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Pretreatment, Enzymatic Hydrolysis, and Fermentation to Ethanol Using a Lignocellulosic Feedstock and Subsequent Recovery of a Value Added Co-product: Pure Crystalline Cellulose

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PRETREATMENT, ENZYMATIC HYDROLYSIS, AND FERMENTATION TO ETHANOL USING A LIGNOCELLULOSIC FEEDSTOCK AND SUBSEQUENT RECOVERY OF A VALUE ADDED CO-PRODUCT: PURE CRYSTALLINE CELLULOSE

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Agriculture and Biosystems Engineering degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidates are necessarily the conclusions of the major department.

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ABSTRACT

PRETREATMENT, ENZYMATIC HYDROLYSIS, AND FERMENTATION TO ETHANOL USING A LIGNOCELLULOSIC FEEDSTOCK AND SUBSEQUENT RECOVERY OF A VALUE ADDED CO-PRODUCT: PURE CRYSTALLINE CELLULOSE

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As more demand for alternatives to petroleum and the industrial world's love of cars increase, cellulosic ethanol will become more important. The ethanol can, of course, be used in the transportation fuel sector, but there is also a potential for co-products to be developed out of the cellulose to ethanol process. Some of these co-products have the potential to replace current petrol products. These co-products may provide the extra revenue generation needed for further investment and development of this industry. This would not only provide better energy independence, but in the United States, it would better satisfy the cellulosic ethanol gallon requirement of the RFS. The present review explores the cellulose to ethanol process and a potential co-product, purified crystalline cellulose (PCC), and potential industrial applications of said co-product.

Chapter 1: Introduction and Literature review

1. Introduction

Research has focused on many of the different parts of the process, improving and innovating on each individual portion of the process. One thing to note, however, is how interconnected each part of the process is to the other parts at continuous and large scale facilities. If one changes one part of the process, it will affect downstream process, either by changing composition or other characteristics of the material. The following is a snapshot of different processes that can be included into a cellulosic ethanol plant. Each method will include its own advantages and disadvantages.

2. Lignocellulosic structure

Biomass cell walls are primarily made up of three components: lignin, hemicellulose, and cellulose. Lignin is a large collection of phenolic polymers. Hemicellulose is a polysaccharide made of xylose linked together with acetic acid and arabinose. Cellulose is a macromolecule of β-linked glucose molecules [2]. All plant cell walls will have these components, differing only on the amount of each component present, therefore any plant material can be used as a feedstock in sugar production. Some common source materials for biomass utilization include: forestry residues; dedicated energy crop, such as miscanthus and switch grass; agricultural residues, such as corn stover; municipal wastes.

2.1 Cellulose

Cellulose is a polysaccharide that is made up of long chain β(1→4) D-glucopyranose units. Cellulose forms the backbone of the plant cell wall. Many cellulose polymers laminate themselves with hemicellulose, glued together with lignin, to form fibrils. These

in turn are arranged to form a lattice in the cell wall. This provides rigidity and strength, but also flexibility for the plant. Purified cellulose has been used industrially for over 150 years. One of the first was the development of cellulose nitrate via reaction with nitric acid [3]. Previous to cellulose nitrate, cotton was used in its native form to produce textiles.

2.2 Hemicellulose

Behind cellulose, hemicellulose is the world's most abundant biopolymer. Hemicellulose is not as homogeneous as cellulose and the abundance of the different molecules that make up hemicellulose will greatly depend on the source. It is also much more amorphous and hydrophilic than that of cellulose. Xylose makes up the majority of the components of hemicellulose with mannan, arabinan, and acetate groups filling out the rest [4].

2.3 Lignin

Lignin can be thought of as the glue that holds the other carbohydrate polymers in the fibril sheets within the cell wall. It is primarily made of three phenylpropane units: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. The amount of each will depend on the biomass it is sourced from [5]. The age-old joke is that you can do anything with lignin except make money. This may change as cellulosic ethanol plants become more viable and profitable. Much research has been dedicated to lignin and using it to make some sort of value added product [6-9]. Some research includes using it for P-F resins in wood binders [10], and even using lignin as a natural sunblock [11].

3. Processing lignocellulose to ethanol

Agricultural residues and wastes are some of the most abundant feedstocks in the United States for use in lignocellulosic ethanol production [12]. Many different feedstocks can be used, each different feedstock having advantages and disadvantages for use. Lignocellulosic feedstocks can vary differently in polysaccharide composition, leading some residues to be favorable over others [13].

The basic concept of converting lignocellulosic biomass to ethanol can be broken down into three main steps: pretreatment, hydrolysis, and fermentation. Pretreatment will either open the structures for access to enzymatic hydrolysis and hydrolyze some of the biomass, or delignify the biomass, depending on which method is used. Enzymatic hydrolysis will convert the longer chain carbohydrates to monomeric sugars. Fermentation will convert the monomeric sugars to ethanol. The arrangement and composition of the cell wall components will dictate which processes are the best choice for each step. Material handling should also play a role in which methods are employed.

3.1. Pretreatment

The goal of pretreatment is to open the structure of the plant cell wall in order to give enzymes access to the cellulose and hemicellulose. The enzyme can then begin to hydrolyze these components into their monomeric form. Pretreatments can be categorized into mechanical pretreatment and chemical pretreatment.

Dilute acid

Dilute acid pretreatment of biomass helps to reduce the recalcitrance of cellulose and makes it more susceptible to enzymatic hydrolysis enzymes. This pretreatment modality

can also solubilize the biomass components in to soluble glucan and xylan, and, depending on severity of pretreatment conditions, can hydrolyze biomass components into monomeric sugars[14]. To detect and quantify the sugars and oligomers released from the biomass, a separation technique is employed. HPLC is a reliable and proven way to separate the hydrolyzate liquid from the biomass and analyze its composition. Sugars can be separated on a chromatography column and detectors can identify and quantify biomass sugars [15].

Auto-hydrolysis

Autohydrolysis is similar to dilute acid hydrolysis in that it uses elevated time and temperature to hydrolyze different biomass components and to reduce the recalcitrance of the cellulose. The process is also followed by an enzymatic hydrolysis where the remaining oligomers are hydrolyzed into monomeric sugar. The difference comes in where autohydrolysis does not use any added chemicals. Since the goal of dilute acid and autohydrolysis is to solubilize biomass components, the pretreatment methods run the risk of generating furans from the degradation of sugar [16]. These chemicals are known fermentation inhibitors. Hydroxymethylfufural (HMF) is a degradation product from glucose and furfural is a degradation product from xylose and arabinose. Depending on the concentration of these inhibitors, removal of HMF and furfural is a desired process inclusion. Many methods can be employed to remove the inhibitors. Lee, Venditti [16] have described a process of using adsorptive activated carbon. Metal oxides can also be employed to remove fermentation inhibitors.

Kraft pulping/alkiline

Most alkaline, or basic, pretreatments have been adapted from the kraft paper industry. Both industries benefit from the removal of lignin [17]. Many of the inhibitory aspects of lignocellulose are associated with lignin [18]. It has been previously shown that the removal of lignin in biomass makes the remaining solids more susceptable to enzymatic hydrolysis [19-22]. The lignin and hemicellulose can be removed from the pulp by centrifugation or simply washed out with water. The black liquor, or lignin rich liquid from processing, is usually concentrated using evaporators. The lignin can then be sold, processed to recover feedstock chemicals, or burned to generate process heat [23].

AFEX and aqueous ammonia

Ammonia fiber explosion (AFEX) is similar to alkaline pretreatments. They also have many different aspects. AFEX employes ammonia mixed with the biomass at elevated temperatures. After the reaction time, the ammonia and biomass are allowed to "explode" at atmospheric pressures. The ammonia from the process can be recovered and used in subsequent pretreatments. The solids provide a very clean glucan for enzymes to digest [24].

Ionic liquid

Ionic liquids (IL) are salts that are liquid below 100°C. Many are liquid at room temperature. This type of pretreatment is a relatively new modality. IL can be either anion or cation and can solubilize cell wall components and can be designed to decrystallize cellulose [2]. Many IL that are used need to be removed from the biomass prior to enzymatic hydrolysis, as they are inhibitory [25]. Shi, Gladden [25] developed a

method in which the dissolved sugars can be enzymatically saccharified with the IL still present in solution. Over 80 % of glucose and over 85 % xylose yield can be achieved using this method. The IL is separated with a liquid-liquid extraction with over 90 % efficiency using boronate complexes.

3.2. Enzymatic hydrolysis

Enzymes are needed in the cellulose to ethanol process to hydrolyze carbohydrates to monomeric sugars that can then be utilized by microorganisms. They are also one of the highest input costs to a commercial sized ethanol facility [26]. Because of this, amongst other reasons, there has been much research into increasing the sugar yields after enzymatic hydrolysis; either by improving pretreatment technologies [14, 27-29], by improving the enzymes themselves [30, 31], or by adding surfactants or detergents to the hydrolysis to improve efficiency [32, 33]. There are several biomass components that can inhibit enzymatic activity, decreasing the efficiency [34], lignin being one of the most significant [20, 22, 35]. Most enzymes used for hydrolysis are made up of a cocktail of several different enzymes that all do a specific thing. 1,4 β d-glucanases cleave the glyosidic bonds of the amorous cellulose regions. Cellobiohydrolases can reduce the ends within crystalline regions and release cellobiose [36]. B-Gluconsidases are the third complementary enzyme that historically make up the cocktail of cellulosic enzymes. This enzyme hydrolyzes cellobiose or the oligosaccharides into monomeric glucose [37].

3.3. Fermentation

Once the biomass components are converted into monomeric sugar, they can undergo fermentation to produce ethanol. The glucose produced from the cellulose is readily

fermentable by most strains of *S. cerevisiae*. This yeast, however, cannot ferment five carbon sugars, such as xylose and arabinose. Genetically modified organism (GMO) strains of many yeasts and bacteria have been constructed to take advantage of the five carbon sugars [38]. Once the ethanol is produced, conventional distillation processes are employed to remove the ethanol from the whole stillage (beer stripping) and then to increase the ethanol concentration (rectifying). At about 95 % ethanol concentration, ethanol and water form an azeotrope. Due to these interactions, another method is required to dehydrate the ethanol [39]. Molecular sieves are employed, and they are composed of synthetic aluminum silicate zeolite resins. These resins have pore sizes that are small enough to allow water to penetrate, but not ethanol [39].

In a cellulosic ethanol plant, after fermentation, the beer goes to distillation. There the first distillation , known as a beer stripper, will drive off any ethanol, water, and volatile compounds. These tops then go on to more distillation and processing. The bottoms of the beer stripper, or whole stillage, are rich in residual carbohydrates, ash, lignin, and any non-volatile compounds. In some plants, the whole stillage is separated into solid and liquid fractions. The liquid, which is rich in non-volatile organic compounds, can be further digested by microbes to produce a biogas. This gas can then be burned on site for energy, lessening the need for natrual gas. The solid cake will be rich in lignin and residual carbohydrates.

4. Crystalline cellulose

Cellulose can be divided into two types: crystalline and amorphous. Crystalline cellulose is a portion of cellulose fibers not readily degraded or hydrolyzed. Purification and isolation of crystalline cellulose can be done by first removing the amorphous regions,

usually by acid hydrolysis or enzymatic hydrolysis, and then further purification/delignification, such as bleaching [40]. Oksman, Etang [41] has described using residue from a wood bioethanol production facility. This is a source of crystalline cellulose that would merit more research. If dilute acid pretreatment is used the residual residue has already gone through an acid hydrolysis followed by an enzymatic hydrolysis. The residual cellulose that is in this stream is already selected to be the crystalline regions of the fibers. As seen above, the residual residues have little use in the production facilities and have no real value except for that of the BTU values when burned. If a solid fuel boiler is not employed at the production facilities, the residue would have to be landfilled. Depending on local laws and regulations, the material may not be suitable for landfill applications. To purify and isolate the cellulose from a dilute acid pretreatment cellulosic ethanol plant, the residual material after the first ethanol distillation (beer stripping) is sent through a solid/liquid separation. The liquid, which is high in organic compounds and acids, can be sent to microbial processing to obtain a biogas. The solid cake is a material well suited for purified crystalline cellulose (PCC) isolation. The isolation method can be done by a base extraction to solubilize the acid-insoluble lignin and subsequent water washing to rinse the lignin out of the pulp. The delignified material can then be bleached to remove any remaining lignin. What is left is a relatively pure, crystalline cellulose stream that has the potential to add more value to the cellulosic ethanol plant than just its burn energy value (Figure 13A). In a base/delignifing pretreatment modality is used then the delignification is done prior to enzymatic hydrolysis. This leaves the step to select the crystalline regions of the cellulose. An acid hydrolysis step can be utilized here and then the pulp can be rinsed as

with the acid pretreatment modality, including any bleaching step to remove residual lignin (Figure 13B). Further processing can then be done in both modalities to reduce the particle size of the material creating nanocrystalline or microcrystalline cellulose.

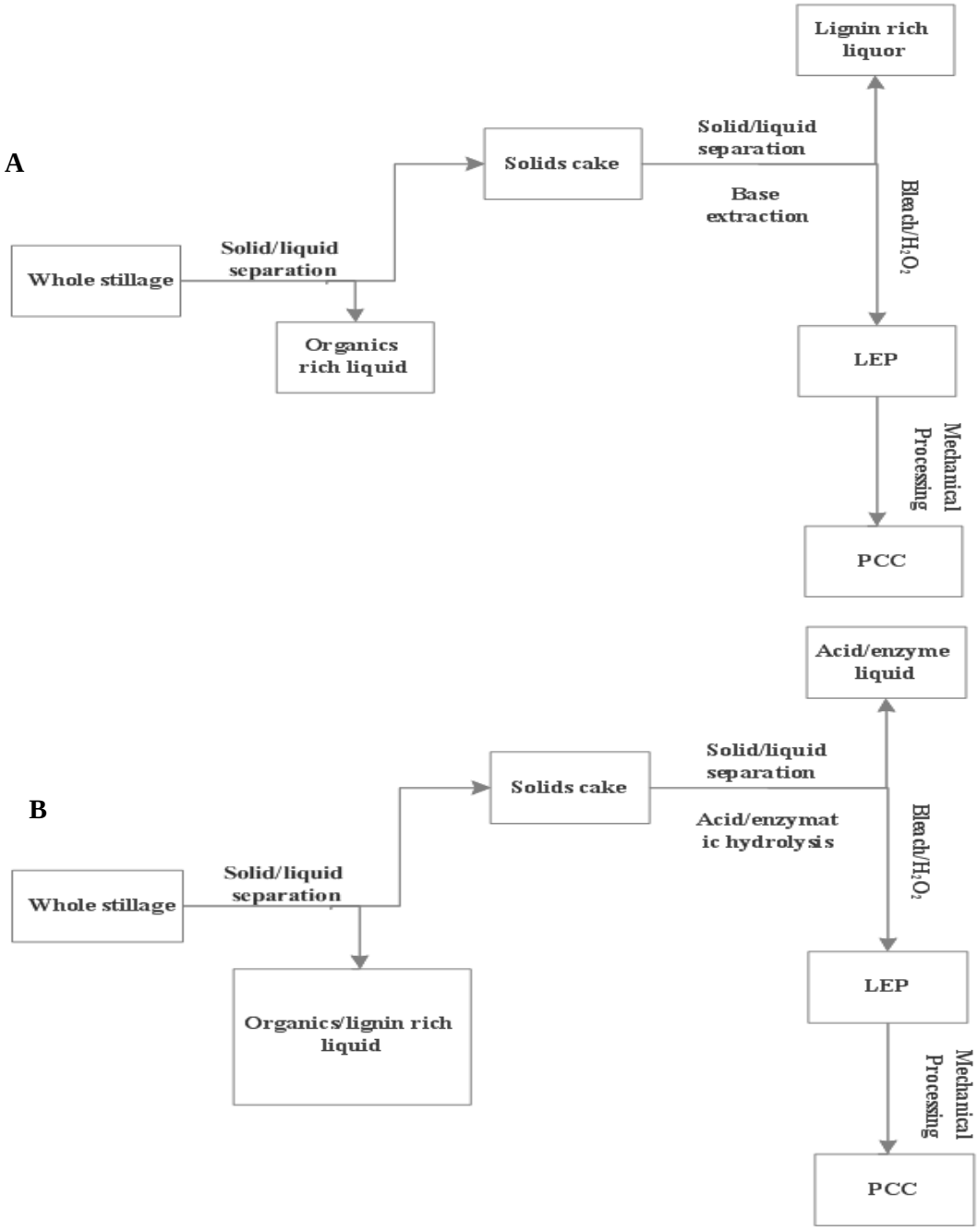


Figure 13. Process flow diagrams of PCC production from an acid hydrolysis pretreatment process (A) and a base/AFEX pretreatment process (B).

4.1. Application of PCC

Pharmaceutical/medical

Purified crystalline cellulose has had uses for many years in the pharmaceutical and medical industries. Bacterial cellulose has been more attractive for many of the applications due to its high purity and crystalline structure [42]. This cellulose is also attractive due to its compatibility to the body and cells, high strength, and its high water binding [43]. Hydrogels are one application being considered for the bacterial cellulose. These gels can be shaped into different geometries. The linked network of cellulose is transparent [44]. Bacterial cellulose is also non-carcinogenic. This makes it an ideal candidate for use in tissue scaffolding and wound dressing [45].

Purified crystalline cellulose from a cellulosic sugar to ethanol plant may have the potential to be used in the same applications. The advantage of the bacterial cellulose is its purity. One disadvantage is that it is not being generated on a large scale. Given the right extractions steps, the cellulose derived from an ethanol plant may be just as pure. The crystallinity is higher in bacterial cellulose; however, there may be a level of acceptable crystallinity for these applications [46]. A cost analysis would have to be performed to analyze the purification and extraction steps versus the revenue generated by selling into the biomedical field.

Films

The cellulose derived and purified from the waste streams of the cellulosic ethanol plant has a high Young's modulus, high surface area and aspect ratio, and high crystallinity [47]. Reinforcement of cast and extruded films are one of the properties PCC could

improve. Inclusion into a cast chitosan film not only creates a biodegradable film suitable for food packaging, but the inclusion of PCC improves tensile strength, water vapor permeability, and particle swelling [48]. Homogenous dispersion of the PCC is one of the problems facing inclusion into low-density polyethylene (LDPE). Although PCC can be highly stable in an aqueous suspension, an organic solvent may have to be used as the dispersing agent in melt extrusion processes. Surface modification of the PCC may be needed to obtain better dispersal. When this is done, PCC significantly improves the properties of LDPE [49].

Absorbent paper

Specialty papers, such as kitchen paper towels, facial tissues, and toilet paper, have wide spread use and represent a significant portion of the total paper pulp industry. The strength of these wetted products is an important requirement, for obvious reasons. PCC can be utilized as an additive that can give increased wet strength while also not using any new paper pulp material [50]. Filler material, or bulking material, is also a good fit for PCC. As well as inclusion into paper board to strengthen the finished product while not having to add increased bulk [51].

5. Conclusion

Purified crystalline cellulose is a potential way that cellulosic ethanol plants can add value to their process. The additional value could make the industry a more attractive investment for others, therefore adding more competition and driving more and better technology. This could, in time, help reduce our dependence on fossil fuels and petrol products.

Chapter 2: Screening Conditions for Acid Pretreatment and Enzymatic Hydrolysis of Empty Fruit Bunches

This chapter has been published previously and can be found at https://doi.org/10.1016/j.indcrop.2016.01.041

Abstract

Empty fruit bunches were received from Teck Guan, Malaysia and were pretreated and enzymatically hydrolyzed to determine the possible sugar recovery from the biomass. Several different conditions were explored in a screening study. Temperature ranged from 100 °C – 150 °C, time ranged from 30 – 90 minutes, and acid loading ranged from 0 – 1.3 % weight acid/weight liquid. The material was then enzymatically hydrolyzed at three different enzyme loadings 1.67 %, 3.33 %, and 6.66 % (g enzyme/g glucan x 100) and total sugar recovery was calculated for both pretreatment and enzymatic hydrolysis. Best pretreatment conditions yielded 81.4 % recovery of hydrolyzed xylan. Best glucan conversions in enzymatic hydrolysis were 74.8 %. These conversions and recoveries make empty fruit bunches a good potential feedstock for cellulosic ethanol.

1. Introduction

Ethanol is an important fuel alternative for use in the transportation sector. Ethanol can be derived from many different sugar sources, including starches from corn. As ethanol becomes more prevalent and widely available, new sources are being sought to replace corn as one of the most used feedstock. Cellulosic ethanol is derived from fermentation of sugars hydrolyzed from cellulose and hemicellulose in plant material, such as agricultural waste and residues. One such feedstock is the lignocellulosic residue that is left over from processing the oil from palm.

Oil palm, *Elaeis guineensis*, is an important oil producing crop for many countries such as Malaysia and tropical regions such as Southeast Asia. The empty fruit bunches (EFB) that are produced after processing the oil from palm, are currently used as a substrate for the cultivation of mushrooms as a manure [52] or burned for the BTU value [53]. The EFB are a fibrous material that is generated after the palm fruit is processed to extract the oil. Fibers are primarily composed of cellulose and hemicellulose, two compounds that can be hydrolyzed into glucose and xylose, which in turn can be fermented into ethanol, and are comparable to a more common cellulosic feedstock, such as corn stover (Table 1). The hemicellulose is composed primarily of xylan with less arabinan making up the composition (Table 1). This is a lower ratio than that of stover.

Table 1. Compositional analysis of raw EFB and corn stover. All values are listed as a percentage of total mass and are averages of 3 samples.

Sample	Structural Inorganics	Non-structural Inorganics	Water Extractives	Ethanol Extractives	Lignin	Glucan	Xylan	Arabinan	Acetyl	Mass Closure
EFB sample	2.61	2.42	3.87	4.79	20.4	33.5	21.5	1.11	4.58	94.8
Corn Stover Sample	5.44	1.33	10.8	2.38	12.8	34.8	23.7	3.34	2.91	97.5

Several pretreatment conditions have been previously suggested [53, 54]. Sulfuric acid will be used in this study. Its benefits have been described before [55]. The goal of pretreatment and enzymatic hydrolysis is to maximize the conversion of the polysaccharide components (glucan and xylan) to monomeric sugars (glucose and xylose) for use in fermentation.

During pretreatment the goal is to maintain conditions severe enough to hydrolyze hemicellulose and cellulose and open the crystalline structures for enzymes to access

without being so severe as to create enzymatic hydrolysis and fermentation inhibitors such as hydroxymethylfurfural (HMF) and furfural. HMF is formed from the dehydration of glucose and furfural from xylose [56]. This study was carried out as a screening study to observe the effect of different pretreatment conditions of EFB and use corn stover as a benchmark. It also focuses on how recalcitrant EFB are in enzymatic hydrolysis under this study's pretreatment conditions.

2. Materials and Methods

2.1. Material

Feedstock

Empty fruit bunches were obtained from Teck Guan, Tawau, Sabah, Malaysia. The feedstock was stored in a cooler at 3 °C**.** Samples were dried in a 40 °C oven and milled using a knife mill fitted with a 1 mm screen. Compositional analyses were done on three sub-samples and are listed in Table 1. The samples were then used in pretreatment.

Pretreatment

Pretreatments were carried out in two Parr 5100 reactors fitted with two stainless steel 1 L jacketed reactor vessels. The sulfuric acid used was 91.2 % sulfuric acid used for Babcock test (Fisher Scientific).

Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out in a BD Falcon 35-1143 Multiwell 12 well plate using NS22146 enzymes (Novozymes). The plates were incubated in a New Brunswick Anova 4300 digital incubator shaker set at 50 °C and 150 rpm.

Analytical testing

HPLC – Liquid samples were loaded into 1 mL HPLC vials after being filtered through a 0.2 µm filter. The vials were loaded onto a carousel which fits into an autosampler (either 717 plus or 2695 separations module from Waters). An aliquot (5 µL) of the sample was injected by the auto-injector onto a reverse phase column (HPX-87H from BioRad Laboratories) maintained at 50 °C. Sulfuric acid at 0.005 M was used as the mobile phase (eluent). The HPLC system was fitted with a refractive index detector (either the 2410 or 2414 model from Waters). The components (sugars, organic acids, and ethanol) were identified and quantified using the Empower software from Waters.

In house compositional analysis follows NREL LAP (nrel.gov) procedures and calculation sheets.

Data analysis

Data were analyzed and graphed on Graphpad Prism software. P values were calculated in Graphpad by one-way ANOVA using Tukey's multiple comparisons test, or by two-way ANOVA using Sidak's multiple comparisons test. Tables and calculations were created using Microsoft Excel.

2.2. Methods

Pretreatment

Ten pretreatments were performed using two, 1 L Parr jacketed reactors. Five conditions were explored as part of this screening study, done pairwise in the two reactors. All reactions were completed with a solids loading of 12.5 % weight of biomass/weight of liquid (w/w). A working mass was kept constant at 700 g. Several conditions were screened and can be found in Table 2. The acid concentrations were loaded as a percentage of the total mass of liquid in the reactor. The combined severity (CS) was calculated using time, temperature and pH [57]:

$$logCS = logR_O - pH$$

R_O is defined as:

$$R_O = t \cdot [(T_H - T_R)/14.75],$$

where t is the time in minutes, T_H is the hydrolysis temperature in °C, and T_R is the reference temperature 100 °C. Once the reactor was loaded and secured, the slurry was brought to the target temperature using steam to heat the jacket of the reactor vessel. The reactor was held at temperature for the target time and cooled to 35 °C in 2 – 3 minutes by running water through the vessel jacket. The slurry was then loaded into 1 L centrifuge tubes and centrifuged for 20 minutes at 4800 x g. The liquor was decanted and sampled in duplicate for analysis. The solids were also sampled for analysis. Both the liquor and solids were retained for enzymatic hydrolysis.

Table 2. Pretreatment conditions and combined severity (CS) factors for EFB. CS values are an average of two pretreatments (n=2).

Condition	Target Time (min)	Target Temperature (°C)	Target acid loading (%)	Target solid/liquid ratio (%)	Target total mass (g)	Combined severity
100°C/90min/0%H+	90	100	0	12.5	700	-4.39
150°C/30min/0%H+	30	150	0	12.5	700	-2.55
100°C/30min/1.3%H+	30	100	1.3	12.5	700	0.20
125°C/60min/0.65%H+	60	125	0.65	12.5	700	0.80
150°C/90min/1.3%H+	90	150	1.3	12.5	700	2.10

Duplicate samples of the liquor were assayed for sugars, acetic acid, and HMF/furfural concentrations by HPLC analysis. Total solids, total dissolved solids, total suspended solids, and density were done on the liquor. The total solids analysis of the solids was determined. Both liquor and solid samples were submitted for compositional analyses. Duplicate liquor samples were sent to a third party laboratory for sulfate analysis. The liquor compositional analyses were used to determine percent of soluble xylan, glucan, and arabinan, monomeric glucose, xylose and arabinose and to determine mass closures around pretreatment. The composition of the raw (starting) biomass feedstock was also determined and is reported in Table 1.

Enzymatic hydrolysis

Each pretreatment condition was enzymatically hydrolyzed in duplicate using NS22146 dosed at 1.67 %, 3.33 %, and 6.66 % (g enzyme/ g glucan of the pretreated solids x 100). The liquor that was separated by centrifugation was used for make-up water. The enzymatic hydrolysis was carried out in 12 well plates with a volume of 8 mL at 17 % solids loading. The enzymatic hydrolysis temperature was 50 °C in a shaking incubator set at 150 rpm for 120 hours. At the end of the enzymatic hydrolysis the samples were

filtered and sugar concentrations were determined by HPLC. Glucose and xylose yields are calculated as a percentage of measured mass over calculated theoretical maximum mass.

Table 3. Yields (amount recovered as a percentage from starting biomass) for each biomass component solubilized in pretreatment. n=2

CS	Lignin	Monomeric Xylose	Soluble Xylan	Furfural	Monomeric Glucose	Soluble Glucan	HMF	Acetic Acid
-4.39	1.24	0.900	2.32	0.00	0.280	0.640	0.0100	12.7
-2.55	1.42	0.790	4.82	0.0300	0.560	0.680	0.00	24.7
0.20	1.74	8.82	26.3	0.0200	0.430	1.68	0.00	29.3
0.80	1.77	10.4	32.8	0.110	0.390	1.66	0.0100	33.9
2.10	4.94	62.8	1.69	16.9	7.07	0.750	0.430	77.5

3. Results and Discussion

3.1. Pretreatment

Table 4. Xylose yield from EFB and corn stover pretreated at a similar CS. Corn stover conditions and yield from Tucker et al. (2003)

Sample	Target Time (min)	Target Temperature (°C)	Target acid loading (%)	CS	Total soluble xylose yield (% theoretical)
EFB	90	150	1.3	2.10	81.4
Corn Stover	1.83	190	1.0	2.05	84.5

This study was done as part of a screening study. These conditions do not represent the most optimal for xylan and glucan conversions. The EFB responded to pretreatment conditions as expected and performed similar to corn stover pretreated at similar CS factors [1] (Table 4). The CS factors ranged from -4.39 to 2.10. Three different enzyme

loadings were used. As expected, the highest enzyme loading yielded the best xylan and glucan conversion. The highest severity pretreatment performed the best in enzymatic hydrolysis. Lower severity pretreatments showed similar results when looking at total sugar recovery, however (Figure 4b). The sample pretreated at 0.08 CS had no statistical difference ($p = 0.2250$) to the sample pretreated at 2.10 CS. This result could be misleading. Although the overall yield had no statistical difference, the higher CS yielded better glucose recovery ($p < 0.0001$). Much of the xylose was driven to furfural in the higher CS. This is a loss of sugar, so when glucose and xylose yields are combined, the

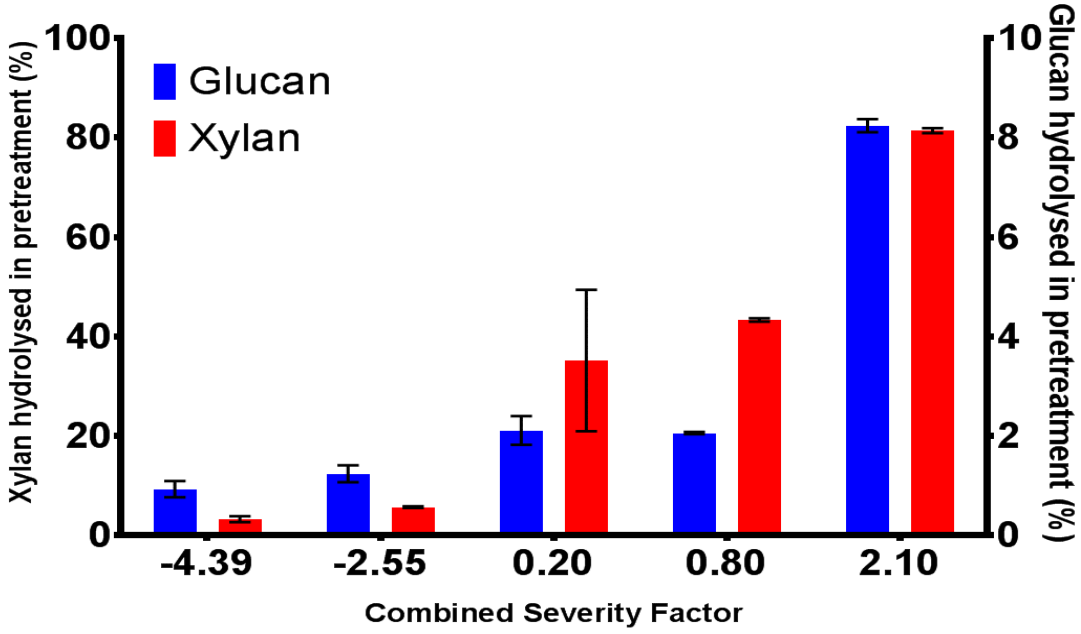


Figure 1. Total xylan and glucan solubilized in pretreatment. Average means are reported (n=2) with error bars representing standard deviation

difference between 0.80 CS and 2.10 CS becomes statistically the same. So while a lower severity may be used to convert xylan to xylose, a more severe pretreatment condition would still be needed to convert the glucan to glucose. Future work could examine more optimal conditions for both xylan and glucan conversions. Other studies could also examine a two stage pretreatment process, wherein the first stage is performed at lower

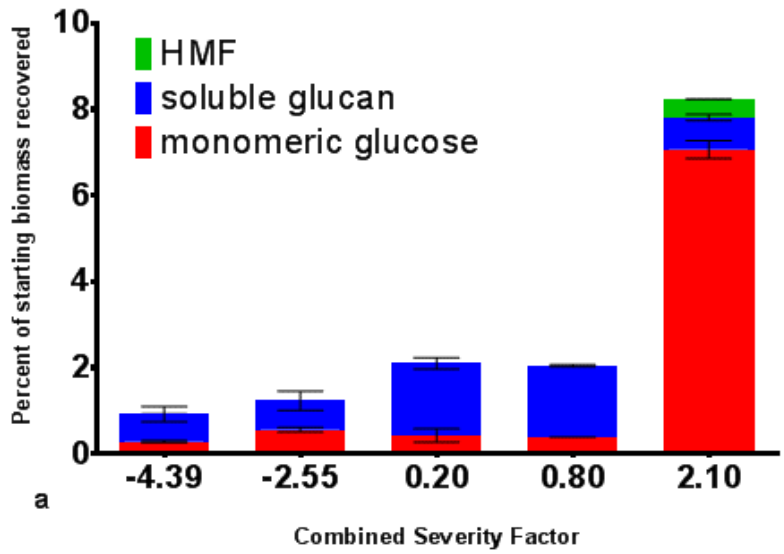
severity to optimize xylan conversion without converting to inhibitors. The second stage could then be more severe to optimize glucan conversion in enzymatic hydrolysis.

The lowest severity pretreatment condition was repeated in this study. The first pretreatment conditions yielded higher enzymatic hydrolysis yields and conversions than higher severity conditions. It was determined to be outliers and the conditions were repeated. After pretreatment, the samples were processed as before. During enzymatic hydrolysis, only the 6.66 % enzyme loading was used. Material from the highest severity was enzymatically hydrolyzed with the new lowest severity pretreatment as a control using the 6.66% enzyme loading. The control yields were lower than what they were in the first enzymatic hydrolysis, so to be able to compare the first enzymatic hydrolysis to the repeat; the control was normalized to the original. This factor was then applied to the calculated yields from the repeat pretreatment.

Table 1 shows the composition of the empty fruit bunches compared to that of raw stover. The inorganic content of the EFB are lower than that of stover. The lignin is higher and the available glucan and xylan are comparable to that of stover. The ethanol extractives

are higher in EFB than stover due to the high residual oil content in the EFB. Lignin was 63 % higher in the EFB. The acetyl component was higher in EFB and arabinan was lower than corn stover.

Analysis of the recovered pretreatment liquor show total recovered xylan peaked at 81 %. Figure 1 shows that the most severe pretreatment also yielded the best recovery of soluble xylan, monomeric xylose, and furfural. The most severe condition converted most of the available xylan to either monomeric xylose (62.8 %) or furfural (16.9 %) with only 1.69 % as soluble xylan (Figure 2a). Figure 2b shows the breakdown of glucan. The most severe condition returned 8.24 % glucan (Figure 1) and of the glucan recovered, 7.07 % was monomeric glucose, 0.750 % was soluble glucan, and 0.430 % was converted to HMF (Figure 2b).



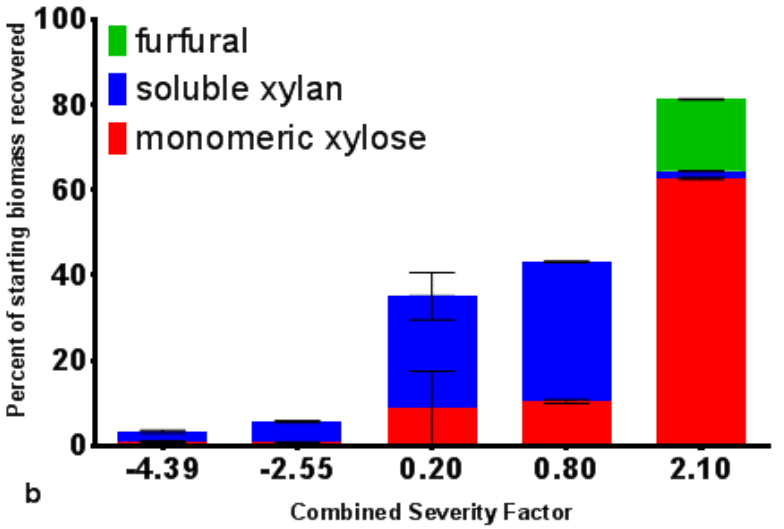


Figure 2. (a) Total xylan recovery broken down into monomeric xylose, soluble xylan, and furfural. Average means are reported (n=2) with error bars representing standard deviation. (b) Total glucan recovery broken down into monomeric glucose, soluble glucan, and HMF. Average means are reported (n=2) with error bars representing standard deviation.

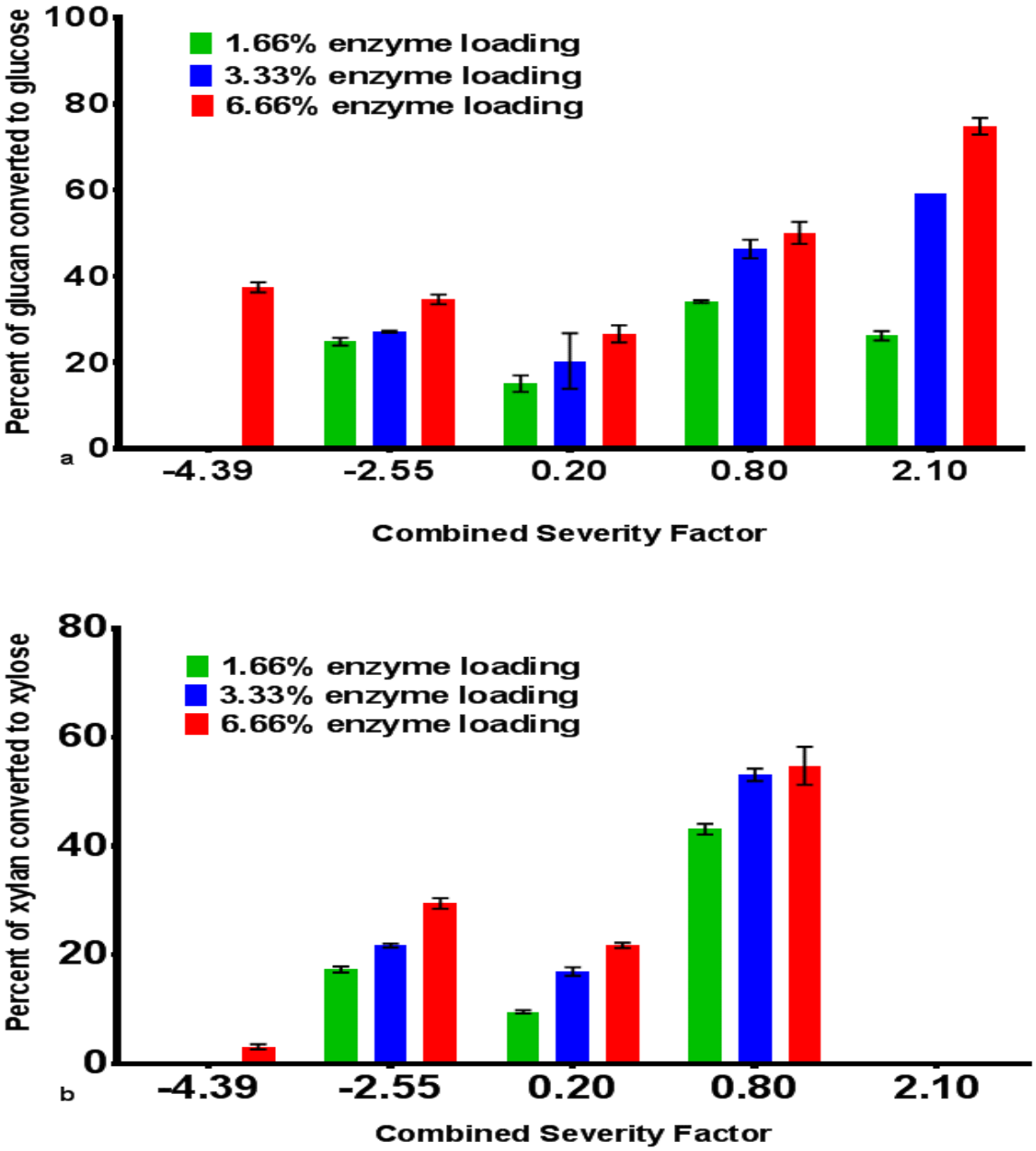


Figure 3. (a) Glucose conversion from glucan after enzymatic hydrolysis. Average means are reported (n=2) with error bars representing standard deviation. (b) Xylose conversion from xylan after enzymatic hydrolysis. Average means are reported (n=2) with error bars representing standard deviation. Yields for -4.39 CS have been normalized to other conditions.

Table 3 shows the liquor composition of the hydrolyzed biomass. This is the percent of each component recovered and hydrolyzed in the liquor. The most severe condition

yielded 77.5 % recovery of the acetyl component as acetic acid. This recovery could represent a possible co-product and revenue stream [58].

3.2. Enzymatic Hydrolysis

After 120 hours, results showed the best glucan to glucose conversion (76.6 %) at the 6.66 % enzyme loading for the highest severity pretreatments (Figure 3a). The best xylan to xylose conversion in enzymatic hydrolysis was seen in the 0.80 CS (54.8 %) (Figure 3b). It should be noted that the majority of xylan was hydrolyzed to either monomeric xylose or furfural in the most severe pretreatment (Figure 2a). This means that there is less xylan to convert during enzymatic hydrolysis, leading to lower xylan to xylose conversion in enzymatic hydrolysis (Figure 3b). This fact gave sugar concentrations below quantification limits when analyzed and were excluded from the figure. Figure 4a shows overall glucose and xylose yields for material saccharified with the 6.66 % enzyme loading. This is the percentage of xylose and glucose recovered from the starting biomass through pretreatment and enzymatic hydrolysis. There was no statistical difference for xylan yield between the -2.55 CS and the 0.20 CS ($p = 0.6434$) and between the 0.80 CS and 2.10 CS ($p = 0.9970$). No statistical difference was observed between the -4.39 CS and the -2.55 CS ($p > 0.9999$) for glucan yield. Figure 4b shows the combined (xylose yield + glucose yield) overall yield for the 6.66% enzyme loading. When looking at Figure 4b, no statistical difference is seen between -4.39 CS and -2.55 CS ($p = 0.4130$), -4.39 CS and -0.20 CS ($p = 0.9662$), -4.39 CS and 0.80 CS ($p = 0.1323$), -2.55 CS and 0.20 CS ($p = 0.5569$), 0.20 CS and 0.80 CS ($p = 0.1569$), and 0.80 CS and 2.10 CS ($p = 0.2250$).

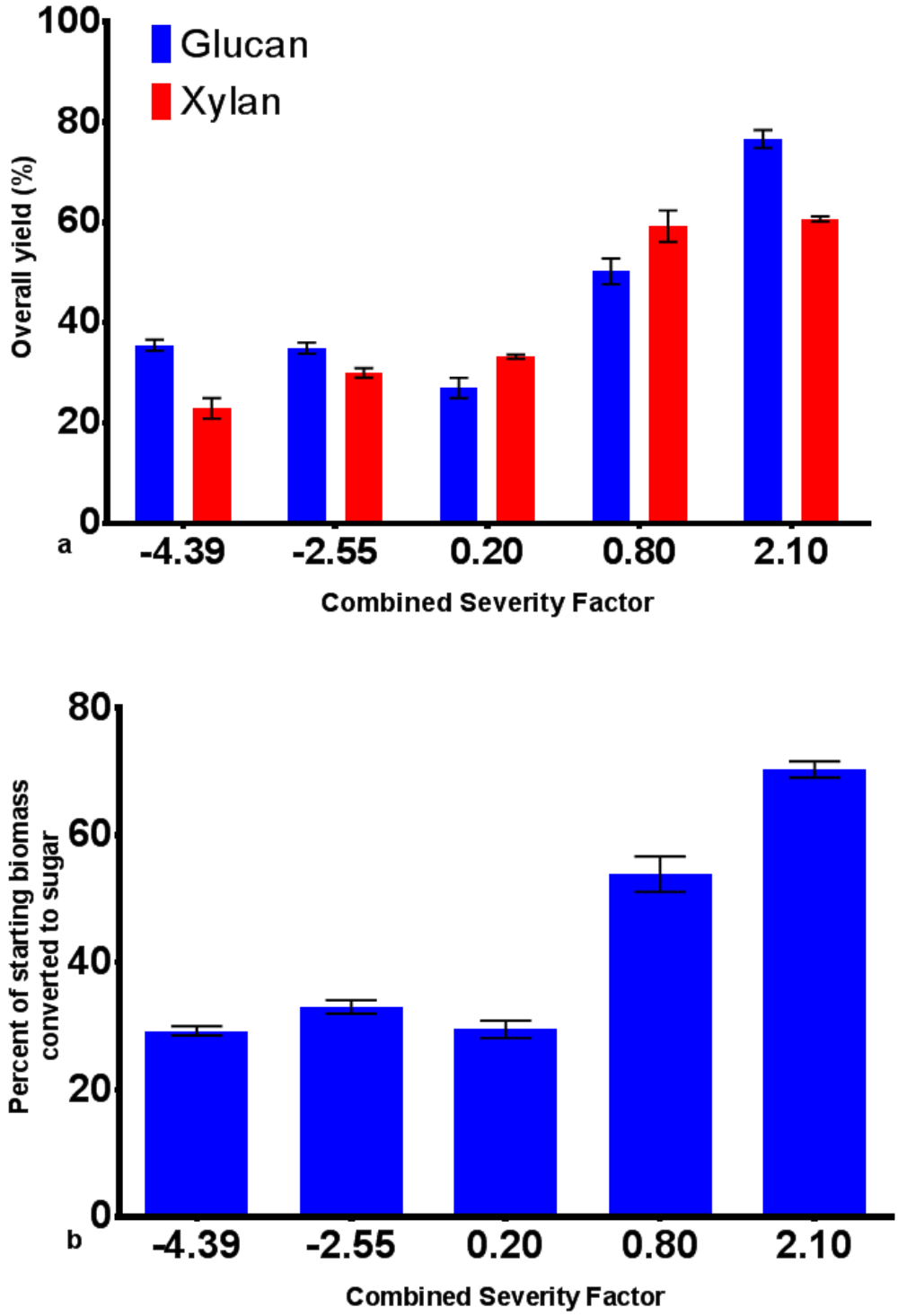


Figure 4. (a) Overall glucose and xylose yield for material enzymatically hydrolyzed at 6.66% enzyme loading. Average means are reported (n=2) with error bars representing standard deviation. Yields for -4.39 CS have been normalized to other conditions. (b) Glucose and xylose yield from starting xylan and glucan in raw biomass. Yields represent sugar recovery from both pretreatment and saccharification using 6.66% enzyme loading. Average means are reported (n=2) with error bars representing standard deviation. Yields for -4.39 CS have been normalized to other conditions.

The ethanol extractives were much higher in the EFB than in corn stover since there is

residual oil on the EFB from the oil extraction process [59]. If this oil could be extracted before pretreatment, it has the potential to be a revenue stream. Removing the oil could have further benefits in enzymatic hydrolysis and fermentation [60]. This was not examined in this study. Another possible co-product from this process is acetic acid. EFB contain 63.5 % more acetyl than that of corn stover and could be used as a revenue stream [61].

4. Conclusions

The most severe pretreatment yielded the best conversion of hemicellulose in pretreatment. The most severe pretreatment also performed the best in enzymatic hydrolysis with regards to glucan conversion to glucose. The highest enzyme loading converted more glucan and xylan to glucose and xylose than the lower two loadings. The xylan and glucan composition of the raw EFB was comparable to that of corn stover. Higher ethanol extractives and acetyl components in the raw EFB compared to corn stover could be used as possible revenue streams. The removal of the residual oil from EFB could also have benefits in enzymatic hydrolysis and fermentation. The pretreatment condition that yielded the best xylan recovery (2.10 CS) performed similar to that of stover pretreated at a similar CS. Future work can include looking in depth at why the lower CS pretreatment conditions didn’t perform as well in enzymatic hydrolysis as the higher CS pretreatments. One possibility would be to observe the lignocellulosic structures under a scanning electron microscope before and after pretreatment at the given conditions. Given that 81.4 % of the available xylan and 74.8 % of the available glucan was recovered, EFB could be a viable feedstock for cellulosic ethanol.

Chapter 3: Lignin extraction of whole stillage from a pilot scale cellulosic ethanol plant

Abstract

Whole stillage obtained from a pilot cellulosic ethanol plant was centrifuged to obtain a lignin rich solid pulp. The lignin was then extracted from the pulp to obtain a relatively clean cellulose stream. Sodium hydroxide was used to extract the lignin from the pulp. Hydrogen peroxide and water were used to wash residual lignin out of the pulp. The greatest removal of lignin was seen at 95.58 ± 1.5 %. Extracting a lignin rich stream while leaving a relatively clean cellulosic stream may provide a co-product opportunity for large scale cellulosic ethanol biorefineries. This could then provide a greater profit for such facilities, facilitating greater investment in this renewable transportation fuel.

1. Introduction

Transportation fuel is one of the leading causes of greenhouse gas emissions in the United States [62]. The United States is also one of the world's largest producers of these emissions [63]. Ethanol has been shown to reduce the amount of greenhouse gas emissions [64]. In the United States, ethanol is primarily produced from field corn. The starch of the kernel is converted to monomeric glucose, which can then be used as a carbon source for yeasts to ferment it to ethanol. While the benefits of using corn ethanol have been shown before [65], a new source of sugars is needed to be able to keep up with demand for transportation fuels. Lignocellulosic feedstocks, which are primarily made of carbohydrates and lignin, can be used as a feedstock to ferment and produce ethanol which can be used to displace the current transportation fuels. There are many challenges associated with the conversion of lignocellulosic sugars to ethanol, however [66]`, one

being the high cost [67]. If there were a way for the lignocellulosic biorefineries to increase the revenue and profit, cellulosic ethanol could be a much more attractive fuel in which a greater number of people and companies would invest.

Cellulosic ethanol is produced from sugars derived from hydrolyzed cellulose and hemicellulose found in the cell wall of plants. The sugars are then fermented. There are many ways to hydrolyze the cellulose and hemicellulose into sugars, each with its own advantages and disadvantages [68]. The material used in this study was generated from a dilute acid pretreatment process. This process was then followed by an enzymatic hydrolysis step. The dilute acid pretreatment process is a balancing act. One must pretreat severe enough to break open the cellulosic structure for enzyme access, but at the same time, keep the severity low enough so as not to degrade the sugars to inhibitory compounds [33]. Because of this balancing act, and due to the recalcitrance of some lignocellulosic feedstocks, there are inevitably residual carbohydrates. These residual carbohydrates can be isolated and purified. They can then be utilized in many processes that could add value to a stream that would otherwise be waste, or as a best case, be burned [69].

Lignin is one of the other main components of lignocellulosic feedstocks. Valorizing lignin is one of the industry's most sought after goals. Much research has been put forth to include lignin as-is [70], purify lignin [10], and include derivatize lignin to value added chemicals [71]. The lignin removed from the cellulosic to ethanol process may provide a good feedstock or value added chemical for many processes and products. Lignin isolated from corn stover may provide better performances in some application when compared to

lignin isolated from other feedstocks. Kalami, Arefmanesh [72] describe lignin derived from corn stover that was able to replace 100% of phenol in phenolic adhesives.

2. Material and Methods

2.1. Materials

Cellulosic whole stillage was obtained from the POET Research Inc cellulosic to ethanol plant, BELL. Cellulosic whole stillage is the term given to the material that is left after the initial distillation step in the cellulosic sugars to ethanol process. It contains residual carbohydrates, acid insoluble lignin, lignin breakdown components, water, and ash.

Sodium hydroxide pellets were bought from Sigma-Aldrich. Hydrogen peroxide was purchased at a local grocery store.

Parr 4600 internal stirred pressure reactors were used for the lignin extraction. Reactions took place in a 100 ml reaction vessel.

Beckman J6B floor centrifuge and Beckman Avanti J-26XP floor centrifuge were used for solid/liquid separation.

Data analyzation and graphical display was done using either Microsoft Excel, SAS JMP 11, or Prism GraphPad 6.

Table 5. Compositional analysis of the lignin cake. Values represent the percent of each analyite.

Sample	Ash	Lignin	Glucan	Xylan	Arabinan	Acetyl	Mass closure
Lignin cake	10.7	46.0	25.2	7.76	0.115	1.06	90.8

2.2. Methods

Feedstock

Cellulosic whole stillage was loaded into 1 L centrifuge tubes. They were then spun at 4500 rpm for 10 min. The supernatant was decanted and the solid layer (lignin cake; Table 5) was emptied into a sample bag. Several centrifuge tube solids were loaded into one bag and mixed well for one homogenous sample. A subsample of the cake was then dried in a 40°C oven until constant mass. The dried cake was then milled using a coffee grinder and the milled material was examined for composition following a modified NREL LAP (NREL/TP-510-42627).

Lignin extraction

Total moisture was determined for the lignin cake. Reactors were loaded to 8 % total solids loading with a total working mass of 70 g. A 50 % w/v solution of NaOH was made by dissolving NaOH pellets in RO water. This solution was then used as make up for the reactions. Sodium hydroxide loading and temperature were independent variables; the DOE can be found in Table 6. All conditions were run in triplicate.

After the reactor was loaded, the reaction vessel was secured to the reactor head and an electric heating mantle was affixed around the vessel. The Parr reactors are controlled via provided software. All reactions were held to 90 min.

After the reactions, the heating mantel was removed and the vessels were doused with a pitcher of water to cool the reaction. Once cool, the lignin cake slurry was loaded into centrifuge tubes and spun at 16000 rpm for 5 min. The initial black liquor was decanted

and collected for further analyzation. The solids were mixed with RO water at 10X volume of the decanted black liquor. This step was repeated ten times to thoroughly wash the biomass. The solids were then washed with a 10X volume of hydrogen peroxide, three times. Then the solids were washed again with RO water. The washed lignin cake then had moisture determined and was milled, once dried, for compositional analysis.

Mass balance

Compositional results were used to calculate mass of each component before and after lignin extraction. Insoluble yields were determined by Eq. 1. Yields are reported as the percentage of the original mass that was not solubilized from the starting feedstock.

$$Insoluble\ biomass\ yiled = \frac{Mass\ insoluble\ biomass\ component\ (g)}{Theoritical \max mass\ of\ biomass\ component\ (g)} * 100$$

Eq. 1.

Data analysis

Yield calculations were made using Excel. SAS JMP 11 was used for statistical comparison. Standard least squares analysis was performed for the given DOE and a prediction profiler was generated from this analysis. Bar graphs were created using Prism GraphPad 6.

Table 6. Design conditions for lignin extraction

Condition	Target time (min)	Target temperature (°C)	Target NaOH loading (% w_{NaOH}/w_{liquid})
1	90	120	3
2	90	100	5
3	90	120	5
4	90	140	7
5	90	120	7
6	90	140	3
7	90	100	3
8	90	100	7
9	90	140	5
10	90	100	3

3. Results

Yield calculations were made using Excel. The data was then loaded into SAS JMP 11 for statistical analysis. A fit least squares analysis was performed using the target temperature, target NaOH concentration, and the interactions between those two variables, including 2nd order interactions. Figure 6 shows the ANOVA table from this analysis. The only significant factor in this model was found to be the target temperature. A prediction profiler was run (Figure 7) and 140°C was found to be the temperature needed to achieve highest lignin solubilization. Target NaOH concentration was included in the analysis even though it was not found to be a significant factor in this model.

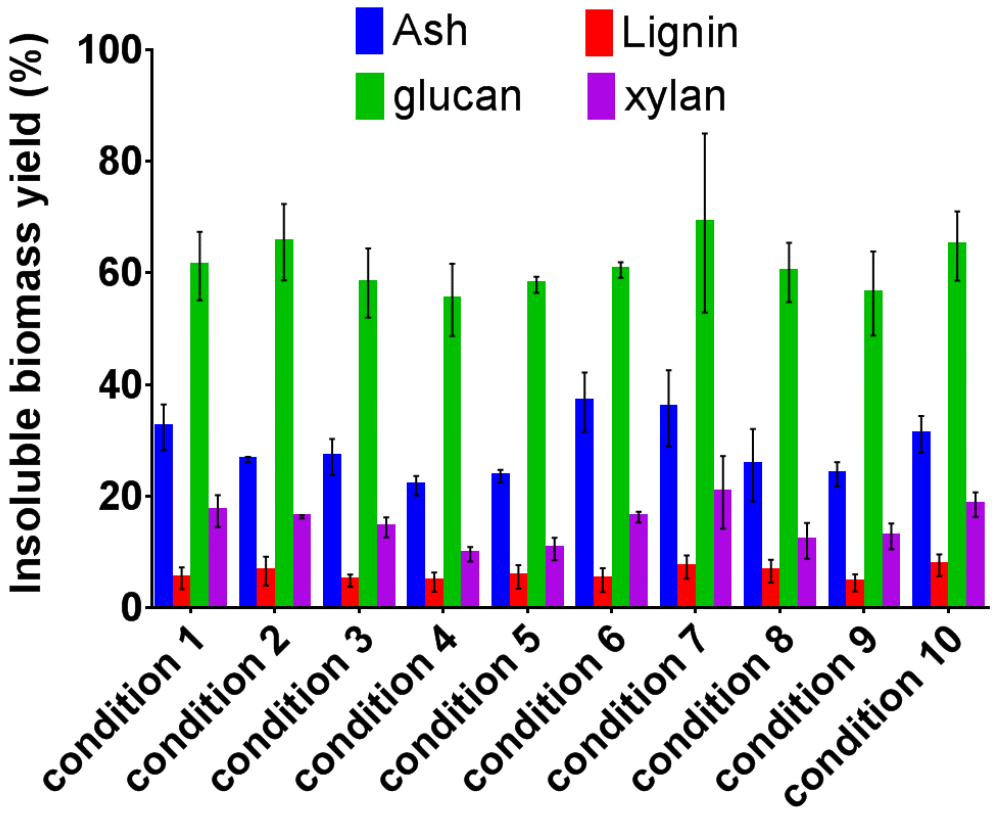


Figure 5. Insoluble biomass yields. Components are calculated using Eq. 1. Error bars represent standard deviation.

Insoluble yields were calculated as Eq. 1. Figure 5 shows the insoluble yields of the biomass components. This is the percentage of each component retained in the biomass relative to the original component loading. Since it is proportional to total mass, as components are taken out at different rates (i.e. lignin), ash's total proportion of the total mass either is diluted or concentrated.

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	38.84821	7.76964	2.7592
Error	30	84.47708	2.81590	Prob > F
C. Total	35	123.32529		0.0363*

Figure 6. ANOVA table from standard least squares analysis of lignin extraction experiment.

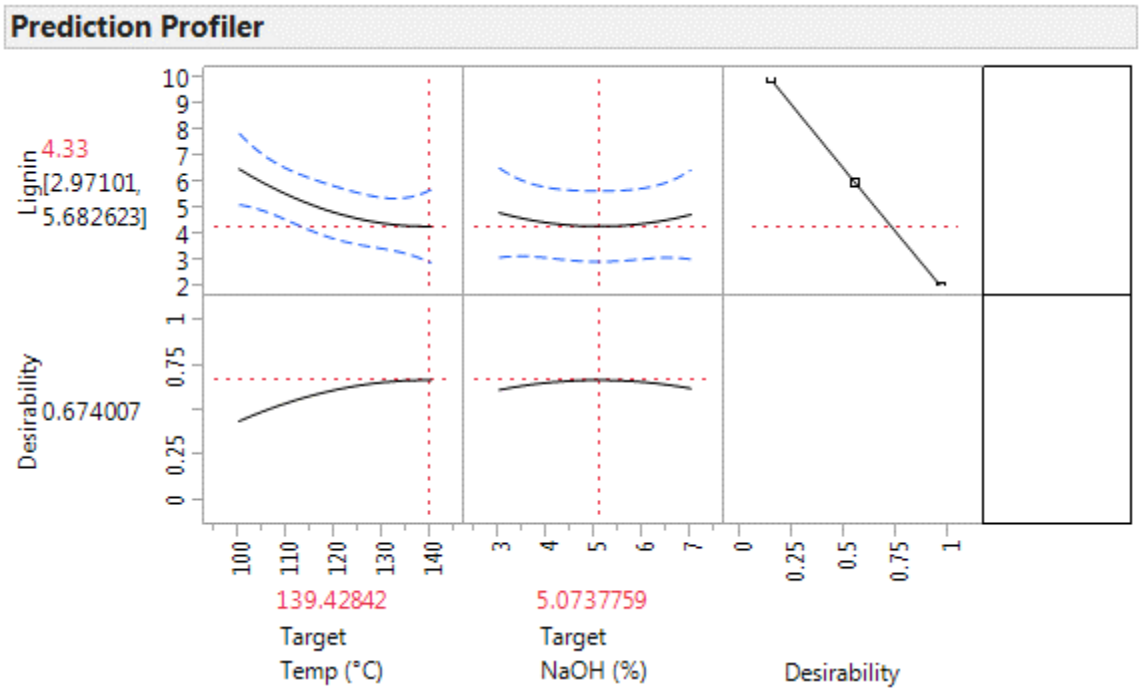


Figure 7. Prediction profiler of the lignin extraction data. Data was fit to standard least square analysis and conditions that yielded highest solubility were sought.

A one-way ANOVA comparison of means with Tukey-Kramer HSD was also performed using the data from Figure 5. Figure 8 shows the connecting letters report for each condition. There was no significant difference found for lignin solubilization for any condition. The glucan yield was statistically different between condition 7 and conditions 3, 4, and 9.

Connecting Letters Report

Level		Mean
condition 10	A	7.5866667
condition 7	A	7.2900000
condition 8	A	6.5433333
condition 2	A	6.5200000
condition 5	A	5.5100000
condition 1	A	5.2466667
condition 6	A	4.9566667
condition 3	A	4.8311111
condition 4	A	4.5800000
condition 9	A	4.4200000

Levels not connected by same letter are significantly different.

Figure 8. Connecting letters report comparing the solubilization yields for lignin. Each condition n = 3.

4. Conclusions

Lignin was removed from the feedstock. With the given data from this experiment, it was decided to use 140°C and a NaOH concentration of 5% (w/v) would be used for conditions in the following experiments. The same lignin cake feedstock was heated and held at 90 min. Larger reactors (Parr 5100) were used for the lignin extraction. A working mass of 700 g was kept for the 1 L reactor vessels. The reactors are similar to the smaller ones but have a steam-jacked vessel for heating samples. The slurry was then processed as before using a centrifuge to separate the black liquor and to wash the cake. The washed cake was then frozen for further mechanical size reduction experiments.

Chapter 4: Production and evaluation of crystalline cellulose from a purified waste stream from a cellulosic ethanol plant

Abstract

Nanocrystalline cellulose was produced from a cellulosic rich waste stream from an industrial cellulosic ethanol plant. Three different methods were used to size reduce the cellulose pulp: homogenization, ultrasonication, and high shear mixing. Laser scattering particle size distribution, XRD, TEM imaging, and optical light microscopy were used to evaluate the size-reduced pulp. Crystallinity index was calculated using the peak height method and the deconvolution method from the XRD data. Pulp crystallinity values were 59.6% and 32.7% for the peak height and deconvolution methods, respectively. The highest crystallinity indexes were found using the homogenizer and the high shear mixer.

1. Introduction

Cellulose is one of the most abundant biopolymers in the word. Cellulose fibers were some of the first materials early humans learned to use and manipulate, turning plants into woven cloths and tools, and later into paper. With much dedication and resources being given to develop bio-renewable products, cellulose's many applications are being reviewed and studied. Nanocrystalline cellulose is generated from cellulose by several different means and can be used in a wide range of products and applications [73]. This research will be focused on generating and testing the quality of nanocrystalline cellulose (NCC) from a cellulosic feedstock to ethanol process which uses dilute acid pretreatment followed by enzymatic hydrolyzation.

Nanocrystalline cellulose is chemically inactive, stable, and has the same structures as the crystalline structures in the larger cellulose fibers, from which the NCC are derived [74].

The NCC are used in pharmaceutical applications as a tablet binder and in food applications as a texturizing agent and as a filler material. Increased interest in using bio-renewable materials has spurred research using NCC in building bio-composite products. The high surface area to volume ratio make NCC an ideal filler in polymers and have been extensively studied [75-77]. One such polymer is polyvinyl alcohol (PVA). This synthetic, water soluble polymer is used to form hydrogels that are non-toxic and biodegradable [78]. This polymer is also non-carcinogenic, making it an ideal candidate for use in medical applications, including tissue scaffolding and wound dressing [45]. One of the current issues with using PVA in these applications is mechanical strength. NCC can be added as a filler material to help strengthen the mechanical properties of the PVA hydrogels. The high surface area of NCC help create good interaction between the fibers and the polymer [79]. Other nanoparticle material can be added to PVA to enhance the mechanical properties, but NCC offers a "green" renewable source with enhanced strength compared to weight of the material, low density, low cost, and low toxicity [80].

The current methods of making NCC involve a process to extract pure cellulose and then a step to remove the amorphous regions. This creates a pure crystalline structure that can then be mechanically processed and concentrated [73]. A clean, de-lignified cellulose pulp (many times a product from the kraft pulping industry) is typically subjected to acid to remove the amorphous regions, leaving a highly crystalline structure [81]. Additionally, the pulp can be subjected to enzymatic digestion or a combination of both acid hydrolysis and enzymatic digestion [82]. Yields for NCC can reach as high as 30 % when acid hydrolysis conditions are optimized [83]. The goal of this research would be to use a purified cellulosic pulp from a cellulosic ethanol plant to extract a highly crystalline

cellulose stream and mechanically process this stream to produce NCC. Dilute acid pretreatments are paired with an enzymatic hydrolysis of the pretreatment slurry. The pretreatment can solubilize over 90 % of the hemicellulose to xylose [84]. Enzymes can then hydrolyze ~60-70 % of the cellulose to glucose [85]. This sugar rich hydrolysate is then fermented to produce ethanol. After distillation, the whole stillage is filter pressed and the liquid is sent to anaerobic digestion. The liquid will consist of unfermented monomeric sugars, soluble oligomeric cellulose and hemicellulose components, acid soluble lignin and nonvolatile lignin degradation products, and organic and inorganic acids [86]. The lignin cake composition will be 30 – 40 % lignin, 9 – 20 % ash, and ~40 % total carbohydrates [86, 87]. The carbohydrate content of the lignin cake should be mostly cellulose, as most all of the hemicellulose will be hydrolyzed to monomeric components. The remaining cellulose will be highly recalcitrant, given the fact that it will have undergone a pretreatment step and an enzymatic hydrolysis step. This recalcitrant, highly crystalline, cellulose will be isolated and processed into NCC.

2. Material and methods

2.1 Material

Feedstock

Cellulosic whole stillage was obtained from POET Research Center, Scotland, SD, USA. The whole stillage was centrifuged and the supernatant was decanted. The solids (lignin cake) were combined into one homogenous sample. The solids were then purified using a sodium hydroxide extraction step. Sodium hydroxide pellets (Sigma-Aldrich) were mixed with RO water to a 5% w/v concentration and loaded with the lignin cake into a 5100 Parr reactor fitted with a 1 L, jacketed, stainless steel reactor vessel. The slurry was held

at 140°C for 90 min. After the reaction, the vessels were cooled to room temperature and the slurry was centrifuged. The top black liquor was decanted and kept for future analysis and stored at -20°C. The solids were washed extensively with RO water and followed by an exhaustive hydrogen peroxide (bought at a local grocery store, 3% v/v solution) rinse to remove any residual lignin. The finished feedstock, referenced for the rest of this thesis as lignin extracted pulp (LEP), was then stored at 4°C. Composition of the lignin cake can be found in Table 7.

Table 7. Compositional analysis of lignin cake and lignin extracted pulp (LEP). All values are listed as a percentage of total mass

Sample	Ash	Lignin	Glucan	Xylan	Arabinan	Acetyl	Mass closure	Total moisture
Lignin cake	10.7	46.0	25.2	7.76	0.115	1.06	90.8	66.1

Homogenizer

A small, ring mounted homogenizer was used for the first mechanical reduction. The homogenizer was a POLY-TRON PT 2100. The intensity setting was set to 30.

Ultrasonication

Ultrasonication was done using an Ultrasonic High – Pressure Chemical Reactor UHiPR (Columbia International). A φ=10 horn was fitted to a 300 ml reactor vessel. The output was set to 99% and it was set to on for 4.0 s and off for 1.0 s.

XRD

Rigaku MiniFlex 600 was used for XRD analysis. The tube voltage was 35 kV and the current was 15 mA. The rotation speed was set for 2°/min, starting at 10° and stopping at 60° with a step of 0.02°.

TEM imaging

Imaging was done using a JOEL JEM-2100 transmission electron microscope.

2.2 Methods

Homogenizer

The lignin extracted pulp was mixed with RO water in a beaker. The percent solids of the LEP was 17.42% and 10.26 g of wet sample were mixed with 200 ml of RO water. Parafilm was stretched over the top of the beaker and the shaft of the homogenizer was punctured through and submerged into the sample. Samples can only be run for 20 min at a time, as per the manufacturer's recommendation. For longer processing times, the homogenizer would be briefly stopped to allow the unit to cool, so as not to cause any damage. Time point samples were taken with a disposable transfer pipette into sample vials.

Ultrasonication

Lignin extracted pulp was mixed with RO water to make a suspension as in the homogenizer experiment. The amount of wet sample used was 9.98 g and it was mixed with 200 ml of RO water. The slurry was loaded into the reactor and the head was bolted to the reactor vessel and the bolts were only hand tightened. The reaction time was set

and once reached, the vessel was unbolted from the horn. Samples were then taken and put into sample vials. After the sample was taken, the vessel was bolted to the horn for longer reaction times.

High shear mixing

Similar to the two previous methods, 10.18 g of wet, LEP was mixed with 200 ml of RO water. This slurry was loaded into the cup of the high shear mixer. A metal lid was affixed to the top of the cup and the mixer was started. Samples were taken from the cup and put into sample vials.

XRD

After processing, the slurried samples were dried at 40°C until constant mass (~48 h). The samples made a thin film and the film was ground using a mortar and pestle. The powdered samples were then loaded onto a glass sample slide and then loaded into the XRD. A blank slide was used as a baseline/signal noise subtraction.

Crystallinity index (CI) using the deconvolution (CI_{devo}) method as described by Park, Baker [46] was calculated from the XRD patterns. OriginPro 2017 (b9.4.0.220) was used to fit the peaks and calculate the peak areas, assuming Gaussian functions. Full width at half maximum (F_{WHM}) were also determined from OriginPro. The broad peak between 15°-20° for all samples was assumed to be the amorphous contribution (Figure 9)[28]. Iterations were repeated until converged with an $R^2 > 0.95$. Crystallinity index was also calculated using the peak height (CI_{ph}) method as described by Segal, Creely [88],

$$CI\ (\%) = \left[\frac{I_{002} - I_{amorphous}}{I_{002}}\right] * 100$$

where I_{002} is the intensity for the crystalline contribution of the biomass and $I_{amorphous\ (am)}$ is the amorphous portion.

Crystal particle size was calculated using the Scherrer equation [46],

$$\tau = \frac{K\lambda}{\beta cos\theta}$$

where τ is the crystallite width in nanometers, K is the Scherrer constant (1 for needle like crystals), β is the width at half maximum (F_{whm}), and λ is the wavelength (1.54178 nm).

TEM imaging

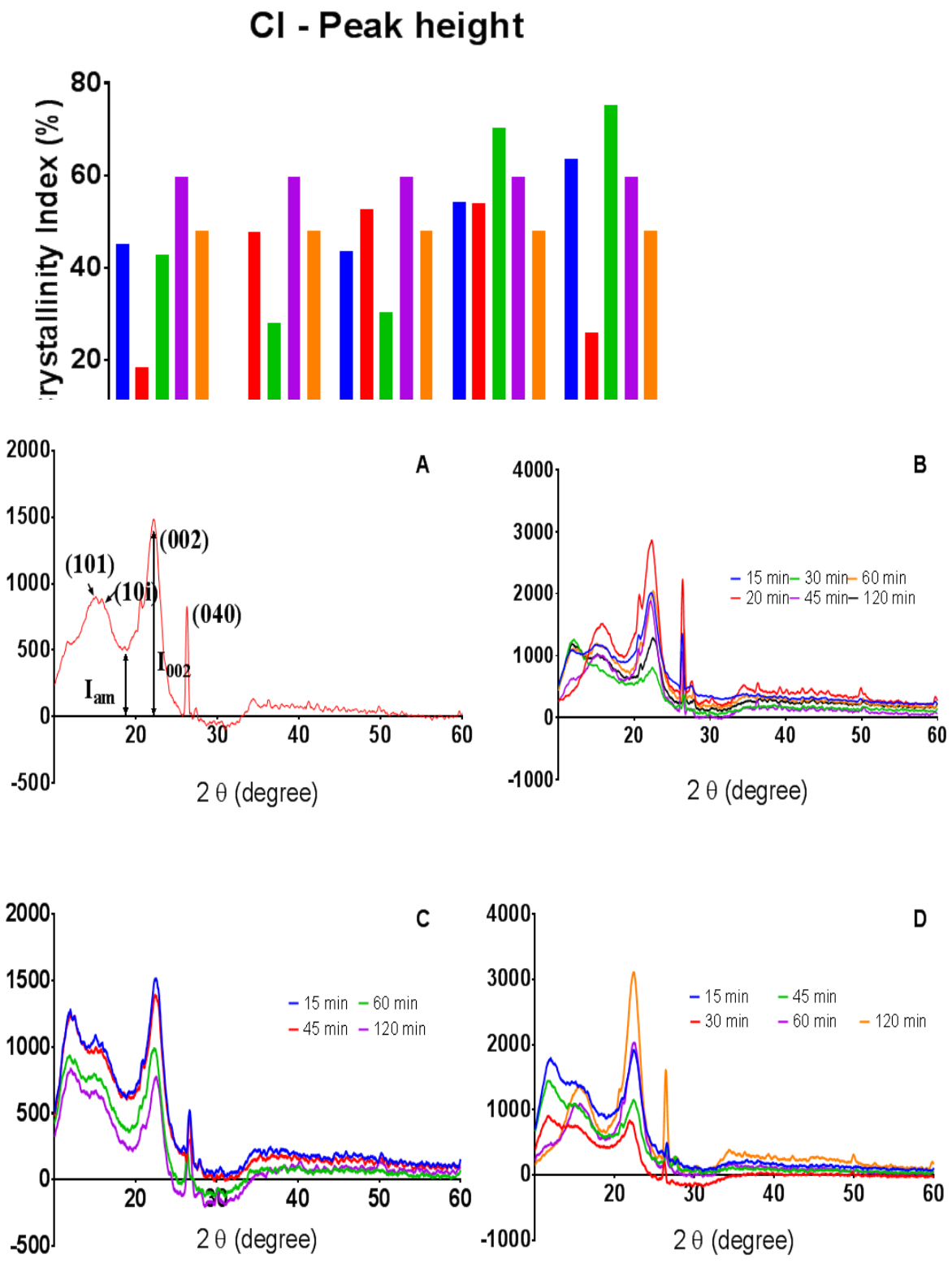
Samples were prepared by drying in a 40°C oven until dry. Samples were then ground into a powder using a mortar and pestle. The powder was then mixed with ethanol. Using a glass pipette, the ethanol sample slurry was added to a copper TEM grid. The grids were then allowed to dry at ambient temperatures overnight.

3. Results and discussion

XRD

Figure 9 shows the XRD patterns for the LEP (A) and the LEP processed and with a homogenizer (B), ultrasonication (C), and high shear mixer (D). Figure 9A also shows the peaks used in the CI_{ph}. The peak height method has been called into question by some [46] and was not the only calculation for CI made. Park, Baker [46], et al, has described using the areas of identified peaks to calculate the CI. Crystalline peak areas are divided by total peak areas and multiplied by 100. This provides a more complete picture of the CI, as biomass samples will generally have more than one peak that contributes to

crystallinity. Figure 9 shows another peak between 26° and 28° that others have identified as a crystalline peak [28]. Figure 10 shows the CI for both the peak height (10A) and deconvolution method (10B). Both calculations show the CI increasing as the LEP is processed in the high shear mixer. Both calculations also show that there is a point when homogenizing the sample where CI decreases. The time at which that occurs differs depending on which calculation method is used. Raw corn stover is more crystalline than



mechanical processing method of the LEP.

Figure 9. XRD spectra for LEP (A), homogenized LEP (B), ultrasonicated LEP (C), and high shear mixed LEP (D). Figures have been smoothed with 15 neighbors on either side of the data and were fit using a 2nd order polynomial.

the LEP. Both the ultrasound and high shear mixer increases the CI over that of the raw

corn stover. There is a disagreement in CI between methods when examining the ultrasonicated samples. The CI_{devo} appears to be decreasing whereas the CI_{ph} appears to increase. Ultrasonication of the LEP showed a lower CI than the LEP except in the 120 min sample.

Homogenization and high shear mixing both increase the CI over that of the raw stover and the LEP. This could indicate that processing the LEP by one of these methods is needed to increase the CI before being used in certain applications.

Particle size was calculated using the F_{WHM} of the identified peaks and using Scherrer equation. Table 8 shows the comparison of the particle width for each processing method. The width seems to loosely correlate with the CI_{devo} ($R^2 = 0.724$). The correlation would agree with previous studies where CI was associated with smaller particle size [89].

Table 8. Particle width calculated from the Scherrer equation of LEP and the different mechanical processing of the LEP. The values at the 2θ represent the diffraction angle of the peaks used for particle width.

Sample	F_{whm}	2θ	Particle width (nm)
Homogenizer	16.0	24.1	0.110
High shear mixing	2.39	22.2	5.31
LEP	2.43	22.0	32.4
Raw corn stover	3.17	22.1	11.2

TEM images

The TEM images from the LEP show porous structures of cellulose (Figure 11**)**. It has been noted that crystalline cellulose is porous in nature [90]. This shows that much of the

lignin has been removed from the cellulose stillage. This fact is confirmed by the mass balance calculations of the lignin extraction step.

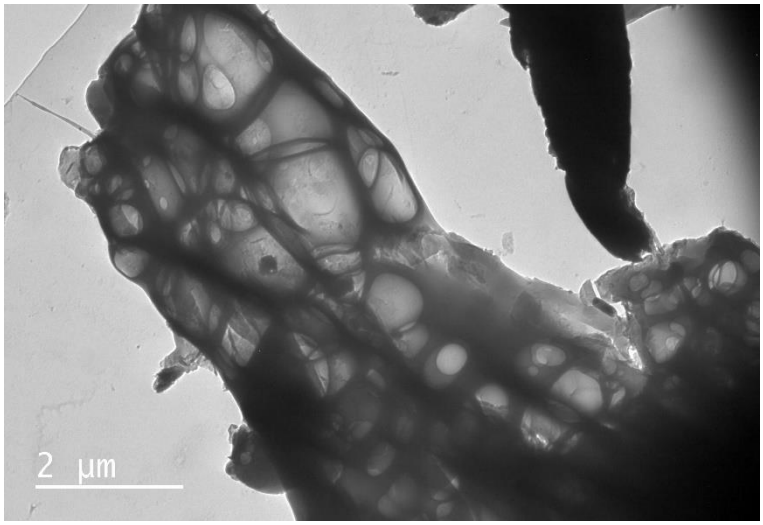


Figure 11. TEM image of LEP.

All three methods of treatment show the porous structure of the material further broken down into more rod like structures. Figure 12A shows a closer image of the rod like structures of the NCC treated with high shear mixing. There is still a significant amount of aggregation in the images. This may be attributed to the manner in which the material was prepared. The treated LEP was dried and then milled using a mortar and pestle. This may have led to larger particles being left in the samples. The image still clearly shows some rod like structures about 5 nm in width and about 75-100 nm in length. Figure 12B shows the structures of material treated with the homogenizer. This image still has significant aggregation of material, but more clearly shows the rod like structures at similar lengths and widths as the high shear mixer.

4. Conclusions

High shear mixing and homogenization are both effective processing steps to increase the crystallinity of a purified cellulose stream. TEM imaging of the processed samples

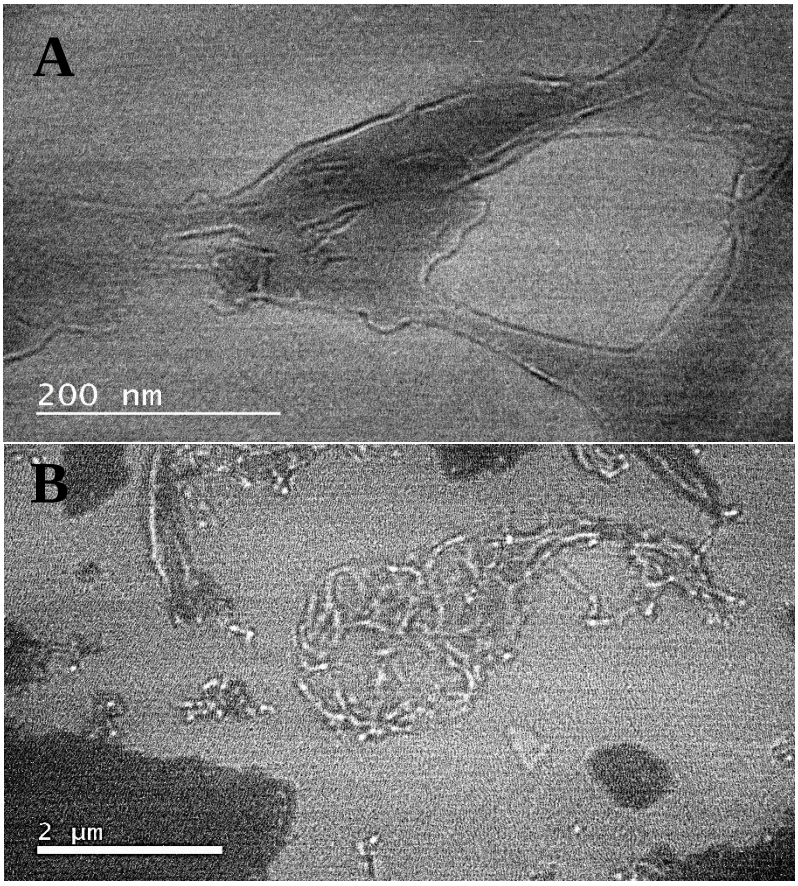


Figure 12. TEM images from LEP that was processed with a high shear mixer for 120 min (A) and with a homogenizer for 30 min (B).

showed rod like structures of NCC and also the porous structure of the lignin extracted pulp. There is a point where homogenizing the lignin extracted pulp lowers the CI. High shear mixing showed an increase in CI as processing time increased. Particle size was loosely correlated with CI_{devo}.

Overall conclusions

Commercial cellulosic ethanol has been the goal of many academic researchers and industry entities for many years now. Using a waste product to make a fuel for transportation is attractive for many reasons. Several studies and industrial attempts have come and gone, but only a few have been able to attempt large scale production. When starch ethanol first started to become profitable, it wasn't just because of the gallons of ethanol sold. Many ethanol plants stayed profitable because of the co-products that they sold, such as DDGS and CO2. If the few commercial cellulosic ethanol plants want to become profitable, value added streams must be researched, demonstrated, and sold just as DDGS in a starch ethanol plant.

Throughout the lignocellulosic to ethanol process, there are many opportunities for improvement, each one with its challenges and rewards. Many of the different sub-processes within the overall process of ethanol production (e.g. unit operations) directly affect downstream unit operations. Changing an upstream process will inevitably change a downstream process, either, amongst other changes, through changing the composition of the feedstock or material, or changing the physical characteristics of the material. This is one challenge that many small scale research models have not had to manage. It is much easier to focus on one unit operation.

The basis of this research did not necessarily focus on how changes to the processes within lignocellulosic to ethanol effect other processes, but rather focus on one method; from raw feedstock, through pretreatment and enzymatic hydrolysis, fermentation and ethanol processing, to finally, value added co-product. My hope in this research is that others will be able to focus on changes to the presented methods and how they affect

downstream processes. Also, the aim of this research was to provide companies that have similar methods to the presented research with a basis on which to advance research within their own company.

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