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Variation of S/G Ratio and Lignin Content in a *Populus* Family Influences the Release of Xylose by Dilute Acid Hydrolysis

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Abstract

Wood samples from a second generation *Populus* cross were shown to have different lignin contents and S/G ratios (S: syringyl-like lignin structures; G: guaiacyl-like lignin structures). The lignin contents varied from 22.7% to 25.8% and the S/G ratio from 1.8 to 2.3. Selected samples spanning these ranges were hydrolyzed with dilute (1%) sulfuric acid to release fermentable sugars. The conditions were chosen for partial hydrolysis of the hemicellulosic fraction to maximize the expression of variation among samples. The results indicated that both lignin contents and S/G ratio significantly affected the yield of xylose. For example, the xylose yield of the 25.8% lignin and 2.3 S/G (high lignin, high S/G) sample produced 30% of the theoretical yield, whereas the xylose yield of the 22.7% lignin and 1.8 S/G (low lignin, low S/G) was 55% of the theoretical value. These results indicate that lignin content and composition among genetic variants within a single species can influence the hydrolyzability of the biomass.

Index Entries: Cell wall chemistry; genetic variation; hybrid poplar; hydrolysis; lignin.

Introduction

Production of fuels and chemicals from biomass crops is limited by the recalcitrance of lignocellulose to hydrolysis into its component pentose and hexose sugars. Two consecutive steps are generally involved in several proposed hydrolysis procedures (*1*). In the first step, dilute acid hydrolysis is used. In the second, the nondissolved solids then are subjected to an enzymatic hydrolysis. Dilute acid hydrolysis serves two purposes. The first one is to generate five-carbon sugars from the hemicellulose fraction; the second, to

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open the structure of the residual cellulose fraction to enhance enzymatic hydrolysis, which generates six-carbon sugars. The extent of dilute acid hydrolysis has strong influence on the efficiency of the overall process.

Plants have varying cell-wall compositions (2). This variation between species (i.e., hardwoods or softwoods) has an influence on their conversion into other feed streams such as paper pulp or sugars. Total lignin content is of interest, as well as the composition of the lignin. One measure of lignin composition is the guaiacyl (G) and syringyl (S) contents. These aromatic subunits determine the type and number of crosslinks. Guaiacyl units can covalently crosslink with up to three other units whereas syringyl units may link to only two (3). Vinzant et al. (4) showed that increased lignin content across 15 tree species decreased the total ethanol fermentation production via dilute acid hydrolysis followed by simultaneous saccharification by enzymes and fermentation (SSF). Chiang and Funaoka (5) showed that the S/G content between native hardwoods and softwoods correlated to their ease during Kraft delignification, with the S units facilitating cleavage of crosslinked bonds. The broad hypothesis is that variations in biomass composition can be controlled through genetic manipulation or breeding, and that these compositional changes will improve conversion of biomass. Conversion improvements can be from direct mass balance considerations (lower lignin may result in higher cellulose and leads to more fermentable hydrolysis sugars), as well as from more subtle composition influences, such as a shift in the S/G ratio. It may also allow milder pretreatment with production of a lower amount of inhibitory byproducts.

Transgenic and recombinant DNA methods have been used to modify lignin content and composition (6). This is achieved by up or down regulating the pathways that lead to the formation of S, G, and other subunits. Suppression of a key lignin pathway in a recombinant *Populus* clone resulted in 45% less lignin (7), faster growth (6), and improved delignification during Kraft pulping (8). Genetic engineering is also beginning to be considered as a means of changing the lignin composition of biomass feedstocks (2). Much of the current work focuses on pulping or hydrolysis differences among different species. Several recombinant plants have shown changes in lignin content and composition including *Populus* (2,9). Pilate et al. (10) indicate that genetically altered composition may not impact tree growth or fitness.

The experiments reported here were part of a larger project in which destructively sampled, clonally replicated field trials provided evidence that carbon allocation is genetically controlled (11). Map-based identification of separate stem and root cell wall chemistry quantitative trait loci supported the hypothesis that carbon partitioning is also genetically controlled. Here, we report on a hydrolysis study of selected members of the above-cited *Populus* family to confirm the hypothesis that lignin composition and lignin content influence hydrolysis sugars production.

Materials and Methods

Biomass Samples

Approximately 300 progeny from a single F_2 segregating hybrid poplar family, Family 331, were established in three clonal replicates at Wallula, WA, and allowed to grow over the course of one growing season in a common garden. Each clonal replicate was completely removed from the site and sampled for component biomass allocation. Subsamples from several stems were preserved and used for the hydrolysis tests. After bark removal, the remaining subsample mass averaged approx 3 g DW. Samples were finely ground in a Thomas–Wiley mill (Thomas Scientific, Swedesboro, NJ) and screened through a 20-mesh screen. Several additional subsamples were obtained from the parental clones (F_1) and were used for establishing and testing the experimental conditions.

Wood Chemistry Analysis

Previously, increment cores were removed from each genotype from pairwise stem and root tissues in an 8-yr-old clonal replicate of Family 331 grown in Clatskanie, OR. These wood samples were subjected to pyrolysis molecular beam mass spectroscopy (pyMBMS) for determination of cell wall lignin content and S/G ratio by the National Renewable Energy Laboratory *(12)*. Lignin content in the stem varied from 20.3 to 27.1% (by weight), with a mean of 24.9%. Root lignin content varied from 14.4 to 25.1%. The simple phenotypic correlation across all genotypes between stem lignin content and root lignin content was nonsignificant ($r = 0.24$). Similar results were obtained for S/G ratio. Stem S/G ratio varied from 1.6 to 2.5; root S/G ratio was lower and varied from 1.1 to 2. Again, the phenotypic correlation between stem and root S/G ratio was nonsignificant ($r = 0.21$). The theoretical xylose yield was calculated based on measured xylan contents from sample composition determined by pyMBMS. Xylan content varied between 15.5 and 18.8% in stems.

Dilute Acid Hydrolysis

Dilute acid hydrolysis was studied in stainless steel reactors. Five identical stainless steel reactors were constructed (316 stainless steel [SS], 1.8 cm ID, 1.9 mm OD, 12 cm length, 30 mL volume with Swagelock fittings). A thermocouple was inserted through one end of the reactor to monitor the temperature inside the biomass–acid mixture. A hot oil bath at 175°C was used to provide heating.

The tested biomass was ground to a uniform size (20-mesh sieve) and loaded into the reactor. A 1% (w/w) solution of sulfuric acid then was added to give a solid content of 1% (w/w). Sample size was 0.75 g.

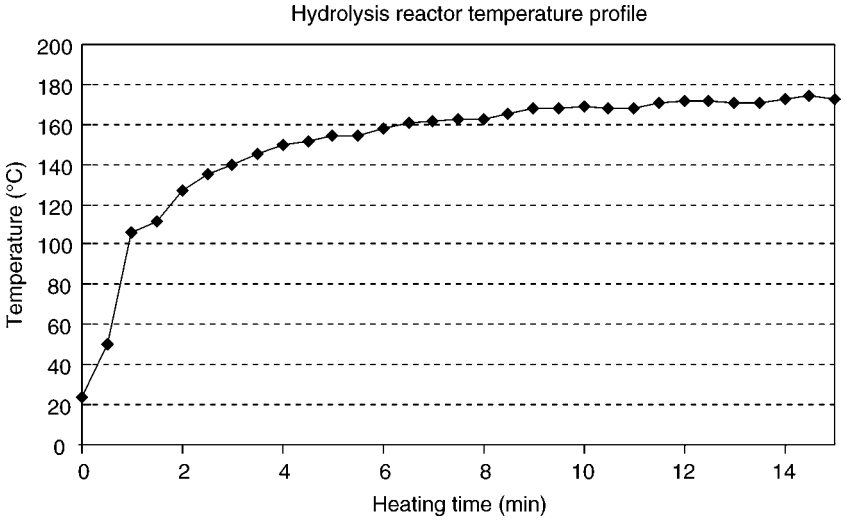

Fig. 1. Typical temperature profile for hydrolysis reactor used in a dilute acid hydrolysis experiment to test xylose yield from hybrid poplar stem samples with varying lignin content and S/G ratios.

The biomass was allowed to soak in this solution for 20 min. The reactors were placed in the preheated oil bath (175°C) for an empirically determined period (12 min). At the end of this period the reactors were quickly removed and placed in ice to quench the reaction. Figure 1 shows a typical temperature profile inside the reactors. The biomass and the liquid were removed from the reactors for analysis. The unreacted biomass solids were separated from the free liquid (larger samples were centrifuged; 0.75 g samples were squeezed dry). The solids were then dried at 90°C to determine the final residual mass and close the mass balance. The liquid samples were filtered and analyzed for xylose and other sugars by high-performance liquid chromatography (HPLC) (RMH Monosaccharide column (Phenomenex), 5 m*M* H_2SO_4 eluant; Waters 2410 RI detector, 10 µL sample). The total mass loss and the quantities of free sugars produced determined the extent of hydrolysis. Each experiment contained a single replicate, as it was not possible to subsample during the dilute acid treatment. Experiments were performed in triplicate.

Statistical Analysis

The raw data was analyzed using ANOVA and JMP statistical packages. To test the statistical significance of the data a simple linear model was used.The model took the form

$$X = a + b(\mathrm{L}\%) + c(\mathrm{S/G}) + d(\mathrm{L}\%)(\mathrm{S/G}) \quad (1)$$

where L% is the percent lignin in stem wood, and S/G is the syringyl to guaiacyl lignin ratio.

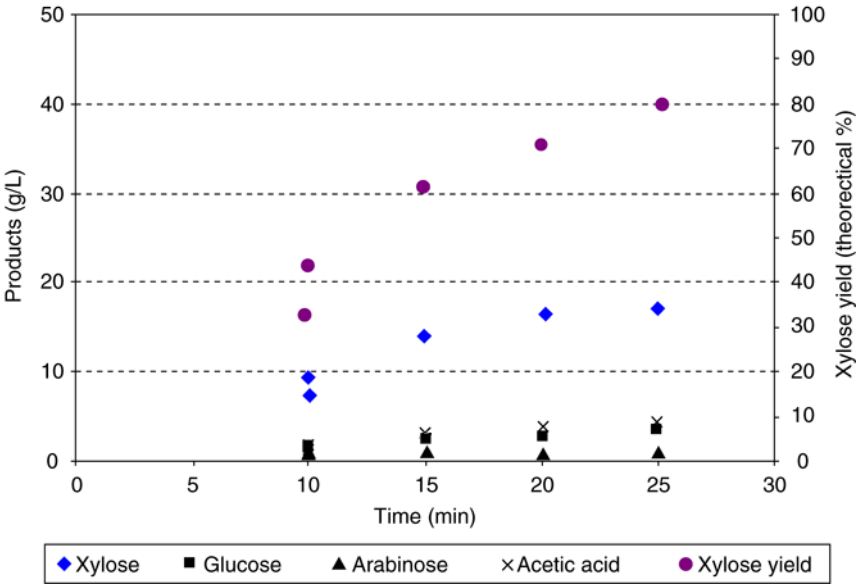

Fig. 2. Hydrolysis sugar production and xylose yield (as % of theoretical) with time for the test parental F_1 clone, 242.

Results and Discussion

Preliminary Experiments

The preliminary experiments to establish experimental conditions were performed on one of the F_1 parental clones (no. 242) in which there was sufficient biomass to use 1.5 g per test. Figure 2 shows these tests with the resulting sugar production and the calculated xylose yield at different hydrolysis times. The xylose yield was calculated using only the xylose produced and based on the total xylan content of the original specific sample. Because the objective was to study the effect of lignin composition on the efficiency of dilute acid hydrolysis, the experimental conditions were chosen for incomplete hydrolysis of the hemicellulosic fraction in order to maximize the expression of variation among samples. From this data we chose a hydrolysis time of 12 min for the comparative experiments.

Comparative Experiments

Dilute acid hydrolysis was performed on a subset of the F_2 *Populus* clones to determine the amount of fermentable sugars that are available from feedstocks of differing cell-wall composition. The selected samples spanning these ranges were hydrolyzed with dilute sulfuric acid (1% by weight) to release fermentable sugars (1% by-weight solids, 175°C maximum, 12-min hydrolysis time). The clones had been analyzed by pyMBMS for lignin content and for S/G ratio. A small subset (five) of the available clones was chosen across the distribution of these two values. The available samples were

Table 1
Variation in Cell-Wall Composition Among the Untreated Samples Used in the Dilute Acid Hydrolysis Experiments and the Resulting Xylose Yield (% theoretical) After Partial Hydrolysis

Sample No.	Factorial design	Lignin (%)	S/G ratio	Xylose yield (%)	Standard deviation
242	Center	24.6	1.9	44.5	0.057
1093	High–high	25.8	2.3	30.1	0.036
1640	High–low	24.8	1.8	39.5	0.056
1910	Low–high	22.7	2.1	28	0.043
1642	Low–low	22.7	1.8	54.9	0.01

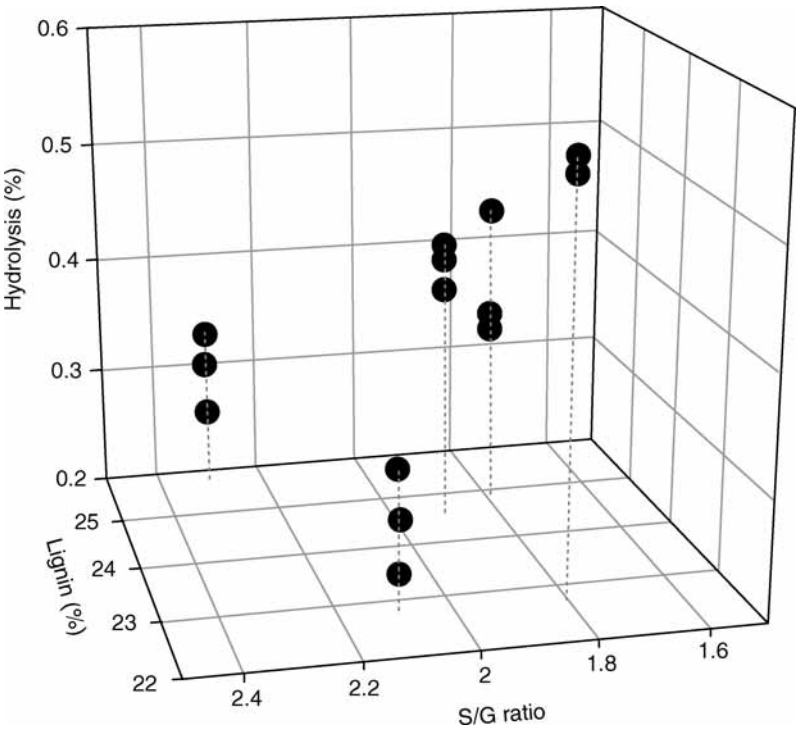

Fig. 3. Effect of lignin content and S/G ratio on xylose yields using a dilute acid hydrolysis. All data points are shown from triplicate runs.

small (most were <3 g) so the comparative hydrolysis experiments were downscaled to consume only 0.75 g of the milled wood chip per experiment, and were performed in triplicate on all five selected clones. Table 1 shows the compositions of the selected clones and the hydrolysis results. The change in hydrolysis results was greater than the replicate error of 5–10%. This was consistent with estimated measurement error of 9% at this small scale. The mass balance closure was greater than 90%. This data is also represented in Fig. 3. The data shows an effect of composition greater than the experimental

Table 2
ANOVA Analysis of Hydrolysis using Lignin% by S/G Ratio Factorial Experiment

Source	DF	Sum of squares	Mean square
Variance in factorial experiment F ratio = 19.87, $p > F$ is 0.0002			
Model	3	0.1065	0.03551
Error	10	0.01788	0.00179
C. Total	13	0.1244	
Lack of fit test F ratio = 3.098, $p > F$ is 0.112, max R^2 = 0.893			
Lack of fit	1	0.004578	0.00458
Pure error	9	0.0133	0.00148
Total error	10	0.01788	

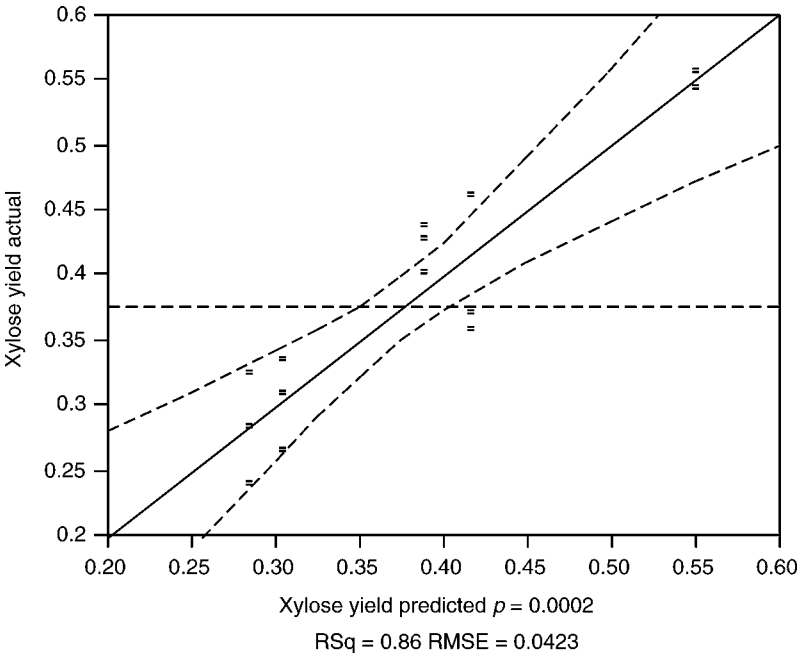

Fig. 4. Response plot of xylose yield–whole model actual vs predicted plot.

variability. The yield was highest at low–low combination of % lignin-S/G ratio and lowest at low–high combination of % lignin-S/G ratio.

Statistical Analysis

The ANOVA analysis presented in Table 2 indicates that the model is significant and that there is no "Lack of Fit" in the model. Figure 4 plots the actual vs predicted xylose yields with the 95% confidence limits. The increment of S/G ratio had a statistically significant negative impact on

xylose yield. The effect of lignin percentage alone is not significant, but the interaction effect of lignin percentage and S/G ratio is significant.

Despite the lack of statistical significance, the magnitude and direction of the lignin percentage effect (decreased xylose yield with increased lignin) is consistent for the mass balance effect. Here the small increase in lignin decreases the potential xylan and the potential xylose yield by a few percent. Vinzant et al. *(4)* showed a significant effect of lignin content on ethanol yield across a variety of hardwood species. This effect was much greater than could be accounted for by the decrease in theoretically available hydrolysis sugars because of increased lignin. This supports the concept that lignin content alone is not the potentially most important factor. The S/G effect and the interaction effect are more interesting and indicate the importance of crosslinking on the ease of degradation.

Conclusions

A small decrease in S/G ratio resulted in statistically significant improvement in the rate of dilute acid hydrolysis. The significance of lignin content alone was not as clear. More data points with higher precision will be needed for a definite conclusion. The combined effect of lignin contents and S/G ratio on the rate of dilute acid hydrolysis; however was significant. These results were obtained despite imperfect distribution of independent variables and significant measurement error because of small sample size.

Interestingly, these results were opposite from our initial intuition. A lower S/G ratio implies more potential for covalent crosslinking and thus would be expected to result in less hydrolysis, not more hydrolysis as seen here. In paper pulping studies, alkali methods for digestion of wood with higher S content gave higher pulp yields. In these poplar samples, acid hydrolysis may break the G bonds more readily. However, a similar effect has been observed in animal digestibility studies *(13)*. Here various forage maize were tested with different compositions and lower S content correlated with more milk and meat production. They speculated that less S residues gave a more crosslinked but thinner cell wall that was easier to degrade. Following these studies, Fontaine et al. *(14)* again showed lower S content in maize associated with improved degradability, and hypothesized that lower S content is typical of a less mature, less lignified cell wall that is more accessible to chemical or enzymatic penetration. However, Reddy et al. *(15)* showed a increased lignin decreased alfalfa digestibility in transgenic lines but that lignin composition did not have an effect.

These results show that natural variation in poplar, whereas relatively narrow, can have a measurable effect on dilute acid hydrolysis. To the best of our knowledge, these results have not been observed for clones within a single species. Taken together, these results imply that genetic engineering or traditional breeding to enhance hydrolysis for release of fermentable sugars would be successful in modifying the composition of biomass crops.

Carbon allocation and carbon partitioning among and within plant tissues ultimately determine the growth and utility of plant materials for various applications including biochemical conversion to fuels and bio-based products as well as options for carbon sequestration. We proposed that cell wall chemistry variations within a single family within a single species could be manipulated to impact potential biomass use. Further work is needed to test if these hydrolysis increases also occur in a subsequent cellulose hydrolysis step. The proof-of-principle hydrolysis experiment reported here demonstrated that changes in cell wall chemistry could significantly enhance the conversion efficiency of the derived biomass feedstocks.

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