2015

THE EFFECTS OF INOCULUM SIZE, AIRFLOW RATE, BULK DENSITY AND PARTICLE SIZE ON THE SCALE-UP OF *PHANEROCHAETE CHRYSOSPORIUM* PRETREATMENT

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THESIS

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

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2015

ABSTRACT OF THESIS

THE EFFECTS OF INOCULUM SIZE, AIRFLOW RATE, BULK DENSITY AND PARTICLE SIZE ON THE SCALE-UP OF *PHANEROCHAETE CHRYSOSPORIUM* PRETREATMENT

The following full-factorial study compared fungal activity on lignocellulosic biomass that was inoculated with three different amounts of fungus, and grown using three different airflow rates. These treatments were compared to a control which consisted of biomass that was not inoculated but was exposed to the same growth conditions in the environmental chamber. The objectives of the following experiment were to determine the inoculum density and airflow rate required to optimize *Phanerochaete chrysosporium* lignin degradation. Additionally, this study quantifies the saccharification yield from the pretreated switchgrass.

The impact of substrate bulk density and substrate particle size on fungal growth were compared to determine if the particle size or the substrate bulk density has the predominant influence on the growth of the fungus, and subsequent pretreatment effectiveness quantified as an increase in glucose yields and lignin degradation. The particle size tests were controlled for bulk density; all three particle sizes were tested at a bulk density of 80 kg/m3. To test the density, three different bale densities were prepared controlling for particle size. The density tests were performed on small-scale bales made of 4 inch cut pieces of switchgrass compressed to the correct density. Therefore; density tests had the same particle size throughout all treatments, and particle size tests had the same density through all treatments. Carbohydrate accessibility post-pretreatment was examined through enzymatic saccharification and determination of glucose yields in the treatments and controls

KEYWORDS: *Phanerochaete chrysosporium*, lignocellulose, density, particle size, biological pretreatment.

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February 19, 2014

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ACKNOWLEDGEMENTS

This thesis and research would have been impossible without the help of several people. First I have to thank my graduate advisor Dr. Sue Nokes for her guidance and advice throughout my undergraduate and graduate career at UK. She gave me a job as a freshman, and instilled an interest that has grown into a passion for engineering and biofuels. I owe huge gratitude to my other committee members: Dr. Michael Montross, and Dr. Knutson for their time and guidance for this research. Huge thanks are in order for Mathew Ruwaya, whom has taught me the ropes throughout my undergraduate career, and helped tremendously for this research. I also have to thank the wonderful Biosystems and Agricultural Engineering Department for giving me a home away from home.

Thank you, to my friends in the department for always trying to include me in volleyball (even when we both know I am never going to show up), and for keeping me smiling on days when I want to pull my hair out. Especially my office mate Bobcat, who somehow dealt with my rants, kept me sane, and most importantly kept me laughing.

Never ending gratitude to my Mom and Dad for their continuing support in all my endeavors and pushing me to reach for the stars; without the lessons about hard work and dedication, I would not be where I am today. Thanks to my friends and family for always supporting me, and always pretending to understand what I do every day when I try and explain the strange details. Thank you Peyton, for always supporting me in every aspect (even when that meant giving up things for yourself). Also, thanks to Titus and Laila for sacrificing our daily walks so I could work on this research and thesis, and giving me big, sloppy kisses when I was feeling down.

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CHAPTER 1:INTRODUCTION

1.1 Lignocellulosic Conversion

Lignocellulosic biomass has become an increasingly feasible feedstock for biofuel production in recent years. In order to make biofuels a realistic replacement or additive for fossil fuels, further research is needed. The Biosystems and Agricultural Engineering Department at the University of Kentucky are developing an on-farm bunker biofuel production system. The on-farm system will allow farmers to use their biomass to produce a crude form of butanol which can be transported to a processing facility for further refinement (Yao and Nokes 2014).

One of the technological roadblocks to the economical use of this new biofuels production system is the recalcitrance of lignocellulose (Kumar, Barrett et al. 2009). To utilize biomass as a source for fuel, the plant cell walls must initially be pretreated either chemically or biologically to break down the lignin and access the carbohydrates in the plant which are the building blocks for biofuel production.

The white rot-fungi *Phanerochaete chrysosporium* produces enzymes that mineralize lignin, cleave carbon-carbon bonds, and depolymerize the lignin biologically, making it an ideal candidate to use during the pretreatment process (Isroi, Millati et al. 2011). To date, lab-scale experiments have been the focus of our research. Optimal conditions for using white rot fungi as a pretreatment method to break down the cellulose and lignin barriers in the plant cell walls have been developed (Asther, Capdevila et al. 1988, Xu, Chen et al. 2001, Gervais and Molin 2003, Asgher, Asad et al. 2006, Shi, Chinn et al. 2008, Said, Chisti et al. 2010, Fontenelle, Corgie et al. 2011). The objective of this study is to develop a larger scaled-up experiment to test the parameters established at the lab scale. Optimizing the growth of the white rot fungi in the biomass samples according to certain essential parameters will lead to increasing lignin degradation, making the next steps in the process much easier. To produce effective lignin-degrading enzymes, the physical conditions have to be optimized for commercial production (Chen, Zhang et al. 2010). Temperature, pH, water activity and moisture content are known parameters which affect the effectiveness of the fungal colony to affect lignin

degradation. We have chosen to focus this study on the effects of aeration rate and inoculation density in the larger bench-scale high-solids biological pretreatment. Another variable known to affect high-solids biological pretreatment is the particle size of the lignocellulose. Our on-farm system will use baled material, and not loose, ground biomass. Laboratory experiments have suggested that the *P. chrysosporium* respond more to particle density rather than particle size, however particle size and bulk density are typically interrelated. Understanding the relationship between particle size and bulk density of bales and quantifying how these affect growth of the fungus is essential to understanding the most effective approach to preparing the substrate for pretreatment. Perhaps grinding the substrate will be necessary, or possibly increasing the bulk density of biomass bales would be sufficient. The majority of the biological pretreatment studies have been conducted on ground biomass, typically between 2-5 mm particle sizes. Because the fungus appears to spread via particle contact, we hypothesize that the improved effectiveness of the pretreatment with smaller particle sizes is really an effect of higher bulk density and not strictly particle size. It is conceivable that because the smaller particle sizes inherently had higher bulk densities they performed better. If this hypothesis is true, the implication is that biomass would not need to be ground to be effectively pretreated; we would just need to control the bulk density at which the material would be provided to the process.

1.2 Project Objectives

The overall goal of this research is to optimize lignocellulose enzymatic digestibility (most likely by degrading lignin) using the white rot fungi *Phanerochaete chrysosporium* while conserving the cellulose and hemicellulose. The process will be conducted in a high-solids environment. The specific objectives are:

i. Quantify the effects of inoculum density and airflow on the growth of *P.chrysosporium* and subsequent enzymatic digestibility of the remaining lignocellulose. Lignin degradation will also be quantified.

ii. Quantify the effect of feedstock bulk density vs. feedstock particle size on the growth of *P.chrysosporium* on switchgrass.

1.3 Organization of Thesis

This thesis is organized into chapters. The first chapter introduces the background knowledge needed for understanding biological pretreatment and the justification for this research. Chapter 1 also includes the overall goals and specific objectives for this research.

The second chapter contains the general literature review for this thesis. The literature review was written to inform a reader new to this topic, and contains more background information than is needed in each of the subsequent chapters.

The third chapter reports on the first objective of this research; quantifying the effects of inoculum density and airflow on the growth of *P.chrysosporium*, and subsequent enzymatic digestibility of the remaining lignocellulose. Chapter 3 includes a summary, introduction, materials and methods, results and discussion, summary and conclusion, future work, and references for this set of experiments.

The fourth chapter discusses the second objective: Quantify the effect of feedstock bulk density vs. feedstock particle size on the growth of *P.chrysosporium* on switchgrass. Chapter four presents an overall summary, an introduction to the recent literature on this topic, materials and methods, results and discussion, summary and conclusion, future work, and references.

CHAPTER 2:LITERATURE REVIEW

2.1 Lignocellulose Chemistry

Lignocellulose is the three-dimensional structural component of plants that is comprised of three main parts: cellulose, hemicellulose, and lignin (Brown and Brown 2013). Along with lignocellulose, plants contain thousands of other components referred to collectively as extractives (Brown and Brown 2013). The makeup of the extractives depends on the type of plant, but generally includes resins, fats and fatty acids, phenolics, phytosterols, and other compounds (Brown and Brown 2013). Extractives can be removed by polar or non-polar solvents such as hot or cold water, ether, benzene, methanol, and other solvents that do not break down the actual biomass (Hu, Sykes et al. 2010).

Cellulose is a homopolysaccharide of glucose, with cellobiose as the main building block of this linear polymer. Cellulose can be either amorphous or crystalline (Brown and Brown 2013). The crystalline form of cellulose is insoluble in most solvents making it difficult to depolymerize (Brown and Brown 2013). Hemicellulose is a heteropolysaccharide composed mainly of hexoses, pentoses, and deoxyhexoses (Brown and Brown 2013). Hemicellulose has a lower degree of polymerization and crystallinity than cellulose, resulting in a lower chemical and thermal stability than cellulose (Brown and Brown 2013).

Lignin is the largest non-carbohydrate component in lignocellulose and is composed of three alcohol monomers: coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol (Brown and Brown 2013). The copolymerization of these three alcohols forms the heterogeneous and cross-linked polymer (Kirk and Farrell 1987). Lignin has both a structural and protective function for the biomass (Brown and Brown 2013). Lignin and hemicellulose form a layer around the cellulose portion of the plant (Brown and Brown 2013). In order to access the carbohydrates in the biomass, it is essential that the lignin and hemicellulose portion of the biomass be broken apart or degraded, while still keeping the important carbohydrates intact for future processing steps.

Different populations of switchgrass will have different compositions in terms of cellulose, hemicellulose, and lignin, and the different parts (nodes, leaves, internodes) of

the switchgrass have different compositions as well. The variety of switchgrass grown on the University of Kentucky farms is Alamo. Alamo switchgrass consists of 26.8% internodes, 3.7% nodes, and 69.5% leaves (Hu, Sykes et al. 2010). The leaf to internodes ratio is 2.7 (Hu, Sykes et al. 2010). Using hot-water extractive determination, Alamo switchgrass contains 16.0% extractives in their internodes, 12.0% extractives in the nodes, 19.7% extractives in the leaves, and 18.4% extractives in the whole plant (Hu, Sykes et al. 2010).

Alamo switchgrass was analyzed for carbohydrate, lignin, and ash content, without extraction. Compared with other populations of switchgrass tested, Alamo switchgrass contained the lowest amount of lignin. Averaging results from several studies on Alamo switchgrass composition the average lignin content was 19.6% in the internodes portion, 22.7% in the nodes portion, 23.0% on the leaves portion, and 22.1% for the whole plant The glucose levels were 44.3% in the internodes, 37.3% in the nodes, 35.6% in the leaves, and 38.0% in the whole plant (Hu, Sykes et al. 2010).

2.2 Pretreatment

The goal of pretreatment is to render the cellulose more accessible to hydrolysis in order to recover the monosaccharides for conversion to biofuels (Kumar, Barrett et al. 2009). Ideal pretreatment of cellulosic material would reduce the amount of intact lignin to less than 12%, reduce cellulose crystallinity, and increase the surface area of exposed cellulose so that cellulases have more binding sites (Hatakka 1983).

Fungal pretreatment is an option as a biological pretreatment of lignocellulose during solid substrate cultivation. Solid substrate cultivation involves the growth of microorganisms in a solid environment, with minimal free water available (Wang, Ivanov et al. 2010). Hyphal fungi have a strong tolerance to lower water activity levels and higher osmotic pressure conditions, making them efficient organisms for solid substrate cultivation (Raimbault 1998). Hyphal fungi have several advantages over other microorganisms for successfully tolerating the growth conditions in the low water environment found in solid substrate cultivation. Fungal hyphae grow through a combination of extension and generation of new hyphae through branching. The hyphae contain a solid mycelium tip that can more easily penetrate the solid substrate. The lignocellulolytic enzymes are excreted at the hyphae tip. In liquid cultivation the

enzymes excreted are diluted and therefore less effective. Solid substrate cultivation occurs in a low water environment so there is little dilution of the enzyme excreted, which allows for more enzyme activity on the substrate (Raimbault 1998).

The most effective biological degradation has been observed using white-rot fungi in aerobic environments (Kirk and Farrell 1987). *Phanerochaete chrysosporium* is a white-rot basidiomycete fungus that degrades lignin while leaving most of the cellulose behind. *P. chrysosporium* has the highest reported rates of lignin degradation (Kirk and Farrell 1987, Li and Zhang 2014). Li and Zhang (2014) tested three different white rot fungi (*P. chrysosporium, V. versicolor*, and strain F11) and chose *Phanerochaete chysosporium* as the optimal strain for degradation (Li and Zhang 2014). Out of three main categories of white rot fungi (those that produce mainly lignin-manganese peroxidase, those that produce mainly manganese peroxidase-laccase, and those that produce mainly lignin peroxidase-laccase) the group containing *P. chrysosporium* (the group that produces mainly lignin-manganese peroxidase) performed the best in degrading lignin (Hatakka 1994). *P. chrysosporium* is a very efficient lignin degrader and produces differing amounts of lignin-degrading enzymes according to adjustment of the culture parameters (Hatakka 1994).

P. chrysosporium cultures initially grow rapidly, but only produce a small amount of lignolytic enzymes during this initial phase (Ray, Saykhedkar et al. 2012). Ray et al, (2012) found that the hyphae of *P.chrysosporium* continue to penetrate the substrate up to fourteen days, but the fungal dry weight decreased after five days of incubation. After the initial growth period (seven days in the Ray et al study), the culture began to produce more lignolytic enzymes (Ray, Saykhedkar et al. 2012).

Quantifying the effectiveness of a given pretreatment method is typically done either by following the pretreatment with enzymatic hydrolysis and subsequently determining the glucose concentration. From the glucose concentration the yield (% of theoretical glucose recovered) can be calculated.

Another common method is to quantify the remaining lignin in the sample; however compositional analysis should be used with caution. Compositional analysis results in a % of lignin in the sample, however cellulose and hemicellulose compositions change also during pretreatment. Therefore the % lignin calculated after pretreatment does not have

the same denominator as the before pretreatment sample, so care should be taken in the interpretation of the results. However, studies have shown that there is a strong relationship between % lignin reduction and glucose yields. Wan and Li (2010) demonstrated that enzymatic hydrolysis is directly affected by the extent of lignin degradation in their study of *Saccharomyces cerevisiae* grown on 5, 10, and 15 mm ground corn stover (Wan and Li 2010). The strong linear correlation [y(% glucose yield) = 1.11x(%lignin reduction) + 19.96] with an R^2 of 0.996 is a strong indication that glucose yield (y) is directly correlated with lignin degradation (x). Cellulose digestibility is higher when the percent of lignin is lower, which supports the use of biological fungal pretreatments that degrade lignin.

Biobleaching or lightening of the substrate has been studied as an effect after pretreatment with white-rot fungi. Lignolytic enzymes, produced from the fungal culture are the key enzymes responsible for substrate bleaching, specifically manganese peroxidase (Kondo, Kurashiki et al. 1994). Therefore, bleaching of the substrate suggests the enzymes that break lignin down are present and active. Screening experiments have been conducted that demonstrate the correlation between MnP activities and bleaching of the substrate (Kondo, Kurashiki et al. 1994). Kondo et al. focused on the role of excreted enzymes during 3-7 day pulp biobleaching using either *Phanerochaete sordida* YK-624, *Phanerochaete chrysosporium*, or *Coriolus versicolor*. Membrane filters were used to prevent direct contact with the fungal culture and the substrate (kraft pulp). MnP activity was positively correlated with high levels of bleaching of the pulp.

2.3 Lignin Degrading Enzymes

P. chrysosporium produces two essential lignin degrading enzymes from different catalytically-distinct families during secondary metabolism (Boominathan and Reddy 1992). The two enzymes are lignin peroxidases (LiP) and manganese peroxidases (MnP) (Boominathan and Reddy 1992). LiP and MnP are heme-containing glycoproteins that require hydrogen peroxide as an oxidant. LiP breaks down non-phenolic lignin by taking one electron and generating a radical. MnP oxidizes Mn(II) to Mn(III) and then oxidizes phenolic compounds to phenoxy radicals (Krishna 2005). The combination of these enzymes breaks down the difficult lignin structure. *P. chrysosporium* produces both of these enzymes when subjected to a nitrogen or carbon limited growth environment.

When *P. chrysosporium* is cultures in a nitrogen-rich environment, LiP and MnP enzymes are not produced in the system (Boominathan and Reddy 1992).

2.4 Temperatures

Although it is important for the surrounding environment temperature to be optimal in terms of best growth of the fungus, it also needs to be at the best temperature for optimal lignolytic enzyme activity. Several studies have confirmed that the optimal temperatures for growth of the fungus and enzyme activity differ. Optimal mycelia growth of *P.chrysosporium* occurs at 37^0C (Asther, Capdevila et al. 1988). The optimal temperature of *P.chrysosporium* for producing the two types of enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP), was reported as 39^0C on steam-exploded wheat straw (Xu, Chen et al. 2001). However the optimal temperature for mycelia growth using *P.chrysosporium* can vary by as much as 25ºC, but the range of temperatures for optimal enzyme activity vary from about 5-10 degrees Celsius (Asther, Capdevila et al. 1988).

2.5 Moisture Content

Initial substrate moisture content is a fundamental parameter for a solid state pretreatment system. Initial substrate moisture content is fundamental to solid substrate cultivation systems because water is involved with the diffusion of solutes, gases and inhibitive metabolites, cell metabolism, and maintaining structure of the plasma membrane in the cells (Gervais and Molin 2003). High substrate moisture content can adversely affect fungal growth. If moisture content is too high, water will fill the voids where air flow is needed for fungal growth and for removal of inhibitors (Fontenelle, Corgie et al. 2011). High water content also leads to particle agglomeration and higher competition from bacteria (Krishna 2005). If the moisture content is too low, microbial growth is also severely hindered (Fontenelle, Corgie et al. 2011). Low moisture content can reduce nutrient diffusion, growth, enzyme stability, and substrate swelling (Krishna 2005).

Initial substrate moisture content is not only fundamental to SSC, but also an essential factor for the optimal growth of *P. chrysosporium*. Initial moisture content of the biomass before fungal inoculation is important for growth initiation. Said et al.

(2010) analyzed *Monascus ruber* fungal growth on long grain rice using different aeration levels and initial moisture contents during solid substrate cultivation. Moisture content produced more effects on the fungal growth of *Monascus ruber* than aeration rate, with levels of moisture below 57.5% wet basis adversely affecting growth. The highest initial moisture level (70%) with 0.2 L/min aeration rate resulted in the highest organism growth (Said, Chisti et al. 2010). These findings indicate that, for the best growth of fungus, the biomass bales should be initially flushed with water prior to inoculation to start with high enough moisture content to support fungal growth. Another study done by Fontenelle et al. (2011) also describes how increasing the initial moisture content compared to dry substrate results in an increase in substrate degradation. High-solids switchgrass degradation parameters were compared among varying bacteria, yeast, and fungi (not specified) with initial moisture contents between 60-75%. A 75% initial moisture content was the optimal amount for the early stages of degradation.

Substrate moisture content also plays an important role in formation of the enzymes that degrade lignin. If the water content is too high (>75% wet basis), enzyme activity will be inhibited due to decreased fungal growth and cessation of LiP synthesis (Asgher, Asad et al. 2006). If the moisture content is too low (< 40% wet basis), the microbial metabolic and enzymatic activity will be inhibited; possibly due to low substrate swelling, high water tension, or reduced solubility of nutrients (Asgher, Asad et al. 2006). The optimal moisture level must be maintained throughout the treatment time in order for the fungal growth and enzyme activity to function synergistically for optimal growth and lignin degradation. Asgher et al. (2006) grew *P. chrysoporium* on corncobs milled to 2 mm for seven days at 37^0 C with moisture contents varying from 50% to 90% wet basis. The optimum moisture level for LiP and fungal growth was 70% and achieved after five days (Asgher, Asad et al. 2006).

Shi et al. (2008) tested the effects of moisture content (65%, 75%, and 80%), salt concentration (no salts, modified without Mn^{2+}, modified salts with Mn^{2+}), and culture time. The dependent variables were lignin degradation, solids recovery, and available carbohydrates. The treatment producing the highest lignin degradation, highest carbohydrate level, and solids recovery was defined as the preferred pretreatment; 75% moisture content without added salts for the longest pretreatment time was found to be

the best pretreatment combination. Shi et al. (2008) also found that the fungal pretreatments with either 75% or 80% moisture content had no significant difference from each other in lignin reduction within the first ten days of treatment. Comparing 65%, 75% and 80% moisture content showed that lignin degradation was highest when the moisture content was either 75% or 80%. The solids recovery and availability of carbohydrates was highest with the 75% moisture content. A moisture content of 65% wet basis was too low for the fungus to metabolize and grow (Shi, Chinn et al. 2008).

2.6 Water Activity

Water activity is another measurement of water than can be used to assess optimal growth conditions. Water activity is an assessment of the energy associated with the water in a given system (Selig, Hsieh et al. 2012). Water relationships in SSC need to be understood because of the heterogeneous state that the system is in (Gervais and Molin 2003). Water activity might be a better measurement for understanding the fermentation water parameters than initial substrate water content because of the complex system between the solid substrate and the liquid. Solids levels in the system will have an effect on the distribution of water within the system (Selig, Hsieh et al. 2012).

Water activity measurements below 0.9 can slow or cease enzymatic and biological processes (Selig, Hsieh et al. 2012). In the study conducted by Gervais and others, they demonstrated the importance of water activity on the growth of two separate filamentous fungi: *Trichoderma viride* TS and *Penicillium Roquefort*. Ten different water activities were tested with growth and sporulation as the responding variables. Growth of the fungi was optimized at .99 for *Trichoderma viride* TS and .97 for *Penicillium Roquefort*. The researchers concluded that water activity is an important parameter to control for optimal growth of the fungi because of the variation in their results with lower water activity levels. If the optimal water activity was altered, a drastic decrease in growth and sporulation occurred, further demonstrating the need to control the water activity of the substrate being used for the SSF (Gervais, Molin et al. 1987).

2.7 Equilibrium Moisture Content

Equilibrium moisture content (EMC) is defined as the amount of water the substrate will retain when it reaches equilibrium within its environment. The EMC is an important characteristic of the feedstock and central to this research because EMC affects the amount of mass transfer occurring during aeration of the treatments. Switchgrass EMC gives important information about how the substrate will perform in various humidity and temperature environments. Godbolt et al. (2013) experimentally determined switchgrass moisture sorption isotherms. The moisture tests were conducted in humidified incubators using salt solutions to control the humidity within the incubators. Switchgrass weighed out to 3 grams was placed inside the incubators above the salt solutions, and weighed daily until a constant weight was observed for three days for each of the temperature and humidity conditions tested. The data collected were used to determine the EMC of switchgrass at varying temperatures and relative humidities. Switchgrass was determined to have an equilibrium moisture content of approximately 12% at 35ºC and 87% relative humidity, 10% at 35°C and 69% relative humidity, and 6% at 35°C and 40% relative humidity (Godbolt, Danao et al. 2013); the environmental conditions used in the study described in this thesis were 35°C and 50% relative humidity. Interpolating the results from the Godbolt et al study suggests the EMC using our environmental conditions is 7%.

2.8 Feedstocks

Feedstocks will perform differently during biological pretreatment due to varying lignin subunits, hemicellulose matrix, or differing extractives (Wan and Li 2011). Optimal fungal pretreatment by white rot fungi is feedstock selective (Wan and Li 2011). Corn stover, wheat straw, soybean straw, switchgrass, and hardwood chips were pretreated with a white rot fungus, *C. subvermispora*, and the %lignin removal and digestibility was evaluated to determine effectiveness of microbial pretreatment (Wan and Li 2011). Fungal pretreatment on wheat straw and soybean straw resulted in much lower %lignin degradation than corn stover and switchgrass. There was little to no levels of lignin degrading enzymes in wheat straw and soybean straw. Glucose yields from corn stover, switchgrass, and hardwood chips were 56%, 37%, and 24%,

respectively. Glucose yields from soybean straw and wheat straw was not improved. Increasing the pretreatment time increased the switchgrass glucose yields to 59% (Wan and Li 2011).

Other feedstocks have also been tested for pretreatment. *Phanerochaete chrysosporium* was grown on cotton stalks, and showed successful pretreatment through %lignin reduction. Scanning electron microscopy and Fourier transform infrared spectroscopy both visually demonstrated hyphae breakdown of the cell wall (Li and Zhang 2014). However, another study utilizing cotton stalks as the feedstock demonstrated similar %lignin degradation, but poor cellulose conversion. *Phanerochaete chrysosporium* was grown on cotton stalks for ethanol production. Although there was a decrease in %lignin following fungal pretreatment, there was no increase in cellulose conversion (Shi, Chinn et al. 2008).

CHAPTER 3: INOCULATION AND AIR-FLOW OPTIMIZATION

3.1 Summary

The following full-factorial study compared fungal activity on lignocellulosic biomass that was inoculated with three different amounts of fungus, and grown using three different airflow rates. These treatments were compared to a control which consisted of biomass that was not inoculated but was exposed to the same growth conditions in the environmental chamber. The objectives of the following experiment were to determine the inoculum density and airflow rate required to optimize *Phanerochaete chrysosporium* lignin degradation. Additionally, this study quantifies the saccharification yield from the pretreated switchgrass.

The percent lignin in the pretreated samples did not differ from each other or from the raw switchgrass. The glucose yields did differ significantly by treatment and by layer in the cultivation. However, the saccharification yields of glucose was lower than expected, with the control treatments releasing an average glucose yield of 5.1% of theoretical, while the treatments released an average of 7.0% of theoretical. Treatment 5 released the highest average glucose yield with 7.8% theoretical. Cultivation temperature between treatments varied due to airflow. The average temperature of the 30 L/min airflow treatment was 5 degrees lower than the average temperature of the 0 L/min airflow treatment. Higher airflows resulted in lower temperature readings of the substrate throughout pretreatment due to the evaporative cooling by the the incoming air.

3.2 Introduction

The vast majority of studies related to the biological pretreatment of lignocellulose using *Phanerochaete chrysosporium* have been conducted at the lab scale, for example 25 g or less of lignocellulose. This study was conducted in 2 kg size containers. The motivation for conducting this study was to determine if the amount of inoculum added to the culture, and the amount of air flow required for optimum growth of the fungal colony differ when the culture conditions are scaled-up from 25 g to 2 kg. “Optimal growth” will be quantified by lignin degradation and sugar yields after saccharification.

Amount and vigor of the inoculum is one of the most important factors in the initial growth of the fungus on the substrate. Sufficient inoculum is essential for the newly

cultured fungus to grow. In order to produce the desired amount of product in a shorter period of time, higher inoculum loadings are generally used for larger scale production (Lonsane, Saucedo-Castaneda et al. 1992). Using a larger amount of inoculum also leads to a faster onset of secondary metabolism, so enzyme production will begin sooner. The high ratio of inoculum to substrate also helps to defend against contamination during the pretreatment process (Lonsane, Saucedo-Castaneda et al. 1992).

Studies have found that air flow through the substrate is necessary during pretreatment and performs multiple functions; it maintains aerobic conditions, removes carbon dioxide, regulates temperature, and helps regulates moisture level (Gervais and Molin 2003, Krishna 2005). The high-solids pretreatment environment is heterogeneous; including liquid, solid, and gaseous phases. Air flow helps resolve heterogeneity issues between the solid, liquid and gas states that typically arise during solid state fermentation by maintaining temperature and moisture balances (Lonsane, Saucedo-Castaneda et al. 1992). This heterogeneous system is complex and difficult to optimize the different parameters due to their diverse interactions (Lonsane, Saucedo-Castaneda et al. 1992).

Not only is oxygen used for fungal growth, degradation of lignin also requires oxygen (Kirk, Schultz et al. 1978, Shi, Chinn et al. 2008). Kirk et al, (1978) found that at low oxygen levels, there was still fungal growth, but very little, if any, degradation of lignin (Kirk, Schultz et al. 1978). The concentration of oxygen in the sample affects the amount of lignin decomposed to CO_2. In an environment with 5% oxygen concentration, there was no attack on the lignin polymer (Kirk, Schultz et al. 1978). However, oxygen concentrations over 5% in the inflow air did not limit the degradation of lignin. Another study determined that oxygen concentrations above 21% do not substantially improve lignin degradation or enzyme activity (Leisola, Ulmer et al. 1983). Oxygen is still a requirement for lignin degradation, but an oxygen concentration greater than that in ambient air was not beneficial, therefore for this research, only air will be pumped into the system. Aeration also helped decrease the pretreatment time required. Increasing aeration and oxygen concentration in the high-solids cultivation decreased the pretreatment time needed to achieve higher reducing sugar yields by one week (Hatakka 1983). *P. sordida* was grown for 14 days in an oxygen atmosphere with reducing sugar

yields of 8.8 g/L, while only 5.4 g/L reducing sugar yields were reached following the same pretreatment time in a normal atmosphere (Hatakka 1983).

Particle size and airflow rates have been shown to affect the production and expression of lignocellulosic enzymes in high-solids cultivation (Gómez, Cuenca et al. 2011). The highest lignin degrading enzyme activity occurred in the treatment with the larger particle size (4.8 mm) and low aeration level (100 ml/min). High aeration levels with the smaller particle size dried out the substrate (Fontenelle, Corgie et al. 2011, Gómez, Cuenca et al. 2011).

According to these researchers, one of the most important variables in biodegradation by fungus is substrate moisture content. Because high aeration rates can dry out the high solids substrate bed, saturated air has been used to help minimize drying while maintaining the optimum substrate moisture level (Fontenelle, Corgie et al. 2011). High aeration for this study was controlled at an optimum 20 L/min in a 50 L bioreactor with 75% moisture content, where the air was humidified by bubbling through a water column to lessen the drying effects on the substrate (Fontenelle, Corgie et al. 2011). Another research study by Lopez et al., (2002) looked at two different white rot fungi *Coriolus vericolor* and *Phanerochaete flavido-alb*. It was found that the lowest aeration rate (3000 mL of sterile air once a week) worked best with the high-solids cultivation (Lopez, Elorrieta et al. 2002). Under low aeration rates, both fungi degraded lignin by a maximum of 30%. Both fungi also had higher lignin degradation rates at the lower aeration rates. (Lopez, Elorrieta et al. 2002).

3.3 Materials and Methods

3.3.1 Feedstock

The substrate for this research consisted of Alamo switchgrass collected directly from the North Farm in Lexington, KY in January 2014. After harvest, the bales of switchgrass were stored in a barn until being moved to the lab for testing. The switchgrass was not sterilized before pretreatment, but rather used straight from the bales for testing.

3.3.2 Environmental Chamber

The environmental chamber was a large walk-in chamber which controlled the temperature and relative humidity of the environment of all containers. All treatments were placed in the same environmental chamber set at 35°C and 50% relative humidity. This air has a humidity ratio of 17.8 grams of water per kg of dry air.

At these conditions the EMC of the substrate (switchgrass) is 7% water content wet basis (Godbolt, Danao et al. 2013). Therefore the substrate (approximately 70% wet basis originally) will easily release water to the air if possible, so we are saturating the air before moving it through the substrate.

3.3.3 Container Design

All experiments were conducted in separate containers for each treatment with their own liquid pumping system and their own aeration supply. Before testing, the bins were sterilized with bleach, cleaned, rinsed, and dried. The containers' dimensions were 0.74 m x 0.52 m x 0.38 m, or approximately 100 L in volume, shown in Figure 1. An elevated perforated metal platform was located at the bottom of the container which allowed the gravity-drained water/inoculum mixture to pool on the bottom while separating the biomass from the liquid to prevent oversaturation of the biomass, shown in Figure 1.

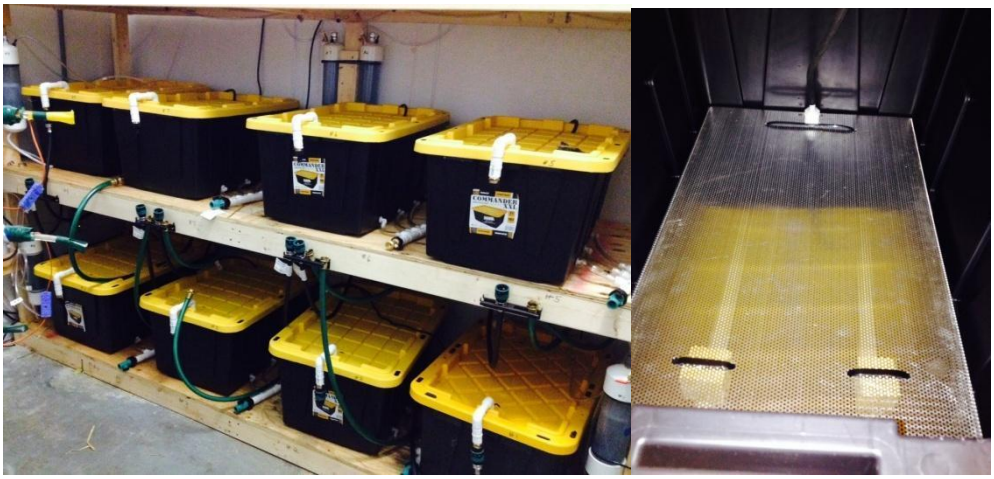


Figure 1: Containers and Perforated Metal Platform inside Containers

The air was conditioned before entering the chamber, then pumped through an adjustable manifold located outside of the container but within the environmental

chamber. The manifold controlled the volumetric flow rate of air to each container. Rotameters were used to adjust the air flow into each container, shown in Figure 2. Each container had its own water column located directly behind the container within the chamber. The heated air was forced through the water column before moving into the container. The aeration system within each container was located under the platform. The air flow tubes connected to the containers underneath the perforated platform. The air passed through two pipes attached to the bottom of the container with holes evenly spaced along the tubes, so the incoming air was also forced through the water pooled on the bottom of the container to further humidify the air before it is passed through the biomass. The objective was to saturate the heated air so that the air flow through the system will remove as little water as possible from the substrate to avoid drying the substrate.

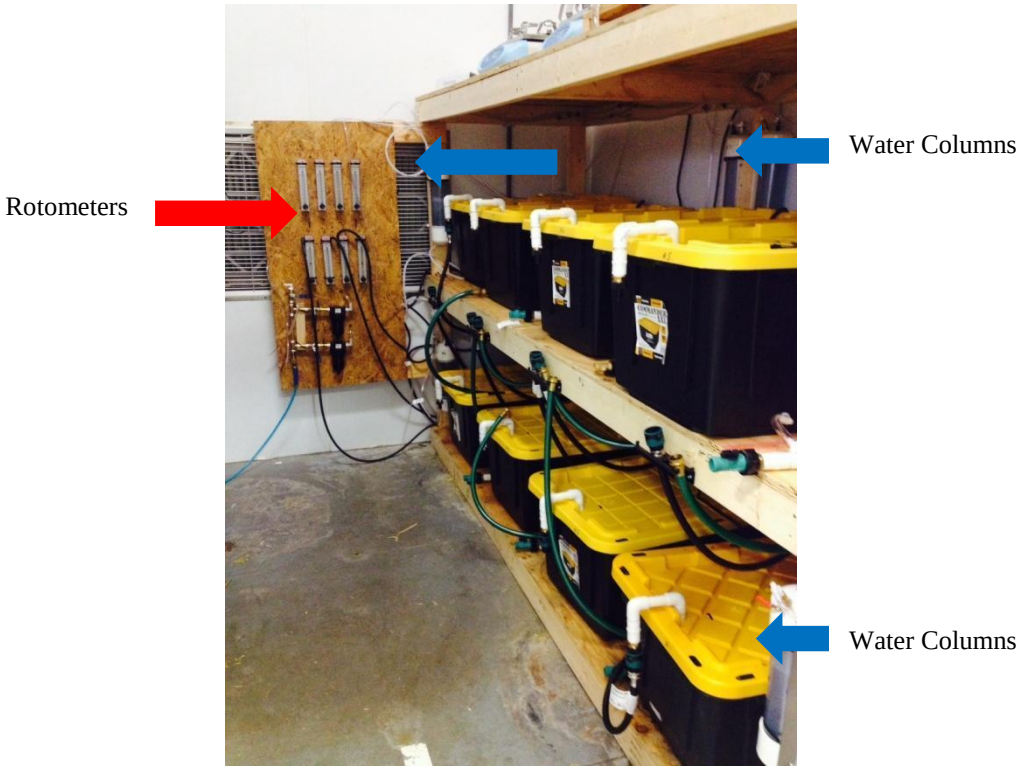


Figure 2: Container and Aeration Manifold

3.3.4 Inoculum Preparation

Phanerochaete chrysosporium (Parent Strain: ATCC 24725) culture was maintained in the lab at -40°C, The inoculum for each experiment was grown using freezer stock culture and growing it on potato dextrose agar plates at 35°C for 7 days. Once an agar plate was completely covered in fungal growth after seven days, a sterile blade was used to remove a section of the agar containing the organism. This section was further divided and placed into a 50 mL centrifuge tube. The sections in the centrifuge tube were mixed by vortexing for 30 seconds with 30 mL of DI water. During mixing, the agar was broken apart and a white precipitate was formed when the cells were released from the agar. The white precipitate was carefully added to the 6 L flask previously prepared and sterilized with dextrose (10g), potato powder (4g), and 1 L DI water. The flask was placed in a shaking incubator at 125 rpm for 72 hours at 37°C. The media used in the 100 L New Brunswick Scientific reactor was prepared in a 20 L container by combining 10L of DI water with 900 g of dextrose, 200 g of potato powder, and 10 mL of Antifoam 204 (Sigma A6426). This mixture was added to the 100 L fermenter along with 89 liters of tap water to fill. The media mixture was sterilized in place in the 100 L fermenter. The sterile media was inoculated with the 1 L previously prepared culture. The following set optimum growth conditions for the 100 L liquid culture were used: 35°C temperature, 10 LPM air, 125 RPM, 3 PSI for three days of growth.

After three days of growth, the fermentation broth was pumped out of the fermenter, filtered through cheesecloth, washed to remove contaminants and excess media, and allowed to drain for ten minutes to allow excess water to drain.

To quantify the inoculum volume, the fungal pellets were placed in a graduated cylinder after draining. Three different volumes were used for treatments; 1000 mL, 500 mL, and 250 mL of fungal pellets per container. The volume of inoculum was converted to a dry weight by drying a known volume in a 105°C oven for 24 hours or until a constant weight was achieved in a previously dried and weighed aluminum pan. The fungal dry matter was approximately 0.03 g/ml for all the samples. The data for moisture content determination and fungal culture concentration are located in Appendix A.

3.3.5 Experimental Design

Each container, described in section 3.3.2, held one treatment, and each treatment was tested in duplicate. Each treatment was subdivided into three sections, referred to as the top (TOP), middle (MID), and bottom layer (BOT), and separated by chicken wire for uniform sampling during the experiment. Each layer contained 500 ± 2.00 g of switchgrass for a total weight of 1500 g. The initial weight of each layer and total weight of each treatment is included in Appendix A.

There were 9 separate treatments summarized by the Figure 3. A full factorial experiment involving three different inoculum amounts (250 ml, 500 ml, 1000 ml) and three different superficial velocities (air flow rates) 0 m/min (0 L/min), 0.06 m/min (15 L/min), and 0.13 m/min (30 L/min) was conducted. These rates were selected to be representative of other research conducted on air flow for fungal growth; such as in Fontenelle et al. (2011), where the air flow rate was found to be optimal at 0.3 m/min (20 L/min) in a 50 L bioreactor with the solid substrate (switchgrass) at 75% moisture content wet basis (Fontenelle, Corgie et al. 2011).

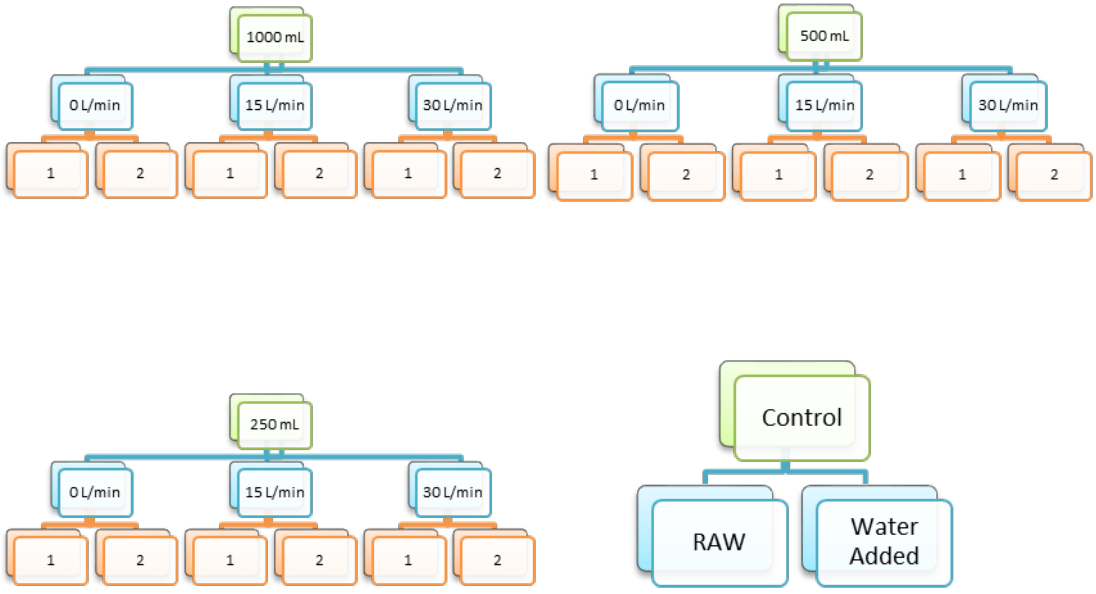


Figure 3: Experimental Design for the Inoculum and Airflow Rate Experiments.

Prior to pretreatment, the three separate layers were placed in a tub and saturated with water to ensure the initial moisture content of 70% and corresponding water activity of at least 0.9 was reached. After 10 minutes the switchgrass was removed from the soaking container and placed into the reactor as described above. An initial temperature and water activity measurement was then taken with the Rotronic Hygropalm water activity meter (model HP23-AW; Rotronic Instrument Corporation, Hauppauge, NY) to ensure the proper water activity was obtained.

The prepared inoculum was placed on the top layer of the switchgrass by hand distributing the fungus evenly. Once this initial pretreatment was completed the containers were checked daily for growth for five days at 35°C and relative humidity of 50% in the chamber. Measurements of water activity and temperature were taken daily for each layer in the container and recorded, and pictures were taken for a visual record of the fungal growth. Every measurement was taken in the same area to assure that any change in temperature or water activity was not due to location within the container. There were two control containers that were not inoculated with fungus. The switchgrass for one control was weighed out, and had no water added. The other control contained the weighted switchgrass which was soaked in water for 10 minutes, removed from water, and placed in the container.

3.3.6 Temperature and Water Activity Measurements

Temperature and water activity were recorded throughout the experiment using a hand-held water activity meter from Rotronic. The Rotronic meter (model HP23-AW; Rotronic Instrument Corporation, Hauppauge, NY) is an indicator with two configurable probe inputs and real time clock. The meter measures temperature in the range of -10℃ to 60℃, 0% to 100% relative humidity, and then displays the water activity from these measurements. The HP23-AW meter was calibrated with lithium chloride standards provided by Rotronic. The quick mode was used for all measurements, with accuracy within ± .005 A_w. The quick mode provided a measurement within about five minutes of start. One measurement was taken from each layer of each treatment bin for every day of pretreatment. Results were recorded and can be found in Appendix A.

3.3.7 Sampling Protocol

Prior to sampling, each layer was divided into 6 equal subsections and each subsection assigned a number. Three subsections in each layer were chosen using a random number generator. One sample (50g) was taken from each selected subsection in the top, middle, and bottom layer. The substrate along with any attached fungal cell growth was collected for destructive testing to determine moisture content, efficacy of enzymatic saccharification, and change in lignin composition. The samples were prepared using the NREL Laboratory Procedure (LAP) "Preparation of Samples for Compositional Analysis" (Hames, Ruiz et al. 2008). The samples were dried in a 45ºC oven for 48 hours, until they achieved a constant weight. The samples were milled so that the samples passed through a 2 mm screen. The mill was cleaned, and allowed to cool between each sample. Once preparation of the samples was complete, they were placed in labeled plastic bags and refrigerated until needed for further analysis. The NREL Protocol "Enzymatic Saccharification of Lignocellulosic Biomass" was used to test for the glucose yields (Selig, Weiss et al. 2008). This procedure was used to quantify the maximum extent of digestibility of the biomass after pretreatment. The higher yield of glucose provided an understanding of a more effective pretreatment based on maximum enzyme loading. Samples were run in triplicate to verify reproducibility. The samples used for saccharification were not extracted. Prior to enzymatic hydrolysis each sample was analyzed for moisture content using an Ohaus MB35 Halogen moisture analyzer. A full description of enzymatic protocol and calculations is provided in Appendix B.

Samples used for lignin analysis were extracted using the NREL Laboratory Procedure (LAP) for "Determination of Extractives in Biomass" protocol (Sluiter, Ruiz et al. 2005) using a Dionex ASE 350 solvent extractor (ASE 350; Thermo Fisher Scientific Inc., Bannockburn, IL). Each sample was extracted by water and ethanol extraction using the following settings: 100°C, 1500 PSI, static time 7 minutes with 3 static cycles, and 150% flush volume. The extractives were not quantified for this analysis, only removed using the protocol. Once water and ethanol extraction was complete, removing any soluble sugars or fungus remaining on the biomass, the samples were air dried in the 45ºC oven for 24 hours, placed in plastic bags and labeled, and refrigerated until

composition analysis was performed. The determination of lignin was performed following the NREL Laboratory Procedure (LAP) for "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter, Hames et al. 2008). Lignin was the only part studied for these tests. A full description of lignin protocol and calculations is provided in Appendix C.

3.4 Statistics

This experiment was conducted using a split plot experimental design. The container was treated as the whole plot, and layer within the container was evaluated as the split plot subunit. A PROC GLM model was used in SAS (version 9.3) with the error term for the whole unit being the interaction term of "rep" x "trt". The main effects tested for the whole plot were inoculum density and airflow rate, and the interaction of inoculum density and airflow rate. The response variable tested was glucose concentration after enzymatic hydrolysis. The split-plot subunit was analyzed for the effect of layer using the interaction of "layer" x"trt".

SAS tests were also run on lignin composition. A PROC GLM model was used in SAS (version 9.3) with treatment analyzed as the whole unit and significance was evaluated with an error term of "rep" x "trt". The response variable tested was lignin concentration after compositional analysis. The split-plot subunit was analyzed for the effect of layer using the interaction of "layer" x"trt".

3.5 Results and Discussion

3.5.1 Fungal Growth

A summary of treatments are summarized in Table 1.

Table 1: Treatment Summary

Treatment	Airflow (L/min)	Inoculum Amount (mL)
1	0	1000
2	15	1000
3	30	1000
4	0	500
5	15	500
6	30	500
7	0	250
8	15	250
9	30	250

Records kept for substrate moisture loss and fungal growth, are summarized in Table 2 and Table 3. If no drying of the substrate was observed (visually) the treatment was assigned a zero. If excessive drying was observed the treatment was assigned a 4. Treatments were considered unsuccessful if the switchgrass became dry (score of 0 to 2 for Table 2), and there was little to no visual fungal growth and no visual hyphae growth (score of 0 to 2 for Table 3).

The initial moisture content of the bales was approximately 75% wet basis before adding inoculum. The 0 m/min (0 L/min) treatments had average temperatures of 35°C, 36°C, 35°C in the top, middle, and bottom layers respectively over the course of experiments. The 0.06 m/min (15 L/min) treatments had average temperatures of 33°C, 33°C, 31°C in the top, middle and bottom layers, respectively. The 0.13 m/min (30 L/min) treatments had average temperatures of 31°C, 30°C, 28°C in the top, middle, and bottom layers, respectively.

Following pretreatment, the moisture content of each treatment at each layer was analyzed. The 0 m/min (0 L/min) treatments had average final moisture contents of 80%, 68%, and 71% in the top, middle, and bottom layers respectively. The 0.06 m/min (15 L/min) treatments had average moisture contents of 75%, 65%, and 65% in the top, middle and bottom layers, respectively. The 0.13 m/min (30 L/min) treatments had average moisture contents of 67%, 65%, and 74% in the top, middle, and bottom layers, respectively. The highest air flow treatment (0.13 m/min or 30 L/min) had a final

average moisture content over all layers of 69% wet basis. The final average moisture content of the treatments with no air flow over all layers was 73%. Overall treatments had a final average moisture content of 70%.

Table 2: Visual Observations of Drying of Substrate Over Pretreatment Time (0-no drying, 4-high drying)

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5
1	0	0	0	0	0
2	0	0	0	1	1
3	0	0	1	1	2
4	0	0	0	0	0
5	0	0	1	2	2
6	0	1	2	3	4
7	0	0	0	0	0
8	1	2	2	3	4
9	2	2	3	4	4

Table 3: Visual Observations of Growth of Fungus Over Pretreatment Time (0-no growth, 4-high growth)

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5
1	0	1	2	3	4
2	0	1	3	4	4
3	3	1	1	1	0
4	1	1	2	3	3
5	1	1	2	2	3
6	0	1	1	1	0
7	0	0	1	1	0
8	0	0	1	1	0
9	1	1	0	0	0

As can be seen from Table 3, the lowest inoculum amounts (treatments 7-9), showed less visual growth along the outside and throughout the bale. The growth along the top was only seen in the areas where the initial fungal cultures were placed, and most 250 mL inoculum treatments showed no sign of hyphal growth throughout the middle and bottom layers of the bale. When air flow was applied, the air flowing through the bales with the smallest amount of inoculum dried out much faster than the other bales with higher amounts of inoculum. Table 2 demonstrates this treatment had moisture issues. At the lowest inoculum level there was less moisture on the top of the substrate (moisture originating with inoculum); causing the treatments with any air flow to dry out much faster than other treatments where more inoculum were added. Overall, these

treatments were considered unsuccessful. This could possibly be caused by the additional moisture content increase when adding the larger amount of inoculum. Assuming the bales started with an initial moisture content of 75%, the 1000 mL treatments added an extra 3% moisture content to the bale for a final initial moisture content of 78%.

Most of the 500 mL inoculum treatments (treatments 4-6) were successful. Typically, they did not dry out as much as the 250 mL treatments, and also showed better growth than the 250 mL treatments. The 500 mL treatments did show hyphae growth into the middle and bottom layers by day 5 of pretreatment.

The 1000 mL treatments (treatments 1-3) were very successful. They had little to no drying of the substrate due to the extra moisture added with the inoculum. The fungal pellets had an initial moisture content of 97%, which added 3% to the moisture content of the top layer of the container. High inoculum treatments took longer to see visual white growth. Generally it took about two days to see any change in the culture on top. Visible white growth might have taken a little longer to see with the higher inoculum levels, but there were increased hyphae, and once the white growth appeared there was a drastic increase in growth on the bale.

When there was no air flow through the bale, the growth of the fungal colony took longer to show signs of visual growth. There was little to no growth after the first day for all of the treatments with no air flow. However, if the treatments had air applied, the white growth appeared sooner.

Daily journal entries for each treatment with visual descriptions are provided in Appendix D. The results of the fungal inoculum dry weight tests are summarized in Table 4. The samples reported an average fungus culture dry weight of .03 grams of fungus per mL of liquid inoculum. As can be seen by the following table, the fungus had an average 97% moisture content. When adding larger amount of inoculum, we were also increasing the moisture content of the substrate by 3%

Table 4: Fungal Culture Initial Moisture Content and Final Dry Weight

Pan Weight	Initial Weight (sample +pan)	Final Weight	Moisture Content	Fungus Culture g/mL
13.9	948.2	36.5	97.6%	0.02
14.0	549.0	28.6	97.3%	0.03
12.1	294.5	19.7	97.3%	0.03
13.8	299.9	21.4	97.3%	0.03
15.3	441.8	26.9	97.3%	0.03
13.9	988.9	43.4	97.0%	0.03
14.0	460.1	28.6	96.7%	0.03

3.5.2 Temperature

The three following figures demonstrate the temperature relationships found by the data. Figure 4 demonstrates every treatment with their own color to show the variability of the temperatures over the five day pretreatment time. The temperatures collected at each layer were averaged to obtain an average temperature for that treatment for every day of pretreatment. The temperatures vary over five days of pretreatment and show no obvious relationship. Varying temperature ranges from about 29ºC to 36ºC on the first day, and 25ºC to 36ºC by the last day. The highest average temperature was treatment 4 (no air flow, 500 mL inoculum) on day 2 at 37°C. The lowest average temperature was treatment 3 (30 L/min air flow, 1000 mL inoculum) on day 4 at 23°C. The bottom three lines on the graph are the treatments that had 30 L/min airflow.

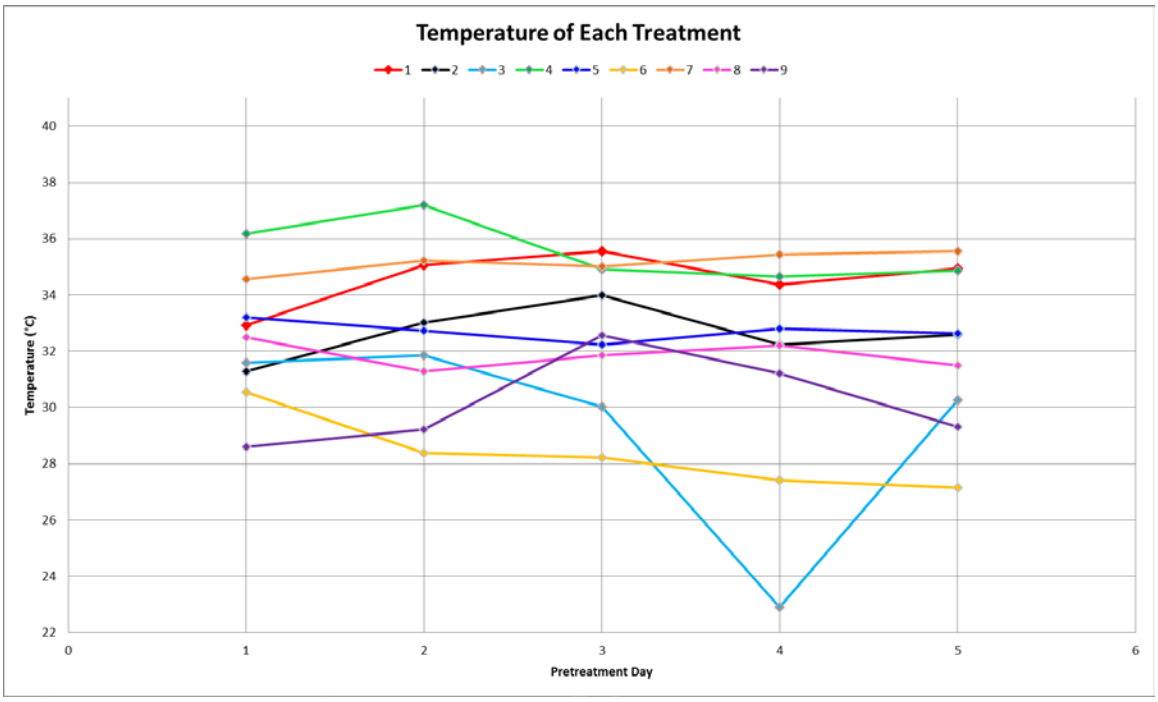


Figure 4: Temperature of All Treatments during Pretreatment Time

Figure 5 demonstrates top, middle, and bottom layers of each treatment. Temperatures recorded in the top layers are all blue; temperatures in the middle layers are all red; and temperatures recorded in the bottom layers are all green. In some cases, such as treatment 3, 8, and 9, the bottom layer had significantly lower temperatures than the other treatments. This graph also shows that all of the higher airflow treatments, no matter what layer, are lower than the treatments with no air flow.

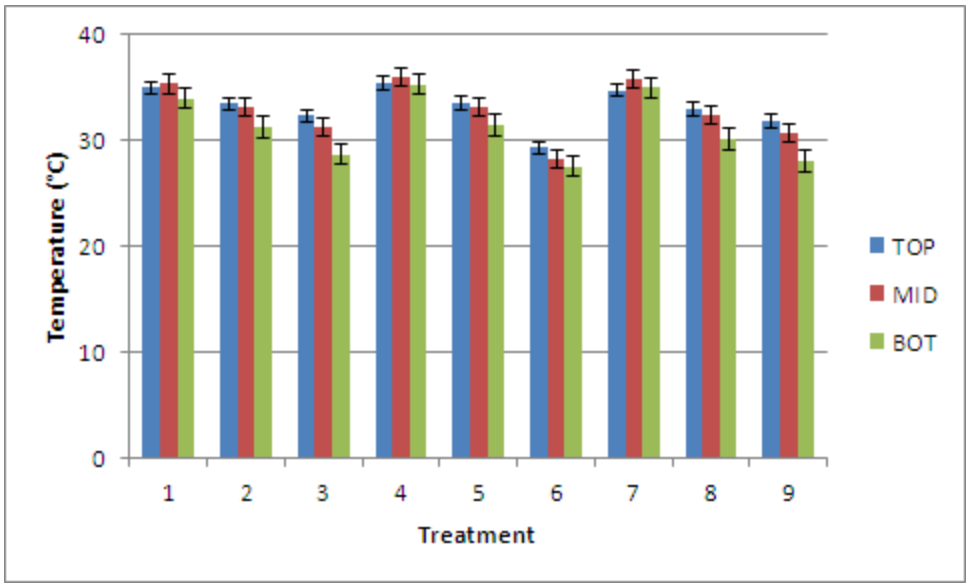


Figure 5: Temperature of Each Layer during Pretreatment Time

There was a difference in temperatures of the bales with different air treatments. Figure 4 demonstrates the temperatures measured over five days of all the treatments of the different air flows; the red section showing the treatments with no air-flow, blue section representing 15 L/min airflow, and green representing treatments with 30 L/min air flow. This figure demonstrates that the treatments with no air-flow had higher temperatures, 15 L/min had medium temperatures, and treatments with 30 L/min had the lowest temperatures and also had the most sporadic temperatures ranges as well. The treatments with no air-flow were closest to the optimum temperature range for fungal growth, 37°C (Asther, Capdevila et al. 1988). The average temperature of the 30 L/min airflow treatment was 5 degrees Celsius lower than the average temperature of the 0 L/min airflow treatment. The treatments with no air flow were optimal for temperature growth of fungus, and should be used in future research.

The drop in substrate temperature can be explained by evaporative cooling caused by the incoming air. The average temperature in the substrate dropped 5°C for the treatments with 0.13 m/min (30 L/min) (30°C) compared to treatments with no air flow (35°C). The following calculations were used to verify that evaporative cooling could be the cause of the temperature drop. Using the psychometric properties of the air, the energy in the air available for removing water from the substrate was equivalent to the

energy necessary to remove the water; therefore evaporative cooling is a plausible explanation for the temperature drop.

The following table summarizes the psychrometric properties used for the calculations.

Table 5: Psychrometric Properties and Other Values

	Avg. substrate Temp	Enthalpy kJ/kg da	Humidity Ratio $gH_2O/kg\ da$	Specific volume m^3/kg_{da}	Specific heat of water kJ/kg·K	Specific heat of air kJ/kg·K
High air flow treatment 30 L/min	30°C	81.5	20	0.886	4.18	1.005

First, the air properties of the high air flow treatment were determined. In the 100 L tub with a 30 L/min air flow rate a complete volumetric change of air occurred every ~3 minutes. The specific heat of dry air at 30°C is 1.005 kJ/kg·K (Henderson, Perry et al. 1997), and the change in temperature was quantified from the difference between the average temperature over the course of the experiments of the high flow system (30°C) (assuming substrate-air temperature equilibrium) and the temperature of the incoming air (35°C). First, the mass flow rate ($\dot{m}$) was calculated.

Equation 1: Mass flow rate of dry air

$$\dot{m}_{da} = Q\rho = \frac{30\ \frac{L}{min}\left(\frac{.001\ m^3}{L}\right)}{.886\ \frac{m^3}{kg}} = .03\ \frac{kg}{min}$$

Where $\dot{m}$ is the mass flow rate of dry air, da represents dry air, Q is the volumetric flow rate of dry air in m^3/min and ρ is the density of dry air.

The amount of water that the air was capable of removing from the substrate was calculated. The change in humidity ratio between the outgoing air of the 30 L/min treatment (20 g H_2O/kg dry air) and the incoming air for the same treatment (17.8 g H_2O/kg dry air) was calculated to be 2.2 g H_2O/kg_{da}. The $\dot{m}$ as calculated above was used to find the amount of water the air can remove per minute as it moved through the substrate. Multiplying this number by the time of the entire pretreatment time results in the potential amount of water removed during pretreatment from the high air flow system.

Equation 2: Potential Water Removed from Aerating the Biomass/Water mixture

$$\dot{m}_{da} \times (\Delta\, humidity\, ratio) =$$

$$.035\ \frac{kg\ dry\ air}{min}\left(20\frac{g\ H_2O}{kg\ dry\ air} - 17.8\frac{g\ H_2O}{kg\ dry\ air}\right)$$

$$= .077\frac{g\ H_2O}{min} . 14{,}286\ \text{min}\ . \frac{kg}{1000\ g}$$

$$= 1.1\ kg\ water\ potentially\ removed\ during\ pretreatment$$

The energy required to remove this water was calculated using the difference in enthalpy of the exit air and entrance air at the high flow rate treatment (81.5 kJ/kg dry air) and the amount of water previously calculated. It took 89.65 kJ to remove the water from the system.

Equation 3: Energy Required to Evaporate Water from Substrate

Heat of vaporization × amount of water removed = $\left(81.5\ \frac{kJ}{kg}\right) 1.1\ kg = 89.65\ kJ$

The specific heat for the biomass and water mixture was calculated from the previous values found. The total biomass and water was found and used for these calculations. The specific heat of the biomass was assumed to be the same as the specific heat of cellulose, 1.25 kJ/kg·K (Blokhin, Voitkevich et al. 2011).

Equation 4: Weighted Average of Specific Heat from Biomass and Water

$$weighted\ average\ of\ specific\ heat\ from\ biomass\ and\ water = \frac{[total\ biomass(specific\ heat\ of\ cellulose)+total\ water(specific\ heat\ of\ water)]}{(total\ biomass+total\ water)}$$

$$Mass\ of\ dry\ substrate\ in\ tub * c_{p_{da}}$$

$$= [1.32\ kg\ \left(1.25\ \frac{kJ}{kgK}\right) + 4.68\ kg\left(4.18\ \frac{kJ}{kgK}\right)]/(1.25 + 4.18)$$

$$weighted\ specific\ heat\ of water\&biomass\ mixture = 3.58\frac{kJ}{kgK}$$

Next, the following equation was used to calculate the temperature change in the biomass.

Equation 5: Calculating Change in Temperature in the Substrate

$$Q = m \cdot c_{pweighted} \Delta T$$

Q was calculated above and is the energy required to remove the water that the air can hold. The total mass of substrate and water is m, and using the weighted c_p we can calculate ΔT.

$$Energy\ required\ to\ evaporate\ \ water\ from\ substrate$$

$$= \ (m_{bm+mwater})(cp_{bm+water})(\Delta T_{bm+water})$$

$$89.65\ kJ = \ (6\ kg)\left(3.58\frac{kJ}{kg \cdot K}\right)(\Delta T)$$

$$\Delta T_{bm+water} = 4.2\ K$$

If the air removes enough water to become saturated, the energy required to do so would lower the biomass/water temperature by 4.2 K or 4.2 °C. This predicted change in temperature is similar to the measured change in temperature of 5°C.

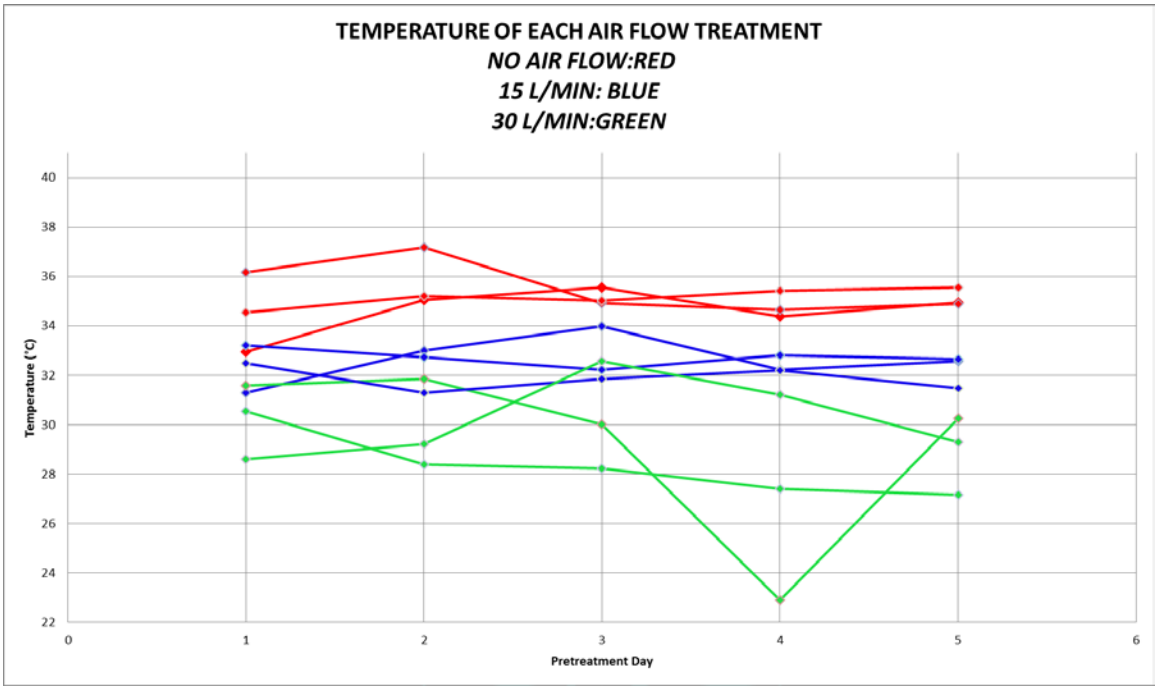


Figure 6: Temperature of Combined Layers of Each Air Flow Treatment during Pretreatment Time

3.5.3 Water Activity

There was an obvious variability in water activity across each layer of the container, but due to time constraints, it was not feasible to take multiple water activity readings throughout the container to follow up on this variability. Visually, I could see the containers drying out, especially along the edges due to edge effect, during higher airflow treatments.

Each of the water activity measurements was taken from the center of the container in each layer, which is where there was generally the least amount of moisture loss. Overall the water activity stayed fairly constant throughout all of the treatments over 5 days in the middle of the bale. The standard deviation of all of the water activity measurements over every day and every treatment was only 0.0034 out of 1.000. The water activity did not differ, but the final moisture content did. The high air flow and low air flow treatments had final moisture contents differing by 6%.

3.5.4 Enzymatic Hydrolysis

The Raw Data collected from the YSI including the sugar yields, enzyme blanks, and corrected sugar yields are in Appendix E. The following table shows the average glucose yields from the top, middle, and bottom layers as one overall average.

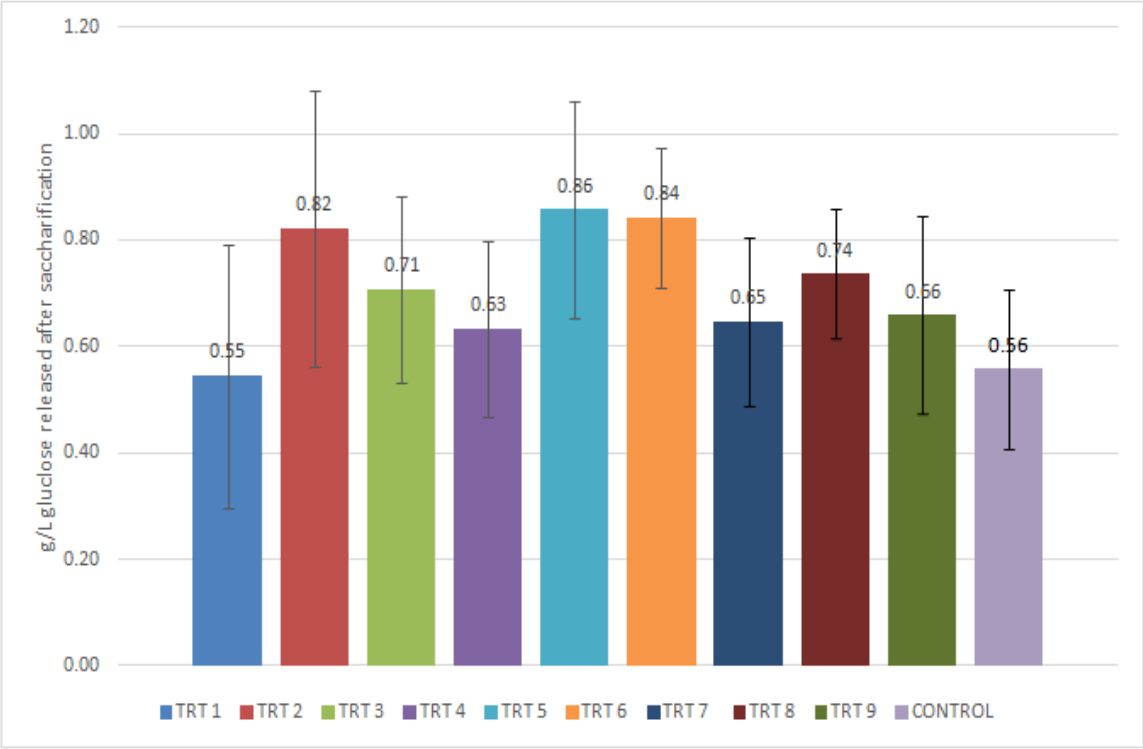


Figure 7: Glucose Yields of All Inoculum and Airflow Treatments and Control

Pertinent SAS codes and data are provided in Appendix E. The results from the Statistical analysis show a difference between treatments ($p < 0.05$). There were significant differences between the treatments, and significant differences between the layers. There was no significance with the interaction of these two. The control containers had an average glucose yield of 0.55 g/L. The lowest glucose yield out of all the treatments was treatment 1 (1000 mL fungus, 0 L/min airflow) with an average of 0.52 g/L. The highest glucose yield was treatment 5 with an average of .86 g/L. The following table shows the ANOVA for this statistical analysis.

Table 6: ANOVA of Treatments and Layers

Source	*DF*	*Type I SS*	*Mean Square*	*F Value*	*Pr > F*
Trt	9	0.80971878	0.08996875	7.04	<.0001
Rep(Trt)	11	0.2881055	0.02619141	2.05	0.07
Layer	4	0.61801235	0.15450309	12.08	<.0001
*Trt*Layer*	16	0.22780247	0.01423765	1.11	0.3999

There was a significant difference between treatments; however, the levels of glucose released were low compared to the theoretical yield. Theoretical glucose yield of all the treatments was 11.0 g glucose/L. Control samples released an average of 5.1% of the theoretical glucose. All treated samples released between 1.5% and 13.7% of the theoretical glucose. The average of all treatments released 7% theoretical glucose.

A different, yet similar study was done utilizing unsterilized corn stover as a substrate to test for lignin degradation using *P. chrysosporium* against other fungal cultures for 14 days. The glucose yield after saccharification in this study was 11.7% of theoretical (Tanjore 2009). Tanjore also found that sterile biomass had statistically higher levels of glucose released, possibly because autoclaving the biomass is in itself a pretreatment. Sterilization gives the fungal pretreatment an optimal environment to grow immediately after inoculation, and also possibly pretreats the biomass due to high temperature and pressure during autoclaving.

Another study utilized *Ceriporiopsis subvermispora* for an 18 day pretreatment. Five mm particle sized switchgrass resulted in glucose yields of 37.15% after enzymatic hydrolysis. Extending the pretreatment time increased glucose yields 10-30% (Wan and Li 2011). This study also had more than 26% lignin loss after pretreatment. The reduced lignin and elevated sugar levels found in Wan and Li's research is closer to the desired outcome for fungal pretreatment. However, Wan and Li's study was conducted on sterilized, 5 mm switchgrass, in a small flask. It is unknown if these results can be maintained in less ideal condition, for example larger volumes, larger particle sizes and unsterilized substrate.

3.6 Conclusion

The objectives of this research were to determine the inoculum density and airflow required to optimize *Phanerochaete chrysosporium* growth and therefore, lignin degradation. Additionally, this study investigated saccharification efficacy under optimal growth conditions. This research has determined that in 1.5 kg containers, forced air is not necessary in order to optimize fungal growth. From the visual results, lignin results, and sugar results; it can be determined that there were no significant improvements in pretreatment with higher air flow through the system. The visual results show a decrease in fungal growth, and drying out of the substrate.

The optimal amount of inoculum studies were inconclusive. The visual results showed more visible growth than higher initial inoculum levels, however, this observation was not supported by any of the lignin composition numbers or glucose yields. Lignin results showed no difference from the control treatments.

CHAPTER 4:DENSTIY AND PARTICLE SIZE

4.1 Summary

The impact of substrate bulk density and substrate particle size on fungal growth were compared to determine if the particle size or the substrate bulk density has the predominant influence on the growth of the fungus, and subsequent pretreatment effectiveness quantified as an increase in glucose yields and lignin degradation. The switchgrass particle sizes tested included: 5 mm milled, 1 inch cut pieces, and 4 inch cut pieces. The particle size tests were controlled for bulk density; all three particle sizes were tested at a bulk density of 80 kg/m^3. To test the density, three different bale densities were prepared: 80 kg/m^3, 120 kg/m^3, and 180 kg/m^3 controlling for particle size (approximately 4 inch pieces). The density tests were performed on small-scale bales made of 4 inch cut pieces of switchgrass placed in an Arbor Press, and compressed to the correct density. Therefore; density tests had the same particle size throughout all treatments, and particle size tests had the same density through all treatments. Carbohydrate accessibility post-pretreatment was examined through enzymatic saccharification and determination of glucose yields in the treatments and controls. The highest density bale treatment (.36 g/L) resulted in higher sugar yield than the lower density treatments (.18 g/L). The smallest particle size resulted in the highest sugar yields out of all of the density and particle size treatments (.40 g/L).

4.2 Introduction

Biochemical conversion of lignocellulosic biomass to fuel requires pretreatment to break down the lignin and provide enzymes access to the polysaccharides. Previous studies have reported that the fungus *Phanerochaete chrysosporium* is an effective biological pretreatment for lignocellulose. However the majority of the biological pretreatment studies have been conducted on ground lignocellulose, typically with particle size between 2-5 mm. Because the fungus appears to grow on the lignocellulose via hyphal contact with the biomass, we hypothesized that the improved effectiveness of the pretreatment with smaller particle sizes may be due to the higher bulk density inherently achieved with smaller particle sizes, and not strictly particle size. If this hypothesis is true, it would imply that biomass would not need to be ground to be

effectively pretreated; as long as the bulk density of the larger particle size material was sufficiently high.

Particle size affects the relationship between fungal growth, the heat and mass transfer during fermentation, and the surface area of the substrate that is available to the fungus. The surface area to volume ratio increases as the particle size decreases, hence smaller particle sizes would provide a larger surface area per unit volume on which the fungus can grow. Additionally, smaller particle sizes provides better nutrition availability for fungal cultures by making it easier for fungi to retrieve nutrients from the substrate and possibly preventing metabolite intermediates into the particles (Wan and Li 2010). However, smaller particle sizes will likely negatively affect mass transfer and could result in substrate agglomeration and aeration issues (Krishna 2005). Larger particle size is better for aeration, but the larger distance between lignocellulose particles makes it more difficult for the fungus to spread to new substrate, unless the density of the material is increased, thereby reducing the distance between particles. Particle size research also suggests there is an effect on enzyme activity and lignin modification or degradation. An increase in surface area could be beneficial for enzyme productivity. Breaking up the plant cell wall structure following pretreatment improves access for cellulases (Donohoe, Selig et al. 2009). Wan and Li weighed out ten grams of three different particle sizes (5mm, 10 mm, and 15 mm) of corn stover and pretreated with a wood-decaying fungi, *Ceriporiopsis subvermispor*. This study found that the largest particle size had significantly less lignin degradation compared to the other two sizes. There was no significant difference between 5 mm and 10 mm corn stover in regards to lignin degradation. Also, the smallest particle size had less cellulose loss (Wan and Li 2010).

Particle size could have an effect on sugar availability after enzymatic hydrolysis. In the same experiments, Wan and Li also tested the sugar yields following biological pretreatment. Following enzymatic hydrolysis, the smaller particle sizes had increasing yields of glucose and xylose. There was not a difference in sugar yields comparing the 5 mm and 10 mm particle sizes (Wan and Li 2010). Reducing the particle size from 15 mm to 10 mm caused a 5-10% increase in glucose yields. Extending pretreatment time also resulted in higher glucose yields from the smaller particle sizes. The enzymatic hydrolysis

yield was highly related to lignin removal, and therefore smaller particle size resulted in effective pretreatment.

Density of the lignocellulose in bales is an important parameter to understand with regard to pretreatment and biofuel production. In order to compete in the energy market, several components of the biofuel production process must be optimized; one of which includes the harvesting of the feedstock. Traditional methods of harvesting typically move hay from the fields in bales, chopped hay, or long loose hay (Kepner, Bainer et al. 1978). Large square balers are an additional option to be used for harvesting.

Large square bales vary in size, but research has been conducted on bales with end dimensions of 1.20 m by 0.9 m, and bale length of 2.44 m (Kemmerer and Liu 2010). Large square bales produce the densest and consistently well-shaped bales (Leisola, Ulmer et al. 1983). The large square bales have a high capacity in the storage and transportation of the substrate (Kemmerer and Liu 2010). With 180 kg/m^3 density bales, a large amount of material that can be transported; a semi-trailer flatbed could transport as many as 42 bales, with a net weight of 19,900 kg, approaching the legal load for a semi-trailer (Kemmerer and Liu 2010). Utilizing large square bales instead of round bales can produce transport loads between 30-60% heavier because of the geometry of the bales, increasing the amount of feedstock moved, and decreasing the fuel used (Lötjönen and Paappanen 2013). The markets for large square bales already exists; so the machinery, transportation, and storage needed are already widely available (Kemmerer and Liu 2010). Baling large square bales allows the tractor to be driven much slower to properly load the baler. With these decreased speeds, there is an increase of efficiency of producing bales, but also an unfortunate trade off with the farmer's time (Kemmerer and Liu 2010).

Higher density bales transport more material in a smaller amount of space; ideally, a higher density bale is essential for economically transporting bales of switchgrass for processing. However, bale density is currently limited by the density the baler can produce; but understanding the limitations can give us an idea of the reasonable densities to expect for current pretreatment with bales. Long loose hay non-mechanically compressed during stacking typically has a density of 65 kg/m^3 (Kepner, Bainer et al. 1980). Typically, large square bales have densities of 130-180 kg/m^3 (Lotjonen and

Paappanen 2013). Using these values, three separate bale densities will be tested for this research: 80 kg/m^3, 120 kg/m^3, and 180 kg/m^3.

This research dealt with bulk density of switchgrass during the particle size tests. Particle size tests required the same bulk density of each variation. This controlled one of the variables of the experiments, and also allowed for direct comparison with the lowest density experiment. Bulk density depends on several characteristics of both the individual particles and the material itself: material composition, particle shape and size, orientation of particles, particle density, particle distribution, moisture content, and applied axial pressure. Switchgrass has a higher loose filled bulk density compared to wheatstraw and corn stover (Chevanan, Womac et al. 2010). The tapped bulk density ranged from 68-323 kg/m^3.

Leaving the substrate in bale form would decrease the energy input for the process. Size reduction is a very energy intensive process; and could contain up to one-third of the energy input in the entire biofuel conversion process (Bitra, Womac et al. 2009). Bitra, Womac et al. tested total specific energy input calculations to grind up switchgrass as a function of screen size, mass feed rate, and rotor speed. Increasing speed from 250 to 500 rpm increased energy inputs 33% for all screen sizes tested (Bitra, Womac et al. 2009). Total specific energy decreased by 20% and effective specific energy consumption decreased by 55% with an increase in screen size from 12.7 to 50.8 mm for switchgrass (Bitra, Womac et al. 2009). As screen size in the tests increased, specific energy decreased; larger particle size resulted in less energy input for the process. Total specific energy decreased gradually by 55% with an increase in mass feed rate from 2 to 11 kg/min switchgrass (Bitra, Womac et al. 2009). Optimizing the system resulted in a knife mill screen size of 25.40 mm, rotor speed of 250 rpm, feed rate 7.6 kg/min and corresponding total specific energy of 7.57 MJ/Mg for switchgrass (Bitra, Womac et al. 2009).

The objectives of this study were to quantify the effect of feedstock bulk density vs. feedstock particle size on the growth of *P.chrysosporium* on switchgrass. Additionally, the density treatments and particle size treatments were separately analyzed for differences in glucose yields following saccharification.

4.3 Materials and Methods

4.3.1 Substrate

The substrate for all of the experiments consisted of switchgrass collected directly from the North Farm in Lexington, KY in January 2014. After harvest, the switchgrass bales were stored in a barn until moved to the lab for testing.

The bulk density samples for this research had variation. The assumptions in the shape of each particle size changed the bulk density measurements. The samples were made up of various parts of the whole switchgrass, and the samples were cut down using a table saw and grinder. Using the table saw and grinder means there will be some included variation among the actual sizes of the pieces of switchgrass. The actual bulk density of each of the treatments will also somewhat differ because of the variation in the particle size and shape. Assuming the particles are solid cylinders results in a much lower final bulk density than assuming the particles are hollow cylinders (Lam, Sokhansanj et al. 2008). For this research, the individual particles of switchgrass were assumed to be solid cylinders instead of hollow.

To test the effect of particle size, three separate particle sizes (10.2 cm (4 inches), 2.54 cm (1 inch), and 0.5 cm (0.2 inches)) were tested with a control for each variation. A table saw was used to cut the baled switchgrass down to 10.2 cm pieces. The 2.54 cm pieces were hand cut. The 0.5 cm sample was obtained using a hammer mill with a 0.5 cm screen. Each treatment was autoclaved for 60 minutes at 121ºC at 15 Pa.

The moisture content of each sample was determined in triplicate using an Ohaus MB35 Halogen moisture analyzer. The average of the three samples was used to determine the amount of DI water to be added to achieve an initial moisture content of 70% for the samples used in the particle size experiment. The equation used for quantifying the final bulk density is as follows:

Equation 6: Bulk Density

$$Final\ Bulk\ Density = \frac{Mass\ of\ the\ Biomass}{Final\ Volume\ of\ Compressed\ Biomass}$$

The bales used to test the effect of density were prepared used the 10.2 cm switchgrass pieces. Three densities were achieved by weighing out the calculated weight in grams of 4 inch switchgrass needed (126g, 189g, 280 g) and compressing this weight into 4"x4"x6" bales by using a Dake Arbor Press. Once compressed down to the desired size, the bales were tied with wire to ensure they would hold the desired density, as shown in Figure 8. To confirm the bulk density, the bales were weighed and measured after forming.

Figure 8: Mini Bale Before Sterilization

Each bale was autoclaved for 60 minutes at 121ºC at 15 Pa. Once the bales were sterilized, they were soaked in DI water for ten minutes to ensure saturation. The bales then were then inoculated and placed into their separate containers in the environmental chamber.

4.3.2 Experimental Design

In order to test the effects of bale density and particle size, each container held its own treatment. Each treatment had four replications, and each treatment had a

corresponding control. The control treatments were prepared exactly as the treatments were, however were not inoculated.

Three particle sizes were tested to test the effects of particle size on fungal growth. Each treatment had a density of 80 kg/m^3. The density was chosen to enable a direct comparison to one of the bale density treatments. To ensure that all the particle size treatments had the correct density, a known amount of switchgrass was weighed and placed in a glass graduated cylinder of known volume. The 0.5 cm particle size treatments required 7.78 g, the 2.54 cm particle size pieces required 18.59 g and the 10.2 cm particle size pieces required 26.28 g to achieve the same density of 80 kg/m^3. Three different sized glass cylinders were used for each particle size, and the radius of each was calculated and used for density calculations. A 2000 mL cylinder (radius of 6.51 cm) was used for the 10.2 cm pieces, a 1000 mL (radius of 5.40 cm) cylinder was used for the 2.54 cm pieces, and a 250 mL cylinder (radius of 3.49 cm) was used for the 0.5 cm pieces. The radius of the container, height of the switchgrass, and weight in switchgrass was used to calculate the density for each treatment. Each treatment needed a height of 2.54 cm for the correct density. In order to obtain the correct height, a weight was used to compress the biomass. The weight used was a plastic container filled with varying amounts of water according to the weight needed. Four locations at the inside surface of the container wall were measured to ensure the correct height was met using the weight placed on top of the substrate. The experimental treatments are summarized in the following table.

Table 7: Summary of density and particle size treatments used in this study

Objective 2 Treatments			
Density (constant particle size of 4'')	Treatment A	80	kg/m3
	Treatment B	120	kg/m3
	Treatment C	180	kg/m3
Particle Size (constant density of 80 kg/m^3)	Treatment D	0.5	cm
	Treatment E	2.54	cm
	Treatment F	10.2	cm

4.3.3 Fungus and Inoculation

Phanerochaete chrysosporium (Parent Strain: ATCC 24725) culture was maintained in the lab from the -40°C freezer stock, and reconstituted by culturing on potato dextrose agar plates at 35°C for 7 days. The culture was regularly maintained using the same lab protocol. Two larger cultures were used for this research: a 6 L flask culture, and a 100 L fermenter culture. Once an agar plate was completely covered in fungal growth after seven days, a sterile blade was used to cut a section of the agar with the cells. This section was cut into more sections and placed into a 50 mL centrifuge tube. The sections in the centrifuge tube were mixed by vortexing for 30 seconds with 30 mL of DI water. During the mixing the agar was broken apart and a white precipitate was formed from the cells being released from the agar. The precipitate was carefully added to the 6 L flask which had been previously prepared with dextrose (10g), potato powder (4g), and 1 L DI water and sterilized at 121ºC, 15 Pa, 30 minutes. The inoculated flask was placed in a shaking incubator at 125 rpm for 72 hours at 37°C.

The media used for the 100 L New Brunswick Scientific reactor inoculum was prepared in a 20 L reactor by combining 10L of DI water with 900 g of dextrose, 200 g of potato powder, and 10 mL of Antifoam 204 (Sigma A6426). This mixture was added to the 100 L fermenter along with 89 liters of tap water to fill the vessel. The media mixture was sterilized in place in the 100 L fermenter. The sterile media was inoculated with the 1 L previously prepared culture. The following set optimum growth conditions for the liquid culture were used: 35°C temperature, 10 LPM air, 125 RPM, 3 PSI for three days of growth.

After three days of growth, the inoculum was removed from the fermenter, filtered through cheesecloth, washed to remove contaminants and excess media, allowed to gravity drain for ten minutes, and placed onto the lignocellulosic substrate. To standardize inoculum amounts, the fungal pellets were measured in a graduated cylinder. A dry weight was determined for inoculum amounts by filtering through cheesecloth, rinsing with water, measured, and drying in a 105°C oven for at least 24 hours until a constant weight was achieved. The fungal dry matter weighed approximately 0.03 g/ml for all the samples. This data for moisture content determination and fungal culture concentration is located in Appendix A.

Inoculum was added to each treatment for quantifying the effects of particle size and density by per gram of biomass; .25 mL of fungus was added for each gram of biomass. The amount of fungal pellets to be added to each treatment was calculated, and each amount was measured using a graduated cylinder. All fungal pellets were spread out on the top layer of each treatment: particle size treatments and bale density treatments.

4.3.4 Fungal Pretreatment

Three separate bale densities were tested for this research: 80 kg/m3, 120 kg/m3, and 180 kg/m3. Each treatment was tested in quadruplicate, with a control bale for each density that did not have any fungal pretreatment added. Once prepared, .25 mL fungus/gram of biomass was measured out, and added to each bale by manually spreading it out on the top layer. Treatment A (80 kg/m^3) had 32 mL of inoculum added, treatment B (120 kg/m^3) had 48 mL inoculum added, and treatment C (180 kg/m^3) had 74 mL inoculum added because the initial substrate weight varied between treatments. Each

bale was placed in one of the containers described in section 4.3.3. The containers, open to the atmosphere, were placed in the environmental chamber at 35ºC and 50% RH for 10 days. Pictures were taken daily of each treatment.

Once the particle size tests were prepared, 43 mL DI water was added to treatment D (101.6 mm particle size), 28 mL DI water was added to treatment E (25.4 mm particle size), and 12 mL DI water was added to treatment F (5 mm particle size). Treatment D (101.6 mm particle size) had 7 mL fungus added, treatment E (25.4 mm particle size) had 5 mL of fungus added, and treatment F (5 mm particle size) had 2 mL fungus added. The flasks were placed in a 35°C and 50% relative humidity controlled environmental chamber for 10 days. Pictures were taken each day.

4.3.5 Sampling Protocol

Throughout the ten day pretreatment period, digital photographs were taken to observe fungal growth on the three different bale density treatments. At ten days, two samples from each of the bales were taken and placed in aluminum pans. The first sample was taken from the top 2 inches of the bale. The second sample was taken from the bottom 2 inches of the bale. Samples were taken from the control bales in the same way. All samples were placed in aluminum pans and dried at 45ºC for 48 hours. Pictures for all test are found in Appendix C.

4.3.6 Analysis

Samples were enzymatically hydrolyzed for 72 hours at 50°C to determine the resulting glucose yields after varying pretreatments to determine pretreatment effectiveness. The hydrolysate was analyzed using an YSI analyzer (YSI 2900D; YSI, Inc.; Yellow Springs, Ohio) to quantify glucose. The enzymatic saccharification was performed following the NREL Laboratory Analytical Procedures (LAP) for Enzymatic Saccharification of Lignocellulosic Biomass protocol (Selig, Weiss et al. 2008). The cellulase enzyme was purchased from American Laboratories Inc. (lot number ALI 14175-04). Protein concentration, cellulose activity, and cellobiose data was previously determined from separate lab studies. Protein concentration was determined from prior lab study using a modified Bradford method provided in Appendix G. Cellobiase activity was determined from prior lab study using a pNPG method for B-Glucosidase provided in Appendix F.

4.3.7 Enzymatic Hydrolysis

The NREL Protocol "Enzymatic Saccharification of Lignocellulosic Biomass" was used to test for the glucose yields (Selig, Weiss et al. 2008). This procedure was used to quantify the maximum extent of digestibility of the biomass after pretreatment. The higher yield of glucose provided an understanding of a more effective pretreatment based on maximum enzyme loading. Samples were run in triplicate to verify reproducibility. Prior to enzymatic hydrolysis each sample was analyzed for moisture content using an Ohaus MB35 Halogen the moisture analyzer. According to previous lab tests, the compositional analysis of switchgrass from the North Farm contained 30% (w/w) cellulose. Using this information, the equivalent of 0.1 grams of original cellulose was weighed out of each sample and added to a 20 mL glass vial with a screw top. The following formula was used to determine the amount in grams of each sample:

Equation 7: Wet Biomass Adjustment for Cellulose

$$.1\ g\ cellulose \times \frac{1\ g\ dry\ biomass}{.3\ g\ cellulase} \times \frac{1\ g\ biomass\ wet}{1 - \left(\%\ moisture\ content / 100\right)} = g\ sample$$

Sodium citrate buffer (5.0 mL), pH 4.8, was added to each vial. 100 ul of 2% sodium azide solution was added to each vial to prevent growth of organisms during digestion. The amount of water sufficient to bring the total volume of each vial to 10.0 mL after edition of enzymes was added to each vial. All samples were assumed to have a specific gravity of 1.000 g/mL. The following equation was used to determine the amount of DI water added to each sample.

Equation 8: DI Water Adjustment for Hydrolysis

$$10\ ml - 5\ ml\ buffer - .1\ ml\ Na\ Azide - 1.0\ ml\ enzyme\ dilution - (g\ measured\ out\ biomass) = ml\ DI\ water$$

After the addition of water, the samples were brought to 50℃ by setting them in a 50°C temperature incubator for ten minutes. Once an equilibrium temperature has been reached, 1 mL of cellulase enzyme was added. The volume of cellulose enzyme was equal to 60 FPU/g cellulose. The cellulose enzyme solution was prepared using a concentration of 1.729 g enzyme per 100 mL DI water. Equation 9 was used to find the enzyme concentration using the previously determined protein content of the enzyme. Enzyme blanks were also prepared that only contained buffer, water and enzyme (no substrate). Once all the samples were prepared, the samples were screw capped and placed in a shaking incubator for 72 hours at 50°C and 150 RPM.

Equation 9: Enzyme Concentration

$$.1\ g\ cellulose \times \frac{60\ FPU}{g\ cellulose} \times \frac{1.0\ mg\ protein}{3.0\ FPU} \times \frac{1.0\ mg\ enzyme}{.1157\ mg\ protein} \times \frac{1\ g}{1000\ mg} = .01729\ g\ enzyme$$

Once the 72 hour incubation was complete, the enzymatic reaction was stopped by placing the samples in a 93°C water bath for 15 minutes. Samples were vortexed and poured into a 2 mL collection vial and centrifuged at 5000 RPM for ten minutes. The supernatant from each sample was subjected to glucose analysis using the YSI glucose analyzer. To determine the glucose from each sample, the glucose concentration from the YSI was used, and any glucose concentrations from the enzyme blanks were subtracted out from the samples for final glucose. Samples were analyzed for glucose using YSI (YSI 2900D; YSI, Inc.; Yellow Springs, Ohio). The YSI was calibrated and standards were run at 9.00 g/L glucose before each analysis. YSI results were validated by comparison to HPLC results of random samples.

4.4 Statistics

The density experimental results were analyzed using a split plot design to test for differences within this treatment. The whole plot was the bale, to which the treatments were applied and depth within the bale was evaluated as the split plot subunit. A PROC GLM model was used in SAS (version 9.3) with treatment analyzed as the whole unit and

the significance was evaluated with an error term of “rep” × “trt”. The response variable tested was glucose concentration after enzymatic hydrolysis. The subunit (rep) was analyzed for the effect of layer and the interaction of “layer” × ”trt”.

The particle size results were analyzed using an ANOVA model in SAS (version 9.3). The response variable tested was glucose concentration after enzymatic hydrolysis.

The density and particle size treatments that overlapped one another were tested against one another using TTEST in excel. The lowest density bale, with four inch pieces at 80 kg/m^3, was tested against the largest particle size treatment, with four inch pieces at 80 kg/m^3. The response variable tested was glucose concentration after enzymatic hydrolysis.

4.5 Results and Discussion

4.5.1 Pretreatment Observations

4.5.1.1 Density Treatment

All the treatment bales had visual white rot growth. Pictures were taken to record growth during the pretreatment period; day ten and are available in Appendix H. A high density bale treatment is shown in Figure 9.

Figure 9: High Density Bale on Day 10 of Pretreatment

By the end of the ten day pretreatment period for the density tests; the highest density bales had the most visual fungal growth (Figure 9). Notice how the sides and top of the bale is completely covered in hyphae and dense white growth. A side by side comparison

of the high density bale and the low density bale on day ten demonstrates that the fungal culture grew well on the high density bale, and somewhat poorly on the low density bale shown in Figure 10. When the highest dense bales were opened for sampling, hyphae and visual white growth could be seen throughout the bale. The medium density bales had results midway between the low and high density bales. When the lower density bales were opened for sampling, there was much less visible growth throughout the bale. All of the bales had obvious bleaching when compared to the control bales; treatment bales were all several shades lighter in color. Treatment bales had similar bleaching results.

Figure 10: Low Density Bale on Day 10 of Pretreatment

The control bales were not inoculated prior to pretreatment. There was minimal growth on the control bales. There were minimal signs of contamination with some visible hyphae growing on the bale. The bales had been autoclaved prior to pretreatment; however, the bales were not cultured in a sterile environment, and may not have been completely sterilized during the autoclave cycle.

4.5.1.2 Particle Size Treatment

All particle size treatments had visual white growth by the end of the ten day pretreatment period. Pictures of the growth on day 10 were recorded and can be seen in Appendix H. The 5 mm particle size took longer to show white growth; the treatments did not have any growth until day 7 and even then it was sparse, but quickly accumulated by day 10. The larger particle sizes had visual white growth by day 5, and continued to growth quickly until sampling was done. By the end of the treatment time, all treatments

had very similar dense white growth along the edges of the containers, as seen in Figure 12, Figure 13, and Figure 14. When the treatments were removed for sampling, it was noted that the middle of the top the biomass did not have any white growth where the weight was placed to ensure the same density for all treatments (Figure 11). This reinforces the need for oxygen transfer to enhance fungal growth. Although there was no growth on this top middle circle, samples were still collected from the total treatment container.

Figure 11: View from the Top of the 4 inch Particle Size Treatment

Figure 12: Day 10 of Pretreatment for 5 mm Treatment

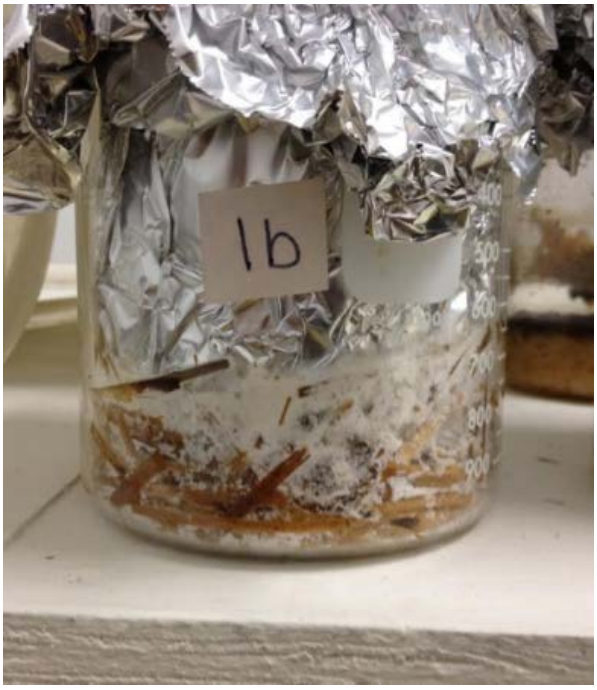


Figure 13: Day 10 of Pretreatment for 1 inch Treatment

Figure 14: Day 10 of Pretreatment for 4 inch Treatment

4.5.2 Enzymatic Hydrolysis

Glucose yields were determined from all of the density treatment and control samples. Raw data for all of the samples and controls are provided in Appendix I. Data are included for the top and bottom layer of each sample. Three enzyme blanks were recorded and the average was subtracted from the treatment samples. The control samples for each of the treatments had similar average glucose yields with very small standard deviations; .54 ± .01 g/L, .60 ± .02 g/L, and .64 ± .06 g/L. The three density treatment samples had less glucose released after hydrolysis than the control samples. The lowest density bale had the least amount of glucose released (.19 g/L). The highest density bale had double the glucose released compared to the lowest density bale, and the most glucose released after hydrolysis out of all of the treatments (.40 g/L), although this was still less than the control bale.

The overall results showed a statistical difference between treatments within the two main experimental treatments; particle size and density of bales. The glucose results for particle size showed no significant difference from the sugar released from the density treatments. Figure 15 shows the box and whisker plot of the two categories and their resulting sugar yields.

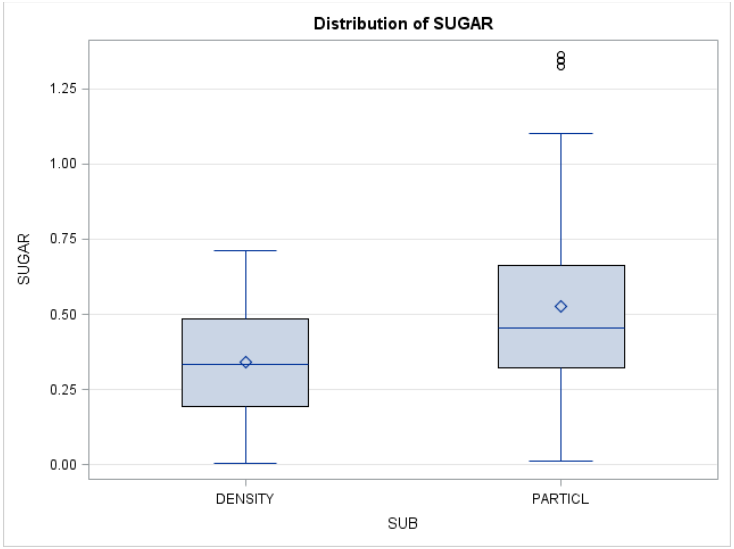


Figure 15: Distribution of Sugar between Density and Particle Size

Two experimental units within treatment main effect shared overlapping values in their separate tests: the lowest bale density test had 101.6 mm particle sizes, and a density of 80 kg/m^3 and the highest particle size tests had 101.6 mm particle sizes with a density of 80 kg/m^3. The overlapping glucose yields were compared using a T-TEST in excel. The null hypothesis was that there was no significant difference between the glucose yields in the density treatment and the particle size treatment, with a hypothesized mean difference of 0. The resulting T-TEST had a low p value (.00018 << .05). The probability that the observed results are due to random chance is very low. There is a significant difference between the particle size and the density glucose yields. Table 8 demonstrates the T-TEST results from excel.

Table 8: TTEST Comparing Treatment A and F

t-Test: Two-Sample Assuming Equal Variances

	Particle Size	*Density*
Mean	0.4	0.178
Variance	0.031	0.014
Observations	12	20
Pooled Variance	0.02	
Hypothesized Mean Difference	0	
df	30	
t Stat	4.267	
P(T<=t) one-tail	9.12E-05	
t Critical one-tail	1.697	
P(T<=t) two-tail	0.0002	
t Critical two-tail	2.042	

Although the two treatments had the same density and particle size, just different forms of compaction, they differed in the amount of substrate weighed out in each treatment to obtain the desired density. There was more switchgrass in the bale treatment, and might have required longer pretreatment time to get the same results as the particle size test. The difference in fungal growth between the particle size and density treatments could be due to the difference in amounts of substrate available. The bale had more total substrate and may have required more time for the fungus to disperse throughout the bale. The bale might have the same amount of glucose yields if the pretreatment time had been extended. Figure 16 shows a side by side comparison of the two common treatments.

Figure 16: Visual Comparison of Two Equivalent Treatments

The density treatments were analyzed for differences between treatments according to glucose yields. The saccharified density treatments resulted in 1-3% of theoretical glucose yield, while the controls released d between 4-6% theoretical. There was a statistical difference between the treatments ($p < .05$). There was no difference between layers, and no significant difference between the interaction of treatments and layers. Results are summarized in Table 9.

Table 9 : ANOVA for Density Treatment with Sugar as Response Variable

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	5	0.61967450	0.12393490	39.70	<.0001
REP(TRT)	9	0.14109648	0.01567739	5.02	0.0165
Layer	1	0.00051429	0.00051429	0.16	0.6955
TRT*Layer	5	0.01190597	0.00238119	0.76	0.6010

T-tests performed using excel showed a difference in the high density treatments against both middle and low density treatments. The null hypothesis being evaluated was that there was no significant difference between the glucose released in the low density treatment compared to the particle size treatment of the same density. The resulting T-test had a p value of 0.03 therefore there was significant difference between the middle density and the high density glucose yields. The high density bale versus the low density bale had a null hypothesis that there is no significant difference between the

glucose yields in the density treatment and the particle size treatment, with a hypothesized mean difference of 0. The resulting TTEST had a low p value (0.0000016<< .05). There was significant difference between the low density and the high density glucose yields; the high density bales resulted in higher glucose yields. Average glucose yields were .36 g/L and .18 g/L for the high and low density treatments, respectively.

The entire sample of the particle size treatments was saccharified and analyzed for differences in glucose yields. There were four replications of the treatments. Each treatment replication was split into three sub samples to saccharify. Glucose yields were determined after saccharification and YSI analysis. Each hydrolysis included three enzyme blanks, which were averaged and subtracted from the treatment results. The raw data can be found in Appendix J. Table 10 demonstrates that there was a statistical difference between the particle size treatments. The smallest particle size tested had the highest glucose yields. The smallest particle size was the only treatment statistically different from any other treatment. The 5 mm particle size treatments released on average 9% of theoretical glucose, while the 1 inch released on average 3%, and the 4 inch pieces released on average 4% theoretical glucose. The 5 mm particle size released over twice as much as the other treatments, with some individual 5mm particle size samples achieving between 10-12% theoretical glucose. However; these levels are still low when comparing to similar research that resulted in theoretical glucose yields as high as 37.15% (Wan and Li 2010).

Table 10: One Way ANOVA for Particle Size Treatments with Sugar as the Response Variable

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	5	3.85676611	0.77135322	19.52	<.0001
REP	2	2.67939111	1.33969556	33.90	<.0001
TRT*REP	-2	0.00000000	0.00000000	0.00	.

Overall low glucose yields following hydrolysis could be attributed to the drying of all of the samples immediately following pretreatment. Research testing the cellulose digestibility as a result of cellulose accessibility was examined. The effects of air drying on corn stover after pretreatment resulted in a substantial decrease in digestibility. Two separate severity levels of pretreatment were analyzed and despite having drastically different pretreatments, after drying both resulted in similar hydrolysis results (Jeoh, Ishizawa et al. 2007). Wang, He et al. studied the effects of air and heat drying on pore volume distributions. Air drying at 25°C caused collapse of pores; specifically larger pores that were equal to the size of the cellulases needed for effective enzymatic hydrolysis (Wang, He et al. 2012). This could explain the similar glucose yields obtained from both density and particle size treatments.

Another study demonstrated the effects of substrate availability on enzymatic hydrolysis. This studied compared the hydrolysis results of oven drying at 50 and 100°C and air-drying. Drying the samples resulted in reduced enzymatic hydrolysis reaction; which was attributed to the hornifying effect that lignocellulosic material undergoes during drying. At 50ºC, there was a 15% reduction in enzyme susceptibility to the substrate. According to this research, drying had a significant impact on the reduction of large pores and formation of smaller pores in the cell walls which did not allow for enzyme accessibility. Therefore enzymatic saccharification did not give an accurate evaluation of pretreatment effectiveness (Esteghlalian, Bilodeau et al. 2001).

4.6 Conclusion

The effect of bale density and particle size was studied to determine if the particle size or the density of the substrate has a greater effect on the growth of the fungus. Pretreatment effectiveness was quantified through, glucose yields during saccharification and lignin degradation post-pretreatment. The particle size tests were all done on substrates in a container that allowed a constant density across all tests (80 kg/m3). Density tests had the same particle size throughout all treatments. Carbohydrate accessibility post-pretreatment was examined through enzymatic saccharification and determination of glucose yields in the treatments and controls.

This research has helped determine the need for future understanding of sample preparation using large particle sizes in regards to drying and milling the samples.

Further tests will validate the hypothesis that the drying of the substrate post-pretreatment caused irreversible pore collapse which resulted in low and non-representative glucose yields. The visual results suggest that at a higher density, the biological pretreatment performs better.

CHAPTER 5:FUTURE WORK

5.1 Inoculation and Air-Flow Optimization

There are several areas for future research regarding these tests. Scaled-up biological pretreatment should undergo more testing to determine optimal parameters and techniques. Longer pretreatment times need to be explored. The five day long pretreatment time could be extended to test for higher glucose yields. The larger scale could require an extended pretreatment period. Although the five day pretreatment time is ideal at the lab scale for the fungus to produce the required enzymes for lignin degradation, there could be issues with fungal growth and lag phase on a larger scale. Low glucose yields could be a result of the need for longer pretreatment times; Yebo and Li (2011) had 10-30% increase in glucose yields when extending pretreatment to 35 days (Wan and Li 2010).

The statistics results showed there was a difference in the layers of the treatments; so inoculation according to layers could be studied. Tests could be run on different inoculation techniques. This current research used a top loading technique; the fungus was spread on the top layer only. Other possible techniques could be spreading the inoculum throughout the bale, and/or dispersing the inoculum along the edges where the pieces of substrate are cut. Treatment of the inoculum before inoculation could be tested as well. There is the possibility of using a sprayer technique with a blended inoculum and spraying over the bales of substrate to evenly coat the substrate. If the sprayer technique is used, the fungal pellets will have to be able to move through the sprayer without clogging. Using a spraying technique could potentially result in more even pretreatment, and also make for easier application of inoculum.

The effective use of unsterilized substrate should undergo further research. Questions to be answered include: is sterilization a pretreatment in itself, can the same yields be acquired through unsterile treatments as in sterile treatments using fungus by changing the conditions and treatment time, and should the biomass undergo sterilization before biological pretreatment?

Variations in optimal temperature and humidity conditions should be studied. Throughout the containers temperature variability may change the optimal temperature

set point. It also could be worthwhile to study the change in temperature throughout the containers over time in several different humidity environments.

5.2 Density and Particle Size

This results of this research are intriguing and should lead to further studies. The excellent fungal growth observed on the bales led to an expectation that the high density bales would produce higher glucose yields than the other treatments. However the results did not follow this expectation. There are several possible explanations of the low sugar yields encountered. Pore collapse from the heat during drying could lead to problems of enzyme accessibility, which would lead to low glucose yields (Esteghlalian, Bilodeau et al. 2001). However, preliminary data in our lab showed no difference in saccharification extents when the post-pretreatment samples were dried compared to undried. The protocol for sample preparation was used on the larger samples before they were milled for analysis (Hames, Ruiz et al. 2008). Air-drying the samples at 45ºC is common practice for prior to grinding the samples for compositional analysis. Understanding the relationship of the layers in large bales to the total bale pretreatment will be important for further scale-up. Further testing on the layering effect in the bales should be conducted. Although this research resulted in no difference between the layers of the bales, these bales were only four inches in height, and a larger bale could have very different results according to layer.

Pretreatment time length could have an effect on future carbohydrate accessibility. Depending on the length of time the substrate is exposed to the pretreatment, certain layers could undergo more efficient pretreatment than other layers. If there is a difference in effective pretreatment times as a function of layers, it would be interesting to study the effectiveness of removing the top layer first while leaving the bottom layers for further pretreatment. Re-inoculation after removing a layer should also be tested.

Different inoculation techniques could also be studied. For this research, the inoculum was placed on the top of the substrate, but there are several other methods that could be utilized on an on farm system: blending the fungal pellets and spraying them with a nozzle, opening up a bale and spreading it throughout several layers at once, or

possibly a continuous inoculation throughout the pretreatment to ensure the fungus is beating out any other competing microbial colonies.

Differences in moisture content due to faster drying at lower densities could have had an effect on the growth of the fungus on the bales. Instead of a low density effect, there could have been a moisture content issue with the low density bales drying out more quickly than the higher density bales. In order to understand the true cause of the poor pretreatment results of the low density bales, salts controlling the moisture content in the containers could be used to examine the same experimental test, ensuring the same moisture through all treatments.

Further scaling up of this system is necessary with larger bales. Optimal pretreatment time with larger bales will have to be determined. Density of the bales could influence the optimal time of pretreatment. Higher density bales might not require as much pretreatment time as a lower density bale would for the same pretreatment efficacy due to higher substrate proximity for the fungus. However, recent research found that a 20-30% glucose yield increase was found by increasing the pretreatment time for switchgrass (Wan and Li 2011). Further understanding of the bale density, pretreatment time, and fungus relationship will need to be determined.

An optimal method to quantify fungal growth would be beneficial for future testing. Comparing the amount of fungal growth with the resulting sugar yields would be important to understand to determine how much growth is needed, and therefore the optimum culture length. Fungal growth and the enzyme activity present in the culture should be quantifies. The relationship between fungal growth and enzymatic activity should be understood to confirm the correlation between white rot growth and delignification.

Feedstocks will perform differently during biological pretreatment due to varying lignin subunits, hemicellulose matrix, or differing extractives (Wan and Li 2011). There is a difference between cornstover and switchgrass glucose yields following enzymatic hydrolysis, 56.50% glucose yield and 37.15% glucose yield, respectively. Switchgrass had a slightly higher amount of hemicellulose present than cornstover, 25.25 ± .22, 22.95 ± 1.32, respectively. Switchgrass also had slightly higher amounts of lignin than cornstover, 22.73 ± .42, 20.18 ±.53, respectively (Wan and Li 2011). The combination of

the higher lignin and hemicellulose levels and possibly a difference in their hemicellulose matrix makeup could make cornstover a slightly more appealing substrate for carbohydrate availability following fungal pretreatment. Understanding the reasoning why cornstover results in higher glucose yields should be understood.

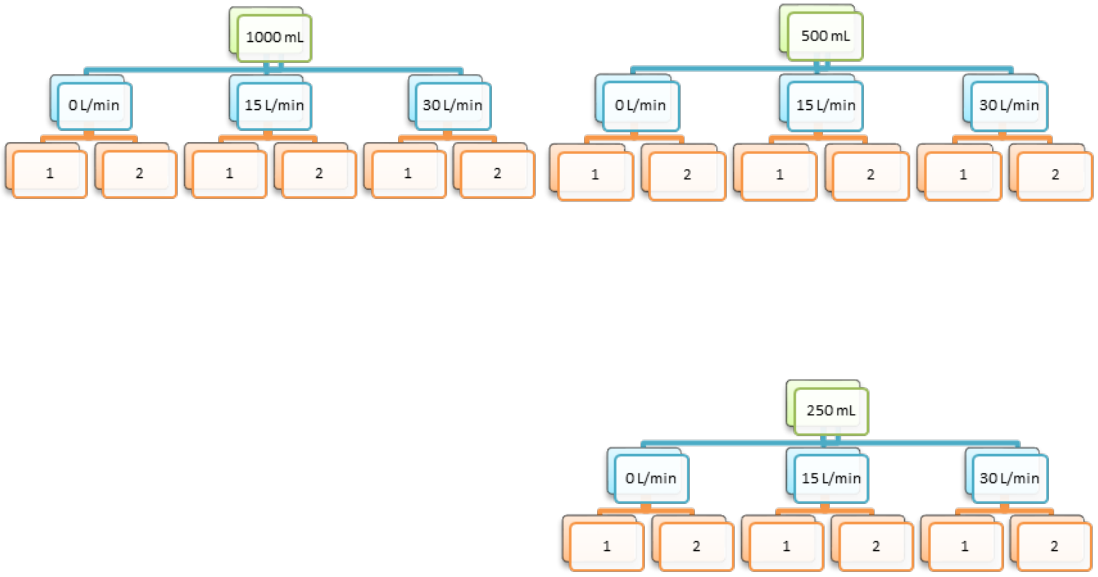
Identical pretreatments on different feedstocks result in differences with digestibility, with over 50% of these differences being accounted for due to cell-wall architecture (Li, Heckwolf et al. 2015)Water retention has been used as a predictor for how a substrate will perform following enzymatic hydrolysis because water retention is an indirect measurement of hydrophobicity, porosity, and polysaccharide accessibility (Li, Heckwolf et al. 2015). The water retention value incorporates several structural differences between different cell-wall types such as the digestible parenchyma cell walls and less-digestible cell walls within vascular bundles and epidermis. In the study by Li et al. (2015), the relationships between cell-wall properties of maize and the hydrolysis yields both prior to and following NaOH pretreatment were correlated. They hypothesize that the positive correlation with ferulate indicated that breaking ferulate cross-links between cell wall polymers is an important outcome of a successful pretreatment. Also, although it is commonly accepted that lignin content is negatively correlated with sugar yields, their study found that the cell wall's response to delignifying pretreatment is not dictated by the initial lignin content (Li, Heckwolf et al. 2015). Their research further indicated that cell wall architecture in different substrates defines how it will respond to different pretreatments. Even if the differing substrates have similar compositions (% lignin, cellulose and hemicellulose), they still may differ in cell wall architecture, and hence their response to a particular pretreatment.

Switchgrass has an equilibrium moisture content (EMC) of about 12% at 35ºC and 87% relative humidity (Godbolt, Danao et al. 2013). Corn stover has the following EMC values at 35ºC and 90% relative humidity: leaves: 22.7%, stalk skin: 20.7%, pith: 24.0%, and stalk 21.6% (Karunanithy, Muthukumarappan et al. 2013). The EMC values suggest that the corn stover holds onto moisture better than the switchgrass, which is a possibility why it performs better. This suggests further testing on the different ways in which we pretreat both substrates. Varying moisture sorption values suggest the amount

of moisture added, relative humidity, and drying methods will be different optimum values for both substrates.

APPENDICES

Appendix A. **Experimental Data**



Objective 1: Moisture Content and Fungal Culture Concentration

Pan Weight	Initial Weight (sample +pan)	Final Weight	Moisture Content	Fungus Culture g/mL
13.9	948.2	36.5	97.6%	0.02
14.0	549.0	28.6	97.3%	0.03
12.1	294.5	19.7	97.3%	0.03
13.8	299.9	21.4	97.3%	0.03
15.3	441.8	26.9	97.3%	0.03
13.9	988.9	43.4	97.0%	0.03
14.0	460.1	28.6	96.7%	0.03

Objective 1: Initial Weight

	Weight (g)			
Sample	**TOP**	**MID**	**BOT**	**Total**
1-1	498	500.2	500.3	1498.5
1-2	500.6	500	499.7	1500.3
2-1	500.3	499.8	500.3	1500.4
2-2	501.9	498.5	499.9	1500.3
3-1	501.1	500	500.1	1501.2
3-2	499.7	500.9	500.7	1501.3
4-1	499.1	499.9	500.4	1499.4
4-2	502	500.3	500.3	1502.6
5-1	500.1	499.1	500.6	1499.8
5-2	498.3	504.4	496.9	1499.6
6-1	501.6	499.6	499.5	1500.7
6-2	518.1	519.6	517.8	1555.5
7-1	499.1	500.5	499.3	1498.9
7-2	499.1	499.6	500.7	1499.4
8-1	500.4	497	500.6	1498
8-2	499.9	500.2	500.3	1500.4
9-1	499.9	500.9	499.6	1500.4
9-2	501.1	501.6	500.3	1503

Objective 1: Treatment Summary

Treatment	Inoculum (mL)	Air Flow (L/min)
1-1	1000	0
1-2	1000	0
2-1	1000	15
2-2	1000	15
3-1	1000	30
3-2	1000	30
4-1	500	0
4-2	500	0
5-1	500	15
5-2	500	15
6-1	500	30
6-2	500	30
7-1	250	0
7-2	250	0
8-1	250	15
8-2	250	15
9-1	250	30
9-2	250	30

Objective 1: Water Activity and Temperature (Day 0-2)

	DAY								
Treatment	0	0	0	1	1	1	2	2	2
	TOP	MID	BOT	TOP	MID	BOT	TOP	MID	BOT
1-1	0.999	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	22.61	23.64	23.49	35.89	36.80	35.39	36.43	37.08	36.00
1-2	1.000	1.000	1.000	1.000	0.998	0.998	0.988	0.992	0.992
	23.25	24.22	23.87	29.85	29.82	29.83	34.13	34.40	32.29
2-1	0.994	0.997	0.998	0.993	0.996	0.993	1.000	1.001	1.000
	24.13	24.46	23.94	30.15	29.13	27.75	34.24	34.23	32.03
2-2	0.996	1.003	0.999	1.000	1.000	1.000	1.000	1.000	1.000
	25.65	24.38	22.55	34.70	33.83	32.12	33.40	33.12	31.02
3-1	0.988	0.991	0.990	0.990	0.992	0.993	0.998	0.998	0.998
	23.85	24.08	24.24	34.53	34.48	32.11	35.69	34.25	31.89
3-2	0.995	0.993	0.996	0.998	0.996	0.998	0.998	0.998	1.000
	23.53	23.71	23.57	31.27	30.02	27.09	31.59	30.42	27.30
4-1	1.004	1.002	1.001	1.000	1.000	1.000	1.000	1.000	1.000
	24.34	24.34	24.48	34.36	34.96	33.85	35.27	35.06	33.72
4-2	0.994	0.995	0.996	0.996	0.994	0.996	0.988	0.993	0.997
	23.84	24.15	24.22	38.03	38.39	37.40	39.47	40.26	39.35
5-1	0.999	1.000	1.001	0.997	0.998	1.000	0.993	0.997	1.001
	24.70	23.43	22.15	33.93	33.70	32.51	33.36	33.44	31.43
5-2	1.003	1.004	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	24.12	24.06	23.92	33.70	33.20	32.21	33.34	33.37	31.42
6-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	24.24	24.39	24.13	31.55	29.87	28.07	30.52	30.07	27.69
6-2	1.000	1.000	1.000	1.000	1.000	1.000	0.997	0.995	1.000
	22.99	23.97	23.33	32.07	31.99	29.73	27.59	26.30	28.08
7-1	0.997	0.997	0.998	0.991	0.995	0.996	0.992	0.993	0.996
	24.61	24.57	24.52	34.96	35.71	33.89	34.42	35.59	34.85
7-2	1.000	1.000	1.000	0.994	1.000	1.000	1.000	1.000	1.000

	24.61	24.57	24.52	35.12	33.28	34.35	34.45	36.13	35.88
8-1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	24.23	24.58	24.34	33.49	33.43	30.67	32.36	32.00	29.36
8-2	1.000	1.000	1.000	1.000	1.000	1.000	0.999	1.000	0.998
	20.00	20.72	18.38	33.32	33.25	30.83	32.66	31.96	29.41
9-1	1.001	1.000	1.000	0.998	0.998	0.998	0.994	0.997	0.998
	23.19	24.00	24.07	28.04	26.64	23.29	31.76	30.12	26.11
9-2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	23.19	24.00	24.07	32.32	31.43	29.84	31.19	29.38	26.79
Control	0.588	0.61	0.582	0.796	0.646	0.622	0.653	0.611	0.596
A: No water	24.13	24.06	23.94	29.41	31.79	33.07	29.21	32.09	33.02
Control B	0.994	0.991	0.993	0.981	0.974	0.978	0.985	0.987	0.99
	19.71	19.25	19.33	34.7	34.9	33.79	34.34	34.56	33.62

Objective 1: Temperature and Water Activity (Day 3-5)

	DAY									
TRT	3	3	3	4	4	4	5	5	5	
	TOP	MID	BOT	TOP	MID	BOT	TOP	MID	BOT	
1-1	0.996	0.997	0.996				0.996	0.992	0.986	Aw
	36.91	37.09	35.45				36.63	36.65	34.54	T (°C)
1-2	1.000	1.000	1.001	0.997	0.999	1.001	0.998	0.997	1.000	Aw
	34.93	35.17	33.75	34.29	34.84	33.98	34.09	34.63	33.12	T (°C)
2-1	0.997	1.000	1.001	0.993	0.995	0.997	1.000	1.000	1.000	Aw
	35.97	36.12	34.96	33.61	33.55	31.27	33.54	34.01	30.87	T (°C)
2-2	1.000	1.001	0.998	1.000	1.001	1.003	0.996	0.998	1.001	Aw
	33.42	32.45	31.00	32.40	32.03	30.47	33.23	32.77	31.05	T (°C)
3-1	0.990	0.990	0.995	0.993	0.995	0.998	0.999	0.998	0.999	Aw
	31.05	31.24	28.25	31.75	30.85	28.99	32.31	31.10	29.04	T (°C)
3-2	0.995	0.989	0.990				0.998	0.997	0.999	Aw
	31.88	30.05	27.65				31.68	30.29	27.14	T (°C)
4-1	1.000	1.001	1.002	1.000	1.000	1.001	1.000	1.001	1.002	Aw
	34.89	35.45	34.59	34.06	35.44	34.91	34.39	35.77	35.11	T (°C)
4-2	0.995	0.989	0.985	0.990	0.986	0.995	0.992	0.993	0.994	Aw
	35.56	34.33	34.62	34.00	34.99	34.49	34.30	35.04	34.66	T (°C)
5-1	0.993	1.001	1.001	0.997	0.998	0.998	0.995	0.995	0.995	Aw
	33.36	32.37	30.57	33.55	32.76	31.32	33.72	33.34	31.55	T (°C)

5-2	1.000	1.000	1.000	1.000	1.000	1.000	0.999	1.002	1.000	Aw
	33.32	33.05	30.65	33.99	33.30	31.91	33.46	32.77	31.01	T (°C)
6-1	1.004	1.000	1.000	0.996	0.998	0.995	0.994	0.994	0.999	Aw
	31.37	30.58	28.00	30.26	29.20	26.64	30.10	29.69	27.47	T (°C)
6-2	1.001	1.003	1.000	1.000	1.000	1.000	1.001	1.002	1.000	Aw
	28.08	24.66	26.60	25.87	25.75	26.75	25.42	23.75	26.47	T (°C)
7-1	1.000	1.000	1.000	1.000	1.000	1.000	0.995	0.994	0.997	Aw
	34.15	35.39	34.06	34.30	35.88	34.51	34.98	36.32	35.19	T (°C)
7-2	0.997	1.000	1.002	0.994	1.001	1.001	0.998	0.999	0.999	Aw
	35.17	36.40	34.89	34.79	36.65	36.42	35.03	36.24	35.55	T (°C)
8-1	0.998	1.001	1.001	1.000	1.000	1.001	1.001	1.001	0.998	Aw
	33.58	32.85	30.09	33.58	32.79	30.38	33.63	32.65	30.60	T (°C)
8-2	1.001	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.002	Aw
	32.52	32.01	29.99	32.90	32.25	31.31	31.98	31.09	28.99	T (°C)
9-1	1.002	1.001	1.001	1.001	1.000	1.001	1.002	1.001	1.002	Aw
	34.40	34.24	32.70	33.87	33.28	31.75	32.02	30.10	27.72	T (°C)
9-2	1.003	1.002	1.001	1.004	1.000	1.000	0.992	0.991	0.994	Aw
	33.23	31.79	29.06	31.87	29.66	26.83	29.81	29.57	26.59	T (°C)
Control A: No water	0.566	0.569	0.561	0.555	0.553	0.549	0.503	0.495	0.497	Aw

	31.84	33.3	34.22	31.85	31.34	33.21	31.97	33.52	34.49	T (°C)
Control B	0.981	0.983	0.995	0.989	0.993	0.995	0.941	0.951	0.962	Aw
	34.47	34.23	32.71	34.01	34.21	30.12	34.04	34.19	31.12	T (°C)

Minimal Salts

(25X Strength Stock)

Dissolve successively:

$Na_3citrate$ (5.5 H_2O) in 750 ml water	150 g
[or $Na_3citrate$ (2 H_2O) in 775 ml water]	125 g
KH_2PO_4, anhydrous	250 g
NH_4NO_3, anhydrous	100 g
$MgSO_4$ (7 H_2O)	10 g
$CaCl_2$ (2 H_2O)	5 g

**dissolve separately in 20 ml water and ad the solution slowly

Biotin stock solution	2.5 m l
Trace element solution (i.e. Wolf's)	5 ml

Store at 4℃

Complete Medium – solid (SELECTION) [300 ml]

Sucrose	3 g
Yeast extract	1.8 g
Casamino acids	1.8 g
Agar	4.5 g

Autoclave

Immediately after cooling medium drops to 50℃ add:

Thiamin (1 g/l)	300 µl (to 1 µg/l)

Hygromycin B (100 mg/ml)	600 µl (to 200 µg/l)
Tecarcillin (150 µg/ml)	

Appendix B. **Enzymatic Hydrolysis Protocol**

The NREL Protocol "Enzymatic Saccharification of Lignocellulosic Biomass" was used to test for the glucose yields (Selig, Weiss et al. 2008). This procedure was used to quantify the maximum extent of digestibility of the biomass after pretreatment. The higher yield of glucose provided an understanding of a more effective pretreatment based on maximum enzyme loading. Samples were run in triplicate to verify reproducibility. Prior to enzymatic hydrolysis each sample was analyzed for moisture content using an Ohaus MB35 Halogen the moisture analyzer. According to previous lab tests, the compositional analysis of switchgrass from the North Farm contained 30% (w/w) cellulose. Using this information, the equivalent of .1 grams of cellulose was weighed out of each sample and added to a 20 mL glass vial with a screw top. The following formula was used to determine the amount in grams of each sample:

Equation 10: Wet Biomass Adjustment for Cellulose

$$.1\ g\ cellulose \times \frac{1\ g\ dry\ biomass}{.3\ g\ cellulase} \times \frac{1\ g\ biomass\ wet}{1 - \left(\%\ moisture\ content / 100\right)} = g\ sample$$

5.0 mL of sodium citrate buffer, pH 4.8, was added to each vial. 100 ul of 2% sodium azide solution was added to each vial to prevent growth of organisms during digestion. The amount of water need to bring the total volume of each vial to 10.0 mL after edition of enzymes was added to each vial. All samples were assumed to have a specific gravity of 1.000 g/mL. The following equation was used to determine the amount of DI water added to each sample.

Equation 11: DI Water Adjustment for Hydrolysis

$$10\ ml - 5\ ml\ buffer - .1\ ml\ Na\ Azide - 1.0\ ml\ enzyme\ dilution - (g\ measured\ out\ biomass) = ml\ DI\ water$$

After the addition of water, the samples are brought to 50°C in an incubator for ten minutes. Once equilibrium temperature has been reached, 1 mL of cellulase enzyme was added. The volume of cellulose enzyme was equal to 60 FPU/g cellulose. The cellulose enzyme solution was prepared using a concentration of 1.729 g enzyme per 100 mL DI water. Equation 3 was used to find the enzyme concentration using the previously determined protein content of the enzyme. Enzyme blanks were also prepared that only contained buffer, water and enzyme. Once all the samples were prepared, they were screw with the caps and placed in a shaking incubator for 72 hours at 50°C and 150 RPM.

Equation 12: Enzyme Concentration

$$1\ g\ cellulose \times \frac{60\ FPU}{g\ cellulose} \times \frac{1.0\ mg\ protein}{3.0\ FPU} \times \frac{1.0\ mg\ enzyme}{.1157\ mg\ protein} \times \frac{1\ g}{1000\ mg} = .01729\ g\ enzyme$$

Once the 72 hour incubation was complete, the enzymatic reaction was stopped by placing the samples in a 93°C water bath for 15 minutes. Samples were vortexed and poured into a 2 mL collection vial and centrifuged at 5000 RPM for ten minutes. The supernatant from each sample was subjected to glucose analysis using the YSI glucose analyzer. To determine the glucose from each sample, the glucose concentration from the YSI was used, and any glucose concentrations from the enzyme blanks were subtracted out from the samples for final glucose. Samples were analyzed for glucose using YSI (YSI 2900D; YSI, Inc.; Yellow Springs, Ohio). The YSI was calibrated and standards were run at 9.00 g/L glucose before each analysis.

Appendix C. **Lignin Analysis Protocol**

The extractives in biomass that are water soluble and ethanol soluble need to be removed prior to lignin analysis. The extractives found in these biomass samples could affect with the downstream analysis of the samples for lignin analysis. If the extractives are not removed, there could be a falsely high lignin number at the end of the total analysis. In order to remove the extractives in the biomass samples, the NREL/TP-510-42619 protocol "Determination of Extractives in Biomass" was used (Sluiter, Ruiz et al. 2005). This method used a two-step extraction with water and ethanol to remove the non-structural material from the samples prior to lignin analysis. Extraction cells were prepared by placing a filter in the bottom of each cell, and the samples were packed and labeled in each extraction cell. The cells were placed into the Dionex ASE 350 system automatic extractor with settings of 1500 PSI, 100C, preheat time 0, heat time 5 minutes, static time 7 minutes, flush volume 150%, purge time 120 seconds, and 3 static cycles. The water extraction was done first on the samples, followed immediately by the ethanol extraction. After the extractions were complete, the samples were left to cool, and then removed from the extraction cells and placed in aluminum tins to dry in a 45°C oven for 24 hours. Quantification of the extractives was not used as a means to save time and resources.

After preparation and extraction, the samples were tested for lignin according to the Laboratory Analytical Procedure for "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter, Hames et al. 2008). Prior to analysis, .5 grams of each sample was weighed out and used for determination of total solids (Sluiter, Hames et al. 2008). The day prior to hydrolysis, crucibles were prepared by drying in a furnace at 575°C for 4 hours. The samples are prepared for analysis and hydrolysis. 3 grams of the previously dried and milled samples were placed in a glass screw cap vial. 3 mL 72% sulfuric acid was added to each sample, and mixed thoroughly. The samples were in the 30°C water bath for one hour, with mixing every 5-10 minutes. The tubes were removed from the water bath, and diluted by adding 84 mL DI water. All samples were autoclaved at 121°C for 60 minutes, and allowed to cool to room temperature. The samples were vacuum filtered through the crucibles previously prepared, and a 50 mL sample of the hydrolyzate was taken. The 50 mL hydrolyzate was used for determination

of acid insoluble lignin using the spectrometer. Duplicate samples measured between .7-1 using a wavelength of 320. Samples were diluted as necessary; recording the dilution for later calculations. The samples in the crucibles were dried in an oven at 105°C for a minimum of 4 hours. The samples were then removed from the oven and cooled in a desiccator for one hour. The weight of the crucible and dry residue was recorded. The crucibles were placed in the furnace at 575°C for 24 hours, and a final weight was recorded. The percent lignin was calculated after finding the acid soluble lignin and acid insoluble lignin. Lignin was calculated from the following equations

Equation 13: Oven Dry Weight Calculation

$$ODW = \frac{weight_{air\ dry\ sample} \times \%total\ solids}{100}$$

Equation 14: Percent Acid Insoluble Residue

$$\%AIR = \frac{weight\ _{crucible\ plus\ AIR} - weight\ _{crucible}}{ODW\ _{sample}} \times 100$$

Equation 15: Percent Acid Insoluble Lignin

$$\%AIL = \frac{\left(weight_{crucible\ plus\ AIR} - weight_{crucible}\right) - \left(weight_{crucible\ plus\ ash} - weight_{crucible}\right) - \left(weight_{protein}\right)}{ODW_{sample}}$$

Equation 16: Percent Acid Soluble Lignin

$$\%ASL = \frac{UVabs \times volume_{filtrate} \times dilution}{\varepsilon \times ODW_{sample} \times pathlength} \times 100$$

Equation 17: Percent Lignin

$$\%Lignin = \%ASL + \%AIL$$

Appendix D. **Inoculum and Air-flow Daily Log**

Treatment 1: 1000 mL, 0 L/min:

Treatment 1(TRT 1), 1000 mL fungal pellets and 0 L/min air flow, was an overall success. Similar results were recorded for both replications of this treatment. Originally the fungal pellets were light brown in color, and about 2-3 mm in diameter. The first day showed no obvious change in fungal growth. They were the same for the most part, no change in color or size. The bale was still moist as well with no signs of drying out. The second day showed growth of the fungal colony. The pellets were now looking whiter, with no signs of contamination or drying out of the bale. Over day three and four the fungus continued to get whiter, and grow over the top and into the middle layer of the bale. The bale did not show signs of moisture loss. By day five, the colony was white; there was substantial hyphae growth into the second layer, and a small amount of growth into the bottom layer as well.

Treatment 2: 1000 mL, 15 L/min:

Treatment 2 (TRT 2), 1000 mL fungal pellets and 15 L/min air flow, was successful. This treatment did very well over the five days. Initially, there was not much change with the pellets. The first day of growth showed very little change from the initial day. The pellets were about the same color and the same size. The bale stayed very moist like the initial day. By the third day, there was a lot of white growth along the top of the bale and where the pellets were. The fourth day showed hyphae growth into the second layer of the bale, with even more white growth on the top layer. During these days there was not much drying out of the bale. There was some visible drying out along the edges but nothing too great. The last day showed an optimal bale for day 5. There was extensive white growth along the top layer and white growth into the second layer as well. There was hyphae growth into the middle layer and some hyphae growth into the third layer. This was an overall very successful treatment.

Treatment 3: 1000 mL, 30 L/min:

Treatment 3(TRT 3), 1000 mL fungal pellets and 30 L/min air flow was not successful. The first day the pellets had a very distinct white color. There were no

visible hyphae, but visible white growth from one day was high. The biomass was not overly dried out even though the air flow was at the highest level to be tested. Water activity was at a good level throughout the levels. The second replication showed no change after one day of growth, no change in color or size. After day 1, the results from both of the replications were very similar. The second day the pellets had more of a brown tint. There was visible hyphae growth along the switchgrass. The biomass itself did not appear to be drying out drastically. Day 3 and 4 were similar in growth, and had very little change in pellets and hyphae. The same brown color was seen throughout the pretreatment. However, by day 4, there is substantial drying out of the bale along the edges, and there is little growth in these areas. Day 5 showed there was a halt in growth, and no growth of fungi colonies or hyphae.

Treatment 4: 500 mL, 0 L/min:

Treatment 4 (TRT 4) was successful. The replications visibly showed a lot of white growth along the first few days, and stayed very moist throughout the entire treatment. The first day showed good white growth. There was a mix of brown and white pellets, with no visible hyphae. The second day there was more brown growth than white. There was hyphae growth along the substrate. The third and fourth day showed an increase in hyphae growth. On the fourth day there was obvious hyphae growth into the middle layer of the tub. There was also some white appearing in spots on the top on the fourth day. When sampling the second replication there was hyphae growth in the middle and bottom layers.

Treatment 5: 500 mL, 15 L/min

This treatment was considered successful, but not the most successful out of the treatments**.** The first replication showed substantial white growth from the initial day to the first day. After this first day, there was not much change in the growth over the pretreatment time. The bale did not dry out much over the course of the pretreatment.

The second replication also had visible white growth on the first day, and showed lots of white growth by the second day, and growth into the second layer as well by extending hyphae. The bale did not dry out over day 2, day 3 and day 4 substantially. The fourth

day showed increasing growth. The bale did not look like it is drying out dramatically on day 5.

Treatment 6: 500 mL, 30 L/min

This treatment was unsuccessful. The first day showed no change in the bale and growth. By the second day, the bale had begun drying out along the edges but showed white growth on the middle portion of the bale. The third and fourth day showed optimal white growth, but only in the middle. The edges were increasingly dry and too dry to accommodate any fungal growth. The last day the bale was very dry and even visibly dried out in the middle. By the last day the initial optimistic white growth was not visible, and contamination was obvious by black and pink growth all along the bale.

The replication (TRT 6-2) bale showed similar results. The bale was substantially dried out by the third day, but there was good white growth in the middle where the pellets were placed initially. The white growth happened early on, but due to the dry bale, there was little expansion of growth. By the end of the pretreatment, the bale was totally dried out, and unable to allow the fungal colony to grow throughout the dry bale.

Treatment 7: 250 mL, 0 L/min

There was little to no growth on the first day, with no drying out and the pellets look about the same as they did initially. The bale stayed very moist on day 2, with a small amount of growth. On day 3, the pellets began to break down with brown and white growth. The bale was still very moist on day 3 and had not lost any moisture. Day 4 showed a little more growth than day 3. On day 5, there was no white growth on the bale, but there were some visible hyphae. There was hyphae growth into the second layer. This was the most successful treatment for the lowest inoculum amount added to the bale. This could be accounted to the higher moisture content allowing the lower amount of fungus to flourish. The other treatments seemed to dry out the fungus quickly, and didn't allow for growth.

Treatment 8: 250 mL, 15 L/min

This treatment was not successful**.** The first days for both of the replications didn't show much change in fungal growth; there was only slight drying out along the edges of the bale. By day 2 of the first replication, the edges of the bales were very dry. There was visible white growth on the third day in the middle of the bale, but still increasingly dry around the edges of the biomass. Day 4 and day 5 showed no more white growth, and the entire bale was very dry even in the center of the bale. It was noted that the visible growth was on the nodes and broken pieces of the switchgrass, with little growth along the stalks of the biomass.

The second bale showed slightly better growth than the first bale. It seemed to maintain a little more moisture, allowing the fungal culture to grow better. Similar to the first replication of this treatment, the white growth appeared on the third day of growth. The fourth day showed more growth along the top layer, and some visible hyphae growth into the second layer of the bale. There was drying out along the edges of the bale, but the middle of the bale the moisture level stayed higher.

Treatment 9: 250 mL, 30 L/min

This treatment had very poor fungal growth for both replications, and had very similar results for both of the experiments. After the first day there was extensive drying of both the bale and the fungal colony. The pellets were very white on the first day, but the bale is drying out a lot, and there was no hyphae growth. By the end of the pretreatment time, there was extensive drying out of the bale, but the pellets that were placed on top look very white. There is a possibility that the straw the pellets were on has had some lignin degradation, but there was no growth into the middle or bottom layers of the bale. The bale did not have the moisture content that the fungal colony needed because of the extensive air flow through the bale. This proves that even though the air is saturated, extensive drying still happens at the highest level.

Appendix E. **Inoculum and Air-Flow Enzymatic Hydrolysis Data**

Trt	Rep		Layer	Glucose	Glucose-Enzyme	Enzyme Blanks
1	1	Blank	Top	0.33	-0.39	0.73
1	1		Top	1.25	0.53	0.7
1	1		Top	0.92	0.20	0.73
1	1		Top	0.88	0.16	
1	1	Blank	Mid	0.14	-0.58	
1	1		Mid	1.15	0.43	
1	1		Mid	1.04	0.32	
1	1		Mid	0.92	0.20	
1	1	Blank	Bot	0.21	-0.51	
1	1		Bot	1.37	0.65	
1	1		Bot	1.26	0.54	
1	1		Bot	1.51	0.79	
1	2	Blank	Top	0.15	-0.57	
1	2		Top	0	-0.72	
1	2		Top	1.06	0.34	
1	2		Top	0	-0.72	
1	2	Blank	Mid	0.15	-0.57	
1	2		Mid	1.32	0.60	
1	2		Mid	1.61	0.89	
1	2		Mid	1.33	0.61	
1	2	Blank	Bot	0.15	-0.57	
1	2		Bot	1.54	0.82	
1	2		Bot	1.58	0.86	
1	2		Bot	1.5	0.78	
2	1	Blank	Top	0.15	-0.57	0.79
2	1		Top	1.23	0.51	0.84
2	1		Top	1.07	0.35	0.82
2	1		Top	1.25	0.53	
2	1	Blank	Mid	0.2	-0.52	
2	1		Mid	1.48	0.76	
2	1		Mid	1.51	0.79	
2	1		Mid	1.59	0.87	
2	1	Blank	Bot	0.16	-0.56	
2	1		Bot	1.62	0.90	
2	1		Bot	1.54	0.82	
2	1		Bot	1.55	0.83	
2	2	Blank	Top	0.46	-0.26	

2	2		Top	1.42	0.70	
2	2		Top	1.51	0.79	
2	2		Top	1.33	0.61	
2	2	Blank	Mid	0.25	-0.47	
2	2		Mid	1.7	0.98	
2	2		Mid	1.72	1.00	
2	2		Mid	1.94	1.22	
2	2	Blank	Bot	0.12	-0.60	
2	2		Bot	1.5	0.78	
2	2		Bot	2.22	1.50	
2	2		Bot	1.57	0.85	
3	1	Blank	Top	0.35	-0.37	
3	1		Top	1.65	0.93	
3	1		Top	1.3	0.58	
3	1		Top	1.5	0.78	
3	1	Blank	Mid	0.23	-0.49	
3	1		Mid	1.67	0.95	
3	1		Mid	1.57	0.85	
3	1		Mid	1.64	0.92	
3	1	Blank	Bot	0.21	-0.51	
3	1		Bot	1.37	0.65	
3	1		Bot	1.38	0.66	
3	1		Bot	1.4	0.68	
3	2	Blank	Top	0.22	-0.50	
3	2		Top	1.2	0.48	
3	2		Top	1.22	0.50	
3	2		Top	1.26	0.54	
3	2	Blank	Mid	0.12	-0.60	
3	2		Mid	1.13	0.41	
3	2		Mid	1.27	0.55	
3	2		Mid	1.38	0.66	
3	2	Blank	Bot	0.21	-0.51	
3	2		Bot	1.57	0.85	
3	2		Bot	1.53	0.81	
3	2		Bot	1.65	0.93	
4	1	Blank	Top	0.22	-0.50	
4	1		Top	1.06	0.34	
4	1		Top	1.13	0.41	
4	1		Top	1.14	0.42	
4	1	Blank	Mid	0.16	-0.56	
4	1		Mid	1.31	0.59	

4	1		Mid	1.44	0.72	
4	1		Mid	1.48	0.76	
4	1	Blank	Bot	0.18	-0.54	
4	1		Bot	1.55	0.83	
4	1		Bot	1.28	0.56	
4	1		Bot	1.74	1.02	
4	2	Blank	Top	0.25	-0.47	
4	2		Top	1.28	0.56	
4	2		Top	1.22	0.50	
4	2		Top	1.34	0.62	
4	2	Blank	Mid	0.23	-0.49	
4	2		Mid	1.3	0.58	
4	2		Mid	1.34	0.62	
4	2		Mid	1.46	0.74	
4	2	Blank	Bot	0.33	-0.39	
4	2		Bot	1.37	0.65	
4	2		Bot	1.46	0.74	
4	2		Bot	1.46	0.74	
5	1	Blank	Top	0.22	-0.50	
5	1		Top	1.44	0.72	
5	1		Top	1.38	0.66	
5	1		Top	1.49	0.77	
5	1	Blank	Mid	0.14	-0.58	
5	1		Mid	1.4	0.68	
5	1		Mid	1.27	0.55	
5	1		Mid	1.3	0.58	
5	1	Blank	Bot	0.1	-0.62	
5	1		Bot	1.64	0.92	
5	1		Bot	1.72	1.00	
5	1		Bot	1.64	0.92	
5	2	Blank	Top	0.42	-0.30	
5	2		Top	1.63	0.91	
5	2		Top	1.47	0.75	
5	2		Top	1.59	0.87	
5	2	Blank	Mid	0.24	-0.48	
5	2		Mid	1.59	0.87	
5	2		Mid	1.78	1.06	
5	2		Mid	1.53	0.81	
5	2	Blank	Bot	0.17	-0.55	
5	2		Bot	2.09	1.37	
5	2		Bot	1.87	1.15	

5	2		Bot	1.56	0.84	
6	1	Blank	Top	0.32	-0.40	
6	1		Top	1.65	0.93	
6	1		Top	1.49	0.77	
6	1		Top	1.34	0.62	
6	1	Blank	Mid	0.25	-0.47	
6	1		Mid	1.68	0.96	
6	1		Mid	1.72	1.00	
6	1		Mid	1.65	0.93	
6	1	Blank	Bot	0.14	-0.58	
6	1		Bot	1.44	0.72	
6	1		Bot	1.57	0.85	
6	1		Bot	1.41	0.69	
6	2	Blank	Top	0.4	-0.32	
6	2		Top	1.87	1.15	
6	2		Top	1.45	0.73	
6	2		Top	1.49	0.77	
6	2	Blank	Mid	0.16	-0.56	
6	2		Mid	1.57	0.85	
6	2		Mid	1.47	0.75	
6	2		Mid	1.43	0.71	
6	2	Blank	Bot	0.13	-0.59	
6	2		Bot	1.65	0.93	
6	2		Bot	1.64	0.92	
6	2		Bot	1.59	0.87	
7	1	Blank	Top	0.18	-0.54	0.73
7	1		Top		-0.72	0.74
7	1		Top	1.2	0.48	0.75
7	1		Top	1.3	0.58	
7	1	Blank	Mid	0.25	-0.47	
7	1		Mid	1.48	0.76	
7	1		Mid	1.36	0.64	
7	1		Mid	1.59	0.87	
7	1	Blank	Bot	0.2	-0.52	
7	1		Bot	1.52	0.80	
7	1		Bot	1.24	0.52	
7	1		Bot	1.72	1.00	
7	2	Blank	Top	0.24	-0.48	
7	2		Top	1.34	0.62	
7	2		Top	1.18	0.46	
7	2		Top	1.09	0.37	

7	2	Blank	Mid	0.27	-0.45	
7	2		Mid	1.37	0.65	
7	2		Mid	1.27	0.55	
7	2		Mid	1.34	0.62	
7	2	Blank	Bot	0.22	-0.50	
7	2		Bot	1.34	0.62	
7	2		Bot	1.39	0.67	
7	2		Bot	1.5	0.78	
8	1	Blank	Top	0.18	-0.54	
8	1		Top	1.38	0.66	
8	1		Top	1.42	0.70	
8	1		Top	1.37	0.65	
8	1	Blank	Mid	0.21	-0.51	
8	1		Mid	1.52	0.80	
8	1		Mid	1.34	0.62	
8	1		Mid	1.33	0.61	
8	1	Blank	Bot	0.19	-0.53	
8	1		Bot	1.66	0.94	
8	1		Bot	1.43	0.71	
8	1		Bot	1.51	0.79	
8	2	Blank	Top	0.17	-0.55	
8	2		Top	1.28	0.56	
8	2		Top	1.51	0.79	
8	2		Top	1.38	0.66	
8	2	Blank	Mid	0.18	-0.54	
8	2		Mid	1.51	0.79	
8	2		Mid	1.36	0.64	
8	2		Mid	1.35	0.63	
8	2	Blank	Bot	0.12	-0.60	
8	2		Bot	1.64	0.92	
8	2		Bot	1.53	0.81	
8	2		Bot	1.69	0.97	
9	1	Blank	Top	0.2	-0.52	
9	1		Top	1.45	0.73	
9	1		Top	1.18	0.46	
9	1		Top	1.23	0.51	
9	1	Blank	Mid	0.18	-0.54	
9	1		Mid	1.54	0.82	
9	1		Mid	1.51	0.79	
9	1		Mid	1.6	0.88	
9	1	Blank	Bot	0.21	-0.51	

9	1		Bot	1.45	0.73	
9	1		Bot	1.53	0.81	
9	1		Bot	1.68	0.96	
9	2	Blank	Top	0.06	-0.66	
9	2		Top	0.97	0.25	
9	2		Top	1.11	0.39	
9	2		Top	1.2	0.48	
9	2	Blank	Mid	0.18	-0.54	
9	2		Mid	1.32	0.60	
9	2		Mid	1.34	0.62	
9	2		Mid	1.43	0.71	
9	2	Blank	Bot	0.13	-0.59	
9	2		Bot	1.48	0.76	
9	2		Bot	1.31	0.59	
9	2		Bot	1.49	0.77	
Control	1		Top	1.33	0.61	
Control	1		Top	1.27	0.55	
Control	1		Top	1.23	0.51	
Control	2		Top	1.56	0.84	
Control	2		Top	1.47	0.75	
Control	2		Top	1.46	0.74	
Control	3		Top	1.18	0.46	
Control	1		Mid	1.19	0.47	
Control	1		Mid	1.18	0.46	
Control	1		Mid	1.18	0.46	
Control	2		Mid	1.11	0.39	
Control	2		Mid	1.11	0.39	
Control	2		Mid	1.09	0.37	
Control	3		Mid	1.07	0.35	
Control	3		Mid	1.1	0.38	
Control	3		Mid	1.14	0.42	
Control	1		Bot	1.33	0.61	
Control	1		Bot	1.34	0.62	
Control	1		Bot	1.3	0.58	
Control	2		Bot	1.23	0.51	
Control	2		Bot	1.23	0.51	
Control	2		Bot	1.31	0.59	
Control	3		Bot	1.53	0.81	
Control	3		Bot	1.49	0.77	
Control	3		Bot	1.5	0.78	

```
proc sort data=sugar;
      by trt rep layer;
run;
proc means data=sugar mean noprint;
      by trt rep layer;
      var sugar;
      output out=data mean=sugar;
run;

proc glm data=data plots(unpack)=all;
      class trt rep layer;
    model sugar=trt rep(trt) layer layer*trt/ss1;
    test h=trt e=rep(trt);
      means trt layer layer*trt;
   run;
```

Inoculum and Airflow Treatments Glucose Yields

The GLM Procedure

Dependent Variable: sugar

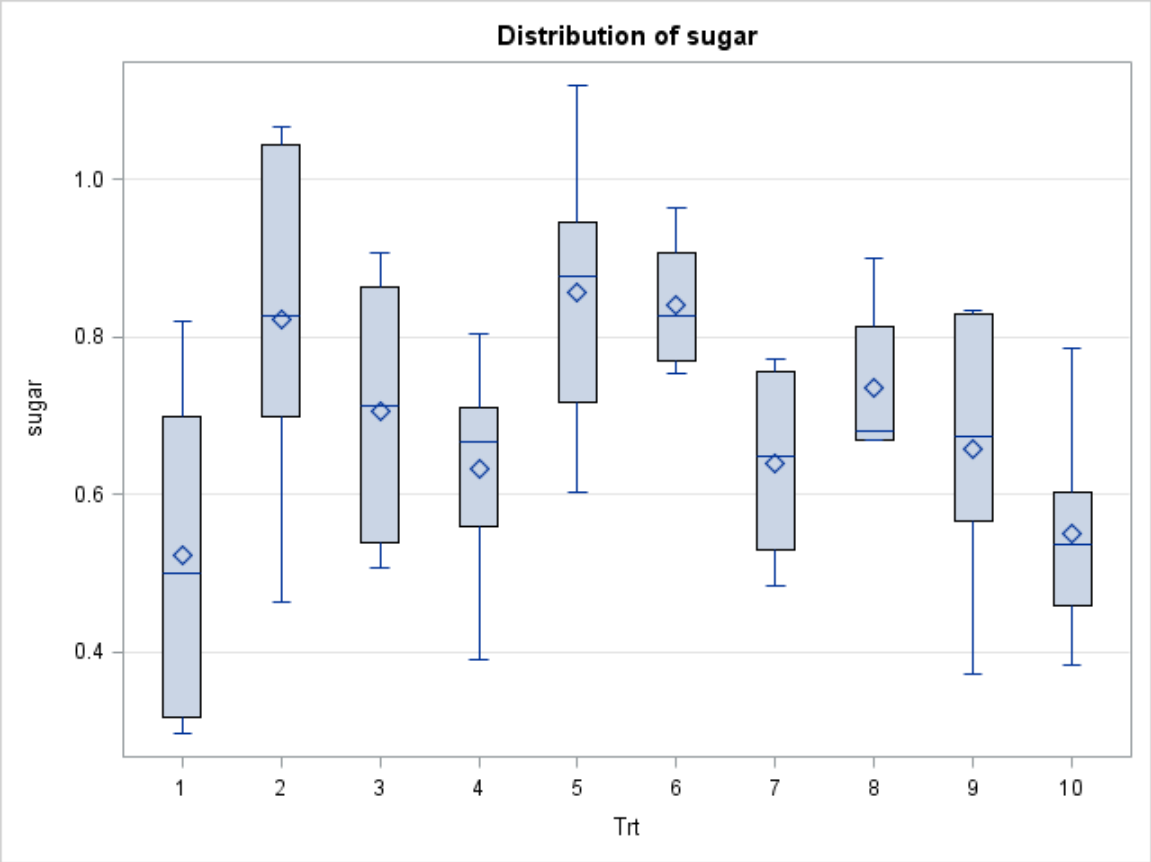
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	40	1.94363915	0.04859098	3.80	0.0007
Error	22	0.28130370	0.01278653		
Corrected Total	62	2.22494286			

R-Square	Coeff Var	Root MSE	sugar Mean
0.873568	16.39182	0.113078	0.689841

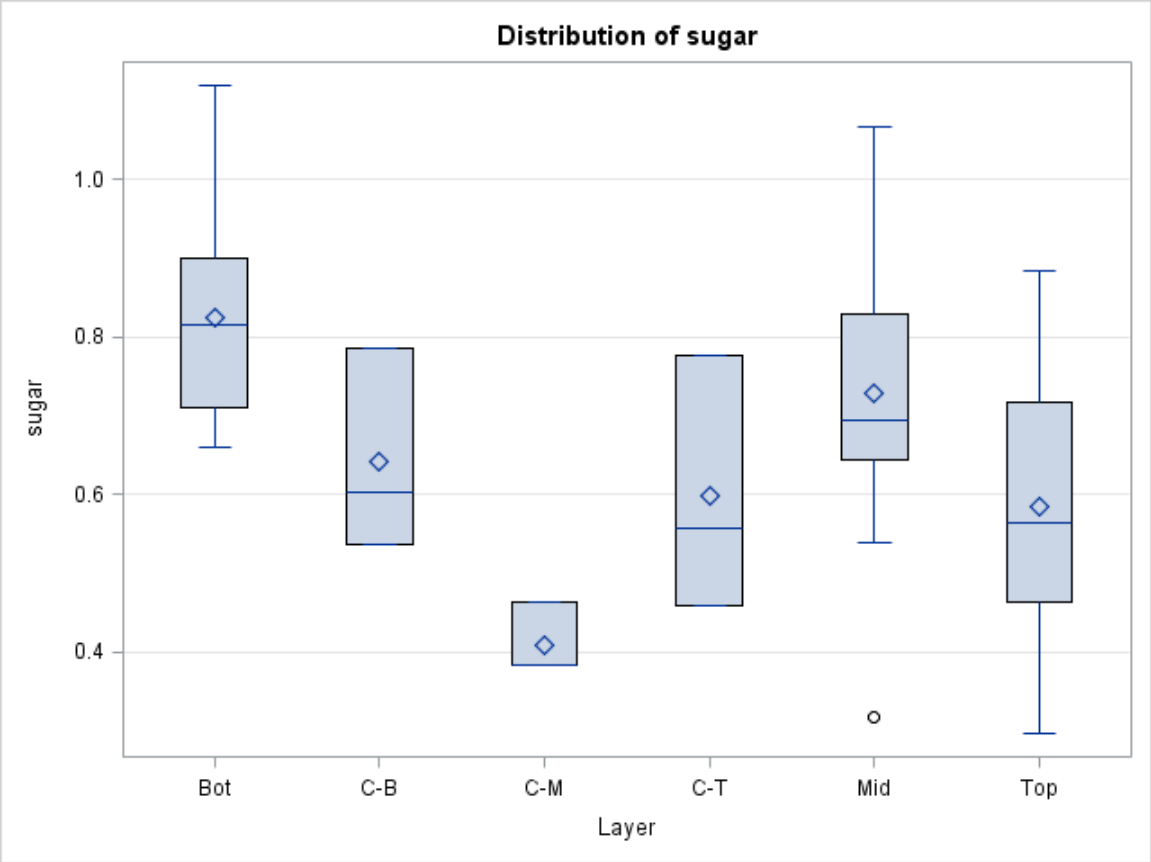
Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	9	0.80971878	0.08996875	7.04	<.0001
Rep(Trt)	11	0.28810556	0.02619141	2.05	0.0734
Layer	4	0.61801235	0.15450309	12.08	<.0001
Trt*Layer	16	0.22780247	0.01423765	1.11	0.3999

Tests of Hypotheses Using the Type I MS for Rep(Trt) as an Error Term

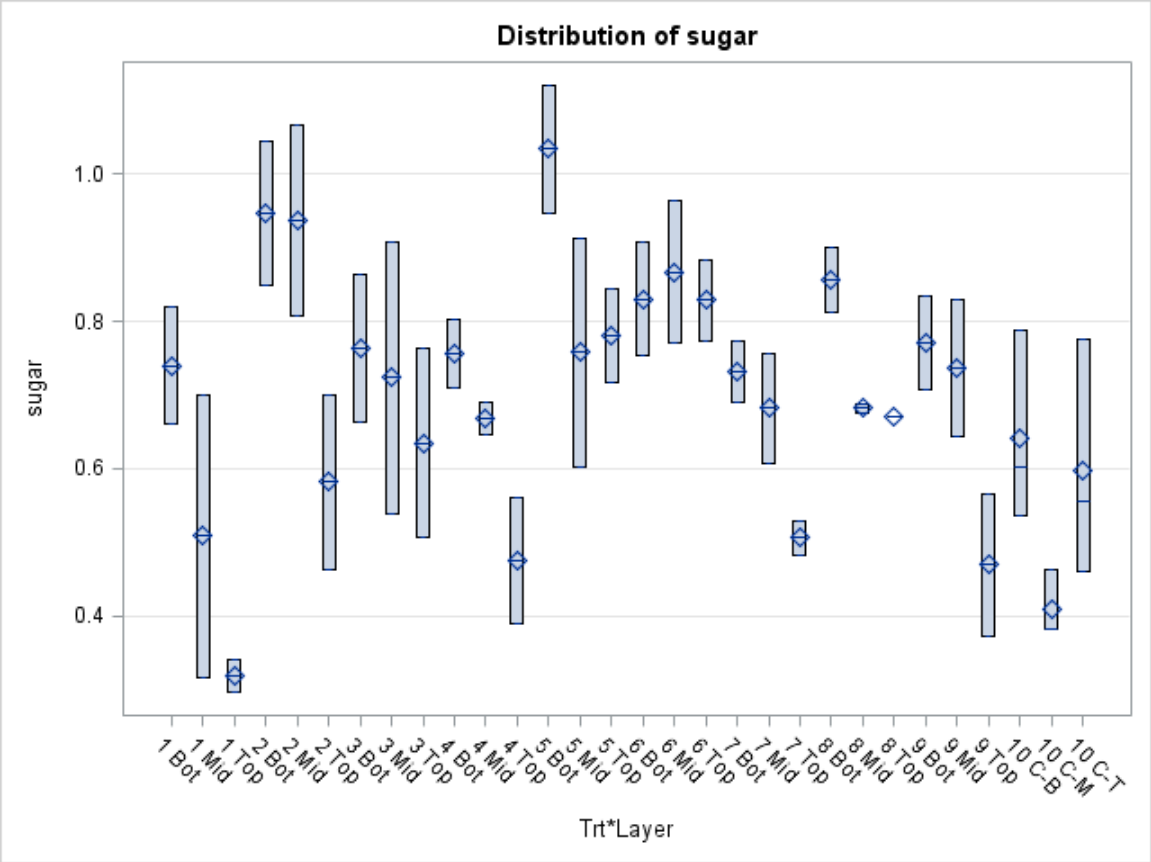
Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	9	0.80971878	0.08996875	3.44	0.0290



Level of Trt	N	sugar Mean	sugar Std Dev
1	**6**	0.52222222	0.23047455
2	**6**	0.82166667	0.22511972
3	**6**	0.70722222	0.16577651
4	**6**	0.63333333	0.14335659
5	**6**	0.85722222	0.18144380
6	**6**	0.84166667	0.08760391
7	**6**	0.64000000	0.11970334
8	**6**	0.73611111	0.09751163
9	**6**	0.65888889	0.17451732
10	**9**	0.55000000	0.15067441



Level of Layer	N	sugar	
		Mean	Std Dev
Bot	**18**	0.82537037	0.12630567
C-B	**3**	0.64222222	0.12945756
C-M	**3**	0.41000000	0.04618802
C-T	**3**	0.59777778	0.16228690
Mid	**18**	0.72907407	0.17331752
Top	**18**	0.58500000	0.17644733



Level of Trt	Level of Layer	N	sugar	
			Mean	Std Dev
1	**Bot**	**2**	0.74000000	0.11313708
1	**Mid**	**2**	0.50833333	0.27105760
1	**Top**	**2**	0.31833333	0.03064129
2	**Bot**	**2**	0.94666667	0.13670731
2	**Mid**	**2**	0.93666667	0.18384776
2	**Top**	**2**	0.58166667	0.16734860
3	**Bot**	**2**	0.76333333	0.14142136
3	**Mid**	**2**	0.72333333	0.25927249
3	**Top**	**2**	0.63500000	0.18149074
4	**Bot**	**2**	0.75666667	0.06599663
4	**Mid**	**2**	0.66833333	0.03064129
4	**Top**	**2**	0.47500000	0.12020815

Level of Trt	Level of Layer	N	sugar	
			Mean	Std Dev
5	**Bot**	**2**	1.03333333	0.12256518
5	**Mid**	**2**	0.75833333	0.21920310
5	**Top**	**2**	0.78000000	0.08956686
6	**Bot**	**2**	0.83000000	0.10842304
6	**Mid**	**2**	0.86666667	0.13670731
6	**Top**	**2**	0.82833333	0.07778175
7	**Bot**	**2**	0.73166667	0.05892557
7	**Mid**	**2**	0.68166667	0.10606602
7	**Top**	**2**	0.50666667	0.03299832
8	**Bot**	**2**	0.85666667	0.06128259
8	**Mid**	**2**	0.68166667	0.00707107
8	**Top**	**2**	0.67000000	0.00000000
9	**Bot**	**2**	0.77000000	0.08956686
9	**Mid**	**2**	0.73666667	0.13199327
9	**Top**	**2**	0.47000000	0.13670731
10	**C-B**	**3**	0.64222222	0.12945756
10	**C-M**	**3**	0.41000000	0.04618802
10	**C-T**	**3**	0.59777778	0.16228690

Appendix F. **B-Glucosidase Enzymatic Activity Assay**

Dr. Nokes Lab Protocol (University of Kentucky – Biosystems & Agricultural Engineering)

Prepared by Bobby Carey and Pedro Vieira Hamann (August 2014)

Principal:

Cellobiose is a disaccharide composed of two glucose units connected by a β (1-4) glycosidic bond. A handful of enzymes can cleave this bond to produce two separate glucose units however most of this activity is attributed to B-Glucosidase. In this Assay a substrate known as 4-Nitrophenyl β-D-glucopyranoside (PNPG) containing the same linkage is hydrolyzed by B-Glucosidase. When the PNPG molecule is cleaved it results in two separate molecules – Glucose and a Nitrophenol (PNP). When the assay is terminated by addition of NaCO_3 the Nitrophenol and the NaCO_3 bond resulting in the intense color change from clear to yellow. The level of absorbance can be correlated to the abundance of free Nitrophenol which is related to the activity of the enzyme. Using a standard curve produced using known concentrations of PNP a measurement of B-Glucosidase activity can be made.

Reagents:

1. 0.05M Na-Citrate Buffer – Begin by preparing a stock of 1M Na-Citrate
    1. 84g Citric Acid Monohydrate C_6 H_8 O_7·H_2 O
    2. 300ml DI H_2 O
    3. Add NaOH until pH = 4.5 (Should be around 15-18g)
        4. Dilute to 400ml and check pH (if necessary add NaOH until pH = 4.5); you now have a 1M stock of Na-Citrate
        5. To make 100 ml of 0.05M Na-Citrate add 5ml 1M Na-Citrate to 95ml DI H_2 O. When diluted to 0.05M, pH should equal 4.8
2. 4mM 4-Nitrophenyl β-D-glucopyranoside (Sigma-Aldrich/500mg) PNPG- add 0.120mg of PNPG to 100ml of 0.05M Na-Citrate Buffer.
3. 4-Nitrophenol solution (Sigma-Aldrich/100ml) PNP

4. Enzyme stock-combine desired amount (g) of enzyme with 100ml DI water. *A good place to start when using lyophilized powder enzyme is 0.5g/l. The goal is to use a stock that will place the assay readings within the range of the standard curve. If the value does not fall in the range, the assay should be reattempted adjusting the enzyme stock or adjusting the volume of enzyme aliquot used for the assay.
5. 1M NaCO_3- combine 10.599 g with 100ml DI. Or if you plant to do the assay again in the near future make a larger volume and store on the bench top.

Assay Procedures:

1. Prepare water bath at 50℃.
2. Prepare standards by combining the following in individual test tubes.

PNP (µL)	DI H_2O (µL)
0	200
5	195
10	190
15	185
20	180
25	175
30	170

*Total volume in each tube should be 200µL

3. Prepare enzyme assay tubes by pipetting 100µL of 4mM PNPG into empty test tube. Prepare in triplicate. Do not add enzyme yet.
4. Prepare control by adding 100µL of 4mM PNPG into empty test tube. If you are testing a range of enzyme dilutions – prepare controls for each dilution. Prepare all controls in triplicate.
5. Place all tubes in rack and into the water bath at 50℃ for 10 minutes to acclimate. *The standard temperature is 37℃ for this assay however if you want to know your enzyme activity at the temperature at which you plan to perform your hydrolysis then perform the assay at that temperature.

6. After the 10 minute acclimation time. Quickly add 100µL of enzyme stock to each assay tube. Do not add enzyme to standards or control.

   *You can use one dilution of enzyme stock or multiple in attempt to ensure readings fall within the standard curve range. But prepare each in triplicate.

7. Incubate in water bath for exactly 30 minutes.
8. After the incubation period is up remove from water bath and quickly add 1ml of 1M NaCO_3 to all tubes. This will stop the reaction.
9. Add 100µL of enzyme dilution to the control. If testing multiple dilutions, ensure the appropriate enzyme dilution is mixed with the corresponding control.

   *No color change should be visually observed in controls after addition of enzyme solution.

10. Poor contents of each tube into cuvette and read absorbance at 400nm.

Analysis:

1. Plot the standard absorbance readings on a graph. Y-axis: Abs at 400nm, and X-axis: µmol of PNP. Fit a line and equation for the standard curve.
2. Determine the average absorbance for the enzyme assay samples then subtract out the average control for the corresponding sample. Using equation from standard curve line determine µmol of PNP for the enzyme assay samples.
3. Divide this number by the reaction time (30min) to determine the international units (IU)in µmol/min. IU are sometimes wrote as pNPGU or PNPGU.
4. Divide that number by the volume of enzyme in the reaction (0.1ml) to determine Units per ml of original stock.
5. Divide that number by mg of protein in the enzyme stock to retain the most useful unit which is in IU/mg protein. Use the Bradford method of protein determination to find mg of protein in the original stock.

Appendix G. **Bradford Method for Protein Determination**

Assay prepared by Pedro Ricardo (University of Kentucky)

Enzyme stock:

3g/l enzyme stock- 0.30 g enzyme powder
100 ml DI

Assay:

1.5 ml Bradford Reagent (Sigma-Aldrich)

50 µl sample

Blank:

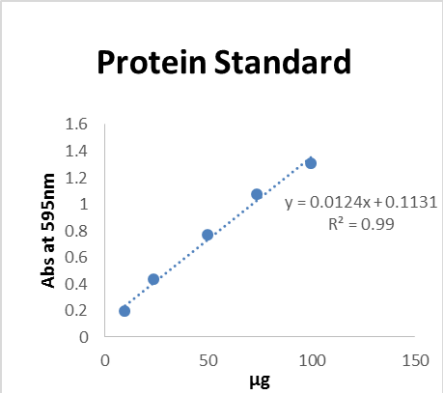
1.5 ml Bradford Reagent (Sigma-Aldrich)

50 µl DI

1. Prepare triplicates of Assay mixtures and a single blank in test tubes. Vortex for 15 seconds or pipet mix.
2. Pipet 1.5 ml of contents of each tube to individual cuvette.
3. Read absorbance at 595 nm. Subtract out blank from each assay sample.
4. Determine mass of protein using the standard curve equation below.
5. Multiply by 20 to determine µg protein/ml of stock.

Note: Curve is only suitable for protein contents up to and not exceeding 2mg/ml.
Original stock may need to be adjusted to fit within the range of the curve.

Following standards prepared by Pedro Ricardo using bovine albumin serum:

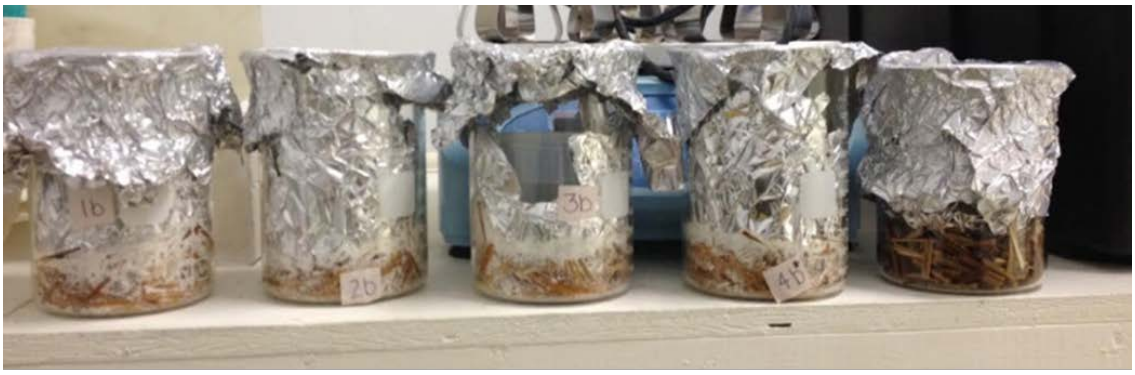


Results for American Laboratories Inc. enzyme:

Stock Enzyme Dilution: 3.0 g/l

Protein: 0.318 mg/ml

Appendix H. **Pictures of Density and Particle Size Tests**


1b
2b
3b
4b


4 CONTROL
1 inch
1B
1 inch
2B
1 inch
3B
1 inch
4B
1 inch


1b


1C
2C
4C


CONTROL C
4 inch
1C
4 inch
2C
4 inch
3C
4 inch


2C




Appendix I. **Density Enzymatic Hydrolysis Data**

```
proc sort data=density;
        by trt rep layer;
run;
proc means data=density mean noprint;
        by trt rep layer;
        var sugar;
        output out=data mean=sugar;
run;

proc glm data=data plots(unpack)=all;
        class trt rep layer;
   model sugar=trt rep(trt) layer layer*trt/ss1;
   test h=trt e=rep(trt);
        means trt layer layer*trt;
  run;
```

The GLM Procedure

Dependent Variable: sugar

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	20	0.77319123	0.03865956	12.39	0.0005
Error	8	0.02497141	0.00312143		
Corrected Total	28	0.79816264			

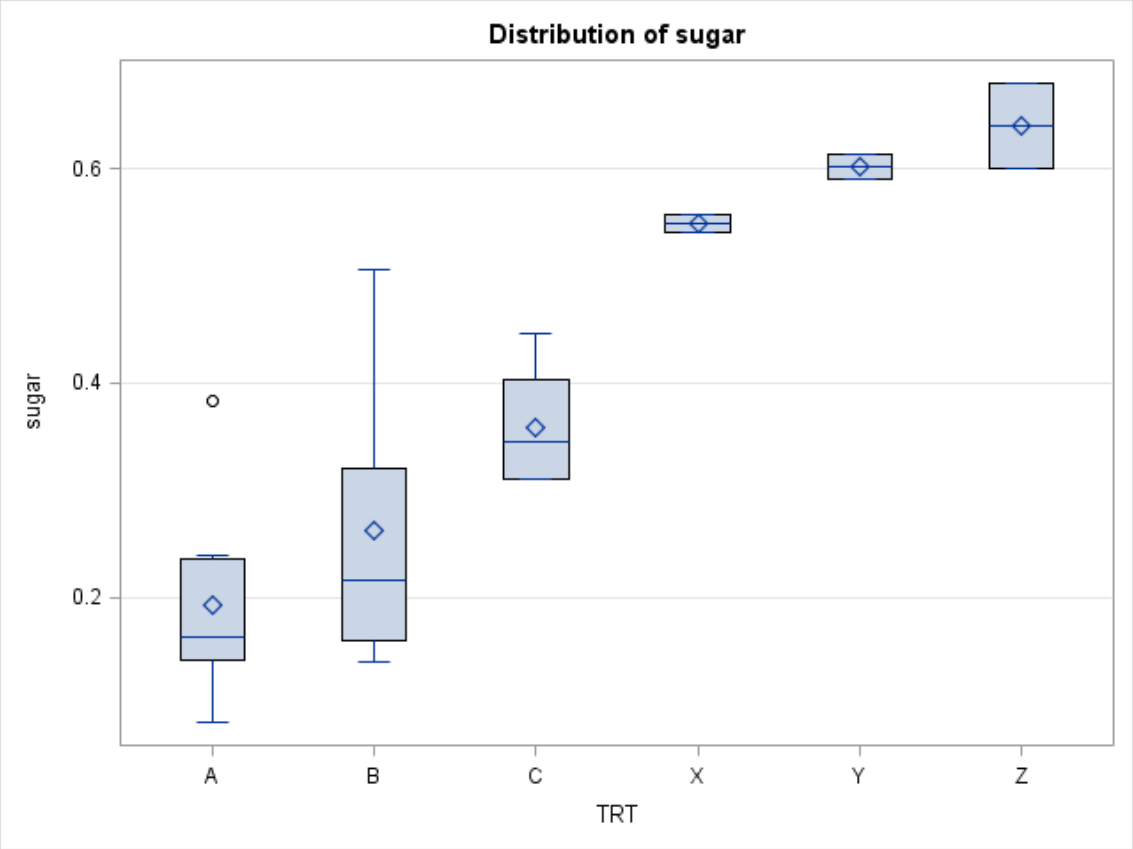
R-Square	Coeff Var	Root MSE	sugar Mean
0.968714	16.46010	0.055870	0.339425

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	5	0.61967450	0.12393490	39.70	<.0001
REP(TRT)	9	0.14109648	0.01567739	5.02	0.0165
Layer	1	0.00051429	0.00051429	0.16	0.6955
TRT*Layer	5	0.01190597	0.00238119	0.76	0.6010

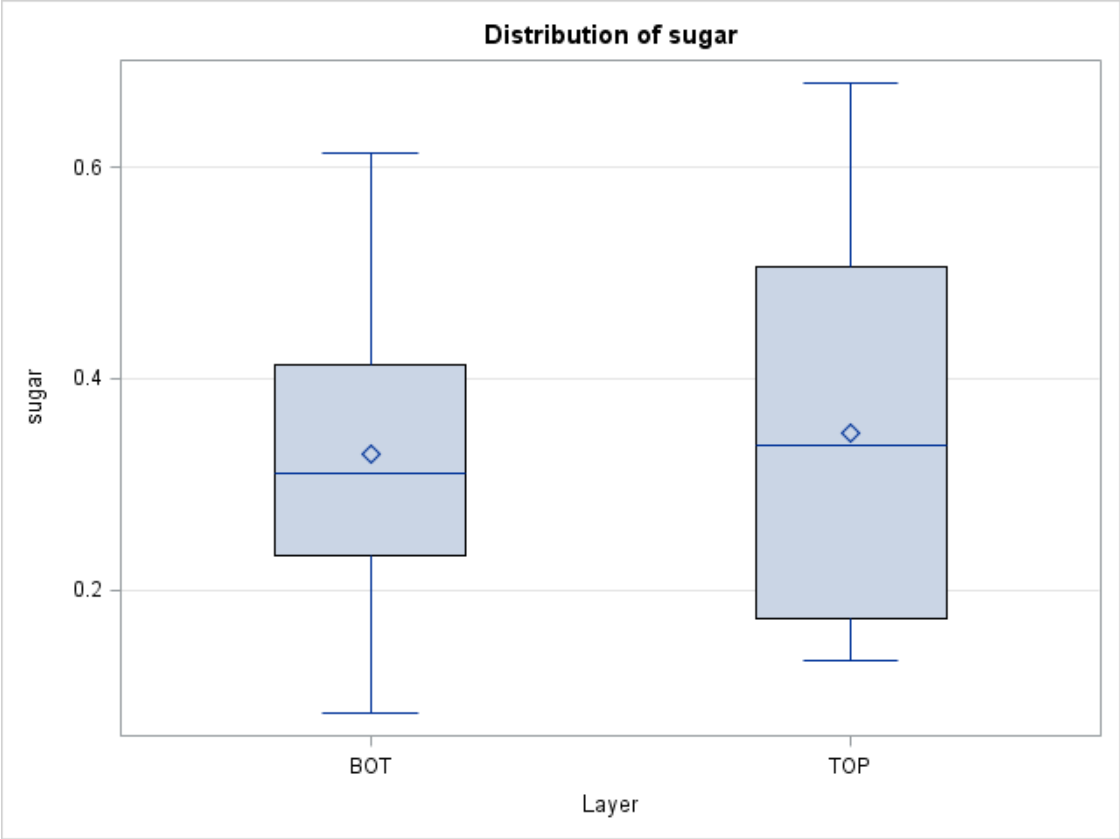
Tests of Hypotheses Using the Type I MS for REP(TRT) as an Error Term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	5	0.61967450	0.12393490	7.91	0.0041

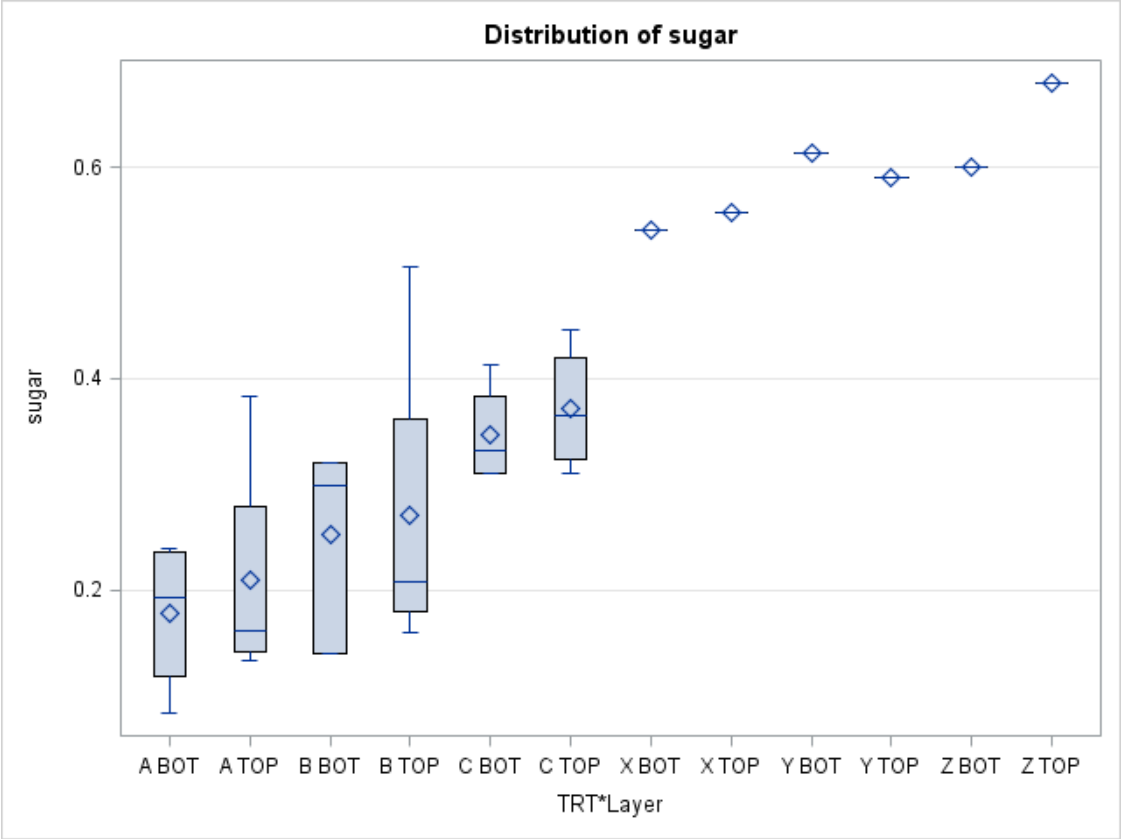
The GLM Procedure



Level of TRT	N	sugar	
		Mean	Std Dev
A	**8**	0.19354167	0.09227477
B	**7**	0.26309524	0.12630954
C	**8**	0.35916667	0.05284749
X	**2**	0.54833333	0.01178511
Y	**2**	0.60166667	0.01649916
Z	**2**	0.64000000	0.05656854



Level of Layer	N	sugar	
		Mean	Std Dev
BOT	**14**	0.32916667	0.16461137
TOP	**15**	0.34900000	0.17789532



Level of TRT	Level of Layer	N	sugar	
			Mean	Std Dev
A	**BOT**	**4**	0.17750000	0.07410578
A	**TOP**	**4**	0.20958333	0.11700249
B	**BOT**	**3**	0.25277778	0.09826740
B	**TOP**	**4**	0.27083333	0.15901025
C	**BOT**	**4**	0.34666667	0.04891413
C	**TOP**	**4**	0.37166667	0.06088848
X	**BOT**	**1**	0.54000000	.
X	**TOP**	**1**	0.55666667	.
Y	**BOT**	**1**	0.61333333	.
Y	**TOP**	**1**	0.59000000	.
Z	**BOT**	**1**	0.60000000	.
Z	**TOP**	**1**	0.68000000	.

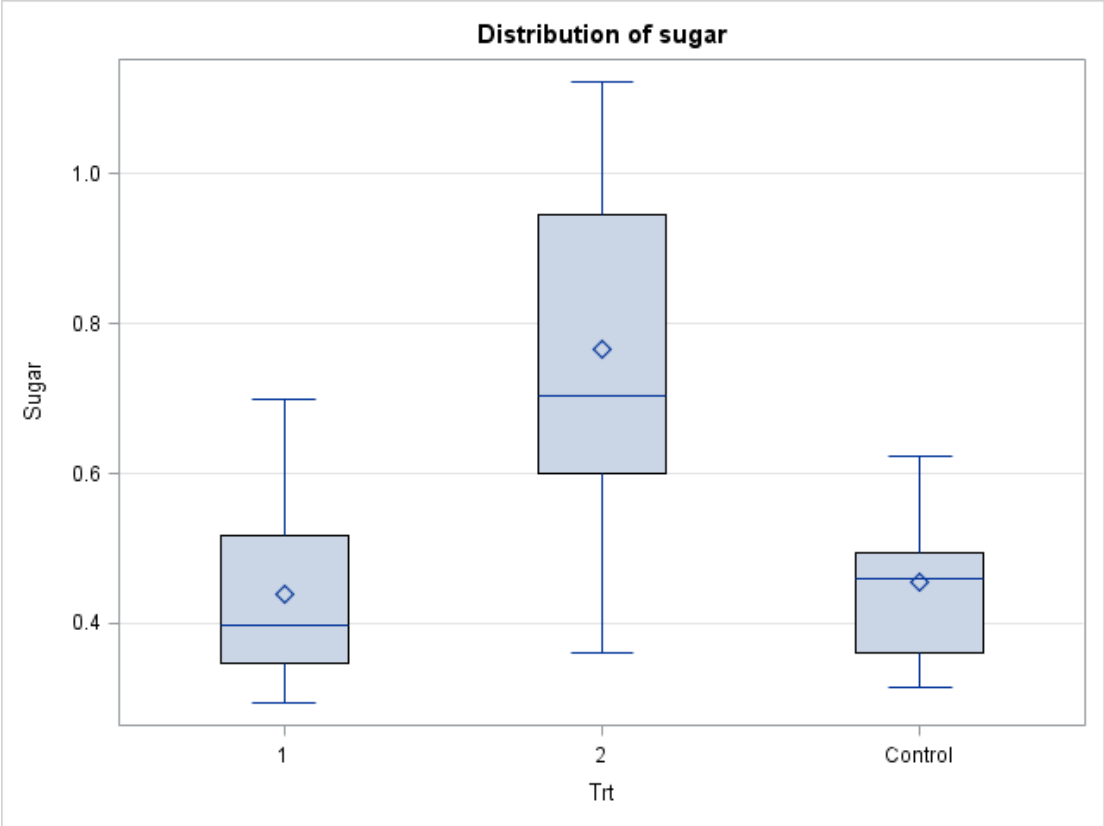
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	14	1.28161235	0.09154374	8.15	0.0004
Error	12	0.13470617	0.01122551		
Corrected Total	26	1.41631852			

R-Square	Coeff Var	Root MSE	sugar Mean
0.904890	19.13488	0.105951	0.553704

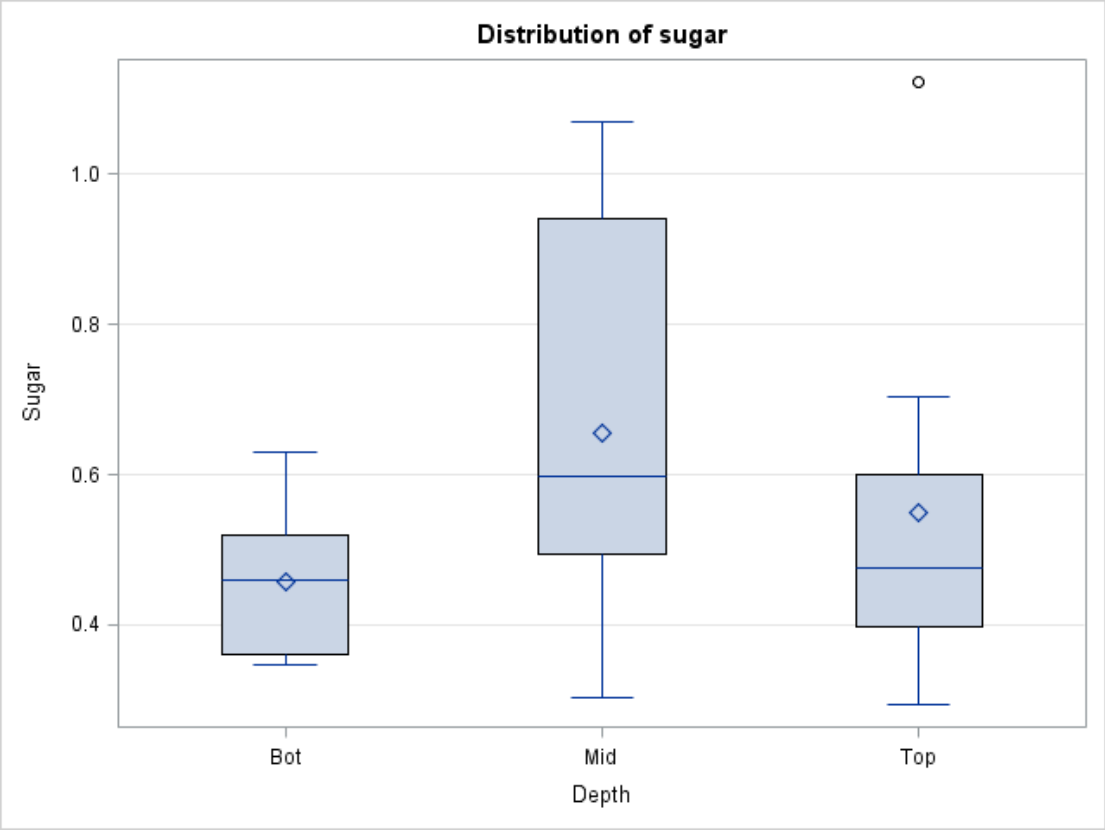
Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	2	0.60915802	0.30457901	27.13	<.0001
Bin(Trt)	6	0.28496049	0.04749342	4.23	0.0161
Depth	2	0.17567654	0.08783827	7.82	0.0067
Trt*Depth	4	0.21181728	0.05295432	4.72	0.0161

Tests of Hypotheses Using the Type I MS for Bin(Trt) as an Error Term					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
Trt	2	0.60915802	0.30457901	6.41	0.0324

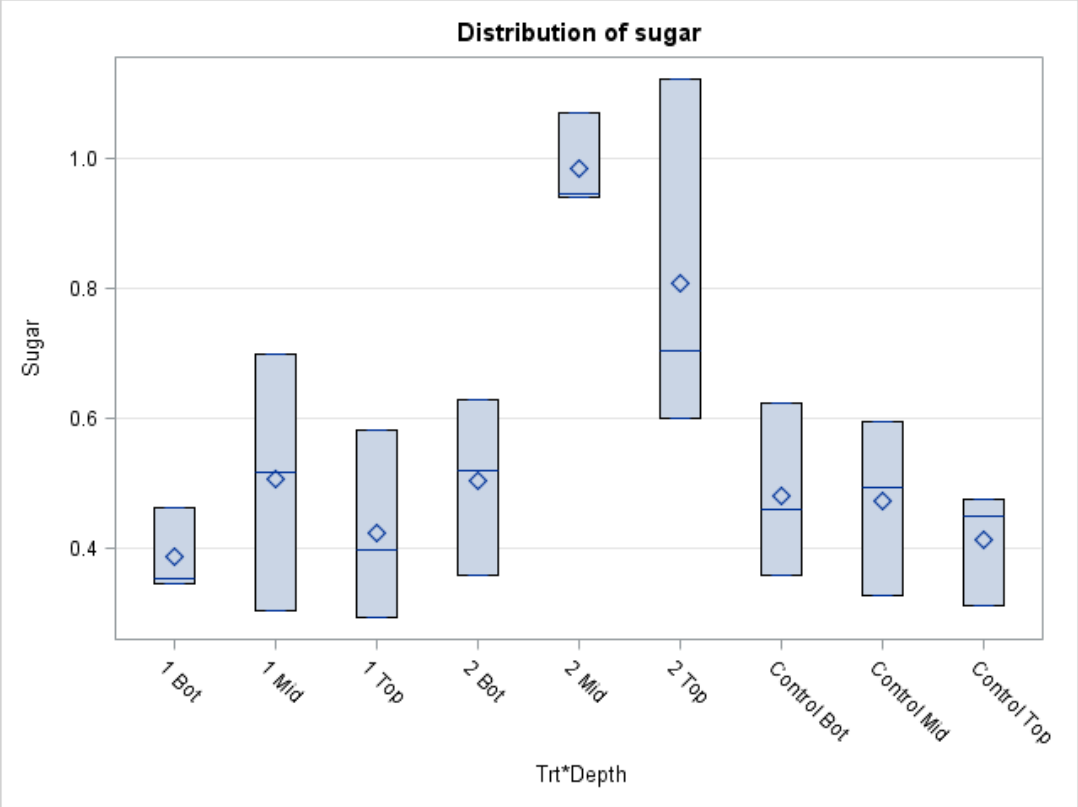
The GLM Procedure



Level of Trt	N	sugar	
		Mean	Std Dev
1	9	0.43962963	0.13822731
2	9	0.76592593	0.26418521
Control	9	0.45555556	0.10951915



Level of Depth	N	sugar	
		Mean	Std Dev
Bot	**9**	0.45740741	0.11366102
Mid	**9**	0.65481482	0.27854339
Top	**9**	0.54888889	0.25411612



Level of Trt	Level of Depth	N	sugar	
			Mean	Std Dev
1	**Bot**	**3**	0.38777778	0.06551788
1	**Mid**	**3**	0.50666667	0.19852232
1	**Top**	**3**	0.42444444	0.14698198
2	**Bot**	**3**	0.50333333	0.13576941
2	**Mid**	**3**	0.98555556	0.07320696
2	**Top**	**3**	0.80888889	0.27717490
Control	**Bot**	**3**	0.48111111	0.13292995
Control	**Mid**	**3**	0.47222222	0.13623237
Control	**Top**	**3**	0.41333333	0.08762293

Appendix J. **Particle Size Enzymatic Hydrolysis Data**

```
proc anova data=particle;
        class trt rep;
   model sugar=trt rep rep*trt;
        means trt rep rep*trt;
  run;
```

The SAS System

The ANOVA Procedure

Class Level Information

Class	Levels	Values
TRT	6	D E F X Y Z
REP	3	1 2 3

Number of Observations Read	45
Number of Observations Used	45

The SAS System

The ANOVA Procedure

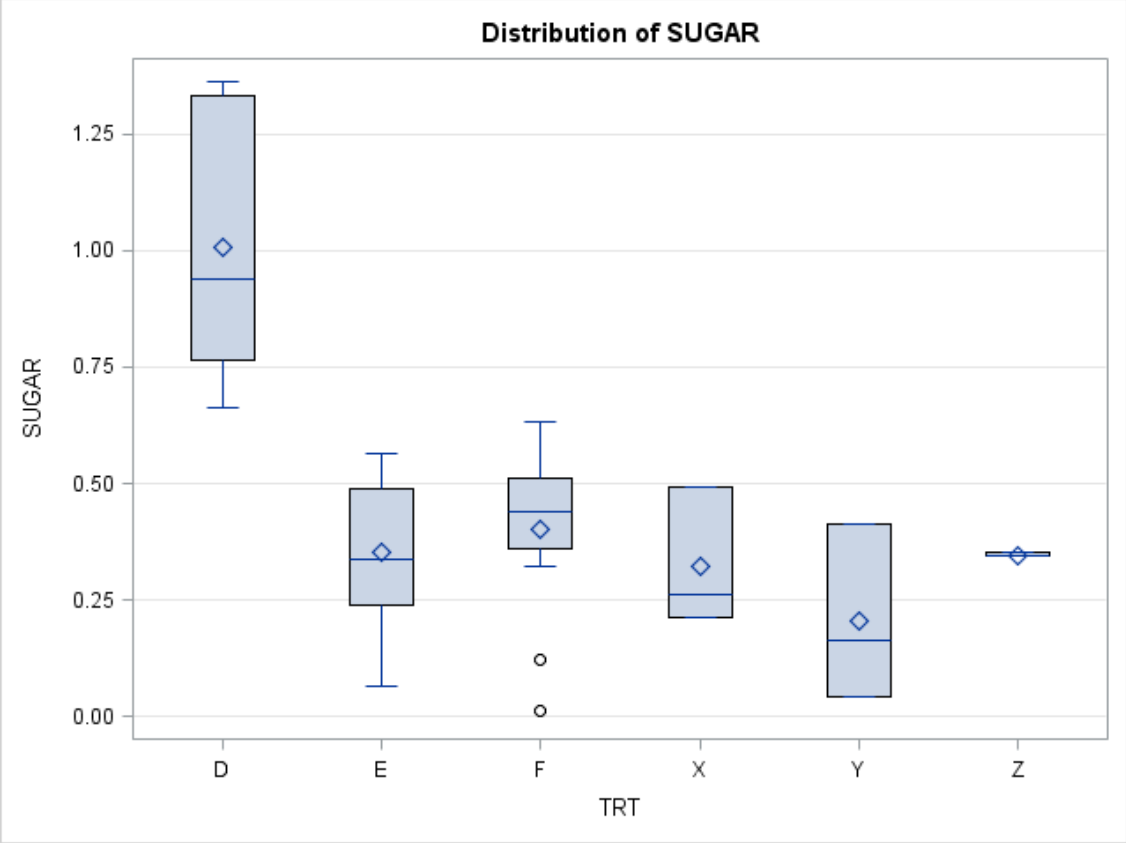
Dependent Variable: SUGAR

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	3.85676611	0.77135322	19.52	<.0001
Error	39	1.54129167	0.03952030		
Corrected Total	44	5.39805778			

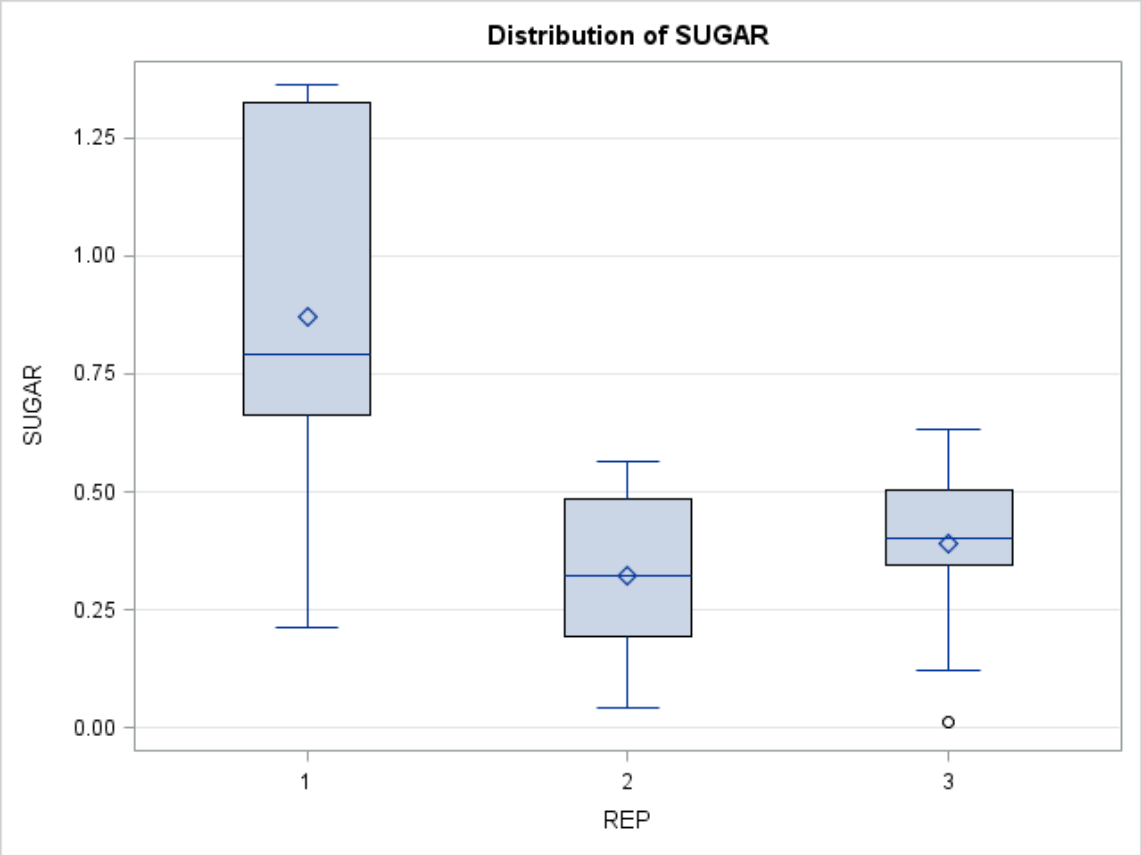
R-Square	Coeff Var	Root MSE	SUGAR Mean
0.714473	37.71446	0.198797	0.527111

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	5	3.85676611	0.77135322	19.52	<.0001
REP	2	2.67939111	1.33969556	33.90	<.0001
TRT*REP	-2	0.00000000	0.00000000	0.00	.

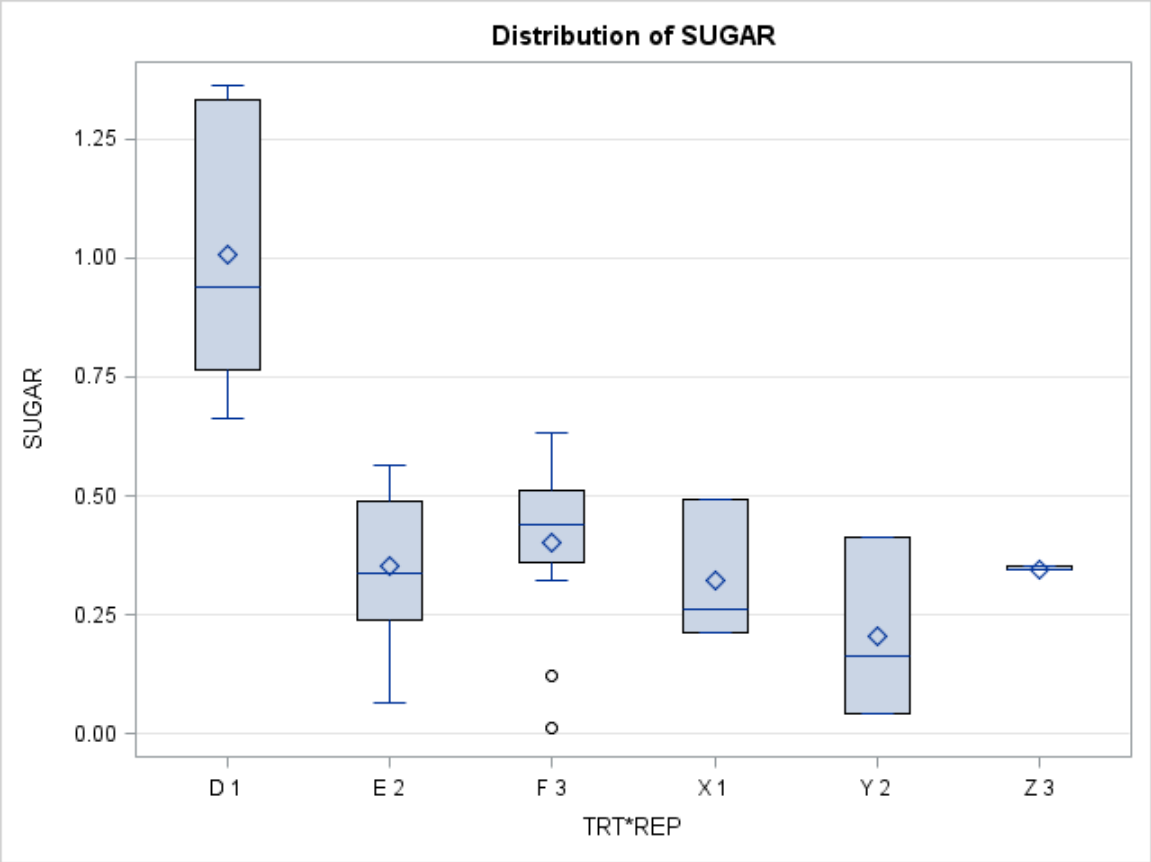
The ANOVA Procedure



Level of TRT	N	SUGAR	
		Mean	Std Dev
D	**12**	1.00666667	0.27370632
E	**12**	0.35083333	0.15475053
F	**12**	0.40000000	0.17525739
X	**3**	0.32333333	0.14933185
Y	**3**	0.20666667	0.18876794
Z	**3**	0.34666667	0.00577350



Level of REP	N	SUGAR	
		Mean	Std Dev
1	15	0.87000000	0.37695522
2	15	0.32200000	0.16573932
3	15	0.38933333	0.15692582



Level of TRT	Level of REP	N	SUGAR	
			Mean	Std Dev
D	**1**	**12**	1.00666667	0.27370632
E	**2**	**12**	0.35083333	0.15475053
F	**3**	**12**	0.40000000	0.17525739
X	**1**	**3**	0.32333333	0.14933185
Y	**2**	**3**	0.20666667	0.18876794
Z	**3**	**3**	0.34666667	0.00577350

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	0.4	0.177833333
Variance	0.030715152	0.014320789
Observations	12	20
Pooled Variance	0.020332056	
Hypothesized Mean Difference	0	
df	30	
t Stat	4.266963097	
P(T<=t) one-tail	9.12408E-05	
t Critical one-tail	1.697260887	
P(T<=t) two-tail	0.000182482	
t Critical two-tail	2.042272456	

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VITA

Amanda Hickman

Education

2012 – Present
University of Kentucky - Lexington, KY, United States
Master of Science: Biosystems and Agricultural Engineering | 3.7/4.0 GPA
Anticipated Graduation Date: Dec 2014
Specializing in Bioprocessing

2008 – 2012
University of Kentucky - Lexington, KY, United States
Bachelor of Science: Biosystems Engineering, | 3.4/4.0 GPA
Specializing in Bioprocessing

Certification

May 2014
University of Kentucky - Lexington, KY, United States
Student Lean Certification: Toyota True Lean: Lean Systems Program

April 2013
Engineer in Training (EIT)- Passed Fundamentals of Engineering Exam

Honors and Awards

2012-Present
Research Assistantship

2010 Fall Semester, 2011 Fall Semester, 2012 Spring Semester, 2012 Fall Semester
Dean's List

2014-Present
Alpha Epsilon Honor Society

Scholarships:

- Hope Scholarship ($1250 per semester)
- KEES Scholarship ($1238 per semester)
- Provost Scholarship ($750 per semester)
- Women and Philanthropy Scholarship, 2012 ($1250 per semester)
- Leslie and David Ludwick Scholarship, 2012 ($500 per semester)

Research Experience

2012 – Present

University of Kentucky - Lexington, KY, United States

Supervisor: Dr. Sue Nokes

Master's Research: Scale up of *P.chysosporium* during solid state pretreatment

- Optimization of solid state pretreatment parameters in scale up design of biomass to biofuel process
- Design of experiments, analysis, and testing procedures
- Designed, tested, and implemented a pilot-scale aeration and water pumping system
- Problem solving: maintain lab equipment, manage and supervise lab technicians
- Utilized SEM technology to visualize lignin degradation and fungal growth
- Development of technical reports and scientific publications
- Presented oral and poster presentations for national engineering conferences

September 2012 – December 2012

University of Kentucky - Lexington, KY, United States

Supervisor: Dr. Gregory Graf

Undergraduate Research

- Developed a library cataloging quickly accessible DNA samples for real time PCR testing
- Designed and implemented user-friendly spreadsheets to identify the specific DNA needed and to located the sample in the DNA library

2008 – 2012

University of Kentucky - Lexington, KY, United States

Supervisor: Dr. Sue Nokes

Undergraduate Research

- Ran biomass pretreatment experiments for biofuel processes and presented results to a group of PI's
- Collected, analyzed, and interpreted data
- Maintained chemical inventory and updated supplies
  Created AutoCAD drawings and maintained, archived and retrieved

  CAD files

Presentations

American Society of Agricultural and Biological Engineers (ASABE) International Conference Montreal, Canada, 2014
Poster Presentation: Scale-up of *Phanerochaete chrysosporium* during solid state pretreatment

Institute of Biological Engineering (IBE) International Conference, Lexington KY, 2014.
Poster Presentation: Optimization of P. chrysosporium during solid state pretreatment

Institute of Biological Engineering (IBE) International Conference, Indianapolis, IN, 2012
Poster Presentation: Real-Time Algae Growth System

Professional Memberships

2013-Present
Kentucky Society of Professional Engineers (KSPE)

2013-Present
American Society of Agricultural and Biological Engineers (ASABE)

2012-Present
Institute of Biological Engineering (IBE)

2012-Present
Order of the Engineer

Leadership Experience

August 2014-December 2014
Teaching Assistant, BAE 102: Introduction to Biosystems Engineering

- Assisted instructor in daily class functions, preparations, and grading of student material for over70 students
- Held weekly office hours for student help
- Administered, assigned, and graded a video project for the students

January 2013- December 2014

Research Coordinator

University of Kentucky - Lexington, KY, United States

Department of Biosystems Engineering

- Managed daily tasks for 3 undergraduate students working in the chemistry lab
- Introduced essential lab protocols to the students for their research

2013-2014

Student Peer Mentor

University of Kentucky - Lexington, KY, United States

Department of Biosystems Engineering

- Met with several undergraduate students during the semester
- Advised, suggested and discussed pertinent courses, professors, and work opportunities

June 2012-August 2012

Summer Camp Leader

Aquabots Summer Camp Kentucky Girls STEM Collaborative

- Lead teams in planning and design of “water-bot” to reach mission milestones
- Assisted in programming the robots for interactive user interface allowing control
- Supervised group of 5-15 girls ages 12-15