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Pretreatment of Loblolly Pine Tree Toppings (Needled) Using DESs

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PRETREATMENT OF LOBLOLLY PINE TREE TOPPINGS (NEEDLES) USING DEEP EUTECTIC SOLVENTS

by

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ABSTRACT

Contemporary industrial development and swift urbanization require environmentally sustainable energy sources. Ethanol made from biomass provides unique environmental and economic strategic benefits and can be considered a safe and clean liquid fuel alternative to fossil fuels. Ethanol's significant advantages, such as low cost, biodegradability, and abundance, make the application of biomass for production of bio-renewable energy favorable. However, biomass must be subjected to pretreatment processes to liberate the components needed for effective enzymatic hydrolysis that converts cellulose to sugars prior to fermentation to create biofuel. Production of value-added co-products besides biofuels, through coordinated bio refinery processes, requires selectivity during pretreatment. The current work concentrates on biomass pretreatment technologies with an emphasis on lignin dissolution using deep eutectic solvents (DES). DES are new 'green' solvents that have a high potential in biomass processing because of their low cost, low toxicity, biodegradability and easy recycling. The present work focuses on the preparation of three types of DES, pretreatment of abundantly available Loblolly pine needles, dissolution of lignin from the biomass and measurement of the mass yield of pine needles treated with different types of DES. The pretreated and raw biomass underwent FTIR analysis, fiber analysis and enzymatic hydrolysis to compare the pretreatment index of different DES on pine needles and also to investigate one of the applications of lignin as a natural dyeing component.

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Author Prathima Gujjula

Date March 2020

DEDICATION

I would like to dedicate this thesis to my father Nagaraju Gujjula who has been with me and picked me on time, supported me morally and financially in each and every phase of my life and Louisiana Tech University for giving me this great opportunity to pursue Masters.

.

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CHAPTER 1

INTRODUCTION

Contemporary industrial development and the swift pace of urbanization call for environmentally sustainable energy sources. Lignocelluloses are the most abundant biomass available on earth. Lignocellulosic biomass is comprised of biopolymers of cellulose, hemicellulose and lignin (Malaeke et al., 2018). Production of biofuels is quite possible by fermenting these polymers (polysaccharides) into ethanol, provided the optimization and pretreatment of biomass that is used as a raw source of polymeric components has occurred (Lee, 1997).The significant uses of lignocelluloses are in the pulp and paper industries, production of fuel alcohol and chemicals, and protein for food and feed using biotechnological processes. Lignin can also be used for various industrial and biomedical applications, including biofuels, chemicals and polymers, and the development of nanomaterials for drug delivery. However, these uses depend on the source, chemical modifications and physicochemical properties. These properties and different preparation methods for lignin-based nanomaterials permit their use as reinforcing agents in nano composites, as well as in drug delivery and gene delivery vehicles for biomedical applications (Figueiredo et al., 2018). In Figure 1-1, various steps involved in the conversion of lignocellulosic biomass into valuable products are illustrated.

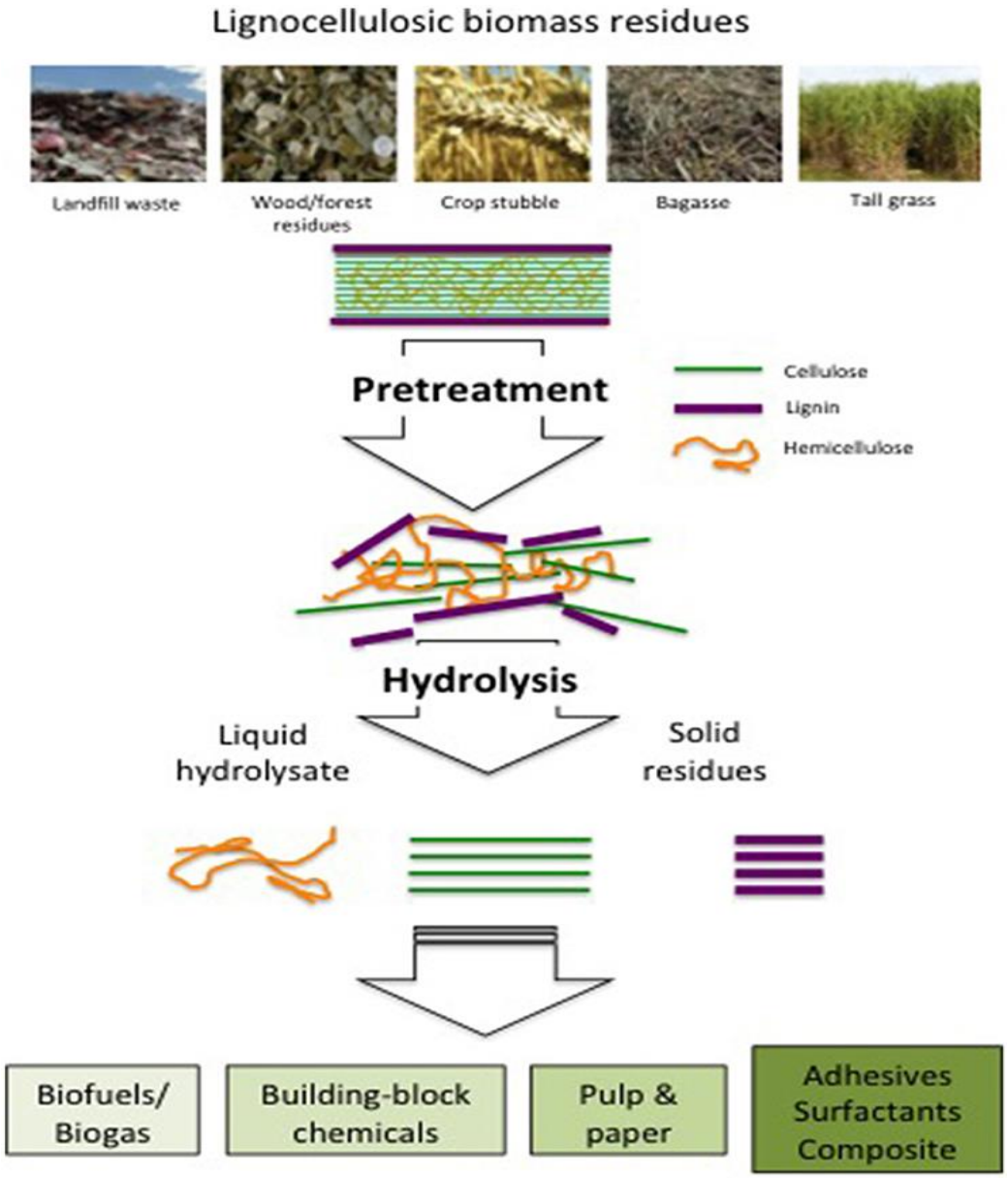

Figure 1-1 Illustration of various steps in the conversion of biomass into value added products

Ethanol made from biomass provides unique environmental and economic strategic benefits, and it can be considered a safe and clean liquid fuel alternative to fossil fuels. Ethanol has significant advantages, such as low cost, biodegradability, and abundance, making application of biomass for production of bio-renewable energy favorable(Panwar

et al., 2011). Nevertheless, the biomass must be subjected to pretreatment processes to liberate the sugars needed for fermentation. Production of value-added co-products alongside of biofuels through integrated biorefinery processes creates the need for selectivity during pretreatment. Some exciting alternative types of solvents are called deep eutectic solvents (DES). DES have low volatility, non-flammability, a wide liquid range, nontoxicity, biocompatibility and biodegradability (Francisco et al., 2012). Unlike ionic liquids (ILs), they are simply prepared at high purities from readily obtainable materials and are inexpensive compared to ILs. In addition, DES are valuable in biofuel processing because they do not typically inactivate enzymes (Lynam et al., 2017).They are less sensitive to water content than ILs are, which makes their use more practical with the wet biomass that would be present at field sites (Francisco et al., 2012).

The present work targeted biomass pretreatment technologies with an emphasis on lignin dissolution using DES that separate a minimum of 20% of the lignin from the biomass. In addition, this research employed FTIR analysis, fiber analysis, enzymatic hydrolysis, and identified color components that could be explored for use as natural dyes.

1.1 Lignocellulosic Materials

Lignocellulosic biomass is a potential resource for the production of value-added products like glucose, ethanol, xylitol, vinegar etc. Lignocellulosic materials are formed by the three main biopolymeric constituents, cellulose, lignin, and hemicelluloses (Hankin and Sands, 1974). Figure 1-2 reveals the lignocellulosic structure, showing cellulose, hemicelluloses, lignin and relevant bonds (Hankin et al., 1971).

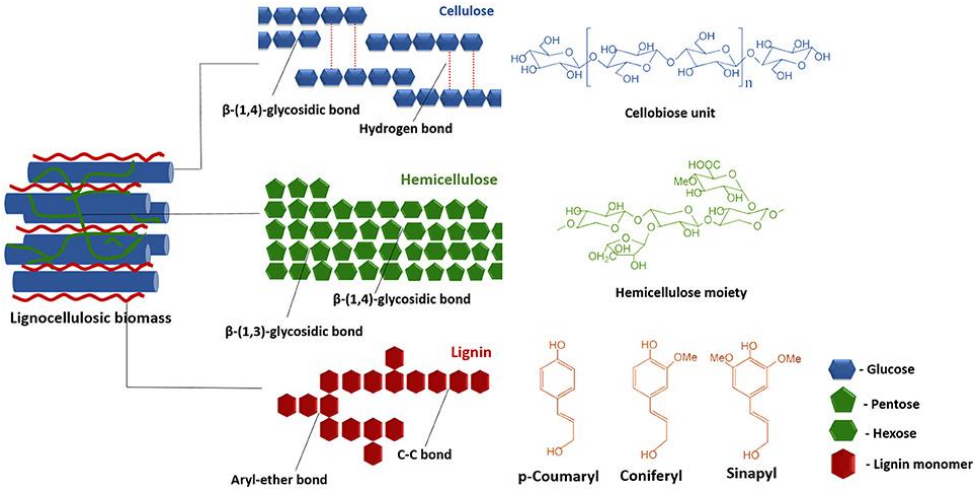

Figure 1-2 Structure of components present in lignocellulosic biomass

1.2.1 Composition

Plant cell walls are the sources of lignocellulosic materials whose structure is represented by the chains of cellulose molecules associated with other biopolymers to form linear structures of high tensile strength known as microfibrils. Layers upon layers of microfibrils make up the cell wall. Each microfibril is about 10 to 20 nm in diameter and may consist of up to 40 cellulose chains. A microfibril is a crystalline and semi-crystalline (amorphous) cellulose core surrounded by hemicelluloses, a branched polymer composed of a mix of mostly pentose sugars (eg. xylose, arabinose) and some hexoses (eg. mannose, galactose, or glucose). In addition to crosslinking individual microfibrils, hemicelluloses also form covalent associations with lignin, a high molecular weight aromatic biopolymer.

1.2.2 Cellulose

Cellulose, the most abundant polysaccharide on earth, is a highly ordered polymer of cellobiose (D-glucopyranosyl-β-1, 4-D-glucopyranose), representing over 50% of wood mass. Approximately $4x10^{10}$ tons of cellulose are produced annually (Coughlan, 1985).

Native cellulose from wood has about 10,000 glycosyl units in linear cellulose chains that are stabilized by numerous strong intermolecular hydrogen bonds between hydroxyl groups of adjacent molecules, as well as intramolecular hydrogen bonds(Puls, 1997). The crystallinity of cellulose presents another challenge to efficient hydrolysis. Figure 1-3, illustrates the structure of cellulose with glycosidic linkages and lattice structure. The high degree of hydrogen bonding that occurs among the sugar subunits within and between cellulose chains forms a 3D lattice-like structure. The highly ordered, water-insoluble nature of crystalline cellulose makes access and hydrolysis of the cellulose chain difficult for aqueous solutions of enzymes. Amorphous cellulose lacks this high degree of hydrogen bonding, thus giving it a structure that is less ordered.

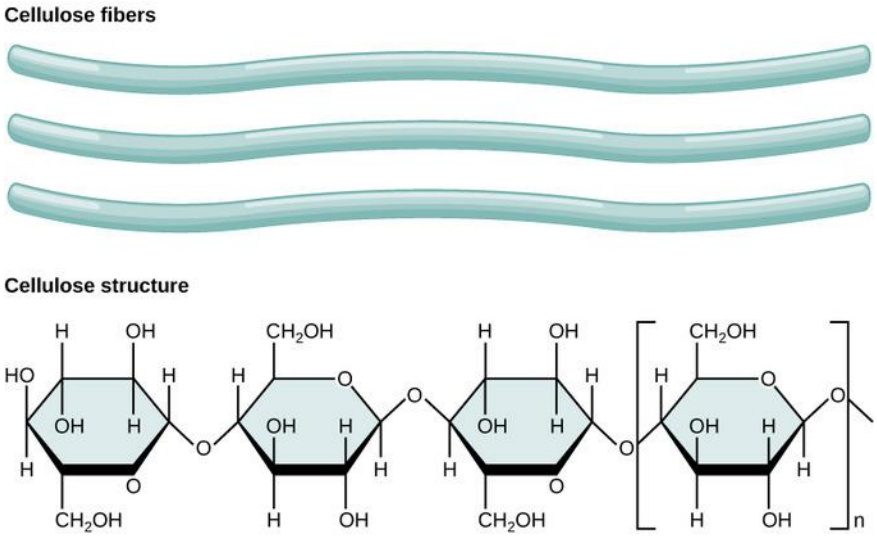

Figure 1-3 Structure of cellulose

1.2.3 Hemicellulose

In Figure 1-4, structure of hemicellulose is revealed, it is a complex, heterogenous mixture of sugars and sugar derivatives that form a highly branched network (Kulkarni et

al., 1999). The monomers that comprise hemicellulose are hexoses (glucose, galactose and mannose) and pentoses (arabinose and xylose). Some monomers, such as galactans, are based on a polymer backbone that is very often homopolymeric with β-1, 4 linkages, Xylan is by far the most important component because of its large quantities in most biomass.

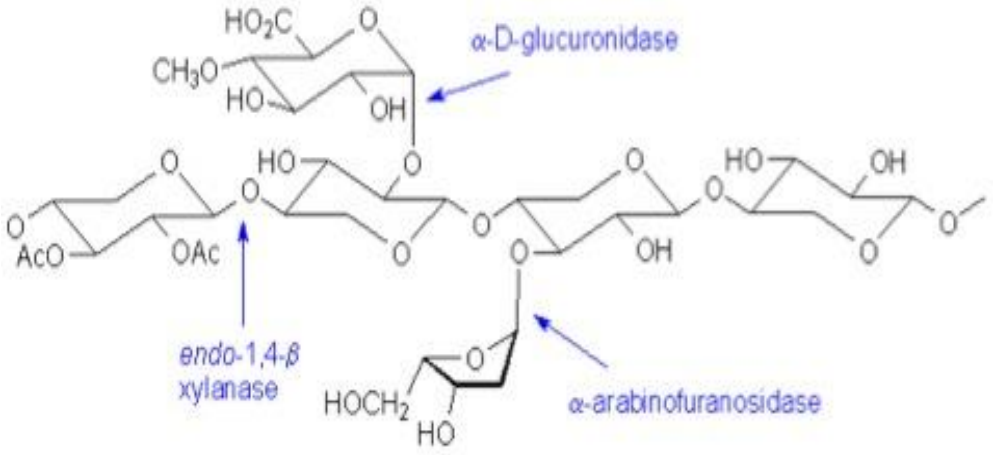

Figure 1-4 Structure of hemicellulose

1.2.4 Lignin

Cellulosic materials also contain lignin, a three-dimensional polymer with phenyl propane units shown in Figure 1-5, held together by ether and carbon-carbon bonds. When plants mature and their cell growth ceases, the middle lamella (the space between the primary walls of adjacent cells) and the secondary cell wall (inside the primary wall) have a large degree of lignin. The lignin strengthens the cell structures by stiffening and holding the fibers of polysaccharides together (Ojumu et al., 2003). It is hydrophobic and highly resistant towards chemical and biological degradation. Lignin content and composition vary among different plant groups. Moreover, the lignin composition varies between the different plant tissues and cell wall layers.

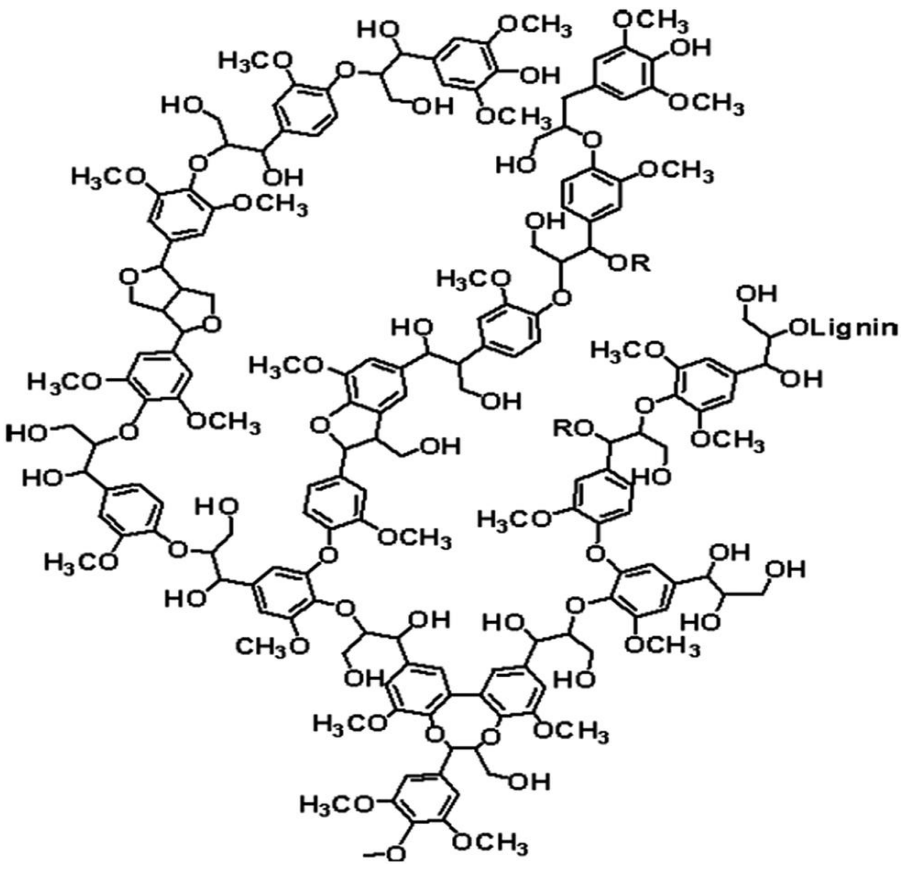

Figure 1-5 Structure of lignin

Other non-structural components of plant tissues include compounds that are extractable with organic solvents such as phenols, tannins, fats and sterols, and water-soluble compounds like sugars, starch, proteins and inorganics (ash). These components usually represent less than 5% of biomass's dry weight (Michael J. Gidley, 2001). The association between polysaccharide (cellulose and hemicellulose) and non-polysaccharide (lignin) components in the structure of plant cell walls is, in great part, responsible for its mechanical and biological resistance (Bon and Ferrara, 2007).

1.2 Pine Needles

Loblolly pine, also known as southern yellow pine, has a rapid growth rate and is the most abundant species in the United States (Nix, 2016). It has significant commercial importance in the pulp and paper industry. In North America, paper production is declining, and recycling of paper is increasing (Miranda and Blanco, 2010). Young Loblolly pine trees shown in Figure 1-6, (10–15 years old) are thus in abundant supply throughout the southeastern United States. This biomass could be pretreated before shipping to biorefineries for production of biofuels or other value-added products.

Figure 1-6 Pine needles of Loblolly pine tree

If a technology that is both safe and green can be discovered to separate lignin from holocellulose, it could enhance production of other bioproducts and possibly be implemented at a local depot or even in the woods or fields. A cellulose- and hemicellulose-enriched product (holocellulose) could then be conveyed to a biorefinery, and the separated lignin could be sent for purification or locally used as a

biofuel. These separated components can be transformed into fuels and chemicals using already established biotechnologies. Dissolution by means of a non-hazardous, green method near the harvesting site could result in the reduction of the volume of material that has to be transported, especially in the case of products that are denser than the untreated biomass. If the products are denser, carbon dioxide produced by transportation would also be reduced with a decrease in the volume of biomass. Initiating this kind of ecofriendly preprocessing method in rural areas would also encourage rural economies that are gifted with biomass resources.

The most common method for the separation of cellulose and lignin in biomass is the Kraft process. As the level of inorganics is low in woody biomass, it has gained prominence. However, wood has other structural uses. The Kraft process is not particularly environmental-friendly, since it releases sulfur-containing volatile organic compounds into the atmosphere (Catalan et al., 2007).

Some of the solvents with an ability to separate lignocellulosic biomass are ILs. ILs, with their extremely low vapor pressures, have novel abilities to dissolve cellulose and lignin both separately and as packed in biomass (Lynam et al., 2012). However, ILs are expensive to produce, the synthesis of most ILs is not eco-friendly, and they are currently unavailable in large scale quantities (Francisco et al., 2013).

1.3 DES

Other possible alternative types of solvents that are unlike traditional technologies are deep eutectic solvents (DES). DES have low volatility, wide liquid range, non-flammability, biocompatibility, non-toxicity and bio-degradability (Francisco et al., 2012). Unlike ILs, they have a straightforward preparation from

highly available materials that gives high purities and they are low cost compared to ILs. In addition, DES usually do not inactivate enzymes, making them helpful in biofuel processing (Gorke et al., 2010). They are less sensitive than ILs to water content, making their use more feasible with the wet biomass found at field sites (Francisco et al., 2012). Drying costs for biomass constitute a considerable portion of pretreatment costs and, if the biomass is not first dried, transportation costs are higher (Lamers et al., 2015).

DES are a homogeneous mixture of two solid-phase chemicals that form a joint super-lattice at a specific molar ratio, called the eutectic composition. The joint super-lattice melts at what is called the eutectic temperature, which is lower than the melting points of the individual components. DES are referred to as deep because the melting point curve has a deep crevice at the eutectic point, since the eutectic temperature is a much lower than the melting points of the pure substances. DES are formed by hydrogen bonding instead of the ionic bonding of ILs (Yiin et al., 2016)at temperatures of 130 °C or less (Francisco et al., 2012). Some safe and economically feasible components used to make DES are formic acid, acetic acid, lactic acid, betaine, and choline chloride (Perez-Sanchez et al., 2013). The safe food additives formic acid, lactic acid, and acetic acid can all be sustainably produced from biomass (Huo et al., 2015);(Yang et al., 2015). Choline chloride is made in large quantities for chicken feed and betaine is generated from sugar beets. (Francisco et al., 2012); (Perez-Sanchez et al., 2013). Work previously done on pretreatment of biomass reports that lignin can be separated from cellulose using DES (Hou et al., 2017); (Lynam et al., 2017); (Vigier et al., 2015).

The present work pursued the pretreatment of the biomass (Loblolly pine needles) with different DES solvents for separation of lignin and cellulosic components of the biomass, and also investigated the byproducts. In this study, mass yield from the pretreatment was calculated and the Fourier transform infrared spectroscopy (FTIR) spectra of the two solid products from the separation analyzed. Enzymatic hydrolysis was performed to find the glucose yield from the pretreated biomass. Fiber analysis was completed to find the composition of fiber remaining in the sample after pretreatment, using a National Renewable Energy Laboratory (NREL) compositional analysis procedure followed by a high-performance liquid chromatography (HPLC) analysis of carbohydrates and insoluble lignin analysis using ultraviolet-visible (UV) spectroscopy.

CHAPTER 2

BACKGROUND

Production of bio-renewable polymers like lignocellulosic compounds for sustainable energy use has gained additional wide-spread importance in the fields of biomedical and transportation industries. Production of biofuels is achievable by hydrolyzing and then fermenting these polymers (polysaccharides) into ethanol, provided the processing and pretreatment of biomass that is used as a raw source of polymeric components has occurred (Lee, 1997). Significant advantages, such as low cost, biodegradability, and abundance, make application of biomass for production of bio-renewable energy favorable. Contemporary industrial developments and the rapid pace of urbanization require environmentally sustainable energy sources. Ethanol made from biomass provides unique environmental and economic strategic advantages, as it can be considered as a safe and clean liquid fuel alternative to fossil fuels (Panwar et al., 2011).

Lignin can be used in various types of batteries, in capacitors, and in fuel cells. The use of lignin in energy storage could decrease cost and toxicity to allow greener energy equipment. (Espinoza-Acosta et al., 2018).

Lignin can also be used for various industrial and biomedical applications. These uses depend on the lignin source and how it has been modified. These properties and different preparation methods for lignin-based nanomaterials influence how lignin can be used as reinforcing agents in nanocomposites, in drug delivery, or as gene delivery systems

for biomedical applications (Figueiredo et al., 2018).In addition, petroleum based asphalt binders can be successfully replaced with 6% of lignin that was precipitated from black liquor for hot mix asphalt, and could be applicable in the low temperature warm mix asphalt process(Arafat et al., 2019).

Lignocellulosic biomass is rich in lignin, which is a widely available under-utilized natural biopolymer due to its low solubility and reactivity characteristics. Despite these drawbacks, a very high solubility of lignin in a resorcinol-choline chloride DES when ultrasound irradiation was used has been reported (Malaeke et al., 2018). As cellulose is less soluble in some DES, lignin can be completely isolated from lignocellulosic biomass.

Biomass can be treated with different solvents; among many others DES are used to separate lignin because of its biodegradability, wide liquid range, non-flammability, nontoxicity, biocompatibility, and low volatility (Lynam et al., 2017).

In the field of sustainable chemistry, the study of new solvents tends to be the greatest challenge. DES represents the principles of green chemistry. These new types of chemicals are suitable for the selective removal of extractives, lignin, or polysaccharides from biomass (Škulcová et al., 2016). Biomass is specifically treated to separate the individual fractions, which following purification can provide products with high yields and purities. (Škulcová et al., 2016).

According to a “Lignin Color Reactions” article (Crocker, 2005) it was reported that there are certain color reactions given by wood and lignified fibers. All the substances that gave high color test results were pure aldehydes. When some of the liquid solution was diluted with alcohol, phloroglucinol, or pyrrole, and a nitro aniline reagent was added, orange, and dark red colors were obtained. Maule reactions were done with eighteen

species of deciduous and coniferous woods, resulting in dark red for deciduous, and pale brown for coniferous species. Other chemicals like chlorine water, weak alkalis, sodium bicarbonate when reacted with lignin also gave color (Crocker, 2005).

Three kinds of DES were facilely prepared in the study of Zhang et al. (2016), and used in the pretreatment of corncob, including monocarboxylic acid: choline chloride, dicarboxylic acid: choline chloride and polyalcohol: choline chloride. The enhanced delignification and subsequent enzymatic hydrolysis efficiency were found to be related to the acid amount, acid strength, and the nature of hydrogen bond acceptors. The X-ray diffraction, scanning electron microscope, and Fourier transform infrared spectroscopy (FTIR) results consistently indicated that the structures of corncob were disrupted by the removal of lignin and hemicellulose in the pretreatment process. In addition, the optimal pretreatment temperature and time were 90 °C and 24 h, respectively. Roles of various DES combinations were investigated, as well as pretreatment temperature and time to better utilize the DES in the pretreatment of lignocellulosic biomass (Zhang et al., 2016). The present study has built on this basic idea to utilize DES for the pretreatment of pine needles in the production of biofuel.

When Hou et al., (2017) treated rice straw with DES, both hydrogen bond acceptors (HBAs) and hydrogen bond donors (HBDs) proved to be important for DES pretreatment efficiency (Hou et al., 2017). DES containing lots of hydroxyl or amino groups with a high intermolecular hydrogen-bond (H-bond) strength exhibited weak biomass deconstruction abilities. The presence of strong electron-withdrawing groups in DES furthered xylan removal, thus delivering higher cellulose digestibility. The relationships between the properties of DES, xylan removal, and cellulose digestibility of pretreated biomass were

established. It was found that xylan removal was negatively correlated with the pKa values of HBDs. The enzymatic cellulose digestibility of the residues was linearly and positively related to xylan removal, instead of to delignification. These results provided a roadmap for rational design of novel DES for biomass pretreatment (Hou et al., 2017). In another study by Zulke et al, (2017), DES were used as a pretreatment solvent on oil palm trunk (OPT) fibers in order to disorder the crystalline cellulose before enzymatic hydrolysis. Of the DES investigated, ethyl ammonium chloride: ethylene glycol (EAC:EG) was found to be the best solvent for the pretreatment of OPT fibers. Zulke et al, (2017) reported that 42% of lignin and 83%of hemicelluloses were removed by EAC:EG after heating at 100°C for 48h (Zulke et al., 2017).

DES obtained from various hydrogen bond donors and acceptors can be used to evaluate the dissolving ability of lignin from biomass. Among various DES, lactic acid: choline chloride showed the optimal lignin extraction ability, since it could dissolve 95% of lignin from biomass at 120°C in 6h(Chen et al., 2019). Another study found a DES-lignin of 95.4% purity was extractable from willow after pretreatment with a 10:1 molar ratio of lactic acid: choline chloride at 120 °C for 18 h (Lyu et al., 2018). Loblolly pine, one of the most abundant trees in the United States, has commercial importance in the paper and pulp industry and has been treated with DES. It was observed that DES can dissolve lignin without inactivating enzymes, a property that makes them valuable in biofuel production (Lynam et al., 2017). From the above inferences it was understood that, DES can be used for the separation of lignin from the biomass. As Loblolly pine is abundant, pine needles can be utilized as byproduct of harvesting the pine in the process of biofuel production.

CHAPTER 3

MATERIALS AND METHODS

3.1 Raw Materials and Chemicals

Loblolly pine needles were collected from the branches of 28-year-old Loblolly pine trees that had been harvested in Ruston LA with the help of Weyerhauser, Inc, employees and then refrigerated. Choline chloride powder (BioReagent, suitable for cell culture, ≥98%), formic acid (reagent grade, ≥95%), acetic acid (reagent grade, ≥99.7%), lactic acid solution (reagent grade, ≥85%), sulfuric acid (reagent grade, 95-98, cellulase (powder) from *Trichoderma reesei* ATCC 26921, hemicellulase (powder) from *Aspergillus niger*, and cellobiase (liquid) from *Aspergillus niger*, were purchased from Millipore Sigma (St.Louis, MO, USA). Sodium azide, 99% min, and sodium citrate dehydrate, 99.0% min, were purchased from Alfa Aesar (Ward Hill, MA, USA). Denatured ethanol (90.5%) was purchased from Duda Energy (Decatur, AL, USA). Nylon membrane discs size 0.45 μm were bought from Foxx Life Sciences (Salem, NH, USA).

3.2 Synthesis of DES

Three types of DES solvents were prepared for pretreatment of pine needle biomass. These were chosen on the basis of their nontoxicity and likelihood for effectiveness in pretreatment. FA:CC was prepared by mixing the formic acid (hydrogen bond donor) and choline chloride(hydrogen bond acceptor) in the mole ratio of 2:1; LA:CC

was prepared by mixing lactic acid with choline chloride in the mole ratio of 10:1; and AA:CC in the mole ratio of 2:1 (Table 3-1). After mixing the two components, mixtures of the solvents were placed in an orbital shaker at 200 RPM at 60°C for 20 min, and were kept for 20 min more if necessary, until a clear solution was seen. The clear DES solvents were then stored at room temperature and remained transparent for several weeks until they were used. In addition, the pHs of the three DES were measured by diluting them with DI water as the acids in the neat solvents gave very low pH values (Table 3-1).These values suggest that the activity of the DES in biomass processing was high, particularly for FA:CC.

Table 3-1 Mole ratio of components in the DES mixtures and pH of DES.

Hydrogen bond donor	**Hydrogen bond acceptor**	**Molar ratio of DES**	**pH of DES**	**pH of DES diluted 10% with water**	**pH of DES diluted 90% with water**
Formic acid	Choline chloride	2:1	-0.92	0.70	1.24
Lactic acid	Choline chloride	10:1	-0.60	0.54	1.91
Acetic acid	Choline chloride	2:1	0.47	1.01	1.84

3.3 Preparation of Biomass for Pretreatment

Pine needles were dried at 60 °C for 24 h and ground. Ground biomass was sieved with numbers 14 and 25 mesh filters to obtain the appropriate size (~1 mm diameter particles) for pretreatment and stored in a closed plastic bag until use.

3.4 Pretreatment of Pine Needles Using DES

Dried and sieved biomass of weight 3 grams was mixed with 30 grams of each of the three types of DES: FA:CC(2:1 ratio), LA:CC(10:1 ratio), AA:CC(2:1ratio) in separate flasks with a magnetic stirrer for continuous stirring and kept in the preheated oil bath for 1 hour at 125°C. The flask with this solution mixture was connected to a condenser to collect liquid from the vapor of the slightly volatile DES in order to maintain the solvent volume constant. Standard errors for the results were found from triplicates of the samples that were done at 125 °C for 1 hour.

3.5 Separation of Biomass and DES

3.5.1 Filtration

Once the pretreated biomass was cooled, biomass was separated from the DES solution using nylon membrane filters (0.44 mm) and a mesh filter membrane. Initially, nylon membrane filters and mesh filters were dried in a drying oven for 30 min and stored in a desiccator. A filtration unit was set up by connecting a Buchner funnel with a filter and a filtration flask to a vacuum pump. Pretreated biomass with the solvent was allowed to pass through the mesh filter; filter cake (filtride) from the filtration was rinsed with 25 ml of ethyl alcohol to wash away any remaining DES on the residues by magnetically stirring for 20 min and filtering with the same mesh filter.

The filtride (biomass) from the above filtration was rinsed with 75 ml of deionized water by magnetically stirring at 50 °C for 20 min. Rinsing with deionized water was done three times to make sure no DES was left in the biomass. Finally rinsing with deionized water was repeated for another 24 h at 50 °C to obtain the clean biomass without any DES. A 0.44 mm nylon filter was used to separate the rinsed biomass with deionized water using a vacuum pump. Filtered biomass was dried at 105 °C for 24 h prior to weighing.

3.5.2 Lignin Precipitation

Filtrate from the vacuum filtration, which was expected to have lignin, DES, and ethyl alcohol was collected into a flask, then 175 ml of deionized water was added to precipitate lignin and separate it from DES, ethyl alcohol and deionized water. Precipitated biomass was separated using a nylon membrane filter and a vacuum pump. After separation, precipitate was dried at 105 °C for 24 h prior to weighing. All the procedures were repeated three times to get triplicates to overcome any lapse in experiments and provide details on measurement error.

3.6 Fourier Transform Infrared Spectroscopy (FTIR)

A Mattson Genesis II FTIR (Mattson Technology, Fremont, Ca, USA) was used to obtain the spectra of pretreated biomass samples. These are the third-generation infrared spectrometers with significantly higher signal to noise ratio, high accuracy for wave numbers and an error range less than 0.01 cm^{-1}. As the standard method to prepare solid samples for FTIR spectrometry is to use KBr pellets, 1 mg of sample was mixed with 100 mg of KBr and a pellet was made with a pellet holder press by applying pressure. Single beam spectra of the samples were collected by running 32 scans with resolution 2 cm^{-1} from wave number 3000 to 800 cm^{-1}. FTIR analysis was performed on both the raw

biomass and pretreated biomass, and precipitated biomass as well. Data analysis of obtained spectra was done using OMNIC software that allows accessing the spectra and calculating peak areas. Table 3-2 shows the vibrations specifically used to evaluate cellulose and lignin content of the samples. Cellulose vibrations used were 1160 cm^{-1} and 1425 cm^{-1} for type I/type II amorphous stretching and crystalline cellulose, respectively. The lignin vibration used was at 1515 cm^{-1}.

Table 3-2 Wavenumber vibration assignments and references

Wavenumber (cm^{-1})	**Band assignment**	**Reference**
1160	Amorphous stretching of cellulose type I and type II	(Shi and Li, 2012)
1425	Crystalline cellulose	(Raj et al., 2015)
1515	Lignin aromatic ring skeletal sketch	(Raj et al., 2015)

3.7 Enzymatic Hydrolysis

NREL's enzymatic saccharification of lignocellulosic biomass LAP 009 protocol (Selig et al., 2008) was followed to understand the saccharification of cellulose from pretreated biomass in order to determine the maximum digestibility possible. Biomass after pretreatment was dried at 105°Cin a drying oven for 24 h. A sample of 0.1 gram of ground biomass was mixed with 5ml of sodium citrate buffer of pH 5.05, and100 μl of 2% sodium azide solution. The total volume was brought to 10 ml by adding an appropriate amount of deionized water as described in the NREL protocol. Subsequently, cellulase, hemicellulase,

and cellobiase were added at a concentration of 5, 14 and 50 units per 0.1 gram of sample, respectively. The mixture of biomass, buffer, sodium azide and enzyme cocktail was kept in the orbital shaker at 200 RPM at 50°C. Samples were collected at 24 h, 48 h and 72 h prior to filtering through a 0.45 μm syringe filter and stored in the refrigerator at 4 °C prior to HPLC analysis. Glucose yield is calculated as a fraction of cellulose present in biomass that was recovered as glucose. The glucose yield was obtained using equation 1.

% glucose yield =

$$\frac{0.9*10mL\ volume\ hydrolysis*glucose\ concentration\ \frac{g}{ml}(corrected\ with\ blanks)}{g\ pretreated\ substrate*\ \ fraction\ cellulose\ in\ pine*\ mass\ yield\ pretreatment} \qquad (1)$$

The correction factor that accounts for the conversion from the biopolymer cellulose to the monomer glucose is 0.9.

3.8 Carbohydrate Analysis Using HPLC

The cellulose, hemicellulose, and lignin content of all the raw and pretreated samples were determined by quantitative saccharification with acid hydrolysis and subsequent HPLC analysis, using NREL protocols LAP/TP-510-426 18 through 22 (Sluiter et al., 2008). For raw biomass, ethanol extraction was carried out to remove the non-structural components of the biomass prior to acid hydrolysis and thus the biomass fraction regarded as extractives was removed from the raw biomass. For pretreated samples, it was assumed that the extractives component had been removed during the pretreatment process, so that these samples directly progressed to acid hydrolysis for compositional analysis.

The concentrations of glucose, xylose, arabinose, galactose, and mannose were quantified using an HPLC (ThermoFisher Scientific, Waltham, MA, USA) equipped with

refractive index detector and an Aminex HPX-87P column 300x7.8 mm, from Bio-Rad. The column temperature was maintained at 80 ˚C and the flow rate was 0.6 ml min^{-1} (DI water). Each experiment was performed in triplicate. Total cellulose release upon acid hydrolysis was determined as the sum of cellobiose and glucose and the total hemicellulose release was determined as the sum of xylose, galactose, arabinose, and mannose. The sum of acid insoluble and acid soluble lignin was represented as total lignin content available in each sample.

3.9 UV Visible Spectroscopy

UV Visible spectroscopy is a quantitative technique to determine the analyte concentration in the sample by absorption of light at a desired wavelength. The sample is dispensed into a quartz cuvette and placed in the path between a light source and a detector. From the Beer Lambert law, the concentration of the compound can be measured from the light absorbed at a desired wavelength with a constant light path length and a known absorption coefficient. The NREL fiber analysis procedure was followed to measure acid soluble lignin from the biomass after acid hydrolysis (Sluiter et al., 2010). A UV-2401PC spectrophotometer was used to analyze acid soluble lignin in the biomass. Once the biomass was pretreated it was hydrolyzed with 70% $H_2 SO_4$ and, with the hydrolysis, lignin in the sample was dissolved. Subsequently, 3000 µl of this sample was measured and diluted 10 times with deionized water. With deionized water as a blank, analytes (lignin) in the sample was measured by absorbance at wavelength 260 nm.

3.10 Soxhlet Extraction

Nonstructural materials from the biomass must be removed from the biomass prior to compositional analysis to prevent any obstructions in later analytical procedures. Water

soluble and ethanol soluble extractives in the biomass can be removed by soxhlet extraction. Biomass of weight 3 grams was placed in a thimble and 200 ml of ethyl alcohol transferred into a conical flask. In Figure 3-1, a picture was taken when the soxhlet extraction setup was made, the thimble with biomass was inserted carefully into a Soxhlet siphon tube and kept above the conical flask with ethyl alcohol.

The whole setup was kept in an oil bath at 80 °C for 24 h, and after 24 h biomass from the thimble was taken out and the loss of biomass measured. The percentage of extractives removed was then calculated.

Figure 3-1 Soxhlet extraction setup

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Mass Yield from DES Pretreatment

When loblolly pine needles were treated with AA:CC, the percentage of mass yield for the biomass was 61.25%, with a standard error of 2.63 %.This mass yield was high when compared to the biomass yields for pretreatment with LA:CC and FA:CC. Average mass yield with LA:CC was lower than FA:CC at 49.55%, however the standard error for LA:CC was 8.32%, so no difference can be confirmed. Table 4-1 represents the mass yield percentages obtained with different DESs solvents and their standard errors. Mass yield for FA:CC was low because the mass of the precipitate from the filtrate was high. Heat required was 0.31 kW-h for the pretreatment process, which is relatively low as more energy can be produced with resultant glucose concentration (Kumar et al., 2019).

Table 4-1 Biomass mass yield obtained with 3 types of DES

DES solvent	Mass yield %	Standard error %
FA:CC	43.65	1.25
LA:CC	49.55	8.32
AA:CC	61.25	2.63

When the biomass was treated with glycerol as a control, no reaction was observed and the biomass remained the same as raw biomass. This result made it clear that the heat treatment alone (1 hour at 125 °C) had no effects on biomass unlike the pretreatment with DES. Interestingly, an intense dark pink color of (#911137 from https://www.color-hex.com/color/911137) was seen when pine needle biomass was treated with LA:CC at 125 °C, 145 °C, 150 °C for 45 min, 1 h and 2 h at these respective temperatures. Further investigation was done to identify the cause of the color by removing the precipitated lignin by centrifugation at 200 RPM giving a decrease in the intensity of the color after the extraction of lignin. Extractives in the biomass were removed using the Soxhlet extraction procedure from NREL and were found to be about 26.10 % of raw biomass. The color was assumed to be due to a modification of lignin compounds in the pine needles. In Figure 4-1, mass yield of the biomass pretreated with three types of DES and precipitated biomass was compared, where FA: CC showed more precipitate when compared to LA: CC and AA: CC.

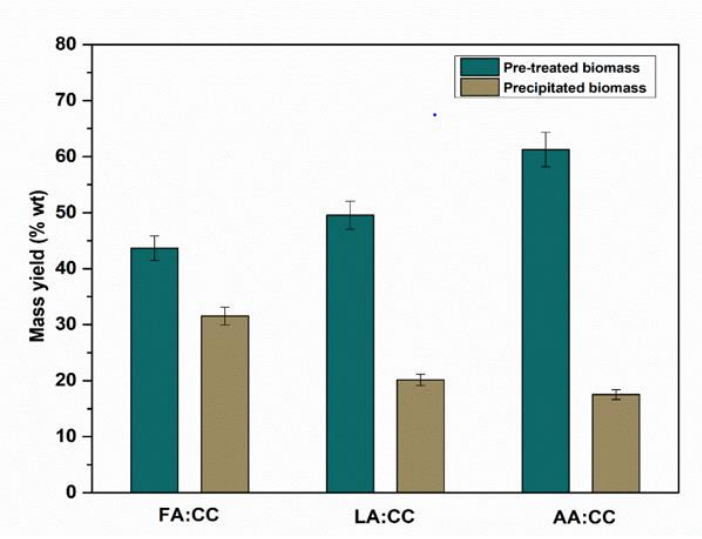

Figure 4-1 Mass yield and precipitated biomass with 3 types of DES

After the separation of biomass filtrate was precipitated by the addition of deionized water, the mass of precipitated biomass (lignin precipitate) shown in Table 4-2, was higher when the biomass was treated with FA:CC, at 31.56% with a standard error of 1.75%. It was 20% with a standard error of 2.87% in the case of LA:CC and 17.51% with a standard error of 0.76% with AA:CC.

Table 4-2 Lignin precipitated with addition of DI water

Type of DES solvent	**Lignin precipitate %**	**Standard error %**
FA:CC	31.56	1.75
LA:CC	20.14	2.87
AA:CC	17.51	0.76

Thus, larger amounts of lignin can be separated from the biomass when it is pretreated with FA:CC and LA:CC compared to AA: CC. The images in Figure 4-2 confirm that the pine needles treated with FA:CC and LA:CC show a greater extent of biomass breakdown compared to AA:CC.

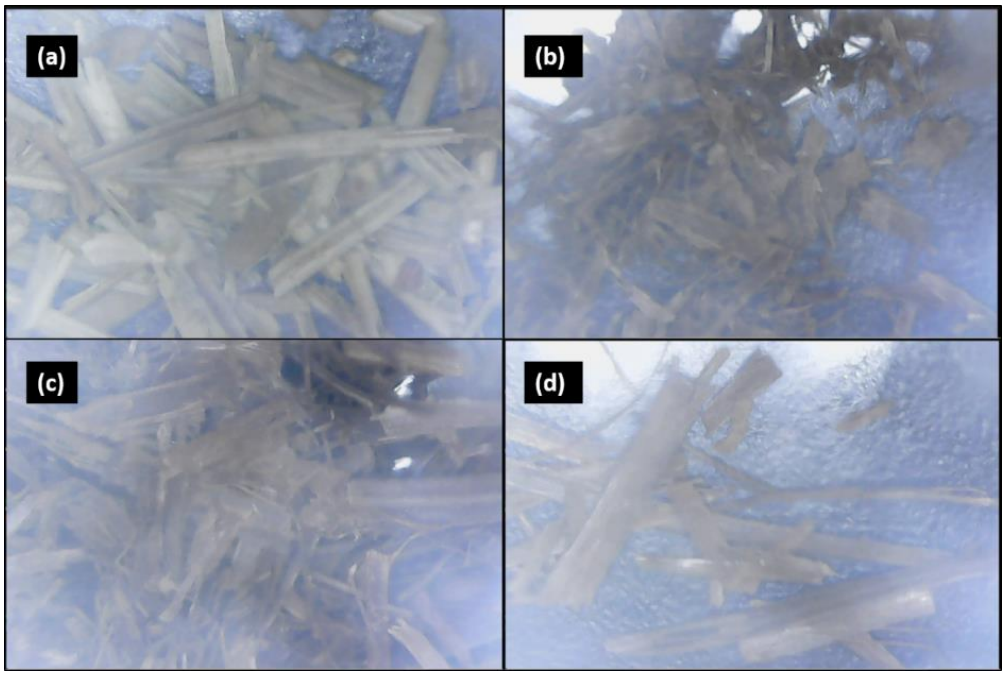

Figure 4-2 Digital microscopic images of biomass with 500x magnification (a) Before pretreatment, (b) After pretreatment with FA:CC, (c) After pretreatment with LA:CC, (d) After pretreatment with AA:CC

4.2 FTIR Analysis of Pretreated Loblolly Pine Needles and Precipitate from Deionized Water

FTIR analysis of raw and pretreated biomass elucidated the transformation in biomass composition and structure in the process of DES pretreatment. FTIR spectra of raw and pretreated biomass and precipitated lignin samples are shown in Figure 4-4, and Figures 4-5. It can be seen from Figure 4-4 that all the pretreated samples showed a FTIR vibration at 1425 cm^{-1} (native type 1 cellulose) and 1160 cm^{-1} (amorphous stretching of cellulose type I and type II) indicating the pretreated biomass still contains considerable amount of crystalline and disordered cellulose. These findings confirm that the structure of the pretreated biomass was obviously changed, resulting in improved enzymatic saccharification.

$$\text{CL ratio} = \frac{Peak_{1425} + Peak_{1160}}{Peak_{1515}} \quad (2)$$

CL ratio is the cellulose to lignin ratio as found from FTIR. $Peak_{1425}$ is the intensity of the vibration at 1425 cm^{-1}. Similarly, $Peak_{1160}$ is the intensity of the vibration at 1160 cm^{-1} and $Peak_{1515}$ is the intensity of the vibration at 1515 cm^{-1}, from the lignin aromatic ring skeletal stretch. The results are shown in Figure 4-3.

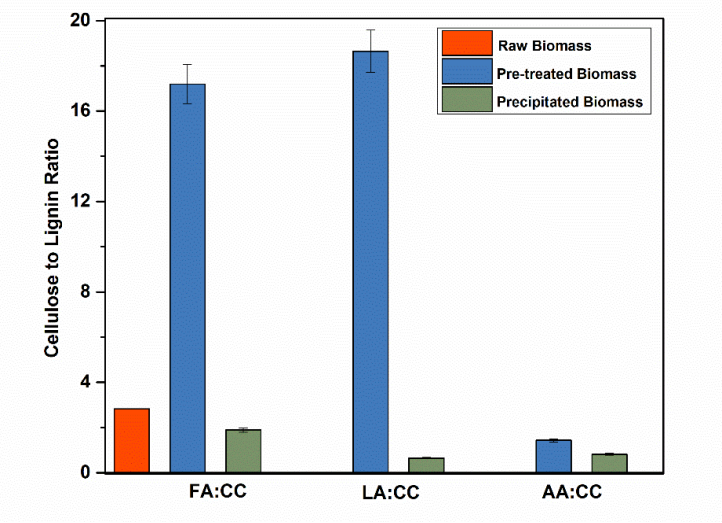

Figure 4-3 Cellulose to lignin ratio of raw pine needles and DES pretreated pine needles

Figure 4-3, calculated from FTIR spectra (equation 2), shows the ratios of intensities of vibrations resulting from cellulose compared to the most prominent lignin vibration. The raw biomass had a low cellulose to lignin ratio. Biomass pretreated with FA:CC and LA:CC had high cellulose to lignin ratios, indicating much more cellulose than lignin after pretreatment. Precipitated lignin from the FA:CC showed align in related characteristics peaks and a low cellulose to lignin ratio, indicating little cellulose compared to lignin. Similarly, precipitated lignin from the LA:CC had a low CL ratio, indicating little cellulose. AA:CC showed less effectiveness in pretreatment when quantified with these FTIR vibrations intensities. Figures 4-4 and 4-5 show sample FTIR spectra for the raw

biomass and the products from DES pretreatment.

An additional method to evaluate the effectiveness of DES pretreatment was chosen using the ratios of the combined areas of relevant cellulose vibrations (1160 cm^{-1} and 1425 cm^{-1}) to that related to lignin (1515 cm^{-1}) for both raw and pretreated samples.

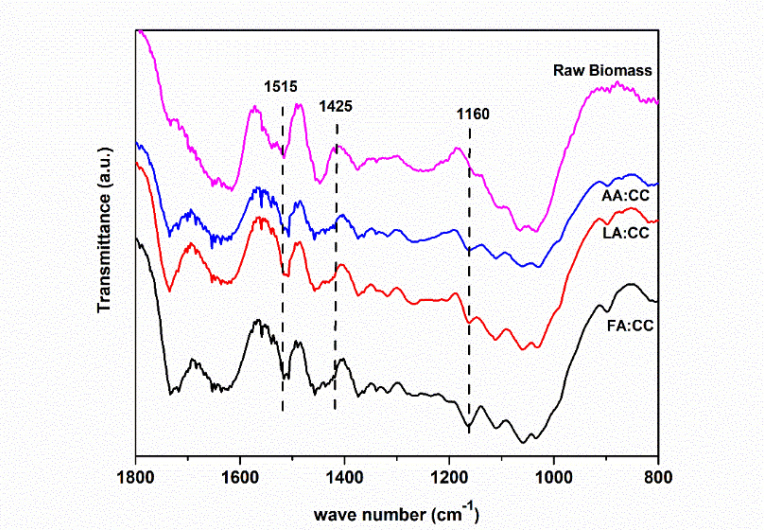

Figure 4-4 FTIR transmission spectra of raw and FA:CC, LA:CC and AA:CC pretreated biomass

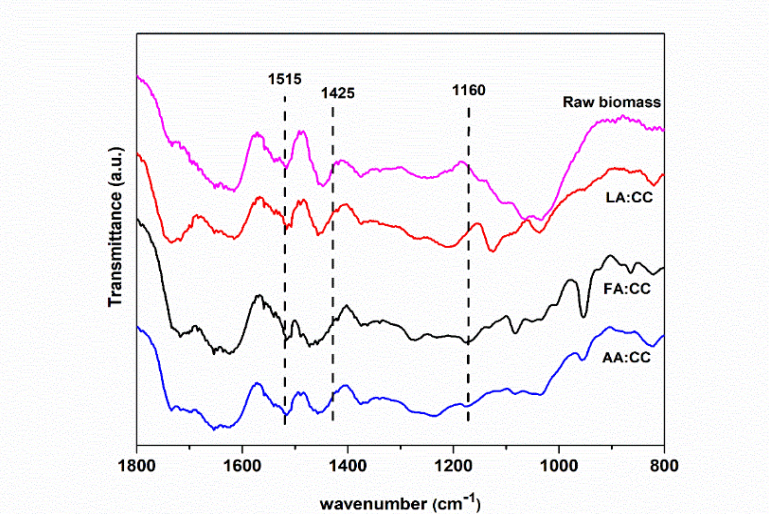

Figure 4-5 FTIR transmission spectra of raw and FA:CC, LA:CC and AA:CC precipitated biomass

Pretreatment Index was calculated using the following equation:

$$\text{PI} = \frac{CI\ ratio_{pretreated\ sample}}{CI\ ratio_{raw\ pine\ needles}} \quad (3)$$

CI ratio is the cellulose to lignin ratio as found from the selected FTIR vibrational area of (1425 cm^{-1}+ 1160 cm^{-1})/ 1515 cm^{-1}.

The calculated pretreatment indexes (PI) in Figure 4-6 clearly show that the biomass treated with FA:CC and LA:CC gave high PIs indicating high cellulose to lignin ratios compared to the PI of the raw biomass. This analysis method also confirmed that lignin in pine needle samples was removed by the DES pretreatment using FA:CC and LA:CC, but to a lesser extent by AA:CC. The low PIs shown from the lignin precipitate by these FA:CC and LA:CC reveal a high lignin content.

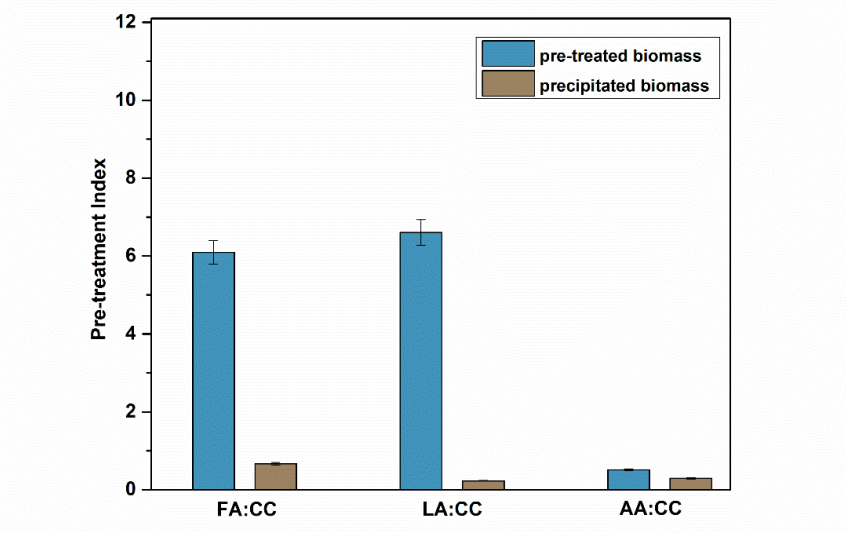

Figure 4-6 Pretreatment index of DES pretreated pine needles

These findings confirm the mass yield data above for three DES, which showed reduced mass yield by these two DES, but higher mass yield after AA:CC pretreatment. FA:CC pretreated pine needles showed the most pronounced lignin peaks, and also resulted in the highest glucose yield after enzymatic hydrolysis, as discussed in the next section.

4.3 Enzymatic Hydrolysis

To evaluate the efficiency of the DES pretreatment to enhance the cellulose accessibility to hydrolytic enzymes, the rates of conversion of cellulose into glucose during enzymatic hydrolysis of raw and pretreated biomass were measured. The glucose yields (the ratio of glucose liberated by enzymatic hydrolysis to glucose that exists as cellulose in the raw or pretreated biomass) as a function of hydrolysis time for pine needles are shown in Figure 4-7.

Figure 4-7 shows glucose yield versus enzymatic hydrolysis time, after pretreatment in various DES at 1 hour at 125 $^{\circ}$C, as well as for raw biomass. As shown in Figure 4-7, a very low concentration of glucose was liberated for the pine needles that did not undergo DES pretreatments. This result indicates the necessity of pretreatment preceding enzymatic hydrolysis to change some structural characteristics of pine needles and to increase cellulose accessibility to hydrolytic enzymes in order to provide high sugar yield, due to the recalcitrant lignocellulosic structure of the biomass. The liberated glucose yield for FA:CC pretreated pine needles was the highest after 72 h. The glucose yield for the rest of the samples lie below the FA:CC pretreated sample.

The mass yield results showed that FA:CC pretreated pine needles had the lowest mass yield compared to the other samples, indicating that increased biomass components removal allowed more enzyme accessibility, and as a result a higher sugar yield was obtained. Among the three pretreated samples AA:CC had the highest mass yield, and all the three DES pretreated samples shows constant hydrolysis glucose yield for 24 h and 48 h that then increased slightly when hydrolyzed for 72 h.

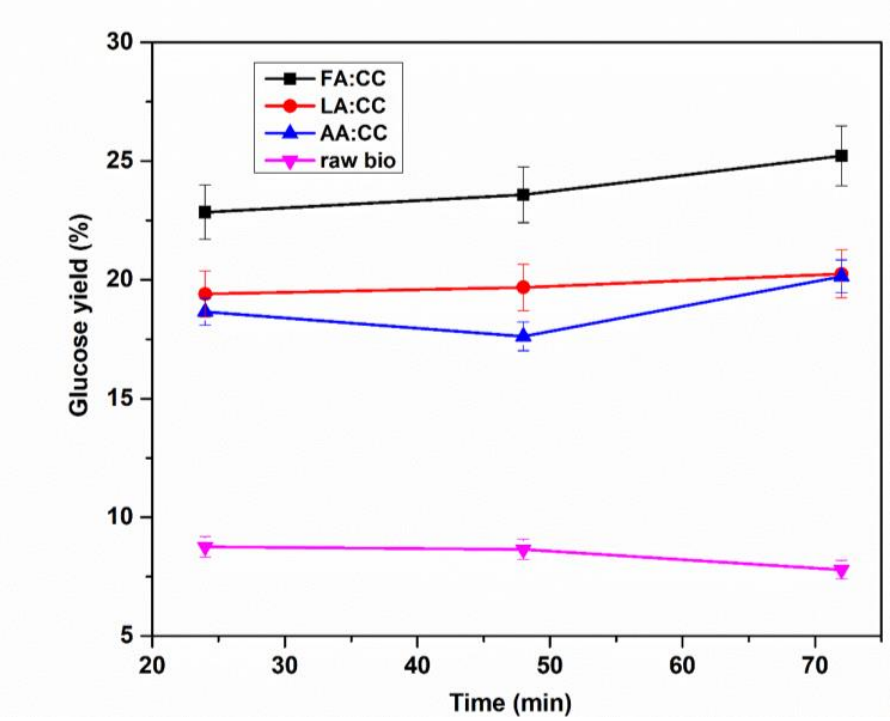

Figure 4-7 Glucose yield of Loblolly pine needles treated with 3 types of DES

In Figure 4-7, standard error bars are shown. The chromatogram from HPLC of the analyzed sugars from enzymatic hydrolysis (Figure 4-8) illustrates the increased sugar concentration in pretreated biomass. Figure 4-9 illustrates the chromatogram obtained with precipitated biomass showing less sugar in biomass treated with FA:CC indicating increased lignin concentration in the precipitated biomass and increased concentration of sugar in the pretreated biomass (the scales for these graphs are different). The precipitated biomass with AA:CC pretreatment exhibits a higher sugar concentration as it is less lignin-concentrated when compared to precipitated biomass from FA:CC and LA:CC. Thus, the chromatograms of pretreated and precipitated biomass suggest that sugar concentration in the pretreated biomass was increased due to separation of lignin from the biomass with DES pretreatment. When compared to raw biomass, all the pretreated pine needle samples showed an increase in glucose yield indicating that the pretreatment of pine needles was

beneficial in the separation. AA:CC was the least efficient, while FA:CC was the most efficient DES for obtaining higher glucose yields during hydrolysis.

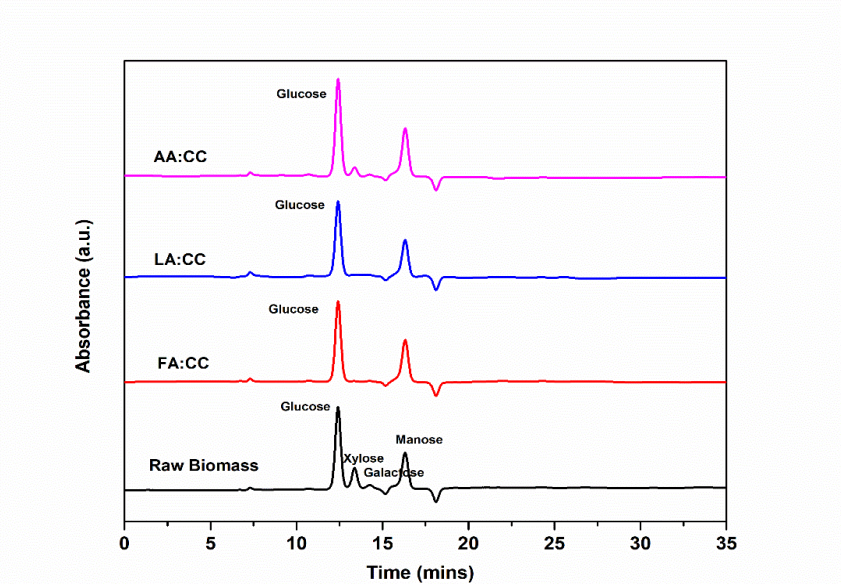

Figure 4-8 Chromatogram of pretreated biomass

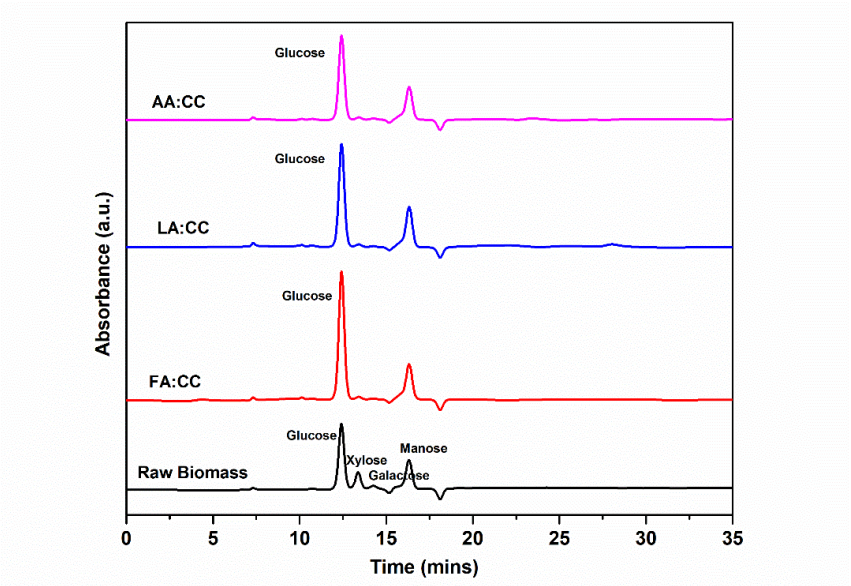

Figure 4-9 Chromatogram of precipitated biomass

4.4 Fiber Analysis

Structural compositions of the samples that were treated with DES were

characterized with a fiber analysis process from NREL, as described above. Raw biomass and biomass pretreated with DES were compared using data obtained from fiber analysis. It was clearly understood from the data that biomass that was pretreated with DES biomass was more cellulosic, and less concentrated in lignin when compared to raw biomass. In the case of AA:CC composition of the pretreated biomass showed negligible change when compared to the respective raw biomass, indicating that no specific biomass fraction was removed selectively during pretreatment. This explains why there was little improvement observed in the enzymatic saccharification of AA:CC pretreated pine needles, when compared to the respective raw biomass sample. In other DES pretreated biomass samples, lignin and hemicellulose concentration decreased, indicating a corresponding increase of the relative cellulose concentration.

When analyzing the composition of pretreated biomass, it was found that the highest change in cellulose and lignin components was observed in biomass pretreated with FA:CC. This result explains the faster enzymatic saccharification obtained. LA:CC pretreated biomass showed improved cellulose and lignin separation compared to biomass treated with AA:CC. Glucose yield obtained after enzymatic hydrolysis would be expected for FA:CC and LA:CC pretreated biomass compared to AA:CC pretreated biomass as FA:CC and LA:CC are more effective than AA:CC.

From the above findings it can be seen that DES solvents can be used for pretreatment of pine needle biomass, resulting in more cellulosic biomass and increased enzymatic saccharification. Regardless of the type of DES, pretreatment does affect enzymatic saccharification. In Figure 4-10, UV visible spectra show the concentration of acid soluble lignin that was expected to dissolve with the acid treatment. The AA:CC

pretreated sample exhibits the highest lignin absorbance, indicating that less lignin was removed.

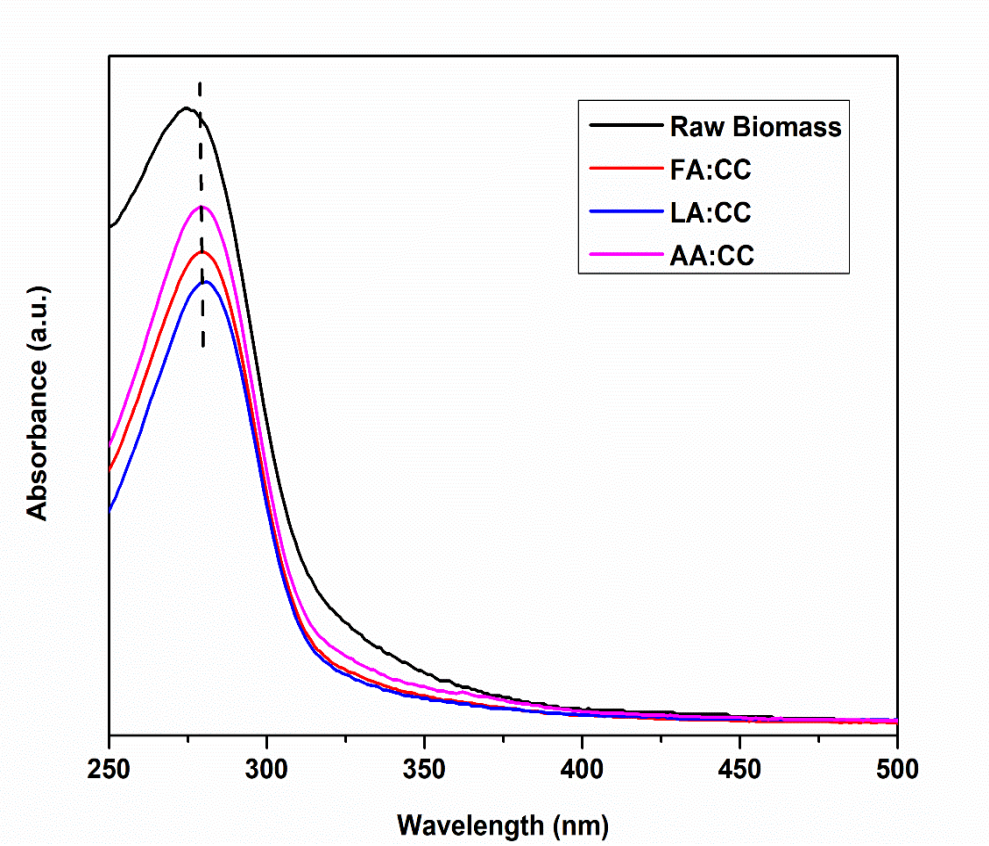

Figure 4-10 UV-Visible spectra of acid soluble lignin

In the pretreated samples ash content was increased compared to relevant raw biomass. It has been reported that these inorganics play a significant role as catalyst in the process, disrupting hydrogen bonds between crystalline cellulose biomacromolecules (Yang H 2014).

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

In the present study three different kinds of DES were synthesized and pretreatment efficacy investigated for each type of DES with Loblolly pine needles. Despite its recalcitrance, pine needle biomass had the potential to act as a substrate for pretreatment with significant DES. From the mass yields obtained it was clear that some portion of lignin was removed from the sample efficiently with one of the DES, FA:CC. Enzymatic hydrolysis data showed that DES pretreatment enhanced the glucose yields significantly after enzymatic saccharification. FTIR analysis of DES-pretreated biomass samples indicated that the enhanced glucose yield was due to removal of lignin during pretreatment, so that the pretreated biomass was rich in cellulosic content. FTIR analysis also confirmed the precipitated lignin was of high purity. Based on the results shown in this study, among the three types of DES, FA:CC pretreatment had the potential to serve as an alternative to existing technologies for biomass pretreatment. FA:CC pretreatment of Loblolly pine needles has potential to enhance the production of biofuels from glucose and also to separate lignin, which has wide range of applications.

5.2 Future Work

An integrated process of pretreatment with DES should be developed, considering the complexity of biomass composition and copious availability of forest residues. Such work could significantly increase the conversion of lignocellulosic biomass to high value products. Various conditions for optimum pretreatment of lignocellulosic biomass should be verified with a range of DES to increase the saccharification and lignin separation for industrial application. Investigation of the application of a lignin component in pine needle modified when treated with DES for use as a color dispersing or coloring agent could introduce new applications for using lignin as a natural dye.

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