Fungal Pretreatment and Enzymatic Hydrolysis of Genetically-modified *Populus trichocarpa*

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Fungal pretreatment of *Populus trichocarpa* wood genetically modified to reduce lignin and alter lignin chemistry is investigated for its effectiveness as an alternative to common pretreatment methods. The goal of this work is to improve biomass utilization for biofuel and biochemical applications by increasing sugar release. Sugar release after enzymatic hydrolysis was measured after various biomass pretreatments (including wood-rot fungus, hot water, and dilute acid). In the wildtype, and in constructs downregulated in PAL, 4CL, and C3H, the fungal pretreatment resulted in substantial improvements in sugar yields, up to 2.4-fold increase in glucose yield and 6-fold increase in xylose yield after enzymatic hydrolysis compared to the unpretreated control. However, the effects of fungal pretreatment were inconsistent, and in genetic lines down-regulated in 4CL, CCoAOMT, CAld5H, and C3H, fungal pretreatment yielded similar or decreased sugar release after enzymatic hydrolysis.

Keywords: Transgenic poplar; Fungal treatment; Ceriporiopsis; Pretreatment; Enzymatic hydrolysis; Lignin

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INTRODUCTION

Rising demand for liquid fuels and the finite supply of fossil fuels are driving research for biofuels and alternative energy. Producing bioethanol from lignocellulosics requires pretreatment steps to increase the reactivity of the biomass prior to enzyme hydrolysis and fermentation of sugars. Biomass pretreatments have been identified as one of the most costly steps in the bioethanol conversion process (Mosier *et al.* 2005). Common pretreatments require harsh chemicals, high temperature, and high energy inputs. Strategies for reducing the costs and complexity of pretreatments are of great interest (Mood *et al.* 2013). In this work, we explore the effects of genetic modification in combination with alternative pretreatments including fungal, hot water, and dilute acid pretreatments, to increase sugar release after enzymatic hydrolysis.

White-rot fungi (named for the bleached appearance of wood in the advanced stages of degradation) have an exceptional ability to selectivity degrade lignin, and in some cases they leave cell wall carbohydrates largely untouched (Eriksson *et al.* 1990; Martínez *et al.* 2005; Wan and Li 2012).

The white-rot species *Ceriporiopsis subvermispora* is particularly well-suited for pretreatment of both hardwoods and softwoods due to its ability to selectively degrade lignin without major degradation to carbohydrates during the initial stage of wood degradation (Blanchette *et al.* 1997; Wan and Li, 2010). Lignin degradation by *C. subvermispora* is largely driven by the extracellular lignin-degrading enzymes manganese peroxidase (MnP) and laccase (Blanchette *et al.* 1997; Tanaka *et al.* 2009; Li and Zheng 2019). Erven *et al.* (2019) demonstrates the main ligninolysis mechanism of single-electron transfer and resulting lignin bond cleavage. Studies show that syringyl (S) lignin may be preferentially degraded over guaiacyl (G) lignin in hardwoods (Choi *et al.* 2006); however, some studies utilizing different tree species are inconclusive due to the additional physical and morphological differences between species (Obst *et al.* 1994). Vasco-Correa *et al.* (2019) reported fungal pretreatment of hardwood, softwood, and miscanthus with *C. subvermispora* followed by enzymatic hydrolysis, and their results indicated that the fungal pretreatment effectiveness was feedstock dependent. More recently, studies utilizing wood from genetically-modified feedstocks have provided further insight into the relationship between lignin chemistry and fungal degradation (Giles *et al.* 2012; Skyba *et al.* 2013). In addition, Gaskell *et al.* (2014) reported increased expression of several oxidoreductases in white rot fungus *Phanerochaete chrysosporium* using genetically-modified high-S-lignin hybrid poplar as a substrate. This suggests an interaction between the lignin and expression of fungal enzymes.

Treatment with white-rot fungi has been shown to reduce electrical energy during mechanical pulping, increase paper strength, and reduce the environmental impact of pulping (Reid *et al.* 2010; Akhtar *et al.* 1997). In addition, wood-degrading fungi have been investigated for lignocellulose biomass pretreatment for the production of biofuels and biochemicals (Wan and Li 2012; Shirkavand *et al.* 2016). Fungal pretreatment has been investigated for many types of lignocellulosic biomass, including softwoods and hardwoods, grasses (*e.g.*, switchgrass, miscanthus), and agricultural residue such a wheat straw, cotton stalks, corn stover, and others (Wan and Li 2012; Rezania *et al.* 2020; Sankaran *et al.* 2020; Vasco-Correa *et al.* 2015, 2016). In general, these studies show that fungal pretreatment degrades lignin, in many cases with limited losses of cell wall carbohydrates, and it increases biomass reactivity when subjected to enzyme hydrolysis to produce monomer sugars (Sawada *et al.* 1995; Keller *et al.* 2003; Shi *et al.* 2009; Salvachúa *et al.* 2011; Wan and Li 2011 2012). While these results are promising, the extended pretreatment times (ranging from 14 to 90 days), losses of cellulose and hemicelluloses, and low hydrolysis yields are all practical barriers to commercialization (Wan and Li 2012; Wang *et al.* 2013).

Genetic engineering has received attention as a means to alter lignin content and composition, and reduce the recalcitrance of lignocellulosic feedstocks for enhanced utilization in biofuel and biochemical applications (Li *et al.* 2014; Wang *et al.* 2018). The objective of this study was to test fungal pretreatment with *C. subvermispora* on genetically-modified *P. trichocarpa* followed by enzymatic hydrolysis. In addition, the effectiveness of *C. subvermispora* as the sole pretreatment, or in combination with hot water and dilute acid pretreatments was tested. To our knowledge this is the first study to report the effects of fungal pretreatment on enzymatic hydrolysis utilizing wood from a genetically-modified tree species. These results will increase the understanding of how lignin content and lignin structure influence fungal degradation and enzymatic hydrolysis.

EXPERIMENTAL

Wood Samples

Wood samples from genetically-altered *P. trichocarpa* (provided by The Forest Biotechnology Group, N. C. State University) were used for fungal pretreatment experiments (Wang *et al.* 2018). Trees were grown in a greenhouse and harvested after 6 to 8 months. Wood from wildtype *P. trichocarpa*, referred to as WT, and 10 genetic lines with down-regulation of the following lignin biosynthesis genes were used:

- Two lines with high and low expression of cinnamate 3-hydroxylase (C3H) referred to as C3H-H and C3H-L.
- Two lines with high and low expression of coniferaldehyde 5-hydroxylase (CAld5H) referred to as CAld5H-H and CAld5H-L.
- Two lines down-regulated in 4-coumarate coenzyme A ligase (4CL) referred to as 4CL and 4CL3,5.
- One line simultaneously down-regulated in C3H and cinnamate 4-hydroxylase (C4H) referred to as C3H:C4H.
- Two lines down-regulated in phenylalanin ammonia lyase referred to as PAL and PAL1-5.
- One line down-regulated in caffeoyl coenzyme A O-methyltransferase (CCoAOMT) referred to as CCoAOMT.

Three stems from each of the *P. trichocarpa* genetic lines and wildtype were selected, and eight 1-inch sections were cut from the bottom of each stem. All stems were dried at 103 +/- 2 °C for 24 h and weighed to obtain the oven-dry weight. One section from each of the three stems in each genetic line was placed into a 20-mL scintillation vial, producing eight samples per genetic line. A sample consisted of one vial containing three 1-inch stem sections. All samples were then conditioned by adding an appropriate volume of distilled water to each vial so that the water:wood ratio was 1.5 after the inoculation procedure. After 24 h, all samples were sterilized by autoclaving at 121 °C for 20 min prior to inoculation. For each genetic line and WT, 4 to 5 samples received the fungal pretreatment and 2 to 3 samples served as sterile controls. Sterilizing the wood samples prior to fungal inoculations was necessary to prevent the growth of any preexisting microorganisms, and thereby isolating the effects of pretreatment with the selected white-rot fungus, *C subvermispora*. The control samples were sterilized in the same fashion as the samples receiving the fungal treatment in order to account for any effects that the sterilization treatment may have on the wood's chemical composition and sugar release after enzymatic hydrolysis. After the selected pretreatment, wood was milled to pass through a 20 mesh screen and extracted for 24 h with 95% ethanol (EtOH) for chemical analysis.

Fungal Pretreatment

C. subvermispora was cultured on a petri dish with 5% malt extract agar and incubated at 26 °C for 7 days. Next a fungal plug was used to inoculate 250 mL of 5% malt extract liquid medium (5% malt extract in deionized water (DI)), and incubated for an additional 30 days at 26 °C. The liquid fungal culture was vigorously shaken to homogenize the fungal mycelium, and 1 mL was directly added to each vial to inoculate the samples receiving fungal pretreatment. Sterile control samples received 1 mL of sterile DI water instead of fungal culture. Plastic caps were sterilized with 70% EtOH, tightened onto the

vial, then loosened one-quarter turn to allow gas exchange. Samples were incubated at 29 °C in darkness for 30 days. An open container of water was kept in the incubator to keep the humidity high. This fungal pretreatment method is based on previous research towards optimizing fungal degradation for small wood samples subjected to *C. subvermispora* (Edmunds *et al.* 2016).

Weight Loss and Cell Wall Component Loss

After the 30-day incubation period, samples were removed from the incubation containers and surface mycelium was gently removed. Samples were dried under vacuum at 40 °C for 48 h then weighed. Percent overall weight loss was calculated using the equation below,

$$\%\, Weight\ Loss = \left[1 - \left[\frac{W_F}{W_o}\right]\right] x\ 100\% \quad eq.1 \tag{1}$$

where W_F is dry weight after fungal pretreatment and W_o is oven dry weight before fungal treatment. Percent component loss for cell wall sugars (glucan, xylan, and mannan) and lignin was calculated by the following equation,

$$\%\, Component\ Loss = \left[1 - \left[\frac{W_F\, x\, \%\, Component_F}{W_o\, x\, \%\, Component_{SC}}\right]\right] x\ 100\% \tag{2}$$

where % $Component_F$ is the weight percent of the cell wall component measured after fungal pretreatment, and % $Component_{SC}$ is the average weight percent of the cell wall component measured in sterile control samples.

Chemical Analysis

High-throughput pyrolysis molecular beam mass spectroscopy (Py-mbms) was used to estimate lignin content and S/G ratio in sterile control and fungal-pretreated transgenic wood samples. Approximately 5 mg of milled and extracted wood from each sample was weighed then pyrolyzed for 2 min at 500 °C. The resulting pyrolysis vapors were quenched by free jet expansion in the molecular beam and transported to the mass spectrometer with helium (2 L/min). Electron impact ionization of 22.5-eV was used to collect ions in the range of *m*/*z* ratios of 30 to 450. To estimate lignin content, the relative intensities of the lignin peaks *m*/*z* = 120, 124, 137, 138, 150, 152, 154, 164, 167, 178, 180, 181, 182, 194, and 210 were summed and multiplied by a correction factor based on Klason lignin values determined on extracted samples before the sterilization procedure for WT and each genetic line. S/G ratios were determined by dividing the sum of the syringyl peaks at 154, 167, 168, 182, 194, 208, and 210 by the sum of the guaiacyl peaks at 124, 137, 138, 150, 164, and 178. A detailed description of the analytical pyrolysis procedure and instrumentation can be found in Sykes *et al*. (2009).

Cell wall carbohydrates were measured on sterile control and fungal-pretreated wood samples by a scaled-down version of the standard NREL Laboratory Analytical Procedure (Sluiter *et al.* 2008). All samples were analyzed in duplicate.

Hot Water and Dilute Acid Pretreatments and Enzymatic Hydrolysis

Fungal-treated and sterile control samples (milled and extracted) were subjected to a combined pretreatment (hot water or dilute acid) and enzymatic hydrolysis process followed by sugar release analysis in 96-well reaction plates using the National Renewable

Energy Laboratory's (NREL) high-throughput biomass protocol (Decker *et al.* 2009). This procedure consists of weighing 5.0 ± 0.3 mg of biomass into each well of a 96-well plate; each sample was measured in triplicate. Sterile control and fungal-treated samples were subjected to hot water or dilute acid pretreatment. This creates sample sets receiving a.) only fungal pretreatment, b.) only hot water pretreatment, c.) only dilute acid pretreatment, d.) sequential fungal+hot water pretreatments, and e.) sequential fungal+dilute acid pretreatments. Sterile control (*i.e.* no pretreatment) served as the control.

For no pretreatment (control) and hot-water pretreatment, 250 µL of DI water was added to each sample. In dilute-acid pretreatment, 200 µL of 0.4 % (w/w) H_2SO_4 was added to each sample. Hot-water and dilute-acid pretreated samples were subjected to a steam reactor at 180 °C for 17.5 min. Next, acid-pretreated samples were neutralized with 20 µL of 3.3% NaOH (w/v) and 30 µL of 1.0 M citrate buffer (pH 5.0) was added. Enzymatic hydrolysis was carried out in all pretreatment conditions by addition of 40 µL of 6% CTec2 enzyme solution (Novozymes, Franklinton NC) in 1.0 M citrate buffer (pH 5.0), and incubated for 70 h at 50 °C. After incubation, samples were diluted 10x with DI water. The solution is then diluted 10x again with either glucose oxidase/peroxidase reagent (GOPOD, Megazymes) or xylose dehydrogenase reagent (XDH, Megazymes) in clear microtiter plates to measure glucose and xylose sugars. Microtiter plates with GOPOD and XDH were then measured with UV/VIS spectrometer read at 510 nm and 340 nm for GOPOD and XDH, respectively. Six-point calibration curves for glucose and xylose were made by including calibration standards in each 96-well plate.

The hot water and dilute acid pretreatment conditions were chosen based on previous work completed at the Department of Energy's National Renewable Energy Laboratory on their high-throughput (HTP) biomass pipeline. These process conditions are meant to provide a sub-optimal pretreatment in order to provide incomplete sugar release so that the digestibility of the feedstocks based on a) their composition as affected by genetic modification and 2) fungal pretreatment effectiveness can be better determined. In addition, a relatively high enzyme loading of 40 µL of 6% CTec2 is meant to reduce effects of enzyme activity, thereby better isolating the effects of pretreatment effectiveness (Decker *et al.* 2015).

Glucose and xylose yield after enzymatic hydrolysis were calculated based on the average chemical composition of sterile control (untreated) wood. This accounted for carbohydrate losses due to fungal degradation, and the following equation was used:

$$\%\frac{Glucose}{Xylose}Yield = \frac{glucose/xylose\ released\ after\ enzymatic\ hydrolsis}{glucose/xylose\ in\ sterile\ control\ sample}\ x\ 100\% \qquad (3)$$

Statistical Analysis

A general linear model (proc GLM) and Tukey's mean separation (SAS, v. 9.4, SAS Institute Inc.) was used to test for significant differences in mean weight loss values after fungal pretreatment between samples of WT, and different genetic constructs. Microsoft Excel was used to calculate the r value and *p* value for correlations between % lignin content and % glucose and % xylose yields.

RESULTS AND DISCUSSION

Chemical Composition of Untreated Wood

There were considerable variations in the chemical composition between the WT and transgenic lines sterile control (untreated) samples. Tables 1 and 2 show total lignin content, S/G ratio, and structural carbohydrate content in the sterile control *P. trichocarpa* samples. Lignin content ranged from 10 wt.% in C3H-L to 22 wt.% in WT, and Py-mbms estimated S/G ratios ranging from 0.3 to 1.8 in Cald5H-L and WT, respectively. The H-lignin content was not measured directly in these samples; however, Wang *et al*. (2018) showed high H-lignin content in C3H down-regulated *P. trichocarpa*. The wood samples used for this work were a sub-set of samples studied by Wang *et al*. (2018). For example, Wang *et al*. (2018) reported C3H3 down-regulated samples to contain 45 wt.% H lignin monomers, and C3H:C4H down-regulated sample to contain 33 wt.% H lignin as measured by 2D NMR. Transgenic lines were categorized based on lignin characteristics into the following groups: slight to moderately reduced lignin (including lines PAL, PAL1-5, 4CL, 4CL3,5, and CCoAOMT); increased G-lignin (including CAld5H-L and CAld5H-H), and significantly reduced lignin content and a concurrent increase in H lignin (including C3H-L, C3H-H, and C3H:C4H) as outlined in Table 1.

Fungal Pretreatment Alone

Weight loss

Weight loss values after the 30-day fungal pretreatment period ranged from 7 to 19 wt.%, and substantial differences were seen between WT with 8.5% weight loss and samples from several transgenic lines (Table 2). The ANOVA results demonstrate that both the fungal pretreatment as well as the genetic line had a significant effect on weight loss ($p < 0.0001$). WT and many transgenic lines fall within the range of reported weight loss values utilizing *C. subvermispora* to treat hardwoods, which ranges from 6 to 15 wt.% after 30 days of treatment (Choi *et al.* 2006; Giles *et al.* 2011; Wan and Li 2011). However, samples from several genetically-modified lines exhibited higher weight loss than is generally reported, indicating that these transgenic lines were less resistant to fungal decay. In addition, no clear correlation between the initial lignin content or the initial S/G ratio and weight loss after fungal degradation was observed.

Cell wall component loss after fungal pretreatment

Lignin selectivity of *C. subvermispora* was high in WT and several other transgenic lines. Also, in general, when weight loss was in the low to moderate range (7 to 15 wt.%), cellulose loss was lower, ranging from 1 to 10 wt.% reduction. High lignin selectivity was expected based on the lignin-selective nature of *C. subvermispora* (Otjen *et al.* 1987). However, when the overall measured weight loss after fungal pretreatment was in the higher range (>17%), cellulose loss was also more substantial, ranging from 21 to 25 wt.%. In these cases, it is likely that the fungal degradation had exceeded the initial lignin-selective decay period, suggesting that these samples were degraded more quickly and to a greater extent.

Based on S/G ratio determined by Py-mbms (Table 1), there was a decrease in the S/G ratio after fungal pretreatment for all samples, with the exception of CCoAOMT, Cald5H-L, and Cald5H-H, where the S/G ratio was unchanged or slightly increased. This suggests that with these exceptions, fungal pretreatment resulted in higher degradation of the S-lignin relative to G-lignin.

Table 1. Chemical Composition of Sterile Control (untreated) and Fungal-Pretreated Wildtype and Genetically-Modified *P. trichocarpa* Wood Samples

Construct	Treatment	N	Glucan	Xylan	Mannan	% Lignin** Content	S/G ratio**	Description
WT	*SC*	*3*	*41.4 (0.3)**	*14.1 (08)*	*2.3 (0.3)*	*21.8 (0.4)*	*1.8 (0.1)*	Wildtype
	F	5	44.9 (0.6)	13.8 (0.7)	2.3 (0.4)	14.5 (0.7)	1.4 (0.1)	
PAL	*SC*	*3*	*42.8 (0.8)*	*13.7 (0.2)*	*2.5 (0.6)*	*18.4 (0.5)*	*1.8 (0.0)*	Slightly to Moderately Reduced Lignin Content
	F	5	45.2 (0.8)	12.6 (1.0)	2.5 (0.6)	14.1 (0.4)	1.5 (0.0)	
PAL1-5	*SC*	*3*	*42.8 (1.3)*	*12.5 (1.4)*	*2.2 (0.4)*	*17.6 (1.3)*	*1.8 (0.1)*	
	F	5	45.3 (0.9)	11.6 (0.7)	2.0(0.5)	14.1 (0.5)	1.6 (0.1)	
4CL	*SC*	*3*	*42.1 (0.2)*	*13.5 (0.2)*	*1.6 (0.6)*	*18.8 (0.1)*	*1.8 (0.1)*	
	F	5	45.5 (1.0)	14.8 (0.5)	1.7 (0.4)	13.5 (0.8)	1.5 (0.1)	
4CL3,5	*SC*	*3*	*43.1 (0.9)*	*11.5 (0.1)*	*2.0 (0.7)*	*19.1 (0.3)*	*1.7 (0.0)*	
	F	5	42.1 (0.9)	14.9 (0.3)	1.7 (0.0)	16.5 (1.0)	1.6 (0.1)	
CCoAOMT	*SC*	*3*	*46.0 (1.5)*	*11.2 (0.9)*	*1.7 (0.3)*	*17.6 (0.3)*	*1.6 (0.1)*	
	F	4	42.3 (0.9)	13.7 (0.8)	1.5 (0.1)	17.5 (0.2)	1.6 (0.1)	
CAld5H-L	*SC*	*2*	*44.1 (1.5)*	*12.0 (0.2)*	*1.8 (0.6)*	*20.9 (0.1)*	*0.3 (0.0)*	Increased G-Lignin
	F	4	42.4 (1.2)	11.7 (0.7)	1.7 (0.2)	18.6 (0.5)	0.4 (0.0)	
CAld5H-H	*SC*	*3*	*45.2 (0.4)*	*12.2 (0.3)*	*2.2 (0.1)*	*20.8 (0.4)*	*1.1 (0.0)*	
	F	5	43.7 (1.8)	12.7 (0.9)	1.8 (0.4)	19.3 (0.9)	1.1 (0.0)	
C3H-L	*SC*	*2*	*42.1 (0.5)*	*16.6 (0.8)*	*2.0 (0.3)*	*9.9 (0.3)*	*0.9 (0.0)*	Severely Reduced Lignin Content and Increased H-Lignin
	F	5	44.2 (0.9)	14.9 (0.5)	1.8 (0.2)	9.2 (0.4)	0.8 (0.1)	
C3H-H	*SC*	*3*	*43.1 (0.3)*	*16.6 (0.9)*	*2.8 (0.4)*	*13.5 (0.3)*	*1.4 (0.0)*	
	F	5	46.8 (0.6)	15.4 (0.8)	2.7 (0.3)	9.8 (0.3)	1.0 (0.1)	
C3H:C4H	*SC*	*3*	*44.0 (0.2)*	*16.1 (0.9)*	*2.9 (0.5)*	*13.5 (0.8)*	*1.2 (0.1)*	
	F	5	44.8 (1.4)	15.3 (0.5)	2.9 (0.6)	11.1 (0.3)	1.1 (0.1)	

* Parenthesis indicate standard deviation; ** Lignin content and S/G ratio measured by Py-MBMS; “SC” denotes “sterile control” and “F” denotes “fungal pretreatment”

Table 2. Overall Weight Loss, and Cell Wall Component (Cellulose, Hemicellulose, and Lignin) Loss after Fungal Pretreatment for Wildtype and Genetically-Modified *P. trichocarpa* Wood Samples

Construct	Overall Wt. Loss (%)	Cell Wall Component Loss (%)			Description
		Cellulose**	Hemicellulose***	Lignin	
WT	8.5 (0.5)* D	0.8 (1.2)	9.9 (6.3)	39.1 (3.1)	Wildtype
PAL	7.1 (3.3) D	1.8 (5.1)	13.5 (7.4)	28.7 (2.9)	Slightly Reduced Lignin Content
Pal1-5	6.8 (0.1) D	1.3 (2.0)	13.6 (7.6)	25.6 (2.5)	
4CL	15.3 (1.1) BC	8.3 (1.0)	5.4 (7.0)	39.3 (4.5)	
4CL3,5	19.4 (1.9) A	21.4 (3.2)	5.2 (8.6)	30.2 (5.4)	
CCoAOMT	18.6 (2.0) A	25.1 (10.8)	6.9 (2.9)	19.3(2.9)	
Cald5H-L	19.1 (1.2) A	22.4 (8.9)	21.1 (5.3)	28.2 (1.2)	Increased G-Lignin
Cald5H-H	17.9 (0.9) AB	21.4 (3.5)	21.2 (10.8)	24.0(3.8)	
C3H-L	14.2 (1.2) C	10.0 (1.6)	22.9 (3.9)	20.3 (4.0)	Severely Reduced Lignin Content and Increased H-Lignin
C3H-H	7.4 (0.3) D	-0.7 (1.1)	13.6 (4.9)	33.1 (2.2)	
C3H:C4H	7.5 (0.9) D	5.8 (2.7)	11.1 (5.0)	23.8 (1.8)	

*Parenthesis indicate standard deviation **Cellulose calculated by glucan; *** hemicellulose calculated by the sum of xylan and mannan; Different letters indicate significant difference (α=0.05) in the mean of overall wt. loss.

These results are consistent with Obst *et al*. (1994) and Davis *et al*. (1994), who reported that as fungal degradation progressed the S/G ratio decreased in several species of hardwoods degraded by several white rot fungi such as *Trametes versicolor* and *P. chrysosporium*. In addition, Choi *et al*. (2006) reported that aspen wood degraded by *C. subvermispora* resulted in cleavage of β-O-4 linkages, and degradation of S lignin appeared to be preferred. Microscopic analysis has demonstrated the decay pattern of white rot fungi as beginning in the cell lumen and progressively moving toward the middle lamella and cell corners (Blanchette 1991). It has been suggested that S lignin reduction and decreased S/G ratio following white rot decay is consistent with preferential degradation of the S-lignin-rich cell wall, as opposed to the G-lignin-rich middle lamella and cell corner (Davis *et al.* 1994; Obst *et al.* 1994).

Degradation of lines with high G lignin

The two Cald5H down-regulated samples had among the highest weight loss with 19% and 18% for Cald5H-L and Cald5H-H, respectively. Both Cald5H down-regulated samples had low S/G ratio values and normal lignin content values (measured by Py-MBMS), indicating elevated G lignin concentrations compared to the WT. For example, Cald5H-L had an estimated S/G ratio of 0.3, as determined by Py-mbms. Previous studies on CAld5H up-regulated poplar with high S lignin content have reported increased decay resistance against *C. subvermispora* (Giles *et al.* 2012; Skyba *et al.* 2013). Giles *et al.* (2012) reported significantly less lignin degradation in high S lignin quaking aspen compared to the control, although overall weight loss was higher due to greater degradation of carbohydrates. Skyba *et al*. (2013) reported significantly reduced weight loss and reduced lignin loss in high S/G ratio samples compared to the control, and hypothesized that lignin with high proportions of S monomers have more β-O-4 linkages, resulting in a more linear lignin polymer than in lignin with higher amounts of G monomers. Based on

this rationale, the Cald5H down-regulated samples examined in this study, may be more highly branched, leading to greater degradation by the fungus. The degree of lignin loss in the Cald5H down-regulated samples was lower (24 to 28 wt.%), while carbohydrate loss was higher than many of the other genetic lines. One possible explanation is that in the latter stages of the fungal pretreatment the white-rot fungi may have relied more heavily on carbohydrate as a carbon source, leading to more vigorous growth and catabolism. It is possible that chemical or physical properties, other than those reported in this study, are altered a result of the genetic modification. Due to the complexity of these various aspects of wood properties and fungal degradation, the reason for the high susceptibility to fungal degradation of the Cald5H samples is difficult to isolate.

Degradation of lines with slightly and moderately reduced lignin content

Compared to WT, significantly higher weight loss was observed in samples 4CL, 4CL3,5 and CCoAOMT with weight losses of 15%, 19%, and 19 wt.%, respectively. These three samples had slight to intermediate reductions in lignin content due to the genetic modification with 19%, 19%, and 18 wt.% initial lignin content for 4CL, 4CL3,5, and CCoaOMT, respectively. This is consistent with the results of Giles *et al.* (2012), who showed that genetically-modified 4CL-reduced poplar wood exhibited greater loss of cellulose during fungal pretreatment. This is consistent with the higher loss of cellulose due to higher fungal growth and ligninolytic activity. In contrast, both PAL down-regulated samples showed weight loss similar to WT indicating that higher levels of lignin content did not significantly alter the decay resistance of the wood.

Degradation of lines with severely reduced lignin content

The transgenic line C3H-L with its significant reduction in lignin exhibited higher weight loss after fungal pretreatment than WT, while the other two lines with significant reductions in lignin, *e.g.*, C3H3-H and C3H:C4H, had similar weight loss to WT. It appears that there were several competing effects in these samples, including reduced lignin content and an increased proportion of H lignin, as well as changes in anatomical cell wall structure in these genetically-modified samples (Miller *et al.* 2019). Table 1 shows that in many lines the S/G ratio decreased after the fungal pretreatment, which was likely caused by relatively higher degradation of the S-lignin-rich secondary cell wall compared to G-lignin-rich middle lamella (Table 1). The individual effects of lignin reduction and incorporation of H lignin, and their interactions, obscure these results.

It is generally agreed that increased lignin content leads to increased resistance towards biological degradation (Kirk *et al.* 1984). However, in this study, there was no clear trend between the initial lignin content and weight loss after fungal pretreatment among various wood samples. This may be an effect of the lignin-specific nature of *C. subvermispora*, which exhibits low cellulolytic capacity (Fernandez-Fueyo *et al.* 2012). As discussed previously, genetic perturbations influence both the chemical and physical properties of both the lignin and carbohydrates. For example, Miller *et al*. (2019) showed significant anatomical differences in a subset of these genetically-modified *P. trichoparpa* specimens. Thus, the effects of changes in wood's lignin content on its resistance to fungal degradation is confounded by these anatomical changes.

Enzymatic Hydrolysis

In a second set of experiments, hot water or dilute acid pretreatments were applied to fungal-treated and sterile control samples. Sugar release following the various

pretreatments and enzymatic hydrolysis were measured. The addition of hot water or dilute acid pretreatment following the fungal pretreatment and then enzymatic hydrolysis yielded mixed sugar release results among the various transgenic samples tested. The ANOVA results demonstrate that pretreatment type had a significant effect on both glucose yield and xylose yield ($p < 0.0001$). Figure 1 shows the percent yield of glucose and xylose following various pretreatments and enzymatic hydrolysis relative to the amount of glucose or xylose in the sterile control samples (*i.e.*, no pretreatment); thus, carbohydrate loss due to the fungal pretreatment was accounted for.

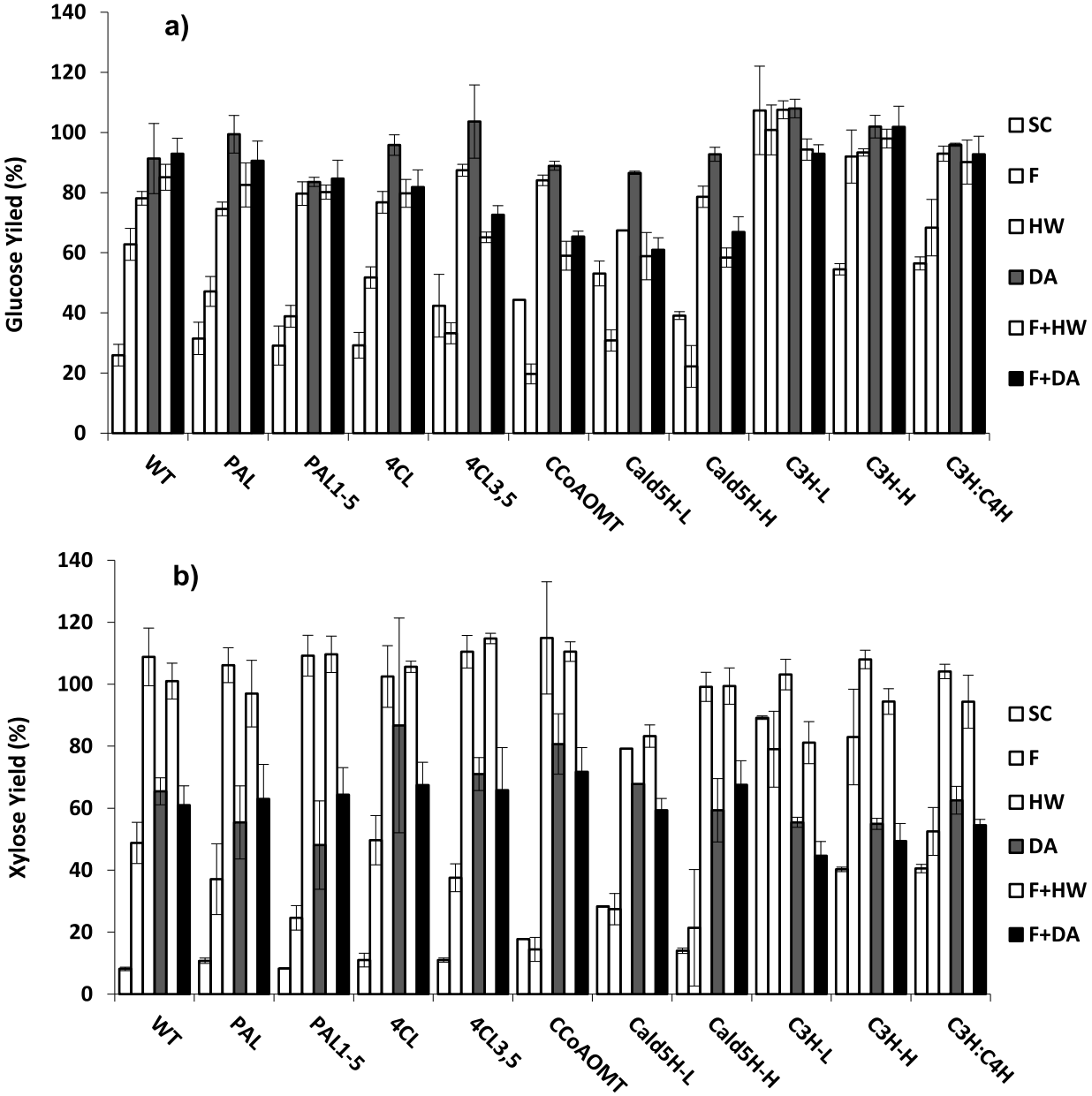

Fig. 1 Yield of a) glucose and b) xylose after 72-h enzymatic hydrolysis for sterile control and various treatment regimes. Yield percent is based on chemical composition of sterile control (untreated) wood; legend abbreviations for the treatments: SC = sterile control (*i.e.* no pretreatment), F = fungal alone, HW = hot water alone, DA = dilute acid alone, F+HW = sequential fungal followed by hot water, and F+DA = sequential fungal followed by dilute acid.

The box plots in Fig. 2 show the distribution of glucose and xylose yields (after pretreatments and enzymatic hydrolysis) for the various pretreatments tested with all sample types (transgenic and wildtype) grouped together. As expected, the SC samples have the lowest average sugar release values. The fungal alone pretreatment resulted in higher average sugar yields; however, there was a large variance among the various transgenic wood samples tested. The hot water alone pretreatment resulted in higher average sugar yields than the fungal alone pretreatment. The dilute acid alone pretreatment resulted in the greatest average glucose yield. However, the xylose yields for both dilute acid alone and sequential fungal and dilute acid pretreatment was lower than hot water alone and sequential fungal and hot water pretreatment. This is likely caused by xylose degradation in acidic conditions. The sequential fungal and hot water pretreatments resulted in similar average glucose and xylose sugar yields to the hot water alone pretreatment.

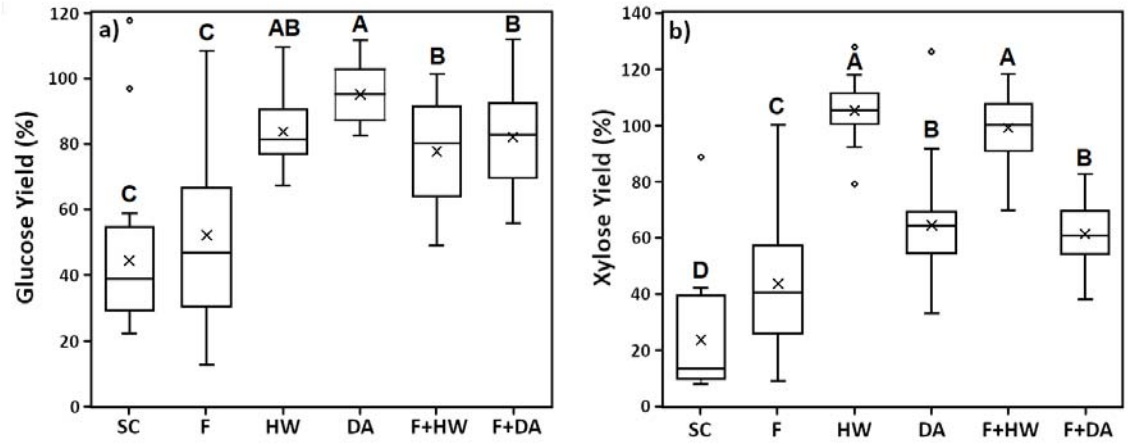

Fig. 2. Box plots showing the distribution of a) glucose yield and b) xylose yield after various pretreatment regimes and 72-h enzymatic hydrolysis. Yield percent based on chemical composition of sterile control (untreated) wood; abbreviations denote the following pretreatments: SC = sterile control (*i.e.* no pretreatment), F = fungal alone, HW = hot water alone, DA = dilute acid alone, F+HW = sequential fungal followed by hot water, and F+DA = sequential fungal followed by dilute acid. Different letters indicate significantly different (α=0.05) mean glucose or xylose yields between the different treatments.

Effect of fungal pretreatment alone

Using only the fungal pretreatment, WT, PAL, 4CL, and C3H-H showed large increases in glucose and xylose yields compared to the sterile controls (Fig 1). WT showed 63% glucose yield and 49% xylose yield after enzymatic hydrolysis, representing 2.4-fold and 6-fold increase compared to the sterile control. Following a similar trend, Vasco-Correa *et al.* (2019) reported a ~35% glucose yields (representing a 4.5-fold increase compared to the control) for hardwood (*Fraxinus americana*) and a ~20% xylose yield (representing a 10-fold increase compared to the control) after a 14-day fungal pretreatment with *C. subvermispora*. The differences in yield between this study and that of Vasco-Correa *et al.* (2019) may be explained by differences in the hardwood species used, differences in the incubation time, as well as the use of sterilized *vs.* non-sterilized feedstocks.

Fungal-treated 4CL resulted in similar yields as WT, while fungal-treated C3H-H resulted in high yields of 91% glucose and 83% xylose, representing yield increases of 1.7-fold for glucose and 2.1-fold for xylose, compared to the sterile control of the same transgenic line.

Fungal-treated lines CCoAOMT, Cald5H-L, and Cald5H-H resulted in lower glucose yields compared to their sterile control counterparts after enzymatic hydrolysis (Fig. 1). These samples had among the highest overall weight loss and glucose reduction during the initial fungal pretreatment, consistent with the easily accessible cellulose fraction being removed by the fungus and leaving behind a more recalcitrant fraction resulting in reduced hydrolysis.

Genetic line C3H-L showed near 100% glucose yield and 89% xylose yield in sterile control samples (*i.e.* no pretreatment), and the fungal pretreatment provided no benefit. This is likely caused by the severe lignin reduction with only 9.9% lignin content and associated low recalcitrance, which were observed in the C3H-L line. Chen and Dixon (2007) reported high enzymatic hydrolysis efficiency (55%) in C3H down-regulated alfalfa with severely reduced lignin content, which is consistent with these results. In addition, Wang *et al.* (2018) reported an increase in sugar release following enzymatic hydrolysis and lower lignin content in genetically-modified *P. trichocarpa*.

Effect of hot water alone and dilute acid alone pretreatments

In most cases, the hot water pretreatment alone resulted in higher yields than the fungal pretreatment alone. Exceptions to this case included C3H-L and C3H-H, where yields of glucose and xylose were similar between fungal pretreatment alone and hot water pretreatment alone. Considering the dilute acid pretreatment alone, yield of glucose was higher in WT and most genetic lines compared to fungal pretreatment alone, and slightly higher or similar to the hot water pretreatment alone. Similar to the hot water only pretreatments, the dilute acid only pretreatment for genetic lines C3H-L and C3H-H yielded similar glucose and xylose yields to that of fungal pretreatment alone. Yields of xylose were consistently lower for the dilute acid pretreatment, which can be attributed to xylose degradation by the acidic pretreatment (Alvira *et al.* 2010).

Effect of sequential pretreatments

As expected, with a few exceptions, fungal pretreatment followed by hot water pretreatment resulted in higher glucose and xylose yields than fungal pretreatment alone. The glucose and xylose yields for the combination of fungal pretreatment followed by hot water pretreatment was essentially the same as hot water pretreatment alone. These results indicate that the beneficial effects of fungal pretreatment were diminished when hot water pretreatment was added. The exceptions to this trend are C3H-L, for which the sterile control (*i.e.*, no pretreatment) and fungal pretreatment alone resulted in the same sugar release as sequential fungal and hot water pretreatment, and C3H-H, where the fungal pretreatment alone produced similar results as the sequential fungal and hot water pretreatment.

Previous research has demonstrated that hot water pretreatment solubilizes hemicelluloses and improves enzymatic hydrolysis yields (Xiao *et al.* 2011). Wang *et al.* (2012) reported that sequential fungal (L. betulina C5617) and liquid hot water (140 to 200 °C) pretreatment on *Populus tomentosa* resulted in 1.15 to 2.66-fold increase of glucose yield after enzymatic hydrolysis compared to hot water pretreatment alone. Wang *et al.* (2012) found that the beneficial effects of sequential fungal and hot water pretreatments

were more pronounced when hot water pretreatment was at a lower temperature due to decreased severity. In the current study, hot water pretreatment was applied at 180 °C for 17.5 min, and if lower temperature or reduced residence time were used, synergistic effects of sequential fungal and hot water pretreatments may have been observed.

The sequential fungal and dilute acid pretreatment showed increased yield of glucose with respect to the fungal pretreatment alone, except in lines C3H-L and C3H-H, in which the results were similar. The yield of xylose was similar between fungal pretreatment alone and sequential fungal and acid pretreatment for WT and C3H:C4H, but higher in the case of fungal pretreatment alone for lines C3H-L and C3H-H. The sequential fungal and dilute acid pretreatment showed no increase in yield compared to dilute acid pretreatment alone. This suggests that the more severe dilute acid pretreatment overwhelmed the benefits of the fungal pretreatment.

For samples 4CL3,5, CCoAOMT, Cald5H-L, and Cald5H-H, for which the fungal pretreatment resulted in significant degradation of the original glucan (18 to 19%), dilute acid pretreatment alone resulted in greater glucose yields than the sequential fungal and dilute acid pretreatment. Similar to the dilute acid pretreatment alone, there was a reduced xylose yield for the sequential fungal and dilute acid pretreatment.

Sugar yield correlations

Lignin content is considered to be one of the main indicators of biomass recalcitrance (Studer *et al.* 2011; Min *et al.* 2012). Previous research has demonstrated an inverse correlation between lignin content and sugar release after enzymatic hydrolysis (Chen and Dixon 2007; Studer *et al.* 2011; Min *et al.* 2012; Wang *et al.* 2018). Similar to these past results, Fig. 3 shows there is a strong correlation between lignin content and sugar release for both glucose (R2 = 0.68) and xylose (R2 = 0.79) after enzymatic hydrolysis for both the fungal alone pretreated and non-pretreated (sterile control) samples.

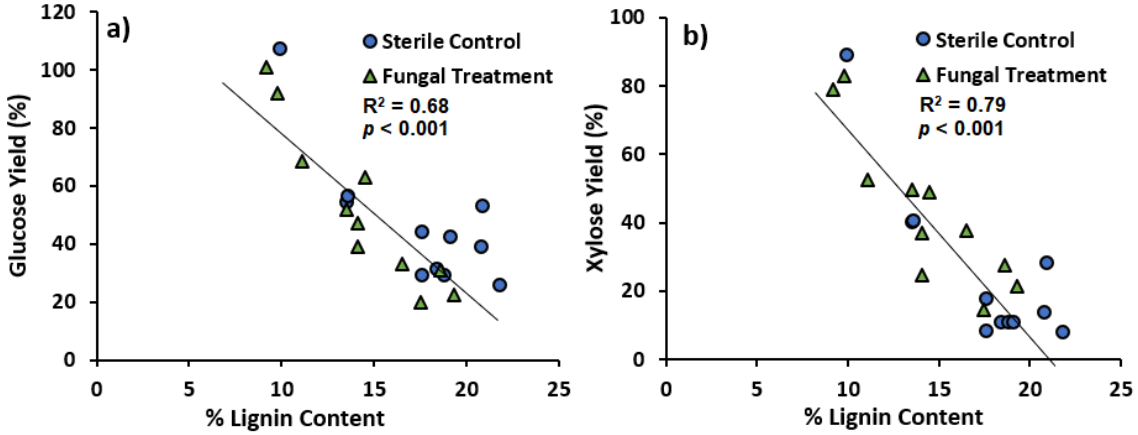

Fig. 3. Correlation between % lignin content and a) glucose yield and b) xylose yield for sterile control and fungal alone treated *P. trichocarpa* samples after enzymatic hydrolysis; glucose and xylose yields are based on chemical composition of untreated (sterile control) wood.

Summary of Pretreatment and Enzymatic Hydrolysis Results

Biomass susceptibility to enzymatic hydrolysis after pretreatments yielded mixed results among the different genetic modifications. These differences can have several different, inter-related mechanisms that include both the initial recalcitrance of the

biomass, wood anatomical differences, and the effectiveness of the fungal treatment. Notably, fungal pretreatment of WT and C3H-H resulted in substantial improvements of sugar yields after enzymatic hydrolysis. Furthermore, transgenic line C3H-L, with its significantly lower lignin content, yielded nearly complete conversion of glucose and xylose without any pretreatment indicating very low initial recalcitrance. These genetic lines exhibit low recalcitrance and could be promising as a renewable feedstock if their silvicultural traits were acceptable. However, severe lignin reductions have been reported to impair growth rate and physiological characteristics of the plant (Coleman *et al.* 2008; Novaes *et al.* 2010; Voelker *et al.* 2010; Li *et al.* 2014). The physiological changes, growth rate, insect and disease resistance, and hardiness must be considered in order to produce commercially attractive feedstock.

It is important to recognize that the time required (generally 14 to 90 days) for fungal pretreatment can be a disadvantage when compared to chemical or physical pretreatment techniques (Shirkavand *et al.* 2016). However, the reduced energy and chemical inputs of fungal pretreatment may offset this disadvantage. In this study, we utilized a 30-day incubation period based on previous work (Edmunds *et al.* 2016) as well as this being a commonly utilized time in previous research. Depending on the initial chemical composition and physical characteristics of the feedstock utilized, a shorter incubation time may yield acceptable results. We suggest the optimization of fungal pretreatment time for different feedstocks as an important topic for further work.

CONCLUSIONS

1. Fungal pretreatment can be effective in improving sugar yield on enzymatic hydrolysis of genetically-modified poplar; however, it is not as efficient as the standard hot-water or dilute-acid pretreatment methods.
2. Sequential pretreatments afford no or minimal improvement over standard individual pretreatments on amount of sugar yield.
3. Genetic lines with severely reduced lignin content provides high glucose yields on enzymatic hydrolysis under all pretreatment conditions, including the control (no pretreatment).

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