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CHANGES IN POPLAR DIGESTIBILITY DUE TO OVEREXPRESSION OF LACCASE GENES

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By

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Author Contribution Statement

Dr. Rebecca Garlock Ong and Dr. Chandrashekhar Joshi coordinated the overall project. Kavitha Satish Kumar performed the transgenic modifications of *Populus trichocarpa*. High-performance liquid chromatography for samples were performed by Sulihat Aloba. Fnu Arunima performed biomass compositional analysis, alkaline pretreatment, and enzymatic hydrolysis. Fnu Arunima wrote this report with input from Dr Rebecca Ong. Dr Rebecca Ong edited this report.

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List of Abbreviations

STTM- Short Tandem Target Mimic

LAC- Laccase enzyme

WT- Wild type (control)

POD-Peroxidase

SDX-Stem differentiating xylem.

HCHL- Hydroxycinnamoyl-CoA hydratase-lyase

MiRna-Micro ribonucleic acid

HTPH- high-throughput pretreatment and hydrolysis

Abstract

Woody biomass, particularly poplar, is a sought-after feedstock for lignocellulosic biofuels because of its year-round availability and high productivity. Because of cell wall components like lignin, poplar is inherently resistant to enzymatic saccharification. The experiment aims to determine whether the controlled overexpression of laccase genes alters the biomass composition and whether it can create poplar that is more digestible than the wildtype.

Three sets of transgenic poplar (*Populus tremula x alba)* samples were evaluated: laccase 18, laccase 27, and STTM. Laccase 18 samples, an target of miRNA397a was overexpressed along with Laccase 27. Short Tandem target Mimic (STTM) technology was adopted to block miRNA397a expression so it will not control laccase expression. Small scale compositional analysis was carried out on the transgenic and wild type poplar, consisting of water and ethanol extraction, followed by acid hydrolysis to measure the lignin and polysaccharide content. Alkaline pretreatment was carried at 3% solids loading and 10mL of 62.5 mM NaOH to make biomass more accessible to enzymatic hydrolysis. The samples were washed three times and dried overnight to determine the pretreatment mass loss. The pretreatment liquor was evaluated for glucose and xylose content using HPLC. Enzymatic hydrolysis was performed on the pretreated biomass at 3% solids loading to convert glucan and xylan to fermentable sugars. The set temperature was 50°C, and the stirring speed was 250 RPM. Samples were collected for HPLC analysis at 24 hours and 72 hours.

When the composition of the laccase 27, laccase 18, and STTM samples were examined, there were lines among all three transgenic modifications that had less lignin than the wild type (control) type samples. Following 72 hours of enzymatic hydrolysis, transgenic samples showed higher glucose release compared to wild type samples, indicating that the transgenic poplar is more digestible than the wild type.

1 Introduction

1.1 Motivation

The increasing demand for biofuels can be met with lignocellulosic biomass, which is a promising feedstock because of its high polysaccharide content that can be broken down to monomeric sugars for conversion by microorganism. Dedicated herbaceous and woody energy crops, such as switchgrass (*Panicum virgatum* L.) and hybrid poplar that are grown for biofuel production should have higher biomass yields and manageable growth. Compared to herbaceous biomass, woody biomass has a higher cellulose content and a higher density, and is available year-round (Tumuluru, 2020). Poplar is promising for biofuel production because of its extensive genetic diversity, available genome sequence, low nutrition demand, and high biomass yield (Bryant et al., 2020). The primary issue with woody biomass is its high lignin content, which makes it harder to convert to biofuels (Polo et al., 2020). One of the ways to tackle this issue is by using genetic modification to obtain plants with less or more readily-degradable lignin. Methods such as alkaline and acidic pretreatments can also be applied to alter lignin content in biomass and make glucose release easier (López et al., 2003; Ragauskas et al., 2014). The motive of pretreatment is making biomass more accessible for hydrolysis, and hydrolysis ensures that targeted polysaccharides like glucan and xylan are converted to fermentable sugars (Noor Idayu Nashiruddin, 2020).

The ultimate objective of this project is to determine whether the controlled overexpression of specific laccase genes (Laccase 27 and Laccase 18) and blocking of miRNA397a expression (STTM) changes the biomass composition and whether these changes produce more digestible poplar than the wildtype in terms of sugar release following enzymatic hydrolysis.

1.2 Components of Plant Cell Wall

The plant cell wall is a naturally occurring nanoscale network structure mostly consisting of glycoproteins, lignin, and polysaccharide polymers like cellulose, hemicellulose, and pectin (**Figure 1.1**). It primarily consists of cellulose arranged into a fibrous structure. The arrangement of cellulose fibers provides plants with high tensile strength and required rigidity (Bidhendi & Geitmann, 2016). Biomass contains crystalline and amorphous forms of cellulose. The majority of cellulose is made up of crystalline cellulose, whereas a tiny amount is made up of amorphous cellulose. The amorphous form of cellulose is more prone to enzymatic breakdown (Béguin & Aubert, 1994).

Hemicellulose is a class of polysachcarides present in nearly all terrestrial plant cell walls (Scheller & Ulvskov, 2010). Unlike cellulose, which is made entirely of glucose, hemicelluloses are made of various sugars, including glucose, mannose, and galactose

(Ebringerová et al., 2005). It interacts with cellulose and lignin through covalent and hydrogen bonds and strengthens the cell wall (Scheller & Ulvskov, 2010).

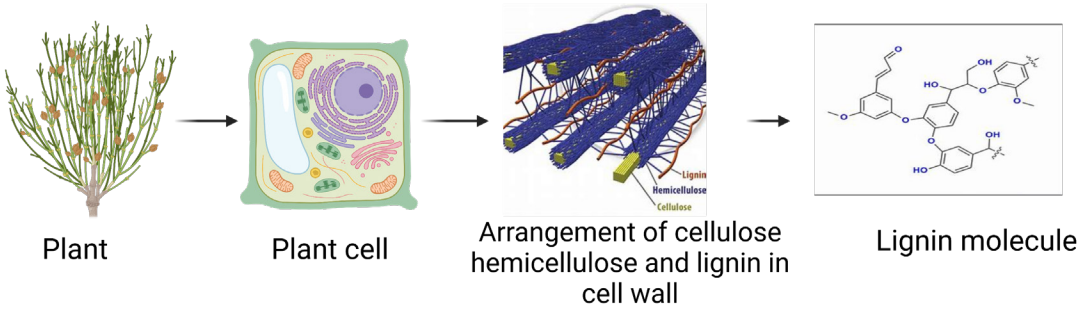


Figure 1.1: Illustration of the arrangement of cellulose, hemicellulose, and lignin in the plant cell wall.

1.2.1 Lignin

Lignification is a process in which lignin is deposited in the cell wall of plant tissues. Lignin is comprised of crosslinked phenolic monomer polymers, and is responsible for plant growth, strength, rigidity, structure, and impermeability (Pérez et al., 2002). Lignin is a highly heterogeneous polymer that is comprised of monolignols connected by various types of crosslinking. Para-coumaryl alcohol (H – *p*-hydroxyphenyl monolignol) (**Figure 1.2**), coniferyl alcohol (G – guaiacyl monolignol) (**Figure 1.2**); sinapyl alcohol (S – syringyl monolignol) (**Figure 1.2**) are the three main monomers present in lignin in terrestrial plants (Boerjan et al., 2003). Softwood plants contain only guaiacyl and *p*-hydroxyphenyl units and while hardwood plants contain all three subunits, providing more structured lignin with high recalcitrance (Bajpai, 2017). These three subunits of lignols are connected by ether linkages and condensed linkages, and the proportion of S, G, and H varies according to species (van der Pol et al., 2014).

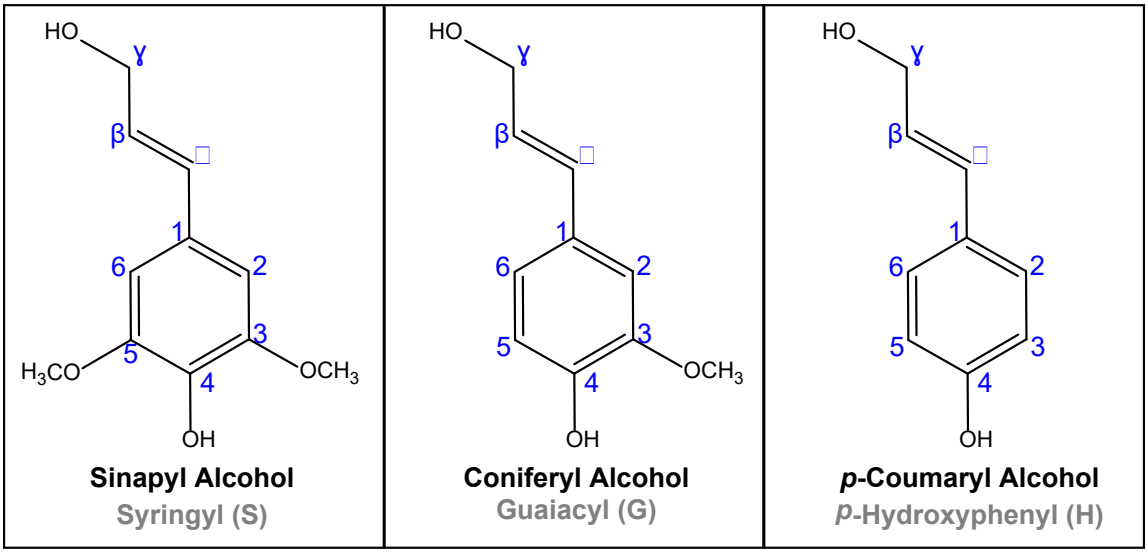


Figure 1.2: Illustration of the structure of p-coumaryl (monolignol), coniferyl alcohol (monolignol) and coniferyl alcohol (monolignol).

1.2.2 Lignin Biosynthesis and Polymerization

Lignin biosynthesis requires three steps: lignin monomer production, transport, and polymerization (**Figure 1.3**). The monolignols originate through the phenylpropanoid biosynthetic pathway. Their biosynthesis begins with the deamination of phenylalanine (or tyrosine), followed briefly by the sequential hydroxylation reactions of the aromatic ring, phenolic *o*-methylation, and reduction of the carboxylic acid group in the side chain to an aldehyde and alcohol by various enzymes (Boerjan et al., 2003). Lignin monomers are created in the cytoplasm and later transferred to the apoplast. In the cell wall, they are oxidized by peroxidase (POD) and laccase (LAC) to form radicals (Alejandro et al., 2012).These radicals polymerize to form substructures with limited types of linkages within the polymer (Ralph et al., 2004).

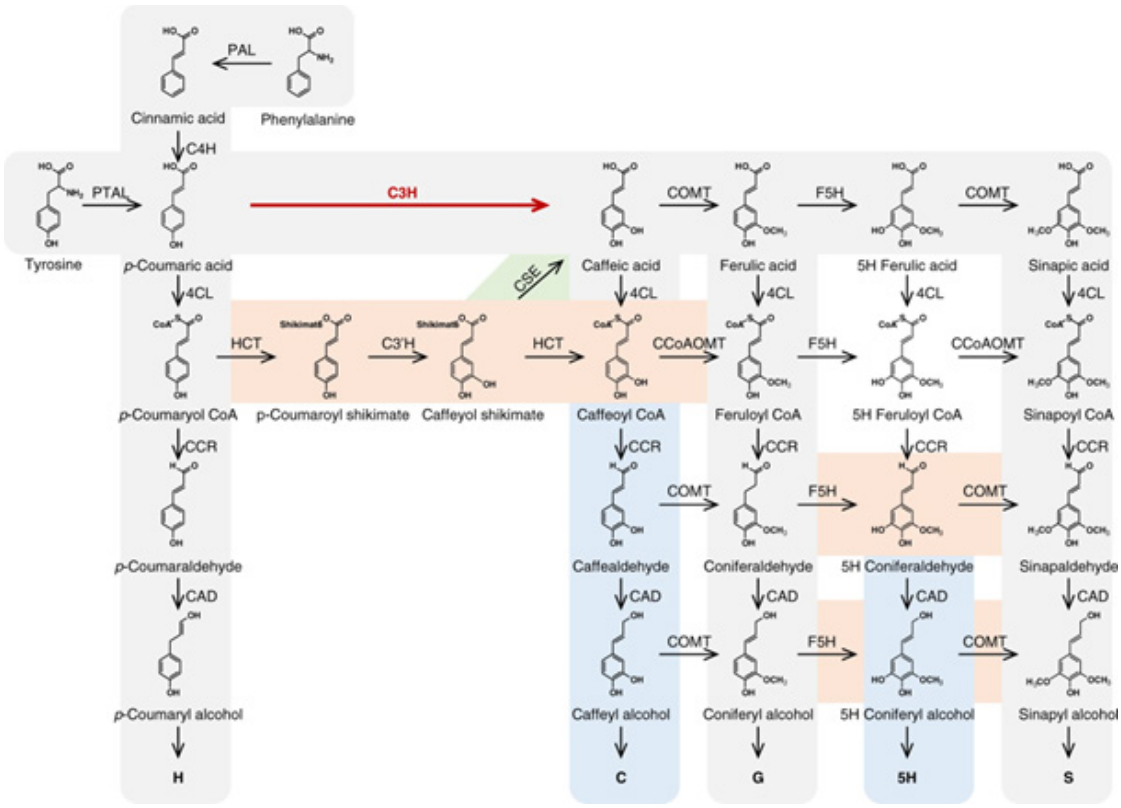
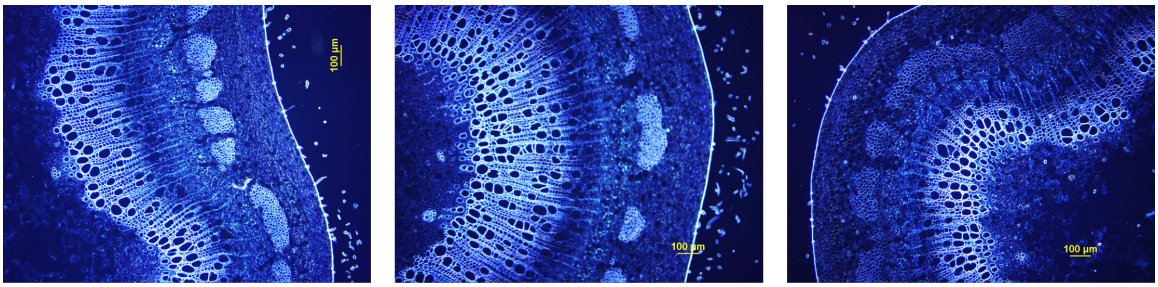


Figure 1.3: Lignin biosynthesis pathway (Barros et al., 2019).

Multi-copper oxidase enzymes called laccases catalyze the oxidation of several substances (phenolics and non-phenolics). The multigene family that encodes laccases is extensively distributed in plant genomes and has a function in oxidizing monolignols to create higher-order lignin, which is important for plant growth and stress responses (Liu et al., 2017). Research on maple sap indicated that laccases catalyze a reaction to polymerize monolignols in the absence of hydrogen peroxide (H_2O_2) (Driouich et al., 1992; Bao et al., 1993). Similar research conducted *in vitro* on suspension cells of sycamore maple, and the xylem of loblolly pine revealed that peroxidases and laccases work together to dehydrate lignin, which results in lignin heterogeneity in plant cell walls (Sterjiades et al., 1992; Bao et al., 1993).

Laccases can change the structure of xylem in poplar (**Figure 1.4)**, with differences depending on the laccase gene targeted. In samples with overexpression of two laccases, laccase 27 overexpression led to similar lignin autofluorescence but increased xylem area compared to the wildtype (**Figure 1.4 A, B**), while laccase 18 overexpression led to reduced lignin autofluorescence in the phloem and sclerenchyma and smaller xylem area in compared to the wildtype (**Figure 1.4 A, C**). Overall, the precise method by which laccases alter poplar lignin structure is complicated and not yet entirely known (Ravi et al., 2017).



(a) (b) (c)

Figure 1.4: Autofluorescence of lignin in poplar stem sections in a) WT b) Laccase 27 c) Laccase 18. Samples were taken from the tenth internode from apex (Kumar, 2017).

1.2.3 Lignin Transgenic Modification

Lignin is a complex polymer that gives plants structural support but also makes it challenging to decompose plant biomass for bioenergy production. As a result, altering plant lignin is a crucial field of research for bioenergy production. One approach to alter lignin structure involved expressing hydroxycinnamoyl-CoA hydratase-lyase (HCHL) isolated from *Pseudomonas fluorescens* in transgenic Arabidopsis. This resulted in an increase in lignin molecular weight and improved saccharification efficiency (Eudes et al., 2012). Downregulating the expression of genes involved in lignin production is another method for lowering the amount of lignin in plants. Several engineering techniques have been developed to lessen or alter lignin's monomeric structure. For instance, when bacterial 3-dehydroshikimate dehydratase (QsuB) was expressed in poplar trees, lignin decreased due to the metabolic flow being diverted away from lignin and toward 3,4-dihydroxybenzoate (Lin et al., 2022). Transgenic and wild-type (WT) poplar trees were cultivated in a different study, and their foliar mechanical and chemical resistance properties were measured. According to the findings there was a change in chemical resistance, but the physical structure remained the same despite low lignin content (Buhl et al., 2017). Another example of lignin modification is the cotton laccase gene (GaLAC1) overexpression in transgenic poplar plants using the cauliflower mosaic virus 35S promoter. Transgenic plants showed a 2.1–13.2-fold increase in laccase activity, while their growth rate and morphological characteristics remained the same (Wang et al., 2008).

Small, single-stranded, non-coding RNA molecules known as microRNA have 21-23 nucleotides (Green et al., 2016). MicroRNAs play an important role in post-transcriptional regulation of gene expression and RNA silencing (Bartel, 2018; Qureshi et al., 2014). MicroRNA targets 3’ untranslated regions of mRNA, leading to degradation. Primary miRNA (later becomes mature) is processed by the enzyme Drosha and forms precursor miRNA. This precursor miRNA reaches cytoplasm, gets processed by dicer, and mature miRNA is generated. Mature miRNA’ is attached to RNA-induced silencing complex, binds to target mRNA, and regulates expression (**Figure 1.5**) (Wu et al., 2018).

Recent studies have demonstrated that miRNAs can modify lignin by targeting genes involved in its manufacture or breakdown. For example, in *Arabidopsis thaliana*, according to a computational investigation, three distinct miRNA target genes encode copper-containing laccases (Abdel-Ghany & Pilon, 2008) and plants grown in different copper concentrations showed accumulation of miR397, miR408 and miR857. It was also revealed that there are other miRNA's that might play a role in copper regulation. In another study to explore the roles of ptr-miR397a and its targets, the laccase gene family was defined, and 49 laccase gene models were discovered, of which 29 were predicted to be targets of ptr-miR397a. They constructed transgenic *P. trichocarpa* that overexpressed Ptr-miR397a. In 9 transgenic lines tested, 17 laccase genes were downregulated, and transgenic lines that showed severe reduction also showed reduced laccase activity. Overexpression of miRNA397a in these transgenic lines also reduced lignin content, but monolignol biosynthetic gene transcripts remained the same (Lu et al., 2013).

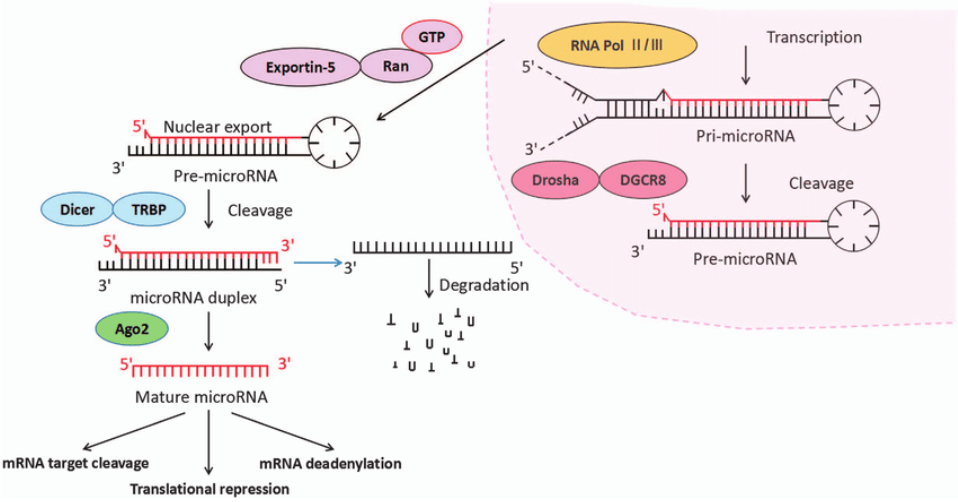


Figure 1.5: The pathway of miRNA regulation of gene expression (Wu et al., 2018)

A genetic technique called a short tandem target mimic (STTM) can also be used to block the expression of target genes. STTM functions by degrading the targeted gene RNA, which results in less protein production. For lignin modification, STTM has been applied to various plant species, including poplar. A study on Arabidopsis developed a short tandem target mimic (STTM), consisting of two brief sequences that mimic small RNA target sites and are separated by a linker. This method caused small RNA-degrading nucleases to cleave the targeted miRNAs. This miRNA blockade prevented it from controlling laccases (Yan et al., 2012). In a different work, STTM393 was developed (*Populus alba* × *Populus glandulosa*) with considerably lower miR393 expression. To suppress the expression of miR393, many genes involved in plant development and wood formation were overexpressed in STTM393 transgenic strains. These findings confirm that using the STTM technique to limit miR393 function can enhance plant growth and biomass production (Chu et al., 2021).

1.3 Overview of Pretreatment

Pretreatment is the process through which woody biomass can be broken down into their individual sugars. Pretreatment techniques work to reduce the plant material's lignocellulose structure and increase the accessibility of the sugars for subsequent enzymatic hydrolysis. Pretreatment modifies the structure of cellulosic biomass and makes it more accessible. Techniques used for pretreatment include ammonia fiber explosion, chemical treatment, biological treatment, and steam explosion (Hsu et al., 1980) (**Figure 1.6**). Cellulose can then be broken down into component sugars using acids or enzymes. Pretreatment aims to disrupt the cellulose structure and break down its lignin structure to provide more accessibility to enzymes for hydrolysis (**Figure 1.7**). Many chemical pretreatments benefit significantly from delignification. Delignification decreases lignin-derived inhibitors, such as phenolics, during the subsequent enzymatic hydrolysis and fermentation (De Assis et al., 2018).

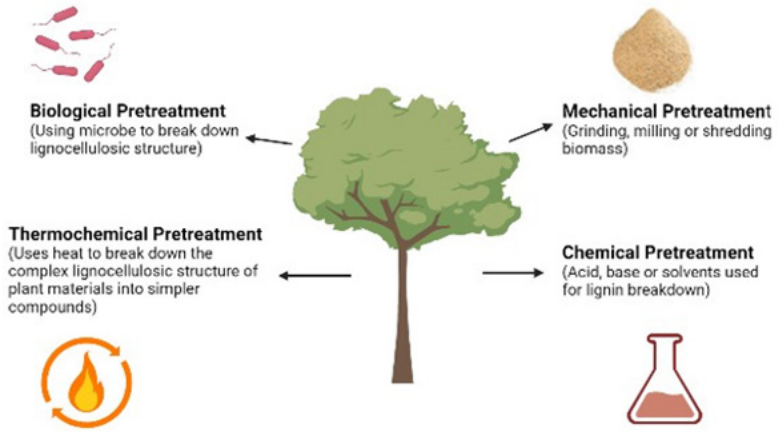


Figure 1.6: Overview of Pretreatment types.

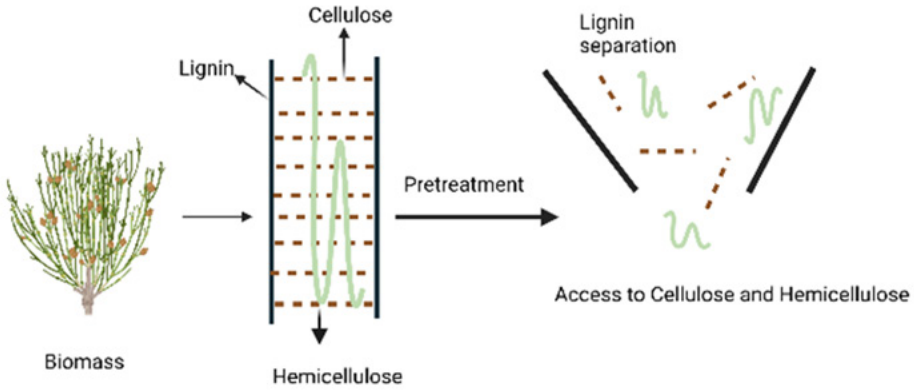


Figure 1.7: Illustration of pretreatment role (based on the figure in (Zahoor, 2014)).

1.3.1 Alkaline Pretreatment

Alkaline pretreatment is one of the most researched pretreatment methods for woody biomass. The woody biomass is soaked in an alkaline solution, usually sodium hydroxide or potassium hydroxide, at defined temperature and pH. The main aim of alkaline pretreatment is to increase the reactivity of polysaccharides during enzymatic hydrolysis by removing lignin from biomass. The pretreatment also can cause swelling of cell walls to make them more accessible for enzymatic degradation. The hypothesized reaction mechanism is degradation of intermolecular ester linkages that crosslink hemicellulose and lignin and degradation of β-O-4 ether linkages within the lignin polymer (Zheng et al., 2009, Kim et al., 2016) (Terrett & Dupree, 2019). Numerous studies have evaluated the use of alkaline pretreatment to improve enzymatic hydrolysis of poplar. For instance, alkaline post-incubation demonstrated a high promotion of the enzymatic hydrolysis yield and had a strong ability to deacetylate and delignify HPAA (Hydrogen Peroxide Pretreated Poplar)-pretreated poplar. Alkaline incubation decreased the HPAA loading and increased pretreatment safety (Wen et al., 2021). In another study, softwood pine and hardwood poplar were pretreated with alkaline NaOH and Na_2CO_3 before being converted into ethanol. For pine and poplar woods, respectively, the NaOH pretreatment at 93 °C produced the most significant increase in ethanol yields compared to untreated woods (Bay et al., 2020). Hardwoods with low lignin content became more digestible after NaOH pretreatment (Ravi et al., 2017). In spite of these benefits, alkaline pretreatment can carry drawbacks. It might produce inhibitors that prevent the growth of microorganisms employed in later stages of fermentation. Furthermore, the alkaline chemicals utilized in the pretreatment process can be dangerous and require proper handling and disposal techniques (Karimi et al., 2013). Alkaline pretreatment mainly works by disrupting the structure of lignin. Biomass exposure to alkaline solution for a longer period cause swelling of cellulose as alkali cleaves hydrolysable bonds of lignin. This change in cellulose structure leads to an increase in porosity, reduced crystallinity, and reduction in degree of polymerization. There are two reactions that occur during that time- saponification and salvation. Saponification breaks down ester bonds between fatty acids and glycerol in lipids while solvation breaks down lignin and hemicellulose structure. They both make the biomass more accessible to enzymes and removes unwanted substances from biomass (Chen et al., 2013; Kumar et al., 2020; Zhao et al., 2020).

Pretreatment ensures that lignin is reduced in biomass while maintaining cellulose and some hemicellulose in biomass. It is followed by enzymatic hydrolysis, that acts on cellulose and hemicellulose and converts them to fermentable sugars (Park & Kim, 2012). Alkaline pretreatment performed under mild conditions gives rise to less inhibitory by-products, these inhibitory products even in small amount can still interfere with total reducing sugar yield (Jönsson & Martín, 2016; Kim et al., 2016; Muhammad

Nauman et al., 2019; Park & Kim, 2012). The washing step is done to remove inhibitors that also contribute in the reduced sugar yield.

In a study conducted on alkaline pretreatment of poplar, the woods were pretreated at 8% NaOH and 0.5M sodium carbonate, and maximum hemicellulose was obtained at 95°C with an increase in glucan content in all samples (Bay et al., 2020). Alkaline pretreatment's sugar yield, solids recovery and lignin removal vary by factors like high solids loading, alkaline concentration, biomass type and temperature. These factors play a role in higher inhibitors and hydrolysate's (Modenbach, 2013).

1.3.2 Enzymatic Hydrolysis

Complex carbohydrates, like cellulose and hemicellulose, can be broken down into simpler sugars by a process called enzymatic hydrolysis. These simpler sugars can then be utilized to make biofuels **(Figure 1.8)** (Modenbach & Nokes, 2013). Enzymes commonly used are cellulases, used for cellulose breakdown and xylanases is used to break down hemicellulose. The enzymes are added to the biomass at proper pH and temperature to enhance enzyme activity (Guo et al., 2023). Although concentrated acids can also be used to break down polysaccharides, enzyme hydrolysis is more selective, has less harmful environmental effects, leads to better yields of the desired sugars, with fewer undesirable byproducts, and consumes less energy (Wang & Lü, 2021). Cellulases have been used to release sugars from various woods, including palm and poplar, and successfully increased the fermentation efficiency (Sathendra et al., 2019; Zhu et al., 2019). The cost of enzymes is the biggest obstacle to enzymatic hydrolysis (Klein-Marcuschamer et al., 2012).

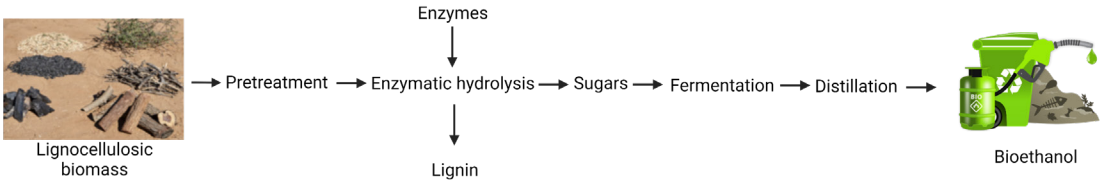


Figure 1.8: Overview of biofuel production using lignocellulosic biomass as feedstock.

1.4 Compositional Analysis of Biomass

Biomass compositional analysis is the process of determining the chemical composition of a biomass sample, typically done to understand its potential as a feedstock for bioenergy production or other applications. The chemical makeup of biomass can be divided into five main categories: cellulose, hemicellulose, lignin, extractives/volatiles,

and ash. The quality of the product and the optimal conversion technique are significantly influenced by biomass chemical characteristics (Williams et al., 2017). The analysis can be conducted using a variety of methods, including wet chemical analysis, spectrophotometry, gas chromatography, and mass spectrometry.

1.4.1 Laboratory Analytical Procedures

There are various wet chemistry procedures to determine biomass composition. The standard laboratory analytical procedure developed by the National Renewable Energy Laboratory (NREL) is divided into three parts: water extraction, ethanol extraction, and acid hydrolysis (Sluiter et al., 2008). Water extraction isolates and measures components of interest, such as sugars, amino acids, organic acids, and other water-soluble chemicals. Ethanol extraction removes waxes, chlorophyll, and lipids. Both extractions are necessary as a preparatory step to remove compounds that can interfere with the characterization of structural carbohydrates and lignin.

National Renewable Energy Laboratory (NREL) uses two equipment to run the water and ethanol extractions, the Soxhlet method and the Accelerated Solvent Extraction method. The Soxhlet method is not popular because of time constraints, high solvent consumption, low selectivity, high-temperature requirements, and loss of volatile compounds (López-Bascón & Luque de Castro, 2020). Rapid extraction of organic compounds from solid samples is also possible with the automated extraction technique known as Accelerated Solvent Extraction. The procedure typically lasts 15 to 45 minutes and can use less solvent than conventional techniques, lowering costs and environmental issues (Mottaleb & Sarker, 2012). Traditional wet chemistry procedures require higher volumes of biomass (300 mg), which is not always available, and can be labor- and time-intensive. Small-scale compositional analysis uses conventional wet chemistry techniques but is scaled down by a factor of 100 to use significantly less material and, in some cases, support a high-throughput pretreatment and hydrolysis (HTPH) screening system (DeMartini et al., 2011).

1.5 Materials

Ethanol (Pharmco-Aaper111000190), Glucose (Tekonova, G5802), Xylose (Sigma-Aldrich, W360600-1KG), Arabinose (Sigma-Aldrich, A3256-100G), Sulphuric acid(Millipore SX1244-5), Sodium azide (Sigma-Aldrich, S2002-100G), Sodium Hydroxide (Aqua Solutions, 221465-2.5KG), Sodium citrate dihydrate (Fisher-Scientific, S279-500), Citric acid monohydrate(Macron Fine Chemicals, 0627-12), Cellic Ctec2 (Novozymes, NS 22257), Hemicellulase (Novozymes, NS 2224). Transgenic and wildtype hybrid poplar 717 samples were obtained from the Department of Biological Sciences at Michigan Technological University (Kumar, 2017).

1.6 Methods

1.6.1 Sample Preparation

Populus tremula x alba clone 717-1B4 genotype (routinely referred as 717) was transformed to overexpress two laccase genes (PtrLAC27 and PtrLAC18), and suppression of miRNA397a using short tandem target mimic (STTM) following the method described previously (Kumar, 2017). Poplar stems were air-dried, debarked, and cut into approximately four to five pieces (with ten to fifteen internodes each. Using a FOSS Cyclotec Mill (Model 1093, FOSS North America, Eden Prairie, MN), the biomass was ground through a 1 mm screen and stored in a desiccator until needed.

1.6.2 Small Scale Compositional Analysis

1.6.2.1 Water and Ethanol Extraction

Before the studies began, the samples were then measured for moisture content using a moisture content analyzer (Sartorius Moisture Analyzer, MA35). Small press n' brew (2.75" x 2.75" p-2241) tea bags (Monterey Bay Herb Co.) were weighed for each triplicate sample, and 0.025 ± 0.0050 g of biomass was placed into each bag. The top third of the tea bag was folded over, and the biomass was dispersed as evenly as possible. The tea bag was then rolled into a jelly roll and secured with a 3-inch piece of 24 AWG tin-coated copper wire. Each jelly roll was inserted in a labeled Hungate vial (16 x 125 mm), which was sealed with a butyl stopper and phenolic cap and left at room temperatue until the next step in the process. Each Hungate vial was filled with distilled water (6 mL). The vials were recapped and positioned in an dry block heating mantle (ISOTEMP 125D-Fisher Scientific) preheated to 100 °C. After around 7 hours, the vials were taken out of the heating blocks and put in crushed ice. Once cooled, the liquids were decanted from the vials, and the volume was determined using a 25 mL serological pipet. The biomass was rinsed twice with 6 mL of distilled water, and the quantities were calculated using a 25 mL serological pipet. The collected water extracts were sterile filtered using 3 mL Luer-lock syringes fitted with 0.2 µm PES filters, into labeled autosampler vials. Samples were stored at -20 °C until later analysis.

Following the water extraction, without removing the jelly rolls, 6 mL of 190-proof ethanol was poured into each tube, and the tubes were then placed in a preheated 70 °C dry block heating mantle. All the tubes were removed from the heat after 16 to 18 hours and put on crushed ice. The ethanol extracts were decanted and discarded once they had cooled. The jelly rolls were then gently taken out of the tubes and unrolled, and the tea bags were spread out in metal pans and dried in an oven for 24 hours at 50-55°C (Precision Econotherm Oven, model 6530, Thermo Electron Corp). Samples were removed from the oven after 24 hours, left to cool in a desiccator, and weighed while

assuming 0% moisture. The samples were kept in anti-static bags until enzymatic hydrolysis.

Calculations for the determination of the biomass total extractives content are as follows:

$$m_{dry}(g) = m_{sample} \times (1 - MC)$$

$$Extractives(\%) = \frac{m_{dry} - (m_{final} - m_{tea})}{m_{dry}} \times 100\%$$

$m_{samples}$ = mass of samples added to tea bag (g)

m_{tea}= mass of tea bag (g)

m_{final}= mass of extracted biomass + tea bag (g)

MC= biomass moisture content- total weight basis (%)

1.6.2.2 Acid Hydrolysis

A 50-well tube rack (ZY-806011, MUHWA) was attached to a shaking incubator (VWR 980150), which was then preheated to 30 °C. The ground biomass samples (approximate 0.025g) were transferred in triplicate to Hungate vials after being weighed using weighing paper. Concentrated 72 percent H_2SO_4 (250 µl) was added to each Hungate vial after the biomass had been loaded. Glass stirrers were inserted into the Hungate vials, which were then shaken at 30 °C and 450 rpm for one hour. In the meantime, a liquid sugar recovery standard (SRS) made up of 2 g/L of glucose, 1 g/L of xylose, and 0.5 g/L of arabinose was created. Duplicate Hungate vials were filled with 6 ml SRS, 250 µL 72 wt% H_2SO_4, capped with a butyl stopper and phenolic cap, and then inverted multiple times to mix the contents thoroughly. Following the concentrated acid hydrolysis, the Hungate vials were taken out of the incubator and sealed with a butyl stopper and phenolic cap. All SRS and sample vials were and placed in an autoclave on a liquid cycle for 60 minutes at 121°C. Following completion of the cycle, the vials were taken out and cooled under running water.

Samples were then filtered using a custom filtration manifold. The manifold consisted of threaded (20-400 GPI) glass vacuum adapters (Chem Glass CG-1049-C-06) with an internal PTFE delivery tube (Chem Glass CG-1049-C-50). The top of the vacuum adapters were fitted with a 24/40 taper joint to a threaded (20-400 GPI) connector (KIMBLE® 747130-2024), connected to a glass filtering funnel assembly (KIMBLE® ULTRA-WARE® 953705-0000). Each glass vacuum adaptor was connected to a 40 mL vial to collect the filtrate. A manifold with attached ball valves was used to link six filtration units to a single vacuum pump.

Glass fiber filters (EMD Millipore, AP40, 25 mm) were pre-ashed at 575 °C for at least 3 hr in a muffle furnace (Type F6000 Furnace-Thermolyne corporation), stored in a desiccator until used, and weighed. The glass filtering funnel was placed on top of the adequately positioned filters, making sure that no filter edges were exposed. A spring clamp was used to secure the base and funnel. Hungate vials were swiftly emptied into the filtration funnel after being shaken to mix the contents for each sample. A 3 mL Luer-lock syringe was used to filter samples of the liquid through a 0.22 µm PES syringe filter and into an autosampler vial after the entire liquid had been drained. Samples were analyzed later by HPLC. A sample of the liquid was also collected for analysis of acid-soluble lignin content (Sluiter et al., 2008).

After collecting the liquid samples for analysis, the 40 mL vials were reconnected to the vacuum adaptor. The Hungate vials were then rinsed with distilled water, and the liquid was then passed to the filtration funnels to ensure no residual biomass remained inside the vials. The funnel interior and the cake of acid-insoluble lignin were rinsed using distilled water. The rinse water was discarded once all the biomass had been filtered. The filter papers were taken from the funnel's base, the filtration funnel was carefully unclamped, and any biomass adhered to it was scraped off with a spatula and put on the filter. The filter papers were dried for 24 hours in labeled metal mini muffin pans at 50–55 °C. The aluminum pans were taken out after some time had passed and left to cool in a desiccator. The filters and residue were weighed after cooling and then put into a muffle furnace at 575 °C for at least four hours. The samples were carefully taken out of the furnace after the specified amount of time, allowed to cool in a desiccator, and then weighed to ascertain the amount of ash in the residue.

Acid hydrolysis liquid samples were evaluated using HPLC. HPLC standards were prepared with three different concentrations for glucose, xylose, arabinose, cellobiose, acetate. The column used was Aminex HPX-87H column. Injection volume was kept at 10µL, flow rate was maintained at 0.6 mL/minute, column temperature was held at 50°C, run time was 20 minutes and the detector used was refractive index.

1.6.3 Alkaline Pretreatment Products Analysis

Biomass was weighed into 15 mL scintillation vials in duplicate at a solids loading of 0.03 g dry biomass per mL solvent (3 w/v%), followed by addition of 10 mL of 62.5 mM NaOH. The vials are put in a shaking incubator at 80 °C for 3 hr and then transferred to 15 mL centrifuge tubes. The samples were centrifuged at 4,000 RPM for 10 minutes using Eppendorf 5810R equipped with a swinging bucket rotor, and the supernatant was removed. Samples for HPLC were collected and passed through a 0.2µm PES syringe filter and transferred to autosampler vials and stored at -20 °C for later analysis by HPLC. The remaining samples in scintillation vials were washed with 10mL distilled water and any residual biomass was collected in 15mL centrifuged tubes. The samples were centrifuged at 12000 RPM for 10 minutes and supernatant was removed, samples were also collected for HPLC analysis after filtering with 0.2µm PES syringe. The

washing was done twice in total. The biomass was transferred to aluminum pans, dried overnight at room temperature, and weighed to determine mass loss during pretreatment. Alkaline pretreatment liquid samples were evaluated using HPLC. HPLC standards were prepared with three different concentrations for glucose and xylose. The column used was Aminex HPX-87H column. Injection volume was kept at 10µL, flow rate was maintained at 0.6 mL/minute, column temperature was held at 50°C, run time was 20 minutes and the detector used was refractive index.

1.6.4 Enzymatic Hydrolysis

For enzymatic hydrolysis, Nalgene racks were installed in a shaking incubator (Innova 42, New Brunswick Scientific) for enzymatic hydrolysis with the temperature set at 50°C and rotation at 250 RPM. Biomass samples were loaded in 20 mL scintillation vials at 3% solids (g dry biomass per mL). Stock sodium citrate buffer was prepared beforehand (1 M, pH 4.3 + 10 mM sodium azide) and 500 µL citrate buffer was added to each vial. The amount of cellulase (Ctec 3, Novozymes) and hemicellulose (Htech3, Novozymes) enzymes used were 52.5 µL and 22.5 µL. The vials were capped and added to the incubator to initiate the hydrolysis reaction. The reaction was allowed to run for 24 hr, at which point, a 600 µL liquid sample was collected, transferred to a microcentrifuge tube and placed in a 95°C heating block (Fisher Scientific Isotemp 125 D) for 10-15 minutes to denature the enzymes. The tubes were allowed to cool at 4°C for 10 minutes and then centrifuged at 13,000 RPM for 5 minutes in a microcentrifuge (Sorvall MC12). The supernatant was filtered using a 0.2 µm PES syringe filter and transferred to autosampler vials to be analyzed later. The liquid sampling process was repeated for the same vials after 72 hrs.

Enzymatic hydrolysis liquid samples were evaluated using HPLC. HPLC standards were prepared with three different concentrations for glucose and xylose. The column used was Aminex HPX-87H column. Injection volume was kept at 10µL, flow rate was maintained at 0.6 mL/minute, column temperature was held at 50°C, run time was 20 minutes and the detector used was refractive index.

The sugar yields were calculated based on the HPLC glucose and xylose concentrations as follows:

$$Glucose\ Conversion\ Yield\ (g/kg) = \frac{Glucose\ hydrolysis\ concentration\ (g/L)}{\text{solids loading (\%)}}$$

$$Xylose\ Conversion\ Yield\ (g/kg) = \frac{Xylose\ hydrolysis\ concentration\ (g/L)}{\text{solids loading (\%)}}$$

2 Results and Discussion

2.1 Compositional Analysis Products

2.1.1 Composition Analysis

Samples of hybrid poplar samples were chosen for each of the overexpressed stem differentiating xylem (SDX) laccases, LAC27, LAC18, and STTM: miR397a (Short tandem target mimic) samples **(Appendix 6.1)**. The debarked poplar samples were individually milled through a 1 mm screen and extracted with water and ethanol to remove water-soluble sugars, water-soluble protein, waxes, chlorophyll, and other substances that would interfere with characterization of structural carbohydrates and lignin (Sluiter et al., 2010). The wildtype samples contained ~5-10% less total extractives compared to the transgenic samples (**Table 3.1**). Often extractives in woody samples are associated with bark content, so it is not entirely clear why the debarked transgenic samples would have such a high extractives content compared to the wildtype.

The highest glucan content was reported for wild type samples (37.23%) followed by laccase 27#3 (35.57%). The lowest was reported for laccase 18#3 (26.51%). The highest XGM (Xylose, galactose, and mannose) was found in wild type samples (16.63%) followed by laccase 27#3 (15.45%), lowest was reported for laccase 18 #3 (12.14%). The highest extractives percentage was obtained for laccase 27#2 (15.03%) and lowest was obtained for wild type samples (4.91%). The highest acid soluble lignin was reported for laccase 18 #5 (2.59%) followed by wild type samples (2.57%). The lowest was reported for laccase 27 #4 (1.73%). The highest acid insoluble lignin was found in wild type samples (22.52%), followed by laccase 18 #4 (20.32%). The lowest was reported for laccase 27 #3 (15.18%).

Table 2.1: Small scale compositional analysis data of wild type and transgenic data. Values are reported as average ± standard deviation. In each row, values with different subscripts are statistically different based on Tukey's HSD Test (95% CI), ($p<0.05$) **(Appendix 6.2).**

	Wild type	**Sttm #2**	**Laccase 27#1**	**Laccase 27#2**	**Laccase 27#3**	**Laccase 27#4**	**Laccase 27#5**	**Laccase 18#1**	**Laccase 18#2**	**Laccase 18#3**	**Laccase 18#4**	**Laccase 18#5**	**Laccase 18#6**
Glucan	37.23 ± 3.25^{a}	31.63 ± 1.65^{def}	32.43 ± 1.20^{cdef}	29.27 ± 1.38^{fg}	35.57 ± 1.46^{bc}	31.72 ± 1.26^{cdef}	33.81 ± 0.78^{bcd}	32.61 ± 1.07^{cde}	28.95 ± 1.67^{efg}	26.51 ± 4.70^{g}	28.40 ± 4.3^{fg}	31.16 ± 1.81^{defg}	29.40 ± 0.30^{defg}
XGM	16.63 ± 0.57^{a}	14.43 ± 1.58^{abcde}	13.85 ± 0.59^{bcdef}	13.63 ± 1.23^{cdef}	15.45 ± 1.77^{abcd}	13.52 ± 0.83^{bcdef}	14.38 ± 0.04^{abcdef}	12.98 ± 0.52^{ef}	12.14 ± 1.40^{ef}	11.13 ± 2.14^{f}	12 ± 0.18^{ef}	12.7 ± 0.07^{def}	12.17 ± 0.04^{def}
Extractives	4.91 ± 2.23^{c}	13.7 ± 2.08^{ab}	14.79 ± 3.6^{ab}	15.03 ± 1.2^{a}	10.5 ± 2.13^{bcd}	12 ± 2.17^{abc}	13.03 ± 1.5^{abc}	10.2 ± 1.9^{bcd}	12.05 ± 2.3^{abc}	11.59 ± 3.4^{abc}	9.92 ± 1.4^{abc}	11.06 ± 2.13^{abcd}	12.86 ± 1.1^{abc}
ASL	2.57± 0.36^{a}	1.88 ± 0.52^{a}	2.06± 0.03^{a}	2.30 ± 0.04^{a}	2.30± 0.034^{a}	1.73± 0.29^{a}	2.14 ± 0.73^{a}	2.16± 0.39^{a}	2.55±0.06^{a}	2.11± 0.78^{a}	1.91± 0.48^{a}	2.59 ± 0.03^{a}	2.50 ± 0.03^{a}
AIL	22.52 ± 1.89^{a}	15.57 ± 2.34^{b}	18.16 ± 0.42^{ab}	17.23 ± 2.69^{ab}	15.18 ± 3.42^{b}	15.75 ± 2.2.1^{ab}	17.62 ± 2.30^{ab}	18.21 ± 2.22^{ab}	19.26 ± 0.82$_{ab}$	18.20 ± 3.22^{ab}	20.32 ± 2.74^{ab}	17.10 ± 4.11^{ab}	15.84 ± 1.95^{ab}

Arabinose, another component of hemicellulose, contributes to fermentable sugar content but in some cases, it can be deemed as undesirable. *Clostridium (Ruminiclostridium) thermocellum* is an anaerobic bacterium capable of high fermentation but it is unable to process hemicellulose like xylose and arabinose. This could lead to low yield and incomplete fermentation (Tafur Rangel et al., 2020).

Acetyl groups are present in proteins, carbohydrates and lipids. It is capable of decreasing sugar yield as well as acting as an inhibitor for microbes responsible for fermentation (Helle et al., 2003; Selig et al., 2009). It can affect cellulose hydrolysis by changing the dimension of cellulose chain and making it difficult to bind to enzymes (Pan et al., 2006).

Another key component is lignin, it is made of phenylpropanoid units. It poses as a barrier in bioethanol production because of its inability to produce monosaccharides during hydrolysis (Yoon et al., 2014). There has been work done in which changes in lignin structure was observed but not in lignin content. In research conducted on hybrid poplar overexpressing Oryza sativa protein, it was observed that no significant lignin content change was observed but a change in S/G ratio was revealed. They concluded that it could have been because of unbalanced induction of lignin biosynthetic genes in transgenic lines (Nuoendagula et al., 2018). In another experiment conducted on

temperature effect on poplar and transgenic poplar, it was observed that three days of high temperature lead to change in lignin content as well (Zhao et al., 2022). In an experiment conducted on (*Populus nigra L.* × *Populus maximowiczii A.*) compositional changes were analyzed. Enzymatic hydrolysis yield for transgenic samples were much higher than the wild type but in field the values between two types were closer. The lignin content an xylan values also altered for transgenic samples indicating that maybe transgenic plants were less recalcitrant (Xiang et al., 2017).

2.2 Alkaline Pretreatment

The poplar samples were deconstructed using dilute alkaline pretreatment (62.5 mM NaOH) at 3% solids loading to increase the enzymatic digestibility of the solids in the following step. The highest solids recovery was obtained for laccase 18#6 (87.8%) and the lowest solids recovery was obtained for laccase 18#5 (63%) **(Figure 3.1)**. Solids recovery determines the efficiency of the process after pretreatment. Higher solids recovery means higher yield and low production costs.

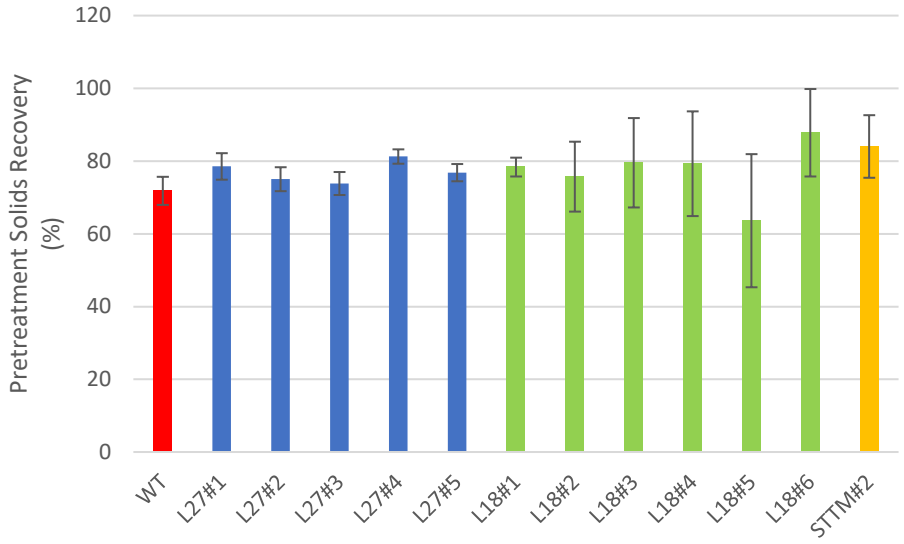


Figure 2.1: Solids recovery of wild type and transgenic samples after alkaline pretreatment. Alkaline pretreatment (62.5 mM NaOH) was conducted at 80 °C for 3 hr. Error bars represent the average glucose yield in the pretreatment liquid ± standard deviation.

The highest glucose pretreatment yield was obtained for laccase 18 #6 (0.036 g glucose/g dry biomass) and the lowest was obtained for laccase 27 #2 (0.0122 g glucose/ g dry biomass) **(Figure 3.2) (Appendix 6.2)**. The highest glucose yield aligns with highest

solids recovery indicating a possibility of laccase 18 #6 having the highest yield after enzymatic hydrolysis.

For XGM (Xylose, galactose and mannose) yield the highest value obtained was for laccase 27 #1 (0.48 g XGM/g dry biomass) and lowest was for laccase 18 #1 (0.19 g XGM/g dry biomass) **(Figure 3.3)**.

Tukey's analysis revealed that many samples did not show a similarity with wild type indicating difference in means. This could indicate a difference in expression and genetic change in samples. Laccase 18 samples showed the most difference when compared to wild type. It could have been because of the overexpression of miRNA397a and laccase 18 being the target laccase gene of the mentioned mRNA. Compositional analysis revealed that the highest glucan content and XGM was obtained for wild type, but it also had the highest acid insoluble content. The plausible explanation for less pretreatment yield (0.16 g glucose/g dry biomass) (0.28 g XGM/g dry biomass) could be because of high lignin content (22%) and low extractives content (4.91%).

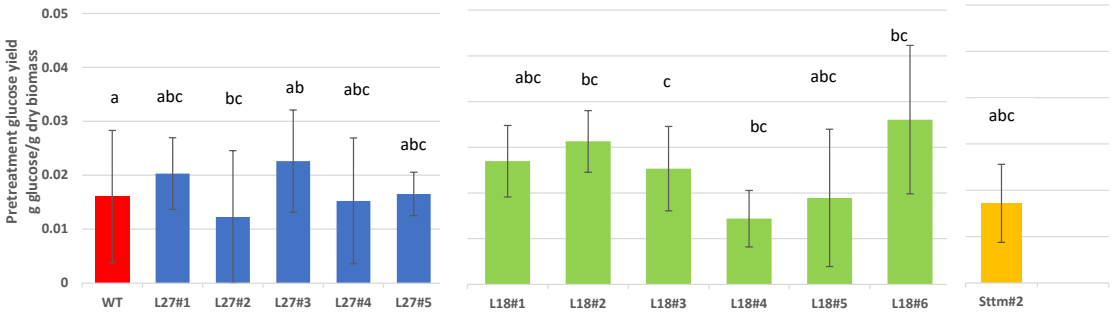


Figure 2.2: Pretreatment glucose yield of wild type and transgenic samples. Alkaline pretreatment (62.5 mM NaOH) was conducted at 80 °C for 3 hr. Error bars represent the average glucose yield in the pretreatment liquid ± standard deviation. Statistical analysis revealed all means are different ($p>0.05$) using ANOVA and Tukey's HSD.

The statistical analysis of xylose yield revealed **(Figure 3.3)** ($p<0.05$) **(Appendix 6.2)** that there is difference between the mean of sample types. This aligns with glucose yield after pretreatment. The actual difference between sample types and their digestibility could be established by enzymatic hydrolysis.

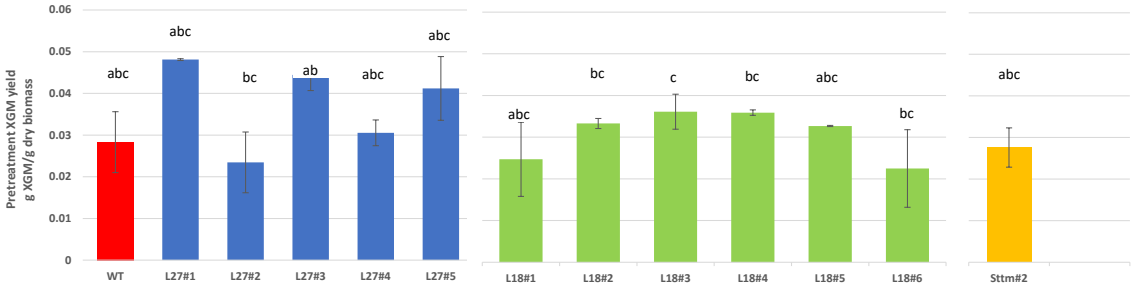


Figure 2.3: Pretreatment xylose yield of wild type and transgenic samples. Alkaline pretreatment (62.5 mM NaOH) was conducted at 80 °C for 3 hr. Error bars represent the average glucose yield in the pretreatment liquid ± standard deviation. Statistical analysis revealed all means are different ($p<0.05$) using ANOVA and Tukey's HSD.

2.3 Enzymatic Hydrolysis

Following pretreatment, washed samples were hydrolyzed at 3% solids loading using 30 mg enzyme (cellulase and hemicellulase) per g solids. Enzymatic hydrolysis works by converting sugars for fermentation. In this polysaccharides are converted to monosaccharides and later used for fermentation by microbes (Modenbach, 2013). The highest overall glucose yield was reported for laccase 27 #5 (108g glucose/g dry biomass) and highest XGM yield was obtained for STTM #2 (123.8 g XGM/g dry biomass).

Laccase 18 is one of the target genes for miRNA37a and miRNA397a has been overexpressed to observe change in lignin content and to confirm if overexpressing a miRNA397a could alter lignin content and confirm that miRNA's control laccase gene expression (Lu et al., 2013). No change in lignin content was observed during analysis of gene manipulation but there was a decrease in S/G ratio in laccase 18 when compared to wild type (Kumar, 2017). Guaiacyl (G) lignin is more rigid than syringyl (S) lignin and is harder to break to increase accessibility for enzymes. Decrease in S/G ratio might lead to higher glucose yield after alkaline pretreatment but there has been evidence of variability in correlation between S/G and lignin in *Populus trichocarpa* (Anderson et al., 2019). This increase of around 7% in laccase 18 glucose yield compared to wild type at 24 hrs. and 72 hrs. **(Figure 3.4) (Figure 3.5)** could be attributed to guaicyl hydrolysis by degradation of β–O–4 bond after alkaline pretreatment and difference in lignin content, wild type lignin content being higher than all other sample types (Yahaya et al., 2020).

The glucose yield observed in laccase 27 was higher than laccase 18, even though it is not a target of miRNA397a, it is still the most expressed laccase gene in SDX (Stem differentiating xylem) of poplar. Laccase 27 showed a decrease in lignin content and the most increase in gene expression (Kumar, 2017). Laccase 27 expression could also be because of altered expression of gene itself as other laccase 27 in SDX did not contribute

to observed results. Another factor could be miRNA397a, it might be suppressing the laccase 27 and changing its expression (Kumar, 2017).

Short Tandem Target Mimic (STTM) samples are gene knockout samples. The target miRNA397a has been suppressed in these samples to identify if miRNA397a plays a role in lignin biosynthesis or laccase gene regulation. These samples had the lowest starting lignin content (15%) and highest glucose yield after hydrolysis among all transgenic samples. The relative gene expression of laccase gene in STTM were downregulated (Kumar et al., 2020). When miRNA393 was knocked down in STTM 393 in, *(P. alba × P. glandulosa)* this led to an increase in secondary growth and more biomass production. This also proved that miRNA393 is a negative regulator of growth and structural alterations like more lignin deposition and loosened cell wall (Chu et al., 2021). In STTM 397a samples lignin was reported to decrease 6% (Kumar et al., 2020). The observed difference in STTM could be because of low expression of miRNA397a in these samples, as miRNA397a controls laccase genes and laccase genes regulate lignin biosynthesis therefore its absence might lead to low lignin content and higher glucose yield.

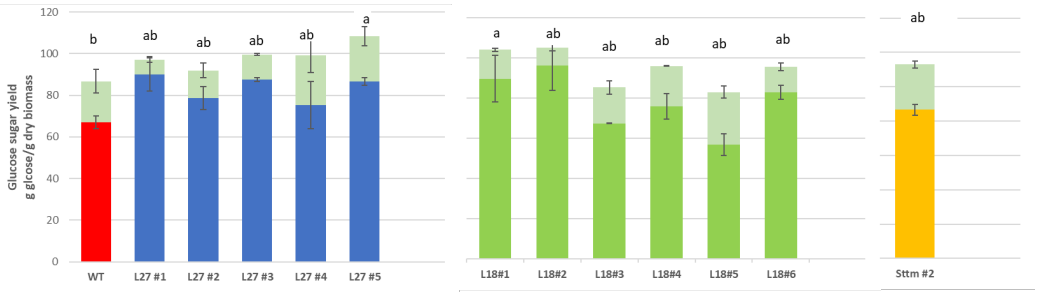


Figure 2.4: Enzymatic hydrolysis of glucose yield of wild type and transgenic samples at 24 and 72 hours. Error bars represent the average glucose yield in the pretreatment liquid ± standard deviation. Statistical analysis revealed all means are different ($p<0.05$).

All the sample types reported to have higher glucose yield at 72 hrs (**Figure 3.4**) but the highest increase of 26.3g glucose/g dry biomass was for STTM #2 (**Appendix 6.3)**.The optimum time for hydrolysis has been reported between 24-72 hrs. for many sample types but these conditions vary by factors like sample size, pH, mixing or product inhibition (Kristensen et al., 2009). One of the plausible reasons behind low glucose yield increase at 72 hrs could be low solids loading. The solids loading was kept at 3% so its plausible that maximum conversion occcured between 24 - 48 hrs.Laccase 27 #5 and Laccase 27 #1 also did not showed any similarities with wild types.

Analysis of xylose yield revealed that the highest xylose release between 24 and 72 hrs. **(Figure 3.5)** of 38.12g glucose/g dry biomass is for wild type samples (**Appendix 6.3**). Laccase 18 #6 showed the lowest release of 2 g glucose/g dry biomass between 24 and 72 hours. The highest overall xylose yield was for STTM samples of 123g glucose/g dry biomass, these samples also showed the highest percent increase in glucose yield. One of

the reasons behind this could be less lignin formation due to blocking of miRNA, and more accessibility of cellulose and hemicellulose to enzymes.

There is not enough research yet on xylose yield and miRNA397a to propose a certain outcome and more research needs to be conducted for further analysis.

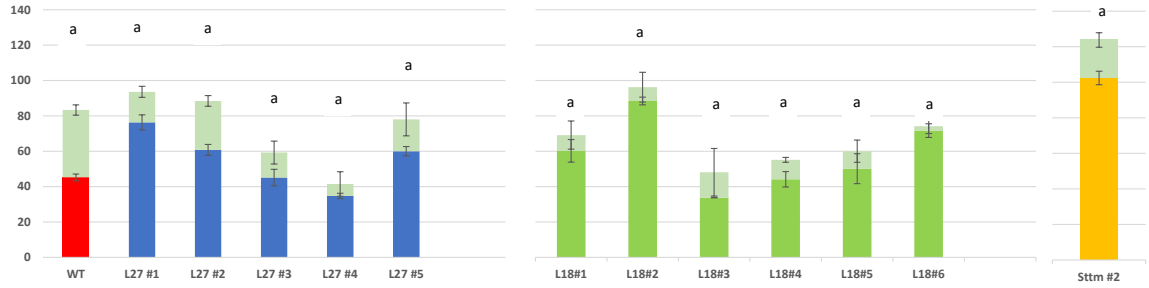


Figure 2.5: Enzymatic hydrolysis of xylose yield of wild type and transgenic samples at 24 and 72 hours. Error bars represent the average xylose yield in the pretreatment liquid ± standard deviation. Statistical analysis revealed all means are similar or maybe there is not evidence to reject the hypothesis ($p>0.05$) using ANOVA.

3 Discussion

The compositional analysis revealed that wild type had the highest glucan content and XGM content but these samples also had highest acid insoluble lignin content. The high acid insoluble lignin content and lowest extractives percent could be a plausible reason behind low alkaline pretreatment yield and low enzymatic hydrolysis yield of wild type samples.

The alkaline pretreatment yield reported highest glucose yield for laccase 18 #6 and lowest for laccase 27 #2. The highest XGM yield was reported for laccase 27 #1and lowest XGM yield was reported for laccase 18 #1. When compared to solids recovery the highest solids recovery was reported for laccase 18 #6 which aligns with highest alkaline pretreatment yield for glucose. If we compare alkaline pretreatment between transgenic modifications and wild type then laccase 27, laccase 18 and STTM reported more yield than wild type for both glucose and XGM. If we compare between transgenic modifications then laccase 18 had higher glucose yield than laccase 27 and STTM.

The enzymatic hydrolysis for glucose yield reported highest overall yield for laccase 27 #5 and highest percent increase was for STTM. The lowest overall glucose yield was reported for laccase 18 #5 and lowest percent increase was for laccase 27 #1. For XGM yield highest overall yield was reported for STTM #2 and highest percent increase was for wild type. The lowest overall XGM yield was reported for laccase 27 #4 and lowest percent increase was reported for laccase 18 #6. If we compare transgenic modifications and wild type then hydrolysis yield for both glucose and XGM were observed higher than wild type. If we compare between transgenic modifications then for glucose yield laccase 27 reported higher values than STTM and laccase 18. For XGM yield STTM reported higher yield than other modifications types.

To summarize, the transgenic samples reported higher pretreatment and hydrolysis yield than wild type. For transgenic lines as individual it is hard to make a trend between compositional analysis, alklaine pretreatment and enzymatic hydrolysis. There are many factors like pH, temperature, standard deviation and many more that play a role in yield determination. A clear trend cannot be observed but none of the high yield samples showed any irregularities like extremely high lignin values or very low glucan content during compositional analysis. It implies that maybe as the experiments proceeded every transgenic samples acted as an individual and maybe more replicates would provide an better explaination and clear observation.

4 Conclusions and Recommendations

The glucose yield for the all the transgenic sample increased more than wild type samples after 24 hours during enzymatic hydrolysis. This may suggest that the substrate is initially impervious to hydrolysis but gradually opens to the enzyme due to structural alterations or other circumstances. In the case of laccase 18, the target of miRNA396a known to regulate gene expression in plants, laccase 18 was more susceptible to high glucose yield than other types but the glucose yield obtained was less than laccase 27. The reason behind it could be high lignin content, S/G ratio or expression of miRNA397a. For Short Tandem Target Mimic the glucose yield increased most between 24 – 48 hours and eventually slowed down. Most plausible explanation for this is since solids loading of biomass is very low, glucose and xylose conversion happened early during the hydrolysis. For laccase 27, the results were surprising as these samples showed higher glucose and xylose loading than laccase 18 thus indicating that maybe laccase 27 is being indirectly regulated by some other factors.

To draw a valid conclusion, it is important to acknowledge that several factors may have influenced glucose production over time, such as substrate availability, enzyme stability, and changes in pH. Therefore, additional research and analysis are necessary to determine the precise reason for the observed differences in glucose yield among the mentioned samples.

In terms of future research, exploring the impact of lignin modification on enzyme stability could be a valuable avenue to pursue. Additionally, since there is limited data available on xylose yield in transgenic plants, further investigations could be conducted to evaluate the potential of transgenic feedstocks for xylose production. Furthermore, an optimization study comparing different transgenic feedstocks to control samples could be undertaken to determine the most efficient option for biofuel production.

Finally, while poplar has shown potential as a feedstock for biofuel production, exploring other hardwood options that could yield similar efficiency would be a significant step forward for the industry. Therefore, research into other hardwood feedstocks that can be used for biofuel production may hold the key to advancing this field further.

5 Reference List

1. Tumuluru, J. S. (2020). Bioenergy Feedstock Types and Properties. In J. S. Tumuluru (Ed.), Biomass Densification: Systems, Particle Binding, Process Conditions, Quality Attributes, Conversion Performance, and International Standards (pp. 1-21). Springer International Publishing. https://doi.org/10.1007/978-3-030-62888-8_1.

2. Bryant, N. D., Pu, Y., Tschaplinski, T. J., Tuskan, G. A., Muchero, W., Kalluri, U. C., Yoo, C. G., & Ragauskas, A. J. (2020). Transgenic Poplar Designed for Biofuels. *Trends in Plant Science*, *25*(9), 881-896. https://doi.org/https://doi.org/10.1016/j.tplants.2020.03.008

3. López, F., Dıaz, M., Eugenio, M., Ariza, J., Rodrıguez, A., & Jiménez, L. (2003). Optimization of hydrogen peroxide in totally chlorine free bleaching of cellulose pulp from olive tree residues. *Bioresource Technology*, *87*(3), 255-261.

4. Ragauskas, A. J., Beckham, G. T., Biddy, M. J., Chandra, R., Chen, F., Davis, M. F., Davison, B. H., Dixon, R. A., Gilna, P., & Keller, M. (2014). Lignin valorization: improving lignin processing in the biorefinery. *science*, *344*(6185), 1246843.

5. Noor Idayu Nashiruddin, N. H. A. M., Roshanida A. Rahman, Nur Izyan Wan Azelee, Daniel Joe Dailin & Shalyda Md Shaarani (2020). Pretreatment and Enzymatic Hydrolysis of Lignocellulosic Biomass for Reducing Sugar Production. In Z. A. Z. C. N. A. R. D. K. P. Binod (Ed.), *Valorisation of Agro-industrial Residues – Volume II: Non-Biological Approaches* (Vol. Volume-II). Applied Environmental Science and Engineering for a Sustainable Future.

6. Bidhendi, A. J., & Geitmann, A. (2016). Relating the mechanics of the primary plant cell wall to morphogenesis. *J Exp Bot*, *67*(2), 449-461. https://doi.org/10.1093/jxb/erv535

7. Béguin, P., & Aubert, J.-P. (1994). The biological degradation of cellulose. *FEMS microbiology reviews*, *13*(1), 25-58.

8. Scheller, H. V., & Ulvskov, P. (2010). Hemicelluloses. *Annu Rev Plant Biol*, *61*, 263-289. https://doi.org/10.1146/annurev-arplant-042809-112315

9. Ebringerová, A., Hromádková, Z., & Heinze, T. (2005). Hemicellulose. In T. Heinze (Ed.), *Polysaccharides I: Structure, Characterization and Use* (pp. 1-67). Springer Berlin Heidelberg. https://doi.org/10.1007/b136816

10. Pérez, J., Munoz-Dorado, J., De la Rubia, T., & Martinez, J. (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International microbiology*, *5*(2), 53-63.

11. Boerjan, W., Ralph, J., & Baucher, M. (2003). Lignin biosynthesis. *Annual Review of Plant Biology*, *54*(1), 519-546.

12. Bajpai, P. (2017). Lignin. In P. Bajpai (Ed.), *Carbon Fibre from Lignin* (pp. 11-15). Springer Singapore. https://doi.org/10.1007/978-981-10-4229-4_2

13. van der Pol, E., Bakker, R., Baets, P., & Eggink, G. (2014). By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels. *Applied Microbiology and Biotechnology*, *98*. https://doi.org/10.1007/s00253-014-6158-9

14. Alejandro, S., Lee, Y., Tohge, T., Sudre, D., Osorio, S., Park, J., Bovet, L., Lee, Y., Geldner, N., & Fernie, A. R. (2012). AtABCG29 is a monolignol transporter involved in lignin biosynthesis. *Current Biology*, *22*(13), 1207-1212.

15. Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P. F., Marita, J. M., Hatfield, R. D., Ralph, S. A., Christensen, J. H., & Boerjan, W. (2004). Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenyl- propanoids. *Phytochemistry Reviews*, *3*, 29-60.

16. Barros, J., Escamilla-Trevino, L., Song, L., Rao, X., Serrani-Yarce, J. C., Palacios, M. D., Engle, N., Choudhury, F. K., Tschaplinski, T. J., Venables, B. J., Mittler, R., & Dixon, R. A. (2019). 4-Coumarate 3-hydroxylase in the lignin biosynthesis pathway is a cytosolic ascorbate peroxidase. *Nature Communications*, *10*(1), 1994. https://doi.org/10.1038/s41467-019-10082-7

17. Liu, Q., Luo, L., Wang, X., Shen, Z. G., & Zheng, L. (2017). Comprehensive Analysis of Rice Laccase Gene (OsLAC) Family and Ectopic Expression of OsLAC10 Enhances Tolerance to Copper Stress in Arabidopsis. *International Journal of Molecular Sciences*, *18*, 209. https://doi.org/10.3390/ijms18020209

18. Driouich, A., Laine, A., Vian, B., & Faye, L. c. (1992). Characterization and localization of laccase forms in stem and cell cultures of sycamore. *Plant Journal*, *2*, 13-24.

19. Bao, W., O'Malley, D. M., Whetten, R., & Sederoff, R. R. (1993). A laccase associated with lignification in loblolly pine xylem. *science*, *260*(5108), 672-674.

20. Sterjiades, R., Dean, J. F., & Eriksson, K. E. (1992). Laccase from Sycamore Maple (Acer pseudoplatanus) Polymerizes Monolignols. *Plant Physiol*, *99*(3), 1162-1168. https://doi.org/10.1104/pp.99.3.1162

21. Ravi, K., García-Hidalgo, J., Gorwa-Grauslund, M. F., & Lidén, G. (2017). Conversion of lignin model compounds by Pseudomonas putida KT2440 and isolates from compost. *Applied Microbiology and Biotechnology*, *101*, 5059 - 5070.

22. Kumar, K. S. (2017). Genetic manipulation of secondary wall-specific laccase genes in poplars (C. Joshi, Trans.). In M. T. University (Ed.).

23. Eudes, A., George, A., Mukerjee, P., Kim, J. S., Pollet, B., Benke, P. I., Yang, F., Mitra, P., Sun, L., & Çetinkol, Ö. P. (2012). Biosynthesis and incorporation of side-chain-truncated lignin monomers to reduce lignin polymerization and enhance saccharification. *Plant Biotechnology Journal*, *10*(5), 609-620.

24. Lin, C.-Y., Geiselman, G. M., Liu, D., Magurudeniya, H. D., Rodriguez, A., Chen, Y.-C., Pidatala, V., Unda, F., Amer, B., Baidoo, E. E. K., Mansfield, S. D., Simmons, B. A., Singh, S., Scheller, H. V., Gladden, J. M., & Eudes, A. (2022). Evaluation of engineered low-lignin poplar for conversion into advanced bioproducts. *Biotechnology for Biofuels and Bioproducts*, *15*(1), 145. https://doi.org/10.1186/s13068-022-02245-4

25. Buhl, C., Meilan, R., & Lindroth, R. L. (2017). Genetic Modification of Lignin in Hybrid Poplar (Populus alba × Populus tremula) Does Not Substantially Alter Plant Defense or Arthropod Communities. *J Insect Sci*, *17*(3). https://doi.org/10.1093/jisesa/iex052

26. Wang, J., Wang, C., Zhu, M., Yu, Y., Zhang, Y., & Wei, Z. (2008). Generation and characterization of transgenic poplar plants overexpressing a cotton laccase gene. *Plant Cell, Tissue and Organ Culture*, *93*(3), 303-310. https://doi.org/10.1007/s11240-008-9377-x

27. Green, D., Dalmay, T., & Chapman, T. (2016). Microguards and micromessengers of the genome. *Heredity*, *116*(2), 125-134. https://doi.org/10.1038/hdy.2015.84

28. Bartel, D. P. (2018). Metazoan MicroRNAs. *Cell*, *173*(1), 20-51. https://doi.org/10.1016/j.cell.2018.03.006

29. Wu, H., Deng, Y., Feng, Y., Long, D., Ma, K., Wang, X., Zhao, M., Lu, L., & Lu, Q. (2018). Epigenetic regulation in B-cell maturation and its dysregulation in autoimmunity. *Cellular and Molecular Immunology*, *15*. https://doi.org/10.1038/cmi.2017.133

30. Abdel-Ghany, S. E., & Pilon, M. (2008). MicroRNA-mediated Systemic Down-regulation of Copper Protein Expression in Response to Low Copper Availability in Arabidopsis*. *Journal of Biological Chemistry*, *283*, 15932 - 15945.

31. Yan, J., Gu, Y., Jia, X., Kang, W., Pan, S., Tang, X., Chen, X., & Tang, G. (2012). Effective small RNA destruction by the expression of a short tandem target mimic in Arabidopsis. *Plant Cell*, *24*(2), 415-427. https://doi.org/10.1105/tpc.111.094144

32. Chu, L., He, X., Shu, W., Wang, L., & Tang, F. (2021). Knockdown of miR393 Promotes the Growth and Biomass Production in Poplar. *Front Plant Sci*, *12*, 714907. https://doi.org/10.3389/fpls.2021.714907

33. Hsu, T. A., Ladisch, M., & Tsao, G. (1980). *Alcohol from cellulose*.

34. De Assis, T., Huang, S., Driemeier, C. E., Donohoe, B. S., Kim, C., Kim, S. H., Gonzalez, R., Jameel, H., & Park, S. (2018). Toward an understanding of the increase in enzymatic hydrolysis by mechanical refining. *Biotechnology for Biofuels*, *11*(1), 1-11.

35. Zahoor. (2014). Pretreatments to Enhance the Digestibility of Wheat Straw. *International Journal of Renewable and Sustainable Energy*, *3(1)*, 26-34. https://doi.org/10.11648/j.ijrse.20140301.15

36. Zheng, Y., Pan, Z., & Zhang, R. (2009). Overview of biomass pretreatment for cellulosic ethanol production. *International Journal of Agricultural and Biological Engineering*, *2*, 51-68.

37. Kim, S., & Holtzapple, M. T. (2006). Delignification kinetics of corn stover in lime pretreatment. *Bioresource Technology*, *97*(5), 778-785.

38. Terrett, O. M., & Dupree, P. (2019). Covalent interactions between lignin and hemicelluloses in plant secondary cell walls. *Current Opinion in Biotechnology*, *56*, 97-104. https://doi.org/https://doi.org/10.1016/j.copbio.2018.10.010

39. Wen, P., Zhang, Y., Zhu, J., Xu, Y., & Zhang, J. (2021). Alkaline post-incubation improves the saccharification of poplar after hydrogen peroxide–acetic acid pretreatment. *Biotechnology for Biofuels*, *14*(1), 151. https://doi.org/10.1186/s13068-021-01999-7

40. Bay, M. S., Karimi, K., Nasr Esfahany, M., & Kumar, R. (2020). Structural modification of pine and poplar wood by alkali pretreatment to improve ethanol production. *Industrial Crops and Products*, *152*, 112506. https://doi.org/https://doi.org/10.1016/j.indcrop.2020.112506

41. Chen, Y., Stevens, M. A., Zhu, Y., Holmes, J., & Xu, H. (2013). Understanding of alkaline pretreatment parameters for corn stover enzymatic saccharification. *Biotechnology for Biofuels*, *6*(1), 8. https://doi.org/10.1186/1754-6834-6-8

42. Kumar, B., Bhardwaj, N., Agrawal, K., Chaturvedi, V., & Verma, P. (2020). Current perspective on pretreatment technologies using lignocellulosic biomass: An emerging biorefinery concept. *Fuel Processing Technology*, *199*, 106244. https://doi.org/https://doi.org/10.1016/j.fuproc.2019.106244

43. Zhao, D., Yu, H., Bao, X., Liu, J., Yuan, H., Wang, Y., & Xue, Z. (2020). Effects of removal of extractives on the chemical composition and mechanical properties of wood. *BioResources*, *15*, 8631-8647. https://doi.org/10.15376/biores.15.4.8631-8647

44. Park, Y. C., & Kim, J. S. (2012). Comparison of various alkaline pretreatment methods of lignocellulosic biomass. *Energy*, *47*(1), 31-35. https://doi.org/https://doi.org/10.1016/j.energy.2012.08.010

45. Jönsson, L. J., & Martín, C. (2016). Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology*, *199*, 103-112. https://doi.org/https://doi.org/10.1016/j.biortech.2015.10.009

46. Muhammad Nauman, A., Irfana, I., Fatima, R., Ahmet, K., & Meisam, T. (2019). Different Pretreatment Methods of Lignocellulosic Biomass for Use in Biofuel Production. In A. Abd El-Fatah (Ed.), *Biomass for Bioenergy* (pp. Ch. 2). IntechOpen. https://doi.org/10.5772/intechopen.84995

47. Modenbach, A. A., & Nokes, S. E. (2013). Enzymatic hydrolysis of biomass at high-solids loadings – A review. *Biomass and Bioenergy*, *56*, 526-544. https://doi.org/https://doi.org/10.1016/j.biombioe.2013.05.031

48. Guo, H., Zhao, Y., Chang, J. S., & Lee, D. J. (2023). Enzymes and enzymatic mechanisms in enzymatic degradation of lignocellulosic biomass: A mini-review. *Bioresour Technol*, *367*, 128252. https://doi.org/10.1016/j.biortech.2022.128252

49. Wang, T., & Lü, X. (2021). Chapter 8 - Overcome saccharification barrier: Advances in hydrolysis technology. In X. Lü (Ed.), *Advances in 2nd Generation of Bioethanol Production* (pp. 137-159). Woodhead Publishing. https://doi.org/https://doi.org/10.1016/B978-0-12-818862-0.00005-4

50. Sathendra, E. R., Baskar, G., Praveenkumar, R., & Gnansounou, E. (2019). Bioethanol production from palm wood using Trichoderma reesei and Kluveromyces marxianus. *Bioresource Technology*, *271*, 345-352.

51. Zhu, J., Chen, L., Gleisner, R., & Zhu, J. (2019). Co-production of bioethanol and furfural from poplar wood via low temperature (≤ 90 C) acid hydrotropic fractionation (AHF). *Fuel*, *254*, 115572.

52. Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B., & Blanch, H. (2012). The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnology and bioengineering*, *109*, 1083-1087. https://doi.org/10.1002/bit.24370

53. Williams, C. L., Rachel, M. E., & Jaya Shankar, T. (2017). Biomass Compositional Analysis for Conversion to Renewable Fuels and Chemicals. In T. Jaya Shankar (Ed.), *Biomass Volume Estimation and Valorization for Energy* (pp. Ch. 11). IntechOpen. https://doi.org/10.5772/65777

54. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008). Determination of structural carbohydrates and lignin in biomass, in: Laboratory Analytical Procedure (LAP). *National Renewable Energy Laboratory*.

55. López-Bascón, M. A., & Luque de Castro, M. D. (2020). Chapter 11 - Soxhlet Extraction. In C. F. Poole (Ed.), *Liquid-Phase Extraction* (pp. 327-354). Elsevier. https://doi.org/https://doi.org/10.1016/B978-0-12-816911-7.00011-6

56. Mottaleb, M. A., & Sarker, S. (2012). Accelerated Solvent Extraction for Natural Products Isolation. *Methods in molecular biology (Clifton, N.J.)*, *864*, 75-87. https://doi.org/10.1007/978-1-61779-624-1_4

57. DeMartini, J. D., Studer, M. H., & Wyman, C. E. (2011). Small-scale and automatable high-throughput compositional analysis of biomass. *Biotechnol Bioeng*, *108*(2), 306-312. https://doi.org/10.1002/bit.22937

6 Appendix

6.1 Samples labels

Table 6.1: Sample details with sample name and Kavitha's key label

Label		Sample name		Kavitha key label
WT		WT		WT
L27#1		Dx Lac 27 1.2		Dx Lac 27 #1
L27#2		Dx Lac 27 a4		Dx Lac 27 #a
L27#3		Dx Lac b.1		Dx Lac 27 #b
L27#4		Dx Lac 27 c.1		Dx Lac 27 #c
L27#5		Dx Lac 27 d.1		Dx Lac 27 #d
L18#1		Dx Lac 18 i-2		Dx Lac 18 #i
L18#2		Dx Lac 18 ii-4		Dx Lac 18 #ii
L18#3		Dx Lac 18 iii-1		Dx Lac 18 #iii
L18#4		Dx Lac 18 vi-2		Dx Lac 18 #vi
L18#5		Dx Lac 18 vii-1		Dx Lac 18 #vii
L18#6		Dx Lac 18 viii-1		Dx Lac 18 #viii
Sttm#2		Sttm2.1		STTM 2.1 & 2.3

6.2 Small scale compositional analysis

Table 6.2: Small scale compositional analysis products after acid hydrolysis.

Transgenic r	Samples	Replicates	Cellobiose	Glucan	XGM	Arabinan	Acetyl	AIL(%)	Extractives	ASL(%)
Wild type	WT#1	A	2.03%	38.14%	17.17%	0.30%	4.20%	19.88%	7.50%	0.03
Wild type	WT#1	B	1.99%	38.15%	17.37%	0.30%	4.30%	22.91%	2.69%	0.02
Wild type	WT#1	C	2.03%	38.12%	17.35%	0.30%	4.30%	23.84%	3.40%	0.03
Wild type	WT#2	A	0.00%	37.19%	16.42%	0.30%	3.90%	24.70%	3.59%	0.03
Wild type	WT#2	B	2.06%	38.81%	16.50%	0.30%	3.80%	22.38%	4.48%	0.02
Wild type	WT#2	C	0.00%	37.77%	17.04%	0.30%	3.80%	20.63%	0.54%	0.03
Wild type	WT#3	A	2.05%	41.57%	16.16%	0.29%	3.80%	24.61%	4.66%	0.03
Wild type	WT#3	B	2.02%	41.37%	17.00%	0.40%	4.00%	19.14%	5.81%	0.02
Wild type	WT#3	C	2.05%	41.39%	16.29%	0.30%	4.01%	24.39%	5.13%	0.03
Wild type	WT#4	A	0.00%	32.40%	16.67%	0.35%	4.00%	21.47%	8.87%	0.03
Wild type	WT#4	B	0.00%	30.17%	15.60%	0.28%	3.80%	23.81%	6.40%	0.03
Wild type	WT#4	C	0.00%	31.67%	16.00%	0.40%	4.00%	22.53%	5.81%	0.03
Laccase 27	L27#1	A	0.00%	33.80%	14.50%	0.40%	3.20%	18.64%	19.01%	0.02
Laccase 27	L27#1	B	0.00%	31.60%	13.40%	0.40%	3.20%	17.88%	12.42%	0.02
Laccase 27	L27#1	C	1.80%	31.90%	13.60%	0.40%	3.00%	17.95%	12.93%	0.02
Laccase 27	L27#2	A	1.76%	27.20%	11.80%	0.40%	2.60%	17.00%	13.97%	0.02
Laccase 27	L27#2	B	1.54%	30.20%	13.30%	0.40%	3.10%	20.14%	15.93%	0.03
Laccase 27	L27#2	C	1.54%	28.20%	12.70%	0.40%	2.90%	15.80%	16.93%	0.02
Laccase 27	L27#2	A	1.55%	29.20%	14.50%	0.50%	2.80%	18.30%	14.62%	0.02
Laccase 27	L27#2	B	1.55%	30.20%	15.00%	0.50%	3.03%	19.37%	15.30%	0.02
Laccase 27	L27#2	C	0.00%	30.80%	14.50%	0.50%	3.00%	12.78%	13.47%	0.02
Laccase 27	L27#3	A	1.87%	34.90%	16.20%	0.50%	3.20%	9.97%	11.76%	0.03
Laccase 27	L27#3	B	1.87%	35.00%	17.00%	0.50%	3.30%	16.44%	9.63%	0.03
Laccase 27	L27#3	C	0.00%	37.30%	17.70%	0.50%	3.40%	18.07%	6.83%	0.01
Laccase 27	L27#3	A	0.00%	36.40%	14.00%	0.40%	3.30%	14.22%	11.01%	0.02
Laccase 27	L27#3	B	0.00%	33.30%	13.30%	0.40%	3.20%	13.14%	11.49%	0.03
Laccase 27	L27#3	C	0.00%	36.50%	14.40%	0.40%	3.10%	19.22%	12.88%	0.03
Laccase 27	L27#4	A	1.65%	31.70%	13.60%	0.40%	3.20%	13.39%	14.12%	0.03
Laccase 27	L27#4	B	1.65%	30.50%	12.60%	0.40%	3.00%	16.08%	9.09%	0.03
Laccase 27	L27#4	C	1.89%	33.00%	14.30%	0.40%	3.20%	17.78%	13.42%	0.01
Laccase 27	L27#5	A	0.00%	33.40%	14.40%	0.30%	3.01%	15.08%	11.78%	0.02
Laccase 27	L27#5	B	0.00%	34.70%	14.30%	0.40%	3.40%	18.22%	14.74%	0.03
Laccase 27	L27#5	C	0.00%	33.30%	14.00%	0.40%	3.20%	19.57%	12.58%	0.03
Laccase 18	L18#1	A	2.16%	32.72%	13.40%	0.50%	3.10%	16.52%	8.97%	0.02
Laccase 18	L18#1	B	2.16%	33.46%	13.10%	0.20%	3.10%	14.93%	10.63%	0.02
Laccase 18	L18#1	C	2.16%	33.21%	13.60%	0.20%	3.10%	17.74%	10.20%	0.03
Laccase 18	L18#1	A	1.86%	32.65%	12.80%	0.20%	3.00%	19.32%	8.10%	0.03
Laccase 18	L18#1	B	1.86%	33.08%	12.80%	0.40%	3.20%	20.05%	13.80%	0.02
Laccase 18	L18#1	C	1.86%	30.52%	12.20%	0.20%	2.80%	20.72%	9.97%	0.02
Laccase 18	L18#2	A	0.00%	29.50%	13.50%	0.40%	3.10%	19.91%	11.77%	0.03
Laccase 18	L18#2	B	0.00%	30.28%	12.20%	0.40%	3.00%	19.52%	14.57%	0.03
Laccase 18	L18#2	C	0.00%	27.07%	10.70%	0.20%	2.80%	18.34%	9.81%	0.02
Laccase 18	L18#3	A	1.90%	29.42%	12.80%	0.40%	2.90%	20.43%	11.68%	0.03
Laccase 18	L18#3	B	1.90%	21.09%	8.90%	0.30%	2.00%	14.51%	8.15%	0.01
Laccase 18	L18#3	C	1.90%	29.02%	12.30%	0.40%	2.80%	19.67%	14.96%	0.03
Laccase 18	L18#4	A	0.48%	27.91%	12.10%	0.50%	2.90%	19.41%	11.12%	0.02
Laccase 18	L18#4	B	1.93%	28.73%	12.20%	0.40%	2.90%	18.15%	8.33%	0.02
Laccase 18	L18#4	C	1.69%	28.57%	11.90%	0.20%	2.60%	23.40%	10.30%	0.01
Laccase 18	L18#5	A	2.12%	33.04%	13.60%	0.30%	3.20%	21.22%	13.52%	0.03
Laccase 18	L18#5	B	1.89%	31.00%	12.50%	0.40%	3.00%	17.08%	9.77%	0.03
Laccase 18	L18#5	C	1.89%	29.43%	12.10%	0.50%	2.90%	13.01%	9.90%	0.03
Laccase 18	L18#6	A	1.59%	29.74%	12.70%	0.40%	3.10%	17.48%	13.85%	0.02
Laccase 18	L18#6	B	1.59%	30.12%	13.20%	0.40%	3.10%	16.38%	11.54%	0.03
Laccase 18	L18#6	C	1.59%	28.35%	12.30%	0.40%	3.10%	13.68%	13.20%	0.02
STTM	Sttm#2	A	0.00%	30.14%	15.90%	0.50%	3.30%	17.72%	17.39%	0.02
STTM	Sttm#2	B	1.82%	34.07%	16.40%	0.50%	3.40%	13.62%	13.48%	0.01
STTM	Sttm#2	C	0.00%	30.61%	15.20%	0.50%	3.30%	12.05%	12.70%	0.01
STTM	Sttm#2	A	2.08%	33.36%	13.10%	0.40%	3.00%	16.96%	11.27%	0.02
STTM	Sttm#2	B	1.85%	30.92%	13.20%	0.20%	3.10%	15.35%	14.61%	0.02
STTM	Sttm#2	C	1.85%	30.67%	12.70%	0.20%	3.00%	17.71%	13.11%	0.03

6.3 Alkaline Pretreatment Products Analysis

Table 6.3: ANOVA and Tukey's Test results for different compositional analysis parameters and p value.

Grouping Information Using the Tukey Method and 95% Confi (Xylose)					
Key labels	N	Mean	Grouping		
WT	8	0.159084	A		
L27#3	4	0.144729	A	B	
STTM#2	4	0.138246	A	B	
L27#5	2	0.135223	A	B	C
L27#1	2	0.129996	A	B	C
L27#2	4	0.128418		B	C
L18#1	4	0.126513		B	C
L27#4	2	0.125321		B	C
L18#6	2	0.12493		B	C
L18#5	2	0.12449		B	C
L18#2	2	0.12262		B	C
L18#4	2	0.114963		B	C
L18#3	2	0.102236			C
Means that do not share a letter are significantly different.					

Grouping Information Using the Tukey Method and 95% Confi (Glucose)					
Key labels	N	Mean	Grouping		
WT	8	0.367516	A		
L27#3	4	0.341829	A	B	
L27#5	2	0.334376	A	B	C
L18#1	4	0.325697	A	B	C
L27#1	2	0.320541	A	B	C
STTM#2	4	0.316489	A	B	C
L18#5	2	0.31551	A	B	C
L27#4	2	0.306828	A	B	C
L18#2	2	0.290015		B	C
L18#6	2	0.288031		B	C
L27#2	4	0.287036		B	C
L18#4	2	0.27943		B	C
L18#3	2	0.246208			C
Means that do not share a letter are significantly different.					

6.4 Enzymatic Hydrolysis Products Analysis

Table 6.4: Statistical data for Enzymatic Hydrolysis at 24 hrs. and 72 hrs.

Grouping Information Using the Tukey Method and 95% Confidence				(72 hrs Glucose)
Key labels	N	Mean	Grouping	
L27#5	2	108.4	A	
L18#2	2	105.115	A	B
L18#1	4	103.957	A	
STTM#2	4	102.376	A	B
L27#3	4	99.622	A	B
L27#4	2	99.22	A	B
L27#1	2	97.209	A	B
L18#4	2	95.917	A	B
L18#6	2	95.379	A	B
L27#2	4	93.948	A	B
WT	8	86.415		B
L18#3	2	85.204	A	B
L18#5	2	82.967	A	B
Means that do not share a letter are significantly different.				

Grouping Information Using the Tukey Method and 95% Confidence			(Xylose at 72 hrs)
Key labels	N	Mean	Grouping
L18#6	2	104.174	A
L27#2	4	96.605	A
L18#2	2	96.25	A
L27#1	2	93.617	A
WT	8	83.336	A
STTM#2	4	80.937	A
L27#5	2	78.06	A
L18#1	4	74.162	A
L18#5	2	60.048	A
L27#3	4	59.284	A
L18#4	2	55.167	A
L18#3	2	48.122	A
L27#4	2	41.493	A
Means that do not share a letter are significantly different.			

7 Copyright documentation

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Figure 1.5: Lignin biosynthesis pathway available under a Creative Commons Attribution 4.0 International License (Barros et al., 2019)
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Figure 1.7: Pathway of miRNA gene regulation available via license: Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International

https://www.researchgate.net/figure/The-pathway-of-miRNA-regulation-of-gene-expression-The-maturation-of-miRNAs-includes-the_fig3_322764105 [accessed 31 March 2023].

Anderson, E. M., Stone, M. L., Katahira, R., Reed, M., Muchero, W., Ramirez, K. J., Beckham, G. T., & Román-Leshkov, Y. (2019). Differences in S/G ratio in natural poplar variants do not predict catalytic depolymerization monomer yields. *Nature Communications*, *10*(1), 2033. https://doi.org/10.1038/s41467-019-09986-1

Bajpai, P. (2017). Lignin. In P. Bajpai (Ed.), *Carbon Fibre from Lignin* (pp. 11-15). Springer Singapore. https://doi.org/10.1007/978-981-10-4229-4_2

Barros, J., Escamilla-Trevino, L., Song, L., Rao, X., Serrani-Yarce, J. C., Palacios, M. D., Engle, N., Choudhury, F. K., Tschaplinski, T. J., Venables, B. J., Mittler, R., & Dixon, R. A. (2019). 4-Coumarate 3-hydroxylase in the lignin biosynthesis pathway is a cytosolic ascorbate peroxidase. *Nature Communications*, *10*(1), 1994. https://doi.org/10.1038/s41467-019-10082-7

Bay, M. S., Karimi, K., Nasr Esfahany, M., & Kumar, R. (2020). Structural modification of pine and poplar wood by alkali pretreatment to improve ethanol production. *Industrial Crops and Products*, *152*, 112506. https://doi.org/https://doi.org/10.1016/j.indcrop.2020.112506

Bryant, N. D., Pu, Y., Tschaplinski, T. J., Tuskan, G. A., Muchero, W., Kalluri, U. C., Yoo, C. G., & Ragauskas, A. J. (2020). Transgenic Poplar Designed for Biofuels. *Trends in Plant Science*, *25*(9), 881-896. https://doi.org/https://doi.org/10.1016/j.tplants.2020.03.008

Chen, Y., Stevens, M. A., Zhu, Y., Holmes, J., & Xu, H. (2013). Understanding of alkaline pretreatment parameters for corn stover enzymatic saccharification. *Biotechnology for Biofuels*, *6*(1), 8. https://doi.org/10.1186/1754-6834-6-8

Chu, L., He, X., Shu, W., Wang, L., & Tang, F. (2021). Knockdown of miR393 Promotes the Growth and Biomass Production in Poplar. *Front Plant Sci*, *12*, 714907. https://doi.org/10.3389/fpls.2021.714907

Helle, S., Cameron, D., Lam, J., White, B., & Duff, S. (2003). Effect of inhibitory compounds found in biomass hydrolysates on growth and xylose fermentation by a genetically engineered strain of S. cerevisiae. *Enzyme and Microbial Technology*, *33*(6), 786-792.

Jönsson, L. J., & Martín, C. (2016). Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology*, *199*, 103-112. https://doi.org/https://doi.org/10.1016/j.biortech.2015.10.009

Kim, J. S., Lee, Y. Y., & Kim, T. H. (2016). A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresource Technology*, *199*, 42-48. https://doi.org/https://doi.org/10.1016/j.biortech.2015.08.085

Kristensen, J. B., Felby, C., & Jørgensen, H. (2009). Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnology for Biofuels*, *2*(1), 11. https://doi.org/10.1186/1754-6834-2-11

Kumar, B., Bhardwaj, N., Agrawal, K., Chaturvedi, V., & Verma, P. (2020). Current perspective on pretreatment technologies using lignocellulosic biomass: An emerging biorefinery concept. *Fuel Processing Technology*, *199*, 106244. https://doi.org/https://doi.org/10.1016/j.fuproc.2019.106244

Kumar, K. S. (2017). Genetic manipulation of secondary wall-specific laccase genes in poplars (C. Joshi, Trans.). In M. T. University (Ed.).

Lu, S., Li, Q., Wei, H., Chang, M.-J., Tunlaya-Anukit, S., Kim, H., Liu, J., Song, J., Sun, Y.-H., Yuan, L., Yeh, T.-F., Peszlen, I., Ralph, J., Sederoff, R., & Chiang, V. (2013). Ptr-miR397a is a negative regulator of laccase genes affecting lignin content in Populus trichocarpa. *Proceedings of the National Academy of Sciences of the United States of America*, *110*. https://doi.org/10.1073/pnas.1308936110

Modenbach, A. (2013). Sodium hydroxide pretreatment of corn stover and subsequent enzymatic hydrolysis: An investigation of yields, kinetic modeling and glucose recovery.

Muhammad Nauman, A., Irfana, I., Fatima, R., Ahmet, K., & Meisam, T. (2019). Different Pretreatment Methods of Lignocellulosic Biomass for Use in Biofuel Production. In A. Abd El-Fatah (Ed.), *Biomass for Bioenergy* (pp. Ch. 2). IntechOpen. https://doi.org/10.5772/intechopen.84995

Nuoendagula, Tsuji, Y., Takata, N., Sakamoto, S., Nakagawa-Izumi, A., Taniguchi, T., Ralph, J., Mitsuda, N., & Kajita, S. (2018). Change in lignin structure, but not in lignin content, in transgenic poplar overexpressing the rice master regulator of secondary cell wall biosynthesis. *Physiol Plant*, *163*(2), 170-182. https://doi.org/10.1111/ppl.12684

Pan, X., Gilkes, N., & Saddler, J. N. (2006). Effect of acetyl groups on enzymatic hydrolysis of cellulosic substrates. *60*(4), 398-401. https://doi.org/doi:10.1515/HF.2006.062

Park, Y. C., & Kim, J. S. (2012). Comparison of various alkaline pretreatment methods of lignocellulosic biomass. *Energy*, *47*(1), 31-35. https://doi.org/https://doi.org/10.1016/j.energy.2012.08.010

Polo, C. C., Pereira, L., Mazzafera, P., Flores-Borges, D. N. A., Mayer, J. L. S., Guizar-Sicairos, M., Holler, M., Barsi-Andreeta, M., Westfahl, H., & Meneau, F. (2020). Correlations between lignin content and structural robustness in plants revealed by X-ray ptychography. *Scientific Reports*, *10*(1), 6023. https://doi.org/10.1038/s41598-020-63093-6

Scheller, H. V., & Ulvskov, P. (2010). Hemicelluloses. *Annu Rev Plant Biol*, *61*, 263-289. https://doi.org/10.1146/annurev-arplant-042809-112315

Selig, M. J., Adney, W. S., Himmel, M. E., & Decker, S. R. (2009). The impact of cell wall acetylation on corn stover hydrolysis by cellulolytic and xylanolytic enzymes. *Cellulose*, *16*, 711-722.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008). Determination of structural carbohydrates and lignin in biomass, in: Laboratory Analytical Procedure (LAP). *National Renewable Energy Laboratory*.

Tafur Rangel, A. E., Croft, T., González Barrios, A. F., Reyes, L. H., Maness, P.-C., & Chou, K. J. (2020). Transcriptomic analysis of a Clostridium thermocellum strain engineered to utilize xylose: responses to xylose versus cellobiose feeding. *Scientific Reports*, *10*(1), 14517. https://doi.org/10.1038/s41598-020-71428-6

Terrett, O. M., & Dupree, P. (2019). Covalent interactions between lignin and hemicelluloses in plant secondary cell walls. *Current Opinion in Biotechnology*, *56*, 97-104. https://doi.org/https://doi.org/10.1016/j.copbio.2018.10.010

Tumuluru, J. S. (2020). Bioenergy Feedstock Types and Properties. In J. S. Tumuluru (Ed.), *Biomass Densification: Systems, Particle Binding, Process Conditions, Quality Attributes, Conversion Performance, and International Standards* (pp. 1-21). Springer International Publishing. https://doi.org/10.1007/978-3-030-62888-8_1

van der Pol, E., Bakker, R., Baets, P., & Eggink, G. (2014). By-products resulting from lignocellulose pretreatment and their inhibitory effect on fermentations for (bio)chemicals and fuels. *Applied Microbiology and Biotechnology*, *98*. https://doi.org/10.1007/s00253-014-6158-9

Wu, H., Deng, Y., Feng, Y., Long, D., Ma, K., Wang, X., Zhao, M., Lu, L., & Lu, Q. (2018). Epigenetic regulation in B-cell maturation and its dysregulation in autoimmunity. *Cellular and Molecular Immunology*, *15*. https://doi.org/10.1038/cmi.2017.133

Xiang, Z., Sen, S. K., Min, D., Savithri, D., Lu, F., Jameel, H., Chiang, V., & Chang, H.-m. (2017). Field-Grown Transgenic Hybrid Poplar with Modified Lignin Biosynthesis to Improve Enzymatic Saccharification Efficiency. *ACS Sustainable Chemistry & Engineering*, *5*(3), 2407-2414. https://doi.org/10.1021/acssuschemeng.6b02740

Yahaya, A., Ursel, H., & Idris, M. B. (2020). Lignin Hydrothermal Liquefaction into Bifunctional Chemicals: A Concise Review. In B. Venko (Ed.), *Biorefinery Concepts, Energy and Products* (pp. Ch. 5). IntechOpen. https://doi.org/10.5772/intechopen.90860

Yoon, S. Y., Han, S. H., & Shin, S. J. (2014). The effect of hemicelluloses and lignin on acid hydrolysis of cellulose. *Energy*, *77*, 19-24. https://doi.org/https://doi.org/10.1016/j.energy.2014.01.104

Zhao, D., Yu, H., Bao, X., Liu, J., Yuan, H., Wang, Y., & Xue, Z. (2020). Effects of removal of extractives on the chemical composition and mechanical properties of wood. *BioResources*, *15*, 8631-8647. https://doi.org/10.15376/biores.15.4.8631-8647

Zhao, X., Li, P., Liu, X., Xu, T., Zhang, Y., Meng, H., & Xia, T. (2022). High temperature increased lignin contents of poplar (Populus spp) stem via inducing the synthesis caffeate and coniferaldehyde. *Front Genet*, *13*, 1007513. https://doi.org/10.3389/fgene.2022.1007513