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Genetically Engineered Poplar Wood Effectively Enhances the Efficiency of Deep Eutectic Solvent-Mediated One-Pot Processing

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Although lignocellulosic biomass is a renewable resource with the potential to replace fossil-derived fuels and chemicals, its recalcitrance, largely due to lignin, limits its utilization. Recent advancements in genetic engineering have produced transgenic trees with reduced lignin content and/or modified lignin structure without compromising growth traits. Here, three engineered poplar varieties are evaluated as feedstocks using a biocompatible one-pot deep eutectic solvent-mediated process that integrates biomass fractionation and enzymatic saccharification within a single reactor, eliminating water washing and reconditioning. All transgenic poplars exhibit higher fermentable sugar yields than wild-type (WT) trees. Notably, QsuB poplar, incorporating 3,4-dihydroxybenzoate in lignin, achieves the highest glucose conversion yield of 91.3% (vs. 73.0% from WT). AT5 and MdCHS3 poplars, incorporating ferulate esters and naringenin, also demonstrate improved glucose yields (86.7 and 84.7%, respectively), confirming reduced biomass recalcitrance. Additionally, residual lignins are valorized via hydrogenolysis into phenolic compounds, with comparable alkylphenol production across all lines. These findings demonstrate that the transgenic poplar lines not only serve as superior feedstocks for sugar conversion but also provide a rich resource for phenolic compound production, enhancing the operational and economic viability of integrated biorefinery processes.

1. Introduction

The average annual global temperature is rising, and extreme weather events are becoming more frequent, leading to reduced agricultural productivity and immense economic losses.[1] The excessive use of fossil-derived resources and the associated greenhouse gas emissions, which define the Anthropocene, are among the main drivers of the current climate crisis and provide a strong impetus to urgently secure alternative resources that mitigate greenhouse gas emissions, particularly carbon dioxide.[2] Lignocellulosic biomass, a natural composite composed largely of cellulose, hemicelluloses, and lignin in the cell walls of plants such as trees and grasses, inherently possesses carbon near-neutrality.[3] This renewable and sustainable resource is abundant, with an estimated 182 billion tons accruing annually on Earth.[4] If lignocellulosic biomass can effectively replace fossil-based fuels and chemicals, it could play a crucial role in addressing the current climate crisis.

Cellulose, hemicellulose, and lignin form individual polymers that are intricately associated with one another by chemical bonds and interactions in the cell wall of lignocellulosic biomass.[5] The inherent complexity of the plant cell wall structure not only hinders the efficient degradation of lignocellulosic biomass, but also lowers the selectivity of the resulting products following chemical treatment.[6] Although numerous chemical-based approaches have been developed to convert plant cell wall components into biofuels and biochemicals, most processes aim to recover and convert only the carbohydrate fractions.[7] Deep eutectic solvents (DESs) are environmentally benign and relatively inexpensive to produce. They have recently gained attention in overcoming the inherent challenges of deploying lignocellulosic biomass by effectively accessing both the carbohydrate and lignin fractions.[8] DESs are commonly prepared by mixing a hydrogen bond acceptor (HBA; e.g., choline chloride and betaine) and a hydrogen bond donor (HBD; e.g., amino acids, diols, and polyols), resulting in a

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mixture with a lower melting point than the individual components, resulting in a liquid phase at ambient temperature.[9] Among the various combinations of HBA and HBD, the mixture of choline chloride and glycerol (ChCl-Gly) has been shown to enable biomass fractionation and conversion in a single vessel, achieving high sugar recovery to produce both ethanol and lignin-derived phenolic compounds.[8] Given that existing technologies for degrading lignocellulosic biomass face challenges in isolating cellulose and lignin, DES treatment is expected to become a promising technique for enabling the recovery of individual polymeric fractions and the subsequent utilization of cell wall components derived from plants.

Lignin, which is primarily derived from three canonical precursors, *p*-coumaryl, coniferyl, and sinapyl alcohols, interacts with cellulose and hemicellulose, providing structural support, enabling long-distance water transportation, and protecting against external pathogens and pests.[10] Although lignin is essential for plant growth, it contributes significantly to the recalcitrance of lignocellulosic biomass toward processing, making it difficult to break down for industrial utilization.[3] However, like cellulose, lignin also has the potential to serve as an eco-friendly source of platform chemicals. Its inherent aromatic ring structure and high carbon content make it suitable for use as a drop-in feedstock in conventional fossil-based chemical industries.[3]

Recent research has focused on manipulating lignin biosynthetic pathways through genetic engineering, either by misregulating the activity of enzymes integral to lignin formation or by strategically engineering the production of alternative phenolic monomers compatible with lignin deposition. These approaches can ultimately produce lignins with desired composition or structural bonding patterns.[11] More specifically, introducing exogenous genes into plants/trees can lead to the accumulation of unique chemical moieties or the deliberate incorporation of ester bonds into the lignin structure.[12] These transgenic techniques enable the creation of lignocellulosic biomass with lignin that is easier to deconstruct chemically.[13] Moreover, evidence suggests that this strategy can help mitigate the recalcitrance of lignocellulosic biomass, enabling more efficient and economical use of all chemical components, and significantly enhancing the feasibility of biorefinery processes.[14]

Herein, three transgenic poplar lines were developed to redirect carbon flux away from the lignin biosynthetic pathway: MdCHS3 (chalcone synthase) introduction leads to the incorporation of naringenin, QsuB (3-dehydroshikimate dehydratase) introduction produces 3,4-dihydroxybenzoate as a pendent group on the lignin, and AT5 (feruloyl-CoA monolignol transferase) allows the incorporation of monolignol ferulates (ML–FA) into the polymeric lignin structure.[12b,c,14] A one-pot process integrating DES treatment and enzymatic hydrolysis was applied to these three transgenic poplar lines. This process fractionated the biomass prior to its conversion to fermentable sugars. Phenolic compounds were produced by hydrogenolysis of the residual solid; their yields were determined to assess the utility of the lignin fraction derived from the one-pot DES treatment. Finally, the yields of fermentable sugars and lignin-derived phenolic compounds from the one-pot process were analyzed in relation to the different lignin characteristics of the three transgenic poplar lines. While previous work demonstrated the potential of DES-based one-pot conversion using a single engineered poplar line (unique from the lines used in this study), the impact of diverse lignin structures on processability remained unexplored. Here, we systematically evaluate three transgenic poplars with distinct lignin modifications to assess how tailored lignin chemistry influences fractionation and sugar release. This work moves beyond proof-of-concept and demonstrates a generalizable strategy for integrating lignin engineering with scalable biomass processing.

2. Results

2.1. Sugar Conversion through the One-Pot Process

Simplifying the biomass conversion steps is crucial for reducing the cost and time required to produce energy and valuable chemicals from lignocellulosic feedstocks.[15] In this study, DES treatment and enzymatic hydrolysis were integrated into a one-pot process enabling the direct production of glucose and xylose without the need for the solids separation or washing steps that are typically required in conventional biomass conversion process; these not only add additional processing steps but also use a significant amount of water, which is a bottleneck to many pretreatment regimes.

DES treatment with the biocompatible ChCl-Gly was previously examined to evaluate the effect of ectopic gene expression in poplar on sugar conversion under varying temperature conditions.[8] The original study found that a reaction temperature of 180 °C was optimal for maximizing the release of fermentable sugars from poplar biomass. A milder temperature of 120 °C also resulted in a significant difference in fermentable sugar production from engineered poplar. The treatment conditions for the three transgenic lines were compared to wild-type (WT) trees grown in parallel under similar conditions.

Figure 1 shows the fermentable sugar yields from WT and the three transgenic poplar samples after a one-pot treatment with ChCl-Gly. As shown in Figure 1A, the transgenic poplar samples, regardless of the lignin modification, released more fermentable sugars than the WT when treated at 180 °C. The glucose conversion of the WT was 73.0%, whereas QsuB poplar exhibited the highest glucose yield at 91.3%, followed by AT5 poplar (86.7%) and MdCHS3 poplar (84.7%). Among the three transgenic poplar lines and the WT, QsuB poplar also had the lowest lignin content (**Table 1**). Considering that the glucan content in QsuB poplar was not substantially different from that of the other two transgenic poplars, the higher glucose release suggests that a lower lignin content plays a crucial role in enhancing glucose conversion efficiency.[16]

It is worth noting that the glucose conversion of the AT5 poplar was also significantly higher than that of the WT poplar (Figure 1A). Considering the marginal difference (1.7%) in total lignin content between AT5 and WT poplar (Table 1), the observed high glucose conversion in AT5 biomass cannot be solely attributed to differences in total lignin content. The introduction of unique ester linkages into the lignin is the likely explanation.

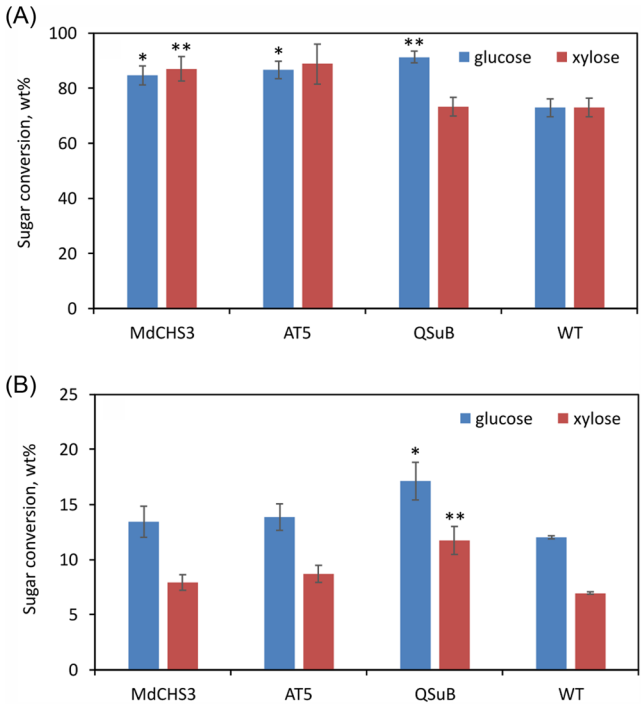

Figure 1. Sugar conversion ratio (%, based on the initial sugar content) of the WT and three transgenic poplar lines after enzymatic hydrolysis. One-pot DES pretreatment conditions: A) 180 °C for 6 h; and B) 120 °C for 3 h. Significance levels from WT: $^{**}p < 0.01$ and $^{*}p < 0.05$.

Table 1. Chemical composition of the WT and transgenic poplars.

	WT	QsuB[a)]	AT5[b)]	MdCHS3[c)]
Carbohydrate (%)				
Glucan	45.1 ± 0.5	45.5 ± 0.3	46.8 ± 0.4	46.7 ± 0.8
Xylan	18.3 ± 0.4	21.8 ± 0.1	20.3 ± 0.2	19.8 ± 0.6
Lignin (%)				
Acid-insoluble	16.7 ± 0.2	11.7 ± 0.2	16.1 ± 0.3	14.9 ± 0.3
Acid-soluble	3.7 ± 0.1	2.8 ± 0.2	2.7 ± 0.1	3.4 ± 0.1

[a)]Data taken from Unda et al.,[12c] (transgenic line 15). [b)]Data taken from Unda et al.,[14] (transgenic line 9). [c)]Data taken from Mahon et al.,[12b] (transgenic line 2).

In the case of MdCHS3 poplar, the results similarly deviate from the conventional understanding of the relationship between lignin content and glucose conversion. The total lignin content of MdCHS3 poplar was 18.3%, which is slightly lower than that of AT5 poplar (18.8%). However, when MdCHS3 poplar was used as a feedstock for the one-pot process, the glucose conversion was 84.7%, lower than that from AT5 poplar (86.7%). Typically, lower lignin content is associated with higher sugar conversion. Factors beyond total lignin content, such as lignin structure and composition, must, therefore, be influencing fractionation efficiency and enzymatic digestibility.

That transgenic poplars exhibited higher sugar release than WT when treated with ChCl-Gly at 180 °C for 6 h warrants further investigation into whether the strategic alteration of lignin also enhances conversion efficiency under milder conditions. To address this, the same WT and transgenic poplars were treated at a lower temperature (120 °C) for a shorter duration (3 h), followed by enzymatic hydrolysis. As shown in Figure 1B, the transgenic poplars demonstrated higher conversion efficiency for both glucose and xylose, reaching up to 42.5%.

Regardless of lignin structural alterations, the engineered poplar samples consistently yielded higher amounts of fermentable sugars than WT, implying that genetic modifications targeting lignin properties can significantly improve the efficiency of biomass conversion processes when using a DES pretreatment regime.

2.2. Characterization of Residual Lignins

The one-pot process resulted in a high conversion of polymeric cellulose and xylan to glucose and xylose following enzymatic hydrolysis. The solid lignin-rich residue was thoroughly characterized to better understand how the various lignin engineering approaches impacted pretreatment efficiency and to explore the possibility of lignin valorization.

The residual lignins were analyzed using 2D heteronuclear single-quantum coherence (HSQC) nuclear magnetic resonance (NMR) to examine the compositional and structural changes induced by alterations in the lignin biosynthetic pathway through gene expression and to identify differences compared to WT lignin induced by the one-pot DES treatment (**Figure 2**). In the aromatic region (Figure 2A), the WT-derived residual lignin showed typical correlations associated with syringyl, guaiacyl, and *p*-hydroxyphenyl units. The correlation peaks are, however, not entirely typical of those from native lignins, presumably because of reactions occurring in the DES system; a more complete interpretation is not possible at this stage. Distinct signals corresponding to *p*-hydroxybenzoate (*p*HB) groups were also observed in the spectrum of the WT residual lignin; lignin acylation by *p*-hydroxybenzoate is a well-established trait in poplar.[17] In the oxygenated-aliphatic region, as shown in Figure 2E, the HSQC NMR spectrum of residual lignin from WT exhibited the usual strong signals corresponding to the sidechain C–H pairs from β-aryl ether (β–O–4, **A**), phenylcoumaran (β–5, **B**), and resinol (β–β, **C**) substructures, indicating that native interunit linkages were preserved. Although the spectrum again deviated from those from native lignins, especially with the new peak labeled $\mathbf{A}^{*}_{\beta}$, that may be from β-ether units γ-acylated by, presumably, glycine in the DES system, an assessment of the relative levels of the key lignin units was gained from the resolved α-H/C peaks from each of those units.

For the HSQC NMR spectrum of the residual lignin stream recovered from MdCH3 poplar (Figure 2B,F), no distinct changes were observed in either the sidechain or aromatic units. A previous study has reported that the expression of MdCHS3 leads to the incorporation and accumulation of very low levels of naringenin into secondary cell wall lignins, as identified by HSQC NMR spectra.[12b] However, no signals corresponding to this flavonoid-derived compound were detected in the DES-treated residual solids, likely due to the cleavage of relatively weak lignin–naringenin linkages during the DES-mediated treatment.

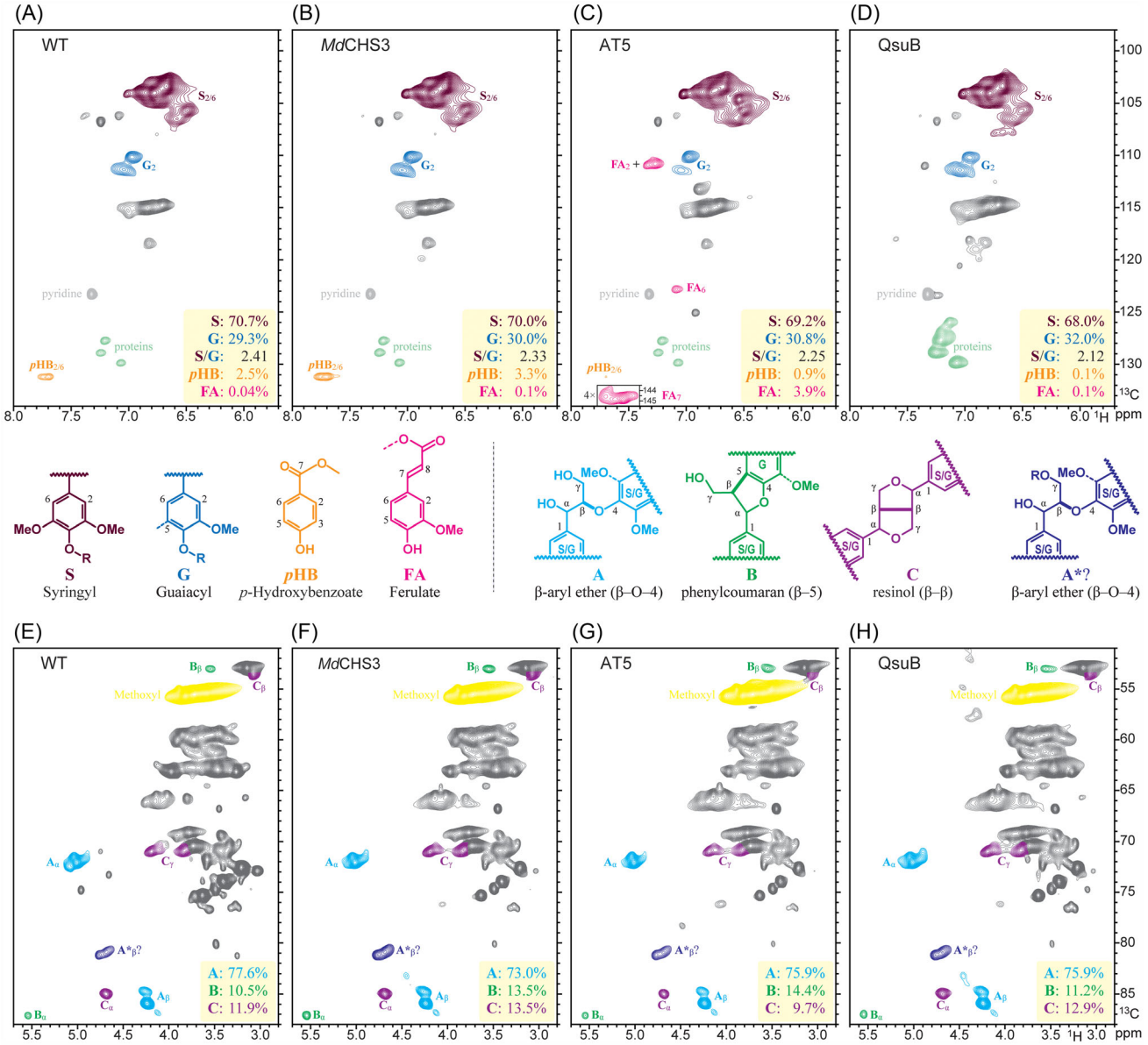

Figure 2. 2D HSQC NMR spectra of residual lignin after enzymatic hydrolysis from WT and transgenic poplars. A–D) Aromatic subregions. E–H) Oxygenated aliphatic subregions. Note that important peaks are colour-coded to the structures, but a full assignment of peaks was not possible at this stage due to uncharacterized reactions occurring during the DES treatment, including the possible formation of some acylated β-ethers **A***; the annotated **A***$_\beta$ peaks match those of guaiacyl γ-acetylated β-ethers, but the **A***$_\gamma$ peaks do not match; they may be from glycine esters. The BD units previously indicating the incorporation of (low levels of) dihydroxybenzoates[15] are not seen at the levels plotted in the NMR spectra presented here. An assessment of the relative levels of the key lignin units was gained from volume-integration of the clearly resolved peaks from each of those aromatic and aliphatic units, as provided in the highlighted tables in the bottom-right of each figure.

The HSQC NMR spectra of residual lignins from both the AT5 and QsuB poplar revealed that their distinct structural features remained following DES treatment. As shown in Figure 2C,G, the HSQC spectrum of AT5-derived lignin exhibited expected signals corresponding to ferulate groups **FA**$_2$ at δ_C/δ_H 111/7.3 ppm (unfortunately overlapped with another unknown component), **FA**$_6$ at δ_C/δ_H 123/7.1 ppm, and **FA**$_7$ at δ_C/δ_H 144/7.5 ppm) that are absent in WT lignin. These peaks represent ferulates linked as end-groups or incorporated via 4-*O*-etherification within the lignin polymer backbone. Given the chemical lability of ML–FA conjugates, lignins enriched in these units could be more amenable to depolymerization, making their valorization relatively easier compared to WT lignin. The strategic incorporation of ML–FA conjugates has indeed been shown to reduce biomass recalcitrance under several pretreatment regimes, including acid and base pretreatments,[18] ionic liquids,[19] copper alkaline peroxide,[20] as well as under full pulping conditions.[21] Although these conjugates typically require less energy to cleave, thereby facilitating lignin fractionation, they were not completely dissociated after DES treatment.

The residual lignin derived from the QsuB poplar clearly exhibited two distinct structural features compared to WT poplar lignin (Figure 2D,H). First, C–H signals corresponding to *p*HB groups were almost entirely absent in the HSQC NMR spectrum, as expected

based on the designed lignin structure. Signals associated with benzodioxane (**BD**) units that previously indicated the incorporation of (low levels of) dihydroxybenzoates[15] was evident at low levels (not shown) at δ_C/δ_H 75/5.0 ppm ($\mathbf{BD}_\alpha$) and 73/4.2 ppm ($\mathbf{BD}_\beta$) in the oxygenated-aliphatic region (Figure 2H). The strategic expression of bacterial 3-dehydroshikimate dehydratase gene (i.e., QsuB) led to the accumulation of **BD** units within the lignin structure, and these remained intact after DES treatment. Although further studies are needed to fully understand the implications of preserving these unique linkages, as with AT5-derived lignin, cell wall engineering presents an exciting opportunity to enhance both carbohydrate utilization and lignin valorization.

Gel-permeation chromatogram (GPC) was employed to analyze the molecular weight distribution of the residual lignin samples after DES-assisted one-pot conversion. The average molecular weights of the residual lignins from the various transgenic poplars were slightly higher than that of WT lignin (**Table 2** and **Figure 3**). Specifically, the average molecular weight of WT lignin was 4,983 Da, whereas that of AT5, MdCHS3, and QsuB lignin was 5,232, 5,520, and 5,730 Da, respectively. Although residual lignins recovered from transgenic poplars exhibited a slightly higher shoulder peak at around 10,000 Da, contributing to the increased average molecular weights, the similar central peaks and overall molecular weight distribution profiles suggest that lignin structural modification did not significantly impact the extent of lignin degradation during the DES treatment.

2.3. Hydrogenolysis of Residual Lignins

Lignin depolymerization is a key approach for lignin valorization, enabling the conversion of lignin macromolecules into renewable building-block chemicals. In this study, the residual lignins obtained from the one-pot conversion process were depolymerized into phenolic compounds via catalytic transfer hydrogenolysis. The resulting phenolic monomers were subsequently identified and quantified (**Figure 4**) by gas chromatography (GC) of the liquid products obtained from the hydrogenolysis of the residual lignins. Reductive depolymerization selectively produced short-chain alkylphenols that can serve as intermediates for the production of sustainable jet fuels and aromatic chemicals.

Quantitative analysis of the 10 identified monomeric phenols revealed that 2,6-dimethoxy-4-propylphenol (4-propylsyringol) was the most abundant phenolic compound, followed by 2,6-dimethoxy-4-ethylphenol (4-ethylsyringol) or 2-methoxy-4-propylphenol (4-propylguaiacol), depending on the feedstocks. The yields of monomeric phenols from transgenic poplar-derived lignins were slightly lower than those from WT poplar (**Table 3**).

Table 2. Molecular weight distribution of residual lignin from the WT and three transgenic poplar lines.

	WT	QsuB	AT5	MdCHS3
Mn (Da)	1,910	2,074	1,984	2,026
Mw (Da)	4,983	5,730	5,232	5,520
Poly dispersity index	2.6	2.8	2.6	2.7

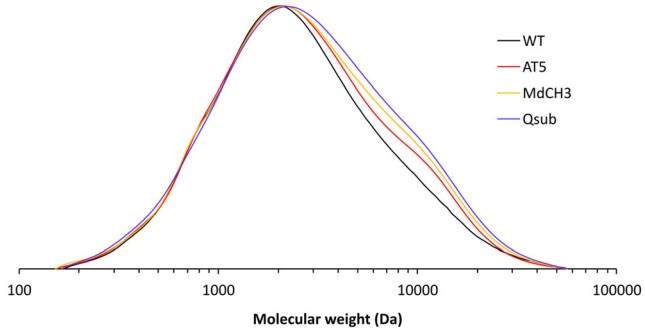

Figure 3. Gel-permeation chromatogram of residual lignin after enzymatic hydrolysis from WT and three transgenic poplar lines.

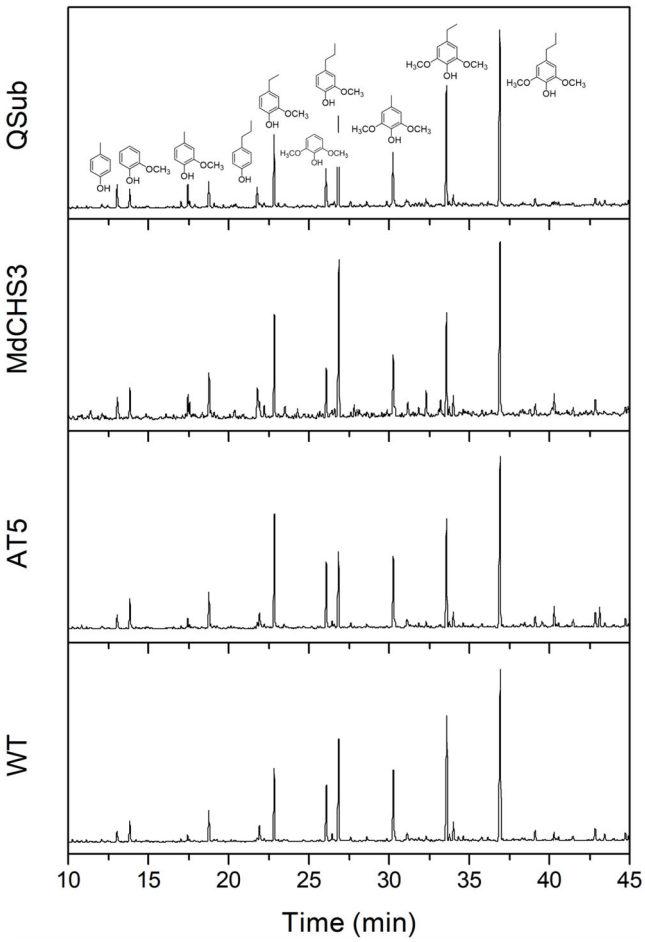

Figure 4. Gas chromatograms of phenolic compounds from hydrogenolysis of residual lignins.

Specifically, the total yield of alkylphenols from WT lignin was 62.5 mg g^{-1} lignin, whereas lignins from transgenic poplar yielded 51.9–55.5 mg g^{-1} lignin. This difference is likely due to variations in lignin composition among the transgenic poplar trees, structural incorporation, and accumulation of new units, which were cleaved during the DES-assisted conversion process. It is also noteworthy that structures enriched in ferulate esters or **BD** units could generate monomers with greater chemical utility or market value upon appropriate lignin depolymerization.

Table 3. Yields of phenolic compounds obtained from hydrogenolysis of residual lignins.

Compounds	Type[a)]	WT	QsuB	AT5	MdCH3
4-Methylphenol	H	1.6 ± 0.0	2.9 ± 0.2	1.5 ± 0.1	2.4 ± 0.1
4-Propylphenol	H	2.5 ± 0.1	2.2 ± 0.1	2.2 ± 0.3	4.1 ± 0.3
H total		4.1	5.8	3.7	6.5
Guaiacol	G	2.5 ± 0.0	2.2 ± 0.1	2.8 ± 0.1	2.6 ± 0.1
4-Methylguaiacol	G	3.3 ± 0.0	2.8 ± 0.1	3.0 ± 0.1	3.7 ± 0.3
4-Ethylguaiacol	G	7.6 ± 0.1	7.2 ± 0.3	9.9 ± 0.4	8.9 ± 0.6
4-Propylguaiacol	G	7.2 ± 0.0	6.1 ± 0.2	4.2 ± 0.2	8.9 ± 0.5
G total		20.6	18.4	19.9	24.1
Syringol	S	5.9 ± 0.0	4.3 ± 0.2	5.4 ± 0.2	4.1 ± 0.3
4-Methylsyringol	S	5.9 ± 0.0	4.7 ± 0.3	4.8 ± 0.2	3.8 ± 0.3
4-Ethylsyringol	S	10.3 ± 0.0	6.7 ± 0.2	5.9 ± 0.4	8.6 ± 0.2
4-Propylsyringol	S	15.7 ± 0.0	13.0 ± 0.2	11.4 ± 0.4	11.1 ± 0.8
S total		37.9	30.6	28.3	24.9
Total		62.6 ± 0.1	54.8 ± 1.7	51.9 ± 2.2	55.5 ± 3.6

[a)]H: hydroxyphenyl unit, G: guaiacyl unit, and S: syringyl unit.

An interesting observation from the distribution of hydrogenolysis products was the lower syringyl-to-guaiacyl (S/G) monomer ratio in residual lignins from transgenic poplars. As shown in Table 3, the S/G ratio of WT lignin was calculated to be 1.84, whereas QsuB, AT5, and MdCHS3 lignins exhibited 1.66, 1.42, and 1.03, respectively; a similar trend is seen in the NMR data even though the two measures are different, NMR profiles the entire lignin, whereas the degradation products derive only from cleavable units. Given that some transgenic poplars (e.g., MdCHS3 poplar) had similar S/G ratios to WT and others (e.g., QsuB poplar) exhibited even higher S/G ratios before processing, these results were somewhat unexpected. Although further investigation is necessary to fully elucidate how each lignin modification strategy affects degradation mechanisms, these results suggest that lignin depolymerization and product distribution can again be tailored through plant cell wall engineering technology.

3. Discussion

The biochemical conversion of lignocellulosic biomass is a promising technology as it enables the separation and recovery of cell wall components, offering a sustainable alternative to petroleum-based compounds in addressing the global climate crisis. Extensive research has focused on producing fermentable sugars from carbohydrates, the most abundant components in the plant cell wall.[22] However, conventional technologies involve a complex process that requires separating cellulose from the cell wall and hydrolyzing it into monomeric glucose.[23] Catalyst-based thermochemical methods are typically employed for cellulose separation and lignin removal, whereas biological processes using cellulolytic enzymes accomplish the breakdown of cellulose into glucose. However, conventional pretreatment approaches necessitate extensive washing and pH adjustment steps before the resulting material can be used as a substrate for enzymatic hydrolysis,[15] inevitably increasing processing costs and time.

The biocompatible DES-based one-pot process used in this study achieved cellulose conversion efficiencies of up to 85% without requiring sample transfers, intermediate washing steps, or additional recovery processes. Although this study was conducted at bench scale (10 wt% solid loadings), we anticipate that solids loading could be increased, although higher viscosity of DESs may introduce mass and heat transfer limitations that require process optimization or new reactor design. The remaining cell wall component, largely lignin, could be readily separated as nearly all fermentable sugars were dissolved into the solvent during the one-pot process. Given that a primary objective of the biorefinery concept is the complete recovery and utilization of all biomass components to enhance economic viability, the DES-based one-pot process presents a significant advancement in sustainable biomass conversion.

As biomass fractionation and conversion technologies continue to advance, such as with the development of the DES-based one-pot process, research is also focusing on engineering new biomass varieties that promote high sugar conversion under the same or milder processing conditions.[24] Gene-engineering-based approaches regulate the expression and enzyme activity involved in the biosynthesis of plant cell walls, enabling the fine adjustment of the content of specific components or structural modifications and alteration of interunit linkage patterns.[3] Although conventional transgenic techniques aimed at manipulating the traditional monomer supply have successfully achieved these objectives, they have often been associated with phenotypic drawbacks, such as reduced overall plant yield or poor cell wall development compared to WT plants.[25]

In this study, three diverse transgenic poplar lines showed no observable phenotypic augmentation during growth compared to WT plants, despite slight differences in cell wall chemical composition.[12b,c,14] This lack of growth penalty is highly desirable, as it demonstrates that cell wall modifications, such as the incorporation of ferulate esters or the addition of naringenin or dihydroxybenzoate units, do not compromise biomass productivity.

The ability to maintain high biomass yield while enhancing processability supports the viability of these transgenic lines as potential feedstocks for scalable and sustainable biorefinery applications.

All three lines demonstrated significantly improved glucose and xylose conversion efficiencies compared to WT when subjected to the one-pot process.

The presence of lignin has been identified as a major factor inhibiting cellulolytic enzyme activity during glucose production from biomass via biological processes.[16] Glucose conversion begins when cellulolytic enzymes bind to cellulose chains. Lignin, which typically surrounds these chains, acts as a physical barrier, preventing efficient enzyme access and limiting binding.[26] Additionally, irreversible adsorption between enzymes and lignin can occur, leading to excessive enzyme consumption beyond that required for complete cellulose hydrolysis.[27] In this study, QsuB poplar, which had the lowest lignin content, exhibited the highest glucose conversion efficiency in the one-pot process. AT5 and MdCHS3 poplars, with slightly lower lignin contents than WT, also achieved high glucose conversion, but not to the same extent as the QsuB lines. These findings suggest an inverse relationship between lignin content and glucose conversion efficiency during enzymatic hydrolysis.[28] Reducing lignin content through gene engineering without negatively impacting plant growth, therefore, represents a promising strategy for optimizing lignocellulosic biomass as a feedstock for efficient glucose production.

The transgenic poplar lines used in this study were designed to alter lignin bonding structures by regulating carbon flux within the biosynthetic pathways for cell wall formation. It was found that QsuB and AT5 lignins introduced new linkages into the lignin backbone, specifically BDs and ferulate esters, respectively.[12c,14] These substituted ether and ester linkages are known to be relatively susceptible to cleavage through milder reactions.[18] Lignins with altered linkages align with the concept of "zip-lignin", which facilitates efficient lignocellulosic biomass decomposition with minimal energy and catalyst consumption.[13]

In the case of MdCHS3 lignin, the target compound, naringenin, is presumed to have formed as a pendent group rather than altering the lignin backbone structure.[12b] Despite this, the MdCHS3 poplar exhibited glucose conversion efficiency comparable to that of AT5 poplar, which likely introduced modifications to both pendent units and the lignin backbone through ester linkages. Considering the relatively lower bond dissociation energy of oxygen-containing linkages, a higher proportion of ester or ether linkages in the lignin backbone can enhance lignin decomposition. These structural modifications, along with reduced lignin content, appear to be key factors influencing glucose conversion in the one-pot process used in this study.

Lignin depolymerization techniques, including hydrogenolysis, can mitigate some of the issues associated with lignin heterogeneity by breaking down its complex structures and dramatically reducing the molecular weight, enabling its conversion into eco-friendly platform chemicals.[8] In this study, the lignin-rich fraction was collected as a residue after the one-pot conversion process and used as a feedstock for lignin hydrogenolysis to produce alkylphenols. As revealed by HSQC NMR analysis, the ChCl-Gly DES treatment did not cause severe structural alterations to the lignin polymer, preserving native interunit linkages, which suggests that the recovered lignin streams can serve as a promising resource for various depolymerization approaches.

Recent advancements in biorefinery technology emphasize the efficient utilization of all biomass components while considering both process feasibility and economic viability. Beyond technical feasibility, economic viability is a key consideration for translating this strategy to industrial applications. The one-pot DES process offers potential cost advantages by eliminating intermediate washing steps, operating under mild conditions, and enabling dual valorization of carbohydrates and lignin. The use of inexpensive, biocompatible solvents and transgenic feedstocks with enhanced digestibility may also reduce both enzyme load and energy requirements. While a detailed techno-economic analysis was not conducted, this work provides a strong foundation for future assessments of process scalability and cost-effectiveness.

In this study, transgenic poplar trees demonstrated their potential as a promising biomass feedstock by achieving higher glucose and xylose conversion than WT poplar through the one-pot process, as well as producing value-added lignin-derived phenolic monomers via hydrogenolysis. Furthermore, the biocompatible DES-based one-pot process exhibited remarkable biomass conversion with a simple operational design. Although further optimization is needed to enhance the sustainability of this approach, the feedstock development and process-friendly technologies demonstrated in this work hold significant potential for future biorefinery.

One important factor influencing the long-term sustainability and economic viability of the DES-based one-pot process is the recyclability of the solvent system. Although this study did not experimentally evaluate solvent recovery, previous reports have shown that ChCl-Gly DES is relatively stable and can be reused for multiple cycles with minimal loss in performance.[29] Recovery of DESs can be achieved through strategies such as evaporation, membrane filtration, or antisolvent-induced precipitation. However, it is important to recognize that solvent reuse may be influenced by the buildup of biomass-derived impurities or structural alterations to the DES components, which could impact performance in subsequent runs. These factors warrant further investigation in future process development and techno-economic evaluations.

4. Conclusions

This study evaluated three recently developed transgenic poplar lines by comparing their performance against the corresponding WT poplar using an innovative DES-based one-pot conversion process. The genetically engineered trees exhibited slightly reduced lignin content and introduced novel types of units, leading to enhanced enzymatic digestibility. When subjected to the one-pot process, these transgenic lines achieved superior glucose and xylose conversion with fewer processing steps, improving the economic feasibility of monomeric sugar production. Additionally, the process facilitated efficient lignin recovery, enabling its further valorization into high-value phenolic monomers via hydrogenolysis. The results highlight the synergy between advanced feedstock development and innovative conversion technologies, demonstrating a promising approach for optimizing lignocellulosic biomass utilization. By integrating genetically modified biomass with an efficient one-pot

process, this study provides valuable insights for advancing sustainable and economically viable biorefinery strategies.

5. Experimental Section

Transgenic Poplars

Three transgenic poplar lines, along with WT, were cultivated at the University of British Columbia greenhouses, and grown for 6 months. The chemical compositions of the three transgenic lines and the associated WT trees are shown in Table 1. Detailed information on the development of these transgenic trees, including construct development, transformations, and growth conditions, has been described previously.[12]

One-Pot Conversion Process (DES Treatment and Enzymatic Hydrolysis)

The DES treatment and enzymatic hydrolysis in the one-pot conversion system were conducted following the methodology previously described.[8] In this study, ChCl-Gly was synthesized and used as the reaction medium for the one-pot conversion process. ChCl and Gly were mixed in a 1:2 molar ratio and heated to 80 °C with continuous stirring until a homogeneous, transparent liquid was formed.

For biomass conversion, 0.3 g of wood powder (sieved to 40 mesh) was mixed with 2.7 g of ChCl-Gly (10 wt% solid loading) in a 20 mL pressure tube (Ace Glass Inc., NJ, USA). The tube was then heated in an oil bath at the desired temperatures and times for 3 h at 120 °C or 6 h at 180 °C. Following treatment, the pretreated slurries were carefully transferred to a 15 mL capacity centrifuge tube, and 10 mL of 50 mM citrate buffer (pH 5.0) was added. Enzymatic hydrolysis was performed by adding cellulase cocktails (CTec3 and HTec, Novozyme, CA, USA) at a loading of 20 mg of protein per gram biomass, followed by incubation in a rotating hybridization oven at 50 °C for 72 h. After enzymatic hydrolysis, the residual lignins were separated from the hydrolysates and recovered for subsequent analyses and hydrogenolysis experiments.

Hydrogenolysis of Residual Lignin

Hydrogenolysis of the residual lignins was performed in a 50 mL batch reactor (Parr Instrument Company, IL, USA). The reaction was carried out using 200 mg of residual lignin and 20 mL of isopropyl alcohol, which were mixed in the reactor vessel in the presence of 20 mg of Ru/C catalyst. The reactor was purged with nitrogen gas and pressurized to 300 psi. The hydrogenolysis reaction was conducted at 300 °C for 1 h with continuous stirring at 300 rpm. To arrest the reaction, the reactor was rapidly cooled using an ice chamber. The solvent was then removed using a vacuum evaporator, and the final product was dissolved in 1 mL of acetone for phenolic compound analysis by GC.

Analytical Methods

After the one-pot conversion, glucose and xylose contents in the hydrolysate were determined using high-performance liquid chromatography (HPLC). Hydrolysate samples were recovered by centrifugation, followed by filtration through a 0.45 μm pore size filter. The analysis was performed using an HPLC (YL9100, Young-Lin, Seoul, Korea) equipped with a Bio-Rad Aminex HPX-87 H column (300 × 7.8 mm) and a refractive index detector. The mobile phase was a 4 mM sulfuric acid solution, with a constant flow rate of 0.6 mL min^{-1}, and the column was maintained at 60 °C.

The molecular weight distribution of the residual lignin from the one-pot conversion system was determined by GPC. Prior to GPC analysis, the residual lignin was acetylated using a 1:1 v/v pyridine-acetic anhydride mixture at 60 °C for 3 h. GPC analysis was conducted using a Shimadzu Prominence LC system (Shimadzu Corp., Kyoto, Japan) equipped with a Shim-pack GPC-804 column (300 × 8 mm) and a UV-VIS detector (wavelength: 254 nm). Tetrahydrofuran was used as the eluent at a flow rate of 1.0 mL min^{-1}, with the column and detector temperatures set to 40 °C. Molecular weight calibration was performed using polystyrene standards (Agilent Technologies, CA, USA) in the range of 162–364,000 g mol^{-1}.

The structural features of residual lignin were analyzed using 2D HSQC NMR spectroscopy. HSQC NMR spectra were acquired using an Avance-600 MHz spectrometer (Bruker Corporation, MA, USA) with the Bruker standard pulse sequence hsqcetgpsi2.2.[26] Detailed experimental conditions were similar to those previously described.[12b,c]

The monomeric phenols accumulating as hydrogenolysis products derived from the residual lignin were identified and quantified using an Agilent 7820A GC coupled with a 5975 mass spectrometric detector (Agilent, CA, USA). A capillary column (HP-5MS UI, Agilent, CA, USA) (30 m × 0.25 mm × 0.25 μm) was employed, with an injection temperature of 270 °C. The oven temperature program began at 70 °C (10 min hold), followed by a ramp to 300 °C at 3 °C min^{-1}, with a 5 min hold at 300 °C.

Statistical Analysis

Statistical analysis was performed using R software (R Foundation for Statistical Computing, Vienna, Austria) to test the null hypothesis regarding sugar yields among WT and transgenic poplars following DES treatment and enzymatic hydrolysis under identical conditions. In this study, the null hypothesis was rejected at a significance level of 0.05.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: biomasses · deep eutectic solvents · one-pot process · renewable resources · transgenic poplar trees

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