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

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THESIS

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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

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Lexington, Kentucky

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

Lexington, Kentucky

2015

ABSTRACT OF THESIS

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 monosacharides can be more easily accessed for fermentation, the lack of which is hindering the economics and widescale 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 environments 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 competeveness 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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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 ℃, 30 ℃, 40 ℃, 50 ℃, and 63 ℃ 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 ($\frac{MW\ carbon}{MW\ total}$) 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 °C and 5%_63 °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_2SO_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.
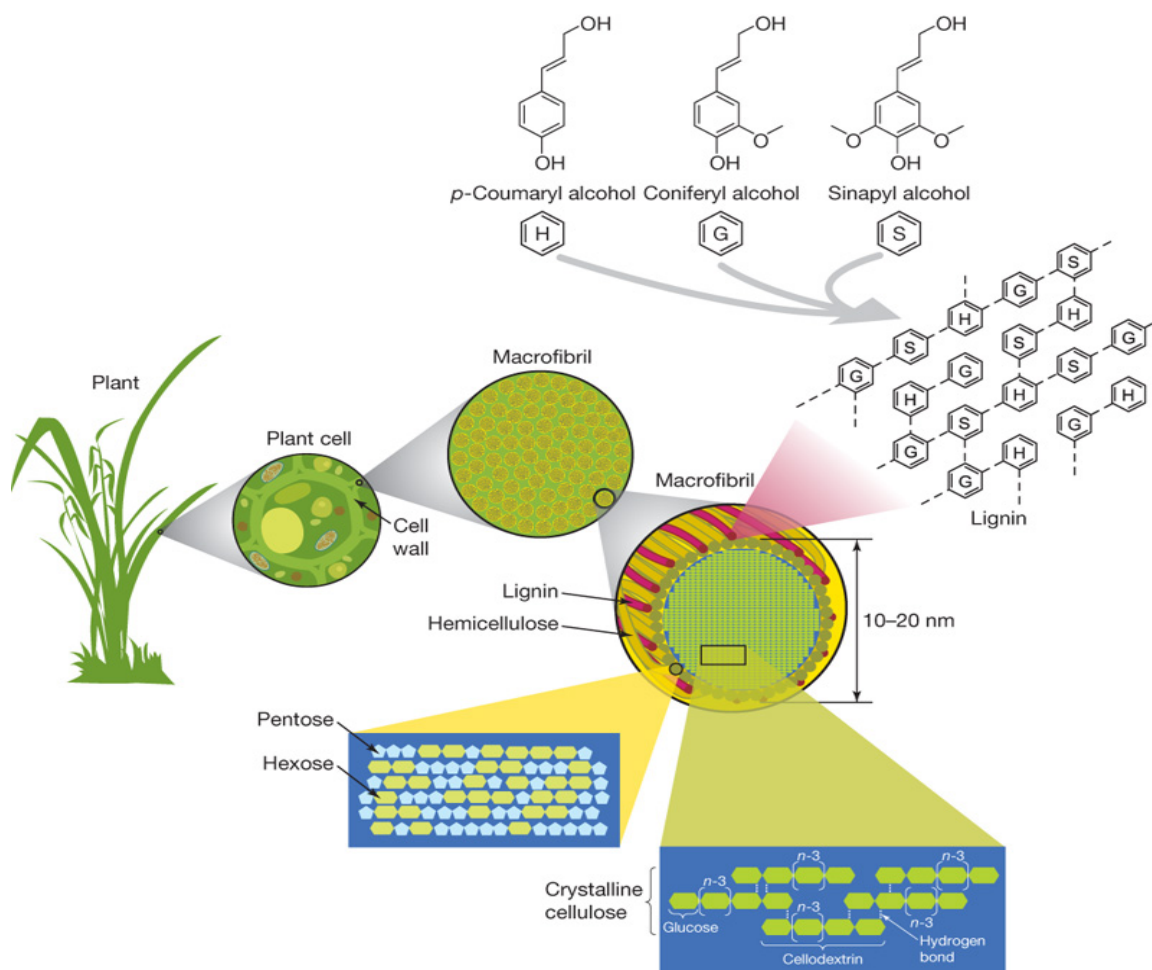
Figure 1.1. An overview of lignocellulose structure.
(from Rubin, E. Genomics of cellulosic biofuels. Nature 454, 841–845 (2008)) [12].
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1.1.2 C. thermocellum

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**].
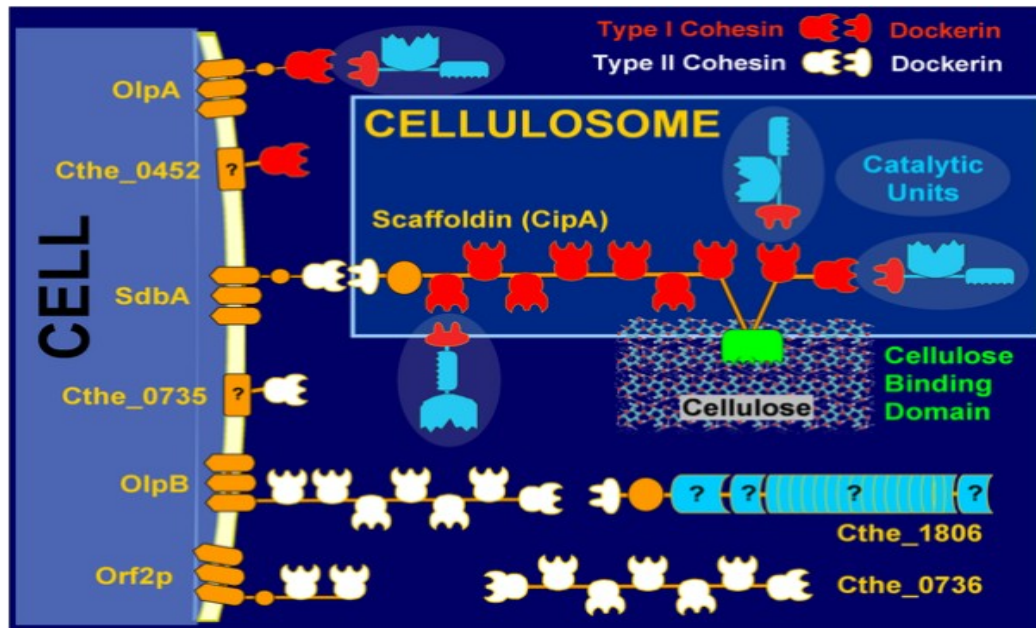
Figure 1.2. Simplified Cellulosome protein architecture.   (from Raman, B. [**14**])

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.

Pi

Glucose -1 -P ← Cellodextrin ← Cellulose
2   1

3ADP   2 NAD+

3ATP   2 NADH

2 pyruvate →3→ 2 lactic acid

2 NADH   2 NAD+
2CoA

2H$_2$

4H$^+$ 7   2Fd ox

6   4   2Fd red   5   2 Formic acid

2 NADH

2 NAD+

2Pi   2CoA   2ADP   2ATP

2 CO$_2$ 2 acetyl -CoA →8→ 2 acetyl -P →9→ 2 acetic acid

2 acetaldehyde   2 ethanol

2NADH   10   11

2NAD+   2NADH   2NAD+
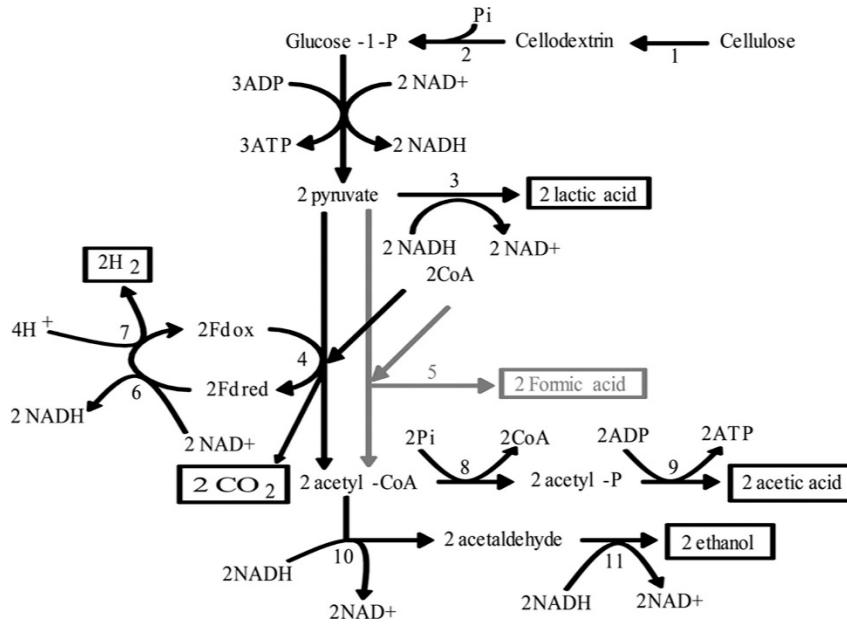
Figure 1.3. Mixed acid fermentation of *C. thermocellum.* (from Tripathi, S. [16])
Reused with permission from American Society for Microbiology.

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

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 researches 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 saccharoperbutylacetonicum* (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. saccharoperbutylacetonicum* 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 CO_2 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.

Component	per L
H_2O	850 ml
Resazurin stock	1.0 ml
Salt T1	50 ml
Salt T2	50 ml
Cysteine	0.5 g
Yeast Extract	2.0 g
Vitamins	10 ml
Modified Metals	5 ml

Table 1.1, continued.

Salt T1	per L
Na$_2$HPO$_4$·12H$_2$O	84 g
Or Na$_2$HPO$_4$	30.60 g

Salt T2	per L
KH$_2$PO$_4$	30 g
NH$_4$Cl	10 g
(NH$_4$)$_2$SO$_4$	10 g
MgCl$_2$·6H$_2$O	1.8 g
CaCl$_2$	0.6 g

Standard Vitamins components	per L
Pyridoxamine 2HCl	100 mg
Riboflavin	200 mg
Thiamine HCl	200 mg
Nicotinamide	200 mg
CaD Pantotheinate	200 mg
Lipoic Acid	100 mg
P-aminobenzoic acid	10 mg
Folic acid	5 mg
Biotin	5 mg
Cobalamin (Co B$_{12}$)	5 mg
Pyridoxal HCl	100 mg
Pyridoxine	100 mg
K$_2$HPO$_4$ or KH$_2$PO$_4$	17.4 g or 13.6 g

Modified metals components	per L
Na$_4$EDTA	500 mg
FeSO$_4$·7H$_2$O	200 mg
ZnSO$_4$·7H$_2$O	10 mg
MnCl$_2$·4H$_2$O	200 mg
H$_3$BO$_3$	20 mg
CoCl$_2$ ·6H$_2$O	20 mg
CuCl$_2$ ·2H$_2$O	1 mg
NiCl$_2$·6H$_2$O	2 mg
Na$_2$MoO$_4$·2H$_2$O	3 mg
Na$_2$WO$_4$·2H$_2$O	10 mg
Na$_2$SeO$_3$	1 mg

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 ℃ 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 ℃, 15 psig for 30 minutes. CO_2 was then bubbled into the media to maintain anaerobic conditions and 50 ml of sterile 8 % Na_2CO_3 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 (amporphous 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 $\pm$ 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 H_2. 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% N_2 gas. The chamber gas was maintained using a mixture of N_2 and H_2 (96% N_2/4% H_2). Prior to importing the work materials, final concentration of H_2 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 H_2 gas reacting on screened containers of pelleted palladium catalyst. Using this method nominal 0 ppm O_2 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.
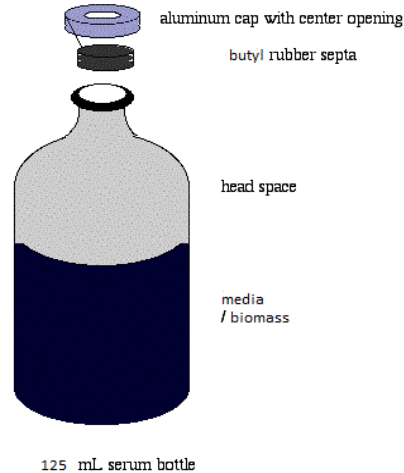
Figure 1.4. Serum bottles used for *C. thermocellum* cultivation on swithgrass and corn stover substrates

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 SO_3 groups (**figure 1.5**). This

column supports the separation of fermentation acids as well as some sugars. The mobile

phase used for this experiement was 5 mM H_2SO_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.
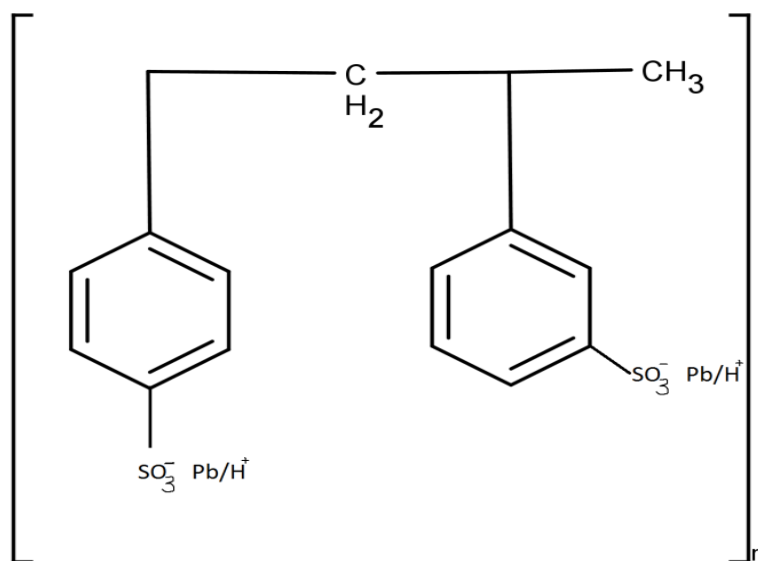
Figure 1.5. A repetitive unit found in the Biorad HPX-87P/H column

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 dectector 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 $\pm$ 5% of one another (William

Sympson, personal communication, Nov. 2014). Initially, glucose spiked media samples of 5

g/L were used to assess the suitablility of the YSI 2900 analyzer for quantifying glucose in

this particular context. Measured concentrations in this preliminary test were found to be

within $\pm$ 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

Figure 1.6. Depiction of treatments in full factorial design
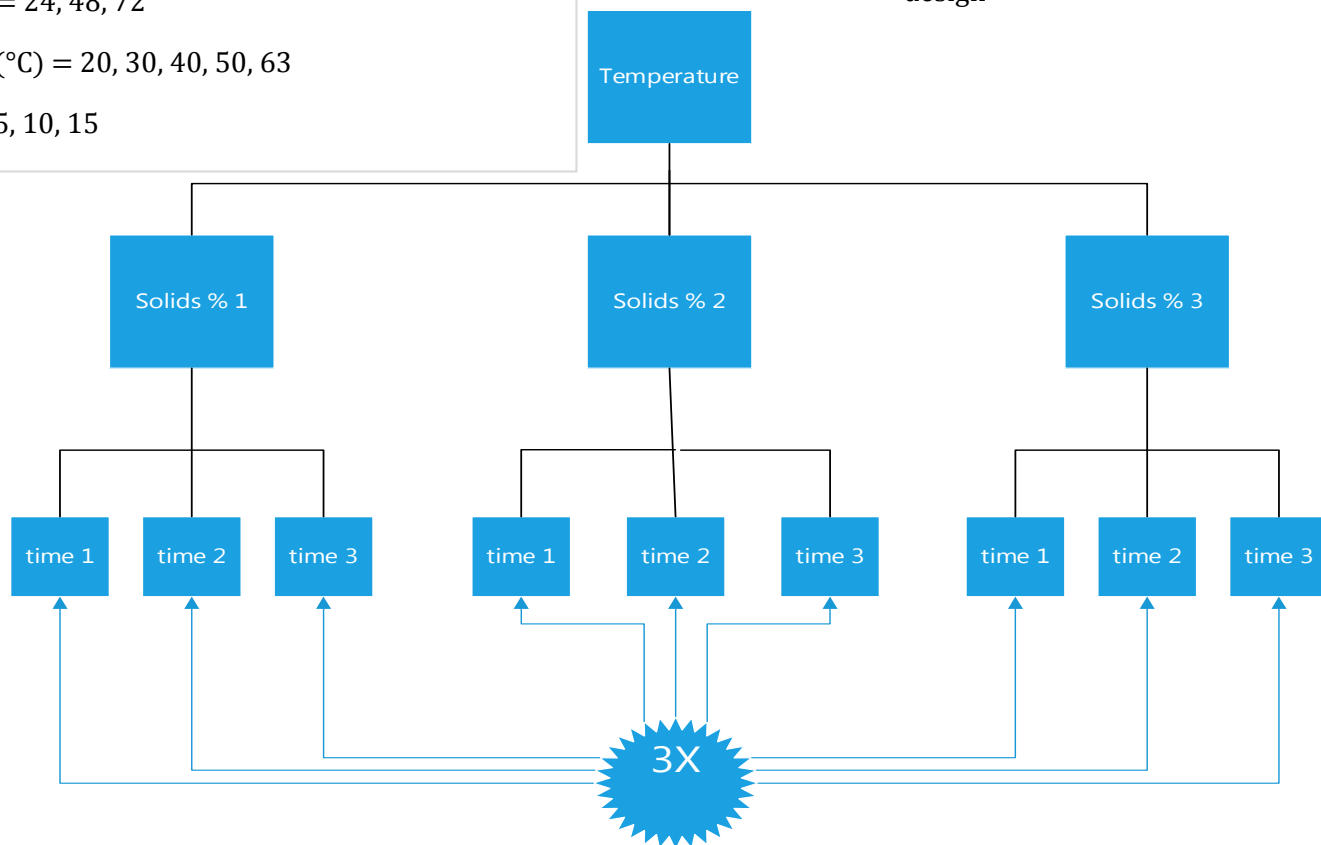
1.4   Results

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 *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 ($\frac{MW\ carbon}{MW\ molecule}$) 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 CO_2 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 *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 *C. thermocellum* on corn stover using solids loadings of 5, 10, and 15% and cultivation temperatures of 20, 30, 40, 50, and 63 °C.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	0.0267	0.0019	28.50	<.0001
Error	30	0.0020	0.0001		
Corrected Total	44	0.0287			

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.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
solids	2	0.0005	0.0003	3.90	0.0313
temp	4	0.0252	0.0063	94.29	<.0001
solids*temp	8	0.0009	0.0001	1.75	0.1269

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 ($\alpha = 0.05$), 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 ($\alpha = 0.05$), which have been highlighted in **figure 1.8,** can be seen as diagonals that travel from top left to bottom right.

					Least Squares Means for effect solids*temp										
					Pr > |t| for H0: LSMean(i)=LSMean(j)										
					Dependent Variable: total_carbon										
i/j	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	0.0124	<.0001	<.0001	0.9936	1	0.0762	<.0001	<.0001	1	1	0.0652	<.0001	0.0002
5_30	1		0.0557	0.0001	<.0001	0.8558	0.9997	0.261	<.0001	<.0001	0.9829	0.9995	0.2307	0.0002	0.0011
5_40	0.0124	0.0557		0.5291	0.0027	0.0005	0.0058	1	0.0868	0.0009	0.0017	0.0051	1	0.706	0.9566
5_50	<.0001	0.0001	0.5291		0.5067	<.0001	<.0001	0.1536	0.999	0.2772	<.0001	<.0001	0.1761	1	0.9999
5_63	<.0001	<.0001	0.0027	0.5067		<.0001	<.0001	0.0003	0.9801	1	<.0001	<.0001	0.0004	0.3429	0.1181
10_20	0.9936	0.8558	0.0005	<.0001	<.0001		0.9996	0.0036	<.0001	<.0001	1	0.9998	0.003	<.0001	<.0001
10_30	1	0.9997	0.0058	<.0001	<.0001	0.9996		0.0389	<.0001	<.0001	1	1	0.0329	<.0001	<.0001
10_40	0.0762	0.261	1	0.1536	0.0003	0.0036	0.0389		0.0145	0.0001	0.0123	0.0341	1	0.2565	0.5909
10_50	<.0001	<.0001	0.0868	0.999	0.9801	<.0001	<.0001	0.0145		0.8754	<.0001	<.0001	0.0172	0.9895	0.8418
10_63	<.0001	<.0001	0.0009	0.2772	1	<.0001	<.0001	0.0001	0.8754		<.0001	<.0001	0.0001	0.1678	0.0485
15_20	1	0.9829	0.0017	<.0001	<.0001	1	1	0.0123	<.0001	<.0001		1	0.0103	<.0001	<.0001
15_30	1	0.9995	0.0051	<.0001	<.0001	0.9998	1	0.0341	<.0001	<.0001	1		0.0288	<.0001	<.0001
15_40	0.0652	0.2307	1	0.1761	0.0004	0.003	0.0329	1	0.0172	0.0001	0.0103	0.0288		0.2891	0.6363
15_50	<.0001	0.0002	0.706	1	0.3429	<.0001	<.0001	0.2565	0.9895	0.1678	<.0001	<.0001	0.2891		1
15_63	0.0002	0.0011	0.9566	0.9999	0.1181	<.0001	<.0001	0.5909	0.8418	0.0485	<.0001	<.0001	0.6363	1	

Figure 1.8. ANOVA p-values from Tukey mutliple 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.
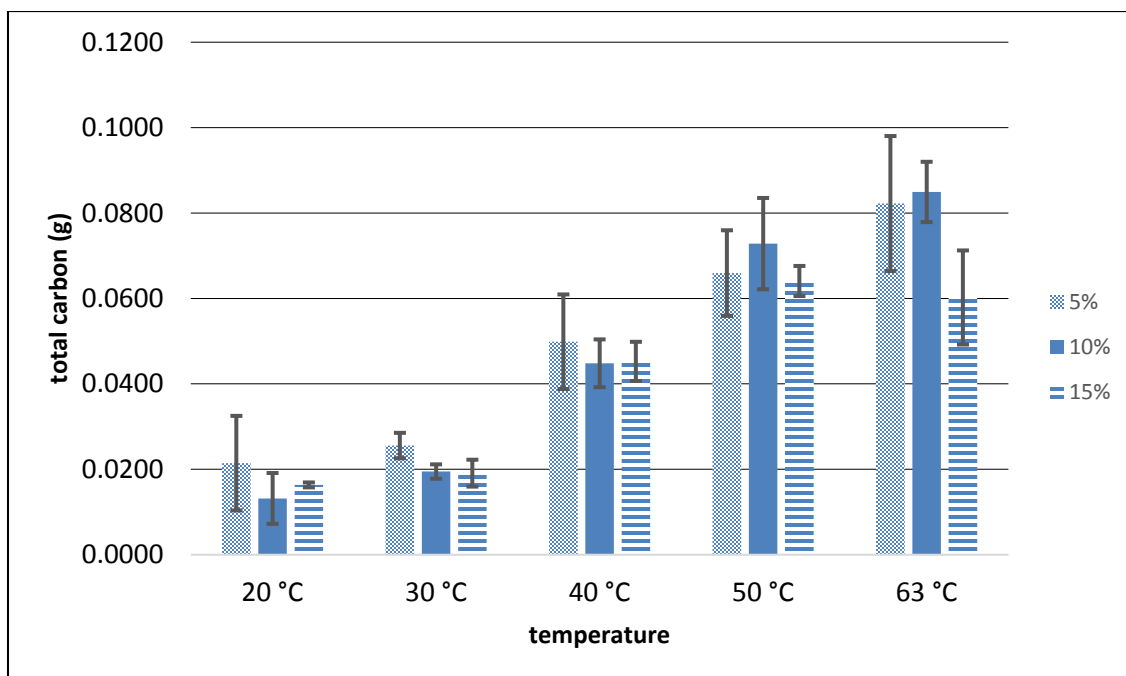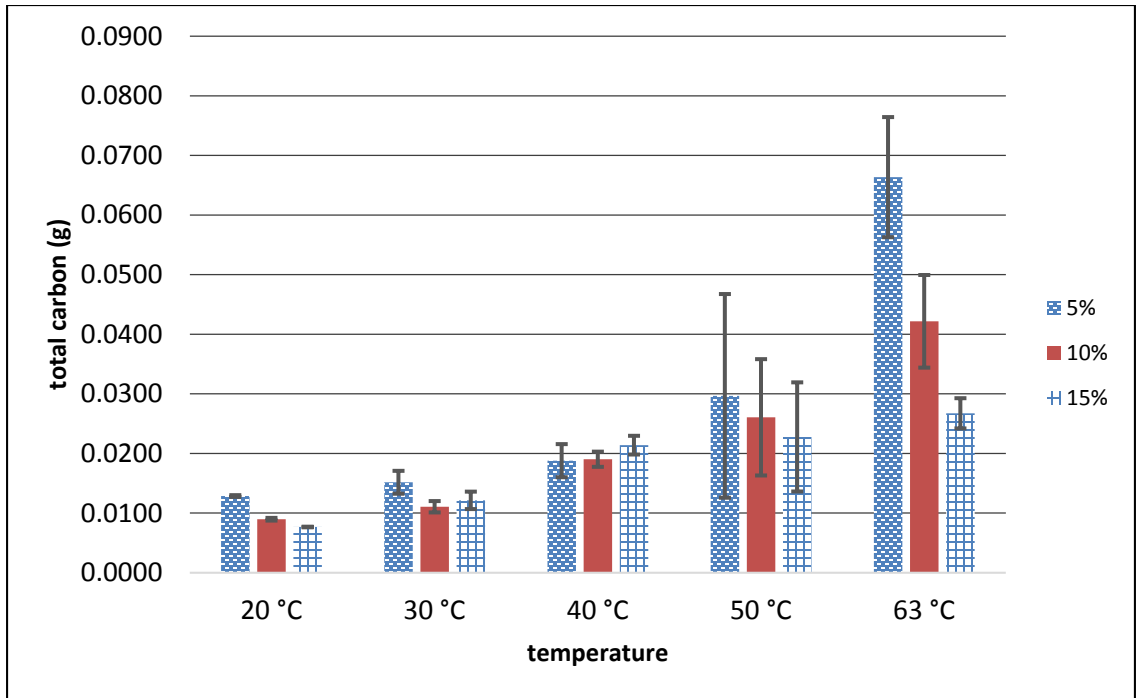
Figure 1.9. Treatment means for the fermentation products from *C. thermocellum* growing on switchgrass, using solids loadings of 5, 10, and 15% and cultivation temperatures of 20, 30, 40, 50, and 63 °C.
Error bars represent $\pm$ 1 standard deviation.

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.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	0.0096	0.0007	15.67	<.0001
Error	30	0.0013	0.0001		
Corrected Total	44	0.0109			

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.

Source	DF	Type I SS	Mean Square	F Value	Pr > F
solids	2	0.0009	0.0004	9.72	0.0006
temp	4	0.0071	0.0018	40.32	<.0001
solids*temp	8	0.0017	0.0002	4.84	0.0007

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 ($\alpha = 0.05$), 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 ($\alpha = 0.05$) 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.**

						Least Squares Means for effect solids*temp									
						Pr > |t| for H0: LSMean(i)=LSMean(j)									
						Dependent Variable: total_carbon									
i/j	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	0.997	1	1	0.7742	0.0018	0.9824	1	0.9968	0.9803	0.7009
5_40	0.998	1		0.7777	<.0001	0.8724	0.9772	1	0.9866	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	0.0199	0.1312	0.9613	0.9915	1
5_63	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	0.0071	<.0001	<.0001	<.0001	<.0001	<.0001
10_20	1	0.997	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	0.978
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
10_63	0.0006	0.0018	0.0106	0.5901	0.0071	<.0001	0.0002	0.0118	0.2192		<.0001	0.0004	0.0347	0.0624	0.2726
15_20	0.9995	0.9824	0.7494	0.0199	<.0001	1	1	0.7236	0.0938	<.0001		0.9999	0.4442	0.3024	0.0716
15_30	1	1	0.9939	0.1312	<.0001	1	1	0.9915	0.4217	0.0004	0.9999		0.9124	0.8013	0.3509
15_40	0.9508	0.9968	1	0.9613	<.0001	0.5982	0.8298	1	0.9999	0.0347	0.4442	0.9124		1	0.9994
15_50	0.8671	0.9803	1	0.9915	<.0001	0.4366	0.6855	1	1	0.0624	0.3024	0.8013	1		1
15_63	0.4291	0.7009	0.972	1	<.0001	0.1201	0.253	0.978	1	0.2726	0.0716	0.3509	0.9994	1	

Figure 1.10. ANOVA p-values from Tukey mutliple 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.
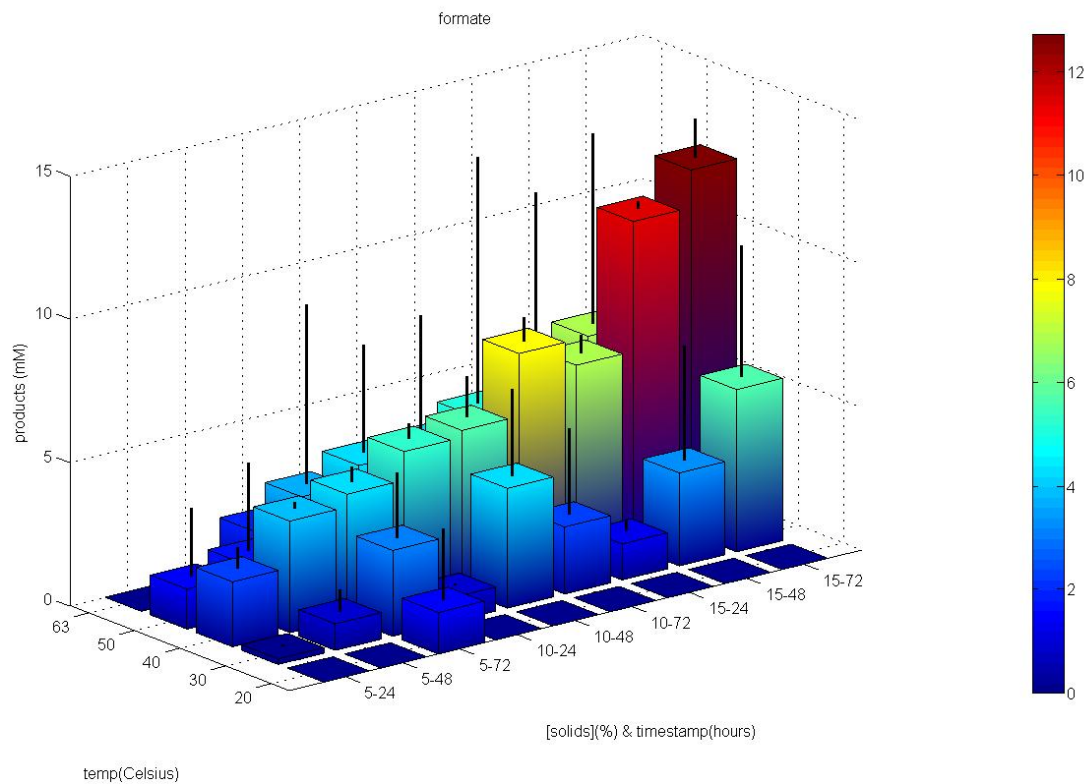
Figure 1.14. Treatment means for formate (mg/L) from *C. thermocellum* cultivation on switchgrass using solids loadings of 5, 10, and 15%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours.
Error bars represent +1 standard deviation.

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**).
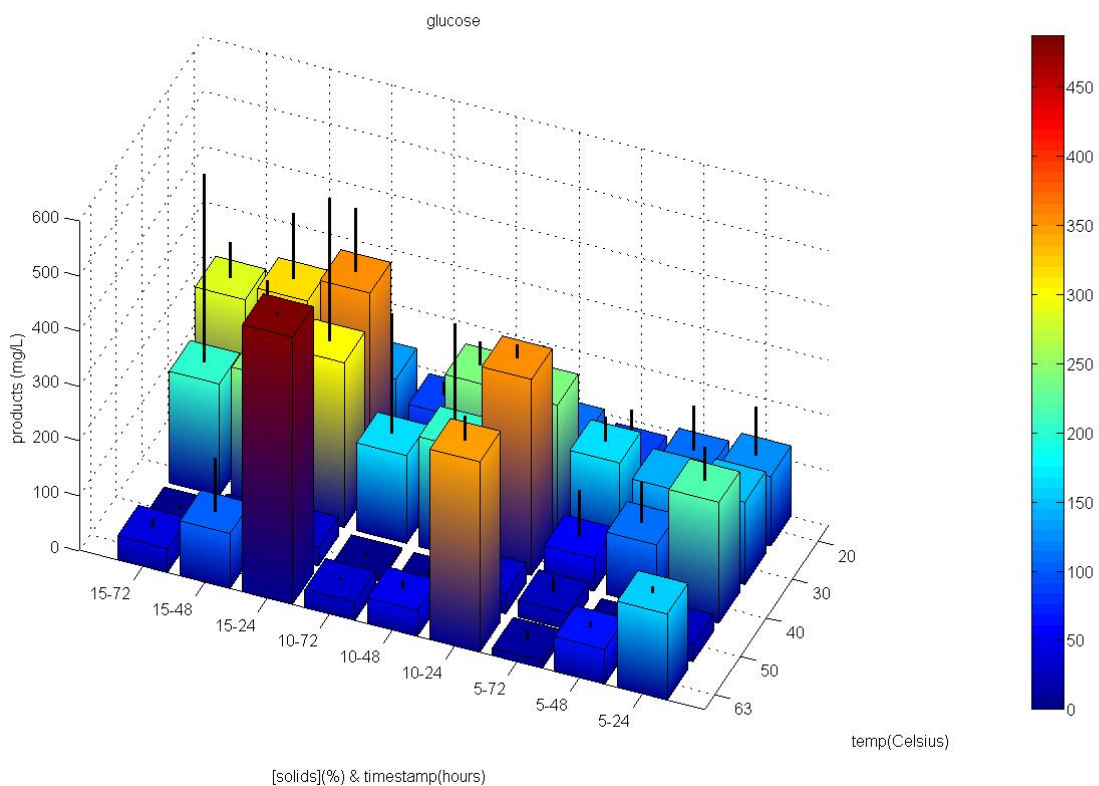
Figure 1.15. Treatment means for glucose (mg/L) from *C. thermocellum* cultivation on corn stover using solids loadings of 5, 10, and 15%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours.
Error bars represent +1 standard deviation.
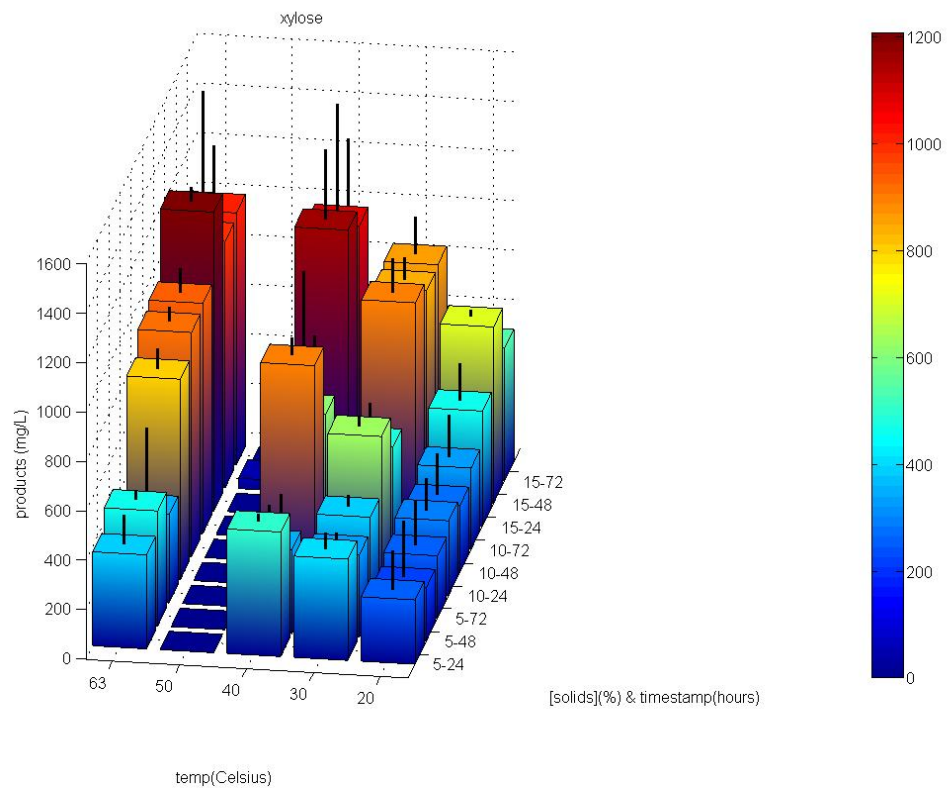
Figure 1.16. Treatment means for xylose (mg/L) from *C. thermocellum* cultivation on corn stover using solids loadings of 5, 10, and 15%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours.
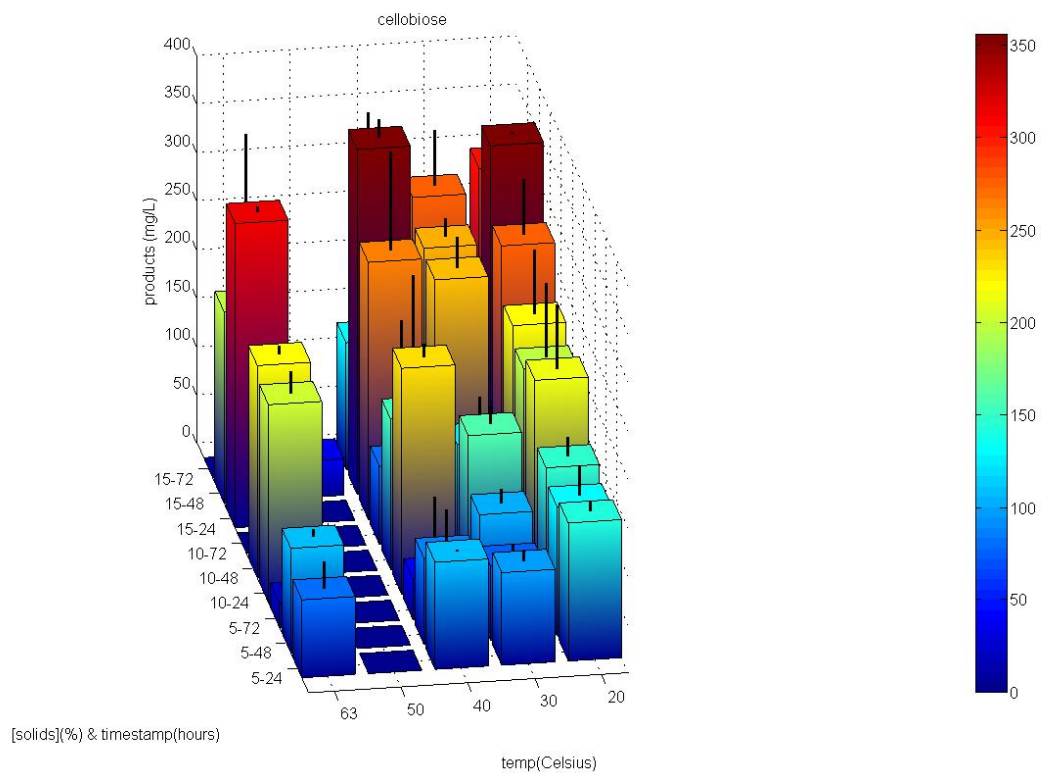Error bars represent +1 standard deviation.
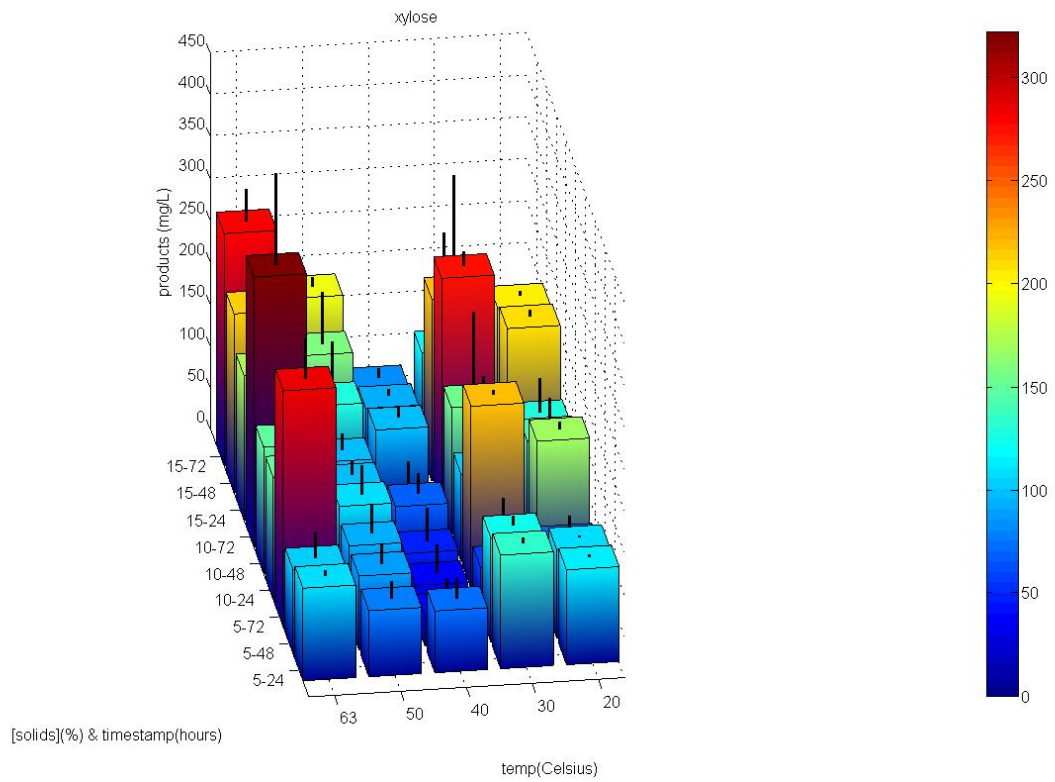
Figure 1.17. Treatment means for the xylose (mg/L) from *C. thermocellum* cultivation on switchgrass using solids loadings of 5, 10, and 15%, cultivation temperatures of 20, 30, 40, 50, 63 °C, and sampling times of 24, 48, and 72 hours.
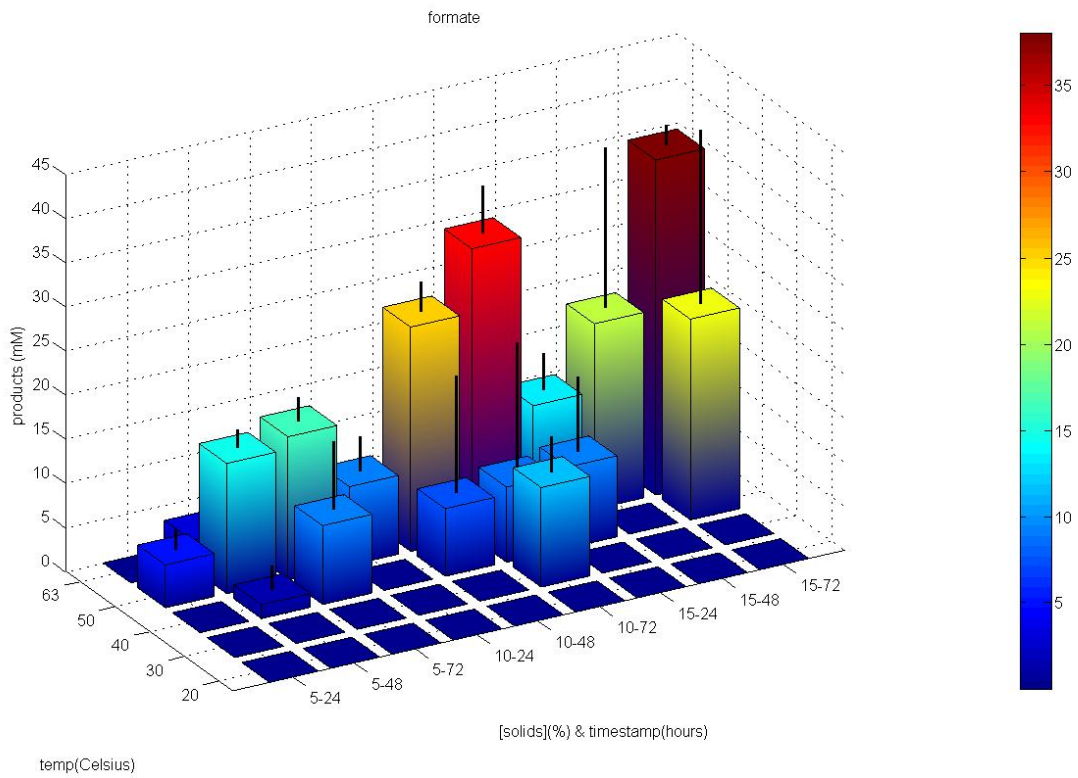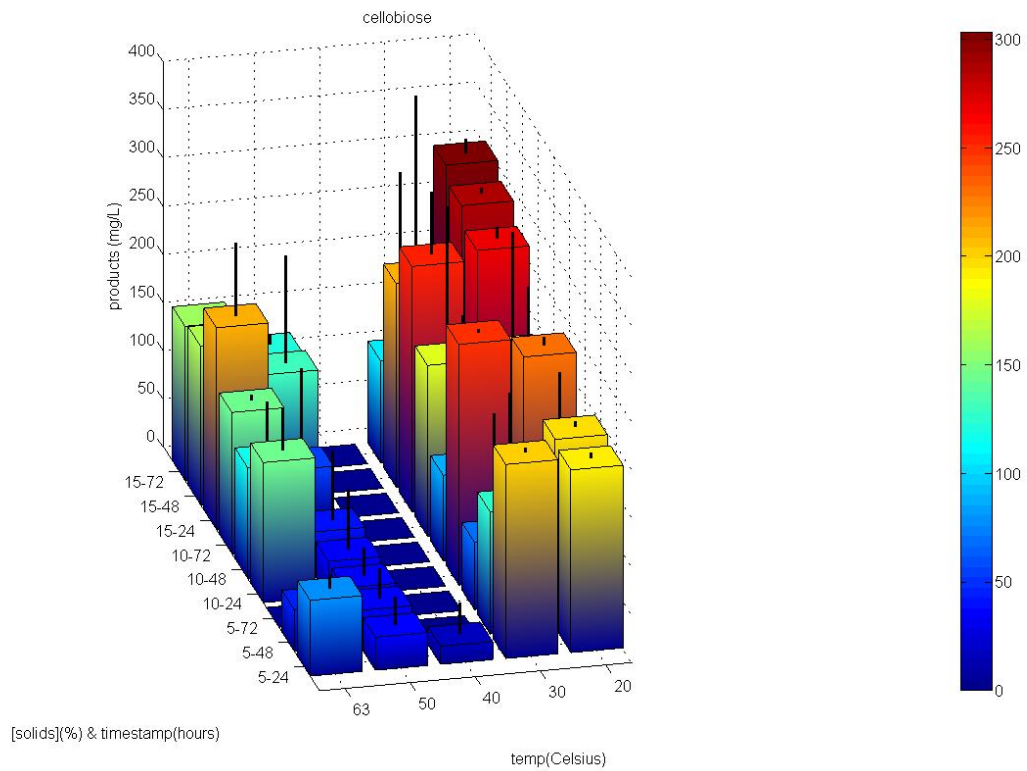Error bars represent +1 standard deviation.

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 I-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 ($\geq 15\%$), 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.

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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 pretreament 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, enyzmes 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 saccharfication 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 liginolytic 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 Ca^{2+}, 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 Mn^{2+} to Mn^{3+} in the presence of peroxide. Mn^{3+} is complexed with an organic acid

chelator such as oxalate or malonate, which are produced as metabolic byproducts of fungal

cell metabolism. This Mn^{3+} : chelator complex diffuses from the surface of the enzyme and

is able to oxidize organic substrates; a process which converts Mn^{3+} back to an Mn^{2+} state.

Organic acid chelators facilitate the release of Mn^{3+} from the enzyme active site and also

serve as a means to stabilize Mn^{3+} with a high redox potential in aqueous solution [49].

Experiments by Mester et al. (1995) [50] showed that addition of exogenous Mn^{2+}

stimulated manganese peroxidase synthesis. Lignin peroxidase and veratryl alcohol

production were in turn inhibited by increased concentration of Mn^{2+}. Absence of Mn^{2+} 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

diffusable 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 feasability 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 saccharificaition 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 ~36 %, compared to ~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, *P. chrysosporium* may actually inhibit enzymatic access to cellulose without some kind of post-pretreatment process to alter mycelia-related obstruction.

2.1.3 Experimental Objectives

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

2.2 Materials and Methods

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.

2.2.2 Thermophile media, Bacterial Inoculum

Thermophile media and *C. thermocellum* inoculum were prepared as described in section 1.2.3 and section 1.2.4, respectively. Any *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 treaments for *P. chrysosporium* pretreatment/sterilization experiment on corn stover.

Ct^P	Pc^L (sterile)	Pc^L (unsterile)
(2)	(12)	(12)
$Pc \rightarrow Ct^P$	$Pc^L \rightarrow Ct$	$63\,°C \rightarrow Pc^L \rightarrow Ct$
($12 \rightarrow 2$)	($12 \rightarrow 2$)	($5 \rightarrow 12 \rightarrow 2$)
$63\,°C \rightarrow Pc \rightarrow Ct^P$	$Ct \rightarrow Pc^L \rightarrow Ct$	$Ct \rightarrow Pc \rightarrow Ct^P$
($5 \rightarrow 12 \rightarrow 2$)	($5 \rightarrow 12 \rightarrow 2$)	($5 \rightarrow 12 \rightarrow 2$)

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* → *P. chrysosporium* → *C. thermocellum* experimental group, *P. chrysosporium* → *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 *C. thermocellum* fermentation. Aluminum foil was used to cover containers during fungal growth phases for any treatment group including a period of *P. chrysosporium* growth.

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

The Ct → Pc → Ct treatment groups required a phase of drying because the moisture content of the corn stover after *C. thermocellum* fermentation was too high for *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 *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. chyrsosporium inoculum

White rot inoculum was prepared in liquid culture containing 4 g/L potato extract and 10 g/L dextrose in DI H_2O (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% water 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.

Equation 2.1: Biomass moisture content (m_c) wet basis

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

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 SO_3 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 H_2SO_4.

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 $\pm$ 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 $\pm$ 0.01 mL of 72% sulphuric acid. All bottles were then placed in a water bath set to 30 $^\circ$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 $\pm$ 0.04 mL of deionized water. Bottles were capped and autoclaved at 121 $^\circ$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 $^\circ$C until a constant weight was achieved, which was usually a period of time >4 hours. After obtaining post 105 $^\circ$C sample weights, crucibles were placed in a furnace set to 575 $^\circ$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

$$\% \text{ AIL} = \frac{\left(\text{Weight}_{\text{crucible plus AIR}} - \text{Weight}_{\text{crucible}}\right) - \left(\text{Weight}_{\text{crucible plus ash}} - \text{Weight}_{\text{crucible}}\right) - \text{Weight}_{\text{protein}}}{\text{ODW}_{\text{sample}}} \times 100$$

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 ε 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

$$\% \text{ ASL} = \frac{\text{UVabs x Volume}_{\text{filtrate}} \text{ x Dilution}}{\varepsilon \text{ x ODW}_{\text{sample}} \text{ x Pathlength}} \text{ x } 100$$

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

$$\text{Dilution} = \frac{Volume_{sample} + Volume_{diluting\ solvent}}{Volume_{sample}}$$

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

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

Carbohydrate analysis requires that approximately 20 mL of hydrolysis liquor be transferred to an Erlenmeyer flask. $CaCO_3$ was used to bring each sample to within a pH of 5-6. The neutralized liquid was then filtered using a 0.2 µm 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:

Equation 2.6: Biomass % Sugar by mass on an extractives free basis.

$$\% \ Sugar_{ext \ free} = \frac{C_{anhydro} \ X \ Volume_{filtrate} \ X \ 1g/1000mg}{ODW_{sample}} \ X \ 100$$

$C_{anhydro}$ is given by $C_{anhydro} = C_{corr}$ x anhydro correction. The anhydro correction is

0.88 for C5 sugars and 0.90 for C6 sugars. Also, $C_{corr} = \frac{C_{HPLC} \ X \ dilution \ factor}{\% \ R_{ave.sugar}/100}$ and $R_{ave. \ sugar}$ is

the average recovery of a sugar recovery standard after dilute acid hydrolysis.

$\% \ R_{sugar} = \frac{conc.detected \ by \ HPLC}{known \ conc.of \ sugar \ before \ hydrolysis}$. 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 treaments 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.
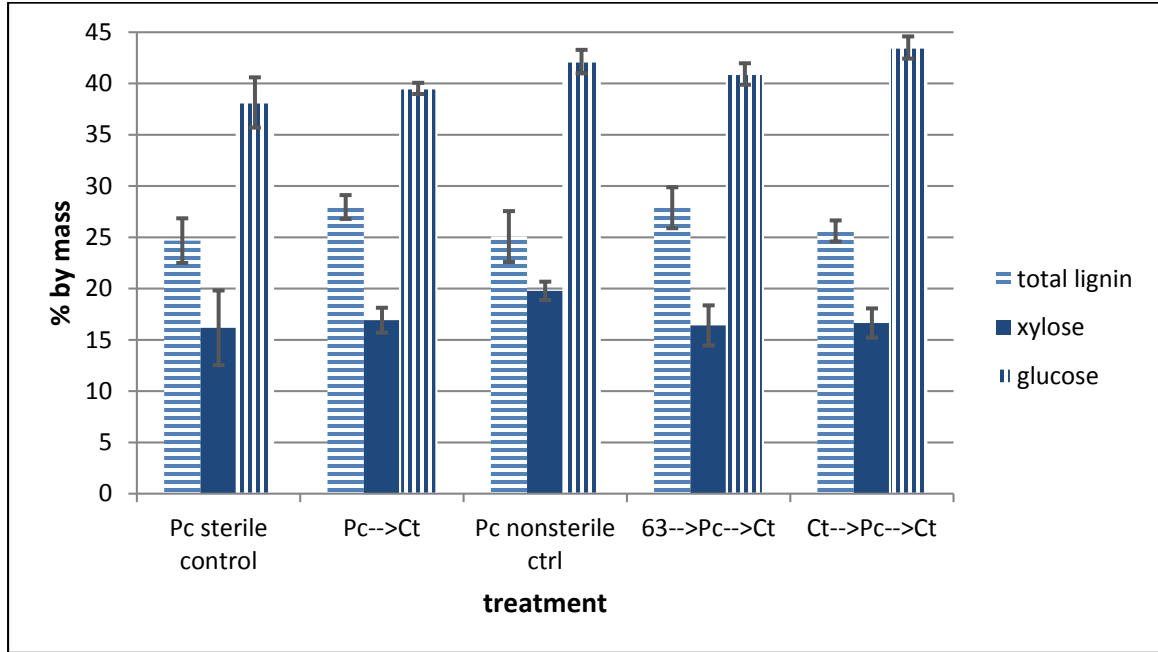

Figure 2.1. Results of NREL compositional analysis of extractives-free corn stover subjected to *P. chrysosporium* inoculation at 75% moisture content wet basis.

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

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	26.11	6.53	1.52	0.2680
Error	10	42.90	4.29		
Corrected Total	14	69.01			

Table 2.3. ANOVA table with treatment as an explanatory variable and glucose % by mass in extractives free corn stover as the response variable.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	53.21	13.30	3.85	0.0380
Error	10	34.58	3.46		
Corrected Total	14	87.79			

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

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	29.45	7.36	3.73	0.0420
Error	10	19.76	1.98		
Corrected Total	14	49.21			

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^L sterile control (2) $Pc^L \rightarrow Ct$ (3) Pc^L nonsterile control (4) 63 °C $\rightarrow Pc^L \rightarrow Ct$ (5) $Ct \rightarrow Pc^L \rightarrow Ct$; 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 treaments 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

64

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[L] (nonsterile), Pc[L]→Ct, and Pc→Ct[P]) 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 (Ct[P]) 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.
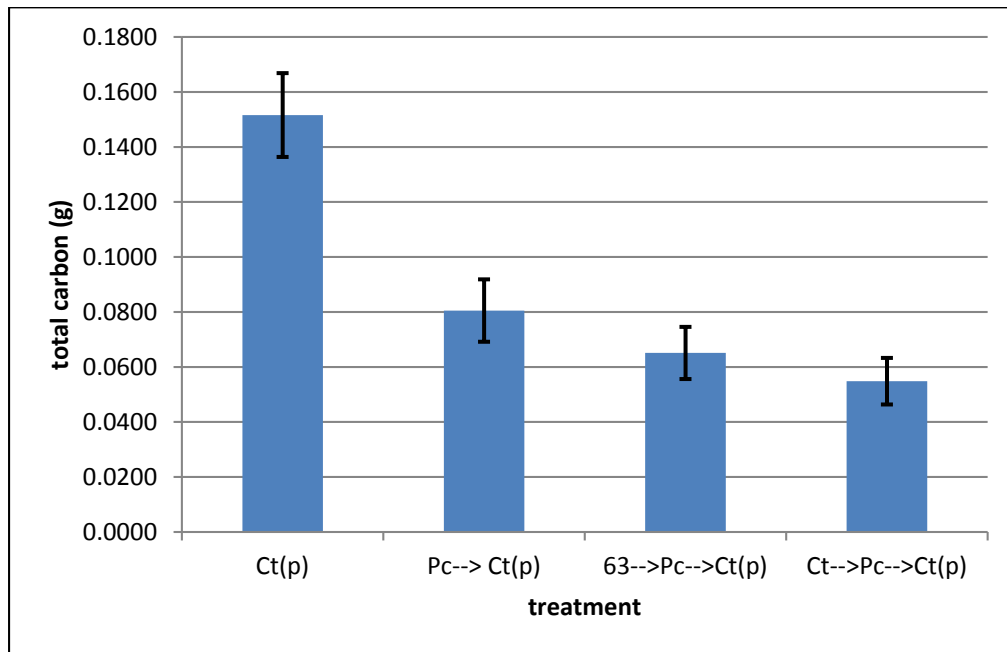

Figure 2.2. Total carbon produced by 48 hour *C. thermocellum* fermentations for 3 experimental groups (Pc→ Ct[P] , 63 °C → Pc → Ct[P], Ct →Pc →Ct[P]) and a control group Ct[P]. Error bars are $\pm$ 1 standard deviation.

Table 2.5. ANOVA results for total carbon produced by 48 hour *C. thermocellum* fermentations for 3 experimental groups (Pc→ Ct^P , 63 °C → Pc → Ct^P, Ct →Pc →Ct^P) and a control group (Ct^P).

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.0172	0.0057	43.90	<.0001
Error	8	0.0010	0.0001		
Corrected Total	11	0.0182			

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 → Pc^L → Ct and Ct → Pc → Ct^P) 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. chrysoporium* 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. chrysoporium* 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.

T-media	Singh et al. 2010
$MgCl_2$	$MgSO_4$
$MnCl_2$	$MnSO_4$
$CuCl_2$	$CuSO_4$
$ZnSO_4$	$ZnSO_4$
$FeSO_4$	$FeSO_4$

Treatments with an initial phase of elevated temperature (63 °C → Pc^L → Ct and 63 °C → Pc → Ct^P) showed varying degrees of successful *P. chrysosporium* proliferation, but did not match the complete colonization and bleaching of material as in the sterile control (Pc^L). 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* extracelluar 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→ Ct^P , and Ct→Pc→ Ct^P 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→ Ct^P 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

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Appendix A. MATLAB code used to display treatment means for *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 *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.

```matlab
%%%%%%%%%%%%%%%%%%%%%%    By : WES 7/17/2014
%%%%%%% Create a surface plot of C. thermocellum activity
%%%%%%% 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');
```

```matlab
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 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_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
```

Note the differences between the above script for ethanol the script for lactate below…

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

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
                                   % 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'}; % 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
```

```matlab
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.

product	treatment	substrate	value	std dev.	units
cellobiose	5_48_20°C	corn stover	350.76	31.51	mg/L
glucose	15_24_63°C	corn stover	486.67	5.77	mg/L
xylose	15_24_63°C	corn stover	1206.46	57.79	mg/L
lactate	15_48_40°C	corn stover	28.79	12.58	mM
formate	15_72_50°C	corn stover	37.99	2.31	mM
acetate	15_72_63°C	corn stover	41.72	6.60	mM
ethanol	15_72_63°C	corn stover	34.32	3.24	mM
sugar	15_24_63°C	corn stover	2006.80	69.27	mg/L
acids	15_72_50°C	corn stover	114.21	6.97	mM
cellobiose	15_72_20°C	switchgrass	302.92	14.71	mg/L
glucose	15_72_50°C	switchgrass	196.67	23.09	mg/L
xylose	10_72_63°C	switchgrass	321.80	110.10	mg/L
lactate	10_72_50°C	switchgrass	2.47	1.73	mM
formate	15_72_40°C	switchgrass	12.70	1.36	mM
acetate	15_72_63°C	switchgrass	27.17	2.08	mM
ethanol	15_48_50°C	switchgrass	7.57	6.86	mM
sugar	15_24_30°C	switchgrass	635.25	133.52	mg/L
acids	15_72_40°C	switchgrass	36.12	5.33	mM

Appendix C. SAS code used for performing ANOVAs in Chapter 1 and Chapter 2.

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

```
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;
```

```
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;
```

```sas
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.

```sas
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
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;
```

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;
```

```
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;
```

```sas
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.

column	flow rate	mobile phase	column temperature	run time	detector	guard column
Biorad 87H	0.4 ml/min	5 mM H_2SO_4	50 °C	35 min	Shdx RI 101	ionic

T-media total background
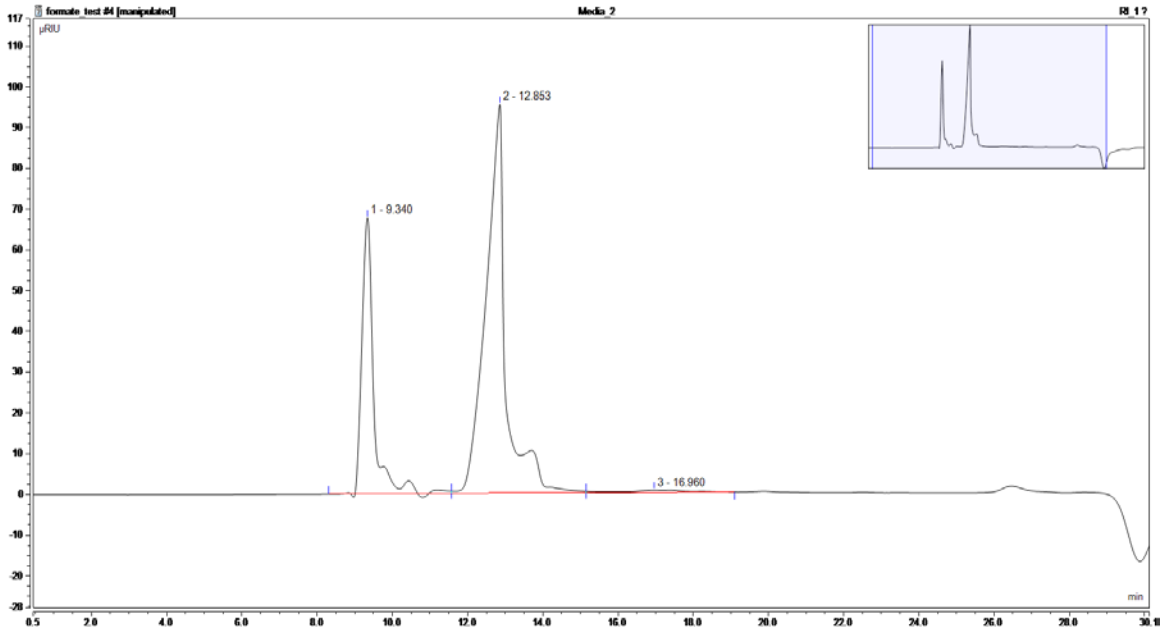
B-vitamins
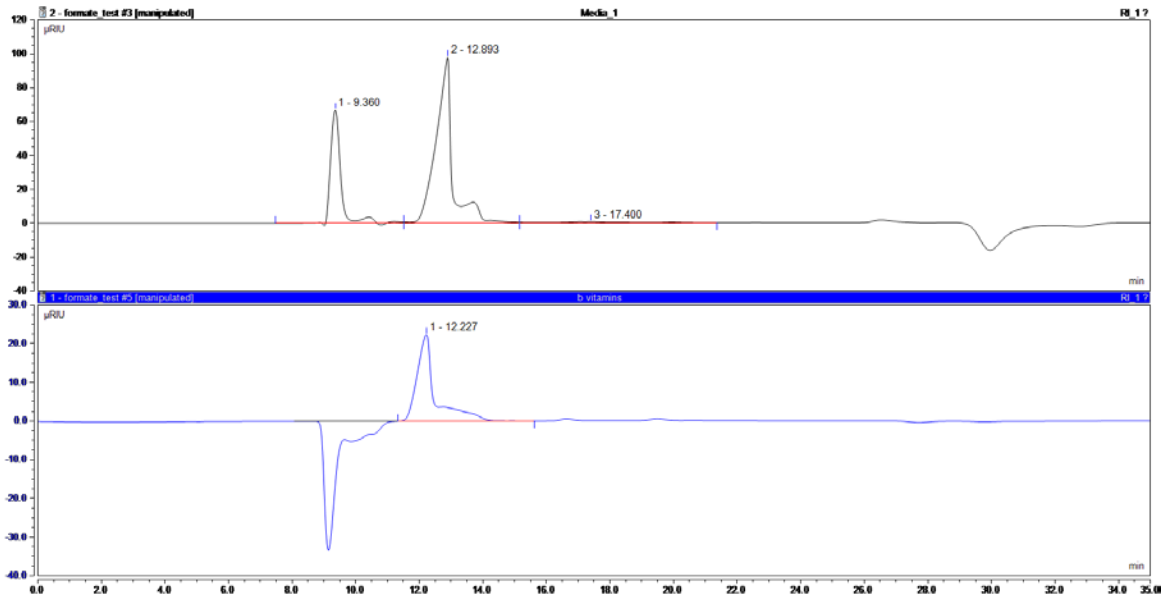
Resauzarin
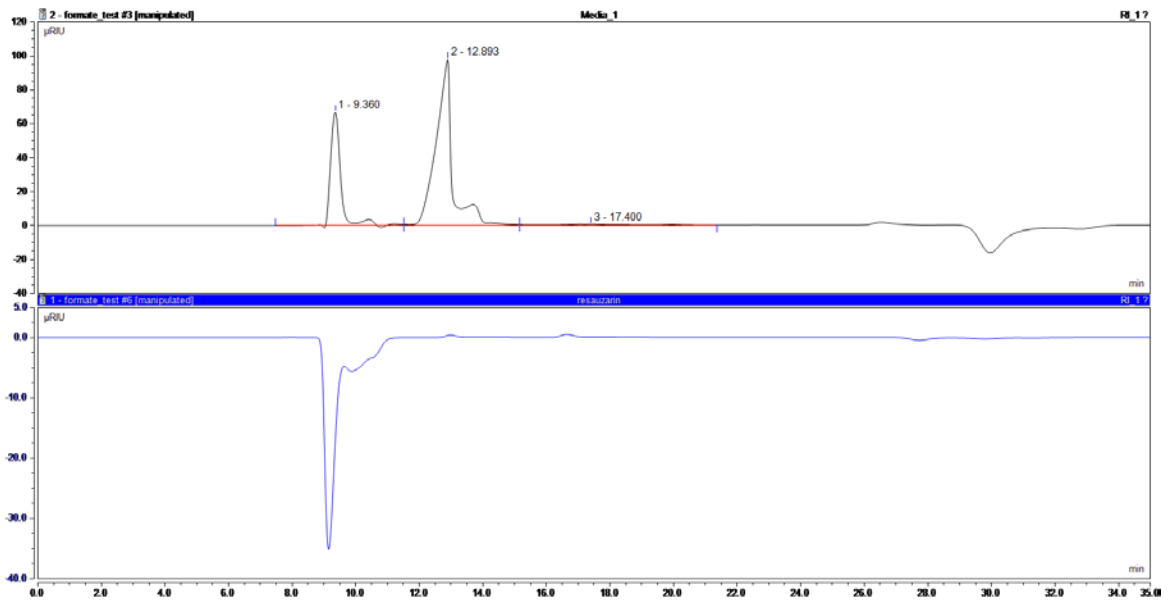
Yeast extract
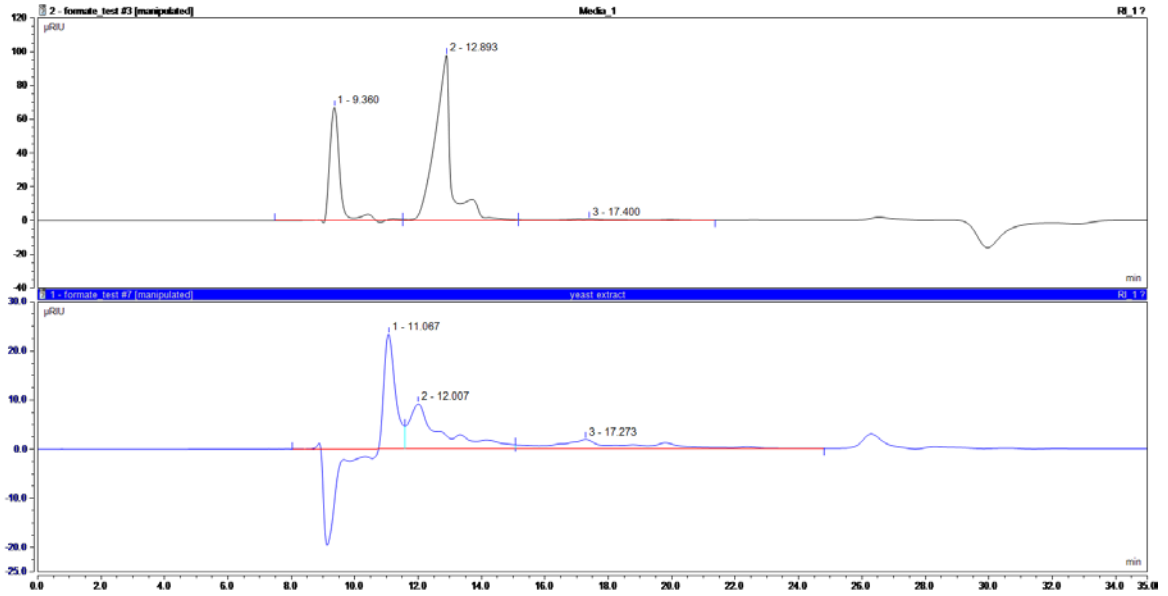
l-cysteine
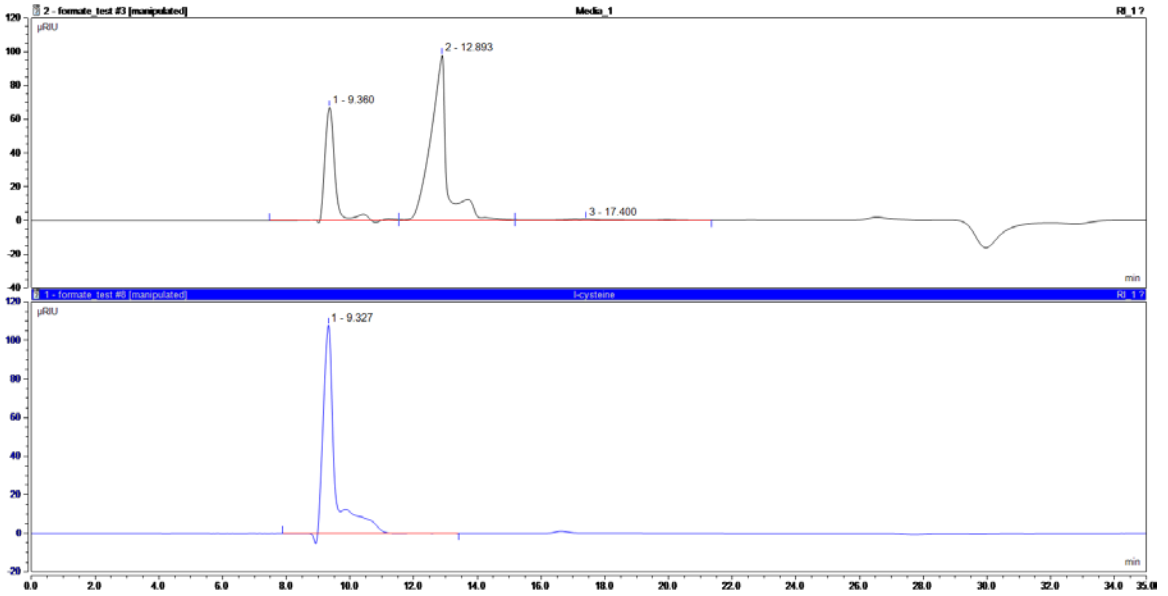
T1 salt
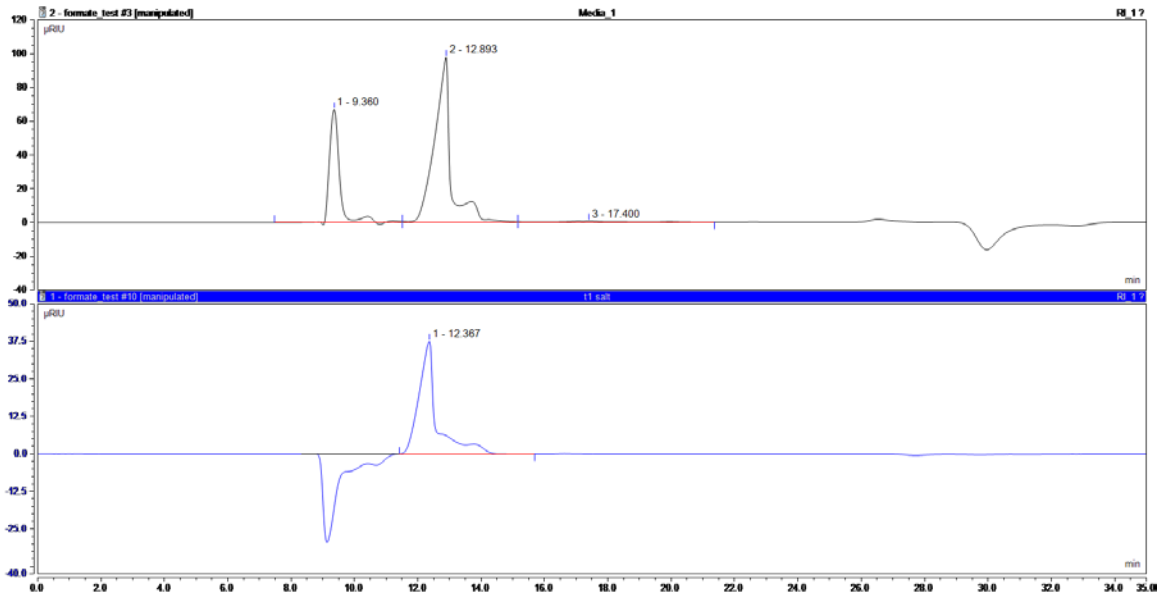
T2 salt
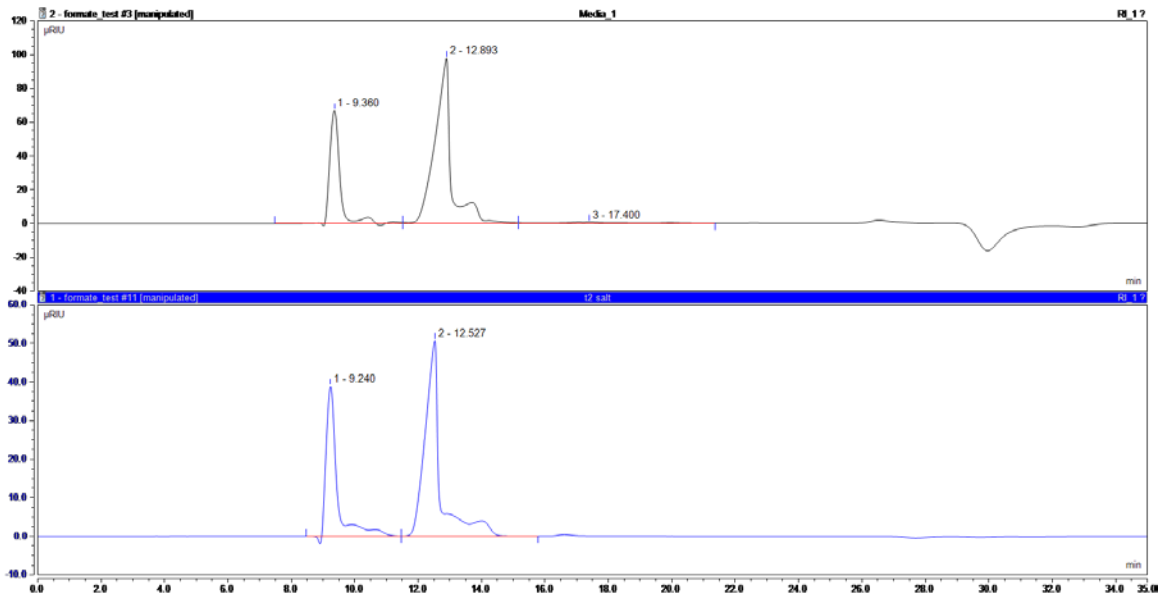
Appendix E. Raw data used in total carbon ANOVAs in Chapter 1 and Chapter 2.

Data used in corn stover ANOVA in Chapter 1.

total_carbon (g)	Solids %	Temp (°C)	Time (hrs)
0.0167	5	20	24
0.0341	5	20	24
0.0135	5	20	24
0.0159	15	20	48
0.0170	15	20	48
0.0161	15	20	48
0.0166	10	20	72
0.0063	10	20	72
0.0166	10	20	72
0.0184	10	30	24
0.0214	10	30	24
0.0186	10	30	24
0.0290	5	30	72
0.0240	5	30	72
0.0237	5	30	72
0.0159	15	30	72
0.0222	15	30	72
0.0192	15	30	72
0.0408	10	40	48
0.0512	10	40	48
0.0424	10	40	48
0.0522	5	40	72
0.0378	5	40	72
0.0596	5	40	72
0.0401	15	40	72
0.0468	15	40	72
0.0489	15	40	72
0.0544	5	50	72
0.0725	5	50	72
0.0709	5	50	72
0.0606	10	50	72
0.0805	10	50	72
0.0774	10	50	72
0.0637	15	50	72
0.0608	15	50	72
0.0678	15	50	72
0.0906	5	63	72
0.0921	5	63	72

0.0640	5	63	72
0.0921	10	63	72
0.0780	10	63	72
0.0847	10	63	72
0.0687	15	63	72
0.0642	15	63	72
0.0478	15	63	72

Data used in switchgrass ANOVA in Chapter 1.

total_carbon (g)	Solids %	Temp (°C)	Time (hrs)
0.0087	10	20	24
0.0091	10	20	24
0.0091	10	20	24
0.0129	5	20	48
0.0130	5	20	48
0.0127	5	20	48
0.0077	15	20	48
0.0077	15	20	48
0.0076	15	20	48
0.0120	10	30	48
0.0101	10	30	48
0.0111	10	30	48
0.0173	5	30	72
0.0146	5	30	72
0.0136	5	30	72
0.0123	15	30	72
0.0106	15	30	72
0.0135	15	30	72
0.0220	5	40	72
0.0174	5	40	72
0.0170	5	40	72
0.0176	10	40	72
0.0200	10	40	72
0.0195	10	40	72
0.0211	15	40	72
0.0231	15	40	72
0.0200	15	40	72
0.0135	15	50	48
0.0318	15	50	48
0.0230	15	50	48
0.0494	5	50	72
0.0197	5	50	72

0.0199	5	50	72
0.0211	10	50	72
0.0198	10	50	72
0.0373	10	50	72
0.0682	5	63	72
0.0555	5	63	72
0.0754	5	63	72
0.0332	10	63	72
0.0466	10	63	72
0.0467	10	63	72
0.0282	15	63	72
0.0238	15	63	72
0.0282	15	63	72

Corn stover treatment means from total carbon ANOVA, Chapter 1.

				temperature main effect ave (g)
solids %	5	10	15	
20 °C	0.0214	0.0132	0.0163	0.0170
30 °C	0.0256	0.0195	0.0191	0.0214
40 °C	0.0499	0.0448	0.0453	0.0466
50 °C	0.0659	0.0728	0.0641	0.0676
63 °C	0.0822	0.0849	0.0602	0.0758
solids main effect ave (g)	0.0490	0.0470	0.0410	

Switchgrass treatment means from total carbon ANOVA, Chapter 1.

	solids %	5	10	15	temperature main effect ave (g)
20 °C		0.0129	0.0090	0.0077	0.0098
30 °C		0.0152	0.0111	0.0121	0.0128
40 °C		0.0188	0.0190	0.0214	0.0197
50 °C		0.0297	0.0261	0.0228	0.0262
63 °C		0.0664	0.0422	0.0267	0.0451
solids main effect ave (g)		0.0286	0.0215	0.0181	

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

sample	xylose (%)	glucose (%)	total_lignin (%)	trt #	trt
1	20.08	40.66	21.91	1	sterile control
2	12.87	36.73	25.52	1	sterile control
3	15.58	37.07	26.58	1	sterile control
4	18.09	40.72	28.02	2	pc-->ct
5	17.00	39.46	27.37	2	pc-->ct
6	15.66	38.39	28.46	2	pc-->ct
7	20.72	44.77	23.94	3	nonsterile ctrl
8	18.92	39.84	26.24	3	nonsterile ctrl
9	19.69	41.80	25.04	3	nonsterile ctrl
10	18.58	43.20	26.68	4	63-->pc-->ct
11	14.79	39.49	28.59	4	63-->pc-->ct
12	15.85	40.07	28.38	4	63-->pc-->ct
13	15.19	44.23	26.87	5	ct-->pc-->ct
14	18.06	42.33	24.89	5	ct-->pc-->ct
15	16.67	43.96	25.11	5	ct-->pc-->ct

Raw data used in total carbon ANOVA, Chapter 2.

#	cellobiose (mg/L)	glucose (g/L)	xylose (mg/L)	lactate (mM)	formate (mM)	acetate (mM)	ethanol (mM)	trt
1	0	0	96.692	18.266	18.529	15.206	1.752	ct(p)
2	0	0	86.327	16.704	12.078	14.329	1.641	ct(p)
3	0	0	100.873	20.797	12.889	16.804	3.070	ct(p)
4	0	0.020	0	1.740	2.581	14.548	2.700	pc->ct(p)
5	0	0	0	2.138	2.870	18.124	4.715	pc-> ct(p)
6	0	0.010	0	1.764	3.217	14.713	3.371	pc-> ct(p)
7	0	0	0	3.900	0	13.905	1.442	63-->pc-->ct(p)
8	0	0	0	3.007	0	9.137	2.912	63-->pc-->ct(p)
9	0	0	0	6.335	0	12.601	0.964	63-->pc-->ct(p)
10	0	0.400	0	0	0	20.823	0.579	ct-->pc-->ct(p)
11	0	0.040	0	3.541	2.602	10.067	2.961	ct-->pc-->ct(p)
12	0	0.040	0	0.064	1.841	18.105	4.093	ct-->pc-->ct(p)

Total carbon for each sample used in total carbon ANOVA, Chapter 2.

sample	total_carbon (g)	trt
1	0.1530	ct(p)
2	0.1357	ct(p)
3	0.1661	ct(p)
4	0.0723	pc->ct(p)
5	0.0934	pc-> ct(p)
6	0.0758	pc-> ct(p)
7	0.0693	63-->pc-->ct(p)
8	0.0542	63-->pc-->ct(p)
9	0.0717	63-->pc-->ct(p)
10	0.0605	ct-->pc-->ct(p)
11	0.0451	ct-->pc-->ct(p)
12	0.0588	ct-->pc-->ct(p)

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:

$$\% \ difference = (sugar_{63} - sugar_T)/(sugar_{63})$$

<u>Corn stover:</u>

Solids_time	20 °C	30 °C	40 °C	50 °C
5_24	-14.2%	6.1%	35.2%	-95.1%
5_48	-28.3%	-13.6%	-14.7%	-99.0%
5_72	22.9%	66.5%	-22.0%	-92.4%
10_24	-54.5%	-24.7%	9.1%	-97.3%
10_48	-50.7%	-26.9%	-17.9%	-99.7%
10_72	-34.2%	-96.9%	-37.9%	-99.3%
15_24	-56.7%	-26.0%	-13.8%	-98.3%
15_48	-6.7%	8.7%	30.4%	-92.1%
15_72	-8.2%	33.6%	-5.8%	-99.4%

Solids_time	20 °C	30 °C	40 °C	50 °C
5_24	-87.5	37.6	216.8	-586.6
5_48	-179.4	-86.4	-93.6	-628.5
5_72	90.6	263.3	-87.1	-365.9
10_24	-741.6	-336.5	124.5	-1324.6
10_48	-593.6	-315.0	-209.2	-1168.1
10_72	-329.6	-934.5	-365.7	-957.8
15_24	-1138.2	-520.8	-277.1	-1973.5
15_48	-86.7	112.3	392.7	-1189.9
15_72	-87.0	356.7	-61.1	-1054.0

Switchgrass:

Soilds_time	20 °C	30 °C	40 °C	50 °C
5_24	40.5%	45.4%	-50.7%	-33.2%
5_48	88.0%	41.8%	-60.7%	-7.3%
5_72	-14.4%	-44.1%	-71.4%	-46.9%
10_24	36.3%	44.8%	-64.6%	-38.3%
10_48	25.3%	-26.5%	-52.7%	-43.3%
10_72	-24.6%	-24.0%	-81.1%	-64.7%
15_24	27.3%	31.0%	-55.9%	-32.3%
15_48	31.1%	12.4%	-56.8%	-18.8%
15_72	4.1%	-33.2%	-67.2%	-8.9%

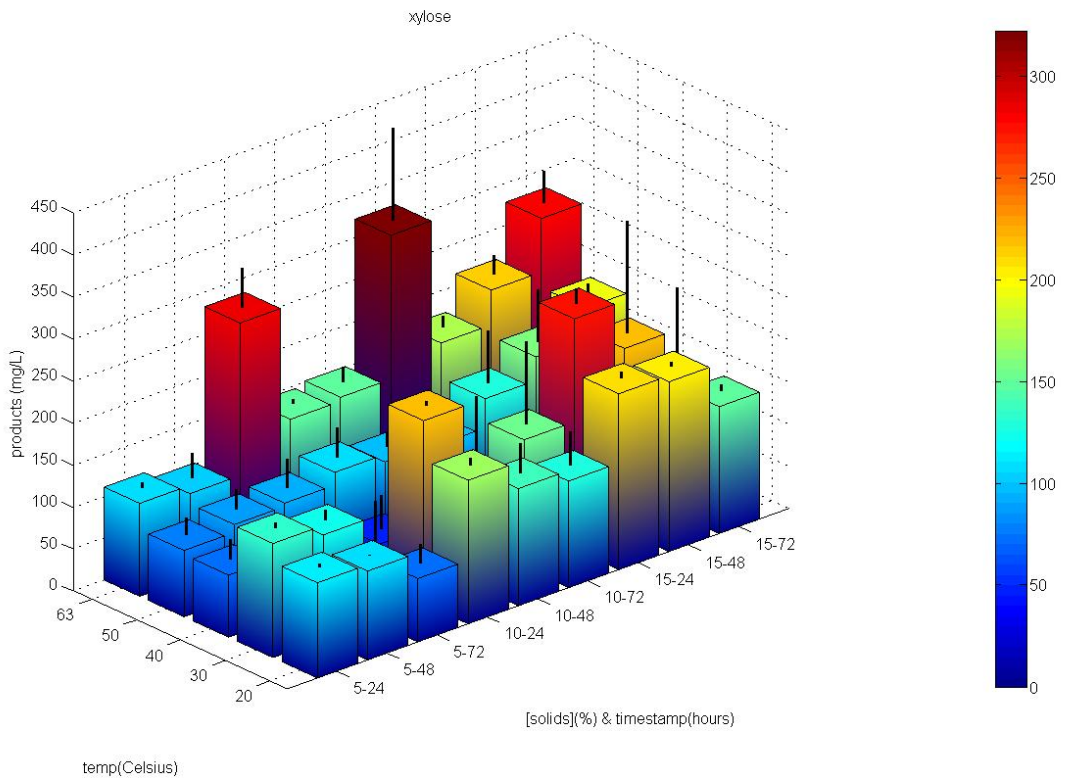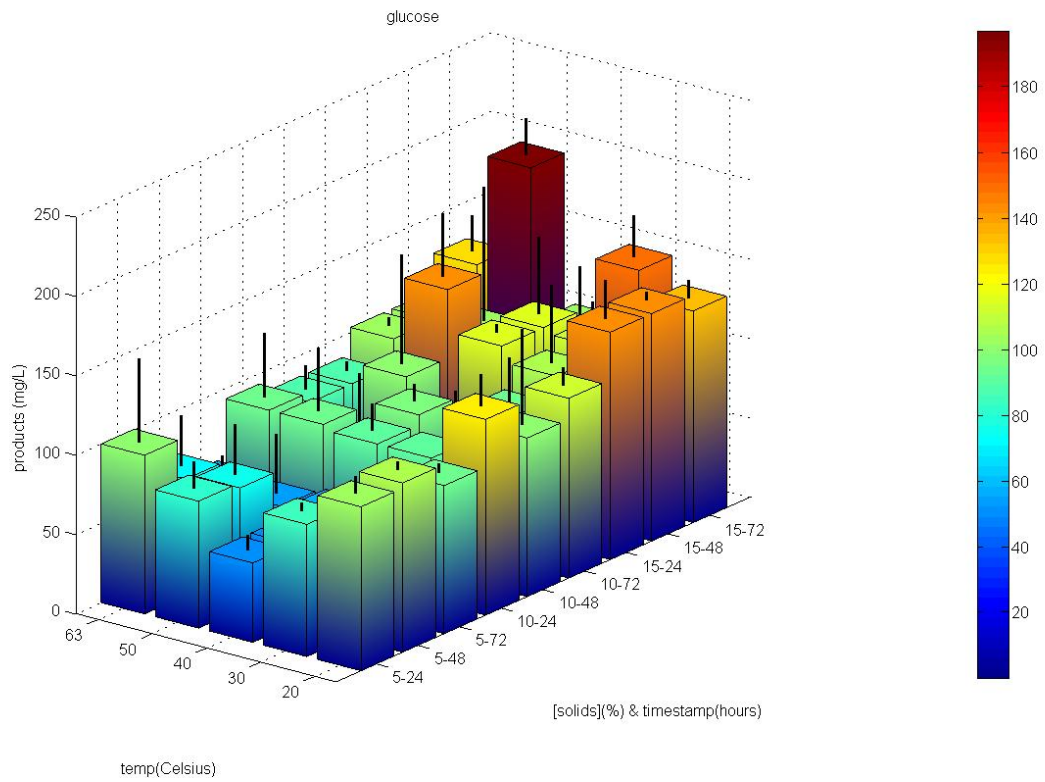| Solids_time	20 °C	30 °C	40 °C	50 °C
5_24	117.2	131.3	-146.6	-96.0
5_48	191.1	90.8	-131.8	-15.8
5_72	-48.7	-149.3	-241.9	-158.7
10_24	139.8	172.3	-248.7	-147.6
10_48	89.1	-93.3	-185.7	-152.6
10_72	-136.8	-133.1	-450.1	-359.4
15_24	132.5	150.3	-270.9	-156.6
15_48	149.6	59.9	-273.9	-90.7
15_72	22.9	-187.6	-379.3	-50.1

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.

cellobiose

cellobiose

95

glucose

glucose

glucose

xylose

xylose

lactate

lactate

formate

formate

acetate

100

acetate

ethanol

101

ethanol

sugar

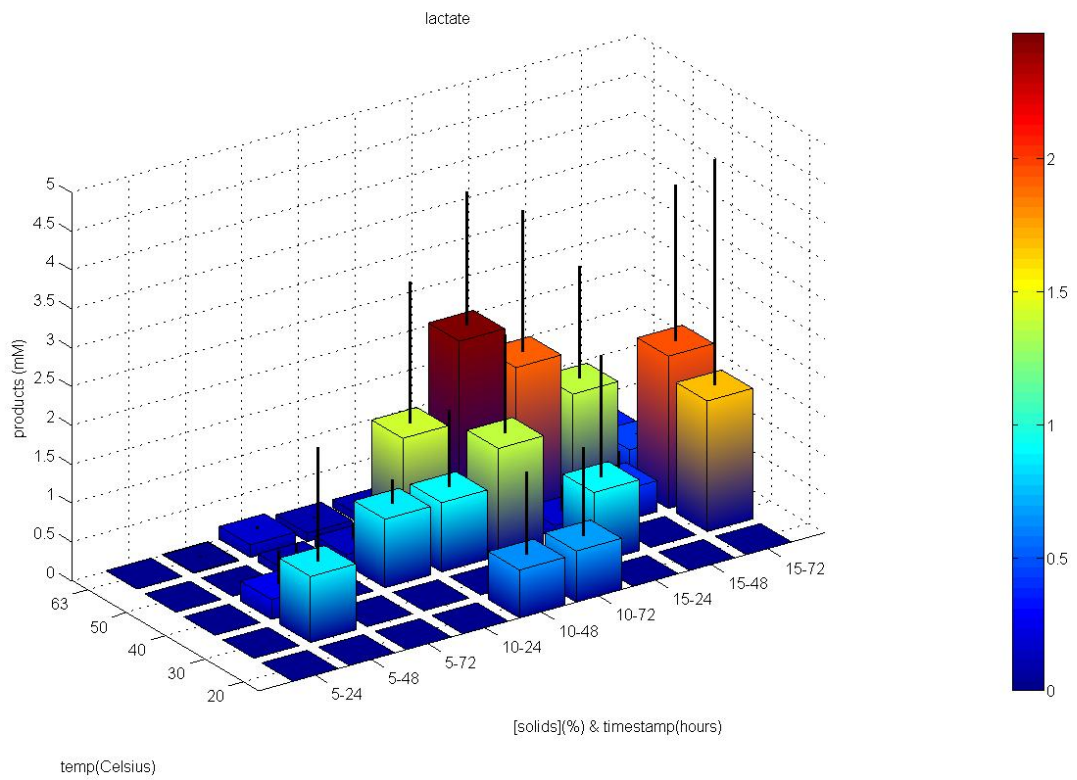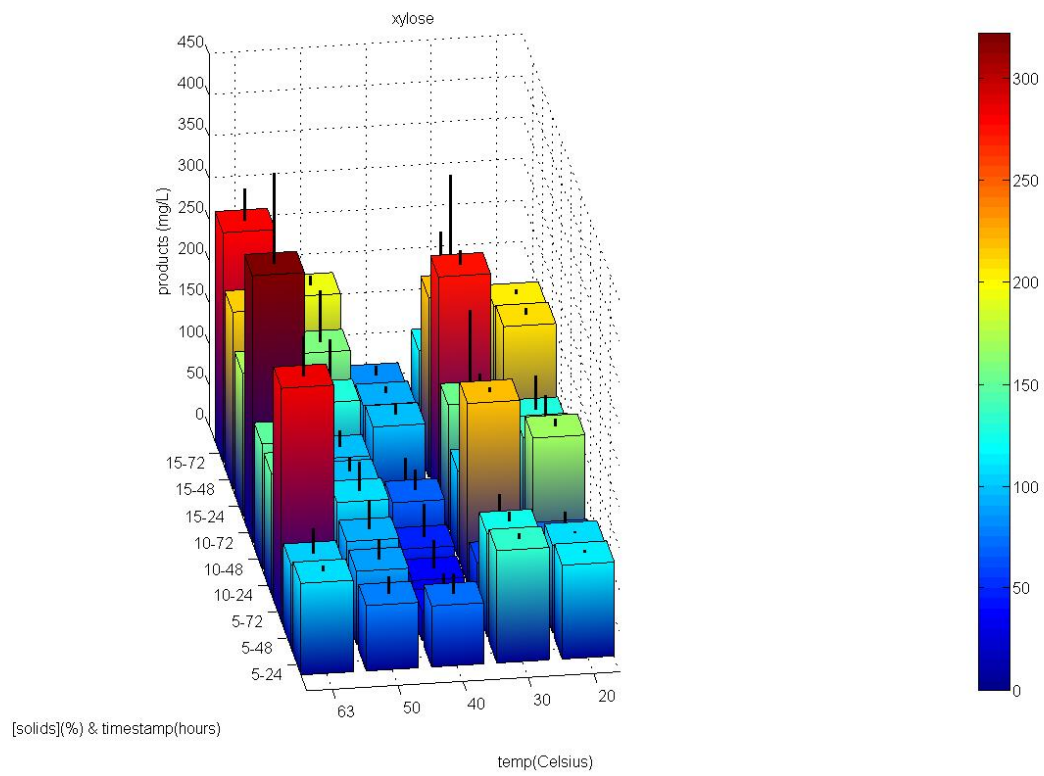sugar

sugar

acids

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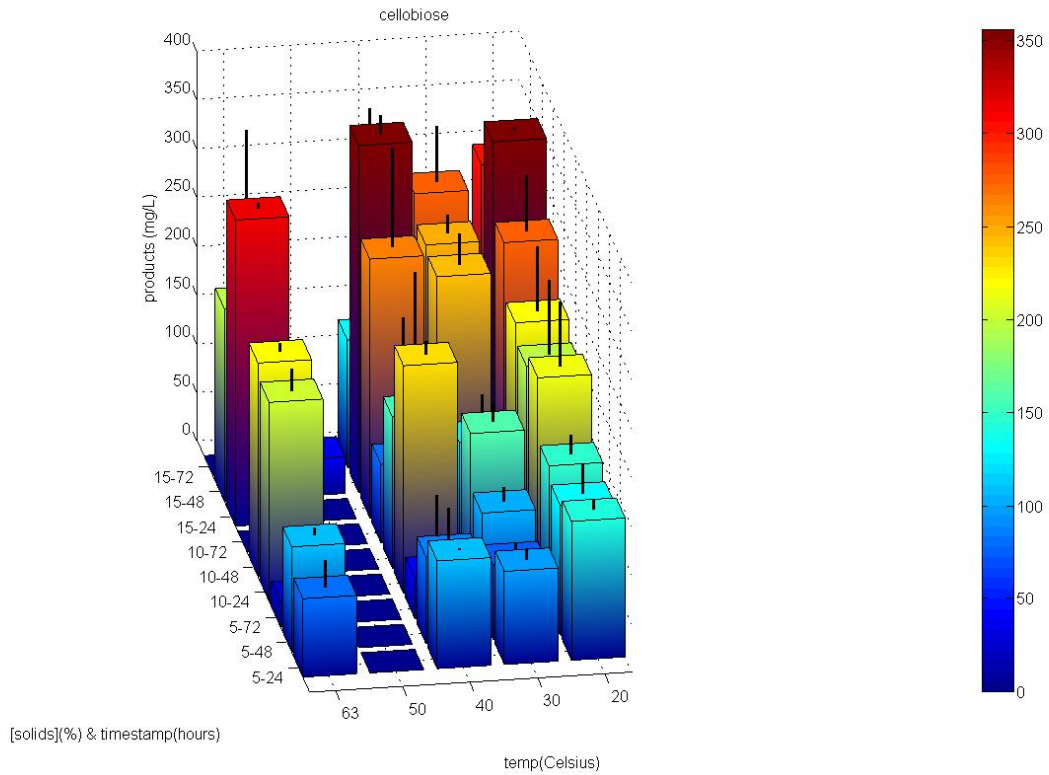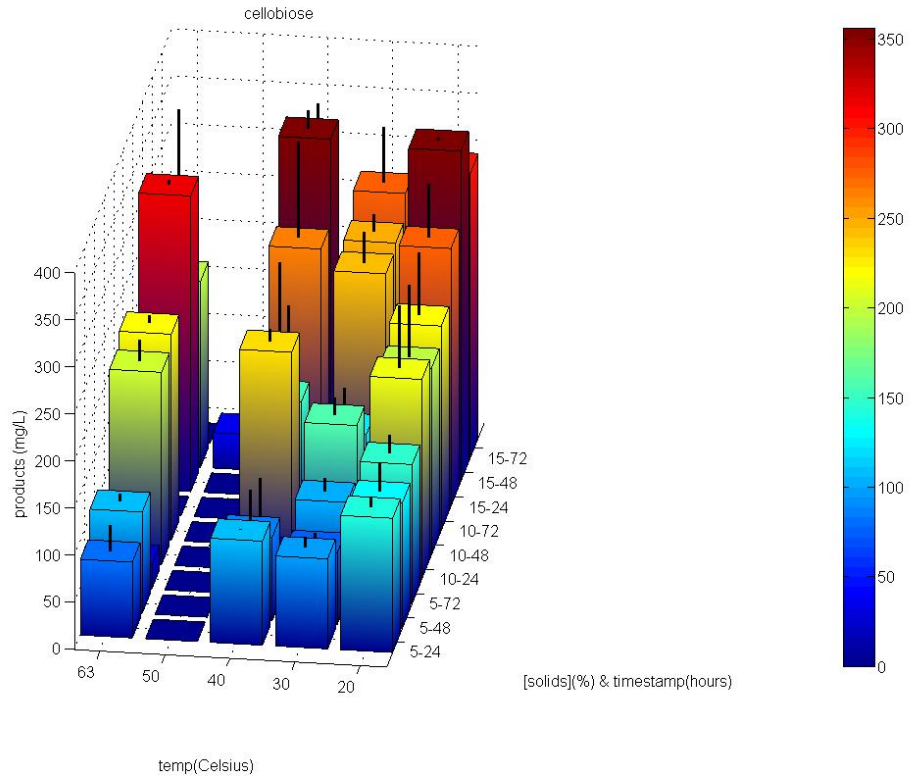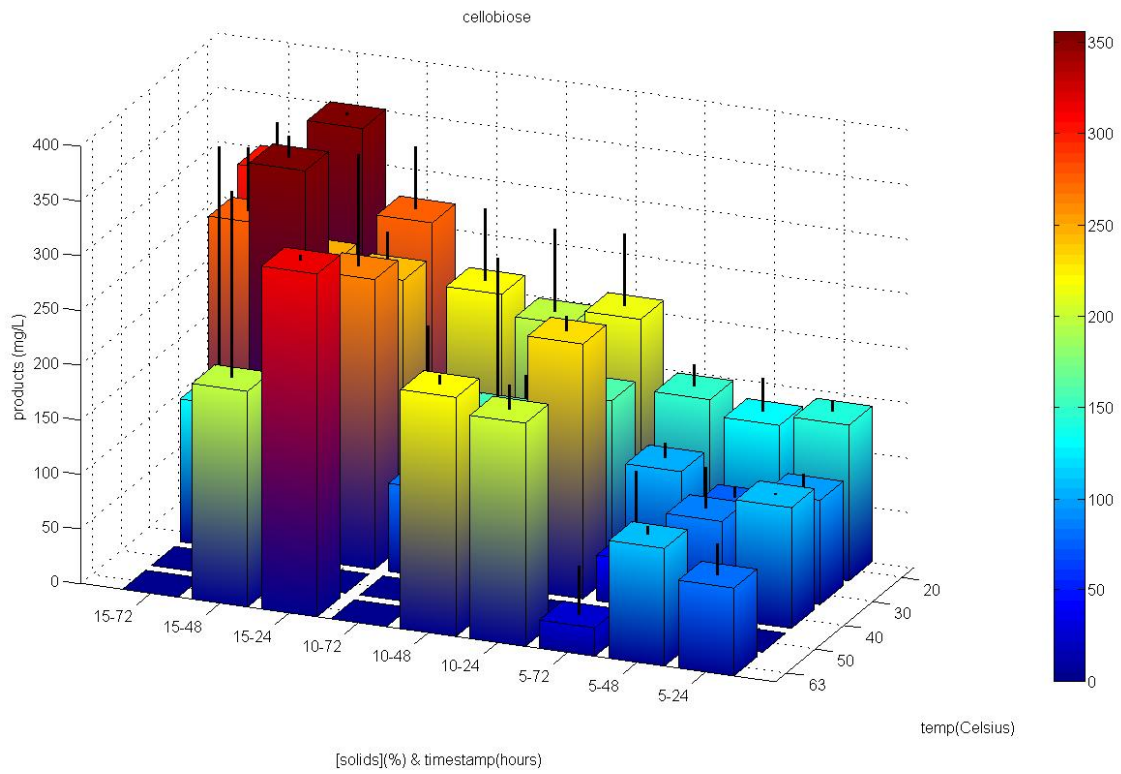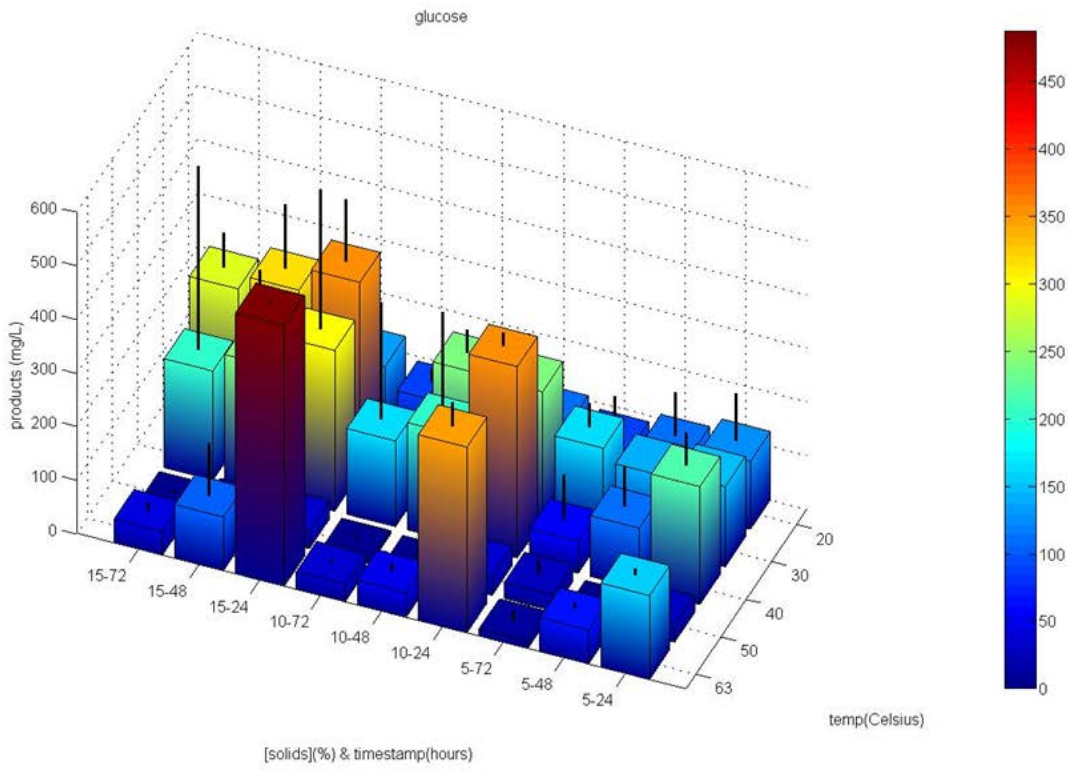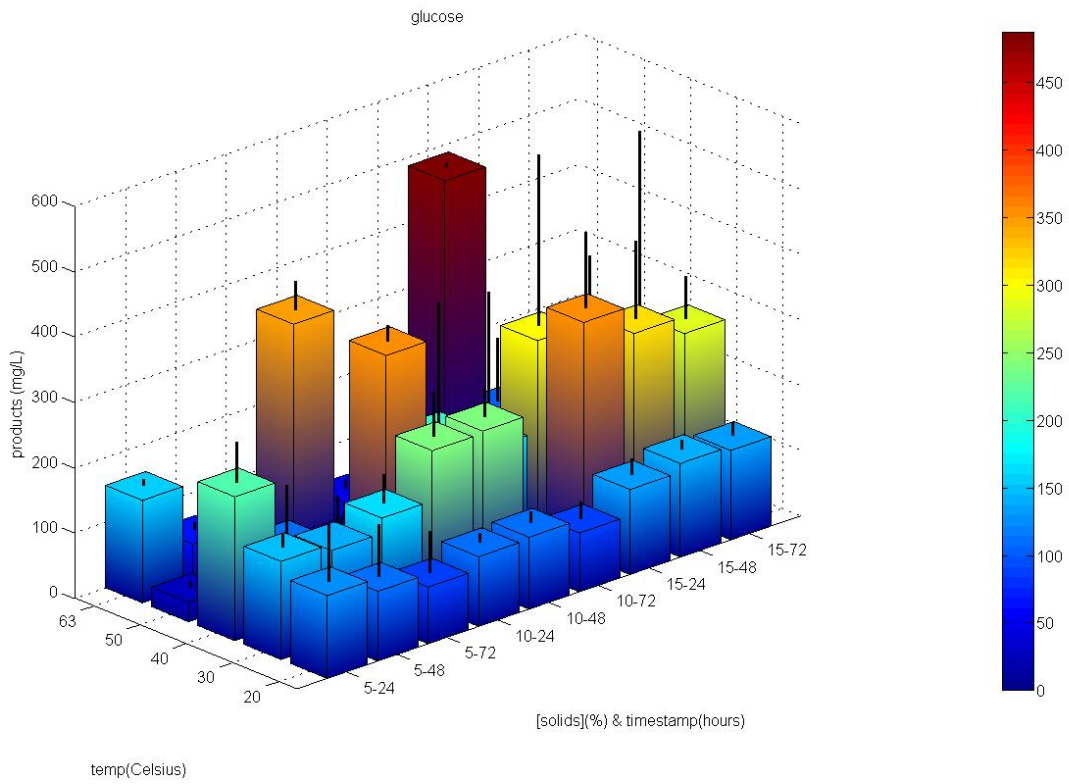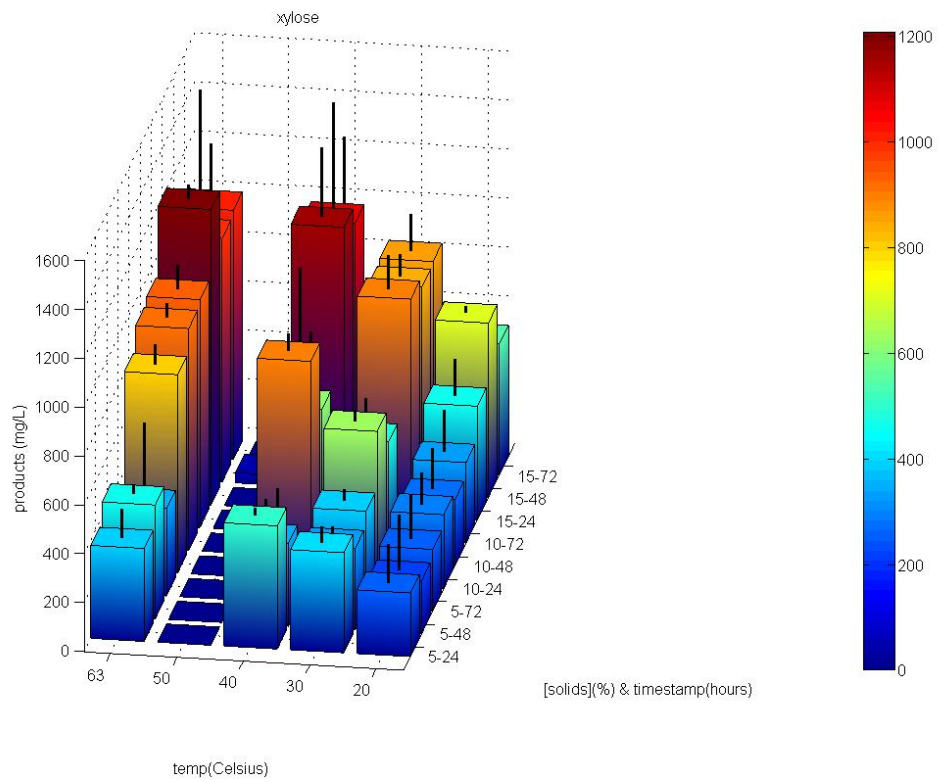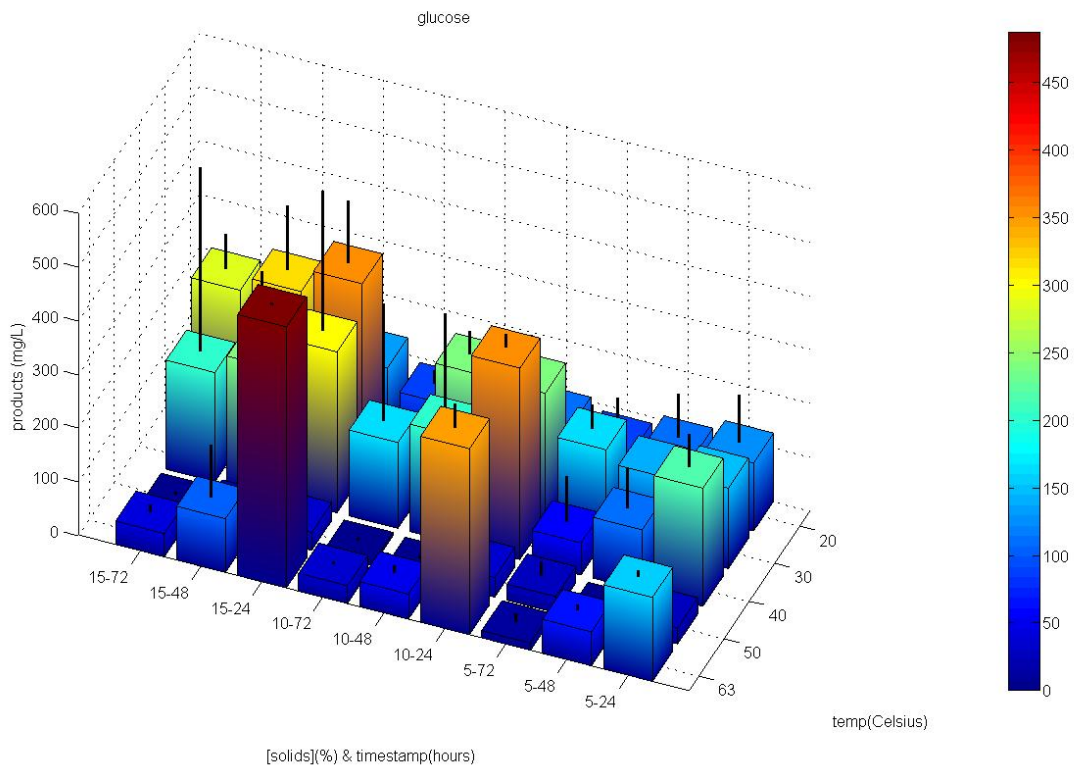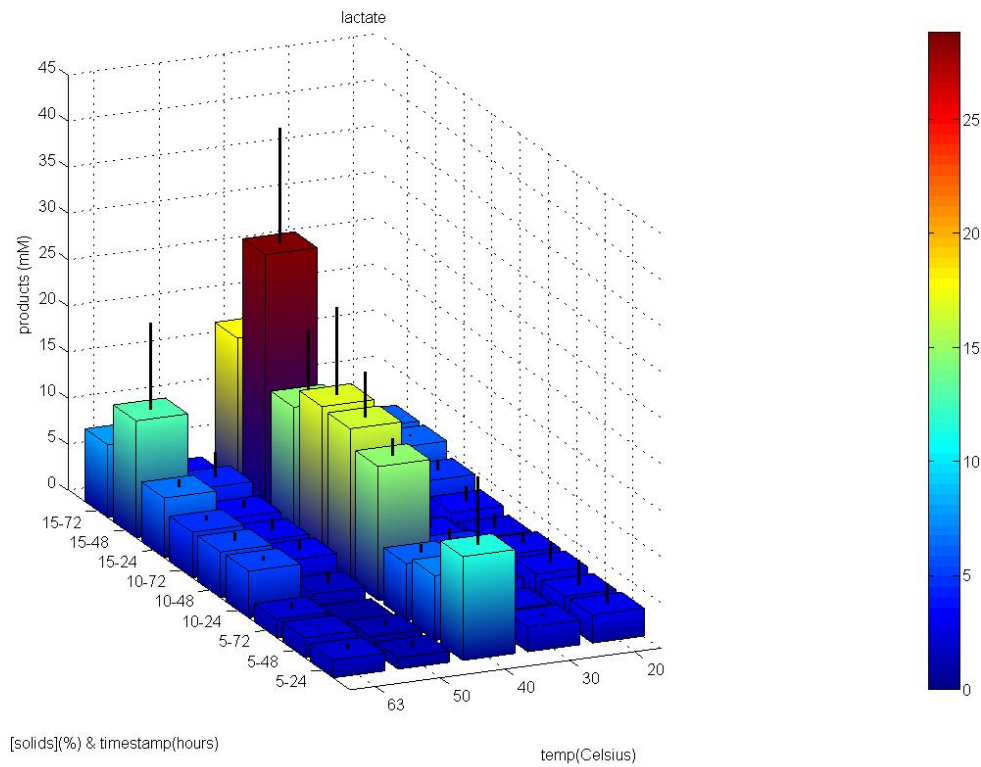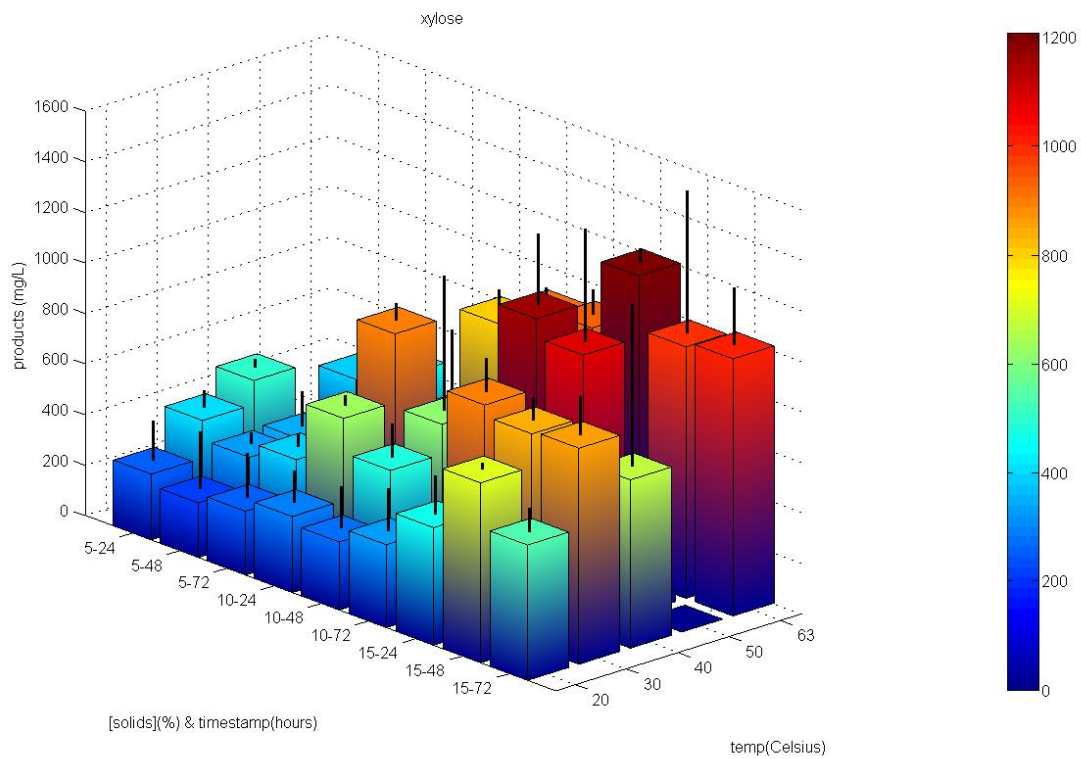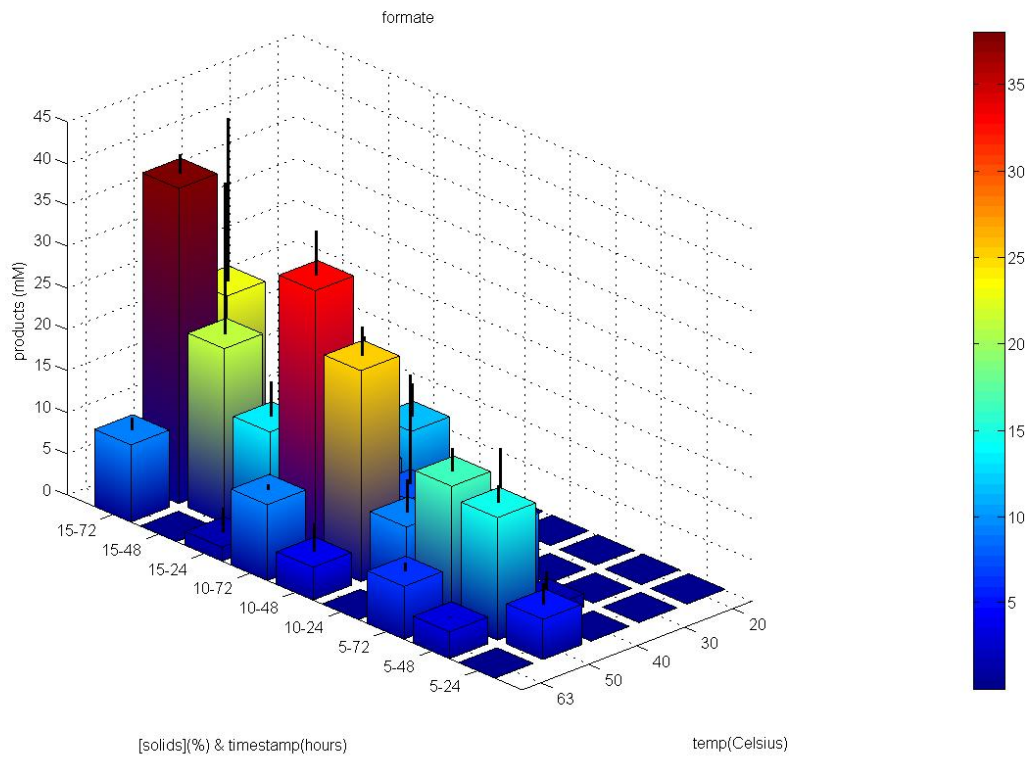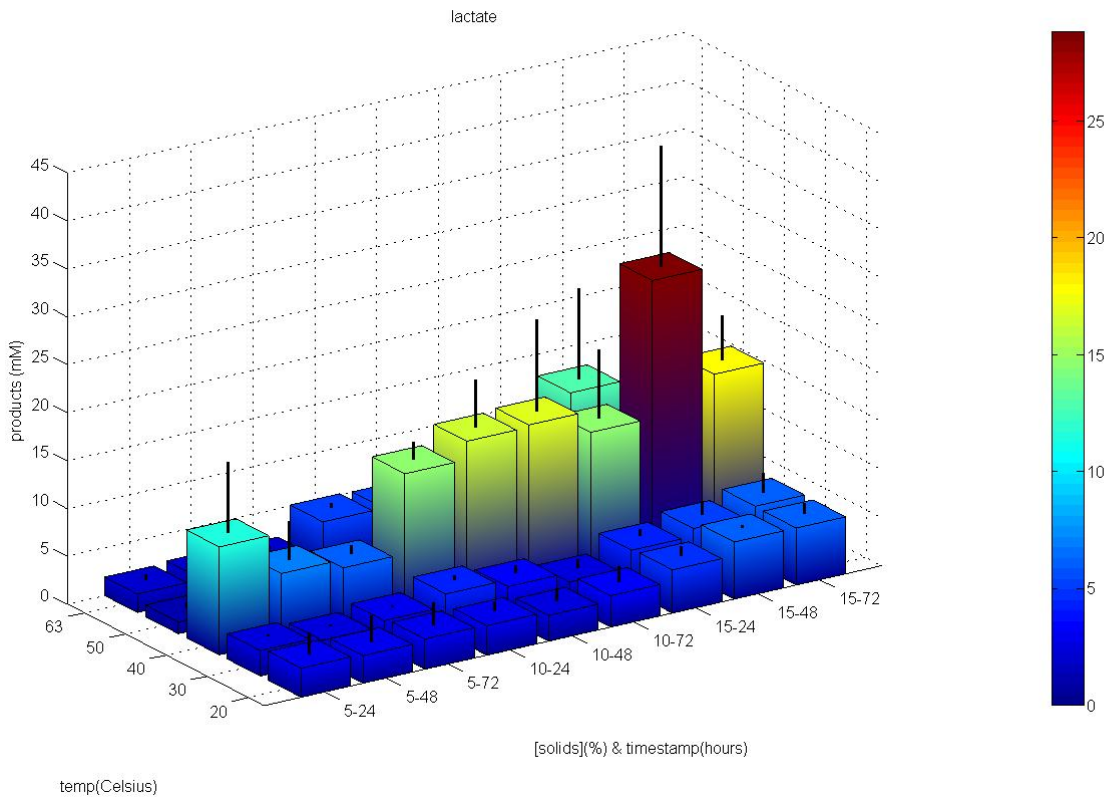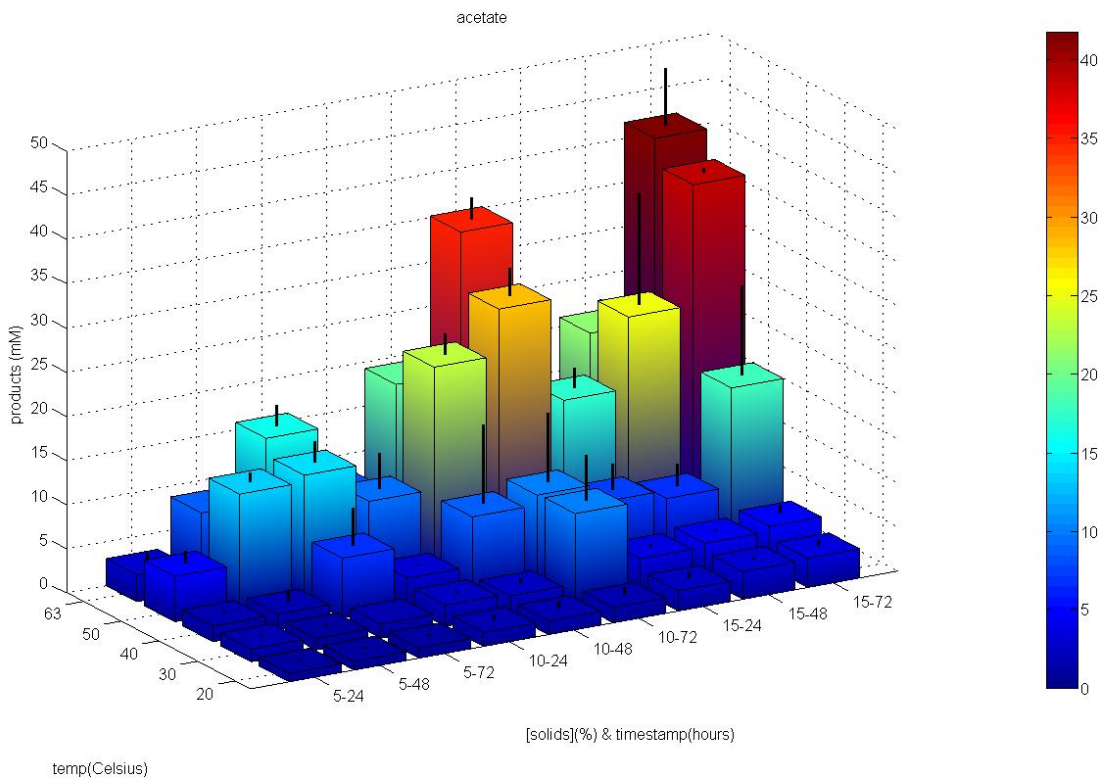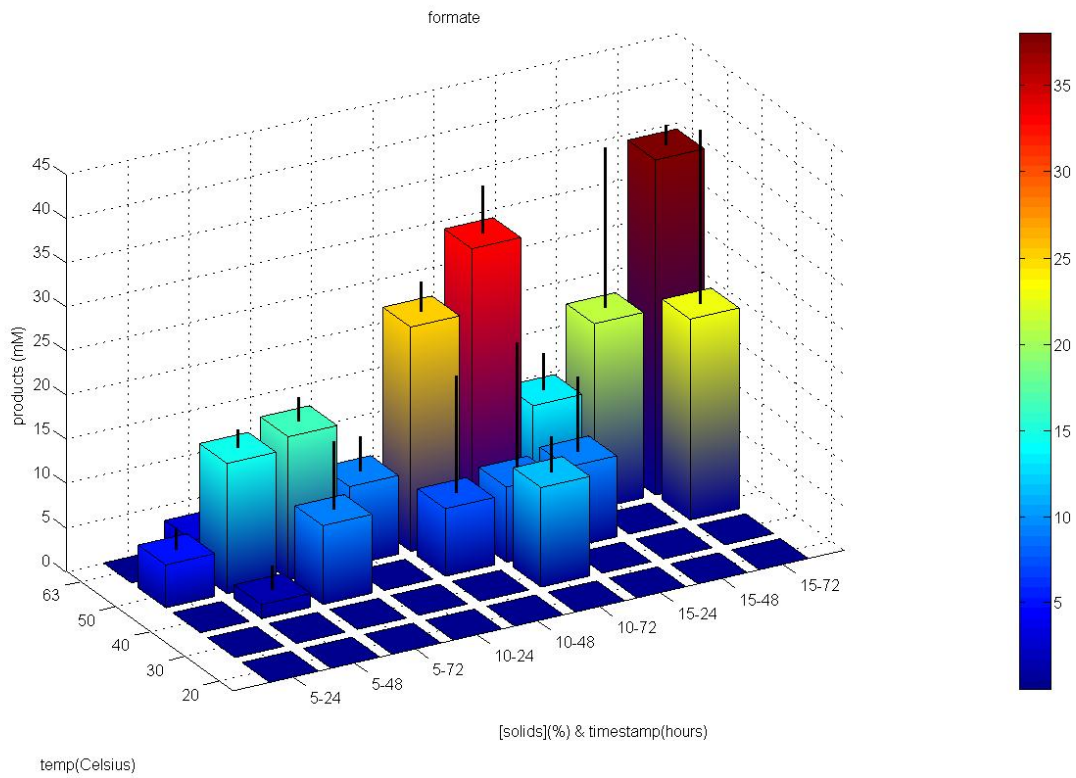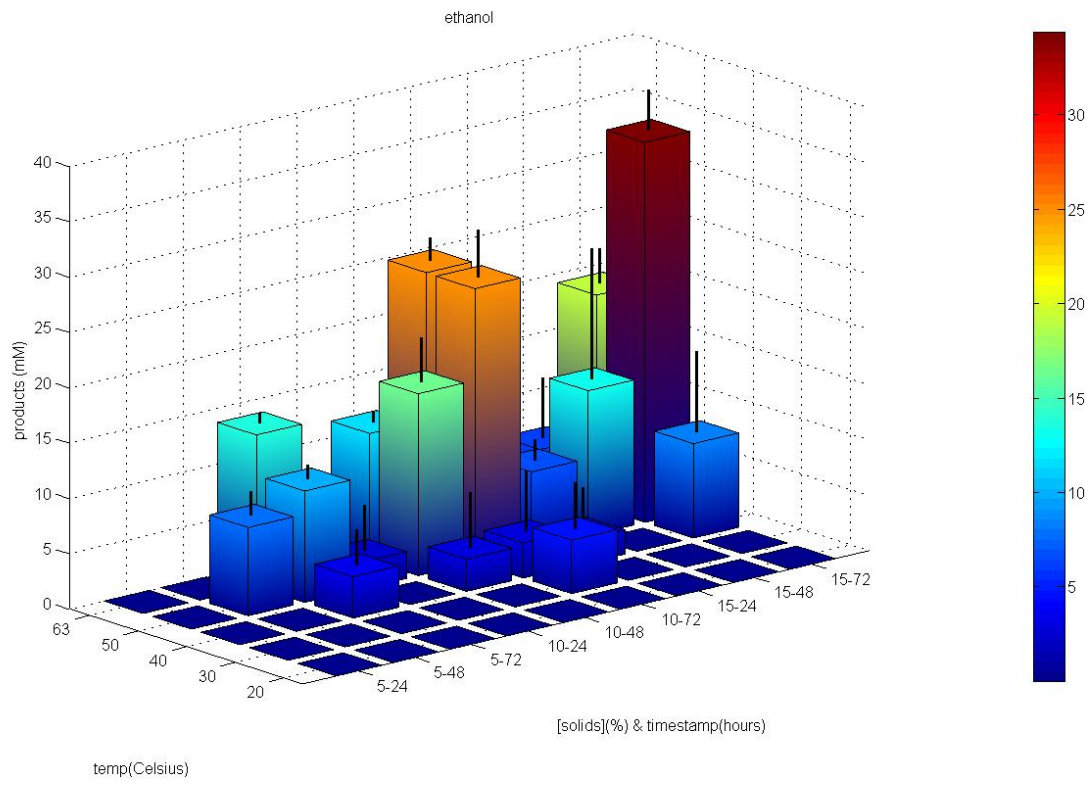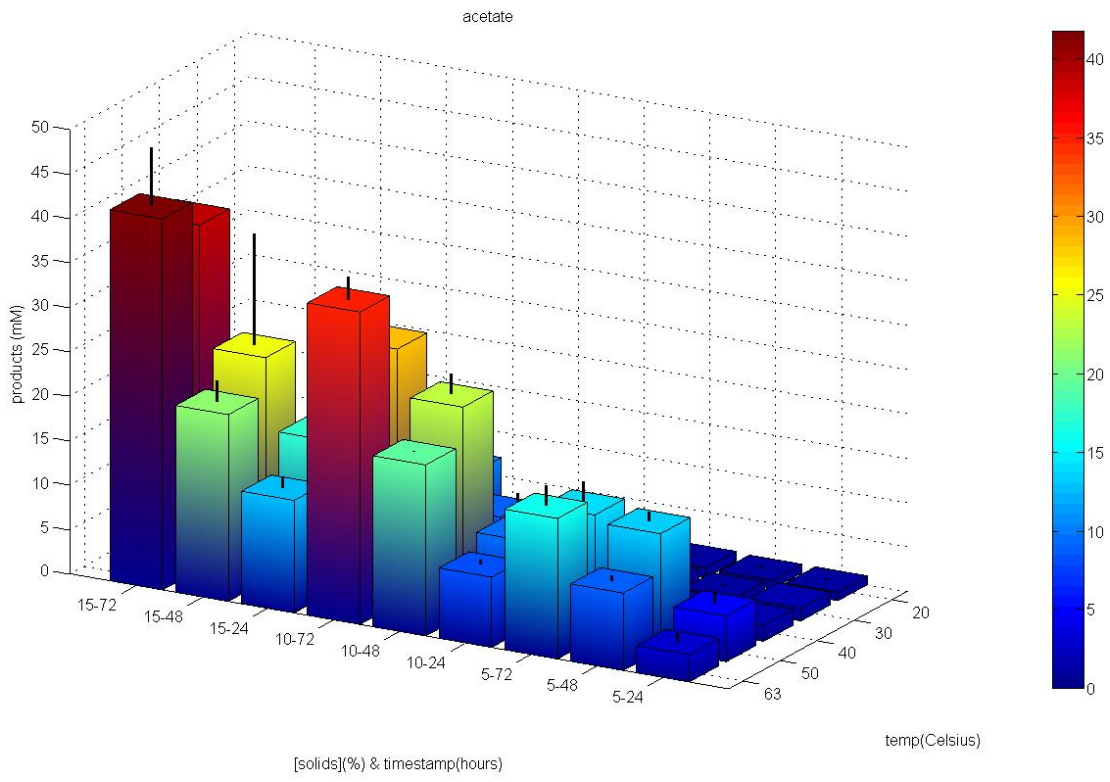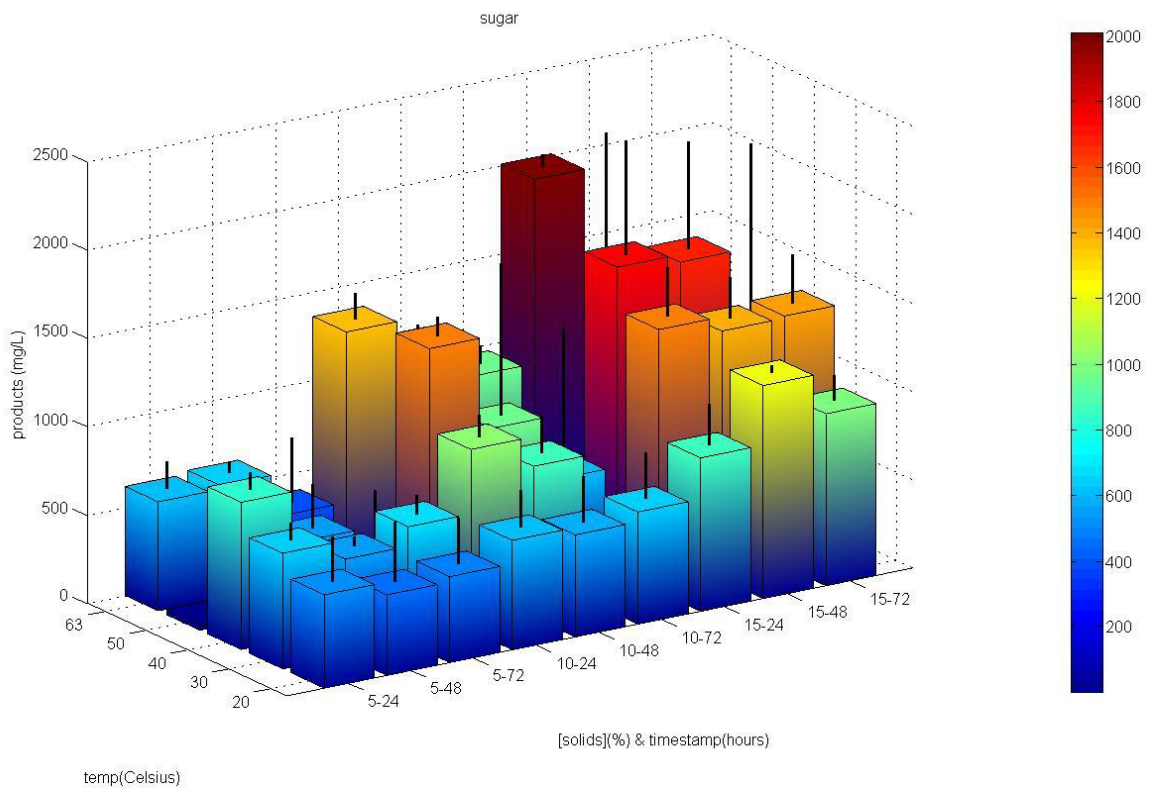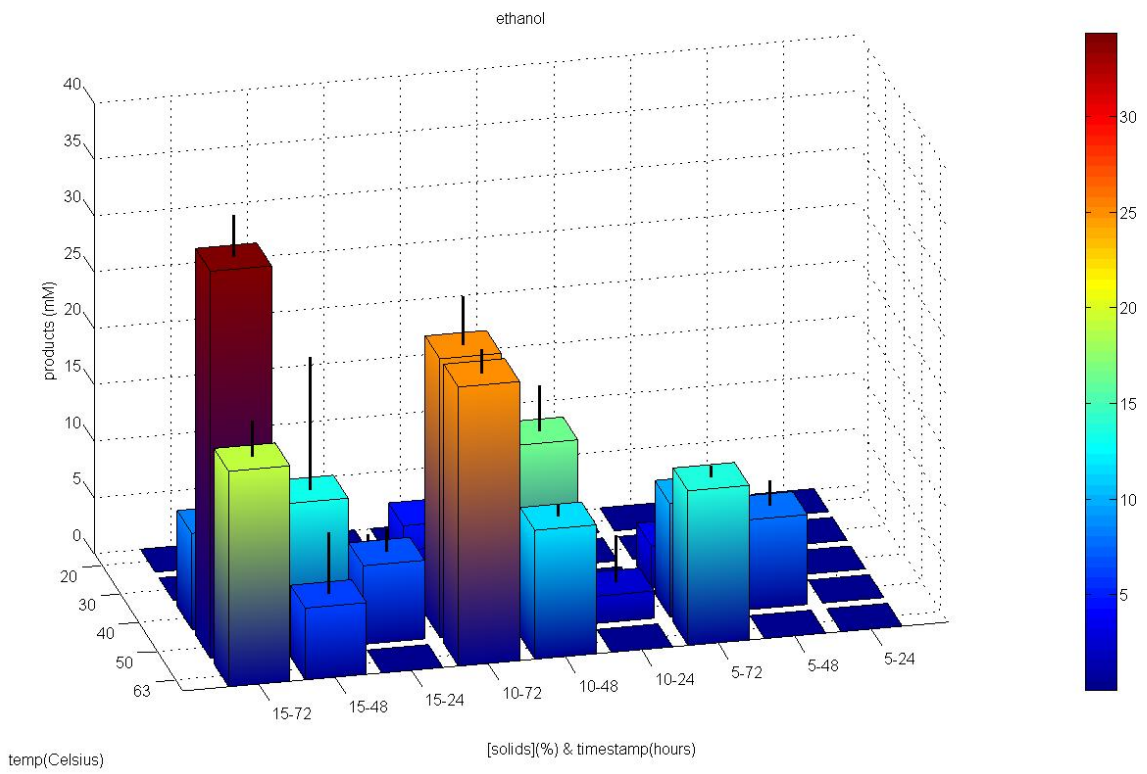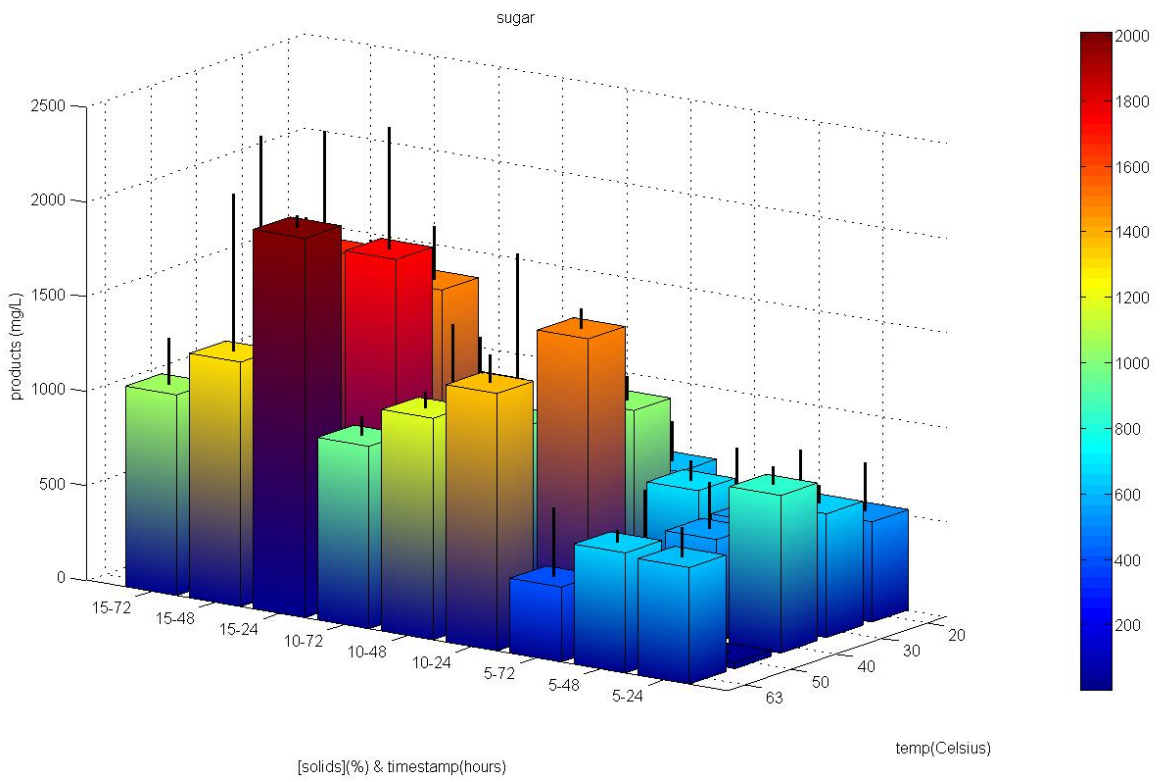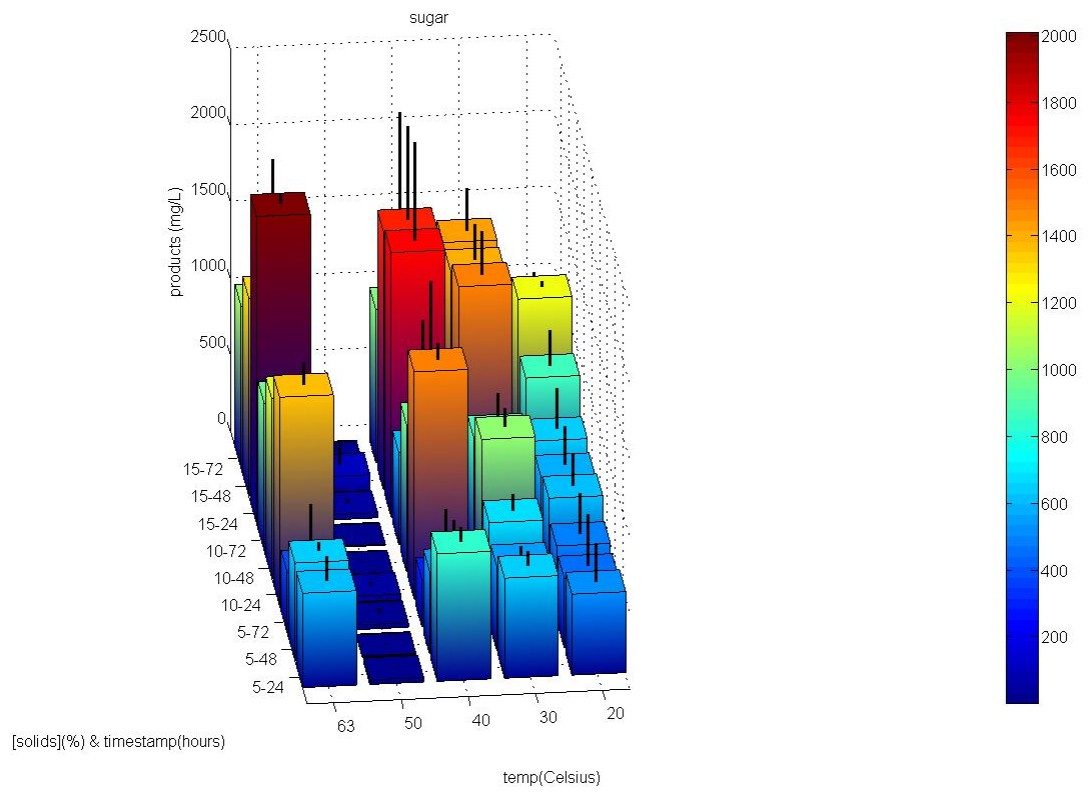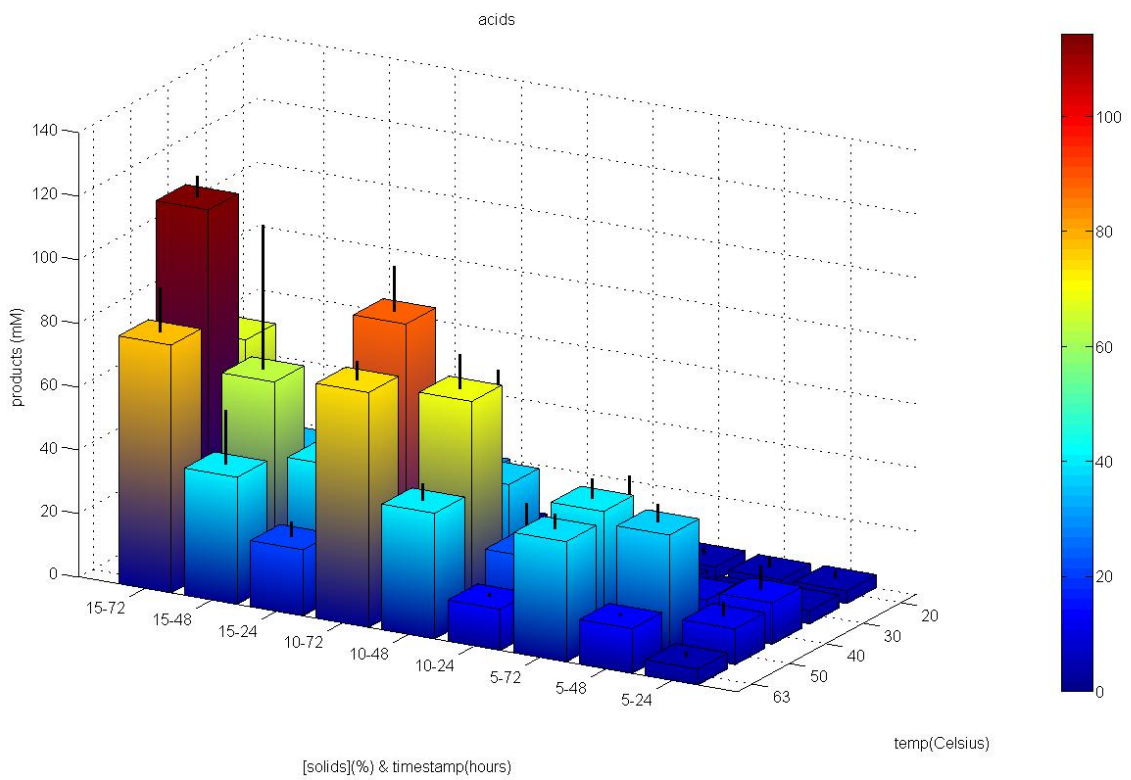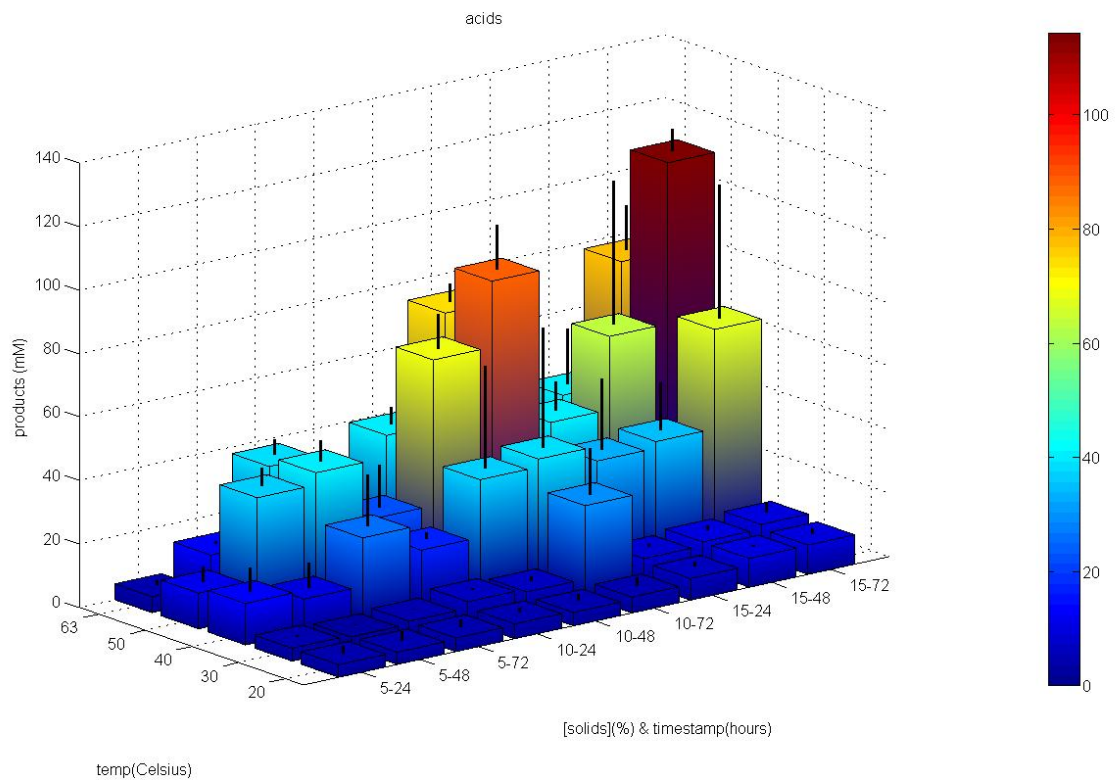
Appendix H. MATLAB graphs of product treatment means produced from corn stover by *C. thermocellum* ATCC 27405 at 5, 10, 15% solids, temperatures of 20 °C, 30 °C, 40 °C, 50 °C, 63 °C, and sampling times of 24, 48, and 72 hrs.
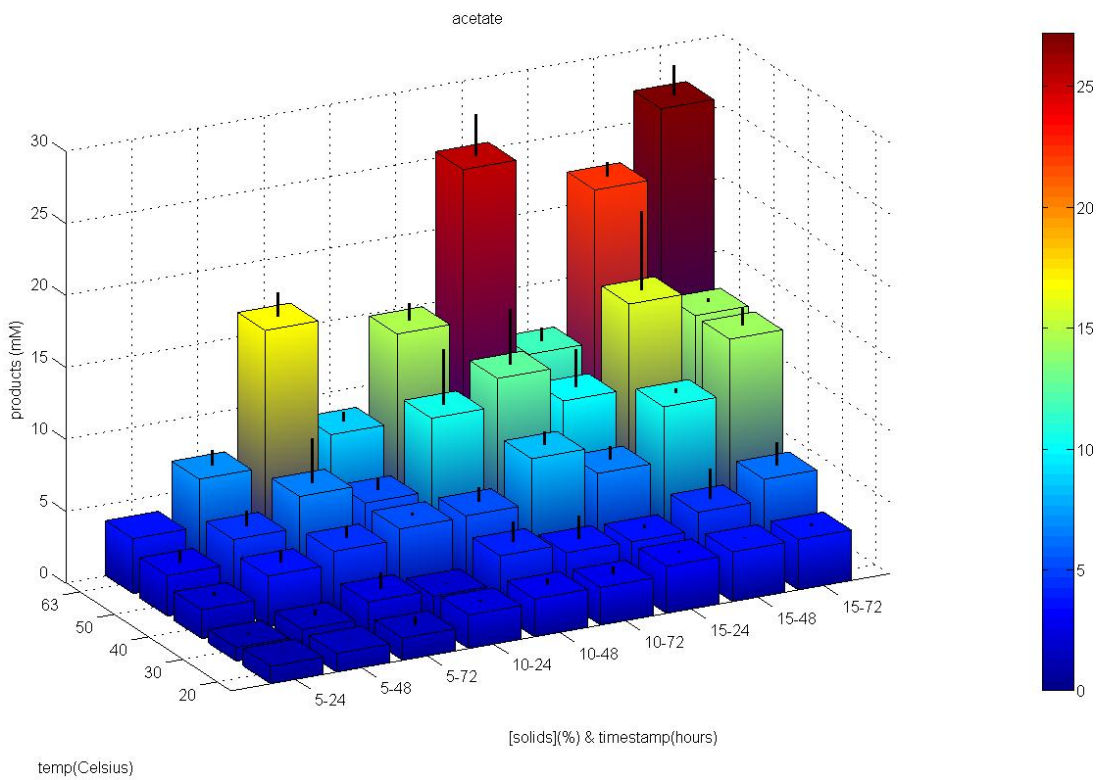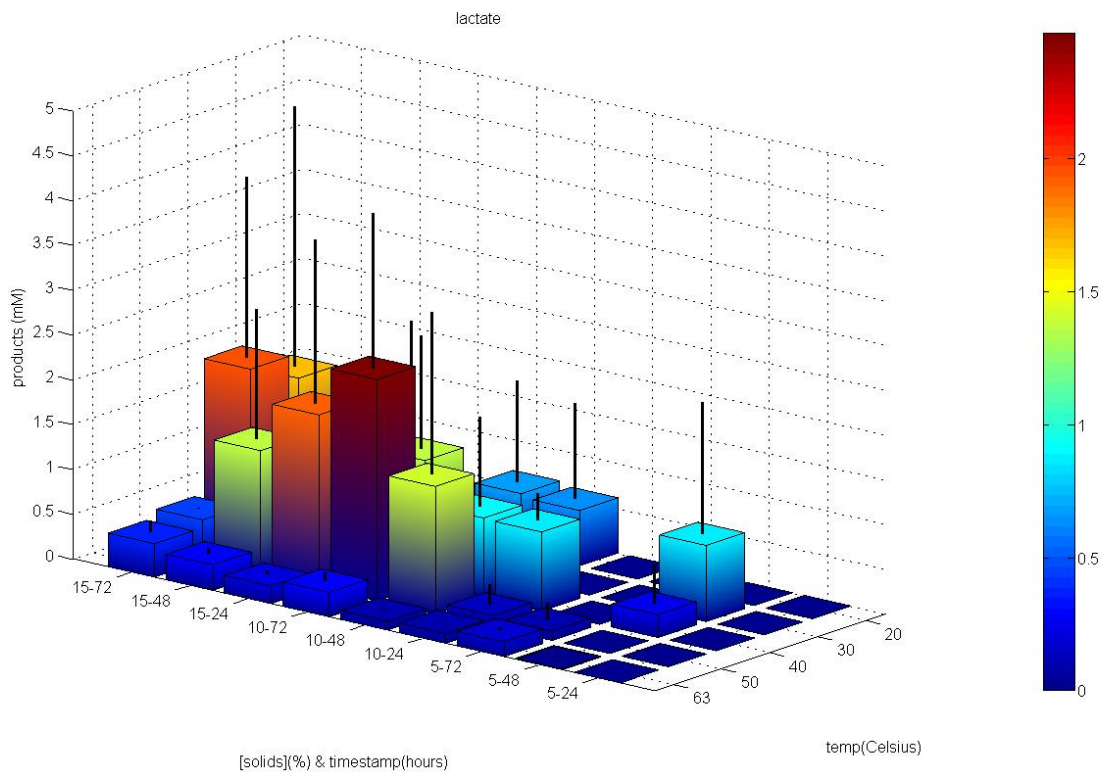
Error bars = + 1 standard deviation.

cellobiose

glucose

glucose

xylose

xylose

products (mg/L)

[solids](%) & timestamp(hours)

temp(Celsius)

lactate

products (mM)

[solids](%) & timestamp(hours)

temp(Celsius)

lactate

acetate

ethanol

ethanol

110

sugar

sugar

111

sugar

acids

112

acids

products (mM)

[solids](%) & timestamp(hours)

temp(Celsius)

REFERENCES

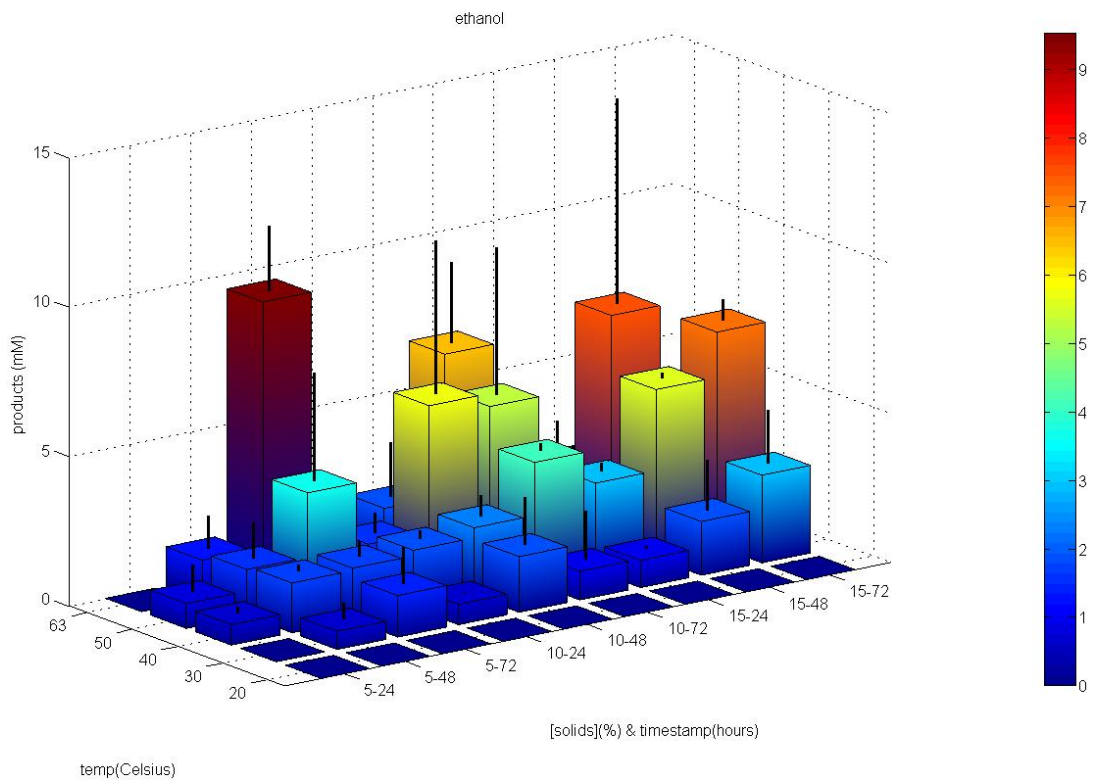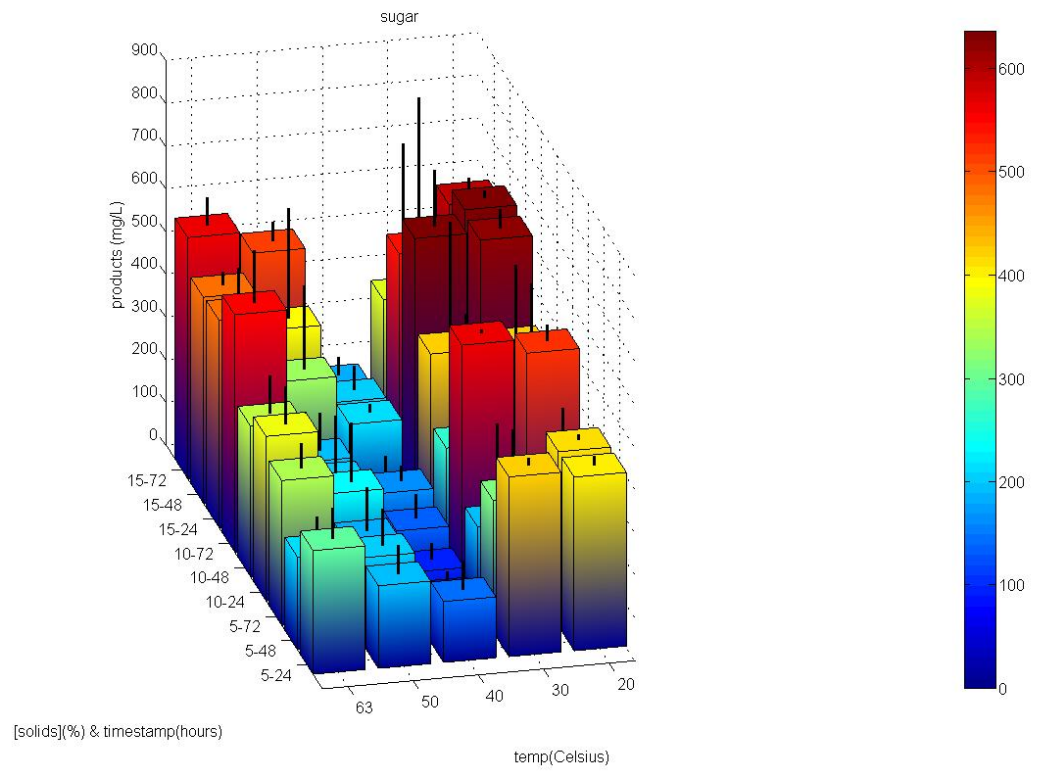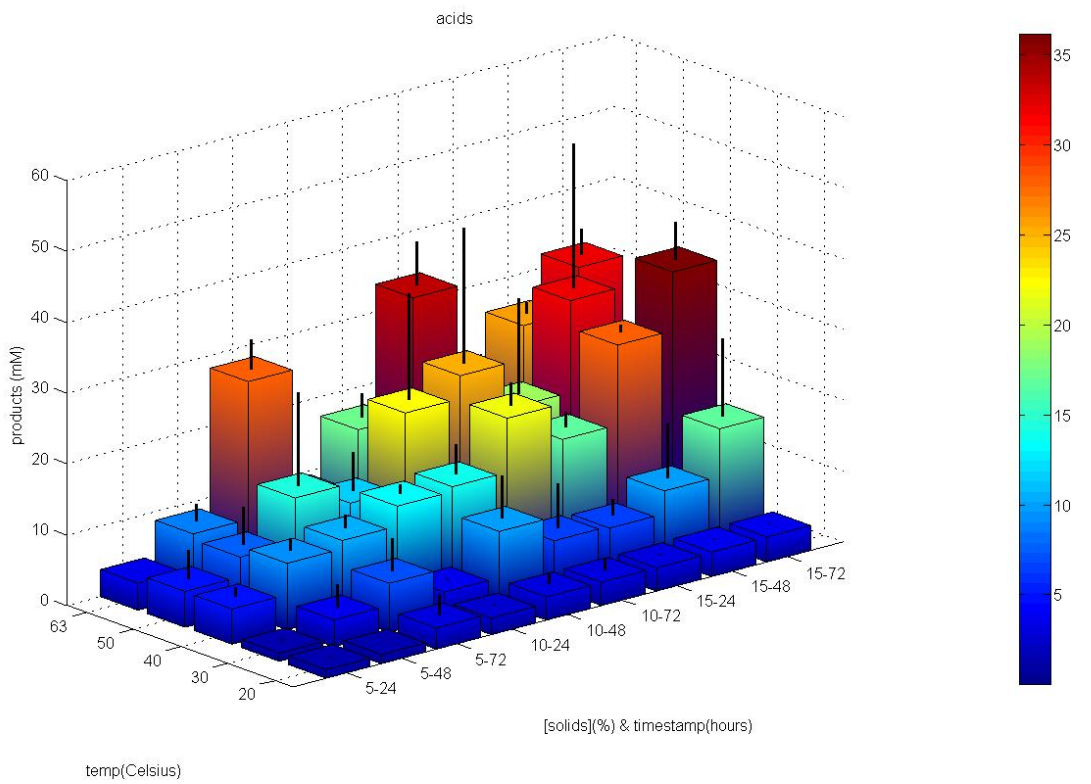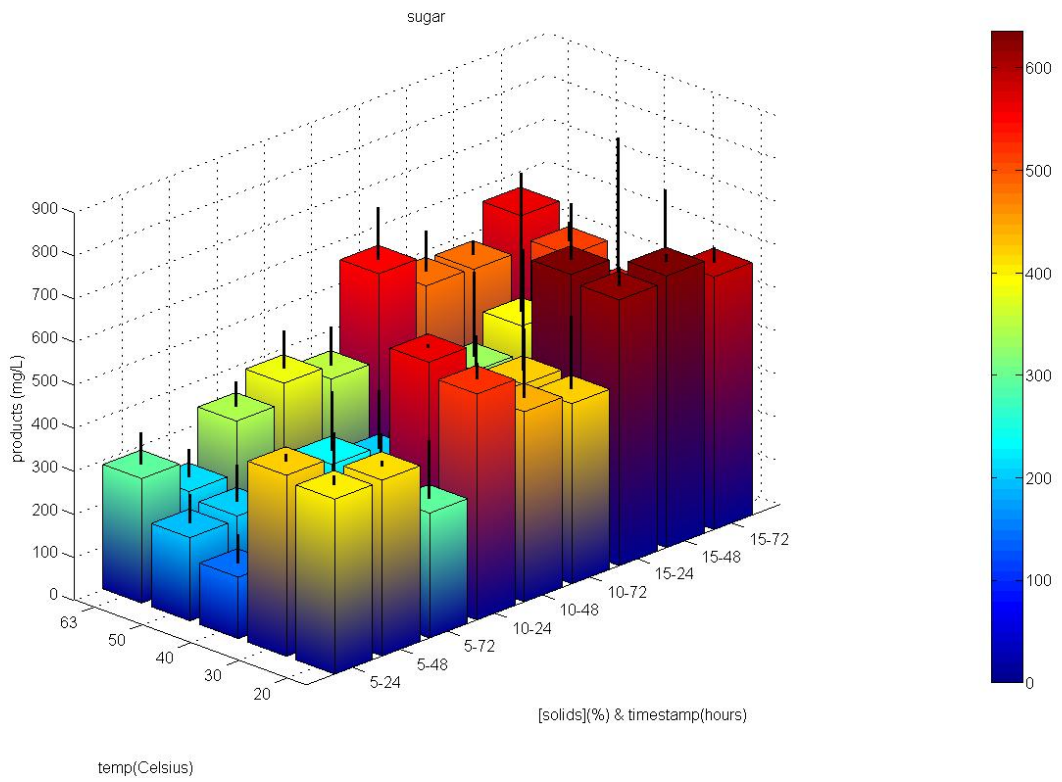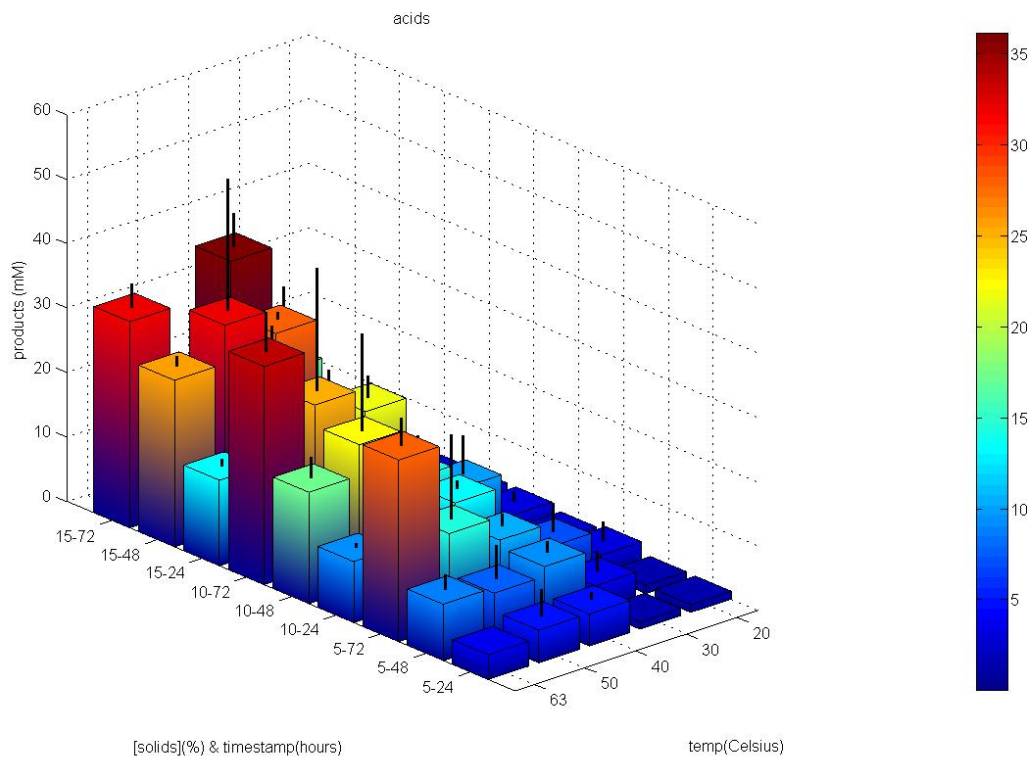[1] Akinosho, H.; Yee, K. (2014). "The emergence of *Clostridium thermocellum* as a high utility candidate for consolidated bioprocessing applications. Frontiers in Chemistry." 2(66) : 1-18.

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- Cancer Research
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- 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   Louisville, KY
Lab Technologist   6/2007 - 6/2011

- Detection of lead by atomic absorption spectroscopy
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