Unlocking Bamboo's Biofuel Potential: A Delignification and Crystallinity Study Using Deep Eutectic Solvent Pretreatment

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Presented By:

Tyler Gambon

Zachary Manfredi

Kathleen Buek

Kathleen Buek

Project Advisor: Professor Michael T. Timko

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ABSTRACT

This study examined the impact of physicochemical pretreatment for biofuel production on *P. nuda* bamboo using ball milling and deep eutectic solvents (DES). The structural and compositional changes of pretreated *P. nuda* were analyzed. It was observed that DES recrystallized *P. nuda* cellulose, while ball milling improved delignification during DES. The project determined that ball milling before and after DES can promote amorphization and delignification. Recommendations were made for potential improvements to our methodology and future DES research opportunities.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

With the escalation of the climate crisis, largely due to the globalizing and industrializing modern landscape powered by fossil fuels, an alternative sustainable fuel source is needed. A sustainable fuel source, such as biofuel, will aim to provide a carbon-neutral solution to its fossil fuel counterpart. Biofuels are produced from the breakdown of biomass into simple sugars and the further fermentation of those sugars. There are various categories of biofuels based on the type of biomass utilized in its production, including first, second, third, and fourth generation. This study focuses on second generation biofuels, which are produced with non-edible lignocellulosic biomass feedstocks.[1]

Lignocellulosic biomass are non-edible crops, as well as agriculture and forestry waste, which is advantageous because they largely avoid the ethical and environmental conflicts associated with using two valuable resources: food and land.[1] The lignocellulosic biomass that this study provides an in-depth analysis of is bamboo, specifically *Phyllostachys nuda (P. nuda)*. Bamboo not only has the advantages of other lignocellulosic biomass, but it is also fast growing, adaptable to a wide range of growing conditions, and widely available.[2] While there are many advantages, it also has its challenges due to its structure and composition, which impede enzymatic breakdown during biofuel production.

Bamboo is composed of three main components: cellulose, hemicellulose, and lignin.[1] The crystalline structure of cellulose limits enzymatic digestibility, making it difficult to reach interior fibers. Lignin provides the structure and rigidity for bamboo, and further protects against degradation, which restricts enzyme access to the desired cellulose components.[3] To overcome these challenges, pretreatment must be utilized to improve the efficiency of subsequent enzyme hydrolysis.

While pretreatments are a necessary step to achieve high yields in second generation biofuel production, they are often a significant driver of production costs and difficult to scale-up in industrial applications.[1] There are distinct pretreatment categories, which target the various challenges to enzymatic breakdown. For bamboo, a combination of physical and chemical pretreatments, or physicochemical, targets both the structural and compositional challenges found in bamboo. Where physical pretreatments, such as ball milling, disrupt the crystalline structure, resulting in amorphous cellulose, the objective of chemical pretreatments is to remove lignin and other unwanted biomass components.[3] There are many chemical pretreatments that target delignification, however the majority are expensive, hazardous, and wasteful. Deep eutectic solvents (DESs), specifically choline chloride-lactic acid (ChCl/LA), however, differ from other chemical pretreatments. DES is inexpensive and safe, as well as considered a “green solvent”, meaning it is biodegradable and recyclable.[3] It is a promising pretreatment method, however it is a viscous solution, making it challenging at times to work with.

The overarching objectives of the laboratory experimentation that was completed for this project, which will be accordingly discussed later, were to ascertain the functional relationship between the order of the physical ball milling and DES physicochemical pretreatments and both the extent of recrystallization and degree of delignification for *P. nuda.* In this manner, this project aimed to gain valuable insights into the structural and compositional changes associated with *P. nuda* bamboo using various pretreatment strategies for biofuel production. Overall, the developed methodology and results from this study will provide the foundation for future research of lignocellulosic biomass in biofuel production, specifically in the context of physicochemical pretreatment with DES.

BACKGROUND

The overarching objectives of the laboratory experimentation that was completed for this project, which will be accordingly discussed later, were to ascertain the functional relationship between the order of the physical ball milling and deep eutectic solvent (DES) physicochemical pretreatments and both the extent of recrystallization and degree of delignification for *P. nuda*. Throughout this chapter, we first discuss the potential growth in popularity of biofuels derived from lignocellulosic biomass in the energy production field through the renewable energy transition. We then provide preliminary information on the physical and chemical properties of lignocellulosic biomass, more specifically bamboo, that makes it a promising and viable candidate for expanded biofuel production, as well as the current challenges faced in commercializing the process. Finally, to encapsulate the scale and scope of our project we provide a comprehensive overview of the current state of research on bamboo feedstock pretreatments, as well as the ball milling and deep eutectic solvent (DES) pretreatments representing the focus of our team's research. Appendix A has more details about the abbreviation nomenclature encountered in this study.

2.1 Biofuels & the Renewable Energy Transition

Amid rising concerns over energy security and the growing pressures of climate change, biofuels have begun to attract global attention as a potential sustainable alternative to conventional fossil fuels.[4] Paleoclimate evidence found in ice cores, tree rings, ocean sediments and other natural repositories suggest that Earth is heating up approximately ten times faster than the average rate of warming following an ice age, with anthropogenic carbon dioxide emissions escalating approximately two hundred and fifty times more rapidly than those caused by natural sources after the last ice age (See Figures 1 and 2).[5] Today, the combustion of fossil fuels that have supported globalization and industrialization add about forty billion metric tons of carbon dioxide into Earth's atmosphere each year, with transportation accounting for about twenty three percent of total energy-related carbon dioxide emissions.[6,7] The primary issue with conventional fossil fuels that are extracted from the Earth's crust, like coal, oil, and natural gas, is that they represent a nonrenewable and carbon positive source of energy, meaning that their usage creates a net addition of carbon and harmful greenhouse gasses into the atmosphere. Biofuels were an established concept long before the discovery of the conventional fossil fuels we know today, and yet the substantial natural reserves of gas, crude oil, and coal at the time of their discovery combined with the corresponding affordability of such fuels made biofuels essentially obsolete.[8] Today, the aforementioned concerns over the environmental destabilization of our planet, rapid depletion of conventional fossil fuel reserves, and escalation of crude oil prices contribute to a renewed emphasis on the development of sustainably-sourced biofuel alternatives.

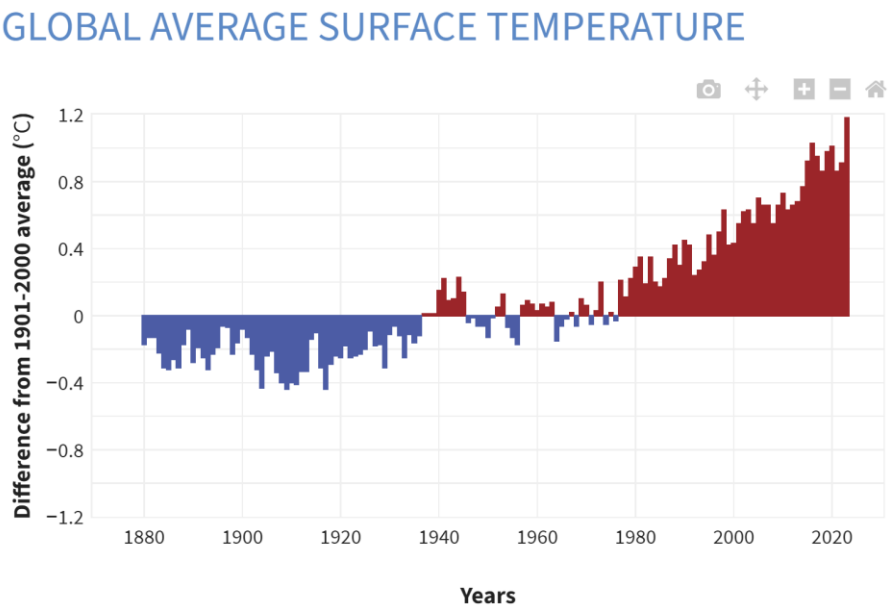


Figure 1. Variation of global average surface temperature relative to the global average surface temperature over the last century (1901-2000).[6]

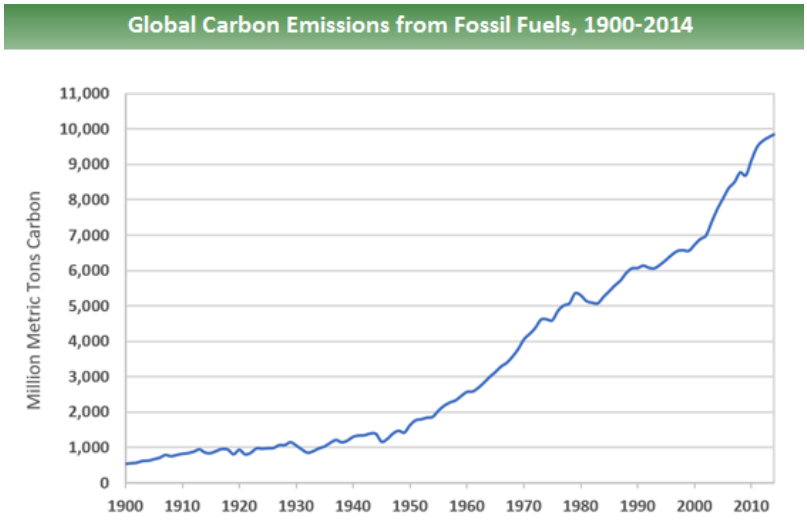


Figure 2. Global carbon emissions from fossil fuels over the time span of 1900-2014.[9]

In contrast, biofuels as a renewable alternative to conventional fossil fuels represent a potentially more carbon-neutral solution to energy security and the transportation sector's contribution to climate change. While biofuels undergo a similar combustion process to conventional fossil fuels that releases greenhouse gasses into the atmosphere, the plants from which those feedstocks are derived also work to accumulate atmospheric carbon dioxide for photosynthesis and growth.[10] As a result, the carbon dioxide emissions generated from the combustion of biofuels could potentially be offset by the uptake of carbon dioxide by these plants in order to perform routine biological functions, thus potentially reducing or eliminating the net addition of carbon dioxide to the atmosphere.

Bioethanol and biodiesel have been linked to a much cleaner combustion process than conventional fossil fuels and generally produce fewer greenhouse gas emissions during their usage.[1] In contrast, the energy density of bioethanol and biodiesel has typically been found to be slightly less than that of gasoline produced from conventional fossil fuels, while also generally coming in at a slightly higher cost for consumers due to their limited market penetration at present. There is also still significant work to be completed to help transform energy and transportation infrastructure for biofuels and ensure compatibility of bioethanol and biodiesel with vehicle internals and overall distribution networks.[1] Nonetheless, there exists an immense potential for expanded usage and commercial growth of sustainably sourced biofuels that can fundamentally re-envision modern energy and transportation landscapes.

Furthermore, acknowledging renewable biofuels as a viable emerging substitute for their carbon-neutrality is just one piece of a complex puzzle. It is also important to understand the various biofuel classifications (first, second, third, and fourth generation) and their key advantages and disadvantages. To begin, first generation biofuels are generally derived from edible energy crops that contain a significant composition of sugar, starch, or edible oils, as well as various animal fats.[1] Common feedstocks therefore include corn, potatoes, sugarcane, vegetable oil, and soybeans.[1] Importantly, edible energy crops also have a reputation for low production costs, are easy to grow, and typically require minimal maintenance. However, since these first-generation biofuel feedstocks are also often edible crops, there exists significant public concern surrounding the tradeoff between producing biofuel feedstock and growing food for direct human consumption.[1] In general, land and water being used to grow feedstock for biofuel production is land and water which would otherwise be used to grow food and provide clean drinking water for communities, thus first generation biofuels pose the risk of contributing to food scarcity, particularly in marginalized and underdeveloped regions of the world. Efforts to develop enough arable cropland to meet future demands for first generation biofuels may also contribute to existing ecological concerns surrounding deforestation and habitat fragmentation, providing yet another reason to remain cautiously optimistic about the current state of first-generation biofuels.

Moreover, second generation biofuels can be characterized as biofuels derived from feedstocks of non-edible lignocellulosic biomass, including nonfood crops, agricultural and forestry residues, and various types of waste biomass.[11] Second generation biofuels accordingly encompass bamboo feedstocks, and as such we will provide a more in-depth overview of second generation biofuels and the various properties of bamboo that make it a promising, although challenging candidate in biofuel research, in the following section.

Moving onward to third generation biofuels, this category primarily consists of various strains of aquatic microalgae feedstocks that are characteristically high in lipid content.[12] Microalgae are a family of aquatic organisms that can be grown year-round on non-arable land and have demonstrated stress resilience in a variety of climates, thus reducing direct competition with meeting global food demand. Microalgae have also been found to have significantly higher photosynthetic efficiencies as compared to traditional terrestrial crops, in effect allowing them to accumulate large quantities of atmospheric carbon dioxide and achieve upwards of sixty percent oil per dry biomass weight.[12] Despite these advantages, there is still significant research to be completed before third generation biofuels could become fully commercialized due to the incredibly high production costs, lack of existing capital equipment for feedstock pretreatment, and the need for nutrient-rich waters and fertilizers that can be harmful to surrounding ecosystems.[12]

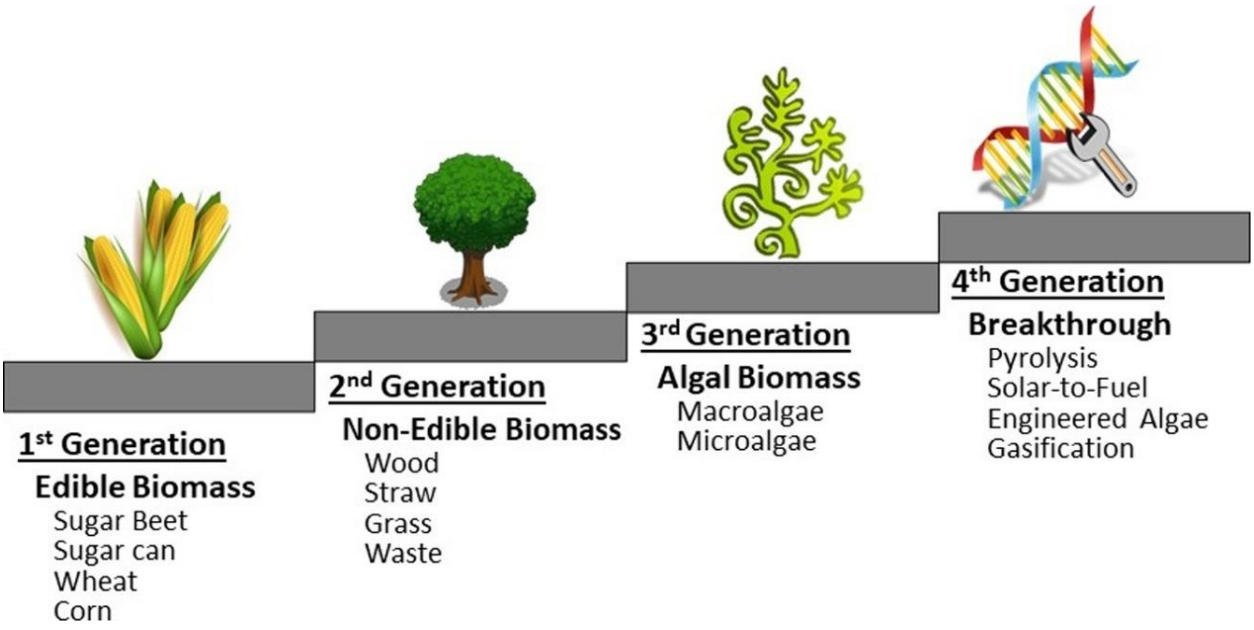


Figure 3. Basic overview and examples of developments in generational biofuels.[13]

Importantly, scientists and researchers with expertise in various disciplines surrounding biofuels have begun to recognize an additional emerging classification of biofuels known as fourth generation biofuels. According to Cornell University, these types of biofuels are derived from genetically modified plants and biomass feedstocks with traits that significantly decrease barriers to cellulosic breakdown and increase yields, thus making it easier to access and process cellulose into simple sugars via enzyme hydrolysis.[8] There is an appreciable variety of likely fourth generation biofuel feedstocks, however, an enormous amount of research still needs to be completed in the fields of genetic engineering and synthetic biology before these feedstocks can enter mainstream commercial use and be leveraged to their fullest potential. Given the issue of food insecurity in relation to first generation biofuels and the extensive research timeline associated with third and fourth generation biofuels, we will next provide an in-depth discussion on the feasibility of lignocellulosic biomass for second generation biofuels as it relates to the focus of our study.

2.2 Second Generation Biomass: Classifications, Advantages, & Challenges

As previously discussed, second generation biofuel feedstocks consist of lignocellulosic biomass from non-edible food crops, as well as waste residue from agriculture and forestry. The composition of lignocellulosic biomass primarily encompasses cellulose and hemicellulose (carbohydrate polymers), lignin (aromatic-rich polymer), and various extractives, the proportions of which vary based upon feedstock.[1] Lignocellulosic biomass feedstocks can be further classified as homogeneous (wood cuttings and wood chips), quasi-homogeneous (agricultural and forestry residues), and non-homogeneous (low-value municipal solid wastes).[1] One of the main highlights of second generation biofuels is that they do not create direct competition with meeting global food demand given that the lignocellulosic feedstocks are non-edible. Additionally, the production of biofuels from agricultural and forestry residues is generally considered to be more beneficial to the environment than first generation biofuels as it reduces the need for development of additional

dedicated cropland, in turn resulting in less habitat fragmentation, less deforestation, and greater preservation of local biodiversity.[1] Production of biofuels from lignocellulosic biomass, however, still has a significant barrier to widespread commercialization due to various technological setbacks. Unfortunately, there is currently an insufficient amount of capital equipment actively in use that can be utilized to effectively process waste byproducts from the conversion process into commercially useful products.[1] This shortage of sufficiently effective pretreatments and associated processing equipment for lignocellulosic biomass represents the primary motivation of our study and will be further discussed in the following section.

The overarching focus of our study centers around a bamboo forestry feedstock known as *Phyllostachys nuda* that can be instrumental in the production of second-generation biofuels. While *P. nuda* is a species native to Southeast Asia, particularly China, Myanmar, and India, it was introduced to the United States in 1907 and can be cultivated extensively in temperate and subtropical regions throughout the world.[2] *P. nuda* can reasonably inhabit geographic regions spanning plant hardiness zones 5-11 (around -20°F-50°F annual extreme minimum temperatures), with warmer, subtropical temperatures generally contributing to more expansive bamboo culm growth than temperate conditions.[14] Bamboo plants have also gained a reputation for their immense diversity and adaptability to a variety of environmental and climatic conditions. One of the highlights of *P. nuda* as a second-generation biofuel candidate is its tolerance to humidity, heat, and verticillium wilt, enabling the plant to grow in permanently humid regions near water bodies, as well as locales with extended dry seasons.[15] *P. nuda* additionally requires occasional to regular water uptake and grows best in well-drained soil that is high in clay content, although it is rather adaptable to different soil conditions.[15] As a result of the resilience and flexibility of *P. nuda*, this species of bamboo is capable of inhabiting widespread geographic and climate distributions. This exceptional versatility, combined with the relatively inexpensive and efficient cultivation practices, and its evergreen growth pattern notably makes *P. nuda* a strong candidate for second generation biofuel production.

2.3 Pretreatment of Lignocellulosic Biomass for Enhanced Biofuel Production

Lignocellulosic biomass, such as the *P. nuda* bamboo that this study focuses upon, are readily available and economical raw material feedstocks. However, due to the inherent composition and crystalline structure of lignocellulosic biomass, the enzyme digestibility of the material is relatively low, meaning it is significantly more difficult to break the biomass down into simpler substances.[3] The general process for biofuel production consists of an enzyme hydrolysis step that results in the conversion of cellulose to simple sugars (primarily glucose) via the enzyme-facilitated cleavage of molecular bonds with water, followed by a subsequent fermentation process to convert the simple sugars to bioethanol.[1] Given the low enzyme digestibility characteristic of lignocellulosic biomass, biofuel production is found to be generally inefficient and energy intensive, thus giving rise to the need for various pretreatments that can improve conversion efficiency through increasing enzyme access to the polysaccharides contained within.[16] Pretreatment of second-generation biomass is necessary for obtaining high yields of simple sugars from cellulose, however, current methods of pretreatment in more widespread usage can also be considerably expensive and give rise to the need for more economical processes.[17] A detailed overview of the overall process can be seen below in Figure 4.

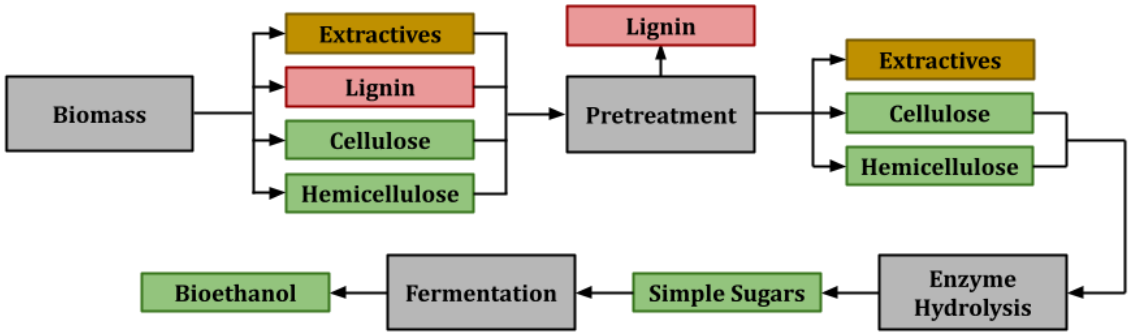


Figure 4. Simplified overview of the conversion of lignocellulosic biomass to bioethanol.

Accordingly, lignocellulosic biomass is primarily composed of cellulose and hemicellulose (carbohydrate polymers), lignin (aromatic-rich polymer), and traces of various extractive compounds as discussed in the previous section. Of these components, only cellulose and hemicellulose can be converted to the fermentable simple sugars needed to produce biofuel.[18] Cellulose polymer chains act as the main structural elements of plant cell walls, and are accordingly made of repeating monomer units called cellobiose.[16] These chains become packed due to the hydrogen and van der Waals bonds linking the long-chained cellulose, in effect forming microfibrils.[3] Most of the cellulose chains in lignocellulosic biomass are oriented in a crystalline structure, with a small fraction being amorphous or unorganized.[3] Notably, cellulose polymer chains are more susceptible to enzymatic breakdown when present in their amorphous form, thus the crystallinity (or lack thereof) of cellulose greatly impacts the enzyme hydrolysis process. Moreover, while hemicellulose, like cellulose, converts into simple sugars, it greatly differs in structure. Hemicellulose is naturally more amorphous and composed of smaller chains, which are easier to break down than cellulose.[3]

Lignin provides the cohesion of the different components of lignocellulosic biomass and is located in the primary cell wall, giving it structure and rigidity.[19] This structure and rigidity is a result of the crosslinking that occurs between the polysaccharides (cellulose and hemicellulose) and lignin via ester and ether linkages. The material strength and hydrophobic nature of lignin allow it to effectively protect the plant from severe water damage and microbial attacks. Unfortunately, due to the high recalcitrance associated with lignin, it is more difficult for enzymes to properly access and hydrolyze the internal cellulose and hemicellulose components of lignocellulosic biomass, effectively inhibiting the conversion process to biofuel.[19] Even without the significant challenges of lignin, the naturally crystalline structure of cellulose polymer chains impede the conversion efficiency.[20] The crystalline structure of cellulose makes it more challenging and time consuming for enzymatic degradation due to the inability to reach interior fibers.[19] Therefore, to more effectively convert lignocellulosic biomass to biofuel, pretreatments must be utilized to depolymerize the cellulose and increase porosity, as well as to remove lignin, as seen in Figure 5.

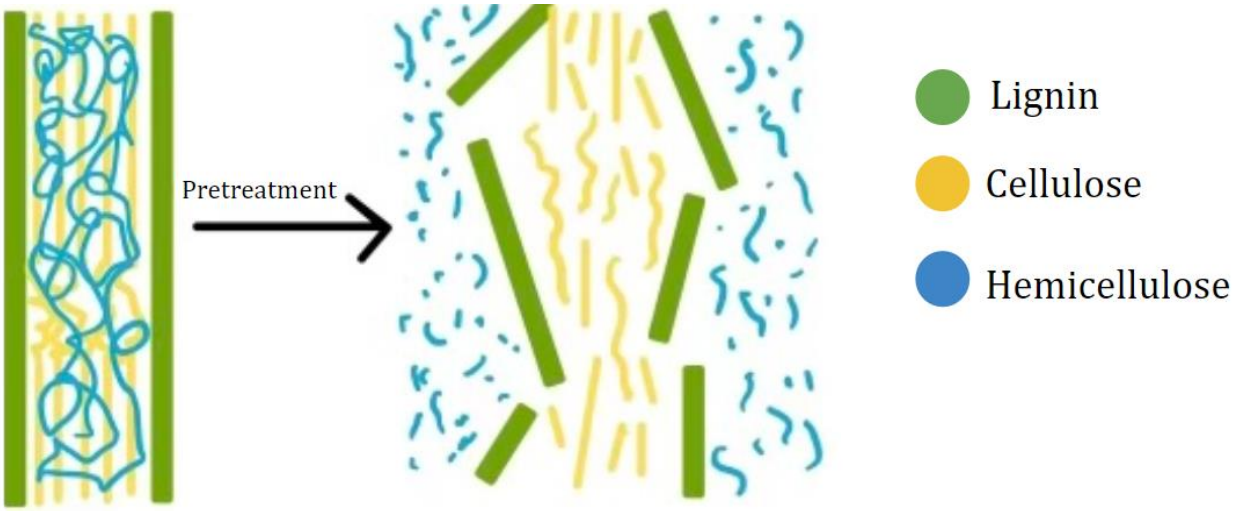


Figure 5. Schematic demonstrating the impact of pretreatments on the various components of lignocellulosic biomass.[16]

Furthermore, there exists a wide variety of biomass pretreatments designed to target different challenges associated with the structure and composition of lignocellulosic biomass. These pretreatments generally fall into two primary categories: physical, chemical, as well as combinations of both types. Biological and electrical pretreatments also exist within research literature, but they are not as commonly utilized for lignocellulosic biomass.[3] Common physical pretreatments for biomass include extrusion, grinding, and ball milling, and target structural changes in the lignocellulosic biomass rather than compositional changes.[3] In the case of dry ball milling for instance, this type of pretreatment "promotes the deconstruction of biomass structure by mechanical energy from impact, compression, attrition, and shear between balls, biomass, and surface reactor." [21] In this manner, physical pretreatments aid in disrupting the crystalline domains of cellulose and breaking up the bulk material to increase access to the polysaccharides within the biomass. In addition, the crystallinity and particle size of the cellulose depends significantly on the duration of the physical pretreatment.[22]

Figure 6. The effect of dry ball milling duration on bamboo fibers.[22]

As seen in Figure 6, the longer duration under physical pretreatment results in finer bamboo powder particles. This further corresponds to higher sugar yields due to improved enzymatic digestibility of the cellulose and hemicellulose, as previously discussed.

While physical pretreatments target the structure of lignocellulosic biomass, chemical pretreatments mainly focus on manipulating the composition to improve conversion efficiency. More specifically, these treatments aim to reduce the amount of lignin in the bulk material, as lignin inhibits polysaccharide accessibility, while maintaining the cellulose composition.[3] Table 1 below highlights some of the most commonly studied chemical pretreatments in the existing literature.

Table 1. Overview of commonly studied chemical pretreatment methods and their associated advantages and disadvantages.

Chemical Pretreatment	Inexpensive	Nonhazardous	Reusable Solvent
Deep Eutectic Solvent (DES)	✓	✓	✓
Alkaline	✗	✓	✗
Organic Solvent	✗	✗	✓
Ionic Liquid	✗	✓	✗
Acid	✓	✗	✗
Co-Solvent Enhanced Lignocellulosic Fractionation (CELF)	✓	✗	✓

There are advantages and disadvantages to the various chemical pretreatments: deep eutectic solvent (DES), alkaline, organic solvent, ionic liquid, acid, and co-solvent enhanced lignocellulosic fractionation (CELF). To start, alkaline pretreatments are primarily utilized for removing components that inhibit cellulose accessibility (lignin and acetyl groups) through the saponification of ester bonds, in effect breaking up some of the cross linkage present in the biomass structure.[3] This method notably uses menial energy consumption, but unfortunately is known to be expensive, requires long residence times, and generates significant amounts of wastewater.[3] Likewise, organic solvent pretreatments remove primarily lignin and hemicellulose from biomass through hydrolyzing the internal bonds in the compounds. Organic solvent pretreatments are additionally able to recover lignin for downstream processing, however, the high volatility of these solvents and expensive separation required for lignin recovery give rise to shortcomings for this method.[3] Ionic liquid pretreatments utilize organic salts with generally large organic cations and small inorganic anions, and like organic solvent pretreatments, are relatively expensive processes.[3] Ionic liquids can effectively dissolve cellulose through the formation of hydrogen bonds between the anions from the ionic liquid and cellulose, but unfortunately require difficult, energy-intensive separations to remove the lignin and hemicellulose remaining in the pretreatment mixture.[3] Further, acid pretreatments for lignocellulosic biomass are amongst some of the most well-studied in the literature and may be carried out under a variety of conditions with both dilute and strong acids. This pretreatment generates hydronium ions that in turn depolymerize hemicellulose into its constituent monomers with the end goal of making the cellulose more accessible for enzyme

digestion.[3] Acid pretreatment methods can also be quite useful for lignin removal. The primary drawback of acid pretreatments is the hazardous and difficult to handle nature of the required chemicals, as well as the potential for formation of various inhibitors that impact microbial growth, and therefore enzyme digestibility of the cellulose.[3] Finally, originally pioneered by researchers at the University of California, Riverside, CELF pretreatments involve the reaction of lignocellulosic biomass with dilute acids in mixtures of tetrahydrofuran and water.[23] The aforementioned co-solvent has been found to make lignin more susceptible to dissolution and acid-catalyzed polymeric breakdown, but can be hazardous in processing environments despite being inexpensive and eco-friendly.[24] In the next section, we will discuss the final pretreatment represented in Table 1: DES, which serves as the main focus area of the studies our team conducted.

To achieve the greatest yield of fermentable simple sugars from lignocellulosic biomass via enzymatic hydrolysis, a combination of physical and chemical pretreatments (physicochemical pretreatment) is most advantageous. Physicochemical pretreatments collaboratively target the main challenges of lignocellulosic biomass: structure and composition. Physical pretreatment breaks down crystalline cellulose to improve access to interior fibers and the chemical pretreatment removes lignin and other components that inhibit enzyme digestibility. It is worth noting that due to the hydrophilic nature of bamboo, the introduction of water may result in the crystallization of amorphous cellulose.[25] As a result, this study conducted by our team worked to better understand the importance behind the order of such physicochemical pretreatments, specifically how this impacts biomass crystallinity and composition in preparation for enzyme hydrolysis.

2.4 Delignification Using Deep Eutectic Solvent (DES) Pretreatment

The focus of this study centers upon, DES, a relatively new and emerging pretreatment in biorefinery research that has gained attention for its affordability, safety advantages (nontoxic, nonvolatile, nonflammable), eco-friendliness, and ease of preparation.[26] The characteristic biodegradability and potential for DES to be recycled and reused has contributed to its reputation as a "green solvent", unlike many other common pretreatment processes. While ionic chemical pretreatments also make use of "green solvents", they require relatively energy-intensive separations to remove lignin and are therefore less eco-friendly.[3]

DESs are ionic solvent systems containing a hydrogen bond acceptor and donor, in which the resulting compound has a lower melting point than its constituents and the solvent is particularly effective at dissolving other substances.[27] DESs differ from ionic liquids in that they form a eutectic mixture from either Lewis or Brønsted acids and bases containing an exceptional diversity of cationic and anionic species, whereas ionic liquids are composed exclusively of one type of discrete cation and anion.[28] DES pretreatments also have a high degree of customizability for different applications due to the various combinations of hydrogen bond acceptors and donors available (See Figure 7 below), as well as their programmable mixing ratios.[26]

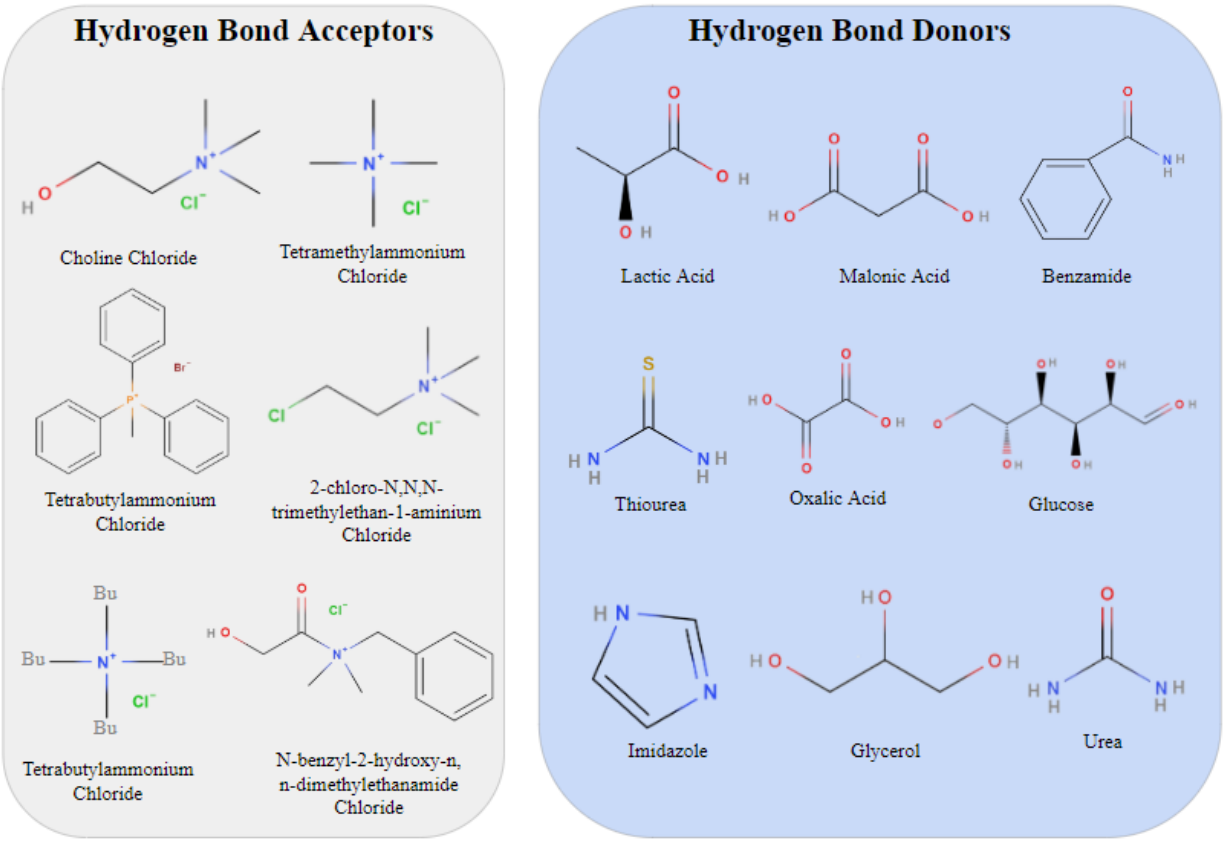


Figure 7. Common hydrogen bond acceptors and donors that make up DESs.[28]

DESs contain relatively large, asymmetrical, and distantly spaced ions, thus giving rise to weaker electrostatic forces between ions, and consequently, a low lattice energy.[28] The low lattice energy and charge delocalization arising from hydrogen bonding are characteristic of DES systems and are responsible for the melting point being significantly lower than that of the original component molecules. Accordingly, DES systems are also found to exhibit much higher viscosities and lower conductivities relative to conventional ionic liquids.[28] While the extensive network of hydrogen bonding leads to higher viscosities via greater intermolecular forces, it is further hypothesized by Smith et al. that the relatively large ion size and free volume in DES systems additionally contributes to the observed high viscosities.[28] While DES is a promising new pretreatment in biorefinery research, the highly viscous solutions can be difficult to transfer to industrial application, leading some experts to suggest the addition of water to such systems to help improve this characteristic.[27]

DES systems can be further classified into types I to IV (See Table 2) based upon the chemical behavior of the complexing agent utilized. The general formula in Eq. 1 below describes a deep eutectic solvent, where Cat^+ represents any ammonium, phosphonium, or sulfonium cation, X^- is a Lewis base (typically a halide anion), Y is a Brønsted acid, and *z* references the number of Y molecules interacting with the anion[28]:

$$Cat^+X^-zY \quad (1)$$

Type I DESs are formed from quaternary ammonium salts and Brønsted acids of the form MCl_x, where M represents one of various metals forming the non-hydrated metal halide.[28] Accordingly, type II DESs differ slightly with a Brønsted acid of the form $MCL_x \cdot yH_2O$ representing hydrated metal halides. Type II DESs are known to be generally less expensive than type I and exhibit specific properties more conducive to industrial scaleup.[28] Furthermore, type III DES systems are characterized by having a Brønsted acid of the form RZ, where RZ represents a hydrogen bond donor. DESs of this subclassification have gained attention for their incredible adaptability and wide range of physical properties rooted in the many options for hydrogen bond donors.[28] Finally, the type IV class of DES systems consists of a complexation between a metal halide and hydrogen bond donor, although these types of DESs are generally less studied in the literature.

Table 2. Overview of DES classifications.[28]

Type	Components	General Formula	Example
I	Metal Salt+Organic Salt	$Cat^+X^-zMCl_x$	$ZnCl_2$+ChCL
II	Metal Salt Hydrate+Organic Salt	$Cat^+X^-zMCl_x \cdot yH_2O$	$CoCl_2 \cdot 6H_2O$+ChCl
III	HBD+Organic Salt	Cat^+X^-zRZ	$C_3H_6O_3$+ChCl
IV	Metal Salt+HBD	$MCl_x + RZ = MCl_{x-1}^+ \cdot RZ + MCl_{x+1}^-$	$ZnCl_2+C_3H_6O_3$

DES pretreatments can selectively target ether and ester linkages between lignin and polysaccharides to effectively remove lignin from the biomass. At the same time, the strong hydrogen bond found in DES solutions decreases the interaction between DES and cellulose, and therefore removes less cellulose during pretreatment than other common methods.[26] Not only does DES have delignification applications, but it also can be used to chelate metals ions, which are found in small quantities in biomass due to bioaccumulation from the soil.[29] Type III DESs (most commonly containing choline chloride with a carboxylic acid-based hydrogen bond donor) can be efficient in the extraction of metal ions due to the formation of chelate complexes with the hydrogen bond donor in the DES.[30] This is an especially important property of type III DESs since certain metal ions can serve as inhibitors to microbial enzyme activity through their redox behavior, although there is still much work to be completed to fully understand such interactions.[31] For the interest of this study, type III DES and its applications were studied (See in Table 3).

Through a literature review of various applications of DES pretreatment on biomass for improved enzymatic digestion, choline chloride (ChCl) and lactic acid (LA) ($ChCl+C_3H_6O_3$) with a high mixing ratio above 1:9, was found to be a viable candidate for treating *P. nuda* bamboo. This DES system can encourage hydrogen bonding, polarity, π-π, and ionic/charge interactions with the lignin component of biomass that ultimately encourage extraction.[32] Additionally, the lactic acid in the DES system has been found to contribute to hemicellulose hydrolysis, and in turn improves lignin extraction and cellulose enzyme accessibility.[32]

Table 3. Applications of type III DESs in biofuel production.

DES	Biomass	Conditions	Results	Reference
ChCl/LA (1:9)	Tortoise-shell Bamboo (*Phyllostachys pubescens)*	Reacted in an 80℃-120℃ oil bath for 2 hours	23.28% lignin recovery at 80℃ for 2hr. 91% lignin recovery at 120℃ for 2hr.	Liu et al.[27]
ChCl/Urea (1:2)	Avicel & Switchgrass (*Panicum virgatum L.)*	Reacted in an 110℃ oil bath for 4 hours	Resulted in a hydrolysis rate of 0.10 $\frac{g}{Lh}$	Xia et al.[33]
ChCl/Gly (1:2)			Resulted in a hydrolysis rate of **0.14** $\frac{g}{Lh}$	
ChCl/LA (1:10)	Corncob	Reacted in an 90℃ oil bath for 24 hours	Removed 86.1% lignin, crystallinity index of 32.3% and a glucose yield of 83.2%	Zhang et al.[34]
ChCl/LA (1:15)			Removed 93.1% lignin, crystallinity index of 30.7% and a glucose yield of 79.1%	
ChCl/Oxalic Acid (1:1)			Removed 98.5% lignin, crystallinity index of 31.6 and a glucose yield of 45.2%	
ChCl/EG (1:2)			Removed 87.6% lignin, crystallinity index of 27.9 and a glucose yield of 85.3%	
ChCl/Malonic Acid (1:1)			Removed 56.5% lignin, crystallinity index of 29.5 and a glucose yield of 61.5%	
ChCl/Gly (1:1)	Sweet Corn (*Zea mays)*	DES to biomass ratio of 16:1. Reacted in an 115℃ oil bath for 6 hours	Glucose yield of 79.1% and a crystallinity index of 29.79	Procentese et al.[35]
ChCl/Urea (1:1)			Glucose yield of 58.6% and a crystallinity index of 36.54	
ChCl/Imidazole (1:10			Glucose yield of 94.0% and a crystallinity index of 40.08	

Choline chloride and lactic acid DES pretreatment on bamboo, specifically *P. nuda* bamboo, is a novel and growing subject matter. While DESs are very viscous, and therefore challenging to work with and eventually scale to industrial applications, they have many benefits, including being inexpensive, non-hazardous, and eco-friendly.[26] As a result, constructing a viable pretreatment methodology and understanding the impacts of sequential arrangement on the physicochemical pretreatment of choline chloride and lactic acid DES is absolutely crucial to furthering research in the field of alternative fuels production.

METHODOLOGY

Throughout this chapter, we provide an extensive overview of the materials and equipment utilized for our project experimentation and a comprehensive discussion detailing each of the steps in our developed pretreatment methodology. Moreover, we have included additional information on the powder X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FT-IR) techniques that were employed in the structural and compositional analysis of our pretreated samples. See Appendix B for more information on the experimental conditions and samples that were collected.

3.1 Materials & Equipment

Table 4. Required chemicals for methodology.

Name	Purity	CAS Number	Quantity	Cost (as of 2024)
Ethanol	100%	64-17-5	1 L	$169.00 (ThermoFisher)
Toluene	>99.5%	108-88-3	4 L	$99.30 (ThermoFisher)
Choline chloride	>98.0%	67-48-1	500 g	$47.00 (TCI Chemicals)
L(+)-Lactic acid	90%	79-33-4	1 L	$147.00 (ThermoFisher)
Microcrystalline cellulose, 90μm	Extra pure	9004-34-6	1 kg	$128.00 (ThermoFisher)
Phyllostachys nuda	N/A	N/A	A lot	N/A (Smithsonian National Zoo, Washington, D.C.)

Table 5. Chemical & equipment overview for each experiment.

Bamboo Fiber Preparation	
Chemicals	*Equipment*
• None	• Bench vise • Saw • Coffee grinder • Sample oven
Soxhlet Extraction of *P. nuda*	
Chemicals	*Equipment*

• Toluene
• Ethanol
	• Lab jack
	• Heating mantle
	  ○ Heat controller
	• Allihn condenser
	• Soxhlet extractor bulb
	• 500 mL two-neck angled round bottom flask
	• Aluminum foil
	• Tygon tubing
	• Cooling water
	• Thermometer
	  ○ Teflon probe holder

Ball Milling Pretreatment	
Chemicals	*Equipment*
• None	• Planetary Ball Mill (Across International, model VQ-N) • 6mm, 10mm, 20mm stainless steel grinding balls
DES Solution Preparation	
Chemicals	*Equipment*

• Choline chloride
• L(+)-Lactic acid
	• 10-dram vial
	• Hot water bath
	  ○ 500 mL beaker
	  ○ Hot plate
	• Analytical balance
	• Micropipettes
	  ○ 100-1000 μL
	  ○ 1000-5000 μL

DES Oil Bath Reaction	
Chemicals ● Choline chloride : Lactic acid	*Equipment* ● Oil hot bath ○ Metal container ○ Silicone oil ○ Hot plate stirrer with temperature probe ● Lab stand ● Clamps ● Thermometer ● Heavy wall pressure vessels (50 mL) ● Vessel stir bars
Extraction/Separation of Solids from DES	
Chemicals ● Ethanol	*Equipment* ● Centrifuge (Thermo Sorvall Legend RT Plus) ● 15 mL centrifuge tubes ● Micropipettes ○ 100-1000 μL ○ 1000-5000 μL ● Büchner funnel ● Whatman 70mm filter papers ● 800 mL sidearm flask ● Vacuum pump ● Spray bottle ● Desiccator

3.2 Bamboo Fiber Preparation

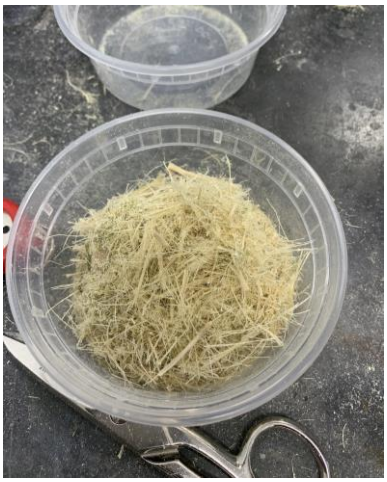
The bamboo used in these trials was obtained by James Walters (CEO of Avos Bioenergy) from Rock Creek Park in Washington, D.C. The species is *Phyllostachys nuda*, as confirmed by Dr. Mark Czarnota (agriculturist at University of Georgia). The *P. nuda* grows along the property line of the Smithsonian National Zoo. The bamboo was stored in GH 222 inside a refrigerator freezer to preserve it from mold and decay.

Figure 8. Bamboo on Rock Creek in Washington, D.C.[36]

To prepare for trials, the bamboo was secured into a bench vise and cut into two-inch pieces using a coping saw. They were then placed into a KitchenAid coffee grinder (model BCG111) and intermittently ground until clumps of fibers were achieved. The fibers were then placed into a round plastic container and dried in a 60°C oven for six hours.

a. Sawing the bamboo stalk in the bench vise.

b. Bamboo loaded into the coffee grinder.



c. Bamboo after coffee grinder.

Figures 9a-c. Preparation of bamboo for Soxhlet extraction.

The microcrystalline cellulose used in this experiment was retrieved from an existing bottle in GH 222 and did not require any preparation steps.

3.3 Soxhlet Extraction of *P. nuda*

While bamboo is mainly made up of cellulose and lignin, minor components such as resins and waxes are also present.[37] Removal of these waxes from different biomass sources like sugarcane bagasse and wheat straw has shown to increase sugar recovery from enzymatic digestibility.[38] Since the motivation of this study is to prepare bamboo for eventual enzymatic hydrolysis into sugars for bioethanol production, a dewaxing protocol was designed based on literature. A very commonly seen method for dewaxing biomass prior to bamboo delignification was Soxhlet extraction. The methods described here were adapted from Li et al.,[39] Li et al.,[40] Ling et al.,[41] Wen et al.,[42] and Bai et al.[43]

Soxhlet extraction involves the removal of organic extractives from solids by continuous reuse of a given volume of pure solvent. This process is utilized by the U.S. EPA to remove semi volatile organic compounds from sludges and wastes for future analysis.[44] A picture of the Soxhlet extraction setup used for this method is shown below.

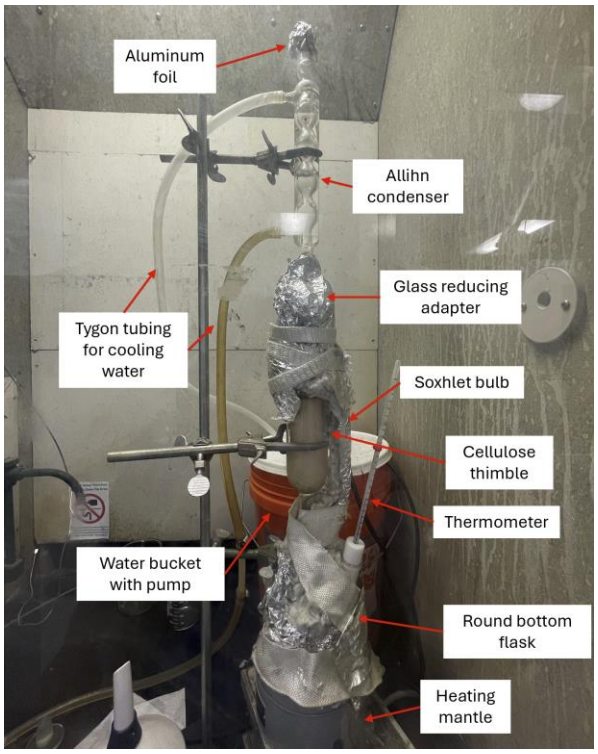


Figure 10. The Soxhlet extraction apparatus inside a fume hood in GH 222.

The setup in Figure 10 consists of (from bottom to top): a heating mantle sand bath containing the double necked round bottom flask with a thermometer in one neck and the Soxhlet extractor bulb in the other; the cellulose thimble filled with dried, ground-up *P. nuda*; connected to a glass reducing adapter to fit the Allihn condenser above. Since there is no cooling water in GH 222, a bucket with a separate pump was used to run cooling water through the condenser. Note the lab jack underneath the heating mantle, used to lower the round bottom flask at the end of the extraction.

To prepare for the Soxhlet extraction, a cellulose thimble was filled with dried ground-up bamboo fibers. The Soxhlet bulb contains about 400 mL before recycling the solvent. To ensure that all the fibers were properly exposed to the solvent, about three inches of head space was left above the fibers (see Figure 11 below). The thimble's empty weight may be taken at this weight if calculation of the wax mass is desired; otherwise, there is no need to mass the thimble.

Figure 11. Headspace above bamboo in the cellulose thimble.

The 500 mL double-necked round bottom flask was then filled with 300 mL toluene and 150 mL ethanol (2:1 ratio of toluene:ethanol). Boiling stones were also added to the round bottom flask to prevent bumping while heating the solvent. This solvent mixture was chosen since the papers cited above used this solvent system for their bamboo extractions. The authors of these papers did not explain why exactly they use this solvent system. One paper mentioned the use of Soxhlet extraction to "remove…the nonpolar extractives fraction" from bamboo.[45] The use of a larger fraction of the less polar toluene to ethanol in this extraction may ensure the removal of all waxes and other mainly nonpolar extractives.

After the solvent was added, the glassware was arranged as depicted above, with aluminum foil lightly covering the top of the Allihn condenser to prevent too much evaporation of the solvent. The neck of the flask, the Soxhlet bulb, and condenser were all secured via clamps to a heavy-duty lab stand. Over the course of two hours, the flask was slowly heated to 79°C. (To speed up the heating process, aluminum foil and other insulation can be added and then later removed once temperature is reached.) The heating level was gradually increased until the vapors could be seen condensing near the middle of the condenser. If the vapors began climbing halfway up the condenser, the heat was lowered slightly. The evaporated solvent then began dripping into the Soxhlet bulb.

After the bulb was about half full of solvent, the solvent with dissolved extractives was recycled back into the flask by a siphoning mechanism. The process then began again as fresh solvent was brought into the bulb. Once at reflux, the extraction was left to run for 8 hours (a programmable outlet timer was used to automatically shut off heat after the time had elapsed). The solvent in the flask at the end of the extraction turned from colorless to a light green, indicating the extractives were removed from the bamboo. The bamboo fibers turned a pale tan color, with all their green color removed. The leftover solvent can then be evaporated via a rotary evaporator and the resultant wax analyzed. For the purposes of this study, the extractives were not analyzed. The thimble, now soaked with leftover solvent, was left to dry in a 60°C oven inside of the fume hood for 24 hours.

3.4 Ball Milling Pretreatment

In its natural state, the cellulose contained within bamboo is crystalline, making it more rigid and less susceptible to enzymatic attack.[18] Therefore, to properly prepare cellulose for ethanol conversion by enzymatic hydrolysis, the cellulose must be changed structurally from crystalline to amorphous. While many pretreatments are available to induce this change, ball milling was selected as a pretreatment due to its low cost, energy requirement, and lack of harmful byproducts. For *P. nuda*, Ekwe et. al showed that with increased duration of ball milling, there was higher conversion of bamboo cellulose to simple sugars by enzyme hydrolysis.[46]

Ball milling was employed to break up the bamboo fibers into a finer powder. A stainless-steel chamber was packed with bamboo fibers and topped with four stainless steel balls. The chamber was secured horizontally into the ball mill and left to rotate at 1200 rpm for 1 hour, 3 hours, or 5 hours. After ball milling, the powder was transferred to plastic containers and labeled accordingly. The ball milled powder was not sieved for a certain particle size, which may have affected the results of subsequent steps.

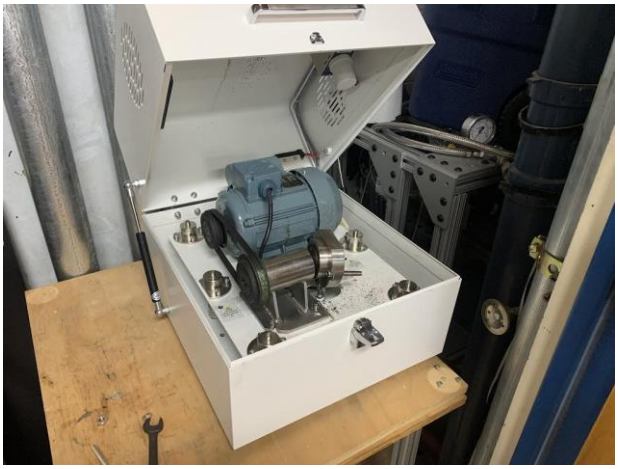


Figure 12. Ball mill apparatus in GH 222.
(Across International Planetary Ball Mill, model VQ-N)

Figure 13. *Phyllostachys nuda* before and after ball-milling pretreatment for 3 hours at 1200 rpm.

3.5 Deep Eutectic Solvent Preparation

A large barrier to converting raw lignocellulosic biomass is its rigid outer lignin structure, as it prevents mechanical and chemical access to the cellulose that can be converted to fuels.[34] Many pretreatments have been explored to remove lignin, such as treatment with alkaline catalysts, acids, steam, and liquid hot water.[47] However, many of these pretreatments have high reagent costs or produce hazardous waste. To reduce this waste, this MQP explored the method of deep eutectic solvent (DES) pretreatment to remove lignin from bamboo.

The DES solvent system chosen for this MQP was choline chloride and lactic acid in a 1:10 molar ratio. To create the DES, a 100 mL beaker was filled with 8.0 g choline chloride and 51.6 g of L(+)-lactic acid and covered with parafilm. In a water bath at 60°C the beaker was lightly swirled until a homogenous, colorless liquid was obtained (about 5 minutes of swirling).[27] The final DES solution had a consistency similar to that of corn syrup.

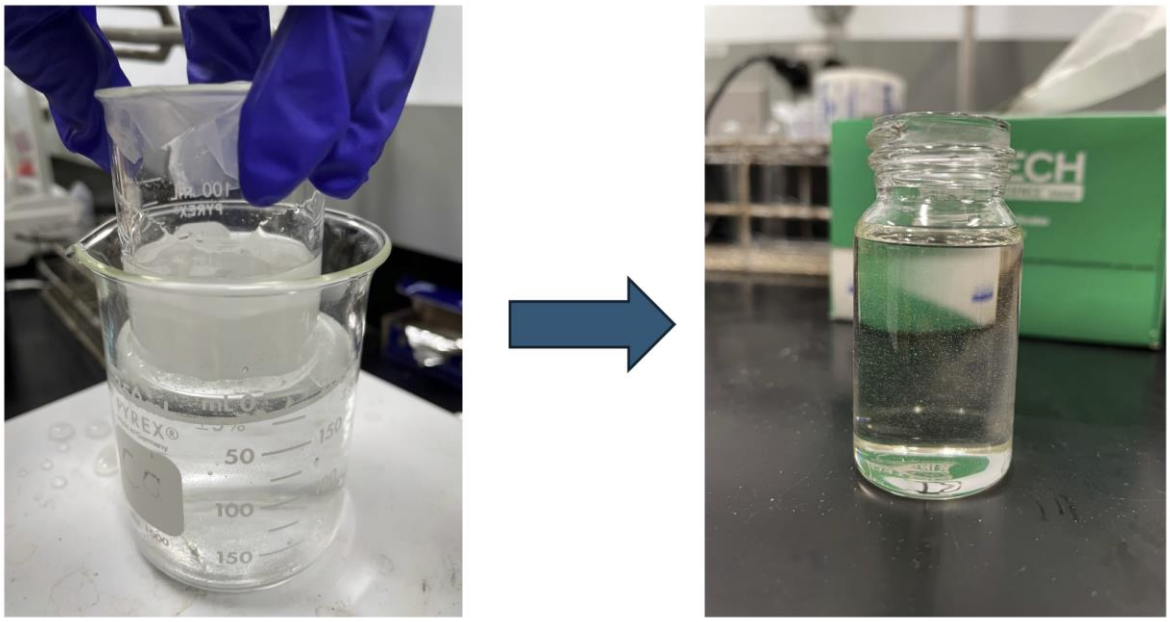


Figure 14. Choline chloride and L(+)-lactic acid deep eutectic solvent before and after mixing at 60°C. Note the end solution tends to contain air bubbles that diffuse out as the solution sits.

3.6 Deep Eutectic Solvent Oil Bath Reaction

To achieve the highest lignin removal from the bamboo, both a certain molar ratio of the two DES components and a certain mass ratio of DES to solid loading must be decided. For this MQP, a molar ratio of 1:10 choline chloride:lactic acid was chosen, along with a mass ratio of 12:1 DES:solid. These ratios were decided as having high success for delignification of eucalyptus and willow in the literature.[48] It was found that a higher mass ratio of DES to solid ratio had better delignification for more crystalline biomass, but due to the volume constraints of the reactors this procedure could only feasibly achieve 12:1 DES:solid ratio.[49]

The DES pretreatment trials were run in triplicate. Three 50 mL heavy wall pressure vessels were loaded with 0.93 g solid (either *P. nuda* or microcrystalline cellulose) and 11.18 g DES solution. A small magnetic stirring bar was added and the teflon cap was secured. The reaction temperature in the pressure vessels should be 110°C. A modified pressure vessel with DES and a thermocouple was used to determine the oil bath temperature to obtain an internal pressure vessel temperature of 110°C.

The figure below shows the setup to allow for DES in three simultaneous vessels. An empty pressure vessel was placed in the middle of a tube holder (yellow) and flipped upside down and clamped to a lab stand. The tube holder could then be rotated to insert each reaction vessel into the oil bath. The height of the tube holder over the oil bath was such that the oil level was above the DES solution level in each pressure vessel. Stirring was arbitrarily set to 190 rpm. Triplicates were run for 2 hours using regular and ball milled microcrystalline cellulose and *P. nuda*. After the duration of the trial, the pressure vessels were promptly removed from the oil bath and placed into an ice bath for 10 minutes to cool.

Figure 15. Deep eutectic solvent delignification pretreatment setup using three heavy wall pressure vessels.

3.7 Aqueous and Solid Phase Separation

The product of the DES pretreatment is a slurry consisting of the DES and DES-soaked solids. After the DES trial has been run and the reactor vessels have cooled, the caps are removed, and the slurry poured into 15 mL plastic centrifuge tubes. Samples were centrifuged at 3500 RPM for 20 minutes. The liquid DES layer on top was decanted into a waste bottle and the solids left inside the tube. Each sample was placed on a massed Whatman 70mm filter paper and put on top of a Büchner funnel and sidearm flask connected to a vacuum. Each sample was rinsed with ethanol (100%) until the ethanol running through the filter became colorless. The samples were then left to dry in a desiccator. After drying, the solids were massed to obtain solids percent yield.

3.8 XRD and FT-IR Analysis

To measure the crystallinity of the solids before and after ball-milling and DES pretreatments, powder X-ray diffraction analysis (XRD) was performed. XRD utilizes X-rays, whose atomic-sized wavelengths permit obtaining information regarding the crystal structure of a given compound.[50] XRD produces X-ray diffractograms, which provide the intensities of characteristic peaks pertaining to the crystallinity and amorphicity of a given compound.

Figure 16. X-ray diffraction apparatus in Goddard Hall.

The XRD diffractogram gives intensity of the X-ray signal plotted against 2θ, a metric of the angle the detector makes with the surface of the compound. The intensity of the peak at ~23 degrees is the (002) peak and is indicative of crystalline structure and is denoted I_{002}. The relative minimum at ~18.5 degrees is the minimum position between (002) and a peak called (101) at ~14 degrees and has intensity I_{am}.[51] To calculate the Segal total crystallinity index (TCI), these intensities were put into the following equation:

$$Segal\ TCI\ (\%) = \frac{(I_{002} - I_{am})}{I_{002}}$$

Note that the authors from which this equation is explained disclose that this method of peak height method should only be a rough approximation of crystallinity used for relative comparisons.[51] Other methods exist for determination of amorphous and crystalline regions from XRD diffractograms, such as deconvolution and amorphous subtraction, but the math is more complicated and beyond the scope of this project.

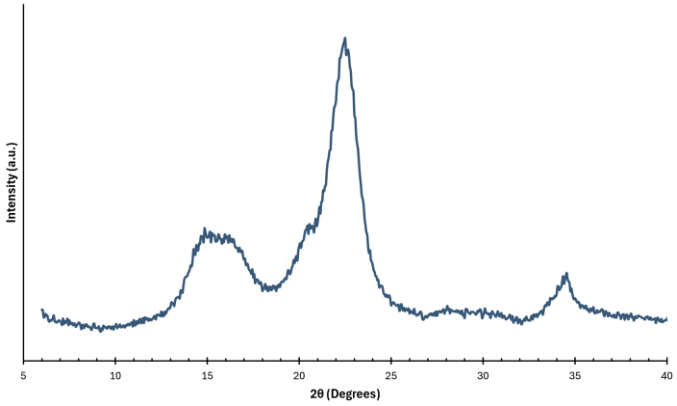


Figure 17. XRD diffractogram of microcrystalline cellulose.

To determine the efficacy of the DES treatment on the *P. nuda* samples, Fourier-transform Infrared Spectroscopy (FT-IR) was utilized. FT-IR makes use of infrared light to expose the bonds within a compound to certain amounts of energy, and then records the energy emitted from the stretching of those bonds. The bonds relating to lignin structure were especially important for determination of approximate lignin content of the solids. While other more directly quantitative methods for lignin composition exist, such as the acetyl bromide method, Klason (insoluble lignin) method, and the NREL acid hydrolysis method, due to supply chain issues the only feasible analytical technique for this MQP was FT-IR.[52,53]

3.9 Development of Methodology

The methodology outlined in this report, while inspired by many online sources, was ultimately developed by the team in GH 222. This section, 3.9, details the various issues we encountered.

3.9.1 Soxhlet Extraction Volume

The Soxhlet extractor bulb used holds ~400 mL of solvent before the siphoning effect recycles back into the main flask. For one trial, only 400 mL of the toluene: ethanol (2:1 volumetric) was for Soxhlet extraction of *P. nuda* fibers. Over the course of the extraction, small amounts of solvent evaporated through the top of the condenser, through the aluminum foil cap (this issue could have been prevented by reducing the heat to the flask to keep the solvent from reaching so high up the condenser). After leaving the lab for a few hours, we discovered that due to the loss of solvent, the flask could not fill up again. The remaining solvent in the flask became superheated, breaking the thermometer tip into the flask and sending superheated vapors through the apparatus.

Figure 18. Left to right: Superheated vapor inside the flask; broken thermometer tip; burnt bamboo wax char inside the round bottom flask.

As a result of this failed extraction, a new batch of *P. nuda* had to be cut and ground in the coffee grinder, and the collected waxes burnt and caked onto the interior of the round bottom flask. At first, the flask was scrubbed with a Brillo pad, using tweezers to guide scrubbing the inside. Eventually, however, the flask was filled with some sand, a stir bar dropped in, and set to spin on top of a stirring plate, rotating the flask so that the bar used the sand to scrub any dirty areas.

3.9.2 Determination of Trial Times

For the sole reason of convenience of processing trials at easy times, we initially set the time for DES reactions at 24 hours. The result of these trials was a very dark DES solution. The slurry had a smoky aroma (like barbecue sauce) and the retrieved product appeared burnt. We instead chose to reduce our reaction times to 2 hours to prevent burning.

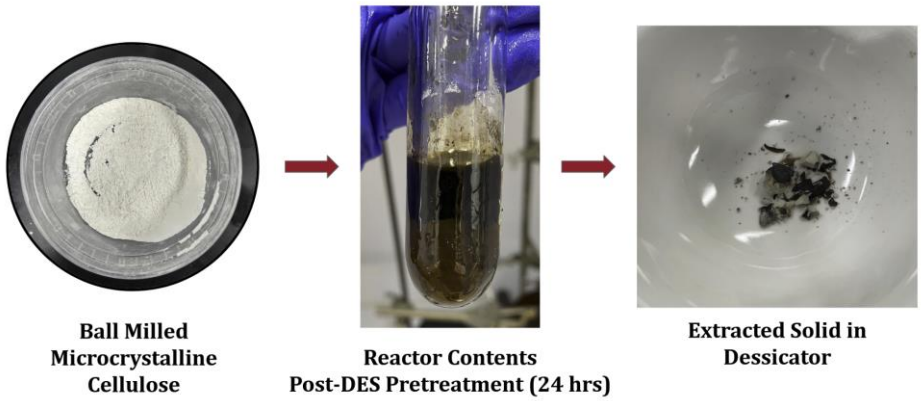


Figure 19. Results from 24-hour DES treatment of ball milled microcrystalline cellulose.

3.9.3 Mass Transfer Limitations of DES Reaction

The reactor vessels used for these DES trials are 15 mL tubes. The stir bars added into the vessels were small and the distance from the vessels to the stir plate affected how well the stir bars could rotate. As a result, after some trials the resultant slurry appeared separated, sometimes with solids not exposed to the DES at all. We do not know for sure if the lack of stirring with some trials affected lignin removal or crystallinity.

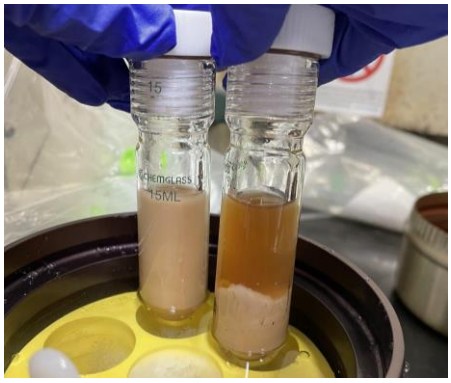


Figure 20. On the left is a well-mixed sample and on the right is a not well-mixed sample after DES pretreatment.

3.9.4 Solid Separation from DES

When we had 24-hour DES trials (as detailed in *3.9.2*), we attempted to separate the solids from the spent DES through filtering with a Büchner funnel and filter papers. Unfortunately, the slurry was incredibly thick and was far too difficult to separate properly through the filtering process. We then attempted to centrifuge once at 3500 RPM for 20 minutes, decant the supernatant DES, and then subsequently rinse with 10 mL ethanol (100%) at 3500 RPM for 5 minutes repeatedly. The samples were rinsed and centrifuged until the ethanol supernatant was colorless. Unfortunately, for all the bamboo trials, the 24-hour trial burnt so much material that the ethanol continued to rinse out material after 5+ centrifuge washes.

After reducing our reaction time, we retained the initial centrifugation step to remove DES and then employed filtering with ethanol. This adjustment to the procedure was much more efficient and effective at separating out the solids from the DES.

EXPERIMENTAL PROCESS

4.1 Laboratory Process Flow Diagram

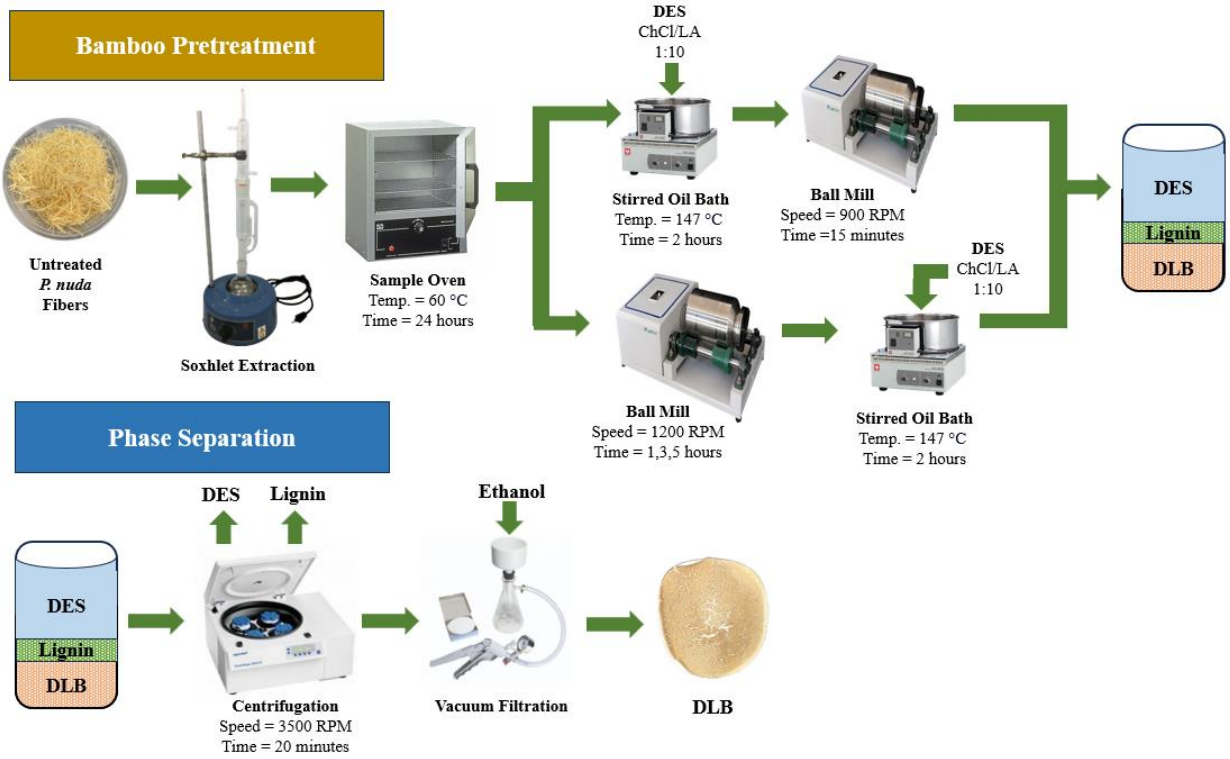


Figure 21. A diagram of the overall process flow from pretreatment to separation.[54–59]

4.2 Laboratory Procedure

4.2.1 Bamboo Fiber Preparation

1. Break the bamboo stalk into small pieces, about 2 in. wide, with a hand saw and a vise.
2. Reduce the small bamboo pieces into fibers using a coffee grinder.
    a. Grind until bamboo is in individual fibers and no chunks remain.

4.2.2 Soxhlet Extraction of P. nuda

1. Mass an empty cellulose extraction thimble.
2. Load the thimble with dried bamboo (powder or fibers) and record the mass.
3. Place the thimble carefully into the Soxhlet apparatus (open side facing up).
4. Set up the Soxhlet extraction apparatus (see Figure 10).
    a. Fill a heating mantle with sand.
    b. Fill a 500 mL two-neck round bottom flask with 300 mL toluene and 150 mL ethanol and add boiling stones.
        i. Note: If you do not use enough solvent, it is possible to not have enough to fill the Soxhlet chamber and cause the oil to become superheated vapor.
    c. Position the heating mantle and round bottom flask on a laboratory jack.

    d. Clamp the Soxhlet extractor bulb just above the top end of one neck on the round bottom flask so that the flask may be jacked up or down.
    e. Clamp a condenser above the Soxhlet extractor bulb, attaching the tubes for cooling water co-currently (cooling water inlet on bottom spout, outlet on top spout).
    f. Cover the top opening of the condenser with aluminum foil.
5. Raise the Jack so that the end of the flask fits snugly into the Soxhlet extractor bulb inlet.
6. Position a thermometer in a Teflon stopper into the second neck of the round bottom flask.
7. Insulate the flask and Soxhlet extractor with aluminum foil, and/or other insulation (mineral wool).
    a. This step is optional – once the apparatus has reached reflux, the insulation is not necessary.
8. Heat the flask to a medium-low heat at first until bubbles are observed on the boiling chips.
    a. This step is important to avoid bumping the solvent.
9. Once boiling (~71°C), raise the temperature to the highest setting.
10. When vapor reaches the condenser and begins falling at a steady rate begin the 8-hour timer.
11. After 8 hours, remove the Soxhlet extracted bamboo from the tumble and dry in the oven for 24 hours.
    a. The oven should be placed inside a fume hood so that the toluene and ethanol fumes are properly vented.

4.2.3 Ball Milling Pretreatment

1. Pack ball mill canister with 4 balls with a diameter of 10 mm at the bottom then fill it ⅔ full of microcrystalline cellulose or bamboo.
2. Secure ball mill canister to the apparatus.
3. Set the ball mill to desired time: 15 minutes, 30 minutes, 1 hour, 3 hours, or 5 hours, and to desired rotation speed: 1200 rpm.
    a. 15 minutes was used for post-DES ball milling of samples.
    b. 30 minutes was used for pre-DES ball milling of microcrystalline cellulose.
    c. 1, 3, and 5 hours were used for pre-DES ball milling of *P. nuda*.
    d. The ball mill will turn off automatically after the desired time.

4.2.4 Deep Eutectic Solvent Preparation

For a 1:10 molar solution of choline chloride:lactic acid, this sample procedure is designed to make 50 mL of DES solution.

1. Mass out 8.02 g of choline chloride.
2. Measure out 42.8 mL of L(+)-lactic acid (or mass 51.74 g of lactic acid).

3. Mix in a dram vial while heating up in a 60 ℃ water bath.
4. Continue heating and mixing until the solution becomes colorless and homogeneous.

4.2.5 Deep Eutectic Solvent Oil Bath Reaction

1. Preheat oil bath to 130 ℃ or reactor vessel to 110 ℃ and set stirring to 190 rpm.
2. Mass empty reactor tube with stir rod and Teflon cap attached for each tube used (labeled 1, 2, 3)
3. Mass out 0.93 g of bamboo powder and put into reactor vessel.
4. Pipette 8 mL (or mass 11.18 g) of DES solution into the reactor vessel.
5. Screw the Teflon caps onto the reactor vessels.
6. Mass the tubes (now full of DES and bamboo).
7. Wipe off the tubes so that there is no residual moisture.
    a. This step is very important! If any water makes contact with the silicone oil bath, the oil will splatter and may cause burns!
8. Clamp the tubes into the oil bath so that the DES is fully submerged into the oil.
9. Note the time placed into the oil bath and leave the tubes to react for 2 hours.
10. After the specified duration, place the reactor tubes into an ice bath for 10 minutes to quench the reaction.
11. Pour the reaction solution into each centrifuge tube (labeled 1, 2, 3).

4.2.6 Aqueous and Solid Phase Separation

Centrifugation

1. Centrifuge the tubes once (3500RPM, 20 minutes).
2. Mass empty dram vials to put DES supernatant in.
3. Decant the DES supernatant solution into dram vials.
4. Mass the reacted DES supernatant inside the vials.
5. Cap the residual solid inside the centrifuge tube.

Vacuum Filtration

1. Mass a piece of filter paper.
    a. Place Whatman 70mm filter paper into the Büchner funnel.
2. Insert a rubber stopper/funnel into a sidearm Erlenmeyer flask with arm extension and center the filter paper.
3. Attach the vacuum hose to the Erlenmeyer sidearm and turn on vacuum.
4. Wash the inside of the centrifuge tube and corresponding reactor with ethanol squirt bottle and spatula tool, then pour contents into filtered funnel.
    a. Ensure that all solids are transferred from the centrifuge tube to the filter paper.
5. Repeatedly wash the solid resting atop the filter paper with ethanol.
6. Repeat step 22 until the ethanol running through the filter is colorless.
7. Allow time for ethanol on filter paper/solid residue to dry in the hood.

8. Transfer filter paper with solid into plastic container.
9. Place filter paper with “wet solid” on crucible inside desiccator and allow sample to sit for ~24 hours.
10. Mass the filter paper with the “dried solid”.
11. Extract dried solid sample into the labeled plastic container.

4.3 Safety Precautions

The methodology detailed in this report makes use of organic solvents with harmful effects if not used with proper safety precaution. The use of proper personal protective equipment (PPE) is always encouraged, such as nitrile gloves and ANSI Z87.1 certified lab glasses/goggles, as well as following and reading information found in the safety data sheets (Seen in Appendix E). It is also important to familiarize oneself with the locations of the emergency phone, safety showers, and fire extinguishers in the lab.

The Soxhlet extraction apparatus should only take place inside a fume hood with the sash closed. Any handling of the solvents (toluene, ethanol) should take place inside the fume hood, with the solvents at room temperature. After running the extraction, the cellulose thimble with the solvent-soaked bamboo is dried in an oven. The oven should be inside the fume hood so that the evaporated toluene and ethanol fumes do not vent into the open air.

For the ball milling steps, if necessary, ear protection should be worn since the ball mill apparatus is quite loud. Otherwise, the ball mill can be stored in a closet with a heavy door to dampen the sound. The ball mill should be avoided while operating since the movement of parts can result in injury. The DES trials require the use of a silicone oil bath set to >110°C. Any reaction vessels must be wiped off for excess moisture, as addition of water into the oil bath can cause splashing of hot oil as the water boils. Care should also be made to prevent personal exposure to the oil bath.

During the XRD analysis of the collected powders, a trained operator (i.e., a graduate student or professor) should be present to ensure proper operation of the apparatus. Since XRD utilizes X-rays, care should be taken to close the windows of the apparatus during running.

Overall, if any safety precautions are uncertain, the lab manager, Professor Timko, the Environmental Health and Safety (EHS) Department, or a skilled graduate student should be contacted for assistance. In the event of an emergency, our team was prepared to take corrective actions to keep ourselves and others safe.

RESULTS & DISCUSSION

5.1 Characterization of Crystallinity of *P. nuda* Using X-Ray Diffraction

To better understand how the crystalline structure of *P. nuda* bamboo is impacted during various stages of the employed pretreatment process as applied in different configurations, our team conducted extensive testing via the powder X-ray diffraction unit located in Goddard Hall. Powder X-ray diffraction was deemed the most suitable analytical technique for this portion of our study because the intensity measurements and diffraction patterns that result from irradiating a sample with X-rays yield direct insights into the crystalline and amorphous structural characteristics of a substance. See Appendices C and D for more information on sample calculations and a breakdown of key datapoints on the diffractograms.

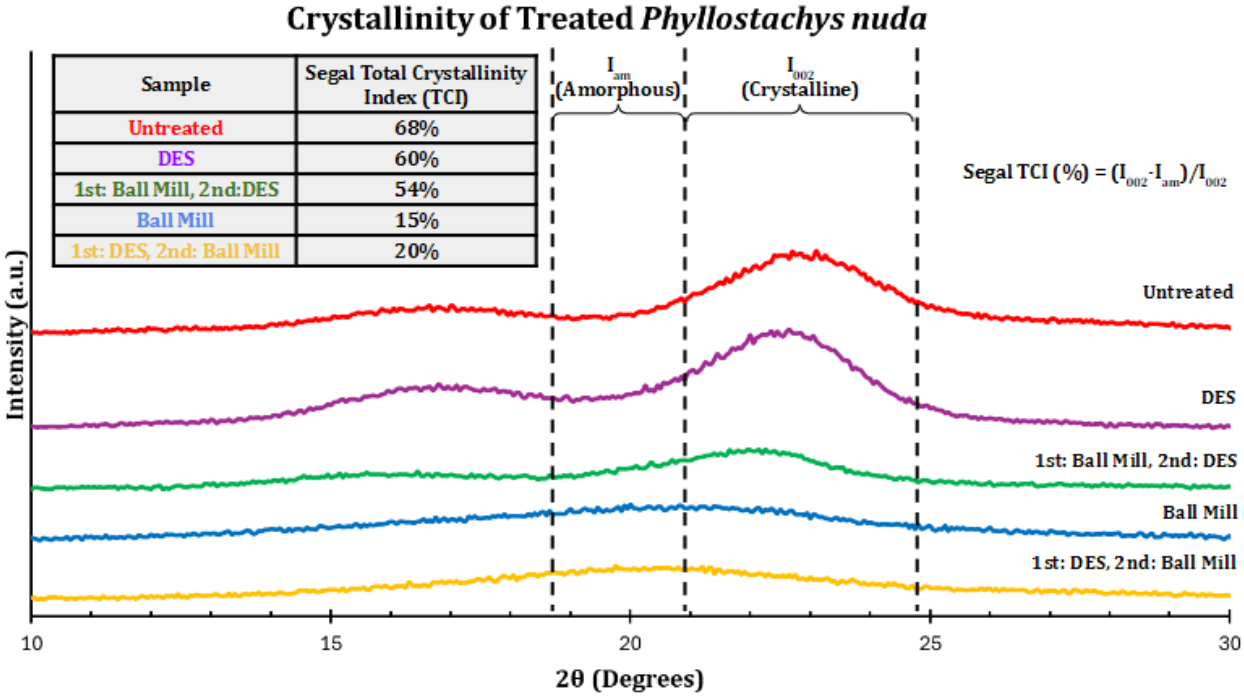


Figure 22. X-ray diffractograms of *P. nuda* bamboo and associated Segal total crystallinity indexes across varied pretreatment strategies.

Figure 22 above includes X-ray diffractograms for untreated *P. nuda* bamboo, as well as corresponding samples for two different pretreatment pathways: DES chemical pretreatment performed prior to physical ball milling pretreatment and DES chemical pretreatment performed after physical ball milling pretreatment. Accordingly, Segal total crystallinity indexes (TCI) have been calculated according to the XRD peak height method described in Park et al.[51] for the purpose of relative crystallinity comparisons between the collected samples. As previously discussed, a more distinct amorphous valley formation paired with a clear crystalline peak in their respective regions outlined in Figure 22 visually indicate a greater degree of crystalline structural characteristics. Here, it can be seen visually and numerically that the untreated *P. nuda* bamboo exhibits a TCI of about 68%, whereas the ball milled *P. nuda* bamboo is represented by a TCI of 15%. This is an expected result and accordingly demonstrates the ability of physical pretreatments,

such as ball milling, to effectively amorphize lignocellulosic biomass and disrupt the highly ordered crystalline structure that makes it more difficult for enzymatic digestion to occur. Interestingly, when performing DES chemical pretreatment after ball milling, the TCI was observed to inflate back upwards to about 54% and showed the partial reformation of the crystalline peak, thus suggesting that the DES solvent has a recrystallization effect on the lignocellulosic biomass samples. Given that a more highly ordered crystalline structure is disadvantageous to enzymatic digestion due to its ability to restrict enzyme access to the more interior cellulose fibers in the biomass, our team proposes that this primary physical and secondary chemical pretreatment pathway in its current state may not be as viable of an option for effective biomass preparation.

On the other hand, simply performing DES chemical pretreatment on *P. nuda* bamboo yielded a corresponding TCI of 60%, roughly within range of the original TCI of untreated *P. nuda* bamboo. Following this DES pretreatment with ball milling caused the sample to become sufficiently amorphous again with a TCI of around 20% and largely flattened X-ray diffractogram like the previous sample that was only ball milled. These results seem to indicate that the negative impacts of DES recrystallization observed in the previously discussed pretreatment pathway may lead to unintended inefficiencies in the enzyme hydrolysis step downstream in the overall biofuel production process. As a result, our findings suggest that it would be beneficial to ensure the inclusion of a physical ball milling pretreatment step following the DES chemical pretreatment to return the lignocellulosic biomass to its amorphous state.

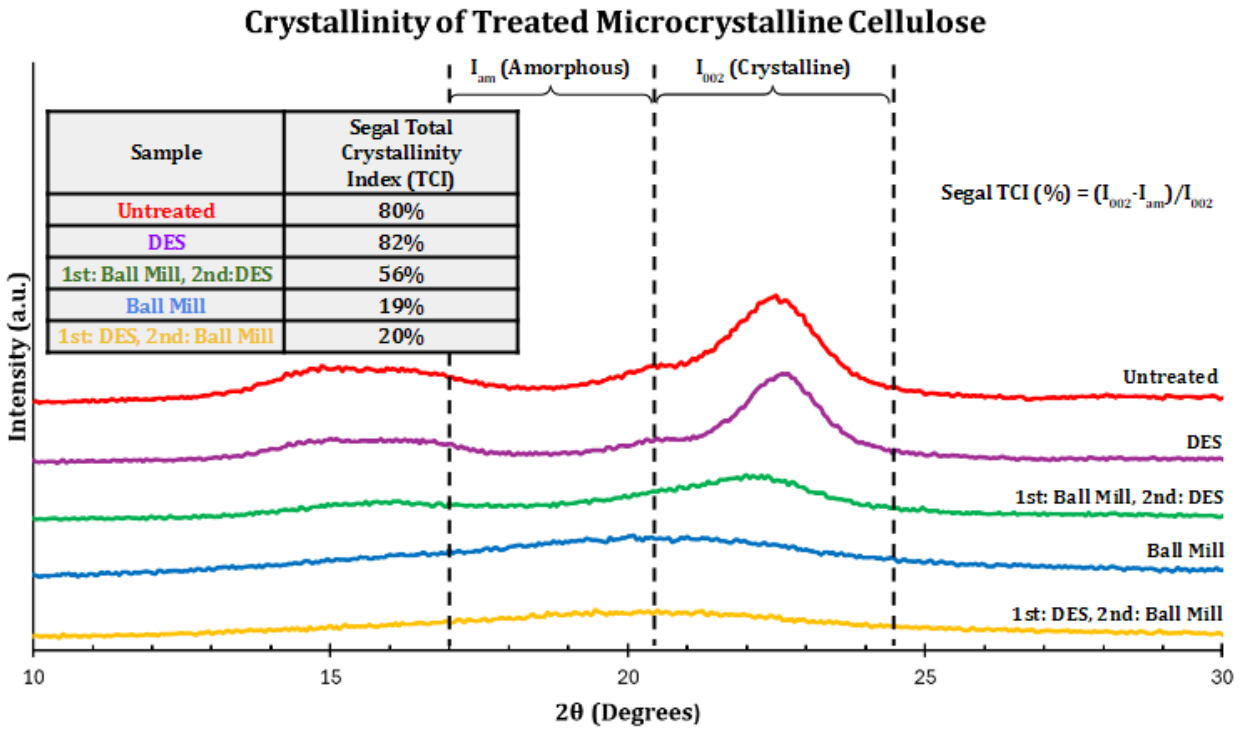


Figure 23. X-ray diffractograms of microcrystalline cellulose (MCC) control trials and associated Segal total crystallinity indexes across varied pretreatment strategies.

Given that the primary structural obstacle of enzyme hydrolysis of lignocellulosic biomass is the high degree of crystallinity in cellulose preventing access to more interior polysaccharide fibers, our team further pretreated a variety of microcrystalline cellulose (MCC) controls to accordingly see how such pretreatment pathways particularly transform the structural characteristics of the cellulose component. As can be seen in Figure 23, our untreated MCC control displayed a significantly high TCI of 80% accompanied by a distinct crystalline peak, which correspondingly diminished to a TCI of about 19% upon ball milling the sample. Once more, this is an expected result in line with the previous results from the *P. nuda* samples as it has been widely accepted that physical ball milling pretreatments significantly disrupt the crystalline domains of cellulosic biomass. We accordingly discovered that subsequent DES chemical pretreatment of ball milled MCC resulted in a new TCI of about 56%, thus demonstrating a recrystallization effect of DES mirroring the results that were observed for our *P. nuda* samples. This accordingly confirms that the negative recrystallization interactions associated with DES do specifically impact the cellulosic components of *P. nuda* bamboo, thus potentially decreasing access to interior fibers and making it much more difficult for enzymatic digestion of cellulose in downstream enzyme hydrolysis.

At the same time, subjecting untreated MCC to DES chemical pretreatment followed by subsequent physical ball milling pretreatment accordingly returned a sample with a much more desirable TCI of 20% and a noticeably flattened and insignificant crystalline peak relative to the other recorded samples. This further confirms our previous results with *P. nuda* bamboo and communicates the importance of considering additional physical pretreatment following DES chemical pretreatment to return the cellulose to a more desirable amorphous state.

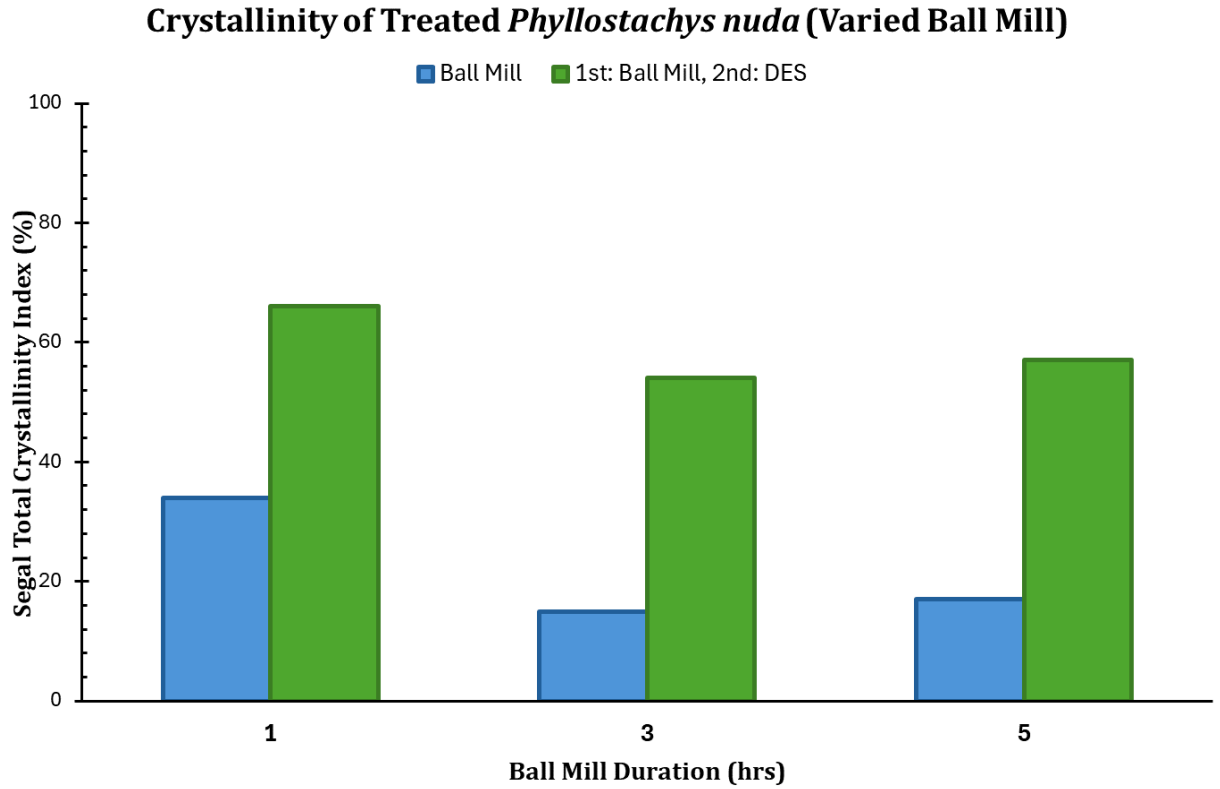


Figure 24. Ball mill duration versus Segal total crystallinity index for *P. nuda* bamboo with and without subsequent DES pretreatment.

Additionally, our team studied the impact of ball mill duration on the crystalline behavior of *P. nuda* bamboo with and without subsequent DES chemical pretreatment to gain more insights into method optimization. In reviewing Figure 24, the TCI of ball milled *P. nuda* bamboo exhibits a significant decrease with an increase in the ball mill duration. This represents an expected result as longer ball mill durations create more intense pretreatment conditions that physically disrupt the crystalline structure of *P. nuda* bamboo. However, when performing subsequent DES chemical pretreatment, it was discovered that the level to which the *P. nuda* samples recrystallize appears to decrease following the longer ball mill durations of three and five hours relative to the one-hour ball mill duration.

While there appears to be a diminishing return to how much the crystalline structure can truly be disrupted around three to five hours, this nonetheless may suggest the ability to reduce the extent that DES recrystallizes lignocellulosic biomass. It is acknowledged that these results must be approached with caution and will require much more extensive research and repetitions to fully confirm the findings, as well as to understand the economic and energy usage tradeoffs associated with the conditional optimization.

5.2 FT-IR Examination of *P. nuda* Delignification with Deep Eutectic Solvent Pretreatment

As previously discussed, the recalcitrant nature of the lignin components of *P. nuda* bamboo necessitates a more in-depth analysis to better understand how the employed physicochemical pretreatment strategies impact the lignin composition of the collected samples. Our team performed a relative compositional analysis using insights from Fourier-transform infrared spectroscopy on the presence of different functional groups and bonding patterns in the samples. Highlighted in Figure 25 below is the most prominent characteristic peak for cellulose around 1035 cm^{-1} that arises due to the stretching of bonds between carbon and oxygen in primary alcohol groups. Additionally, three characteristic peaks representing the aromatic skeletal vibrations of lignin (1425 cm^{-1}, 1515 cm^{-1}, 1600-1650 cm^{-1}) have also been presented as defined by Yang et al.[60] See Appendix D for more information on key datapoints for the FT-IR spectra.

To begin, the characteristic peak for cellulose was present in all of the tested *P. nuda* samples and the untreated MCC control, thus providing evidence that the classification of the peak at 1035 cm^{-1} as cellulose is reliable. On the other hand, the three characteristic peaks for aromatic lignin displayed relative intensities of approximately 0.093, 0.049, and 0.056 for untreated *P. nuda* bamboo, which accordingly decreased to 0.024, 0.023, and 0.030, respectively, after DES chemical pretreatment. This result indicates that the selected DES is behaving as intended in the pretreatment strategy and is successfully removing lignin from *P. nuda* given the significant decreases in the lignin absorbance peaks.

Additionally, it was discovered that the combined physicochemical pretreatment of DES after initial ball milling resulted in a *P. nuda* sample with an even larger decrease in the characteristic lignin absorbance peaks to around 0.008, 0.004, and 0.011, respectively. Here, the identified wavenumbers of 1425 cm^{-1}, 1515 cm^{-1}, and 1600-1650 cm^{-1} encompass portions of the FT-IR spectrum that largely flatten out or cease to form distinct peaks, thus further providing evidence of the increasing extent of lignin removal. These initial results suggest that performing physical ball milling prior to DES chemical pretreatment has a synergistic effect on the final lignin composition. In this manner, it is likely that the ball milling initially breaks up the crystal structure of bamboo in a way that allows the DES to better access and break apart the ether and ester cross linkages between lignin and the polysaccharides contained within. As a result, the DES chemical pretreatment can more easily remove lignin and has a stronger impact on final lignin composition

in the sample. It appears that through both visual and numerical comparison via relative intensities that the DES may have been able to remove roughly 90-95% of the lignin. However, more advanced compositional analysis, such as the acetyl bromide method, will be required to gain an accurate estimate of post-treatment lignin composition.

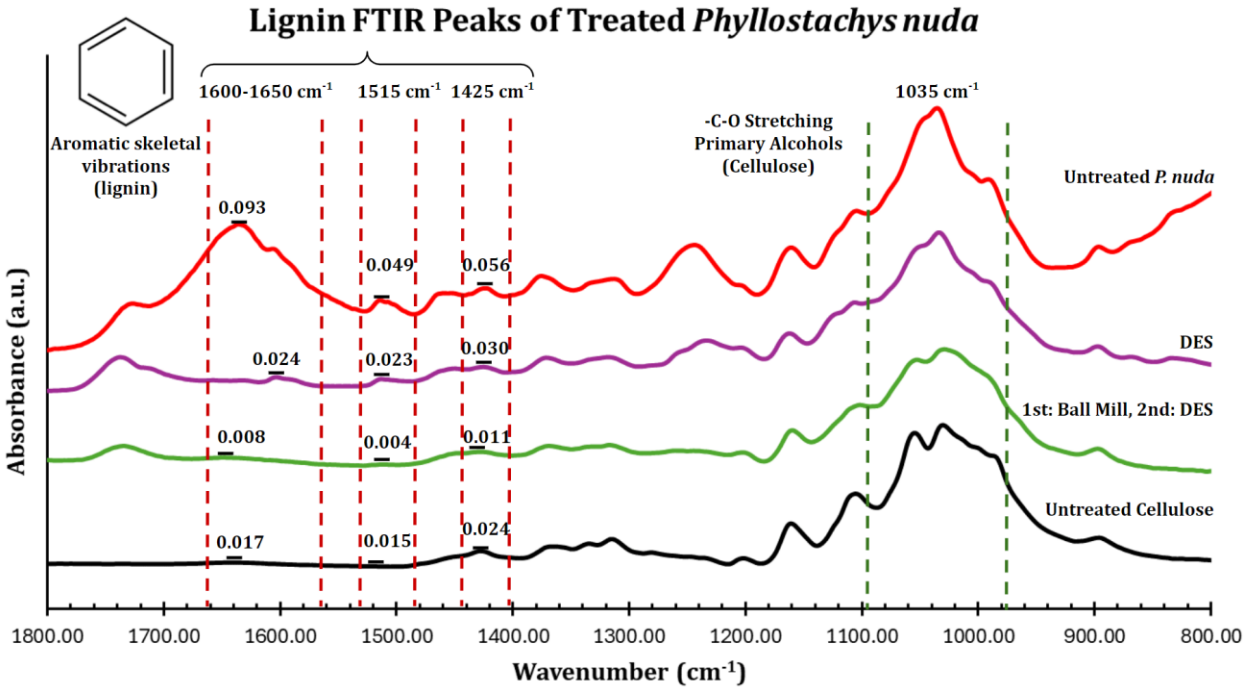


Figure 25. FT-IR absorbance curves for *P. nuda* bamboo across varied pretreatment strategies. Relative peak intensities have been provided at three key wavenumber regions for lignin identification.

Furthermore, it is worth noting that the untreated cellulose sample did exhibit relative intensities for the studied lignin absorbance peaks that surpassed that of the combined physicochemical pretreatment sample discussed previously. This is likely due to inconsistencies in equipment calibration leading to significant variation in obtained readings and different baselines (although adjustments were made to standardize to a single minimum baseline in our analysis), as well as the fact that the calculated absorbance values were already relatively low to begin with. Further work should take care to utilize a more reliable numerical method for compositional analysis as discussed previously. However, the untreated cellulose sample spectrum does still provide a reliable control for visually analyzing peak changes in the above FT-IR spectra.

Our team further studied the impact of ball milling duration prior to DES chemical pretreatment to better understand its synergistic effect in lignin removal. Figure 26 below contains the FT-IR results for this analysis. The relative absorbance peak intensities characteristic of aromatic lignin generally appeared to decrease from the untreated *P. nuda* sample to the one-hour ball mill prior to DES chemical pretreatment, and finally to the three-hour ball mill prior to DES chemical pretreatment. In line with our previous findings, these preliminary results seem to initially suggest that smaller ball mill durations do not disrupt the crystalline structure of *P. nuda* bamboo as significantly, and therefore the DES cannot access and break apart the ester and ether cross linkages as reliably relative to longer durations. However, we additionally found that the

relative intensities of the aromatic lignin absorbance peaks for the *P. nuda* bamboo sample that was ball milled for five hours before DES chemical pretreatment surprisingly increased from the three-hour ball mill sample.

While it was indeed expected that the relative intensities would continue to decrease due to the more intense physical pretreatment conditions, it is hypothesized that the numerical comparison via relative intensities is likely not as reliable for these samples due to contamination of lactic acid from the DES around wavenumbers of 1220 cm^{-1}, 1370 cm^{-1}, and 1740 cm^{-1}. This may have occurred due to insufficient ethanol rinsing of the samples during the vacuum filtration segment of our extraction process. The three-hour ball mill and DES chemical pretreatment sample from Figure 26 originally displayed contamination at these wavenumbers and was accordingly re extracted and rinsed thoroughly with ethanol, thus resulting in the disappearance of these lactic acid peaks at the wavenumbers of interest. In any case, combined physicochemical pretreatment appears to demonstrate significant promise given how the shape and distinctiveness of the characteristic peaks for aromatic lignin transform significantly relative to the untreated control.

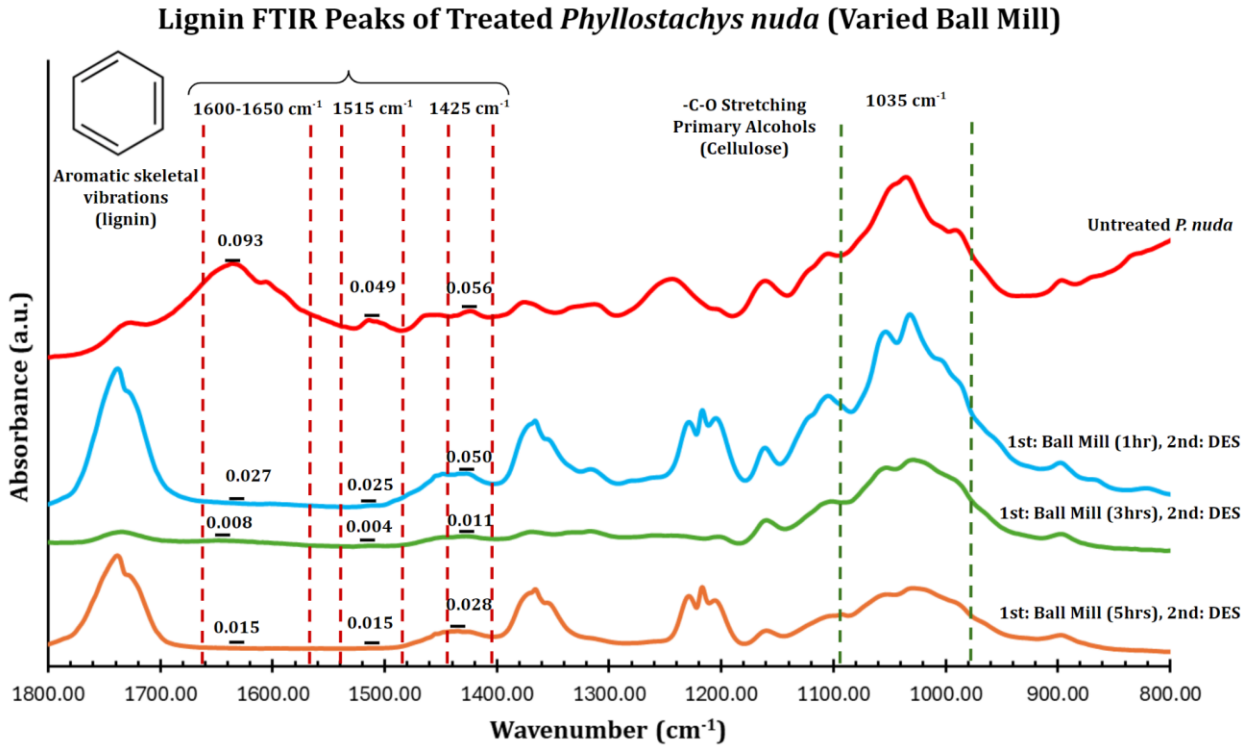


Figure 26. FT-IR absorbance curves for *P. nuda* bamboo using varied ball mill durations. Relative peak intensities have been provided at three key wavenumber regions for lignin identification.

5.3 Impact of Pretreatment Process Variations on Material Balance

As previously mentioned, the highly viscous nature of DES systems makes them particularly difficult to handle and implement despite their many benefits. In our experimentation, we observed that the high viscosity of the DES made the sample runs particularly susceptible to loss from the transfer between containers during the extraction and separation stages. Material balances were completed on the system for each sample trial to account for the total system loss, solid recovery, and DES recovery. We additionally performed a simple reactor recovery control trial to further understand the extent of loss in our pretreatment strategy. This involved the loading of the oil bath reactors with Soxhlet-extracted *P. nuda* bamboo fibers and DES followed by immediate extraction without performing any heating or reaction on the reactor loading. This allowed our team to specifically analyze the extent of material loss occurring due to transfer steps associated with the extraction and separation stages while isolating out any changes to the system from the reaction.

The recovery of the aqueous solution or reacted DES is incredibly important due to the unique, eco-friendly ability of DESs to be recycled for future usage. However, the high viscosity of DESs made it challenging to work with, especially during transfer and separation. This phenomenon is viewed in the following Figure 27.

Figure 27. Highly viscous aqueous solution decanted after centrifuging.

Figure 27 shows the decanted supernatant liquid highlights the transfer issues and potential for loss associated with the collection of residues on the sides of the dram vial container, which was also a common theme for the reactors and centrifuge tubes. Residual DES supernatant often left this viscous residue on the solid precipitate samples as well, thus giving rise to the need for additional vacuum filtration with ethanol to rinse the solids. While the mixture of ethanol and residual DES was collected and has the potential to be separated and further recycled, this was beyond the scope of our study and was simply considered as an additional loss.

As can be seen in Figure 28 below, the average solid and DES recoveries were recorded for both MCC and *P. nuda* samples under various pretreatment strategies. The reactor recovery control trial was completed with a solid recovery of 84.20% and DES recovery of 62.00%, while the exclusively DES-treated *P. nuda* bamboo fibers exhibited a solid recovery of 81.32±8.61% and DES recovery of 40.27±9.61%. Here, the solid recoveries are quite similar and within error of each other, however, the DES recoveries of the two conditions differ considerably. The extracted compounds from the DES chemical pretreatment seem to contribute to a more viscous product residue as compared to the original prepared DES. This in turn complicates the later stages of the extraction and separation process, and inherently results in more loss to container transfers and residual collecting on the extracted bamboo fibers.

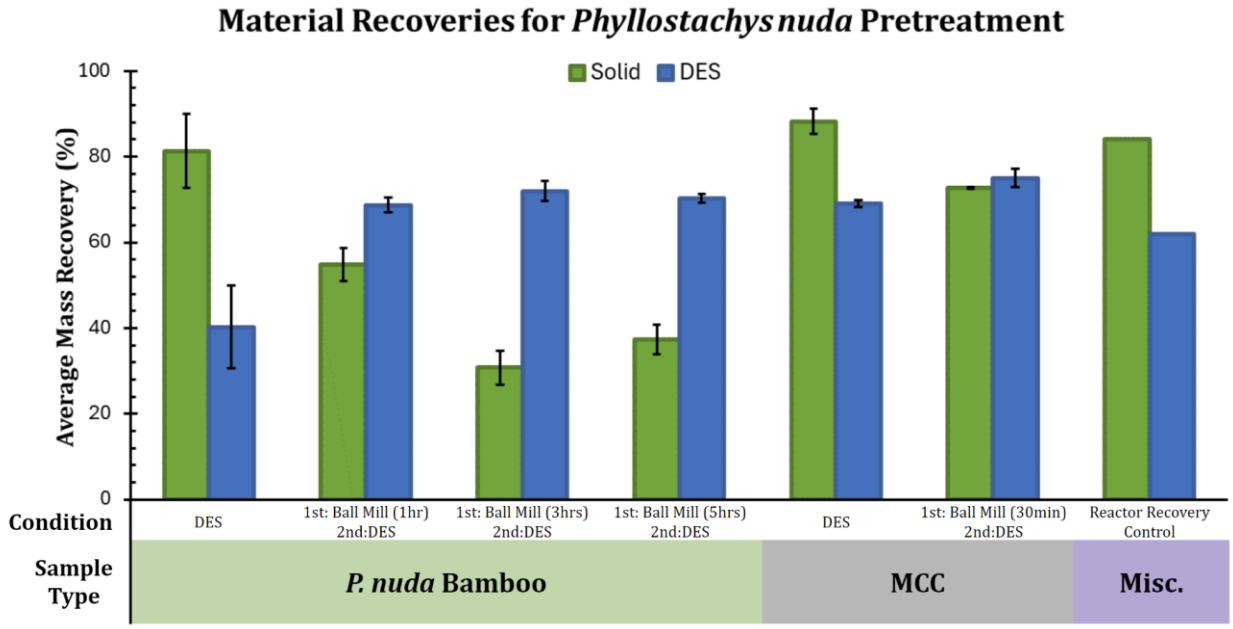


Figure 28. Average percentage recoveries of the solid and DES product phases for various pretreatment conditions and sample types.

In addition, our team found that the trend in solid mass recovery was found to be inverse to that of DES mass recovery for *P. nuda* bamboo as the conditions were altered from exclusively DES pretreatment to five hours of ball milling. The associated decrease in solid mass recovery with increases in ball milling duration are most likely due to the inherent difficulty associated with particle shape and size. More specifically, it was far easier and convenient to properly account for large bamboo fibers in the material balance during the extraction and separation stages as opposed to the finer bamboo powders characteristic of pretreatments with ball milling. At the same time, given the previous results which suggest that preliminary ball milling aids in the effectiveness of subsequent DES chemical pretreatment, increasing amounts of lignin removed from the original solid feedstock would result in much of those extractives partitioning into the DES product phase.

While specific quantities of lignin removed were not determined, it was inferred from visual analysis of the FT-IR spectra that DES removed potentially around 90-95% of initial lignin found in *P. nuda*, which is composed of about 26% lignin as assessed from prior testing. Altogether, this means that the decreasing trend in solid recovery of *P. nuda* bamboo seen in Figure 28 can likely be explained by a combination of particle size and morphology, as well as desired partitioning of solid lignin into the DES product phase. Accordingly, the rising trend in DES mass recovery corresponds to the increase in lignin removal, meaning that more extractives are partitioning into the DES product phase following longer initial ball mill durations. On the other hand, the same general trends for solid and DES mass recoveries can be observed for the MCC samples in Figure 28, although the contrasts between the two for each treatment condition are much less significant. This is likely due to the lack of delignification occurring, in effect reducing the impact of extractives partitioning into the DES product phase.

Table 6. Average total mass loss for various pretreatment strategies.

Sample Type	Condition	Average Total Mass Loss (%)
P. nuda	DES	56.58 ± 8.73
P. nuda	1st: Ball Mill (1hr), 2nd: DES	32.33 ± 1.91
P. nuda	1st: Ball Mill (3hrs), 2nd: DES	31.18 ± 2.26
P. nuda	1st: Ball Mill (5hrs), 2nd: DES	32.22 ± 0.89
MCC	DES	29.48 ± 0.45
MCC	1st: Ball Mill (30min), 2nd: DES	25.14 ± 1.95
P. nuda	Reactor Recovery Control	36.33

In general, the exclusively DES-treated *P. nuda* bamboo fibers were found to have the greatest average total mass loss at approximately 56.58±8.73% (See Table 5), significantly more than that of the reactor recovery control trial at 36.33%. Again, this is primarily due to the fact that extractives partitioning into the DES product phase make it progressively more difficult to properly extract and separate the different phases post-DES due to the high viscosity. Further, the ball milled *P. nuda* bamboo samples all generally exhibited average total mass losses around 31-32%, significantly less than that of the exclusively DES-treated *P. nuda* bamboo fibers. This is likely a result of the appreciable amount of residue that would collect on the extracted fibers, in turn resulting in the need for additional ethanol rinsing to wash it away during the vacuum filtration step. In any case, future work should consider new avenues for improving the extraction and separation stages of the physicochemical pretreatment process.

In general, minimizing loss and closing the material balance is an important factor for process design, especially for future scale-up endeavors. While recovering reacted DES is essential to reducing waste, as well as having a circular economy for the biofuel process, recovering the solid is crucial for high sugar yields, and therefore high biofuel yields. Maintaining a high recovery of this solid is a key aspect in creating an efficient and high-yield process in the production of biofuels.

CONCLUSIONS & RECOMMENDATIONS

Altogether, through extensive sample analysis via powder X-ray diffraction and Fourier-transform infrared spectroscopy, our team was able to gain valuable insights into the structural and compositional changes associated with *P. nuda* bamboo using various pretreatment strategies for biofuel production. Importantly, it was discovered that performing DES chemical pretreatment on *P. nuda* bamboo following an initial ball milling step to disrupt the crystalline structure appears to result in a negative recrystallization effect from the DES interactions. The recrystallization of the cellulosic components of *P. nuda* could therefore contribute to a less effective enzyme hydrolysis step downstream due to the inability to easily access interior cellulose fibers. Thus, an additional physical pretreatment step carried out post-DES is highly recommended to restore the amorphous, easily digestible domains of cellulose. Accordingly, our team also found that the degree to which the DES recrystallizes *P. nuda* bamboo seems to decrease in response to an increase in ball milling duration prior to chemical pretreatment. While this may present an opportunity to reduce the negative impacts of recrystallization, more work will be required to confidently confirm these results and better understand the economic and energy usage tradeoffs associated with these process conditions.

Furthermore, our results also seemed to indicate that the prepared DES of lactic acid and choline chloride did in fact behave as intended as evidenced by the reduction in shape and intensity of the characteristic FT-IR peaks of aromatic lignin. Interestingly, we found that performing ball milling as a physical pretreatment prior to the DES chemical pretreatment responsible for removing lignin seemed to have a synergistic effect on the delignification results. This suggests that physical pretreatment prior to DES chemical pretreatment likely breaks up the crystalline structure in such a way as to make it easier for the DES to properly interact with and break the ester and ether cross linkages between lignin and the internal polysaccharides. Increasing the extent of the ball milling duration appeared to also have an additional impact via evidence of increased delignification, likely since the overall bulk structure of *P. nuda* bamboo is more significantly disrupted under increasingly intense process conditions. As a result, our team recommends that the ideal order of operations for maximizing downstream enzyme hydrolysis effectiveness would be to perform physical ball milling pretreatment both before and after DES chemical pretreatment to optimize lignin removal and maintain the amorphous form of the desired cellulose.

Overall, our team developed a preliminary methodology for the pretreatment of lignocellulosic biomass. To start, our team recommends that instead of using FT-IR for lignin composition analysis, teams should instead utilize the acetyl bromide method. We also highly recommend that future work be conducted to continue iterating upon and improving the extraction and separation stages after pretreatment with the goal of minimizing total average mass loss and improving product phase recoveries. Finally, a major avenue of research that our team believes to be deserving of future attention and resources is the potential to recycle DES used in the chemical pretreatment process. Recycling of the DES has been sparsely studied in the literature, and with even lesser attention in understanding the order of pretreatment operations. It would therefore be of particular interest to better understand how proper recycling of the DES can be achieved and implemented in the existing methodology to improve its eco-friendliness.

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APPENDICES

Appendix A: Abbreviation Nomenclature

Table 7. Definitions of abbreviations used in this report.

Abbreviation	Definition
DES	Deep Eutectic Solvent
CELF	Co-Solvent Enhanced Lignocellulosic Fractionation
ChCl	Choline Chloride
LA	Lactic Acid
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
MCC	Microcrystalline Cellulose
P. nuda	*Phyllostachys nuda*
DLB	Delignified Bamboo
TCI	Total Crystallinity Index
XRD	Powder X-ray Diffraction
FT-IR	Fourier-transform Infrared Spectroscopy

Appendix B: Experimental Sampling Matrix

Table 8. Experimental Sampling Matrix

Condition	*P. nuda*	MCC
No Treatment	✓	✓
Soxhlet Only	✓	
Ball Mill 30min	✓	✓
Ball Mill 1hr	✓	
Ball Mill 3hrs	✓	
Ball Mill 5hrs	✓	
1st: Ball Mill (30min) 2nd: DES		✓
1st: Ball Mill (1hr) 2nd: DES	✓	
1st: Ball Mill (3hrs) 2nd: DES	✓	
1st: Ball Mill (5hrs) 2nd: DES	✓	
DES	✓	✓
1st: DES 2nd: Ball Mill (15min)	✓	✓

Appendix C: Sample Calculations for TCI and Mass Recoveries

For a pretreated *P. nuda* sample (1st: Ball Mill 3hrs, 2nd: DES), the Segal total crystallinity index (TCI) was calculated in the following manner:

$$I_{002} = 1284\ (Crystalline\ peak\ intensity)$$
$$I_{am} = 596\ (Amorphous\ valley\ intensity)$$

$$Segal\ TCI = \frac{I_{002} - I_{am}}{I_{002}}$$
$$Segal\ TCI = \frac{(1284) - (596)}{(1284)} = 0.5358\ (53.58\%)$$

Additionally for the first of the triplicate runs completed for the pretreated *P. nuda* sample under the same conditions, the material balance calculations were performed as follows:

$$Solid\ Loading\ Mass = 0.9245\ g$$
$$DES\ Loading\ Mass = 11.3631\ g$$
$$Total\ Loading\ Mass = 0.9245\ g + 11.3631\ g = \ 12.2876\ g$$

$$Recovered\ Solid\ (Dried) = 0.3077\ g$$
$$Recovered\ DES/Aqueous = 8.4655\ g$$
$$Total\ Recovered\ Mass = 0.3077\ g + 8.4655\ g = 8.7732\ g$$

$$Solid\ Recovery = \frac{0.3077\ g}{0.9245\ g} * 100 = 33.28\%$$
$$\frac{DES}{Aqueous}\ Recovery = \frac{8.4655\ g}{11.3631\ g} * 100 = 74.50\%$$
$$Total\ Loss = \frac{12.2876\ g\ -\ 8.7732\ g}{12.2876\ g} * 100 = 28.60\%$$

Appendix D: Peak Height and Baseline Data for XRD and FT-IR

Table 9. Summary of XRD peak heights and baselines for tested samples.

Sample Type	Condition	Crystalline Peak (I_{002})	Amorphous Valley (I_{am})	Baseline
P. nuda	No Treatment	2556	828	166
	Soxhlet Only	2157	1064	160
	Ball Mill 1hr	2337	1531	215
	Ball Mill 3hrs	1276	1082	186
	Ball Mill 5hrs	1403	1162	179
	1st: Ball Mill (1hr) 2nd: DES	2287	788	215
	1st: Ball Mill (3hrs) 2nd: DES	1284	596	149
	1st: Ball Mill (5hrs) 2nd: DES	2242	969	193
	DES	2968	1197	149
	1st: DES 2nd: Ball Mill (15min)	1186	946	171
MCC	No Treatment	3917	799	204
	Ball Mill 30min	1767	1440	219
	1st: Ball Mill (30min) 2nd: DES	1815	799	192
	DES	3245	598	174
	1st: DES 2nd: Ball Mill (15min)	1224	984	192

Table 10. Summary of FT-IR peak heights for aromatic lignin and baselines for tested samples.

Sample Type	Condition	$1425\ cm^{-}$	$1515\ cm^{-1}$	$1600 - 1650\ cm^{-1}$	Baseline
P. nuda	No Treatment	0.056	0.049	0.093	0.00327
	Soxhlet Only	--	--	--	0.00139
	1st: Ball Mill (1hr) 2nd: DES	0.050	0.025	0.027	0.00988
	1st: Ball Mill (3hrs) 2nd: DES	0.011	0.004	0.008	-0.01068
	1st: Ball Mill (5hrs) 2nd: DES	0.028	0.015	0.015	0.00113
	DES	0.030	0.023	0.024	0.00349
MCC	No Treatment	0.024	0.015	0.017	0.00441
	1st: Ball Mill (30min) 2nd: DES	--	--	--	0.00709
	DES	--	--	--	0.00838

Appendix E: Safety Data Sheets

The SDS are shown below, listed for the following chemicals used in this study: Choline Chloride,[61] Ethanol,[62] Lactic Acid,[63] Microcrystalline Cellulose,[64] and Toluene.[65]

Choline Chloride [TCI Chemicals]

Ethanol [ThermoFisher Scientific]

L(+)-Lactic Acid [ThermoFisher Scientific]

Microcrystalline Cellulose [Thermofisher Scientific]

Toluene [Thermofisher Scientific]