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Storage of Round and Square Switchgrass Bales: Effect of Storage Time and Fungal Inoculation on Saccharification Efficiency

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Storage of Round and Square Switchgrass Bales: Effect of Storage Time and Fungal Inoculation on Saccharification Efficiency

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Abstract

To produce fermentable sugars from lignocellulosic biomass feedstock, severe pretreatment conditions are needed (either high acid concentration, temperature, or retention times). High severities can produce toxic byproducts which inhibit enzymatic hydrolysis or fermentation. In order to reduce pretreatment severities (and thus increase enzyme and fermentation efficiency), the white-rot fungus *Pleurotus ostreastus* was seeded into square and round bales of Kanlow switchgrass (*Panicum virgastum* L.) and left in the field over a period of 9 month. The laccase producing fungus is believed to selectively degrade lignin, a common plant structural polymer, which can function as an enzymatic inhibitor. Samples were taken from different elevations within the bale 3, 5, 7, and 9 months after harvesting. These samples were treated at three different severities with liquid hot water pretreatment. Compositional analysis was done on the pretreated biomass, which was then enzymatically hydrolyzed with cellulases (endoglucanase and beta-glucosidase) after being washed. The yields (total recovered sugars over total present) were calculated and compared along five different variables: fungal treatment, storage time, pretreatment severity, sampling depth, and washing volumes. The results of the study found significant effects for sampling time ($p=.0024$) and pretreatment severity ($p<.0001$), but found no such significance in the effects of washing ($p=.6624$) and sampling depth ($p=.0693$). Results regarding the fungal inoculation were inconclusive, but provided the basis for the creation of experiments to be carried out in future work.

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Contents

Table of Figures

List of Tables

1. Introduction

The current production of ethanol in the United States is done through the fermentation of sugars derived from starch or sucrose, mostly from corn (Bothast et al., 2005). However ethanol derived from food sources such as corn has several negative consequences on food prices and land and water resource use, such as land and water (Searchinger et al., 2008). One alternative solution to these 'first generation' attempts at producing biofuel from corn is the usage of cellulosic sources for the sugar needed for fermentation, known as 'second generation' biofuel (Sims et al., 2010). Cellulosic sources, such as wood or grasses, do not compete with food crops for resources and are a more sustainable approach to satisfy the demand for renewable fuels. Native plants which require little irrigation or maintenance are ideal feedstock sources for second generation biofuels, as such material does not compete or consume as many resources during cultivation as food crops do. If such a biomass source could be used then many of the issues with first generation biofuel could be solved.

However, lignocellulosic biomass, such as switchgrass,displays a tightly woven plant cell wall that contains lignin. Lignin is a complex polyphenol molecule composed of multiple subunits which can vary between plant species (Ruiz-Duenas et al., 2009). The role of lignin in the plant cell walls protect the cellulose fibers from hydrolytic attack, which is the process intrinsic to second-generation biofuels manufacturing to extract fermentable sugars from biomass. Unlike cellulose, lignin has a unique composition from species to species. Lignin can be extracted from biomass separately from cellulose and hemicellulose. A technique common to the pulping and forestry industries uses low-boiling solvents and high temperatures to separate out the fundamental components of biomass (Quesada-Medina et al. 2010). Though these techniques were first developed for woody biomass, the basic principles apply across all types,

including herbaceous crops. With solvents, such as acetone and ethanol, combined with sufficiently long reflux times, lignin can be removed from plant cell walls (Obst et al., 1998). The extracted lignin produced though solvent extraction is known as milled wood lignin. Additional extractives, like oils and other plant metabolites, are also present in the lignin-containing fractions. Lignin removal, or isolation, can be achieved using more advanced techniques, such as centrifugal fractionation. However if solvents, such as alkaline sodium sulfide, or white liquor, are used, the lignin recovered from the biomass separation is considered to be physically different from the lignin within the biomass; this new configuration of lignin is called Kraft lignin (Obst et al., 1988). It is important to note that most lignin extraction techniques do not remove all of the lignin in one pass, as the variety of lignin components within a given biomass require sequential extraction techniques.

The National Renewable Energy Laboratory has methods for the determination of total lignin content of biomass, which can provide a baseline for further study. This technique, as highlighted in Sluiter and Sluiter 2010, does not determine which types of lignin are present or what their properties may be. Instead it provides a mass of lignin present in the biomass. Further detailed characterization and quantification requires a multitude of analytical techniques, such as nuclear magnetic resonance, gas chromatography, and mass spectrometry. A variety of detection techniques can be employed to determine the structure and property of different lignin types. Through analysis, typically mass spectrometry, the structural units that compose lignin (known as H, G, and S types) can be found and the ratio of each type within biomass can be used as a basis for classification (Crestini et al., 2011). The bonds between lignin subunits can be broken by a multitude of techniques and analyzed by gas chromatography. However this separation

technique only separates the major subunits and does not fully quantify the nature of the lignin molecule (Parkas et al., 2007).

As lignin degradation compounds are considered as the leading cause of biomass recalcitrance in second-generation biofuel production platform, much research has focused on how to neutralize or remove lignin present in the biomass. The pulp and paper industry has decades of experience in the field of removing lignin and detoxifying plant biomass processing streams via Kraft pulping (Bajpai et al., 2012). The problems faced in the pulp and paper industry such as large (over 50%) amounts of biomass waste, transportation, and storage are well known and are similar to the obstacles faced by second generation biofuel plants (Towers et al. 2007).

However, many lignin extraction techniques exist, which can be applied as part of the biomass pretreatment, depending on the results of the lignin characterization. For example, laccases, a common class of enzyme with lignin degrading properties, do not act on the lignin molecule itself, but rather the on the subunits which make up the compound (Chen et al., 2012). Different types of laccase have preference for different subunits, and without proper quantification it would be impossible to determine and characterize these enzymes, or perform the necessary pretreatment procedure. In order for a good pretreatment or enzymatic hydrolysis step to take place, it will be necessary to remove the types of lignin which are specifically inhibitory towards cellulases as well as to understand the chemical alterations that occur to the lignin after chemical pretreatment.

Likewise, genetic modification of species with high biomass productivity to alter their lignin content (usually in order to lower it) requires accurate and precise measurements of the nature of the lignin within the plant cell wall (Lubieniechi et al., 2013). Delignification as a whole is not

considered as important as removing the right types of lignin structures, which again requires sufficiently advanced detection mechanisms to gauge the effectiveness of any given pretreatment. For example, the ratio between G and S lignin can have significant effects on the saccharification of switchgrass (though this does not mean that this relationship holds true for all biomass). These changes do not always effect plant physiology in obvious ways, and thus more advanced detection methods are needed (Fu et al., 2011).

Recalcitrance of biomass can be addressed through lignin removal, which results in a cellulose and hemicellulose matrix ready for glucose release through saccharification (Du et al., 2010, Zeng et al. 2014). The saccharifiedstream, containing glucose and xylose-containing fermentable sugars, can be useful as a source for fermentation. The nature of pretreatments, which are essential prior to saccharification, can vary widely both in terms of cost and efficiency. These pretreatment technologies can significantlyalter biomass, resulting in ensuing processing streams that are laden with chemicals, which are toxic to further downstream processing steps that require yeast or other fermenting organisms. These chemicals or byproducts are also inhibitory to saccharification enzymes (Rajan and Carrier, 2014). This poses an additional challenge to the second-generation biofuel platform, as not only must cellulose must be saccharified into glucose, but the unwanted byproducts formed during the process must also be removed if the process is to be successful in generating ethanol or other biobased products (Chandel et al., 2011).

There exists numerous pretreatment technologies, with one such pretreatment process being liquid hot water pretreatment (LHW). A LHW pretreatment consists of holding a processed amount of biomass at a high temperature and controlled pH for a set amount of time(Kim et al., 2009). LHW opens the tightly woven plant cell wall through the solubilization

of hemicellulos, resultingin cellulose being more receptive to enzymatic digestion or saccharification. Unfortunately, LHW pretreatment produces unwanted byproducts, with furans and furan derivatives being primary products. Concentration of furan and furan derivatives in pretreatment hydrolyzate can be decreased by reducing LHW temperature; however, doing so also decreases the eventual recovery of glucose, as the plant cell wall has not released as much hemicellulose, decreasing the ability of the enzymes to saccharify cellulose.On the other hand, decreasing pretreatment temperatures and processing times, often referred to as a combined severity factor (Abatzoglou et al. 1992), represent an energy cost for the process. If these processing temperatures and times could be reduced, making the overall biomass deconstruction process less energy intensive, but without a decrease in saccharification yields, the complete process would be more sustainable.

An approach to reducing pretreatment severity while still achieving 70%-90% glucose recovery, is to modify the composition of the biomass prior to pretreatment, by selectively removing lignin or hemicellulose. To this end a number of different strategies have been attempted, among them the use of lignin and hemicellulose degrading fungi to digest those parts of the biomass while leaving the cellulose fraction untouched (Gupta et al., 2011). The overarching goal of this research project is to design a processing stream that would enable saccharification of biomass at a low pretreatment severity, through a combination of pretreatment and storage technology.

2. Literature review

2.1 Fungal pretreatment

Both bacterial and fungal organisms can degrade lignin, though fungal degradation has been shown to be the superior mechanism in terms of yields and thus is the primary area of focus for research (Sakdaronnagong et al.2012). Fungal influence on cellulosic biomass has been studied, both on the large (farm) scale and small (lab).Farm studies tended to only look at composition of biomass after storage with biofuels not as a primary focus for the research. Fungal pretreatment tends to cause decreases in total organic matter, but increases the cellulose component of the biomass by selectively degrading lignin (Gupta et al.,2011). This lignin degradation can be accompanied by holocellulose degradation as well. While cellulose content may be increased by the removal of lignin, the total amount of cellulose can only either decrease or remain constant. Effective fungal pretreatment relies on optimizing the species and incubation conditions in order to increase overall cellulose content as well as total yields.

The use of fungus for biomass pretreatment has been reported for wheat straw. Interestingly, wheat straw is very similar to switchgrass in terms of composition (AFDC Biomass database). Wheat straw and switchgrass lignin have similar S/G ratio, corresponding to the ratio of S, syringyl phenylpropanoid units, and G, guaiacyl subunits found in lignin polymers. S/G ratios of 0.63 for wheat straw and 0.68 for switchgrass have been reported (Zeng et al.,2013 and Huet al., 2010).

Studieswith the fungi*Pleurotus. ostreatus* have shown that carbohydrate lossis slower when conducted in a laboratory scale environment with only 5% carbohydrate loss after 63 days (Vane et al.,2001), as compared to 42% loss reported for field environments (Adamovicet al. 1998). In rice straw, *P. ostreatus* degraded 48% of the hemicellulose present in the control group over a period of 48 days (Taniguchiet al.,2005). A similar result was reported with 52% degradation of

hemicellulose over 21 days on the laboratory scale, with only 22% cellulose and 27% lignin degradation (Salvachua, 2011). This indicates that *P. ostreatus* has, across multiple types of biomass and growing conditions, a preference for the degradation of hemicellulose. This may be attributed to competing organisms that are able to degrade other parts of the cell wall after the fungus removes the lignin, as the digestibility of the biomass as a whole is lowered (Zadrazilet al.,2011).

Relative to other white rot fungi*P. ostreatus* was found to be more selective towards lignin by virtue of not degrading as much cellulose as other fungus, which could be up to 58% for some other tested species such as *P. chysoporium* (fast growing) and *T. versicolor* (Taniguchiet al., 2005). Overallyields of fungal pretreated biomass, however, have to account for the lost saccharification potential as a result of the degradation of carbohydrates, as well as the expensive cost of fungal spawn (Sainoset al., 2006). While up to 52% of the available cellulose was solubilized, only 33% of the total possible glucose was recovered; the low glucose recovery could possibly be attributed to degradation of cellulose by *P. ostreatus*during pretreatment, making fungal pretreatment alone unfeasible for biofuel production despite its low cost (Taniguchiet al., 2005).

A multitude of other fungi have also been used for the removal of lignin from biomass. Some examples include *Phanerochaete chrysosporium, Ceriporiopsis subvermispora, Coriolus versicolor*, and *Pleurotus eryngi*, on substrates such as corn stalks, cotton residues, bamboo, beech wood, and wheat straw (Sawada et al.1995, Itohet al. 2003, Zhanget al. 2007, Camareroet al. 1994, Hattakaet al. 1983). Quantities of lignin removed in each case were both species and substrate dependent, and the types of lignin removed (when such analysis was done) also varied between species and substrate (Camarero et al.,1994). Saccharification yields were increased

between 1.6 (on bamboo) and 2.3 (on beech wood) times the amount that could be achieved without pretreatment (Itoh et al.2003, Zhanget al. 2007). Camarero et al., 1994, reported that, although lignin degrading peroxidase enzymes were not known to be produced by the *Pleurotus* family of fungi, there was still preferential degradation of phenolic lignin by the species.

The decomposition of lignin is accomplished by the white-rot fungi *Basidiomycetous* (Lundellet al., 2010). This is accomplished through the secretion of a multitude of metabolizing proteins and acids, among them being peroxidases and laccases. In biological pretreatment, either the fungus itself or laccases/peroxidases derived from fungi typically are used, followed by an alkaline wash (Heap et al.2014). This wash step is considered to be additional pretreatment, and is not the same as washing procedure proposed by the National Renewable Energy Laboratories (NREL) (Kelleret al. 2002).

Basidiomycete's ligninolytic activity is largely a function of the lignin peroxidases (LiP) as a correlation exists between the expression of LiP and weight loss in biomass. It is suspected that LiP can account for an extra 17.6 milligramsper gram of released reducing sugars during enzyme hydrolysis per gram of pretreated biomass, compared to no correlation to active laccases (Pinto et al.2012). Laccase activity has not as of yet been correlated to greater amounts of saccharification in enzymatic hydrolysis.

The degradation reaction provides additional benefits to biomass prior to pretreatment and saccharification such as reduction in particle size and changes in biomass composition which can improve enzymatic hydrolysis yields. Fungal pretreatment can also reduce the quantity of organic acids, a known enzyme inhibitor (Balan et al.2008). Additionally, some studies on *Arabidopsis*have shown that the degradation of lignin is not proportional to future saccharification yields but dependent on the biomass composition, specifically the lignin and

carbohydrate types present (Cooket al. 2015). It is known that lignin is the main barrier to rumen digestion and studies have shown that delignification of biomass can increase the digestibility of the remaining cellulose by up to 99% depending on the biomass (Mukherjee and Nandi , 2004). The biomass source is important, as studies on *Cypromeria japonica*on recalcitrant cedar wood have shown that even highly effective lignin removing fungi, up to 45% removal in cedar after 20 weeks,was unable to make the biomass more digestible than rice straw. In this study plain rice straw was easier to saccharify even with 45% of the cedar's lignin removed (Okanoet al. 2005).

2.2 Storage losses

Biomass stored in bales has a natural rate of degradation over time. Dry mass losses from switchgrass stored for a year or more can be accompanied by mass losses as high as 40% (Mooneyet al., 2012). These losses can be mitigated by protective measures such as tarps, bale size and shape, moisture content while harvesting, and short storage times (Mooneyet al., 2012). Mass losses are partially the result of losing moisture after harvesting, depending on the moisture content of the harvested material when baling and can account for as much as three quarters of all mass lost during storage(Monti et al., 2009). All these methods have an associated cost per ton as well as other incidental costs such as energy consumed during harvesting and greenhouse gas emissions produced (Kumar et al.,2007). Mass rate loss slows down over time and tends to reach a constant value near the end of the storage time. In addition, dry matter loss has a tendency to increase with time and total precipitation, at a decreasing rate (Larsonet al., 2010).Thus while certain procedures (such as covering the bales) does improve eventual saccharification yields, the cost of these storage options must be taken into consideration when considering the entire processing chain of lignocellulosic ethanol.

Reasons for storage losses, other than water, are typically because of digestion of the biomass by natural plant-matter consuming organisms. Harvesting biomass while dry (< 40% moisture content) tends to result in high losses during cutting and baling, while harvesting while wet (>60%) tends to promote biological growth which can convert the biomass into CO_2 (Emery and Mosier, 2012). In addition, because it has been shown that high moisture contents can deliver better delignification (and thus better saccharification), harvesting at higher moisture contents should be seen as preferable (Shi et al.2008). The weathered layer in particular suffers large mass losses over time due to exposure to moisture, with up to 23% more decrease in available material from bagasse when compared to unweathered biomass (Sanderson et al.,1997). The consortia of organisms, which use biomass as a substrate, aremade up ofby microbial organisms. Studies in biomass storage are well known for common animal feed crops and likewise not much information exists on the storage properties intended for other uses (Emery and Mosier,2012). Storage losses are affected by a number of variables. Data regarding specifics with respect to causes and effects between planting, harvesting, and baling conditions tends to be highly variable across the literature. Storage technique can also affect enzymatic hydrolysis yields and alter the profile of sugars and byproducts formed in the bioprocessing chain, as storage area (either in the field or in a barn) had significant effects on the lignin composition of switchgrass (Djioleu et al.,2014). These changes might increase or decrease the success of fungal colonization (both natural and intentional) and thus must be considered as white rot fungi require lignin to become established (and thus low-lignin storage methods might actually hamper pretreatment yields). The same study also found no significance in the glucose compositions of the bales independent of storage area, whereas other components (xylose and organic acids) were found to have significantly different values after pretreatment. This combination of storage and compositional

change (or no change) has to be considered in any biofuel processing chain trying to use stored biomass.

2.3 Liquid hot water pretreatment (LHW)

Liquid hot water pretreatment (LHW) (also called hydrothermal pretreatment) is a non-acid using autohydrolysis method for pretreating biomass which does not require acid-resistant steel reactors or feedstock reduction to sizes below 3 mm (van Walsum et al., 1996). In comparison to other pretreatment technologies, such as acid hydrolysis, LHW pretreatment offers minimization of losses of oligosaccharides (Wyman et al., 2005) and decreases the need to mitigate the generation of with acidic and other waste products (Laser et al., 2002).

Typical enzymatic hydrolysis yields range from 35% to 80% recovery of available cellulose. These results occur at higher temperatures and solubilize many xylo-oligomers. Furans are another byproduct of LHW pretreatment, which can be inhibitory in downstream hydrolysis and fermentation reactions (Kimet al., 2008). The pH during LHW is kept in a range between 4 to 7, in order to reduce the number of sugar degradation products formed during the reaction. As the pH of water itself changes with temperature, this restricts the upward bound of LHW treatments to around 200 °C (Kimet al., 2009). LHW treatment works by the process of hydrothermolysis to separate and solubilize hemicellulose, thereby disrupting the cell wall and rendering the biomass easier for enzymes to digest. Temperatures are maintained at supercritical levels by means of pressurized reactor vessels. In addition, temperatures and times tended to have no effect on the results of the subsequent enzymatic hydrolysis (Mosieret al., 2005).

Optimization for LHW pretreatment requires adjusting the temperature, time, and pH for which the biomass is exposed (Mosieret al., 2005). In this study, the optimization process resulted in an

eventual 90% recovery of maximum possible glucose from the substrate (corn stover) and 88% of maximum possible ethanol during fermentation. These results are confirmed by multiple similar studies on different biomass types (Perezet al. 2008, Yuet al. 2010). A well-optimized LHW pretreatment process for switchgrass specifically was found to be similar, with recoveries of glucose up to 100% (Kimet al., 2008). In comparison with other pretreatment technologies, LHW pretreatment does not disrupt lignin structures and does not significantly reduce the crystalline structure cellulose, two mechanisms which are important for enzymatic saccharification (Mosieret al., 2005). In addition when compared with other pretreatment methods, LHW pretreatment requires higher temperatures and pressures (and thus higher energy requirements), and for certain types of biomass (softwoods) LWH is less effective as a pretreatment option for reasons that are currently not understood (Mosier et al., 2005).

2.4 Enzymatic Hydrolysis

To break down the complex polysaccharide cellulose, enzymes known as cellulases are used. These enzymes are usually derived from cellulose digesting organisms such as the fungus *Trichoderma ressei (*Holtzappleet al.,1990*).* One type of enzyme alone is incapable of digesting the entire cellulose structure and a number of unique enzymes are needed to remove specific linkages on the cellulose polymer, such as endo-cellulases (endo-beta-1,4,-glucanase), exo-cellulase (exo-beta-1,4-glucan glucohydrolyase, which hydrolyses the non-reducing end, and exo-beta-1,4-glucan cellobiohydrolase, which uses the reducing end and dominates), and occasionally xylanases (to remove residual hemicellulose and make the cellulose more accessible to other enzymes) (Holtzappleet al.,1990). The combination of enzymes is needed in order to completely break down the cellulose molecule into free sugars which can be readily consumed and converted by fermenting organisms.

Cellulases are inhibited both competitively and noncompetitively by many of the byproducts of chemical pretreatment. Common cellulase inhibitors include other soluble sugars, phenolic compounds, organic acids, furans, and furan derivatives (Kimet al., 2011). Typically, the most inhibitory compounds to cellulases are in the lowest concentration after chemical pretreatment, with (from strongest to weakest inhibition effects) the major compounds being lignin derivatives, furan compounds, and organic acids(Jing et al.,2009). In addition to pretreatment byproducts the carbohydrates themselves can act as inhibitors. Cellubiose, a common product of pretreatment, is very inhibitory to glucanases (Holtzappleet al.,1990) but is largely broken down in the enzyme cocktail mixtures available commercially. Mechanisms of inhibition of cellulases can differ based on the enzymes exact function but generally the rate limiting step is the solubilzation of insoluble cellulose (Himmel et al., 2007).

3. Research gaps

In the field of biofuel research , there are numerous studies on the lab scale (usually <.1 kg) regarding the use of fungus to pretreat biomass prior to saccharification (Sawada et al. 1995, Itoh et al. 2003, Zhang et al. 2007, Camarero et al. 1994, Hatakka et al. 1983). Studies on biomass in excess of .1 kg stored with fungus are either not related to biofuel (Mukherjee and Nandi, 2004), focused in efforts to isolate or study lignin degrading enzymes specifically (Pinto et al. 2012), or related to the livestock industry (Zadrazil, 1997). Switchgrass has been studied as a feedstock for biofuel (Sanderson et al., 2006), had been stored alongside *Pleurotus osteratus* (Liu et al., 2013), and has been saccharified after storage both with (Liu et al., 2013) and without (Mitchell and Schmer, 2012). In all these cases, however, the studies have never exceeded 100 g in total biomass storage. This means that each case was done in highly idealized conditions, where the temperature, moisture content and biological activity of the material was regulated in ways that are not reflective of a true bioprocessing operation. These studies never exceeded 90 days in terms of storage time, and never approached more than .1kg of total mass storage over that same period of time. Moreover these studies are frequently conducted at high (>80%) moisture content during the storage periods. In terms of storage alone, with no fungal treatment, there are a multitude of studies on switchgrass over long periods (greater than 100 days) of time (Monti et al., 2009, Larson et al., 2010, Djioleu et al., 2014, Sanderson et al., 1997). These studies do not focus on saccharification or biofuel, however, and thus their results do not provide information on processes such as pretreatment or enzymatic hydrolysis. Thus there is a meaningful research gap between the two types of study: fungal pretreatment studies and long term storage studies. For second generation biofuel processing plants to gain traction it is important that there be adequate research not only in the laboratory, but in the field as well. This study, which examines the effect of storage in biomass saccharification for long terms, large

masses, and fungal treatments, is one step closer towards bridging this gap in the research efforts in second generation lignocellulosic biofuels.

4. Objectives

Currently many studies regarding the storage of biomass are either done on the small scale (typically <0.1 kilograms of biomass) or are done for other purposes, such as in the agriculture industry where the digestibility and storage/silage of feed for livestock is an important issue, especially regarding minimizing the mass losses incurred during harvesting and storage (Emery et al., 2012). There is a need to find affordable, easy to implement technology in second generation biofuel processes in order to achieve better saccharification (and thus profits). One such promising technique has been the combination of storage and fungal inoculation with biomass, which has been shown to increase digestibility of switchgrass in ruminal fluid by 30 to 59 percent (Zadrazil et al., 1997). Numerous laboratory studies have been conducted over a wide variety of organisms and substrates. Some examples of such fungal studies include the species *Phanerochaete chrysosporium, Ceriporiopsis subvermispora, Coriolus versicolor*, and *Pleurotus eryngi*, on substrates such as corn stalks, cotton residues, bamboo, beech wood, and wheat straw (Sawada et al., 1995, Itoh et al., 2003, Zhang et al., 2007, Camarero et al., 1994, Hatakka et al., 1983). However these studies have been conducted at the laboratory scale, in sanitary conditions with small mass samples. It has been yet to be shown that the promising results shown for fungal degradation of biomass is translatable to a farm-scale process, which would be the next necessary step if the technology is to be applied in the second generation biofuel refinery industry.

There are two major objectives of this study. The first is to determine the saccharification potential of the bales which supported fungal growth, which in this study was accomplished in square bales. Enough switchgrass was harvested for the creation of 12 bales, 8 of which received a 2% by mass fungal inoculation of *P. osteratus*. These bales were then stored in an open field for 270 days, with samples from one set of bales taken at after 90, 150, 210, and 270 days. After

sampling the bales were considered compromised and no longer used for the study. After the sampling was done, each sample was pretreated at either 180 C or 200 C in a liquid hot water pretreatment reactor, and then hydrolyzed with cellulase enzymes to recover the glucose. The amount of glucose recovered was then compared to the amount of glucose available in the form of cellulose, allowing for the calculation of saccharification efficiencies.

The second objective of the study was to determine the saccharification potential of bales stored for 270 days, which in this study was done in round bales. Enough switchgrass was harvested for the creation of 12 bales. These bales were stored in an open field for 270 days with samples from the bales taken after 90, 150, 210, and 270 days. In addition analysis was conducted in an open air field on both bale types to determine the byproduct and xylo oligmer profiles of the hydrolysates, in order to better quantify the changes which occur within square and round bales as a result of storage. After sampling the bales were considered compromised and no longer used for the study. After the sampling was done, each sample was pretreated at either 180 C or 200 C in a liquid hot water pretreatment reactor, and then hydrolyzed with cellulase enzymes to recover the glucose. The amount of glucose recovered was then compared to the amount of glucose available in the form of cellulose, allowing for the calculation of saccharification efficiencies.

To attempt to prove or disprove these objectives, a null hypothesis (n0) was used that both storage time and fungal inoculation would have no effect. An alpha of .05 was used as the threshold to determine whether or not we failed to reject n0 or not.

5. Materials and Methods

5.1. Experiment Overview

Kanlow switchgrass was grown and cut in December of 2012. Before baling, fungal inoculation of the bales was done by spreading commercial P. osteratus spawn on top of the biomass. The switchgrass was then baled and stored in the open field for 90, 150, 210, and 270 days. After storage, the biomass was pretreated with LHW at either 180 C for 20 minutes or 200 C for 10 minutes. The biomass was washed with 5 volumes (liter to gram) of water and hydrolyzed with a cellulase enzyme cocktail. The resultant release of carbohydrates was quantified and the saccharification efficiencies determined through HPLC.

5.2. Source of Biomass and *Pleurotus osteratus* Application

The source of the switchgrass (*Panicum virgatum* var. Kanlow) was from the experimental field of Oklahoma State University South Centeral Research Station in Chichasha, Oklahoma. The switchgrass was cut in December of 2012 and left to dry for 3 to 5 days before baling. During this period the *P. osteratus* spawn (Sylvan Inc, Kittanning, PA) was manually spread on some of the switchgrass, with some receiving no spawn as to serve as controls. The spawn was applied to match a 2% loading, or about 2 kg per 100 kg of biomass.

5.3. Square and round bales

The square bale study used 8’ (2.44 m) by 4’(1.22 m) by 3’(.91 m) (length by width by height) dimensions and the switchgrass was baled with AGCO high-density balers (Hessen, KS) on December 13, 2012. The moisture content of the bales prior to baling was 8.9%, dry basis. Twelve bales were made for each study. The square and round bales were transported to Stillwater, OK two days after baling for the study. The bales were stored outside in the field with no cover or protection. Each bale was spaced one to two metersapart, and left on a wooden forklift pallet to separate them from the ground. Sampling was conducted using a hay corer that

was 2 in (.05 m) in diameter and 3 ft (.9 m) long, mounted on a handheld electric drill. Figure 1 presents how samples were drawn from evenly spaced locations within square bales.The sampling was done as shown in Figure 1. Samples were taken from three elevations and 3 horizontal locations. The top, middle, and bottom cores were combined for testing. A 6 inch 'margin' was used around the edge of the bales, such that all bottom samples were 6 inches from the bottom, and all top samples were six inches from the top. In addition all left and right horizontal samples were 6 inches from the edges. This 'margin' was used because the biomass closest to the surface of the bales receives the most weathering, and is not representative of the whole bale. In total there were 64 unique sampling points, which were combined into 10 composite samples based on the vertical location of the sample. After moisture content and compositional analysis had been conducted, all samples were combined for the enzymatic hydrolysis step of the experiment.

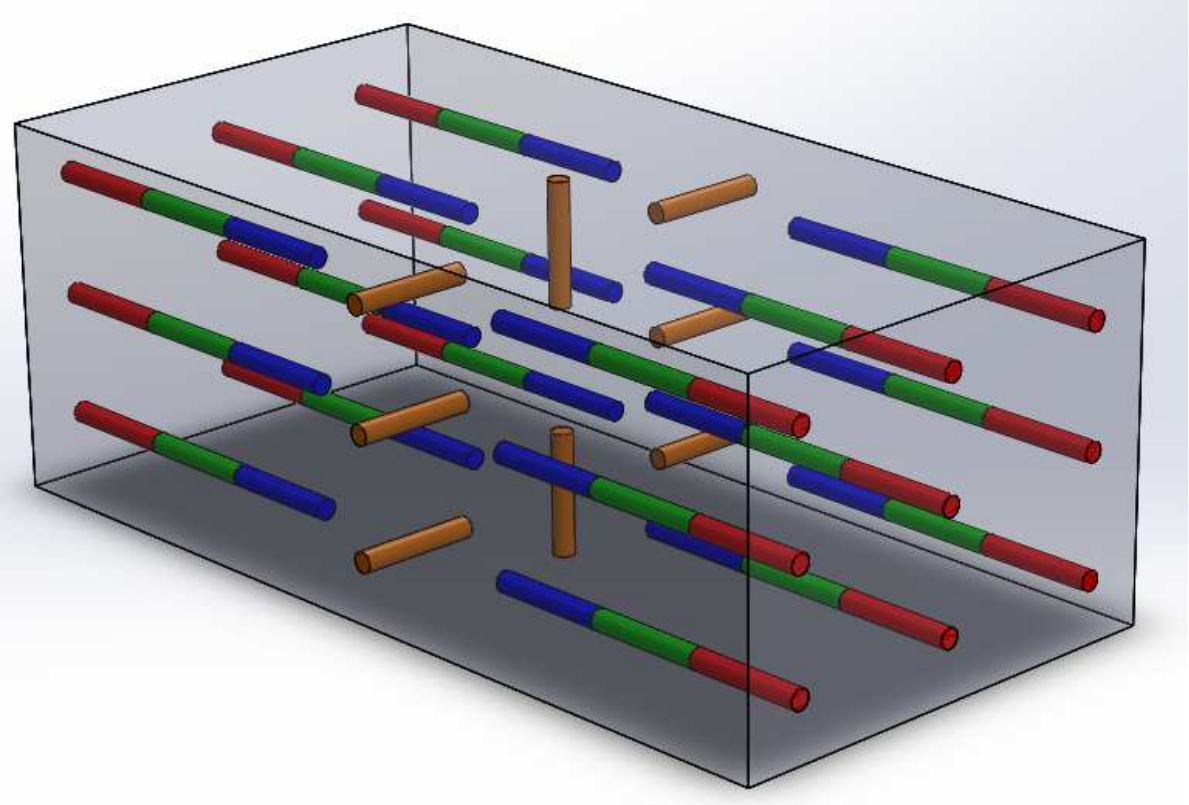


Figure 1: Sampling regime for square bales. Image reproduced with permission of Mengxing Li, Oklahoma State University collaborator on the project.Colors correspond to sampling depth.

The round bales were also prepared with Kanlow switchgrass, in the same location, but on December 19, 2013. AGCO high-density balers (Hessen, KS) were used to make the round bales.Bales were 5 feet by 5 feet (1.52 meter by 1.52 meter) (diameter by height). Bales were transported from Chichasha to Stillwater no more than two days after baling. Bales were left to stand on wooden pallets in the field. The sampling regime is shown in Figure 2. Cores were taken one foot (.31 m) and two feet deep (.61 m) into the bales. Sampling was conducted using a hay corer that was 2 in (.05 m) in diameter and 3 ft (.9 m) long, mounted on a handheld electric drill. The sampling points from the left and right were both started 1 foot in from the bale, and core samples were spaced such that 8 total distinct perimeter sampling points existed. A total of 48 samples were collected. Samples from the 1 foot depth category were placed together, and

samples from the 2 foot depth category were similarly combined. All samples were stored at 4 °C in a walk-in cold room after collection to prevent further degradation of the biomass.

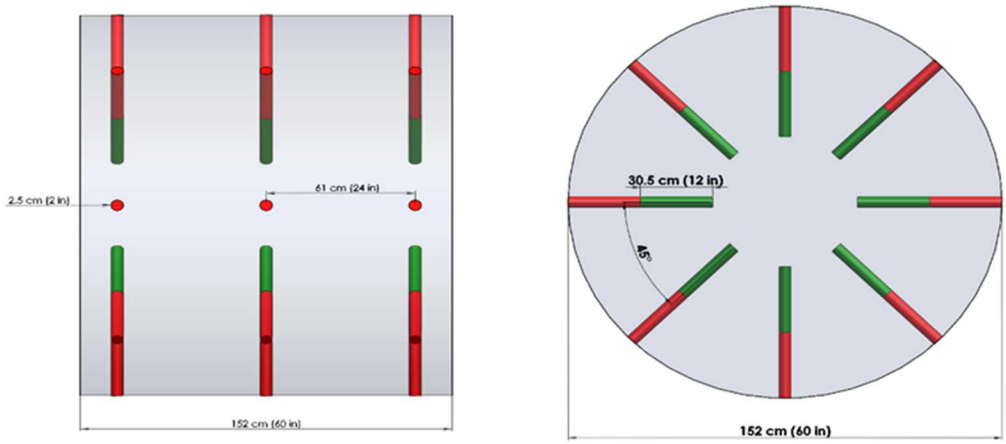


Figure 2 Round bale sampling regime. Reproduced with permission by Mengxing Li, a Graduate Student collaboratorin the project from OK State. The left image shows the side view of the bales, and the right image shows the head-on view of the bales. Color represents sampling depth, with red being the samples taken within 1 foot of the surface of the bale, and green samples being taken from between 1 foot and 2 feet within the bale.

5.4. Biomass Characterization

Compositional analysis was performed at Oklahoma State University (Stillwater, OK) at the end of the storage periods for both studies, through the months of October and November. Compositional analysis of the biomass before and after liquid hot water pretreatment was conducted using the NREL standard protocol for herbaceous crops (Sluiter and Sluiter 2010). Total solids content of the biomass was determined following Sluiter et al. (2008) and the structural carbohydrates and lignin content were calculated using the method in Hymanet al. (2007). Extraction of the solid portion of the biomass was done using deionized water and 190 proof ethanol (Pharm CO-AAPER Brookfield, CT) with an Accelerate Solvent Extractor, ASE® 300 system (Dionex Corporation, Sunnyvale, CA). The sugars and lignin content of the biomass were determined using an acid soluble lignin test (ASL) and the analysis of the hydrolysates was performed using a 205 nm UV-VIS spectrophotometer (Cary 50 Bio, Varian Inc, Palo Alto, Ca). Detection and quantification of the carbohydrates were performed with a refractive index detector (RID) and a Bio-rad Aminex HPX-87 P column (Bio-Rad, Sunnyvale, CA,), using a high-pressure liquid chromatography (HPLC) instrument as described (Fredericket al., 2013) The HPLC eluent was deionized water flowing at a rate of 0.6 ml/min at a temperature of 86 °C. Each sample analysis took 30 minutes.

Digestibility of the biomass was done using 30 ml of 72% sulfuric acid along with 1 gram of biomass, shaken in a 30 °C water bath. Quantification of the glucose content of the biomass was done using an YSI 2900 Biochemistry Analyzer (YSI Life Sciences Inc, Yellow Springs, OH) with an immobilized enzyme membrane. Moisture content was found using an Ohaus MB45 Moisture Analyzer (Pine Brook, NJ) both before and after pretreatments.

5.5. Liquid Hot Water Pretreatment

The pretreatment was carried out in a 1 liter bench top stirred reaction vessel (Parr Series 4520, Parr Instrument Company, Moline, Il,) with a propeller agitator and a 1 kW electrical heater. The reactor volume was loaded as following: 425 grams of deionized water and 75 (dry weight) gram of switchgrass, which was ground and sifted through a 13 mm screen by a hammer mill (Model E9506, Bliss Industries, Ponca City OK). A reaction time of 10 minutes and temperature of 200 °C was used for the first treatment group, and a reaction time of 20 minutes at 180 °C for the second treatment group. For both pretreatments, the agitation of the reactor was set at 300 RPM and there was only manual agitation during cooling.

The severity of each pretreatment was calculated, which is the severity pretreatment equation (1) (Dogaris et al. 2009). The factor R0 is the severity number, the variable t is the pretreatment time, and the variable T is the pretreatment temperatures in degrees C. The severity factor for each pretreatment was used to quantify that the 180 C treatment was less severe than the 200 C. Higher SV factors typically result in biomass that has higher saccharification efficiencies (Laser et al. 2002, Perez et al. 2008, Mosier et al. 2005). The two different SV factor pretreatments were conducted to determine if the effect of storage had different effects based on severity. The SV factor for the 180 treatment was calculated to be 3.66, whereas the SV factor calculated for the 200 C treatment was 3.94.The pretreatment hydrolysate and solids were separated using a vacuum filter with a Buchner funnel and Whatman filter paper (Whatman PLC, Brentford UK). The solid portion in the square bales were washed with 2 liters of deionized water after separation to remove any residual carbohydrates or pretreatment hydrolysates in the sample. For round bales, the biomass samples were washed with 5 times their mass of water (approximately 375 ml of water). Since the round bales were the only ones washed with this volume, only the wash waters from the round bales were saved. The solids and hydrolysates were stored at 4 °C

until needed. All pretreatments were conducted through the months of November and December, and were received for enzymatic hydrolysis in late December.

$$Log(R_0) = Log(t \cdot e^{\frac{T-100}{14.75}}) \textbf{(1)}$$

5.6. Enzymatic Hydrolysis

Hydrolysis was carried out in 30 ml reaction vessels. All enzyme reactions were conducted at 50 °C in a 100 RPM shaking water bath (Thermo Electron Corporation, Winchester, VA). All enzyme reactions used a 4.8 sodium citrate buffer solution (EMD Gibbstown, NJ). All water used in this and other experiments were from a Direct-Q system (Millipore, Billerica, MA) that displayed 12.2 M Ω resistance. Three combinations of enzymes were used in the enzymatic assay experiment: Accelerase ® 1500 (Genencor, Cedar Rapids IA), Cellic® CTec2 and HTec2 and Novozyme 188(Novozyme, Franklington, NC), which is cellobiase from *Aspergillus niger*, combined with Celluclast, which is cellulase from *Trichoderma reesei* ATCC 26921 (Novozyme, Franklington, NC). For the enzymatic assay, each enzyme was loaded to 60 filter paper units (FPU) per gram glucan in the biomass. One filter paper unit is equal to the quantity of enzyme needed to produce 2 mg of reducing sugar from 50 mg of filter paper (4%) in 60 minutes. FPU is unique to each enzyme. In assays performed on the enzyme batches, Accellerase was found to be between 11 and 12 FPU/ml, Ctec and Htec were determined to be between 20 to 22 FPU/ml, and Celluclast and Novozyme were evaluated to be between18-20 FPU/ml.

Four sets of controls were done in accordance with NREL cellulase activity measuring standards (Adney and Baker 2008). The first set of controls contained unpretreated biomass together with the enzyme. The second control contained no biomass and only enzyme. The third control was a set of samples with only biomass and no enzyme. The fourth set consisted of filter

paper and enzyme, with no biomass. Ctec and Htec enzyme cocktails contain a small amount of latent glucose in the batch, and the total concentration for that enzyme solution was subtracted from all the totals in the enzymes done for those sample sets (such that all the data only shows sugar released because of the enzyme and not what was already present). These three controls apart from Ctec and Htec samples showed no signs of saccharification. The hydrolyzed filter paper was used as the 'maximum' amount of theoretical yield possible for the enzymes. This was in accordance with NREL cellulase activity measuring standards (Adney and Baker 2008).

Since composition and moisture content were known for each set of biomass and to ensure analytical uniformity, each sample was loaded to ensure that only 100 mg of glucan was present in each hydrolysis tubes. For the filter paper controls, 100 mg of filter paper was added and it was assumed that the conversion yielded 100% glucan. Each individual condition had its own moisture content and glucan content, however, which meant that the amount of total biomass added for each sample could vary from as low as 150 mg to as high as 900 mg. The total volumes of each reaction vessel were adjusted with water to make sure that the final reaction volume was 10 ml. Thus, while each reaction vessel was loaded to 1% glucan loading (100 mg in 10000 mg of water), the total mass loading in each vessel was dependent on the biomass composition. Enzymatic hydrolysis was conducted for 24 hours. Aliquots of hydrolysate were saved and analyzed on HPLC and YSI and then stored at -4 °C.

5.7. High Performance Liquid Chromatography Analysis

High Performance Liquid Chromatography (HPLC) was conducted to determine carbohydrate content and carbohydrate degradation products present in the liquid hydrolysates of the pretreatment and enzymatic hydrolysis solutions. All the methods used for separation was based on previously published techniques (Spacil et al. 2008 and Djioleu et al. 2014). The

carbohydrates (glucose and xylose) were detected with a Waters 2414 Refractive Index Detector (RID, Milford, MA) after being passed through a Waters 2695 separation module with a Shodex (Waters, Milford MA) precolumn (SP-G, 8 μm, 6 x 60 mm). The method used to achieve separation used deionized water eluting at 0.2 ml per minute, and the column was heated to 85°C, using a Waters (WAT038040) external heater. Organic acids and other liquid hydrolysis byproducts were detected on a similar Waters 2695 separation module with a Bio-Rad (Hercules, CA) Aminex HPX-87H Ion Exclusion column (7.8 mm x 30 mm). The column was heated to 55°C and the mobile phase was 0.005M sulfuric acid flowing at a rate of 0.6 ml per minute. The byproducts were detected using a Waters 296 Photodiode Array Detector (PDA); all byproducts were detected at the wavelength of 280 nm. All injections were 2.5 μl.

5.8. Quantification

HPLC chromatograms were used to quantify the amounts of the known standards that were present in each analyzed sample. The standards used for carbohydrate detection were D-(+) glucose from Alfa-Aesar (Ward Hill, MA), and standards used for organic acid byproduct detection (furfural, HMF, acetic acid, and formic acid) were also from Alfa-Aesar. Typical calibration curves for the organic acids are presented in Figure 3. The YSI Biochemical Analyzer was self-calibrating and was set to calibrate to a 2% threshold every 4 samples or 30 minutes, whichever came first. HPLC calibration was done once at the start of every set of samples.

5.9. Scanning Electron Microscope (SEM)

SEM imaging was used to determine whether or not there was any evidence of any fungal growth on the biomass for the initial and final sampling periods. Samples were dried and mounted on aluminum specimen stands with double coated, carbon conductive PELCO tabs (Ted Pella, INC., Redding, CA). After mounting, each sample was sputter-coated using a Polaron/emitech SC7620 Sputter Coater (Quorum Technologies, Ltd. Esast Sussex, UK) at a

thickness of 1-2 nm of gold. A FEI Nova Nanolab duo-beam SEM/FIB SEM was used at 30 kV to view the samples (FEI Company, Hillsboro, OR). SEM images were only taken of the biomass prior to pretreatment, and only for the bales at the beginning and end of the testing period. Only square bales were examined by SEM. Scanning Electron Microscope images were conducted using a FEI Nova Nanolab duo-beam SEM/FIB system at 30 kV, courtesy of the Arkansas Nano & Bio Materials Characterization Facility at the University of Arkansas, supported by the National Science Foundation and the State of Arkansas.

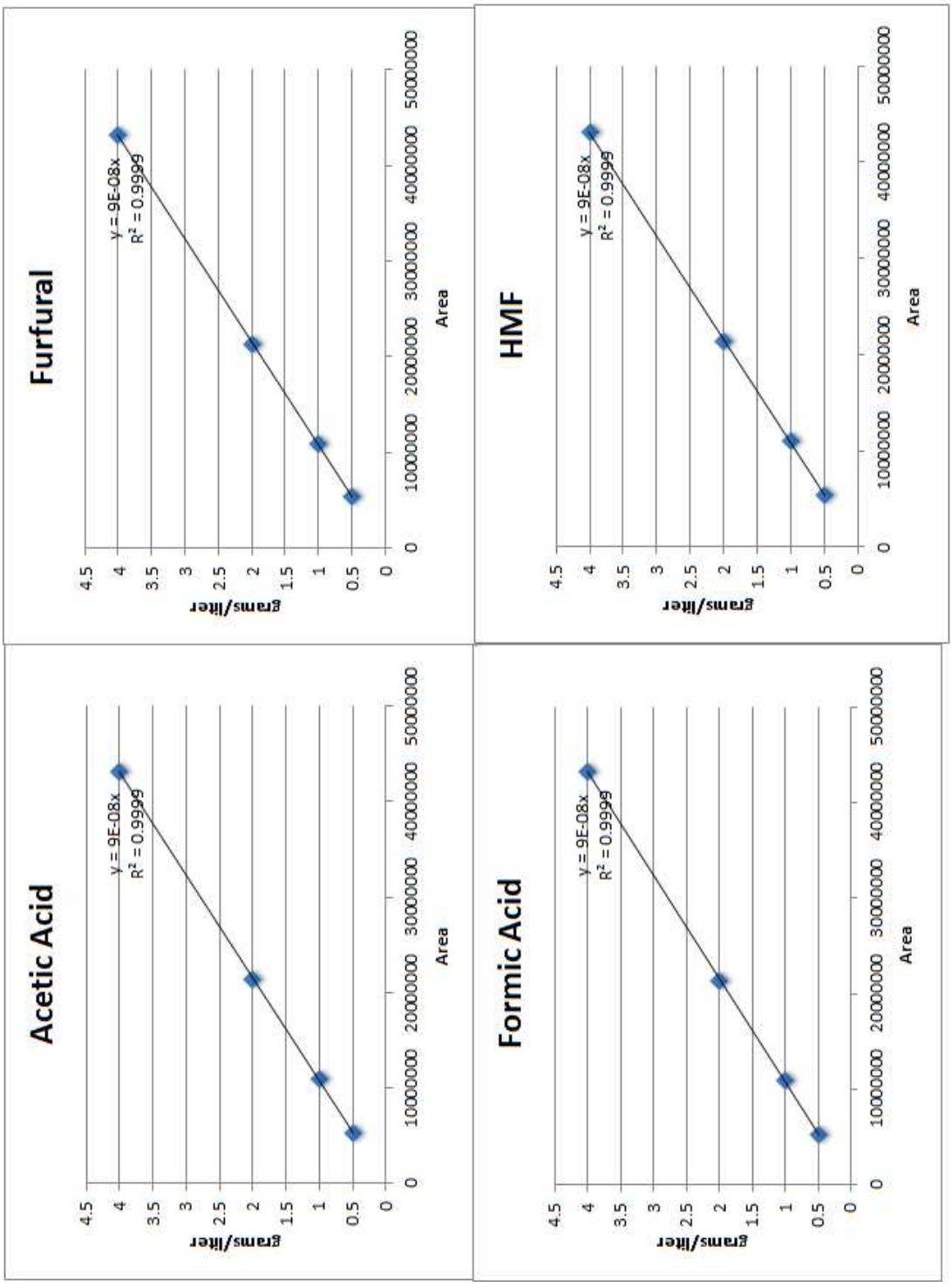


Figure 3 Calibration curves needed for byproducts quantification. Area refers to the area under the curve in each chromatogram, and the concentration which corresponds to that reading. Calibrations were done on a Waters 2695 Separations module with aBio-Rad Aminex HPX-87H Ion Exclusion column (7.8 mm x 30 mm).

5.9. Statistical Analysis

All data points were measured in triplicate and each experiment was done either in duplicate or triplicate. Where applicable, linear regression and Student's t-test or Tukey's test was used using JMP 11.0 software. The alpha value for all tests was 0.05. Graphs were constructed with either JMP 11.0 or Excel 2007. Two-factor analysis of variances (ANOVA) was used to find significance of the data for the storage study, with $P<0.05$ values being considered significant.

6. Results and Discussion

6.1. Square Bales

6.1.1. Moisture Content

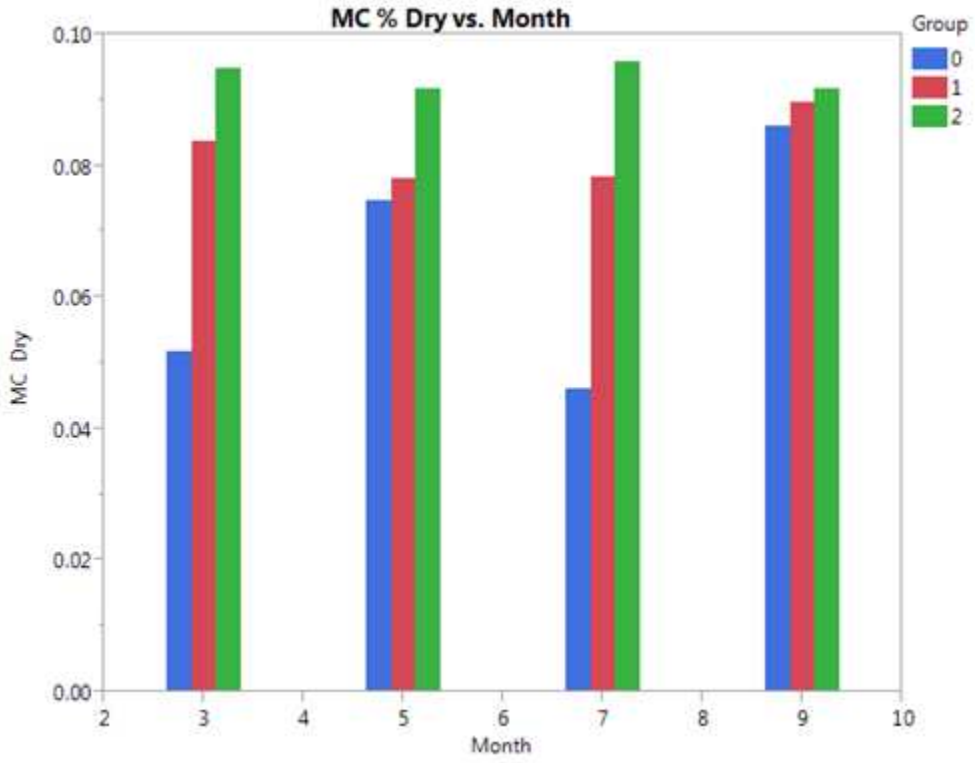


Figure 4 Moisture content as a function of sampling month. The blue bars represent the control samples, whereas the red and green bars (Group 1 and Group 2) are the fungal treated samples.

Prior to the LHW pretreatment each bale's moisture content was determined. The data in figure 4 represents the moisture content for all the sampling elevations combined. This data was only found once per bale, and thus there are no replications and statistical analysis on the data cannot be conducted. However these average values are not unusual for bales of switchgrass and all fall below the 20 percent mark, which is known to be a threshold for causing degradation of organic matter within the bales (Huhnke 2003). The bales were separated into three groups: Group 0, the control, and Group 1 and Group 2, which received the 2% by mass fungal inoculation.

6.1.2. Compositional Analysis

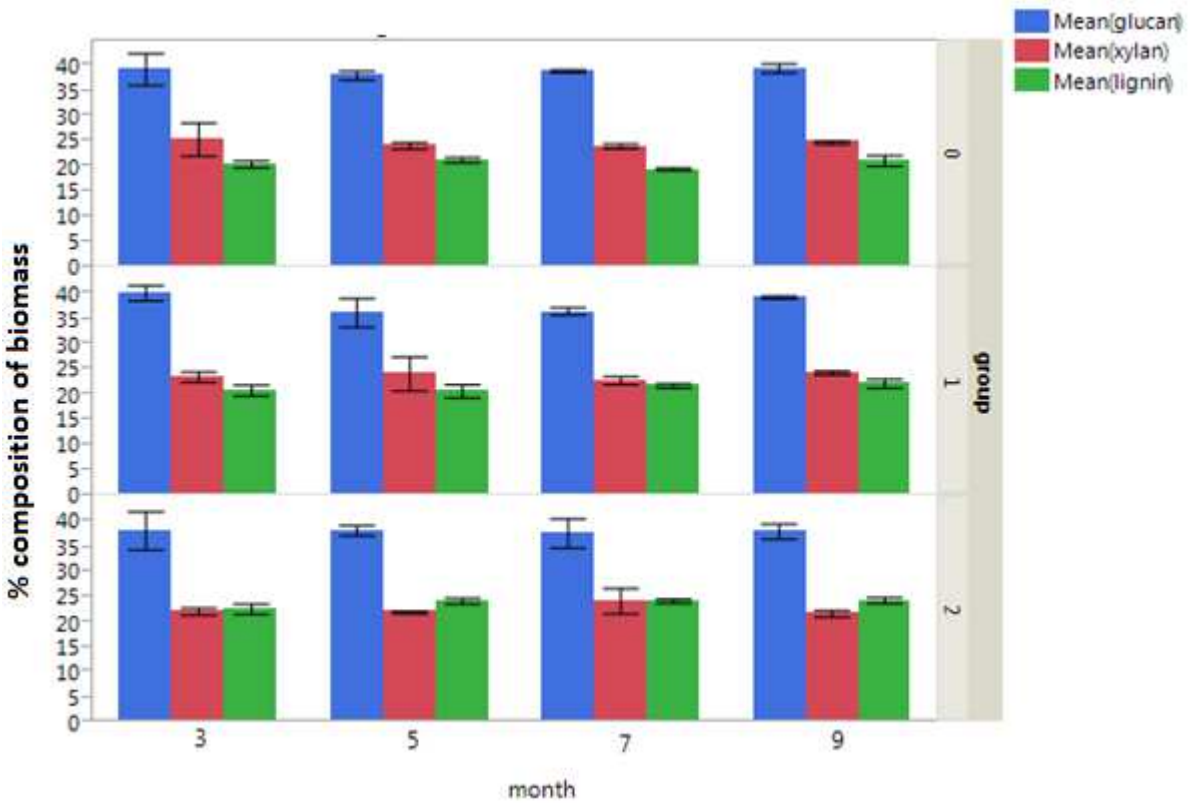


Figure 5 Compositional analysis (mean glucan, xylan and lignin content) of control and fungal inoculated (Group 1 and Group 2) square bales as a function of storage time. The blue bars represent glucan, the red xylan, and the green lignin. The group refers to the treatment that particular bale received. Group 0 is the control, Group 1 and Group 2 are the two sets of bales which received fungal spawn during baling

Figure 5 presents compositional analysis for square bales before pretreatment. The X-axis represents the number of months after baling during which each sample was taken, while the Y-axis is the percentage of glucan, xylan, lignin that is present in the total biomass. Group 0 is the control, and Group 1 and 2 are the *Pleurotus ostreatus* treated bales. Comparisons between treatment times were not performed since there was only one experimental unit for each condition. However, it can be observed from the data that within each bale there is relative composition homogeneity, as there was not a significant amount of variation within bales. This was an important observation as samples were taken throughout the bale, as shown in Figure 1.

Since the samples were taken from different locations within the bales, the average composition was relatively homogenous, indicating that environmental variances did not have a significant effect on localized composition. This does not account for the 6 inch margin used while sampling.

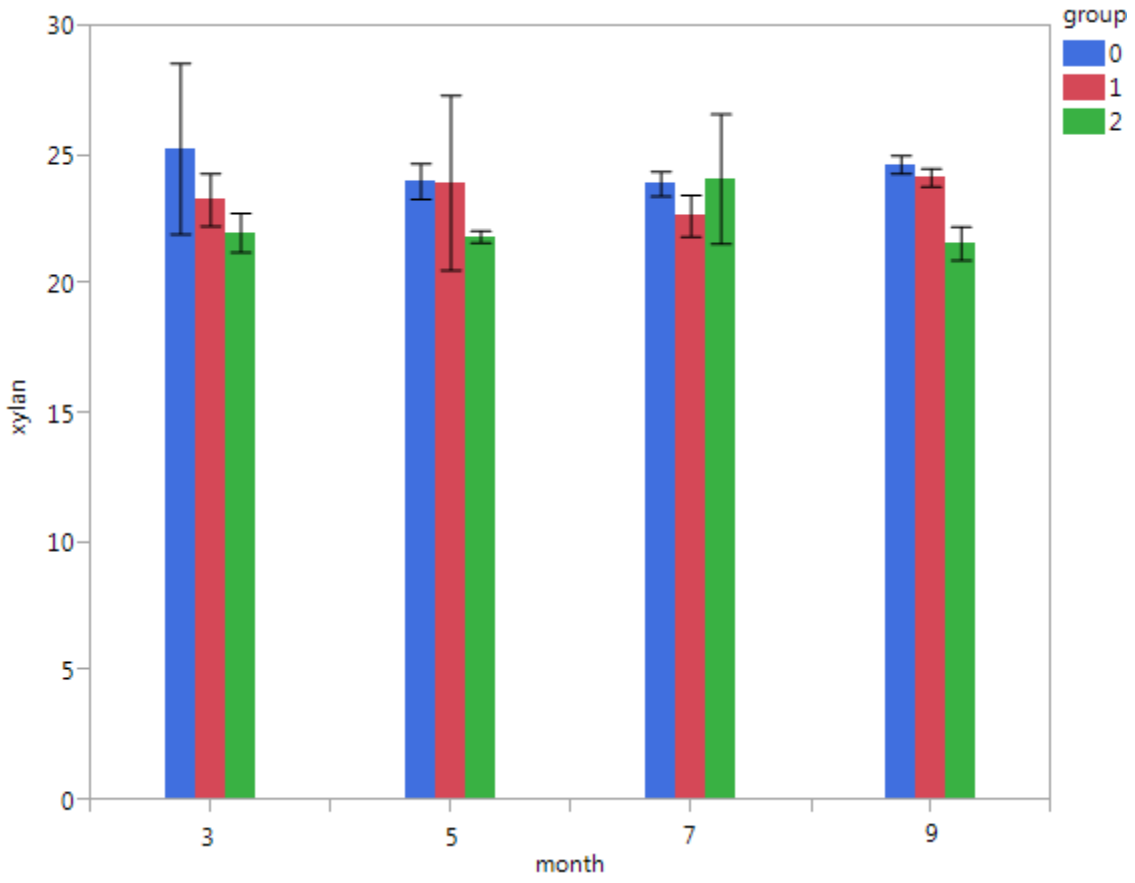


Figure 6 Xylan composition of control and inoculated (Group 1 and Group 2) square bales as a function of storage time, before pretreatment. Each colored bar represents a different treatment group: blue (Group 0) is the control, Group 1 and Group 2 both received the same amount of fungal spawn initially.

Figure 6 is showing the xylan content as a percentage of total biomass in each sample, by group and sampling date. Group 0 is the control, and Group 1 and 2 are the *Pleurotus ostreatus* treated bales. It can be observed in Figure 5 that xylan composition across all three groups accounted for 20 to 30 percent of biomass composition, with the lowest values obtained in Group

2. On the other hand, the xylan content for Group 1 bales was similar to those of the control, possibly indicating that the fungus did not colonize the bales, and/or did not metabolize xylan for growth. These results are inconclusive however as there were too few replications to perform statistical analysis. The most meaningful observation is that compositions of xylan present in the biomass did not show the same degree of removal present in the laboratory scale studies (Gupta et al. 2011).

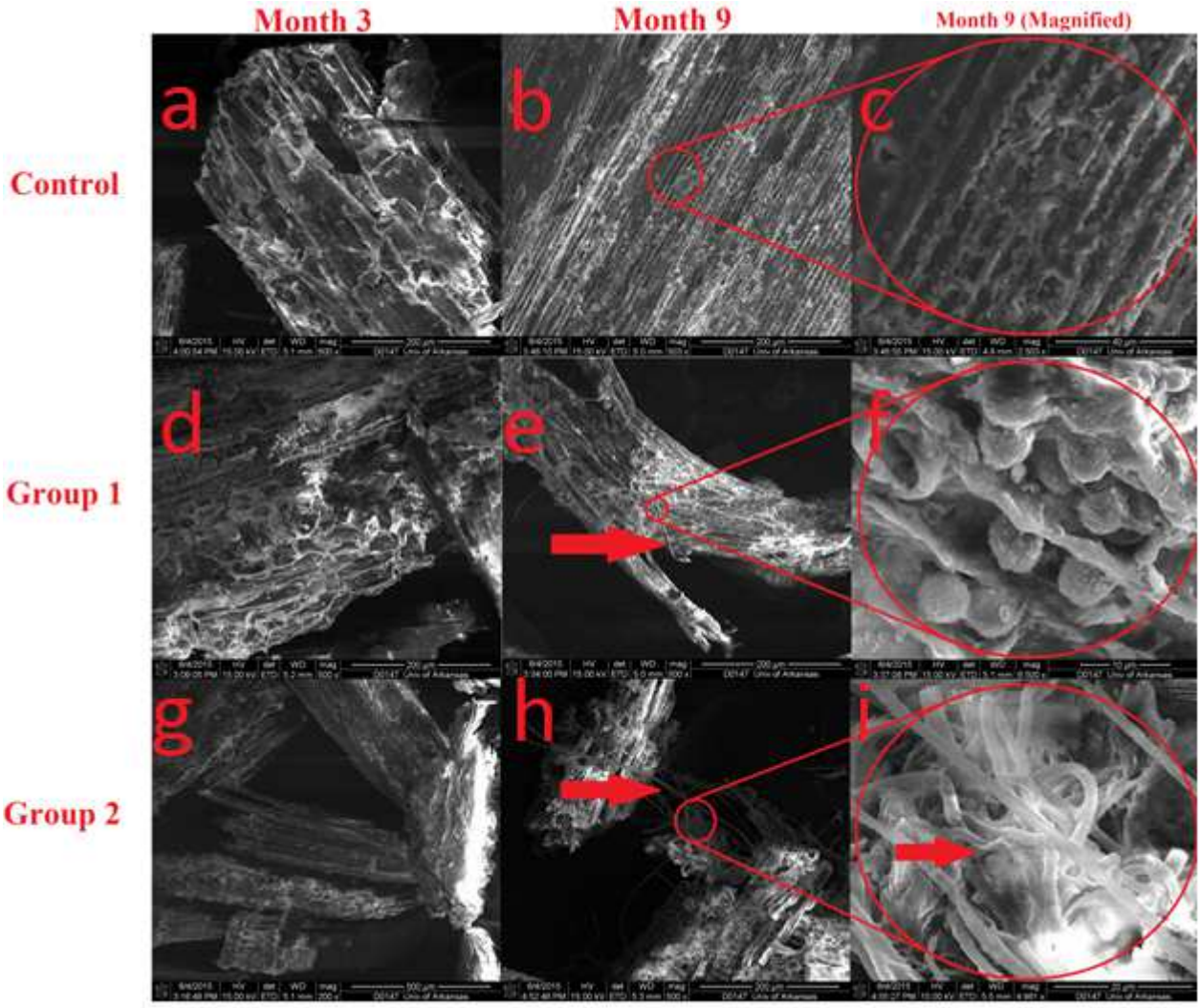


Figure 7 Scanning Electron Microscopy (SEM) images of control,Figure 7a, 7b, and 7c are the control. Figure 7d, 7e, and 7f are the Group 1 bales. Figure 7g, 7h, and 7i are the Group 2 bales.Figure 7f and 7i show (red arrow) the presence of fungal spores on the switchgrass.

In order to perform a qualitative check to determine whether or not *P. ostreatus* grew, electonr microscope images were taken of the biomass at the beginning and end of the study. Scanning Electron Microscopy (SEM) images of control, Group 1 and Group 2 biomass are presented in Figure 7. The intermediate samples (months 5 and 7) were not imaged. It should be noted that between the six biomass samples 104 images were taken over a four hour SEM session. Images that were found to be representative of the rest of the sample were selected for

assembling figure 5. The control group, both on month 3 and month 9 sampling times, showed no signs of fungal hyphae or spore formation, as shown in Figures 7a, 7b, and 7c. Biomass sampled on month 3 showed no signs of fungal hyphae or spores, both in the control and treated groups as seen in Figures 7a, 7d, and 7g. The formation of fungal spores was found only on the older biomass (month 9) in both Group 1 and Group 2 samples as seen in Figures 7e and 7h. The fungus in Figure 7e was growing rather than just surviving as evidenced by the presence of fungal spores on the month 9 biomass but not the month 3 biomass. Group 1 biomass showed some signs of colonization, with individual fungal hyphae and spore formation apparent on the surface of the biomass. This does not mean the fungus was present on the surface of the bales, however. The fungal filaments in this group of biomass were minimal and there was not much fungal biomass in comparison to the switchgrass biomass. This can be seen in Figure 7f where the biomass is still visible beneath the fungal hyphae. The presence of spores indicates a growing fungus but the total colonization appeared minimal. Group 2 bales showed significant colonization, however, as can be seen in Figure 7i, where in places biomass was not visible beneath the fungus hyphae. Through visual identification, it would appear that fungus mass was more present in Group 2 biomass than that of Group 1. Visual traits of the spores and fungal reticulum in the SEM images were similar to that observed in other white-rot fungi species (Luet al., 2009). The extent of colonization in this case was purely qualitative and descriptive and no quantitative work was done to estimate the degree of colonization of the fungus. Although *P. osteratus* spores were placed during baling, it is possible, however, that the fungal species observed is not *P. osteratus* , as fruiting bodies were not detected nor were any genomic testing done to confirm the presence of *P. osteratus*.

6.1.3. Pretreated Biomass

The biomass shown in Figure 5 was subsequently pretreated in 180 Cand 200 °C water. Figure 8 (below) presents the compositional analysis of pretreated bales. The two X-axis represents the temperature of the liquid hot water (LHW) pretreatment (180and 200 °C) as a function of the four sampling months. Each pretreatment was done in duplicate. Each colored bar represents a specific fraction of the biomass, being either glucan, xylan, or lignin. The secondary Y-axis represents the grouping of each bale, with Group 0 being the control and Groups 1 and 2 being the fungal treated bales. For the sake of clarity data similar to what is presented in Figure 7 is also shown in Table 2.

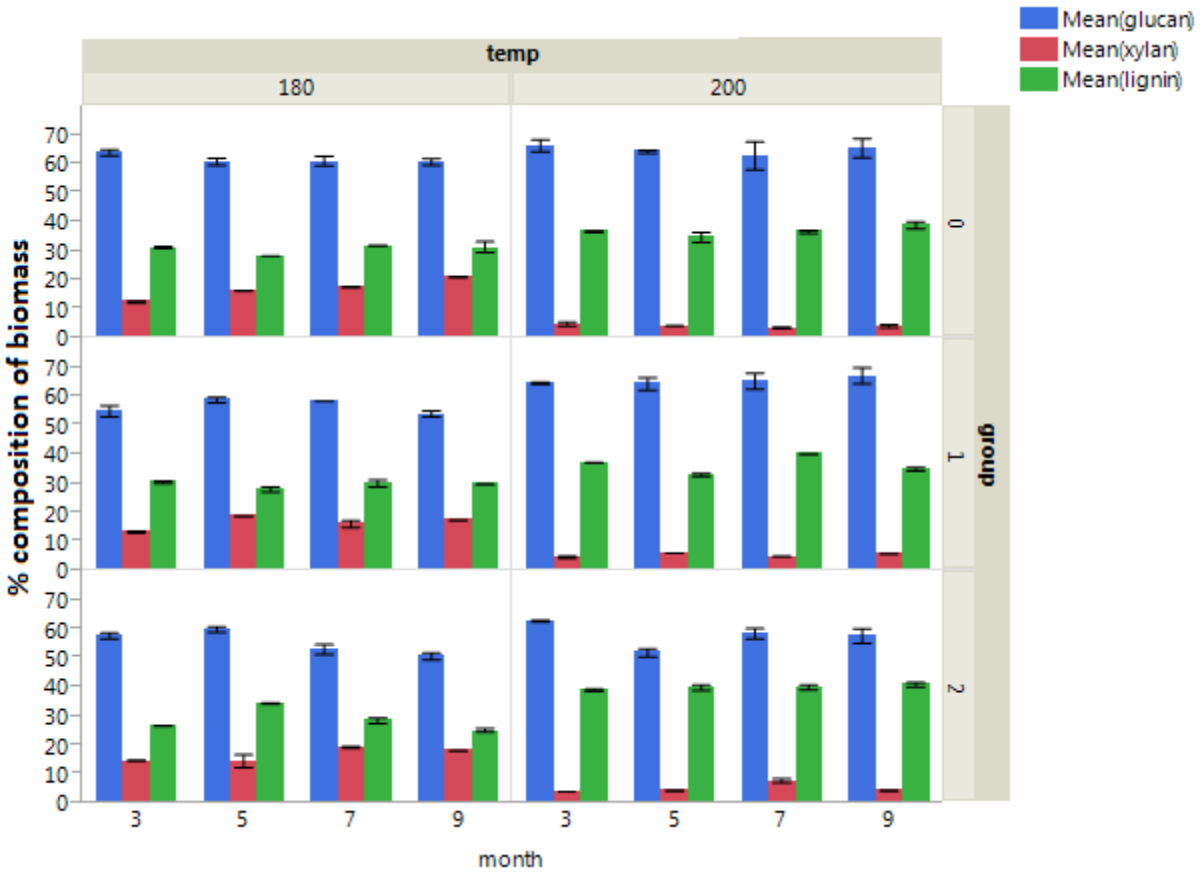


Figure 8 Compositional analysis of pretreated biomass. The y-axis is the percent (out of 100) of each component found in the pretreated biomass at either 180 or 200 Celsius. The blue bar represents the detected ratio of glucan family molecules, the red bar the detected amounts of the xylan family molecules, and the green bar represents lignin molecules. The data is separated into the 3 treatment groups, with 0 being the control, and Group 1 and Group 2 being the two fungal treated bales. The x-axis is time, separated into sampling months.

Table 1 Composition of bales after pretreatment. The data is separated into the 3 treatment groups, with 0 being the control, and Group 1 and Group 2 being the two fungal treated bales. The sample time is represented by the months column (3, 5, 7, and 9). The top table is the pretreatment conducted at 180 degrees Celsius, whereas the bottom table is at 200 degrees Celsius.

Temp		180 C					
		Glucan		Xylan		Lignin	
month	group	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
3	0	63.94	1.0818	12.185	0.3748	31.09	0.3041
	1	54.97	1.9304	13.105	0.3323	30.37	0.3889
	2	57.72	0.9970	14.430	0.19799	26.56	0.15556
5	0	60.78	1.1950	16.140	0.09192	28.11	0.007071
	1	58.81	1.0182	18.515	0.13435	27.87	0.8485
	2	59.95	0.9758	14.270	2.291	34.31	0.19799
7	0	61.01	1.6971	17.330	0.2263	31.72	0.12021
	1	58.39	0.02828	15.875	1.1809	29.95	1.2021
	2	52.97	1.7466	19.10	0.2828	28.34	0.9617
9	0	60.72	1.1384	20.87	0.18385	31.22	1.9021
	1	54.03	1.0889	17.180	0.08485	29.58	0.08485
	2	50.57	1.2021	17.845	0.09192	25.05	0.6293
Temp		200 C					
		Glucan		Xylan		Lignin	
month	group	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
3	0	66.32	2.121	4.56	0.7212	36.52	0.2333
	1	64.62	0.3677	4.37	0.3394	37.13	0.04950
	2	62.84	0.3257	3.84	0	38.88	0.4667
5	0	64.22	0.5091	3.905	0.03536	34.54	1.7819
	1	64.42	2.143	5.81	0.02828	32.9	0.4808
	2	51.85	1.4284	4.1	0.2121	39.72	0.9475
7	0	62.85	4.815	3.165	0.2616	36.51	0.5444
	1	65.33	2.786	4.625	0.03536	40.13	0.10606
	2	58.45	1.7961	7.52	0.6930	39.83	0.8415
9	0	65.53	3.295	3.74	0.5233	38.78	1.2092
	1	67.27	2.744	5.49	0.07071	34.87	0.6293
	2	57.71	2.517	4.045	0.13435	40.78	0.7849

Each pretreatment was only done once, thus the data here is inconclusive and no statistical analysis was conducted. It was observed from the data in table 1 that the control in month 9 samples had higher glucan content when compared to that from both treatment groups.

Group 1 glucan composition after pretreatment was similar to that of the control. As opposed to Group 1, Group 2 samples at months 3, 5, and 9 showed lower glucan averages after pretreatment when compared to those of the control. LHW pretreatment selectively solubilizes hemicellulose (Mosier et al., 2005), confirming that the amounts of glucan and lignin remained unchanged, while the amounts of xylan decreased. Because of LHW's selectivity, increases in glucan and lignin are attributed to decreases of xylan. There was more xylan left over in the pretreated Group 2 biomass than that of the pretreated control biomass. This could be the result of structural changes in the biomass, caused either by weathering or organisms, such as fungi. It is known that structural changes, such as lignin modification can improve saccharification yields (Li et al. 2008) and that the modification of hemicelluloses in plant cell walls can likewise have a positive effect on biomass digestibility and cellulose accessibility (Abramson et al. 2010). The exact nature and mechanisms behind the results here are unknown but the most likely cause is a structural change in the biomass, rather than a compositional one. This is the likely reason why even though the compositions heading into pretreatment were similar, the resultant compositions after pretreatment showed differences. As the pretreatment was conducted only once however it is also possible that this is a result of natural variation.

Table 2: Organic acid profile of prehydrolysates. The mean values for three pretreatment byproducts (acetic acid, formic acid, and furfural) are shown below divided into sampling month, temperature, and treatment group. Group 0 was the control, with Group 1 and Group 2 being the fungal treated bales. 180 degree C pretreatment hydrolysate is the left table, and 200 degree C pretreatment hydrolysate is on the right.

LHW Prehydrolysate Byproducts (g/l)

		180 C			200 C		
		Acetic	Formic		Acetic	Formic	
Month	Group	Acid	Acid	Furfural	Acid	Acid	Furfural
3	0	0.19406	0.3619	0.5961	0.6081	0.4969	2.0303
	1	0.3481	0.2563	1.0230	0.4727	0.3537	2.390
	2	0	0	0	0.3134	0.3832	1.5267
5	0	0.2840	0.3444	0	0.6148	0.4714	1.8149
	1	0.2554	0.2445	1.8148	0.2372	0.3015	0.8058
	2	0	0.2139	0	0.4647	0.3653	2.093
7	0	0.3735	0.3814	0	0.6256	0.5237	3.674
	1	0	0.19691	0	0.5121	0.3630	2.209
	2	0.09913	0.3984	0	0.4424	0.3668	0.8103
9	0	0.18013	0.2025	0	0.7458	0.3504	2.9520
	1	0.2366	0.14803	0	0	0	0
	2	0	0.04913	0	0.2976	0.3058	1.8627

As with table 1, all the data shown here was from a single LHW preatreatment, and thus the differences in the means are inconclusive. Overall more byproducts were formed at 200 °C compared to 180 °C; however, a higher process temperature resulted in more mass conversion as more total grams per liter of byproducts were formed at 200 C when compared to 180C, which is consistent with previous reported literature (Liamsakul et al. 1994). Samples from each bale were only pretreated once (no replications on pretreatments and thus no standard deviations).

The control bales (Group 0), on average, higher total mass of byproducts, mostly in furfural; the maximum concentration of furfural observed was 4 gram per liter). As compared to Jing et al. 2009, organic acids, formic and acetic, were both in concentrations considered to be

below typical inhibition amounts (<1 g/l) and furfural concentrations were within the 0 to 10% inhibition range (between 0 and 4 g/l). The concentrations of organic acids and other byproducts formed from pretreatment cannot be potential causes for the lower enzymatic hydrolysis yields that were observed during analysis, as all concentrations were well below the individual inhibition thresholds. However this does not account for possibly synergistic affects. Similar studies done on Kanlow switchgrass (Suryawati et al. 2008) showed a somewhat similar by-product profile, with little (0.2 g/l) HMF formation. Furfural (0.9 g/l) and acetic acid formation (3.6 g/l) were different from our results, which showed between 0.2 g/l and 0.8 g/l for acetic acid and between 0.5 and 4.0 g/l for furfural (at the 200 °C severity). The large spread in the profile of byproducts between previously published literature and our own data does not suggest that either is wrong, but that even at the same severity the temperature and residence times of the biomass in the reactor can produce byproduct profiles at the end which can vary significantly. Regardless, the concentrations that were formed in this series of pretreatments would not be inhibitory at normal concentrations and would be present in even more dilute concentrations in the enzymatic hydrolysis steps.

There have been no studies on byproduct profiles of biomass pretreated after fungal and LHW treatment, therefore it is difficult to determine whether or not these observed differences and similarities are expected or not. The fungal treatment is assumed to digest hemicellulose preferentially, thus we would expect a lower total concentration of hemicellulose degradation products in the prehydrolysate (furfural and hydromethylfufural). What we see instead is that bales with lower xylan content did not necessarily correspond to lower quantities of byproducts. This indicated, on the other hand, that although there was less hemicellulose to convert, the conversion of the existing hemicellulose was perhaps enhanced. This could be due to the fact that

the hemicellulose that was present was more accessible than in the control, perhaps due to the decay of the cell structure but not necessarily the cell composition. With respect to the compositional analysis data, even though group 1 showed no difference from the control, the total inhibitors formed were still lower.

If Group 2 supported spawn growth, it is possible that processed Group 2 biomass would show differences in sugar yields and enzymatic inhibitors, while Group 1 would be more similar to the control group. Based off the decreased xylan content in Group 2 bales, we would expect greater yields in the enzymatic hydrolysis step as lower hemicellulose contents typically correlates to higher saccharification of the cellulose to glucose. This is partially the reason for selecting LHW as a pretreatment, as the removal of hemicellulose results directly in cellulose being more susceptible to enzymatic attack. (Zhang et al. 2013). Thus we would expect samples with low xylan content to produce a higher percentage recovery of glucose over those with higher xylan content.

6.1.4. Enzymatic Hydrolysis

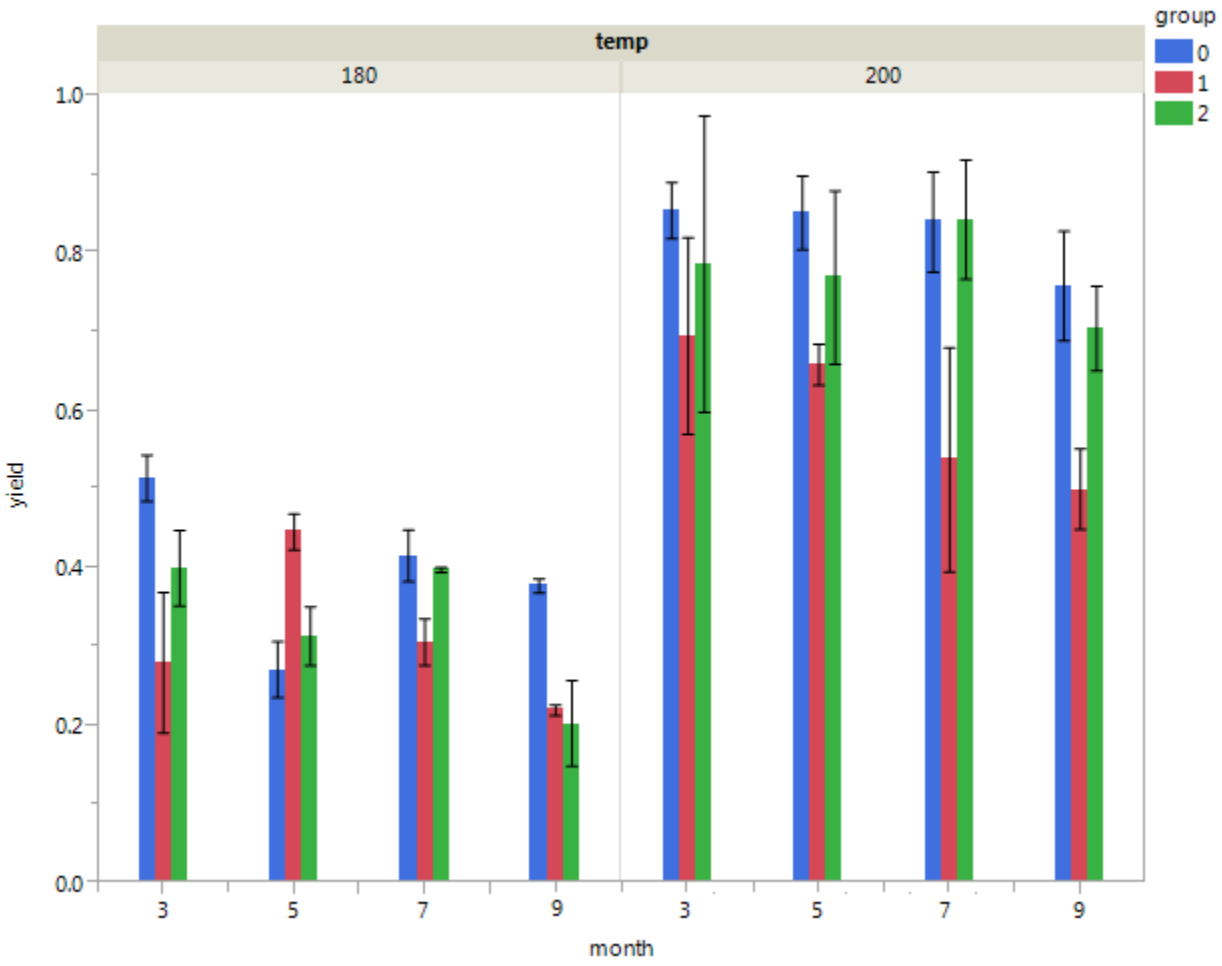


Figure 9 Yield (g/g) of sugar from enzymatic hydrolysis of pretreated biomass. The three treatment groups are 0 (control) and the two fungal treated groups (Group 1 and Group 2). The sampling dates were 3, 5, 7, and 9 months after baling. The yield is expressed as a percentage of what was recovered over what was available.Yield represents the percentage of available glucan available which was hydrolyzed in each study.

As a function of time at the 180 C treatment, Group 0 and Group 1 both showed no effect (p=.293 and p=.208) and Group 2 showed some effect (p=.0239). At the 200 C treatment, time was significant only in Group 1 bales (p=.570, p=0.0133, p=0.583 for Group 0, 1, and 2 respectively). 200 C produced higher average yields in every case (highest p=.0509). Group 1 showed significant differences from Group 0 and Group 1 at all levels past the first sampling

date month 3 (highest p=.0421). This means that after month 3, the Group 1 bales were significantly different than the control. All the data can be seen in figure 9. It should be noted that the compositional analysis of Group 1 was closer to that of the control; however, the enzymatic hydrolysis results suggest that Group 1 displayed different properties from the control, whereas Group 2 hydrolysis results suggest there existed no difference between Group 2 bales and the control in terms of accessibility to the enzyme. These results imply that biomass composition, including the resultant enzymatic hydrolysis yields, are not a predictor of fungal growth in and of themselves; it is more than likely that a total mass balance of xylan within the bales, as opposed to the mass fraction, would be a better indicator of fungal growth. Monitoring the dry matter loss within bales is in fact one of the most common methods for tracking bale degradation (Monti et al. 2009). Moreover, analytical procedures exist for the detection and quantification of fungal mycliem within biomass (Matcham et al. 1985) which could be used in future studies to get a better estimate of the success in colonization after the initial fungal spawn is introduced to the bale. Due to the fact that initial and final bales masses were not measured, the only data available in terms of bale mass in this experiment was the composition of the biomass. This is clearly not sufficient to describe the properties of switchgrass after storage and with or without fungal treatment. The lack of a distinct trend or correlation between composition amounts and final saccharification yields could possibly have been avoided through gravimetric analysis.

The increase in hydrolysis yields given a higher severity pretreatment (200 °C) is consistent with literature results at the same conditions across multiple types of biomass such as sugar cane bagasse, wheat straw, and corn stover (Laser et al. 2002, Perez et al. 2008, Mosier et al.2005). The lower severity pretreatment (180 °C) shows variation of between 20% and 50%

recoveries, with 180 °C being a pretreatment temperature typically too low to achieve any significant amounts of solubilization of hemicellulose (Liu et al.2003), which is the primary benefit of LHW pretreatment. The yields at both treatment temperatures themselves are not abnormal, but the variation between them, especially in the case of the Group 1 bales, indicate that there could be a large amount (between 20 and 90 %) in recovery values for full scale field studies. This variability could be caused by a number of uncontrolled variables, such as variable amounts of sun exposure, differences during baling, moisture content variation, or presence or non-presence of other biomass degrading microorganisms within the bales. It is known that differences in storage location and baling technique can result in swings of composition in hay as large as 14% (Rotz and Abrams, 1988).

There appeared to be no difference between the storage times and the yields of sugar recovered at each step. Though storage studies have shown decreased or increased overall glucose content and digestibility given longer storage periods, this study was conducted over 9 months, approximately 270 days, with samples collected every 90 days as in bale quality studies (Vane et al. 2001, Taniguchi et al. 2005, Salvachua et al. 2011). A similar storage study done for 7 months showed degradation of extractives between 8 and 11 % (Wiselogel et al. 1996). A different variety of switchgrass (Cave-in-Rock) stored outside for 9 months showed a 9% decrease in cellulose content (Agblevor et al. 1996). There is a limited about of research available on the storage of switchgrass for biofuel (Mitchell and Schumer 2012) and the combination of storage together with saccharification over such a long period of storage time is unique to this study.

What is clear from the data is that the composition of the biomass (Figures 5, 6, and 8) are not predictors of enzymatic hydrolysis yields (Figure 8). Even with the same composition as

the control, Group 1 bales performed differently than the control group when it came to sugar recovery. Likewise, the Group 2 bales, which were compositionally different from the control, performed equally as well in the enzymatic hydrolysis stage. Since there is evidence of fungal colonization on the biomass (Figure 7) for both Group 1 and Group 2 bales, we know for certain then that the compositional analysis of said bales is not sufficiently informative to tell us the condition of the switchgrass, at least in regards to enzymatic digestion. As far as the fungal treatment is concerned, it is difficult to make one general statement regarding the results, but it is safe to say that the fungal treated bales all either performed worse or equally as well as the control in regards to saccharification. This would indicate that, at best, the fungal treatment has done nothing, and, at worst, the fungal treatment has decreased overall sugar recovery in the switchgrass. In order to prove this definitively it would be necessary to have tracked the dry matter loss as well in each bale, as it is very likely the fungal treated bales (with fungal growth as well) would have experienced a more rapid mass decay when compared to the control. Coupled with the compositional analysis, it would be possible to do a full mass balance of the system such that the losses could be tracked with enough accuracy to state if the overall effect of the fungal treatment was a net positive or negative (or no effect). Although there are hints that the structure of the switchgrass in the treated bales is different from those of the control, the current data available for square bales attests to the fact that the fungal treatment at the very least did not improve the accessibility of the biomass to enzymatic attack.

6.2. Round Bales

The second study, from early March of 2014 to late September of 2014,used 1 ton (1000 kg) round bales of Kanlow switchgrass. These bales were stored the same way as the square bales, outside and on wooden pallets. All experimental procedures (compositional analysis, pretreatment, and enzymatic hydrolysis) that were conducted on the square bales were likewise conducted on the round bales. Round bales were sampled at 2 depths: between the surface and 1 foot deep, and between 1 foot deep and 2 feet deep. The pretreated biomass was also washed prior to being enzymatically hydrolyzed, a step omitted in the square bale study. Fungal spawn was originally included in the round bales, but visual inspection after the study was finished seemed to suggest that there was no colonization of the bales by the fungi used to inoculated them, as there was no typical evidence of fungal growth such as visible white hyphae. The results below detail the composition of the biomass prior to enzymatic hydrolysis, and finally the accessibility of the round bale biomass to enzymatic attack.

As to the prior work there was no quantification of dry matter loss over time. The only mass balance which exists is for the pretreatment into enzymatic hydrolysis steps, which is not indicative of the total mass of each cell wall component present in the biomass.

6.2.1. Moisture Content

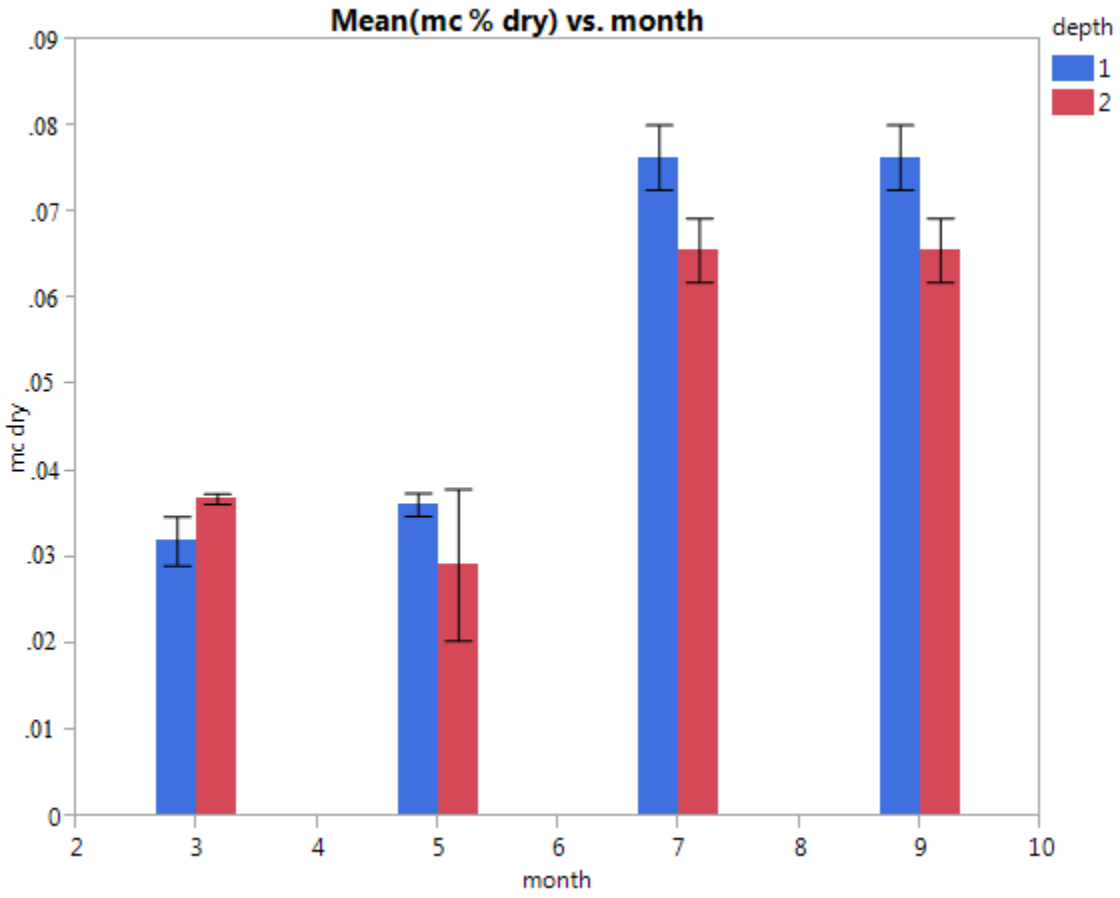


Figure 10 Moisture content as a function of time for the round bales. The blue bars were samples taken within the first foot of the surface of the bale. The red bars were samples taken between 1 foot deep and 2 feet deep.

As with the square bales, moisture content had a positive trend with time for both depth 1 ($p<.0001$) and depth 2 ($p = .0016$). For month 3, depth 2 had a statistically higher moisture content ($p=.0433$), whereas for months 7 and 9 depth 1 had a statistically higher moisture content ($p=.0244$, $p=.0206$). Month 5 showed no difference between depth 1 and depth 2 ($p=.2462$). This data is consistent with the idea that over time, bale integrity drops and allows for greater amounts of water to infiltrate.

6.2.2. Compositional Analysis

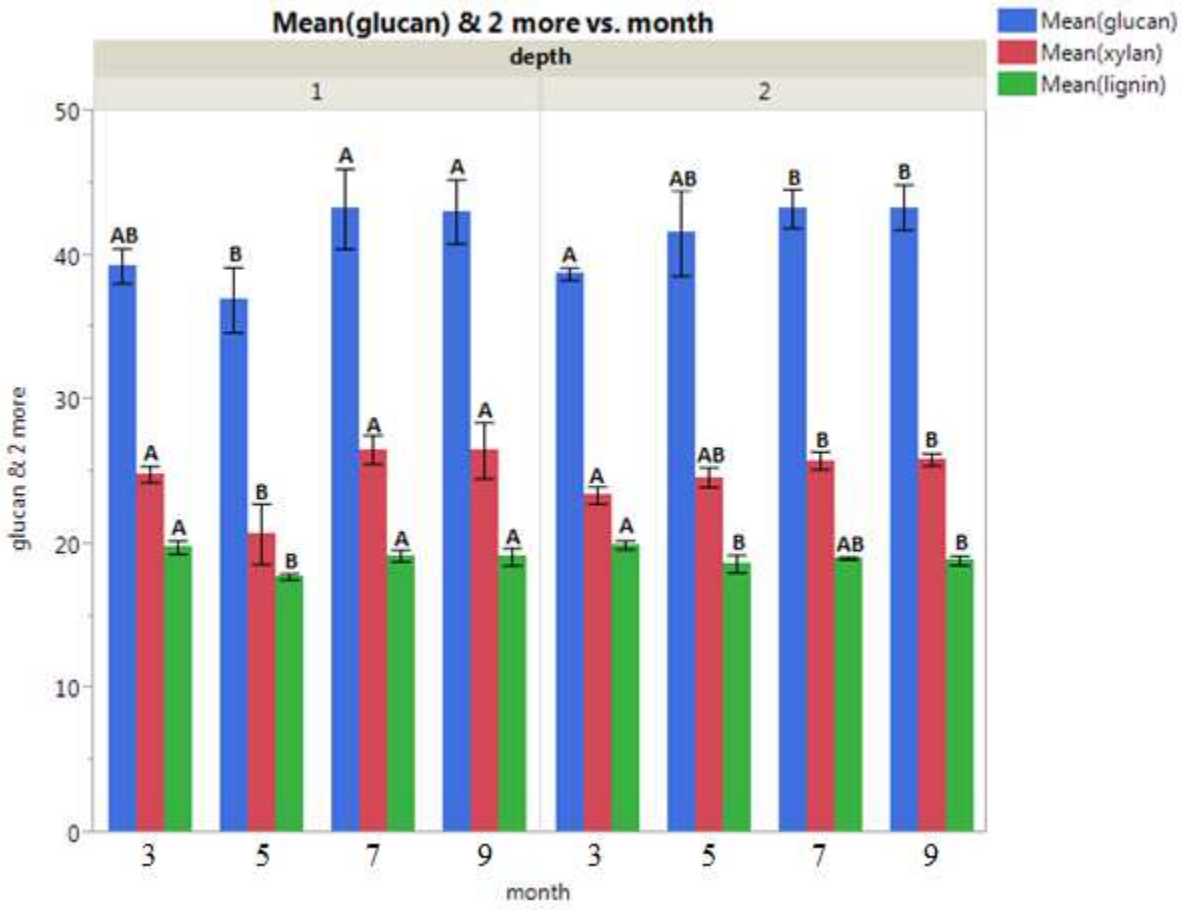


Figure 11 Compositional analysis (mean glucan, xylan and lignin content) of the round bales function of storage time. The blue bars represent glucan, the red xylan, and the green lignin. Month represents the sampling time. Depth signifies where in the bale the biomass came from (depth 1 or depth 2). The letters denote significant differences are a function of sample time, but only between the same sampling depths.

Figure 11 presents the glucan, xylan, and lignin content of stored round bales as a function of storage time. Prior to pretreatment, there was no evidence of sampling depth making a difference in the composition (lowest p=.586). Sampling month made no difference in lignin composition (p=.284) or but did make a difference in glucan (p=.0008) and xylan (p=.0146). In the analysis time was the only factor that affected the composition. It is important to note that all

analyzed components were relative, which implied that the total masses may have changed significantly, but their overall ratio would have remained relatively constant.

6.2.3. Digestibility of Bales

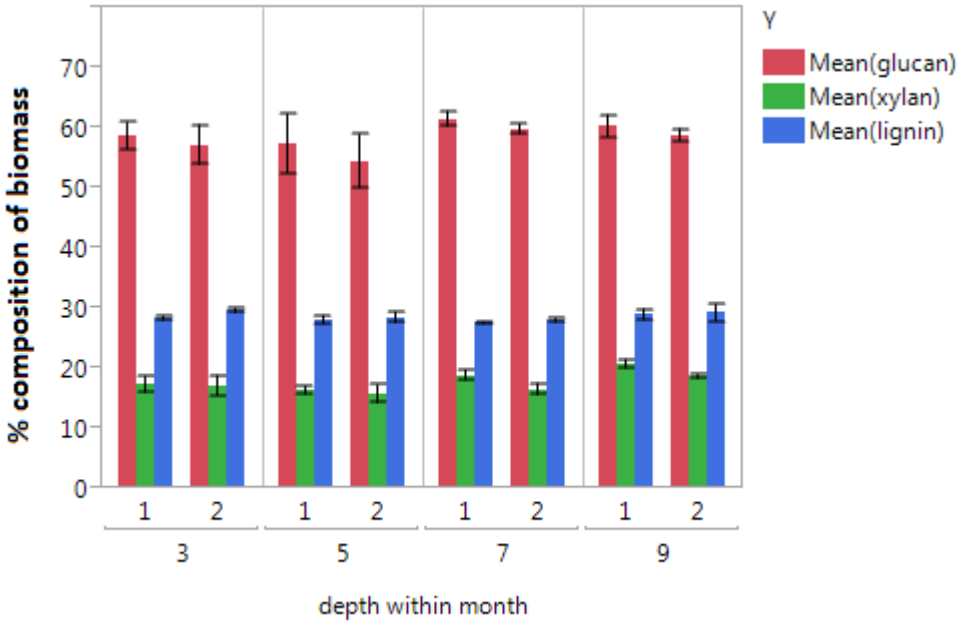


Figure 12 Compositional analysis of pretreated solids as a function of sampling date sampling depth. This biomass was pretreated at 180 Celsius. The Y-axis is the percent (out of 100) of each component which was present after pretreatment, with the red bars being glucan family molecules, the blue bars being lignin, and the green bars being xylan family molecules.

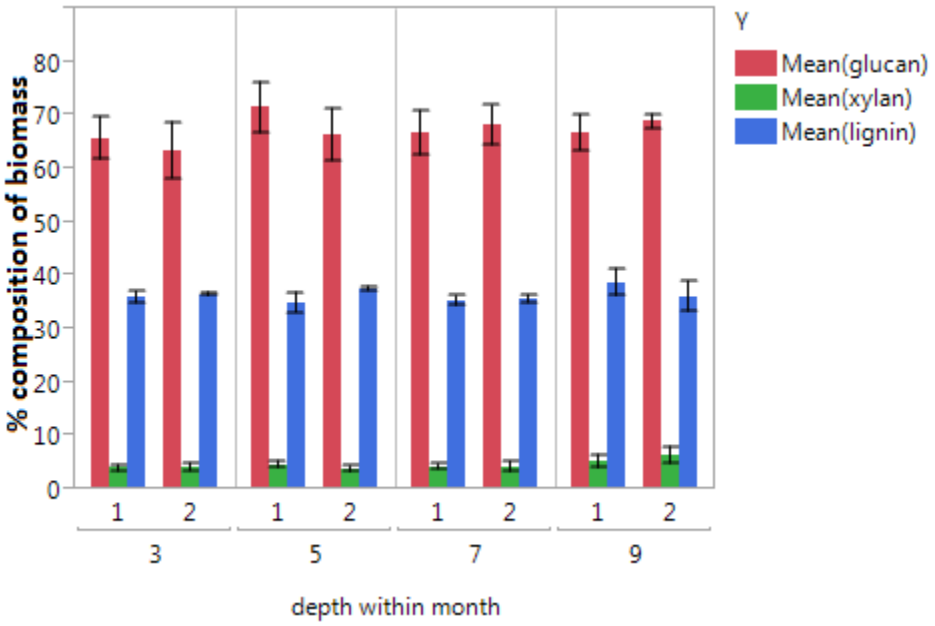


Figure 13 Compositional analysis of pretreated solids as a function of sampling date sampling depth. This biomass was pretreated at 200 degrees Celsius. The Y-axis is the percent (out of 100) of each component which was present after pretreatment, with the red bars being glucan family molecules, the blue bars being lignin, and the green bars being xylan family molecules.The four sampling months (3,5,7, and 9) are divided into the two sampling depths and shown side by side.

Figure 12 and Figure 13 presents the composition analysis of the biomass after 180 °C or 200 °C liquid hot water (LHW) pretreatment. Considering only the variable of time (month) and controlling for sampling depth and temperature, there were no observed differences between the treatments (p=.2557 for depth and p=.1139 for sampling month). When controlling for everything but temperature, between 180 °C or 200 °C pretreatment temperatures, all time and depths were significantly different in glucan and lignin composition (p=<.0001). The 200 °C pretreated samples all showed significantly lower averages of xylan compared to the 180 °C samples (p<.0001) and also showed difference across sampling month (p=.0009). From this data, we concluded that the starting point for all the bales, at all sampling times, had similar composition after pretreatment, as depth and time of sampling did not have any statistical

significance on composition (lowest p=.195). Therefore even the detected compositional differences in the raw biomass should not be relevant passed the pretreatment step, as statistically all the biomass treated at the same temperature was statistically identical. The total amounts of each component within the bales, however, were not determined, as no total mass recordings were obtained for the initial and final periods during sampling. However, from this data it was concluded that pretreatment at 200 °C solublized more xylose than that of 180 C. Higher xylose solubilization at pretreatment temperatures of 200 °C is consistent with previously reported literature (Liu et al. 2003)and with our prior study with square bales. Higher xylose solubilization at pretreatment temperatures of 200 °C holds across all sampling dates, which implies a pretreatment temperature of 180 °C is sufficiently high to solubilize as much xylose as 200 °C. Likewise, the lack of significance in sampling depths indicates that the switchgrass within each bale was homogenous in composition both before and after pretreatment, regardless of sampling depth.

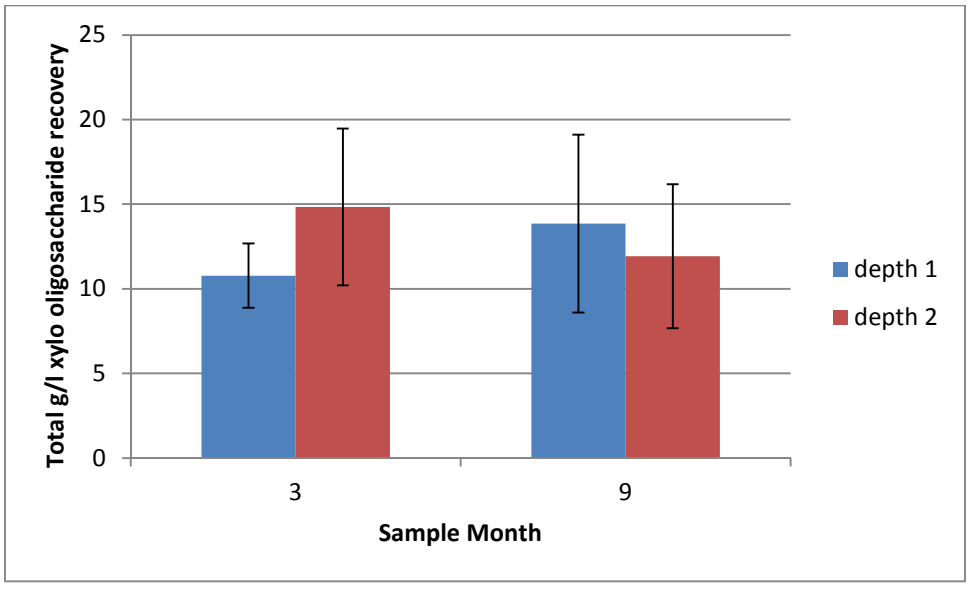
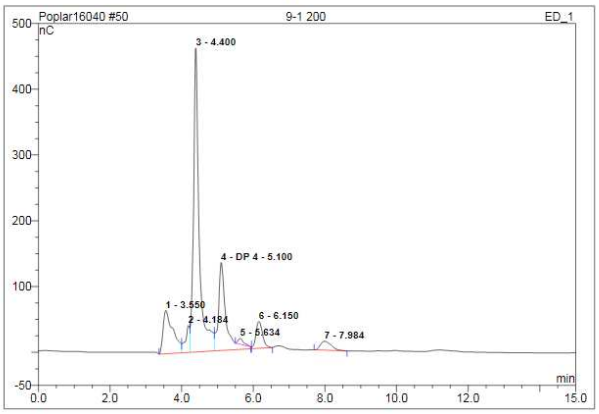


Figure 14 Total xylo oligosaccharides recovered in grams/liter in the pretreatment hydrolysate at both sampling depths. Only the 200 °C pretreatment temperature is shown

here as no significant quantities of xylo oligomers were detected in the 180 °C pretreatment samples. Though there was xylose present in 180 °C pretreated samples, the totals were less than 1 g/l and there were no detected oligomers in those prehydrolysates.

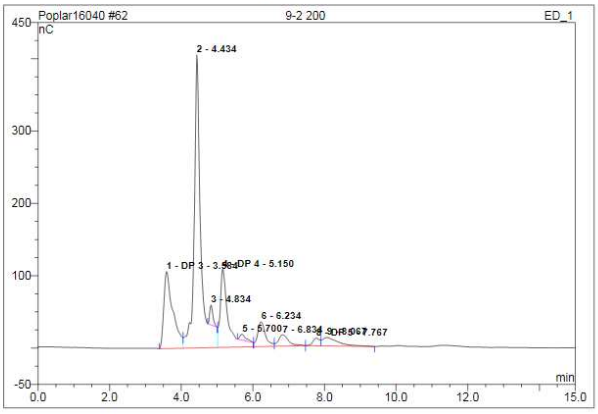
Figure 14 presents the xylo oligosaccharides recovered in 200 °C pretreatment hydrolyzates at both sampling depths. It was observed that the 200 °C hydrolyzate yielded both a wider variety of xylooligmers along with a greater amount of total xylose (over 10 gram/liter) when compared to that from the 180 °C results, which produced less than 1 gram total of xylo oligosaccharides after pretreatment. Though differences existed in the types of oligosaccharides formed, the total amounts between sampling depths were statistically similar. Neither sampling depth nor sample time significantly affected the xylo oligomer totals after pretreatment. These results indicated that whereas the total amounts and quantities of xylo oligomers were similar between samples, the polysaccharides breakdown proceeded differently between sampling depth and sampling time. This difference is indicative of a structural change in the biomass rather than that of a chemical nature, as evidenced by similar compositions in the original biomass. The observed differences in xylooligomer profiles must be due to degradation rather than composition, as all biomass samples had approximately the same quantity of xylan solubilization after pretreatment.

Month 3 Depth 1 200 C



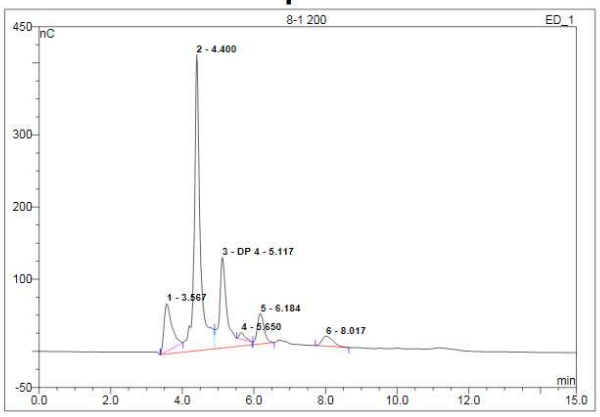
No.	Ret.Time min	Peak Name	Height nC	Area nC*min	Rel.Area %	Amount	Type
1	3.55	n.a.	65.678	21.008	13.21	n.a.	BM
2	4.18	n.a.	40.931	5.744	3.61	n.a.	M
3	4.40	n.a.	462.170	84.705	53.25	n.a.	M
4	5.10	DP 4	133.897	32.672	20.54	n.a.	M
5	5.63	n.a.	8.707	1.583	1.00	n.a.	Rd
6	6.15	n.a.	40.852	8.690	5.46	n.a.	MB
7	7.98	n.a.	13.515	4.673	2.94	n.a.	BMB
Total:			765.750	159.075	100.00	0.000	

Month 3 Depth 2 200 C



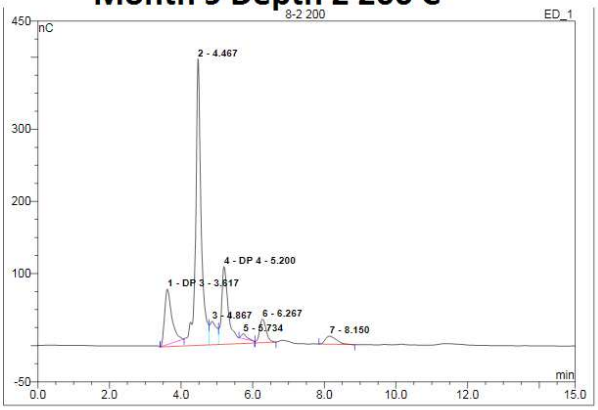
No.	Ret.Time min	Peak Name	Height nC	Area nC*min	Rel.Area %	Amount	Type
1	3.58	DP 3	106.361	31.135	18.03	n.a.	BM
2	4.43	n.a.	405.119	86.190	49.90	n.a.	M
3	4.83	n.a.	27.621	2.820	1.63	n.a.	Rd
4	5.15	DP 4	107.877	28.908	16.74	n.a.	M
5	5.70	n.a.	7.948	1.541	0.89	n.a.	Rd
6	6.23	n.a.	34.263	8.797	5.09	n.a.	M
7	6.83	n.a.	15.596	5.087	2.94	n.a.	Mb
8	7.77	DP 5	10.443	2.321	1.34	n.a.	bM
9	8.07	n.a.	11.650	5.929	3.43	n.a.	MB
Total:			726.878	172.726	100.00	0.000	

Month 9 Depth 1 200 C



No.	Ret.Time min	Peak Name	Height nC	Area nC*min	Rel.Area %	Amount	Type
1	3.57	n.a.	65.813	16.454	10.97	n.a.	Ru
2	4.40	n.a.	410.232	84.427	56.31	n.a.	BM
3	5.12	DP 4	125.921	33.121	22.09	n.a.	M
4	5.65	n.a.	9.351	1.743	1.16	n.a.	Rd
5	6.18	n.a.	41.816	9.249	6.17	n.a.	MB
6	8.02	n.a.	13.983	4.932	3.29	n.a.	BMB
Total:			667.115	149.927	100.00	0.000	

Month 9 Depth 2 200 C



No.	Ret.Time min	Peak Name	Height nC	Area nC*min	Rel.Area %	Amount	Type
1	3.62	DP 3	77.007	17.864	12.94	n.a.	Ru
2	4.47	n.a.	397.536	74.415	53.92	n.a.	BM
3	4.87	n.a.	32.659	7.287	5.28	n.a.	M
4	5.20	DP 4	107.820	26.224	19.00	n.a.	M
5	5.73	n.a.	6.729	1.235	0.89	n.a.	Rd
6	6.27	n.a.	32.633	6.962	5.04	n.a.	MB
7	8.15	n.a.	11.125	4.030	2.92	n.a.	BMB
Total:			665.509	138.017	100.00	0.000	

Figure 15 Chromatograms of the oligomers with the peak integration tables below. Both the depths and beginning and end sampling times are shown, only for 200 degrees Celsius. The retention time for xylose is around 4.4 minutes, then the xylose dimer at around 5.1 minutes, then the xylose trimer at 6.1 minutes.

Despite the totals between the beginning and end bales being similar (Figure 11), the individual profiles within each bale (and within the depths of each bale) were identical. Depth 2

samples had more quantifiable peaks in each chromatogram (9 to 7 in the month 3 bales and 7 to 6 in the month 9 bales) and the response of the detection in each bale was distinct. The overall total amount of xylose present was similar, but it appeared as though the degradation from long xylose polymers to shorter chains occurred differently rates within the bales. Overall this finding indicates that there might be structural differences within the bales that are not apparent when looking strictly at the xylan content of the biomass. The 'gross' quantity of xylose is not a good indicator of the similarity of the biomass. LHW pretreatment, also known as autohydrolysis, has been known to produce differing xylo oligosaccharide profiles based on the severity of the pretreatment used (Carvalheiro et al. 2004). Retention time in the reactor rather than pretreatment temperature was known to cause the observed shifts in the profiles. The results of this study show that most of the xylo oligosaccharides were accounted for in the first few polymer chains (DP1, DP2, DP3, DP4, and DP5). This is expected as LHW is known for solubilizing and breaking up hemicellulose, but the exact extent to which LHW treatment was effective at breaking down long xylose chains was not consistent across sample types. In summary it can be said that the degree of degradation was not the same between bales coming out of pretreatment, even if the net total amount of xylan removed was identical.

6.2.4. Enzymatic Hydrolysis Results

Using least square linear regression with JMP 11.0's modeling software a series of effects tests were done on the four variables in the experiment: storage time (months), pretreatment temperature, sampling depth, and washing/not washing. The results found that washing had no effect ($p=.6624$), sampling depth had little effect ($p=.0693$), and storage time and temperature had the largest effects ($p=.0024$ and $p<.0001$ respectively). In this case the null hypothesis can be rejected for storage time and pretreatment temperature, but the data fails to disprove n0 for sampling depth and washing. The individual means were tested with ANOVA and the results are shown below.

6.2.5. Analysis of sample dates

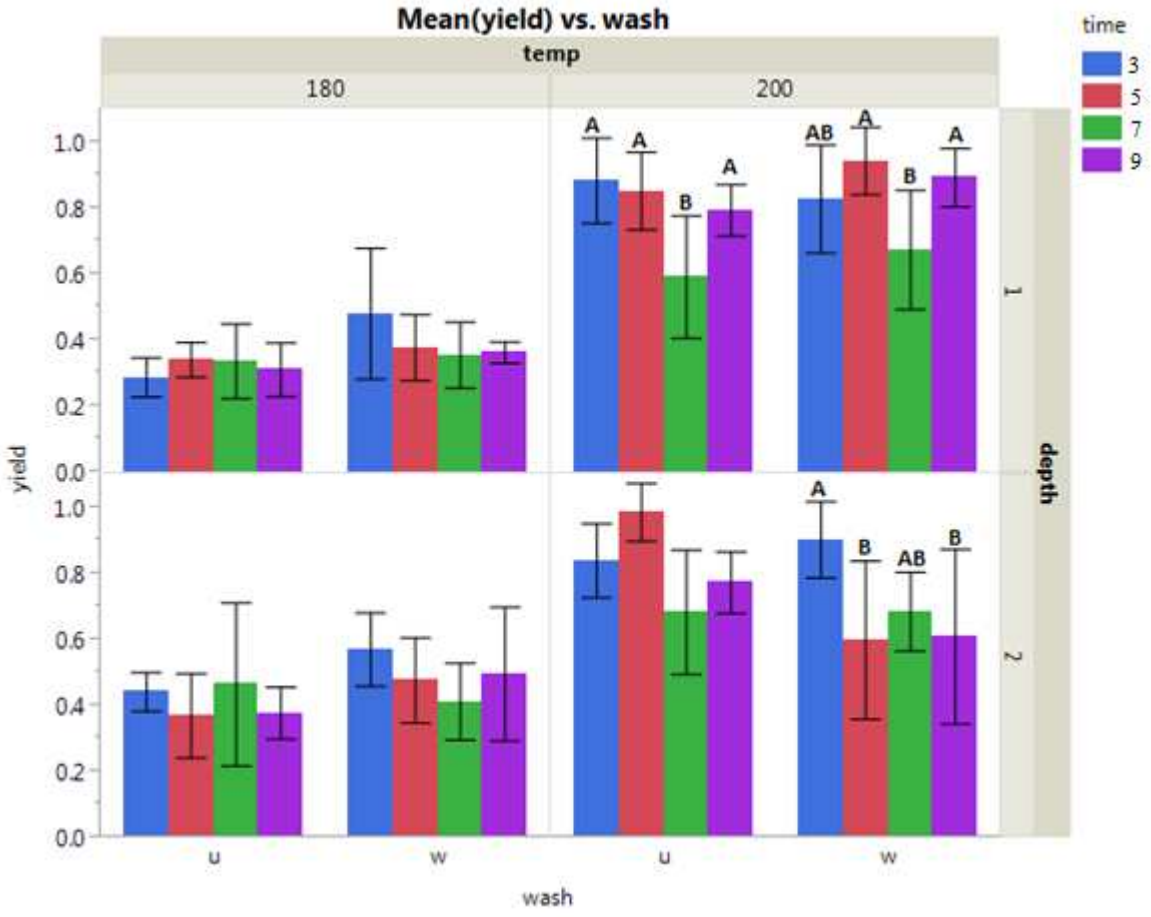


Figure 16 Yield (g/g) of sugar as a function of sampling date. Washed biomass is denoted with the letter w, while unwashed biomass is denoted with the letter u. Time is in months. Temperatures is in Celsius. Depth 1 and depth 2 correspond to the sampling depth from which the biomass was taken.

Figure 16 presents the sugar yield in terms of gram per gram recovered in 180 °C and degrees 200 °C pretreatment hydrolyzates at both sampling depths, including washed and non-washed biomass. There were no significant differences determined between sampling date and enzymatic hydrolysis yields for the 180 °C pretreatment temperatures (lowest p=.0564). However, there were significant differences associated with sampling time for some of the 200 °C pretreatment samples. Washed samples at the depth 2 showed the most significant downward trend (p=.0057) when compared to the other 3 sample sets at 200 C. This indicated that washing

and pretreatment temperatures enhanced the effects that storage had on the bales in regards to saccharification. The reasons why biomass which was sampled deeper in the bales showed a greater decline in glucose recovery, but not necessarily glucose content, is that biomass from the surface of the bale is potentially more weathered, and thus already partially degraded and easier to access. This relationship specifically examined in the depth data comparisons later in the thesis it should be noted that a difference in enzymatic accessibility between depths over time was observed, namely that biomass closer to the surface (depth 1) did not differ in enzymatic accessibility as much as biomass deeper in the bale (depth 2).

6.2.6. Analysis of washing

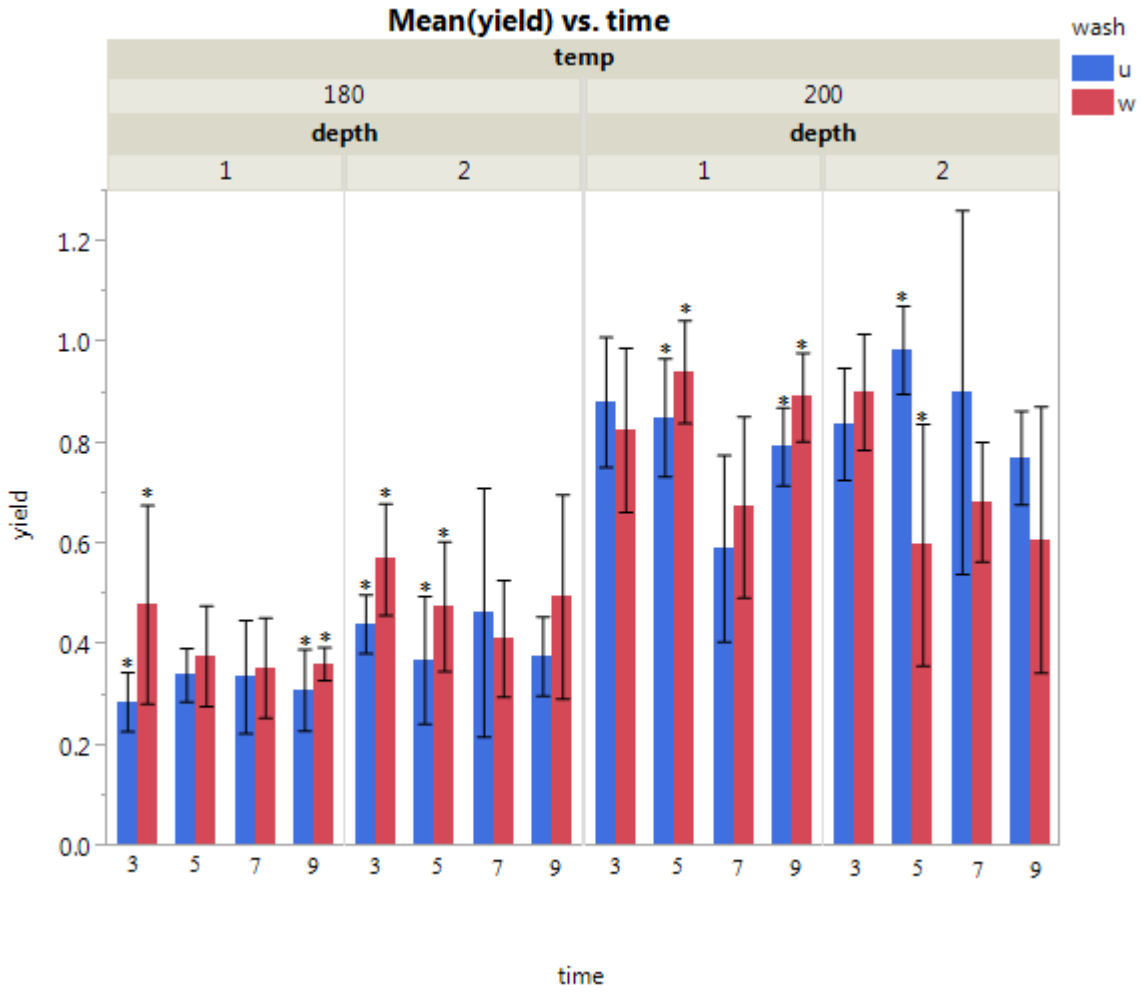


Figure 17 Yield (g/g) of sugar as a function of washing. Yield is in grams recovered over grams available. Time is in months. Washed biomass is denoted with a w, while unwashed biomass is denoted with a u. Time is in months. Temperatures is in Celsius. Sampling depth denotes the area within the bale the biomass was taken from (depth 1 or depth 2).

Figure 17 presents the sugar yield in terms of gram per gram recovered in 180 °C and 200 °C pretreatment hydrolyzates at both sampling depths, including washed and non-washed biomass. The solids loading (1%) was sufficiently small such that the wash step should not of had a significant effect given how diluted the samples were. The increase in carbohydrate recovery in washed biomass samples is similar to published literature (Frederick et al. 2013), though the relationship in this work is surprising given the very dilute (1 in 100) ratio between

the mass (and thus the pretreatment hydrolysate) and the reaction volume. It was not expected that there would be sufficient pretreatment hydrolysate left within the biomass at such a small concentration that any enzymatic inhibition would occur. Thus, it might be the case that the act of washing alters the biomass in some way other than diluting the pretreatment hydrolysate, which may be the cause of the observed differences in enzymatic hydrolysis yields between washed and unwashed samples.

6.2.7. Analysis of temperature

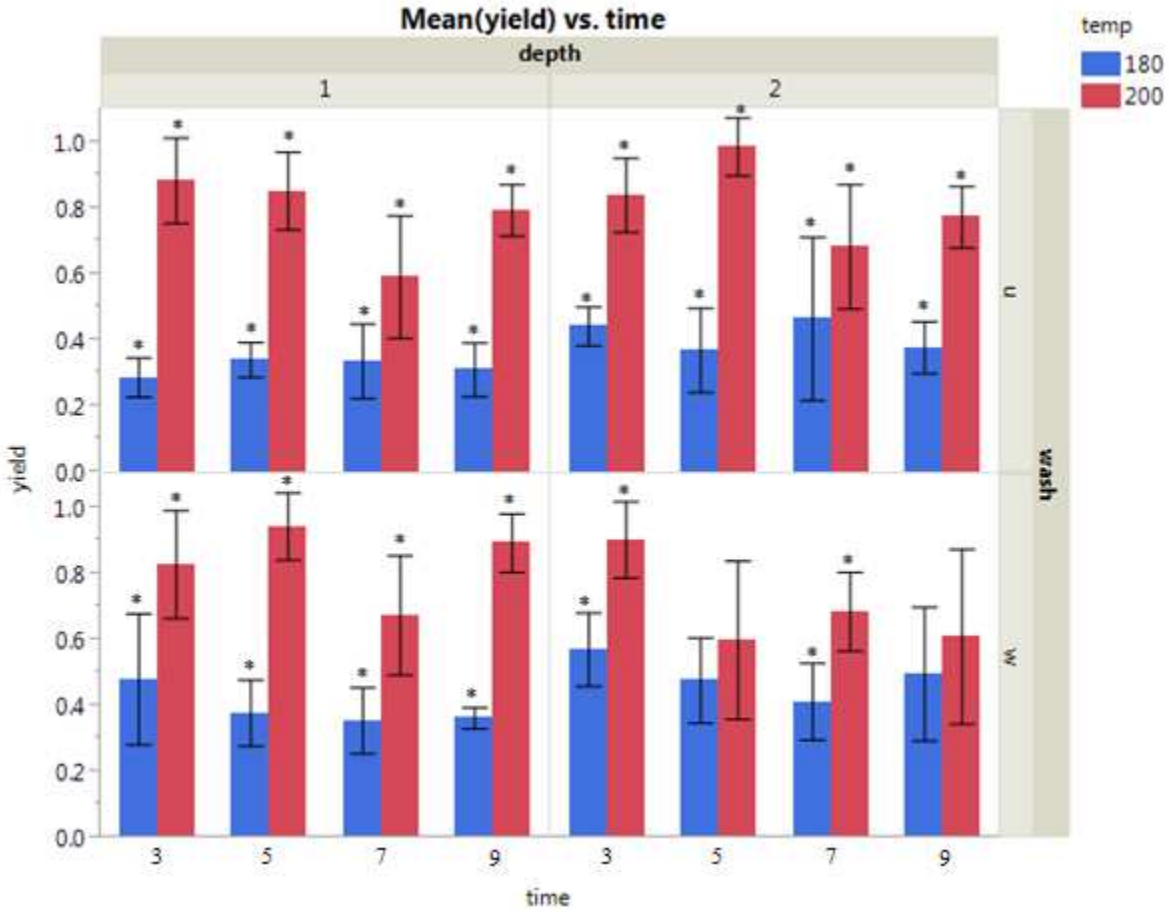


Figure 18 Yield (g/g) of sugar as a function of pretreatment temperature. Washed biomass is denoted with a w, while unwashed biomass is denoted with a u. Time is in months. Temperatures is in Celsius.

Figure 18 presents the sugar yield in terms of gram per gram recovered as a function of pretreatment temperatures (180 °C and 200 °C). For nearly every sample, 200 °C pretreatment produced significantly greater average yields of glucose when compared to the less severe 180 °C pretreatment (lowest p=.008). There were two cases where there was no difference between 180 C and 200 C, at sampling months 5 and 9 at depth 2 washed (p=.203 and p=.340 respectively).This indicated that washing the deeper sampled biomass improved the 180 °C pretreated biomass saccharification sufficiently that it became statistically similar to that of the

200 °C pretreatment. This relationship was also aided by the lower amount of saccharification achieved in the month 9 bales as well, which brought the sugar recoveries of the 200 °C pretreated samples closer to those of those pretreated at 180 °C. These results are consistent with Figure 15, which shows that the later month 9 bales for 200 °C were lower than those from month 3.

These results were consistent with the data found in both the composition analysis and in the pretreatment compositional analysis: 180 °C and 200 °C pretreated biomass both had different physical properties going into the next step of the process (after LHW preatreatment), consequently both sets of biomass responded differently to the saccharification processing chain. There were some conditions, such as washed and stemming from depth 2 that produced a significant difference in enzymatic yields but the general trend across all treatment groups was statistical similarity rather than a difference.

6.2.8. Analysis of sampling depth

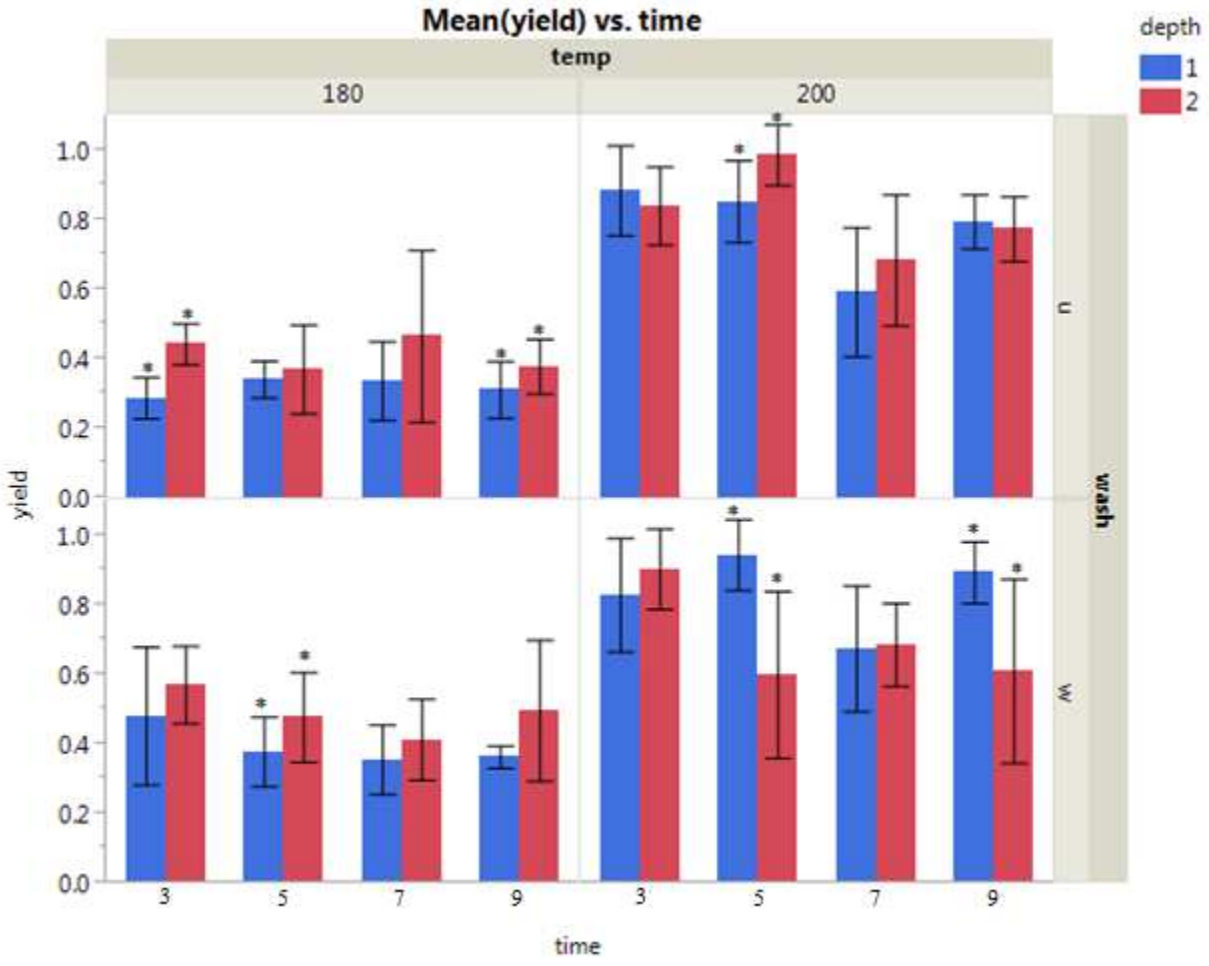


Figure 19 Yield (g/g) of sugar as a function of sampling depth. Washed biomass is denoted with a w, while unwashed biomass is denoted with a u. Time is in months. Temperatures is in Celsius.

Figure 19 presents the sugar yield in terms of gram per gram recovered as a function of sampling depth. Overall depth effects showed no consistent trend, though the relatively low p value (.0693) does suggest some effect might exist. In combination with storage (Figure 16) it was observed that sampling depth combined with storage time did play a role in overall saccharification yields. This implied that, for a given month, sampling depth did not matter because bales were homogenous throughout with respect to enzymatic saccharification. Across

the study period, sampling depth did matter where for the 200 °C pretreated biomass, washed biomass showed a significant decrease over time and over sampling depth in regards to enzymatic digestibility, on the other hand unwashed biomass showed no trends regarding sampling depth or sample time.

6.3. Average Precipitation (Round and Square)

The amount of precipitation which fell during the months of storage was found using the Oklahoma Mesonet network of weather monitoring stations, at their Stillwater station (lat 36-07-15 long 97-05-42).

Table 3 Precipitation over sampling dates, separated into month and year. Squares bales, where fungal colonization was observed, were conducted in 2013, whereas the round bales where no growth was observed were conducted in 2014.

Inches of rain	March	**April**	May	June	July	August	September	October	Total
Month	3	**4**	5	6	7	8	9	10	
2014	1.21	**0.84**	**0.65**	6.29	3.98	2.01	4.19	2.18	21.35
2013	1.12	**5.33**	**6.22**	3.95	5.57	2.25	1.16	1.88	27.48

Precipitation alone is not an indicator of bale degradation, but together with temperature and humidity can be used to estimate the dry matter loss over time (Huhnke 2003). Wetter, warmer weather is preferable to hot, dry weather. The Spring of 2014 in particular was very hot and dry, where 2013 was wetter and more temperate. In addition the 2013 bales were square, which are known to be more susceptible to weather unlike round bales (Macdonald and Clark 1987). Thus the square bales, which were studied in 2013, provided conditions hospitable to fungal growth.The dry year of 2014 could be an explanation for no observable difference was present between the fungi treated and non-fungi treated round bales.

7. Conclusions

7.1 Storage Study

There were no significant changes in composition between bales during storage. This is not an unexpected finding as previous storage studies of switchgrass, such as in Wiselogel et al. (1996) and Adler et al. (2006), showed that the biomass composition changed only slightly between all plant cell wall components, frequently less than 5%, and at a decreasing rate over time. Compositional changes are possible, but often not considered as a significant contributor to bale chemistry after baling (Monti et al. 2009). Our study showed that, as far as composition of each set of biomass is concerned, there was little change over time; it should be noted that dry matter loss, however, could occur.

The most significant differences in the study did not come from compositional analysis, but from enzymatic hydrolysis results, where large significant differences could be determined between different samples, which had statistically similar compositions. This suggested that the differences determined from enzymatic hydrolysis yields were not due to compositional changes within the biomass, but possibly due to structural biomass changes. The structural change of switchgrass bales over time had previously been reported. Switchgrass bales stored in an unprotected environment for up to nine months were reported to have decreases in extractives of switchgrass of up to 11% (Wiselogel et al. 1996). Moreover, it has been reported that multiple types of white-rot fungi could degrade structural proteins in non-woody biomass, such as wheat straw (Agosin et al. 1985), and that different types of lignin were degraded at different rates, depending on storage conditions (Shinners et al. 2010). Thus it is possible that, while composition of biomass did not change in terms of its ratio of cellulose/lignin/xylan/ash, the structural integrity of biomass could have been modified over time. In this work, there were no

significant changes in swichgrass composition as a function of time, but analysis of biomass in terms of its ability to release carbohydrates after enzymatic hydrolysis showed significant differences, as shown in Figure 16. It was observed that switchgrass, while compositionally very similar over time, released carbohydrates more readily in the earliest sampling dates as opposed to the latest. The by-product profile of the prehydrolysates for each sample was relatively similar. The pretreatment was not the source of the differences observed in enzyme yields, as the overall levels of inhibitory byproducts formed were not sufficiently large to inhibit the enzymatic reactions. Acetic and formic acid were less than 1 g/l, while furfural was less, than 5 g/l; concentrations of 5 g/l or greater would be required to confer inhibition starting (Jing et al. 2009). On the other hand, the oligomer profiles varied as a function of time, as shown in Figure 15, and these are known to be inhibitory to enzymatic hydrolysis (Qing et. a 2010).

The only differences in sampling dates came from the 200 °C treated group, and at depth 2 samples, which indicated that if sampling date had an effect on biomass recalcitrance, it was only at the more severe pretreatment conditions. This is consistent with the literature wherein higher temperatures, usually corresponding to higher severities of LHW pretreatment give better and a wider range of saccharification results (Hendriks and Zeeman 2009). This means that whatever structural differences that may exist within the biomass during storage either through decomposition or otherwise are only evident at higher severities.

7.2Water

In regards to the differences in precipitation between the round and square bales, it can be said that the lower amount of precipitation in 2014 when compared to 2013 could be the explanation for why none of the fungal inoculum grew in the round bales, which were assembled in 2014, a year with low precipitation. It has been reported that high levels of precipitation (over 65 cm) correspondedto high levels of bale degradation (Wiselogel et al. 1996).As opposed to field set-ups, laboratory fungal biomass deconstruction experimentsrequire additional water and oxygen to foster fungal growth. Reports on addition of components consist of: saturation of initial growth medium with water up to 75% (Vane et al. 2001); addition of water to the inoculum to sustain growth with up to 1:3 mass to volume ratio(Salvachua et. a 2011); and, the periodic addition of water to maintain the biomass at 80% (wet basis), (Balan et al. 2008). Moisture content for the biomass in laboratory scale experiments can be as high as 90% (wet basis), but most published laboratory work on the cultivation of *P. ostreatus* is conducted at 60 % to 70 % moisture content. In comparison, our biomass never exceeded 10 % moisture content, and in fact averaged around 6 %, which were conditions that were not conducive to foster *P. ostreatus* growth. In that respect we were cultivating the fungus in extremely dry conditions, without additional mineral supplementation, and in potentially oxygen deprived environments.

7.3Biomass Recalcitrance

Though there were observed differences in the biomass digestibility, there was no trend associated with the application of the white-rot fungus inoculum and the digestibility of the biomass. Though specific data points did perform better (or worse) within the two treatment groups, it was not possible to quantify whether this was natural variability, or a direct result of the fungal treatment. Since there were not enough replications of the square or round experimental units (1 control and 2 treatment bales) there is insufficient data to state whether or

not the differences in the averages are significant or not. Qualitative analysis of the biomass in the square bales confirmed the growth of fungus through the observation of both white fruiting bodies of the mushroom as well as the mycelia within the bale but no quantitative analysis such as determining fungal composition or doing mycelia counts was conducted. Therefore it can be said that the fungus did successfully colonize the material, but the extent and progress of the fungus cannot be quantified due to the experimental setup not allowing for such data to be collected on a monthly basis.

However, two general patterns seem present in the data. Within the square fungal treated bales that were enzymatically hydrolyzed there typically were only two levels of significance (A and B), with the data tending to either be statistically similar to the control group or lower. For 200 C, the fungal treated bales did not outperform the control group. For 180 °C, there was only one data set (group 1, month 4), which outperformed the control group in enzymatic hydrolysis, and all other treatment groups were statistically equal to or lower than the control, as shown in Figure 9. Since the compositions of all Groups (0, 1, and 2) were statistically similar, as were the compositions of the biomass after pretreatment, this indicated that there was no structural change in the biomass which made it more susceptible to enzyme attack (or at least no more susceptible than the control already was). The most likely reason for this is simply that the biomass was not affected by the addition of fungal spawn, even if the fungus was observed growing within the biomass. This could be because the fungus tends to grow locally, rather than distributed equally throughout the bale, and the bale sampling method takes an average of multiple points within the bale (some of which might have no fungi). This would mean simply that cultivating the fungi requires more than just spreading the spawn evenly though the bale. The difficulties in achieving uniform fungal growth in biomass are well studied outside of bales (Adamovic et al. 1998, Balan

et al. 2008). Ultimately the investment needed to provide a suitable environment for fungal cultures on biomass would likely be cost prohibitive, thus while our study did not provide ideal conditions for fungal growth, it did provide a more realistic expectation of fungal viability in bales stored as they would be on a biofuels farm.

In regards specifically to the hypothesis that fungal treated bales would lower the pretreatment severity needed to hydrolyze sugars, the experiment was inconclusive. At best we showed that the introduction of fungal treatment decreased overall enzyme yields while showing no difference in biomass composition, though statistically even these results were only true for a few select data sets. There were no significant differences in regard to storage time and composition, but there were significant differences between the ability of the enzyme to hydrolyze the biomass that was 'older'. This suggests a non-compositional based factor which is inhibiting enzymatic activity at some level within the biomass, a change which is present after it has been left outside after harvest for some time (9 months). This change was present in both round and square bales though to a different extent in each. The round bales, which are known to shed water more effectively, showed no signs of significant differences between treated (with mushroom) and untreated controls, and there was no visual identification of fungal growth within these bales either. One possible reason for this was the dryness and inaccessibly of round bales to oxygen and water, which is an intended feature of their shape. Square bales, with their greater surface area and more water-retaining shape, did show signs of fungal growth (in fruiting bodies), but this growth was localized and not throughout the entire bale.

Between different bales there were multiple incidences of significant differences between enzymatic hydrolysis yields, which shows that even in bales being considered to be the same 'unit', there are large amounts of inherent experimental variability within them. These

differences were more pronounced at the 200 °C pretreatment temperatures, with a decreasing amount of significance the older the bales were (the older the bales were, the more their data looked like other data from similarly aged bales). This is consistent with literature findings regarding storage of biomass for long periods of time, which show that the dry matter loss (and thus decomposition) of biomass over time follows a decreasing rate as a function of time (Larson et al. 2010).

8. Suggestions for Future Work

With these results in mind, there are several clear directions to take subsequent studies to alleviate the natural variability within the bales as well as to maximize the likelihood of fungal colonization. One important factor to take into account is the dry matter loss within each bale, which was not tracked in this study but is a fundamental data type that is used in similar storage studies in order to ensure both mass closure (once the mass balance in known) within the bales as well as to provide a metric for degradation of the bales over time. Without that data it is impossible to conduct a full 'yield' analysis on how much sugar exists within a single bale, as we have no baseline for total starting sugars present (as cellulose). In addition to taking mass loss over time, bales should likely be stored indoors and be supplied (at least initially) with water after baling. The addition of water and storage indoors removes two key uncontrollable variables of the experiment (precipitation and water content within the bales at harvest). With these two changes, the differences between square and round bales would likely lessen (or disappear) as we have removed the source of variability (precipitation) which favors fungal growth in one type of bale but not the other. Additionally more replications of each condition would help to reduce the overall variability by having a larger sample size. This is especially important for the control bales. Given the large experimental units (1000 kilograms) however it is possible that together

with funding and land availability, getting more units is difficult. It would then be meaningful to consider smaller bales instead; much like has been done already (Monti et al. 2009).Smaller bales with the same amount of biomass would allow for more replications and more easily controlled experimental conditions, which would both increase the consistency and decrease the variability in the data at the expense of having data which is less applicable to the industry. There is currently another follow-up study being conducted using smaller bales with controlled moisture content.

The results have shown that fungal inoculation of switchgrass bales can produce significant changes in enzymatic hydrolysis of sugars, though the levels are lower rather than higher in terms of grams of glucose yielded per gram of cellulose present. While this work is different from currently published work regarding fungal pretreatment (which has been shown to increase yields), for the reasons discussed prior (water, storage time, storage method) our study showed either no effect or the opposite effect on large bales of biomass. Storage times only had effects on switchgrass at the more severe pretreatment conditions, though the more severe pretreatments also produced significantly more sugar recoveries overall.

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9. Appendix

Table 1: Glucose recovery masses in total grams for 180 C in round bales

Glucose Recoveries for Round Bales (grams)

Each reaction vessel was loaded to .1 gram of cellulose

	180 C							
	Depth 1				Depth 2			
	Unwashed			Washed	Unwashed			Washed
bale	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
1	0.024218	0.000877	0.026044	0.004479	0.0406	0.004975	0.044092	0.002401
2	0.029644	0.0033	0.024963	0.001698	0.020425	0.001092	0.060725	0.007965
3	0.036406	0.005879	0.043918	0.008998	0.039109	0.006508	0.032963	0.002318
4	0.033646	0.012822	0.039428	0.002213	0.035184	0.000421	0.073859	0.002989
5	0.028672	0.008549	0.047913	0.005486	0.043876	0.008184	0.036479	0.000439
6	0.040428	0.000681	0.045623	0.003949	0.045219	0.004386	0.035633	0.005633
7	0.02018	0.002143	0.026483	0.005586	0.035238	0.001426	0.048222	0.001237
8	0.034867	0.002423	0.036321	0.002278	0.047575	0.004076	0.035212	0.006131
9	0.0334	0.001335	0.039611	0.001141	0.048165	0.000617	0.066842	0.000718
10	0.03222	0.002259	0.042929	0.001779	0.045515	0.001692	0.046808	0.009643
11	0.044617	0.001035	0.036144	0.007431	0.065872	0.003333	0.054109	0.000455
12	0.024817	0.000746	0.031725	0.000282	0.030773	0.001192	0.035059	0.000972

Table2: Glucose recovery masses in total grams for 200 C in round bales

200 C							
Depth 1				Depth 2			
Unwashed			Washed	Unwashed			Washed
Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
0.101806	0.010807	0.096504	0.00955	0.09276	0.007588	0.085386	0.017837

0.076099	0.006694	0.104934	0.005606	0.059875	0.004268	0.086899	0.003597
0.081324	0.002314	0.078434	0.002806	0.08458	0.002453	0.08104	0.003862
0.072416	0.007225	0.080102	0.009271	0.086869	0.006719	0.037373	0.002353
0.087964	0.003287	0.088774	0.000669	0.088196	0.005699	0.093459	0.012122
0.074911	0.01145	0.085893	0.009405	0.101098	0.004655	0.0493	0.010791
0.056847	0.006537	0.075354	0.000952	0.052017	0.009307	0.063473	0.009587
0.08218	0.007932	0.097276	0.000834	0.06945	0.007674	0.04116	0.009042
0.075088	0.00156	0.062992	0.006688	0.070759	0.001391	0.086038	0.015012
0.094825	0.006149	0.092019	0.003567	0.103365	0.002348	0.03543	0.006785
0.039557	0.000476	0.038991	0.009317	0.114113	0.004816	0.060921	0.009991
0.083674	0.003666	0.090401	0.000841	0.075486	0.002513	0.08884	0.002686

Table 3: Glucose recoveries in mass for square bales at 180 C and 200 C

Glucose Recoveries (gram)

Each reaction vessel was loaded to .1 gram of cellulose

	180 C		200 C	
bale	Mean	Std Dev	Mean	Std Dev
1	0.051366	0.002918	0.085337	0.003558
2	0.027066	0.003559	0.085029	0.004679
3	0.041506	0.003275	0.08388	0.006334
4	0.037666	0.000906	0.075751	0.006968
5	0.027944	0.00893	0.069412	0.012494
6	0.044545	0.002278	0.065741	0.002596
7	0.030543	0.002947	0.053691	0.014229
8	0.021875	0.000667	0.049962	0.005113
9	0.039921	0.004815	0.078532	0.018831
10	0.031311	0.003727	0.076825	0.011015
11	0.039704	0.000326	0.084195	0.007569
12	0.020234	0.00546	0.070359	0.005386