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

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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.

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Signed *Date*

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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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