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Mushroom Inoculation on Switchgrass Feedstock during Storage: Effects of Subsequent Pre-processing for Intended Biofuels Production

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Mushroom Inoculation on Switchgrass Feedstock during Storage: Effects of Subsequent Pre-processing for Intended Biofuels Production

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Abstract

The objective of this project was to examine the effect of fungal treatment and liquid hot water pretreatment of switchgrass combine in view of increasing glucose release. The fungal treatment consisted of incubating *Pleurotus ostreatus* in square switchgrass bales, at 50% moisture content for 25 days, 54 days, and 82 days. The digestibility of the switchgrass biomass was subsequently evaluated using Accelerase® 1500 enzyme. Lignin is an important barrier to enzymatic hydrolysis, and it was stipulated that incubation with *P. ostreatus* would disrupt plant cell walls, resulting in enhanced saccharification. Three different concentrations of *P. ostreatus* were evaluated: 0%, 2%, and 3% by weight. Maximum digestibility was observed in switchgrass pretreated with 3% *P. ostreatus* for 25 days but not yet pretreated in hot water, which resulted in 39% saccharification as opposed to 32% with that of 0% *P. ostreatus*. This indicated that the fungal inoculation facilitated structural carbohydrate release. Switchgrass samples collected after solid state fermentation with *P. ostreatus* were subjected to liquid hot water (LHW) pretreatment at 200°C for 10 min, and 180°C for 30 min, and the prehydrolyzates were washed with 5X volumes of water, before being used for enzymatic analysis. Overall, LHW pretreatment enhanced the enzymatic digestibility of fungal fermented switchgrass. For fermented switchgrass samples pretreated using LHW, at 200°C for 10 min, maximum saccharification of 82% was obtained for the samples inoculated with 3% *P. ostreatus* and stored for 82 days. However, there were no significant differences between the other conditions. Enzymatic hydrolysis was also performed for the washed LHW pretreated switchgrass samples. Washing prehydrolyzates after LHW pretreatment was supposed to enhance its enzymatic digestibility; however in this study, a significant overall reduction in enzymatic digestibility was observed as compared to the non-washed samples.

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1. Introduction

Petroleum resources are non-renewable and are creating uncertainty with climate due to increased CO_2 concentrations. Therefore, there is an urgent need for alternative solutions to produce non-petroleum based fuels or other bio-based products. In addition, an increase in awareness regarding climate and environmental change (due to use of fossil fuels) has attracted more researchers to search for environmental alternative energy sources (Souza et al., 2017). Bioenergy is under significant consideration over the last decade (Liu et al., 2013; and Souza et al., 2017). Lignocellulosic biomass, such as energy crops, wood, and agricultural waste are available in abundance and could serve as feedstock for fuel production (Himmel et al., 2007; and Saini et al., 2015). In recent past years, a lot of attention has been paid to the fermentation of lignocellulosic derived sugars to ethanol (Lin et al., 2006); ethanol has many advantages as it is considered the cleanest source in terms of liquid fuels (Lin et al., 2006; and Liu et al., 2015). The term "second generation" applies to fuels that are produced from lignocellulosic biomass and not from starch or sugar cane-derived carbohydrates. On the other hand, "first generation" term is used to describe liquid fuels derived from food crops like, corn, sugarcane, cereals etc. Their production and production processes are deeply studied and understood (Sims et al., 2010).

Second generation biofuels have the potential to become important in the ethanol production arena (Searchinger et al., 2008). Also different sources have been used from time to time to meet the world's ethanol needs (Sims et al., 2010). For example, Brazil used lignocellulosic sources to produce ethanol in the first decade of the 21st century to meet biofuel needs. Lignocellulosic sources, such as grasses, wood and agricultural wastes do not compete with food crops in terms of resources and are more sustainable, thereby satisfying the demand for renewable fuels. Once established, herbaceous crops, such as switchgrass, demand less irrigation, fertilizer inputs, or maintenance and are thus an ideal feedstock for

cellulosic ethanol production. Kanlow switchgrass (*Panicum virgatum* L.) has low water and nutrient requirements, is quite robust on marginal lands and displays high productivity, making it a desirable feedstock source (Sanderson et al., 2006; and Liu et al., 2013).

However, the conversion of sugars from lignocellulosic to ethanol is not as effortless as that of starch to ethanol. Lignocellulosic biomass, like switchgrass, displays chemical complexity which is partly due to a tightly wounded structure, rendering lignocellulosic materials challenging to be used as alternative carbohydrate sources (Zheng et al., 2009). Unfortunately, the carbohydrates that are contained in lignocellulosic biomass are trapped in a complex structure that is made up of three components: cellulose, hemicellulose and lignin. Cellulose forms the backbone of all plant cell walls (Kim et al., 2009; and Liu et al., 2013). On the other hand, lignin is a complex molecule that provides rigidity to the plant cell wall, while protecting cellulose from hydrolytic attack (Kim et al., 2009). It is important to note that lignin composition is plant specific where hemicellulose acts as a bond between cellulose and lignin. Tightly woven plant cell walls can release carbohydrates through pretreatment followed by enzymatic hydrolysis. There are numerous existing pretreatment technologies to alter the structure of biomass such as, chemical, physico-chemical, and mechanical pretreatments. It is important to note that the goal of any pretreatment is the disruption of the biomass structure such that it is no longer recalcitrant by increasing cellulose and hemicellulose accessibility (Salvachua et al., 2011; and Zheng et al., 2009).

In addition to chemical, physico-chemical, and mechanical pretreatment, there exists an option of fungal pretreatment, also known as biological pretreatment, based on the capacity of some organisms to consume lignin and enhance carbohydrates accessibility (Salvachua et al., 2011; and Kim et al., 2009). Specialized fungal strains can be used to develop scalable and environment-friendly delignification methods that are less energy consuming. Biological pretreatment can also be used in combination with other pretreatments

like liquid hot water (LHW) to achieve higher enzymatic digestion. LHW pretreatment solubilizes the hemicellulose fraction of biomass, with minimal formation of sugar degradation products like hydroxymethylfurfural (HMF) and furfural. It allows the enzymes to access the cellulose, which had been made more reactive by the solubilization of hemicellulose fraction. Moreover, in LHW pretreatment processes, there is no addition of any chemicals whatsoever, rendering this unit operation environmentally friendly. It is likely that large scale biological delignification and subsequent LHW pretreatment of biomass would ensure higher enzymatic digestibility, in an efficient and sustainable manner.

2. Literature Review

2.1 Pretreatments

The primary source of ethanol production in the United States is through fermentation of sugars derived from food sources, such as corn and sugarcane (Sims et al., 2010). Since this approach can have negative consequences on food prices and supply (Li and Khraisheh, 2010), efforts are being made to secure carbohydrates from non-food sources (Balan et al., 2008). Biofuels produced from various lignocellulosic materials, such as so-called 'energy grasses' (grown as a low cost and low maintenance crops for the production of biofuels), wood, agricultural or forest residues, have the potential to be valuable alternatives of carbohydrate sources that can be fermented into ethanol, complementing and offsetting gasoline production (Kumar et al., 2009). The three basic steps in the production of ethanol from biomass are: 1) pretreatment of biomass to alter the complexity of the structure; 2) enzymatic hydrolysis to convert complex sugars into single sugars for the production of fermentable sugars; and, 3) anaerobic fermentation of sugars to produce liquid biofuel ethanol (Faga et al., 2010; Pessani et al., 2011; and Wyman et al., 2005). The main component that limits enzymatic breakdown of carbohydrates is lignin.

Among lignocellulosic biomasses used for the production of biofuels, switchgrass has been widely favored due to its high yield, low fertilizer and water requirements and high nutrition-use efficiency (McLaughlin and Walsh et al., 1998; Keshwani et al., 2009; and Searchinger et al., 2008). Switchgrass has been used as a potential feedstock that has immense potential for the production of ethanol (Krishna and Chowdary et al., 2000; Stenberg et al., 1998; and Sanderson et al., 2006). Switchgrass is called a warm season crop because it grows best in warm conditions and it is normally planted from April to mid June (Garland et al., 2008). It can be planted at planting depth of ¼ inch (6 mm) to no deeper than ½ inch (13 mm) in tilled or no-tilled seedbed but it grows better in unbedded fields from past

row crops. Moreover, it can be harvested multiple-times in a year using conventional balers for harvesting (Garland et al., 2008).

Switchgrass has high cellulose content and widely adapted for different soils and climate conditions. Switchgrass yields of up to 10 tons per acre per year (usual yields are 6 to 7 tons/acre, 5400 to 6400 kg per ha per year) have been reported, which can yield up to 500 gallons per acre (5000 liter per ha) of ethanol (Garland et al., 2008). By comparison, the usual annual conversion for corn-based ethanol is around 85 gallons per ton. It is important to note that switchgrass is resistant to many insects and diseases, decreasing the need for fertilizer and insecticides. In a study conducted at the University of Tennessee, researchers and farmers confirmed the above listed traits (Garland et al., 2008). From the University of Tennessee study, the recommended varieties of switchgrass for the production of ethanol were Kanlow and Alamo (Garland et al., 2008). In summary, switchgrass is a widely adapted lignocellulosic biomass that can be used for the production of ethanol or other bio-based products due its wide adaptability, low cost, high yield, and high cellulose content (Garland et al., 2008).

However, many physicochemical and compositional factors hinder the conversion of cellulose, which is present in switchgrass, to sugars (Kumar et al., 2009). As mentioned above, the conversion of lignocellulosic biomass to ethanol is not as straightforward as that of starch to ethanol (Kumar et al., 2009). Lignocellulose material is made of plant cell walls that contain cellulose, hemicelluloses and lignin (Jeon et al., 2010; and Monrroy et al., 2011) that are tightly woven together, conferring strength to cell walls. On average, cellulose and hemicelluloses make up to 50 to 75% of the plant cell wall structure on a dry weight basis. These polysaccharides can be converted into sugars and then into alcohols, such as ethanol and butanol (Swana et al., 2011). Because of their interwoven plant cell wall structure, the polysaccharides are not easily converted to their corresponding monosaccharides, making

their conversion to ethanol challenging. For the conversion of biomass to ethanol, pretreatment of lignocellulose feedstocks is required to remove the barrier created by the association of hemicelluloses and lignin with the cellulose polysaccharide (Foyle et al., 2007). Pretreatment makes the structure of the biomass less crystalline, removes hemicellulose, and lignin and increases the surface area of the biomass (Wyman et al., 2005). Figure 1, highlights the effects of pretreatment on biomass plant cell walls. The interwoven plant cell wall structure of biomass becomes reformed or altered after the pretreatment, allowing for hydrolyzing enzymes to gain better access to targeted cell wall polymers.

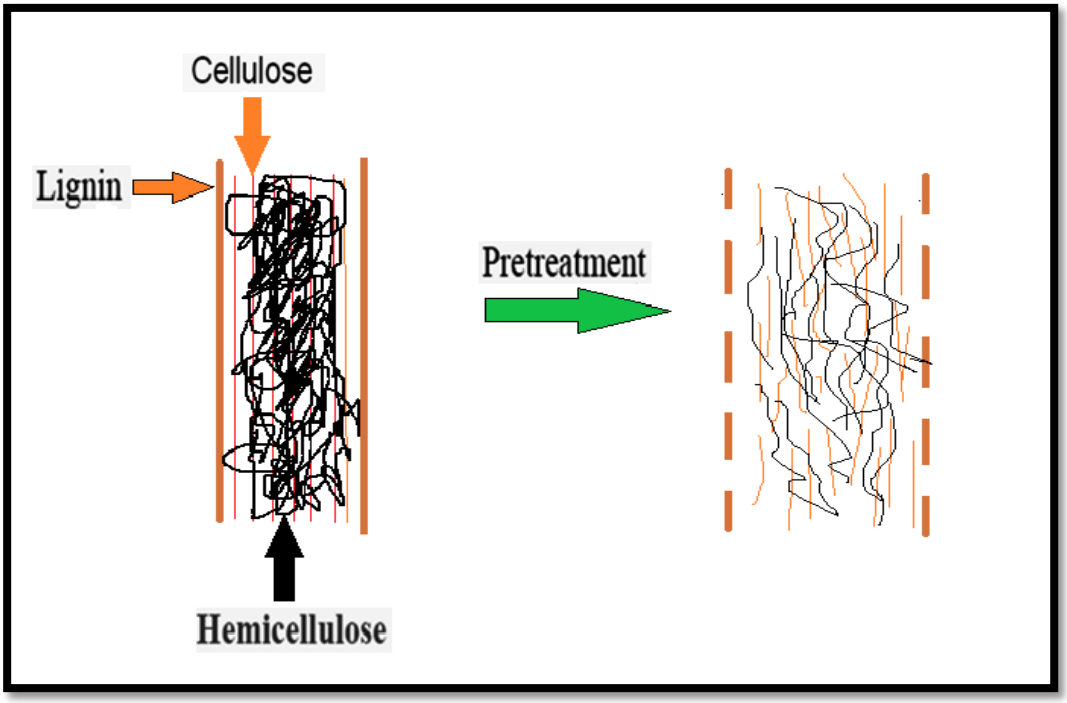

Figure 1: Goals of pretreament on lignocellulosic biomass (Inspired by Hsu et al., 1980; and Mosier et al., 2005).

A flow chart (Figure 2) explains the process of obtaining fermentable single sugars from switchgrass biomass. It explains the step-by-step procedure to get single sugars from a complex biomass structure. Fungal pretreatment as shown in the flow-chart below degrades lignin and LHW pretreatment degrades hemicellulose.

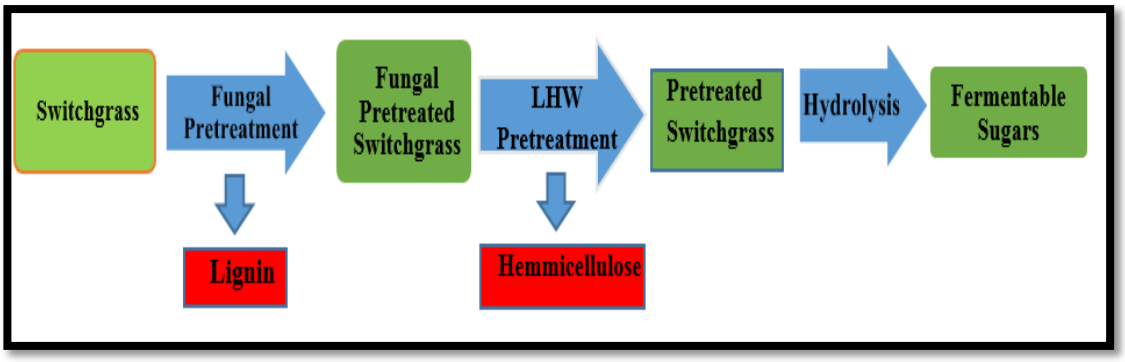

Figure 2: Process of obtaining fermentable sugars from switchgrass.

2.2 Fungal pretreatment

Various types of pretreatments, such as physical, chemical, biological or their combination can be done to alter or disrupt the complex structure of biomass, enhancing hydrolysis rates (Kumar et al., 2009). Biological pretreatments, such as fungal, and physical-chemical pretreatment, like liquid hot water, enhance biomass saccharification (Faga et al., 2010; and Hatakka et al., 1983). Fungal pretreatment has been conducted with three types of fungi: white rot, brown rot and soft rot (Aguair, et al., 2014). Of the three types of fungi, white-rot fungi (*Trametes versicolor*) are the most effective at degrading lignin. Moreover, biological pretreatment by white-rot fungi is low in cost, sustainable and environment friendly; however, it is not a rapid pretreatment as delignification rates are slow, making the process last between 15 and 45 days (Ryu, 2013). Figure 3, below explains the process of biological pretreatment (via lignin degradation by fungi) of lignocellulosic biomass.

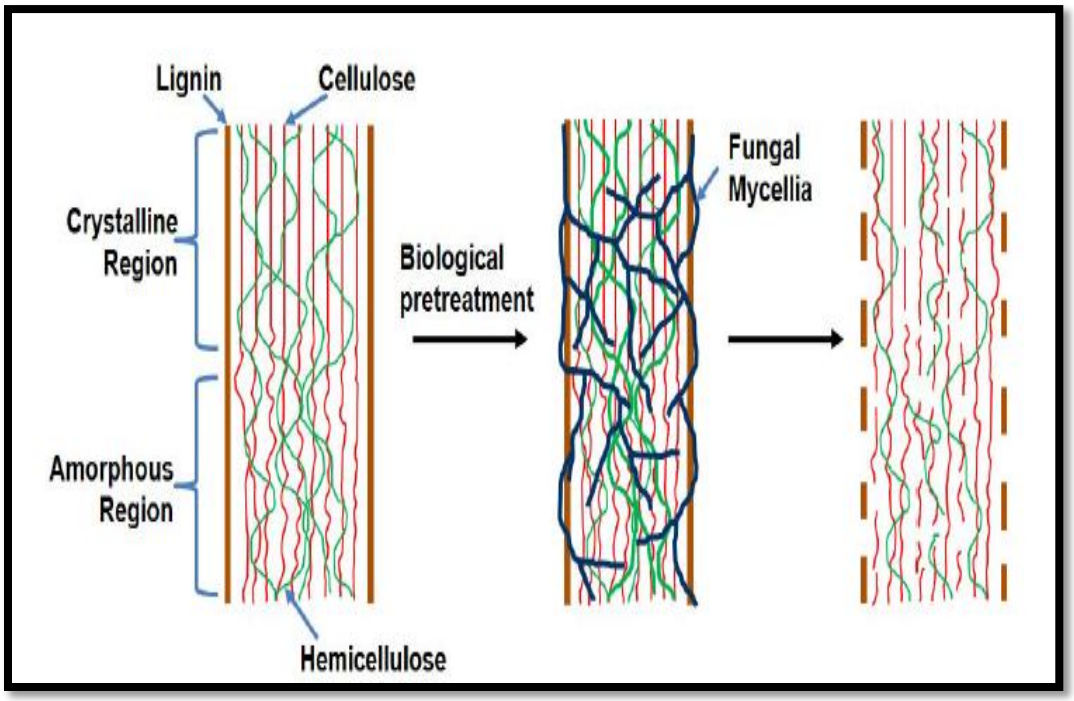

Figure 3: Process of biological pretreatment of lignocellulosic biomass (Isroi et al., 2011).

Relative to other white-rot fungi that were evaluated, *Pleurotus ostreatus* was reported to selectively degrade lignin instead of cellulose; this is an important trait because 50% of other white-rot fungi species metabolize cellulose (decreasing the eventual sugar yield for ethanol production) as well as lignin (Taniguchi et al., 2005). *P. ostreatus* is an edible mushroom widely cultivated in the world and it has various food, medicinal and nutraceutical applications and properties (Carrasco-González et al., 2017). Fungal pretreatment with white-rot fungi species, such as a selection of *Pycnoporus sp*. and *P. ostreatus*, were reported to increase enzymatic hydrolysis efficiency of switchgrass by decreasing its lignin content (Liu et al., 2013; and Taniguchi et al., 2005 and 2010). Liu et al. (2013) reported that the switchgrass lignin content was decreased by 30% without any significant loss of cellulose and hemicelluloses after 36 days of cultivation period. Concomitantly, after a 36-day pretreatment period with *P. ostreatus*, enzymatic hydrolysis was 50% higher than that of the control (Liu et al., 2013). It was observed that total organic matter decreased during fungal pretreatment and this was positively correlated with lignin

degradation, thereby increasing the hollocellulose to lignin (H/L) ratio (Gupta et al., 2011). It was interesting to note that the total cellulose content slightly decreased or remained constant (Gupta et al., 2011), and this was important because cellulose must be conserved as much as possible.

Three white-rot fungi, *Pleurotus florida*, *Coriolopsiscaperata sp. and Ganoderma sp.*, were studied on sugarcane bagasse (Deswal et al., 2014). After a 15-day of inoculation, period, it was observed that incubation with *P. florida* resulted in maximum delignification, (7.91%); interestingly, it was reported that after a 25-day incubation period, delignification was arrested in all three tested white-rot fungi (Deswal et al., 2014). Fungal or biological treatment efficiency can be measured in two ways: 1) yield of sugars from biomass (efficiency of enzymes); and, 2) conversion of sugars from biomass (efficiency of pretreatments). Out of 19 white-rot fungi tested, *P. ostreatus,* was one of the fungi that degraded lignin, increasing the efficiency of enzymatic saccharification. Pretreatment with *P. ostreatus* for 35 days resulted in 35% of the wheat straw being converted to reducing sugars, of which 74% was glucose. On the other hand, if pretreatment is carried out in an (oxygen environment), such as with *Phanerochaete sordida* 37 or *Pycnoporus cinnabarinus* 115, the treatment time could be reduced by one week (Hatakka et al., 1983). Taniguchi et al. (2005) conducted a study on rice straw to evaluate the effect of biological treatment with four white-rot fungi, - *Phanerochaete chrysosporium*, *Trametes versicolor*, *Ceriporiopsis subvermispora,* and *P. ostreatus,* Taniguchi et al., (2005), reported that *P. ostreatus* preferentially degraded lignin over hemicellulose. Moreover, cellulose content was 83% of that of untreated rice straw. The action of the fungi resulted in a hemicellulose decrease by 48%, while that of cellulose was 17%. Taniguchi et al. (2005) also reported that, after 60 days of pretreatment, 41% lignin was degraded and 25% weight losses were incurred. *P. ostreatus* was incubated on cotton stalks; once again lignin degradation was preferred over cellulose

degradation (Hadar et al., 1993). Although *P. ostreatus* degrades preferentially lignin over cellulose, their slow growth rates as compared to other white–rot fungi present a major drawback.

2.3 Liquid hot water pretreatment (LHW)

The objective of the pretreatment step is to open up the cell wall structure in order to make it more accessible to saccharification enzymes, which are critical for the conversion of cell wall carbohydrate polymers to fermentable sugars (Mosier et al., 2005; and Frederick et al., 2016). Improving pretreatment efficiency has the potential to lower the cost of ethanol production (Lee et al., 1995). Liquid hot water (LHW) pretreatment, also called hydrothermal pretreatment, is not considered as a chemical pretreatment because no additional chemicals are added to the biomass containing pretreatment vessels. LHW separates and solubilize hemicellulose, thereby modifying the plant cell wall structure; hence, biomass becomes more accessible to enzymatic hydrolysis (Frederick et al., 2016). Generally, LHW pretreatment does not lead to the generation of degradation products, decreasing the need of mitigating these compounds (Laser et al., 2002). Frederick et al. (2016), studied the effect of hot water treatment in conjunction with storage practices and reported that pretreatment resulted in solubilization of hemicellulose without significant generation of enzyme inhibitors and eventually enhancing enzymatic accessibility. However, it should be noted that Frederick et al. (2016) only tested the system through enzymatic hydrolysis; it is possible that fermentation inhibitors could have been generated. In summary, LHW pretreatment increases cellulose's availability to cellulase enzyme consortiums. Disadvantages of LHW pretreatment include the lack of lignin solubilization thereby does not significantly alter the complexity of lignocellulosic biomass (Mosier et al., 2005). As compared to other types of pretreatments, such as dilute acid pretreatment that requires high temperature, LHW requires both high pressure and temperatures, increasing the energy consumption of this unit operation.

Moreover, LHW may not be well suited for biomass with high lignin content, such as hardwoods (Mosier et al., 2005).

Effects of washing biomass after LHW but prior to enzymatic saccharification were also reported and results demonstrated that there could be need for washing the LHW pretreated biomass prior to saccharification steps (Garlock et al., 2011; and Li et al., 2013). Garlock et al. (2011) reported that post-washing the LHW samples, and sulfur dioxide (SO_2) pretreated samples exhibited 83% higher glucose yield for unwashed samples. Garlock et al. (2011) also reported similar results for xylose release with other type pretreatments. Li et al. (2013) stated that washing of switchgrass (*Panicum virgatum)* with water was done to remove residual ionic liquid content and observed that washing was successful in removing residual ionic liquid. However, a certain amount will always stay in the biomass. The removal of ionic residual liquid significantly improved the sugar release from switchgrass (Li et al., 2013). On the other hand, Frederick et al., (2016) reported that washing of 5, 6 and 9 month *P. ostreatus* stored samples that were located at 0.6 m inside the bale, reduced saccharification returns by at least 20%. This effect was not observed for outer layer of the bale for 5 and 9 month samples. Frederick et al., (2016) provided no explanation as to the reasons why exterior and interior samples responded differently to rinsing. In addition, Frederick et al. (2016) suggested that the washing step can be eliminated without affecting saccharification efficiency and it will result in saving a great amount of water in the industry.

There are numerous reports on the use of LHW to pretreat biomass prior to enzymatic hydrolysis. LHW was used to pretreat poplar, where saccharification reached 50%, as compared to 3% without pretreatment (Kim et al., 2009). LHW pretreatment was compared to that of steam to pretreat sugar cane bagasse; xylan recovery was highest with LHW at 200°C and 2 min, as compared to that of solely saturated steam. Overall, xylan recovery was higher for any given temperature and time with LHW pretreatment, as compared to that of

solely steam (Laser et al., 2002). LHW was used to pretreat corn stover; 90% cellulose was converted to glucose and 88% efficiency was achieved during fermentation of glucose and xylose to ethanol (Mosier et al., 2005). This latter study highlighted the fact that LHW pretreatment affected the composition of the biomass, as well as the physical appearance at the microscopic level. It was proposed that pH during LHW pretreatment should be kept between 4 and 7, using potassium hydroxide to minimize the formation of sugar degradation products (Kim et al., 2009). Moreover, Kim et al. (2009) reported that temperature and time had no effect on yields, while saccharification results were independent of temperature and time (Mosier et al., 2005). LHW pretreatment had the potential of releasing carbohydrates, but final yields were deemed dependent on initial composition and LHW operating conditions (Hendriks et al., 2009).

2.4 Storage

Switchgrass is a perennial crop that is under extensive study for its use as a cellulosic feedstock. If year-long switchgrass supply chains are to be established, economically viable storage protocols will need to be developed that will mitigate biomass loss, while maintaining biomass integrity. There are few studies that have addressed biomass storage losses, which is currently attributed microbial growth (Sanderson et al., 1997; Emery and Mosier et al., 2012; and Frederick et al., 2016). Loss of dry matter is positively correlated with precipitation and bale contact to the soil (Emery and Mosier et al., 2012; and Frederick et al., 2016). Frederick et al. (2016) reported that lignin and structural carbohydrates, such as glucan and xylan, were 10% or less during a 9 month long uncovered round bale study. This group reported that 0.28 m rainfall, occurring during the storage period between months 5 and 7, resulted in accelerated loss of biomass components, as compared to other storage periods where rainfall was less. With respect to the integration of storage and LHW pretreatment, a 5% increase in glucan, content was reported as a function of storage time (Frederick et al., 2016). Frederick

et al. (2016) reported that length of storage period did affect saccharification, where samples stemming from the interior of the bale returned 20% less glucose.

Sanderson et al. (1997) also reported that there were large differences in dry mass loss for two studies conducted in one identical year but at different locations; dry matter loss differences in these two studies were correlated to the amount of precipitation. In the Sanderson et al. (1997) study, the outer layer of switchgrass was exposed to precipitation (as well as convective and radiation-mediated evaporation) and its dry matter content was lower than that of the inner layer (Sanderson et al., 1997). Wiselogel et al. (1996) reported that there were less compositional changes observed in switchgrass that was not exposed to high rainfall as compared to highly exposed switchgrass. The loss of dry matter was also influenced by harvesting methods, storage time and cover (Mooney et al., 2012). However, these losses were eliminated by modifying bale composition and using some protective measures. In addition to the dry matter losses, compositional changes were also observed during switchgrass storage for 26 weeks (Wiselogel et al., 1996). Samples of fresh and stored switchgrass were analyzed for their extractive content, where results showed changes as a function of time. It was determined that the outer layer and the unweathered portion afforded losses of 11% and 8% of extractive content, respectively (Wiselogel et al., 1996). Storage conditions affect the lignin content of the biomass. Lignin content was greater in switchgrass stored in field conditions, as compared to that of barn storage; however, increases in lignin content were mostly due to loss of extractives and carbohydrates (Djioleu et al., 2014). Substantial losses of carbohydrates (51% glucan and 39% xylan) were observed when switchgrass was stored unprotected and uncovered in an open field conditions (Bitra et al., 2013). The lignin composition in the biomass affects the enzymatic hydrolysis and saccharification efficiency, as lignin degradation gives rise to phenolic compounds, which impede enzymatic activity (Rajan and Carrier et al., 2014). The effect of storage on biomass

composition must be considered before starting any experiment or production either at small scale or large scale. Djioleu et al. (2014) reported that controlled storage of switchgrass could be beneficial in cost reduction of biofuel production processes. In summary, biomass storage procedures that minimize the loss of fermentable carbohydrates, could be an important component in developing economically viable, resilient and practical biomass supply chains.

3. Research Gaps

Storage studies were initially conducted to minimize the mass losses of biomass stored for livestock feeding; this work provided insight as to the response of biomass to storage. From a bioenergy perspective, small scale studies on fungal pretreatment of biomass, such as switchgrass and rice or wheat straw, to remove lignin, have been previously conducted (Hatakka et al., 1983; Emery et al., 2012; and Itoh et al., 2003). Although these small scale studies were conducted with less than 0.1 kg of biomass, their results showed that lignin was degraded to a higher degree than hemicellulose and cellulose (Frederick et al., 2016). These small scale studies indicated that glucose yields were enhanced when switchgrass was pretreated with various types of fungi; where white-rot fungi were reported as the most effective at degrading lignin (Aguair, et al., 2014). Specifically, *Pleurotus ostreatus*, a white-rot fungus, incubated with switchgrass during storage for different incubation periods showed sufficient promise to warrant farm scale studies (Liu et al., 2013; and Taniguchi et al., 2005).

From a bioenergy perspective, fungal pretreatments are appealing; however, they have inherent drawbacks. Fungal pretreatments are time-consuming, where their duration can range from days to months. Moreover, the extent of delignification cannot be controlled in fungal pretreatments. Therefore, fungi-based pretreatment calls for trade-offs between higher delignification, loss of cellulose and pretreatment duration. Although these studies (Hatakka et al., 1983; Emery et al., 2012; and Itoh et al., 2003) were initially conducted to minimize the mass losses of biomass stored for livestock feeding, they nonetheless provided insight as to storage responses.

An Oklahoma State University study was conducted in 2013-2015 where round and square bales were inoculated with *P. ostreatus* and stored in un-protected field conditions at exposed to climate variations. The proposed study is a follow up of the study conducted by

Frederick et al. (2016) at University of Arkansas in collaboration with Oklahoma State University. Frederick et al. (2016) studied the effect of fungal (*P. ostreatus*) inoculation and storage on carbohydrates recovery after LHW and enzymatic hydrolysis on uncovered round bales stored in an open field for 9 months. Unfortunately, under these experimental conditions, *P. ostreatus* did not grow, which was most likely due to the very dry weather that occurred during the experiment; basically Frederick's study resulted in developing knowledge on the effect of field storage on bale bioprocessing. Results showed that there was no significant compositional change observed during storage; however, bale mass as a function of time was not monitored, yielding solely information in terms of relative proportions of the components. On the other hand, Frederick et al. (2016) reported variations in enzymatic hydrolysis results between samples which were determined to display similar compositions during storage periods, questioning whether changes in switchgrass structure could occur. Decreases in glucose were reported from saccharification as a function of storage time (Frederick et al., 2016).

Therefore, studies have yet to be conducted that will document the structural changes of *P. ostreatus* inoculated biomass during storage and how these changes will affect its saccharification. A need for storage studies where temperature and moisture content remain such that *P. ostreatus* growth occurs, elucidating whether or not this solid-state fermentation technique can act as a pretreatment. Lignin concentration of biomass could decrease upon incubation with *P. ostreatus* affecting the structure and composition, which in turn could facilitate the biochemical conversion yields and efficiency.

Studies, where *P. ostreatus* is cultured in a controlled environment, could shed light on biomass storage strategies and their relationship to saccharification. Thus, this study is based on the work of Frederick et al. (2016) where smaller square bales that could be

controlled for temperature and moisture to ensuring *P. ostreatus* growth to determine if, indeed, stored biomass could be more amendable to saccharification.

4. Objectives

Energy or fuel consumption has increased rapidly in the last decade due to increases in world population and intensified industrialization, Transportation across the world is almost completely dependent on fossil fuel (Balat et al., 2011). Second generation biofuels are an alternative source of energy as they do not rely on food crops for their carbohydrate sources. However, second generation biofuels require economically viable technology to remove lignin and enhance saccharification efficiency. Taking advantage of storage operations, switchgrass can be incubated with fungi to jump start its cell wall deconstruction, possibly enhancing saccharification (Faga et al., 2010; and Hatakka et al., 1983).

The goal of this project is to examine the effect that oyster mushrooms, *Pleurotus ostreatus*, incubated with switchgrass bales during storage operations have on cell wall components. It is anticipated that these storage periods will enhance saccharification. Twenty seven square Kanlow switchgrass bales (12 x 18 x 36 inches) (0.38 x 0.46 x 0.91 m) were stored after inoculation with *P. ostreatus* in an enclosed building with no air-condition for 25, 54, and 82 days. After storing with *P. ostreatus*, samples were taken from bales and stored in the bags. Fungal pretreated bales were then ground and subjected to liquid hot water pretreatment. There are two major objectives of this study.

1) To determine the saccharification potential of raw switchgrass incubated with 0%, 2%, and 3% *P. ostreatus* stored in bales for 25, 54 and 82 days.
2) To determine the saccharification potential of switchgrass incubated with 0%, 2%, and 3% *P. ostreatus* stored in bales for 25, 54 and 82 days that was further pretreated with Liquid Hot Water (LHW).

5. Material and Methods

Many of the the methods, instruments, standards etc. used in this study have also been used by other researchers, Kalavathy Rajan, Angele Djioleu, Noaa Frederick and Gurshagan Kandhola in the same lab under the same professor and academic advisor Dr. Danielle Julie Carrier.

5.1 Experimental design overview

The effects of two different types of pretreatments--fungal pretreatment, and liquid hot water pretreatment--on the sacharification of switchgrass were analyzed in this study. A quick overview of all the tasks and analyses conducted in the study are listed below.

1. Harvest of biomass (switchgrass) at Chickasha, Oklahoma by Oklahoma State University co-operators.
2. Biological/fungal pretreatment of switchgrass was performed with *P. ostreatus* at three different compositions--0%, 2%, and 3% and then stored for 25 days, 54 days, and 82 days (replications = 3) in an enclosed building.
3. Liquid Hot Water (LHW) pretreatment was performed after biological pretreatment at two different severities (replications = 3):
    a. Low temperature longer time (LTLT; 180°C for 30 min); and
    b. High temperature short time (HTST; 200°C for 10 min)
4. Fungal fermented and LHW pretreated switchgrass samples were washed with water to observe the effect of washing on enzymatic digestibility.
5. After all the pretreatments, samples were subjected to enzymatic hydrolysis.
6. HPLC was used for analysing the samples.

5.2 Source of biomass

The biomass used in the study was switchgrass (*Panicum virgatum* var. Kanlow). The biomass was grown and harvested from an experimental field at Oklahoma State University

South Central Research Station in Chickasha, Oklahoma (longitude of 97.9° and latitude of 35.1°).

5.3 Switchgrass characterization

Characterization of switchgrass biomass was performed by co-workers (Shelyn Gehle and Mark R. Wilkins) at Oklahoma State University, Stillwater, Oklahoma using standard protocol developed for herbaceous crops by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2010). The composition of the biomass was defined by quantifying the concentrations of cellulose, hemicellulose, and lignin. This was done to allow for the determination of the effects of pretreatments on compositions of different compounds in switchgrass. An accelerated solvent extractor (ASE® 300 system, Dionex Corporation, Sunnyvale, CA) and soxhlet extractors were used for the determination of water and ethanol extractives, respectively. Determination of lignin and carbohydrate content of the biomass was calculated following Hyman et al. (2007). A refractive index detector (RID) and a Bio-rad Aminex HPX-87 P column (Bio-Rad, Sunnyvale, CA,) were combined to High Performance liquid chromatography (HPLC) for the quantification of structural carbohydrates. Each sample was analyzed for 30 minutes with deionized water flowing at the rate of 0.6 ml/min. The column was maintained at a temperature of 85°C. Digestibility of switchgrass was determined at the University of Arkansas, Fayetteville, AR. A two-stage acid hydrolysis was performed using concentrated sulfuric acid (EMD Millipore, Gibbstown, NJ) to digest the biomass and determine its total glucan content prior to enzymatic hydrolysis. A YSI 2900 Biochemistry Analyser (YSI Life Sciences Inc, Yellow Springs, OH) was used as an enzyme membrane for the quantification of glucose after the digestibility step. Digestibility was calculated as:

$$\%\ Enzymatic\ digestibility = \frac{C_A}{C_B}\ x\ 100 \quad \text{.....Eqn. 1}$$

where

C_A = the final glucose concentration (g/L) in the enzymatic hydrolyzates, and

C_B = initial glucose concentration (g/L) in the acid hydrolyzates.

5.4 Biological pretreatment

The biological pretreatment study was conducted at Oklahoma State University, Stillwater, Oklahoma in an enclosed building with no air conditioning. Twenty seven square Kanlow switchgrass bales (12 x 18 x 36 inches) (0.38 x 0.46 x 0.91 m) were set up as shown in Figure 4. The bales were divided as follows: nine bales each inoculated with 0%, 2%, and 3% *P. ostreatus* growing millet. The inoculum was prepared using a known mass of fungi *P. ostreatus* mixed with proso millet. All of the inoculations were done by weight on wet basis. For example in 2% *P. ostreatus* treatment group, fungal inoculum was equal to 2% of the total weight of the bale with 50% moisture content. The treated bales were hung from steel racks (see Figure 4) in the shed and stored for a total of 82 days. Biomass samples were taken at 25 days, 54 days, and 82 days. At each sampling period nine bales were removed and sampled. Sampling procedure is explained in section 5.4.

Figure 4: The full set of bales hanging at Oklahoma State University, Stillwater, Oklahoma

Prior to mushroom incubation, bale weights were recorded. Following initial mass recording, before inoculation the bales were completely immersed in a tub of water (96 x 18 inches) (2.44 x 0.46 m) for a period of 24 hours; bales were completely saturated with water (see figure 5A). The bales were turned over after one hour of soaking and then turned over again after twelve hours and kept for another 11 hours in the tub to insure that each bale reached the desired moisture content of 50%. Bales were placed on saw horses to allow the excess water to drain after 24 hour soaking period. After allowing the excess water to drain, *P. ostreatus* growing millet was inoculated in each bale at four locations, as depicted in Figures 5B. A dual-sided bale splitting stand was used for the fungal inoculation and each bale was split into four parts and one-fourth of the inoculum was poured on the exposed faces of each part (see figure 5B). Before starting the fungal inoculation, wires holding the bale together were removed and each bale was divided into four parts. This procedure was repeated for all 27 bales to complete the *P. ostreatus* inoculation process.

Figure 5: Bales being soaked prior to inoculation (Figure A); incubation of *P. ostreatus* growing millet in the bales (Figure B).

Since *P. ostreatus* requires a moisture content of at least 50% to grow, an automatic watering system was developed to maintain the required moisture content at all times throughout the bales. To maintain that moisture content of the bales at 50% throughout the experiment, drip hose and thermocouple wires were placed in the bales. Bales were suspended from metal supports as shown in Figure 6. After fully inoculating the bales, the ratchet straps were attached and tightened on alternate sides. The weight of the bales was measured in real time using strain gauges, which were connected to two NI USB-6225 data loggers (National Instrumentation Corporation, Austin, TX). A LabVIEW computer program was developed to read and calculate the moisture content of the bales based on their measured wet mass (the dry weight of the bale was computed from the starting mass and moisture content of the bale). If the calculated value of moisture content was below 50%, the program activated the pump and water was added to the bales. Interior bale temperature was monitored at four points to avoid the possibility of overheating. Overall, approximately a quarter-mile of T-type thermocouple wire (OMEGA Engineering, INC., Stamford, CT) was used. The weight and temperature of the bales was recorded at intervals of 30 seconds. A typical experimental bale is shown in Figure 6.

Figure 6: An example of bales hanging on the rack with instrumentation.

5.5 Sampling

During each sampling period, three bales from each condition where a total of nine bales were sampled. Four core samples were taken from each bale during the sampling process. Bales were removed from the racks and sampling processing was initiated. Drip hose rings and wires were removed from the bales to access fungal growth. After, visually observing the fungal growth, biomass was realigned to perform sampling. Pictures were also taken to document the fungal growth. Cylindrical core samples were extracted from each bale using a 2 inch (5 cm) diameter core tube, 36 inch (91 cm) long, rotated at 500 rpm using an electric drill (Model DS4000, Makita U.S.A., La Mirada, CA). In the first step of sampling, the face of the core-tube was placed on the surface of the bale. The location of the sample was 4 inch horizontally and 3 inch vertically from each corner. In the second step the drill was operated to insert the core into the whole length of the bale (one end to other). In the third step, sample biomass from inside the core was placed in a bag. Four samples were taken

from each of the four corners of the bale. After sampling, each sample was placed in a tared bag, taken to the lab and dried at 55°C for 72 hours.

5.6 Liquid hot water pretreatment (LHW)

LHW was performed on all *P. ostreatus* inoculated switchgrass and this was performed at the Oklahoma State University, Stillwater, OK. After sampling of *P. ostreatus* inoculated bales, samples were placed in a large drying oven at 55°C and dried for atleast 72 hours before being ground. After drying, all four samples from each bale were ground using a Thomas-Willey mill (Model 4, Arthur H. Thomas Co., Philadelphia, PA) and passed through a 3 mm screen prior to LHW pretreatment. These samples were stored in Ziploc bags at 4°C until further used for LHW pretreatment.

For LHW pretreatment, samples stored at 4°C were used and 80 g of dry biomass was loaded into the reactor to which deionized water was also added, bringing the pretreament volume to 500 mL. The pretreatment was performed at two severities i.e. 1) high severity of 3.94, and 2) low severity of 3.83, using the severity scale described below. To achieve high and low severities biomass was pretreated at 200°C for 10 min and at 180°C for 30 min respectively. A 1 liter bench top stirred reaction vessel (Parr Series 4520, Parr Instrument Company, Moline, IL) was used to perform the LHW pretreatment with a propeller agitator. To achieve the required high temperature 1 kW electrical heater was connected to the reaction vessel. The reactor agitated at 300 rpm for both pretreatments and only manually agitated during cooling down period. The propeller speed was 300 rpm for both conditions. The pretreatment time interval was started when the target temperature was reached. When the pretreatment time was completed, the heating unit was turned off and after a cool down period, prehydrolyzates and wash waters were collected, placed in glass containers, and stored at 4°C until needed. After LHW, samples were washed with water to analyze the effect of washing on enzymatic hydrolysis. Results of washed and unwashed samples were

compared in order to determine the effect of washing on LHW pretreated samples. Pretreatment hydrolyzates and solids were separated using vacuum filtration where a Buchner funnel and filter paper (Whatman PLC, Brentford UK) were used. Equation (2) was used to calculate pretreatment severity (Dogaris et al., 2009).

$$Log(R_0) = Log\left(t \cdot e^{\frac{T-100}{14.75}}\right) \text{.....Eqn. 2}$$

where

R_0 = the severity number

t = pretreatment time (min)

T = pretreatment temperatures (°C).

5.7 Washing

Bale samples taken at 25 days, 54 days, and 82 days, were washed with 0.1 N sodium hydroxide at 10% solid loading (Mohanram et al., 2015). To perform this experiment, 10 g dry switchgrass was washed with 100 mL of 0.1 N NaOH followed by water rinsing twice with 3.5 mL of water. After liquid hot water (LHW) pretreatment, the pretreated solids were washed with water at 19% (w/v) solid loading. 80 g dry solids was loaded with 420 g of water in beaker to perform the experiment.

5.8 Compositional analysis

Chemical analysis was performed according to the National Renewable Energy Laboratory (NREL) standard protocol for herbaceous crops (Sluiter et al., 2010) prior to enzymatic hydrolysis. The samples were digested with 72% sulfuric acid at room temperature for 1 h followed by hydrolysis in an autoclave at 121°C, for 1 h, with 4% sulfuric acid before subjecting them to enzymatic hydrolysis. Glucose concentration of the acid hydrolyzates was determined and used as the baseline for estimating the digestibility of pretreated switchgrass.

5.9 Enzymatic hydrolysis

Enzymatic hydrolysis was performed on both raw and LHW pretreated switchgrass. Enzymatic hydrolysis performed at the University of Arkansas. Sodium citrate buffer solution, at pH 4.8, was used to perform the enzymatic digestibility (EMD Gibbstown, NJ). The protocol developed by Sluiter et al. (2010) was carried out to determine the amount glucan present in the biomass. With biomass composition and moisture content determined, switchgrass samples equivalent to 100 mg of glucan were placed in the reaction vials. Due to the fact that samples displayed different moisture contents, masses placed in reaction vials varied accordingly. On average, *P. ostreatus* treated biomass exhibited approximately 10% moisture content, while LHW pretreated biomass displayed more than 60% moisture content. Vials were placed in a shaking water bath (Thermo Electron Corporation, Winchester, VA) that was set at 100 RPM and kept at 50°C. The enzyme used for hydrolysis was Accelerase ® 1500 (Genencor, Cedar Rapids, IA) at an enzyme loading of 30 FPU/g of glucan. Water (18.2 M Ω resistance) was obtained from a Direct-Q system (Millipore, Billerica, MA). In summary, total volume of each reaction vial was 10 mL and was composed of: 0.5 mL of enzyme, 4.5 mL of citrate buffer and 5.0 mL of water. The reaction vials were placed in a 50°C water bath agitated at 100 rpm and incubated for 24 h. After enzymatic hydrolysis, the liquid hydrolyzates were analyzed by HPLC and then stored at -4°C.

5.10 High performance liquid chromatography (HPLC) analysis

HPLC was used to determine the carbohydrate content. Liquid hydrolyzates stemming from raw switchgrass and both LHW pretreatment conditions were analyzed by HPLC. A Waters 2695 Separation module with a 2414 Refractive Index Detector (RID) (Waters, Milford, MA) was used for the detection of carbohydrates, such as glucose and xylose. The HPLC system was equipped with Shodex (Waters, Milford MA) precolumn (SP-G, 8 μm, 6 x 60 mm) and column Sugar SP0810 (8 μm, 6 x 300 mm). Water, flowing at a rate of 0.2

ml/min was the mobile phase. To achieve separation, the column was heated up to 85°C, using an external heater (Waters, Milford, MA). Organic acids and byproducts present in liquid hydrolyzates were detected using a Waters 2695 Separation module, with a Waters 296 Photodiode Array Detector (PDA). The column used was a Bio-Rad (Hercules, CA) Aminex HPX-87H Ion Exclusion column (7.8 mm x 300 mm). The column was heated to 55°C and the eluent was 0.005M sulfuric acid, flowing at a rate of 0.6 mL/min. Every method used in this study was based on the earlier published techniques (Spacil et al., 2008; and Djioleu et al., 2014). Prior to HPLC analysis, all samples were filtered using a syringe filter to remove solids. HPLC analysis was used to quantify the released carbohydrates, using known standard samples. The standards used for carbohydrate detection such as glucose, xylose, arabinose, mannose and galactose were purchased from Alfa-Aesar (Ward Hill, MA), and standards used for organic acid byproduct detection, such as furfural, hydroxymethylfurfural, acetic acid, and formic acid, were also secured from Alfa-Aesar. Spreadsheets (Excel 2007) were used to perform calculations in the analysis of the data obtained from the HPLC chromatograms, using calibration curves obtained using standards. Using the area under the calibration curve, a slope was calculated as shown in Figure 7. The concentration of each analyte was computed as the area from the HPLC chromatograms divided by slope. Oligomers of xylose and glucose, i.e., xylan and glucan, in wash water samples were also analyzed using the HPLC. The wash water samples were subjected to 4% sulphuric acid hydrolysis to depolymerize the xylan and glucan to their respective monomeric sugars (NREL/TP-510-42623, 2008). All the experiments were repeated three times. For oligomer analysis a different sugar oligomers column (HPLC Carbohydrates Analysis Column) was used. This column was an Aminex® HPX-42A Carbohydrate Ion Exclusion column (7.8 mm x 300 mm) and it was equipped with a Bio-Rad (Hercules, CA) precolumn (30 x 4.6 mm). All other conditions used for oligomer analysis were similar to that of monomeric sugar analysis.

Glucose		
g/L	Area	Slope
1.25	7248101	6000000
2.5	15005023	
5	29791023	
10	59295759	
Xylose		
g/L	Area	Slope
0.78	3562740	4000000
6.25	27359950	
12.5	57063231	
25	112041851	
Arabinose		
g/L	Area	Slope
0.78	3337208	4000000
6.25	26888987	
12.5	54547307	
25	106149836	
Mannose		
g/L	Area	Slope
0.78	3941640	5000000
6.25	29095424	
12.5	58264552	
25	115630130	
Cellobiose		
g/L	Area	Slope
0.78	3980933	5000000
6.25	30270149	
12.5	60428089	
25	122290365	

Formic Acid		
g/L	Area	Slope
0.625	335764	764762
2.5	1733436	
5	2434858	
10	8395596	
Acetic Acid		
g/L	Area	Slope
0.625	276182	531273
2.5	821255	
5	2844394	
10	5348936	
HMF		
g/L	Area	Slope
0.325	3163665	9000000
0.625	6299525	
1.25	12270131	
2.5	24763021	
5	50791347	
10	83689545	
Furfural		
g/L	Area	Slope
0.325	3289124	10000000
0.625	5618043	
1.25	13219780	
2.5	23786683	
5	50452573	
10	100220195	

Figure 7: Calibration curves were used to determine the slope needed for byproducts quantification. Area refers to the area under the curve in each chromatogram.

5.11 Scanning electron microscope (SEM)

Samples were air dried prior to analysis. Dried samples were placed on aluminum specimen with double coated, carbon conductive PELCO tabs (Ted Pella, INC., Redding, CA). Then samples were sputter coated with gold coating (2 nm thickness) with the help of a sputter coater Polaron/emitech SC7620 (Quorum Technologies, Ltd. Esast Sussex, UK). Sample pictures were captured using FEI duo-beam SEM/FIB (FEI Company, Hillsboro, OR) SEM at 30 kV.

5.12 Statistical analysis

The samples were pretreated in triplicate and the enzymatic hydrolysis was also performed in triplicate. Statistical analysis was conducted on JMP 12 and JMP 13 software using linear regression, Student's t-test and Tukey's HSD test. Two-factor analysis of variance (ANOVA) was also used to calculate significant difference between the treatments, with $\alpha = 0.05$. Effects were considered as significantly different for $p \leq 0.05$. Excel 2011 was

also used, depending upon the complexity of the data. The resulting graphs were produced using the JMP software and the Excel spreadsheet. The specific statistical tests that were used will be mentioned when presenting the results each of the analysis.

6. Results and Discussion

Quarterly progress reports of this study were submitted to the granting agency (South Central Sun Grant Center) written by authors Amandeep Singh, Julie Carrier, Kalavathy Rajan, Mark Wilkins, Shelyn Gehle, and Michael Buser. This study was conducted in collaboration with Oklahoma State University.

6.1 Effects of mushroom inoculation during storage on digestibility of switchgrass

Enzymatic digestibility of switchgrass was calculated after storage with *P. ostreatus* and LHW pretreatment. Effect of *P. ostreatus* pretreatment were studied and the results are explained in the following sections.

6.1.1 Digestibility of fungal pretreated biomass

A significant fungal growth was observed in this study as compared to the previous study by Frederick et al. (2016). Growth of *P. ostreatus* in bales stored under controlled conditions can be easily observed in Figure 8.

Figure 8: Growth of *P. ostreatus* in bales stored under controlled conditions.

Data for fungal treated samples (Figure 9, and Appendix; Table 1), which were not subjected to washing after sampling, did not show any significant increase in enzymatic digestibility of switchgrass at any of all three fungal inoculation. It should be noted that these biomass samples were not pretreated with liquid hot water (LHW), but were directly subjected to enzymatic hydrolysis. It was observed from the data that for 0% and 2% fungal inoculation, enzymatic digestibility decreased with increased in storage duration from 25 days to 82 days. In fact at longest duration period (i.e. 82 days), enzymatic digestibility was decreased by 34% compared to that of the control. However, at 3% fungal inoculation results were slightly different than at the other two loading conditions. At 3% loading, enzymatic digestibility was not constant: it was highest with 25 days of storage, and it decreased with further (up to 54 days) followed by an increase from 54 days to 82 days.

The combined effects of percent fungal inoculum and storage duration on enzymatic digestibility of stored switchgrass (that was not pretreated with LHW) are shown in Figure 9. After 25 days of incubation with *P. ostreatus*, maximum digestibility of 39% and 37% was obtained at 3% and 2% of fungal inoculum, respectively; however, there were no significant differences between the treatments. Overall, storage with *P. ostreatus* negatively affected sugars yields obtained after enzymatic hydrolysis; this was especially marked during the 82 days storage period of the experiments with 2% inoculum.

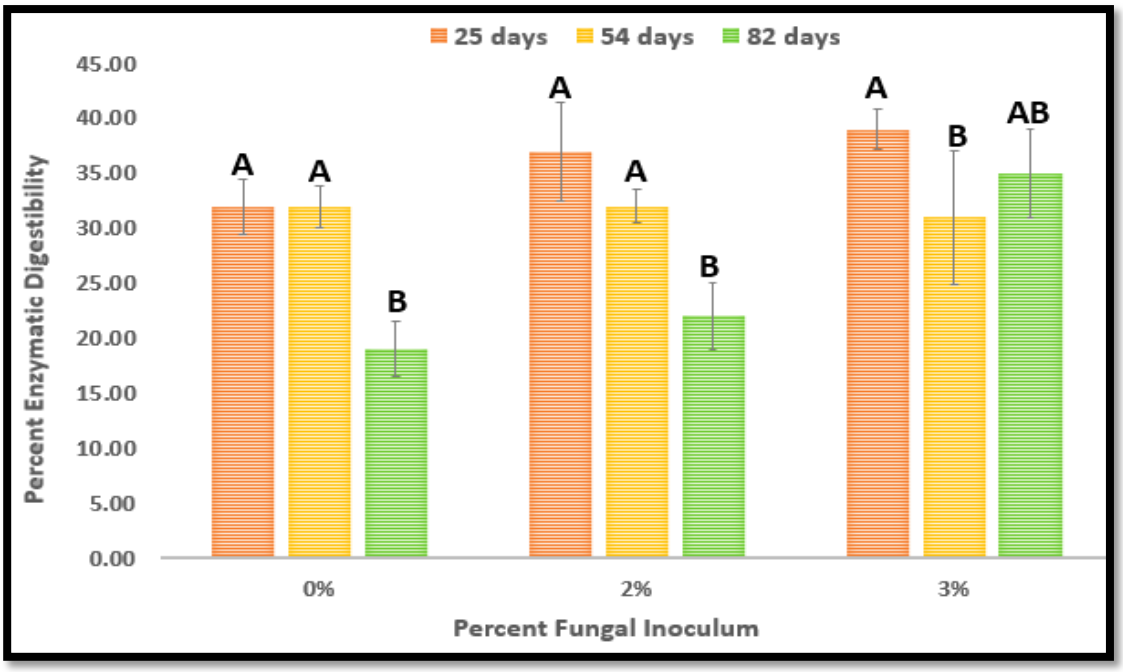

Figure 9: Percentage enzymatic digestibility of fungal (*P. ostreatus*) incubated samples after enzymatic hydrolysis. Results are presented in terms of fungal inoculation as a function of storage period. Significantly different bars are connected with different letters. Statistical analysis was conducted on JMP 13 software using Tukey's HSD test. Effects were considered significantly different for $p \leq 0.05$. Mean and standard deviation, *n=3*.

6.1.2 Digestibility of alkali washed fungal pretreated biomass

Fungal fermented samples were subjected to alkali wash with 0.1N sodium hydroxide at 10% solid loading and then enzymatically hydrolyzed. Figure 10 represents the combined effect of fungal inoculum, alkali wash, and storage duration on enzymatic hydrolysis. Among the washed samples, as shown in Figure 10, longer storage periods for *P. ostreatus* incubated material improved sugar recovery, as compared to that observed with 25 days incubated samples. In the absence of fungal inoculum, a similar pattern of enzymatic digestibility was observed, which indicated that fungal loading had no impact on switchgrass saccharification. Overall, in this work, the alkali wash was not effective in improving digestibility of fungal fermented switchgrass and even lead to decreases in enzymatic hydrolysis efficiency. The difference and variation in enzymatic digestibility between the washed and unwashed biomass is contrasted in Figures 9 and 10. Alkali wash

has been reported to solubilize lignin loosened by *P. ostreatus* during storage due to higher pH and therefore, improving cellulose accessibility for subsequent enzymatic hydrolysis processes. However, in this work, alkali wash led to a significant reduction in enzymatic digestibility of the *P. ostreatus* incubated switchgrass. Overall, enzymatic digestibility of washed biomass was found to be significantly lower than that of non-washed material.

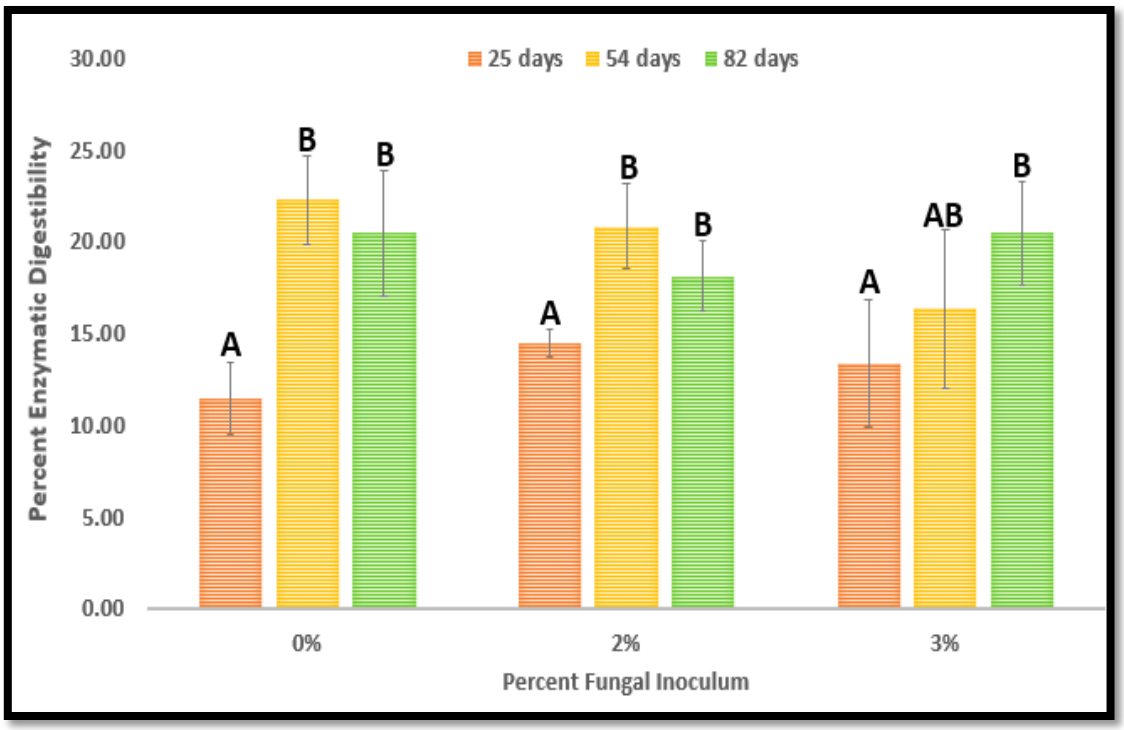

Figure 10: Percentage enzymatic digestibility of fungal (*P. ostreatus*) incubated samples washed with 0.1N sodium hydroxide at 10% solid loading prior to enzymatic hydrolysis. Results are presented in terms of fungal inoculation as a function of storage period. Significantly different bars are connected with different letters. Statistical analysis was conducted on JMP 13 software using Tukey's HSD test. Effects were considered significantly different for $p \leq 0.05$. Mean and standard deviation, *n=3.*

6.2 Effects of solid state fermentation during storage and subsequent hot water pretreatments on switchgrass digestibility

P. ostreatus incubated samples were further subjected to LHW pretreatment after sampling. After LHW samples were subjected to enzymatic hydrolysis.

6.2.1 Enzymatic digestibility of unwashed *P. ostreatus* and LHW pretreated switchgrass

Fungal fermented switchgrass samples were subjected to LHW, at two severities – (1) high temperature short time (HTST), corresponding to a severity of 3.94 were obtained by pretreating switchgrass at 200°C for 10 min, and (2) low temperature longer time (LTLT), corresponding to a severity of 3.83 were attained with pretreatment at 180°C for 30 min. Samples incubated for 82 days with 3% *P. ostreatus* and further exposed to high severity LHW treatment resulted in a maximum enzymatic digestibility of 82%; this post-harvest processing technique enabled a 17% increase over switchgrass incubated for 25 days without *P. ostreatus* (Figure 11, Appendix; Table 2). However, a similar enzymatic digestibility (slightly lower but not statistically significant) was achieved without *P. ostreatus* but stored for 82 days. It can be observed from Figure 11 that digestibility was higher with increases in incubation duration, but there were no significant differences observed between the three storage periods. Our results are in accordance with Mosier et al. (2005); Kristensen et al. (2008); and Laser et al. (2002). Mosier reported that maximum of 80% cellulose was hydrolyzed to glucose with 30 FPU/g glucan enzyme loading at 0% fungal loading. Data was also analyzed to see if percent fungal inoculation effected the enzymatic digestibility. However, no significant difference was observed between any of the conditions ($p = 0.9875$) (Table1).

Table: 1 Results from ANOVA (JMP 12 software) with enzymatic digestibility as the dependent variable and LHW treatment severity (200°C for 10 min, vs. 180°C for 30 min) as the main factor. For unwashed samples pretreated with LHW.

Source	α	DF	F Ratio	p
200°C for 10 min	0.05	51	0.0126	0.9875
180°C for 30 min	0.05	51	1.4067	0.2543

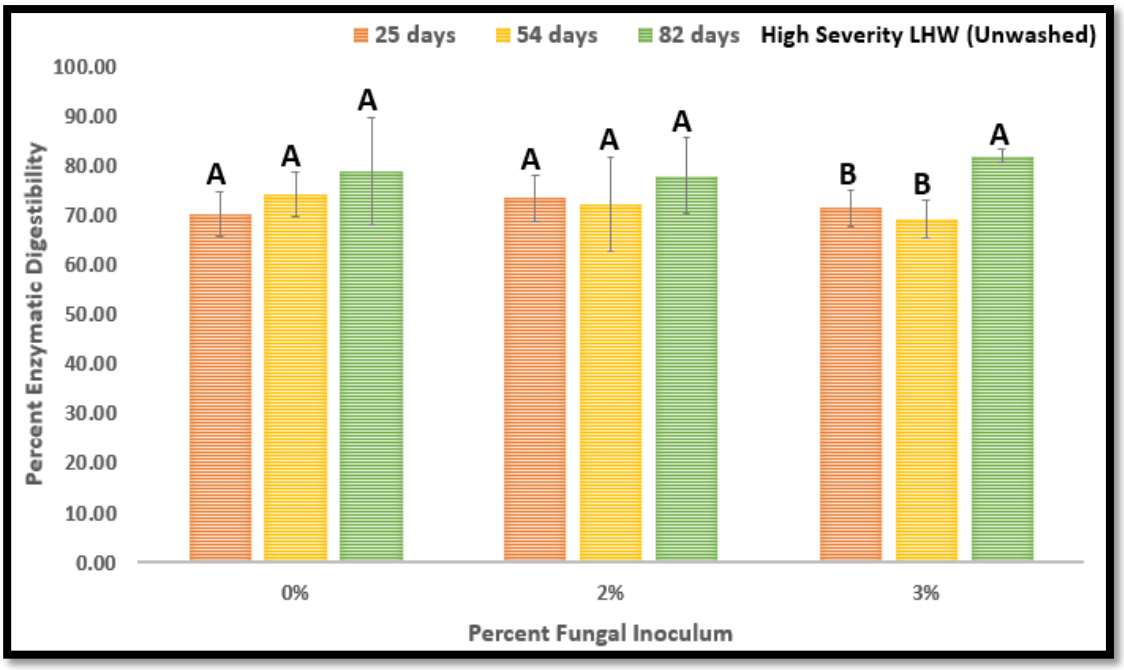

Figure 11: Percentage enzymatic digestibility of fungal (*P. ostreatus*) incubated and liquid hot water (HTST) (200°C for 10 min) pretreated samples after enzymatic hydrolysis. Results are presented in terms of fungal inoculation as a function of storage period. Biomass was hydrolyzed directly without prior rinsing. Significantly different bars are connected with different letters. Statistical analysis was conducted on JMP 13 software using Tukey's HSD test. Effects were considered significantly different for $p \leq 0.05$. Mean and standard deviation, *n=3.*

On the other hand, samples incubated with *P. ostreatus* and subjected to low severity LHW pretreatment resulted in maximum saccharification efficiency of 66%, which was obtained with switchgrass stored for 54 days without *P. ostreatus* (Figure 12, Appendix; Table 4). Unlike high severity pretreated samples, enzymatic digestibility significantly increased in low severity pretreated switchgrass at 0% fungal inoculation from 25 days to 54 days of storage period. However, a reduction in digestibility was determined after 82 days of storage; digestibility values were similar to those obtained after 25 days of storage, as depicted in Figure 12. At the other two fungal loadings, 2% and 3%, digestibility decreased with an increase in incubation time. These results are presented in Figure 12. Moreover, increase in fungal inoculation did not result in higher enzymatic digestibility (Figure 12, and Table 1). When comparing HTST and LTLT, an increase of 18% in

enzymatic digestibility was noted for HTST as compared to LTLT, and these results are consistent with studies reported by (Laser et al., 2002; and Mosier et al., 2005).

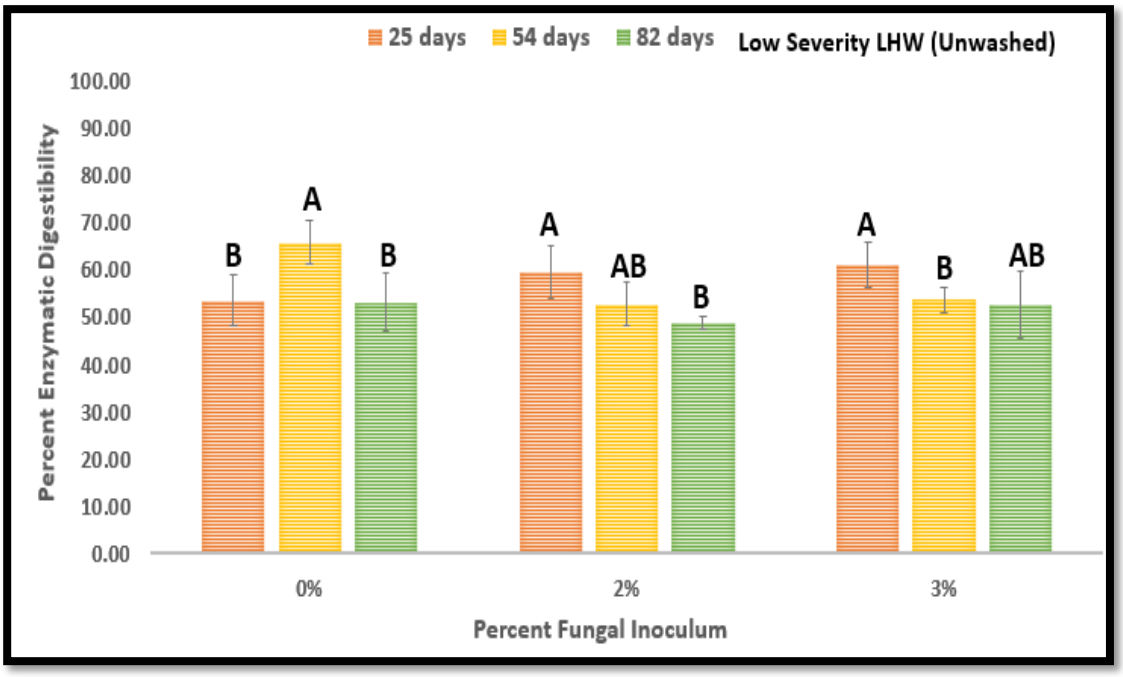

Figure 12: Percentage Enzymatic digestibility of fungal (*P. ostreatus*) incubated and liquid hot water (LHW) (180°C for 30 min) pretreated samples after enzymatic hydrolysis. Results are presented in terms of fungal inoculation as a function of storage period. Biomass was hydrolyzed directly without prior rinsing. Significantly different bars are connected with different letters. Statistical analysis was conducted on JMP 13 software using Tukey's HSD test. Effects were considered significantly different for $p \leq 0.05$. Mean and standard deviation, *n=3.*

6.2.2 Enzymatic digestibility of washed *P. ostreatus* and LHW pretreated switchgrass

Liquid Hot Water (LHW) pretreatment is known to improve cellulose accessibility of enzymes by solubilizing hemicellulose. However, during the LHW process various byproducts like, organic acids, furans, phenolics, and aldehydes are also generated (Klinke et al., 2004; and Du et al., 2010). Acetic acid can be generated during LHW pretreatment processes due to the solubilization of hemicellulose. Other inhibitory byproducts like furfural, formic acid, levelunic acid and HMF (5-hydroxymethyl-2-furaldehyde) can also be generated due to the degradation of free pentoses and hexoses, which are extracted from the

biomass during the pretreatment process (Brodeur et al., 2011). Many of these by-products can adversely affect ensuing enzymatic hydrolysis and microbial fermentation processes (Kim et al., 2011). It is desirable to keep the concentration of sugar degradation byproducts, HMF, furans, and organic acids, formed during the LHW pretreatment process below 1 g/L due to adverse effects observed in subsequent processing steps. For example, furfural, at a concentration greater than 2 g/L, is toxic to fermentation microorganisms (Kim et al., 2011). In addition to byproduct formation, LHW pretreatment results in the production of a solid residue, composed mainly of hemicellulose and lignin, which is left behind on the surface of the biomass and that is in need of removal prior to enzymatic saccharification. Removing the solid layer from the surface of the biomass was known to enhance enzyme accessibility of cellulose (Brodeur et al., 2011). Specifically, Kim et al., (2009) reported that lignin degradation compounds formed during the LHW pretreatment needed to be removed because, these compounds inhibited cellulolytic enzymes, reducing their enzymatic hydrolysis efficiency. At high temperature pretreatments, lignin residues also underwent a phase change and deposited on the surface of the biomass in the form of phenolic compounds (Selig et al., 2007; and Xu et al., 2007). Based on these reports it can be concluded that, washing of chemically pretreated biomass is highly recommended in order to remove the cellulosic and lignin degradation byproducts from the surface of the biomass and to improve the efficiency of downstream processes. Thus, switchgrass biomass recovered after LHW pretreatment was washed with 5X volumes of water and the compounds removed in the wash water were determined using HPLC analysis.

After incubation with *P. ostreatus* and subsequesnt HTST or LTLT hot water pretreatments, switchgrass samples were washed with 5X volumes water with respect to biomass prior to saccharification as washing after physico-chemical pretreatment is reported to improve the enzymatic digestibility (Brodeur et al., 2011 and Kim et al., 2009)).

Figure 13 presents the combined effect of fungal inoculum, wash, and HTST pretreatment as a function of percent *P. ostreatus* inoculation and of storage time on enzymatic hydrolysis. With the exception of 2% *P. ostreatus* inoculation for 25 and 54 days, enzymatic hydrolysis was not statistically different; at 2% *P. ostreatus* inoculation for 25 and 54 days, glucose returns were in fact lower. Moreover, a significant overall reduction in enzymatic digestibility was noted as compared to samples that were not washed, as previously shown in (Figure 11, and 13).

Table: 2 Results from ANOVA (JMP 12 software) with enzymatic digestibility as the dependent variable and LHW treatment severity (200°C for 10 min, vs. 180°C for 30 min) as the main factor. For washed samples pretreated with LHW.

Source	**α**	**DF**	**F Ratio**	**p**
200°C for 10 min	0.05	51	2.689	0.0769
180°C for 30 min	0.05	51	0.4046	0.6694

Higher fungal inoculation has been reported to degrade more lignin and foster enzymatic hydrolysis processes but no significant difference was observed between control bales and fungal inocubated bales pretreated at both low severity, and high severity (Figure 13 and 14, and Table 2).

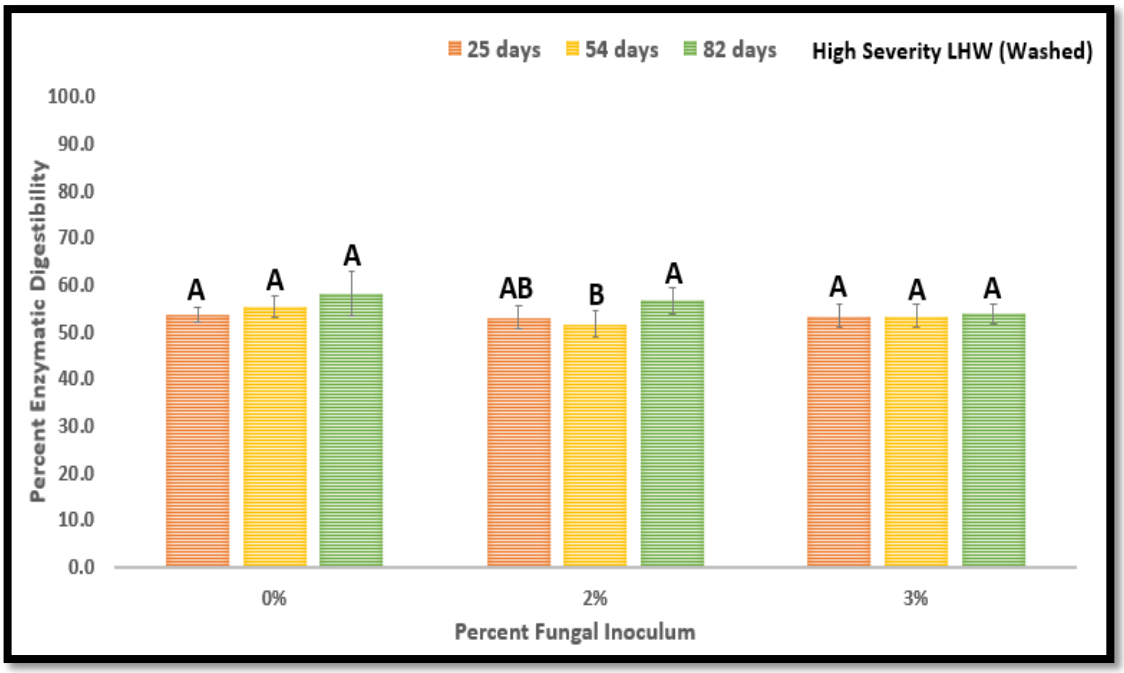

Figure 13: Percentage enzymatic digestibility of fungal (*P. ostreatus*) and liquid hot water (200°C for 10 min) pretreated samples after enzymatic hydrolysis. Results are presented in terms of fungal inoculation as a function of storage period. Biomass was washed with 5X volumes of water prior to enzymatic hydrolysis. Significantly different bars are connected with different letters. Statistical analysis was conducted on JMP 13 software using Tukey's HSD test. Effects were considered significantly different for $p \leq 0.05$. Mean and standard deviation, *n=3.*

Figure 14 presents the combined effect of fungal inoculum, storage duration, wash and LTLT pretreatment on enzymatic hydrolysis. As for HTST pretreated samples, washing significantly also reduced switchgrass digestibility for LTLT pretreated samples. An increase of 22% digestibility was observed for unwashed samples when compared to that of washed samples. With the exception of 0% *P. ostreatus* loading and stored for 25 days and 82 days, all saccharification results were statistically similar. Moreover, for all LTLT conditions, saccharification was lower than that calculated for HTST pretreated samples.

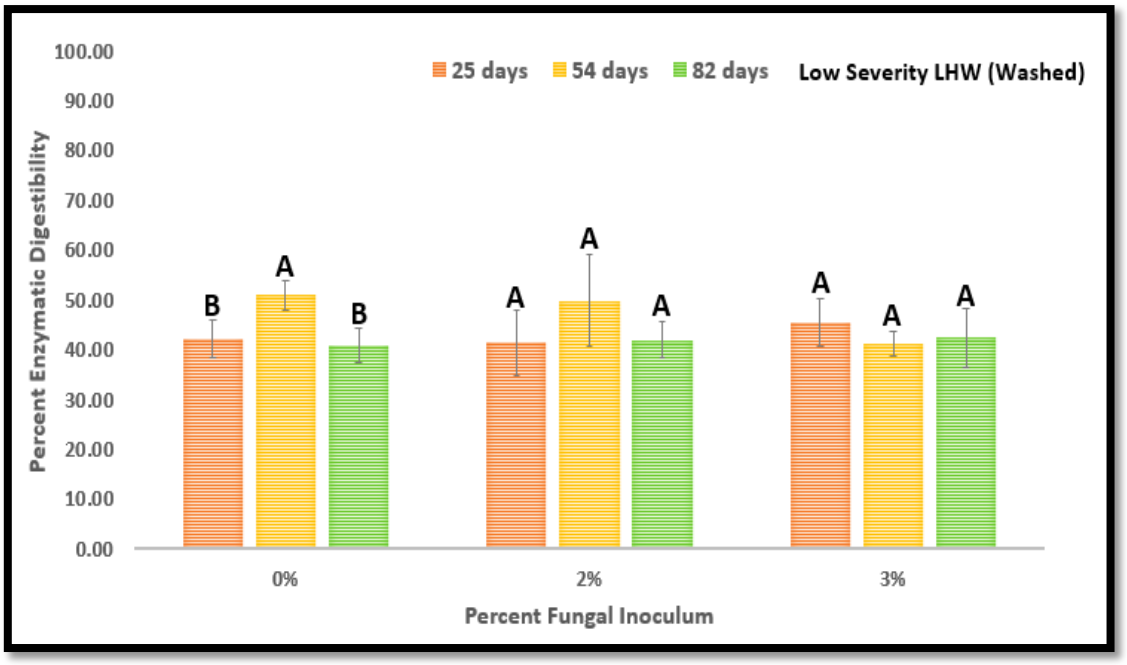

Figure 14: Percentage enzymatic digestibility of fungal (*P. ostreatus*) incubated and liquid hot water (180°C for 30 min) LTLT pretreated samples after enzymatic hydrolysis. Results are presented in terms of fungal inoculation as a function of storage period. Biomass was washed with 5X volumes of water prior to enzymatic hydrolysis. Significantly different bars are connected with different letters. Statistical analysis was conducted on JMP 13 software using Tukey's HSD test. Effects were considered significantly different for $p \leq 0.05$. Mean and standard deviation, *n=3.*

6.3 Wash water Analysis for sugars, sugar oligomers, organic acids and furans

6.3.1 Wash water analysis

The wash water was analyzed on High Performance Liquid Chromatography (HPLC) mainly for different compounds namely, xylose, cellobiose, glucose, formic acid, acetic acid, furfural and HMF. Oligomers of xylose and glucose, were estimated by subjecting the wash water samples to acid hydrolysis and by measuring the resultant monomeric sugars on HPLC.

6.3.2 Wash water analysis for sugars

Three monomeric sugars were detected in wash water samples namely, xylose, cellobiose and glucose for both HTST and LTLT conditions. At HTST xylose was determined to be in higher concentrations than the other two sugars, as shown in the Table

1. Higher xylose concentrations were also reported by Frederick et al. (2016). Moreover, the results show that at all three fungal inoculations and three different storage periods, 0%, 2%, and 3% and 25 days, 54 days, and 82 days respectively, the concentrations of xylose were approximately 1.00 g/L with a maximum of 1.46 g/L at 2% fungal inoculation stored for 82 days and minimum of 1.04 g/L at 2% fungal inoculation stored for 25 days.

Data was also analyzed to see if there was a significant difference between different storage conditions with respect to fungal inoculation. It was noted that at 0% *P. ostreatus* inoculation, maximum xylose concentration was observed in switchgrass stored for 25 days and it significantly decreased with increase in duration to 54 days. However, there was increase in xylose concentration in samples stored for maximum duration but not significantly and this value was also not significantly lower than that of lowest incubated samples (Table 1). On the other hand, at 2%, none of the three storage periods were significantly different from each other. Moreover, at 3% inoculation only 25 days and 82 days stored samples were significantly different.

As compared to the HTST LHW pretreated samples, the concentration of xylose was the lowest for the LTLT pretreated samples, where a maximum of 1.18 g/L of xylose was observed at 2% fungal inoculation stored for 25 days. The xylose concentration was less than 1 g/L for all the other storage conditions. At 0% and 3% *P. ostreatus* inoculation no storage periods were significantly different from each other and at 2%, only xylose concentration for 25 days and 54 days storage were significantly different.

Glucose was the second most common monomeric sugar found in the wash water in HTST pretreated samples with maximum concentration of 0.45 g/L at 3% fungal inoculation stored for 82 days and minimum of 0.21 g/L at 2% *P. ostreatus* inoculation stored for 25 days. None of conditions were significantly different. On the other hand, at LTLT pretreatment glucose concentrations were approximately 0.25 g/L for all cases,

except at 2% fungal inoculation stored for 25 and 82 days. Moreover at 2% glucose concentration of 25 days stored switchgrass was significantly higher than other two higher storage durations and all other condition were significantly not different from each other for LTLT.

Cellobiose concentrations were the least of all free sugars observed within the HTST pretreated samples, with a maximum of 0.28 g/L at 3% fungal inoculation, stored for 82 days and minimum of 0.09 g/L at 2% fungal inoculation, stored for 25 days. Also, it can be easily observed from the table that the cellobiose concentration increased with increase in storage duration. Moreover at 2% and 3%, concentrations of 54 and 82 days stored samples were significantly higher than that of lowest stored samples but not from each other and at 0% concentrations, the longest stored samples were significantly higher from other two conditions. On the other hand, cellobiose concentration was significantly higher for the LTLT pretreated samples, as compared to HTST samples. Moreover between the conditions, only the shortest stored and longest stored samples at 2% were significantly different in concentration from each other. Maximum concentration was 1.06 g/L at 2% fungal inoculation, stored for 25 days and minimum was 0.19 g/L at 2% fungal inoculation, stored for 82 days.

Table 3: Compositional analysis of wash water from the liquid hot water pretreatment. Mean concentrations of sugars are listed (n=3) with the standard deviation in parentheses. For each analyte measured (in columns), different letters indicate significant differences ($p \leq 0.05$) between storage periods. The different colors of the letters indicate distinct sub-groups (by inoculation concentration) that were compared separately. Statistical analyses were conducted on JMP 12 software using Student's T-Test.

Liquid Hot Water Pretreatment (LHW)		**HTST (200 C, 10 min) LHW treatment group**			**LTLT (180 C, 30 min) LHW treatment group**		
Fungal inoculation	**Storage period**	**Xylose (g/L)**	**Cellobiose (g/L)**	**Glucose (g/L)**	**Xylose (g/L)**	**Cellobiose (g/L)**	**Glucose (g/L)**
0%	25 days	1.33 ± 0.09 **(A)**	0.15 ± 0.05 (B)	0.25 ± 0.04 **(A)**	0.75 ± 0.18 **(A)**	0.46 ± 0.17 (A)	0.24 ± 0.02 **(A)**
	54 days	1.09 ± 0.10 **(B)**	0.19 ± 0.02 (B)	0.41 ± 0.15 **(A)**	0.90 ± 0.17 **(A)**	0.56 ± 0.19 (A)	0.28 ± 0.08 **(A)**
	82 days	1.18 ± 0.10 **(AB)**	0.28 ± 0.06 (A)	0.29 ± 0.11 **(A)**	0.68 ± 0.21 **(A)**	0.59 ± 0.13 (A)	0.23 ± 0.07 **(A)**
2%	25 days	1.04 ± 0.43 **(A)**	0.09 ± 0.60 **(B)**	0.21 ± 0.10 **(A)**	1.18 ± 0.26 **(A)**	1.06 ± 0.31 **(A)**	0.49 ± 0.13 **(A)**
	54 days	1.21 ± 0.05 **(A)**	0.22 ± 0.40 **(A)**	0.33 ± 0.13 **(A)**	0.87 ± 0.23 **(A)**	0.70 ± 0.15 **(A)**	0.28 ± 0.09 **(A)**
	82 days	1.46 ± 0.28 **(A)**	0.27 ± 0.20 **(A)**	0.40 ± 0.05 **(A)**	0.46 ± 0.07 **(B)**	0.19 ± 0.04 **(B)**	0.15 ± 0.02 **(B)**
3%	25 days	1.16 ± 0.09 **(A)**	0.12 ± 0.04 **(B)**	0.30 ± 0.04 **(A)**	0.72 ± 0.02 **(A)**	0.34 ± 0.02 **(A)**	0.25 ± 0.00 **(A)**
	54 days	1.29 ± 0.05 **(AB)**	0.22 ± 0.04 **(A)**	0.45 ± 0.18 **(A)**	0.67 ± 0.02 **(A)**	0.47 ± 0.23 **(A)**	0.24 ± 0.04 **(A)**
	82 days	1.40 ± 0.09 **(B)**	0.28 ± 0.03 **(A)**	0.45 ± 0.02 **(A)**	0.72 ± 0.19 **(A)**	0.48 ± 0.18 **(A)**	0.21 ± 0.02 **(A)**

6.3.3 Wash water analysis for sugar oligomers

Oligomer content of wash water was determined by HPLC for both pretreatment conditions. LHW pretreatment enhanced enzymatic digestibility by dissolving hemicellulose to soluble sugar oligomers (Kim et al., 2009). The xylan concentration was determined to be more than glucan concentration in both pretreatment conditions, as shown in Table 2. Moreover, the xylan concentration was more in the LTLT, as compared to HTST samples.

At high temperature, the maximum xylan removed was 0.50 g/L at 0% fungal inoculation fermented for 25 days and minimum was 0.05 g/L same treatment group but incubated for 54 days. In addition, no other conditions were significantly different in terms of xylan concentration. On the other hand, at LTLT xylan concentration values were significantly higher than that of at HTST, the maximum concentration being 3.38 g/L at 3% fungal inoculation stored for 25 days and minimum was 2.66 g/L at same fungal inoculum but stored for 82 days. Xylan concentrations, in the LTLT wash water group, decreased for all fungal inoculation treatments with the increase in storage time but not significantly, from 25 to 82 days. Hemicellulose hydrolyzed during LHW pretreatment was recovered as monomeric sugars and more than 90% monomeric sugars were recovered in a study conducted by Mosier et al. (2005).

Glucan values at LTLT pretreatment were also higher as compared to the HTST group. At high temperature, glucan concentrations were not significantly different and were also close to zero for a few treatment conditions. The maximum glucan concentration of 0.07 g/L was observed at 3% fungal inoculum, stored for 25 days. The maximum glucan concentration for LTLT group was 0.35 g/L and the minimum was 0.01 g/L. Moreover at LTLT, the glucan concentration decreased with an increase in storage duration. In addition, this decrease was significant for the 3% inoculation (Table 2). Thus, it can be concluded

that a greater xylan proportion was solubilized during both LHW pretreatment as compared to glucan, which in turn leads to the presence of higher concentrations of xylose or xylose oligomers in the wash water. Also with the LTLT treatment, xylan was mostly removed in the form of xylose oligomers; and, with the HTST treatment, the xylose oligomers were converted to xylose, which was also applicable to the glucose oligomers. A HPLC chromatogram for sugar oligomers was used for oligomer analysis. The Chromatogram below depicts the sugar oligomers observed during the wash water analysis; and, this chromatogram was for LTLT pretreatment condition i.e. 180°C for 30 min. Xylose oligomers peaked at 52.06 minutes as shown in Figure 15, and glucose oligomers were detected and quantified at 48.55 minutes. Figure 15 also states that concentrations of glucose oligomers were less compared to xylose oligomers as explained before.

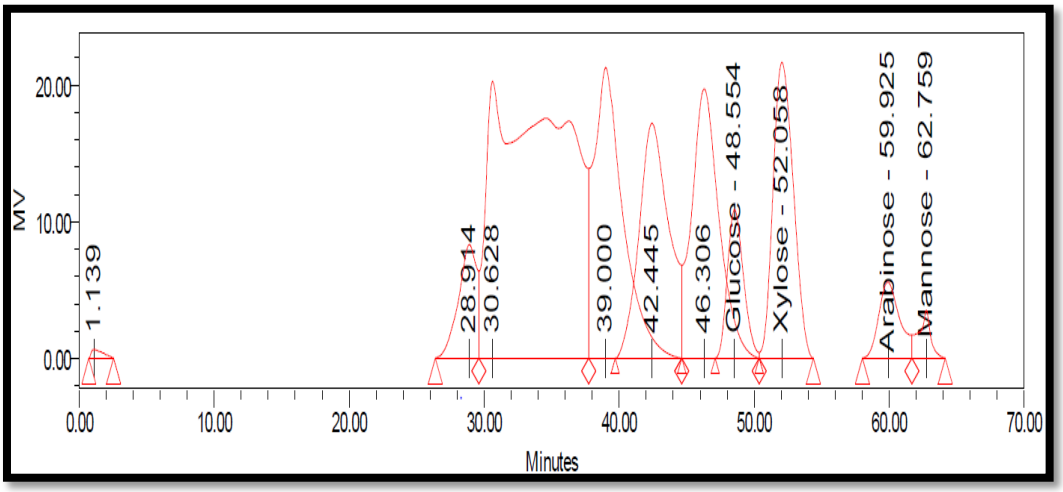

Figure 15: HPLC chromatogram of sugar oligomers at 180°C for 30 min.

Table 4: Compositional analysis of wash water from the liquid hot water pretreatment. Mean concentrations of sugar oligomers are listed (n=3) with the standard deviation in parentheses. For each analyte measured (in columns), different letters indicate significant differences ($p \leq 0.05$) between storage periods. The different colors of the letters indicate distinct sub-groups (by inoculation concentration) that were compared separately. Statistical analyses were conducted on JMP 12 software using Student's T-Test.

Liquid Hot Water Pretreatment (LHW)		**HTST (200 C, 10 min) LHW treatment group**		**LTLT (180 C, 30 min) LHW treatment group**	
Fungal inoculation	**Storage period**	**Xylan (g/L)**	**Glucan (g/L)**	**Xylan (g/L)**	**Glucan (g/L)**
0%	25 days	0.50 ± 0.08 **(A)**	0.02 ± 0.03 (A)	3.23 ± 0.09 **(A)**	0.17 ± 0.07 (A)
	54 days	0.05 ± 0.25 **(B)**	0.00 ± 0.00(A)	2.90 ± 0.15 **(A)**	0.11 ± 0.05 (A)
	82 days	0.24 ± 0.22 **(AB)**	0.03 ± 0.05 (A)	2.74 ± 0.81 **(A)**	0.12 ± 0.01 (A)
2%	25 days	0.06 ± 0.07 **(A)**	0.05 ± 0.08 **(A)**	3.31 ± 0.46 **(A)**	0.02 ± 0.04 **(A)**
	54 days	0.20 ± 0.26 **(A)**	0.00 ± 0.00 **(A)**	2.71 ± 0.07 **(A)**	0.11 ± 0.04 **(B)**
	82 days	0.25± 0.13 **(A)**	0.00 ± 0.00 **(A)**	2.69 ± 0.34 **(A)**	0.16 ± 0.03 **(B)**
3%	25 days	0.28 ± 0.15 **(A)**	0.07 ± 0.09 **(A)**	3.38 ± 0.41 **(A)**	0.35 ± 0.14 **(A)**
	54 days	0.31 ± 0.16 **(A)**	0.00 ± 0.00 **(A)**	2.94 ± 0.41 **(A)**	0.15 ± 0.05 **(B)**
	82 days	0.18 ± 0.09 **(A)**	0.00 ± 0.00 **(A)**	2.66 ± 0.57 **(A)**	0.15 ± 0.05 **(B)**

6.3.4 Wash water analysis for furans

Two main furan compounds -- namely, furfural and HMF--that have been reported to affect the digestibility of lignocellulosic biomass, were also analyzed. Concentration of both the compounds for the HTST pretreatment (200/10) group was quite low, as compared to other compounds like acids and sugars. Concentration of HMF was lower than 0.1 g/L for all the conditions. The concentrations of HMF for LTLT pretreatment (180/30) group on an average was 0.02 g/L, which is lower than that observed for the 200/10 group. On the other hand, the concentrations of furfural were 0.25 g/L, which could be related to the fact that LHW pretreatment led to higher solubilization and degradation of xylan, increasing furfural in the wash water; nevertheless, the concentrations were not statistically different. Moreover higher severity may also lead degradation sugar into by-products like furfural compounds and organic acids (Frederick et al., 2016). Furfural concentrations were approximately 0.2 g/L for all 200/10 wash water samples, except for two conditions, 0% for 82 days and 3% for 54 days, as shown in Table 3. Concentrations of both components at LTLT and HTST provided no statistical difference.

Table 5: Compositional analysis of wash water from the liquid hot water pretreatment. Mean concentrations of furans are listed (n=3) with the standard deviation in parentheses. For each analyte measured (in columns), different letters indicate significant differences ($p \leq 0.05$) between storage periods. The different colors of the letters indicate distinct sub-groups (by inoculation concentration) that were compared separately. Statistical analyses were conducted on JMP 12 software using Student's T-Test.

Liquid Hot Water Pretreatment (LHW)		**HTST (200 C, 10 min) LHW treatment group**		**LTLT (180 C, 30 min) LHW treatment group**	
Fungal inoculation	**Storage period**	**Furfural (g/L)**	**HMF (g/L)**	**Furfural (g/L)**	**HMF (g/L)**
0%	25 days	0.28 ± 0.05 **(A)**	0.08 ± 0.01 (A)	0.21 ± 0.04 **(A)**	0.02 ± 0.01 (A)
	54 days	0.23 ± 0.21 **(A)**	0.10 ± 0.04 (A)	0.29 ± 0.11 **(A)**	0.02 ± 0.02 (A)
	82 days	0.30 ± 0.12 **(A)**	0.09 ± 0.01 (A)	0.28 ± 0.08 **(A)**	0.02 ± 0.00 (A)
2%	25 days	0.27 ± 0.17 **(A)**	0.07 ± 0.04 **(A)**	0.27 ± 0.04 **(A)**	0.02 ± 0.00 **(A)**
	54 days	0.29 ± 0.08 **(A)**	0.05 ±0.02 **(A)**	0.26 ± 0.02 **(A)**	0.03 ± 0.00 **(A)**
	82 days	0.21 ± 0.05 **(A)**	0.08 ± 0.00 **(A)**	0.20 ± 0.02 **(A)**	0.02 ± 0.01 **(A)**
3%	25 days	0.28 ± 0.09 **(A)**	0.09 ± 0.01 **(A)**	0.20 ± 0.02 **(A)**	0.03 ± 0.01 **(A)**
	54 days	0.17 ± 0.03 **(A)**	0.06 ± 0.03 **(A)**	0.25 ± 0.08 **(A)**	0.02 ± 0.01 **(A)**
	82 days	0.27 ± 0.06 **(A)**	0.09 ± 0.02 **(A)**	0.30 ± 0.04 **(A)**	0.03 ± 0.00 **(A)**

6.3.5 Wash water analysis for organic acids

Two organic acids were mainly tracked in the wash water samples: acetic acid and formic acid. It was determined that acetic acid concentrations for HTST pretreatment (200/10) were higher than those of formic acid. A maximum acetic acid concentration of 1.02 g/L was recorded in wash waters stemming from the 2% fungi inoculation and stored for 25 days. A maximum formic acid concentration of 0.71 g/L was recorded in wash waters stemming from 3% fungi inoculation and stored for 82 days. Only HTST formic acid concentrations were found to be significant. At 0%, 25 day inoculated samples were significantly lower in concentrations as compared two other groups. Moreover, all treatment conditions were significant at 2% from each other except the lowest stored samples and at 3%, shortest and longest stored samples were significantly different from each other. On the other hand, for acetic acid only one condition, 54 days inoculated samples at 2% (HTST) were significantly different from other samples. Previous work indicates that higher LHW pretreatment severity leads to increased solubilization of hemicellulose from the biomass and thus resulting in the production of higher concentrations of acetic acid (Kim et al., 2011; and Brodeur et al., 2011).

A similar trend was observed for 180/30 LHW pretreated samples. Acetic acid concentration was observed to be greater than that of formic acid. However, concentration of both the acids were found to be low as compared to the 200/10 treatment group, which is plausible because lower pretreatment severity could lead to lower degradation of pentose and hexose sugars to organic acids and a higher process temperature resulted in more mass conversion. Overall, the concentration of sugars was more than acids as well as furans for both pretreatment groups.

Table 6: Compositional analysis of wash water from the liquid hot water pretreatment. Mean concentrations of organic acids are listed (n=3) with the standard deviation in parentheses. For each analyte measured (in columns), different letters indicate significant differences ($p \leq 0.05$) between storage periods. The different colors of the letters indicate distinct sub-groups (by inoculation concentration) that were compared separately. Statistical analyses were conducted on JMP 12 software using Student's T-Test.

Liquid Hot Water Pretreatment (LHW)		**HTST (200 C, 10 min) LHW treatment group**		**LTLT (180 C, 30 min) LHW treatment group**	
Fungal inoculation	**Storage period**	**Formic acid (g/L)**	**Acetic acid (g/L)**	**Formic acid (g/L)**	**Acetic acid (g/L)**
0%	25 days	0.40 ± 0.03 **(A)**	0.98 ± 0.04 (A)	0.31 ± 0.03 **(A)**	0.60 ± 0.07 (A)
	54 days	0.70 ± 0.01 **(B)**	0.78 ± 0.32 (A)	0.35 ± 0.04 **(A)**	0.69 ± 0.01 (A)
	82 days	0.67 ± 0.10 **(B)**	0.99 ± 0.09 (A)	0.35 ± 0.01 **(A)**	0.69 ± 0.08 (A)
2%	25 days	0.44 ± 0.03 **(B)**	1.02 ± 0.10 **(A)**	0.29 ± 0.02 **(A)**	0.56 ± 0.60 **(A)**
	54 days	0.36 ± 0.06 **(B)**	0.50 ± 0.16 **(B)**	0.35 ± 0.09 **(A)**	0.80 ± 0.70 **(A)**
	82 days	0.69 ± 0.10 **(A)**	0.97 ± 0.07 **(A)**	0.23 ± 0.08 **(A)**	0.57 ± 0.40 **(A)**
3%	25 days	0.42 ± 0.08 **(B)**	0.88 ± 0.16 **(A)**	0.35 ± 0.07 **(A)**	0.57 ± 0.08 **(A)**
	54 days	0.50 ± 0.10 **(B)**	0.68 ± 0.23 **(A)**	0.28 ± 0.06 **(A)**	0.67 ± 0.10 **(A)**
	82 days	0.71 ± 0.09 **(A)**	0.98 ± 0.12 **(A)**	0.31 ± 0.04 **(A)**	0.68 ± 0.05 **(A)**

6.4 Scanning electron microscope (SEM) analysis

Scanning Electron Microscope (SEM) was used to observe the effects of washing on LHW pretreated samples. Washing overall reduced the enzymatic digestibility of LHW pretreated bales. The reduction associated with washing was 22% in the case of LTLT LHW pretreated samples and 20% in the case of HTST LHW pretreated samples. Since reduction due to washing was quite high, it is very important to find the cause of this reduction and SEM analysis was used with an aim to find the reason for this reduction.

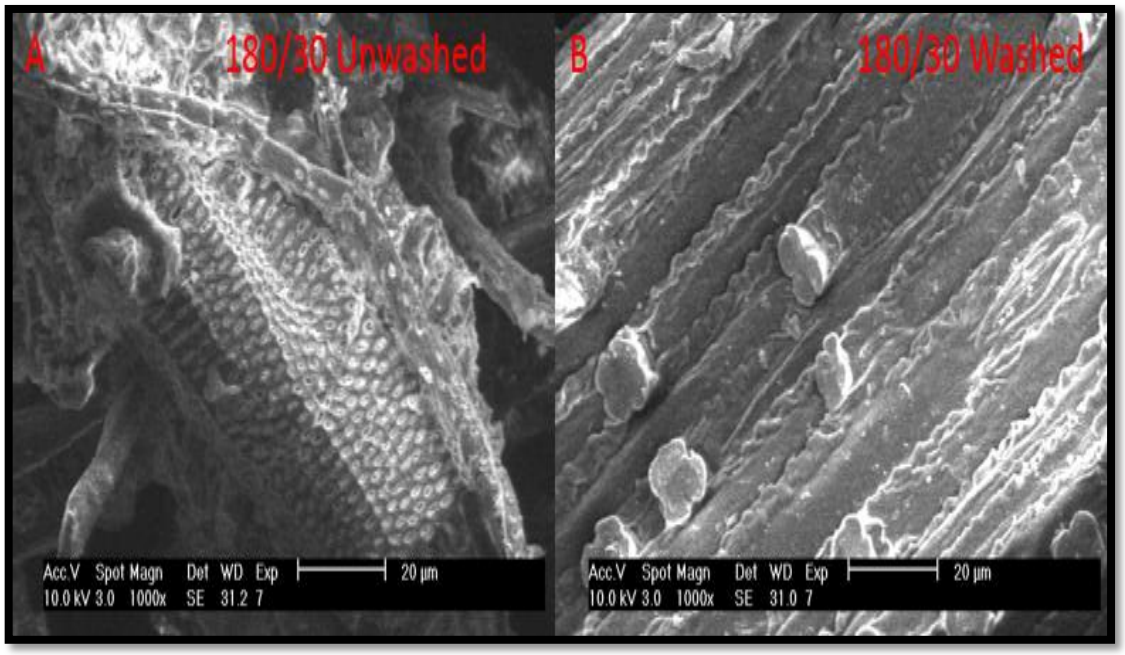

Figure 16: Scanning Electron Microscope (SEM) pictures of LTLT low severity pretreated samples**.**

Bales stored for 54 days with 3% *P. ostreatus* resulted in maximum difference in enzymatic digestibility between washed and unwashed material and were analyzed by SEM. Enzymatic hydrolysis of lignocellulose biomass is highly dependent on structural features of the biomass after pretreatments (Fan et at., 1981; and Zhu et al., 2008). Therefore, it is very important to increase the surface area and pore volume of the biomass to improve cellulose

accessibility (Torget et al., 1991). On the other hand, SEM analysis of LTLT pretreated bales as shown in Figure 16 showed that plant cell wall structure collapses after washing of LHW pretreated samples due to the application of gravitational force (*g*-force) during vacuum filtration. These closed pores, in figure 16, may have reduced the cellulose accessibility to enzymes and resulted in less enzymatic digestibility, as compared to the unwashed samples.

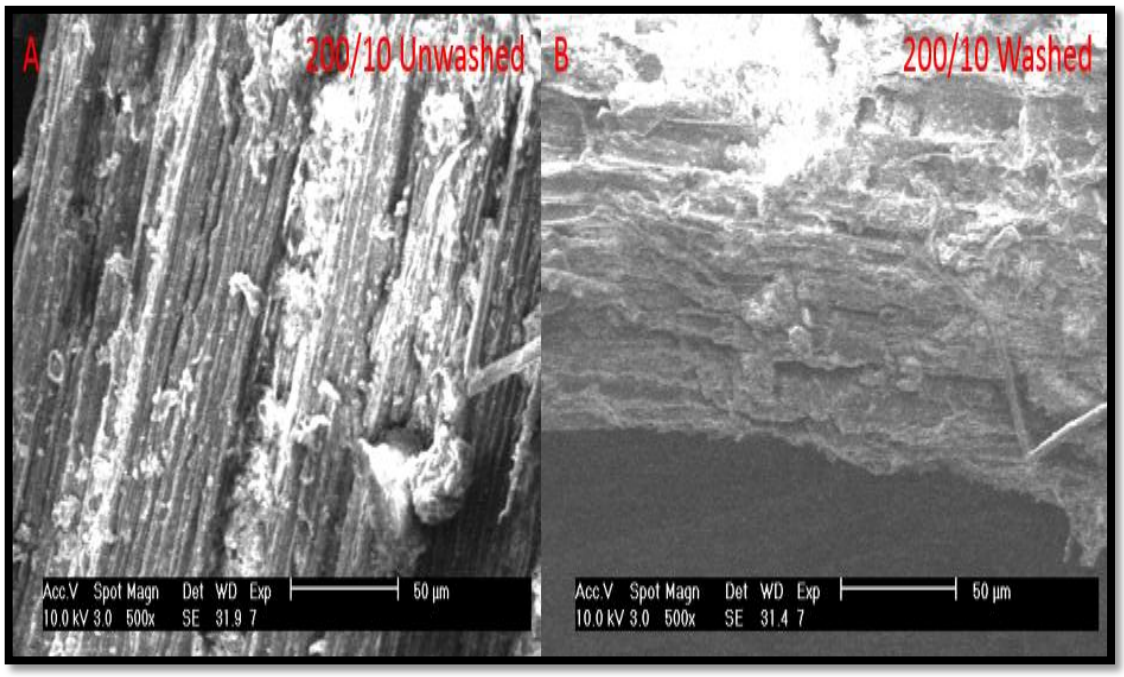

Figure 17: Scanning Electron Microscope (SEM) pictures of HTST high severity LHW pretreated samples.

However, there were no discernible differences between the SEM images (Figures 17A, 17 B) of the washed and unwashed material from the HTST LHW pretreated samples, indicating that the observed differences in saccharification efficiencies between washed and unwashed material could not be attributed to macroscopic differences. According to our results, it was observed that increasing storage time beyond 25 days resulted in decreases of enzymatic digestibility, which was opposite to our hypothesis. However, our results are similar with earlier studies reported by Deswal et al. (2014) and Meza et al. (2006). Deswal

reported that after 25 days of fermentation, no significant difference in lignin degradation was observed which could be the reason for the decrease in pores nature of biomass.

Alkali wash with 0.1 N sodium hydroxide at 10% solid loading, was reported to improve enzymatic accessibility, but in this work, alkali wash did not improve the enzymatic hydrolysis efficiency. A study on alkaline pretreatment of sugarcane bagasse was conducted by Aiello et al. (1995) in which they compared untreated sugarcane bagasse only washed with water, and sugarcane bagasse treated with 0.25 N NaOH at 100ºC for 30 min and at 20% solid loading followed by three times washing with water. This group reported that untreated biomass was as good as treated biomass for cellulose production.

LHW pretreatment of stored bales on the other hand, overall improved the saccharification efficiency. Wan et al. (2011) reported similar results as ours. He reported that LHW (170 ºC for 3 min) combined with fungal pretreatment improved glucose yields of soybean straw and maximum glucose yield was 64.25%. Another study by Wang et al. (2012) on populus tomentosa reported similar results of combined pretreatment with LHW and white-rot fungi. LHW at 200ºC for 30 min and populus tomentosa co-treated with *Lenzites betulina C5617* increased enzymatic hydrolysis efficiency by 2.66 times. He studied the LHW pretreatment with another white-rot fungus as well and found out that the overall combination of LHW and fungal pretreatment enhanced the enzymatic digestibility of lignocellulosic biomass.

The HTST LHW pretreated material gave 18% higher digestibility as compared to the LTLT LHW pretreated material. The reason for this increase in enzymatic digestibility could possibly be attributed to degradation or removal of the hemicellulose fraction (Frederick et al., 2016; Mosier et al., 2005; Wang et al., 2012; and Zeng et al., 2007). Frederick et al. (2016)

reported 97% greater yield for high severity HTST (200°C/10 min) pretreatment, as compared LTLT low severity (180°C/20 min) pretreatment, but hydrolysis efficiency decreased with increases in storage duration. In our results, maximum digestibility was obtained with control bales that were stored for 54 days or that were incubated with *P. ostreatus* (2% and 3%) *for* 25 days. Therefore, results from this study indicate that if biomass needs to be stored for periods greater than 25 days, fungal inoculation is not recommended.

On another note, washing of LTLT pretreated samples had negative effects and resulted in 22% decrease in overall digestibility. Our results are consistent with those of Frederick et al. (2016) in terms of 0% *P. ostreatus* incubation, where washing biomass prior to enzymatic hydrolysis was deleterious to saccharification yields.

Kim et al. (2009) studied the effects of both hot water washing and washing with water at room temperature on sugar recovery. He reported that subsequent hot water washing (80-90°C) and filtering steps removed up to 35% of the initial lignin from the pretreated biomass. Hot water washing was reported to enhance sugar recovery by 27%; moreover, room temperature washing was also reported to enhance sugar recovery by 10%. One more study reported by Shi et al. (2009) talks about washing and heat-wash of corn stalks after fungal pretreatment only. Washing with water and heat-wash was done in order to inactivate fungus and removal of lignin derivatives (Mes-Hartree et al., 1983; and Dekker et al., 1988). However, washing with water did not improve the cellulose conversion efficiency and in fact concentration of cellobiose in hydrolysates was less as compared to unwashed samples. Heat-wash treatment on the other hand significantly improved the cellulose conversion. In heat-wash treatment, first they autoclaved the biomass for 10 min and then washed with water. Increase in cellulose conversion might be because of the release of lignin derivatives due to heating. In

addition, he explained that mycelia of fungus were tightly attached and washing with water was insufficient for its removal. Our results on the other hand, are not exactly consistent with these results published by Kim et al., (2009); and Shi et al., (2009). According to our results, washing of LHW pretreated samples reduced enzymatic hydrolysis and mainly removed xylose oligomers from LTLT LHW pretreated samples and organic acids from HTST LHW pretreated material, as observed in the HPLC analysis of the wash water.

7. Conclusions

Lignocellulosic biomass is a potential feedstock for ethanol production. However, the main challenge for cellulose conversion from lignocellulosic biomass is its structural complexity. We studied switchgrass storage with *P. ostreatus* and also observed the effects of LHW pretreatment switchgrass digestibility. Following are some important conclusions based on the results from our study. Storage with white-rot fungi has been reported to improve the saccharification efficiency but storage with *P. ostreatus* did not improve the saccharification efficiency in this study. Maximum digestibility was achieved with 25 days of storage/fermentation.

- LHW pretreatment of stored bales resulted in overall increase in the saccharification efficiency.
- Fungal + low severity LHW overruled the effects of fungal pretreatment. 25 days of storage with fungi and 54 days of storage without fungi gave similar digestibility.
- Fungal + high severity LHW pretreatment resulted in higher enzymatic digestibility. On the other hand, there was no significant differences between all the pretreatment conditions.
- Washing led to significant decrease in digestibility. At high severity there was 7% decrease in enzymatic digestibility as compared to low severity LHW pretreatment. Therefore, washing step should be eliminated. Elimination of this step would foster enzymatic hydrolysis processes and also save huge amount of water when these processes employed at commercial scale.

8. Future Work

Switchgrass that is to be later fermented should be stored in controlled environment conditions to maximize the possibility of desired (innoculated) fungal growth. Alternately, results also show that storing biomass under controlled conditions for longer periods without inoculation resulted in similar saccharification efficiencies compared to those of biomass stored with *P. ostreatus* for short storage periods. In this study, the monitoring of the control (bales without *P. ostreatus*) served as a baseline. Liquid hot water pretreatment at high temperature and short time (or 200°C) should be performed in preference to liquid hot water at 180°C, as it resulted in higher enzymatic digestibility.

This work combined with that of Frederick et al. (2016) indicate that the washing step should be eliminated. Omission of this step fosters higher digestibility and saves water. This is especially important as water consumption would be important when these processes are deployed to commercial scales.

9. References

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Appendix 1: Enzymatic digestibility data

Table 1: Enzymatic digestibility of unwashed fungal pretreated biomass.

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
1	1	21.50	82	0
1	2	21.18	82	0
1	3	20.00	82	0
2	1	17.86	82	0
2	2	19.83	82	0
2	3	18.60	82	0
3	1	18.22	82	0
3	2	16.54	82	0
3	3	16.47	82	0
4	1	15.76	82	2
4	2	18.25	82	2
4	3	18.64	82	2
5	1	18.18	82	2
5	2	28.84	82	2
5	3	18.55	82	2
6	1	33.49	82	2
6	2	30.12	82	2
6	3	29.41	82	2
7	1	30.22	82	3
7	2	35.60	82	3
7	3	34.04	82	3
8	1	34.43	82	3
8	2	43.43	82	3
8	3	31.28	82	3
9	1	38.21	82	3
9	2	37.64	82	3
9	3	32.93	82	3
10	1	35.23	54	0
10	2	29.69	54	0
10	3	29.54	54	0
11	1	34.92	54	0
11	2	30.80	54	0
11	3	23.11	54	0
12	1	31.73	54	0
12	2	38.09	54	0
12	3	35.21	54	0

Table 1: Enzymatic digestibility of unwashed fungal pretreated biomass (Cont.).

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
13	1	29.39	54	2
13	2	31.52	54	2
13	3	31.18	54	2
14	1	32.58	54	2
14	2	34.38	54	2
14	3	33.28	54	2
15	1	30.84	54	2
15	2	30.23	54	2
15	3	31.02	54	2
16	1	30.61	54	3
16	2	29.99	54	3
16	3	25.30	54	3
17	1	31.57	54	3
17	2	34.69	54	3
17	3	33.74	54	3
18	1	34.56	54	3
18	2	30.25	54	3
18	3	28.27	54	3
19	1	30.63	25	0
19	2	29.48	25	0
19	3	33.13	25	0
20	1	36.45	25	0
20	2	29.72	25	0
20	3	29.38	25	0
21	1	31.43	25	0
21	2	33.98	25	0
21	3	33.36	25	0
22	1	34.71	25	2
22	2	36.06	25	2
22	3	36.73	25	2
23	1	35.89	25	2
23	2	41.50	25	2
23	3	36.05	25	2
24	1	37.50	25	2
24	2	37.33	25	2
24	3	37.01	25	2
25	1	39.36	25	3
25	2	38.05	25	3
25	3	40.23	25	3

Table 1: Enzymatic digestibility of unwashed fungal pretreated biomass (Cont.).

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
26	1	43.17	25	3
26	2	39.28	25	3
26	3	38.44	25	3
27	1	34.97	25	3
27	2	35.60	25	3
27	3	37.02	25	3

Table 2: Enzymatic digestibility of unwashed fungal and LHW (200°C for 10 min, HTST) pretreated switchgrass.

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
1	1	77.78	82	0
1	2	74.68	82	0
2	1	68.76	82	0
2	2	71.99	82	0
3	1	99.03	82	0
3	2	81.11	82	0
4	1	77.72	82	2
4	2	74.15	82	2
5	1	67.21	82	2
5	2	75.20	82	2
6	1	83.58	82	2
6	2	89.10	82	2
7	1	83.93	82	3
7	2	79.95	82	3
8	1	82.29	82	3
8	2	81.80	82	3
9	1	82.10	82	3
9	2	80.90	82	3
10	1	80.41	54	0
10	2	75.48	54	0
11	1	68.35	54	0
11	2	70.46	54	0
12	1	72.77	54	0
12	2	77.84	54	0
13	1	77.78	54	2

Table 2: Enzymatic digestibility of unwashed fungal and LHW (200°C for 10 min, HTST) pretreated switchgrass (Cont.).

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
13	2	76.07	54	2
14	1	75.91	54	2
14	2	80.15	54	2
15	1	54.67	54	2
15	2	67.66	54	2
16	1	72.40	54	3
16	2	74.33	54	3
17	1	66.76	54	3
17	2	70.85	54	3
18	1	64.86	54	3
18	2	65.59	54	3
19	1	68.61	25	0
19	2	67.46	25	0
20	1	75.13	25	0
20	2	76.02	25	0
21	1	64.63	25	0
21	2	69.84	25	0
22	1	75.49	25	2
22	2	67.54	25	2
23	1	70.76	25	2
23	2	72.03	25	2
24	1	81.18	25	2
24	2	72.89	25	2
25	1	77.92	25	3
25	2	71.47	25	3
26	1	71.49	25	3
26	2	69.86	25	3
27	1	67.38	25	3
27	2	69.97	25	3

Table 3: Enzymatic digestibility of washed fungal and LHW (200°C for 10 min, HTST) pretreated switchgrass.

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
1	1	57.00	82	0
1	2	61.80	82	0
2	1	63.22	82	0
2	2	61.00	82	0
3	1	51.89	82	0
3	2	53.76	82	0
4	1	52.95	82	2
4	2	54.06	82	2
5	1	60.18	82	2
5	2	57.95	82	2
6	1	57.73	82	2
6	2	56.82	82	2
7	1	57.14	82	3
7	2	54.76	82	3
8	1	54.98	82	3
8	2	52.02	82	3
9	1	52.70	82	3
9	2	51.19	82	3
10	1	54.42	54	0
10	2	51.61	54	0
11	1	57.59	54	0
11	2	54.69	54	0
12	1	56.82	54	0
12	2	56.71	54	0
13	1	52.58	54	2
13	2	53.61	54	2
14	1	48.02	54	2
14	2	55.05	54	2
15	1	49.09	54	2
15	2	51.62	54	2
16	1	57.17	54	3
16	2	55.18	54	3
17	1	51.96	54	3
17	2	53.35	54	3
18	1	52.32	54	3
18	2	50.33	54	3
19	1	54.09	25	0
19	2	53.28	25	0

Table 3: Enzymatic digestibility of washed fungal and LHW (200°C for 10 min, HTST) pretreated switchgrass Cont.).

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
20	1	55.54	25	0
20	2	54.51	25	0
21	1	53.30	25	0
21	2	50.74	25	0
22	1	53.28	25	2
22	2	49.91	25	2
23	1	54.69	25	2
23	2	50.41	25	2
24	1	55.19	25	2
24	2	54.83	25	2
25	1	56.75	25	3
25	2	54.05	25	3
26	1	55.27	25	3
26	2	51.65	25	3
27	1	50.83	25	3
27	2	51.63	25	3

Table 4: Enzymatic digestibility of unwashed fungal and LHW (180°C for 30 min, LTLT) pretreated switchgrass.

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
1	1	45.40	82	0
1	2	46.52	82	0
2	1	59.01	82	0
2	2	60.14	82	0
3	1	53.87	82	0
3	2	54.05	82	0
4	1	47.98	82	2
4	2	50.28	82	2
5	1	49.62	82	2
5	2	49.75	82	2
6	1	47.02	82	2
6	2	48.25	82	2
7	1	59.68	82	3
7	2	59.01	82	3
8	1	43.93	82	3
8	2	43.62	82	3
9	1	55.30	82	3
9	2	54.33	82	3
10	1	70.16	54	0
10	2	70.13	54	0
11	1	66.32	54	0
11	2	68.04	54	0
12	1	60.71	54	0
12	2	59.98	54	0
13	1	50.38	54	2
13	2	53.87	54	2
14	1	57.35	54	2
14	2	57.54	54	2
15	1	45.45	54	2
15	2	51.69	54	2
16	1	49.04	54	3
16	2	55.67	54	3

Table 4: Enzymatic digestibility of unwashed fungal and LHW (180°C for 30 min, LTLT) pretreated switchgrass (Cont.).

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
17	1	53.62	54	3
17	2	56.76	54	3
18	1	53.48	54	3
18	2	53.45	54	3
19	1	46.18	25	0
19	2	48.84	25	0
20	1	59.12	25	0
20	2	59.64	25	0
21	1	53.11	25	0
21	2	54.02	25	0
22	1	65.46	25	2
22	2	67.13	25	2
23	1	57.57	25	2
23	2	58.98	25	2
24	1	54.96	25	2
24	2	53.67	25	2
25	1	65.88	25	3
25	2	65.13	25	3
26	1	62.67	25	3
26	2	62.14	25	3
27	1	56.94	25	3
27	2	53.59	25	3

Table 5: Enzymatic digestibility of washed fungal and LHW (180°C for 30 min, LTLT) pretreated switchgrass.

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
1	1	36.15	82	0
1	2	37.37	82	0
2	1	41.99	82	0
2	2	41.82	82	0
3	1	43.83	82	0
3	2	44.02	82	0
4	1	47.30	82	2
4	2	45.86	82	2
5	1	38.79	82	2
5	2	39.09	82	2
6	1	41.72	82	2
6	2	39.45	82	2
7	1	47.79	82	3
7	2	48.97	82	3
8	1	34.81	82	3
8	2	36.16	82	3
9	1	42.88	82	3
9	2	43.64	82	3
10	1	49.61	54	0
10	2	50.48	54	0
11	1	48.69	54	0
11	2	49.32	54	0
12	1	56.68	54	0
12	2	51.22	54	0
13	1	62.40	54	2
13	2	51.81	54	2
14	1	57.55	54	2
14	2	47.14	54	2
15	1	40.62	54	2
15	2	39.45	54	2
16	1	38.10	54	3
16	2	38.96	54	3
17	1	43.52	54	3
17	2	42.96	54	3
18	1	40.26	54	3
18	2	43.23	54	3
19	1	38.21	25	0
19	2	39.28	25	0

Table 5: Enzymatic digestibility of washed fungal and LHW (180°C for 30 min, LTLT) pretreated switchgrass (Cont.).

Bale Number	Replication	Enzymatic Digestibility (%)	Storage Duration (Days)	Percentage Fungal Inoculum (%)
20	1	44.15	25	0
20	2	39.04	25	0
21	1	44.94	25	0
21	2	47.63	25	0
22	1	45.21	25	2
22	2	50.44	25	2
23	1	41.66	25	2
23	2	43.50	25	2
24	1	34.39	25	2
24	2	33.19	25	2
25	1	46.92	25	3
25	2	41.85	25	3
26	1	49.88	25	3
26	2	51.13	25	3
27	1	38.37	25	3
27	2	44.48	25	3