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INVESTIGATION OF PHANEROCHAETE CHRYSOSPORIUM AND CLOSTRIDIUM THERMOCELLUM FOR IMPROVED SACCHARIFICATION OF LIGNOCELLULOSE UNDER NONSTERILE CONDITIONS

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INVESTIGATION OF *Phanerochaete chrysosporium* AND *Clostridium thermocellum* FOR IMPROVED SACCHARIFICATION OF LIGNOCELLULOSE UNDER NONSTERILE CONDITIONS

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biosystems and Agricultural Engineering in the College of Engineering at the University of Kentucky

By

William Evan Simon

Lexington, Kentucky

Director: Dr. Sue Nokes, Professor and Chair, Biosystems & Agricultural Engineering

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2015

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INVESTIGATION OF PHANEROCHAETE CHRYSOSPORIUM AND CLOSTRIDIUM THERMOCELLUM FOR IMPROVED SACCHARIFICATION OF LIGNOCELLULOSE UNDER NONSTERILE CONDITIONS

Current research efforts are directed at developing competitive processes that can utilize lignocellulose as a feedstock for biorefineries. The purpose of this study was to investigate methods of processing lignocellulosic material so that its monosaccharides can be more easily accessed for fermentation, the lack of which is hindering the economics and wide-scale adoption of lignocellulosic biorefining. The monosaccharides are of interest because they can be used by Clostridium beijerinckii downstream of P. chrysosporium and C. thermocellum in a sequential bioprocess to produce butanol. Butanol is an attractive biofuel because it can be utilized without modifying current transportation infrastructure. Butanol is also used as a starting material in organic synthesis.

In the first study, the potential for C. thermocellum’s (ATCC 27405) cellulase system to operate outside its optimal temperature range in a high-solids environment was assessed by quantification of the fermentation products lactate, acetate, and ethanol and by quantification of xylose, glucose, and cellobiose remaining.

Additionally, the lignin degrading white-rot fungus Phanerochaete chrysosporium RP 78 was investigated as a potential pretreatment for lignocellulose. Elevated temperatures required for Clostridium thermocellum fermentation were examined as a means to improve poor competitiveness that is characteristic of P. chrysosporium on unsterile corn stover substrate.

KEYWORDS: Phanerochaete chrysosporium, lignocellulosic bioprocessing, Clostridium thermocellum, biological pretreatment, 2nd generation biofuels.

William E. Simon

July 20, 2015
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July 20, 2015
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Chapter 1: *C. thermocellum* ATCC 27405 fermentation activity profile

Quantification of fermentation products and sugar accumulation by *Clostridium thermocellum* ATCC 27405 under varying solids concentrations, temperatures, and durations of the fermentation

Abstract

The thermophilic anaerobe and potential bioprocessing bacterium, *Clostridium thermocellum*, has been studied extensively since its discovery in the 1970’s because of its high level of cellulolytic activity. Through continued study of *C. thermocellum*’s genomic, transcriptomic, proteomic, and metabolomic responses to varying biomass sources, it is believed *C. thermocellum* can be used to process lignocellulosic biomass on an industrial scale [1, 2]. The effect of varying cultivation temperature and lignocellulosic solids concentration on the metabolic products produced by *C. thermocellum* has yet to be studied extensively. In this study, solids concentrations of 5%, 10%, and 15% were examined. For culture temperature, levels of 20 °C, 30 °C, 40 °C, 50 °C, and 63 °C were considered and fermentation liquids were sampled at 24, 48, and 72 hours after inoculation with *C. thermocellum* ATCC 27405. *C. thermocellum*’s optimal cellulase activity has been shown to occur near 60 °C [3], however no temperature response curve exists in the literature for its cellulase activity. The primary goal of this investigation was to obtain comprehensive fermentation data for *C. thermocellum* functioning in different environments for use in optimizing a bioprocessing system seeking to convert lignocellulosic biomass to butanol. C5 and C6 sugars can be utilized by the butanol producer *C. beijerinckii* [4], which is envisioned to be downstream of *C. thermocellum* in a liquid energy carrier production process. It is hypothesized that *C. thermocellum* could potentially function at an "acceptable level" in terms of economic competitiveness for a bioprocessing system under conditions that would normally be considered sub-optimal for *C. thermocellum* functioning.
in monoculture, which could mean energy savings and therefore cost reduction for a
conversion scheme. Cellulosomal products cellobiose, glucose, and xylose, as well as the
metabolic products lactate, acetate, formate, and ethanol were quantified using HPLC. An
ANOVA was used to assess whether a difference existed between treatment means based on
maximum total carbon recovered from each treatment (regardless of time of occurrence),
which was calculated as the carbon weighted \( \frac{MW_{\text{carbon}}}{MW_{\text{total}}} \) sum of the carbon molecules in
the seven products listed above. Mean total carbon recovered in products of the
cellulosome and metabolism was greatest at 10\%_63 \^\circ C and 5\%_63 \^\circ C for corn stover and
switchgrass, respectively.
1.1 Introduction

1.1.1 Lignocellulose

Interest in biofuels production has experienced a resurgence in recent years due to the rising difficulty of maintaining petroleum production rates. Much of the world is dependent on liquid fuels to power transport, so producing liquid fuels could be viewed as mandatory, considering the previous investment in infrastructure. Conversion of biomass to biofuels represents a process that can displace petroleum fuels, and can be done by thermochemical methods or through bioprocessing [5]. One school of thought views bioconversion as having greater potential than thermochemical methods because it requires smaller energy inputs and capital investment [6].

Biochemical conversion involved producing liquid biofuels from the microbial metabolism of cellulose and hemicellulose, two of the most abundant biopolymers on Earth. While still under development, second generation biofuels (lignocellulosic) are of interest because they utilize feedstocks which are not a source of food for humans, unlike first generation biofuels.

Most dry plant matter is composed of lignocellulose and is one of the most abundant materials on earth, making it an object of interest for bio-resource utilization. The sugar polymers cellulose and hemicellulose, and the phenolic polymer lignin, are the 3 different components which together make up lignocellulose (see figure 1.1). Cellulose is composed of 6 carbon glucose molecules joined by β 1-4 glycosidic linkages. The hydrogen bonding that occurs between cellulose chains is also a significant factor in the resistance of crystalline cellulose to depolymerization. Hemicellulose, the second most prevalent polymer in lignocellulose, is made up of 5 and 6 carbon sugars which include arabinose, mannose, glucose, xylose, and galactose. Hemicellulose gives the entire cellulose-
hemicellulose-lignin network more rigidity as well as serving as a connector between cellulose and lignin fibers. The third component, lignin, is a water insoluble polymer constructed from three different phenolic alcohols: coniferyl alcohol, sinapyl alcohol, and p-coumaryl alcohol. There is a variation in the ratio of these alcohols in the lignin polymer among different types of plants [7].

The structure of lignocellulosic material can be understood as being arranged into multiple levels of structure: microfibrils are composed of the cellulose, hemicellulose, and lignin polymers and these microfibrils are arranged to form macrofibrils which provide stability to the plant cell wall. In addition to its structural role, lignin also protects plant structures from microbial attack, blocking access to the sugar polymers that can be degraded and used in cellular metabolism. Lignin is said to be a major contributor to "biomass recalcitrance", defined as the resistance cell walls have towards enzymatic and microbial attack. It is this property of plants that is largely responsible for the high cost of lignocellulose conversion. Due to lignocellulose being protected by hemicellulose and lignin, only 20% of its theoretical maximum yield can be obtained from enzymatic hydrolysis without appropriate pretreatment [8]. Thus, lignin modification/removal is a major focus of lignocellulosic processing research [9].

In addition to lignin content, other factors which limit the biodegradability of lignocellulose include crystallinity of cellulose and available surface area [3]. Pretreatment is a critical step in lignocellulose processing and is aimed at affecting one or more of the aforementioned factors. A pretreatment process can be chemical or physical in nature, or a combination of the two. The goal of pretreatment is currently believed to be increasing the surface area of cellulose by 1) removing the lignin seal, 2) solubilizing hemicellulose, 3) disrupting crystallinity, and/or 4) increasing pore volume [10]. The choice of pretreatment is important not only because of the cost but because of the effect it will have on all other
downstream processing operations. A pretreatment can be considered ideal if it avoids the need for size reduction, preserves hemicellulose fractions, is low in cost, minimizes inhibitor production, and requires minimal energy inputs. Recovery of co-products (lignin and hemicellulose) may also need to occur in order to make a process economically feasible [11].

In one particular study [4], corn fiber was treated with dilute H$_2$SO$_4$ and the resulting hydrolyzate was fermented using C. beijerinckii. The study examined adding various levels of inhibitor compounds at the start of each fermentation and examining the effect on ABE solvent production. Two of the acid inhibitor compounds resulted in 10% ABE yield when compared to the control, with inhibitor levels at <0.5 g/L. Inhibitors were selected based on a list of compounds which are known to be generated during acid hydrolysis of biomass. The significant effect these inhibitors, which are generated from various pretreatments, have on C. beijerinckii illustrates why it is important to consider downstream processes when choosing a pretreatment.
1.1.2 *C. thermocellum*

*Clostridium thermocellum*, a thermophilic, anaerobic gram-positive bacterium, has the highest known rate of cellulose utilization of any bacterium [13]. Because of this fact, it is hypothesized that *C. thermocellum* can be used effectively in a bioprocessing system which transforms biomass into biofuels.

*C. thermocellum* has the ability to hydrolyze crystalline cellulose through the utilization of a large membrane bound protein complex known as the cellulosome. The cellulosome itself is composed of one or more scaffoldin proteins called CipA (see figure 1.2), which have the ability to bind multiple catalytic subunits along its length. These catalytic subunits include endoglucanases, cellobiohydrolases, and xylanases which work together towards attacking insoluble lignocellulosic substrates. Catalytic subunits possess a cohesin protein module which binds to a dockerin 1 module on the CipA protein. A plug and socket mechanism is an analogy that has been used to describe cohesin-dockerin interactions, the strength of which rivals those of high affinity antigen-antibody interactions. A different isoform of the dockerin (dockerin 2) module tethers each CipA molecule to the cell by binding to a cohesin 2 domain. The CipA protein also possesses a carbohydrate binding domain (CBD) which keeps each CipA molecule attached to substrate [3, 14].
Cellulose is hydrolyzed into cellobiose and cellodextrins by the cellulosome with the intent that these molecules will be transported into the cell and used in metabolism by *C. thermocellum*. The primary products of this metabolism are lactate, acetate, and ethanol. The yield ratios of fermentation products is dependent on many properties of the fermentation, including substrate [15]. *C. thermocellum*’s ability to produce ethanol directly from cellulose would lead one to believe *C. thermocellum* could be used alone in a biomass to biofuels process, however the rate of production of ethanol is quite low compared to yeast [3] and the overall process is inefficient due to accessory acid production (lactate and acetate, see **figure 1.3**). *C. thermocellum* also lacks the ability to metabolize pentose sugars, which are released through the catabolic activity of its cellulosomes.

**Figure 1.2.** Simplified Cellulosome protein architecture. (from Raman, B. [14])
Of the major catalytic subunits produced by *C. thermocellum*, the cellulase CelS is the only exoglucanase produced. *C. thermocellum* has one of the most studied cellulosomal subunits as well as the first subunit to be studied at the transcriptional level [14, 17]. According to Dror et al. [17], CelS is growth rate dependent under conditions of cellobiose and/or nitrogen limitation. Maximum levels of CelS expression were achieved by raising the dilution rate to a threshold value, at which time the level of CelS transcription did not increase further. In the treatments subjected to nitrogen limitation, cellobiose was in excess and CelS transcript levels were still determined by the dilution rate, indicating the importance of growth rate in CelS regulation as opposed to cellobiose availability. Elevated growth rates would tend to be supported by elevated cellobiose levels, which makes CelS repression in the face of elevated growth rates a logical cellular response for limiting cell energy expenditure.

In a later study by Dror et al. [18], the production of endoglucanases CelB, CelG, and CelD were found to be inversely proportional to cell growth rate. These enzymes had lower

**Figure 1.3.** Mixed acid fermentation of *C. thermocellum*. (from Tripathi, S. [16]) Reused with permission from American Society for Microbiology.
levels of expression as growth rate increased. The same study found that a xylanase, XynC, was independent of growth rate and was expressed at relatively stable levels regardless of cell growth rates. Persistent expression of xylanses helps the bacterium continually remove structural hemicellulose xylan, which it cannot utilize and gives better enzymatic access to cellulose.

Currently, industrial lignocellulosic bioprocessing utilizes *Zymomonas mobilis*, *Saccharomyces cerevisiae* or *Escherichia coli* along with hydrolytic enzymes from *T. reesei* to access fermentable sugars within biomass [1]. The above organisms are favored primarily because their biochemistry has been extensively studied and methods of genetic manipulation have been developed for these particular organisms. A major drawback of using these organisms is that they do not tolerate the elevated temperatures more suitable for the functioning of exogeneous hydrolytic enzymes.

One proposed strategy for circumventing the limitations of separate enzymatic and fermentation steps is the use of consolidated bioprocessing. In consolidated bioprocessing (CBP), enzyme production, hydrolysis, and fermentation are all combined into one reactor and these processes can be thought of as occurring simultaneously [19]. CBP can be contrasted with sequential bioprocessing, which seeks to utilize multiple organisms in series to produce the desired end products. In the case of CBP, reducing the number of unit operations is a way to lower the cost of a process. *C. thermocellum*, with its ability to produce saccharifying enzymes and ethanol, is viewed as a promising candidate organism for CBP of lignocellulosic biomass [19]. Acceptable benchmark characteristics for a CBP organism, in terms of hydrolysis rate and product titer have been quoted as >1g glucose released/L/h and 40 g product/L respectively [20]. Greater than 90% theoretical conversion efficiency is also desired. Currently, no organism occurring in nature meets these specifications, so a presently unknown organism would have to be discovered or an
existing one genetically modified in order to reach the targets. Engineering a native cellulase producer to become a viable solvent producer is thought to be an easier problem than the reverse: engineering a solvent producer for cellulase production [1].

*C. thermocellum* is an attractive organism because it has demonstrated superior enzymatic hydrolysis efficiency when compared with free cellulases. However, wild type *C. thermocellum* can only tolerate 5g/L ethanol before it is inhibited at a significant level. As fermentation products accumulate, the cell membrane fluidity is increased to levels that become detrimental to cellular health. A target for the genetic engineering approach would be to modify *C. thermocellum*'s membrane composition so that it is better able to tolerate higher ethanol titers without experiencing a deleterious effect on membrane integrity [1], yet still grow and metabolize at rates similar to the wild type.

At the time of this writing, *C. thermocellum* is still unproven in an industrial setting because of prohibitively high processing costs [1]. Starting in the early 1980's, much work has been done to understand the biochemistry and genetic regulation of *C. thermocellum* [3, 21, 14, 22, 13]. The strength of *C. thermocellum*'s cellulolytic activity makes it a candidate for use in a bacterial co-culture, which is viewed as a way to potentially lower process costs by minimizing the number of unit operations [1]. A bacterial co-culture is a system that uses organisms possessing a synergistic metabolic relationship which has superior processing ability to any monoculture of the involved organisms [23]. The following paragraphs will summarize some of the small scale promise which *C. thermocellum* has shown to date.

In one particular study [24], a co-culture of *C. thermocellum* and *T. aotearoense* produced glucose concentrations of 13.65 ± 0.45 g/L from cassava pulp, which was 1.75 and 1.17 fold greater than controls of each organism in monoculture. *S. cerevisiae* was then introduced with its superior ethanol producing capability. It should be mentioned that both
*C. thermocellum* and *T. aotearoense* both have the ability to produce hydrogen [24], which gives added value to the process because the secondary products are also valuable.

Engineering a process so that all products have market value is a general guiding principle when trying to develop co-cultures for bioprocessing [23].

The fact that *C. thermocellum* has the ability to hydrolyze hemicellulose, yet does not possess the ability to metabolize pentoses has led researchers to try co-culture by pairing it with an organism which can metabolize pentoses. In one study [25], *C. thermocellum* was used in conjunction with *C. beijerinckii* to produce butanol from corn stover. The concentration of reducing sugars obtained via *C. thermocellum*’s cellulolytic activity was twice that of untreated material (20 mg/g biomass vs 10 mg/g biomass). It is known that the butanol producer *C. beijerinckii* has the ability to metabolize xylose in addition to glucose, albeit with a preference for glucose [4].

The co-culturing of *C. thermocellum* with a butanol producer (*C. beijerinckii, C. acetobutylicum*, or *Clostridium saccharoperbutylicum* (strain N1-4)) on model crystalline cellulose (Avicel) has been studied recently [26]. Simultaneous addition of the butanol producer and *C. thermocellum* (0 hour incubation time) resulted in little production of butanol (0.3 g/L) when the co-culture was maintained at 30 °C. This result was anticipated by researchers seeing that *C. thermocellum* has optimal cellulase activity at temperatures greater than 60 °C [3] and strain N1-4 of *C. saccharoperbutylicum* lacks cellulolytic activity. Alternatively, when strain N1-4 was introduced after at least 24 hours of *C. thermocellum* pre-culture at 60 °C, all of the cultures produced over 3.9 g/L of butanol from 20 g/L of crystalline cellulose, with strain N1-4 producing the highest titer of 7.9 g/L after 9 days of incubation. Proteomic studies of *C. thermocellum*’s cellulosome indicated that the protein content of the cellulosome was dependent on the culture substrate type [14, 27]. These studies suggest that an initial adaptation period is needed for
C. thermocellum to tailor its cellulosomal proteins for effective substrate utilization, which is seen in the above study where *C. thermocellum* is given a pre-culture phase.

1.1.3 Experimental Objectives

For a bioprocessing system seeking to utilize *C. thermocellum*, it has yet to be determined under what process conditions this organism will function for the benefit of the process as a whole. This optimum may not coincide with *C. thermocellum*'s individual optimum conditions. The objectives of this study were to quantify sugar hydrolysis and fermentation products produced by *C. thermocellum* over a range of temperature conditions and solids loadings. The purpose of this objective was to obtain data for developing a fermentation activity profile, which will aid in the design of a lignocellulosic bioprocessing system.
1.2 Materials and methods

1.2.1 Organism

*C. thermocellum* ATCC 27405 was obtained from ATCC (American Type Culture Collection) and was stored at -80 °C. Stock culture was prepared by growing the contents of the ATCC ampule in thermophile media liquid culture with a filter paper carbon source for 48 hours. One ml of inoculum culture was distributed to CO₂ purged 15 ml glass vials containing 20% v/v glycerol, which were subsequently stored at -80 °C.

1.2.2 Substrate

The corn stover used was variety Becks 6175 AM, which was harvested in the fall of 2013. It was grown at the C. Oran Little Research Center in Woodford County, KY and was ground to equal to or less than 5 mm using a hammer mill. Switchgrass was the quicksand variety, which was released by the Quicksand, Kentucky Plant Materials Center as a germplasm release in 1987 (source, USDA). The switchgrass was harvested in the fall of 2012. It was grown at the C. Oran Little Research Center in Woodford County, KY and was ground to equal to or less than 5 mm using a hammer mill.

1.2.3 Thermophile media

The composition of the thermophile media (T media) is shown in table 1.1.

Table 1.1. Composition of the media used for *C. thermocellum* cultivation.

<table>
<thead>
<tr>
<th>Component</th>
<th>per L</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>850 ml</td>
</tr>
<tr>
<td>Resazurin stock</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Salt T1</td>
<td>50 ml</td>
</tr>
<tr>
<td>Salt T2</td>
<td>50 ml</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Vitamins</td>
<td>10 ml</td>
</tr>
<tr>
<td>Modified Metals</td>
<td>5 ml</td>
</tr>
</tbody>
</table>
Table 1.1, continued.

<table>
<thead>
<tr>
<th>Salt T1</th>
<th>per L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>84 g</td>
</tr>
<tr>
<td>Or</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>30.60 g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Salt T2</th>
<th>per L</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>30 g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>10 g</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>10 g</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>1.8 g</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.6 g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Standard Vitamins components</th>
<th>per L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxamine 2HCl</td>
<td>100 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>200 mg</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>200 mg</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>200 mg</td>
</tr>
<tr>
<td>CaD Pantotheinate</td>
<td>200 mg</td>
</tr>
<tr>
<td>Lipoic Acid</td>
<td>100 mg</td>
</tr>
<tr>
<td>P-aminobenzoic acid</td>
<td>10 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>5 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>5 mg</td>
</tr>
<tr>
<td>Cobalamin (Co B₁₂)</td>
<td>5 mg</td>
</tr>
<tr>
<td>Pyridoxal HCl</td>
<td>100 mg</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>100 mg</td>
</tr>
<tr>
<td>K₂HPO₄ or KH₂PO₄</td>
<td>17.4 g or 13.6 g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Modified metals components</th>
<th>per L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂EDTA</td>
<td>500 mg</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>200 mg</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>10 mg</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>200 mg</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>20 mg</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>20 mg</td>
</tr>
<tr>
<td>CuCl₂·2H₂O</td>
<td>1 mg</td>
</tr>
<tr>
<td>NiCl₂·6H₂O</td>
<td>2 mg</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>3 mg</td>
</tr>
<tr>
<td>Na₂WO₄·2H₂O</td>
<td>10 mg</td>
</tr>
<tr>
<td>Na₂SeO₃</td>
<td>1 mg</td>
</tr>
</tbody>
</table>
It should be noted that the components resazurin, modified metals, vitamins, salt T1 and salt T2 are all pre-made. Modified metals and vitamins were stored in a 4 °C refrigerator and the rest were stored at room temperature. After mixing the components, T-media was adjusted to a pH of 6.7 using 1 M NaOH and autoclaved at 121 °C, 15 psig for 30 minutes. CO₂ was then bubbled into the media to maintain anaerobic conditions and 50 ml of sterile 8 % Na₂CO₃ buffer was added for each liter of media.

1.2.4 C. thermocellum inoculum

*C. thermocellum* ATCC 27405 inoculum was cultured in 125 ml serum bottles at 63 °C with 60 ml of thermophile media and four filter paper strips (amorphous cellulose) approximately 2” x 0.5” in size. Inoculum was monitored daily and considered to be in a state of readiness once all the filter paper had been completely degraded. Filter paper degradation typically took 2 days and upon completion the remaining culture registered an OD 600 reading of ~0.7 with respect to a media blank. In the rare instance that the filter paper degradation took longer than 2 days, then the virility of the *C. thermocellum* culture was considered unsatisfactory, and the culture was abandoned. In such cases, more inoculum was taken from the -80 °C freezer and a new serum bottle was inoculated with this new stock.

1.2.5 Sample preparation

Using air-dried corn stover or switchgrass samples were first weighed out in 125 ml serum bottles to 3.3 g of feedstock to within ± 0.05 g. The moisture content of air-dry corn stover was found to be approximately 10-11% through the use of a moisture analyzer (Ohaus MB35); 3.3 g was used as a target mass to obtain approximately 3 g of dry matter per replicate. After weighing, all serum bottles were loosely capped by shaping aluminum
foil squares over the bottle tops. The bottles were then autoclaved at 121 °C for 30 minutes at 15 psig and were subsequently allowed to cool to room temperature.

1.2.6 Sample inoculation, Use of Glove Box

The following items were loaded into an anaerobic glove box (Labconco protector):

- Autoclaved serum bottles with material;
- Previously prepared thermophile media in an Erlenmeyer flask with a custom manifold, w/gasing port clamped with adjustable clamps;
- Pipette and battery-powered pipetter;
- 20 mm aluminum seals, for use with serum bottles;
- Previously autoclaved butyl rubber stoppers for serum bottles;
- Seal clamping tool;
- Serum bottle containing *C. thermocellum* inoculum;
- 1 ml syringe, for distributing inoculum;
- 70% ethanol for glove sterilization. The glove box working gloves were sterilized with 70% ethanol during the purge cycle.

The above items were all placed onto a built-in sliding tray found inside the glove box purge chamber. The purge chamber functions as a buffer between the working chamber and the outside air and maximizes the life of the working chamber H₂. Prior to initiation of an automatic purging routine, the tops from the serum bottles were removed so and the liquids transferred to screw cap bottles to avoid having the vacuum in the chamber syphon liquid out of the bottles. The strength of the vacuum in the purge chamber was set to approximately -25 in Hg. The vacuum of -25 in Hg was not capable of removing butyl rubber stoppers from unsealed serum bottles.

The automated purge routine consisted of 4 successive evacuation and fill cycles using a vacuum pump. The backfill gas port was connected to a cylinder of >99.9% N₂ gas. The chamber gas was maintained using a mixture of N₂ and H₂ (96% N₂/4% H₂). Prior to importing the work materials, final concentration of H₂ in the working chamber was brought to 1% to create a buffer against air leaking into the glove box, which occurs slowly.
over time. Solenoid valves on the gas lines were controlled by the embedded system in the
glove box circuitry, which synchronizes backfilling and vacuum operation. The method of
oxygen removal consisted of H₂ gas reacting on screened containers of pelleted palladium
catalyst. Using this method nominal 0 ppm O₂ was achieved and confirmed using an oxygen
analyzer. Air flow over the palladium was maintained by the use of computer-sized electric
fan drawing air through a steel box containing the palladium sheets (12”x 4”), which were
mounted perpendicular to the direction of air flow. Two such reactive apparati were
deployed in this particular glove box. Palladium catalyst was regenerated before work
began by heating in an oven at 105 °C for 48 hours.

Media was first added to the lignocellulosic samples under anaerobic atmosphere to
achieve the desired solids concentration (w/w) of 5%, 10%, or 15%. After addition of
media, 1 ml of logphase C. thermocellum ATCC 27405 inoculum was added to each sample
replicate via sterile syringe. Finally, the serum bottles (figure 1.4) were capped with rubber
stoppers and clamped with 20 mm aluminum seals. Samples were permitted to incubate at
the desired temperature level (20 °C, 30 °C, 40 °C, 50 °C, or 63 °C) for either 24, 48, or 72
hours. At the end of the specified incubation period, serum bottles were sampled using a 1
ml syringe to draw out fermented liquids. Extracted liquids were stored in 1.5 ml plastic
centrifuge tubes at -80 °C in preparation for HPLC analysis.
1.2.7 HPLC sample preparation

Prior to HPLC analysis, all samples were 'conditioned' by two cycles of thaw and refreeze in order to break down cellulosomal proteins accumulated during fermentation. In preliminary experiments, HPLC column pressure was higher than desired and accumulated proteins were assumed to be part of the cause. After thawing, sample tubes were spun in a centrifuge at 14.8 x g for 30 minutes. Next, 1 ml of supernatant was transferred to a clean 1.5 ml centrifuge tube and subjected to another centrifuge treatment at 14.8 x g for 30 minutes. Approximately 900 µl of this secondary supernatant was transferred to a 1.5 ml glass, screw cap HPLC vial. Samples were analyzed as quickly as the HPLC lab queue would permit; if prepared samples could not be analyzed immediately, they were stored at 4 °C until analysis could begin. All samples were analyzed within two days.

1.2.8 HPLC method

The HPLC system used was a Dionex Ultimate 3000, controlled using Chromeleon 7 software over a USB connection. The column used was a Biorad Aminex HPX-87H column, which is composed of a divinylbenzene polymer with attached SO₃ groups (figure 1.5). This
column supports the separation of fermentation acids as well as some sugars. The mobile phase used for this experiment was 5 mM H$_2$SO$_4$ in DI water, filtered through a 0.2 µm filter. An initial attempt to use a Biorad HPX-87P column for sugar separation failed because of overpressure, which resulted in column failure. 87P is recommended by Biorad for separating xylose and glucose, which is why it was the first choice for sugar analysis. The 87P column chemistry differs from that of 87H in that Pb atoms are present in the 87P column. In the 87H column these lead atoms are substituted with hydrogen atoms, which means 87P and 87H have very different pore space available for compounds traveling through the column; the atomic number of hydrogen is 1 and the atomic number of lead is 82.

Figure 1.5. A repetitive unit found in the Biorad HPX-87P/H column

Once the column was installed inside the column compartment, the flow rate of the mobile phase was set to 0.200 ml/min and allowed to flow for 30 minutes, after which the flow rate was increased to 0.300 ml/min. This was repeated until the desired operating flow rate of 0.400 ml/min was achieved. Before the start of analysis the mobile phase at 0.400 ml/min was permitted to flush the column for at least 30 minutes and the RI detector
output was examined to check for a stable starting baseline. If the baseline was not 0 µRIU (refractive index unit), the initial flushing process was allowed to continue. The column compartment was maintained at a temperature of 50 °C and the sample compartment at 8 °C. The injection volume was 100 µl for standards and samples.

All samples were screened for the presence of cellobiose, glucose, xylose, lactate, acetate, ethanol, and formate. Six levels of standards were prepared consisting of the following: for acids the six levels were – 100 mM, 50 mM, 25 mM, 10 mM, 5 mM, and 1 mM; and for sugars the levels were 5 g/L, 2.5 g/L, 1.25 g/L, 0.5 g/L, 0.25 g/L, and 0.05 g/L. The lowest level standard registered component peak heights of only 2-3 µRIU, which is close to the lower limit of the Shodex 101 detector of 0.25 µRIU. All data fit within the range prescribed by the standard curve. Standards were injected at the beginning and end of each run of 27 samples to illustrate the extent of peak drifting over the course of the run. Component peak drift was observed to be between 0.1 and 0.2 minutes and did not present a significant difficulty for peak identification.

1.2.9 Media background and an unidentified peak of interest

An unidentified peak (retention time ~20 minutes), the height of which varied linearly with the solids concentration, was found with the switchgrass treatments. This compound was present at all temperatures and showed the same aforementioned behavior in all treatments. It was present in amounts comparable to neighboring fermentation acid peaks, which had similar retention times. This peak should be identified via mass spectrometry, as it is expected to be an overflow metabolite. *C. thermocellum* is known to dump amino acids out of the cell as overflow metabolites [28].

A total media background was collected to rule out the possibility of this compound originating in the media itself. The compound of interest was not detected in the media.
Individual T-media component contributions to the HPLC background (background pertaining to Biorad Aminex HPX-87H) are shown in Appendix D.

1.2.10 YSI 2900 glucose analyzer

Background peaks produced by components in the media obscured glucose on the chromatogram during HPLC analysis. Glucose was ultimately quantified using a YSI 2900 analyzer, which uses a chemical reaction that produces an electrical current proportional to the amount of glucose present in a sample. Previous comparisons between HPLC and the YSI 2900 for glucose showed that values were within ± 5% of one another (William Sympson, personal communication, Nov. 2014). Initially, glucose spiked media samples of 5 g/L were used to assess the suitability of the YSI 2900 analyzer for quantifying glucose in this particular context. Measured concentrations in this preliminary test were found to be within ± 5% of the nominal concentration.

1.3 Experimental Design and Statistical Analysis

In the two full factorial experiments (one for corn stover, one for switchgrass) 135 serum bottles (125 ml) were assigned to 45 treatments, allowing for 3 replicates per treatment, varying the factors of temperature (20 °C, 30 °C, 40 °C, 50 °C, 63 °C), solids concentration w/v (%) (5%, 10%, 15%), and length of fermentation (24 hr., 48 hr., 72 hr.). Experimental values for these process parameters were chosen after consulting the literature [11, 29] and making an assumption about probable conditions that a lignocellulosic processing system involving C. thermocellum would use. Temperature was randomly assigned in order to construct treatment groups. After adding media and inoculum, serum bottles were placed in a stationary incubator and remained there for the
amount of time specified by the treatment. Fermentation liquids were sampled at the designated time using a 1 ml syringe. Duplicate samples were stored for each replicate in 1.5 ml centrifuge tubes at -80 °C in preparation for HPLC analysis for sugars and fermentation acids.

Data were statistically analyzed using a full factorial treatment structure for each set of experiments using the `proc glm` statement in SAS® statistical software. If a significant difference between at least one pair of treatment means was detected, a Tukey test was carried out to examine differences in total carbon among all combinations of treatment means. The time sample with the maximum value for products was utilized in the ANOVA (analysis of variance), which considered temperature and solids concentration as main effects and total carbon produced by the fermentation as the dependent variable. Depictions of product treatment means with +1 standard deviation error bars were generated with MATLAB software using the native `bar3` function. MATLAB-derived graphs are shown in Appendix G and Appendix H. The MATLAB code is given in Appendix A.
full factorial architecture for both switchgrass and corn stover

time (hours) = 24, 48, 72

temperature (°C) = 20, 30, 40, 50, 63

solids (%) = 5, 10, 15
1.4 Results

The corn stover treatment means for % solids and cultivation temperature are presented in figure 1.7. ANOVA results for the corn stover experiment are presented in table 1.2 and table 1.3. The model used total carbon liberated from the lignocellulose during fermentation by \textit{C. thermocellum} as the response variable, whereas solids concentration and temperature were considered explanatory variables. Total carbon (grams) was calculated by multiplying each product concentration by its appropriate carbon mass fraction $\left(\frac{MW_{\text{carbon}}}{MW_{\text{molecule}}}\right)$ and summing over all products. Total carbon concentrations (g/L) were then multiplied by the volume of media used for a particular replicate. From figure 1.3 stoichiometry, for every mole of ethanol or acetate produced, one mole of CO2 is also produced, and this fact was accounted for in the total carbon calculation. Total carbon was considered an indirect metric for measuring cellulosomal activity in this experiment.
Figure 1.7. Treatment means for the fermentation products from \textit{C. thermocellum} on corn stover using solids loadings of 5, 10, and 15\% and cultivation temperatures of 20, 30, 40, 50, and 63 °C.

Table 1.2. SAS output for corn stover ANOVA, total model for \textit{C. thermocellum} on corn stover using solids loadings of 5, 10, and 15\% and cultivation temperatures of 20, 30, 40, 50, and 63 °C.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>14</td>
<td>0.0267</td>
<td>0.0019</td>
<td>28.50</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>30</td>
<td>0.0020</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>44</td>
<td>0.0287</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.3. SAS output for corn stover ANOVA, main effects for *C. thermocellum* on corn stover using solids loadings of 5, 10, and 15% and cultivation temperatures of 20, 30, 40, 50, and 63 °C.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type I SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>solids</td>
<td>2</td>
<td>0.0005</td>
<td>0.0003</td>
<td>3.90</td>
<td>0.0313</td>
</tr>
<tr>
<td>temp</td>
<td>4</td>
<td>0.0252</td>
<td>0.0063</td>
<td>94.29</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>solids*temp</td>
<td>8</td>
<td>0.0009</td>
<td>0.0001</td>
<td>1.75</td>
<td>0.1269</td>
</tr>
</tbody>
</table>

Table 1.2 shows that the overall model for corn stover total carbon is statistically significant. Table 1.3 reveals that the two independent variables, solids concentration and temperature, are each significant in this particular model (α = 0.05), however the interaction between the two variables was not statistically significant. The greatest mean total carbon was seen in the 10% 63 °C treatment group. The 5% 63 °C treatment had an almost equal total carbon mean and was not statistically different from 10% 63 °C.

A post-ANOVA tukey test was done to examine the differences among treatment means grouped by main effect. When grouped by the main of effect solids loading, neither the 15% nor the 5% group had a mean significantly different from the 10% group. The 5% and 15% groups were however significantly different from each other. When grouped by the main effect of temperature, treatments at 20 °C and 30 °C as well as 50 °C and 63 °C did not differ significantly. All other possible pairs were deemed statistically different. A multiple comparison Tukey test was also done, which examined all possible pairs of the 15 treatments (solids_temperature) considered by the ANOVA. A distinct pattern of significant pairs (α = 0.05), which have been highlighted in figure 1.8, can be seen as diagonals that travel from top left to bottom right.
Figure 1.8. ANOVA p-values from Tukey multiple comparison test between all possible treatment pairs (solids%_temperature) for total carbon from *C. thermocellum* fermentation on corn stover.

The switchgrass treatment means for solids loading and temperature are presented in figure 1.9. ANOVA results for the switchgrass experiment are presented in table 1.4 and table 1.5. The ANOVA modeling was identical to that used above for the *C. thermocellum* corn stover fermentation.

<table>
<thead>
<tr>
<th>i/j</th>
<th>5_20</th>
<th>5_30</th>
<th>5_40</th>
<th>5_50</th>
<th>5_63</th>
<th>10_20</th>
<th>10_30</th>
<th>10_40</th>
<th>10_50</th>
<th>10_63</th>
<th>15_20</th>
<th>15_30</th>
<th>15_40</th>
<th>15_50</th>
<th>15_63</th>
</tr>
</thead>
<tbody>
<tr>
<td>5_20</td>
<td>1</td>
<td>0.0124</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.9936</td>
<td>1</td>
<td>0.0762</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>1</td>
<td>1</td>
<td>0.0652</td>
<td>&lt;.0001</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>5_30</td>
<td>1</td>
<td>0.0557</td>
<td>0.0001</td>
<td>&lt;.0001</td>
<td>0.8558</td>
<td>0.9997</td>
<td>0.261</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.9829</td>
<td>0.9995</td>
<td>0.2307</td>
<td>0.0002</td>
<td>0.0011</td>
<td></td>
</tr>
<tr>
<td>5_40</td>
<td>0.0124</td>
<td>0.0557</td>
<td>0.5291</td>
<td>0.0267</td>
<td>0.0005</td>
<td>0.0058</td>
<td>1</td>
<td>0.0868</td>
<td>0.0009</td>
<td>0.0017</td>
<td>0.0051</td>
<td>1</td>
<td>0.706</td>
<td>0.9566</td>
<td></td>
</tr>
<tr>
<td>5_50</td>
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Figure 1.9. Treatment means for the fermentation products from *C. thermocellum* growing on switchgrass, using solids loadings of 5, 10, and 15% and cultivation temperatures of 20, 30, 40, 50, and 63 °C. Error bars represent ± 1 standard deviation.

Table 1.4. Switchgrass ANOVA, total model for *C. thermocellum* on switchgrass using solids loadings of 5, 10, and 15% and cultivation temperatures of 20, 30, 40, 50, and 63 °C.

<table>
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<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
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<th>Pr &gt; F</th>
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</tbody>
</table>
Table 1.5. Switchgrass ANOVA, main effects for *C. thermocellum* on switchgrass using solids loadings of 5, 10, and 15% and cultivation temperatures of 20, 30, 40, 50, and 63 °C.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type I SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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Table 1.4 shows that the overall model for switchgrass total carbon in hydrolysis and fermentation products is statistically significant, so at least one treatment differs from the others statistically. Table 1.5 reveals that the main effects of solids concentration and temperature are each significant in this particular model (α = 0.05), so solids concentration averaged over all temperatures affects total product carbon, and temperature averaged over all solids concentration also affects product carbon. The interaction between the two explanatory variables was also significant for switchgrass substrate, unlike corn stover. The greatest mean total carbon was seen in the 5% 63 °C treatment, which is not surprising given that optimal cellulosomal activity is known to occur above 60 °C [3] and end product inhibition of cellulosomes is more prominent at higher solids loadings [21].

A post-ANOVA Tukey test was done to examine the differences among treatment means grouped by main effect. An interaction plot confirmed that the interaction between both main effects was significant.

When grouped by the main of effect solids loading, neither the 10% nor the 15% group had a mean significantly different from each other in terms of mean total carbon. The 5% solids loading treatment was significantly different from both the 10% and 15% group. When grouped by the main effect of temperature, treatments of 40 °C, 30 °C, and 20 °C did
not differ significantly. The main effect group 63 °C was significantly different from all others. 50 °C was significantly different from 20 °C, 30 °C, and 63 °C, but not significantly different from 40 °C.

A multiple comparison Tukey test was also done, which examined all possible pairs of the 15 treatments (solids_temperature) considered by the ANOVA. A distinct pattern of significant pairs (α = 0.05) can be seen, which have been highlighted in figure 1.10. This pattern of significant pairs is different from the pattern observed for corn stover in figure 1.8.

\[
\begin{array}{cccccccccccccccc}
ij & 5_20 & 5_30 & 5_40 & 5_50 & 5_63 & 10_20 & 10_30 & 10_40 & 10_50 & 10_63 & 15_20 & 15_30 & 15_40 & 15_50 & 15_63 \\
5_20 & 1 & 0.998 & 0.1718 & <.0001 & 1 & 1 & 0.9971 & 0.5061 & 0.0006 & 0.9995 & 1 & 0.9508 & 0.8671 & 0.4291 \\
5_30 & 1 & 1 & 0.3611 & <.0001 & 1 & 0.9971 & 0.7742 & 0.0018 & 0.9824 & 1 & 0.9968 & 0.9803 & 0.7009 & 1 & 1 & 0.972 \\
5_40 & 0.998 & 1 & 0.7777 & <.0001 & 0.8724 & 0.9772 & 1 & 0.3686 & 0.0106 & 0.7494 & 0.9939 & 1 & 1 & 0.972 \\
5_50 & 0.1718 & 0.3611 & 0.7777 & <.0001 & 0.0357 & 0.0866 & 0.8013 & 1 & 0.5901 & 0.2199 & 0.1312 & 0.9613 & 0.9915 & 1 & 1 & 0.972 \\
5_63 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 & <.0001 \\
10_20 & 1 & 0.967 & 0.8724 & 0.0357 & <.0001 & 1 & 0.8534 & 0.1541 & <.0001 & 1 & 1 & 0.5982 & 0.4366 & 0.1201 \\
10_30 & 1 & 1 & 0.9772 & 0.0866 & <.0001 & 1 & 0.971 & 0.3118 & 0.0002 & 1 & 1 & 0.8298 & 0.6855 & 0.253 \\
10_40 & 0.9971 & 1 & 1 & 0.8013 & <.0001 & 0.8534 & 0.971 & 0.9899 & 0.0118 & 0.7236 & 0.9915 & 1 & 1 & 0.972 \\
10_50 & 0.5061 & 0.7742 & 0.9866 & 1 & <.0001 & 0.1541 & 0.3118 & 0.9899 & 0.2192 & 0.0938 & 0.4217 & 0.9999 & 1 & 1 & 0.972 \\
10_63 & 0.0006 & 0.0018 & 0.0106 & 0.5901 & 0.0071 & <.0001 & <.0001 & 0.0002 & 0.0118 & 0.2192 & <.0001 & 0.0004 & 0.0347 & 0.0624 & 0.2726 \\
15_20 & 0.9995 & 0.9824 & 0.7494 & 0.0357 & <.0001 & 1 & 1 & 0.7236 & 0.0938 & <.0001 & 0.9999 & 0.4442 & 0.3024 & 0.0716 \\
15_30 & 1 & 1 & 0.9993 & 0.0312 & <.0001 & 1 & 1 & 0.9915 & 0.4217 & 0.0004 & 0.9999 & 0.9124 & 0.8013 & 0.3509 \\
15_40 & 0.9508 & 0.9824 & 1 & 0.9613 & <.0001 & 0.5982 & 0.8013 & 1 & 0.9999 & 0.0047 & 0.4442 & 0.9124 & 1 & 0.9994 \\
15_50 & 0.9671 & 0.9803 & 1 & 0.9615 & <.0001 & 0.4366 & 0.6658 & 1 & 1 & 0.0024 & 0.3024 & 0.9013 & 1 & 1 & 0.972 \\
15_63 & 0.4291 & 0.7009 & 0.971 & 1 & <.0001 & 0.1201 & 0.253 & 0.978 & 1 & 0.2726 & 0.0716 & 0.3509 & 0.9994 & 1 & 1 & 0.972
\end{array}
\]

Least Squares Means for effect solids*temp

\( Pr > |t| \) for H0: LSMean(i)=LSMean(j)

Dependent Variable: total_carbon

**Figure 1.10.** ANOVA p-values from Tukey multiple comparison test between all possible treatment pairs (solids%_temperature) for total carbon from *C. thermocellum* fermentation on switchgrass.

In this study, a temperature dependent depletion of cellobiose was observed for both substrates, with a corresponding spike in formate concentration at the same cultivation temperature at which the depletion occurred. This phenomenon was observed to occur at 50 °C for corn stover (see figure 1.11 and figure 1.12) and at 40 °C for switchgrass (see figure 1.13 and figure 1.14).
Figure 1.11. Treatment means for cellobiose (mg/L) from *C. thermocellum* cultivation on corn stover using solids loadings of 5, 10, and 15%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours. Error bars represent +1 standard deviation.
Figure 1.12. Treatment means for formate (mg/L) from *C. thermocellum* cultivation on corn stover using solids loadings of 5, 10, and 15%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours. Error bars represent ±1 standard deviation.
Figure 1.13. Treatment means for cellobiose (mg/L) from *C. thermocellum* cultivation on switchgrass using solids loadings of 5, 10, and 15%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours. Error bars represent +1 standard deviation.
Figure 1.14. Treatment means for formate (mg/L) from \textit{C. thermocellum} cultivation on switchgrass using solids loadings of 5, 10, and 15\%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours. Error bars represent +1 standard deviation.

Glucose was also largely absent at 50 °C for corn stover treatments (see figure 1.15). Xylose concentrations were unexpectedly low at 50 °C and 40 °C for corn stover and switchgrass treatments respectively (see figure 1.16 and figure 1.17).
Figure 1.15. Treatment means for glucose (mg/L) from *C. thermocellum* cultivation on corn stover using solids loadings of 5, 10, and 15%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours. Error bars represent +1 standard deviation.
Figure 1.16. Treatment means for xylose (mg/L) from *C. thermocellum* cultivation on corn stover using solids loadings of 5, 10, and 15%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours. Error bars represent +1 standard deviation.
Figure 1.17. Treatment means for the xylose (mg/L) from *C. thermocellum* cultivation on switchgrass using solids loadings of 5, 10, and 15%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours. Error bars represent +1 standard deviation.
1.5 Discussion

The absence of xylose for both substrates is strange because *C. thermocellum* is reported to be incapable of pentose utilization [3] and xylanases are known to be expressed constitutively by *C. thermocellum* in its cellulosome when grown on various model and real world substrates [14]. However, a proteomic study [14] showing constitutive xylanase expression used data from *C. thermocellum* cellulosome production at 60 °C, not 50 °C when grown on pretreated switchgrass.

Although formate synthesis has been observed for *C. thermocellum* strain l-1-B, several studies of *C. thermocellum* ATCC 27405 fermentations, each examining different growth conditions, have failed to detect the presence of formate post fermentation [30, 31]. In a 2006 study by Sparling et al. [32], formate was detected when *C. thermocellum* ATCC 27405 was cultured using cellobiose as a carbon source. mRNA products from putative genes related to formate synthesis were confirmed using RT-PCR (reverse transcriptase polymerase chain reaction).

Elevated formate production at 50 °C could indirectly be the cause of elevated solvent production seen in the 50 °C corn stover treatments. In a 2010 study by Xu et al. [33], the effect of organic acid products formate, acetate, and lactate on cellulosomal activity was investigated. In this particular study, reducing sugar concentration was used to assess cellulosomal activity. With pH held constant, it was found that cellulolytic activity of the cellulosome was stimulated (positive feedback loop) by formate, acetate, and lactate at concentrations below 100 (0.1 M), 200 (0.2 M), and 50 mM (0.05 M) respectively. Formate levels above 0.5 M, acetate above 1 M, and lactate above 0.5 M were shown to be inhibitory to cellulosomes. Eighty percent of cellulosomal activity was lost when formic acid, acetic acid, and lactic acid reached levels of 0.1 M, 0.2 M, and 0.1 M respectively and pH of the growth media was allowed to decrease. These conditions caused pH levels to drop below
5.0, which is well below the optimal pH of 6.5 for cellulosomal activity [3]. Ultimately higher cellulosomal activity will lead to more sugar available for cellular metabolism and solvent production. The study reported here was conducted as part of the BRDI project (Biomass and Resource Development Initiative), the primary goal of which is to produce butanol from lignocellulosic biomass using C. beijerinckii. If the goal is to maximize solvent production from C. beijerinckii, then maximizing the cellulolytic activity of C. thermocellum, while minimizing its sugar consumption, is the outcome to keep in mind when choosing process conditions. One should also be cognizant that sugar utilized for cellular metabolism at 30 °C will not be replenished through action of the cellulosome in the same way as it is at 63 °C. However, C. thermocellum’s cellulosome retains about 25% of its peak activity at 35 °C [30], as can also be seen in figure 1.7 and figure 1.9. In short, favorable sugar concentrations at higher temperature should receive more weight as viable process options.

A recent study [34] of C. thermocellum determined oligomeric cellulose hydrolysis products lost from the biofilm were 13.7% and 29.1% of the total substrate carbon hydrolyzed, respectively, for low (44 g/L) and high (202 g/L) cellulose loadings. For the type of processing system being proposed in this study, it is advantageous to minimize C. thermocellum’s metabolism of its own hydrolysis products. As seen in the study mentioned [34], the majority of the carbon liberated by the cellulosomes never passes through the biofilm, and this is still a major problem that needs to be addressed.

If high sugar yields are a desired outcome of a C. thermocellum fermentation, then 50 °C and 40 °C are not viable process parameters for corn stover and switchgrass, respectively.

Also, for a given solids loading and temperature, sugar concentration declined after 24 hours in corn stover treatments; this trend was not observed for switchgrass, where sugar concentration generally increased with fermentation time (see Appendix G and
Appendix H). This is likely due to lower biomass recalcitrance with corn stover when compared to switchgrass.

Both switchgrass and corn stover substrates were ground to 5 mm particle sizes, however, corn stover had a greater tendency to turn to dust during the grinding process, so it can be said that the effective particle size was less than 5 mm for corn stover. This discrepancy is believed to be partially responsible for the statistically significant and insignificant main effect interaction seen for switchgrass and corn stover, respectively. The smallest corn stover dust particles were determined to be 50-75 µm in diameter through a series of mesh screen filters.

If accessory acids production is also an important goal then the choice of optimal solids loading, temperature, and duration is not as simple as if one is considering only sugar yields. Also, *C. thermocellum* is more susceptible to osmotic stress and pH changes from metabolic acid accumulation [22] at higher solids loadings (≥15%), even though higher solids can yield greater sugar concentrations. Metabolic inhibition from osmotic stress could be mitigated by a periodic or continuous flushing regime. If maximizing sugar is the lone concern, then 15%_63°C_24hr is recommended for corn stover and 15%_63°C_72hr for switchgrass.
1.6 References


Chapter 2: Improving *P. chrysosporium* RP78 competitiveness

Investigation of a sequential bioprocessing system using *Phanerochaete chrysosporium* and *Clostridium thermocellum* under nonsterile conditions

Abstract

The white rot fungus *Phanerochaete chrysosporium* is a promising candidate for the bioprocessing of lignocellulosic biomass because of the oxidative enzymes it produces [35]. These enzymes, known as fungal peroxidases, have been proven to oxidize a variety of organic substrates, including pesticides, polyaromatic hydrocarbons, polychlorinated biphenyls, and other halogenated aromatics (e.g. dioxins), as well as trinitrotoluene (TNT) [36]. *P. chrysosporium* has also been used successfully in small scale processing of pulp and paper mill effluent [37]. *P. chrysosporium*’s extracellular oxidative enzymes have the ability to break down the aromatic polymer lignin [38], making it a promising organism for pretreatment of lignocellulosic biomass. *P. chrysosporium* is known to have a low tolerance to toxicity [39], so for the purpose of pretreating lignocellulose, its ability to grow in the presence of other micro-organisms is an issue to be addressed. The goal of this study was to examine the potentially beneficial competitive effect of a preliminary *C. thermocellum* fermentation on *P. chrysosporium*’s ability to grow on the same unsterile biomass post fermentation. Elevated temperature alone (63 °C) was also examined to separate the effect of temperature and fermentation on subsequent *P. chrysosporium* growth. No significant difference in lignin % was detected for any of the treatments with respect to control. Mold growth was evident in all experimental treatments expect for the *C. thermocellum* "pretreated" replicates and sterile controls. Reduced mold growth was observed in the temperature only pretreated group and *P. chrysosporium* growth was also observed over ~50 % of the material. Over-rinsing may have occurred after the *C.
thermocellum fermentation, removing salts that *P. chrysosporium* needs for its metabolism [40], which could explain the lack of *P. chrysosporium* growth after fermentation.

Mycotoxins from field-derived molds [41] are also a possible factor in the experimental results.
2.1 Introduction

2.1.1 Lignocellulose Processing

In the introduction section of Chapter 1, various aspects of lignocellulose utilization were discussed including 1) motivation for lignocellulose utilization, 2) importance of pretreatment and its effect on cellulose conversion, 3) the effect of pretreatment on downstream processes, and 4) the general structure of lignocellulose. This section is an addendum to the first section and will discuss enzyme cost as well as genetic modification of feedstocks, which are important topics related to lignocellulosic resource utilization.

In lignocellulosic bioprocessing, enzymes are used in order to saccharify cellulose and hemicellulose polymers for fermentation. The cost of enzyme has been deemed a significant and underestimated contributor to the overall cost of biofuels production [42]. Klein-Marcuschamer et al. [42] analyzed the cost of enzyme for ethanol production based on four different scenarios, ranging from an unrealistic best-case to an unoptimized typical case. These scenarios are as follows: (1) The theoretical maximum yield based on conversion of all C5 and C6 sugars present in corn stover; (2) the yield based on conversion of C6 sugars at a 95% efficiency, but not C5 sugars; (3) the yield based on conversion of all C5 and C6 sugars after a saccharification cellulose conversion of 70%; and (4) the yield based on conversion of C5 and C6 sugars expected from a typical saccharification and a typical fermentation using engineered yeast. Based on these scenarios the authors concluded that enzymes would contribute between $0.60 and $1.30 per gallon of ethanol produced from corn stover, which is higher than most literature values. This cost projection was made with the assumption that poplar is freely available as a substrate for enzyme production by *Trichoderma reesei*. 
Cost will vary depending on the loading of enzyme used, the type of biomass being utilized, and the desired duration of the saccharification process. Bioprocessing using organisms which produce their own cellulases and hemicellulases (i.e. C. thermocellum) is seen as a way to reduce enzyme costs because enzymes are produced and utilized within one unit operation [1]. Traditionally, cellulases are produced in a completely separate process using Trichoderma reesei [42]. Consolidated bioprocessing (CBP), which by definition uses a single microorganism (typically genetically modified) [19], has been suggested as an efficient and economical method for producing low value products from lignocellulose; however, CBP is not being used commercially for commodity chemical production [23].

In addition to metabolic engineering of microbes for more efficient fermentations, the genetic engineering of the lignocellulosic biomass itself has been conceived as a way to improve the economics of biomass conversion. In a 2011 experiment [43], researchers used RNAi (RNA interference) to down-regulate the switchgrass caffeic acid O-methyltransferase gene, which codes for a crucial enzyme in the lignin synthesis pathway. The presence of lignin in cell walls negatively impacts the conversion of biomass to sugars. Enzyme down-regulated plants required milder pretreatment and 300-400% lower cellulase loadings to match control product levels in a simultaneous saccharification and fermentation process utilizing yeast. Ethanol yield was also increased up to 38% using conventional biomass fermentation processes. A C. thermocellum fermentation was also used to assess the cellulose accessibility in transgenic plants with respect to a control. The control switchgrass had $27.2 \pm 0.84\%$ of the cellulose remaining, while the transgenic line had only $14.1 \pm 2.1\%$ of the cellulose remaining. The results supported the observed higher yields of fermentation products for the COMT transgenic line of switchgrass. The transgenic line showed normal growth and development. Overall, genetic modification of lignocellulose
aims to decrease the recalcitrance of the biomass to microbial and/or chemical processing. A high degree of recalcitrance from the presence of lignin is commonly considered the major hurdle hindering the economic success of biomass to biofuels processes [9].

2.1.2 *P. chrysosporium*

*P. chrysosporium* is a white rot fungus that has the ability to degrade the abundant biopolymer lignin [38]. *P. chrysosporium* possesses an extracellular system of oxidative enzymes [35], which includes various isoforms of manganese peroxidases and lignin peroxidases. The term "white rot" refers to the white cellulose left behind by *P. chrysosporium* growing on biomass [44]. Although other white rot fungi exist, *P. chrysosporium* is viewed as the model white rot fungus because its lignolytic enzyme complex is considered more complete in its lignin degradation capability than other strains [38]. This claim of dominance is evidenced by the fact that *P. chrysosporium* was the first white rot fungus to have its genome sequenced [44].

Consistent production of LDPs (lignin degrading peroxidases) presents a major challenge to using *P. chrysosporium* as an organism in lignocellulosic processing. Often the level of enzyme production is unstable and the total level of enzyme production is low. Many studies have been conducted to optimize the cultural conditions of *P. chrysosporium* for LDP production. Sporulation and maintenance of *P. chrysosporium* can be achieved using potato dextrose agar. However, high levels of LDPs production have been linked to secondary metabolic processes brought on by carbon or nitrogen scarcity. A study by Zacchi et al. [45] showed that lignin decomposition in a liquid culture medium containing 24 mM nitrogen was only 25-35% of that containing only 2.4 mM nitrogen. The nitrogen concentration for optimal enzyme production has been reported to be below 4 mM [46].

In solid substrate cultivation, one study [47] reported that 75% moisture content wet basis is the optimal moisture content for ligninolytic activity in *P. chrysosporium*
growing on cotton stalks. Shi et al. [47], found that if the moisture content was too high
limitations were imposed on oxygen transfer, as well as an increased susceptibility of the
culture to bacterial contamination.

One important parameter in determining extracellular peroxidase activity of *P. chrysosporium* is pH [48]. High pH has been shown to cause reversible inactivation of
peroxidases. Activity was reclaimed after the pH was lowered from 9 to 6 while adding 50
mM Ca²⁺, which has been determined to be essential for maintaining the proper
conformation of the enzyme active site.

Analysis of the *P. chrysosporium* genome [44] has revealed the presence of five
isoforms of the manganese peroxidase enzyme. Manganese peroxidase has the ability to
oxidize Mn²⁺ to Mn³⁺ in the presence of peroxide. Mn³⁺ is complexed with an organic acid
chelator such as oxalate or malonate, which are produced as metabolic byproducts of fungal
cell metabolism. This Mn³⁺ : chelator complex diffuses from the surface of the enzyme and
is able to oxidize organic substrates; a process which converts Mn³⁺ back to an Mn²⁺ state.
Organic acid chelators facilitate the release of Mn³⁺ from the enzyme active site and also
serve as a means to stabilize Mn³⁺ with a high redox potential in aqueous solution [49].
Experiments by Mester et al. (1995) [50] showed that addition of exogenous Mn²⁺
stimulated manganese peroxidase synthesis. Lignin peroxidase and veratryl alcohol
production were in turn inhibited by increased concentration of Mn²⁺. Absence of Mn²⁺ led
to inhibition of manganese peroxidase production but encouraged the production of lignin
peroxidase and veratryl alcohol.

According to recent analyses of the *P. chrysosporium* genome, the presence of ten
lignin peroxidase genes have been detected [44]. Unlike manganese peroxidases, lignin
peroxidases oxidize lignin in proximity to the enzyme active site and do not utilize a
diffusible oxidant. Veratryl alcohol is required for lignin peroxidase activity. Veratryl
alcohol prevents the lignin peroxidase enzyme from remaining in an oxidized state after it has reacted with lignin. Like manganese peroxidase, lignin peroxidase function is also dependent on the production of peroxide by glyoxal oxidase [38].

*P. chrysosporium* as a lignocellulosic pretreatment is not a new idea. A 2012 study [51] examined the feasibility of pretreating corn stalks using *P. chrysosporium* prior to enzymatic saccharification and H$_2$ production. Sterilized 5 mm ground corn stalks were pretreated for 15 days using *P. chrysosporium* and saccharification was conducted using crude cellulases from *Trichoderma viride*. Bio-H$_2$ was then produced using a thermophilic bacterium, *Thermoanaerobacterium thermosaccharolyticum*. Maximum saccharification was 47.3 % of theoretical, 20.3 % higher when compared with a control, which was not pretreated with fungus. The yield of H$_2$ gas was 80.3 ml/g corn stalk, which is deemed promising based on other bio-hydrogen production studies.

In one study [52], researchers used *P. chrysosporium* to pretreat wheat straw in a hydrogen production process. Wheat straw was milled and passed through a screen with 0.45 mm diameter holes. Fungal pretreatment was performed in 250 ml Erlenmeyer flasks filled with 5 g of wheat straw at a moisture content of 75 %. Pretreatment lasted 12 days and resulted in lignin removal of 28.5 ± 1.3 %. The wheat straw was then subjected to cellulases from *Trichoderma atroviride* in a simultaneous saccharification and fermentation (SSF) process with the hydrogen producer *Clostridium perfringens*. Hydrogen production on pretreated wheat straw was approximately 1.8 times that of the unpretreated group. Liquid fermentations were performed in 100 ml Erlenmeyer Flasks with 0.5 g of dry pretreated wheat straw and a mineral salt solution (5 % solids loading). Also in this study, the duration of *P. chrysosporium* pretreatment and its effect on hemicellulose and cellulose depletion was examined. Treatments of 0, 3, 6, 9, 12, 15, 18, and 21 days were subjected to compositional analysis to determine the extent of lignin removal and the percentage of
hemicellulose and cellulose remaining after *P. chrysosporium* pretreatment. The 21 day treatment group had a lignin removal value of \( \sim 36\% \), compared to \( \sim 29\% \) for the 12 day treatment. The researchers concluded that there was minimal benefit in terms of extra lignin reduction for 21 day treatment when considering the additional loss of holocellulose consumed during the longer pretreatment. Pretreatment with *P. chrysosporium* consumes valuable cellulose and hemicellulose, so finding a pretreatment time that adequately removes/alters lignin while preserving sugar chains is desired. In this study, 12 day pretreatment was considered by the researchers to be an optimum for lignin reduction and sugar preservation.

The previously mentioned article [52] did not discuss the fate of mycelia after pretreatment, which was demonstrated in a 2007 study [47] to have a significant effect on the extent of subsequent cellulose hydrolysis. Cellulose hydrolysis efficiency was actually lower for submerged culture and SSF pretreated groups when compared with controls, which were comprised of untreated material at 75% solids content and 3 mm particle size. Commercial enzymes were used for cellulose hydrolysis and the ethanol producer was *Saccharomyces cerevisiae* ATCC 24859. Ethanol yields (g/g substrate) were also lower for the pretreated groups, which logically follows from the fact that a lower cellulose hydrolysis efficiency was observed from the treated groups and *P. chrysosporium* consumes up to 60% substrate hemicellulose and 44% substrate cellulose for its metabolism [47]. Ethanol yield was statistically unaffected by the *P. chrysosporium* pretreatment when compared to the untreated controls. The actual treatment groups consisted of 1) 14 day PC pretreatment with unaltered mycelia after pretreatment 2) 14 day PC pretreatment followed by a sterile water wash of 3x 100 ml volumes 3) 14 day PC pretreatment followed by a hot water wash (121 °C, 3 x 100 ml). The hot water wash treatment showed an improvement in cellulose conversion when compared to the washed and unwashed treatments. These data suggest
that on certain substrates, \textit{P. chrysosporium} may actually inhibit enzymatic access to cellulose without some kind of post-pretreatment process to alter mycelia-related obstruction.

\subsection*{2.1.3 Experimental Objectives}

The hypothesis pertaining to this study is that \textit{P. chrysosporium}'s enzymatic activity, acting on biomass lignin, can function as a pretreatment which will facilitate access to hemicellulose and cellulose for \textit{C. thermocellum}'s enzyme complex. Elevated temperature and \textit{C. thermocellum} fermentation were also examined as ways to enable and improve competitiveness of \textit{P. chrysosporium} growth on unsterile substrate.

\section*{2.2 Materials and Methods}

\subsection*{2.2.1 Substrate}

The corn stover used was from the corn variety Becks 6175 AM, which was harvested in the fall of 2013 from the C. Oran Little Research Center in Woodford County, KY. After harvesting, the stover was air dried and was ground to 5 mm using a hammer mill.

\subsection*{2.2.2 Thermophile media, Bacterial Inoculum}

Thermophile media and \textit{C. thermocellum} inoculum were prepared as described in section 1.2.3 and section 1.2.4, respectively. Any \textit{C. thermocellum} inoculation was carried out in an anaerobic glove box as described in 1.2.6.
2.2.3 Sample preparation

Table 2.1 shows a list of the experimental treatments. Superscripts depict destructive sampling points as well as what type of data for which the sample was analyzed (metabolic products or lignin). The duration of each treatment phase (days) is listed in parentheses.

Table 2.1. Complete list of treatments for P. chrysosporium pretreatment/sterilization experiment on corn stover.

<table>
<thead>
<tr>
<th>Step Description</th>
<th>Ct(^p) (2)</th>
<th>Pc(^l) (sterile) (12)</th>
<th>Pc(^l) (unsterile) (12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pc (\rightarrow) Ct(^p)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(12 (\rightarrow) 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63 °C (\rightarrow) Pc (\rightarrow) Ct(^p)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5 (\rightarrow) 12 (\rightarrow) 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63 °C (\rightarrow) Pc (\rightarrow) Ct(^p)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5 (\rightarrow) 12 (\rightarrow) 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Substrate = corn stover for all treatments, \(^l\) = lignin sampling point, \(^p\) = product sampling point. Duration of each treatment step is given in days. All C. thermocellum fermentations were carried out at a 5% solids loading with T media. All treatments used unsterile stover unless otherwise noted. Grey arrows represent steps that are cutoff by destructive sampling and are included for completeness.

Each sample consisted of 5.5 ± 0.01 g of air-dried corn stover. P. chrysosporium controls were prepared in 250 ml Erlenmeyer flasks, while the other groups (C. thermocellum control, C. thermocellum \(\rightarrow\) P. chrysosporium \(\rightarrow\) C. thermocellum experimental group, P. chrysosporium \(\rightarrow\) C. thermocellum experimental group, and sterile P. chrysosporium control) were prepared in 250 ml bottles. All glassware was autoclaved at 121 °C for 30 minutes @ 15 psig prior to sample weighing. Butyl rubber stoppers were
used, along with the extractor screw caps, which were present to prevent metabolic gas pressure from removing the stoppers during the \textit{C. thermocellum} fermentation. Aluminum foil was used to cover containers during fungal growth phases for any treatment group including a period of \textit{P. chrysosporium} growth.

One treatment substituted the initial fermentation with a period of time in a 63 °C oven (i.e. 63 °C $\rightarrow$ \textit{P. chrysosporium} $\rightarrow$ \textit{C. thermocellum}) to separate the effect of elevated air temperature from fermentation by \textit{C. thermocellum} on the subsequent effectiveness of \textit{P. chrysosporium} treatment.

The \textit{Ct} $\rightarrow$ \textit{Pc} $\rightarrow$ \textit{Ct} treatment groups required a phase of drying because the moisture content of the corn stover after \textit{C. thermocellum} fermentation was too high for \textit{P. chrysosporium} growth (>80% wet basis). Bottles were open and dried at 40 °C in an oven and then cooled to room temperature prior to inoculation with \textit{P. chrysosporium}. It should be noted that 45 °C is the maximum drying temperature allowed by the NREL lignin quantification protocol [53]. Initially, bottles were placed on their sides in the 40 °C oven to expose more of the material surface area to the heat. Bottles were also rotated once a day until dry in order to reorient the material and speed up the drying process. Any treatment requiring rehydration had initial weights recorded for material and bottles so that material loss from the fermentation phase could be quantified for moisture content calculations. Any rehydration was done using sterile DI to achieve a moisture content of 75%.

Substrate drying was accomplished using a butyl rubber stopper with syringe needle in/out ports. Bench-top air lines were hooked to the in ports for drying the material. Air passed through a 0.2 µm filter upstream of the syringe air ports. The air-out port used a larger gauge needle than the air-in to reduce air pressure in the bottle. Excessive pressure sometimes occurred as material dried and the substrate began to swirl inside the bottles. Material blocking the outlet port on the stopper was the main cause of
this pressure build-up. Air flow rates were held at the lowest rate possible to avoid over-
pressurizing.

2.2.4 *P. chrysosporium* inoculum

White rot inoculum was prepared in liquid culture containing 4 g/L potato extract
and 10 g/L dextrose in DI H₂O (inoculum broth). One liter of inoculum broth was prepared
in a 6 L Erlenmeyer flask and autoclaved at 121 °C for 30 minutes @ 15 psig. Spores from
the inoculum prepared on corn stover were added to the broth and the flask was covered
with sterile aluminum foil and maintained at 37 °C in a shaker incubator, rotating at 125
rpm for 48 hours. Mycelia pellets were harvested and used to inoculate the appropriate
experimental groups. Pellets were homogenized in a blender prior to inoculation and
rinsed with three 100 ml aliquots of DI water to remove residual soluble sugar from the
inoculum broth. Three grams of wet, rinsed pellets were used to inoculate each replicate.
Pellets were determined to be ~97% water by mass and pellets were considered 100% 
waters for purposes of moisture content calculations. Inoculated samples were all brought
to a moisture content of 75 % wet basis. Moisture content ($m_c$) is determined using the
following equation.

\[
m_c = \frac{mass_{water}}{mass_{total}} = \frac{mass_{water}}{mass_{water} + mass_{dry\ matter}}
\]

Equation 2.1: Biomass moisture content ($m_c$) wet basis

Liquid inoculum was composed predominately of mycelia, which were able to begin
regrowing immediately upon introduction to a solid substrate. This approach is contrasted
with using spore inoculum, which requires an initial lag period to transition from a dormant
state to an actively growing state.
2.2.5 HPLC sample preparation

Prior to HPLC analysis, all samples (C. thermocellum fermentation products) were prepared for analysis using two cycles of thaw and refreeze in order to remove particulates accumulated during fermentation. After thawing, sample tubes were spun in a centrifuge (thermo scientific) at 14.8 x g for 30 minutes. Next, 1 ml of supernatant was transferred to a clean 1.5 ml centrifuge tube and subjected to another centrifuge treatment at 14.8 x g for 30 minutes. Approximately 900 µl of this secondary supernatant were transferred to a 1.5 ml glass, screw cap HPLC vial. Samples were analyzed as quickly as the HPLC lab queue would permit; if samples could not be analyzed immediately, they were stored at 4 °C until analysis could begin.

2.2.6 HPLC method

The HPLC system used was a Dionex Ultimate 3000 (Hercules, CA), controlled using Chromeleon 7 software over a USB connection. The column used for fermentation products was a Biorad Aminex HPX-87H column, which is composed of a divinylbenzene polymer with attached SO₃ groups, with H guard column. This column supports the separation of fermentation acids as well as some sugars. The mobile phase used for this experiment was 5 mM H₂SO₄.

Once the column was installed inside the column compartment, the flow rate of the mobile phase was set to 0.200 ml/min and allowed to flow for 30 minutes, after which the flow rate was increased to 0.300 ml/min. This was repeated until the desired operating flow rate of 0.400 ml/min was achieved. Before the start of analysis, the mobile phase with a flow rate of 0.400 ml/min was permitted to flush the column for at least 30 minutes and the refractive index (RI) detector output was examined to check for a stable starting baseline. If the baseline was not 0 µRIU, the initial flushing process was continued. The
column compartment was maintained at a temperature of 50 °C and the sample compartment at 8 °C. The injection volume was 100 µl for standards and samples.

NREL acid digests (see 2.2.7) were screened for the presence of cellobiose, glucose, and xylose, whereas *C. thermocellum* fermentation samples were screened for cellobiose, glucose, xylose, lactate, acetate, ethanol, and formate. Six levels of standards were prepared consisting of the following: for acids the six levels were – 100 mM, 50 mM, 25 mM, 10 mM, 5 mM, and 1 mM. For sugars: 5 g/L, 2.5 g/L, 1.25 g/L, 0.5 g/L, 0.25 g/L, and 0.05 g/L. The lowest level standard registered component peak heights of only 2-3 µRIU, which is close to the lower limit of the Shodex 101 refractive index detector of 0.25 µRIU. All data fit within the range prescribed by the standard curve. Standards were injected at the beginning and end of each run of 27 samples to illustrate the extent of peak drifting over the course of the run. Peak drift was observed to be between 0.1 and 0.2 minutes and did not present a significant difficulty for peak identification.

### 2.2.7 Lignin and Sugar analysis

The NREL (National Renewable Energy Laboratory) laboratory analytical procedure (LAP) "Determination of Structural Carbohydrates and Lignin in Biomass" [53] was used to determine the extent of lignin removal with respect to the control. In addition to quantifying acid soluble and acid insoluble lignin, this protocol was also used to quantify structural as well as non-structural carbohydrates. Prior to the day on which acid hydrolysis was to be carried out, filter crucibles (w/filter) were placed in a furnace set to 575 °C for 24 hours in order to "ash" the crucibles. After ashing, the crucibles were placed in a desiccator and allowed to cool for at least one hour prior to weighing to the nearest 0.1 mg. Prior to analysis the sample material was exhaustively extracted first with water at 80 °C, and then ethanol at 70 °C using a Dionex ASE 350 extractor system. After extraction,
samples were allowed to air dry at room temperature for 24 hours. Material with significant extractives content can cause irreproducible lignin results, so extraction of material prior to analysis is mandatory [53].

After drying, 0.3 g ± 0.01g of each sample was weighed out; this weight is referred to as the air dry weight. The oven dry weight (ODW) is the air dry weight multiplied by the total solids in the sample. Biomass samples to be analyzed for composition were weighed into 125 ml glass bottles and treated with 3 ± 0.01 mL of 72% sulphuric acid. All bottles were then placed in a water bath set to 30 °C for the duration of one hour, with intermittent mixing every 5-10 minutes using a glass stir rod. After the 60-minute hydrolysis, bottles were removed from the water bath and samples were diluted to a 4% acid concentration by adding 84.00 ± 0.04 mL of deionized water. Bottles were capped and autoclaved at 121 °C for one hour.

Autoclaved hydrolysis solutions were vacuum filtered through the previously weighed filter crucibles. The filtrate from each sample was saved and stored in a 50 ml plastic tube for acid soluble lignin and carbohydrate analysis. The filtered solids were washed with a minimum of 50 ml of fresh deionized water. The crucibles and acid insoluble residue were dried at 105 °C until a constant weight was achieved, which was usually a period of time >4 hours. After obtaining post 105 °C sample weights, crucibles were placed in a furnace set to 575 °C for 24 hours. Again crucibles were allowed to cool in a desiccator for one hour and then each sample, (crucibles + ash), were weighed to the nearest 0.1 mg. Percent acid insoluble lignin (% AIL) was calculated using the following equation.

**Equation 2.2**: Calculation of biomass acid insoluble lignin (% AIL) on a mass basis

\[
\text{% AIL} = \frac{(\text{Weight}_{\text{crucible plus AIR}} - \text{Weight}_{\text{crucible}}) - (\text{Weight}_{\text{crucible plus ash}} - \text{Weight}_{\text{crucible}}) - \text{Weight}_{\text{protein}}}{\text{ODW}_{\text{sample}}} \times 100
\]
NREL studies have shown that only a very small fraction of the protein condenses into the residue, so the weight of the protein was neglected in this study.

Determination of acid soluble lignin should be done within 6 hours of the filtering step. A UV-Visible spectrophotometer was used to determine the amount of acid soluble lignin in each sample. Hydrolysis liquor from each sample had its absorbance measured in a polystyrene cuvette at the NREL recommended wavelength for corn stover, which is 320 nm. Samples should be diluted and reanalyzed if the absorbance value is not within 0.7 - 1.0, relative to a DI water blank. All samples were measured in duplicate. The percent acid soluble lignin can be calculated using the formula below. The value of \( \varepsilon \) for corn stover at the recommended wavelength is 30 L/ g cm. Volume of the filtrate = 86.73 mL and Pathlength = pathlength of the UV-Vis cell, which was 1 cm for this experiment.

**Equation 2.3:** Calculation of biomass acid soluble lignin (% ASL) on a mass basis

\[
\text{% ASL} = \frac{\text{UVabs} \times \text{Volumefiltrate} \times \text{Dilution}}{\varepsilon \times \text{ODWsample} \times \text{Pathlength}} \times 100
\]

**Equation 2.4:** Definition of the dilution factor for calculation of %

\[
\text{Dilution} = \frac{\text{Volume}_{\text{sample}} + \text{Volume}_{\text{diluting solvent}}}{\text{Volume}_{\text{sample}}}
\]

**Equation 2.5:** The total amount of biomass lignin on an extractives free basis

\[
\text{% Lignin}_{\text{ext free}} = \text{% AIL} + \text{% ASL}
\]

Carbohydrate analysis requires that approximately 20 mL of hydrolysis liquor be transferred to an Erlenmeyer flask. CaCO₃ was used to bring each sample to within a pH of 5-6. The neutralized liquid was then filtered using a 0.2 \( \mu \)m filter attached to a syringe and the liquid transferred to an HPLC autosampler vial. Sugar samples were analyzed for the
presence of xylose and glucose. The percentage of each sugar on an extractives free basis is defined by the following:

\[ \% \text{Sugar}_{\text{ext free}} = \frac{C_{\text{anhydro}} \times \text{Volume}_{\text{filtrate}} \times 1g/1000mg}{ODW_{\text{sample}}} \times 100 \]

\(C_{\text{anhydro}}\) is given by \(C_{\text{anhydro}} = C_{\text{corr}} \times \text{anhydro correction}\). The anhydro correction is 0.88 for C5 sugars and 0.90 for C6 sugars. Also, \(C_{\text{corr}} = \frac{C_{\text{HPLC}} \times \text{dilution factor}}{\% \text{Rave.sugar} / 100}\) and \(\% \text{Rave.sugar}\) is the average recovery of a sugar recovery standard after dilute acid hydrolysis.

\[ \% \text{R}_{\text{sugar}} = \frac{\text{conc.detected by HPLC}}{\text{known conc.of sugar before hydrolysis}} \]

The sugar quantification of the NREL procedure calls for the quantification of the sugars lost during dilute acid hydrolysis.

The NREL protocol is a classical "wet" method used for biomass determination. Standard wet chemical methods have the advantage of reliability when used to analyze various feedstocks, however they are labor intensive and costly, and consequently are not feasible for use on an industrial scale. Infrared spectroscopy is being examined as a faster, lower cost method which can be used in place of current chemical methods. Fourier transform infrared spectroscopy (FTIR) has been used to determine the composition of lignocellulosic biomass [54]. Use of infrared spectroscopy for biomass determination has the advantages of being non-destructive as well as inexpensive in terms of cost per sample. One of the difficulties currently hindering the implementation of infrared spectroscopy at this time is the need for multivariate statistical models, which are used to process the spectral data. These models often lead to a very accurate prediction of one component (e.g. lignin), while another component is grossly misestimated (e.g. xylan). Typically a large population (>100 samples) is needed in order to develop a reliable calibration model. For
research purposes, chemical methods are still preferred, however this may change if IR analysis achieves sufficient accuracy for all biomass components [55].

2.3 Experimental Design and Statistical Analysis

Table 2.1 in section 2.2.3 shows a list of the experimental treatments. Superscripts depict destructive sampling points as well as what type of data for which the sample was analyzed (metabolic products or lignin). The duration of each treatment phase (days) is listed in parentheses.

Treatment biomass samples were subjected to NREL compositional analysis (L superscript) or *C. thermocellum* fermentation liquids were analyzed for cellobiose, glucose, xylose, lactate, acetate, ethanol, and formate using HPLC (P superscript). Data were statistically analyzed for each set of experiments using the *proc glm* statement in SAS® statistical software. If a significant difference between at least one pair of treatment means was detected, a Tukey test was carried out to examine differences in total carbon or biomass composition (xylose, glucose, or lignin) among all combinations of treatment means. A complete list of treatments for the *P. chrysosporium* pretreatment/sterilization experiment on corn stover is given in Table 2.1 in section 2.2.3.
2.4 Results

Tables 2.2, 2.3, and 2.4 present the ANOVA results for each dependent variable affected by the main effect of treatment type. Treatment means calculated from the NREL data are shown in figure 2.1.

![Figure 2.1](image)

**Figure 2.1.** Results of NREL compositional analysis of extractives-free corn stover subjected to *P. chrysosporium* inoculation at 75% moisture content wet basis.

**Table 2.2.** ANOVA table with treatment as an explanatory variable and xylose % by mass in extractives free corn stover as the response variable.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>4</td>
<td>26.11</td>
<td>6.53</td>
<td>1.52</td>
<td>0.2680</td>
</tr>
<tr>
<td>Error</td>
<td>10</td>
<td>42.90</td>
<td>4.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>14</td>
<td>69.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3. ANOVA table with treatment as an explanatory variable and glucose % by mass in extractives free corn stover as the response variable.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>4</td>
<td>53.21</td>
<td>13.30</td>
<td>3.85</td>
<td>0.0380</td>
</tr>
<tr>
<td>Error</td>
<td>10</td>
<td>34.58</td>
<td>3.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>14</td>
<td>87.79</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4. ANOVA table with treatment as an explanatory variable and lignin % by mass in extractives free corn stover as the response variable.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>4</td>
<td>29.45</td>
<td>7.36</td>
<td>3.73</td>
<td>0.0420</td>
</tr>
<tr>
<td>Error</td>
<td>10</td>
<td>19.76</td>
<td>1.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>14</td>
<td>49.21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2 shows that there was no significant difference among treatments in terms of % xylose on a mass basis. **Table 2.3** shows that there was a significant difference among treatments in terms of % glucose on a mass basis. A Tukey test was carried out and of the five treatments: (1) Pc$^i$ sterile control  (2) Pc$^i$ $\rightarrow$ Ct  (3) Pc$^i$ nonsterile control  (4) 63 °C $\rightarrow$Pc$^i$ $\rightarrow$ Ct  (5) Ct $\rightarrow$ Pc$^i$ $\rightarrow$ Ct; only treatments 1 and 5 were found to be statistically distinguishable (figure 2.1) in terms of glucose % on a mass basis. All other possible pairs of treatments were not significantly different from one another. A Tukey test was unable to detect a difference between treatment means regarding total lignin by mass %, even though the p-value ($\alpha = 0.05$) in **table 2.4** suggests that at least one pair of treatment means is
statistically different. Inoculated sterile controls had extensive bleaching from *P. chrysosporium* growth, so the lignin results are not as expected. It is possible that the lignolysis was not induced or was marginally induced.

All treatments designated to begin with a period of *P. chrysosporium* growth (Pc→Ct, Pc→CtP, and CtP) exhibited signs of contamination with a mold possessing a "cobweb-like" morphology. Several attempts were made to grow pure *P. chrysosporium* cultures without success. It is problematic that most molds thrive at >70% moisture content wet basis because this preference is also shared by *P. chrysosporium* [47].

From figure 2.2 it can be seen that all three experimental treatments were different from control (CtP) in terms of total carbon, however, the experimental treatments were not significantly different from each other. Each experimental treatment had a mean total carbon that was approximately half or less than half that of the control.

![Figure 2.2](image_url)  
**Figure 2.2.** Total carbon produced by 48 hour *C. thermocellum* fermentations for 3 experimental groups (Pc→CtP, 63 °C → Pc→CtP, Ct→Pc→CtP) and a control group CtP. Error bars are ± 1 standard deviation.
Table 2.5. ANOVA results for total carbon produced by 48 hour *C. thermocellum* fermentations for 3 experimental groups (Pc → CtP, 63 °C → Pc → CtP, Ct → Pc → CtP) and a control group (CtP).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>0.0172</td>
<td>0.0057</td>
<td>43.90</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>0.0010</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>11</td>
<td>0.0182</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.5 Discussion

Mold that is harmful to livestock (typically *Fusarium*) may be present in corn silage as well as a high percentage of stalks and stover [41]. Molds are known to produce general toxins (mycotoxins) as part of their secondary metabolism [41], which could in part explain the inability of *P. chrysosporium* inoculum to proliferate on unsterile corn stover. With between 400-500 known mycotoxins [41], many of whose production is poorly understood, it is difficult to assess chemically what is occurring in this culture. Once produced, most mycotoxins are not destroyed by heat, time, or fermentation. Also, the relationship between mold/mycotoxin is reported to be less than straightforward. It is possible to have visible mold and not have any mycotoxins; Conversely, it is also possible to not see any visible mold and have relatively high levels of mycotoxins present [41].

Treatments with a *C. thermocellum* phase first (Ct → PcL → Ct and Ct → Pc → CtP) were washed and air-dried prior to rehydration with DI water to 75% moisture content and inoculation with *P. chrysosporium*. These treatments did not show signs of *P.*
chrysosporium growth, however, no growth of a mold contaminant was evident. This sequential process appears to have some effectiveness in terms of repressing P. chrysosporium's fungal competition. Substrate remaining at the end of the C. thermocellum fermentation was rinsed with three 50 ml aliquots of sterile DI water to remove media salts prior to P. chrysosporium inoculation. An attempt to grow P. chrysosporium on unwashed material was unsuccessful if following a C. thermocellum fermentation. Inorganic salts are known to help induce the lignin degrading peroxidase system of Phanerochaete chrysosporium [40]; over-rinsing of the material may have occurred resulting in excessive loss of salts necessary for mycelial proliferation. Inorganic salts reported to be important for inducing lignolysis by P. chrysosporium are also present in T-media (table 2.6) used to culture C. thermocellum. There may be an optimal amount of rinsing that will leave a sufficient level of beneficial inorganic salts.

Table 2.6. Inorganic salts shown to affect P. chrysosporium extracellular enzyme activity.

<table>
<thead>
<tr>
<th>T-media</th>
<th>Singh et al. 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>MgSO₄</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>MnSO₄</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>CuSO₄</td>
</tr>
<tr>
<td>ZnSO₄</td>
<td>ZnSO₄</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>FeSO₄</td>
</tr>
</tbody>
</table>

Treatments with an initial phase of elevated temperature (63 °C → Pcḷ → Ct and 63 °C → Pc → Ctₚ) showed varying degrees of successful P. chrysosporium proliferation, but did not match the complete colonization and bleaching of material as in the sterile control (Pcḷ). In general, the advancement of P. chrysosporium growth is easy to spot with the naked eye because of the enzymatic activity of P. chrysosporium's extracellular oxidases bleaches any biomass [35] in which it comes into contact. In these temperature-pretreated treatments, the bleaching was seen in varying portions of the material but never occurred
over the material in its totality. The bleached material formed a very defined boundary with the unbleached material. It is possible that 63 °C is not severe enough so that the mold seen in previous treatments is sufficiently weakened. As mentioned previously, there is probably a good reason for this which lies in the metabolites (mycotoxins) being produced by the mold present in the corn stover material. *P. chrysosporium* was repeatedly shown to grow unabated and completely on material that had been autoclaved at 121 °C and 15 psig for 30 minutes. Extensive *P. chrysosporium* conidia (spore) formation was only seen in the sterile control treatment.

One hypothesis as to why Pc→CtP, and Ct→Pc→CtP had lower mean total carbon than control is that the Pc treated material effectively did not receive pretreatment due to lack of *P. chrysosporium* growth and the latter was actually a secondary fermentation (easily accessible sugars already used in the primary fermentation). What is surprising is the 63 °C→Pc→CtP treatment had a lower total mean carbon than control; this is surprising because partial bleaching of the material was observed, indicating lignolytic activity. One reason for this result might be that metabolites produced during *P. chrysosporium* growth may be toxic to *C. thermocellum* or mycelia were physically blocking cellulosomal access to the substrate. A 2007 study [47] showed *P. chrysosporium* mycelial mass to be a significant factor influencing the extent of subsequent cellulose hydrolysis efficiency. Cellulose hydrolysis efficiency was actually lower for submerged culture and SSF Pc pretreated groups when compared with controls comprised of untreated material.
2.6 References


Appendices

Appendix A. MATLAB code used to display treatment means for \textit{C. thermocellum} growing on switchgrass and corn stover substrates.

This was the program used to generate all three-dimensional bar graphs in this document. Each figure has its own dedicated script and the only differences between scripts are the locations accessed by \textit{xlsread} and the source file for means and error. A further generalization of this routine would be writing it as a function which takes 'sheet name' and error variables as arguments. Comments are prefaced with a \% character.

%%%%%%    By : WES 7/17/2014
%%%%% Create a surface plot of \textit{C. thermocellum} activity
%%%%% \textit{C. thermocellum} products, under various Temperature and [solids]

products = xlsread('means.xlsx' , 'ethanol'); \% import the hplc data for [product]

% define levels for different fermentation conditions
Temp = [20 30 40 50 63]; \% levels of temperature in celsius
solids_time = [1 2 3 4 5 6 7 8 9]; \% placeholders for solids_timestamp strings
labels = {'5-24' '5-48' '5-72' '10-24' ...
'10-48' '10-72' '15-24' '15-48' '15-72'}; \% array of strings that will be used to label the y-axis
labels2 = {'20' '30' '40' '50' '63'};

set(gca, 'YTick', 1:9 , 'YTickLabel', labels); \% label the y-axis with appropriate strings

xlabel('temp') \% label the x-axis
ylabel('[solids] & timestamp') \% label the y-axis
zlabel('products') \% label the z-axis

figure(1) \% create a figure
h = bar3(products, 'detached'); \% create a 3d bar graph w/detached bars
title ethanol

for n = 1:length(h) \% this loop defines the coloring gradient of the bars
    zdata = get(h(n),'ZData');
    set(h(n),'CData',zdata,...
        'FaceColor','interp');
end

% use a preset colormap
colorbar % display a colorbar to the right of the graph

set(gca, 'YTick', 1:9, 'YTickLabel', labels); % label the y-axis with strings appropriate
set(gca, 'XTick', 1:5, 'XTickLabel', labels2); % label the x-axis with appropriate strings

xlabel('temp(Celsius)') % label the x-axis
ylabel('[solids](\%) & timestamp(hours)') % label the y-axis
zlabel('products (mM)') % label the z-axis

hold on;

e_ethanol = xlsread('i_am_error.xlsx', 'ethanol');  
% read in excel file with margin of error  
% values, error bars are +/- 1 std dev

labels_x = [1 2 3 4 5]; % create numerical arrays for use in error bar generation
labels_y = [1 2 3 4 5 6 7 8 9];

for i = 1:length(labels_x)       % nested loop for generating error bars
  for k = 1:length(labels_y)
    xV = [labels_x(i); labels_x(i)]; % x array for error bar placement
    yV = [labels_y(k); labels_y(k)]; % y array for error bar placement
    zMin = products(k,i); %+ e_ethanol(k,i); % calculate lower confidence limit
    zMax = products(k,i) + e_ethanol(k,i); % calculate upper confidence limit
    zV = [zMin , zMax];
    % plot points defining line of error bar and draw error bar
    g = plot3(xV, yV, zV, '-k');
    set(g, 'LineWidth', 2); % define width of error bar
  end
end

end
Note the differences between the above script for ethanol the script for lactate below...

products = xlsread('means.xlsx', 'lactate'); % import the hplc data for [product]

% define levels for different fermentation conditions
Temp = [20 30 40 50 63]; % levels of temperature in celsius
solids_time = [1 2 3 4 5 6 7 8 9]; % placeholders for solids_timestamp
labels = {'5-24' '5-48' '5-72' '10-24' ...
'10-48' '10-72' '15-24' '15-48' '15-72'}; % array of strings that will be used to label the y-axis
labels2 = {'20' '30' '40' '50' '63'}; % strings for x-axis labels

set(gca, 'YTick', 1:9 , 'YTickLabel', labels); % label the y-axis with appropriate strings
xlabel('temp') % label the x-axis
ylabel('[solids] & timestamp') % label the y-axis
zlabel('products') % label the z-axis

figure(1) % create a figure
h = bar3(products, 'detached'); % create a 3d bar graph w/detached bars
title lactate

for n = 1:length(h) % this loop defines the coloring gradient of the bars
  zdata = get(h(n),'ZData');
  set(h(n),'CData',zdata,...
    'FaceColor','interp');
end

% use a preset colormap
colorbar % display a colorbar to the right of the graph

set(gca, 'YTick', 1:9 , 'YTickLabel', labels); % label the y-axis with appropriate strings
set(gca, 'XTick', 1:5 , 'XTickLabel', labels2); % label the x-axis with appropriate strings
xlabel('temp(Celsius)') % label the x-axis
ylabel(['solids'](%) & timestamp(hours')) % label the y-axis
zlabel('products (mM)') % label the z-axis

hold on;

e_lactate = xlsread('i_am_error.xlsx' , 'lactate');
    % read in excel file with margin of error
    % values, error bars are +/- 1 std dev

labels_x = [1 2 3 4 5]; % create numerical arrays for use in error bar
    % generation
labels_y = [1 2 3 4 5 6 7 8 9];

for i = 1:length(labels_x) % nested loop for generating errorbars
    for k = 1:length(labels_y)
        xV = [labels_x(i); labels_x(i)]; % x array for error bar
            % placement
        yV = [labels_y(k); labels_y(k)]; % y array for error bar
            % placement
        zMin = products(k,i); %+ e_lactate(k,i); % calculate lower confidence
            % limit
        zMax = products(k,i) + e_lactate(k,i); % calculate upper confidence
            % limit
        zV = [zMin , zMax];

        % plot points defining line of error bar and draw error bar
        g = plot3(xV, yV, zV, '-k');

        set(g, 'LineWidth', 2); % define width of error bar
    end
end
Appendix B. Greatest product means produced by *C. thermocellum* ATCC 27405 growing on switchgrass and corn stover at solids loadings 5, 10, 15% and temperatures 20 °C, 30 °C, 40 °C, 50 °C, 63 °C.

<table>
<thead>
<tr>
<th>product</th>
<th>treatment</th>
<th>substrate</th>
<th>value</th>
<th>std dev.</th>
<th>units</th>
</tr>
</thead>
<tbody>
<tr>
<td>cellobiose</td>
<td>5_48_20°C</td>
<td>corn stover</td>
<td>350.76</td>
<td>31.51</td>
<td>mg/L</td>
</tr>
<tr>
<td>glucose</td>
<td>15_24_63°C</td>
<td>corn stover</td>
<td>486.67</td>
<td>5.77</td>
<td>mg/L</td>
</tr>
<tr>
<td>xylose</td>
<td>15_24_63°C</td>
<td>corn stover</td>
<td>1206.46</td>
<td>57.79</td>
<td>mg/L</td>
</tr>
<tr>
<td>lactate</td>
<td>15_48_40°C</td>
<td>corn stover</td>
<td>28.79</td>
<td>12.58</td>
<td>mM</td>
</tr>
<tr>
<td>formate</td>
<td>15_72_50°C</td>
<td>corn stover</td>
<td>37.99</td>
<td>2.31</td>
<td>mM</td>
</tr>
<tr>
<td>acetate</td>
<td>15_72_63°C</td>
<td>corn stover</td>
<td>41.72</td>
<td>6.60</td>
<td>mM</td>
</tr>
<tr>
<td>ethanol</td>
<td>15_72_63°C</td>
<td>corn stover</td>
<td>34.32</td>
<td>3.24</td>
<td>mM</td>
</tr>
<tr>
<td>sugar</td>
<td>15_24_63°C</td>
<td>corn stover</td>
<td>2006.80</td>
<td>69.27</td>
<td>mg/L</td>
</tr>
<tr>
<td>acids</td>
<td>15_72_50°C</td>
<td>corn stover</td>
<td>114.21</td>
<td>6.97</td>
<td>mM</td>
</tr>
<tr>
<td>cellobiose</td>
<td>15_72_20°C</td>
<td>switchgrass</td>
<td>302.92</td>
<td>14.71</td>
<td>mg/L</td>
</tr>
<tr>
<td>glucose</td>
<td>15_72_50°C</td>
<td>switchgrass</td>
<td>196.67</td>
<td>23.09</td>
<td>mg/L</td>
</tr>
<tr>
<td>xylose</td>
<td>10_72_63°C</td>
<td>switchgrass</td>
<td>321.80</td>
<td>110.10</td>
<td>mg/L</td>
</tr>
<tr>
<td>lactate</td>
<td>10_72_50°C</td>
<td>switchgrass</td>
<td>2.47</td>
<td>1.73</td>
<td>mM</td>
</tr>
<tr>
<td>formate</td>
<td>15_72_40°C</td>
<td>switchgrass</td>
<td>12.70</td>
<td>1.36</td>
<td>mM</td>
</tr>
<tr>
<td>acetate</td>
<td>15_72_63°C</td>
<td>switchgrass</td>
<td>27.17</td>
<td>2.08</td>
<td>mM</td>
</tr>
<tr>
<td>ethanol</td>
<td>15_48_50°C</td>
<td>switchgrass</td>
<td>7.57</td>
<td>6.86</td>
<td>mM</td>
</tr>
<tr>
<td>sugar</td>
<td>15_24_30°C</td>
<td>switchgrass</td>
<td>635.25</td>
<td>133.52</td>
<td>mg/L</td>
</tr>
<tr>
<td>acids</td>
<td>15_72_40°C</td>
<td>switchgrass</td>
<td>36.12</td>
<td>5.33</td>
<td>mM</td>
</tr>
</tbody>
</table>
Appendix C. SAS code used for performing ANOVAs in Chapter 1 and Chapter 2.

SAS code for generating treatment means shown in MATLAB generated figures. Only code for corn stover is shown for all SAS programs.

```sas
PROC IMPORT OUT = WORK.cellobiose DATAFILE= "C:\Users\E\Desktop\ct_workbook_cs.xlsx" DBMS = xlsx REPLACE;
    SHEET = "cellobiose";
    GETNAMES = YES;
RUN;

proc sort data = cellobiose;
    by sample;
run;

proc means data = cellobiose mean std;
    title cellobiose;
    class sample;
    var _20_CR _30_CR _40_CR _50_CR _63_CR;
run;

PROC IMPORT OUT = WORK.glucose DATAFILE= "C:\Users\E\Desktop\ct_workbook_cs.xlsx" DBMS = xlsx REPLACE;
    SHEET = "glucose";
    GETNAMES = YES;
RUN;

proc sort data = glucose;
    by sample;
run;

proc means data = glucose mean std;
    title glucose;
    class sample;
    var _20_CR _30_CR _40_CR _50_CR _63_CR;
run;

PROC IMPORT OUT = WORK.xylose DATAFILE= "C:\Users\E\Desktop\ct_workbook_cs.xlsx" DBMS = xlsx REPLACE;
    SHEET = "xylose";
    GETNAMES = YES;
RUN;

proc sort data = xylose;
    by sample;
run;
```
```plaintext
proc means data = xylose mean std;
title xylose;
class sample;
var _20_CR _30_CR _40_CR _50_CR _63_CR;
run;

PROC IMPORT OUT = WORK.lactate DATAFILE="C:\Users\E\Desktop\ct_workbook_cs.xlsx"
   DBMS = xlsx REPLACE;
   SHEET = "lactate";
   GETNAMES = YES;
RUN;

proc sort data = lactate;
by sample;
run;

proc means data = lactate mean std;
title lactate;
class sample;
var _20_CR _30_CR _40_CR _50_CR _63_CR;
run;

PROC IMPORT OUT = WORK.acetate DATAFILE="C:\Users\E\Desktop\ct_workbook_cs.xlsx"
   DBMS = xlsx REPLACE;
   SHEET = "acetate";
   GETNAMES = YES;
RUN;

proc sort data = acetate;
by sample;
run;

proc means data = acetate mean std;
title acetate;
class sample;
var _20_CR _30_CR _40_CR _50_CR _63_CR;
run;

PROC IMPORT OUT = WORK.ethanol DATAFILE="C:\Users\E\Desktop\ct_workbook_cs.xlsx"
   DBMS = xlsx REPLACE;
   SHEET = "ethanol";
   GETNAMES = YES;
RUN;

proc sort data = ethanol;
by sample;
run;
```
proc means data = ethanol mean std;
title ethanol;
class sample;
var _20_CR _30_CR _40_CR _50_CR _63_CR;
run;

PROC IMPORT OUT = WORK.formate DATAFILE="C:\Users\E\Desktop\ct_workbook_cs.xlsx"
   DBMS = xlsx REPLACE;
   SHEET = "formate";
   GETNAMES = YES;
RUN;

proc sort data = formate;
by sample;
run;

proc means data = formate mean std;
title formate;
class sample;
var _20_CR _30_CR _40_CR _50_CR _63_CR;
run;

SAS code for generating the total carbon ANOVA tables for Chapter 1.

PROC IMPORT OUT = corn_stover DATAFILE = "C:\Users\E\Desktop\anova_cs.xlsx"
   DBMS = xlsx REPLACE;
   SHEET = "Sheet2";
   GETNAMES = yes;
RUN;

proc print data = corn_stover;
title
run;

proc sort data = corn_stover;
by solids temp;
run;

proc means data = corn_stover;
by solids temp;
**SAS code for generating the biomass composition ANOVA tables for Chapter 2.**

```sas
PROC IMPORT OUT = corn_stover_ob2 DATAFILE = "C:\Users\E\Desktop\ob2_cs.xlsx" DBMS = xlsx REPLACE;
SHEET = "sugar_lignin";
GETNAMES = yes;
RUN;

proc print data = corn_stover_ob2;
title run;

proc sort data = corn_stover_ob2;
by trt;
run;

proc means data = corn_stover_ob2;
by trt;
var xylose glucose total_lignin;
run;

proc glm data = corn_stover_ob2;

var total_carbon;
run;

proc glm data = corn_stover;
class solids temp;
   model total_carbon = solids temp solids*temp;
lsmeans solids temp solids*temp / pdiff = all;
run;
```
class trt;
    model total_lignin = trt;
lsmeans trt / pdiff = all;
run;

proc glm data = corn_stover_ob2;
class trt;
    model glucose = trt;
lsmeans trt / pdiff = all;
run;

proc glm data = corn_stover_ob2;
class trt;
    model xylose = trt;
lsmeans trt / pdiff = all;
run;

SAS code for generating the total carbon ANOVA tables for Chapter 2.

PROC IMPORT OUT = corn_stover_ob2_totalcarbon   DATAFILE = "C:\Users\E\Desktop\ob2_cs.xlsx"
   DBMS = xlsx REPLACE;
   SHEET = "total_carbon_ob2";
   GETNAMES  = yes;
RUN;

proc print data = corn_stover_ob2_totalcarbon;
   title
run;

proc sort data = corn_stover_ob2_totalcarbon;
by trt;
run;

proc means data = corn_stover_ob2_totalcarbon;
by trt;
var total_carbon;
run;

proc glm data = corn_stover_ob2_totalcarbon;
class trt;
    model total_carbon = trt;
means trt / pdiff = all;
run;
Appendix D. T-media HPLC Background for Biorad 87H.

The table below displays the run parameters. All individual media components are juxtaposed with the total background.

<table>
<thead>
<tr>
<th>column</th>
<th>flow rate</th>
<th>mobile phase</th>
<th>column temperature</th>
<th>run time</th>
<th>detector</th>
<th>guard column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biorad 87H</td>
<td>0.4 ml/min</td>
<td>5 mM H$_2$SO$_4$</td>
<td>50 °C</td>
<td>35 min</td>
<td>Shdx RI 101</td>
<td>ionic</td>
</tr>
</tbody>
</table>

**T-media total background**
B-vitamins

Resauzarin
T1 salt

T2 salt
Appendix E. Raw data used in total carbon ANOVAs in Chapter 1 and Chapter 2.

Data used in corn stover ANOVA in Chapter 1.

<table>
<thead>
<tr>
<th>total_carbon (g)</th>
<th>Solids %</th>
<th>Temp (°C)</th>
<th>Time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0167</td>
<td>5</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>0.0341</td>
<td>5</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>0.0135</td>
<td>5</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>0.0159</td>
<td>15</td>
<td>20</td>
<td>48</td>
</tr>
<tr>
<td>0.0170</td>
<td>15</td>
<td>20</td>
<td>48</td>
</tr>
<tr>
<td>0.0161</td>
<td>15</td>
<td>20</td>
<td>48</td>
</tr>
<tr>
<td>0.0166</td>
<td>10</td>
<td>20</td>
<td>72</td>
</tr>
<tr>
<td>0.0063</td>
<td>10</td>
<td>20</td>
<td>72</td>
</tr>
<tr>
<td>0.0166</td>
<td>10</td>
<td>20</td>
<td>72</td>
</tr>
<tr>
<td>0.0184</td>
<td>10</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>0.0214</td>
<td>10</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>0.0186</td>
<td>10</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>0.0290</td>
<td>5</td>
<td>30</td>
<td>72</td>
</tr>
<tr>
<td>0.0240</td>
<td>5</td>
<td>30</td>
<td>72</td>
</tr>
<tr>
<td>0.0237</td>
<td>5</td>
<td>30</td>
<td>72</td>
</tr>
<tr>
<td>0.0159</td>
<td>15</td>
<td>30</td>
<td>72</td>
</tr>
<tr>
<td>0.0222</td>
<td>15</td>
<td>30</td>
<td>72</td>
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Data used in switchgrass ANOVA in Chapter 1.
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Corn stover treatment means from total carbon ANOVA. Chapter 1.
Switchgrass treatment means from total carbon ANOVA. Chapter 1.

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Raw data for sugar and total lignin on an extractives-free basis. Chapter 2.

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Raw data used in total carbon ANOVA, Chapter 2.

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<th>#</th>
<th>cellubiose (mg/L)</th>
<th>glucose (g/L)</th>
<th>xylose (mg/L)</th>
<th>lactate (mM)</th>
<th>formate (mM)</th>
<th>acetate (mM)</th>
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Total carbon for each sample used in total carbon ANOVA, Chapter 2.

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Appendix F. Total sugar % difference between each treatment and the corresponding treatment at 63 °C and total sugar absolute difference (mg/L) between each treatment and corresponding treatment at 63 °C.

Tables for both corn stover and switchgrass are shown. % difference was calculated using the following equation:

\[
\text{% difference} = \frac{(\text{sugar}_{63} - \text{sugar}_T)}{\text{sugar}_{63}}
\]

**Corn stover:**

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<td>-14.7%</td>
<td>-99.0%</td>
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**Switchgrass:**

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Switchgrass:

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Appendix G. MATLAB graphs of product treatment means produced from corn stover by *C. thermocellum* ATCC 27405 at 5, 10, 15% solids, temperatures of 20 °C, 30 °C, 40 °C, 50 °C, 63 °C, and sampling times of 24, 48, and 72 hrs.

Error bars = + 1 standard deviation.
Appendix H. MATLAB graphs of product treatment means produced from corn stover by *C. thermocellum* ATCC 27405 at 5, 10, 15% solids, temperatures of 20 °C, 30 °C, 40 °C, 50 °C, 63 °C, and sampling times of 24, 48, and 72 hrs.

Error bars = +1 standard deviation.
REFERENCES


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EDUCATION:

University of Louisville
B.S. Chemistry, 2006
GPA: 3.74

University of Kentucky
M.S. Biosystems and Agricultural Engineering, 2015
GPA: 3.62

RESEARCH EXPERIENCE:

University of Louisville

• Cancer Research
• Carried out cell culture work and mutagenesis studies
• Transformed cells with plasmid to knockout selected DNA repair polymerase
• Managed a colony of hairless mice, set up breeding pairs in order to obtain offspring with a desired KO genotype. Genotyped animals using PCR and gel electrophoresis.

Louisville Health Department

• Detection of lead by atomic absorption spectroscopy
• Disease detection by PCR amplification / UV spectrophotometry
University of Kentucky                              Lexington, KY
Graduate Research Assistant                       8/2011 – 7/2015

• Thesis title: “Investigation of *Phanerochaete chrysosporium* and
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