INTERACTION BETWEEN *CLOSTRIDIUM THERMOCELLUM* ATCC 27405 AND *THERMOANAEROBACTERIUM SACCHAROLYTICUM* DSM 7060 ON CELLULOSIC AND LIGNOCELLULOSIC SUBSTRATES: SUBSTRATE ATTACHMENT, CONCENTRATION DYNAMICS, AND ETHANOL PRODUCTION

by

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Bachelor of Science Biology, Ryerson University 2012

A thesis

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Master of Applied Science

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AUTHOR’S DECLARATION

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ABSTRACT

Second generation biofuel research and bioethanol production via consolidated bioprocessing has the potential to become a viable alternative to finite fossil fuel reserves. Further advances have to be made in order to make this biotechnology more economically feasible. The focus of this study was to use cellulolytic *C. thermocellum* in a consortium with hemicellulolytic *T. saccharolyticum* to investigate the dynamics of their interaction with respect to cellulosic and lignocellulosic substrate attachment, respective numbers, and extent of solid substrate hydrolysis and desired end product formation. *T. saccharolyticum*'s partial adherence to cotton and switchgrass has demonstrated limited need for substrate colonization and hence reduced competition with *C. thermocellum*. Real-time PCR analysis indicated that *T. saccharolyticum* can proliferate under low carbon supplementation, and efficiently utilize the metabolites produced from *C. thermocellum*'s hydrolysis of cotton and switchgrass. The interaction between the two thermophiles on both substrates demonstrated a potential for increased bioethanol production.

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Dedicated to my late grandfather,

Bogdan Miscevic.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CBD	Cellulose binding domain
CBP	Consolidated bioprocessing
CEM	Cellulose-enzyme-microbe complex
DSM	Deutsche Sammlung von Mikroorganismen
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DNA	Deoxyribonucleic acid
HCS	High carbon supplement
HPLC	High performance liquid chromatography
LCS	Low carbon supplement
qPCR	Quantitative polymerase chain reaction
SHF	Separate hydrolysis and fermentation
SSCF	Simultaneous saccharification and co-fermentation

CHAPTER 1: INTRODUCTION

1.1 The conventional energy problem

Currently with an equivalent global consumption of over 11 billion tonnes of oil in fossil fuels per year and crude oil vanishing at a rate of 4 billion tonnes per year, it is estimated, considering the world population increase as a major source of influence, that our known oil deposits could be completely depleted by the end of this century (Shafiee and Topal, 2009). The International Energy Agency (IEA) has predicted that the world's crude oil supply will peak in 2014, followed by a steadily decline in the upcoming years (Evans, 1999). Other estimates state that 64 of the 98 oil-producing countries have already reached their peak oil production in the last few years (Scragg, 2009). The world depends on a regular supply of energy, meaning that whatever the statistics are for the finite fossil fuel reserves, alternative sources of energy need to be introduced.

Aside from the shrinking fossil fuel supply, the conventional method used to extract fossil fuels has a tremendous negative effect on the environment. Carbon dioxide (CO_2) that is emitted causes the pollution of air and contributes to 55% of global warming (Scragg, 2009). According to the IEA, the annual carbon dioxide discharge from the use of coal, gas, and oil were above 23 Gt in the year 2000 having risen to 15.7 Gt in 1973 from 0 Gt in pre-industrial period (IEA, 2002). Nitrogen oxides (NO_x) produced during burning of fossil fuels can contribute to ozone depletion and on a molecule-to-molecule basis it absorbs infrared radiation 200 times more than carbon dioxide (Hameed and Dignon, 1992; Khalil and Rasmussen, 1989). Release of sulphur dioxide (SO_2) during combustion of fossil fuels can generate acid precipitation which has a wide range of negative effects on waters, flora, and fauna (Scragg, 2009; Van Breeman *et al.*, 1982). Finally, discharge of radioactive substances such as uranium

and thorium into the atmosphere can increase the risk of lung cancer (Hameed and Dignon, 1992; Van Breeman *et al*., 1982). Thus, it is essential to search for more sustainable and environmentally friendly techniques for energy production.

To date, there are various renewable energy sources that are considered to be alternatives to fossil fuels. These alternatives include (but are not limited to) wind or solar power, hydroelectricity, geothermal power, and biomass (Kallos and Apostolopoulou, 2007). Out of many renewable energy sources present globally, biofuels generated from biomass are generally more sustained, have the ability to be stored for transportation, and contain zero carbon footprint (Kallos and Apostolopoulou, 2007).

First generation biofuels are type of biofuels that extract energy from crops such as sugarcane, sugarbeet, maize, wheat, soybean, and soyflower (Scragg, 2009). However, the main issue with this type of biofuel is that it requires large areas of land to completely replace fossil fuels gas, petrol and diesel (Scragg, 2009). Therefore, there is not an adequate amount of land to successfully grow sufficient energy crops without competing with food crops for land which causes increase in food prices as a result. For these specific reasons, second generation biofuels are under development. The second generation biofuels are produced from lignocellulosic biomass and wastes which tend to provide better yields per hectare and thus do not compete with food crops (Sims and Taylor, 2008; Damartzis and Zabaniotou, 2011). In addition to food competitions, they also require less fertilizer and pesticide usage, contain a more satisfying energy balance, and reduce the amount of greenhouse gas emission than first generation biofuels (Sims and Taylor, 2008). According to Kallos and Apostolopoulou, cellulosic bioethanol has 1.25 times more megajoule (MJ) of energy output than sugar ethanol and 10 times MJ of energy output than corn ethanol (Kallos and Apostolopoulou, 2007). Furthermore, cellulosic ethanol

produces only 1.36 kilograms of CO_2 per 130 MJ of energy delivered, whereas sugar ethanol produces 5.44 kilograms of CO_2, and corn ethanol 9.53 kilograms of CO_2 (Kallos and Apostolopoulou, 2007).

The term *lignocellulose* was first introduced by Edwards John Bevan (1856-1921) and Charles Fredrick Cross (1855-1935), who were leading British chemists in cellulose technology in the late 19th century (Kamm *et al.*, 2006). Lignocellulosic biomass is currently the most abundant feedstock on Earth for biofuel production (Lynd *et al.*, 2008). Some examples of lignocellulosic substrates include wood chips, bagasse, agave, grasses, and paper sludge (Lynd *et al.*, 2002). In 2005, it was estimated that the total combustible renewables and waste consumption was approximately 1,149 Mtoe, with 94% of this material being lignocellulosic biomass (Sims and Taylor, 2008). Although abundant and renewable, only limited amount of this particular feedstock can actually be utilized in practice. Lignocellulosic structure itself is very tough to break down due to the strong β-1-4 glycosidic bonds between the monomer sugars (Sims and Taylor, 2008). In addition, current technology is limited when it comes to fermenting both pentose and hexose sugars present in cellulose and hemicelluloses components. Therefore, it is essential to the future of second generation biofuels to develop a system that will be able to utilize all the available sugars present in the lignocellulosic structure, as well as optimizing bio-ethanol production by using renewable biocrops that are environmentally friendly.

1.2 Scope of work, objectives, and thesis outline

Much attention has been given to the strictly anaerobic thermophile *C. thermocellum*, more specifically how its cellulolytic and ethanologenic abilities can be used in converting biomass into an applicable energy source. However, the model bacterium *C. thermocellum* pure cultures are often limited to the amount of available sugars that they can ferment from the lignocellulosic substrate. In other words, being capable of fermenting only hexose sugars, *C. thermocellum*'s chances of reaching a sufficient abundance of ethanol end-product are slim (Lynd *et al.*, 2002). The addition of anaerobic thermophilic bacterium, *T. saccharolyticum*, and its ability to ferment pentose sugars found in hemicellulose, can increase the potential of valuable end products (Maki *et al.*, 2009). By applying this co-culture to a promising biocrop, such as switchgrass, this study intended to fill in an important gap in the lignocellulosic biomass research.

The scope of the research was focused on studying the relationship between *C. thermocellum* and *T. saccharolyticum* and how their interaction can be utilized to develop ethanol production from cellulosic and lignocellulosic substrates. This was based on three main objectives of the research: (i) comparing morphology and analyzing the interaction between the two thermophilic anaerobes in their attachment capabilities to various carbon sources, (ii) examining the competitive vs. co-operative behaviour based on physiology tests and relative numbers on different substrates, and (iii) investigating the extent of solid substrate solubilisation and end product formation.

This thesis is divided into four specific chapters. Chapter 1 lays down the framework of the project as far as the reasoning and motivation behind this particular work is concerned. This chapter also describes the goals and general expectations of the work. Chapter 2 offers

background information on some of the key elements and terms of the research through literature review. The experimental approach, results, and discussion of the present study are presented in chapter 3. Finally, chapter 4 deals with general conclusions and recommendations for future research in this particular field.

1.3 Expectations and hypothesis

Due to the fact that hardly any detailed microscopy and physiology testing has been performed on *T. saccharolyticum* DSM 7060, it would be of great interest to compare morphological differences and metabolic production to its cellulolytic counterpart. Morphological differences would reveal easier differentiation between *C. thermocellum* and *T. saccharolyticum* with future co-culture imaging. Furthermore, microbe attachment to different biomass substrates has been extensively researched in the past. *C. thermocellum*'s ability to bind to cellulosic and lignocellulosic materials has been covered by Lamed and Bayer (1988), Raman *et al.* (2011), and Dumitrache *et al.* (2013); the results show that this anaerobic microbe can adhere to various cellulosic sources at different rates. In addition, *C. thermocellum* is well known to occupy carbon material such as cellulose and cellobiose, with a growth rates of 0.23 h^{-1} and 0.35 h^{-1}, respectively (Dror *et al.*, 2003). Attachment capabilities of *T. saccharolyticum* are still largely unknown, and there is no record in literature which points to *Thermoanaerobacterium* strains binding to cellulosic or lignocellulosic sources. This part of the study also aimed to determine whether or not there is any physical competition between *C. thermocellum* and *T. saccharolyticum* when it comes to substrate colonization. Consequently, due to *T. saccharolyticum*'s lack of cellulose-binding domains (CBDs) it is expected that this strain has limited attachment to carbon material.

The competitive vs. cooperative aspect of the research has shed light on the interaction dynamics between *C. thermocellum* and *T. saccharolyticum*. To measure this interaction, the carbon supply in the medium had to be effectively controlled. First, yeast extract is considered to be unfeasible and uneconomical for cultivation of cellulolytic bacteria; therefore, minimal amount of this carbon source was used for this experimentation. Second, using lower yeast

extract and sugar supplements have provided a more accurate carbon sharing between the two thermophiles in continuous-flow preparations. This experimentation was organized by preparing a continuous-flow system and using high carbon supplement medium for obtaining successful initial growth of both thermophiles then switching the medium to minimal carbon supplement to then compare the respective numbers of each bacterium. A quantitative analysis by the use of a real-time PCR has provided prosperity measurement of each bacterium during the nutritional change. Competitive interaction between the two thermophiles would be indicated by one bacterium outcompeting the other nutritionally (digesting more carbon available over the other). On the other hand, a cooperative interaction would be demonstrated by a lack of significant change or a stable recovery in numbers of each thermophile after the high-to-low carbon supplement medium switch. Previous research by Liu and colleagues (1996) has shown that *T. saccharolyticum* is known to utilize pentose and hexose type sugars. This parallel sugar metabolism hints towards a lowered competition between *C. thermocellum* and *T. saccharolyticum* since *C. thermocellum* can only metabolize hexose sugars. Therefore, the expectations for this part of the research are that *C. thermocellum* will efficiently hydrolyze the carbon substrates and the pentose metabolites released will be fermented by *T. saccharolyticum*, thus alluding to a more cooperative interaction in consortium.

The extent of carbon solubilization and ethanol production, the final two parts of the study, are to reveal the true value of cellulosic utilization and end product formation from this particular co-culture. Research on measurement of solid carbon degradation with both cellulolytic and hemicellulolytic thermophiles in co-culture is limited; however, previous experimentation suggested cooperative relationship between the two anaerobes will result in

higher ethanol yield than in *C. thermocellum* monoculture due to the additional fermentation of pentose sugars by *T. saccharolyticum* (Suib, 2013).

It was hypothesized that *T. saccharolyticum* will exhibit little substrate attachment and will also utilize sugars produced by *C. thermocellum* hydrolysis of cellulosic and lignocellulosic material and as a result, increase ethanol production in both cases.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to biofuels

Biofuel, in general terms, is fuel that is generated from dead organic material by living organisms via carbon fixation (Lal, 2005; Barber, 2008). Biofuels are produced through biochemical reactions, which can be carried out in industrial or laboratory setting that use decomposed organic matter (known as biomass). Today, approximately 2% of the world's transportation fuel is either derived from ethanol fermentation or processing of plant oils to generate biodiesel (Koonin, 2006). Furthermore, biofuels can be characterized into three specific groups: i) *first generation* biofuels – fuel derived from sugar, starch, or vegetable oil, ii) *second generation* biofuels – fuel derived from lignocellulosic biomass (woody crops), grasses, or agricultural waste, and (iii) *third generation* biofuels – fuel derived from algae (Antizar-Ladislao and Turrion-Gomez, 2008; Naik *et al*., 2010). It is important to note that the structure of the fuel does not change between generations, but rather the source from which the fuel is derived. These types of biofuels have the capability to generate a wide variety of fuel, such as bio-alcohols (ethanol, propanol, and butanol), biodiesel, bio-ethers, and biogas (Naik *et al*., 2010). Analyses of various biofuels showed that cellulosic ethanol (second generation biofuel) delivers more energy per 1 mega joule (MJ) than fossil fuel energy (a ten-fold increase in energy output) (Kallos and Apostolopoulou, 2007). Finally, second generation biofuels are projected to account for over 90% of total biofuel production by the year 2050 due to its higher efficiency, feedstock availability, and not being in competition with the food industries (Bardle and Abadi, 2010; IEA, 2010).

2.2 Lignocellulose

Lignocellulose is the most abundant renewable natural resource and inexpensive substrate available for conversion to fuels (Demain, 2005). This substrate is composed of cellulose, hemicellulose, and lignin that constitute an essential part of woody cell walls of plants. Cellulose $(C_6H_{10}O_5)_n$ is an organic polymer (polysaccharide) that is composed of thousands of repeating D-glucose molecules which are linked by β-1-4 glycosidic bonds (Dauenhauer *et al*., 2007). This particular straight chain polymer can be predominately found in nature as a structural component in the cell wall of plants and many forms of algae (Baldan *et al*., 2001). Cellulose can make up 35-50% of plant dry weight and is the most abundant component of plant biomass (Lynd *et al.*, 2002). Hemicellulose is a heteropolymer that is made up of simple sugars, for example, xylose, galactose, mannose, and arabinose (Ronan, 2011). Hemicellulose accounts for 20-35% total dry weight of lignocellulose (Zavrel *et al*., 2009). Last, lignin can comprise up to 5% to 30% of plant dry weight and is considered to be more resistant organic polymer within which cellulose fibres are entangled (Lynd *et al*., 2002). One particular measure of lignin composition is a ratio of its guaiacyl (G) and syringyl (S) moieties (Davison *et al.*, 2006). Guaiacyl units can covalently crosslink with three other units and syringyl can link with only two units (Davison *et al.*, 2006). The S/G ratio has a large effect on processability and delignification of lignocellulosic material (Ohra-aho e*t al.*, 2013). Thus, lignocellulosic substrates with an increased S/G ratio are more easily delignfied and more ideal for biofuel use (Lima *et al.*, 2008).

Cellulolytic microorganisms contain specific enzymes that enable them to efficiently degrade cellulose substrates. These endoglucanase and exoglucanase enzymes are called *cellulases* and are known to break or cleave the β-1-4 glycosidic bonds within a cellulose polymer (see section 2.7.1) (Demain *et al.*, 2005). Interestingly, the ability of microorganisms to

degrade cellulose is widely distributed in different environmental conditions such as pH, oxygen content, temperature, and salinity (among others). Due to various environmental conditions, cellulolytic microorganisms (both fungi and bacteria) can fall under both aerobe and anaerobe groups.

2.3 Anaerobic cellulose hydrolysis

Some anaerobic bacteria contain a protein complex called *cellulosome* (see section 2.7.2) that is used for the degradation of cellulose. This protein complex is located on the outside of the cell wall and is composed of up to 11 subunits consisting of enzymatic cellulase and non-enzymatic regions (Shwartz, 2001). Cellulosomes are capable of breaking the β-1-4 glycosidic bonds in the cellulose fibre and this cleavage releases independent glucose molecules from the chain in a process called *hydrolysis* (Kim, 1995). Anaerobic cellulolytic bacteria have evolved to use cellulosomes due to their advantages over cell-free enzymes. The cellulosome allows the cell to directly regulate and control the ratio of expression of enzymatic subunits, which directly affects the rate of hydrolysis (Norsker *et al.*, 1999). In addition, the physical distance between the cell and the cellulose substrate is subsequently reduced (Lynd *et al.*, 2002). Currently, the specific anaerobes that are known to contain a cellulosome complex are: *Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens*, *Clostridium acetobutylicum*, *Clostridium cellulolyticum*, *Clostridium cellulovorans*, *Clostridium josui*, *Clostridium papyrosolvens*, *Clostridium thermocellum*, and *Ruminococcus flavefaciens* (Shoseyov *et al.*, 1992; Kakiuchi *et al.*, 1998; Ding *et al.*, 1999; Pages *et al.*, 1999; Nolling *et al.*, 2001).

2.4 Anaerobic sugar fermentation

Anaerobic *fermentation* is the biological conversion of sugar molecules into alcoholic derivatives such as ethanol (Bai *et al.,* 2008). It is important to state that from all cellulose-degrading microorganism identified, *Clostridium thermocellum* has the highest hydrolyzing rate (see section 2.7) (Lu *et al.*, 2006; Sparling *et al*., 2006). First, the liberated cellulose and cellobiose products from the hydrolysis step are used by specific types of microorganisms and, in the absence of oxygen, are fermented into desired end products via the Embden-Meyerhof pathway (Figure 2.1). In other words, the degraded cellulose products, such as glucose and cellodextrins (two or more glucose monomers), are transported into the bacterial cell for metabolism. Glycolysis generates pyruvate molecules from sugars and these pyruvate molecules are then fermented to produce ethanol (producing acetaldehyde intermediate in the process) (Ronan, 2011). *C. thermocellum* is known to produce two major end-products: ethanol and acetate. On a cellulose source such as a cotton fibre, the two products contain a final ratio of 2:1 (ethanol to acetate) (Dumitrache *et al*., 2013).

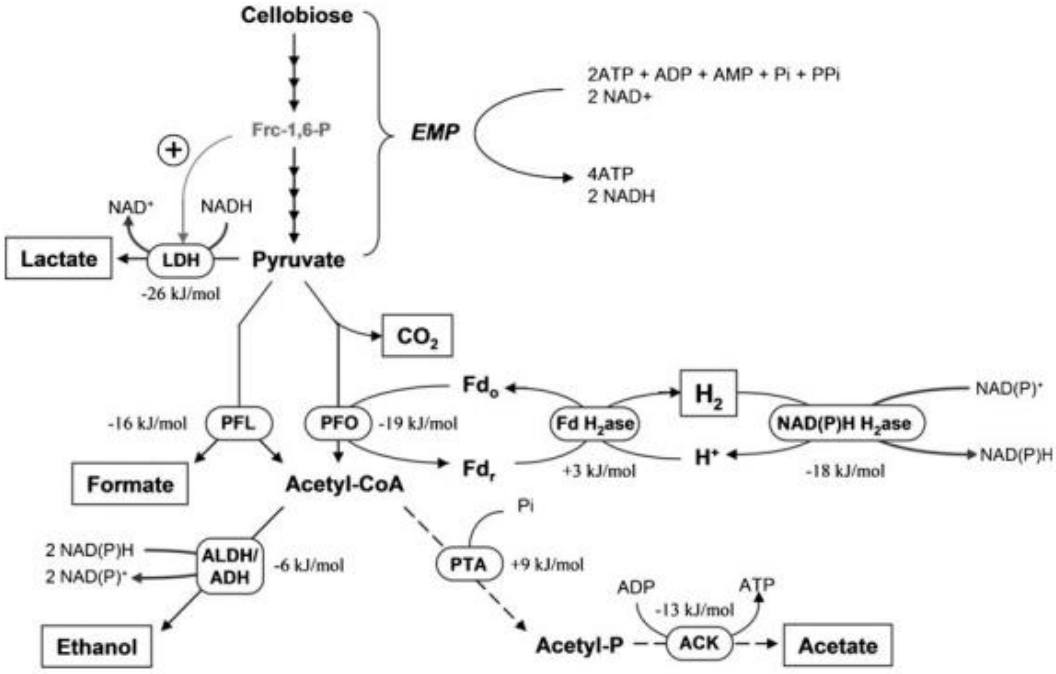

Figure 2.1: Carbon metabolism in *C. thermocellum* ATCC 27405. *Solid lines* indicate verified enzyme pathway activity; *dashed lines* are genetically deduced pathway not verified by enzyme activity. Abbreviations : LDH, lactate dehydrogenase (fructose-1,6-bisphosphate activated); PFL, pyruvate:formate lyase; PFO,pyruvate:fd oxidoreductase; ALDH/ADH, acetaldehyde dehydrogenase/alcohol dehydrogenase; PTA, phosphotransacetylase; ACK, acetatekinase; Fd-H2ase, ferredoxin hydrogenase; NAD(P)H H2ase, NAD(P)Hhydrogenase (Adopted from Rydzak *et al.*, 2011).

2.5 Early ethanol production methods and challenges

For successful biological conversion of cellulosic biomass into fuels, four biologically mediated events must occur: (i) cellulase enzyme production, (ii) hydrolysis of cellulose and other polysaccharides (if present), (iii) fermentation of soluble cellulose sugar products, and (iv) fermentation of soluble hemicelluloses sugar products (Lynd *et al.*, 2002). Prior to the 1980s, ethanol yield was achieved by performing each of the four events individually by a process known as separate hydrolysis and fermentation (SHF) (Figure 2.2) (Lynd *et al.*, 2002). Microorganisms used in steps i and ii were generally fungal microbes such as *Trichoderma reseei* and *Trichoderma viride*, due to their most efficient cellulase production at the time

(Taherzageh and Karimi, 2007). In 1988, Wright and colleagues developed a method to combine hydrolysis and fermentation into one step using mixed yeast cultures, known as simultaneous saccharification and fermentation (SSF) (Wright *et al.*, 1988). However, cellulase production and hemicelluloses sugar fermentation occurred in two additional and individually separate steps (Lynd *et al.*, 2002). In the early 1990s, cellulase production was successfully joined to SSF to develop a new process called simultaneous saccharification and cofermentation (SSCF) (Wyman and Goodman, 1993). Finally, in 1996 Dr. Lee Lynd and colleagues from Dartmouth College were able to develop a one-step biological conversion of biomass into ethanol in a process called direct microbial conversion (DMC), which was later termed consolidated bioprocessing (CBP) (Lynd *et al.*, 2002). No single microbe is capable of successfully performing CBP; however there are few microorganisms that have been considered as potential candidates (see section 2.6).

Two of the early major challenges facing ethanol production were the plant resistance to hydrolysis and land use concerns (Lynd *et al.*, 2005). As plants evolve they develop stronger structures and become more resistant to degradation as a result. Due to this phenomenon, increased costs for heat and cellulase enzymes are required. Furthermore, the land use concerns relate to food vs. fuel debate since many acres of land are necessary to cultivate and harvest crops for bioethanol production.

2.6 Consolidated bioprocessing

Consolidated bioprocessing (CBP) is a biological conversion of lignocellulose into desired products that combines cellulase production, lignocellulose hydrolysis, and hexose and pentose fermentation into a single step (Lynd et al., 2005). One potential CBP candidate is *Clostridium thermocellum*, due to its cellulolytic and fermentation capabilities (see section 2.7).

This microorganism contains a more effective cellulosome complex that is capable of hydrolyzing cellulose at a faster rate than other cellulosome-containing microbes and at the same time fermenting glucose or six-carbon (hexose) sugars into ethanol (Sparling *et al.*, 2006). It is worth mentioning that to date no other natural occurring microbes have been isolated that can compete with *C. thermocellum*'s efficiency to carry out CBP (Sizova *et al.*, 2011; Mazzoli, 2012). Figure 2.2 illustrates the effectiveness of CBP in comparison to other ethanol production methods.

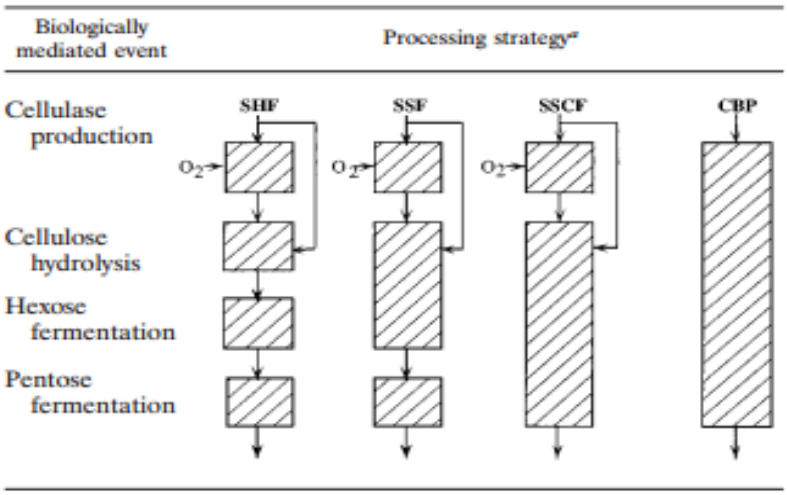

Figure 2.2: Consolidated bioprocessing with respect to SHF, SSF, and SSCF processes (Adopted from Lynd *et al*., 2002)

In addition to the time saving advantage of CBP, this process also offers the potential for lowering cost and increasing overall efficiency (He e*t al.*, 2011). Unnecessary expenses can be avoided when using CBP due to the fact that this process requires less spending on cellulase enzymes and other raw material that are used in ethanol production. Total cost for biological processing of hydrolysis and fermentation individually (which includes the cost for yield lost, utilities, raw materials, and capital and related spending) is about US$0.19 per gallon (US$0.05/L), which is more than fourfold larger than US$0.04 per gallon(US$0.01/L) projected for CBP (Lynd *et al*., 2005).

2.7 *Clostridium thermocellum*

A model organism for cellulose degradation is the *C. thermocellum* ATCC 27405 strain (Dumitrache *et al.,* 2013, Pers. Comm.). Along with being an anaerobe, this strain is amenable to genetic engineering and can thrives in high temperature environments (thermophilic) at around 60°C and an optimum pH range between 6.5 and 7.2 (Argyros *et al.*, 2011; Dumitrache *et al.*, 2013, Pers. Comm.). In addition, *C. thermocellum* is a Gram-positive, spore forming bacterium that is capable of hydrolyzing cellulose; fermenting hexose sugar oligomers to ethanol and other products such as organic acids, carbon dioxide (CO_2), and hydrogen gas (H_2) (Dumitrache *et al.*, 2013, Pers. Comm.).

The interest of *C. thermocellum* in the biotechnological application in biofuel research is due to several of factors. First, this particular microbe is currently the number one high utility candidate for use in consolidated bioprocessing applications (Akinosho *et al.*, 2014). Second, *C. thermocellum* is an anaerobic microorganism. This specific characteristic provides great advantage to the technology since of most expensive steps in industrial fermentations is that of providing adequate oxygen transmission (Demain *et al*., 2005). Third, aside from being anaerobic, this bacterium is also thermophilic. Having an optimal temperature around 60°C, the possibility for contamination is greatly reduced (Demain *et al*., 2005). Fourth, growth at high temperatures promotes the recovery of ethanol. Fifth, more of the cellulose substrate can be converted to ethanol due to anaerobic ability to possess low cell yield (Demain *et al*., 2005). Last, the release of carbon dioxide into the atmosphere during fermentation is recycled by growth of plants. Hence ethanol-based fuel does not contribute to the mobilization of carbon from the deep geosphere into the biosphere and possible global warming as in the case of fossil fuels.

2.7.1 Cellulase system

The cellulase system is composed of series of enzymes that are produced by cellulolytic organisms in order to metabolize cellulose (Demain *et al.*, 2005). Specifically, the *C. thermocellum* cellulase complex displays a high activity on crystalline cellulose, which refers to its ability to completely solubilize crystalline forms of cellulose such as cotton or avicel (Demain *et al*., 2005). This unique complex of enzymes is comprised of: (i) endo-β-glucanases, responsible for random breakdown of cellulose fibre; (ii) exoglucanases that generate cellobiose; (iii) cellobiose phosphorylase which breaks down cellobiose into glucose and glucose-1-phosphate; (iv) cellodextrin phosphorylase, whose function is to phosphorylyze β-1,4-oligoglucans; and (v) two β-glucosidases that hydrolyze cellobiose to glucose (Demain *et al.*, 2005).

2.7.2 Cellulosome

The cell bound, multi-enzyme complex called the cellulosome is a typical structural property found in many anaerobic cellulolytic strains such as those belonging to *Closridium* spp. and *Ruminococcus* spp., and is considered to be the most efficient biochemical system for cellulose degradation (Mazzoli, 2012). This particular protein complex is attached to the outside of the cell wall and contains both enzymatic (cellulase) and non-enzymatic regions of up to 11 subunits long (Schwarz, 2001). Early studies in Massachusetts Institute of Technology speculated that the true activity of cellulase system belongs to a larger aggregate with a molecular weight of more than 1.5 x 10^6 g/mol (Johnson, 1983). This allowed researchers to further investigate this aggregate and to eventually purify a cellulosome complex for the first time in 1983 from a *C. thermocellum* strain (Bayer *et al*., 1994).

Today, the majority of cellulosome components has been identified and genetically mapped (Figure 2.3). The cellulosome multi-subunit complex contains various domains that have their specific function, and as a whole contribute to the biological degradation of cellulose. These domains include a three repeat S-layer homology (SLH) domain that is directly attached to the cell wall of the cellulolytic microorganism. Next, an anchoring protein physically connects the SLH repeats to the 1,850 amino acid long scaffoldin protein, also known as cellulosome-integrating protein (CipA) (Lamed *et al*., 1987). This attachment is made possible through the binding of the Type II cohesion domain on the anchoring protein and Type II dockerin domain on the CipA, which requires calcium (Ca^{+}) (Demain *et al.*, 2005). Furthermore, according to researchers at the Weizmann Institute of Science in Israel, the interaction between cohesins and dockerins is one of the strongest found in nature (disassociation constants $<10^{-9}$ M) (Mechaly *et al*., 2001).

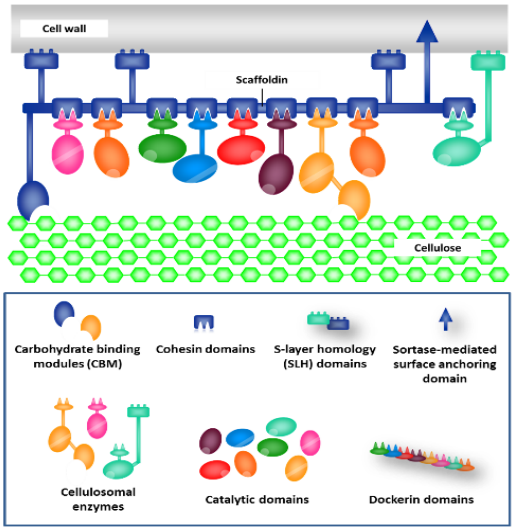

Figure 2.3: Simplistic model of a typical cellulosomal structure (Adapted from Mazzoli, 2012).

Recent studies have suggested that linker regions between cohesins and scaffoldin proteins play an essential role when it comes to cellulosome plasticity and catalytic efficiency (Garcia-Alvarez *et al*., 2011; Molinier *et al*., 2011). Moving on, along the length of the CipA protein are multiple cohesion domains as well as cellulose-binding modules or cellulose-binding domains (CBDs). The multiple

cohesion domains are called Type I cohesion domains and they are bound to a Type I dockerins of various enzymatic components of the cellulosome (Carere *et al*., 2008), which includes cellobiohydrolase (Shwarz, 2001). In addition, the CBDs mediate the attachment of the bacterial cell to the cellulose fibre. However, it is important to state that the underlying mechanism of how cellulosomes actually attach to cellulose substrates is still unknown (Liu *et al*., 2008).

During active growth, the cellulosome structure is attached with strong affinity to the surface of the cell as well as the solid substrate. This complex interaction is known as the cell-cellulosome-cellulose interaction (Waller *et al.*, 2012). In late-stationary phase, *C. thermocellum* is known to release its cellulosome altogether (Demain *et al*., 2005). This cellulosome release during fermentation is believed to be necessary since it would allow for the development of new cellulosomes with modified contents. Another theory relating to this phenomenon is that controlled release of cellulosomes during growth may serve as a process to release *C. thermocellum* from its substrate, leaving liberated cellulosome to further proceed in hydrolyzing cellulose (Bayer and Lamed, 1986).

Finally, not all cellulolytic microorganisms such as *C. thermocellum* actually produce cellulosomes. Research is still weak in explaining why this may be the case, and very little is currently known regarding the mechanisms by which non-cellulosome forming microbes attach to and metabolize cellulose fibres (Wang *et al*., 2011).

2.7.3 Cellulose-binding domain and cellulose degradation

Cellulose-binding domains are crucial components of the cellulosome structure that promote cellulase stable binding to cellulose. However, it is important to note that different CBDs appear to target different sites on crystalline cellulose (Carrard *et al*., 2000). The CipA CBD from cellulolytic *C. thermocellum* binds many more sites of the cellulose substrate than CBDs from other cellulolytic

bacteria such as *Cellulomonas fimi* and *Trichoderma reseei* (Carrard *et al*., 2000). The *C. thermocellum* CipA CBD belongs to the family IIIa (out of 10 families identified) and binds to crystalline cellulose with a K_d of 0.4 μM and a maximum binding capacity of 10 mg of CBD per 1 gram of microcrystalline cellulose (Morag *et al*., 1995). In addition, the maximum binding capacity for amorphous cellulose is 20-fold higher. Furthermore, even though the precise mechanism of CBD attachment to cellulose substrate is a mystery, it is believed that cellulose binding likely involves interaction between planar amino acids and two adjacent glucose chains of cellulose (Shimon *et al*., 2000). The binding itself appears to be extremely stable, although the cellulose degrading enzymes may undergo lateral diffusion on the substrate surface (Bayer *et al*., 1998). Recent studies have shown that CBDs could also be able to induce conformational changes in the quaternary structure of cellulosomes via direct interaction with linker segments of cohesion domains (Yaniv *et al*., 2012).

2.8 Challenges with monoculture ethanol production

Despite a wide range of positive attributes, *C. thermocellum* does contain a drawback in its ability to ferment to high ethanol yields. Even though this strain has been regarded as the highest ethanol producer from all the cellulolytic microorganisms, it still generates relatively low levels of ethanol ranging from 0.08 to 0.29 g ethanol per gram of glucose equivalents (Linger and Darzins, 2013). Other studies have reported highest concentration of ethanol produced by *C. thermocellum* at levels below 30 g/L (Rani e*t al.*, 1996). One of the main reasons for the latter issue is related to its native ability to ferment specific groups of carbohydrates. *Clostridium thermocellum* is known to ferment only hexose sugars, that is, six carbon sugars such as glucose (Lynd *et al.*, 2002). Five carbon sugars (pentose) such as xylose (found in hemicellulose) are not fermented by this microbe. Xylan (a xylose polysaccharide) makes up the majority of hemicellulose found in lignocellulose material (see

section 2.2). Hence, a xylose-fermenting process must also occur in order to improve ethanol production as a whole (Shaw *et al.*, 2008).

2.9 *Thermoanaerobacterium saccharolyticum*

The second microorganism of focus, *Thermoanaerobacterium saccharolyticum*, shows much similarity to *C. thermocellum.* It is also an anaerobic, Gram-positive non-sporulating bacterium that thrives in high temperature environments (between 45 and 66°C) and a pH range of 4.0 and 6.5 (Shaw *et al.*, 2006). However, unlike *C. thermocellum*, it is capable of fermenting almost all types of sugar derivatives found in lignocellulosic biomass, including xylose sugars. Several xylan-degrading enzymes have been isolated from this microorganism including an acetyl xylan esterase, a β-xylosidase, α-glucuronidase, and a cell associated xylanase (Liu *et al*., 1996). In addition, numerous studies were performed on *T. saccharolyticum* resulting in its classification as a microbe with an ability to ferment high ethanol yields. This production has a maximum titer of 37 g/L, which for a thermophilic anaerobe is the highest reported (Shaw *et al.*, 2008). Nonetheless, cellular morphology and substrate attachment capabilities of *T. saccharolyticum* strains are still widely unknown.

2.9.1 Xylose fermentation

A xylose sugar monomer is considered to be the main building block of hemicellulose heteropolymer found in certain types of plants (Hilz *et al*., 2007). In addition, two xylose sugar subunits (connected by β-1-4 bonds) form a disaccharide known as xylobiose (Hilz *et al*., 2007).

Like *C. thermocelum*, all *T. saccharolyticum* are described to have a mixed fermentation pathway, producing mainly ethanol, organic acids, hydrogen, and carbon dioxide (Lacis *et al*.,

1991; Altarcas *et al*., 2001). Xylose fermentation seen in Figure 2.4 shows the typical metabolic pathway in non-cellulolytic utilization of this five-carbon sugar.

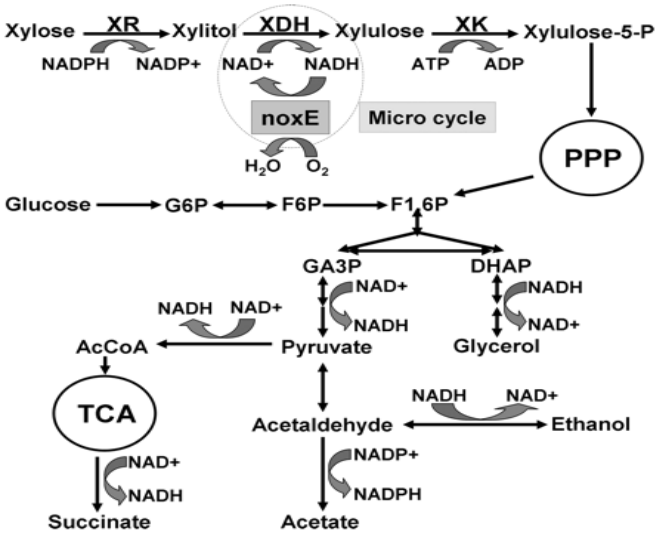

Figure 2.4: Xylose metabolism showing key enzymes and identified cofactors in central break down of this sugar. Abbreviations: XR, xylose reductase; XDH, xylitol dehydrogenase; XK, xylulokinase; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6P, fructose-1,6-bisphosphate; PPP, pentose phosphate pathway; GA3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; AcCoA, acetylcoenzyme A; TCA, tricarboxylic acid cycle (Adopted from Zhang *et al*., 2011).

2.10 Co-culture research

One of the earliest co-culture studies with *Clostridium thermocellum* dates back to 1977 when Weimer and Zeikus from University of Wisconsin examined fermentation of cellulose by using *Clostridium thermocellum* in the presence of *Methanobacterium thermoautotrophicum*. In this study it was discovered that cellulase activity in *C. thermocellum* appeared earlier in co-culture and that more biohydrogen was produced than in mono-culture (Weimer and Zeikus, 1977). A few years later, *C. thermocellum* was tested with *Zymomonas mobilis*; however, only about 27% of the cellulose substrate was converted to ethanol (Saddler *et al.*, 1981). The *Z. mobilis* was capable of fermenting only glucose, fructose, and sucrose but not other forms of

sugars (Tojo, 2013). A similar research with *C. thermocellum* and *Clostridium thermohydrosulfuricum* co-culture on Solka Floc cellulose generated ethanol/acetate ratio of greater than 7 (Ng *et al.*, 1981). However, *C. thermohydrosulfurican* was unable to utilize xylan. Therefore, xylose and the majority of hemicellulose were left un-fermented by these previous microorganisms.

Once a genetically engineered strain of *C. thermocellum* was developed, co-culture research focused on improving cellulose degradation. When inoculating *C. thermocellum* and non-cellulolytic *Clostrodium thermopalmarium* on cellulose (filter paper), results displayed an increase in hydrolysis by 7-fold (from 1.23 g/L to 8.59 g/L utilized cellulose) (Geng *et al.*, 2009). This left the question of how co-cultures involving cellulolytic and non-cellulolytic microorganisms influence hydrolysis and ethanol production on applicable lignocellulosic material and not just laboratory cellulose sources.

Geng and colleagues from Xi'an Jiaotong University in PR China stated that, "The mechanism of cooperation and competition between two strains [in co-culture analysis] should be studied further by comparing monoculture versus co-cultures" (Geng *et al.*, 2009). Understanding the way the two microorganisms *interact* with one another has the potential to fill in the gap in co-culture research and improve bio-ethanol production as a whole (Burrel *et al.*, 2004; Salimi and Mahadevan, 2013).

2.10.1 Requirements for successful co-culture

A potential CBP organism or effective co-culture must be able to meet three main requirements: (i) able to hydrolyze cellulose and hemicelluloses; (ii) able to ferment both hexose and pentose sugars; and (iii) able to produce ethanol at high yield (Shaw, 2008). Since *C.*

thermocellum is capable of utilizing only the six carbon sugars (hexoses) and not the five carbon sugars (pentoses), which are derived from cellulose and hemicellulose (Figure 2.5) the use of co-culture or mixed culture systems is of significant interest. Previous research showed that *C. thermocellum* and *Thermoanaerobacter* spp. strains are capable of working together by fermenting more sugar molecules and as a result significantly increase the production of ethanol end-product (Svetlitchnyi *et al.*, 2013; Jiang *et al.*, 2013). Figure 2.5 illustrates how the interaction would be carried out:

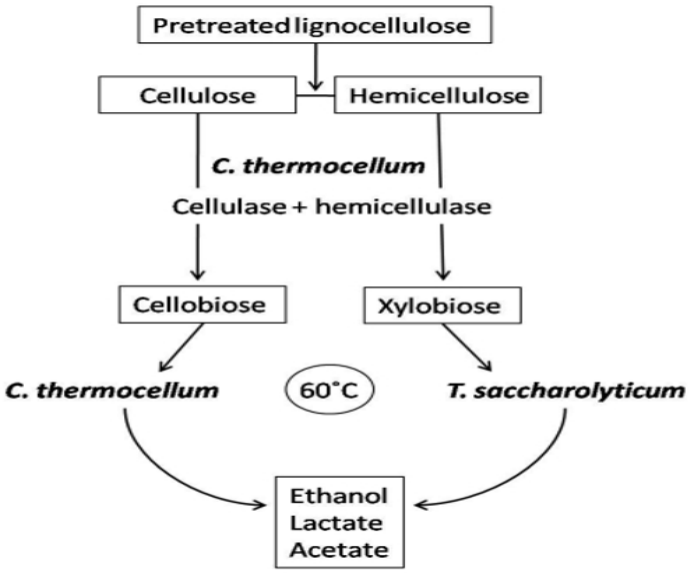

Figure 2.5: Simplified process using *C. thermocellum* and *T. saccharolyticum* in co-culture for ethanol production (Adopted from Maki *et al*., 2009).

2.10.2 *Clostridium thermocellum* and *Thermoanaerobacterium saccharolyticum*

As can be seen in Figure 2.5, a cellulolytic *C. thermocellum* produces two disaccharide molecules: cellobiose (two glucose molecules) and xylobiose (two xylose molecules). The non-cellulolytic *T. saccharolyticum* strain helps during the fermentation stage by converting the five-carbon sugar xylose into desired end products. A study performed by Bungay (1982) showed that *Clostridium thermosaccharolyticum* (now classified as *T. saccharolyticum*) has the potential to

utilize cellobiose faster than *Clostridium* spp. thus having the ability to prevent any build-up that may be caused by the inhibitory or repressive actions of this disaccharide. Therefore, this particular bacterial combination may lead to a closely associated, syntrophic, and quite stable dual culture (Wiegel, 1980).

2.11 Carbon catabolite repression and co-culture sugar utilization

Carbon catabolite repression (CCR) is a general mechanism that dictates the order in which types of sugars are utilized by a microorganism (Tsakraklides *et al.*, 2012). Carbon catabolite repression in some bacteria allows for quick adaption to a readily metabolisable carbohydrate energy source first. This adaptation is achieved via inhibition of synthesizing enzymes involved in catabolism of sugars that are not mainly preferred (Slonczewski, 2014). In other words, the cell's CCR system is responsible for making sure that the energy expenditure on sugar import and eventual metabolism will be guided to the source of carbon that is most readily available and allows for most rapid growth (Brückner and Titgemeyer, 2002; Gorke and Stulke, 2008). CCR can become a barrier for lignocelluloses-derived sugar mixtures in consortia due to the fact that different microorganisms will compete for the *same* type of sugar source and thus create unwanted competition (Vinuselvi *et al.*, 2012). In addition, the accumulation of unused substrates may complicate extraction of desired end-products downstream (Vinuselvi *et al.*, 2012).

Studies on species belonging to the Clostridia class (which includes both *C. thermocellum* and *T. saccharolyticum*) show that these microorganisms utilize glucose substrates via Histidine-containing Protein (HPr) and cell membrane transporter called Phosphotransferase System (PTS) (Tsakraklides *et al.*, 2012). Fortunately, carbon catabolite repression in *T. saccharolyticum*

shows that this microorganism uses an alternative cell membrane transporter called ATP-Binding Cassette (ABC) when utilizing xylose (Tsakraklides *et al.*, 2012). Therefore, glucose and xylose utilization or transport in *T. saccharolyticum* can occur in parallel due to the presence of two separate sugar transporters located within its cell membrane. As a result, this tends to lower the competition with *C. thermocellum* for glucose and generates a more efficient co-culture partnership.

2.12 Economics of cellulosic bio-ethanol production

Typical lignocellulosic biomass found on the North American continent contains roughly 45% glucan (one straight chain of D-glucose molecules) (Lavoie *et al.*, 2012). This amount of stored glucose can lead to a production of about 313 litres (L) of ethanol per ton of raw biomass (Lee and Lavoie, 2013). Since the market value is US$0.68/L, with a production price approximately US$0.30/L, the cellulosic bio-ethanol production value is US$212/ton ($192/metric tonne) of biomass (Lee and Lavoie, 2013). According to Patzek and colleagues from University of California, bio-ethanol fuel has a gasoline gallon equivalency (GGE) value of 1.5 US gallons (5.7 L) (Patzek *et al.*, 2005). In other words, it takes 1.5 gallons (5.7 L) of bio-ethanol to produce the energy equivalent of 1 gallon (3.8 L) of gasoline. Hence, one crucial way to optimize bio-ethanol production and in turn make it more economically competitive with fossil fuel production is to utilize *all* sugars present in lignocellulosic material, not just glucose sugars.

Currently, regular engines on vehicles can use up to 25% anhydrous ethanol (E25) blend with regular gasoline and no engine modification is required (Costa and Sodre, 2010). However, engines that use 85% (E85) blend and 100% (E100) ethanol require structural changes. The

average cost to produce a modified flex fuel engine for a new vehicle is approximately US$100 (Anderson and Sallee, 2011). This value can range up to US$500 depending on the type of the engine (Anderson and Sallee, 2011).

2.13 Cellulose Substrates

One of the most important features of cellulose is its crystalline structure (Lynd e*t al.*, 2002). Roughly 30 different separate cellulose molecules are put together into larger protofibrils, which are assembled into larger units called microfibrils, and these are in turn are the components of cellulose fibres (Lynd *et al.*, 2002). Avicel and Sigmacell are commonly used sources of cellulose in laboratories for batch processing. Avicel, for example, is a microcrystalline cellulose powder and outside research settings it can be used in food preparations (Wolf *et al.*, 1984). Microcrystalline celluloses are nearly pure cellulose in composition, possess a straight, non-bending structure and have the ability to get completely utilized or hydrolyzed.

2.13.1 Cotton fibre

Over the past few year cotton has started to gain more attention as a valuable carbon source candidate for continuous-flow experimentation and imaging. Cellulose component of cotton can range anywhere from 85% to 100%; however, once bleached most commercially available cotton balls will yield up to 99% pure cellulose (Tripp and Rollins, 1952). The majority of cellulose that is found in cotton fiber is assembled in the secondary wall and for this reason is considered to be both physically and economically the most important portion of the fiber (Tripp and Rollins, 1952). The diameter of one cotton fiber is from 10 µm to 20 µm, making it an excellent size for microscope imaging with cellulolytic microorganisms (Figure 2.6).

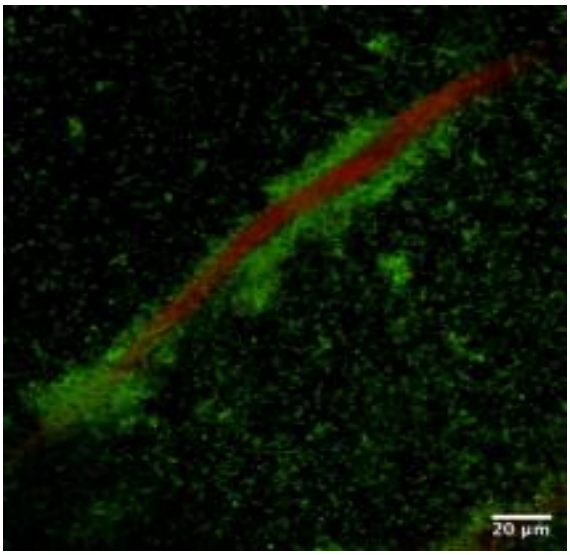

Figure 2.6: Live cell image of *C. thermocellum* ATCC 27405 monoculture grown in the flow cell (60˚C) under anaerobic conditions. Cotton fiber can be seen in brown and colonization of cells (green) around the fiber. Cell were stained with SP-DilC_{18}(3) (Adopted from Wang *et al.*, 2013).

2.14 Lignocellulosic substrates

Lignocellulosic biomass is biological material that is composed of cellulose, hemicelluloses, and lignin polymers (see section 2.2). This type of material is organic in nature and may be used as a source of energy and/or chemicals (Scragg, 2009). Biomass in the form of wood, specific crops, crop residues and organic wastes meet both sustainable and renewable criteria due to their ability to become replanted (Scragg, 2009). Theoretically, biofuel from biomass can be regarded as carbon dioxide neutral since any carbon dioxide discharged during their combustion had to be fixed first from the atmosphere during photosynthesis stage. However, in the cultivation, harvesting, preparation, and transportation of biomass, the use of fossil fuels may be required and thus making them more accurately 85-93% carbon neutral (Parikka, 2004; Venturi and Venturi, 2003). There are five general categories of solid biofuels or second generation biofuels: i) agricultural residues (corn stover, crop straws, and bagasse); ii) herbaceous crops (perennial grasses); iii) short rotation woody crops; iv) forestry residues; and v)

waste paper and other wastes (municipal and industrial) (Limayem and Ricke, 2012). From the latter categories, perennial grasses are regarded as the best option as a source for energy since they are readily available energy feedstock, relatively easy to grow and harvest, can grow on marginal lands, and the yield per acre is greater than for wood (Igboanugo *et al.*, 2013).

2.14.1 Perennial grasses

Perennial grasses are plants that are able to live for more than two years (Kephart *et al.*, 1992). They have been used in the past as fodder crops but have recently been gaining more attention as a valuable energy crop source due to a high cellulose and hemicelluloses contents (Scragg, 2009). In 2003, a study performed by Lewandowski and colleagues isolated various perennial grass sources as optimum substrates for energy production. In this study, trials with sixteen perennial grasses were performed for energy use in both USA and Europe. The criteria used in the selection as an energy crop were: i) suitable for the climate in the region; ii) easily propagated; iii) a consistent and high yield per hectare; iv) positive balance of energy; v) the crop can be cultivated in a suitable manner; vi) resistance to pests and diseases; vii) broad genetic diversity; viii) harvesting possible with existing technology; ix) perennial; and x) competitive on cost with food crops (Lewandowski *et al.*, 2003). Results have shown that from the sixteen different perennial grasses, four have been chosen as the suitable energy crops and they are miscanthus (S/G ratio of 0.70), switchgrass (S/G ratio of 0.68), reed canary grass (unknown S/G ratio), and giant reed (S/G ratio of 1.13). They have been chosen based on their yield, establishment time, photosynthetic pathway, fertilizer use, water supply, pesticide use, establishment cost, pest/disease content, day/length of growth, plantation longevity, energy content, and energy output (Lewandowski *et al.*, 2003).

Furthermore, one of the most important characteristics of perennial grasses is the type of metabolism used. The two types of metabolism are C4 and C3 and they refer to the pathways used to incorporate carbon dioxide during photosynthesis process (Scragg, 2009). The C4 metabolism, rather than C3 metabolism, is preferred since C4 plants are more efficient at higher light exposure and temperature (Scragg, 2009). C4 plants also have a lower moisture content, need less fertilizer input and as far as water requirement, are twice as efficient (Scragg, 2009). According to Alan Scragg (2009) "(Switchgrass) is perhaps the best choice as it is a perennial C4 grass, drought-tolerant, gives high yields, and can be harvested once a year".

2.14.1.1 Switchgrass

Panicum virgatum, or better known as switchgrass, is a perennial bunchgrass that can be commonly found in temperate to subtropics regions of North America (south of 55th parallel) (Price and Casler, 2014). Researchers have first considered switchgrass as a good source for biofuel production in the early 1990s due to its wide adaptability in temperate climates and ability to produce moderate to high yields on marginal farmlands (Robertson *et al.*, 2011). The more research is performed on this crop, the more attention it is given when it comes to being utilized for ethanol and butanol production. Switchgrass can produce almost 1200 gallons of ethanol per acre (~11,000 L/hectare) whereas conventional corn or maize can produce about 400 gallons per acre (~3,700 L/hectare) (Kent, 2012). In addition, switchgrass also uses much less water for irrigation purposes than maize crops. It can use anywhere from 1.9 to 9.0 gallons of water per gallon of ethanol and maize requires anywhere from 10 to 324 gallons of water per one gallon of ethanol produced (Wu *et al*., 2008). According to Schmer and colleagues at the University of Nebraska, switchgrass crops in Minnesota could provide feedstock to produce

540% more energy than energy consumed in its production and cut down greenhouse gas (GHG) emissions by 94% when compared to gasoline (Schmer *et al.*, 2007). Therefore, switchgrass has a great potential to overcome the challenge of land use concerns and simultaneously create more sustainable and environmentally friendly energy.

Last, previous research has shown that using the SSCF method with a genetically modified yeast (*S. cerevisiae* 424A – LNH ST) inoculum, switchgrass has presented ethanol yields of 32.1g/L and sugar conversions of glucan and xylan of 80.3% and 84.3%, respectively (Monti, 2012). Thus, it would be interesting to see how switchgrass digestion using bacterial co-culture and CBP method would compare to ethanol yield values in the literature.

CHAPTER 3: EXPERIMENTAL

3.1 Introduction

For the past century, the global economics, geopolitical statuses, social class, and almost all human life on the planet continue to rely primarily on a single natural resource called fossil fuels. Unfortunately, fossil fuels are a non-renewable energy source and due to an exponential increase in global population, energy consumption has dramatically increased as a result. According to some prominent studies, it is predicted that complete depletion of fossil fuel reserves may be reached within next few decades (Radolfi *et al.*, 2009). Consequently, there is an unavoidable need for a more renewable and in turn sustainable alternative energy source.

The growing interest in bioethanol originates from a combination of different elements which include security of supply, climate change, and accelerated increase in oil prices (Rosillo-Calle and Johnson, 2010). This particular interest is continually supported by evidence that, if adequate management practises are applied, the social, economic, and environmental benefits of bioethanool production can overcome its potential negative environmental impacts (Rosillo-Calle and Johnson, 2010). The current mode of bioethanol production is achieved mainly from cellulosic food sources such as corn and sugar cane. Growing these edible biocrops is considered to be a strain on agricultural lands and a direct cause of food price inflation (Inderwildi and King, 2009). However, fuels that are derived from lignocellulosic biomass – the fibrous and generally inedible part of the plant structure – present a substitute to conventional energy sources (Burkheisser, 2010). Cellulose and hemicellulose portions of the lignocellulosic structure are found in plant cell walls and are polysaccharides composed of energy-rich sugars that can be converted into ethanol through bio-processing. In addition, lignocellulosic biomass is an

attractive bioenergy feedstock due to supplies being domestically and globally sufficient (Burkheisser, 2010).

One promising bioenergy crop that has been receiving attention in the recent years is a perennial herbaceous plant called *Panicum virgatum* or commonly referred to as switchgrass. In general, switchgrass is a C4 plant that has a potential for being a biofuel feedstock due to its ability to grow in a diverse range of climates, less water requirement for irrigation, high yield rates, and low fertilizer demand compared to other common biocrops (Fike *et al.*, 2005). Other management advantages of switchgrass include low nitrogen, phosphorus, and potassium requirements, as well as, acid soil tolerance and low harvest costs (Smith and Greenfield, 1979; Morris *et al.*, 1982; Jung *et al.*, 1990; Wright, 1990).

Plants have evolved an efficient system for resisting biodegradation of their structural support by microbial species (Himmel *et al.*, 2007). Thus, current methods used to break down lignocellulosic biomass into simple sugars are considered to be ineffective and therefore establish the main barrier to producing bioethanol at quantities and prices that are competitive with petroleum (Burkheisser, 2010). Chemical pre-treatment and enzyme addition used for hydrolyzing this resistant plant wall barrier are costly and time consuming process. One solution to this particular problem was solved by the invention of consolidated bioprocessing (CBP). This process involves the use of a genetically modified *Clostridium thermocellum*, a thermophilic anaerobe that is able to hydrolyze cellulosic biomass – with already present cellulose degrading enzyme complex - into useful sugar products and can simultaneously ferment these sugars into ethanol (Yee *et al.*, 2014). Hence, CBP remains a fair alternative due to the potential of lowered

costs related to reducing unit operations and enzyme additives (Lynd *et al.*, 2002; Olson *et al.*, 2012).

Despite *C. thermocellum*'s positive CBP attributes, major hurdles still remain with this microbe's inability to produce sufficient levels of ethanol and lack of metabolic capability to ferment pentose sugars found in lignocellulosic material (Linger and Darzins, 2013). Recent studies have shown that the anaerobic thermophilic, hemicellulosic bacterium *Thermoanaerobacterium saccharolyticum* contains valuable properties that make it a potential member for a CBP consortium because *T. saccharolyticum* can ferment pentose and other biomass-derived sugars and has a highly effective metabolic activity allowing it to produce ethanol with a maximum titer (Shaw *et al.*, 2008). Thus, studying the nature of *C. thermocellum* and *T. saccharolyticum* co-culture interaction and their combined ability to produce higher ethanol yield is an important step in improving CBP and lignocellulosic bioethanol production as a whole.

The objectives of this study were: (i) to compare the respective morphologies and examine the interaction between *C. thermocellum* and *T. saccharolyticum* in their abilities to bind to different carbon substrates, (ii) to investigate competitive vs. co-operative behaviour physiologically and numerically on various carbon sources between the two thermophiles, and (iii) to determine the extent of substrate solubilisation and high-value product formation using HPLC analysis.

3.2 Methodology

Bacterial strains and chemicals used

Clostridium thermocellum ATCC 27405 was donated by Dr. Paul Weimer (University of Wisconsin) and was maintained in the laboratory for three generations in RM medium (Ozkan *et al.*, 2001) with added Avicel PH-101 cellulose substrate. *Thermoanaerobacterium saccharolyticum* DSM 7060 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). All the reagent chemicals used were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and VWR International (Radnor, PA, USA). Fluorescent stains have been purchased from Life Technologies Inc. (Burlington, ON, CA) while compressed gas was acquired from Linde Canada Ltd. (Concord, ON, CA). Last, ultra-pure water used in preparation of assays and medium was produced with a Milli-Q Gradient system purchased from EMD Millipore (Billerica, MA, USA).

Culture media

Batch systems

Batch culture medium for all tests were prepared in 30 mL glass crimp-sealed vials (VWR, ON, CA) capped with rubber stoppers (Bellco Glass Inc., NJ, USA) with buffer modified DSMZ Medium 122 containing (per litre of sterile water): 0.5 g urea, 1.43 g KH_2PO_4, 2.5 g K_2HPO_4, 2 g yeast extract, 4 g Na_2-β-glycerophosphate • $4H_2O$, and 0.002 g oxygen indicator resazurin. The media were autoclave-sterilized (121˚C, 2 hours) and vacuum sparged with pure nitrogen gas for 10 minutes (40 second vacuum, 20 second nitrogen sparging intervals). Sterile mixtures of 0.9 mL salt and minerals (1.3 g $MgCl_2$ • $6H_2O$, 0.13 g $CaCl_2$ • $2H_2O$, 0.02 g $FeSO_4$ •

$7H_2O$), 0.1 mL vitamin solution (0.01 g pyridoxamine dihydrochloride, 0.002 g P-aminobenzoic acid, 0.001 g biotin, 0.001 g vitamin B12, 0.002108 g thiamine, 0.0025 g nicotinic acid, 0.00025 g α-lipoic acid) and 0.1mL trace elements (0.001256109 g $MnCl_2$ • 4H2O, 0.0005 g $ZnCl_2$, 0.000125 g $CoCl_2$ • $6H_2O$, 0.000125 g $NiCl_2$ • $6H_2O$, 0.000125 g $CuSO_4$ • $5H_2O$, 0.000125 g H_3BO_3, 0.000125 g Na_2MoO_4 • $2H_2O$), with sterile 1 g L-cysteine HCl (reducing agent).

Morphology tests The medium for *C. thermocellum* (3 mL inoculum) was supplemented with 10 g/L cellobiose and the medium for *T. saccharolyticum* (3 mL inoculum) was prepared with 10 g/L xylose supplement.

Physiology tests The respective media contained supplements of these sugars at 5 g /L. The pH was ~6.5 before 3 mL inoculation of *C. thermocellum* ATCC 27405 and *T. saccharolyticum* DSM 7060 in monoculture and both volumes combined in co-culture batch medium.

Continuous-flow systems

Continuous-flow testing were separated into four separate experiments and each experiment medium was conducted in 1800 mL sterile glass water bottles where the concentrations of urea, KH_2PO_4, K_2HPO_4, Na_2-β-glycerophosphate • $4H_2O$, and resazurin was added and sealed with rubber caps. After autoclave sterilization (121˚C, 2 hours), the medium vessel was cooled down under sparging with pure nitrogen for a period of six hours. Using 10 mL syringe (containing 0.2µL syringe filters), sterile mixtures of trace elements, vitamins, salt and minerals, and L-cycteine HCl (reducing agent) were added to the medium afterwards (at the same final concentrations as in batch systems mentioned previously).

Substrate attachment capabilities Continuous-flow medium was prepared for four types of analyses: i) *T. saccharolyticum* DSM 7060 on cotton; ii) *T. saccharolyticum* DSM 7060 on switchgrass; iii) co-culture on cotton; and v) co-culture on switchgrass as mentioned above with an additional 0.3 g/L cellobiose and 10 g/L xylose additives. A detailed analysis of attachment by *C. thermocellum* has been described by Dumitrache *et al.* (2013).

Competition vs. cooperation test and end product analysis Continuous-flow media for a further series of experiments had different yeast extract and sugar supplements, depending on the experiment performed and as detailed in Table 3.1. Yeast extract was added before autoclave sterilization and sugar supplementation was added after autoclave sterilization to prevent charring.

Table 3.1: Culture media preparation for each continuous-flow experiment with respect to HCS and LCS composition

Experiment	High Carbon Supplement (HCS)			Low Carbon Supplement (LCS)		
	Yeast Extract (g/L)	Cellobiose (g/L)	Xylose (g/L)	Yeast Extract (g/L)	Cellobiose (g/L)	Xylose (g/L)
1. *C. thermocellum* ATCC27405 on cotton fibre	2.0	0.3	N/A	0.3	0.0	N/A
2. *C. thermocellum* ATCC27405 on switchgrass	2.0	0.3	N/A	0.3	0.0	N/A
3. Co-culture* on cotton fibre	2.0	0.3	10	0.3	0.0	0.0
4. Co-culture* on switchgrass	2.0	0.3	10	0.3	0.0	0.0

* Co-culture containing *C. thermocellum* ATCC 27405 and *T. saccharolyticum* DSM 7060 together

Continuous-flow design

Flowcell reactor

Continuous-flow (Figure 3.1) experiments with lignocellulosic substrate were prepared with a flowcell reactor that contained 0.51 g midseason untreated switchgrass processed with 6

mm screen (kindly provided by Dr. Julie Paye from Dartmouth College, USA) and those with cellulosic substrates were prepared with 0.13 g of commercially available cotton (substrate attachment capability experiment – *T. saccharolyticum* DSM 7060 on cellulose trial and competition vs. cooperation tests – *C. thermocellum* on cotton and co-culture on cotton)

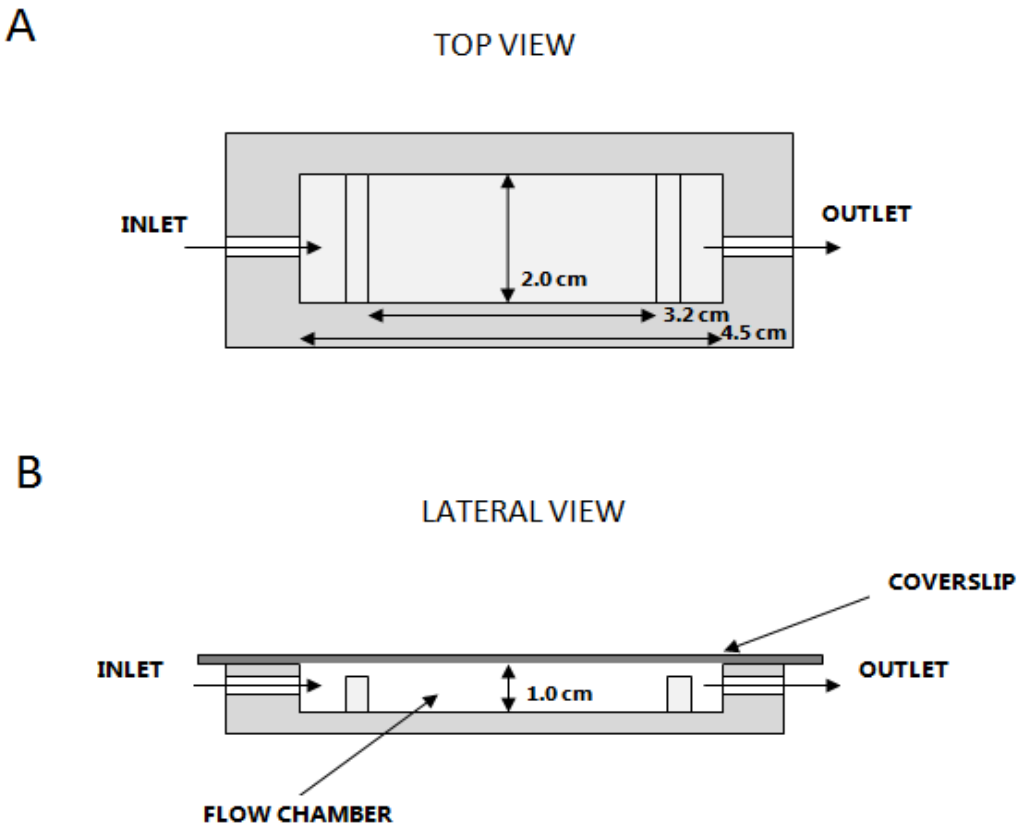

Figure 3.1: Schematic of flow cell reactor design displaying top view (A) and lateral view (B).

Continuous-flow system

The general arrangement of the components in all of the continuous-flow experiments (such as medium, flow cell reactor, and effluent sample) in the anaerobic cabinet (Type B vinyl anaerobic chamber; Coy Laboratory Products, MI, USA) can be seen in Figure 3.2 and Figure

3.3. Unidirectional flow was from the medium to the effluent flask (2 litre), and was maintained by a peristaltic pump (model 205S, Watson Marlow, Cornwall, England). Silicon tubing (0.188 x 0.313 x 0.063 in., I.D x O.D x Wall, VWR, ON, CA) along with polycarbonate adapters were used to link all the components in the anaerobic chamber. Prior to using the culture media, all the tubing and the flowcell reactor were sterilized with a mixture of 10% sodium hypochlorite and 90% distilled water for 1 hour (118 mL/hour) and washed with sterile water for 12 hours (33 mL/hour) with continuous-flow. *C. thermocellum* ATCC 27405 and *T. saccharolyticum* DSM 7060 was inoculated from the respective batch sources via sterile syringe and needles (BD size 25G, Fisher Scientific, ON, CA). The flow of the medium was stopped for a period of 1 hour to allow for substrate colonization (60˚C incubation). Regular flow was then resumed (10 mL/hour).

Substrate attachment capabilities Each of the four continuous-flow experiments was performed as described above. Experiment 1 – *T. saccharolyticum* on cotton, experiment 2 – co-culture on cotton, experiment 3 – *T. saccharolyticum* on switchgrass, and experiment 4 – co-culture on switchgrass had incubation growth for 3 days before confocal microscopy imaging.

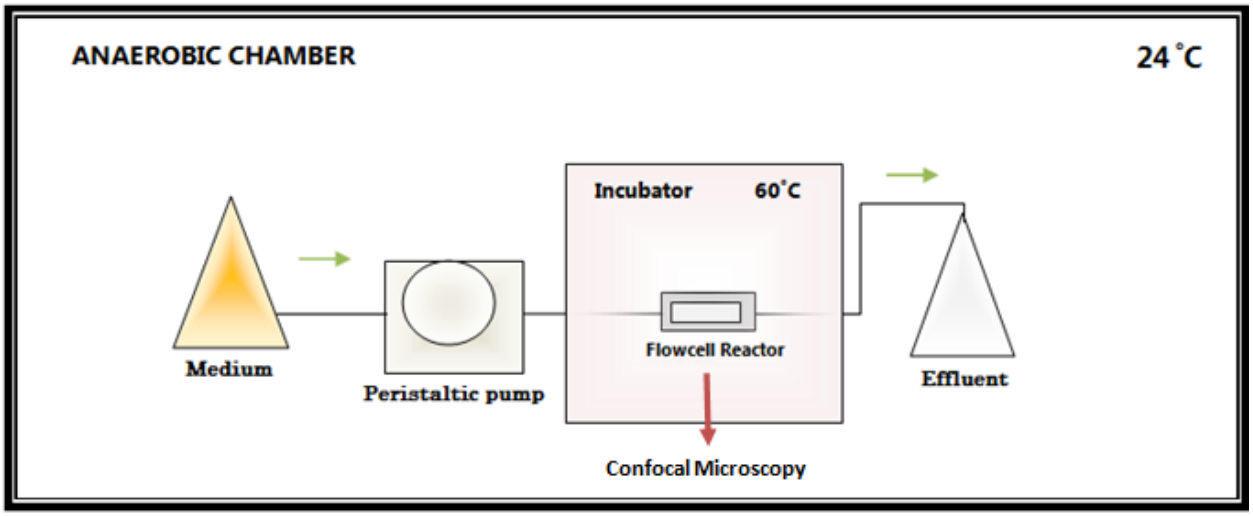

Figure 3.2: Schematic illustration of the flow cell system design for the four substrate attachment experiments. The system was used under normal atmosphere in an anaerobic chamber where a ~60˚C incubator was used for the growth of anaerobic, thermophiles *C. thermocellum* ATCC 27405 and *T. saccharolyticum* DSM 7060 (flow rate of 10 mL/h). Confocal microscopy was used for imaging of the strains and substrates.

Concentration dynamics The four separate continuous flow experiments: Experiment 1 – *C. thermocellum* on cotton, experiment 2 – *C. thermocellum* on switchgrass, experiment 3 – co-culture on cotton, and experiment 4 – co-culture on switchgrass were each tracked over a six day period at two day intervals. General schematic can be seen in Figure 3.3:

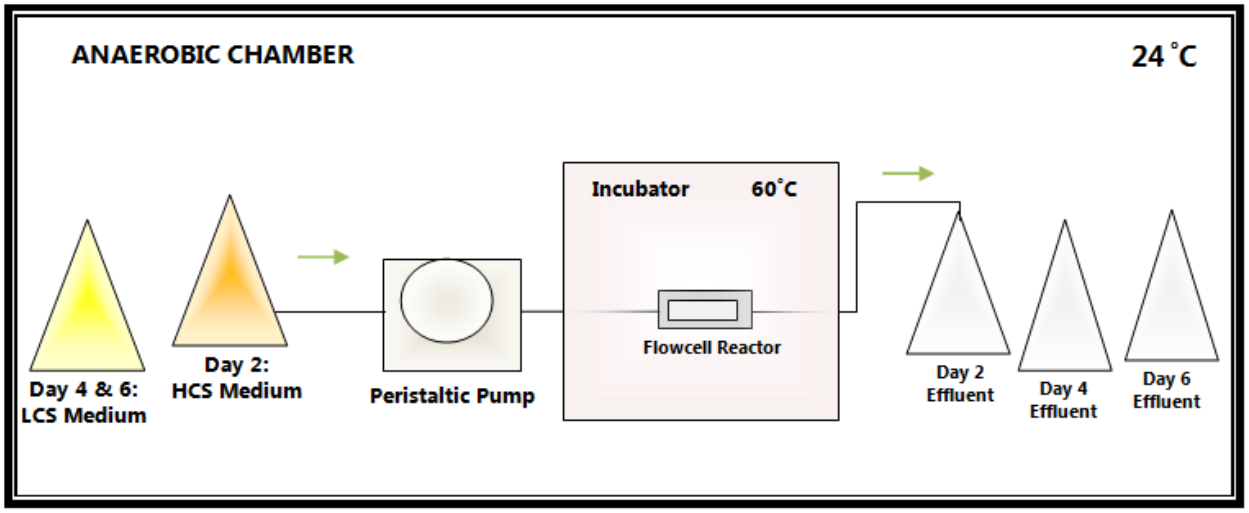

Figure 3.3: An overall continuous flow cell system used for the four concentration dynamics and HPLC experiments. A high carbon supplement (HCS) medium was used for the first two days for culture growth (60˚C, 10 mL/hour flow rate). After 2 days of incubation, sample effluent was collected for analysis and HCS medium was switched to low carbon supplement (LCS) medium for the next four days (two day effluent sample collecting intervals). Temperature of anaerobic chamber was around room temperature. See Table 3.1 for HCS and LCS medium contents for each experimental trial.

For these series of continuous-flow experiments, DSMZ-Medium 122 along with high carbon supplement (contents seen in Table 3.1) was used as a source of nutrients for the first two days of culture medium flow in order to grow *C. thermocellum* and *T. saccharolyticum* successfully in 60˚C incubation. Effluent samples were collected for real-time PCR and HPLC analysis, as described under the respective headings below. The culture medium was then switched to DSMZ-Medium 122 with low carbon supplement (LCS) and further day 4 and day 6 samples were collected for similar analysis.

Imaging

Epifluorescent Microscopy

Morphology comparison This comparison experiment was stained and imaged by a protocol described by Kepner and Pratt (1994). A 1 mL bacterial sample from both *C. thermocellum* and *T. saccharolyticum* batch systems was extracted and the cells were targeted with 0.5 µL SYBR® Gold Nucleic Acid Stain (Life Technologies, CA, USA) and diluted to 1 in 100 with ultrapure water. The dilution sample was then left in dark for 15 minutes before vacuum filtration on a pre-wetted polycarbonate membrane filter with 0.2 micron pore size and 25 mm diameter (Fisher Scientific, ON, Canada). Images were immediately obtained with a Nikon Eclipse 80i fluorescent microscope (Nikon Instruments Inc., ON, CA) using X60 oil immersion objective.

Confocal Laser Scanning Microscopy (CLSM)

Substrate attachment Imaging of cell attachment to different substrates were acquired *in-situ* with a Nikon Eclipse 80i-C1 confocal laser scanning microscope following the same procedure as described by Dumitrache *et al.* (2013). Syto 9 (2 µL) stain was used to target cells while cellulose fibres (in case of cotton experiments) and lignocelluloses (for switchgrass experiments) were targeted by a 15 µL carbohydrate binding lectin wheat germ agglutinin (WGA) conjugated

with a far-red fluorescent Alexa Fluor® 633 (AF633) dye. Syto 9 and WGA-AF33 were prepared as a mixture with ultrapure water and the stain was then applied with a syringe and needle into the flowcell tubing (inlet end) (Figure 3.1). Fifteen minutes were allowed for stain binding to cells and substrate (in the dark), with the medium flow turned off. The flow was then resumed (peristaltic pump turned on) for 20 minutes to remove excess dye and the flowcell reactor was sealed at both inlet and outlet ends, removed from anaerobic cabinet, and placed onto the microscope stage for imaging.

DNA extraction and real-time PCR quantification

Competition vs. cooperation experiments The genomic DNA of both *C. thermocellum* and *T. saccharolyticum* was extracted using InstaGene™ Matrix with specifically formulated 6% w/v Chelex resin (Bio-Rad Laboratories Inc., ON, Canada) as stated by the manufacturer's protocol. Extracted genomic DNA was stored in a -20˚C freezer until further use. Quantitative PCR (qPCR) was performed on both *C. thermocellum*'s and *T. saccharolyticum*'s extracted DNA using specifically designed forward and reverse primers (Life Technologies Inc., CA, USA) and probes (Biosearch Technologies, CA, USA). See Table 3.2 for sequence information.

Table 3.2: Individual sequence of the designed forward primer, reverse primer, and probe used for amplification of *C. thermocellum* and *T. saccharolyticum* used in real-time PCR analysis

Bacterial Sample	Forward Primer	Reverse Primer	Probe
C. thermocellum ATCC 27405*	5'-GTTATCACTGTTGAAGAAGC-3'	5'-TTCTTGTCTGTAATGAGGAT-3'	5' d HEX-AATGGGCACAAACCTCGAAA-BHQ-1 3'
T. saccharolyticum DSM 7060**	5'-AGATCGAAGAGACTACATCA-3'	5' -AGAGCATCTTCTATTCTGTG-3'	5' d FAM – TTACTGCAACACCGCCAGAA-BHQ-1 3'

* Sequenced from Nucleotide UT Sequence
** Sequenced from cpn60 gene

Each 15.5 µL qPCR reaction contained 2 µL of template DNA and 13.5 µL of master mix which was composed of *C. thermocellum*'s forward primer, reverse primer, and probe (20 µL, 20 µL, 13.8 µL respectively); *T. saccharolyticum*'s forward primer, reverse primer, and probe (20 µL, 20 µL, and 13.8 µL respectively); iQ™ Multiplex Powermix (Bio-Rad Laboratories Inc., ON, Canada) (345 µL) which contains dNTPs, 11 mM $MgCl_2$, iTaq DNA Polymerase, and additional stabilizers; and sterilized water (142.6 µL). Standard curves for *C. thermocellum* (R^2 of 0.94) and *T. saccharolyticum* (R^2 of 0.99) were generated for the standard dilutions of 1:2, 1:200, 1:20,000, and 1:2,000,000. Once the standard curves were developed, effluent samples from experimentations outlined in Figure 3.3 were collected for qPCR analysis. Each sample reaction was prepared in the same way as mentioned above.

All qPCR samples were analyzed with a LightCycler® 2.0 Instrument (Hoffmann-La Roche Limited, ON, Canada). The samples first went through an initialization step at 95˚C for 3 minutes followed by 10 seconds of denaturation at 95˚C. Primer annealing was programmed for another 10 seconds at 55˚C, followed by elongation time of 30 seconds at 72˚C. The annealing temperature was set at 57˚C for a period of 50 seconds. The whole qPCR reaction was programmed for a total of 39 cycles.

High performance liquid chromatography analysis

Samples that were collected for physiology test and effluent samples from continuous-flow experiments for end-product analysis were transferred into 1.5 mL cryovials (Sigma-Aldrich, MO, USA) and acidified to pH 2 by adding 1.5 mmol HCl. The sample was further centrifuged for 5 minutes at 10,000 rpm. Around 800 µL of supernatant was removed and pipetted into 1 mL HPLC vials (Cole-Parmer, QC, CA). End products of interest were xylose, cellobiose and ethanol for *T. saccharolytium*'s physiology test in batch and ethanol, acetate,

lactate, formate, xylose, and cellobiose for continuous-flow (pure and co-culture) end product analysis. These specific products were analyzed by high performance liquid chromatography (HPLC) on Perkin Elmer Series 200 HPLC System (Woodbridge, ON, CA) and a carbohydrate analysis Aminex® HPX-87H column (Bio Rad Laboratories Inc., ON, CA) with 9 µm particle size and 300 x 7.8 mm dimension. The solution used for the mobile phase was 5 mM H_2SO_4 and was introduced into the column at a flow rate of 0.5 mL/min with a column temperature of 60˚C (Yee *et al.*, 2012). In addition, to allow for the detection of all soluble constituents present, each sample was run for the period of 28 minutes. The data were analyzed and final concentrations were calculated by the use of TotalChrom software program. A standard curve was generated for the mixtures of different standard concentrations of 50, 20, 10, 5, 2, 1, and 0.5 mM. The standards were also run separately at a concentration of 20 mM to determine their retention time values.

Solid substrate utilization

The initial substrate mass (S_i) of both cotton and switchgrass (for all four continuous-flow experiments) was determined by dry weight measurements after incubating substrates for three hours in an oven at 120˚C. The final mass of both substrates remaining in the flow cell reactor at day 6 was also determined by dry weight measurements. In addition, day 2, day 4, and day 6 effluent samples were treated with NaOH (0.2 N final concentration) for one hour at 50˚C to digest cellular biomass (but not cellulose itself) (Dumitrache *et al.*, 2013). Once treated, the samples were then filtered on 934-AH Borisilicate glass fiber filters (Sterilitech Corporation, WA, USA) to remove undigested residual substrate particles and baked to determine dry weight of substrate lost to the effluent (S_f). Total solid substrate (S_t) utilization was calculated as follows: $S_t = S_i - S_f$.

3.3 Results

Cell morphology

C. thermocellum ATCC 27405 and *T. saccharolyticum* DSM 7060, grown separately in batch system in a similar medium composition, were imaged using epifluorescence microscopy. The morphology comparison can be seen in Figure 3.4.

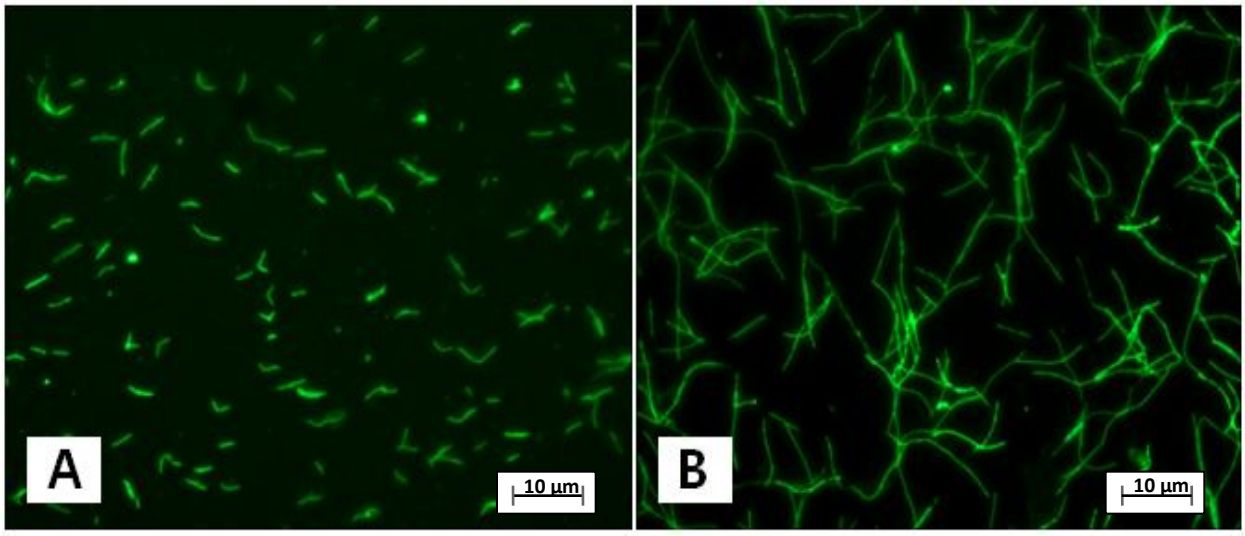

Figure 3.4: Epifluorescence microscopy showing A) *C. thermocellum* ATCC 27405 cells gown in batch (2 g/L yeast extract with 10 g/L cellobiose supplement) and B) *T. saccharolyticum* DSM 7060 (2 g/L yeast extract with 10 g/L xylose supplement), showing that the latter had a more elongated cell morphology that flocked together. Cells (1:100 dilution) were stained with SYTO9 green stain and observed under 60x objective lens.

It can be seen that *T. saccharolyticum* (8-15 µm cell size) has elongated cells, while those of C. *thermocellum* (3-5 µm) were smaller and rod shaped. *T. saccharolyticum* cells tend to undergo higher amount of intertwining or linking together than the individually separate *C. thermocellum* cells. It also appears that *T. saccharolyticum* DSM 7060 does not show any sporulation.

Substrate attachment capabilities of *T. saccharolyticum*

Four separate continuous-flow experiments have been carried out to test the binding efficiency of *T. saccharolyticum* to cellulosic and lignocellulosic materials. Figure 3.5 illustrates the images gathered with CLSM of *T. saccharolyticum* attachment on cotton (cellulose), switchgrass (lignocellulose), as well as its relative spatial distribution when present together with *C. thermocellum* in co-culture.

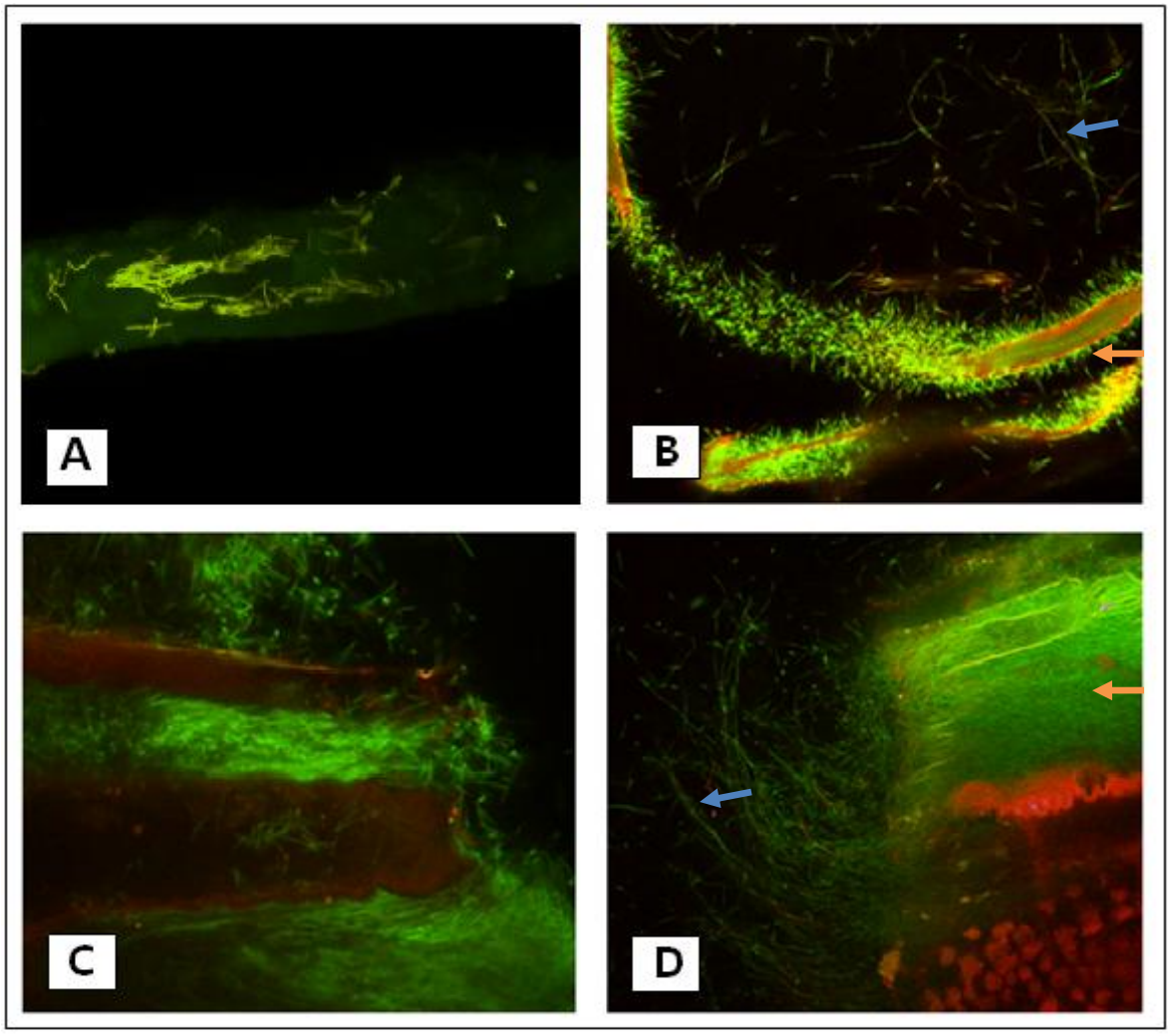

Figure 3.5: Attachment of *T. saccharolyticum* DSM 7060 on cellulosic (cotton) substrate in A) pure culture and B) in co-culture with *C. thermocellum* ATCC 27405, and on lignocellulosic (switchgrass) substrate in C) pure culture and D) in co-culture with *C. thermocellum* ATCC 27405. Blue arrow: *T. saccharolyticum* DSM 7060. Orange arrow: *C. thermocellum* ATCC 27405.

Figure 3.5A and Figure 3.5C show that *T. saccharolyticum* monoculture can in fact bind to both carbon substrates, and this attachment appears to be non-specific for both substrates. However, both images shown here reveal partial binding. Comparison with co-culture in Figure 3.5B and Figure 3.5D show that *C. thermocellum* cells occupy more substrate surface area and *T. saccharolyticum* tend to be more free floating and in close proximity to *C. thermocellum*. The blue arrows on Figure 3.5B and Figure 3.5D indicate where *T. saccharolyticum* is most likely located compared to *C. thermocellum*, shown by orange arrows.

T. saccharolyticum DSM 7060 physiology test

An HPLC analysis was performed to show yet undetermined physiology of *T. saccharolyticum* DSM 7060 strain in monoculture and in comparison with *C. thermocellum* in co-culture (Figure 3.6). The results revealed that *T. saccharolyticum* DSM 7060 is capable of utilizing both cellobiose (2.88 mM remaining at the end of 3 day incubation period) and xylose (4.44 mM remaining) sugars in parallel without the interference of carbon catabolite repression. In other words, both pentose and hexose sugars are fermented simultaneously without significant preference for one type of sugar over the other. When in co-culture, *T. saccharolyticum* could utilize xylose more efficiently, with 0.4 mM remaining at the end of the incubation period. On the other hand, *C. thermocellum*'s ability to utilize cellobiose decreased in co-culture when comparing to monoculture, 6.59 mM to 1.16 mM respectively. Ethanol production increased in co-culture (4.89 mM) by 23% when comparing to overall ethanol production in *C. thermocellum* monoculture (3.97 mM). Last, it should be noted that all three batch samples experienced a drop in pH after inoculation and 3 day incubation period (*C. thermocellum* monoculture – decrease of ~0.08; *T. saccharolyticum* monoculture – decrease of ~0.61; and co-culture – decrease of ~0.37).

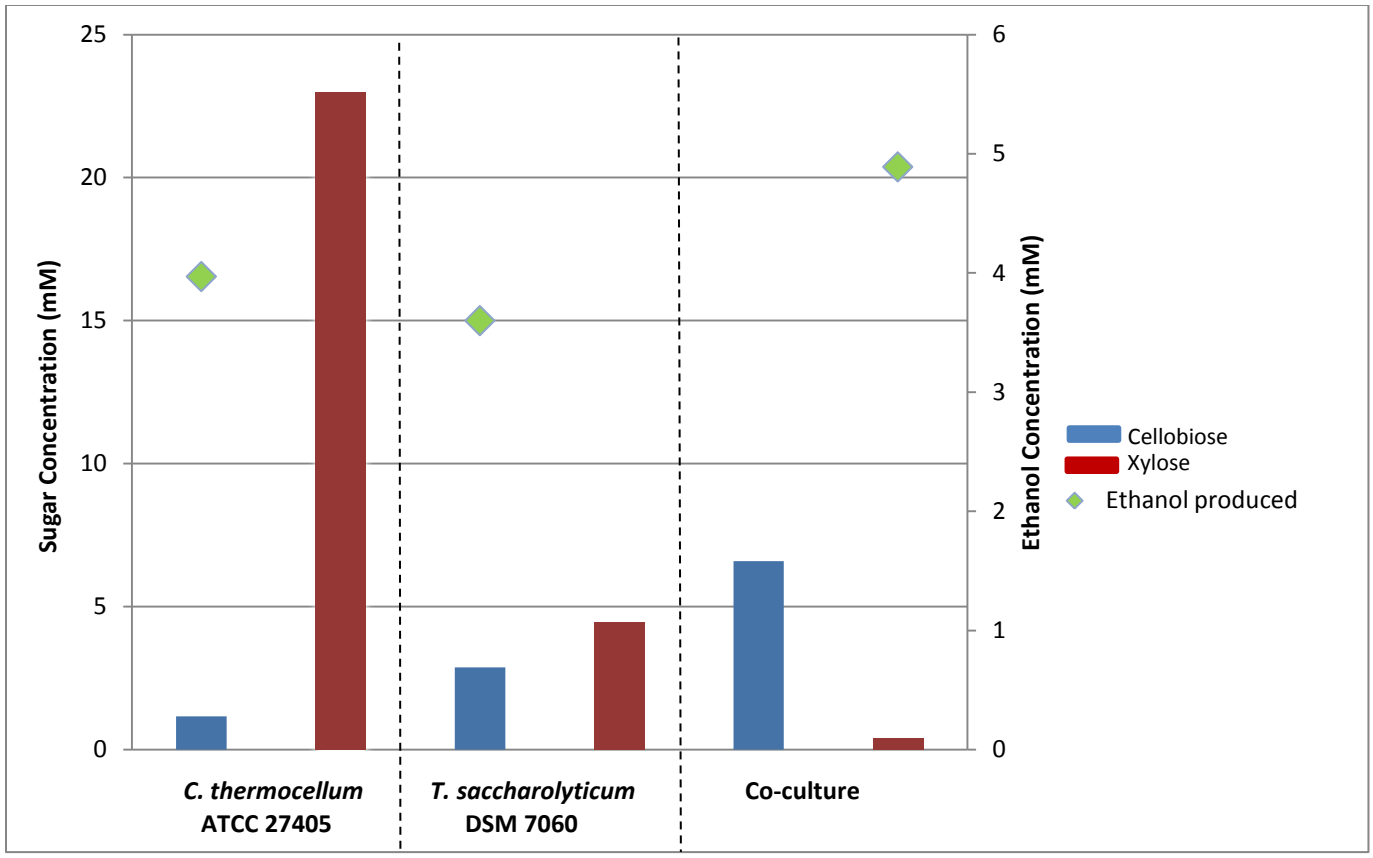

Figure 3.6: Comparing sugar utilization and ethanol production between *C. thermocellum* ATCC 27405 and *T. saccharolyticum* DSM 7060 monoculture and co-culture samples in batch system with 5 g/L cellobiose and 5 g/L xylose as sugar supplements in medium. pH before inoculation: ~6.5. The pH after inoculation and 3 days incubation period: *C. thermocellum* ATCC 27405 – 6.42; *T. saccharolyticum* DSM 7060 – 5.89; Co-culture – 6.13.

Concentration dynamics: Competition vs. cooperation analysis

Experiment 1 - C. thermocellum on cotton

Quantitative PCR analysis (Figure 3.7) shows that the DNA concentration (see Table 3.3 at the end of the results section) of *C. thermocellum* for the first two days of continuous-flow on cotton with high carbon supplement (HCS) (2 g/L yeast extract with 0.3 g/L cellobiose) was 408 ng/µL (C_p = 26.93). Once low carbon supplement (LCS) (0.3 g/L yeast extract with no cellobiose) was introduced that concentration number changed to 355 ng/µL (C_p = 27.27) for day 4, a decrease of nearly 13%. However, at day 6 under the same LCS medium, the concentration has steadily increased to 378 ng/µL (C_p = 27.01). Concentration (ng/µL) values were determined

by creating a standard curve that measured known dilution concentrations and their corresponding crossing point (C_p).

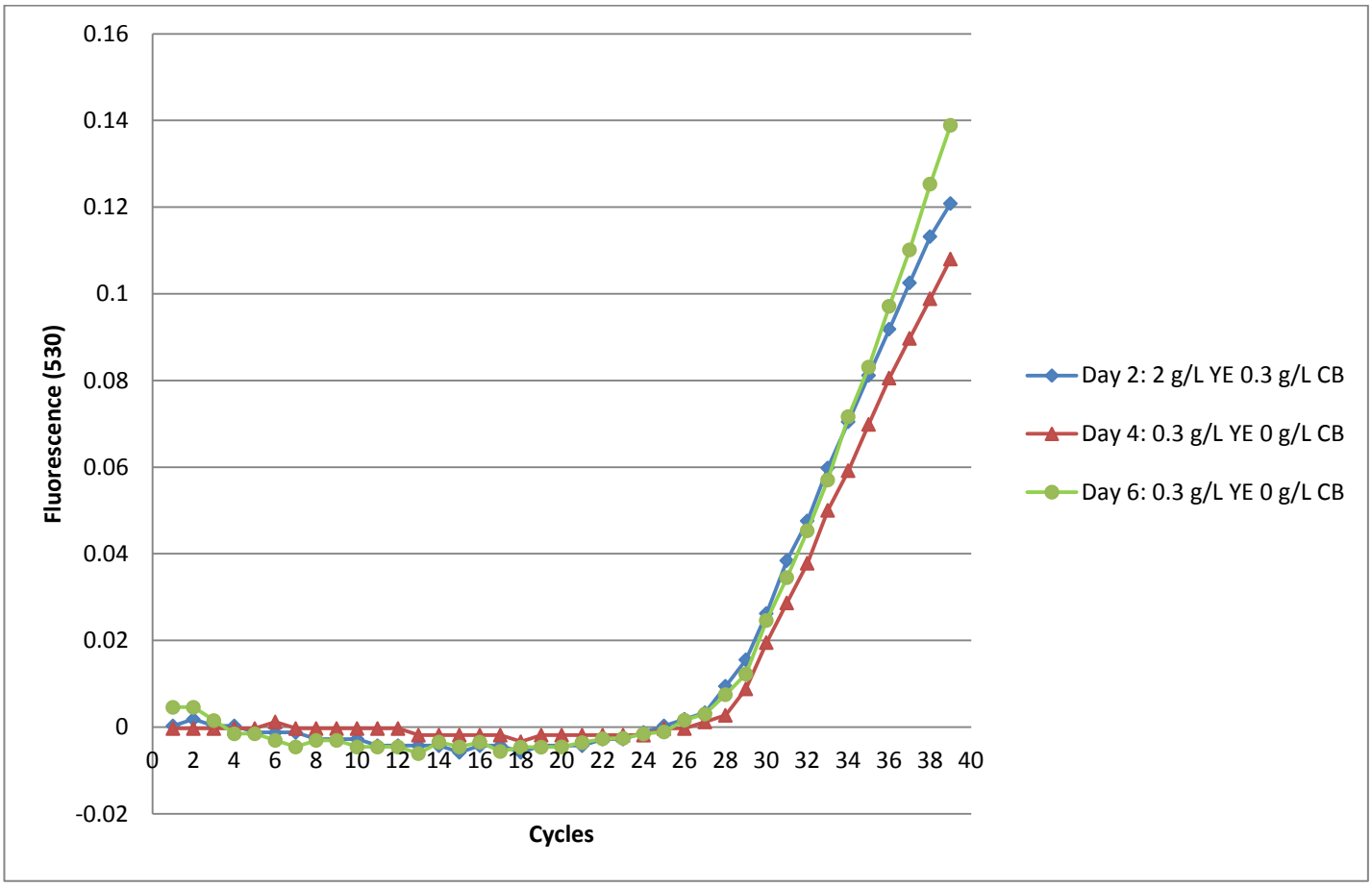

Figure 3.7: qPCR analysis of *C. thermocellum* ATCC 27405 monoculture flowcell abundance on cotton with respect to HCS (2 g/L yeast extract and 0.3 g/L cellobiose) and LCS (0.3 g/L yeast extract without cellobiose) over the course of 6 days

Experiment 2 – C. thermocellum on switchgrass

Within the first two days, *C. thermocellum* DNA concentration with HCS medium reached 639 ng/µL (C_p = 20.48). Next qPCR analysis at day 4, with medium switched to LCS, has dropped the latter concentration value to 509.1 ng/µL (C_p = 24.77). This is a decrease of 20.3% in DNA concentration value. At day 6, much like in day 6 in cotton experiment, the DNA

concentration of this bacterium gradually increased to 528.3 ng/µL (C_p = 24.19), which represents an increase of 3.8%.

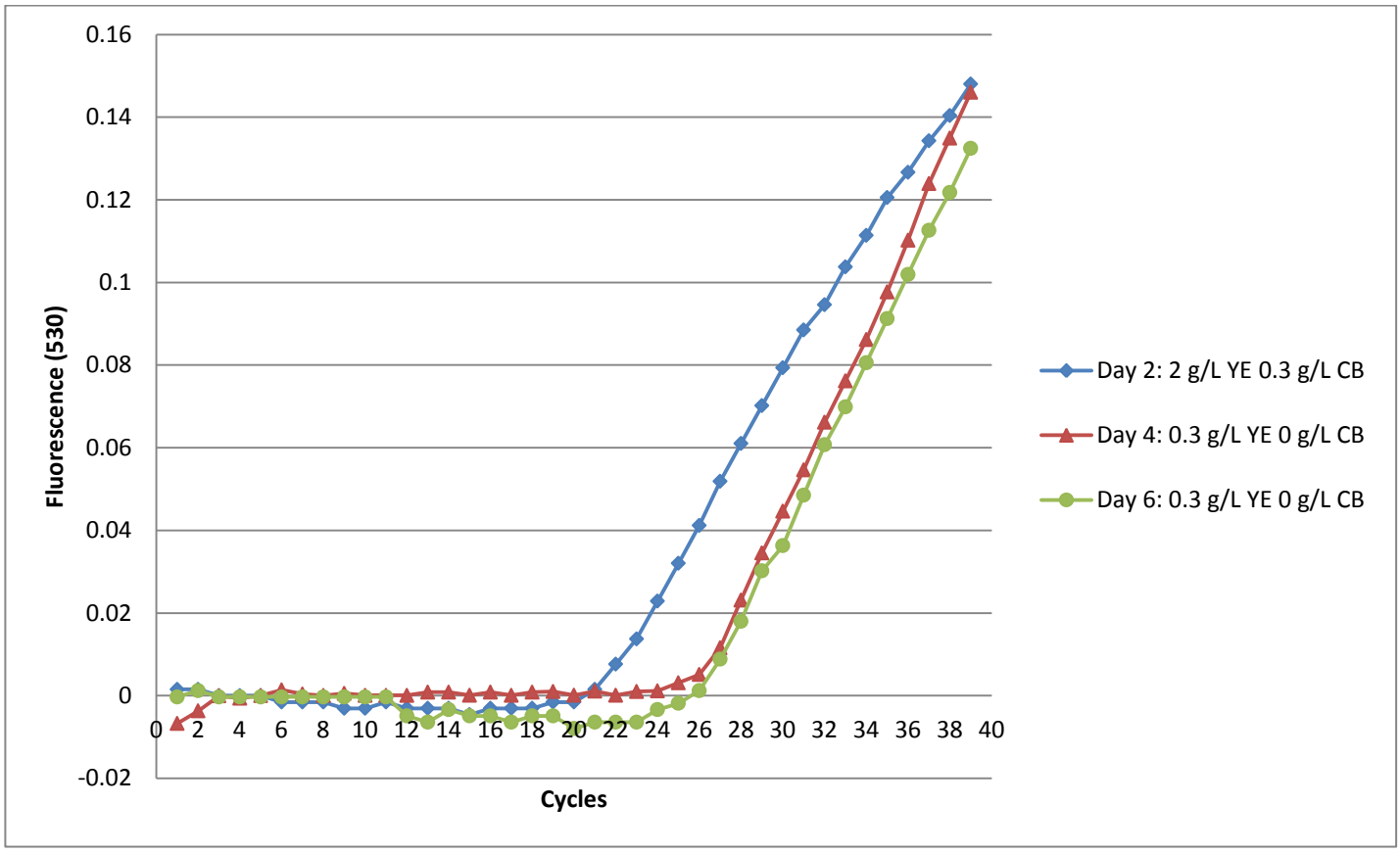

Figure 3.8: qPCR analysis of *C. thermocellum* ATCC 27405 monoculture flowcell DNA abundance on switchgrass with respect to HCS (2 g/L yeast extract and 0.3 g/L cellobiose) and LCS (0.3 g/L yeast extract with no cellobiose) over the course of 6 days

Experiment 3 – Co-culture on cotton

Figure 3.9 presents the results of the co-culture investigations over a period of six days. Both *C. thermocellum* and *T.saccharolyticum* exhibit greatest abundance at day 2 (591.8 ng/µL – C_p 21.21 and 578.6 ng/µL C_p 21.36 respectively) with HCS as the medium source. These values drop to 463 ng/µL (C_p = 25.26) in day 4 for *C. thermocellum* and to 122 ng/µL (C_p = 31.27) for *T. saccharolyticum*. Finally at day 6, *C. thermocellum* DNA concentration dropped further to 441.5 ng/µL (C_p = 25.65) and *T. saccharolyticum* increased slightly to 133.4 ng/µL (C_p = 31.12).

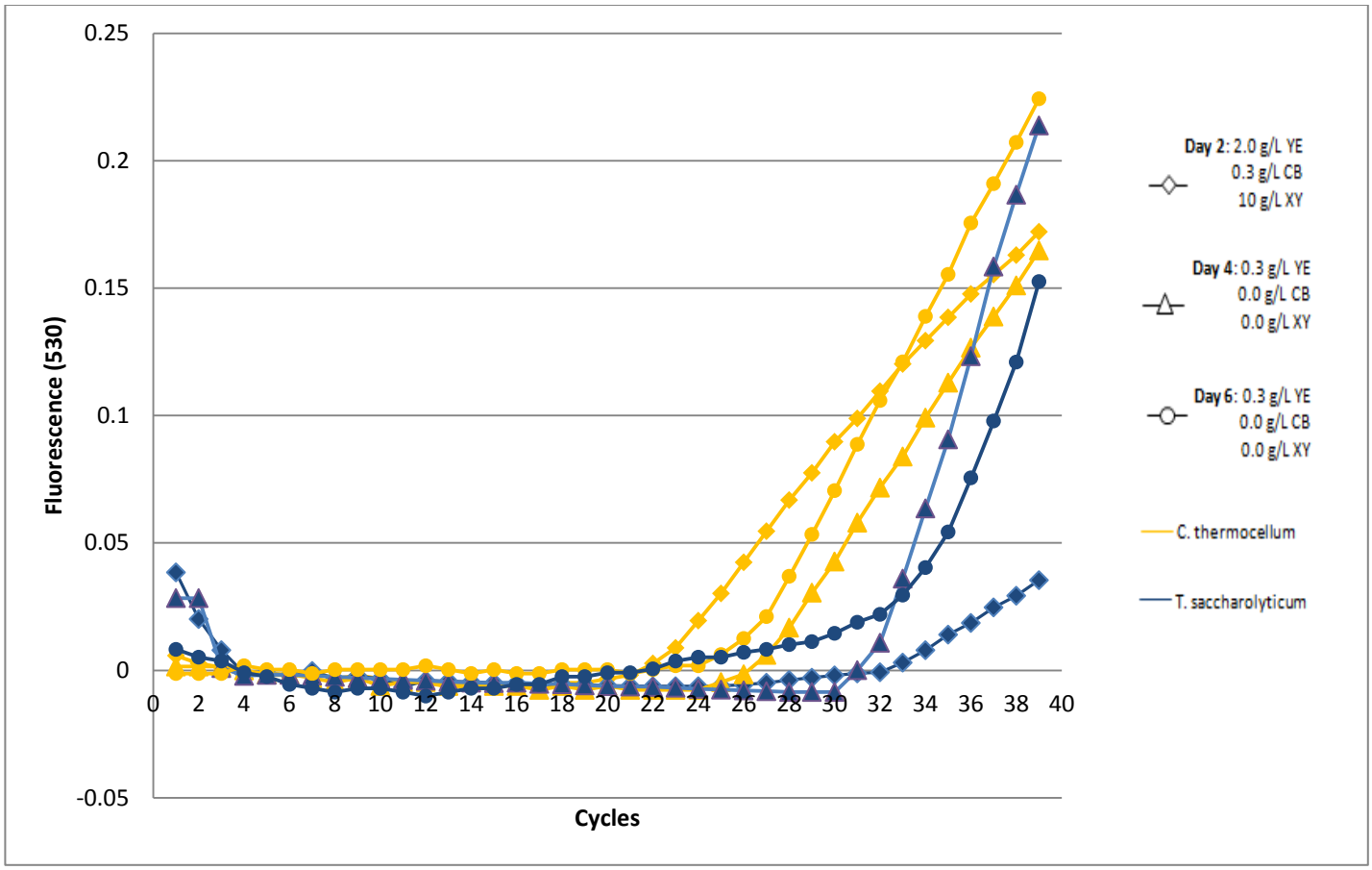

Figure 3.9: qPCR analysis of *C. thermocellum* ATCC 27405 and *T. saccharolyticum* DSM 7060 flowcell co-culture DNA abundance on cotton with respect to HCS (2.0 g/L yeast extract, 0.3 g/L cellobiose, and 10 g/L xylose) and LCS (0.3 g/L yeast extract with no sugar supplement) over the course of 6 days. See Table 3.2 for numerical analysis

Experiment 4 – Co-culture on switchgrass

Figure 3.10 shows that the concentrations of *C. thermocellum* (641 ng/µL - C_p 20.46) and *T. saccharolyticum* (315 ng/µL - C_p 30.40) with HCS at day 2 decreased to 543 ng/µL (15.3% drop) and 73.5 ng/µL (76.7 % drop), respectively by day 4 when the medium has been switched to LCS. These values tend to mimic cotton substrate concentration pattern at day 6 since values of *C. thermocellum* dropped slightly to 524 ng/µL (C_p = 23.63) and *T. saccharolyticum* recovered to 112 ng/µL (C_p = 33.49). Data for crossing points (C_p), DNA concentrations, and solid carbon remaining at the end of the experimentation can be found in Table 3.3.

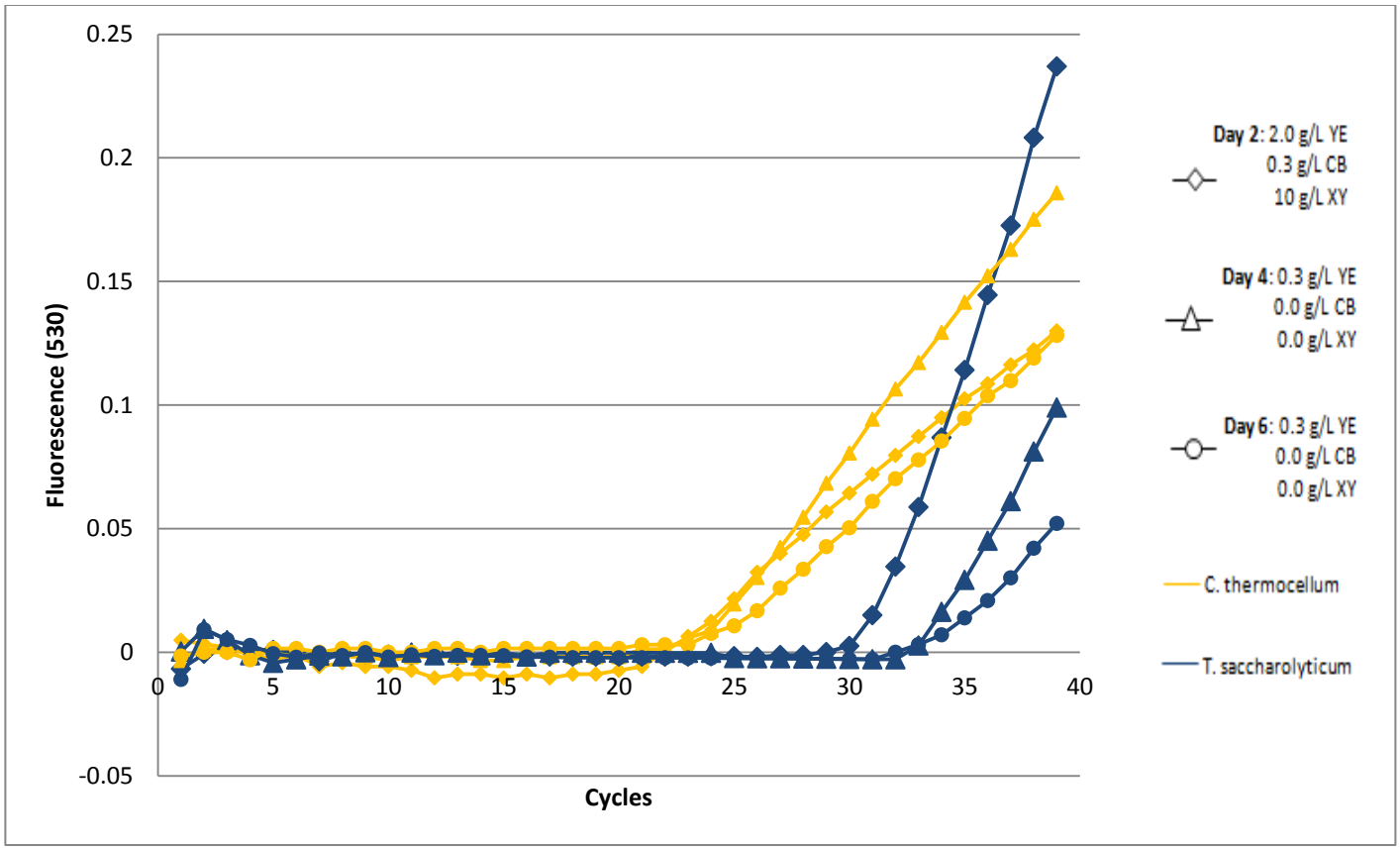

Figure 3.10: qPCR analysis of *C. thermocellum* ATCC 27405 and *T. saccharolyticum* DSM 7060 flowcell co-culture DNA abundance on switchgrass with respect to HCS (2.0 g/L yeast extract, 0.3 g/L cellobiose, and 10 g/L xylose) and LCS (0.3 g/L yeast extract with no sugar supplement) over the course of 6 days

End product analysis

The end product analysis, as determined with high performance liquid chromatography (HPLC) can be seen in Figure 3.11, showing that *C. thermocellum* growing on cotton exhibited least amount of ethanol production variation, fermenting 4.06 mM ethanol at day 2 HCS medium, 3.64 mM at day 4 LCS medium, and 3.59 mM at day 6 LCS medium. These results differed from *C. thermocellum* on switchgrass since more ethanol was produced at day 2, day 4, and day 6 (7.77 mM, 6.31 mM, and 6.27 mM respectively). Co-culture end product investigation have shown that for day 2 ethanol production was 4.86 mM, dropping to 3.72 mM at day 2, and spiking up to 5.09 mM at day 6. Lastly, co-culture on switchgrass results displayed highest amount of ethanol yield of 8.95 mM at day 2, after plummeting to 3.8 mM ethanol for day 6 the system started to recover at day 6 with ethanol production at 4.53 mM. Table 3.4 displays other by-products of fermentation and hydrolysis that were generated from the four continuous-flow experiments.

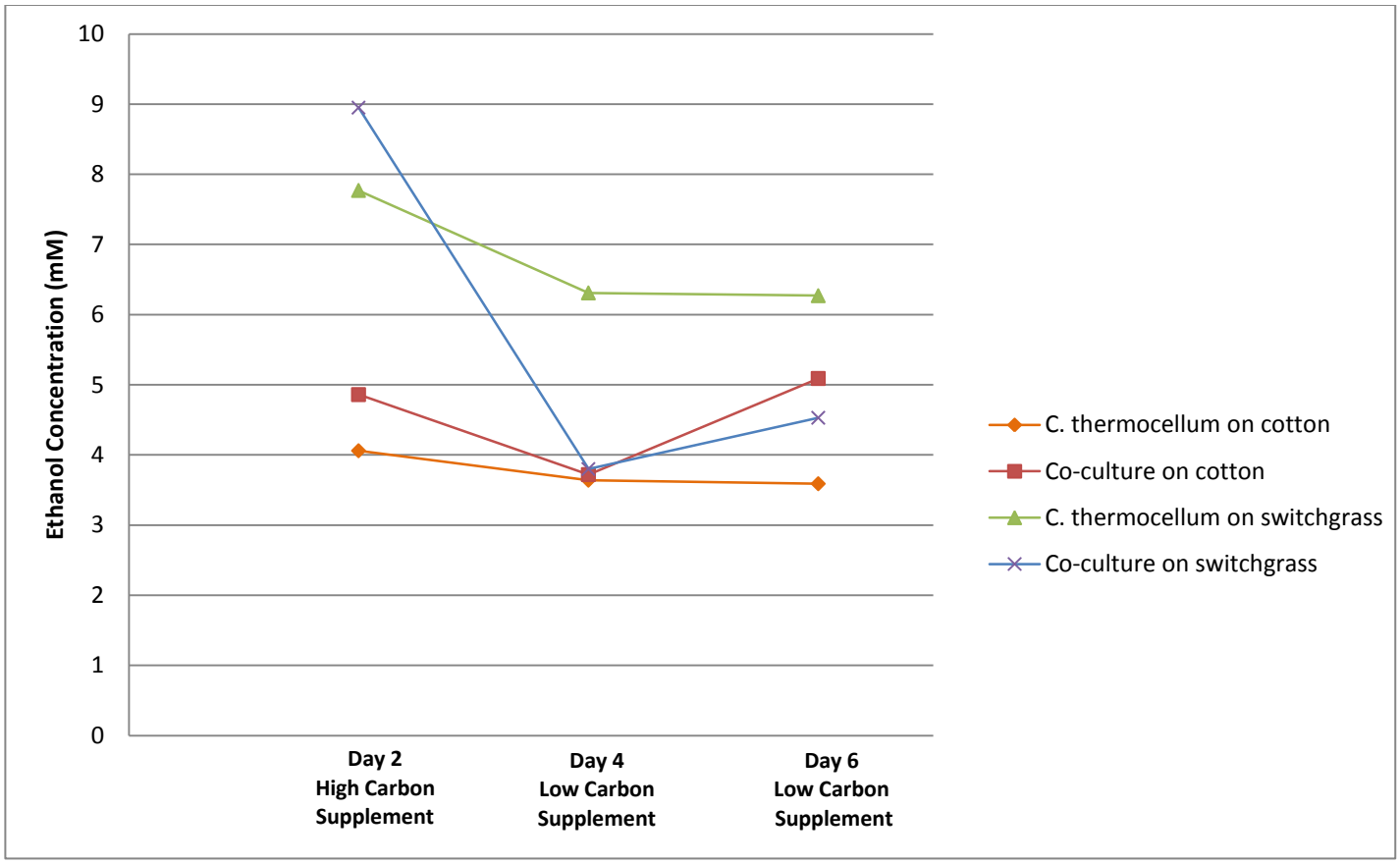

Figure 3.11: HPLC results displaying ethanol production for *C. thermocellum* ATCC 27405 monoculture on cotton and switchgrass with HCS (2g/L yeast extract and 0.3 g/L cellobiose) for first two days and LCS (0.3 g/L yeast extract with no sugar additive) for the next four days, and the same time periods and carbon sources with *T. saccharolyticum* DSM 7060 in co-culture with HCS (2g/L yeast extract, 0.3 g/L cellobiose, and 10 g/L xylose) and LCS (2g/L yeast extract with no sugar additives) in flowcell systems

Table 3.4: Peak concentrations of other end products generated from fermentation and hydrolysis processes

	Acetate (mM)	Lactate (mM)	Formate (mM)	Cellobiose (mM)	Xylose (mM)
***C. thermocellum* on cotton**					
Day 2	0.93	0.04	6.78	2.91	N/A
Day 4	ND	ND	3.31	3.50	N/A
Day 6	1.12	0.41	ND	3.26	N/A
***C. thermocellum* on switchgrass**					
Day 2	3.37	0.01	5.33	1.79	4.44
Day 4	2.81	ND	2.01	2.73	ND
Day 6	2.60	ND	2.69	ND	ND
Co-culture on cotton					
Day 2	0.53	0.03	7.80	1.58	N/A
Day 4	1.28	0.02	2.33	ND	N/A
Day 6	5.65	ND	ND	ND	N/A
Co-culture on switchgrass					
Day 2	4.83	ND	6.66	1.26	0.77
Day 4	ND	ND	2.17	ND	3.90
Day 6	ND	ND	2.09	1.01	3.79

Total solid substrate hydrolysis

The initial mass of dry cotton was 0.13g and the initial mass of switchgrass was 0.51g. The total amount of substrate remaining after experimentation for two control monoculture trials was 0.05g (~62% hydrolyzed) and 0.25g (~51 % hydrolyzed) for cotton and switchgrass respectively (Table 3.3). Co-culture experiments with *T. saccharolyticum* resulted in solid substrate residue of 0.03g (~75% hydrolyzed) and 0.21g (~59% hydrolyzed) (Table 3.3). Co-culture on cotton resulted in increased degradation by 13% and on switchgrass by 8%. Therefore, co-culture trials have suggested partial increase in both cellulosic and lignocellulosic degradation when compared to *C. thermocellum* in monoculture.

Table 3.3: Summary of the quantitative results for qPCR, HPLC, and solid carbon remaining at the end of the four individual continuous-flow experiments

	Continuous-flow experiment																	
	C. thermocellum on cotton (0.13 g dry mass)			*C. thermocellum* on switchgrass (0.51 g dry mass)			Co-culture on cotton (0.13 g dry mass)						Co-culture on switchgrass (0.51 g dry mass)					
	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6	Day 2		Day 4		Day 6		Day 2		Day 4		Day 6	
	C. thermocellum ATCC 27405			*C. thermocellum* ATCC 27405			C.t*	T.s**	C.t*	T.s**	C.t*	T.s**	C.t*	T.s**	C.t*	T.s**	C.t*	T.s**
Crossing point (C_p)	26.93	27.27	27.01	20.48	24.77	24.19	21.21	21.36	25.26	31.27	25.65	31.12	20.46	30.40	22.88	34.00	23.63	33.49
DNA concentration (µg/µL)	408	355	378	639	509.1	528.3	591.8	578.6	463	122	441.5	133.4	641	315	543	73.5	524.4	112
Ethanol Produced (mM)	4.06	3.64	3.59	7.77	6.31	6.27	4.86		3.72		5.09		8.95		3.80		4.53	
Total solid substrate remaining (g)	0.05 (~62% hydrolyzed)			0.25 (~51% hydrolyzed)			0.03 (~75% hydrolyzed)						0.21 (~59 % hydrolyzed)					

* *C. thermocellum* ATCC 27405
** *T. saccharolyticum* DSM 7060

3.4 Discussion

Cell morphology

The first experiment that was performed was epifluorescent staining (0.5 µL SYBR® Gold Nucleic Acid Stain) to observe the general morphological characteristics of *C. thermocellum* ATCC 27405 and *T. saccharolyticum* DSM 7060 in batch culture (DSMZ – Medium 122 with 2 g/L yeast extract, 5 g/L cellobiose and 5 g/L xylose) (Figure 3.4). Cellobiose was used as the main carbon source for the growth of *C. thermocellum* due to its high preference and maximum growth rates exhibited on this particular sugar (Ng and Zeikus, 1982; Zhang and Lynd, 2005). This morphology test was of great importance since it would lay down a general platform for identification and classification of *T. saccharolyticum* as cellular structure, which was yet to be described in literature. Figure 3.4 shows that *T. saccharolyticum* does not contain the typical Clostridia family rod shape, but it is roughly 3 times larger when compared to *C. thermocellum*. The morphological characteristics of *T. saccharolyticum* are more elongated and tend to interlink with other cells in batch system more than *C. thermocellum*.

In addition, a previous study performed by Lee *et al.* (1993) on various unknown strains of *Thermoanaerobacterium* spp. has revealed that one strain B6A-RI^T contains much longer rod shaped cells and once nutrients become limiting or once they reach their stationary phase, become more elongated and are more prone to aggregation. These morphological characteristics are viewed as a mechanism of survival under competitive or limiting nutrient conditions. Typical DSM Medium 122 (ingredients described in the methodology section) with limited yeast extract of 2 g/L in batch system does generate nutritionally unfavourable conditions for *T.*

saccharolyticum that mimic those same morphological indicators of B6A-RI^T mentioned in the past. However, these conditions do provide an easier identification from *C. thermocellum* which was of great value in the subsequent substrate attachment analysis.

Substrate attachment capabilities of *T. saccharolyticum*

The ability of *C. thermocellum* to bind to carbon material has been extensively researched and examined (Bayer *et al.,* 1983; Lynd e*t al.,* 2002; Wang *et al.*, 2013; Dumitrache e*t al.*, 2013). It is hypothesized that one reason for efficient *C. thermocellum* attachment to cellulosic substrates is due to it enzymatic and cell-bound complex called the cellulosome (Lynd *et al.*, 2002). However, since *T. saccharolyticum* does not contain this enzymatic complex, its attachment to carbon material was questionable. Furthermore, understanding the degree of binding for this hemicellulosic bacterium in co-culture with *C. thermocellum* can provide more insight in the colonization competition and overall spatial distribution of these two thermophiles.

Figure 3.5 shows confocal microscopy results that were obtained from four separate continuous-flow experiments where *T. saccharolyticum*'s binding ability to cellulosic and lignocellulosic (both substrates stained with Syto9 with WGA-AF633) materials were tested in monoculture and in consortium. Figure 3.5A and Figure 3.5C present *T. saccharolyticum* images on cotton and switchgrass, respectively. To note, cotton fibre was used as a cellulosic substrate for all continious-flow experiments due to its high cellulose content and its ability to pack well into a flowcell reactor. Moving on, in both examples (cotton and switchgrass) it can be noticed that this thermophile only partially binds to the carbon substrates. Figure 3.5C indicates the bacterium's accumulation in the regions between switchgrass fibres. There are few cells visible on the lignocellulosic material, however in much smaller numbers than *C. thermocellum*. Similar

results can be seen in Figure 3.5A on cotton material where the fibres are only partially colonized by *T. saccharolyticum* cells when compared to full colonization in the case of the cellulosic *C. thermocellum* (Dumitrache e*t al.*, 2013). These results show that in consortium *T. saccharolyticum* should not be of significant competition for substrate colonization and thus should not interfere with *C. thermocellum*'s ability to hydrolyze carbon sources.

C. thermocellum and *T. saccharolyticum* in co-culture on cellulosic material and lignocelluosic material can be viewed in Figure 3.5B and 3.5D, respectively. Research in the past has demonstrated that *C. thermocellum* is capable of fully colonizing cellulosic (Dumitrache *et al.*, 2013) material; however, similar research with lignocellulosic substrate is very limited in literature so far. The two confocal images show that *C. thermocellum* has likely colonized both cotton fibre and switchgrass (indicated by the orange arrow) and *T. saccharolyticum* can be seen in the background (indicated by the blue arrow). The *T. saccharolyticum* strain can be identified by its elongated shape and interlinking appearance in the empty regions of the objective field (as described in previos morphology experiment as well). These results provide significant information to the spatial distribution between the two thermophiles. Knowing that *T. saccharolyticum* does not significantly interfere with the actions of hydrolyzing *C. thermocellum*, it can be proposed that *T. saccharolyticum* will likely utilize the metabolites produced by *C. thermocellum* for subsequent fermentation.

These results support the suggested role of cellulose-binding modules within the cellulosome complex in *C. thermocellum* and its role in forming cellulose-enzyme-microbe (CEM) complex. The cellulosome complex is indeed vital for the adherence of this thermophile to cellulosic and lignocellulosic material. Further research on cellular signalling and CEM

interaction with cellulose itself needs to be performed in order to completely understand microbe attachment to carbon substrates.

T. saccharolyticum DSM 7060 physiology test

Due to the lack of published research on DSM 7060 strain, it was an objective to perform experiments to assess the extent of carbon metabolism and sugar utilization preferences for this hemicellulolytic thermophile. Figure 3.6 shows the results of three separate batch system experiments on 5 g/L xylose and 5 g/L cellobiose (with 2 g/L yeast extract) as the main carbon sources, in both monoculture and co-culture with *C. thermocellum*. These findings provided a further look into variability of *Thermoanaerobacterium* spp. ethanol yield on different sugar substrates. In monoculture DSM 7060 strain produced 3.6 mM of ethanol, compared to literature where 0.46 mM of ethanol was generated in mutant strain *T. saccharolyticum* JW/SL-YS485 ALKI on the same sugar supplement (Bardsley *et al.*, 2008), 1.35 mM of ethanol in *T. saccharolyticum* HKO7 (Δ *hfs* Δ*ldh*) on 10 g/L glucose (Shaw *et al.*, 2009), and 5.5 mM of ethanol in *Thermoanaerobacterium* strain DSM 18780 inoculated with 10 g/L cellobiose (Almarsdottir *et al.*, 2011).

In addition to presenting the degree of ethanol yield in DSM 7060 strain, Figure 3.6 also shows whether or not carbon catabolite repression (see section 2.11) plays any significant role in co-culture with the hexose-only fermenting bacterium, *C. thermocellum*. As mentioned earlier, a microorganism preferring one type of sugar to the other can become an issue of competition in particular co-culture (Görke and Stülke, 2008). However, physiology test of DSM 7060 strain hint that carbon catabolite repression does not play a significant role in a medium with both hexose and pentose sugars since this strain is capable of fermenting both sugars simultaneously.

In other words, it is able to co-utilize all sugars without serious competition with *C. thermocellum.* Therefore, *T. saccharolyticum* DSM 7060 is a valuable co-culture partner for *C. thermocellum* in increasing ethanol yield. This increase can be seen in Figure 3.6 co-culture batch experiment with an overall ethanol production of 4.89 mM, an improvement in yield by approximately 23.2% compared to *C. thermocellum* monoculture (ethanol yield of 3.97 mM). Last, it should be noted that due to a pH drop from ~6.5 to 6.13 in co-culture (caused by the production of acid by-products), it is likely that *C. thermocellum* levels have dropped which might explain the high concentrations of cellobiose (6.59 mM) in the batch system. The latter issue is not present with continuous-flow systems, due to constant culture media replenishment at a dilution rate of ~10 mL/hour.

Competition vs. cooperation interaction: Real-time PCR analysis

Quantitative PCR results of continuous-flow experiments can be seen in Figure 3.7 (*C. thermocellum* on cotton), Figure 3.8 (*C. thermocellum* on switchgrass), Figure 3.9 (Co-culture on cotton), and Figure 3.10 (Co-culture on switchgrass). This experimental analysis was performed to describe the degree of competition vs. cooperation between the cellulolytic and hemicellulolytic bacteria. Due to the use of minimal yeast extract at day 4 and day 6, this experiment was to test whether or not *T. saccharolyticum* is truly capable of utilizing *C. thermocellum* metabolites, and as a result generate more ethanol.

Yeast extract is a complex nutrient source that includes carbon, vitamins, nitrogen, and various amino acids that are often used to cultivate different cellulolytic and ethanologenic microorganisms (Ronan, 2011). However, due to the high cost and extraneous steps used to produce yeast extract, its use in consolidated bioprocessing industry will be uneconomical

(Maddipati *et al.*, 2011). Thus, one of the goals to improving consolidated bioprocessing is to progress away from yeast supplemented culture media (Lynd *et al.*, 2005).

Specific primer and probe construction of each strain used has enabled the development of PCR analysis in real-time to quantify DNA concentrations of one or both strains over a course of 6 days (sampled every two days). For all four experiments, the idea was to obtain a successful growth for the first two days with high carbon supplement (HCS) medium (high amount of yeast extract and sugar additives) and then switch the medium to low carbon supplement (LCS) medium (minimum yeast extract required for growth and no sugar additives) for the next four days. Minimum yeast extract of 0.3 g/L was used for LCS medium since research in the past has demonstrated that is the minimal amount required for sufficient *C. thermocellum* growth.

The continuous-flow experiment where *C. thermocellum* monoculture was inoculated in a flowcell reactor filled with 0.13 grams of dry commercial cotton (Figure 3.7) shows that qPCR results for day 2, HCS medium, contains highest amount of *C. thermocellum* with a DNA concentration of 408 ng/µL. Once the medium was switched to LCS, this DNA concentration value dropped to 355 ng/µL at day 4, which is a decrease of ~13.0%. It is clear that high yeast extract and sugar supplement does play an important part in sustaining *C. thermocellum* numbers. The amount of high carbon supplement being introduced into the reactor was greater than the amount of sugar being hydrolyzed and metabolized by *C. thermocellum*. However, tracking DNA concentration for the next two days shows a steady increase in *C. thermocellum* numbers at day 6 with a value of 378 ng/µL and an increase of ~6.5% from day 4. This steady increase hints that gradual *C. thermocellum* recovery is occurring and that this cellulolytic strain is perfectly capable of surviving on cotton fibre solubilisation alone, with little yeast extract and

no sugar supplement necessary. This means that after four days under LCS medium, *C. thermocellum* eventually starts to utilize cotton fibres at a rate that helps it flourish in numbers.

The second continuous-flow experiment was designed to grow *C. thermocellum* on 0.51 grams of unpretreated midseason lignocellulosic switchgrass (Figure 3.8). The flow reactor exposed to the medium with HCS for the first two days generated *C. thermocellum* DNA concentrations of 639 ng/µL. This is a significant increase to the *C. thermocellum* numbers for the first two days on cotton. This may be due to the higher amount of carbon material present in this experiment ~0.19 grams of digestible cellulose present since switchgrass is composed of ~37% cellulosic material (Kumar *et al.*, 2011). Also, past research has shown that switchgrass hydrolysis is known to generate 4 times less cellobiose than hydrolysis of pure cellulose (Basen *et al.*, 2014).This particular disaccharide has been known to inhibit cellulase activity and thus slow down the rate of cellulose breakdown (Johnson e*t al.*, 1982; Gruno *et al.*, 2004; Teugjas and Väljamäe, 2013). More specifically, it is the exoglucanase (Cel S) and endoglucanse cellulase activity that is constrained by the presence of cellobiose (Demain *et al.*, 2005). According to the research performed by Zhao *et al.* (2014), it is suggested that steric hindrance is created between cellobiose and tryptophan residue that is located near the active site of cellobiohydrolase, which blocks cellulose fibre from binding to active site of cellulase. Thus, microbe abundance is known to increase under low cellobiose concentrations. Furthermore, much like in day 4 of the cotton results, after the medium has been switched to LCS, the DNA concentration of *C. thermocellum* dropped down by ~20.3% to 509 ng/µL. Although, unlike the results seen in experiment one, day 6 results showed a smaller increase in DNA concentration of ~3.8% with 528.3 ng/µL. One explanation for such outcome may be due to the more robust nature of lignocellulosic material (see chapter 2). Since lignin is considered harder to digest due to the strong polymer bonds that

keep it in place, cellulosomes have a tougher time breaking them apart (Sanderson, 2011). In addition, due to a lowered carbon supplement, energy for the recovery in numbers is also diminished.

The third continuous-flow experiment included both *C. thermocellum* and *T. saccharolyticum* in co-culture on 0.13 grams of dry commercial cotton (Figure 3.9). The *C. thermocellum* DNA concentration after two days of HCS medium resulted in 591.8 ng/µL and *T. saccharolyticum* DNA concentration of 578.6 ng/µL. There is an abundant increase in DNA concentration when comparing co-culture concentrations to *C. thermocellum* monoculture at day 2. The reason for this increase in numbers seems somewhat unclear; however, one possible explanation may be due to lowering of inhibitory effects caused by cellobiose produced via hydrolysis of cotton (Table 3.4). The cellobiose concentration in *C. thermocellum* monoculture on cotton was 2.91 mM and in co-culture on cotton was 1.58 mM. Thus, the presence of *T. saccharolyticum* may have added to the fermentation of cellobiose and thus faster elimination from the system. Day 4 LCS medium results for experiment 3 co-culture on cotton, showed a drop in numbers for both *C. thermocellum* and *T. saccharolyticum*, with concentrations of 463 ng/µL (decrease of 21.8%) and 122 ng/µL (decrease of 78.9%) respectively. A greater drop in *C. thermocellum*'s DNA concentration in co-culture compared to monoculture on cotton after the medium switch may be due to less efficient attachment on cotton. Figure 3.5 shows *T. saccharolyticum* is capable of partially colonizing cellulosic material. This partial colonization may have interfered with *C. thermocellum*'s ability to recover.

There are different potential explanations for the drastic drop in *T. saccharolyticum* numbers. First, *T. saccharolyticum* is dependent on *C. thermocellum*'s metabolism in LCS

medium and thus a drop in *C. thermocellum* numbers would result in a drop in *T. saccharolyticum*'s numbers as well. Second, the physiology test in Figure 3.6 has shown that *T. saccharolyticum* prefers xylose slightly over cellobiose and thus the switch from 10 g/L xylose to 0 g/L xylose may have caused a nutritional strain to this hemicellulolytic bacterium. Day 6 concentrations resulted in a slight dip in numbers of *C. thermocellum* with a DNA concentration of 441.5 ng/µL (decrease of ~4.6% from day 4) and *T. saccharolyticum* increase in abundance to 133.4 ng/µL (increase of ~9.3% from day 4).

The continual decrease in *C. thermocellum* numbers by day 6 might have been caused by a minor carbon competition during nutritional limitation. In low carbon environments, *C. thermocellum* is producing carbon source for both itself and its *T. saccharolyticum* partner; however, due to *T. saccharolyticum*'s faster metabolic rate (maximum specific growth rate of 0.37 h^{-1}), it will tend to ferment more sugars produced than *C. thermocellum* does (Shaw *et al.*, 2008). However, *C. thermocellum* should eventually reach a stable growth rate and the numbers will start to increase in co-culture as well. The fact that *T. saccharolyticum* DNA concentration has increased by approximately 9.3% shows that this strain is capable of successfully utilizing *C. thermocellum*'s metabolites as the only carbon source.

The last continuous-flow experiment was a co-culture on 0.51 grams of switchgrass as the carbon feedstock (Figure 3.10). Here it can be seen that DNA concentrations of *C. thermocellum* and *T. saccharolyticum* under HCS medium at day 2 is 641 ng/µL and 315 ng/µL, respectively. The numbers of *C. thermocellum* are very similar to the ones in monoculture on switchgrass (639 ng/µL), hinting no significant change. The abundance of *T. saccharolyticum* was lower than in day 2 of experiment 3 on cotton (578.6 ng/µL), possibly due to longer time for

C. thermocellum to break the lignin structure apart and expose hemicellulosic material and digestible cellulose. Furthermore, once LCS was introduced, DNA concentrations of both strains have decreased to 543 ng/µL (drop of 15.3%) in *C. thermocellum* and 73.5 ng/µL (drop of 77%) in *T. saccharolyticum* at day 4. The latter concentrations mimic those seen in day 4 in experiment 3 co-culture on cotton.

Last, in day 6 LCS medium *C. thermocellum* results appeared similar to those in experiment 3. The abundance of *C. thermocellum* DNA dropped only slightly to 524.4 ng/µL (decrease of 3.4% from day 4). On the other hand, *T. saccharolyticum* DNA concentrations increased by a surprisingly 52% from day 4 to 112 ng/µL at day 6. These findings suggest very efficient co-culture cooperation due to the fact that *T. saccharolyticum* has drastically increased in numbers over sugars produced by cellulolytic metabolism of *C. thermocellum*. The rapid increase in concentration of *T. saccharolyticum* DNA on switchgrass can be explained by the fast growth of this bacterium and introduction of pentose sugars from degradation of lignocellulosic switchgrass. Once more pentose sugars are available, *T. saccharolyticum* will lower its competition with *C. thermocellum* for hexose sugars. In addition, DNA concentrations of *C. thermocellum* should eventually increase over time because of this factor as well.

Solid substrate hydrolysis and end product analysis

The final bioethanol yield and other by-products of fermentation and hydrolysis can be examined in Figure 3.11, Table 3.3, and Table 3.4. Samples that have been collected from the four previously mentioned continuous-flow experiments for qPCR analysis were also used for HPLC investigation. For experiment 1 - *C. thermocellum* on cotton, ethanol productions at day 2 (HCS), day 4 (LCS), and day 6 (LCS) resulted in: 4.06 mM, 3.64 mM, and 3.59 mM,

respectively. These findings have confirmed those of qPCR in Figure 3.7 since they follow the similar pattern with DNA concentration values. Due to high number of carbon supplement at day 2, metabolic activity of this cellulolytic thermophile is at its peak and has produced highest amount of ethanol. Trace amounts of acetate and lactate were also present at day 2 and day 6. Day 2 has generated a surprising amount of formate (6.78 mM), which was uncommon in literature for *C. thermocellum* to produce formate at such high concentration on cellulosic materials (Patni and Alexander, 1971; Biswas *et al.*, 2014). According to Sparling *et al.* (2006), the presence of *pfl, fnr,* and *adhE* genes in C. *thermocellum* ATCC 27405 strongly suggests that this bacterium is capable of utilizing catabolic pathway which involves pyruvate to acetyl-CoA + formate (Figure 2.1) and thus fluctuate in its formate production depending on environmental conditions. Once the pH drops below 6.5, formate producing gene, *pfl*, gets activated in *C. thermocellum* ATCC 27405 (Sparling *et al.*, 2006).

Cellobiose concentrations have also varied from day 2 (2.91 mM), day 4 (3.50 mM), and day 6 (3.26 mM). These values indicate a slight drop in cellobiose fermentation after the HCS-to-LCS medium switch, which gradually picks up at day 6. Last, when it comes to the extent of solid substrate hydrolysis, experiment 1 has demonstrated that *C. thermocellum* was capable of degrading roughly 62% of the original solid cellulosic material over the 6-day incubation.

Experiment 2 – *C. thermocellum* monoculture on switchgrass has generated similar end product results to experiment 1 with few differences. In general, ethanol yield was greater on switchgrass over the course of six days: day 2 (7.77 mM), day 4 (6.31 mM), and day 6 (6.27 mM). These numbers do follow similar DNA concentration pattern over the course of the experiment. Ethanol concentrations remained relatively constant from day 4 to day 6 due to

similar DNA concentrations at these days, indicating metabolic activity did not drastically change. Ethanol to acetate ratios remained constant at 1:2.3 on average for all 3 samples with minimum lactate production, which compares to research performed in the past on lignocellulosic biomass (Yee *et al*., 2014). Research performed by Yee *et al*. (2014) also confirmed lower formate production on transgenic unwashed switchgrass feedstock. Furthermore, xylose concentrations of 4.44 mM were only detected at day 2. Solid substrate hydrolysis has shown that *C. thermocellum* was able to degrade approximately 51% of the original switchgrass mass. Other research with Clostridia class bacteria have shown similar degradation rates on various other lignocellulosic substrates such as unpretreated, unwashed cornstalk (56% mass loss), corn cob (63% mass loss), and wheat straw (59% mass loss) (Cao *et al.*, 2014).

Experiment 3 – Co-culture on cotton have produced many different end products compared to monoculture. The increased DNA concentration of *C. thermocellum* with the addition of *T. saccharolyticum* has generated more ethanol yield in all 3 samples than *C. thermocellum* alone: day 2 (4.86 mM), day 4 (3.72 mM), and day 6 (5.09 mM). Cellobiose concentration at day 2 (1.58 mM) was ~54.3% lower than in monoculture (2.91 mM), which demonstrates co-culture with *T. saccharolyticum* enhanced fermentation of this sugar. Day 4 DNA concentrations showed a significant decrease in numbers for both *C. thermocellum* (21.8 % decrease) and *T. saccharolyticum* (78.1 % decrease) which help explain why there is virtually no cellobiose present at day 4 LCS medium because whatever little sugar has been hydrolyzed by *C. thermocellum* was instantaneously utilized by both strains due to increased partial competition for this sugar. Ethanol concentration dropped as well by ~23.5%. However, due to the higher *T. saccharolyticum* numbers at day 6, ethanol concentrations spiked by 35% (5.09 mM) exceeding

the original 4.86 mM from day 2. The latter results show that the hemicellulolytic *T. saccharolyticum* is capable of utilizing cellulolytic *C. thermocellum*'s metabolites and as a result raise ethanol production levels. Since there is no evidence that the genome of *T. saccharolyticum* contains a gene encoding for formate dehydrogenase and thus inability to produce formate as described by Shaw *et al.* (2008a), the levels of this end product have remained fairly unchanged compared to monoculture. Finally, comparing solid substrate hydrolysis with monoculture in experiment 1, it was demonstrated that there was a 13% increase in degradation of cotton once *T. saccharolyticum* was added. These results can be explained, once again, due to the increased abundance of *C. thermocellum* throughout the whole 6 day experimentation period.

Experiment 4 – Co-culture on switchgrass ethanol production is: day 2 (8.95 mM), day 4 (3.80 mM), and day 6 (4.53 mM). Compared to monoculture on switchgrass, ethanol yield followed a similar pattern with a drop following HCS-to-LCS medium switch; however, at day 6 there was a significant increase in ethanol (by 16.1%) in co-culture. As seen in co-culture on cotton, there was a notable increase in *T. saccharolyticum* DNA concentration (increase by 53%), which resulted in a more rapid fermentation and ethanol recovery overall. Lactate levels remained minimal and formate concentration showed similar fluctuations as seen in monoculture on switchgrass. However, due to the presence of xylose (generated from *C. thermocellum*'s hydrolysis of lignocellulose) there was less competition for sugar between the two thermophiles and hence there was more cellobiose present in the day 6 sample. Moreover, hydrolysis of solid switchgrass by monoculture and co-culture mimic those on cotton in a sense that there is more degradation recorded with co-culture (8% increase). Relative DNA concentration of *C. thermocellum* was higher in co-culture than monoculture, which can be expected to lead to increased hydrolysis.

Concluding remarks

Naturally occurring microbial ecosystems, changed and revised constantly through evolution, are almost exclusively composed of communities or (also known as) consortia (Bernstein and Carlson, 2012). These mixed communities can be found in a wide range of environmental conditions and can metabolize various complex organic substrates that, as a result, convert these substrates into useful forms of energy. A common consortial interaction strategy uses cooperative measures through division of labour (Crespi, 2001; Briones and Raskin, 2003). This division of labour permits lateral or sequential processing of substrates and can generally be considered as enhancing productivity and nutrient (carbon) recycling (Bernstein and Carlson, 2012). The carbon flow through the biosphere serves as an energy transfer between community members in a non-competitive manner that is established on metabolic functionality (Bernstein and Carlson, 2012). This template enables parallel processing of carbon substrates and can in turn be used to develop a consortium that is capable of simultaneously fermenting various types of sugars.

In principle, it is possible to take advantage of these microbial consortia and apply their metabolic capabilities to some of the problems relating to urban, agricultural, and industrial waste. Landfill space to manage this waste is become evermore limiting and expensive (Demain *et al.*, 2005). Thus a potential solution to this problem is the microbial conversion of biomass into alternative energy source such as ethanol. This study has shown that using cellulolytic *C. thermocellum* in co-culture with hemicellulolytic *T. saccharolyticum*, with low carbon supplement, can increase the levels of carbon substrate hydrolysis and ethanol production as a result. Furthermore, the lack of *T. saccharolyticum*'s substrate attachment and the lack of

metabolic competition between the two thermophiles suggest this co-culture can be a valuable consortium for improving second generation biofuel production.

In conclusion, a remaining challenge is that the natural processes found in microbial ecosystems are typically slow compared to those required in industrial bioprocesses, and therefore further research is necessary to eventually have improved control over these processes but at a much faster and efficient rate.

CHAPTER 4: OVERALL CONCLUSIONS AND FUTURE WORK

Due to a finite nature of fossil fuels, there is a growing demand for a more sustainable energy source alternative. Bio-ethanol production from CBP technology has been presented as a viable and more environmentally friendly solution. However, efforts in improving this biotechnology and hence increase levels of bioethanol production are still necessary to maximize its economical effectiveness.

The results of this study suggested that the metabolic relationship between *C. thermocellum* and *T. saccharolyticum* can contribute to higher ethanol yield and the dynamics of their interaction can be used to further enhance CBP technology. Revisiting the objectives; cell morphology has shown that *T. saccharolyticum* DSM 7060 is generally 3 times larger than *C. thermocellum* and due to this visible morphological difference assessment of monoculture and co-culture physical substrate attachment was possible. The hemicellulolytic organism has shown poor adherence to both cellulosic and lignocellulosic substrates and thus the majority of its sugar utilization does not necessarily depend on direct (physical) contact with the carbon structures. The lack of interaction between *T. saccharolyticum* and carbon material does not influence hydrolysis rates and does confirm cellulosome-directed binding in the case of cellulolytic microbes such as *C. thermocellum*. In addition, *C. thermocellum* is capable of attaching to lignocellulosic material such as switchgrass in a similar fashion as to cellulosic cotton substrates. The relative numbers of the two strains in co-culture when cultivated on cotton or switchgrass suggest that *T. saccharolyticum* is able to utilize sugar produced from *C. thermocellum*'s hydrolysis and eventually increase the production of ethanol and, potentially, hydrolysis of carbon material (due to increased fermentation of inhibiting cellobiose). Hydrolysis rates of both

cellulosic and lignocellulosic material were increased in co-culture experiments. Overall, these results revealed the potential, and need for future work to optimize lignocellulose-to-ethanol conversions. Topics that should be considered for future research include the following:

Co-culture research involving other lignocellulosic substrates

It is also important to analyze how *C.thermocellum* and *T. saccharolyticum* interact on other forms of lignocellulosic sources. The results obtained from switchgrass can be used to test the limits of bio-ethanol production and cellulosic hydrolysis on other promising substrates such as corn stover, *Miscanthus* grass species, wood chips, and municipal waste. For example, *Miscanthus giganthus* has shown average annual harvestable yields of 45 tonnes per hectare, compared to annual yield of 19.8 tonnes per hectare for switchgrass (Heaton *et al.*, 2008). Thus, it would be interesting to see the comparison between switchgrass and *M. giganthus* with respect to cellulose utilization and bio-ethanol production.

Genetically modified switchgrass

Research performed by Shen *et al.* (2013) has demonstrated that overexpression of transcription factor PvMYB4 can reduce carbon deposition into lignin and increase levels of anaerobic thermophilic ethanol production by 2.6-fold. In addition, a study by Yee *et al.* (2014) has shown that downregulation of the caffeic acid O-methyl transferase (COMT) gene reduced lignin content and increased the S/G ratio. Using *C. thermocellum* in their experiments, this group showed a 90% increase in ethanol yield compared to the wild-type switchgrass. Hence, future research on genetically modified switchgrass with *C. thermocellum* and *T. saccharolyticum* in co-culture may reveal interesting new possibilities.

Lignin utilization

A recent study by Beauchet *et al.* (2012) showed that it is possible to convert 10 to 20% of lignin by weight into value added products such as catechol, guaiacol, and other phenolic compounds used for bioplastic production. In addition, it was also shown that it is possible to convert part of lignin into a desirable hydrocarbon fuel such as Jet A-type fuels used in the aviation industry (Shabtai *et al.,* 1998). Therefore, further analysing the availability of switchgrass's lignin conversion into desirable end products may lead to more financially feasible processes.

Bio-hydrogen production

Previous studies have shown that co-culture research has a great potential to increase the production of bio-hydrogen (Weimer and Zeikus, 1977; Liu *et al.,* 2008; Geng *et al.,* 2010). The study performed by Liu and colleagues showed that *C. thermocellum* JN4 strain and *Thermoanaerobacterium thermosaccharolyticum* GD17 strain, when placed in co-culture on microcrystalline cellulose, caused bio-hydrogen production to increases about 2-fold (from 0.8mol H_2 to 1.8mol H_2) (Liu *et al.,* 2008). Understanding the nature of this interaction can aid in developing more effective methods to further increase the levels of bio-hydrogen yield.

Solid, liquid, and gas phase carbon utilization in co-culture

The use of *C. thermocellum* and *T. saccharolyticum* in this study has provided insight on the extent of solid substrate hydrolysis on both cellulose and lignocellulose material. Future research can be open to analyzing the total metabolic activity, including liquid and gas phase carbon. This study would help contribute to determining how much carbon is actually being utilized and provide further insight into the extent of carbon balance analysis in consortium.

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