and human waste sources (Fan et al. 2004; Hussy et al. 2003; Kapdan and Kargi 2006; Lay 2000; Mizuno et al. 1997; Nandi and Sengupta 1998; Oh et al. 2003).

Biohydrogen production by dark fermentation (Das and Veziroglu 2001; Levin et al. 2004; Nath and Das 2004; Wu et al. 2005) has been demonstrated as a viable approach in converting organic compounds (like carbohydrates) into H₂ energy with a variety of anaerobic bacteria (Haruhiko et al. 2001). However, the hydrogen production rate and yield must be further upgraded to increase the economical feasibility of bioH₂ production (Hallenbeck and Benemann 2002; Wu et al. 2005). Previous investigators have suggested strategies for the enhancement of bioH₂ production, including redirection of metabolic pathways to avoid butyrate as an end product, gas sparging to remove H₂ and/or CO₂, maintenance of low partial pressure of hydrogen in the head gas for efficient removal of product, and finally some combination of light and dark production (Collet et al. 2005; Mahyudin et al. 1997; Park et al. 2005; Nath and Das 2004).

Although fermentative H₂ production has been intensively studied, very few research works have analyzed bioH₂ production from molecular genetic aspects (Fang et al. 2006; Chang et al. 2006; Li et al. 2007). In particular, no studies to date have linked the characteristics of hydrogen production to multiple molecular parameters of the copy number of the hydrogenase gene (*hydA*), mRNA transcripts of *hydA*, transcripts of 16s ribosomal RNA as well as total DNA and RNA. Since few studies of this nature have been conducted, initial work in our laboratory has focused batch reactor experiments using pure cultures of *Clostridium butyricum* CGS5 in a defined medium, PM medium. This organism has been described in previous works, and both culture and hydrogen production characteristics have been well described by Chen et al. (2005); thus, it provided a useful model to determine if mRNA transcripts of the *hydA* gene, hydrogen production, characteristics of growth phase based on both RNA and DNA would corroborate earlier biochemical studies.

Materials and methods

Strain source and fermentable condition

C. butyricum CGS5 (accession no. AY540109) was maintained by culture transfer every 72 h between experiments using PM medium. For hydrogen production experiments, 5 ml of a 72-h culture of *C. butyricum* CGS5 was injected into 100 ml of sterile and oxygen-free PM culture medium (Chen et al. 2005) in a 150-ml serum vial batch bioreactor and then incubated at 37°C in the water bath, and biogas was

continuously collected. Biogas was also collected by syringe at each sampling time and H₂ and CO₂ concentrations were measured by gas chromatography. The initial pH of medium was 7.2 and the culture pH was not controlled over the course of the experiment. One-milliliter samples were taken at designated times to measure cell density (DU 7400 Spectrophotometer, Beckman). The sample was then centrifuged, supernatant removed, and the pH measured (Orion Research Inc). The supernatant was preserved for the determination of total sugar concentration (Dubois et al. 1956). DNA and RNA were extracted from each corresponding pellet.

Cell density, sugar, and gas determinations

The cell concentration was calculated from absorbance at 600 nm (Van Ginkel and Logan 2005). Total sugar concentration was determined by the phenol–sulfuric acid method (Dubois et al. 1956). The total biogas was collected by water displacement and gas capture. The biogas products were analyzed by gas chromatography (GC) using a thermal conductivity detector (Hewlett Packard 5890 Series II, TCD). The carrier gas was argon at a flow rate of 15.3 ml/min and the capillary column (0.53 mm×30 m) packed with carboxen-1010 (PLOT, Supelco, USA). The injection, oven, and detector temperatures were 70°C, 50°C, and 175°C, respectively. Volatile fatty acids and ethanol were also detected by GC using flame ionization detector (Shimadzu GC-14A, FID). Nitrogen was used as the carrier gas at a flow rate of 20 ml/min and the capillary column (0.53 mm×15 m) packed with FON (contains polyethylene glycol and 2-nitrotterephthalic acid, Stabilwax-DA, Vercotech Inc.). A ramping temperature program was used beginning with an initial column temperature of 90°C for 2 min, after which the temperature was increased at a rate of 10°C/min to get the final temperature of 210°C for 1 min. The injection and detector temperature was 250°C.

Primer and probe design

Ten hydrogenase gene (*hydA*) sequences for Clostridia spp. (accession numbers: CAU15277, AF148212, AB016775, AB016820, AB035092, CAU09760, AP003193, AE007516, NC003366, and AB159510) from the NCBI (National Center for Biotechnology Information) database were aligned using ClustalW (Workbench, SDSC, UCSD) to form 5′–3′ and 3′–5′ consensus sequences. The alignment of eleven 16s rRNA gene sequences from Clostridia spp. (accession numbers: M59113, X81021, U16147, AJ237756, AJ 427628, AY458857, X68178, AY699288, AY534872, Y18184, and AY442812) from the NCBI database was also performed using ClustalW (Workbench, SDSC, UCSD). These were then submitted to Primer 3

(Workbench, SDSC, UCSD) to develop the primer and probe sets designated as HGf, HGr, and HGPr for the hydrogenase gene and 16Sf, 16Sr, and 16SPr for the 16S ribosomal gene (Table 1). The *hydA* and 16s rDNA amplicon sequences were screened using BLAST (NCBI) to determine cross reactivity. The primer sets were synthesized by Sigma Genosys (Woodlands, TX, USA), and the probe sets were synthesized by Biosearch Technologies (Novato, CA, USA). Amplicon fragment sizes of hydrogenase (*hydA*) and 16s rRNA gene were 259 and 182 bp, respectively.

DNA and RNA extraction

Total DNA extraction was carried out following the procedures of Yu and Mohn (1999). The extracted crude nucleic acid was re-suspended in 50 μ l of TE buffer or sterile distilled water to quantify DNA concentration. Extraction of total RNA was carried out using a similar protocol combined with the RQ1 RNase-free DNase kit (Promega Corporation, Madison, WI, USA) for DNA removal.

Reverse transcriptase PCR

Reverse transcription was carried out using a GeneAmp RNA PCR kit (Applied Biosystems, Foster, CA, USA). For each reaction, 3 μ l of sample was added to the master mixture which was made according to manufacturer's instructions. The cycle process for reverse transcriptase polymerase chain reaction (RT-PCR) was 45°C for 15 min, 99°C for 5 min, and 5°C for 5 min using GeneAmp PCR system 2700 (Applied Biosystem, Foster, CA, USA).

Quantitative PCR amplification

Quantitative polymerase chain reaction (qPCR) reactions were conducted using Rotor Gene 3000 (Corbett Research, Melbourne, Australia) employing a total volume of 25 μ l with 5 μ l of sample. The qPCR master mixture contained 1 \times PCR buffer, 3.5 mM of MgCl₂, 200 μ M of dNTP, 300 nM of each primer, 100 ng/ μ l bovine serum albumin, 50 nM probe, and 1 U of *Taq* DNA polymerase. Each sample was run in triplicate. The qPCR cycling included an

initial holding time of 2 min at 95°C, followed by 40 cycles of denaturation at 95°C for 20 s and annealing at 58.3°C for 45 s. The median chromosome size for Clostridia was used to determine the MW of the chromosome. Based on this calculated value, concentrations for the standard curves were derived (Methrel et al. 1990).

Results

The standard curve was established to calculate the qPCR detection at various time intervals. The median chromosome size for Clostridia was used to determine the MW of the chromosome, based on which DNA concentrations for the standard curves were derived (Methrel et al. 1990). We obtained a linear relationship between the serial-diluted DNA concentrations and the number of cycles needed to reach the detection threshold (C_T). The detection limit for 16s ribosomal RNA gene was 500 copies/5 μ l or 5×10^5 copies/100 ml of culture media, while the limit for *hydA* was 5×10^4 copies/5 μ l or 5×10^7 copies/100 ml of culture media. In Fig. 1, examples of typical standardization curves achieved for the *hydA* and the 16s ribosomal gene are shown using different concentrations of direct extracts of *C. butyricum* CGS5. It can be seen that the PCR efficiency as measured by the coefficient of variation for the *hydA* gene was 0.9 which was below that achieved for the 16s ribosomal gene. The mean efficiency across all the standard curves generated in this study was 0.9 ± 0.02 and 0.97 ± 0 for *hydA* and 16s rDNA, respectively. It was likely that the decrease in efficiency for the *hydA* was due to the larger amplicon size and the use of total cell DNA extracts (Devers et al. 2004; Fang et al. 2006; Zhang and Fang 2006). While the efficiency we achieved for the *hydA* gene was lower than optimum, reproducibility was excellent, target copy number of cells made the detection limit, and the efficiency was acceptable.

The cell dry weight increased until 47 h, while sucrose decreased steadily until 68 h (Fig. 2). Approximately, 75% of the sugar was utilized by the time additional sugar was added at 68 h. Cell dry weight did not increase after the addition of sugar at 68 h, although the decrease in biomass stopped. The additional nutrient supplied did not result in

Table 1 Primer and probe sets used to detect the hydrogenase gene and 16s ribosomal RNA gene for *Clostridium butyricum* CGS5

Primers and probes	Sequence (5'–3')	Specificity	Reference
HGf	AAGAAGCTTTAGAAGATCCTAA	Hydrogenase gene	This study
HGr	GGACAACATGAGGTAAACATTG	Hydrogenase gene	This study
HGPr	FAM-TATGGCACCAGCTGTTAGAACATCTATGGGT-TAMRA	Hydrogenase gene	This study
16Sf	AGCGTTGTCCGGATTACTG	Clostridia spp. 16s rRNA gene	This study
16Sr	TTCGCCACTGGTATTCTTCC	Clostridia spp. 16s rRNA gene	This study
16SPr	FAM-GGCGTAAAGGGAGCGTAGGTGGATATTT-BHQ1	Clostridia spp. 16s rRNA gene	This study

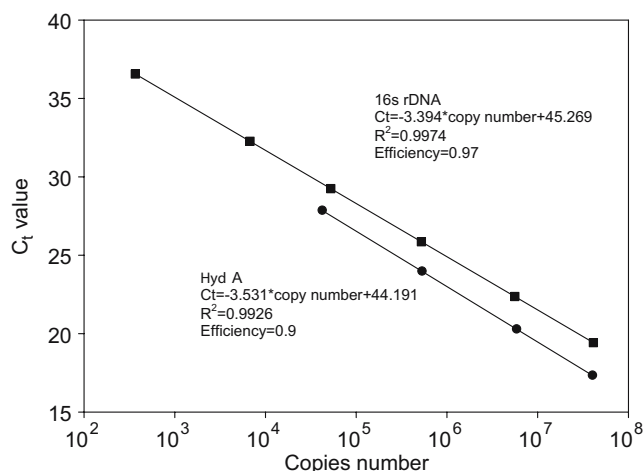


Fig. 1 The standard curves of *hydA* and 16s rDNA gene detection achieved using total DNA extractions of *Clostridium butyricum* CGS5

patterns of cell growth or nutrient utilization observed during the earlier growth period (i.e., between 0 and 34 h in Fig. 1).

The concentrations of total RNA and DNA (Fig. 3a) followed the same pattern over the course of the experiment, which lagged behind sucrose utilization by about 4 h. It was clear that maximum production of total RNA and DNA occurred 47 h after the initiation of the experiment. The extended lag time was likely due to addition of a 72-h culture which might have already undergone sporulation (Wrigley et al. 1995) as shown in Fig. 2. As would be expected, sucrose utilization increased more rapidly (Fig. 3b) than total DNA or RNA, and initial hydrogen production lagged slightly behind that of total DNA and RNA. The rapid rise in hydrogen gas after the addition of sucrose at 68 h suggested that hydrogenase was still present. Additionally, the findings suggested that increases in transcription, measured as cDNA, of the *hydA* gene would occur slightly later than initial DNA increases (Fig. 4a,b).

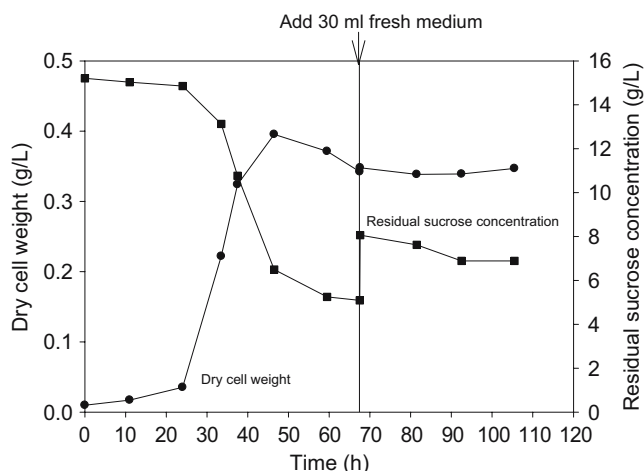


Fig. 2 Sucrose utilization and dry cell weight by *C. butyricum* in hydrogen production batch culture experiments

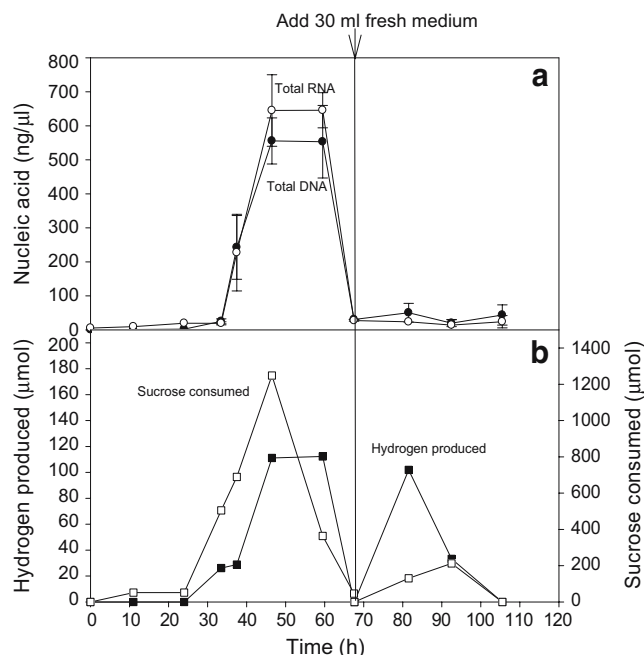


Fig. 3 Time course profile of **a** concentration of total RNA and DNA extracted from *C. butyricum* CGS5 and **b** sucrose consumption and hydrogen production during the dark fermentation experiment

In Fig. 4a, cell dry weight and *hydA* copy number tracked each other as would be expected. Transcripts, measured as cDNA, of the *hydA* gene increased later than cell biomass (dry cell weight), and the increase was initially far smaller (Fig. 4a,b). The 500-fold amplification of *hydA* transcripts was measured at 47 h, suggesting that transcription of this operon occurred during late log phase and was short lived quickly returning to initial levels at 60 h.

Another peak in cDNA (transcripts) of the *hydA* gene was seen at 68 h (Fig. 4b), which corresponded to the time at which 30 ml of fresh medium was added, suggesting the response was almost instantaneous or the increase was not due to the additional substrate. The number of transcripts of the *hydA* gene produced may be cyclical in fashion with the magnitude becoming smaller over time, if no new substrate was supplied. In our case, the additional substrate was supplied very late in the growth phase precluding us from discerning how rapidly transcript production from the *hydA* could rebound when variables were examined independently. The utilization of the additional sucrose should have produced additional growth which would have peaked at approximately 115 h, if the initial patterns of increase were followed. However, the changes in cell biomass, *hydA* genes, and transcripts as well as sucrose utilization and hydrogen production over time after the addition of fresh medium were quite different.

A negative feedback mechanism has been demonstrated if hydrogen was allowed to build up in the headspace (Park et al. 2005). However, in these studies, this negative

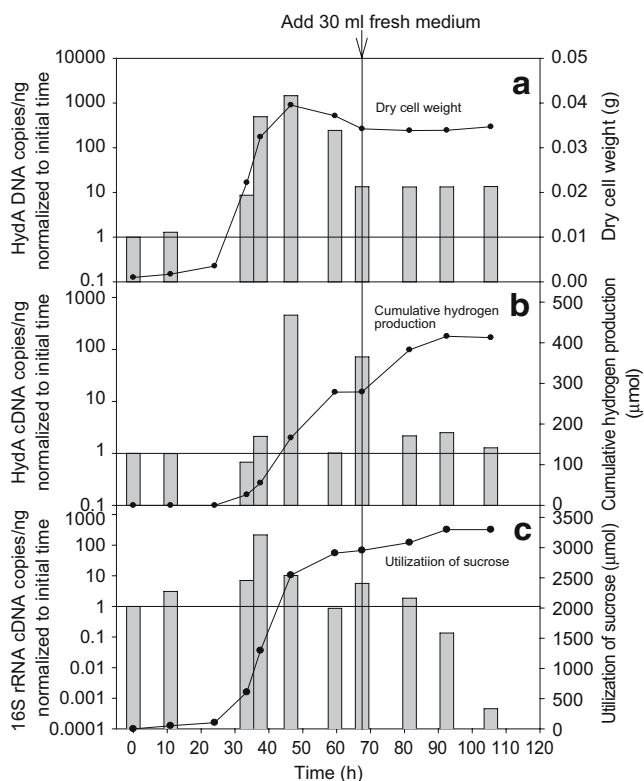


Fig. 4 Occurrence of *hydA* gene copy number, transcripts of the *hydA* gene (measured as cDNA) and 16S RNA transcripts (measured as cDNA) of *C. butyricum* in batch reactor culture experiments. Copy numbers per nanogram of total DNA and copy numbers per nanogram of cDNA were normalized to time zero. **a** *hydA* copy number from total DNA and dry cell weight per 100 ml of batch culture. **b** *hydA* copy number from cDNA and hydrogen production in 100 ml of batch culture. **c** 16S rRNA copy number from cDNA and sucrose utilization in 100 ml of batch culture

feedback mechanism was unlikely because of the manner in which hydrogen gas was removed (see Fig. 1). In Fig. 3b, the continued production of hydrogen and a low level of transcript production from the *hydA* gene (Fig. 4b) suggested that enzyme was still available. In Fig. 4c, it can be seen that cDNA for 16S ribosomal gene increased prior to the increase the transcripts of the *hydA* gene again suggesting that growth precedes the initiation of hydrogen production. The effect of the new media addition can be seen in the continued increase in sugar utilization at 68 h and a modest increase in 16S ribosomal RNA measured as cDNA. However, the rate of sucrose utilization (138.67 μmol/h) between 38 and 48 h was considerably greater than that seen (5.77 μmol/h) between 60 and 70 h which likely relates to the cell age of the culture or a reduction in culture pH (data not shown). Also, when the transcripts between the 16S ribosomal gene and the *hydA* gene were compared (Fig. 4b,c), a cyclical pattern for both was seen over the time course.

In Fig. 5, the maximum activity for hydrogen production was 0.0874 fmol H₂/h/cell. A second peak, 0.0602 fmol

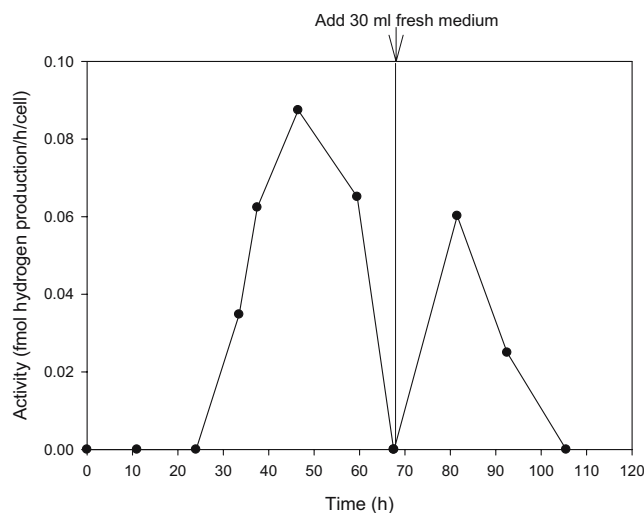


Fig. 5 Hydrogen production activity (fmol/h/cell) by *C. butyricum* over the course of the batch culture experiments

H₂/h/cell, was achieved between 68 and 82 h which corresponded to the addition of fresh media. The majority of hydrogen production would have concluded by 47 h without the addition of new medium. Comparing the first maxima of hydrogen production in Fig. 6 with the increase in *hydA* gene copy number and the 16S ribosomal gene transcripts (Fig. 4b,c) clearly showed that increases in these measurements occurred prior to the H₂ maximum, but *hydA* transcripts more closely mirrored the activity for H₂ production.

In Fig. 6, cumulative cell growth ends after 60 h, and hydrogen production also stopped. Hydrogen gas production was reinitiated after the addition of fresh medium (additional substrate), and the response was rapid, again indicating that enzyme hydrogenase was likely to have been present in the cells. Calculated cell concentration per liter did increase after the addition of new media, while DNA concentration stopped declining and remained stable over the rest of the

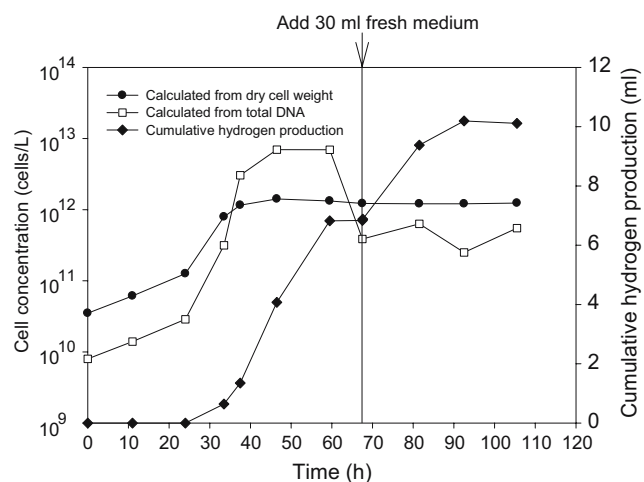


Fig. 6 Relationship among cumulative dry cell weight, total DNA, and hydrogen production of *C. butyricum* in batch culture experiments

study. If the experiment had been extended longer, the addition of 30 ml of medium might have identified additional growth or it may be that cell function had switched to mainly hydrogen production.

Lastly, examining the ratios of *hydA* cDNA to *hydA* gene copy and *hydA* cDNA with 16s rRNA copies (shown in Fig. 7) may provide more insight into what was actually occurring in the cell culture than examining the individual molecular variables. Both produced very similar patterns. Importantly, *hydA* transcripts increased, while those of the *hydA* gene copies and 16s rRNA transcripts remained the same or decreased, respectively. Thus, it did appear that the culture was no longer producing precursors to cell growth, but rather had centered on hydrogen production. Hence, the ratio data for *hydA* transcripts to those of 16s rRNA tracked the pattern of hydrogen production shown in Fig. 3.

Discussion

Overall, this work has shown that transcripts of the *hydA* gene and 16s rRNA can be measured over the course of a batch bioreactor experiment and easily used to anticipate, validate, or augment biochemical studies. This research also provided insight into molecular processes which underlie biohydrogen production by *C. butyricum*. Most importantly, examining the ratio of the *hydA* transcripts to gene, *hydA* and to transcripts of 16s rRNA strongly suggested that the addition of substrate late in the growth phase did not stimulate additional growth, but only hydrogen production.

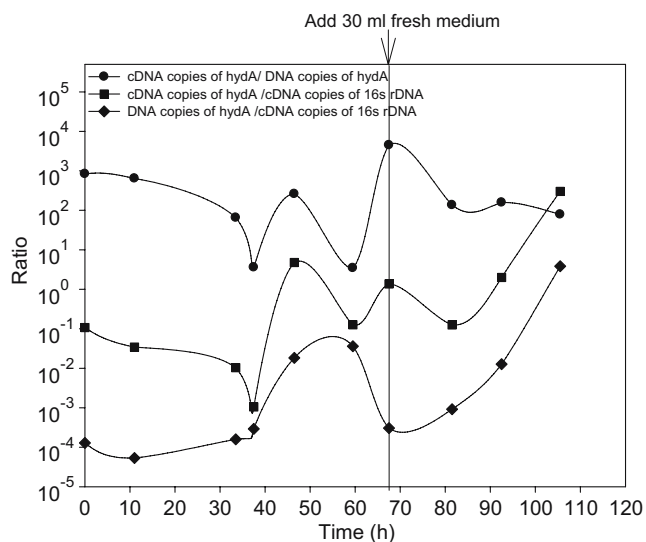


Fig. 7 The ratio of *hydA* transcripts (measured as *hydA* cDNA copies) to *hydA* gene copy number, *hydA* transcripts (measured as *hydA* cDNA copies) to 16s transcripts (as cDNA) of *C. butyricum* CGS5, and *hydA* genes to 16s transcripts (as cDNA) of *C. butyricum* CGS5

The ability to rapidly obtain qPCR data suggested that molecular indices could be combined with other physical and chemical parameters in fermentation reactors to more quickly optimize processes in biotechnology.

The qPCR calibration curve data for *hydA* were similar to that published by Fang et al. (2006), but the level of detection for the *hydA* primer and probe sets used in this study had a detection limit of one log less with a corresponding lower efficiency of 0.9 (Fig. 1). Studies have examined 16s rDNA and functional genes qPCR efficiencies which ranged from 0.94 to 0.99 (Chang et al. 2006; Fang et al. 2006), and our data was slightly outside of that range.

A variety of approaches to normalize mRNA transcripts have been published in the literature. Chang et al. (2006) correlated hydrogenase A (*hydA*) transcripts with hydrogen production in media supplemented with brewery waste. They normalized the mRNA transcript data by standardizing the amount of total RNA in each qPCR reaction. These investigators did not quantify transcripts produced to copy number but rather expressed valued as $1/C_t$ over the 48-h experiment. Although these investigators worked with mixed cultures, by following the cDNA of *hydA* and 16s rRNA over time and amplifying and sequencing those of gene fragments, they were able to identify that *C. pasteurianum*-like was the dominant strain in the batch culture reactor and correspondingly produced the greatest amount total hydrogenase transcripts as measured by $1/C_t$. While *C. saccharobutylicum*-like hydrogenase strains produced more *hydA* transcripts based on total RNA concentrations, *C. butyricum* also initially produced less *hydA* transcripts than 16s rRNA but after 70 h, produced far more (Fig. 7). Expression of the results as ΔC_t values meant that the results of Chang et al. (2006) were not directly comparable with those from our study. Overall, in this research and that using $1/C_t$ demonstrated similar trends in increases of *hydA* and 16s rRNA over the experiment.

Alternatively, Shin et al. (2006) normalized mRNA transcript copy number of botulism neurotoxin type A (BoNT/A) (*cntA*) to those for 16s ribosomal RNA. These researchers examined five different strains of *C. botulinum* and described the production of *cntA* transcripts as relative expression. They found that 80% of the strains expressed the gene in late log phase of the growth cycle, while one clinical isolates KNIH1 produced the toxin early in growth phase, but at a substantially lower level. In the present study, different amounts of cDNA were added to each qPCR reaction, but each was normalized to total RNA or DNA in the nucleic acid extraction volume of 100 μ l (Fig. 3). Furthermore, to examine the change in mRNA transcripts of *hydA* measured as cDNA, results were normalized to time zero (Fig. 4). Thus, the presentation of

data in our study was based on log-fold change of cDNA of the target gene which was similar to relative expression used by Shin et al. (2006) as opposed to $1/C_t$. Often, the variation in copy numbers among replicates has made the use of C_t or $1/C_t$ advantageous because of the lower variability. The drawback being the interpretation data across studies, whereas relative expression or log-fold change in copy number was easily compared.

A number of variables have been shown to affect the production of hydrogen by Clostridia. Each strain has demonstrated slightly different optima in regard to pH, substrate utilization, and by product production, but general trends were clear (Chen et al. 2005; Ferchichi et al. 2005; Nath and Das 2004; Van Ginkel and Logan 2005; Wu et al. 2005). Growth conditions have been shown to influence both hydrogen production and rate of hydrogen production (Ferchichi et al. 2005) which was also seen in this study when additional sucrose was added at 68 h. Acid conditions depress hydrogen production, especially if produced by the culture as opposed to the addition of acid from another source (Van Ginkel and Logan 2005). Thus, the production of acid or solvent is important to hydrogen production. In our study, pH was not controlled which may also have been an explanation of why the addition of sucrose did not result in the same increases of DNA or RNA as seen earlier in the time course. Although this investigation focused on monitoring changes in *hydA* genes and its transcripts as well as 16s rRNA, Dürre et al. (2002) was able to identify some of the promoters in *Clostridium* species involved in switching from acidogenesis to solventogenesis. Combining the study of *hydA* transcript production with transcription of the promoters identified in the work of Dürre et al. (2002) could provide additional understanding of hydrogen production because the solventogenic phase required more NAD(P)H than can be produced by glycolysis causing the reoxidation of ferroxdoxin which in turn reduced hydrogen production.

In conclusion, *hydA* transcripts measured as cDNA followed the patterns suggested in earlier works (Haruhiko et al. 2001; Van Ginkel and Logan 2005) and relative to the *hydA* gene and 16s rRNA transcripts, continued to increase after the addition of fresh medium (Fig. 7). Examining the ratios of the various components of growth and specific gene function, 16s rRNA and *hydA* genes and transcripts (Fig. 7) provided a clearer view of how the culture functioned as opposed to examining the changes in these individual variables (Figs. 3 and 4). Even though pH was not controlled in this study *hydA* transcripts increased relative to the other nucleic acid measures, which suggested that pH did not affect hydrogen production.

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