

WHITE ROT FUNGI *PLEUROTUS OSTREATUS*
PRETREATMENT ON SWITCHGRASS TO ENHANCE
ENZYMATIC HYDROLYSIS AND ETHANOL
PRODUCTION

By
MENGXING LI

Bachelor of Science in Food Science and Technology

Henan University of Technology

Zhengzhou, Henan, China

2013

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2015

WHITE ROT FUNGI *PLEUROTUS OSTREATUS*
PRETREATMENT ON SWITCHGRASS TO ENHANCE
ENZYMATIC HYDROLYSIS AND ETHANOL
PRODUCTION

Thesis Approved:

Dr. MARK R. WILKINS

Thesis Adviser

Dr. MICHAEL BUSER

Dr. HASAN ATIYEH

Dr. STEPHEN MAREK

ACKNOWLEDGEMENTS

I would like to express sincere gratitude to my advisor, Dr. Mark Wilkins for the knowledge and training I got during my Master study. He offered me the research assistantship to make my study possible in the US. I am the one who like to have my own ideas and to do things in my way. He respected my ideas, encouraged and guided me when I met setbacks. Dr. Wilkins pushed me in the right way thus I managed to finish specific research goals in the set timeline. He was always kind and helpful to me, his knowledge and character influenced me a lot.

The deep gratitude is extended to my committee members, Dr. Michael Buser, Dr. Stephen Marek and Dr. Hasan Atiyeh. Dr. Buser and his lab members Collin Craige, Drew Sutterfield, and Thomas Moore provided help for baling and sampling. Dr. Marek gave instructions on isolation of the *Pleurotus* strain used in my study in Chapter 5. I would like to thank Dr. Julie Carrier and her student Noaa Frederick from University of Arkansas for their work on biomass hydrolysis. Dr. Nurhan Dunfrod and her lab members Dr. Meizhen Xie, Nan Zhou and Qichen Ding were really helpful with me working on the biomass extraction instrument. I would like to appreciate the work conducted by Jiaqi Peng and Dr. Zhongdong Liu from Henan University of Technology, China for the SEM study on my fungal treated samples. Most of my work was done in our lab in ATRC. I got good training with experiment skills and research experiences from lab members. Lab manager Mark Gilstrap, lab members Oscar Pardo Planas, Shelyn

Gehle, Dr. Atiyeh's student Dr. Kan Liu, Xiao Sun, all of you were like a family around me. I could not forget the moments you helping me working on my problems, I could not have finished my Master thesis without your help.

Apart from the people aforementioned, I met many interesting people and made a lot of friends during my stay at OSU. I could always find someone around to talk to when I felt stressed and find someone to share the happiness when I had some great news. All these make me have a splendid life in the past two years.

My deepest gratitude belongs to my family in China. All accomplishments I achieved today are resulted from the love and support from my parents and my sister. Though they are far away in China, they always care me the most and wish me the best.

Name: MENGXING LI

Date of Degree: JULY, 2015

Title of Study: WHITE ROT FUNGI *PLEUROTUS OSTREATUS* PRETREATMENT ON SWITCHGRASS TO ENHANCE ENZYMATIC HYDROLYSIS AND ETHANOL PRODUCTION

Major Field: BIOSYSTEMS ENGINEERING

Abstract: Biofuels and high value chemicals derived from cellulosic biomass are good substitutes for petroleum energy and are more environmentally beneficial than corn or soybean based biofuels. The high cost of biomass conversion to biofuels is the main impediment for large scale biofuel production, in which pretreatment is one of the most expensive processing steps. White rot fungal pretreatment shows good potential to efficiently degrade lignin and enhance enzymatic hydrolysis and fermentation under mild environmental conditions. In this thesis biological pretreatment was applied to switchgrass with an emphasis on white rot fungi-associated pretreatment. Two specific studies were conducted: *Pleurotus ostreatus* pretreatment on large switchgrass bales (either square or round bales) in a natural environment and lab scale *P. ostreatus* pretreatment in a controlled environment. For the study in a natural environment, *P. ostreatus* was applied to large switchgrass bales and stored for 9 months. Sampling was done at three months, five months, seven months and nine months after fungus applied. Fungal treated samples were subjected to composition analysis, hydrothermolysis pretreatment and enzymatic hydrolysis. Fungal treated square bales had a lignin fraction ranging from 1.8% to 3.2% higher than untreated bales. There were no clear trends for composition of hydrothermolysis pretreated samples or glucose yield from enzymatic hydrolysis. Fungus did not grow in round bales and bale moisture contents varied between 6% and 11%, which was too low for fungal growth. Glucan and lignin contents of stored samples increased and xylan contents decreased from March to May and were constant from May to September. For enzymatic hydrolysis of hydrothermolysis (200 °C/10 min or 180 °C/20 min) treated samples, there were no significant differences among samples of unwashed and washed samples, which indicates washing is not necessary. For the lab study in a controlled environment, switchgrass was treated with varied initial inoculum loading and substrate moisture content. Results showed that no ethanol was produced during SSF of untreated switchgrass. For fungal treated samples, after 80 days fungal pretreatment, samples with 75% substrate moisture content and 5 ml initial inoculum loading had the highest lignin degradation, 52%, and the highest ethanol yield, 45%.

Keywords: white rot fungi, large scale, pretreatment

TABLE OF CONTENTS

Chapter	Page
LIST OF TABLES	ix
LIST OF FIGURES	x
1. Introduction.....	1
1.1. References	8
2. Literature review	13
2.1. Effect of biomass storage on biomass saccharification and fermentation	13
2.2. Anaerobic pretreatment on biomass	14
2.3. White rot fungi pretreatment process parameters	16
2.4. Biochemical mechanism of biomass degradation by white rot fungi	18
2.5. Involvement of white rot fungi in biomass pretreatment	21
2.5.1. White rot fungi pretreatment individually or in combination with other microorganisms in biological pretreatment	21
2.5.2. White rot fungi combined with physical pretreatments	24
2.5.3. White rot fungi combined with chemical pretreatments	25
2.5.4. White rot fungi combined with physiochemical pretreatments	26
2.6. Effect of white rot fungi pretreatment on biomass structural changes	27
2.7. References	31
3. Research objective and summary of studies	39
4. Oyster mushroom (<i>Pleurotus ostreatus</i>) pretreatment on large switchgrass bales in a natural storage environment.....	41
4.1. Abstract	41
4.2. Introduction.....	43
4.3. Methods.....	48
4.3.1. Field work	48
4.3.1.1. <i>Pleurotus ostreatus</i> spawn application and sampling	48
4.3.1.2. Rainfall and temperature	54
4.3.1.3. Moisture content	54
4.3.2. Laboratory analysis	55
4.3.2.1. Biomass characterization	55
4.3.2.2. Hydrothermolysis.....	56
4.3.2.3. Mass balance analysis of samples after hydrothermolysis pretreatment	57
4.3.2.4. Sugars, byproducts and degradation products analysis.....	58
4.3.2.5. Enzymatic hydrolysis	60
4.3.3. Statistical analysis	61
4.4. Results and discussion	62

4.4.1. Square bales study.....	62
4.4.1.1. Rainfall and temperature files during fungal pretreatment	62
4.4.1.2. Moisture content in fungal pretreatment	62
4.4.1.3. Composition after fungal pretreatment	67
4.4.1.4. Composition of pretreated solids after hydrothermolysis pretreatment	73
4.4.1.5. Mass balances after hydrothermolysis pretreatment	75
4.4.1.6. Degradation products in prehydrolyzate after hydrothermolysis pretreatment	76
4.4.1.7. Enzymatic hydrolysis	82
4.4.2. Round bales study	84
4.4.2.1. Rainfall and temperature files during storage time	84
4.4.2.2. Moisture file during storage time	84
4.4.2.3. Composition of samples during storage time	88
4.4.2.4. Composition of pretreated solids after hydrothermolysis pretreatment	91
4.4.2.5. Mass balances after hydrothermolysis pretreatment	93
4.4.2.6. Degradation products in prehydrolyzate after hydrothermolysis pretreatment	93
4.4.2.7. Enzymatic hydrolysis	100
4.5. Conclusions	103
4.6. Acknowledgements	103
4.7. References	105
5. Lab scale white rot fungi <i>Pleurotus ostreatus</i> pretreatment to enhance simultaneous saccharification and fermentation of switchgrass	111
5.1. Abstract	111
5.2. Introduction	113
5.3. Methods	115
5.3.1. Switchgrass feedstock	115
5.3.2. Fungus strain and inoculum	116
5.3.3. Fungal pretreatment	117
5.3.4. Biomass compositional analysis	118
5.3.4.1. Structural carbohydrates and lignin determination	118
5.3.5. SSF	120
5.3.5.1. Enzymes, yeast strain and inoculum	120
5.3.5.2. Yeast strain	121
5.3.5.3. SSF conditions	121
5.3.6. Ligninolytic enzymes extraction and activity assay	124
5.3.6.1. Enzymes extraction	124
5.3.6.2. Activity assay	124
5.3.7. SEM	125
5.3.8. Statistical analysis	125
5.4. Results and discussion	126
5.4.1. <i>P. ostreatus</i> isolation	126
5.4.2. <i>P. ostreatus</i> colonization	126
5.4.3. Preliminary tests	130

5.4.3.1. Effect of sterilization and grinding on ethanol yield	130
5.4.3.2. Effect of enzymes adding on ethanol yield	131
5.4.3.3. Effect of high initial inoculum loading on ethanol yield	131
5.4.4. Fungal pretreatment with a completely randomized 2*2 factorial design	135
5.4.4.1. Biomass dry matter loss and biomass components loss	136
5.4.5. SSF of fungal treated samples with 2*2 factorial design	140
5.4.5.1. Glucose yield	140
5.4.5.2. Ethanol concentration	140
5.4.5.3. Ethanol yield	142
5.4.5.4. Overall ethanol yield	145
5.4.5.5. Xylose yield	147
5.4.6. SEM	154
5.5. Conclusions	156
5.6. Acknowledgements	158
5.7 References	159
6. Future work	164

LIST OF TABLES

Table	Page
Table 4.1. Square bale top, middle, bottom and other samples combining procedure.....	51
Table 4.2. Round bale 1 ft, 2 ft samples combining procedure.....	53
Table 4.3. Moisture contents (% wet basis) of top, middle and bottom samples of 12 square bales	66
Table 4.4. Initial composition of square bale and round samples	69
Table 4.5. Biomass components (glucan, xylan and lignin) fraction of fungal treated square bale samples.....	70
Table 4.6. Glucan, xylan and lignin fraction of hydrothermolysis pretreated square bale samples.....	74
Table 4.7. Glucan, xylan and lignin fraction round bale samples during storage	90
Table 4.8. Glucan, xylan and lignin fraction of hydrothermolysis pretreated square bale samples.....	94
Table 5.1. Composition of fungal treated samples at different fungal pretreatment time.....	138

LIST OF FIGURES

Figure	Page
Fig. 1.1. Biomass conversion to ethanol process via sugar platform.	2
Fig. 4.1. Square bales and sampling locations of square bale	50
Fig. 4.2. Round bales and sampling locations of round bale	52
Fig. 4.3. Rainfall accumulation at certain period after <i>P. ostreatus</i> spawn applied to square bales	63
Fig. 4.4. Daily average air temperature after mushroom spawn applied to square bales.	64
Fig. 4.5. Moisture variability of 12 square bales samples.	65
Fig. 4.6. Limited fungus colonization on square bales	72
Fig. 4.7. Glucan (A), xylan (B) recovery of pretreated square bales samples under hydrothermolysis condition 200 °C/min	77
Fig. 4.8. Glucan (A), xylan (B) recovery of pretreated square bales samples under hydrothermolysis condition 190 °C/15min	78
Fig. 4.9. Glucan (A), xylan (B) recovery of hydrothermolysis pretreated square bales samples under hydrothermolysis condition 180 °C/20min	79
Fig. 4.10. Degradation products acetic acid (A), formic acid (B) and furfural (C) yields after hydrothermolysis pretreatment at 200/10.	80
Fig. 4.11. Degradation products acetic acid (A), formic acid (B) and furfural (C) yields after hydrothermolysis pretreatment at 180/20	81

Fig. 4.12. Glucose yield of pretreated solids with hydrothermolysis pretreatment condition 200/10 (A) and 180/20 (B) for square bale samples	83
Fig. 4.13. Rainfall accumulation at certain period after round bales storage started	85
Fig. 4.14. Daily average temperature after round bales storage started	86
Fig. 4.15. Moisture content of square bale samples	87
Fig. 4.16. Dried <i>P. ostreatus</i> spawn and no fungus growing on round bales	89
Fig. 4.17. Glucan (A) and xylan (B) recovery after hydrothermolysis pretreatment at 200/10 for round bale samples	96
Fig. 4.18. Glucan (A) and xylan (B) recovery after hydrothermolysis pretreatment at 180/20 for round bale samples	97
Fig. 4.19. Degradation products yields after hydrothermolysis pretreatment at 200/10 for round bale samples	98
Fig. 4.20. Degradation products yields after hydrothermolysis pretreatment at 180/20 for round bale samples	99
Fig. 4.21. Glucose yield of unwashed (A) and washed (B) pretreated solids after hydrothermolysis pretreatment at 200/10 for round bale samples	101
Fig. 4.22. Glucose yield of unwashed (A) and washed (B) pretreated solids after hydrothermolysis pretreatment at 180/20 for round bale samples	102
Fig. 5.1. <i>P. ostreatus</i> mushroom spawn, pure culture and clamp connection	127
Fig. 5.2. CFUs from <i>P. ostreatus</i> mycelial suspension cultivation on MBCD plate for 7 days.....	128
Fig. 5.3. Fungal colonization on switchgrass samples with 50% MC, 20 ml inoculum.....	129

Fig. 5.4. Ethanol yield (% theoretical) during SSF for fungal treated samples in preliminary tests	132
Fig. 5.5. Ethanol yield (% theoretical) during SSF for fungal treated samples without enzyme addition in preliminary tests	133
Fig. 5.6. Ethanol yield (%theoretical) during SSF for samples with high initial inoculum loading in preliminary tests.....	134
Fig. 5.7. Biomass dry matter loss (A), cellulose loss (B), hemicellulose loss (C), lignin loss (D) during an 80 days fungal pretreatment	139
Fig. 5.8. Ethanol concentration (g/L) during SSF under a completely randomized 2*2 factorial design.....	141
Fig. 5.9. Ethanol yield (% theoretical) during SSF under a completely randomized 2*2 factorial design.....	144
Fig. 5.10. Overall ethanol yield (% theoretical) under a completely randomized 2*2 factorial design.....	148
Fig. 5.11. Xylose yield (%theoretical) during SSF without enzymes addition in preliminary tests	149
Fig. 5.12. Xylose yield (% theoretical) during SSF under a completely randomized 2*2 factorial design.....	151
Fig. 5.13. Laccase (A), MnP (B) activities during an 80 day fungal pretreatment under a completely randomized 2*2 factorial design	153
Fig. 5.14. SEM images	155

CHAPTER I

1. Introduction

Lignocellulosic biomass is one of the most abundant and renewable resources on earth (Sánchez & Cardona, 2008; Sokhansanj et al., 2009). Cellulosic ethanol produced from biomass could be a substitute for petroleum energy in future decades. Cellulosic ethanol exhibits energetic, economic and environmental advantages over starch or sugar based ethanol. Cellulosic ethanol does not require food sources as a substrate, makes use of agricultural residues and reduces greenhouse gas emissions (Krylova et al., 2008; Lashinsky & Schwartz, 2006; Wang et al., 1999). Though extensive research has been completed on the conversion of biomass to ethanol for the last three decades (Duff & Murray, 1996; Reshamwala et al., 1995; Salvachúa et al., 2011; Sánchez & Cardona, 2008; Shi et al., 2009; Taniguchi et al., 2005; Wilkins et al., 2007; Wright, 1988), various challenges associated with implementing large scale biomass ethanol production from biomass still remain (Bruce, 2013; Cheng & Timilsina, 2011; Sarkar et al., 2012).

Through the sugar platform, biomass conversion to biofuels usually requires four steps, pretreatment, enzymatic hydrolysis, fermentation and downstream processing (Fig 1.1). Pretreatment is a process to disrupt recalcitrant structure of lignocellulosic biomass in order to improve enzymatic hydrolysis efficiency (Mosier et al., 2005). As shown in Fig. 1.1, pretreatment could be involved with mechanical, physico and/or chemical process,

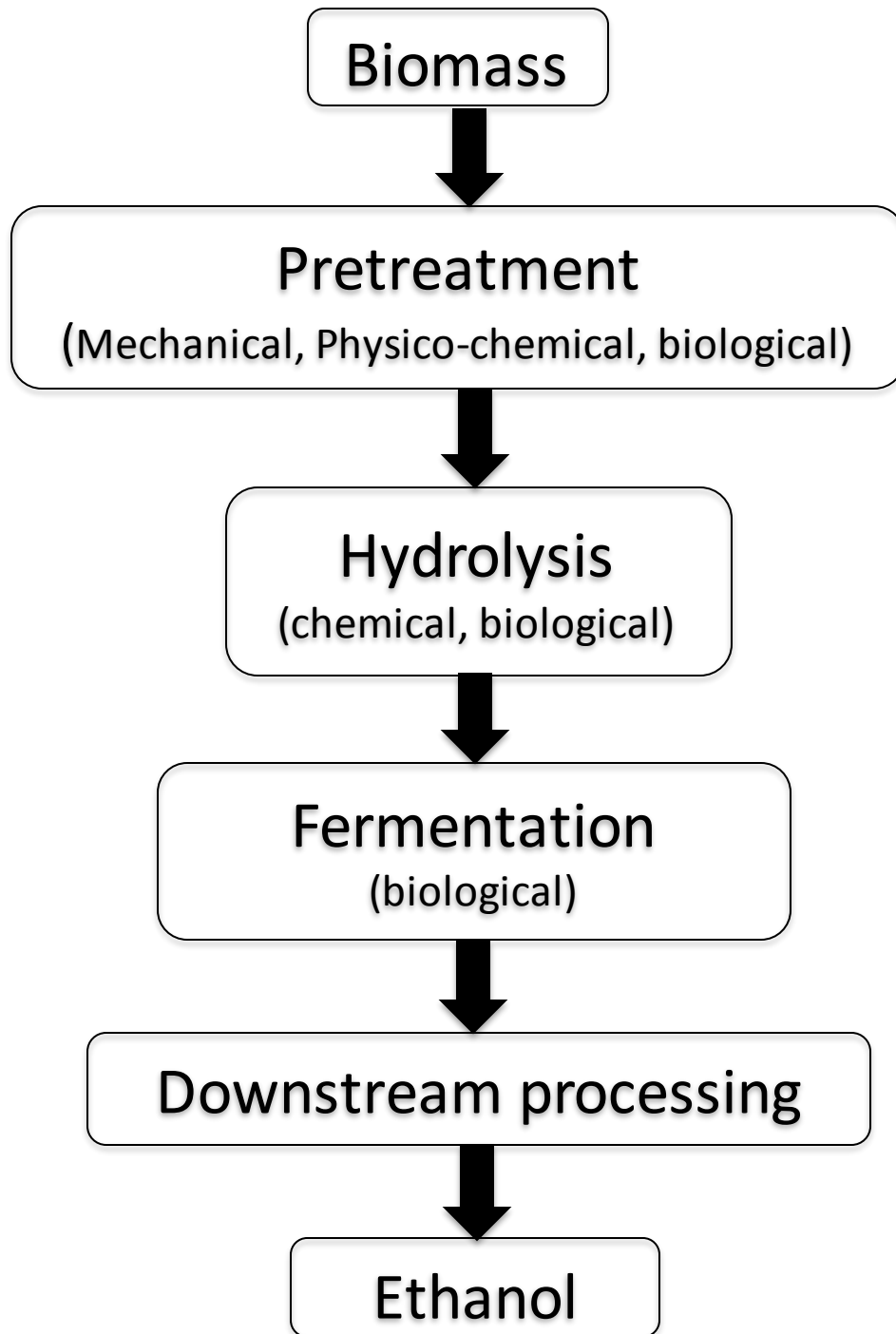


Fig. 1.1. Biomass conversion to ethanol process via sugar platform.

biological process or a combination of these processes. Hydrolysis usually applies chemical or biological processes to hydrolyze polymeric sugars into monomeric sugars, which are utilized by fermentative microorganisms to produce ethanol in the fermentation step. Downstream processing is used to concentrate ethanol from fermentation to obtain fuel ethanol (Kumar et al., 2009). Pretreatment is one of the most costly processing steps in bioethanol production from lignocellulosic biomass that constitutes almost 20% of the entire cost, developing a more efficient biomass pretreatment could make bioethanol production more economically viable (Agbor et al., 2011; Alvira et al., 2010; da Costa Sousa et al., 2009; Wan & Li, 2012).

The term 'lignocellulosic biomass' is often used to describe the material that composes the plant cell wall, which primarily includes cellulose (30–50%), hemicellulose (15–35%), and lignin (10–30%). Cellulose is a polysaccharide linked by beta-1, 4-glycosidic bonds that can be digested by cellulase and beta-glucosidase to produce glucose, a fermentable sugar for bioethanol production. Hemicellulose is a highly branched short polymer composed of xylose, arabinose, glucose, galactose and/or mannose. Lignin fills the space in the cell wall between cellulose, hemicellulose and pectin components (Sánchez, 2009). Lignin is covalently linked to hemicellulose and crosslinks different plant polysaccharides, conferring mechanical strength to the cell wall. Lignin locks cellulose and hemicellulose in the hetero matrix of the plant cell wall (Liu et al., 2013; Wyman et al., 2004). As a result of the organization and interaction between these polymeric structures, the plant cell wall is naturally recalcitrant to biological degradation (Himmel et al., 2007). Lignins are a group of highly branched phenylpropanoid polymers found in terrestrial plants and are generally considered to be

one of the most important limiting factors in the enzymatic hydrolysis process (Vanholme et al., 2010). Pretreatment is often required to liberate sugars from lignocellulosic biomass and decrease lignin content for subsequent hydrolysis and fermentation.

Pretreatment is regarded as a process to reduce particle size (Cadoche & López, 1989; Chandra et al., 2007), reduce lignin content and crystallinity of cellulose (Hall et al., 2010), and increase biomass surface area (Burns et al., 1989; Huang et al., 2010). Delignification is a key factor in determining enzyme digestibility and fermentable sugars yield (Chandra et al., 2007; Zhu et al., 2008). Ding et al. (2012) applied confocal laser scanning microscopy and atomic force microscopy on corn stover cell walls to characterize the nanoscale structure. This research confirmed that lignin affected enzyme digestibility by physically impeding or nonspecifically absorbing enzymes. Their study found that after delignification, cellulases bonded at the cell wall surfaces, and penetrated the Parenchyma type secondary cell walls from its innermost side. Their observations suggested that delignification renders the cell wall more accessible to fungal cellulases (Ding et al., 2012).

An ideal pretreatment should have a low capital and operative cost, be adaptive to a wide range of lignocellulosic materials and result in high recovery of biomass components that are easily segregated and in usable and separate forms for conversion to ethanol (Agbor et al., 2011). In order to efficiently access the energy potential of the substrate, physical and/or chemical pretreatment methods have been widely investigated (Canam et al., 2013; Galbe & Zacchi, 2007; Mosier et al., 2005; Taherzadeh & Karimi, 2008; Wyman et al., 2005b). For biological pretreatment, only a few studies have been reported (Liggenstoffer et al., 2014; Richard et al., 2001; Wan, 2011).

Physical pretreatment processes use intensive energy without lignin removal, but are relatively insensitive to physical and chemical characteristics of biomass (Chandra et al., 2007). Chemical pretreatment processes show strong ability of solubilizing hemicellulose and lignin, thus enhancing enzymatic accessibility to the cellulose; however, a drawback is the required neutralization of chemical residues prior to hydrolysis (Chandra et al., 2007). Physiochemical pretreatment processes combining both chemical and physical processes have been widely investigated in recent years. Dilute acid, lime, ammonia fiber explosion (AFEX), steam explosion and hydrothermolysis are typical physiochemical pretreatment processes and were reviewed in Wyman et al. (2005a). Ethanol yields of 70% to 90% have been achieved through physiochemical pretreatment processes (Faga et al., 2010; Schacht et al., 2008; Suryawati et al., 2009). Despite the high ethanol yields that have resulted from physiochemical pretreatment processes, physiochemical pretreatment processes usually involve harsh operating conditions (high pressure and/or high temperature), high energy input and/or require a corrosion-resistant pressure reactor, which makes pretreatment one of the most costly steps in biomass conversion to biofuels.

Biological pretreatment processes have received attention in recent years due to their mild pretreatment conditions and their reliability to work on different plant biomass types (Cook et al., 2014). In terms of the long pretreatment time requirement, biological pretreatment could be incorporated into biomass storage; therefore, long microbial pretreatment time would no longer be an issue. Natural processes such as ensilaging and ruminant fungi digestion showed strong abilities to degrade biomass, but also resulted in considerable sugar loss (Chen et al., 2007; Liggenstoffer et al., 2014). Microorganisms

mainly employed in biomass degradation and specifically associated with lignin degradation are mainly brown rot, white rot and soft rot fungi (Alvira et al., 2010). Brown rot fungi refer to fungi that preferentially degrade cellulose and hemicellulose and modify lignin only to a limited extent (Singh-Arora & Kumar-Sharma, 2010). Less is known about the soft rot fungi degradative enzyme systems, but their degradative mechanisms have been reviewed along with the degradative enzymatic and non-enzymatic systems known to exist in brown rot and white rot fungi (Goodell et al., 2008). Soft rot fungi were found to cause microscopic cavities within the secondary cell wall of wood (Blanchette et al., 2004). White rot fungi are believed to be the most efficient lignin degraders among fungi (Sánchez, 2009; Singh-Arora & Kumar-Sharma, 2010). White rot fungi, as a kind of saprophytic fungi, can degrade all plant cell wall components, including lignin. Some species are lignin-selective, meaning they preferentially remove lignin from wood leaving pockets of white, degraded cells that consist entirely of cellulose, while others degrade lignin and cellulose simultaneously (Blanchette, 1991). Lignin-selective white rot fungi have the natural ability to access the energy-rich components of biomass and degrade lignin more than cellulose and hemicellulose (Ufot, 2010).

However, previous studies showed white rot fungi pretreated biomass usually led to a fermentable sugar and ethanol yield between 30% and 70% (Bak et al., 2009; Wan & Li, 2010; Xu et al., 2010), which is lower than that of successful physicochemical pretreatments. Controlled partial biological degradation is believed to offer tangible energy and cost benefits to the whole biofuel process (Ray et al., 2010). In order to realize effective pretreatment, white rot fungi pretreatment is proposed to be applied on

biomass first to decrease the severity of subsequent thermochemical or thermomechanical pretreatments, or even eliminate the necessity of energy-intensive pretreatment (Balan, 2014; Shi et al., 2009). White rot fungi contribute to delignification, create more access for enzymes to approach cellulose and hemicellulose, improve cellulose digestibility, decrease pretreatment energy costs and result in higher fermentable sugar yields (Bak et al., 2009; Liu et al., 2013; Wan & Li, 2012). Incorporation of white rot fungi into physical and/or chemical pretreatment before hydrolysis of biomass is a promising method to degrade lignin and liberate sugar from the plant cell matrix in a method that is much milder than thermochemical or thermomechanical methods.

1.1. References

- Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B. 2011. Biomass pretreatment: Fundamentals toward application. *Biotechnology Advances*, **29**(6), 675-685.
- Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology*, **101**(13), 4851-4861.
- Bak, J.S., Ko, J.K., Choi, I.-G., Park, Y.-C., Seo, J.-H., Kim, K.H. 2009. Fungal pretreatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw. *Biotechnology and Bioengineering*, **104**(3), 471-482.
- Balan, V. 2014. Current challenges in commercially producing biofuels from lignocellulosic biomass. *International Scholarly Research Notices*, **2014**.
- Blanchette, R.A. 1991. Delignification by wood-decay fungi. *Annual Review of Phytopathology*, **29**(1), 381-403.
- Blanchette, R.A., Held, B.W., Jurgens, J.A., McNew, D.L., Harrington, T.C., Duncan, S.M., Farrell, R.L. 2004. Wood-destroying soft rot fungi in the historic expedition huts of Antarctica. *Applied and Environmental Microbiology*, **70**(3), 1328-1335.
- Bruce, S.L. 2013. Ethanol supply chain and industry overview: more harm than good?, PhD diss., Massachusetts Institute of Technology.
- Burns, D.S., Ooshima, H., Converse, A.O. 1989. Surface area of pretreated lignocellulosics as a function of the extent of enzymatic hydrolysis. *Applied Biochemistry and Biotechnology*, **20-21**(1), 79-94.
- Cadoche, L., López, G.D. 1989. Assessment of size reduction as a preliminary step in the production of ethanol from lignocellulosic wastes. *Biological Wastes*, **30**(2), 153-157.
- Canam, T., Dumonceaux, T.J., Record, E., Li, Y. 2013. White-rot fungi: the key to sustainable biofuel production? *Biofuels*, **4**(3), 247-250.
- Chandra, R.P., Bura, R., Mabee, W., Berlin, d.A., Pan, X., Saddler, J. 2007. Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics? in: *Biofuels*, (Eds.) L. Olsson, B.K. Ahring, Vol. 108, Springer. New York, pp. 67-93.
- Chen, Y., Sharma-Shivappa, R.R., Chen, C. 2007. Ensiling agricultural residues for bioethanol production. *Applied Biochemistry and Biotechnology*, **143**(1), 80-92.

- Cheng, J.J., Timilsina, G.R. 2011. Status and barriers of advanced biofuel technologies: A review. *Renewable Energy*, **36**(12), 3541-3549.
- Cook, C., Francocci, F., Cervone, F., Bellincampi, D., Bolwell, P.G., Ferrari, S., Devoto, A. 2014. Combination of pretreatment with white rot fungi and modification of primary and secondary cell walls improves saccharification. *BioEnergy Research*, 1-12.
- da Costa Sousa, L., Chundawat, S.P.S., Balan, V., Dale, B.E. 2009. 'Cradle-to-grave' assessment of existing lignocellulose pretreatment technologies. *Current Opinion in Biotechnology*, **20**(3), 339-347.
- Ding, S.-Y., Liu, Y.-S., Zeng, Y., Himmel, M.E., Baker, J.O., Bayer, E.A. 2012. How does plant cell wall nanoscale architecture correlate with enzymatic digestibility? *Science*, **338**(6110), 1055-1060.
- Duff, S.J., Murray, W.D. 1996. Bioconversion of forest products industry waste cellulose to fuel ethanol: a review. *Bioresource Technology*, **55**(1), 1-33.
- Faga, B.A., Wilkins, M.R., Banat, I.M. 2010. Ethanol production through simultaneous saccharification and fermentation of switchgrass using *Saccharomyces cerevisiae* D5A and thermotolerant *Kluyveromyces marxianus* IMB strains. *Bioresource Technology*, **101**(7), 2273-2279.
- Galbe, M., Zacchi, G. 2007. Pretreatment of lignocellulosic materials for efficient bioethanol production. in: *Biofuels*, (Eds.) L. Olsson, B.K. Ahring, Vol. 108, Springer. New York, pp. 41-65.
- Goodell, B., Yuhui, Q., Jellison, J. 2008. Fungal decay of wood: Soft rot-brown rot-white rot. in: *Development of Commercial Wood Preservatives*, (Eds.) T.P. Schultz, H. Miltz, M.H. Freeman, B. Goodell, D.D. Nicholas, Vol. 982, American Chemical Society. Washington, DC, pp. 9-31.
- Hall, M., Bansal, P., Lee, J.H., Realff, M.J., Bommarius, A.S. 2010. Cellulose crystallinity—a key predictor of the enzymatic hydrolysis rate. *FEBS journal*, **277**(6), 1571-1582.
- Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D. 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science*, **315**(5813), 804-807.
- Huang, R., Su, R., Qi, W., He, Z. 2010. Understanding the key factors for enzymatic conversion of pretreated lignocellulose by partial least square analysis. *Biotechnology Progress*, **26**(2), 384-392.

- Krylova, A.Y., Kozyukov, E.A., Lapidus, A.L. 2008. Ethanol and diesel fuel from plant raw materials: A review. *Solid Fuel Chemistry*, **42**(6), 358-364.
- Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & Engineering Chemistry Research*, **48**(8), 3713-3729.
- Lashinsky, A., Schwartz, N. 2006. How to beat the high cost of gasoline. Forever! in: *Fortune on CNNMoney.com, January*, Vol. 24.
- Liggenstoffer, A.S., Youssef, N.H., Wilkins, M.R., Elshahed, M.S. 2014. Evaluating the utility of hydrothermolysis pretreatment approaches in enhancing lignocellulosic biomass degradation by the anaerobic fungus *Orpinomyces* sp. strain C1A. *Journal of Microbiological Methods*, **104**(0), 43-48.
- Liu, J., Wang, M.L., Tonnis, B., Habteselassie, M., Liao, X., Huang, Q. 2013. Fungal pretreatment of switchgrass for improved saccharification and simultaneous enzyme production. *Bioresource Technology*, **135**(0), 39-45.
- Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, **96**(6), 673-686.
- Ray, M.J., Leak, D.J., Spanu, P.D., Murphy, R.J. 2010. Brown rot fungal early stage decay mechanism as a biological pretreatment for softwood biomass in biofuel production. *Biomass and Bioenergy*, **34**(8), 1257-1262.
- Reshamwala, S., Shawky, B., Dale, B. 1995. Ethanol production from enzymatic hydrolysates of AFEX-treated coastal bermudagrass and switchgrass. *Applied Biochemistry and Biotechnology*, **51-52**(1), 43-55.
- Richard, T., Proulx, S., Moore, K., Shouse, S. 2001. Ensilage technology for biomass pre-treatment and storage. *ASAE Annual International Meeting, Sacramento, CA*.
- Salvachúa, D., Prieto, A., López-Abelairas, M., Lu-Chau, T., Martínez, Á.T., Martínez, M.J. 2011. Fungal pretreatment: An alternative in second-generation ethanol from wheat straw. *Bioresource Technology*, **102**(16), 7500-7506.
- Sánchez, C. 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances*, **27**(2), 185-194.
- Sánchez, Ó.J., Cardona, C.A. 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology*, **99**(13), 5270-5295.
- Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K. 2012. Bioethanol production from agricultural wastes: An overview. *Renewable Energy*, **37**(1), 19-27.

- Schacht, C., Zetzl, C., Brunner, G. 2008. From plant materials to ethanol by means of supercritical fluid technology. *The Journal of Supercritical Fluids*, **46**(3), 299-321.
- Shi, J., Sharma-Shivappa, R.R., Chinn, M., Howell, N. 2009. Effect of microbial pretreatment on enzymatic hydrolysis and fermentation of cotton stalks for ethanol production. *Biomass and Bioenergy*, **33**(1), 88-96.
- Singh-Arora, D., Kumar-Sharma, R. 2010. Lignolytic Fungal Laccases and Their Biotechnological Applications. *Applied Biochemistry and Biotechnology*, **160**(6), 1760-1788.
- Sokhansanj, S., Mani, S., Turhollow, A., Kumar, A., Bransby, D., Lynd, L., Laser, M. 2009. Large-scale production, harvest and logistics of switchgrass (*Panicum virgatum* L.)-current technology and envisioning a mature technology. *Biofuels, Bioproducts & Biorefining*, **3**(2), 124.
- Suryawati, L., Wilkins, M.R., Bellmer, D.D., Huhnke, R.L., Maness, N.O., Banat, I.M. 2009. Effect of hydrothermolysis process conditions on pretreated switchgrass composition and ethanol yield by SSF with *Kluyveromyces marxianus* IMB4. *Process Biochemistry*, **44**(5), 540-545.
- Taherzadeh, M., Karimi, K. 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. *International Journal of Molecular Sciences*, **9**(9), 1621-1651.
- Taniguchi, M., Suzuki, H., Watanabe, D., Sakai, K., Hoshino, K., Tanaka, T. 2005. Evaluation of pretreatment with *Pleurotus ostreatus* for enzymatic hydrolysis of rice straw. *Journal of Bioscience and Bioengineering*, **100**(6), 637-643.
- Ufot, U.F. 2010. Expression and characterisation of a novel manganese peroxidase from *Phlebia radiata*, PhD diss., University of Sussex.
- Vanholme, R., Demedts, B., Morreel, K., Ralph, J., Boerjan, W. 2010. Lignin biosynthesis and structure. *Plant Physiology*, **153**(3), 895-905.
- Wan, C. 2011. Microbial pretreatment of lignocellulosic biomass with *Ceriporiopsis subvermispota* for enzymatic hydrolysis and ethanol production, PhD Diss., The Ohio State University.
- Wan, C., Li, Y. 2012. Fungal pretreatment of lignocellulosic biomass. *Biotechnology Advances*, **30**(6), 1447-1457.
- Wan, C., Li, Y. 2010. Microbial pretreatment of corn stover with *Ceriporiopsis subvermispota* for enzymatic hydrolysis and ethanol production. *Bioresource Technology*, **101**(16), 6398-6403.

- Wang, M., Saricks, C., Santini, D. 1999. Effects of fuel ethanol use on fuel-cycle energy and greenhouse gas emissions, Argonne National Laboratory. Argonne, IL, pp. 32.
- Wilkins, M.R., Widmer, W.W., Grohmann, K., Cameron, R.G. 2007. Hydrolysis of grapefruit peel waste with cellulase and pectinase enzymes. *Bioresource Technology*, **98**(8), 1596-1601.
- Wright, J.D. 1988. Ethanol from biomass by enzymatic hydrolysis. *Chemical Engineering Progress*, **84**(8), 62-74.
- Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y. 2005a. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. *Bioresource Technology*, **96**(18), 2026-2032.
- Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y. 2005b. Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology*, **96**(18), 1959-1966.
- Wyman, C.E., Decker, S.R., Himmel, M.E., Brady, J.W., Skopec, C.E., Viikari, L. 2004. Hydrolysis of cellulose and hemicellulose. in: *Polysaccharides: Structural Diversity and Functional Versatility*, (Ed.) S. Dumitriu, Marcel Dekker. New York, pp. 995-1033.
- Xu, C., Ma, F., Zhang, X., Chen, S. 2010. Biological pretreatment of corn stover by *Irpex lacteus* for enzymatic hydrolysis. *Journal of Agricultural and Food Chemistry*, **58**(20), 10893-10898.
- Zhu, L., O'Dwyer, J.P., Chang, V.S., Granda, C.B., Holtzapple, M.T. 2008. Structural features affecting biomass enzymatic digestibility. *Bioresource Technology*, **99**(9), 3817-3828.

CHAPTER II

2. Literature review

Biological pretreatment processes have received attention in recent years due to their mild pretreatment conditions and their reliability to work on different plant biomass types (Cook et al., 2014). In terms of the long residence time requirement, biological pretreatment could be incorporated into biomass storage; therefore, long microbial pretreatment time would no longer be an issue. Biomass storage, anaerobic microorganism pretreatment on biomass, and aerobic microorganism pretreatment on biomass, specifically white rot fungi associated pretreatment, are reviewed as follows.

2.1. Effect of biomass storage on biomass saccharification and fermentation

Biomass storage methods for forage are similar to biomass storage methods for biofuel production. Previously, a few studies on switchgrass storage mainly focused on the dry matter loss, which is associated with improving forage quality and stability. Sanderson et al. (1997) conducted six months storage of switchgrass under outside, unprotected conditions and got a dry matter loss of 13% of the original weight. Although storage for forage and for biofuel production may have some differences, the fundamental requirements (e.g. biomass feed stocks used, overcoming biomass recalcitrance, etc.) are similar and the infrastructure and facilities for forage storage could be adapted to biomass storage for biofuel production. The effects of different storage

methods on biomass saccharification and fermentation have been the subject of a few studies. Djioleu et al. (2014) conducted a study with 65 days field storage and barn storage of switchgrass prior to dilute acid pretreatment and enzymatic hydrolysis. Their results showed that barn storage led to greater preservation of fermentable sugars and reduced production of fermentation inhibitors compared to unprotected storage. Richard et al. (2001) did ensilaging of corn stover for 8 weeks and observed hemicellulose degradation of 0.7% and a relative cellulose and lignin accumulation (1.8% and 0.5%, respectively) in moisture levels ranging from 53% to 80%. Digman et al. (2007) applied chemicals (alkali, acid and oxidizing agents) to switchgrass during storage. Their results showed that conversion of cellulose to ethanol was 12% higher for acid pretreated switchgrass and 13% higher for lime pretreated switchgrass compared to untreated samples. Another study by the same authors conducted dilute acid pretreatment on reed canarygrass and switchgrass at farm scale and obtained 19% and 7% higher ethanol after SSF, respectively, than with untreated biomass, (Digman et al., 2010).

2.2. Anaerobic pretreatment on biomass

Ensilaging has been widely used for forage preservation and improvement of forage digestibility. Ensilaging is used for high moisture forages that are liable to spoilage by aerobic microorganisms. The process involves storing forage anaerobically, which results in the growth of epiphytic lactic acid bacteria (Pitt et al., 1985). Richard et al. (2001) did bench scale ensilage of corn stover for 8 weeks and found a slight degradation of hemicellulose and a relative accumulation of cellulose and lignin. The effect of ensilaging corn stover on the saccharification and fermentation to ethanol was not investigated in their study (Richard et al., 2001). The effect of ensilaging biomass to

improve sugar yield is limited. Chen et al. (2007) observed 5% to 10% sugar yield increases for ensilaged agricultural residues compared to untreated residues while the degradation of cellulose and hemicellulose were up to 10%. Large scale ensilage processes use enclosed systems (Harrison & Fransen, 1991), which increase process complexity. Also, the microorganisms working during ensilaging consume mainly carbohydrates and no substantial lignin. Ensilage is not regarded normally as a stand-alone and efficient pretreatment method (Canam et al., 2013b).

Rumen gut fungi require mild conditions and show the potential consolidation of pretreatment, hydrolysis and fermentation in one step. Due to their rich reservoir of cellulolytic enzymes and enzyme complexes, anaerobic gut fungi are proposed as microorganisms for consolidating bioprocessing for their ability to mediate both biomass saccharification and conversion to ethanol or other bioenergy products (Haitjema et al., 2014). Grenet et al. (1993) isolated *Piromyces communis* from rumen fungi and cultivated this fungus on untreated and ammonia treated wheat straw. They found that *P. communis* degraded 15% of untreated and 23% of ammonia treated straw. Rezaeian et al. (2005) conducted rumen fungi cultivation on barley straw in vitro over 14 days. Their results showed that dry matter losses were 38% on rumen fungal treated barley straw. Ligenstoffer et al. (2014) performed hydrothermolysis pretreatment on corn stover and switchgrass and applied the anaerobic rumen fungus *Orpinomyces* sp. strain C1A. Their results showed that strain C1A metabolized 25% of untreated switchgrass and 24% of untreated corn stover.

2.3. White rot fungi pretreatment process parameters

Inoculum for white rot fungi could be either spores or fungal mycelia. Using *Pleurotus ostreatus* as an example, this fungus has the life cycle of spore to spore stage, which is universal in pileate basidiomycetes. Spores germinate and produce mycelium, mycelia grow and permeate throughout the natural substrate, producing fruiting primordia and further growing into bigger fruit bodies, which is accompanied by the formation of basidiospores (Rajaratnam et al., 1987). In commercial *Pleurotus* cultivation as well as other white rot fungi cultivation, the mycelium is usually inoculated into seeding materials called “spawn”, which contains some wet grains as the substrate (Rajaratnam et al., 1987). Spawn is used to make the *Pleurotus* strain stable (Royse, 2003). Compared to liquid inoculum, spawn is much easier to handle and cheaper for large scale application.

Incubation temperature affects fungal growth and enzyme production, and optimal temperature varies from organism to organism (Asgher et al., 2009). Most white rot fungi pretreatments were conducted in a temperature range of 20 °C to 30 °C, while efficient delignification was obtained in a temperature range of 25 °C to 30 °C. Wan and Li (2010b) found 28 °C was the optimal temperature for improving subsequent enzymatic hydrolysis after testing *Ceriporiopsis subvermispora* pretreatment on corn stover in a temperature range of 4 °C to 37 °C. Fernández-Fueyo et al. (2014) analyzed the differences in the transcription levels of ligninolytic peroxidase genes when culturing *P. ostreatus* by decreasing temperature from 37 °C to 10 °C or adjusting pH from 5.5 to 3. They found most of the peroxidase genes were downregulated and the enzyme activity was decreased when temperature and pH were decreased.

In most cases of biomass treatment by white rot fungi, white rot fungi were cultivated in a solid state, which means microorganisms were cultivated in solids with the absence (or near absence) of free water (Barrios-González, 2012; Glassey & Ward, 2015). Optimal moisture for efficient biomass degradation differs among different fungi. High moisture decreases oxygen penetration while low moisture may lead to poor nutrient accessibility (Pandey, 2003). Miles and Chang (2004) reported that fungus mycelia could not grow with a moisture content of 20%. *Phanerochaete chrysosporium* solid state cultivation required a moisture range of 40% to 90% (Asgher et al., 2006). Wan and Li (2010b) conducted pretreatment on corn stover in a moisture range of 45% to 80% and found that the highest glucose and xylose yield were both obtained at 75% moisture content.

Wan and Li (2012) reported that moisture (70% to 80%) and particle size (0.5mm-10 mm) of the feedstock, good heat dissipation and air circulation, media supplements (Mn^{2+} , high carbon/nitrogen ratio) and pretreatment time were critical for fungal growth and metabolism to achieve good performance, while complete decontamination was not necessary. However, the extensive degradation of hemicelluloses was a crucial prerequisite for efficient lignin removal since hemicelluloses are covalently associated with lignin (Dinis et al., 2009). In the studies of Xu et al. (2010), during a 150 day *Irpex lacteus* pretreatment on corn stover, the saccharification ratio (saccharification ratio is based on holocellulose in the raw corn stover, holocellulose is the fraction constituting fibrous cellulose wrapped in hemicellulose-pectin (Segato et al., 2014), simply a combination of cellulose and hemicellulose (Silverstein et al., 2007)) reached 66% at 25 d, then increased slowly until 60 d and started to decrease dramatically

due to holocellulose loss of 26% at 60 days and 63% at 150 days. Holocellulose degradation, especially hemicellulose degradation, was dominant during the early stage of fungal growth (0–5 day) while no lignin degradation was observed. Thereafter, active lignin degradation was observed from day 5 to 10 with a rate higher than that of holocellulose degradation (Xu et al., 2010). Previous studies indicated lignin degrades simultaneously with holocellulose for many white rot fungi, which results in a lower sugar yield. For an efficient fungal treatment, excessive fungal pretreatment time should be avoided.

It is well known that lignin peroxidase, manganese peroxidase, and laccase are the three major oxidative enzymes secreted by white rot fungi (Eriksson et al., 1990). Enhancing ligninolytic enzymes production and catalytic activity by optimizing physiochemical parameters are important. Parenti et al. (2013) found wheat straw extract induced laccase activity in *P. ostreatus*. Mäkelä et al. (2013) obtained elevated lignin peroxidase and manganese peroxidase activities on high nitrogen medium using the white rot fungus *Phlebia radiata*. In their study the laccase activity was improved on high Cu^{2+} amended medium. However, Cu^{2+} concentration as high as 10 mM completely stopped laccase production when using *T. gibbosa* to degrade wheat straw (Knežević et al., 2014).

2.4. Biochemical mechanism of biomass degradation by white rot fungi

Lignin is chemically difficult to degrade because it is a three-dimensional polymer interconnected through diverse carbon-carbon and other bonds that are not hydrolyzable under biological conditions. Plant cell wall degrading enzymes have been classified into enzyme systems such as cellulolytic, ligninolytic and xylanolytic, which

reflect the nature of the polymers that these enzymes breakdown (Canam et al., 2013b). Lignin is amorphous in nature, lacks stereoregularity, and is not susceptible to hydrolytic attack. Despite its resistant nature, it can be degraded by various organisms, particularly, white rot fungi (Arora & Sharma, 2010).

Despite the complexity of lignocellulosic biomass, white rot fungi can break down the rigid structure through the interaction of low molecular weight chemicals, hydrolytic enzymes and ligninolytic enzymes. Ligninolytic enzymes are primarily responsible for lignin depolymerization. However, high molecular weight ligninolytic enzymes cannot penetrate the cell wall due to the low permeability of wood at the initial stages of degradation, indicating that low molecular weight chemicals catalyze lignin depolymerization at the initial stages (Ferraz et al., 2003). There were reports indicating that lipid radicals such as peroxy and acyl radicals derived from manganese peroxidase-dependent lipid peroxidation might serve as small, diffusible agents for initiating lignin depolymerization in sound wood (sound wood refers to wood of intact structure, non-decayed wood) where ligninolytic enzymes cannot penetrate (Bao et al., 1994; Jönsson et al., 1998; Kapich et al., 1999). Other low molecular weight chemicals, primarily reactive oxygen species, are also small enough to penetrate substrate that is too compact for enzymes to access. The production of hydrogen peroxide from highly reactive hydroxyl radicals ($\text{OH}\cdot$), which are oxidizing species, leads to covalent bond cleavage in both lignin and cellulose (Canam et al., 2013a). Other notable redox enzymes that mainly belong to ligninolytic enzymes, such as manganese peroxidase and laccase, catalyze similar non-specific breakage of covalent bonds in lignocellulose (Dong et al., 2014). Hydrolytic enzymes, unlike redox enzymes, recognize and catalyze the cleavage of

specific glycosidic bonds within lignocellulose to release metabolizable sugar molecules. Enzymes capable of degrading carbohydrates act synergistically to hydrolyze cellulose, which include endo-(1, 4)- β -glucanase (endocellulase), cello-biohydrolase (exocellulase) and β -glucosidases (Canam et al., 2013a). These enzymes recognize and act upon the glycosidic linkages, including endo-xylanases, endo- α -L-arabinase, endo-mannanase, β -galactosidase and corresponding β -glucosidases (ten Have & Teunissen, 2001).

Ligninolytic enzymes are composed mainly of laccase, lignin peroxidase and manganese peroxidase (Levin et al., 2008). Lignin peroxidase (also “ligninase”, EC 1.11.1.14) involves the oxidative cleavage of non-phenolic aromatic lignin moieties and similar compounds (Hermoso et al., 2004). Lignin peroxidases are strong oxidants that interact directly with non-phenolic lignin structures to cleave them, but cannot penetrate the small pores in sound lignocellulose. Manganese peroxidase (EC 1.11.1.13) is an enzyme secreted to aid lignin degradation, catalyzing the chemical reaction that oxidizes numerous phenolic compounds, especially syringyl (3, 5-dimethoxy-4-hydroxyphenyl) and vinyl side-chain substituted substrates in the presence of Mn^{2+} (Hermoso et al., 2004). Manganese-dependent peroxidases produce small, diffusible strong oxidants that can penetrate the substrate. Laccases (EC 1.10.3.2) are copper-containing oxidase enzymes that act on phenols and similar molecules, performing one-electron oxidation (Hermoso et al., 2004). Ferulic acid and p-coumaric acids that are esterified to hemicellulose sugars constitute another limitation to biodegradation of lignocellulosic walls and thus feruloyl esterase produced by white rot fungi is another key enzyme in the delignification process. Most feruloyl esterases have been shown to act synergistically

with cellulases, xylanases and pectinases to break down complex plant cell walls by cleaving covalent bonds between lignin and hemicellulose (Hermoso et al., 2004).

However, lignin peroxidase, manganese peroxidase, and laccase are not always present during fungal pretreatment of biomass. Enzymes are activated during fungal pretreatment based on the time of pretreatment, the type of biomass and the fungus used. In *C. subvermispora* pretreatment on corn stover conducted by Wan and Li (2010a), enzyme activity measurements did not detect lignin peroxidase. Manganese peroxidase and laccase were the two major lignin degradation enzymes detected and showed different activity during different pretreatment periods. Manganese peroxidase activity was 238 IU/ kg and 125 IU/ kg when the same white rot fungus *C. subvermispora* with the same pretreatment time, was acting upon on sugarcane bagasse and pine (*Pinus taeda*), respectively (Costa et al., 2005; Guerra et al., 2003).

2.5. Involvement of white rot fungi in biomass pretreatment

2.5.1. White rot fungi pretreatment individually or in combination with other microorganisms in biological pretreatment

Extensive research has discussed the structural changes, mass loss, lignin degradation of biomass and enhanced saccharification and fermentation after white rot fungi pretreatment. Taniguchi et al. (2005) applied four different white rot fungi *P. ostreatus*, *P. chrysosporium*, *Trametes versicolor* and *C. subvermispora* for pretreatment of rice straw. All of these fungi consumed cellulose while removing lignin in which *P. ostreatus* conserved the most cellulose while degrading lignin. Lee et al. (2007) evaluated the effects of biological pretreatment on the Japanese red pine (*Pinus densiflora*) after

exposure to three white rot fungi: *Ceriporia lacerata*, *Stereum hirsutum*, and *Polyporus brumalis*. Their results showed that total weight loss and chemical composition changes in Japanese red pine were well correlated with the ligninolytic enzyme activities related with lignin and cellulose degradation in these fungi. For *S. hirsutum* treated Japanese red pine chips, the sugar yield increased by 21 % compared to non-pretreated samples. Keller et al. (2003) reported that a saccharification yield of 36% was obtained from corn stover pretreated with *Cyathus stercoreus* for 29 days when hydrolysis was conducted at a cellulase loading of 60 FPU/g glucan for 136 h, which was about 4 times that of the untreated. A saccharification yield of 66.4% was obtained with fungus *Irpex lacteus* treated corn stover for 25 days when hydrolysis was conducted at a cellulase loading of 20 FPU/g solid for enzymatic hydrolysis (Xu et al., 2010).

However, excessive time of white rot fungi pretreatment can result in a decreased saccharification yield (saccharification means glucan conversion to glucose). Shrestha et al. (2008) used *P. ostreatus* to pretreat corn fiber. Pretreatment time beyond 2 weeks resulted in reduced saccharification yield compared to that of the control. Keller et al. (2003) reported that pretreatment of corn stover with *P. chrysosporium* for 29 days did not significantly increase saccharification yield compared to the control, probably due to an excessive pretreatment time. Even for a 14 day fungal pretreatment of cotton stalk by *P. chrysosporium*, no significant increase in cellulose conversion was observed for both submerged and solid state fungal-treated cotton stalks compared to untreated corn stalks (Shi et al., 2009).

Of all the white-rot basidiomycetes, *P. chrysosporium* is most often used as a model strain due to its high generation rates and complete ligninolytic enzyme complex

(Singh & Chen, 2008). However, the presence of lignin selective degradation and enhanced saccharification in *P. chrysosporium* is controversial. Bak et al. (2009) treated rice straw by *P. chrysosporium* under submerged conditions for 30 days and improved the ethanol yield by 2 times compared to that of untreated through simultaneous saccharification and fermentation. However, taking into consideration both considerable cellulose loss and low solid loading of submerged cultivation, *P. chrysosporium* was considered non-lignin selective and may not be effective for fungal pretreatment to improve the saccharification yield of biomass feedstocks (Wan & Li, 2012). The cellulose loss with prolonged pretreatment time may be explained by the effect of cellobiohydrolases for degrading crystalline cellulose. A genomic analysis on 33 basidiomycetes showed that white rot species possess multiple ligninolytic class II peroxidases (PODs) (PODs refers to lignin peroxidase and manganese peroxidase) and expanded suites of enzymes attacking crystalline cellulose (Riley et al., 2014).

White rot fungi pretreatments aforementioned were monoculture degradations on biomass, which usually led to a saccharification yield of around 30% to 70% (Wan, 2011). These yields are relatively low compared to that of successful physical and/or chemical pretreatments. Due to the powerful features of microbial consortia such as comprehensive enzymes, increased efficiency and stability relative to monoculture, symbiotic consortia that mimic the synergistic communities in natural environments were proposed for lignocellulosic biofuel production (Yao & Nokes, 2013; Zuroff & Curtis, 2012). Enhanced enzyme activities and production were observed in several studies. Gutierrez-Correa and Tengerdy (1998) cultivated *Trichoderma* and *Aspergillus* species together in solid state fermentation and got 35-45% increase in xylanase and 20-142%

increase in cellulase compared to single cultures of either partner. Stoilova and Krastanov (2008) performed *T. versicolor* and *Aspergillus niger* co-culture that synthesized 8.4 times more laccase than a monoculture of *T. versicolor*. Dwivedi et al. (2011) applied paired culturing of a *Penicillium oxalicum* mutant and *P. ostreatus* and obtained 58% and 33% higher levels of xylanase and laccase production, respectively. Previously isolated microbial consortia were capable of degrading 50-90% lignocellulose biomass, however, the interplay of species in these consortia was too complex for large scale industrial system design (Zuroff & Curtis, 2012).

Systematic searches for fungal diversity via high-throughput assessment have been done to discover new strains that could enhance biomass pretreatment, saccharification and fermentation. Shrestha et al. (2011) isolated nine fungi most frequently cultivated from miscanthus and found that four of the nice species caused miscanthus biomass loss of 12% or higher in four weeks. Berrin et al. (2012) isolated wood decaying fungi from forests and found the secretome of strain *T. gibbosa* showed higher activity on crystalline cellulose than that of commercial *T. reesei* cellulase cocktail. Also, *T. gibbosa* showed laccase activity, which *T. reesei* did not have. Their results showed that fungi convert lignocellulose through a multi enzyme process involving numerous carbohydrate-active enzymes (Cantarel et al., 2009) and oxidative enzymes.

2.5.2. White rot fungi combined with physical pretreatments

Traditional physical pretreatments are usually mechanical pretreatments, which have the function of reduction of particle size and cellulose crystallinity in order to

increase the specific surface area and reduce the degree of polymerization (Kumar et al., 2009). A combination of chipping, grinding or milling is an indispensable first step to pretreat biomass. Ultrasonic pretreatment has the advantages of less reaction time and high energy, which can degrade biomass in a very short period of time such as 30 min in the study of Yu et al. (2009). Ultrasonic pretreatment and subsequent white rot fungus pretreatment with *P. ostreatus* were performed to enhance enzymatic hydrolysis of rice hulls. In Yu et al. (2009) the combined pretreatments led to a significant increase of lignin degradation compared to either ultrasonic or white rot fungus pretreatment alone. After combined pretreatment with ultrasonic waves (250 W, 30 min) and *P. ostreatus* (18 days), glucose yield was 4.2 times higher than that of sole fungal pretreatment (18 days). Ligninase analyses and SEM observations indicated that the enhancement of efficiency could have resulted from structure disruption of the rice hulls during the ultrasound pretreatment step (Yu et al., 2009). Yachmenev et al. (2009) indicated that higher enzymatic hydrolysis yields after ultrasound pretreatment could be explained by cavitation effects of the ultrasonic field. The effects of ultrasound pretreatment include mechanical impacts produced by the collapse of cavitation bubbles and the enhanced transportation of enzyme macromolecules toward the substrate surface (Yachmenev et al., 2004). Another study applied low intensity ultrasound to increase bleaching effect on cotton by laccase (Basto et al., 2007).

2.5.3. White rot fungi combined with chemical pretreatments

Yang et al. (2013) used *Trametes velutina* and subsequent alkali pretreatment on triploid poplar. Their results showed that the digestibility of cellulose was improved 19.5% with a dry mass loss of 53.4% after a 16 week fungal pretreatment compared to an

untreated sample. White rot fungi enhancement of the efficiency of chemical pretreatment was confirmed by Yu et al. (2010b). Yu et al. (2010b) applied NaOH and H₂O₂ treatment to digest corn straw at room temperature for 24 h after pretreating corn straw with *Echinodontium taxodii* for 15 days. The reducing sugar yield was increased by 50.7% compared to NaOH and H₂O₂ treatment alone. However, the adsorbed cellulase decreased by 24.8 % compared to NaOH and 3% H₂O₂ pretreatment alone after 72 h of enzymatic hydrolysis, which may result from unproductive adsorption of cellulase on lignin that could impede desorption of cellulase. Similar results were also found by Gregg and Saddler (1996). Thakur et al. (2013) reported white rot fungi and subsequent mild acid or dilute alkali pretreatment on wheat straw and banana stems. The process resulted in lignin removal of 49%, which was comparatively higher than that for cellulose, 12%, and for hemicellulose, 21%, with a 49% dry matter loss. Wang et al. (2013) pretreated poplar using white rot fungus *T. velutina* combined with chlorite treatment or dilute acid treatment, separately. Subsequent enzymatic hydrolysis results showed that lignin rather than hemicellulose was dominant in impeding enzymatic hydrolysis. López-Abelairas et al. (2013) used *I. lacteus* and subsequent mild alkali pretreatment on wheat straw. The ethanol yield was 74%, which is similar to that for biomass pretreated with steam explosion.

2.5.4. White rot fungi combined with physiochemical pretreatments

White rot fungus *I. lacteus* and subsequent catalytic fast pyrolysis on corn stover showed that aromatic hydrocarbons yield increased from 10.03% to 11.49% and the coke yield decreased from 14.29% to 11.93% (Yu et al., 2013). White rot fungi pretreatment on corn stover reduced the reaction temperature and the gas contamination (SO_x) of

pyrolysis, making pyrolysis easier by making the structure less compact (Yang et al., 2010). Baba et al. (2011) conducted white rot fungus *Phellinus* sp. and ethanolysis pretreatment on cedar wood. The saccharification yield reached 41% compared to 10% of only ethanolysis pretreatment.

There are also studies applying physiochemical pretreatment prior to fungal pretreatment. Wan and Li (2011) performed liquid hot water extraction and fungal pretreatment with *C. subvermispota* to pretreat different biomass materials. Hot water extraction (85 °C for 10 min) partially removed water soluble extractives and subsequently improved fungal degradation of wheat straw, while it had little or no effect on the fungal degradation of corn stover and soybean straw. However, corn stover was effectively degraded by fungal pretreatment alone. Their results indicated that a mild hot water extraction or liquid water extraction pretreatment worked synergistically with fungal degradation for some recalcitrant feedstocks. However, exhaustive hot water extraction substantially improved the fungal degradation of wheat straw, but not of soybean straw, which may result from their different recalcitrance (Wan & Li, 2011).

2.6. Effect of white rot fungi pretreatment on biomass structural changes

Lignin and hemicellulose content, cellulose crystallinity, degree of polymerization and specific surface area are believed to be the factors affecting enzymatic hydrolysis (Membrillo et al., 2008). Characterization of structural changes contributes to a better understanding of how white rot fungi degrade lignin as well as consume holocellulose. Characterization tools include Fourier transform infrared spectroscopy (FTIR), ¹³carbon-nuclear magnetic resonance analysis (¹³C-NMR), X-ray diffraction (XRD), scanning

electrical microscopy (SEM) analysis and Brunauer, Emmet and Teller (BET) surface area analysis.

Corn stover was pretreated by white rot fungi and then pyrolyzed by Yang et al. (2010). In FTIR spectra the peaks indicative of lignin decreased in fungi pretreated samples. The increment in intensity ratio illustrated cellulose crystallinity decreasing in fungi pretreated samples. FTIR spectra changes indicated that fungal pretreatment could destroy lignin structure and decrease the crystallinity of cellulose in corn stover.

Yang et al. (2013) conducted treatments on triploid poplar with *T. velutina* and alkaline fractionation. Solid-state NMR spectra demonstrated that *T. velutina* depolymerized carbohydrates through an attack at C1 and C4 indicative of cellulose and also have a preference to cleave the C₆-CH₂OH group to open the cellulose crystalline structure to an amorphous structure. The total specific area measured by BET showed specific area increased from 1.7 m²/g (control) to 10.6 m²/g (8 weeks treated sample), however, prolonged incubation to 16 weeks did not further increase the BET area. Correspondingly, the cellulose conversion to glucose did not further increase.

XRD is used to characterize the crystallinity of cellulose. Bak et al. (2009) found that crystallinity increased from 54% to 60% after a 15 day *P. chrysosporium* pretreatment on rice straw, which indicates the cellulose portion was relatively more exposed than untreated straw.

The productive adsorption of cellulase on cellulose is the first step in the cellulose hydrolysis reaction and is a prerequisite for efficient cellulose hydrolysis (Xu et al., 2010; Yu et al., 2010a; Yu et al., 2010b). Adsorption of cellulase to cellulose is closely related

to the accessible cellulose surface area (Rollin et al., 2011). In the work of Yang et al. (2013), the total specific surface area changes measured by BET showed that alkaline fractionation after white rot fungus pretreatment clearly increased the BET surface area from 1.7 to 4.8 m²/g. Similar phenomena were also observed when corn stover was biodegraded with *I. lacteus* and corn straw was treated with the combination of white-rot fungi and alkaline/oxidative pretreatments. However, prolonging incubation time to 16 weeks did not further increase the BET surface area of substrate (Xu et al., 2010; Yu et al., 2010a; Yu et al., 2010b).

The structural changes described above are in line with the compositional changes. Composition analysis showed that during 30 days biodegradation, white-rot fungi mainly degraded hemicellulose (hemicellulose loss 24.4-34.9%), which is embedded in the lignin-polysaccharide matrix of biomass and plays a significant role in the structure of corn stover cell walls by both covalent and non-covalent associations. By degrading hemicellulose, the structural integrity and complexity of corn stover were disrupted, making the lignocellulose easier to pyrolyze (Yang et al., 2013).

Taniguchi et al. (2005) observed morphological modifications that were induced by pretreatment with *P. ostreatus* by using scanning electron microscopy (SEM) to obtain insight into the structural modification in rice straw. *P. ostreatus* pretreatment induced structural loosening of cells with a simultaneous increase in porosity. In addition, partial exposure of cellulose networks was observed in 60 day pretreated rice straw, which supports the results that half of the Klason lignin (acid insoluble lignin) was removed. The SEM observations showed that the pretreatment with *P. ostreatus* resulted in an

increase in susceptibility of rice straw to enzymatic hydrolysis due to partial degradation of lignin that is responsible for preventing penetration of cellulase in the rice straw.

In conclusion, white rot fungi show strong ability to degrade lignin and to disrupt rigid biomass structure, thus enhancing subsequent saccharification and fermentation. However, compared to successful physio-chemical pretreatment which resulted in an ethanol yield of 70% to 90%, the ethanol yield of 10% to 60% resulted from white rot fungal pretreatment is still low. To obtain an efficient pretreatment, white rot fungal pretreatment is suggested to be combined with physio-chemical pretreatment to obtain a better pretreatment than white rot fungal pretreatment or physio-chemical pretreatment alone. In previous lab scale studies, *P. ostreatus* was capable of pretreating biomass to enhance biofuel production during biomass storage. However, there are no published studies regarding the feasibility of *P. ostreatus* pretreatment being incorporated into large switchgrass bale storage. There have only been some chemical pretreatment studies reported on biomass during biomass storage. Given the advantages of *P. ostreatus* storage over chemical pretreatment such as mild pretreatment conditions and no washing step needed after fungal pretreatment, study of large scale *P. ostreatus* pretreatment on switchgrass during storage is necessary to develop an efficient fungal pretreatment.

2.7. References

- Arora, D.S., Sharma, R.K. 2010. Ligninolytic fungal laccases and their biotechnological applications. *Applied Biochemistry and Biotechnology*, **160**(6), 1760-1788.
- Asgher, M., Asad, M., Legge, R. 2006. Enhanced lignin peroxidase synthesis by *Phanerochaete chrysosporium* in solid state bioprocessing of a lignocellulosic substrate. *World Journal of Microbiology and Biotechnology*, **22**(5), 449-453.
- Asgher, M., Azim, N., Bhatti, H.N. 2009. Decolorization of practical textile industry effluents by white rot fungus *Coriolus versicolor* IBL-04. *Biochemical Engineering Journal*, **47**(1), 61-65.
- Baba, Y., Tanabe, T., Shirai, N., Watanabe, T., Honda, Y., Watanabe, T. 2011. Pretreatment of Japanese cedar wood by white rot fungi and ethanolysis for bioethanol production. *Biomass and Bioenergy*, **35**(1), 320-324.
- Bak, J.S., Ko, J.K., Choi, I.-G., Park, Y.-C., Seo, J.-H., Kim, K.H. 2009. Fungal pretreatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw. *Biotechnology and Bioengineering*, **104**(3), 471-482.
- Bao, W., Fukushima, Y., Jensen Jr, K.A., Moen, M.A., Hammel, K.E. 1994. Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase. *FEBS Letters*, **354**(3), 297-300.
- Barrios-González, J. 2012. Solid-state fermentation: physiology of solid medium, its molecular basis and applications. *Process Biochemistry*, **47**(2), 175-185.
- Basto, C., Tzanov, T., Cavaco-Paulo, A. 2007. Combined ultrasound-laccase assisted bleaching of cotton. *Ultrasonics Sonochemistry*, **14**(3), 350-354.
- Berrin, J.-G., Navarro, D., Couturier, M., Olivé, C., Grisel, S., Haon, M., Taussac, S., Lechat, C., Courtecuisse, R., Favel, A. 2012. Exploring natural fungal biodiversity from tropical and temperate forests to improve biomass conversion. *Applied and Environmental Microbiology*, **81**(14), 6483-6490.
- Canam, T., Dumonceaux, T.J., Record, E., Li, Y. 2013a. White-rot fungi: the key to sustainable biofuel production? *Biofuels*, **4**(3), 247-250.
- Canam, T., Town, J., Iroba, K., Tabil, L., Dumonceaux, T. 2013b. Pretreatment of lignocellulosic biomass using microorganisms: Approaches, advantages, and limitations. in: *Sustainable Degradation of Lignocellulosic Biomass—Techniques, Applications and Commercialisation*, (Eds.) A.K. Chandel, S. Silvério da Silva, InTech. Rijeka, Croatia, pp. 181-206.

- Cantarel, B.L., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V., Henrissat, B. 2009. The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Research*, **37**(1), 233-238.
- Chen, Y., Sharma-Shivappa, R.R., Chen, C. 2007. Ensiling agricultural residues for bioethanol production. *Applied Biochemistry and Biotechnology*, **143**(1), 80-92.
- Cook, C., Francocci, F., Cervone, F., Bellincampi, D., Bolwell, P.G., Ferrari, S., Devoto, A. 2014. Combination of pretreatment with white rot fungi and modification of primary and secondary cell walls improves saccharification. *BioEnergy Research*, 1-12.
- Costa, S., Gonçalves, A., Esposito, E. 2005. Ceriporiopsis subvermispora used in delignification of sugarcane bagasse prior to soda/anthraquinone pulping. *Applied Biochemistry and Biotechnology*, **122**(1-3), 695-706.
- Digman, M., Shinnars, K., Muck, R., Dien, B. 2010. Pilot-scale on-farm pretreatment of perennial grasses with dilute acid and alkali for fuel ethanol production. *Transactions of the ASABE*, **53**, 1007-1014.
- Digman, M.F., Shinnars, K.J., Dien, B.S., Hatfield, R., Li, X., Muck, R., Weimert, P. 2007. On-farm pretreatment technologies for improving enzymatic degradability of cellulose and hemicellulose present in perennial grass. *ASABE Annual International Meeting*, Minneapolis, MN.
- Dinis, M.J., Bezerra, R.M.F., Nunes, F., Dias, A.A., Guedes, C.V., Ferreira, L.M.M., Cone, J.W., Marques, G.S.M., Barros, A.R.N., Rodrigues, M.A.M. 2009. Modification of wheat straw lignin by solid state fermentation with white-rot fungi. *Bioresource Technology*, **100**(20), 4829-4835.
- Djioleu, A.C., Sverzut, C.B., Martin, E.M., Childress, E., Johnson, C., West, C.P., Carrier, D.J. 2014. Effects of harvest and storage of switchgrass on the recovery of carbohydrates during dilute acid pretreatment and enzymatic hydrolysis. *Forage and Grazinglands*, **12**(1), 1-6.
- Dong, Y.-C., Dai, Y.-N., Xu, T.-Y., Cai, J., Chen, Q.-H. 2014. Biodegradation of chestnut shell and lignin-modifying enzymes production by the white-rot fungi *Dichomitus squalens*, *Phlebia radiata*. *Bioprocess and Biosystems Engineering*, **37**(5), 1-10.
- Dwivedi, P., Vivekanand, V., Pareek, N., Sharma, A., Singh, R.P. 2011. Co-cultivation of mutant *Penicillium oxalicum* SAU E-3.510 and *Pleurotus ostreatus* for simultaneous biosynthesis of xylanase and laccase under solid-state fermentation. *New Biotechnology*, **28**(6), 616-626.

- Eriksson, K.E.L., Blanchette, R.A., Ander, P. 1990. *Microbial and Enzymatic Degradation of Wood and Wood Components*. Springer-Verlag, New York.
- Fernández-Fueyo, E., Castanera, R., Ruiz-Dueñas, F.J., López-Lucendo, M.F., Ramírez, L., Pisabarro, A.G., Martínez, A.T. 2014. Ligninolytic peroxidase gene expression by *Pleurotus ostreatus*: differential regulation in lignocellulose medium and effect of temperature and pH. *Fungal Genetics and Biology*, **72**, 150-161.
- Ferraz, A., Córdova, A.M., Machuca, A. 2003. Wood biodegradation and enzyme production by *Ceriporiopsis subvermispota* during solid-state fermentation of *Eucalyptus grandis*. *Enzyme and Microbial Technology*, **32**(1), 59-65.
- Glassey, J., Ward, A.C. 2015. Solid state fermentation. in: *Diversity, Dynamics and Functional Role of Actinomycetes on European Smear Ripened Cheeses*, (Eds.) N. Bora, C. Dodd, N. Desmases, Springer. New York, pp. 217-225.
- Gregg, D.J., Saddler, J.N. 1996. Factors affecting cellulose hydrolysis and the potential of enzyme recycle to enhance the efficiency of an integrated wood to ethanol process. *Biotechnology and Bioengineering*, **51**(4), 375-383.
- Grenet, E., Bernalier, A., Jamot, J., Fonty, G. 1993. Degradation of untreated and anhydrous ammonia-treated wheat straw by two strains of rumen anaerobic fungi. *Annales De Zootechnie*. pp. 180-180.
- Guerra, A., Mendonça, R., Ferraz, A. 2003. Molecular weight distribution of wood components extracted from *Pinus taeda* biotreated by *Ceriporiopsis subvermispota*. *Enzyme and Microbial Technology*, **33**(1), 12-18.
- Gutierrez-Correa, M., Tengerdy, R.P. 1998. Xylanase production by fungal mixed culture solid substrate fermentation on sugar cane bagasse. *Biotechnology Letters*, **20**(1), 45-47.
- Haitjema, C.H., Solomon, K.V., Henske, J.K., Theodorou, M.K., O'Malley, M.A. 2014. Anaerobic gut fungi: Advances in isolation, culture, and cellulolytic enzyme discovery for biofuel production. *Biotechnology and Bioengineering*, **111**(8), 1471-1482.
- Harrison, J., Fransen, S. 1991. Silage management in North America. in: *Field Guide for Hay and Silage Management in North America.*, (Eds.) K.K. Bolson, J.E. Baylor, M.E. McCullough, National Feed Ingredients Association. Arlington, VA.
- Hermoso, J.A., Sanz-Aparicio, J., Molina, R., Juge, N., González, R., Faulds, C.B. 2004. The crystal structure of feruloyl esterase A from *Aspergillus niger* suggests evolutive functional convergence in feruloyl esterase family. *Journal of Molecular Biology*, **338**(3), 495-506.

- Jönsson, L.J., Palmqvist, E., Nilvebrant, N.O., Hahn-Hägerdal, B. 1998. Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor*. *Applied Microbiology and Biotechnology*, **49**(6), 691-697.
- Kapich, A.N., Jensen, K.A., Hammel, K.E. 1999. Peroxyl radicals are potential agents of lignin biodegradation. *FEBS Letters*, **461**(1-2), 115-119.
- Keller, F.A., Hamilton, J.E., Nguyen, Q.A. 2003. Microbial Pretreatment of Biomass. *Applied Biochemistry and Biotechnology*, **105**(1), 27-41.
- Knežević, A., Stajić, M., Vukojević, J., Milovanović, I. 2014. The effect of trace elements on wheat straw degradation by *Trametes gibbosa*. *International Biodeterioration & Biodegradation*, **96**, 152-156.
- Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & Engineering Chemistry Research*, **48**(8), 3713-3729.
- Lee, J., Gwak, K., Park, J., Park, M., Choi, D., Kwon, M., Choi, I. 2007. Biological pretreatment of softwood *Pinus densiflora* by three white rot fungi. *Journal of Microbiology*, **45**(6), 485.
- Levin, L., Herrmann, C., Papinutti, V.L. 2008. Optimization of lignocellulolytic enzyme production by the white-rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology. *Biochemical Engineering Journal*, **39**(1), 207-214.
- Liggenstoffer, A.S., Youssef, N.H., Wilkins, M.R., Elshahed, M.S. 2014. Evaluating the utility of hydrothermolysis pretreatment approaches in enhancing lignocellulosic biomass degradation by the anaerobic fungus *Orpinomyces* sp. strain C1A. *Journal of Microbiological Methods*, **104**(0), 43-48.
- López-Abelairas, M., Pallín, M.Á., Salvachúa, D., Lú-Chau, T., Martínez, M., Lema, J. 2013. Optimisation of the biological pretreatment of wheat straw with white-rot fungi for ethanol production. *Bioprocess and Biosystems Engineering*, **36**(9), 1251-1260.
- Mäkelä, M.R., Lundell, T., Hatakka, A., Hildén, K. 2013. Effect of copper, nutrient nitrogen, and wood-supplement on the production of lignin-modifying enzymes by the white-rot fungus *Phlebia radiata*. *Fungal Biology*, **117**(1), 62-70.
- Membrillo, I., Sánchez, C., Meneses, M., Favela, E., Loera, O. 2008. Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by *Pleurotus ostreatus* strains. *Bioresource Technology*, **99**(16), 7842-7847.

- Miles, P.G., Chang, S.-T. 2004. *Mushrooms: Cultivation, Nutritional Value, Medicinal Effect, and Environmental Impact*. CRC press, Boca Raton, FL.
- Pandey, A. 2003. Solid-state fermentation. *Biochemical Engineering Journal*, **13**(2–3), 81-84.
- Parenti, A., Muguerza, E., Redin Iroz, A., Omarini, A., Conde, E., Alfaro, M., Castanera, R., Santoyo, F., Ramírez, L., Pisabarro, A.G. 2013. Induction of laccase activity in the white rot fungus *Pleurotus ostreatus* using water polluted with wheat straw extracts. *Bioresource Technology*, **133**, 142-149.
- Pitt, R., Muck, R., Leibensperger, R. 1985. A quantitative model of the ensilage process in lactate silages. *Grass and Forage Science*, **40**(3), 279-303.
- Rajaratnam, S., Bano, Z., Miles, P.G. 1987. *Pleurotus* mushrooms. Part I A. Morphology, life cycle, taxonomy, breeding, and cultivation. *Critical Reviews in Food Science & Nutrition*, **26**(2), 157-223.
- Rezaeian, M., Beakes, G.W., Chaudhry, A.S. 2005. Relative fibrolytic activities of anaerobic rumen fungi on untreated and sodium hydroxide treated barley straw in vitro culture. *Anaerobe*, **11**(3), 163-175.
- Richard, T., Proulx, S., Moore, K., Shouse, S. 2001. Ensilage technology for biomass pre-treatment and storage. *ASAE Annual International Meeting, Sacramento, CA*.
- Riley, R., Salamov, A.A., Brown, D.W., Nagy, L.G., Floudas, D., Held, B.W., Levasseur, A., Lombard, V., Morin, E., Otilar, R., Lindquist, E.A., Sun, H., LaButti, K.M., Schmutz, J., Jabbour, D., Luo, H., Baker, S.E., Pisabarro, A.G., Walton, J.D., Blanchette, R.A., Henrissat, B., Martin, F., Cullen, D., Hibbett, D.S., Grigoriev, I.V. 2014. Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi. *Proceedings of the National Academy of Sciences*, **111**(27), 9923-9928.
- Rollin, J.A., Zhu, Z., Sathitsuksanoh, N., Zhang, Y.H.P. 2011. Increasing cellulose accessibility is more important than removing lignin: A comparison of cellulose solvent-based lignocellulose fractionation and soaking in aqueous ammonia. *Biotechnology and Bioengineering*, **108**(1), 22-30.
- Royse, D.J. 2003. Cultivation of oyster mushroom. College of Agricultural Sciences, Pennsylvania State University, University Park, pp. 1-12.
- Sanderson, M.A., Egg, R.P., Wiseloge, A.E. 1997. Biomass losses during harvest and storage of switchgrass. *Biomass and Bioenergy*, **12**(2), 107-114.

- Segato, F., Damásio, A.R., de Lucas, R.C., Squina, F.M., Prade, R.A. 2014. Genomics review of holocellulose deconstruction by *Aspergilli*. *Microbiology and Molecular Biology Reviews*, **78**(4), 588-613.
- Shi, J., Sharma-Shivappa, R.R., Chinn, M., Howell, N. 2009. Effect of microbial pretreatment on enzymatic hydrolysis and fermentation of cotton stalks for ethanol production. *Biomass and Bioenergy*, **33**(1), 88-96.
- Shrestha, P., Rasmussen, M., Khanal, S.K., Pometto, A.L., van Leeuwen, J. 2008. Solid-substrate fermentation of corn fiber by *Phanerochaete chrysosporium* and subsequent fermentation of hydrolysate into ethanol. *Journal of Agricultural and Food Chemistry*, **56**(11), 3918-3924.
- Shrestha, P., Szaro, T.M., Bruns, T.D., Taylor, J.W. 2011. Systematic search for cultivatable fungi that best deconstruct cell walls of Miscanthus and sugarcane in the field. *Applied and Environmental Microbiology*, AEM. 02996-10.
- Silverstein, R.A., Chen, Y., Sharma-Shivappa, R.R., Boyette, M.D., Osborne, J. 2007. A comparison of chemical pretreatment methods for improving saccharification of cotton stalks. *Bioresource Technology*, **98**(16), 3000-3011.
- Singh, D., Chen, S. 2008. The white-rot fungus *Phanerochaete chrysosporium*: conditions for the production of lignin-degrading enzymes. *Applied Microbiology and Biotechnology*, **81**(3), 399-417.
- Stoilova, I., Krastanov, A. 2008. Overproduction of laccase and pectinase by microbial associations in solid substrate fermentation. *Applied Biochemistry and Biotechnology*, **149**(1), 45-51.
- Taniguchi, M., Suzuki, H., Watanabe, D., Sakai, K., Hoshino, K., Tanaka, T. 2005. Evaluation of pretreatment with *Pleurotus ostreatus* for enzymatic hydrolysis of rice straw. *Journal of Bioscience and Bioengineering*, **100**(6), 637-643.
- ten Have, R., Teunissen, P.J. 2001. Oxidative mechanisms involved in lignin degradation by white-rot fungi. *Chemical Reviews*, **101**(11), 3397-3414.
- Thakur, S., Shrivastava, B., Ingale, S., Kuhad, R., Gupte, A. 2013. Degradation and selective ligninolysis of wheat straw and banana stem for an efficient bioethanol production using fungal and chemical pretreatment. *3 Biotech*, **3**(5), 365-372.
- Wan, C. 2011. Microbial pretreatment of lignocellulosic biomass with *Ceriporiopsis subvermispora* for enzymatic hydrolysis and ethanol production, PhD Diss., The Ohio State University.

- Wan, C., Li, Y. 2011. Effect of hot water extraction and liquid hot water pretreatment on the fungal degradation of biomass feedstocks. *Bioresource Technology*, **102**(20), 9788-9793.
- Wan, C., Li, Y. 2012. Fungal pretreatment of lignocellulosic biomass. *Biotechnology Advances*, **30**(6), 1447-1457.
- Wan, C., Li, Y. 2010a. Microbial delignification of corn stover by *Ceriporiopsis subvermispota* for improving cellulose digestibility. *Enzyme and Microbial Technology*, **47**(1-2), 31-36.
- Wan, C., Li, Y. 2010b. Microbial pretreatment of corn stover with *Ceriporiopsis subvermispota* for enzymatic hydrolysis and ethanol production. *Bioresource Technology*, **101**(16), 6398-6403.
- Wang, W., Yuan, T., Cui, B., Dai, Y. 2013. Investigating lignin and hemicellulose in white rot fungus-pretreated wood that affect enzymatic hydrolysis. *Bioresource Technology*, **134**(0), 381-385.
- Xu, C., Ma, F., Zhang, X., Chen, S. 2010. Biological Pretreatment of Corn Stover by *Irpex lacteus* for Enzymatic Hydrolysis. *Journal of Agricultural and Food Chemistry*, **58**(20), 10893-10898.
- Yachmenev, V., Condon, B., Klasson, T., Lambert, A. 2009. Acceleration of the enzymatic hydrolysis of corn stover and sugar cane bagasse celluloses by low intensity uniform ultrasound. *Journal of Biobased Materials and Bioenergy*, **3**(1), 25-31.
- Yachmenev, V.G., Blanchard, E.J., Lambert, A.H. 2004. Use of ultrasonic energy for intensification of the bio-preparation of greige cotton. *Ultrasonics*, **42**(1), 87-91.
- Yang, H., Wang, K., Wang, W., Sun, R.-C. 2013. Improved bioconversion of poplar by synergistic treatments with white-rot fungus *Trametes velutina* D10149 pretreatment and alkaline fractionation. *Bioresource Technology*, **130**(0), 578-583.
- Yang, X., Zeng, Y., Ma, F., Zhang, X., Yu, H. 2010. Effect of biopretreatment on thermogravimetric and chemical characteristics of corn stover by different white-rot fungi. *Bioresource Technology*, **101**(14), 5475-5479.
- Yao, W., Nokes, S.E. 2013. The use of co culturing in solid substrate cultivation and possible solutions to scientific challenges. *Biofuels, Bioproducts and Biorefining*, **7**(4), 361-372.
- Yu, H., Du, W., Zhang, J., Ma, F., Zhang, X., Zhong, W. 2010a. Fungal treatment of cornstalks enhances the delignification and xylan loss during mild alkaline

pretreatment and enzymatic digestibility of glucan. *Bioresource Technology*, **101**(17), 6728-6734.

Yu, H., Zhang, X., Song, L., Ke, J., Xu, C., Du, W., Zhang, J. 2010b. Evaluation of white-rot fungi-assisted alkaline/oxidative pretreatment of corn straw undergoing enzymatic hydrolysis by cellulase. *Journal of Bioscience and Bioengineering*, **110**(6), 660-664.

Yu, J., Zhang, J., He, J., Liu, Z., Yu, Z. 2009. Combinations of mild physical or chemical pretreatment with biological pretreatment for enzymatic hydrolysis of rice hull. *Bioresource Technology*, **100**(2), 903-908.

Yu, Y., Zeng, Y., Zuo, J., Ma, F., Yang, X., Zhang, X., Wang, Y. 2013. Improving the conversion of biomass in catalytic fast pyrolysis via white-rot fungal pretreatment. *Bioresource Technology*, **134**(0), 198-203.

Zuroff, T.R., Curtis, W.R. 2012. Developing symbiotic consortia for lignocellulosic biofuel production. *Applied Microbiology and Biotechnology*, **93**(4), 1423-1435.

CHAPTER III

3. Research objective and summary of studies

The overall objective of the project was to develop fungal pretreatment protocols using *Pleurotus ostreatus* during switchgrass storage. Two specific studies: an on farm study and a lab scale study were conducted.

- 1) Large scale study (Chapter 4). White rot fungus pretreatment with *Pleurotus ostreatus* was conducted on large switchgrass square or round bales in a natural environment and hydrothermolysis pretreatment on fungal pretreated biomass were done. Commercial *P. ostreatus* spawn was used as the inoculum. Moisture and fungal colonization in square or round bales were studied to see if square or round bales could be used as candidates for fungal pretreatment. The effect of fungal pretreatment during storage on biomass composition change was studied to determine if fungal pretreatment achieved delignification. After hydrothermolysis, composition of pretreated switchgrass and inhibitors in prehydrolyzate were analyzed to ascertain if the severity of hydrothermolysis can be lowered. Enzymatic hydrolysis of pretreated biomass was done to see if fungal pretreatment facilitated higher glucose yield.
- 2) Lab scale study (Chapter 5). Lab scale *P. ostreatus* pretreatment in a controlled environment was conducted. *P. ostreatus* mycelial suspension was used as the inoculum. The effects of inoculum loading and substrate moisture content on

switchgrass delignification and ethanol production through simultaneous saccharification and fermentation were studied.

CHAPTER IV

4. Oyster mushroom (*Pleurotus ostreatus*) pretreatment on large switchgrass bales in a natural storage environment

4.1. Abstract

Biomass storage is an important factor affecting the feedstock supply chain for commercial biofuel production. Application of fungal pretreatment on biomass during storage could utilize the long storage time and degrade biomass to facilitate subsequent hydrolysis. In a two year storage study, oyster mushroom (*Pleurotus ostreatus*) spawn was applied to large square and round switchgrass bales to conduct fungal pretreatment in a natural storage environment. *P. ostreatus* was applied in December and switchgrass bales were stored for nine months in an open, unprotected field. Samples were taken at three months, five months, seven months and nine months after fungus applied. Samples were subjected to hydrothermal pretreatment and enzymatic hydrolysis. Results for square bales showed that controls had from 1.8% to 3.2% less lignin than that of fungal treated bales, which may be due to moisture not homogenously distributing through the bale and low temperature (below 10 °C) for the first three months. There were no clear trends for composition changes and glucose yields of hydrothermolysis treated samples. Results of round bales showed round bales could not retain rain, which resulted in a moisture content of 6% to 11%, thus may have led to no fungus grew in the treated bales.

Glucan, xylan and lignin content were similar throughout storage time. After hydrothermolysis, glucan, xylan and lignin contents were similar for all samples. Based on these findings, *P. ostreatus* pretreatment on unprotected large square or round switchgrass bales in a natural environment did not enhance subsequent enzymatic hydrolysis. For large scale *P. ostreatus* pretreatment, system control is necessary to realize homogenous fungus colonization throughout the bales.

4.2. Introduction

Lignocellulosic biomass as feedstock for ethanol production has been the subject of considerable interest. Switchgrass is a perennial plant native to the United States, and it grows widely in temperate regions. Due to its cold tolerance, drought resistance and ability to grow on marginal lands, switchgrass is regarded as a sustainable bioenergy crop (Kumar & Sokhansanj, 2007; Sanderson et al., 1996; Schmer et al., 2008; Yuan et al., 2008). Regarding environmental effects, switchgrass ethanol can provide greater greenhouse gas reduction and less agrichemical pollution than corn grain ethanol or soybean biodiesel (Kumar & Sokhansanj, 2007; Tilman et al., 2006).

Given the net energy and carbon dioxide balance, in temperate regions, biofuel production from lignocellulosic biomass represents the best choice among different biomass feedstocks if key technical hurdles can be scaled (Tilman et al., 2006; Yuan et al., 2008). In the biomass feedstocks supply chain, logistics of harvesting, storing and transporting of biomass to supply cost-competitive biorefineries is a great challenge (Digman et al., 2010). Biomass storage facilities stockpile massive amounts of material to ensure continuous operation and account for a large fraction of the cost of biomass energy generation (Cheng, 2010; Rentizelas et al., 2009). Biomass storage methods for forage are similar to biomass storage methods for biofuel production. Although the applications are different, the fundamental requirements are similar, and the infrastructure and facilities for forage storage could be adapted to biomass storage for biofuel production. Previously, a few studies on switchgrass storage mainly focused on improving forage protein content. Sanderson et al. (1997) conducted six months storage of switchgrass under outside unprotected conditions and got a dry matter loss of 13% of

the original weight. The effect of different storage methods on biomass saccharification and fermentation have been the subject of a few studies. Lab scale *Ceriporiopsis subvermispota* pretreatment on corn stover significantly enhanced the enzymatic degradability by 2–3-fold after 90-d wet storage (Cui et al., 2012). At large scale, Djioleu et al. (2014) stored switchgrass for 65 days in the field and in a protected barn prior to dilute acid pretreatment and enzymatic hydrolysis. Their results showed that barn storage led to greater preservation of fermentable sugars and reduced production of fermentation inhibitors compared to unprotected field storage. Liu et al. (2013) conducted 3 month wet storage and steam explosion pretreatment on corn stover and got a glucose yield of 87% after enzymatic hydrolysis of pretreated sample.

It may be worthwhile to apply some pretreatments during biomass storage to achieve positive effects on biofuel production. Pretreatments that added organic acids, salts, and/or ammonia to biomass to improve forage digestibility and inhibit unwanted microorganisms development have been used (Koegel et al., 1985; Lalman, 2002; Ward & Ward, 1987). Digman et al. (2007) applied chemicals (alkali, acid and oxidizing agents) to switchgrass during storage. Their results showed that conversion of cellulose to ethanol was 12% higher for acid pretreated switchgrass and 13% higher for lime pretreated switchgrass than that of controls. However, there are safety issues for on-farm chemicals application, especially for corrosive chemicals such as sulfuric acid.

Biological pretreatment happening during on-farm storage is mild and could utilize storage time since biological pretreatments require longer incubation time than physiochemical treatments. Ensilaging has been widely used for forage preservation and improvement of forage digestibility. Richard et al. (2001) did bench scale ensilage on

corn stover for 8 weeks and found a slight degradation of hemicellulose and a relative accumulation of cellulose and lignin. The effect of ensilaging corn stover on the saccharification and fermentation to ethanol was not investigated in their study (Richard et al., 2001). The effect of ensilaging biomass to improve sugar yield is limited. Chen et al. (2007) observed 5% to 10% sugar yield increase for ensilaged agricultural residues subjected to enzymatic hydrolysis compared to untreated residues. Large scale ensilage processes use enclosed systems (Harrison & Fransen, 1991), which increase process complexity. Also, the microorganisms working during ensilaging consume mainly carbohydrates, which are the main substrate for ethanol production. Therefore, ensilaging has limited pretreatment efficiency when applying to biomass storage for biofuel production.

Current industrial harvest and storage practices predominately use unprotected dry materials to reduce transportation and labor costs (Richard et al., 2001). Applying aerobic microorganisms will not require enclosed systems. The organisms predominantly responsible for lignocellulose degradation in nature are fungi, and the most rapid degraders in this group are basidiomycetes (Rabinovich et al., 2004; ten Have & Teunissen, 2001). White rot basidiomycete fungi are among the best lignin degraders (ten Have & Teunissen, 2001). The multiple enzyme systems (ligninolytic enzyme system and hydrolytic enzyme system) of white rot fungi and their production of hydrogen peroxide and hydroxyl radicals have been found to effectively unpack polysaccharide components from layers of lignin (Balan et al., 2008; Leonowicz et al., 1999). There have been many publications and industrial scale processes concerning white rot fungi application for bioremediation and biodegradation. In terms of energy cost, introducing lignocellulose

degrading enzymes to biopulping and biobleaching has allowed considerable electrical power savings (Pérez et al., 2002). Laccase, one of the ligninolytic enzymes white rot fungi secrete, has been widely used in industrial processes that require lignin bleaching (Riva, 2006). Wan and Li (2010) achieved 45% higher glucose yield for corn stover samples subjected to a 35 day white rot *Ceriporiopsis subvermispora* fungal pretreatment than untreated samples. Shi et al. (2009) investigated *Phanerochaete chrysosporium* solid state cultivation (14 days) pretreatment on corn stalks and proposed that fungal pretreatment can extensively reduce the severity of chemical pretreatments.

Pleurotus ostreatus, the most commercially important strain within genus *Pleurotus* for the cultivation of oyster mushrooms, is one of the white rot fungi that exhibit ability to degrade the lignin fraction of biomass. *P. ostreatus* widely grows in temperate climates, which are also what switchgrass is widely adapted in. Taniguchi et al. (2005) investigated the pretreatment of *P. ostreatus* on rice straw and found that 41% of Klason lignin (acid insoluble lignin) was removed while 17% of cellulose and 48% of hemicellulose were removed. Balan et al. (2008) treated rice straw with *P. ostreatus* for over 23 days prior to ammonia fiber expansion (AFEX) pretreatment and achieved 15% higher glucan conversion than rice straw pretreated with AFEX alone.

Hydrothermolysis utilizing pressurized liquid hot water has shown good effects to pretreat switchgrass to enhance subsequent saccharification and fermentation. After pretreatment, high cellulose and hemicellulose solubilization have been achieved (Mosier et al., 2005; Yang & Wyman, 2004). An ethanol yield from 70% to 88% based on the glucan available in untreated switchgrass was achieved using hydrothermolysis-pretreated switchgrass subjected to simultaneous saccharification and fermentation (Faga

et al., 2010; Suryawati et al., 2008). An 88% ethanol yield based on glucan and xylan available in the pretreated biomass was achieved using hydrothermolysis-pretreated corn stover that was hydrolyzed and fermented by a yeast that can produce ethanol from glucose and xylose (Mosier et al., 2005). However, to conduct a successful hydrothermolysis, the operating temperature should be in a range from 170 °C to 210 °C and the corresponding hold time should be 10 to 30 minutes, resulting in a high cost of pretreatment. Also, some soluble fermentation and enzyme inhibitors such as furfural and 5-hydroxy-methyl-furfural (HMF) are produced that decrease the efficacy of enzymatic hydrolysis and fermentation (Jing et al., 2009; Kim et al., 2011; Yang & Wyman, 2004). Severity is a term that links the operating temperature and reaction time during hydrothermolysis (Dogaris et al., 2009; Hendriks & Zeeman, 2009) and is related to the cost of hydrothermolysis. The lower severity is, the lower the pretreatment cost will be.

The objective of this study was to apply *P. ostreatus* pretreatment during switchgrass storage to obtain a low severity hydrothermolysis pretreatment. Under a natural storage environment, fungal treatment was done in a two year study using square bales in 2013 and round bales in 2014. Commercial *P. ostreatus* spawn was applied to the switchgrass right before baling. Sampling was performed after three months, five months, seven months and nine months. Fungal treated samples were subjected to hydrothermolysis and enzymatic hydrolysis.

4.3. Methods

4.3.1. Field work

4.3.1.1. *Pleurotus ostreatus* spawn application and sampling

Square bales were started at December 2012 and round bales were started at December 2013. *P. ostreatus* spawn application procedure and sampling times were similar for square and round bales. Kanlow switchgrass (*Panicum virgatum* var. Kanlow) was harvested from the experimental field of Oklahoma State University South Central Research Station, Chickasha, Oklahoma. After harvesting, switchgrass was left as a windrow in the field to let switchgrass naturally dry for 3 to 5 days. Prior to baling, switchgrass from the windrows was taken as the initial samples. The moisture contents for initial square bale samples and initial round bale samples were 8.9% and 8.4%, respectively. After sampling, fungal treatment was applied by manually spreading *P. ostreatus* spawn (Sylvan Inc, Kittanning, PA, USA) to some switchgrass windrows at a rate of 2 kg spawn/100 kg grass; meanwhile, some other windrows were not applied with spawn and used as controls. Twelve bales, 4 for control and 8 for treatment, were made and transported to Stillwater, OK within two days after baling. Bales were stored in an open, unprotected area in an experimental field at Oklahoma. After fungus applied, three bales (one control and two fungal treated) were sampled at three months (termed as March sample), five months (termed as May sample), seven months (termed as July sample) and nine months (termed as September sample), respectively.

For the square bale study, switchgrass in windrows was transformed into twelve 2.4 m×1.2 m×0.9 m (converted from 8 ft ×4 ft × 3 ft, Length×Width×Height) square

bales with an AGCO high-density baler (Hesston, KS, USA) on December 13, 2012. Twelve square bales were stored in the field next to each other under the same environment condition. Bales 1, 5 and 9 were used for sampling in March, bales 2, 6 and 10 were used for sampling in May, bales 3, 7 and 11 were used for sampling in July and bales 4, 8 and 12 were used for sampling in September. Fig. 4.1A shows how the square bales were placed on the ground. The square bales sampling locations are shown in Fig. 4.1B using the method described by Moore et al. (2014). Samples were taken using a hay corer, which was a 0.05 m (2 in) diameter and 0.9 m (3 ft) long probe mounted on an electric drill. For 0.3 m (1 ft), 0.6 m (2 ft) and 0.9 m (3 ft) samples, the corer was drilled perpendicular into the bale surface 0.3 m, 0.6 m and 0.9 m, respectively. Fig. 4.1C and Fig. 4.1D show the distance of the sampling location to the edge of the bale. A total of 62 samples were taken to represent the whole bale. All 62 samples were used for moisture content measurement. Top, middle, bottom and other samples were collected in a way shown in Table 4.1. All 18 samples from the top, middle and bottom locations were combined into top, middle and bottom samples for composition analysis. All 62 samples were combined for hydrothermolysis pretreatment.

For the round bale study, switchgrass was transformed into twelve 1.5 m × 1.5 m (converted from 5 ft × 5 ft, Diameter × Width) round bales using the South Central Research Station's John Deere round baler JD568 (East Moline, IL, USA) on December 19, 2013. Twelve square bales were stored in the field next to each other under the same environment condition. Fig. 4.2 A shows how the round bales were placed on the ground. The round bales sampling scheme is shown in Fig. 4.2B. Fig. 4.2 C and 4.2D show the distance of sampling locations to the edge of the bale. Samples were taken using a hay

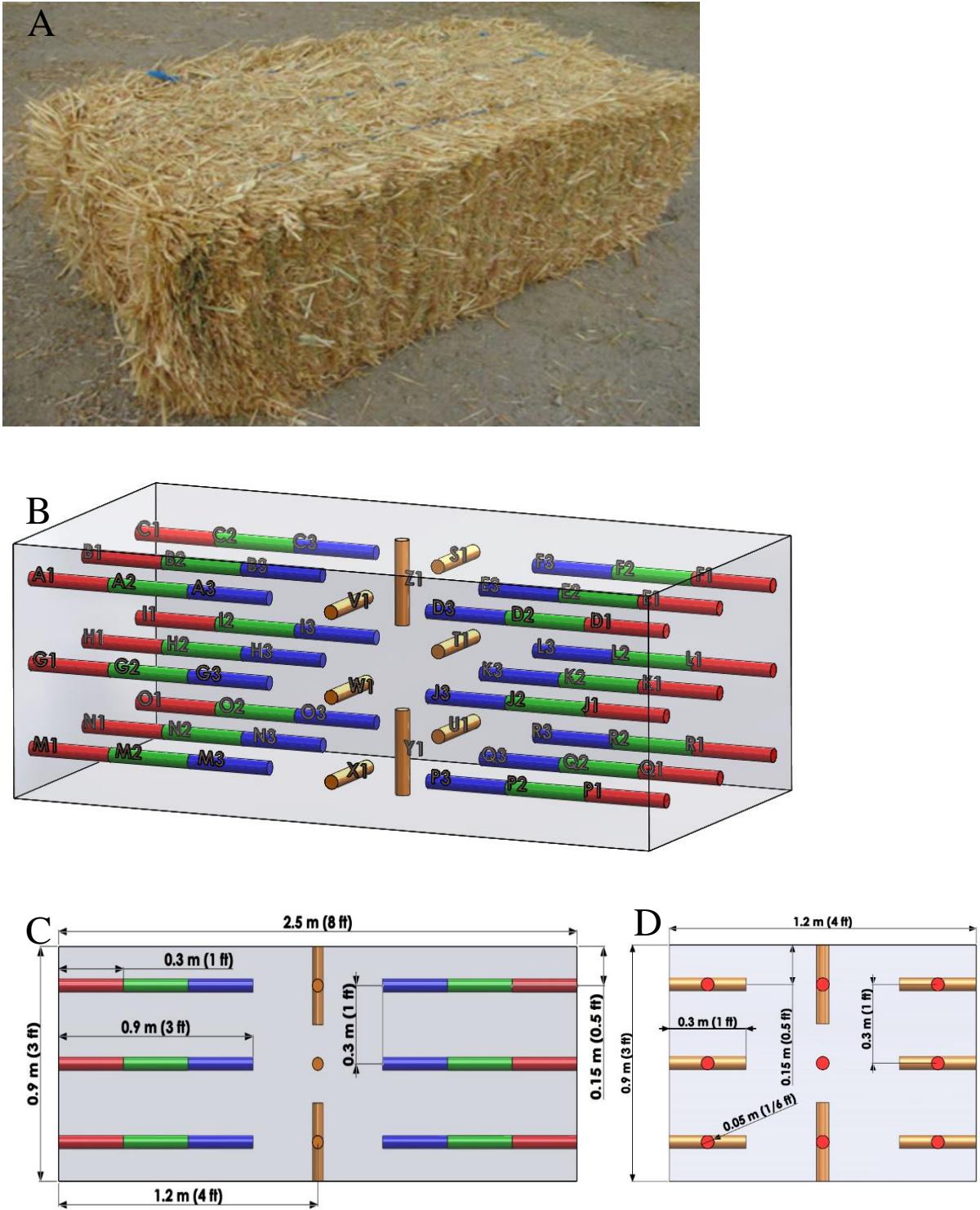


Fig. 4.1. Square bales and sampling locations of square bale. A, large square bale, B, scheme of sampling locations of square bale, C, front view of the scheme, D, side view of the scheme. Numbers 1, 2, 3 after the letters (A-Z) in B denotes the sampling depth 0.3 m (1ft), 0.6 m (2ft), 0.9 m (3ft), respectively.

Table 4.1. Square bale top, middle, bottom and other samples combining procedure

Sample	Sample No.
Top	A1, B1, C1, A2, B2, C2, A3, B3, C3, D1, E1, F1, D2, E2, F2, D3, E3, F3
Middle	G1, H1, I1, G2, H2, I2, G3, H3, I3, J1, K1, L1, J2, K2, L2, J3, K3, L3
Bottom	M1, N1, O1, M2, N2, O2, M3, N3, O3, P1, Q1, R1, P2, Q2, R2, P3, Q3, R3
Other	S1, T1, U1, V1, W1, X1, Y1, Z1

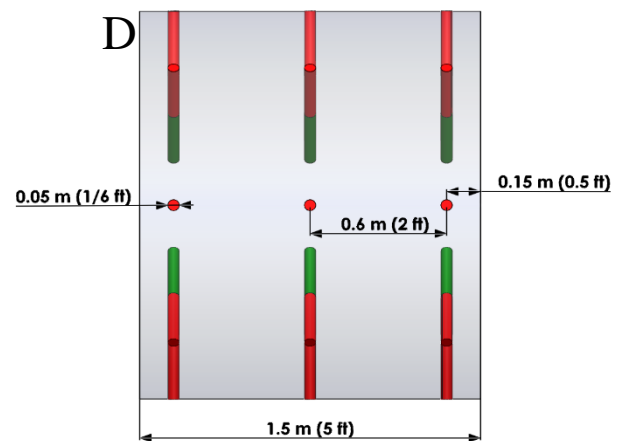
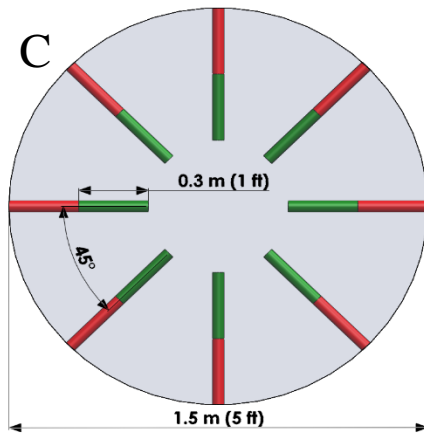
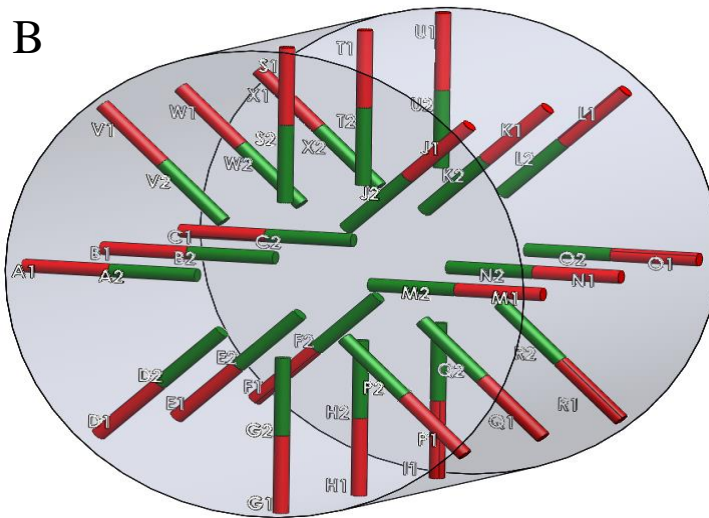


Fig. 4.2. Round bales and sampling locations of round bale A, large round bale, B, scheme of sampling locations of round bale, C, front view of the scheme, D, side view of the scheme. Numbers 1, 2 after the letters (A-X) in B denotes the sampling depth 0.3m (1ft), 0.6m (2ft), respectively.

Table 4.2. Round bale 1 ft, 2 ft samples combining procedure

Sample	Sample No.
1ft	A1, B1, C1, D1, E1, F1, G1, H1, I1, J1, K1, L1, M1, N1, O1, P1, Q1, R1, S1, T1, U1, V1, W1, X1
2ft	A2, B2, C2, D2, E2, F2, G2, H2, I2, J2, K2, L2, M2, N2, O2, P2, Q2, R2, S2, T2, U2, V2, W2, X2

corer. A total of 48 samples were taken to represent the whole bale. Since samples taken from round bales expose to different environmental conditions (air temperature, humidity), it is of interest to see the effect of sampling depth on composition change and subsequent pretreatment and enzymatic hydrolysis. For 0.3 m (1 ft) and 0.6 m (2 ft) samples, the corer was drilled perpendicular into the bale surface 1 ft and 2ft, respectively. Three 1 ft samples and three 2 ft samples were taken every 45 ° along the round bale surface. A total of 24 samples were taken to represent the whole bale.

4.3.1.2. Rainfall and temperature

Rainfall and temperature data were obtained from Oklahoma Mesonet (<http://www.mesonet.org/>). Mesonet data from the Stillwater station was retrieved. Rainfall data from Mesonet during storage were cumulatively summed to represent the rainfall bales received as storage time increased. Average air temperature data from Mesonet were used to represent the temperature.

4.3.1.3. Moisture content

Samples were dried for 3 to 5 days in a dryer (Thomas & Betts, Memphis, TN, USA) at a temperature range of 70 °C to 90 °C immediately after sampling. Moisture content was measured based on the weight loss on a wet basis according to Anonymous (2002). Samples were ground to 2 mm using a Thomas-Wiley mill (Model 4, Arthur H. Thomas Co., Philadelphia, PA, USA) equipped with a 2 mm sieve. Ground samples were stored in a 4 °C walk-in cold room until use.

4.3.2. Laboratory analysis

4.3.2.1. Biomass characterization

For square bale samples, top, middle and bottom samples were separately subjected to composition analysis. For round bale samples, 1 ft and 2 ft samples were separately subjected to composition analysis. Switchgrass composition analysis was done according to NREL standard protocols (Sluiter et al., 2007; Sluiter et al., 2005). Total solids content of biomass was determined according to the method described by Sluiter et al. (2008). The structural carbohydrates and lignin content were determined using the method described by Sluiter et al. (2007) after biomass extraction according to the method of Sluiter et al. (2005). Biomass was extracted by deionized water followed by 95% (v/v) ethanol using an Accelerated Solvent Extractor, ASE® 300 system (Dionex Corporation, Sunnyvale, CA, USA). Water and ethanol extractives were air dried in the fume hood until weight was constant. For structural sugars and lignin determination, acid soluble lignin (ASL) after acid hydrolysis of biomass was analyzed at 205 nm using a UV-Vis spectrophotometer (Cary 50 Bio, Varian Inc, Palo Alto, CA, USA). Concentrations of sugars produced from acid hydrolysis of structural carbohydrates were measured using HPLC (Model 1100, Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID) and a Bio-Rad Aminex HPX-87P column (Bio-Rad, Sunnyvale, CA, USA). Deionized water was used as an eluent at a flow rate of 0.6 ml/min and a column temperature of 85 °C. The total run time for each sample was 30 min.

4.3.2.2. *Hydrothermolysis*

Hydrothermolysis was conducted in a 1-L bench top stirred reactor and pressure vessel (Parr Series 4520, Parr Instrument Company, Moline, IL, USA) equipped with a propeller agitator, a 1 kW electrical resistance heater and a temperature controller. The reactor was filled with 75 g dry switchgrass and 425 g deionized water to achieve a 15% dry matter mixture.

Three reaction conditions were applied: temperature 200 °C with reaction time 10 min (200/10), temperature 190 °C with reaction time 15 min (190/15), and temperature 180 °C with reaction time 20 min (180/20). Severity is a term that combines the treatment temperature (T) and residence time (t) in one value and provides an approximate indication of the treatment conditions (Dogaris et al., 2009). Severity was calculated using equation 4.1 and the logarithmic severities of the three hydrothermolysis conditions are 3.94 for condition 200/10, 3.82 for condition 190/15 and 3.66 for condition 180/20, respectively. Reaction time was defined as the time the reaction was held at the desired temperature. The agitator was set at 300 rpm throughout the reactions. Temperature profiles were recorded during the reaction. At the end of the reaction time, the reactor was immediately disconnected from the heating unit and cooled in an ice bath. The agitator was rotated manually until temperature decreased to 55 °C. Pretreated slurry was removed from the reactor completely and filtered by vacuum filtration using a Buchner funnel lined with Whatman filter paper #5 (Whatman PLC, Brentford, UK). After filtration, the obtained solids termed as pretreated solids were washed with deionized water to remove inhibitors such as furfural and 5-HMF. The obtained liquid portion termed as prehydrolyzate was stored at 4 °C for analysis of sugars and inhibitors.

For square bale samples, all the samples taken from each bale at different fungal pretreatment time were combined and subjected to hydrothermolysis pretreatment condition 200/10, 190/15 and 180/20, respectively. For washing step after hydrothermolysis pretreatment on square bale samples, pretreated solids were washed repeatedly with a total of 2 L of deionized water. The washed solids and prehydrolyzate were then stored in airtight plastic bags at 4 °C until hydrolysis and degradation products analysis.

For round bales, 1ft samples, 2 ft samples were subjected to hydrothermolysis pretreatment 200/10 and 180/20, respectively. For round bale samples, the solids were washed repeatedly with an amount equal to five times of pretreated solids (wet basis) to remove inhibitors. Unwashed and washed samples were then stored in airtight plastic bags at 4 °C until they were transported to the University of Arkansas for hydrolysis and degradation products analysis.

Severity (R_0) (Dogaris et al., 2009; Overend & Chornet, 1989) was calculated using equation 4.1,

$$\log(R_0) = \log\left(t \cdot e^{\left(\frac{T-100}{14.75}\right)}\right) \quad 4.1$$

where t, reaction time (min) and T, temperature (°C).

4.3.2.3. *Mass balance analysis of samples after hydrothermolysis pretreatment*

For square bale samples, sugars and mass balance analysis were done for samples with hydrothermolysis conditions 200/10, 190/15 and 180/20. Degradation products analysis was done with hydrothermolysis 200/10 and 180/20 due to time limit. Overall

mass balances were conducted as described by Ramachandriya et al. (2014). Pretreated solids were subjected to structural carbohydrates analysis according to the method of Sluiter et al. (2007). Sugars and degradation products were analyzed as follows.

4.3.2.4. Sugars, byproducts and degradation products analysis

During hydrothermolysis pretreatment, acetyl groups of hemicelluloses form acetic acid while hexoses and pentoses are degraded into hydroxymethylfurfural (HMF) and furfural, respectively (Shuai et al., 2010). Successive decomposition of HMF produces formic acid and levulinic acid (Shuai et al., 2010). Prehydrolyzate was analyzed for glucose, xylose, galactose, arabinose and mannose and degradation products acetic acid, formic acid, 5-HMF and furfural by HPLC (Sluiter et al., 2006).

Sugars analysis in prehydrolyzate was performed according to the method of Sluiter et al. (2006), where 10 µl of each sample were analyzed by HPLC with a RID (Agilent 1100, Santa Clara, CA, USA) and a Bio-Rad Aminex HPX-87P column (Bio-Rad, Sunnyvale, CA, USA). Deionized water was used as an eluent at a flow rate of 0.6 ml/min and a column temperature of 85 °C. The total run time for each sample was 30 min. The HPLC with Chemstation software (Agilent Technologies) was calibrated at five levels using known concentrations of compounds before being used to quantitate the concentration of compounds.

Glucan and xylan recovery were calculated using equations 4.2 and 4.3, respectively.

$$\text{Glucan recovery} = \frac{[\text{Glucan}]_{\text{pretread solids}} + [\text{Glucan}]_{\text{prehydrolyzate}}}{[\text{Glucan}]_{\text{samples put in hydrothermolysis}}} \quad 4.2$$

where $[Glucan]_{pretread\ solids}$, glucan content of pretreated solids (g),
 $[Glucan]_{prehydrolyzate}$, glucan content in prehydrolyzate (g),
 $[Glucan]_{samples\ put\ in\ hydrothermolysis}$, glucan content of samples put in
hydrothermolysis (g).

$$\text{Xylan recovery} = \frac{[Xylan]_{pretread\ solids} + [Xylan]_{prehydrolyzate}}{[Xylan]_{samples\ put\ in\ hydrothermolysis}} \quad 4.3$$

where $[Xylan]_{pretread\ solids}$, xylan content of pretreated solids, $[Xylan]_{prehydrolyzate}$,
xylan content in prehydrolyzate, $[Xylan]_{samples\ put\ in\ hydrothermolysis}$, xylan content of
samples put in hydrothermolysis.

Degradation products (organic acids, furfural, and 5-hydromethylfurfural (HMF)) analysis was done by the University of Arkansas according to Frederick et al. (2014), which was originally adapted from Spáčil et al. (2008). Organic acids were detected on a Waters 2695 separation module with a Bio-Rad (Hercules, CA) Aminex HPX-87H ion exclusion (7.8 mm × 30 mm) column at 55 °C. The mobile phase consisted of 0.01 N H₂SO₄ with a flowrate of 0.6 ml/min. The organic acids, furfural, and 5-hydromethylfurfural (HMF) were detected at a UV wavelength of 280 nm, while acetic and formic acids were detected at 210 nm. All organic acid concentrations were quantified using a Waters 2996 photodiode array detector. An Acquity Waters UPLC fitted with a 2.1 mm × 50 mm C18 1.7 μm column (Milford, MA, USA) was used for phenolics detection and quantification. A Waters UPC UV detection module, adjusted at wavelengths between 210 and 280 nm using a methanol and water gradient solvent

system ramping from 15% water to 85% over the course of 8 min was used to detect the phenolics. The injections had a volume of 2.5 μ l, and analysis was conducted at 50 $^{\circ}$ C.

Degradation products yield was calculated as g degradation products produced through hydrothermolysis per kg dry switchgrass put into hydrothermolysis using equation 4.4.

$$\text{Degradation product yield} = \frac{[\text{Degradation product}] \times V_{\text{predrolyzate}}}{75} \quad 4.4$$

where $[\text{Degradation product}]$, degradation product concentration in prehydrolyzate (g/L), $V_{\text{predrolyzate}}$, volume of prehydrolyzate (ml), 75, dry switchgrass put in hydrothermolysis pretreatment (g).

4.3.2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was performed by the University of Arkansas according to Selig et al. (2008) with a few modifications. A reaction volume of 10 ml containing 1% glucan for each sample was used. The working volume of 10 ml included 5 ml of 0.1 M sodium citrate buffer with pH 4.9. Accellerase 1500 (Dupont, Rochester, NY, USA) was used at a 60 FPU/g glucan. The reaction was conducted at 50 $^{\circ}$ C with stirring at 180 rpm over a 24 h period. Glucose was measured at the end of 24 h hydrolysis using HPLC (Waters 2695, Milford, MA, USA) equipped with a Shodex (Waters, Milford, MA, USA) precolumn (SP-G, 8 μ m, 6 \times 60 mm) and Shodex column (SP0810, 8 μ m, 6 \times 300 mm). The water mobile phase, eluting at 0.2 ml/min, was heated to 85 $^{\circ}$ C, using a Waters (WAT038040) external heater. Glucose was detected using a Waters 2414 RID (Milford, MA, USA).

Glucose yield was calculated as g glucose obtained per g glucose available in 0.1 g glucan using equation 4.5.

$$\text{Glucose yield} = \frac{[\text{Glucose}] \times 10}{1000 \times 0.1 \times 1.11} \quad 4.5$$

where [Glucose], glucose concentration (g/L), 10, hydrolysis reaction volume (mL), 1000, conversion factor from mL to L, 0.1, glucan put in the reaction (g), 1.11, mass conversion factor of glucan hydrolysis to glucose (g/g).

4.3.3. Statistical analysis

There was no statistical analysis done on square bales since there was only one control bale. Round bales did not have any fungus growing on them, thus the round bale study was considered as a storage study with three replicates, which will be mentioned in 4.4.2. Analysis of variance (ANOVA) was conducted to study the effects of sampling depth and storage time on composition after storage; the effects of sampling depth, storage time and washing on composition after hydrothermolysis pretreatment; and the effects of sampling depth, storage time, pretreatment temperature and time and washing on composition after hydrothermolysis pretreatment glucose yield after hydrolysis using the generalized linear model (GLM) in SAS release 9.3 (SAS, Cary, NC, USA). Multiple comparison tests were performed with Tukey's test at a 95% confidence interval.

4.4. Results and discussion

4.4.1. Square bales study

4.4.1.1. Rainfall and temperature files during fungal pretreatment

As shown in Fig. 4.3, there was no rainfall in the first 7 days after *P. ostreatus* spawn was applied. There was 0.017 m rainfall in the first 30 days and 0.05 m in the first 60 days. The accumulated rainfall was 0.12 m prior to the 3 month sampling in March. After the March sampling, considerable rainfall was received before and after the May sampling. Temperature files are shown in Fig. 4.4. The March sampling was done at 83 days after applying the spawn. Air temperature showed high variation. For most of the days before the March sampling, the air temperature was below 10 °C. Kashangura (2008) reported at temperature 10 °C, *P. ostreatus* mycelia would be damaged.

4.4.1.2. Moisture content in fungal pretreatment

The initial moisture content of square bale samples at baling was 8.9%. Moisture content variability in the 12 bales is shown in Fig. 4.5. The moisture contents expressed as mean and standard deviation of 12 bales are shown in Table 4.3. For March samples (bales 1, 5 and 9) shown in Fig. 4.5, for most of the top samples of three bales, moisture contents were around 50% with a standard deviation from 5% to 8%, and for most of the middle and bottom samples of these three bales, the moisture contents were around 25% with a standard deviation from 4% to 8%. For May samples shown in Fig. 4.5, the top samples moisture contents increased to around 70% with a standard deviation of 3%, the middle samples moisture contents were around 55%, the bottom samples moisture contents were around 40%. For bales 2, 6, 10, standard deviations of middle samples and

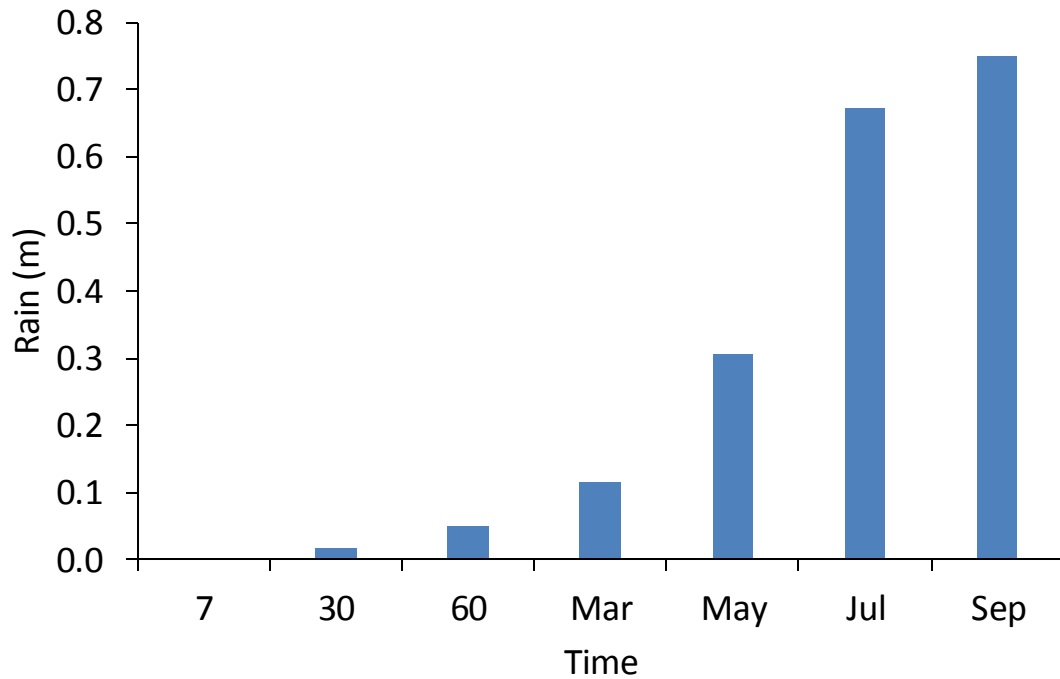


Fig. 4.3. Rainfall accumulation at certain period after *P. ostreatus* spawn applied to square bales. Numbers on x-axis indicate number of days after sampling. Mar denotes day 83 when first sampling done, May denotes day 144 when second sampling done, Jul denotes day 223 when third sampling done, Sep denotes day 275 when fourth sampling done.

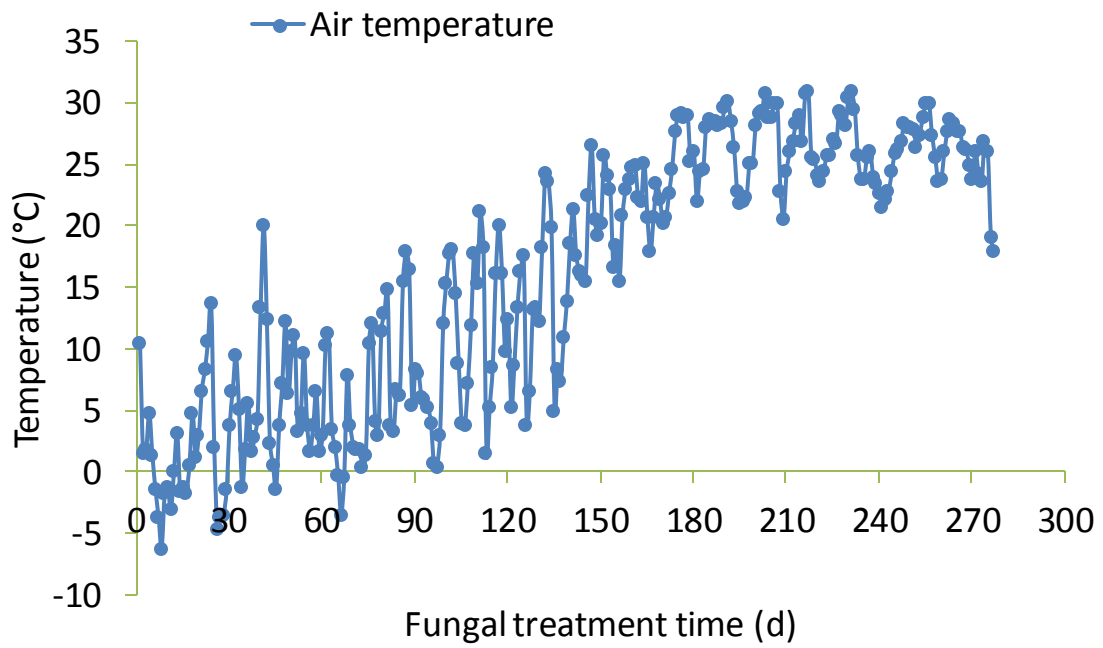


Fig. 4.4. Daily average air temperature after mushroom spawn applied to square bales.

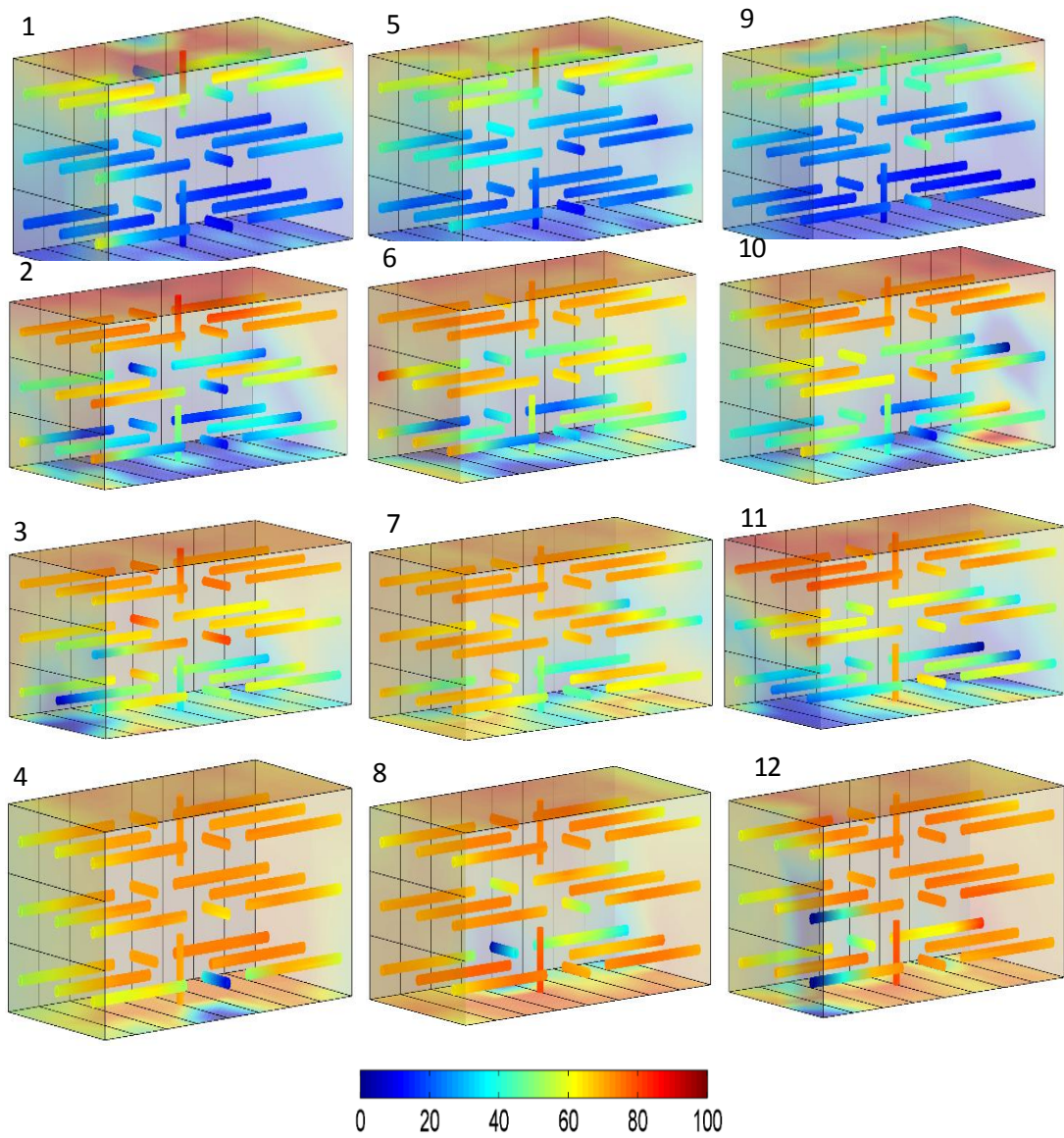


Fig. 4.5. Moisture variability of 12 square bales samples. Number on the top left corner of each bale is bale number. The bar with the scale 0 to 100 refers to the moisture content (%) of each sampling location.

Table 4.3. Moisture contents (% wet basis) of top, middle and bottom samples of 12 square bales

Bale No.	Top sample	Middle sample	Bottom sample
1	58.66±5.29	23.97±7.26	20.73±7.84
5	55.85±5.22	28.11±8.12	24.05±8.13
9	48.61±7.92	23.14±4.23	15.37±3.79
2	71.13±3.04	60.77±9.00	40.21±16.01
6	71.52±3.79	54.11±15.36	42.49±14.29
10	72.45±3.32	56.67±13.22	33.17±13.95
3	70.25±1.63	62.86±6.43	50.53±12.79
7	69.71±4.21	66.01±8.72	58.35±11.79
11	72.03±5.63	60.69±9.74	40.32±15.15
4	70.50±3.57	70.19±4.82	68.50±6.75
8	71.10±4.46	71.79±3.63	68.81±8.68
12	71.29±4.46	68.35±14.20	65.09±14.31

Values listed are average of two replicates ± standard deviation.

bottom samples were higher than top samples, which is consistent with the increased cumulative rain from March to May. The moisture content gradient from top to bottom indicates that water was received by the top samples and went deep into the bottom while during this process moisture was not homogeneously distributed. For top samples of July samples and top samples of September samples, the moisture contents were around 70%, similar to the top samples of May samples while both middle and bottom samples moisture contents kept increasing and the standard deviation was lower than that of May samples.

4.4.1.3. *Composition after fungal pretreatment*

Initial square bale sample composition is shown in Table 4.4. Fungal treated samples compositions of different sampling locations (top, middle and bottom) and different sampling times (Mar, May, July and September) are shown in Table 4.5. Overall glucan/xylan/lignin fractions shown are the means of glucan/xylan/lignin top, middle and bottom samples. Treatments were expected to have higher glucan and xylan fraction and lower lignin fraction than that of controls since *P. ostreatus* selectively consumes lignin. There was no clear trend observed showing selective lignin degradation happening during fungal pretreatment during 9-month storage.

There was no clear trend for glucan fractions of top and middle samples. For glucan fraction of bottom samples, treatment bales had a higher glucan fraction ranging from 1.7% to 7.8% than that of control bales throughout storage time. For overall glucan fractions, except that treatment bales had 2.5% higher glucan fraction than control for

March samples, treatments had from 2.0 % to 10% lower glucan fraction than controls for the other three sampling times.

For xylan fractions, there was no clear trend for top samples and bottom samples. while for middle samples, xylan fractions for treatments were from 6.8% to 15.9% lower those of controls throughout storage. The overall xylan fraction of treatments was from 4.4% to 7.6% lower than that of controls.

Lignin fractions showed a clear trend. Treatment bales showed higher lignin than controls for all samples except for the similar fraction of control and treatment samples (20.87% and 20.75%) for March. For top samples, treatments were from 8.2% to 16.2% higher than controls throughout the storage time. For middle samples treatments were from 9.4% to 11.5% higher than controls throughout the storage time. For bottom samples, except that treatment was 0.6% lower than control for March samples, treatment showed from 13.7% to 15.7% higher lignin fraction than that of control bales for top, middle and bottom samples. For lignin fractions of overall samples, control bales were from 8.9% to 16.4% higher than treatment bales. Compared to the considerable biomass component degradation reported in previous lab scale white rot fungi pretreatment on various substrates (Shi et al., 2009; Taniguchi et al., 2005; Wan & Li, 2011b; Xu et al., 2010), glucan, xylan and lignin changes here were minimal.

Treatment bales showing lower glucan and xylan fractions and higher lignin fraction than controls may suggest that more glucan and xylan were consumed in control bales than in treatment bales. To the best of the author's knowledge, there are no published studies using white rot fungi pretreatment on switchgrass or any other biomass

Table 4.4. Initial composition of square bale and round samples

Biomass components	Composition (% dry basis)	
	Square	Round
Glucan	35.92 ± 0.09	39.03 ± 0.20
Xylan	22.63 ± 0.17	24.56 ± 0.29
Galactan	1.94 ± 0.03	2.48 ± 0.03
Arabinan+Mannan	7.28 ± 0.06	2.95 ± 0.25
Lignin	20.34 ± 0.27	19.71 ± 0.35
Extractives	11.21 ± 2.19	10.17 ± 0.07
Ash	3.41 ± 0.00	1.72 ± 0.09

Values listed are average of two replicates ± standard deviation.

Table 4.5. Biomass components (glucan, xylan and lignin) fraction of fungal treated square bale samples

		Composition (% dry basis)					
Components	Location		Mar	May	Jul	Sep	
Glucan	Top	Control	36.07	37.43	39.12	40.13	
		Trt	40.86±1.52	37.05±0.67	34.02±0.27	39.09±0.51	
	Middle	Control	39.12	37.42	38.55	39.49	
		Trt	40.22±0.53	34.60±0.09	34.05±0.17	38.71±1.66	
	Bottom	Control	39.01	38.98	38.85	38.36	
		Trt	35.96±0.35	35.31±2.39	35.86±2.20	37.72±0.82	
	Overall	Control	38.07	37.94	38.84	39.33	
		Trt	39.02±2.39	35.66±1.31	34.64±1.33	38.51±0.96	
	Xylan	Top	Control	23.4	23.71	23.35	24.77
			Trt	23.53±0.33	24.14±0.59	22.45±0.42	23.33±0.19
Middle		Control	26.82	23.46	24.28	24.23	
		Trt	22.54±0.07	21.39±0.35	21.91±0.29	22.59±0.68	
Bottom		Control	23.26	24.75	23.99	24.88	
		Trt	21.78±0.46	21.52±0.73	24.10±0.98	22.61±0.15	
Overall		Control	24.49	23.97	23.87	24.63	
		Trt	22.62±0.67	22.35±1.07	22.82±1.19	22.84±0.40	
Lignin		Top	Control	36.07	37.43	39.12	40.13
			Trt	40.86±1.52	37.05±0.67	34.02±0.27	39.09±0.51
	Middle	Control	39.12	37.42	38.55	39.49	
		Trt	40.22±0.53	34.60±0.09	34.05±0.17	38.71±1.66	
	Bottom	Control	39.01	38.98	38.85	38.36	
		Trt	35.96±0.35	35.31±2.39	35.86±2.20	37.72±0.82	
	Overall	Control	38.07	37.94	38.84	39.33	
		Trt	39.02±2.39	35.66±1.31	34.64±1.33	38.51±0.96	

Trt denotes treatment. Treatment values listed are mean of two replicates ± standard deviation.

feedstocks. At lab scale white rot fungus *C. subvermispora* pretreatment on switchgrass biomass feedstocks in controlled environment at bench scale resulted in 2% cellulose degradation, 14% hemicellulose degradation and 27% lignin degradation (Wan & Li, 2011a). Richard et al. (2001) reported small biomass components fraction changes (1.8% cellulose fraction increase, 0.5% lignin fraction hemicellulose fraction increase and 0.7% hemicellulose fraction decrease) during 11 weeks of bench scale ensilage on corn stover. Their study indicated hemicellulose degradation and a relative accumulation of cellulose and lignin.

There were mushroom fruiting bodies or fungus mycelia observed from outside and inside square bales, which are shown in Fig. 4.6. The morphology of the mushroom shown in Fig. 4.6A appeared to be *P. ostreatus*. However, the white mycelia shown in Fig. 4.6 B and C were not homogeneously distributed through the bales, which may result from the moisture variation (Fig. 4.5). Most of *P. ostreatus* cultivation for efficient biomass degradation in lab scale or for oyster mushroom production in large scale are usually conducted under the conditions of temperature ranging from 20 °C to 30 °C and substrate moisture ranging from 40% to 90% (Balan et al., 2008; Taniguchi et al., 2005; Wang et al., 2001). The moisture contents for all samples of top position of all bales were over 50%, which is enough for fungus to grow and metabolize, but the moisture contents for middle and bottom March samples were only 20%.

The temperature was below 10 °C for most of the first 90 days after fungus application. Kashangura (2008) studied temperature-growth relationship of *P. ostreatus* on 9 representative strains and found that the minimum temperature that resulted in mycelia extension was 10 °C and no mycelia occurred at 37 °C for all strains. This study



Fig. 4.6. Limited fungus colonization on square bales. A: Mushroom fruiting body on bale 10. B and C: Fungus mycelia colonization inside square bales.

also found that *P. ostreatus* exposure to 38 °C led to lack of mycelia viability. Zharare et al. (2010) also found that a temperature of 35 °C was detrimental to mycelial growth for *P. ostreatus*. The lack of a homogenous mycelia distribution throughout bales may explain why *P. ostreatus* did not achieve considerable delignification, which resulted lignin fractions of treatment bales being higher than that of control bales.

4.4.1.4. Composition of pretreated solids after hydrothermolysis pretreatment

Glucan, xylan and lignin fractions of hydrothermolysis-pretreated switchgrass under conditions 200/10, 190/15 and 180/20 are shown in Table 4.6. For pretreated samples under condition 200/10, the values of glucan, xylan and lignin fractions were similar to that of a previous study (Suryawati et al., 2008). Glucan fractions of treatments were from 0.1% to 3.9% lower than that of controls. For xylan fractions, treatment was 10% lower than that of control for March samples and was from 26% to 91% higher than controls for the other sample times. For lignin fractions, treatments were from 4.1% to 9.5% higher than controls for March, May and July samples while treatments were 2.5% lower than control for September samples.

For pretreated samples under condition 190/15, glucan fraction of treatment was 2.4% higher than that of control for March samples, while for July, May and September samples, glucan fractions of treatment were from 2.8 % to 3.5% higher than that of control. There was no clear trend for xylan fractions. Xylan fractions of treatment were from 4.1% to 17% lower than that of control among samples taken in March, May and July while xylan fraction of treatment was 32% higher than that of treatment for September sample. For lignin fractions, control and treatment were similar for March

Table 4.6. Glucan, xylan and lignin fraction of hydrothermolysis pretreated square bale samples

hydrothermolysis condition		Composition (% dry basis)				
		Mar	May	Jul	Sep	
200/10	Glucan	Control	66.32	64.22	62.84	65.53
		Trt	63.73±1.26	58.13±8.88	61.89±4.86	62.49±6.76
	Xylan	Control	4.56	3.91	3.17	3.74
		Trt	4.10±0.37	4.96±1.21	6.07±2.05	4.77±1.02
	Lignin	Control	36.51	34.54	36.50	38.78
		Trt	38.00±1.24	36.31±4.8	39.98±0.21	37.82±4.18
190/15	Glucan	Control	57.71	64.64	61.61	60.71
		Trt	59.08±6.25	62.36±1.32	59.06±4.16	59.03±4.12
	Xylan	Control	9.05	7.43	8.22	10.85
		Trt	9.87±1.21	7.74±0.38	9.67±2.43	7.37±0.82
	Lignin	Control	32.02	31.91	32.84	31.21
		Trt	31.94±1.56	35.65±4.79	34.34±2.39	33.80±3.16
180/20	Glucan	Control	63.94	60.77	61.01	60.71
		Trt	56.34±1.95	59.38±0.81	55.68±3.84	50.57±2.45
	Xylan	Control	12.19	16.13	17.33	20.87
		Trt	13.77±0.94	16.40±3.00	17.49±2.28	17.51±0.47
	Lignin	Control	31.09	28.10	31.71	31.21
		Trt	28.46±2.69	31.09±4.55	29.15±1.14	27.32±3.21

Trt denotes treatment. Treatment values listed are mean of two replicates ± standard deviation.

samples and treatments were from 4.5% to 11% higher than controls for May, July and September samples.

For pretreated samples under condition 180/20, glucan fractions of treatments were from 2.3% to 16.7% lower than those of controls for all sampling times. For xylan fractions, treatments were from 9% to 13% higher than those of controls for March, May and July samples, while treatment was 16% lower than that of control for September samples. For lignin fractions, treatment was 8.1% lower than that of control for July sample while for the other three sampling times, treatments were from 8.1% to 12% higher than those of controls.

Among hydrothermolysis condition 200/10, 190/15 and 180/20, either for glucan fractions or lignin fractions, differences between control and treatment were below 20% (most differences were below 10%). This could have resulted from the minimal changes of biomass components in fungal treatment samples compared to the controls. The considerable xylan changes could be mainly caused by natural variation since xylan is more easily dissolved during pretreatment.

4.4.1.5. Mass balances after hydrothermolysis pretreatment

Glucan and xylan recoveries after hydrothermolysis pretreatment 200/10 are shown in Fig. 4.7. Glucan recoveries (Fig. 4.7A) ranged from 67% to 86%. Xylan recoveries (Fig. 4.7B) ranged from 24% to 35%. Xylan recoveries in July samples were the highest among all sampling times for both control and treatment samples. Glucan and xylan recoveries after hydrothermolysis pretreatment 190/15 are shown in Fig. 4.8. Glucan recoveries (Fig. 4.8A) ranged from 64% to 86%. Xylan recoveries (Fig. 4.8B)

ranged from 48% to 67%. Glucan recoveries for March samples were the highest among different sampling times. Glucan and xylan recovery after hydrothermolysis pretreatment 180/20 are shown in Fig. 4.9. Glucan recoveries (Fig. 4.8A) ranged from 68% to 86%. Xylan recoveries (Fig. 4.8B) ranged from 68% to 86%. For March samples, glucan and xylan recoveries were similar between controls and fungal treated samples. For samples of the other sampling times, fungal treated samples showed higher glucan recoveries ranging from 12% to 20% and xylan recoveries ranging from 11% to 20% than controls. Xylan fractions of hydrothermolysis condition 200/10 were the lowest and xylan fractions of hydrothermolysis condition 180/20, which is consistent with the study of Pérez et al. (2007).

4.4.1.6. Degradation products in prehydrolyzate after hydrothermolysis pretreatment

The yields of degradation products acetic acid, formic acid and furfural after hydrothermolysis pretreatment 200/10 are shown in Fig. 4.10. Control samples showed higher yield for all degradation products than treatment samples. For acetic acid yield (Fig. 4.10A), controls or treatments were similar among the first three sampling times while control was higher than treatment for September samples. Formic acid yield (Fig. 4.10B) showed a similar trend to that of acetic acid yield. For furfural yield (Fig.4.10C), the July and September samples showed higher furfural yield than March and May samples

The yields of degradation products acetic acid, formic acid and furfural after hydrothermolysis pretreatment 180/20 are shown in Fig. 4.11. For acetic acid and formic acid yields (Figs. 4.11A and 4.11B), control samples showed higher yield than fungal

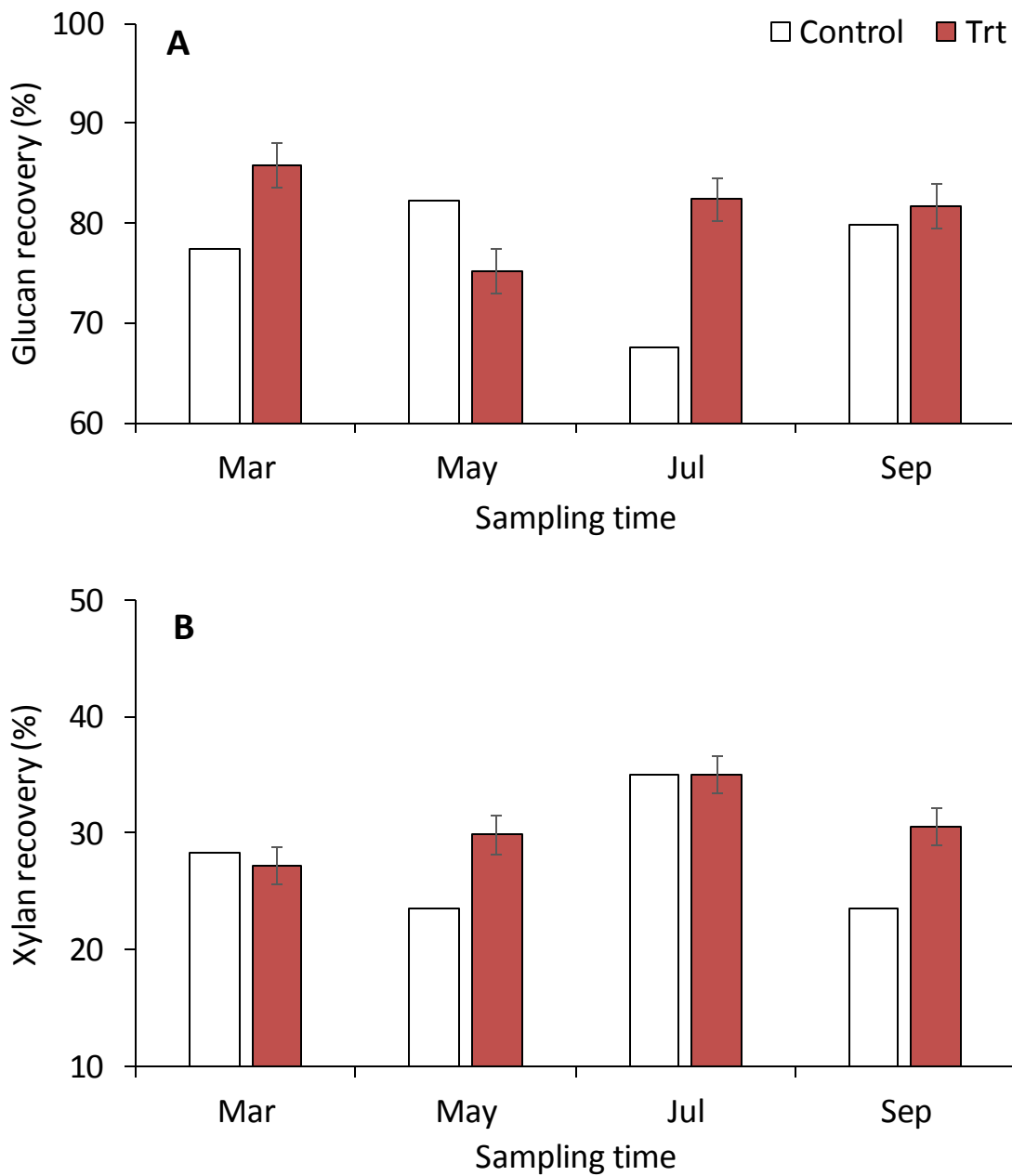


Fig. 4.7. Glucan (A), xylan (B) recovery of pretreated square bales samples under hydrothermolysis condition 200 °C/min. Control denotes samples without mushroom spawn applied. Trt denote samples with mushroom spawn applied. Error bars equal mean \pm one standard error.

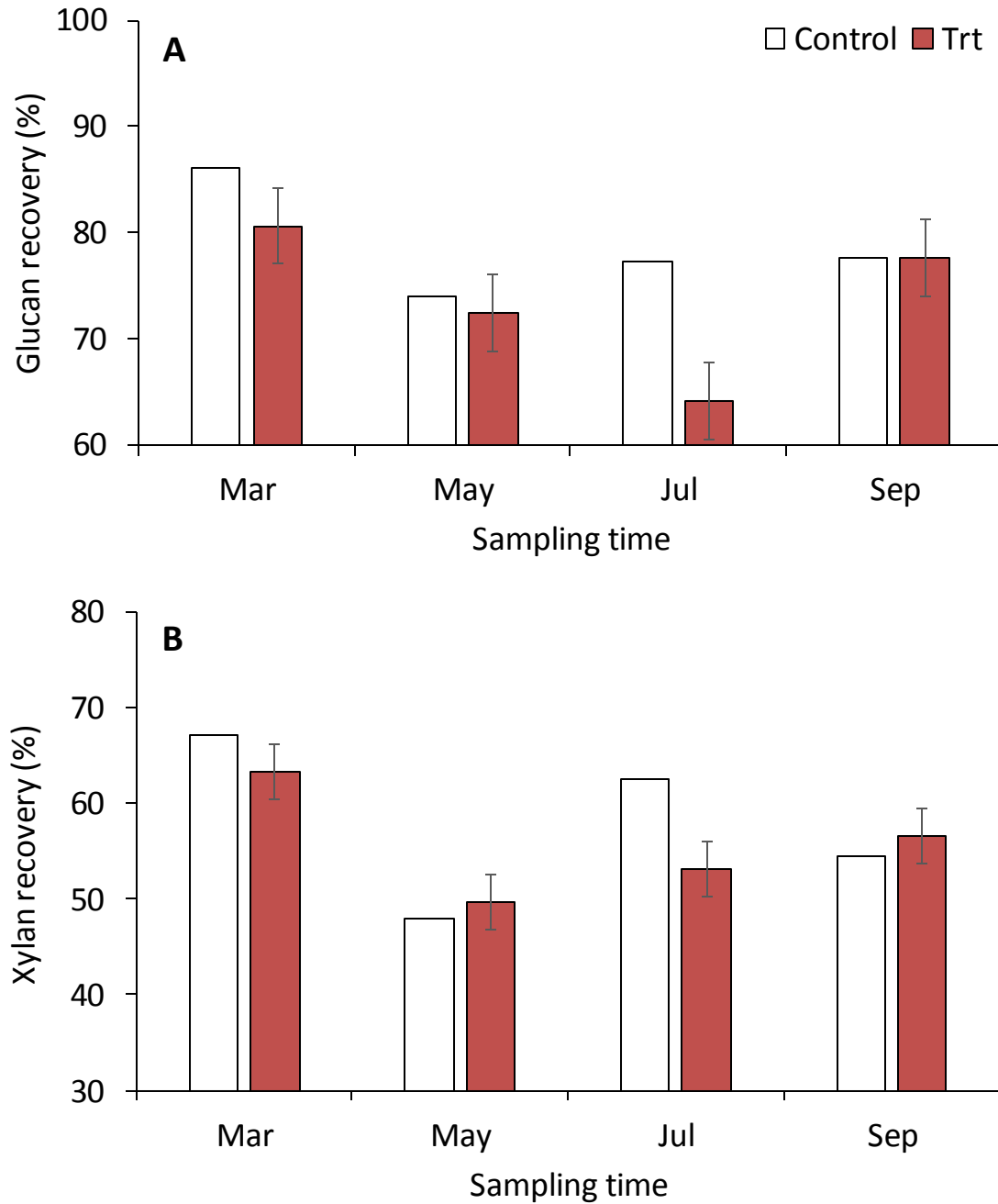


Fig. 4.8. Glucan (A), xylan (B) recovery of pretreated square bales samples under hydrothermolysis condition 190 °C/15min. Control denotes samples without mushroom spawn applied. Trt denote samples with mushroom spawn applied. Error bars equal mean \pm one standard error.

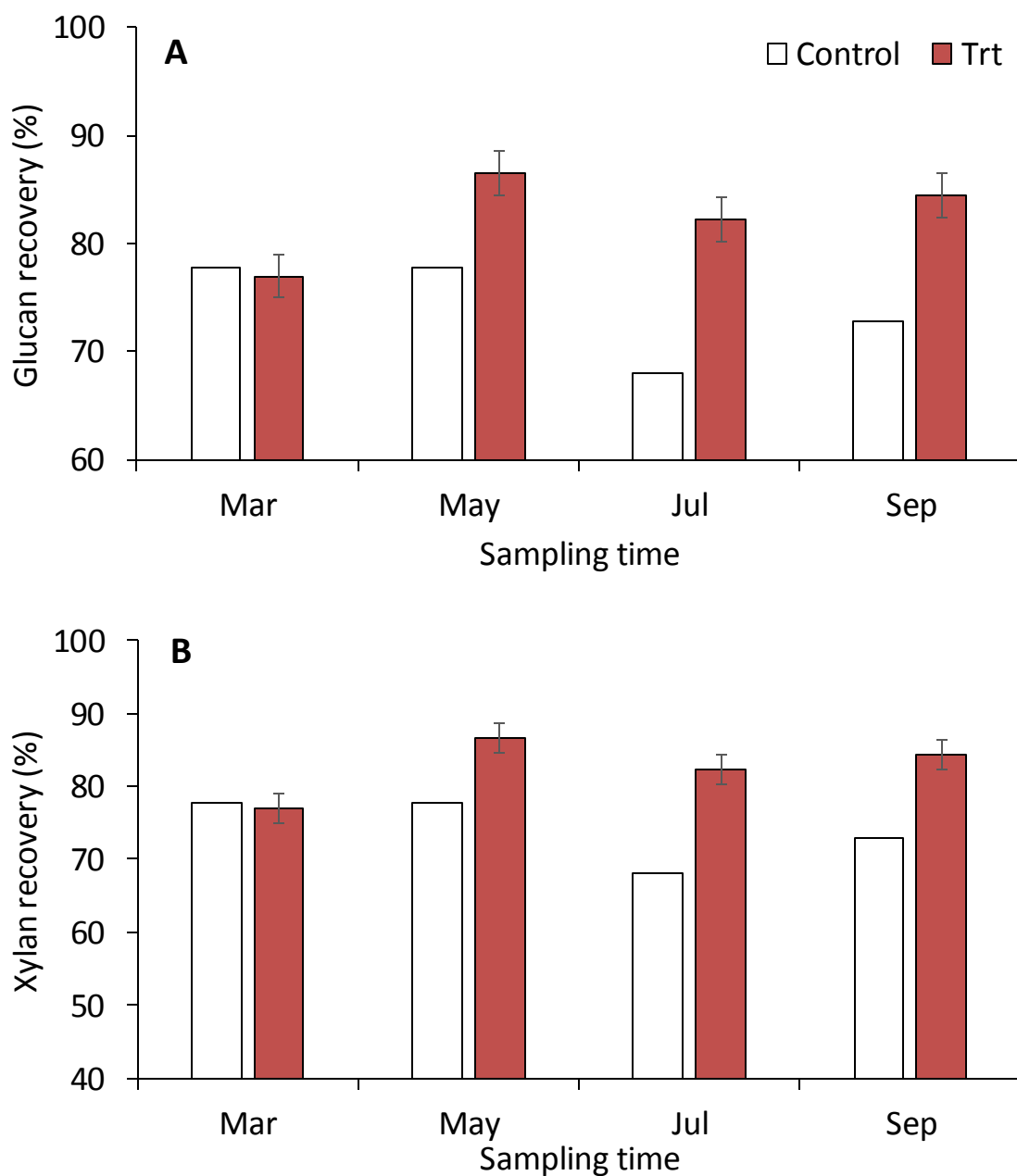


Fig. 4.9. Glucan (A), xylan (B) recovery of hydrothermolysis pretreated square bales samples under hydrothermolysis condition 180 °C/20min. Control denotes samples without mushroom spawn applied. Trt denote samples with mushroom spawn applied. Error bars equal mean± one standard error.

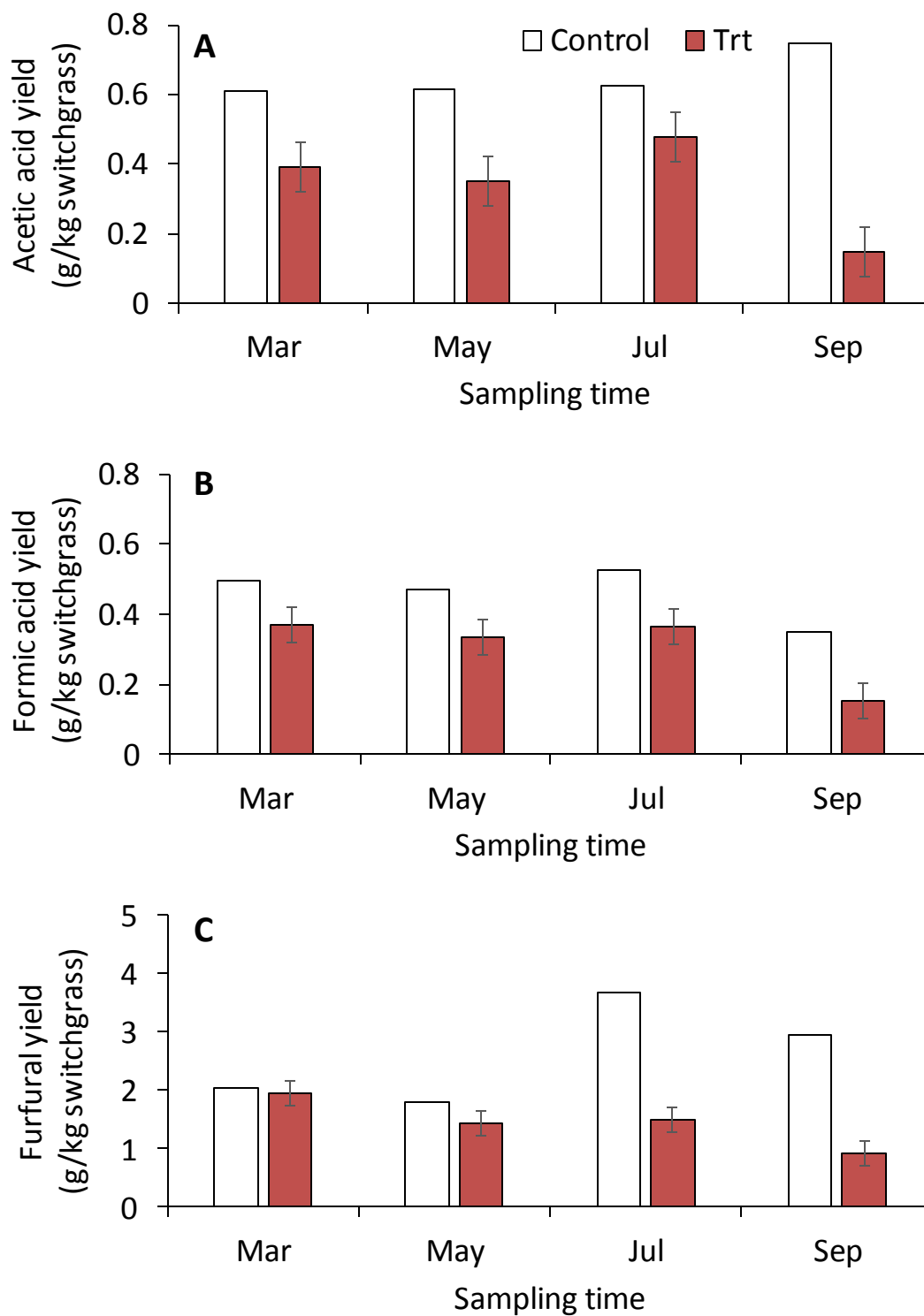


Fig. 4.10. Degradation products acetic acid (A), formic acid (B) and furfural (C) yields after hydrothermolysis pretreatment at 200/10. Error bars equal mean \pm one standard error.

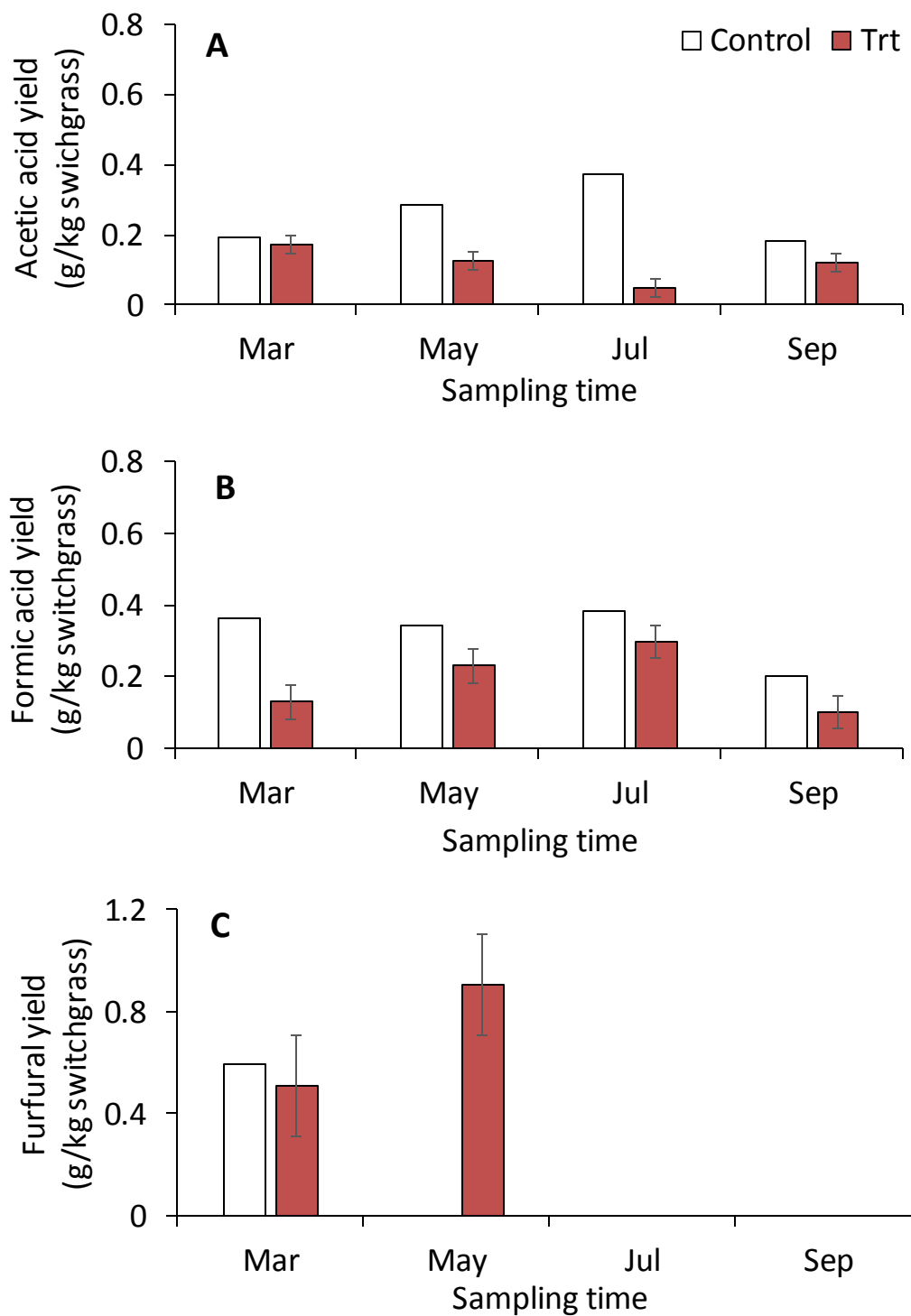


Fig. 4.11. Degradation products acetic acid (A), formic acid (B) and furfural (C) yields after hydrothermolysis pretreatment at 180/20. Furfural yield of control samples at May, Jul, Sep and treatment samples at Jul, Sep were not detected. Error bars equal mean \pm one standard error.

treated samples for all sampling times. For furfural yield (Fig. 4.11C), there was no clear trend. No furfural was detected for controls for July and September samples.

4.4.1.7. Enzymatic hydrolysis

Glucose yields of pretreated solids after hydrothermolysis pretreatment 200/10 and 180/20 are shown in Fig. 4.12. For the hydrolysis of 200/10 pretreated solids (Fig. 4.12A), controls showed from 16% to 20% higher glucose yield than treatment for all sampling times. Glucose yield decreased slightly as fungal pretreatment time increased. For the hydrolysis of 180/20 pretreated solids (Fig. 4.12B), control samples showed higher glucose yield than that of fungal treated samples except for May samples. There have been no published studies on large scale white rot fungal pretreatment to enhance enzymatic hydrolysis and fermentation. For lab scale white rot fungal pretreatment, there were various studies conducting white rot fungi pretreatment under optimal pretreatment condition (e.g., substrate moisture 50% to 75% and incubation temperature 25 °C to 30 °C, pretreatment time 10 days to 150 days depending on different fungi), glucose yields from 40% to 70% were obtained (Bak et al., 2009; Taniguchi et al., 2005; Wan & Li, 2010; Yu et al., 2010). The environment conditions in this study were not optimal for *P. ostreatus* to grow, thus lignin degradation did not happen and glucose yield was not improved.

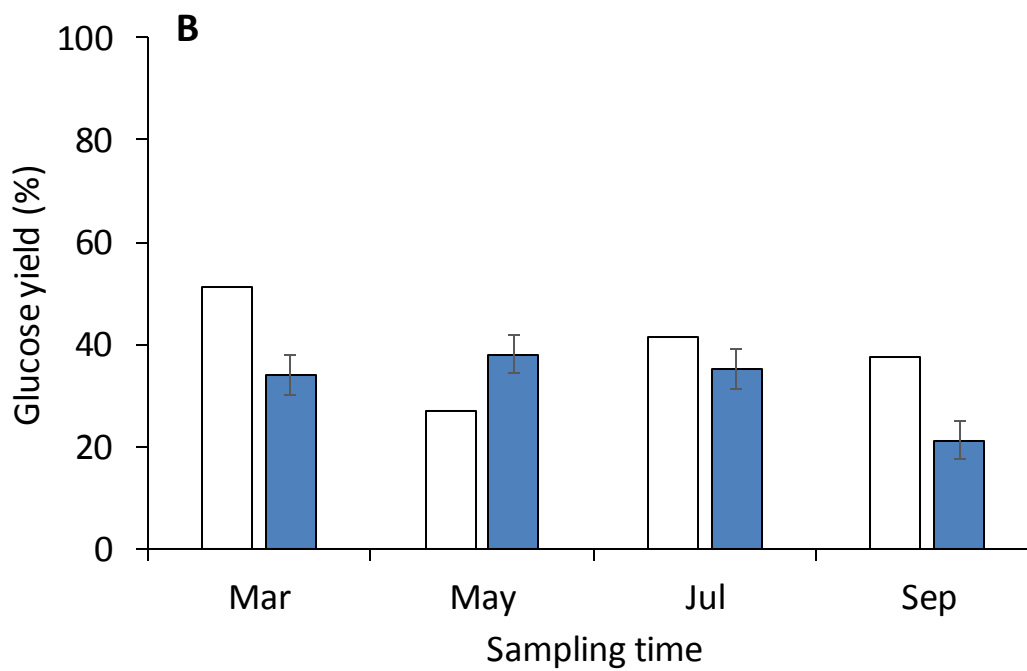
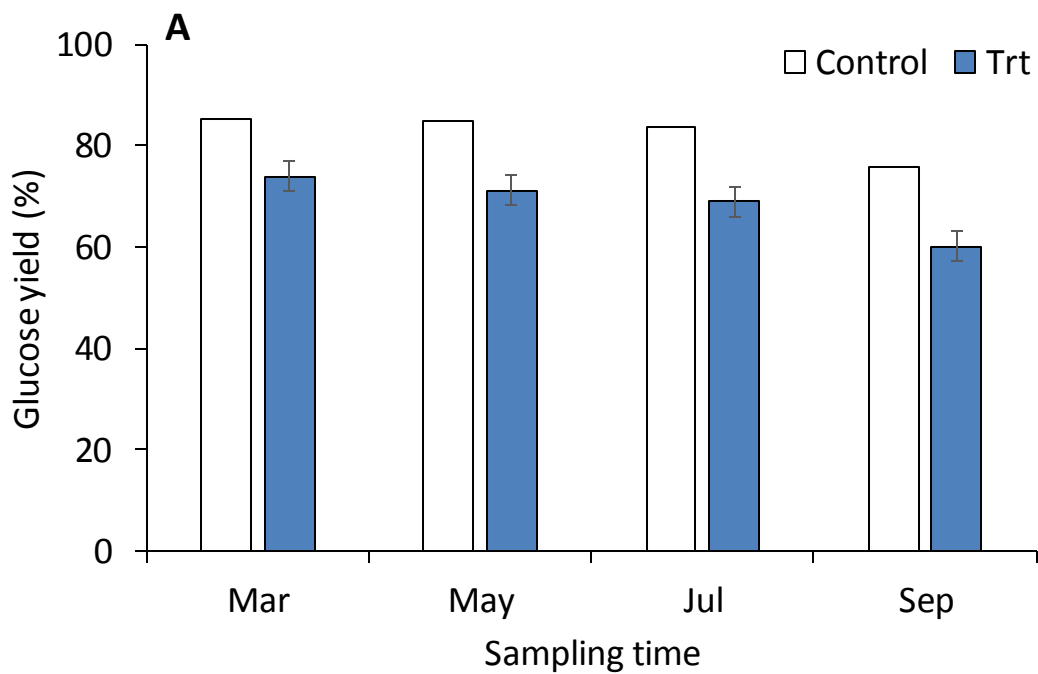


Fig. 4.12. Glucose yield of pretreated solids with hydrothermolysis pretreatment condition 200/10 (A) and 180/20 (B) for square bale samples. Error bars equal mean \pm one standard error.

4.4.2. Round bales study

4.4.2.1. Rainfall and temperature files during storage time

As shown in Fig. 4.13, there was a rainfall accumulation of 0.013 m the first 7 days after the mushroom spawn was applied. There was 0.015 m rainfall accumulation in the first 30 days and 0.020 m in the first 60 days. The accumulated rainfall before the March sampling was 0.035 m. After March sampling, more rainfall was received and the rainfall before the May sampling was 0.077 m. There was considerable rainfall after the May sampling.

Temperature files are shown in Fig. 4.14. The March sampling was done at 86 days after applying the spawn. The average air temperature showed high variation and was lower than 10 °C for most of the days before the first sampling, which is similar to the temperature condition of square bales.

4.4.2.2. Moisture file during storage time

The initial moisture content of square bale samples at baling was 8.4%. Moisture content files are shown in Fig. 4.15. The moisture contents of all bales were in the range of 6% to 11% with standard deviations from 1.7% to 5.4%. Based on the initial moisture content of 8.4%, round bales did not retain any rain. This was expected since the flakes (flake, 1-2 inch thick layer of grass, (Blood et al., 2007)) were wrapped in circles when baling round bales. Square bales shown in Fig. 4.5 showed higher moisture content since square bale was wrapped in a way that the flakes were perpendicular to the ground, which made it easier for the rain to permeate the bale from the top into the bottom.

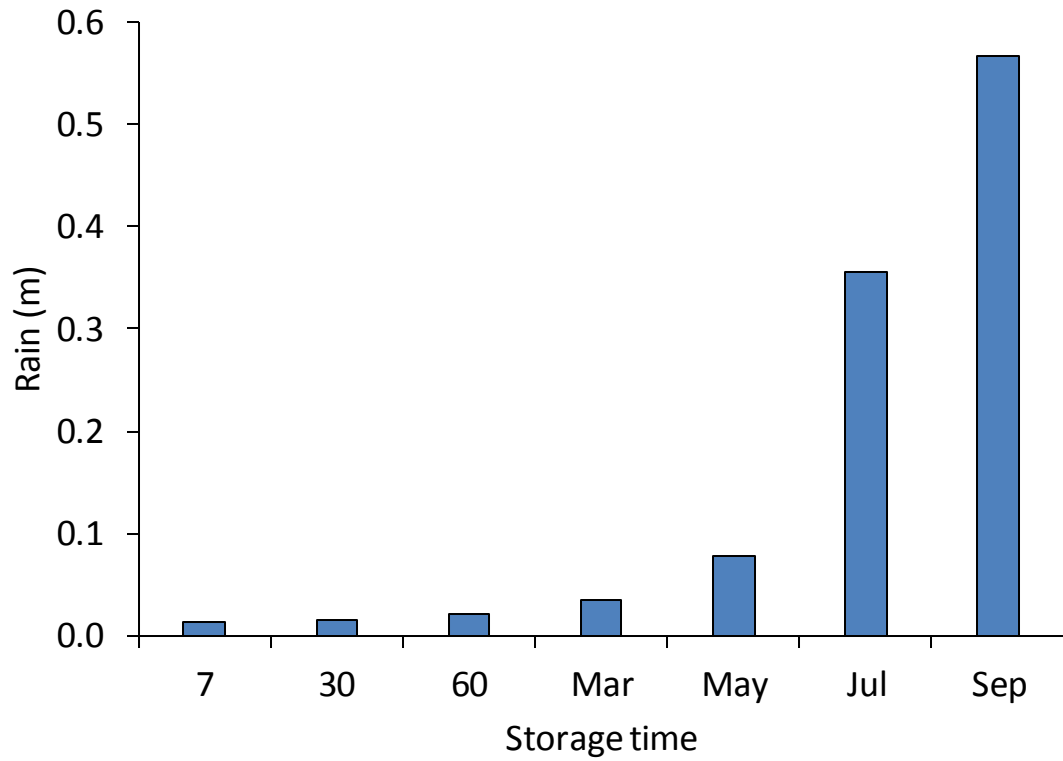


Fig. 4.13. Rainfall accumulation at certain period after round bales storage started. Numbers on x-axis indicate number of days after sampling. On X axis, 7, 30, 60 denotes the days after round bale storage started. Mar denotes day 86 when first samples done, May denotes the day 138 when second sampling done, Jul denotes day 231 when third sampling done, Sep denotes day 310 when fourth sampling done.

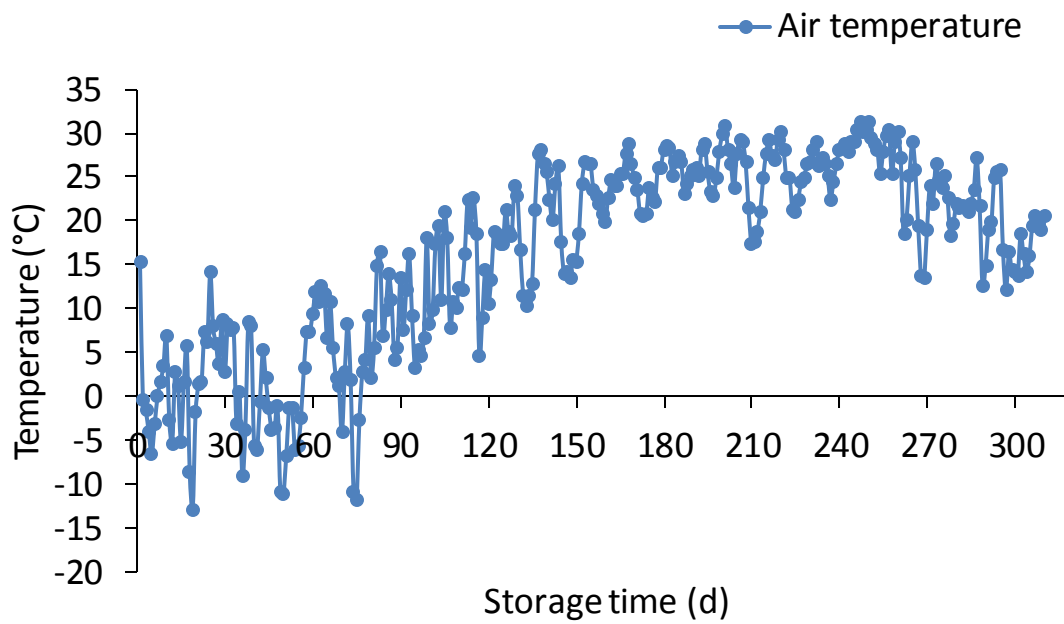


Fig. 4.14. Daily average temperature after round bales storage started.

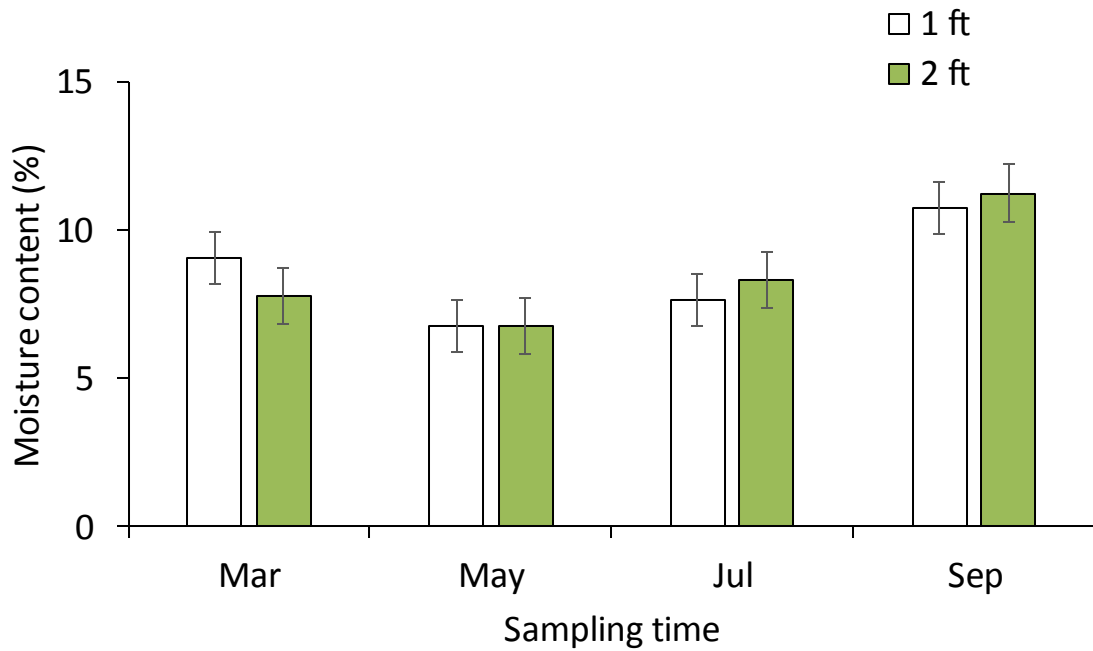


Fig. 4.15. Moisture content of square bale samples. Control denotes samples without mushroom spawn applied. 1 ft denotes sample taken from 0.3 m (1 ft) from round bale surface, 2 ft denotes sample taken from 0.6 m (2 ft) from round surface. Error bars equal mean \pm one standard error.

After dismantling the bales, dry fungal spawn were found in the bales, which are shown in Figs 4.16 A and 4.16 B. The low moisture of 6% to 11% shown in Fig. 4.15 was presumably why *P. ostreatus* did not live and grow.

Due to no fungal growth, the round bale study was regarded as a dry storage study. Three bales from each sampling time were used as triplicates to determine the effect of storage time on biomass degradation, hydrothermolysis pretreatment and enzymatic hydrolysis.

4.4.2.3. Composition of samples during storage time

Initial round bale sample composition is shown in Table 4.1. Glucan, xylan and lignin fractions are shown in Table 4.7. Glucan, xylan and lignin fractions were similar among all sampling times. For 1 ft samples, From March to May, glucan fractions increased by 11%, xylan fractions increased by 10% and lignin fraction decreased by 7%. For 2 ft samples, From March to May, glucan fractions increased by 12%, xylan fractions increased by 10% and lignin fraction decreased by 7%. For both 1 ft samples and 2 ft samples, statistical analysis showed glucan or xylan fractions of March samples were significantly lower than that of May, July and September samples while there were no significant differences for glucan, xylan or lignin fractions among May, July and September samples. Between 1 ft and 2 ft samples, there were no significant differences for glucan, xylan or lignin fractions among all samples times. This phenomenon indicates sampling depth had no effect on composition changes during 9 months of storage.



Fig. 4.16. Dried *P. ostreatus* spawn and no fungus growing on round bales. A: dried *P. ostreatus* spawn in bale 6, B: dried *P. ostreatus* spawn in bale 11.

Table 4.7. Glucan, xylan and lignin fraction round bale samples during storage

		Mar	May	Jul	Sep
1 ft	glucan	39.31±1.20	43.28±1.33	43.28±2.76	43.05±2.23
	xylan	24.87±0.56	25.83±0.62	26.58±0.99	26.51±1.94
	lignin	19.82±0.46	18.85±0.11	19.23±0.41	19.16±0.62
2 ft	glucan	38.74±0.41	43.28±1.33	43.28±1.33	43.36±1.57
	xylan	23.44±0.62	25.83±0.62	25.83±0.62	25.88±0.43
	lignin	20.22±0.31	18.85±0.11	18.85±0.11	18.89±0.33

Values listed are mean of two replicates ± standard deviation.

Minimal biomass components losses during storage were also observed in other studies. Wiseloge et al. (1996) observed 4% cellulose loss and 1.4% hemicellulose loss during 26 weeks storage using large round bales in outside, unprotected field. Shinnery et al. (2006) stored switchgrass indoors for 11 months with a moisture content of 49% and the dry matter loss was only 1.95%. For round bales, moisture content of 6% to 11% and very few switchgrass composition changes throughout storage time indicated that round bales are good for preserving switchgrass hay. In a natural environment, round bales did not receive or retain rain.

4.4.2.4. Composition of pretreated solids after hydrothermolysis pretreatment

Glucan, xylan and lignin fractions of pretreated switchgrass samples are shown in Table 4.8. The values of glucan, xylan and lignin fractions in hydrothermolysis pretreated samples were similar to a previous study (Suryawati et al., 2008). Composition of 1 ft and 2 ft samples were analyzed to determine the effect of sampling depth on composition. For samples from all sampling times pretreated with hydrothermolysis 200/10, there were no differences for glucan fraction, xylan fraction or lignin fraction between 1 ft samples and 2 ft samples. For unwashed samples of all sampling times pretreated with hydrothermolysis 180/20, there were no differences for glucan fraction, xylan fraction or lignin fraction between 1 ft samples and 2 ft samples. For washed samples from all sampling times pretreated with hydrothermolysis 180/20, there were no differences for glucan fraction or lignin fraction between 1 ft samples and 2 ft samples while for xylan fractions 1 ft sample was significantly higher than that of 2 ft samples. The xylan differences may be caused by natural experimental errors since xylan is easily dissolved during hydrothermolysis.

A washing step is necessary to remove inhibitors produced during hydrothermolysis (Frederick et al., 2014). For glucan fractions of 1 ft samples pretreated with hydrothermolysis 200/10, there were no significant differences between unwashed and washed samples for March, July and September samples. Washed samples had higher glucan than unwashed samples for May. For lignin fractions of 1 ft samples pretreated with hydrothermolysis 200/10, there were no significant differences between unwashed and washed samples for March, May and July samples. There was higher lignin in unwashed samples than in washed samples for September. For glucan fraction of 1 ft samples pretreated with hydrothermolysis 180/20, there were significant differences between unwashed and washed samples for all sampling times. Glucan fractions of unwashed samples were lower than those of washed samples. For lignin fraction of 1 ft samples pretreated with hydrothermolysis 180/20, there were no significant differences between unwashed and washed samples for March, May, July or September samples.

For glucan fractions and lignin fractions of 2 ft samples pretreated with hydrothermolysis 200/10, there were no significant differences between unwashed and washed samples for March, May, July or September samples. For glucan fractions and lignin fractions of 2 ft samples pretreated with hydrothermolysis 180/20, washed samples had more glucan than unwashed for March, but there were no significant differences for May, July or September samples between unwashed and washed samples. For lignin fractions of 2 ft samples pretreated with hydrothermolysis 180/20, unwashed samples were significantly lower than washed samples for March while there were no significant differences for May, July or September samples between unwashed and washed samples.

After washing, it is clear that xylan fraction decreased since xylan fractions of unwashed samples were significantly higher than that of washed samples for all sampling times.

For round bales, there were no changes in glucan or lignin fractions at different pretreatment time throughout storage time, which resulted from the minimal composition changes of stored samples. Xylan changes were caused by experiment errors since xylan is easily dissolved during hydrothermolysis pretreatment.

4.4.2.5. Mass balances after hydrothermolysis pretreatment

Glucan and xylan recoveries for 1 ft and 2 ft samples after hydrothermolysis pretreatment 200/10 are shown in Fig. 4.17. Glucan recoveries (Fig. 4.17A) were similar between 1 ft and 2 ft samples among all sampling times. Xylan recoveries (Fig. 4.17B) were similar between 1 ft and 2 ft samples for all sampling times while July samples showed the highest recoveries among all sampling times.

Glucan and xylan recoveries for 1 ft and 2 ft samples after hydrothermolysis pretreatment 180/20 are shown in Fig. 4.18. Glucan recoveries (Fig. 4.18A) were similar between 1 ft and 2 ft samples among all sampling times, in which the July samples had the lowest glucan recoveries. Xylan recoveries (Fig. 4.18B) were similar between 1 ft and 2 ft samples among all sampling times.

4.4.2.6. Degradation products in prehydrolyzate after hydrothermolysis pretreatment

Degradation products acetic acid, formic acid, furfural and 5-HMF after pretreatment 200/10 are shown in Fig. 4.19. For 1 ft samples (Fig. 4.19A), acetic acid, formic acid and furfural yield were the highest for July samples while 5-HMF yield was

Table 4.8. Glucan, xylan and lignin fraction of hydrothermolysis pretreated square bale samples

		Mar	May	Jul	Sep	
200	unwashed	Glucan	63.33±3.78	58.68±4.96	63.10±5.26	63.42±4.02
		Xylan	6.65±0.79	5.96±1.17	6.45±1.28	7.00±1.33
		Lignin	35.96±1.49	35.01±1.39	35.30±0.77	40.07±2.51
	washed	Glucan	65.74±2.95	71.47±3.68	66.74±4.11	66.79±3.94
		Xylan	3.96±0.42	4.68±0.46	4.25±0.51	5.24±0.82
		Lignin	35.95±1.51	37.57±1.18	35.65±1.23	34.55±1.98
1ft	unwashed	Glucan	52.80±3.77	52.64±3.92	56.81±3.81	53.76±2.61
		Xylan	20.32±1.49	19.76±1.46	21.27±1.34	22.48±1.74
		Lignin	28.29±1.02	28.03±1.06	27.53±1.06	27.49±0.85
	washed	Glucan	58.67±3.21	57.37±5.21	61.44±4.37	60.29±3.90
		Xylan	17.26±2.08	16.27±2.15	18.82±1.84	20.68±2.12
		Lignin	28.25±0.60	28.02±0.67	27.51±0.53	28.61±0.73
200	unwashed	Glucan	60.72±3.46	59.42±4.18	63.23±4.22	64.34±3.29
		Xylan	6.19±1.45	4.98±1.52	6.34±1.28	7.59±1.13
		Lignin	36.94±1.74	37.40±1.64	35.51±1.56	34.13±2.12
	washed	Glucan	63.49±4.06	66.41±4.50	68.13±4.26	68.90±3.55
		Xylan	4.17±0.76	3.79±0.76	4.26±0.69	6.41±1.43
		Lignin	36.56±1.11	37.57±1.18	35.65±1.23	34.55±1.98
2ft	unwashed	Glucan	50.73±3.88	52.25±3.73	55.55±3.67	57.34±3.66
		Xylan	18.92±1.47	19.58±1.17	20.14±1.20	20.69±1.44
		Lignin	28.24±1.06	27.51±0.56	26.97±0.51	28.87±1.05
	washed	Glucan	57.14±3.31	54.43±5.29	59.77±4.83	58.83±4.35
		Xylan	17.09±1.66	15.68±1.82	16.46±1.89	18.56±1.95
		Lignin	29.55±1.07	28.40±0.93	27.95±0.88	27.55±1.09

Values listed are mean of two replicates ± standard deviation.

lower than other degradation product yields for all sampling times. For 2 ft samples (Fig. 4.19B), furfural yield of July samples was higher than that of other sampling times while for other degradation products there were no clear trends.

Degradation product yields after pretreatment 180/20 are shown in Fig. 4.20. For 1 ft samples (Fig. 4.20A), furfural and 5-HMF yields were both the highest for March samples. Acetic acid yield increased with increasing storage time. Formic acid yield was similar among all sampling times. For 2 ft samples, furfural and 5-HMF yields were both the highest for March samples. There were no clear trends for formic acid and acetic acid changes throughout storage time.

Djioleu et al. (2014) conducted a large round bale storage study and subsequently did dilute acid pretreatment and enzymatic hydrolysis on stored samples. Field storage decreased production of 5-HMF and increased production of furfural relative to barn storage. Their study did not show if storage had an effect on inhibitors. In this study, there are no clear trends for degradation products yields over storage time.

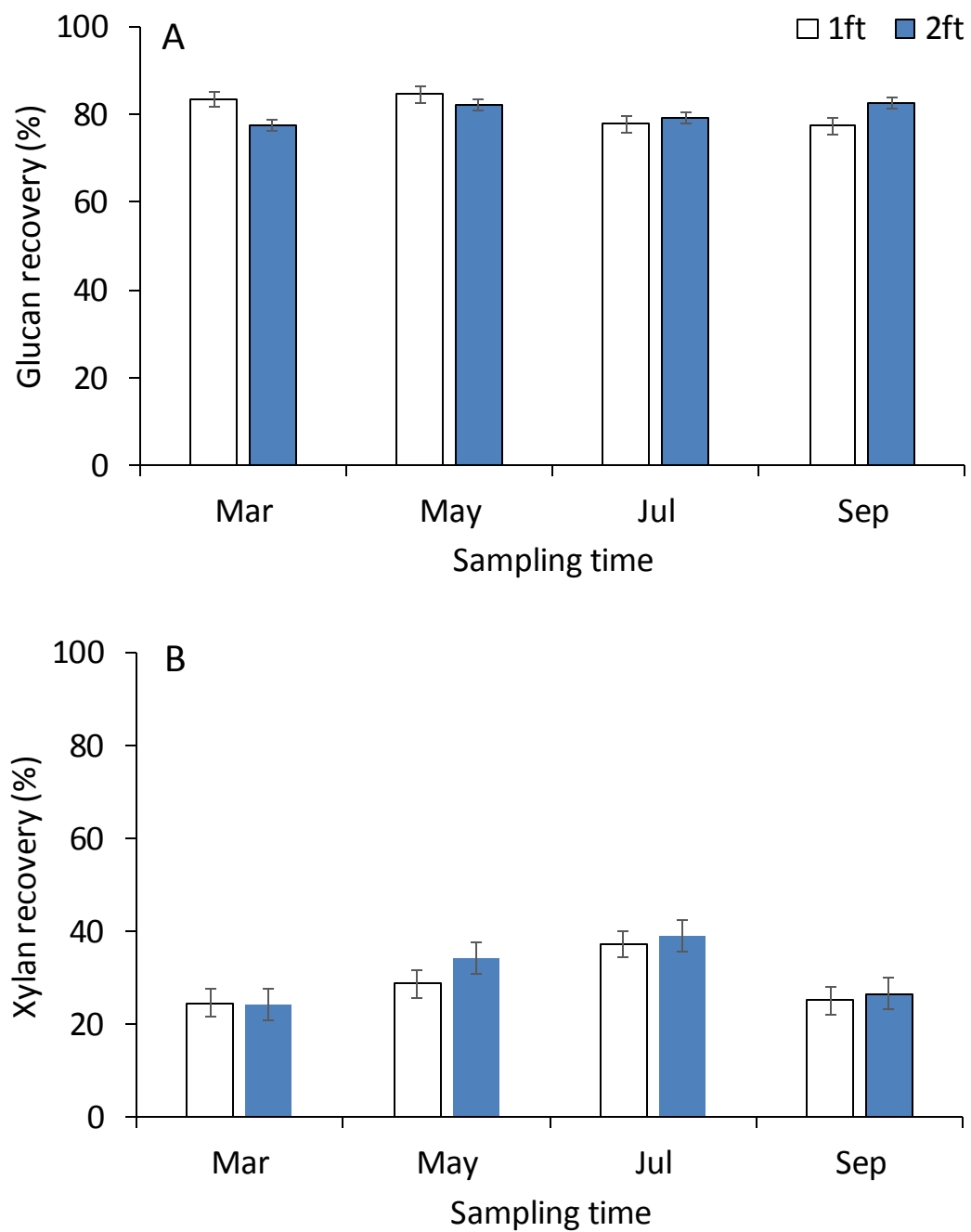


Fig. 4.17. Glucan (A) and xylan (B) recovery after hydrothermolysis pretreatment at 200/10 for round bale samples. Error bars equal mean \pm one standard error.

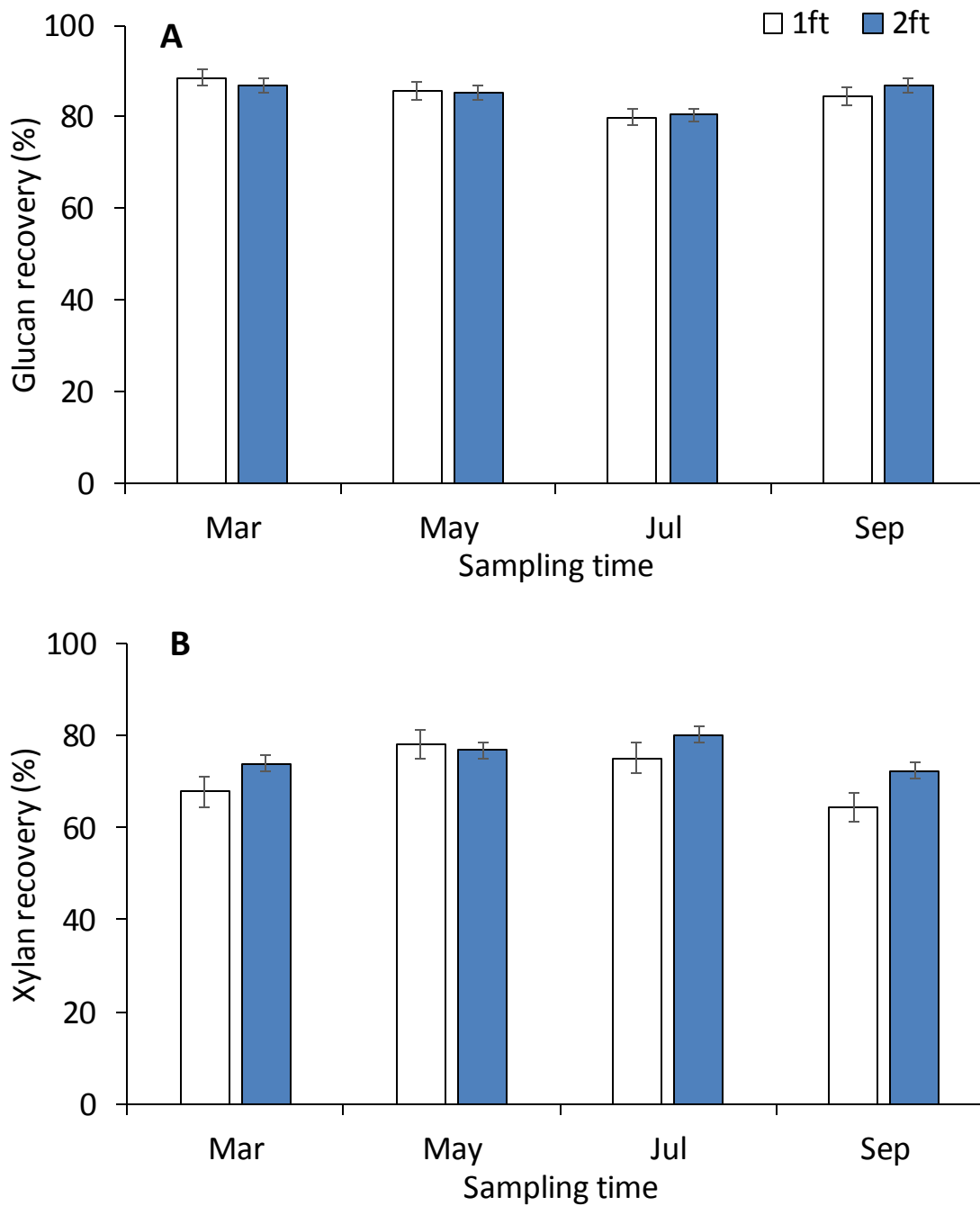


Fig. 4.18. Glucan (A) and xylan (B) recovery after hydrothermolysis pretreatment at 180/20 for round bale samples. Error bars equal mean \pm one standard error.

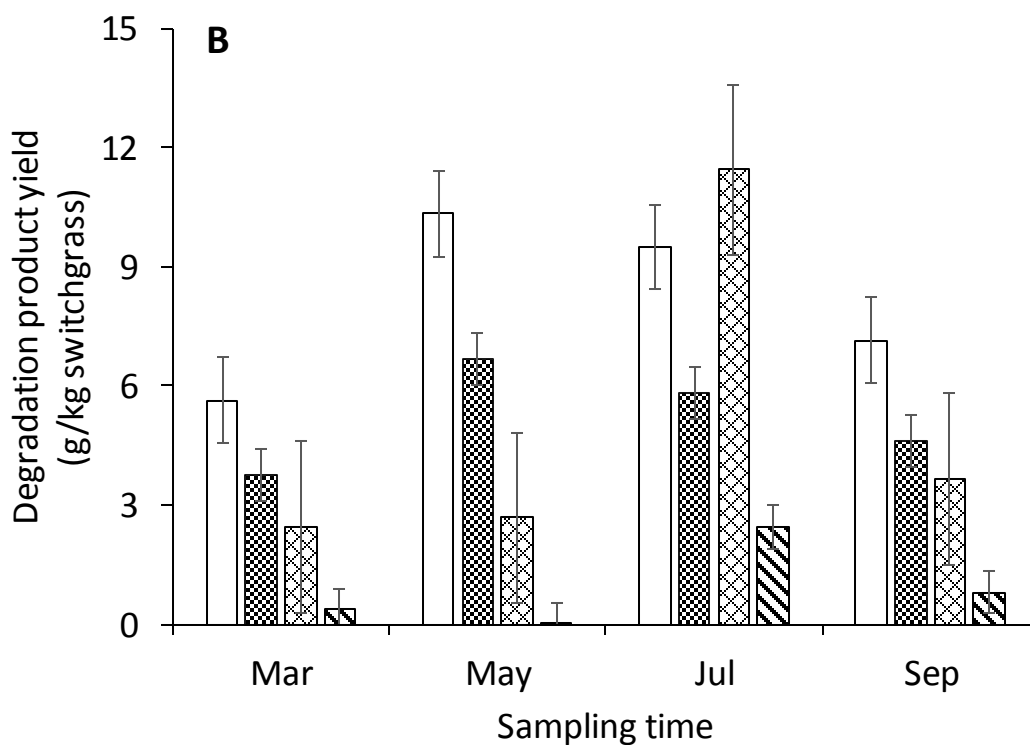
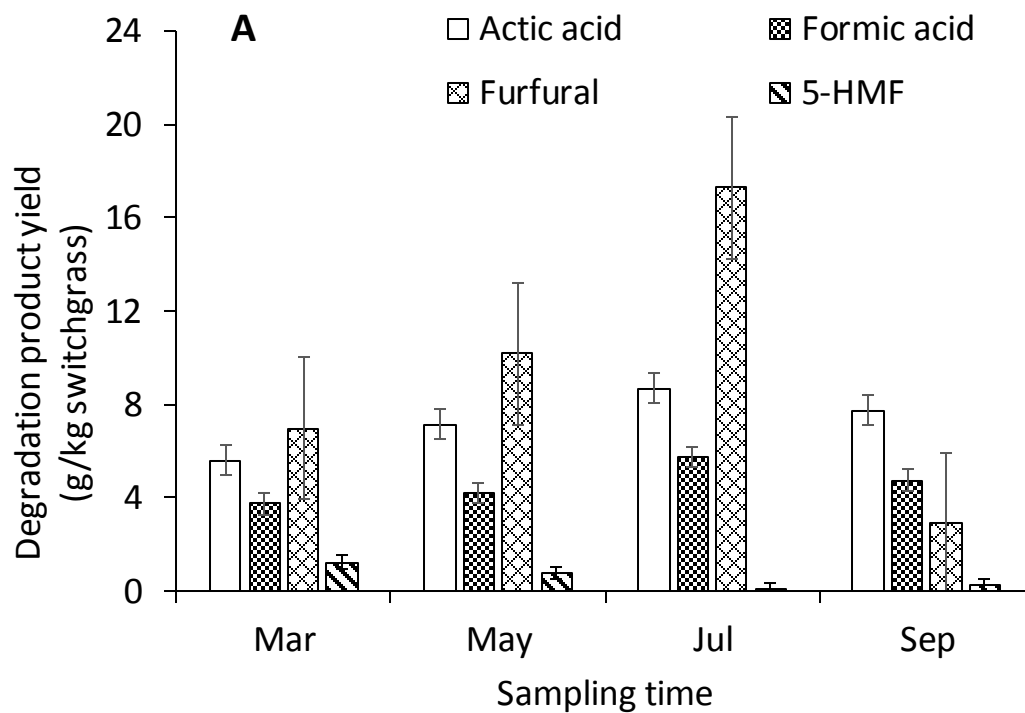


Fig. 4.19. Degradation products yields after hydrothermolysis pretreatment at 200/10 for round bale samples. Degradation products of 1 ft samples are shown in A and degradation products of 2 ft samples are shown in B. Error bars equal mean \pm one standard error.

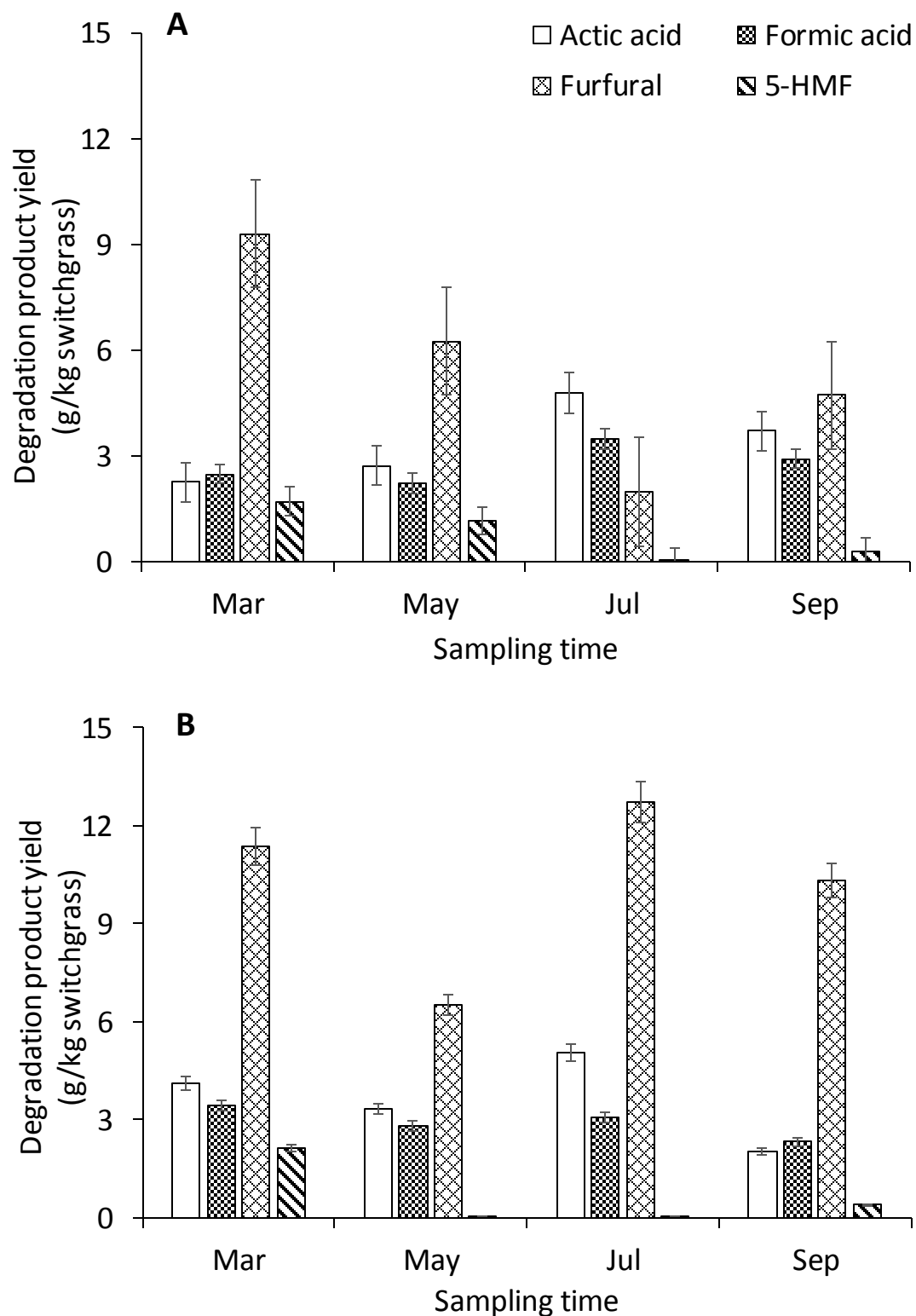


Fig. 4.20. Degradation products yields after hydrothermolysis pretreatment at 180/20 for round bale samples. Degradation products of 1 ft samples are shown in A and degradation products of 2 ft samples are shown in B. Error bars equal mean \pm one standard error.

4.4.2.7. *Enzymatic hydrolysis*

Glucose yields of unwashed and washed pretreated solids after hydrothermolysis pretreatment 200/10 are shown in Fig. 4.21. Glucose yields for 200/10 unwashed samples (Fig. 4.21A) were similar between 1 ft and 2 ft samples among all sampling times except for July samples where 2 ft samples had 40% higher glucose yield than 1 ft samples. For 200/10 washed samples (Fig. 4.21B), glucose yield of 2 ft samples were 1.6 times higher than that of 1 ft samples for May samples. For March, July and September samples, there were no clear trends. Comparing the glucose yield of unwashed and washed March samples, unwashed 1 ft samples had slightly higher yield than that of washed 1 ft samples while unwashed 2 ft samples had slightly lower glucose yield than washed 2 ft samples. For 2 ft samples taken at May, July and September, unwashed samples had higher glucose yield than washed samples.

For all samples, statistical analysis showed that there are no significant differences between unwashed samples and washed samples, which indicates that washing did not increase glucose yield. Various lab studies conducted washing after hydrothermolysis and found that washing significantly increased glucose yield (Frederick et al., 2014; Mosier et al., 2005; Suryawati et al., 2008).

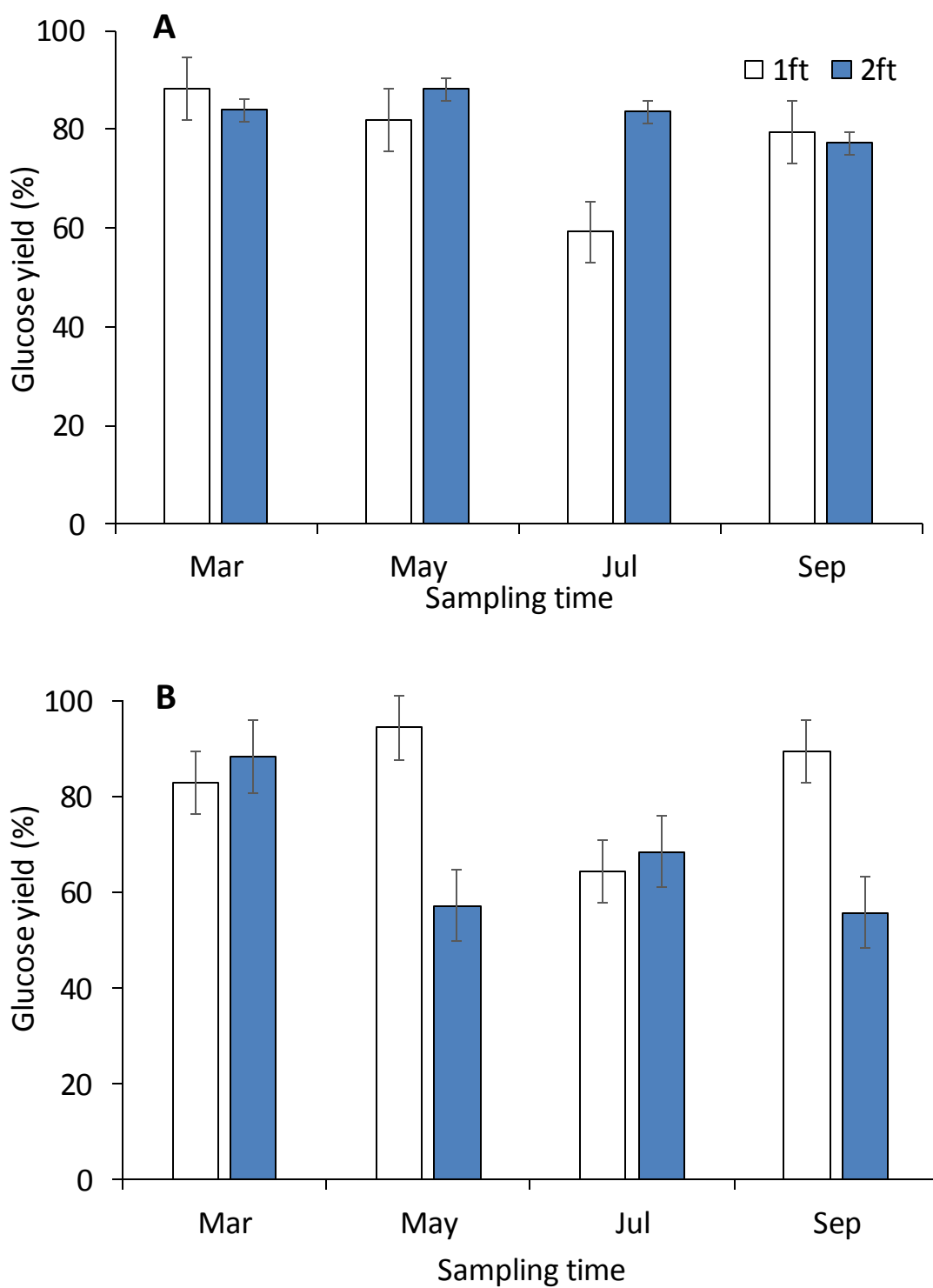


Fig. 4.21. Glucose yield of unwashed (A) and washed (B) pretreated solids after hydrothermolysis pretreatment at 200/10 for round bale samples. Error bars equal mean \pm one standard error.

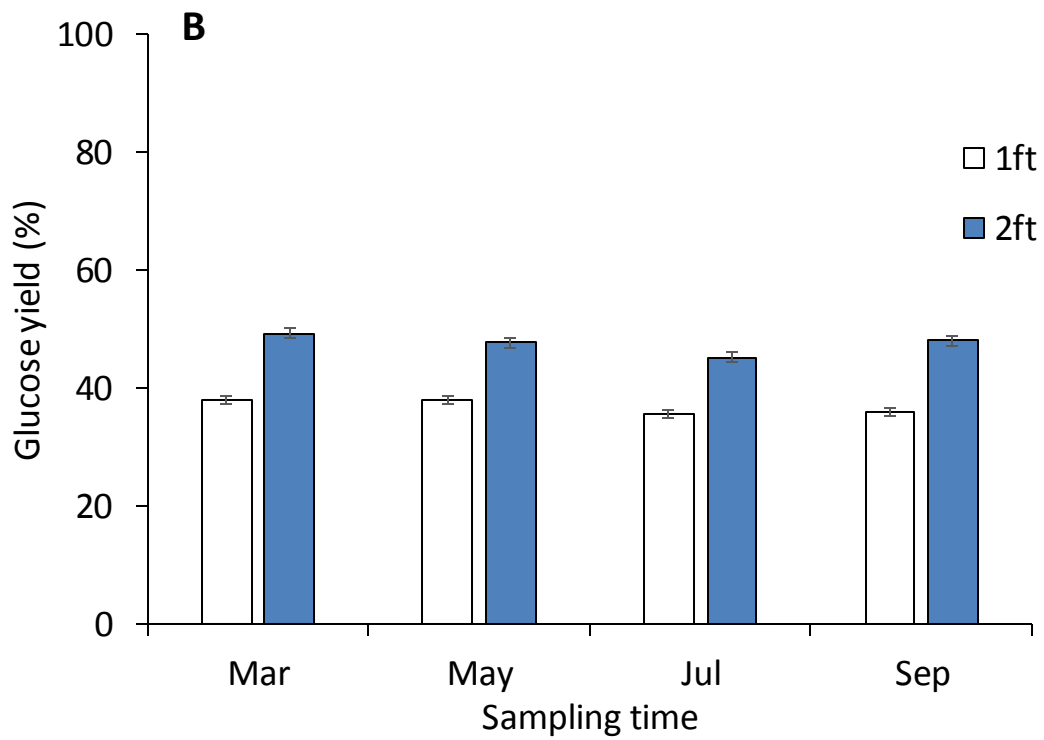
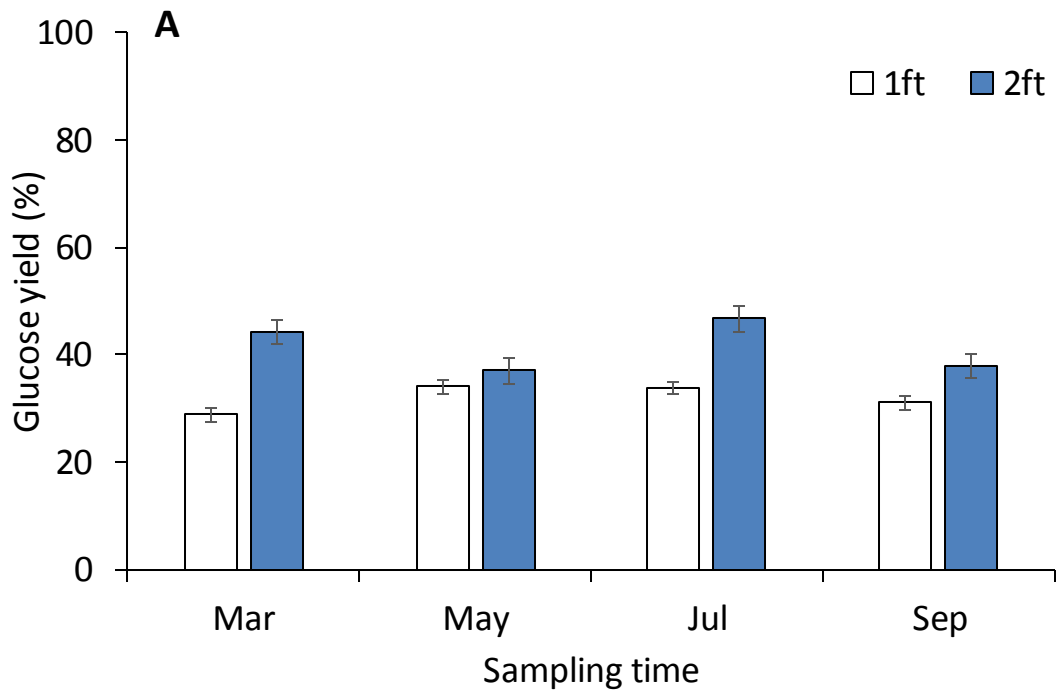


Fig. 4.22. Glucose yield of unwashed (A) and washed (B) pretreated solids after hydrothermolysis pretreatment at 180/20 for round bale samples. Error bars equal mean \pm one standard error.

4.5. Conclusions

Square bales showed ability to receive and retain rain. Moisture in square bales did provide water for fungus to grow, but the moisture was not homogeneously distributed, which resulted in the fungal mycelia failing to run homogeneously through the bale. For square bale samples, fungal treated samples showed from 1.8% to 3.2% higher lignin fractions than untreated samples. There were no clear trends for composition change of hydrothermolysis pretreated samples. There were no clear trends observed for glucan or xylan recovery under hydrothermolysis conditions 200/10, 190/15 and 180/20. For glucose yield of 200/10 pretreated sample, control samples showed from 6% to 14% higher yield than treated samples. There was no clear trend for glucose yields of 180/20 pretreated samples.

Round bales did not receive and retain rain, which resulted in a moisture content of 6% to 11%. It is presumable that *P. ostreatus* could not live with a substrate moisture content of 6% to 11%. However, the low moisture content made round bales a good dry storage method to preserve switchgrass hay, since the glucan, xylan and lignin fractions were similar throughout storage time.

For square bale or round bale, *P. ostreatus* inoculation timing and physical parameters such as temperature and substrate moisture content should be controlled to make *P. ostreatus* grow and degrade switchgrass.

4.6. Acknowledgements

This research was supported by the South Central Sun Grant Initiative, the Oklahoma Bioenergy Center and the Oklahoma Agricultural Experiment Station. We

would like to thank Dr. Michael Buser, Collin Craige, Drew Sutterfield, and Thomas Moore (Department of Biosystems and Agricultural Engineering, OSU) for their work on biomass harvesting, baling, sampling, grinding. Special thanks to Collin Craige for drawing the sampling locations and moisture gradient of square and round bale. The work on pretreated biomass hydrolysis and degradation products analysis in prehydrolyzate done Noaa Frederick and Dr. Julie Carrier (Department of Biological and Agricultural Engineering, University of Arkansas) was greatly appreciated. I owe thanks to Oscar Pardo Planas and Shelyn Gehle for provided assistance on biomass composition analysis and hydrothermolysis pretreatment. I also appreciate the assistance of Dr. Meizhen Xie and Dr. Nurhan Dunford (Robert M. Kerr Food & Agricultural Products Center, OSU) with training of biomass extraction.

4.7. References

- Anonymous. 2002. *S358. 2, Moisture Measurement—Forages*. American Society of Agricultural and Biological Engineers, St. Joseph, MI.
- Bak, J.S., Ko, J.K., Choi, I.-G., Park, Y.-C., Seo, J.-H., Kim, K.H. 2009. Fungal pretreatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw. *Biotechnology and Bioengineering*, **104**(3), 471-482.
- Balan, V., Costa Sousa, L., Chundawat, S.S., Vismeh, R., Jones, A.D., Dale, B. 2008. Mushroom spent straw: a potential substrate for an ethanol-based biorefinery. *Journal of Industrial Microbiology & Biotechnology*, **35**(5), 293-301.
- Blood, D., Studdert, V., Gay, C. 2007. *Saunders Comprehensive Veterinary Dictionary, 3rd ed.* Elsevier, St. Louis, MO.
- Chen, Y., Sharma-Shivappa, R.R., Chen, C. 2007. Ensiling agricultural residues for bioethanol production. *Applied Biochemistry and Biotechnology*, **143**(1), 80-92.
- Cheng, J. 2010. *Biomass to Renewable Energy Processes*. CRC Press, Boca Raton, FL.
- Cui, Z.F., Wan, C.X., Shi, J., Sykes, R.W., Li, Y.B. 2012. Enzymatic Digestibility of Corn Stover Fractions in Response to Fungal Pretreatment. *Industrial & Engineering Chemistry Research*, **51**(21), 7153-7159.
- Digman, M., Shinnars, K., Muck, R., Dien, B. 2010. Full-scale on-farm pretreatment of perennial grasses with dilute acid for fuel ethanol production. *BioEnergy Research*, **3**(4), 335-341.
- Digman, M.F., Shinnars, K.J., Dien, B.S., Hatfield, R., Li, X., Muck, R., Weimert, P. 2007. On-farm pretreatment technologies for improving enzymatic degradability of cellulose and hemicellulose present in perennial grass. *ASABE Annual International Meeting*, Minneapolis, MN.
- Djioleu, A.C., Sverzut, C.B., Martin, E.M., Childress, E., Johnson, C., West, C.P., Carrier, D.J. 2014. Effects of harvest and storage of switchgrass on the recovery of carbohydrates during dilute acid pretreatment and enzymatic hydrolysis. *Forage and Grazinglands*, **12**(1), 1-6.
- Dogaris, I., Karapati, S., Mamma, D., Kalogeris, E., Kekos, D. 2009. Hydrothermal processing and enzymatic hydrolysis of sorghum bagasse for fermentable carbohydrates production. *Bioresource Technology*, **100**(24), 6543-6549.
- Faga, B.A., Wilkins, M.R., Banat, I.M. 2010. Ethanol production through simultaneous saccharification and fermentation of switchgrass using *Saccharomyces cerevisiae*

- D5A and thermotolerant *Kluyveromyces marxianus* IMB strains. *Bioresource Technology*, **101**(7), 2273-2279.
- Frederick, N., Zhang, N., Ge, X., Xu, J., Pelkki, M., Martin, E., Carrier, D.J. 2014. Poplar (*Populus deltoides* L.): The effect of washing pretreated biomass on enzymatic hydrolysis and fermentation to ethanol. *ACS Sustainable Chemistry & Engineering*, **2**(7), 1835-1842.
- Harrison, J., Fransen, S. 1991. Silage management in North America. in: *Field Guide for Hay and Silage Management in North America.*, (Eds.) K.K. Bolson, J.E. Baylor, M.E. McCullough, National Feed Ingredients Association. Arlington, VA.
- Hendriks, A.T.W.M., Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*, **100**(1), 10-18.
- Jing, X., Zhang, X., Bao, J. 2009. Inhibition performance of lignocellulose degradation products on industrial cellulase enzymes during cellulose hydrolysis. *Applied Biochemistry and Biotechnology*, **159**(3), 696-707.
- Kashangura, C. 2008. Optimisation of the growth conditions and genetic characterisation of *Pleurotus* species, PhD diss., University of Zimbabwe.
- Kim, Y., Ximenes, E., Mosier, N.S., Ladisch, M.R. 2011. Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. *Enzyme and Microbial Technology*, **48**(4-5), 408-415.
- Koegel, R.G., Straub, R.J., Shimmers, K.J. 1985. Ammonia treatment of large and small hay packages. *Transactions of the ASAE-American Society of Agricultural Engineers (USA)*, **28**(3), 726-730.
- Kumar, A., Sokhansanj, S. 2007. Switchgrass (*Panicum virgatum*, L.) delivery to a biorefinery using integrated biomass supply analysis and logistics (IBSAL) model. *Bioresource Technology*, **98**(5), 1033-1044.
- Lalman, D.L. 2002. *Ammoniation of low quality roughages*. Division of Agricultural Sciences and Natural Resources, Oklahoma State University.
- Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Wojtaś-Wasilewska, M., Cho, N.-S., Hofrichter, M., Rogalski, J. 1999. Biodegradation of lignin by white rot fungi. *Fungal Genetics and Biology*, **27**(2-3), 175-185.
- Liu, Z.-H., Qin, L., Jin, M.-J., Pang, F., Li, B.-Z., Kang, Y., Dale, B.E., Yuan, Y.-J. 2013. Evaluation of storage methods for the conversion of corn stover biomass to sugars based on steam explosion pretreatment. *Bioresource Technology*, **132**(0), 5-15.

- Moore, T., Buser, M.D., Miller, E.A., III, V.P.S., Huhnke, R.L. 2014. Bale sampling tools used in OSU's BRDI project *2014 ASABE and CSBE/SCGAB Annual International Meeting*, Montreal, QC, Canada.
- Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M.R. 2005. Optimization of pH controlled liquid hot water pretreatment of corn stover. *Bioresource Technology*, **96**(18), 1986-1993.
- Overend, R., Chornet, E. 1989. Steam and aqueous pretreatments: are they prehydrolyses. in: *Wood Processing and Utilization*, (Eds.) J.F. Kennedy, G.O. Phillips, P.A. Williams, Ellis Horwood. Chichester, UK, pp. 395-400.
- Pérez, J., Muñoz-Dorado, J., de la Rubia, T., Martínez, J. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International Microbiology*, **5**(2), 53-63.
- Pérez, J.A., González, A., Oliva, J.M., Ballesteros, I., Manzanares, P. 2007. Effect of process variables on liquid hot water pretreatment of wheat straw for bioconversion to fuel-ethanol in a batch reactor. *Journal of Chemical Technology and Biotechnology*, **82**(10), 929-938.
- Rabinovich, M., Bolobova, A., Vasilchenko, L. 2004. Fungal decomposition of natural aromatic structures and xenobiotics: a review. *Applied Biochemistry and Microbiology*, **40**(1), 1-17.
- Ramachandriya, K.D., Wilkins, M., Pardo-Planas, O., Atiyeh, H.K., Dunford, N.T., Hizirolu, S. 2014. Simultaneous saccharification and fermentation of Eastern redcedar heartwood and sapwood using a novel size reduction technique. *Bioresource Technology*, **161**(0), 1-9.
- Rentizelas, A.A., Tolis, A.J., Tatsiopoulos, I.P. 2009. Logistics issues of biomass: The storage problem and the multi-biomass supply chain. *Renewable and Sustainable Energy Reviews*, **13**(4), 887-894.
- Richard, T., Proulx, S., Moore, K., Shouse, S. 2001. Ensilage technology for biomass pre-treatment and storage. *ASAE Annual International Meeting, Sacramento, CA*.
- Riva, S. 2006. Laccases: blue enzymes for green chemistry. *Trends in Biotechnology*, **24**(5), 219-226.
- Sanderson, M., Reed, R., McLaughlin, S., Wullschleger, S., Conger, B., Parrish, D., Wolf, D., Taliaferro, C., Hopkins, A., Ocumpaugh, W. 1996. Switchgrass as a sustainable bioenergy crop. *Bioresource Technology*, **56**(1), 83-93.
- Sanderson, M.A., Egg, R.P., Wiseloge, A.E. 1997. Biomass losses during harvest and storage of switchgrass. *Biomass and Bioenergy*, **12**(2), 107-114.

- Schmer, M.R., Vogel, K.P., Mitchell, R.B., Perrin, R.K. 2008. Net energy of cellulosic ethanol from switchgrass. *Proceedings of the National Academy of Sciences*, **105**(2), 464-469.
- Selig, M., Weiss, N., Ji, Y. 2008. *Enzymatic Saccharification of Lignocellulosic Biomass*. National Renewable Energy Laboratory, Golden, CO.
- Shi, J., Sharma-Shivappa, R.R., Chinn, M.S. 2009. Microbial pretreatment of cotton stalks by submerged cultivation of *Phanerochaete chrysosporium*. *Bioresource Technology*, **100**(19), 4388-4395.
- Shiners, K., Boettcher, G., Muck, R., Weimer, P., Casler, M. 2006. Drying, harvesting and storage characteristics of perennial grasses as biomass feedstocks. *Proceedings of the American Society of Agricultural and Biological Engineers International (ASABE)*. pp. 1-19.
- Shuai, L., Yang, Q., Zhu, J., Lu, F., Weimer, P., Ralph, J., Pan, X. 2010. Comparative study of SPORL and dilute-acid pretreatments of spruce for cellulosic ethanol production. *Bioresource technology*, **101**(9), 3106-3114.
- Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Wolfe, J. 2008. *Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples*. National Renewable Energy Laboratory, Golden, CO.
- Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. 2006. *Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples*. National Renewable Energy Laboratory, Golden, CO.
- Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2007. *Determination of Structural Carbohydrates and Lignin in Biomass*. National Renewable Energy Laboratory, Golden, CO.
- Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. 2005. *Determination of Extractives in Biomass*. National Renewable Energy Laboratory, Golden, CO.
- Spáčil, Z., Nováková, L., Solich, P. 2008. Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography. *Talanta*, **76**(1), 189-199.
- Suryawati, L., Wilkins, M.R., Bellmer, D.D., Huhnke, R.L., Maness, N.O., Banat, I.M. 2008. Simultaneous saccharification and fermentation of Kanlow switchgrass pretreated by hydrothermolysis using *Kluyveromyces marxianus* IMB4. *Biotechnology and Bioengineering*, **101**(5), 894-902.

- Taniguchi, M., Suzuki, H., Watanabe, D., Sakai, K., Hoshino, K., Tanaka, T. 2005. Evaluation of pretreatment with *Pleurotus ostreatus* for enzymatic hydrolysis of rice straw. *Journal of Bioscience and Bioengineering*, **100**(6), 637-643.
- ten Have, R., Teunissen, P.J. 2001. Oxidative mechanisms involved in lignin degradation by white-rot fungi. *Chemical Reviews*, **101**(11), 3397-3414.
- Tilman, D., Hill, J., Lehman, C. 2006. Carbon-negative biofuels from low-input high-diversity grassland biomass. *Science*, **314**(5805), 1598-1600.
- Wan, C., Li, Y. 2011a. Effectiveness of microbial pretreatment by *Ceriporiopsis subvermispota* on different biomass feedstocks. *Bioresource Technology*, **102**(16), 7507-7512.
- Wan, C., Li, Y. 2010. Microbial pretreatment of corn stover with *Ceriporiopsis subvermispota* for enzymatic hydrolysis and ethanol production. *Bioresource Technology*, **101**(16), 6398-6403.
- Wan, C.X., Li, Y.B. 2011b. Effectiveness of microbial pretreatment by *Ceriporiopsis subvermispota* on different biomass feedstocks. *Bioresource Technology*, **102**(16), 7507-7512.
- Wang, D., Sakoda, A., Suzuki, M. 2001. Biological efficiency and nutritional value of *Pleurotus ostreatus* cultivated on spent beer grain. *Bioresource Technology*, **78**(3), 293-300.
- Ward, M., Ward, J. 1987. Ammoniation of warm-season grass hay for gestating beef cows. *Journal of Animal Science*, **65**(2), 359-365.
- Wiseloge, A., Agblevor, F., Johnson, D., Deutch, S., Fennell, J., Sanderson, M. 1996. Compositional changes during storage of large round switchgrass bales. *Bioresource Technology*, **56**(1), 103-109.
- Xu, C., Ma, F., Zhang, X., Chen, S. 2010. Biological pretreatment of corn stover by *Irpex lacteus* for enzymatic hydrolysis. *Journal of Agricultural and Food Chemistry*, **58**(20), 10893-10898.
- Yang, B., Wyman, C.E. 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. *Biotechnology and bioengineering*, **86**(1), 88-98.
- Yu, H., Zhang, X., Song, L., Ke, J., Xu, C., Du, W., Zhang, J. 2010. Evaluation of white-rot fungi-assisted alkaline/oxidative pretreatment of corn straw undergoing enzymatic hydrolysis by cellulase. *Journal of Bioscience and Bioengineering*, **110**(6), 660-664.

Yuan, J.S., Tiller, K.H., Al-Ahmad, H., Stewart, N.R., Stewart, C.N. 2008. Plants to power: bioenergy to fuel the future. *Trends in Plant Science*, **13**(8), 421-429.

Zharare, G.E., Kabanda, S.M., Poku, J.Z. 2010. Effects of temperature and hydrogen peroxide on mycelial growth of eight *Pleurotus* strains. *Scientia Horticulturae*, **125**(2), 95-102.

CHAPTER V

5. Lab scale white rot fungi *Pleurotus ostreatus* pretreatment to enhance simultaneous saccharification and fermentation of switchgrass

5.1. Abstract

The white rot fungus *Pleurotus ostreatus* has exhibited the ability to selectively degrade lignin. Switchgrass with 50% or 75% moisture content (MC) was statically pretreated by *P. ostreatus* at 28 °C for a total of 80 days. Sampling was conducted every 20 days during pretreatment. Liquid culture was used as the inoculum and the fungus loading was either 5 mL (20,000 CFUs) or 20 mL (80,000 CFUs) per 100 g dry basis switchgrass for each MC. After fungal pretreatment, simultaneous saccharification and fermentation (SSF) was performed using yeast *Saccharomyces cerevisiae* D₅A. The highest lignin degradation was 52% and the highest ethanol yield during SSF was 45% for samples with 75% MC and 5 mL inoculum after 80 days pretreatment. Meanwhile, the highest xylose yield observed was 15.6% for samples with 75% MC and 5 mL inoculum at fungal pretreatment of 40 days. Scanning electronic microscopy (SEM) images showed tiny pits and enlarged holes and breakages on fungal treated samples. The physical parameters of *P. ostreatus* cultivation, biomass degradation and ethanol

production data in this study could provide reference for large scale *P. ostreatus* pretreatment on switchgrass.

5.2. Introduction

Lignocellulosic biomass feedstocks have been considered to be sustainable, abundant, and environmentally beneficial energy sources (Limayem & Ricke, 2012; Tilman et al., 2006). Switchgrass can produce significantly greater quantities of energy per unit of land compared to other lignocellulosic biomass feedstocks while using moderate inputs (Schmer et al., 2008).

In the process of converting lignocellulosic biomass to fermentable sugars, pretreatment is viewed as one of the most difficult and expensive processing steps (Dibble et al., 2011). Pretreatment also shows great potential for improvement of efficiency and lowering of cost by decreasing the biomass recalcitrance to saccharification and making cellulose more accessible (Chen & Dixon, 2007; Kumar et al., 2009). A low cost and advanced pretreatment process is crucial to the commercial production of cellulosic ethanol (Alvira et al., 2010). Prevalent pretreatment technologies are physical and/or chemical pretreatments that are usually associated with high temperatures and/or high pressures, and high input of chemicals (Digman et al., 2007). Pretreatment technologies such as liquid hot water, steam explosion, dilute acid and alkaline pretreatment have high energy needs and/or require large amounts of chemical inputs. These factors not only result in high pretreatment cost, but they also can result in production of soluble inhibitors that hamper enzyme and yeast activity for enzymatic hydrolysis and/or fermentation (Jing et al., 2009). The presence of inhibitors requires a washing step or another means of inhibitor neutralization to be added (Kim et al., 2011).

Agriculturally produced feedstocks are harvested seasonally and require storage from several weeks to several months in order to establish a year-round biomass supply for a cellulosic ethanol plant (Athmanathan et al., 2014). Biomass wet storage serving as pretreatment has been proposed as a cost effective alternative to increase biomass degradability and feedstock susceptibility to enzymatic hydrolysis (Digman et al., 2010b; Richard et al., 2001). Wet storage for several months is reported to increase the degradability of biomass by either allowing milder pretreatment or possibly eliminating the need for pretreatment at the biorefinery, thereby providing better return for the farmer (Digman et al., 2010a). However, on-farm wet storage is limited to ambient temperature and pressure conditions.

Biological pretreatments are promising pretreatment technologies that operate under mild conditions with low energy use and produce no inhibitors (Wan & Li, 2010a). Disadvantages of fungal pretreatment on biomass include long incubation time, high moisture content (MC) requirement and consumption of carbohydrates (van Kuijk et al., 2015). The requirements of mild conditions and long incubation time make it possible to incorporate biological pretreatment into biomass wet storage.

Some white rot fungi are capable of degrading lignin while leaving the cellulose and hemicellulose relatively intact (Eggert et al., 1996; Mukherjee & Nandi, 2004). The multiple enzymes (ligninolytic enzyme systems and hydrolytic enzyme systems) and small molecules like hydrogen peroxide and hydroxyl radicals that are secreted by white rot fungi have been found to effectively unpack the polysaccharide components from layers of lignin (Balan et al., 2008; Leonowicz et al., 1999). *Pleurotus ostreatus*, as the most commercially important strain for the cultivation of oyster mushrooms, is one of the

white rot fungi that exhibit ability to degrade the lignin fraction of biomass. Taniguchi et al. (2005) investigated the pretreatment of rice straw by *P. ostreatus* and found that 41% of Klason lignin (acid insoluble lignin) was removed while 17% of cellulose and 48% of hemicellulose were removed. *P. ostreatus* grows widely in temperate climates, which switchgrass is also widely adapted in.

To apply *P. ostreatus* pretreatment on switchgrass during storage, substrate MC is one of the most important factors. Also, fungus inoculum size affects the cost of fungal pretreatment. This study investigated the effect of varying substrate MC and white rot fungi inoculum size on switchgrass degradation and ethanol yield through simultaneous saccharification and fermentation (SSF). The ligninolytic enzymes activities of *P. ostreatus* under different treatment conditions were also evaluated.

5.3. Methods

5.3.1. Switchgrass feedstock

Kanlow switchgrass (*Panicum virgatum* var. Kanlow) was harvested from the Oklahoma State University Plant and Soil Sciences Research Center and ground with a hammer mill fitted with a 13 mm screen (Model E9506, Bliss Industries, Ponca City, OK, USA). Switchgrass was stored at 4 °C until it was used.

For preliminary tests described later in this chapter, samples that were sterilized and ground, sterilized and not ground, not sterilized and ground, and not sterilized and not ground were used to determine the effects of sterilization and grinding on ethanol yield after fungal pretreatment. The term “sterilized” referred to samples autoclaved at 121 °C for 30 min. The term “not sterilized” referred to fungal treated samples that were

directly introduced to SSF without autoclaving. The term “ground” referred to fungal treated samples that were ground to a size of 5 mm in an aseptic blender. The term “not ground” referred to fungal treated 13 mm samples directly introduced to SSF.

5.3.2. Fungus strain and inoculum

P. ostreatus as grain spawn was purchased from Mushroom Mountain (Easley, SC, USA). The spawn was stored at room temperature for over one month until the fruiting bodies grew out. Several internal tissue pieces excised from the fruiting body were taken and placed on MBCD medium (malt extract 15 g/L, yeast extract 1 g/L, tryptone 1 g/L, sucrose 1 g/L, Benlate 50 SP (containing 50% benomyl) 10 mg/L, Botran 75 WP (containing 75% dichloran) 2mg/L, chloramphenicol 100 mg/L) to isolate *P. ostreatus* and suppress the growth of bacteria and other unwanted fungi. After 5 days of incubation at 28 °C in the dark, the plate was observed under stereomicroscope and hyphae with clamp connections were found at the periphery of the colony. Clamp connections are a key characteristic of most Agaricomycetes Basidiomycota, the class of fungi to which *P. ostreatus* belongs. The isolated fungus was used in this study.

P. ostreatus was subcultured by cutting one agar plug of hyphal tip from the growing margin of the isolation culture and transferring it into the centers of new MBCD plates. For *P. ostreatus* culture maintenance, cultures sealed with wax film were incubated at 28 °C for 5 days and then kept at 4 °C for storage. Monthly subculture was conducted. Before using the fungus in an experiment, subculturing was done twice at 28 °C to obtain vigorous *P. ostreatus* cultures.

For liquid fungus inoculum preparation, five agar plugs with *P. ostreatus* mycelium from the plate cultures were inoculated into a 250 mL Erlenmeyer flask containing 30 mL of liquid medium (malt extract 15 g/L, sucrose 2 g/L, yeast extract 0.8 g/L, CuSO₄ 0.01mg/L) with foam plug closures (Parenti et al., 2013). After static incubation at 28 °C for 10 days in the dark, the liquid cultures from different flasks were combined and blended in an aseptic blender for 3 cycles of 15 seconds with 1 minute interval between cycles to obtain a homogeneous mycelial suspension as the inoculum (Patel et al., 2009; Tinoco et al., 2011). A portion of the mycelial suspension was reserved and diluted in series to measure the viable fungus concentration present as colony-forming units (CFUs).

5.3.3. Fungal pretreatment

Deionized water was added to 100 g dry basis switchgrass to obtain MCs of 50% or 75% to start fungus pretreatment in one quart glass jars. The cap of each glass jar was replaced by fiberglass to block microorganisms and ensure aeration. The jars were shaken vigorously to mix biomass and water and sterilized at 121 °C for 30 min twice with an overnight interval. After the samples were cooled to room temperature, two fungus loading levels: 5 mL and 20 mL of mycelial suspension with 4,000 CFUs/mL, were inoculated onto sterilized switchgrass at two MC levels (50% and 75%). For switchgrass samples with 50% MC, either 5 mL of mycelial suspension (20,000 CFUs) were added to 100 g dry basis switchgrass and 95 g deionized water, or 20 mL of mycelial suspension (20,000 CFUs) and 80 g deionized water were added to 100 g dry basis switchgrass. For switchgrass samples with 75% MC, either 5 mL of mycelial suspension and 295 g deionized water, or 20 mL of mycelial suspension and 280 g deionized water were added

to 100 g dry basis switchgrass. The same amount of liquid broth without fungus was added to sterilized switchgrass as controls. All jars containing 75% moisture switchgrass were placed in one incubator with saturated sodium chloride (NaCl) solution for maintaining humidity and all jars containing 50% moisture switchgrass were placed in another incubator with saturated magnesium nitrate ($Mg(NO_3)_2$) solution for maintaining humidity. The jars were shaken every three days by hand to make the mycelium completely colonize the biomass. The experiment was run in triplicate. Fungal pretreatment was conducted for a total of 80 days and sampling was conducted every 20 days. Samples were subjected to biomass composition analysis, SSF and ligninolytic enzymes extraction and activity assay.

5.3.4. Biomass compositional analysis

5.3.4.1. Structural carbohydrates and lignin determination

Raw switchgrass and fungal treated switchgrass from each sampling period were further ground to 2 mm for composition analysis using a Thomas-Wiley mill (Model 4, Arthur H. Thomas Co., Philadelphia, PA, USA). Switchgrass composition analysis was determined by NREL standard methods. Total solids content of biomass was determined according to Sluiter et al. (2008). The structural carbohydrates and lignin content were determined using the method described by Sluiter et al. (2007) after biomass extraction according to Sluiter et al. (2005). For biomass extraction, biomass was extracted by deionized water followed by 190 proof ethanol using an Accelerated Solvent Extractor, ASE® 300 system (Dionex Corporation, Sunnyvale, CA, USA). Water and ethanol extractives were air dried in a fume hood until the weight was constant. For structural

sugars and lignin determination, acid soluble lignin (ASL) after acid hydrolysis of biomass was analyzed at 205 nm using a UV-Vis spectrophotometer (Cary 50 Bio, Varian Inc, Palo Alto, CA, USA). Sugars produced from acid hydrolysis of structural carbohydrates were analyzed using HPLC (Model 1100, Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID) and a Bio-Rad Aminex HPX-87P column (Bio-Rad, Sunnyvale, CA, USA). Deionized water was used as an eluent at a flow rate of 0.6 ml/min and a column temperature of 85 °C. The total run time for each sample was 30 min. Dry matter loss and cellulose, hemicellulose and lignin losses were defined as the percentage of reduction after pretreatment of the corresponding fraction in the raw biomass. Dry matter loss and cellulose, hemicellulose and lignin losses were calculated using equations 5.1, 5.2, 5.3 and 5.4, respectively.

$$\text{Dry matter loss} = 100 - \frac{[\text{Dry matter left}_d]}{100} * 100\% \quad 5.1$$

where $[\text{Dry matter left}_d]$, dry matter left after fungal pretreatment time d (g), 100, initial dry matter put into fungal pretreatment (g).

$$\text{Cellulose loss} = 1 - \frac{[\text{Dry matter left}_d] * [\text{Cellulose fraction}_d]}{100 * [\text{Cellulose fraction}]} * 100\% \quad 5.2$$

where $[\text{Dry matter left}_d]$, dry matter left after fungal pretreatment time d (g),

$[\text{Cellulose fraction}_d]$, cellulose fraction of fungal treated samples after different fungal pretreatment time, 100, initial dry matter put into fungal pretreatment (g),

$[\text{Cellulose fraction}]$, initial cellulose fraction of switchgrass samples put into fungal pretreatment.

$$\text{Hemicellulose loss} = 1 - \frac{[\text{Dry matter left}_d] * [\text{Hemicellulose fraction}_d]}{100 * [\text{Hemicellulose fraction}]} * 100\%$$

5.3

where $[\text{Dry matter left}_d]$, dry matter left after fungal pretreatment time d (g), $[\text{Cellulose fraction}_d]$, cellulose fraction of fungal treated samples after fungal pretreatment time d, 100, initial dry matter put into fungal pretreatment (g), $[\text{Cellulose fraction}]$, initial cellulose fraction of switchgrass samples put into fungal pretreatment.

$$\text{Lignin loss} = 1 - \frac{[\text{Dry matter left}_d] * [\text{Lignin fraction}_d]}{100 * [\text{Lignin fraction}]} * 100\%$$

5.4

where $[\text{Dry matter left}_d]$, dry matter left after fungal pretreatment time d (g), $[\text{Lignin fraction}_d]$, lignin fraction of fungal treated samples after fungal pretreatment time d, 100, initial dry matter put into fungal pretreatment (g), $[\text{Lignin fraction}]$, initial lignin fraction of switchgrass samples put into fungal pretreatment(%).

5.3.5. SSF

5.3.5.1. Enzymes, yeast strain and inoculum

Celluclast 1.5 L and Novozym 188 (Novozyme, Franklinton, NC, USA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The cellulase activity of Celluclast 1.5 L was determined using the filter paper assay (Adney & Baker, 1996). Cellobiase activity of Novozym 188 was determined by adding 1 g of 5% (w/w) enzyme solution to 9 g of 10 g/L cellobiose solution in 50 mM pH 4.8 sodium acetate buffer and incubating them in 50 °C for 3 min. Glucose produced was determined by a

hexokinase glucose assay reagent from Sigma–Aldrich (G3293, St. Louis, MO, USA).

One CBU of cellobiase activity is defined as two μmol of glucose released per min. Other analytical grade chemicals required for the assays were purchased from Sigma–Aldrich (St. Louis, MO, USA).

5.3.5.2. *Yeast strain*

Saccharomyces cerevisiae D₅A yeast was used for SSFs. The yeast was maintained at 4 °C and subcultured periodically on YPD agar slants containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar. Prior to fermentation, 100 ml preculture was prepared by transferring a loopful of yeast cells into YPD medium containing 10 g/L yeast extract, 20 g/L peptone and 50 g/L glucose in a 250 mL baffled flask. The flask was incubated anaerobically at 37 °C at 250 rpm until an optical density (OD) of a range of 14-17 was obtained. Then, yeast cells were concentrated to obtain an initial OD of 50 as the yeast inoculum by centrifuging the cells at 3,500 rpm for 10 min twice in a bench-top centrifuge (Sorvall, Legend RT, Asheville, NC, USA) and washing with 0.89% (w/v) sterile sodium chloride solution between two centrifugations.

5.3.5.3. *SSF conditions*

SSF was conducted following the procedures outlined by Ramachandriya et al. (2014) with a few modifications. The SSF experiments were performed in 250 ml shake flask reactors with a working volume of 100 ml operating anaerobically on an orbital shaker incubator (New Brunswick Scientific) at 200 rpm and 37 °C. The medium was of 8% solids loading (solids refers to fungal treated samples on dry basis), enzymes loading of 7.5 FPU/g dry biomass Celluclast 1.5L and 15 CBU/g dry biomass Novozym 188,

containing 10 ml of 10× yeast fermentation medium, 5 ml of 1 M sodium citrate buffer at pH 5.5 and 1 ml of 50 OD concentrated yeast cell suspensions. 10× yeast fermentation medium consisted of 100 g/L yeast extract and 200 g/L of peptone. During SSF, samples (1.8 ml) were taken at 0, 6, 12, 18, 24, 36, 48, 60, 72, 96, and 120 h. Samples were centrifuged at 13,000 rpm for 10 min and the supernatant was decanted and filter sterilized using 0.22 µm nylon filters (VWR International, West Chester, PA, USA) prior to product analysis. Ethanol, acetic acid, glycerol, succinic acid, glucose and xylose concentrations were measured using HPLC (1100 Series, Agilent, Santa Clara, CA, USA) with an Aminex HPX-87H column (BioRad, Hercules, CA, USA) and a refractive index detector (RID). The eluent was 0.005 M sulfuric acid flowing at a rate of 0.6 ml/min with a 30 min run time. The HPLC with Chemstation software (Agilent Technologies) was calibrated at five levels using known concentrations of compounds before being used to quantitate the concentration of compounds.

Ethanol yield (% theoretical) during SSF was calculated as the percentage of the theoretical yield based on the biomass put in SSF using equation 5.5.

$$\text{Ethanol yield(\% theoretical)} = \frac{[EtOH_t]}{0.511 * (f[Biomass] * 1.11)} * 100\% \quad 5.5$$

where [EtOH_t], ethanol concentration produced at time t (g/L), 0.51, mass conversion factor of glucose to ethanol (g/g), f, fraction of glucan in dry solids (g/g), [Biomass], initial concentration of solids (8 g/L), and 1.11, mass conversion factor of glucan hydrolysis to glucose (g/g).

Xylose yield during SSF as the percentage of the theoretical yield based on the biomass put in SSF was calculated using equation 5.6.

$$\text{Xylose yield} = \frac{[\text{Xylose}_t]}{(f[\text{Biomass}] * 1.14)} * 100\% \quad 5.6$$

where $[\text{Xylose}_t]$, xylose concentration produced at time t (g/L), f , fraction of xylan in dry solids (g/g), $[\text{Biomass}]$, initial concentration of solids (8 g/L), and 1.11, mass conversion factor of xylan hydrolysis to xylose (g/g).

Overall ethanol yield was calculated as the percentage of the theoretical yield based on the biomass put in fungal pretreatment using equation 5.7.

$$\text{Overall ethanol yield} = (\text{Ethanol yield}(\% \text{theoretical})) * (\text{Glucan preservation}) \quad 5.7$$

where (ethanol yield (%theoretical)), ethanol yield (%theoretical) during SSF obtained in equation 5.1, (Glucan preservation), the percentage of glucan preserved after fungal pretreatment.

Glucan preservation was calculated using equation 5.8.

$$\text{Glucan preservation} = \frac{[\text{Dry matter left}_d] * [\text{Glucan fraction}_d]}{100 * [\text{Glucan fraction}]} * 100\% \quad 5.8$$

where $[\text{Dry matter left}_d]$, dry matter left after fungal pretreatment time d (g), $[\text{Glucan fraction}_d]$, cellulose fraction of fungal treated samples after fungal pretreatment time d (%), 100, initial dry matter put into fungal pretreatment (g), $[\text{Glucan fraction}]$, initial glucan fraction of switchgrass samples put into fungal pretreatment (%).

5.3.6 Ligninolytic enzymes extraction and activity assay

5.3.6.1. Enzymes extraction

Laccase and manganese peroxidase (MnP) enzymes were extracted as follows: 1 g (dry basis) of fungal treated sample was added to 50 mL sterile deionized water and agitated at 150 rpm for 30 min. Then, the sample was centrifuged at 10,000 rpm for 3 min and the supernatant obtained was utilized for the enzyme activities assay (Gómez et al., 2011).

5.3.6.2. Activity assay

Laccase and MnP activities were determined colorimetrically as described previously (Parenti et al., 2013; Santoyo et al., 2008). Laccase activity was determined by detecting the product of the oxidation of 2, 6-dimethoxyphenol (DMP, $\epsilon_{468} = 49,600 \text{ M}^{-1} \text{ cm}^{-1}$). The reaction mixture contained 450 μL of supernatant described above and 500 μL of 10 mM DMP in 100 mM acetate buffer (pH 5.0). The reaction was performed for 1 minute at room temperature before the variation in the absorbance at 468 nm (A_{468}) was recorded. After measuring A_{468} , 50 μL of MnSO_4 was added to the sample and incubated for 1 min at room temperature before adding 30 μL of an H_2O_2 solution containing 22.5 μL of 30% H_2O_2 in 10 ml deionized water. The reaction was allowed to proceed for an additional minute before the A_{468} was measured. MnP activity was estimated as the difference between the two activity measurements. One unit of enzyme activity was defined as 1 μmol of the substrate oxidized per min. The activity of enzyme extract was determined as IU/g dry switchgrass. All assays were performed in

duplicate using a UV-Vis spectrophotometer (Cary 50 Bio, Varian Inc, Palo Alto, CA, USA).

5.3.7. SEM

SEM (Quanta FEG 250, FEI, The Netherlands) images were taken for controls and 80 days fungal treated switchgrass samples. Samples were taken from the glass jar and left to dry in fume hood for at least 2 days until the moisture content was around 5%. Then the samples were stored in airtight bags and transported to China for SEM analysis. Before being placed in the SEM, samples were dried in a 45 °C convection oven until samples weights were constant. After that, samples were mounted on a stub and sputter coated with 5 nm Au/Pd. The images were acquired with an acceleration voltage of 10 kV.

5.3.8. Statistical analysis

Preliminary tests were run before a completely randomized 2*2 factorial design was constructed. Analysis of variance (ANOVA) was conducted to study the effect of substrate MC and inoculum loading on biomass degradation, ethanol yield, xylose yield and ligninolytic enzyme production using the generalized linear model (GLM) procedure in SAS release 9.3 (SAS, Cary, NC, USA). Multiple comparison tests were performed with Tukey's test at a 95% confidence interval.

5.4. Results and discussion

5.4.1. *P. ostreatus* isolation

The procedure to clean up *P. ostreatus* pure culture and observe clamp connection is shown in Fig. 5.1. The clamp connection is visible as a lateral bulge in the hyphal wall adjacent to a transverse septum. The clamp connection is the structure that maintains the dikaryon and also rearranges nuclear order by undergoing conjugate nuclear division by one haploid nucleus of each mating type paired together (Webster & Weber, 2007).

5.4.2. *P. ostreatus* colonization

As shown in Fig. 5.2, the liquid mycelial suspension had a concentration of 4,000 CFUs/ml. Fig. 5.3 shows how *P. ostreatus* colonized on switchgrass over time using the treatment of 50% MC and 5 ml inoculum as an example. The mycelia growing out on the switchgrass were transferred to new MBCD plates and cultivated at 28 °C for 7 days. The periphery of the colony was observed under stereomicroscope to check clamp connection to ensure that *P. ostreatus* was colonizing on the substrate instead of other unwanted microorganisms. For moistened switchgrass with 5 ml or 20 ml inoculum, it took 16 or 18 days for the switchgrass to be completely colonized by *P. ostreatus*. Compared to other white rot fungi, the growth rate of *P. ostreatus* is slow. Taniguchi et al. (2005) used moistened wheat bran as solid substrate to cultivate and compare the growth rate of four different white rot fungi. They found that cell mass accumulation reached 350 mg/g dry matter for *P. ostreatus* at 18-20 days compared to 500-550 mg/g dry matter for *Ceriporiopsis subvermispota* at 18-20 days, 200 mg/g dry matter for *Phanerochaete*

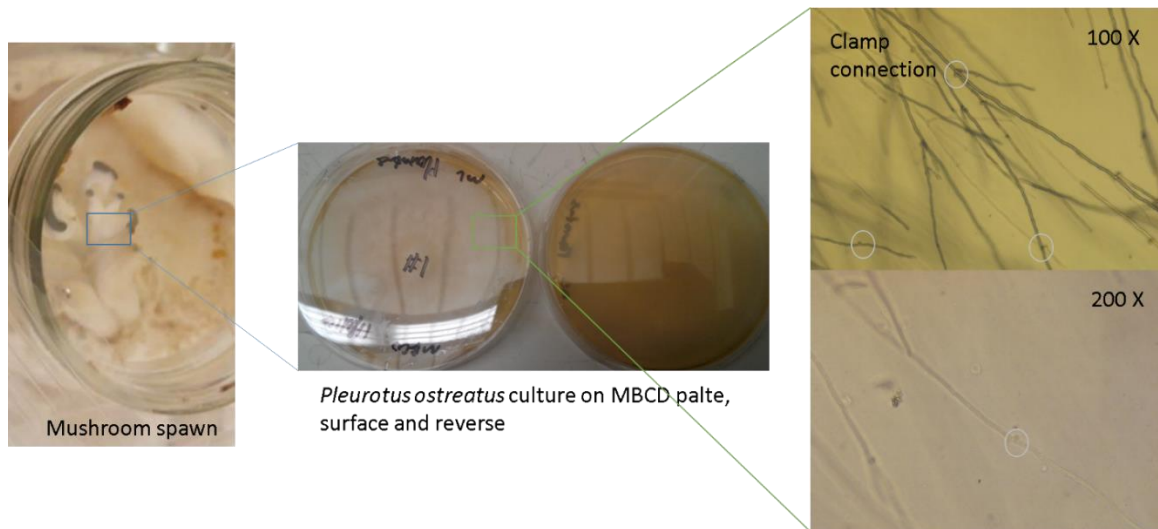


Fig. 5.1. *P. ostreatus* mushroom spawn, pure culture and clamp connection (Clamp connections were circled in the images of 100x and 200x magnification).

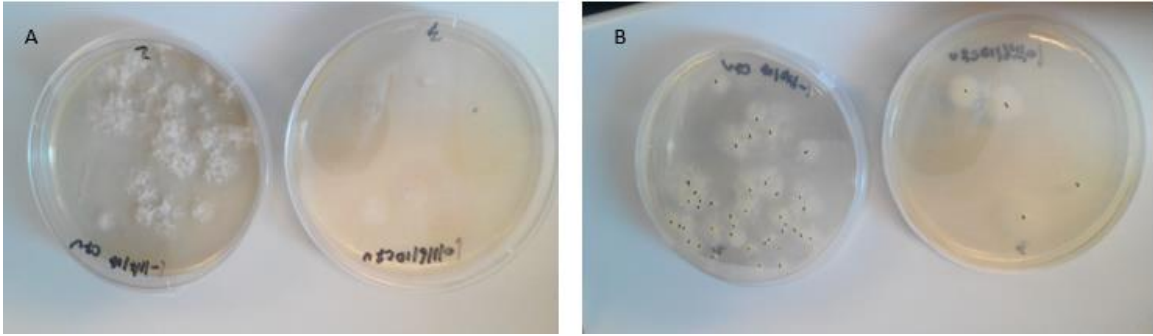


Fig. 5.2. CFUs from *P. ostreatus* mycelial suspension cultivation on MBCD plate for 7 days. A: plate surface, B: plate reverse. Left: 10^2 dilutions of mycelial suspensions, Right: 10^3 dilutions of mycelial suspensions.



Fig. 5.3. Fungal colonization on switchgrass samples with 50% MC, 20 ml inoculum. A: raw switchgrass, B: control, C: fungal colonization at one week, D: fungal colonization at two weeks, E: fungal colonization at four weeks, F: fungal colonization at eight weeks.

chryso sporium at 3-5 days and 450-500 mg/g dry matter for *Trametes versicolor* at 10 days.

5.4.3. Preliminary tests

To the best of our knowledge, no previous study used *P. ostreatus* to do solid state pretreatment on switchgrass. Therefore, some preliminary tests were done to gain some knowledge of *P. ostreatus* growth and corresponding ethanol production through SSF of fungal treated samples.

5.4.3.1. Effect of sterilization and grinding on ethanol yield

Samples used for fungal pretreatment were passed through a 13 mm sieve. It was not known whether or not 13 mm samples needed to be ground to a smaller size to obtain good SSF results. Also, it was of interest to determine if the enzymes secreted by *P. ostreatus* during fungal pretreatment would synergistically work with cellulase and yeast during SSF. In this test, a 30 days fungal pretreatment was applied to samples with 50% MC and 75% MC both with 20 ml inoculum. The fungal pretreatment test was set for 30 days based on previous fungal pretreatment studies (Taniguchi et al., 2005; Wan & Li, 2011) After fungal pretreatment, samples were subjected to SSF as described in section 5.3.5. There were four different treatments (sterilized and ground, sterilized and not ground, not sterilized and ground, not sterilized and not ground). Ethanol yield was used to evaluate the differences among the four treatments. As shown in Fig. 5.4A, there were no significant differences for ethanol yield at 24 h among different treatments of samples with 50% MC. For samples with 75% MC shown in Fig. 5.4B, significant differences existed for ethanol yield at 24 h among different treatments. Samples with the treatment

of sterilized, not ground had the highest ethanol yield. Based on these results, fungal treated samples were not ground, but sterilized before being subjected to SSF in subsequent tests.

5.4.3.2. Effect of enzymes adding on ethanol yield

In this test, samples with 50% and 75% MC were both inoculated with 20 ml inoculum and pretreated by *P. ostreatus* for 30 days. Fungal treated samples were subjected to SSF, but without adding enzymes. As shown in Fig. 5.5, the highest ethanol yields were 3.6% at 24 h for both 50% MC and 75% MC samples. Without adding enzymes in SSF, the ethanol yield was very low, compared to ethanol yield of 23% at 24 h for samples with 50% MC and 20 ml inoculum (Fig. 5.4A, samples not ground, sterilized) and 29% at 24 h for samples with 75% MC and 20 ml inoculum (Fig. 5.4B, samples not ground, sterilized).

5.4.3.3. Effect of high initial inoculum loading on ethanol yield

The effect of initial inoculum loading on ethanol yield after SSF was unknown for *P. ostreatus* pretreatment on switchgrass. High initial inoculum loading was applied to switchgrass with three MC levels; 33%, 50%, 75%. For 33% MC samples, 50 ml liquid fungus inoculum was added to 100 g sterilized switchgrass (dry basis). For 50 % MC samples preparation, 100 ml liquid fungus inoculum was added to 100 g sterilized switchgrass (dry basis). For 75 % MC samples preparation, 100 ml liquid fungus inoculum and 200 ml sterilized deionized water were added to 100 g sterilized switchgrass (dry basis).

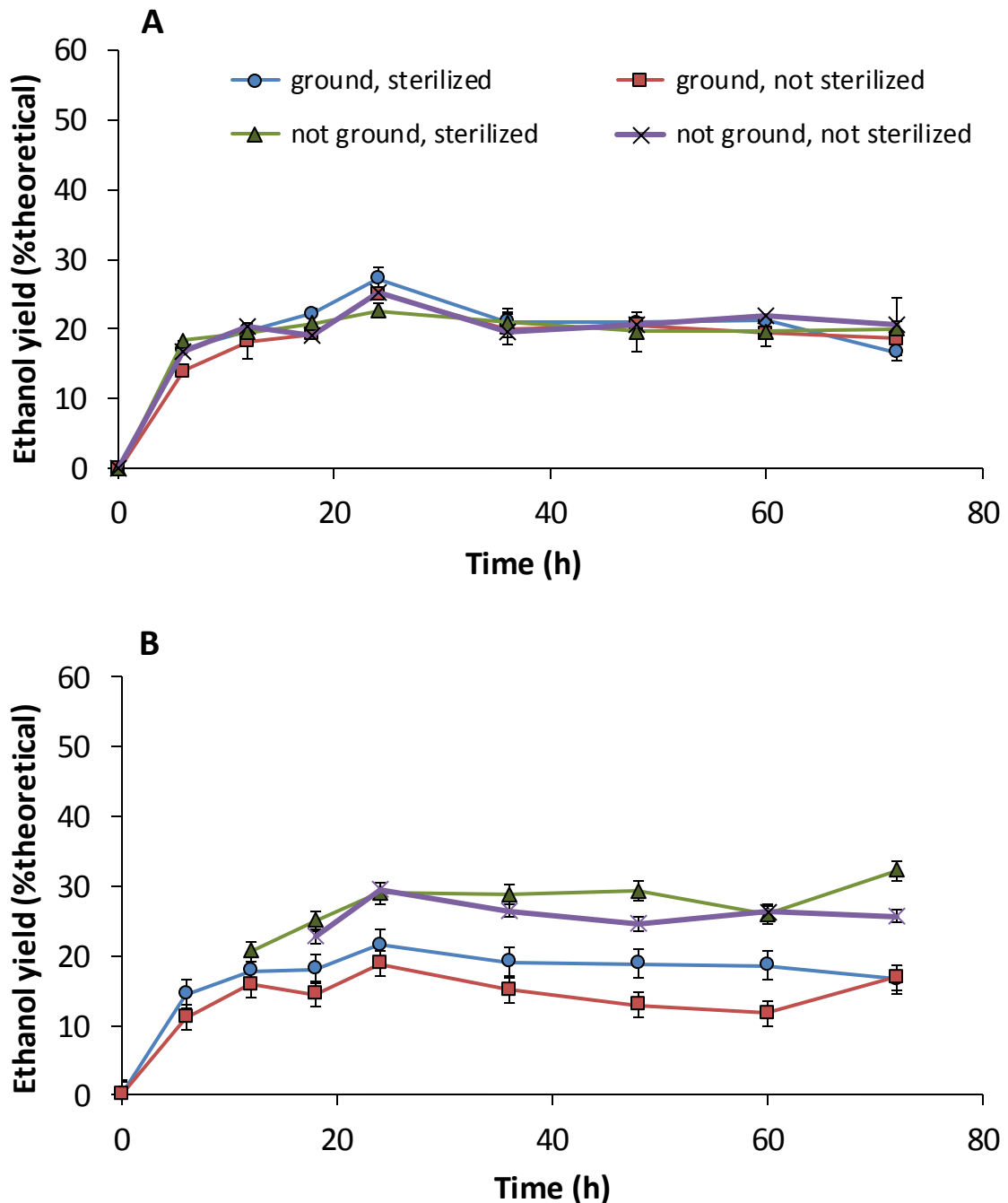


Fig. 5.4. Ethanol yield (% theoretical) during SSF for fungal treated samples in preliminary tests. A: samples with 50% MC and 20 ml inoculum pretreated by fungus for 30 days; B: samples with 75% MC and 20 ml inoculum pretreated by fungus for 30 days. Error bars equal mean \pm one standard error. In Fig 5.4B, data were not available for samples at 6h and 12h for 75% MC, not ground, sterilized samples and for samples at 6h, 12h and 18h for 75% MC, not ground, not sterilized samples since the samples at these times were not become well liquefied.

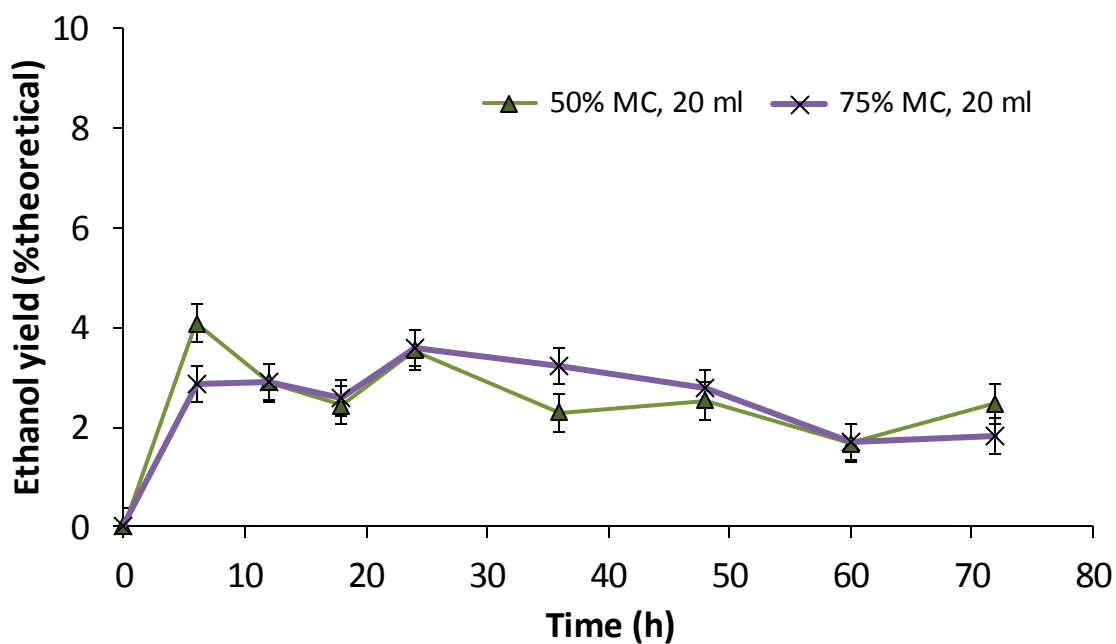


Fig. 5.5. Ethanol yield (% theoretical) during SSF for fungal treated samples without enzyme addition in preliminary tests. Samples with 50% MC and 75% were both inoculated with 20 ml inoculum and pretreated by *P. ostreatus* for 30 days. Error bars equal mean \pm one standard error.

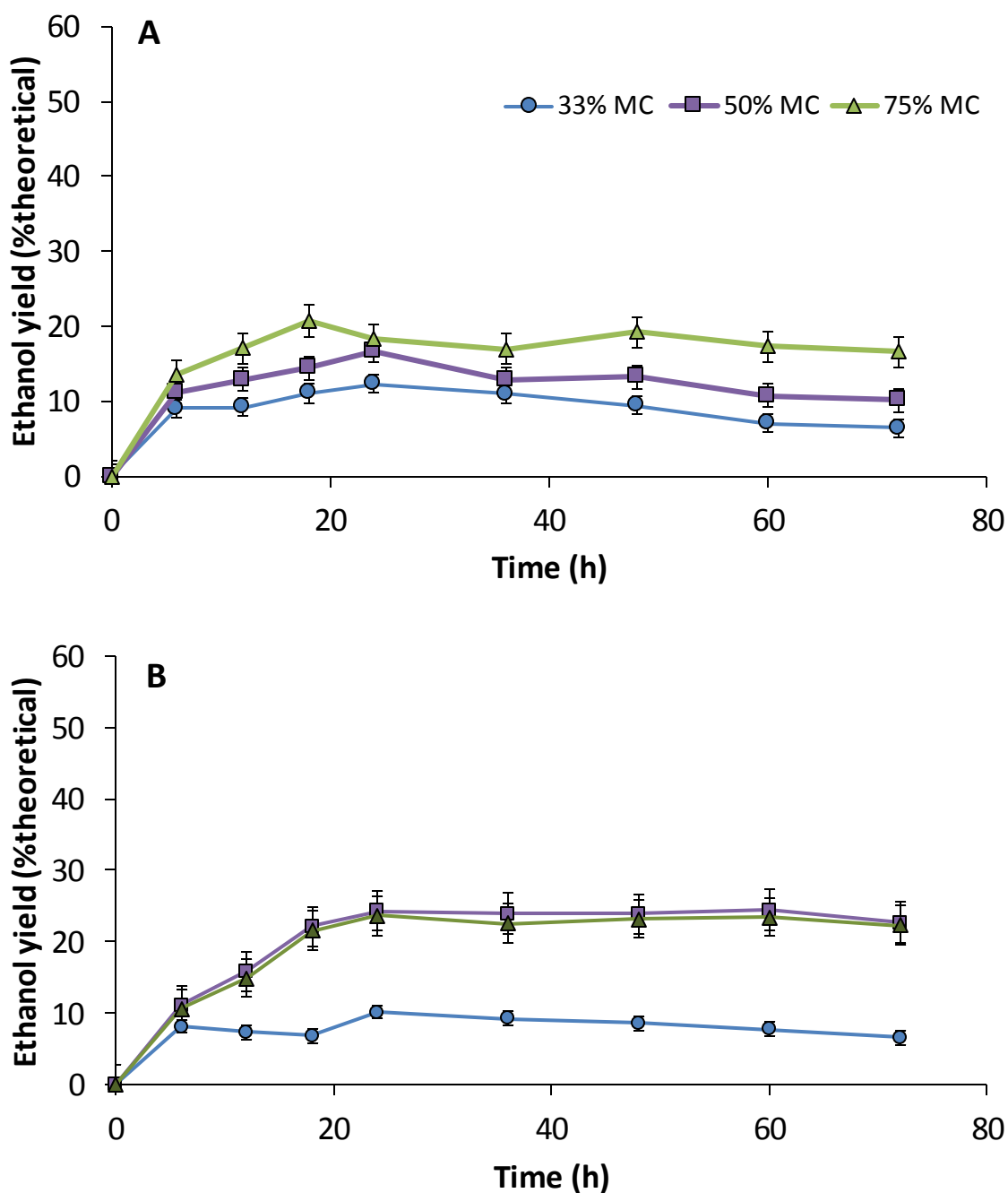


Fig. 5.6. Ethanol yield (%theoretical) during SSF for samples with high initial inoculum loading in preliminary tests. Samples with high initial inoculum loading were treated under MC of 33%, 50% and 75% by *P. ostreatus* for 30 days (A); Samples with high initial inoculum loading were treated under MCs of 33%, 50% and 75% by *P. ostreatus* for 30 days (B). Error bars equal mean \pm one standard error.

For fungal treated samples of 30 days shown in Fig. 5.6A, samples with 75% MC had the highest ethanol yield while samples with 33% MC had the lowest yield. For fungal treated samples of 60 days shown in Fig. 5.6B, there were no significant differences between ethanol yield of samples with 75% MC and samples with 50% MC. For samples with 33% MC, the ethanol yield from 60 days fungal treated samples was lower than that from 30 days fungal treated samples.

As shown in Fig. 5.6A, the ethanol yield for 75% MC samples was 21%, the ethanol yield for 50% MC samples was 18%, and the ethanol yield for 33% MC samples was 12%. In the previous experiment, the ethanol yield of sterilized and not ground samples was 23% for 50% MC samples (Fig. 5.4A) and 32% for 75% MC samples (Fig. 5.4B); therefore, higher fungus loading of 100 ml inoculum did not contribute to a higher ethanol yield compared to that of 20 ml inoculum. However, increasing the initial inoculum loading is beneficial to hasten full colonization of the mycelium, which should be helpful to reduce the time non-colonized substrate is exposed to competitors such as weed molds and bacteria.

5.4.4. Fungal pretreatment with a completely randomized 2*2 factorial design

Based on the results of the preliminary tests, a completely randomized 2*2 factorial design was constructed. Two moisture contents, 50% and 75%, and two initial inoculum loadings, 5 ml and 20 ml, were used.

5.4.4.1. Biomass dry matter loss and biomass components loss

As the fungus grew, nutrients from switchgrass were taken for fungal growth and metabolism, which caused dry matter loss and corresponding biomass components loss. Compositions of fungal pretreated samples during an 80 day fungal pretreatment are shown in Table 5.1. Cellulose, hemicellulose and lignin represented the biomass components. Cellulose equals the content of glucan and hemicellulose is the sum of the xylan, galactan, arabinan and mannan fractions (Ramachandriya et al., 2014). As shown in Fig. 5.7, biomass dry matter loss as well as cellulose, hemicellulose and lignin loss increased over pretreatment time. Dry matter loss shown in Fig. 5.7A was less than 20% for all treatments until 40 days, and then reached as high as 43% at 80 days for the treatment of 75%, 5ml inoculum. Cellulose degradation shown in Fig. 5.7B was less than 21% for all treatments until 60 days, and then it increased up to 45% at 80 days for the treatment of 75%, 5ml inoculum. Hemicellulose loss shown in Fig. 5.7C increased to a maximum of 60% at 80 days for the treatment of 75% MC, 5 ml inoculum, which was the highest among all treatments. The lowest hemicellulose degradation at 80 days was 45% for the treatment of 50% MC, 5 ml inoculum. As shown in Fig. 5.7D, the highest lignin degradation of 52% also happened with the treatment of 75% MC, 5 ml inoculum at 80 days. The strong lignin degradation ability of *P. ostreatus* was also found in a previous study (Taniguchi et al., 2005), For a 14 days fungal pretreatment on rice straw with 60% MC, *P. ostreatus* degraded 30% Klason lignin (acid insoluble lignin) as well as 32% cellulose.

For samples treated by *P. ostreatus* for 20 days, there were no significant differences among treatments for cellulose loss; however, statistical analysis showed

significant differences among treatments for hemicellulose and lignin loss. Hemicellulose loss at 20 days was the highest for samples with 50% MC and 20 ml inoculum. Lignin loss at 20 days was the highest for samples with 75% MC and 20 ml inoculum. For the samples treated by *P. ostreatus* for 40 days, cellulose losses for samples with 50% MC were higher than those for samples with 75% MC. Among all different treatments, both hemicellulose and lignin loss were the highest for samples with 75% MC and 5 ml inoculum.

There was a steep increase in dry matter loss (Fig. 5.7A), cellulose loss (Fig. 5.7B) and hemicellulose loss (Fig. 5.7C), but a slight increase in lignin loss (Fig. 5.7D) from 60 days to 80 days for fungal pretreatment on all treatments. Before 60 days, cellulose loss (Fig. 5.7B) increased much more slowly than hemicellulose and lignin loss (Figs. 5.7C and 5.7D). Statistical analysis showed no significant differences for lignin losses among all treatments between 60 days and 80 days while the differences in cellulose and hemicellulose loss were significant, which indicated that prolonged fungal pretreatment from 60 days to 80 days did not facilitate further lignin degradation, but did result in continuous consumption of polymeric sugars. No significant differences were observed for cellulose degradation in samples treated by fungus for 20 days, 40 days and 60 days, but significant differences existed for hemicellulose and lignin degradation.

A lignin degradation pattern similar to this study was observed in *P. chrysosporium* treatment on corn stalks after 10 days in a 14 days treatment (Shi et al., 2009). Wan (2011) observed that lignin degradation was 2.9% at 7 days and 25.4% at 14 days in *C. subvermispora* degraded corn stover, then increased very slowly to 39.2% at 42 days. This may indicate that white rot fungi degrade hemicellulose and lignin easier

Table 5.1. Composition of fungal treated samples at different fungal pretreatment time

	Treatment	Cellulose	Hemicellulose	Lignin
Raw	NA	42.77	25.71	17.31
20	50% MC, 5 ml	40.60±0.93	27.94±1.45	18.26±0.58
	50% MC, 20 ml	38.62±1.21	24.17±1.01	18.39±0.19
	75% MC, 5 ml	42.34±1.87	25.00±1.30	17.59±0.52
	75% MC, 20 ml	39.66±1.26	24.18±0.39	17.60±0.39
40	50% MC, 5 ml	42.06±0.77	27.44±3.44	18.36±0.18
	50% MC, 20 ml	42.00±1.73	24.96±1.42	16.18±1.23
	75% MC, 5 ml	47.42±1.24	23.92±0.44	13.81±0.83
	75% MC, 20 ml	46.16±1.98	25.77±3.34	17.41±1.80
60	50% MC, 5 ml	47.45±4.08	26.88±0.53	18.11±3.24
	50% MC, 20 ml	45.49±3.74	22.63±1.23	13.78±1.54
	75% MC, 5 ml	52.94±1.22	21.87±0.59	12.18±0.66
	75% MC, 20 ml	48.78±4.82	22.31±2.18	11.56±1.10
80	50% MC, 5 ml	34.73±0.71	20.44±1.18	17.22±0.14
	50% MC, 20 ml	38.27±6.02	19.58±2.74	16.39±0.88
	75% MC, 5 ml	40.59±6.32	14.32±2.55	11.34±1.20
	75% MC, 20 ml	42.27±2.22	15.76±1.80	12.08±1.51

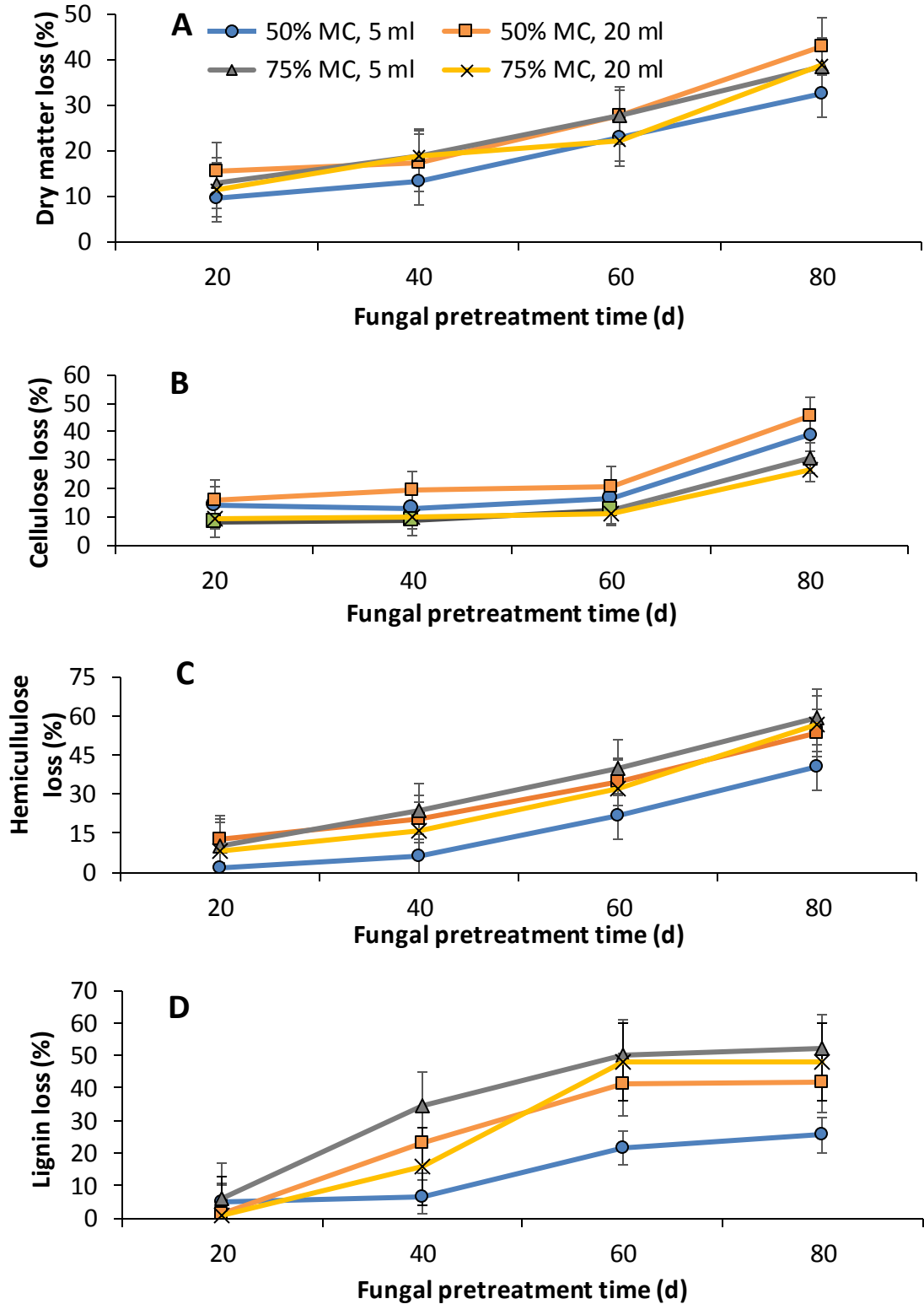


Fig. 5.7. Biomass dry matter loss (A), cellulose loss (B), hemicellulose loss (C), lignin loss (D) during an 80 days fungal pretreatment. Error bars equal mean \pm one standard error.

than cellulose at the early stage (20 days in this study), then start utilizing cellulose and hemicellulose instead of lignin at some critical point (60 days in this study).

5.4.5. SSF of fungal treated samples with 2*2 factorial design

5.4.5.1. Glucose yield

Glucose concentrations in SSF solutions for all treatments at different fungal pretreatment times were around 0.1 g/L. For some of the samples, there was no glucose detected; thus, it was assumed that glucose was not accumulated in SSF and glucose was converted to ethanol by yeast as it was hydrolyzed from glucan.

5.4.5.2. Ethanol concentration

Ethanol concentrations during SSF for the completely randomized 2*2 factorial design are shown in Fig. 5.8. As shown in Fig. 5.8A, for 20 days fungal treated samples, the concentrations of all treatment conditions were around 3 g/L. There were no significant differences for ethanol concentrations among different treatment conditions. For 40 days fungal treated samples shown in Fig. 5.8B, the ethanol concentration of treatment 75% MC, 5 ml inoculum was highest (6.6 g/L at 24 h) among all treatments. Statistical analysis showed differences in ethanol concentrations between 75% MC, 5 ml inoculum and the other three treatments and there were no differences among the other three treatments. For 60 days fungal treated samples (Fig. 5.8C), there were no significant differences for ethanol concentrations at 48 h between samples with 75% MC, 5 ml inoculum and samples with 50% MC, 20 ml inoculum. There were also no significant differences for ethanol concentrations at 48 h between samples with 75% MC, 20 ml inoculum and samples with 50% MC, 5 ml inoculum. Ethanol concentrations at 48 h of

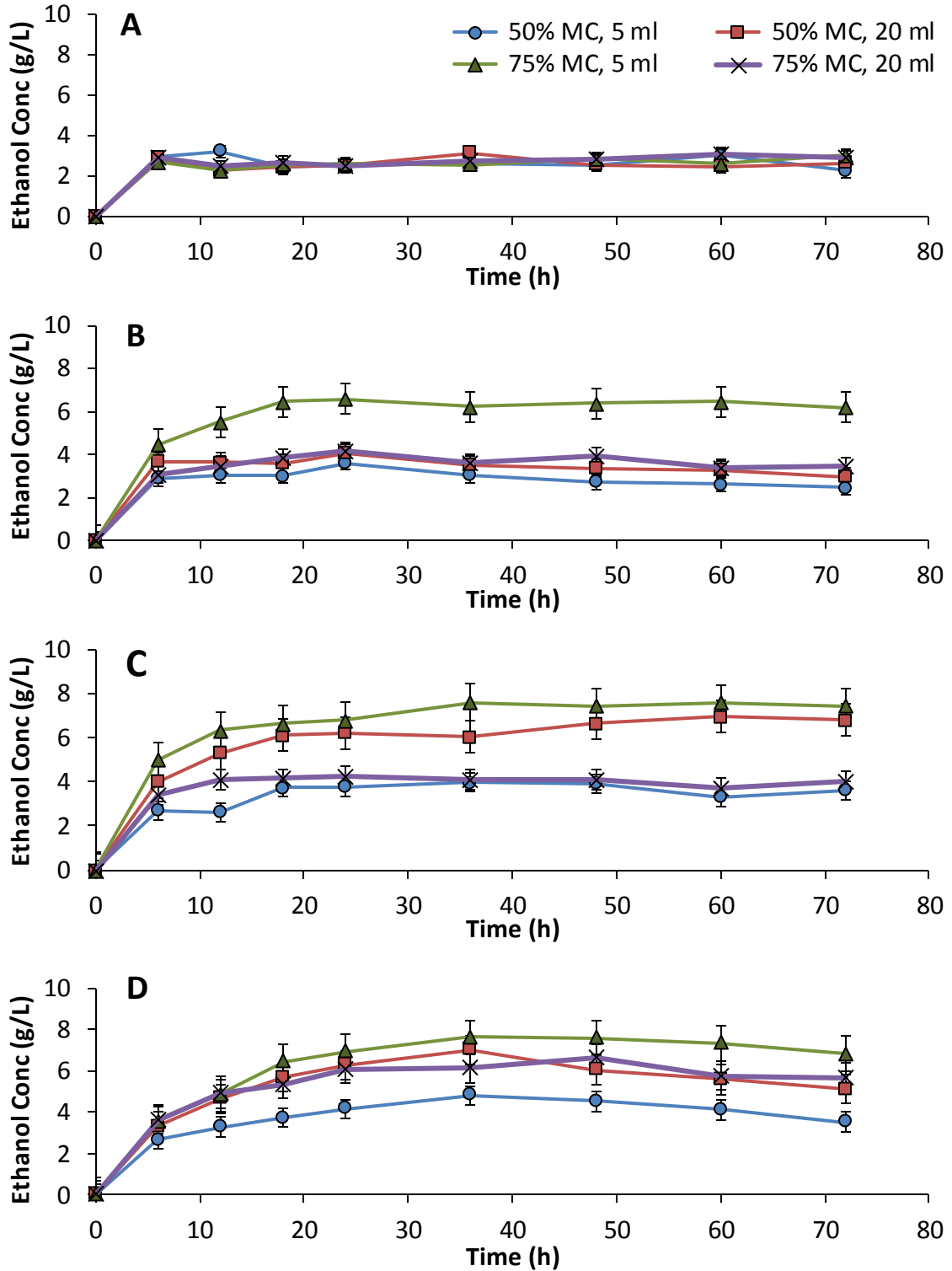


Fig. 5.8. Ethanol concentration (g/L) during SSF under a completely randomized 2*2 factorial design, samples were treated by *P. ostreatus* for 20 days (A), 40 days (B), 60 days (C), 80 days (D). Error bars equal mean \pm one standard error.

samples with 75% MC, 5 ml inoculum and samples with 50% MC, 20 ml were significantly higher than those of samples with 75% MC, 20 ml inoculum and samples with 50% MC, 5 ml

For 80 days fungal treated samples (Fig. 5.8D), significant differences existed for ethanol concentrations at 36 h among all treatments with 75% MC, 5 ml inoculum having the highest ethanol concentration of 7.6 g/L. The 60 days fungal treated samples with 75% MC, 5 ml inoculum also had an ethanol concentration of 7.6 g/L.

5.4.5.3. Ethanol yield

Controls subject to SSF did not become liquefied and there was no ethanol detected in SSF. Ethanol yields during SSF for the completely randomized 2*2 factorial design are shown in Fig. 5.9. The trends of ethanol yield in Fig. 5.9 were similar to those of ethanol concentration shown in Fig. 5.8. For 20 days fungal treated samples after 12 h (Fig. 5.9A), the maximum ethanol yields were 14% for samples with 50% MC, 5 ml inoculum, 18% for samples with 50% MC, 20 ml inoculum, 15% for samples with 75% MC, 5 ml, 17% for samples with 75% MC, 20 ml inoculum, respectively. There were no significant differences among these ethanol yields. Since the fungus had just fully colonized the substrate before sampling in all treatments, it was expected the ethanol yield would be similar for all treatments.

For SSFs with samples pretreated by *P. ostreatus* for 40 days (Fig. 5.9B), 60 days (Fig. 5.9C) and 80 days (Fig. 5.9D), samples with 75% MC, 5 ml inoculum showed the highest ethanol yield for all treatments. Correspondingly, samples with 75% MC, 5 ml inoculum showed the highest hemicellulose and lignin loss (Figs. 5.9C and 5.9D). Since

plant biomass has high complexity of lignin and cell wall matrix (Donohoe et al., 2008), it is expected that hemicellulose and lignin loss liberate cellulose thus increasing the accessibility of SSF. On the other hand, fungal pretreatment showed strong ability to disrupt biomass structure and decrease the cellulose crystallinity (Bak et al., 2009). For the ethanol yields during SSF for 40 days fungal treated samples (Fig. 5.9B), treatment 75% MC, 5 ml inoculum showed the highest ethanol yield, 30%, at 18 h while the other three treatments were not significantly different. For SSFs with samples pretreated by *P. ostreatus* for 60 days (Fig. 5.9C), there were no significant differences for ethanol yields at 18 h between samples with 75% MC, 5 ml inoculum and samples with 50% MC, 20 ml inoculum. The highest ethanol yields were obtained at 80 days for all treatments (Fig. 5.9D). The 80 days yields at 36 h were 31% for samples with 50% MC, 5 ml inoculum, 41% for samples with 50% MC, 20 ml inoculum, 45% for samples with 75% MC, 5 ml inoculum, 34% for samples with 75% MC, 20 ml inoculum, respectively. There were no significant differences between samples with 75% MC, 20 ml inoculum and samples with 50% MC, 5 ml inoculum, and between samples with 75% MC, 5 ml inoculum and samples with 50% MC, 20 ml inoculum. Although the ethanol concentrations were the same as 7.6 g/L for 60 days samples and 80 days samples with treatment 75% MC, 5 ml inoculum, the ethanol yields were different since the glucan preservation were different. Ethanol yield increased as fungal pretreatment time increased, which was consistent with increased hemicellulose and lignin loss as fungal pretreatment time increased. The removal of lignin and hemicellulose from biomass facilitates exposure of crystalline cellulose to hydrolytic enzymes (Shi et al., 2009; Sun & Cheng, 2002).

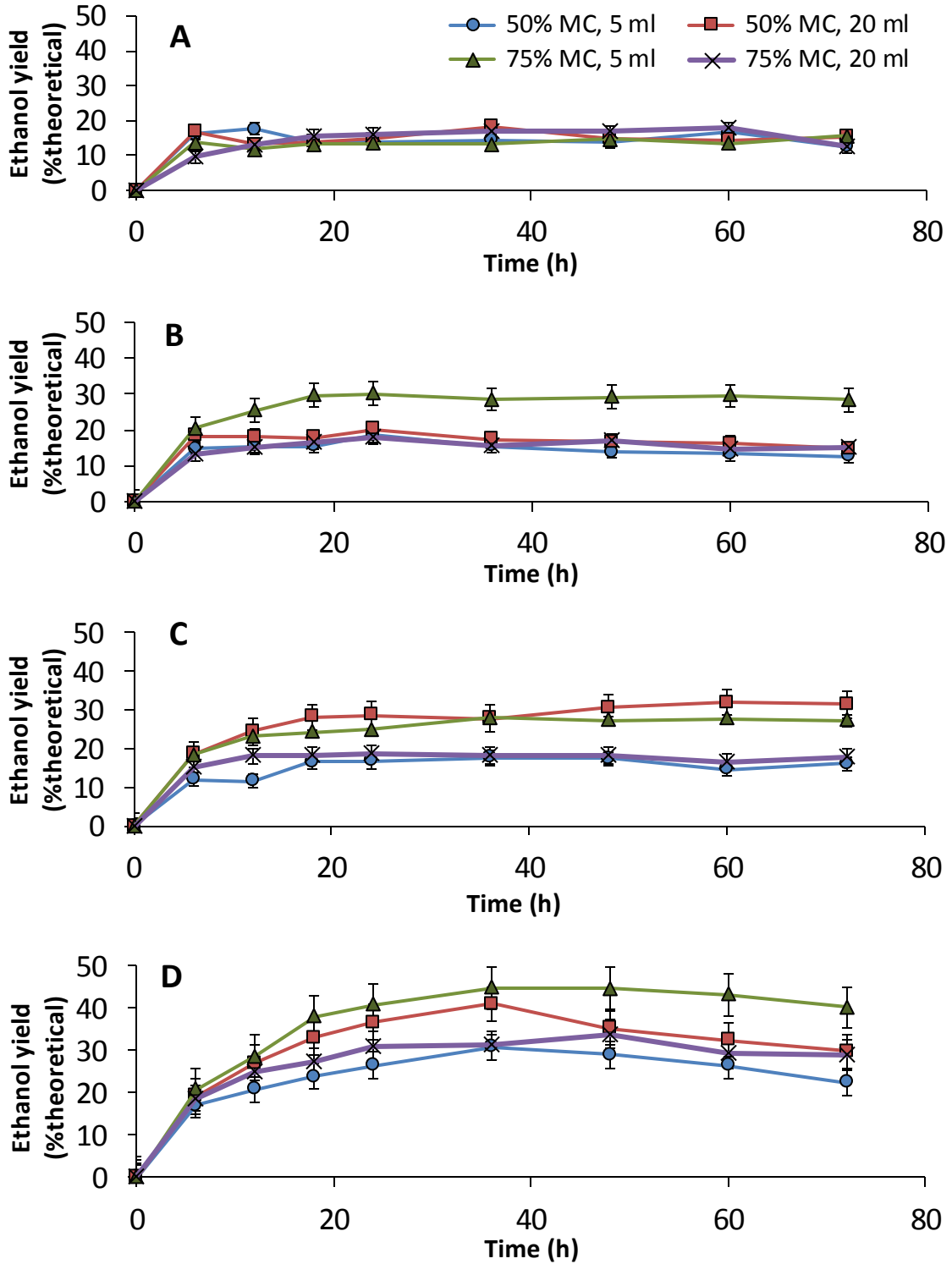


Fig. 5.9. Ethanol yield (% theoretical) during SSF under a completely randomized 2*2 factorial design, samples were treated by *P. ostreatus* for 20 days (A), 40 days (B), 60 days (C), 80 days (D). Error bars equal mean \pm one standard error.

The highest ethanol yield, 45%, was lower than that of previous studies; however, in previous studies ethanol yields of 10-20% were achieved in untreated samples. Wan and Li (2010b) obtained an ethanol yield of 57.8% after 35 days fungal pretreatment by *C. subvermispora* and SSF on 5 mm corn stover with 75% MC while the ethanol yield was 15.9% for untreated corn stover. Bak et al. (2009) obtained 63% ethanol yield on *P. chrysosporium* treated rice straw in submerged cultivation, their study did not show the ethanol yield for untreated rice straw. The glucose yield of fungal treated rice straw reached 65% while the glucose yield was 23% for untreated samples (Bak et al., 2009). In this study, when raw switchgrass was subjected to SSF, no ethanol was produced, which may indicate that switchgrass is more difficult to convert to ethanol than corn stover or rice straw.

The maximum ethanol yields were obtained at 36 h for 80 days samples while the maximum ethanol yields were obtained at 18 h for 40 days and 60 days samples and at 12 h for 20 days samples. This phenomenon indicated that more time was required for 80 days fungal treated samples to reach maximum ethanol production than for 20, 40 or 60 days fungal treated samples.

5.4.5.4. Overall ethanol yield

Overall ethanol yield was calculated by multiplying the biomass glucan preservation after fungal pretreatment by ethanol yield during SSF. The overall ethanol yield gives an assessment of the ethanol yield from switchgrass through the process of fungal pretreatment and SSF.

For the overall ethanol yield for *P. ostreatus* treated 20 days samples (Fig. 5.10A), the pattern was similar to ethanol concentration (Fig. 5.8A) and ethanol yield during SSF (Fig. 5.9A). Statistical analysis showed there were no significant differences for the overall ethanol yields at 12 h among the different treatments of 20 days fungal treated samples (Fig. 5.10A). For the overall ethanol yield at 18 h for 40 days samples (Fig. 5.10B), there were significant differences for the overall ethanol yields among all treatments with 75% MC, 5 ml inoculum having the highest overall ethanol yield of 25%, followed by treatment 50% MC, 20 ml with 20%, then treatment 50% MC, 5 ml with 16%, and treatment 75% MC, 20 ml with 11%. This trend was unlike the SSF ethanol yields for 40 days fungal treated samples (Fig. 5.9B), in which treatment 75% MC, 5 ml inoculum showed the highest ethanol yield while the other three treatments had no significant differences. This was due to the fact that cellulose (glucan) loss (Fig. 5.7B) did not have the same trend as ethanol yield during SSF. The overall ethanol yields for 60 days fungal treated samples are shown in Fig. 5.10C. The treatment of 75%, 20 ml inoculum at 24 h had a greater overall ethanol yield (15%) than that of 40 days samples (11%), but for the other three treatments, there were no significant differences between 40 days fungal treated samples (Fig. 5.10B) and 60 days fungal treated samples (Fig. 5.10C). The overall ethanol yields for 80 days fungal treated samples (Fig. 5.10D) were the highest among all fungal treatment times. The treatment 75%, 5 ml inoculum showed the highest overall ethanol yield (31%) and the treatment 75%, 20 ml inoculum showed the lowest (19%).

5.4.5.5. Xylose yield

Xylose yield was measured since it is not metabolized by *S. cerevisiae* D5A. Xylose was detected during SSF of fungal pretreated biomass. Xylanase has been detected in *P. ostreatus* (Membrillo et al., 2008; Qinghe et al., 2004). Since Celluclast® 1.5 L hydrolyzes (1,4)-beta-D-glucosidic linkages in cellulose and other beta-D-glucans (Novozymes, 2012), xylose should only be produced by xylanase degradation on switchgrass during fungal pretreatment. For the preliminary test, as shown in Fig. 5.11A, for SSF without enzyme addition on 30 days fungal treated samples, xylose yield reached 4.3% at 48 h for 50% MC samples and 5% at 24 h for 75% MC samples. As shown in Fig. 5.11B, for SSF with enzymes addition on 30 days fungal treated samples, xylose yield reached 15% at 24 h for 75% MC samples and 8% at 24 h for 50% MC samples. The xylose yield differences between these tests were caused by the addition of Celluclast 1.5 L and Novozym 188. This observation indicates that there is xylanase activity in Celluclast 1.5 L and Novozym 188 since xylose yield increased as SSF time increased, as shown in Fig. 5.11B. Overall, the xylose present during SSF could be due to xylanase secreted by *P. ostreatus* and xylanase in Celluclast 1.5 L and Novozym 188 degrading xylan into monomeric xylose. Similar results were found in a previous study. Mosier et al. (2005) conducted liquid hot water pretreatment on corn stover and pretreated corn stover with the same brands of enzymes and similar dosages to those in this study. Their results showed that xylose yield reached a range of 20% to 65% from pretreatment temperature from 170 °C to 200 °C.

As shown in Fig. 5.12, fungal treated samples with 75% MC showed higher xylose yield than that of samples with 50% MC. This may have resulted from higher

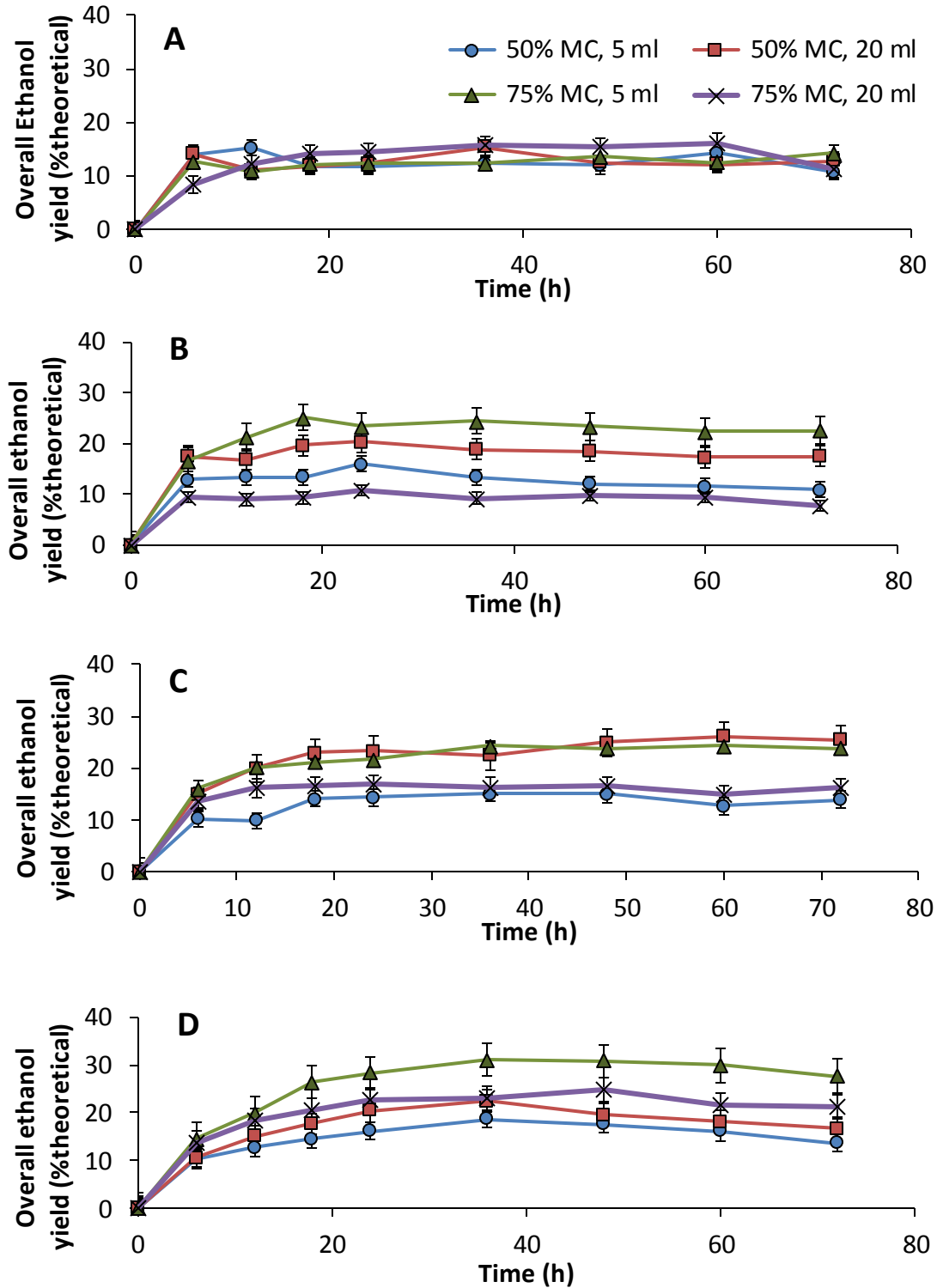


Fig. 5.10. Overall ethanol yield (% theoretical) under a completely randomized 2*2 factorial design, samples were treated by *P. ostreatus* for 20 days (A), 40 days (B), 60 days (C), 80 days (D). Error bars equal mean \pm one standard error.

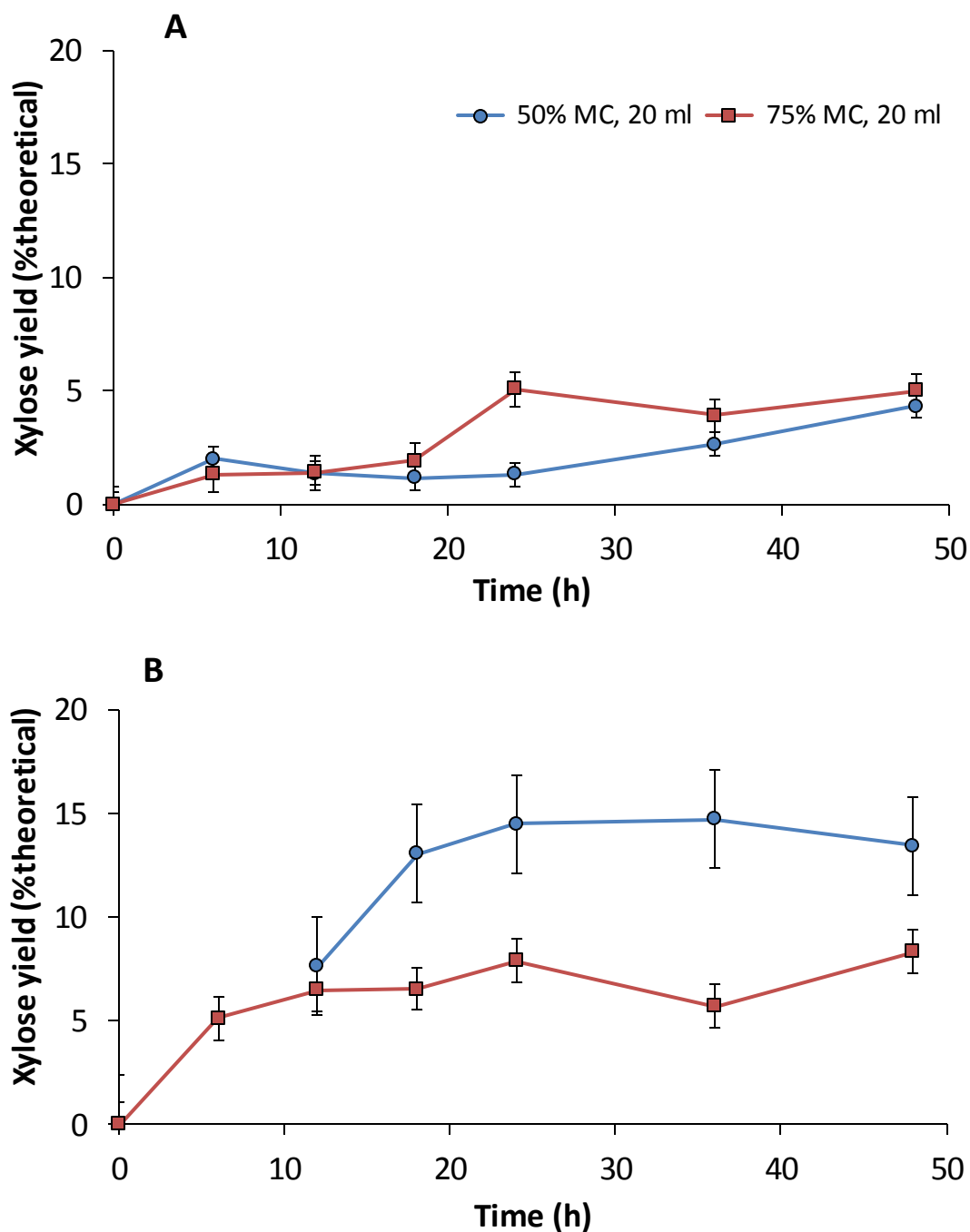


Fig. 5.11. Xylose yield (%theoretical) during SSF without enzymes addition in preliminary tests. Samples with 50% MC and 75% were both inoculated with 20 ml inoculum and pretreated by *P. ostreatus* for 30 days and fro 60 days (B). Error bars equal mean \pm one standard error. In Fig 5.11B, Data points of 0 h, 6 h of samples with 75% MC, 20 ml was not available. Error bars equal mean \pm one standard error.

fungus metabolism at 75% MC than at 50% MC. Fungal pretreatment (Taniguchi et al., 2005) and liquid hot water pretreatment (Mosier et al., 2005) induces cell wall structure loosening and perforation on the surface of biomass, which improves exposure of cellulose networks and enzymes access.

As shown in Fig. 5.12A for 20 days samples, the maximum xylose yields were obtained at 36 h for all treatments while statistical analysis showed that xylose yield from samples with 75% MC, 20 ml inoculum was higher than that of the other three treatments. As shown in Fig. 5.12B, for 40 days fungal treated samples, xylose yield reached a maximum of 16% for samples with 75% MC, 5 ml inoculum. Statistical analysis showed there were no differences between xylose yields of samples with 50% MC, 5 ml inoculum and samples with 50% MC, 20 ml inoculum. For all treatments, the xylose yields from 60 days samples (Fig. 5.12C) were higher than those from 80 days samples (Fig. 5.12D). This result may be caused by xylose consumption by *P. ostreatus* for metabolism right after xylose was produced. Since there were xylose monomers released in SSF and 40% to 70% of hemicellulose was preserved in fungal pretreated biomass, it is predictable that more ethanol would be produced by adding xylanase into hydrolysis and applying hexose and pentose co-fermenting microorganisms in SSF.

5.4.6. Ligninolytic enzymes activities

Lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase are known to be the three main enzymes responsible for lignin degradation in white rot fungi. Laccase and MnP are regarded as the main ligninolytic activities in *P. ostreatus*, while LiP has not been detected in *P. ostreatus* (Santoyo et al., 2008).

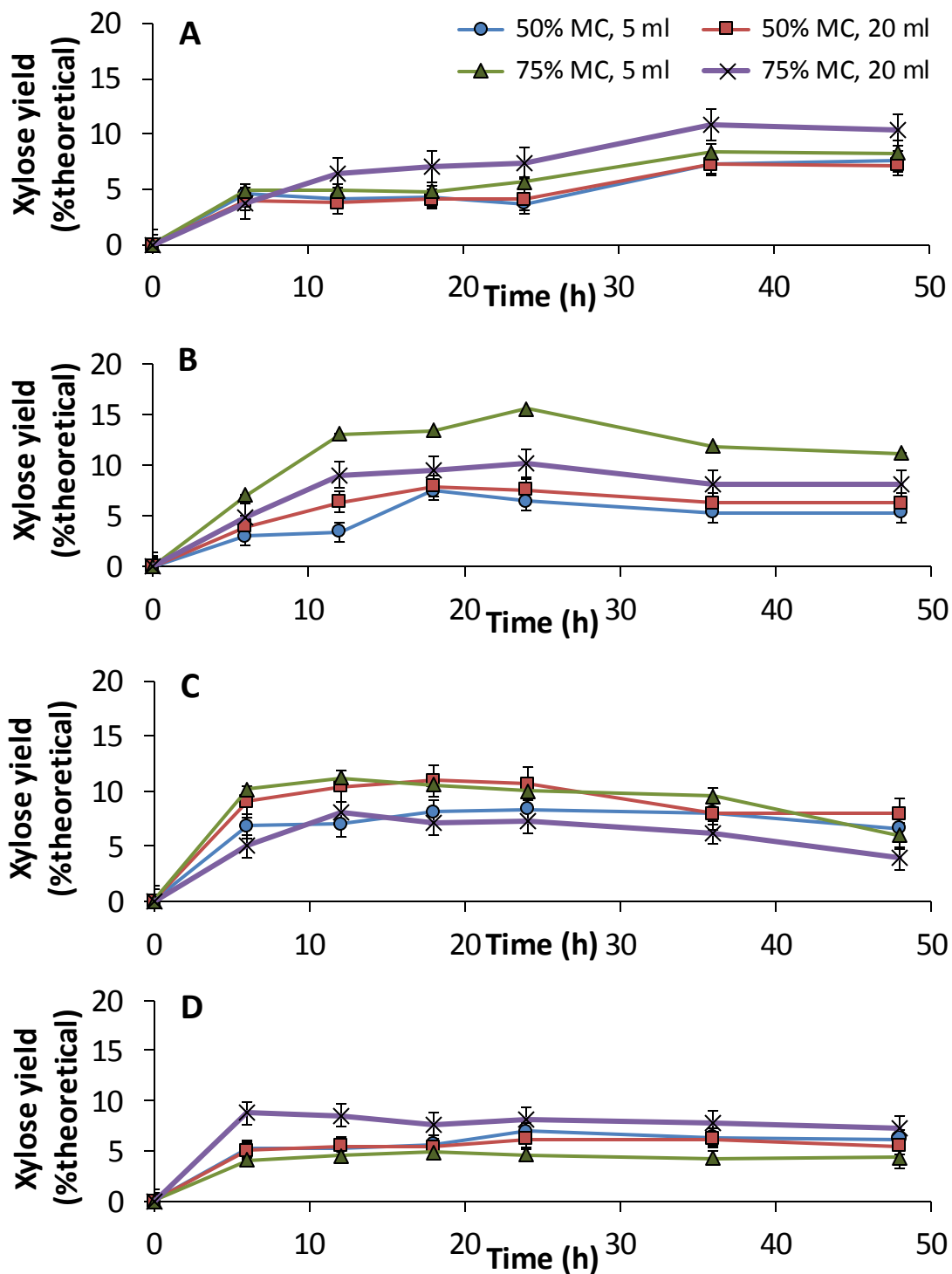


Fig. 5.12. Xylose yield (% theoretical) during SSF under a completely randomized 2*2 factorial design, samples were treated by *P. ostreatus* for 20 days (A), 40 days (B), 60 days (C), 80 days (D). Error bars equal mean \pm one standard error.

For all treatments, laccase activities are shown in Fig. 5.13A and MnP activities are shown in Fig. 5.13B. Laccase activity increased from 20 days to 40 days, was constant from 40 days to 60 days, and decreased at 80 days for samples with 50% MC, 5 ml inoculum. For samples with 75% MC, 5 ml inoculum, laccase activity increased as fungal pretreatment time increased. There were no apparent patterns in laccase activity for samples with 50% MC, 20 ml inoculum and samples with 75% MC, 20 ml inoculum. For all treatments, MnP activities showed a trend to increase and then decrease as fungal pretreatment time increased. For samples with 50% MC, 5 ml inoculum and samples with 50% MC, 20 ml inoculum, MnP activity reached the maximum at 60 days and then decreased. For samples with 75% MC, 5 ml inoculum and samples with 75% MC, 20 ml inoculum, MnP activity reached the maximum at 40 days and then decreased. Similar results were observed for MnP activity in the study of Wan and Li (2010a). During a 28 day *C. subvermispora* pretreatment on corn stover, MnP activity reached a maximum value of 2.2 IU/g at 28 days and then decreased to 0.3 IU/g at 28 days. In this study, for fungal treated samples at 20 days, laccase activity was equal to MnP activity for samples of 75%, 5 ml inoculum while laccase activity was higher than MnP activity for the other three treatments. This was consistent with the study of Wan and Li (2011), in which laccase activity was higher than MnP activity for a 18 days *C. subvermispora* treatment on switchgrass.

Laccase activity was lower than that observed in the study of Wan and Li (2011). The low laccase activity may be due to the fungal pretreatment not being supplied with sufficient copper since there was only 0.05 mg/L copper supplied in the liquid broth. Laccases are multicopper oxidases (Giardina et al., 2010). The addition of copper 1.5

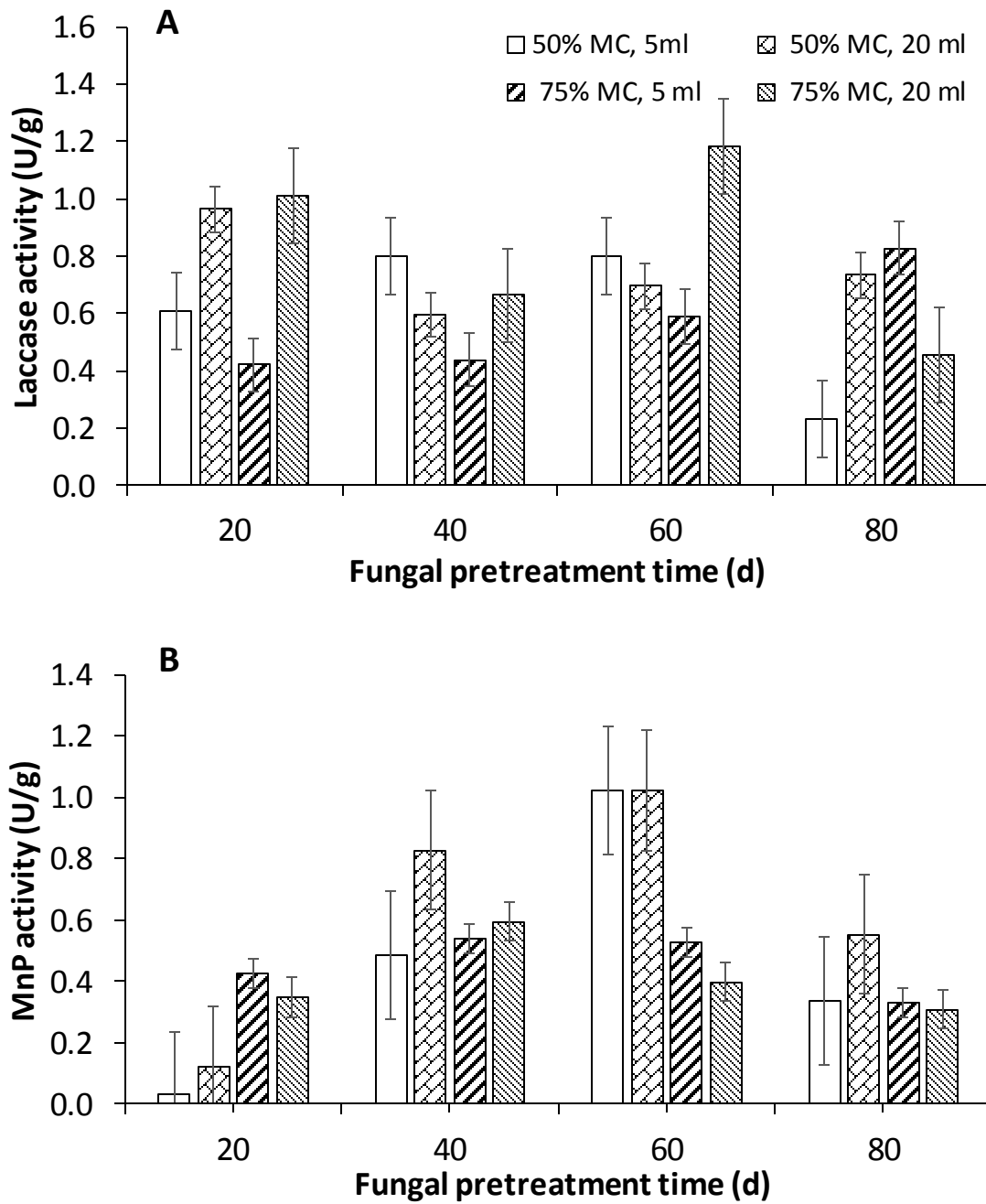


Fig. 5.13. Laccase (A), MnP (B) activities during an 80 day fungal pretreatment under a completely randomized 2*2 factorial design.

mM was observed to cause a 20 fold increase in laccase activity compared to a culture without addition of copper (Mäkelä et al., 2013). The activity of MnP was similar to the study of Wan and Li (2011). Their study found that adding Mn^{2+} did not show a positive effect on lignin degradation when using *C. subvermispora* to degrade wheat straw. Shi et al. (2009) also found that supplemental Mn^{2+} did not necessarily increase lignin degradation when using *P. chrysosporium* to degrade corn stalks, which may indicate supplemental Mn^{2+} may be not necessary to increase the MnP activity.

5.4.6. SEM

SEM images are shown in Fig. 5.14. Controls (Fig. 5.14A) showed an intact, contiguous surface structure. As shown in Fig. 5.14B, samples were heavily degraded and the outer layer was peeled off. Fig. 5.14C shows switchgrass wrapped by fungal mycelia with various tiny pits widely distributed along the vessel walls of switchgrass. Fig. 5.14D shows the sizes of the tiny pits ranging from 1.24 to 1.69 μm . Fig. 5.14E shows enlarged pores and breakages, which may indicate fungus “drill” tiny pits first and enlarge the pores which resulted in the large cracks. Fig. 5.14F shows a large deep breakage with a length of 70 μm . It may indicate that *P. ostreatus* consumed the outer layer of the cell wall to get access to the internal area, which may explain why *P. ostreatus* consumed considerable hemicellulose along with the consumption of lignin; although, *P. ostreatus* is regarded as a lignin selective fungus. Tiny pits and breakages similar to those observed in this study were also observed using SEM by Wang et al. (2012) doing electrolyzed water pretreatment on switchgrass.

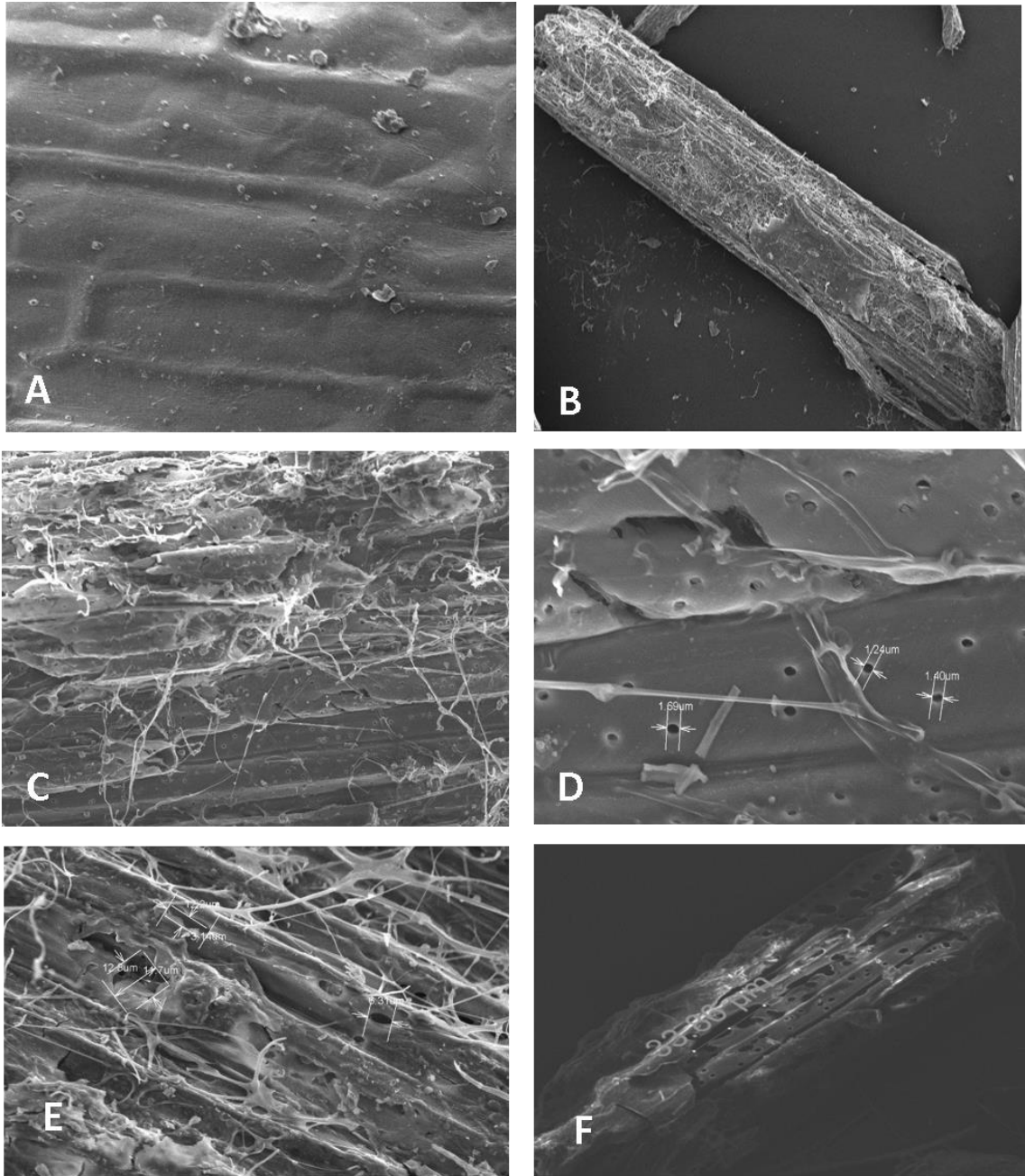


Fig. 5.14. SEM images. A: controls (untreated switchgrass), magnification at 600 \times ; B-F samples were treated by *P. ostreatus* for 80 days. B: treatment with 50% MC, 5ml inoculum at 60 \times magnification; C: treatment with 50% MC, 5ml inoculum at 400 \times magnification. D: treatment with 50% MC, 20ml inoculum at 2000 \times magnification; E: treatment with 75% MC, 5ml inoculum at 1000 \times magnification; F: treatment with 75% MC, 20ml inoculum at 1200 \times magnification. Holes shown on D has a diameter range of 1.24 μm to 1.69 μm . Rectangular holes (12.6 μm *11.7 μm and 12.2 μm *3.1 μm) and hole of length 6.31 μm are shown on E. Breakage shown on F has a length of 33.88 μm .

The surface morphology changes seen in the SEM images indicate that *P. ostreatus* induced perforation of the outer layer of switchgrass from tiny pits to large breakages and expose internal areas to improve the accessibility of fungus. According to the assumption that cellulose and hemicellulose are trapped in the barrier of lignin as the outer layer (Balan et al., 2008), the degradation mechanism of *P. ostreatus* is to degrade partial lignin first and expose cellulose and hemicellulose, which caused significant hemicellulose loss first (Fig. 5.11) then cellulose loss (Fig. 5.10) as fungal pretreatment time increased.

5.5. Conclusions

There was no ethanol production during SSF of untreated switchgrass. After fungal pretreatment, there was no need to further grind 13 mm samples since an improvement in ethanol yield was not observed after grinding. Sterilization is necessary before introducing fungal treated samples to SSF.

Fungus did not grow well on samples with 33% MC and the corresponding ethanol yield was much lower than that of samples with 50% and 75% MC. 50% MC and 75% MC were good moisture conditions for fungal pretreatment. 33% MC was not recommended for the moisture condition of large scale fungal pretreatment.

For fungal treated samples with high fungus loading, higher fungus loading did not contribute to a higher ethanol yield. This finding may lower the cost for industrial scale application. However, higher initial inoculum loading usually leads to faster fungus colonization on samples than lower fungus loading, which helps to fight unwanted microorganisms contamination, especially under the circumstance that large scale

application has no sterilization step. One of the disadvantages of *P. ostreatus* is its longer incubation period to fully colonize substrate compared to other white rot fungi. An appropriate fungus loading should be explored for large scale application.

Under completely randomized 2*2 factor design, 80 days fungal treated samples showed the highest lignin degradation and ethanol yield for all treatments during an 80 day fungal pretreatment. For 80 days fungal treated samples, the samples with 75% MC and 5 ml inoculum showed the highest lignin degradation of 52% and ethanol yield of 45% (at 36 h during SSF), the samples with 75% MC and 20 ml inoculum had lignin degradation of 48% and ethanol yield of 33% (at 36 h during SSF), the samples with 50% MC and 20 ml inoculum had lignin degradation of 42% and ethanol yield of 41% (at 36 h during SSF), the samples with 50% MC and 5 ml inoculum showed the lowest lignin degradation of 25% and ethanol yield of 30% (36 h during SSF). For overall ethanol yield, 80 days fungal treated samples showed the highest during an 80 day fungal pretreatment. Treatment of 75% MC and 5 ml inoculum led to the highest overall ethanol yield 31% (at 36 h during SSF), treatments of 75% MC and 20 ml inoculum and 50% MC and 20 ml inoculum both led to the ethanol yield 23% (at 36 h during SSF), treatment of 50% MC and 5 ml inoculum led to the lowest overall ethanol yield 19% (at 36 h during SSF).

Monomeric xylose was detected after samples were treated by fungus. Hemicellulose loss increased with increased fungal pretreatment time; therefore an optimal fungal pretreatment should be investigated in order to obtain the highest xylose production if a xylose fermenting organism is used.

SEM images showed *P. ostreatus* caused small poles and then enlarged the poles to large breakages on switchgrass, which was consistent with trends of lignin loss, hemicellulose loss, and cellulose loss trend.

5.6. Acknowledgements

This research was supported by the South Central Sun Grant Initiative and the Oklahoma Agricultural Experiment Station. The SEM images and nitrogen content analysis were done by Jiaqi Peng and Dr. Zhongdong Liu (Department of Food Science and Technology, Henan University of Technology, China), which was greatly appreciated. I owe thanks to Oscar Pardo Planas and Shelyn Gehle for provided assistance on SSF. I appreciate the instructions of Dr. Stephen Marek (Department of Entomology and Plant Pathology, OSU) with isolation and cultivation of *P. ostreatus*. I would like to thank Dr. Ajay Kumar (Department of Biosystems and Agricultural Engineering, OSU) for providing switchgrass materials. I also appreciate the assistance of Dr. Meizhen Xie and Dr. Nurhan Dunford (Robert M. Kerr Food & Agricultural Products Center, OSU) with training on biomass extraction.

5.7 References

- Adney, B., Baker, J. 1996. *Measurement of Cellulase Activities*. National Renewable Energy Laboratory, Golden, CO.
- Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology*, **101**(13), 4851-4861.
- Athmanathan, A., Emery, I., Kuczek, T., Mosier, N. 2014. Impact of temperature, moisture, and storage duration on the chemical composition of switchgrass, corn stover, and sweet Sorghum Bagasse. *BioEnergy Research*, 1-14.
- Bak, J.S., Ko, J.K., Choi, I.-G., Park, Y.-C., Seo, J.-H., Kim, K.H. 2009. Fungal pretreatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw. *Biotechnology and Bioengineering*, **104**(3), 471-482.
- Balan, V., Costa Sousa, L., Chundawat, S.S., Vismeh, R., Jones, A.D., Dale, B. 2008. Mushroom spent straw: a potential substrate for an ethanol-based biorefinery. *Journal of Industrial Microbiology & Biotechnology*, **35**(5), 293-301.
- Chen, F., Dixon, R.A. 2007. Lignin modification improves fermentable sugar yields for biofuel production. *Nature Biotechnology*, **25**(7), 759-761.
- Dibble, D.C., Li, C., Sun, L., George, A., Cheng, A., Çetinkol, Ö.P., Benke, P., Holmes, B.M., Singh, S., Simmons, B.A. 2011. A facile method for the recovery of ionic liquid and lignin from biomass pretreatment. *Green Chemistry*, **13**(11), 3255-3264.
- Digman, M., Shinnars, K., Muck, R., Dien, B. 2010a. Pilot-scale on-farm pretreatment of perennial grasses with dilute acid and alkali for fuel ethanol production. *Transactions of the ASABE*, **53**, 1007-1014.
- Digman, M.F., Shinnars, K.J., Casler, M.D., Dien, B.S., Hatfield, R.D., Jung, H.-J.G., Muck, R.E., Weiner, P.J. 2010b. Optimizing on-farm pretreatment of perennial grasses for fuel ethanol production. *Bioresource Technology*, **101**(14), 5305-5314.
- Digman, M.F., Shinnars, K.J., Dien, B.S., Hatfield, R., Li, X., Muck, R., Weimert, P. 2007. On-farm pretreatment technologies for improving enzymatic degradability of cellulose and hemicellulose present in perennial grass. *ASABE Annual International Meeting*, Minneapolis, MN.
- Donohoe, B.S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B. 2008. Visualizing lignin coalescence and migration through maize cell walls following

- thermochemical pretreatment. *Biotechnology and Bioengineering*, **101**(5), 913-925.
- Eggert, C., Temp, U., Eriksson, K.-E. 1996. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Applied and Environmental Microbiology*, **62**(4), 1151-1158.
- Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., Sannia, G. 2010. Laccases: a never-ending story. *Cellular and Molecular Life Sciences*, **67**(3), 369-385.
- Gómez, S.Q., Cuenca, A.A., Flores, Y.M., Rodríguez, J.N.G., Jurado, A.T. 2011. Effect of particle size and aeration on the biological delignification of corn straw using *Trametes* sp 44. *BioResources*, **7**(1), 0327-0344.
- Jing, X., Zhang, X., Bao, J. 2009. Inhibition performance of lignocellulose degradation products on industrial cellulase enzymes during cellulose hydrolysis. *Applied Biochemistry and Biotechnology*, **159**(3), 696-707.
- Kim, Y., Ximenes, E., Mosier, N.S., Ladisch, M.R. 2011. Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. *Enzyme and Microbial Technology*, **48**(4-5), 408-415.
- Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & Engineering Chemistry Research*, **48**(8), 3713-3729.
- Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Wojtaś-Wasilewska, M., Cho, N.-S., Hofrichter, M., Rogalski, J. 1999. Biodegradation of lignin by white rot fungi. *Fungal Genetics and Biology*, **27**(2-3), 175-185.
- Limayem, A., Ricke, S.C. 2012. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. *Progress in Energy and Combustion Science*, **38**(4), 449-467.
- Mäkelä, M.R., Lundell, T., Hatakka, A., Hildén, K. 2013. Effect of copper, nutrient nitrogen, and wood-supplement on the production of lignin-modifying enzymes by the white-rot fungus *Phlebia radiata*. *Fungal Biology*, **117**(1), 62-70.
- Membrillo, I., Sánchez, C., Meneses, M., Favela, E., Loera, O. 2008. Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by *Pleurotus ostreatus* strains. *Bioresource Technology*, **99**(16), 7842-7847.

- Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M.R. 2005. Optimization of pH controlled liquid hot water pretreatment of corn stover. *Bioresource Technology*, **96**(18), 1986-1993.
- Mukherjee, R., Nandi, B. 2004. Improvement of in vitro digestibility through biological treatment of water hyacinth biomass by two *Pleurotus* species. *International Biodeterioration & Biodegradation*, **53**(1), 7-12.
- Novozymes. 2012. Celluclast® 1.5L product data sheet.
- Parenti, A., Muguerza, E., Redin Iroz, A., Omarini, A., Conde, E., Alfaro, M., Castanera, R., Santoyo, F., Ramírez, L., Pisabarro, A.G. 2013. Induction of laccase activity in the white rot fungus *Pleurotus ostreatus* using water polluted with wheat straw extracts. *Bioresource Technology*, **133**, 142-149.
- Patel, H., Gupte, A., Gupte, S. 2009. Effect of different culture conditions and inducers on production of laccase by a basidiomycete fungal isolate *Pleurotus ostreatus* HP-1 under solid state fermentation. *BioResources*, **4**(1), 268-284.
- Qinnghe, C., Xiaoyu, Y., Tiangui, N., Cheng, J., Qiugang, M. 2004. The screening of culture condition and properties of xylanase by white-rot fungus *Pleurotus ostreatus*. *Process Biochemistry*, **39**(11), 1561-1566.
- Ramachandriya, K.D., Wilkins, M., Pardo-Planas, O., Atiyeh, H.K., Dunford, N.T., Hiziroglu, S. 2014. Simultaneous saccharification and fermentation of Eastern redcedar heartwood and sapwood using a novel size reduction technique. *Bioresource Technology*, **161**(0), 1-9.
- Richard, T., Proulx, S., Moore, K., Shouse, S. 2001. Ensilage technology for biomass pre-treatment and storage. *ASAE Annual International Meeting, Sacramento, CA*.
- Santoyo, F., González, A.E., Terrón, M.C., Ramírez, L., Pisabarro, A.G. 2008. Quantitative linkage mapping of lignin-degrading enzymatic activities in *Pleurotus ostreatus*. *Enzyme and Microbial Technology*, **43**(2), 137-143.
- Schmer, M.R., Vogel, K.P., Mitchell, R.B., Perrin, R.K. 2008. Net energy of cellulosic ethanol from switchgrass. *Proceedings of the National Academy of Sciences*, **105**(2), 464-469.
- Shi, J., Sharma-Shivappa, R.R., Chinn, M.S. 2009. Microbial pretreatment of cotton stalks by submerged cultivation of *Phanerochaete chrysosporium*. *Bioresource Technology*, **100**(19), 4388-4395.
- Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Wolfe, J. 2008. *Determination of Total Solids in Biomass and*

Total Dissolved Solids in Liquid Process Samples. National Renewable Energy Laboratory, Golden, CO.

- Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2007. *Determination of Structural Carbohydrates and Lignin in Biomass*. National Renewable Energy Laboratory, Golden, CO.
- Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. 2005. *Determination of Extractives in Biomass*. National Renewable Energy Laboratory, Golden, CO.
- Sun, Y., Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*, **83**(1), 1-11.
- Taniguchi, M., Suzuki, H., Watanabe, D., Sakai, K., Hoshino, K., Tanaka, T. 2005. Evaluation of pretreatment with *Pleurotus ostreatus* for enzymatic hydrolysis of rice straw. *Journal of Bioscience and Bioengineering*, **100**(6), 637-643.
- Tilman, D., Hill, J., Lehman, C. 2006. Carbon-negative biofuels from low-input high-diversity grassland biomass. *Science*, **314**(5805), 1598-1600.
- Tinoco, R., Acevedo, A., Galindo, E., Serrano-Carreón, L. 2011. Increasing *Pleurotus ostreatus* laccase production by culture medium optimization and copper/lignin synergistic induction. *Journal of Industrial Microbiology & Biotechnology*, **38**(4), 531-540.
- van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W. 2015. Fungal treated lignocellulosic biomass as ruminant feed ingredient: A review. *Biotechnology Advances*, **33**(1), 191-202.
- Wan, C. 2011. Microbial pretreatment of lignocellulosic biomass with *Ceriporiopsis subvermispota* for enzymatic hydrolysis and ethanol production, PhD Diss., The Ohio State University.
- Wan, C., Li, Y. 2011. Effectiveness of microbial pretreatment by *Ceriporiopsis subvermispota* on different biomass feedstocks. *Bioresource Technology*, **102**(16), 7507-7512.
- Wan, C., Li, Y. 2010a. Microbial delignification of corn stover by *Ceriporiopsis subvermispota* for improving cellulose digestibility. *Enzyme and Microbial Technology*, **47**(1-2), 31-36.
- Wan, C., Li, Y. 2010b. Microbial pretreatment of corn stover with *Ceriporiopsis subvermispota* for enzymatic hydrolysis and ethanol production. *Bioresource Technology*, **101**(16), 6398-6403.

Wang, X., Feng, H., Li, Z. 2012. Pretreatment of switchgrass with electrolyzed water and a two-stage method for bioethanol production. *Biotechnology and Bioprocess Engineering*, **17**(3), 624-633.

Webster, J., Weber, R. 2007. *Introduction to fungi*. Cambridge University Press, Cambridge, UK.

CHAPTER IV

6. Future work

The white rot fungi decay mechanism is not clear. Previous work mostly focused on the mechanism of cellulose conversion to glucose. The multiple enzyme systems in white rot fungi or natural consortia indicate the potential to design biological treatment that consolidates pretreatment and hydrolysis in one step. Characterization of genomic, transcriptomic and biochemical levels of different white rot fungi species, not just *P. chrysosporium*, would contribute to target identification for monoculture improvement and consortia construction.

Plant cell walls are very complex. Research on the cellular, subcellular, ultrastructural and even molecular level changes in the cell wall during white rot fungi pretreatment could reveal diverse catalytic reactions acting on biomass as well as the consequences of white rot fungi pretreatments. Plant cell wall structure changes induced by fungal pretreatment also accelerate an increased knowledge of lignin degradation enzyme systems and white rot fungi decay mechanisms.

A relatively long pretreatment time is required for white rot fungi pretreatment, which is a drawback compared to prevalent thermomechanical and thermochemical pretreatments. Simultaneous on-farm storage with white rot fungi pretreatment is a good option for the long time incubation based on the lignin degradation and enhanced ethanol

production in a controlled environment in chapter 5. Among the various aforementioned pretreatment methods, white rot fungi combined with mild chemical or physiochemical pretreatment methods (i.e. white rot fungi pretreatment prior to chemical and thermochemical pretreatment) would be the most efficient and promising method for large scale commercial operation. More thought should be put into efficiently integrating fungal pretreatment into the current biomass supply chain. There are challenges in system control for large scale fungal treatment. Moisture content of substrate seems to be the most sensitive factor affecting biomass pretreatment based on the results in Chapter 5. A moisture control system should be designed to achieve homogenous moisture distribution through switchgrass bale, which can make fungal treated samples sent to biorefinery are of the same quality. When applying lab scale pretreatment at large scale, fire risk in large switchgrass bales due to active microbial activity should be addressed. Modelling of the moisture and temperature gradients in the bale would be necessary to realize an efficient fungal pretreatment effect.

Vita

Mengxing Li

Candidate for the Degree of

Master of Science

Thesis: WHITE ROT FUNGI *PLEUROTUS OSTREATUS* PRETREATMENT ON SWITCHGRASS TO ENHANCE ENZYMATIC HYDROLYSIS AND ETHANOL PRODUCTION

Major Field: Biosystems Engineering

Biographical:

Born in Dengfeng, Henan, China on March 24, 1990, the son of Jianshe Li and Xiu'e Wang, raised in Dengfeng and graduated from Zhengzhou No.11 High School, Henan.

Education:

Completed the requirements for the Master of Science in Biosystems Engineering at Oklahoma State University, Stillwater, Oklahoma in July, 2015.

Completed the requirements for the Bachelor of Science in Food Science and Technology at Henan University of Technology, Zhengzhou, China in 2013.

Experiences:

Graduate research assistant, Oklahoma State University, Stillwater, OK, August 2013 to July 2015.

Undergraduate research assistant, Henan University of Technology, September 2010 to July 2013.

Professional Memberships:

American Society of Agricultural and Biological Engineers (ASABE).

Institute of Food Technologists (IFT).