INVESTIGATION OF *PLEUROTUS OSTREATUS* PRETREATMENT ON SWITCHGRASS FOR ETHANOL PRODUCTION

By

SHELYN GEHLE SLAVENS

Bachelor of Science in Biosystems Engineering

Oklahoma State University

Stillwater, Oklahoma

2014

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July 2016

INVESTIGATION OF *PLEUROTUS OSTREATUS*

PRETREATMENT ON SWITCHGRASS FOR

ETHANOL PRODUCTION

Thesis Approved:

Dr. MARK R. WILKINS

Thesis Adviser

Dr. MICHAEL BUSER

Dr. STEPHEN MAREK

ACKNOWLEDGEMENTS

I would like to thank the USDA South Central Sun Grant Program for providing funding for this project (Grant No. 2011-51110-31044). I would especially like to thank my advisor, Dr. Wilkins, and my committee members, Dr. Buser and Dr. Marek, for all their help and guidance on this project. I would like to thank Collin Craige for all his help with the equipment design and set-up, inoculation and sampling, and other help throughout the bale storage period. I would like to thank Nyoman Sulastri, Dr. Ning Wang, and Mike Veldman for their help with the electronics. The building storage space for the bale study was provided by the OSU Division of Agricultural Sciences and Natural Resources and Rocky Walker at the Stored Products Research and Education Center. I would like to thank Dr. Nurhan Dunford's group for help and use of the Dionex ASE; Dr. Niels Maness and Donna Chrz for uses of laboratory space and supplies during the Soxhlet extractions. I would also like to thank Oscar Pardo-Planas for all his help in the laboratory, especially with the SSF preparation and sampling. Finally, I would like to thank my husband and our families for their ongoing support over the last two years.

Acknowledgements reflect the views of the author and are not endorsed by committee members or Oklahoma State University.

Name: SHELYN GEHLE SLAVENS

Date of Degree: JULY 2016

Title of Study: INVESTIGATION OF *PLEUROTUS OSTREATUS* PRETREATMENT ON SWITCHGRASS FOR ETHANOL PRODUCTION

Major Field: BIOSYSTEMS ENGINEERING

Abstract:

Fungal pretreatment using the white-rot fungus *Pleurotus ostreatus* on switchgrass for ethanol production was studied. In a small-scale storage study, small switchgrass bales were inoculated with fungal spawn and automatically watered to maintain moisture. Sampled at 25, 53, and 81 d, the switchgrass composition was determined and liquid hot water (LHW) pretreatment was conducted. Fungal pretreatment significantly decreased the xylan and lignin content; glucan was not significantly affected by fungal loading. The glucan, xylan, and lignin contents significantly decreased with increased fungal pretreatment time. The effects of the fungal pretreatment were not highly evident after the LHW pretreatment, showing only changes based on sampling time. Although other biological activity within the bales increased cellulose degradation, the fungal pretreatment successfully reduced the switchgrass lignin and hemicellulose contents.

In a laboratory-scale nutrient supplementation study, copper, manganese, glucose, or water was added to switchgrass to induce production of ligninolytic enzymes by *P. ostreatus*. After 40 d, ligninolytic enzyme activities and biomass composition were determined and simultaneous saccharification and fermentation (SSF) was conducted to determine ethanol yield. Laccase activity was similar for all supplements and manganese peroxidase (MnP) activity was significantly less in copper-treated samples than in the other fungal-inoculated samples. The fungal pretreatment reduced glucan, xylan, and lignin content, while increasing extractable sugars content. The lowest lignin contents occurred in the water-fungal treated samples and produced the greatest ethanol yields. The greatest lignin contents occurred in the copper-fungal treated samples and produced the lowest ethanol yields. Manganese-fungal and glucose-fungal treated samples had similar, intermediate lignin contents and produced similar, intermediate ethanol yields. Ethanol yields from switchgrass were increased significantly by fungal pretreatment.

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CHAPTER I

1. Research objectives and brief descriptions of studies

The overall project objective was to determine if treating switchgrass prior to storage with a *Pleurotus ostreatus* fungal pretreatment would decrease lignin contents and improve ethanol yields. The project included two studies: a small-scale storage study and a laboratory-scale study. The small-scale storage study objective was to determine if fungal pretreatment of switchgrass during storage would achieve significant delignification over time. The laboratory-scale study objective was to determine the effects of adding trace metals or nutrients to the fungal pretreatment on the induction of ligninolytic enzymes and the resulting ethanol production.

1) Small-scale storage study (Chapter 3): Fungal pretreatment with *P. ostreatus* was conducted on small switchgrass bales to facilitate delignification. An automatic control system was developed to continuously record bale weight and internal temperature, while adding water to the bales to maintain a 50% bale moisture content. The project treatment design included three storage periods, three inoculation loading rates, and three replications. Once bales were removed from storage, samples were collected and compositional analysis and hydrothermolysis pretreatment were conducted to determine if fungal pretreatment facilitated greater glucose yield.
2) Laboratory-scale study (Chapter 4): Copper, manganese, and glucose additions for the induction of ligninolytic enzymes, laccase and manganese peroxidase, were studied.

Each solution was added to switchgrass, which was inoculated with *P. ostreatus* liquid mycelia and incubated for 40 d. Samples were taken for the determination of ligninolytic enzyme activities. The effects of the chemical additions on the biomass composition and ethanol yield from simultaneous saccharification and fermentation were evaluated.

CHAPTER II

2. Literature Review

2.1. Switchgrass harvest and storage

Switchgrass (*Panicum virgatum*) is considered a good lignocellulosic biomass for the production of ethanol in the southern United States. It is a native, warm-season, perennial grass species with high drought tolerance and high biomass yield potential, which requires low inputs of fertilizers and pesticides (Adler et al., 2006; Djioleu et al., 2014; Mooney et al., 2012). Switchgrass, a C4 grass, has low protein and organic acid concentrations, but high levels of total carbohydrates (Dien et al., 2006).

Harvest timing significantly affects switchgrass composition. As the grass matures, there are changes in the soluble sugar quantity and ease of extracting glucans. One study showed that the percent of soluble glucose recovered peaked in the mid-growth (anthesis) stage. The cell wall glucose and non-glucose sugar concentrations, as well as the concentration of lignin, increased with maturity (Dien et al., 2006). These composition difference corresponds to a change in sugar location and concentration within the plant and is a response to environmental and physiological cues. (Adler et al., 2006; Dien et al., 2006).

In most climates, the highest biomass yields are achieved with a single harvest during this mid-growth stage. However, in areas where the climate is less variable, a two-

cut harvest system produces higher overall yields (Inman et al., 2010). Switchgrass is usually harvested after a frost (late fall to early winter), which also effects the composition. Adler et al. (2006) reported that in switchgrass ash content, soluble carbohydrate and storage carbohydrate (starch) concentrations decreased while Klason lignin and cell wall carbohydrate concentrations increased while the grass was in the field during the winter.

Storage conditions also significantly affect biomass quality and composition. Bales stored outside and unprotected can have significant dry matter loss depending on the climate, which is partially due to leaching of soluble components within the biomass. Storing bales inside results in significantly lower dry matter losses; however, inside storage of switchgrass bales is not economically feasible (Sanderson et al., 1997; Shinners et al., 2010). Dry matter loss for a bale can be calculated as the dry bale weight at harvest minus dry bale weight at destruction, divided by dry bale weight at harvest (Mooney et al., 2012). Storage method has a significant effect on the lignin concentration due to loss of extractives and carbohydrates (Djioleu et al., 2014).

2.2. Lignocellulosic biomass

Lignocellulosic biomass consists of cellulose, hemicellulose, pectin, and lignin composites. Lignin strongly inhibits the saccharification (breaking down into simple sugars) of cell wall polysaccharides (Weng et al., 2008). Lignin can be classified into softwood lignin, hardwood lignin, and grass lignin based on the monomer units chemical structure. Lignin is composed of chemically distinct subunits known as monolignols. The most common monolignols are *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S). The abundance of each of the monolignols varies among species, individual plants, and cell types (Weng et al., 2008). Grass lignin is composed of guaiacyl-, syringyl-, and *p*-

hydroxyphenylpropane units, with *p*-coumaric acid (5-10%) esterified to the terminal hydroxyl groups of some of the propyl side chains (Higuchi, 1990). Switchgrass typically contains 12-23% lignin on a dry basis (Lindsey et al., 2013; Sanchez, 2009).

2.2.1. Current pretreatment technologies

In order to utilize carbohydrates in biomass for ethanol production, biomass must be pretreated to improve the accessibility of carbohydrates to hydrolytic enzymes. A generalized schematic of pretreatment is shown in Figure 2.1. There are chemical pretreatment methods, including dilute acid, alkaline, oxidative (using hydrogen peroxide or peracetic acid), organic solvents (organosolv), and ionic liquid pretreatment. There are several thermochemical pretreatment methods, including steam explosion, ammonia fiber explosion (AFEX), CO_2 explosion, wet oxidation (WO), and liquid hot water (LHW). There are also combinations of thermochemical pretreatments and biological pretreatments (Mood et al., 2013).

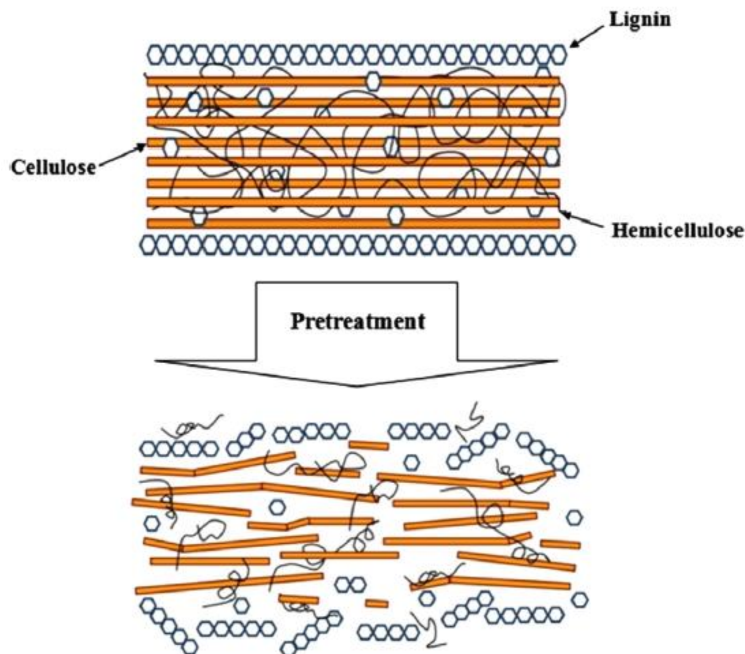

Figure 2.1. Schematic of lignocellulosic biomass pretreatment; reproduced with permission from Mood et al. (2013)

General thermal pretreatment involves heating biomass to increase cellulose hydrolysis. At temperatures above 150-180°C (depending on the composition), hemicellulose and lignin begin to solubilize. Hydrolysis of hemicellulose forms acids, while solubilization of lignin results in phenolic compounds. Severe pretreatment conditions promote the condensation and precipitation of soluble lignin compounds and soluble hemicellulosic compounds, such as furfural and HMF (Hendriks & Zeeman, 2009).

Steam explosion pretreatment is a hydrothermal pretreatment that uses pressurized steam (20-50 bar, 160-270°C) for several minutes, after which the vessel is suddenly depressurized. This explosive decompression separates the fibers, resulting in partial hemicellulose hydrolysis and solubilization and lignin redistribution. Large particle sizes can be treated, which is one main advantage of this pretreatment over others (Alvira et al., 2010; Mood et al., 2013).

Alkali pretreatments use sodium, potassium, calcium and ammonium hydroxides or lime to break apart the lignin and hemicellulose. These pretreatments can be operated at low temperatures, but require longer residence times and neutralization before fermentation (Mood et al., 2013). Kumar and Wyman (2009) reported less sugar degradation from a lime pretreatment than a dilute acid pretreatment.

Acid pretreatments can be done with low (dilute) acid concentrations at high temperatures or high acid concentrations at lower temperatures. Although high acid concentrations require less energy, these pretreatments result in fermentation inhibitor production and degrade monosaccharides. The main inhibitors produced are HMF (5-hydroxymethylfurfural) and furfural (2-furfuralaldehyde). Dilute acid pretreatments are more commonly used because lower concentrations of fermentation inhibitors are

generated (Mood et al., 2013). Dilute acid pretreatments can be operated at high temperatures (180°C) for shorter times (10-15 min) or lower temperatures (120°C) for longer times (30-90 min) (Alvira et al., 2010; Saha et al., 2005).

Liquid hot water (LHW) pretreatment uses high temperatures (160-220°C) and pressure to keep water in the liquid state and in contact with the biomass for a specific residence time. The slurry generated through the LHW pretreatment process consists of a solid fraction (enriched cellulose and water-insoluble materials), a liquid fraction (water and solubilized hemicellulose), and few or no inhibitors (Mood et al., 2013). Ko et al. (2015) performed LHW pretreatment on hardwood at increasing severities. They observed an increase in recovered lignin with increased pretreatment severity. They attributed this response to cellulose and lignin remaining in the recovered solid while xylan was solubilized into the liquid portion. This increase in lignin recovery could have been caused by condensation reactions of lignin with other degradation products. They also reported a decrease in acid soluble lignin (ASL) and an increase in acid insoluble lignin (AIL) with greater pretreatment severity, increasing the AIL/ASL ratio with increasing severity. AIL and ASL percentages were determined using the two-stage acid hydrolysis method outlined by Sluiter et al. (2012). Yasuda et al. (2001) described the ASL portion as the low molecular weight and hydrophilic derivatives of lignin.

2.2.2. Combined biological and thermochemical pretreatment

Combinations of biological pretreatment with other pretreatments have been found to increase glucose yield compared to thermochemical pretreatments. Ma et al. (2010) combined pretreatment by the fungus *Echinodontium taxodii* with mild acid (0.25% H_2SO_4) pretreatment on water hyacinth. They reported a 2.11-fold increase in reducing

sugar yield compared to mild acid pretreatment only at 25°C for 30 min. Taniguchi et al. (2010) investigated the effects of steam explosion (1.5 MPa for 1 min) on rice straw before applying a *P. ostreatus* fungal treatment, reporting glucose yield versus treatment time. They were able to reduce the required treatment time to achieve a 31% net glucose yield from 60 d to 36 d.

Wang et al. (2012) combined *Lenzites betulina* or *Trametes ochracea* and liquid hot water pretreatments on Chinese white poplar (*Populus tomentosa*). They tested LHW conditions at 140, 160, 180, and 200°C for 30 min after fungal pretreatment. They determined that the biomass co-treated with *L. betulina* and LHW at 200°C/30 min resulted in a 1.15-fold increase in glucose yield over LHW at 200°C/30 min only. This was a 3.84-fold increase over *L. betulina* pretreatment only. They also determined that the biomass co-treated with *T. ochracea* and LHW at 200°C/30 min resulted in a 1.12-fold increase in glucose yield over LHW at 200°C/30 min only.

Wan and Li (2011) studied the effects of LHW pretreatment of corn stover or soybean straw at 170°C for 3 min before fungal pretreatment with *Ceriporiopsis subvermispora*. With LHW pretreatment alone, there was not significant degradation of cellulose, hemicellulose, or lignin. However, LHW pretreatment followed by fungal pretreatment significantly increased lignin degradation for both substrates. For corn stover, which can be degraded by *C. subvermispora*, the combined treatment increased lignin and hemicellulose degradation by 13 and 20%, respectively, over fungal pretreatment only. Soybean straw cannot be degraded by *C. subvermispora* alone; however, the combined pretreatment resulted in lignin degradation of 36.7% and hemicellulose degradation of

41.42%. The cellulose digestibility was increased by 30% for the combined pretreatment of soybean straw over no LHW or fungal pretreatment.

2.3. Fungal pretreatment

Fungal pretreatment is one possible alternative to thermochemical pretreatment methods currently being used. White-rot, brown-rot, and soft-rot fungi are capable of degrading lignocellulosic biomass and have many advantages over thermochemical pretreatment processes. Simple techniques, low energy requirements, reduced processing costs and waste, and reduced inhibitors are some of the advantages of fungal pretreatment over thermochemical pretreatment for ethanol production. However, loss of cellulose and hemicellulose are major issues attributed to fungal pretreatment. White-rot fungi have been found to be highly selective lignin degraders, achieving high lignin degradation and low cellulose degradation, making them the most promising group of fungi for pretreatment (Wan & Li, 2012).

Several white-rot fungi have been studied for pretreatment of lignocellulosic biomass, including *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Coriolus versicolor*, and *Ceriporiopsis subvermispora*. Table 2.1 shows a variety of fungal pretreatment studies that have been previously reported. White-rot fungi use hydrolases that gradually degrade cellulose while lignin is mineralized for use in fungal growth by high-oxidation potential class II peroxidases (PODs), which include lignin peroxidase (LiP) and manganese peroxidase (MnP). Some white-rot fungi also produce the ligninolytic enzyme laccase (Riley et al., 2014).

White-rot fungi are generally characterized by high laccase activity, except for *P. chrysosporium, Phanerochaete carnosa,* and *Auricularia delicata*, which do not produce

laccase (Riley et al., 2014). Lignin degrading fungi can be categorized based on their enzyme production patterns into one of three groups: (i) LiP-MnP group (e.g. *P. chrysosporium*), (ii) MnP-laccase group (e.g. *P. ostreatus*), or (iii) LiP-laccase group (e.g. *Phlebia ochraceofulva*) (Dwivedi et al., 2011). LiP (also known as ligninase, EC 1.11.1.14) is involved in the oxidative cleavage of non-phenolic aromatic lignin compounds. MnP (EC 1.11.1.13) aids lignin degradation by catalyzing the oxidation of syringyl and vinyl side-chain substituted compounds in the presence of Mn^{2+}. Laccases (EC 1.10.3.2) are copper-containing oxidases that perform one-electron oxidations of phenolic compounds (Wan & Li, 2012).

Table 2.1. Fungal pretreatment studies, sorted by substrate

Fungal species	Substrate	Summary of Results	Reference
Trametes versicolor, *Echinodontium taxodii*	Bamboo culms	Increased sugar yield and decreased lignin by: 5.15-fold and 12.0% for *T. versicolor*, 8.76-fold and 29.1% for *E. taxodii*	Zhang et al. (2007b)
Coriolus versicolor	Bamboo residues	Increased saccharification rate by 2.34-fold	Zhang et al. (2007a)
Ceriporiopsis subvermispora, *Dichomitus squalens*, *Pleurotus ostreatus*, *C. versicolor*	Beech wood	Lignin loss: 21.7% for *D. squalens*, 17.7% for *C. versicolor*, 10.3% for *P. ostreatus*, 13.0% for *C. subvermispora*	Itoh et al. (2003)
Lenzites betulina, *Trametes ochracea*	Chinese white poplar	Increased glucose yields, over LHW alone:1.5-2.66-fold for *L. betulina*, 1.12-2.25-fold *T. ochracea*	Wang et al. (2012)
E. taxodii	Chinese willow, China-fir	Lignin degradation (120 d) and increased enzymatic hydrolysis ratios by: 45.6% and 4.7-fold for Chinese willow, 39.8% and 6.3-fold for China-fir	Yu et al. (2009a)
Trametes hirsute yj9	Corn stover	Degraded 71.5% lignin and reached 74% enzymatic digestibility (42 d)	Sun et al. (2011)
C. subvermispora	Corn stover	Lignin degradation of 19.48% at 60% moisture, 29.54% at 75% moisture, and 31.33% at 85% moisture; glucose yield of 66.61% and xylose yield of 38.30% at 35 d, 75% moisture	Wan and Li (2010)
C. subvermispora	Corn stover, soybean straw	Increased lignin and hemicellulose degradation: 13% and 20% for corn stover, 36.7% and 41.4% for soybean straw	Wan and Li (2011)
Ganoderma lucidum, *T. versicolor*, *E. taxodii*	Corn straw	Lignin loss (30 d) and reducing sugar yield increase: 54.6 and 29.3% for *T. versicolor*, 32.7 and 26.5% for *G. lucidum*, 42.2 and 50.7% for *E. taxodii*	Yu et al. (2010)
Phaerochaete chrysosporium	Cotton stalks	Decreased lignin 19.4-35.5%	Shi et al. (2009)
P. chrysosporium, *P. ostreatus*, *Pleurotus pulmonarius*, *Trametes* sp.	*Eucalyptus grandis* sawdust	Efficiency of cellulose hydrolysis: *P. ostreatus* 16.7%, *P. pulmonarius* 15.4%, *Trametes* sp. 10.1%, *P. chrysosporium* 2.8%	Castoldi et al. (2014)
Pycnoporus cinnabarinus	*Lantana camara*, *Prosopis juliflora*	Increased the holocellulose to lignin ratio (degraded lignin) after 15 d: 14.62% for *L. camara*, 17.28% for *P. juliflora*	Gupta et al. (2011)
I. lacteus, *E. taxodii*	Moso bamboo	Reduced lignin by: 13% for *I. lacteus*, 29% for *E. taxodii*	Zeng et al. (2012)
P. ostreatus	Rice hulls	Increased total soluble sugars and glucose yields (over fungal only): 3.3 and 4.2-fold for ultrasonic, 5.8 and 6.5-fold for H_2O_2	Yu et al. (2009b)
P. ostreatus	Rice straw	Selectively degraded lignin and hemicellulose fractions by 41% and 48%, glucose yield 32%	Taniguchi et al. (2005)
P. ostreatus	Rice straw	Reduced time required to obtain 33% net glucose yield from 60 to 36 d	Taniguchi et al. (2010)
Pleurotus florida, *Coriolopsis caperata*, *Ganoderma* sp. rckk-02	Sugarcane bagasse	Degraded lignin after 15 d and increased sugar yield by: 7.91% and 2.36-fold for *P. florida*, 5.48% and 1.50-fold for *C. caperata*, 5.58% and 1.72-fold for *Ganoderma*	Deswal et al. (2014)
P. chrysoporium, *Lentinula edodes*, *P. ostreatus*	Sugarcane bagasse	Degraded cellulose, hemicellulose, and lignin by: 67.0, 88.6, and 93.4% for *P. chrysosporium*; 15.2, 73.5, and 87.6% for *L. edodes*; 16.3, 64.4, and 84.9% for *P. ostreatus*	Dong et al. (2013)
Pycnoporus sp. SYBC-L3	Switchgrass	Reduced lignin by 35% and glucose yield of ~60% at 36 d	Liu et al. (2013)
E. taxodii	Water hyacinth	Increased reducing sugar yield by 1.13-2.11-fold and ethanol yield 1.34-fold	Ma et al. (2010)
D. squalens, *Fomitopsis pinicola*, *G. lucidum*, *Lenzites betulinus*, *Pleurotus eryngii*, *P.ostreatus*, *T. versicolor*	Wheat straw	Lignin degradation after 14 d: *D. squalens* 34.1%, *F. pinicola* 32.4%, *L. betulinus* 28.3%, *G. lucidum* 19.9%, *T. versicolor* 20.9%, *P. eryngii* 14.5%, *P. ostreatus* 12.5%	Knežević et al. (2013)
Poria subvermispora, *I. lacteus*	Wheat straw	Hemicellulose degradation, lignin degradation, and glucose yield after 21 d: 36%, 30%, and 69% for *P. subvermispora*; 26%, 34%, and 66% for *I. lacteus*	Salvachua et al. (2011)

Higuchi (1990) reported that lignin decayed by white-rot basidiomycetous fungi has decreased contents of methoxyl groups and β-O-4 substructures, with increased contents of oxygen and aliphatic and aromatic (mostly benzoic) carboxylic groups. It has been suggested that three degradative reactions are utilized by white-rot basidiomycetes (Higuchi, 1990; Kirk & Chang, 1975):

1. Oxidative cleavage of propyl side chains between α- and β-carbons, forming benzoic acids.
2. Cleavage of β-aryl ether bonds and modification of side chain structures.
3. Degradation of aromatic nuclei through oxidative ring opening.

P. ostreatus, also known as the oyster mushroom, is a white-rot basidiomycetous fungus that produces laccase and MnP as part of its ligninolytic enzyme complex. Growing in shelf-like clusters on dead logs and living trees, the oyster mushroom is a common edible mushroom with thick, white flesh. The fungus starts as mycelia, which later produces fruiting bodies. The fungal spawn running (mycelia spreading across the substrate) usually occurs at a higher temperature than fruiting; most strains list 8-18°C as the ideal temperature for the formation of fruiting bodies. Curvetto et al. (2002) allowed their *P. ostreatus* spawn run to occur at 24±1°C for 15-20 d.

P. ostreatus has been found to be moderately selective in the degradation of the lignin fraction of lignocellulosic biomass (Adamovic et al., 1998; Agosin & Odier, 1985; Taniguchi et al., 2005), but will start degrading the cellulose portion with prolonged pretreatment times (Wan & Li, 2012). Adamovic et al. (1998) investigated the chemical composition changes of wheat straw treated with *P. ostreatus*. They concluded that the cell wall components had different rates of degradation when subjected to *P. ostreatus*

enzymes. The rates of degradation of hemicellulose, cellulose, and lignin were 0.902, 0.290 and 0.450 (% day^{-1}), respectively.

2.4. Ligninolytic enzymes

2.4.1. Laccase

Laccases are dimeric or tetrameric glycoproteins, with four copper atoms per monomer. The copper sites are categorized into three groups: blue copper center (Type-1), normal copper center (Type-2), and coupled binuclear copper centers (Type-3). The arrangement of the four copper atoms is shown in Figure 2.2. The copper center structure and properties also affect the redox potential of the laccase enzyme. Laccases from basidiomycetes (white-rot fungi) are high-redox potential laccases. Bacterial and plant laccases have low-redox potential (Dwivedi et al., 2011).

Laccases catalyze the one-electron oxidation of *ortho*- and *para*-diphenols and aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical (Ardon et al., 1996; Higuchi, 1990). After receiving four electrons, the enzyme donates them to molecular oxygen to form water. There are three major steps in laccase catalysis:

1. Type-1 Cu reduction by the reducing substrate,
2. Internal electron transfer from Type-1 Cu to Type-2 and Type-3 Cu trinuclear cluster,
3. Reduction of oxygen to water at Type-2 and Type-3 Cu (Thurston, 1994; Yaropolov et al., 1994).

The overall reaction is: $4RH + O_2 \rightarrow 4R^{\bullet} + 2H_2O$ (Dwivedi et al., 2011).

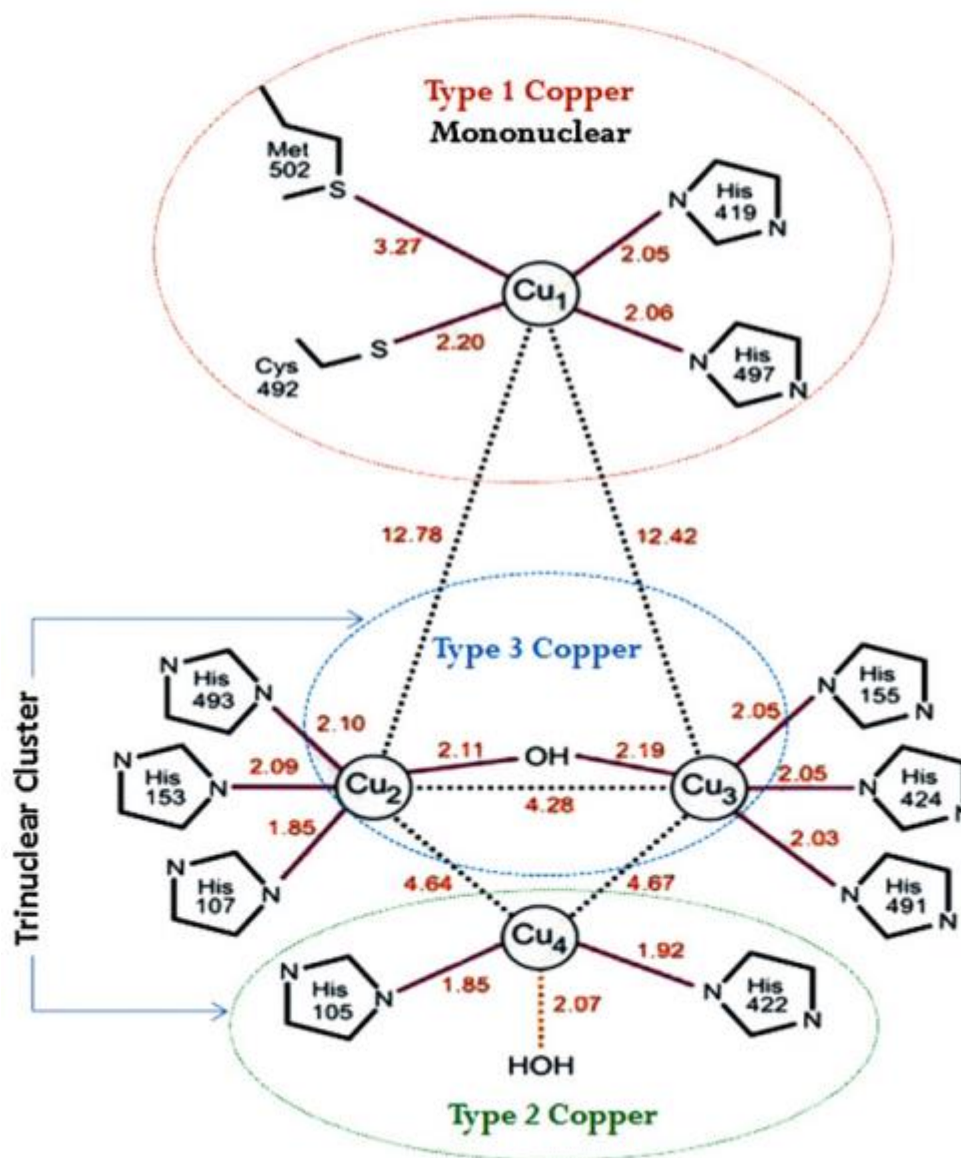

Figure 2.2. Schematic representation of copper coordination centers within a laccase monomer; reproduced with permission from Dwivedi et al. (2011)

The induction of laccase has been widely studied, especially by the addition of $CuSO_4$. Collins and Dobson (1997) studied the effects of adding copper to *Trametes versicolor* cultures. They reported that the addition of 400 μM $CuSO_4$ resulted in 18-fold greater laccase activity. Baldrian and Gabriel (2002) investigated the laccase activity effects of adding cadmium, copper, silver, mercury, lead, zinc, or hydrogen peroxide to cultures of *P. ostreatus*. Addition of silver (1 mM $AgNO_3$), mercury (1 mM $HgCl_2$), lead

(1 mM $Pb(NO_3)_2$), zinc (1 mM $ZnSO_4$), and hydrogen peroxide (0.5 M) decreased laccase activity. They found that addition of cadmium and copper increased laccase enzyme activity by 18.5-fold (at 2 mM $Cd(NO_3)_2$) and 3.7-fold (at 5 mM $CuSO_4$), respectively. They also reported that addition of copper resulted in slower mycelial growth. Baldrian et al. (2005) also reported an increase in laccase activity with addition of cadmium and copper.

Galhaup and Haltrich (2001) studied the addition of copper and other trace metals on laccase activity of *Trametes pubescens*. They reported a more than 23-fold increase in laccase activity when copper (2 mM) was added; there was a 5-fold increase in laccase activity when manganese (1 mM) was added. They also showed that partial inhibition of growth occurred at concentrations above 1 mM Cu; although, laccase activity also increased with increasing Cu concentrations above 1 mM. Klonowska et al. (2001) reported that laccase activity from *Marasmius quercophilus* was increased 10-fold with the addition of 5 mg/L $CuSO_4$.

Tinoco et al. (2011) described that in the presence of lignin, adding copper (0.5 mM $CuSO_4$) resulted in a laccase activity of 12 U ml^{-1} after 108 h. Although it has been previously reported that adding copper reduces *P. ostreatus* growth at higher concentrations (Galhaup & Haltrich, 2001; Tychanowicz et al., 2006), Tinoco et al. (2011) reported that adding 0.5 mM $CuSO_4$ during the middle of the exponential growth phase significantly increased laccase production.

Tychanowicz et al. (2006) reported that copper concentrations up to 10.0 mM did not inhibit growth of *Pleurotus pulmonarius* or significantly increase laccase activity. However, 10.0-25.0 mM copper concentrations did slow growth of the fungus while

increasing laccase activity 8-fold when compared to the control. No growth was observed when the copper concentration was more than 40 mM, even after 20 d. Mäkelä et al. (2013) reported a 20-fold increase in laccase activity for *Phlebia radiata* when adding copper (1.5 mM) to the base media. However, they also reported that $CuSO_4$ concentrations over 0.5 mM notably suppressed MnP activity.

2.4.2. Manganese peroxidase

Production of MnP is limited to certain basidiomycetous fungi. The catalytic cycle of MnP is shown in Figure 2.3, which is initiated by the binding of H_2O_2 to the ferric MnP enzyme. MnP uses Mn^{2+} as an electron donor, requiring two molecules for every cycle. MnP is sensitive to high concentrations of H_2O_2, which cause a reversible inactivation of the enzyme (Hofrichter, 2002). *P. ostreatus* has been found to produce multiple isozymes, including MnP1, MnP3, and MnPL in liquid cultures, and MnP2 and MnP3 in solid cultures (Kamitsuji et al., 2004).

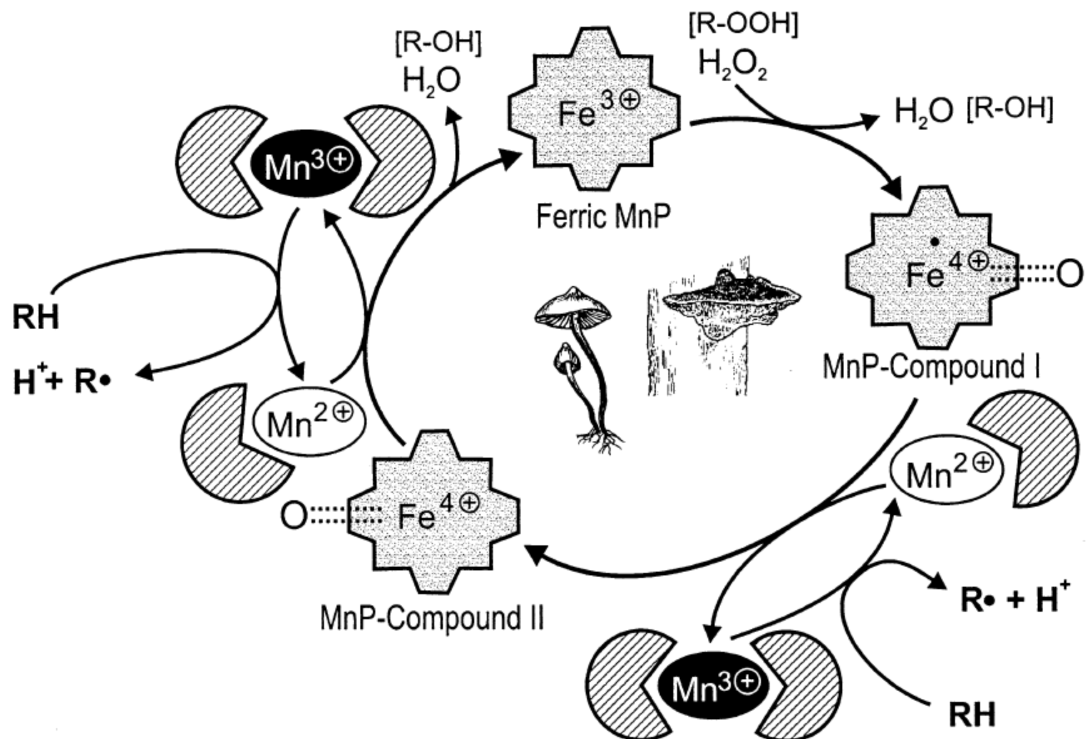

Figure 2.3. The catalytic cycle of manganese peroxide (MnP); reproduced with permission from Hofrichter (2002)

Multiple studies have been done for the addition of manganese (specifically $MnSO_4$) on the induction of MnP. Kerem and Hadar (1993) studied the mineralization rate of [^{14}C]-lignin by *P. ostreatus* supplemented with $MnSO_4$. They reported that *P. ostreatus* grown in Mn-deficient media reached 11% mineralization of the total [^{14}C]-lignin after 27 d. However, *P. ostreatus* grown in media with 73 μM Mn and 730 μM Mn reached 14% and 16% mineralization after 27 d, respectively. Cohen et al. (2001) studied the addition of Mn^{2+} to cotton stocks inoculated with *P. ostreatus*. They found that the addition of 730 μM Mn^{2+} significantly increased lignin degradation. Giardina et al. (2000) reported that addition of 100 μM $MnSO_4$ caused a 10-fold increase of MnP activity for *P. ostreatus* grown on wood sawdust.

Kamitsuji et al. (2005) reported that adding 270 μM $MnSO_4$ enabled extracellular MnP activity to be detected from *P. ostreatus*. They also studied the effects of excess H_2O_2

on isozyme activities. In the presence of excess H_2O_2, MnP2 isozyme rapidly lost activity. However, the presence of Mn^{2+} prevented this inactivation. Therefore, the addition of H_2O_2 must also be accompanied by the addition of $MnSO_4$. Kamitsuji et al. (2004) reported that addition of manganese (0.27 mM $MnSO_4$) resulted in a 10-fold increase in MnP activity from *P. ostreatus*.

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CHAPTER III

3. Evaluation of *Pleurotus ostreatus* degradation of switchgrass lignin in a controlled storage environment

3.1. Abstract

Fungal pretreatment of lignocellulosic biomass is an alternative to thermochemical pretreatment for delignification in the production of biofuels. Small switchgrass bales were inoculated with *Pleurotus ostreatus* to determine if the fungal treatment would significantly degrade lignin and hemicellulose while in storage. After inoculation the bale moisture content was maintained at 50% using an automated moisture restoration system. The test design included three fungal loading rates, three storage periods, and three replications for a total of twenty-seven square bales used in the study. The fungal loading rates were 0%, 2%, or 3% (w/w on wet basis). The storage periods were 25, 53, and 81 d. The samples were analyzed for biomass composition and underwent liquid hot water (LHW) pretreatment. Based on the biomass composition, the fungal loading did not have a significant effect on glucan content. However, lignin and xylan recoveries were significantly less in the 2% and 3% fungal loading bales (71.6% lignin and 64.4% xylan) than in the 0% fungal loading bales (77.5% lignin and 70.1% xylan). Increased fungal pretreatment time significantly decreased glucan, xylan, and lignin recoveries. The effects of fungal pretreatment based on fungal loading were not evident after LHW pretreatment,

showing only differences based on sampling time. Xylan recovery was greater in the less severe LHW pretreatment, indicating that fungal pretreatment did not reduce the necessary LHW severity for increasing cellulose hydrolysis. Delignification and hemicellulose degradation occurred, at greater rates than in the control, during fungal pretreatment.

3.2. Introduction

Conversion of biomass to biofuels has become incredibly important since the implementation of the Renewable Fuel Standard Program in 2005 and its expansion by the Energy Independence and Security Act of 2007. This act set long-term goals for the production of renewable fuels, especially cellulosic and advanced biofuels (US Environmental Protection Agency, 2015). Many biomass conversion facilities utilize thermochemical processes, mostly gasification, pyrolysis, or direct liquefaction. Some facilities use biochemical processes for biomass conversion, mostly anaerobic digestion and fermentation. Fermentation is ideal for biomass with high sugar, starch, or cellulose content, with ethanol as the product (Demirbaş, 2001).

When looking at the overall sustainability of bioethanol production, the biomass-to-ethanol yield (volume EtOH per mass of feedstock) at the biorefinery and the delivered feedstock cost are two key parameters. The delivered feedstock cost is affected by moisture content because wet biomass increases the cost of transportation and energy requirements (for drying), while decreasing biomass stability and energy content (Inman et al., 2010). Larger year-round biomass conversion facilities will need to store biomass to be able to continue production between growing seasons (Sanderson et al., 1997). In order to achieve an economically feasible biomass-to-ethanol yield, lignocellulosic biomass must be pretreated to degrade the lignin fraction and increase the yield of glucose through hydrolysis (Hamelinck et al., 2005).

The use of a white-rot fungus to selectively degrade the lignin fraction of biomass is an alternative to thermochemical pretreatment. Fungal pretreatment of biomass during storage at the biorefinery would reduce energy consumption and waste compared to other

pretreatment techniques (Sanchez, 2009; Wan & Li, 2012). The white-rot fungus *Pleurotus ostreatus* has been found to selectively degrade lignin in lignocellulosic crop residues, including wheat straw (Adamovic et al., 1998; Agosin & Odier, 1985), cotton stalks (Hadar et al., 1993), and rice straw (Taniguchi et al., 2005). However, *P. ostreatus* is considered a moderately selective lignin degrader, because cellulose loss has been reported with longer treatment times (Wan & Li, 2012).

The purpose of this study is to determine if pretreating switchgrass with *P. ostreatus* prior to bale storage will result in significant lignin and hemicellulose degradation over time. The effect of the fungal pretreatment was determined based on the composition of the biomass after storage and liquid hot water (LHW) pretreatment. Using fungal pretreatment in place of LHW or in conjunction with less severe conditions during LHW for reducing lignin and hemicellulose contents would be ideal. Hemicellulose content can be estimated by the xylan recovery; increased xylan recovery generally leads to lower hydrolysis of cellulose (Chandra et al., 2007; Öhgren et al., 2007).

3.3. Materials and Methods

3.3.1. Automatic watering system

An automatic watering system was developed to maintain a minimum bale moisture content during bale storage of 50%, in order to promote fungal growth. This system consisted of load cells to continuously record bale weights, thermocouples to monitor temperature inside the bales, and solenoid valves connected to a pump to add water when necessary.

3.3.1.1. Bale weight and moisture content

During storage, individual bales were hung on individual load cells to continuously weigh the bales over time. Tacuna Systems (Denver, CO) AmCells STL-200 S-Type Alloy Steel load cells with a maximum load of 200 lb. (90.72 kg) were used. The load cells output 3.0 mV/V, which was amplified using differential bridge amplifiers (LTC1250, Linear Technology Corp., Milpitas, CA). The input voltage was 10 V with a gain resistance of 36 kΩ, which is shown in Figure 3.1.

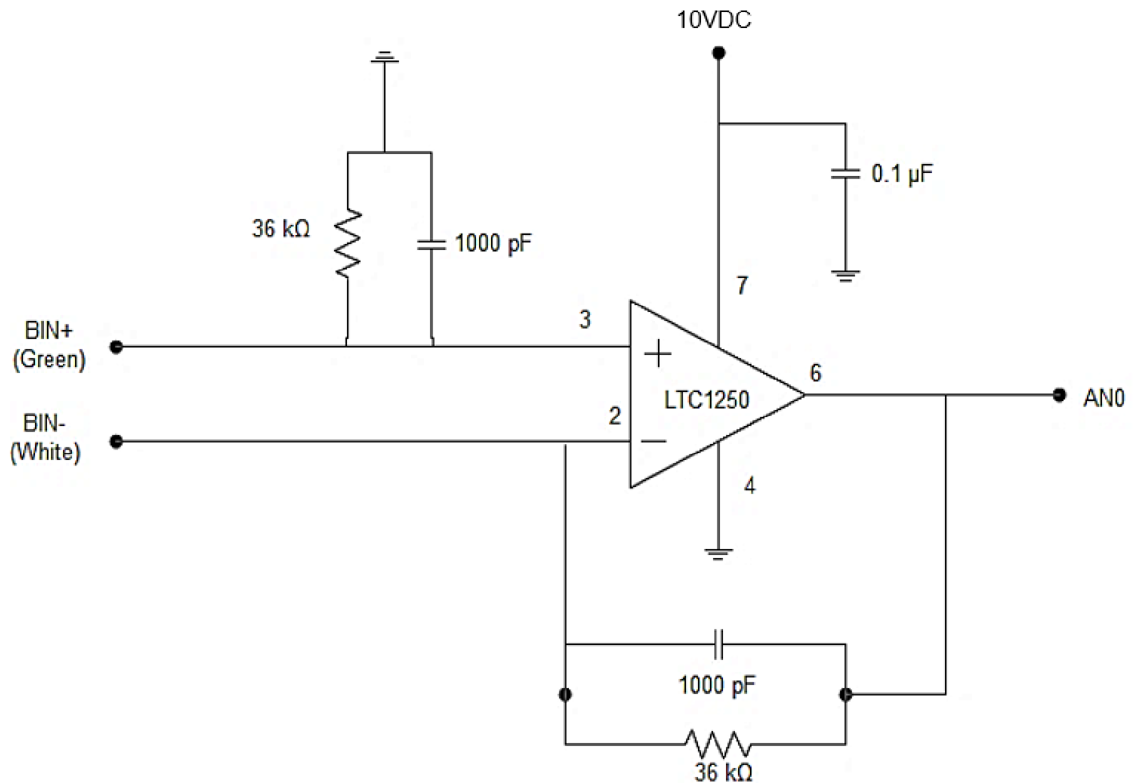

Figure 3.1. Load cell amplifier circuit diagram

Each load cell was calibrated using five combinations of weights, ranging from 24.7 to 202 lb. (11.2 to 91.63 kg). The following linear equation was used to calculate weight:

$$x = \frac{(V_i - b_i)}{m_i} \tag{3.1}$$

where *V* is the voltage reading from the load cell, *i* is the load cell number and *x* is the weight (lb.). The linear coefficients, *m* and *b*, for each load cell are shown in Table 3.1.

Table 3.1. Load cell calibration coefficients

i	*m*	*b*	*i*	*m*	*b*	*i*	*m*	*b*
1	0.03159	0.02248	10	0.03115	0.04073	19	0.03129	0.02167
2	0.03159	0.01758	11	0.03152	0.02081	20	0.03120	0.03775
3	0.03158	0.02156	12	0.03156	0.01281	21	0.03144	0.02725
4	0.03114	0.02970	13	0.03135	0.01833	22	0.03125	0.02354
5	0.03132	0.02378	14	0.03078	0.02218	23	0.03136	0.04845
6	0.03115	0.01393	15	0.03094	0.00707	24	0.03130	0.04362
7	0.03141	0.08713	16	0.03127	0.01834	25	0.03129	0.06206
8	0.03129	0.01925	17	0.03119	0.03086	26	0.03141	0.06303
9	0.03118	0.01504	18	0.03104	0.02159	27	0.03113	0.02986

The following equations were used to calculate the bale weight and moisture content from the load cell voltage:

$$\text{bale} = \frac{\text{V}-\text{b}}{\text{m}} - \text{comp} \tag{3.2}$$

$$\text{MC} = \frac{\text{bale}-\text{dry}+(\text{dry}*\text{M}_0)}{\text{bale}} \tag{3.3}$$

For equation 3.2, *V* is the load cell voltage reading; *m* and *b* are the load cell calibration values from Table 3.1; and *comp* is the weight of the straps, angle iron frames, and hosing (21.036 lb., 9.542 kg). For equation 3.3, *dry* is the weight of the bale before soaking, and M_0 is the initial moisture content of the bale before soaking (set as 9.25%). The initial moisture content was determined by weighing four full bales, placing them in a dryer at 55°C for 72 h, and weighing the bales again.

3.3.1.2. Relays, solenoid valves, and pump

To automatically add water when the bale moisture content drops below the moisture content set point or the internal bale temperature exceeds the temperature set point, relays were used to control solenoid valves and a pump connected to a water storage tank. Omron PCB G5L-5V relays (OMRON Global, Kyoto, Japan) were connected to

Spartan Scientific (Boardman, OH) Series 3923 Stackable solenoid valves (circuit diagram in Figure 3.2). The solenoid valves required a 24 V power supply and were stacked together with one inlet. A CountyLine (Deluxe Spot Sprayer, Tractor Supply Company, Brentwood, TN) 113.6 L, 7.95 L/min (30 gal, 2.1 gpm) sprayer pump was connected to a relay as shown in Figure 3.2 where pump would replace the solenoid value, with a 12 V, 8.5 A power supply.

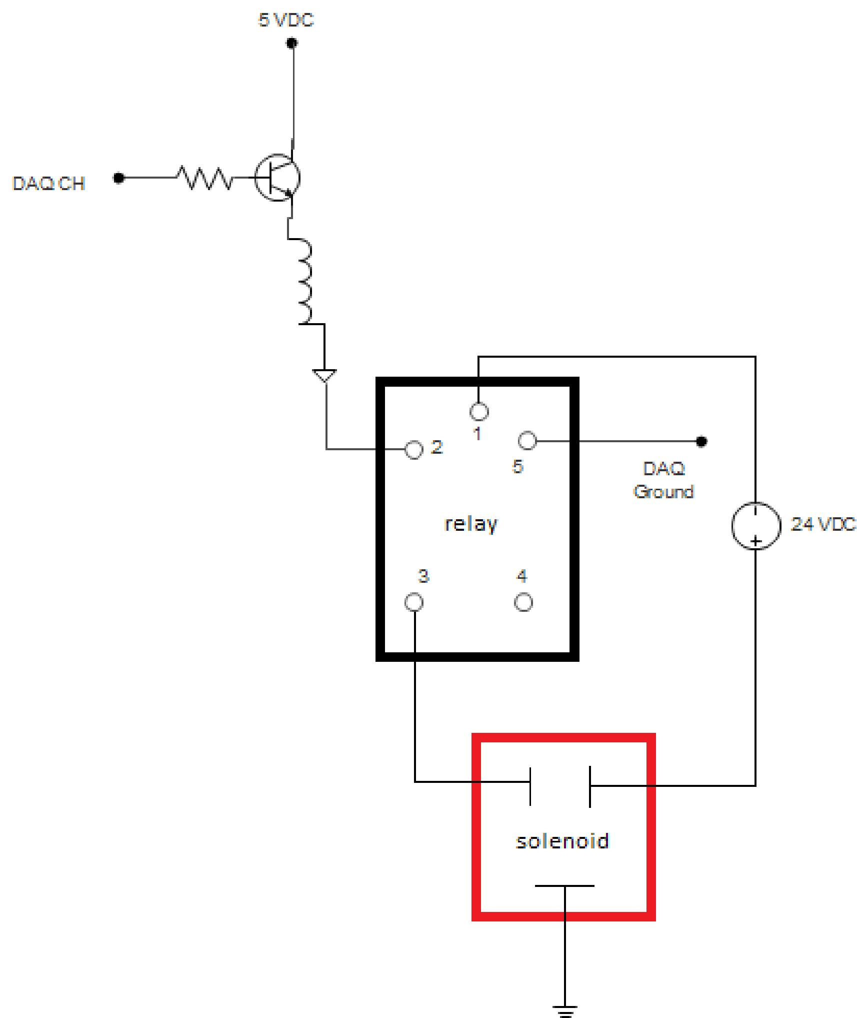

Figure 3.2. Relay to solenoid circuit diagram

3.3.1.3. Temperature measurements

A HTM2500LF series (TE Connectivity Measurement Specialties) temperature and relative humidity (RH) module was used to measure ambient temperature and relative humidity. This thermistor type sensor estimated the temperature (in degrees Kelvin) using a change of resistance value. Equations 3.4, 3.5, and 3.6 were used to calculate the temperature and relative humidity; V is the voltage reading. For equation 3.5, the temperature calibration coefficients were provided by TE Connectivity Measurement Specialties (a=8.54942E-04, b=2.57305E-04, c=1.65368E-07).

Temperature:

$$R = \frac{10000V}{5000-V} \tag{3.4}$$

$$T\ (°C) = \frac{1}{a + b\ ln\,R + c(ln\,R)^3} - 273 \tag{3.5}$$

Relative Humidity:

$$RH\% = 0.0375(V \times 1000) - 37.7 \tag{3.6}$$

T-type thermocouples were used to measure temperature inside the bales. Each bale had four thermocouple wires, for a total of 108 thermocouples. Because of the relatively large number of thermocouples used in the study, differential wiring to provide cold junction compensation was not used. Instead, Monolithic Thermocouple Amplifiers with Cold Junction Compensation (AD594, Analog Devices Inc., Norwood, MA) were used to allow the thermocouples to be measured relative to the data logger ground. The thermocouple amplifier output was 10 mV/°C. The thermocouple amplifier circuit diagram was provided in Figure 3.3. The thermocouple amplifiers were connected to a 24 V power supply. Temperature readings over time are shown Appendix 6.5.

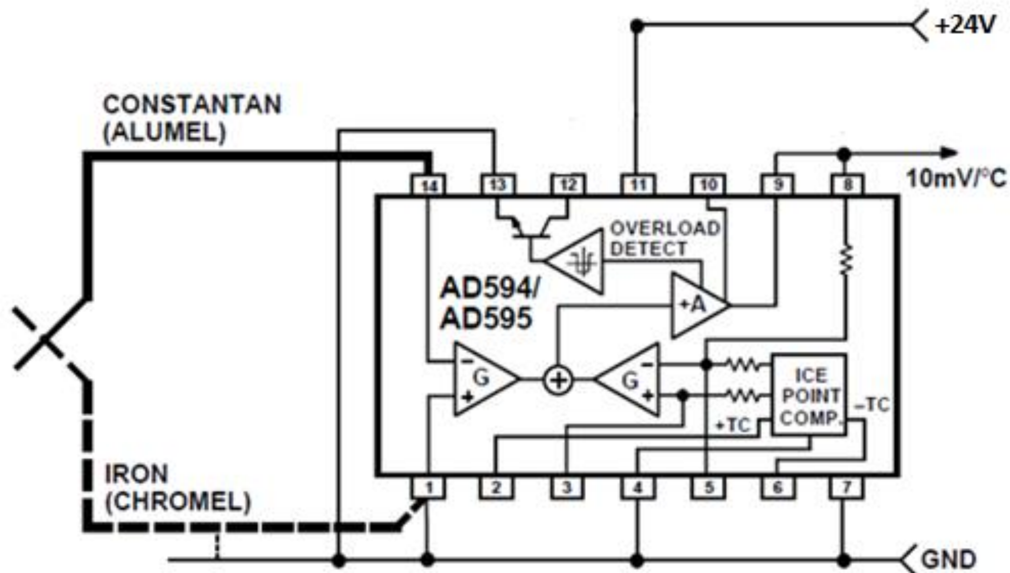

Figure 3.3. Thermocouple amplifier circuit (AD594, Analog Devices Inc., Norwood, MA)

3.3.1.4. Data acquisition and programming

NI USB-6225 data loggers (National Instruments Corporation, Austin, TX) were selected for the project because each logger has 80 analog inputs and 24 digital outputs; two data loggers were used. The thermocouples and load cells for fifteen bales were connected to one data logger and the thermocouples and load cells for the other twelve bales were connected to the second data logger. The ambient temperature and RH sensor was connected to the data logger with fifteen bales. The solenoid valves and pump relays were connected to the digital outputs. The pump relay had a digital output from both data loggers. Each data logger was connected to a different computer. LabVIEW (Austin, TX) was loaded on each computer and was used to interface and control the data loggers.

The LabVIEW virtual instrument (VI) was developed to control the system, which needed to read the load cell and thermocouple voltages, calculate the moisture content and temperature, and turn on relays for a solenoid valve and pump if the moisture content or temperature was outside of the acceptable range. Based on the experiment configuration,

four separate programs were developed to account for the configuration changes that occurred when bales were removed from the system after each defined storage period. During the first 25 d, one computer and data logging system was dedicated to bales 1-15, and the other computer and data logging system was dedicated to bales 16-27. After the first storage period when nine bales were removed from storage, the second computer and data logging system was modified to handle only the monitoring and control algorithms related to bales 16-18. After 53 d another nine bales were removed from the storage study and the first computer and data logging system was modified to handle only the monitoring and control algorithms related to bales 1-9, and the second computer and data logging system was turned off.

Each of the load cells, thermocouples, and the ambient temperature/relative humidity sensor were connected to the data logger analog input channels and the relays for the solenoid valves and pump were connected to the data logger digital output channels; the specific wiring configurations were provided in Appendix 6.1 (Figure 6.1 and Figure 6.2). The DAQ Assistant within LabVIEW was setup on a primary software loop to read each of these voltages continuously. Although the voltages are continuously read, single data points for each analog input are only collected on 60 s intervals using a time delay block. Once a voltage measurement was collected from the DAQ Assistant, the output was routed to a signal splitter. The splitter outputs correspond to a defined DAQ Assistant signal order which are assigned to corresponding variables.

Temperature was calculated for each of the thermocouple voltage readings by multiplying the thermocouple voltage by 100. The resulting temperature was in units of degrees Celsius. To reduce the data noise, running averages of the last 10 readings were

calculated. Temperature readings for every thermocouple were digitally recorded every 60 s. If an individual average temperature reading exceeded 55˚C then a digital flag was set to indicate that the bale temperature was too hot and that water would need to be added to the specific corresponding bale to keep the bale temperature below the threshold.

Moisture content was calculated from each of the load cell voltages using equations 3.2 and 3.3. The moisture content was calculated every 60 s. If the moisture content was below 50%, a digital flag was set to indicate that the bale moisture content was low and additional water was needed.

Ambient temperature and relative humidity were calculated for each voltage reading from the HTM2500LF series Temperature and Relative Humidity (RH) module using equations 3.4 and 3.5 and equation 3.6, respectively.

If a digital bale temperature (above 55°C) or moisture (below 50%) flag was set, the corresponding bale required additional water. Once it was determined that a specific bale needed water, the digital channel wired to the relay/pump circuit was set to 5V to turn the pump on. The control algorithms for the relay/bale water valve circuits were constructed so that only one circuit was active per data logger at any given time. This control algorithm was designed to water bales initially in an ascending bale order (if multiple bales are identified as needing water at the same time and no other bales are in the watering que) and in a first-in first-out queuing sequence. For example, if bales 2 and 8 were identified as needing water at the same time and no other bales were in the watering que, the digital channel wired to the relay/valve circuit for bale 2 would be set to 5V to turn the bale 2 valve on. This bale 2 water valve would remain open until the moisture content was calculated as being above 50%. Then the digital channel wired to the relay/valve circuit

for bale 2 would be set to 0V to turn the bale 2 valve off. Then a similar process would be used to water bale 8. If bale 1 was identified as needing water while bale 2 was watered, bale 8 would be watered first, then bale 1.

Remote viewing and sending system warning text messages were an essential component of the monitoring and digital control system. TeamViewer (Göppingen, Germany) was installed on both computers to allow them to be remotely viewed and controlled. TeamViewer was coupled with a custom Excel VBA macro and LabVIEW to remotely monitor the system and for the system to send the investigator text messages when the bale temperatures were above the threshold of 55°C. Additional details corresponding to the LabVIEW and Excel VBA macros were provided in Appendix 6.2 and 6.3.

3.3.2. Bale set-up

Twenty-seven small square bales (0.38 x 0.46 x 0.91 m) of Kanlow switchgrass (grown in Stillwater, OK at the Oklahoma State University EFAW station and harvested in November 2014) were used. The test configuration included three fungal loading rates, three storage periods, and three replications. The loading rates included: nine controls with no fungus (2% millet only by weight on wet basis), nine lower fungal loading (2% by weight on wet basis), and nine higher fungal loading (3% by weight on wet basis). The fungal spawn was grown on millet, so the controls also had millet added to determine how the added nutrients affected the growth of the native microbial population within the bale. The bales were stored inside an enclosed building with no central air, so the indoor air temperature was relatively close to the outside ambient temperature. The study was conducted in Stillwater, OK; started on June 1, 2015, and ended on August 21, 2015.

3.3.2.1. Inoculation procedure

The pure culture was isolated from a strain of *P. ostreatus* called HK-35 (Sylvan Inc., Dayton, NV) using MCBD agar plates (also known as Basidiomycete Isolation Medium). The Basidiomycete Isolation Medium contained 10 g/L malt extract, 1 g/L yeast extract, 1 g/L tryptone, 16 g/L agar, 0.01 g/L Benlate 50 SP (DuPont, Wilmington, DE), 0.005 g/L Botran 75 WP (DuPont New Zealand Ltd., Manukau City, New Zealand), 0.1 g/L chloramphenicol, and 0.2 g/L streptomycin. The Benlate 50 SP (50% benomyl) inhibits ascomycete fungi, Botran 75 WP (75% dichloran) inhibits zygomycete fungi, and chloramphenicol and streptomycin inhibit bacteria.

The *P. ostreatus* grain spawn was grown on proso millet. The grain spawn was started as spawn jars, which were inoculated about three weeks before the bales were inoculated. The spawn jars (only millet and water) were autoclaved twice (20 min, 121°C, liquids cycle) with an overnight cooling interval. After cooling, the autoclaved grain was inoculated with 8-10 agar plugs from a pure agar *P. ostreatus* plate culture. The jars were shaken randomly once per day for one week, until the entire jar was colonized (fungal spawn was attached to all of the grain).

Bags containing 970 g of proso millet, 30 g sawdust pellets (to activate the ligninolytic enzymes), 3 g wollastonite (to reduce clumping), and 1 L water with fungicide. The fungicide solution was made by mixing 50 mg Benlate 50 SP (DuPont, Wilmington, DE) in 5 L deionized water. The bags had vents to allow only air to pass. The bags were autoclaved twice (20 min, 121°C, liquids cycle) with an overnight cooling interval, then a spawn jar was poured into each bag. The bags were heat-sealed and shaken randomly, then laid flat so that the vent was uncovered. The grain spawn bags were shaken every other day

for five days, then every day for the next week; the grain spawn was used to inoculate the bales on day 12 of fungal growth. The bales that were inoculated with grain only had the same mixture of millet, sawdust, wollastonite, and fungicide solution.

Twenty-four h before inoculation, the bales were weighed and tagged with a numbering label and then placed in five 8 ft. x 18 in. (2.44 x 0.457 m) swimming pools with water to soak, as shown in Figure 3.4. The bales were placed in the pools and water was added until the pool was full. The bales were pushed down as necessary and flipped over after about 1 h. The bales were flipped again after 12 h to insure that the initial bale moisture content was greater than 50%.

Figure 3.4. Picture of bales soaking prior to inoculation

Three storage racks were constructed to hold the bales. Each rack (1.85 x 1.28 x 2.44 m) was designed to suspend nine bales from load cells, conserving space and allowing for continuous data acquisition; an image of a completed rack is shown in Figure 3.5 (drawings in Figure 6.16 and Figure 6.17). Lightweight corrugated 0.658 m x 3.66 m steel roofing panels were placed under each row of bales to divert extra water dripping from the bales to one end of the test stand, where the water was allowed to pool and evaporate.

Figure 3.5. Test stand storage rack for hanging the bales

The bales were removed from the water and set on saw horses to allow excess water to drain. The bales were then moved to the right side of a dual-sided bale splitting stand (Figure 3.6, Figure 6.15), which was constructed to elevate the bale and allow sections to be moved as needed. Two ratchet straps were laid out on the left side of the stand and the wires holding the bale together were removed. Approximately one-fifth of the bale was flipped over (rotated 180° vertically) onto the ratchet straps. The inoculant was weighed into tared four metal pans; each pan contained 25% of the total inoculant weight (Table 3.2). One pan of inoculant (grain or grain/fungus) was spread over the bale flake, within approximately one-inch of the edges. The thermocouple labeled “A” and a drip hose ring was placed so that they were in the center of the bale. The drip hose rings were constructed using drip hose with six-inch emitter spacing with another hose attached; the hose was cut so that each ring included four emitters, with a diameter of approximately 7.5 in. (19 cm).

The next section of the bale was flipped over, with one person holding the hose and wire in place. This was repeated for the next three bale sections.

After the bale was completely inoculated, the support frames (Appendix 6.6, Figure 6.18 and Figure 6.19) were held on each side while the ratchet straps were connected. The straps were tightened by alternating sides, while insuring that the hosing and thermocouple wires were not smashed. The bale was then moved to one of the storage racks (Figure 3.5) and hooked to the load cell. More drip hose was laid on top of the bale and connected in a serpentine pattern to provide water to the entire bale. This was repeated for all 27 bales and the final set-up is shown in Figure 3.7.

Figure 3.6. Bale splitting stand for fungal inoculation; showing a bale split and partially inoculated

Table 3.2. Bale and inoculate weight for each test bale

Bale	Loading		Bale Weight (kg)	Fungus (Grain) Added (kg)
1	2%	grain	18.12	0.368
2	2%	grain	21.14	0.426
3	2%	grain	18.23	0.365
4	2%	fungus/grain	19.07	0.383
5	2%	fungus/grain	20.41	0.410
6	2%	fungus/grain	19.28	0.386
7	3%	fungus/grain	20.05	0.607
8	3%	fungus/grain	16.69	0.503
9	3%	fungus/grain	18.12	0.548
10	2%	grain	18.96	0.386
11	2%	grain	18.48	0.372
12	2%	grain	18.82	0.380
13	2%	fungus/grain	18.51	0.374
14	2%	fungus/grain	22.02	0.444
15	2%	fungus/grain	19.19	0.388
16	3%	fungus/grain	19.23	0.579
17	3%	fungus/grain	18.69	0.567
18	3%	fungus/grain	20.91	0.632
19	2%	grain	23.90	0.484
20	2%	grain	18.60	0.374
21	2%	grain	19.69	0.396
22	2%	fungus/grain	23.86	0.480
23	2%	fungus/grain	19.66	0.396
24	2%	fungus/grain	19.10	0.385
25	3%	fungus/grain	18.12	0.547
26	3%	fungus/grain	22.02	0.662
27	3%	fungus/grain	19.16	0.579

Figure 3.7. Picture of the automated watering system with all 27 bales in storage

3.3.2.2. Sampling

An untreated bale control was collected as the combined core samples from the center of five bales from the same harvesting period at the beginning of the storage period. The storage study bales were sampled at 25, 53, and 81 d after inoculation; three bales for each fungal loading condition were removed after each storage period (nine total bales removed per storage period). Each bale was removed from the storage rack and taken to a processing table where the ratchet straps and metal bale supports were removed. The bales were systematically opened at the inoculation locations. At the first inoculation location in a given bale, the switchgrass flakes were spread apart so the drip hose rings could be removed and pictures could be taken to visually document if fungal growth was present. This process was repeated for all four inoculation locations within the bale. Next, the bale flakes were realigned and a single ratchet strap placed in the middle of the bale was used to recompress the bale. A Makita DS4000 (Makita U.S.A., La Mirada, CA) 13 mm drill

(rated at 500 rpm) equipped with a 2” (5.08 cm) diameter by 3-ft. (0.914 m) long core tube was used to core sample the bales (Figure 6.20). Four core samples were collected from each bale. When looking at the 12” x 18” (0.3048 x 0.4572 m) bale face the core sample locations were about 4” (10.2 cm) horizontally and 3” (7.6 cm) vertically from each corner of the bale. The full length of the bale was sampled. A core tube plunger was used to extract the sample from the core tube. Each individual sample was collected in a pre-weighed, pre-labeled sample bag. The top of each sample bag was folded and stapled. Then each sample was weighed on a model ML802E/2003 Mettler Toldeo balance (Columbia, MD) to determine the wet weight that was used in calculating the sample moisture content after storage. This process was repeated four times for each of the nine bales removed from storage after each storage period.

After each storage period, all the collected core samples were placed in a large drying oven at 55°C for at least 72 h. After 72 h, the core samples were removed from the oven and weighed to obtain the dry sample weight used in calculating the core sample moisture content after storage. The post storage core sample moisture contents were calculated using the following equation:

$$\text{DM\% (completely dry)} = \frac{\frac{\text{dry}-\text{bag}}{\text{wet}-\text{bag}}}{1+\left(\frac{1-\text{DM}_{\text{final}}}{\text{DM}_{\text{final}}}\right)} \times 100\% \qquad (3.7)$$

where *wet* is the starting weight of the sample, *dry* is the weight after 72 h in the 55°C oven, *bag* is the weight of the sampling bag, and DM_{final} is the dry matter content of the combined samples after grinding and drying completely in a 105°C oven. The individual bale post-storage moisture content was determined by averaging the four individual bale moisture contents. The standard deviation of the four core samples per bale were also calculated. The four dried core samples from each bale were ground using a Thomas-Wiley

mill (Model 4, Arthur H. Thomas Co., Philadelphia, PA) through a 3 mm screen and combined for biomass composition analysis and hydrothermolysis pretreatment. After the samples were ground, they were stored in GMD Reclosable Poly Bags (size G8x10, 2 mm) at 4°C until needed.

3.3.3. Laboratory analysis

3.3.3.1. Biomass composition analysis

For each bale sample, the moisture content after grinding (to determine DM_{final}) and the ash content of a 2 g subsample were determined based on the "convection oven method" outlined by Sluiter et al. (2008) and ash procedure outlined by Sluiter et al. (2005a). Two-4 g subsamples for each bale in the first and second sampling periods were extracted using a Dionex ASE 300 Accelerated Solvent Extractor, according to Sluiter et al. (2005b). Two-4 g subsamples for each bale in the third sampling period and the untreated control were extracted using Soxhlet extractors, also according to Sluiter et al. (2005b). Each subsample was extracted first with water, then ethanol.

The water and ethanol extractives contents were determined by the mass of the extractives residue after forced-air drying of the extracted liquids in the fume hood. The extracted biomass was allowed to air dry, and the carbohydrate and lignin contents were determined using a two-stage acid hydrolysis according to Sluiter et al. (2012). The carbohydrate concentrations were determined using High Performance Liquid Chromatography (HPLC) (1100 Series, Agilent, Santa Clara, CA, USA) with an Aminex HPX-87H column (BioRad, Hercules, CA, USA) and a refractive index detector (RID). The eluent was deionized water flowing at a rate of 0.6 ml/min and a column temperature of 85˚C. The total run time for each sample was 30 min. The HPLC with ChemStation

software (Agilent Technologies) was calibrated at five levels using known concentrations of cellobiose, glucose, xylose, galactose, mannose, and arabinose before being used to quantify the concentration of these compounds.

Recoveries in composition were calculated based on equation 3.8:

$$\text{recovery} = \frac{\text{DM}_{\text{final}} * f_{\text{sample}}}{\text{DM}_{\text{initial}} * f_{\text{control}}} \tag{3.8}$$

where DM_{final} is the final mass (dry basis), $DM_{initial}$ is the starting mass (dry basis) found in Table 3.5, f_{sample} is the glucan, xylan, or lignin fraction of the sample, and $f_{control}$ is the glucan, xylan, or lignin fraction of the untreated (raw) biomass control.

3.3.3.2. Hydrothermolysis pretreatments

Liquid hot water (LHW) pretreatment of the biomass was completed with 80 g (dry basis) of the bale sample (subsampled from the combined samples) and deionized water to a total mass of 500 g in a 1-L bench top stirred reactor and pressure vessel (Parr Series 4520, Parr Instrument Company, Moline, IL, USA). The Parr reactor was equipped with a propeller agitator, a 1 kW electrical resistance heater and a temperature controller.

Two reaction conditions were evaluated: temperature of 200˚C with a 10 min reaction time (200/10) and temperature of 180˚C with a 30 min reaction time (180/30). The severity factor R_0 of the hydrothermolysis was calculated based on equation 3.9.

$$\log(\text{R}_0) = \log(\text{t} \cdot \exp(\frac{\text{T}-100}{14.75})) \tag{3.9}$$

where *t* is reaction time (min) and *T* is temperature (°C) (Dogaris et al., 2009; Overend & Chornet, 1989). The logarithmic severities were calculated as 3.94 for condition 200/10 and 3.83 for condition 180/30. Reaction time was considered to be time that the reactor was held at the desired temperature. The propeller speed was set to 300 rpm for both reaction conditions. The temperature, reactor pressure, propeller speed, and heater setting

was recorded every 2 min over the entire duration of the reaction. At the end of the reaction period, the vessel was immediately disconnected from the heating unit and placed into an ice bath. The pressure was slowly released once the temperature dropped below 100˚C, and the vessel was removed from the ice bath after the temperature was below 55˚C.

The pretreated biomass slurry was removed from the reaction vessel and filtered by vacuum filtration using a Buchner funnel lined with Whatman filter paper #4 (Whatman PLC, Brentford, UK). After filtration, subsamples of the solids (~65 g, placed in a tared Ziploc bag) and the prehydrolyzate liquid (10 mL) were taken. The remaining solids were washed, to remove inhibitors of enzymatic hydrolysis and fermentation such as HMF and furfural (Wan and Li, 2011), with five-times the mass of solids of deionized water and filtered again. The unwashed and washed biomass, prehydrolyzate, and wash water samples were stored at 4˚C before being sent to the University of Arkansas for analysis of enzymatic digestibility.

A 10 g subsample of the unwashed and washed biomass samples were allowed to air dry, and the carbohydrate and lignin contents were determined using a two-stage acid hydrolysis according to Sluiter et al. (2012). The same HPLC procedure was used to determine the carbohydrate concentrations as was used for the biomass composition.

Pretreatment recoveries for glucan, xylan and lignin were calculated using equation 3.10:

$$\text{recovery} = \frac{\text{DM}_{\text{recovered}} * f_{\text{pretreat}}}{\text{DM}_{\text{in}} * f_{\text{extractives}}} \qquad (3.10)$$

where $DM_{recovered}$ is the total recovered biomass after washing, DM_{in} is the biomass going into the reactor (80 g), $f_{extractives}$ is the composition fraction of the biomass with extractives, and $f_{pretreat}$ is the composition fraction after pretreatment of glucan, xylan, or lignin.

3.3.4. Statistical analysis

Analysis of variance (ANOVA) was calculated ($p<0.05$) using the generalized linear model (GLM) in SAS 9.4 (SAS, Cary, NC) and multiple comparison tests on the treatment means were performed using Tukey's Honest Significant Difference test at a 95% confidence interval. For the biomass composition recoveries, the glucan, xylan, and lignin fraction recoveries and dry matter loss were the dependent variables and the sampling time and fungal loading rate were the independent variables. For the pretreatment recoveries after washing, the glucan, xylan, and lignin fraction recoveries were the dependent variables and the sampling time, fungal loading rate, and pretreatment severity were the independent variables.

3.4. Results and Discussion

3.4.1. Visual observations of fungal growth

After 25 d of storage, the control bales showed evidence of deterioration, as illustrated by the darkening of the biomass shown in Figure 3.8. Fungal growth was evident in the 2% and 3% fungal bales, with white fungal mycelia visible (Figure 3.9 and Figure 3.10). After 53 d of storage, the control bales showed further evidence of deterioration (Figure 3.11). The fungal growth within the 2% bales had a gray tint to the mycelia and switchgrass (Figure 3.12). Fungal growth was still occurring in the 3% fungal bales (Figure 3.13). At the end of 81 d of storage, the bale storage conditions had become detrimental for fungal growth. The control bales were black (Figure 3.14), indicating that the biomass

had deteriorated. The 2% fungal (Figure 3.15) and 3% fungal (Figure 3.16) bales no longer had any visible fungal growth.

Figure 3.8. Bale 19 (0% fungal loading, 25 d), deterioration of switchgrass is evident

Figure 3.9. Bale 24 (2% fungal loading, 25 d), fungal growth visible (white)

Figure 3.10. Bale 25 (3% fungal loading, 25 d), fungal growth visible (white)

Figure 3.11. Bale 12 (0% fungal loading, 53 d), deterioration of switchgrass is apparent

Figure 3.12. Bale 14 (2% fungal loading, 53 d), less fungal growth visible

Figure 3.13. Bale 17 (3% fungal loading, 53 d), visible fungal growth (white)

Figure 3.14. Bale 2 (0% fungal loading, 81 d), deteriorated switchgrass

Figure 3.15. Bale 6 (2% fungal loading, 81 d), no visible fungal growth, shows deterioration

Figure 3.16. Bale 9 (3% fungal loading, 81 d), no visible fungal growth, shows deterioration

3.4.2. Biomass composition

The moisture content of bales after inoculation was 55-60% (Table 3.3). Approximately 10 d passed before all the bales needed to be constantly watered to maintain the moisture content of 50%. Table 3.4 shows the moisture contents of the bales after sampling, which were used to calculate the dry matter losses in Table 3.5. The initial dry matter content was based on the untreated control moisture content in Table 3.4. The biomass composition of the fungal pretreated switchgrass is shown in Figure 3.17.

Table 3.3. Bale moisture content calculated from load cell voltage after inoculation

Bale	M.C.	Bale	M.C.	Bale	M.C.
1	59%	10	57%	19	55%
2	58%	11	57%	20	57%
3	59%	12	57%	21	57%
4	57%	13	58%	22	57%
5	55%	14	56%	23	56%
6	57%	15	58%	24	57%
7	58%	16	56%	25	59%
8	58%	17	56%	26	57%
9	59%	18	58%	27	58%

Table 3.4. Average moisture content of bales after sampling (completely dry)

Sampling	Fungal Loading	Moisture Content
untreated control		13.5% ± 0%
25 d	0%	63.8% ± 2.5%
	2%	65.3% ± 0.3%
	3%	64.1% ± 1.3%
53 d	0%	65.9% ± 1.5%
	2%	68.9% ± 1.8%
	3%	65.9% ± 0.6%
81 d	0%	70.3% ± 2.3%
	2%	73.4% ± 0%
	3%	72.4% ± 1.7%

Table 3.5. Average initial and final dry matter weights and the percentage of dry matter loss for all bales

Sampling	Fungal Loading	Initial Dry Matter (kg)	Final Dry Matter (kg)	Dry Matter Loss (%)
25 d	0%	18.2	13.5	26%
	2%	18.3	13.2	28%
	3%	17.4	12.8	26%
53 d	0%	16.5	11.2	32%
	2%	17.5	10.8	38%
	3%	17.3	10.7	38%
81 d	0%	16.8	10.0	41%
	2%	17.2	9.1	47%
	3%	16.1	8.9	45%

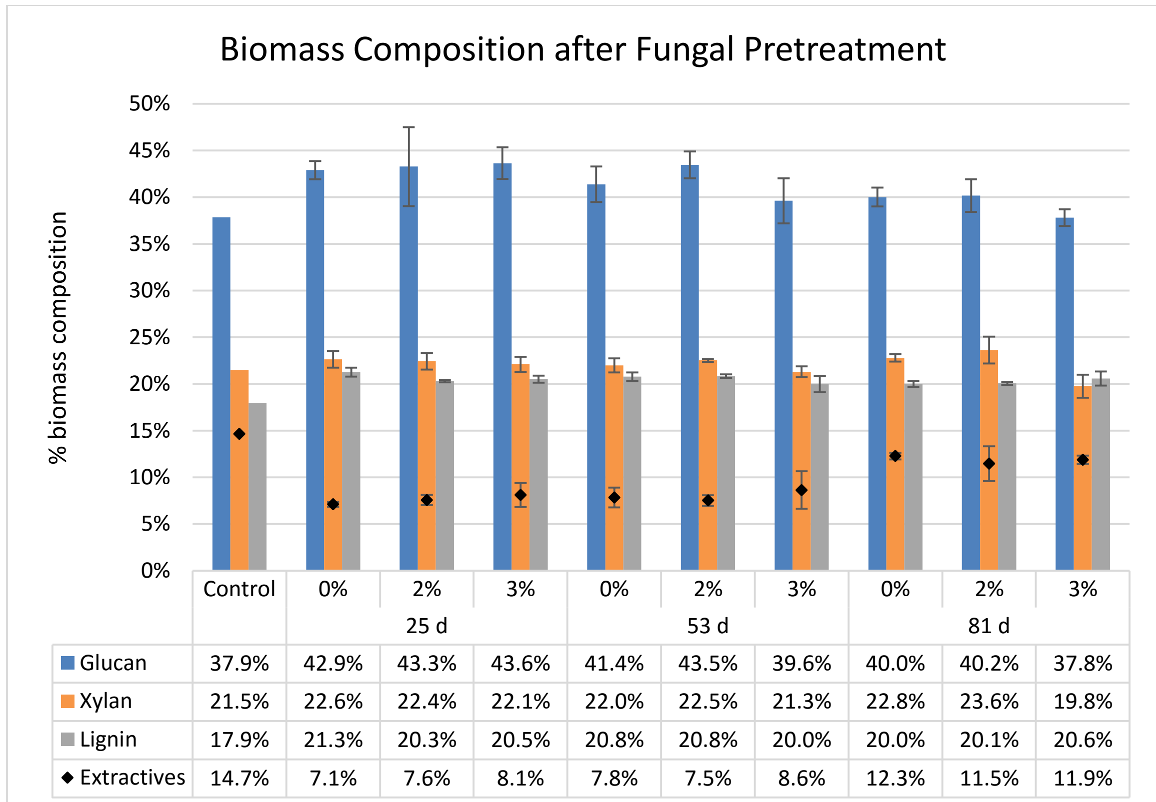

Figure 3.17. Biomass composition of fungal pretreated switchgrass with extractives; the error bars represent one standard deviation

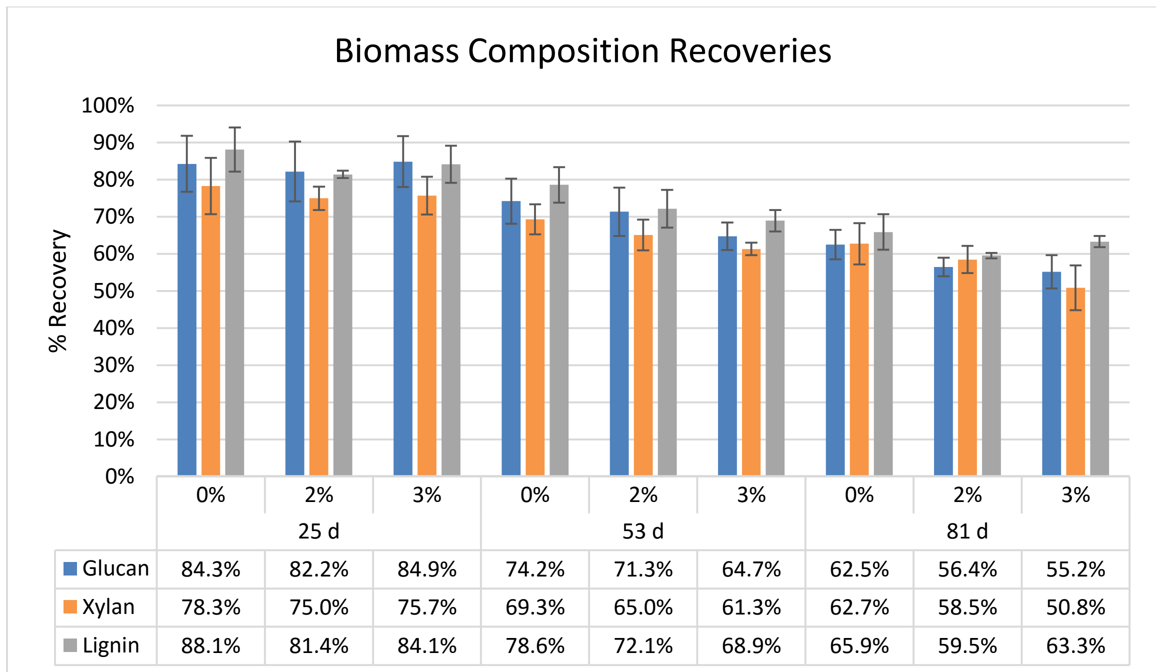

Figure 3.18. Biomass composition losses; the error bars represent one standard deviation

Statistical analysis was completed on the glucan, xylan, and lignin recoveries (Figure 3.18) and dry matter loss (Table 3.5). There was not a significant interaction between sampling period and fungal loading for glucan, xylan or lignin recoveries or dry matter loss. The sampling period had a significant effect on glucan recoveries. The 25 d samples had the greatest mean glucan recovery and the 81 d samples had the least mean glucan recovery, with all means being significantly different from one another (Table 3.6).

Table 3.6. Tukey's test (α=0.05) groupings of glucan recovery for sampling period and fungal loading

Sampling	Mean	Grouping	Fungal Loading	Mean	Grouping
25 d	0.83762	A	0%	0.73631	A
53 d	0.70072	B	2%	0.69978	A
81 d	0.58023	C	3%	0.68246	A

Sampling period and fungal loading had significant effects on xylan recovery. The 25 d samples had the greatest mean xylan recovery and the 81 d samples had the least mean xylan recovery, with all means being significantly different from one another. The 3% fungal loading was significantly less than the 0% fungal loading for xylan recovery; the 2% fungal loading xylan recovery was not significantly different from the others (Table 3.7).

Table 3.7. Tukey's test (α=0.05) groupings of xylan recovery for sampling period and fungal loading

Sampling	Mean	Grouping	Fungal Loading	Mean	Grouping
25 d	0.76296	A	0%	0.70099	A
53 d	0.65220	B	2%	0.66152	A, B
81 d	0.57340	C	3%	0.62606	B

Sampling period and fungal loading had significant effects on lignin recovery. The 25 d samples had the greatest lignin recovery and the 81 d samples had the least lignin recovery, with all means being significantly different from one another. Lignin recovery in the 0% fungal loading samples was significantly greater than in the 2% and 3% fungal

loading samples, which were not significantly different; the 2% fungal loading samples had the least lignin recovery (Table 3.8).

Table 3.8. Tukey's test (α=0.05) groupings of lignin recovery for sampling period and fungal loading

Sampling	Mean	Grouping	Fungal Loading	Mean	Grouping
25 d	0.84534	A	0%	0.77519	A
53 d	0.73207	B	3%	0.72101	B
81 d	0.62886	C	2%	0.71007	B

Sampling period and fungal loading had significant effects on dry matter loss. The 81 d samples had the greatest dry matter loss and the 25 d samples had the least dry matter loss, with all means with all means being significantly different from one another. Dry matter loss in the 2% fungal loading samples was significantly greater than in the 0%; the 3% fungal loading samples were not significantly different from the others (Table 3.9).

Table 3.9. Tukey's test (α=0.05) groupings of dry matter loss for sampling period and fungal loading

Sampling	Mean	Grouping	Fungal Loading	Mean	Grouping
81 d	0.44158	A	2%	0.37613	A
53 d	0.36080	B	3%	0.36472	A,B
25 d	0.26778	C	0%	0.32931	B

Since glucan recovery decreased with time, this may be the result of the fungal growth or other biological activity within the bales. However, the addition of fungus had an effect on the xylan and lignin recoveries. There was greater xylan recovery in the grain control bales than in the fungal-inoculated bales. The grain control had significantly greater lignin recovery than the fungal-inoculated bales and the highest lignin degradation occurred with longer treatment times. Since dry matter loss is a result of the loss of biomass components, it also increased with time and fungal pretreatment. The decrease in recovery for the fungal-inoculated bale indicates that *P. ostreatus* fungus has greater selectivity toward the degradation of xylan and lignin, rather than the degradation of glucan.

3.4.3. Pretreatments

The pretreated biomass composition analysis results for glucan, xylan, and lignin fractions are shown in Figure 3.19, Figure 3.20, and Figure 3.21, respectively.

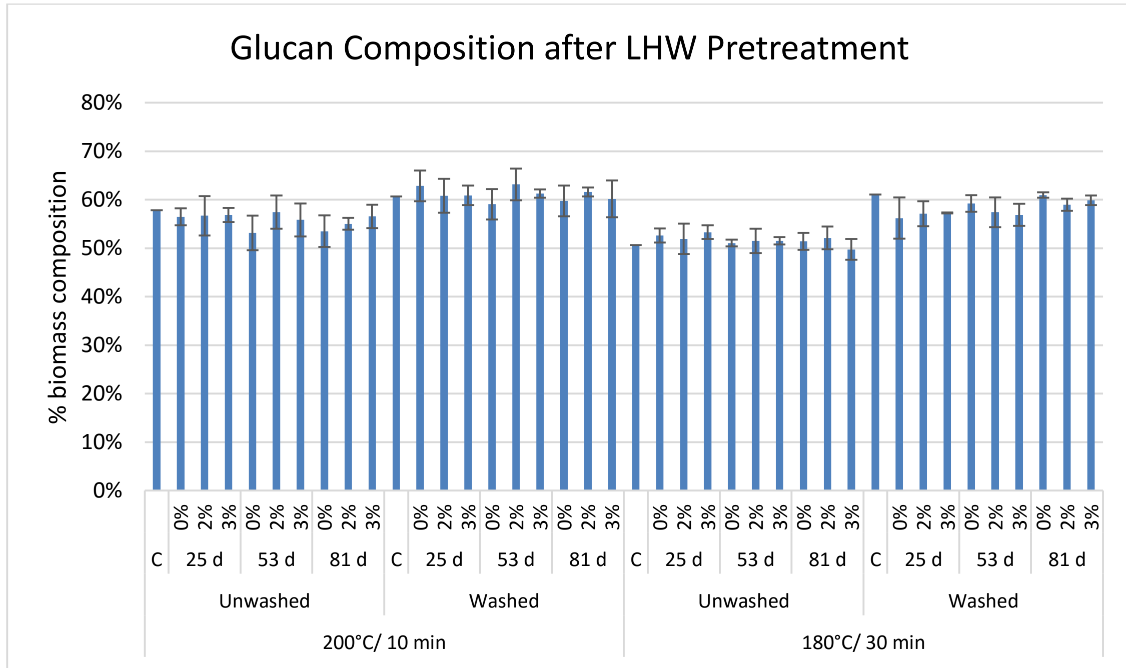

Figure 3.19. Glucan composition percentages of fungal pretreated bales after liquid hot water pretreatment; the error bars represent one standard deviation

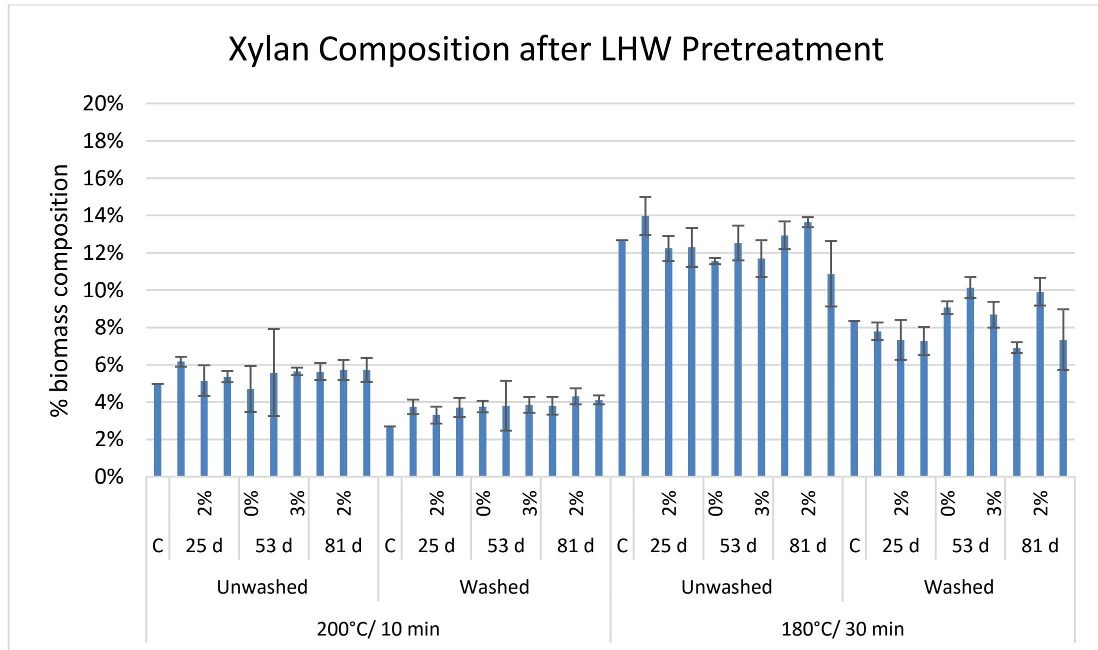

Figure 3.20. Xylan composition percentages of fungal pretreated bales after liquid hot water pretreatment; the error bars represent one standard deviation

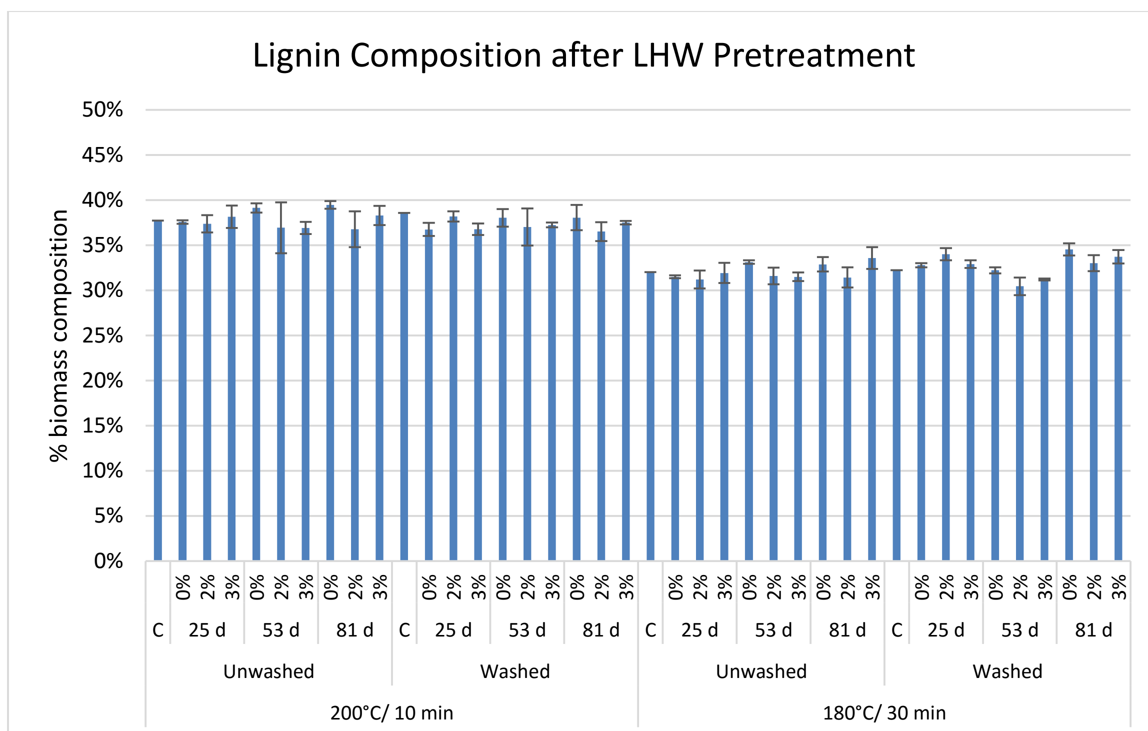

Figure 3.21. Lignin composition percentages of fungal pretreated bales after liquid hot water pretreatment; the error bars represent one standard deviation

Statistical analysis was completed on the washed pretreated biomass recoveries. The pretreatment severity and sampling period were significant for lignin recovery. The more severe pretreatment condition (200/10) had a significantly greater lignin recovery. Comparisons for sampling showed three groups for lignin recovery (Table 3.10). The lignin recovery of the untreated control was significantly greater than the other bale samples for fungal loading. The lignin recoveries being greater than 100% may be the result of acid insoluble compounds being formed by the fungus during growth or experimental error.

There was a significant interaction between sampling time and fungal loading (p=0.0013) for lignin recovery. The LSMEANS comparison of this interaction is shown in Figure 3.22, where matching letters indicate the values that are not significantly different. The lignin recovery of the untreated control was significantly greater than all samples

except for the 0%, 81 d samples. This indicates that less lignin was present in the LHW pretreated biomass after fungal pretreatment.

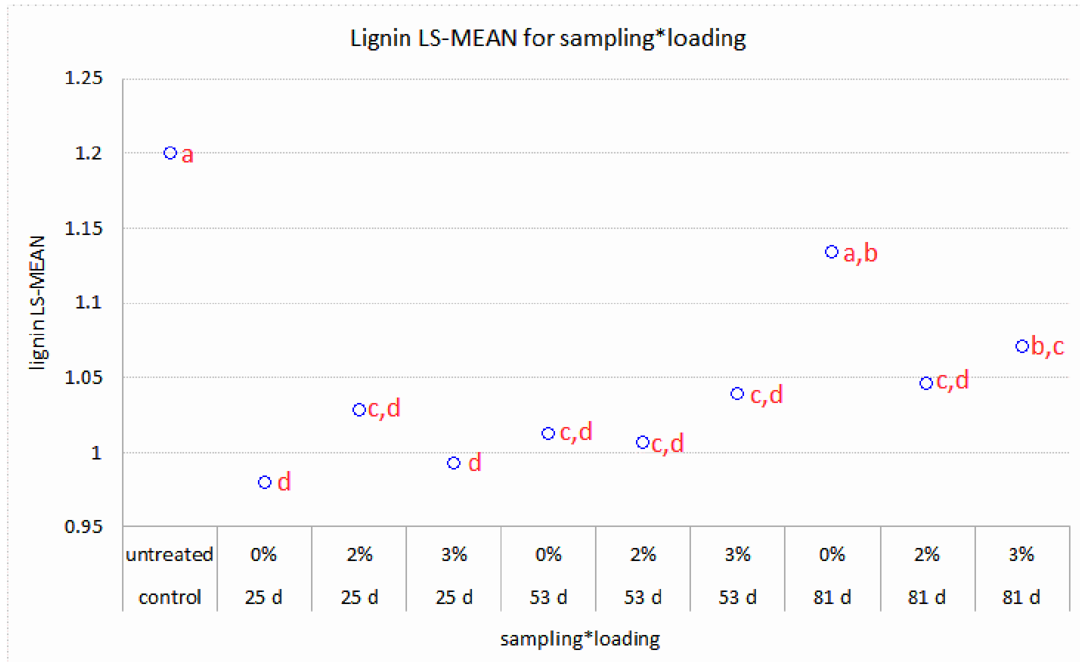

Figure 3.22. LS-MEANS comparison of the sampling time and fungal loading rate interaction for lignin recovery after pretreatment; matching letters indicate no significant difference

There were no significant interactions between sampling time and fungal loading for glucan or xylan recoveries. Sampling period was significant for glucan recovery. There were three groupings for glucan recovery based on sampling period, shown in Table 3.10. A Tukey's comparison of glucan recovery based on loading showed that only the 3% fungal loading was not significantly less than the control.

Table 3.10. Tukey's test (α=0.05) results for glucan, xylan, and lignin recovery after pretreatment based on sampling

	Glucan		Xylan		Lignin	
Sampling	**Mean**	**Grouping**	**Mean**	**Grouping**	**Mean**	**Grouping**
Untreated	0.97655	A	0.15575	A	1.20025	A
81 d	0.94524	A, B	0.17861	A	1.08370	B
53 d	0.87101	B, C	0.15719	A	1.01981	C
25 d	0.83617	C	0.15776	A	1.00040	C

Only the pretreatment severity was significant for xylan recovery. The less severe pretreatment condition (180/30) had a significantly greater xylan recovery.

In the case of pretreatment severity, the less severe condition of 180°C for 30 min had greater xylan recoveries and less lignin recoveries. The greatest lignin recovery occurred in the untreated control sample, which was expected because it had not been previously treated with water or fungus. The least glucan and lignin recoveries after LHW pretreatment occurred with the 25 d samples.

The effects of fungal pretreatment on glucan, xylan, and lignin recoveries based on fungal loading were not evident after LHW pretreatment, showing only differences based on sampling time. The greatest glucan recovery of the stored bales occurred with the 3%, 81 d samples; however, these samples also had significantly greater lignin recovery than many of the other fungal-inoculated samples. It is unclear why glucan and lignin recoveries increased with increased storage time. The untreated control had the greatest glucan recovery, but it also had the greatest lignin content after LHW pretreatment, which would reduce enzymatic digestibility.

After LHW pretreatment, the xylan recovery was not significantly affected by fungal pretreatment. Higher xylan recovery in the solids after pretreatment results is lower enzymatic hydrolysis of cellulose by cellulase (Chandra et al., 2007; Öhgren et al., 2007). The less severe (180/30) LHW pretreatment resulted in greater xylan recovery than the more severe (200/10) LHW pretreatment (Figure 3.20). Therefore, the fungal pretreatment was not effective in reducing the necessary severity of LHW pretreatment for similar xylan recoveries.

3.5. Conclusions

Significant fungal growth was achieved in the 2% and 3% fungal loading bales. The fungal-inoculated bales had significantly greater xylan and lignin degradation over

time than the grain control bales when comparing the biomass composition before LHW pretreatment. Since dry matter loss is a result of the degradation of glucan, xylan, and lignin, it also increased with time and fungal pretreatment. After LHW pretreatment, there was not a significant difference in glucan, xylan, or lignin content for the different fungal loadings. Increased xylan recovery results in lower hydrolysis of cellulose; the xylan recovery was greater for the less severe LHW pretreatment, so the fungal pretreatment was not effective in reducing the necessary severity of LHW pretreatment. It is important to note that while there were differences between the storage times for glucan composition, the native biological activity within the bales may have caused glucan in all the bales to degrade at similar rates since there was not a difference between the fungal loadings.

Fungal pretreatment time negatively affected the glucan composition, while simultaneously increasing the lignin and xylan degradation. Although, after liquid hot water pretreatment, the longer fungal pretreatment times resulted in greater glucan recovery, as well as greater lignin recovery. Fungal pretreatment of switchgrass with white-rot fungi like *P. ostreatus* is feasible, but further research needs to be completed.

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CHAPTER IV

4. Laboratory-scale study of copper, manganese, or glucose addition on the induction of ligninolytic enzymes produced by *Pleurotus ostreatus* during fungal pretreatment of switchgrass

4.1. Abstract

Pleurotus ostreatus produces laccase and manganese peroxidase (MnP) to selectively degrade lignin. Copper and manganese have been reported to increase production of laccase and MnP, respectively. The effects of adding copper, manganese, or glucose to switchgrass on the growth and ligninolytic enzyme production of *P. ostreatus* were evaluated in this project. Solutions of copper, manganese, or glucose, along with water, were tested with and without fungal inoculum at 75% moisture for 40 d at 28°C. Ligninolytic enzyme activities and biomass compositions were determined after the pretreatments. Simultaneous saccharification and fermentation (SSF) were conducted with the pretreated biomass. There were no significant differences between the solutions for laccase activity, but MnP activity of copper-treated samples were significantly less than the other fungal samples. Fungal pretreated samples had significantly less glucan, xylan, and lignin recoveries than the controls. The extractable sugars content of fungal-inoculated biomass was significantly greater than in the controls. Ethanol yield during SSF

corresponded with lignin degradation in the fungal-inoculated samples. Water-fungal samples had the greatest lignin degradation and ethanol yield, while the copper-fungal samples had the lowest lignin degradation and ethanol yield. Manganese-fungal and glucose-fungal treated samples had similar lignin contents and ethanol yields. Ethanol yield during SSF was significantly increased by fungal pretreatment compared to no pretreatment. Water alone was the more effective than the copper, manganese, and glucose solutions added to the fungal treatments. Fungal pretreatment with *P. ostreatus* provided significant lignin degradation to increase ethanol yield from switchgrass.

4.2. Introduction

White-rot fungi have been found to selectively degrade lignin by producing ligninolytic enzyme complexes. The three major ligninolytic enzymes excreted by white-rot fungi are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Wan & Li, 2012). Lignin degrading fungi can be categorized based on their enzyme production patterns into one of three groups: (i) LiP-MnP group, (ii) MnP-laccase group, or (iii) LiP-laccase group (Dwivedi et al., 2011). *Pleurotus ostreatus* is in the second enzyme production group, mainly excreting laccase and MnP. Laccases are copper-containing, cell wall localized glycoproteins that consume O_2 to oxidize monolignols in lignin (Boerjan et al., 2003). MnP is a heme glycoprotein that catalyzes the oxidation of Mn^{2+} to Mn^{3+} in the presence of H_2O_2 (Giardina et al., 2000).

Several studies have shown that laccase and MnP are activated in the presence of copper (Baldrian & Gabriel, 2002; Collins & Dobson, 1997; Galhaup & Haltrich, 2001) and manganese (Giardina et al., 2000; Kamitsuji et al., 2005), respectively. The addition of $CuSO_4$ has been widely studied for the induction of the laccase gene and laccase activity (Baldrian et al., 2005; Tinoco et al., 2011). However, studying the effect of laccase induction on biomass lignin degradation is less common. The addition of $MnSO_4$ has been studied for the effects of its induction of MnP on lignin content. Kerem and Hadar (1993) and Cohen et al. (2001) reported significant lignin degradation with the addition of manganese.

Soden and Dobson (2001) studied the effects of copper, manganese, and glucose addition on laccase gene expression in *Pleurotus sajor-caju* (Psc). They found that the

addition of glucose (60 mM) resulted in a 20.4-fold increase in Psc *lac1* gene transcript levels, an 11.8-fold increase in Psc *lac2* gene transcript levels, and a 28.7-fold increase in Psc *lac4* gene transcript levels.

A laboratory-scale study was developed to determine the impact of adding copper, manganese, or glucose to switchgrass during fungal pretreatment with *P. ostreatus* on ligninolytic enzyme production and ethanol yield during simultaneous saccharification and fermentation.

4.3. Materials and Methods

To evaluate the effects of nutrient addition, sixteen jars were filled with 50 g (dry basis) switchgrass. The desired moisture content for all jars was 75%. Four substrate treatments were evaluated: 1 mM $CuSO_4$, 1 mM $MnSO_4$, 3.33 g/L glucose, and water only. The four treatment replications were as follows: three jars for each treatment were inoculated with 5% liquid *P. ostreatus* inoculum and one control jar for each treatment did not have fungal inoculum.

4.3.1. Liquid inoculum prep

P. ostreatus mycelia were grown in liquid medium (15 g/L malt extract, 2 g/L sucrose, and 0.8 g/L yeast extract) at 28°C for 6 d. Six 250 mL flasks containing 25 mL liquid medium were autoclaved (liquids cycle, 20 min). To each of five flasks (one left as a control, five flasks were used to observe variations in mycelial growth), one 2 mL tube of bead-beat mycelial homogenate was added. The bead-beat mycelial homogenate was prepared as follows:

1. Three 3 mm glass beads and one 6 mm glass bead were added to a 2 mL screw cap microcentrifuge tube, and autoclaved (gravity cycle, 20 min, 121°C).
2. Two pure culture mycelia-covered MCBD (Basidiomycete Isolation Medium) agar plugs (~0.9 cm diameter) were added to the tube, along with 1 mL sterilized Milli-Q® water.
3. The tube was placed in the bead-beater for 30 s at 5,000 rpm to form a mycelial homogenate slurry.

After *P. ostreatus* completely colonized the surface of the liquid medium (Figure 4.1), the two flasks with the most growth (upon visual inspection) were used for the liquid inoculum preparation. Each flask (25 mL) was poured into a separate sterile 50 mL centrifuge tube and centrifuged in a bench-top centrifuge (Sorvall, Legend RT, Asheville, NC, USA) for 10 min at 3,750 rpm. The inoculum was washed using a procedure similar to Tinoco et al. (2011), with different final volume ratios. The liquid was decanted and the tube was filled to 25 mL with a 0.89% w/v NaCl solution. The tubes were mixed, then centrifuged for 6 min and the liquid was decanted. The tube was filled to 25 mL with the NaCl solution, mixed, and centrifuged for 10 min. After decanting, sterile deionized water was added to a total volume of 20 mL and the tubes were vortexed to mix the mycelia. A 5 mL pipette tip with the end cut off was used to add 2.5 mL of the mycelial inoculum to each jar of switchgrass. For the jars with no inoculum, 2.5 mL of deionized water was added.

Figure 4.1. *P. ostreatus* liquid inoculum flasks

4.3.2. Fungal pretreatment

The following solutions were prepared for addition to switchgrass to reach the desired moisture content. A 1 mM solution of $CuSO_4$ was prepared by adding 0.1497 g copper(II) sulfate pentahydrate in 0.6 L deionized water. A 1 mM solution of $MnSO_4$ was prepared by adding 0.1017 g manganese(II) sulfate monohydrate in 0.6 L deionized water. A 3.33 g/L (18.5 mM) solution of glucose was prepared by adding 1.9984 g of glucose in 0.6 L deionized water.

Kanlow switchgrass grown in Stillwater, OK at the Oklahoma State University EFAW station and harvested in November 2012 was used for this study. Switchgrass was coarsely chopped by a bale grinder prior to use. The moisture content of switchgrass used for the fungal pretreatment was determined as 10.84% based on the "convection oven method" outlined by Sluiter et al. (2008), so 56.08 g of switchgrass was added to each jar.

To achieve the desired moisture content of 75%, 141.42 mL of water (or copper, manganese, or glucose solution) needed to be added to each jar, plus 2.5 mL of either fungal inoculum or sterile deionized water after autoclaving. The jars of switchgrass and solutions were autoclaved twice at 121˚C for 30 min (liquids cycle), with an overnight interval between autoclaving periods. Once the jars cooled to room temperature, the 2.5 mL of *P. ostreatus* inoculum or deionized water was added under sterile conditions.

The jars were shaken and placed in an incubator chamber at 28˚C for 40 d. A saturated sodium chloride (NaCl) solution was placed inside the chamber to maintain a relative humidity of 75%. The jars were shaken every 2-3 days to distribute the mycelia. An example of the fungal growth is shown in Figure 4.2.

Figure 4.2. Jar 6 before and after shaking on day 21

4.3.3. Ligninolytic enzyme activity

4.3.3.1. Enzyme extraction

The activities of laccase and manganese peroxidase (MnP) enzymes were determined from samples of the fungal treated switchgrass after 40 d. The ligninolytic enzymes were extracted using a procedure similar to the procedure outlined by Gomez et al. (2012). In a large centrifuge tube, 1 g (dry basis) of the fungal treated sample was added to 50 mL sterile DI water and agitated at 150 rpm for 30 min. The samples were centrifuged in a bench-top centrifuge (Sorvall, Legend RT, Asheville, NC, USA) at 3,750 rpm for 10 min and the supernatant was used for the activity assay.

4.3.3.2. Activity assay

The laccase and MnP enzymatic activities were determined colorimetrically (Parenti et al., 2013; Santoyo et al., 2008). The activity of laccase was determined by detecting the oxidation product of 2,6-dimethoxiphenol (DMP, $\varepsilon_{468} = 49{,}600\ M^{-1}cm^{-1}$). The reaction mixture contained 450 µL supernatant and 500 µL 10 mM DMP in 100 mM acetate buffer (pH 5.0). The starting absorbance at 468 nm (A_{468}) was recorded. The reaction occurred for 1 min at room temperature before A_{468} was recorded. Then, 50 µL of 0.1 mM $MnSO_4$ in acetate buffer was added to the reaction mixture and incubated at room temperature for 1 min. Finally, 30 µL of a H_2O_2 solution (22.5 µL of 30% H_2O_2 in 10 mL deionized water) was added to the mixture. The reaction proceeded for an additional 1 min and A_{468} was recorded. Manganese peroxidase activity was estimated as the difference between the two activity measurements. One unit of enzyme activity was defined as 1 µmol of the substrate oxidized per min. The activity of enzyme extract was determined as IU/g

dry switchgrass. All assays were performed in duplicate using a UV-2100 spectrophotometer (Cole-Parmer, Vernon Hills, IL).

The ligninolytic enzyme activities were calculated using equation 4.1:

$$\text{Activity (U/g)} = \frac{A_{468} * V_{ex}}{V_{sup} * 49.6 * g} \tag{4.1}$$

where A_{468} is the absorbance reading (for laccase or MnP), V_{ex} is the volume of water used in the enzyme extraction (50 mL), V_{sup} is the volume of the enzyme extraction supernatant added to the reaction (0.45 mL), 49.6 is the converted extinction coefficient for DMP, and g is the mass of the sample on dry basis (g).

4.3.4. Biomass compositional analysis

After samples were taken for the enzyme activity assay, the jars were autoclaved for 30 min at 121°C to stop all biological activity. Samples of the autoclaved biomass were taken and air-dried for compositional analysis. The dried samples were ground using a coffee grinder and the fine dust particles were sifted out. For each sample, the moisture content after grinding and the ash content were determined based on the "convection oven method" outlined by Sluiter et al. (2008) and ash procedure outlined by Sluiter et al. (2005a). The samples were extracted using Soxhlet extractors according to Sluiter et al. (2005b), first with water, then ethanol. The water and ethanol extractives contents were determined by the mass of the extractives residue after forced-air drying of the extracted liquids in the fume hood.

Extracted biomass was allowed to air dry in the fume hood, and the carbohydrate and lignin contents were determined using a two-stage acid hydrolysis according to Sluiter et al. (2012). Carbohydrate concentrations were determined using HPLC (1100 Series,

Agilent, Santa Clara, CA, USA) with an Aminex HPX-87H column (BioRad, Hercules, CA, USA) and a refractive index detector (RID). The eluent was deionized water flowing at a rate of 0.6 ml/min and a column temperature of 85˚C. The total run time for each sample was 30 min. The HPLC with ChemStation software (Agilent Technologies) was calibrated at five levels using known concentrations of cellobiose, glucose, xylose, galactose, mannose, and arabinose before being used to quantify the concentration of these compounds.

The biomass composition recoveries were calculated using equation 4.2:

$$\text{recovery} = \frac{\text{DM}_{\text{final}} * f_{\text{sample}}}{\text{DM}_{\text{in}} * f_{\text{raw}}} \tag{4.2}$$

where DM_{final} is the final mass (dry basis), DM_{in} is the starting mass (dry basis), f_{sample} is the glucan, xylan, or lignin fraction of the extracted biomass sample, and f_{raw} is the glucan, xylan, or lignin fraction of the untreated (raw) biomass control.

4.3.5. Simultaneous saccharification and fermentation

Samples of the biomass were taken from the jars after autoclaving and dried in an oven at 105°C overnight to determine the moisture content. The autoclaved biomass was then used for simultaneous saccharification and fermentation (SSF).

4.3.5.1. Enzymes

Celluclast 1.5 L and Novozyme 188 (Novozymes, Franklinton, NC) were purchased from Sigma-Aldrich (St. Louis, MO). The cellulase activity of Celluclast 1.5 L was determined using the filter paper assay developed by Adney and Baker (1996). The cellobiase activity of Novozyme 188 was determined by adding 100 µL of 5% (w/w) enzyme solution to 900 µL of 10 g/L cellobiose solution in 50 mM sodium citrate buffer

(pH 4.8). An enzyme control was also prepared by mixing 900 μL of citrate buffer with 100 μL enzyme solution. The tubes were incubated at 50°C for 3 min, then boiled for 5 min to stop the enzymatic activity. Glucose produced was determined by High Performance Liquid Chromatography (HPLC) (1100 Series, Agilent, Santa Clara, CA, USA) with an Aminex HPX-87H column (BioRad, Hercules, CA, USA) and a refractive index detector (RID). The eluent was deionized water flowing at a rate of 0.6 ml/min and a column temperature of 85˚C, with a total run time of 30 min. One CBU of cellobiase activity was defined as two μmol of glucose released per min.

4.3.5.2. Yeast culture

Saccharomyces cerevisiae D_5A yeast was used for the SSFs. The yeast was maintained at 4°C and subcultured periodically on YPD agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar. The day before fermentation, 100 mL of preculture was prepared by transferring a loopful of yeast cells into sterile YPD medium containing 10 g/L yeast extract, 20 g/L peptone, and 50 g/L glucose in a 250 mL baffled flask. The flask was incubated anaerobically at 37°C at 250 rpm until reaching an optical density (OD, absorbance at 600 nm) in the range of 14-17. The yeast cells were then concentrated to an initial OD of 50 for the yeast inoculum by centrifuging the cells at 3,500 rpm for 10 min twice in a bench-top centrifuge (Sorvall, Legend RT, Asheville, NC, USA) and washing with 0.89% (w/v) sterile sodium chloride solution between centrifugations, then adding the appropriate volume of sterile deionized water (Dowe and McMillan, 2001).

4.3.5.3. SSF conditions

SSF was conducted following the procedure outlined by Ramachandriya et al. (2014) with modifications to the enzyme loading. SSF of each fungal pretreatment jar was done in duplicate as blocks (1-16 in each SSF set). The experiments were conducted in 250 mL baffled flasks, sealed with a rubber stopper fitted with a one-way valve (Check valve, Fisher Scientific, Pittsburgh, PA); with a working volume of 100 mL, operating anaerobically on an orbital shaker (New Brunswick Scientific) at 200 rpm and 37°C. The flasks contained a solids loading of 8% (fungal treated samples on dry basis), an enzyme loading of 7.5 FPU/g dry biomass Celluclast 1.5 L (823 μL) and 15 CBU/g dry biomass Novozyme 188 (385 μL), 10 mL of 10x yeast fermentation (YP) medium, 5 mL of 1 M sodium citrate buffer (pH 5.5), and 1 mL of OD 50 concentrated yeast cells suspension. The 10x yeast fermentation medium contained 100 g/L yeast extract and 200 g/L peptone.

During SSF, samples (~1.8 mL) were taken at 0, 6, 12, 18, 24, 36, 48, 60, 72, 96, and 120 h. The pH of samples was recorded, then the samples were centrifuged at 13,000 rpm for 10 min. The supernatant was decanted and filter sterilized using 0.22 μm nylon filters (VWR International, West Chester, PA). A one-fifth dilution of the supernatant was analyzed for cellobiose, glucose, xylose, succinic acid, lactic acid, glycerol, acetic acid, and ethanol concentrations using HPLC (1100 Series, Agilent, Santa Clara, CA, USA) with an Aminex HPX-87H column (BioRad, Hercules, CA, USA) and a refractive index detector (RID). The eluent was 0.005 M sulfuric acid flowing at a rate of 0.6 ml/min with a 30 min run time. The HPLC with ChemStation software (Agilent Technologies) was calibrated at five levels using known concentrations of the compounds listed above.

Ethanol yield (% theoretical) during SSF was calculated as the percentage of the theoretical yield based on the glucan fraction of the starting biomass used for SSF, according to equation 4.3:

$$\text{Ethanol yield (\% theoretical)} = \frac{[EtOH_t]}{0.511*(f[biomass]*1.11)} * 100\% \qquad (4.3)$$

where $[EtOH_t]$ is the ethanol concentration at time t (g/L), 0.511 is the mass conversion from glucose to ethanol (g/g), f is the glucan fraction in the dry solids (g/g), [*biomass*] is the initial concentration of solids (8 g/L), and 1.11 is the mass conversion factor for glucan hydrolysis to glucose (g/g).

Xylose yield during SSF was calculated as the percentage of the theoretical yield based on the starting biomass used for SSF, using equation 4.4:

$$\text{Xylose yield} = \frac{[Xylose_t]}{(f[biomass]*1.11)} \qquad (4.4)$$

where $[Xylose_t]$ is the xylose concentration at time t (g/L), f is the xylan fraction in the dry solids (g/g), [*biomass*] is the initial concentration of solids (8 g/L), and 1.11 is the mass conversion factor for xylan hydrolysis to xylose (g/g).

Glucan preservation was calculated as the glucan recovery after pretreatment, using equation 4.5:

$$\text{Glucan preservation} = \frac{DM_{final}*f_t}{50*f_{in}} \qquad (4.5)$$

where DM_{final} is the dry matter left after fungal pretreatment (g), f_t is the glucan fraction after fungal pretreatment (%), 50 is the initial dry matter put into the fungal pretreatment (g), and f_{in} is the initial glucan fraction of the switchgrass samples (%).

Overall ethanol yield was calculated as the percentage of the theoretical yield based on the biomass after fungal pretreatment, according to equation 4.6:

$$\text{Overall ethanol yield} = (\text{Ethanol yield (\% theoretical)}) * (\text{Glucan preservation}) \quad (4.6)$$

where (Ethanol yield (% theoretical)) is the ethanol yield during SSF obtained in equation 4.1, and (Glucan preservation) is the glucan recovery after pretreatment.

4.3.6. Statistical analysis

Analysis of variance (ANOVA) was calculated ($p<0.05$) using the generalized linear model (GLM) in SAS 9.4 (SAS, Cary, NC) and multiple comparison tests were performed using Tukey's Honest Significant Difference test at a 95% confidence interval. For the enzyme activity tests, laccase and MnP activities were the dependent variables and the solution was the independent variable. For the biomass composition recoveries, the extractives content and glucan, xylan, and lignin fractions were the dependent variables and the solution and fungal addition were the independent variables. For the ethanol yield during SSF, the ethanol yield at 24 h was the dependent variable and the solution and fungal addition were the independent variables.

4.4. Results and Discussion

4.4.1. Ligninolytic enzyme activity

The average ligninolytic enzyme activities for laccase and MnP of the three fungal inoculated jars for each solution is shown in Figure 4.3. The controls had no enzyme activity.

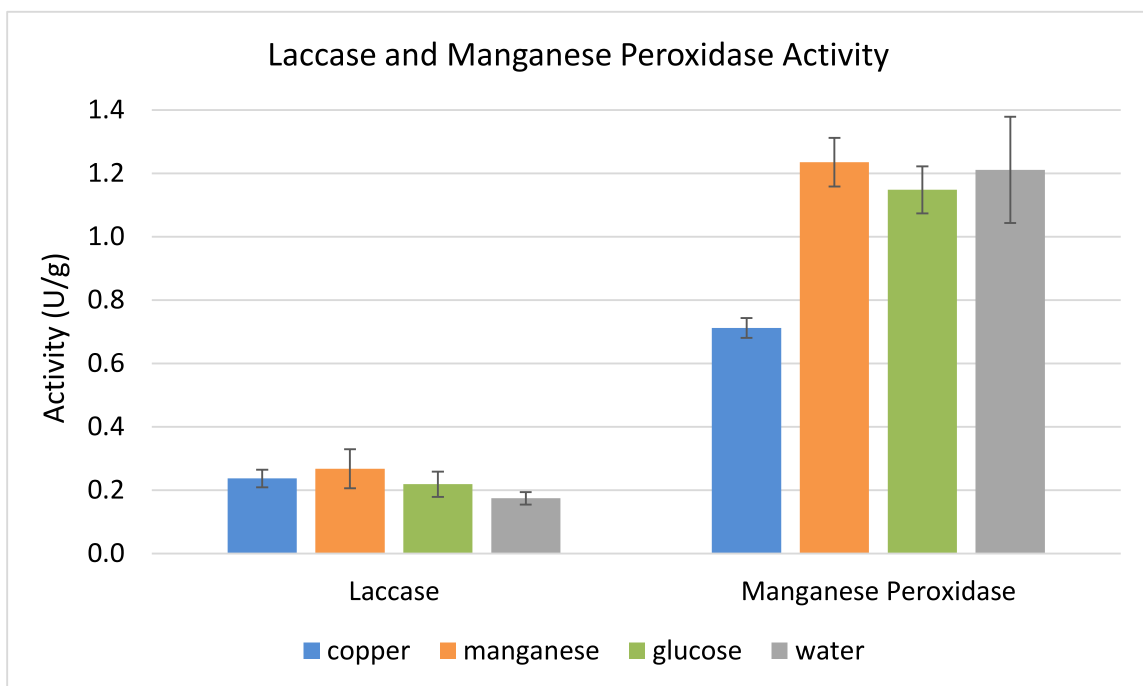


Figure 4.3. Ligninolytic enzymes laccase and manganese peroxidase activities in fungal inoculated switchgrass with the addition of copper, manganese, glucose and water; the error bars represent one standard deviation

Since the control samples had no enzyme activity, the statistical analysis was completed on both enzyme activities for the effect of the solution only. The solution added had a significant effect on both enzyme activities. Comparison of the solutions showed that only the manganese treated sample had significantly greater laccase activity than the water treated samples. The MnP activity with the addition of copper was significantly less than with the addition of the other solutions (Table 4.1). Manganese addition resulted in the greatest activity for both enzymes.

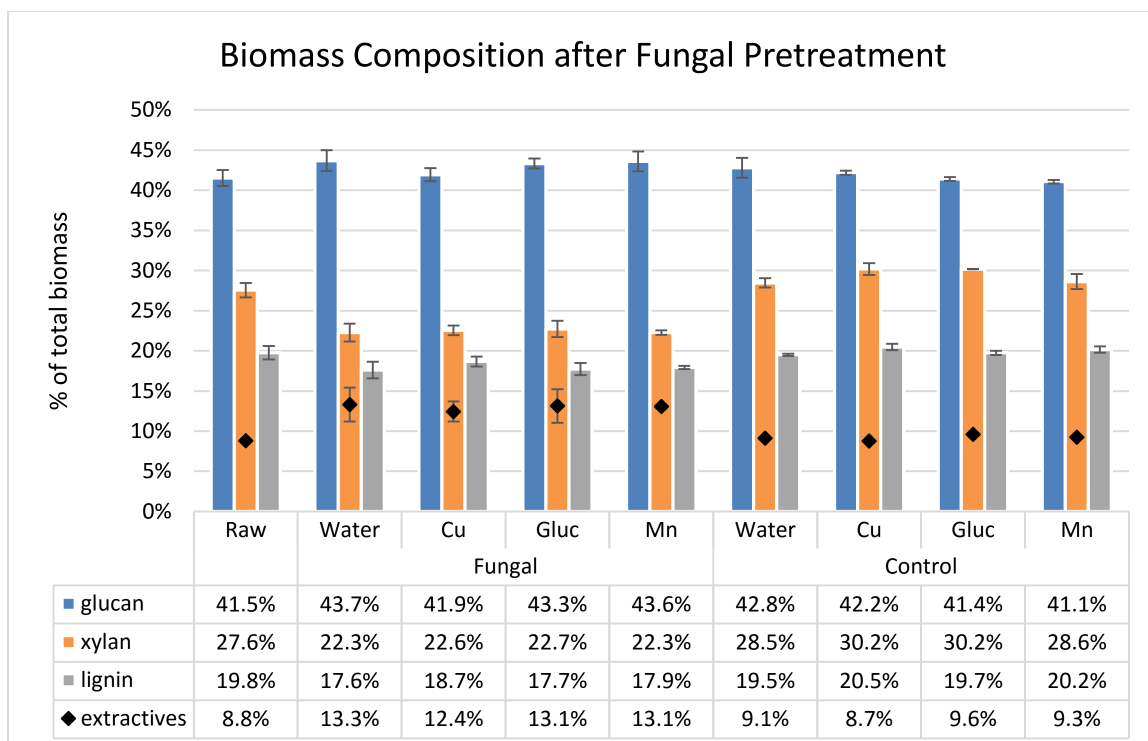
Table 4.1. Tukey's test (α=0.05) results for ligninolytic enzyme activities based on the solution added to switchgrass with fungal inoculation

Laccase activity			Manganese peroxidase activity		
Solution	**Mean**	**Grouping**	**Solution**	**Mean**	**Grouping**
Manganese	0.24083	A	Manganese	1.11171	A
Copper	0.21316	A, B	Water	1.08976	A
Glucose	0.19683	A, B	Glucose	1.03314	A
Water	0.15679	B	Copper	0.64089	B

The reduced MnP activity for the addition of copper was consistent with the suppression of MnP activity by concentrations of $CuSO_4$ over 0.5 mM as reported by Mäkelä et al. (2013). However, laccase activity was not effected greatly by the addition of copper, which was contrary to previously reported results (Baldrian & Gabriel, 2002; Mäkelä et al., 2013; Palmieri et al., 2000). Reduced MnP activity resulted in reduced lignin degradation, which corresponds to the greater lignin recovery in the copper treated fungal-inoculated samples than in the other fungal-inoculated samples (Figure 4.4).

4.4.2. Biomass composition

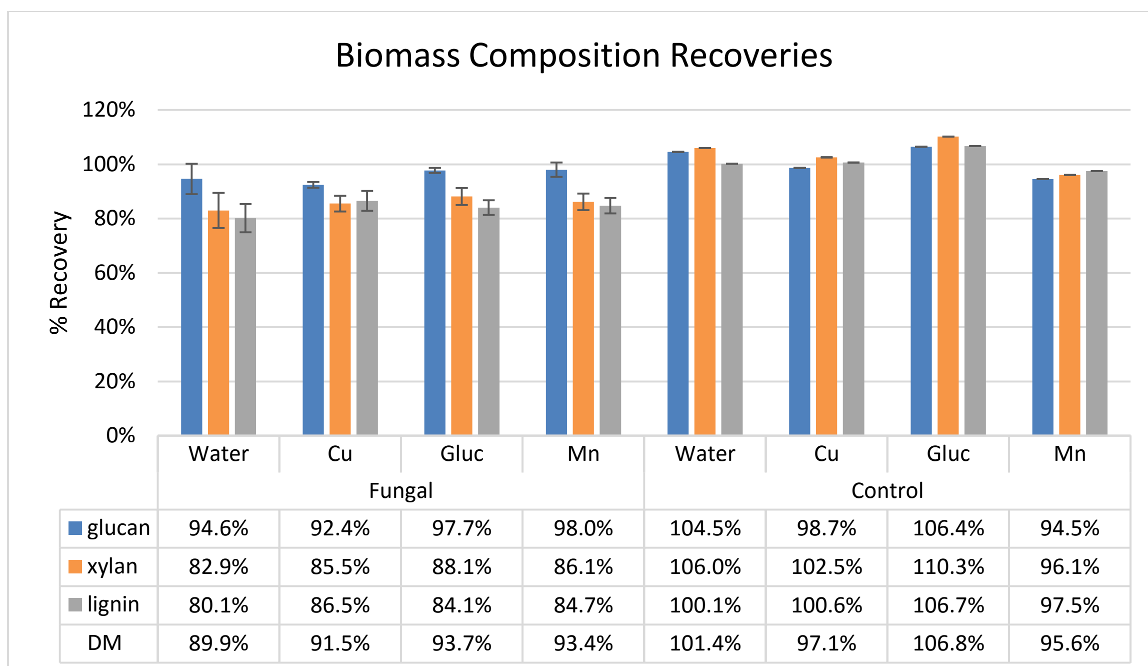
The biomass compositional fractions used for recovery calculations are shown in Figure 4.4. The statistical analysis was completed on the glucan, xylan, lignin, and extractives recoveries (Figure 4.5).



	Raw	Water	Cu	Gluc	Mn	Water	Cu	Gluc	Mn
		Fungal				Control			
glucan	41.5%	43.7%	41.9%	43.3%	43.6%	42.8%	42.2%	41.4%	41.1%
xylan	27.6%	22.3%	22.6%	22.7%	22.3%	28.5%	30.2%	30.2%	28.6%
lignin	19.8%	17.6%	18.7%	17.7%	17.9%	19.5%	20.5%	19.7%	20.2%
extractives	8.8%	13.3%	12.4%	13.1%	13.1%	9.1%	8.7%	9.6%	9.3%

Figure 4.4. Biomass composition after fungal pretreatment; the error bars represent one standard deviation

For all fractions, the difference between the solutions was not significant. There was a significant difference between the fungal-inoculated jars and the controls for all compositional fractions tested. The mean glucan, xylan and lignin recoveries were significantly less in biomass from the fungal-inoculated jars than in biomass from the control jars. The mean extractives were significantly greater in biomass from the fungal-inoculated jars than in biomass from the control jars. The reduction in glucan, xylan, and lignin in the fungal-inoculated samples indicates that the fungus partially degraded all fractions of the biomass. The increase in extractives may be due to hydrolysis of these fractions by the enzymes the fungus excreted, resulting with the hydrolysis products being extracted.



	Fungal				Control			
	Water	Cu	Gluc	Mn	Water	Cu	Gluc	Mn
glucan	94.6%	92.4%	97.7%	98.0%	104.5%	98.7%	106.4%	94.5%
xylan	82.9%	85.5%	88.1%	86.1%	106.0%	102.5%	110.3%	96.1%
lignin	80.1%	86.5%	84.1%	84.7%	100.1%	100.6%	106.7%	97.5%
DM	89.9%	91.5%	93.7%	93.4%	101.4%	97.1%	106.8%	95.6%

Figure 4.5. Biomass composition recoveries after fungal pretreatment; the error bars represent one standard deviation

4.4.3. Simultaneous saccharification and fermentation

Based only on the appearance of the flasks, the fungal inoculation had a positive effect on the ability of the enzymes to hydrolyze cellulose. As you can see in Figure 4.6, the biomass in the fungal-inoculated flask became somewhat liquefied, while the control remained solid. This trend was observed for all four solutions. However, the copper treated fungal-inoculated flasks did not liquefy as well as the other solution treatments.

Figure 4.6. SSF flasks at 72 h; flask 1: water only with fungal inoculation, flask 16: water only control

4.4.3.1. Ethanol yield

Figure 4.7 shows the average ethanol yield (% theoretical) for each treatment. For the fungal-inoculated samples, the highest ethanol yield (maximum 16.87%) was achieved with the water only treatments; the lowest yield was achieved by the copper treated samples (maximum 12.84%). The manganese and glucose fungal-inoculated samples were approximately the same for all time points, based on the graph. The peak fungal yield to control yield ratios for ethanol production at 24 h are shown in Table 4.2. The glucose control had an ethanol yield approximately 1.5% higher than the other controls as a result of free glucose in the biomass. The additional glucose was accounted for in the theoretical ethanol yield calculation for the glucose control.

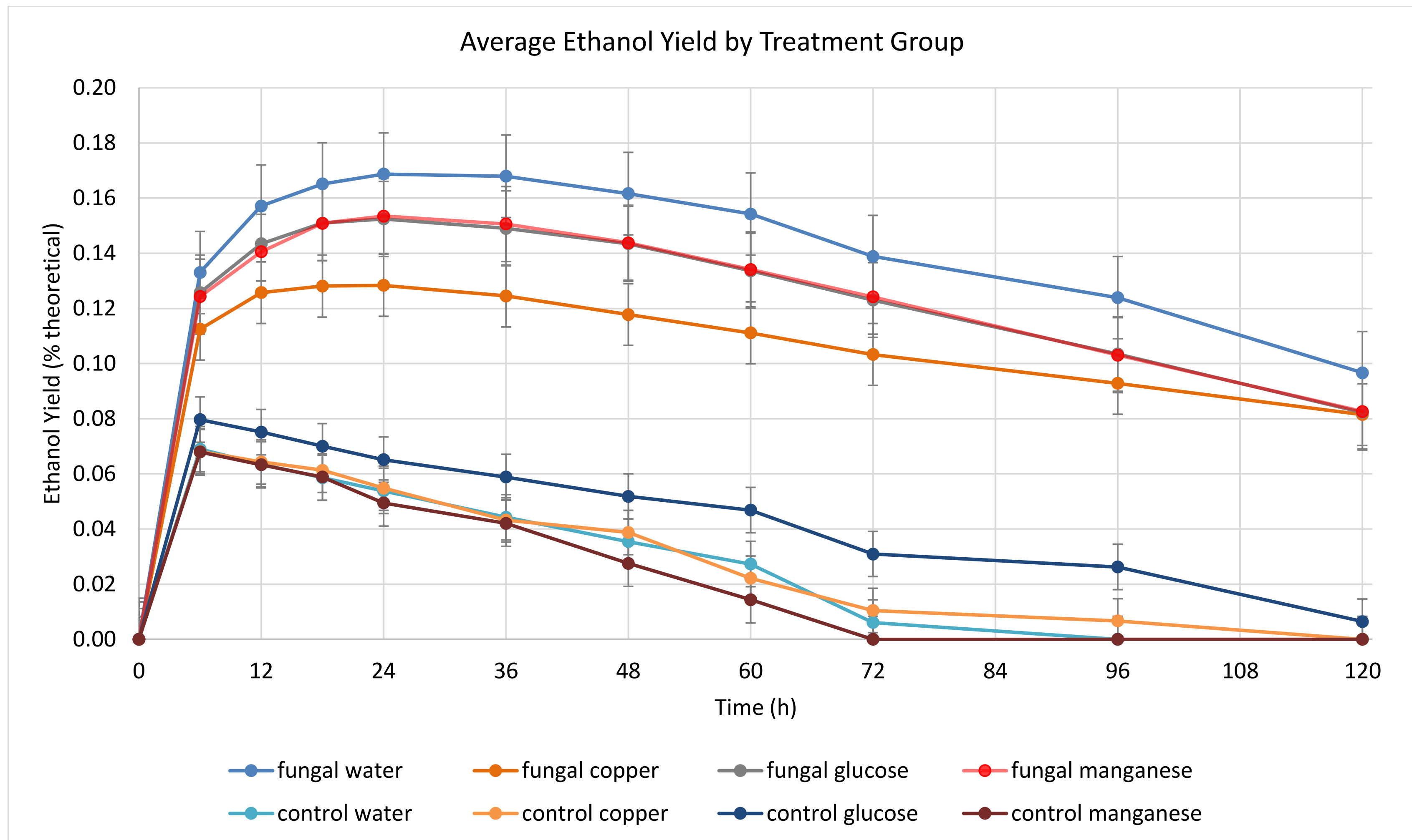


Figure 4.7. Ethanol yield (% theoretical) averaged by treatment group; the error bars represent one standard deviation

Table 4.2. Fungal to control ratio of ethanol yield at 24 h

Solution	Fungal/Control Yield
Water	3.133
Manganese	3.103
Copper	2.341
Glucose	2.340

Statistical analysis was completed on the ethanol yield (% theoretical) for the 24 h sample, when the maximum yield occurred. There was no significant interaction between solution and fungal-inoculation. Fungal-inoculation was the significant main effect for the ethanol yield at this time. Comparison of the solutions showed that only the water samples had significantly greater yields than the copper samples (Table 4.3). The ethanol yield from the fungal-inoculated samples was significantly greater than the controls.

Table 4.3. Tukey's test (α=0.05) results for ethanol yield for fungal-inoculated samples based on solution

Solution	Mean	Grouping
Water	0.168667	A
Manganese	0.153467	A, B
Glucose	0.152433	A, B
Copper	0.128333	B

The interaction between solution and fungal-inoculation was determined by a LSMEANS comparison: only the water-fungal samples had significantly greater ethanol yields than the copper-fungal samples. At 24 h, the control samples were not significantly different from each other.

Lignin degradation was not significantly different for the different solutions. However, the greatest lignin degradation corresponds to the greatest ethanol yield for the water-fungal samples, while the least lignin degradation corresponds to the least ethanol yield for the copper-fungal samples.

While the water-fungal samples had the lowest lignin recovery, they also had the lowest xylan recovery and lower glucan recovery than the glucose or manganese fungal-

inoculated samples. Although these samples had lower glucan recovery, the ethanol production was maximized by the lignin degradation.

Although the manganese-fungal samples had the greatest laccase and MnP activities, it had the second greatest lignin recovery (behind copper). However, the greatest glucan recovery was also achieved with these samples, which may be a result of higher selectivity to lignin over glucan. The lower ethanol yields for the manganese-fungal and the glucose-fungal compared to the water-fungal samples were most likely due to the lower lignin degradation.

The glucose-fungal samples had the greatest xylan recovery, the second greatest glucan recovery, and the second lowest lignin recovery. The addition of glucose seemed to reduce the glucan and xylan utilization of the fungus during growth. It also resulted in only slightly different laccase and MnP activities than water. Glucose-fungal samples had similar ethanol yields to those of the manganese-fungal samples, which had nearly the same glucan and lignin recoveries.

The copper-fungal samples had the lowest glucan recovery and the greatest lignin recovery. The decrease in lignin degradation compared to water is likely the result of the MnP inhibition. The copper-fungal samples had approximately 42% less MnP activity than the manganese-fungal samples. The greater lignin content and reduction in glucan recovery is likely responsible for these samples having the lowest ethanol yield of the fungal-inoculated samples.

4.4.3.2. Glucose and cellobiose concentrations

The glucose and cellobiose concentrations over time are shown in Figure 4.8 and Figure 4.9, respectively. The steady concentration of glucose after 12 h indicates that there

was inhibition of the yeast; normal yeast activity would have resulted in a greater decrease in glucose concentrations and steady ethanol production after 24 h, which decreased (Figure 4.7). The increase in cellobiose concentration over time indicates that the cellobiase enzyme was inhibited, which is responsible for converting cellobiose to glucose. The enzyme and yeast inhibitions are responsible for the decrease in ethanol yield over time.

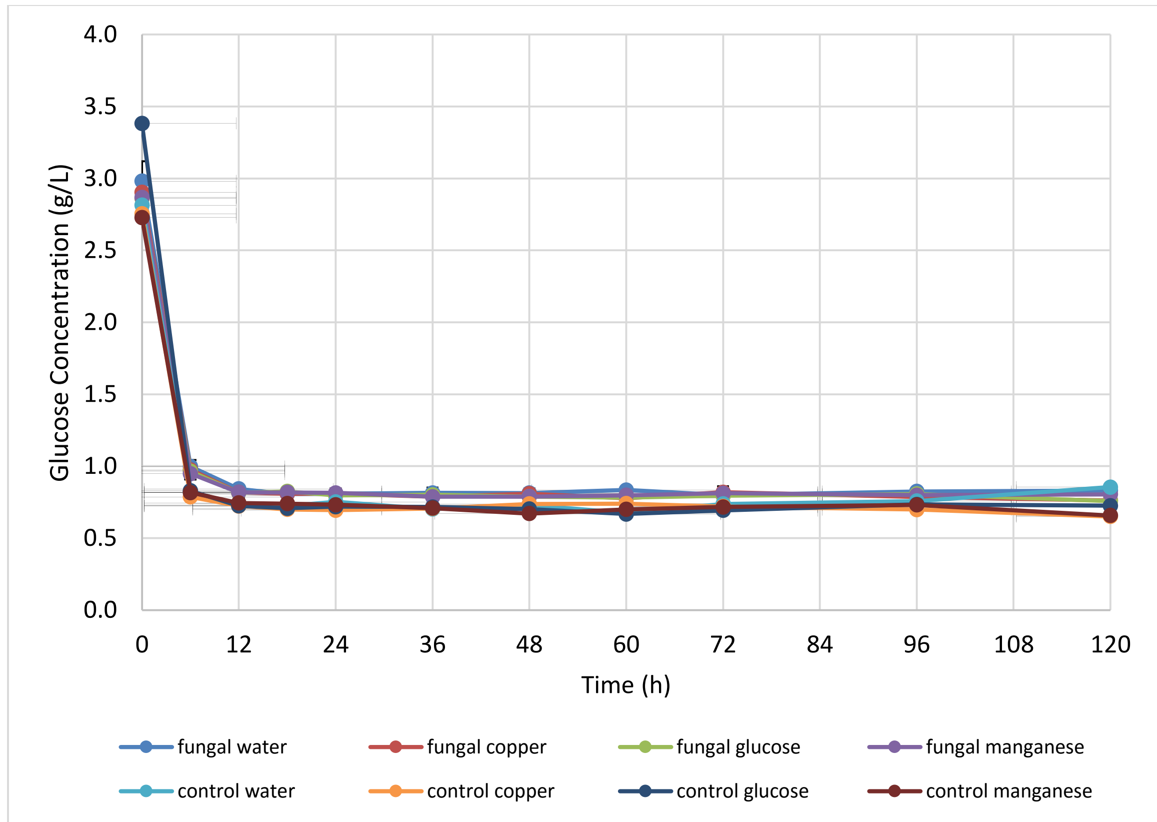


Figure 4.8. Glucose concentration during simultaneous saccharification and fermentation, grouped by treatment

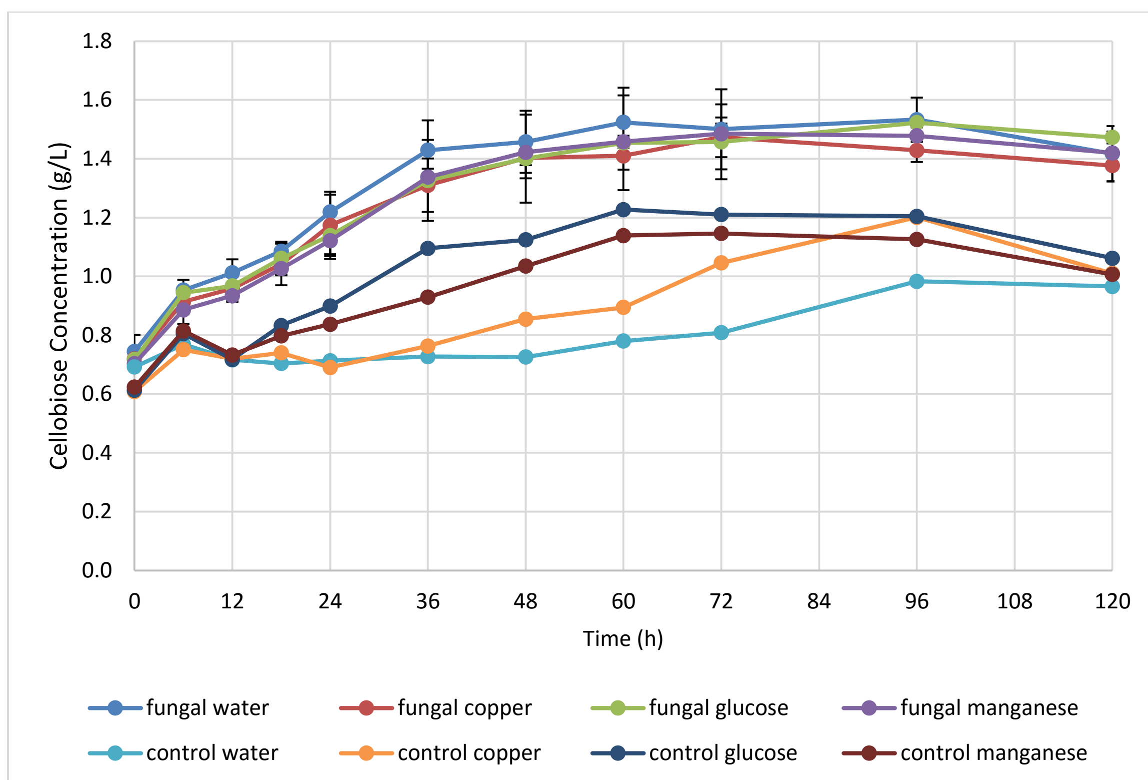


Figure 4.9. Cellobiose concentration during simultaneous saccharification and fermentation, grouped by treatment; the error bars represent one standard deviation

4.5. Conclusions

Addition of copper, manganese, glucose, or water had similar effects on laccase activity. However, addition of 1 mM $CuSO_4$ had a negative effect on MnP activity of *P. ostreatus*. Addition of copper, manganese, glucose, or water did not have different effects on biomass composition after fungal pretreatment. However, fungal inoculation significantly decreased the glucan, xylan, and lignin fractions in switchgrass compared to the control of no fungal inoculation. This indicates that *P. ostreatus* degraded the lignin while also hydrolyzing some glucan and xylan.

Addition of *P. ostreatus* mycelia to switchgrass had a significant effect on the enzymatic digestibility during SSF. Fungal-inoculated jars had a 2.1 to 3.1-fold increase in ethanol yield (% theoretical) compared to controls at the maximum production time (24 h).

Addition of copper in fungal-inoculated samples resulted in less ethanol yield than other fungal-inoculated samples, which was likely the result of less MnP activity leading to less lignin degradation. The addition of manganese or glucose in fungal-inoculated samples had similar ethanol yields to one another, while fungal-inoculated samples with only water added had the greatest ethanol yield.

Since water alone was more effective than addition of copper, manganese, and glucose solutions to the fungal treatments, it is evident that the switchgrass contained the necessary nutrients for lignin degradation. The addition of copper, manganese, or glucose did not increase lignin degradation in switchgrass by *P. ostreatus*. However, addition of *P. ostreatus* did provide significant lignin degradation to increase ethanol yield from switchgrass compared to no pretreatment.

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CHAPTER V

5. Future Work

In order for fungal pretreatment of switchgrass during storage to be effective, the microbial load in the bales will need to be reduced. Pasteurization of bales to reduce the native biological activity before fungal inoculation would likely decrease the losses experienced in the control bales from our study. Currently, mushroom farms pasteurize biomass by placing it in a sealed container and injecting steam for a certain period.

Improvements of the inoculation procedure and the automatic watering system need to be made. The inoculation procedure could be changed so that the bales do not need to be split apart, reducing biomass losses, labor, and equipment costs. Metal spikes could be used to add liquid fungal inoculum throughout the bale. Changes in inoculation procedure require changes in the current watering system. Watering the bale from the top may allow more water to stay in bale, because the flowrate of water out of the bales was an issue with the system described in this paper. If water was applied with sprinklers, it is possible that less water would come out of the bottom of the bales. In this case, moisture sensors (possibly soil moisture probes) would be essential to determine the exact moisture content inside the bale. The bale temperature could be monitored at a single point, because the temperature was stable throughout the bales.

Future study of fungal pretreatment in switchgrass will also need to involve determining the ideal treatment time to minimize cellulose and hemicellulose loss while

achieving appropriate lignin degradation. Since the goal of pretreatment is to maximize cellulose hydrolysis for ethanol production, fungal pretreatment should be ended once cellulose and hemicellulose begin to significantly degrade.

6. APPENDICES

6.1. Data logger wiring

The data loggers were wired following screw terminal pinout diagram shown in Figure 6.1, according to the configurations in Figure 6.2.

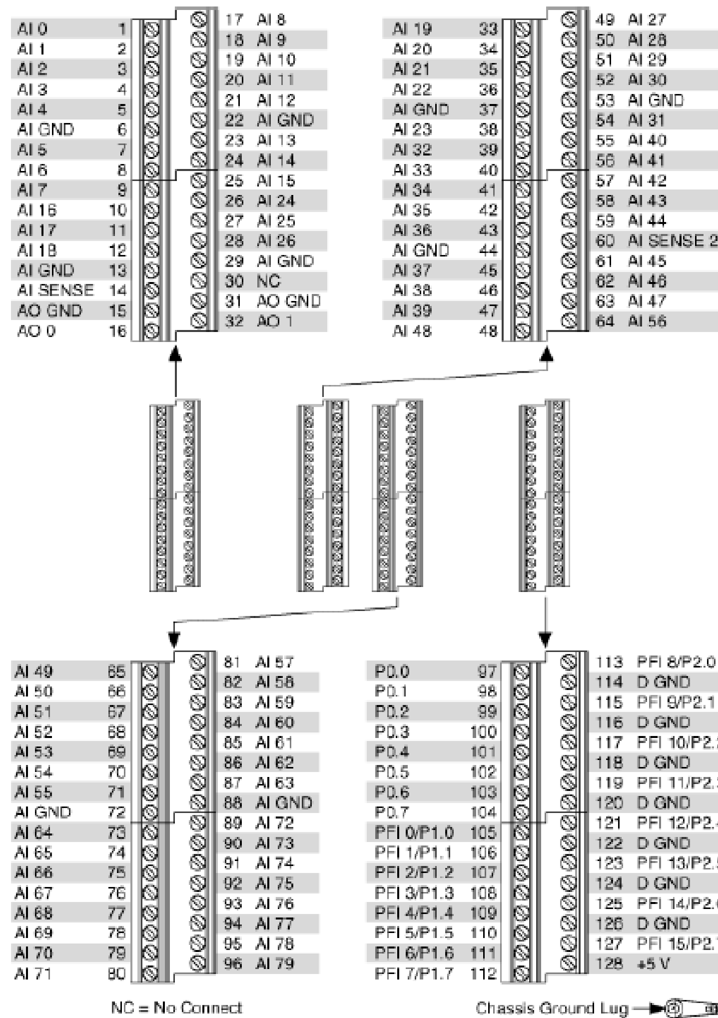


Figure 6.1. Screw terminal pinout for NI USB-6225 data logger

DAQ 1					
Sensor	DAQ channel	Channel #	Sensor	DAQ channel	Channel #
Ground	AI GND	6	4a	27	49
Temp	79	96	4b	28	50
RH	78	95	4c	29	51
SG 1	0	1	4d	30	52
SG 2	1	2	5a	31	54
SG 3	2	3	5b	32	39
SG 4	3	4	5c	33	40
SG 5	4	5	5d	34	41
SG 6	5	7	6a	35	42
SG 7	6	8	6b	36	43
SG 8	7	9	6c	37	45
SG 9	8	17	6d	38	46
SG 10	9	18	7a	39	47
SG 11	10	19	7b	40	55
SG 12	11	20	7c	41	56
SG 13	12	21	7d	42	57
SG 14	13	23	8a	43	58
SG 15	14	24	8b	44	59
pump	P0.0	97	8c	45	61
R 1	P0.1	98	8d	46	62
R 2	P0.2	99	9a	47	63
R 3	P0.3	100	9b	48	48
R 4	P0.4	101	9c	49	65
R 5	P0.5	102	9d	50	66
R 6	P0.6	103	10a	51	67
R 7	P0.7	104	10b	52	68
R 8	P1.0	105	10c	53	69
R 9	P1.1	106	10d	54	70
R 10	P1.2	107	11a	55	71
R 11	P1.3	108	11b	56	64
R 12	P1.4	109	11c	57	81
R 13	P1.5	110	11d	58	82
R 14	P1.6	111	12a	59	83
R 15	P1.7	112	12b	60	84
Ground	D GND	114	12c	61	85
			12d	62	86
1a	15	25	13a	63	87
1b	16	10	13b	64	73
1c	17	11	13c	65	74
1d	18	12	13d	66	75
2a	19	33	14a	67	76
2b	20	34	14b	68	77
2c	21	35	14c	69	78
2d	22	36	14d	70	79
3a	23	38	15a	71	80
3b	24	26	15b	72	89
3c	25	27	15c	73	90
3d	26	28	15d	74	91

DAQ 2					
Sensor	DAQ channel	Channel #	Sensor	DAQ channel	Channel #
Ground	AI GND	72	19a	24	26
SG 16	0	1	19b	25	27
SG 17	1	2	19c	26	28
SG 18	2	3	19d	27	49
SG 19	3	4	20a	28	50
SG 20	4	5	20b	29	51
SG 21	5	7	20c	30	52
SG 22	6	8	20d	31	54
SG 23	7	9	21a	32	39
SG 24	8	17	21b	33	40
SG 25	9	18	21c	34	41
SG 26	10	19	21d	35	42
SG 27	11	20	22a	36	43
pump	P0.0	97	22b	37	45
R 16	P0.1	98	22c	38	46
R 17	P0.2	99	22d	39	47
R 18	P0.3	100	23a	40	55
R 19	P0.4	101	23b	41	56
R 20	P0.5	102	23c	42	57
R 21	P0.6	103	23d	43	58
R 22	P0.7	104	24a	44	59
R 23	P1.0	105	24b	45	61
R 24	P1.1	106	24c	46	62
R 25	P1.2	107	24d	47	63
R 26	P1.3	108	25a	48	48
R 27	P1.4	109	25b	49	65
Ground	D GND	114	25c	50	66
			25d	51	67
16a	12	21	26a	52	68
16b	13	23	26b	53	69
16c	14	24	26c	54	70
16d	15	25	26d	55	71
17a	16	10	27a	56	64
17b	17	11	27b	57	81
17c	18	12	27c	58	82
17d	19	33	27d	59	83
18a	20	34			
18b	21	35			
18c	22	36			
18d	23	38			

Legend
load cells
thermocouples
relays

Figure 6.2. Wiring configuration for each data logger

6.2. LabVIEW program

Each VI consisted of a front panel and a block diagram. The front panel (Figure 6.3) shows all of the calculated values for the moisture contents, load cell voltages, bale weights, and bale temperatures, as well as indicators for the relays and a waveform chart of the moisture contents. The block diagram contains the data acquisition loop (Figure 6.4) and calculations necessary for the temperature (Figure 6.5), moisture content (Figure 6.6), and ambient temperature (Figure 6.7). The relays were controlled using the script and control loops shown in Figure 6.8 and Figure 6.9, respectively.

Figure 6.3. LabVIEW virtual instrument front panel

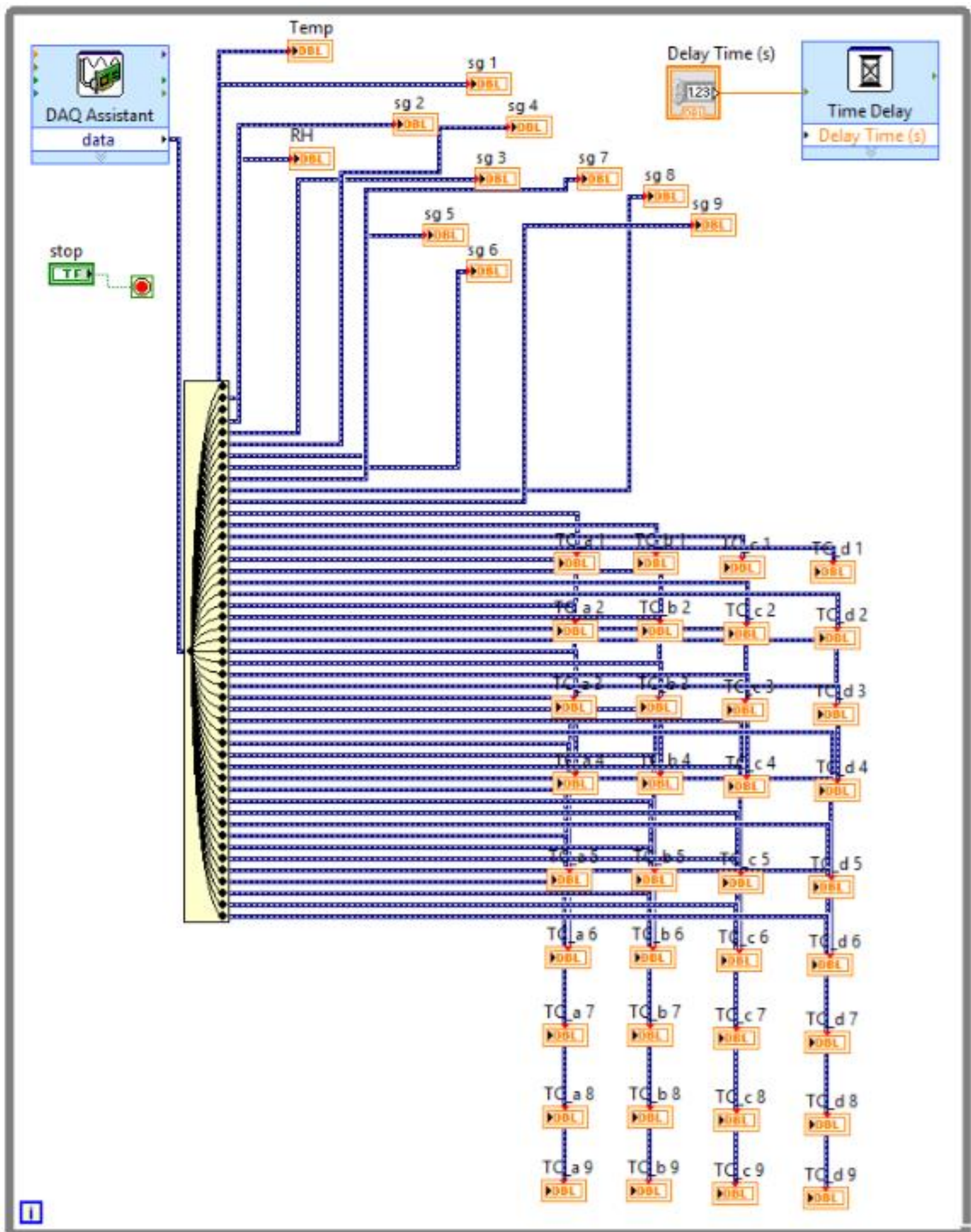


Figure 6.4. Data acquisition and signal splitting loop

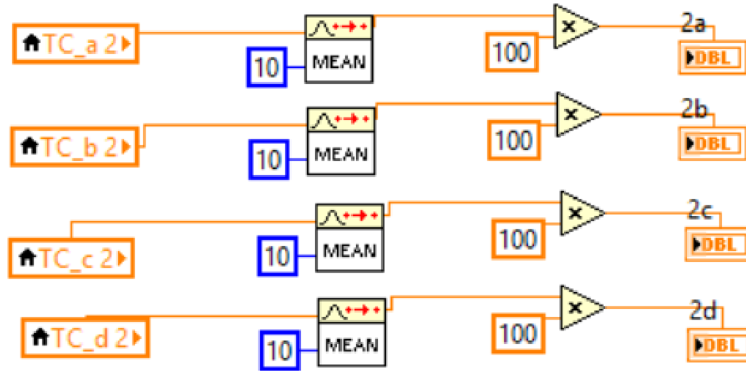


Figure 6.5. Thermocouple calculator

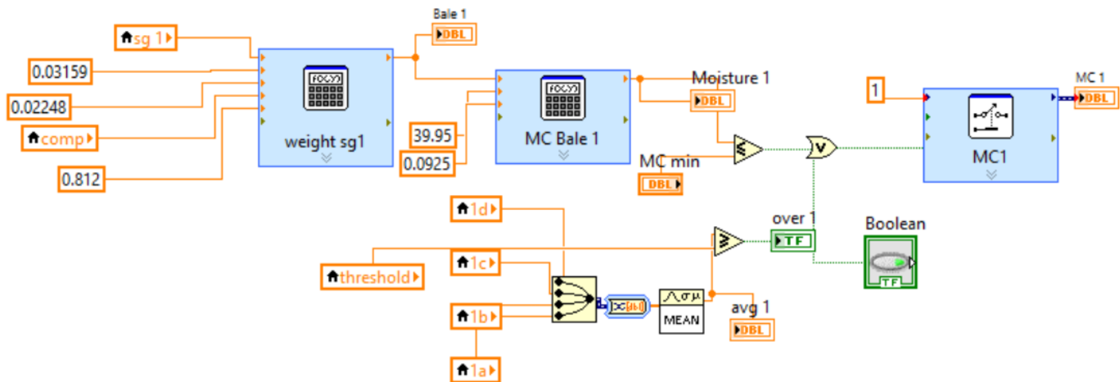


Figure 6.6. Moisture content and temperature average calculator

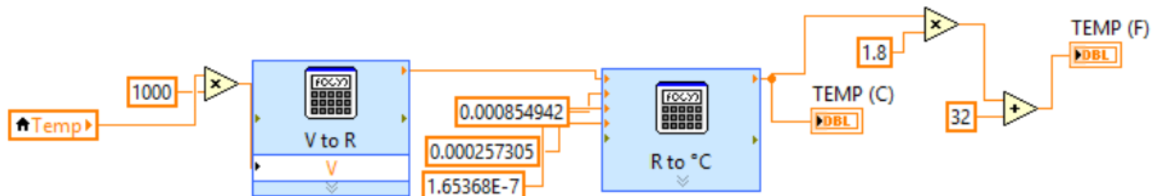


Figure 6.7. Ambient temperature calculator

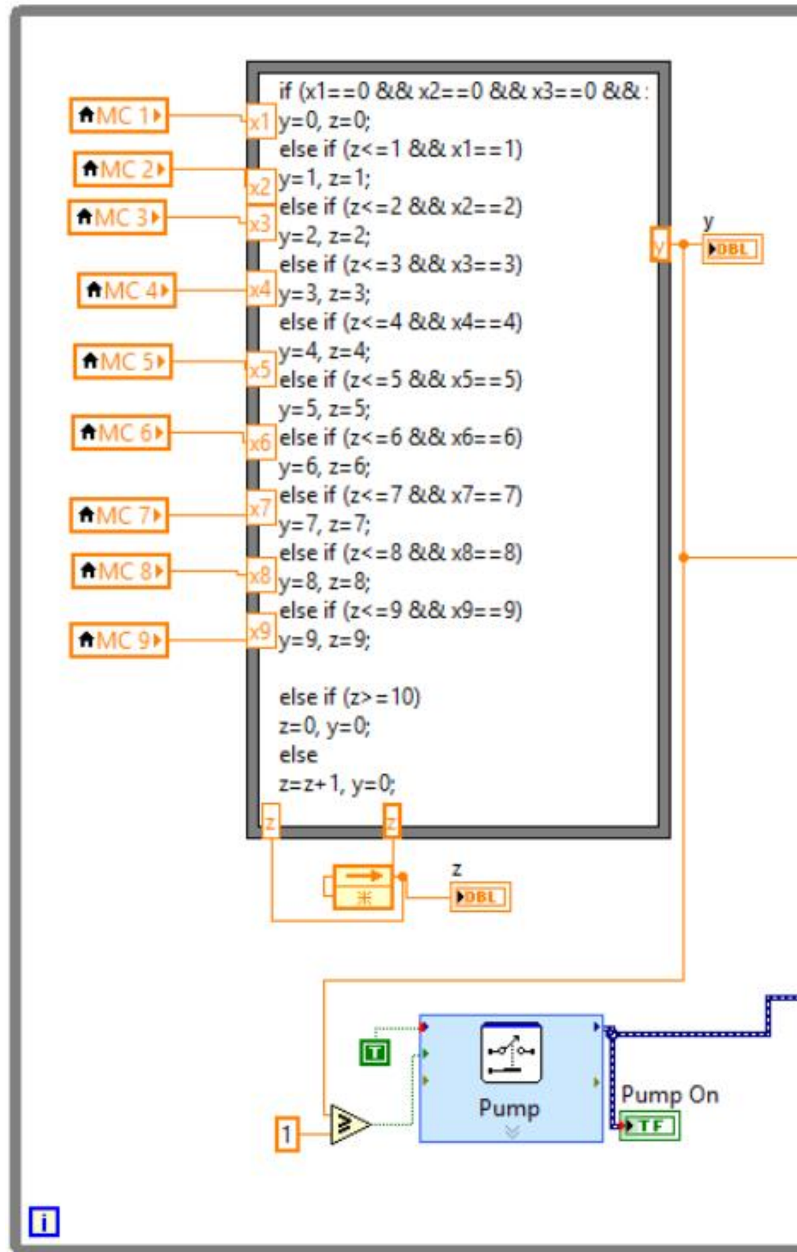


Figure 6.8. Relay loop control script box

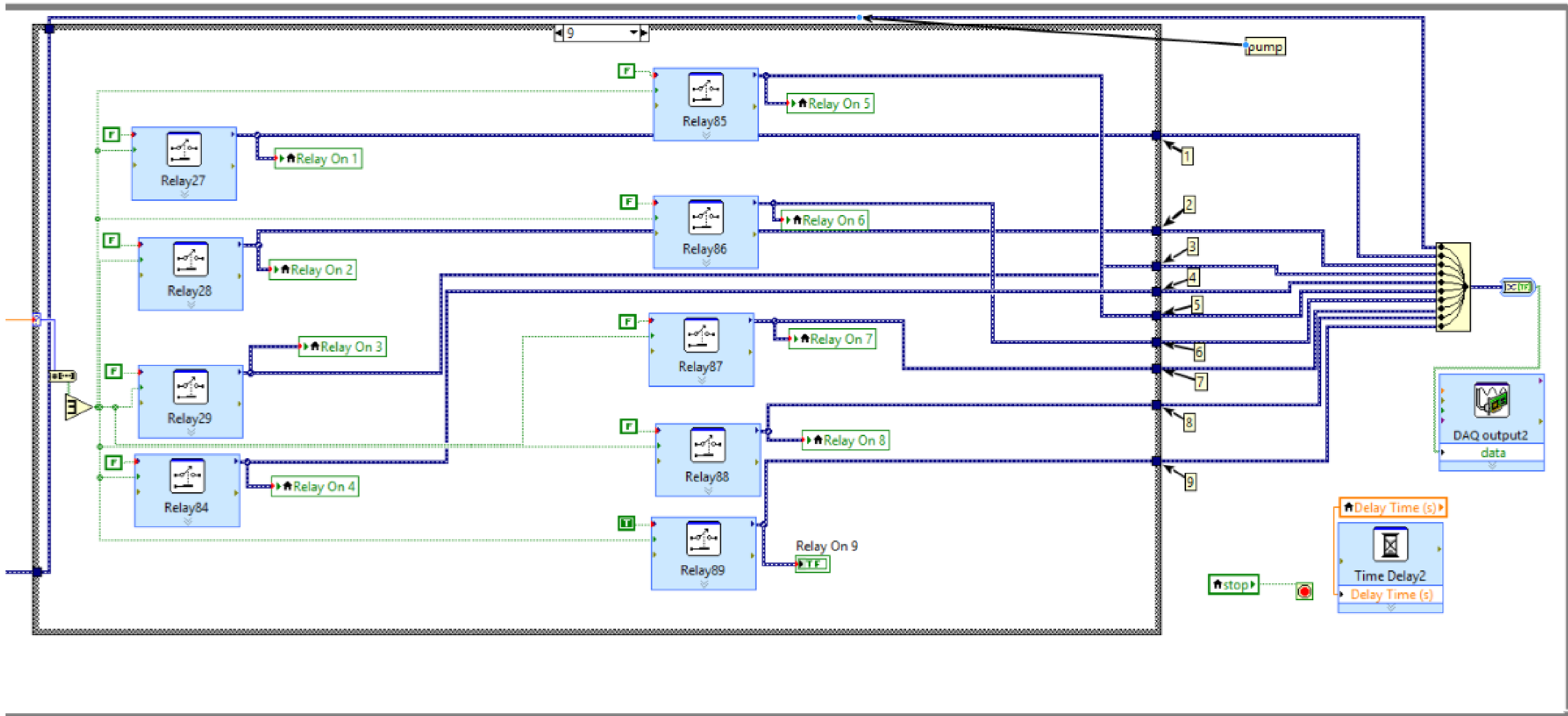


Figure 6.9. Relay control loop selector box

6.3. Excel VBA programming

Text messages were sent when the temperature was read above 55°C to warn that water may need to be added. The following Excel VBA program and the LabVIEW program loop shown in Figure 6.10 were used to send an email with this warning. The email address was formatted as a phone number, so that it would be received as a text message. Each bale had a VBA Sub program for determining the appropriate message to send.

```
Sub TextTemp16()
Application.WindowState = xlMinimized
   Dim OutApp As Object
   Dim OutMail As Object

   Set OutApp = CreateObject("Outlook.Application")
   Set OutMail = OutApp.CreateItem(0)

   On Error Resume Next

   With OutMail
      .To = "##########@mms.att.net"
      .CC = ""
      .BCC = ""
      '.Subject =
      .Body = "Temperature 16 is too high!!!"
      .Send
   End With
   On Error GoTo 0

   Set OutMail = Nothing
   Set OutApp = Nothing

Application.Quit
End Sub
```

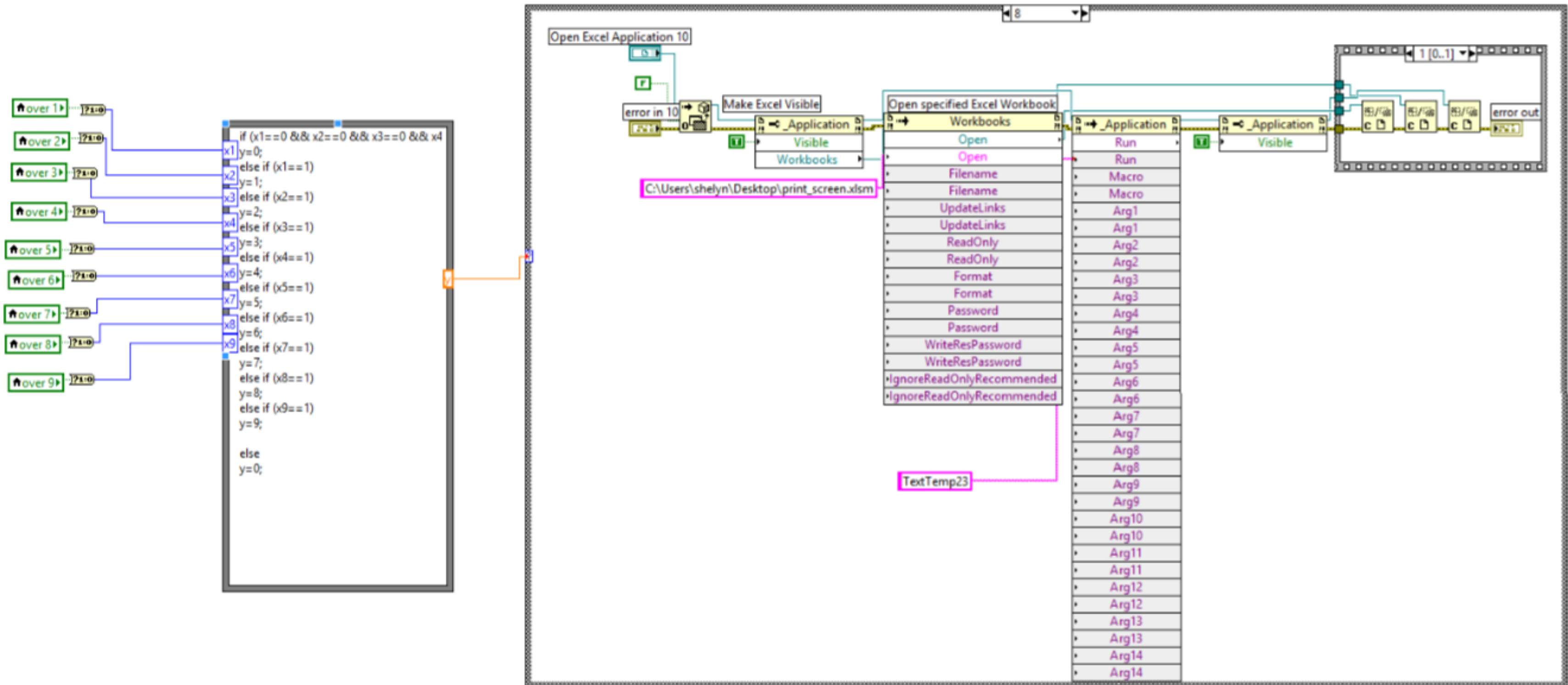


Figure 6.10. Text message LabVIEW program

6.4. MATLAB moisture content calculation

MATLAB was used to calculate the moisture content of the bales over time using the load cell voltage readings. Each set of bale voltage readings was sorted into a separate Excel file and the appropriate MATLAB script was executed.

Bales 1-9

```
sga = readtable('sg_data_a.xlsx');
sg1=sga{:,2};
sg2=sga{:,3};
sg3=sga{:,4};
sg4=sga{:,5};
sg5=sga{:,6};
sg6=sga{:,7};
sg7=sga{:,8};
sg8=sga{:,9};
sg9=sga{:,10};

w1=39.95;
w2=46.6;
w3=40.2;
w4=42.05;
w5=45.00;
w6=42.5;
w7=44.2;
w8=36.8;
w9=39.95;
c=21.036;
m0=0.0925;
m1=0.03159;
m2=0.03159;
m3=0.03158;
m4=0.03114;
m5=0.03132;
m6=0.03115;
m7=0.03141;
m8=0.03129;
m9=0.03118;
b1=0.02248;
b2=0.01758;
b3=0.02156;
b4=0.0297;
b5=0.02378;
b6=0.01393;
b7=0.08713;
b8=0.01925;
b9=0.01504;

s1=size(sga,1);
for i = 1:s1;
```

```
MC1(i,1)=((((sg1(i,1)-b1)/m1)-c)-w1+(w1*m0))/(((sg1(i,1)-b1)/m1)-
     c);
MC2(i,1)=((((sg2(i,1)-b2)/m2)-c)-w2+(w2*m0))/(((sg2(i,1)-b2)/m2)-
     c);
MC3(i,1)=((((sg3(i,1)-b3)/m3)-c)-w3+(w3*m0))/(((sg3(i,1)-b3)/m3)-
     c);
MC4(i,1)=((((sg4(i,1)-b4)/m4)-c)-w4+(w4*m0))/(((sg4(i,1)-b4)/m4)-
     c);
MC5(i,1)=((((sg5(i,1)-b5)/m5)-c)-w5+(w5*m0))/(((sg5(i,1)-b5)/m5)-
     c);
MC6(i,1)=((((sg6(i,1)-b6)/m6)-c)-w6+(w6*m0))/(((sg6(i,1)-b6)/m6)-
     c);
MC7(i,1)=((((sg7(i,1)-b7)/m7)-c)-w7+(w7*m0))/(((sg7(i,1)-b7)/m7)-
     c);
MC8(i,1)=((((sg8(i,1)-b8)/m8)-c)-w8+(w8*m0))/(((sg8(i,1)-b8)/m8)-
     c);
MC9(i,1)=((((sg9(i,1)-b9)/m9)-c)-w9+(w9*m0))/(((sg9(i,1)-b9)/m9)-
     c);
end

m_A = table(MC1,MC2,MC3,MC4,MC5,MC6,MC7,MC8,MC9);
writetable(m_A, 'moisture_content_a.dat');
```

Bales 10-15

```
sgb = readtable('sg_data_b.xlsx');
sg10=sgb{:,2};
sg11=sgb{:,3};
sg12=sgb{:,4};
sg13=sgb{:,5};
sg14=sgb{:,6};
sg15=sgb{:,7};

w10=41.8;
w11=40.75;
w12=41.5;
w13=40.8;
w14=48.55;
w15=42.3;
c=21.036;
m0=0.0925;
m10=0.03115;
m11=0.03152;
m12=0.03156;
m13=0.03135;
m14=0.03078;
m15=0.03094;
b10=0.04073;
b11=0.02081;
b12=0.01281;
b13=0.01833;
b14=0.02218;
b15=0.00707;

s2=size(sgb,1);
for i=1:s2;
```

```
MC10(i,1)=((((sg10(i,1)-b10)/m10)-c)-w10+(w10*m0))/(((sg10(i,1)-
     b10)/m10)-c);
MC11(i,1)=((((sg11(i,1)-b11)/m11)-c)-w11+(w11*m0))/(((sg11(i,1)-
     b11)/m11)-c);
MC12(i,1)=((((sg12(i,1)-b12)/m12)-c)-w12+(w12*m0))/(((sg12(i,1)-
     b12)/m12)-c);
MC13(i,1)=((((sg13(i,1)-b13)/m13)-c)-w13+(w13*m0))/(((sg13(i,1)-
     b13)/m13)-c);
MC14(i,1)=((((sg14(i,1)-b14)/m14)-c)-w14+(w14*m0))/(((sg14(i,1)-
     b14)/m14)-c);
MC15(i,1)=((((sg15(i,1)-b15)/m15)-c)-w15+(w15*m0))/(((sg15(i,1)-
     b15)/m15)-c);
end

m_B = table(MC10,MC11,MC12,MC13,MC14,MC15);
writetable(m_B, 'moisture_content_b.dat');
```

Bales 16-18

```
sgc = readtable('sg_data_c.xlsx');
sg16=sgc{:,2};
sg17=sgc{:,3};
sg18=sgc{:,4};

w16=42.4;
w17=41.2;
w18=46.1;
c=21.036;
m0=0.0925;
m16=0.03127;
m17=0.03119;
m18=0.03104;
b16=0.01834;
b17=0.03086;
b18=0.02159;

s3=size(sgc,1);
for i = 1:s3
MC16(i,1)=((((sg16(i,1)-b16)/m16)-c)-w16+(w16*m0))/(((sg16(i,1)-
     b16)/m16)-c);
MC17(i,1)=((((sg17(i,1)-b17)/m17)-c)-w17+(w17*m0))/(((sg17(i,1)-
     b17)/m17)-c);
MC18(i,1)=((((sg18(i,1)-b18)/m18)-c)-w18+(w18*m0))/(((sg18(i,1)-
     b18)/m18)-c);
end

m_C = table(MC16,MC17,MC18);
writetable(m_C, 'moisture_content_c.dat');
```

Bales 19-27

```
sgd = readtable('sg_data_d.xlsx');
```

```
sg19=sgd{:,2};
sg20=sgd{:,3};
sg21=sgd{:,4};
sg22=sgd{:,5};
sg23=sgd{:,6};
sg24=sgd{:,7};
sg25=sgd{:,8};
sg26=sgd{:,9};
sg27=sgd{:,10};

w19=52.7;
w20=41.00;
w21=43.4;
w22=52.6;
w23=43.35;
w24=42.1;
w25=39.95;
w26=48.55;
w27=42.25;
c=21.036;
m0=0.0925;
m19=0.03129;
m20=0.0312;
m21=0.03144;
m22=0.03125;
m23=0.03136;
m24=0.0313;
m25=0.03129;
m26=0.03141;
m27=0.03113;
b19=0.02167;
b20=0.03775;
b21=0.02725;
b22=0.02354;
b23=0.04845;
b24=0.04362;
b25=0.06206;
b26=0.06303;
b27=0.02986;

s4=size(sgd,1);
for i = 1:s4
MC19(i,1)=((((sg19(i,1)-b19)/m19)-c)-w19+(w19*m0))/(((sg19(i,1)-
     b19)/m19)-c);
MC20(i,1)=((((sg20(i,1)-b20)/m20)-c)-w20+(w20*m0))/(((sg20(i,1)-
     b20)/m20)-c);
MC21(i,1)=((((sg21(i,1)-b21)/m21)-c)-w21+(w21*m0))/(((sg21(i,1)-
     b21)/m21)-c);
MC22(i,1)=((((sg22(i,1)-b22)/m22)-c)-w22+(w22*m0))/(((sg22(i,1)-
     b22)/m22)-c);
MC23(i,1)=((((sg23(i,1)-b23)/m23)-c)-w23+(w23*m0))/(((sg23(i,1)-
     b23)/m23)-c);
MC24(i,1)=((((sg24(i,1)-b24)/m24)-c)-w24+(w24*m0))/(((sg24(i,1)-
     b24)/m24)-c);
MC25(i,1)=((((sg25(i,1)-b25)/m25)-c)-w25+(w25*m0))/(((sg25(i,1)-
     b25)/m25)-c);
```

```
MC26(i,1)=((((sg26(i,1)-b26)/m26)-c)-w26+(w26*m0))/(((sg26(i,1)-
     b26)/m26)-c);
MC27(i,1)=((((sg27(i,1)-b27)/m27)-c)-w27+(w27*m0))/(((sg27(i,1)-
     b27)/m27)-c);
end

m_D = table(MC19,MC20,MC21,MC22,MC23,MC24,MC25,MC26,MC27);
writetable(m_D, 'moisture_content_d.dat')
```

6.5. Bale temperature

The ambient temperature and relative humidity are shown in Figure 6.11. The internal temperatures for Bales 1-9, 10-18, and 19-27 are shown in Figure 6.12, Figure 6.13, and Figure 6.14, respectively. Each data point represents a 1 h average.

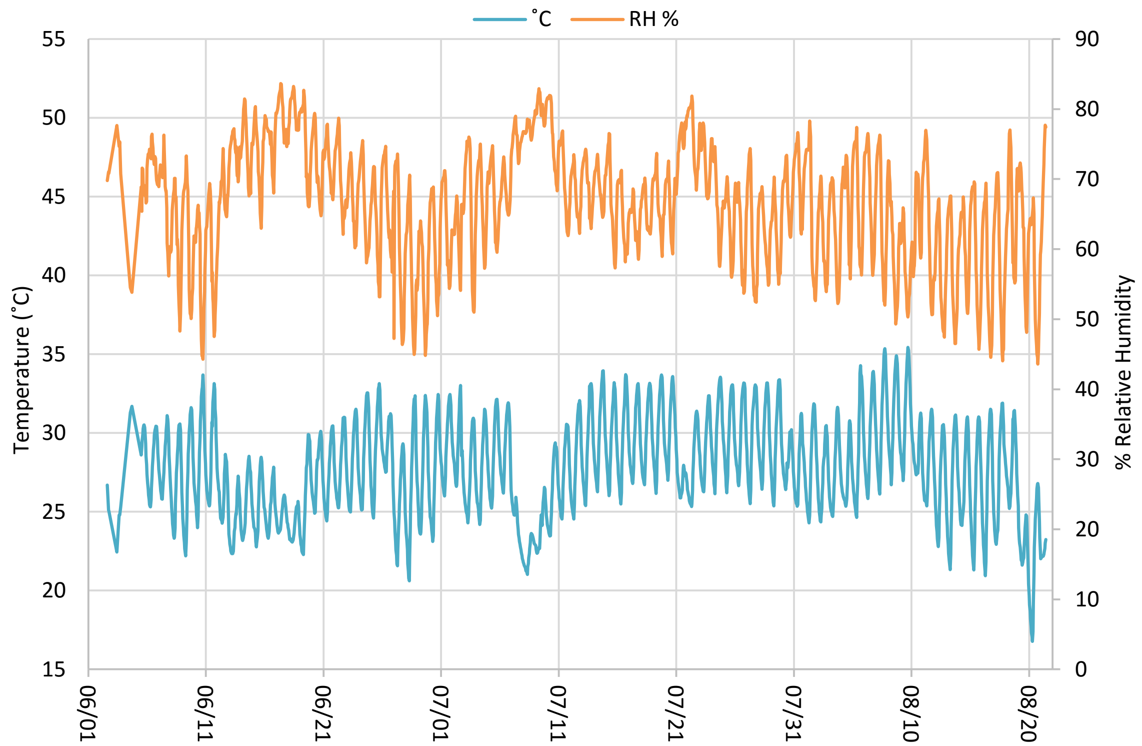


Figure 6.11. Ambient temperature and relative humidity over time

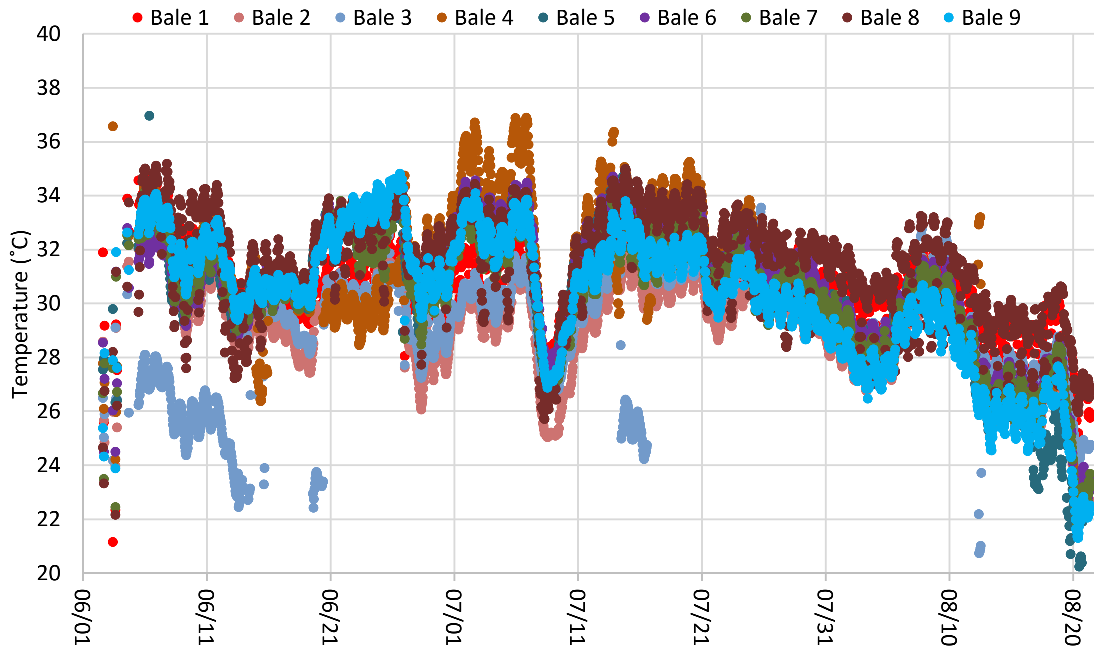


Figure 6.12. Bales 1-9 internal temperatures (81 d)

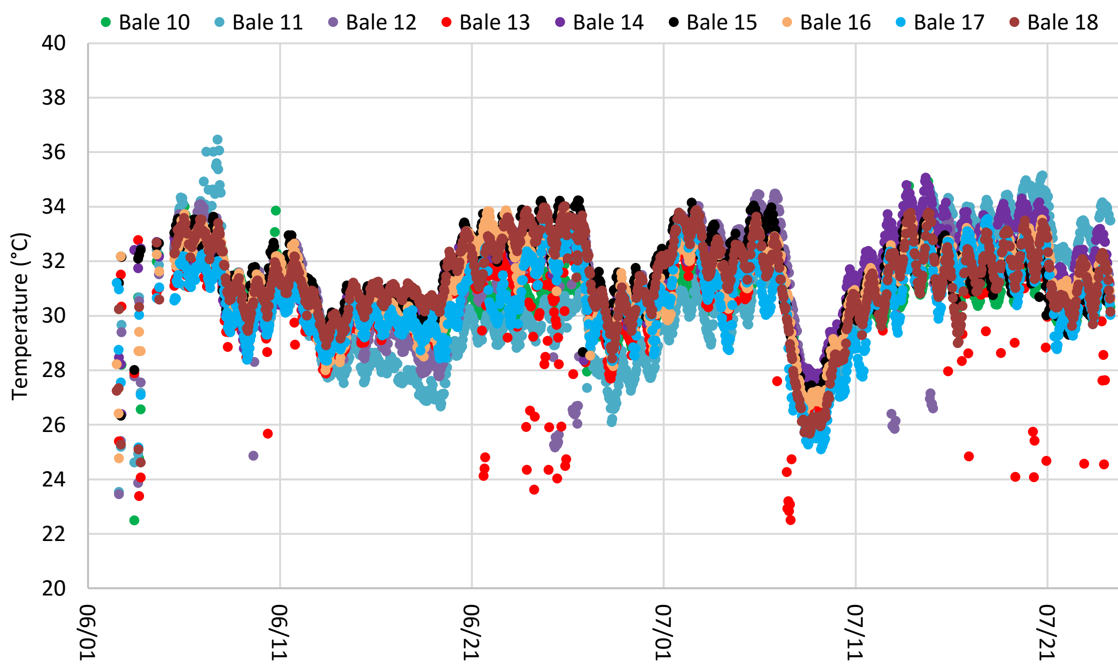


Figure 6.13. Bales 10-18 internal temperatures (53 d)

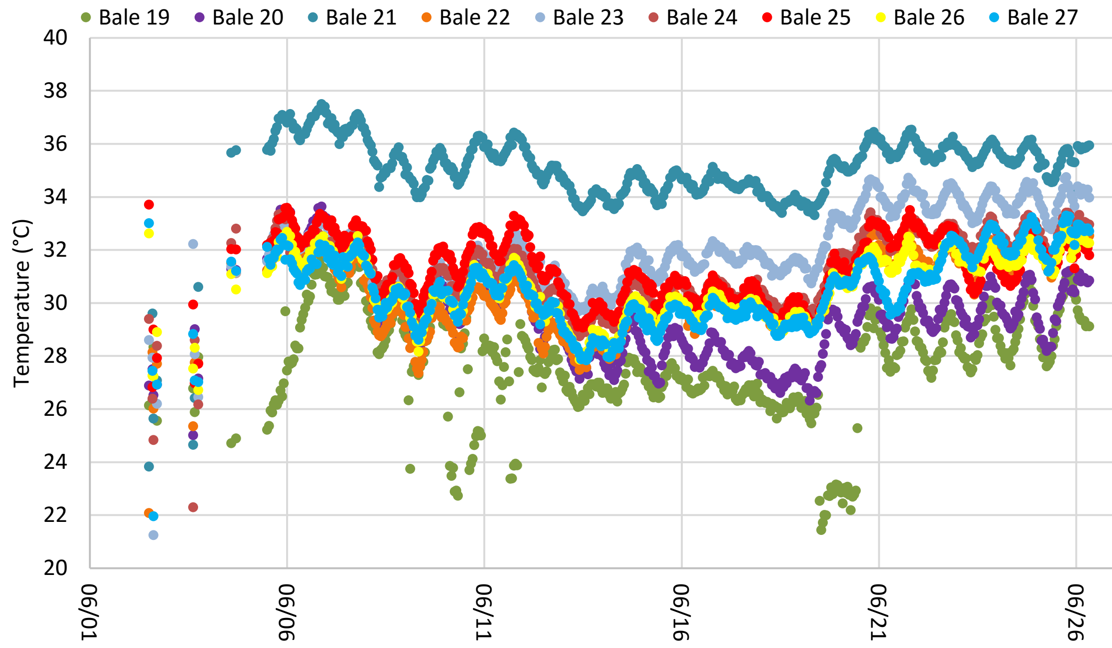


Figure 6.14. Bales 19-27 internal temperatures (25 d)

6.6. Storage and sampling equipment

The SolidWorks drawing used to construct the bale splitting stand is shown in Figure 6.15. Plywood was used for platform, which was attached to the bar indicated by the arrows. The bale storage racks were constructed using the drawing in Figure 6.16. Figure 6.17 shows an angled view of the bale storage rack. The bale supports used to hold the bales together with ratchet straps are shown in Figure 6.18 and Figure 6.19. The core tube used for sampling is shown in Figure 6.20.

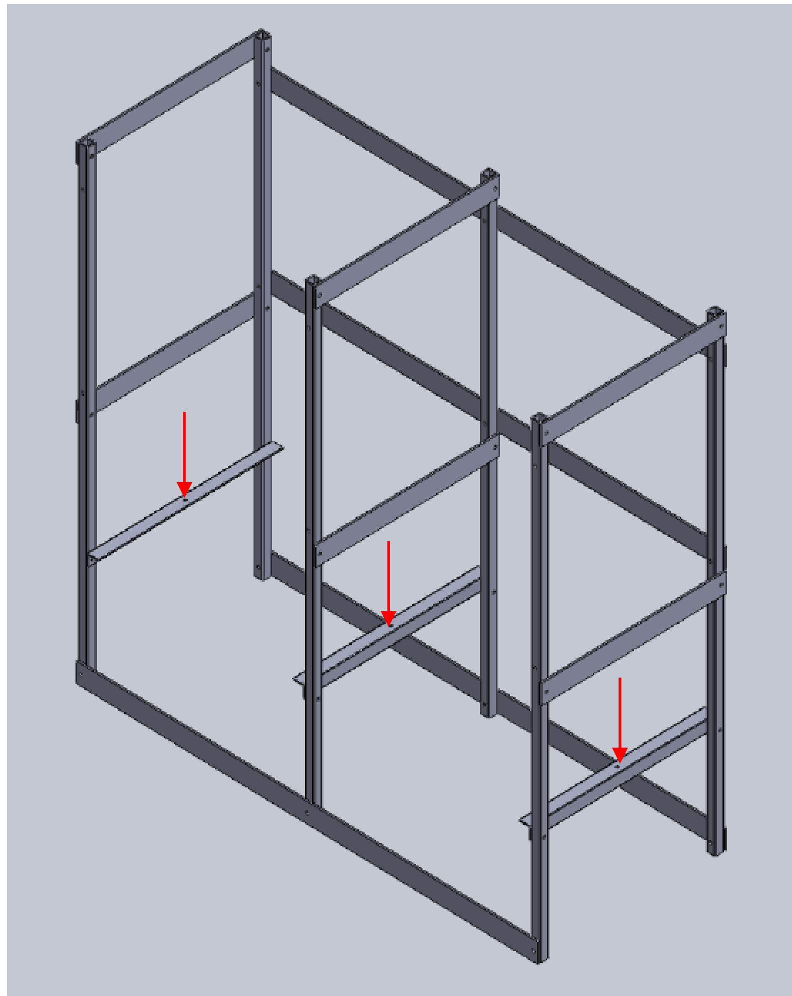
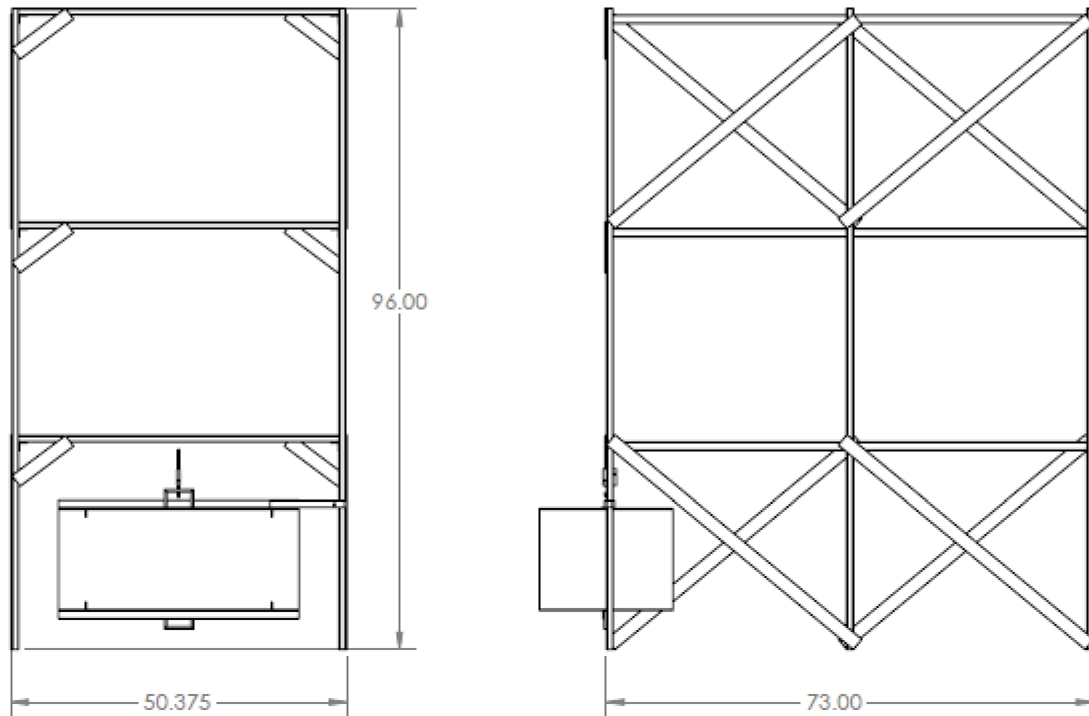


Figure 6.15. Bale splitting stand for fungal inoculation, plywood platform attached at arrows



ITEM NO.	PART NUMBER	DESCRIPTION	QTY.
1	supportBeam	1" x 96" Quare Tubing	6
2	angleIronSupport	1" x 73" Angle Iron	6
3	hangingBeam	1" x 48" square tubing	9
4	brace	3/16" x 2" x 10" Flat Plate	9
5	sideBrace	3/16" x 2" x 48" Flat plate	8
6	turnStop		1

Figure 6.16. SolidWorks drawing of the bale storage rack, with dimensions (inches)

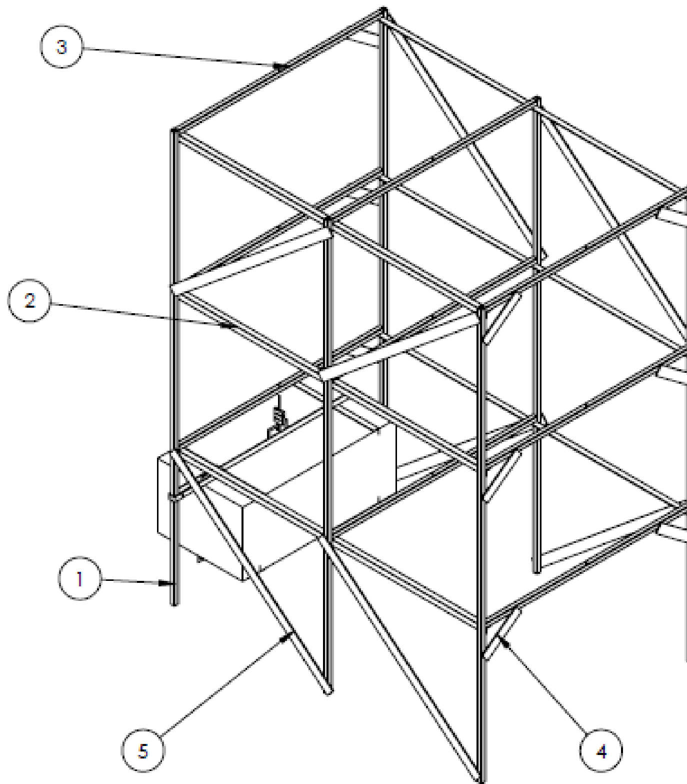
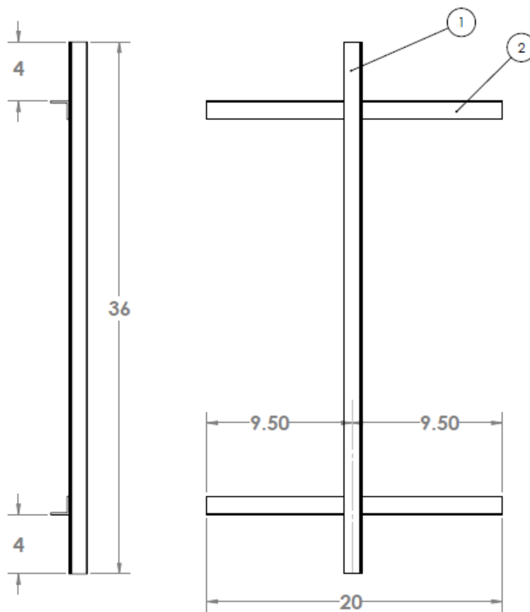
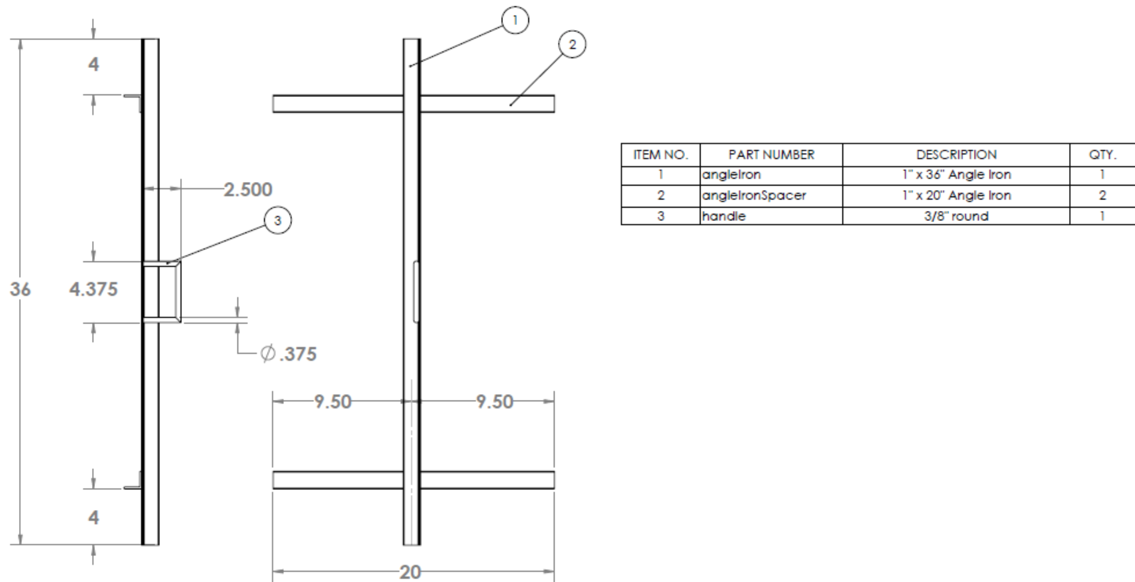


Figure 6.17. Angled view of the bale storage rack



ITEM NO.	PART NUMBER	DESCRIPTION	QTY.
1	angleIron	1" x 36" Angle Iron	1
2	angleIronSpacer	1" x 20" Angle Iron	2
3	handle	3/8" round	1

Figure 6.18. Bottom bale support drawing, dimensions in inches



ITEM NO.	PART NUMBER	DESCRIPTION	QTY.
1	angleIron	1" x 36" Angle Iron	1
2	angleIronSpacer	1" x 20" Angle Iron	2
3	handle	3/8" round	1

Figure 6.19. Top bale support drawing, dimensions in inches

Figure 6.20. Core sampling tube SolidWorks drawing, dimensions in inches

VITA

Shelyn Gehle Slavens

Candidate for the Degree of

Master of Science

Thesis: INVESTIGATION OF *PLEUROTUS OSTREATUS* PRETREATMENT ON SWITCHGRASS FOR ETHANOL PRODUCTION

Major Field: Biosystems Engineering

Biographical:

Grew up in Wann, Oklahoma; daughter of Jeff Gehle and Shelly Honeycutt; graduated from Oklahoma Union High School in So. Coffeyville, Oklahoma.

Education:

Completed the requirements for the Master of Science in Biosystems Engineering at Oklahoma State University, Stillwater, Oklahoma in July 2016.

Completed the requirements for the Bachelor of Science in Biosystems Engineering at Oklahoma State University, Stillwater, Oklahoma in May 2014.

Experience:

Graduate Research Assistant, Oklahoma State University, Stillwater, OK, August 2014 to July 2016.

Undergraduate Research Assistant, Oklahoma State University, Stillwater, OK, October 2013 to July 2014.

Undergraduate Research Assistant, National Renewable Energy Laboratory, Golden, CO, May to August 2013.

Professional Memberships:

American Society of Agricultural and Biological Engineers (ASABE)