Natural acetylation impacts carbohydrate recovery during deconstruction of *Populus trichocarpa* wood

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**Abstract**

**Background:** Significant variation in the inherent degree of acetylation naturally exists in the xylem cell walls of *Populus trichocarpa*. During pretreatment, endogenous acetate hydrolyzes to acetic acid that can subsequently catalyze the breakdown of poplar wood, increasing the efficiency of biomass pretreatment.

**Results:** Poplar genotypes varying in cell wall composition were pretreated in 0.3% H2SO4 in non-isothermal batch reactors. Acetic acid released from the wood was positively related to sugar release during pretreatment ($R \geq 0.9$), and inversely proportional to the lignin content of the poplar wood ($R = 0.6$).

**Conclusion:** There is significant variation in wood chemistry among *P. trichocarpa* genotypes. This study elucidated patterns of cell wall deconstruction and clearly links carbohydrate solubilization to acetate release. Tailoring biomass feedstocks for acetate release could enhance pretreatment efficiencies.

**Keywords:** Acetate, Acetyl group, Xylan, Pretreatment, Biorefinery, Advanced biofuels

**Background**

In recent decades, there has been widespread global interest in developing dedicated bioenergy feedstocks [1]. Sustainable and economically viable production of purpose-grown lignocellulosic feedstocks is requisite on the ability to generate crops with suitable composition and significant biomass yield to maximize land-use efficiency. Biochemical conversion of lignocellulosic biomass can yield valuable bioproducts, including ethanol, organic acids, lignin-derived coproducts, butanol, and hydrogen gas [2]. Tailoring lignocellulosic feedstocks for emerging biorefineries, through classical breeding or biotechnological application, could offer a means to improve the economy and feasibility of commoditizing these bioproducts [3]. Poplar wood has emerged as a promising biofuel feedstock, as these trees have large native ranges, inherently possess the ability to grow on marginal sites, and are highly productive.

Cell wall ultrastructure and composition dictate the utility of poplar and other lignocellulosic feedstocks for bioconversion applications. Lignocellulosic feedstocks are largely recalcitrant to breakdown into utilisable sugars because of the compact structure of crystalline cellulose microfibrils, lack of substrate porosity, and high lignin concentrations [4]. Consequently, harsh pretreatment regimes are often required to separate the carbohydrates from lignin in the cell wall complex and provide sufficient accessibility for biochemical conversion. Some pretreatments, notably those using dilute acid processes, solubilize hemicelluloses into the reaction liquor, where they degrade or are discarded as a waste stream [5, 6]. As such, the hemicelluloses, comprising up to 30% of the secondary cell wall of poplar [7], are an underutilized fraction of biomass. Studies of their dissolution and degradation are therefore required in order to improve estimates of product yield in the biorefinery process.

Hemicelluloses are highly branched mixed-carbohydrate polysaccharides with a degree of polymerization of approximately 200 [8]. Xylan, which has a backbone of β-(1→4)-linked xylosyl residues, is the
main hemicellulose in poplar. Acetate groups decorate xylopyranosyl residues, such that acetate makes up approximately 5% (w/w) of poplar wood [9].

To date, the biological and structural roles of acetate substitution are uncertain. Acetylation may prevent aggregation of xylan precursors during their biosynthesis and transport to the cell wall because of its associated steric forces [10]. In the assembled secondary cell wall, xylan acetylation has been shown to increase chain stiffness and impact the flexural properties of wood, including modulus of rupture and modulus of elasticity [11, 12]. Recent research is uncovering the regularity of acetate substitution, and suggests that acetate groups face towards the lignified region of the cell wall to improve cohesion between lamellae [13, 14].

Acetate has been the subject of research because of its effect on wood processing, pulping, and bioconversion. Acetate corrodes metal, decreases fiber swelling, and inhibits growth of fermentative microorganisms [15–17]. Studies also suggest that acetate from lignocellulose inhibits growth of fermentative microorganisms [15–17]. In most lignocellulosic substrate pretreatment regimes, a key goal is to remove hemicelluloses from the cell wall matrix and offer a means to liberate and extract the recalcitrant lignin polymer [19, 20]. Although acetate is removed during alkaline pretreatment, it remains part of biomass during most other types of pretreatment [21]. Currently, dilute acid pretreatment, which hydrolyzes glycans and disrupts hydrogen bonding between cell wall polymers, is the most commonly considered pretreatment. Xylan deconstruction usually occurs in three phases during dilute acid pretreatment. In the first phase, fast- and slow-reacting xylan are dissolved and hydrolyzed to oligomers [22]. Next, oligomers are further hydrolyzed to individual xylose monomers, and acetate hydrolyzes to acetic acid [23, 24]. During the third phase, xylose monomers dehydrate to furfural, while hexitose monomers degrade to 5-hydroxymethyl-2-furaldehyde (HMF) [25].

Acetic acid derived from acetate in wood has both positive and negative effects on biomass conversion. Acetic acid alone can be an effective agent for selective delignification [26]. The powerful dissolving effect of acetic acid led to the establishment of acetosolv pretreatment for hardwood, softwood, and agricultural residues [27–31]. For example, pretreatment of beechwood with 1% acetic acid was shown to be as effective as raising the reaction temperature by 20 °C [32].

In contrast, during biochemical pretreatment regimes that employ enzymatic digestion, acetate increases the overall enzyme load required to effectively convert woody feedstocks [18]. Acetate accumulating above 100 mM in pretreatment slurries was shown to be inhibitory to downstream fermentative microorganisms [33]. These negative effects have prompted several studies focused on acetate removal from pretreated biomass prior to enzymatic conversion [34–36]. In one study, transgenic Arabidopsis stems with 32% less total cell wall acetate content yielded 70% higher ethanol production by fermentation compared to wild-type stems [37]. This study highlights the importance of acetate content in lignocellulosic biorefinery processes, as acetate has been shown to be both positively or negatively correlated with sugar release in previous studies depending on the pretreatment and hydrolytic method employed. Herein, the dissolution of xylan, glucan, and acetate groups during pretreatment of poplar wood are explored.

Results
Wood sampling and degree of acetylation
Wood sampled from 200 unrelated 5-year-old Populus trichocarpa individuals grown in a common garden had an average acetate content of 5.2 ± 0.3% (w/w ± SD, extractives-free dry weight), with a high of 6.7% and low of 3.5% w/w. Regression analysis of several wood chemistry traits of the trees determined whether acetate content correlates with any of the primary chemical features of the wood (Table 1; Additional file 1: Table S1). There were positive correlations between xylose, mannos, and rhamnose and acetate content (R = 0.40, 0.28, and 0.25, respectively), whereas glucose and galactose contents were inversely correlated with acetate content (R = −0.40, −0.16). Acid-soluble lignin and acetate were positively correlated (R = 0.35; Table 1). The Klass lignin (acid-insoluble lignin) or arabinose content of the wood samples were not significantly associated with acetate (R = 0.07, 0.02), nor were the 5-year growth traits, including total tree biomass (R = 0.03).

Table 1 Associations between wood acetate content and other cell wall components or traits among P. trichocarpa genotypes

| Trait                  | R   | p value | n  |
|------------------------|-----|---------|----|
| Glucose                | −0.40 | <0.001 | 208 |
| Xylose                 | 0.40 | <0.001 | 203 |
| Lignin (acid-soluble)  | 0.35 | <0.001 | 207 |
| Mannose                | 0.28 | <0.001 | 202 |
| Rhamnose               | 0.25 | <0.001 | 205 |
| Galactose              | −0.16 | 0.004 | 204 |
| Lignin (Klass)         | 0.07 | 0.20   | 208 |
| Total tree biomass     | 0.03 | 0.67   | 232 |
| Arabinose              | 0.02 | 0.78   | 208 |

* R Pearson correlation coefficient; p value, test statistic; n number of observations. Values are the average of three technical replicates. Raw data are presented in Additional file 1: Table S1.
NMR

Figure 1 is a 2D $^1$H–$^{13}$C-correlated (HSQC) NMR spectrum of poplar xylem. Acetate groups are positioned on xylopyranosyl and mannoxyranosyl residues. Considerable amounts of xylopyranosyl residues are O-acetylated, whereas the majority of xylan structures are non-acetylated. The peaks of 2-O-Ac-β-d-Xylp C2/H2 at 73.5/4.64 ppm and a 3-O-Ac-β-d-Xylp C3/H3 at 75.0/4.94 ppm can be easily recognized. Poplar inherently displays moderate levels of 2,3-di-$^3$H at 75.0/4.94 ppm can be easily recognized. Poplar wood was then pretreated at 180 °C in acetic acid (0–9% v/v; Table 2). Total xylose and glucose release increased incrementally with higher concentrations of acetic acid. Values for total effective xylose and glucose release are the sum of monomeric, oligomeric, and dehydrated forms (furfural or HMF). Following 70-min pretreatment at 180 °C in water with 0% acetic acid, 84 mg/g xylose and 13 mg/g glucose formed. This result is comparable to that from the temperature optimization experiment described above. Sugar release was only significantly different between hot pressurized liquid water and 3% acetic acid for monomeric xylose. Following the addition of 6% acetic acid, total effective xylose release increased to 148 mg/g and glucose to 28 mg/g. At 9% acetic acid concentration, xylose and glucose release were 153 and 29 mg/g, respectively. Each 3% increase of acetic acid resulted in an increase in furfural and HMF product.

Next, we pretreated poplar wood for varying time periods and sulphuric acid concentrations (without exogenous acetic acid). Twelve “regimes” consisting of 10, 30, or 60 min pretreatment supplemented with 0.0, 0.1, 0.3, or 0.6% (w/w) sulphuric acid were examined. Table 3 shows the percent wood dissolved or degraded versus the percent solid wood residue. Wood dissolution was less than 40 mg/g for regimes 1–5. Thirty-minutes pretreatment using 0.3% catalyst dissolved the same mass of wood as 60 min of uncatalyzed pretreatment (regimes 7 and 9). Thirty-minutes pretreatment using 0.6% catalyst...
Fig. 1  HSQC 2D-NMR spectrum obtained on total cell wall material from poplar wood
yielded as much sugar as 60-min pretreatment using 0.1% sulphuric acid (regimes 8 and 10). The increase in degradation products at regimes 11 and 12 was accompanied by a decrease in dissolved sugars. Increasing severity further decreased the proportion of oligomers to monomers.

A key consideration in the selection of pretreatment time and the concentration of sulphuric acid was the partitioning of acetate into its three possible forms: as acetate attached to wood (WR), dissolved and attached to short xylooligosaccharides (XOS), and as acetic acid (AA) (Table 3). As pretreatment severity increased, acetylated xylan hydrolyzed to produce acetylated XOS. Thereafter, these acetylated XOS hydrolyzed to acetic acid and xylose (or low DP XOS). Mild pretreatments resulted in very little acetic acid liberation; harsher pretreatments resulted in high acetic acid concentrations, with very little acetate remaining on XOS or wood. Based on our original mass

Table 2 Acetic acid catalyzes the hydrolysis of wood polysaccharides during pretreatment

| Added acetic acid | Xylose release (mg/g) | Total effective xylose release (mg/g) | Glucose release (mg/g) | Total effective glucose release (mg/g) |
|-------------------|-----------------------|--------------------------------------|------------------------|---------------------------------------|
|                   | Monomeric | Oligomeric | Furfural | Monomeric | Oligomeric | HMF | Monomeric | Oligomeric | HMF |
| 0.0               | 4.01 ± 1.42a | 59.1 ± 7.62a | 3.61 ± 1.60a | 84.4 ± 19.1a | 1.40 ± 0.16a | 10.2 ± 1.67a | 0.41 ± 0.08a | 13.0 ± 0.97a |
| 3.2               | 7.93 ± 1.05b | 56.3 ± 8.60a | 10.8 ± 4.84b | 97.4 ± 18.9a | 2.52 ± 0.77ab | 14.9 ± 2.81a | 1.16 ± 0.53a | 19.1 ± 3.95ab |
| 6.3               | 11.2 ± 2.28c | 72.1 ± 19.4a | 21.4 ± 9.12a | 148 ± 25.9a | 3.36 ± 0.76bc | 19.1 ± 3.85b | 1.61 ± 0.91a | 27.7 ± 3.90bc |
| 9.4               | 9.90 ± 3.60a | 74.0 ± 22.9a | 31.0 ± 16.3a | 153 ± 44.2b | 2.24 ± 0.87c | 22.7 ± 27.2b | 3.14 ± 1.74a | 29.4 ± 4.23c |

All values are in milligrams per gram ± standard error of the mean. Total effective sugar release is the sum of all three fractions, with degradation products converted on a molar ratio to xylose. Pretreatments were at 180 °C for 70 min. Superscripts indicate statistical significance at p value < 0.05.
balance, all acetate in wood hydrolyzed to acetic acid at the highest pretreatment severity. Under these conditions, 60 mg/g acetate was released from wood. We therefore chose suitable pretreatment conditions based on acetate release, as well as carbohydrate solubilization and degradation. Regime 7—pretreatment in 0.3% sulphuric acid catalyst for 30 min—provided the “middle ground” for acetate partitioning whereby acetic acid, acetylated wood, and acetylated XOS were present in approximately equal fractions. Regime 7 dissolved on average 28% (w/w) of wood, including two-thirds of the available xylan and one-twentieth of the available glucan (Table 3).

Comparing acetate and sugar release in different wood samples
Having established the impact of acetic acid on poplar wood solubilization, we evaluated the impact of native acetate in 19 different poplar wood samples using the sulphuric acid-catalyzed pretreatment regime 7. Samples came from the natural population and had known cell wall chemistries and similar ultrastructural properties (density, fiber dimensions, and crystallinity; data not shown).

Pretreatment sugar release is shown in Table 4. Overall sugar yield and the oligomer-to-monomer rati0 (O:M) of xylose and glucose varied twofold. Poplar wood samples released 63–184 mg xylose, and 6–22 mg glucose per gram of extractives-free, oven-dried wood. Monomeric xylose release ranged from 3–11 mg/g, whereas oligomeric xylose amounted to 60–140 mg/g. Monomeric glucose release ranged between 0.2 and 1.6 mg/g, whereas oligomeric glucose ranged from 6 to 20 mg/g. Individuals with high xylose release also released high quantities of glucose.

Figure 3a shows the relationship between xylose and acetic acid during pretreatment. There is a strong linear correlation between acetic acid and monomeric xylose (R = 0.95). The oligomeric xylose versus acetic acid curve followed a hyperbolic shape that plateaued at 140 mg/g xylose oligomers (Fig. 3a). Oligomeric and monomeric glucose also correlated linearly with acetic acid (R = 0.89 and 0.91; Fig. 3b). Figure 3c plots sugar degradation products, HMF and furfural, against acetic acid in pretreatment liquor. Higher acetic acid was not associated with higher furfural or HMF formation.

Table 4 demonstrates how acetate in wood partitioned into three phases. Following pretreatment, it may exist as free acetic acid, or remain linked to dissolved XOS or on wood residues. This acetate partitioning, unique to each sample, suggests a wood chemistry basis for autohydrolysis. If all acetate groups were released in a sample, the resulting solution would be less than 1 mg/g in acetic acid. Sample number 19, for example, had the lowest overall acetate, which partitioned between free acetic acid and wood residue in a 1:6 ratio. In contrast, sample 4 had the highest overall acetate in a 1:2:2 ratio. Acetate levels in samples 16 and 11 were in the middle range, with 1:1:3 and 1:1:2 acetate partitioning, respectively (acetic acid:XOS:solid wood residue). Acetate partitioning clearly reflected different wood chemistries.

Evaluation of factors affecting acetyl-to-acetic acid hydrolysis
Simple linear regressions can describe acetate partitioning based on other wood chemistry traits (Table 5). Lignin content was inversely correlated with acetate-to-acetic

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**Table 3** Dissolution and degradation of *P. trichocarpa* wood at various dilute acid pretreatment regimes

| Regime | Time (min) | H₂SO₄ | Dissolved sugars | Wood residue | Degraded | Xyl O:M | Glc O:M | Acetate |
|--------|------------|-------|------------------|--------------|----------|--------|---------|---------|
|        |            |       |                  |              |          |        |         | AA      | XOS    | WR     | AA:XOS:WR |
| 1      | 10         | 0.0   | 33               | 930          | ND       | 0.6    | 1.1     | ND      | ND     | 50     | 1.0:ND:83 |
| 2      | 10         | 1.0   | 34               | 910          | ND       | 1.8    | 1.1     | ND      | ND     | 48     | 1.0:ND:34 |
| 3      | 10         | 3.0   | 33               | 880          | ND       | 7.1    | 1.0     | ND      | ND     | 50     | 1.0:ND:36 |
| 4      | 10         | 6.0   | 33               | 880          | ND       | 3.2    | 1.2     | ND      | ND     | 50     | 1.0:ND:31 |
| 5      | 30         | 0.0   | 38               | 880          | ND       | 100    | 2.2     | ND      | ND     | 50     | 1.0:ND:83 |
| 6      | 30         | 1.0   | 140              | 840          | ND       | 4.5    | 2.1     | ND      | ND     | 41     | 1.0:4.0:13.7 |
| 7      | 30         | 3.0   | 280              | 740          | ND       | 1.1    | 1.1     | ND      | ND     | 15     | 1.2:1.0:1.6 |
| 8      | 30         | 6.0   | 380              | 720          | ND       | 0.5    | 0.5     | ND      | ND     | 37     | 1.0:5.4:6.9 |
| 9      | 60         | 0.0   | 280              | 720          | ND       | 20     | 4.8     | ND      | ND     | 21     | 1.0:5.4:12.2 |
| 10     | 60         | 1.0   | 370              | 700          | ND       | 0.4    | 0.3     | ND      | ND     | 5.5    | 1.0:5.4:13.3 |
| 11     | 60         | 3.0   | 340              | 690          | ND       | 74     | 0.2     | ND      | ND     | 24     | 1.0:5.4:13.3 |
| 12     | 60         | 6.0   | 210              | 680          | 230      | 0.8    | 0.3     | ND      | ND     | 58     | 1.0:5.4:13.3 |

Pretreatment temperature was 180 °C. H₂SO₄ sulphuric acid, Glu glucose, Xyl xylose, O:M oligomer-to-monomer ratio; Acetate partitions into acetic acid (AA), dissolved acetate on xylooligosaccharides (XOS), and acetate on wood residue (WR). ND not detected (less than 5% w/w). Values are the average of three technical replicates. Soluble lignin and minor sugars arabinose, rhamnose and galactose are not included in the mass balance. All values except ratios are mg/g.
acid hydrolysis \( R = -0.6, p = 0.01 \). Conversely, there was no significant correlation between native wood cellulose and acetate hydrolyzed \( R = 0.34, p = 0.027 \). The degree of inherent cell wall acetylation did not correlate with the extent of acetate released during pretreatment \( R = 0.14, p = 0.56 \), nor did minor variations in starting initial moisture content correlate with acetate-to-acetic acid hydrolysis \( R = -0.12, p = 0.62 \).

**Discussion**

**Wood sampling and degree of acetylation**

In a survey of wood samples from over 200 *P. trichocarpa* accessions growing in a common garden, the overall average degree of acetylation for xylan was approximately 0.6, consistent with earlier findings [13, 39]. In poplar, approximately 90% of acetate is attached to xylopyranosyl units, and it appears that poplar lignin is not significantly acetylated (Fig. 1). The positive relation between xylose and acetate in wood was therefore expected (Table 1), as acetate is added to xylan during xylan biosynthesis in relatively fixed proportions [40].

There is a moderately significant correlation between xylose and acetate content—a trend that may reflect differential degrees of substitution on individual xylopyranosyl units or blocks of xylan. For example, the degree of xylan substitution is not uniform across xylopyranosyl residues. Each xylopyranosyl residue can effectively have zero, one, or two acetate groups decorating the xylose unit. In contrast, some “blocks” of the xylan polymer may have higher localized acetate content relative to others. Another degree of uncertainty in the xylose-acetate trend is the presence of other acetylated polymers in the cell wall. Xyloglucans and glucomannans are additional hemicelluloses in poplar wood that can also be acetylated; however, both of these comprise less than 5% of normal poplar wood composition [9]. These factors explain some of the uncertainty in the xylose-acetate trend recorded in Table 1.

The glucose-acetate trend noted in Table 1 agrees with previously noted relationships between xylose and cellulose in *P. trichocarpa* [7]. Table 1 records statistically significant trends between acetate and cell wall carbohydrates: arabinose, rhamnose, galactose, and mannose. It could be that hemicellulose acetylation couples with the deposition of other cell wall components such as pectin. Acid-soluble lignin and acetate deposit in related amounts, but this is not the case with acid-insoluble lignin (Table 1). Acid-soluble lignins possess ether-linkages and this could indicate an association with cell wall acetate. The bulk of lignin (acid-soluble, >80% w/w) does

### Table 4 Xylose, glucose, and acetate release and partitioning following pretreatment

| Sample number | Xylose Total | O:M | Glucose Total | O:M | Acetate AA | XOS | WR | AA: XOS: WR |
|---------------|-------------|-----|---------------|-----|------------|-----|----|-------------|
| 1             | 148 ± 2.0   | 11.7| 218 ± 2.0     | 17.8| 13.0 ± 0.4| 26.0 ± 0.3| 14.4 ± 1.0| 1.0:2.0:1.1 |
| 2             | 140 ± 14.9  | 15.2| 184 ± 3.3     | 21.2| 13.2 ± 0.2| 19.0 ± 0.4| 19.3 ± 3.5| 1.0:5.1:1.5 |
| 3             | 136 ± 2.1   | 16.8| 167 ± 0.3     | 23.5| 12.7 ± 0.2| 22.0 ± 0.9| 25.3 ± 8.9| 1.0:1.7:2.0 |
| 4             | 135 ± 6.2   | 14.9| 151 ± 0.5     | 18.7| 12.4 ± 0.6| 27.1 ± 1.4| 25.8 ± 5.1| 1.0:2.2:1.1 |
| 5             | 134 ± 8.3   | 15.9| 182 ± 1.4     | 21.7| 12.3 ± 0.9| 20.0 ± 1.2| 20.9 ± 0.3| 1.0:1.6:1.7 |
| 6             | 129 ± 6.3   | 14.3| 132 ± 0.9     | 14.6| 12.7 ± 0.7| 17.7 ± 1.2| 19.7 ± 0.2| 1.0:1.4:1.5 |
| 7             | 126 ± 15.1  | 14.8| 151 ± 3.4     | 17.8| 11.5 ± 2.8| 17.3 ± 1.4| 22.2 ± 5.0| 1.0:1.5:1.9 |
| 8             | 120 ± 14.2  | 17.7| 145 ± 2.3     | 23.1| 10.7 ± 1.7| 17.0 ± 1.5| 25.2 ± 4.7| 1.0:1.6:2.3 |
| 9             | 118 ± 4.7   | 16.3| 158 ± 2.0     | 20.5| 10.4 ± 0.9| 22.7 ± 2.3| 19.5 ± 1.0| 1.0:2.2:1.9 |
| 10            | 118 ± 15.9  | 15.2| 150 ± 2.5     | 17.3| 10.5 ± 3.8| 17.8 ± 2.0| 26.2 ± 1.8| 1.0:1.7:2.5 |
| 11            | 115 ± 17.5  | 13.6| 158 ± 2.5     | 14.9| 11.4 ± 2.4| 14.6 ± 1.8| 26.6 ± 3.1| 1.0:1.3:2.3 |
| 12            | 115 ± 3.5   | 15.5| 100 ± 0.3     | 18.4| 8.7 ± 0.5 | 15.5 ± 2.0| 29.7 ± 5.9| 1.0:1.8:3.4 |
| 13            | 113 ± 35.2  | 13.6| 163 ± 6.4     | 14.9| 12.2 ± 3.6| 12.0 ± 1.7| 18.9 ± 1.2| 1.0:1.0:1.5 |
| 14            | 113 ± 3.5   | 22.9| 167 ± 0.6     | 38.9| 11.6 ± 0.5| 15.8 ± 2.4| 24.2 ± 3.6| 1.0:1.4:2.1 |
| 15            | 108 ± 13.3  | 14.1| 146 ± 3.1     | 14.5| 10.7 ± 2.5| 18.9 ± 3.0| 25.0 ± 2.6| 1.0:1.8:2.3 |
| 16            | 107 ± 8.6   | 16.4| 106 ± 1.1     | 17.3| 10.1 ± 1.2| 14.2 ± 2.4| 28.7 ± 10.9| 1.0:1.4:2.8 |
| 17            | 100 ± 22.2  | 12.5| 147 ± 2.8     | 13.3| 9.4 ± 2.8 | 18.4 ± 3.3| 27.9 ± 4.6| 1.0:1.9:2.9 |
| 18            | 84 ± 5.8    | 14.9| 117 ± 0.8     | 16.2| 8.1 ± 0.9 | 11.5 ± 2.2| 29.3 ± 5.3| 1.0:1.4:3.6 |
| 19            | 63 ± 30.7   | 12.8| 63 ± 3.3      | 13.4| 5.6 ± 4.7 | ND         | 34.2 ± 10.7| 1.0:ND:6.1  |

All values, except ratios, are listed as mg/g ± standard error of the mean. Total xylose and glucose release include the corresponding sugar in its oligomeric and monomeric form. O:M oligomer-to-monomer ratio. Acetate partitions into acetic acid (AA), dissolved acetate on xylooligosaccharides (XOS), and acetate on wood residue (WR). ND not detected.
not correlate with acetate content (Table 1). This could be
due to the timed deposition of secondary-wall-specific
lignin versus acetate in the secondary cell wall. Lignifica-
tion occurs after acetylated xylans are synthesized and
shuttled to the apoplast [41].

In this study of 200 individual poplar trees representing
over 100 unrelated genotypes, there was no link between
wood acetate and total biomass accrued (Table 1).
This trait independence implies that possibilities for
selectively breeding for wood acetate while maintaining
biomass yield is indeed possible [42].

Dilute acid pretreatment of poplar wood

Table 2 shows that pretreating poplar wood in acetic acid
increases its solubilization. Acetic acid alone was effec-
tive in catalyzing glucose and xylose release, i.e., that
acetate’s organosolv capacity is expeditious for sugar
release. For example, 4% acetic acid or above facilitates
5% lignin removal during hardwood pretreatment [43].
In another study, acetic acid boosted glucan and xylan
yields by up to 50% in natural Populus variants [44]. As
with other acid catalysts, excessively high concentrations
of acetic acid result in yield loss from degradation. Each
3% increment of acetic acid resulted in a twofold increase
in furfural products. The buildup of undesirable furfural
is preventable using a flow-through system, where excess
acetate acidi coming from the wood would be con-
trolled [48].

Previous studies have shown that endogenous ace-
tic acid can catalyze the breakdown of hemicelluloses,
depending on the pretreatment employed for process-
ing the biomass [23, 24, 43, 45–47]. However, there has
been no quantification of the proportion of acetate con-
verted to acetic acid. Cell wall chemistry and ultrastruc-
ture affect the proportion of acetate release; this was
demonstrated when wheat straw released one-third as
much acetic acid as hybrid poplar chips following the
same pretreatment [39]. The goal of our next analysis was
to pinpoint these components. Table 4 presents data for
acetate partitioning in wood samples varying in cell wall
chemistry, and Table 5 ties these data in regression analy-
ses to cellulose, lignin, acetate, and the moisture content
of wood. Nineteen distinct P. trichocarpa genotypes were
pretreated identically.

Figure 3 shows that, at the selected pretreatment con-
tdition, acetic acid did not correlate with the degradation
of carbohydrates into HMF and furfural. Instead, HMF
and furfural formation likely depended on sulfuric acid
concentrations [20]. Therefore, in order to optimize sugar
release, high levels of inherent acetic acid are desirable in
the pretreatment, as they correlate well with sugar release
but not degradation, as is consistent with prior findings
[48].

Table 4 records levels of acetate bound to xylan on
wood residue, dissolved in solution on XOS, or hydro-
lyzed to acetic acid. The absence of acetate on xylose oligo-
saccharides in sample 15 implies that the formation
of acetic acid can precede xylan hydrolysis during pre-
treatment. Xylose monomers may be formed either by
direct degradation of xylan in the wood or by depolymer-
ization of the solubilized oligomeric xylan [49]. High-
pressure environments could limit acetate hydrolysis;
the formation of volatiles such as acetic acid occurs less prominently in high-pressure reactions [50]. Moreover, dilute acid pretreatment on hardwood at atmospheric pressure completely removed acetate groups [51].

Under the mild pretreatment conditions employed, acetate partitioning is useful for studying wood deconstruction. A high pretreatment severity would result in all acetate groups being hydrolyzed to acetic acid (Table 3). In the present pretreatment, one-sixth to one-third of acetate groups were hydrolyzed to acetic acid (Table 4). Examining the partitioning of acetate into the three different pools, it is apparent that a higher proportion of acetate is retained in the wood residue than in the dissolved fraction. This suggests that less-acetylated xylan is more easily removed from the secondary cell wall during pretreatment, and leads to the interpretation that cell wall acetylation could be a factor distinguishing slow- and fast-reacting xylan [52]. Table 4 suggests that fast-reacting xylan has a degree of acetylation of 0.35, and slow-reacting xylan has a degree of acetylation of 0.73. Previous studies have speculated that slow-reacting xylan retains its acetate substituents and is “contaminated with” or “embedded within” lignin [22, 53]. In addition, wood samples with a higher total amount of acetate did not release the highest amount of acetic acid. This implies that there are additional factors limiting the removal of highly acetylated xylan from the cell wall.

Factors affecting xylan dissolution

Our observations show that wood with higher lignin content generally released less xylan and acetic acid (Table 5), ultimately decreasing pretreatment sugar yield. This finding agrees with Timmel [54], who compared xylan removal in aspen and elm wood. Elm contains 15% more lignin than aspen and treatment with aqueous potassium hydroxide removed the entire proportion of aspen xylan, but only one-fourth that in elm. An explanation for these results is two-tiered: First, lignin retains xylan in the wood residue by non-covalent interactions and, second, dissolved lignin interferes with xylan dissolution [55]. Poplar xylan has high acetate content; thus, the effect of lignin on wood recalcitrance is more pronounced than in other feedstocks such as sugarcane bagasse [56].

Recalcitrance of xylan to pretreatment depends upon non-covalent or covalent interactions as well as mechanical entanglement of xylan with itself or other cell wall polymers [57]. The findings of the current study suggest that lignin and xylan interact, and that acetate content influences the interaction between these two major cell wall polymers. The amount of acetate hydrolyzed inversely correlates with total lignin content ($R = -0.6$; Table 5), and this supports previous findings that lignin increases biomass recalcitrance to pretreatment [58]. That acetylated xylan forms complexes with lignin in aqueous pretreatment slurries can be explained by hydrophobic effects. In sufficient quantity, hydrophobic or van der Waals interactions can facilitate intermolecular adhesion in the secondary cell wall [55, 59]. The removal of acetate from solid wood residue and their dissolution as XOS could be enthalpy-driven and highly affected by non-covalent interactions.

Conclusions

Acetate endogenous to woody biomass could improve sugar release during pretreatment, an effect also noted by Ewanick et al. [60]. Acetate hydrolysis did not vary with wood cellulose content, which is consistent with the hypothesis that interactions between acetate and the cellulose microfibril are minimal [61]. Results from this and other studies also show that some acetate is retained in xylan in woody biomass during pretreatment [62]. Maximizing acetate release may be one way to increase sugar yield without increasing sugar degradation (Fig. 3). For example, acetate tends to be associated with slow-reacting xylans more than fast-reacting xylans (Table 4). Finally, there is more acetic acid in the liquid stream following pretreatment of lower-lignin wood samples (Table 5). Tailoring pretreatments by taking into consideration these compositional relationships could increase the effectiveness of biomass refining processes.

This study provides insight into the deconstruction of P. trichocarpa during pretreatment. The catalytic potential of acetic acid released from the cell wall is controllable by altering reaction time and changing acid concentration. Strong correlations among woods from 19 individuals suggest that acetate aids in dissolving hemicelluloses during pretreatment, but only if hydrolyzed to free acetic acid. Our findings suggest that highly acetylated xylan is more difficult to remove from wood samples, as feedstocks with higher lignin released less acetic acid into solution. This relates the catalytic effect of acetate in poplar wood to its lignin content. These findings demonstrate the importance of considering cell wall acetate.

Table 5 Possible factors affecting acetyl-to-acetic acid conversion during dilute acid pretreatment of poplar wood

| Factor         | $R$  | $p$ value | $n$ |
|----------------|------|-----------|-----|
| Lignin         | -0.59| 0.010     | 18  |
| Cellulose      | 0.34 | 0.027     | 18  |
| Total acetate  | 0.14 | 0.560     | 19  |
| Wood moisture  | -0.12| 0.620     | 19  |

$R$ Pearson correlation coefficient; $p$ value represents the test statistic; $n$ number of observations (each is the average of three technical replicates). Cellulose estimates based on glucose content.
when evaluating potential bioenergy crops. As acetylated xylan is the major hemicellulose present in the secondary xylem of most dicot species, this knowledge is applicable to other lignocellulosic biofuel feedstocks, such as shrub willow and eucalyptus.

**Methods**

**Wood processing and compositional analysis**

*Populus balsamifera* subsp. *trichocarpa* individuals were grown in a common garden established by the British Columbia Ministry of Forests at the University of British Columbia [63]. Two hundred of 500 available individuals planted in Totem Field in June 2008 were harvested in March 2012 according to McKown et al. [64]. Cookies were cut 6” from the base, and wood was processed to remove bark and pith. The wood specimens were then ground in a Wiley-mill fit with 40-mesh screen and divided into technical replicates. Samples were stored at −20 °C until subjected to pretreatment.

Wood composition was determined by two-stage acid hydrolysis (Klason method), according to Cullis [65]. Briefly, 3 mL of 72% sulphuric acid was added to 200 mg of extractives-free ground wood in a reaction flask at room temperature. The reaction was stirred every 3 min for 2 h. Nanopure water was used to dilute the reaction to a final sulphuric acid concentration of 4%. Carbohydrates were hydrolyzed in an autoclave at 121 °C for 1 h. High-performance anion-exchange liquid chromatography quantified constituent sugars. The neutral sugars separated on a Carbopak-PA1 anion-exchange resin using an AS50 autosampler, a G50 gradient pump, and an ED50 electrochemical detector (Dionex, USA). Isocratic elution in deionized water occurred over 35 min; next, a linear gradient ramping to 0.5 M NaOH for 10 min washed out strongly adsorbing components. From 45–60 min, a mobile phase of pure water equilibrated the resin for the next injection. Peaks were manually integrated and quantified against sugar standards. Molar stoichiometrics accounted for mass loss following the hydrolysis of polysaccharides into monosaccharides (0.90 for hexose sugars, 0.88 pentose sugars), and acetic acid to acetate (0.98). Lignin was recovered as acid-insoluble (Klason) and acid-soluble lignin. Acid-insoluble lignin was the hydrolysate retentate in a medium-coarseness sintered glass crucible. Acid-soluble lignin in the filtrate was estimated by Beer–Lambert’s Law using an absorbance at 205 nm and extinction coefficient of ε = 110 L/g/cm [66].

To quantify acetate, a saponification reaction from Browning [67] was conducted. Sodium hydroxide (0.2 M) reacted with acetone-extracted wood samples at a 2% solids loading. The reaction was incubated at 120 °C with constant shaking (500 rpm) for 75 min. Sulphuric acid (72% w/w) acidified each sample to pH 2 ± 1 and cooling took place in an ice bath for 5 min. Centrifuging samples at 13,000g for 2 min separated solid and liquid phases. The supernatant eluted through a 0.45 μm filter into a 2-mL glass vial. Samples were injected onto an HPX-87H column (Aminex, USA) on a high-pressure liquid chromatography instrument equipped with an ASI-100 Autosampler, a P60 HPLC Quaternary Gradient Pump, and a PDA-100 photodiode array detector set to 205 nm (Dionex, USA). The mobile phase was 5 mM sulphuric acid at a flow rate of 0.7 mL/min. Acetic acid peaks were integrated manually and their areas measured against standards of known concentration.

**NMR**

NMR analysis was performed as described in the recent protocol [68]. Briefly, plant biomass was air-dried to a constant moisture content and cryogenically pre-ground for 2 min at 30 Hz using a Retsch (Newton, PA, USA) MM301 mixer mill. The pre-ground cell walls were extracted with distilled water, followed by 80% ethanol, using ultrasonication. Isolated cell walls (200 mg) were then finely milled using a Retsch PM100 planetary ball mill for 70 in 10-min intervals with 5-min interval breaks. Approximately 30–60 mg of extracts-free, ball-milled plant cell wall material was transferred to a 5-mm NMR tube, and 500 μL of premixed DMSO-d$_6$/pyridine-d$_5$ (4:1) was added directly into the NMR tube containing individual samples. The NMR solvent mixture was carefully introduced (via a syringe), spreading it from the bottom of the NMR tube, along the sides, and towards the top of the sample. The NMR tubes were then placed in an ultrasonic bath and sonicated for 1–5 h, until the gel became homogeneous; the final sample height in the tube was ~4 to 5 cm. 2D $^1$H–$^{13}$C HSQC spectra were acquired using a standard Bruker pulse program (hsqcetgpsisp2.2). The NMR spectra had the following parameters typical for plant cell wall samples: spectra were acquired from 10 to 0 ppm in F2 ($^1$H) using 2800 data points for an acquisition time (AQ) of 200 ms, an interscan delay (D1) of 1 s, 200–0 ppm in F1 ($^{13}$C) using 560 increments (F1 acquisition time 8 ms) of 56 scans, with a total acquisition time of 11 h. Processing used typical matched Gaussian apodization in F2 and squared cosine-bell in F1. Interactive integrations of contours in 2D HSQC plots were carried out using Bruker’s TopSpin 3.5 (Mac) software, as was all data processing.

**Pretreatment of wood—autohydrolysis, acetic-acid-catalyzed, and sulphuric-acid-catalyzed**

Wood flour and pretreatment liquid (dilute water, acetic or sulphuric acid) were added into reactors at 5% (w/w) solids loading. Reaction vessels with a capacity of 15 mL were stainless steel cylinders with a bolt-screw fitting on either end. Prior to closing the reactors, both fittings were sealed with polytetrafluoroethylene tape. The reactors
were vortexed and preincubation at 60 °C for 60 min to ensure impregnation of the wood sample with pretreatment solvent followed. Non-isothermal pretreatment was conducted in a Lindberg Blue M laboratory gravity oven (Thermo Scientific, USA) at 180 °C. At time zero, heating in the oven began. Pressure inside the reactor was measured by attaching a 2000 psi pressure gauge (Ashcroft, USA) to a bolt-screw fitting via 30 cm of stainless steel tubing. All reactions were quenched in an ice bath.

Analysis of solid and liquid phases after autohydrolysis

Following reactions, the pretreatment liquors and wood residue were transferred to polypropylene tubes wrapped in aluminum foil to prevent furan degradation by ultraviolet light. Samples were stored at 4 °C for a maximum of three days prior to HPLC analysis. Monosaccharides were quantified by high-performance anion-exchange chromatography columns as described above. Oligosaccharides underwent secondary acid hydrolysis, where the original reaction hydrolysates were autoclaved in 2.5% (w/w) sulphuric acid at 121 °C for 60 min, and the carbohydrates were again quantified using high-performance anion-exchange chromatography, and the difference between total sugars in the secondary hydrolysates and monosaccharides in the original reaction hydrolysate was determined to be the oligosaccharide fraction. Separation of the acetic acid, furfural and 5-hydroxymethylfurfural (hydroxymethylfurfural, HMF) was achieved on an Aminex HPX-87H column (BioRad, USA) as described above. After washing, acetate in wood residue was quantified by saponification as described above. Acid-soluble sugars in wood residue were determined using the Klaip method described above.

Additional file

Additional file 1: Table S1. Data used to calculate associations presented in Table 1. All values except biomass are in percent extractives-free dry weight. Biomass is in kilograms.

Abbreviations

AA: acetic acid; DP: degree of polymerization; XOS: xylooligosaccharide; R: Pearson’s correlation coefficient; w/w: weight over weight; v/v: volume over volume; HPLC: high-performance liquid chromatography; HMF: 5-Hydroxymethyl-2-furaldehyde; O:M: oligomer-to-monomer ratio; SD: standard deviation; WR: wood residue.

Authors’ contributions

AMJ performed experiments, HK preformed NMR analysis, SDM conceptualized the work, AMJ, HK, JR, and SDM wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of supporting data

All data generated or analyzed during this study are included in this published article and its Additional file 1.

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