Understanding the Regulation of the Metabolic Network Associated with Fermentative Hydrogen Production in *Clostridium butyricum*

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B. Eng. (Biology Engineering)

M.Sc. (Biochemistry and Molecular Biology)

Thesis submitted for the degree of

Doctor of Philosophy

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December 2011

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ABSTRACT

Hydrogen is an environmentally friendly and high energy carrier that could be a potential replacement for depleting fossil fuels. Fermentative hydrogen production (FHP) has received great interest in recent decades, as it offers a potential means of producing H_2 from a variety of renewable and waste resources via a low energy process. However, the commercial application of the FHP process has been hampered by its low yields. It becomes important to obtain a better understanding of metabolic pathways and their regulation in H_2-producing microorganisms. The aim of this work was to improve fundamental knowledge of the central metabolic flux network associated with FHP using *Clostridium butyricum*, and to restructure metabolic pathways to enhance hydrogen production yield.

The metabolic network model was firstly constructed for *C. butyricum* and metabolic flux analysis (MFA) using this model was applied to predict metabolic flux distribution under varying initial glucose concentrations and operational pHs when the specific growth rate was chosen as the objective function. MFA results suggested that pH has a more significant effect on hydrogen production yield compare to the initial glucose concentration. These results also suggested that the phosphoenolpyruvate (PEP), pyruvate and Acetyl CoA (AcCoA) nodes are not rigid nodes. MFA was found to be a useful tool to provide valuable information for optimization of the fermentative hydrogen production process and for future design of metabolic engineering strategies.

The butyrate formation pathway was blocked using a shuttle plasmid pMTL007 containing a group II intron designed for targeting *hbd*, which encodes β-hydroxybutyryl-CoA dehydrogenase in the *C. butyricum* W5 genome. A method for transforming the plasmid into *C. butyricum* was developed. Fermentation studies

showed that the resulting *hbd*-deficient strain M3-1 performed less H_2 production with a substantial increase in ethanol production compared to the wild type strain W5; while under nitrogen sparging conditions, M3-1 exhibited increased H_2 production with the simultaneous decrease of ethanol production. These results indicated that H_2 production by *C. butyricum* may compete for reduced nicotinamide adenine dinucleotide (NADH) with the ethanol formation pathway. Homologs of all three subunits of a bifurcating hydrogenase from *Thermotoga maritime* were amplified from the strain W5, indicating that W5 may possess a potential bifurcating hydrogenase which utilized reduced ferredoxin and NADH simultaneously to produce H_2.

The ethanol formation pathway was blocked by disrupting the acetaldehyde CoA dehydrogenase (ACDH) domain on *aad* (encoding a bifunctional aldehyde-alcohol dehydrogenase) using pMTL007C-E2. Fermentation studies showed that the *aad*-deficient strain M6 produced 484% more lactate, 32% more acetate, 9% less butyrate and 78% less H_2 than the wild type strain W5; while with the addition of sodium acetate (NaAc) to the culture of M6, carbon flux to the lactate formation pathway was redirected to the butyrate formation pathway, resulting in the increase of final H_2 yield from 0.94 mol/mol glucose to 1.65 mol/mol glucose.

The results from this study have provided a better understanding of the metabolic flux network associated with hydrogen production by *C. butyricum*, and developed a genetic and metabolic approach to the enhancement of hydrogen production yield.

Declaration

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1. Cai G, Jin B, Monis P & Saint C. 2011. Metabolic flux network and analysis of fermentative hydrogen production. Biotechnology Advances. 29:375-387.

2. Cai G, Jin B, Saint C & Monis P. 2010. Metabolic flux analysis of hydrogen production network by *Clostridium butyricum* W5: Effect of pH and glucose

concentrations. International Journal of Hydrogen Energy. 35:6681-6690.

3. Cai G, Jin B, Saint C & Monis P. 2011. Genetic manipulation of butyrate formation pathways in *Clostridium butyricum*. Journal of Biotechnology. 155:269-274.

4. Cai G, Jin B, Monis P & Saint C. Redirection of biochemical pathways for hydrogen production in *Clostridium butyricum*. Submitted to Environmental Science and Technology.

Guiqin Cai

Signed *Date*

Acknowledgements

First of all, I would like to thank Dr. Joan M. Kelly, as she gave me hope to study in Australia and the chance to meet Associate Professor Bo Jin, who is my principal supervisor.

Nothing can really express my gratitude towards Bo Jin for his guidance and support throughout my PhD candidature. He not only strongly supported my scholarship application, but also provided me with outstanding support for the present thesis. Without his kind help, it would be impossible for me to finish the research on time.

I would also like to thank my co-supervisors Dr Paul Monis and Professor Christopher Saint for sharing their knowledge and providing valuable support for the present thesis.

I am grateful to all the people whose knowledge and experience have been so valuable to the success of my work. I acknowledge Professor Sang Yup Lee (KAIST, Korea) provided us the MetaFluxNet software program for metabolic flux analysis. I acknowledge Professor Nigel P. Minton (University of Nottingham, England) for providing plasmids pMTL007, pMTL007C-E2 and *E. coli* CA434, as well as his knowledge of clostridia gene knockout. I also acknowledge my labmates in the

Bionanotechnology Laboratory (previously Water Environment Biotechnology Laboratory): Dr Giuseppe Laera and Dr Hongjie An; PhD candidates Adrian Hunter and Florian Zepf; Intern students Isa Kolbe, Nicholas Kwok and Nishcha Chopra, and postgraduate student Ming Dai for all their help on this project. I also appreciate reviewers from each journal where I submitted my papers, as their comments made me progress.

Finally, I would like to give my special thanks to my husband Feng Lin for his support and understanding, and to my parents and parents-in-law for their encouragement from China during my PhD candidature.

INTRODUCTION

With rising oil prices and increasing concerns over climate change, there is an overwhelming need to find alternative energy sources. In this new situation, hydrogen is being recognised as a potential energy carrier and substitute for depleting fossil fuels, since its combustion generates higher energy than that of fossil fuels and produces water as the only by-product with no emission of greenhouse gases or other pollutants (Veziroglu and Sahin, 2008).

Hydrogen can be produced by conventional industrial processes, such as steam reforming, coal gasification and water electrolysis. However, the usage of exhaustible energy sources and intensive energy consumption are the major disadvantages of these methods. By comparison, biological hydrogen production processes seem to be the most cost-effective due to the use of renewable sources such as biomass (Antonopoulou et al., 2008; Ntaikou et al., 2008; Westermann et al., 2007) and waste materials (Jayalakshmi et al., 2008; Kapdan and Kargi, 2006; Li et al., 2008). These processes include direct and indirect biophotolysis, photo fermentation and fermentative hydrogen production (FHP). Among them, the FHP process has the highest energy efficiency as it can use renewable and waste carbon sources to produce H_2 as well as other valuable by-products with less energy consumed (Manish and Banerjee, 2008).

Although the FHP process has attracted great research interest in recent decades, hydrogen production yields and rates are still low in FHP, accompanied with

relatively high capital cost (Liu et al., 2008b). The low yield is associated with production of other reduced end products including lactate, butyrate and ethanol. The formation of these products involves oxidation of NADH, which can alternatively be utilized to produce H_2. It was proposed that H_2 yield may be further improved by eliminating the formation of some reduced products (Wang, 2008). However, without a detailed understanding of regulation in complex metabolic networks in the H_2-producing microorganisms, it can be rather difficult to predict which approaches could ultimately succeed in substantial enhancement of H_2 yields. Defined as the speed of metabolism, metabolic flux represents the degree of engagement of different pathways in a metabolic network. In this regard, the determination of metabolic flux is of central importance to understanding of metabolic network regulation.

Previously, failure to improve the low yield in the FHP process has been due to the unavailability of genetic tools for some H_2-producing microorganisms. For example, *Clostridium butyricum* is well-known as one of the common bacteria used for fermentative hydrogen production; however, it has been recognized as a genetically inaccessible species (Gonzalez-Pajuelo et al., 2005). Recently, a promising technology, the ClosTron system, has been developed and successfully applied to mutagenesis in several species of *Clostridium* excluding *C. butyricum* (Heap et al., 2010). If this technology can be extended to *C. butyricum*, it will facilitate the development of metabolic engineering strategies for H_2 production by this species.

The aim of this study was to improve fundamental knowledge of the central metabolic flux network associated with the FHP process using *C. butyricum*, and to restructure the metabolic pathways to enhance hydrogen production yield. Accordingly, specific objectives are to

1) understand the enzymatic and metabolic reactions,
2) analyze how metabolic fluxes respond to varying environmental conditions during the FHP process: initial glucose concentrations and operational pHs,
3) eliminate some end-product formation pathways and examine their effects on metabolism of *C. butyricum*,
4) redirect metabolic pathways to achieve increased H_2 production.

This thesis contains *six chapters*, of which four chapters (Chapter 1, 3, 4 and 5) comprise the main body.

Chapter 1 is a comprehensive literature review, which summarizes fundamentals of biochemical reactions and enzymatic activities of FHP, discusses key operational factors influencing metabolism, and reviews recently developed and applied technologies for metabolic flux analysis. *Chapter 2* contains a general introduction to the experimental materials and methods used in this study. Specific details of the methods are given in the relevant chapters. In *Chapter 3*, an *in silico* metabolic-flux model was constructed for the anaerobic glucose metabolism of *C. butyricum* W5 to evaluate metabolic flux distribution in the FHP network. The effects of initial glucose

concentration and operational pH value on the fractional flux were investigated. In *Chapter 4*, a conjugation system was developed for *C. butyricum* and applied to inactivated *hbd* (encoding β-hydroxybutyryl-CoA dehydrogenase) involved in butyrate formation. The effect of *hbd* inactivation on end-product metabolism during FHP by *C. butyricum* was examined and metabolism of *C. butyricum hbd*-deficient strain under N_2 sparging and non-sparging conditions was compared. In addition, homologs of all three genes encoding subunits of the *Thermotoga maritime* hydrogenase E were amplified, which extends the understanding of the mechanism of H_2 production by *C. butyricum*. Results from Chapter 3 and Chapter 4 provide important information for the design of metabolic engineering strategies for the enhancement of H_2 production. Therefore, in *Chapter 5*, blockage of the ethanol formation pathway by disruption of *aad* (encoding aldehyde-alcohol dehydrogenase) using the same method developed in Chapter 4 are described, and biochemical pathways were redirected for H_2 production by a combination of genetic and metabolic approaches. *Chapter 6* draws the conclusions from Chapter 3 to 5 and provides some recommendations for future investigation of this project.

Chapters of 1, 3 and 4 have been published in refereed academic journals. Chapter 5 has been submitted to a refereed academic journal. All the papers are closely related to the research field of this work.

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CHAPTER 1

Literature review

METABOLIC FLUX NETWORK AND ANALYSIS OF FERMENTATIVE HYROGEN PRODUCTION

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Biotechnology Advances 2011, 29:375-387

STATEMENT OF AUTHORSHIP

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Biotechnology Advances 2011, 29:375-387

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Saint, C.

Manuscript evaluation.

I give consent for G. Cai to present this paper for examination towards the Doctor of Philosophy.

Signed *Date*

Cai, G., Jin, B., Monis, P. & Saint, C. (2011) Metabolic flux network and analysis of fermentative hydrogen production.
Biotechnology Advances, v. 29 (4), pp. 375-387

CHAPTER 2

MATERIAL AND METHODS

Materials and methods are generally described in this chapter. Specific materials and methods are given in details in the relevant chapters.

Table 2.1 Strains and plasmids

Organism/plasmid	Feature	Source/reference
Strains		
Clostridium butyricum W5	Wild-type	Laboratory strain/ Wang et al., 2007
Escherichia coli DH5α	*E. coli* cloning strain	Laboratory strain
E. coli CA434	*E. coli* HB101 carrying R702	The University of Nottingham
Plasmid		
pMTL007	*E. coli*/*Clostridium* shuttle vector	The University of Nottingham/ Heap et al., 2007
pMTL007C-E2	pMTL007 containing FRT-flanked *ermB* RAM	The University of Nottingham/ Heap et al., 2010

1 Microorganisms and Maintenance

The bacterial strains used in this thesis are listed in Table 2.1. *Clostridium butyricum* W5 was isolated from activated sludge from a sewage treatment plant in South Australia and identified by 16S rDNA sequencing (GenBank accession number: DQ831124) and the RapID ANA II system (Remel, Inc., Lenexa, KS) in our previous research. The strain was grown on Tryptone Soya Agar anaerobically in ANAEROGEN system (AN2005, Columbia, HBA, Oxoid) at 37 ℃ for 24 h and stored at 4 ℃. *E. coli* strains were cultured in Luria-Bertani (LB) broth at 37 ℃ for 12 h and stored in LB broth supplemented with 10% glycerol at -20 ℃.

2. Media

All media described in this section was prepared in Milli-Q water (Millipore Ultra-Pure Water System) and autoclaved at 121 ℃ for 15 min before use.

2.1 Luria-Bertani (LB) broth and agar

LB broth used in this study consisted of 1% (W/V) tryptone (Oxoid), 0.5% (w/v) yeast extract (Oxoid) and 0.5% (w/v) NaCl, pH7.0. LB agar was LB broth supplemented with 1.8% (w/v) agar. LB broth and agar were used to culture *E. coli* strains.

2.2 Tryptone Soya Broth (TSB) and agar (TSA)

Tryptone Soya Broth (TSB) (CM0129, Columbia, HBA, Oxoid) was prepared following the manufacturer's instructions and used as the seed culture medium. Tryptone Soya Agar (TSA) was TSB supplemented with 1.8% (w/v) agar.

2.3 Media for fermentation

Two different media (MY and YL) were used in the fermentation system. MY medium consisted of: Na_2HPO_4-$7H_2O$, 12.8 g/L; KH_2PO_4, 3 g/L; NaCl, 0.5 g/L; NH_4Cl, 1 g/L; $MgSO_4$, 2 mmol/L; $CaCl_2$, 0.1 mmol/L; yeast extract, 3 g/L and glucose. YL medium contained 10 g/L "LAB-COMCO" powder (LP0029, Columbia, HBA, Oxoid), 3 g/L yeast extract (LP0021, Columbia, HBA, Oxoid) and glucose. Glucose was added separately after autoclaving in a range of 4-10 g/L depending on experimental design. MY medium was mainly used to investigate *C. butyricum* fermentation in response to pH and glucose variation. YL medium was used to compare fermentation performances between *C. butyricum* W5 wild type strain and its mutants under different environmental conditions.

3 Fermentation System Set-up

3.1 Bottle test

Preliminary experiments were carried out at 37 ℃ with initial pH7.0 in refitted 500

mL bottles, which were closed with a rubber stopper equipped with two tubes to allow gas purging and biogas emission respectively. The working volume was 500 mL and 10% (v) seed culture was used as the inoculum. Biogas was collected by the water release method.

Fig 2.1 Bottle test device

3.2 Bioreactor system

Batch fermentation experiments were performed in a laboratory-scale batch bioreactor BioFlo110 (New Brunswick Scientific, USA) with a working volume of 1.8 L at 39 ℃, and 300 rpm. The anaerobic environment was maintained by sealing all the connections with silicone oil. The inoculum was transferred with a sterile hypodermic disposable syringe. Nitrogen purging, for maintaining the anaerobic environment of the fermentation system, was carried out after the inoculation. Biogas produced was collected by the water release method. The fermentation conditions were varied according to experimental requirements.

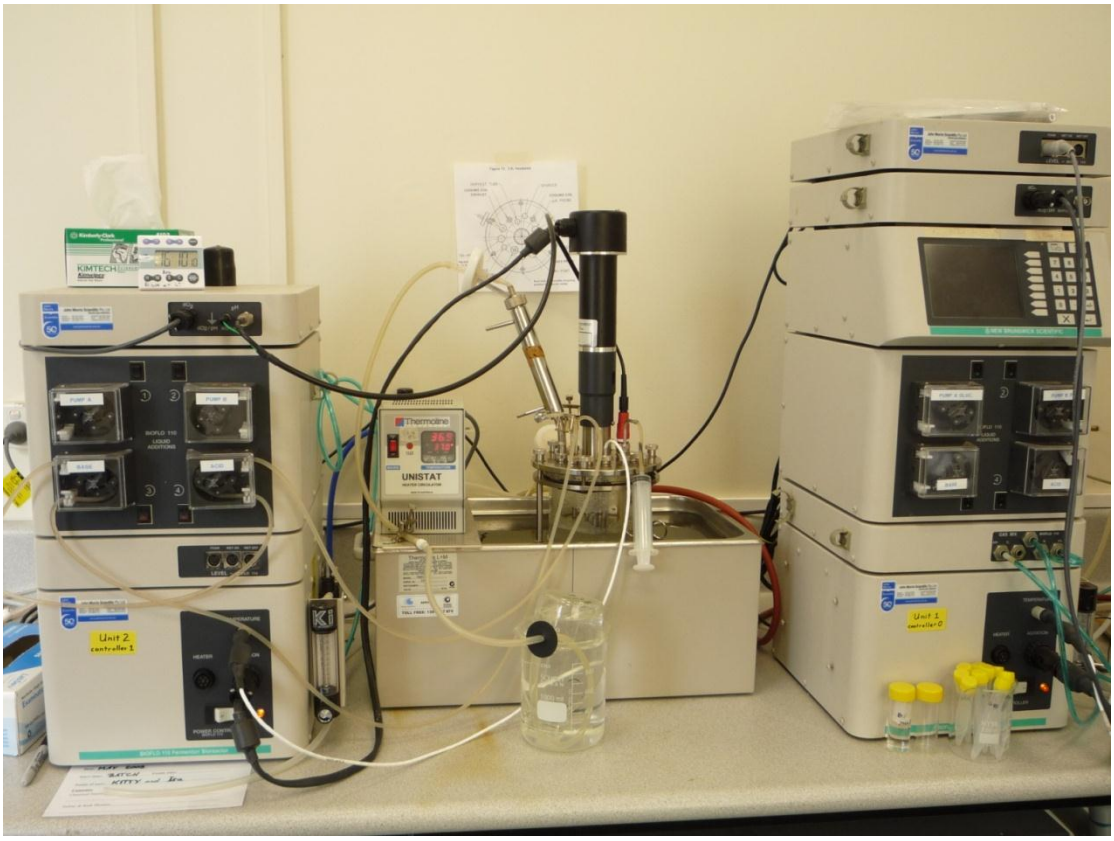

Fig 2.2 Bioreactor system used in this study

4 Sample Collection and Preparation

Biogas and fermentation broth in the bioreactor were collected at certain intervals for measuring cell growth and extracellular metabolite concentrations. Fermentation broth samples were centrifuged at 4000 rpm for 20 min. Pellets were washed twice with Mill-Q water and dried at 55 ℃ for more than 48 h for biomass measurement. The supernatant from the above centrifugation was diluted 10 times and further filtered through a 0.22 μm membrane for HPLC analysis.

5 Chemical Analysis

Cell concentrations were measured at 600 nm using a spectrophotometer (Lambda 20, Perkin-Elmer, USA). Biomass was weighed after drying using a balance (Mettler AT250). The carbon content of the biomass was measured by a Sercon 20-20 isotope ratio mass spectrometer connected with an elemental analyzer (Sercon-GSL).

Metabolite concentrations were analyzed by HPLC and GC. The HPLC analysis used a ROA Organic Acid Column (Phenomenex, 300×7.8) and a refractive index detector (Varian, Model 350). The mobile phase was 4 mM H_2SO_4 at a flow rate of 0.5 mL/min and the column temperature was 50 ℃. The total biogas volume was measured, while the concentration of H_2 and CO_2 was analyzed on a CP-3800 gas chromatograph (Varian Inc. CA, USA) equipped with a thermal conductivity detector. The GC column was packed with Molecular Sieve 5A and Hayesep Q. The working temperatures of injector, detector, and column were maintained at 50, 120 and 50 ℃, respectively.

6. Metabolic Flux Analysis

Metabolic flux analysis (MFA) was performed using software MetaFluxNet (Lee et al., 2003). The metabolic network of *C. butyricum* consisted of 32 fluxes and 26 intracellular metabolites. Five of the fluxes, including uptake rate of glucose, production rates of lactate, acetate, butyrate and ethanol, were directly calculated from experimental data over the exponential growth phase and were used as constrains in MFA. When the specific growth rate was chosen as the objective function, the solution gave the values of the unknown metabolic fluxes in the network. To understand how environmental conditions affect carbon flux, flux partitioning at key nodes was analyzed. The flux entering each node was normalized to 100. Flux partitioning provides a means to observe how bacterial regulates flux distribution in the metabolic network under a set of environmental conditions. A list of reactions used in the MFA model is provided in Chapter 3.

7. Molecular Techniques

7.1 Genome DNA extraction

Genome DNA from pure cultures was extracted by GenElute^{TM} Bacterial Genomic DNA Kit (Sigma).

7.2 PCR reactions

The PCR machine used in this research was a Mastercycler ep Gradient S thermocycler (Eppendorf, Westbury, NY). Reaction products were verified by 1.0% (w/v) agarose gel electrophoresis.

7.2.1 Amplification of a partial hbd gene from the genome of C. butyricum W5

PCR primer sets BHBD-F and BHBD-R (Table 2.2) were designed according to the known sequence of β-hydroxybutyryl-CoA dehydrogenase gene (*hbd*) in other strains of *C. butyricum* (NCBI webpage) using the software Primer 5.0. They were used to amplify a 450 bp product of a partial *hbd* gene from *C. butyricum* W5. PCR mix contains 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM BHBD-F and –R, 0.1 units Taq DNA polymerase and 200 pg/μl genomic DNA. The reaction conditions were performed as follows: 94 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 1 min, 50 ℃ for 30 sec and 72 ℃ for 30 sec, and finally 72 ℃ for 10 min.

7.2.2 Amplification of potential bifurcating Fe-hydrogenase subunits in W5

Three PCR primer sets (BFeHS-1-S and BFeHS-1-A, BFeHS-2-S and BFeHS-2-A, BFeHS-3-S and BFeHS-3-A), as shown in Table 2.2, were designed from three subunits of a potential bifurcating Fe-hydrogenase from *C. butyricum* E4 str. BoNT E BL5262 using the software Primer 5.0. PCR mix contains 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM each primer, 0.1 units Taq DNA polymerase and 200 pg μl^{-1} genomic DNA. The reaction conditions were performed as follows: 94 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 1 min, 50 ℃ for 30 sec and 72 ℃ for 1 min, and finally 72 ℃ for 10 min.

7.2.3 Amplification of a partial aad gene from the genome of W5

PCR primer sets AADa-f and AADa-r (Table 2.2) were designed according to the known sequence of a bifunctional aldehyde-alcohol dehydrogenase gene (*aad*) in other strains of *C. butyricum* (NCBI webpage) using the software Primer 5.0. PCR

using primer sets AADa-f and AADa-r was performed to amplify a 348 bp product of a partial *aad* gene from *C. butyricum* W5. PCR mix contained 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM AADa-f and AADa-r, 0.1 units Taq DNA polymerase and 200 pg μl^{-1} genomic DNA. The reaction conditions were performed as follows: 94 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 1 min, 52 ℃ for 30 sec and 72 ℃ for 2 min, and finally 72 ℃ for 10 min.

Table 2.2 Primers used for target gene amplification in this study

Oligonucleotide	Sequence(5'-3')	Target
BHBD-F	TTAGCTGCTGACTGCGATTT	*hbd*
BHBD-R	AGGTCCCATTGGGTGGTT	*hbd*
BFeHS-1-S	TATAATAGACAGCTTCTTATTGC	Fe-hydrogenase gene
BFeHS-1-A	AGTGACTTTACTTTTGCCTTTGT	Fe-hydrogenase gene
BFeHS-2-S	ATACAGCAGACAAGAAGATATACCC	Fe-hydrogenase gene
BFeHS-2-A	AAGAACAGTAGGCTTTCCAAACAAT	Fe-hydrogenase gene
BFeHS-3-S	GTGATTAAGACTAATACACCGAAAC	Fe-hydrogenase gene
BFeHS-3-A	ATACCACGCATTCCTACAAACTG	Fe-hydrogenase gene
AADa-f	CTCCAGAAGGAATAATCGGATGG	*aad*
AADa-r	CTATTTCTTCGCCTTTAAGGATG	*aad*

7.3 Agarose gel electrophoresis

The size and quality of genome DNA and DNA fragments generated from PCR or restriction enzyme digestion were determined by electrophoresis at room temperature using 0.8~1.0% (w/v) agarose gels stained with 1×SYBR Safe ™ nucleic acid stain (Invitrogen, Carlsbad, CA, USA). PCR products were mixed with 6×loading buffer (Geneworks, SA, Australia) and electrophoresed in 1×TAB buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) at 100V with an Owl electrophoresis chamber connected to an EC25090 electrophoresis power supply. After electrophoresis, DNA was visualised on a Dark Reader transilluminator and the image was captured using a

GeneFlash gel documentation system (Medos Company Pty. Ltd, Australia).

7.4 DNA digestion and ligation

After digestion with *Hin*d III and *Bsr*G I, the PCR product was ligated into *Hin*d III/*Bsr*G I-digested pMTL007 linear vector by Fast Ligation Kit (Bio-lab).

7.5 DNA transformation

Plasmid DNA (0.5 μl) or 5 μl ligation mixture (2 ng/μL) was added to a vial of 100 μl *E. coli* competent cells and incubated on ice for 20 min. Cells were then heat shocked at 42 ℃ for 1 min and immediately placed on ice for 2 min. Then 400 μl of pre-warmed LB broth was added to the cells and incubated at 37 ℃ for 1 h with horizontal shaking at 150 rpm for initial cell recovery. Transformants were then cultured overnight on LB agar containing 12.5 μg/ mL chloramphenicol. Colonies were randomly picked and tested by plasmid digestion and electrophoresis for the correct sized fragment.

7.6 Plasmid extraction

Colonies with correct inserts were inoculated into 5 mL LB broth containing 12.5 μg/ mL chloramphenicol and incubated overnight at 37 ℃. Plasmid DNA was isolated with the Spin Miniprep Kit (Sigma), following the manufacturer's instructions. Final elution volume was 50 μl in sterile Milli-Q water.

7.7 Gene knockout using the Clostron system

7.7.1 Intron re-targeting and plasmid construction

The target site was identified and PCR primers (modified IBS, EBS1d and EBS2 as shown in chapter 4 and 5) for intron re-targeting were designed using a computer algorithm, the access of which is provided as part of the TargeTron^{TM} Gene Knockout System kit (http://www.sigmaaldrich.com). Splicing by Overlap Extension PCR (SOE PCR) was used to amplify a 350 bp intron fragment following the manufacturer's procedure (TargeTron^{TM} Gene Knockout System kit). The re-targeted intron was

inserted into a Clostron plasmid and then transformed into *E. coli* DH5α by heat-shock. The constructed plasmid which contains the new targeting region generated by PCR was confirmed by restriction analysis of plasmid DNA from transformed cells using *Hin*d III and *Bsr*G I, followed by sequencing.

7.7.2 Conjugation procedures

The *E. coli* donor strain CA434 was first transformed with the re-targeted plasmid. The strain obtained was then grown overnight in LB broth and a 1 ml aliquot was centrifuged at 12000 rpm for 1 min. The supernatant was decanted and the cells were gently resuspended in 1 ml of sterile phosphate buffered saline (PBS). Centrifugation was repeated and the harvested cells were resuspended in a total volume of 100 µl of overnight culture of *C. butyricum* grown in TSB medium. This mating mix was then spotted onto a well-dried TSB plate which was then incubated anaerobically overnight. The bacterial growth was harvested by flooding the agar surface with 1 ml of PBS and resuspension of the biomass with a sterile spreader. The cell suspension was plated onto TSA plates supplemented with 250 µg/ml cycloserine and 15 µg/ml thiamphenicol to select for transformants.

7.7.3 Intron integration

Transformant colonies from the re-streak plate were used to inoculate 1 ml of anaerobic TSB supplemented with 7.5 µg/ml thiamphenicol, followed by induction with 1 mM IPTG. Cells were then washed in 0.5 ml PBS, re-suspended in 1 ml TSB, and incubated for 3 h to recover. Finally, the integration mixture was spread on TSA plates supplemented with 2.5 µg/ml erythromycin to select for intron integration.

7.8 DNA sequencing

PCR products and plasmid inserts were sequenced by SouthPath and Flinders Sequencing Facility (Adelaide, SA, Australia). Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) program to search

the National Center for Biotechnology Information (NCBI) sequence database (http://www.ncbi.nlm.nih.gov/BLAST/).

8. Enzyme Assays

For measurement of intracellular enzyme activity, cells were harvested by centrifugation at 4000 rpm, 4 ℃ for 20 min and washed twice with cold 10 mM Tris-HCl (pH7.5). The washed cells were resuspended in the same buffer containing 1 mM 1,4-dithiothreitol (DTT) and sonicated on ice for five cycles (a working period of 30 s in a 30 s interval for each cycle) at 40% of maximum power output by an ultrasonic disruptor (Branson B15). The cell debris was removed by centrifugation at 132 000 rpm, 4 ℃ for 20 min, and the supernatant was used for enzyme activity determination.

BHBD activity was measured at 345 nm by monitoring the decrease in NADH concentration resulting from β-hydroxybutyryl-CoA formation from acetoacetyl-CoA (Hartmanis and Gatenbeck 1984). The cell extract was added to the mixture containing 100 mM MOPS buffer (pH 7.0), 1 mM 1,4-dithiothreitol, 0.1 mM acetoacetyl-CoA, and 0.15 mM NADH. Decrease in the absorbance was monitored in the sample solution and control solution, from which acetoacetyl-CoA was omitted. The enzyme activity was determined by calculating the difference in the slope values of the sample and control, and using the molar extinction coefficient of 6,220 $M^{-1}cm^{-1}$.

The protein content was determined according to Bradford's method, using bovine serum albumin as a standard for calibration.

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CHAPTER 3

METABOLIC FLUX ANALYSIS OF HYDROGEN PRODUCTION NETWORK BY CLOSTRIDIUM BUTYRICUM W5: EFFECT OF PH AND GLUCOSE CONCENTRATIONS

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International Journal of Hydrogen Energy 2010, 35:6681-6690

STATEMENT OF AUTHORSHIP

METABOLIC FLUX ANALYSIS OF HYDROGEN PRODUCTION NETWORK BY CLOSTRIDIUM BUTYRICUM W5: EFFECT OF PH AND GLUCOSE CONCENTRATIONS

International Journal of Hydrogen Energy 2010, 35:6681-6690

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I give consent for G. Cai to present this paper for examination towards the Doctor of Philosophy.

Signed *Date*

Cai, G., Jin, B., Saint, C. & Monis, P. (2010) Metabolic flux analysis of hydrogen production network by Clostridium butyricum W5: Effect of pH and glucose concentrations.
International Journal of Hydrogen Energy, v. 35 (13), pp. 6681-6690

CHAPTER 4

GENETIC MANIPULATION OF BUTYRATE FORMATION PATHWAYS IN CLOSTRIDIUM BUTYRICUM

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Journal of Biotechnology 2011, 155:269-274

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GENETIC MANIPULATION OF BUTYRATE FORMATION PATHWAYS IN CLOSTRIDIUM BUTYRICUM

Journal of Biotechnology 2011, 155:269-274

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I give consent for G. Cai to present this paper for examination towards the Doctor of Philosophy.

Signed *Date*

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Interpreted data; manuscript evaluation.

I give consent for G. Cai to present this paper for examination towards the Doctor of Philosophy.

Signed *Date*

Cai, G., Jin, B., Saint, C. & Monis, P. (2011) Genetic manipulation of butyrate formation pathways in Clostridium butyricum.
Journal of Biotechnology, v. 155 (3), pp. 269 - 274

CHAPTER 5

A GENETIC AND METABOLIC APPROACH TO REDIRECTION OF BIOCHEMICAL PATHWAYS OF CLOSTRIDIUM BUTYRICUM FOR ENHANCING HYDROGEN PRODUCTION

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Manuscript to be submitted.

STATEMENT OF AUTHORSHIP

A GENETIC AND METABOLIC APPROACH TO REDIRECTION OF BIOCHEMICAL PATHWAYS OF CLOSTRIDIUM BUTYRICUM FOR ENHANCING HYDROGEN PRODUCTION

Submitted manuscript to Environmental Science & Technology

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Jin, B.
Manuscript evaluation; acted as corresponding author.

I give consent for G. Cai to present this paper for examination towards the Doctor of Philosophy.

Signed *Date*

Monis, P.
Manuscript evaluation.

I give consent for G. Cai to present this paper for examination towards the Doctor of Philosophy.

Signed *Date*

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Manuscript evaluation.

I give consent for G. Cai to present this paper for examination towards the Doctor of Philosophy.

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A genetic and metabolic approach to redirection of biochemical pathways of *Clostridium butyricum* for enhancing hydrogen production

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Abstract

Clostridium butyricum, a well-known H_2 producing bacterium, produces lactate, butyrate, acetate, ethanol, and CO_2 as its main by-products from glucose. The conversion of pyruvate to lactate, butyrate and ethanol involves oxidation of NADH. It was hypothesised that the NADH could be increased if the formation of these end-products can be eliminated, resulting in enhancing H_2 yield. Herein, this study

aimed to establish a genetic and metabolic approach for enhancing H_2 yield via redirection of metabolic pathways of a *C. butyricum* strain. The ethanol formation pathway was blocked by disruption of *aad* (encoding aldehyde-alcohol dehydrogenase) using a ClosTron plasmid. However, elimination of ethanol formation appeared to be unfavourable for hydrogen production, while significantly improving lactate production. With the addition of sodium acetate, the resulting *aad*-deficient mutant showed approximately 20% enhanced performance in hydrogen production. This work demonstrated the possibility of improving hydrogen yield by eliminating unfavourable end-products ethanol and lactate.

Keywords: *Clostridium butyricum*, ethanol formation pathway, *aad* disruption, NaAc addition, fermentative hydrogen production

Introduction

H_2 has been recognised as an ideal source of clean energy, as it has the highest gravimetric energy density of any of the known fuels and its combustion produces water as the sole by-product (*1*). Fermentative H_2 production is an exciting R&D area that offers a potential means of producing H_2 from a variety of renewable resources, even waste or wastewaters via a low energy required continuous bioprocess. However, a typically low H_2 yield associated with the H_2 production process continues to be a challenge for developing a viable industrial process. Enormous research efforts have been mounted to improve H_2 productivity and yield in the last two decades, mostly

focussed on the online reduction of H_2 partial pressure, the optimization of process conditions, and the isolation of wild pure and/or mixed cultures (*1-2*). Nevertheless, additional approaches have been suggested, such as to alter metabolism via metabolic engineering.

Clostridium butyricum is a Gram-positive, spore-forming, anaerobic bacterium used for fermentative hydrogen production. It has been widely acknowledged that *Clostridium* species possess an additional pathway that allows them to produce hydrogen from NADH under specific conditions (*2-3*). The evolution of H_2 from NADH by a potential bifurcating hydrogenase has been hypothesised (4). This enzyme is capable of oxidizing NADH and ferredoxin simultaneously to produce H_2 when hydrogen partial pressure is low (*4*). NADH is usually generated during glycolysis of glucose. During fermentation, various extracellular metabolites including acetate, butyrate, lactate and ethanol can be produced in association with production of H_2 and biomass. The conversion of pyruvate to butyrate, lactate and ethanol involves oxidation of NADH. The NADH concentration should increase if the formation of the reduced end-products can be blocked. H_2 evolution through the NADH pathway is driven by the necessity of deoxidizing the residual NADH of metabolic reactions such as $NADH + 2Fd^{red} + 3H^+ \rightarrow NDH^+ + 2Fd^{ox} + 2H_2$ (where Fd^{red} indicates reduced ferredoxin and Fd^{ox} indicates oxidized ferredoxin). Thus, if metabolic reactions can be controlled to increase the amount of residual NADH, the H_2 yield may be enahnced further.

C. butyricum W5, a recently isolated hydrogen producer, is capable of using glucose, starch and molasses to produce hydrogen (*5-6*). *C. butyricum* W5 grown on glucose produced hydrogen and carbon dioxide along with a diversity of acids (lactate, acetate and butyrate) and a small amount of ethanol (*7*). The butyrate formation pathway is recognized as the main competing pathway during hydrogen production because it consumes more NADH than other pathways, reducing the yield of hydrogen (*2-3*). Hallenbeck (2009) suggested that elimination of the butyrate pathway may improve hydrogen production by *Clostridium sp* (*2*). In our recent study, we inactivated *hbd* (encoding β-hydroxybutyryl-CoA dehydrogenase) in an isolated wild strain of *C. butyricum* W5 to eliminate the butyrate formation pathway, resulting in a significant increase in ethanol production and a slight decrease in H_2 yield compared with the wild type strain (*8*). It seems that high butyrate production accompanies high hydrogen production, whereas high ethanol production is associated with low hydrogen production. Thus, we hypothesised that blocking the ethanol formation pathway may be more beneficial for enhancing hydrogen production yield.

The ethanol formation pathway consists of two reductive steps from acetyl-CoA to acetaldehyde and then to ethanol, which are catalyzed by acetaldehyde CoA dehydrogenase (ACDH) and alcohol dehydrogenase (ADH), respectively. The *aad* gene of *C. butyricum* encodes a bifunctional aldehyde-alcohol dehydrogenase, which has ACDH and ADH activities. This study aimed to examine whether H_2 yield can be

enhanced via reducing the ethanol formation. Herein, this paper describes the successful interruption of the ethanol formation pathway by inactivation of *aad* using the ClosTron system. We for the first time investigated and used a genetic and metabolic approach of blocking the ethanol formation pathway in conjunction with addition of sodium acetate to imorove H_2 production yield. We experimentally investigated how these genetic and metabolic alterations could affect metabolism during fermentative hydrogen production. From our view, *aad* inactivation using the ClosTron system to block ethanol formation pathway of *Clostridium sp*. with an attempt to enhance H_2 yield has not yet been reported in the literature.

Materials and Methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. *C. butyricum* W5 was isolated from activated sludge from a sewage treatment plant in South Australia and identified by 16S rDNA sequencing (GenBank accession number: DQ831124) and the RapID ANA II system previously (*5*). *C. butyricum* W5 was cultured in Tryptone Soya Broth (TSB) (CM0129, Columbia, HBA, Oxoid) and on Tryptone Soya Agar (TSA), and cultivated in an anaerobic jar at 37 ℃. The anaerobic gas atmosphere was created by ANAEROGEN (AN2005, Columbia, HBA, Oxoid) in the anaerobic jar. *Escherichia coli* strains were cultured in L-broth and on L-agar at 37 ℃. For *E. coli* carrying the retargeted plasmid, the medium was supplemented with chloramphenicol (12.5 μg ml^{-1}).

Table 1 Strains and plasmids

Organism/plasmid	Feature	Source/reference
Strains		
Clostridium butyricum W5	Wild-type	Laboratory strain/ Wang et al., 2007
Escherichia coli DH5α	*E. coli* cloning strain	Laboratory strain
E. coli CA434	*E. coli* HB101 carrying R702	The University of Nottingham
C. butyricum M6	*aad*::intron	This study
Plasmid		
pMTL007C-E2	*E. coli*/*Clostridium* shuttle vector	The University of Nottingham/ Heap et al., 2010
pMTL007C-E2::Cbu-aad-683a	pMTL007C-E2 with intron targeted to *aad*	This study

Intron re-targeting and plasmid construction

The target sites were recognized. PCR primers (modified IBS, EBS1d and EBS2 as shown in Table 2) for intron re-targeting were designed using a computer algorithm. The access of the PCR primers is provided with purchase of the TargeTron^{TM} Gene Knockout System kit. Re-targeted intron was amplified using universal EBS, and modified IBS, EBS1d and EBS2 primers according to the manufacturer's procedure

(TargeTron™ Gene Knockout System kit). The re-targeted intron was introduced into pMTL007C-E2 by Ligation after digestion of the plasmid and the intron with *Hin*d III and *Bsr*G I. The new targeting region in the plasmid was verified by sequencing. The re-targeted plasmid was named as pMTL007C-E2:Cbu-*aad*-683a, *i.e.*, the intron on this plasmid is targeted to insert in the antisense orientation after base 683 of the *aad* ORF of *C. butyricum*.

Mutant generation

Mutants in *aad* were generated according to the ClosTron system and procedures using the re-targeted plasmid (9). Transformation of the plasmid to *C. butyricum* W5 was accomplished by conjugation as described previously (*8*). Transconjugant colonies were selected by resistance to thiamphenicol. Transconjugants were used to inoculate anaerobic TSB supplemented with thiamphenicol to select for the re-targeted plasmid. After inducing intron expression with IPTG, *aad* mutants were isolated on TSA plates supplemented with erythromycin.

PCR screening to confirm intron insertion into aad

PCR primer sets AADa-f and AADa-r (Table 2) were designed according to the known sequence of *aad* in other strains of *C. butyricum* (NCBI webpage) using the software Primer 5.0. PCR using primer sets AADa-f and AADa-r was performed to confirm intron insertion in the *aad*, yielding a ~2200 bp product in the presence of the intron and a 348 bp product in the absence of the intron. PCR mix contained 1× PCR

buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM AADa-f and AADa-r, 0.1 units Taq DNA polymerase and 200 pg μl^{-1} genomic DNA. The reaction conditions were performed as follows: 94 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 1 min, 52 ℃ for 30 sec and 72 ℃ for 2 min, and finally 72 ℃ for 10 min.

Table 2 Primers used for DNA Amplification in this Study

Oligonucleotide	Sequence(5'-3')
AADa-f	CTCCAGAAGGAATAATCGGATGG
AADa-r	CTATTTCTTCGCCTTTAAGGATG
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC
IBS	AAAAAAGCTTATAATTATCCTTAACTGACTTTACAGTGCGC CCAGATAGGGTG
EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTTTACA GCTAACTTACCTTTCTTTGT
EBS2	TGAACGCAAGTTTCTAATTTCGATTTCAGTTCGATAGAGG AAAGTGTCT

Bioreactor experiments and sample analysis

Batch fermentations were performed in a laboratory-scale batch bioreactor BioFlo110 (New Brunswick Scientific, USA) with a working volume of 1.8 L, at 39 ℃, pH 6.5 and 300 rpm. Fermentation medium contained 5 g L^{-1} glucose, 3 g L^{-1} yeast extract (LP0021, Columbia, HBA, Oxoid) and 10 g L^{-1} "LAB-COMCO" powder (LP0029,

Columbia, HBA, Oxoid). For sodium acetate (NaAc) addition experiments, the fermentation medium was supplemented with 2 g/L NaAc. The anaerobic environment was maintained by sealing all the connections with silicone oil. The inoculum was transferred with a sterile hypodermic disposable syringe. Nitrogen purging for maintaining the anaerobic environment of the medium was carried out after the inoculation. Biogas produced was collected by the water release method. Sample preparation and analyses were performed as described by Cai et al. (*7*).

Results and Discussion

Generation of aad-deficient strains

Disruption of *aad* in *C. butyricum* W5 was accomplished following the protocols outlined in the methods section. An antisense insertion site was selected that would yield an unconditional disruption. This site is 683 bp downstream of the predicted start codon and resides in the ACDH domain on *aad*. The pMTL007C-E2 was used because it contains FRT-flanked *ermB* RAM and can be used for multiple gene disruption (*9*). Erythromycin resistance (Em^R) colonies were confirmed to be *aad*-deficient mutants by PCR using primers flanking the 683/684 site of *aad*. PCR results showed a 348 bp product in the wild type strain and a 2.2 kb product in a mutant strain M6, indicating that the expected 1.85 kb intron was inserted in *aad* of the mutant M6 genome (Figure 1).

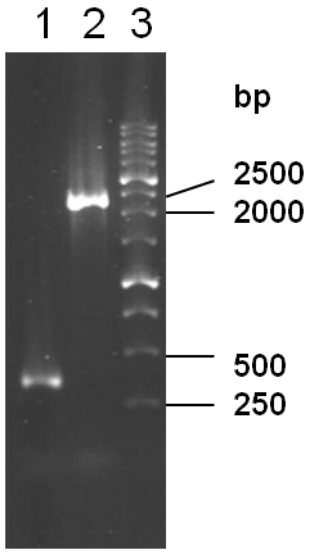

Figure 1. Confirmation of *aad* gene disruption in the genome of *C. butyricum* by PCR. PCR from the genome of wild type strain W5 (lane 1); PCR from the genome of mutant M6 (lane 2); 1 kb marker (lane 3).

This study presents another example of the successful application of the ClosTron system in gene disruption in *C. butyricum*. Although intron target specificity and intron insertion efficiency are the two main features that make it attractive to genetically manipulate *Clostridium* species (*10-11*), another two important factors need to be considered in order to successfully disrupt genes in *C. butyricum*,. Firstly, selection of a target site plays a key role in specific insertion, which is important to determine whether or not the gene could be successfully disrupted. Initially, a sense insertion site, which is 1137 bp downstream of the predicted start codon, was used. However, the re-targeted intron failed to insert itself into the selected target site. Thus, we selected another antisense insertion site that could target to the correct site. Furthermore, we found that selection of an appropriate plasmid is another important

step, as which will not only affect the efficiency of gene transfer, but also make it promising for screening the re-targeted plasmid. For example, plasmid pMTL007C-E2 rather than pMTL007 contains a *lacZ*α ORF, which can be replaced with a re-targeted intron. Clones containing successfully re-targeted plasmid can be easily distinguished from those which contained the parental plasmid by a while instead of blue colony color on plates supplemented with X-gal. In term of this, it is more efficient to screen re-targeted pMTL007C-E2 compared to re-targeted pMTL007.

End-product metabolism of the aad-deficient and wild type strains

To examine the effect of *aad* inactivation on end-product metabolism, batch fermentations of the wild type and the *aad-deficient* mutant (M6) strains were performed under the same conditions as described in the method section. It was exciting to note that the strain M6 did not produce ethanol, which indicates that interruption of the ACDH domain of *aad* successfully blocked the ethanol formation pathway. The function of AAD (referred to as AdhE in some reports) varies among different microorganisms. Green and Bennett (*12*) reported that inactivation of the ADH domain on *aad* in *C. acetobutylicum* resulted in more ethanol production and less butanol production. It was suggested that AAD plays an important role in butanol production. In *Escherichia coli*, AdhE shows ACDH and ADH activities, and catalyzes the conversion of acetyl-CoA into ethanol (*13*). Similar function of AdhE in *Lactococcus lactis* was reported by Arnau et al. (*14*). They found that a mutant with

*adh*E disruption (MGKAS15) produced reduced amounts of ethanol under anaerobic conditions compared to the wild type strain. Based on our results, it would be assumed that AAD in *C. butyricum* W5 may be responsible for ethanol production rather than butanol production. Another possibility that cannot be ruled out is that the ADH domain of *aad* may play an important role in butanol production and other ADHs may be responsible for ethanol production, since there are several ADHs in *Clostridium* species. Since ACDH catalyses the first step in the production of ethanol from acetyl-CoA, inactivating the ACDH domain should completely block ethanol formation, which is the case for the ACDH deficient mutant M6 in this study.

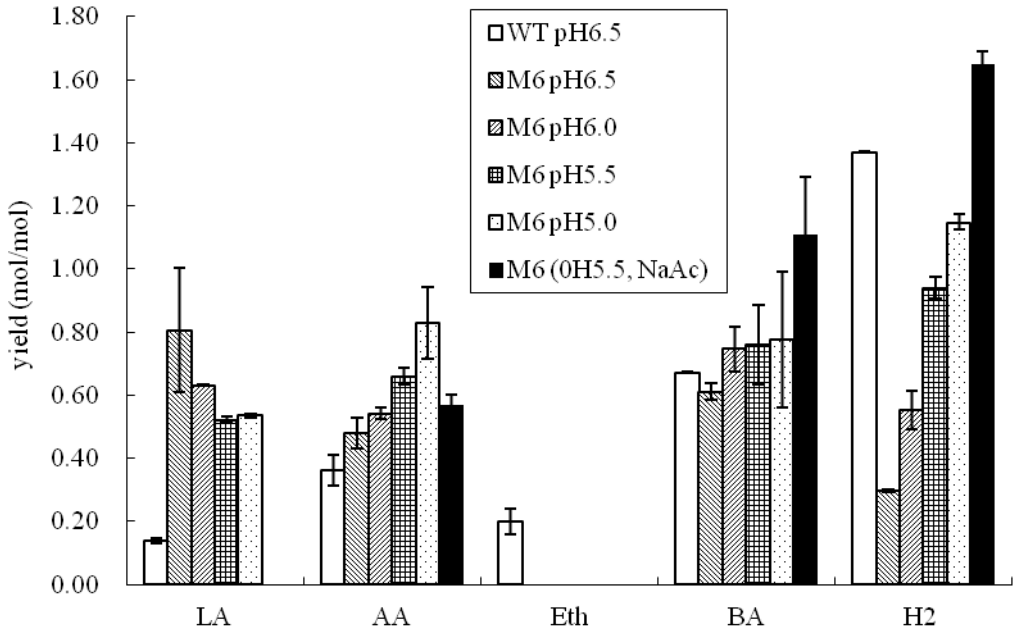

Figure 2. Product yields during fermentation using the wild type strain (W5) and the *aad*-deficient strain (M6). LA, lactic acid; AA, acetic acid; Eth, ethanol; BA, butyric acid; H_2, hydrogen. (Value ± SD for n = 2 samples).

Results from the fermentation experiments showed that the mutant strain M6 performed a enormously enhanced lactate production over 484% and as well as 32% more acetate production, while generating 78% less hydrogen and 9% less butyrate compared with the wild type strain at an operational pH 6.5 (Figure 2), which was unexpected. These unwanted results suggested that blockage of the ethanol pathway could redirect the metabolic flux towards the lactate formation pathway. A similar result was observed in cultures of *Lactococcus lactis* MGKAS15 (*adh*E deficient), which showed an enhanced lactate production under anaerobic conditions (*14*). It was interesting to note that the enhanced lactate production resulted in a significant reduction in H_2 yield accompanied by reduced butyrate production. Our findings from this and previous studies (*7-8*) could reveal that elimination of butyrate formation pathway may not be a promising approach to the enhancement of H_2 yield.

Enhancing hydrogen production by the aad-deficient strain

Our results stated above may reveal that the reduction of lactate production is a key step to enhance hydrogen production by the mutant strain M6. According to our recent metabolic flux analysis, we found that operational pH is a crucial parameter which affected lactate production and less lactate was produced at a low pH (*7*). When pH was decreased from 6.5 to 5.5, final lactate yield decreased from 0.81 mol/mol glucose to 0.52 mol/mol glucose. As pH was further decreased to 5.0, lactate production increased slightly (0.54 mol/mol glucose). This result could be linked to the decrease in the activity of NAD-independent lactate dehydrogenase (iLDH)

responsible for lactate utilization when pH was lower than 5.5 (*15*). By contrast, the yields of other metabolites including acetate, butyrate and hydrogen, increased with the decrease in operational pH from 6.5 to 5.0. However, the final hydrogen yield observed in the mutant strain M6 was still lower than that of the wild type strain, and the lactate yield was still high compared to that of the wild type strain.

Here, we could assume that the hydrogen yield should be improved if the lactate produced during the fermentation can be reutilized by the hydrogen producing strain. The role of acetate in the utilization of lactate has been reported in several *Clostridium* species. Thus, we added sodium acetate (NaAc) into the fermentation medium in order to examine its effect on the utilization of lactate with respect to operational pH. Analytical data showed that NaAc inhibited the cell growth when pH was controlled at 5.0, while NaAc improved cell growth when pH was controlled at 5.5 or above (data not shown). The profiles of metabolite production from glucose with NaAc addition at pH 5.5 are shown in Fig. 3. Lactate concentration increased during the first 11 hours and reached a maximum value of 1.41 g/L, and then followed a lactate reduction phase down to the initial value. This indicates that lactate produced in the early stage of fermentation was consumed completely during the late stage of fermentation. Thus, we may conclude that addition of NaAc can substantially reduce lactate formation by the mutant strain M6, while leading to an pronounced increase in butyrate production, reaching a maximum value of 2.53 g/L at 24 h. Importantly, the mutant strain M6 grown in the fermentation medium with additional

NaAc performed a significant enhancement in H_2 yield. The final hydrogen yield increased from 0.94 mol/mol glucose to 1.65 mol/mol glucose with the addition of NaAc under operational pH of 5.5.

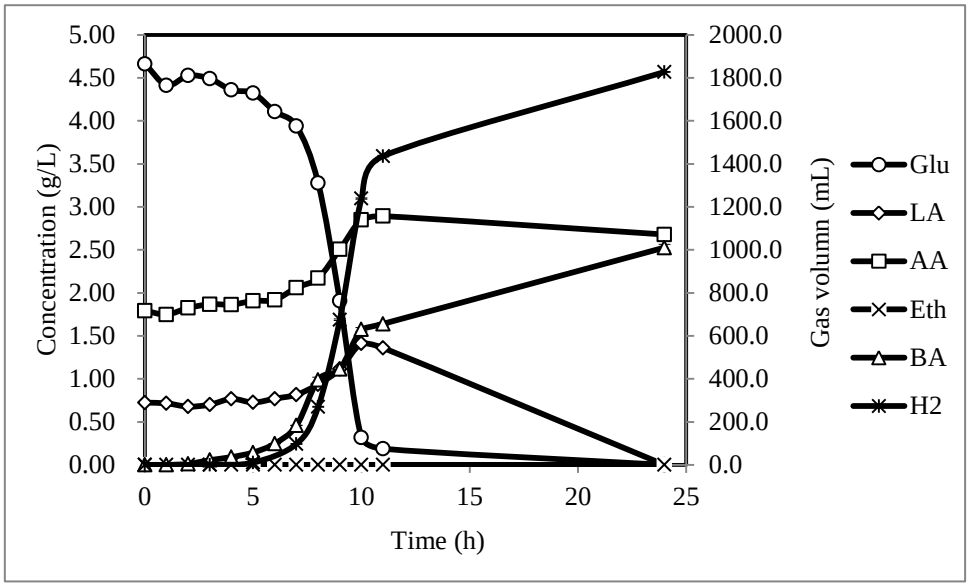

Figure 3. Time course profile of glucose consumption and product formation during fermentation using M6 under operational pH of 5.5 with the sodium acetate addition. LA, lactic acid; AA, acetic acid; Eth, ethanol; BA, butyric acid; Glu, glucose; H_2, hydrogen. (Value ± SD for n = 2 samples).

From the results as stated above, it can be seen that with the addition of NaAc (or acetate) *C. butyricum aad*-deficient strain M6 was able to utilize endogenetic end-product lactate to produce butyrate and hydrogen when glucose was nearly consumed. However, this phenomenon was not obvious when NaAc (or acetate) was not added. This suggested that the added NaAc instead of the endogenetic end-product acetate may play a role in redirection of metabolic fluxes, leading to a

significant lactate consumption by the *C. butyricum aad*-deficient strain M6. Whilst the role of acetate and the pathway of lactate metabolism were not clearly clarified, the requirement for acetate in lactate utilization by *C. beijerinckii* (*16*) and *C. tyrobutryicum* (*17*) has been reported in previous studies. The role of acetate in the utilization of lactate by *C. acetobutylicum* was defined as an alternative electron acceptor and acetate was completely consumed at the end of fermentation (*15*). However, acetate consumption appeared to be minor when lactate was utilized in this study. Results from this study demonstrated that the added NaAc (or acetate) can affect the cell growth (maximum OD_{600nm} increased from 3.15 to 5.02), and improve butyrate and gas production (Fig 2). Similar results had already been noted during glycerol fermentation by *C. butyricum* (*18-19*). They suggested that acetate acted as an indirect proton acceptor during the early stages of fermentation, but did not serve as an energy source. It is worthwhile to point out that the acetate measured within 5h fermentation was not the endogenetic end-product acetate, as acetate formation could only take place after 6 h (Fig. 3). The re-utilization of lactate may be explained by the essential reactions to consume excess oxidized nucleotides (NAD^+) generated through stimulation of butyrate formation by acetate supplementation, leading to increased hydrogen production.

Our results show that hydrogen production yield was improved merely due to pH decrease and NaAc addition. To clarify if the *aad* disruption could contribute to hydrogen production improvement, we conducted comparison trials with NaAc

addition using the wild type *C. butyricum* W5 strain and the *hbd* defficient mutant. The latter was genetically modified to eliminate butyrate production (*8*). It was found that the added NaAc did not significantly influence hydrogen production in both strains (data not shown). Therefore, it can be concluded that hydrogen production was enhanced via redirection of metabolic pathways as a result of *aad* disruption and NaAc addition.

In conclusion, the ethanol formation pathway can be blocked by disruption of *aad* encoding aldehyde-alcohol dehydrogenase using a ClosTron plasmid. However, elimination of targeting metabolic pathway appeared to be unfavourable for hydrogen production, while redirecting metabolic reaction towards lactate production. The resulting *aad*-deficient mutant with the addition of sodium acetate showed approximately 20% enhanced performance in hydrogen production. Overall results from this study revealed that the combined genetic and metabolic approach could be a promising way to promote process performance of fermentative hydrogen production.

Acknowledgements

We thank the scholarship for Guiqin Cai given by the University of Adelaide, Australia, financial and technical support from Bionanotechnology Laboratory: Water, Energy and Materials at the University of Adelaide, and Prof. Nigel P. Minton from University of Nottingham, UK for providing plasmid pMTL007C-E2 and *E. coli* CA434 strain,

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CHAPTER 6

CONCLUSIONS AND RECOMENDATIONS

1. Conclusions

1.1. Development of an in silico metabolic network model for C. butyricum

Although fermentative hydrogen production by strict anaerobes has been widely reported, there is a lack of information related to metabolic flux distribution in the metabolic production system. The *in silico* central metabolic flux model for anaerobic glucose fermentation was developed for the first time for hydrogen production by *C. butyricum* W5 based on fermentation data and previous reports. A computer program MetaFluxNet was used to predict the metabolic flux distribution during fermentative hydrogen production. The results showed that the constructed model can properly predict the phenotypic behavior of *C. butyricum* W5 during fermentative hydrogen production, when the specific growth rate was chosen as an objective function. Metabolic flux analysis (MFA) indicated that carbon partitioning at three key nodes (PEP, pyruvate and AcCoA nodes) in the strain W5 was affected in varying degrees by operational pH and initial glucose concentration. It was concluded that none of the three nodes are rigid nodes. This provides important information for future design of metabolic engineering strategies.

1.2. Extension of the ClosTron technology to C. butyricum, a genetically inaccessible species

C. butyricum has been recognized as a genetically inaccessible species (Gonzalez-Pajuelo et al., 2005). A promising technology, the ClosTron system, has

been recently developed and successfully applied to mutagenesis in seven species of *Clostridium*: *C. acetobutylicum*, *C. botulinum*, *C. difficile*, *C. beijerinckii*, *C. sporogenes, C. perfringens* and *C. sordellii* (Heap et al., 2010). In this study, a conjugation system for *C. butyricum* was developed. The ClosTron plasmids (pMTL007 and pMTL007C-E2) containing a group II intron were designed for targeted chromosomal insertions, and were used to disrupt a β-hydroxybutyryl-CoA dehydrogenase gene (*hbd*) and a bifunctional aldehyde-alcohol dehydrogenase gene (*aad*) respectively. Gene disruption was confirmed by PCR using primers flanking the target sites. This work opens the possibility to genetically modify *C. butyricum*, and will facilitate the development of metabolic engineering strategies for the production of valuable metabolites.

1.3. Extending the understanding of the mechanism of H_2 production by C. butyricum

Comparing metabolism of the wild type and the mutant strains showed that inactivation of *hbd* blocked the butyrate formation pathway and affected the production of ethanol and H_2, while inactivation of *aad* blocked the ethanol formation pathway and affected the production of lactate and H_2. PCR amplification of three subunits of a potential bifurcating hydrogenase showed that, in addition to the well-studied ferredoxin-dependent hydrogenase, *C. butyricum* may also possess a bifurcating hydrogenase that utilizes ferredoxin and NADH simultaneously to produce H_2. These findings elucidated the competitive mechanism between production of H_2 and other by-products (lactate, butyrate and ethanol). Finally, the

metabolic fluxes were redirected from the lactate formation pathway to the butyrate formation pathway by the addition of NaAc during fermentation using the *aad*-deficient strain M6, leading to an increase in hydrogen yield by 20% and butyrate yield by 65% compared to those of the wild type strain W5.

2. Recommendations

2.1. Developing biomass constituting equations for C. butyricum

In the absence of literature data, the biomass constituting equation of *C. butyricum* in this study was derived from the *C. acetobutylicum* model. The *in silico* central metabolic flux model containing this biomass constituting equation appears to be unsuitable for the prediction of metabolic flux distribution during fermentative hydrogen production using the mutants generated in this study (data not shown). This may be due to solvent-induced biomolecular changes occurring in the mutants. Thus further experiments need to be performed to obtain data on specific biomass composition for *C. butyricum*. The biomass constituting equation contains macromolecules (such as protein, DNA, RNA, lipids and cell wall), solutes, ions, cofactors, as well as ATP. The methods of biomass constituting equation development for an organism have been reviewed by Senger (2010).

2.2. Isolation and characterization of the potential bifurcating hydrogenase in C. butyricum

In order to confirm the existence of the potential bifurcating hydrogenase in *C. butyricum*, crude cell and membrane extracts can be prepared as described previously (Kim et al., 2007; Soboh et al., 2004) and assayed to determine hydrogenase activities, as well as the source of the reductant for H_2 production. The crude extract that displays synergistic uses of reduced ferredoxin and NADH can be further applied to a series of chromatography columns for purification of the enzyme (Soboh et al., 2004). The purified enzyme would then be characterized for its molecular weight, enzyme activity, and amino acid composition.

2.3. Further improving hydrogen yield and productivity

The genetic approach developed in this study has been proved as a promising biotechnological tool to restructure the metabolic pathways towards improving hydrogen yield. Further studies on process optimization to maximize the process efficiency and productivity are needed. Nitrogen sparging experiments showed that hydrogen production increased under low partial pressure of hydrogen, indicating that it is possible to further enhance yield and rate of hydrogen production by the *C. butyricum* wild type strain W5 and the mutant strain M6 via lowering partial pressure of hydrogen in the bioreactor. Several attempts to reduce H_2 partial pressure have been made by gas sparging (Mizuno et al., 2000), lowering the operating pressure of the reactor (Mandal et al., 2006), steam stripping of H_2 from the reactor (Van Groenestijn et al., 2002), or employing other membrane-based processes (Liang et al., 2002; Nielsen et al., 2001; Teplyakov et al., 2002). In addition, substantial

enhancement of hydrogen production can be expected through optimization of bioreactor design (Nath and Das, 2004).

2.4. Evaluating industry wastes as carbon sources for H_2 production

One of the major factors that hinder the commercial application of H_2 production is the high production cost associated with substrates. The use of industry wastes as substrates for H_2 production has brought the FHP process to the focus. *C. butyricum* W5 has been shown to produce H_2 from glucose, starch and molasses. It would be attention-grabbing to evaluate industry wastes as carbon sources for H_2 production using *C. butyricum* wild type and its mutant strains. Waste materials include starch containing wastes, cellulose containing wastes, food industry wastes and wastewater, and waste sludge from wastewater treatment plants (Kapdan and Kargi, 2006). The biological hydrogen production from industry wastes can be served as an environmental friendly waste treatment process.

Contamination poses another major problem in the pure-culture hydrogen fermentation with non-sterile wastes as substrates. Due to economic concern, mixed culture hydrogen fermentation processes are more applicable. Since *Clostridium* sp have been found to be dominant in most H_2-producing mixed cultures, the metabolic network model constructed in this study can serve a basis for developing a mixed culture metabolic network model, which can be further used to predict the effect of various environmental conditions on hydrogen yields (Chaganti et al., 2011).

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