Effect of aging on lignin content, composition and enzymatic saccharification in *Corymbia* hybrids and parental taxa between years 9 and 12.

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Abstract

Corymbia (a eucalypt) is an important forestry genus and a potential lignocellulosic bioenergy feedstock. The composition of the lignocellulosic cell wall significantly impacts pretreatment efficiency and conversion to biofuel but is variable and changes with age. In this study, we estimated Klason lignin content, composition, and monosaccharide (glucose and xylose) release after enzymatic saccharification of untreated and hydrothermally pretreated biomass from *Corymbia* parental species *C. torelliana* (CT), *C. citriodora* subsp. *variegata* (spotted gum; CCV), and interspecific F1 hybrids (CT x CCV) at ages 9 and 12 years from planting. Analysis of lignin composition derived from syringyl/guaiacyl monolignols (S/G) found significant differences among taxa, with CT S/G ratios (2.2 and 2.0) being significantly lower than CCV (2.6 and 2.3) or hybrids (2.5 and 2.3) at ages 9 and 12 respectively. In general, enzymatic saccharification yields from untreated biomass were significantly different among taxa, with CT (113 and 75 mg g⁻¹) and hybrids (108 and 81 mg g⁻¹) yielding significantly higher glucose from untreated biomass than CCV (82 and 56 mg g⁻¹) at ages 9 and 12 respectively. Comparison of traits within taxa between ages 9 and 12 found S/G ratios and glucose yields from untreated biomass were significantly lower in CT, CCV and hybrid taxa. In conclusion, the formation of lignocellulosic cell walls is complex, influenced by genetics and age of material, requiring optimization of rotation age for biofuel production and other industrial processes.

Keywords: *Corymbia*, saccharification, lignin, S/G, bioproducts, biofuels

1. Introduction

Lignocellulosic (woody) biomass is an important industrial resource, grown for the production of lumber, pulp and paper, and high-value biomaterials [1,2]. Recently, lignocellulose from high production short rotation trees has become an attractive biomass source for producing biofuels based on the ease of management, energy content, and high concentrations of cellulose [3,4]. However, lignocellulose is difficult to deconstruct and traits desirable for other industrial processes, such as cellulose crystallinity for pulping or lignin content for timber, inhibit enzymatic hydrolysis. Indeed, cellulose crystallinity reduces the reactive surface area for efficient saccharification [5,6], hemicellulose surrounds cellulose microfibrils within a matrix of difficult to ferment 5-carbon sugars [7,8], and lignin (the greater contributor to biomass recalcitrance) restricts enzymatic access to polysaccharides and non-specifically binds cellulases [9,10]. Therefore, lignocellulose must be either mechanically or chemically pretreated in order to be efficiently converted into biofuel [11].

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72 The 3-dimensional structure of lignin lends itself to prevent enzymatic access to polysaccharides within the cell
73 wall [12]. Lignin is synthesized through oxidation and polymerization of three monolignols: *p*-hydroxyphenyl
74 (H), guaiacyl (G) and syringyl (S), and the S/G ratio of lignin composition is important for deconstruction, as S
75 subunits are only able to cross-link with two monolignols (creating a less branched structure) and their bonds are
76 generally more reactive and can be broken down more easily by industrial processes [13–15]. As each biomass
77 component contributes to the overall recalcitrance of biomass to pretreatment and hydrolysis, an improved
78 understanding of wood formation would allow for more efficient biofuel conversion. Wood formation is a
79 complex polygenic process, dependant on genotype, transcription, enzyme kinetics, chemical reactions, and
80 environmental factors such as rainfall and soil conditions [16–18]. Also, wood composition changes with tree
81 age [19], necessitating the need to discover the optimal harvesting age for biomass in order to maximize its
82 efficiency for industrial processes. While tree harvest age typically varies between 3 and 30 years (depending
83 on its use) [20,21], the majority of wood formation research has focused on early stage development, with
84 changes that occur after 9 years of age being less researched.

85 The wood properties of *Eucalyptus*, being the most widely planted hardwood tree in the world, have been
86 extensively researched, both at the phenotypic and genotypic levels [22–24], and are a potential crop for
87 lignocellulosic biofuels [25]. The closely related *Corymbia* genus (another eucalypt) has been less researched,
88 but is the most harvested hardwood tree in Queensland Australia, and has been deployed overseas in Brazil,
89 South Africa and China for commercial use [26,27]. *Corymbia* also possess desirable pulping and timber
90 qualities, and may be suitable as a bioenergy crop based on their S/G lignin composition (2.5) [28], high glucan
91 content (48.5%) [29], amenability to a wide range of environments [26,30], and high biomass production
92 achieved through controlled-cross interspecific hybrids (127-287% increased diameter) [27].

93 The aim of this study is to investigate the effect of aging on economically important biofuel traits among
94 *Corymbia* parental species *C. torelliana* (CT), and *C. citriodora* subsp. *variegata* (CCV; spotted gum), and their
95 controlled-cross F1 interspecific hybrids (CT x CCV). The investigated traits were Klason lignin content (%
96 mass fraction), lignin composition (S/G ratio), total enzymatic saccharification yields from untreated and
97 hydrothermally pretreated biomass, and proportions of glucose and xylose (G/X) released from each sample.
98 Environmental influences from rainfall and soil type were minimized by sampling trees within a single forestry
99 trial site. Investigation of alternation in biofuel traits as trees age will inform optimal harvesting age and require
100 less harsh pretreatments to deconstruct biomass.

2. Materials and Methods

2.1 Sample collection

Sapwood frass was collected from CT (n=12), CCV (n=8) and interspecific F1 *Corymbia* hybrids (CT x CCV; n=13) located at the Queensland Department of Agriculture and Fisheries (DAF) Amamoor trial site (26°21.415 S; 152°31.844 E, Altitude: 154 m above sea level, Soil: black dermosol 35 cm depth; annual rainfall mean (1913-1946) 1090 mm; humid sub-tropical climate), located near Gympie, Queensland. Sample collection occurred at 107 months (age 9) from planting and again from the same trees at 144 months (age 12). Sapwood frass was extracted by drilling into the tree under the bark layer at a height of 1.3 m with a 16 mm wood boring drill bit on the north facing aspect with a modified funnel and a paper bag (taking care to avoid knots and tension wood). Each bag was air dried in an air-conditioned room for 14 days then held in a climate-controlled room at 4°C until use.

2.2 Lignin content and composition

Lignin content and S/G composition were determined in duplicate using pyrolysis molecular beam mass spectroscopy (pyMBMS) at the National Renewable Energy Laboratory (NREL) in Golden, Colorado as described by Sykes et al [31]. This robust technique has been successfully used to estimate lignin content and S/G ratio in poplar [32,33] corn stover [34], and *Eucalyptus* [35]. In brief, total Klason lignin content was estimated from pyMBMS peaks m/z = 120, 124, 137, 138, 150, 152, 154, 164, 167, 178, 180, 181, 182, 194 and 210, relative to carbohydrate peaks m/z = 114 (hemicellulose), 98, 126, and 144 (cellulose). S/G ratios were determined by summing the syringyl peaks 154, 167, 168, 182, 194, 208, and 210 and dividing by the sum of guaiacyl peaks 124, 137, 138, 150, 164, and 178 [31]. Several lignin peaks were omitted in the syringyl or guaiacyl summations due to individual peaks having associations with both S and G precursors [36]. The summed lignin intensities from the pyMBMS provide relative differences between samples. In order to relate these relative differences to industry standard Klason values, species specific correction factors were developed by determining the amount of Klason lignin [37] within a CT and CCV sample (collected from DAF germplasm in Gympie, Queensland), then dividing each sample's pyMBMS lignin estimation by their Klason lignin value. The correction factors were then applied to their representative taxa. Additionally, Klason lignin content was estimated from a *Populus* standard (NIST 8492, in biological duplicate) for validation (Klason lignin: 23.7% $\pm$ 0.2%); 24% $\pm$ 1%). As the two correction factors were similar (CT=0.35 and CCV=0.34) the hybrid lignin values were corrected using the CT correction factor.

2.3 Hydrothermally pretreated enzymatic hydrolysis

Enzymatic saccharification of hydrothermally pretreated biomass samples was conducted at NREL in Golden Colorado as described by Selig et al [38]. In brief, 5.0 +/- 0.2 mg of 20-mesh milled biomass was pretreated in 250 mm³ of water, held at 180°C for 17.5 min using custom designed and built 96-well reactor plates, sealing, and pressure control. Triplicate samples were digested with commercial cellulase (Ctec2, Novozymes; 70 mg g⁻¹ biomass) for 70 h at 50°C. The hydrolysates were analysed for glucose and xylose using glucose oxidase/peroxidase and xylose dehydrogenase assays, respectively [33].

2.4 Saccharification of untreated wood: sample preparation

Particle size reduction was performed prior to untreated saccharification by aliquoting biomass into standard-format plastic labware for use with automation. In 2.0 cm³ polyethylene vials (Sarstedt VWR 72.609.001) containing three ceramic beads (yttrium stabilized zirconia, 5mm, Inframat Advanced Materials, Manchester, CT, USA), the wood samples were ground for 5 min (2.5 min grind, 60 sec rest, repeat) cycle using the Joint BioEnergy Institute (Sandia National Laboratories) Biomass Preparation System robot created at Labman Automation Ltd. (North Yorkshire, UK). This robot was also used to dispense the ground wood, with a target mass of 5 ± 0.2 mg per well, into 2 cm³ 96-well polypropylene deep-well blocks (Corning Costar 3961). The samples were dispensed using three replicate aliquots dispersed across three separate 96-well blocks.

Samples were ethanol extracted by adding 1.0 cm³ aliquot of 80% ethanol to each well in the 96-well blocks using a Biomek FX liquid-handling robot equipped with an AP96 multichannel pod (Beckman Coulter, Brea, CA, USA). Each block was sealed with peelable heat seal (Agilent 24210-001) using an Agilent PlateLoc heat sealer set to 175°C for 3 sec, and incubated in a thermostatically controlled room at 37°C for 24 h. Following ethanol extraction, the Circulating Reservoir (Beckman Coulter PN 989299) and vacuum manifold (Beckman Coulter PN A16097) on the Biomek FX were used, respectively, to supply aliquots of ultrapure water to, and discard ethanol aliquots (containing extractives) until reaching an ethanol concentration of less than 1% in 820 mm³ final volume per well. De-starching was found to be unnecessary as no additional sugars were released when representative wood samples prepared as described were treated with amylase (data not shown).

2.5 Saccharification of untreated wood

Saccharification was initiated by using to the Biomek FX to add to each well 180 mm³ of an enzyme solution

containing an 8.2:1 v:v ratio of Cellic CTec2 : HTec2 (Novozymes, Franklinton, NC, USA) in 29.4 g L⁻¹ citrate buffer pH 5.0. The enzyme loading was approximately 0.1 g g⁻¹ glucan, empirically chosen to maximize observed differences between representative wood samples known to have low and high cellulose content (data not shown). Each 96-well block was sealed, again with peelable seal and incubated at 55°C for 24 h without agitation in a Thermos oven (Thermo Scientific, Waltham, MA, USA). Following the saccharification, the 96-well blocks were centrifuged for two minutes using an Eppendorf 5810R centrifuge set to 3000 x g. The Biomek FX was used to transfer from each block three 100 mm³ aliquots, one each into separate 0.45 µm filter plates (Whatman, 7700-1301, GE Healthcare Bio-sciences, Pittsburgh, PA, USA), which were in turn centrifuged at 3000 x g for 3 min to filter the aliquots into 200 mm³ PCR plates (Bio-Rad, Hercules, CA, USA, HSP9601) that were subsequently sealed using pierceable aluminium heat seal (Agilent 06644-001) applied by a PlateLoc sealer set to 175°C for 4 sec.

2.6 High-performance liquid chromatography (HPLC) analysis of glucose and xylose

The HPLC analysis was performed using an Agilent 1260 Infinity system (Agilent, Santa Clara, CA, USA) equipped with a Bio-Rad 300 mm x 7.8 mm Aminex 87H column (Bio-Rad, Hercules, CA, USA) with a Bio-Rad cation H guard column. The Agilent 1260 refractive index detector was kept at 35°C. The samples were measured using an isocratic 0.39 g L⁻¹ sulphuric acid eluent, prepared using HPLC grade water (Honeywell, Morristown, NJ, USA) and 98% sulphuric acid (Millipore, Billerica, MA, USA) at 600 mm³ min⁻¹ and 60°C for 16 min. Standards were prepared in a volumetric flask using Supelco (Supelco, Bellefonte, PA, USA) monosaccharides in 0.39 g L⁻¹ sulphuric acid, and were diluted to create a seven-point calibration curve. The range of concentrations used for the glucose standards were between 0.03125-4.0 g L⁻¹; and those for xylose were between 0.0156-2.0 g L⁻¹. The standards were measured at the beginning, middle, and end of every 96-well plate. De-ionized water blanks were inserted to the sample queue before and after each set of standards. The concentration of glucose and xylose in the unknowns was calculated using the Chemstation software package, by integrating the area under each peak.

2.7 Data analysis

Trait data was assessed for outliers (using median average deviation) and erroneous data points as determined by >20% relative standard error. Outliers and erroneous data points were removed from the datasets, with each trait then being visualized prior to analysis using a histogram to assess normality. As none of the traits fulfilled

the requirements for parametric testing, non-parametric statistics were used. Untreated and hydrothermally pretreated saccharification data was collected using two separate methods at two different laboratories so no comparisons were made across datasets. To compare traits among species separately at sample ages 9 and 12, a rank-based Kruskal-Wallis test was used [39]. Any trait that showed significant differences (p-value <0.05) among groups was further tested using Mann-Whitney U-tests [40]. To assess wood composition changes that occur within each sample group between ages 9 and 12, each trait was analysed using paired Mann-Whitney U-tests. All analyses were carried out using Rstudio (version 3.0.2).

3. Results

Data summaries (trait mean and standard deviation) and all test statistic values are found in Tables 1-2.

3.1 Lignin S/G composition

Analysis of pyMBMS lignin compositional data using a Kruskal-Wallis test revealed significant differences among *Corymbia* samples at ages 9 ($\chi^2=16.0$, $p=3.4 \times 10^{-4}$) and 12 ($\chi^2=21.1$, $p=2.6 \times 10^{-5}$), each being considered separately (Fig.1). Comparison of 9-year-old samples found that the S/G ratio of CT (2.2 ± 0.1) was significantly lower ($p<0.001$) than CCV (2.6 ± 0.1) and hybrids (2.5 ± 0.2) (Table 1). Testing at age 12 again revealed the same trend, with the S/G ratio of CT (2.0 ± 0.0) being significantly lower ($p<0.001$) than CCV (2.3 ± 0.1) and hybrids (2.3 ± 0.1) (Table 2), with the overall difference at each time-point between CT and other taxa being ~ 0.3 . Comparison of S/G ratios within CT, CCV and hybrid groups (, Fig.1) at ages 9 and 12 revealed a significant decrease ($p< 0.01$) in lignin S/G ratios over time. Interestingly, the magnitude of each change for each group mean was approximately the same, decreasing by 0.2-0.3. This result, along with the paired Mann-Whitney test statistic (V) values for each taxa (77, 36, and 91 respectively), can be interpreted as for every comparison (33 trees in total), in every instance (except the top ranked CT tree that remained constant), each tree's 12-year S/G ratio was lower than its 9-year S/G ratio.

“Table 1 here”

3.2 Lignin Content

Analysis of Klason lignin content (Fig.2) among taxa at age 9 found no significant differences ($\chi^2=5.5$, $p=0.06$) among taxa (Table 1). However, at age 12 there was a significant difference among taxa ($\chi^2=6.8$, $p=0.03$), where CT lignin content was significantly lower ($23\% \pm 1$, $W= 30$, $p<0.05$) than hybrids ($24\% \pm 1$) and CCV

was not significantly different than either taxa ($23\% \pm 3$, $p>0.05$) (Table 2). Comparison within each taxa between ages 9 and 12 found a significant difference in CCV samples, where lignin content decreased from 25% to 23% ($V=36$, $p=0.008$), while no significant differences ($p>0.05$) were found within CT or hybrid samples.

Table 2 here”3.3 Untreated *Corymbia* glucose and xylose yields

Analysis of saccharification as determined by HPLC from enzymatic hydrolysis of untreated *Corymbia* wood samples revealed significant differences among taxa for glucose yield (Fig.3A), and the proportions of glucose and xylose (Fig.3C) released from biomass at ages 9 (Table 1) and 12 (Table 2). At age 9, glucose release among taxa was significant ($\chi^2=6.38$ $p=0.04$), with CCV biomass yielding significantly lower glucose (82 ± 26 mg g^{-1} , $p=0.04$) than both CT (113 ± 24 mg g^{-1}) and hybrid trees (108 ± 25 mg g^{-1}) (Table 1). At age 12, glucose release among taxa was significant ($\chi^2=9.95$ $p=6.9 \times 10^{-3}$) (Table 2), where CCV again releases significantly less glucose (56 ± 11 mg g^{-1} ; $p<0.001$) than hybrid samples (81 ± 13 mg g^{-1}), whereas CT (75 ± 25 mg g^{-1}) was not significantly different ($p>0.05$) to either taxa (Fig.3A). Comparison of xylose yields at age 9 found significant differences among taxa ($\chi^2=8.15$ $p=2.0 \times 10^{-3}$), with CCV biomass releasing a significantly higher amount of xylose (13 ± 3 mg g^{-1} ; $p<0.01$) than CT trees (9 ± 3 mg g^{-1}). However at age 12 (Table 2) there were no significant differences ($p>0.05$) among *Corymbia* trees for xylose released from untreated samples, each yielding an average of approximately 9 mg g^{-1} of biomass (Fig.3B).

Investigation of G/X ratio found a significant difference ($\chi^2=16.81$, $p=2.0 \times 10^{-4}$) among taxa for proportion of monosaccharides released from untreated biomass (Fig.3C). At age 9, the average CT G/X ratio was highest (14 ± 5), followed by hybrids (11 ± 3), and CCV (6 ± 2) which was statistically lower than both CT and hybrids ($p<0.001$). At age 12, comparison of G/X ratio among taxa was significant ($\chi^2=7.72$, $p=0.02$), again with the G/X ratio of CCV being significantly lower (7 ± 2 , $p<0.001$) than hybrids (10 ± 3). The G/X ratio of CT (10 ± 6) was not significantly different to either taxa (Fig.3C).

Assessment of saccharification results within taxa between ages 9 and 12 found significant decreases in glucose (Fig.3A) and xylose yields within each *Corymbia* taxon. Assessment of glucose yield revealed that significantly less glucose was detected after enzymatic saccharification at age 12 as compared to age 9 for CT (change in yield (Δ)= -38 mg g^{-1} ; $V=75$, $p=0.002$), CCV (Δ = -26 mg g^{-1} ; $V=33$, $p=0.04$) and hybrid (Δ = -27 mg g^{-1} ; $V=81$, $p=0.01$) samples. Investigation of xylose yields revealed the same trend, where CCV (Δ = -5 mg g^{-1} ; $V=36$, $p=0.008$) and hybrid (Δ = -2 mg g^{-1} ; $V=78$, $p=0.02$) samples released significantly less xylose at age 12

than age 9 (Tables 4-5). While CT xylose release remained constant ($9 \pm 3 \text{ mg g}^{-1}$) at ages 9 and 12, the G/X ratio significantly decreased from 14 ± 5 to $10 \pm 6 \text{ mg g}^{-1}$ ($V=66$, $p=0.03$), respectively (Fig.3C).

3.4 Pretreated *Corymbia* glucose and xylose yields

There were no significant differences in glucose release among *Corymbia* groups at either ages 9 or 12 (Fig.4A).

The total amount of glucose released was variable within taxa; at age 9, glucose release from CT, CCV, and hybrids ranged between 286 ± 136 , 205 ± 78 , $314 \pm 114 \text{ mg g}^{-1}$ ($\chi^2=4.07$, $p=0.13$) respectively (Table 1), which were not significantly different ($p>0.05$) than glucose values at age 12 (310 ± 63 , 270 ± 64 , $284 \pm 58 \text{ mg g}^{-1}$, respectively) (Table 2).

Comparison of xylose release from pretreated biomass at age 9 among taxa was significant ($\chi^2=6.89$, $p=0.03$), with CCV xylose release ($96 \pm 10 \text{ mg g}^{-1}$, $p<0.01$) being significantly lower than hybrids ($123 \pm 24 \text{ mg g}^{-1}$), while CT xylose release ($130 \pm 51 \text{ mg g}^{-1}$) was not significantly different to either taxa (Fig.4B). At age 12, there was no significant differences among CT, CCV or hybrid taxa (115 ± 32 , 107 ± 16 , $109 \pm 31 \text{ mg g}^{-1}$, respectively), or significant differences within taxa between ages 9 and 12. Comparison of G/X ratios among taxa and within taxa at both ages found no significant differences at age 9 (2.8 ± 0.8 , 2.6 ± 0.8 , 2.7 ± 0.9 , respectively) or age 12 (2.2 ± 0.4 , 2.2 ± 0.8 , 2.6 ± 0.7 , respectively) (Fig.4C).

4. Discussion

Polysaccharides represent some of the highest value targets during biomass deconstruction, and lignin content and composition most significantly affect the efficiency at which these polymers can be extracted [1].

PyMBMS measures lignin content and composition by pyrolyzing small amounts of biomass then quantifying the resulting phenolic structures to determine the original amount and composition of lignin present [31].

Assessment of Klason lignin composition within our three *Corymbia* taxa using pyMBMS found significant differences among the proportions of pyrolyzed phenolic subunits derived from either S or G monolignols.

Within each age considered separately, the S/G ratio of CT was significantly lower than CCV and hybrid samples, by a consistent amount of approximately 0.3 (Fig 1). Although the relative difference among samples is small, these findings are consistent with those reported by Lupoi et al [28], which included these samples within a much larger dataset. In-depth investigation of how lignin composition changes between ages 9 and 12 years within CT, CCV and hybrid trees revealed a significant drop in S/G ratios that occurred within 32 of 33 samples tested. This significant result is particularly impactful for the pulp and paper industries where S/G

ratios are monitored for their increased productivity during Kraft pulp manufacture [41,42]. Small gains in productivity afforded by high S/G ratios would result in greater profitability margins.

Although the genetic pathways for lignin formation have been extensively investigated, particularly by pulp and bioenergy industries through the use of transgenic gene manipulation [43], little is known about how lignin composition changes over time. Studies conducted thus far have mainly focused on early stages of plant growth, where lignin formation outpaces cellulose biosynthesis [44]. The significant and consistent decrease of S/G lignin over time in this study is surprising, considering several studies across a variety of plant systems, including non-woody biomass [45] have suggested the opposite. Research into biomass compositional changes to occur over time in *E. globulus* by Rencoret et al [19] found S lignin was the last and most dominant monolignol to deposit. Biomass sampling of an *E. globulus* clone at 1 month, 18 months, and 9 years from planting revealed H lignin was the first monolignol to deposit during hardwood cell wall formation, followed by G lignin, and finally S lignin as evidenced by 9-year biomass with the highest S/G ratios. As such, β -aryl ether bonds were the most abundant linkage found within *E. globulus* woody biomass, consistent with other compositional analyses involving eucalypts. Similar results have been reported within pine species, where monolignol deposition within the cell wall occurred in the same order in which it was synthesized, starting with H lignin, followed by G and S subunits [46]; although *Eucalyptus* and pine represented two distinct types of wood (hardwood and softwood, respectively), each with different lignin compositions. Softwood, derived from gymnosperm biomass, is primarily composed of H and G lignin, whereas hardwood's (angiosperm) main component is S lignin, with lesser amounts of G and H [13]. *Corymbia*, like *Eucalyptus*, is a hardwood taxon whose lignin is primarily linked through β -aryl ether bonds [47], but our data shows that S lignin deposition declines between ages 9 and 12. The mechanism for this result is unclear, particularly as Wu et al [48] found that latewood formation correlated with S lignin deposition, which should continue increasing with tree age. It is possible that the S lignin gene pathway could be suppressed during late stage growth, but transcriptomic data at each time point would be required to confirm. The collection of *Corymbia* samples at early ages would help assess at what age the biomass reaches its S lignin maxima, information that could be factored into rotation ages for pulp harvesting or other industrial processes.

S/G lignin composition is an important eucalypt pulping trait, demonstrated by del Río et al [49] with *E. globulus* biomass. The authors found that S/G ratios, as predicted by pyrolysis gas chromatography/mass spectroscopy, were positively correlated with pulp yield, reasoning that β -aryl ether linkages produced from S

unit dimerization are more reactive during alkaline pretreatment, thereby increasing pulp yield. S lignin also generates fewer cross linkages than G lignin resulting in a less branched lignin structure [32]. The impact of S/G ratios on alkaline pulping efficiency has also been well characterized in a number of *Populus* studies [50,51]. However, the reactivity of S lignin could be dependent on pretreatment, as Davison et al [14] reported that lower S/G ratios in *Populus* biomass promoted xylose release after dilute acid hydrolysis. Regardless of pretreatment, lignin composition is an important pulping trait and considering the overlap between pulp production and biofuel synthesis [52,53], it is reasonable to expect that lignin S/G ratios would also affect efficiency of biomass conversion. Indeed, assessment of structural biomass characteristics in *Eucalyptus* species found that a high S/G ratio promoted delignification during kraft pretreatment and resulted in more efficient enzyme hydrolysis [54].

An important parameter for efficient biofuel production is enzymatic accessibility to polysaccharides for hydrolysis [12,54]. Analysis of this data set revealed significant differences among CT, CCV and hybrid glucose and xylose yields from untreated at ages 9 and 12 years (Fig.3). At either age, CCV glucose release was lowest, despite similar lignin content among taxa and relatively high S/G ratio (as compared to CT). Analysis within each taxa found both glucose and xylose yields significantly decreased between ages 9 and 12 years (with exception to xylose yield from CT samples which remained constant over time). Considering that pulp yield positively correlates with density and diameter breast height measurements [55], both of which increase with age, it is expected that monosaccharide recovery should increase with tree age. However, tree harvesting for pulp manufacture occurs after wood fibres have matured which affords them desirable consumer traits such as mechanical strength and resistance to shearing and tearing stresses [56]. As such, wood maturation processes may result in decreased enzymatic saccharification of untreated biomass as polysaccharides become more cross-linked which decreases surface area and reduces access for enzymatic adsorption. However, aging can have a varied effect on wood composition with respect to polysaccharide content. Investigation of early harvest age of *Populus* and *Eucalyptus* wood from two rotation ages (1-3 years and 10-13 years) found conflicting results. Although *Eucalyptus* samples on longer rotations released lower amounts of glucose but higher amounts of xylose, poplar samples displayed the opposite trend [57]. Miranda and Pereira [58], analysing *E. globulus* trees, also reported variation in pulping yields between 2, 3 and 6 years of age. Measured glucose and xylan content varied significantly over time, with a drop in available monosaccharides occurring between ages 2 and 3, which then rebounded at age 6. These studies, in conjunction with our data, suggest that polysaccharide content is highly variable among hardwood species at different ages. After hydrothermal pretreatment, each *Corymbia*

taxa released similar amounts of glucose that was unaltered by aging. This was also true for xylose yield (with exception to significantly higher yield from hybrid biomass as compared to CCV). Interestingly, despite variations in G/X ratios within the untreated saccharification dataset, the G/X ratio from pretreated biomass were similar among taxa and at different ages, ranging between 2.2 and 2.8. This consistency could be attributed to relatively constant glucan and xylan abundance in the cell wall, and attributes that negatively impact saccharification such as cellulose crystallinity and cellulose-hemicellulose cross-linkages, which change with wood fiber maturation, are lost during pretreatment. This is especially true of the hemicellulose matrix that is deconstructed by hydrothermal pretreatment [5].

Surprisingly, neither Klason lignin content nor composition significantly influenced the amount of glucose or xylose obtained from untreated or hydrothermally pretreated wood samples. Although untreated glucose and xylose yields negatively correlate with the S/G ratios between ages 9 and 12, the linear regression of monosaccharide release as predicted by Klason lignin content (% mass fraction) and composition (S/G) was not significant. Pyrolysis of un-extracted biomass, where extractives and resins remain, while unlikely to influence determination of S/G ratios (as these are based on integration of specific pyMBMS peaks), can influence calculation of lignin content [31]. When extractives are present in biomass, pyrolysis of the extractive parent ions result in fragmentation that can fall into the aromatic region associated with lignin molecules. If this occurs, pyMBMS can result in overestimation of lignin content. While extractives are preferentially deposited into heartwood [59] and eucalypt bark is rich in extractives [60], only sapwood (representing the major component of *Corymbia* biomass at age 9 (75-82%) [61] was collected and analysed from these samples, along with the *Populus* control. Alternatively, lignin content may not have significantly affected saccharification due to lack of variation within the genus. Transgenic manipulation of lignin content to produce extreme values has shown the positive effect of lignin removal on saccharification yield [62], but to discover these effects within a natural population, Studer et al [32] selected 47 extreme lignin phenotypes from a screening pool of 1,100 undomesticated *Populus trichocarpa* trees. Extreme lignin phenotypes were subjected to enzymatic hydrolysis after hot water pretreatment at increasing temperatures (and an untreated control), finding that lignin content negatively correlated with sugar release with a stronger relationship when lignin S/G ratio was below 2.0. Alternatively, the negative effects of lignin on saccharification were negated when S/G ratios were >2.0. The average trait mean for *Corymbia* S/G lignin composition was not lower than 2.0, providing a possible explanation for the non-significant impact of lignin content on saccharification.

An interesting observation during analysis was the relationship of hybrid traits in relation to parental taxa. Normally within an F1 hybrid population, traits are intermediate to those of the parents [63], as is the case for a number of tree species, including poplars [64] and eucalypts [65]. Examination of 9-year-old biofuel traits finds that *Corymbia* hybrids are intermediate for traits: S/G composition, lignin content, untreated glucose yield, untreated xylose yield, untreated G/X ratio, pretreated xylose yield, and pretreated G/X ratios (Table 1). Transgressive traits, an important factor for the ecological adaptation of hybrids [66], are typically much less common. Although significant differences were not detected, the sole instance of hybrids outperforming both parents at age 9 was for measured glucose yield after hydrothermal pretreatment. At age 12 (Table 2), fewer hybrid traits were intermediate to the parental taxon (e.g. pretreated glucose and xylose yields) and maybe trending towards transgressive values, as evidenced by: Klason lignin content, untreated glucose yield, and pretreated G/X ratios. Unfortunately, without larger sample sizes, these trends cannot be fully investigated but suggest that differences in biomass composition of hybrids may not be immediately apparent and may become transgressive as hybrid trees mature. Eucalypts, including *Corymbia*, being relatively undomesticated and maintaining high levels of heterozygosity through outbreeding, have seen large gains from breeding and deployment of transgressive hybrids, particularly within the pulp and paper industry [67]. A shift from the selection of volume to the objective trait wood quality saw a rapid reduction (20%) in wood specific consumption (WSC) for pulp yield through deployment of *E. grandis* hybrids. Second generation crossing with *E. globulus* resulted in a further 20% reduction in WSC [68], and similar gains were reported for *E. globulus* hybridizations with *E. urophylla* [69]. Once our understanding of the complexity of biomass formation and its recalcitrance to deconstruction is advanced, the same rapid gains may be achieved for biofuel trait selection and hybrid deployment.

5. Conclusion

This dataset and analysis represents the first longitudinal characterization of *Corymbia* biomass for biofuel traits. Despite the close genetic relationship between parental species CT and CCV with their F1 hybrid offspring, significant differences were observed for industrially important biomass traits. Analysis of Klason lignin content, composition and enzymatic saccharification among *Corymbia* taxa at ages 9 and 12 years illustrates the inherent complexity of the formation of the lignocellulosic cell wall, demonstrating that genetics alone can be poor predictors of chemical traits, which are significantly influenced by age of material. Given the

impact of each biomass component on industrial processes, optimization of harvest age for lignocellulosic feedstocks will maximize pretreatment efficiency and biomass conversion for biofuels.

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Table 1 Trait summary of *Corymbia* taxa aged 9 years. Each species trait comparison was completed using a Kruskal-Wallis test (significant test statistic values are bolded), followed by a Mann-Whitney U Test. Significant differences found between species are illustrated by letter codes (a,b), where taxa that do not share letters are significantly different from one another with the lowest significant trait value also including a symbol to denote the level of significance.

9 Year old Trait	CCV Mean ±SD (σ)	CT Mean ±SD (σ)	Hybrid Mean ±SD (σ)	Kruskal-Wallis Test Result: p- value (χ^2 test statistic)
S/G Ratio	2.6 ± 0.1 (a)	2.2 ± 0.1 (*** b)	2.5 ± 0.2 (a)	3.4x10⁻⁴ (16.0)
Klason Lignin Content (% mass fraction)	25 ± 2	23 ± 2	25 ± 1	0.06 (5.5)
Untreated Glucose Yield (mg g⁻¹ biomass)	82 ± 26 (* b)	113 ± 24 (a)	108 ± 25 (a)	0.04 (6.38)
Untreated Xylose Yield (mg g⁻¹ biomass)	13 ± 3 (b)	9 ± 3 (** a)	10 ± 3 (ab)	0.02 (8.15)
Untreated Glucose:Xylose Yield Ratio	6 ± 2 (*** b)	14 ± 5 (a)	11 ± 3 (a)	2.0 x10⁻⁴ (16.81)
Pretreated Glucose Yield (mg g ⁻¹ biomass)	205 ± 78	286 ± 136	314 ± 114	0.1 (4.07)
Pretreated Xylose Yield (mg g⁻¹ biomass)	96 ± 10 (** a)	130 ± 51 (ab)	123 ± 24 (b)	0.03 (6.89)
Pretreated Glucose:Xylose Yield Ratio	2.6 ± 0.8	2.8 ± 0.8	2.7 ± 0.9	0.3 (2.30)

* p-value <0.05; ** p-value <0.01; *** p-value <0.001

CT-*Corymbia torelliana*; CCV- *Corymbia citriodora* subsp. *variegata*; Hybrid- Interspecific *Corymbia* hybrid (CT x CCV)

Table 2 Trait summary of *Corymbia* taxa aged 12 years. Each species trait comparison was completed using a Kruskal-Wallis test (significant test statistic values are bolded), followed by a Mann-Whitney U Test. Significant differences found between species are illustrated by letter codes (a,b), where taxa that do not share letters are significantly different from one another with the lowest significant trait value also including a symbol to denote the level of significance.

12 Year old Trait	CCV Mean ±SD (σ)	CT Mean ±SD (σ)	Hybrid Mean ±SD (σ)	Kruskal-Wallis Test Result: p- value (χ^2 test statistic)
S/G Ratio	2.3 ± 0.1 (b)	2.0 ± 0.0 (*** a)	2.3 ± 0.1 (b)	2.6x10⁻⁵ (21.1)
Klason Lignin Content (% mass fraction)	23 ± 3 (ab)	23 ± 1 (* a)	24 ± 1 (b)	0.03 (6.8)
Untreated Glucose Yield (mg g⁻¹ biomass)	56 ± 11 (*** a)	75 ± 25 (ab)	81 ± 13 (b)	0.0069 (9.95)
Untreated Xylose Yield (mg g ⁻¹ biomass)	9 ± 2	9 ± 3	8 ± 2	0.8 (0.26)
Untreated Glucose:Xylose Yield Ratio	7 ± 2 (*** a)	10 ± 6 (ab)	10 ± 3 (b)	0.02 (7.72)
Pretreated Glucose Yield (mg g ⁻¹ biomass)	270 ± 64	310 ± 63	284 ± 58	0.34 (2.14)
Pretreated Xylose Yield (mg g ⁻¹ biomass)	107 ± 16	115 ± 32	109 ± 31	0.80 (0.45)
Pretreated Glucose:Xylose Yield Ratio	2.2 ± 0.8	2.2 ± 0.4	2.6 ± 0.7	0.6 (0.82)

* p-value <0.05; ** p-value <0.01; *** p-value <0.001

CT-*Corymbia torelliana*; CCV- *Corymbia citriodora* subsp. *variegata*; Hybrid- Interspecific *Corymbia* hybrid (CT x CCV)

Figure 1

Figure 2

Figure 3

Figure 4

Figure Captions

Fig. 1. *Corymbia* syringyl/guaiacyl (S/G) lignin composition at ages 9 and 12 (years). S/G ratios were determined by pyrolysis molecular beam mass spectroscopy (pyMBMS). *Corymbia* taxa and age (years) are denoted on the x-axis. Black bars represent the median S/G ratio for each species and age, with the surrounding boxes representing the interquartile range. Non-overlapping notches suggest informally a significant (95% confidence) difference exists between median values. CCV- *Corymbia citriodora* subsp. *variegata*; CT- *Corymbia torelliana*; Hybrid- Interspecific *Corymbia* hybrid (CT x CCV)

Fig. 2. *Corymbia* Klason lignin content at ages 9 and 12 (years), expressed as percent mass fraction of biomass. Percentages were calculated from corrected values collected from pyrolysis molecular beam mass spectroscopy (pyMBMS). *Corymbia* taxa and age (years) are denoted on the x-axis. Black bars represent the median lignin content for each species and age, with the surrounding boxes representing the interquartile range. Non-overlapping notches suggest informally a significant (95% confidence) difference exists between median values. CCV- *Corymbia citriodora* subsp. *variegata*; CT- *Corymbia torelliana*; Hybrid- Interspecific *Corymbia* hybrid (CT x CCV)

Fig.3. Enzymatic saccharification results of untreated *Corymbia* biomass at ages 9 and 12 (years) for glucose (A), xylose (B) and glucose:xylose ratio (C). Sugar quantification was performed using high performance liquid chromatography. Glucose and xylose yields are expressed in mg of monosaccharide per g biomass. *Corymbia* taxa and age (years) are denoted on the x-axis. Black bars represent the median glucose release for each species and age, with the surrounding boxes representing the interquartile range. Non-overlapping notches suggest informally a significant (95% confidence) difference exists between median values. CCV- *Corymbia citriodora* subsp. *variegata*; CT- *Corymbia torelliana*; Hybrid- Interspecific *Corymbia* hybrid (CT x CCV)

Fig. 4. Enzymatic saccharification results of hydrothermally pretreated *Corymbia* biomass at ages 9 and 12 (years) for glucose (A), xylose (B) and glucose:xylose ratio (C). Sugar quantification was performed using high performance liquid chromatography. Glucose and xylose yields are expressed in mg of monosaccharide per g biomass. *Corymbia* taxa and age (years) are denoted on the x-axis. Black bars represent the median glucose release for each species and age, with the surrounding boxes representing the interquartile range. Non-overlapping notches suggest informally a significant (95% confidence) difference exists between median values. CCV- *Corymbia citriodora* subsp. *variegata*; CT- *Corymbia torelliana*; Hybrid- Interspecific *Corymbia* hybrid (CT x CCV)