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BIOFUELS (HYDROGEN AND ETHANOL) PRODUCTION OF *CLOSTRIDIUM* BY CELLULOSE BIOCONVERSION

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BIOFUELS (HYDROGEN AND ETHANOL) PRODUCTION OF *CLOSTRIDIUM* BY CELLULOSE BIOCONVERSION

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

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Abstract

In view of the realization that fossil fuels reserves are limited, the dramatically increasing consumption of fossil fuels raised concerns for the energy security in the recent decades. Alternative energy generating options are being explored. Biological methods for producing sustainable biofuels converted from cellulose such as hydrogen and bio-ethanol have the potential to provide a sustainable method to meet the requirements of the new generation energy. However, huge challenges still exist to improve the efficiency of cellulose bioconversion process and thus reduce the economic cost to enable large-scale application of biofuels using this strategy. This study aimed to improve the bioconversion process of cellulose to produce biofuels including hydrogen and ethanol, by investigating the biofuel production of mesophilic and thermophilic *Clostridia* under different conditions and revealing the mechanisms behind the high bioconversion efficiency.

Hydrogen gas production via dark fermentation of cellulose has been investigated as a potential source of renewable energy. The model strain of mesophilic *Clostridium* species, *Clostridium cellulolyticum*, is capable of both cellulose hydrolysis and H_2 production, which make it a potential candidate in producing hydrogen from cellulose under a Consolidated Bioprocessing (CBP) scheme. However, it has been reported that *C. cellulolyticum* was considered as a sluggish cellulolytic bacterium, which means that the efficiency of bioconversion of cellulose still need to be improved. In the beginning of this study, the effects of different initial cellulose concentrations on cellulose degradation rates and hydrogen productions of *C. cellulolyticum* were examined. The results indicated that culturing *C. Cellulolyticum* on slow released substrates (e.g., cellulose but not

cellobiose) can reduce the accumulation of intermediate products (e.g., glucose and/or pyruvate). The restriction of substrate availability can balance the metabolism rate of intermediate products in *C. Cellulolyticum*, which can relieve it from catabolite repression and improve the hydrogen production efficiency. Further transcriptional analysis indicated that cellulosomal genes were down-regulated along with the increase of cellulose concentrations, however, the expression level of other genes related to central metabolic pathway peaked at 7 g/L cellulose. Our study agreed well with previous studies, and provided detailed transcriptional information of the carbon metabolism of *C. cellulolyticum*.

To further improve the hydrogen production and cellulose degradation, a co-cultured consortium composed of *C. cellulolyticum* and *Desulfovibrio vulgaris* Hildenborough was developed and it was optimized to achieve the best possible hydrogen production rate in this study. The co-culture can produce 3.3 mol H_2 mol^{-1}glucose comparing to the 1.8 mol H_2 mol^{-1} glucose from the mono-culture with a much more efficient cellulose degradation process. Our results suggested that lactate may be the carbon source that *C. cellulolyticu*m provides to *D. vulgaris* Hildenborough by degrading cellulose, so the lactate concentration in the co-culture can be kept at a low level and the catabolic pathway of lactate production in *C. cellulolyticu*m will keep going and result in low NADH/NAD+ ratios. This efficient regulation of carbon flow of *C. cellulolyticu*m enable the strain to achieve a high cell abundance in the co-culture, which in turn, promotes the efficient cellulose degradation in the co-culture system. Further analysis indicated that *D. vulgaris* Hildenborough does not only use lactate to produce H_2 in the system, but also be able to help *C. cellulolyticum* attach on cellulose fibers to speed up

the cellulose degradation process. These results provide comparable characterizations of the cellulolytic and hydrogen-producing capabilities of the mono-culture and co-culture systems, and the identification of ecological relationship between these two organisms will contribute to the future improvements of the hydrogen-producing efficiency using this approach.

In order to enhance cellulosic bioethanol production from thermophilic anaerobic bacteria, we also obtained the ethanol adapted strains of *Clostridium thermocellum* LQR1 and *Thermoanaerobacter ethanolicus* X514 through long term evolution. The evolved LQR1 and X514 strains were able to resistant to 5.4% and 3.9% ethanol respectively. Even though the parent strain had a greater biomass than most of the ethanol-tolerant derivatives when cultured in the absence of ethanol, the ethanol-evolved LQR1 can produce more ethanol than its ancestor. When using ethanol evolved LQR1 as the cellulose degrader, 15% more ethanol can be produced than the parent strain when co-cultured with parent X514. These results demonstrate that ethanol resistance can be developed by adaptive evolution and ethanol production can be promoted using ethanol evolved strains.

In summary, this study provided novel insights of the improvement of cellulose bioconversion process to produce biofuels, such as hydrogen and ethanol, which could be of merit for the application of *Clostridia* in to the industrial field and make progress with the production of second generation biofuels from the lignocellulose biomass.

Keywords: **biofuels, bioconversion of cellulose; *Clostridium cellulolyticum*; *Desulfovibrio vulgaris* Hildenborough; hydrogen production; cellulose degradation; cellulose metabolism; microarray; microorganism interaction;**

***Clostridium thermocellum* LQR1; *Thermoanaerobacter ethanolicus* X514; ethanol production; ethanol tolerance adaptation; long term evolution**

Chapter 1: Introduction

1.1 Introduction to Sustainable biofuels

In view of the realization that fossil fuels reserves are limited, the dramatically increasing consumption of fossil fuels raised concerns for the energy security in the recent decades. Alternative energy generating options are being explored. Biological methods for producing sustainable biofuels such as bio-ethanol, bio-diesel, hydrogen (H_2), methane, among others have the potential to provide a sustainable method to improve energy security.

According to report of IEA 2016, about 81% of world total primary energy supplies were produced from the fossil fuels including petroleum oil, natural gas and coal. In the year 2015, 67% of the United States electricity which accounts for 18% production of the worldwide was generated from fossil fuels. In China, 70% of electricity (24% electricity production in the world) was generated from fossil fuels. As we all known, the fossil fuels are nonrenewable energy sources which have finite reserves on the earth. Considering the shrinking crude oil reserves, a global energy shortage as well as the global climate change and energy security will be the concerns in future. These issues may not only cause the potential nation energy security but also impact the future life of our whole human society. Scientific researchers around the world are investing endless efforts and money to develop alternative energy such as solar, wind and bioenergy to change the energy supplies from nonrenewable to sustainable.

Biofuels which are produced from plant materials and biomass which were so called bio-based materials become increasingly relevant as a potential sustainable alternative to fossil fuels. Biofuels have already commercially entered the market, driven

among other kinds of energy supplies by their potential to improve energy security and contribute to climate change mitigation. The liquid biofuels such as ethanol and biodiesel which not only have higher energy density but also are more compatible with current situation are superior to other renewable energy forms including the biogas and solar/wind electricity (Liao, Mi et al. 2016).

Biofuels, such as ethanol and biodiesel as mentioned above, are currently produced from the products of conventional food crops (i.e. the starch, sugar and oil feedstocks) from soybean, wheat, corn, sugar cane, oilseed rape and palm oil. In addition, there are other biomass resources including lignocellulosic biomass, algae and municipal solid waste (i.e. tires) to produce biofuels. However, many of the biofuels so called first generation biofuels that are currently being supplied have been criticized as unsustainable because of their potential threaten to the traditional crops on their production places (Naik, Goud et al. 2010). Many of researchers fear that a major switch to biofuels from such crops would create a direct competition with their use for our food and animal feed, and the adverse impacts on our food security, natural environment and land use. Although ethanol and biodiesel currently have been produced in an efficient way from the food resources such as maze and sugar cane, there are strong concerns that more and more land originally used for the food production will be brought under biofuel crops leading to impacts on the global food market and food security especially in those from low-income net food importing countries.

To overcome this challenge and support biofuel development, new researches were conducted including non-edible lignocellulosic biofuel technologies, with responsible policies and economic instruments to ensure that biofuel commercialization

is sustainable. So-called second-generation biofuels are now being produced from a much broader range of non-food related materials including the dedicated energy crops such as switchgrass, the co-products of food products and lignocellulosic materials. The non-edible lignocellulosic materials are the most promising materials considered as natural and renewable resource. The global annual production of plant biomass is about 200 Gt, in which over 67% of dry mass is in the form of lignocellulose and from where 8-20 Gt of the primary biomass can be potentially obtained for biofuel production

1.2 Biofuel production from lignocellulosic biomass

Lignocellulosic biomass is organic material that derived from biological origin and usually contains high amount of lignin, hemicellulose, cellulose and pectin and a relatively low content of monosaccharides, starch, protein or oils (Wu, McLaren et al. 2010). Lignocellulolytic microorganisms can degrade lignocellulosic biomass, such as cellulose, hemicellulose and even lignin, using a variety of enzymes and utilize them as substrate to support their metabolisms (Lynd, Weimer et al. 2002, Doi 2008). This process can solve the problem of recalcitrance of cellulosic biomass which has been an impediment to the widespread of utilization of this important resource. Enzymes and mechanisms that involved in this process have been revealed by extensive studies of these lignocellulolytic degraders (Lynd, Van Zyl et al. 2005, Doi 2008), but only a few microorganisms and enzymes among them have been used in the biofuel production, for the reason that the cost and conversion efficiency of such a system are still big challenges for large scale utilization in the industry (Klein-Marcuschamer, Oleskowicz-Popiel et al. 2012, Balan 2014, Liao, Mi et al. 2016). The mechanisms and strategies for

microorganisms to degrade lignocellulose into biofuels will be overviewed in the following sections.

1.2.1 Lignocellulose composition and bio-degrading mechanisms

Lignocellulose is a complex substrate and the main constituents of lignocellulosic biomass are cellulose, hemicellulose and lignin as shown in Figure 1.1. The composition of lignocellulose differs in the percentages of these three main components for different materials (Betts, Dart et al. 1991). Therefore, the biodegradation of lignocellulose does not only depend on the microbial population that charge with the biodegradation of the lignocellulose, but also the substrate composition of the lignocellulose biomass which may require wide range of enzymes and environmental conditions.

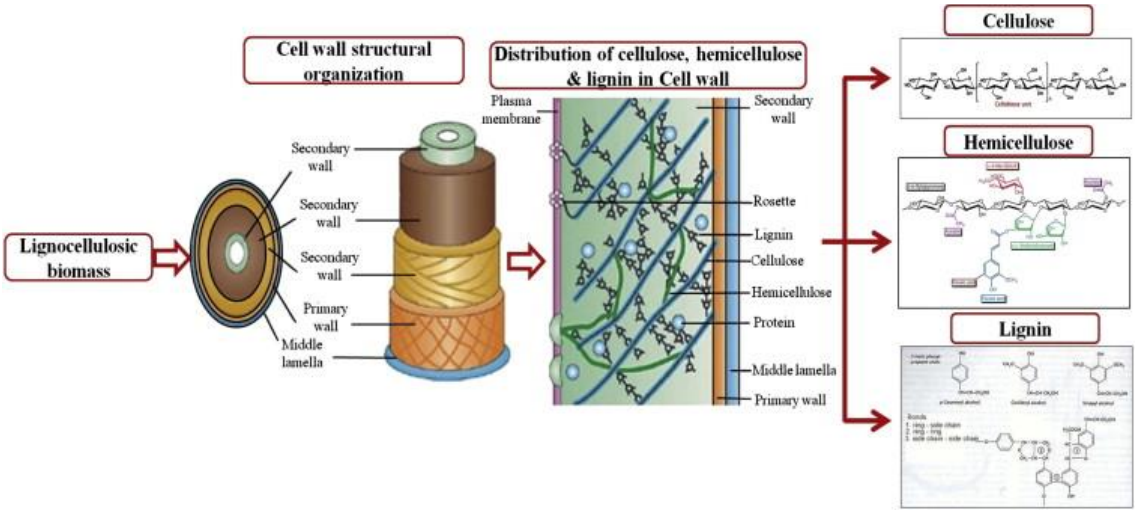


Fig. 1.1 The representative framework of lignocellulose biomass (Menon and Rao 2012). Cellulose, hemicellulose and lignin are the three major components in the lignocellulose biomass. The structural complexities increase from cellulose to lignin, as well as the difficulties for the microorganisms to enzymatic hydrolyze them.

Cellulose is a linear polymer of glucose linked through α-1,4-linkages and the degree of polymerization is very high, which usually ranges from 500 to 25,000 (Leschine 1995). In the natural environment, the microcrystalline structures of cellulose make it difficult to dissolve or hydrolysis. To efficiently degrade cellulose, there are at least three

classes of enzymes are required, which are generally called cellulases (Figure 1.1). The first class is the endoglucanases that can randomly cleave intermonomer bonds and break cellulose chain into oligosaccharides of varied lengths, which reduces the molecular size and creates accessible chain ends. Actually, the ability of the endoglucanases to create new ends in the complex cellulose crystalline structure is the rate limiting step in the hydrolysis process of cellulose, which creates positions for exoglucananses to attack and thus break the cellulose chain (Malherbe and Cloete 2002). The second class is the exoglucanases that remove glucose or cellobiose (dimers) from the end of the cellulose oligosaccharides chain. This class of enzymes release glucose or glucose dimers (cellobiose), and they are also called 4-β-glucosidase and cellobiohydrolases. The third class of the enzymes is the β-glucosidase that can hydrolyze glucose dimers into individual glucose units (Lynd, Weimer et al. 2002, Malherbe and Cloete 2002). These enzymes can act together to hydrolysis cellulose, and complexed cellulase systems allow greater coordination between the different cellulose hydrolyzing enzymes.

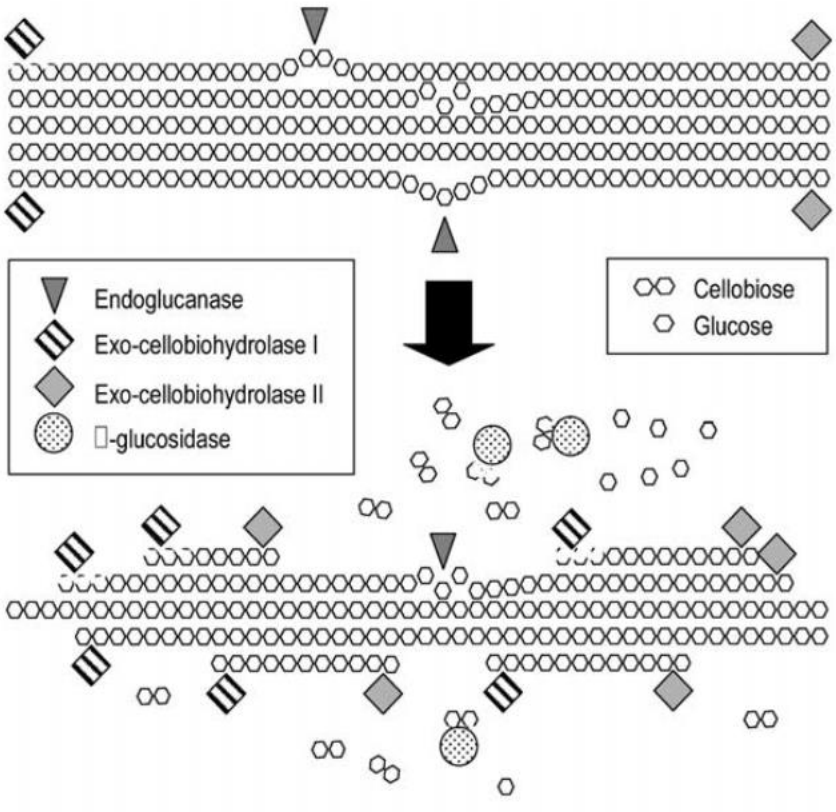


Fig. 1.2 A simplified cellulase system that completes hydrolysis process of cellulose. Consorted action of all three types of enzymes (endoglucanase, exoglucanase and β-glucosidase) are required to degrade cellulose efficiently. Figure adapted from (Malherbe and Cloete 2002)

Hemicellulose is a heteropolysaccharide branched with the degree of polymerization of 100 to 200, and it is more soluble than cellulose (Malherbe and Cloete 2002). The heterogeneous polymers usually compost of different pentoses, hexoses and sugar acids (Girio, Fonseca et al. 2010). Due to the complexity and heterogeneity of hemicellulose, more types of enzymes are required for the hydrolysis of hemicellulose than those for cellulose. Since xylan is the most hemicellulose component of grass, xylanase is the best studied enzymes involved in the hydrolysis process (Kuhad, Singh et al. 1997). Like cellulose biodegradation, the degradation of hemicellulose also requires enzymes for depolymerization and polymer ends cleavage. For the hydrolysis of xylan,

endo-xylanases breaks the internal bonds in the main chains of xylan, while exo-xylanases attacks the β-1,4-xylose linkages and clip off xylooligosaccharides and xylobioses from the end. Other enzymes such as β-xylosidase are needed to further hydrolysis polymers or dimers to xylose (Gilbert, Stalbrand et al. 2008). During the xylan hydrolysis process, additional enzymes are needed to remove the side chains on xylose, since they can block the action of xylanases for efficient and complete hydrolysis. Enzymes such as α-glucuronidase, acetylxylan esterase feruloylesterases can remove the side chains and increase the accessibility of xylanase to breakdown the chains (Gilbert, Stalbrand et al. 2008).

Lignin is highly irregular and complex macromolecule consisting of phenylpropanoid subunits which can be classified into *p*-hydroxyphenyl (H-type), guaiacyl (G-type) and syringly (S-type) units (Malherbe and Cloete 2002, Vanholme, Demedts et al. 2010). Besides the chemically complexity, the crosslinking structure makes lignin extremely insoluble and lacking in hydrolysable linkage. And unlike cellulose or hemicellulose, there are no repeating subunits are present in the chain of lignin, which makes it an extremely difficult substrate for enzymatic depolymerization (Reid 1995). The difficulty for microorganisms to digest lignin also creates barriers for the bio-degradation of lignocelluloses biomass because the enzymes that can decompose of cellulose and hemicellulose cannot get access to the target positions that wrapped in the complex structure of lignin (Balan 2014). Only certain fungi such as white-rot fungi can extensively biodegrade lignin by mineralization of it whereas brown-rot fungi can modify it while removing other carbohydrates in the lignocellulose biomass (Reid 1995). For the white-rot fungi to decompose of lignin, the key enzymes are phenol oxidases,

such as lignin peroxidase, manganese peroxidase and laccases. While the former two enzymes oxidize the substrate with intermediate cation radical formation, laccases can oxidize lignin substructures with the formation of oxygen radicals (Leonowicz, Matuszewska et al. 1999, Malherbe and Cloete 2002). In nature, for microorganisms that cannot overcome barrier that lignin caused, they can obtain energy from intermediates released from the lignocellulose by the lignin degradable microorganisms, such as the white-rot fungi (Malherbe and Cloete 2002).

Although the general enzymatic hydrolysis process of cellulose can be described as above, the systems between the aerobic and anaerobic fungi and bacteria is different. Usually, aerobic fungi and bacteria comprise non-complexed cellulase systems while anaerobic bacteria (especially *Clostridium spp*.) and fungi contain complexed cellulase systems (Tomme, Warren et al. 1995). The non-complexed system that secrets of the cellulose hydrolysis enzymes into the culture medium and the enzymes will function without interacting with other hydrolases. In contrast, for complexed systems, also known as cellulosomes, the cellulose hydrolyzing enzymes are contained in membrane-bound enzyme complexes which can adhere to the cell wall of the microorganisms (Fontes and Gilbert 2010). Cellulosomes create a dynamic and heterogeneous enzyme systems that allow greater coordination between the different cellulose hydrolyzing enzymes. The loss of degradation intermediates will be restricted due to dynamic environmental conditions. Also, the adhesion from cellulosomes to the cell wall also prevents substrate loss from diffusion or uptake by other microorganisms. This gives cellulosomes an advantage in terms on bio-degradation efficiency of the cellulose over the non-complexed

systems, where the loss of the secreted enzymes and degradation intermediates might be detrimental to overall process efficiency (Schwarz 2001, Malherbe and Cloete 2002).

1.2.2 Bioconversion of lignocellulose into biofuels

Pretreatment is a crucial step in the production of biofuels from lignocellulose. As mentioned in the last section, lignin in lignocellulose biomass are the barriers for the microorganisms to access more easily degradable cellulose and hemicellulose. Pretreatment step can solubilize or separate the major components of lignocellulose to increase the conversion efficiency by rendering the digestible components for microorganisms (Malherbe and Cloete 2002). Many studies of pretreatment methods have be published (Wyman, Dale et al. 2005) during the last few decades and they can be generally classified into several categories: biological, mechanical, chemical methods, and various combinations thereof (Menon and Rao 2012). The choice of pretreatment method should consider the overall compatibility of feedstocks, enzymes and microorganism, and the economic cost as well as the environmental impact.

To convert the pretreated lignocellulose biomass into biofuels, there are four events occur during the enzymatic hydrolysis process: hydrolase production, enzymatic hydrolysis of cellulose and hemicellulose, hexose fermentation and pentose fermentation (Lynd, Weimer et al. 2002). Based on how these four events are consolidated, different processing configurations can be summarized into four types as shown in **Figure 1.3**: separate hydrolysis and fermentation (SHF), simultaneous scarification and fermentation (SSF), simultaneous scarification and co-fermentation (SSCF) and consolidated bioprocessing (CBP). SHF perform the enzymatic hydrolysis and fermentation sequentially. Even though all the steps in SHF can be carried out at their own optimum

conditions, the accumulated product of cellobiose and glucose strongly inhibit the cellulase activities (Gruno, Valjamae et al. 2004, Wu, McLaren et al. 2010). SSF is a combination of enzymatic hydrolysis and fermentation of lignocellulosic material and they are conducted in the same reactor which can eliminate the accumulation of the hydrolysis products (Olofsson, Bertilsson et al. 2008). In SSF, the cellulase production and fermentation of hemicellulose hydrolysis products are conducted in two additional separate steps. This process has been proven to have a significant economic advantage over SHF in terms of product yield, enzyme dose and equipment settings (Zhong, Karberg et al. 2003, Olsson, Soerensen et al. 2006, Tomas-Pejo, Oliva et al. 2008, Rana, Eckard et al. 2014). However, the different optimal temperatures and pH required for these combined two steps are the main problems for SSF (Wu, McLaren et al. 2010). The key to maximize the efficiency of SSF is to select hydrolases and fermenting enzymes with close optimum temperatures and pHs. However, most microorganisms need a lower optimum temperature than hydrolases, which makes hydrolysis of cellulose a limiting step in SSF. The third strategy is NSSF, a two-step process in which saccharification and fermentation of both cellulose and hemicellulose hydrolysis products occur simultaneously (Lynd, Weimer et al. 2002). Given the ability to simultaneously ferment six- and five-carbon sugars, NSSF presented several advantages compared with SSF, including higher ethanol yield, shorter residence time and less enzyme input (Wu and Lee 1998, Oh, Kim et al. 2000). CBP consolidate all four steps together and accomplished all the hydrolysis and fermentation simultaneously in a single step. Compared with the other three strategies, CBP offers the potential of lower production costs and less time required to complete a fermentation cycle than processes featuring dedicated cellulase production.

However, at this time there is no anaerobic organism available that can carry out CBP efficiently (Liao, Mi et al. 2016), but efforts have been made to use metabolic engineering of cellulolytic microorganisms or even non-cellulolytic organisms to enable consolidated bioprocessing (Lynd, Van Zyl et al. 2005). Hence, the better understanding of physiology and metabolism of lignocellulose-degrading microorganisms is crucial to realize industrial application to product biofuels from lignocellulose.

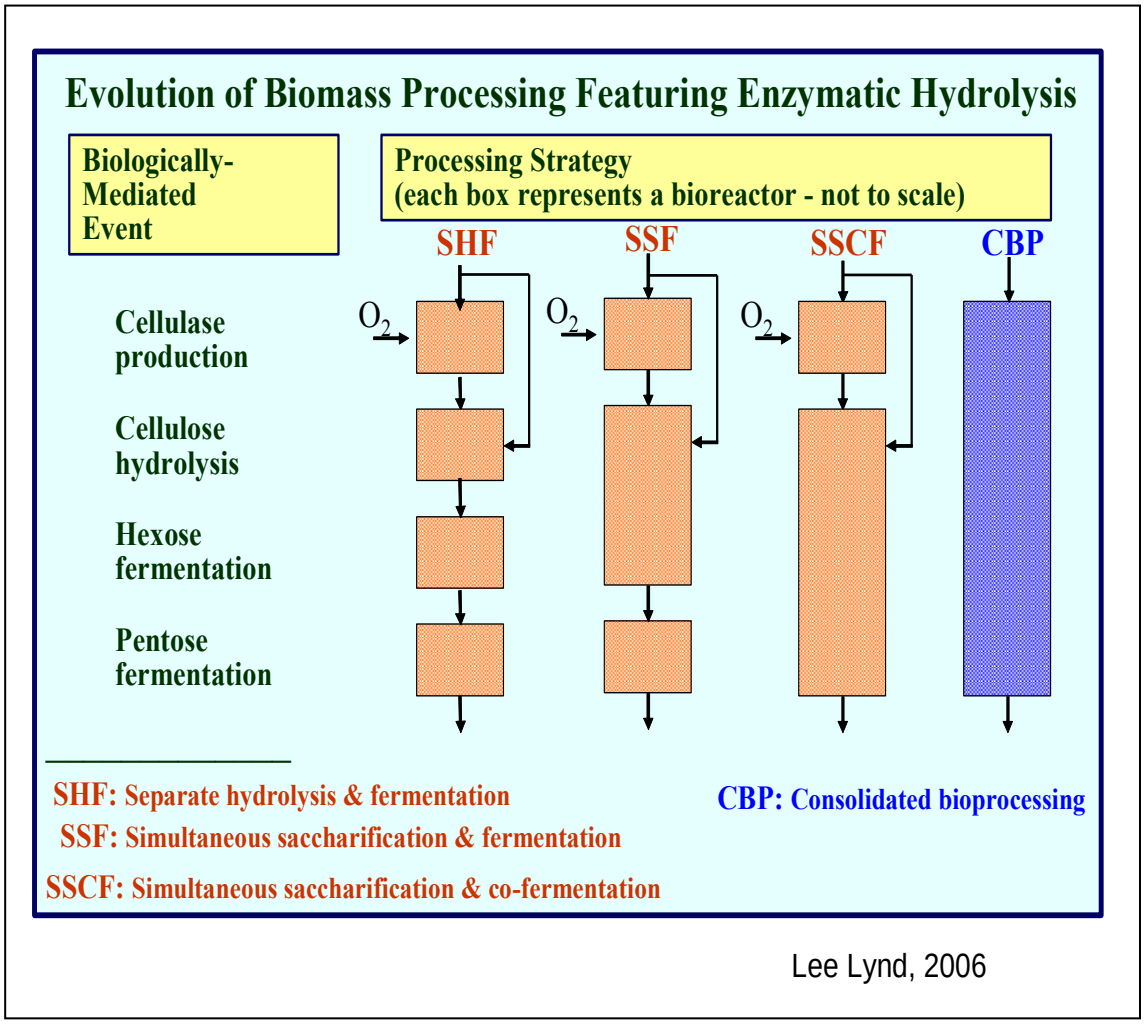


Fig. 1.3 Development of lignocellulose biomass processing configurations. The four platforms differ from each other by how the biologically mediated events are consolidated (Lynd, Greene et al. 2006).

1.3 *Clostridium cellulolyticum*: model organism of mesophilic cellulolytic clostridia

Microorganisms that can degrade lignocellulose are the key players in the production of biofuels from lignocellulose biomass, and in nature they participate as an indispensable group in the carbon cycling process, where lignocellulose is a major component. Within the domain of bacteria, only seven phyla contain cellulolytic organisms (Desvaux 2005), and about 80% of the isolated cellulolytic bacteria can be found in only two phyla: Actinobacteria and Firmicutes, where the latter phylum contains all the gram-positive anaerobic cellulolytic bacteria. To be specific, the majority of these bacteria are found in the class *Clostridia*, order *Clostridiales*, family *Clostridiaceae*, genus *Clostridium* (Desvaux 2005). *Clostridium cellulolyticum* ATCC 35319 (formerly identified as the strain H10) is a cellulolytic bacterium belongs to the genus *Clostridum*. It was first isolated from decayed grass and is considered as the model of mesophilic cellulolytic clostridia (Petitdemange, Caillet et al. 1984).

1.3.1 Physiology of Clostridium cellulolyticum

Clostridium cellulolyticum H10 is a Gram-positive mesophilic cellulolytic bacillus, which usually has a rod-shape that is 3-6 μm long and 0.6-1 μm wide with peritrichous flagella. The cell wall is typical cell walls of gram-positive species and spores can be found when the strain is cultured on cellulose media, but rarely on other carbohydrate media. The growth temperature of *C. cellulolyticum* H10 is from 25°C to 45°C, with the optimum growth temperature of 34°C (Petitdemange, Caillet et al. 1984). This bacterium can grow on many substrates: moderate growth on cellulose, xylan, glucose, xylose; weak growth on some sugars found in the hemicelluloses, such as arabinose, fructose, galactose, mannose and ribose; and no growth with adonitol, amygdalin, dulcitol, erythritol, glycerol,

glycogen, inositol, inulin, lactose, maltose, mannitol, melezitose, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, and trehalose. (Petitdemange, Caillet et al. 1984, Desvaux 2005). Questions like how *C. cellulolyticum* metabolizes these carbohydrates and what the preferences are especially when different sugars presented at the same time, lead to more and more studies on the physiology and metabolism of this microorganism (Stülke and Hillen 1999, Doi, Kosugi et al. 2003, Li, Tschaplinski et al. 2012, Xu, Huang et al. 2013). The extensive studies of the structure, genetics, and functions in *C. cellulolyticum* , makes it a model of mesophilic cellulolytic Clostridia (Desvaux 2005).

The attractive feature of *C. cellulolyticum* is its capability of anaerobic fermentation of lignocellulosic biomass into lactate, ethanol and H_2, which can be used of biofuels as an alternative energy source (Giallo, Gaudin et al. 1983, Li, Tschaplinski et al. 2012). In other words, *C. cellulolyticum* can perform lignocellulose hydrolysis and fermentation at the same time so it can be used in the consolidated bioprocessing (CBP), which combined these two processes into one reactor to reduce the cost of separated processing steps. Compared to thermophilic bacteria, which can also convert lignocellulose into biofuels, *C. cellulolyticum* can reduce cost by avoid maintaining high temperature for the thermophilic bacteria to perform fermentation of the hydrolysis products of lignocellulose. The efficiency of the bio-energy yields of cellulose fermentation by *C. cellulolyticum* depends on growth phase, substrate limitation, nutrient condition (Desvaux, Guedon et al. 2001). Therefore, optimization of the metabolic conditions for *C. cellulolyticum* to produce biofuels such as H_2 and ethanol attracts great attentions. The proposed genome sequencing of *C. cellulolyticum* would provide important information regarding the metabolic and regulatory pathways responsible for

cellulose degradation and biofuel production. Such information is also critical for the design of process control strategies for the improvement and optimization of bio-energy production. Further, this information will allow more robust and comprehensive design strategies for genomics approaches such as DNA microarrays and mass spectrometry-based proteomics aimed at evaluating and monitoring the cellulose fermentation and energy production processes carried out by *C. cellulolyticum***.**

1.3.2 The cellulosome of C. cellulolyticum

Cellulosomes are protein complexes assembled from mostly extracellular enzymes present in many cellulolytic microorganisms (Bayer, Belaich et al. 2004, Bayer, Lamed et al. 2008). The *C. cellulolyticum* cellulosome is organized around a specialized integrating protein called *Cip*C, where different catalytic components bind to it. Studies have identified 148 putative carbohydrate active enzymes in the genome, including 90 putative glycoside hydrolases, 4 putative polysaccharide lysases and 15 putative carbohydrate esterases (Blouzard, Coutinho et al. 2010). These genes are widely distributed along the 4.1-Mb genome and found in numerous operons (Hemme, Mouttaki et al. 2010). Thus, efficient cellulolysis should rely upon a global regulatory network which controls such many transcripts, but this regulatory mechanism remains not fully understood (Xu, Huang et al. 2015). Cellulosome is present on the bacteria surface to perform the cellulose depolymerization. The cellulosomes structure not only provides direct and specific adhesion to the substrate, but also insures an efficient uptake of soluble cello-oligosaccharides before they diffuse in the extracellular milieu (Shoham, Lamed et al. 1999). The cellulosomes have an efficient cellulolytic activity by allowing optimum concerted activity and synergism of the cellulases while avoiding non-productive

adsorption of the enzyme. The cellulosome structure can also avoid competition between cellulases for the sites of adsorption and allow optimal processive of the cellulase along the cellulose fiber (Schwarz 2001, Desvaux 2005)

In *C. cellulolyticum*, most cellulosomal genes are clustered in a DNA fragment, namely the *cel* cluster, which includes 12 identified genes, among which, the first gene is the scaffolding *cip*C gene (Desvaux 2005). CipC is a modular scaffolding protein without catalytic activities, consisting of a cellulose-binding domain, two hydrophilic X-modules with unknown functions and eight type I cohesion domains. Given the scaffolding structures, how the cellulosome attaches to the *C. cellulolyticum* cell surface is yet to be uncovered. It may involve novel mechanism that may not be the same as any confirmed attachment mechanism from other cellulolytic organisms such as *C. thermocellum* and *C. cellulovorans.* The catalytic components of *C. cellulolyticum* cellulosome are the cellulases and they all display similar mechanisms to hydrolyse the β-1,4-glycosidic bonds by acid catalysis (Davies and Henrissat 1995). The various cellulases processing complementary activities together with appropriate proximity are thought to be the reason why the cellulosome can degrade crystalline cellulose efficiently (Lynd, Weimer et al. 2002).

1.4 Foci of this study

Lignocellulose, which makes up nearly of plant biomass, is a major renewable energy resource based on its quantity and availability. Biofuels including hydrogen and ethanol converted from cellulose (the major component of lignocellulose) by microorganisms are more environmental friendly and can reduce considerable transportation based on the resource availabilities, therefore they have great potentials to be a sustainable alternative of the fossil fuels. However, the biofuels required substantial resources and technologies

to produce and there is still a challenge to improve the efficiency of cellulose bioconversion process, therefore, it is not commercially available on the market as a replacement of traditional fuels. Studies on cellulotic *Clostridia*, which is a consolidated bioprocessing enabling candidate, will not only help us to understand the mechanisms and physiologies behind the cellulose bio-degradation process but also provide potential strategies to improve the efficiency of the bioconversion process of cellulose. In previous studies, the cellulolytic *Clostridia*, including *C. cellulolyticum*, were comprehensively studied on their physiology and applications on the production of biofuels and it have been pointed out that their efficiency of the biofuel production from cellulose is still low. Many efforts have been made to increase the biofuel production rate while lower the economic cost. Among these efforts, the defined mixture could be a promising process to improve the efficiency of bioconversion process of cellulose, comparing to the mono-culture of a single microorganism. This study aimed to improve the bioconversion process of cellulose to produce biofuels including hydrogen and ethanol, by investigating the biofuel production of mesophilic and thermophilic *Clostridia* under different conditions and revealing the mechanisms behind the higher bioconversion efficiency. Major results are presented in the following 3 chapters (2-4).

Chapter 2 presents an investigation on the effects of different carbon loads on hydrogen production of *C. cellulolyticum*, an extensively studied model of cellulolytic microorganism. It was identified that the slow-released carbon source such as cellulose could be a better choice for *C. cellulolyticum* in terms of hydrogen production than fast-released carbon source (i.e. cellobiose). In addition, a comprehensive transcriptomic level study of the impacts of different carbon loads on *C. cellulolyticum* was conducted in this

paper, which provides more detailed in-depth understanding of the carbon metabolism of *C. cellulolyticum*.

Chapter 3 presents the development of a high hydrogen yield consortium composed of *C. cellulolyticum* and *Desulfovibrio vulgaris* Hildenborough, which provides a potential alternative for efficient cellulose degradation and hydrogen production. First, the optimized culture condition for hydrogen production was determined. Then, the interactions within the consortium were comprehensively studies from different perspectives (both physiologic and transcriptomic levels). Further, the bioconversion of cellulose to hydrogen was improved by the defined mixture we provided, and the mechanism of why the consortium is more efficient was explored. We also proposed a conceptual model to illustrate what is the function of *Desulfovibrio vulgaris* Hildenborough in the cellulose degradation. These results provide comparable characterizations of the cellulolytic and hydrogen-producing capabilities of the mono-culture and co-culture systems, and the identification of ecological relationship between these two organisms will contribute to the future improvements of the hydrogen-producing efficiency using this approach.

Chapter 4 presents the development of ethanol adapted strains of *Clostridium thermocellum* LQR1 and *Thermoanaerobacter ethanolicus* X514 through long term evolution. Interestingly, after the adapted strains that can tolerant high concentration of ethanol were obtained, we found that some strains grew slower without the exogenous ethanol after long term adaptation. More importantly, the ethanol production of the co-cultured *Clostridium thermocellum* LQR1 and *Thermoanaerobacter ethanolicus* X514 was significantly improved after the long-term ethanol adaptation.

In summary, this study provided novel insights of the improvement of cellulose bioconversion process to produce biofuels, such as hydrogen and ethanol, which could be of merit for the application of *Clostridia* in to the industrial field and make progress with the production of second generation biofuels from the lignocellulose biomass.

Chapter 2: Effects of carbon loads on hydrogen production of *Clostridium cellulolyticum* H10

2.1 Abstract

Hydrogen gas production via dark fermentation of cellulose has been investigated as a potential source of renewable energy. Some microorganisms, such as *Clostridium* species, are capable of both cellulose hydrolysis and H_2 production. However, both H_2 yield and cellulose degradation efficiency remain very low with *Clostridium* species. In this study, the effects of different initial cellulose concentration on cellulose degradation rate and hydrogen production of *Clostridium cellulolyticum* H10 were examined. The *C.cellulolyticum* was cultured on 1,4,7,10,15 g/L cellulose and cellobiose respectively, and the results showed that the highest hydrogen production was obtained when *C. cellulolyticum* was cultured on 7g/L cellulose. The cellulose degradation ratio decreased along with the increase of substrate concentrations. Glucose is the main intermediate product accumulated when *C. cellulolyticum* was cultured on high concentration of cellobiose, however, culturing *C. cellulolyticum* on cellulose can relieve the accumulation of glucose. The microarray data showed that cellulosomal genes were down regulated along with the increase of cellulose concentrations. The expression level of other genes related to carbon metabolism (e.g., pyruvate kinase, lactate dehydrogenase and hydrogenase) peaked at 7g/L cellulose. The results indicated that high loads of carbon are unfavorable for *C. cellulolyticum*, and culturing *C. cellulolyticum* on slow released substrates (e.g., cellulose but not cellobiose) can reduce the accumulation of intermediate products (e.g., glucose and/or pyruvate). The restriction of substrate availability can balance the metabolism rate of intermediate products in *C. cellulolyticum*, which can

relieve H10 from catabolite repression and improve the hydrogen production. The future work will focus on further understanding how to improve the carbon utilization ability of *C. cellulolyticum* thus enhance the hydrogen production.

Keywords: *Clostridium cellulolyticum*, initial carbon loads, hydrogen production, cellulose degradation, cellulose metabolism, microarray, gene co-expression network

2.2 Introduction

In view of the realization that fossil fuels reserves are limited, alternative energy generating options are being explored. Biological methods for producing fuels such as ethanol, diesel, hydrogen (H_2), methane, among others have the potential to provide a sustainable energy system. H_2 has been proposed as a clean and efficient energy carrier, with the highest energy content per unit weight of any known fuel (Das and Veziroglu 2001). It is the only common fuel that is not chemically bound to carbon and burning hydrogen does not contribute to the greenhouse effect, ozone depletion, or acid rain (Nath and Das 2004). Among the various processes comprising biological production of hydrogen, fermentative H_2 production appears to be promising as it is non-polluting and can be produced biologically from renewable resources, including cellulosic biomass. The key technique obstacle of utilizing these sustainable cellulosic materials is how to develop a low-cost technology to produce H_2 on a large-scale (Li, Xu et al. 2014). The fermentation of cellulose and its subunits (cellobiose) to H_2 can be implemented by consolidated bio-processing (CBP) (Lynd, Van Zyl et al. 2005), where saccharolytic enzymes production, cellulose hydrolysis, and fermentation of sugars produced during the degradation of cellulose occur in one step.

Since the cellulosic biomass is the most abundant sustainable material on earth, the cellulosic biofuels become a promising alternatives of fossil fuels as the research of affordable and clean sustainable energy fuels continues (Lynd, Weimer et al. 2002). Cellulolytic clostridia play an important role in carbon cycling in terms of cellulose decomposition (Leschine 1995). *Clostridium cellulolyticum* ATCC 35319, formerly identified as the strain H10 is a cellulolytic mesophilic Gram-positive bacterium isolated

from decayed grass by Petitdemange et al.(Petitdemange, Caillet et al. 1984). *C. cellulolyticum* is capable of degrading crystalline cellulose via an extracellular cellulosome and utilizing the sugars (cellubiose and glucose) from cellulose degradation to acetate, lactate, ethanol, CO_2 and H_2 (Desvaux 2005). With mesophilic *Clostridium* species, Ren *et al*. (2007) characterized the cellulolytic and hydrogen-producing activities of six species, and observed that *C. cellulolyticum* catalyzed the highest H_2 production from cellulose, with yield of 1.7 mol H_2 mol $^{-1}$ hexose (Ren, Ward et al. 2007). Therefore, *C. cellulolyticum* could be the potential model system of the H_2 production directly from cellulosic biomass with industrial relevance. However, the cellulolytic capability and metabolic productivity of *C. cellulolyticum* are still not able to satisfy the requirements of industrial production.

Fermentation of cellulosic materials at high concentration is economically critical in industrial process which can reduce the operational cost and lower energy input (Kristensen, Felby et al. 2009). However, in the natural environment, the *C. cellulolyticum* is more likely under substrate-limiting conditions since it is hard for the bacterium to find a niche with plenty carbon sources and other nutrients, thus, it has become well adapted to the nutrients limited conditions and was reported that compared to a rich medium it is more likely favorable to the mineral salt medium for a better control of the carbon flow (Payot, Guedon et al. 1998, Desvaux, Guedon et al. 2000). Previously, it has been reported that *C. cellulolyticum* was considered as a sluggish cellulolytic bacterium which was mostly attributed to its cellulosic system (Petitdemange, Caillet et al. 1984, Petitdemange, Tchunden et al. 1992, Tchunden, Petitdemange et al. 1992). However, according to the extensive study of enzymological properties of *C.*

cellulolyticum cellulosome, there is no direct evidence that its limiting factor of cellulose degradation is its cellulolytic system (Desvaux 2005). In fact, it has been reported that high concentration of substrate may cause the cellulose degradation drops rapidly, due to the accumulation of NADH and pyruvate to toxic level (Guedon, Desvaux et al. 1999, Desvaux, Guedon et al. 2000). There are studies reported that the impacts of different carbon load (both cellulose and cellubiose) on *Clostridia* from the metabolic perspectives (Strobel 1995, Guedon, Desvaux et al. 1999, Desvaux, Guedon et al. 2000), the impacts of carbon loads on transcriptional level still remains unknown.

In this study, we hypothesized that culturing *Clostridium cellulolyticum* H10 on slow released substrates (e.g., cellulose but not cellobiose) can reduce the accumulation of intermediate products (e.g., glucose and/or pyruvate). The restriction of substrate availability can balance the metabolism rate of intermediate products in *C. cellulolyticum* H10, which can relieve H10 from catabolite repression and improve the hydrogen production. To test these hypotheses, we cultured the *C.cellulolyticum* with different substrate under different concentration, microarray analysis were also conducted to study the transcriptional differences under different substrate load. A gene co-expression network was constructed to study the co-occurrence patterns of some key genes of carbon metabolism under different carbon loads. The results indicated that in terms of hydrogen production of *C. cellulolyticum*, cellulose is a better carbon source than cellobiose. In addition, the microarray data showed that cellulosomal genes were down regulated along with the increase of cellulose concentrations. The expression level of other genes related to carbon metabolism (e.g., pyruvate kinase, lactate dehydrogenase and hydrogenase) peaked at 7g/L cellulose. The microarray data indicated that high loads of carbon are

unfavorable for *C. cellulolyticum* since the expression level of carbon metabolism genes were decreased under high cellulose concentration.

2.3 Materials and Methods

2.3.1 Microorganisms and media

Clostridium cellulolyticum (ATCC 35319) was obtained from the American Type Culture Collection (ATCC). Unless otherwise stated, the components of DCB-1 medium (per liter of distilled deionized water) were NaCl 1.0 g; $MgCl_2 \cdot 6\ H_2O$ 0. 5g; KH_2PO_4 0.2 g; NH_4Cl 0.3 g; KCl 0.3 g; $CaCl_2 \cdot 2H_2O$ 0.015 g; resazurin (0.1% solution) 0.25 ml; L-cysteine 0.1 g; trace element solution 1.0 ml; wolin vitamin solution 1.0 ml and Se/Wo solution 1.0 ml. Mineral medium was buffered by 2.52 g L^{-1} $NaHCO_3$ and pH was adjusted by mixed gas of N_2 and CO_2.

Trace element solution contained the following (per liter): HCl (25% solution, w/w) 10 ml; $FeCl_2 \cdot 4H_2O$ 1.5 g; $CoCl_2 \cdot 6H_2O$ 0.19 g; $MnCl_2 \cdot 4H_2O$ 0.1 g; $ZnCl_2$ 0.07 g; H_3BO_3 0.006 g; $Na_2MoO_4 \cdot 2H_2O$ 0.036 g; $NiCl_2 \cdot 6H_2O$ 0.024 g; $CuCl_2 \cdot 2H_2O$ 0.002 g. Wolin vitamin solution contained (per liter): biotin 20 mg; folic acid 20 mg; pyridoxine hydrochloride 100 mg; riboflavin 50 mg; thiamine 50 mg; nicotinic acid 50 mg; pantothenic acid 50 mg; vitamin B_{12} 1 mg; p-aminobenzoic acid 50 mg; thioctic acid 50 mg. Se/Wo solution contained (per liter): $Na_2SeO_3 \cdot 5H_2O$ 6 mg; $Na_2WO_4 \cdot 2H_2O$ 8 mg; NaOH 0.5 g.

C. cellulolyticum was maintained by the routine transfer of 10% (v/v) inoculum into fresh DCB-1 medium containing 5 g/Lcellobiose. The reductant used for DCB-1 medium was 0.1 g/L L-cysteine, the serum bottles used for anaerobic culture were flushed with N_2/CO_2 closed with butyl rubber stoppers and aluminum seals.

2.3.2 Batch experiments

C. cellulolyticum H10 was cultured under five different substrate concentrations for both cellobiose and cellulose (1, 4, 7, 10 and 15 g /L) with initial pH 6.8 at 39 °C. All batch experiments were performed by using a small liquid volume relative to the head space volume (1:7; v/v) to reduce the inhibition caused by H_2 accumulation(Hallenbeck and Benemann 2002). In addition, gas was released and the headspace was flushed with filtered N_2 every 12 hours. The total inoculum was 10% by volume from an exponentially growing culture. The batch experiments were performed in an incubator shaker (120 rpm). An abiotic control without inocula was also included, and all tests were run in triplicate. Zero hour samples were harvested immediately post-inoculation.

2.3.3 Measurement of cell growth, cellulose degradation, sugar consumption and fermentation products

Cell growth on cellubiose as carbon source was profiled by monitoring OD600 with a spectrophotometer, and time-course samples were taken for HPLC analysis to measure the amount of residual sugars at the corresponding time point. Cell growth on cellulose was determined by cellular protein measurement. Samples (1 ml) were harvested (10000g, 5min), lysed by 0.2N NaOH/1% (w/v) SDS solution for 60 mins at room temperature, and followed by neutralization with 0.8 N HCl. The total protein in the supernatant was quantified with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), using bovine serum albumin as a standard.

Any remaining pellets not dissolved by NaOH were stored at -20 °C for subsequent cellulose analysis. Cellulose concentration was determined as described previously (Huang and Forsberg 1990). Residual cellulose was put in boiling water bath

for 30 min and washed to remove noncellulosic materials then solubilized in 67% sulfuric acid as described by Updegraff (Updegraf.Dm 1969). Cellulose was then quantified by using the phenol-sulfuric acid method for sugars with glucose as the standard (Dubois, Gilles et al. 1951, Dubois, Gilles et al. 1956). The concentration of soluble sugar was determined similarly after filtration of the supernatant through a 0.2 µm syringe filter.

Product biogas composition (H_2 and CO_2) was measured using a gas chromatograph (Agilent 6890N; Agilent Technologies, CA, USA) equipped with a thermal conductivity detector. Hydrogen measurements were conducted with a molecular sieve column (HP-PLOT MoleSieve/5A, Agilent Technologies, CA, USA) with nitrogen as the carrier gas. For CO_2 analysis, a HP-PLOT Q column was used with helium as carrier gas. Gas concentrations were quantified by comparing peak area values to those of a standard curve prepared from known concentrations of H_2 and CO_2.

For metabolites analysis, samples were centrifuged and then supernatant was filtered through 0.2 µm membranes and stored at -20 °C. The concentrations of lactate, acetate and ethanol were analyzed by a HPLC with an Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA) equipped with an ion-exclusion column (Aminex HPX- 87H; 300 mm × 7.8 mm; Bio-Rad, Hercules, CA, USA) operating at 55°C detected by a refractive index detector using a water 410 refractometer, and quantified using a standard curve. The mobile phase consisted of 0.025% sulfuric acid at a flow rate of 0.6 ml/minute.

2.3.4 Global transcriptomic analysis

2.3.4.1 Sample collection

C. cellulolyticum was cultured under five different substrate concentrations of cellulose (1, 4, 7, 10 and 15 g /L). Each concentration had three biological replicates. Cell samples (10ml) were collected at the mid-exponential phase by 10 min centrifugation at 5000g at 4°C. The cell pellets collected were immediately frozen with liquid nitrogen and then stored at -70°C. Total cellular RNAs were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA samples were purified by use of an RNeasy mini-kit (Qiagen), and RNA samples were treated with on-column RNase-free DNase I (Qiagen) to digest genomic DNA. RNA samples were quantified by Nanodrop ND-1000 spectrophotometry at wavelengths of 260 and 280 nm; RNA integrity was estimated by running agarose gels.

2.3.4.2 Microarray hybridization and data analysis

13, 098 probes with 50 nt in length were designed to cover 94% protein encoding genes in *C. cellulolyticum* and then sent to manufacture 8-array slides by Agilent. For each RNA sample, 0.6 µg purified total RNA was reversely transcribed to cDNA using Reverse transcriptase III (Invitrogen) and fluorescent dye Cy3 was used for labeling. Genomic DNA (gDNA), which was isolated from *C. cellulolyticum* with CTAB method (Zhou, Bruns et al. 1996), was labeled by fluorescent dye Cy5 using Klenow DNA polymerase. 1.5 µg qualified gDNA was used for each labeling reaction and the resulting product was used for 1 slide (8 hybridizations). All labeled cDNA and gDNA were purified and dried before hybridization process by using QIAquick PCR purification reagents (Qiagen). Labeled cDNA and gDNA were mixed together in the hybridization buffer containing 8% formamide then denaturing at 95°C for 3 min, followed with 30 min incubation at 37°C and loading onto the array. Hybridization was carried out at 20 rpm 67°C for 22 hours.

The slides were washed and scanned using NimbleGen MS200 (Roche) with the flowing settings (two-channel scanning, 2 μm scanning resolution, 100% laser strength, 30% gain percentage).

Microarray data analysis was performed using limma package in R(Ritchie, Phipson et al. 2015). First, the signal of probes with fluorescent dye were screened as previously described (single-to-noise ratio (SNR)>2) (He and Zhou 2008). Second, the mean signals of each probe were applied to background correction by subtraction, within-array normalization by loess, and then between-array normalization by quantile.

2.3.5 Construction of gene co-expression network

The microarray datasets from different initial cellulose load samples were used to construct gene co-expression network based on random matrix theory (RMT) (Luo, Zhong et al. 2006), which was described in the pipeline developed in our lab (Deng, Jiang et al. 2012). For each spot in the microarray, the log transformation of the normalized Cy3/Cy5 ratio was calculated and used as the gene signal intensity for that specific spot. For each gene (spot) there was a positive signal intensity for each sample if the gene was expressed in that sample. The genes were not considered if they were not expressed in more than half of the samples to assure the accuracy and confidence of the correlation between the gene signals. The correlations between the eligible genes were calculated from the signal intensities of these genes. To detect strong and significant relationships between the gene expression patterns, random matrix theory was used to determine the best cutoff value of the correlations. The link between two genes were kept in the network only when the correlations between these genes were equal or larger than the cutoff determined by the RMT. In the final network, the nodes represent genes and the links

represent the kept correlations between the genes, which indicate a co-expression pattern. A gene was called a hub of the network if the number of links connecting this gene to others was the highest in the network. Usually the hub gene plays important roles in the co-expression network as it may have regulatory effect on its neighbor genes, or it may represent a group of genes that have similar functions in the samples provided.

2.4 Results and discussion

2.4.1 Effect of different carbon loads on the growth and hydrogen production of C. cellulolyticum

Growth of *C. cellulolyticum* was examined on both soluble (cellobiose) and insoluble substrates (cellulose). *C. cellulolyticum* were cultured on 1,4,7,10,15 g/L cellulose and cellobiose respectively. The results showed that the highest biomass was obtained when the *C. cellulolyticum* was cultured on 4g/L on cellobiose, which was consistent with previous reported results that when the *C. cellulolyticum* was batch cultured on 14.62mM cellobiose (approximately 5g/L), residual cellobiose was observed (4.24mM) and growth was limited (Guedon, Desvaux et al. 1999). However, the highest biomass was obtained at 7g/L when it was cultured on cellulose (Fig. 2.1). The results indicated that when the cellulose concentration is lower than 7g/L, the biomass increased with the initial cellulose amount increase, however after the maximum biomass was obtained, with above 7g/L cellulose the biomass was decreased (Fig 2.1 B). Interestingly, when the *C. cellulolyticum* were cultured on cellobiose, even though the maximum biomass was obtained with 4g/L cellobiose, the biomass maintained at a similar level with increased initial cellobiose amount (Fig 2.1 A). Since the growth on cellobiose was limited when the carbon loads were higher than 4g/L, the result indicated that when the *C. cellulolyticum* was cultured

on the high concentration of carbon source (i.e. 7 g/L), the cell growth may better when cultured on the slow-released carbon source cellulose than the fast-released carbon source cellobiose. These results were consisted with previous reported that compared to the cellobiose which was considered as a model of soluble cellodextrin, the *C. cellulolyticum* is more likely adapted to a cellulolytic lifestyle with the pure cellulose as a carbon source closely related to the lignocellulosic compounds (Desvaux 2005).The hydrogen production of the *C. cellulolyticum* under different carbon load was also examined. For all the substrate concentrations tested, the hydrogen production from cellulose are higher than cellobiose except when the carbon load is 1 g/L (Fig 2.2). The results suggested that *C. cellulolyticum* produced the highest amount of hydrogen with 7g/L cellulose as substrate which is significantly higher than the hydrogen produced cultured on 7g/L cellobiose ($p < 0.05$). According to the ANOVA test, hydrogen produced on 7g/L cellulose was significantly higher than the hydrogen produced on 1g/L and 15 g/L cellulose, however, it did not show significance when compared to 4g/l and 10 g/L cellulose. Therefore, in terms of hydrogen production, it was concluded that cellulose is a better carbon source rather than cellobiose. The amount of hydrogen produced from sugar fermentation depends on the catabolic pathways used by the *C. cellulolyticum* (Ren, Xing et al. 2007). A higher hydrogen yield associated with acetate production, of the different carbon loads tested in this study, the highest H_2 production was obtained when the *C. cellulolyticum* was cultured on 7 g/L cellulose. These results were consistent with the stoichiometry mentioned above, because under this concentration *C. cellulolyticum* produced the highest amount of acetate 1.9 g/L (Table 2.1). These results were also consistent with previous studies. When acetate was the main end products (39-58%) the

C. cellulolyticum can produce large amount of H_2 (Desvaux, Guedon et al. 2001). It was reported that when *Clostridium phytofermentans* was cultured on cellobiose, 58% of metabolites were ethanol, but no H_2 data were provided (Warnick, Methe et al. 2002). Another study tested cellulolytic and hydrogen-producing activities of six mesophilic *Clostridium* species showed that the *C. cellulolyticum* and *C. populeti* cultured on cellulose obtained the higher H_2 production than other three cellulose-degrading strains with higher percentage of acetate production, while *C. phytofermentans* showed the lowest H_2 production with the most ethanol produced (Ren, Ward et al. 2007). However, the performance of each strain may depend on the type of substrates. For example, it was reported that the acetate to ethanol ratio produced by *Clostridium lentocellum* increased when cultured on crude biomass instead of pure cellulose, but no H_2 data were reported (Ravinder, Ramesh et al. 2000, Lynd, Weimer et al. 2002).

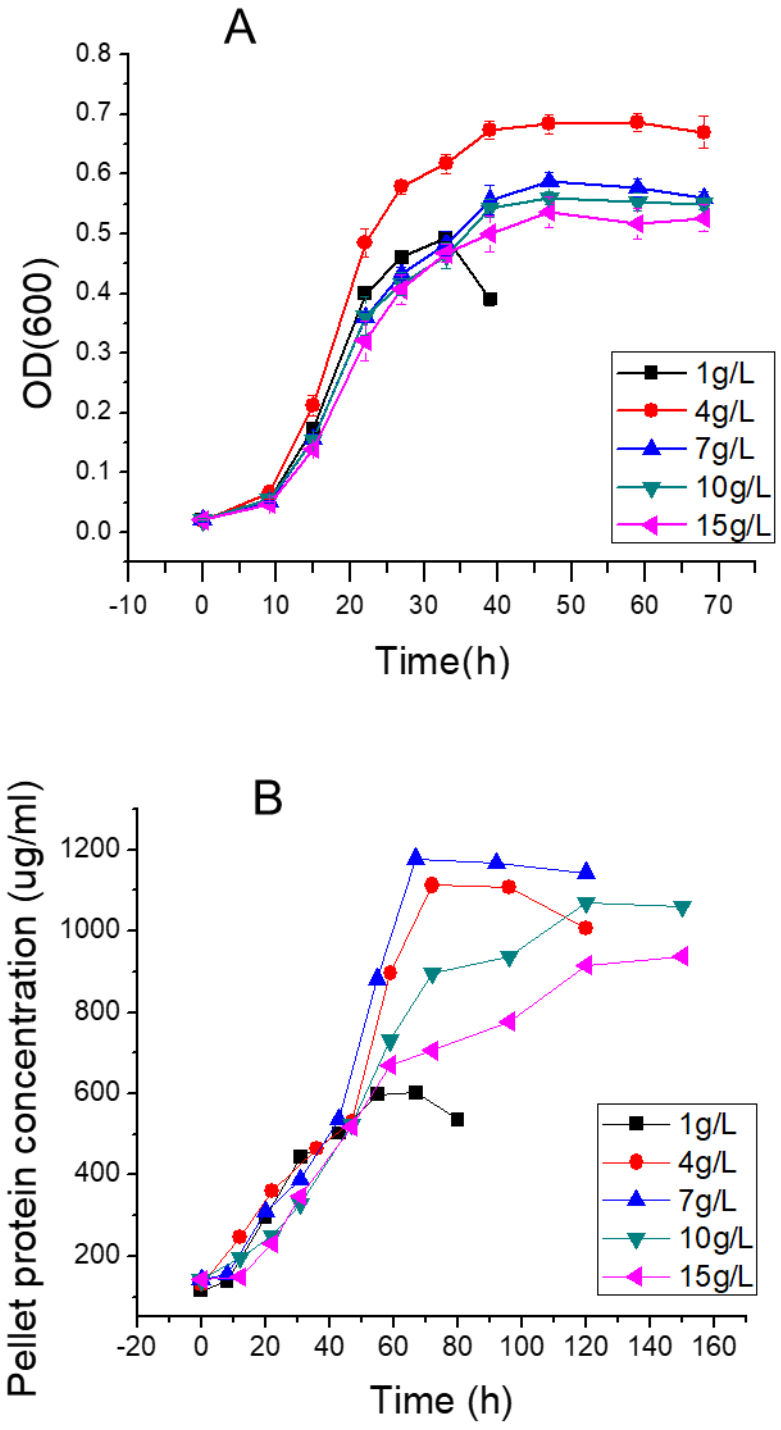


Fig. 2.1 Growth curve of *C. cellulolyticum* under different carbon loads cellobiose(A) and cellulose (B average of triplicates)

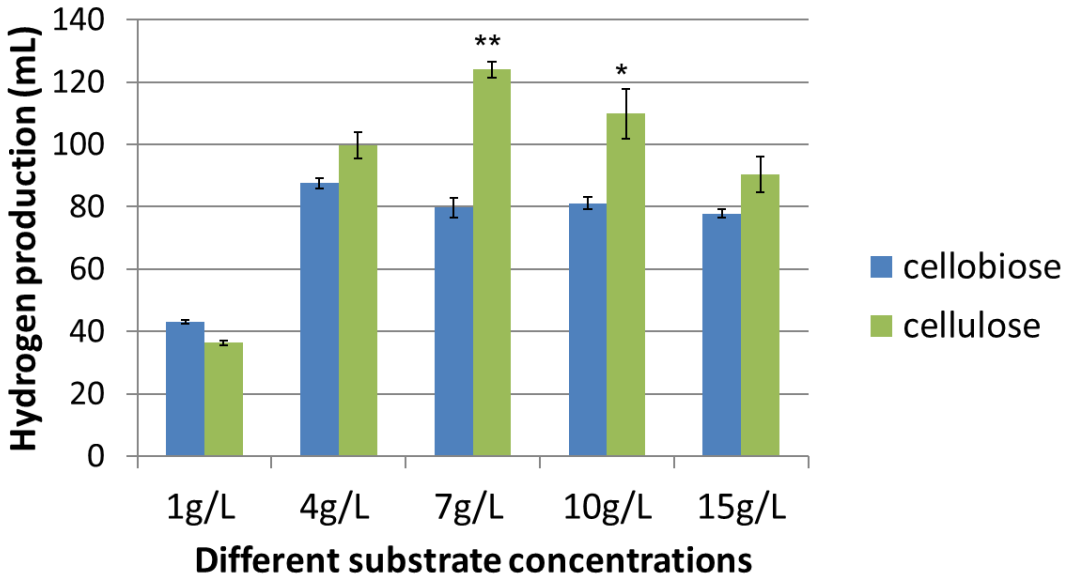


Fig. 2.2 Hydrogen production of *C. cellulolyticum* under different carbon loads (cellobiose and cellulose) (** $p<0.05$, *$p<0.1$ for t-test comparing the hydrogen produced from the same concentration of cellulose and cellobiose)

2.4.2 End products concentrations of different carbon loads

To compare the metabolic behavior of *C. cellulolyticum* under different carbon sources, the end products of each conditions were examined. The final levels of acetate, lactate and ethanol were measured. In general, the amount of each end products of fermentation increased with initial cellulose added (Table 2.1), however, beyond 7g/L initial cellulose and up to the highest 15 g/L cellulose concentration, the concentration of end products did not increase, conversely, decreased to a lower level. The hydrogen production decreased as well (Fig. 2.2). When the *C. cellulolyticum* was cultured on cellobiose, the production of end products showed at the same trend but the turn over concentration of cellobiose is 4 g/L.

The results also showed that there is more than 80% cellobiose was not utilized when the *C. cellulolyticum* was cultured with 15 g/L cellobiose, and 1.94 g/L glucose was accumulated but very low mount of pyruvate was observed. In the contrast, when the *C.*

cellulolyticum was cultured on high concentration of cellulose, there are higher amount of pyruvate was accumulated and less glucose accumulated than cellobiose.

In order to investigate the impacts of intermediate products on the carbon utilization in the system, the dynamics of glucose and pyruvate concentrations from different time point under high carbon load (both cellulose and cellobiose 15 g/L) were also examined. The results showed that when *C. cellulolyticum* was cultured on 15 g/L cellobiose, glucose was accumulated with the cell growth from the beginning, and the final concentration reached more than 10mM, however, the concentration of pyruvate was extremely low which is less than 0.1mM (Fig 2.3A). The concentration of glucose and pyruvate produced when *C. cellulolyticum* was cultured on cellulose showed a different trend on cellobiose. Interestingly, the extracellular pyruvate was first accumulated when the carbon source is cellulose, and after the concentration of extracellular pyruvate reached 1.5mM, the accumulation of glucose was observed in the system (Fig 2.3B). The leak of pyruvate to the medium indicated an intracellular accumulation of pyruvate which suggested that the PFO can not support the carbon flow derived from glycolysis, resulting an accumulation of glucose in the system (Guedon, Payot et al. 1999, Desvaux, Guedon et al. 2001). In addition, unlike the extracellular pyruvate concentration did not change too much afterward, the glucose concentration increased long with the fermentation process.

Considering that cellobiose is the main product of cellulose hydrolysis, as a consequence, bacterial growth on cellobiose was comprehensively studied to represent the growth on cellulose. In addition, since it is more easily to observe the metabolic behaviors with a soluble substrate than an insoluble one, cellobiose has been used widely

in the literatures to simulate the bacterial growth on cellulose and justify the use of cellobiose to study the physiology of *Clostria* species and many other different cellulolytic microorganisms (Ng and Zeikus 1982, Giallo, Gaudin et al. 1983, Helaszek and White 1991, Lou, Dawson et al. 1997, Guedon, Payot et al. 1999, Desvaux 2005). However, in this study, the results suggested that there are different metabolic behaviors when the *C. cellulolyticum* was cultured on the same amount of cellulose and cellobiose. First, when the *C. cellulolyticum* was cultured on cellulose, a very small amount of cellobiose was accumulated, the accumulation of glucose is also at a low level compared to the same time point when *C. cellulolyticum* was cultured on cellobiose. Second, more lactate was produced when the *C. cellulolyticum* was cultured on cellobiose than cellulose, conversely, more acetate was produced when the *C. cellulolyticum* was cultured on cellulose (Table 2.1). That maybe the reason why there is more hydrogen produced when the substrate is cellulose. It was reported that when the *C. cellulolyticum* was cultured on high concentration of substrates, it was considered as a sluggish carbon utilizer by limited carbon consumption and subsequent limited growth, and the metabolic flux analysis suggested that an inefficiently regulated carbon flow causing the self-intoxication of bacterial metabolism (Guedon, Desvaux et al. 1999, Desvaux 2005). It was observed that a shift from an acetate–ethanol fermentation to a lactate–ethanol fermentation when the substrate of *C. cellulolyticum* were changed from cellulose to cellobiose. This shift of fermentation end products also consistent with the hydrogen production mentioned above, when the *C. cellulolyticum* was cultured on cellobiose, more lactate was produced and less acetate was produced coupled with less hydrogen production than it was cultured on cellulose. In this situation, we can question that why there is a shift of fermentation when

C. cellulolyticum was cultured on cellulose and cellobiose. In fact, the lactate products always indicated a pyruvate overflow. The production of lactate can decrease the NADH/NAD+ ratio which played a very important role in the regulation of electronic fluxes. The lactation production associated with a decrease in NADH was reported, which enables growth resumption of *C. cellulolyticum* in batch cultures on cellobiose (Payot, Guedon et al. 1999). It was reported that when the *C. cellulolyticum* was cultured on cellulose the NADH/NAD+ ratio is always lower than 1, whereas a ratio of as high as 1.51 was obtained with cellobiose as a carbon source (Desvaux, Guedon et al. 2000, Desvaux, Guedon et al. 2001). Thus, our results indicated that the cellulose as a slow released substrate may be a better carbon source of *C. cellulolyticum* than cellobiose in terms of relieving the bacterium from the catabolic repression.

Table 2.1 Concentrations (g/L) of fermentation products of *C. cellulolyticum* under different carbon loads (cellobiose and cellulose)

Substrate Concentration (cellobiose)	Cellobiose	Glucose	Pyruvate	Lactate	Acetate	Ethanol
15g/L	12.7	1.94	0.01	1.23	0.94	0.18
10g/L	8.16	1.57	0.01	1.4	0.91	0.16
7g/L	5.53	1.37	0.02	1.43	0.93	0.2
4g/L	0.38	0.04	0.02	1.45	1.52	0.23
1g/L	N.D.	N.D.	N.D.	0.04	0.47	N.D.

Substrate Concentration (cellulose)	Cellobiose	Glucose	Pyruvate	Lactate	Acetate	Ethanol
15g/L	0.95	0.72	0.19	0.7	1.64	0.18
10g/L	0.75	0.58	0.17	0.77	1.51	0.13

7g/L	0.75	0.45	0.08	1.08	1.9	0.2
4g/L	N.D.	N.D.	N.D.	0.23	1.76	0.19
1g/L	N.D.	N.D.	N.D.	N.D.	0.44	N.D.

The cellulose degradation ratio was also determined with different initial cellulose load. The results showed that, not surprisingly, when the initial cellulose concentrations are higher than 7 g/L, the percentage of cellulose degradation drops rapidly. The results indicated that the higher cellulose concentration the lower cellulose degradation ratio (Fig S2.1). The cellulose degradation decreased dramatically after 40 hours of fermentation when the *C. cellulolyticum* was cultured on 15 g/L cellulose. The decreased cellulose degradation was observed at the same time point with the glucose accumulation (Fig 2.3B), which indicated that glucose may be the key intermediate product that slows down the cellulose degradation. These results consistent with previous studies that the release of soluble sugars may inhibit both cell growth and cellulase production resulting in low cellulose degradation (Petitdemange, Tchunden et al. 1992, Desvaux 2005).

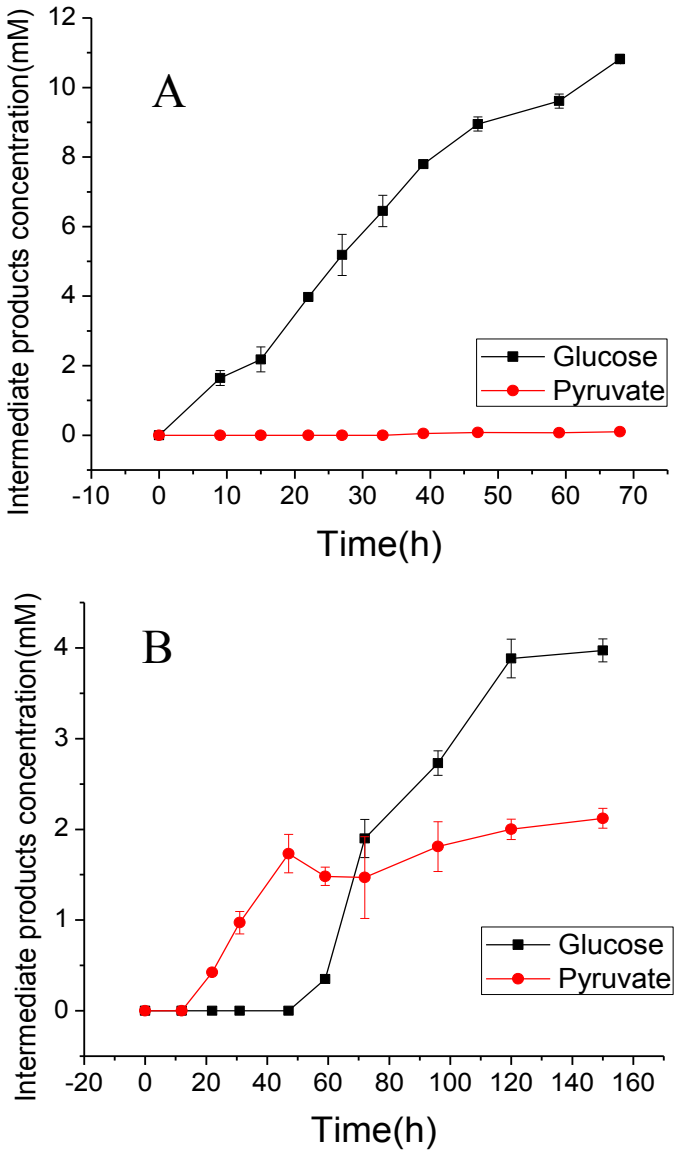


Fig. 2.3 Extracellular concentration of glucose and pyruvate in 15g/L cellobiose (A) and cellulose (B)

2.4.3 Microarray analysis of C. cellulolyticum cultured under different cellulose concentrations

To explore an overall picture of the impacts of different cellulose loads on the growth of *C. cellulolyticum*, we conducted transcriptomic analysis for all the 5 different initial cellulose concentrations with gene expression microarrays. We cultivated *C.*

cellulolyticum in defined medium with different initial cellulose and collected cells at the middle of exponential phase. The sampling on different initial cellulose not only provides an opportunity to detect the carbon load- dependent gene expression and regulations, but also the chance to analyze the correlations of genes of interested by constructing co-expression gene networks.

Microarray hybridization of different initial cellulose load samples was conducted on 8- array Agilent slides designed by our lab which contains 13098 50nt length probes which covered 94% protein encoding genes in *C. cellulolyticum.* Since we try to get the differentially expressed genes under different initial cellulose load, it is not fair to set any specific cellulose concentration as the control to calculate the relative express level of the target genes, in this study, we just normalized the raw signal intensities of each gene to show the differential expression level under the different initial cellulose load. Briefly, the probes signal intensities of cDNA were first normalized by gDNA, then this adjusted cDNA signal intensities of each probe were divided by the sum of total cDNA signal intensities of the whole array to get the normalized signal intensities we used for the calculations in this study. After the normalization, we consider the signal intensities of each probe were comparable with-arrays and between-arrays.

Detrended correspondence analysis (DCA) of was conducted to see the overall similarity of the microarray gene expression profile for the different initial cellulose load samples (Fig. 2.4). The results showed that the samples from different cellulose concentrations separate well along with the changes of cellulose concentration. The samples from lower cellulose concentration (1, 4 and 7 g/L) were well clustered together at the left side of DCA1 according to their initial cellulose concentration respectively,

however, the samples from 10 and 15 g/L were not clustered as well as the low cellulose concentrations.

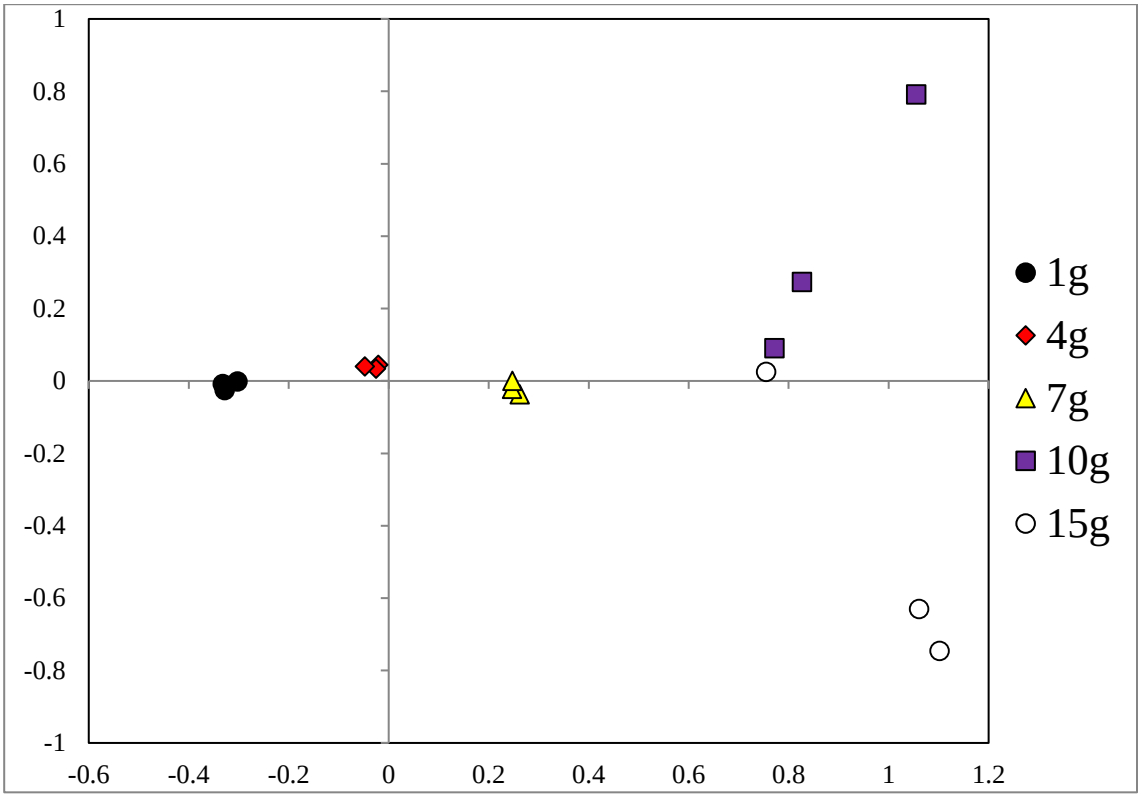


Fig. 2.4 Detrended correspondence analysis (DCA) of the transcriptional changes. Overall similarity of the microarray gene expression profile for different cellulose concentration samples (1,4,7,10 and15 g/L)

In the previous studies, the investigation of *C. cellulolyticum* metabolism by using both batch cultures and continuous cultures under different conditions with cellulose or cellobiose as the substrate. There are 3 key metabolic node were revealed: (1) the cellulosome node which regulates of the entering carbon flow; (2) the phosphor-glucomutase (PGM) which regulates the carbon flow towards the central metabolism, and (3) the pyruvate ferredoxin oxidoreductase (PFO) metabolic node in regulation of energetic and electronic fluxes (Desvaux 2005).

Since in the previous studies, researchers only investigated the carbon metabolism of *C. cellulolyticum* on the physiology level but no study on the transcriptomic level was performed. In this study, we conducted the microarray analysis with different initial cellulose load and investigated related genes expression level related to the carbon metabolisms. In *C. cellulolyticum*, most cellulosomal genes are clustered together in a so called *cip-cel* operon contains 12 genes (*cipC–cel48F–cel8C–cel9G–cel9E–orfX–cel9H–cel9J–man5K–cel9M–rgl11Y–cel5N*) within an approximately 26 kb long DNA fragment (Pages, Valette et al. 2003). The results showed that the gene expression level of *cip-cel* operon which encodes the cellulosome in *C. cellulolyticum* decreased along with the increase of cellulose concentration (Fig. 2.5). These results suggested that high cellulose concentration will inhibit the gene expression level of cellulosome.

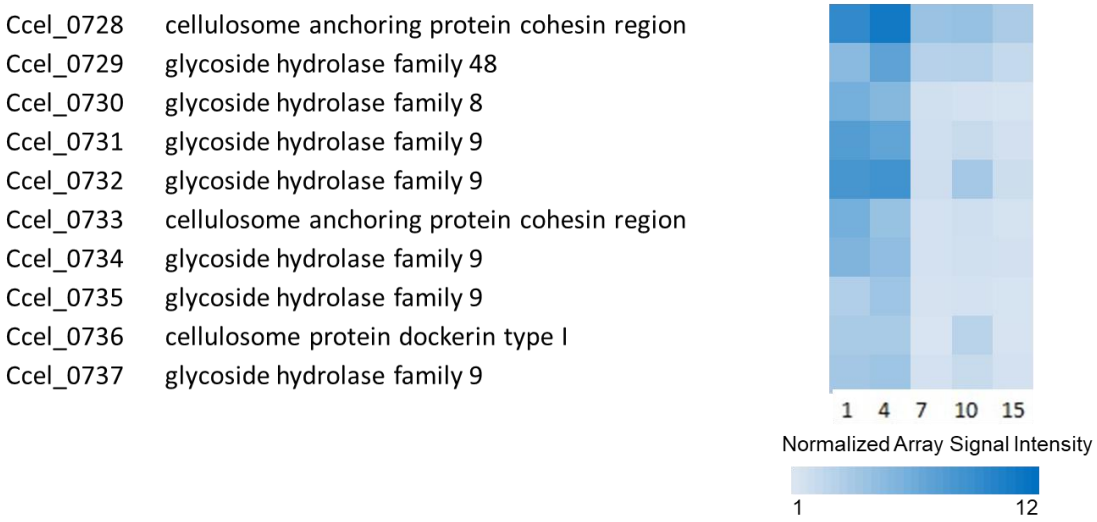


Fig. 2.5 Cellulosomal genes (*cip-cel*) differentially expressed under different concentrations of cellulose.

The expression level of other cellulosomal genes not clustered on *cip-cel* operon were also analyzed under different initial cellulose load (Fig. 2.6). Unlike the previous

results of *cip-cel* operon encoded the cellulosome in *C. cellulolyticum* which showed the decreased gene expression level along with the increase of cellulose concentration, the expression level of glycoside hydrolase varies along with the increase of cellulose concentration, however, most of them showed the same trend with *cip-cel* operon whose gene expression level are decreased along with the increase of carbon load, except Ccel_0740 and Ccel_0750. Overall results suggested that the higher carbon load will inhibit the gene express level of cellulosomal genes in *C. cellulolyticum*. Expression level of non-hydrolysis cellulosomal genes under different cellulose load were also investigated (Fig. S2.2). Those genes contained the cellulosome anchoring protein and carbon hydrate binding protein which are not directly hydrolyze the cellulose but very important in the cellulose degradation reaction. The results consisted with expression level of cellulosomal genes, and the gene expression level are decreased along with the increase of carbon load.

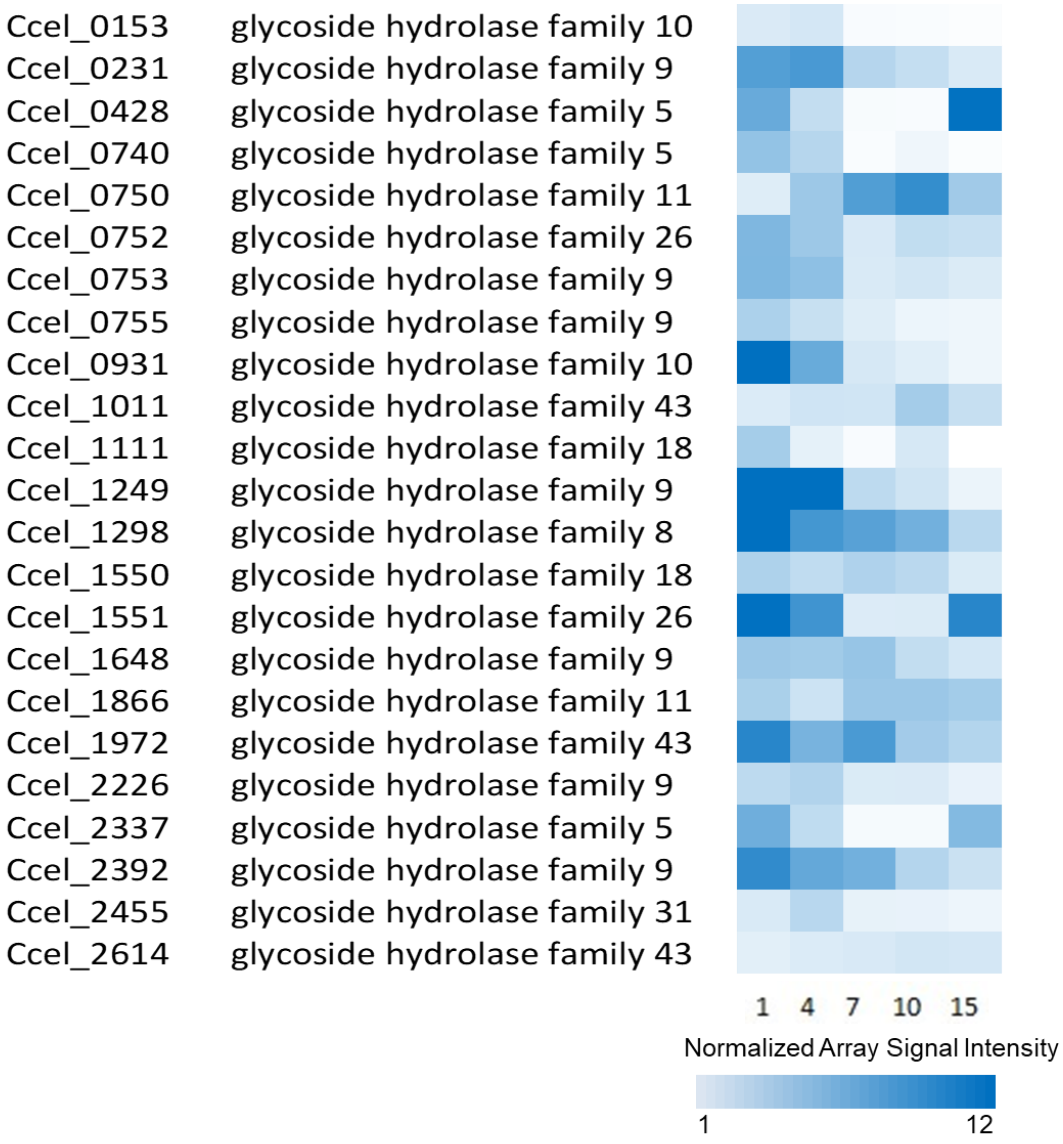


Fig. 2.6 Cellulosomal genes differentially expressed under different cellulose concentration.

The overall signal intensities of genes involved in central metabolic pathway were investigated and mapped (Fig. 2.7). In general, the signal intensities of genes involved in carbon catabolism peaked at cellulose concentration of 7 g/L except the glycosyltransferase Ccel_2109. The signal intensities of other key genes directly related to carbon metabolism such as pyruvate kinase, lactate dehydrogenase, acetate kinase and alcohol dehydrogenase are all increased along with the cellulose concentration when the

carbon load is lower than 7g/L, and decreased along with the cellulose concentration when the carbon load is higher than 7g/L. In addition, signal intensities of the hydrogenase Fe- only which are related to hydrogen production also showed the same trend and peaked at 7 g/L cellulose concentration. There are multiple copies of pyruvate ferredoxin oxidoreductase in *C. cellulolyticum*, except the Ccel_0554 and Ccel_1390 did not show the same trend with other genes, the signal intensities of rest copies of this gene were also peaked at 7 g/L cellulose concentration. Interestingly, the gene expression level of central metabolic pathway did not show the same trend of cellulosomal genes we mentioned above. The results indicated that *C. cellulolyticum* may have different regulation patterns for these two parts of genes, even though they all played important roles in the cellulose metabolism.

There were lots of studies presented carbon regulations of *C. cellulolyticum* from the metabolic perspectives (Payot, Guedon et al. 1998, Guedon, Payot et al. 1999, Desvaux, Guedon et al. 2000, Desvaux, Guedon et al. 2001, Desvaux 2005), however, this study was the first comprehensive study of the impacts of carbon loads level on transcriptional level. In the first metabolic investigation in 1983, *C. cellulolyticum* was cultured on cellobiose with an acetate-lactate fermentation with low substrate conversion rate (Giallo, Gaudin et al. 1983), because of the early entry of stationary phase of the cell growth. When the *C. cellulolyticum* was cultured on cellulose, it was reported that the productions of acetate, ethanol and lactate were lower and the rate of cellulose degradation declined over time (Giallo, Gaudin et al. 1985). The rate-limiting factor of cell growth was identified as the cellulolysis rate (Petitdemange, Tchunden et al. 1992, Tchunden, Petitdemange et al. 1992), therefore, the *C. cellulolyticum* was characterized

as a sluggish celllulasic bacteria (Desvaux, Guedon et al. 2001). However, after extensive study of *C. cellulolyticum* cellulosome, there was no direct evidence that the limiting factor of cellulose degradation is its cellulolytic system (Desvaux 2005). The studies found out that the cultivation of *C. cellulolyticum* on a defined mineral salts medium clearly increase the cellulose degradation compared to the complex medium (Desvaux, Guedon et al. 2000). Their results also indicated that when the initial cellulose concentrations were higher than 6.7 g/L, the cellulose degradation dropped dramatically which was consistent with our study, which indicated that the *C. cellulolyticum* is not able to handle high concentration of substrates which was proved by this study from the transcriptional level that higher carbon load will inhibit the gene express level of cellulosomal genes in *C. cellulolyticum*. In this study, it was observed that there was an extracellular accumulation of pyruvate followed by an accumulation of glucose when the *C. cellulolyticum* was cultured on high concentration of cellulose. The lactate produced by *C. cellulolyticum* was lower when it was cultured on 10g/L and 15 g/L than 7g/L, which means PFO and LDH was not able to utilize the carbon derived from glycolysis under high cellulose concentration. These results were also consistent with the microarray data that the expression levels of pyruvate kinase, lactate dehydrogenase were decreased when the cellulose concentration was higher than 7 g/L. As mentioned above, the lactate production can decrease the NADH/NAD+ ratio which can help *C. cellulolyticum* regulate the electronic flux to release the accumulation of pyruvate (Guedon, Desvaux et al. 1999, Guedon, Payot et al. 1999). Although PFO is clearly an important and sensitive metabolic node for the regulation of both energetic and electronic fluxes, it was no direct experimental evidence showed that pyruvate was the direct cause of growth inhibition

(Desvaux 2005). However, previous studies still attempted to improve the cellulose degradation by regulation of *C. cellulolyticum*pyruvate metabolic node. Guedon (Guedon, Desvaux et al. 2002) constructed a metabolic engineered strain of *C. cellulolyticum* which contains pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis*. The extra engineered ethanol production pathway released the pyruvate accumulation in *C. cellulolyticum*, led to a 150% increase in cellulose consumption which indicated that the release of accumulation of pyruvate of *C. cellulolyticum* can improve the cellulose degradation. Therefore, in the flowing work, this study tried to release the pyruvate accumulation by adding other microorganisms to improve the cellulose degradation and hydrogen production.

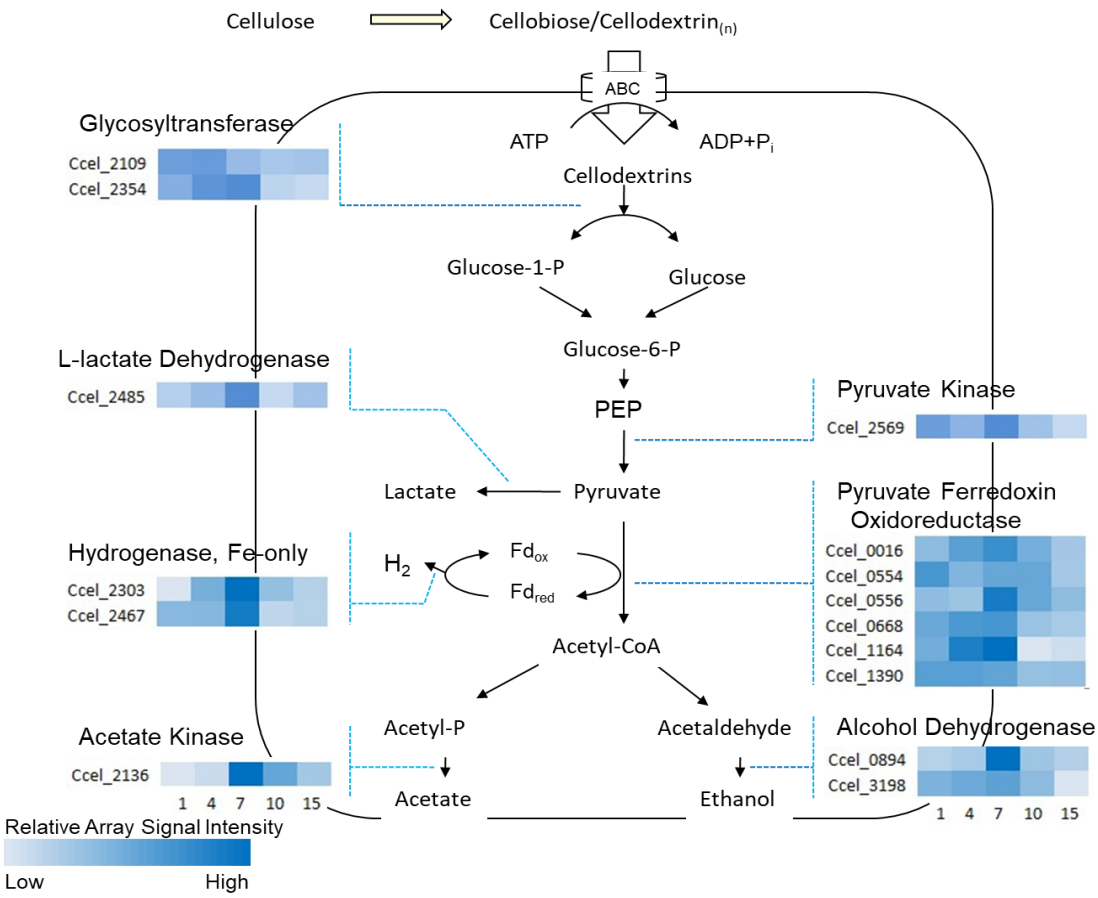


Fig. 2.7 Expression of genes involved in central metabolic pathway under different concentrations of cellulose.

2.4.4 Gene co-expression network of C. cellulolyticum

In order to further understand the transcriptional response to different initial carbon loads, a gene co-expression network of *C. cellulolyticum* was constructed with the microarray data of 15 samples of 5 different initial cellulose concentrations. The resulting network contained a total 1068 genes that were partitioned into 21 sub networks (modules with more than four genes) (Fig. 2.8). As expected that genes from the same operon such as *cip-cel* tend to link together in the subnetwork contains the functionally related genes. In another word, functionally related genes may cluster together in functional categories (modules), further insights into gene interactions in these categories can be obtained by examining the clustered modules (Luo, Yang et al. 2007, Zhou, He et al. 2010). Furthermore, in the gene co-expression network, it provides a method to predict the function of hypothetical genes due to the fact that functionally related genes are more likely connected to each other in the gene co-expression networks (Luo, Yang et al. 2007). For example, the genes from *cip-cel* operon were linked together as showed in Fig 2.9. *Cip-cel* operon was involved in the largest module were tightly linked (first neighbor) to 52 genes which contained 6 hypothetical proteins (Cel_0060, Cel_0167, Cel_0402, Cel_0813, Cel_1050 and Cel_3254) and 4 function unknown proteins (Cel_1109, Cel_2042, Cel_2066 and Cel_2479) (Table S3.3). Therefore, these genes were predicted to be involved in "energy production and conversion" and could be functionally involved in cellulose degradation process. Interestingly, the gene Cel_3254 which was annotated to encode a hypothetical protein, was closely connected with the genes from *cip_cel* operon (marked red in Fig 2.9). In the future work, a mutation of Cel_3254 may be constructed to test its function whether related to the cellulose degradation. There was

another sub-network as showed in Fig. S2.3, there are two unknown functional genes are linked with Ccel_2467 hydrogenase, which are Ccel_0695 (predicted to encode an aldo/keto reductase) and Ccel_1945 (predicted to encode a CoA-substrate-specific enzyme activase) could be functionally involved in hydrogen production. The genes within in this sub-network were listed in Table S3.4. There were 2 hypothetical proteins included in this sub-network Cel_2664 and Cel_1335, which could be functionally involved in electron transfers of hydrogen production process.

Another purpose for the construction of gene co-expression network was to find out the regulator which may regulate the carbon metabolism in *C. cellulolyticum*. Unfortunately, the genes involved in central metabolic pathway including the pyruvate kinase, lactate dehydrogenase, acetate kinase and alcohol dehydrogenase were not found in the co-expression network, which indicated that those functional genes may not be regulated together with other genes but distinct regulated depends on the culture conditions. However, the gene co-expression network may still indicate the importance of genes based on the number of links for each gene. Ccel_0559 (two components transcriptional regulator, winged helix family) and Ccel_2855 (transcriptional regulator, TetR family) were the examples of genes with the highest number of connections (46 links) (Fig. S2.3). In addition, there were more than 20 connections linked to genes from *cip-cel* operon which encodes the cellulosome of *C. cellulolyticum* confirmed the importance of *cip-cel* operon in the cellulose degradation process. These results suggested that the analysis of gene co-expression network can provide useful information for understanding gene function and interactions when the *C. cellulolyticum* was cultured in the different initial cellulose.

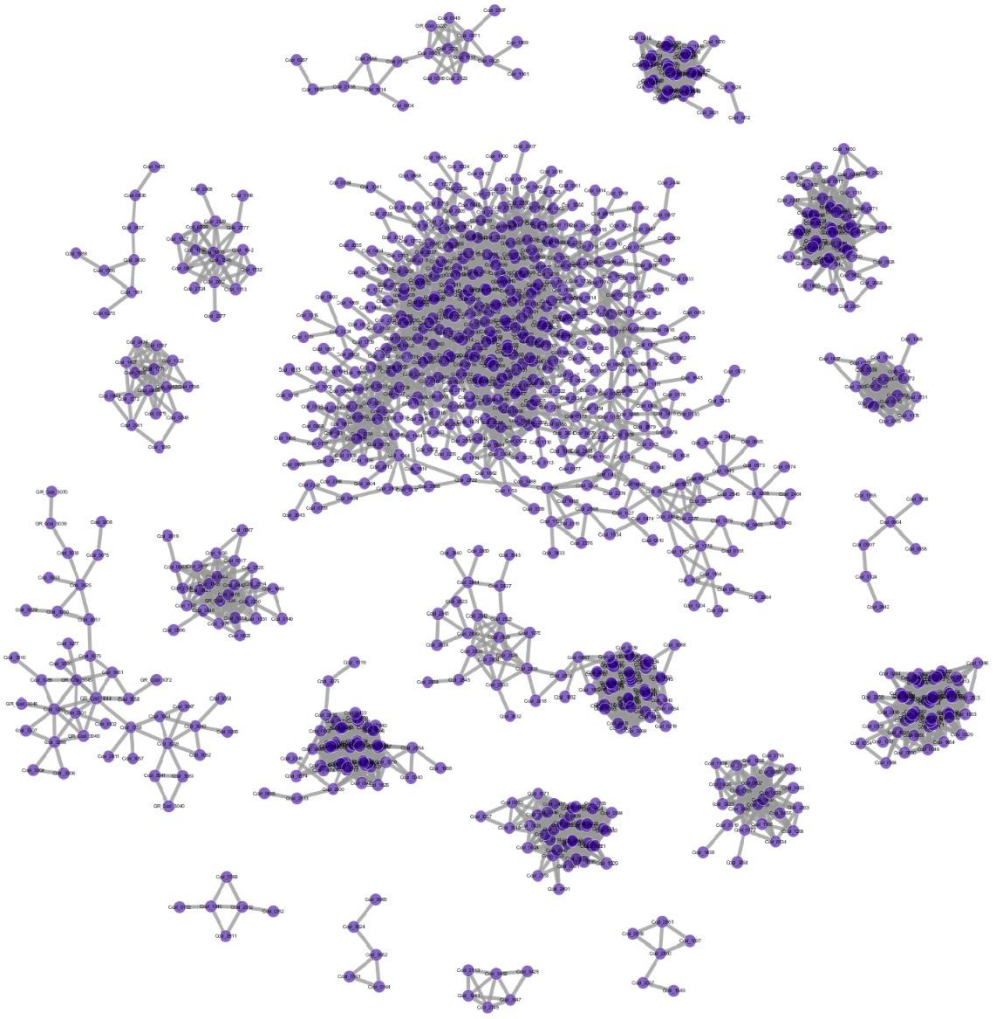


Fig. 2.8 *C. cellulolyticum* co-expression network from different initial cellulose concentration microarray profile.

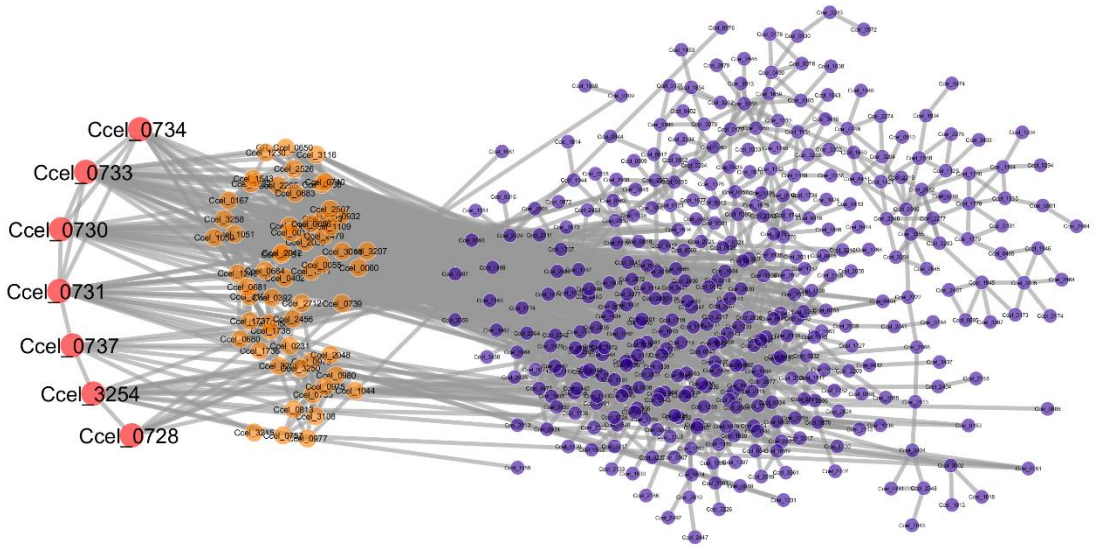


Fig. 2.9 Gene co-expression sub-network of *C. Cellulolyticum* contains *cip-cel* operon

2.5 Conclusion

Our results indicated that culturing *C. Cellulolyticum* on cellulose but not cellobiose, can reduce the accumulation of intermediate products (e.g., glucose and/or pyruvate). The restriction of substrate availability can balance the metabolism rate of intermediate products in *C. Cellulolyticum*, which can relieve it from catabolite repression and improve the hydrogen production. The transcriptional analysis indicated that cellulosomal genes were down regulated along with the increase of cellulose concentrations, however, the expression level of other genes related to central metabolic pathway (e.g., pyruvate kinase, lactate dehydrogenase and hydrogenase) peaked at 7g/L cellulose, which indicated that even though they played an important role in cellulose degradation process, these genes are regulated differently with the cellulosome related genes.

Chapter 3: Biohydrogen production from cellulose by a *Clostridium cellulolyticum* and *Desulfovibrio vulgaris* Hildenborough co-culture

3.1 Abstract

Hydrogen has been considered as clean, efficient, renewable energy in the future. However, the production of hydrogen from lignocellulosic biomass by consolidated bioprocess (CBP) remains challenging largely due to low hydrogen yields and inefficient biomass degradation. In this study, a co-culture of *Clostridium cellulolyticum* H10 and *Desulfovibrio vulgaris* Hildenborough was developed for efficient cellulose degradation and high-yield H_2 production from cellulose using a CBP strategy. The impacts of temperature, initial pH, cellulose concentration, yeast extract concentration and ferrous concentration on hydrogen gas production by the co-culture were investigated. Under optimized conditions (39 °C, initial pH 7.0, 7.5 mg L^{-1} $FeCl_2 \cdot 4H_2O$, no yeast extract, and 4 g cellulose L^{-1}), cellulose degradation, H_2 production, volatile fatty acids and alcohols, soluble sugars, and the cell populations were monitored in the co-culture and both mono-cultures. The data showed that the co-culture performed better in terms of cellulose degradation and H_2 production (3.3 mol H_2 mol^{-1}glucose) than the mono-culture (1.8 mol H_2 mol^{-1} glucose) with 4 g cellulose L^{-1} as the sole carbon source. Experiments were conducted with co-culture microarray analyses and physiology analyses to study the interactions of the two species in this high hydrogen yield bacterial consortium. The results showed that *D. vulgaris* Hildenborough not only use lactate to produce H_2 in the system and proton could be the potential electron acceptor, also be able to help *C. cellulolyticum* colonize on cellulose to speed up the cellulose degradation process. The microarray data showed that *C. cellulolyticum* genes involved in cellulose degradation

(glycoside hydrolase genes, cellulosome protein dockerin genes and cellulosome anchoring protein cohesin region genes) were up-regulated under co-culture conditions. However, the genes related to pyruvate /acetate –CoA metabolic node (lactate dehydrogenase and pyruvate-ferredoxin oxidoreductase) were down regulated compared to the mono-culture. It was observed that, in the co-culture, the cell population of *C. cellulolyticum* was increased about 100% more than mono-cultured *C. cellulolyticum*. In addition, the production of lactate reduced to 3mM in the co-culture system from 18 mM in the mono-cultured *C. cellulolyticum*, suggesting that the adding of *D. vulgaris* Hildenborough to remove the lactate produced by *C. cellulolyticum* and enable the growth resumption from an inefficiency regulated carbon flow. The results suggested that in the co-culture system, the rate-limiting step was no longer the pyruvate /acetate –CoA metabolic node which is rate-limiting step in the mono-culture, but the cellulose degradation process.

Keywords: *Clostridium cellulolyticum*, *Desulfovibrio vulgaris* Hildenborough, co-culture, hydrogen production, cellulose degradation, interaction, microarray, metabolic regulation

3.2 Introduction

Cellulose, which accounts for 35–50% (w/w) of biomass, is a major renewable resource based on its quantity and availability (Schwarz 2001). The fermentation of cellulose and its subunits (cellobiose and glucose) to H_2 can be implemented by consolidated bio-processing (CBP) (Lynd, Van Zyl et al. 2005), where cellulase production, cellulose hydrolysis, and fermentation occur in one step. The current bottleneck of this strategy is the hydrolysis of cellulose and the low yield of H_2. The maximum of H_2 yield from microbial fermentation of biomass is 4 mol H_2 mol $^{-1}$ hexose, which can be achieved when only acetate and carbon dioxide are produced. However, this theoretic yield cannot be achieved experimentally when more reduced organic compounds, such as lactate and ethanol, are produced as fermentation products, because these represent end products of metabolic pathways that bypass the major hydrogen-producing reaction in carbohydrate fermentations (Logan, Oh et al. 2002, Angenent, Karim et al. 2004).

Several microbial consortia capable of both cellulose hydrolysis and H_2 production, including the genus *Clostridium* have been used in the biohydrogen fermentation of cellulose. Researchers have tested various *Clostridium* for H_2 production from cellulose, but the H_2 yields were generally low (Levin, Islam et al. 2006, Ren, Ward et al. 2007). With mesophilic *Clostridium* species, Ren *et al*. (2007) characterized the cellulolytic and hydrogen-producing activities of six species, and observed that *Clostridium cellulolyticum* catalyzed the highest H_2 production from cellulose, with yield of 1.7 mol H_2 mol $^{-1}$ hexose. However, high H_2 yields are generally associated with the fermentation product of acetate, and the low H_2 yields are associated with reduced end products, such as alcohols and lactate. Therefore, by taking advantage of their specific

metabolic capacities, it is expected that a co-culture of *Clostridium cellulolyticum* and another microbial species capable of producing from lactate offers a promising strategy to improve the conversion efficiency of cellulose to hydrogen by taking advantage of their specific metabolic capacities.

The objective of this research was to improve the cellulose degradation rate and hydrogen production rate and yield of cellulose by minimizing electron loss in the non-hydrogen-producing fermentative reactions. A co-cultured consortium containing *Clostridium cellulolyticum* and *Desulfovibrio vulgaris* Hildenborough was established for biohydrogen production from cellulose. The results showed that, under optimized conditions, the co-culture could produce 3.21 mol H_2 mol $^{-1}$ hexose with 4 g cellulose L^{-1}, it is more efficient than the mono-cultured *C. cellulolyticum* 2.05 mol H_2 mol $^{-1}$ hexose. In the co-culture system, *D. vulgaris* Hildenborough not only ferments lactate, producing acetate, carbon dioxide, and H_2, but also helps *C. cellulolyticum* colonizing on the cellulose fiber and releasing excess carbon flow to speed up cellulose degradation process. These data provide a comparable characterization of the cellulolytic and hydrogen-producing capabilities of the mono-culture and co-culture systems, and identification of ecological relationship between these two organisms in the system. This study will also contribute to improvements of the hydrogen-producing efficiency, and the defined mixture strategy can be commercialized and applied in the industrial processes of hydrogen production.

3.3 Materials and methods

3.3.1 Microorganisms and media

Clostridium cellulolyticum (ATCC 35319) and *Desulfovibrio vulgaris* Hildenborough (ATCC 29579) were obtained from the American Type Culture Collection (ATCC). The components of DCB-1 medium used is descripted in Chapter 2. Mineral medium was buffered by 2.52 g L^{-1} $NaHCO_3$ and pH was adjusted by mixed gas of N_2 and CO_2. *C. cellulolyticum* was maintained by the routine transfer of 10% (v/v) inoculum into fresh DCB-1 medium containing 5 g/L cellobiose. *D. vulgaris* Hildenborough was maintained by the routine transfer 5% (v/v) inoculum into fresh DCB-1 medium containing 50 mM lactate and 20 mM sulfate.

The DCB-1 medium was used for the batch experiments with Solka Floc (powdered cellulose; International Fiber Co. Urbana, Ohio, USA) in various amounts as specified in Results to examine H_2 production in the mono-culture of *C. cellulolyticum* and co-culture of *C. cellulolyticum* and *D. vulgaris* Hildenborough from cellulosic substrate. No sulfate was added to the co-culture medium in order to inhibit the H_2S production by *D. Vulgaris* Hildenborough.

3.3.2 Experimental design

All batch experiments were performed with mono- and co-cultures using large head space, using a small liquid volume relative to the head space volume (1:7; v/v) to reduce the inhibition caused by H_2 accumulation (Hallenbeck and Benemann 2002), intermittent gas release and flushed by filtered N_2 every 12 hours. The optimum conditions tests of temperature, initial pH, cellulose concentration and ferrous concentration for co-culture were carried out, respectively. The total inoculum was 10% by volume from an

exponentially growing culture (the inoculum ratio of *C. cellulolyticum* and *D. vulgaris* Hildenborough was 1:1 in co-culture). The batch experiments were performed in a shaker and the agitation was kept constant at 120 rpm. The control without inocula was also included, and all tests were run in triplicate. Zero hour samples were harvested immediately post-inoculation and used to determine the amounts of end products and sulfate carried-over to the culture bottles with the inocula.

All the analytical procedures for measuring cell growth, cellulose degradation, sugar consumption and fermentation products was described in Chapter 2. Briefly, Product biogas composition (H_2 and CO_2) was measured using a gas chromatograph (Agilent 6890N; Agilent Technologies, CA, USA) equipped with a thermal conductivity detector. Hydrogen measurements were conducted with a molecular sieve column (HP-PLOT MoleSieve/5A, Agilent Technologies, CA, USA) with nitrogen as the carrier gas. For CO_2 analysis, a HP-PLOT Q column was used with helium as carrier gas. Gas concentrations were quantified by comparing peak area values to those of a standard curve prepared from known concentrations of H_2 and CO_2. For metabolites analysis, samples were centrifuged and then supernatant was filtered through 0.2 μm membranes and stored at -20°C. The concentrations of lactate, acetate and ethanol were analyzed by a HPLC with an Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA) equipped with an ion-exclusion column (Aminex HPX- 87H; 300 mm × 7.8 mm; Bio-Rad, Hercules, CA, USA) operating at 55°C detected by a refractive index detector using a water 410 refractometer, and quantified using a standard curve. The mobile phase consisted of 0.025% sulfuric acid at a flow rate of 0.6 ml/minute.

3.3.3 Real-time quantitative PCR.

To quantity the cell numbers of each strain in the co-culture and mono-culture, real time PCR was performed using the iQ^{TM}5 Multicolor Real-Time PCR Detection System (BioRad Laboratories, Herculues, CA, USA). The abundance of each strain in the mono-culture and co-culture system was measured by quantifying the copy number of a hypothetical protein gene from each strain: *C. cellulolyticum* CC238 and *D. vulgaris* Hildenborough DVH0012. For gene CC238, amplification using the forward primer 5'-TAAGCCTTCAGTTCAGTTTG-3' and reverse primer 5'-TCTTCCACCAGTTTACCAG-3' resulted in a 209 bp fragment. For gene DVH0012, amplification using the forward primer 5'-TGCCGCCTCTTCTGTCTCCG-3' and reverse primer 5'-CATCCGTCTTCCTGTTCCTCCC-3' gave a 229 bp fragment. An iTaq SYBR Green Supermix kit (BioRad Laboratories, Herculues, CA, USA) was used under the following reaction conditions: 95℃ for 3min followed by 40 cycles of 95℃ for 10s and 58℃ for 30s. Those products with copy numbers between 10^2 and 10^8 were used as gradient templates to generate standard curves. Gnomic DNA was extracted from co-cultured and mono-culture at various time points and used as the template for quantitative PCR.

3.3.4 Global transcriptomic analysis

3.3.4.1 Oligonucleotide probe design and microarray construction

DNA Microarrays covering 3169 of 3490 annotated gene sequences of *C. cellulolyticum* were constructed with 70-mer nucleotide probes (He, Wu et al. 2005). Gene specific, inclusive and exclusive group-specific oligonucleotide probes were designed by a new version of the computer program CommOligo (Li, He et al. 2005).

All designed oligonucleotides were synthesized commercially without modification by MWG Biotech Inc. (High Point, NC). The concentration of oligonucleotides was adjusted to 100 pmol/µl. Oligonucleotide probes were prepared in 50% (vol/vol) dimethyl sulfoxide (Sigma) and spotted onto UltraGAPS glass slides (Corning Life Sciences, Corning, NY) by use of a BioRobotics Microgrid II microarrayer (Genomic Solutions, Ann Arbor, MI). Each oligonucleotide probe had two replicates on a single slide. In addition, six different concentrations (11, 22, 45, 90, 180, and 360 ng/µl) of genomic DNA were spotted (eight replicates for each of the six concentrations on a single slide) as additional positive controls. After the oligonucleotide probes were printed, they were fixed onto the slides by UV cross-linking (600 mJ of energy), according to the protocol of the manufacturer of the UltraGAPS glass slides.

3.3.4.2 Total RNA, genomic DNA isolation and fluorescence labeling

Total RNAs from both co-culture and mono-culture were isolated from the samples collected at mid-log phase using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA samples were then purified by an RNeasy minikit (Qiagen), and treated with on-column RNase-free DNase I (Qiagen) to digest genomic DNA. RNA samples were quantified by Nanodrop ND-1000 spectrophotometry at wavelengths of 260 and 280 nm; RNA integrity was estimated by running agarose gels. To generate labeled cDNA, 10 µg purified total RNA was used with a previously described protocol (Thompson, Beliaev et al. 2002). Briefly, 10 µg random primers (3.0 µg/l; Invitrogen) was used for priming, and fluorescent dye Cy5 was used for labeling. After labeling, cDNA was then purified with a PCR purification kit (Qiagen) and

concentrated with an SPD1010 Speedvac apparatus. The efficiency of labeling was measured by Nanodrop ND- 1000 spectrophotometry.

Genomic DNA was isolated from *C. cellulolyticum* with CTAB method (Zhou, Bruns et al. 1996). Genomic DNA, which was used as the control, was labeled with fluorescent dye Cy3 as described previously (Chhabra, He et al. 2006). Cy5-labeled cDNA and Cy3-labeled gDNA were dried and stored at -20 ℃ before hybridization.

3.3.4.3 Microarray hybridization and data analysis

To hybridize microarray slides, the Cy5-labeled cDNA and the Cy3-labeled gDNA were mixed together and dissolved in a hybridization solution which contained 50% formamide, 5× SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10% SDS, and 0.1 mg/ml herring sperm DNA (Invitrogen). The mixture was then incubated for 98°C for 3 min to denature the DNAs and then kept at 65°C and applied to microarray slides (Hemme, Fields et al. 2011). Array hybridizations, washing, and drying were carried out with TECAN hybridization station (TECAN HS4800, TECAN Group Ltd, Durham, NC, USA). The slides were scanned for the fluorescence intensities of both the Cy5 and Cy3 fluorophores by a ScanArray Express microarray analysis system (Perkin Elmer, Boston, MA).

To determine the signal intensity of fluorescence for each spot, 16-bit scanned TIFF images were analyzed by ImaGene 6.1 software (Biodiscovery Inc., El Segundo, CA) to quantify spot signal, spot quality, and background fluorescence intensities. Data analysis included the following major steps. (a) Empty spots (with a signal-to-noise ratio [SNR] less than 2.0), poor spots, and negative spots were flagged according to the instructions of the software and were removed in the subsequent analysis. (b) The net

signal of each spot was calculated by subtracting the background signal and adding a pseudo-signal of 100 to obtain a positive value. If the resulting net signal was less than 50, then a value of 50 was used. (c) The total signal intensity for all microarrays (slides) was normalized with the gDNA signal (Cy3 signal), and a normalization factor was calculated for each slide. (d) Both Cy5 and Cy3 signal intensities of each spot were adjusted by multiplying by the normalization factor calculated above, and the calculated Cy5 signal of each probe presents the normalized signal intensity. (e) The transcriptional level of each open reading frame (ORF) was calculated as the ratio (R) of Cy5/Cy3, and the genes detected in at least two out of three replicates were kept for statistical analysis. (f) For comparative genomic analysis, the control and treatment conditions were defined so that the ratios Rc and Rt were defined for each gene under control and treatment conditions, respectively. The $\log_2$(Rt/Rc) value was then calculated for each gene. (g) Finally, to assess the significance of gene expression with the treatment, a Z score was calculated by using the following equation: $Z=\log_2 (Rt/Rc)/\sqrt{(0.25+\sum variance)}$, where 0.25 is a pseudo-variance term. Typically, a Z score cut off of >1.5 was used for significant changes.

3.3.5 Cell attachment assay

Late-exponential phase cells (10ml) of each strain *C. cellulolyticum* and *D. vulgaris* Hildenborough were transferred to anaerobic tubes containing 20ml fresh DCB-1 medium with cellulose (final cellulose concentration is 4 g/L). The tubes were incubated at 37°C for 24 hours. Samples were collected at different time points (0.5, 2, 4, 10, 24h) during this period to measure the number of cells in the supernatant and the ones attached on the cellulose by measuring cell protein concentrations. Samples were centrifuged

1000rpm for 2 mins to separate the cellulose from the supernatant. A ratio of cells attached on the cellulose and in the supernatant was calculated. Experiments were also conducted in the same conditions using the mono-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough separately.

3.3.6 Lactate utilization assay

C. cellulolyticum and *D. vulgaris* Hildenborough were co-cultured with 1g/L cellulose as substrate. Different amounts of lactate (0, 3, 6, 10mM) was added into the co-culture system, and samples were collected after 5 days incubation to measure the concentrations of end products and hydrogen yield. Experiments were also conducted in the same conditions using the mono-cultured *C. cellulolyticum*.

3.3.7 Calculations

The volume of biogas produced by batch fermentation was calculated by the total volume of culture bottle deducted by the volume of fermentation medium and corrected to standard conditions (273K, 101kPa). The cumulative hydrogen production per liter of culture was calculated according to the biogas produced from 20ml of fermentation medium in each test. The hydrogen yield from cellulose and the extent of cellulose utilization was estimated as described (Geng, He et al. 2010) from 150 ml of fermentation medium.

3.4 Results

3.4.1 Optimization of culture conditions

The impacts of temperature, initial pH, cellulose concentration and ferrous concentration on hydrogen gas production by the co-culture of *C. cellulolyticum* and *D. vulgaris* Hildenborough were investigated.

Co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough were grown in a batch culture with DCB-1 medium using 10 g/L Solka Floc cellulose as the carbon source. For temperature test, cells were cultured at pH 7.0, and at 30°C, 34°C, 37°C, 39°C, or 41°C. For the initial pH test, cells were cultured at 39°C, and at initial pH 6.6, 6.8, 7.0, 7.2, 7.4, or 7.6. The hydrogen yield of the collected biogas within 120h was determined (Fig. S3.1). About 2.1 L hydrogen per liter medium was achieved with cells cultured under 39°C with initial pH 7.0, and the hydrogen production dropped to about 1.6L/L medium when temperature was 30°C or initial pH was 7.6.

Co-cultured cells were grown on DCB-1 medium at 39°C and initial pH 7.0 with seven concentrations of Solka Floc: 1, 4, 7, 10, 13, 18 and 25 g/L cellulose. The percentage of degraded cellulose and hydrogen production within 120h was determined (Fig. S3.2). The results indicated that around 95% degradation was achieved with less than 4 g/L initial cellulose, but this percentage dropped and reached 57% with the highest cellulose concentration (25 g/L). When cellulose concentration was lower than 7 g/L, hydrogen production increased with the initial cellulose concentration, achieving 2.1 L /L medium with initial cellulose concentration of 7 g/L, but the hydrogen production did not show significant increases with an increase in cellulose concentration. Such a succession of increase indicated that approximately the same amount of hydrogen was produced so that hydrogen production performance was close to their maximum at and above 7 g/L cellulose added. In fact, the maximum amount of cellulose was hydrolyzed by the co-culture system increased with initial cellulose amount, however, the hydrogen production remained quite constant around 19 mM from 20ml medium at cellulose concentrations above 7 g/L. Even though hydrogen production was increased with lower

than 7 g/L cellulose, the hydrogen yield was significantly decreased at 7 g/L cellulose and above, when cellulose concentration lower than 4 g/L, the hydrogen yield from the co-culture system was larger than 3 mol H_2 /mol hexose (Table S3.1).

Fe is the key molecular ion in the active site of hydrogenase. The concentrations of ferrous will affect the activity of hydrogenase in the cells. The original $FeCl_2 \cdot 4H_2O$ concentration in the DCB-1 medium is 1.5 mg/L, different $FeCl_2 \cdot 4H_2O$ concentrations were tested in the co-culture using 4 g/L cellulose without yeast extract in order to improve hydrogen production of the co-culture. The results indicated that hydrogen production increased from 0.7 to 2.0 L/L medium as the concentration of $FeCl_2 \cdot 4H_2O$ from 0 to 7.5 m g/L. As the $FeCl_2 \cdot 4H_2O$ concentration increased to 15m g/L, the hydrogen production did not show continuously increase (Fig. S3.3). The results showed that when 7.5 m g/L $FeCl_2 \cdot 4H_2O$ was added, the co-culture can achieve the highest hydrogen production.

3.4.2 Increased cellulose degradation and hydrogen production in co-cultured C. cellulolyticum and D. vulgaris Hildenborough

Under optimized conditions (39℃, initial pH 7.0, 7.5 m g/L $FeCl_2 \cdot 4H_2O$ and 4 g/L cellulose), the rate of cellulose degradation in the co-culture was much faster than in the mono-culture. About 10% of the cellulose was degraded in the first 12 hours in both the mono-culture and the co-culture. The cellulose in the co-culture was then hydrolyzed and coupled with a large amount of hydrogen production in the following 20 hours. Almost 90% of the cellulose in the co-culture was hydrolyzed in 50 hours, while the mono-culture only utilized about 50% (Fig. 3.1A).

The hydrogen production was significantly higher in the co-culture system than with the *C. cellulolyticum* mono-culture in terms of yield and production rate (Fig. 3.1B). The co-culture also performed better in terms of cellulose degradation and H_2 production (3.21 mol H_2 mol^{-1} glucose) than the mono-culture (2.05 mol H_2 mol^{-1} glucose) with 4 g/L cellulose as the sole carbon source, which was about 1.6 folds of mono-culture from the same amount of cellulose. By 36 hours the co-culture produced a comparable amount hydrogen gas as the mono-culture did in 80 hours.

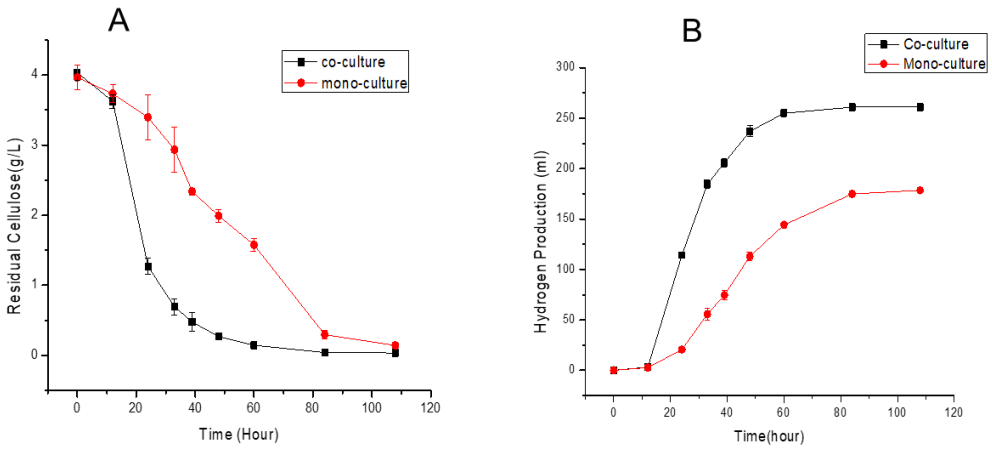


Fig. 3.1 Residual cellulose concentration (A) and Hydrogen production (B) in mono-cultured of *C. cellulolyticum* and co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough with 4 g/L cellulose.

3.4.3 End products and soluble sugars production

The end products such as acetate, lactate and ethanol concentration were investigated in both mono-cultured of *C. cellulolyticum* and co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough. When *C. cellulolyticum* was mono-cultured on cellulose, lactate and acetate were the main end products, and lactate was accumulated from the beginning of the incubation. However, in the co-culture, no lactate was detected in the first 12 hours (Fig. 3.2). this may be because *D. vulgaris* Hildenborough can use lactate as a carbon source. Lactate in the co-culture remained low concentration (less than 3 mM) compared

to the 17 mM observed in the mono-culture. Acetate was the main end product for the co-culture, and a high concentration of acetate was observed in the co-culture (27 mM) compared to the 18 mM produced by the mono-culture. Interestingly, a very low concentration of ethanol (less than 1 mM) was produced in the mono-culture compared to the co-culture (6 mM), which may be because of too much acetate accumulated in the co-culture system which caused the end product feedback, the carbon flow switched to ethanol production.

The H_2 yield from sugar fermentation depends on the catabolic pathway used by a bacterium. H_2 production is coupled with the acetate and butyrate production pathways and not coupled directly with ethanol and lactate production. Therefore, the theoretical maximum H_2 yield would be achieved when the acetate is the only end products, and reduced H_2 yield occurs when fermentation products shifts to ethanol and lactate. In present study, *C. cellulolyticum* produced high percentage of lactate (about 50%) when mono-cultured on the cellulose. After a lactate user *D. vulgaris* Hildenborough was introduced into the system, since the *D. vulgaris* Hildenborough can utilize lactate produced by *C. cellulolyticum* to produce acetate coupled with hydrogen production, the co-culture produced higher percentage of acetate (78%) and very low percentage of lactate (10%), while the co-culture achieved higher H_2 production and yield on the cellulose than the mono-culture. These findings are consistent with the stoichiometry mentioned above. This present study did not detect high ethanol concentration in the end products described by previous study (Desvaux, Guedon et al. 2000) that may because the performance of *C. cellulolyticum* differ with other cellulose types, media and culture conditions.

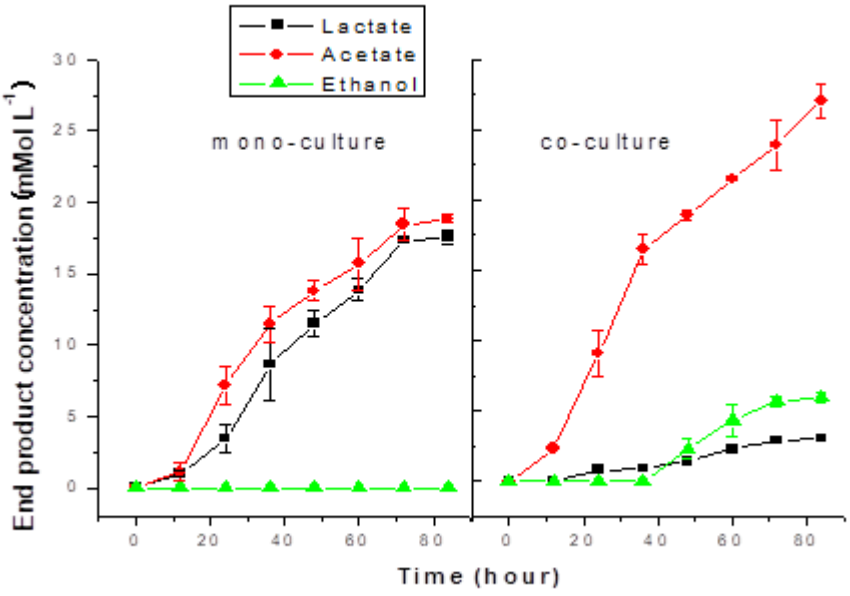


Fig. 3.2 End products concentration of mono-cultured of *C. cellulolyticum* and co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough with 4 g/L cellulose.

Soluble sugars in both co-culture and mono-culture decreased first as the cellobiose in the inocula were used up. The sugar concentration then increased due to cellulose degradation. It was observed that there are different patterns of sugar accumulations in co-culture and mono-culture. In the co-culture system, the concentration of soluble sugars first increased after 12 hours of incubation rather than the 24 hours in the mono-culture, then the concentration of soluble sugars in the co-culture decreased along with the incubation, however, in the mono-culture, the sugar concentration was accumulated to a high level in the system (Fig. 3.3). These results suggested that, in the co-culture system, the cellulose was degraded faster than the mono-culture, which may cause the accumulation of soluble sugars at the beginning of the incubation. The difference of sugar concentration at the end of the incubation is probably because the

carbon flow was excessive in the mono-culture system, which resulted in the accumulation of soluble sugars. However, in the co-culture system, the *D. vulgaris* Hildenborough can help *C. cellulolyticum* release the pyruvate accumulation and all the soluble sugars were used for cellular metabolism (e.g., hydrogen production) in the co-culture. In another word, the carbon metabolism in co-culture system is more efficient than the mono-culture.

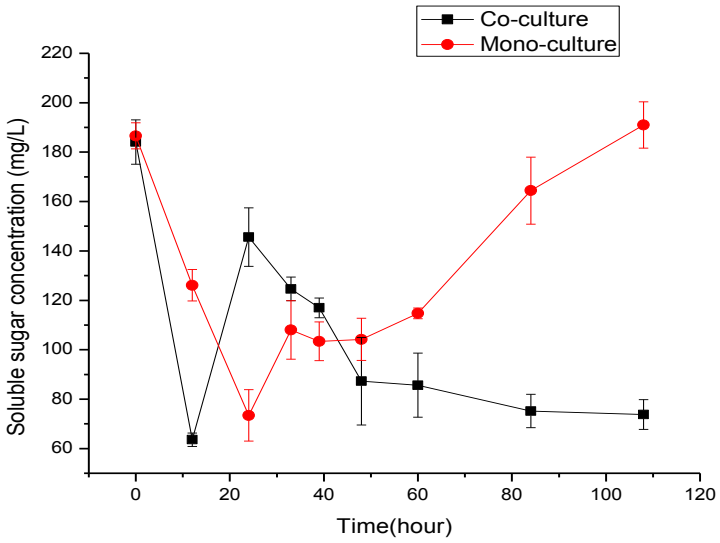


Fig. 3.3 Soluble sugar concentration of mono-cultured of *C. cellulolyticum* and co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough with 4 g/L cellulose.

3.4.4 Cell populations in co-culture and mono-culture

Cell populations of *C. cellulolyticum* in both mono-culture and co-culture were investigated (Fig. 3.4). The cell numbers of *C. cellulolyticum* in the stationary phase of the co-culture was doubled comparing to the mono-culture, and *C. cellulolyticum* grow much faster in the co-culture, it reached the stationary phase at a higher cell density in about 40 hours rather than the more than 60 hours of mono-culture. This also may be the reason why faster cellulose degradation was observed in the co-culture.

The cell population of *D. vulgaris* Hildenborough in the co-culture system was also measured. The results showed the *C. cellulolyticum* was dominant in the co-culture system and the *D. vulgaris* Hildenborough population increased in the first 12 hours then kept a relatively low abundance in the system. In the co-culture, cellulose is the substrate of *C. cellulolyticum* whereas *D. vulgaris* Hildenborough depends on the end products produced by *C. cellulolyticum* for growth. In the early phase of co-culture (0-12h), the small amount of lactate and sulfate present mainly in the inocula in the media enable *D. vulgaris* Hildenborough to grow faster than *C. cellulolyticum*. However, the sulfate from inoculums was consumed quickly, limiting the growth of *D. vulgaris* Hildenborough and caused the low abundance afterward. Since the *D. vulgaris* Hildenborough do not have the more favored electron acceptor (sulfate) in the system, and its growth was based on the lactate produced by *C. cellulolyticum*, this explains why it has much lower abundance than *C. cellulolyticum* in the co-culture system.

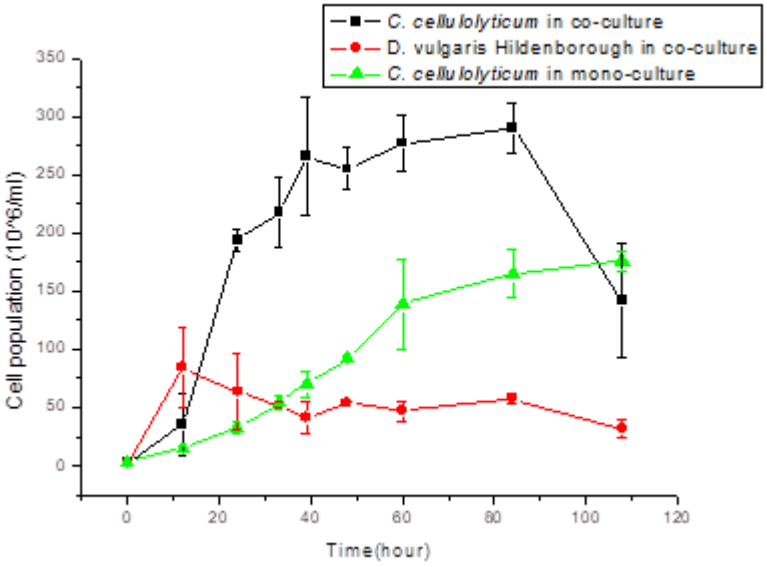


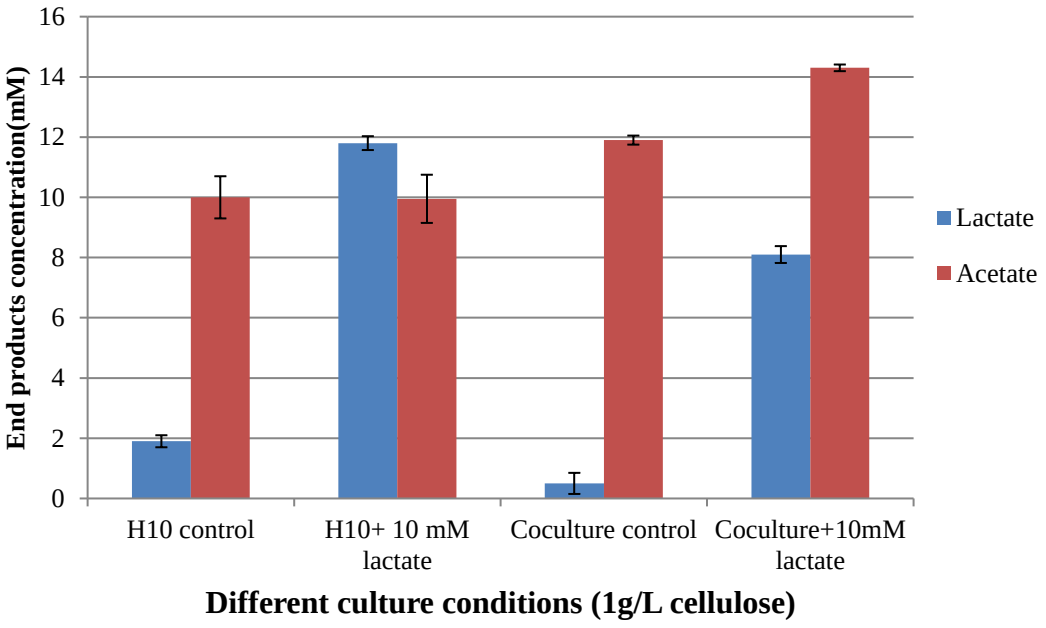
Fig. 3.4 Cell population of *C. cellulolyticum* and *D. vulgaris* Hildenborough in co-culture and *C. cellulolyticum* in mono-culture.

3.4.5 D. vulgaris Hildenborough could use lactate to produce H_2 in the system

To examine whether the *D. vulgaris* Hildenborough can utilize the lactate in the co-culture system, the lactate utilization assay was conducted. Under carbon starvation conditions (1g/L cellulose), the cellulose could not offer enough carbon for *C. cellulolyticum* and *D. vulgaris* Hildenborough. When lactate was added into the co-culture system, if *D. vulgaris* Hildenborough can use lactate, the added lactate would be used, since the lactate produced by *C. cellulolyticum* from cellulose is not enough for its growth.

10mM lactate was added to the mono-cultured of *C. cellulolyticum* and co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough, the lactate addition was omitted in the control. In the mono-cultured *C. cellulolyticum* when the extra lactate is not added, the bacterium produced about 2 mM lactate, when the 10 mM lactate was added to the system, the lactate at the end of fermentation is 12 mM, which indicated that mono-cultured *C. cellulolyticum* with 10mM lactate produced the same amounts of lactate and acetate as without lactate one from cellulose (Fig. 3.5). The results suggested that added lactate would not affect the pattern of lactate and acetate produced by *C. cellulolyticum.* When *C. cellulolyticum* and *D. vulgaris* Hildenborough were co-cultured together without additional lactate, the lactate concentration in the medium was about 1mM, however, when the lactate was added to the co-culture, the final concentration of lactate after incubation is 8mM which is lower the lactate added (10mM) (Fig. 3.5). The results indicated that extra lactate added to the co-culture system was utilized by *D. vulgaris* Hildenborough because the lactate was not utilized when it was added to the *C.*

cellulolyticum mono-culture. The hydrogen and acetate production were also investigated when the additional lactate was added to the carbon starving system, the results showed that more acetate and hydrogen was produced (Fig. 3.5), interestingly, more hydrogen was produced when more lactate was added to the co-culture system when the added lactate was added increased from 8mM to 13mM which was significantly higher than without adding lactate (Fig 3.5). All these results supported that *D. vulgaris* Hildenborough can use the lactate and produce hydrogen in the co-culture system. However, even there were evidence showed that *D. vulgaris* Hildenborough may use the lactate to produce hydrogen, it was barely thermodynamically favorable for this reaction. That was the reason why in this study the head space was flushed by N_2 every 12 hours to remove the accumulation of hydrogen in the system. Also, there was still possibility that *D. vulgaris* Hildenborough may directly use pyruvate to produce ethanol or acetate associated with hydrogen production (Pankhania, Spormann et al. 1988, Keller, Rapp-Giles et al. 2014).



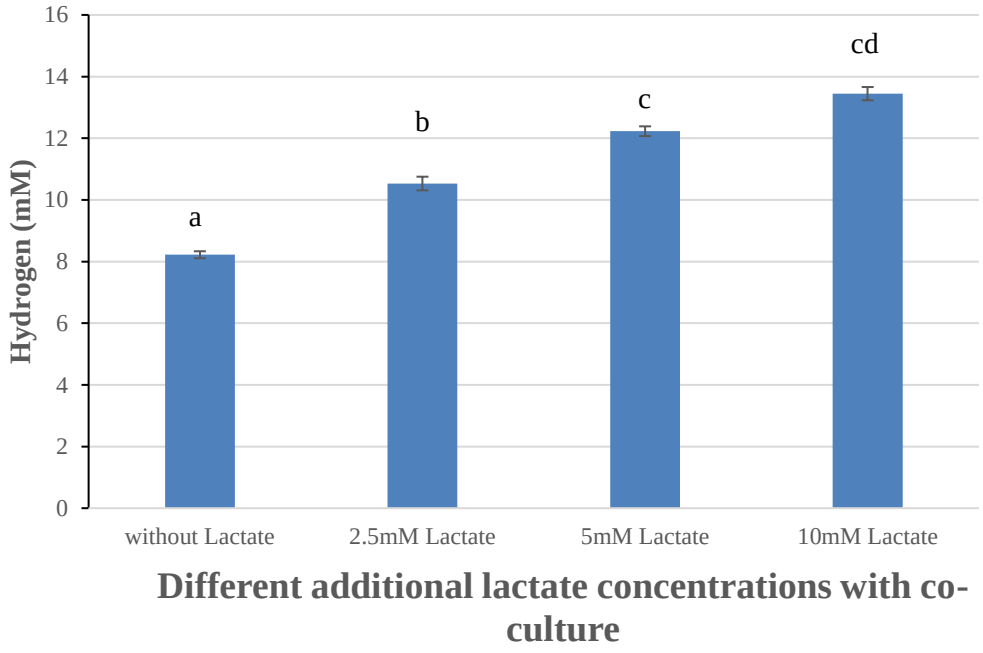


Fig. 3.5 End products concentrations and hydrogen production in the lactate utilization assay with extra different concentration of additional lactate ($p<0.05$ ANOVA test).

3.4.6 Microarray analysis of C. cellulolyticum under co-culture and mono-culture

To explore an overall picture of gene expression level of *C. cellulolyticum* under co-culture conditions, we conducted transcriptomic analysis of *C. cellulolyticum* under both mono-culture and co-culture conditions with gene expression microarrays. We consider the *C. cellulolyticum* under co-cultured condition as the treatment and mono-cultured condition as the control. Differentially expressed genes (DEGs) in treatment to the control are identified as genes with a log2 fold-change above 1 for up-regulated genes (or below -1 down-regulated genes) and an adjusted p value below 0.05. In general, about 2500 genes in co-cultured *C. cellulolyticum* were detected and about 1300 genes detected in mono-cultured *C. cellulolyticum.* In total, more than 600 genes up-regulated, and about 100 genes down-regulated under the co-culture conditions comparing to the mono-culture conditions.

In the up-regulated genes, except large numbers of genes encode hypothetical proteins and function unknown proteins, the genes involved in cellulose degradation (glycoside hydrolase genes, cellulosome protein dockerin genes and cellulosome anchoring protein cohesin region genes) were up-regulated under co-culture conditions (Table 3.1).

Table 3.1 Differentially expressed genes of *C. cellulolyticum* (up-regulated) related to cellulose degradation under co-culture conditions.

Gene name	Annotation	Log R	Z score
Ccel_0730	glycoside hydrolase family 8	4.46	5.65
Ccel_0731	glycoside hydrolase family 9	5.49	7.37
Ccel_0732	glycoside hydrolase family 9	5.26	9.25
Ccel_0733	cellulosome anchoring protein cohesin region	6.84	11.14
Ccel_0735	glycoside hydrolase family 9	5.57	9.22
Ccel_0736	cellulosome protein dockerin type I	4.96	9.18
Ccel_0737	glycoside hydrolase family 9	5.64	9.92
Ccel_0739	cellulosome protein dockerin type I	5.05	9.24
Ccel_0740	glycoside hydrolase family 5	4.79	8.25
Ccel_0881	carbohydrate-binding, CenC-like protein	5.85	10.11
Ccel_1543	cellulosome anchoring protein cohesin region	5.07	9.95
Ccel_1550	glycoside hydrolase family 18	3.21	5.67
Ccel_1551	glycoside hydrolase family 26	4.51	8.68
Ccel_1655	cellulosome protein dockerin type I	4.09	7.48
Ccel_1656	carbohydrate binding family 6	5.72	10.53
Ccel_1809	cellulosome protein dockerin type I	2.49	3.9

We also observed that the genes related to hydrogen production were up-regulated (Table 3.2).

Table 3.2 Differentially expressed genes of *C. cellulolyticum* (up-regulated) related to hydrogen production under co-culture conditions.

Gene name	Annotation	Log R	Z score
Ccel_2232	hydrogenase, Fe-only	2.35	4.56
Ccel_2233	Respiratory-chain NADH dehydrogenase domain	2.61	5.04
Ccel_1069	cytochrome b5	3.05	5.9
Ccel_1070	hydrogenase (NiFe) small subunit HydA	2.89	5.5
Ccel_1071	nickel-dependent hydrogenase large subunit	2.31	4.25
Ccel_1072	hydrogenase maturation protease	2.45	4.7
Ccel_3363	hydrogenase accessory protein HypB	4.28	7.7
Ccel_1102	4Fe-4S ferredoxin iron-sulfur binding domain protein	4.26	8.06
Ccel_1327	4Fe-4S ferredoxin iron-sulfur binding domain protein	3.45	6.56
Ccel_1691	NADH/Ubiquinone/plastoquinone (complex I)	2.08	3.92
Ccel_0642	NAD(P)H dehydrogenase (quinone)	3.5	6.97

Surprisingly, the genes related to central metabolic pathway were down-regulated under the co-culture condition than mono-culture. Those genes including Ccel_2485 L-lactate dehydrogenase, Ccel_2136 acetate kinase and Ccel_0668 pyruvate ferredoxin oxidoreductase (PFO）as well as Ccel_2275 glyceraldehyde-3-phosphate dehydrogenase (Table 3.3).

Table 3.3 Differentially expressed genes of *C. cellulolyticum* (down-regulated) related to central metabolic pathway under co-culture conditions.

Gene name	Annotation	Log R	Z score
Ccel_2485	L-lactate dehydrogenase	-2.6	-1.5
Ccel_0668	pyruvate ferredoxin/flavodoxin oxidoreductase	-1.3	-1.5

Ccel_2136	acetate kinase	-3.5	-2.5
Ccel_2275	glyceraldehyde-3-phosphate dehydrogenase, type I	-2.8	-2.1
Ccel_1888	glycogen/starch synthase, ADP-glucose type	-1.4	-2

When *C. cellulolyticum* was cultivated in sealed flasks without shaking or pH regulation and where fermentation gases accumulated. In such conditions, H10 was described as a sluggish cellulolytic microorganism (Giallo, Gaudin et al. 1985). The first metabolic studies performed in batch cultures suggested nutrients limitation and/or by-products inhibition as the reason for the limited growth of *C. cellulolyticum* (Giallo, Gaudin et al. 1983), however, most recently studies suggested that a self-intoxication of bacterial metabolism resulting from an inefficiency regulated carbon flow. There are three key nodes (Fig.S3.4) in the cellulose hydrolysis processes: (i) cellulose degradation(regulation of cellulosome); (ii) the glucose 1-phosphate/ glucose 6-phosphate node which is the branch point controls the carbon flow directed to glycolysis (into the cell) or dissipates carbon excess towards the formation of cellodextrins and glycogens (polysaccharides outside of the cell); (iii) the pyruvate /acetate –CoA metabolic node which is essential to the regulation of electronic and energetic fluxes (Desvaux, Guedon et al. 2001, Desvaux, Guedon et al. 2001).

In a previous study, the *C. cellulolyticu*m was characterized by limited carbon consumption and subsequent limited growth, and the metabolic flux analysis suggested that an inefficiently regulated carbon flow caused the self-intoxication of bacterial metabolism (Desvaux 2005). When the *C. cellulolyticum* is mono-cultured on cellulose, the inefficient regulation of carbon flow into cells resulted in high NADH/NAD+ ratios, which were correlated with the inhibition of glyceraldehydes-3-phosphate dehydrogenase

and low growth rate (Payot, Guedon et al. 1998). In this case, the speed control step of carbon flow was Node 3, and the accumulation of pyruvate will negative feedback to reduce the cellulose consumption and result in low cellulose degradation ratio.

However, the production of lactate associated with a decrease in NADH will enable growth resumption of *C. cellulolyticu*m, and the lactate production serves as an additional catabolic pathway for *C. cellulolyticu*m to regulate the excesses of the carbon and NADH produced (Payot, Guedon et al. 1999). In the co-culture system, the *D. vulgaris* Hildenborough was added to remove the lactate produced by *C. cellulolyticum* and promote more lactate produced from pyruvate resulted in the release of pyruvate accumulation and lower NADH/NAD+ ratios. In the mono-culture, because of the accumulation of pyruvate, the Node 3 is the speed control step and *C. cellulolyticum* has a strong desire to regulate the exceed pyruvate resulted in high expression on the lactate dehydrogenase and PFO. However, in the co-culture system, the lactate was remove by *D. vulgaris* Hildenborough, the Node 3 was no longer speed control step, the Node 1 (cellulose degradation step) became the speed control step resulted in the up-regulation on cellulosome genes (such as glycoside hydrolase genes, cellulosome protein dockerin genes and cellulosome anchoring protein cohesin region genes) and down-regulation on genes for Node 3 (such as lactate dehydrogenase and PFO) and the Node 2 glyceraldehyde-3-phosphate dehydrogenase (less carbon was dissipated from the cell to environment to form polysaccharides outside of the cell) (Fig. 3.6). All these microarray data were consistent with our physiology data of co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough.

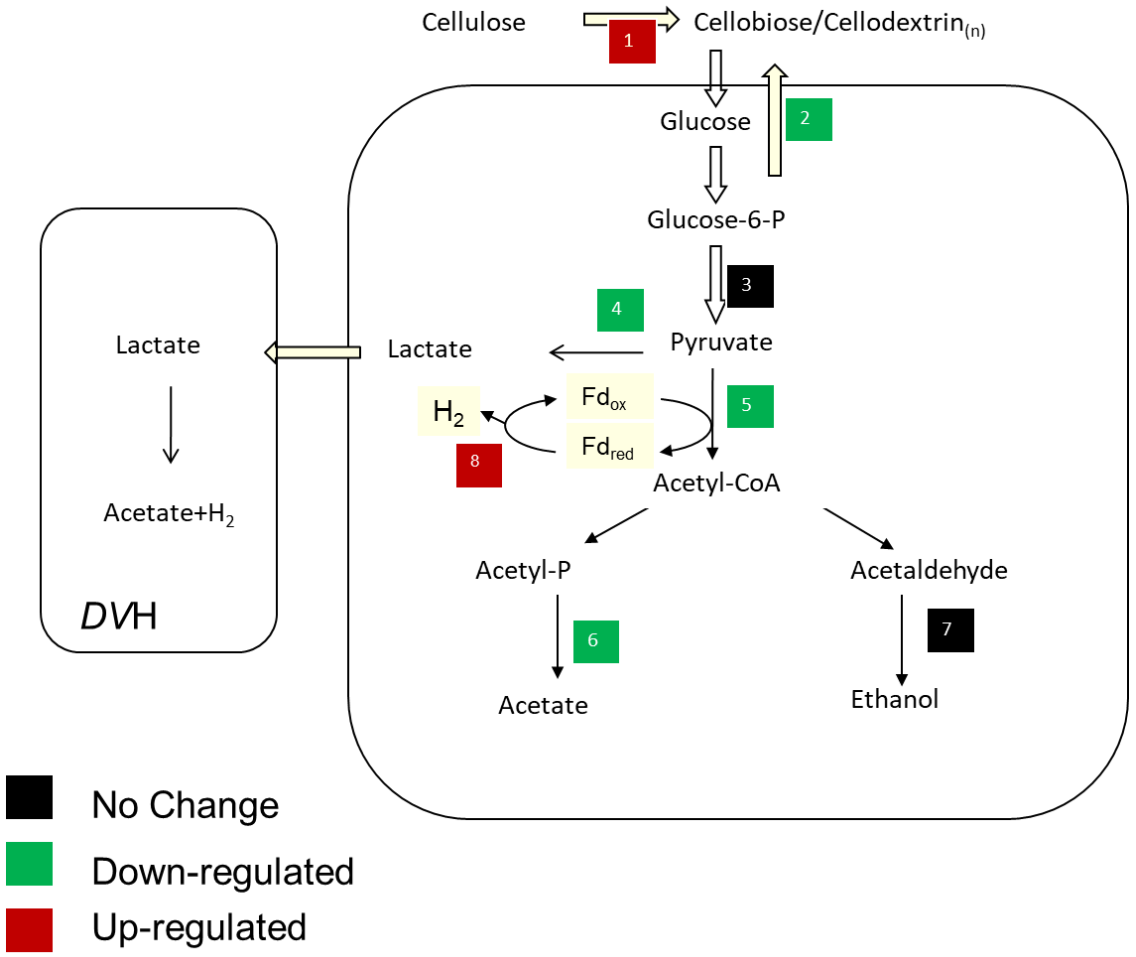


Fig. 3.6 Gene regulations of *C. cellulolyticum* in co-culture system (1. Cellulosomal related genes, 2. Glyceraldehyde-3-phosphate dehydrogenase, 3. Pyruvate Kinase, 4. L-lactate Dehydrogenase, 5. Pyruvate Ferredoxin Oxidoreductase (PFO), 6. Acetate Kinase, 7. Alcohol Dehydrogenase, 8 Hydrogenase, Fe-only)

3.4.7 D. vulgaris Hildenborough could help C. cellulolyticum colonize on cellulose

From the microarray data of differential expressed genes in *C. cellulolyticum* under the co-culture conditions, interestingly, we observed that the genes related to sporulation were upregulated (Table S3.2). As we known, the sporulation usually was a method that bacterium used to overcome the unfavorable conditions, however, under the co-cultured conditions the *C. cellulolyticum* grow very well in terms of the cell density and productivity we present above. We can question that why there are so many sporulation

related genes up regulated in the co-cultured conditions compare to the mono-culture? It has been reported that in *Clostridium thermocellum*, attachment to cellulose fibers may trigger the sporulation process (Wiegel and Dykstra 1984). In *C. cellulolyticum*, it is reported that the sporulation can be triggered under high concentration of cellulose and low pH value in the system and the entry into stationary phase which may inhibit the metabolic production (Desvaux and Petitdemange 2002), indicating a possible relationship between sporulation and cellulose degradation.

As all cellulosomes described so far, the *C. cellulolyticum* cellulosome is organized around a specialized scaffolding protein CipC, and this structure is present on the bacterial cell surface, and adhesion of the cell to cellulose appears to be required for rapid and efficient cellulose hydrolysis. Cellulose colonization of *C. cellulolyticum* is proposed to occur through a cyclic process of adhesion colonization-release–readhesion (Fig. S3.5) (Desvaux 2005). It is indicated that during the cyclic process of cellulose colonization, sporulation could be an efficient way of the release process from the cellulose fibers. The higher gene expression level may indicate more cellulose colonization- release activities in the co-culture system. The *D. vulgaris* Hildenborough was well studied as a biofilm former (Clark, Edelmann et al. 2007), thus we propose that *D. vulgaris* Hildenborough can help the *C. cellulolyticum* on cellulose colonization. The aggregates of cell and cellulose in the co-culture system were observed (Fig S3.6), however, there is still no direct evidence to show that *D. vulgaris* Hildenborough played an important role in the cellulose colonization in the co-culture.

To study the ability of cell attachment on cellulose in both mono-culture and co-culture, the ratio of cell protein concentration in the cellulose phase and liquid phase were

calculated. The stationary phase of mono-cultured *C.cellulolyticum* and *D. vulgaris* Hildenborough were collected and added to three sets of fresh media contains cellulose (final cellulose concentration is 4g/L), respectively (one set for mono-culture of *C.cellulolyticum*, one for *D. vulgaris* Hildenborough and one for co-cultured *C.cellulolyticum* and *D. vulgaris* Hildenborough). Inoculated co-culture bottle and mono-culture bottles were then incubated under 39℃, samples were taken from different time points, and samples were separate to cellulose phase and liquid phase. Cell protein concentrations in each phase were measured and the ratio was calculated (Fig 3.7）

The results showed that when *C.cellulolyticum* and *D. vulgaris* Hildenborough was mono-cultured with cellulose separately, the ratio of cell attachment on the cellulose were different. The ratio is higher in *C.cellulolyticum* than *D. vulgaris* Hildenborough as expected since there are cellulosome on the surface of *C.cellulolyticum* which can specific attach on the cellulose. Thus, it is expected that if there is no interaction between *C.cellulolyticum* than *D. vulgaris* Hildenborough the cell attachment ratio should be a value in between mono-cultured *C.cellulolyticum* than *D. vulgaris* Hildenborough separately. However, the results showed that in the co-culture system, more bacteria attached on the cellulose, the cell attachment ratio is much higher in the co-culture than the mono-cultured *C.cellulolyticum* (Fig 3.7), which is not expected. The results indicated that there are cell to cell interactions between *C.cellulolyticum* than *D. vulgaris* Hildenborough in the co-culture system to promote the cell attachment on the cellulose, and this could also be the reason that the cellulose was degraded much faster in the co-culture system than the mono-culture.

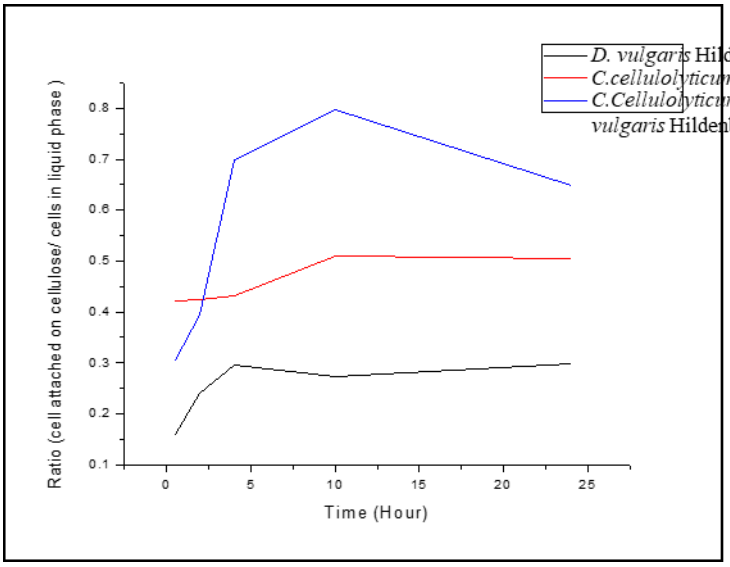


Fig. 3.7 Cell attachment ratio in mono-cultured *C.cellulolyticum* and *D. vulgaris* Hildenborough and co-cultured *C.cellulolyticum* and *D. vulgaris* Hildenborough

To obtain more information of cell attachment in the co-culture system, samples from both mono-culture and co-cultured *C.cellulolyticum* were collected at early stationary phase to get the cell image with cellulose by SEM. The SEM images also indicated that there is a potential biofilm layer on the cellulose surface (Fig 3.8). The *C.cellulolyticum* was considered as a sluggish cellulolytic bacteria in the early studies (Giallo, Gaudin et al. 1985), which was attributed mostly to its cellulosome system that need to be further studied genetically and biochemically to improve the cellulose fermentation (Tchunden, Petitdemange et al. 1992). However, there was no direct evidence that its limiting factor of cellulose degradation is its cellulolytic system (Desvaux 2005). *C.cellulolyticum* was reported that adhesion of the cell to cellulose was required for efficient cellulose degradation which means that it was essentially for the cellulosome of *C.cellulolyticum* contact with cellulose (Bayer, Kenig et al. 1983). In addition, the release of cells at the end of growth always suggested the exhaustion of accessible cellulose (Gelhaye, Petitdemange et al. 1992, Gelhaye, Petitdemange et al.

1993). Therefore, when the initial cellulose concentration is lower than 6.7 g/L, the slowdown of cellulose degradation was because of the change in distribution of the cellulose fibers (Desvaux, Guedon et al. 2000). Since the cellulosome of *C.cellulolyticum* has to attach to the cellulose fibers to be functional, the growth was not limited by the substrate concentration but the number of available adhesion sites on the cellulose fibers during the cellulose fermentation process. It means the more cellulose was degraded, the less opportunities cells could find new adhesion sites for the cellulosic system attaching on the cellulose fibers. That was the reason why the bacteria was not able to participate in the residual cellulose degradation at the end of the fermentation (Desvaux, Guedon et al. 2000, Desvaux and Petitdemange 2002, Desvaux 2005).

In this study, the addition of *D. vulgaris* Hildenborough may help *C.cellulolyticum* to attach on the residual cellulose in the medium. It was observed that cell-cellulose aggregates were formed in the co-culture system (Fig S3.6), which can obviously increase the chance to help the cells finding the new adhesion site and attaching on the cellulose fibers, since under this condition, the cellulose concentration within the aggregates formed by the co-culture was much higher than the cellulose concentration in the supernatant. Moreover, along with the cellulose hydrolyzation within the aggregates, more cellulose fibers in the supernatant may get involved, therefore, the *C.cellulolyticum* in the co-culture can access to the new colonization sites on the fresh cellulose fiber much easier than its mono-culture, which may explain why the cellulose degradation was much faster in the co-culture system.

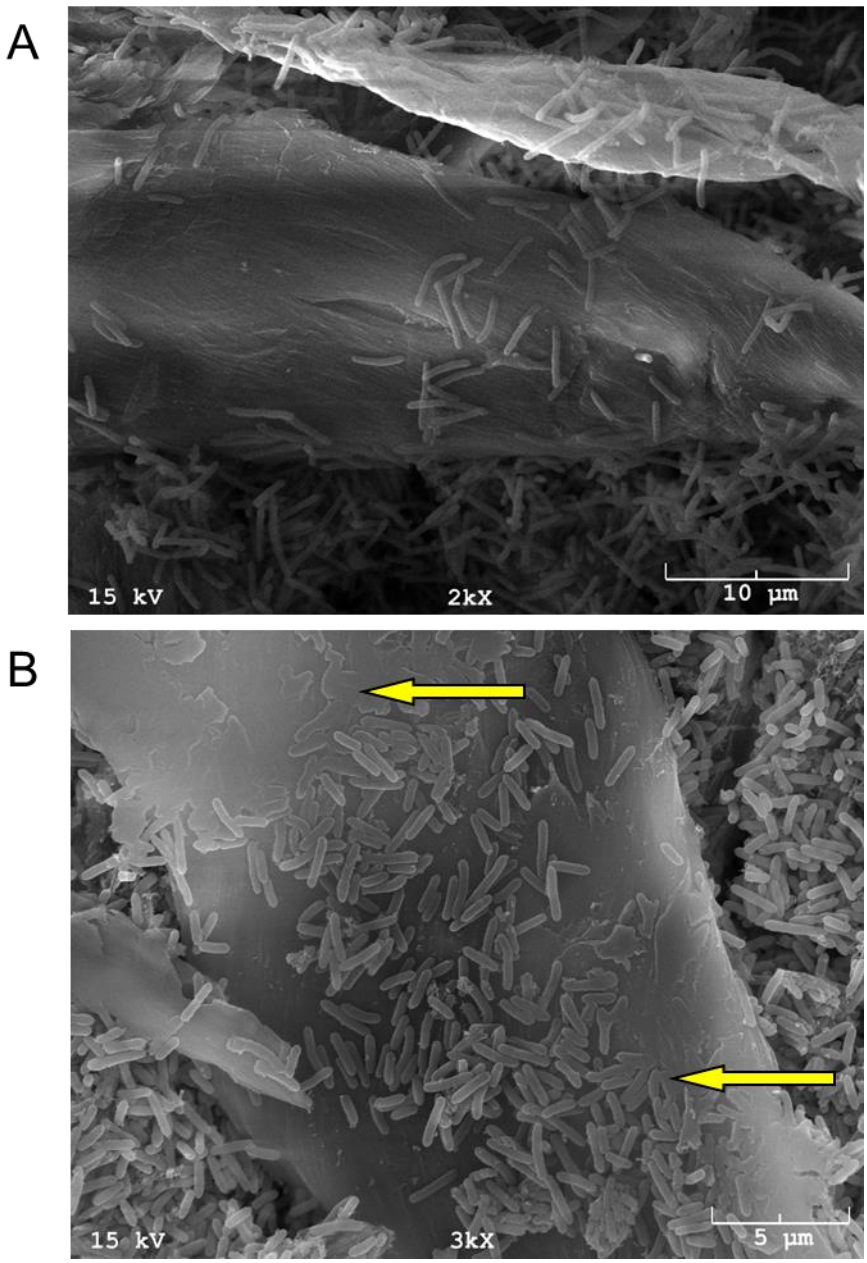


Fig. 3.8 SEM images of cell colonization on cellulose (A is mono-culture *C.cellulolyticum*, B is co-cultured *C.cellulolyticum* and *D. vulgaris* Hildenborough, arrows indicated the potential bio-film)

All the results including the microarray analysis (the up-regulation of the genes related to cellulosome and sporulation process) indicated that in the co-culture system the cyclic process of adhesion colonization-release–readhesion is more active than the mono-culture which may explain why the co-culture system can degrade cellulose much faster

than the mono-culture. According the results we have, a model of the cell attachment in the co-culture system was proposed (Fig 3.9). Briefly, the *C.cellulolyticum* first attached on the cellulose fibers, and it is the primary colonizer on the cellulose, then, *D. vulgaris* Hildenborough get involved and make a secondary colonization happened with more cellulose fibers. In this case, a fully functional cell-cellulose aggregates containing both *C.cellulolyticum* and *D. vulgaris* Hildenborough were formed and then dispersed by sporulation to attach on more cellulose fibers.

Hydrogen production from dark fermentation is a promising approach since there are lots of low cost substrates available such as lignocellulose biomass. Previous studies tried to enhance hydrogen production by metabolic engineering. It was reported that overexpression of the hydrogenase on *Clostridium paraputrificum* M-21 and *Clostridium tyrobutyricum* JM1 can improve about 1.5-1.7 fold hydrogen production (Morimoto, Kimura et al. 2005, Jo, Jeon et al. 2010). In addition, there were other studies tried to delete the lactate dehydrogenase gen (*ldh*) to block the lactate production to increase the hydrogen production on *Clostridium pefringens* strain W11 and *Thermoanaerobacterium aotearoense* (Li, Lai et al. 2010, Wang, Zong et al. 2011). However, the substrate used in those studies mentioned above were soluble sugars but not lignocellulose biomass. Moreover, for *C.cellulolyticum*, as discussed above the lactate production could help the cell to balance the electronic flux, it could not be efficient to block the lactate production pathway to increase hydrogen production. In this study, the strategy to improve hydrogen production is to improve the conversation efficiency of cellulose to hydrogen by taking advantage of their specific metabolic capacities. *C.cellulolyticum* can produce lactate, acetate and ethanol, therefore, a co-culture was

introduced in this study to minimize the electron loss in the non-hydrogen-producing fermentative reactions.

In previous study, there were also co-cultures reported to improve cellulose degradation and biofuels production. However, the widely used strategy is using the combination of cellulose user to produce soluble sugars and saccharolytic microorganisms to produce biofuels. It was reported that the co-culture of *C. cellulolyticum* and *Clostridium acetobutylicum* (can not utilize cellulose) was able to produce 350 mg/L butanol by using cellulose as substrate (Salimi, Zhuang et al. 2010, Salimi and Mahadevan 2013). Recently, another study reported that the co-culture of *Clostridium termitidis* which can breakdown cellulose into glucose and produce hydrogen and *Clostridium beijerinckii* which can not utilize cellulose but can produce hydrogen from glucose can increase the hydrogen production from cellulose. In their study, the hydrogen production of co-culture was increased to 2.54 mol hydrogen mol^{-1} hexose from 1.45 mol hydrogen mol^{-1} hexose of the mono-culture (Gomez-Flores, Nakhla et al. 2017). It was also reported that the co-culture of *C. cellulolyticum* and *Citrobacter amalonaticus* can enhance the hydrogen production from corn stover, this co-culture was established by using *C. cellulolyticum* to utilize corn stover to produce glucose and xylose which were used by hydrogen producing bacterium *Citrobacter amalonaticus* (Zhang, Lai et al. 2016*)*.

Compared to previous study mentioned above, this study more focused on the co-culture which can utilize the end products produced by *C. cellulolyticum*. Other studies only considered *C. cellulolyticum* as a cellulose degrader and co-cultured with sugar fermenter to produce hydrogen or butanol (Salimi, Zhuang et al. 2010, Salimi and

Mahadevan 2013, Zhang, Lai et al. 2016, Gomez-Flores, Nakhla et al. 2017), but did not attempt to solve the problem that *C. cellulolyticum* can not handle high concentration of carbon source which is the most critical limitation of *C. cellulolyticum* application. Therefore, in this study, the aim of established co-culture system not only can produce more hydrogen but also fix the inefficient carbon regulation of *C. cellulolyticum*.

As discussed in Chapter 1, the hydrogen production was associated with acetate production, when lactate and ethanol were the end products, no hydrogen was produced. Therefore, two different strategies were developed. First strategy was adding a acateate user into the system which consume the acetate making the *C. cellulolyticum* producing more acetate and hydrogen; the other strategy was adding a lactate user which can produce hydrogen from lactate, thus more hydrogen can be produced and *C. cellulolyticum* can get efficient carbon regulation by lactate production. Different combinations of co-culture were tested in this study. The co-culture of *C.cellulolyticum* and acetate user of *Geobacter sulfurreducens* or *Rhodopseudomonas palustris* were conducted, however, since H_2 could be utilized by both *Geobacter* and *Rhodopseudomonas* as electron accepter, there were no significant hydrogen production increase in the co-cultures (data not shown) than mono-cultured *C.cellulolyticum*. Other combinations of co-culture were conducted with lactate user of *Shewanella* and *Desulfovibrio*. Without extra electron accepter, the results showed that the co-culture of *C.cellulolyticum* and with *Shewanella putrefaciens* W3-18-1 or *Shewanella oneidensis* MR-1 can both increase the hydrogen production than the mono-cultured *C.cellulolyticum* (Fig. S3.7). However, the hydrogen production with 10g/L cellulose of co-culture with *Shewanella* can only produce about 1.25 L hydrogen per liter medium

which was much less than the 2 L hydrogen per liter medium produced by the co-culture with *Desulfovibrio vulgaris* Hildenborough. The reason for these results were because that *Desulfovibrio* played multiple roles in the co-culture, not only the lactate user but also the helper on cellulose colonization of *C.cellulolyticum*. This was also the reason in this study the hydrogen yield from cellulose was 3.21 mol hydrogen mol^{-1} hexose which is much higher than the 2.54 mol hydrogen mol^{-1} hexose reported in the other *C.cellulolyticum* and *Clostridium beijerinckii* co-culture (Gomez-Flores, Nakhla et al. 2017).

The *C. cellulolyticu*m is the model organism of mesophilic cellulolytic *Clostridia*. This organism has been characterized as a sluggish cellulolytic bacterium in the early studies of cellulose hydrolysis (Giallo, Gaudin et al. 1985) . A previous study also showed the improved cellulolytic performance of *C. cellulolyticu*m that with initial cellulose concentrations less than 6.7 g L^{-1}, more than 85% degradation occurred in 5 days (Desvaux, Guedon et al. 2000). However, the cellulose degradation rate was still not comparable with the thermophilic cellulolytic *Clostridia*. In the present study, cellulolytic performance of co-cultured *C. cellulolyticu*m and *D. vulgaris* Hildenborough was further improved, with initial cellulose concentration 4 g L^{-1} more than 95% degradation occurred in 3 days. The cellulose degradation process was affected by the cellulosome concentration and production. The cellulosome efficiency in the co-culture may be higher than mono-culture since more abundant *C. cellulolyticu*m and faster growth observed in the co-culture according to the cell population investigated by RT-PCR which suggested that more cellulosome may be produced by the *C. cellulolyticu*m since the high cell density in the co-culture system. Thus, the co-culture system can degrade cellulose faster

than the mono-culture, potentially not only because the *D. vulgaris* Hildenborough can utilize the lactate (or pyruvate) which may release the accumulation of pyruvate in cellulose metabolism for *C. cellulolyticu*m, but also that there are more cellulosome were synthesized and more efficient cellulose colonization cyclic process of adhesion colonization-release–readhesion in the co-culture. The flowing work of this study will be using the continuous culture with pH regulation to further improve hydrogen production and cellulose degradation of the co-culture. Since the cell abundance of *D. vulgaris* Hildenborough in the co-culture was relatively low, a re-inoculation process may be needed to maintain a relative high level of *D. vulgaris* Hildenborough abundance in the continuous culture system.

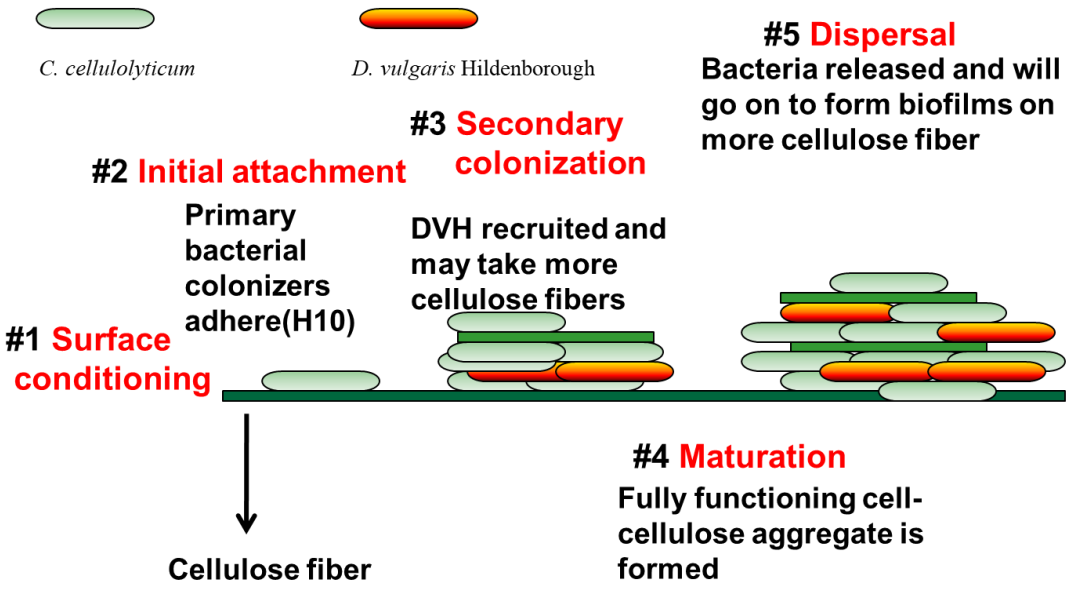


Fig. 3.9 Scheme of the model of cellulose colonization in the co-cultured *C.cellulolyticum* and *D. vulgaris* Hildenborough

3.5 Conclusions

In this study, a co-culture consisted of *C. cellulolyticu*m and a lactate user *D. vulgaris* Hildenborough was constructed for high hydrogen yield and efficient cellulose degradation. Basically, while *C. cellulolyticu*m provides the carbon source, most likely lactate, to *D. vulgaris* Hildenborough by degrading cellulose, therefore, the lactate concentration in co-culture remains a low level. The lactate production in *C. cellulolyticu*m will release the accumulation of pyruvate and result in an efficient regulation of carbon flow to achieve high cell abundance of *C. cellulolyticu*m in the co-culture. Under optimized conditions (39°C, initial pH 7.0, 4 g/L cellulose, 7.5 mg L^{-1} $FeCl_2 \cdot 4H_2O$ and no yeast extract), the co-cultured *C. cellulolyticu*m and *D. vulgaris* Hildenborough could produce 3.21 mol H_2 mol^{-1} glucose, which is more efficient than the mono-cultured *C. cellulolyticum* 2.05 mol H_2 mol^{-1} glucose, and 95% cellulose was degraded within 72 hours in the co-culture system. Besides, the *D. vulgaris* Hildenborough can not only use lactate to produce H_2 in the system but also help *C. cellulolyticum* colonize on cellulose which make the co-culture system more efficiently in terms of cellulose degradation and hydrogen production.

Chapter 4: Ethanol production of *Clostridium thermocellum* LQR1 and *Thermoanaerobacter ethanolicus* X514 developed through long term ethanol tolerance adaptation

4.1 Abstract

Thermophilic anaerobic bacteria have a potential for efficiently converting inexpensive substrates like cellulose to industrial ethanol by microbial consortia through consolidated bioprocessing (CBP). Previous studies have shown that the consortium of *Clostridium thermocellum* LQR1 and *Thermoanaerobacter ethanolicus* X514 produce higher amounts of ethanol than other consortia. Many *Clostridium* and *Thermoanaerobacter* strains, however, are sensitive to even moderate concentrations of ethanol (< 1%), resulting in a low final ethanol yield. To develop strains of *Clostridium* and *Thermoanaerobacter* that are resistant to high ethanol concentrations, we used the ethanol tolerance adaptation method. LQR1 and X514 were cultured with 0.5% ethanol, followed by repeated transfers with small increases in ethanol concentration every 10 transfers for 500 generations. After 500 generations, evolved LQR1 and X514 strains were resistant to 5.4% and 3.9% ethanol, concentrations at which the parent strain could not grow. Ethanol concentrations were then fixed at 2% and 3% and cultures were transferred every 7 generations. Single colonies were isolated from the LQR1 and X514 ethanol resistance cultures at 800 generations. The parent strain had a greater biomass after 48 hours growth in the absence of ethanol than the ethanol-tolerant derivative. Interestingly, isolated ethanol resistant LQR1 grew significantly faster under 2% ethanol than in the absence of ethanol but X514 did not. LQR1 ethanol resistant strains were also able to produce more ethanol than the parent strain when co-cultured with parent X514. However, the parent

X514 was able to produce more ethanol than the ethanol resistant X514. The results demonstrate that ethanol resistance can be developed by repeated transfers and more ethanol can be produced using ethanol resistant strains. The whole genome sequences of evolved LQR1 strains were available and there were 214 mutations observed in LQR1 T3 strain as well as 359 mutations in LQR1 T13 strain. Future work will focus on the functions of observed whole genome mutations of the ethanol-resistant mutant strains and understanding possible carbon flow shift from the parent strains.

Keywords: *Clostridium thermocellum* LQR1; *Thermoanaerobacter ethanolicus* X514; cellulose; ethanol production; ethanol tolerance adaptation; long term evolution

4.2 Introduction

Bioethanol has been considered as one of the most important renewable sustainable energy alternative to the fossil fuels, particularly as a liquid transport fuel (Agarwal 2007, Himmel, Ding et al. 2007, Carroll and Somerville 2009). However, the current bioethanol that dominates the market was mostly derived from food crops such as sugarcane and maize which was considered as first general biofuels that is unsustainable because of the competition of the farm land and water resources with the food supply (Demain, Newcomb et al. 2005, Chakravortya, Magne et al. 2008). Recently, researchers have focused more on the conversion of lignocellulosic biomass such as cellulose to bioethanol which is considered more environmentally sustainable (Lynd 2008). Compared to the food-derived bioethanol, cellulosic based bioethanol is a more sustainable and feasible alternative because of the abundance of cellulosic biomass on the earth.

However, the bioconversion of cellulosic feedstock is still the major challenge since the lack of an effective process to produce cellulosic bioethanol which typically involved multiple steps of enzymatic cellulose hydrolysis and microbial ethanologenic fermentation. The consolidated bio-processing (CBP) (Lynd, Weimer et al. 2002)concept has been proposed as a promising strategy to combine saccharolytic enzymes production, cellulose hydrolysis, and fermentation of sugars into one single bioconversion step which could lead to the largest reduction in cost for the cellulosic bioethanol production (Lynd, Laser et al. 2008).

It has been reported that the co-culture of cellulolytic and saccharolytic microorganisms could be a promising method to convert cellulose to bioethanol (He, Hemme et al. 2011, Jiang, He et al. 2013). In the chapter 3, this study also constructed a

mesophilic consortium which can effectively degrade the cellulose to hydrogen. Compared to mesophilic fermentation, thermophilic fermentation offers several advantages converting cellulosic biomass to bioethanol including: the high cellulose utilization rate, high temperature which facilitates ethanol removal and recovery, and less chance of contamination (Demain, Newcomb et al. 2005). Previous studies have shown that the consortium of *Clostridium thermocellum* LQR1 and *Thermoanaerobacter ethanolicus* X514 produce higher amounts of ethanol than other consortia (He, Hemme et al. 2011). However, as is the case in many microorganisms, *Clostridium thermocellum* LQR1 and *Thermoanaerobacter ethanolicus* X514 are sensitive to high concentration of ethanol (>1%), which may reduce the cell vitality and decrease membrane integrity (Shaw, Podkaminer et al. 2008, Taylor, Tuffin et al. 2008, Timmons, Knutson et al. 2009). Cellular tolerance to ethanol can be derived by adaptive evolution of the wild-type strains via long time exposure to exogenous ethanol has been reported(Timmons, Knutson et al. 2009, Goodarzi, Bennett et al. 2010, Lin, Ji et al. 2013).

Therefore, the objective of this study was to develop strains of *Clostridium* and *Thermoanaerobacter* that are resistant to high ethanol concentrations by using the long term adaptive evolution by evolving under increasing concentrations of exogenous ethanol to enhance cellulosic bioethanol production. The results showed that the ethanol tolerance can be adapted and more ethanol was produced by the adapted *Clostridium thermocellum* LQR1 strains.

4.3 Materials and Methods

4.3.1 Microorganisms and media

C. thermocellum strain LQR1 (ATCC 35609) was obtained from the American Type Culture Collection. *Thermoanaerobacter sp.* strain X514 was maintained in our laboratory culture collection which was originally isolated from the deep subsurface in the Piceance Basin, CO(Roh, Liu et al. 2002). Strain X514 has been deposited at the American Type Culture Collection (ATCC BAA-938). The components of DCB-1 medium used is descripted in Chapter 2. Mineral medium was supplemented with yeast extract when needed and the medium was buffered by 2.52 g L^{-1} $NaHCO_3$ and pH was adjusted by mixed gas of N_2 and CO_2. *C. thermocellumstrain* LQR1 was maintained by the routine transfer of 10% (v/v) inoculum into fresh DCB-1 medium containing 20mM cellobiose. *Thermoanaerobacter sp.* strain X514 was maintained by the routine transfer into fresh DCB-1 medium containing 40mM glucose.

4.3.2 Adaptive evolution for improved ethanol tolerance

C. thermocellum strain LQR1 and *Thermoanaerobacter sp.* X514 were mono-culture anaerobically in DCB-1 medium with 5g/L yeast extract at 60 °C without shaking separately supplied with 20mM cellobiose for LQR1 as carbon source and 40mM glucose as carbon source for X514, respectively. For ethanol adaptation evolution, sequential transfer was employed under exogenous ethanol. The wild type strain of LQR1 and X514 was initially inoculated separately into DCB-1 containing 0.5% (v/v) ethanol in triplicates. When OD_{600} reached the maximum, cultures were immediately transferred (10% (v/v) inoculum) into fresh 0.5%-ethanol DCB-1 medium. The transfer was repeated until OD600 reached a reproducible maximum value, cells were inoculated into 1%-ethanol

medium. The cycle was repeated with increasing ethanol concentrations. The cycle was repeated with increasing ethanol concentration for approximately 500 generations over 6 months (treatment lines). After that LQR1 and X514 were repeated transferred under a certain concentration of exogenous ethanol for another 300 generations. The ancestor of both *C. thermocellumstrain* LQR1 and *Thermoanaerobacter sp.* X514 were transferred without exogenous ethanol as the control (control lines) to examine the impacts of repeated transfers. After about 800 generation, single colonies were isolated from the mixed cultures of mutant pools and ethanol production was tested. Further experiments confirmed that the ethanol tolerance phenotypes of both LQR1 and X514 were inheritable and stable after culturing for at least 60 generations in ethanol-free medium.

4.3.3 Cellulose fermentation

Cellulose fermentation experiments were initiated by a 10% (v/v) inoculum of log-phase cultures (OD600 ~ 0.5) grown on cellobiose or glucose. Mono-cultures were inoculated with *C. thermocellum* only and co-cultures were inoculated with *Thermoanaerobacter sp.* strain X514 in addition to *C. thermocellum*. 10 g/L Solka Floc cellulose (International Fiber Co., Urbana, OH) with 0.5% yeast extract was used as the cellulosic substrate.

4.3.4 Whole-genome DNA sequencing

Genomic DNA was isolated with CTAB (hexadecyltrimethylammonium bromide) method and purified with phenol/chloroform (Zhou, Bruns et al. 1996). Illumina sequencing of genomic DNA from evolved colony isolates *C. thermocellum* LQR1 T3, LQR1 T13 and the ancestor isolate. 1 µg DNA from each sample was used for sequencing library preparation with (KAPA Hyper Prep Kit (KR0961-v2.15, Kapabiosystems) following fragmentation (~ 300bp) with sonication. The sequencing was conducted with

Hiseq3000 PE150. Illumina reads were aligned to the *Clostridium thermocellum* LQR1 DSM2360 reference sequence in NCBI (OP016502) and mutation (SNP and Indel) calls were performed with Geneious R9.1.5 (Biomatters Limited). Final calls of mutations were manually checked by viewing the alignments in Geneious.

4.3.5 Analytical procedures

To monitor the production of fermentation end products, samples (1 mL) from the culture were taken at the end of fermentation via degassed sterile syringes and filtered through Millipore GSWP 0.20-μm filters prior to analysis. Ethanol and other end products were determined with HPLC (Agilent Technologies, Santa Clara, California) following instructions previously described in Chapter 2.

4.3.6 Statistical analysis

Differences in ethanol yields between cultures were analyzed using the one-way analysis of variance (ANOVA). If significant differences were indicated by a probability value less than 0.05 in ANOVA analysis, post hoc comparisons were performed using Tukey's test to further identify the pairs of cultures with significant difference as indicated by a probability value less than 0.05. Statistical analyses were performed with JMP 7.0.1 for Windows (SAS Institute Inc., Cary, North Carolina) as described previously (He, Lokken et al. 2009).

4.4 Results and Discussions

4.4.1 Quick Ethanol adaptation of C. thermocellumstrain LQR1 and Thermoanaerobacter sp. X514

After 500 generation of ethanol adaptation, the highest ethanol resistance concentration of *C. thermocellumstrain* LQR1 was 5.4% and 3.9% for *Thermoanaerobacter* sp. X514.

Since the ethanol adapted population were exposed to high exogenous ethanol for about 6 months, the growth in normal DCB-1 medium without exogenous ethanol was examined to test its growth under normal conditions. The results indicated that even though the adapted population of *C. thermocellumstrain* LQR1 can tolerant to a higher concentration of exogenous ethanol than adapted population of *Thermoanaerobacter* sp. X514, the biomass of adapted LQR1 did not show significant decrease when it was cultured without ethanol comparing to the ancestor (Fig. 4.1A). The OD_{600} of adapted LQR1 population G10 which can tolerant to 5.4% ethanol is about 0.9 when cultured without exogenous ethanol comparing to the 1.0 of ancestor. However, the OD_{600} of adapted X514 population F10 was only about 0.5 which is about the half of the 1.0 of ancestor (Fig 4.1B).

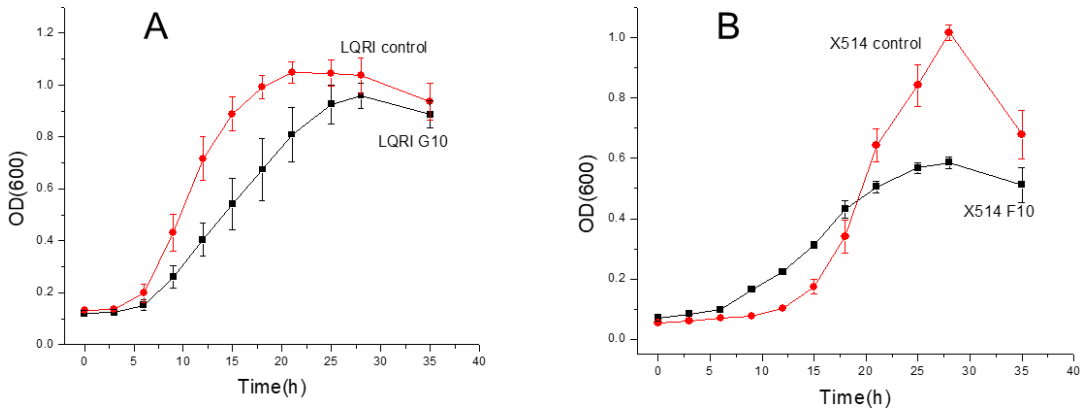


Fig. 4.1 Growth curve of *C. thermocellumstrain* LQR1 G10 and control (G10 could tolerant 5.4% ethanol) (A) and *Thermoanaerobacter* sp. X514 F10 and control (F10 could tolerant 3.9% ethanol)

After the quick adaption to the high ethanol concentration, we have to find an ethanol concentration that could stress the cells but at the same time will not kill all the cells to fix the ethanol resistance features of LQR1 and X514. The adapted train of LQR1 and X514 which can grow with the 5.4% and 3.9% exogenous ethanol from 500

generation were used to determine the ethanol concentration we are going to use. For LQR1, the results showed that that the ethanol resistance strains grow similarly when ethanol concentration was under 1%, and when ethanol concentration reached 2% the growth was slightly inhibited, and when ethanol concentration went up to 4%, the growth were significantly inhibited, thus, 2% ethanol concentration was used as the treatment concentration for LQR1 in the following evolution experiments (Fig. S4.1). For X514, the results showed that the ethanol resistance strains grow similarly when ethanol concentration was under 1.5% and when ethanol concentration reached 3% the growth was slightly inhibited so 3% ethanol concentration was used as the treatment concentration for X514 in the following evolution experiments (Fig. S4.2).

4.4.2 Single colony isolation from ethanol adapted population

Single colonies were isolated from the ethanol resistance long term evolution mix-cultures at 800 generations, *C. thermocellumstrain* LQR1 from 2% exogenous ethanol and *Thermoanaerobacter* sp. X514 from 3% exogenous ethanol, respectively. Growth curve and end products of the isolated colonies were examined.

4.4.2.1 Colonies isolated from ethanol adapted C. thermocellumstrain LQR1 population

Single colonies were isolated from both treatment lines (2% exogenous ethanol) and control lines (no ethanol added but only repeated transfers). The isolates were cultured under normal DCB-1 medium with no exogenous ethanol, and the end products concentrations of different isolation were investigated. The results showed that the ethanol productions of colonies from control and treatment lines are higher than ancestor. In addition, the end products profile of lactate, acetate, ethanol was different between ancestor and treatment (Fig. 4.2). Furthermore, the hydrogen productions of treatment

colonies are also lower than ancestor may because of the potential mutations of key genes of the ethanol adapted isolations (Fig 4.3).

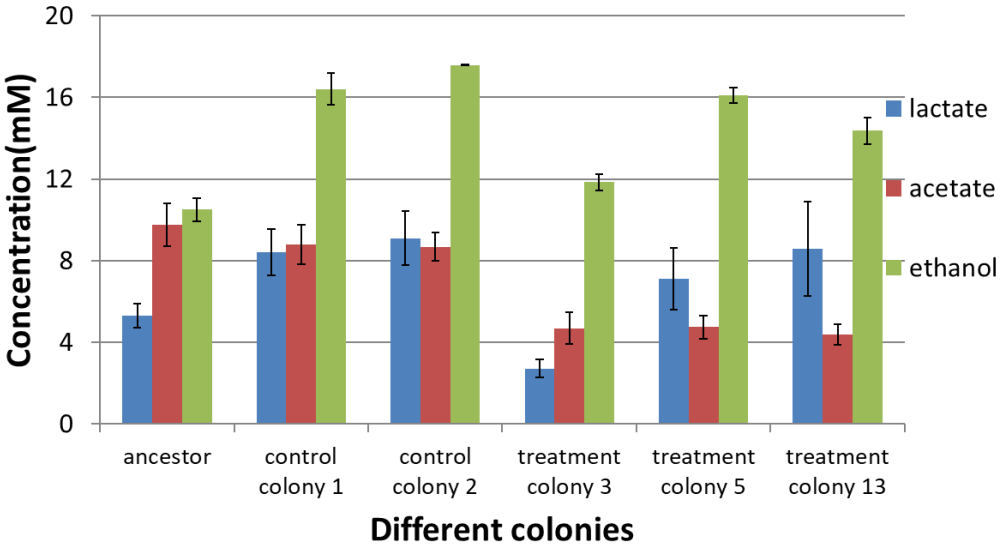


Fig. 4.2 End products (lactate, acetate, ethanol) concentration of different *C. thermocellumstrain* LQR1 isolations from ancestor, control lines and treatment lines (no exogenous ethanol)

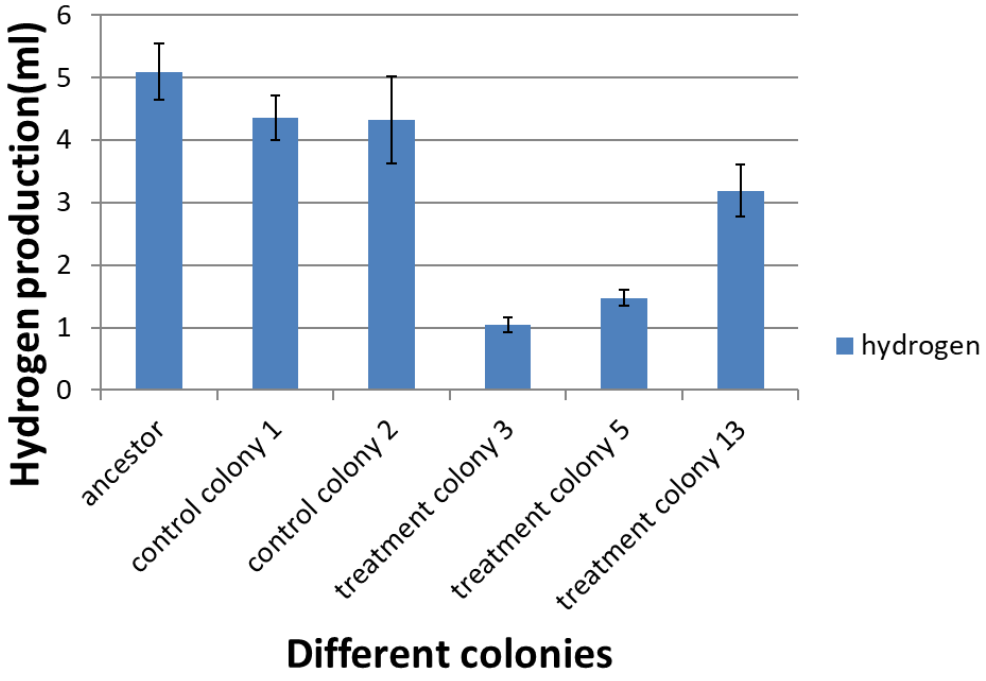


Fig. 4.3 Hydrogen production of different *C. thermocellumstrain* LQR1 isolations from ancestor, control lines and treatment lines (no exogenous ethanol)

The growth curve of isolates was investigated under normal condition and with exogenous ethanol. Since many isolations were tested, the detailed data were not present here. Interestingly, there were 2 isolations represent different phenotypes when they were cultured with exogenous ethanol. The growth curve of LQR1 treatment colony #3, #13 and LQR1 ancestor were showed in Fig 4.4. The results indicated that the ancestor strain had a greater biomass after 48 hours growth in the absence of ethanol than the ethanol tolerant derivative. As we expected, the ethanol tolerant derivative grew well in 4% ethanol rather than no significant growth of parent strain. Interestingly, the treatment colony #3 grow much slower than #13 and ancestor when the exogenous ethanol is absence in the medium. Thus, another growth tests of treatment strains #3 and #13 with different exogenous ethanol concentration was conducted.

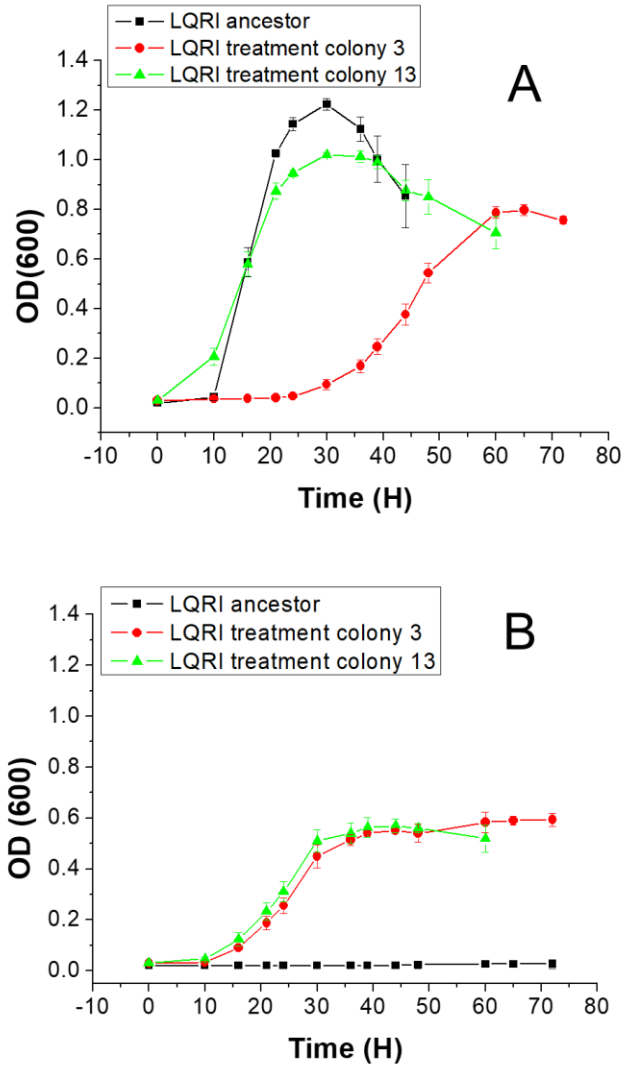


Fig. 4.4 Growth curve of *C. thermocellumstrain* LQR1 parent strain and ethanol-tolerant derivative in absence of ethanol (A) and 4% ethanol (B)

To examine the growth pattern of LQR1 treatment strains under exogenous ethanol, the starins were exposed to different ethanol concentration in DCB-1 medium. The results showed that the LQR1 treatment colonies #3 has shorter lag phase when it was cultured with higher concentration of ethanol, however, the other treatment colonies #13 did not show this feature. The results suggested that even though these two ethanol-

tolerant derivatives were all adapted to the high ethanol stress, there were probably two different evolution directions between them. (Fig. 4.5).

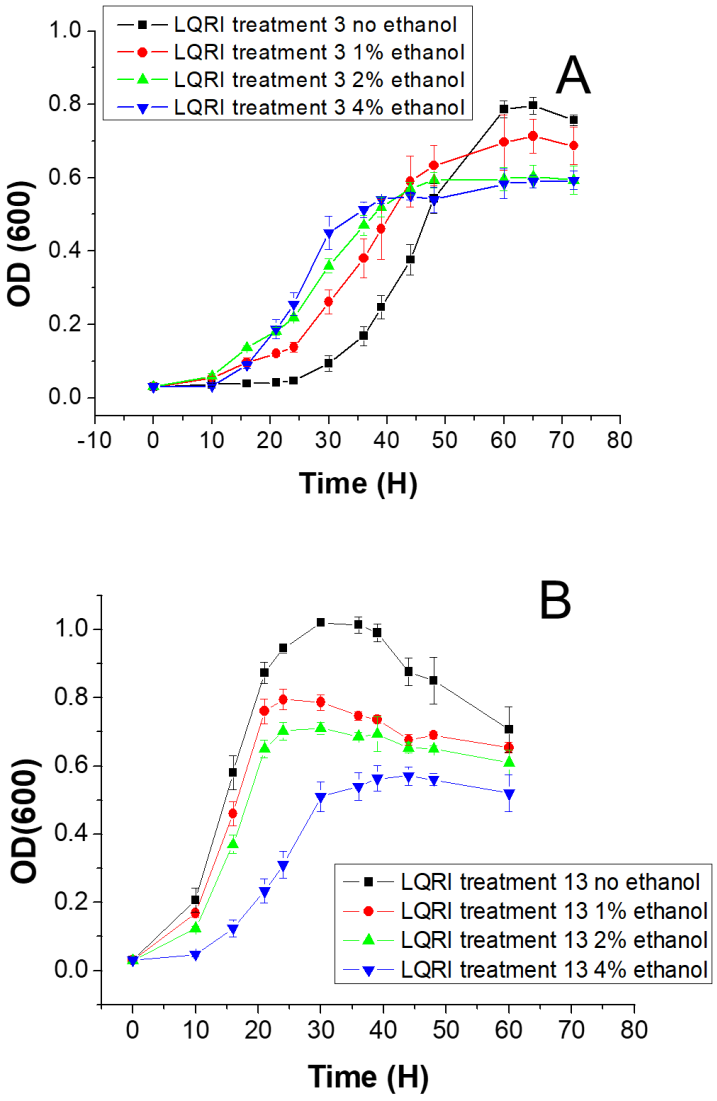


Fig. 4.5 Growth curve of *Clostridium thermocellum* LQR1 treatment colony #3 (A) and colony #13 (B) under different concentrations of ethanol

4.4.2.2 Colonies isolated from ethanol adapted Thermoanaerobacter sp. X514 population

Single colonies were isolated from both treatment lines (3% exogenous ethanol) and control lines from the repeat transferred *Thermoanaerobacter* sp. X514. Similar procedures were conducted as LQR1. The biomass of the X514 ethanol tolerant derivative were comparable or even greater than ancestor after 60 hours growth in the absence of ethanol. In addition, the X514 ethanol tolerant derivative grew well in 3% ethanol rather than no significant growth of parent strain. However, there was no significant lag phase when ethanol treated colonies are transferred to normal medium, also they did not show faster growth rate when cultured with ethanol than in absence of ethanol like the LQR1 treatment strain #3 did (Fig. S4.3). The end products concentrations of different X514 isolations were also investigated. The results showed that, unfortunately, the ethanol productions of control lines and treatment lines are lower than the ancestor (Fig 4.6). Thermophilic Gram-positive anaerobes (TGPAs) such as certain *Thermoanaerobacter* and *Clostridium* species are of interest in producing solvents (e.g., ethanol, butanol and isopropanol) from lignocelluloses under a Consolidated Bioprocessing (CBP) scheme (Lin, Ji et al. 2013). One strategy to develop cellular tolerance to solvents produced by the TGPAs is the adaptive evolution of the wild-type strains by exposing them to exogenous solvents for a long term repeated transfers (Goodarzi, Bennett et al. 2010). However, via this adaptive evolution strategy, solvent-tolerant strains usually produce less solvent than ancestors (Goodarzi, Bennett et al. 2010, Brown, Guss et al. 2011), this negative correlation between tolerance and productivity represents a major bottleneck in strain development. Thus, the reduction in ethanol yield of X514 ethanol tolerant derivatives was not unexpected, however, we were surprised that the LQR1 ethanol tolerant derivatives can produce more ethanol than the wild type.

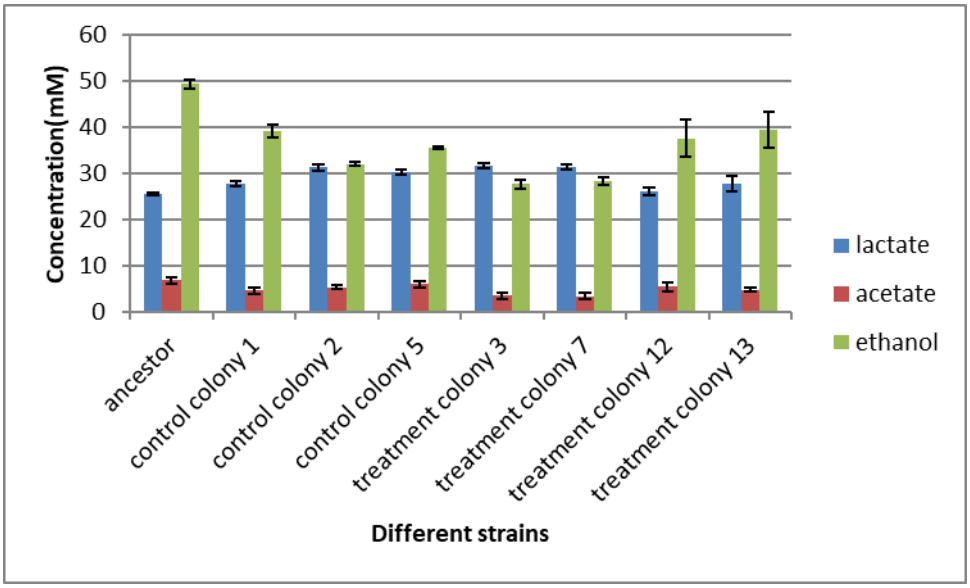


Fig. 4.6 End products (lactate, acetate, ethanol) concentration of different *Thermoanaerobacter* sp. X514 isolations from ancestor, control lines and treatment lines (no exogenous ethanol)

4.4.3 Mutations in evolved C. thermocellumstrain LQR1 strains

It was observed that dramatically improved ethanol resistance in ethanol evolved strains *C. thermocellumstrain* LQR1 T3 and LQR1 T13. As a first step in identifying the genetic bases of ethanol adaptation, the genomes of LQR1 T3，LQR1 T13 and the ancestral LQR1 were sequenced.

Sequences of these genomes covered 99.99% of *Clostridium thermocellum* LQR1 DSM2360 reference sequence in NCBI (OP016502) with average sequencing depths of 253x for the ancestor, 270x for the LQR1 T3 and 247x for the LQR1 T13 (Table 4.1). Comparing to the ancestor, in LQR1T3, there were total 214 mutations including 156 protein encoding mutations and 58 mutations in non-coding region were identified. In LQR1 T13, there were total 359 mutations detected including 286 protein encoding mutations and 73 mutations in non-coding region. Besides these specific mutations, 10

mutations including 5 protein encoding mutations were common in the ancestor, LQR1 T3 and LQR1 T13 (Table S4.1), and these results indicated the genetic variation between the LQR1 strain used in this study and the type strain genome originally deposited in the NCBI database. Interestingly, even though there were more than 200 mutations in each evolved strain, only 9 mutations were identified in both LQR1 T3 and LQR1 T13 (Table S4.2). Moreover, in these 9 common mutations, there was no SNP mutation but all deletion or insertion mutations, which indicated that LQR1 T3 and LQR1 T13 may evolved differently on genetic bases.

It would be interesting to look at the distribution of SNPs in the genome of evolved LQR1 T3 and LQR1 T13, particularly those genes contain multiple number of mutations. Evolved LQR1 T3 and LQR1 T13 both can resistant to high concentration of ethanol but LQR1 T3 has shorter lag phase with higher ethanol concentrations. It would be great if there was genetic evidence to explain the difference of phenotypes. Therefore, the genes with more than one SNP from evolved LQR1 T3 and LQR1 T13 were summarized and compared in a heatmap (Table 4.2), the darker the orange color means the higher the variant frequency. The results showed that LQRI_1851 encoding Glucokinase had total 5 mutations and LQRI_2548 encoding a hypothetical protein showed 4 mutations. Within these genes, LQRI_0681 encoding the zinc/iron permease was observed (Table 4.2). Although it was not clear that the role of iron transportation in stress tolerance, mutations in iron transport related genes were commonly detected in the evolutionary adaptation to stress conditions. Mutations in iron related genes were detected in *E. coli* evolution with butanol or salt stress (Dragosits, Mozhayskiy et al. 2013) as well as salt adapted *D. vulgaris* Hildenborough (Zhou, Hillesland et al. 2015).

Despite our increased knowledge of physiological adaptation, the genetic basis of microbial evolution remains unclear. In this study, more than 200 mutations were observed with 800 generation evolved strains, which was significantly higher than the 11 mutations identified in previous *D. vulgaris* Hildenborough evolutionary adaptations (Zhou, Hillesland et al. 2015). Interestingly, even in this study, evolved LQR1 T3 (214 mutations) and LQR1 T13 (359 mutations) were both resistant to high concentration of ethanol, the mutations in different evolved strain were differ greatly. One of the oldest questions in evolution is that the relationship between the number of mutations and the fitness effects (Dettman, Rodrigue et al. 2012). Even though there were lots of studies reported that few mutations associated with dramatic phenotype changes or fitness improvement (Herring, Raghunathan et al. 2006, Zhou, Hillesland et al. 2015), results from this study indicated the linkage between the significantly increased ethanol resistance and large number of mutations.

There were too many mutations in the genomes of evolved LQR1 T3 and LQR1 T13, further analysis should be conducted to determine the contributions of individual mutations by site-directed mutagenesis and the following phenotype test.

Table 4.1 Summary of sequenced genomes of *C. thermocellumstrain* LQR1 strains

Strain name	read length	# of sequences	# of sequences aligned to the reference genome	mean coverage	stdev of coverage
Ancestor	150	8338800	7585691	253.0	23.1
LQR1T3	150	8870860	8085357	270.0	26.0
LQR1T13	150	8540030	7422369	247.9	23.1

Table 4.2 Summary of genes with multiple mutations identified in ethanol evolved strain LQR1 T3 and LQR1 T13 (orange color represents the variant frequency)

Locus_tag	Affected Genes	Mutation type	Position	Amino Acid Change	LQR1 T13	LQR1 T3
LQRI_0016	VanW family protein	SNP	18465	A -> T	0.00	0.99
LQRI_0030	sporulation peptidase YabG	Deletion	32566		0.15	0.00
LQRI_0030	sporulation peptidase YabG	Deletion	32567		0.84	0.99
LQRI_0269	TraB determinant protein	SNP	287538	A -> T	0.87	0.00
LQRI_0309	acetolactate synthase, large subunit, biosynthetic type	SNP	333110	G -> S	0.99	0.00
LQRI_0309	acetolactate synthase, large subunit, biosynthetic type	SNP	333743	A -> T	0.00	0.99
LQRI_0317	DNA-directed RNA polymerase subunit beta	SNP	341301	T -> M	0.99	0.00
LQRI_0317	DNA-directed RNA polymerase subunit beta	SNP	341319	A -> V	0.97	0.00
LQRI_0318	DNA-directed RNA polymerase subunit beta	SNP	345340	D -> N	0.21	0.00
LQRI_0318	DNA-directed RNA polymerase subunit beta	SNP	346432	L -> F	0.49	0.00
LQRI_0380	protein of unknown function DUF1847	SNP	410538	K -> E	0.90	0.00
LQRI_0380	protein of unknown function DUF1847	Insertion	410784		0.90	0.00
LQRI_0406	integral membrane sensor signal transduction histidine kinase	Insertion	445323		0.00	0.12
LQRI_0406	integral membrane sensor signal transduction histidine kinase	SNP	445889	A -> V	0.64	0.00
LQRI_0439	glycoside hydrolase family 18	SNP	490812	T -> M	0.99	0.00
LQRI_0439	glycoside hydrolase family 18	Insertion	491706		0.00	0.99
LQRI_0455	ribosomal protein L22	SNP	505926	R -> K	0.99	0.00
LQRI_0455	ribosomal protein L22	SNP	506211	Q -> R	0.13	0.00
LQRI_0545	hypothetical protein	SNP	590391	R -> K	0.00	0.98
LQRI_0545	hypothetical protein	Deletion	590391		0.99	0.00
LQRI_0587	methyl-accepting chemotaxis sensory transducer with Cache sensor	SNP	639635	H -> Y	0.97	0.00
LQRI_0681	zinc/iron permease	Deletion	764577		0.66	0.00

LQRI_0681	zinc/iron permease	SNP	764816	G -> D	0.88	0.00
LQRI_0696	Dockerin type 1 protein	SNP	788679	M -> I	0.67	0.00
LQRI_0772	CRISPR-associated protein	Deletion	879681		0.00	1.00
LQRI_0772	CRISPR-associated protein	Insertion	880216		0.84	0.00
LQRI_0829	hypothetical protein	Deletion	945260		1.00	0.00
LQRI_0829	hypothetical protein	SNP	945721	V -> L	0.99	0.00
LQRI_0868	2-isopropylmalate synthase	SNP	997468	R -> K	0.00	0.97
LQRI_0920	(p)ppGpp synthetase I, SpoT/RelA	SNP	1063118	E -> K	0.11	0.00
LQRI_0920	(p)ppGpp synthetase I, SpoT/RelA	SNP	1063680	A -> V	0.90	0.00
LQRI_1067	major facilitator superfamily MFS_1	SNP	1266788	G -> E	0.99	0.00
LQRI_1067	major facilitator superfamily MFS_1	SNP	1267237	R -> G	0.99	0.00
LQRI_1069	Adenosylhomocysteinase	SNP	1268652	D -> G	0.94	0.00
LQRI_1069	Adenosylhomocysteinase	SNP	1269348	M -> T	0.99	0.00
LQRI_1094	nicotinate phosphoribosyltransferase	SNP	1296393	L -> F	0.00	0.17
LQRI_1200	Acetate kinase	SNP	1413328	V -> I	0.00	0.99
LQRI_1200	Acetate kinase	SNP	1413423	N -> K	0.99	0.00
LQRI_1215	sporulation integral membrane protein YtvI	SNP	1431770	F -> L	0.90	0.00
LQRI_1282	Carbamoyl-phosphate synthase large subunit glutamine-dependent	SNP	1504429	S -> G	0.14	0.00
LQRI_1282	Carbamoyl-phosphate synthase large subunit glutamine-dependent	SNP	1505842	R -> C	0.99	0.00
LQRI_1828	Cellulose 1,4-beta-cellobiosidase, Cellulase	Substitution	2113628	N -> S	0.00	0.98
LQRI_1847	Alcohol dehydrogenase	Insertion	2144912		0.00	0.97
LQRI_1851	Glucokinase	Insertion	2149156		0.00	0.36
LQRI_1851	Glucokinase	Insertion	2149156		0.99	0.64
LQRI_1851	Glucokinase	Deletion	2149342		1.00	0.00
LQRI_1851	Glucokinase	SNP	2149777	P -> S	1.00	0.00
LQRI_1851	Glucokinase	Insertion	2150122		0.00	0.23
LQRI_2017	Long-chain-fatty-acid--CoA ligase	SNP	2354065	R -> Q	0.99	0.00
LQRI_2017	Long-chain-fatty-acid--CoA ligase	SNP	2355412		0.00	0.99

LQRI_2031	ADP-ribosylation/Crystallin J1	SNP	2371738	A -> V	0.90	0.00
LQRI_2060	glutamate synthase alpha subunit domain protein	SNP	2402275	N -> S	0.11	0.00
LQRI_2060	glutamate synthase alpha subunit domain protein	Insertion	2402488		0.21	0.00
LQRI_2063	Glutamate synthase (NADPH)	SNP	2405291	P -> L	0.13	0.00
LQRI_2063	Glutamate synthase (NADPH)	Deletion	2405916		0.21	0.00
LQRI_2118	diguanylate cyclase and metal dependent phosphohydrolase	SNP	2462249	A -> T	0.00	0.99
LQRI_2124	6-deoxyerythronolide-B synthase, 3-oxoacyl-(acyl-carrier-protein) reductase	SNP	2471337	P -> L	0.92	0.00
LQRI_2124	6-deoxyerythronolide-B synthase, 3-oxoacyl-(acyl-carrier-protein) reductase	Insertion	2473083		0.85	0.00
LQRI_2352	Phosphonate-transporting ATPase	SNP	2736104	K -> E	1.00	0.00
LQRI_2411	hypothetical protein	SNP	2803105	M -> T	0.00	0.99
LQRI_2411	hypothetical protein	SNP	2803132	A -> V	0.98	0.00
LQRI_2466	Glutamate 5-kinase	SNP	2857012	E -> G	0.91	0.00
LQRI_2466	Glutamate 5-kinase	SNP	2857049	V -> I	0.00	0.98
LQRI_2473	peptidase S16 lon domain protein	SNP	2862892	I -> V	1.00	0.00
LQRI_2548	hypothetical protein	SNP	2961180	N -> S	0.69	0.00
LQRI_2660	Mannose-1-phosphate guanylyltransferase, Phosphoglucosamine mutase	Insertion	3101427		0.49	0.00
LQRI_2736	Dockerin type 1 protein	Deletion	3179393		1.00	0.00
LQRI_2736	Dockerin type 1 protein	SNP	3179455	T -> A	0.98	0.00
LQRI_2999	Phosphonate-transporting ATPase	SNP	3479863	T -> I	0.00	0.98
LQRI_2999	Phosphonate-transporting ATPase	SNP	3479882	H -> Q	0.99	0.00

Abbreviation: SNP, single-nucleotide polymorphis

4.4.4 Ethanol production of co-cultured C. thermocellumstrain LQR1 and Thermoanaerobacter sp. X514

To investigate the ethanol production of co-cultured *C. thermocellumstrain* LQR1 and *Thermoanaerobacter* sp. X514 from cellulose, different combinations of LQR1 and X514 isolations were used. The results showed that there are significant differences on ethanol production between the LQR1 wild type strain and LQR1 ethanol resistant strains. The wild type combination (X514wt+LQR1wt) produced about 160mM ethanol. When the ethanol resistant LQR1 strains co-cultured with wild type X514, the co-culture could produce more than 180mM ethanol. However, all the co-culture combinations which contains ethanol resistant X514 showed a reduction on the ethanol production regardless which LQR1 strains were involved. These results indicated that ethanol yield of this co-culture system more likely depends on the performance X514, the ethanol resistance X514 just produced about one third ethanol of X514 wild type, however, there were more than 15% increase when the X514 wild type co-cultured with ethanol resistant LQR1 strains (Fig. 4.7).

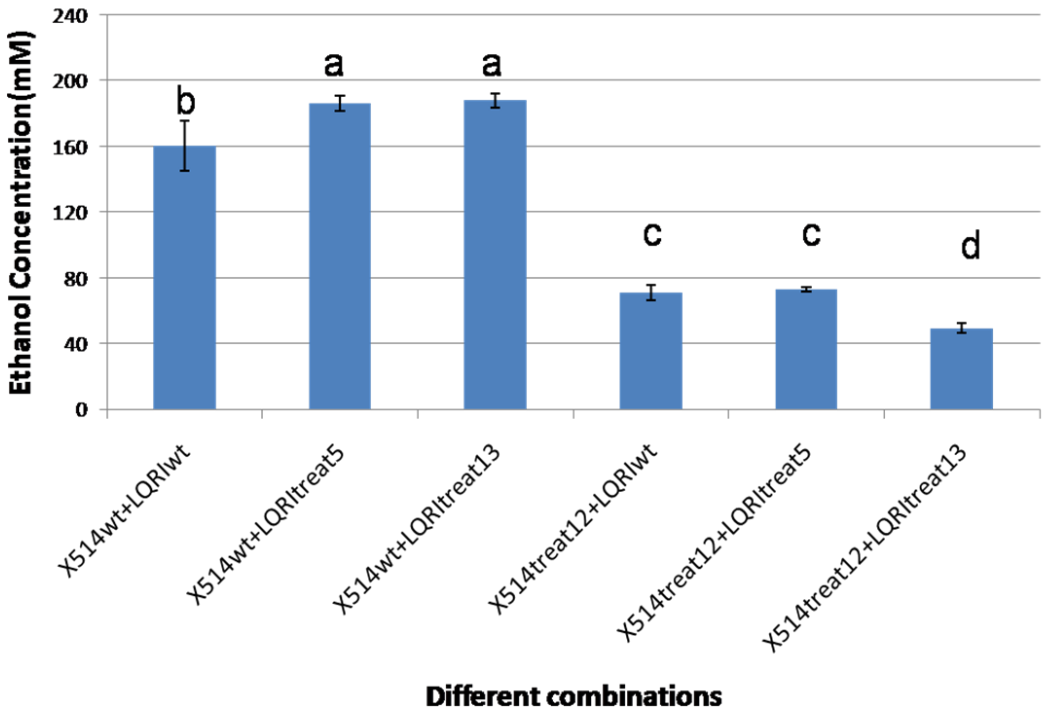


Fig. 4.7 Ethanol production under different combinations of *C. thermocellumstrain* LQR1 and *Thermoanaerobacter* sp. X514 ($p<0.05$)

The results indicated that the adaptive evolution strategy worked well on both LQR1 and X514 in terms of ethanol tolerance, however, in terms of ethanol production, this strategy worked well on LQR1 but not X514 which exhibited a reduction on ethanol yield. It is probably because that in the co-culture system the ethanol producer mainly was X514, and LQR1 mainly played a role of cellulose degrader. Ethanol was the main end products of X514, during the adaptive evolution process, the ethanol production may decrease since the negative feedback of high ethanol added in the media. In contrast, in the co-culture system, the role of LQR1 was degrading cellulose not producing ethanol, the evolved strains may more adapted with the condition of high ethanol concentration. Actually, the co-culture of LQR1 and X514 can produce relatively high concentration of ethanol, that maybe the reason why under this condition LQR1 ethanol resistant strains

can performance better in terms of ethanol production when co-cultured with X514 wild type.

Microorganisms can evolve resistance to specific stress factors such as salt, alcohol and temperature, which help them thrive in the conditions which non-evolved strain can not grow (Voordeckers, Kominek et al. 2015). Tolerance to high concentrations of ethanol is an industrial relevant phenotype since microorganism especially yeast was widely used in industrial bio-ethanol production. There were lots of studies have investigated the alcohol adaptation (Tomas, Welker et al. 2003, Tomas, Beamish et al. 2004, Alper, Moxley et al. 2006, Atsumi, Wu et al. 2010, Kim, Kim et al. 2016, Kitichantaropas, Boonchird et al. 2016), however, most of them were focused on yeast few on the *Thermoanaerobacter* and *Clostridium* species. Moreover, the molecular mechanisms that underlie adaptation to the stress factors were poorly understood. In the previous study, it was reported that *Thermoanaerobacter* X514 was evolved to tolerance of 2% ethanol and eventually 6% ethanol by genetic engineering, however, the ethanol production was also lower than ancestor which was consistent with the results of this study (Lin, Ji et al. 2013).

Clostridium thermocellum, a celluloytic thermophilic anaerobe can rapidly solubilize cellulose biomass which has potential for commercial application in converting cellulose to ethanol. It can produce ethanol as well as organic acids from cellulose, but growth and fermentation is inhibited when the ethanol concentration is above 10g/L (Lynd, Weimer et al. 2002). It was reported that *C. thermocellum* strain 27405 have been adapted to tolerate 8% ethanol (Williams, Combs et al. 2007), however, this study was more focused on comparing the membrane proteomic profiles for wild type and ethanol

evolved strains but no ethanol production and cellulose degradation data available for the evolved strain. Timmons et al. (Timmons, Knutson et al. 2009) proposed that the increased ethanol tolerance of *C. thermocellum* strain 27405 evolved strain was due to the change of member rigidity to reduce the ethanol fluidizing effect, however, the genetic basis for the increased ethanol tolerance for evolved strains of *C. thermocellum* was not clear. It was reported that a mutated bifunctional acetaldehyde-CoA/alcohol dehydrogenase gene may lead to improved ethanol tolerance in *C. thermocellum* strain 27405 (Brown, Guss et al. 2011), interestingly, in this study, it was observed that mutations on gene LQRI_1818 encoding acetaldehyde dehydrogenase in evolved LQRI T13 strain and mutations on gene LQRI_1847 encoding alcohol dehydrogenase in both LQR1 T13 and LQR1 T3 strain which was consistent with the Brown's study.

Microbial ethanol tolerance was proposed to be a complex process (Lovitt, Longin et al. 1984) which have been suggested that no single gene can endow the microorganisms with the ethanol tolerance (Stephanopoulos 2007, Alper and Stephanopoulos 2009). Some key genetic changes that confer enhanced ethanol tolerance was reported in *Saccharomyces cerevisiae* (Hong, Lee et al. 2010). Although the ethanol tolerance generally associated with membrane protein changes, ethanol tolerance may also limited by electron flux and carbon metabolism (Brown, Guss et al. 2011). In the future work of this study may firstly identify the membrane changes of the evolved LQR1 strains then

further analyze the mutations observed in the evolved strains to finally identify and characterize the functions of genes link to desired phenotypes.

4.5 Conclusions

In this study, ethanol tolerant strains of *Clostridium thermocellum* LQR1 and *Thermoanaerobacter ethanolicus* X514 were obtained by adaptive evolution of long term exposure to exogenous ethanol. The evolved LQR1 and X514 strains were able to resistant to 5.4% and 3.9% ethanol, concentrations at which the parent strain could not grow. The parent strain produced a greater biomass in the absence of ethanol than most of the ethanol-tolerant derivatives. In addition, it was observed that isolated ethanol resistant LQR1 grew significantly faster with exogenous ethanol than without. Interestingly, the ethanol evolved LQR1 can produce more ethanol than ancestor, however, the ethanol evolved X 514 showed a reduction on ethanol yield compared to the ancestor. The whole genome sequences of evolved LQR1 strains were available and more than 200 mutations were observed. When co-culture LQR1 and X514 on cellulose, 15% more ethanol can be produced using ethanol evolved LQR1 than the parent strain when co-cultured with parent X514.

Chapter 5: Summary and Output

Biofuels which are produced from plant materials and biomass which were so called bio-based materials become increasingly relevant as a potential sustainable alternative to fossil fuels. Many of the biofuels so called first generation biofuels that are currently being supplied have been criticized as unsustainable because of their potential threaten to the traditional crops on their production places (Naik, Goud et al. 2010). To address this challenge and support biofuel development, new research was conducted including non-edible lignocellulosic biofuel technologies, with responsible policies and economic instruments to ensure that biofuel commercialization is sustainable. Lignocellulose is a major renewable energy resource based on its quantity and availability. Biofuels converted from cellulose (the major component of lignocellulose) by microorganisms are more environmental friendly and sustainable alternatives to fossil fuels based on the resource availabilities. In previous studies, the cellulolytic *Clostridia,* including *C. cellulolyticum,* were comprehensively studied on their physiology and applications on the production of biofuels. However, huge challenges still exist to improve the efficiency of cellulose bioconversion process and thus reduce the economic cost to enable large-scale application of biofuels using this strategy. This study investigated the biofuel production from cellulose of mesophilic and thermophilic *Clostridia* under different conditions and revealing the mechanisms behind the high bioconversion efficiency. Based on the observed results, several outcomes and/or mechanisms about how to improve the efficiency of cellulose bioconversion process were revealed.

First, this work investigated the effects of different carbon loads on hydrogen production of *C. cellulolyticum,* an extensively studied mesophilic model microorganism.

Previous studies primarily focused on the physiology level of carbon metabolism but failed to explore the mechanisms on transcriptional level (Payot, Guedon et al. 1998, Guedon, Desvaux et al. 1999, Desvaux, Guedon et al. 2000) because of the lack of high through put microarray system. In this study, our results provide insights from physiology level that the restriction of substrate availability can balance the metabolism rate of intermediate products in *C. cellulolyticum*, which can relieve *C. cellulolyticum* from catabolite repression and improve the hydrogen production. In addition, our results indicated that the slow-released carbon source (i.e. cellulose) could be a better choice for *C. cellulolyticum* in terms of hydrogen production than fast-released carbon source (i.e. cellobiose), Furthermore, a comprehensive transcriptomic level study of the impacts of different carbon loads on *C. cellulolyticum* was conducted, which provided more detailed in-depth understanding of the carbon metabolism of *C. cellulolyticum*. Moreover, a co-expression gene network was constructed which can provide useful information for understanding gene function and interactions when the *C. cellulolyticum* was cultured in the different initial cellulose.

Second, a defined mix consortium composed of a cellulolytic microorganism *C. cellulolyticum* and a lactate user *Desulfovibrio vulgaris* Hildenborough was developed to exhibit the efficient cellulose degradation and high hydrogen yield. Previous studies usually use a cellulolytic *Clostridium* co-culture with a sugar fermenter to construct the consortium for cellulose bioconversion to biofuels (He, Hemme et al. 2011). However, in this study, the defined mixture showed outstanding hydrogen yield than the consortium previously reported, which is 3.3 mol H_2 mol^{-1}glucose that is 1.6 folds of the mono-cultured *C. cellulolyticum*. In addition, 95% cellulose was degraded within 72 hours

which is much more efficient than the mono-culture. More importantly, the interactions between two microorganisms in this defined mix to were comprehensively studied and a conceptual model to illustrate what is the function of *Desulfovibrio vulgaris* Hildenborough in the cellulose degradation was proposed. Our results suggested that adding of *D. vulgaris* Hildenborough to remove the lactate produced by *C. cellulolyticum* resulted in more lactate production which serves as an additional catabolic pathway for *C. cellulolyticum* to regulate the excesses pyruvate accumulation and lower NADH/NAD+ ratios will enable growth resumption of *C. cellulolyticum* from an inefficiently regulated carbon flow caused by the self-intoxication of bacterial metabolism. Besides, *D. vulgaris* Hildenborough does not only use lactate to produce H_2 in the system, but also be able to help *C. cellulolyticum* colonize on cellulose to speed up the cellulose degradation process. These data provide a comparable characterization of the cellulolytic and hydrogen-producing capabilities of the mono-culture and co-culture systems, and identification of ecological relationship between these two organisms in the system. This study will also contribute to improvements in the hydrogen-producing efficiency, and the defined mixture strategy can be commercialized and applied in the industrial processes of hydrogen production.

Third, ethanol tolerant strains of *Clostridium thermocellum* LQR1 and *Thermoanaerobacter ethanolicus* X514 were obtained by the strategy of long term evolution were obtained. The evolved LQR1 and X514 strains were able to resistant 5.4% and 3.9% ethanol respectively. Previous studies reported that the solvents tolerance thermophilic gram-positive anaerobes developed by adaptive evolution strategy usually produce less solvent than their wild type (Goodarzi, Bennett et al. 2010, Brown, Guss et

al. 2011). However, in our study, the ethanol-evolved LQR1 can produce more ethanol than its wild type, and interestingly, the ethanol adapted LQR1 has shorter lag phase when it was cultured with higher concentration of ethanol which suggested that there was a trade-off between the biomass and the ethanol resistance ability. More importantly, when ethanol evolved LQR1 was used as the cellulose degrader, 15% more ethanol can be produced than the parent strain when co-cultured with parent X514. These results demonstrate that ethanol resistance can be developed by adaptive evolution and ethanol production can be promoted using ethanol evolved strains. Moreover, there is a possibility to apply this strategy into the industrial processes of ethanol production from bioconversion of cellulose.

In summary, this study provided novel insights of the improvement of cellulose bioconversion process to produce biofuels, such as hydrogen and ethanol, which could be of merit for the application of *Clostridia* in to the industrial field and make progress with the production of second generation biofuels from the lignocellulose biomass.

Those results from this study and other associated projects that I have involved are largely reflected in my publications (published, in press, in preparation) as they are listed below:

1. **Wenbin Liu** et al. "Biohydrogen production from cellulose by the co-culture of *Clostridium cellulolyticum* H10 and *Desulfovibrio vulgaris* Hildenborough" (draft)
2. **Wenbin Liu** et al. "The effects of carbon loads on hydrogen production and cellulose degradation of *Clostridium cellulolyticum*" (draft)
3. **Wenbin Liu** et al. "Ethanol production by thermophilic bacteria based on ethanol tolerance development" (In preparation)
4. **Wenbin Liu** et al. "Biodiversity and productivity in competitive communities" (In preparation)
5. LIN lu, SONG houhui, TU qichao, QIN yujia, ZHOU aifen, **LIU wenbin**, HE zhili, ZhOU jizhong, and XU jian. The *Thermoanaerobacter Glycobiome* reveals mechanisms of pentose and hexose co-utilization in bacteria. PLoS Genetics, 2011, 7(10): e1002318.

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Appendix A: Supplementary Tables

Table S3.1 Hydrogen yield of co-culture at different cellulose concentration

Table S3.2 Differentially expressed genes of *C. cellulolyticum* (up-regulated) related to sporulation under co-culture conditions.

Table S3.3 Genes tightly linked with *cip-cel* operon in the co-expression network

Table S3.4 Genes linked with hydrogenase in the co-expression sub-network

Table S4.1 Common mutation identified in *C. thermocellum* ancestor, LQR1 T3 and LQR1 T13

Table S4.2 Common mutation identified in *C. thermocellum* LQR1 T3 and LQR1 T13

Table S3.1 Hydrogen yield of co-culture at different cellulose concentration

Cellulose (g)	Used Cellulose(g)	Produced H2(mM)	M H2/ M glucose
0.2(1 g/L)	0.19	4.7	3.85
0.8(4 g/L)	0.78	16.6	3.34
1.4(7 g/L)	1.32	19.1	2.28
2.0(10 g/L)	1.76	18.8	1.67
2.6(13 g/L)	2.18	19.2	1.38
3.6(18 g/L)	2.69	19.3	1.12
5.0(25 g/L)	2.88	18.9	1.02

Table S3.2 Differentially expressed genes of *C. cellulolyticum* (up-regulated) related to sporulation under co-culture conditions.

Gene name	Annotation	Log R	Z score
Ccel_0292	stage II sporulation protein R	3.55	6.92
Ccel_0490	sporulation protein YhbH	2.89	5.51
Ccel_0572	sporulation protein YqfC	3.23	6.18
Ccel_1878	sporulation integral membrane protein YlbJ	2.73	5.27
Ccel_1895	stage IV sporulation protein B	2.68	5.21
Ccel_1913	stage III sporulation protein AD	3.2	6.18
Ccel_1914	stage III sporulation protein AC	2.5	4.76
Ccel_1915	stage III sporulation protein AB	3.5	6.66
Ccel_2280	stage V sporulation protein AE	3.46	6.65
Ccel_2281	stage V sporulation protein AD	2.05	3.81
Ccel_2282	stage V sporulation protein AC	3.11	5.96

Table S3.3 Genes tightly linked with *cip-cel* operon in the co-expression network

Gene name	Annotation
Ccel_1738	carboxyl transferase
Ccel_1543	cellulosome anchoring protein cohesin region
Ccel_1736	Conserved carboxylase region
Ccel_1737	biotin/lipoyl attachment domain-containing protein
Ccel_2479	protein of unknown function DUF1294
Ccel_2265	ABC-type bacteriocin transporter
Ccel_0402	hypothetical protein
Ccel_0231	glycoside hydrolase family 9
Ccel_3108	sodium/hydrogen exchanger
Ccel_3109	TrkA-C domain protein
Ccel_2050	flagellar motor switch protein FliG
Ccel_2048	ATPase FliI/YscN
Ccel_2042	protein of unknown function DUF1078 domain protein
Ccel_2033	flagellar biosynthetic protein FliR
Ccel_0150	extracellular solute-binding protein family 1
Ccel_0167	hypothetical protein
Ccel_2712	beta-lactamase domain-containing protein
Ccel_0735	glycoside hydrolase family 9
Ccel_0739	cellulosome protein dockerin type I
Ccel_1044	Methyltransferase type 11
Ccel_0740	glycoside hydrolase family 5
Ccel_1249	glycoside hydrolase family 9
Ccel_2066	protein of unknown function DUF1290
Ccel_1230	Carbohydrate binding family 6
Ccel_0751	PpiC-type peptidyl-prolyl cis-trans isomerase
Ccel_0392	chaperonin GroEL
Ccel_0975	Acyl transferase
Ccel_0973	beta-lactamase
Ccel_0757	ribosomal protein S10
Ccel_1051	transcriptional regulator, AraC family
Ccel_1050	hypothetical protein
Ccel_0980	amino acid adenylation domain protein
Ccel_0977	amino acid adenylation domain protein
Ccel_2507	adenylate cyclase
Ccel_2526	methyl-accepting chemotaxis sensory transducer
Ccel_3116	two component transcriptional regulator, LytTR family
Ccel_0932	Carbamoyl-phosphate synthase L chain ATP-binding
Ccel_1811	glycosyl transferase family 2

Ccel_0813	hypothetical protein
Ccel_3207	sulfatase
Ccel_3013	germination protein, Ger(x)C family
Ccel_0011	CDP-diacylglycerol/serine O-phosphatidyltransferase
Ccel_3215	anthranilate phosphoribosyltransferase
Ccel_0060	hypothetical protein
Ccel_1109	protein of unknown function DUF1646
Ccel_0056	single-strand binding protein
Ccel_0055	ribosomal protein S6
Ccel_0686	3-oxoacyl-(acyl-carrier-protein) synthase 2
Ccel_0684	3-oxoacyl-(acyl-carrier-protein) reductase
Ccel_0680	regulatory protein DeoR
Ccel_0683	malonyl CoA-acyl carrier protein transacylase
Ccel_0681	fatty acid/phospholipid synthesis protein PlsX

Table S3.4 Genes linked with hydrogenase in the co-expression sub-network

Gene name	Annotation
Ccel_2664	hypothetical protein
Ccel_0801	ribosomal-protein-alanine acetyltransferase
Ccel_0294	ATPase, P-type (transporting), HAD superfamily, subfamily IC
Ccel_1204	phosphoglucosamine mutase
Ccel_1164	pyruvate flavodoxin/ferredoxin oxidoreductase domain protein
Ccel_1335	hypothetical protein
Ccel_0191	Appr-1-p processing domain protein
Ccel_1779	thioredoxin reductase
Ccel_1778	amine oxidase
Ccel_1780	putative signal transduction protein with CBS domains
Ccel_3203	transport system permease protein
Ccel_2277	peptidase T-like protein
Ccel_3355	MscS Mechanosensitive ion channel
Ccel_2482	drug resistance transporter, EmrB/QacA subfamily
Ccel_2545	glutamate synthase (NADPH), homotetrameric
Ccel_3354	protein of unknown function DUF951
Ccel_2546	oxidoreductase FAD/NAD(P)-binding domain protein
Ccel_1660	cytochrome c biogenesis protein transmembrane region
Ccel_1427	zinc/iron permease
Ccel_0610	pseudouridine synthase, RluA family
Ccel_2464	Pyrrolo-quinoline quinone
Ccel_1946	aspartyl-tRNA synthetase
Ccel_0465	hydrogenase expression/formation protein HypE
Ccel_0174	protein of unknown function DUF710
Ccel_3205	phosphopentomutase
Ccel_0173	hemerythrin-like metal-binding protein
Ccel_0695	aldo/keto reductase
Ccel_1945	CoA-substrate-specific enzyme activase
Ccel_2467	hydrogenase, Fe-only
Ccel_3367	NADH-ubiquinone oxidoreductase chain 49kDa

Table S4.1 Common mutation identified in *C. thermocellum* ancestor, LQR1 T3 and LQR1 T13

Position	locus_tag	Affected Genes	Mutation Type	Amino Acid Change
404497	intergenic region		Insertion	
404528	intergenic region		Deletion	
408223	LQRI_0377	Uroporphyrinogen decarboxylase (URO-D)	SNP	I -> N
408298	LQRI_0377	Uroporphyrinogen decarboxylase (URO-D)	Insertion	
479418	intergenic region		Insertion	
1497808	intergenic region		Insertion	
2149129	LQRI_1851	Glucokinase	Insertion	
2177144	LQRI_1877	helix-turn-helix domain protein	Insertion	
2838093	LQRI_2450	hypothetical protein	Insertion	
3489925	intergenic region		Insertion	

Table S4.2 Common mutation identified in *C. thermocellum* LQR1 T3 and LQR1 T13

Position	locus_tag	Affected Genes	Mutation Type	LQR1 T13	LQR1 T3
32567	LQRI_0030	sporulation peptidase YabG	Deletion	0.84	0.99
946628	intergenic region		Deletion	1.00	0.11
1634656	LQRI_1406	hypothetical protein	Insertion	0.99	1.00
1881899	LQRI_1620	iron (metal) dependent repressor, DtxR family	Insertion	0.94	0.99
2149156	LQRI_1851	Glucokinase	Insertion	0.99	0.64
2321477	intergenic region		Deletion	0.13	0.99
2441144	intergenic region		Deletion	0.16	0.17
2441144	intergenic region		Insertion	0.10	0.15
3047875	intergenic region		Deletion	1.00	0.98

Appendix B: Supplementary Figures

Fig. S2.1 Cellulose degradation ratio of *C. cellulolyticum* H10 with different initial cellulose load (average of triplicates)

Fig. S2.2 Non-hydrolysis cellulosomal genes differentially expressed under different cellulose concentration

Fig. S2.3 A sub-network of *C. cellulolyticum* contains Ccel_2467 hydrogenase

Fig. S2.4 Connectivity distribution of C. cellulolyticum co-expression network

Fig. S3.1 Hydrogen yield of co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough at different temperature (A) and initial pH value (B) with 10 g/L cellulose

Fig. S3.2 Hydrogen production and cellulose degradation ratio of co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough with different cellulose concentration

Fig. S3.3 Hydrogen production of co-culture at different Fe ion concentration

Fig. S3.4 3 metabolic nodes in cellulose metabolism of C. cellulolyticum (adapted from Guedon, Desvaux et al. 2002)

Fig. S3.5 Scheme of the model of cellulose colonization by C. cellulolyticum (adapted from (Desvaux 2005))

Fig. S3.6 Aggregates in co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough (A is mono-culture, B is co-culture)

Fig. S3.7 Hydrogen production of co-cultured *C. cellulolyticum* with *Shewanella putrefaciens* W3-18-1 and *Shewanella oneidensis* MR-1 without additional electron accepter

Fig.S4.1 Ethanol concentration test of *Clostridium thermocellum* LQR1 ethanol-tolerant derivative

Fig S4.2 Ethanol concentration test of *Thermoanaerobacter ethanolicus* X514 ethanol-tolerant derivative

Fig S4.3 Growth curve of *Thermoanaerobacter ethanolicus* X514 parent strain and ethanol-tolerant derivative in absence of ethanol (A)and 3% ethanol (B)

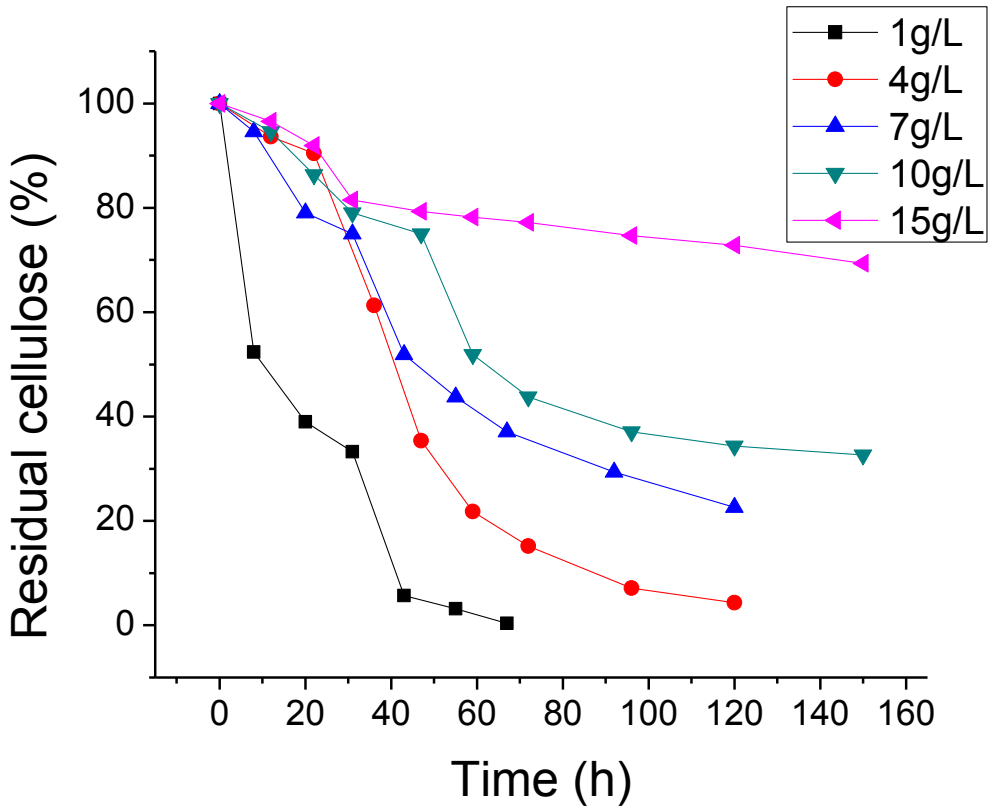


Fig. S2.1 Cellulose degradation ratio of *C. cellulolyticum* H10 with different initial cellulose load (average of triplicates)

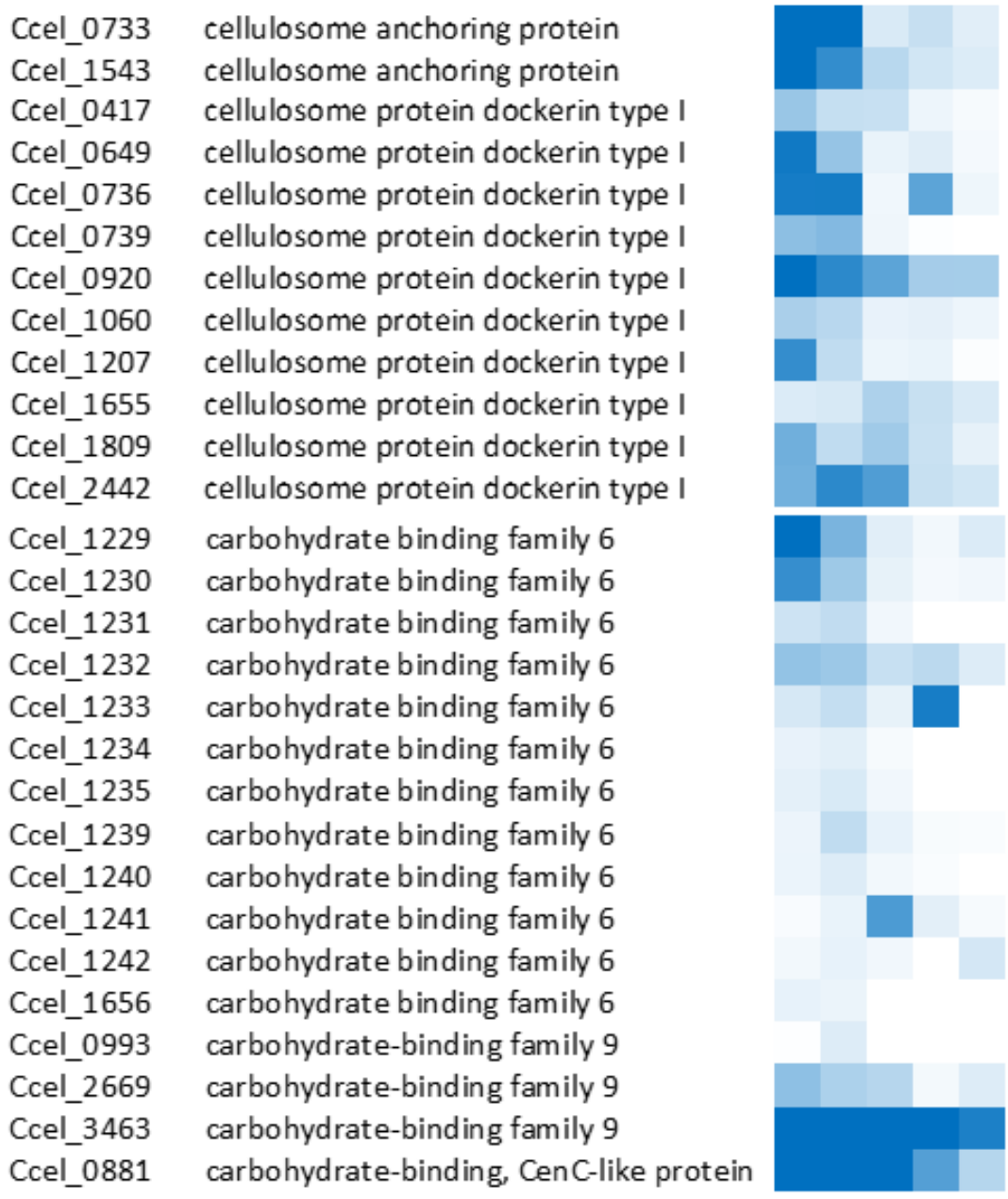


Fig. S2.2 Non-hydrolysis cellulosomal genes differentially expressed under different cellulose concentration

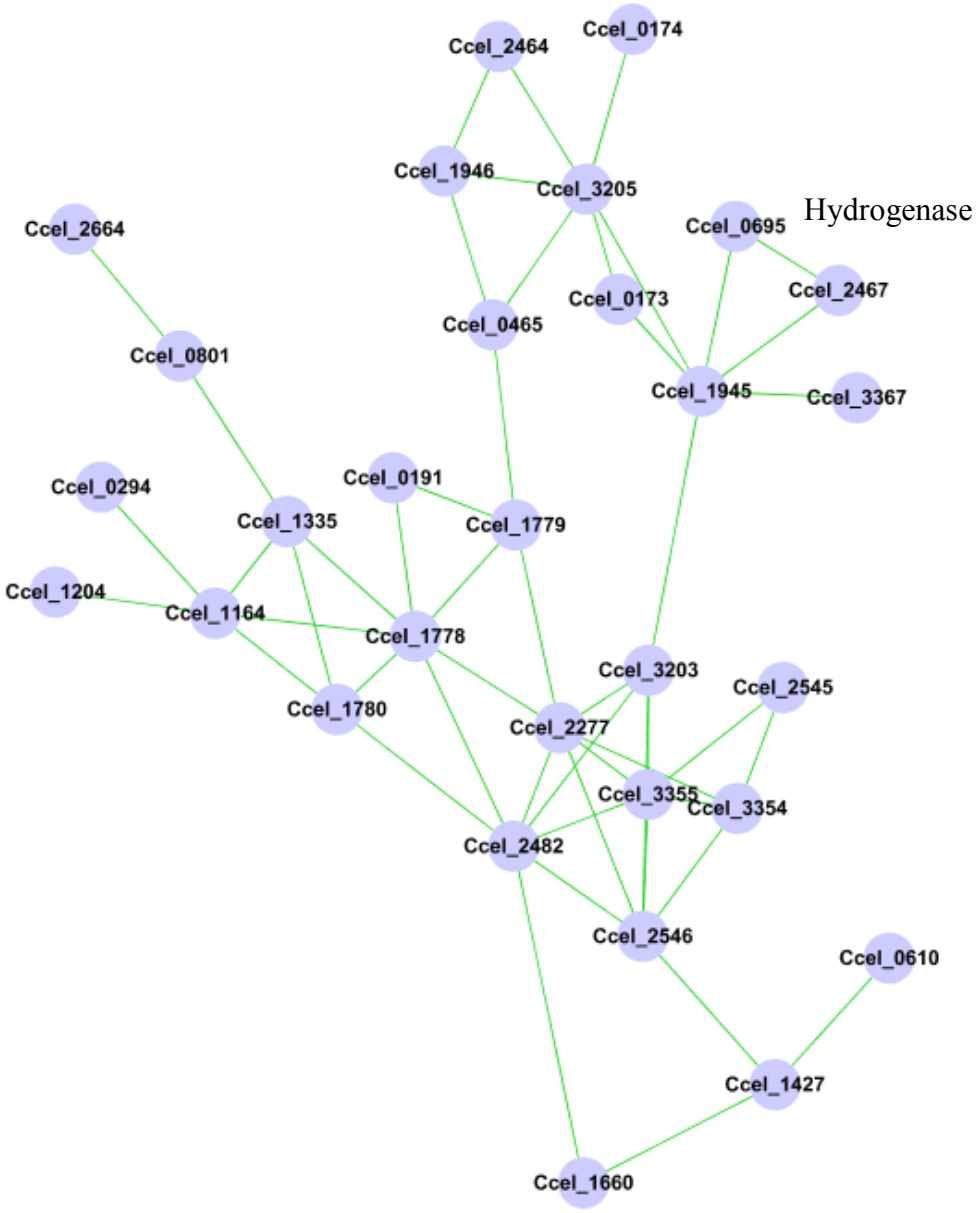


Fig. S2.3 A sub-network of *C. cellulolyticum* contains Ccel_2467 hydrogenase

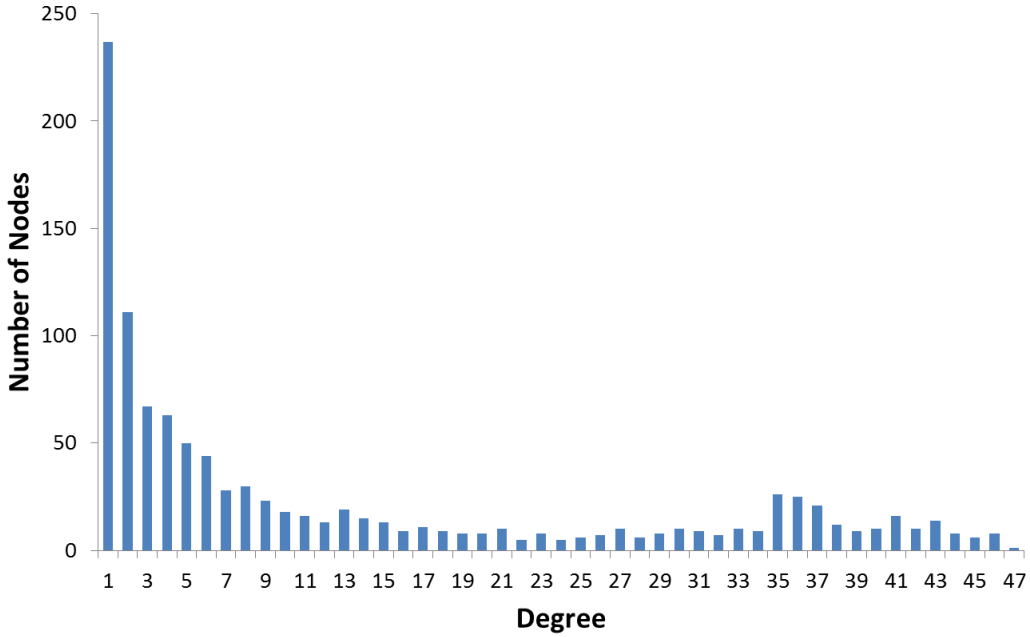


Fig. S2.4 Connectivity distribution of *C. cellulolyticum* co-expression network

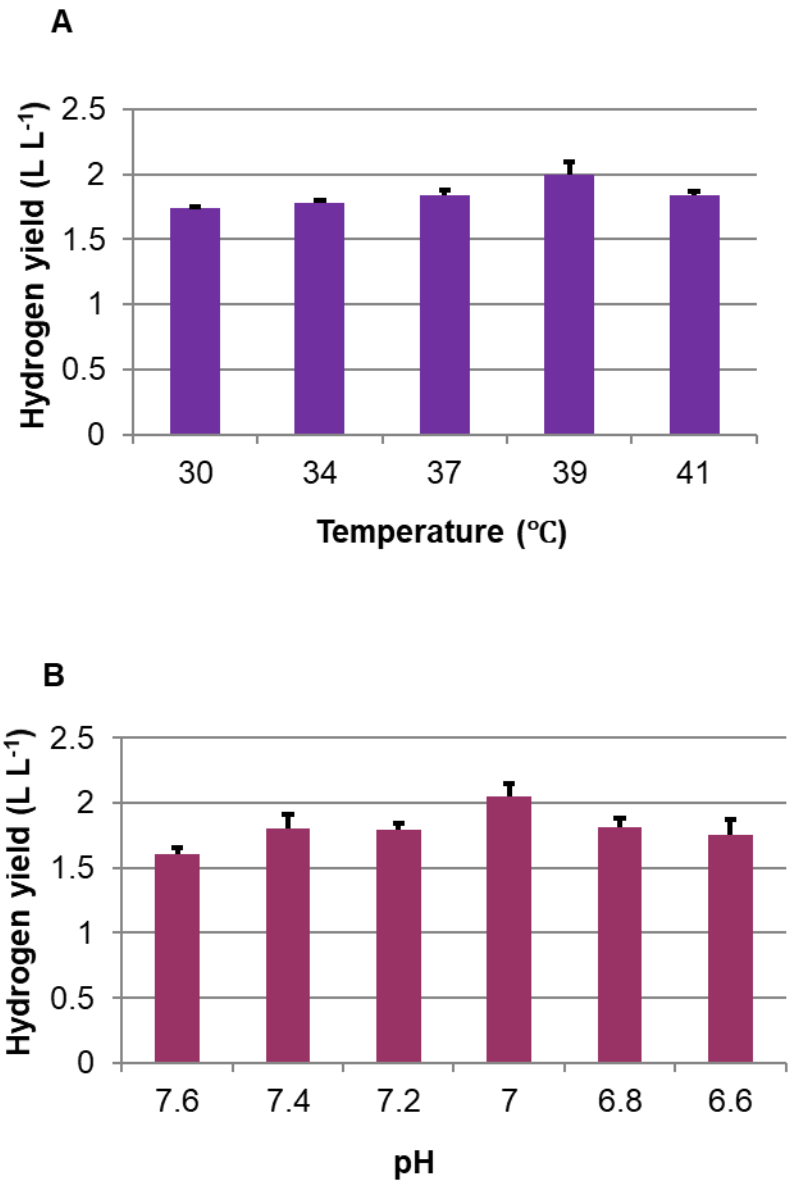


Fig. S3.1 Hydrogen yield of co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough at different temperature (A) and initial pH value (B) with 10 g/L cellulose

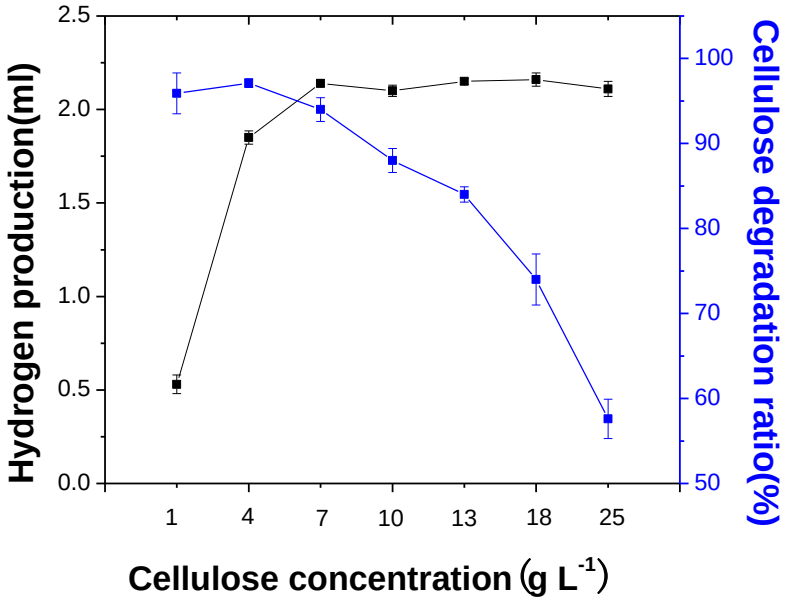


Fig. S3.2 Hydrogen production and cellulose degradation ratio of co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough with different cellulose concentration

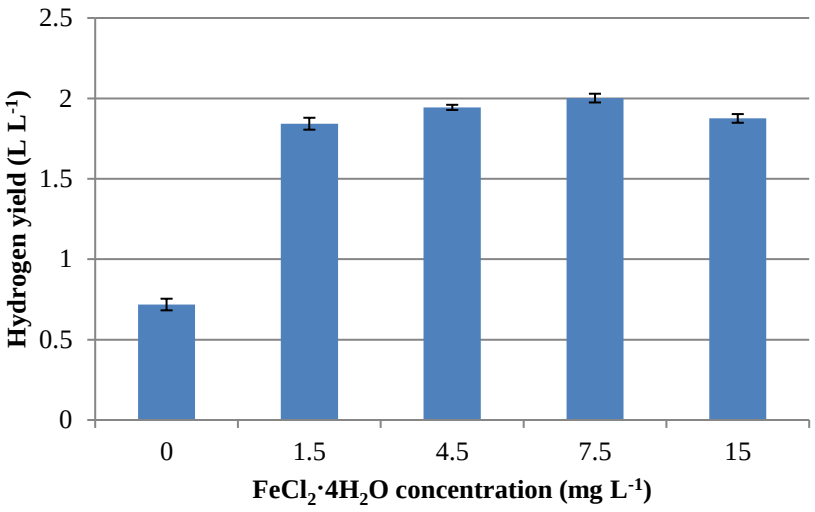


Fig. S3.3 Hydrogen production of co-culture at different Fe ion concentration

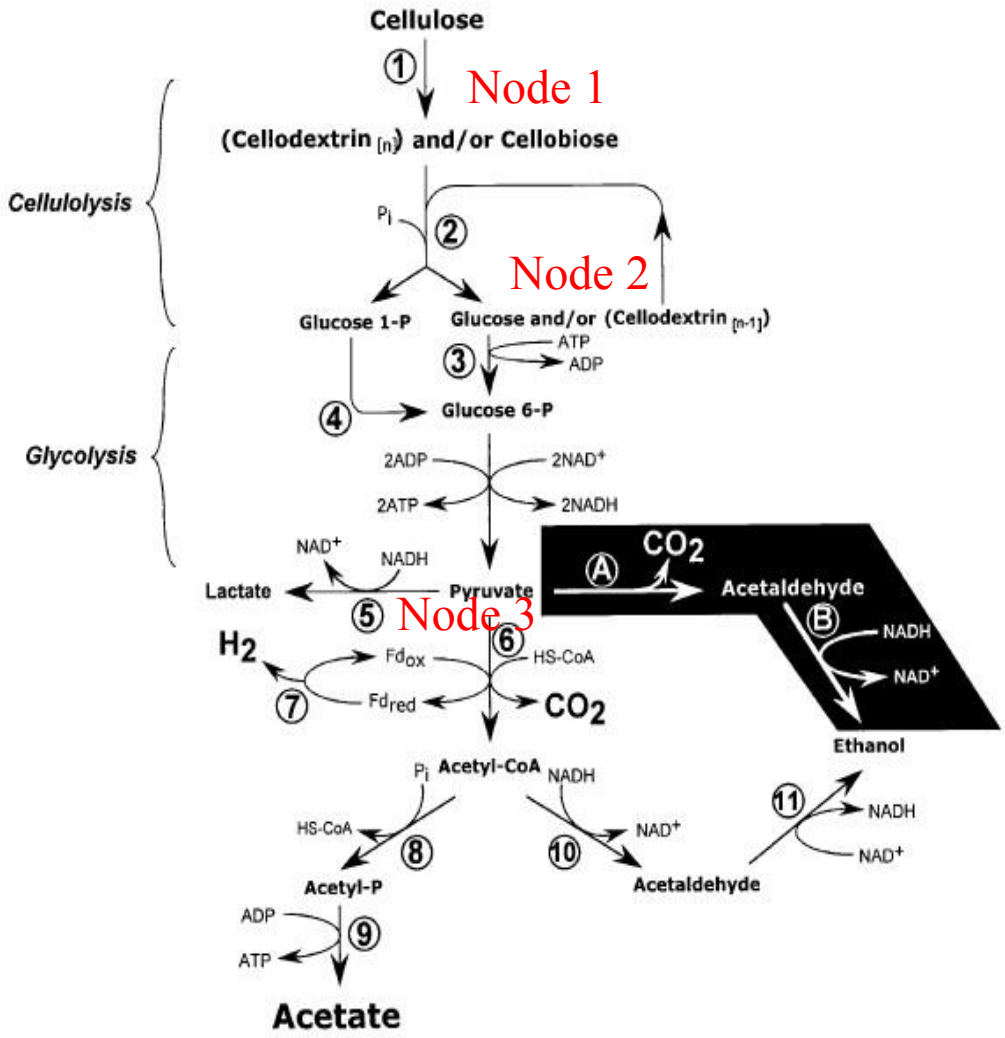


Fig. S3.4 3 metabolic nodes in cellulose metabolism of *C. cellulolyticum* (adapted from (Guedon, Desvaux et al. 2002))

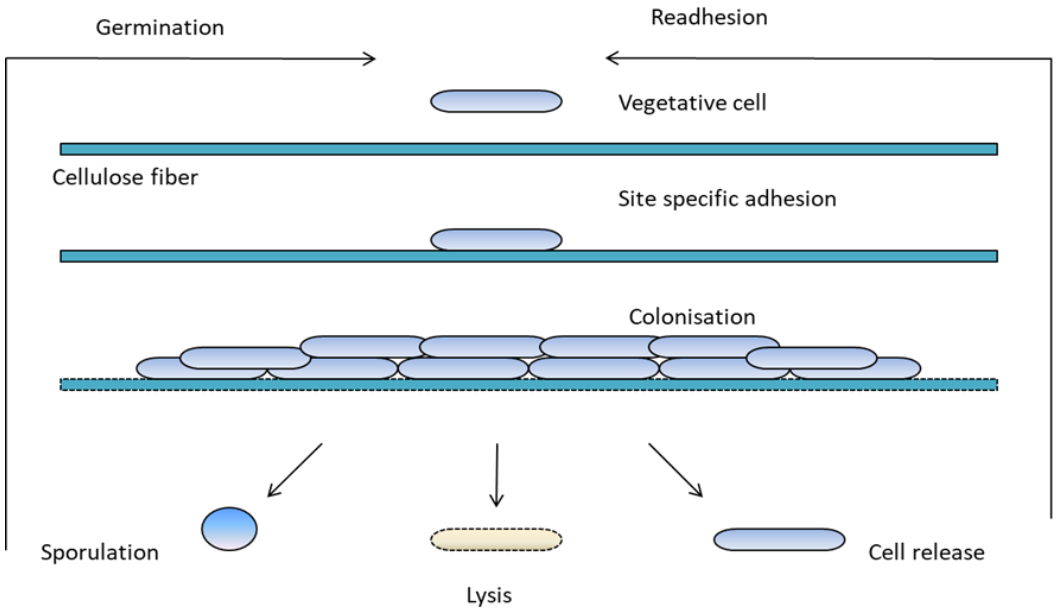


Fig. S3.5 Scheme of the model of cellulose colonization by *C. cellulolyticum* (adapted from (Desvaux 2005))

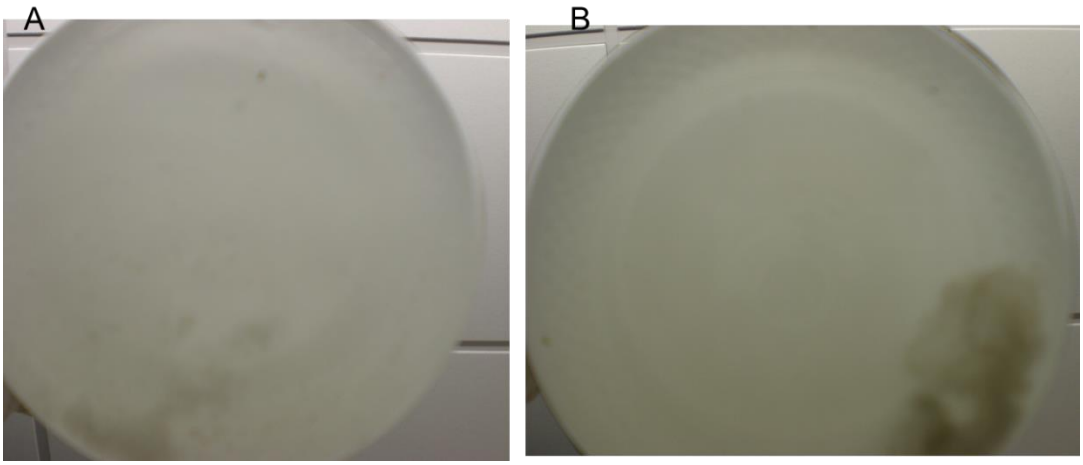


Fig. S3.6 Aggregates in co-cultured *C. cellulolyticum* and *D. vulgaris* Hildenborough (A is mono-culture, B is co-culture)

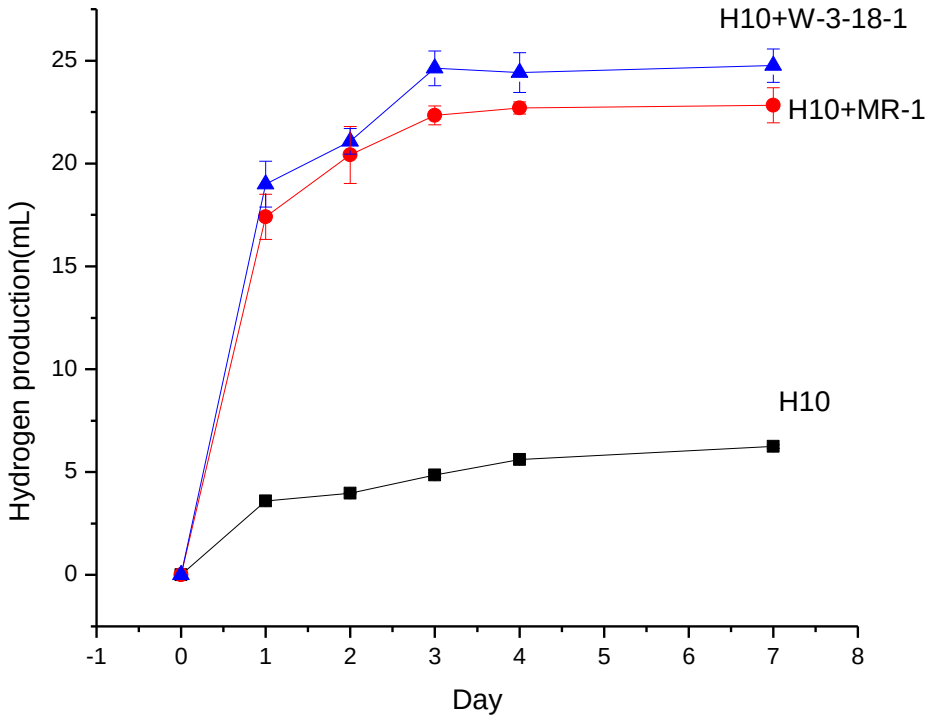


Fig. S3.7 Hydrogen production of co-cultured *C. cellulolyticum* with *Shewanella putrefaciens* W3-18-1 and *Shewanella oneidensis* MR-1 without additional electron accepter

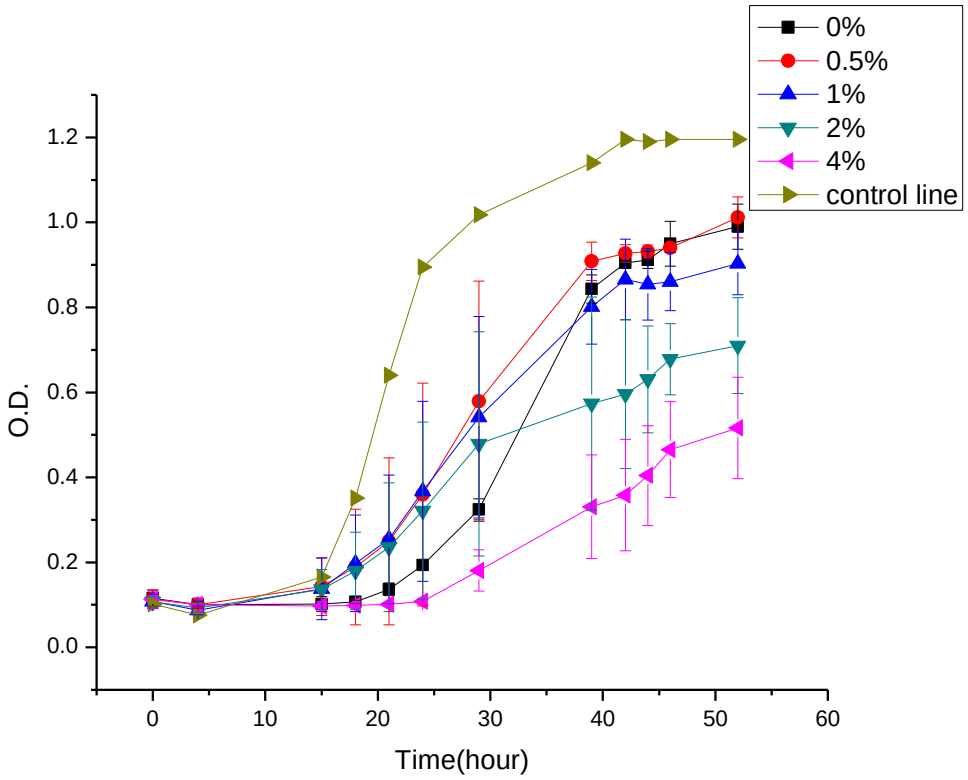


Fig.S4.1 Ethanol concentration test of *Clostridium thermocellum* LQR1 ethanol-tolerant derivative

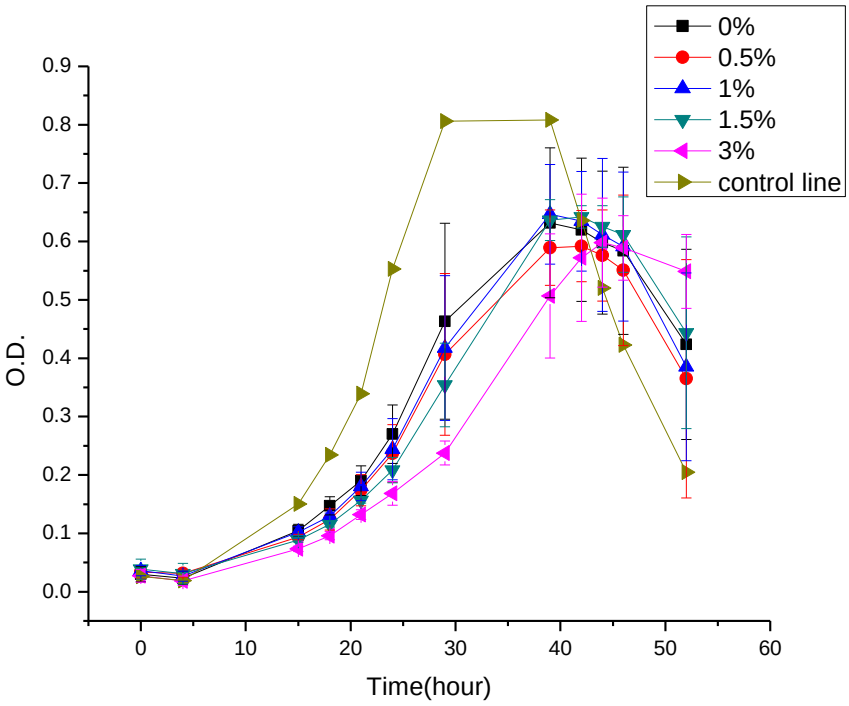


Fig S4.2 Ethanol concentration test of *Thermoanaerobacter ethanolicus* X514 ethanol-tolerant derivative

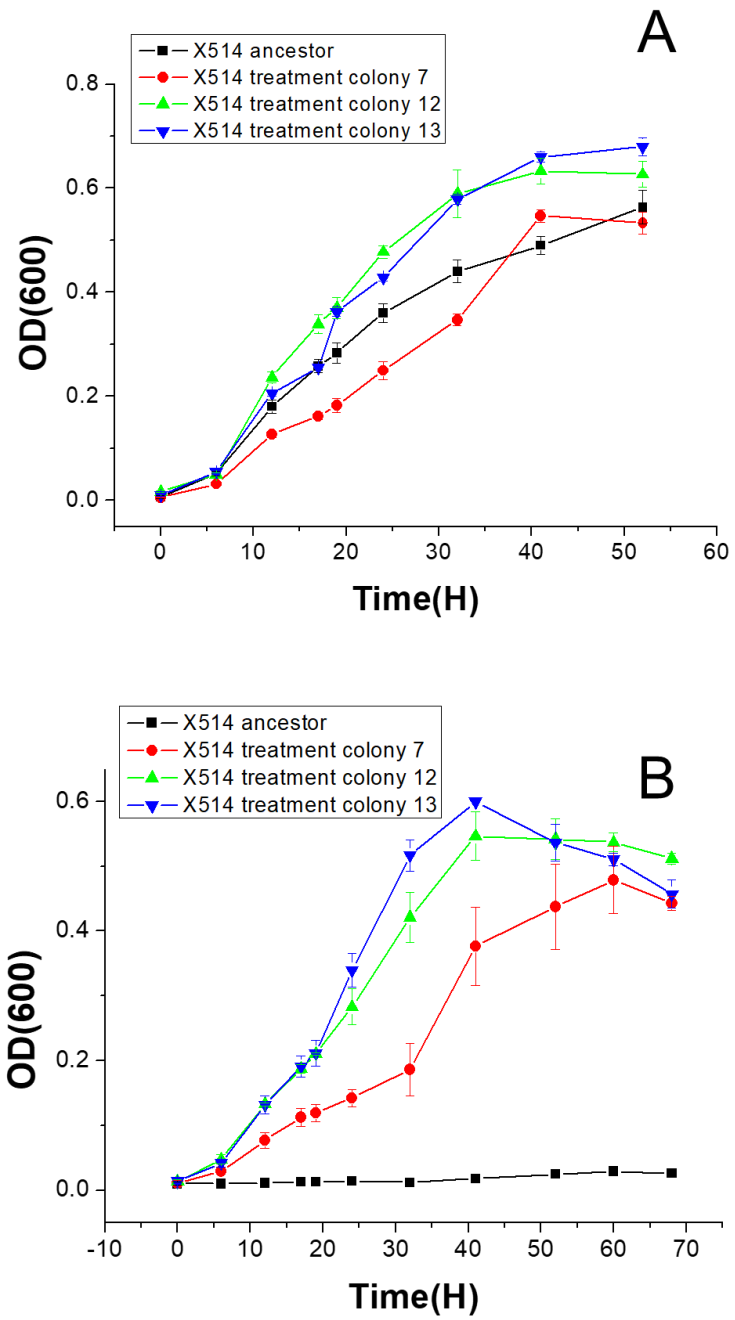


Fig S4.3 Growth curve of *Thermoanaerobacter ethanolicus* X514 parent strain and ethanol-tolerant derivative in absence of ethanol (A) and 3% ethanol (B)