Genome-Scale Characterization of Metabolic Interactions in a Clostridial Co-culture for Consolidated Bioprocessing

by

Fahimeh Salimi

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Graduate Department of Chemical Engineering and Applied Chemistry
University of Toronto

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Abstract

The co-culture of Clostridium acetobutylicum and Clostridium cellulolyticum can offer a potential CBP approach for producing commodity chemicals from cellulosic biomass. This thesis examines the nature of interactions between these two species in this co-culture. An expanded genome-scale metabolic model of C. acetobutylicum, which incorporates thermodynamic and metabolomic data, was presented. Flux variability analysis showed the presence of alternate carbon and electron sinks that allows for different carbon assimilation patterns and ranges of product formation rates. The genome-scale metabolic model of C. cellulolyticum was presented and validated against experimental data, and was able to predict the metabolism of C. cellulolyticum on cellulose and cellobiose. A genome-scale model of the clostridial co-culture metabolism was developed by integrating C. cellulolyticum and C. acetobutylicum metabolic models, and was used to analyze the integrated physiology of this co-culture; the simulation results showed that at high cellulose concentrations, the model is not able to capture the C. cellulolyticum growth arrest, suggesting that the removal of cellobiose inhibition by C. acetobutylicum is not the main factor that improves cellulose degradation in the co-culture. Experimental methods were developed to characterize the cellulolytic activity, the co-culture metabolism and the metabolic interactions in this co-culture. Comparative qPCR analyses and
characterization of the metabolism in this clostridial co-culture along with the mono-cultures revealed that significant increase in the rate of cellulose hydrolysis can be achieved using the co-culture due to the synergy between the two clostridial species. It is likely that C. acetobutylicum improves the cellulolytic activity of C. cellulolyticum in the co-culture through exchange of metabolites, such as pyruvate, enabling it to grow and metabolize cellulose under suboptimal co-culture conditions. An in vivo metabolite analysis of C. cellulolyticum suggested a limitation on the lactate transportation pathway that leads to intracellular lactate accumulation and the growth arrest.
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List of Non-Standard Abbreviations Used

CA: *Clostridium acetobutylicum*
CC: *Clostridium cellulolyticum*
CBP: Consolidated bioprocessing
CGM: Clostridial growth medium
DS: Degree of synergism
DMMM: Dynamic multispecies metabolic modeling
FBA: Flux balance analysis
FVA: Flux variability analysis
GAM: Growth-associated maintenance energy
GPR: Gene-protein-reaction
NGAM: Non-growth associated maintenance
TOC: Total organic carbon
Chapter 1 - Introduction

1.1 Cellulosic butanol, an alternative biofuel

Increasing world energy demands and environmental concerns due to fossil fuel consumption motivate considerable efforts toward the development of sustainable and renewable energy resources such as solar energy, wind energy, geothermal energy, energy from biomass and biofuel. Biofuels are attractive substitutes for fossil fuels, and can help significant reduction in greenhouse gas emissions. Liquid biofuels such as biodiesel, bioethanol and biobutanol as well as gaseous biofuels, like hydrogen and methane, can be produced from biomass. Compared to gaseous energy carriers, liquid energy carriers present advantages due to their storage, transportation and usage considerations (Brown and Brown, 2013, Dürre, 2007, Lee et al., 2008b).

Biodiesel is derived from vegetable oils, animal fats or waste cooking oils, and can be used in pure form or as a blend in diesel engines. These oils are converted to fatty acid methyl esters (FAME), via a transesterification with alcohols like methanol and ethanol, resulting in glycerol as a process by-product (Meng et al., 2008). Bioethanol is currently the main liquid biofuel, and can be produced from sugar/starch containing biomass, such as sugar cane and corn starch, or lignocellulosic biomass. Bioethanol is mainly being used as a fuel additive, but hydrated ethanol, an azeotrope mixture of 93% ethanol and 7% water, is also used in ethanol-powered vehicles (Carere et al., 2008). Compared to gasoline, ethanol is less toxic to humans, and has a higher octane number, higher heat of vaporization, and lower flame temperature, and thus burns with greater efficiency and produces less smog (Demain et al., 2005).

Currently most of the worldwide bioethanol production is derived from fermentation of either starch (corn, wheat, cassava) or sugar (sugarcane, sugar beets), and *Saccharomyces cerevisiae* is the most commonly used microorganism in large scale bioethanol production plants. However, the main concerns in these approaches are the high cost of feedstock as well as the competition with food resources (Carere et al., 2008, Karakashev et al., 2007). Another alternative approach is the production of ethanol from cellulosic feedstock (Demain et al., 2005), and commercial-scale cellulosic ethanol plants are operating and under construction (Brown and Brown, 2013). A variety of approaches are currently under commercial development for producing cellulosic
biofuels, including: (1) catalytic pyrolysis and hydrotreating to hydrocarbons; (2) gasification and Fischer-Tropsch synthesis to hydrocarbons; (3) gasification and methanol-to-gasoline synthesis; (4) dilute acid hydrolysis, fermentation to acetic acid, and chemical synthesis to ethanol; (5) enzymatic hydrolysis to ethanol; and (6) consolidated bioprocessing of biomass to ethanol (Brown and Brown, 2013).

Biobutanol has recently been introduced as a direct substitute of gasoline. Application of biobutanol as a biofuel instead of bioethanol brings several advantages: 1) butanol can be used in pure form or can be blended with gasoline in any concentration, while ethanol can be blended only up to 85%; 2) utilization of butanol either as a biofuel or as a fuel additive does not require significant modifications in existing car engines; 3) butanol is less volatile, so it is a safer fuel; 4) it is not hygroscopic and is less corrosive, so it is possible to use current existing facilities for storage, transportation and distribution purposes; 5) butanol has a higher energy content; thus the mileage to gasoline ratio increases, and 6) some butanol derivatives such as isobutanol, 3-methyl-1-butanol and 2-methyl-1-butanol, which have higher octane number, are suitable fuel additives (Dürre, 2007, Lee et al., 2008c).

Until 1950, about two thirds of the world’s butanol supply was produced using the fermentation of Clostridium acetobutylicum, and the Acetone-Butanol-Ethanol (ABE) process was the second major large-scale biological process after ethanol. However, due to the increasing substrate costs and low crude oil prices, petrochemical production became more feasible (Dürre, 2007). In the ABE process, C. acetobutylicum converts carbohydrate substrates (e.g., sugars and starch) to a mixture of acetone, butanol and ethanol in the ratio of 3:6:1, and the total solvent production in the range of 16 to 24 g/L, with solvent yield of 29-34% (18-25% for butanol only) (Soucaille, 2010). High substrate cost, low product concentration, because of the product toxicity, and high product recovery costs are the main factors that influence the economical feasibility of this process (Karacashev et al., 2007, Green, 2011). With the revival of interest in solventogenic clostridia to produce biofuels, novel processes for the production of petrol, jet and diesel through the integration of an extractive ABE fermentation and chemical catalysis, and conversion of alcohols and acetone into ketones by a palladium-catalysed alkylation have been developed (Anbarasan et al., 2012). Moreover, metabolic engineering approaches have been applied to develop transformant strains of C. acetobutylicum to convert acetone into isopropanol,
and to produce a mixture of isopropanol, butanol and ethanol (IBE) as a biofuel (Jang et al., 2013, Dusseaux et al., 2013, Lee et al., 2012).

Low product titer due to solvent toxicity is a significant issue in the ABE process, which limits the carbohydrate utilization and solvent productivity, while the total solvent concentration of 20 g/L inhibits the metabolism of clostridia (Jones and Woods, 1986, Green, 2011). Butanol is more toxic than the other solvents since it interacts with the phospholipid components of the cell membrane, and increases the membrane fluidity; therefore, all membrane associated functions, such as transport processes and membrane-bound ATPase activity, are interrupted (Lee et al., 2008c). The identification of solvent tolerance genes in *C. acetobutylicum* has been investigated to overcome this barrier (Borden and Papoutsakis, 2007, Tomas et al., 2003).

Four major solvent producing species include *C. acetobutylicum* ATCC 824, *Clostridium saccharobutylicum*, *Clostridium beijerincki* and *Clostridium saccharoperbutylicum* (Keis et al., 2001, Du et al., 2013, Kosaka et al., 2007). Although *C. acetobutylicum* ATCC 824 is the most studied and manipulated strain, different strains from these four main species groups have been developed and used based on their performance on different feedstocks (Green, 2011). Industrial mutant strains of *C. beijerincki*, strain BA101 with butanol titer of 19.6 g/L, and *C. acetobutylicum* mutant strain EA2018, with butanol titer of 14.4 g/L, have been developed through chemical mutagenesis (Annous and Blaschek, 1991, Green, 2011). Metabolic engineering tools have also been employed toward improving butanol formation in *C. acetobutylicum*, to reach butanol titer of about 17 g/L (Harris et al., 2000, Harris et al., 2001, Jiang et al., 2009), and to introduce butanol formation pathway in non-natural host organisms (Connor and Liao, 2009, Alper and Stephanopoulos, 2009, Knoshaug and Zhang, 2009), such as engineered *Escherichia coli* strain to produce butanol with high titer of 30 g/L (Shen et al., 2011). In a recent study, a two stage immobilized column reactor system, with immobilized *C. acetobutylicum* DSM 792, integrated with *in situ* solvent extraction has been investigated for continuous ABE production from a sugar mixture (glucose, mannose, galactose, arabinose, and xylose) comparable to lignocellulose hydrolysates (Bankar et al., 2013), where high sugar mixture conversion of 93.7%, solvent productivity of 10.85 g L⁻¹ h⁻¹ and the solvent yield of 0.38 g g⁻¹ were achieved using this system.
Although high oil prices make the biological butanol production process feasible, or even superior to petrochemical processes (Dürre, 2007), this process can be further improved. Butanol has been produced by the fermentation of various substrates including molasses, whey permeate, and corn (Jones and Woods, 1986, Lee et al., 2008c). Currently, sugars (e.g., molasses, sugar cane and sugar beet) as well as corn starch are the substrates being used in new fermentation plants (Dürre, 2007), while corn starch comprises about 79% of the solvent production cost (Green, 2011). Since these substrates compete with food, and the cost of substrate has a significant effect on the process economic feasibility, the utilization of cellulosic biomass, which is an abundant low-cost feedstock and does not compete with food, as well as agricultural residues and municipal solid waste have been suggested (Ezeji et al., 2007a, Mes-Hartree and Saddler, 1982, Qureshi et al., 2008d, Ezeji et al., 2007b). In a two-stage process, pre-treated cellulosic biomass is hydrolyzed, and then in the ABE process, the hydolysates will be fermented by *C. acetobutylicum* to butanol (Lee et al., 1985).

Butanol production from various cellulosic feedstocks has also been investigated. Fermentation of enzyme treated corn fiber hydrolysate (ETCFH) and sulfuric acid treated corn fiber hydrolysate (SACFH) by *C. beijerinckii* BA101, after removing some of the fermentation inhibitors using resins, has resulted in the production of about 9 g/L ABE with a yield ranging from 0.35 to 0.39 g g\(^{-1}\) (Qureshi et al., 2008a). In a recent study on the ABE production from wheat straw hydrolysate, it has been shown that addition of furfural and hydroxymethyl furfural (sugar degradation products) to *C. beijerinckii* P260 culture improves the ABE productivity, producing 25.0 g/L ABE from wheat straw hydrolysate with a yield of 0.42 g g\(^{-1}\) (Qureshi et al., 2012, Qureshi et al., 2007). Further improvements in the butanol productivity have been achieved through the removal of fermentation inhibitors from enzymatically hydrolyzed cellulosic biomass (Qureshi et al., 2008c, Wang and Chen, 2011), simultaneous hydrolysis and fermentation (Qureshi et al., 2008d, Qureshi et al., 2008b), and *in situ* solvent recovery (Ezeji et al., 2004b, Ezeji et al., 2004a, Qureshi, 2010).

However, the cost of enzymatic hydrolysis is still a significant factor influencing the economic feasibility of cellulosic butanol (Green, 2011, Du and Yang, 2012). New strains of *C. acetobutylicum* capable of hydrolyzing hemicellulose and converting it to solvents have also been isolated recently (Berezina et al., 2008). Although *C. acetobutylicum* genome contains a large cellulosomal gene cluster, these genes are not expressed sufficiently, and *C. acetobutylicum*
is not able to grow on cellulose (Nolling et al., 2001, Sabathe et al., 2002). Recently, the expression of \textit{Clostridium cellulolyticum} cellulase-encoding genes in \textit{C. acetobutyllicum} has been investigated, but it has been suggested that the lack of chaperons involved in the cellulase secretion leads to the cytoplasmic cellulase accumulation and the growth inhibition in \textit{C. acetobutyllicum} (Mingardon et al., 2011).

\subsection*{1.2 Rationale}

An alternative method for the production of biobutanol from cellulosic biomass in a consolidated bioprocessing approach is the use of mesophilic clostridial co-culture. \textit{C. acetobutyllicum} shows an effective capability to ferment hemicelluloses and cellulose derived sugars such as cellobiose, mannose, arabinose, xylose, glucose, and galactose to acetone, butanol, and ethanol (Yu et al., 1984, Fond et al., 1986, Ali et al., 2004). Thus, the co-culture of this bacterial species with a mesophilic cellulose degrading bacterium can be an efficient approach. \textit{C. cellulolyticum} is a cellulolytic, mesophilic bacterium which is able to solubilize crystalline cellulose in pretreated hardwood (Demain et al., 2005).

The co-culture of \textit{C. cellulolyticum} with \textit{C. acetobutyllicum} has been studied previously, and it has been shown that cellulolytic activity is the limiting factor in the co-culture fermentation since most of the cellulase activity products are consumed by \textit{C. acetobutyllicum}. The fermentation products are mainly butyric acid along with butanol, acetic acid and ethanol, and the lack of glucose, which is required for solvent production due to low cellulolytic activity, was hypothesized to be the reason for acid accumulation (Jones and Woods, 1986, Petitdemange et al., 1983). Furthermore, three times more cellulosic material was degraded in the co-culture compared to the mono-culture of \textit{C. cellulolyticum}, suggesting the presence of a synergism between these two species. Hence, the analysis of this effect can be valuable for optimizing the rate of cellulosic material degradation.

Genome-scale metabolic models of microorganisms from different domains of life have been developed and been applied for analyses of metabolism in pure cultures. However, systems biology of microbial co-cultures will extend our knowledge on pure culture physiology to microbial co-cultures, where metabolic interactions along with inter-species transport of metabolites are present. System-level understanding of the \textit{C. cellulolyticum} and \textit{C.}
acetobutylicum co-culture metabolism, which can be applied for biobutanol production from cellulosic biomass, facilitates the analyses and design of strategies for process and metabolic optimization, thus improving the biobutanol production rate. Therefore, the development of computational methods as well as experimental methods to investigate the interactions between microorganisms in this co-culture, based on the community genome sequences and physiology, is beneficial for the ultimate engineering of the co-culture. Consequently the focus of this thesis is the development of such methods, as outlined in the following objectives.

1.3 Research objectives

It is hypothesized that the genome-scale metabolic analysis of the *C. cellulolyticum* and *C. acetobutylicum* co-culture can assist in designing strategies for improving the yield and productivity of the biobutanol production rate, which is the desired product in this consolidated bioprocessing approach. Thus, the eventual goal of this study is to develop methods for the analysis of metabolism in this co-culture, and to investigate the inter-species metabolic interactions for enhancing the metabolic rate and biofuel synthesis. The research method involves both computational methods for the development of genome-scale models of metabolism, and experimental approaches for the examination of microbial physiology. The following objectives were investigated:

1. To develop the genome-scale metabolic model of *C. acetobutylicum* ATCC 824 that includes thermodynamic and metabolomic constraints, and to achieve thermodynamically feasible phenotypes.

This objective is fulfilled through developing an updated genome-scale metabolic model of *C. acetobutylicum*, acquiring intracellular metabolite concentrations in *C. acetobutylicum* cultures, and incorporating these metabolomic and thermodynamic data into this metabolic model (chapter 3).

2. To develop the genome-scale metabolic model of *C. cellulolyticum*.

3. To apply the developed metabolic models of the pure cultures to develop the genome-scale model of metabolic interactions in the co-culture.

These two objectives are fulfilled through developing the genome-scale metabolic model of *C. cellulolyticum*, and combining this model with aforementioned *C. acetobutylicum* model to
obtain the co-culture metabolic model (chapter 4). This metabolic model was used to analyze the integrated physiology of the co-culture.

4. To characterize the co-culture metabolism by developing molecular biology methods. We investigated metabolic interactions in this co-culture by developing a comparative qPCR method, and dynamically characterized the co-culture physiology and microbial composition using this qPCR method (chapter 5).

5. To examine the growth arrest event in C. cellulolyticum. We developed a method for the extraction and quantitative analysis of intracellular metabolites in C. cellulolyticum cultures under high and low cellulose concentrations; particularly to test the hypothesis that there is a link between pyruvate accumulation and the growth arrest event at high cellulose concentrations (chapter 6).

1.4 Thesis outline

Chapter 2 provides an overview on the research in this field.

Chapter 3 addresses the first objective, where an expanded genome-scale metabolic model of C. acetobutylicum is presented. This study has been published in the conference proceedings below:


Affiliations: 1 - Department of Chemical Engineering and Applied Chemistry, University of Toronto, 2 - Department of Biological Sciences, University of Alberta

Contributions: FS designed and conducted the in silico and in vitro experiments, analyzed the data, and drafted the manuscript. RM\(^2\) and DW\(^2\) carried out the NMR analyses. RM conceived of the experiments and helped to draft the manuscript.
Chapter 4 presents the genome-scale models of *C. cellulolyticum* metabolism as well as the clostridial co-culture metabolism, which point to the second and third objectives. This work has also been published as a research paper:


**Affiliations:** 1 - Department of Chemical Engineering and Applied Chemistry, University of Toronto, 2 - Present Address: Novo Nordisk Foundation Center for Biosustainability, Denmark.

**Contributions:** FS conceived of the study, reconstructed the metabolic network, conducted the modeling, and drafted the manuscript. KZ carried out the DMM simulations. RM conceived of the study and helped to draft the manuscript.

In chapter 5, we developed and presented methods for the co-culture experiments to characterize the cellulolytic activity and the co-culture metabolism, and to investigate the fourth objective. This study has been published as a research paper:


**Affiliations:** Department of Chemical Engineering and Applied Chemistry, University of Toronto

**Contributions:** FS conceived of the study, designed and conducted the experiments, and drafted the manuscript. RM conceived of the study and helped to draft the manuscript.

Chapter 6 describes the efforts to further investigate and understand the mechanism behind the growth arrest event in *C. cellulolyticum*. To explore the fifth objective, we designed and conducted an *in vivo* metabolite analysis. This study is being prepared for submission as a short research note. Finally, chapter 7 summarizes the results and conclusions of this thesis, and offers recommendations for future studies in this field.
Chapter 2 - Background and Literature Review

2.1 Consolidated bioprocessing of cellulosic biomass

Cellulose is the most abundant component of plant biomass, which is almost exclusively found in plant cell wall and composes 35 to 50% of plant dry weight. A matrix of other structural biopolymers, mainly hemicelluloses and lignin, which form 20 to 35% and 5 to 30% of plant dry weight accordingly, embeds cellulose fibres (Lynd et al., 2002). Cellulosic biomass is a plentiful, inexpensive and renewable feedstock for the production of biofuels (Lynd et al., 2008). Cellulosic feedstock is an abundant resource with relatively low cost, but the main technological factor, which hampers the extensive utilization of this supply, is the lack of low-cost technology for hydrolysis of the highly ordered, insoluble structure of cellulose (Lynd et al., 2002). The products of this hydrolysis are readily soluble sugars. Most of the research on cellulose hydrolysis has been focused on the genetics, biochemistry and process development using fungi, mostly *Trichoderma reesei*, for the conversion of cellulose to sugars (Demain et al., 2005).

Efficient pre-treatment approaches to release the polysaccharides, cellulose and hemicellulose, from lignocellulosic structure, and make them available for hydrolysis and then fermentation are also key factors in the process of lignocellulosic biomass. Lignocellulosic material is hard to hydrolyze since it is associated with hemicelluloses, and is enclosed by a lignin seal, hence, has a highly ordered and firmly packed structure. Pre-treatment increases the surface area of cellulose, eliminates the lignin seal, solubilises hemicelluloses, and disrupts the crystallinity of cellulose (Demain et al., 2005). Various pre-treatment methods include steam pre-treatment with or without acid catalyst, wet oxidation and thermal hydrolysis, which are often at high temperature and pressure. No microorganisms are currently known to efficiently use liberated lignin, which is an aromatic polymer (Karakashev et al., 2007).

Four biological steps are involved in the conversion of cellulosic biomass into biofuel via enzymatic hydrolysis: a) production of saccharolytic enzymes, cellulase and hemicellulase; b) the hydrolysis of the produced polysaccharides present in pre-treated biomass; c) fermentation of hexoses, and d) fermentation of pentose sugars (D-xylose, L-arabinose) present in the hydrolyzate to desired products (Lynd et al., 2008). Figure 2-1 shows the development of biomass processing approaches. Different process configurations can be categorized based on the
degree of integration of these four steps. Separate hydrolysis and fermentation (SHF), involves four separate process stages using four different biocatalysts. Simultaneous saccharification and fermentation (SSF) integrates hydrolysis and fermentation of cellulose hydrolyzates into one process step, while cellulase production and fermentation of hemicellulase hydrolysis products take place in two additional stages. Simultaneous saccharification and co-fermentation (SSCF) includes two process stages; production of saccharolytic enzymes takes place in one step, while hydrolysis of pre-treated biomass along with the co-fermentation of both cellulose and hemicellulose hydrolysis products occur in another step (Lynd et al., 2002).

![Figure 2-1. Various biomass-processing schemes: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP) (Lynd et al., 2002).](image)

In consolidated bioprocessing (CBP), all of above mentioned steps occur in one bioreactor using a single microorganism or a microbial consortium which is able to ferment pretreated biomass without adding saccharolytic enzymes (Lynd et al., 2002). Consolidated bioprocessing is an efficient approach to decrease the product cost, as a 75% decrease in the cost of biological process and a 50% reduction of overall process cost is achievable comparing to a hypothetical advanced simultaneous saccharification and co-fermentation process (SSCF) for ethanol production (Lynd et al., 2008). Furthermore, use of complex cellulase systems and enzyme-
Microbe synergy may improve biomass solubilisation using CBP (Olson et al., 2012, Lu et al., 2006). Presently, Mascoma is proposing to construct a commercial cellulosic ethanol plant using CBP approach to convert hardwood pulpwood feedstock into ethanol (Brown and Brown, 2013).

Microorganisms with both rapid conversion of cellulose and high product yield, productivities and titers required for CBP have not been identified in nature and need to be developed (Lynd et al., 2008, Olson et al., 2012, Hasunuma et al., 2013). To realize this aim, two strategies can be applied: the native strategy improves the product formation capabilities, such as yield and titer in natural cellulolytic microorganisms, while the recombinant strategy involves engineering non-cellulolytic organisms with high product yields so that they will express heterologous cellulase and be able to utilize cellulose (Lynd et al., 2002).

Developing genetic modification tools, and constructing genetically modified strains that produce a desired fuel with high yield, titer and robustness under industrial conditions are the main challenges of the native strategy (Olson et al., 2012). The native strategy has been applied to *Thermoanaerobacterium saccharolyticum*, a free-enzyme bacterium that ferments xylan (but not cellulose) and biomass-derived sugars; by eliminating the acetate and lactate formation pathways, the engineered strain has produced 37 g/L ethanol, with high yield of 0.46 grams of ethanol per gram of substrate (Shaw et al., 2008). Genetic tools also have been developed for other free-enzyme bacterial systems, such as *Clostridium phytofermentans* (Tolonen et al., 2009), *Clostridium japonicus* (Gardner and Keating, 2010), *Thermoanaerobacter ethanolicus* (Peng et al., 2006), and *Geobacillus thermoglucosidasius* (Cripps et al., 2009). Recently, anaerobic cellulolytic bacteria capable of degrading untreated lignocellulosic biomass, such as *Anaerocellum thermophilum* and *Caldicellulosiruptor* species, have been screened and studied as potential CBP organisms (Yang et al., 2009, Svetlitchnyi et al., 2013).

Among cellulosome forming bacteria, genetic manipulation systems have been developed for *C. cellulolyticum* and for *C. thermocellum* (Li et al., 2012, Lynd et al., 2005, Tripathi et al., 2010, Argyros et al., 2011, Higashide et al., 2011, Cui et al., 2013). An acid-deficient mutant strain of *C. thermocellum* has been created, by deleting lactate dehydrogenase (Ldh) and phosphotransacetylase (Pta) pathways (Argyros et al., 2011), to improve ethanol formation by redirecting intracellular carbon and electron flows. Subsequently, co-culturing the two Acid-deficient mutant strains of *C. thermocellum* and *T. saccharolyticum* (Shaw et al., 2008) has
resulted in the fermentation of 92 g/L cellulose and the production of 38 g/L ethanol with undetectable acetic and lactic acids (Argyros et al., 2011). This study demonstrates the potential of an engineered co-culture as a CBP solution for cellulosic ethanol. However, in contrast to *S. cerevisiae*, the main disadvantage of thermophilic clostridia is the low ethanol tolerance (Karakashev et al., 2007). Although, ethanol-adapted strains of *C. thermocellum* that can tolerate and grow in the presence of 50 g/L added ethanol have been evolved by serial transfer, where biochemical assays have identified the mutation of the coenzyme specificity of acetaldehyde-CoA/alcohol dehydrogenase gene, from NADH-dependent activity to NADPH-dependent activity, as the reason behind the improved ethanol tolerance (Brown et al., 2011, Williams et al., 2007).

Recently, a targeted gene inactivation system for *C. cellulolyticum* has been developed and applied to disrupt the paralogous lactate dehydrogenase and malate dehydrogenase genes, resulting in a significant increase in ethanol formation from cellulose (Li et al., 2012). Moreover, gene transfer systems for *C. cellulolyticum* have also been investigated (Jennert et al., 2000, Tardif et al., 2001), and applied for the production of ethanol (Guedon et al., 2002) and isobutanol (Higashide et al., 2011) from cellulose. Heterologous expression of pyruvate decarboxylase and alcohol dehydrogenase genes from *Zymomonas mobilis* has led to 53% and 150% increase in ethanol formation and cellulose consumption in the *C. cellulolyticum* batch culture, respectively (Guedon et al., 2002). Furthermore, the feasibility of isobutanol production from cellulose in a CBP has been demonstrated through metabolic engineering of *C. cellulolyticum* to divert its 2-keto acid intermediates toward alcohol formation, and the synthesis of 660 mg/L isobutanol from cellulose. However, the lack of an inducible gene expression system has been deemed as a significant barrier in expressing new pathways in *C. cellulolyticum* (Higashide et al., 2011). These studies suggest metabolic engineering approaches can significantly improve the metabolism of *C. cellulolyticum* as a potential CBP organism. Table 2-1 provides a summary of the recent studies on cellulase producing microorganisms for developing native CBP strategies.
Table 2-1. Recent studies on cellulase producing microorganisms for developing native CBP strategies (adapted from (Olson et al., 2012)).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Relevant genotype</th>
<th>Substrate</th>
<th>Substrate conc. (g/L)</th>
<th>Conversion (%)</th>
<th>Product</th>
<th>Titer (g/L)</th>
<th>Metabolic yield, (%)</th>
<th>Rate (gL⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. thermocellum M1570 and T.saccharolyticum ALK2 co-culture</td>
<td>M1570 ∆ldh ∆pta ALK2 ∆ldh ∆pta ∆ack</td>
<td>Avicel</td>
<td>92.2</td>
<td>90</td>
<td>Ethanol</td>
<td>38.1</td>
<td>82</td>
<td>0.26</td>
</tr>
<tr>
<td>C. cellulolyticum H10</td>
<td>Wildtype</td>
<td>Cellulose MN301</td>
<td>50</td>
<td>26</td>
<td>Ethanol</td>
<td>0.51</td>
<td>7</td>
<td>1.1E-03</td>
</tr>
<tr>
<td>C. phytofermentans ATCC 700394</td>
<td>Wildtype</td>
<td>AFEX-corn stover</td>
<td>8</td>
<td>81</td>
<td>Ethanol</td>
<td>2.8</td>
<td>85</td>
<td>1.2E-02</td>
</tr>
<tr>
<td>C. thermocellum DSM 1313</td>
<td>Wildtype</td>
<td>Avicel</td>
<td>19.5</td>
<td>90</td>
<td>Ethanol</td>
<td>1.3</td>
<td>15</td>
<td>1.8E-02</td>
</tr>
<tr>
<td>C. cellulolyticum CC-pMG8</td>
<td>Wildtype</td>
<td>Cellulose MN301</td>
<td>50</td>
<td>64</td>
<td>Ethanol</td>
<td>0.83</td>
<td>5</td>
<td>1.8E-03</td>
</tr>
<tr>
<td>Geobacillus sp.R7</td>
<td>Wildtype</td>
<td>Prairie cord grass</td>
<td>10.8</td>
<td>ND</td>
<td>Ethanol</td>
<td>0.035</td>
<td>ND</td>
<td>2.1E-04</td>
</tr>
<tr>
<td>T. hirsuta</td>
<td>Wildtype</td>
<td>Rice straw</td>
<td>10.3</td>
<td>ND</td>
<td>Ethanol</td>
<td>3</td>
<td>ND</td>
<td>3.1E-02</td>
</tr>
<tr>
<td>C. japonicus Ueda 107</td>
<td>Wildtype</td>
<td>Avicel</td>
<td>10</td>
<td>ND</td>
<td>Ethanol</td>
<td>0.0002</td>
<td>ND</td>
<td>4.2E-06</td>
</tr>
<tr>
<td>C. phytofermentans ATCC 700394</td>
<td>Wildtype</td>
<td>Filter paper</td>
<td>10</td>
<td>63</td>
<td>Ethanol</td>
<td>2.9</td>
<td>81</td>
<td>4.3E-03</td>
</tr>
<tr>
<td>C. cellulolyticum pWH320</td>
<td>Wildtype</td>
<td>Sigma 50</td>
<td>10</td>
<td>ND</td>
<td>Isobutanol</td>
<td>0.66</td>
<td>ND</td>
<td>4.6E-03</td>
</tr>
<tr>
<td>C. japonicus MSB280</td>
<td>Wildtype</td>
<td>Avicel</td>
<td>10</td>
<td>ND</td>
<td>Ethanol</td>
<td>0.0035</td>
<td>ND</td>
<td>7.3E-05</td>
</tr>
<tr>
<td>C. thermocellum M1570</td>
<td>Wildtype</td>
<td>Avicel</td>
<td>19.5</td>
<td>94</td>
<td>Ethanol</td>
<td>5.6</td>
<td>60</td>
<td>7.8E-02</td>
</tr>
<tr>
<td>C. acetobutylicum 7</td>
<td>Wildtype</td>
<td>Grass</td>
<td>30</td>
<td>ND</td>
<td>Butanol</td>
<td>0.6</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Developing CBP organisms by a recombinant strategy has also been studied in engineered strains of *E. coli*, *Klebsiella oxytoca*, *Zymomonas mobilis* and the yeast *S. cerevisiae* (Lynd et al., 2002, Lynd et al., 2008, Hasunuma et al., 2013). Although, *S. cerevisiae* and *Z. mobilis* are promising ethanol producers, native strains of *S. cerevisiae* and *Z. mobilis* are not able to utilize pentoses (xylose and arabinose), so genetically modified pentose utilizing strains of *S. cerevisiae* have been developed (Karakashev et al., 2007, Matsushika et al., 2009). However, engineered yeasts ferment xylose slowly, and usually only when glucose is completely consumed. Subsequently, engineered yeast strains that co-ferment mixtures of xylose and cellobiose have been developed (Ha et al., 2011), in which cellobiose hydrolysis takes place inside yeast cells, minimizing glucose repression of xylose fermentation, and improving ethanol yield.

Furthermore, high level heterologous expression and secretion of two exocellulase enzymes (CBHI & CBHII, 4-5% of total cell protein), which is required for an industrial process, has been achieved in *S. cerevisiae* (Ilmen et al., 2011). However, most studies on heterologous cellulase expression in yeasts have been conducted under aerobic conditions with high cell density, so it is necessary to evaluate these systems under anaerobic conditions and under industrial conditions required for CBP (Olson et al., 2012). Moreover, production of up to 7.6 g/L ethanol from phosphoric acid swollen cellulose (PASC) using recombinant strain of *S. cerevisiae*, capable of direct fermentation of cellulosic material in anaerobic systems with low cell density, has been reported (Den Haan et al., 2007, Hasunuma and Kondo, 2012, Yamada et al., 2011). A cellulolytic strain of *B. subtilis*, which grows on cellulose and converts it to lactate, has also been developed by over-expression of *B. subtilis* endoglucanase BsCel5 and produced about 3.1 g/L lactate with a yield of 60% of the theoretical maximum (Zhang et al., 2011). To summarize, beside the substantial achievements obtained in microbial cellulose utilization in recent years, there are still basic questions to be answered yet; the optimal operational conditions for microbial cellulose utilization, such as temperature, pH, substrate particle size, and the application of microbial communities are not known. Furthermore, the effectiveness of microbial cellulose conversion (e.g., rate and yield) compared to cell-free enzymatic systems is not well-understood (Olson et al., 2012). Table 2-2 provides a summary of the recent studies on non-cellulolytic microorganisms for developing recombinant CBP strategies.
Table 2-2. Recent studies on the production of chemicals from cellulose through SSF and recombinant CBP strategies (adapted from Hasunuma et al., 2013).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genes</th>
<th>Substrate</th>
<th>Enzyme added</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. coagulans 36D1</td>
<td>None</td>
<td>96 g L(^{-1}) cellulose</td>
<td>15 FPU g-cellulose(^{-1}) biocellulase W</td>
<td>80 g L(^{-1}) lactic acid</td>
</tr>
<tr>
<td>B. coagulans DSM2314</td>
<td>None</td>
<td>154.9 g L(^{-1}) lime-pretreated wheat straw</td>
<td>98 mg g-biomass(^{-1}) cellulase GC220</td>
<td>39.6 g L(^{-1}) lactic acid</td>
</tr>
<tr>
<td>B. coagulans P4-102B</td>
<td>Dldh, DalsS, GlyDH/ (D121N/F245S)</td>
<td>40 g L(^{-1}) lignocellulose</td>
<td>7.5 FPU g-cellulose(^{-1}) cellulase</td>
<td>2.0 g L(^{-1}) h(^{-1}) lactic acid</td>
</tr>
<tr>
<td>B. subtilis168</td>
<td>B. subtilis EG (BsCel5), DalsS</td>
<td>7.0 g L(^{-1}) amorphous cellulose</td>
<td>None</td>
<td>3.1 g L(^{-1}) lactic acid</td>
</tr>
<tr>
<td>C. glutamicum pCCT-engD</td>
<td>C. cellulovorans engD</td>
<td>15 g L(^{-1}) barley β-glucan</td>
<td>A. aculeatus β-glucosidase</td>
<td>0.17 g L(^{-1}) Glutamic acid</td>
</tr>
<tr>
<td>C. glutamicum ATCC 13032</td>
<td>C. cellulovorans celE, cbpA</td>
<td>Carboxymethyl cellulose</td>
<td>None</td>
<td>Not determined</td>
</tr>
<tr>
<td>E. coli MG1655</td>
<td>C. japonicas cel3A and gly43F, Bacillus sp. cel, C. stercoranium xyn10B, FAEE biosynthesis pathway</td>
<td>55 g L(^{-1}) IL-treated switchgrass</td>
<td>None</td>
<td>71 mg L(^{-1}) Fatty acid ethyl esters</td>
</tr>
<tr>
<td>E. coli DH1 ΔadhE</td>
<td>C. japonicas cel3A and gly43F, Bacillus sp. cel, C. stercoranium xyn10B, butanol biosynthesis pathway</td>
<td>55 g L(^{-1}) IL-treated switchgrass</td>
<td>None</td>
<td>28 mg L(^{-1}) n-butanol</td>
</tr>
<tr>
<td>E. coli MG1655</td>
<td>C. japonicas cel3A and gly43F, Bacillus sp. cel, C. stercoranium xyn10B, pinene biosynthesis pathway</td>
<td>55 g L(^{-1}) IL-treated switchgrass</td>
<td>None</td>
<td>1.7 mg L(^{-1}) pinene</td>
</tr>
<tr>
<td>L. plantarum NCIMB8826</td>
<td>C. thermocellum celA Dldh1</td>
<td>2 g L(^{-1}) cellohexaose</td>
<td>A. aculeatus β-glucosidase</td>
<td>1.27 g L(^{-1}) lactic acid</td>
</tr>
<tr>
<td></td>
<td>pCU-CelA/ΔldhL1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. plantarum NCIMB8826</td>
<td>C. thermocellum celA Dldh1</td>
<td>2 g L(^{-1}) barley β-glucan</td>
<td>A. aculeatus β-glucosidase</td>
<td>1.47 g L(^{-1}) lactic acid</td>
</tr>
<tr>
<td></td>
<td>pCU-CelA/ΔldhL1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Recently, several studies have demonstrated the potential of microbial co-cultures as CBP solutions for cellulosic ethanol and butanol production. As described earlier, an engineered co-culture of two acid-deficient mutant strains of \textit{C. thermocellum} and \textit{T. saccharolyticum} (Shaw et al., 2008) has resulted in the fermentation of 92 g/L cellulose in 6 days, and the production of 38 g/L ethanol with high yield of 0.41 g/g substrate and undetectable acetic and lactic acids (Argyros et al., 2011). In the above mentioned co-culture, it has been shown that the metabolism of \textit{C. thermocellum} rather than its cellulolytic capacity is the limiting factor in the cellulose metabolism and ethanol formation, as the co-culture has shown significantly improved ethanol yield and titer compared to the mono-culture of \textit{C. thermocellum}. However, the reason for this improved co-culture metabolism is not clear, and the secretion of some metabolites by \textit{C. thermocellum} that can be later utilized by the other species in the co-culture as well as prevention of the overflow metabolism in \textit{C. thermocellum} through rapid consumption of soluble sugars by \textit{T. saccharolyticum} have been deemed as the possible explanations for this enhancement. In another study, the co-culture of \textit{Acremonium cellulolyticus}, a mutant hyper cellulase producer filamentous fungus, and \textit{S. cerevisiae} has been employed to produce cellulosic ethanol with titer and yield of 35.1 g/L and 0.19 g/g substrate respectively (Park et al., 2012), whereas process variables, such as inoculation time for each species and medium composition, have been optimized in this one-pot ethanol production. Although significant improvements in ethanol titers have been achieved using co-cultures, the ethanol yield and productivity still require further improvement.

The co-culture of \textit{C. thermocellum} and the mesophilic \textit{Clostridium saccharoperbutylacetonicum} strain N1-4 has been investigated for producing butanol from crystalline cellulose (Nakayama et al., 2011), and optimizing operational parameters has resulted in the production of 7.9 g/L butanol. However, no information has been provided on cellulose metabolism and the cellulolytic activity in this co-culture. It has been suggested that a quorum-sensing mechanism may control and induce the solventogenesis in \textit{C. saccharoperbutylacetonicum}, which is a different mechanism from other butanol producing clostridial species (Kosaka et al., 2007, Nakayama et al., 2011). The sequential co-culture of \textit{C. thermocellum} and \textit{C. acetobutylicum} has also been investigated (Yu et al., 1985, Nakayama et al., 2011), whereas the co-culture produced only organic acids. In a recent study, the co-culture of a xylanase producing thermophilic strain of \textit{Kluyvera} and \textit{Clostridium} sp. strain BOH3 could directly convert xylan to 1.2 g/L butanol in
6 days (Xin and He, 2013), which has been comparable to the butanol titer obtained through separate hydrolysis (using xylanase) and fermentation by Clostridium sp. strain BOH3.

2.2 Biobutanol production using a clostridial co-culture, a CBP approach

An alternative method for the production of biobutanol from cellulosic biomass in a consolidated bioprocessing approach is the use of mesophilic clostridial co-culture. Clostridium acetobutylicum shows an effective capability to ferment hemicellulose derived sugars as well as cellulose derived sugars such as cellobiose, mannose, arabinose, xylose, glucose, and galactose to acetone, butanol, and ethanol (Yu et al., 1984, Fond et al., 1986, Ali et al., 2004). Thus, the co-culture of this bacterial species with a mesophilic cellulose degrading bacterium can be an efficient approach. Clostridium cellulolyticum is a cellulolytic, mesophilic bacterium which is able to solubilize crystalline cellulose in pretreated hardwood (Demain et al., 2005).

The simplified scheme of the clostridial co-culture fermentation on biomass hydrolyzates, cellulose and hemicellulose, is presented in Figure 2-2. The co-culture of C. cellulolyticum with C. acetobutylicum has been studied previously, and it has been shown that cellulolytic activity is the limiting factor in the co-culture fermentation since most of the cellulase activity products are consumed by C. acetobutylicum (Petidemange et al., 1983, Jones and Woods, 1986). The fermentation products are mainly acids with small amounts of ethanol and butanol, and the lack of glucose, which is required for solvent production due to low cellulolytic activity, seems to be the reason for acid accumulation. Furthermore, three times more cellulosic material was degraded in the co-culture compared to the mono-culture of C. cellulolyticum. The utilization of cellulose hydrolysis products, and the removal of their repressive effects have been deemed to be the reasons for this improved cellulolytic activity in the co-culture (Mitchell et al., 1995). This fact suggests the presence of a synergism between these two species, where the interactions between the two species in the co-culture result in an improved metabolic activity compared to their pure cultures. Hence, the analysis of this effect can be valuable for optimizing the rate of cellulosic material degradation.
The cellulolytic mesophilic *C. cellulolyticum* synthesizes a cellulosome, which is an extracellular multi-enzymatic complex, and degrades cellulose to glucose and soluble cellodextrins (mainly cellobiose) with the use of this cellulosome. This extracellular complex comprises a variety of cellulases, hemicellulase, and pectinase, which are arranged on a protein scaffold (CipC) (Guedon et al., 1999b, Desvaux, 2005b, Desvaux, 2005a). The cellulosomes are located on the cell surface and facilitate both cell adhesion as well as cellulolytic activity on the cellulose fibres. Different aspects of this cellulosome complex, including structure, regulation, extracellular assembly, and enzymatic activity have been characterized and reviewed. However, the regulation of the cellulosomal gene expression and the mechanism of secretion, attachment, and arrangement of this complex on the cell surface still need more investigation (Desvaux, 2005a).

![Figure 2-2. The simplified scheme of the clostridial co-culture fermentation on biomass hydrolyzates (cellulose and hemicellulose); (1): cellulase, (2): hemicellulase enzymes produced by *C. cellulolyticum*.](image-url)
Limited growth and catabolic activity on cellulose is the common feature of the currently known cellulolytic bacteria (Desvaux, 2005b). Previously, heterologous expression of pyruvate decarboxylase and alcohol dehydrogenase genes from Zymomonas mobilis has led to 180% and 150% increase in cell concentration and cellulose consumption in the C. cellulolyticum batch culture on fibrous cellulose (Guedon et al., 2002). This recombinant strain has shown a prolonged growth on cellulose, with the same growth rate as the wild strain, and produced ethanol, acetate and lactate. Improving the cellulolytic and catabolic activity of C. cellulolyticum by metabolic engineering can facilitate the shift of co-culture metabolism to the solventogenic phase. However, it has been demonstrated that the central metabolism of C. cellulolyticum rather than the cellulose hydrolysis rate is the limiting step in cellulose metabolism; therefore, the improvement of C. cellulolyticum metabolism, which is not adapted to high catabolic rates, instead of the catalytic activity of the cellulosome, should be the main focus in order to improve its cellulolytic activity (Guedon et al., 2002, Desvaux, 2005b). Therefore, genome-scale metabolic modeling of C. cellulolyticum metabolism can bring deep insights into the bottleneck pathways and facilitate the improvement of this cellulolytic activity using in silico generated hypotheses which can be tested experimentally.

Furthermore, developing a genome-scale model of metabolism in this co-culture can assist in designing strategies for improving the yield and productivity of biobutanol, which is the desired product in this CBP. Moreover, by means of the developed model of co-culture metabolism, it would be possible to examine the in silico production of other valuable chemicals using genetically engineered strains, and to characterize the critical pathways for their production (Lee and Papoutsakis, 1999). Developing viable strains through system-level metabolic engineering, using genome sequence information and metabolic engineering tools for clostridia, has been proposed as an essential part of developing commercial biobutanol process, along with fermentation and solvent recovery process developments, as depicted in Figure 2-3 (Lee et al., 2008c).
2.2.1 *Clostridium acetobutylicum* fermentation

*C. acetobutylicum* is a Gram-positive, solvent producing, anaerobic bacterium which is able to convert a variety of sugars, polysaccharides and oligosaccharides to organic acids and solvents. In this fermentation, the main products are acetate, butyrate, ethanol, butanol, acetone, carbon dioxide, and hydrogen (Hartmanis and Gatenbeck, 1984). In a typical fermentation of this bacterium, two distinct phases are observed. In the acidogenic phase, acetate, butyrate, hydrogen, and carbon dioxide are produced; during this phase, the bacterium grows rapidly and due to the accumulation of acids, the medium pH drops. In the stationary growth phase, the metabolic shift to solventogenesis takes place. During solventogenic phase, the consumption of acids, as well as carbohydrates, and production of neutral solvents result in an increase in the medium pH (Jones and Woods, 1986). Since comprehensive knowledge on the metabolism is required for
developing a genome-scale metabolic model, the central metabolic pathways of *C. acetobutylicum* are described in the subsequent section.

The ClosTron, a gene knockout system has been developed for *C. acetobutylicum* and other clostridia, which facilitates further functional genomic studies and the construction of multiple knock-out mutants (Heap et al., 2010, Heap et al., 2007, Lehmann et al., 2012, Tracy BP and ET, 2009). Moreover, plasmid-based gene over expression systems have been developed recently (Dong et al., 2010, Soucaille et al., 2008, Heap et al., 2009), providing a platform for metabolic engineering of *C. acetobutylicum*. The genome sequence of *C. acetobutylicum* has also been completed (Nolling et al., 2001), and genome wide transcriptome studies on different physiological aspects of this species, such as sporulation, stationary-phase events and solvent stress, have been conducted (Alsaker and Papoutsakis, 2005, Jones et al., 2008, Janssen et al., 2012). Therefore, systems biology approaches for strain development and the improvement of *C. acetobutylicum* metabolic activity seems to be promising. However, difficult genetic accessibility of clostridia, limited number of available genetic methods and lack of knowledge on the regulatory mechanisms have restricted the success of metabolic engineering studies on *C. acetobutylicum* to improve the butanol productivity (Lee et al., 2008c, Lutke-Eversloeh and Bahl, 2011, Papoutsakis, 2008).

### 2.2.1.1 Central metabolism of *Clostridium acetobutylicum*, characterization of enzymes, pathways and genes

The central biochemical pathways of *C. acetobutylicum* involved in the conversion of carbohydrates to hydrogen, carbon dioxide, acids, and solvents are presented in Figure 2-4. In the primary metabolism of *C. acetobutylicum*, hexose and pentose sugars are converted to pyruvate, ATP and NADH (Lee et al., 2008c). Hexoses are metabolized via glycolysis to pyruvate, 2 mol of ATP and 2 mol of NADH. However, pentoses are metabolized via the pentose phosphate pathway and 3 mol of pentose generates 5 mol of ATP and 5 mol of NADH. Pyruvate is decarboxylated in the presence of coenzyme A (CoA) by pyruvate-ferredoxin oxidoreductase to generate carbon dioxide, acetyl-CoA and reduced ferredoxin (Meinecke B et al., 1989). The produced acetyl-CoA is the major intermediate in both acid and solvent production pathways. Bacterial-type ferredoxins are low molecular weight carrier proteins composed of two [4Fe-4S] clusters which can both accept and donate electrons in very low potential reactions.
In *C. acetobutylicum*, reduced ferredoxin is the physiological electron donor of the iron containing hydrogenase which transfers electrons to protons as final electron acceptors, and produces hydrogen (Guerrini et al., 2008, Calusinska et al., 2010). NADH-ferredoxin oxidoreductase is another enzyme which facilitates redox reactions between NAD$^+$ and ferredoxin, whereas NADPH-ferredoxin oxidoreductase controls the production of NADPH, which is essential in biosynthesis pathways, from reduced ferredoxin (Jungermann et al., 1973). Furthermore, the presence of another low molecular weight nonsulfur protein, rubredoxin, has been shown. Unlike ferredoxin, no specific electron acceptor for reduced rubredoxin has been identified (Jones and Woods, 1986). *C. acetobutylicum* is also able to convert pyruvate to lactate under certain conditions; although this pathway is a less efficient alternative for NAD$^+$ regeneration and energy production, where the activity of hydrogenase enzyme has been blocked using carbon monoxide, or in iron deficient cultures where the levels of ferredoxin and the iron containing hydrogenase are low. Moreover, reducing hydrogenase activity and redirecting the electron flow for the regeneration of NAD(P) pool in *C. acetobutylicum* has been demonstrated to increase the butanol formation (Lutke-Eversloh and Bahl, 2011). Increasing the hydrogen partial pressure, gassing with carbon monoxide and addition of artificial electron carriers, for instance methyl viologen and neutral red, are some of the techniques that have been employed to drive the metabolism of *C. acetobutylicum* towards butanol formation by decreasing hydrogenase activity (Girbal and Soucaille, 1998, Girbal and Soucaille, 1994, Girbal et al., 1995).
Figure 2-4. Central biochemical pathways of Clostridium acetobutylicum (adapted from (Desai et al., 1999)). Enzymes involved in different pathways are given next to the arrows: HYDA (hydrogenase), PTA (phosphotransacetylase), AK (acetate kinase), THL (thiolase), BHBD (B-hydroxybutyryl-CoA dehydrogenase), CRO (crotonase), BCD (butyryl-CoA dehydrogenase), PTB (phosphotransbutyrylase), BK (butyrate kinase), AADC (Acetoacetate decarboxylase), CoAT (acetoacetyl-CoA: acetate/butyrate: CoenzymeA transferase), ADC (acetoacetate decarboxylase), AAD (aldehyde/alcohol dehydrogenase) and BDHA&B (butanol dehydrogenase A & B).
Conversion of acetyl-CoA to acetate is catalyzed by two enzymes, phosphotransacetylase (PTA) and acetate kinase (AK), and the genes pta and ack encoding these enzymes are organized in one operon (Boynton et al., 1996b). The acetate production is a significant pathway in the acidogenic fermentation stage and generates ATP for the cell growth. These enzymes are present in all anaerobic bacteria that consume acetyl-CoA to produce ATP via substrate level phosphorylation (Hartmanis and Gatenbeck, 1984). Thiolase (THL) catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, the precursor of four carbon products in *C. acetobutylicum* metabolism, and hence it has a significant effect on the relative amounts of the two-carbon products (acetate and ethanol) to three- and four-carbon products (butanol and acetone) (Bennett and Rudolph, 1995). In acidogenic phase, thiolase competes with phosphotransacetylase for the available pool of acetyl-CoA to form butyrate and acetate respectively (Wiesenborn et al., 1988, Wiesenborn et al., 1989a). In comparison to the formation of acetic acid, butyric acid production yields 3 mol of ATP, while 4 mol of ATP is made during acetate formation. Butyrate production is neutral in NADH formation and consumption but acetate formation leads to an increase in the NADH level.

The condensation reaction is highly sensitive to the presence of micromolar amounts of CoA due to competitive inhibition by it, and the ratio of CoA to acetyl-CoA may be the most important factor affecting the net rate of acetyl-CoA condensation to acetoacetyl-CoA by thiolase (Wiesenborn et al., 1988). Reducing thiolase sensitivity towards CoA through protein engineering has also been studied recently, which resulted in improved alcohol titers (Mann and Lutke-Eversloh, 2013). In *C. acetobutylicum*, thiolase activity has a broad range of pH values from 5.5 to 7, which is the range of internal pH, so change in internal pH does not seem to have an important role in regulation of thiolase activity. During solventogenesis, thiolase competes with acetaldehyde dehydrogenase for the available pool of acetyl-CoA and it affects the ratio of butanol plus acetone to ethanol; furthermore, its specific activity is highest in this phase, showing that thiolase might be somewhat inducible (Bennett and Rudolph, 1995).

In the next three steps, acetoacetyl-CoA is reduced to butyryl-CoA. The first step involves the B-hydroxybutyryl-CoA dehydrogenase (BHBD) which catalyzes the reduction of acetoacetyl-CoA by NAD(P)H. The *hbd* gene encoding BHBDH has been cloned and sequenced, while analysis of the chromosomal region including *hbd* shows that the five open reading frames (*hbd, crt, bcd, etfB* and *etfA*) are transcribed as a transcription unit, and form a BCS (butyryl-CoA synthesis)
operon (Boynton et al., 1996a, Bennett and Rudolph, 1995). Subsequently, the \( \text{crt} \) gene encoding crotonase (CRO) catalyzes the dehydration of 3-hydroxybutyryl CoA to crotonyl-CoA. In the next step, \( \text{bcd} \) gene encodes butyryl-CoA dehydrogenase (BCD) that catalyzes the reduction of carbon-carbon double bond in crotonyl-CoA to form butyryl-CoA, a key branch point in acid and solvent formation. Open reading frames \( \text{etfB} \) and \( \text{etfA} \) located downstream of \( \text{bcd} \), show homology to those encoding the \( \alpha \) and \( \beta \) subunits of the electron transfer flavoprotein; these findings suggest that BCD in clostridia may interact with the electron transfer flavoprotein (Boynton et al., 1996a). Unlike \( \text{Clostridium kluyveri} \), there is no evidence that the exergonic BCD reaction in \( \text{C. acetobutylicum} \) is energetically coupled with the endergonic reduction of ferredoxin by NADH, catalyzed by BCD/etf complex (Lutke-Eversloh and Bahl, 2011). Energy conservation through the flavin-based electron bifurcation (FEB) has been considered as a mode of energy generation in \( \text{C. kluyveri} \) and some other anaerobes (Herrmann et al., 2008, Buckel and Thauer, 2013).

Butyryl-CoA is converted to butyrate by phosphotransbutyrylase (PTB) and butyrate kinase, and the genes encoding these enzymes, \( \text{ptb} \) and \( \text{buk} \), form an operon (Walter et al., 1993). It has been shown that when a continuous culture of \( \text{C. acetobutylicum} \), producing a significant amount of butyrate, is sparged with carbon monoxide, a high butyrate consumption rate coincides with butanol production, but not acetone formation (Meyer et al., 1986). Carbon monoxide inhibits dehydrogenase activity, and redirects the electron flow to alcohol production pathways. The exclusive production of butanol and not acetone shows that CoA transferase enzyme is not active under this condition, and suggests that the butyrate uptake is catalyzed by the reversal of the PTB-butyrate kinase pathway (Wiesenborn et al., 1989b, Lehmann et al., 2012). In the physiological range of pH, 5.5 to 7, PTB is highly sensitive to pH changes in the butyryl phosphate production direction, and its activity is too low below pH 6. This fact suggests a feedback control loop to slow or stop the butyrate formation by butyrate kinase pathway. But this enzyme is less sensitive to pH change in butyryl-CoA formation direction, so this pathway can act in the butyrate uptake direction at low external pH condition.

Acetoacetate decarboxylase (AADC) and acetoacetyl-CoA: acetate/butyrate: CoenzymeA transferase (short: CoA transferase) are involved in the production of acetone from acetoacetyl-CoA. The \( \text{adc} \) gene coding acetoacetate decarboxylase is organised in a monocistronic operon whereas the \( \text{ctf} \) genes, encoding CoA transferase, with an aldehyde/alcohol dehydrogenase (aad)
form an operon named sol operon (Fischer et al., 1993). The complete sol operon is induced or derepressed before the onset of solventogenesis. CoA transferase from C. acetobutylicum has been purified and consists of two subunits which are involved in the uptake of acetate and butyrate, and the production of corresponding CoA derivatives (Wiesenborn et al., 1989a). The \( K_m \) values of CoA transferase for acetate and butyrate were determined to be 1.2M and 0.6M respectively, which are higher than intracellular concentrations of acetate and butyrate, and may suggest a regulatory system that enables the cells to tolerate the toxic environment caused by increased levels of acids (Wiesenborn et al., 1989a, Dürre et al., 1995). Acetoacetate decarboxylase catalyzes the decarboxylation of acetoacetate via the formation of a Schiff base intermediate in the catalytic centre of the enzyme, and produces acetone and carbon dioxide (Highbarger et al., 1996).

At the onset of solventogenesis, substrate uptake rate slows down, because large amounts of acids are accumulated, and must be consumed to prevent the dissipation of proton motive force across the cell membrane. Accordingly, CoA transferase converts acids to butyryl-CoA and acetyl-CoA, which are converted to ethanol and butanol by the subsequent action of the aldehyde/alcohol dehydrogenase. Therefore, it seems logical for the cell to arrange these two enzymes in one transcription unit, sol operon. Acetoacetate decarboxylase is required to provide a thermodynamic gradient for the initial reaction of butyryl-CoA formation, since this reaction is less thermodynamically favourable. This requirement for the decarboxylase might be the reason that the ade gene is organised in a monocistronic adjacent operon (Dürre et al., 1995).

Ethanol and butanol are produced in two reduction steps from acetyl-CoA and butyryl-CoA respectively. Butanol formation is catalyzed by several enzymes. In addition to the AAD, two butanol dehydrogenase isozymes are involved in butanol formation. RNA analysis shows a sequential order in the induction of different butanol dehydrogenase genes. BDH1 enzyme is mostly involved in low concentration of butanol formation at the onset of solventogenesis, but BDH2 and AAD contribute predominantly to butanol production at higher concentrations, and ensure continued butanol production during solventogenesis (Dürre et al., 1995, Chen, 1995, Welch et al., 1989, Walter et al., 1992, Petersen et al., 1991). Welch et al. have characterized two butanol dehydrogenases with different cofactor requirements and different pH ranges in C. acetobutylicum ATCC 824. The NADH dependent BDH is more active at lower pH with a pH maximum at 5.5 and reduced activity at higher pH, while the NADPH dependent one is more
active at higher pH, and its activity decreases when pH drops below 8.0, so the NADH dependent BDH is more active in the intracellular pH range.

NADH dependent butanol dehydrogenases, BDH1 and BDH2, can be considered BDH isozymes. BDH1 is 2.2-fold more active with butyratedehyde than acetaldehyde, while BDH2 is 46-fold more active with butyratedehyde (Petersen et al., 1991). These isozymes are primarily NADH dependant but show some NADPH activity. Although the bdhA and bdhB genes coding these isozymes are located in tandem on the chromosome, RNA studies have shown that they do not form an operon (Walter et al., 1992). The detection of bdh mRNA and BDH activity at approximately the same time implies that the BDHs are initially regulated at the transcriptional level (Walter et al., 1992).

The gene (aad) encoding the aldehyde/alcohol dehydrogenase (AAD) has been identified as a part of sol operon with two ctf genes (Nair et al., 1994, Nair and Papoutsakis, 1994). The transformation of aad gene to the mutant M5 of C. acetobutylicum, which lacks acetoacetate decarboxylase, CoA transferase and butyraldehyde dehydrogenase, restores the butanol formation without any acetone production or significant increase in ethanol production. This suggests that AAD’s main role is in butyraldehyde dehydrogenase activity, and it does not play a significant role in ethanol production (Nair and Papoutsakis, 1994). The second NADH dependant alcohol/aldehyde dehydrogenase has also been identified in alcohologenic cultures of C. acetobutylicum and not in solventogenic cultures (Fontaine et al., 2002). An alcohologenic culture is observed in continuous cultures when grown at neutral pH under conditions of high NAD(P)H availability and produces butanol and ethanol but no acetone. Both aad genes reside on a megaplasmid whose loss produces degenerated mutants that produce acids and little or no solvents (Cornillot et al., 1997). The sol operon, located on the megaplasmid, plays a significant role in solventogenesis, and is induced or derepressed at the onset of solventogenesis. The presence of a common regulatory factor for butanol and acetone production has been suggested (Grupe and Gottschalk, 1992). A CoA linked butyraldehyde/acetaldehyde dehydrogenase has been characterized and purified in C. acetobutylicum B643. The $K_m$ values for both acetyl-CoA and butyryl-CoA have been almost identical, but the relative turnover rate for butyryl-CoA to acetyl-CoA is 5.5; therefore, this enzyme might be responsible for the final molar ratio of 6:1 for butanol to ethanol during this fermentation (Palosaari and Rogers, 1988).
Metabolic engineering studies of *C. acetobutylicum* have been conducted to improve butanol yield, titer and productivity. The inactivation of acetate and butyrate formation pathways (Green et al., 1996), and the combination of CoA transferase downregulation with AAD overexpression (Tummala et al., 2003, Sillers et al., 2009) have shown to improve alcohol formation. The B-hydroxybutyryl-CoA dehydrogenase (BHBD)-negative mutant has produced ethanol with a yield of 0.38 g/g substrate, while the inactivation of entire butyrate/butanol pathways had no significant effect on the growth of *C. acetobutylicum*, indicating the flexibility of the central metabolism towards product pattern modifications (Lehmann and Lutke-Eversloh, 2011). Disrupting the acetate and acetone formation pathways significantly reduced acetate and solvent production (Lehmann et al., 2012), while the megaplasmid-deficient mutant, which lacks the solvent producing genes, has shown enhanced hydrogen production rate (Oh et al., 2009).

### 2.2.1.2 Metabolic shift from acidogenesis to solventogenesis

The onset of solventogenesis coincides with endospore formation; both processes start when cells are exposed to severe acid stress. Low pH and accumulation of acids in the cell environment lead to the presence of massive amounts of undissociated acids; these undissociated acids are membrane permeable, and will dissipate the proton gradient across the cell membrane which causes cell death. Sporulation is a time consuming process that enables cells to survive the harsh environmental condition. On the other hand, the conversion of acids to neutral solvents and decreasing the acids production rate provide cells with an opportunity to prevent cell death by the proton gradient dissipation and formation of mature endospores, before the solvent concentration reaches the toxic level (Dürre et al., 1995).

In batch cultures, the level of undissociated butyric acid correlates with the induction of solvent production (Hüsemann and Papoutsakis, 1989). The kinetic studies of acid and solvent formation, at various pH values between 4.5 and 6.0 in batch cultures, show that at lower pH, solvents are the main products, but at higher pH, only acids are produced. The effect of pH can be correlated with undissociated butyric acid concentration in the medium. Between pH 4.5 to 5.5, solvent formation starts at undissociated butyric acid concentration between 1.6 and 1.9 g/l, while at pH 6.0, in spite of high acid concentrations, the undissociated butyric acid concentration does not exceed 0.8 g/l, and no significant amount of solvents are formed (Monot et al., 1984). The intracellular pH in growing clostridia is higher than external pH due to the activity of
membrane bound ATPase in proton translocation, and it increases the undissociated acetate and butyrate concentrations inside the cell (Hüsemann and Papoutsakis, 1989, Monot et al., 1984).

In contrast to batch cultures, the level of undissociated butyric acid does not correlate with solventogenesis in continuous cultures, and high intracellular ATP concentration has been suggested as an important factor in the kinetic regulation of several key enzymes such as phosphotransbutyrylase (PTB) and thiolase. ATP is an inhibitor to the PTB and inactivation of this enzyme redirects the carbon flow from butyryl-CoA to the production of butanol instead of butyrate (Hüsemann and Papoutsakis, 1989). Grupe et al. analyzed the continuous cultures of C. acetobutylicum DSM1731 during the shift from acidogenic state to solventogenic state, and noticed an increase in intracellular butyric acid concentration, followed by a decrease in acid production along with acid uptake at the onset of solventogenesis (Grupe and Gottschalk, 1992). Furthermore, the intracellular ATP concentration reached a minimum before the commencement of solventogenesis; probably due to the ATP-consuming proton extrusion process accomplished by F0-F1ATPase at higher ΔpH conditions. The NADH plus NADPH pool showed a drastic increase until the induction of solventogenesis followed by stable oscillatory changes in culture redox potential in steady state solventogenic culture. The shift to solventogenic phase decreases the ATP demand for pumping protons out; thus, regeneration of the ATP pool as well as the decrease in the NAD(P)H concentration weaken the signals for solventogenesis, and the level of solventogenic enzymes decrease, acids are produced, and the cell enters another shift induction cycle (Grupe and Gottschalk, 1992).

Several studies have been conducted to determine the shift inducing conditions and increase the solvent yield (Zhao et al., 2005, Girbal et al., 1995, Girbal and Soucaille, 1994, Vasconcelos et al., 1994, Sauer and Dürre, 1995); however, the signals which trigger the solventogenesis are yet to be known. The shift could be attributed to a combination of several signals responsible for transcriptional regulation. DNA supercoiling also has been proposed as a transcriptional sensor that responds directly to the environmental stimuli (Dürre et al., 1995). In a recent study (Wietzke and Bahl, 2012), the redox-sensing transcriptional repressor protein Rex has been identified as a transcriptional regulator of solventogenesis in C. acetobutylicum; in response to the cellular NADH/NAD⁺ ratio, Rex controls the expression of the genes involved in the butanol formation pathway. The Rex-negative mutant produced significantly higher amounts of butanol and ethanol, while the solventogenesis started earlier than in the wild type, and acetone
and hydrogen formation were significantly decreased. Also, the expression of NADH dependant alcohol dehydrogenase was significantly higher in the Rex mutant. These findings offer new metabolic engineering targets for improving the butanol production in *C. acetobutylicum*.

### 2.2.2 *C. cellulolyticum* fermentation

The cellulolytic mesophilic *C. cellulolyticum* synthesizes cellulosome, which is an extracellular multi-enzymatic complex, and degrades cellulose to glucose and soluble cellodextrins (mainly cellobiose) with the use of this cellulosome, where the β-1,4-glycosidic bonds of the cellulose polymer are hydrolyzed by general acid catalysis (Desvaux, 2005b). This extracellular complex comprises a variety of cellulases, hemicellulase, and pectinase, which are arranged on a protein scaffold (CipC) (Guedon et al., 1999b, Desvaux, 2005b, Desvaux, 2005a, Perret et al., 2004). So far, eight cellulosomal enzymes, including seven cellulases and one pectinase, have been biochemically characterized (Desvaux, 2005a). The cellulosomes are located on the cell surface and facilitate both cell adhesion as well as cellulolytic activity on the cellulose fibres. Different aspects of this cellulosome complex including structure, regulation, extracellular assembly, and enzymatic activity have been characterized and reviewed; however, the regulation of the cellulosomal gene expression and the mechanism of secretion, attachment, and arrangement of this complex on the cell surface still need more investigation (Abdou et al., 2008, Desvaux, 2005a). Compared to other mesophilic cellulosome-forming clostridia, such as *C. acetobutylicum* and *C. cellulovorans*, *C. cellulolyticum* contains fewer carbohydrate-active enzyme genes (CAZyme; 149 genes), involved in the degradation, creation and modification of glycosidic bonds (Cantarel et al., 2009, Xu et al., 2013). However, it has the most diverse cellulosomal genes (65 genes) which have been identified in four clusters spread along its genome (Xu et al., 2013).

In a recent study, it has been demonstrated that the cellulosomes of *C. cellulolyticum* differ in their protein compositions and are acting synergistically on complex substrates, such as straw, whereas higher enzyme diversity advances the degradation efficiency of the cellulosomes (Fendri et al., 2009). The cellulosome of *C. cellulolyticum* is smaller (600kDa) and less complex than its counterpart in *C. thermocellum* (2.0 to 6.5 MDa) (Desvaux, 2005b). Also, in addition to cell-associated cellulosomes, *C. cellulolyticum* produces cell-free cellulosomes, and it has been proposed that the degree of cell-association, cellulose adhesion, and the composition of
cellulosomes are regulated by the substrate characteristics (Mohand-Oussaid et al., 1999); for instance, in contrast to cellulose-grown cells, about 90% of the xylanase activity is not cell-associated when *C. cellulolyticum* is cultivated on xylan, and the composition of cellulosomes obtained from xylan and cellulose-grown cells are different.

Previous reports have shown that the cellulosome of *C. thermocellum* is strongly inhibited by cellobiose, while glucose has an insignificant inhibitory effect. Furthermore, adding β-glucosidase which hydrolyses cellobiose, to the *C. thermocellum* culture has been shown to increase cellulose degradation rate and remove the adverse effects of cellobiose inhibition by hydrolyzing it to glucose. Cellobiose inhibits the function of *C. thermocellum* cellulosome through a competitive inhibitory mechanism (Kruus et al., 1995). So far, cellobiose inhibitory effect on the Cel5A, an endoglucanase component of the *C. cellulolyticum* cellulosome has been established (Fierobe et al., 1991). Furthermore, it has been shown that glucose has a significant derepressing effect on the production of cellulosome in *C. cellulolyticum*, and in contrast to cellobiose, it does not have any inhibitory effect on its catalytic activity (Petitdemange et al., 1992). In a recent study, it has been shown that the expression of cellulases in *C. cellulolyticum*, at both transcription and protein levels, is induced by glucose, while the expression of cellulosomal genes are repressed under cellobiose and xylose, through a carbon catabolite repression mechanism (Xu et al., 2013). In *C. cellulolyticum* cultures on cellulose mixed with 0.5-8.0 g/L of glucose, glucose addition has led to a decrease in the culture lag phase, and the peak cellulose degradation rate has increased compared to the control culture on cellulose (0.854 g/L.day and 0.607 g/L.day respectively), with less than 0.5 g/L of glucose in the culture.

The metabolism of cellobiose, as the main product of cellulose hydrolysis by *C. cellulolyticum* is demonstrated in Figure 2-5 (Desvaux, 2005b). *C. cellulolyticum* is also able to grow on celloolodextrins (cellobiose to cellohexaose), xylan, glucose, xylose, and weakly on hemicellulose-derived sugars, such as arabinose, fructose, galactose, mannose and ribose. The uptake of sugars through an ATP-Binding Cassette (ABC) transport system followed by their metabolism through glycolysis pathways results in the production of pyruvate, NADH and ATP. Pyruvate is decarboxylated in the presence of coenzyme A (CoA) by pyruvate-ferredoxin oxidoreductase to generate carbon dioxide, acetyl-CoA and reduced ferredoxin, where electrons are then transferred from the reduced ferredoxin to the dehydrogenase, NADH-ferredoxin oxidoreductase and NADPH-ferredoxin oxidoreductase pathways. Furthermore, the ratio of H₂/CO₂ higher than
one has been observed in *C. cellulolyticum* cultures which confirms the transfer of electrons from NADH to hydrogenase pathway through ferredoxin reduction (Desvaux et al., 2000).

It has been previously shown (Desvaux et al., 2000) that in pH controlled batch cultures of *C. cellulolyticum* on a defined medium, the distribution of carbon flow depends on the initial cellulose concentration. For concentrations less than 6.7 g/L of cellulose, acetate, ethanol, CO₂ and H₂ were shown to be the main fermentation end products and more than 91% of cellulose was observed to be degraded. At higher cellulose concentrations, from 6.7 g/L up to 29.1 g/L, the carbon flow is redirected from ethanol and acetate towards lactate and extracellular pyruvate. In addition, in batch cultures of *C. cellulolyticum* on high cellulose concentration, it has been shown that the peak of pyruvate formation coincides with the start of lactate formation, and this pyruvate accumulation in the *C. cellulolyticum* culture shows that the rate of cellulose catabolism is higher than the rate of pyruvate consumption. Also it has been suggested that the cellulose hydrolysis depends on the concentration of *C. cellulolyticum*, which remains constant at and above 6.7 g/L of cellulose (Desvaux et al., 2000).
Figure 2-5. Central metabolic pathways of Clostridium cellulolyticum (adapted from (Desvaux, 2005b)); 1: pyruvate-ferredoxin oxidoreductase; 2: hydrogenase; 3: NADH-ferredoxin oxidoreductase; 4: lactate dehydrogenase; 5: acetaldehyde dehydrogenase; 6: alcohol dehydrogenase; 7: phosphotransacetylase; 8: acetate kinase.
Cellulose metabolism mainly occurs when *C. cellulolyticum* is attached to the substrate (Gelhaye et al., 1993), but in the batch cultures of *C. cellulolyticum* at high cellulose concentrations, cell growth terminates before substrate depletion (Desvaux et al., 2000). Moreover, it has been shown that re-inoculating a fresh culture of *C. cellulolyticum* at high cellulose concentration of 29.1 g/L, where substrate is not fully consumed, significantly improves the cellulose solubilization and cell biomass yield compared to a classical batch culture (Desvaux et al., 2000). This result indicates that the incomplete cellulose catabolism is not due to either the limitation of adhesion sites on cellulose fibers or product inhibition. At high cellulose concentrations, the likely explanation for the incomplete cellulose consumption is the lack of control on carbon uptake flow and an imbalanced metabolism, leading to the accumulation of intracellular metabolites and self-intoxication of the cells, eventually resulting in a growth arrest (Desvaux et al., 2000, Desvaux, 2005b). Similarly, extracellular pyruvate formation has been reported in *C. thermocellum* cultures at high cellulose and cellobiose concentrations, which evidences the overflow metabolism (Levin et al., 2006).

Limited growth and catabolic activity on cellulose is the common feature of the currently known cellulolytic bacteria (Desvaux, 2005b). Improving the cellulolytic and catabolic activity of *C. cellulolyticum* by metabolic engineering (Guedon et al., 2002) can facilitate the shift of the coculture metabolism to the solventogenic phase. However, it has been demonstrated that the central metabolism of *C. cellulolyticum* rather than the cellulose hydrolysis rate is the limiting step in cellulose metabolism; therefore, the improvement of *C. cellulolyticum* metabolism which is not adapted to high catabolic rates, instead of the catalytic activity of the cellulosome, should be the main focus in order to improve its cellulolytic activity (Guedon et al., 2002, Desvaux, 2005b).

Recently, a targeted gene inactivation system for *C. cellulolyticum* has been developed and applied to disrupt the paralogous lactate dehydrogenase and malate dehydrogenase genes, resulting in a significant increase in ethanol formation from cellulose (Li et al., 2012). Moreover, gene transfer systems for *C. cellulolyticum* have also been investigated (Jennert et al., 2000, Tardif et al., 2001), and applied for the production of ethanol (Guedon et al., 2002) and isobutanol (Higashide et al., 2011) from cellulose. Heterologous expression of pyruvate decarboxylase and alcohol dehydrogenase genes from *Zymomonas mobilis* has led to a 53% and 180% increase in ethanol formation and cellulose consumption in the *C. cellulolyticum* batch
culture, respectively (Guedon et al., 2002). Furthermore, the feasibility of isobutanol production from cellulose in a CBP has been demonstrated through metabolic engineering of *C. cellulolyticum* to divert its 2-keto acid intermediates toward alcohol formation, and the synthesis of 660 mg/L isobutanol from cellulose. However, the lack of an inducible gene expression system has been deemed as a significant barrier in expressing new pathways in *C. cellulolyticum* (Higashide et al., 2011). These studies suggest metabolic engineering approaches can significantly improve the metabolism of *C. cellulolyticum* as a potential CBP organism. Table 2-3 presents some attributes of the *C. cellulolyticum* genome and the calculated number of orthologous genes, which has been derived using reciprocal BLAST (Altschul et al., 1990) of *C. cellulolyticum* and *C. acetobutylicum* genome sequences. About 180 of the 1148 orthologous genes currently exist in the reconstructed *C. acetobutylicum* metabolic network (iFS700).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>The genome sequence length</td>
<td>3.9 Mb</td>
</tr>
<tr>
<td>Number of protein coding gene</td>
<td>3283</td>
</tr>
<tr>
<td>The calculated number of orthologs among <em>C. acetobutylicum</em> and <em>C. cellulolyticum</em></td>
<td>1148</td>
</tr>
<tr>
<td>The calculated number of orthologous genes that exist in <em>C. acetobutylicum</em> model</td>
<td>180</td>
</tr>
</tbody>
</table>

### 2.3 Genome-scale metabolic modeling

A wealth of genomic, proteomic, metabolomic and other types of biological data have been generated using high-throughput technologies, and it is now possible to use this information to investigate the phenotypic characteristics of microorganisms (Edwards et al., 2002, Hyduke et al., 2013, Bordbar et al., 2012). Furthermore, system-level quantitative analysis of cellular metabolism using *in silico* modeling approaches facilitates the generation of new knowledge, via iterative modification of *in silico* models and experimental designs. This knowledge can be applied for designing strains with improved metabolic activities for industrial applications (Sang et al., 2005, Curran et al., 2012, Mahadevan et al., 2005, Anesiadis et al., 2011, Yim et al., 2011).
Figure 2-6 illustrates a schematic process of the genome-scale microbial metabolic model development. This iterative process involves two main stages. In the first, using the knowledge of the metabolism of an organism, annotated genome sequence data, along with experimentally driven biochemical and physiological data, the metabolic network is reconstructed. This reconstructed network forms the modeling platform, and in the second stage together with other physiological constraints, is analyzed using a mathematical technique (i.e., Flux Balance Analysis). Cell biomass synthesis reaction, which is derived from cellular chemical composition, as well as maintenance and growth energy requirements, must be incorporated into this model (Senger, 2010). The model is refined until the modeling results validate the experimental growth yield, product secretion rates, substrate uptake rates, and gene knockout experiments (Orth et al., 2010, Cheung CY et al., 2013). This biochemically and genomically accurate model will then be applied to study the fluxome of the metabolic network and generate testable hypotheses (Chen et al., 2011, Choon et al., 2012, Martinez et al., 2013).

![Diagram of metabolic model development process](image-url)

Figure 2-6. The process of metabolic model development (adapted from (Covert et al., 2001a)).
2.3.1 Metabolic network reconstruction

Through the metabolic network reconstruction process, genes, proteins and metabolites, which are involved in the metabolism of an organism, are identified, classified and interrelated to create a network (Feist et al., 2006). This reconstructed network forms the backbone of a metabolic model. Automated approaches for reconstruction of metabolic networks have also been developed in order to accelerate the pace of reconstruction process (Alves and Buck, 2007, Notebaart et al., 2006). However, inefficiencies of these automated methods due to some problems, such as existence of mis-annotations, non-standard protein identifier designations along with the use of incomplete and unspecific identifiers, and the presence of organism specific reactions or pathways highlight the need for manual curation of these automated reconstructed networks to increase their accuracy (Francke et al., 2005).

A schematic illustration of the metabolic network reconstruction procedure is shown in Figure 2-7. Annotated genome sequences are the scaffolds of the metabolic networks, and are available from various databases such as KEGG (http://www.genome.jp/kegg), TIGR (http://www.tigr.org), EBI (www.ebi.ac.uk), NCBI (www.ncbi.nlm.nih.gov), and so forth. The annotation of sequenced genomes involves the identification of genes in a genome, and assigning either predicted or known functions to these identified gene products (Reed et al., 2006), using various computational methods such as BLAST (Altschul et al., 1990) or FASTA (Pearson and Lipman, 1988).

In the next step, the annotated genes and proteins will be associated to the reactions by means of database searches using protein name, EC number or any other assigned identifiers. Some of these biochemical databases include BRENDA (Schomburg et al., 2004), KEGG (Kanehisa and Goto, 2000), Biocyc (Karp et al., 2005) and Expasy proteomic server (Artimo et al., 2012, Gasteiger et al., 2003) as well as TransportDB (Ren et al., 2007) which provides information on transporter proteins in various sequenced genomes. Various levels of information such as substrate and co-enzyme specificity of proteins, charged molecular formula of metabolites, stoichiometric coefficients of the reactions, and the reaction directionalities, which are based on thermodynamic and biochemical data, are required to achieve an accurate metabolic network (Reed et al., 2006). Further metabolic details can be found in scientific literature and textbooks. Gene-Protein-Reaction (GPR) associations also should be defined using Boolean rules for
connecting the reactions in a metabolic network to the genes and proteins which are catalyzing those reactions in a biological system. These GPR associations are useful for linking various types of data, such as genomic, proteomic and fluxomic data (Reed et al., 2006, Francke et al., 2005).

Pathway analysis, for filling in missing metabolic activities or metabolic gaps, is a significant step in metabolic network reconstruction. The metabolic capabilities must be in agreement with the physiology of the organism; for example, if proline is not an essential amino acid in the growth medium, then the metabolic network must include the complete proline biosynthesis pathways, even though some of enzymes are missing in the genome annotation. Thus, it can result in annotation of some genes, which were missed in the annotation process, or in re-annotation of other incorrectly annotated genes (Osterman and Overbeek, 2003, Green and Karp, 2004). Therefore, the central metabolism pathways as well as the biosynthesis pathways of macromolecule building blocks such as amino acids, lipids and nucleotides should be examined. Sequence similarity and orthology searches, phylogenetic relationships, gene context comparisons between different organisms along with experimental evidence for functional associations, such as transcription pattern data, should be considered to achieve high-quality function assignments to the gene products (Francke et al., 2005).
A metabolic network continuously evolves as more knowledge on different aspects of a biological system becomes available, and like the annotation process, it is never complete. Once a metabolic network is reconstructed, its capabilities must be evaluated and further refined. In the network evaluation step, the results of *in silico* analyses using flux balance approach are compared to the available physiological and biochemical information. The metabolic model must be able to generate precursor metabolites, cell biomass components and the cultivation products of an organism; furthermore, the network behaviour should be compatible with experimental results such as cellular phenotypes, growth requirements, and genetic perturbation data. Network evaluation often results in completion of network gaps and refinements to the network or model. Network reconstruction can also be used to evaluate the consistency of various data sets, and to generate experimentally testable hypotheses (Reed et al., 2006).

### 2.3.2 Flux-balance analysis (FBA)

A reconstructed metabolic network can be represented in textual, graphical or mathematical forms. The mathematical representation, which is a stiochiometric matrix \((S)\), is advantageous since it facilitates quantitative analysis of the network using various computational methods (Price et al., 2004). Each row of this matrix corresponds to a metabolite, each column stands for a biochemical reaction, and each element expresses the stiochiometric coefficient of the metabolites in various reactions. Various features of the metabolic network, such as metabolite connectivity and systemic reactions, can be readily known by the analysis of the \(S\) matrix (Reed et al., 2006, Schilling et al., 2000a), and metabolic phenotypes can be characterized as the metabolic flux distributions through the metabolic network which are derived using mathematical modeling approaches (Edwards et al., 2002).

Although vast biological data are available, the lack of detailed kinetic information limits the development of dynamic models of cellular metabolism (Chassagnole et al., 2002); therefore, instead of attempting to calculate the exact metabolic network fluxes, constraint-based modeling approach narrows the range of possible flux distribution under steady state conditions, and thus constrains the possible phenotypes (Edwards et al., 2002). In flux balance analysis (FBA), the constraints are defined by the stoichiometry of metabolic reactions, and flux distributions that satisfy these constraints are derived by optimizing a particular objective function such as growth...
rate or ATP production (Edwards et al., 2002). The FBA is based on the mass conservation law for each metabolite in the network.

Figure 2-8 illustrates an example of defining mass balance constraints (Palsson, 2001); a flux balance for each metabolite \( X_i \) in the network yields a dynamic mass balance equation for each metabolite.

\[
\frac{dX_i}{dt} = V_{syn} - V_{deg} - V_{use} \pm V_{trans}
\]  \hspace{1cm} (1)

The growth stoichiometry and maintenance requirements can be derived from literature data, so if \( b_i \) is the net transport rate of extra-cellular metabolites \( (X_i) \) into the system boundaries, equation (1) can be rewritten as:

\[
\frac{dX_i}{dt} = V_{synth} - V_{deg} - V_{use} + b_i
\]  \hspace{1cm} (2)

Equation (3) represents all the mass balance equations in a matrix form, where \( X \) is an \( m \) dimensional vector that describes the amount of metabolites inside the system boundary, \( v \) is an \( n \) dimensional vector of metabolic reaction fluxes, \( S \) is the \( m \times n \) stoichiometric matrix, and \( b \) is
the vector of exchange fluxes, which transport extracellular metabolites into and out of system boundary.

\[ \frac{dX}{dt} = S.v + b \]  

(3)

Vallino et al. have shown that the steady-state approximation of the metabolite concentrations is a valid approximation. Since the intracellular metabolite concentrations and metabolic fluxes dynamics are rapid (Vallino and Stephanopoulos, 1993), the metabolic transients are very fast in comparison to the time constants of the cell growth. Thus, at a steady-state condition, the accumulation term is set to zero, and the following equation is derived:

\[ S.v + I.b = 0 \]  

(4)

Where \( I \) is the identity matrix, and according to this equation, no metabolite accumulation takes place in the metabolic system, since on time scales longer than the doubling time, all the consumption, formation and transport reactions are balanced (Palsson, 2001). Rearranging the terms of equation (4) yields the following equation, in which \( S' \) is the stoichiometric matrix that includes all metabolic, transport and exchange reactions, and \( v' \) is the vector of all metabolic and exchange fluxes.

\[ S'.v' = 0 \]  

(5)

Since all of the reactions in the metabolic system are balanced, all vectors that satisfy Equation (5), the null space of \( S' \), are steady-state flux distributions, which meet these constraints. These flux distributions describe the cellular phenotypes under different genetic and environmental conditions, although many of these flux vectors are not physiologically feasible, and additional constraints must be imposed to further limit the solution space (Price et al., 2004). In addition to the stoichiometric constraints, flux capacity constraints (Yang et al., 2008), and thermodynamic constraints to generate thermodynamically feasible fluxes through the metabolic network (Henry et al., 2007) have been applied. Reaction directionalities and capacity constraints on the fluxes are introduced as linear inequalities \( \alpha \leq v_i \leq \beta \), where the irreversible reaction fluxes are constrained by setting the lower flux limits to zero, while reversible reactions are not constrained.
Finally, a particular steady-state flux distribution, in this underdetermined system where the number of fluxes often exceeds the number of metabolites, can be determined by applying an optimization approach, for maximization or minimization of an objective function such as growth rate and ATP production (Burgard and Maranas, 2003, Schuetz et al., 2007). Thus, solving the linear programming (LP) formulated optimization problem subject to all constraints will result a specific set of steady-state fluxes, which meet the objective function as well. FBA has been applied for quantitative prediction and analysis of phenotypic behaviours of various microorganisms from various life domains (Varma and Palsson, 1994, Mahadevan et al., 2006, Feist et al., 2006, Oh et al., 2007, Bro et al., 2006).

2.4 Conclusion

Several studies have recently demonstrated the potential of microbial co-cultures as CBP solutions for cellulosic ethanol and butanol production, however little is known about the co-culture physiology and inter-species metabolic interactions. Such knowledge will be valuable in designing co-culture systems with improved yield and productivity of the desired biochemicals in a CBP approach.

Therefore, genome-scale characterization of metabolic interactions in the co-culture of C. cellulolyticum and C. acetobutylicum can bring deep insights into the bottleneck pathways, and facilitate the improvement of this cellulolytic activity using \textit{in silico} generated hypotheses which can be tested experimentally. Furthermore, developing a genome-scale model of metabolism in this co-culture can assist in designing strategies for improving the yield and productivity of biobutanol, which is the desired product in this CBP. Moreover, by means of the developed model of co-culture metabolism, it would be possible to examine the \textit{in silico} production of other valuable chemicals using genetically engineered strains, and to characterize the critical pathways for their production (Lee and Papoutsakis, 1999).

Thus, the eventual goal of this study is to develop methods for the analysis of metabolism in this co-culture, and to investigate the inter-species metabolic interactions for enhancing the metabolic rate and the synthesis of biochemicals from cellulose. The research method involves both computational methods for the development of genome-scale metabolic models, and experimental approaches for the examination of the microbial physiology.
Chapter 3
Understanding *Clostridium acetobutylicum* ATCC 824 Metabolism Using Genome-Scale Thermodynamics and Metabolomics-based Modeling

This chapter contains material published in the conference proceedings below:


**Affiliations:** 1 - Department of Chemical Engineering and Applied Chemistry, University of Toronto, 2 - Department of Biological Sciences, University of Alberta.

**Contributions:** FS designed and conducted the *in silico* and *in vitro* experiments, analyzed the data, and drafted the manuscript. RM\(^2\) and DW\(^2\) carried out the NMR analyses. RM\(^1\) conceived of the experiments and helped to draft the manuscript.

The permission for the reproduction of this material has been granted by the International Federation of Automatic Control (IFAC). In addition to the original document, supplementary information to this chapter including the list of non-gene associated reactions in *C. acetobutylicum* model as well as the method for the metabolomic study (intracellular metabolite extraction and NMR analysis) are provided in Appendices A and B.

### 3.1 Abstract

Biobutanol has a potential application as a biofuel and can replace gasoline as an alternative fuel. However, the productivity of the biobutanol process has to be increased significantly, before it can be commercialized. *Clostridium acetobutylicum* has been the primary microbial host used for biobutanol production. Consequently, systems biology based genome-scale metabolic model of *C. acetobutylicum* metabolism is essential to optimize the biobutanol
production rate via genetic and process optimization and metabolic engineering. An updated genome-scale metabolic model of *C. acetobutylicum* ATCC824 consists of 700 genes, 709 reactions, 29 exchange reactions, and 679 metabolites, which covers 16.3% of ORFs and is more comprehensive compared to two other existing models. This metabolic network was used as a platform for simulating phenotypes using the constraint-based modeling approach. Flux variability analysis shows the presence of alternate carbon and electron sinks that allows for different carbon assimilation patterns and ranges of product formation rates. Incorporation of metabolomic and thermodynamic data into this model results in thermodynamically feasible flux distributions. We apply an NMR based approach to quantify 31 intracellular metabolites in both metabolic phases. The total molar concentration of metabolites was approximately 245 mM, where asparagine was the most dominant metabolite.

### 3.2 Introduction

*Clostridium acetobutylicum* is a Gram-positive, solvent producing, anaerobic bacterium which is able to convert a variety of sugars to organic acids and solvents. In a typical fermentation of this bacterium, two distinct phases are observed; in the acidogenic phase, the bacterium grows rapidly and produces acetate, butyrate, and hydrogen, whereas in the stationary growth phase the metabolic shift to solventogenesis takes place (Jones and Woods, 1986). Several studies have been conducted to increase the solvent yield; The ClosTron, a gene knockout system for the genus *clostridia* has been developed that facilitates further functional genomics studies (Heap et al., 2007), and genome wide transcriptome studies on this species have been performed (Alsaker and Papoutsakis, 2005). Therefore, a systems biology approach that allows the integration of these genome-scale data and the improvement of *C. acetobutylicum* metabolic activity provides a promising method for optimizing the biobutanol production process.

System-level quantitative analysis of cellular metabolism using *in silico* modeling approaches facilitates the generation of new knowledge, via iterative modification of *in silico* models and experimental designs. This knowledge can be applied for designing strains with improved metabolic activities for industrial applications (Sang et al., 2005). Flux balance analysis (FBA) has been applied for analysis and quantitative prediction of phenotypic behaviours of various microorganisms across different biological domains. More recently, FBA has been extended to include genome-scale thermodynamic and metabolomic data (Wang et al., 2006, Henry et al.,
2007, Zamboni et al., 2008) in order to refine the metabolic models and shed light on various aspects of the metabolism.

Lee et al., have reconstructed a genome-scale metabolic model of *C. acetobutylicum* with 11.2% ORF coverage, and applied this model to predict the metabolic behaviours in acidogenic and solventogenic phases using FBA (Lee et al., 2008a). Senger et al., has also presented another genome-scale metabolic model of *C. acetobutylicum* with 12.6% ORF coverage, and applied genetic algorithm to derive a constraint based on the specific proton flux state to further restrict the solution space in FBA, and predict the pH of the batch culture during the acidogenic phase (Senger and Papoutsakis, 2008a, Senger and Papoutsakis, 2008b).

However, these models do not incorporate thermodynamic and metabolomic data analyses. These additional constraints can be applied to further limit the solution space. In this study, an expanded genome-scale metabolic model of *C. acetobutylicum* is presented. We applied thermodynamics-based metabolic flux analysis (Henry et al., 2007), to obtain thermodynamically feasible flux distributions, along with metabolomic data analysis to study the metabolism of *C. acetobutylicum*.

### 3.3 Methods

#### 3.3.1 *C. acetobutylicum* metabolic network reconstruction

The MetaFluxNet®, version 1.82 was the platform used for the model reconstruction ([http://mbel.kaist.ac.kr/lab/mfn/](http://mbel.kaist.ac.kr/lab/mfn/)). The reconstructed network model was then exported to a Systems Biology Markup Language model (SBML; [http://sbml.org](http://sbml.org)), for further analyses using COBRA toolbox (Becker et al., 2007a) in MATLAB environment (The MathWorks™). The automated annotated genome sequence of *C. acetobutylicum* ATCC824 was downloaded from TIGR-CMR database ([http://www.tigr.org/](http://www.tigr.org/)) and was used as a framework for GPR assignments.

The 4.13 Mb-length genome sequence of ATCC824 (Nolling et al., 2001) comprises 4273 ORFs, and the GPR assignments were mostly based on this genome annotation; in addition, the automated metabolic network of ATCC824, reconstructed using Pathway Tools software version 11.5 and MetaCyc version 11.1 ([http://biocyc.org/biocyc-pgdb-list.shtml](http://biocyc.org/biocyc-pgdb-list.shtml)), was used for confirmation of GPR assignments or re-annotations after manual inspection. The model was
reconstructed manually on the metabolic roles in the existing annotation. Biochemical databases such as KEGG (http://www.genome.jp/kegg/) and the enzyme nomenclature database were consulted for more information on pathways, metabolites, and enzymes (http://ca.expasy.org/enzyme/).

The elementally and charge balanced reaction equations were mostly obtained from the BIGG database (http://bigg.ucsd.edu/) and the SimPheny™ database (Genomatica Inc. San Diego, CA); furthermore, the chemical composition and charge of some particular metabolites, which did not exist in the aforementioned databases, were determined using ACD/LogD Sol Suite version 11.0 (http://www.acdlabs.com/) at the physiological pH of 6.7, which is consistent with the intracellular pH of *C. acetobutylicum* (Jones and Woods, 1986). Transport reactions were included in the network according to the transport protein information in KEGG and TransportDB (http://www.membranetransport.org/) databases as well as the available physiological data.

Pathway completion and gap filling were done manually so that the network produces ATP along with all cell biomass building blocks, including amino acids, solute pool, nucleotides, cofactors, lipids and polysaccharides, from available substrates in a defined growth medium (Monot et al., 1982). The BLAST search using UniProt Blast tool (http://www.uniprot.org/) was applied to assign predicted functions to some missing ORFs in the original annotation as well as some ORF re-annotations. The GPR assignments also were done using available literature data. Further network evaluation and pathway completion were done using computational analyses (FBA) of the network using COBRA toolbox; however, network gaps in some poorly characterized pathways can exist even after performing the network evaluation step.

Non-gene associated reactions were added in order to fill the gaps or to meet the physiological requirements according to the available literature data. The cell biomass macromolecular composition was assumed to be identical to the *Bacillus subtilis* (Lee et al., 2008a, Oh et al., 2007), where the composition of nucleotides, protein, lipids and trace elements as well as the required polymerization energy were adapted from the model developed by Lee *et al.* The cell biomass synthesis equation, which includes 40 mmol ATP per gram DCW per hour as the growth associated maintenance energy (GAM) (Lee et al., 2008a, Senger and Papoutsakis, 2008a), was included in the network as a demand reaction.
3.3.2 Flux balance analysis (FBA) and flux variability analysis (FVA)

Metabolic phenotypes can be characterized as the metabolic flux distributions through the metabolic network derived using mathematical modelling approaches (Edwards et al., 2002). The flux balance analysis (FBA) is based on the mass conservation law for each metabolite in the network. Vallino et al. have shown that the steady-state approximation of the metabolite concentrations is a valid approximation, where due to the intracellular metabolite concentrations and rapid metabolic fluxes, the metabolic transients are very fast in comparison to the time constants of the cell growth (Vallino and Stephanopoulos, 1993).

In FBA, the constraints are defined by the stoichiometry of metabolic reactions, and flux distributions that satisfy these constraints, in this underdetermined system, are derived by optimizing a particular objective function such as growth rate or ATP production (Schilling et al., 1999). Thus, solving the linear programming (LP) problem subject to all constraints will result in a specific set of steady-state fluxes, which meet the objective function as well. In the C. acetobutylicum network, an additional constraint is required due to the presence of cyclic acid uptake pathways (Desai et al., 1999). This constraint relates the acetate and butyrate uptake rates based on the enzyme kinetic and selectivity data and metabolite concentrations. The butyrate and acetate concentrations were obtained from chemostat data (Meyer and Papoutsakis, 1989).

\[
\frac{\text{Rate}_{\text{butyrate uptake}}}{\text{Rate}_{\text{acetate uptake}}} = 0.315 \frac{[\text{butyrate}]}{[\text{acetate}]}.
\]  

(1)

However, alternate flux distributions can be achieved using a linear programming technique, under the same optimal condition and objective value (Mahadevan and Schilling, 2003). These alternate flux distributions give an idea about the existence of alternate pathways in the metabolic network. In flux variability analysis (FVA), the optimal objective value is calculated by FBA, and then each flux is maximized and minimized under the same FBA constraints as well as the fixed optimal objective value. The obtained ranges of flux variability demonstrate the feasible fluxes due to alternate optimal solutions in the metabolic network (Mahadevan and Schilling, 2003).
3.3.3 Thermodynamics-based metabolic flux analysis (TMFA)

In addition to the stoichiometric constraints, thermodynamic constraints have been imposed to further limit the solution space (Henry et al., 2007). Thermodynamics-based metabolic flux analysis (TMFA) integrates thermodynamic constraints into a constraint-based metabolic model to generate thermodynamically feasible fluxes through the metabolic network and to provide data on feasible metabolite activity ranges and $\Delta_r G'$ (Henry et al., 2007). The $\Delta_r G'^0$ of the metabolic reactions, required for thermodynamic constraints, were estimated using an improved group contribution method (Jankowski et al., 2008).

3.3.4 Batch cultivation and metabolomic samples

Batch cultivations of *C. acetobutylicum* ATCC 824 were conducted anaerobically at 37 °C in 1.5 litre fermenters on Clostridial Growth Medium (CGM), which contains per litre of distilled water: KH$_2$PO$_4$: 0.75 g; K$_2$HPO$_4$: 0.75 g; MgSO$_4$: 0.348 g; MnSO$_4$.1H$_2$O: 0.01 g; FeSO$_4$.7H$_2$O: 0.01 g; yeast extract: 5.0 g; glucose: 50 g; NaCl: 1.0 g; para amino benzoic acid: 0.004 g; asparagine: 2.0 g and (NH$_4$)$_2$SO$_4$: 2 g (Desai, 1999). The pH of the medium was adjusted to 5.5 before inoculation.

Cell biomass concentration was monitored by measuring OD at 600 nm, while the samples were diluted to keep the optical density below 1.0. Product concentrations were measured using an HPLC system (Biorad Aminex HPX87H column) at 20 °C and 0.5 ml.min$^{-1}$ of 5 mM H$_2$SO$_4$ as eluent. The *C. acetobutylicum* metabolomic samples were taken from three biological replicates in two growth phases, and quenched using liquid nitrogen (Wang et al., 2006). Intracellular metabolite extraction from cell cultures was done according to the method described by Maharajan *et al.*, (Prasad Maharjan and Ferenci, 2003) using hot methanol; the extracted metabolites were then analyzed and quantified by NMR Spectroscopy, at the University of Alberta.
3.4 Results and discussion

3.4.1 Genome-scale network reconstruction and flux balance analysis

The specifications of the iFS700 genome-scale model along with two other previously available genome-scale metabolic models of *C. acetobutylicum* ATCC 824 are illustrated in Table 3-1. The iFS700 includes 709 reactions, 679 metabolites and 700 genes, and has a higher ORF coverage comparing to the other existing models. Furthermore, a number of genes on ATCC 824 annotation file were identified to be mis-annotated in the TIGR-CMR database, and were highlighted by the discrepancies among various database annotations. These genes have been re-annotated with a defined confidence level in this model.

The comparison of the existing model reactions is depicted in Figure 3-1. The major differences in these network reactions are related to fatty acid and phospholipid metabolism, purines and pyrimidines metabolism, and transport reactions. Integration of these three available models will be useful to obtain a comprehensive model of *C. acetobutylicum* metabolism and to develop a universal model (Lee et al., 2008a). Both iJL432 (Lee et al., 2008a) and iRS474 (Senger and Papoutsakis, 2008a) have been reconstructed mostly based on the KEGG database, and are similar in size; while iFS700 is mainly based on TIGR-CMR database. Also, Senger et al. have applied a semi-automated reverse engineering approach for metabolic network reconstruction and gap filling, but iJL432 and iFS700 are mainly manually curated networks.

![Figure 3-1. Venn diagram comparison of the existing model reactions.](image-url)
<table>
<thead>
<tr>
<th>Model Features</th>
<th>iFS700</th>
<th>iRS474</th>
<th>iJL432</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes</td>
<td>700</td>
<td>474</td>
<td>432</td>
</tr>
<tr>
<td>Reactions</td>
<td>711</td>
<td>552</td>
<td>502</td>
</tr>
<tr>
<td>None-gene associated reactions</td>
<td>69</td>
<td>NA</td>
<td>71</td>
</tr>
<tr>
<td>Metabolites</td>
<td>679</td>
<td>422</td>
<td>479</td>
</tr>
<tr>
<td>ORF coverage %</td>
<td>16.3</td>
<td>12.6</td>
<td>11.2</td>
</tr>
<tr>
<td>Re-annotated ORFs</td>
<td>180</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Another major difference of this model is the representation of the TCA cycle. The TCA cycle of *C. acetobutylicum* is incomplete and lacks several key enzymes. The degenerate TCA cycle has been found in many prokaryotes, but in those cases, the pathways of 2-oxoglutarate, succinyl-CoA and fumarate syntheses proceed in both reductive and oxidative directions (Nolling et al., 2001). It was previously suggested that 2-oxoglutarate, which is a key precursor in amino acid biosynthesis pathways, is being produced through either reductive counter clockwise direction of TCA cycle (Nolling et al., 2001, Lee et al., 2008a) or from ornithine through the reverse direction of arginine biosynthesis pathway and urea cycle (Senger and Papoutsakis, 2008a).

However, a recent flux profiling experiment by Amador-Noguez *et al.* demonstrates that the TCA cycle in *C. acetobutylicum* is bifurcated (Amador-Noguez et al., 2011). This suggested that 2-oxoglutarate is being produced through the oxidative side of the TCA cycle from oxaloacetate, despite the putative lack of citrate synthase, and succinate production from oxaloacetate is through the reductive direction of TCA cycle via malate and fumarate. Therefore, citrate synthase was included as a non-gene associated pathway in this model. In *C. acetobutylicum* the succinate dehydrogenase, which reduces fumarate to succinate, is missing and it is assumed in this model that another unknown dehydrogenase catalyzes this reaction (Nolling et al., 2001). Furthermore, the enzyme involved in succiny-CoA formation is missing. This genome encodes a CoA transferase which catalyzes the acetyl-CoA and butyryl-CoA formations in solventogenic phase, and it is plausible that this enzyme also catalyzes the succiny-CoA formation from succinate (Nolling et al., 2001).
To analyse the reconstructed metabolic network, and to simulate the growth and metabolism in a continuous culture (Meyer and Papoutsakis, 1989), FBA was conducted using maximization of cell biomass as the objective function. The results showed that the predicted growth rate is in good agreement with the reported growth rate ($\mu=0.44 \text{ hr}^{-1}$ compared to the experimental value of $\mu=0.43 \text{ hr}^{-1}$); however, the cell produced only acetate, hydrogen and CO$_2$ \textit{in silico}. Therefore, in order to investigate the possibility of the alternate flux distributions, flux variability analysis was performed (Mahadevan and Schilling, 2003).

### 3.4.2 Flux variability analysis

The results of FVA under various sets of stoichiometric, thermodynamic and metabolomic constraints are demonstrated in Figure 3-2a. In these analyses, the growth rate has been fixed to its predicted value by FBA ($\mu=0.44 \text{ hr}^{-1}$). The FVA determines the maximum and minimum values for each flux that can satisfy the model constraints as well as the exact growth rate. In all cases, there are significant flux variation ranges for the product formation rates, and all of the experimental chemostat flux data fall in these model-predicted ranges.

The alternate optimal solutions are due to the presence of equivalent pathways in the metabolic network (Price et al., 2002, Mahadevan and Schilling, 2003). There were 191 reactions with significant variation ranges (above 0.1 mmol.gDCW$^{-1}$. h$^{-1}$), which can be examined for identification of the alternate pathways using extreme pathway analysis (Schilling et al., 2000b). However, alternate pathways are equivalent routes for the production of metabolites, under the same objective value, and cannot describe the various fluxes through these alternative product pathways. These results suggest that these various products act as alternative carbon and electron sinks in \textit{C. acetobutylicum} metabolism. All of these pathways are thermodynamically feasible, and consequently, the fluxes through them are perhaps determined by the regulatory network resulting in different product patterns. The iFS700 model was able to predict the growth rate and flux variability in a chemostat and incorporate metabolomic data.

Furthermore, it is metabolically feasible to have both acids and solvents produced simultaneously in the exponential growth phase (Harris et al., 2000). We applied FVA under predicted maximum growth and butanol formation rates, and these results are depicted in Figure 3-2b. These results suggest that hydrogen and acetate formation pathways are essential under maximum butanol formation rate, while butyrate, ethanol, lactate, acetone and acetoin
pathways can be eliminated. A previous study has also shown that higher amount of butanol is produced in iron limited cultures (Peguin and Soucaille, 1995). Lactate formation pathway is a less efficient alternative for NAD\(^+\) regeneration and energy production, when the activity of hydrogenase enzyme has been blocked or in iron deficient cultures where the levels of ferredoxin and the iron containing hydrogenase are low (Jones and Woods, 1986). Also, in CO-sparged culture of *C. acetobutylicum*, where the activity of hydrogenase enzyme has been blocked using carbon monoxide, a drastic increase in lactic acid formation has been reported (Datta and Zeikus, 1985).

Lee *et al.* (Lee et al., 2008a) have also applied FBA to analyze this metabolic system. Maximization of cell biomass has been the objective function for modelling the acidogenic phase, and minimization of metabolic adjustment (MOMA) (Segre et al., 2002) has been applied to simulate the solventogenic phase. It has been shown that the QP-based approach results are significantly dependant on the reference flux distribution which is obtained from FBA (Mahadevan and Schilling, 2003). So the predicted solventogenic fluxes will be dependent on the predicted fluxes in the acidogenic phase, where there are significant differences in the alternative optimal solutions as illustrated in Figure 3-2.
Figure 3-2. The range of product flux variations for *C. acetobutylicum* metabolic products. FVA under stoichiometric, thermodynamic and metabolomic constraints (A), and FVA under predicted maximum growth and butanol formation rates (B).
3.4.3 Thermodynamics-based metabolic flux analysis

In order to further constrain the model solution space and improve the model predictability, thermodynamic along with metabolomic information were integrated into the model using TMFA. Thermodynamic data of the model reactions were estimated by group contribution method (Jankowski et al., 2008). The upper and lower bound for metabolite concentrations were set at 250 mM and $10^{-9}$ mM; for the measured intracellular metabolites which are presented in Table 3-2, the concentrations were restricted to the observed ranges, and extracellular metabolites were fixed at the measured concentrations. TMFA along with metabolomic constraints predicted the same growth rate and flux ranges obtained using the FBA and FVA. So, all of the reactions involved in the in silico growth, which are not part of internal loops, are thermodynamically feasible. Furthermore, by analyzing the model-predicted ranges for the reaction Gibbs free energies ($\Delta_r G'$) putative regulatory sites (Kummel et al., 2006) in C. acetobutylicum metabolic network can be identified.

3.4.4 Metabolomic data analysis

Knowledge of the intracellular metabolite concentrations can be valuable for further characterizing the metabolic shift and shift inducing conditions in C. acetobutylicum metabolism. Thirty one intracellular metabolites were quantified, and the list of some of these metabolites, with the error estimates, and some of the extracellular concentrations are presented in Table 3-2. The total molar concentration of metabolites was approximately 245 mM which is comparable with the E. coli metabolite pool of 300 mM (Bennett et al., 2009). Measured metabolites are mainly amino acids, which are present at the same level in both phases. The most dominant metabolite after glucose was asparagine, followed by alanine and glutamate. This is in contrast to the E. coli metabolome, where glutamate was the most abundant compound (Bennett et al., 2009). Nucleotides and redox coenzymes could be identified but were present in very low amounts, so could not be quantified. In terms of the significance of the changes in metabolite concentrations, butyrate and acetate concentrations showed a significant increase in solventogenic phase, while phenylalanine, 2-oxoglutarate and fumarate showed significant decrease during solventogenesis consistent with their role as precursors for cell biomass synthesis.
Table 3-2. Intracellular and extracellular metabolite concentrations in acidogenic and solventogenic phases. Standard deviations are based on three biological replicates, as presented in the parentheses.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Acidogenic phase</th>
<th>Solventogenic phase</th>
<th>Significant variation in intracellular concentrations between two phases, at 95% confidence level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intracellular</td>
<td>Extracellular</td>
<td>Intracellular</td>
</tr>
<tr>
<td></td>
<td>(mM)</td>
<td>(mM)</td>
<td>(mM)</td>
</tr>
<tr>
<td>Glucose</td>
<td>221 (47)</td>
<td>270 (2)</td>
<td>160 (54)</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.99 (0.36)</td>
<td>6 (5)</td>
<td>2.62 (0.34)</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.07 (0.04)</td>
<td>0.15 (0.1)</td>
<td>0.05 (0.02)</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0.81 (0.38)</td>
<td>10 (9)</td>
<td>5.12 (1.44)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.12 (0.01)</td>
<td>2 (9)</td>
<td>0.34 (0.11)</td>
</tr>
<tr>
<td>Formate</td>
<td>0.21 (0.05)</td>
<td>0.12 (0.1)</td>
<td>0.11 (0.02)</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.39 (0.11)</td>
<td>4 (7)</td>
<td>0.30 (0.05)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.09 (0.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>1.73 (0.46)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.25 (0.25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>14.1 (3.75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>0.17 (0.05)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.53 (0.09)</td>
<td>0.21 (0.15)</td>
<td></td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>0.07 (0.02)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>0.02 (0.01)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.01 (7×10⁻⁴)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>0.29 (0.02)</td>
<td>0.21 (0.11)</td>
<td></td>
</tr>
<tr>
<td>Isocitrate</td>
<td>0.18 (0.06)</td>
<td>0.60 (0.41)</td>
<td></td>
</tr>
</tbody>
</table>

3.5 Conclusion

Flux balance analysis cannot predict the complex metabolism of *C. acetobutylicum* properly, and additional constraints are required to narrow down the solution space. Previously, specific proton flux state (Senger and Papoutsakis, 2008b) was developed for this purpose; here we incorporated thermodynamic and metabolomic data. Although it did not improve the predictability of the model, it allows for thermodynamically feasible flux distributions. On the other hand, incorporating the regulatory network in the genome-scale model is necessary for describing the metabolic shift from acidogenic to solventogenic phase (Covert et al., 2001b).
The intracellular metabolome of *C. acetobutylicum* was quantified in two phases, but a dynamic metabolome profiling experiment is necessary to trace the intracellular metabolite concentration changes over time and during the metabolic shift.
Chapter 4
Genome-scale Metabolic Modeling of a Clostridial Co-culture for Consolidated Bioprocessing

This chapter contains material from our publication:

Affiliations: 1 - Department of Chemical Engineering and Applied Chemistry, University of Toronto, 2 - Present Address: Novo Nordisk Foundation Center for Biosustainability, Denmark.

Contributions: FS conceived of the study, reconstructed the metabolic network, conducted the modeling, and drafted the manuscript. KZ carried out the DMM simulations. RM conceived of the study and helped to draft the manuscript.

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4.1 Abstract
An alternative consolidated bioprocessing approach is the use of a co-culture containing cellulolytic and solventogenic clostridia. It has been demonstrated that the rate of cellulose utilization in the co-culture of *Clostridium acetobutylicum* and *Clostridium cellulolyticum* is improved compared to the mono-culture of *C. cellulolyticum* suggesting the presence of synergy between these two species. However, the metabolic interactions in the co-culture are not well understood. In order to understand the metabolic interactions in the co-culture, we developed a genome-scale metabolic model of *C. cellulolyticum* comprising of 431 genes, 621 reactions, and 603 metabolites. The *C. cellulolyticum* model can successfully predict the chemostat growth and byproduct secretion with cellulose as the substrate. However, a growth arrest phenomenon, which occurs in batch cultures of *C. cellulyoticum* at cellulose concentrations higher than 6.7 g/L, cannot be predicted by dynamic flux balance analysis due to the lack of understanding of the underlying mechanism. Furthermore, these genome-scale metabolic models of the pure cultures have been integrated using a community modeling framework to develop a dynamic model of metabolic interactions in the co-culture. Co-culture
simulations suggest that cellobiose inhibition cannot be the main factor that is responsible for improved cellulose utilization relative to mono-culture of *C. cellulolyticum*.

### 4.2 Introduction

In consolidated bioprocessing (CBP), all four biological steps involved in the conversion of cellulosic biomass, which includes production of saccharolytic enzymes, the hydrolysis of the polysaccharides present in pre-treated biomass, and fermentation of hexose and pentose sugars present in the hydrolyzate to desired products occur in one bioreactor. CBP uses a single microorganism or a microbial consortium without the external addition of saccharolytic enzymes (Lynd et al., 2002). Consolidated bioprocessing has been proposed to decrease the product cost by eliminating substrate costs, utility, capital and other costs related to the cellulase production stage. However, microorganisms with both rapid conversion of cellulose and high-titer product formation capabilities required for CBP do not currently exist and need to be developed (Lynd et al., 2005); current strategies for this purpose include the native strategy which improves the product formation capabilities, such as yield and titer in natural cellulolytic microorganisms, while the recombinant strategy involves engineering non-cellulolytic organisms with high product yields so that they will express heterologous cellulase and be able to utilize cellulose (Lynd et al., 2005).

Clostridia are among the metabolically diverse group of bacteria and include both cellulolytic and solventogenic members. This metabolic diversity motivates an alternative method for the production of biobutanol from cellulosic biomass in a consolidated bioprocessing approach using a mesophilic clostridial co-culture. *C. acetobutylicum* shows an effective capability to ferment hemicellulose derived sugars as well as cellulose derived sugars such as cellobiose, mannose, arabinose, xylose, glucose, and galactose to acetone, butanol, and ethanol (Yu et al., 1984, Fond et al., 1986); therefore, the co-culture of this bacterial species with a mesophilic cellulose degrading bacterium can be an alternative option for CBP.

A simplified scheme of the clostridial co-culture fermentation on cellulose is presented in Figure 4-1. *C. acetobutylicum* is a Gram-positive, solvent producing, anaerobic bacterium which is able to convert a variety of sugars to organic acids and solvents. In a typical fermentation of this bacterium, two distinct phases are observed; in the acidogenic phase, the bacterium grows rapidly and produces acetate, butyrate, and hydrogen, whereas in the
stationary growth phase, the metabolic shift to solventogenesis takes place (Jones and Woods, 1986). \textit{C. cellulolyticum}, which is cellulolytic mesophilic bacterium, synthesizes cellulosome, which is an extracellular multi-enzymatic complex, and degrades cellulose to glucose and soluble celdextrins (mainly cellobiose) with the use of this cellulosome. This extracellular complex comprises a variety of cellulases, hemicellulase, and pectinase, which are arranged on a protein scaffold (CipC) (Guedon et al., 1999b, Desvaux, 2005a). The cellulosomes are located on the cell surface and facilitate both cell adhesion as well as cellulolytic activity on the cellulose fibers. However, in comparison to \textit{C. thermocellum}, the cellulosome of \textit{C. cellulolyticum} is smaller and less complex (Felix and Ljungdahl, 1993).

![Figure 4-1. The scheme of the clostridial co-culture fermentation on cellulose. \textit{C. cellulolyticum} adheres to the cellulose fibers using cellulosome and hydrolyzes cellulose to cellobiose (cellb) and glucose (glc), which can be metabolized by \textit{C. cellulolyticum} and \textit{C. acetobutylicum} in the co-culture. The produced pyruvate (pyr) can also be fermented as a carbon source by \textit{C. acetobutylicum}. The co-culture of \textit{C. cellulolyticum} with \textit{C. acetobutylicum} has been studied previously, and it has been shown that cellulolytic activity is the limiting factor in the co-culture fermentation since most of the cellulase activity products are consumed by \textit{C. acetobutylicum}. The fermentation products are mainly butyric acid along with butanol, acetic acid and ethanol, and the lack of glucose, which is required for solvent production due to low cellulolytic activity, was hypothesized to be the reason for acid accumulation (Jones and Woods, 1986,}
Petitdemange et al., 1983). Furthermore, three times more cellulosic material was degraded in the co-culture compared to the mono-culture of *C. cellulolyticum*; due to the utilization of cellulase activity products and the elimination of their repressive effects (Mitchell et al., 1995), suggesting the presence of synergy between these two species. Hence, the analysis of this effect can be valuable for optimizing the rate of cellulosic material degradation.

Limited growth and catabolic activity on cellulose is the common characteristic of the currently known cellulolytic bacteria (Desvaux, 2005b). Improving the cellulolytic and catabolic activity of *C. cellulolyticum* by metabolic engineering (Guedon et al., 2002) can facilitate the shift of co-culture metabolism to the solventogenic phase. However, it has been demonstrated that the central metabolism of *C. cellulolyticum*, rather than the cellulose hydrolysis rate, is the limiting step in cellulose metabolism; therefore, the improvement of *C. cellulolyticum* metabolism which is not adapted to high catabolic rates, instead of the catalytic activity of the cellulosome is critical in order to improve its cellulolytic activity (Guedon et al., 2002, Desvaux, 2005b). Therefore, genome-scale metabolic modeling of *C. cellulolyticum* metabolism can provide an improved understanding of the bottleneck pathways, and facilitate the improvement of this metabolic and cellulolytic activity using *in silico* generated hypotheses which can be tested experimentally. Furthermore, developing a genome-scale model of metabolism in this co-culture can assist in designing strategies for improving the yield and productivity of bioproducts via CBP.

Lee et al., has reconstructed a genome-scale metabolic model of *C. acetobutylicum* with 11.2% ORF coverage, and applied this model to predict the metabolic behavior in acidogenic and solventogenic phases using FBA (Lee et al., 2008b). Senger et al., has also presented another genome-scale metabolic model of *C. acetobutylicum* with 12.6% ORF coverage, and applied genetic algorithm to derive a constraint based on the specific proton flux state to further restrict the solution space in FBA, and predict the pH of the batch culture during the acidogenic phase (Senger and Papoutsakis, 2008b).

In this study, we present the genome-scale metabolic model of *C. cellulolyticum* H10, which comprises 431 genes, 621 reactions, 603 metabolites, and covers 12% of ORFs. Also, we developed an updated genome-scale metabolic model of *C. acetobutylicum* ATCC824 which consists of 700 genes, 712 reactions, and 679 metabolites, covering 16.3% of ORFs and is more comprehensive compared to two other existing models. These metabolic networks were used as
platforms for simulating phenotypes using the constraint-based modeling approach. C. cellulolyticum model (iFS431) was able to predict the physiology during growth in the presence of cellulose and cellobiose. These models were used to develop a model of co-culture metabolism by combining these metabolic models of the pure cultures using the DMMM framework (dynamic multi-species metabolic modeling framework, also illustrated in Appendix C, Figure A1) (Zhuang et al., 2011). This co-culture model was used to analyze the integrated physiology of this co-culture, and the results showed that at high cellulose concentrations, the model is not able to capture the C. cellulolyticum growth arrest, suggesting that cellobiose inhibition removal by C. acetobutylicum is not the main factor that improves cellulose degradation in the co-culture. Further investigation to understand the mechanism behind this growth arrest is required to improve the model predictions at high cellulose concentrations as well as predictions of the co-culture physiology.

4.3 Methods

4.3.1 C. acetobutylicum and C. cellulolyticum metabolic network reconstructions

The automatically annotated genome sequence of C. acetobutylicum ATCC824 was downloaded from TIGR-CMR database (http://www.tigr.org/) and was used as a framework for gene-protein-reaction (GPR) assignments. The 4.13Mb-length genome sequence of ATCC824 (Nolling et al., 2001) comprises 4273 ORFs, and the GPR assignments were mostly based on this genome annotation; in addition the automated metabolic network of ATCC824, reconstructed using Pathway Tools software version 11.5 and MetaCyc version 11.1 (http://biocyc.org/biocyc-pgdb-list.shtml), were used for confirmation of GPR assignments or re-annotations after manual inspection.

The model was reconstructed manually based on the metabolic roles in the existing annotation, and biochemical databases such as KEGG (http://www.genome.jp/kegg/) and the enzyme nomenclature database were consulted for more information on pathways, metabolites, and enzymes (http://ca.expasy.org/enzyme/). Transport reactions were included in the network according to the transport protein information in KEGG and TransportDB (http://www.membranetransport.org/) databases as well as the available physiological data. The cell biomass synthesis equation of C. acetobutylicum, which includes 40 mmol ATP per gram
DCW per hour as the growth associated maintenance energy (GAM) (Lee et al., 2008b, Senger and Papoutsakis, 2008b) was included in the network as a demand reaction. *C. cellulolyticum* H10 Metabolic network was reconstructed based on the genome annotation available on JGI database (http://www.jgi.doe.gov/) for GPR associations, and using the available literature and experimental data. To find the transporter genes in the *C. cellulolyticum* H10 genome reciprocal blast was conducted, where the entire amino acid sequences were blasted against Transport Classification Database (http://www.tcdb.org/) at the cut off e-value equal of $10^{-5}$. The candidate orthologous genes then were assigned to the corresponding transport reactions. Non-growth associated maintenance (NGAM) for growth of *C. cellulolyticum* on cellobiose has been reported to be 2.2 mmol ATP per gram DCW per hour and 2.9 mmol ATP per gram DCW per hour for growth on cellulose (Desvaux et al., 2001b).

The MetaFluxNet® version 1.82 was the platform used for the model reconstructions (http://mbel.kaist.ac.kr/lab/mfn/), and the reconstructed network models were then exported to Systems Biology Markup Language models (SBML; http://sbml.org), for further analyses using COBRA toolbox (Becker et al., 2007b) in MATLAB environment (The MathWorks™). The elementally and charge balanced reaction equations were mostly obtained from the BIGG database (http://bigg.ucsd.edu/) and SimPheny™ database (Genomatica Inc. San Diego, CA); furthermore, the chemical composition and charge of some particular metabolites, which did not exist in the aforementioned databases, were determined using ACD/LogD Sol Suite version 11.0 (http://www.acdlabs.com/) at the physiological pH of 6.7, which is consistent with the intracellular pH of *C. acetobutylicum* (Jones and Woods, 1986).

Pathway completion and gap filling were done manually so that the networks produce ATP along with all cell biomass building blocks, including amino acids, solute pool, nucleotides, cofactors, lipids and polysaccharides, from available substrates in their defined growth media (Monot et al., 1982, Guedon et al., 1999b). The BLAST search using UniProt BLAST tool (http://www.uniprot.org/) was applied to assign predicted functions to some missing ORFs in the original annotations as well as some ORF re-annotations. The GPR assignments also were done using available literature data. Further network evaluation and pathway completion were done using the COBRA toolbox; however, network gaps in some poorly characterized pathways can exist even after performing the network evaluation step. Non-gene associated
reactions were added in order to fill the gaps or to meet the physiological requirements according to the available literature data manually and using FBA.

These reactions are detailed in the supplementary information (Appendix A, available at http://onlinelibrary.wiley.com/doi/10.1002/biot.201000159/full#supp-info). The cell biomass macromolecular compositions were assumed to be identical to the *Bacillus subtilis* (Lee et al., 2008b, Oh et al., 2007), where the composition of nucleotides, protein, lipids and trace elements as well as the required polymerization energy were adapted from the model developed by Lee *et al.* (Lee et al., 2008b).

### 4.3.2 Flux balance analysis (FBA)

Metabolic phenotypes can be predicted by calculating the flux distributions through the metabolic networks using constraint-based modeling (Feist et al., 2009, Price et al., 2004, Henry et al., 2007). The flux balance analysis (FBA) is based on the mass conservation law for each metabolite in the network. Vallino *et al.* (Vallino and Stephanopoulos, 1993) have shown that the steady-state approximation of the metabolite concentrations is a valid approximation, where due to the intracellular metabolite concentrations and rapid metabolic fluxes, the metabolic transients are very fast in comparison to the time constants of the cell growth. In FBA, the constraints are defined by the stoichiometry of metabolic reactions, and flux distributions that satisfy these constraints, in this underdetermined system, are derived by optimizing a particular objective function such as growth rate or ATP production (Schilling et al., 2000a). Thus, solving the linear programming (LP) problem subject to all constraints will result in a specific set of steady-state fluxes, which maximizes the objective function as well. In the *C. acetobutylicum* network, an additional constraint is required due to the presence of cyclic acid uptake pathways (Desai et al., 1999). This constraint relates the acetate and butyrate uptake rates based on the enzyme kinetics and selectivity data and metabolite concentrations. The butyrate and acetate concentrations were obtained from chemostat data (Meyer and Papoutsakis, 1989); \( \text{Rate}_{\text{butuptake}} \) and \( \text{Rate}_{\text{acuptake}} \) are the rate of butyrate and acetate uptake, and \( C_{\text{butyrate}} \), \( C_{\text{acetate}} \) are extracellular butyrate and acetate concentration.

\[
\frac{\text{Rate}_{\text{butuptake}}}{\text{Rate}_{\text{acuptake}}} = 0.315 \frac{C_{\text{butyrate}}}{C_{\text{acetate}}}
\]  (1)
However, alternate flux distributions can be achieved, using the linear programming technique, under the same optimal condition and objective value (Mahadevan and Schilling, 2003). These alternate flux distributions provide information on the existence of alternate or redundant pathways in the metabolic network. In flux variability analysis (FVA), the optimal objective value is calculated by FBA, and then each flux is maximized and minimized under the same FBA constraints as well as the fixed optimal objective value.

4.3.3 Co-culture model development

The scheme of cellulose fermentation by clostridial co-culture has been illustrated in Figure 4-1. *C. cellulolyticum* can attach to the cellulose fibrils via the carbohydrate binding domains of the cellulosome, which is an extracellular multi-enzymatic complex located on the cell surface of *C. cellulolyticum* (Desvaux, 2005a). Due to the cellulytic activity of the cellulosome, soluble sugars are released where cellobiose is the major product of cellulose hydrolysis (Guedon et al., 1999b), and it has been shown that glucose constitutes 30% of the cellulose hydrolysis products (Giallo et al., 1985). So in this study, we assumed the following stoichiometry for the cellulase reaction, which takes place in *C. cellulolyticum* extracellular compartment by means of the cellulosome complex, in the model:

\[ G_{cell} + 0.65H_2O \rightarrow 0.35Cellb + 0.3glc \]  \hspace{1cm} (2)

The co-culture system dynamics can be described using the following equations (3-11), which are based on the dynamic multi-species metabolic modeling framework (Zhuang et al., 2011) (also illustrated Appendix C). The Michaelis-Menten kinetic parameters of cellulose solubilisation and metabolism by *C. cellulolyticum* have been reported previously, where \( v_{max} \) is 2.91 mmol/gDCW.h (Desvaux et al., 2001c), and \( K_m \) is equal to 0.8 g hexose equivalent per litre (Giallo et al., 1985). It has been assumed that cellobiose inhibits the function of *C. cellulolyticum* cellulosome through a competitive inhibitory mechanism, similar to what has been demonstrated for the *C. thermocellum* cellulosome (Kruus et al., 1995); the \( K_i \) value for competitive inhibition of the endogluconaseA component of the cellulosome is equal to 11 mM (Fierobe et al., 1991), and the \( K_i \) value of about 2 mM has been reported for the *C. thermocellum* cellulosome (Kruus et al., 1995).
\[ V_{\text{cellulase}} = \frac{v_{\text{max}} C_{\text{Gcell}}}{(1 + \frac{C_{\text{cellb}}}{k_i}).K_m + C_{\text{Gcell}}} \] (3)

\[ \frac{dC_{\text{Gcell}}}{dt} = -V_{\text{cellulase}} X_{cc} \] (4)

\[ \frac{dC_{\text{cellb}}}{dt} = 0.35V_{\text{cellulase}} X_{cc} - V_{\text{cellb}} X_{cc} \] (5)

\[ \frac{dC_{\text{glc}}}{dt} = -V_{\text{glc}} X_{cc} - V_{\text{glc}} X_{cc} + 0.3V_{\text{cellulase}} \] (6)

\[ \frac{dC_{\text{pyr}}}{dt} = V_{\text{pyr}} X_{cc} - V_{\text{pyr}} X_{cc} \] (7)

\[ \frac{dX_{ca}}{dt} = \mu_{ca} X_{ca} \] (8)

\[ \frac{dX_{cc}}{dt} = \mu_{cc} X_{cc} \] (9)

Max \( \mu_{ca} \)

Subject to: \( S_{ca}. V_{ca} = 0 \)

\[ V_{\text{glc}} \leq \frac{15.1C_{\text{glc}}}{3.56 + C_{\text{glc}}}, \quad V_{\text{cellb}} \leq \frac{2.47C_{\text{cellb}}}{1 + C_{\text{cellb}}}, \quad V_{\text{pyr}} \leq \frac{5.91C_{\text{pyr}}}{1 + C_{\text{pyr}}} \]

Max \( \mu_{cc} \)

Subject to: \( S_{cc}. V_{cc} = 0 \)

\[ V_{\text{glc}} \leq \frac{6.01C_{\text{glc}}}{20.22 + C_{\text{glc}}}, \quad V_{\text{cellb}} \leq \frac{5.01C_{\text{cellb}}}{20.22 + C_{\text{cellb}}} \]

Where \( C_{\text{Gcell}}, C_{\text{cellb}}, C_{\text{glc}}, C_{\text{pyr}}, C_{\text{ac}}, C_{\text{lac}} \) are cellulose, cellobiose, glucose, pyruvate, acetate and lactate concentrations, \( V \) is reaction rate obtained from FBA, and \( X_{ca} \) and \( X_{cc} \) are \( C. acetobutylicum \) and \( C. cellulolyticum \) biomass concentrations. Kinetic data for \( C. cellulolyticum \) growth on cellobiose (Desvaux et al., 2001b, Guedon et al., 2000) and glucose (Giallo et al., 1983), and \( C. acetobutylicum \) growth on cellobiose (Lopez-Contreras et al., 2003), glucose (Yang and Tsao, 1994, Houston and Papoutsakis, 1989), pyruvate (Janati-Idrissi et al., 1989), acetate and lactate (Diez-Gonzalez et al., 1995) were incorporated into the constraints. For
modeling cellulose fermentation in batch and the co-culture, the upper limit of cellobiose uptake rate has been set to 0.76 mmol/gDCW.h to fit the experimental data (Desvaux et al., 2001c). Hence, $\mu^c$ and $\mu^{cc}$ were obtained by solving the FBA problem for each species in equations (10-11), using maximization of growth rates as the objective functions, and the integration of these ODEs was performed using established methods in MATLAB (Covert et al., 2008).

4.4 Results and discussion

4.4.1 C. cellulolyticum metabolic network analysis

4.4.1.1 Central metabolism and energetics

The genomic features and specifications of C. cellulolyticum H10 (iFS431) metabolic network are shown in Figure 4-2a and b. Aligning C. cellulolyticum and C. acetobutylicum genomes using reciprocal BLAST at e-value cut off of $10^{-2}$ suggests the existence of 1142 putative orthologous genes between these two species, out of which 180 of them are currently present in the C. acetobutylicum metabolic network. The iFS431 network includes 603 metabolites, 621 reactions associated to 431 genes, and 68 reactions have been added as non-gene associated reactions in order to fill the gaps or to meet the physiological requirements according to the available literature data (Guedon et al., 1999b). This reconstruction is mainly based on the genome annotation available on JGI database. The TCA cycle and central metabolism pathways of C. cellulolyticum have not been characterized yet; however, this species is capable of producing succinate (Giallo et al., 1983). Therefore, succinate dehydrogenase is included in the model as a non-gene associated reaction in the TCA cycle. Like C. acetobutylicum, the TCA cycle of C. cellulolyticum also seems to be bifurcated, and it is missing succinate CoA ligase.

C. cellulolyticum degrades cellulose to glucose and soluble celloextrins (mainly cellobiose) using its extracellular cellulosome. It has been suggested that the bioenergetic cost of cellobextrin transport is independent of the celloextrin degree of polymerization (Lynd et al., 2002), and for C. cellulolyticum 2 mol ATP is being consumed per each mole of substrate transported through ABC transport system. The energetic cost of cellulosome biosynthesis has been included in the GAM value. Furthermore, there is an ATP gain associated with
phosphorylhetic cleavage of the celldextrins (EC. 2.4.1.20, EC 2.4.1.49), which can explain the higher cell biomass yield on cellobiose compared to glucose (Lynd et al., 2002).

### Table

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>The genome sequence length</td>
<td>4.1 Mb</td>
</tr>
<tr>
<td>Number of protein coding genes</td>
<td>3575</td>
</tr>
<tr>
<td>Number of genes included in the reconstructed network</td>
<td>431</td>
</tr>
<tr>
<td>ORF coverage (%)</td>
<td>12</td>
</tr>
<tr>
<td>The calculated number of orthologs among <em>C. acetobutylicum</em> and <em>C. cellulolyticum</em></td>
<td>1148</td>
</tr>
<tr>
<td>The calculated number of orthologous genes that exist in <em>acetobutylicum</em> model</td>
<td>C. 180</td>
</tr>
</tbody>
</table>

**Reactions/Metabolites**

<table>
<thead>
<tr>
<th>Reaction Type</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of model reactions</td>
<td>621</td>
</tr>
<tr>
<td>Gene associated reactions</td>
<td>513</td>
</tr>
<tr>
<td>Non-gene associated reactions</td>
<td>71</td>
</tr>
<tr>
<td>Exchange reactions</td>
<td>37</td>
</tr>
<tr>
<td>Transport reactions</td>
<td>45</td>
</tr>
<tr>
<td>Total number of metabolites</td>
<td>603</td>
</tr>
<tr>
<td>Number of extracellular metabolites</td>
<td>46</td>
</tr>
</tbody>
</table>

**Figure 4-2.** (a) Distribution of reactions in *C. cellulolyticum* metabolic network, and (b) the genomic features and specifications of *C. cellulolyticum* H10 metabolic network.
This higher yield is also predicted by the model. Growth associated maintenance energy (GAM) was estimated using the available chemostat data (Guedon et al., 1999b) by minimizing the error between the model-predicted growth rate and the experimental data at various dilution rates on cellobiose, as shown in Figure 4-3e. The estimated GAM value of 15 mmol ATP per gram DCW per hour was included in the cell biomass synthesis equation (supplementary Table, at http://onlinelibrary.wiley.com/doi/10.1002/biot.201000159/full#supp-info).

4.4.1.2 Analysis of by-product secretion patterns in C. cellulolyticum during growth on cellobiose

The FBA was used to simulate the flux distributions in the reconstructed network in continuous cultures of C. cellulolyticum on cellobiose and cellulose (Guedon et al., 1999b, Desvaux et al., 2001b). The maximization of growth rate was selected as the objective function, and the results of FBA for the growth on cellobiose are shown in Figure 4-3d. The growth simulation on cellobiose demonstrates that acetate formation rate is significantly higher than the experimental data. C. cellulolyticum is a fermentative anaerobe and produces energy through the acetate formation pathway. To investigate the reason for high acetate flux and the presence of futile cycles, FVA on the acetate formation pathway under the same conditions and predicted growth rate of 0.116 h\(^{-1}\) were conducted. The FVA showed that for this growth rate, the minimum required ATP formation and acetate flux is 4.49 mmol/gDCW.h and rejects the presence of futile cycles. Another explanation for this can be the existence of alternate energy production pathways in C. cellulolyticum, as it has been shown recently that this genome encodes an energy-converting [NiFe] hydrogenase (Calusinska et al., 2010), which can couple the exergonic hydrogen formation reaction to the formation of a proton or sodium-ion motive force and energy conservation through oxidative level phosphorylation (Hedderich, 2004).

In addition, the model did not predict pyruvate formation while it has been observed that pyruvate is being produced at high dilution rates or carbon fluxes (Guedon et al., 2002), and it has been suggested that limited pyruvate-ferredoxin oxidoreductase (PFO) activity which cannot support high pyruvate flow is the reason of this pyruvate overflow (Guedon et al., 1999b). Furthermore, high pyruvate affinity of PFO (\(k_m=0.57 \text{ mM}\)), as well as the low pyruvate affinity of lactate dehydrogenase (LDH, \(k_m=4.5 \text{ mM}\)), can explain the intracellular pyruvate accumulation and overflow (Guedon et al., 1999b). Also, as shown in Figure 4-3a, simulation of the C. cellulolyticum metabolism without any constraint on the LDH and PFO, at high
cellobiose uptake rate (1.86 mmol/gDCW.h), showed that pyruvate will not be produced if there is no capacity constraint on these two reactions. Therefore, the upper bound for the LDH flux was set to the maximum observed lactate production rate, which is 0.5 mmol/gDCW.h (Guedon et al., 1999b), and by minimizing the error in predicted pyruvate formation rate, the upper bound for the flux through PFO was found to be 5.45 mmol/gDCW.h. The constraint on hydrogen formation flux has been set to $q_{H_2} = 4q_{cellb}$ based on the fermentation stoichiometry (Guedon et al., 1999b), and simulation results with expression of pyruvate decarboxylase (PDC) pathway showed that PDC pathway will not be active unless there is a constraint on hydrogen formation rate. Furthermore, Figure 4-3b and c show that removing these constraints on PFO, LDH and hydrogen production pathways decrease the model prediction accuracy.
(B) Model prediction with only H₂ constraint

(C) Model prediction without LDH constraint
Figure 4-3. Model predicted metabolism of *C. cellulolyticum* in chemostat culture on cellobiose. (a) model predicted cellobiose metabolism with no constraint on H$_2$ formation, LDH and PFO pathways; (b) model predicted cellobiose metabolism with no constraint on LDH and PFO pathways (c) model predicted cellobiose metabolism with no constraint on LDH pathway; (d) Model predicted product formation rates of *C. cellulolyticum* on cellobiose, D=0.12 h$^{-1}$, and comparison with the experimental data (Guedon et al., 1999b); (e) Goodness of fit for *C. cellulolyticum* growth rate data on cellobiose at various dilution rates (Guedon et al., 1999b) and GAM=15 mmol/gDCW.h ; bm, celb, eth, ac, lac, pyr stand for cell biomass, cellobiose, ethanol, acetate, lactate and pyruvate.
4.4.1.3 Analysis of *C. cellulolyticum* growth physiology on cellulose

In order to validate the model prediction, which was calibrated using cellobiose growth data, we compared the model simulations with cellulose as the substrate with experimental data. Modeling results show that the *i*FS431 model is able to appropriately predict the cellulose metabolism, growth rates and product formation rates, as depicted in Figure 4-4a and e. We used cellobiose growth data for prediction of the GAM energetic parameter, and then used this calibrated model to predict the cellulose growth data. However, due to the cellulosome synthesis cost, the GAM value for growth on cellulose could be higher. As this cost has not been considered in the model, which was calibrated on cellobiose, the model over-predicts the growth rates in the Figure 4-4a. The rate of acetate formation is higher when *C. cellulolyticum* H10 is growing on cellulose, while it has higher growth rate on the cellobiose; this discrepancy might be related to the ATP cost of cellulosome biosynthesis, which is higher for the growth on cellulose, and leads to more ATP formation via acetate production pathway. The acetate and ethanol formation rates are properly predicted by the model; however lactate and pyruvate secretion rates, which are significantly less than cellobiose growth case, could not be captured by the model. The impact of imposing the constraints on PFO, LDH and hydrogen production pathways on the model prediction of cellulose metabolism are illustrated in Figure 4-4b to d, to support the presence of these constraints.
(B) Model predictions with no constraints

(C) Model predictions with only H₂ constraint
Figure 4-4. Model predicted cellulose metabolism in chemostat culture by *C. cellulolyticum*. (a) Model prediction of *C. cellulolyticum* growth rate on cellulose at various experimental dilution rates (Desvaux et al., 2001c); (b) model predicted cellulose metabolism with no constraint on H\textsubscript{2} formation, LDH and PFO pathways; (c) model predicted cellulose metabolism with no constraint on LDH and PFO pathways; (d) model predicted cellulose metabolism with no constraint on LDH pathway; (e) *C. cellulolyticum* model predictions on cellulose as carbon source and D=0.08 h\textsuperscript{-1}, and comparison with the experimental data (Desvaux et al., 2001c); bm, cel, eth, ac, lac, pyr stand for cell biomass, cellulose, ethanol, acetate, lactate and pyruvate, and it has been assumed that cellobiose is the major product of cellulose hydrolysis.
4.4.1.4 Analysis of *C. cellulolyticum* batch growth on cellulose

We also applied dynamic flux balance analysis (dFBA) (Mahadevan et al., 2002) to model the metabolism of *C. cellulolyticum* in a batch culture on cellulose (Desvaux et al., 2001a). The results of this simulation with the initial cellulose concentration of 6.7 g/L and initial cell biomass concentration of 20 mg/L are shown in Figure 4-5a. Under these conditions, *C. cellulolyticum* degrades approximately 80% of the cellulose and shifts to its stationary growth phase before complete cellulose exhaustion, which is due to a growth arrest and is thought to be caused by the accumulation of an intracellular metabolite (Desvaux et al., 2000). This intracellular growth arrest, which has been shown to happen when the cellulose concentration is higher than 3.9 g/L, cannot be captured using FBA; however, in the exponential growth phase, the initial cellulose degradation and cell biomass growth patterns are well predicted by the model.

Modeling this phenomenon requires the knowledge of the underlying growth arrest mechanism and is a topic for further experimental studies. The results of dFBA for simulating the cellulose hydrolysis by *C. cellulolyticum* in a batch culture at initial cellulose concentration of 3.9 g/L, where no growth arrest occurs, are depicted in Figure 4-5b; the model is able to predict the cellulose hydrolysis accurately and there is a good agreement between the model prediction and the experimental data (Desvaux et al., 2000); also, the predicted final cell biomass concentration of 364 mg/L is in accordance with the reported experimental value of 340 mg/L. Moreover, in batch cultures of *C. cellulolyticum* glucose and cellobiose accumulation of up to 1.5 mM has been observed, which is predicted by the model as well (Desvaux et al., 2001a).
4.4.2 Genome-scale metabolic modeling of the co-culture and strategies for improving the co-culture metabolism

Cellulose metabolism mainly occurs when \textit{C. cellulolyticum} is attached to the substrate (Gelhaye et al., 1993), but in the batch cultures of \textit{C. cellulolyticum} at high cellulose concentrations, cell growth terminates before substrate depletion (Desvaux et al., 2000).
Furthermore, it has been shown that re-inoculating a fresh culture of *C. cellulolyticum*, when substrate is not fully consumed, significantly improved the cellulose solubilisation and cell biomass yield. This result indicates that the incomplete metabolism is not due to either the limitation of adhesion sites on cellulose fibers or product inhibition. At high cellulose concentrations, the likely explanation for the incomplete cellulose consumption is that the lack of control on carbon uptake flow and an imbalanced metabolism leads to the accumulation of intracellular metabolites and self-intoxication of the cells (Desvaux et al., 2000, Desvaux, 2005b).

Previous studies suggest that the cellobiose metabolic capacity is a limiting factor in the cellobiose metabolism by *C. cellulolyticum*, since it is not able to metabolize high concentrations of cellobiose as the cells may not be able to efficiently control the cellobiose uptake (Guedon et al., 1999a). The co-culture study of the *C. cellulolyticum* and *C. acetobutylicum* has shown improved cellulose fermentation, and the co-culture has been able to degrade entirely, a high amount of cellulose at a higher rate, compared to the monoculture of *C. cellulolyticum*. Hence, it can be hypothesized that cellobiose uptake by *C. acetobutylicum* improves the cellulose degradation process, and *C. acetobutylicum* can contribute in cellulose hydrolysis by preventing the cellobiose overflow, which is the major product of the cellulose hydrolysis, and the associated inhibitory effect on *C. cellulolyticum*. Therefore, it is crucial to know how the products of cellulose hydrolysis, cellobiose and glucose (Giallo et al., 1985), impact the cellulosome activity and cellulose metabolism by the co-culture.

Previous reports have shown that the cellulosome of *Clostridium thermocellum* is strongly inhibited by cellobiose, while glucose has an insignificant inhibitory effect. Furthermore, adding β-glucosidase which hydrolyses cellobiose, to the *C. thermocellum* culture has been shown to increase cellulose degradation rate and remove the adverse effects of cellobiose inhibition by hydrolyzing it to glucose. Cellobiose inhibits the function of *C. thermocellum* cellulosome through a competitive inhibitory mechanism (Kruus et al., 1995). So far, the cellobiose inhibitory effect on the Cel5A, an endoglucanase component of the *C.cellulolyticum* cellulosome has been established (Fierobe et al., 1991). Furthermore, it has been shown that glucose has a significant derepressing effect on the production of the cellulosome in *C. cellulolyticum*, and in contrast to cellobiose, it does not have any inhibitory effect on its catalytic activity (Petitdemange et al., 1992). *C. acetobutylicum* has extracellular
endoglucanase and cellobiase activities during its growth on celllobiose (Lee et al., 1985), therefore improving the cellobiase activity and expression in *C. acetobutylicum*, which leads to the hydrolysis of celllobiose to glucose, and thus, reducing the celllobiose inhibitory effect on the cellulosome activity can improve the cellulose degradation rate in this co-culture.

4.4.2.1 Analysis of the co-culture for butanol production

Further interaction in this co-culture is related to the pyruvate metabolism; *C. cellulolyticum* typically secretes pyruvate as a result of its metabolic imbalance, which can be metabolized by *C. acetobutylicum* in this co-culture. *C. cellulolyticum* secretes pyruvate as the result of a limited pyruvate consumption rate and a comparatively higher carbon catabolism rate (Guedon et al., 1999a), while intracellular pyruvate accumulation could be the explanation for the growth arrest at high cellulose concentrations (Guedon et al., 2002). Also, it has been shown that providing *C. acetobutylicum* with pyruvate as the sole carbon source results in the growth and production of mainly acetate and butyrate (Janati-Idrissi et al., 1989). The major products of pyruvate fermentation by *C. acetobutylicum* are acetate, butyrate and butanol, and neither acetate nor butyrate is re-utilized.

The effects of pyruvate on glucose fermentation by *C. acetobutylicum* have also been investigated before, and it is shown that both substrates can be fermented simultaneously (Junelles et al., 1987). Therefore, a potential strategy for improving celllobiose metabolic capacity is through the redirection of the celllobiose toward secreted pyruvate, which can be later metabolized by *C. acetobutylicum*. In the study of co-culture fermentation of cellulose (Petitdemange et al., 1983), high level of acids and low titer butanol have been reported. This observation can be explained by secretion of pyruvate by *C. cellulolyticum*, which affects the *C. acetobutylicum* metabolism and reduces the solvent formation; although the pyruvate formation in the co-culture was not measured. Moreover, it has been shown that addition of pyruvate to the glucose culture under pH-controlled condition represses the solventogenic pathways in *C. acetobutylicum* (Junelles et al., 1987). So, based on this information, additional physiological and genetic manipulations are required to enhance the biobutanol production from cellulose by the co-culture.

Figure 4-6 depicts the model-predicted cellulose hydrolysis profiles in the co-culture, and these profiles have been compared to the simulated mono-culture profiles at various extents of
cellulbiose inhibition on cellulose hydrolysis rate. At a high cellulose concentration of 20 g/L, dFBA is not able to capture the *C. cellulolyticum* growth arrest; however, qualitative comparison of these two hypothetical profiles shows that, even at the $K_i$ value of 2 mM, the cellulbiose removal by *C. acetobutylicum* does not improve the rate of cellulose degradation in the co-culture; so, this result implies that cellulbiose inhibition removal by *C. acetobutylicum* is not the main factor that improves cellulose degradation in the co-culture. The model prediction suggests that the concentration of cellulbiose in the mono-culture is mostly less than 1 mM, which cannot significantly slow down the cellulose hydrolysis through the assumed competitive inhibition mechanism, and this $K_i$ value of 2 mM is still too high for any significant inhibition due to cellulbiose. Also, the experimental profiles of cellulbiose and glucose concentrations have not been reported, and it is possible that a higher level of cellulbiose accumulation takes place in the batch culture at this high cellulose concentration (Desvaux et al., 2000). In addition, we have assumed a competitive inhibition mechanism for the modeling of cellulbiose inhibitory effect on *C. cellulolyticum*, and it is possible that a different inhibitory mechanism affects the cellulosome activity. Hence, these simulations highlight the importance of the *C. cellulolyticum* cellulosome characterization. Further studies should investigate the underlying mechanism associated with the growth arrest phenomenon in *C. cellulolyticum* at high cellulose concentration.
4.5 Concluding remarks

Metabolic models have been used in the past to characterize *C. acetobutylicum* metabolism; however, flux balance analysis cannot predict the complex metabolism of *C. acetobutylicum* properly, and additional constraints are required to narrow down the solution space. Previously, the specific proton flux state (Senger and Papoutsakis, 2008b) was developed for this purpose. On the other hand, incorporating the regulatory network in the genome-scale model seems to be necessary to describe the metabolic shift from acidogenic to solventogenic phase (Covert et al., 2001b). In this work, we present the first genome-scale model of *C. cellulolyticum* metabolism that has been validated against the experimental data, and is able to predict the metabolism of *C. cellulolyticum* on cellulose and cellobiose.

Furthermore, this model of *C. cellulolyticum* metabolism has been integrated with a *C. acetobutylicum* model to develop a genome-scale model of the clostridial co-culture metabolism. The results of our simulations suggest that 1) removal of cellobiose inhibition is
not the main factor responsible for improved cellulose utilization in the co-culture, and 2) *C. cellulolyticum* is primarily adapted to low carbon flow and availability (Guedon et al., 1999a). These results also motivate the need for metabolic engineering strategies to enable *C. cellulolyticum* to handle high carbon flows. The inability of *C. cellulolyticum* to metabolize high concentrations of cellobiose (Guedon et al., 1999a) is a significant barrier to the development of commercial CBP process using clostridia. In order to overcome this limitation, there is a need to improve the cellobiose uptake capacity of *C. cellulolyticum*, assuming that the cellulosome function is not the limiting step (Desvaux, 2005b). Genome-scale models of *C. cellulolyticum* and co-culture modeling framework, as presented here, will be valuable for further optimization of this co-culture for improved cellulose degradation and biobutanol production. This metabolic model of the co-culture can also be easily extended to incorporate hemicellulose metabolism for growth on more complex cellulosic substrates.

### 4.6 Nomenclature for chapter 4

- $\mu^i$: Specific growth rate of organism $i$ (hr$^{-1}$)
- $X^i$: Cell biomass concentration of organism $i$ (gDW.l$^{-1}$)
- $C_j$: Metabolite concentration of metabolite $j$ (mmol)
- $C_{Gcell}$: Cellulose concentration (mmol)
- $C_{cellb}$: Cellobiose concentration (mmol)
- $C_{glc}$: Glucose concentration (mmol)
- $C_{pyr}$: Pyruvate concentration (mmol)
- $C_{ac}$: Acetate concentration (mmol)
- $C_{lac}$: Lactate concentration (mmol)
- $v_{max}$: Maximum specific uptake rate of cellulose (mmol/gDW.hr)
- $V^i_j$: Specific uptake or secretion rate of metabolite $j$ by the organism $i$ (mmol/gDW.hr)
- $V^i$: Vector of reaction fluxes of organism $i$ (mmol/gDW.hr)
- $V_{cellulase}$: Cellulose hydrolysis rate (mmol/gDW.hr)
\( c_i \)  
FBA objective vector of the \( i^{th} \) organism

\( S^i \)  
The stoichiometric matrix of organism \( i \)

\( S_j^i \)  
The sub-set of stoichiometric matrix of organism \( i \) corresponding to metabolite \( j \)

\( k_i \)  
cellobiose inhibition constant

\( k_m \)  
Michaelis-Menten constant

\( \text{Rate}_{\text{butuptake}} \)  
Rate of butyrate uptake by \( C.\ acetobutylicum \)

\( \text{Rate}_{\text{acuptake}} \)  
Rate of acetate uptake by \( C.\ acetobutylicum \)

\( \mathbf{ub}^i \)  
vector of upper-bound capacity constraints on the fluxes of organism \( i \)

\( \mathbf{lb}^i \)  
vector of lower-bound capacity constraints on the fluxes of organism \( i \)
Chapter 5
Characterizing Metabolic Interactions in the Clostridial Co-culture

This chapter includes the material published as a research paper in BMC Biotechnology journal:

Affiliations: Department of Chemical Engineering and Applied Chemistry, University of Toronto

Contributions: FS conceived of the study, designed and conducted the experiments, and drafted the manuscript. RM conceived of the study and helped to draft the manuscript.

The supplementary experiments and information to this chapter are presented in Appendix D, including the qPCR assay development, examination of the specificity of the interactions between C. cellulolyticum and C. acetobutylicum in the co-culture, identifying potential exchanged metabolites in the co-culture, assessment of C. cellulolyticum growth on the supernatant of C. acetobutylicum culture, and experimental characterization of C. acetobutylicum metabolic activity under co-culture conditions.

5.1 Abstract

Background: Clostridial co-culture containing cellulolytic and solventogenic species is a potential consolidated bioprocessing (CBP) approach for producing biochemicals and biofuels from cellulosic biomass. It has been demonstrated that the rate of cellulose utilization in the co-culture of Clostridium acetobutylicum and Clostridium cellulolyticum is improved compared to the mono-culture of C. cellulolyticum (Petitdemange et al., 1983). However, the metabolic interactions in this co-culture are not well understood. To investigate the metabolic interactions in this co-culture, we dynamically characterized the physiology and microbial composition using qPCR.

Results: The qPCR data suggested a higher growth rate of C. cellulolyticum in the co-culture compared to its mono-culture. Our results also showed that in contrast to the mono-culture of C. cellulolyticum, which did not show any cellulolytic activity under conditions similar to those
of co-culture, the co-culture did show cellulolytic activity even superior to the *C. cellulolyticum* mono-culture at its optimal pH of 7.2. Moreover, experiments indicated that the co-culture cellulolytic activity depends on the concentration of *C. acetobutylicum* in the co-culture, as no cellulolytic activity was observed at low concentration of *C. acetobutylicum*, and thus confirming the essential role of *C. acetobutylicum* in improving *C. cellulolyticum* growth in the co-culture. Furthermore, a butanol concentration of 350 mg/L was detected in the co-culture batch experiments.

**Conclusion:** These results suggest the presence of synergism between these two species, while *C. acetobutylicum* metabolic activity significantly improves the cellulolytic activity in the co-culture, and allows *C. cellulolyticum* to survive under suboptimal co-culture conditions, which do not allow *C. cellulolyticum* to grow and metabolize cellulose independently. It is likely that *C. acetobutylicum* improves the cellulolytic activity of *C. cellulolyticum* in the co-culture through exchange of metabolites such as pyruvate, enabling it to grow and metabolize cellulose under suboptimal co-culture conditions

**5.2 Background**

In consolidated bioprocessing (CBP), all four biological steps involved in the conversion of cellulosic biomass, which includes production of saccharolytic enzymes, hydrolysis of the polysaccharides present in pre-treated biomass, and fermentation of hexose and pentose sugars present in the hydrolyzate to desired products, takes place in one bioreactor using a single microorganism or a microbial consortium without the external addition of saccharolytic enzymes (Lynd et al., 2002). Consolidated bioprocessing has been proposed to decrease the production cost by eliminating the costs associated with the cellulase production stage. CBP requires microorganisms with both rapid conversion of cellulose and high product yield, productivities and titers, while such microbes have not been identified in nature yet and need to be developed (Lynd et al., 2005, Lynd et al., 2008, Olson et al., 2012, Hasunuma et al., 2013). To realize this aim, two strategies have been applied: the native strategy which improves the product formation capabilities, such as yield and titer in natural cellulolytic microorganisms (Li et al., 2012, Shaw et al., 2008, Argyros et al., 2011, Yang et al., 2009, Svetlitchnyi et al., 2013, Higashide et al., 2011), and the recombinant strategy that involves engineering non-cellulolytic
organisms with high product yields so that they will express heterologous cellulase and be able to utilize cellulose (Lynd et al., 2002, Hasunuma and Kondo, 2012, Hasunuma et al., 2013).

An alternative method for the production of biobutanol and biochemicals from cellulosic biomass in a consolidated bioprocessing approach is the use of mesophilic clostridial co-culture. *Clostridium acetobutylicum* shows an effective capability to ferment cellulose derived sugars as well as hemicellulose derived sugars, such as cellobiose, mannose, arabinose, xylose, glucose, and galactose to acetone, butanol, and ethanol (Yu et al., 1984, Fond et al., 1986, Ali et al., 2004, Lee et al., 2012). Thus, the co-culture of this bacterial species with a mesophilic cellulose degrading bacterium can be an efficient approach. *Clostridium cellulolyticum* is a cellulolytic, mesophilic bacterium which is able to solublize crystalline cellulose in pretreated hardwood (Demain et al., 2005). A simplified scheme of this clostridial co-culture fermentation on cellulose is presented in Figure 5-1. *C. cellulolyticum* synthesizes cellulosome, which is an extracellular multi-enzymatic complex, and degrades cellulose, with the use of this cellulosome, to glucose and soluble cellooligos (mainly cellobiose), which can be fermented by both species in the co-culture.

![Figure 5-1. The scheme of the clostridial co-culture fermentation on cellulose. *C. cellulolyticum* adheres to the cellulose fibers using a cellulosome and hydrolyzes cellulose to cellobiose (cellb) and glucose (glc), which can be metabolized by *C. cellulolyticum* and *C. acetobutylicum* in the co-culture. The produced pyruvate (pyr) can also be fermented as a carbon source by *C. acetobutylicum.*](image)
The co-culture of *C. cellulolyticum* with *C. acetobutylicum* has been studied previously, and it has been shown that cellulolytic activity is the limiting factor in the co-culture fermentation since most of the cellulase activity products are consumed by *C. acetobutylicum*. The fermentation products have been mainly butyric acid along with butanol, acetic acid and ethanol, and the lack of glucose, which is required for solvent production due to low cellulolytic activity, was hypothesized to be the reason for acid accumulation (Jones and Woods, 1986, Petidemange et al., 1983). Furthermore, three times more cellulosic material was degraded in the co-culture compared to the mono-culture of *C. cellulolyticum* due to the utilization of cellulase activity products and the elimination of their repressive effects (Mitchell et al., 1995), suggesting the presence of synergism between these two species. The analysis of this effect can be valuable for optimizing the rate of cellulosic material degradation.

Therefore, in this study, we investigated metabolic interactions in this co-culture by developing a comparative qPCR analysis of the co-culture and mono-cultures of *C. cellulolyticum* and *C. acetobutylicum*. Investigation of the metabolism in this clostridial co-culture along with the mono-cultures revealed that significant increase in the rate of cellulose hydrolysis can be achieved using the co-culture and making use of the synergism existing between the two clostridial species. It is likely that *C. acetobutylicum* improves the cellulolytic activity of *C. cellulolyticum* in the co-culture through exchange of metabolites such as pyruvate, enabling it to grow and metabolize cellulose under suboptimal co-culture conditions. This clostridial co-culture can offer a considerable potential CBP approach for producing commodity chemicals from cellulosic biomass, taking advantage of *C. acetobutylicum* metabolic potential in converting sugars to variety of chemicals.

5.3 Methods

5.3.1 Strains and media

*C. acetobutylicum* ATCC 824 was grown in clostridial growth medium (CGM) containing per litre: 0.75 g of KH$_2$PO$_4$, 0.75 g of K$_2$HPO$_4$, 1 g of NaCl, 0.01 g of MnSO$_4$, 0.004 g of *p*-aminobenzoic acid, 0.348 g of MgSO$_4$, 0.01 g of FeSO$_4$, 2 g of asparagine, 5 g of yeast extract, 2 g of (NH$_4$)$_2$SO$_4$ and 50 g of glucose (pH 5.8). *C. cellulolyticum* ATCC 35317 was maintained and cultivated on a modified CM3 medium containing per litre: KH$_2$PO$_4$, 1.5 g; K$_2$HPO$_4$, 2.9 g; (NH$_4$)$_2$SO$_4$, 1.3 g; MgCl$_2$.6H$_2$O, 1 g; CaCl$_2$, 0.15g; FeSO$_4$, 1.25 mg; resazurin, 1 mg;
cysteine hydrochloride, 1 g; fibrous cellulose (medium, Sigma-Aldrich, C6288), 7.5 g, and 2 g of yeast extract (pH 7.2).

The co-culture medium was composed of 0.75 g of KH$_2$PO$_4$, 1 g of MgCl$_2$.6H$_2$O, 0.15 g of CaCl$_2$, 1 mg of resazurin, 1 g of cysteine hydrochloride, 0.01 g of MnSO$_4$, 0.004 g of p-aminobenzoic acid, 10 mg of (NH$_4$)$_2$Mo$_7$O$_{24}$, 0.01 g of FeSO$_4$, 2 g of asparagine, 3 g of yeast extract, 1 g of (NH$_4$)$_2$SO$_4$ and 20 g of fibrous cellulose per litre of medium. All pre-cultures were incubated at 37°C without shaking in serum bottles to reach the total protein concentration of 15 to 20 mg/L (Bio-Rad Protein Assay, 500-0006).

**5.3.2 Co-culture experiments**

The co-culture experimental set-up is depicted in Figure 5-2. All batch cultivations were conducted in 5 L bioreactors with a working volume of 2 L at 37°C, where agitation was set at 200 rpm, and the volume of *C. cellulolyticum* inoculum at exponential growth phase formed 10% of the total volume. The *C. cellulolyticum* pre-cultures were transferred twice on the co-culture medium with 7.5 g/l of cellulose before inoculation into bioreactors, where the cell biomass concentration in the bioreactor inocula was between 6×10$^8$ and 8×10$^8$ cells/mL. For the co-culture batches, *C. cellulolyticum* was first cultivated for 48 hours at pH 7.0; then, 200 mL of *C. acetobutylicum* culture at exponential phase was centrifuged and the cell pellets were suspended in 20 mL of CGM medium and inoculated into the bioreactor. The pH then was adjusted and maintained at 6.0 using 1N H$_2$SO$_4$ and 3N NaOH. The bioreactors were kept under anaerobic conditions by continuous sparging of nitrogen gas. The same pH adjustment and medium were applied for the *C. cellulolyticum* mono-culture batch unless for the mono-culture batch at pH 7.2 that was conducted on CM3 medium with no pH profile.
Figure 5-2. The co-culture experimental set-up. *C. cellulolyticum* was cultivated at the initial pH of 7.0 for two days, and pH was adjusted to 6.0 after inoculating *C. acetobutylicum* into the co-culture at 48 hr.

### 5.3.3 Analysis

Concentrations of sugars, organic acids and alcohols were measured using a Dionex Ultimate 3000 HPLC (Bio-Rad, Hercules, CA) equipped with a UV monitor (210 nm) and a Shodex RI-101 differential refractive index detector. The products were separated using a Bio-Rad HPX-87H cation-exchange column, where 10 mM H$_2$SO$_4$ was used as the mobile phase (0.6 mL/min flow rate, 60°C column temperature, 20 µL injection volume). To account for the extracellular proteins, amino acids and amino compounds in cell cultures (Guedon et al., 1999b), and to estimate the overall carbon recovery, total organic carbon (TOC) concentrations of the sample supernatants were quantified using Total Organic Carbon Analyzer (TOC-V$_{CPH}$/CPV, Shimadzu, Japan), with the lower detection limit of 0.5 mg/L using standard TOC catalyst. Cellulose concentration was measured using Updegraff method (Updegraff, 1969). Cellulose pellets were washed using acetic acid-nitric acid reagent and water to remove the non-cellulosic material; cellulose then was quantified using anthron in a colorimetric method. The results of cellulose assay and TOC measurements showed that 24±4% (based on 7 data points) of the total carbon was used for CO$_2$ formation, which was consistent with our previous model prediction (Salimi et al., 2010).
To measure protein, 0.5 ml of cell culture was centrifuged at 8,000 g for 2 min and washed with 0.9% (wt/vol) NaCl. The pellet was resuspended in 0.5 ml of 0.2 N NaOH, and this suspension was placed in a boiling water bath for 10 min. After cooling, the hydrolyzed sample was centrifuged as described above, and the total solubilized protein concentration in supernatants was measured using Bio-Rad Protein Assay (500-0006). Quantification of the population of each species in the co-culture was conducted using quantitative PCR (qPCR) method, which includes DNA extraction, PCR amplification of 16S rRNA gene and detection using fluorescent dyes, and is elaborated in following sections. Total cell quantifications of *C. acetobutylicum* were also conducted using hemacytometry method.

### 5.3.3.1 Primer design and qPCR standard plasmid DNA preparation

The qPCR primers were designed to target the 16S rRNA genes in *C. acetobutylicum* (CA_Cr001) and *C. cellulolyticum* (Cc_el_R0007) as reported in Table 5-1. The qPCR standard plasmid solutions for each target species were prepared by cloning the purified PCR products into pCR®2.1-TOPO® vector using TOPO-TA Cloning® kit (Invitrogen™, K4500-01). In the first step, fresh PCR products were purified using GeneJET PCR Purification Kit (Fermentas, K0701, K0702); the nucleic acid concentrations in purified PCR products were measured to be 67 and 50 ng/µl for *C. acetobutylicum* and *C. cellulolyticum*, respectively, using NanoDrop 1000 (Thermo Scientific) spectrophotometer, and PCR products purity were verified using agarose gel electrophoresis. Plasmid extraction from the positive clones was conducted using GenElute Plasmid Miniprep kit (Sigma-Aldrich, USA). All of the extracted plasmids were sequenced at the University of Toronto Sanger Sequencing Facility, and the plasmids with right inserts were selected and applied for qPCR calibrations.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target species</th>
<th>Primer sequence (5'-&gt;3')</th>
<th>Product length</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA2 (forward)</td>
<td><em>C. acetobutylicum</em></td>
<td>CTTGTTGGTGAGGTAACGG</td>
<td>386 bp</td>
<td>60°C</td>
</tr>
<tr>
<td>CA2 (reverse)</td>
<td><em>C. acetobutylicum</em></td>
<td>CACTCCAGACATCCAGTTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC2 (forward)</td>
<td><em>C. cellulolyticum</em></td>
<td>TACAGGGGGATAACACAGG</td>
<td>348 bp</td>
<td>60°C</td>
</tr>
<tr>
<td>CC2 (reverse)</td>
<td><em>C. cellulolyticum</em></td>
<td>CGTGGGTTATTCTTCAGGTAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
There are eleven 16S rRNA genes in *C. acetobutylicum* genome (CA_Cr001, CA_Cr004, CA_Cr007, CA_Cr010, CA_Cr013, CA_Cr016, CA_Cr019, CA_Cr022, CA_Cr025, CA_Cr028, CA_Cr033), and eight 16S rRNA genes in *C. cellulolyticum* genome (Ccel_R0007, Ccel_R0018, Ccel_R0088, Ccel_R0084, Ccel_R0059, Ccel_R0012, Ccel_R0024, Ccel_R0036). Multiple sequence alignment of the 16S rRNA genes for each species using ClustalW2 (ClustalW2) showed identical amplification regions for each set of 16S rRNA genes, therefore 8 amplicons with the same size are being produced per copy of *C. cellulolyticum* genome in a qPCR reaction, and 11 amplicons with the same size are being produced per copy of *C. acetobutylicum* genome in a qPCR reaction; these facts also were verified by observing single peaks in melting curve analysis as well as single DNA bands for each species in agarose gel electrophoresis runs, and must be considered in qPCR quantifications of each species. Therefore, to calculate the concentration of cells (cell/mL) in cultures using qPCR data of the 16S rRNA gene quantifications (copies/µL), the number of 16S rRNA genes in the genome (n_{16s}), the volume of the culture from which genomic DNA was extracted (V_c), and the volume of extracted DNA (400 µL) were taken into account, as below:

\[
\text{Concentration (cell/mL)} = \frac{\text{concentration (copies/µL)} \times 400 \, \mu\text{L} \times \text{dilution factor}}{V_c \times n_{16s}}
\]

The value for n_{16s} was equal to 11 for *C. acetobutylicum*, and equal to 8 for *C. cellulolyticum* cell quantifications.

For each qPCR run a new standard curve was made using fresh dilutions, where the standard curve concentrations of 10^8 copies/µL to 10^1 copies/µL were prepared by making serial 1:10 dilutions starting with the 10^9 copies/µL plasmid solution. More details about the qPCR method development are presented in Appendix D.

### 5.3.3.2 Genomic DNA isolation

Initially the Mo Bio Laboratories UltraClean® Soil DNA Isolation Kit was applied to extract DNA from the samples. However, it was demonstrated that there was no correlation between culture size and the DNA yield using this kit for co-culture samples, as the same amount of DNA was extracted from 0.2, 1, 2 and 5 mL of cultures; in fact this kit has been aimed for soil samples where the concentrations of cell biomass is typically low, and therefore there was a chance to miss significant amount of DNA in the co-culture samples. Consequently, DNA
isolation using Norgen bacterial genomic DNA isolation kit (Norgen, #17900) was tested (Appendix Figure A5). It was found that there was a correlation between the sample size (in the range of 0.2 to 0.5 mL) and DNA yield using Norgen kit, while it had a higher DNA yield compared to the Mo Bio kit. Therefore, the Norgen kit was chosen for DNA isolations; culture samples (0.5 mL) were centrifuged at 13000 g for 2 minutes, and the cell pellets were used for DNA isolations following the standard kit protocol, where 400 μL elution buffer was used lastly to elute DNA in two steps (200 μL for each dilution step).

5.3.3.3 qPCR reaction preparation, detection and quantification

The qPCR amplification was performed using 2 μL of tenfold diluted sample genomic DNA and 18 μL of a master mix in a total reaction volume of 20 μL. A master mix was prepared for each qPCR run whereby the reagents for the individual reactions were combined prior to plate preparation in order to minimize errors. Master mixes were prepared by combining 10 μL of SsoFast™ EvaGreen® Supermix (Bio-Rad, #172-5200), 1 μL of each primer with final concentration of 0.25 μM, and 6 μL of water for each reaction, and 2 μL of DNA solution was added to each reaction in a 20 μL reaction volume.

The qPCR amplifications and detections were carried out in a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). The qPCR program had the following protocol: 1) initial denaturation at 98 ℃ for 3 min, 2) 40 cycles of denaturation at 98 ℃ for 5 sec, 3) annealing and extension at 60 ℃ for 10 sec followed by a plate read; afterward a melting curve analysis from 65 to 95 ℃ measuring fluorescence every 0.5 ℃. For all qPCR runs, the qPCR signals were analyzed at a standard pre-determined threshold of 1E+03 RFU which was in the exponential amplification phase and above the background fluorescence noise for all the qPCR runs. The quantification cycles (C_q or C_T), the cycle number where fluorescence increases above the threshold, were used to find the DNA copy numbers (automatically calculated from the standard curve). To examine the quality of qPCR assays, standard curve with coefficient of determination (R^2) > 0.980, high amplification efficiency (90–110%), and consistency across replicate reactions were considered.
5.4 Results and discussion

5.4.1 Experimental characterization of *C. cellulolyticum* cellulolytic activity at high cellulose concentration, addressing the pH effect

In the previous co-culture study (Petitdemange et al., 1983), the pH effect on *C. cellulolyticum* growth and cellulolytic activity has not been addressed. Therefore, to investigate the pH effect on *C. cellulolyticum* growth and cellulolytic activity in a mono-culture at high cellulose concentration of 20 g/L, two batch cultures of *C. cellulolyticum* were conducted: one batch at pH of 7.2, which is an optimal pH for *C. cellulolyticum* growth and cellulolytic activity (Desvaux et al., 2001a), and another batch with the same pH profile as the co-culture run, i.e. initially at pH of 7.0 for 2 days followed by a pH switch to 6.0. The profiles of cellulose solubilization and cell biomass concentration are shown in Figure 5-3. In the mono-culture at pH of 7.2, TOC data showed that 1.5 g/L of carbon was solubilized after 14 days, which is equivalent to 4.9 g/L of cellulose degraded, assuming 24% of carbon flow goes towards CO₂ formation; this amount of degraded cellulose was comparable to 5.1 g/L reported for the *C. cellulolyticum* mono-culture at the same initial cellulose concentration (Desvaux et al., 2000, Petitdemange et al., 1983). Also, qPCR data showed that cells reached a stationary growth phase after 9 days with a maximum cell density of $5 \times 10^9$ cell/mL and a specific growth rate of 1.23 day⁻¹.

In contrast, the mono-culture run under co-culture pH profile did not show any cellulose solubilization during the batch (Figure 5-3), and cells had a very low growth rate of 0.16 day⁻¹ (87% decrease) compared to the mono-culture batch at pH of 7.2. The effect of pH on *C. cellulolyticum* growth and cellulolytic activity at low cellulose concentration of 3.7 g/L has been addressed previously (Desvaux et al., 2001a), and it has been shown that *C. cellulolyticum* is significantly affected by acidic pH, where a pH drop from 7.0 to 6.4 leads to 75% lower cell biomass concentration. It has been suggested that acidic pH hampers cell biomass formation, likely through direct effect of pH on a cellular constituent such as an enzyme or a transport protein, rather than cellulose degradation capability in *C. cellulolyticum*. This argument is supported by the observation that the flux through cellulose degradation reaction remains
almost unvarying, in the range of 1.69 to 1.84 mmol (g cell. h)-1, while pH varies between 6.4 to 7.0 (Desvaux et al., 2001a). However, regardless of the mechanism of inhibition, this pH effect must be considered when comparing cellulolytic activity and C. cellulolyticum growth and metabolism in the mono-culture and co-culture.

Figure 5-3. The total solubilized carbon and cell biomass profiles in the C. cellulolyticum mono-culture experiments. The cell biomass profile in the mono-culture at pH of 7.2 (circle), TOC profile in the mono-culture at pH of 7.2 (triangle), cell biomass profile in the mono-culture under the co-culture pH profile (rhombus), and TOC profile in the mono-culture under the co-culture pH profile (square). The co-culture pH profile includes the initial pH of 7.0 for two days, and pH adjustment to 6.0 at 48 hr. Cell biomass concentrations were calculated from qPCR data, taking into account that there are eight 16S rRNA genes in C. cellulolyticum genome.

5.4.2 Experimental characterization of the co-culture metabolism

To prepare the co-culture medium for the first co-culture experiment A, all media components including the iron solution were autoclaved. The profile of cellulose degradation in this co-culture is shown in Figure 5-4a. At an initial cellulose concentration of 20 g/L, which is equivalent to 6.08 g/L of final TOC concentration, assuming full degradation of cellulose and considering 24% of the total carbon would be used toward CO2 formation, 82% of cellulose was degraded after 28 days in the co-culture experiment A. Compared to the mono-culture at the optimal pH of 7.2, cellulose degradation showed 82% improvement as shown in Figure 5-5, whereas no cellulose degradation was observed in the mono-culture run under co-culture pH profile. These results confirm that C. acetobutylicum metabolic activity significantly improves the cellulolytic activity in the co-culture, and in fact makes it possible for C. cellulolyticum to
Figure 5-4. The cellulose solubilization in the co-culture and mono-culture experiments. The profiles of cellulose degradation and cell biomass formations in the co-culture experiment A, with autoclaved iron solution (a) and in the co-culture experiment B, with filter sterilized iron solution (b). The time profiles of *C. cellulolyticum* (circle), *C. acetobutylicum* (square) and cellulose solubilization in terms of total organic carbon in each co-culture (triangle) at initial cellulose concentration of 20 g/L and under pH profile, along with cellulose solubilization in terms of total organic carbon in *C. cellulolyticum* mono-culture under the same co-culture conditions (rhombus). The co-culture pH profile includes the initial pH of 7.0 for two days, and pH adjustment to 6.0 at 48 hr, as shown by arrows. Figures (a) and (b) each show one data set representative of two independent experiments, with both showing comparable results. Cell biomass concentrations were calculated from qPCR data, taking into account that there are eleven 16S rRNA genes in *C. acetobutylicum* genome, and eight 16S rRNA genes in *C. cellulolyticum* genome.
survive under suboptimal co-culture conditions, which do not allow it to grow and metabolize cellulose independently. Furthermore, to check and ensure iron sufficiency in the co-culture medium, the co-culture medium was prepared by filter sterilizing the ferrous sulphate solution (experiment B), rather than autoclaving as for co-culture experiment A, to avoid potential iron oxidation during medium preparation. The results of this experiment are shown in Figure 5-4b; while the cellulolytic activity was high in the co-culture experiment B, and *C. cellulolyticum* growth rate was still as high as experiment A, but cellulose degradation declined by 38% compared to experiment A, as presented in Figure 5-5. Cellulose degradation in each batch was estimated from carbon balance and TOC measurements, taking into account that 24% of the total carbon has been utilized for CO₂ formation, as described in the analysis section. These results confirmed that the co-culture was not under iron limiting condition, and the presence of more ferrous ions had an adverse effect on the co-culture cellulolytic activity.

![Figure 5-5. Degradation of cellulose in the co-culture experiments A and B, the *C. cellulolyticum* mono-culture experiment at the optimal pH of 7.2 and the *C. cellulolyticum* mono-culture experiment under the co-culture pH profile, 5 days after reaching the maximum *C. cellulolyticum* concentration in the culture (light grey) and after 28 days (dark grey). The co-culture pH profile includes the initial pH of 7.0 for two days, and pH adjustment to 6.0 at 48 hr. Error bars show the ranges based on two sets of experimental data.](image)

The mechanism underlying such a synergy between the two clostridial species is not clearly understood. Hence, in order to understand the nature of interactions between *C. acetobutylicum* and *C. cellulolyticum*, that improve cellulose solubilization, we used qPCR to track the population of each species in the batch cultures. Figure 5-4a and Figure 5-4b show the dynamic profiles of each species population in the co-culture batches. *C. cellulolyticum* cell biomass concentration reached to $3 \times 10^9$ cell/mL in the co-cultures, which was the same value for maximum cell biomass concentration in the mono-culture runs; however the growth dynamics was significantly faster in the co-cultures compared to the mono-culture run under the same pH.
profile. *C. cellulolyticum* growth rate in the co-culture was comparable to its growth rate in mono-culture under optimal pH condition of 7.2. Also, it could be observed from all co-culture TOC profiles that cellulose solubilization started after *C. cellulolyticum* had reached its late exponential growth phase.

*C. acetobutylicum* cell biomass profiles are also shown in Figure 5-4, where the initial decrease in cell biomass concentration in the co-culture could be attributed to the lack of available sugars for *C. acetobutylicum* to grow while *C. cellulolyticum* had been in the lag phase. Furthermore, a considerable growth could be observed for *C. acetobutylicum*, when *C. cellulolyticum* had reached its maximum concentration in the co-culture, where possibly more sugars became available in the co-culture to support *C. acetobutylicum* growth. Although *C. acetobutylicum* cell biomass concentration showed significant increases at some points over the course of co-culture, it was fluctuating and did not remain constant. It has been suggested that in the microbial communities growing on cellulosic material, where there is a competition between cellulose-adherent cellulolytic microorganisms and non-adhered microbes for cellulose hydrolysis products, cellulose-adherent cellulolytic microorganisms are possibly successful winners (Lynd et al., 2005), and this phenomenon could explain the limited growth of *C. acetobutylicum* in the co-culture.

*C. acetobutylicum* shows cellobiase and endoglucanase activities during growth on cellobiose, glucose, xylose and mannose, but is not able to hydrolyze crystalline cellulose due to lack of the required enzymatic activities (Lee et al., 1985, Sabathe et al., 2002, Kovacs et al., 2013). Sabathe et al. have reported the production of a high molecular mass cellulosome in *C. acetobutylicum* (Sabathe et al., 2002). Since *C. acetobutylicum* is unable to grow on crystalline cellulose as the sole carbon source, *C. acetobutylicum* has been cultivated on cellobiose (20 g/L), as the main carbon source, along with microcrystalline cellulose (10 g/L) for the adsorption of the cellulosomal protein components. Proteins purified from the culture supernatant as well as proteins eluted from cellulose have been tested for the cellulolytic activity. No cellulolytic activity on Avicel, and very low levels of activity on carboxymethyl cellulose (CMC) and phosphoric acid swollen cellulose (PASC) have been observed (Sabathe et al., 2002). Recently, heterologous expression of three *C. thermocellum*-derived cellulosome components, and the production of functional mini-cellulosomes in *C. acetobutylicum* has been reported (Kovacs et al., 2013). Furthermore, our experiments showed that *C. acetobutylicum*
did not grow on fibrous cellulose under the co-culture conditions (Figure 5-7). Therefore, the improved cellulolytic activity in the co-culture cannot be attributed to *C. acetobutylicum* cellulolytic activity. However, *C. acetobutylicum* is able to ferment cellobiose and cellulose-derived sugars, and the improved cellulolytic activity in co-culture can be attributed to the role of *C. acetobutylicum* in consuming sugars and preventing the carbon overflow toward *C. cellulolyticum*, as *C. cellulolyticum* is unable to metabolize high concentrations of cellobiose (Guedon et al., 1999a).

Also, degree of synergism (DS), defined as the activity of a mixture of components divided by the sum of the component activities evaluated separately (Lu et al., 2006), in this co-culture can be estimated as the ratio of *C. cellulolyticum* growth rates in co-culture and mono-culture under pH profile, and was equal to 7.99. However, the co-culture DS determined based on the cellulolytic activities in the co-culture and the mono-culture would be substantially high, which indicates the presence of a strong synergism in this clostridial co-culture. The DS value of 5 or higher is not very common in enzyme-microbe cellulose hydrolysis systems and has been observed under some conditions (Zhang and Lynd, 2004, Hu et al., 2011). Moreover, in this co-culture on fibrous cellulose, observed maximum cellulose degradation rate of 0.108 g/(L. h) is comparable with a cellulose degradation rate of 0.15 g/(L. h) in *C. thermocellum* culture on crystalline cellulose, which shows one of the highest cellulose utilization rates among cellulolytic microorganisms (Lu et al., 2006).

Since cellulolytic bacteria are unable to grow at low intracellular pH, under acidic environment the pH gradient (ΔpH) across the cell membrane is high; consequently, the intracellular dissociation of fermentation acids, which are membrane permeable in undissociated form, and the intracellular accumulation of acid anions lead to anion toxicity, which is the likely reason of growth inhibition under acidic condition (Russell and Wilson, 1996, Flythe and Russell, 2006). Furthermore, it has been shown that presence of lactate and acetate ions in an acidic medium leads to a significant decline of glutamate synthesis in *Clostridium sporogenes* MD1, which inhibits the bacterial growth (Flythe and Russell, 2006). Also, for *E. coli* culture at pH 6, incubation of cells with 8 mM acetate for 20 min was shown to result in intracellular accumulation of acetate anions (240 mM), and reduced level of intracellular glutamate pools (Roe et al., 1998). Furthermore, in mildly acidic *E. coli* cultures (pH of 6), inhibition of methionine biosynthesis by acetate (8 mM) and the toxicity of accumulated homocysteine have
been indicated as the cause of growth inhibition by acetate under weak acid stress (Roe et al., 2002). Addition of methionine to this culture can restore *E. coli* growth rate to some significant extent. This effect has been also reported for other organic acids.

In this clostridial co-culture, the synergy could be attributed to the exchange of some growth precursors and cell biomass constituents between *C. acetobutylicum* and *C. cellulolyticum*, which potentially enables the cellulolytic organism to grow and metabolize cellulose under acidic pH condition. *C. acetobutylicum* is a fermentative bacterium which is able to grow well under acidic conditions in acidogenic and solventogenic growth phases. The results of co-culture experiments under a low concentration of *C. acetobutylicum*, which are presented in the following section, also provide support for the role of *C. acetobutylicum*.

### 5.4.3 Experimental characterization of the co-culture metabolism with low concentration of *C. acetobutylicum*

To investigate if a high initial concentration of *C. acetobutylicum* contributes to its low growth in the co-culture, and if *C. acetobutylicum* growth in the co-culture is affected by the ratio of cells to the cellulose hydrolysate, a co-culture experiment was conducted with 1% of the initial concentration of *C. acetobutylicum*. Two mL of *C. acetobutylicum* culture at exponential growth phase was centrifuged and the cell pellets were suspended in 2 mL of CGM medium and inoculated into the bioreactor. The results of this experiment are presented in Figure 5-6, where no cellulose degradation was observed in either biological replicates. Also, *C. cellulolyticum* as well as *C. acetobutylicum* did not grow in the co-culture. This experiment confirmed that the role of *C. acetobutylicum* in the metabolism of cellulose by the co-culture is associated with the population of *C. acetobutylicum*, and can be attributed to the exchange of some metabolites between the two species.

Furthermore, the metabolic behaviour of *C. acetobutylicum* under the co-culture conditions was investigated, as discussed in the following section. This study confirmed the metabolism of pyruvate and the released sugars by *C. acetobutylicum* in the clostridial co-culture, and that the observed oscillations in the *C. acetobutylicum* concentration in the co-cultures could be due to the slow release of sugars by *C. cellulolyticum* that can lead to starvation cycles for *C. acetobutylicum* in the co-culture.
5.4.4 Experimental characterization of *C. acetobutylicum* metabolic activity under co-culture conditions

To further investigate the metabolic behaviour and the role of *C. acetobutylicum* in the clostridial co-culture, mono-culture batches of *C. acetobutylicum* were conducted under the co-culture conditions. The results of these experiments are presented in Figure 5-7a and b. In the first mono-culture experiment (Figure 5-7a), *C. acetobutylicum* was cultivated on the co-culture medium with 20 g/L cellulose and no glucose added to the culture. The qPCR data as well as the cell quantification of *C. acetobutylicum* using hemacytometry showed a significant reduction in the cell population over the course of the batch, due to the lack of glucose and cell starvation.

In the second mono-culture batch (Figure 5-7b), after 12 days of cultivation and cell starvation in the absence of glucose, 1 g/L of glucose was added to the culture. As a result a drastic increase in the cell biomass concentration was observed in the culture, followed by a decline due to cell starvation after consuming the entire added glucose in 24 hrs. Furthermore, to assess

---

Figure 5-6. The profiles of cellulose degradation and cell biomass formations in the co-culture experiment with low concentration of *C. acetobutylicum*. The time profiles of *C. cellulolyticum* (circle), *C. acetobutylicum* (square) and cellulose solubilization in terms of total organic carbon (triangle) in the co-culture experiment. The co-culture pH profile includes the initial pH of 7.0 for two days, and pH adjustment to 6.0 at 48 hr. The figure shows one data set representative of two independent experiments, with both showing comparable results. Cell biomass concentrations were calculated from qPCR data, taking into account that there are eleven 16S rRNA genes in *C. acetobutylicum* genome, and eight 16S rRNA genes in *C. cellulolyticum* genome.
*C. acetobutylicum* growth and metabolism on pyruvate, 1 g/L of pyruvate was added to the culture on day 19th, while the cells had been starving for previous 6 days, and a 10-fold increase in the cell biomass concentration was observed, confirming metabolism of pyruvate by *C. acetobutylicum*. However, pyruvate was not consumed entirely (HPLC data showed that only 0.1 g/L pyruvate had been consumed), and the main fermentation products were acetate and butyrate. Higher cell biomass concentrations obtained using qPCR method can be attributed to the spore formation in *C. acetobutylicum* cultures, as both viable and non-viable spores in addition to dead cells are quantified using qPCR (Rawsthorne et al., 2009) in contrast to hemacytometry. Also, the similar trends observed in the cell biomass profiles using both qPCR and hemacytometry methods verified the appropriateness of the developed qPCR method.

Moreover, a cell biomass yield of 22.1 g cell/mol glucose has been reported for *C. acetobutylicum* (Meyer and Papoutsakis, 1989). Therefore, the increase in the *C. acetobutylicum* cell concentration, as a result of adding 1 g/L glucose to the culture, assuming 3.5 ×10⁹ cells per mg cell protein in the culture (Giallo et al., 1985), can be calculated as follows:

\[
\text{Increase in the cell concentration} = 3.5 \times 10^9 \text{ (cell/mg protein)} \times 0.6 \text{ (mg protein/mg cell)} \times 0.12 \text{ (mg cell/mg glucose)} = 2.5 \times 10^8 \text{ cell/mg glucose}
\]

This value is comparable to the increase in the *C. acetobutylicum* concentration obtained from the qPCR data (1.5×10⁹ cell/mg glucose), and the hemacytometry data (6.0×10⁷ cell/mg glucose) in this *C. acetobutylicum* culture (Figure 5-7b). Moreover, this study confirmed the metabolism of pyruvate and the released sugars by *C. acetobutylicum* in the clostridial co-culture, and that the observed oscillations in the *C. acetobutylicum* concentrations in the co-culture experiments could be due to the slow release of sugars by *C. cellulolyticum*, which can lead to starvation cycles for *C. acetobutylicum* in the co-culture.
Figure 5-7. *C. acetobutylicum* biomass profiles in the mono-cultures under co-culture conditions. (a) *C. acetobutylicum* was cultivated on the co-culture medium at pH of 6.0 and 20 g/L cellulose without glucose/pyruvate addition, and characterized using qPCR (rhombus) and hemacytometry (square). (b) *C. acetobutylicum* culture on the co-culture medium at pH 6.0 and 20 g/L cellulose. 1 g/L glucose was added at day 12, and 1 g/L pyruvate was added to the culture on day 19, as shown by arrows. Cell biomass concentrations were calculated from qPCR data, taking into account that there are eleven 16S rRNA genes in *C. acetobutylicum* genome.
5.4.5 Analysis of product formation in the co-culture

Figure 5-8 shows the ranges for co-culture and mono-culture product concentrations after 28 days. As it can be noted, acetate, ethanol, lactate, butyrate, and butanol were the main products of the fermentation in the co-culture. Butyrate appeared after *C. acetobutylicum* inoculation in the co-culture, but its concentration remained low. Neither acetate nor butyrate uptake, which are the characteristics of the solventogenic phase in *C. acetobutylicum* metabolism, was observed in this co-culture. At high cellulose concentration, *C. cellulolyticum* produces lactate as its main product along with acetate and ethanol (Desvaux et al., 2000). The lactate uptake, observed in the co-culture batches, coincided with butanol formation (Appendix Figure A7). The lactate uptake can be related to *C. acetobutylicum* metabolic activity, as *C. acetobutylicum* ATCC824 has been shown to co-ferment lactate and glucose (Bahl et al., 1986).

It has been previously shown (Desvaux et al., 2000) that in pH controlled batch cultures of *C. cellulolyticum* on a defined medium, the distribution of carbon flow depends on the initial cellulose concentration. For concentrations less than 6.7 g/L of cellulose, acetate, ethanol, CO₂ and H₂ were shown to be the main fermentation end products and more than 91% of cellulose was observed to be degraded. At higher cellulose concentrations, from 6.7 g/L up to 29.1 g/L, carbon flow is redirected from ethanol and acetate towards lactate and extracellular pyruvate. In addition, in batch cultures of *C. cellulolyticum* on high cellulose concentration, it has been shown that the peak of pyruvate formation coincides with the start of lactate formation, and this pyruvate accumulation in the *C. cellulolyticum* culture shows that the rate of cellulose catabolism is higher than the rate of pyruvate consumption. Also it has been suggested that the cellulose hydrolysis depends on the concentration of *C. cellulolyticum*, which remains constant at and above 6.7 g/L of cellulose (Desvaux et al., 2000).
Furthermore, it has been shown that re-inoculating a fresh culture of *C. cellulolyticum* at high cellulose concentration of 29.1 g/L, where substrate is not fully consumed, significantly improves the cellulose solubilization and cell biomass yield compared to a classical batch (Desvaux et al., 2000). This result indicates that the incomplete cellulose catabolism is not due to either the limitation of adhesion sites on cellulose fibers or product inhibition. At high cellulose concentrations, the likely explanation for the incomplete cellulose consumption is the lack of control on carbon uptake flow and an imbalanced metabolism leading to the accumulation of intracellular metabolites and self-intoxication of the cells, eventually resulting in a growth arrest (Desvaux et al., 2000, Desvaux, 2005b). Similarly, extracellular pyruvate formation has been reported in *C. thermocellum* cultures at high cellulose and cellobiose concentrations, which evidences the overflow metabolism (Levin et al., 2006).

In our experiments, the maximum concentration of *C. cellulolyticum* in co-culture experiments was the same as the mono-culture experiment under optimal pH of 7.2; however, the cellulose degradation was improved up to 82% (Figure 5-5), which confirms the role of *C. acetobutylicum* in cellulose degradation, while *C. cellulolyticum* has reached the stationary growth phase. We observed pyruvate accumulation of 0.029 g/L in the mono-culture batch under the co-culture pH profile and 0.004 g/L in the mono-culture batch at pH of 7.2. In the co-culture replicates, maximum pyruvate concentration of 0.17 g/L was observed, which was
taken up later during the course of experiments coinciding with butyrate formation in the cocultures. Our previous modeling studies have suggested that limited pyruvate-ferredoxin oxidoreductase (PFO) activity, which cannot support high pyruvate flow, results in pyruvate overflow (Guedon et al., 1999b). Hence, a potential explanation for pyruvate secretion in *C. cellulolyticum* cultures is the limitation on the pyruvate consumption rate and a comparatively higher carbon catabolism rate, and due to inefficient regulation of entering carbon flow (Guedon et al., 1999a). Furthermore, intracellular pyruvate accumulation could be the explanation for the growth arrest at high cellulose concentrations (Guedon et al., 2002), at which cells switch to stationary growth phase before substrate depletion.

Pyruvate uptake in the co-culture can be explained by the capability of *C. acetobutylicum* to metabolize pyruvate. It has been also reported that providing *C. acetobutylicum* with pyruvate as the sole carbon source results in the growth and production of mainly acetate and butyrate (Janati-Idrissi et al., 1989). In another co-culture study, the removal of *C. cellulolyticum* metabolic products such as pyruvate and their associated inhibitory effects, by *Rhodopseudomonas palustris* in the co-culture of *C. cellulolyticum* and *R. palustris* has been reported as the underlying reason for the improved cellulose degradation and bacterial growth in this co-culture (Jiao et al., 2012). *C. cellulolyticum* growth on cellulose has been shown to be severely inhibited by pyruvate; where about a 60% decrease in the cell biomass concentration in the presence of 2 mM (176 mg/L) pyruvate has been observed in *C. cellulolyticum* monoculture at initial pH of 7.2 (Jiao et al., 2012). Therefore, pyruvate removal by *C. acetobutylicum* and alleviating its inhibitory effect can be a contributing factor in the improved growth of *C. cellulolyticum* and its boosted cellulolytic activity in the co-culture.

The major products of pyruvate fermentation by *C. acetobutylicum* are acetate, butyrate and butanol, and neither acetate nor butyrate is reutilized. The effects of pyruvate on glucose fermentation by *C. acetobutylicum* have also been investigated before, and it has been shown that both substrates can be fermented simultaneously (Junelles et al., 1987). Furthermore, cellobiose and glucose were only detected at the early stage of batches, which could have been present in the pre-cultures inoculated into the bioreactors, and were taken up in 24 hours. Cellobiose and glucose could not be detected in the course of co-cultures, which indicated their immediate consumption in the co-culture. In conclusion, in this study we showed a strong synergism between the two species of clostridia in the co-culture, and found that *C.
acetobutylicum enables C. cellulolyticum to grow under a suboptimal co-culture environment. This synergy can be attributed to the production of some growth pre-cursors, and future metabolomic studies of this co-culture can identify such metabolites.

5.5 Conclusions

Examining the metabolism in this clostridial co-culture along with the mono-cultures revealed that a significant increase in the rate of cellulose hydrolysis can be achieved using the co-culture and making use of the synergism existing between the two clostridial species. It is likely that C. acetobutylicum improves the cellulolytic activity of C. cellulolyticum in the co-culture through exchange of metabolites such as pyruvate, enabling it to grow and metabolize cellulose under suboptimal co-culture conditions. This clostridial co-culture can offer a considerable potential CBP approach for producing commodity chemicals from cellulosic biomass, taking advantage of C. acetobutylicum metabolic potential in converting sugars to variety of chemicals.
Chapter 6
Investigation of the Growth Arrest Event in C. cellulolyticum
Through an In vivo Metabolite Analysis

6.1 Background

As discussed in chapter 4, the knowledge of the underlying growth arrest mechanism at high cellulose concentration is required for modeling C. cellulolyticum and the co-culture metabolism. Previous study of cellulose fermentation in the continuous cultures of C. cellulolyticum has shown that with increasing amounts of cellulose, at a dilution rate of D=0.048 h\(^{-1}\), cell biomass concentration rises in proportion to the cellulose concentration in the feed stream; however, at and above 7.6 g/L of cellulose, the steady state cell biomass concentration levels off (Desvaux et al., 2001c). Furthermore, an increase in acetate/ethanol ratio as well as an increase in lactate and extracellular pyruvate formation has been observed while shifting from cellulose limited to cellulose sufficient conditions. This shift has been accompanied by a decrease in both \(Y_{\text{ATP}}\) and \(Y_{\text{X/S}}\) as well as an increase in \(q_{\text{ATP}}\), showing the occurrence of an uncoupling growth phenomenon. Therefore, the fact that some metabolized cellulose and ATP are no longer associated with cell biomass formation under cellulose sufficient conditions, along with concomitant increase in lactate formation and pyruvate leakage, suggests the accumulation of an intracellular inhibitory compound(s) that may also explain the establishment of steady state conditions under substrate excess conditions (Desvaux et al., 2001c).

Also, in batch cultures of C. cellulolyticum at different initial cellulose concentrations, the growth arrest event has been shown to limit the maximum cell biomass concentration and cellulolytic activity at high cellulose concentrations, as illustrated in Figure 6-1. It has been suggested that during the course of evolution, C. cellulolyticum bacteria have been exposed to low carbon availability conditions, and have evolved with no need for regulating the entering carbon flow. Therefore, when C. cellulolyticum is growing on easily metabolizable carbon sources such as cellobiose and cellulose, the high carbon flow leads to pyruvate overflow and accumulation of intracellular metabolites and consequently early growth cessation (Guedon et al., 2002). This observation highlights the importance of conducting a metabolomic analysis on C. cellulolyticum. Therefore, in this study, we developed a method for the extraction and
comparative analysis of intracellular metabolite concentrations in *C. cellulolyticum* cultures under high and low cellulose concentrations; particularly, to test the hypothesis that there is a link between intracellular pyruvate accumulation and the growth arrest event at high cellulose concentrations.

![Graph](image)

**Figure 6-1.** The percentage of cellulose degradation (circle), maximum cell biomass concentration (rhombus) and maximum rate of cellulose degradation (square) obtained in 120 h in batch cultures of *C. cellulolyticum* as a function of initial cellulose concentration (Desvaux et al., 2000).

### 6.2 Methods

#### 6.2.1 Experimental characterization of *C. cellulolyticum* cellulolytic activity at high and low cellulose concentrations

Batch cultures of *C. cellulolyticum* were conducted on CM3 medium containing per litre: KH$_2$PO$_4$, 0.75 g; K$_2$HPO$_4$, 0.75 g; (NH$_4$)$_2$SO$_4$, 1.3 g; MgCl$_2$.6H$_2$O, 1 g; CaCl$_2$, 0.15g; FeSO$_4$, 1.25 mg; resazurin, 1 mg; cysteine hydrochloride, 1 g; fibrous cellulose (medium particle size, Sigma-Aldrich, C6288), 20 g or 2 g, and 2 g of yeast extract. The pH was controlled at 7.2, which is the optimal pH for *C. cellulolyticum* growth and cellulolytic activity (Desvaux et al., 2001a). The profiles of cell biomass concentration for a typical batch with 20 g/L cellulose are shown in Figure 6-2a. The protein assay along with qPCR analysis showed that cells reached a stationary growth phase after 9 days with a maximum cell density of $5 \times 10^9$ cell/mL (Figure 6-
These data also verified the appropriateness of the protein assay for profiling the cell biomass concentration in the *C. cellulolyticum* culture for metabolomic samplings.

![Graph showing protein concentration and biomass concentration over time](image)

**Figure 6-2.** (a) *C. cellulolyticum* cell biomass profiles obtained from protein assay and qPCR analyses on 20 g/L cellulose. Arrows indicate the metabolomic sampling points, (b) final concentrations of the products in each set of batches with 20 g/L and 2 g/L initial cellulose concentrations (for dynamic profiles see Figure 6-3). Error bars show the ranges in two duplicate experiments.

The main fermentation products in the batch cultures with 20 g/L and 2 g/L cellulose were acetate, ethanol, formate, lactate, pyruvate and succinate, as shown in Figure 6-2b (measured by HPLC analysis as it was explained in chapter 5). Under high cellulose concentration of 20 g/L, more carbon flow has been oriented towards acetate and lactate formations compared to the batches under low cellulose concentration of 2 g/L.
6.2.2 Method for the metabolomic study

*C. cellulolyticum* was cultivated on modified CM3 medium at pH of 7.2, under low cellulose concentration (2 g/L) or high cellulose concentration (20 g/L), as described in the previous section. Metabolomic samples were taken during the exponential and stationary growth phases from *C. cellulolyticum* batch cultures under high and low cellulose concentrations, as represented by arrows on Figure 6-2a. The procedure for intracellular metabolome extraction was as follows:

1. 20 mL of culture was passed quickly through 47 mm nylon filters (Millipore, HNWP04700, 0.45 µm), and the filter-bound cells were quenched by immediately dropping the filter into 2.5 mL of -20°C extraction solvent: 40:40:20 acetonitrile/methanol/water (Amador-Noguez et al., 2011, Lu et al., 2010).

2. The quenched cells were vortex mixed and extracted at -20°C for 30 min.

3. The extraction solvent was transferred into eppendorf tubes; the residual cells were washed from filter with 1 mL extraction solvent, and it was combined with previous extraction solvent.

4. The mixture of extraction solvent and cell debris was centrifuged for 5 min at 13000 g and 4°C, and the supernatant was stored at 4°C.

5. The pellet was resuspended in 600 µL of extraction solution, and extracted at -20°C for 15 min, followed by centrifugation for 5 min at 13000 g and 4°C.

6. The two supernatants were combined, and then frozen using liquid nitrogen and stored at -80°C.

7. For the targeted LC/MS (Liquid Chromatography/Mass Spectrometry) analysis of metabolomic samples, 0.5 mL of each metabolomic sample was dried using a vacuum dryer at 30°C for 2 h, and the dried samples were then resuspended in 0.4 mL HPLC grade water. In contrast, the original method has used drying under nitrogen for sample preparation (Lu et al., 2010).

The targeted analyses of pyruvate and lactate concentrations were conducted using reversed-phase ion-exclusion liquid chromatography coupled to an Orbitrap mass spectrometer. An LC/MS method was developed for analyzing the metabolomic samples using a HPX-87H HPLC column (300 x 7.8 mm, 9 µm particle size), where the mobile phase was 0.1% formic acid in water, with a flow rate of 0.15 mL/min at room temperature. The mass spectrometry was
conducted in negative ion mode with the scan range of 50-900. The limit of detection (LOD) was 50 ng/mL (for both pyruvate and lactate), and pyruvate and lactate loss of 57% and 2% were observed in 5.0 ppm solutions using this method.

### 6.2.3 Calculation of intracellular lactate concentration

*C. cellulolyticum* is a rod-shaped bacterium which is 3–6 µm long and 0.6–1 µm wide (Desvaux, 2005b), so the cell volume of 4.7 (µm)³ was approximated for *C. cellulolyticum* based on its geometry. The specific cell volume of *C. cellulolyticum* was also estimated using the *E. coli* specific cell volume (2.15 µL/mg dry cell (Park et al., 2011)), with a correction factor (Cₖ) of 6.7 which was based on the cell volume ratios (*E. coli* cell volume is 0.6–0.7 (µm)³ (Kubitschek, 1990)). The intracellular volume of the cells in that 20 mL metabolomic sample culture (V₊), was calculated using sample size (20 mL), cell biomass concentration (mg protein/L) and protein content of *C. cellulolyticum* (0.62 g protein/g cell biomass dry weight (Giallo et al., 1985)) as follows:

\[
V_{\text{int}} = 20 \text{ (mL)} \times \text{cell concentration (mg protein/L)} \times 2.15 \text{ (µL/mg dry cell}) \times C_f/0.62
\]

The intracellular concentration \(C_{\text{int}}^m\) of each metabolite \(m\) was then calculated from the concentration of the metabolite in that 4.1 mL extraction solvent \(C_{\text{solv}}^m\) and \(V_{\text{int}}\):

\[
C_{\text{int}}^m \text{ (g/L)} = C_{\text{solv}}^m \text{ (g/L)} \times 4.1 \text{ (mL)} /V_{\text{int}} \text{ (mL)}
\]

For example, in sample a-5 with the cell concentration of 370 mg protein/L:

\[
V_{\text{int}} = 20 \times 10^{-3} \text{ (L)} \times 370 \text{ (mg protein/L)} \times 2.15 \text{ (µL/mg dry cell}) \times 6.7/0.62 \text{ (mg protein/mg cell biomass dry weight)} = 0.17 \text{ mL}
\]

Intracellular lactate concentration = lactate concentration in the solvent (0.45 g/L) × 4.1 (mL) /0.17 (mL) = 11 (g/L)
6.3 Results and discussion

The cell biomass formation profiles in *C. cellulolyticum* batch cultures with 20 g/L and 2 g/L cellulose are presented in Figure 6-3a, b, e and g, where arrows show the metabolomic sampling points. At the high cellulose concentration of 20 g/L, cells reached a stationary growth phase after 8 days, with a maximum cell biomass concentration of 0.7 g/L, assuming 0.62 g protein/g cell dry weight of *C. cellulolyticum* (Giallo et al., 1985). Notably, drastic increases in the concentration of pyruvate and lactate were observed in the cultures with 20 g/L cellulose. These increases were coinciding with the growth arrest and the switch to a stationary growth phase, which was in agreement with previous reports (Desvaux et al., 2000). However, in the batch cultures at 2 g/L cellulose, the cells reached the stationary growth phase after cellulose depletion, as has been reported for cultures on cellulose concentrations below 6.7 g/L (Desvaux et al., 2000).

Table 6-1 presents the results of the LC/MS analysis of the metabolomic samples. As cells were transitioning from exponential growth to the stationary growth phase (e.g., from a-1 to a-5), in the batches from a high cellulose concentration of 20 g/L, intracellular accumulation of lactate was observed in each set of metabolomic samples. The intracellular lactate concentration reached 9.4 g/L after 5 days of growth in the culture. In addition, the intracellular lactate concentration reached 13 g/L in the stationary growth phase. In contrast, in the batches with low cellulose concentration of 2 g/L, no intracellular lactate accumulation was observed.

In addition, no intracellular pyruvate accumulation was observed in all of the metabolomic samples under low and high cellulose concentrations, which rejected the hypothesis that intracellular pyruvate accumulation is involved in the growth arrest event. However, this observation implies a possible connection between the lactate accumulation and the growth arrest in *C. cellulolyticum* at high cellulose concentration.
Figure 6-3. The profiles of products and cell biomass formations in the batches from which metabolomic samples were taken: a, b at 20 g/L, and e, g at 2 g/L initial cellulose concentration. Arrows show the metabolomic sampling points; e.g., sample a-1 is the sample #1 of batch “a” as indicated by an arrow.
Table 6-1. LC/MS analysis of the metabolomic samples. Cell biomass formation profiles and sampling points in batches a, b, c, and g are presented in Figure 6-3; e.g., sample a-1 is the sample #1 of batch “a” as indicated by an arrow. ND: not detectable.

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<th>Sample ID</th>
<th>Days after starting to grow</th>
<th>Cell biomass concentration (g protein/L)</th>
<th>Lactate conc. in 4.1 mL extraction solvent (g/L)*</th>
<th>Intracellular lactate conc. (g/L)</th>
<th>Extracellular lactate conc. (g/L)</th>
<th>Intracellular pyruvate conc. (g/L)</th>
<th>Extracellular pyruvate and lactate detection limit (mg/L)**</th>
<th>Extracellular pyruvate conc. (mg/L)</th>
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<td>8</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>e-1</td>
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<td>5</td>
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<td>e-2</td>
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<td>ND</td>
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<td>ND</td>
<td>5</td>
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</tr>
<tr>
<td>e-3</td>
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<td>ND</td>
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<td>ND</td>
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<td>ND</td>
<td>ND</td>
<td>0.3</td>
<td>ND</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>g-2</td>
<td>2</td>
<td>0.07</td>
<td>ND</td>
<td>ND</td>
<td>0.3</td>
<td>ND</td>
<td>6</td>
<td></td>
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<tr>
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<td>0.07</td>
<td>ND</td>
<td>ND</td>
<td>0.3</td>
<td>ND</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

*All values were determined with an average accuracy of ±10%.

*Intracellular lactate concentrations were calculated from lactate concentrations in 4.1 mL extraction solvent, using Equation (2).

**Intracellular pyruvate and lactate detection limit = 50 (ng/mL) × 4.1 mL extraction solvent/V_int, and V_int for each sample was obtained using Equation (1).
Previous kinetic analysis of the fermentation products in *C. cellulolyticum* culture with pH controlled at 7.2 has also shown accelerated lactate formation while the culture is transitioning to the stationary growth phase on high cellulose concentration of 29.1 g/L. Lactate formation is coinciding with pyruvate formation in the culture and the growth arrest (Desvaux et al., 2000), suggesting accumulation of an intracellular compound under high carbon flow conditions as an explanation for the growth arrest event (Guedon et al., 1999a). Furthermore, consecutive re-inoculations of this culture with fresh inoculum, while cells had reached the stationary growth phase, has significantly increased the cell biomass concentration and cellulose degradation in the *C. cellulolyticum* culture with 29 g/L cellulose, verifying that growth arrest event is not due to end product inhibitions or availability of adhesion sites (Desvaux et al., 2000). The lactate concentration in this culture at re-inoculation points has been in the range of 1.8 to 6.2 g/L, which confirms that *C. cellulolyticum* growth is not inhibited in the presence of lactate. Furthermore, since, at this extracellular pH of 7.2, only 0.045% of the total lactate concentration is in undissociated membrane permeable form, it indicates that trans-membrane migration of lactate is not the reason for the intracellular lactate accumulation.

Moreover, a recent metabolic engineering study of *C. cellulolyticum* (Li et al., 2012), in which lactate dehydrogenase and malate dehydrogenase genes have been disrupted using targeted mutagenesis, has shown that the double mutant ferments more cellulose compared to the wild-type species growing on 10 g/L cellulose. Thirty eight percent increase in cellulose degradation has been reported, and the mutant produces very little lactate and acetate, while the ethanol yield has increased 9 times, compared to the wild-type species (Li et al., 2012). In another study, heterologous expression of pyruvate decarboxylase and alcohol dehydrogenase genes from *Zymomonas mobilis* in *C. cellulolyticum* has resulted in a prolonged growth of *C. cellulolyticum*, a 180% increase in cellulolytic activity, a 48% decrease in lactate production, and a 60% decrease in pyruvate production, while the concentration of acetate and ethanol increased by 93 and 53% respectively (Guedon et al., 2002).

Also, it has been shown that the lactate formation flux in *C. cellulolyticum* has a weak correlation with *in vitro* LDH activity, which can be related to regulation effects imposed by intracellular metabolites (Guedon et al., 1999b). Based on these results and our observations, it can be hypothesized that the likely explanation for the improved cellulolytic activity and cell
biomass formations in the above mutant and recombinant strains, compared to the wild-type strain, is the decreased level of lactate production and consequently decreased level of intracellular lactate accumulation. It is likely that there is a limit on the lactate transportation pathway in *C. cellulolyticum* that leads to intracellular lactate accumulation and growth cessation. This hypothesis can be tested by over-expressing the lactate transporter gene in *C. cellulolyticum*.

In addition, the metabolomic samples were also examined for the presence of acetyl CoA (exact mass of 809.1258 u), glucose 6-phosphate (exact mass of 260.0297 u), NADH (exact mass of 65.1248 u), coenzyme A (exact mass of 767.1152 u), glyceraldehyde 3-phosphate (exact mass of 169.998 u), and 1,3 diphosphoglycerate (exact mass of 265.6593 u) in the samples, but no peaks were detected for any of the mentioned compounds. It is possible that some volatile compounds have been evaporated during the vacuum drying step. The ideal procedure was to analyze the samples without further processing (i.e., drying) to minimize the errors, but the HPLC column used in this study was not compatible with methanol in the solvent. Also, this HPLC column and method might have not been suitable for separating and detecting these compounds.

### 6.3.1 Profiling intracellular pH\(_i\) at high cellulose concentration

To investigate the effect of intracellular lactate accumulation on the intracellular medium acidity, and to address if an intracellular pH drop is involved in the growth arrest event in *C. cellulolyticum*, extracellular pH (pH\(_e\)) and intracellular pH (pH\(_i\)) were estimated using Henderson-Hasselbalch equation:

\[
\text{pH}_e = \text{pK}_a + \log_{10}\left(\frac{[\text{lac}^-]}{[\text{lactic acid}]}\right) \\
\text{pH}_i = \text{pK}_a + \log_{10}\left(\frac{[\text{lac}^-]}{[\text{lactic acid}]}\right)
\]

Where the pK\(_a\) of lactic acid is 3.86 and the extracellular pH (pH\(_e\)) was 7.2. Total lactic concentration ([lact] + [lactic acid]) was obtained from HPLC (extracellular) and LC/MS (intracellular) measurements. The undissociated lactic acid concentration ([lactic acid]) was deemed to be equal inside and outside the cells, since the undissociated forms of fermentation acids are membrane permeable, therefore, they can pass freely across the cell membrane. The
estimated intracellular pH \( i \) at each metabolomic sampling point is presented in Table 6-2. In all metabolomic samples obtained from \( C.\ cellulolyticum \) batch cultures with high cellulose concentration of 20 g/L, the estimated intracellular pH \( i \) was in the range of 7.1 to 7.8, which was consistent with the previously reported intracellular pH \( i \) in \( C.\ cellulolyticum \) (Russell and Wilson, 1996, Desvaux, 2005b). Therefore, the acidification of intracellular medium in \( C.\ cellulolyticum \) has not been the reason for the growth arrest event. The intracellular lactic acid accumulation was mainly in form of lactate anion, as only 0.02% of total lactic acid was found in undissociated form in this range of intracellular pH \( i \). Furthermore, as discusses earlier, at this pH \( c \) of 7.2, only 0.045% of the total lactate concentration is in the undissociated membrane permeable form, which indicates that the trans-membrane migration of lactate is not the reason for the intracellular lactate accumulation.

Table 6-2. \( C.\ cellulolyticum \) intracellular pH values estimated from intracellular and extracellular lactate concentrations in metabolomic samples, obtained from \( C.\ cellulolyticum \) batch cultures with high cellulose concentration of 20 g/L. Total lactic concentration \([\text{lac}] = [\text{lactic acid}]\) was obtained from HPLC (extracellular) and LC/MS (intracellular) measurements*.

<table>
<thead>
<tr>
<th>Sample id</th>
<th>Cell biomass concentration (g/L)</th>
<th>Total intracellular lactate conc. (g/L)**</th>
<th>Total extracellular lactate conc. (g/L)</th>
<th>Undissociated lactic acid conc. (mg/L)</th>
<th>pH ( i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-3</td>
<td>0.19</td>
<td>0.5</td>
<td>0.3</td>
<td>0.1</td>
<td>7.3</td>
</tr>
<tr>
<td>a-4</td>
<td>0.43</td>
<td>7.0</td>
<td>2.9</td>
<td>1.3</td>
<td>7.6</td>
</tr>
<tr>
<td>a-5</td>
<td>0.37</td>
<td>11</td>
<td>3.9</td>
<td>1.8</td>
<td>7.6</td>
</tr>
<tr>
<td>b-1</td>
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<td>2.1</td>
<td>0.6</td>
<td>0.3</td>
<td>7.7</td>
</tr>
<tr>
<td>b-2</td>
<td>0.37</td>
<td>7.1</td>
<td>2.0</td>
<td>0.9</td>
<td>7.7</td>
</tr>
<tr>
<td>b-3</td>
<td>0.43</td>
<td>9.4</td>
<td>3.8</td>
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<td>7.6</td>
</tr>
<tr>
<td>b-4</td>
<td>0.41</td>
<td>13</td>
<td>4.0</td>
<td>1.8</td>
<td>7.7</td>
</tr>
<tr>
<td>c-2</td>
<td>0.23</td>
<td>0.4</td>
<td>0.5</td>
<td>0.2</td>
<td>7.1</td>
</tr>
<tr>
<td>d-2</td>
<td>0.27</td>
<td>8.7</td>
<td>2.4</td>
<td>1.1</td>
<td>7.8</td>
</tr>
</tbody>
</table>

*All values were determined with an average accuracy of ±10%.

**Intracellular lactate concentrations were calculated using Equation (2), as presented in Table 6-1.

Intracellular anion accumulation and toxicity under acidic pH has also been previously shown to be the reason for the growth inhibition in cellulolytic bacteria as well as \( E.\ coli \) cultures. Cellulolytic bacteria are unable to grow at low intracellular pH, and it has been shown that under an acidic environment, where the pH gradient (\( \Delta \text{pH} \)) across the cell membrane is high, the intracellular dissociation of fermentation acids, which are membrane permeable in undissociated form, and the intracellular accumulation of acid anions lead to anion toxicity,
which is the likely reason of growth inhibition under acidic conditions (Russell and Wilson, 1996, Flythe and Russell, 2006).

Furthermore, it has been shown that presence of lactate and acetate ions in an acidic medium leads to a significant decline in glutamate synthesis in Clostridium sporogenes MD1, which inhibits the bacterial growth (Flythe and Russell, 2006). Also, in E. coli cultures at pH 6, incubation of cells in 8 mM acetate for 20 min resulted in intracellular accumulation of acetate anions (240 mM), and reduced the level of intracellular glutamate pools (Roe et al., 1998). Furthermore, in mildly acidic E. coli cultures (pH 6), inhibition of methionine biosynthesis by acetate (8 mM) and the toxicity of accumulated homocysteine have been indicated as the cause of growth inhibition by acetate under weak acid stress (Roe et al., 2002), where addition of methionine to this culture could restore the E. coli growth rate to some significant extent. This effect has been also reported for other organic acids.

In conclusion, intracellular lactate accumulation along with the intracellular pH data in C. cellulolyticum cultures at high cellulose concentration of 20 g/L suggests that lactate anion accumulation and toxicity, possibly through its inhibitory effect on the biosynthesis of a growth precursor, is the likely reason for the growth arrest event in C. cellulolyticum. This lactate accumulation might be due to a limit on the lactate transportation pathway in C. cellulolyticum.

6.4 Conclusion

C. cellulolyticum produces significant amounts of lactate when it is growing under high cellulose concentrations; however, its growth and metabolism is limited due to the growth arrest event. In this study, we propose and hypothesize that there is a limit on the lactate transportation pathway in C. cellulolyticum which leads to intracellular lactate accumulation and the growth arrest. This hypothesis can be tested by over-expressing the lactate transporter gene in C. cellulolyticum, using existing gene transfer techniques for C. cellulolyticum ATCC 35319 (Jennert et al., 2000, Tardif et al., 2001, Guedon et al., 2002), and investigating its effects on the metabolism of C. cellulolyticum at high cellulose concentrations. Overcoming the growth arrest barrier and improving the lactate production in C. cellulolyticum culture would promote it as a potential CBP approach for cellulosic lactate production. In addition, developing methods for targeted LC/MS analysis of other metabolites involved in the central metabolism of C. cellulolyticum will assist in obtaining a more comprehensive picture of the
growth arrest phenomenon in *C. cellulolyticum*, and identifying other potential metabolites which might be playing roles in the growth arrest event.
Chapter 7
Summary, conclusions and recommendations for future work

7.1 Summary and conclusions

The eventual goal of this study was to develop methods for the analysis of metabolism in the co-culture of *C. acetobutylicum* and *C. cellulolyticum*, and to investigate the inter-species metabolic interactions that enhance the metabolic rate and biofuel synthesis. To address this goal, the following objectives were defined and investigated:

1. To develop a genome-scale metabolic model of *C. acetobutylicum* ATCC 824 that includes the thermodynamic and metabolomic constraints, and to achieve thermodynamically feasible phenotypes.

An updated genome-scale metabolic model of *C. acetobutylicum* ATCC824 was developed, which consists of 700 genes, 712 reactions, and 679 metabolites, covering 16.3% of ORFs, and is more comprehensive compared to two other existing models. This metabolic network was used as a platform for simulating phenotypes using the constraint-based modeling approach. Flux variability analysis showed the presence of alternate carbon and electron sinks that allows for different carbon assimilation patterns and ranges of product formation rates.

We applied an NMR based approach to quantify 31 intracellular metabolites in both acidogenic and solventogenic phases. The total molar concentration of metabolites was approximately 245mM, where asparagine was the most dominant metabolite. Incorporation of these metabolomic data along with thermodynamic data into this model resulted in thermodynamically feasible flux distributions. However, flux balance analysis cannot predict the complex metabolism of *C. acetobutylicum* properly, and additional constraints are required to narrow down the solution space. Incorporating the regulatory network in the genome-scale model seems to be necessary for describing the metabolic shift from acidogenic to solventogenic phase.

2. To develop the genome-scale metabolic model of *C. cellulolyticum*, and to apply the developed metabolic models of the pure cultures to develop the genome-scale model of metabolic interactions in the co-culture.
The genome-scale metabolic model of *C. cellulolyticum* H10, which comprises 431 genes, 621 reactions, 603 metabolites, and covers 12% of ORFs, was developed and used as a platform for simulating phenotypes using the constraint-based modeling approach. *C. cellulolyticum* model (iFS431) was able to predict the physiology during growth in the presence of cellulose and cellobiose. The model of co-culture metabolism was developed by combining these metabolic models of the pure cultures. This co-culture model was used to analyze the integrated physiology of this co-culture, and the results showed that at high cellulose concentrations, the model is not able to capture the *C. cellulolyticum* growth arrest, suggesting that cellobiose inhibition removal by *C. acetobutylicum* is not the main factor that improves cellulose degradation in the co-culture. Also, these simulations highlighted the importance of the *C. cellulolyticum* cellulosome characterization. Further investigation to understand the mechanism behind this growth arrest is required for improving the model predictions at high cellulose concentrations as well as predictions of the co-culture physiology.

Genome-scale models of *C. cellulolyticum* and the co-culture modeling framework, as presented here, will be valuable for further optimization of this co-culture for improved cellulose degradation and biobutanol production. This metabolic model of the co-culture can also be easily extended to incorporate hemicellulose metabolism for growth on more complex cellulosic substrates. The comprehensive genome-scale model of the co-culture metabolism can be applied to devise methods for design and optimization of environmental, process, and genetic parameters; and for analyzing the impact of the genetic perturbations (gene additions or gene deletions) as well as environmental parameters such as medium composition, on the metabolic activity of the clostridial co-culture to enhance the desired product formation.

3. To characterize the co-culture metabolism by developing molecular biology methods.

Clostridial co-culture containing cellulolytic and solventogenic species is a potential consolidated bioprocessing (CBP) approach for producing biochemicals and biofuels from cellulosic biomass. It has been demonstrated that the rate of cellulose utilization in the co-culture of *C. acetobutylicum* and *C. cellulolyticum* is improved compared to the mono-culture of *C. cellulolyticum* (Petitdemange et al., 1983). However, the metabolic interactions in this co-culture are not well understood. To investigate the metabolic interactions in this co-culture, we dynamically characterized its physiology and microbial composition using qPCR.
Therefore, in this study, we investigated metabolic interactions in this co-culture by developing a comparative qPCR analysis of the co-culture and mono-cultures of *C. cellulolyticum* and *C. acetobutylicum*. Investigation of the metabolism in this clostridial co-culture along with the mono-cultures revealed that a significant increase in the rate of cellulose hydrolysis can be achieved using the co-culture and making use of the synergism existing between the two clostridial species. The qPCR data suggested a higher growth rate of *C. cellulolyticum* in the co-culture compared to its mono-culture.

Our results also showed that in contrast to the mono-culture of *C. cellulolyticum*, which did not show any cellulolytic activity under conditions similar to those of the co-culture, the co-culture did show cellulolytic activity even superior to the *C. cellulolyticum* mono-culture at its optimal pH of 7.2. Moreover, experiments indicated that the co-culture cellulolytic activity depends on the concentration of *C. acetobutylicum* in the co-culture, as no cellulolytic activity was observed at a low concentration of *C. acetobutylicum*, thus confirming the essential role of *C. acetobutylicum* in improving *C. cellulolyticum* growth in the co-culture. Furthermore, a butanol concentration of 350 mg/L was detected in the co-culture batch experiments.

These results suggest the presence of synergism between these two species, while *C. acetobutylicum* metabolic activity significantly improves the cellulolytic activity in the co-culture, and allows *C. cellulolyticum* to survive under suboptimal co-culture conditions, which do not allow *C. cellulolyticum* to grow and metabolize cellulose independently. It is likely that *C. acetobutylicum* improves the cellulolytic activity of *C. cellulolyticum* in the co-culture through exchange of metabolites such as pyruvate, enabling it to grow and metabolize cellulose under suboptimal co-culture conditions. This clostridial co-culture can offer a considerable potential CBP approach for producing commodity chemicals from cellulosic biomass, taking advantage of the *C. acetobutylicum* metabolic potential to convert sugars to a variety of chemicals.

We also observed a significant increase in acetate and ethanol production in the co-culture compared to the mono-culture of *C. cellulolyticum* at high cellulose concentrations, where the carbon flow was redirected from lactate to acetate and ethanol formations. The produced acetate can be further reduced to ethanol by integrating this CBP with a chemical reduction unit.
4. To examine the growth arrest event in *C. cellulolyticum*

The knowledge of the underlying growth arrest mechanism at high cellulose concentration is required for modeling *C. cellulolyticum* and the co-culture metabolism. It has been suggested that during the course of evolution, *C. cellulolyticum* bacteria have been exposed to low carbon availability conditions, and have evolved with no need for regulating the entering carbon flow; therefore, when *C. cellulolyticum* is growing on easily metabolizable carbon sources such as cellubiose and cellulose, it has been suggested that the high carbon flow leads to pyruvate overflow and accumulation of intracellular metabolites, and consequently, early growth cessation (Guedon et al., 2002). This premise highlights the importance of conducting a metabolomic analysis on *C. cellulolyticum*. Therefore, in this study, we developed an LC/MS method for the extraction and comparative analysis of intracellular metabolite concentrations in *C. cellulolyticum* cultures under high and low cellulose concentrations; particularly, to test the hypothesis that there is a link between intracellular pyruvate accumulation and the growth arrest event at high cellulose concentrations.

No intracellular pyruvate accumulation was observed in all of the metabolomic samples under low and high cellulose concentrations, which rejected the hypothesis that intracellular pyruvate accumulation is involved in the growth arrest event. However, in the batches with a high cellulose concentration of 20 g/L, intracellular accumulation of lactate was observed in each set of metabolomic samples as cells were transitioning from exponential growth to the stationary growth phase. These results suggest a possible connection between the lactate accumulation and the growth arrest event in *C. cellulolyticum* at high cellulose concentration.

Intracellular lactate accumulation along with the intracellular pH data in *C. cellulolyticum* cultures at high cellulose concentration of 20 g/L suggest that lactate anion accumulation and toxicity, possibly through its inhibitory effect on the biosynthesis of a growth precursor, is a possible reason for the growth arrest event in *C. cellulolyticum*. We propose and hypothesize that this lactate accumulation might be due to a limit on the lactate transportation pathway in *C. cellulolyticum* which leads to intracellular lactate accumulation, and may account for the growth arrest.
7.2 Engineering significance

The first genome-scale metabolic model of *C. cellulolyticum* was presented in this study. This model has been validated against the experimental data, and is able to predict the metabolism of *C. cellulolyticum* on cellobiose and cellulose. Also, we presented an updated genome-scale metabolic model of *C. acetobutylicum*, which has a higher ORF coverage compared to other existing models. However, incorporation of the regulatory network seems to be necessary to model the complex metabolism of *C. acetobutylicum*. These genome-scale metabolic models are providing platforms for future analyses and optimization of the metabolism in these two clostridial species, using *in silico* generated hypotheses that can be tested experimentally.

Furthermore, this model of *C. cellulolyticum* metabolism has been integrated with the *C. acetobutylicum* model to develop the first genome-scale model of the clostridial co-culture metabolism. The results of our simulations suggest that 1) removal of cellobiose inhibition is not the main factor responsible for improved cellulose utilization in the co-culture, and 2) *C. cellulolyticum* is primarily adapted to low carbon flow and availability (Guedon et al., 1999a). These results also motivate the need for metabolic engineering strategies to enable *C. cellulolyticum* to handle high carbon flows. This co-culture model will be valuable for further analyses and optimization of the co-culture for the production of various biochemicals in this consolidated bioprocessing approach.

We also developed and validated the first qPCR method for the characterization of the clostridial co-culture physiology, dynamics and composition. This method will be valuable for the future analyses of the interactions between these two species, and for devising methods to improve the metabolic and cellulolytic activity in this co-culture. Optimization of the co-culture medium and experimental conditions seems to be necessary, as small variations in the medium preparation resulted in different cellulolytic activities in our co-culture experiments. Moreover, we used a novel method to investigate the growth arrest event in *C. cellulolyticum* through a comparative *in vivo* metabolite analysis. We proposed a hypothesis that there is a limit on the lactate transportation pathway in *C. cellulolyticum*, which leads to intracellular lactate accumulation and the growth arrest. The knowledge of the growth arrest mechanism in *C. cellulolyticum* metabolism is an essential requirement for improving its metabolic and cellulolytic activity, under industrial conditions with high cellulose concentrations.
7.3 Future work

1- Optimization of the co-culture medium and experimental conditions
The co-culture of *C. acetobutylicum* and *C. cellulolyticum* can be further improved and optimized for cellulose degradation and biochemical production. In a recent study, it has been shown that the expression of cellulases in *C. cellulolyticum*, at both transcription and protein levels, is induced by glucose, while the expression of cellulosomal genes are repressed under cellobiose and xylose, through a carbon catabolite repression mechanism (Xu et al., 2013). In *C. cellulolyticum* cultures on cellulose, glucose addition led to a decrease in the culture’s lag phase. The peak cellulose degradation rate increased compared to the control culture on cellulose (0.854 g/L.day and 0.607 g/L.day respectively), with less than 0.5 g/L of glucose in the culture. Therefore, adding a small amount of glucose to the co-culture in a fed-batch system may benefit cellulose degradation in the co-culture, and it needs to be investigated.

Recently, the medium conditions have been optimized for *C. cellulolyticum* cultivation on cellobiose (Wang et al., 2013). In the co-culture of *C. acetobutylicum* and *C. cellulolyticum*, the medium composition, initial cellulose concentration, the impact of the co-culture pH profile, and the duration of *C. cellulolyticum* culture before the addition of *C. acetobutylicum* should be explored. We examined the metabolism of the co-culture under low concentrations of *C. acetobutylicum*, and demonstrated its drastic effect on the co-culture cellulosolytic activity. The impact of initial concentration of both species on the co-culture metabolism can be further investigated and optimized. This study also might be helpful in reducing the observed long lag phase in the co-culture experiments.

2- Developing LC/MS methods for untargeted metabolomic analysis of the growth arrest event in *C. cellulolyticum*
Potential compounds and metabolites unidentified in this thesis, which may play a role in the growth arrest event, can be identified by conducting untargeted analysis of the LC/MS data. Also, methods for targeted LC/MS analysis of other metabolites involved in the central metabolism and cofactors such as NAD$^+$ can be developed and employed, to obtain a more comprehensive picture of the growth arrest phenomenon in *C. cellulolyticum*. 
3- $^{13}$C labelling experiment to trace the carbon distribution pattern in the co-culture

To test the hypothesis that *C. acetobutylicum* can improve cellulose hydrolysis in the co-culture by preventing the cellobiose overflow towards *C. cellulolyticum* metabolism, a $^{13}$C labelling experiment and $^{13}$C metabolic flux analysis (MFA) can be applied.

4- Comparative transcriptomic study of *C. cellulolyticum*

Further understanding of the growth arrest mechanism in *C. cellulolyticum* can be obtained by comparing the gene expression profiles under low and high cellulose concentrations, and transcriptional profiling of the genes involved in the central metabolism of *C. cellulolyticum* during the switch from exponential phase to stationary phase and growth arrest. RNAseq and qPCR methods can be applied for this purpose. Also, profiling gene expression in the co-culture can provide us with further understanding of the interactions between the two species in the co-culture.

5- Further investigation of the specificity of the metabolic interactions between the two clostridial species in this co-culture

In order to examine the specificity of the synergism in the clostridial co-culture, and to find out if other microbial species would also have similar metabolic interactions with *C. cellulolyticum* in a co-culture, we tried an *E. coli* strain C and *C. cellulolyticum* co-culture system. No significant improvement in the co-culture cellulolytic activity compared to the control *C. cellulolyticum* culture was observed. Further details and the results of this experiment are presented in Appendix D-6. However, this experiment has been conducted under unregulated pH conditions and the final pH in all bottles was about 5.5, which is below the optimal pH range for cellulolytic activity. Therefore, running this co-culture under pH-controlled conditions and the pH profile that has been used for clostridial co-culture would be more insightful. Such a co-culture would also be interesting as a potential CBP for producing other biochemistry.

6- Modeling the regulatory network of *C. acetobutylicum*

In a recent study (Wietzke and Bahl, 2012), the redox-sensing transcriptional repressor protein Rex has been identified as a transcriptional regulator of solventogenesis in *C. acetobutylicum*; in response to the cellular NADH/NAD$^+$ ratio, Rex controls the expression of the genes
involved in the butanol formation pathway. The Rex-negative mutant produced significantly higher amounts of butanol and ethanol. The solventogenesis started earlier than in the wild type, and acetone and hydrogen formation were significantly decreased. Also, the expression of NADH dependant alcohol dehydrogenase was significantly higher in the Rex mutant. These findings offer new metabolic engineering targets for improving the butanol production in \textit{C. acetobutylicum}. Furthermore, incorporating the regulatory network in the genome-scale model of \textit{C. acetobutylicum} seems to be necessary for describing the metabolic shift from acidogenic to solventogenic phase.

7- Over-expression of PFO (pyruvate ferredoxin oxidoreductase) and the lactate formation pathway in \textit{C. cellulolyticum}

The rationale behind this experiment is based on the finding that the lactate production pathway may be constrained. Simulation of the \textit{C. cellulolyticum} metabolism without any constraint on the LDH (lactate dehydrogenase) and PFO (pyruvate ferredoxin oxidoreductase), at high cellobiose uptake rate (1.86 mmol/gDCW.h) has shown that pyruvate will not be produced if there is no capacity constraint on these two reactions (Chapter 4). It has been suggested that limited PFO activity which cannot support high pyruvate flow is the reason for this pyruvate overflow (Guedon et al., 1999b). Furthermore, high pyruvate affinity of PFO (k_m=0.57mM), which results in PFO saturation, as well as low pyruvate affinity of lactate dehydrogenase (LDH, k_m=4.5mM), where there is a significant difference in the \(V_{\text{max}}/K_{\text{m}}\) ratios (5.02×10\(^{-3}\) min\(^{-1}\)mg\(^{-1}\) for PFO and 0.182×10\(^{-3}\) min\(^{-1}\)mg\(^{-1}\) for LDH), can explain the intracellular pyruvate accumulation and overflow (Guedon et al., 1999b). Also, it has been shown that the lactate formation flux has a weak correlation with \textit{in vitro} LDH activity, which can be related to the regulation effects imposed by intracellular metabolites (Guedon et al., 1999b).

In addition, in batch cultures of \textit{C. cellulolyticum} with a high cellulose concentration, it has been shown that the peak of pyruvate formation coincides with the start of lactate formation, and this pyruvate accumulation in the \textit{C. cellulolyticum} culture shows that the rate of cellulose catabolism is higher than the rate of pyruvate consumption (Desvaux et al., 2000); therefore, a potential strategy for improving the cell biomass yield and consequently cellulose hydrolysis is the reduction of pyruvate accumulation, which has been identified as one of the growth inhibitory metabolites (Guedon et al., 2002) by over-expression of LDH and lactate
transformation pathway. The secreted lactate by the transformant strain can be later fermented by *C. acetobutylicum* in the co-culture for butanol production (Bahl et al., 1986).

In another approach, over-expression of a low affinity PFO in *C. cellulolyticum*, which is a limiting pathway in *C. cellulolyticum* metabolism, can improve the cell density and cellulose degradation; as it has been shown that the cellulose depolymerization by the cellulosome is not the limiting step in the cellulolytic activity of *C. cellulolyticum* and improving the metabolism of *C. cellulolyticum* should be the main focus (Desvaux, 2005b). A metabolic engineering approach has been applied previously through heterologous expression of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) from *Zymomonas mobilis*, to reduce the pyruvate accumulation, and it increased the cell biomass formation (180%) and cellulose hydrolysis (150%) in batch culture of *C. cellulolyticum* on cellulose. Also, in a very recent work, Higashide *et al.* (2011) applied a metabolic engineering approach to synthesize isobutanol from cellulose using a *C. cellulolyticum* transformant strain.

8- Analysis of H₂ production in the co-culture of *C. cellulolyticum* and *C. acetobutylicum* M5 mutant

It has been shown that the *C. acetobutylicum* M5 mutant, which does not bear the solventogenic genes, has a higher H₂ yield (Oh et al., 2009). Therefore, the co-culture of this mutant with *C. cellulolyticum* can be a promising approach for producing biohydrogen from cellulosic biomass in a consolidated bioprocessing approach. Furthermore, engineering the redox balance and down-regulating the NADH formation from reduced ferredoxin, through ferredoxin-NADH oxidoreductase (FNOR) pathway, will improve redox balance through hydrogen formation, and result in the higher hydrogen formation in *C. acetobutylicum* and *C. cellulolyticum*. 
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Appendix A: supplementary information for chapter 3


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[c] : tdeACP <=> tmrs2eACP | Fatty acid and phospholipid metabolism |
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[c] : hdeACP <=> tpalm2eACP | Fatty acid and phospholipid metabolism |
| 32       | 
[c] : octeACP <=> toctd2eACP | Fatty acid and phospholipid metabolism |
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[c] : uaagtmda + (5) gly --> uaagtmdga + (5) h2o | Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides |
| PPTGS2   | 
[c] : uaagtmdga --> pptg1 + h + udcdp | Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides |
| G3PCT    | 
[c] : ctp + gly3p + h --> cdpglyc + ppi | Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides |
| UGT_BS   | 
[c] : (0.01) 12dgr_CA + (2) udpg --> (0.01) d12dg_CA + (2) h + (2) udp | Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides |
| LIPO4S24_CA | 
[c] : (2400) cdpglyc + d12dg_CA --> (2400) cmp + (2400) h + lipo4-24_CA | Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides |
| LIPO1S24_CA | 
[c] : (2400) cdpglyc + d12dg_CA + (2400) udpg --> (2400) cmp + (4800) h + lipo1-24_CA + (2400) udp | Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides |
| LIPO2S24_CA | 
[c] : (2400) cdpglyc + d12dg_CA + (2400) uacgam --> (2400) cmp + (4800) h + lipo2-24_CA + (2400) udp | Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides |
| LIPO3S24_CA | 
[c] : (2400) ala-D + (2400) atp + (2400) cdpglyc + d12dg_CA + (2400) h2o --> (2400) amp + (2400) cmp + (4800) h + lipo3-24_CA + (2400) ppi | Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides |
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<td>[c] : h + ethap + h2o --&gt; etha + pi</td>
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Appendix B: supplementary information for chapter 3

B-1 Extraction of intracellular metabolites from *Clostridium acetobutylicum* cell cultures

Intracellular metabolite extraction from the cell cultures was performed according to the method described by Maharajan *et al.* (2003). Briefly, the bacterial cultures were centrifuged at 5000 x g for 20 min at room temperature. The pellets were further washed 2-3 times with milliQ water and centrifuged to remove traces of the culture medium. The pellets were resuspended in 0.025 mL milliQ water after respective dry weight measurements and were further subjected to extraction with hot methanol. To the cell suspensions, 0.75-1.0 mL of hot Methanol: water (2:1 v/v) was added. After thorough mixing, the tubes were incubated for 30 min at 70°C with brief vortexing at regular intervals. After incubation, the suspensions were centrifuged (Eppendorf centrifuge) for 10 min (maximum speed) and the supernatant was transferred to a new tube. The metabolite extracts (supernatant) was further dried and stored in the freezer at -20°C until further analysis.

B-2 NMR sample preparation

The dried samples were reconstituted in 250 μL H₂O. 50 μL of 50 mM NaH₂PO₄ buffer (pH 7), 35 μL of D₂O and 15 μL of a standard buffer solution (11.66 mM DSS (disodium-2,2-dimethyl-2-silapentane-5-sulphonate and 0.47% NaN₃ in H₂O) was added to the sample. The sample was vortexed for 1 minute and sonicated for 30 minutes, and then transferred to a standard Shigemi microcell NMR tube for subsequent spectral analysis.

B-3 NMR spectroscopy

All ¹H-NMR spectra were collected on a 500 MHz Inova (Varian Inc., Palo Alto, CA) spectrometer equipped with a 5 mm HCN Z-gradient pulsed-field gradient (PFG) room-temperature probe. ¹H-NMR spectra were acquired at 25 °C using the first transient of the TN-NOESY presaturation pulse sequence, which was chosen for its high degree of quantitative accuracy. Spectra were collected with 256 transients using a 4 s acquisition time and a 1 s recycle delay.
B-4 NMR compound identification and quantification

All FIDs were zero-filled to 64k data points and subjected to line broadening of 0.5 Hz. The singlet produced by the DSS methyl groups was used as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. All $^1$H-NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 6.0 (Chenomx Inc., Edmonton, AB). The Chenomx NMR Suite software allows for qualitative and quantitative analysis of an NMR spectrum by manually fitting spectral signatures from an internal database to the spectrum. Specifically, the spectral fitting for each metabolite was done using the standard Chenomx 500 MHz metabolite library. Typically 90% of all visible peaks were assigned to a compound and more than 90% of the spectral area could be routinely fit using the Chenomx spectral analysis software. Most of the visible peaks are annotated with a compound name. It has been previously shown that this fitting procedure provides absolute concentration accuracies of 90 % or better. Each spectrum was processed and analyzed by at least two NMR spectroscopists to minimize compound misidentification and misquantification. We used sample spiking to confirm the identities of assigned compound. Sample spiking involves the addition of 20-200 μM of the suspected compound to selected clostridium samples and examination whether the relative NMR signal intensity changed as expected.

B-5 Method for thermodynamics-based metabolic flux analysis (TMFA) formulation

Thermodynamics-based metabolic flux analysis (TMFA) integrates thermodynamic constraints into a constraint-based metabolic model to generate thermodynamically feasible fluxes through the metabolic network and to provide data on feasible metabolite activity ranges and $\Delta_r G'$ (Henry et al., 2007). The $\Delta_r G^0$ of the reactions, required for thermodynamic constraints, are estimated using an improved group contribution method (Jankowski et al., 2008) and are adjusted for the cytosol ionic strength. The mixed integer linear constraints include mass balance equations for each metabolite in the network, and the second law of thermodynamics on each reaction considering the uncertainties in $\Delta_r G^0$ values. Furthermore, a flux variability analysis (FVA) analysis of the E.coli metabolic model iJR904 demonstrated that thermodynamic constraints can restrict the net flux through the thermodynamically infeasible loops to zero, while the effect of ionic strength was shown to be in the range of uncertainties in
\( \Delta_r G^0 \) values. Also, a thermodynamic variability analysis (TVA) analysis introduced the reactions with highly negative \( \Delta_r G^0 \) values as candidates for regulatory control points (Henry et al., 2007).

Assuming a closed system and constant pressure, according to the second law of thermodynamics, a reaction occurs only in the direction of negative \( \Delta_r G \), so \( \Delta_r G < 0 \ \forall \ r > 0 \) and \( \Delta_r G > 0 \ \forall \ r < 0 \), where \( r \) is the reaction rate, and a negative rate means the flux in the backward direction (Kummel et al., 2006). The following formulation is applied in TMFA (Henry et al., 2007):

**Maximization of an objective function (e.g., growth rate)**

**Subject to:**

\[
N \cdot v = 0 \tag{a-1}
\]

\[
0 \leq v_i \leq z_i v_{\text{max}}, \quad \{i = 1, \ldots, r\}, \tag{a-2}
\]

\[
\Delta_r G_i' - K + K z_i < 0 \quad \{i = 1, \ldots, r \mid \Delta_r G_i^0 \text{ is known}\} \tag{a-3}
\]

\[
\Delta_r G_i^0 + R T \sum_{j=1}^{m} n_{ij} \ln(x_j) = \Delta_r G_i', \quad \{i = 1, \ldots, r + L \mid \Delta_r G_i^0 \text{ is known}\} \tag{a-4}
\]

\[
\Delta_r G_i' - K y_i < 0, \quad \{i = r, \ldots, r + L\}, \tag{a-5}
\]

\[
y_i + \sum_{j=1}^{r} \alpha_{i,j} z_j \leq \sum_{j=1}^{r} \alpha_{i,j} \quad \{i = r, \ldots, r + L\} \tag{a-6}
\]

Where \( N \) is the m \( \times \) r stoichiometric matrix, and \( v \) is the 1 \( \times \) r flux vector. The total number of reactions (r) is higher than this value in the metabolic model since each reversible reaction is split into separate forward and backward directions, so all reactions have zero or positive fluxes. The \( z_i \), a binary value for each reaction, is zero if the flux is equal to zero, and is equal to one if the flux is positive. The \( v_{\text{max}} \) is the upper flux limit, which is set at a very large value (1000 mmol/grDCW. hr). Equations (a-3, a-4) ensure that the flux distributions and activity profiles satisfy the second law of thermodynamics. The constant \( K \) is a very large positive value that always satisfies Equation (a-3) when \( v_i \) and \( z \) are equal to zero.

Equation (a-4) is the Gibbs free energy equation, and \( \ln(x_j) \) is the natural logarithm of the activity of compound \( x_j \) and \( n_{ij} \) is the stoichiometric coefficient of that compound in the \( i \)th
reaction. The reactions for which $\Delta_r G^0$ cannot be estimated are lumped into overall reactions with known $\Delta_r G^0$, and equations (a-5, a-6) are the thermodynamic constraints for these reactions. The $\alpha_{ij}$ is equal to one if the reaction $j$ is one of the reactions which compose the reaction $i$, and $L$ is the number of them. The $\ln(x_j)$, $v_i$, $\Delta_r G_i$ are the continuous independent variables, and $z_i$ and $y_i$ are the binary variables of this optimization problem. The TMFA results can be validated using the metabolomic data. We used the group contribution method to determine the $\Delta_r G^0$ of all reaction (Jankowski et al., 2008).
Figure A1. Dynamic Multi-species Metabolic Modeling framework (DMMM) (Zhuang et al., 2011) used for developing the clostridial co-culture metabolic model. In this model, $\mu$ is the cell biomass growth rate, $V^A_i$ and $V^B_i$ are the fluxes of metabolite $i$ predicted by genome-scale metabolic models of each species in the co-culture, $X$ is the cell biomass concentration, $[S_i]$ is the concentration of $i^{th}$ metabolite, and $K_s^i$ is the saturation constant for $i^{th}$ metabolite.
Appendix D: supplementary information for chapter 5

D-1 Primer design and qPCR standard plasmid DNA preparation

The qPCR primers have been designed to target the 16S rRNA genes in *C. acetobutylicum* (CA_Cr001) and *C. cellulolyticum* (Ccel_R0007) (Table A2). Primers were designed using OligoAnalyzer3.1 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/), and Sequence Massager (http://www.attotron.com/cybertory/analysis/seqMassager.htm). Criteria for designing primers were as follows:

1- Primers size should be 19-24 bp, and qPCR amplicon size should be 250-300 bp.
2- Primers GC content must be 40-60%.
3- Melting temperature (T_m) for reverse and forward primers should be the same and not differ more than 2°C.
4- At both ends of the primer sequences, it is preferable to have G/C, and no long stretch of any base is desired.
5- Long stretches of G/C (longer than 3) in the last 5 bases of primers 3’ end must be avoided.
6- Hairpins melting temperatures must be far below primers melting temperature.
7- Hetero-dimers and homo-dimers size must be smaller than 1/3 of the primer sequence and homo/hetero-dimers formation ΔG must be higher than -9 kcal/mol.
8- 16S rRNA genes of both species must be aligned to find conserved regions using BLASTn (http://www.ncbi.nlm.nih.gov/blast), and the primers must be in the regions with at least two mismatches for each primer to ensure reaction specificity.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target species</th>
<th>Primer sequence (5’-3’)</th>
<th>Product length</th>
<th>T_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA2 (forward)</td>
<td><em>C. acetobutylicum</em></td>
<td>CTTGTGGTGGAGGTAACGG</td>
<td>386 bp</td>
<td>60°C</td>
</tr>
<tr>
<td>CA2 (reverse)</td>
<td><em>C. acetobutylicum</em></td>
<td>CACTCCAGACATCCAGTTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC2 (forward)</td>
<td><em>C. cellulolyticum</em></td>
<td>TACAGGAGGATAACACAGG</td>
<td>348 bp</td>
<td>60°C</td>
</tr>
<tr>
<td>CC2 (reverse)</td>
<td><em>C. cellulolyticum</em></td>
<td>CGTGGCTTATCCCTCACGTAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primer stocks of 50µM in 10mM Tris buffer with 0.1mM EDTA were stored at -20°C and working stocks of 5µM were used in PCR reactions per following protocol:
Table A3. PCR protocol.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time</th>
<th>Temperature (°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>5min</td>
<td>95</td>
<td>x1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>30sec</td>
<td>95</td>
<td>x30</td>
</tr>
<tr>
<td>Annealing</td>
<td>30sec</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>30sec</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Adding an A overhang</td>
<td>10 min</td>
<td>72</td>
<td>x1</td>
</tr>
</tbody>
</table>

The qPCR standard plasmid solutions for each target species were prepared by cloning the purified PCR products into pCR®.2.1-TOPO® vector using TOPO-TA Cloning® kit (Invitrogen™, K4500-01). In the first step, fresh PCR products were purified using GeneJET PCR Purification Kit (Fermentas, K0701,K0702); the nucleic acid concentrations in purified PCR products were measured to be 67 and 50 ng/µl for *C. acetobutylicum* and *C. cellulolyticum* respectively, using NanoDrop 1000 (Thermo Scientific) spectrophotometer, and PCR products purity were verified by gel electrophoresis.

To set up the cloning reaction, 4 µL of this purified PCR product was incubated with 1 µL of TOPO® vector solution for 5 min at room temperature; subsequently 2 µL of the cloning reaction product was incubated with TOP10® OneShot Chemically Competent *E.coli* cells on ice for 10 minutes. The mixture was then heat-shocked at 42°C for 30s and transferred immediately on ice. After adding 250 µL of S.O.C medium the culture was cultivated for 1 hour in an incubator at 200 rpm and 37°C.

To screen for positive clones, 20 µL, 50 µL and 200 µL of the culture were plated on LB agar plates containing 50 mg/L kanamycin and X-gal (40 µL of 40 mg/mL in DMF) spread on the surface of the solid agar medium. Plates were incubated overnight at 37°C, and refrigerated at 4°C on the next morning. Potential positive clones appearing as white colonies were examined and verified by running colony PCR and gel electrophoresis. Afterward, 3 positive clones for each species were selected and transferred into 5 mL of LB (containing 50 mg/L kanamycin) liquid medium to be cultivated overnight at 37°C and 200 rpm. Glycerol stocks of the clones were prepared by putting 800 µL of each cloned culture into 200 µL of 70% glycerol solution and stored in a -80°C freezer.

Plasmid extraction was conducted using the GenElute Plasmid Miniprep kit (Sigma-Aldrich, USA) and 2 mL of clone cultures. All of the extracted plasmids were sequenced at the University of Toronto Sanger Sequencing Facility, and the plasmids with right inserts were
selected and applied for qPCR calibration. The Plasmid DNA solutions were homogenized by flicking the eppendorf tube several times and then spinning the tube in a mini-centrifuge. The concentration of plasmids in each solution was measured using 1 µL of each sample on a NanoDrop 1000 (Thermo Scientific) spectrophotometer in duplicates, and they are shown in Table A4.

<table>
<thead>
<tr>
<th>Species</th>
<th>Plasmid</th>
<th>Plasmid Size (bp)</th>
<th>Nanodrop Concentration (ng/µL)</th>
<th>Gene Copies/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. acetobutylicum</em></td>
<td>CA4(1)</td>
<td>4286</td>
<td>60.2</td>
<td>1.28E+10</td>
</tr>
<tr>
<td><em>C. cellulolyticum</em></td>
<td>CC10</td>
<td>4248</td>
<td>85.4</td>
<td>1.83E+10</td>
</tr>
</tbody>
</table>

**D-2 qPCR reaction preparation**

Primer stocks of 50 µM in 10 mM Tris buffer with 0.1 mM EDTA were stored at -20°C, and aliquots of 5 µM primer stocks were prepared and used in qPCR reaction preparations to circumvent potential degradation of the primer sequences in multiple freeze-thaws. The qPCR amplification was performed using 2 µL of tenfold diluted sample genomic DNA and 18 µL of a master mix in a total reaction volume of 20 µL. A master mix was prepared for each qPCR run whereby the reagents for the individual reactions were combined prior to plate preparation in order to minimize error. Master mixes were prepared by combining 10 µL of SsoFast™ EvaGreen® Supermix (Bio-Rad, #172-5200), 1 µL of each primer with final concentration of 0.25 µM, and 6 µL of water for each reaction.

The qPCR reaction was prepared in White Individual PCR Tubes™ – Low Tube Strip (Bio-Rad, USA), each strip containing eight 0.2 mL reaction wells. 18 µL of the master mix was put into each well, and then 2 µL of DNA was added to each well with a fresh pipette tip, and the full strip was capped with Ultra Clear Cap Strips (Thermo Scientific, USA). The negative controls (NTC) were placed in the same strip that samples were placed in, and they only contained an 18 µL volume as no DNA was added to these wells. The qPCR amplifications and detections were carried out in a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). The qPCR protocol took place following the protocol in Table A5. Running qPCR temperature gradient tests on both plasmid solutions showed that 60°C was the optimal annealing temperature (T_a) for qPCR runs.
Table A5. qPCR protocol.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time</th>
<th>Temperature</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial Denaturation, slow ramp</td>
<td>3min</td>
<td>98°C</td>
<td>x1</td>
</tr>
<tr>
<td>rate to 2°C/sec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stage 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation, slow ramp rate</td>
<td>5sec</td>
<td>98°C</td>
<td>x40</td>
</tr>
<tr>
<td>to 2°C/sec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annealing and Extension</td>
<td>10sec</td>
<td>T_a</td>
<td></td>
</tr>
<tr>
<td><strong>Plate Read</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stage 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melting Curve, increment 0.5°C for</td>
<td>65-95°C</td>
<td></td>
<td>x1</td>
</tr>
<tr>
<td>5sec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plate Read</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D-3 qPCR calculations and quantifications

To calculate the concentration of DNA copies in the plasmid standard solutions (Table A4), the total plasmid size including the length of the pCR®2.1-TOPO® vector (3.9 kbp) was taken into account, and it was assumed that there is 1 gene copy per 1 molecule of plasmid. Also, 1 mol of bp = 660 g (Farrell, 2010), and Avogadro’s Constant = 6.023×10^23 molecules/mol.

For example, in CA4(1) plasmid standard solution, using the data from Table A4:

The mass of one CA4(1) plasmid molecule = 4286 (bp) × 660 (g/mol bp) /6.023 × 10^{23} (bp/mol bp) = 4.69 × 10^{-18} (g/copy)

So, the quantity of plasmids in 1 μL plasmid solution, with a concentration of 60.2 ng/μL, is obtained by:

60.2 × 10^{-9} (g/μL)/4.69 ×10^{-18} (g/copy) = 1.28 × 10^{10} (copies/μL).

Furthermore, there are eleven 16S rRNA genes in C. acetobutylicum genome (CA_Cr001, CA_Cr004, CA_Cr007, CA_Cr010, CA_Cr013, CA_Cr016, CA_Cr019, CA_Cr022, CA_Cr025, CA_Cr028, CA_Cr033), and eight 16S rRNA genes in C. cellulolyticum genome (Ccel_R0007, Ccel_R0018, Ccel_R0088, Ccel_R0084, Ccel_R0059, Ccel_R0012, Ccel_R0024, Ccel_R0036). Multiple sequence alignment of the 16S rRNA genes for each species using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) showed identical amplification regions for each set of 16S rRNA genes, therefore 8 amplicons with the same size are being produced per copy of C. cellulolyticum genome in a qPCR reaction, and 11
amplicons with the same size are being produced per copy of *C. acetobutylicum* genome in a qPCR reaction; these facts also were verified by observing single peaks in melting curve analysis as well as single DNA bands for each species in agarose gel electrophoresis runs. Therefore, to calculate the concentration of cells (cell/mL) in samples using qPCR data of the 16S rRNA gene quantifications (copies/µL), the number of 16S rRNA genes in the genome (n_{16s}), the volume of the culture from which genomic DNA was extracted (V_c), and the volume of extracted DNA (400 µL) must be taken into account, as below:

\[
\text{Concentration (cell/mL)} = \frac{\text{concentration (copies/µL)} \times 400 \, \mu\text{L} \times \text{dilution factor}}{V_c \times n_{16s}}
\]

V_c was 0.5 mL, n_{16s} was equal to 11 for *C. acetobutylicum*, and equal to 8 for *C. cellulolyticum*.

After the plasmid standards were prepared, a 10^9 copies/µL solution of each plasmid was made and stored in a -20°C freezer (2 µL DNA in 20 µL final volume), along with 1 tube of the stock 10^{10} copies/µL plasmid solution. For each qPCR run a new standard curve was made using fresh dilutions, where the standard curve concentrations of 10^8 copies/µL to 10^1 copies/µL were prepared by making serial 1:10 dilutions starting with the 10^9 copies/µL plasmid solution (5 µL DNA in 45 µL of sterile UV treated DNase/RNase-Free distilled water).

A qPCR amplification plot shows the fluorescent signal, which is proportional to the amount of amplified product in the tube, versus cycle number. The amplification plot shows two phases, an exponential phase followed by a plateau phase. During the exponential phase, the amount of the PCR product approximately doubles in each cycle. As the reaction precedes the reaction components are being consumed, and eventually one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase.

For all qPCR runs, the qPCR signals were analyzed at a standard pre-determined threshold of 1E+03 RFU which was in the exponential amplification phase and above the background fluorescence noise for all the qPCR runs. The quantification cycles (C_q or C_T), the cycle number where fluorescence increases above the threshold, were used to find the DNA copy numbers (automatically calculated from the standard curve). For each qPCR run, a standard curve was constructed by plotting the log of the starting quantity in standard plasmid serial dilutions (10^8 copies/µL to10^1 copies/µL) against the C_T values obtained during amplification of each dilution.
Table A6. qPCR standard curves used for the quantifications of *C. acetobutylicum* (CA) and *C. cellulolyticum* (CC) in the co-culture and mono-culture experiments.

<table>
<thead>
<tr>
<th>Run date</th>
<th>Target species</th>
<th>$R^2$</th>
<th>Amplification efficiency (%)</th>
<th>slope</th>
<th>y-intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>08/09/2011</td>
<td>CA</td>
<td>0.997</td>
<td>96.3</td>
<td>-3.4</td>
<td>38.5</td>
</tr>
<tr>
<td>17/01/2012</td>
<td>CA</td>
<td>0.993</td>
<td>106.4</td>
<td>-3.2</td>
<td>36.3</td>
</tr>
<tr>
<td>16/05/2012</td>
<td>CA</td>
<td>0.993</td>
<td>100.7</td>
<td>-3.3</td>
<td>37.8</td>
</tr>
<tr>
<td>20/07/2012</td>
<td>CA</td>
<td>0.996</td>
<td>97.9</td>
<td>-3.4</td>
<td>39.6</td>
</tr>
<tr>
<td>01/11/2012</td>
<td>CA</td>
<td>0.996</td>
<td>90.4</td>
<td>-3.6</td>
<td>40.1</td>
</tr>
<tr>
<td>07/09/2011</td>
<td>CC</td>
<td>0.996</td>
<td>95.1</td>
<td>-3.4</td>
<td>37.3</td>
</tr>
<tr>
<td>01/12/2011</td>
<td>CC</td>
<td>0.996</td>
<td>98.1</td>
<td>-3.4</td>
<td>38.2</td>
</tr>
<tr>
<td>16/01/2012</td>
<td>CC</td>
<td>0.995</td>
<td>90.0</td>
<td>-3.6</td>
<td>40.0</td>
</tr>
<tr>
<td>20/07/2012</td>
<td>CC</td>
<td>0.997</td>
<td>93.6</td>
<td>-3.5</td>
<td>38.6</td>
</tr>
<tr>
<td>12/06/2012</td>
<td>CC</td>
<td>0.987</td>
<td>99.7</td>
<td>-3.3</td>
<td>36.8</td>
</tr>
</tbody>
</table>

To examine the quality and optimality of qPCR assay, for accurate and reproducible quantification of the genomic DNA samples, following criteria have been applied:

- Linear standard curve with coefficient of determination ($R^2$) > 0.980.
- High amplification efficiency (90–110%), where Amplification efficiency, E, is calculated from the slope of the standard curve ($E = 10^{-1/\text{slope}}$), and % Efficiency = ($E - 1$) × 100%.
- Consistency across replicate reactions

Furthermore, to verify the qPCR product specificity a melt-curve analysis was conducted after each amplification stage, as shown in Figure A2. Melting curve analyses for *C. cellulolyticum* qPCR runs showed two peaks (Figure A2b). Therefore, to ensure the qPCR product purity, a gel electrophoresis run was conducted on the qPCR products, where only a single band with the right size (348 bp) was obtained, as shown in Figure A3. This test confirmed that the small peak on the melting curve might be due to the secondary structure of the qPCR products, and not due to the formation of primer-dimers or other products in qPCR runs. To account for intraplate variability (pipetting error) duplicates of each sample and standard were run, and the average of these two values was taken, whereas the measurements were repeated for the samples with C$_T$ ranges larger than one.
Figure A2. Derivative melting curves for (a) a *C. acetobutylicum* qPCR assay, (b) *C. cellulolyticum* qPCR assays using standard plasmid solution (10^5 copies/µL) and isolated genomic DNA.
D-4 qPCR primers cross-specificity test

As it was discussed in the previous section, the qPCR standard curve is linear as there is a linear relationship between $C_T$ and DNA sample size. However, in DNA mixtures where the concentration of target DNA is low, and in presence of high copy numbers of non-target DNA, some non-linearity effects are observed in qPCR analyses; this phenomenon is due to non-specific PCR reactions that lead to lower $C_T$ values in dilute DNA samples.

To address this matter, cross-specificity tests were conducted on mixtures of CA4(1) and CC10 plasmids. Plasmid combinations of $10^x+10^y$ copies/µL were prepared, where $0 \leq x, y \leq 8$ and $x+y=8$, and $10^x$ is the concentration of CA4(1) and $10^y$ is the concentration of CC10 in the mixture. Master mixes were prepared using both sets of primers for the two species, and qPCR runs were conducted on each set of plasmid combinations. With CA4(1) as the target plasmid, the data showed that at high concentrations of CC10 (at and above $10^6$ copy/µL), $C_T$ is decreasing while decreasing the concentration of CA4(1) from $10^2$ to 1 copy/µL. The same trend was observed when targeting CC10 in the plasmid mixtures, and the results of these tests are presented in Figure A4. Although in actual qPCR runs the target DNA molecules are
genomic DNA rather than small plasmid molecules, where the PCR target is highly available and this availability may increase the odds of non-specific PCR reactions, this non-linearity can lead to over-quantification of each species in unknown samples.

![Graph](image)

**Figure A4.** Cross-specificity tests for *C. acetobutylicum* (a) and *C. cellulolyticum* (b) plasmids using qPCR plasmid combinations in contrast to the standard curves. Plasmid combinations of $10^x + 10^y$ copies/µL were prepared, where $0 \leq x, y \leq 8$ and $x+y=8$, and $10^x$ is the concentration of CA4(1) and $10^y$ is the concentration of CC10 in the mixture.

To resolve this issue, primer specificity tests were conducted using pure genomic DNA solutions; pure *C. acetobutylicum* and *C. cellulolyticum* genomic DNA solutions with 6.7 and 6.4 ng/µL of DNA, respectively, were applied. Negative control qPCR experiments on pure non-target genomic DNAs resulted in CT values far higher than the maximum significant CT limits, corresponding to $10^1$ copy/µL of target plasmids for each species, and therefore were outside the range of valid data for qPCR quantifications. Consequently, in order to tackle non-
specific qPCR reactions, the concentration of genomic DNA in all unknown samples were kept below 6 ng/µL, and qPCR assays were conducted on 10 times diluted DNA samples. All the genomic DNA dilutions were stored at -20°C before qPCR analyses.

**D-5 Genomic DNA isolation**

Initially the Mo Bio Laboratories UltraClean® Soil DNA Isolation Kit was applied to extract DNA from the samples; 5 mL of culture was filtered through Millipore Sterivex™ filters (0.22 µm pore size) and filters were frozen at -80°C before DNA extraction. However, it was demonstrated that there was no correlation between culture size and the DNA yield using this kit for co-culture samples, as the same amount of DNA was extracted from 0.2, 1, 2 and 5 mL of cultures; in fact this kit has been aimed for soil samples where the concentrations of cell biomass is typically low, and therefore there was a chance to miss significant amount of DNA in the co-culture samples. A Comparison of *C. acetobutylicum* and *C. cellulolyticum* biomass profiles in a co-culture using genomic DNA obtained by Norgen and Mo-Bio DNA isolation kits are presented in Figure A5.

Consequently, DNA isolation using Norgen bacterial genomic DNA isolation kit (Norgen, #17900) was tested, and it was found that there was a correlation between the sample size (in the range of 0.2 to 0.5 mL) and DNA yield using Norgen kit, while it had a higher DNA yield compared to the Mo Bio kit. Therefore, the Norgen kit was chosen for DNA isolations; culture samples (0.5 mL) were centrifuged at 13000 g for 2 minutes, and the cell pellets were used for DNA isolations following the standard kit protocol, where 400 µL elution buffer was used lastly to elute DNA in two steps (200 µL for each dilution step).
Figure A5. A comparison of *C. acetobutylicum* (a) and *C. cellulolyticum* (b) quantifications in a co-culture experiment using Norgen and Mo-Bio DNA isolation kits.
D-6 Examination of the specificity of the interactions between *C. cellulolyticum* and *C. acetobutylicum* in the co-culture

In order to examine the specificity of the synergism in the clostridial co-culture, and to find out if another microbial species would also have similar metabolic interactions with *C. cellulolyticum* in a co-culture, we tried *E. coli* strain C and *C. cellulolyticum* co-cultures. Co-culture experiments were conducted on VM medium (Higashide et al., 2011) in serum bottles, with 3 bottles of *C. cellulolyticum* as control experiment. *E. coli* was inoculated to the co-culture bottles simultaneously at the beginning of batch or after 4 days (sequential) into *C. cellulolyticum* cultures. The t-test analysis of the TOC data after 28 days showed that the difference in the mean values of the two groups (*C. cellulolyticum* mono-culture and sequential co-culture) was not great enough to reject the possibility that the difference is due to random sampling variability, and there was not a statistically significant difference between the input groups (P = 0.133). Therefore, no significant improvement in the co-culture cellulolytic activity compared to the control *C. cellulolyticum* culture was observed (data presented in Figure A6).

Also, inoculation of *E. coli* to the *C. cellulolyticum* culture simultaneously, led to no cellulolytic activity in the co-culture, possibly due to immediate pH drop in the culture. However, this experiment has been conducted under unregulated pH condition and the final pH in all bottles were about 5.5, which is below the optimal pH range for cellulolytic activity, therefore running this co-culture under pH controlled condition and the pH profile that has been used for clostridial co-culture would be more insightful.

![Figure A6. Cellulolytic activities in the sequential co-culture of *E. coli* and *C. cellulolyticum* and the mono-culture of *C. Cellulolyticum* (control).](image)
D-8 Time profiles of the product formations in the clostridial co-culture experiment

[Graph showing time profiles of various products such as lactate, acetate, butyrate, and ethanol for replicate 1.]
D-9 Identifying potential exchanged metabolites in the co-culture

To address this question if any metabolite exchange in the co-culture enables *C. cellulolyticum* to grow and degrade cellulose in the co-culture, the extracellular metabolite pools were characterized in the co-culture and mono-culture samples; from each batch two samples in early and mid exponential growth phases, as demonstrated by arrows in Figure A8, were analyzed using NMR technique at Chenomx Laboratory (http://www.chenomx.com/). The data
are reported in Table A7, and the concentrations of 38 compounds have been measured in total of 8 co-culture and mono-culture samples.
Figure A8. (a,b) The extracellular metabolomic samples 1, 2, 4 and 5 were taken from co-culture experiments set A (replicates a, b) as shown by arrows. The time profiles of *C. cellulolyticum* (circle), *C. acetobutylicum* (square) and cellulose solubilization in terms of total organic carbon in each co-culture (triangle). (c) samples 6, 7 were taken from *C. cellulolyticum* mono-culture experiment at pH of 7.2, and samples A and B were taken from *C. cellulolyticum* mono-culture experiment under pH profile (data has also been presented in Figure 5-3), where the cell biomass profile in the mono-culture at pH of 7.2 (circle), TOC profile in the mono-culture at pH of 7.2 (triangle), cell biomass profile in the mono-culture under pH profile (rhombus), and TOC profile in the mono-culture under pH profile (square).
Comparing the metabolite concentrations in the mono-culture supernatants, in the mono-culture under pH profile, the concentration of negatively charged amino acids, aspartate and glutamate, have increased significantly; this observation is consistent with the glutamate efflux in C. sporogenes MD1 culture under acidic pH conditions and in presence of acetate, lactate, and propionate ions (Flythe and Russell, 2006), while under acidic pH conditions and in absence of weak acids neither glutamate efflux nor growth inhibition of C. sporogenes cells by intracellular anion accumulation have been observed. Furthermore, it has been shown that in E. coli culture under acidic pH of 6.0, the presence of weak acids such as 8 mM acetate leads to accumulation of 232 mM acetate ions in the cytoplasm, which results in the decrease in other anion pools such as glutamate and aspartate to compensate, however, it has not been clear if the decrease in intracellular glutamate pool is due to its rapid metabolism or efflux (Roe et al., 1998). With the exception of arginine and histidine, which their biosyntheses are linked to glutamate, most of amino acids had significantly higher concentrations in mono-culture under pH profile, as it is presented in Table A8; moreover, betaine, choline, which are derived from serine, and isovalerate, a Leucine derivative, as well as pyruvate, uracil, hypoxanthine, isobutyrate (intermediate metabolites in purine and pyrimidine pathways) have increased; these data suggest an overflow metabolism in the mono-culture under pH stress which has hampered the cell growth and cellulolytic activity.
Table A7. The extracellular metabolite concentrations from NMR analysis of the co-culture and monoculture samples at Chenomx; samples are explained in Figure A8.

<table>
<thead>
<tr>
<th>Sample id:</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Hydroxybutyrate</td>
<td>41</td>
<td>40</td>
<td>36</td>
<td>34</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acetate</td>
<td>2394</td>
<td>4092</td>
<td>5682</td>
<td>7928</td>
<td>1693</td>
<td>2764</td>
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### Table A8. The extracellular metabolite concentrations in mono-culture samples.

<table>
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<tr>
<th></th>
<th>Control, mono-culture at pH 7.2</th>
<th>Mono-culture under pH profile</th>
<th>Percentage of change</th>
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Comparing the mono-culture under pH profile and the co-culture samples indicated considerable changes in concentration of 2-hydroxybutyrate (propanoate metabolism), acetoin (butanoate metabolism) and glycerol (Table A9). The concentration of pyruvate was also significantly lower in the co-culture samples, which implies less pyruvate overflow and a better balanced metabolism. However, it should be noted that since co-culture samples 4 and 5 have been analyzed freshly and overall had higher metabolite concentrations compared to samples 1 and 2, which had been thawed few times before NMR analysis, and so potential degradation of metabolites could have led to the heterogeneity in co-culture samples. Considering co-culture
samples 1 and 2 only, the concentration of choline, glutamate, glycine, hypoxanthine, isoleucine, isobutyrate, isovalerate, leucin, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine are lower compared to the mono-culture under pH profile, which indicates higher flux through cell biomass synthesis and consequently cellulolytic activity in the co-culture.

Table A9. The extracellular metabolite concentrations in the co-culture and mono-culture samples.

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<td>Isobutyrate</td>
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<td>Pyruvate</td>
<td>87±48</td>
<td>30±19</td>
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D-10 Assessment of *C. cellulolyticum* growth on the supernatant of *C. acetobutylicum* culture

To further explore the metabolic interactions in this co-culture, the mono-culture of *C. cellulolyticum* on the supernatant of *C. acetobutylicum* culture was tested. In this experiment, *C. acetobutylicum* was cultivated on the co-culture medium containing 3 g/L glucose until the entire glucose was consumed in the overnight culture in 5×100 mL serum bottles. The culture was then filtered using 0.2µ Sterivex™ filters, and the filtered supernatant was used as the medium for the cultivation of *C. cellulolyticum* in bioreactors. 20 g/L of fibrous cellulose was added to each bioreactor containing 250 mL filtered supernatant, and inoculated with 25 mL of 5-day old *C. cellulolyticum* culture, where pH was adjusted and controlled at 6.0. The concentration of *C. cellulolyticum* was monitored for 28 days in the batch culture; however, no significant cell biomass growth was detected in either bioreactor replicate experiments.
Appendix E: supplementary information for chapter 6

Figure A9. The standard curve of lactate concentration versus peak area obtained from LC-MS mass chromatograms.