Metabolic engineering of cellulolytic \textit{Clostridium cellulovorans} for biofuel production directly from cellulosic biomass

Dissertation

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By

Xiaorui Yang, B.S.

Graduate Program in Molecular Genetics

The Ohio State University

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Dissertation Committee:

Shang-Tian Yang, advisor
Ana Alonso
Erich Grotewold
Jeffrey Chalmers
Abstract

With worldwide energy crisis, biofuel has been proposed as a renewable, sustainable and environmentally-friendly fuel to replace petroleum fuel or be used as fuel additive. Currently, ethanol fuel from corn takes the major market share of biofuel. With many advantageous properties, n-butanol is considered to be a more advanced fuel than ethanol. To reduce the feedstock cost as well as avoid competing with human food, the second-generation biofuel is being developed to produce ethanol and butanol fuels from sustainable and low-cost lignocellulosic biomass, pretreated with chemical and biological hydrolysis processes prior to fermentation. Recently, consolidated bioprocessing (CBP) has been proposed to produce the third-generation biofuel directly from lignocellulosic biomass, combining cellulase production, cellulose hydrolysis, and fermentation into a single-step process. CBP can greatly simplify the process and reduce the cost.

*Clostridium cellulovorans*, a cellulolytic acid-producing anaerobe, was chosen to be the host of metabolic engineering for biofuel production directly from cellulosic biomass. To introduce butanol synthesis pathway into *C. cellulovorans*, its transformation method was developed at first. Understanding its restriction-modification (RM) systems, as well as establishment of methylation system were shown to be the key factors for successful transformation of *C. cellulovorans*. Particularly, two RM systems, *Cce743I* and *Cce743II* were determined with the details of their restriction specificities and
methylation specificities. An in vivo methylation system, expressing M. Cce743I and M. Cce743II in E. coli, was then established to protect plasmids from being degraded. With the proper methylated plasmids, an engineered strain of C. cellulovorans, producing n-butanol and ethanol directly from cellulosic biomass by expressing an aldehyde/alcohol dehydrogenase 2 (adhE2), was obtained. Basic fermentation studies with this engineered C. cellulovorans were then performed, including medium optimization, comparison of fermentation bioreactors, and comparison of wild-type and engineered C. cellulovorans. The results showed the fermentation performance and metabolic flux distributions were greatly affected by these factors. To further enhance biofuel production, methyl viologen, an artificial electron carrier was added into the fermentation broth to increase NADH availability, since n-butanol and ethanol production was usually limited by NADH availability. The result showed that biofuel production was increased sharply, and acids production was inhibited greatly by the addition of optimal concentration of methyl viologen from the beginning of fermentation with glucose, cellobiose, or cellulose as the carbon substrates.

In summary, C. cellulovorans was metabolically engineered to produce n-butanol and ethanol directly from cellulosic biomass, with the development of its transformation method for the first time. In addition, the engineered C. cellulovorans could produce 1.6 g/L n-butanol directly from cellulose, which is the highest, compared to other wild-type and engineered cellulolytic strains. This project provided a promising platform for the production of biofuel and other value-added products directly from lignocellulosic biomass.
Dedication

This document is dedicated to my family.
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Vita

June 1987………………………………………………………………..Born-Lanzhou, P.R. China
2009…………………………………………….B.S. Biotechnology, Zhejiang University, P. R. China
2009- 2014…………………Graduate Teaching/Research Associate, Department of
Molecular Genetics/ Chemical and Biomolecular Engineering, The Ohio State University

Publications


Fields of Study

Major Field: Molecular Genetics

Also studied in Chemical and Biomolecular Engineering
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Chapter 1: Introduction

Butanol is a four-carbon alcohol with a formula of C$_4$H$_9$OH. There are four isomeric structures for butanol, including n-butanol, isobutanol, 2-butanol, and tert-butanol. Among them, n-butanol and isobutanol were proposed as superior biofuel in addition to important solvent and intermediate for chemical synthesis (Dürre, 2007; Lee et al., 2008; Nigam and Singh, 2011). n-Butanol was once produced primarily by ABE (acetone-butanol-ethanol) fermentation, which was the second largest biotechnological process in the early 20$^{th}$ century. However, ABE fermentation was replaced by petrochemical methods, using oxo synthesis process, Reppe synthesis process, and crotonaldehyde hydrogenation process, due to the low titer of butanol and high cost of feedstock from mid 20$^{th}$ century (Dürre, 2007; Lee et al., 2008). However, with the decreased supply but increased demand of petroleum fuels, production of renewable, sustainable and environmental-friendly biofuels by ABE fermentation was re-examined.

With decades of research efforts, a considerable knowledge of physiology and pathways of microorganisms has lead tremendous progress on microbial biofuel production. Currently, bioethanol dominates the biofuel market, with most of the gasoline mixed with 10% ethanol in the U.S. However, compared to ethanol fuel, butanol fuel has many superior physical properties, making it a better choice of biofuel. Firstly, butanol
fuel has a much higher energy density than that of ethanol fuel, releasing more energy per gallon fuel. Secondly, the air/fuel ratio for butanol burning is closer to that for gasoline, compared to ethanol fuel. Thirdly, the heat of vaporization for butanol is much lower than that for ethanol, enhancing its safety property. Moreover, butanol can be blended in any concentration with gasoline without any modification of current vehicle engines, and can be transported in existing infrastructure (Lee et al., 208). Therefore, butanol, compared to ethanol, is considered to be a superior biofuel in many respects.

Biological production of n-butanol by ABE fermentation using Clostridia species has a long history as a significant industrial fermentation process. Clostridia are Gram-positive, rod-shaped, spore-forming, anaerobic bacteria. Solventogenic Clostridia used for traditional ABE fermentation included *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*. ABE fermentation by these solventogenic Clostridia usually undergoes two stages, acidogenic stage and solventogenic stage. During acidogenic stage, acids, such as acetic acid and butyric acid, in addition to H₂ and CO₂, are produced. During solventogenic stage, acids are reassimilated to produce solvents, including acetone, ethanol, and butanol. In recent years, hyper-butanol production strains were isolated or developed by continuous culture, evolutionary engineering and metabolic engineering. However, ABE fermentation by these solventogenic Clostridia still suffered from several drawbacks, impacting economics of butanol production. One of the major factors is substrate cost. Butanol production by those solventogenic Clostridia relies on the utilization of conventional carbon substrates, such as molasses, whey permeate, and corn (Jones and Woods, 1986;
Ezeji and Blaschek, 2008; Lee et al., 2008). It was pointed out that the feedstock accounted for up to 79% of the overall cost of ABE fermentation (Pfromm et al., 2010). With the increased prices of the conventional feedstocks, ABE fermentation cannot compete well with petrochemical routes for butanol production. Therefore, the utilization of low-cost and environmentally friendly feedstock becomes important.

Lignocellulosic biomass is the most abundant biomass on earth. Bioconversion of renewable lignocellulosic biomass to biofuel not only can lower the feedstock cost, but also avoid the concerns of potential competition with human food. Lignocellulosic biomass could be obtained from agricultural residues and forestry residues, consisting of 40-50% cellulose, 25-30% hemicellulose and 15-20% lignin and other components (Knauf and Moniruzzaman, 2004; Somerville et al., 2010; Menon and Rao, 2012). Due to the structure of lignocellulosic biomass and crystalline structure of cellulose, bioconversion of lignocellulosic biomass to second-generation biofuel, particularly ethanol, involved pretreatment, cellulases and hemicellulases production, fermentation of hexoses and pentoses, and downstream processing (Sun and Cheng, 2002; Margeot et al., 2009; Menon and Rao, 2012). Within these steps, cellulase production is expensive, making lignocellulosic ethanol less economic (Lynd et al., 2008).

While efforts to improve the pretreatment of lignocellulosic biomass are continuously being made, the idea of consolidated bioprocessing (CBP) has been developed, considered as an ideal approach for the production of third-generation biofuel, that generates biofuel directly from cellulosic substrates, by combining cellulases production, cellulose hydrolysis, and biofuel production via fermentation in a single step.
(Olson et al., 2012). In this way, the processes of biofuel production can be greatly simplified and the cost of fermentation can be greatly reduced. Efforts were made to use native cellulolytic microbes to produce ethanol (Guedon et al., 2002; Argyros et al., 2011; Jin et al., 2011; Tolonen et al., 2011) and butanol (Berezina et al., 2008; Virunanon et al., 2008; Berezina et al., 2009). However, no effective native cellulolytic biofuel-producer was found, combining both fast growth on cellulosic substrates and substantial production of biofuel. To achieve CBP by combining both cellulolytic and solventogenic properties, studies of co-fermentation of cellulolytic microbes with solventogenic microbes were done (Nakayama et al., 2011; Minty et al., 2013; Zuroff et al., 2013). Though significant progress of co-fermentation was made during recent years, still a lot of issues need to be addressed, including different growth condition requirements (e.g., pH, temperature, media composition, aerobic/anaerobic conditions), the balance (competition vs. cooperation) between two species, extra chemical (e.g. butyric acid) addition to induce biofuel production for solventogenic bacteria, extra cellulase (e.g., endoglucanase) addition for cellulosic biomass degradation, extra sugars (e.g. glucose) addition for the growth of solventogenic bacteria, extra growth stage (e.g., sequential co-culture). Overall, CBP by co-fermentation was still problematic, and particularly difficult for scale-up for industrial application.

Therefore, metabolic engineering of microorganisms becomes an alternative and more effective method, either by incorporating cellulases into biofuel producers, or by incorporating biofuel synthesis pathways into cellulolytic organisms, or by the combination of both. With respect to the first strategy, cellulases or minicellulosomes
were expressed in *Saccharomyces cerevisiae* for producing ethanol from cellulosic biomass (Wen et al., 2009; Tsai et al., 2010; Yanase et al., 2010; Yamada et al., 2011), and in *C. acetobutylicum* (Kim et al., 1994; Sabathé and Soucaille, 2003; Perret et al., 2004; Mingardon et al., 2005). However, the biotechnology of heterologous expression and assembly of cellulosomes with efficient enzyme activity was still immature, due to the highly organized and complex structure of native cellulosomes. The results showed that either a very low amount of ethanol was produced from engineered yeast, or no true cellulose hydrolysis ability was obtained by *C. acetobutylicum* for butanol production.

To express and assemble minicellulosome heterologously, many factors need to be considered, including enzyme stability, enzyme activity, expression ratios, enzyme interactions, synergetic effect, surface display. On the contrary, the introduction of biofuel pathway into cellulolytic microbes is an alternative option. It was reported that isobutanol synthesis pathway was incorporated into cellulolytic *C. cellulolyticum*, the strain now capable of producing 0.66 g/L isobutanol from cellulose (Higashide et al., 2011). Considering the developed genetic tools of *E. coli*, incorporation of both cellulases and biofuel synthesis pathway into *E. coli* provided another option. Engineered *E. coli* with cellulolytic and hemicellulolytic activities could produce very low amount of biofuel (less than 0.1 g/L fatty acid ethyl esters (biodiesel), 0.028 g/L butanol, or 0.0017 g/L pinene (a precursor to a potential jet fuel)) from pretreated switchgrass (Bokinsky et al., 2011). Overall, the production of third-generation biofuel by CBP is still in its infancy, although significant progress has been made to understand the fundamentals of microbial cellulose utilization and various strategies of CBP have been explored.
1.1 Project goal and specific tasks

The overall project goal is to develop a cellulolytic strain capable of producing high level of biofuel directly from lignocellulosic biomass, by genetically engineering cellulolytic *C. cellulovorans* for n-butanol and ethanol production, followed by fermentation, and enhanced biofuel production by metabolic process engineering (shown in Figure 1.1).

Task 1: Genetical engineering of the cellulolytic *C. cellulovorans* for biofuel production directly from cellulose

Acid-producing cellulolytic *C. cellulovorans* was chosen to be the engineering host to express n-butanol and ethanol synthesis genes, because of its effective utilization of cellulose with cellulosomes. Since transformation of *C. cellulovorans* was particularly difficult, due to under-developed genetic tools for Clostridia and unreported transformation method of *C. cellulovorans*, initial transformations based on conventional electroporation and conjugation methods for Clostridia were unsuccessful. Therefore, restriction-modification system of *C. cellulovorans* was investigated, followed by the establishment of an *in vivo* methylation system, using native methyltransferases to protect plasmids from being degraded. With this, a transformation method of *C. cellulovorans* was established for the first time. And a biofuel synthesis pathway was successfully introduced into *C. cellulovorans*, generating a new strain of *C. cellulovorans*, expressing *adhE2* gene, capable of producing n-butanol and ethanol directly from cellulose. The development of the transformation method of *C. cellulovorans* and generation of the
engineered strain producing n-butanol and ethanol directly from cellulose are presented in Chapter 3.

**Task 2. Fermentation of the engineered *C. cellulovorans***

The fermentation kinetics of the engineered *C. cellulovorans* were analyzed in details in Chapter 4. Specifically, media optimization, analysis of fermentation kinetics using various nitrogen and carbon sources, analysis of the effects of different fermentation bioreactors, and comparison of fermentation patterns between wild-type and engineered *C. cellulovorans* were performed to understand metabolic flux and fermentation properties of engineered *C. cellulovorans*.

**Task 3. Metabolic process engineering for enhanced biofuel production by the engineered *C. cellulovorans***

To further enhance n-butanol and ethanol production by the engineered *C. cellulovorans*, metabolic process engineering was performed without genetic modification. The effects of adding an artificial electron carrier (methyl viologen) on alcohol and acid production during fermentation of the engineered *C. cellulovorans* were studied. It was shown that the addition of methyl viologen significantly enhanced n-butanol and ethanol production and inhibited acids production, with increasing NADH availability. Particularly, the optimal timing for adding methyl viologen and the optimal concentrations of methyl viologen using various carbon substrates were determined. The results are presented in Chapter 5.
1.2 Significance and major impacts

n-Butanol is an important chemical intermediates, with estimated annual production of 10-12 billion pounds (Donaldson et al., 2007), and an expansion of 3% per year (Kirschner, 2006). It could be used for the synthesis of butyl acrylate and methacrylate esters for latex surface coating, enamels, and lacquers, or for the synthesis of butyl glycol ether, butyl acetate and plasticizers. It could also be used as solvent for antibiotics, vitamins, and hormones. Besides for chemical synthesis, it was also proposed as an ideal choice of a replacement of gasoline, or fuel additive (Lee et al., 2008). The Energy and Independence and Security Act (EISA) passed by the US Congress and the Renewable Fuel Standards (RFS) published mandated at least 36 billion gallons of biofuel to be blended into transportation fuel by 2022. Among the 36 billion gallons, 15 billion gallons were proposed to be conventional biofuel (e.g., corn starch ethanol), and 16 billion gallons were required to be cellulosic biofuels from cellulosic feedstocks. Currently, ethanol fuel is produced with the largest market share, as a fuel additive for gasoline. However, since butanol fuel has more advanced properties, growing interest has been raised on developing of butanol fuel by the government and multiple companies (e.g., BP, DuPont, Cobalt, Gevo, Green Biologics, and Chevron Oronite). The current cellulosic biofuel, so-called second-generation biofuel, is produced by the conversion of lignocellulosic biomass to sugars by chemical and biological pretreatments, followed by conventional fermentation. Because of the high cost of the pretreatment in addition to complicated process, the third-generation biofuel directly from lignocellulosic biomass by CBP is proposed. However, the development of biotechnology for CBP is still in
infancy, and continuous efforts are required. This project aimed to develop a strain, which could produce high level of biofuel directly from lignocellulosic biomass by metabolic engineering. In this project, for the first time, a cellulolytic microbe was engineered to produce n-butanol and ethanol directly from cellulose. Though the biofuel production is still far from commercial applications, it provides a promising platform for further enhanced production of biofuel or other value added products directly from cellulosic biomass.

1.3 References


Figure 1.1 Overview of project goal and major tasks
Chapter 2: A review: current progress in CBP for biofuel production

Abstract

Due to the worldwide energy crisis, biofuel production from cellulosic biomass has caught people’s attentions. To integrate the process of cellulosic biomass hydrolysis and fermentation into a single-step process, consolidated bioprocessing (CBP) has been proposed for biofuel production directly from cellulosic biomass. Since there is no effective cellulolytic biofuel-producers in nature, efforts of metabolic engineering and co-fermentation to produce cellulosic biofuel were made in recent years. To metabolically engineer the CBP microbes, either biofuel synthesis pathway can be incorporated into cellulolytic microbes, or cellulases or minicellulosomes can be expressed in biofuel producers. During this process, the availability of the genetic tools for the hosts is the key to success, particularly the gene transfer technology. Since many of the CBP hosts are non-type microbes, lacking developed genetic tools, gene transfer is often the bottleneck. On the other hand, instead of genetic modification of the microbes, co-fermentation is another technology to achieve CBP, by culturing solventogenic microbes and cellulolytic microbes together. However, there is still many problems to be overcome, mainly due to the complicated biological interactions between different strains. Overall, in this review, we will mainly focus on the current advancement of metabolic engineering and co-fermentation technologies for biofuel production via CBP, which may provide a guide for the future.
2.1 Introduction

With limited supply of petroleum fuels, there is a growing interest of producing sustainable biofuels from renewable biomass. Butanol, a four-carbon alcohol, is considered as a superior biofuel. Compared to ethanol fuel, which has been widely used as fuel additive currently, butanol has a higher energy density, and similar properties as gasoline, including energy density, air-fuel-ratio, heat of vaporization, and octane number as shown in Table 2.1 (Lee et al., 2008). Energy density evaluates the amount of energy stored in certain volume of fuel. Air-fuel ratio refers to the ratio of air to fuel for complete burn of the fuel. Heat of vaporization refers to the amount of heat to be absorbed to vaporize certain quantity of fuel. Octane number measures the performance of the fuel. The higher it is, the more compression it can withstand. In addition to these fuel properties, butanol can be transported via existing fuel infrastructure, and can be blended with gasoline in higher concentration in vehicle engines. Therefore, nowadays there is more attention of producing butanol as biofuel. Currently, butanol is still produced via petrochemical methods due to its relatively low cost, compared to microbial fermentation. However, biobutanol production from renewable biomass via fermentation was revisited in recent years.

The first-generation biofuels were mainly produced from food crops, such as corn and sugar cane. However, such feedstocks for biofuel production are not only expensive, but can also compete with human food, raising many of the ethics issues. Therefore, the focus has turned to producing the second-generation biofuels from cellulosic biomass, which is the most abundant biomass on earth. By utilizing cellulosic biomass as the
feedstocks, the cost of feedstock can decrease greatly, while avoiding the issues of competition with human food. However, due to the crystalline structure of cellulose, cellulosic biomass is hardly to be utilized directly by most of the microbes. Developing technologies that could convert cellulosic biomass into biofuels is quite challenging.

Current technology involves chemical pretreatment, enzymatic hydrolysis, prior to microbial fermentation. Among many of the steps, enzymatic hydrolysis is quite expensive, because of the high cost of enzymes (Carroll and Somerville, 2009). Therefore, CBP becomes attractive (Olson et al., 2012), in which the enzymatic hydrolysis and fermentation are combined into a single process step to greatly simplify the process and reduce the cost. To achieve CBP, a microbe must be able to utilize cellulosic biomass directly and to produce biofuel at high titer, yield and productivity. Unfortunately, such microbes were not found in nature. Therefore, metabolic engineering becomes a powerful tool to modify the genetic pathways of the potential hosts. There are two main strategies. One is to incorporate biofuel synthesis pathway into cellulolytic microbes. The other is to introduce cellulases into biofuel-producers. However, genetic manipulations of many of the microbes are the bottleneck during metabolic engineering, due to the under developed genetic tools and the lack of the understanding the genetics of the microbes. Therefore, developing efficient genetic tools for those potential hosts is one of the keys to generate a microbe with both of the properties of degrading cellulosic biomass and producing substantial biofuels. In addition to metabolic engineering, co-fermentation is an alternative strategy to achieve CBP without genetic modification of
microbes by co-culturing cellulolytic microbes with biofuel-producers. Here we provide an updated overview of advances of CBP.

2.2 Cellulolytic microbes

Cellulolytic microbes including bacteria and fungi were isolated from various habitats, such as soil, compost, sludge, rotten grass, and rotten wood. These microbes can degrade cellulosic biomass by encoding either non-complexed cellulases or complexed cellulases. Aerobic bacteria and fungi often encode non-complexed cellulases, while anaerobic bacteria often encode complexed cellulases, which are called cellulosomes. Several representative cellulolytic bacteria and fungi with genome sequenced (Schwarz et al., 2001; Tamaru et al., 2010; Olson et al., 2012) are listed in Table 2.2. Among many of these cellulolytic microbes, aerobic fungi T. reesei, and anaerobic bacteria C. cellulolyticum, C. cellulovorans and C. thermocellum are notable for their ability to utilize cellulose efficiently, and thus are most studied. Therefore, these microbes have great potential to be used for industrial application, involving degradation of cellulosic biomass.

Cellulosomes are multi-enzyme complexes displayed on the cell surface that can degrade cellulose efficiently. Cellulosomes of C. cellulovorans, C. cellulolyticum and C. thermocellum are most studied (Gal. et al., 1997; Fierobe et al., 1999; Gold et al., 2007; Tamuru et al., 2010 and 2011). Current model of cellulosome proposed that cellulosomes are composed of scaffolding proteins and catalytic proteins. Scaffolding proteins are anchored on the cell surface, containing cellulose-binding domains and cohesion
domains. Catalytic proteins contain catalytic domains and dockerin domains. Scaffolding proteins and catalytic proteins can interact with each other via dockerin-cohesion interactions (Bayer et al., 2004). One scaffolding protein may have multiple cohesion domains, and thus can interact with multiple catalytic proteins. Genome analysis of cellulosomal genes of *C. cellulovorans*, *C. cellulolyticum* and *C. thermocellum* revealed that they encoded 57, 65, and 84 cellulosomal genes, respectively (Tamuru et al., 2010 and 2011). Therefore, cell display of cellulosomes can be very complicated. And the case can be even more complicated, considering the induced expression of different cellulosome proteins by different cellulosic carbohydrates. Therefore, mimicking the cellulosomes by heterologous overexpression in target hosts is nearly impossible. Only minicellulosomes containing 2-4 subunits have been heterologously overexpressed and studied, as discussed in cellulase engineering in biofuel-producers below.

It is also interesting that a large cellulosomal gene cluster has been found in *C. acetobutylicum*, which is a well-known solventogenic bacterium producing acetone, ethanol and butanol. However, *C. acetobutylicum* cannot grow on cellulosic substrate, suggesting the absence of functional cellulosome, though several cellulosomal proteins were detected (Sabathé et al., 2002). The expression, function and evolution of the cellulosomal genes are not clear. Nevertheless, CipA and CipC, scaffolding proteins in *C. acetobutylicum* were often cloned and overexpressed for minicellulosome assembly (Sabathé and Soucaille, 2003; Mingardon et al., 2005; Chanal et al., 2011). In addition, one strain of *C. acetobutylicum* capable of growing on grass was isolated (Berezina et al.,
Therefore, *C. acetobutylicum* is still a promising host for butanol fuel production via CBP.

### 2.3 Cellulolytic biofuel-producers

Among those cellulolytic microbes, the ones capable of producing either ethanol or butanol are summarized in Table 2.3. *C. cellulolyticum* could produce about 0.5 g/L ethanol from cellulose (Petitdemange et al., 1984; Guedon et al., 2002). *C. thermocellum* could produce 1.4 g/L ethanol from cellulose (Argyros et al., 2011). And *C. phytofermentans* could produce about 2.8 g/L ethanol from AFEX-treated corn stover (Warnick et al., 2002; Jin et al., 2012; Tolonen et al., 2011). In addition to the strains capable of producing ethanol from cellulosic biomass, four other Clostridia species capable of producing butanol were isolated. However, they could only produce less than 0.04 g/L butanol (Virunanon et al., 2008). Interestingly, a strain of *C. acetobutylicum* isolated was found to be able producing 0.6 g/L butanol from grass (Berezina et al., 2008). As discussed above, though *C. acetobutylicum* encodes a large cellulosomal gene cluster, most of their strains could not grow on cellulose. Allover, though several wild-type cellulolytic microbes capable of producing ethanol or butanol were isolated, the biofuel titers were still too low to be applied in industry. Further efforts are needed to engineer these strains to boost their biofuel production from cellulose.
2.4 Cellulase engineering in biofuel-producers

Though cellulose is the most abundant biomass on earth, it is rather difficult to be used as carbon source due to its crystalline structure. No single enzyme could hydrolyze cellulose. Multiple enzymes are required for cellulose hydrolysis, including endoglucanase, exoglucanase, and β-glucosidase. Endoglucanases randomly cleave internal bonds at the amorphous sites of cellulose chain (Schwarz 2001; Teeri, 1997). Exoglucanases cleaves from the end, generating cellobiose. β-glucosidases cleave the cellobiose into glucose. Anaerobic cellulolytic bacteria are known to hydrolyze cellulose with cellulosomes, such as *C. thermocellum*, *C. cellulosorans*, and *C. cellulolyticum*. And aerobic cellulolytic fungi are known to hydrolyze cellulose with secreted enzymes, such as *T. reesei*. Therefore, they are often used as the sources for cellulases cloning and heterologous expression in yeast or solventogenic Clostridia for biofuel production directly cellulosic biomass.

Several studies have successfully incorporated cellulases/minicellulosomes in *S. cerevisiae* for ethanol production directly from cellulosic biomass (shown in Table 2.4). For example, an endoglucanase from *T. reesei* and the β-glucosidase from *Saccharomyces fibuligera* were co-expressed in *S. cerevisiae*, leading to 1.0 g/L ethanol produced from phosphoric acid-swollen cellulose (PASC) (Den Haan et al., 2007). Compared to expression of free cellulases, surface display of functional cellulases or minicellulosomes in yeast was widely studied. For example, codisplay of three types of cellulolytic enzymes, including *T. reesei* endoglucanase II, exoglucanase II and *Aspergillus aculeatus* β-glucosidase I on yeast cell surface enabled the engineered yeast
to produce 3 g/L or 2.1 g/L ethanol directly from PASC (Fujita et al., 2004; Yanase et al., 2010). Interestingly, generation of a diploid yeast strain, expressing endoglucanase, exoglucanase and β-glucosidase could greatly enhance ethanol production, producing 7.5 g/L ethanol from PASC or pretreated rice straw (Yamada et al., 2011). In addition to co-expression of several cellulases in one yeast strain, co-fermentation of yeast strains with individually expressing cellulases was also explored. For example, *T. aurantiacus* EGI, *T. reesei* exoglucanase CBHII and *Aspergillus aculeatus* β-glucosidase BGLI was expressed individually in yeast cells. And the three types of yeast cells were co-cultured for ethanol production from PASC, producing 2.1 g/L ethanol with the optimized EGI:CBHII:BGLI ratio of 6:2:1 (BaeK et al., 2012). With the increasing understanding of Clostridia cellulosomes, assembly of functional minicellulosomes on yeast cell surface was also investigated.

By mimicking the structure of Clostridia cellulosomes, surface assembly of a trifunctional minicellulosome on the cell surface of yeast was reported, by expressing a miniscaffolding protein, and three types of cellulases, including endoglucanase, exoglucanase, and β-glucosidase. The engineered yeast was reported to produce 1.8 g/L ethanol from PASC (Wen et al., 2010). Since subunits of native cellulosomes are assembled in a highly organized manner with the strict control of their expression ratios, efforts were also made assemble the functional minicellulosome by controlling the cellulase subunit ratios. For example, it was reported a functional minicellulosome was assembled, by growing a synthetic yeast consortium with four different engineered yeast strains. The yeast strains either displayed a trifunctional scaffolding protein (SC) with
three cohesion domains from *C. thermocellum, C. cellulyticum*, and *Ruminococcus flavefacience*, or one of the secreted cellulases (endoglucanase (EG), exoglucanase (CBH), β-glucosidase (BGL)) with dockerin domains. The three types of secreted cellulases could specifically interact with the three cohesion domains of the scaffolding protein via cohesion-dockerin interactions to form a highly organized minicellulosome on the yeast surface. By manipulating the population ratio of the yeast strains, 1.87 g/L ethanol was produced with a SC:EG:CBH:BGL ratio of 7:2:2:2 (Tsai et al., 2010). In a word, progress was made to incorporate cellulases/minicellulosomes into yeast for ethanol production from cellulosic biomass, though the ethanol titers from cellulosic biomass so far were still unsatisfactory.

In spite of incorporation of cellulases/minicellulosomes into yeast for ethanol production, display of functional minicellulosomes into *C. acetobutylicum* for n-butanol production from cellulosic biomass was also studied (shown in Table 2.5). *C. acetobutylicum* was proposed to be an attractive host for consolidated bioprocessing. It was not only because *C. acetobutylicum* was conventionally used for ABE (acetone-butanol-ethanol) fermentation with high butanol production level (Lütke-Eversloh and Bahl, 2011), but also a large cellulosomal gene cluster was found in the genome of *C. acetobutylicum*, though it is unable to grow on cellulosic substrates (Sabathé et al., 2002). An endoglucanase *engB* from *C. cellulovorans* was expressed in *C. acetobutyricum* heterologously, with more than 4-fold activity towards carboxymethyl cellulose (CMC) (Kim et al., 1994). In addition, several studies of secretion of heterologously expressed cellulases from *C. cellulyticum* in *C. acetobutylicum* were made. It showed that
cellulases Cel5A, Cel8C, Cel9M could be secreted in active form, losing their C terminal dockerin domains, while secretion of Cel48F, Cel9E and Cel9G were unsuccessful, probably due to lack of specific chaperones (Mingardon et al., 2011). Similarly, heterologously expressed and secreted mannanase Man5K was also shown to be unstable, losing its N-terminal dockerin domain (Mingardon et al., 2005). However, the unstable or deleterious secretion of heterologous cellulases could be rescued by grafting a scaffoldin domain to the cellulases in some cases (Mingardon et al., 2005; Chanal et al., 2011; Fierobe et al., 2012). Furthermore, heterologous expression of a chimeric miniscaffolding, containing one truncated scaffolding from C. cellulolyticum, and one from C. thermocellum, which could interacted with dockerin domains from C. cellulolyticum and C. thermocellum (Perret et al., 2004). In addition, in vivo formation of a homologous minicellulosome CipA, containing a cellulose-binding domain, and two cohesion domains, in C. acetobutylicum was demonstrated (Sabathé and Soucaille, 2003). Besides C. acetobutylicum, C. beijerinckii, another conventional solventogenic bacteria used for ABE fermentation, was engineered to overexpress cellulase CelA or CelD from the fungus Neocallimastix patriciarum. The recombinant strains showed clear halos on agar plates containing CMC upon staining with Congo red, but could not grow on CMC or crystalline cellulose (López-Contreras et al., 2001). In brief, though various studies were performed to express cellulas/minicellulosomes into solventogenic Clostridia for butanol production from cellulosic biomass, few active cellulase was expressed, and no cellulase or minicellulosome was displayed on cell surface. More importantly, no true cellulose activity was observed in the engineered strains. It seemed
that heterologous expression and cell surface display of cellulases/minicellulosomes in solventogenic Clostridia were much more difficult, compared to that in yeasts.

All in all, these examples suggested that heterologous expression and assembly of cellulosomes in yeast and solventogenic Clostridia with efficient enzyme activity were difficult, due to the highly organized and complex structure of native cellulosomes. To express and assemble cellulosome heterologously, many factors need to be considered, including enzyme stability, enzyme activity, expression ratios, enzyme interactions, synergetic effect, surface display.

2.5 Incorporation of biofuel pathways into cellulytic microbes

Instead of overexpression of cellulases or minicellulosomes in solventogenic microbes, incorporation of biofuel pathways into cellulytic microbes is another strategy to engineer CBP microbes. For example, an isobutanol synthesis pathway was introduced into *C. cellulolyticum*, which could then produce 0.66 g/L isobutanol from cellulose (Higashide et al., 2011). In our study, n-butanol synthesis pathway was overexpressed in *C. cellulosorans*, generating a recombinant strain that could produce 1.6 g/L n-butanol directly from cellulose. Though there are only a few such studies, with the progress of isolation of more cellulytic microbes and development of their generic tools, it is believed that more cellulytic microbes can be engineered to produce biofuel in future.
2.6 Genetic manipulation for strain development

Genetic manipulations, such as plasmid overexpression, gene knock-out, gene knock-in, gene silencing, always involve gene transfer technology into microbes for biofuel production. However, genetic manipulation in non-type or non-developed strains could be very difficult, often as the bottleneck during metabolic engineering. This could happen to many strains used as the hosts for biofuel production. Difficult gene transfer into these non-type microbes is mainly due to two factors. One is the firm cell wall structure, particularly for the gram-positive strains. Cell wall weakening treatment may overcome this problem (Tyurin et al., 2004; Pyne et al., 2013). The other is the restriction-modification system of the host, restricting the incoming foreign DNAs by recognizing the foreign methylation patterns. To overcome this problem, heat-shock treatment (Lin and Blaschek, 1984), deletion of restriction sites in DNAs (Lee et al., 1992; Purdy et al., 2002), knock-out of the restriction enzymes (Cui et al., 2012; Dong et al., 2010), or proper methylation of the DNAs (Mermelstein et al., 1993; Jenert et al., 2000; Davis et al., 2000; Purdy et al., 2002; Lin et al., 2010; Zhang et al., 2012; Pyne et al., 2013) were investigated.

Among these methods, cell-wall weakening and heat shock method do not require the information of the restriction enzyme information, but they are very limited, only effective for certain strains. And all the other three methods all require the information of restriction enzymes and even the methyltransferases. By knowing the target sites of the restriction enzymes, the target sites in the DNAs used for transfer could be deleted by spontaneous mutations. However, this method does not apply to all, if the DNA contains
too many target sites. On the other hand, knock-out the restriction enzymes could avoid the trouble of modification of the DNAs used for transfer, but the prerequisite is that the DNA transfer method is available to transfer the knock-out plasmid, even if the efficiency is low. Therefore, compared to these methods, development of methylation methods seems to be more universal. To methylate plasmids, first of all, the restriction activities could be analyzed by restricting the plasmid with the cell lysate. Secondly, the putative restriction enzymes could be analyzed by REBASE (http://rebase.neb.com/rebase), if the genome is sequenced. Thirdly, the restriction target sites are usually the same as the methylation sites in their genome. Therefore, their genome methylation pattern could provide complementary information for the restriction sites. Then according to the information of restriction sites, proper methyltransferases could be chosen for methylation. They could be commercial ones recognizing partial or full target sequences, or the native ones, usually encoded in the same operon as the restriction enzymes. With proper DNA methylation, gene transfer efficiency could increase significantly.

Overall, restriction-modification systems are very common defensive machineries in bacteria. However, the transformation method and efficiency may be strain dependent. Therefore, understanding each of their methylation-restriction systems is important. In addition, development of shuttle plasmids, including plasmid replicons, promoters, and antibiotic resistant genes is also very important for gene transfer technology. All in all, continuous development of genetic tools will contribute to the key process of strain development.
2.7 Co-fermentation

Recently, co-fermentation technology was widely applied to produce biofuel directly from cellulosic biomass, by growing solventogenic and cellulolytic organisms together (summarized in Table 2.6). Particularly, Clostridia co-fermentations were widely studied. For example, solventogenic *C. acetobutylicum* was co-cultured with cellulolytic *C. cellulolyticum* or *C. thermocellum* for fermentation directly from cellulose. However, butyric acid was produced as the major product. No or a little butanol (less than 1 g/L) was produced by *C. acetobutylicum*, and only about 1.0 g/L ethanol was produced by *C. cellulolyticum* and *C. thermocellum* in the co-fermentation system (Petitdemange et al., 1983; Yu et al., 1985; Nakayama et al., 2011). In addition, co-fermentation of *C. thermocellum* and *C. beijerinckii* could produce about 1 g/L ethanol and 2 g/L butanol (Nakayama et al., 2011). It was proposed that only when a critical level of butyric acid was reached, solventogenic phase occurred in solventogenic Clostridia. Therefore, butyric acid was added into the co-fermentation system of *C. acetobutylicum* and *C. thermocellum*, resulting in increased butanol production (2.5 g/L) from cellulose (Yu et al., 1985). To avoid the problem of solventogenic induction by extra addition of butyric acid, *C. saccharoperbutylicum* N1-4 was chosen to replace *C. acetobutylicum* or *C. beijerinckii* for co-fermentation, which is also a butanol hyperproducer with a different solventogenic induction mechanism. Co-fermentation of *C. saccharoperbutylicum* N1-4 with *C. thermocellum* produced much higher butanol (7.9 g/L) from cellulose without the addition of butyric acid (Nakayama et al., 2011). Besides the *Clostridia* co-fermentations for butanol production, an evolutionarily
engineered *C. thermocellum* strain with *hpt, ldh*, and *pta* gene deletions was co-cultured with *Thermoanaerobacterium saccharolyticum* ALK2 strain, which produced 38 g/L ethanol (Argyros et al., 2011).

In addition to bacterial co-fermentations, fungal-bacterial co-fermentations for biofuel production directly from cellulosic biomass were also investigated. For example, co-fermentation of cellulolytic *C. phytofermentans* and *S. cerevisiae cdt-1* could produce ~6 g/L ethanol. However, this result was identical to the result of *C. phytofermentans* monoculture, due to the limited hydrolysis productivity. Therefore, extra endoglucanase from *Trichoderma viride* (400 mg/L) was added, which caused dramatic increased ethanol production (22 g/L) (Zuroff et al., 2013). In addition, co-fermentation of the cellulolytic fungus *T. reesei* and the engineered *E. coli* strain produced 1.88 g/L isobutanol from pretreated corn stover (Minty et al., 2013).

Though significant progress of co-fermentation has been made over the years, there are still a lot of problems that impede further development of this technology and its industrial application, involving many complicated the biological interactions between different strains. For example, different growth condition requirements (e.g., pH, temperature, media compositions, and aerobic/anaerobic condition), the balance between two species (cooperation vs. competition), extra chemical addition (e.g., butyric acid) to induce solventogenic phase, extra cellulase addition for cellulosic biomass degradation, extra sugars addition for bacteria growth, extra growth stage. Overall, there is still a long way to go and apply co-fermentation for industrial production of biofuel directly from cellulosic biomass.
2.8 Conclusion

Production of cellulosic biofuel via CBP for industrial application requires progress across many disciplines. Though it is still in its infancy, tremendous progress has been made, including isolation of potential cellulolytic biofuel-producing hosts, metabolic engineering of the hosts, development of their genetic tools and co-fermentation. Long with the progress in these areas, there are fascinating opportunities to produce substantial biofuel from cellulosic biomass directly.

2.9 References


with optimized cellulase expression. Biotechnol Biofuels. 4:8.


<table>
<thead>
<tr>
<th>Fuel property</th>
<th>Gasoline</th>
<th>Butanol</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy density (MJ/L)</td>
<td>32</td>
<td>29.2</td>
<td>19.6</td>
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<tr>
<td>Air-fuel ratio</td>
<td>14.6</td>
<td>11.2</td>
<td>9</td>
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<tr>
<td>Heat of vaporization (MJ/kg)</td>
<td>0.36</td>
<td>0.43</td>
<td>0.92</td>
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<tr>
<td>Octane number</td>
<td>86-94</td>
<td>87</td>
<td>116</td>
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</table>

Table 2.1 Fuel properties of gasoline, butanol and ethanol
<table>
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<tr>
<th>Organisms</th>
<th>Anaerobic/aerobic</th>
<th>Mesophilic/thermophilic</th>
<th>Isolated source</th>
<th>Cellulases</th>
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<tr>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cytophaga hutchinsonii</td>
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<td>Non-complexed</td>
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<td>Non-complexed</td>
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</tbody>
</table>

Table 2.2. Representative species of sequenced cellulolytic bacteria and fungi.
<table>
<thead>
<tr>
<th>Organisms</th>
<th>Carbon source</th>
<th>Ethanol /butanol (g/L)</th>
<th>Isolation source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. cellulolyticum</em></td>
<td>cellulose</td>
<td>0.5 (ethanol)</td>
<td>decayed grass</td>
<td>Petitdemange et al., 1984;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Guedon et al., 2002</td>
</tr>
<tr>
<td><em>C. thermocellum</em></td>
<td>cellulose</td>
<td>1.4 (ethanol)</td>
<td></td>
<td>Argyros et al., 2011</td>
</tr>
<tr>
<td><em>C. phytofermentans</em></td>
<td>AFEX-treated corn stover</td>
<td>2.8 (ethanol)</td>
<td>Forest soil</td>
<td>Warnick et al., 2002; Jin et al.,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2012; Tolonen et al., 2011</td>
</tr>
<tr>
<td>4 <em>Clostridia</em> strains</td>
<td>cellulose</td>
<td>&lt;0.04 (butanol)</td>
<td>Cow dung, bagasses, decayed grass</td>
<td>Virunanon et al., 2008</td>
</tr>
<tr>
<td><em>C. acetobutylicum</em> 7</td>
<td>grass</td>
<td>0.6 (butanol)</td>
<td></td>
<td>Berezina et al., 2008</td>
</tr>
</tbody>
</table>

Table 2.3 Wild-type cellulolytic biofuel-producers
<table>
<thead>
<tr>
<th>Cellulase expression</th>
<th>Cellulase formation</th>
<th>Ethanol</th>
<th>Carbon source</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG (T. reesei)</td>
<td>Codisplay on cell surface</td>
<td>3.0 g/L</td>
<td>PASC</td>
<td>A cell surface display system based on α-agglutinin</td>
<td>Fujita et al., 2004</td>
</tr>
<tr>
<td>CBH (T. reesei)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BGL (A. aculeatus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EG (T. reesei)</td>
<td>Free enzymes</td>
<td>1.0 g/L</td>
<td>PASC</td>
<td></td>
<td>Den Haan et al., 2007</td>
</tr>
<tr>
<td>BGL (Saccharomycopsis fibuligera)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EG (T. reesei)</td>
<td>Codisplay on cell surface</td>
<td>2.1 g/L</td>
<td>PASC</td>
<td></td>
<td>Yanase et al., 2010</td>
</tr>
<tr>
<td>CBH (T. reesei)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BGL (A. aculeatus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EG (T. reesei)</td>
<td>Codisplay on cell surface</td>
<td>7.5 g/L</td>
<td>PASC</td>
<td>Diploid strain</td>
<td>Yamada et al., 2011</td>
</tr>
<tr>
<td>CBH (T. reesei)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BGL (A. aculeatus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EG (Thermoascus aurantiacus)</td>
<td>Display of single cellulase on cell surface followed by co-culture</td>
<td>2.1 g/L</td>
<td>PASC</td>
<td>EG:CBH: BGL= 6:2:1</td>
<td>Baek et al., 2012</td>
</tr>
<tr>
<td>CBH (T. reesei)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BGL (A. aculeatus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CipA3 (C. thermocellum)</td>
<td>Display of trifunctional minicellulosome</td>
<td>1.8 g/L</td>
<td>PASC</td>
<td></td>
<td>Wen et al., 2010</td>
</tr>
<tr>
<td>EG (T. reesei)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBH (T. reesei)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BGL (A. aculeatus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scaf (C. thermocellum, C.cellulolyticum, Ruminococcus flavefaciens)</td>
<td>Display of Scaf on cell surface, or secretion of either EG, CBH, or BGL, followed by co-culture</td>
<td>1.87 g/L</td>
<td>PASC</td>
<td>Scaf:EG: CBH:BGL= 7:2:4:2</td>
<td>Tsai et al., 2010</td>
</tr>
<tr>
<td>EG CelA (C. thermocellum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBH CelE (C. cellulolyticum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BGL (T. aurantiacus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4 Cellulase engineering in *S. cerevisiae*
<table>
<thead>
<tr>
<th>Cellulase expression</th>
<th>Cellulase formation</th>
<th>Enzyme activity</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG engB (<em>C. cellulovorans</em>)</td>
<td>Free enzyme</td>
<td>Increased activity (4-fold) towards CMC</td>
<td></td>
<td>Kim et al., 1994</td>
</tr>
<tr>
<td>Cel5A; Cel8C; or Cel9M (<em>C. cellulolyticum</em>)</td>
<td>Truncated free enzyme</td>
<td>Enhanced activity on CMC agar plates</td>
<td>Lost the C-terminal dockerin domain</td>
<td>Mingardon et al., 2011</td>
</tr>
<tr>
<td>Cel9E; Cel48F; or Cel9G (<em>C. cellulolyticum</em>)</td>
<td>N/A</td>
<td>N/A</td>
<td>Showed inhibitory effect on cell growth; No transformant obtained</td>
<td>Mingardon et al., 2011</td>
</tr>
<tr>
<td>CipC (<em>C. acetobutylicum</em>)-Cel48F (<em>C. cellulolyticum</em>)</td>
<td>Chimeric free enzyme</td>
<td>Bound to cellulose; active towards PASC</td>
<td></td>
<td>Chanal et al., 2011</td>
</tr>
<tr>
<td>mannanase man5K (<em>C. cellulolyticum</em>)</td>
<td>Truncated free enzyme</td>
<td>Active towards galactomannan</td>
<td>Lost the N-terminal dockerin domain</td>
<td>Mingardon et al., 2005</td>
</tr>
<tr>
<td>miniCipC1 (<em>C. acetobutylicum</em>) &amp; man5K(<em>C. cellulolyticum</em>)</td>
<td>Coexpressed free enzymes</td>
<td>Active towards galactomannan; bound to cellulose</td>
<td>full-length Man5K bound to CipC1</td>
<td>Mingardon et al., 2005</td>
</tr>
<tr>
<td>miniCipC1 (<em>C. cellulolyticum</em>); Scaf3 (<em>C. cellulolyticum</em> &amp; <em>C. thermocellum</em>)</td>
<td>Secreted scaffoldin proteins</td>
<td>Actively interact with dockerin domains</td>
<td></td>
<td>Perret et al., 2004</td>
</tr>
<tr>
<td>mini-CipA (<em>C. acetobutylicum</em>)</td>
<td>Secreted free enzyme</td>
<td>Bound to cellulose; interacted with Cel48A from <em>C. acetobutylicum</em></td>
<td></td>
<td>Sabathé and Soucaille, 2003</td>
</tr>
</tbody>
</table>

Table 2.5 Cellulase engineering in *C. acetobutylicum*
<table>
<thead>
<tr>
<th>Organisms</th>
<th>Carbon source</th>
<th>Ethanol &amp; butanol (g/L)</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial coculture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. cellulolyticum</em> <em>C. acetobutylicum</em></td>
<td>cellulose</td>
<td>0.9 (ethanol)</td>
<td>0.3 g/L reducing sugars present; sequential coculture</td>
<td>Petitdemange et al., 1983.</td>
</tr>
<tr>
<td><em>C. thermocellum</em> <em>C. acetobutylicum</em></td>
<td>cellulose</td>
<td>1.0 (ethanol) 2.5 (butanol)</td>
<td>1.0 g/L reducing sugar present; sequential coculture; butyric acid (3 g/L) addition</td>
<td>Yu et al., 1985.</td>
</tr>
<tr>
<td><em>C. thermocellum</em> <em>C. acetobutylicum</em></td>
<td>cellulose</td>
<td>~1 (ethanol) 0.7 (butanol)</td>
<td>Sequential coculture</td>
<td>Nakayama et al., 2011</td>
</tr>
<tr>
<td><em>C. thermocellum</em> <em>C. beijerincki</em></td>
<td>cellulose</td>
<td>~1 (ethanol) 2 (butanol)</td>
<td>Sequential coculture</td>
<td>Nakayama et al., 2011</td>
</tr>
<tr>
<td><em>C. thermocellum</em> <em>C. saccharoperbutylacetonicum N1-4</em></td>
<td>cellulose</td>
<td>~1.5 (ethanol) 7.9 (butanol)</td>
<td>Sequential coculture</td>
<td>Nakayama et al., 2011</td>
</tr>
<tr>
<td><em>C. thermocellum ev. Δhpt Δald Δpta T. saccharolyticum ALK2</em></td>
<td>cellulose</td>
<td>38 (ethanol)</td>
<td>Eliminated acetic acid and lactic acid production</td>
<td>Argyros et al., 2011</td>
</tr>
<tr>
<td>Fungal-bacterial coculture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. phytofermentans</em> <em>S.cerevisiae cdt-I</em></td>
<td>cellulose</td>
<td>22 (ethanol)</td>
<td>Endoglucanase addition (400 mg/L); controlled oxygen transport</td>
<td>Zuroff et al., 2013</td>
</tr>
<tr>
<td><em>T. reesei</em> <em>E. coli</em></td>
<td>AFEX pretreated corn stover</td>
<td>1.88 (isobutanol)</td>
<td></td>
<td>Minty et al., 2013</td>
</tr>
</tbody>
</table>

Table 2.6 Co-fermentations for biofuel production directly from cellulosic biomass
Chapter 3: Metabolic engineering of Clostridium cellulovorans: Restriction-modification system analysis and in vivo methylation protection for enhanced transformation

Abstract

Clostridium cellulovorans 743B, a cellulolytic bacterium producing butyric acid, is of particular interest as a host for biofuel production by fermentation. However, the gene transfer system of C. cellulovorans was not available. In the present study, two restriction-modification (RM) systems, Cce743I and Cce743II were determined with their restriction specificities and methylation specificities. An in vivo methylation system, expressing M. Cce743I and M. Cce743II in E. coli, was then established to protect plasmids from being degraded. With plasmid methylated prior to electrotransformation, recombinant strains were obtained, harboring the plasmid with pCB102 replicon from C. butyricum, expressing an aldehyde/alcohol dehydrogenase 2 (adhE2) for biofuel production. By expressing adhE2, 1.42 g/L butanol and 1.60 g/L ethanol were produced by the new strain of C. cellulovorans, using crystalline cellulose as carbon source. For the first time, an effective transformation method was developed for metabolic engineering of C. cellulovorans, and a new strain producing n-butanol and ethanol directly from cellulose was obtained, providing a promising platform for biofuel production from cellulosic biomass.
3.1 Introduction

Nowadays there is a growing interest in developing novel fermentation for biofuel production from renewable biomass and understanding the metabolism and genetics of the microorganisms involved (Ezeji et al., 2007; Lee et al., 2008; Paoutsakis, 2008). Ethanol has been widely used as a fuel additive in the U.S., blended with gasoline. And n-butanol has been proposed as a better choice of biofuel (Lee et al., 2008). ABE fermentation using solventogenic Clostridia to convert sugars into acetone, butanol and ethanol was once the second largest industrial fermentation process, and has received renewed interest in recent years. Furthermore, in order to avoid the competition with human food as well as to offer improved sustainability and reduce the cost of feedstocks, efficient utilization of cellulosic biomass has drawn more and more attention (Durre, 2011; Green, 2011). Current industrial bioconversion of cellulosic biomass requires physical, chemical, and enzymatic treatments prior to fermentation. However, these treatments are problematic because of the costly cellulase enzymes as well as downstream processing required for substrate detoxification, and product concentration and purification (Jonsson et al., 2013; Menon and Rao, 2012).

In recent years, consolidated bioprocessing (CBP), which proposed discrete conversion from cellulosic biomass to desired products in a single step without adding enzymes, can greatly simplify the processes and reduce the costs (Olson et al., 2012). Since there is no effective native cellulolytic microbes capable of producing substantial biofuel directly from cellulose (Guedon et al, 2002; Argyros et al., 2011; Jin et al., 2011; Tolonen et al., 2011; Virunanon et al., 2008; Berezina et al., 2008; Berezina et al., 2009), metabolic engineering becomes a powerful tool. For example, several studies expressed
heterologous cellulases or minicellulosomes in *S. cerevisiae*, producing 1.8 g/L~7.5 g/L ethanol directly from cellulosic biomass (Fujita et al., 2004; Wen et al., 2009; Yanase et al., 2010; Tsai et al., 2010; Yamada et al., 2011; BaeK et al., 2012; ). In addition, heterologous expression and assembly of minicellulosomes in solventogenic Clostridia have also been explored for n-butanol production from cellulosic biomass (Kim et al., 1994; Sabathé and Soucaille, 2003; Perret et al., 2004; Mingardon et al., 2005; Chanal et al., 2011; Mingardon et al., 2011; Fierobe et al., 2012). However, none of these recombinant *C. acetobutylicum* could grow on cellulosic biomass. It seemed that expression and secretion of cellulases could be deleterious in many cases, and grafting of a scaffoldin domain to the cellulases could sometimes rescue it (Mingardon et al., 2005; Mingardon et al., 2011; Chanal et al., 2011; Fierobe et al., 2012), suggesting lack of specific chaperones. Thus, expression and surface display of functional cellulases/minicellulosomes in solventogenic Clostridia was much more difficult than that in yeast.

In addition to cellulase engineering, biofuel synthesis pathway could also be integrated in cellulolytic microbes, such as *C. cellulosyticum, C. thermocellum* and *C. cellulovorans*, which can utilize cellulose efficiently because of their cellulosome structures (Tamaru et al., 2011). A recent study showed that an iso-butanol synthesis pathway was introduced into *C. cellulosyticum*, by overexpressing five heterologous enzymes to produce up to 0.66 g/L isobutanol from crystalline cellulose (Higashide et al., 2011). However, there was no example of metabolic engineering of cellulolytic bacteria for the production of n-butanol directly from cellulose. In this study, we chose *C.*
cellulovorans as the host to incorporate the heterologous n-butanol synthesis pathway from *C. acetobutylicum*.

*C. cellulovorans*, isolated from a wood chip pile, was capable of utilizing various substrates, including cellulose, xylan, pectin, cellobiose, glucose, maltose, galactose, sucrose, lactose, and mannose (Sleat et al., 1984). It was found to encode 57 cellulosomal genes (Tamatu et al., 2010). Compared to *C. cellulolyticum* and *C. thermocellum*, *C. cellulovorans* has the advantage of the simpler metabolic engineering process for n-butanol production, since it already encodes the butyric acid synthesis pathway (see Figure 3.1). Therefore, only one gene, aldehyde/alcohol dehydrogenase 2 (*adhE2*), needs to be overexpressed in *C. cellulovorans*, as already demonstrated in a recent study done in *C. tyrobutyricum*, a butyric acid producing bacteria, which was able to produce a large amount of n-butanol from glucose and xylose (Yu et al., 2011). In addition, the expression of *adhE2* could also lead to ethanol production, due to the substrate similarity between acetyl-CoA and butyryl-CoA. Therefore, *C. cellulovorans* is a promising host for biofuel production from cellulosic biomass by metabolic engineering, in terms of its efficient utilization of cellulosic biomass and its existing metabolic pathway.

However, a transformation method for *C. cellulovorans* was unavailable. In fact, genetic tools for Clostridia species were most under-developed, due to insufficient understanding of their genetics. Particularly, transformation of Clostridia was often hindered by their restriction modification (RM) systems, the defensive machineries to degrade foreign DNAs. There are four types of RM systems, including type I, type II, type III, and type IV, consisting of restriction (RE) subunits and methyltransferase (MT) subunits, and also specificity (S) subunits in type I RM systems. These subunits work
coordinately, cleaving specific sequence by REs, while protecting their own genomic DNA by marking it with methyl group by MTs (Tock and Dryden, 2005). RM systems are widespread in bacteria. About 95% of the genome-sequenced bacteria contain RM systems, and about 5000 REs and 8000 MTs have been described in REBASE (rebase.neb.com) (Roberts et al., 2010). Numerous studies have shown that proper DNA methylation prior to transformation would significantly enhance the possibility of successful transformation by overcoming the restriction barrier (Davis et al., 2000; Elhai et al., 1997; Jennert et al., 2000; Lin et al., 2010; Mermelstein et al., 1993; Rego et al., 2011; Suzuki et al., 2011; Purdy et al., 2002; Pyne et al., 2013; Zhang et al., 2012).

In this work, in order to metabolically engineer *C. cellulovorans* for biofuel production, an effective transformation method was established by first determining the RM systems and developing an *in vivo* plasmid methylation method. This study showed that proper methylation was the key factor for electrotransformation of *C. cellulovorans*. A new strain capable of producing n-butanol and ethanol directly from cellulose was obtained. To our knowledge, this is the first report of metabolic engineering of *C. cellulovorans*, which provides a promising platform for cellulosic biofuel production.

### 3.2 Materials and Methods

#### 3.2.1 Bacteria strains and plasmids

The strains and plasmids employed in this study are summarized in Table 3.1.

#### 3.2.2 Cultural media and bacteria growth

*E. coli* was grown in Luria–Bertani (LB) medium (Fisher Scientific, US). *C.*
Cellulovorans was grown in modified medium 520 (DSMZ), which contained (per liter): (NH₄)₂SO₄, 1.3 g; KH₂PO₄, 1.5 g; K₂HPO₄·3H₂O, 2.9 g; MgCl₂·6H₂O, 0.2 g; CaCl₂·2H₂O, 75 mg; FeSO₄·7H₂O, 1.25 mg; resazurin, 1 mg; yeast extract, 2 g; tryptone, 4 g; cysteine-HCl, 0.5 g. The medium was adjusted to pH 6.0 and sparged with N₂ gas before autoclaving. Then, sterile 50× trace elements, 50× Na₂CO₃ (10% w/v), and 15 g/L glucose, cellobiose or cellulose were added before use. 1000× trace elements contained: HCl (25%; 7.7 M), 10 ml; FeCl₂·4H₂O, 1.5 g; ZnCl₂, 70 mg; MnCl₂·4H₂O, 100 mg; H₃BO₃, 6 mg; CoCl₂·6H₂O, 190 mg; CuCl₂·2H₂O, 2 mg; NiCl₂·6H₂O, 24 mg; Na₂MoO₄·2H₂O, 36 mg; add H₂O to 1 liter. It was diluted 20 times to make 50× trace elements. Colonies of C. cellulovorans were grown at 37 °C on medium plates (1% agar) in the anaerobic chamber. Media 520 was supplemented with 15 µg/ml thiamphenicol for transformant selection. E. coli was grown aerobically at 37 °C with agitation at 250 rpm in LB media supplemented with chloramphenicol (30 µg/ml) or tetracycline (30 µg/ml).

3.2.3. Characterization of the restriction profile by restriction assay

Crude extract of C. cellulovorans was prepared using the method modified from previously described (Azeddoug and Reysset, 1991). 100 ml exponential-phase (OD600 = 0.5-0.6) cells were harvested by centrifugation. They were then washed once and resuspended in 1 ml lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10 mM β-mercaptoethanol). Cells were disrupted with glass beads (0.1 mm) in a Mini Beadbeater (Biospec) for 30 s with a 30 s interval on ice, repeated 10 times. The cell debris was removed by centrifugation at 13,200 rpm for 30 min at 4 °C. The lysate was adjusted to
contain 50 mM NaCl. After 10 min on ice, the lysate was centrifuged at 13,200 rpm for 10 min at 4 °C. The same volume of glycerol was mixed with the supernatant, stored at -20 °C. For restriction assay, 0.5-1 µg plasmid substrate was incubated with 3 µl cell extract at 37 °C in the digestion buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 0.01% BSA) in 25 µl reaction volume. All the manipulations were handled in anaerobic condition.

3.2.4. Cloning methyltransferases

Genomic DNA of *C. cellulovorans* prepared using QIAGEN Genomic-tips kit (QIAGEN, US) served as the DNA template to amplify *M. Cce*743I and *M. Cce*743II by PCR. Specific primers with T7 promoter on the front and *Nco*I restriction site on the end were designed as follows:

TAATACGACTCACTATAGGGAGACCGGCATGAGGATAGTTTTATGAGTAAGA (forward primer); ATTCCATGGCTACACCTCCATTTCTTTTAACAAATC (reverse primer). PCR was performed with Phusion high-fidelity PCR master mix with HF buffer (NEB, US), under following thermocycling conditions: 98 °C for 3 minutes for initial denaturation; 98 °C for 10 s for denaturation, 55 °C for 30 seconds for annealing, 72 °C for 1 min and 15 s for extension, for amplification with 35 cycles; 72 °C for 5 minutes for final extension, and ended with 10 °C hold. The PCR product size was about 2.5 kb. The purified PCR product was then cut by *Nco*I restriction enzyme (NEB, US), and ligated into the distal *Pvu*II site and *Nco*I site of plasmid pACYC184 by T4 ligase (NEB, US) to generate the new plasmid, namely pXY1. Plasmid pACYC184 was prepared from *E. coli*
K12 ER2420/pACYC184 (NEB, US). pXY1 plasmid was then confirmed by amplification of cloned methyltransferases by PCR, as well as enzyme digestion by NcoI and BamHI with expected patterns. The recombinant pXY1 plasmid lost its chloramphenicol resistance due to the removal of partial chloramphenicol resistance genes by PvulII and NcoI enzyme digestion, but retained its tetracycline resistance gene for selection.

3.2.5 In vivo methylation

Once pXY1 plasmid was confirmed, it was then transformed into competent E. coli DH10β (NEB, US), which had mrr, hsdRMS, and mcrBC deletions and was thus not sensitive to methylated DNA, to generate a new strain E. coli pXY1 with M. Cce743I and M. Cce743II activities. Successful generation of E. coli pXY1 was confirmed by plasmid preparation using QIAGEN Genomic-tips kit (QIAGEN, US), followed by PCR of cloned methyltransferases as well as enzyme digestion by NcoI and BamHI as previously described. For plasmid methylation in vivo, pMTL 80000 series shuttle plasmids, including pMTL82151-adhE2, pMTL83151-adhE2, pMTL84151-adhE2, and pMTL85151-adhE2 (Yu et al., 2012) were then transformed into competent E. coli pXY1. Plasmids prepared from these strains by QIAPrep spin miniprep kit (Qiagen, US) were subjected to restriction assay to test its protection effect or used for the transformation of C. cellulovorans.
3.2.6 Preparation of E. coli competent cells

Competent E. coli pXY1 was prepared as previously described (Seidman et al., 1997) with minor modifications. E. coli pXY1 was inoculated into 10 ml LB media with tetracycline (30 µg/ml) for overnight growth. Overnight cell culture was then transferred to 300 ml LB media with tetracycline (30 µg/ml) to grow until OD600 = 0.37-0.45. Cells were then aliquoted into sterile 50 ml centrifuge tubes and were incubated on ice for 5-10 min. Cells were then collected by centrifugation at 1600×g at 4 °C for 7 min, followed by supernatant removal. 10 ml sterile ice-cold CaCl₂ solution, containing 60 mM CaCl₂, 15% glycerol, 10 mM PIPES (pH 7), was added to each centrifuge tube to resuspend cells. After washing and centrifugation again at 1600×g at 4 °C for 5 min, supernatant was poured off and each pellet was resuspended in 10 ml ice-cold CaCl₂ solution. Cell culture was then incubated on ice for 30 min. Cells were combined and centrifuged at 1600×g at 4 °C for 5 min, and resuspended in 1 ml CaCl₂ solution. Aliquots were stored in microcentrifuge tubes, 30 µl each, in a -80 °C freezer.

3.2.7 Characterization of the methylation specificity by bisulfite conversion assay

Genomic DNA of C. cellulovorans prepared as described above was treated by bisulfite reagent using Epimark bisulfite conversion kit (NEB, US). After treatment, methylation pattern could be further analyzed by PCR and DNA sequencing. Specifically, two pairs of primers for amplification of Cce743I sites and Cce743II sites were designed. Forward primer (GTGGATGTAGAAGTGAGAATGTTGGATAAG) and reverse primer (CaCTCCCCCATCACACCTCAaAaTATTAAaCACaC) could cover
both strand sequences of \textit{Cce743II} sites, and one strand sequence of \textit{Cce743I} site. Forward primer (AGGTGAAGGtGAGGGTAAAAAttTtGTGAGGAAttG) and reverse primer (TCCCACATCCTTTTTCCACTTAACCTTACACT) could cover the other strand sequence of \textit{Cce743I} site (shown in Table 3.3). After bisulfite treatment, double stranded genomic DNA became single stranded, and unmethylated cytosine was converted to uracil, while methylated cytosine remained unchanged. Because of such changes, primer design was modified according to the sequences of the sense strand as template. In addition, it was proposed that no methylation took place in the primer regions. Therefore, the cytosines in the forward primer region were designed to be thymines, and the adenines were designed in the antisense strand to be base-paired with uracil in the sense strand in the reverse primers. The changes in the primers (t in the forward primers, and a in the reverse primers) were marked as small letters in red color. Since bisulfite treatment of genomic DNA could cause broken DNA, the PCR products by these primers were designed to be about 300 bp. And the bisulfite-treated genomic DNA needed to be purified to remove extra bisulfite by the kit provided for successful PCR reaction. In addition, specific polymerase, Epimark hot start taq (NEB, US), was needed for PCR from bisulfite-converted DNA with high AT content under following thermocycling conditions: 95 °C for 30 s for initial denaturation; 95 °C for 30 s for denaturation, 55 °C for 30 s for annealing, 68 °C for 30 s for extension, for amplification with 40 cycles; 68 °C for 5 min for final extension, and ended with 10 °C hold. The PCR products were confirmed by DNA gel electrophoresis. The confirmed PCR products were then purified prior to DNA sequencing for methylation pattern analysis. By comparing the sequencing results of the PCR products with their original sequences, the methylation pattern at
single-nucleotide resolution was determined. Specifically, cytosines in the original sequences read as thymines were without methylation, while cytosines in the original sequences read as cytosines were methylated.

3.2.8 Transformation

Electroporation was performed as previously described (Jennert et al., 2000) with minor modifications. Wild-type *C. cellulovorans* was grown in modified 520 media with glucose as carbon substrate. 100 ml cells at the exponential phase (OD600 = 0.5) were harvested by centrifugation at 4500 rpm at 4 °C for 10 min. Supernant was poured off, and the pellet was washed once with 50 ml ice-cold electroporation buffer (5 mM sodium phosphate buffer containing 270 mM sucrose and 1 mM MgCl, pH 7.4) purged with nitrogen. Washed cells were again collected by centrifugation at 4500 rpm at 4 °C for 10 minutes. Supernatant was removed and cell pellet was resuspended in 800 µl electroporation buffer, which was then aliquoted into microcentrifuge tubes with 400 µl cells. 10 µl *in vivo* methylated plasmids (5-8 µg) prepared as described above were mixed with 400 µl cell suspension, incubating on ice for 10-15 min. The mixture was then transferred to a pre-chilled electroporation cuvette (0.2 cm inter-electrode distance). Cells were pulsed once at 1.8 kV, 25 µF, 800 Ω (Bio-Rad Gene Pulser). The resulting pulse duration was 4-6 ms. The cuvette was moved to ice immediately after pulse, incubating for 10-15 min. Totally, two aliquots of cells were transformed to enhance the possibility of obtaining transformants. The cell suspensions were then combined, transferred to a tube with 2-3 ml modified 520 media with glucose or cellobiose, incubating at 37 °C for recovery. After overnight incubation, obvious bubbles in the
media could be observed, suggesting electrotransformed cells were recovered to an active state. 15-20 ml 520 media with glucose or cellobiose as carbon source was added, supplemented with 15 µg/ml thiamphenicol for selection. When obvious growth was observed (3-7 days), 1 ml cells was inoculated to 20 ml modified 520 media with cellobiose, supplemented with 30 µg/ml thiamphenicol. This step was repeated once if cells grew well. If cells could not grow during transfer, it suggested the transformants were false positive. The actively growing cells were then concentrated to 1 ml, spreading on 5 agar 520 media plates with 15 µg/ml thiamphenicol for colony growth. Actively growing wild-type *C. cellulovorans* served as control. All the manipulations were handled in anaerobic condition.

### 3.2.9 Plasmid preparation and confirmation

To verify that the plasmid had been transformed into *C. cellulovorans*, plasmid purification from *C. cellulovorans* was performed. 25 ml cells grown in modified 520 media with glucose were collected, and were treated by 20 mg/ml lysozyme from chicken egg (Sigma, US) in P1 buffer (miniprep kit, QIAGEN, US) at 37 °C for 5 h prior to plasmid purification. The concentration of prepared plasmid was very low due to the low copy-number replicon of it, thus was difficult to be observed by DNA gel electrophoresis. Therefore, the purified DNA from *C. cellulovorans* transformant was then transformed into competent *E. coli* DH5α for further plasmid replication. The plasmid was prepared from *E. coli* DH5α for verification. Firstly, the plasmid was verified by PCR using specific primers flanking *adhE2* (M13f:
TGTAATACGACGGCCAGT; M13r: GGAAACAGCTATGACCGC). Secondly, the plasmid was verified by enzyme digestion by BamHI and SacII. Plasmid was not sequenced, since adhE2 enzyme assay and fermentations could further confirm its existence and function in C. cellulovorans.

3.2.10 adhE2 enzyme activity assays

The butyraldehyde dehydrogenase activity and butanol dehydrogenase activity of adhE2 were evaluated by monitoring NADH consumption, adapted from the method described previously (Durre et al., 1987) with minor modifications. This method has been widely used for measurement of adhE2 enzyme activity in bacteria for biofuel production. Wild-type or transformed C. cellulovorans was grown in 100 ml modified 520 media with glucose as carbon substrate. Cells at the exponential phase (OD600 = 0.5) were harvested by centrifugation at 4500 rpm 4 °C for 10 min. Supernatant was removed and cell pellet was resuspended in 1.5 ml Tris-HCl buffer (0.1 M Tris-HCl, pH 7.5, 1 mM dithiothreitol). The cells were then disrupted with glass beads (0.1 mm) in a Mini Beadbeater (Biospec) for 30 s with a 30 s interval on ice, repeated 10 times. Supernatant was collected by centrifugation at 15,000×g for 20 min at 4 °C. All these manipulations were handled in anaerobic condition.

To measure the butyraldehyde dehydrogenase activity of adhE2 in the cell lysate, reaction buffer with pH 6.0, containing 0.2 mM butyryl-CoA 1 mM dithioerythritol, 0.27 mM NADH, 72 mM semicarbazide hydrochloride and 67 mM Tris-HCl was prepared. To measure the butanol dehydrogenase activity of adhE2 in the cell lysate, reaction
buffer with pH 7.8, containing 11 mM butyraldehyde, 0.23 mM NADH, and 77 mM Tris-HCl was prepared. To start the reaction, 190 µl reaction buffer was added into each well on a 96-well plate (Fisher Scientific, US). Four wells were used for each sample; one well for negative control without cell lysate, and three wells for triplicates of enzyme activity test. 10 µl cell lysate was added and mixed with the reaction buffer in three of the wells. NADH consumption was monitored every 12 s for 5 min at 365 nm. During the first 5 min of reaction, NADH was consumed almost at a constant speed. The slope of NADH reading value relative to time was used to calculate the enzyme activity.

Protein concentration of the cell lysate was determined using the Bio-Rad protein assay kit (Bio-Rad, US) with bovine serum albumin (BSA) as standard. Specifically, Bio-Rad concentrated dye reagent was diluted and filtered following the protocol. BSA standards with 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml and 0.5 mg/ml were prepared. 200 µl dye reagent was added into 7 wells of a 96-well plate (Fisher Scientific, US). Cell lysate was diluted 10 times by distilled water. 10 µl cell lysate or BSA standard was mixed with the dye reagent in the wells for incubation at room temperature for 5-10 minutes. Color reaction was monitored at 595 nm. This experiment was triplicated to minimize the error. The protein concentration of cell lysate could be then calculated, based on the linear relationship between the reading values and BSA standard concentrations.

Enzyme activity was calculated on the basis of a molar NADH extinction coefficient of 3.4 cm⁻¹mM⁻¹. One unit of enzyme activity was defined as the amount of enzyme converting 1 µmol NADH per minute under the reaction conditions. The equation used to calculate the enzyme activity is: enzyme activity = the slope of NADH
consumption \times 0.2/(3.4\times0.7)/\text{protein mass}, \text{ where 0.2 is the total reaction volume (0.2 ml), 3.4 is the NADH extinction coefficient (3.4 cm}^{-1}\text{mM}^{-1}), and 0.7 is the light path of the reaction buffer (0.7 cm).

3.2.11 Fermentation analysis

Unless otherwise noted, batch fermentations with \textit{C. cellulovorans}/83151-\textit{adhE2} were carried out in serum bottles containing 50 ml medium 520 supplemented with 30 \mu g/ml thiamphenicol. The medium pH was maintained between 6.0 and 7.0 by adding NaOH solution. Samples were taken twice per day to monitor cell growth, substrate consumption and production of ethanol, butanol, acetic acid and butyric acid during the fermentation. Cell density was analyzed by measuring the optical density of cell suspension at 600 nm using a spectrophotometer (Shimadzu, Columbia, MD, UV-16-1). Ethanol, butanol, acetic acid, and butyric acid, were analyzed with a gas chromatograph (GC) (GC-2014 Shimadzu, Columbia, MD) equipped with a flame ionization detector (FID) and a 30.0 m fused silica column (Stabilwax-DA, 0.25 mm film thickness and 0.25 mm ID, Restek, Bellefonte, PA) with a standard curve. The GC was operated at an injection temperature of 200 °C with 1 \mu L of sample injected with an auto injector (AOC-20i, Shimadzu). The column temperature was initially held at 80 °C for 3 min, then increased at a constant rate of 30 °C per min to 150 °C, and held at 150 °C for 3.7 min.

To quantify cellulose, it was first hydrolyzed with Accellerase 1500 cellulase (Dupont Industrial Biosciences, New York, US), and then determined by measuring the released glucose by high performance liquid chromatography (HPLC, LC-20AD, Shimadzu, Columbia, MD) with an organic acid column (Bio-Rad HPX-87, ion exclusion
organic acid column, 300 mm × 7.8 mm). Specifically, cellulose sample (1 ml) was washed with distilled water prior to autoclaving. After autoclaving, the cellulose pellet was washed again with distilled water. Afterwards, it was resuspended in 1 ml distilled water at pH 4.5, and was hydrolyzed with 20 µl Accellerase 1500 cellulase at 50 °C for 7 days. Samples were centrifuged at 13,200 rpm for 15 min in 1.5 ml microcentrifuge tubes and diluted 10 times with distilled water prior to analysis on HPLC. HPLC was run at 45 °C using 0.007N H₂SO₄ (0.7 ml H₂SO₄ in 2 L distilled water) as the eluent at a flow rate of 0.6 mL/min. 15 µl sample was injected by an automatic injector (SIL-10Ai) and the running time was set at 40 min. A refractive index (RI) detector (Shimadzu RID-10A) was set at the range of 200 to detect the organic compounds in the sample. The HPLC column was installed in a column oven (CTO-10A) with temperature controlled at 45 °C. Peak height was used to calculate the concentration of sugars in the sample based on the peak height of standard sample.

3.3 Results

3.3.1 Genome analysis of RM system of C. cellulovorans

The genome of C. cellulovorans published in 2010 consists of 4254 ORFs. By analyzing its genome sequences in silico on REBASE, 12 operons were found, consisting of putative MT subunits, RE subunits, and S subunits (shown in Figure 3.2). The proteins in the same operon are generally transcriptionally coupled and functionally related. Among these operons, three candidates were selected (marked with ★ in Figure 3.2), containing at least a RE and a MT working in pairs. They are type I, type II, and type III
RM systems, respectively. The details of gene locus, functions, and putative target sequences are summarized in Table 3.2. The type I RM system consisted of a RE, a MT, and an S subunit, possibly recognizing the EcoKI sites (5’-AACGTGC-3’; 5’-GCACGTT-3’), based on protein sequence homology of the RE subunit with other organisms. Since the RE, MT, and S subunits were encoded in the same operon, it was hypothesized that they recognized the same site and work coordinately. The type II RM system contained two REs and two MTs in the same operon. The first RE subunit, namely R. Cce743I, may have the same specificity as LlaJI, recognizing 5’-GACGC-3’ and 5’-CGGTC-3’, based on the protein sequence homology with other organisms. The second RE subunit, namely R. Cce743II, possibly has the same specificity as LlaI, recognizing 5’-CCAGG-3’ and 5’-CCTGG-3’, based on the protein sequence blast. The MT subunit at the end of the operon (M. Cce743II) seemed to work in pairs with R. Cce743II, since it was homologous with NlaX (5’-CCNGG-3’), recognizing the same sites as R. Cce743II. Therefore, the MT subunit after R. Cce743II in the operon (M. Cce743I) was hypothesized to work in pairs with R. Cce743I, though no protein sequence similarity with other organisms was found. The type III RM system contained a RE, a MT and a SAM (the substrate to offer methyl group during methylation) protein. However, there was no clear knowledge of their specificity. Among these three RM systems, candidates were narrowed down to the type II operon, since type II RM systems are the most common and dominant.
3.3.2 Characterization of the restriction profile by restriction assay

To test the hypothesis that *R. Cce*743I and *R. Cce*743II restricted foreign DNAs, restriction assay was performed. The cell lysate of *C. cellulovorans* was prepared and incubated with plasmid pMTL82151-*adhE2*, one of the plasmids used for transformation of *C. cellulovorans* for the overexpression of *adhE2*. The DNA digestion patterns were predicted (as shown in Figure 3.3) by analyzing cutting sites in the plasmid sequence by NEB Cutter (http://tools.neb.com/NEBcutter2/). If only *R. Cce*743I worked, the plasmid was predicted to be cut into 4 pieces, one around 7 kb and the other three around or less than 500 bp. If only *R. Cce*743II played a role of restriction, the plasmid would be cut into 11 pieces, with two around 3.2 kb, and the other nine around or less than 500 bp. If both *R. Cce*743I and *R. Cce*743II were functional, the plasmid was predicted to be cut into 15 pieces, with one around 3.2 kb, one around 2.8 kb, and the other thirteen around or less than 500 bp, of which the pattern would be similar to that cut by *Cce*743II. The actual result of the restriction assay showed that there were bands around 3 kb (shown in Figure 3.4), indicating *C. cellulovorans* possessed specific restriction activities. However, it was hard to distinguish the possibility of restriction by *Cce*743II from the possibility of restriction by both *Cce*743I and *Cce*743II from the results of gel electrophoresis. Further identification from other perspective, such as identification of its methylation pattern, would be needed.
3.3.3 Characterization of the methylation specificities and determination of the functional RM systems

To further distinguish the possibilities between restrictions by single R. CceI 743II and by both R. CceI 743I and R. CceI 743II, methylation pattern of the native genome sequences were analyzed. Since the pairing MT and RE recognize the same specific sequence, by determining the methylation specificity, restriction specificity would be clear. The methylation pattern was analyzed by bisulfite conversion assay. Bisulfite can convert cytosine to uracil, unless the cytosine is protected by methylation and thus will remain unchanged. After bisulfite conversion of the genomic DNA, the modified DNA was subjected to PCR to amplify specific sequences covering CceI 743I and CceI 743II sites, followed by DNA sequencing. Therefore, the methylation pattern with single-base pair resolution could be analyzed. It should be noted that after bisulfite conversion, several changes of the genomic DNA took place. Firstly, unmethylated cytosine, which was a major portion of the genomic DNA, were converted to uracil. Secondly, bisulfite treatment could break down genomic DNA to short pieces. Thus, PCR products larger than 500 bp were hardly amplified. In this study, PCR products were designed to have a length around 300 bp. Thirdly, the double-stranded DNA became single-stranded, since most of the base pairs were disrupted by cytosine conversions. Thus, primers were designed to use only one strand (sense strand) as template. In addition, the forward primers were designed to replace cytosine with thymine, and the reverse primers were designed to replace guanine with adenine. Since the methylation patterns of both strands for each cutting site may be different, primers were designed to cover four sequences, including both strands of CceI 743I and CceI 743II (5’-GACGC-3’; 5’-GCGTC-3’; 5’-
CCAGG-3'; 5'-CCTGG-3'). The analysis result showed that both Cce743I and Cce743II sites were methylated (shown in Table 3.3), suggesting that both RM systems were functional. Particularly, the external cytosines in both strands of Cce743II site were methylated (5'-mCCTGG-3'; 5'-mCCAGG-3'), while the external cytosine in one strand of Cce743I site was methylated (5'-GACGmC-3'), leaving the other stand unmethylated (5'-GCGTC-3'). In addition, except for the cytosine at these restriction sites, all other cytosines in the region of amplification were all sequenced as thymines, indicating that all other cytosines in the region were unmethylated. Taken all together, an operon encoding two RM systems (Cce743I and Cce743II) in C. cellulovorans defending foreign DNAs were identified with the details of the restriction sequences and methylation specificity. However, no commercial methyltransferases recognizing either of these two sites were available. Therefore, in vitro methylation of commercial methyltransferases prior to transformation for plasmid protection as previously done (Jenert et al., 2000) was impossible in this case. To overcome this problem, an in vivo methylation system must be established to protect plasmids from being degraded during the transformation process.

3.3.4 Establishment of the transformation method of C. cellulovorans

A series of pMTL80000 Clostridia shuttle plasmids with various Gram-positive replicons (shown in Table 3.1) were used to overexpress adhE2 from C. acetobutylicum (Yu et al., 2012). pMTL82151-adhE2 harbors pBP1 replicon from C. botulinum; pMTL83151-adhE2 harbors pCB102 replicon from C. butyricum; pMTL84151-adhE2 harbors pCD6 replicon from C. difficile; and pMTL85151-adhE2 harbors pIM13 replicon
from Bacillus subtilis. adhE2 gene was expressed under the constitutive thiolase promoter Pthl from C. tyrobutyricum. All four plasmids were almost the same, except for the different replicons. Replicons are the key factors to control the plasmid replication, plasmid copy number, and plasmid stability, thus resulting in different transformation efficiency. In addition, the different sequences of replicons also affected the number of RM cleavage sites in the plasmids. As shown in Table 3.4, these plasmids contained no EcoKI sites, but 13-16 Cce743I and Cce743II sites. Studies have shown that the transformation efficiency decreased dramatically when the number of the unprotected sites increased, and transformation was particularly difficult when there were more than 4 cleavage sites (Elhai et al., 1997). Since there were at least 13 identified cleavage sites in the shuttle plasmids, it was almost impossible to obtain transformants without circumvention of the restriction barrier. This conclusion was further confirmed by the initial transformation without methylation. Electroporation and conjugation were employed for transformations. No matter what conditions were tested (e.g., physical condition of cells, buffer pH, addition of cell-wall-weakening reagent, electricity voltage, electricity capacity, electricity resistance, pulse frequency), transformations without proper methylation of the plasmids were never successful. Therefore, a transformation method combining plasmid methylation to overcome the restriction barrier must be established based on the RM systems identified above (Cce743I and Cce743II).

Considering that bacterial operon is usually a functioning unit with a cluster of genes with related functions, M. Cce743I and M. Cce743II encoded within the same operon of R. Cce743I and R. Cce743II were proposed to methylate the same sites to protect its own genomic DNA. Since no commercial methyltransferases was available for
in vitro methylation, cloning native M. Cce743I and M. Cce743II for in vivo methylation could be another solution for plasmid protection. In vivo methylation was shown to successfully protect plasmids and increase transformation efficiency (Mermelstein and Papoutsakis, 1993; Jenert et al., 2000; Davis et al., 2000; Purdy et al., 2002; Pyne et al., 2013), by transforming the plasmid from the E.coli expressing methyltransferase(s). In vivo methylation had many advantages over in vitro methylation. Firstly, it reduced the cost without using commercial methyltransferase. Secondly, it increased the experimental efficiency without enzymatic treatment, purification and concentration. Thirdly, the transformation efficiency with in vivo plasmid methylation was usually a little higher than that with in vitro plasmid methylation. Lastly, by using native methyltransferase for in vivo methylation could avoid negative side effects. Therefore, M. Cce743I and M. Cce743II from the genomic DNA of C. cellulovorans were cloned into a vector pACYC184 to be transformed into E. coli. pACYC 184 was frequently used for in vivo methylation, since it had a low-copy-number Gram-positive replicon (p15A), which could co-exist in cells with plasmids of the ColE1 compatibility group (e.g., pMTL 80000 plasmids). It contained two antibiotic resistance genes, including chloramphenicol resistance gene and tetracycline resistant gene. Since the pMTL 80000 shuttle plasmids tested for overexpression in C. cellulovorans had chloramphenicol resistant gene, the chloramphenicol resistance gene in pACYC184 plasmid needed to be disrupted. As shown in Figure 3.5, partial chloramphenicol resistance gene was removed at the distal PvuII site and NcoI site, and was replaced with M. Cce743I and M. Cce743II genes under T7 promoter. Thus, a plasmid, namely pXY1, compatible with shuttle plasmid in E. coli expressing M. Cce743I and M. Cce743II was generated.
To further establish the \textit{in vivo} methylation system, pXY1 was transformed into \textit{E. coli}. It was noted that normal \textit{E. coli} strains used for cloning, such as DH5\(\alpha\), was not suitable for amplification of plasmid with methyltransferase activity. \textit{E. coli} strains, such as DH10\(\beta\), TOP10, or stellar cells, with disruption in \textit{mcrA}, \textit{mcrBC}, or \textit{mrr}, allowed methylated DNA to not be recognized as foreign, which were necessary when cloning methylated DNA. In this case, pXY1 was transformed into competent \textit{E. coli} DH10\(\beta\), for expression of M. \textit{Cce743I} and M. \textit{Cce743II}, generating a new strain of \textit{E. coli} XY1. Chemical competent \textit{E. coli} XY1 was prepared, and was ready to transform the pMTL80000 series plasmids for methylation \textit{in vivo} (shown in Figure 3.6). To verify the protection effect of the \textit{in vivo} methylation system, restriction assay was performed. Specifically, methylated pMTL82151-\textit{adhE2}, or pMTL83151-\textit{adhE2} was prepared from \textit{E. coli} XY1. And unmethylated pMTL82151-\textit{adhE2}, or pMTL83151-\textit{adhE2} was prepared from \textit{E. coli} DH5\(\alpha\), served as controls. The unmethylated or the methylated plasmid was incubated with the cell lysate of \textit{C. cellulovorans} for restriction for 0 h and 20 h, respectively. The result of the restriction assay by gel electrophoresis showed that without methylation, the plasmid pMTL82151-\textit{adhE2} was restricted, showing the similar pattern as in Figure 3.7. However, with methylation, the plasmid remained intact (shown in Figure 3.7, left panel). Similar results could be seen using pMTL83151-\textit{adhE2} plasmid as the DNA substrate (shown in Figure 3.7, right panel). Therefore, an \textit{in vivo} methylation system with the plasmid protection effect was established.

By employing the \textit{in vivo} methylation system, the methylated pMTL80000 series plasmids were prepared and used for electroporation. At 1.8 kV, 25 \(\mu\)F, and 800 \(\Omega\) (in 0.2 cm cuvette), transformants harboring pMTL83151-\textit{adhE2} (\textit{C. cellulovorans}/83151-
The C. cellulovorans/83151-adhE2 transformant was transferred in 520 media with thiamphenicol and subcultured twice to rule out the possibility of false positive, followed by picking single colony from agar plates. C. cellulovorans are obligate anaerobic bacteria, requiring very restricted anaerobic condition in anaerobic chamber. In addition, it grew better on 1% agar plates, instead of 1.5% agar plates, in modified 520 media. Commercial Reinforced Clostridial Medium (RCM) with high nitrogen contents is usually good for growth of Clostridia. However, C. cellulovorans or its transformants could not grow well in RCM media or agar plates. The reason was not clear. Moreover, it was harder to grow the C. cellulovorans transformants on agar plates, compared to wild-type C. cellulovorans, probably caused by the antibiotic pressure. Therefore, to grow single colony of C. cellulovorans transformants, cells needed to be concentrated prior to plating. In this case, it was difficult to grow electrotransformed cells, even after an extensive recovery period, directly on plates for selection. It was thus also difficult to estimate the transformation efficiency by counting the colony numbers on plates.

Once the single colony of C. cellulovorans transformant was picked, plasmid verification was performed. Since the plasmids prepared directly from C. cellulovorans transformant was invisible in the agarose gel electrophoresis, due to the low copy number of the replicon, it was therefore transformed into E. coli for replication, followed by plasmid purification and plasmid confirmation. The plasmid was confirmed by amplification of adhE2 by PCR and enzyme digestion by BamHI and SacII with the expected pattern (shown in Figure 3.8). Therefore, a transformation method combining plasmid methylation to overcome the restriction barrier was established. This is the first
time successful transformation of *C. cellulovorans* with an overexpression plasmid has been achieved.

### 3.3.5 Enzyme activity assay

To further confirm the *adhE2* expression in *C. cellulovorans*, enzyme activity assay was performed. Since *adhE2* is a bifunctional enzyme, the butyraldehyde dehydrogenase activity (converting butyryl-CoA to butyraldehyde) as well as butanol dehydrogenase activity (converting butyraldehyde to butanol) were measured. As shown in Figure 3.9, the butyraldehyde dehydrogenase activity (0.1619 U/mg) of *adhE2* increased about 27-fold, while butanol dehydrogenase activity (0.0774 U/mg) of *adhE2* increased about 7-fold in the strain *C. cellulovorans*\!/83151-*adhE2*, compared to the wild-type control. It should be mentioned that the measured enzyme activities could not represent the absolute values of adhE2’s activity *in vivo*. Firstly, the assay was performed *in vitro*, and could be affected by many factors, such as buffer condition, temperature and handling. Secondly, when measuring butyraldehyde dehydrogenase activity using butyryl-CoA as the substrate, the reaction from butyraldehyde to n-butanol was also carried out. However, it was hard to evaluate the amount of NADH consumed for the second reaction. Therefore, the value of adhE2 enzyme activity was a relative one that was used for comparison with the wild-type control. Overall, the results confirmed that overexpressed *adhE2* was functional in the engineered strain.
3.3.6 n-Butanol and ethanol production by engineered C. cellulovorans

Since the heterologously expressed adhE2 was functional in the engineered C. cellulovorans, it was expected to see ethanol and n-butanol production from cellulose in fermentation by the recombinant strain. Therefore, fermentation in serum bottles using cellulose as carbon substrate was performed. The result showed that 1.42 g/L butanol and 1.60 g/L ethanol were produced by C. cellulovorans/83151-adhE2 when grown on 15 g/L crystalline cellulose (shown in Figure 3.10). This result not only represented the first example of successful metabolic engineering of C. cellulovorans, but also showed a promising platform for biofuel production from cellulosic biomass.

3.4 Discussion

In this study, in the attempts to metabolically engineer C. cellulovorans for n-butanol and ethanol production directly from cellulosic biomass, it was found that proper methylation of plasmids was the key to make the transformation successful. In particular, two RM systems (Cce743I and Cce743II) in C. cellulovorans were identified, and the native MTs protecting these two sites were further cloned from C. cellulovorans and expressed in E. coli for in vivo methylation. By using the methylated plasmids, a new strain of C. cellulovorans was obtained, capable of expressing adhE2 and further producing 1.42 g/L butanol from crystalline cellulose. This study not only developed an effective transformation method for C. cellulovorans, combining electrotransformation and plasmid methylation, but also obtained a strain of C. cellulovorans capable of producing n-butanol and ethanol from cellulose. It is possible that by further metabolic engineering and process engineering of this strain, a much higher n-butanol titer could be
achieved.

In recent years, a series of pMTL80000 *Clostridium* shuttle plasmids were generated and had been successfully transformed into *C. acetobutylicum*, *C. difficile*, *C. botulinum*, and *C. tyrobutyricum*, though the transformation efficiency varied (Heap et al., 2009; Yu et al., 2012). However, in this study, among the four plasmids, only pMTL83151-adhE2 was successfully transformed into *C. cellulovorans*, suggesting that pCB102 replicon from *C. butyricum* was functional in *C. cellulovorans*. In addition, since adhE2 under the thiolase promoter (Pthl) from *C. tyrobutyricum* was actively expressed, indicating that Pthl from *C. tyrobutyricum* was also functional in *C. cellulovorans*. To develop the genetic tools of transformation of *Clostridia*, more systemic studies about the shuttle plasmids with various Gram-positive replicons and strong promoter are needed in future.

Besides the lack of developed shuttle plasmids, difficult transformation of *Clostridia* may be also due to their Gram-positive cell wall with thick peptidoglycan layer. Approaches to weaken the cell wall have been shown to improve the transformation efficiency mildly (about 10-fold) in *C. thermocellum* (Tyurin et al., 2004) and in *C. pasteurianum* (Pyne et al., 2013), on the basis that they were transformable without cell-wall-weakening treatments. Furthermore, this method was strain sensitive. Thus, a more universal method was needed.

Besides the factors of shuttle plasmids and cell-wall structures, RM systems existing in the bacteria as defensive machineries seemed to be the most dominant factor in affecting the transformation efficiency (Roberts et al., 2010). In recent years, because of the increased sequenced bacteria genomes as well as the increased RM studies, RM
systems became better understood. Putative RM systems in various frequently-used Clostridia species were analyzed *in silico* on REBASE (shown in Table 3.5). The analysis suggested that RM systems were common defensive machineries in Clostridia, resulting in low transformation efficiency. To achieve successful transformations of Clostridia, circumvention of their restriction barriers was the key. Recently, successful identifications of the restriction endonucleases in Clostridia led to the studies to overcome the restriction barriers, employing different methods. The first one is to disrupt the restriction endonucleases in the genomes (Cui et al., 2012; Dong et al., 2010) using the ClosTron system (Heap et al., 2007) to allow the acceptance of unmethylated plasmids by the host. However, this strategy worked only when the transformation methods were available to enable the transfer of the ClosTron plasmids. The second one used plasmids with no or less restriction sites recognized by RM systems in the host to avoid plasmid degradation (Lee et al., 1992; Purdy et al., 2002). The number of restrictions sites in the plasmids could affect the transformation efficiency. Transformants could be obtained using plasmids with less than 4 unprotected sites in certain strains. However, the transformation efficiency decreased as an exponential function of the number of unprotected sites (Elhai et al., 1997). This may be one of the reasons that for some *Clostridia* transformation, plasmid methylation was not necessary. However, using plasmids with no or less restriction sites, or reducing restriction sites may not apply to all plasmids and strains. The third approach is to temporarily inactivate the RM system by heat (Lin and Blaschek, 1984) to facilitate transformation. However, this method was only reported to be effective to certain strains. Last, methylation of plasmids prior to transformation is used to enhance the transformation efficiency. The key for this strategy
to success is the utilization of proper MTs, recognizing the same restriction sites as the REs in the host. Successful transformation was shown to be facilitated significantly by plasmid methylation or even to be absolutely dependent on plasmid methylation in several Clostridia species as no transformants could be obtained without methylation (see Table 3.6). In the present study, plasmid methylation was necessary to facilitate the transformation of *C. cellulovorans*, from no transformant (zero success) without methylation to a few transformants after methylation. Although several other studies had used *in vitro* or *in vivo* methylation to increase Clostridia transformation efficiency, this study identified and utilized native methyltransferases for methylation for the first time. Methylation with native methyltransferases could avoid unknown negative side effects. The knowledge about the RM systems obtained in this study should also contribute to future metabolic engineering of other hardly-transformed but valuable Clostridia for industrial applications.

### 3.5 Conclusion

In summary, the RM systems (*Cce743I* and *Cce743II*) of *C. cellulovorans* were identified by genome analysis, restriction assay, and bisulfite assay with the details of gene sequences, gene functions, restriction specificity, and methylation specificity. An *in vivo* methylation system, employing native M. *Cce743I* and M. *Cce743II*, was established to protect plasmids from being degraded. Methylation of shuttle plasmids significantly enhanced transformation, making the transformation of *C. cellulovorans* possible. For the first time, a recombinant strain of *C. cellulorovans* producing n-butanol and ethanol from crystalline cellulose was obtained by expressing *adhE2*. To our
knowledge, this is the first example of metabolic engineering of microbes for n-butanol and ethanol production directly from cellulose, providing a promising platform for biofuel production from cellulosic biomass.

3.6 References


<table>
<thead>
<tr>
<th>Strains/plasmids</th>
<th>Relevant characteristics</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. cellulovorans 743B</td>
<td>DSM 3052</td>
<td>DSMZ</td>
</tr>
<tr>
<td>C. cellulovorans/83151- adhE2</td>
<td>C. cellulovorans with plasmid pMTL83151- adhE2</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli DH10β</td>
<td>Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- Φ80lacZΔM15 recA1 relA1 endA1 supG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC)</td>
<td>NEB</td>
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<td>E. coli DH5α</td>
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<td>E. coli XY1</td>
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<tr>
<td>E. coli XY1/83151-adhE2</td>
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<tr>
<td>E. coli XY1/84151-adhE2</td>
<td>E. coli XY1 with pMTL84151-adhE2</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli XY1/85151-adhE2</td>
<td>E. coli XY1 with pMTL85151-adhE2</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
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<tr>
<td>pMTL82151-adhE2</td>
<td>ColE1 ori; pBP1 ori; CmR; Pthl::adhE2</td>
<td>Yu et al., 2012</td>
</tr>
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<td>Yu et al., 2012</td>
</tr>
<tr>
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<td>Yu et al., 2012</td>
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<tr>
<td>pMTL85151-adhE2</td>
<td>ColE1 ori; pLM13 ori; CmR; Pthl::adhE2</td>
<td>Yu et al., 2012</td>
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<tr>
<td>pACYC184</td>
<td>p15A ori; CmR; TcR</td>
<td>NEB</td>
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<td>pXY1</td>
<td>p15A ori; TcR; PT7::M.Cce7431&amp; M.Cce743II</td>
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Table 3.1 Strains and plasmids used in this study
<table>
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<td></td>
<td>3231780..3232556</td>
<td>SAM protein</td>
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Table 3.2 Analysis of the putative target sites of the candidate RM system
Table 3.3 Analysis of the methylation specificity. After bisulfite treatment of the genomic DNA, the cytosine was converted to uracil. Therefore, cytosine was replaced by thymine in the forward primers, while guanine was replaced by adenine in the reverse primers. The small "t" in the primers originally was cytosine in the sense strand. The small "a" in the primers originally was guanine in the antisense strand.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Methylation sites</th>
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<tr>
<td>f: GTGGA&lt;sup&gt;t&lt;/sup&gt;TGTAT&lt;sup&gt;A&lt;/sup&gt;GAAGTGAGAATGTGGGtATAAG</td>
<td>Cce743II: m&lt;sup&gt;C&lt;/sup&gt;CCTGG; m&lt;sup&gt;C&lt;/sup&gt;CCAGG</td>
</tr>
<tr>
<td>r: CaCTCCCCATCACACCTCaAaTATTAaCACaC</td>
<td>Cce743I: GACG&lt;sup&gt;m&lt;/sup&gt;C</td>
</tr>
<tr>
<td>f: AGGTTGAAGtGAGGGTAAAAAttTtGTGGAGGAttG</td>
<td>Cce743I: GCGTC</td>
</tr>
<tr>
<td>r: TCCCACATCCCTTTTCCACTAACTTACACT</td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
<td>Size (bp)</td>
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<td>----------</td>
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<td>pMTL82151-adhE2</td>
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<td>pMTL84151-adhE2</td>
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<td>pMTL85151-adhE2</td>
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Table 3.4 Analysis of restriction sites of the shuttle plasmids. Results were analyzed by NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/).
<table>
<thead>
<tr>
<th>Organisms</th>
<th>Genome size (Mbp)</th>
<th>Genes</th>
<th>Putative REs</th>
<th>Putative MTs</th>
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<tr>
<td><em>C. acetobutylicum</em> ATCC 824</td>
<td>3.94</td>
<td>3672</td>
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<td><em>C. beijerinckii</em> NCIMB 8052</td>
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<td><em>C. cellulovorans</em> 743B</td>
<td>5.26</td>
<td>4254</td>
<td>6</td>
<td>13</td>
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<td><em>C. difficile</em> 630</td>
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<td>3897</td>
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<tr>
<td><em>C. ljungdahlii</em> DSM 13528</td>
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<td><em>C. pasteurianum</em> BC1</td>
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<td>4</td>
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<tr>
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<td>7</td>
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<td>3.84</td>
<td>3137</td>
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<td>12</td>
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</tbody>
</table>

Table 3.5 Preliminary analysis of putative RM in Clostridia. Results were analyzed by REBASE (http://rebase.neb.com)
Table 3.6 The effects of methylation on transformation efficiency. Except for *C. difficile*, all strains were transformed by electroporation and the transformation efficiency was calculated as the number of transformants per µg DNA. For *C. difficile*, transformation was done by conjugation, and the transformation efficiency was calculated as the number of conjutrasnformants per donor cell (*E. coli*).
Figure 3.1 Metabolic pathways in *C. cellulovorans* with heterologous butanol and ethanol synthesis pathways (shown in dotted lines) for butanol and ethanol production by overexpressing aldehyde-alcohol dehydrogenase 2 (*adhE2*).
Figure 3.2 Genome analysis of the putative RM systems in *C. cellulovorans* by REBASE ([http://rebase.neb.com](http://rebase.neb.com)). Twelve operons were found, consisting of putative RE, MT, and S subunits. The operons marked with stars (★) were the candidates for further analysis.
Figure 3.3 Predictions of restriction assay. The sequence of pMTL82151-\textit{adhE2} was input into NEB cutter (http://tools.neb.com/NEBcutter2/) to analyze the restriction pattern of \textit{Cce743I}, \textit{Cce743II}, and both.
Figure 3.4 Characterization of the restriction profile by restriction assay (1% agarose gel). The plasmid (pMTL82151-\textit{adhE2}) was restricted by the cell lysate of \textit{C. cellulovorans} after overnight incubation. Bands around 3 kb were observed. Lane 1, 1 kb DNA Ladder (NEB); Lane 2, the plasmid in the digestion buffer without the addition of cell extract; Lane 3, the plasmid was incubated with the cell extract for 0 h; Lane 4, the plasmid was incubated with the cell extract for 2 h; Lane 5, the plasmid was incubated with the cell extract for 20 h.
Figure 3.5 Generation of pXY1 plasmid by cloning native M. Cce743I and M. Cce743II into pACYC 184 vector. Amplified M. Cce743I and M. Cce743II with T7 promoter was ligated into pACYC 184 at the distal site of PvuII and NcoI site to generate pXY1.
Figure 3.6 Establishment of the in vivo methylation system with M. Cce743I and M. Cce743I activities. pXY1 was transformed into E. coli DH10β to generate E. coli XY1. Plasmid (e.g., pMTL82151-adhE2) was further transformed into competent E. coli XY1 for methylation in vivo, followed by plasmid purification.
Figure 3.7 Restriction assay showing the protection effect of *in vivo* methylation system (1% agarose gel). The unmethylated and methylated plasmids pMTL82151-*adhE2* (left panel) and pMTL83151-*adhE2* (right panel) were incubated with the cell lysate of *C. cellulovorans* for restriction assay. After incubation for 20 h, the unmethylated plasmids were restricted, showing a major band around 3 kb, while the methylated plasmids remained unchanged, compared to the negative control. Lane 1, 1 kb DNA Ladder (NEB); Lane 2, unmethylated plasmid incubated with the cell lysate for 0 h (negative control); Lane 3, unmethylated plasmid incubated with the cell lysate for 20 h; Lane 4, methylated plasmid incubated with the cell lysate for 0 h (negative control); Lane 5, methylated plasmid incubated with the cell lysate for 20 h.
Figure 3.8 Confirmation of the plasmid in *C. cellulovorans*/83151-**adhE2** (1% agarose gel). The plasmid was extracted from *C. cellulovorans*/83151-**adhE2**, followed by transformation into *E. coli* DH5α and then plasmid purification from *E. coli* for confirmation by PCR and enzyme digestion. Lane 1, positive control of PCR of **adhE2** (2.8 kb); Lane 2, PCR of **adhE2** (2.8 kb); Lane 3, 1 kb DNA Ladder (NEB); Lane 4, enzyme digestion by *Bam*HI and *Sac*II (4.5 kb + 2.6 kb).
Figure 3.9 Enzyme activities of adhE2. The butyraldehyde dehydrogenase activity and butanol dehydrogenase activity of adhE2 in C. cellulovorans were measured. The butyraldehyde dehydrogenase activity of the WT and the mutant strain C. cellulovorans/83151-adhE2 were 0.006 U/mg and 0.1619 U/mg, respectively. The butanol dehydrogenase activities of the WT and C. cellulovorans/83151-adhE2 were 0.0112 U/mg and 0.0774 U/mg, respectively (n ≥ 3).
Figure 3.10 *C. cellulovorans* / 83151-adhE2 produced butanol and ethanol. 1.42 g/L n-butanol and 1.60 g/L ethanol were produced when grown on crystalline cellulose (15 g/L) in 252 h (n = 3). No ethanol or butanol production was observed in the wild-type *C. cellulovorans*. 
Chapter 4: Biofuel production directly from cellulosic biomass by fermentation with *Clostridium cellulovorans/83151-adhE2*

Abstract

Biofuel has been considered as a renewable, sustainable and environmentally-friendly fuel to replace petroleum fuel or be used as fuel additive. Consolidated bioprocessing (CBP) has been proposed to produce the third-generation biofuel directly from lignocellulosic biomass, combining cellulase production, cellulosic hydrolysis, and fermentation into a single-step process. Cellulolytic *Clostridium cellulovorans* has been engineered to produce n-butanol and ethanol by expressing *adhE2*. To further understand the fermentation properties of the engineered *C. cellulovorans*, the detailed fermentation kinetics was studied in this Chapter. Specifically, growth media was optimized to enhance cell growth and thus facilitate biofuel production. And fermentation in bioreactor with constant agitation and pH control led to relatively higher n-butanol and acids production but relatively lower ethanol production, compared to the result of fermentation in serum bottles. In addition, the carbon flux of the engineered *C. cellulovorans* shifted from C4 towards C2 products, which was different from that of wild-type *C. cellulovorans*. In summary, basic fermentation conditions for *C. cellulovorans* were developed, and the fermentation kinetics of the engineered *C. cellulovorans* were studied.
4.1 Introduction

Nowadays, with limited supply but increased demand of petroleum fuels, it is indispensable to develop economical, sustainable and environmental-friendly biofuels, such as ethanol and butanol fuel. While biofuels production technology continues to improve, cellulosic biofuels are of particular interest. Cellulosic biomass is the most abundant biomass. The utilization of cellulosic biomass for biofuel production not only can lower the feedstock cost, but also avoid the competition with human food. However, due to the crystalline structure of cellulose, it is hard to be utilized. The current technology converting cellulosic biomass into biofuel includes the following steps: feedstock pretreatment, cellulase production, saccharification, fermentation, and downstream product recovery. Within these steps, cellulase production is expensive and contributes to $0.30 per gallon ethanol, based on a best-case estimate (Lynd et al., 2008). To decrease the cost of conversion of cellulosic biomass to sugars, decreasing cellulase cost by increasing cellulase efficiency is one possible scenario. On the other hand, CBP that incorporates cellulase production, hydrolysis, and fermentation in a single process step provides another improvement possibility.

Since there is no native cellulolytic biofuel-producer that could produce substantial biofuel from cellulosic biomass (Guedon et al, 2002; Argyros et al., 2011; Jin et al., 2011; Tolonen et al., 2011; Virunanon et al., 2008; Berezina et al., 2008; Berezina et al., 2009), co-fermentation technology was applied to produce biofuel, such as n-butanol (Nakayama et al., 2011), isobutanol (Minty et al., 2013), and ethanol (Zuroff et al., 2013), directly from cellulosic biomass. In addition to co-fermentation, cellulase engineering in yeast for ethanol production (Fujita et al., 2004; Den Haan et al., 2007;
Wen et al., 2009; Tsai et al., 2010; Yanase et al., 2010; Yamada et al., 2011; BaeK et al., 2012) and *C. acetobutylicum* for butanol production (Kim et al., 1994; Sabathé and Soucaille 2003; Perret et al., 2004; Mingardon et al., 2005) were investigated.

Considering the difficulty of expressing cellulases/minicellulosomes in biofuel producers, the strategy incorporating biofuel synthesis pathways into cellulolytic microbes was also explored. An isobutanol synthesis pathway was introduced into cellulolytic *C. cellulolyticum*, by overexpressing five heterologous enzymes, that could produce up to 0.66 g/L isobutanol from cellulose (Higashide et al., 2011). In addition, *E. coli* were engineered to express cellulases and biofuel synthesis pathways, producing 0.071 g/L fatty acid ethyl esters (biodiesel), 0.028 g/L butanol, or 0.0017 g/L pinene (a precursor to a potential jet fuel) from pretreated switchgrass (Bokinsky et al., 2011). Furthermore, a strain of cellulolytic *C. cellulovorans* was engineered to produce about 1.4 g/L butanol from cellulose adhE2 gene (shown in Chapter 3).

In this study, fermentation kinetics of the engineered *C. cellulovorans* was analyzed in details. Specifically, how media compositions and bioreactor affected the fermentation kinetics, and how the fermentation kinetics of *C. cellulovorans* changed with the expression of *adhE2* were all investigated.

### 4.2 Materials and Methods

#### 4.2.1 Cultural media and bacteria growth

WT *C. cellulovorans* and *C. cellulovorans/83151-adhE2* were cultured in media 320 (DSMZ) or modified media 520 (DSMZ). Cells were grown in modified media 520, containing (per liter): (NH₄)₂SO₄, 1.3 g; KH₂PO₄, 1.5 g; K₂HPO₄·3H₂O, 2.9 g;
MgCl₂·6H₂O, 0.2 g; CaCl₂·2H₂O, 75 mg; FeSO₄·7H₂O, 1.25 mg; resazurin, 1 mg; yeast extract, 2 g; tryptone, 4 g; cysteine-HCl, 0.5 g. The pH of the media was adjusted to 6.0 and the media was sparged with N₂ gas before autoclaving. Sterile 50× SL-10 trace elements, 50× Na₂CO₃ (10% w/v), and carbon source were added before use. Media 320 contained (per liter): K₂HPO₄·3H₂O, 1.0 g; NH₄Cl, 1.0 g; KCl, 0.5 g; MgSO₄·7H₂O, 0.5 g; resazurin, 1 mg; trypticase peptone, 0.5 g; yeast extract, 0.5 g; cysteine-HCl, 0.15 g. Media was sparged with 80% N₂ and 20% CO₂ gas mixture prior to autoclaving. Then sterile 50× SL-10 trace elements, 100× Na₂CO₃ (10% w/v), 50× Na₂S·9H₂O (0.75% w/v), and carbon source were added before use. The final pH was about 7.0. 1000× SL-10 trace elements contained (per liter): HCl (25%; 7.7 M), 10 mL; FeCl₂·4H₂O, 1.5 g; ZnCl₂, 70 mg; MnCl₂·4H₂O, 100 mg; H₃BO₃, 6 mg; CoCl₂·6H₂O, 190 mg; CuCl₂·2H₂O, 2 mg; NiCl₂·6H₂O, 24 mg; Na₂MoO₄·2H₂O, 36 mg. Then, it was diluted 20 times to make 50× trace elements. When serum bottles were used for fermentation, less sugar was added (around 15 g/L glucose, cellobiose, or cellulose). When bioreactor was used for fermentation, more sugar was added (around 40-50 g/L glucose or cellobiose, 15-20 g/L avicel). When avicel (a commercial name of cellulose) was used as the main carbon source, a small amount of glucose/cellobiose (around 3 g/L) was added to initiate the cell growth for overnight. Modified Media 520 was supplemented with 30 µg/mL thiamphenicol when growing C. cellulosorans/83151-adhE2.

4.2.2 Fermentation

Fermentation was performed either in serum bottle with 50 mL cell culture or bioreactor with 500 mL cell culture. 10% seed (OD = 0.5-1.0) was inoculated. Cells in
serum bottles were grown in an incubator at 37 °C. When cells were grown on glucose or cellobiose, samples were taken twice per day from serum bottles to monitor cell growth and metabolites production. When cells were grown on avicel, samples were taken once per day from serum bottles to monitor metabolites production. After each sampling, pH was adjusted by adding NaOH solution to maintain the pH between 6.0-7.0.

When bioreactor was used for fermentation, the growth temperature was controlled at 37 °C, and the pH was controlled at 6.5 by adding ammonia solution automatically with a pH controller and pump. Samples were taken twice per day (when grown on glucose/cellobiose) or once per day (when grown on avicel) to monitor cell growth and metabolites production.

4.2.3 Analytical methods

Cell density was analyzed by measuring the optical density of cell suspension at 600 nm using a spectrophotometer (Shimadzu, Columbia, MD, UV-16-1). Acids and alcohols including acetic acid, butyric acid, ethanol and n-butanol were measured with a gas chromatograph (GC) (GC-2014 Shimadzu, Columbia, MD) equipped with a flame ionization detector (FID) and a 30.0 m fused silica column (Stabilwax-DA, 0.25 mm film thickness and 0.25 mm ID, Restek, Bellefonte, PA). The GC was operated at an injection temperature of 200 °C with 1 µL of sample injected with an auto injector (AOC-20i, Shimadzu). The column temperature was initially held at 80 °C for 3 min, then increased at a constant rate of 30 °C per min to 150 °C, and held at 150 °C for 3.7 min. Samples were centrifuged at 13,200 rpm for 5 min in 1.5 ml microcentrifuge tubes and and supernatant was subjected to GC.
Glucose and cellobiose were quantified by high performance liquid chromatography (HPLC, LC-20AD, Shimadzu, Columbia, MD) with an organic acid column (Bio-Rad HPX-87H, ion exclusion organic acid column, 300 × 7.8 mm). Cellulose sample (1 ml) was washed by distilled water prior to autoclaving. After autoclaving, the cellulose pellet was washed again with distilled water. Afterwards, it was resuspended in 1 ml distilled water at pH 4.5, and was hydrolyzed with 20 µl Accellerase 1500 cellulase (Dupont Industrial Biosciences, New York, US) at 50 °C for 7 days with standards, followed by measurement of glucose by HPLC. Samples were centrifuged at 13,200 rpm for 15 min in 1.5 ml microcentrifuge tubes and diluted 10 times with distilled water prior to analysis on HPLC. HPLC was run at 45 °C using 0.007N H₂SO₄ (0.7 ml H₂SO₄ in 2 L distilled water) as the eluent at a flow rate of 0.6 mL/min. 15 µl sample was injected by an automatic injector (SIL-10Ai) and the running time was set at 40 min. A refractive index (RI) detector (Shimadzu RID-10A) was set at the range of 200 to detect the organic compounds in the sample. The HPLC column was installed in a column oven (CTO-10A) with temperature control at 45 °C. Peak height was used to calculate concentration of sugars in the sample based on the peak height of standard sample.

4.2.4 Butyric acid tolerance assay and butanol tolerance assay

*C. cellulovorans* was inoculated into 520 media with glucose as carbon substrate, and grew to OD600=1.0. 20% active cell culture was inoculated into 520 media supplemented with different concentrations of butyric acid or butanol. In butyric acid tolerance assay, 0 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, and 25 g/L butyric acid were tested. In butanol tolerance assay, 0 g/L, 4 g/L, 6 g/L, 8 g/L and 10 g/L butanol were tested. The
cell growth was monitored every several hours by measuring OD600.

4.3 Results

4.3.1 Media optimization

Media 320 and media 520 were suggested by DSMZ, from which *C. cellulovorans* 743B strain was purchased, for growth. The recipes are listed in Table 4.1. Fermentation kinetics in the two media was compared with glucose, cellobiose and cellulose as carbon sources (see Figure 4.1). The major products were acetic acid and butyric acid. Though it was reported that trace of lactic acid and formic acid were also produced (Sleat et al., 1984), they were all under detectable level and could be ignored. Therefore, only acetic acid, butyric acid, or later ethanol and butanol from the newly developed strain were shown. It was observed that the OD600 of cells in media 320 was much lower than that in media 520, resulting in much lower acids production, regardless of the carbon source used. This was further confirmed with the analysis of specific growth rates, acid titers, yields and productivities (shown in Figure 4.2). There were several possible reasons. Firstly, the cysteine-HCl in media 520 (0.5 g/L) was higher than that in media 320 (0.15 g/L). Cysteine-HCl was susceptible to oxidation and served as a reducing reagent in many other Clostridia medium. Since *C. cellulovorans* is an obligate anaerobe, the amount of cysteine-HCl could be critical to maintain the anaerobic environment, thus affecting the cell growth. Secondly, the nitrogen sources in media 520 (tryptone 4 g/L, yeast extract 2 g/L) were also much higher than that in media 320 (trypticase peptone 0.5 g/L, yeast extract 0.5 g/L). The amount of nitrogen sources was important for cell growth. Based on the fermentation kinetics, acid production seemed to
be positively correlated to cell growth. Lower nitrogen sources in media 320 could be one of the factors leading to low cell growth. Lastly, the buffer capacities were also largely different. Media 520 contained both KH$_2$PO$_4$ and K$_2$HPO$_4$·3H$_2$O with a stronger buffer capacity, compared to media 320, containing only K$_2$HPO$_4$·3H$_2$O. *C. cellulovorans* is an acid producing strain. Thus, the pH of media could drop very quickly. It was also observed that when pH was lower than 5.5 or even 6.0, cell growth was inhibited, entering an inactive state. When pH was adjusted twice or even less per day manually, low pH could be a problem. Therefore, it was concluded that media 520 was a better choice for *C. cellulovorans* fermentation.

Corn steep liquor, a by-product of corn wet-milling, containing proteins, carbohydrates, and organic acids (e.g., lactic acid), vitamins, and minerals (White and Johnson, 2003). Since it contains about 50% proteins in the dry weight, is an inexpensive nitrogen source to replace yeast extract or peptone. It was used for a wide variety of microbial fermentations (Lawford and Rousseau, 1997; Maddipati et al., 2011; Saxena et al., 2012; Jang et al., 2013). To test the effect of corn steep liquor on fermentation of *C. cellulovorans* in serum bottles, 30 g/L corn steep liquor was used to replace yeast extract and tryptone in 520 media. As shown in Figure 4.3, the fermentation result of *C. cellulovorans* when corn steep liquor was used was comparable to that when yeast extract and tryptone were used. To analyze fermentation kinetics of *C. cellulovorans*, synthetic media with yeast extract and tryptone were used in this study. However, corn steep liquor was shown to be an alternative inexpensive nitrogen source for fermentation of *C. cellulovorans*. 
4.3.2 Fermentation of WT C. cellulovorans in bioreactor

With the optimized growth media, the fermentation kinetics of WT C. cellulovorans using glucose, cellobiose, and cellulose as substrates in bioreactor was analyzed (see Figure 4.4). The result showed that about 10 g/L butyric acid and 2.0-2.5 g/L acetic acid were produced from about 34 g/L glucose or 52 g/L cellobiose consumed. Though the final titers of butyric acid and acetic acid from glucose and cellobiose were very close, cellobiose consumption was much higher than glucose consumption, leading to lower product yield. The yield of butyric acid and acetic acid from cellobiose was 0.206 g/g and 0.037 g/g, respectively, compared to 0.299 g/g and 0.061 g/g from glucose. Since the cell growth on cellobiose was comparable with that on glucose, the deceased yield might be due to cellobiose breakdown. It was observed that when cellobiose was used as the carbon source, there is only trace of glucose detected in the medium (less than 0.5 g/L), suggesting most of the cellobiose was uptaken directly, or it was degraded into glucose, which was then uptaken very soon, without diffusing into the medium. When cellulose was used as carbon source, about 5.5 g/L butyric acid, and 1.7 g/L acetic acid were produced, with the yield of 0.367 g/g and 0.113 g/g. Even though the acid titers were lower, compared to those from glucose and cellobiose, the yields were much improved. This result also suggested that less carbon flux towards cell growth. Therefore, the improved yields were the results of the balance between more energy consumption for cellulose degradation and less energy consumption for cell growth. In order to hydrolyze cellulose, the expression of cellulosomal proteins had to be induced first, followed by cellulose degradation and sugar uptake. When cellulose was used as the carbon source, only trace of cellobiose and glucose detected in the medium, suggesting
most of the cellobiose and glucose degraded from cellulose was uptaken very soon, without diffusing into the medium. Also it was observed that the cells took about one day to start to consume cellulose, and the fermentation process was much slower than that using glucose and cellobiose as substrates, taking about 10 days to consume about 15 g/L cellulose. Even so, *C. cellulovorans* was adapted to utilize cellulose efficiently, producing butyric acid as the main product.

### 4.4.3 Measurement of cellulose

Cellulose is a polysaccharide consisting linear chains of glucose units. It was white powder, with no solubility in water. Therefore, to measure the cellulose kinetics after fermentation, the key step is to degrade it to produce glucose, which can be measured by HPLC. There are many ways to degrade cellulose, mostly reported for cellulosic biomass pretreatment, including acid treatment, alkali treatment, followed by cellulase treatment. Acid treatment and alkali treatment were used mainly for lignin breakdown as well as partial cellulose degradation. However, the cellulose used for fermentation in this study was crystalline cellulose. Therefore, acid and alkali treatment were not necessary. In addition, since *C. cellulovorans* cells were attached to cellulose during fermentation, acid or alkali may cause some bias during treatment. Therefore, cellulase treatment was chosen. Two kinds of commercial cellulases were tested, including Cellic CTec2 and Accellerase 1500 cellulase. Standard samples with various concentrations were subject to cellulase hydrolysis, followed by quantification by HPLC. The result showed that samples hydrolyzed by Cellic CTec2 showed multiple peaks, while samples hydrolyzed by Accellerase 1500 cellulase produced single glucose peak,
though hydrolysis was not complete. Therefore, Accellerase 1500 cellulase was chosen for cellulose quantification with further hydrolysis condition modification. To improve cellulose hydrolysis, three factors were considered, including the amount of cellulase loading, removing of cell debris from cellulose, pH for hydrolysis. Autoclaving and repeated cellulose washing were applied prior to hydrolysis to facilitate the cell removal from cellulose to increase the accessibility of cellulose. And the amount of cellulase loading was increased from 1 µl suggested by the protocol to 10 µl (Condition I) and 20 µl (Condition II). The results of cellulose quantification in two conditions are shown in Table 4.2 and Figure 4.5A. The hydrolyzed cellulose concentrations were all proportionally correlated to the initial cellulose loadings with at least 0.98 R² value. However, hydrolysis under condition II was better (about 83% hydrolysis), compared to that under condition I (about 58% hydrolysis). The fermentation samples were then measured for test, with the fermentation kinetics shown in Figure 4.5B. The result of measured cellulose concentration at 0 h was close to actual cellulose loading, suggesting that this method was valid for fermentation samples as well. Therefore, a method to measure cellulose was established.

4.3.4 Butyric acid tolerance

To test butyric acid tolerance of C. cellulovorans, the effect of various concentrations of butyric acid on cell growth were investigated. As shown in Figure 4.6, cell growth in terms of OD600 and specific growth rate could tolerate with up to 10 g/L butyric acid. When butyric acid concentration increased to 15 g/L or higher, cell growth was greatly inhibited. However, the result of tolerance assay could not accurately indicate
the maximum titer of butyric acid during fermentation. This was because in tolerance assay, butyric acid was added from the beginning, when the cell density was very low with OD600 around 0.2, enhancing its inhibitory effect. In addition, the cells did not undergo adaption. However, during real fermentation process, cell density increased with butyric acid concentration, which also allowed an adaptive process. Therefore, the potential maximum butyric acid titer of fermentation of \textit{C. cellulovorans} would be higher than 10 g/L.

\textbf{4.3.5} \textit{n-Butanol tolerance}

To evaluate the \textit{n}-butanol-producing potential of the engineered \textit{C. cellulovorans}, \textit{n}-butanol tolerance assay was conducted as well. As shown in Figure 4.7, cell growth was inhibited when 8 g/L or higher \textit{n}-butanol was present. Since \textit{n}-butanol was added from the beginning, when the cell density was very low (OD600\textasciitilde{}0.2). In addition, cells did not undergo adaptation process prior to the test. Therefore, \textit{n}-butanol tolerance should be higher than the boundary tested in the tolerance assay.

\textbf{4.3.6 Initial fermentation of \textit{C. cellulovorans}/83151-\textit{adhE2} in serum bottles}

To genetically engineer \textit{C. cellulovorans} for biofuel production, a plasmid harboring \textit{adhE2} gene was successfully transformed into it, and the recombinant strain \textit{C. cellulovorans}/83151-\textit{adhE2} was confirmed to have \textit{adhE2} enzyme activity and \textit{n}-butanol and ethanol production were detected (as discussed in Chapter 3). Initial fermentation in serum bottles was performed, with the kinetics shown in Figure 4.8. The recombinant \textit{C. cellulovorans} produced 1.61-1.68 g/L butanol, 2.0 g/L ethanol, 0.6-0.84 g/L butyric acid
and 2.2-2.33 g/L acetic acid from 13.6 g/L glucose or 20.6 g/L cellobiose. Higher cellobiose consumption than glucose consumption was observed, leading to lower yields from cellobiose, which was consistent with phenomenon observed in fermentations by wild-type *C. cellulovorans*. When cellulose was used as the carbon substrate, 1.42 g/L butanol and 1.6 g/L ethanol in addition to 0.9 g/L butyric acid and 2.62 g/L acetic acid were produced. It is clear that the recombinant strain produced a significant amount of alcohol with decreased acid production, compared to the wild-type strain.

### 4.3.7 Improved n-butanol titer with fermentation in bioreactor

To scale up, fermentations in bioreactor with 500 ml working volume were tested. When cells were grown in bioreactor, pH was maintained at 6.5 by monitoring pH and adding ammonia solution automatically with agitation. The fermentation kinetics are shown in Figure 4.9. The result showed that 2.81 g/L butanol, 1.4 g/L ethanol, 2.06 g/L butyric acid and 3.76 g/L acetic acid were produced from 22.5 g/L glucose. And 4.55 g/L butanol, 2.74 g/L ethanol, 2.37 g/L butyric acid, and 4.57 g/L acetic acid were produced with 51.3 g/L cellobiose consumed. It seemed that acids production and alcohols production from cellobiose were much better than that from glucose, which was different when fermentations were conducted in serum bottles. However, since cellobiose consumption was much higher than glucose consumption, the metabolites yields from cellobiose were still lower. When cellulose was utilized as the carbon source, 1.68 g/L butanol, 0.62 g/L ethanol, 1.87 g/L butyric acid, and 3.0 g/L acetic acid were produced. Compared to serum bottle fermentation, n-butanol production was significantly improved in bioreactor.
4.3.8 Comparison of fermentation in serum bottle and bioreactor

To compare the fermentation patterns in serum bottle and in bioreactor in details, the metabolite titer and yield are summarized in Table 4.3. Since cell growth was much better and sugar consumption was higher in bioreactor with pH maintained at 6.5, n-butanol titer and acetic acid titer increased proportionally with sugar consumption, with similar n-butanol and acetic acid yields. However, ethanol yield decreased about 50%, while butyric acid yield increased about 2 fold in bioreactor. It was reported agitation facilitated H$_2$ release from fermentation broth, which affected NADH production, and thus resulted in dramatic change of the acid/alcohol ratios in \textit{C. thermocellum} and \textit{C. acetobutylicum} \cite{Zertuche1982,Doremus1985,Lamed1988}. The hydrogenase reaction occurs through ferredoxin in the organism, which could transfer electron to either H$^+$ or NAD$^+$ to form H$_2$ or NADH. Since the electron transfer reactions are reversible, NADH formation can be positively affected by the level of dissolved H$_2$ in the fermentation broth. Since formation of alcohols require extra NADH, less dissolved H$_2$ caused by agitation facilitated acid production, leading to increased the acid/alcohol ratio in bioreactor. In addition, the C2/C4 ratio decreased from 1.5-1.8 to 0.9-1.0, suggesting a carbon flux shift from C2 products to C4 products. It was also noticed that the ethanol/butanol ratio decreased 40%-70% in bioreactor, suggesting butanol production was preferred over ethanol production. The changes of metabolites production are also summarized in Figure 4.10. In summary, butanol production was improved, while the production of acids was also increased in bioreactor. To further enhance alcohols production and decrease acids production, an artificial electron carrier can be added, which will be discussed in Chapter 5.
4.3.9 Comparison of fermentation of WT and C. cellulovorans/83151-adhE2

Since an alcohol dehydrogenase was introduced in C. cellulovorans, the metabolite production pattern changed significantly, as summarized in Table 4.4. All the data were collected from fermentations done in bioreactor. Since ethanol and n-butanol were produced, acid production changed significantly. Butyric acid production decreased 65%-80%, while acetic acid titer increased 50%-125%. In addition, C2/C4 ratio increased 2.5-4.5 fold in the engineered stain (shown in Figure 4.11), suggesting a shift of carbon flux from C4 products to C2 products. Except that, the total metabolites yields of the wild-type and the recombinant C. cellulovorans were very similar, about 0.36-0.40 g/g, 0.24-0.26 g/g, and 0.48 g/g from glucose, cellobiose and cellulose, respectively. Therefore, the redistribution of metabolite production might be because of lack of driving force, limiting the carbon flux from C2 to C4 products. To further enhance butanol production, NADH availability can be increased and the butanol synthesis pathway can be strengthened.

4.4 Discussion

Media optimization was important for fermentation and can enhance cell growth and metabolite production significantly without engineering the strains (Balusu et al., 2004; Cha et al., 2004; Sim et al., 2008). In this study, two media were compared, with significant differences in buffering capacity, nitrogen source, and reducing reagents. Though C. cellulovorans was an acid-producing bacterium, it could not tolerate pH lower than 5.5. Therefore, the buffering capacity of the media was very important, when the pH could not be adjusted constantly. Obviously, the buffering capacity of modified 520
media was much better than that of 320 media, with a pair of buffering salts. Besides buffering capacity, nitrogen source was another important factor, effecting cell growth. As shown in the fermentation kinetics, metabolite production was correlated with cell growth, suggesting that slow cell growth could limit metabolite production. Therefore, the amount of nitrogen source in modified 520 media was significantly increased to support cell growth. Moreover, reducing reagent may be the most critical factor in this case. *C. cellulovorans* was an obligate anaerobic bacterium. Little amount of oxygen could inhibit its growth. Resazurin was used as an oxygen indicator in the media. Upon the exposure of oxygen in the media, it would turn from pink to red or even dark blue. Purging the media with nitrogen in addition to boiling helped remove most of oxygen from the media, but not all of it. Therefore, addition of reducing reagent was critical to enable anaerobic environment by reducing oxygen in the media. The reducing reagents in 320 media were 0.15 g/L cysteine-HCl and 0.15 g/L Na₂S·9H₂O. During the preparation of 320 media, it was observed that oxygen was hardly removed from the media with pink color, even though it was boiled for about 2 h while purging nitrogen in addition to autoclaving, suggesting less than enough reducing reagents present. Instead, when 0.5 g/L cysteine-HCl was added in modified 520 media, by purging nitrogen gas for 5 min-30 min in addition to autoclaving, oxygen was removed without any pink color in the media, suggesting 0.5 g/L cysteine-HCl was sufficient to keep the media in obligate anaerobic condition. By combining all these factors, acid titer, yield, and productivity by *C. cellulovorans* were significantly increased about 1.5-10 fold, through media optimization.
*C. cellulovorans* could utilize various carbon sources, including cellulose, xylan, pectin, cellobiose, glucose, maltose, galactose, sucrose, lactose, and mannose (Sleat et al., 1984). In this study, fermentation kinetics of wild-type *C. cellulovorans* grown on glucose, cellobiose, and cellulose were analyzed with butyric acid and acetic acid as the major metabolic products. In addition, butyric acid production was much higher than acetic acid, suggesting a preferred carbon flux towards butyryl-CoA, thus potential butanol production. It was also observed, when different carbon sources were used, the titer, yield and productivity were different, due to different mechanisms for sugar degradation and consumption. The structure and working model of cellulosome of *C. cellulovorans* has been proposed (Tamaru et al., 2010; 2011), consisting of scaffoldin proteins and catalytic proteins. In addition, several studies showed that the expression patthern of cellulosomal genes varied with carbon substrates, and catabolite repression of cellulosomal genes was observed when cells were grown in glucose or cellobiose (Tamaru et al., 2002; Han et al., 2003; Han et al., 2004; Cho et al., 2010). Therefore, utilization of different carbon substrates involved induced cellulases expression, carbon substrate degradation, sugar uptake, resulting in different patterns of cell growth and metabolites production. In spite of these, it was also illustrated that metabolite production was in a linear relationship with sugar consumption. In other word, metabolite production in *C. cellulovorans* increased proportionally with sugar consumption and was growth associated. When *C. cellulovorans* was engineered to produce alcohols, acid and alcohol production still increased proportionally with sugar consumption, unlike other native solventogenic bacteria, which usually undergo two stages of metabolite production (acidogenic stage and solventogenic stage).
In order to produce butanol from cellulosic biomass, *adhE2* gene was overexpressed in *C. cellulovorans* by establishing a transformation method described in Chapter 3. The fermentation kinetics of the engineered *C. cellulovorans* were analyzed in details in this chapter. Firstly, the differences of fermentations in serum bottles and bioreactor were compared. When the engineered strain was grown in serum bottles with periodical pH adjustment, 1.4-1.6 g/L butanol was produced from glucose, cellobiose and cellulose. When the engineered strain was grown in bioreactor with constant pH, 1.7-4.5 g/L butanol was produced from glucose, cellobiose, and cellulose. In addition, less ethanol but more acetic acid and butyric acid were produced in bioreactor. The differences of fermentation results might be caused by constant pH control and agitation. Better pH control facilitated cell growth and sugar consumption, resulting to higher metabolites production. Agitation could help to remove H$_2$ from media, which changed the acid/alcohol ratio by affecting NADH formation. From the perspective of butanol production, bioreactor was a better choice for fermentation of engineered *C. cellulovorans*. Secondly, the differences of fermentations between wild-type and engineered *C. cellulovorans* were compared. The wide-type strain produced 5.5-10.5 g/L butyric acid and 1.7-2.5 g/L acetic acid, while the engineered strain produced 1.9-2.4 g/L butyric acid and 3.0-4.6 g/L acetic acid in addition to ethanol and butanol from glucose, cellobiose and cellulose. It was obvious that with the expression of *adhE2* gene, less butyric acid was produced but more acetic acid was produced, suggesting that increased carbon flux from C4 products towards C2 products. Since formation of ethanol, butanol and butyryl-CoA molecules from acetyl-CoA all require NADH, NADH availability constrained the flux from acetyl-CoA to butyryl-CoA and butanol production, resulting in
the shift of carbon flux from C4 to C2. To solve this problem, increasing the availability of NADH could be an effective solution. The effects of the addition of artificial electron carrier on the enhancement of alcohols production and inhibition of acid production through increasing NADH availability will be discussed in Chapter 5.

Besides the issue of NADH, butanol tolerance may be another limiting factor. Butanol is toxic to cells, and *Clostridium* strains could not tolerate greater than 2% butanol (Knoshaug and Zhang, 2009; Liu and Qureshi, 2009). In this study, it was shown that *C. cellulovorans* could not tolerate more than 6 g/L butanol. However, whether cells undergo butanol adaption before the test, and the density and the physiological condition of cells could affected the result of butanol tolerance assay. Therefore, the engineered *C. cellulovorans* has the potential to produce higher butanol during optimized fermentation process. To obtain butanol tolerant strains, mutagenesis, metabolic engineering, evolutionary engineering by continuous culture, and serial enrichment approaches were shown to be effective (Lin and Blaschek, 1983; Hermann et al., 1985; Liyanage et al., 2000; Tomas et al., 2003; Alsaker et al., 2004).

### 4.5 Conclusion

In this chapter, fermentations of wild-type and engineered *C. cellulovorans* were investigated in details. 520 media was first shown to facilitate cell growth with sufficient buffering capacity, reducing reagent, and nitrogen source, compared to 320 media. With the optimized growth media, the engineered *C. cellulovorans* produced substantial n-butanol and ethanol in addition to butyric acid and acetic acid, though the carbon flux shifted from C4 products to C2 products, compared to the wild-type strain. In addition,
faminations of the engineered *C. cellulovorans* in bioreactor produced much higher butanol, acetic acid and butyric acid, but less ethanol, compared to those in serum bottles, suggesting that constant pH control and agitation are important factors affecting the acid/alcohol ratio. And redistributions of the carbon flux from C2 to C4 products were also observed in bioreactor. In conclusion, the basic fermentation kinetics of the engineered *C. cellulovorans* was studied in this chapter, providing a foundation for further metabolic engineering and metabolic process engineering for enhanced production of butanol and ethanol in future.

4.6 References


Zuroff TR, Xiques SB, Curtis WR. 2013. Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic Clostridium phytofermentans/yeast co-culture. Biotechnol Biofuels. 6(1):59
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Table 4.1 Comparison of 320 media and modified 520 media
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<td>2.33</td>
<td>4.57</td>
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<td></td>
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<tr>
<td><strong>Yield (g/g)</strong></td>
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<td></td>
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<tr>
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<td>0.088</td>
<td>0.055</td>
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</tr>
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<td>0.088</td>
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<td>Butyric acid</td>
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<td>0.04</td>
<td>0.044</td>
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<td>0.125</td>
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<td><strong>Acid/alcohol</strong></td>
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<td>2.12</td>
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<td>0.99</td>
<td>1.80</td>
<td>1.02</td>
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<tr>
<td><strong>Ethanol/Butanol</strong></td>
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<td>0.43</td>
<td>1.09</td>
<td>0.63</td>
<td>1.15</td>
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Table 4.3 Comparisons of fermentation results using serum bottle (n=3) and bioreactor (n=1)
<table>
<thead>
<tr>
<th></th>
<th>glucose</th>
<th>celllobiose</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>adhE2</td>
<td>WT</td>
</tr>
<tr>
<td><strong>Titer (g/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>-</td>
<td>1.40</td>
<td>-</td>
</tr>
<tr>
<td>Butanol</td>
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<tr>
<td>Acetic acid</td>
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</tr>
<tr>
<td><strong>Yield (g/g)</strong></td>
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<tr>
<td>Ethanol</td>
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<td>0.054</td>
<td>-</td>
</tr>
<tr>
<td>Butanol</td>
<td>-</td>
<td>0.126</td>
<td>-</td>
</tr>
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<td>Butyric acid</td>
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<td>0.083</td>
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Table 4.4 Comparisons of fermentation results by wild-type and engineered strain (n=1)
Figure 4.1 Comparison of 320 media and modified 520 media on fermentation kinetics (n=1)
Figure 4.2 Comparison of 320 media and 520 media on cell growth and acids production. A, Specific growth rate of *C. cellulovorans* in 320 media or 520 media; B, Acids titer of *C. cellulovorans* in 320 media or 520 media; C, Acids yield of *C. cellulovorans* in 320 media or 520 media; D, Acids productivity of *C. cellulovorans* in 320 media or 520 media. (n=1)
Figure 4.3 Comparison of yeast extract & tryptone with corn steep liquor on fermentation kinetics using glucose as carbon source. A, Fermentation kinetics using yeast extract & tryptone as nitrogen source; B, Fermentation kinetics using corn steep liquor as nitrogen source. (n=1)
Figure 4.4 Fermentation kinetics and yield of wild-type *C. cellulovorans* in bioreactor. A and B, Fermentation kinetics and yield using glucose as carbon source; C and D, Fermentation kinetics and yield using cellobiose as carbon source; E, Fermentation kinetics using cellulose as carbon source. (n=1)
Figure 4.4 continued
Figure 4.4 continued
Figure 4.5 Cellulose measurement. A, cellulose measurement using standards under two conditions (n=1); B, cellulose measurement of fermentation samples under condition II (n=3).
Figure 4.6 Butyric acid tolerance. A, Effect of different concentrations of butyric acid on OD600; B, Effect of different concentrations of butyric acid on specific growth rate. (n=1)
Figure 4.7 Butanol tolerance. A, Effect of different concentrations of butanol on OD600; B, Effect of different concentrations of butanol on specific growth rate. (n=2)
Figure 4.8 Fermentation kinetics and yield of *C. cellulovorans* 83151-***adhE2*** in serum bottles. A and B, Fermentation kinetics and yield using glucose as carbon source; C and D, Fermentation kinetics and yield using cellobiose as carbon source; E and F, Fermentation kinetics and yield using cellulose as carbon source. (n=3)
Figure 4.8 continued

**C**

Cellobiose (g/L) vs. OD and Products (g/L) vs. Time (h)

- **Cellobiose**
- **Acetic acid**
- **Butyric acid**
- **OD**
- **Ethanol**
- **Butanol**

**D**

Cellobiose (g/L) vs. Products (g/L)

- **Acetic acid**
- **Butyric acid**
- **Ethanol**
- **Butanol**

Equations for Product-Yield Correlation:

- Acetic acid: $y = -0.0948x + 3.6158$, $R^2 = 0.98891$
- Butyric acid: $y = -0.0396x + 1.2279$, $R^2 = 0.91716$
- Ethanol: $y = -0.0879x + 3.0428$, $R^2 = 0.88065$
- Butanol: $y = -0.0805x + 2.5944$, $R^2 = 0.97795$
Figure 4.8 continued

![Graph E](image)

![Graph F](image)

- Cellulose/Glucose (g/L)
  - Cellulose
  - Acetic acid
  - Butyric acid
  - Ethanol
  - Butanol

- Products (g/L)
  - Acetic acid
  - Butyric acid
  - Ethanol
  - Butanol

- Time (h)
  - 0, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264

- Equation:
  - $y = -0.1824x + 2.9852$
  - $R^2 = 0.94924$
  - $y = -0.0641x + 0.9852$
  - $R^2 = 0.93852$
  - $y = -0.1184x + 1.8263$
  - $R^2 = 0.8604$
  - $y = -0.1032x + 1.4992$
  - $R^2 = 0.95651$
Figure 4.9 Fermentation kinetics and yield of *C. cellulovorans/83151-adhE2* in bioreactor. A and B, Fermentation kinetics and yield using glucose as carbon source; C and D, Fermentation kinetics and yield using cellobiose as carbon source; E, Fermentation kinetics using cellulose as carbon source. (n=1)
Figure 4.9 continued

**C**

- **Cellobiose (g/L)**
- **Acetic acid**
- **Butyric acid**
- **OD**
- **Ethanol**
- **Butanol**

**D**

- **Butanol**
- **Ethanol**
- **Butyric acid**
- **Acetic acid**

Equations and R² values:

\[
y = -0.0876x + 5.2078, \quad R^2 = 0.97386
\]

\[
y = -0.055x + 3.4429, \quad R^2 = 0.98251
\]

\[
y = -0.0442x + 2.6946, \quad R^2 = 0.97777
\]

\[
y = -0.076x + 5.5374, \quad R^2 = 0.99655
\]
Figure 4.9 continued

![Graph showing the concentration of products over time](image-url)

- **Products (g/L)**
  - Acetic acid
  - Butyric acid
  - Ethanol
  - Butanol

- **Time (Day)**
  - 0
  - 2
  - 4
  - 6
  - 8
  - 10
  - 12
  - 14

- **Products (g/L)**
  - 0.0
  - 0.5
  - 1.0
  - 1.5
  - 2.0
  - 2.5
  - 3.0
  - 3.5
Figure 4.10 Comparison of serum bottle and bioreactor for fermentation of *C. cellulovorans/83151-adhE2*. A, Comparison of butanol titer; B, Comparison of the ethanol/butanol ratio; C, Comparison of the acid/alcohol ratio; D, Comparison of the C2/C4 ratio.
Figure 4.11 Comparison of the C2/C4 ratio by wild-type and engineered *C. cellulovorans*
Chapter 5: The effects of methyl viologen on biofuel production by fermentation

with engineered *Clostridium cellulovorans*

Abstract

Consolidated bioprocessing (CBP) has been proposed to produce biofuel directly from lignocellulosic biomass. Cellulolytic *Clostridium cellulovorans* was engineered to produce n-butanol and ethanol (see Chapter 3) with fermentations kinetics studied (see Chapter 4). Though n-butanol titer from cellulose produced by the engineered *C. cellulovorans* is the highest to date, compared to other isolated or engineered cellulolytic butanol producers, there is still much room to improve. Instead of using metabolic engineering, metabolic process engineering without genetic modifications was employed to improve n-butanol and ethanol production. In this Chapter, an artificial electron carrier, methyl viologen, was used to increase NADH availability. Since n-butanol and ethanol production was usually limited by NADH availability, increasing NADH by the addition of methyl viologen showed great impact on metabolism of the engineered *C. cellulovorans*, increasing production of n-butanol and ethanol as well as inhibiting production of acetic acid and butyric acid. Particularly, the optimum timing and concentration of methyl viologen were determined. In general, with the addition of methyl viologen, biofuel production by the engineered *C. cellulovorans* was greatly enhanced, showing a great potential for high level biofuel production directly from cellulosic biomass.
5.1 Introduction

Consolidated bioprocessing (CBP) was proposed to produce biofuel directly from cellulosic biomass. Compared to traditional fermentation process from cellulosic biomass, CBP avoided the problematic and expensive steps of cellulase production and cellulase hydrolysis. Thus, it greatly simplified the process and reduced the cost. The current CBP development is still in its infancy stage. Since there was no effective native cellulolytic biofuel-producers (Berezina et al., 2008; Virunanon et al., 2008; Berezina et al., 2009; Argyros et al., 2011; Jin et al., 2011; Tolonen et al., 2011; Guedon et al, 2012), current researches mainly focus on co-fermentation (Nakayama et al., 2011; Minty et al., 2013; Zuroff et al., 2013) and metabolic engineering (Kim et al., 1994; Sabathe et al., 2003; Perret et al., 2004; Mingardon et al., 2005; Wen et al., 2009; Tsai et al., 2010; Yanase et al., 2010; Higashide et al., 2011; Yamada et al., 2011), or by combining both strategies (Bokinsky et al., 2011). Particularly, an engineered strain \textit{C. cellulovorans} capable of producing n-butanol and ethanol directly from cellulose was generated by overexpressing \textit{adhE2} gene (Chapter 3), and fermentation studies of this strain showed that 1.7 g/L butanol and 0.6 g/L ethanol could be produced from crystalline cellulose (Chapter 4). The butanol titer from cellulose produced by the engineered \textit{C. cellulovorans} was the highest to date, compared to other strains. However, there is still room for improvement.

Metabolic engineering and metabolic process engineering are the two major ways to boost biofuel production. On one hand, metabolic engineering enhances biofuel production by altering metabolic pathway genetically. Down regulation of acetic acid or butyric acid synthesis pathway or a solvent formation repressor \textit{solR} gene was shown to
enhance butanol production (Green et al., 1996; Harris et al., 2000; Harris et al., 2001; Yu et al., 2011; Zhang et al., 2012). In addition, up regulation of acetone-formation genes, aldehyde/alcohol dehydrogenase, or stress response genes were shown to enhance butanol production effectively (Alsaker et al., 2004; Mermelstein et al., 1993; Nair and Papoutsakis, 1994; Harris et al., 2000, 2001; Tomas et al., 2003; Tummala et al., 2003).

Moreover, it was shown that the redirection of metabolic flux towards preferred chemical synthesis could be achieved by creating extra internal driving forces via redox engineering based on genetic modification of enzyme mechanism or balancing redox state (Sánchez et al., 2005; Bond-Watts et al., 2011; Shen et al., 2011; Lim et al., 2013a; 2013b; Shi et al., 2013).

On the other hand, redistributing metabolic flux towards desired products could also be achieved by metabolic process engineering without genetic modifications via controlling oxidation-reduction potential. Examples include carbon monoxide flushing (Datta and Zeikus, 1985; Meyer et al., 1986), iron limitation (Junelles et al., 1988; Peguin and Soucaille, 1995), utilization of more reduced substrates (Vasconcelos et al., 1994; Nakeshimada et al., 2002; Li et al., 2010; Yu et al., 2011), and addition of artificial electron carriers (Rao and Mutharasan, 1987; Peguin et al., 1994; Girbal et al., 1995; Peguin and Soucaille, 1995; Park et al., 1999; Jiang et al., 2009; Choi et al., 2012). Among them, the addition of artificial electron carrier was shown to be the most effective and relatively simple approach to boost ethanol and butanol production by increasing NADH availability.

It was shown that butanol production in solventogenic clostridia was usually limited by the availability of NADH (Srivastava and Volesky, 1991; Fontaine et al.,
The expression of adhE2 gene enabled C. cellulovorans to produce ethanol and n-butanol, the formation of which required extra NADH. However, it was also observed that the carbon flux had shifted from C4 to C2 products. This might be caused by the competition between NADH consumption for alcohols production and conversion of acetyl-CoA to butyryl-CoA. Therefore, to balance NADH availability, the pathway converting acetyl-CoA to butyryl-CoA was weakened for ethanol and butanol production. This might be a major limiting factor for higher ethanol and butanol production by the engineered C. cellulovorans. To increase NADH availability, an artificial electron carrier, methyl viologen, which was widely used for fermentation of C. acetobutylicum, was tested on alcohol production by the engineered C. cellulovorans in this study.

5.2 Materials and Methods

5.2.1 Cultural media and bacteria growth

C. cellulovorans/83151-adhE2 was cultured in modified media 520 (DSMZ), containing (per liter): (NH4)2SO4, 1.3 g; KH2PO4, 1.5 g; K2HPO4·3H2O, 2.9 g; MgCl2·6H2O, 0.2 g; CaCl2·2H2O, 75 mg; FeSO4·7H2O, 1.25 mg; resazurin, 1 mg; yeast extract, 2 g; tryptone, 4 g; cysteine-HCl, 0.5 g. pH was adjusted to 6.0 and media was purged with N2 gas before autoclaving. Sterile 50× SL-10 trace elements, 50× Na2CO3 (10% w/v), and carbon source were added before use. 1000× SL-10 trace elements contained (per liter): HCl (25%; 7.7 M), 10 mL; FeCl2·4H2O, 1.5 g; ZnCl2, 70 mg; MnCl2·4H2O, 100 mg; H3BO3, 6 mg; CoCl2·6H2O, 190 mg; CuCl2·2H2O, 2 mg; NiCl2·6H2O, 24 mg; Na2MoO4·2H2O, 36 mg. It was diluted 20 times to make 50× trace
elements. Around 15 g/L glucose, cellobiose, or cellulose was provided as carbon source. When cellulose was used as the main carbon source, a little amount of glucose/cellobiose (around 3 g/L) was added to initiate the cell growth. Media was supplemented with 30 µg/ml thiamphenicol.

5.2.2 Fermentation with or without methyl viologen addition

Fermentation was performed in serum bottles with 50 mL working volume. 10% seed at active stage (OD = 0.5-1.0) was inoculated. Various concentrations of methyl viologen (0 µM, 100 µM, 250 µM, 500 µM or 750 µM) were added in the growth media. Cells were grown in an incubator at 37°C. When cells were grown on glucose or cellobiose, samples were taken twice per day from serum bottles to monitor cell growth and metabolites production. When cells were grown on cellulose, samples were taken once per day from serum bottles to monitor metabolites production. The medium pH was adjusted after each sampling to maintain the pH between 6.0-7.0 by adding NaOH solution. When methyl viologen was added, pH was controlled between 6.0-6.5.

5.2.3 Analytical methods

Cell density was analyzed by measuring the optical density of cell suspension at 600 nm using a spectrophotometer (Shimadzu, Columbia, MD, UV-16-1). Acids and alcohols including acetic acid, butyric acid, ethanol and n-butanol were measured with a gas chromatograph (GC) (GC-2014 Shimadzu, Columbia, MD) equipped with a flame ionization detector (FID) and a 30.0 m fused silica column (Stabilwax-DA, 0.25 mm film thickness and 0.25 mm ID, Restek, Bellefonte, PA). The GC was operated at an injection
temperature of 200 °C with 1 μL of sample injected with an auto injector (AOC-20i, Shimadzu). The column temperature was initially held at 80 °C for 3 min, then increased at a constant rate of 30 °C per min to 150 °C, and held at 150 °C for 3.7 min. Samples were centrifuged at 1,3200 rpm for 5 min in 1.5 ml microcentrifuge tubes and the supernant was subjected to GC.

Glucose and cellobiose were quantified by high performance liquid chromatography (HPLC, LC-20AD, Shimadzu, Columbia, MD) with an organic acid column (Bio-Rad HPX-87H, ion exclusion organic acid column, 300×7.8 mm). Cellulose sample (1 ml) was washed by distilled water prior to autoclaving. After autoclaving, the cellulose pellet was washed again by distilled water. Afterwards, it was resuspended in 1 ml distilled water at pH 4.5, and was hydrolyzed by 20 µl Accellerase 1500 cellulase (Dupont Industrial Biosciences, New York, US) at 50 °C for 7 days with standard samples, followed by measurement of glucose by HPLC. Samples were centrifuged at 1,3200 rpm for 15 min in 1.5 ml microcentrifuge tubes and was diluted 10 times with distilled water prior to analysis on HPLC. HPLC was run at 45 °C using 0.007N H₂SO₄ (0.7 ml H₂SO₄ in 2 L distilled water) as the eluent at a flow rate of 0.6 mL/min. 15 µl sample was injected by an automatic injector (SIL-10Ai) and the running time was set at 40 min. A refractive index (RI) detector (Shimadzu RID-10A) was set at the range of 200 to detect the organic compounds in the sample. The HPLC column was installed in a column oven (CTO-10A) with temperature control at 45 °C. Peak height was used to calculate concentration of sugars in the sample based on the peak height of standard sample.
5.3 Results

5.3.1 Effects of methyl viologen on fermentation from glucose

Various approaches of metabolic process engineering were applied to enhance production of desired products by controlling redox potential. Among these approaches, the addition of methyl viologen was the most effective. Since most of the studies of methyl viologen were done in *C. acetobutylicum*, the effect of methyl viologen on fermentation of the engineered *C. cellulovorans* was unknown. Particularly, when different carbon substrates were used, the effect of methyl viologen might vary due to their different reducing power. Therefore, the concentration of methyl viologen with the best effect when glucose was used as carbon source was investigated. The production of ethanol, butanol, acetic acid, and butyric acid in addition to cell growth and glucose usage were analyzed, when 0 µM, 100 µM, 250 µM, or 500 µM methyl viologen was added from the beginning of fermentation. The results (Figure 5.1; Table 5.1) showed that alcohols production increased, while acids production decreased sharply with the addition of 100-250 µM methyl viologen. Specifically, when 100 µM methyl viologen was added, 3.3 g/L ethanol, 1.8 g/L butanol, 0.6 g/L acetic acid, and 0.2 g/L butyric acid were produced, compared to 0.7 g/L ethanol, 0.6 g/L butanol, 1.9 g/L acetic acid and 1 g/L butyric acid produced without the addition of methyl viologen. The titers of ethanol and butanol increased about 4-fold and 2-fold, while the titers of acetic acid and butyric acid decreased about 65% and 85%, respectively. Since glucose consumption did not change much, similar change of metabolites yields were observed with the addition of methyl viologen. When 250 µM methyl viologen was added, 2.6 g/L ethanol, 2.4 g/L butanol, 0.5 g/L acetic acid, and 0.1 g/L butyric acid were produced. Compared to the
result with 100 µM addition of methyl viologen, more butanol was produced, though less ethanol was produced. In addition, when 250 µM methyl viologen was added, the yields of ethanol, acetic acid, and butyric acid were lower than those when 100 µM methyl viologen was added, with a comparable butanol yield. However, when a higher concentration (500 µM) of methyl viologen was used, both ethanol and butanol titers were much lower than those when 100 µM or 250 µM methyl viologen was added. This was caused by the toxic effect of high concentration of methyl viologen. 500 µM methyl viologen greatly inhibited cell growth, while 100 µM and 250 µM methyl viologen affected cell growth in a relatively minor way. Comparing the effects of different concentrations of methyl viologen, 250 µM methyl viologen seemed to be most effective, in terms of butanol production.

5.3.2 Effects of methyl viologen when added after overnight cell growth

The inhibitory effect of methyl viologen on cell growth limited the application of methyl viologen for higher alcohol production. Therefore, whether the inhibitory effect on cell growth could be relieved was tested, by adding methyl viologen at a later growth stage. After overnight incubation (18 h), cell growth was entering the stationary phase from the exponential phase, with OD 600 at ~ 2-3. Then, various concentrations of methyl viologen were added to the fermentation broth for test. The results (Figure 5.2, Table 5.2) showed that by adding methyl viologen after overnight cell growth, the toxic effect was eliminated. Cell growth, monitored by OD600, was comparable, when 0 µM-750 µM methyl viologen was added. The ethanol titer was similar, when 250 µM, 500 µM, or 750 µM methyl viologen was added, about 2.8-3.0 g/L. Butanol titer reached to
2.3 g/L with the addition of 250 µM methyl viologen, while about 2 g/L butanol was produced with the addition of 500 µM or 750 µM methyl viologen. It was noted that without growth inhibitory effect, a higher concentration of methyl viologen did not induce higher ethanol and butanol production as expected. In addition, the ethanol and butanol production with the addition of 250 µM, 500 µM, and 750 µM methyl viologen after overnight cell growth were comparable to that with the addition of 250 µM methyl viologen at the beginning of fermentation. Moreover, much more acetic acid was produced (1.3-1.5 g/L) with the addition of methyl viologen after overnight cell growth, compared to the acetic acid produced (0.4 -0.6 g/L) with the addition of methyl viologen from the beginning, regardless of the concentration tested. Therefore, considering all these factors, the addition of 250 µM methyl viologen at the beginning of fermentation had the best effect. Unless otherwise noted, methyl viologen was added at the beginning in the following experiments.

5.3.3 Effects of methyl viologen on fermentation from cellobiose

Since different sugars may require different amount sof methyl viologen to achieve the best effect, different concentrations of methyl viologen were added, when cellobiose served as the carbon source. As shown in Figure 5.3 and Table 5.3, ethanol and butanol titers were significantly increased with the addition of 100-500 µM methyl viologen. Specifically, the addition of 100 µM or 250 µM had similar effects on alcohol and acid production, producing 1.9 g/L ethanol, 2.6-2.8 g/L butanol, 0.3-0.5 g/L acetic acid, and 0.1-0.2 g/L butyric acid. The addition of 500 µM methyl viologen induced similar production of butanol, acetic acid and butyric acid, but higher ethanol production
(2.6 g/L). Interestingly, the addition of 100-500 µM methyl viologen did not inhibit cell growth, when cellobiose was used, suggesting cellobiose as the carbon substrate could somehow release the toxic effect of methyl viologen. From the prospective of butanol production, the addition of 100 µM methyl viologen was slightly better.

5.3.4 Effects of methyl viologen on fermentation from cellulose

Similarly, the effects of methyl viologen were investigated when cellulose was served as the carbon source. As expected, the addition of methyl viologen could enhance alcohol production and inhibit acid production significantly (as shown in Figure 5.4 and Table 5.4). Specifically, when 100 µM methyl viologen was added, 1.9 g/L ethanol and butanol were produced, almost 2-fold of that without the addition of methyl viologen. Only 0.4 g/L acetic acid and 0.2 g/L butyric acid were produced. However, when a higher concentration of methyl viologen was added, less ethanol and butanol were produced. When 250 µM methyl viologen was used, 1.5 g/L ethanol and 1 g/L butanol were produced. When 500 µM methyl viologen was applied, only 1 g/L ethanol and 0.8 g/L butanol were produced, which were even lower than that with no methyl viologen addition. Even though the cell growth was hardly monitored by measuring OD600, because of cell attachment to the insoluble cellulose, the inhibitory effect of methyl viologen on cell growth was suggested with lower ethanol and butanol production when higher concentrations (250-500 µM) of methyl viologen were used. Taken together, when cellulose was used as the carbon source, the addition of 100 µM methyl viologen had the best effect on butanol production.
The effects of methyl viologen on alcohol and acid production are summarized in Table 5.5. The total alcohol production with methyl viologen was 1.7-3.8-fold of that without methyl viologen. In contrast, the total acid production with methyl viologen was 20%-29% of that without methyl viologen. In addition, the acid/alcohol ratio decreased from 0.95-2.22 to 0.12-0.16. Overall, the addition of optimal concentration of methyl viologen caused sharp increase of alcohol production and decrease of acid production on fermentation by engineered *C. cellulovorans*.

### 5.4 Discussion

In this chapter, the dramatic effects of methyl viologen on the metabolism of engineered *C. cellulovorans* were studied. Consistent results were observed as done in *C. acetobutylicum*, alcohol production increased and acid production decreased. Two possible biological mechanisms were proposed (as shown in Figure 5.5), by which methyl viologen could increase alcohol production by increasing NADH availability (Rao and Mutharasan, 1987; Peguin et al., 1994). The conversion of pyruvate to acetyl-CoA requires ferredoxin (Fd) reduction. Ferredoxins are iron-sulfur proteins that mediate electron transfer. The reduced ferredoxin (FdH₂) is oxidized by hydrogenase, which releases electron to form hydrogen, and regenerates Fd. In addition, FdH₂ could also transfer electron to NAD⁺ to form NADH. However, this reaction is reversible and is greatly inhibited by the presence of NADH. When methyl viologen was added, blue color of the media was observed, indicating that the reduction of methyl viologen took place. The oxidation of reduced methyl viologen required hydrogenase, competing with FdH₂ for active sites (Yu and Wolin, 1969; Adams et al., 1980; Adams and Mortenson, 1984;
Peguin et al., 1994). Therefore, to regenerate Fd, the alternative mechanism to oxidize FdH₂ was activated, by transferring electron to NAD⁺. It was also shown that in the presence of methyl viologen, the enzyme activity of NAD:Fd oxidoreductase increased 60-fold, accelerating the reaction of electron transfer from FdH₂ to NAD⁺ to form NADH (Peguin et al., 1994). In this case, NADH formation took place via FdH₂. Similar effects were observed by carbon monoxide flushing (Datta and Zeikus, 1985; Meyer et al., 1986), which could inhibit the enzyme activity of hydrogenase, resulting in enhanced NADH formation via FdH₂. The other possible mechanism was that the reduced methyl viologen could directly transfer the electron to NAD⁺ to form NADH (DiCosimo et al., 1981). In this case, NADH was formed directly from reduced methyl viologen. The effects of methyl viologen on enhanced alcohol production could be due to a combination of both mechanisms. But no matter what mechanism was preferred, the outputs of both mechanisms were the same. That is NAD⁺ received the electrons that used to be transferred to H⁺, resulting in less H₂ produced but more NADH formed, which could then be used for alcohol production.

The results also showed that a higher concentration of methyl viologen could inhibit cell growth, resulting in lower alcohol production, of which the phenomenon was relatively obvious when glucose or cellulose was used as carbon source. The possible mechanisms of cell growth inhibition were still not clear. One of the possibilities was that ATP was the byproduct of acid synthesis pathway. Thus, inhibited acid production resulted in inhibited ATP production, which greatly affected cell growth and other catabolism/metabolism in a negative way. In addition, the effect of methyl viologen on decreasing acid production was remarkable. Interestingly, acids production was all at a
very low level, regardless of the concentration of methyl viologen tested. The exact reason was not clear. But one of the explanations could be that increasing availability of NADH required the turnover of NAD+ by alcohol formation, resulting in carbon flux shift from acid formation to alcohol formation.

To overcome the toxicity of methyl viologen, the effects of the addition of methyl viologen at different time were studied. The addition of methyl viologen from the beginning of fermentation showed increased alcohol production and decreased acid production within a certain range of concentration of methyl viologen. When a higher concentration was used, cell growth could be disrupted, resulting in less alcohol production improvement. On the contrary, when methyl viologen was added after overnight cell growth, growth inhibitory effect was eliminated, but alcohols production did not increase when a higher concentration of methyl viologen was tested, suggesting that the ability of butanol production or butanol tolerance might be limiting factors. The maximum alcohol production was similar to that when methyl viologen was added from the beginning. In addition, during the cell growth without methyl viologen addition, a considerable amount of acetic acid was produced. Therefore, it was suggested to add methyl viologen from the beginning of fermentation. In addition, when the growth inhibitory effect was relieved by adding methyl viologen overnight or using cellobiose as carbon substrate, the addition of a higher concentration of methyl viologen did not induce significant higher production of alcohols, suggesting an effective upper limit existed. At the end, the optimal concentration of methyl viologen addition was determined. 250 µM, 100 µM, and 100 µM were the optimal concentrations of methyl viologen when glucose, cellobiose, and cellulose was used as carbon substrates, respectively. In a next stage,
agricultural wastes could be used as feedstock to produce ethanol and butanol directly by this engineered *C. cellulovorans*. The compositions of agricultural wastes vary between plant species, and tissue types, and thus are more complex. The test of optimal concentration of methyl viologen is needed, when different feedstock is used.

To further solve the problem of the negative effect caused by methyl viologen, fermentation with high cell density, and continuous fermentation using fibrous-bed bioreactor (FBB) could be effective solutions. High-cell-density fermentation was usually performed to increase productivity of desired products by concentrating cells to a high density (Riesenberg and Guthke, 1999; Tashiro et al., 2005) by membrane filtration or centrifugation. This strategy could also be used for fermentation with methyl viologen to overcome the toxic effect on cell growth. During the cell condensing process, cells grow without the addition of methyl viologen. Then highly condensed cells could be inoculated for fermentation with methyl viologen for ethanol and butanol production. Similarly, fermentation in FBB could be performed to grow cells to high density. FBB was designed to immobilize cells in the fibrous material to reach a high density for better production of desired products (Huang et al., 2004; Liang et al., 2012; Shi et al., 2012; Chen et al., 2013; Wei et al., 2013; Lan et al., 2013). Therefore, engineered *C. cellulovorans* can be first grown in FBB without adding methyl viologen. When cells grow to a high density, methyl viologen can be added for ethanol and butanol production. In this way, inhibitory effect on cell growth by methyl viologen can be ignored, and at the same time, higher ethanol and butanol production are expected due to the high cell density.
5.5 Conclusion

Adding methyl viologen in the fermentation of engineered *C. cellulosorans* was an effective method of metabolic process engineering method to enhance ethanol and butanol production. Though the effect of methyl viologen was widely studied in other species, such as the solventogenic *C. acetobutylicum*, this was the first time to study its effect in engineered *C. cellulosorans* for biofuel production directly from cellulosic biomass. The addition of methyl viologen could shift the carbon flux from acid production towards alcohol production, by increasing NADH availability. 250 µM, 100 µM, and 100 µM were shown to the optimal concentration of methyl viologen to increase alcohol production 2-4 fold, when glucose, cellobiose, and cellulose were used as carbon sources, respectively. Therefore, it is expected that a higher level of ethanol and butanol can be produced directly from cellulosic biomass with further metabolic engineering and metabolic process engineering.

5.6 References


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Carbon monoxide gasing leads to alcohol production and butyrate uptake without acetone formation in continuous cultures of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 24:159-167.


Butanol production from
crystalline cellulose by cocultured Clostridium thermocellum and Clostridium saccharoperbutylacetonicum N1-4. Appl Environ Microbiol. 77(18), 6470-5.


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<th>MV (µM)</th>
<th>Ethanol Titer (g/L)</th>
<th>Ethanol Yield (g/g)</th>
<th>Butanol Titer (g/L)</th>
<th>Butanol Yield (g/g)</th>
<th>Acetic acid Titer (g/L)</th>
<th>Acetic acid Yield (g/g)</th>
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Table 5.1 Effects of methyl viologen (added from the beginning) using glucose (n=1)

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<th>MV (µM)</th>
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<th>Ethanol Yield (g/g)</th>
<th>Butanol Titer (g/L)</th>
<th>Butanol Yield (g/g)</th>
<th>Acetic acid Titer (g/L)</th>
<th>Acetic acid Yield (g/g)</th>
<th>Butyric acid Titer (g/L)</th>
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Table 5.2 Effects of methyl viologen (added after overnight cell growth) using glucose (n=1)

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<th>Butanol Titer (g/L)</th>
<th>Butanol Yield (g/g)</th>
<th>Acetic acid Titer (g/L)</th>
<th>Acetic acid Yield (g/g)</th>
<th>Butyric acid Titer (g/L)</th>
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Table 5.3 Effects of methyl viologen (added from the beginning) using cellobiose (n=2)

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<th>Ethanol Yield (g/g)</th>
<th>Butanol Titer (g/L)</th>
<th>Butanol Yield (g/g)</th>
<th>Acetic acid Titer (g/L)</th>
<th>Acetic acid Yield (g/g)</th>
<th>Butyric acid Titer (g/L)</th>
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Table 5.4 Effects of methyl viologen (added from the beginning) using cellulose (n=2)
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<th>Acetic acid (g/L)</th>
<th>Butyric acid (g/L)</th>
<th>Total alcohol (g/L)</th>
<th>Total acid (g/L)</th>
<th>Alcohol /acid</th>
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<td>0.65</td>
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<td>8.46</td>
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<td>6.59</td>
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Table 5.5 Summary of effects of methyl viologen using glucose, celllobiose and cellulose
Figure 5.1 Effects of methyl viologen (added from the beginning) on fermentation of the engineered *C. cellulovorans* using glucose as carbon substrate. A, MV effect on ethanol production; B, MV effect on butanol production; C, MV effect on acetic acid production; D, MV effect on butyric acid production; E, MV effect on cell growth; F, MV effect on glucose consumption. (n=1)
Figure 5.2 Effects of methyl viologen (added after overnight cell growth) on fermentation of engineered *C. cellulovorans* using glucose as carbon substrate. A, MV effect on ethanol production; B, MV effect on butanol production; C, MV effect on acetic acid production; D, MV effect on butyric acid production; E, MV effect on cell growth; F, MV effect on glucose consumption. (n=1)
Figure 5.3 Effects of methyl viologen (added from the beginning) on fermentation of engineered *C. cellulovorans* using cellobiose as carbon substrate. A, MV effect on ethanol production; B, MV effect on butanol production; C, MV effect on acetic acid production; D, MV effect on butyric acid production; E, MV effect on cell growth; F, MV effect on cellobiose consumption. (n=2)
Figure 5.4 Effects of methyl viologen (added from the beginning) on fermentation of engineered *C. cellulovorans* using cellulose as carbon substrate. A, MV effect on ethanol production; B, MV effect on butanol production; C, MV effect on acetic acid production; D, MV effect on butyric acid production; E, MV effect on cellulose consumption. (n=2)
Figure 5.5. Working mechanisms of increased NADH availability by methyl viologen.
Chapter 6: Conclusions and Recommendations

6.1 Conclusions

6.1.1 Genetically engineering of C. cellulovorans for biofuel production directly from cellulosic biomass

*C. cellulovorans* is a notable cellulolytic microbe with outstanding ability to degrade cellulose and hemicellulose by encoding cellulosomal genes. Though its cellulosomal genes were widely studied or heterologously expressed in other microbes, metabolic engineering of *C. cellulovorans* was not reported. In this project, *C. cellulovorans* was chosen to be the engineering host to express the n-butanol synthesis pathway from *C. acetobutylicum* to produce biofuel directly from cellulosic biomass. Compared to other widely studied cellulolytic microbes, such as *C. cellulolyticum* and *C. thermocellum*, *C. cellulovorans* had the advantages of having the native butyric acid synthesis pathway. Therefore, only one aldehyde/alcohol gene, adhE2 was needed for n-butanol production, which greatly simplified the experimental process to overexpress the pathway from acetyl-CoA to butyryl-CoA. In addition, a similar strategy had been applied to *C. tyrobutyricum*, a butyric acid producing microbe, to produce high level of n-butanol by overexpressing *adhE2* (Yu et al., 2011), which further increased the viability of this strategy.
However, overexpression of *adhE2* in *C. cellulovorans* was not as easy as expected, lacking the established transformation method. The initial trials of transformation were all unsuccessful, using various conditions of electroporation and conjugation, indicating that the regular transformation methods for *Clostridia* were not working for *C. cellulovorans*. This phenomenon was not rare. *Clostridium* is a genus of Gram-positive bacteria, with widely branched species. Transformation methods for *Clostridia* species were not systemically studied, until a series of shuttle plasmids were developed with relatively high transformation efficiency using the transformation methods reported (Heap et al., 2009; Yu et al., 2012). However, the relatively well developed transformation method applied only to limited *Clostridia* species, such as *C. acetobutylicum, C. botulinum, C. difficile, C. perfringens, C. tyrobutyricum*. Since the transformation method and efficiency for *Clostridia* was usually strain-dependent, no universal method was developed. Since regular or modified *Clostridia* transformation method was not working for *C. cellulovorans*, suggesting a new transformation needs to be established.

With intensive literature review, unknown restriction-modification (RM) systems were proposed to be the major factor. RM system, as defensive machinery, is widespread in bacteria to prevent the invasion of foreign DNA. Methyltransferases mark its own DNA, and restriction enzymes recognize and degrade foreign DNAs without autologous methylation pattern or with foreign pattern. Proper methylation of plasmids showed significant increase of transformation efficiency (Mermelstein et al., 1993; Elhai et al., 1997; Davis et al., 2000; Jennert et al., 2000; Purdy et al., 2002; Lin et al., 2010; Rego et al., 2011; Suzuki et al., 2011; Zhang et al., 2012; Pyne et al., 2013). Particularly, no
transformants, or very few transformants, could be obtained in several *Clostridia* species without proper methylation of plasmids (Mermelstein et al., 1993; Davis et al., 2000; Jennert et al., 2000; Purdy et al., 2002; Pyne et al., 2013), suggesting it may be the key factor for some difficult *Clostridia* transformation.

Therefore, to overcome the restriction barrier of *C. cellulovorans* to enhance transformation, the RM system of *C. cellulovorans* was studied. Unlike other studies to identify the restriction activity by analyzing the restriction pattern of plasmids, we started with analyzing the genome sequences with bioinformatics. Two pairs of RM systems (*Cce*743I and *Cce*743II) in the same operon were targeted. With further restriction assay and bisulfite assay, *Cce*743I and *Cce*743II RM systems were identified with the restriction specificity, methylation specificity, and sequences of restriction enzymes and methyltransferases. With all this information, establishing a methylation method became easier. Since no commercial methyltransferases available recognize the same sites, the native methyltransferases were cloned and expressed in *E. coli* for methylation *in vivo*. The protection effect of the *in vivo* methylation system was shown to be effective by restriction assay. With the effective methylation method, plasmids were methylated prior to transformation. A transformant was obtained, harboring plasmid pMTL83151-adhE2. With the functional adhE2, confirmed by enzyme activity, *C. cellulovorans*/83151-adhE2 was capable of producing n-butanol and ethanol directly from cellulose. It was the first example of metabolic engineering of *C. cellulovorans* for n-butanol and ethanol production with the established transformation method, providing a promising platform for biofuel production directly from cellulosic biomass.
6.1.2 Fermentation of C. cellulovorans/83151-adhE2

Once the engineered strain of *C. cellulovorans* producing n-butanol and ethanol was obtained, it was important to understand its fermentation properties. Since there was no report of fermentation of *C. cellulovorans*, the basics needed to be explored, including media composition, fermentation conditions, and fermentation kinetics. Though media optimization does not change the strain properties and can sometimes be ignored, it could greatly improve the fermentation results in some cases. In this project, utilization of 320 media and modified 520 media showed great differences on the fermentation results. Modified 520 media with better buffering capacity and reducing capability provided a better growth environment for *C. cellulovorans*, thus resulting in much better fermentation results.

Fermentations were also conducted using various carbon substrates, including glucose, cellobiose, and cellulose. Utilization of different carbon substrates involved induced expression of enzymes for carbon substrate degradation and sugar uptake, resulting in different fermentation patterns. But, the common phenomenon was that metabolite production in *C. cellulovorans* increased proportionally with sugar consumption and was growth associated, which was different from the solventogenic *Clostridia* with acidogenic and solventogenic stages. Additionally, fermentation kinetics in the lab-scale bioreactors and serum bottles were compared. The results showed that fermentation of engineered *C. cellulovorans* in bioreactor produced higher n-butanol and acids, but relatively low ethanol, compared to that in serum bottles, probably due the effects of constant agitation and pH control. In addition, it was found that carbon flux shifted from C4 products towards C2 products in the engineered *C. cellulovorans*,

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compared to that of wild-type \textit{C. cellulosorans}. It was proposed that the carbon flux shift was caused by rebalancing NADH usage. It was known that NADH was required for ethanol and n-butanol synthesis, which outcompeted the NADH used for conversion from acetyl-CoA to butyryl-CoA, resulting in weakened carbon flux towards C4 products, and strengthened carbon flux towards C2 products. In conclusion, the basic conditions for fermentation of wild-type and engineered \textit{C. cellulosorans} were developed, and fermentation kinetics of engineered \textit{C. cellulosorans} grown on various carbon substrates were analyzed, providing a base for further improvement of n-butanol and ethanol production from cellulosic biomass.

\textit{6.1.3 Enhanced biofuel production and inhibited acid production with methyl viologen addition}

n-Butanol and ethanol production was usually limited by the availability of NADH, which could be increased by metabolic engineering or metabolic process engineering. In this project, effects of the addition of methyl viologen as an artificial electron carrier on biofuel production by the engineered \textit{C. cellulosorans} were studied. Methyl viologen was proposed to increase NADH availability by two possible mechanisms. One was reduced methyl viologen outcompeted native reduced ferredoxin with hydrogenase, resulting in increased NADH formation via reduced ferredoxin. The other was NADH could be formed directly from reduced methyl viologen. No matter what mechanism worked, the result was less hydrogen and more NADH. The results showed that addition of methyl viologen could enhance alcohol production and inhibit acid production significantly. This result was constant when it was used for fermentation
of other *Clostridia* (e.g., *C. acetobutylicum*, *C. tyrobutyricum*). However, different species or different carbon substrates had different optimal concentrations of addition of methyl viologen, since methyl viologen had growth inhibitory effects, and also an effective maximum threshold existed. The optimal addition timing, in addition to the optimal concentration used for fermentation of engineered *C. cellulovorans* when grown on glucose, cellobiose and cellulose was determined. This study provided a metabolic process engineering method to enhance n-butanol and ethanol production without genetic modification of the engineered *C. cellulovorans*. It was believed that with further metabolic engineering, evolutionary engineering or metabolic process engineering, a much higher level of biofuel could be produced by the engineered *C. cellulovorans*.

### 6.2 Recommendations

#### 6.2.1 Further metabolic engineering of *C. cellulovorans* for higher biofuel production

*adhE2* gene has been transformed into *C. cellulovorans* for n-butanol and ethanol production directly from cellulosic biomass in this project. About 1.4 g/L ethanol and 2.8 g/L butanol were produced from glucose, 2.7 g/L ethanol and 4.6 g/L butanol were produced from cellobiose, and 0.6 g/L ethanol and 1.7 g/L butanol were produced from cellulose in bioreactor. Though this study showed the highest butanol production directly from cellulose, there is still much room to improve. It was reported that with *adhE2* overexpression in butyric acid producing *C. tyrobutyricum*, 1.1 g/L butanol was produced from glucose. In addition, when *adhE2* was overexpressed in the *ack* (acetate kinase) knockout mutant of *C. tyrobutyricum*, butanol titer was increased to 10.0 g/L (Yu et al., 2011), suggesting the great impact of *ack* gene in metabolic flux. Similarly, knock out of
the acid synthesis pathway could potentially increase n-butanol production in the engineered *C. cellulovorans*. It should be noticed that the acetic acid synthesis pathway and butyric acid synthesis pathway produced ATP, which were essential for cell growth. Knockout of both acid synthesis pathways at the same time may not work.

To further enhance n-butanol production in engineered *C. cellulovorans*, n- butanol synthesis pathway could be further strengthened. For example, the genes converting acetyl-CoA to butyryl-CoA (e.g., *thl*) could be overexpressed to drive the metabolic flux towards butyryl-CoA. Or, *adhE2* overexpression could be further strengthened, by inserting multiple copies in the shuttle plasmid, or by using a stronger promoter. In addition, to stabilize the heterologous gene expression in *C. cellulovorans*, and to reduce the cost of using antibiotics, shuttle plasmids with more stability need to be applied, or heterologous genes can be inserted into the genome of *C. cellulovorans*. However, whether developing shuttle plasmids with more stability, developing stronger promoter, or expressing heterologous genes by knock in, the genetic tools of *C. cellulovorans* must be advanced, which is also essential for metabolic engineering of other *Clostridia* for biofuel production.

### 6.2.2 Further evolutionary engineering and metabolic process engineering for higher biofuel production

In Chapter 4 and Chapter 5, basics of fermentation of wild-type and engineered *C. cellulovorans* were developed, and biofuel production was much improved by addition of methyl viologen. However, it was believed that further evolutionary engineering and metabolic process engineering could bring the biofuel production to a higher level, in
addition to the further metabolic engineering discussed above. In Chapter 4, butanol tolerance of \textit{C. cellulovorans} was tested. The result showed that \textit{C. cellulovorans} could not grow well in the presence of more than 8 g/L butanol added from the beginning. Because of the limitations of butanol tolerance assay, it could not measure the true butanol tolerance accurately, since during fermentation process, butanol is produced with the cell growth, allowing better cell growth and adaptation to butanol. Therefore, the true butanol tolerance is usually higher than that measured by butanol tolerance assay. Solventogenic \textit{Clostridia} can usually produce 10-20 g/L butanol, and the engineered \textit{C. tyrobutyricum} could produce 10 g/L butanol, suggesting a higher butanol tolerance of \textit{C. cellulovorans}. To improve butanol tolerance of \textit{C. cellulovorans}, mutagenesis, metabolic engineering, evolutionary engineering by continuous culture, and serial enrichment approaches displayed effectiveness (Lin and Blaschek, 1983; Hermann et al., 1985; Liyanage et al., 2000; Tomas et al., 2003; Alsaker et al., 2004). Besides these, our lab showed that continuous culture in fibrous-bed bioreactor (FBB) could facilitate cell growth with high cell density, and thus facilitate cell adaptation to generate butanol tolerant mutant. Butanol tolerant mutant of \textit{C. acetobutylicum} was obtained by FBB-based cell adaptation, increasing butanol titer from 12-13 g/L to about 24 g/L (data not published). This provides an excellent method to generate a butanol tolerant mutant of engineered \textit{C. cellulovorans} for much higher butanol production.

FBB technology can not only be used for adaptation of butanol tolerant mutant of engineered \textit{C. cellulovorans}, it could be also used for regular fermentation for higher biofuel production. Unlike other solventogenic \textit{Clostridia} or \textit{C. tyrobutyricum}, \textit{C. cellulovorans} is a slowly-growing bacteria, probably because of its long-time evolution
growing in cellulosic biomass. Therefore, the OD600 at stationary stages are much lower. In addition, the results in Chapter 5 showed that methyl viologen could great enhance n-butanol and ethanol production, but inhibit cell growth to some extent. Therefore, by increasing cell density by FBB, biofuel production is predicted to be improved, and the toxic effects of methyl viologen could be alleviated if methyl viologen is added. Similarly, other methods that could increase cell density, such as high-cell-density fermentation by centrifugation of cell culture or by filter membrane, may greatly improve biofuel production.

### 6.2.3 Fermentation of engineered *C. cellulovorans* with agricultural residues or forestry residues as carbon sources for biofuel production

The ultimate goal of this project is to produce biofuel directly from lignocellulosic biomass from agricultural and forestry wastes to reduce the cost of feedstocks and increase the sustainability of biofuel production, without competing with human food. Though fermentations of the engineered *C. cellulovorans* for biofuel production have been done using glucose, cellobiose, and cellulose as carbon substrates, fermentations from agricultural residues or forestry residues were not tested. Since the compositions of agricultural residues or forestry residues are more complex, different from plant species and even from different tissues, fermentation kinetics may be different. In addition, utilization of agricultural residues or forestry residues by engineered *C. cellulovorans* still requires physical or chemical pretreatment to breakdown or remove lignin, which could not be used by *C. cellulovorans*, but encapsulates cellulose and hemicellulose, making them hard to be contacted and utilized. Therefore, the
pretreatment process of agricultural residues or forestry residues for fermentation by engineered *C. cellulovorans* needs to be developed. It should be noted that such pretreatment is different from the pretreatment for fermentation by regular solventogenic *Clostridia*. The purpose of pretreatment for regular fermentation is to break down lignin, and degrade cellulose and hemicellulose into shorter polymers for further enzymatic hydrolysis. Acid pretreatment is usually used for this purpose, which typically produced significant amount of toxic compounds, somewhat inhibiting the following fermentation. In some cases, a detoxification process is needed. In addition, after the hydrolysis process, the supernatant containing soluble sugars is used for fermentation. However, the purpose of pretreatment for fermentation by engineered *C. cellulovorans* is to break down lignin to make cellulose and hemicellulose accessible. Therefore, a different pretreatment process with less toxic compounds produced can be used, such as alkali pretreatment. And, the pellet containing unsoluble cellulose and hemicellulose is used for fermentation. In conclusion, to further reduce the feedstock cost and enhance the sustainability of biofuel production, the fermentation process of engineered *C. cellulovorans* with agricultural residues or forestry residues as carbon sources needs to be further developed for biofuel production.

6.3 References


**Bibliography**


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