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Lignocellulose modifications by brown rot fungi and their effects, as pretreatments, on cellulolysis

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ABSTRACT

Brown rot fungi *Gloeophyllum trabeum* and *Postia placenta* were used to degrade aspen, spruce, or corn stover over 16 weeks. Decayed residues were saccharified using commercial cellulases or brown rot fungal extracts, loaded at equal but low endoglucanase titers. Saccharification was then repeated for high-yield samples using full strength commercial cellulases. Overall, brown rot pretreatments enhanced yields up to threefold when using either cellulase preparation. In the best case, aspen degraded 2 weeks by *G. trabeum* yielded 72% glucose-from-cellulose, a 51% yield relative to original glucan. A follow-up trial with more frequent harvests showed similar patterns and demonstrated interplay between tissue modifications and saccharification. Hemicellulose and vanillic acid (G6) or vanillin (G4) lignin residues were good predictors of saccharification potential, the latter notable given lignin's potential active role in brown rot. Results show basic relationships over a brown rot time course and lend targets for controlling an applied bioconversion process.

1. Introduction

Brown rot fungi are a group of wood-degrading fungi with a mechanism relevant for industrial bioconversion. These fungi remove nearly 100% of carbohydrates from wood, but they usually remove little lignin (Eriksson et al., 1990). Brown rot fungi are theorized to initiate decay with a non-enzymatic pretreatment step (Koenigs, 1974; Jensen et al., 2001) that has potential to be chemically reproduced and hastened. Many brown rot species also do not secrete cellobiohydrolase enzymes and lack their genes (Valášková and Baldrian, 2006; Martinez et al., 2009), and many have difficulty efficiently degrading pure cellulose substrates like standard filter paper or delignified wood (Highley, 1980; Blanchette, 1983). This suggests an active role for lignin and a potential for exploiting synergies using simpler, non-traditional hydrolytic suites. Collectively, these biological traits encourage research into the basic brown rot mechanism and into industrial alternatives that harness or emulate these fungi.

Aspects of the brown rot mechanism remain unclear and are often studied individually rather than as part of a system. Brown rot research has focused primarily on early modifications that rapidly reduce wood strength (Winandy and Morrell, 1993; Curling et al., 2002), a key decay issue in load-bearing lumber. Brown rot fungi are theorized to use a non-enzymatic mediated pretreatment combining iron (Fe^{2+}) and hydrogen peroxide to produce hydroxyl radicals (Goodell et al., 1997). Reactive oxygen species (ROS) may play a direct role in demethylating, oxidizing, and cleaving lignin (Kirk, 1975; Filley et al., 2002; Yelle et al., 2008), and brown rot is characterized by early carbohydrate depolymerization starting with side-chain hemicelluloses (Curling et al., 2002; Irbe et al., 2006). Relative to bioconversion interests, however, this is only the first step in metabolizing wood – a pretreatment before saccharification. Although Schilling et al. (2009) showed that early wood modifications made by brown rot fungi broadly facilitate enzymatic saccharification, its underlying mechanism remains unclear and the best application scenario has not been investigated. Therefore, research applying brown rot for bioconversion offers two benefits: (1) improvement in commercial potential, and (2) opportunity to study the mechanism as output from a system rather than reconstruction from individual observations.

Exploiting brown rot fungi for biomass conversion has been attempted in the past, either by inoculating with a fungus as a biological pretreatment or by using a chemical mimick to pretreat wood. Ray et al. (2010) used *Coniophora puteana* as a biological pretreatment to degrade Scots pine blocks for 15 days, then halted

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degradation and saccharified the degraded material. They estimated that subsequent saccharification yields of glucose likely exceeded 70% at only 9% wood mass loss, although lignin and holocellulose contents were inferred from published values and not measured. As another direct pretreatment approach, Fissore et al. (2010) inoculated Radiata pine with *Gloeophyllum trabeum* and further pretreated this decayed wood with organosolv to attain up to 70% glucan-to-glucose yield. Rasmussen et al. (2010) inoculated corn stover with *G. trabeum*, and, after an aerobic growth period, grew *G. trabeum* anaerobically to encourage fermentation. Although surprisingly high ethanol conversion yields have been published for white rot fungi (Okamoto et al., 2010), yields from *G. trabeum* in this case were low and *Saccharomyces cerevisiae* was added to aid fermentation. Finally, as a brown rot mimick, Rättö et al. (1997) used Fenton reagents to pretreat spruce, which improved saccharification yields of glucose from 12.2% to 35.2% for commercial cellulases.

These approaches applying brown rot fungi or their presumed mechanism to commercial bioconversion must promise competitive yields (>85%) versus other pretreatments such as dilute acid (Saha, 2004; Aden and Foust, 2009; Kazi et al., 2010), or else offer consolidation potential or other operating cost savings that would compensate lower single batch yields. Approaches requiring addition of extra processing steps are likely not practical. In all cases, reliable assessments require both accounting for sugars lost over the course of processing and defining relationships between tissue modifications and saccharification efficiency in commercially-relevant processes. Our goals with this study were to track both soluble and embedded carbon fractions in relevant plant feedstocks over a time course of fungal pretreatment and enzymatic saccharification, and to correlate promising yields with the timing of relevant cell wall modifications.

2. Methods

2.1. Feedstocks

Aspen, spruce and corn stover were selected as viable bioenergy feedstocks that also represent a range of plant types, useful for making comparisons. Aspen (*Populus* sp.) and spruce (*Picea* sp.) 19 mm cubes were cut from single, non-treated boards acquired from a local lumber supplier in Minneapolis, Minnesota, USA. Corn stover (*Zea mays*) was acquired from a farm co-operative in Pine City, Minnesota, and stems and leaves were homogenized in a Wiley mill to pass through a 40 mesh screen. Wood was oven-dried at 100 °C for 48 h to determine initial weight. Stover powder was partitioned into 1 g aliquots and oven-dried for addition to soil-block microcosms. All substrates were then autoclaved at 121 °C for 1 h to sterilize.

2.2. Feedstock degradation

Wood blocks were degraded using the ASTM soil-block jar method (ASTM, 2007, D 1413-07) for 1, 2, 4, 8, or 16 weeks using one of two test brown rot fungi, *G. trabeum* (Fr.) Murr. (isolate ATCC 11539) or *P. placenta* (Fr.) M.J. Larsen & Lombard (isolate MAD 698). Five blocks were added per jar for a time series, and single-block harvests were made aseptically. Corn stover was degraded in modified soil-block jars. The 1 g aliquot of stover was added to a 15 ml PTFE centrifuge tube cut in cross section so that the lip of the tube, thrust into the soil between two inoculated birch feeder strips, was flush with the soil surface. This allowed fungal inoculum to grow over the lip of the tubes and down into the stover, with one tube per soil-block jar. Incubation was at room temperature in the dark. All fungus-feedstock treatments were replicated five times ($n = 5$), totaling 50 stover jars and 20 wood cube jars. Included also were 15 non-inoculated jars with fungus-free agar and a replicate set for each feedstock to serve as time zero, non-decayed control material.

Wood blocks were harvested and weighed fresh and a small portion was oven-dried to determine weight loss. The large portion was air-dried at 30 °C for 7 d, Wiley milled to 40 mesh, and stored over desiccant prior to saccharification. In the case of stover powders, characterization of carbohydrate and lignin fractions was used instead of weight loss to infer decay progress, and powders were air-dried and stored over desiccant, similar to wood samples.

2.3. Feedstock and product characterization

For all samples, lignin, cellulose, hemicelluloses, and solubilized glucan and xylan were determined. This was done gravimetrically or via high performance liquid chromatography (HPLC) as oven-dry weight percents, following an earlier protocol (Schilling et al., 2009), with the exception that acid-insoluble lignin was ashed at 575 °C for stover in order to subtract inorganics (specifically silica) from the acid-insoluble lignin fraction. Solublized glucan and xylan fractions were measured in enzyme-free saccharification blanks. This fraction was included in calculations of glucan-to-glucose saccharification yields (useful for determining overall percent-of-original yields) or subtracted during calculations of cellulose-to-glucose yields (useful for determining specifically the effect of pretreatment in enhancing enzyme hydrolysis).

Cellulose crystallinity was measured using X-ray diffraction (XRD) in five replicates from time zero material and for each replicate sample harvested at weeks 8 and 16. Corn stover was heavily degraded by *G. trabeum* and there was not enough material for XRD in weeks 8 and 16. Measurements were made using standard volumes of air-dried 40 mesh powders. Crystallinity was measured using a Bruker-AXS D5005 (Siemens AG, Erlangen, Germany) XRD and at a voltage of 45 kV and current of 40 mV. The samples were scanned over a range from $2\theta = 5°–40°$ at a scan rate of 0.048°/s. Following Segal et al. (1959) setting I_{002} as the intensity of the crystalline peak at about $2\theta = 22.5°$ and I_{am} as the intensity at $2\theta = 18.7°$, crystallinity was calculated as an index (CrI) using peak area comparisons, an approach verified by Howell et al. (2009).

Lignin demethylation and side chain oxidation were measured over the time series using ^{13}C-tetramethylammonium hydroxide (^{13}C-TMAH) thermochemolysis (Filley et al., 2002). Single culture decomposition experiments have demonstrated that during both brown and white rot decay the relative proportion of permethylated vanillic acid (G6) to vanillin (G4) (G6/G4), or also commonly termed acid to aldehyde- Ac/Al, increases progressively (Filley et al., 2002). This increase is thought to be indicative of oxidative alteration of the alpha carbon on the propanoid chain in lignin which decomposes to vanillic acid during TMAH thermochemolysis. ^{13}C-TMAH thermochemolysis and analysis was performed in-line with a Pyr-4a pyrolysis unit (Shimadzu Scientific Instruments, Columbia, MD, USA) interfaced to a GC17A gas chromatograph with peak detection by a QP5050A quadrupole mass spectrometer (Shimadzu). Samples were weighed (≈150 μg) into 3 mm platinum buckets containing 200 ng of an eicosane internal standard. Next, 3 μl of a 50 wt% solution of ^{13}C-TMAH in degassed, dionized water was added. The bucket was placed in the Pyr-4a at room temperature for 5 min under a He gas stream, then heated to 350 °C. The injector base of the Pyr-4a was maintained at 320 °C and with a split flow of 20/1. Chromatographic separation was performed on an RTx-5 (Restek Corporation, Bellefonte, PA, USA) fused silica column (30 m, 0.25 mm i.d., film thickness 0.25 μm). The oven was ramped from 60 °C (1 min stationary) to 300 °C at 7 °C min^{-1} with a hold time of 15 min. Mass spectra were collected over a range from 40 to 550 amu.

Chromatograms were analyzed for methylated syringyl (S), guaiacyl (G), and p-coumaryl (P) phenols in order to determine the extent their relative yields, lignin side chain oxidation, and extent of fungal demethylation (Filley, 2003). The methylated phenols released in the TMAH chemolysis (Fig. S1) were analyzed for their original aromatic methoxyl/hydroxyl content by determining the number of ^{13}C-labeled methyl groups added during the procedure using mass spectrometry. Analysis permits, for example, resolution of whether observed 3,4-dimethoxy structures are of native guaiacyl (as 3-methoxy, 4-hydroxy substitution), or orthodihydroxy (3,4-dihydroxy substitution) that are produced due to progressive brown rot demethylation of lignin.

2.4. Enzymes and saccharification

Celluclast 1.5L (Sigma, St. Louis, MO, USA) and Novozyme 188 (Sigma) were used to saccharify degraded wood or stover. Although no hemicellulases were added, Celluclast 1.5L has been shown to have strong hemicellulase activity (Rodriguez et al., 2005). Combined with the fact that hemicellulase activity remains in fungal crude extracts, hemicellulose saccharification yields are included in the results. Cellulase extracts from the two test fungi were used to saccharify substrates in order to compare gains in saccharification efficiency provided by brown rot pretreatments and to ensure that pretreatment effects were robust and not specific only to brown rot enzymes. Fungal extracts were acquired from solid-state cultures and concentrated/desalted following Tewalt and Schilling (2010), with aspen, spruce, or stover powder substituting for the 212 g of pine used previously. Protein concentrations, measured using Protein Assay Kit I (Bio-Rad, Hercules, CA, USA), were used to determine efficacy of concentrating and desalting steps.

Because these brown rot extracts have little or no activity on crystalline cellulose when using the standard filter paper units (FPU) assay, commercial and crude extracts were loaded at equal endoglucanase activity. Endo-β-glucanase activity was measured on azo-dyed carboxymethylcellulose (CMC) (Megazyme, Bray, Ireland) and quantified as CMC units, or CMCU (Valášková and Baldrian, 2006). β-glucosidase activity was measured as p-nitrophenyl-β-D-glucopyranoside (Sigma) units (pNPGU), as outlined previously (Tewalt and Schilling, 2010). Commercial cellulase activity was evaluated using an additional assay with Whatman #1 filter paper (Whatman, Kent, UK) and expressed as FPU (Adney and Baker, 1996). Standard loading potentials are, in each case, relative to 1 g of cellulose. The endoglucanase-limited loadings for *G. trabeum* and *P. placenta* extracts were 1880 and 720 CMCU, respectively. In order to test synergy specifically between pretreatment and brown rot enzymes, commercial enzymes were added at these low endoglucanase titers, which are respectively 50% and 19% of the CMCU in the recommended full charge of 60 FPU (Sluiter et al. 2006). Novozyme 188, the β-glucosidase, was added at 41.2 and 15.6 pNPGU, respectively, to remove limitation of cellobiose hydrolysis. To assess yields at full strength enzyme loading, the two best-yield samples were selected for each test fungus for saccharification at 60 FPU (in this case 3727 CMCU) and at 64 pNPGU. Saccharification conditions were with rotation (68 rpm) at 50 °C, for 12 days ensuring completion.

2.5. Sugar analysis and yield

Hydrolysates from saccharified feedstocks were analyzed via HPLC on an Aminex HPX-87P column (Bio-Rad) following an earlier protocol (Schilling et al., 2009), and hydration conversion factors were applied to hydrolyzed carbohydrates. Soluble glucan fractions were measured from the enzyme-free blanks, and used toward calculating sugar yields as absolute cellulose-to-glucose conversion after pretreatment as well as the yields of sugars relative to the initial contents in the feedstocks. A detailed description of yield calculations was previously published (Schilling et al., 2009).

2.6. Targeted time series

Glucose yields after saccharification were most promising for *G. trabeum* degraded aspen; therefore, a second decay series was conducted in ASTM microcosms, as before. Harvests were made around the time point of highest yields in the first experimental time series (day 14), with harvests made every 2 d starting on day 8 and ending on day 34. Residues were harvested and handled in the same manner as before, but only characterizing weight-based lignin, cellulose, and xylan fractions and saccharifying using full strength Celluclast 1.5L and Novozyme 188.

3. Results & discussion

In the case of woody feedstocks, aspen and spruce blocks were well-degraded by week 16, and carbohydrates were removed sooner than lignin, typical of brown rot. Average weight loss by week 16 was higher in wood degraded by *G. trabeum* than by *P. placenta* (Table 1). In spruce, there was greater removal of hemicellulose, particularly side chain hemicellulose (arabinan + galactan), than of cellulose, a characteristic of brown rot in conifer wood (eg, Curling et al., 2002). In aspen, however, where xylan is more prevalent than mannan and where side chain content is 60% lower than in spruce, the hemicellulose and cellulose removal rates were similar. This may relate to constitutive production of hemicellulase enzymes adapted for mannan-rich conifer wood and not for aspen, or may reflect the difference in chemical assembly and the sequence of deconstruction by these fungi. It is notable that the rate of decay was generally faster in aspen than in spruce, suggesting the latter. In this case, trials including aspen, such as Yelle et al. (2011), would lend useful comparisons for understanding the decay mechanism.

Cellulose crystallinity, as CrI, decreased by week 8 and again at week 16 in both wood types and for both test fungi (Table 2), with the exception that CrI increased in spruce degraded by *P. placenta* at week 8. This increase occurred at a point when *P. placenta* had removed only 8% of glucan, as opposed to 28% from aspen, and at a time point when *G. trabeum* had removed 40% and 55% of glucan from spruce and aspen, respectively. This increase in CrI during early holocellulose attack, followed by reduction in crystallinity, may relate to the accessibility of amorphous cellulose early in decay and matches the patterns of glucan loss and CrI reported by Howell et al. (2009) for brown rot fungi (including the same *G. trabeum* isolate) degrading pine.

Lignin side chain oxidation and demethylation were common in wood degraded by *P. placenta* or *G. trabeum* (Table 2). Both fungi exhibited progressive side chain oxidation, as identified by an increase in the ratio of permethylated vanillic acid (G6) to vanillin (G4) and permethylated syringic acid (S6) to syringaldehyde (S4), termed Ac/Al ratio or G6/G4 and S6/S4 (Filley, 2003). In aspen, which contains both syringyl (S) and guaiacyl (G) monomer types, G showed more progressive oxidation during brown rot than S and a steeper increase than G in decaying spruce. Demethylation was evident, as well, in both aspen and spruce, and this is typical during brown rot (Filley et al., 2002). Although progressive demethylation was evident in G7, G8, G14, G15, S4, S6, S7, and S15, the steepest and best-fit correlations with sugar hydrolysis yields were G4 and G6, shown in Table 2. Like those changes seen for the G6/G4 ratio in aspen and spruce, G6 in aspen exhibited much greater demethylation values compared to spruce in both the controls

Table 1
Wood percent weight loss (standard error, $n = 5$), and carbon fractions in aspen or spruce blocks decayed as a pretreatment over a 16-week time series by either *Gloeophyllum trabeum* or *Postia placenta*, two brown rot fungi.

Fungus/wood	Week	% Weight loss	Adjusted[a] (wt%)						
			Lignin	Glucan	Xylan	Galactan	Arabinan	Mannan	Sum hemicellulose
G. trabeum/aspen	0	–	27.1	42.1	8.4	1.4	2.8	3.7	16.2
	1	1.0 (2.7)	27.6	43.2	10.6	0.7	0.8	1.8	14.0
	2	18.8 (2.1)	22.3	29.5	7.3	1.0	1.2	0.9	10.5
	4	28.6 (2.7)	24.7	24.6	5.1	0.5	0.4	0.5	6.5
	8	38.1 (2.1)	24.9	18.8	3.4	0.5	1.0	0.6	5.4
	16	57.9 (3.4)	20.9	9.1	1.9	0.6	0.2	0.2	2.9
P. placenta/aspen	0	–	27.1	42.1	8.4	1.4	2.8	3.7	16.2
	1	0.2 (0.6)	28.6	38.4	8.6	0.7	1.9	1.8	13.0
	2	1.7 (0.9)	21.8	40.6	10.2	0.7	2.1	2.1	15.1
	4	0.8 (1.5)	19.4	41.0	10.4	0.7	2.4	2.6	16.0
	8	17.9 (3.3)	18.5	30.3	8.3	0.7	2.0	2.0	13.0
	16	34.2 (4.5)	15.0	22.2	5.4	0.5	0.9	1.0	7.7
G. trabeum/spruce	0	–	31.7	36.0	5.8	5.7	5.0	9.4	25.8
	1	3.8 (4.1)	30.7	39.2	3.2	0.8	1.4	9.0	14.5
	2	10.7 (5.6)	33.5	32.7	3.5	1.6	1.1	6.6	12.8
	4	19.5 (4.2)	30.8	29.2	2.6	0.5	0.4	5.2	8.7
	8	35.9 (2.9)	26.3	21.7	1.7	0.4	0.9	3.9	6.9
	16	53.6 (2.2)	21.4	12.2	1.1	0.7	0.6	2.6	5.0
P. placenta/spruce	0	–	31.7	36.0	5.8	5.7	5.0	9.4	25.8
	1	0.0 (2.2)	27.5	43.4	5.7	1.4	1.4	12.0	20.6
	2	0.2 (1.4)	30.5	42.6	4.3	1.1	1.3	10.7	17.3
	4	7.0 (2.4)	30.6	36.8	3.8	1.3	1.1	8.8	15.0
	8	15.7 (2.7)	29.9	33.0	3.2	0.8	0.8	6.7	11.6
	16	36.5 (4.8)	29.8	18.2	1.9	0.7	0.42	3.6	6.6

[a] Duplicate characterizations from homogenized powder - equal parts of five separately-decayed replicates. Data adjusted to compensate mass loss by multiplying by the average fraction of weight remaining in blocks (oven-dry).

Table 2
Cellulose crystallinity index (CrI), lignin side chain oxidation (G6/G4; S6/S4), and selected lignin demethylation (standard deviations) in wood decayed by *G. trabeum* or *P. placenta*, measured by XRD or ^{13}C-TMAH thermochemolysis. CrI replication was $n = 5$, while TMAH data is from duplicate analyses of homogenized wood powder.

Fungus/wood	Week	CrI (%)	S/G[a]	G6/G4[b]	S6/S4[c]	%dm G14 + 15[d]	%dm S15[e]	%dm G4[f]	%dm G6[g]
G. trabeum/aspen	0	56.4 (3.9)	1.6 (0.1)	0.3 (0.0)	0.3 (0.0)	1.1 (0.2)	2.7 (0.5)	5.5 (0.6)	18.7 (1.5)
	2	–	1.8 (0.6)	0.4 (0.2)	0.7 (0.4)	4.1 (0.8)	10.0 (1.7)	14.7 (1.5)	46.1 (1.7)
	4	–	1.6 (0.4)	0.6 (0.3)	0.6 (0.2)	4.6 (0.8)	10.2 (1.4)	14.0 (1.6)	37.7 (0.8)
	8	48.0 (3.2)	2.2 (0.6)	1.1 (0.2)	1.1 (0.4)	4.7 (0.8)	8.9 (1.7)	14.4 (1.5)	47.5 (1.7)
	16	40.5 (4.4)	1.9 (0.4)	1.5 (0.3)	1.1 (0.2)	5.7(0.8)	14.4 (4.6)	15.6 (1.6)	52.1 (0.8)
P. placenta/aspen	0	56.4 (3.9)	1.6 (0.1)	0.3 (0.0)	0.3 (0.0)	1.1 (0.2)	2.7 (0.5)	5.5 (0.6)	18.7 (1.5)
	2	–	1.6 (0.2)	0.3 (0.1)	0.4 (0.1)	1.5 (1.1)	0.9 (1.7)	7.2 (1.5)	20.9 (0.4)
	4	–	1.4 (0.4)	0.6 (0.4)	0.5 (0.2)	3.8 (0.8)	3.9 (2.0)	9.8 (1.5)	23.8 (0.8)
	8	52.3 (1.9)	1.8 (0.2)	1.4 (0.2)	0.8 (0.1)	2.9 (1.0)	6.7 (1.2)	12.0 (1.6)	41.4 (0.5)
	16	50.4 (3.4)	1.9 (0.3)	1.2 (0.3)	0.9 (0.1)	2.5 (1.6)	6.7 (0.8)	12.6 (0.6)	42.9 (1.3)
G. trabeum/spruce	0	48.5 (0.7)	–	0.3 (0.0)	–	1.1 (0.3)	–	4.5 (0.5)	11.4 (3.6)
	1	–	–	0.5 (0.2)	–	1.3 (0.2)	–	6.3 (1.6)	10.9 (3.5)
	2	–	–	0.7 (0.1)	–	2.1 (0.5)	–	8.4 (1.6)	14.9 (1.3)
	4	–	–	0.7 (0.3)	–	4.1 (0.6)	–	10.7 (1.8)	18.2 (1.8)
	8	45.4 (3.9)	–	0.8 (0.1)	–	5.7 (0.9)	–	11.5 (1.3)	17.2 (0.6)
	16	38.2 (4.4)	–	0.8 (0.1)	–	8.0 (3.7)	–	15.4 (3.7)	21.4 (1.0)
P. placenta/spruce	0	48.5 (0.7)	–	0.3 (0.0)	–	1.1 (0.3)	–	4.5 (0.5)	11.4 (3.6)
	1	–	–	0.5 (0.2)	–	1.2 (0.2)	–	4.3 (1.6)	10.9 (3.5)
	2	–	–	0.7 (0.1)	–	2.1 (0.5)	–	8.2 (1.6)	14.8 (1.3)
	4	–	–	0.6 (0.2)	–	3.0 (1.2)	–	6.8 (1.9)	14.1 (2.8)
	8	51.1 (3.2)	–	0.7 (0.3)	–	5.3 (1.0)	–	11.8 (1.5)	17.7 (1.1)
	16	40.8 (4.2)	–	0.7 (0.1)	–	5.7 (1.6)	–	13.4 (0.9)	19.0 (1.0)

[a] Syringyl/Guaicyl (S/G) ratio.
[b] Ac/Al in G and S (spruce has no syringyl units).
[c] Ac/Al in G and S (spruce has no syringyl units).
[d] %demethylation of averaged G14 and G15.
[e] %demethylation of S15 in the angiosperm, aspen.
[f] %demethylation of G4 and G6. Note: week 1 aspen samples were not available in enough volume for analyses.
[g] %demethylation of G4 and G6. Note: week 1 aspen samples were not available in enough volume for analyses.

and during the course of decay. This indicates that the G6 released by TMAH in non-degraded aspen is more proportionately derived from a catecholic (dihydroxyl structure) that may serve as a linkage to carbohydrates in aspen. Additionally, as the final increase in demethylation of G6 is higher for aspen, there is either progressive release of the dihydroxyl structure or greater demethylation. Full characterization of wood lignin modifications is provided as a supplemental table (Table S1).

Table 3
Cellulose-to-glucose[a] (wt%) (standard deviation) from duplicate saccharification (at equal CMCU[b]) of homogenized decayed feedstocks after decay up to 16 weeks by either *Gloeophyllum trabeum* or *Postia placenta*.

Enzyme source	Week	Fungus/feedstock pretreatment			
		Gt[c] aspen	*Pp* aspen	*Gt* spruce	*Pp* spruce
Native[d]	0	4.3 ± 0.5	2.9 ± 0.6	3.3 ± 0.3	0.7 ± 0.0
	1	3.3 ± 0.3	1.6 ± 0.2	3.9 ± 1.1	1.3 ± 0.0
	2	8.1 ± 0.9	1.6 ± 0.3	7.1 ± 0.3	1.2 ± 0.0
	4	7.9 ± 1.1	1.5 ± 0.3	4.0 ± 1.5	0.9 ± 0.2
	8	8.0 ± 1.5	3.2 ± 0.9	4.1 ± 0.7	2.2 ± 0.3
	16	7.8 ± 1.6	1.6 ± 0.2	3.8 ± 0.9	2.8 ± 0.4
Celluclast + novozyme	0	24.7 ± 0.9	8.6 ± 0.2	13.3 ± 0.4	6.3 ± 0.2
	1	26.8 ± 0.3	10.3 ± 0.6	12.7 ± 0.6	9.6 ± 0.3
	2	**47.8 ± 1.3**[e]	10.7 ± 0.1	11.6 ± 0.1	8.9 ± 0.2
	4	42.9 ± 0.3	14.1 ± 0.1	15.1 ± 0.3	11.7 ± 0.7
	8	41.8 ± 1.3	**29.3 ± 0.5**[e]	19.2 ± 0.8	19.3 ± 1.3
	16	36.2 ± 1.5	18.1 ± 0.2	12.1 ± 0.5	14.3 ± 0.8

[a] Calculated by subtracting soluble glucan measured in enzyme-free blanks.
[b] Loaded at equal CMCU, within a fungal species, forcing the Celluclast FPU to 65% and 24% of a full strength (Sluiter et al., 2006) in *G. trabeum* and *P. placenta*, respectively.
[c] *Gt* = *G. trabeum*, *Pp* = *P. placenta*.
[d] 'Native' is crude extract from solid-state cultures of the same fungus used to pretreat material.
[e] Means in bold selected to re-saccharify at full FPU strength (Section 3).

Table 4
Hemicellulose sugar yield (wt%) (standard deviation) from duplicate saccharification (at equal CMCU) of homogenized decayed feedstocks after decay up to16 weeks by either *Gloeophyllum trabeum* or *Postia placenta*.

Enzyme source	Week	Fungus/feedstock pretreatment			
		Gt[a] aspen	*Pp* aspen	*Gt* spruce	*Pp* spruce
Native[b]	0	15.2 ± 0.4	6.5 ± 1.6	5.2 ± 0.2	1.6 ± 0.3
	1	11.2 ± 0.4	3.3 ± 0.4	13.4 ± 0.6	9.5 ± 0.3
	2	20.9 ± 0.3	8.2 ± 0.6	17.1 ± 1.0	10.3 ± 0.2
	4	21.7 ± 2.0	6.7 ± 0.5	32.6 ± 2.2	11.2 ± 0.8
	8	25.1 ± 1.2	10.0 ± 1.1	18.7 ± 1.0	18.3 ± 0.4
	16	15.7 ± 2.5	14.8 ± 0.4	19.7 ± 0.1	9.8 ± 0.3
Celluclast + novozyme	0	17.3 ± 0.6	12.0 ± 0.1	5.0 ± 0.6	4.6 ± 0.1
	1	18.4 ± 0.8	11.6 ± 0.6	12.6 ± 0.5	8.2 ± 0.0
	2	**39.1 ± 1.4**[c]	11.6 ± 0.5	13.6 ± 0.3	7.6 ± 0.2
	4	31.5 ± 1.1	11.0 ± 0.2	20.4 ± 0.9	6.3 ± 0.3
	8	39.0 ± 1.5	**16.4 ± 0.4**[c]	14.9 ± 0.1	14.7 ± 0.7
	16	26.7 ± 0.2	20.8 ± 0.0	6.0 ± 0.4	13.5 ± 0.7

[a] *Gt* = *G. trabeum*, *Pp* = *P. placenta*.
[b] 'Native' is crude cellulase extracted fromsolid-state cultures of the same fungus used to pretreat the material.
[c] Means in bold selected to re-saccharify at full FPU Celluclast + novozyme.

In material pretreated by brown rot fungi, saccharification was enhanced up to threefold, irrespective of enzyme source (Tables 3 and 4). In fresh never-dried wood, yields could perhaps be improved even further. This suggests real potential as a pretreatment and demonstrates a robust interaction, regardless of enzyme source, as shown previously (Tewalt and Schilling, 2010). In most cases, highest glucose yields relative to initial glucan content were achieved prior to week 16. The optimal harvest point would be when the fungus has 'loosened' the cell wall sufficiently without metabolizing the more accessible carbohydrates, leaving recalcitrant polymers behind. When we repeated the saccharification trial at full strength FPU for our 'best yield' week 2 and 8 aspen samples degraded by *G. trabeum* and *P. placenta*, respectively (Table 3, bold), the glucan-to-glucose yields were 72.2% for *G. trabeum* and 50.4% for *P. placenta*. This approaches 85% glucose yield from glucan, an accepted industrial bioconversion target (Saha, 2004). When expressed per original glucan content, the yields are lower, however, at 50.5% and 36.2%, respectively. While a cost analysis would help determine if lower yields were potentially acceptable due to process cost savings, we feel this would be premature without first screening a larger pool of candidate fungi, beyond the familiar group of pest brown rot species, for yield potential.

Relationships between tissue modifications and saccharification lend insight into basic mechanisms of decay, and they can help target certain characteristics to be used in predicting pretreatment severity or perhaps in calculating a severity factor, as done for chemical pretreatments (Abatzoglou et al., 1992). Characters having a steep slope and strong correlation with saccharification yields in early decay would seem best for predictive capacity in the case of brown rot. From our data, mass loss would be a robust measure of progress along a decay continuum, but hemicellulose components show a steeper decay slope in early stages, particularly in spruce, and might be a more practical measurement in milled or otherwise fractionated biomass. In aspen, however, side chain content is lower and glucan and hemicellulose losses track one another over a more gradual drop. CrI was also a poor predictor of saccharification efficiency, similar to other studies using plant biomass instead of pure cellulose substrates (eg, Puri, 1984; Kim and Holtzapple, 2006). Conversely, lignin removal and demethylation were good predictors of saccharification potential, particularly G4 in spruce and G6 in aspen. In spruce, correlations with the increase to maximum of saccharification yields for G4 gave R^2 of 0.88 and 0.77 for *G. trabeum* and *P. placenta*, respectively, which were higher than R^2 values from hemicellulose. In aspen, R^2 for *P. placenta* for G6 was 1.00. Although saccharification yields peaked at the first

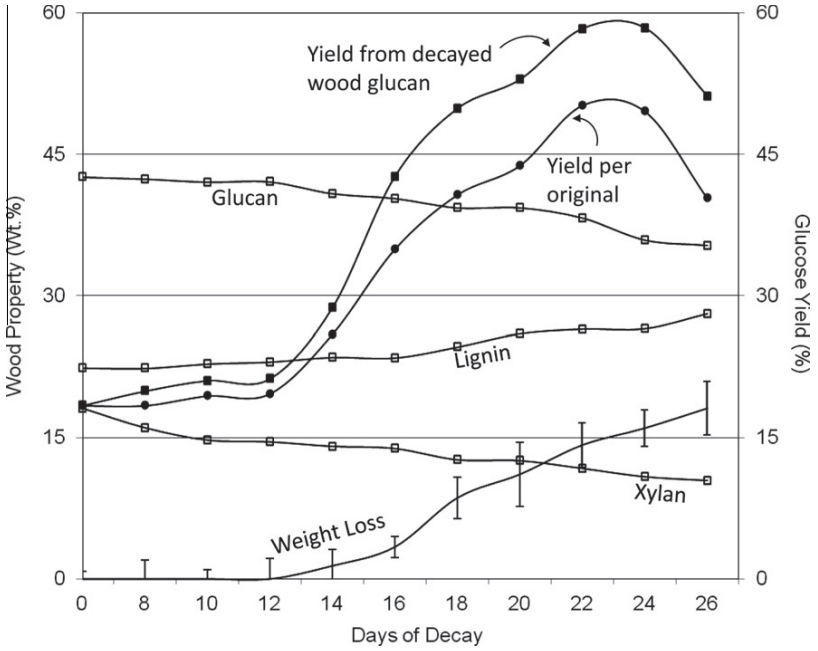

Fig. 1. Yields of glucose (per glucan) after saccharification of aspen pre-degraded by *G. trabeum* over a time series. Yields relative to initial diverge due to progressive metabolism of glucan by the fungus during the course of decay. Weight percent carbon fractions are plotted along the same time series. These wt% were not adjusted for mass loss in order to show characters as they would be measured in a batch process using fractionated biomass.

Table 5
Non-adjusted[a] lignin, glucan and hemicellulose fractions, plus saccharification yields (standard deviation) in corn stover decayed over a 16-week time series by a brown rot fungus, either *Gloeophyllum trabeum* or *Postia placenta*.

Fungus	Week	Constituent (Non-adjusted[a] Wt%)								
		Lignin	Glucan	Xylan	Galactan	Arabinan	Mannan	Sum Hemicellulose	Native[a,b] Cellulose-to-glucose	*Cell/Novo* Cellulose-to-glucose
G. trabeum	0	25.9	32.6	8.3	1.0	4.0	0.4	13.7	7.1 (0.2)	29.2 (1.2)
	1	24.5	30.8	7.2	0.8	3.3	0.4	11.7	3.9 (0.1)	30.8 (0.2)
	2	24.4	30.5	7.5	0.8	3.7	0.4	12.4	12.7 (2.4)	31.4 (0.4)
	4	26.8	28.6	6.9	0.5	3.2	0.4	11.0	16.5 (0.1)	38.7 (0.8)
	8	33.2	22.3	5.1	0.7	1.7	0.4	7.9	15.4 (0.2)	44.0 (0.0)
	16	39.6	14.0	3.0	0.3	2.1	0.7	6.1	11.5 (0.1)	21.9 (0.0)
P. placenta	0	25.9	32.6	8.3	1.0	4.0	0.4	13.7	6.1 (0.6)	12.8 (0.6)
	1	26.5	32.7	8.5	0.9	3.3	0.7	13.4	3.2 (0.4)	27.1 (1.6)
	2	25.5	33.3	7.9	0.7	3.2	0.4	12.2	4.1 (0.1)	25.7 (0.4)
	4	24.3	33.0	8.5	0.8	3.2	0.4	12.9	3.2 (0.6)	24.0 (0.6)
	8	26.4	33.6	7.9	0.5	3.5	0.5	12.4	1.7 (0.3)	23.8 (0.8)
	16	24.7	32.6	8.3	0.7	3.1	0.4	12.5	1.8 (0.3)	25.0 (0.1)

[a] Duplicates run on homogenized milled powder from five separately-degraded replicates. Weight% data could not be adjusted to reflect density loss because it was decayed as a loose powder. The trend of each constituent is therefore relative to other constituents.
[b] "Native" is brown rot extracts, and "Cell/Novo" is Celluclast and Novozyme, not at full FPU.

G6 data point for *G. trabeum*, this was the steepest change of any variable (18.7 to 46.1%). In addition to the potential for using targeted demethylation analyses as a pretreatment severity indicator, this relationship between certain TMAH lignin products and saccharification potential may lend insight into the specific lignin modifications that enable brown rot. Lignin removal exceeded 30% in some cases and, coupled with the supposed requirement of lignin for most brown rot fungi to efficiently degrade crystalline cellulose, suggests lignin fragments may actively contribute or participate mechanistically.

Saccharification yields were tracked with more resolution in our follow-up trial with *G. trabeum* on aspen. Yields are expressed both relative to the original glucan content and relative to glucan remaining after fungal pretreatment and metabolism (Fig. 1). Yields peaked at day 24 relative to post-decay glucan (58.4%) and at day 22 relative to original glucan (50.2%). By targeting this more thorough time series, we increased resolution to better predict when you would achieve the highest yield, showing divergence relative to initial and post-decay glucan after a short incubation period. The later timing of the optimum harvest point in the follow-up trial also highlights run-to-run variability that can occur when using a fungus. This variability supports the idea that finding reliable, predictive tissue characters is useful for inferring pretreatment severity in future batch processing of material.

Finally, corn stover weight loss could not be quantified, and analyses were hindered in cases of extensive degradation. Characterization indicated significant decay by *G. trabeum* but not by *P. placenta* (Table 5), an observation supported both by CrI

which remained unchanged at 48.5 and by subsequent trials showing no weight loss in decay studies using whole corn stalks (unpublished data). For stover lignin, which notably contains significant p-hydroxyphenyl (P) units, the control started off with higher G6/G4 than S6/S4, but the G monomers exhibited a rapid drop in value at week 1 and both S and G monomers then exhibited progressive increases. This potentially indicates an initial consumption or polymerization of acidic compounds present in the stover. During wood decay, demethylated G6 often decreases initially and then accumulates with a methoxy at position 3 as a result of microbial oxidation (Filley, 2003; Filley et al., 2002). Interestingly, saccharification of stover after 1 week of colonization with minor degradation by *P. placenta* resulted in more than a twofold increase in yield relative to time zero, but only for commercial enzymes and not for crude extracts. It is unclear, in general, how these variables relate to decay capacity or saccharification potential, but it is clear that these natural decay pathways vary significantly among different plant tissue types.

4. Conclusion

A threefold increase in saccharification yields in this study encourages research into brown rot pretreatments of lignocellulosic tissues. Tissue modifications, including those to lignin, also show promise for predicting pretreatment severity and timing harvest, and lend insight into basic mechanisms of brown rot. It is notable that aspen gave best yields. Brown rot fungi are often associated with conifers rather than with angiosperms like aspen and studies typically focus on conifer substrates. This makes sense for extrapolating natural phenomena, but our results encourage testing non-conifer substrates to draw mechanistic comparisons and to extend applicability to a broader range of feedstocks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2012.04.018.

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