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Production of Fermentable Sugars from Energy Cane Bagasse

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PRODUCTION OF FERMENTABLE SUGARS FROM ENERGY CANE BAGASSE

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To my beloved parents

Without whom

I would not have come so far.

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ABSTRACT

Lignocellulosic biomass contains cellulose and hemicellulose which are composed of hexose and pentose sugars. These sugars can be used in the sustainable production of fuels and chemicals. However, the recalcitrant nature of lignocellulosic biomass makes this conversion a challenging process. An effective pretreatment can remove lignin, solubilize the hemicellulose, decrease cellulose crystallinity, and prepare the biomass for enzymatic hydrolysis and conversion into green renewable chemicals. The research study presented in this dissertation addressed some of the challenges associated with the conversion of lignocellulosic biomass into green fuels and chemicals. This study was divided into three main goals.

The first goal was to optimize a liquid ammonium hydroxide pretreatment for energy cane bagasse for maximum sugar yields via Response Surface Methodology (RSM). Optimum pretreatment conditions for maximum glucose yield were 208°C, for 36 min and ammonium hydroxide to biomass ratio of 0.4:1. A yield of 30.77 g glucose and 3.99 g xylose was predicted per 100 g of untreated biomass (dry weight). The quadratic models were found reliable within the design space.

The second goal of this study was to evaluate the interaction effect of cellulase (Cellic® CTec2), xylanase (Cellic® HTec2), and laccase along with a non-ionic surfactant (Tween® 80) on the cellulose digestibility of unwashed and post-washed pretreated substrate. Highest cellulose digestibilities observed were 84.30% and 97.10% for values set within the design range for the unwashed and washed biomass, respectively. Optimum enzymatic hydrolysis conditions for unwashed substrate were 19.39% CTec2, 12.04% HTec2, 46.32 IU/g laccase, and 10.15% Tween® 80; and for washed substrate were 16.90% CTec2, 14.17% HTec2, 34.64 IU/g laccase, and 14.86%Tween® 80.

The third and last goal of this research study involved assessing six hydrophobic imidazolium-based ionic liquids in the liquid-liquid extraction and recovery of non-sugar compounds (i.e., phenolic

compounds, organic acids, and furans) from enzymatically hydrolyzed dilute ammonia pretreated energy cane bagasse hydrolysates. Phenolic compounds were considerably removed from the hydrolysates by all six ionic liquids, followed by furfural and 5-HMF; however, formic acid and acetic acid failed to partition. No more than two regenerations of these ionic liquids are recommended.

CHAPTER 1

INTRODUCTION

1.1. Lignocellulosic Biomass

Concerns about the depletion of non-renewable resources for the production of energy and chemicals, along with climate changes and global warming have become incentives for finding sustainable resources (Alvira et al., 2010, Aita and Kim, 2010). Among the available sustainable resources, lignocellulosic biomass has received great interest since it makes up 50% of the world total available biomass (Sánchez and Cardona, 2008). In addition to energy, lignocellulosic biomass can be used as building blocks for the production of bio-chemicals and bio-fuels. Furfural, succinic acid and levulinic acid are a few examples of these chemicals with applications in the food, pharmaceutical and cosmetic industries (Cherubini and Ulgiati, 2010). Bio-based products can replace petroleum-based chemicals (Fahd et al., 2012). Around 87% of the world energy consumption comes from fossil fuels (Ullah et al., 2015). While combustion of fossil fuel accounts for a total CO_2 emission of 28.9 billion tons annually, biomass energy production is considered to be almost carbon neutral (Ullah et al., 2015). According to a study supported by the U. S. Department of Energy (DOE) and the U. S. Department of Agriculture (USDA), the United States has the capacity to support the production of 1.3 billion dry tons of biomass annually. This is sufficient to replace 30% of petroleum consumption (Perlack et al., 2005).

Energy crops, woody biomass and agricultural residues (i.e., energy cane bagasse, corn stover, rice husks) are examples of lignocellulosic biomass that can be used in the production of bio-chemicals and bio-fuels, and improve the land efficiency by increasing the fuel production per acre land area (Zabed et al., 2016). Utilization of marginal land and grasslands for the production of lignocellulosic biomass would benefit the farmers and local agricultural markets (Su et al., 2015). Thermochemical and biochemical pathways are two different pathways through which lignocellulosic biomass can be converted into bio-

fuels and bio-chemicals. Thermochemical pathways involve the thermal treatment of biomass which includes combustion, gasification and pyrolysis processes. Biochemical pathways utilize the fermentable sugars available in lignocellulosic biomass during a fermentation process for the production of bio-fuels and bio-chemicals (Kudakasseril Kurian et al., 2013; Ullah et al., 2015). By the use of thermochemical or biochemical pathways, one ton of dry biomass with approximately 20 GJ energy can be converted to some form of energy carrier with almost 6.5 GJ energy. This translates to a conversion efficiency of 35% currently achieved with existing technologies. Conversion efficiencies can be further improved through research and adopting proper strategies (Duku et al., 2011).

1.2. Chemical Composition of Lignocellulosic Biomass

Lignocellulosic material is mainly composed of cellulose, hemicellulose and lignin (Zabed et al., 2016). The amount of each of these components varies based on the feedstock (Table 1.1). Cellulose is the main component of the plant cell wall and it is also the most abundant bio-polymer in nature. Depending on the source, lignocellulosic biomass contains around 40-50% cellulose (Tye et al., 2016). Cellulose is made up of glucose monomers that are linearly linked by strong β-1,4 glycosidic bonds (Tye et al., 2016). The average number of glucose units in a cellulose chain is defined as degree of polymerization which is around 10,000 in native biomass (Bao et al., 2011). These links along with hydrogen bonds and van der Waals forces contribute to the formation of cellulose microfibers. Random orientation of these microfibers results in the formation of amorphous and crystalline regions that make up the structure of cellulose (Taherzadeh and Karimi, 2008). Crystalline regions, with packed and highly ordered microfibers, contribute to the insolubility of cellulose in water and in most organic solvents, and add to the resistance of cellulose to chemical and biological degradation (Haghighi Mood et al., 2013).

Table 1.1. Composition analysis of different feedstocks. Adopted from (Menon and Rao, 2012).

Feedstocks	Chemical Composition (% dry weight)		
	Cellulose	Hemicellulose	Lignin
Barley hull	34	36	19
Barley straw	36–43	24–33	6.3–9.8
Bamboo	49–50	18–20	23
Banana waste	13	15	14
Corn cob	32.3–45.6	39.8	6.7–13.9
Corn stover	35.1–39.5	20.7–24.6	11.0–19.1
Cotton	85–95	5–15	0
Cotton stalk	31	11	30
Coffee pulp	33.7–36.9	44.2–47.5	15.6–19.1
Douglas fir	35–48	20–22	15–21
Eucalyptus	45–51	11–18	29
Hardwood stems	40–55	24–40	18–25
Rice straw	29.2–34.7	23–25.9	17–19
Rice husk	28.7–35.6	11.96–29.3	15.4–20
Wheat straw	35–39	22–30	12–16
Wheat bran	10.5–14.8	35.5–39.2	8.3–12.5
Grasses	25–40	25–50	10–30
Newspaper	40–55	24–39	18–30
Sugarcane bagasse	25–45	28–32	15–25
Sugarcane tops	35	32	14
Pine	42–49	13–25	23–29
Poplar wood	45–51	25–28	10–21
Olive tree biomass	25.2	15.8	19.1
Jute fibers	45–53	18–21	21–26
Switchgrass	35–40	25–30	15–20
Grasses	25–40	25–50	10–30
Winter rye	29–30	22–26	16.1
Oilseed rape	27.3	20.5	14.2
Softwood stem	45–50	24–40	18–25
Oat straw	31–35	20–26	10–15
Nut shells	25–30	22–28	30–40
Sorghum straw	32–35	24–27	15–21
Water hyacinth	18.2–22.1	48.7–50.1	3.5–5.4

Hemicellulose connects the cellulose microfibers together and to the lignin, and provides a physical barrier for cellulose (Figure 1.1). Hemicellulose makes up 25-30% of the total dry biomass (Menon and Rao, 2012). Unlike cellulose, hemicellulose has a heterogenic and branched structure composed of several pentose monomers (β-D–xylose and α-L–arabinose) and hexose monomers (β-D–glucose, α-D–galactose and β-D–mannose). Uranic acids such as glucuronic acid and methyl glucuronic acid can also be found in the hemicellulose structure (Zheng et al., 2014). Hemicellulose backbone chain is commonly composed of D–xylose (90%) and L–arabinose (10%) which are connected through β-1,4 bonds (Menon and Rao, 2012). The ratio of monomeric sugars can vary depending on the source of biomass. For example, hemicellulose in hardwoods and agricultural residues has more xylose and less mannose and galactose units as compared to softwoods (Sun et al., 2016). Compared to cellulose, hemicellulose has a lower degree of polymerization (around 100-200) and lacks a crystalline structure. Additionally, the chemical bonds connecting the monomers in hemicellulose are less strong as the chemical bonds present in cellulose, thus making it is less resistant to thermal and chemical decomposition (Karimi and Taherzadeh, 2016; Zabed et al., 2016).

Lignin is the second most abundant polymer in nature and it is composed of phenylpropane units including coniferyl alcohol, coumaryl alcohol and sinapyl alcohol (Aita and Kim, 2010). Lignin is responsible for providing the plant structural support and acts as the glue that holds the cellulose and hemicellulose together (Aita and Kim, 2010). Lignin is insoluble in water; however, it becomes soluble at temperatures above 180 °C (Grabber, 2005). Lignin solubilization is dictated by the specific combinations of lignin precursors (*p*-coumaryl, coniferyl, sinapyl alcohol) (Behera et al., 2014). Ferulic acids and *p*-coumarate links between lignin, cellulose and hemicellulose are one of the main factors contributing to the recalcitrance of the lignocellulosic material (Aita and Kim, 2010). Among the various types of lignocellulosic biomass, softwood has the highest lignin content (30-60%) as compared to hardwood (30-

55%), grassy biomass (10-30%), and agricultural residue (3-15%) (Zabed et al., 2016). Less lignin content is desirable in biomass for energy and chemical production, thus one of the scopes for breeding new feedstock for energy purposes is to contain less lignin (Taherzadeh and Karimi, 2008).

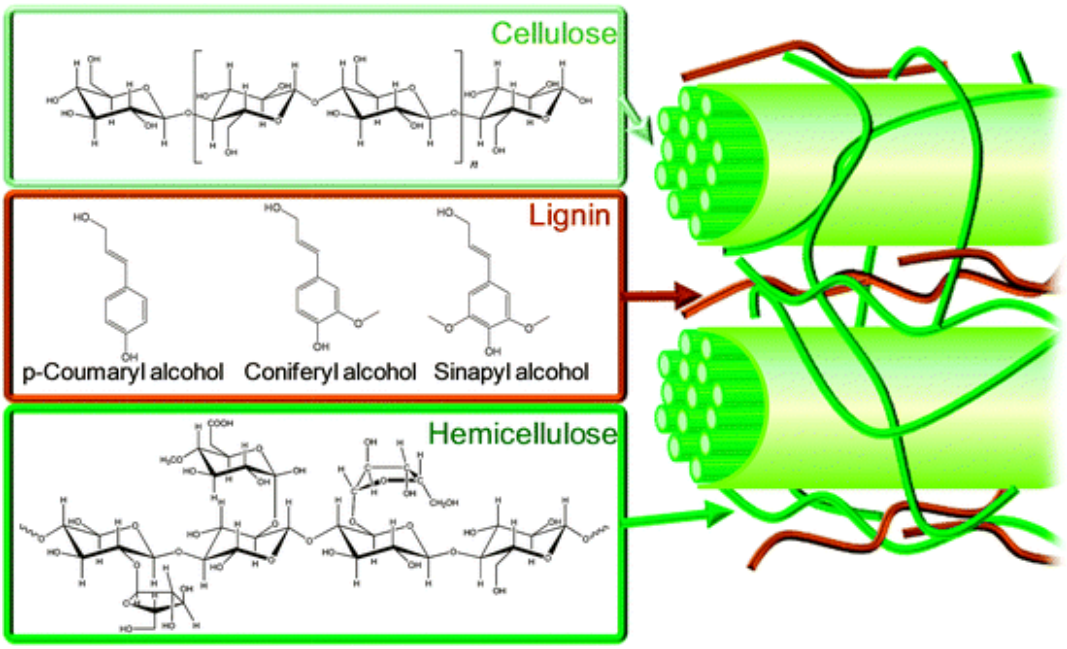

Figure 1. 1. Composition of Lignocellulosic Biomass (Patel et al., 2016).

1.3. Energy Cane

Energy cane is a cross breed between commercial sugarcane and its wild ancestors. Compared to sugarcane, energy cane has higher fiber content and less fermentable sugars (M. Fouad et al., 2015). It is more resistant to cold and disease and requires less water input (Kim and Day, 2011). All these features have made energy cane a potential energy crop. HO 02-113 is a non-commercial energy cane variety developed by the U. S. Department of Agriculture-Agricultural Research Service (USDA-ARS) in Houma, LA and the Louisiana State University Agricultural Center Sugar Research Station in St. Gabriel, LA. This variety has a fiber content of 257 g/kg (dry basis) and yields 83.30 Mg/ha (dry basis) as compared to sugarcane commercial variety LCP 85-384 which has a fiber content of 117 g/kg (dry basis) and yields 59.20 Mg/ha (dry basis) (Salassi et al., 2014; Bischoff et al., 2008). In Louisiana, planting energy cane in August can improve the fiber yield by 2.40 Mg ha^{-1} when compared to planting in September or October

(Viator and Richard, 2012). Cold resistance of energy cane helps the crop to survive winter temperatures and be ready for harvesting anytime between January and March (Kim and Day, 2011). Energy cane is composed of 26% fiber consisting of 43% cellulose, 24% hemicellulose and 22% lignin. It also contains 53.60% juice (wet basis), which has 9.80% sugars (mainly sucrose) (Kim and Day, 2011).

1.4. Pretreatment of Lignocellulosic Biomass

Lignocellulosic biomass is not readily available for the production of fuels and chemicals in its natural form due to its recalcitrance nature (Sánchez and Cardona, 2008). The recalcitrance nature of biomass can be attributed to the lignin content, ester linkages between lignin and hemicellulose, and the crystalline structure of cellulose (Karimi and Taherzadeh, 2016; Wei et al., 2009). Therefore, some form of pretreatment is required in order to improve access to the polymeric sugars. An optimum pretreatment should remove lignin, solubilize the hemicellulose and decrease the crystallinity of cellulose (Kumar and Wyman, 2013). Nonetheless, an optimum pretreatment should preserve the maximum amount of sugars while minimizing the formation of by-products (Galbe and Zacchi, 2007; Palmqvist and Hahn-Hägerdal, 2000a; Alvira et al., 2010). These by-products including carboxilic acids, phenolic compounds and furans are generated from the degration of cellulose, hemicellulose and lignin during harsh pretreatment conditions and have shown inhibitory effects during downstream processes (e.i., enzymatic hydrolysis, microbial fermentation). Pretreatments are categorized into three main groups, physical, chemical and biological. Based on the substrate, each pretreatment has inherent advantages and disadvantages.

1.4.1. Physical pretreatment

Grinding, extrusion and irradiation are the most common types of physical pretreatment. During grinding, reduction in particle size increases the surface area and reduces the crystallinity of biomass. This is achieved by using different types of mills, rollers and grinders until biomass particle sizes are not larger than 2 mm. High energy input of this method makes it economically less favorable (Hendriks and Zeeman, 2009). In extrusion, biomass is passed through an extruder under cumulative pressure and friction, and

ultimately the biomass is subjected to a sudden pressure release. This would result in the depolymerization of lignocellulosic polymers and particle size reduction (Silva Ortiz and de Oliveira Jr, 2014; Lamsal et al., 2010). High energy consumption, safety issues and the generation of unwanted by-products are downsides associated with extrusion (Williams et al., 1997). Most common forms of irradiation include microwave irradiation, gamma-ray irradiation and ultrasonic irradiation. These methods are not specific for lignin removal but they can alter the crystallinity of cellulose and also increase the surface area of biomass, with minimal effect on the hemicellulose content (Mohammad and Keikhosro, 2008).

1.4.2. Chemical pretreatment

1.4.2.1. Steam explosion

Steam explosion is the explosive decomposition of biomass, processed under high pressure with saturated steam (160–260 °C and 0.7–4.8 MPa) and followed by a sudden decrease in pressure (Zheng et al., 2014). By-products or inhibitory compounds can be formed with this pretreatment technology (García-Aparicio et al., 2006a). However, low pollution, low energy requirements and low cost for steam recovery has made steam explosion pretreatment one of the few pretreatment technologies applied at full scale (Zheng et al., 2014). A downside to this pretreatment is the need for expensive reactors and safety precautions due to the use of high steam pressure and temperatures (Rouches et al., 2016).

1.4.2.2. Liquid hot water pretreatment

Most of the hemicellulose and some of the lignin is removed by liquid hot water pretreatment. Water acts as an acid at the applied temperatures (160–240 °C) due to the generation of H^+ ions (Dien et al., 2006). By-products such as acetic acid and other weak organic acids can be formed from the acetyl groups and uronic acids present in the hemicellulose (Silva Ortiz and de Oliveira Jr, 2014). These acids further solubilize the hemicellulose and the formation of by-products. Alkaline compounds can be added to keep the pH within an optimum range so that unwanted reactions are avoided (Mosier et al., 2005). Liquid hot

water pretreatment is not as corrosive as acid pretreatment which reduces both capital and process costs (Sun et al., 2016). Flow-through bioreactors, co-current or counter current reactors are the most common reactors used with this technology (Mosier et al., 2005).

1.4.2.3. Acid pretreatment

Acids (organic and inorganic) can be used in their concentrated (up to 70% at mild temperatures) or diluted (less than 3% at high temperatures) form to completely dissolve the hemicellulose and to partially remove the lignin (Patel et al., 2016). By-products are generated with this pretreatment technology so additional steps such as neutralization and washing (for the removal of by-products or inhibitory compounds) are inevitable prior to enzymatic hydrolysis (Zabed et al., 2016). Industrial-scale applications of acid pretreatment are hindered by concerns related with environmental safety and corrosion challenges. Additional challenges include acid and water consumption, as well as the disposal of gypsum which is produced during the neutralization step (Sun and Cheng, 2002).

1.4.2.4. Ionic liquids pretreatment

Ionic liquids are made of asymmetrically packed organic cations and inorganic or organic anions. Altering the composition of anions and cations results in the production of a variety of ionic liquids with different solvent properties (Sun et al., 2016; Clough et al., 2015). Ionic liquids are referred to as "green solvents" due to their low vapor pressure, non-toxicity, recyclability, thermal stability, and high solvent power (Rabemanolontsoa and Saka, 2016). Hydrophilic alkyl-chain imidazolium ionic liquids are a group of ionic liquids most studied for the purpose of pretreatment of lignocellulosic biomass. Ionic liquids can dissolve the cellulose at low to moderate temperatures and at ambient pressure (Feng and Chen, 2008). Oxygen and hydrogen atoms from the hydroxyl groups present in the cellulose interact with the ionic liquids by accepting or donating electrons resulting in the solubilization of the cellulose (Feng and Chen, 2008). The dissolved cellulose has less hydrogen bond which translates to a less crystalline and a more

porous structure of cellulose (Zheng et al., 2014). Dissolved cellulose can be recovered from the ionic liquid solution by the addition of an anti-solvent such as water (Feng and Chen, 2008). Some of the most successfully studied ionic liquids in dissolving cellulose are 1-allyl-3-methylimidazolium-chloride ([AMIM]Cl), 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) and methylimidazolium-acetate ([EMIM]Ac) (Haghighi Mood et al., 2013). The high production cost associated with ionic liquids makes their regeneration process a must.

1.4.2.5. Alkaline pretreatment

Alkaline pretreatments (i.e., lime, sodium carbonate, sodium hydroxide, ammonium hydroxide) are effective due to the saponification of the ester links present between carbohydrates and lignin which results in the removal of lignin (Zheng et al., 2014). Grassy biomass is more prone to lignin removal by alkaline pretreatment as compared to woody biomass (Kim et al., 2016). Alkali pretreatments are considered to be more cost effective as compared to other pretreatment technologies and produce less amounts of by-products or inhibitory compounds (Chen et al., 2012).

Ammonia-based pretreatment is a major group within alkali pretreatments that can selectively remove the lignin and dissolve the hemicellulose by altering their degree of polymerization (Kim, 2013, Kim et al., 2003). Ammonia-based pretreatment also increases the porosity and surface area of the biomass (Salvi et al., 2010; Rabemanolontsoa and Saka, 2016). In addition to being non-corrosive, non-pollutant and non-toxic, ammonia-based pretreatments can be performed under a wide range of residence times, temperatures and ammonia to biomass ratios (Rouches et al., 2016). However, ammonia recovery is an energy intensive process (Haghighi Mood et al., 2013).

There are different types of ammonia-based pretreatments as these technologies are versatile in terms of the process residence time and temperature (Park and Kim, 2012). Soaking in aqueous ammonia (Itoh et al.) utilizes low temperatures (40-90 °C) and long residence times (hours) at ambient pressure to

efficiently remove the lignin while preserving the hemicellulose and the cellulose (Jurado et al., 2013). Ammonia soaking yields the second highest cellulose conversion after sodium hydroxide pretreatment (Bali et al., 2015). Soaking rice straw and barley in aqueous ammonia at 60 °C for 24 h resulted in a cellulose digestibility of 85% and 95%, respectively (Park and Kim, 2012). Soaking sugarcane bagasse in aqueous ammonia at 70 °C for 12 h under 1:10 solid to liquid ratio resulted in a 95% cellulose digestibility

Ammonia recycle percolation pretreatment (ARP) utilizes a fixed bed reactor in a flow through mode to prevent the re-precipitation of dissolved lignin on the surface of biomass. ARP is often run at 150–210 °C and 2.3 MPa pressure with 10–15% ammonium hydroxide (Kim et al., 2016). Using a percolation reactor under the common reaction conditions removed up to 80% of the lignin from sugarcane bagasse (Kim et al., 2016). Lignin removal is accompanied by hemicellulose loss due to the high temperatures associated with this technology (Li and Kim, 2011). The hydroxyl groups in ammonium hydroxide cleave the ether and ester bonds connecting the lignin and the hemicellulose resulting in their solubilization (Aita and Kim, 2010).

Low liquid ammonia pretreatment (LLA) is another pretreatment method from ammonia-based technologies which uses less aqueous ammonia. The process is done at relatively lower temperatures (around 30 °C) than ARP and at ambient pressure with residence times lasting for several weeks (Li and Kim, 2011). This method can be used as a lignin removal pretreatment during ensilage of lignocellulosic biomass (Patel and Kumar, 2016).

There are other ammonia-based pretreatment methods in which anhydrous ammonia is used. Low moisture anhydrous ammonia (LMAA) eliminates water use. This prevents liquid reactions from taking place and facilitates downstream processes (Yoo et al., 2014). For example, washing is not required as the amount of moisture and ammonia left in the biomass is negligible. The mild reaction conditions associated with this pretreatment saves on energy requirements (Yasuda et al., 2014). LMAA treated corn stover

resulted in no microbial growth and carbohydrate loss during a 90-day storage, while lignin was significantly removed (Yang and Rosentrater, 2016).

Ammonia fiber expansion (AFEX) is another method that uses anhydrous ammonia. Biomass comes in contact with anhydrous ammonia at moderate temperatures (60–120 °C) and high pressure (15–20atm) for a short residence time (less than an hour) followed by an abrupt pressure release. The abrupt release of pressure causes the breakdown of the biomass structure due to ammonia expansion (Kim et al., 2016; Bals et al., 2011). AFEX has shown to work best on grassy biomass and agricultural residues and it is not as effective on hardwoods (Mosier et al., 2005). It has minimum effect on sugar degradation. However, it is an energy intensive method due to high pressure requirements (Mosier et al., 2005).

1.4.3. Biological pretreatment

Biological pretreatment requires the use of microorganisms, mostly white-rot fungi, in order to degrade lignin and hemicellulose with minimum cellulose loss (Karimi and Chisti, 2015). These microorganisms are capable of producing lignin degrading enzymes such as laccases and peroxidases. Cellulose enzymatic conversion yields from biological pretreatments are lower as compared to chemical pretreatments. However, due to the absence of chemicals, biological pretreatments are environmentally friendly and require a comparatively lower capital cost (Rouches et al., 2016). Biological pretreatments have been used in combination with other pretreatments to improve enzymatic hydrolysis (Sun and Cheng, 2002). Optimization of pretreatment parameters such as moisture content, temperature, aeration, and nutrient supplementation can improve glucose yields from biologically pretreated biomass (Rouches et al., 2016).

1.5. Generation of Inhibitory Compounds during Pretreatment

These compounds are generated during harsh pretreatment conditions from the degradation of lignin, cellulose and hemicellulose (Figure 1.2), and pose inhibitory effects on the activity of enzymes and growth

of fermenting microorganisms (Taherzadeh and Karimi, 2008). The nature of these compounds can be different based on the composition of biomass, biomass type and severity of pretreatment applied (Redding et al., 2011). These compounds can be categorized into three different groups including furan derivatives (furfural and 5-hydroxymethyl furfural (5-HMF)), carboxylic acids (i.e., formic acid, acetic acid) and phenolic compounds (Behera et al., 2014).

Furfural can be produced from the degradation of pentose and hexose sugars while 5-HMF is only produced from the degradation of hexose sugars. Furan derivatives can inhibit glycolysis through deactivation of the enzyme dehydrogenase (Phitsuwan et al., 2013). Presence of furfural and 5-HMF, at concentrations as low as 1 g/L, can inhibit the growth of microorganisms during fermentation processes (Carter et al., 2011). Furfural is often present in larger amounts as compared to 5-HMF and it is more toxic to microorganisms (Chandel et al., 2007). The decomposition rate of furfural is 4 times faster than 5-HMF; therefore, 5-HMF exerts a longer inhibitory effect (Ask et al., 2013). Some studies have reported a synergistic effect of toxicity between furfural and HMF while contradictory results are presented by others (Behera et al., 2014).

Organic acids such as formic acid, acetic acid and levulinic acid result from the hydrolysis of acetyl groups present in the hemicellulose. Higher pretreatment temperatures and longer residence times favor the production of organic acids (Carter et al., 2011). 5-HMF can also be oxidized to levulinic acid (Almeida et al., 2007). Organic acids can enter the cytoplasm of the cell in their un-dissociated form and generate protons inside the cell. The generated protons will alter the pH gradient of the cell cytoplasm and force the cell to pump out the excess amount of protons using ATP. Subsequently, less ATP will be available for cell growth (Phitsuwan et al., 2013). Furthermore, the acidic conditions of the cell cytoplasm can be fatal to microorganisms (Larsson et al., 1999). A concentration above 100 mmol/L of acetic acid, formic acid or levulinic acid has shown inhibitory effects during ethanol fermentation (Larsson et al.,

1999). Formic acid is a stronger inhibitor (due to its low Pk_a) and it is produced by furfural degradation (Almeida et al., 2007).

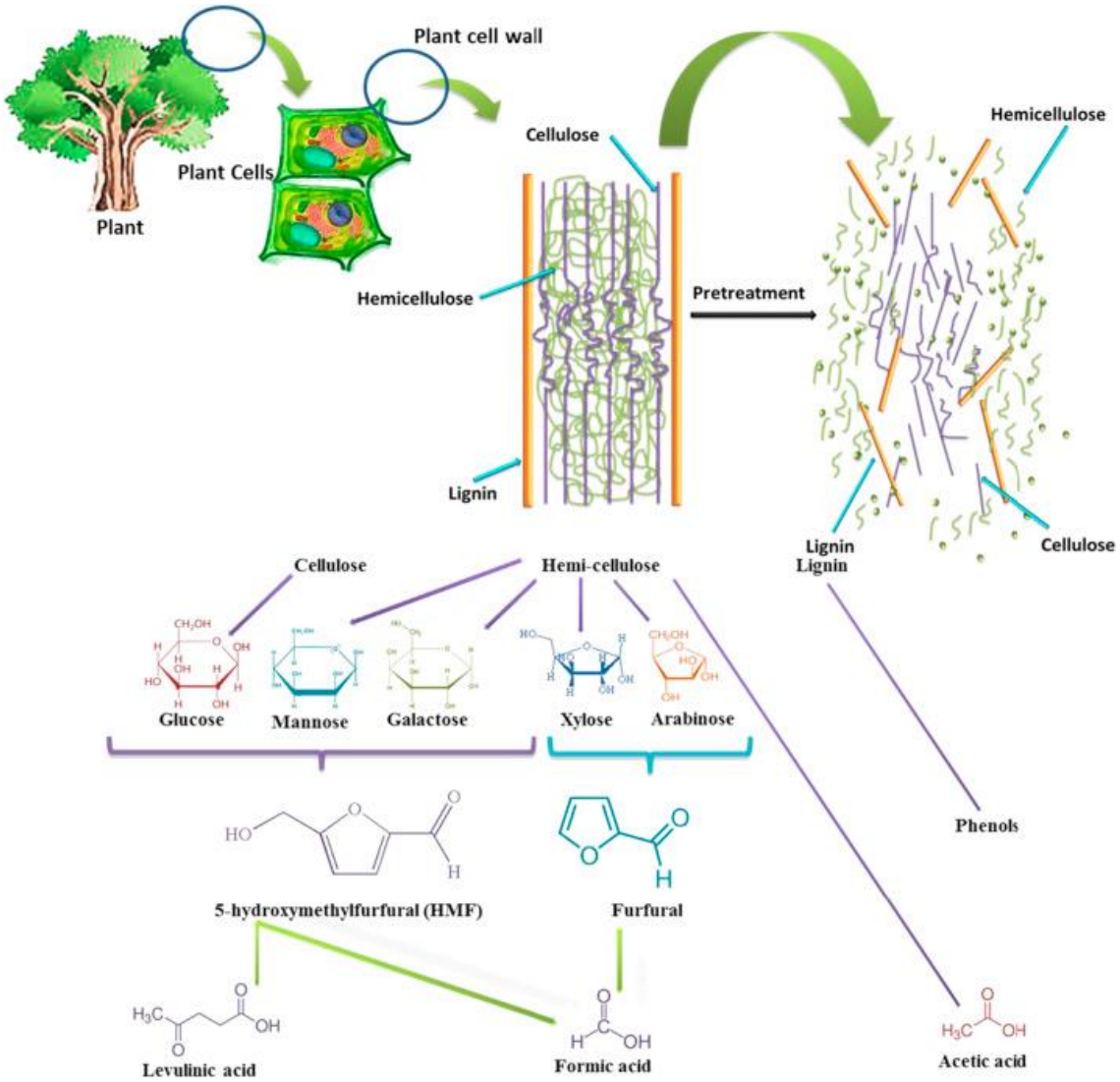

Figure 1. 2. Degradation Compounds from Pretreated Lignocellulosic Biomass (Patel et al., 2016).

Phenolic compounds are generated from the degradation of lignin and are phenol monomers with different aliphatic functional groups including ketones, aldehydes and acids (Alvira et al., 2010). Phenolic compounds are present in lower concentrations as compared to organic acids, furfural and 5-HMF (Klinke et al., 2002). Cinnamic acid, vanilic acid, 4-hydroxybenzaldehyde, vanillin, synringaldehyde, syringic acid, and cathecol are some of the most commonly found phenolic compounds derived from lignin (Almeida et al., 2007). It is suggested that these compounds can inhibit the growth of microbial cells

during fermentation by interfering with the selective transportation of enzymes and substances through their cell membrane (Palmqvist and Hahn-Hägerdal, 2000b). Phenolic compounds can also unproductively bind to the hydrolyzing enzymes (Almeida et al., 2007). Concentrations of 1 g/L of 4-hydroxybenzoic acid can cause a 30% reduction in ethanol fermentation yields from *Saccharomyces cerevisiae* (Ando et al., 1986).

1.6. Enzymatic Hydrolysis of Lignocellulosic Biomass

The highest glucose yields that can be achieved with untreated biomass using excessive amounts of enzymes will not exceed 20% (Mosier et al., 2005). Despite the improvement in the digestibility of lignocellulosic material after pretreatment, the complex structure of lignocellulosic biomass still requires the use of enzymes to yield maximum carbohydrate conversions. Enzyme loadings, the use of accessory enzymes and the presence of inhibitory compounds can affect carbohydrate conversion yields during enzymatic hydrolysis (Bussamra et al., 2015). Washing the substrate after pretreatment (to reduce the concentration of inhibitory compounds) along with the right balance of enzyme loadings and the addition of surfactants can enhance the enzymatic digestibility of lignocellulosic materials (Bussamra et al., 2015).

1.6.1. Cellulase

Cellulase is the main enzyme used in the hydrolysis of pretreated lignocellulosic biomass. It breaks down the strong β-1,4 glycosidic bonds between the glucose monomers of cellulose. Cellulase is composed of three groups of enzymes, endoglucanases, exoglucanases and β-glucosidases (Figure 1.3). Exoglucanases generate cellobiose by breaking down the oligosaccharides that result from endoglucanase activity. Cellobiose is further hydrolyzed into two glucoses molecules by β-glucosidase (Juturu and Wu, 2014). Accessory enzymes (i.e., xylanase, pectinase, laccase, feruroyl esterase, lytic polysaccharide monooxygenase) can be used in combination with cellulase to improve the digestibility of pretreated biomass. There might be a degree of synergism between cellulase and these enzymes. Synergism is

defined as the ratio of the product yield when enzymes are used together to the sum of the yield of their products when they are used separately. Degree of synergy in biomass digestibility depends on the ratio of accessory enzymes to cellulase, biomass physiochemical properties, type of pretreatment applied, and enzymatic hydrolysis conditions (Kumar and Wyman, 2009).

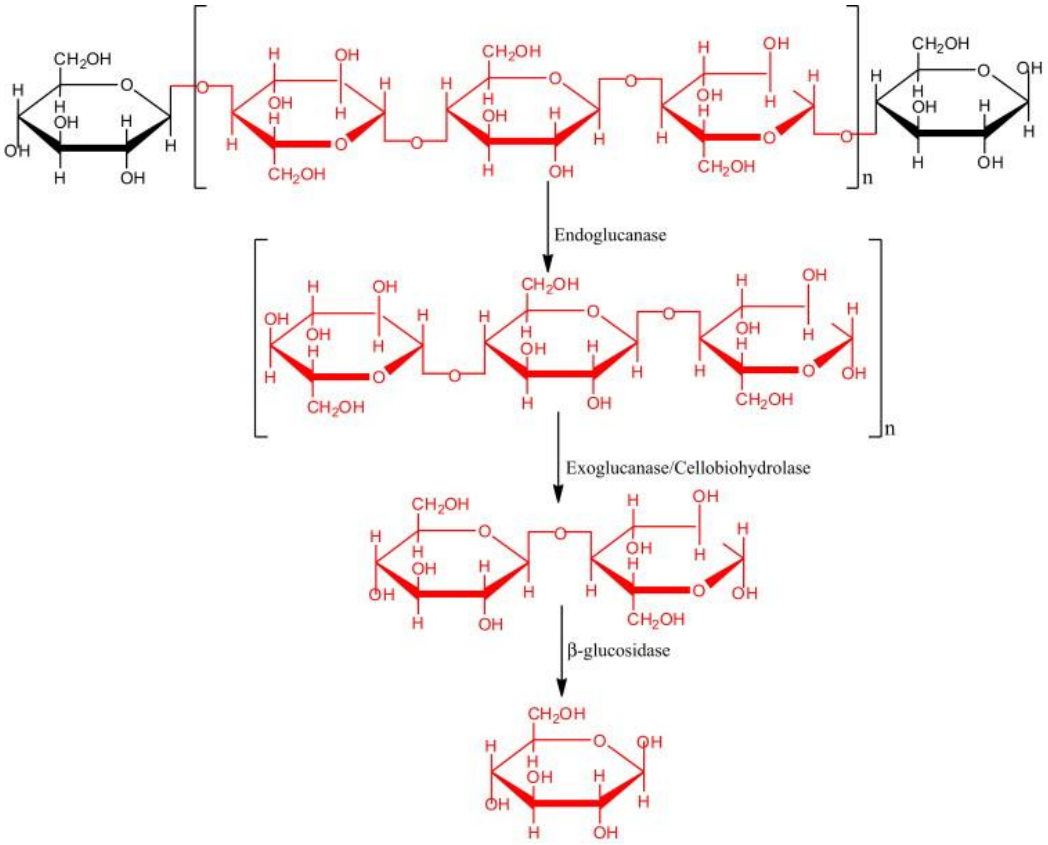

Figure 1. 3. Cellulose Degrading Enzymes (Juturu and Wu, 2014).

1.6.2. Xylanase

Xylanase are a group of enzymes that degrade the hemicellulose. The main enzymes involve in the hydrolysis of hemicellulose are endoxylanase and β-xylosidase. Endoxylanases generate xylo-oligosaccharides which are further cleaved to xylose by β-xylosidase (Juturu and Wu, 2012). Xylanase is the most studied accessory enzyme that can increase cellulose accessibility by hydrolyzing and removing the hemicellulose barrier. There is also a synergistic effect between xylanase and cellulase (Juturu and Wu, 2012). Xylanase can boost the digestibility of cellulose regardless of the hemicellulose content of the

biomass (Hu et al., 2013). The ratio of loading xylanase to cellulase is very important in order to gain the highest synergistic effect. For example, a combination with molar ratio of xylanase to endoglucanase of 75%:25% has shown the greatest synergistic effect (degree of synergy of 3.6) in hydrolysing sugarcane bagasse that was pretreated with liquid hot water at 121 °C for 20 min (Beukes et al., 2008). To achive the best enzymatic hydrolysis results and benefit from the synergy between the enzymes, commercially available enzymes ussually contain an optimum balance of different types of cellulase and xylanase in their mixed cokctail. For example, Celluclast 1.5 L FG from Novozyme contains 65 FPU cellulase, 12 IU/g β-glucosidase and 660 IU/g xylanase (García-Aparicio et al., 2006b). Spezyme CP from Genencor contains 58.20 FPU/ml cellulase, 128 IU/g β-glucosidase, 2622 IU/g xylanase, 22.60 IU/g α-arabinofuranosidase, 7.30 IU/g β-xylosidase, and 0.39 IU/g α-galactosidase (Dien et al., 2006). Accelerase 1000 from Gennecor contains 93 FPU cellulase, 1632 IU/g β-glucosidase and 849 IU/g xylanase (Lin et al., 2011). Li et al. (2014) investigated the effect of xylanase addition on the enzymatic hydrolysis yield of sugarcane bagasse pretreated under different conditions incuding steam explosion with 2% sodium hydroxide for 1 h and 2% hydrogen peroxide for 1 h. They reported that replacing 20% of cellulase with xylanase improved the glucose yield by 9.5% as compared to control samples with no xylanase. Intrestingly, they observed no improvement in the enzymatic hydrolysis when they studied the same synergistic effect on 2% sulfuric acid steam exploded sugarcane bagasse. This means that the type of pretreatment plays an important role in the synergysitic effet between enzymes during enzymatic hydrolysis. Song et al. (2016) reported that xylanase addition can increase the outside surface area of cellulose by removing the hemicellulose as well as the inside surface area of cellulose by exposing the lignocellulose pores that are covered under the layer of hemicellulose. Xylanase has shown to improve cellulose digestibility in untreated lignocellulosic material. In one study, it was reported that an enzyme loading of 0.20 (g/g cellulose) cellulase in combination with 0.20 (g/g cellulose) xylanase improved

enzymatic hydrolysis yieds by 133%, 164% and 545%, for untreated corncob, corn stover, and rice husks; respectively (Song et al., 2016). Li et al. (2015) compared the addition of surfactants (BSA, PEG 6000 and Tween® 80) versus the addition of xylanase in the enzymatic hydrolysis yield of ammonia pretreated bamboo (26 wt. % of aqueous ammonia at 70 °C for 72 h and solid: liquid ratio of 1:10). They reported that the combined addition of 1 mg/g (DM) xylanase with 10 FPU/g (DM) cellulase was more efficient (76.30% glucose yield) than the separate addition of some of the surfactants (BSA , 53.80% glucose yield,; PEG 6000, 56.90% glucose yield; and Tween® 80, 57.40% glucose yield). However, the addition of these surfactant to the cellulase-xylanase combination resulted in an increase in the glucose yield with Tween® 80 being the most effective surfactant (88.50% glucose yield) followed by BSA (86.80% glucose yield) and PEG 6000 (86.00% glucose yield).

1.6.3. Laccase

Lignin is known to hinder the enzymatic hydrolysis of lignocellulosic biomass (Taherzadeh and Karimi, 2008). This can happen through several mechanisms. Lignin can act as a physical barrier to enzymes by preventing them from reaching their substrates (cellulose and hemicellulose). Lignin can block cellulase activity by non-productively binding to cellulase. Furthermore, lignin-derived products including phenolic compounds generated during pretreatment can also inhibit cellulase activity (Kim et al., 2003).

Laccase, lignin peroxidase and manganese peroxidase are used in biological delignification (Van Dyk and Pletschke, 2012). Laccase are multicopper-containing phenoloxidases and catalyze the oxidation of phenols, anilines and aromatic thiols at the expense of molecular oxygen. Laccase and other lignin biodegrading enzymes are used in biological pretreatments or in combination with other pretreatments. The addition of laccase as an accessory enzyme during enzymatic hydrolysis can potentially remove lignin, oxidize the phenolic compounds and enhance enzymatic hydrolysis yields (Qiu and Chen, 2012).

Laccase can also oxidize the aromatic ring of lignin. This would create microspores in the biomass where cellulase can go through and hydrolyze the substrate (Qiu and Chen, 2012). There might also be a synergistic effect between laccase and cellulase. In one study, the simultaneous use of laccase with cellulase resulted in a 60% cellulose digestibility of date palm biomass, while sequential application of these enzymes resulted in a lower enzymatic digestibility of 45.60% (Al-Zuhair et al., 2013). There is a downside to laccase treatment. Some studies have revealed its negative effect on enzymatic hydrolysis yield due to its inhibitory effect on β-glucosidase activity (Tabka et al., 2006; Moreno et al., 2013). Oliva-Taravilla et al. (2015) reported that addition of 10 IU/g (dry base) laccase to the enzymatic hydrolysis of acid pretreated wheat straw resulted in a 10% decrease in enzymatic digestibility.

1.6.4. Surfactants

Surfactants are one of the most common additives used in the bioconversion of lignocellulosic materials. They have been used during different processes including pretreatment, enzymatic hydrolysis and recycling of enzymes after enzymatic hydrolysis (Sun and Cheng, 2002). Surfactants, especially non-ionic, have been reported to increase the enzymatic digestibility of biomass (Eriksson et al., 2002). Surfactants can improve enzyme stability by preventing enzyme degradation caused by heat or agitation shear force, by improving enzyme recyclability, by lowering irreversible binding, and by deactivating enzymes after enzyme-substrate formation (Ouyang et al., 2010). Surfactants can also modify the structure of biomass by creating micropores on its surface thus expanding the surface area of the substrate (Sun and Cheng, 2002). For example, Tween® 20 assisted in the generation of pores of 10-50 nm in the cell wall of pine wood chips which allowed for 3 to 3.6 times more cellulose to be adsorbed onto the biomass surface (Seo et al., 2011). Surfactants can prevent unproductive binding of lignin to cellulase (Kristensen et al., 2007). Addition of 0.20% (w/v) Tween® 80 during the enzymatic hydrolysis of organosolv-pretreated lodgepole pine reduced the adsorption of lignin to cellulase by 60% (Tu et al., 2009). Addition

of 5 g/L of Tween® 80 L resulted in a 7.50% improvement in enzymatic hydrolysis yield of maize straw pretreated with 2% sodium hydroxide at 80 ° C for 1 h (Chen et al., 2008). Alkasrawi et al. (2003) found that the addition of 2.5 g/L of Tween® 20 to steam pretreated spruce wood chips decreased the amount of enzyme loading by 50% without affecting the final glucose yield. The type of pretreatment as well as the type of biomass and its particle size can influence the effectiveness of surfactants on enzymatic hydrolysis. Acid pretreated wheat straw showed a better response to the addition of surfactants as compared to ammonia pretreated straw (Kristensen et al., 2007). Menegol et al. (2014) reported that the addition of non-ionic surfactants (i.e., Tween® 20, Tween® 80, PEG 4000) increased the hydrolysis yield of larger sized particles of elephant grass thus saving on the energy costs associated with fine grinding.

1.6.5. Washing

Washing is usually used as the simplest method that can remove the inhibitory compounds (organic acids, furan derivatives and phenolic compounds) generated during pretreatment and eliminate their inhibitory effect on the activity of enzymes and microbial growth (Rajan and Carrier, 2014). Thus, washing of pretreated biomass can improve enzymatic hydrolysis yields (Qin et al., 2013). Xing et al. (2016) observed that post-washing of bisulfite pretreated corncob removed the organic acids, furans and phenolic compounds completely and increased the cellulose digestibility of the pretreated biomass by 53.80%. Qin et al. (2013) reported that post-washing of corn stover pretreated with 20% (w/w) aqueous ammonia at 180 °C for 30 min and 20% solid loading removed the inhibitors and increased the glucose yield from 56% to 82%. Rajan and Carrier (2014) reported that washing of corn stover pretreated with 10% sulfuric acid at 140 °C for 30 min resulted in the removal of 87% of formic acid, 64% of acetic acid, 86% of furfural, and 87% of HMF, with a final cellulose conversion of 85%. Toquero and Bolado (2014) compared the effect of four types of pretreatment (dilute hydrochloric acid, dilute sodium hydroxide, alkaline peroxide, and liquid hot water) on the enzymatic hydrolysis of wheat straw. They observed

negligible changes in the chemical composition (cellulose, hemicellulose and lignin) of the pretreated material as a result of washing. Although washing removed all the inhibitory compounds generated during pretreatment, the effect of washing on glucose yield was not consistent. In the case of hot water pretreated wheat straw, washing caused no increase in cellulose digestibility due to ineffectiveness of the pretreatment method in removing the lignin. In the acid pretreated wheat straw, washing caused a 2-fold increase in glucose yield. Washing of sodium hydroxide pretreated wheat straw increased glucose yields by 19.20% whereas alkaline peroxide pretreated wheat straw improved glucose yields by 35.20%.

1.7. Detoxification of Enzymatic Hydrolysates

It is widely accepted that removal of inhibitory compounds (organic acids, furans derivatives and phenolic compounds) can significantly improve the fermentability of the enzymatically hydrolyzed biomass (Palmqvist and Hahn-Hägerdal, 2000a). Detoxification methods should be applied to decrease the concentration of these non-sugar compounds to levels below those that will have a negative effect on downstream processes (Canilha et al., 2012). Detoxification methods can be categorized into physical, physicochemical and biological. There might be a need to combine several detoxification strategies to reach the target concentration level for inhibitors or non-sugar compounds as each of these strategies have some inherent shortcomings (Ranjan et al., 2009). Sugar losses while applying detoxification strategies to pretreated biomass hydrolysates should be negligible (Mussatto and Roberto, 2004).

After an effective detoxification method is implemented, the recovery of these inhibitors or non-sugar compounds need to be considered as they can serve as building-blocks to many bio-based products including drugs, fuels and polymers (Ranjan et al., 2009). Furfural, one of the major inhibitors of enzymatic hydrolysis and fermentation of lignocellulosic material is ranked among the top 30 value added products (Bozell and Petersen, 2010). Organic acids such as formic acid, succinic acid and lactic acid also

have many applications in the production of polymers and bio-degradable plastics (Van Nguyen et al., 2016). Detoxification methods can be categorized as physical, physicochemical and biological.

1.7.1. Physical methods of detoxification

Some of the non-sugar compounds generated and/or released during pretreatment and enzymatic hydrolysis are volatile (i.e., acetic acid, furfural vanillin) and can be removed by evaporation (Mussatto and Roberto, 2004). Membrane adsorption is another popular physical detoxification method which has the advantage of keeping the hydrolysate and extraction solvent separated. This would prevent the potential toxicity of the solvent to microorganisms while the non-sugar compounds are being removed. Grzenia et al. (2012) used 15% alamine 336 in oleyl alcohol and a microporous polypropylene membrane to extract inhibitors from the hydrolysate of sulfuric acid pretreated corn stover. They reported that unlike some of the other detoxification methods, acetic acid was effectively removed along with other inhibitors while no sugar losses were observed. Percentage extractions for acetic acid, furfural and 5-HMF were 97%, 47%, and 94%; respectively.

1.7.2. Physicochemical methods of detoxification

1.7.2.1. Overliming

Overliming is the most popular detoxification method due to its efficiency and economic feasibility (Zabed et al., 2016). The process starts with an increase in the pH followed by a pH adjustment to the level required by fermentation (Canilha et al., 2012). The acidic pH of the hydrolysate is neutralized by the addition of calcium hydroxide or sodium hydroxide. As a result, furfural and 5-HMF are removed. However, calcium carbonate is generated and a centrifugation step must be added in order to remove the precipitant (Palmqvist and Hahn-Hägerdal, 2000a). Both precipitation and centrifugation steps can result in sugar losses (Chandel et al., 2011). Millati et al. (2002) investigated the effect of overliming on the detoxification of dilute sulfuric acid pretreated bagasse hydrolysate. It was reported that at pH 5.5, while

furan derivatives were significantly removed, phenolic compounds were reduced only by 30% and acetic acid concentration remained untouched. Increasing the pH of the hydrolysate to 12 resulted in the degradation of sugars by 70%. Increasing the temperature from 25 °C to 60 °C resulted in a slightly higher extraction of furans; however, a 60% glucose loss was observed. Overliming detoxification of sulfuric acid pretreated sugarcane bagasse hydrolysate with calcium hydroxide at pH 9 and at 60°C for 30 min resulted in a 69% furan removal and 35% removal of phenolic compounds. However, 15% of the total sugars were lost during the process (Nilvebrant et al., 2001).

1.7.2.2. Ion exchange resins

Ion exchange resins are effective in removing furans, organic acids and phenolic compounds (Canilha et al., 2012). They can also be regenerated and reused (Villarreal et al., 2006). However, ion exchange resins can result in sugar losses, pressure build-up and long processing times. Scale-up is also not feasible (Nilvebrant et al., 2001). There are three types of ion-exchange resins, anion, cation and neutral resins. The type of ion exchange resin used is usually selected based on the type of inhibitors present in the hydrolysate and the pH of the hydrolysate (Carvalheiro et al., 2005). Anion exchange resins are better at removing organic acids and furans (at pH around 5) while cation exchange resins have yielded better results in removing phenolic compounds (at pH around 10) (Carvalheiro et al., 2005). Chandel et al. (2007) compared the effect of activated charcoal (granular activated charcoal ca. 2.5 mm at pH 5.5, ratio to hydrolysate of 1:10, mixed for 1 h at room temperature), ion-exchange resin (industrial resin DIAION (HPA 25) at room temperature for 1 h), overliming (calcium hydroxide for 1 h at pH 10), and laccase treatment (100 IU/g enzyme loading at 100 rpm for 4 h at 30 °C) on the detoxification of hydrocloric acid prereated sugarcane bagasse hydrolysate (2.5% (v/v) hydrocloric acid at 140 °C for 30 min at a solid to liquid ratio of 1:10). It was reported that among all these methods, ion-exchange resin most effectively removed furans (63.40%), acetic acid (85.20%) and phenolic compounds (75.80%). Activated charcoal

was the next most effective detoxification method and removed 38.70% of furans, 46.80% acetic acid and 57% of phenolic compounds. Overliming removed 45.80% of furans and 35.87% of phenolic compounds with no effect on acetic acid. Laccase treatment removed 77.50% of phenolic compounds with no effect on acetic acid and furan concentrations. Minimum sugar losses were observed with laccase treatments.

1.7.2.3. Activated charcoal

This method can effectively remove phenolic compounds at a relatively low cost and without causing major sugar losses (Trinh et al., 2014). There are factors that affect the efficiency of this method including the ratio of charcoal to hydrolysate (ranging from 1% to 30%), pH of the hydrolysate, residence time, and temperature (Trinh et al., 2014). Ratio of activated charcoal to hydrolysate is one of the most important factors affecting detoxification. Parajó et al. (1996) reported that by increasing the rate of hydrolysate to activated charcoal from 10 g/g to 400 g/g the extraction of phenolic compounds from hardwood hydrolysate increased from 25% to 75%. Opposite observations have been reported by Rodrigues et al. (2003) where increasing the concentration of activated charcoal from 1% (w/w) to 30% in sulfuric acid pretreated sugarcane bagasse hydrolysate (100 mg sulfuric acid/g of dry sugarcane bagasse at 121°C for 10 min) resulted in the removal of 94% of the phenolic compounds and in a 98% increase in sugar losses. Activated charcoal works better at higher temperatures (Mussatto and Roberto, 2004). Gurgel et al. (1995) observed that reducing the temperature of sugarcane hydrolysate (pretreated with impregnation for 16 h at 35 °C in 35 mM sulfuric acid followed by steam explosion at 190 °C for 5 min) from 80 to 35 °C resulted in a 70% reduction in the elimination of phenolic compounds.

1.7.2.4. Liquid-liquid extraction

Common solvents used are ethyl acetate, chloroform and trichloroethylene. Zhuang et al. (2009) used liquid-liquid extraction for removing inhibitors from wheat straw hydrolysate (pretreated with 4% (v/w) hydrochloric acid at 140 °C for 1 h). They reported that ethyl acetate at a solvent to hydrolysate (v/v) ratio

of 3:1 for an extraction time of 5 min removed 90.36% of furfurals, 77.44% of phenolic compounds and 96.29% of acetic acid while less than 2% sugar losses were observed. Volatility, recovery and flammability of solvents as well as their toxicity to microorganisms are the major concerns for using this method for detoxification (Cantarella et al., 2004).

1.7.3. Ionic liquids

Ionic liquids are salts with a melting point below 100 °C and are composed of asymmetrically packed anions and cations (Mohammad Fauzi and Amin, 2012). Figure 1.4 shows the structure of the most commonly used cations, anions and the alkyl chain used in the production of ionic liquids. Ionic liquids are referred to as "green solvents" due to their non-toxicity, negligible vapor pressure, high thermal stability, and recyclability. They are able to extract organic and inorganic materials and can form a separate phase during extraction due to their immiscibility with water. Miscibility of ionic liquids with water is prominently dictated by their anion type (Mohan et al., 2015). The length of alkyl chain and the degree of coordination of ions are the other factors affecting water miscibility of ionic liquids (Mohan et al., 2015). Small changes in the type of anions and cations, as well as the alkyl side chain will lead to significant differences in the ionic liquid properties including water miscibility, hydrophilicity, hydrophobicity, and chemical affinity with other compounds, density, and viscosity (Mohan et al., 2015).

Imidazolium-based ionic liquids including (1-Methyl-3-octylimidazolium hexafluorophosphate [OMIM][PF_6], 1-Butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6], 1-Methyl-3-octylimidazolium tetrafluoroborate [OMIM][BF_4], 1-hexyl-3-methylimidazolium tetrafluoroborate [HMIM][PF_4], 1-Methyl-3-octylimidazolium bis (trifluoromethylsulfonyl) imide [OMIM][NF_2], and 1-Butyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide [BMIM][NF_2]) have some features that make them good candidates for liquid-liquid extraction.

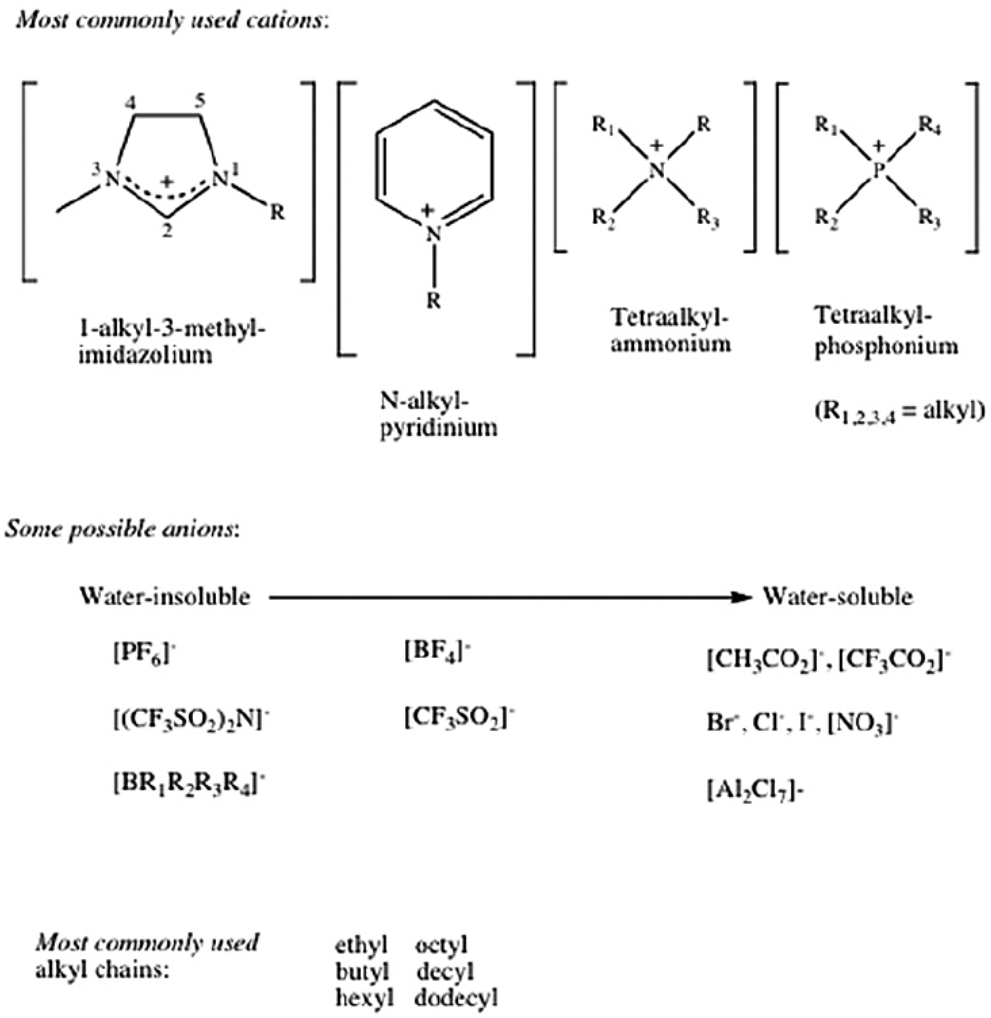

Figure 1. 4. Most Commonly Used Cation and Anions in the Production of Ionic Liquids (Seddon et al., 2000).

They are non-toxic, non-corrosive, not miscible with water, and are stable in air and water with a relatively low viscosity (Zhang et al., 2015). They have three different anions and two cations with different alkyl chains available for each of the anions. As mentioned above, the variety of cations, anions and the length of alkyl chains contribute to their difference in hydrophobicity/hydrophilicity and subsequently their extraction properties. Ionic liquids have been mostly evaluated as a pretreatment method for the solubilization of cellulose and to decrease its crystallinity (Brandt et al., 2013; Carvalho et al., 2015). They have been evaluated as catalysts to decrease the reaction temperature during pyrolysis (Qiu and Chen, 2012; Zhang et al., 2015; Qiu et al., 2012; Shill et al., 2011). Recovery and reusability is

important when working with ionic liquids. The thermal stability of ionic liquids and the difference between their vapor pressure as compared to that of the extractants can make the recovery of the extractants easier through simple methods such as distillation and evaporation without forming azeotropic solutions (Wu et al., 2009). The feasibility of recovering ionic liquids can be different based on their chemical properties. For example, water immiscibility of hydrophobic ionic liquids will make the recovery process more feasible as compared to hydrophilic ones (Wu et al., 2009). A desired ionic liquid for the detoxification of hydrolysates should possess a maximum solubility for inhibitors and minimum solubility for sugars (Mohammad Fauzi and Amin, 2012).

1.7.4. Recovery and regeneration of ionic liquids and extractives

1.7.4.1. Distillation and evaporation

The high boiling temperature of ionic liquids and their heat stability make distillation (vacuum distillation and steam distillation) and evaporation suitable methods for their recovery. Distillation and evaporation can be applied to solutions containing volatile compounds. Solvent extraction is one of the methods that uses solvents such as water, diethyl ether and hexane to extract the compounds from the ionic liquids or to induce a phase separation and facilitate their recovery (Mai et al., 2014). Extraction of solutes by supercritical CO_2 is another method that does not require intensive energy consumption. Blanchard and Brennecke (2001) were able to recover a variety of organic compounds including benzoic acid, phenol and aniline from [BMIM][PF_6] using supercritical carbon dioxide.

1.7.4.2. Membrane filtration

Reverse osmosis and nanofiltration have been successfully applied in the recovery of ionic liquids. However, there might be a need to use multiple membranes and different flow rates and pressures to reach a recovery above 97% (Kurt et al., 2010). Crystallization is another method used in the recovery of ionic liquids. Reducing the temperature below the crystallization point of the ionic liquid makes the separation

easier (Mohammad Fauzi and Amin, 2012). Hayyan et al. (2010) used this method for the recovery of glycerine from a glycerine-ionic liquid solution by reducing the temperature below 20 °C.

1.7.4.3. Physical adsorption

Physical adsorption is a promising method for the recovery of ionic liquids as the separation is relatively low cost and the regeneration and the reuse of the adsorbent is possible. Vijayaraghavan et al. (2009) investigated the recovery of 1-Butyl-3-methylimidazolium Chloride [BMIM][Cl] using two activated charcoals (SPS-200 and SPC-100) and an ion-exchange resin. It was reported that the ion exchange resin absorbed 8.96 times more ionic liquid as compared to the activated charcoal (Mai et al., 2014). Separation of hydrophilic ionic liquids from aqueous phase is possible using activated charcoal. However, the efficiency of recovery is affected by factors such as pH of the aqueous phase, size and hydrophilicity of the ions as well as the chemical features of the surface of activated carbon (Mai et al., 2014).

1.7.5. Biological methods

Biological methods involve the application of microorganisms and enzymes to degrade the inhibitory compounds (Canilha et al., 2012). Laccase and phenoloxidases can oxidize phenolic compounds (Parawira and Tekere, 2011). Disadvantages of this method include the high cost associated with enzymes as well as the long process times. Some microorganism can also degrade inhibitory compounds including white-rot fungi. They are mostly used as *in situ* delignification of biomass (Parawira and Tekere, 2011). Increasing the tolerance of microorganisms through adaptation and genetic modification is another alternative method which does not reduce the concentration of inhibitory compounds but eliminates their inhibitory effect on microbial growth. Palmqvist and Hahn-Hägerdal (2000b) reported that treatment of hydrolysates from steam pretreated willow tree with filamentous soft-rot fungus, *Trichoderma reesei*,

resulted in a 30% reduction in the total phenolic content and improved the fermentation yield. Although cellulose loss was negligible no effect was observed on the removal of acetic acids and furans.

1.8. Goals of this Study

The goals of this study are presented in three sections and included:

1. Optimization of a liquid ammonium hydroxide pretreatment for energy cane bagasse for maximum sugar yields. Optimized pretreatment parameters included temperature, residence time and biomass to ammonium hydroxide ratio. The interaction effect of pretreatment parameters on lignin removal, hemicellulose solubilization and cellulose digestibility were also evaluated.
2. Assessing the interaction effect of enzymes (cellulase, xylanase and laccase) with or without the addition of a surfactant (Tween® 80) on the cellulose digestibility of washed and unwashed dilute ammonia pretreated energy cane bagasse.
3. Evaluating the effect of six hydrophobic imidazolium-based ionic liquids on the liquid-liquid extraction of non-sugar compounds (phenolic compounds, formic acid, acetic acid, furfural, and 5-hydroxymethyl-2-furaldehyde (5-HMF)) and sugar recovery from dilute ammonia pretreated energy cane bagasse hydrolysates. Studies on the regeneration and reusability of ionic liquids were also conducted.

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CHAPTER 2

OPTMIZATION OF LIQUID AMMONIA PRETREATMENT VARIABLES FOR MAXIMUM ENZYMATIC HYDROLYSIS YIELD OF ENERGY CANE BAGASSE

2.1. Introduction

Cellulose and hemicellulose from lignocellulosic material can be utilized in the sustainable production of bio-based fuels and chemicals (Kim et al., 2003). However, the crystalline structure of cellulose and the presence of lignin tightly linked to the hemicellulose create a rigid structure that resists bioconversion (Aita et al., 2011). A pretreatment technology is needed in order to disrupt the recalcitrant nature of lignocellulosic biomass (Haghighi Mood et al., 2013). Unless some type of pretreatment is performed prior to enzymatic hydrolysis, no more than 20% cellulose digestibility is achievable (Mosier et al., 2005).

There are still inherent shortcomings with each type of pretreatment available to date (Pérez et al., 2008). Among all the available technologies, ammonia-based pretreatments have some advantages in that they are non-corrosive, non-pollutant and non-toxic, remove lignin, and preserve most carbohydrates with minimal generation of by-products. These by-products (i.e., furans, carboxylic acids, phenolic compounds) can inhibit enzymes and microorganisms during enzymatic hydrolysis and downstream fermentation processes (Behera et al., 2014). Ammonia-based pretreatment technologies are versatile in terms of applied temperature, residence time and ammonia to biomass ratio. In addition, the volatility of ammonia facilitates its recovery post pretreatment. Residual amounts of ammonia can serve as a nitrogen source for microorganisms during fermentation (Wyman et al., 2005; Salvi et al., 2010a; Kim et al., 2008; Tae Hvun et al., 2006; Salvi et al., 2010b). Ammonia-based pretreatments can increase the porosity and surface area of the biomass due to swelling. Furthermore, ammoniation of the methoxyl groups present in lignin prevents the lignin from non-productively binding to cellulases (Yoo et al., 2011).

Choosing the right crop for a bioconversion process is crucial since 60-75% of the total cost of biofuel production is allotted to the purchase price of the feedstock (Salassi et al., 2014). Energy cane has great potential as an energy crop and it is a cross breed between sugarcane and its wild ancestors. It is more resistant to cold, drought and disease than sugarcane (Kim and Day, 2011). Variety Ho 02-113 (used in this study) is a non-commercial energy cane variety with a replanting period of seven years compared to three years for sugarcane (Salassi et al., 2014). This variety yields 9% Brix in pressed juice, a fiber content of 264 g/kg (dry basis) and a cane yield of 88.9 t/ha (dry basis) as compared to sugarcane commercial variety LCP 85-384 which has 13.5% Brix in pressed juice, a fiber content of 135 g/kg (dry basis) and a cane yield of 69.2 t/ha (dry basis) (Kim and Day, 2011; Knoll et al., 2013).

Response surface methodology (RSM) is a statistical modeling technique that determines a multivariate equation by utilizing quantitative data from an appropriately designed experiment (Bas and Boyacı, 2007). This modeling technique solves the equation by finding an optimal response. RSM has become a practical method for optimizing different chemical and biochemical processes (Bas and Boyacı, 2007). The main advantage of RSM is minimizing the number of experimental trials required to evaluate the effect of multiple variables as well as their interactions (Kim and Han, 2012).

This study is the first one to evaluate the interactive effect of liquid ammonia pretreatment variables including temperature, residence time, and ammonium hydroxide to biomass ratio on the glucose and xylose yields from energy cane bagasse. Subsequently, these variables were optimized for maximum sugar yield (glucose, xylose) using RSM modeling technique.

2.2. Material and Methods

2.2.1. Biomass

Energy cane non-commercial variety Ho 02-113 was bred in Houma, LA through collaboration between the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) and

the Sugar Research Station at Louisiana State University Agricultural Center in St. Gabriel, LA. Energy cane was harvested at the Sugar Research Station and the entire plant was milled three times using a roller press (Farrel Company, Ansonia, CT) to extract the juice. The remaining solids or fibrous material (bagasse) was oven dried at 45 °C overnight to a final moisture content of 10%. Partially dried energy cane bagasse was milled (Wiley Mill, Arthur Thomas Co, PA), sieved (2 mm mesh sieve) and stored at -20 °C until further use.

2.2.2. Experimental design and statistical analysis

Central Composite Design (CCD) was employed utilizing the software Design-Expert 9.0.3 (State Ease Inc., Minneapolis, MN) to assess the effect of temperature, residence time and ammonium hydroxide to biomass ratio on the glucose yield of pretreated energy cane bagasse. CCD consists of 2^k factorial points, 2k axial points (±α), and six center points for replications, where k is the number of variables. The range and levels of independent variables are shown in Table 2.1 Range of independent variables (temperature, residence time and ammonium hydroxide to biomass ratio) were selected based on published literature and preliminary studies. A total of 20 experiments were performed in duplicate and shown in Table 2.2 Center points of the design were replicated six times to estimate the pure error sum of squares. A quadratic polynomial equation (Equation 2.1) was assumed to approximate the true function.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i \quad + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i \; X_j + \epsilon_i \qquad (2.1)$$

Where Y, response variable; X_i and X_j, the explanatory variables; β_0, constant coefficient; β_{ij}, two factor interaction coefficient; ϵ, random error. Significance of each coefficient was evaluated with analysis of variance (ANOVA).

Xylose yields collected from experimental data were used to statistically optimized the pretreatment conditions for maximum xylose yield (independently from glucose yield) using the software Design-Expert 9.0.3.

Table 2.1. Coded level of independent variables in the central composite design (CCD).							
			Coded level				
Variables	Unit	Coding	-α[a]	-1	0	+1	+α[a]
Temperature	°C	A	160	172	190	208	220
Residence Time	min	B	30	36	45	54	60
Ammonium Hydroxide[b] to Biomass Ratio	v/w	C	0	0.10	0.25	0.40	0.50
[a]Axial distance = $\sqrt[4]{N}$, where N is the number of experiments of the factorial design. [b]H_4OH, 28% v/v solution							

2.2.3. Liquid ammonia pretreatment

Liquid ammonia pretreatment of energy cane bagasse was carried out in a 4 L stirrer reactor (Autoclave Engineers, Erie, PA). Experiments were carried out at ammonium hydroxide (NH_4OH, 28% v/v solution, Fisher Scientific, Pittsburgh, PA) to dried biomass to water ratio of 0-0.5:1:20. Pretreatment temperatures ranged from 160 to 220 °C and residence times ranged from 30 to 60 min. Residence time was monitored once the desired temperature had been reached. At the end of each pretreatment, the bioreactor was cooled down to room temperature using cold water spray to accelerate the process. The pretreated material was passed through a stainless steel sieve (0.2 mm mesh) to separate the liquid and solid fractions. The solid fraction was used for chemical composition analysis and enzymatic hydrolysis assessments. All pretreatments were carried out in duplicate and the mean values calculated.

2.2.4. Chemical composition analysis

Untreated energy cane bagasse and liquid ammonia pretreated energy cane bagasse samples (solid fraction) were analyzed for glucan, xylan, lignin, arabinan, mannan, and also ash content following NREL's Laboratory Analytical Procedures (LAP TP-510-42618, 42619, 42622). NREL reference material 8491 (for sugarcane bagasse) was analyzed as an internal sample to ensure the accuracy of the procedures.

2.2.5. Enzymatic hydrolysis

Enzyme concentrations used were kept constant for all the treatments in order to evaluate the effect of

pretreatment on biomass digestibility. Spezyme CP (Genencor, Danisco US Inc., Rochester, NY, USA), and Novozyme 188 (Sigma–Aldrich, Inc., St. Luis, MO, USA) were used in combination during enzymatic hydrolysis. Activity of the enzymes were measured following Ghose, (1987) method for cellulase activity and Bailey et al. (1992) method for xylanase activity. Subsequently, Spezyme CP at 30 FPU/g glucan and Novozyme 188 at 30 CBU/g glucan were used in enzymatic hydrolysis of samples following NREL LAP TP-51043629. A biomass loading of 5% (w/v) was mixed with 0.1 M sodium citrate buffer (pH 4.8) and incubated at 50 °C in a shaker incubator (Amerex Instruments Inc., Lafayette, CA) at 150 rpm for 72 h. Samples were taken at 0 h (before the addition of enzymes), 24 h, 48 h, and 72 h. Samples were frozen at -20 °C until further analysis. Enzymatic hydrolysis experiments were carried out in triplicate and the mean values calculated.

2.2.6. Chemical composition of hydrolyzed samples

All collected samples from enzymatic hydrolysis were centrifuged at 10,000 rpm (Spectrafuge 24D, Labnet International Inc., Woodbridge, NJ) for 5 min and filtered (0.2 μm Syringe Filters, Environmental Express Inc., Mt. Pleasant, SC). Properly diluted samples were analyzed for sugars by high performance liquid chromatography (HPLC) (Agilent 1200 Series) with a BioRad Aminex HPX-87P (P), lead form, 3000 × 7.8 mm (ID), 9 μm column and a differential refractive index detector (G1362A Agilent).

Equation 2.2 and 2.3 as described in NREL's LAP TP-510-43630 was used to calculate percent theoretical yield for cellulose and hemicellulose, respectively.

$$\% \text{ Theoretical Cellulose Digestibility} = \frac{[Glucose]+1.053[Cellobiose]}{1.111\ f\ [Biomass]} \times 100\% \qquad (2.2)$$

$$\% \text{ Theoretical Hemicellulose Digestibility} = \frac{[Xylose]\ 0.9 + 0.9[Arabinose]}{1.136\ f\ [Biomass]} \times 100\% \qquad (2.3)$$

Where [Glucose], residual glucose concentration (g/L); [Cellobiose], residual cellobiose concentration (g/L); 1.053, multiplication factor that converts cellobiose to equivalent glucose; 1.111, converts cellulose to equivalent glucose; [Biomass], dry biomass concentration at the beginning of the fermentation (g/L); *f*,

cellulose fraction in dry biomass (g/g). [Xylose], residual xylose concentration (g/L); [Arabinose], residual arabinose concentration (g/L); 1.136, converts xylan to equivalent xylose.

Glucose and xylose yields were reported as the mass of glucose or xylose released per 100 g of untreated biomass after enzymatic hydrolysis and it was calculated using equation 2.4 as shown below.

Glucose (xylose) yield = 100 × recovered solids × 1.111(1.136) *f*× % theoretical cellulose (hemicellulose) digestibility (2.4)

2.3. Results and Discussion

2.3.1. Effect of liquid ammonia pretreatment on the chemical composition of biomass

Results from the compositional analysis of energy cane bagasse before and after pretreatment are shown in Table 2.2 Untreated energy cane bagasse was composed of 40.14 ±0.16% glucan, 24.23 ± 0.51% xylan, 2.76 ± 0.04% arabinan, 24.41 ± 0.37% lignin (5.97 ± 0.16 acid soluble, 18.44 ± 0.21 acid insoluble), 3.76 ± 0.62 extractives, and 4.7 ± 0.04% ash (dry weight). These results are in agreement with those reported by Aita et al. (2011) and others (Aita et al., 2011; Qiu et al., 2012; Knoll et al., 2013; Benjamin et al., 2014).

2.3.1.1. Recovered solids

All pretreatments caused considerable solid losses. A quadratic model was significant (p<0.01) for determining the effect of all variables on the solid recovery of pretreated samples with R^2=0.97 and adjusted R^2=0.94 (Table A.1). Recovered solids ranged from 55.50% to 74.01% (Table 2.2) depending on the severity of the pretreatment conditions. Similar findings for solid recovery (75.90%) of energy cane bagasse pretreated with dilute ammonia at 160 °C for 60 min and ammonium hydroxide to biomass to water ratio of 0.5:1:8 has been reported by Aita et al. (2011).

Table 2.2. Composition analysis and sugar yields of energy cane bagasse.

Samples	Experimental Variables			Recovered Solids (%w/w)	Glucan (%w/w)	Xylan (%w/w)	Lignin (%w/w)	Cellulose Digestibility (% w/w)	Hemicellulose Digestibility (% w/w)	Glucose Yield (g/ 100 g of untreated biomass)	Xylose Yield (g/ 100 g of untreated biomass)	Glucan Loss (%w/w)	Xylan Loss (%w/w)	Lignin removal (%w/w)
	Temperature (°C)	Time (min)	[a]Ratio											
Untreated	[b]N/A	N/A	N/A	100	40.14	24.23	24.41	9.27	4.36	4.13	1.22	0	0	0
1	208	54	0.3:1:20	62.74	61.10	2.20	23.52	69.36	57.43	29.54	0.91	4.49	94.18	66.85
2	190	45	0.5:1:20	63.50	58.41	8.69	22.32	72.54	68.51	29.91	4.29	7.59	76.77	64.23
3	190	30	0.25:1:20	67.09	54.57	14.89	13.98	55.66	49.30	22.65	5.63	8.77	57.93	42.85
4	208	54	0.1:1:20	55.50	63.19	2.06	16.86	66.05	52.86	25.74	0.71	12.62	95.19	42.74
5	190	45	0:1:20	59.22	59.08	18.39	16.61	59.56	28.28	23.12	3.50	12.95	56.13	44.88
6	208	36	0.1:1:20	56.81	62.07	6.57	18.11	76.11	79.83	29.82	3.40	12.14	84.29	46.86
7	190	45	0.25:1:20	64.34	56.33	16.79	17.14	71.75	58.67	28.89	7.22	9.71	54.50	50.22
8	190	45	0.25:1:20	62.90	57.62	15.57	17.85	67.45	61.00	27.16	6.81	9.71	58.77	51.11
9	220	45	0.25:1:20	56.42	64.70	1.77	23.77	74.34	52.02	30.15	0.65	9.06	95.79	60.83
10	190	45	0.25:1:20	62.34	58.11	12.60	17.09	66.21	59.43	26.65	5.31	9.75	66.92	48.54
11	172	54	0.4:1:20	69.11	52.33	20.74	15.79	64.73	46.15	26.02	7.48	9.89	39.63	49.69
12	208	36	0.4:1:20	62.91	58.20	7.81	19.90	75.20	75.35	30.59	4.23	8.78	87.26	56.86
13	172	36	0.1:1:20	68.63	54.00	17.78	12.54	41.77	36.76	17.20	5.11	7.67	48.59	39.40
14	190	60	0.25:1:20	67.65	54.02	14.06	18.56	62.01	53.56	25.18	5.78	8.95	59.95	57.01
15	172	54	0.1:120	72.88	48.01	14.90	12.38	57.13	51.86	22.21	6.42	12.83	54.26	41.27
16	190	45	0.25:1:20	65.86	55.25	14.70	16.45	65.08	54.52	26.31	6.04	9.34	59.24	49.35
17	160	45	0.25:1:20	74.01	48.60	23.12	11.97	46.89	37.07	18.74	7.19	10.37	27.94	40.51
18	172	36	0.4:1:20	71.09	50.65	22.39	11.02	48.19	42.55	19.28	7.74	10.30	32.96	35.96
19	190	45	0.25:1:20	63.29	57.03	15.93	17.86	67.75	56.85	27.17	6.46	10.08	57.55	51.43
20	190	45	0.25:1:20	63.67	56.99	15.25	18.45	71.78	58.71	28.94	6.53	9.60	59.12	53.41

Note: Data shown are the mean value (n=2 for pretreatments and n=3 for enzymatic hydrolysis; standard error < 5%).

[a] Ammonium hydroxide (NH_4OH, 28% v/v solution): bagasse (dry weight): water.

[b] Not applicable.

Ammonium hydroxide to biomass ratio had a significant positive effect on solid recovery. However, our model showed that temperature had the most significant effect (as confirmed by the strong negative correlation observed, -0.88) on recovered solids (Table 2.3). The interactive effect of temperature and time on solid recovery at an ammonium hydroxide to biomass ratio of 0.25:1 (Figure 2.1) showed that the amount of recovered solids decreased with the increase of pretreatment temperature. However, residence time had no significant effect on recovered solids. Qiu et al. (2014) reported the same significant effect of pretreatment temperature on the solid recovery of ionic liquid pretreated energy cane bagasse. Jiang et al. (2015) observed a linear relationship between the severity of liquid hot water pretreatment and solid recovery of cotton stalk. Solid loss in ammonia-based pretreatments can be attributed mostly to hemicellulose solubilization followed by lignin removal. In our study, this was confirmed by the high correlation (-0.81) seen between xylan loss and solid recovery followed by a correlation of -0.40 between lignin removal and solid recovery (Table 2.3).

Table 2.3. Correlation coefficients between variables and responses.

	Temperature	Time	Ammonium Hydroxide to Biomass Ratio	Glucose Yield	Lignin Removal	Xylan Loss	Glucan Loss	Recovered Solids	Xylose Yield
Temperature	1	0	0	0.77	0.60	0.93	-0.15	-0.88	-0.79
Time	0	1	0	0.17	0.34	0.10	0.04	0.02	-0.12
Ammonium Hydroxide to Biomass Ratio	0	0	1	0.34	0.53	0	-0.65	0.23	0.17
Glucose Yield	0.77	0.17	0.34	1	0.80	0.72	-0.22	-0.66	-0.39
Lignin Removal	0.60	0.34	0.53	0.80	1	0.62	-0.62	-0.40	-0.42
Xylan Loss	0.93	0.10	0.02	0.72	0.62	1	-0.20	-0.81	-0.88
Glucan Loss	-0.15	0.04	-0.65	-0.22	-0.62	-0.20	1	-0.11	0.14
Recovered Solids	-0.88	0.02	0.23	-0.66	-0.40	-0.81	-0.11	1	0.75
Xylose Yield	-0.79	-0.12	0.17	-0.39	-0.42	-0.88	0.14	0.75	1

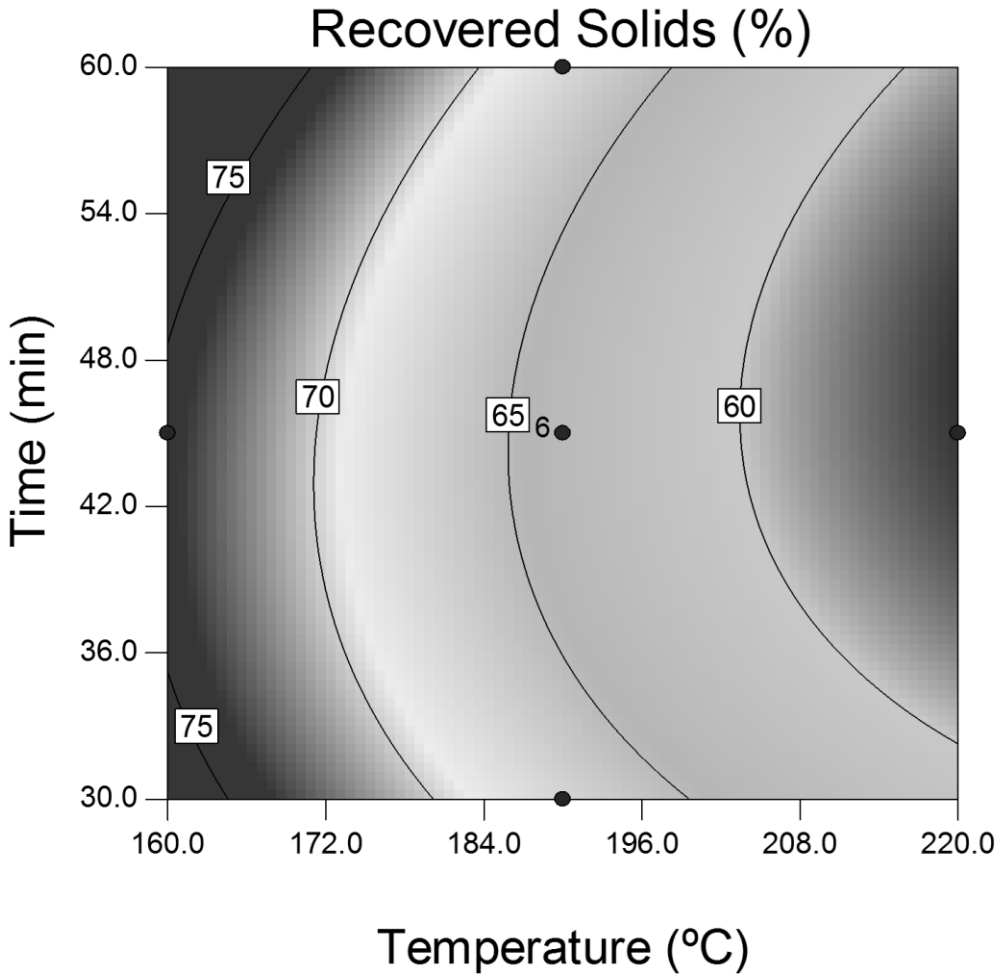

Figure 2. 1. Contour plot of the combined effect of temperature and time on percent recovered solids at an ammonium hydroxide to biomass ratio of 0.25:1.

2.3.1.2. Lignin removal

Lignin is known for its underlying recalcitrant nature in biomass. Thus lignin removal is one of the main factors defining the effectiveness of a pretreatment. Lignin was significantly removed by all the pretreatment conditions evaluated in this study. Lignin removal ranged from 35.96% to 66.85% (Table 2.2). A 2FI model (two factor interaction model) was significant for the effect of pretreatment variables on lignin removal (p<0.01) with R^2=0.91 and adjusted R^2=0.87 (Table A.2). The interaction effect of temperature and ammonium hydroxide to biomass ratio as well as the interaction effect of residence time

and ammonium hydroxide to biomass ratio posed a significant effect on the lignin removal of pretreated samples (Table A.2). For example, increasing the ammonium hydroxide to biomass ratio from 0:1 to 0.5:1 at a residence time of 40 min resulted in an increase in lignin removal from 44.88% to 64.23%.

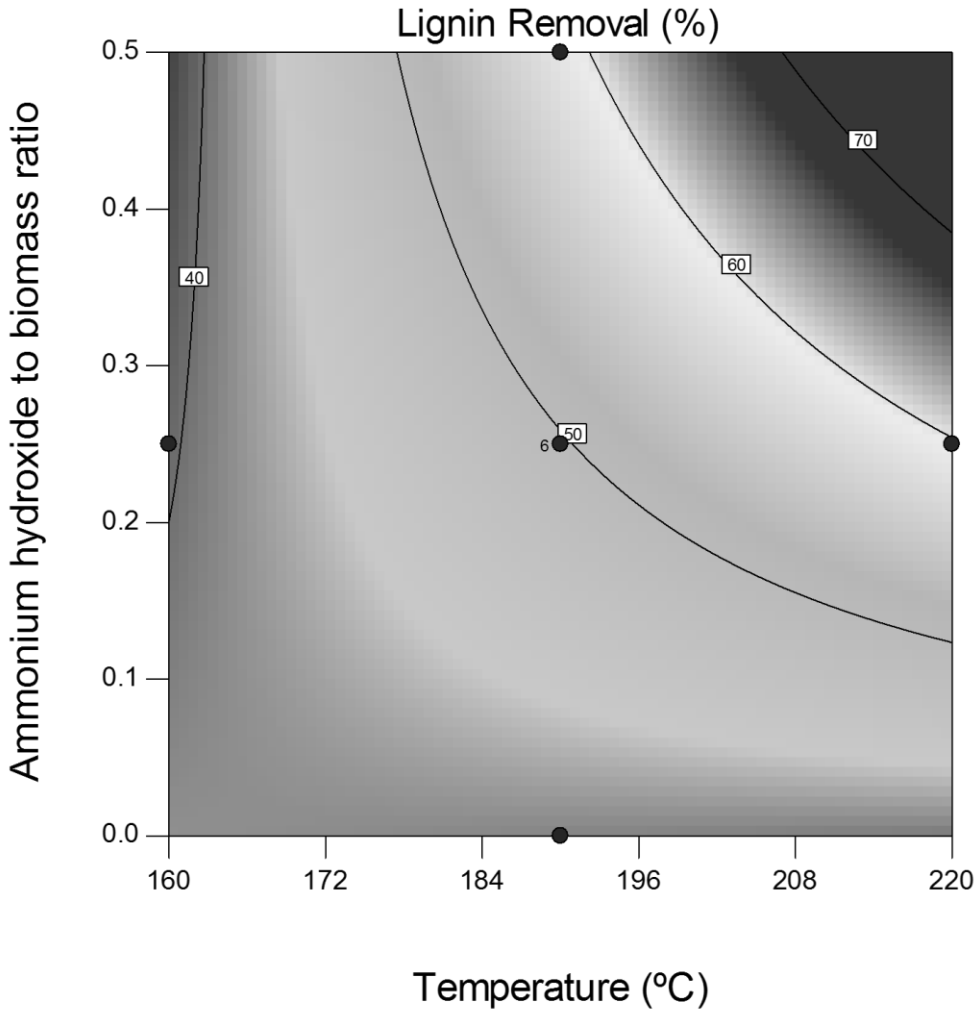

Figure 2. 2. Contour plot of the combined effect of temperature and ammonium hydroxide to biomass ratio on lignin removal at 45 min residence time.

Similarly, at an ammonium hydroxide to biomass ratio of 0.25:1 and a residence time of 40 min, increasing the temperature from 160°C to 220 °C increased lignin removal from 40.51% to 60.83%, respectively. Ammonia-based pretreatments are known to preferentially remove the lignin as compared to some other methods such as acid and hydrothermal pretreatments (Gírio et al., 2010). Ammonia removes

the lignin from the lignocellulosic matrix through the saponification of ester bonds present between the lignin and the carbohydrates (Kim et al., 2003). However, the location of the lignin within the biomass combined with its hydrophobicity and its strong poly-ring bonds make the complete removal of lignin difficult to achieve (Kim et al., 2003). Qin et al. (2013) reported the effect of ammonia on the dissociation of ester linkages between lignin and hemicellulose, the disruption of the C-O bonds of guaiacyl units in lignin, and the ammonolysis of the acetyl groups present in hemicellulose in ammonia treated corn stover. In our study, lignin removal was enhanced by the addition of ammonium hydroxide at any pretreatment temperature and at 45 min residence time (Figure 2.2).

This is due to the effect of ammonia in reducing the solubilization temperature of lignin (Aita et al., 2011). Ko et al. (2009) reported a maximum of 60.60% lignin removal for rice straw soaked in 20% w/w ammonia for 12 h at 70 °C. Chen et al. (2012) reported a 48.00% lignin removal and a 10.00% cellulose loss for microwave-assisted ammonia pretreated sorghum bagasse at 160 °C for 1 h and at a biomass, ammonium hydroxide (28% v/v solution) and water ratio of 1:0.5:8, respectively.

2.3.1.3. Xylan Loss

Alkaline pretreatments including ammonia-based pretreatments work non-selectively toward lignin and can result in the removal of the hemicellulose along with the lignin (Gírio et al., 2010). A positive correlation of 0.62 was observed between xylan loss and lignin removal meaning that more xylan was being solubilized as more lignin was removed. Dilute ammonia pretreatment of sorghum bagasse at 160 °C, for 60 min, and at an ammonium hydroxide to biomass ratio of 0.14:1 resulted in a considerable loss of hemicellulose (35.00%) simultaneously with 44.00% lignin removal (Salvi et al., 2010). In general, the harsh pretreatment conditions required to achieve an effective lignin removal do not overlap with the milder conditions under which hemicelluloses can be preserved Gírio et al. (2010). In our study, a linear model was significant (p<0.01) for xylan loss with R^2 = 0.88 and adjusted R^2 = 0.86. Temperature was the

only variable posing a significant effect on the response at 0.01 significance level. Figure 2.3 shows the contour plot of the combined effect of pretreatment temperature and residence time on xylan loss. Confirmed by the strong correlation observed (0.93), increasing the temperature from 160 °C to 220 °C resulted in xylan losses ranging from 27.94% to 95.79%, respectively. Xylan solubilization starts around 180 °C and increases as temperature goes up (Hendriks and Zeeman, 2009).

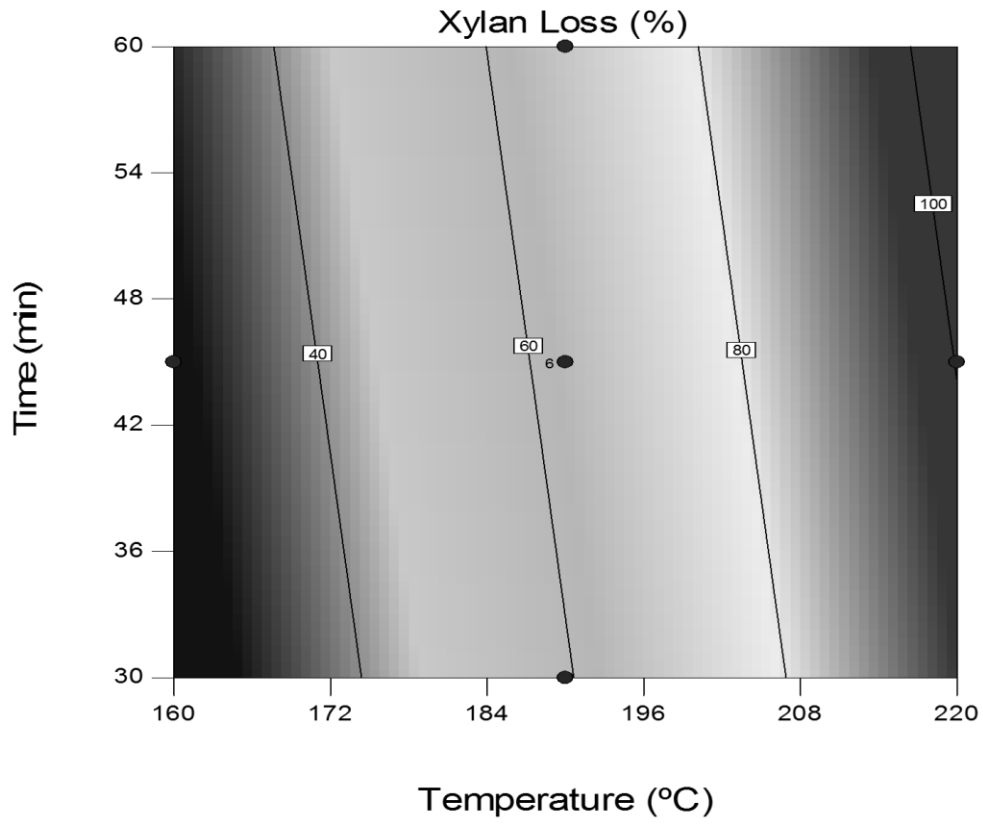

Figure 2. 3. Contour plot of the combined effect of pretreatment temperature and residence time on xylan loss at 5% (w/w) solid loading with ammonium hydroxide to biomass ratio of 0.25:1.

Bahcegul et al. (2012) reported an improvement in hemicellulose extraction from cotton stock when alkaline pretreatment temperatures were increased. Removal of hemicellulose in liquid hot water pretreated sugarcane bagasse was increased from 8.40% to 100.00% when temperatures were increased from 140 °C to 200 °C (Yu et al., 2013). Kim et al. (2009) reported retaining 80.00% of the hemicellulose

and 100.00% of the cellulose in the solid fraction of liquid ammonia pretreated corn stover by using lower temperatures (30-80 °C) and longer residence times (4-24 h). Pérez et al. (2008) reported that no hemicellulose remained in the solid fraction when the temperature of the liquid hot water pretreatment of wheat straw was increased to 220 °C.

Extensive hemicellulose removal during hydrothermal pretreatment of biomass leads to structural changes in the biomass and modification of lignin distribution in the biomass (Donohoe et al., 2008). A 2D NMR spectra from aqueous ammonia pretreated miscanthus showed that ammonia pretreatment resulted in the deacetylation of the xylan backbone. Moreover, ammonia was shown to hydrolyze the carboxyl-ester linkages between the lignin units and the hemicellulose. This would leave the pretreated biomass with high amounts of cellulose (Qin et al., 2013).

2.3.1.4. Glucan loss

Cellulose sensitivity to temperature is less as compared to hemicellulose and lignin. However, the more severe the ammonia pretreatment the more cellulose is removed (Jung et al., 2011). As compared to the losses observed with xylan and lignin, only a small amount of glucan (4.49% to 12.95%) was lost during pretreatments with ammonium hydroxide. Aita et al. (2011) reported a 9.00% sugar loss in energy cane bagasse pretreated at 160 °C for 60 min with ammonium hydroxide (1:0.5:8 ratio of dry biomass to ammonium hydroxide to water). A quadratic model was found significant for the effect of pretreatment parameters on glucan loss (p<0.01) with R^2 = 0.99 and adjusted R^2 = 0.98 (Table A.3). Ammonium hydroxide to biomass ratio was found to be the dominant variable followed by temperature. The effect of residence time was only effective at the quadratic level. Interactive effect of all variables was found to be significant for glucan loss (p<0.01).

2.3.2. Statistical analysis and model fitting

Glucose yield was used to fit the model using Design–expert 9.0.3. The quadratic model was chosen due to the observed high adjusted R-squared and predicted R-squared values (Table 2.4). Credibility of the fitted quadratic model was confirmed by lack of the fit test (Table 2.4). Coefficients of the second order polynomial equation (Equation 2.1) for glucose yield were calculated by Design-expert 9.0.3 and the final equation (Equation 2.5) is presented below.

$$\text{Glucose yield} = +27.51 + 3.64A + 0.83B + 1.64C - 2.04AB - 1.05A^2 - 1.24B^2 \quad (2.5)$$

Where, A: Temperature (°C), B: Time (min), C: Ammonium hydroxide to biomass ratio.

Table 2.4. Analysis of variance (ANOVA) for response surface quadratic model for the effect of pretreatment variables on glucose yield g/ 100 g (dry weight) of untreated biomass.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	
Model	297.45	9	33.05	38.52	< 0.0001	significant
A-Temperature	180.79	1	180.79	210.69	< 0.0001	
B-Time	9.50	1	9.50	11.07	0.0077	
C-Ammonium Hydroxide	36.62	1	36.62	42.67	< 0.0001	
AB	33.46	1	33.46	38.99	< 0.0001	
AC	0.084	1	0.084	0.098	0.7607	
BC	2.27	1	2.27	2.64	0.135	
A^2	15.82	1	15.82	18.43	0.0016	
B^2	22.11	1	22.11	25.76	0.0005	
C^2	1.45	1	1.45	1.69	0.2223	
Residual	8.58	10	0.86			
Lack of Fit	2.21	5	0.44	0.35	0.8644	not significant
Pure Error	6.37	5	1.27			
Std. dev.	0.93		R-squared	0.972		
Mean	25.74		Adj R-squared	0.947		
Coefficient of Variation%	3.60		Pred R-squared	0.915		
PRESS	26.1		Adeq precision	20.73		

Analysis of variance (Table 2.4) was performed to evaluate the significance of the developed model as well as each of the coefficients (p-value<0.01). P-value of the model was less than 0.0001 meaning that there is only 0.01% chance that the model was fit due to the noise. Additionally, all the pretreatment parameters had a significant relationship with the response while only time and temperature interaction was significantly effective at 99% confidence interval. Reliability and precision of the experiment was confirmed by a low coefficient of variation (3.60). A 20.73 signal to noise ratio was gained from our model. Adequate precision represents the signal to noise ratio with a minimum desired number of four. Additionally, a strong correlation between actual and predicted data was confirmed by the high correlation coefficient (R2 =0.97) observed, meaning the model fails to explain only 3% of the total variance. The normality of residuals was confirmed by the normal probability plot of the studentized residuals as shown in Figure 2.4.

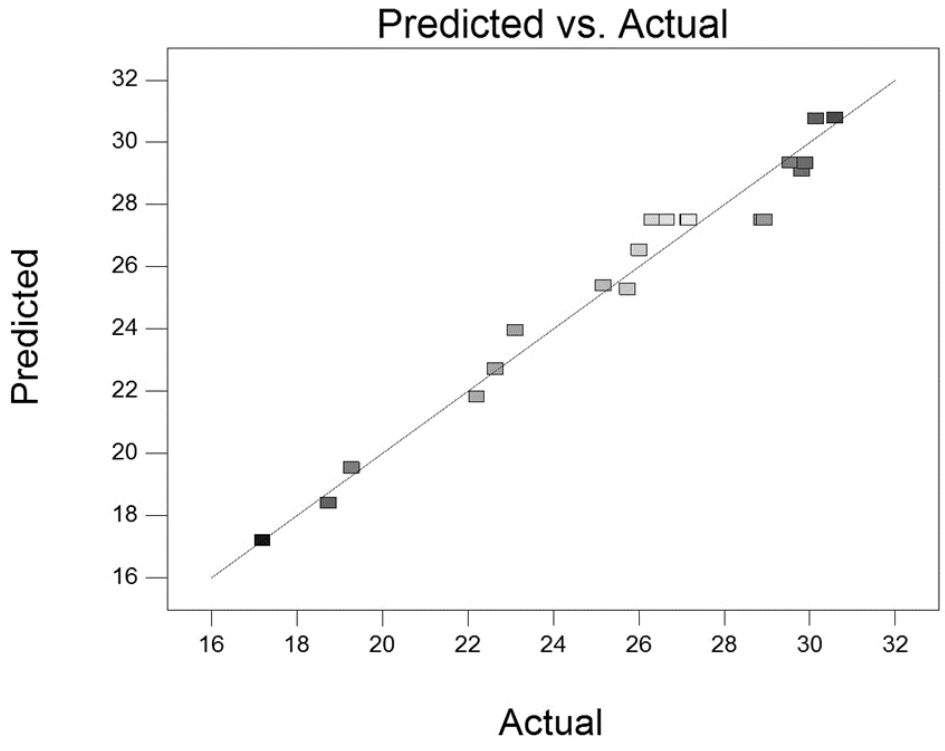

Figure 2. 4. Predicted versus actual values.

2.3.3. Cellulose and hemicellulose digestibility and sugar yield

Cellulose digestibility of our samples ranged from 41.77% to 76.11% as compared to 9.27% in untreated samples. Three-dimensional response surface graphs were plotted based on the developed quadratic model within the range of experimental design (Figure 2.5 and Figure 2.6). All the pretreatment variables had a significant effect on glucose yield (p<0.01). Among the variables, temperature had the highest correlation (0.77) (Table 2.3). At lower temperatures, for example 160 °C, glucose yield increased from 9.14 g/L to 21.53 g/L as residence time increased from 30 to 60 min. However, at higher temperatures, extending the residence time had a negative effect on the glucose yield (Figure 2.5). The negative effect of residence time was more significant as temperatures increased from 190°C to 220 °C. At 220 °C, increasing the residence time from 30 to 60 min resulted in a drastic drop in glucose yield from 32.41 g/L to 17.22 g/L. Longer residence times at high temperatures favor the formation of degrading products form lignocellulosic components. Harsh pretreatment conditions degrade pentose and hexose sugars into furfural and 5-hydroxylmethyl furfural, respectively (Jiang et al., 2015). Moreover, hydrolysis of acetyl groups presents in the hemicellulose results in the production of organic acids, mainly acetic acid, formic acid and levulinic acid (Nichols et al., 2008). Xylo-oligomers, from the incomplete break down of hemicellulose, can compete with cellulose over the binding to hydrolyzing enzymes thus blocking their activity (Zhang and Viikari, 2012) As illustrated in Figure 2.5, the combination of a higher temperature and a shorter residence time improved the glucose yield (larger slope of the response line) as compared to the combination of a lower temperature and a longer residence time. Our results are in agreement with those reported by Qin et al. (2013) on ammonia pretreatment of corn stover where glucose yield was primarily affected by temperature. They also reported the same less significant effect of residence time, at temperatures below 180 °C, as compared to other pretreatment variables (temperature, ammonia concentration and solid loading). A cellulose digestibility of 86.80% was reported in corn stover

pretreated at 180 °C for 60 min with 20% w/w ammonium hydroxide and hydrolyzed with 60 FPU cellulase loading (Qin et al., 2013). A similar dominant effect of temperature as compared to acid concentration and residence time was reported in RSM optimization of dilute phosphoric acid pretreatment of corn stover (Avci et al., 2013). Increasing the ratio of ammonium hydroxide to biomass also resulted in a better cellulose digestibility (Figure 2.6). For example, pretreatment of energy cane bagasse at 190 °C for 45 min containing no ammonium hydroxide resulted in a glucose yield of 23.15 g glucose /100 g (dry weight) untreated biomass and 44.88% lignin removal. However, glucose yield was improved to 28.89 and 29.91 g glucose/100 (dry weight) untreated biomass when ammonium hydroxide was added to

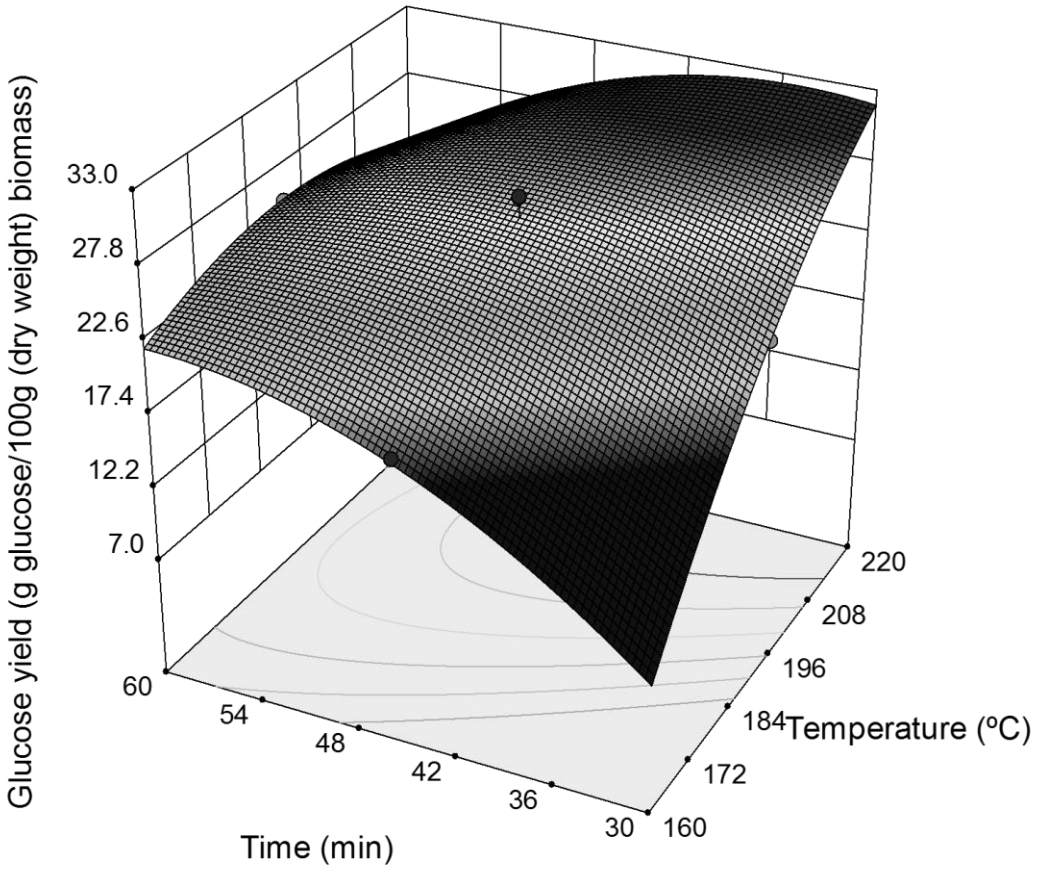

Figure 2. 5. 3 Dresponse surface plot of the effects of pretreatment temperature and residence time on glucose yield (g glucose/ 100 g (dry weight) untreated biomass) at ammonium hydroxide to biomass ratio of 0.25:1.

the mix at an ammonium hydroxide to biomass ratio of 0.25:1 and 0.5:1, respectively. This can be explained by the effect of ammonium hydroxide on lignin removal with observed values of 53.41% and 64.23%, respectively.A negative effect was observed between recovered solids and glucose yield. By decreasing the recovered solids from 74.01% to 55.50%, the corresponding glucose yield increased from 18.74 to 25.74 g glucose /100 g (dry weight) untreated biomass. This was expected as decreasing the recovered solids in the pretreated samples can be translated to higher lignin removal and hemicellulose solubilization. Similar observations were reported by Hongdan et al. (2013) in liquid hot water pretreatment of sugarcane bagasse. It was reported that increasing the pretreatment temperature from 160

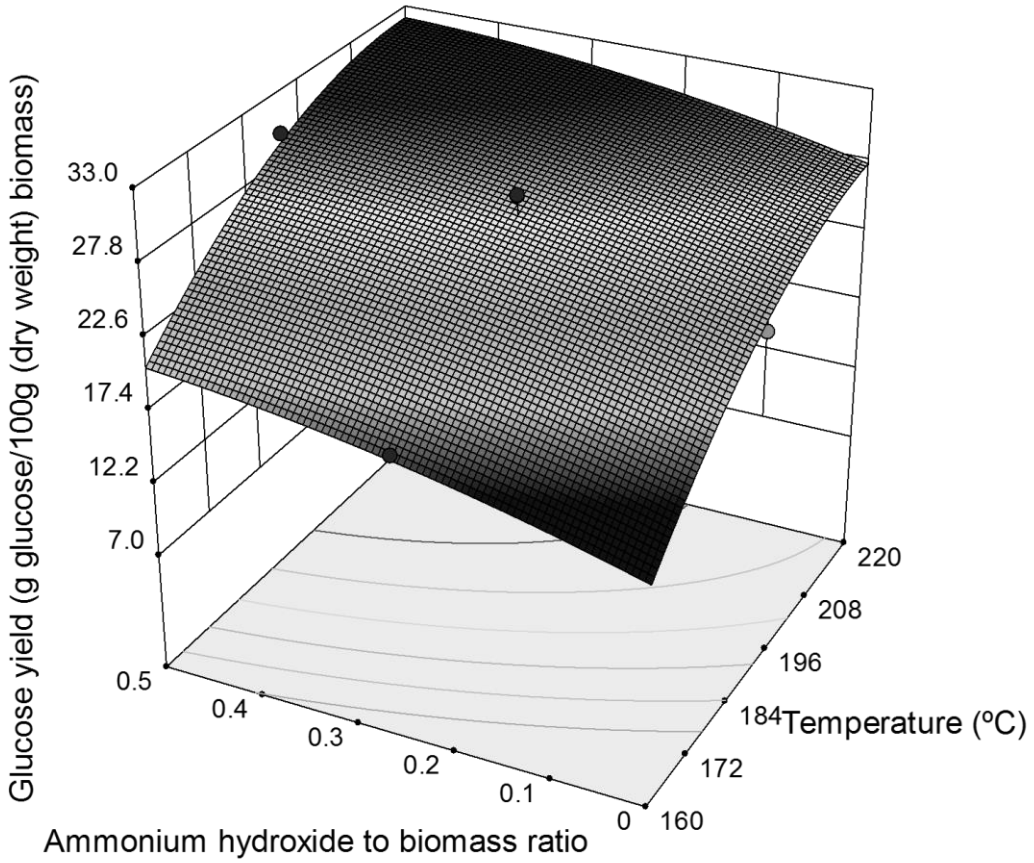

Figure 2. 6. 3D response surface plot of the effects of ammonium hydroxide to biomass ratio and pretreatment temperature at 45 min residence time.

to 200 °C resulted in a decrease in recovered solids from 86.16% to 62.60%. The decrease in solid recovery was accompanied by an increase in sugar yield as well as the generation of by-products (acetic acid, furfural and 5-hydroxymethyl furfural) in the pretreatment hydrolysate. Glucose yield showed the highest positive correlation with lignin removal (0.81) followed by hemicellulose loss (0.72) (Table 2.3), indicating the importance of removing lignin to improve enzymatic digestibility (Jeoh et al., 2007; Hendriks and Zeeman, 2009). Yu et al. (2013) and Gao et al. (2013) also reported that lignin removal had a more prominent effect on the enzymatic digestibility of sugarcane bagasse regardless of pretreatment type. Ko et al. (2009) reported a strong correlation (R^2 =0.93) between lignin removal and cellulose digestibility in aqueous ammonia soaking of rice straw. Kim and Han (2012) reported a high degree of correlation (R^2 =0.88) between lignin removal and enzymatic digestibility of alkaline (sodium hydroxide) pretreated rice straw. Ammonia can disrupt the linkages between lignin, cellulose and hemicellulose leaving the cellulose available to enzymes (Li and Kim, 2011). Furthermore, lignin can non-productively bind to cellulases or decrease the reaction rate by interfering during enzymatic hydrolysis (Zabed et al., 2016; Saini et al., 2016). Therefore, removing lignin enhances the enzymatic digestibility of cellulose.

Studies have shown that as important as the lignin removal is on improving enzyme digestibility, location and distribution of lignin plays an equally important role. Donohoe et al. (2008) reported that increasing the temperature above the lignin phase transition (120-200 °C) during thermochemical pretreatment of biomass can cause the molten lignin to merge into larger droplets and migrate to the surface of the plant cell walls. After pretreatment, these relocated lignin droplets can deposit on the surface of the biomass and provide a physical barrier for the hydrolyzing enzymes (Kaparaju and Felby, 2010). Aita et al. (2011) observed drastic morphological changes in aqueous ammonia pretreated energy cane bagasse that included disruption of the rigid structure of the biomass, increase in the surface area of the

biomass as well as the formation of micropores. All these changes favor enzyme accessibility to the pretreated biomass which translates into higher cellulose digestibility.

It was also reported that since cellulose and hemicellulose are physically connected, removal of hemicellulose can potentially enlarge the pore sizes present on the cellulose structure and increase cellulose accessibility to hydrolyzing enzymes (Kaparaju and Felby, 2010). Cao and Aita (2013) reported a cellulose digestibility of 66.00% for ammonium hydroxide pretreated sugarcane bagasse at 1:20:0.5 bagasse to water to ammonium hydroxide ratio with 3% Tween® 80 and hydrolyzed with 30 FPU Spezyme CP along with 30 CBU Novoxyme 188. Kim and Lee (2005) reported a 53.60% solid recovery after pretreatment of corn stover with ammonia recycle percolation (15 wt% ammonia, 170 °C, 5 mL/min flow rate and 2.3 MPa), 84.70% lignin removal and 60.00% hemicellulose solubilization resulting in a 93.30% cellulose digestibility with 60 FPU cellulase enzyme activity. Despite the fact that hemicellulose provides a physical barrier for cellulases, more hemicellulose removal does not necessarily translate to better cellulose digestibility. Yang et al. (2013) studied the correlation between hemicellulose removal and cellulose digestibility on hulless oat straw and observed that the highest bioconversion was achieved while still 33.00% hemicellulose remained intact.

Hemicellulose digestibility of our samples ranged from 28.28% to 79.83% as compared to 4.40% in untreated samples (Table 2.2). Hemicellulose digestibility of pretreated samples was lower than those observed for cellulose. This can be attributed to the composition of the enzymes cocktail used in this study which mostly contained cellulases over xylanases. Xylose yields of pretreated samples were low and ranged from 0.71 to 7.74 g xylose/ 100g (dry weight) untreated biomass. The low xylose yields stemmed from the low amounts of hemicellulose present in the biomass post ammonium hydroxide pretreatment. A xylose yield of 7.74 g xylose/100g (dry weight) untreated biomass was achieved when 67.04% of the xylan was preserved at 172 °C, for 36 min, and at an ammonium hydroxide to biomass ratio of 0.4:1, and

resulted in a xylose digestibility of 42.55% (Table 2.2). However, under these pretreatment conditions only 35.96% of the lignin was removed which resulted in a poor cellulose digestibility (48.19%) and a low glucose yield (19.28 g glucose/100g (dry weight) untreated biomass). A negative correlation was observed between glucose yield and xylose yield (Table 2.3) as pretreatment conditions for their optimum sugar yields did not overlap.

A quadratic model was fitted ($p<0.01$) based on the xylose yield of our samples with $R^2 = 0.95$ and adjusted $R^2 = 0.91$ (Table A.4). The model showed that temperature and the ratio of ammonium hydroxide to biomass were the most significant variables ($p<0.05$). Figure 2.7 shows the contour plot effect of temperature and ammonium hydroxide to biomass ratio on xylose yield. Pretreatment temperature had a negative and close to linear relationship with xylose yield (correlation of -0.79) (Table 2.3). The opposite was observed for pretreatment temperature and glucose yield having a strong positive correlation (0.77) (Table 2.3). An indication that while increasing the pretreatment temperature improved the glucose yield, it caused a decrease in xylose yield. Yu et al. (2013) reported an increase in total xylose yield (including monomeric sugars and xylo-oligomers) by increasing the temperature of liquid hot water pretreatment of sugarcane bagasse up to 160 °C. However, increasing pretreatment temperature above 180 °C resulted in a significant decrease in the xylose yield while xylan loss was also increased. They also observed that the same increase in pretreatment temperature (from 160°C to 180 °C) resulted in a drastic increase in the generation of degrading products from hemicellulose. Additionally, by the same increase in temperature, the concentration of xylo-oligomers in the hydrolysate was decreased in favor of an increase in the concentration of xylose; meaning that no oligomeric sugars were found in the pre-hydrolysate as the pretreatment temperature approached 200 °C. The drop observed in the xylose yield can be justified by the inhibitory effect of these compounds on the hydrolyzing enzymes. There are other strategies suggested to preserve both pentose and hexose sugars for maximum total sugar yield. Kim and Lee (2005)

investigated the effect of a two-stage pretreatment process (hot water followed by ammonia percolation) in corn stover. Liquid hot water pretreatment (190 °C, 30 min) removed 84.00% of the xylan which can be recovered from the liquid fraction. The lignin-rich solid fraction was further treated with ammonia recycle percolation (170 °C, 60 min, at 2.3 MPa and 15% NH_3 at 5 mL/min of flow rate) resulting in a 75.00% lignin removal. A 93.60% cellulose digestibility was observed with the two-stage pretreatment process as compared to 94.40% cellulose digestibility for the one-stage ammonia recycle percolation pretreatment.

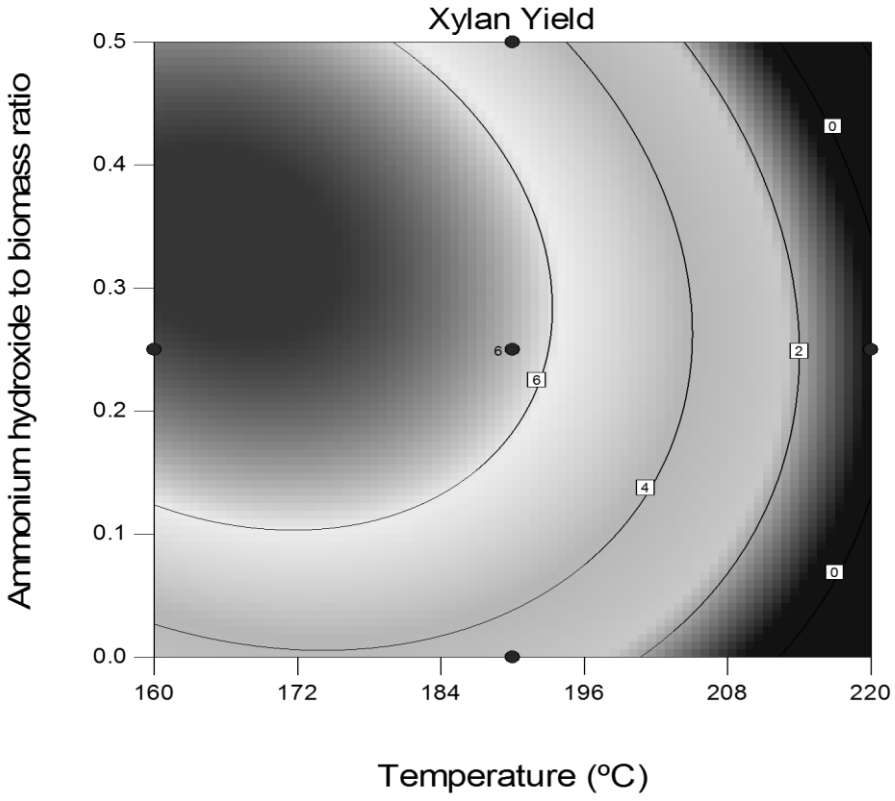

Figure 2. 7. Contour plot of the combined effect of pretreatment temperature and ammonium hydroxide to biomass ratio on xylose yield (g xylose/100 g (dry weight) untreated biomass at 45 min residence time.

The highest combined total amount of fermentable sugars (glucose and xylose) was achieved at 190 °C, for 45 min, and at an ammonium hydroxide to biomass ratio of 0.25:1, where 28.89 g of glucose along

with 7.22 g of xylose were obtained for every 100 g (dry weight) of untreated biomass (Table 2.2). Under these conditions, about 50.22% lignin was removed and 54.50% of xylan was solubilized.

2.3.4. Optimization of process conditions and experimental validation

Optimal pretreatment conditions for maximum glucose yield (as our foremost sugar) were predicted using Design-expert 9.0.3. Data related to the predicted points as well as the results from the experimental evaluation of these points are summarized in Table 2.5. Based on these defined conditions, the optimum pretreatment parameters for maximum glucose yield were 208 ^{o}C, for 36 min and at an ammonium hydroxide to biomass ratio of 0.4:1. The predicted value for glucose yield was 30.77 ± 0.93 g glucose/100 g (dry weight) untreated biomass.

Based on our model for xylose yield, the maximum xylose yield within our experiment design range was predicted as 9.10 g xylose/100 g (dry weight) untreated biomass at 160 °C, for 60 min and at an ammonium hydroxide to biomass ratio of 0.31:1. However, the glucose yield obtained under these conditions was 23.34 g glucose/100g (dry weight) untreated biomass. Experimental results fell within the 95% confidence interval of the predicted value which supported the reliability and robustness of our model (Table 2.5).

Table 2.5. Predicted versus experimental results.

Response	Predicted mean	Observed	Std. dev.	SE Pred.	95% PI low	95% PI high
Glucose Yield (g/100 g (dry weight) untreated biomass)	30.77	30.62	0.96	1.20	28.10	33.43
Xylose Yield (g/100 g (dry weight) untreated biomass)	3.99	4.20	0.66	0.84	2.12	5.87
Recovered Solid (%)	63.71	62.51	1.23	1.59	60.17	67.24
Glucan Loss (%)	8.76	9.01	0.24	0.32	8.12	9.45
Xylan Loss (%)	83.27	84.14	7.48	8.25	65.54	100.28
Lignin Removal (%)	59.18	60.26	3.05	3.91	50.73	67.63

Note: Data shown represent the mean values (n=2; Standard error < 5%).

2.4. Conclusions

Pretreatment parameters including temperature, residence time and ammonium hydroxide to biomass ratio were optimized for maximum sugar yields from energy cane bagasse. Temperature was found to be the most effective variable having the greatest impact on the response followed by ammonium hydroxide to biomass ratio. A quadratic model was fitted (P value < 0.01) with the experimental values and the generated equation was used to find the optimum value for each variable. The highest glucose yield was predicted to be 30.77 g glucose/ 100 g (dry weight) untreated biomass with a xylose yield of 3.99 g xylose/100 g (dry weight) untreated biomass obtained at 208^{o}C for 36 min and at an ammonium hydroxide to biomass ratio of 0.4:1. A separate quadratic model (P value < 0.01) was fitted for the experimental values of xylose yield. Based on the model, the maximum xylose yield was predicted to be 9.10 g xylose/100 g (dry weight) untreated biomass obtained at 160°C for 60 min and at an ammonium hydroxide to biomass ratio of 0.31:1. However, these pretreatment conditions resulted only in a predicted glucose yield of 23.34 g glucose/ 100 g (dry weight) untreated biomass. Results of actual experiments carried out at the optimal points from our models fell within 95% confidence interval of the predicted value. The low xylose yields observed in this study can be attributed to the substantial amounts of xylan being lost during pretreatment due to solubilization.

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CHAPTER 3

INTERACTIVE EFFECT OF CELLULASE, XYLANASE, LACCASE, TWEEN® 80 AND POST-WASHING ON THE CELLULOSE DIGESTIBILITY OF DILUTE AMMONIA PRETREATED ENERGY CANE BAGASSE

3.1. Introduction

Cellulose, the most abundant bio-polymer, can be exploited for the sustainable production of bio-fuels and bio-chemicals. The main challenge is to decrease its crystallinity and increase its accessibility by removing the lignin and by dissolving the hemicellulose, the other two main structures present in the lignocellulosic biomass (Salvi et al., 2010). Without some type of biomass pretreatment (physical, chemical or biological), a cellulose conversion of no more than 20% can be achieved (Ko et al., 2009).

Research studies have indicated that in addition to cellulases (a mixture of endoglucanases, cellobiohydrolases and β-glucosidases), the use of accessory enzymes (i.e., xylanase, pectinase, laccase, feruloyl esterase, lytic polysaccharide monooxygenase) can enhance the enzymatic digestibility of pretreated biomass (Bhattacharya and Pletschke, 2015; Gonçalves et al., 2015; Sun et al., 2015; Tabka et al., 2006; Uppugundla et al., 2014; Wan and Li, 2010; Laothanachareon et al., 2015). Xylanase can increase cellulose accessibility by hydrolyzing the hemicellulose and eliminating its physical barrier (Zhang and Viikari, 2012). Moreover, addition of xylanase can address the issues related to the presence of solubilized hemicellulose, hemicellulose-derived products and xylo-oligomers (Zhang and Viikari, 2012). These compounds are known to have inhibitory effects on cellulase activity by blocking its accessibility to cellulose (Qing et al., 2010; Zhang and Viikari, 2012). Additionally, xylanase has shown synergistic effects with cellulase (Juturu and Wu, 2012; Gonçalves et al., 2015). Synergistic effect of the combined use of cellulase and xylanase during the enzymatic hydrolysis of untreated corncob, corn stover and rice straw improved hydrolysis yields by 133%, 164% and 545%, respectively, as compared to control samples which were hydrolyzed by cellulase only (Song et al., 2016). Li et al (Song et al., 2016) also

observed a synergistic effect between cellulase and xylanase when hydrolyzing sugarcane pretreated by different methods (steam exploded, hydrogen peroxide and sodium hydroxide); however, it was observed that the degree of synergism was dependent on the substrate chemical composition and its xylose content. Cellulose crystallinity and the molecular structure of enzymes are the other factors affecting the degree of synergism (Jia et al., 2015). Laccase, a multicopper-containing phenoloxidase, can potentially assist with cellulose digestibility by oxidizing lignin and phenolic compounds (Chen and Qiu, 2010; Chen et al., 2012). There might also be a synergistic effect between laccase and cellulase. Al-Zuhair et al. (2013) reported that simultaneous application of laccase and xylanase along with cellulase during the enzymatic hydrolysis of date palm biomass resulted in a 60% cellulose conversion as compared to 45.60% conversion achieved under sequential applications of these enzymes and a 5.60% conversion by using just cellulase. Some studies have illustrated a decrease in the final glucose yield of lignocellulosic material as a result of laccase treatment (Moreno et al., 2013; Oliva-Taravilla et al., 2015). Therefore, further studies are required to stablish the best combination of accessory enzymes that conforms to the type of the biomass as well as the type and severity of the pretreatment applied.

Non-ionic surfactants such as Tween® 80 have been reported to increase enzymatic digestibility of biomass in the presence of reduced enzyme loadings (Eriksson et al., 2002; Kristensen et al., 2007, Liu et al., 2011; Menegol et al., 2014; Yang et al., 2011). However, the activity of surfactants depends on the type of pretreatment used. For example, acid pretreated wheat straw showed a better response to the addition of non-ionic surfactant (poly ethylene glycol) when compared to ammonia pretreated straw (Yang et al., 2011). Jin et al. (2016) reported that a 0.50% loading of Tween® 80 enhanced enzymatic digestibility of steam exploded reed by 1.7 fold as compared to samples without added surfactant. Yang et al. (2011) showed that Tween® 80 increased the activity of endoglucanase and exoglucanase by preventing deactivation of adsorbed cellulase during their interaction with the substrate. Furthermore,

addition of Tween® 80 can increase sugar yields of larger size biomass particles thus reducing the energy required for fine grinding of the biomass (Menegol et al., 2014).

Washing is considered the simplest method for the removal of compounds (i.e., lignin derivatives, organic acids, furans) that are generated or released during the pretreatment of biomass materials (Kumar and Wyman, 2009; Soares et al., 2011; Toquero and Bolado, 2014). These non-sugar compounds can have a negative effect on downstream processes (i.e., enzymatic hydrolysis, fermentation). Post-washing is effective especially at high solid loadings or when the presence of non-sugar compounds is high (Rajan and Carrier, 2014).

In this study, the interactive effect of enzymes (cellulase, xylanase and laccase) with or without the addition of a surfactant (Tween® 80) on the cellulose digestibility of washed and unwashed dilute ammonia pretreated energy cane bagasse was assessed using Response Surface Methodology (RSM). RSM is a statistical modeling technique that uses quantitative data generated from an appropriately designed experiment to determine a multivariate equation (Baş and Boyacı, 2007). Energy cane, a cross breed between sugarcane and its wild ancestors, has interesting features as a potential energy crop. Compared to sugarcane, it has higher fiber content (264 g/kg versus 135 g/kg (dry basis)) and higher cane yield (88.90 t/ha versus 69.20 t/ha (dry base)). It is more resistant to cold and disease and requires less water input (Knoll et al., 2015; Kim and Day, 2011).

3.2. Material and Methods

3.2.1. Biomass

Energy cane non-commercial variety Ho 02-113 was bred in Houma, LA through collaboration between the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) and the Sugar Research Station at Louisiana State University Agricultural Center in St. Gabriel, LA. Energy cane was harvested at the Sugar Research Station and was milled three times using a roller press (Farrel

Company, Ansonia, CT) to remove the juice. The milled solid fraction is called bagasse. Bagasse was oven dried at 45 °C to a 10% final moisture content, milled (Wiley Mill, Arthur Thomas Co, PA), sieved (2 mm mesh sieve), and stored at -20 °C until further use.

3.2.2. Dilute ammonia pretreatment

Energy cane bagasse was pretreated with liquid ammonium hydroxide (28% v/v) in a 4 L stirrer reactor (Autoclave Engineers, Erie, PA) at 208ºC for 36 min, and at an ammonium hydroxide to biomass to water ratio of 0.4:1:20. The pretreatment conditions used in this study had been previously optimized for maximum sugar yields using RSM. The pretreated slurry was pressed to recover the solid fraction. The solid fraction was divided in two parts, half was washed with deionized water (6 volume) and the other half was kept unwashed. The biomass was dried at 45 °C in an oven to a final moisture content below 10%. Composition analysis of untreated and pretreated energy cane bagasse was performed following NREL's Laboratory Analytical Procedures (LAP TP-510-42618, 42619, 42622). NREL reference material 8491 (for sugarcane bagasse) was analyzed as an internal sample to ensure the accuracy of the procedures.

3.2.3. Experimental design and data analysis

Central Composite Design (CCD) and RSM were employed utilizing the software Design-Expert 9.0.3 (State Ease Inc., Minneapolis, MN) to design the experiment, to analyze the interaction effect of variables (cellulase, xylanase, laccase, and surfactant) on the response (glucose yield) and to find the optimum combination that would result in the highest glucose yield. CCD consists of 2^k factorial points, 2k axial points (±α), and six center points for replications, where k is the number of variables. A total of 30 experiments were performed in duplicate for washed an unwashed dilute ammonia pretreated energy cane bagasse (Table 3.1). A quadratic polynomial equation (Equation 3.1) was assumed to approximate

the true function. Center points of the design were replicated six times to estimate the pure error sum of squares.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i \ + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i \ X_j + \epsilon_i \quad \text{(Equation 3.1)}$$

Where Y, is the response variable; X_i and X_j, the explanatory variables; β_0, constant coefficient; β_{ij}, two factor interaction coefficient; ϵ, random error. Significance of each coefficient was evaluated with analysis of variance (ANOVA).

Table 3. 1. Coded level of independent variables in the central composite design (CCD).

		Coded level					
Variables	Unit[a]	Coding	-α[b]	-1	0	+1	+α[b]
Cellulase (CTec2)	% w/w glucan	A	1.5	6.12	10.75	15.4	20
Xylanase (HTec2)	% w/w glucan	B	0	3.75	7.5	11.25	15
Laccase	IU g-1biomass	C	0	12.5	25	37.5	50
Surfactant (Tween®80)	% w/w glucan	D	0	3.75	7.5	11.25	15

[a]All biomass weights are reported on dry basis.
[b]Axial distance = $\sqrt[4]{N}$, where N is the number of experiments of the factorial design.

3.2.4. Enzymatic hydrolysis and sample analysis

Cellulase (Cellic® CTec2) and xylanase (Cellic® HTec2) were obtained from Novozymes (Novozymes A/S, Bagasvaerd, Denmark). Laccase from *Rhus vernicifera* and Tween® 80 were purchased from Sigma (Sigma–Aldrich, Inc., St. Luis, MO, USA). Enzyme assays were done to measure cellulase, xylanase and β-glucosidase activities of CTec2 and HTec2. Cellulase activity for CTec2 (132 FPU mL^{-1}) and HTec2 (90.75 FPU mL^{-1}), and β-glucosidase activity for CTec2 (3229 IU mL^{-1}) and HTec2 (12.61 IU mL^{-1}) were measured following the Ghose method (Ghose, 1987). Xylanase activity of Ctec2 (12100 IU mL^{-1}) and Htec2 (56045 IU mL^{-1}) were determined according to Bailey et al. (1992). Laccase activity (50 U mL^{-1}) was measured using syringaldazine as substrate based on the Ride method (Ride, 1980). Biomass at 8% (w/w) was mixed with 0.10 M sodium citrate buffer and the pH was adjusted to 4.8. Corresponding

amounts of enzymes and Tween® 80 were then added to each flask. Flasks were incubated at 50 °C in a shaker incubator (Amerex Instruments Inc., Lafayette, CA) at 180 rpm for 72 h. Samples were taken at 0 h (before the addition of enzymes), 24 h, 48 h, and 72 h and kept at -20 °C until further analysis. Experiments were done in duplicate. All collected samples were centrifuged at 10,000 rpm (Spectrafuge 24D, Labnet International Inc., Woodbridge, NJ) for 5 min and filtered (0.2 μm Syringe Filters, Environmental Express Inc., Mt. Pleasant, SC). Samples were diluted accordingly and analyzed for sugars by high performance liquid chromatography (HPLC) (Agilent 1200 Series) with a BioRad Aminex HPX-87P (P), lead form, 3000 × 7.8 mm (ID), 9 μm column and a differential refractive index detector (G1362A Agilent). Theoretical yields for cellulose were calculated using Equation 3.2 as described in NREL's LAP TP-510-43630.

$$\% \text{ Theoretical Cellulose yield} = \frac{[\text{Glucose}]+1.053[\text{Cellobiose}]}{1.111\ f\ [\text{Biomass}]} \times 100\% \qquad \text{(Equation 3.2)}$$

Where [Glucose], residual glucose concentration (g/L); [Cellobiose], residual cellobiose concentration (g/L); 1.053, multiplication factor that converts cellobiose to equivalent glucose; [Biomass], dry biomass concentration at the beginning of the hydrolysis (g/L); f, cellulose fraction in dry biomass (g/g).

3.2.5. Mass balance

Mass balances were calculated for washed and unwashed dilute ammonia pretreated energy cane bagasse. Liquid and solid streams of pretreated and enzymatically digested biomass were analyzed for lignin, cellulose, hemicellulose, monomeric sugars, and total solids by NREL procedures as described above. Oligomeric sugars present in the liquid streams were further hydrolyzed to their monomeric form as described in NRELS's LAP TP-510-42618 (Sluiter, 2008). Glucan and xylan values in the solid fraction were reported as glucose and xylose using the conversion factor of 1.111 for glucan to glucose and 1.135 for xylan to xylose conversion.

3.3. Results and Discussion

3.3.1. Biomass composition analysis

Composition analysis of untreated energy cane bagasse resulted in 40.14 ± 0.16% glucan, 24.23 ± 0.51% xylan, 2.76 ± 0.04% arabinan, 24.41 ± 0.37% lignin (5.97 ± 0.16 acid soluble, 18.44 ± 0.21 acid insoluble), 3.76 ± 0.62% extractives, and 4.70 ± 0.04% ash (dry basis). Results are in agreement with those reported by others (Aita et al., 2011; Qiu et al., 2012). 62.51% of solids were recovered after pretreatment while about 84.14% of the initial xylan and 60.26% of the initial lignin were removed. Only 9.01% glucan was lost. Post-washing of pretreated bagasse caused negligible changes to the chemical composition of the biomass.

3.3.2. Cellulose digestibility, statistical analysis and model fitting

Harsh pretreatment conditions favor the generation of compounds such as organic acids (i.e., formic acid, acetic acid, levulinic acid) which result from the degradation of hemicellulose. Furan derivatives (i.e., furfural and 5-hydroxymethyl-2-furaldehyde (HMF)) can be generated from the breakdown of cellulose and hemicellulose, and phenolic compounds (i.e., vanillic acid, syiringaldehyde, coniferyl alcohol) can be derived from the degradation of lignin (Chandel et al., 2013). These compounds can be inhibitory to enzymes by their nonproductive binding and to microorganisms by inhibiting microbial growth through several mechanisms including altering the pH gradient of the cell (Klinke et al., 2002; Panagiotou and Olsson, 2007). Several reports have demonstrated the positive effect of washing on the elimination of these compounds and on the improvement of enzymatic conversions of washed samples (Frederick et al., 2014; Toquero and Bolado, 2014; Pengilly et al., 2015). Our study is in agreement with those observations in that washing had a positive effect on the cellulose digestibility of the dilute ammonia pretreated energy cane bagasse (Table 3.2). For each combination of enzymes and surfactant, cellulose digestibility was significantly higher for washed samples as compared to unwashed samples. However,

the improvement observed was different based on the treatment. For example, washing the pretreated substrate and hydrolyzing it with 1.50% CTec2, 7.50% HTec2, 25 IU/g laccase, and 7.5% Tween® 80 improved cellulose digestibility from 47.70% to 84.60%. However, this 77.36% improvement in cellulose digestibility was reduced to 8.91% when the washed and unwashed substrates were hydrolyzed with a combination of 15.38% CTec2, 3.75% HTec2, 37.50 IU/g laccase and 11.25% Tween® 80. It seems that increasing the amount of cellulase (CTec2) loading resulted in a less prominent effect of washing on the cellulose digestibility of pretreated samples. Percent cellulose digestibility ranged from 82.20% to 94.20% in washed samples and from 47.70% to 83.90% in unwashed samples. Frederick et al. (2014) observed that post-washing of dilute acid pretreated poplar increased glucose yields by 5.3 folds. Toquero et al. (2014) investigated the effect of four types of pretreatment on the enzymatic hydrolysis of wheat straw and reported that post-washing (at a dry biomass to water ratio of 1:10) successfully removed impurities regardless of the type of pretreatment. Rajan and Carrier (2014) removed 87% of formic acid, 64% of acetic acid, 86% of furfural, and 87% of 5-hydroxymethyl-2-furaldehyde after washing dilute acid pretreated wheat straw Qin et al. (2013) observed a higher glucose yield from post-washed liquid ammonia pretreated corn stover as compared to unwashed samples. It was suggested that in addition to lignin removal, the removal of soluble compounds including ligno-phenolic compounds was beneficial in enhancing enzymatic digestibility. Rajan and Carrier (2014) reported that the positive effect of washing on glucose yields was more pronounced when solid loadings exceeded 2%, which simply translates to higher concentration of inhibitors with increased biomass loadings.

Table 3. 2. Cellulose digestibility of unwashed and washed dilute ammonia pretreated energy cane bagasse after 72 h enzymatic hydrolysis.

Samples	Experimental Variables				Cellulose Digestibility %	
	Ctec 2 % (w/w)	Htec 2 % (w/w)	Laccase IU/g (w/w)	Tween®80 % (w/w)	Unwashed Biomass	Washed Biomass
1	20	7.50	25	7.50	81.45	92.64
2	15.38	11.25	12.50	11.25	73.71	91.80
3	6.13	11.25	37.50	3.75	67.50	87.36
4	1.50	7.50	25	7.50	47.70	84.60
5	15.38	11.25	12.50	3.75	66.69	86.88
6	6.13	11.25	12.50	11.25	56.16	86.40
7	10.75	7.50	25	0	63.99	82.92
8	10.75	7.50	25	7.50	67.68	88.08
9	15.38	3.75	12.50	11.25	79.47	90.36
10	15.38	11.25	37.50	11.25	83.88	94.20
11	10.75	7.50	25	7.50	67.95	88.08
12	6.13	3.75	12.50	3.75	58.95	83.40
13	10.75	7.50	25	15	71.10	88.20
14	6.13	11.25	12.50	3.75	67.41	85.68
15	10.75	7.50	25	7.50	67.77	87.96
16	15.38	3.75	12.50	3.75	68.04	86.40
17	10.75	7.50	25	7.50	66.60	87.96
18	6.13	3.75	37.50	11.25	49.41	82.92
19	10.75	7.50	25	7.50	66.51	87.48
20	6.13	11.25	37.50	11.25	61.74	89.76
21	10.75	15	25	7.50	71.55	89.52
22	15.38	3.75	37.50	11.25	82.53	89.88
23	6.13	3.75	12.50	11.25	51.39	83.16
24	10.75	7.50	50	7.50	66.15	88.80
25	10.75	0	25	7.50	58.50	82.68
26	15.38	3.75	37.50	3.75	64.62	85.08
27	15.38	11.25	37.50	3.75	70.92	88.08
28	10.75	7.50	25	7.50	66.69	87.84
29	6.13	3.75	37.50	3.75	49.32	82.20
30	10.75	7.50	0	7.50	65.07	87.60

Cellulase (CTec2) showed the highest correlation coefficient with cellulose digestibility of unwashed material (0.8) as compared to washed material (0.6) indicating a close to linear relationship. Analysis of variance (ANOVA) was performed to evaluate the significance of the developed models as well as the linear, quadratic and interactive effect of variables on the response (Tables 3.3 and 3.4). A quadratic model

was significant (p-value<0.0001) for unwashed and washed dilute ammonia pretreated energy cane bagasse. All models passed the lack of fit test. High determination coefficients were observed for unwashed ($R^2 = 0.99$) and for washed ($R^2 = 0.99$) pretreated biomass. This indicates the strong correlation between actual and predicted values. For both models, the predicted determination coefficient (Pred R^2) was in agreement with the adjusted determination coefficient (Adj R^2). Precision of reliability of models were confirmed by the low coefficient of variation% and high signal to noise ratio (Adeq precision), an indication that this model can be used to navigate within the design space.

Table 3. 3. Analysis of variance (ANOVA) for the response surface quadratic model for unwashed substrate.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	2496.64	14	178.33	433.06	< 0.0001	significant
A-CTec2	1592.18	1	1592.18	3866.45	< 0.0001	
B-Htec2	206.39	1	206.39	501.19	< 0.0001	
C-Laccase	4.39	1	4.39	10.65	0.0052	
D-Tween® 80	63.57	1	63.57	154.37	< 0.0001	
AB	116.64	1	116.64	283.25	< 0.0001	
AC	24.95	1	24.95	60.59	< 0.0001	
AD	340.40	1	340.40	826.63	< 0.0001	
BC	64.16	1	64.16	155.81	< 0.0001	
BD	22.33	1	22.33	54.22	< 0.0001	
CD	40.83	1	40.83	99.16	< 0.0001	
A^2	11.54	1	11.54	28.03	< 0.0001	
B^2	7.89	1	7.89	19.15	0.0005	
C^2	4.17	1	4.17	10.13	0.0062	
D^2	0.24	1	0.24	0.59	0.4561	
Residual	6.18	15	0.14			
Lack of Fit	3.96	10	0.40	0.89	0.5897	not significant
Pure Error	2.21	5	0.44			
Standard deviation	0.64		R^2	0.99		
Mean	66.02		Adj R^2	0.99		
Coefficient of variation%	0.97		Pred R^2	0.98		
PRESS	26.01		Adeq precision	79.59		

All the variables had a significant effect on the response of washed substrate ($p< 0.05$) except for laccase. However, the interactive effect of all the variables was significant ($p< 0.05$) for both unwashed and washed biomass.

Table 3. 4. Analysis of variance (ANOVA) for the response surface quadratic model for washed substrate.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	263.66	14	18.83	499.81	< 0.0001	significant
A-CTec2	95.52	1	95.52	2535.05	< 0.0001	
B-Htec2	68.14	1	68.14	1808.42	< 0.0001	
C-Laccase	2.53	1	2.53	67.28	< 0.0001	
D-Surfactant	48.05	1	48.05	1275.30	< 0.0001	
AB	4.28	1	4.28	113.72	< 0.0001	
AC	0.20	1	0.20	5.37	0.0350	
AD	16.40	1	16.40	435.31	< 0.0001	
BC	8.82	1	8.82	234.10	< 0.0001	
BD	1.51	1	1.51	40.15	< 0.0001	
CD	1.37	1	1.37	36.33	< 0.0001	
A^2	0.85	1	0.85	22.61	0.0003	
B^2	5.65	1	5.65	149.87	< 0.0001	
C^2	0.14	1	0.14	3.70	0.0738	
D^2	9.51	1	9.51	252.32	< 0.0001	
Residual	0.57	15	0.038			
Lack of Fit		10	1.04	0.62	0.7561	not significant
Pure Error		5	1.12			
Standard deviation	0.19		R^2	0.99		
Mean	87.26		Adj R^2	0.99		
Coefficient of variation%	0.22		Pred R^2	0.99		
PRESS	2.17		Adeq precision	87.68		

Design-expert 9.0.3 was used to calculate the coefficients of the second order polynomial equation for glucose digestibility of unwashed (Equation 3.3) and washed substrate (Equation 3.4).

Final equation for unwashed substrate in terms of coded factors (Equation 3.3)

Glucose yield = $+67.20 + 8.15A + 2.93B + 0.43C + 1.63D – 2.70AB + 1.25AC + 4.61AD + 2.0BC – 1.18BD + 1.60CD – 0.65A^2 – 0.54B^2 – 0.39C^2$

Final equation for washed substrate in terms of coded factors (Equation 3.4)

Glucose yield = $+87.9 + 1.99A + 1.68B + 0.32D + 1.41D – 0.52AB – 0.11AC + 1.01AD + 0.74BC + 0.31BD +0.29CD + 0.18A^2 – 0.45B^2 – 0.59D^2$

Where A: Ctec2, B: Htec2, C: Laccase, D: Tween® 80.

Equations (3.3) and (3.4) were then solved for (a) the minimum levels of independent variables required for at least 65% cellulose digestibility, and (b) no limit set for independent variables within the experiment design range to yield the highest possible cellulose digestibility (Table 3.5).

Table 3. 5 Mathematically calculated values for independent variables using polynomial Equations 3 and 4 based on the constraints and the desired response (Design-expert 9.0.3).

Samples	Constraints	CTec2	HTec2	Laccase	Tween® 80	Cellulose Digestibility%
Unwashed	Y	1.50	4.90	0	0	68.00
(Equation 3.3)	Z	19.39	12.04	46.32	10.15	84.30
Washed	Y	11.90	2.00	0	4.78	86.00
(Equation 3.4)	Z	16.90	14.17	34.64	14.86	97.10

Y: Variables set for minimum possible for the response above 65%.
Z: Variables set within the range of experiment for the highest response.

Validity of the predicted response was confirmed by conducting actual experiments at the assigned values for each variable. All the experimental results fell within the 95% confidence interval of the model predicted values. Highest cellulose digestibility values observed with minimum loading of enzymes and Tween® 80 were 68% and 86% for unwashed and washed materials, respectively. Maximum cellulose digestibility values observed with no constraints set for the variables within the range of experimental design were 84.30% and 97.10% for unwashed and washed materials, respectively.

3.3.3. Effect of xylanase

Xylanase can hydrolyze the hemicellulose (i.e., xylobiose, xylotriose) and expose more of the cellulose to cellulase. In the absence of xylanase, these compounds would otherwise bind unproductively to cellulase and reduce cellulose conversion (Jia et al., 2015). Li et al. (2014) also reported a positive correlation between xylobiose and xylotriose concentrations present in the hydrolysate and the effect of xylanase addition. The breakdown of these compounds by xylanase subsequently eliminated their inhibitory effect on cellulase and contributed to better enzymatic conversion yields (Zhang and Viikari, 2012; Qing and Wyman, 2011; Zhang et al., 2012; Ximenes et al., 2010).

Xylanase (HTec2) addition improved cellulose digestibility; however, it was more effective on the unwashed biomass (Figures 3.1 and 3.2). At 1.50% CTec2 loading (g/g glucan, dry basis), addition of HTec2 from 0 to 15% (g/g glucan, dry basis) improved cellulose digestibility of the unwashed biomass by 101.90%; whereas, for the washed biomass only a 14% increase was observed. Qin et al. (2013) observed the same trend in aqueous ammonia pretreated corn stover. Their results suggested that when there is a higher concentration of hemicellulose, lignin and their derivatives in the hydrolysate, the addition of xylanase can be more effective in improving cellulose digestibility

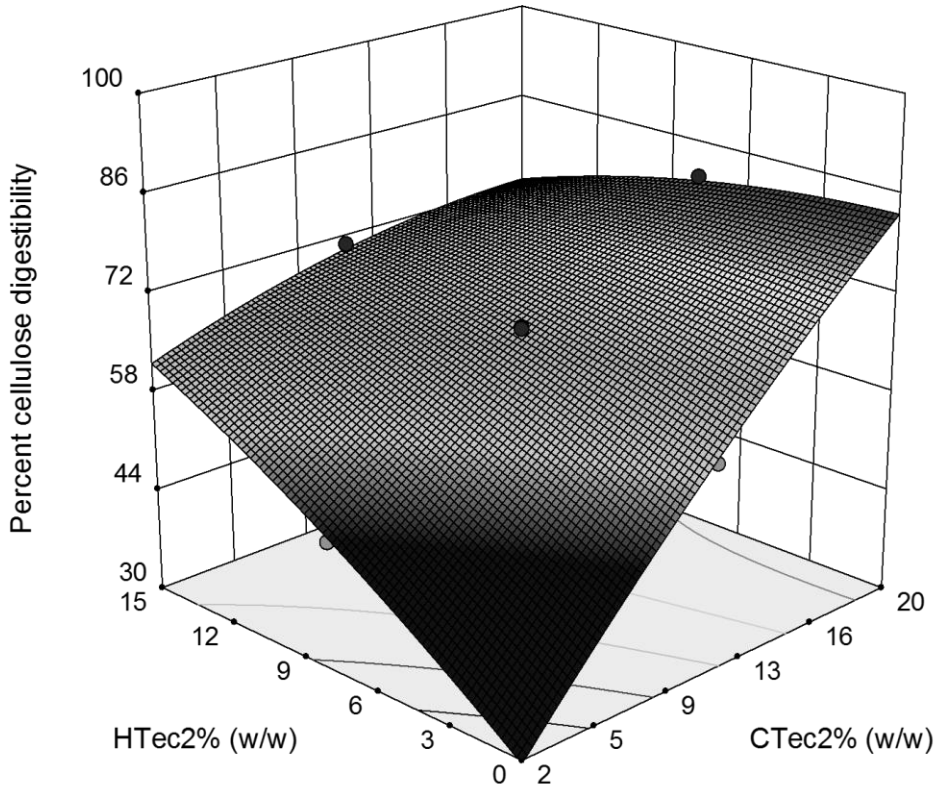

Figure 3. 1. 3D response surface plot of the effect of cellulase (CTec2) and xylanase (HTec2) on cellulose digestibility at 25 IU/g laccase loading for unwashed dilute ammonia pretreated energy cane bagasse.

We also observed that a large portion of the hemicellulose was lost during pretreatment (84.14%); therefore, what was left could not have possibly provided a strong physical barrier to cellulase. However,

studies have shown that even at low concentrations of hemicellulose, removing the remaining hemicellulose has a linear correlation with the release of glucose (Kumar and Wyman, 2009). Therefore, regardless of the hemicellulose content in the pretreated biomass, xylanase can still be added as an accessory enzyme in order to boost sugar yields (Hu et al., 2013). Hu et al. (2011) suggested that in addition to hemicellulose removal, fiber porosity and biomass swelling helped improve glucose yields. At 20% CTec2 loading, increasing HTec2 loading from 0 to 15% (g/100g glucan, dry basis) resulted in a 10.90% decrease in cellulose digestibility for unwashed samples. Our results are in agreement with those reported by Kumar and Wyman (2009).

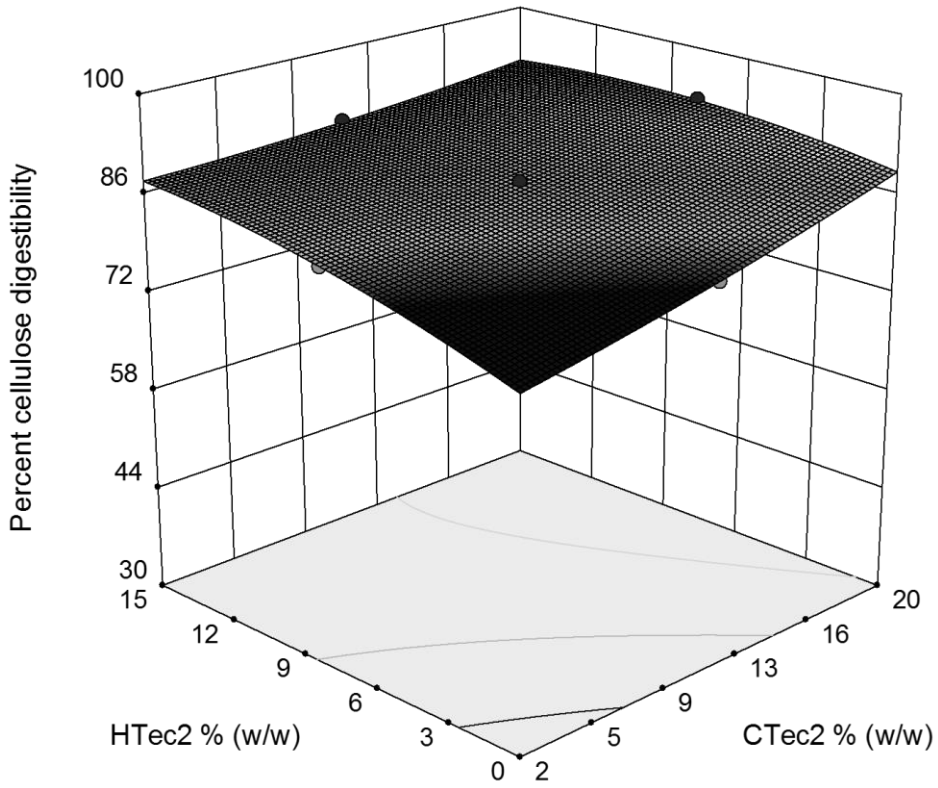

Figure 3. 2. 3D response surface plot of the effect of cellulase (CTec2) and xylanase (HTec2) on cellulose digestibility at 25 IU/g laccase loading for washed dilute ammonia pretreated energy cane bagasse.

A similar trend was also observed by Pengilly et al. (2015) for CTec2 and HTec2 when used on steam-pretreated sweet sorghum bagasse. They observed that with increasing concentrations of HTec2, HTec2 competed with CTec2 for cellulose binding sites thus resulting in a decrease in cellulose digestibility. Sun et al. (2015) suggested that by increasing the loading of accessory enzymes (β-glucosidase, xylanase and lytic polysaccharide monooxygenases) the synergistic effect shifts to co-hydrolysis. Qin et al. (2013) observed that a xylanase loading of more than 12.50 mg protein/ g glucan had no significant effect on cellulose digestibility. It is clear that there is an optimum loading point for xylanase addition. Therefore, effective loadings of xylanase should be considered based on the type of biomass, pretreatment method and enzymatic hydrolysis conditions used, such as cellulase loadings and the use of accessory enzymes.

3.3.4. Effect of laccase

Cellulase activity can be reduced in the presence of phenolic compounds which are derived from the degradation of lignin (Kim et al., 2013). As a result, removing phenolic compounds generated during pretreatment can enhance enzymatic hydrolysis and boost sugar yields of pretreated biomass (Moreno et al., 2012). Laccase (usually used in biological delignification) can potentially act as a detoxifier by oxidizing phenolic compounds but it has no effect on other inhibitory compounds including weak acids and furan derivatives (Moreno et al., 2012; Van Dyk and Pletschke, 2012) Laccase had a significant effect on the response for washed and unwashed biomass ($p<0.05$). The interaction effect of laccase and other variables (cellulase, xylanase and Tween® 80) was also significant ($p<0.05$) for both washed and unwashed materials (Figures 3.3 and 3.4). Addition of laccase from 0 to 50 IU/g biomass at lower cellulase loadings (1.50% CTec2) caused a 13% reduction in cellulose digestibility in unwashed biomass. This can be attributed to the inhibitory effect of laccase on β-glucosidase activity. Oliva-Taravilla et al. (2015) observed a 10% reduction in enzymatic digestibility of wheat straw when laccase was loaded above 10 IU/g substrate. Oliva-Taravilla et al. (2015) also reported that addition of 2 IU/ml laccase decreased the

activity of β-glucosidase by 7%. In our samples, the presence of inhibitory compounds in the unwashed substrate (as compare to the washed substare) enhanced the negative effect of laccase on β-glucosidase activity. In addition to β-glucosidase inhibition at high laccase loadings, laccase competes with cellulase over the binding sites of cellulose (Oliva-Taravilla et al., 2015). We observed that the addition of laccase at higher cellulase loadings (20% CTec2) resulted in a slight improvement in glucose yields. The slight increase can be attributed to the oxidation of lignin-derived compounds and phenolic compounds by laccase. This explains the higher improvement observed in the unwashed substrate (9.10%) as compared to the washed substrate (1.20%) as more phenolic compounds are expected to be present in the unwashed biomass that could be oxidized and eliminated by laccase. Also, a higher cellulase loading translates into a higher concentration of β-glucosidase in the hydrolysate; therefore, cellulose digestibility is less affected by the binding of laccase to β-glucosidase.

Lignin from different plants with different chemical structures respond differently to laccase treatment. Moilanen et al. (2011) reported a 12% increase in enzymatic hydrolysis of steam pretreated spruce after laccase treatment; however, the same laccase treatment caused a 17% decrease in enzymatic hydrolysis of steam pretreated giant reed. They found that laccase-modified spruce had a higher binding affinity to cellulase while the opposite was observed for laccase-treated reed. Moreno et al. (2012) used laccase treatment to detoxify steam explosion pretreated wheat straw and reported a slight decrease in glucose recovery when the laccase treatment was performed before enzymatic hydrolysis. The opposite was reported by Qiu and Chen (2012).They found that enzymatic digestion of steam explosion pretreated wheat straw was increased either when laccase treatment took place before or was paired simultaneously with enzymatic hydrolysis.

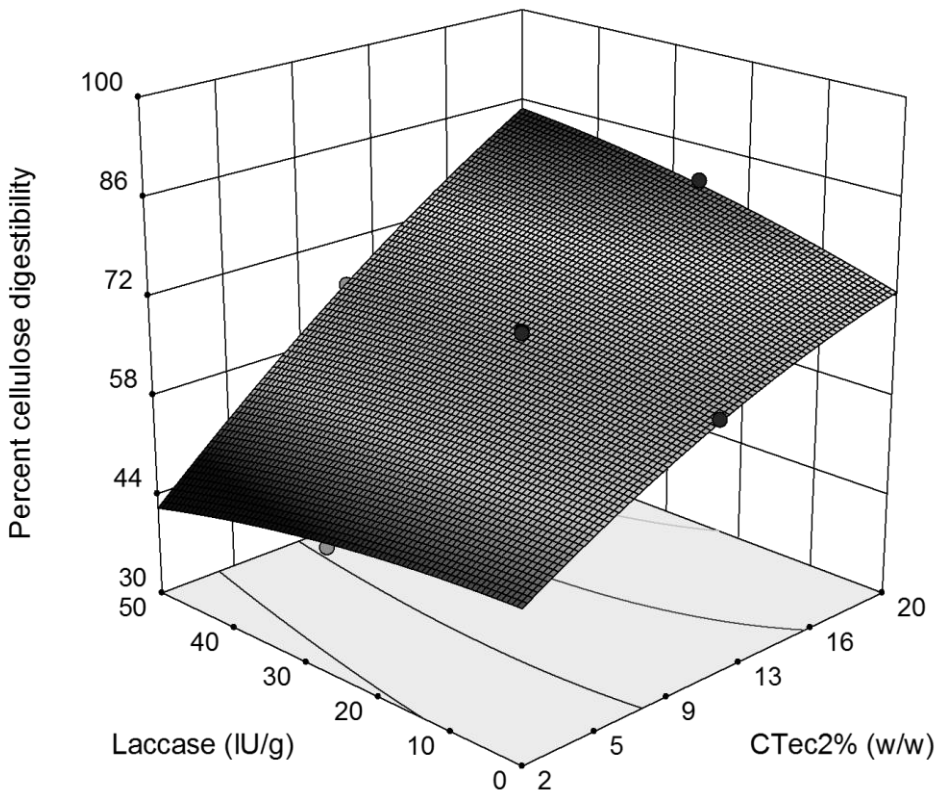

Figure 3. 3. 3D response surface plot of the effects of cellulase (CTec2) and laccase on cellulose digestibility at 7.50% xylanase (HTec2) loading for unwashed dilute ammonia pretreated energy cane bagasse.

Laccase treatment of steam-exploded wheat straw reduced the concentration of total phenolics in the slurry by more than 50%; however, because of the negative effect of laccase on glucose recovery, the treatment was found to be beneficial only at high solid loadings (more than 20%). In general, based on the chemical structure of lignin, laccase treatment can breakdown lignin into different compounds that might enhance their cellulase-blocking activity instead of reducing it (Moilanen et al., 2011). According to Tejirian and Xu (2011) oligomeric phenolics released after laccase treatment had a stronger inhibitory effect on cellulase than simple phenolics. In addition to lignin removal and oxidation of phenolic compounds, modification of the lignin surface with laccase could reduce non-productive binding of lignin

to cellulase (Moilanen et al., 2011). Laccase treatment can also increase cellulose accessibility by micropores formation through the oxidization of the aromatic rings present in lignin (Qiu and Chen, 2012).

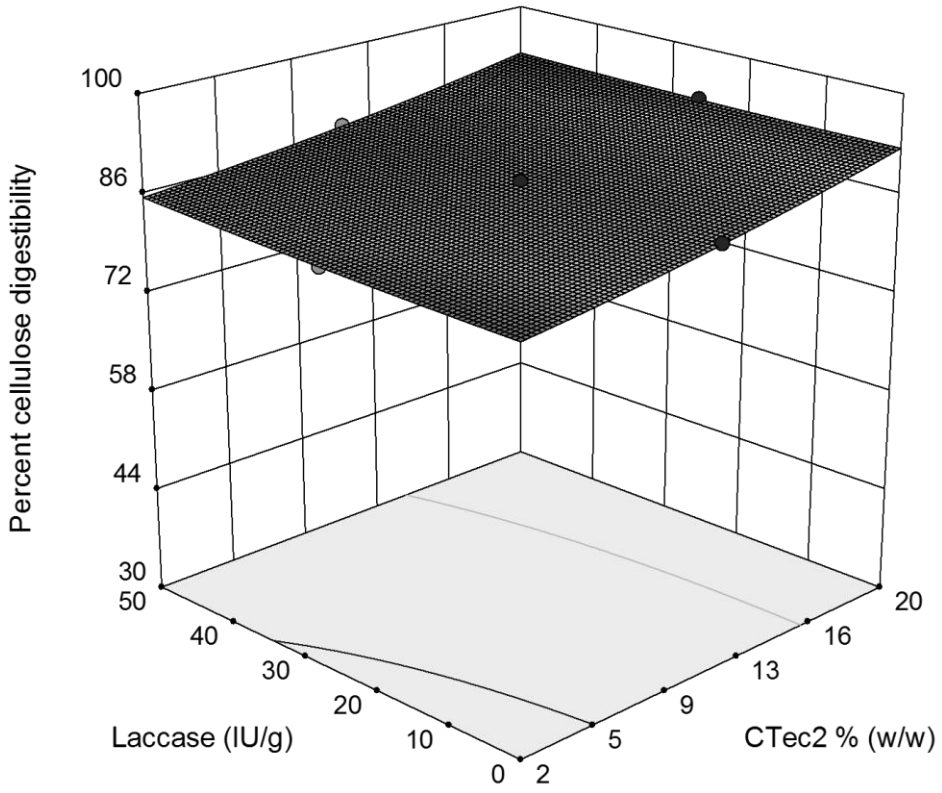

Figure 3. 4. 3D response surface plot of the effects of cellulase (CTec2) and laccase on cellulose digestibility at 7.50% xylanase (HTec2) loading for washed dilute ammonia pretreated energy cane bagasse.

3.3.5. Effect of surfactant

Tween® 80 had a significant effect on the response for washed and unwashed substrate ($p < 0.05$). The interactive effect of Tween® 80 with other variables was also significant as reported in Tables 3.3 and 3.4. Cellulose digestibility improved in both washed and unwashed biomass by the addition of Tween® 80, with the effect being more significant in the unwashed material (Figures 3.5 and 3.6). Surfactants can improve cellulose digestibility by lowering the irreversible binding of enzymes after enzyme-substrate formation, by modifying the substrate structure and by expanding the surface area of

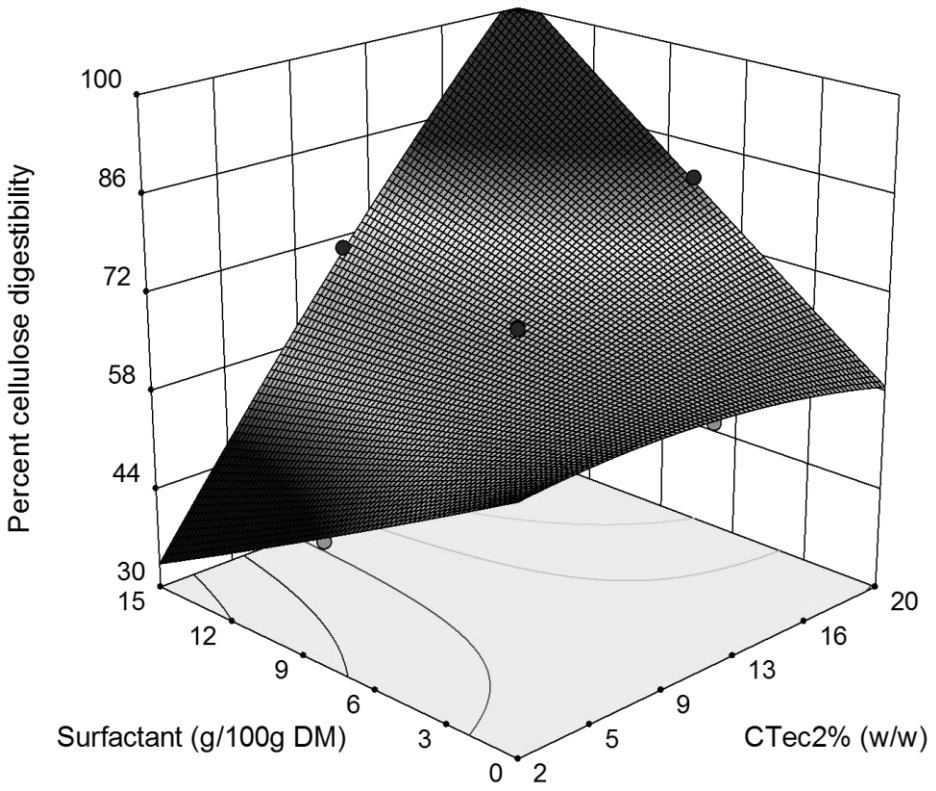

Figure 3. 5. 3D response surface plot of the effects of cellulase (CTec2) and surfactant (Tween®□80) on cellulose digestibility at 25 (IU/g) laccase loading for unwashed dilute ammonia pretreated energy cane bagasse.

the substrate (Kristensen et al., 2007; Menegol et al., 2014; Yang et al., 2011; Sun and Cheng, 2002). Furthermore, surfactants can increase the adsorption of enzymes on the enzyme-substrate interface by reducing the interfacial tension between enzyme and substrate and by increasing substrate hydrophilicity (Menegol et al., 2014). Kristensen et al. (2007) indicated that the hydrophobic part of the surfactant binds to the hydrophobic sites of the lignin and phenolic compounds, while the hydrophilic portion of the surfactant interacts with the solution and wards off enzymes from the surface of lignin. This steric repulsion would prevent unproductive binding of lignin to cellulase. Liu et al. (2011) found that Tween® 80 (above its critical micelle formation (CMC)) makes strong interactions with the enzyme and forms a micelle through which both enzyme activity and stability are preserved. This is especially important in

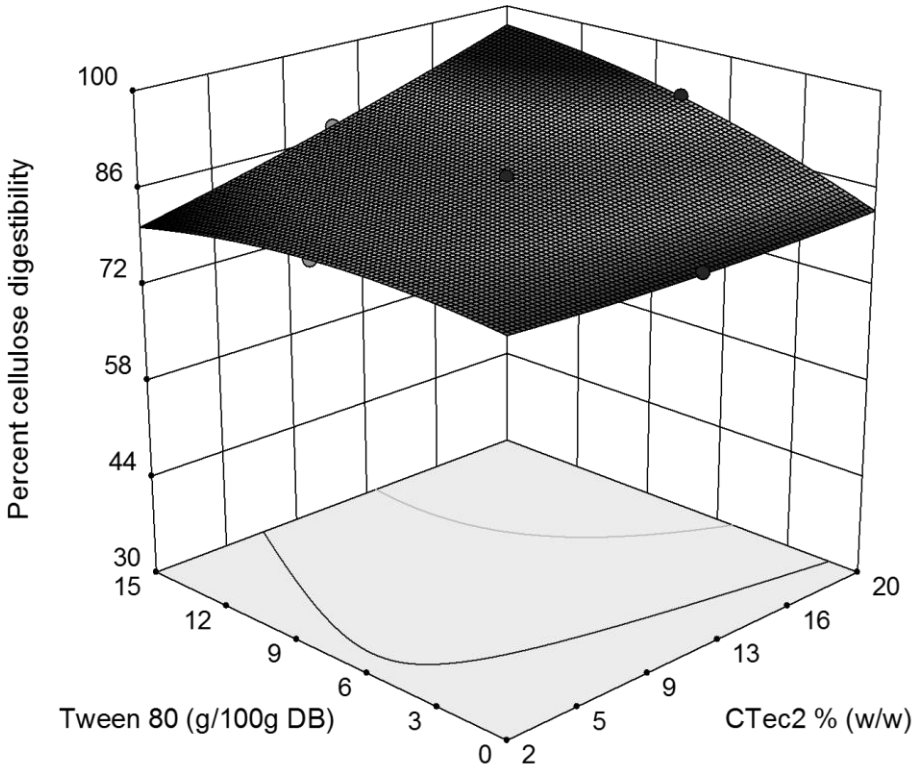

Figure 3. 6. 3D response surface plot of the effects of cellulase (CTec2) and surfactant (Tween® 80) on cellulose digestibility at 25 (IU/g) laccase loading for washed dilute ammonia pretreated energy cane bagasse.

unwashed biomass where the concentration of inhibitory compounds is high. Our results indicated that combining high concentrations of Tween® 80 (15% w/w) and high CTec2 loadings (20% w/w) worked best in improving cellulose digestibility. Addition of Tween® 80 at low and high CTec2 loadings resulted in different responses in cellulose digestibility. For example, at 20% CTec2 loading (highest cellulase loading), increasing the concentration of Tween® 80 from 3.80% to 11.30% resulted in an increase in cellulose digestibility by 36.20% and 7.80% for unwashed and washed biomass, respectively. Interestingly, the same increase in Tween® 80 concentration at 1.5% CTec2 loading (lowest cellulase loading), resulted in a 25.03% and 1.20 % decrease in cellulose digestibility in unwashed and washed material, respectively. At any constant concentration of Tween® 80, increasing the cellulase loading

improved the digestibility of cellulose. This improvement was more prominent at 15% Tween® 80 where increasing cellulase loading from 1.50% to 20% resulted in a 128% and 15% increase in cellulose digestibility in unwashed and washed biomass, respectively. Inconsistencies on the effect of non-ionic surfactants (including Tween® 80) on enzymatic hydrolysis yields have been reported by Zhou et al. (2015). They observed that high concentrations of surfactants enhanced enzymatic hydrolysis of dilute acid pretreated substrate; however, the same high concentrations posed inhibitory effects on the hydrolysis of pure cellulose.

3.3.6. Mass balance for optimized cellulase, accessory enzymes and Tween® 80

Mass balance of the optimized process for cellulose, hemicellulose and lignin was calculated based on an initial 110 g dry weight of untreated biomass for washed and unwashed dilute ammonia pretreated energy cane bagasse (Table 3.6). Optimum enzymatic hydrolysis conditions for unwashed substrate were 19.39% CTec2, 12.04% HTec2, 46.32 IU/g Laccase, and 10.15% Tween® 80. Optimum enzymatic hydrolysis conditions for washed substrate were 16.90% CTec2, 14.17% HTec2, 34.64 IU/g Laccase, and 14.86% Tween® 80.

Untreated energy cane bagasse contained 40.14% cellulose, 24.22% hemicellulose and 24.41% lignin. The remaining 11.23% contained mostly ash, proteins and organic acids. Pretreatment with liquid ammonium hydroxide resulted in a 62.51% solid recovery. High hemicellulose solubilization (84.14%) and effective lignin removal (60.26%) contributed to the amount of recovered solids. Considering both solid and liquid fractions, there was a mass loss of 18.95% for the total combined amounts of glucose, xylose and lignin in the unwashed biomass as compared to 19.26% in the washed biomass after pretreatment.

Table 3. 6. Mass balance for washed and unwashed dilute ammonia pretreated energy cane bagasse and hydrolysates.

Component	Untreated Energy Cane Bagasse (110 g)	Pretreatment*			Enzymatic Hydrolysis**			Digestibility %
		Liquid Fraction (g)	Solid Fraction (g)	Total (g)	Liquid Fraction (g)	Solid Fraction (g)	Total (g)	
Unwashed Biomass								
Glucose (g)	49.06	2.41	44.64	47.05	37.63	6.32	43.95	84.30
Xylose (g)	30.26	18.90	4.79	23.69	4.22	0.42	4.64	88.10
Lignin (g)	26.85	4.63	10.68	15.31	ND	9.54	9.54	
Total (g)	106.17		60.11	86.05			58.13	
Loss (%)				18.95			3.29	
Washed Biomass								
Glucose (g)	49.06	2.85	44.29	47.14	43	0.59	43.59	97.10
Xylose (g)	30.26	19.45	4.18	23.63	4.10	ND	4.10	100
Lignin (g)	26.85	5.05	9.90	14.95	ND	9.17	9.17	
Total (g)	106.17		58.37	85.72			56.86	
Loss (%)				19.26			2.6	

All reported weights are on dry basis.
ND: Not detectable.
*= Pretreatment conditions: 208 °C, 36 min, ammonium hydroxide to biomass to water ratio of 0.4:1:20.
**= Enzymatic hydrolysis conditions for unwashed substrate: CTec2:19.39%, HTec2: 12.04%, Laccase: 46.32 IU/g, Tween®80:10.15%.
**= Enzymatic hydrolysis conditions for washed substrate: CTec2:16.90%, HTec2: 14.17%, Laccase: 34.64 IU/g, Tween®80:14.86%.

The mass losses observed can be attributed to the evaporation of volatile compounds and the formation of compounds such as organic acids, furans and phenolic compounds from the thermal degradation of cellulose, hemicellulose and lignin in the presence of ammonium hydroxide. Relatively lower mass losses were observed following the enzymatic hydrolysis of unwashed (3.29%) and washed (2.60%) dilute ammonia pretreated energy cane bagasse. This can be attributed to the depolymerization and solubilization of lignin by laccase.

3.4. Conclusion

The use of enzymes (cellulase, xylanase and laccase) and a surfactant (Tween® 80) had a significant effect on the cellulose digestibility of unwashed and washed dilute ammonia pretreated energy cane bagasse. Optimum values for each of these parameters were calculated statistically and confirmed experimentally. Highest cellulose digestibility values observed for unwashed and washed biomass were 84.30% and 97.10%, respectively. The addition of accessory enzymes and Tween® 80 had a more significant effect on improving the cellulose digestibility of the unwashed material over the washed material. Increases in cellulose digestibilities by 75.85% for the unwashed biomass and by 12.74% for the washed biomass were observed. Optimum enzymatic hydrolysis conditions for unwashed substrate were 19.39% CTec2, 12.04% HTec2, 46.32 IU/g Laccase, and 10.15% Tween® 80. Optimum enzymatic hydrolysis conditions for washed substrate were 16.90% CTec2, 14.17% HTec2, 34.64 IU/g Laccase, and 14.86% Tween® 80.

The addition of accessory enzymes (xylanase, laccase) and Tween® 80 during enzymatic hydrolysis of dilute ammonia pretreated energy cane bagasse can substitute the need for washing the pretreated biomass for the removal of inhibitory compounds prior to enzymatic hydrolysis. However, additional research is needed on the synergistic effect of various other accessory enzymes and their interaction with surfactants in order to find the most efficient enzyme cocktail tailored for a particular biomass and pretreatment technology.

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CHAPTER 4

EVALUATION OF IMIDAZOLIUM-BASED IONIC LIQUIDS FOR THE REMOVAL OF NON-SUGAR BY-PRODUCTS FROM ENZYMATICALLY HYDROLYZED DILUTE AMMONIA PRETREATED ENERGY CANE BAGASSE

4.1. Introduction

Lignocellulosic biomass is an abundant and renewable resource for the production of bio-based fuels and chemicals (Cherubini, 2010). Lignocellulosic material is mainly composed of cellulose, hemicellulose and lignin. Among lignocellulosic biomass, energy cane has some promising features that makes it a potential energy crop. It is a cross breed between commercial and wild sugarcane with higher fiber content, lower water input requirement, higher cold resistance, and higher biomass yield as compared to sugarcane (Kim and Day, 2011). In order to utilize the lignocellulosic material for the production of bio-based products, cellulose and hemicellulose must be accessible to hydrolyzing enzymes. However, the recalcitrant nature of cellulose, which is guarded by hemicellulose and lignin, hinders its enzymatic conversion (Aita et al., 2011b). This puts emphasis on the value of developing an effective pretreatment to remove the lignin and solubilize the hemicellulose while the maximum amount of sugars is preserved (Kumar et al., 2009). Among all types of pretreatments, ammonia-based pretreatments allow for the vast removal of lignin and for the preservation of most of the cellulose (Aita et al., 2011b). Regardless of the important role pretreatment plays in improving enzymatic hydrolysis by breaking down the structure of lignocellulosic biomass, harsh pretreatment conditions can promote the degradation of the lignocellulosic components. This results in the generation of compounds with inhibitory effects on enzyme activity and microbial growth (Jönsson and Martín, 2016). The nature and concentration of these pretreatment by-products or non-sugar compounds are defined by the type of biomass and its chemical composition as well as the type and severity of the pretreatment itself (Mitchell et al., 2014; Ko et al., 2015). Under severe pretreatment conditions, degradation of pentose and hexose sugars results in the formation of 2-furaldehyde (furfural)

and 5-hydroxymethyl-2-furaldehyde (5-HMF), respectively (Wang et al., 2015). Furfural can be further degraded into formic and levulinic acids, while 5-HMF into formic acid (Jönsson and Martín, 2016). The presence of furfural and 5-HMF, at concentrations as low as 1 g/L, can inhibit the growth of microorganisms during fermentation (Carter et al., 2011). Other non-sugar compounds include acetic acid which is generated from the hydrolysis of acetyl groups found in the hemicellulose. Phenolic compounds (gallic, 4-hydroxybenzoic, chlorogenic, vanillic, caffeic, syringic, coumaric, sinapic and trans-cinnamic) are another group of by-products which are released from the partial degradation of lignin (Jönsson and Martín, 2016). Under oxidative conditions, phenolic compounds can further oxidize into organic acids (Klinke et al., 2002). In addition to pretreatment, enzymatic hydrolysis also contributes to the formation and the release of trapped lignocellulosic oligosaccharides, aliphatic and organic acids as well as furan derivatives and phenolic compounds (Martín et al., 2002; García-Aparicio et al., 2006; Gurram et al., 2011).

The use of solvents or chemicals is the preferred option for the removal of the above mentioned non-sugar compounds from hydrolysates (Jönsson and Martín, 2016; Jönsson et al., 2013a). Some of these methods include chemical neutralization, overliming and the use of polymers, flocculants, ion exchange resins, and activated charcoal (Kamal et al., 2011; Jönsson et al., 2013b; Mussatto and Roberto, 2004; Palmqvist and Hahn-Hägerdal, 2000). Once recovered, these non-sugar compounds can be used as building blocks for the production of value-added products including biopolymers, biochemicals and pharmaceuticals, a sustainable alternative from petroleum-derived chemicals (Carter et al., 2011; Ranjan et al., 2009).

Ionic liquids are salts that stay in the liquid form at ambient temperatures due to their asymmetrically packed ions, have low vapor pressure and can solubilize most organic and inorganic compounds (Liu et al., 2005). These features make them great candidates for liquid-liquid extractions as substitution for

conventional solvents. Imidazolium-based ionic liquids (1-Methyl-3-octylimidazolium hexafluorophosphate [OMIM][PF_6], 1-Butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6], 1-Methyl-3-octylimidazolium tetrafluoroborate [OMIM][BF_4], 1-hexyl-3-methylimidazolium tetrafluoroborate [HMIM][PF_4], 1-Methyl-3-octylimidazolium bis (trifluoromethylsulfonyl) imide [OMIM][NF_2], and 1-Butyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide [BMIM][NF_2]) are stable in air and water and are not miscible with water, are non-toxic, non-corrosive, and have a relatively low viscosity. They have three different anions and two cations with different alkyl chains available for each of the anions. The presence of cations, anions and alkyl chains allow for their difference in hydrophobicity. Ionic liquids have been mostly used during pretreatment for the purpose of fractionating the biomass into cellulose, hemicellulose and lignin (Brandt et al., 2013; Carvalho et al., 2015; Qiu et al., 2012; Qu et al., 2016; Shill et al., 2011; Zhang et al., 2015). They have also been evaluated as catalysts to decrease the reaction temperature during pyrolysis. One of the major concerns of working with ionic liquids is their high cost as compared to conventional solvents; however, ionic liquid recyclability compensates for the cost-related challenge. It also addresses the environmental concerns often attributed with using traditional solvents (Fauzi and Amin, 2012). Furthermore, the feasibility of recovering ionic liquids can be different based on their chemical properties. For example, recovery of hydrophobic ionic liquids is more feasible than hydrophilic ones due to their immiscibility with water (Wu et al., 2009).

To date, no work has been done to assess the extractability of non-sugar compounds using ionic liquids as solvents from enzymatic hydrolysates of energy cane bagasse. The ideal ionic liquid should have a high selectivity and partition coefficient for all the main non-sugar compounds (formic acid, acetic acid, furfural, 5-HMF, and phenolic compounds) found in the hydrolysates. Regeneration of the ionic liquid itself as well as the recovery of the extracted compounds should be feasible. Nonetheless, ionic liquids should not remove or hydrolyze any sugars from the aqueous phase as sugar loss should be minimized. In

this study, the effect of six imidazolium-based ionic liquids on the removal and recovery of non-sugar compounds from dilute ammonia pretreated energy cane bagasse hydrolysates was investigated.

4.2. Material and Methods

4.2.1. Biomass

Energy cane non-commercial variety (HO 02-113) was bred in Houma, LA through the collaboration between the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) in Houma, LA and the Louisiana State University Agricultural Center Sugar Research Station in St. Gabriel, LA. Harvested energy cane was passed through a roller press (Farrel Corporation, Ansonia, CT) to remove the juice. The left over solid material is referred to as bagasse. Bagasse was dried in an oven at 45 °C to a final moisture content of almost 10%. Partially dried bagasse was milled (Wiley Mill, Arthur Thomas Co, PA) and sieved (2 mm mesh sieve) prior to storage at -20 °C until further use.

4.2.2. Pretreatment and composition analysis

Energy cane bagasse was pretreated in a 4L stirrer reactor (Autoclave Engineers, Erie, PA) at 208 °C, for 36 min, and at an ammonium hydroxide (28% v/v NH_4OH solution, Fisher Scientific, Pittsburgh, PA) to biomass to water ratio of 0.4:1:20. The pretreatment conditions used in this study had been previously optimized for maximum sugar yields using Response Surface Methodology (RSM) and the software Design-Expert 9.0.3 (State Ease Inc., Minneapolis, MN). The pretreated slurry was pressed to separate the liquid and solid fractions. Half of the solid material was washed with 6 volume deionized water (Samples 1 and 2) and the other half was kept unwashed (Samples 3 and 4). Both parts were oven dried at 45 °C to reduce the moisture content below 10%. Composition analysis of energy cane bagasse was performed for untreated and pretreated biomass following NREL's Laboratory Analytical Procedures (LAP TP-510-42618, 42619, 42622). NREL reference material 8491 (for sugarcane bagasse) was analyzed as an internal sample to ensure the accuracy of the procedures. All the experiments were run in duplicate.

4.2.3. Enzymatic hydrolysis

Cellulase (Cellic® CTec2) and xylanase (Cellic® HTec2) were provided by Novozymes (Novozymes A/S, Bagasvaerd, Denmark). Cellulase activity of CTec2 (132 FPU mL^{-1}) and HTec2 (90.75 FPU mL^{-1}), and β-glucosidase activity for CTec2 (3229 IU mL^{-1}) and HTec2 (12.61 IU mL^{-1}) were measured according to the Ghose method (Ghose, 1987) Xylanase activity of Ctec2 (12100 IU mL^{-1}) and Htec2 (56045 IU mL^{-1}) were determined following the Bailey method (Bailey et al., 1992). Tween® 80 was added to improve cellulose digestibility and enzyme stability. Laccase was added to decrease the inhibitory effect of lignin-degraded compounds. Laccase from *Rhus vernicifera* were purchased from Sigma (Sigma–Aldrich, Inc., St. Luis, MO, USA). Laccase activity (50U mL^{-1}) was measured using syringaldazine as substrate as described by Ride (Ride, 1980). Enzymatic hydrolysis of samples were performed at 8% w/w solid loading (dry based) in a 0.1 M sodium citrate buffer solution. The final pH was adjusted to 4.8 with concentrated hydrochloric acid. The enzymatic hydrolysis conditions used are presented in Table 4.1. The amount of CTec2, HTec2, Tween® 80, and laccase used were previously optimized for maximum sugar yields using RSM and the software Design-Expert 9.0.3. Two different criteria were considered in optimization of our enzymatic hydrolysis parameters for each group of washed and unwashed samples. First, the minimum values of enzymatic hydrolysis parameters that still yield a cellulose digestibility above 65% were selected (Samples 1 and 3). Second, the values were selected with no constraints for highest cellulose digestibility (Samples 2 and 4).

Flasks were placed in a shaker incubator (Amerex Instruments Inc., Lafayette, CA) for 72 h at 50 °C and at 180 rpm. Samples were collected at 0 h (before the addition of enzymes), 24 h, 48 h, and 72 h and kept at -20 °C until further analysis. All collected samples were centrifuged at 10,000 rpm (Spectrafuge 24D, Labnet International Inc., Woodbridge, NJ) for 5 min and filtered (0.2 μm Syringe Filters, Environmental Express Inc., Mt. Pleasant, SC). Samples were diluted accordingly and analyzed for sugars,

organic acids, HMF, and furfurals by high performance liquid chromatography (HPLC) and total phenolics by Alvira et al. (2013). Experiments were done in duplicate and mean values presented.

Table 4. 1. Enzymatic hydrolysis conditions for washed and unwashed dilute ammonia pretreated energy cane bagasse.

Samples		CTec2 % w/w glucan	HTec2 %w/w glucan	Tween® 80 % w/w biomass	Laccase IU g^{-1} (dry biomass)
Unwashed Dilute Ammonia Pretreated Bagasse-Enzyme Hydrolysate	Sample 1[a]	12.10	1.90	4.89	0
	Sample 2[b]	19.53	8.30	22.34	11.10
Post-Washed Dilute Ammonia Pretreated Bagasse-Enzyme Hydrolysate	Sample 3[a]	1.50	3.90	0	0
	Sample 4[b]	15.37	5.99	22.17	14.30

[a] Variables set for cellulose digestibility above 65%.
[b] Variables set for maximum cellulose digestibility.

4.2.4. Ionic liquid solvent extraction

Ionic liquids were purchased from Iolitec (Tuscaloosa, AL) and included 1-Methyl-3-octylimidazolium hexafluorophosphate ([OMIM][PF_6]), 1-Butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]), 1-Methyl-3-octylimidazolium tetrafluoroborate ([OMIM][BF_4]), 1-hexyl-3-methylimidazolium tetrafluoroborate ([HMIM][PF_4]), 1-Methyl-3-octylimidazolium bis (trifluoromethylsulfonyl) imide ([OMIM][NF_2]), and 1-Butyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide ([BMIM][NF_2]). Ionic liquid to hydrolysate ratios (v/v) of 1:3, 1:2 and 1:1 were evaluated. The ionic liquid to hydrolysate ratio of 1:1 was chosen for further experiments as the

other two ratios showed poor extraction results (data not shown). Enzymatic hydrolysate samples of unwashed dilute ammonia pretreated bagasse (only) were centrifuged and filtered (0.2 µm) followed by the separate addition of equal volumes of each ionic liquid. Hydrolysates from washed dilute ammonia pretreated energy cane bagasse were not used during the detoxification studies with ionic liquids because of the low concentrations of non-sugar compounds present. Samples were stirred vigorously for 5, 10, 15, and 20 min and centrifuged for 5 min at 10,000 rpm to separate the two layers. A clear line was formed between the two layers with the hydrolysate remaining on the top layer and the ionic liquid at the bottom layer. Approximately, 1 ml of the top layer (aqueous layer) was pipetted out and used for further analysis of sugars and non-sugar compounds. The bottom layer (ionic liquid layer) was used to study the recovery and reusability of ionic liquids. Experiments were done in triplicate at room temperature without pH adjustment. Collected samples were analyzed for sugars, organic acids, HMF, and furfurals by HPLC and total phenolics by Alvira et al. (2013).

4.2.5. Regeneration of ionic liquids

Regeneration of ionic liquids was done to assess their reusability. Based on the method published by Fan et al. (2008), ionic liquids were mixed with 0.1 M NaOH solution (stripping solution) for 30 min. This step was repeated three times. Recovered ionic liquids were washed with distilled water to remove any remaining sodium hydroxide solution. Washed ionic liquids were oven dried at 75 °C for two days and re-used.

4.2.6. Analytical methods

Collected samples were analyzed for sugars (glucose, xylose, arabinose, mannose) by HPLC (Agilent 1200 Series) with a BioRad Aminex HPX-P87P (PI), lead form, 300 mm × 7.8 mm (ID), 9 µm column at 80°C and a differential Refractive Index Detector (G1362A Agilent). The eluent was deionized HPLC water at a flow rate of 0.8 mL/min, and 20µL injection volume. Simultaneous analysis of organic acids,

furfural and 5-HMF were performed using HPLC (Agilent 1200 series) with a Shimadzu VP-ODS column (250 mm x 4.6 mm I.D., Shimadzu, Kyoto, Japan) and a guard column (GVP-ODS, 10 mm x 4.6 mm ID). Two eluents were used. The first eluent was a sulfuric acid solution at pH 2.5 and the second eluent was methanol. Flow rate was maintained at 0.35 ml/min. The Diode Array Detector (G1315B Agilent) was set at 210 nm (UV spectra: 200 nm to 600 nm range) and the column temperature at 40 °C. Sample injection was set at 20µL. Total phenolic content of the samples was measured following a slightly modified version of the Folin-Ciocalteau method by Alvira et al. (2013). Extraction efficiency (E) was calculated using Equation 4.1. All the values are the mean of three replicate with standard deviation.

$$E = = \frac{C_o - C_e}{C_o} \times 100 \qquad \text{Equation (4.1)}$$

Where C_o and C_e (mg/l) are the initial and equilibrated concentrations of the inhibitor ion in the aqueous phase, respectively.

4.3. Results and Discussion

4.3.1. Chemical composition of biomass

Energy cane bagasse was composed of approximately 40.14 ±0.16% glucan, 24.23 ± 0.51% xylan, 2.76 ± 0.04% arabinan, 24.41 ± 0.37% lignin (5.97 ± 0.16 acid soluble, 18.44 ± 0.21 acid insoluble), 3.76 ± 0.62 extractives, and 4.70 ± 0.04% ash (dry basis). Results were comparable to those published by Aita et al. (2011a) and Qiu et al. (2012). Liquid ammonia pretreatment removed 84.14% of the hemicellulose along with 60.23% of the lignin. Only a 9.01% glucan loss was observed. Approximately, 62.51% of the total solids were recovered after pretreatment.

4.3.2. Composition analysis of hydrolysate

Composition analysis of dilute ammonia pretreated energy cane bagasse hydrolysates after 72 h enzymatic digestion is presented in Table 4.2. Washing of dilute ammonia pretreated energy cane bagasse prior to enzymatic hydrolysis removed almost all of the non-sugar compounds (formic acid, acetic acid,

furfural, 5-HMF, phenolic compounds) present in the biomass. Therefore, less concentrations of these compounds were present in the hydrolysate of washed biomass as compared to unwashed biomass. A similar effect of washing on the final concentration of non-sugar compounds was observed by Toquero and Bolado (2014). In addition to washing, the differences observed in the concentration of non-sugar compounds can be attributed to the differences in their enzymatic hydrolysis conditions. For example, hydrolysate samples with laccase in their enzyme cocktail (Samples 2 and 4) contained less concentrations of phenolic compounds. Especially in unwashed substrates, the addition of laccase allowed for the removal of 68.07% of the phenolic compounds. Laccase treatment has been studied as a lignin removal treatment as well as a detoxification strategy after pretreatment. Laccase is known to oxidize phenolic compounds. Further polymerization of these oxidized compounds will produce less soluble and non-toxic chemicals (Jurado et al., 2009). Ludwig et al. (2013) was able to remove 82% of the phenolic compounds from the slurry of organo-solvent (ethanol) processed wheat straw using immobilized laccase. Oliva-Taravilla et al. (2015) also observed that laccase treatment of steam-exploded wheat straw caused an 80% drop in the concentration of phenolic compounds after 24 h hydrolysis. Moreno et al. (2012) used laccase treatment as a biological detoxification method and reported a significant removal in phenolic compounds; however, the treatment was not effective on weak organic acids and furan derivatives. Addition of laccase in the enzymatic hydrolysate of dilute ammonia pretreated energy cane bagasse had no effect on the removal of organic acids. This is in agreement with what was reported by Chandel et al. (2007). They observed that laccase treatment of hydrolysate from acid treated sugarcane bagasse reduced the total phenolic compounds by 77.50% while acetic acid and furan concentrations remained intact. The same ineffectiveness of laccase treatment on the removal of furfural and 5-HMF was observed in our study. However, the addition of laccase during enzymatic hydrolysis reduced the concentration of phenolic compounds in the hydrolysate thus assisting during the detoxification step with ionic liquids.

Table 4. 2. Composition analysis of dilute ammonia pretreated energy cane bagasse hydrolysates.

Samples		Glucose (g/L)	Xylose (g/L)	Formic Acid (g/L)	Acetic Acid (g/L)	Furfural (g/L)	5-HMF (g/L)	Total Phenolic Compounds (g/L)
Unwashed Dilute Ammonia Pretreated Bagasse-Enzyme Hydrolysate	Sample 1[a]	30.35±0.14	3.70±0.04	3.17±0.66	4.26±0.98	1.63±0.14	0.76±0.07	6.42±0.22
	Sample 2[b]	37.63±0.44	3.41±0.84	3.66±0.51	4.41±0.12	1.75±0.03	0.81±0.34	2.05±0.17
Post-Washed Dilute Ammonia Pretreated Bagasse-Enzyme Hydrolysate	Sample 3[a]	38.09±0.08	3.70±0.11	0.18±0.62	ND	ND	ND	0.43±0.01
	Sample 4[b]	43.00±0.95	3.42±0.62	ND	0.29±0.00	ND	ND	ND

[a] Variables set for cellulose digestibility above 65%.
[b] Variables set for maximum cellulose digestibility.
ND: None detected.

Tween® 80 had no effect in removing organic acids, furans or phenolic compounds from the hydrolysates. Oliva-Taravilla et al. (2015) reported similar observations with polyethylene glycol (a non-ionic surfactant). However, it was shown that due to the surface activity of Tween® 80 its presence improved fermentation yields by sequestering inhibitors through micelles formation (Lee et al., 2015). The high concentrations of formic acid and furfural in the dilute ammonia pretreated energy cane bagasse hydrolysates can be explained by the degradation of hemicellulose during pretreatment. Formic acid and furfural are by-products of xylose degradation (Rajan and Carrier, 2014).

4.3.3. Effect of ionic liquids on sugar losses

Analysis of ionic liquid-treated hydrolysate samples for glucose and xylose content showed negligible losses due to the lack of solubilization of the sugars into the ionic liquids. However, an increase in sugar loss was observed only after 10 min of mixing for all the ionic liquids evaluated. [BMIM][NF_2] resulted in the highest sugar loss observed at 3.80% (Sample 2) and 3.17% (Sample 1) in hydrolysate with an initial glucose content of 37.63 g/L and 30.35 g/L, respectively. Other ionic liquids with detectable sugar loss were [HMIM][BF_4] at 2.60% (Sample 2) and 2.18% (Sample 1) and [BMIM][PF_6] at 1.91% (Sample 2) and 1.83% (Sample 1). The order observed for the solubility of sugars in the ionic liquids matched the strength of their hydrogen bonding. [NF_2] anion has the strongest hydrogen bonds (3.40 kcal·mol^{-1}) followed by [BF_4] (3.30 kcal·mol^{-1}) and [PF_6] (2.40 kcal·mol^{-1}) (Katsyuba et al., 2013). Crosthwaite et al. (2004) observed the same trend in affinity of these ionic liquids with alcohols. These results are comparable to ours because similar to carbohydrates, solubilization of alcohols happens through hydrogen bonding of the ionic liquid with the solvent. Low percentages in sugar losses are desirable as sugars are the main carbon source for microorganisms during fermentation. Therefore, keeping sugar losses to a minimum is a must during the removal of the non-sugar compounds. The results observed were as expected as the ionic liquids used in our experiments are hydrophobic in nature. In order for a carbohydrate

to be soluble in an ionic liquid, a hydrogen bonding capacity between the anion of an ionic liquid and the hydroxyl group of a carbohydrate must take place (Lateef et al., 2012). Carneiro et al. (2012) investigated the solubility of monomeric sugars in both hydrophobic and hydrophilic ionic liquids and observed that regardless of the hydrophobicity or hydrophilicity of the ionic liquid, solubility of sugars was as follows: fructose > xylose > glucose > galactose. Although the xylose content in the hydrolysate samples was low (3.41-3.70 g/L, due to the substantial solubilization of hemicellulose during dilute ammonia pretreatment), minimal loss of xylose as that of glucose was observed. Based on our results, anions had a dominant effect on sugar loss as compared to cations. In ionic liquids containing the same anion, the one with a shorter alkyl chain caused the most sugar loss due to their high solubility. On the other hand, a cation with a longer alkyl chain has higher hydrophobicity and results in less amount of sugars being lost to solubilization (Remsing et al., 2008).

4.3.4. Extraction of phenolic compounds

Phenolic compounds were extracted by almost all of the ionic liquids used in this study (Figure 4.1). [OMIM][NF_2] was the most effective solvent with 82.62% (Sample 1) and 81.30% (Sample 2) extractions. The second best ionic liquid in extracting phenolic compounds was [BMIM][NF_2] with an extraction of 76.14% (Sample 1) and 73.33% (Sample 2). Lowest percent extractions were obtained with [BMIM][PF_6] at 27.12% and at 23.36% for Sample 1 and Sample 2, respectively. High extractability of phenolic compounds using hydrophobic ionic liquids has been reported by others. Archana et al. (2016) assessed the removal of phenolic compounds using encapsulated room temperature ionic liquids and were able to remove 92.50% of the phenolic compounds from waste water at optimized conditions for mixing time (4 h), mixing speed (600 rpm) and temperature (70 °C). Fan et al. (2008) investigated the effect of 1-methyl-3-alkylimidazolium hexafluorophosphate [C*n*MIM][PF_6] (*n* = 4, 6, 8) and 1-methy-3-alkylimidazolium tetrafluoroborate [C*n*MIM][BF_4] (*n* = 6, 8) for the extraction of phenolic compounds from waste water

and observed that the nature of the ionic liquids as well as the chemical structure of the phenolic compounds can greatly affect the extraction efficiency. Maximum extractability of phenolic compounds by ionic liquids takes place when they are in their un-dissociated form. This promotes hydrogen bonding and hydrophobic interactions between phenolic compounds and ionic liquids (Fan et al., 2008; Nosrati et al., 2011). The pH of our samples was close to 5 which is below the Pka of phenol (around 10). Most of the phenolic compounds have high Pka values (above 7) (Hanai et al., 1997). This means that all the phenolic compounds were in their un-dissociated form favoring their extractability. Nosrati et al. (2011) also reported a high extraction of phenolic compounds by ionic liquids. They were able to extract 85% of phenolic compounds from waste water using [BMIM][HSO_4] in combination with a hydrophobic polytetrafluorethylene (PTEF) membrane filter. Khachatryan et al. (2005) used a ratio of 3:1 (v/v) of aqueous solution to ionic liquid ([BMIM][PF_6]) to extract phenolic compounds and reported considerable extractions after 10 min of mixing. However, phenol itself was not entirely extracted. They reported that pH and the un-dissociated form of phenolic compounds influenced their extraction from aqueous solutions using [BMIM][PF_6].

Hydrogen bonding capacity and hydrophobic interactions are the two main mechanisms of extraction between ionic liquids and phenols (Fan et al., 2008; Poole and Poole, 2010). In our study, $[NF_2]^-$ anion was the most hydrophobic anion and had the strongest hydrogen bonding capacity in the set of ionic liquids tested (Freire et al., 2007; Katsyuba et al., 2013). This explains the highest extractibility of phenolic compounds observed by $[NF_2]^-$ containing ionic liquids. Fan et al. (2008) reported that when two ionic liquids had the same cation with the same alkyl chain, ionic liquids with anions of $[BF_4]^-$ extracted more phenolic compounds as compared to $[PF_6]^-$ anions due to their stronger hydrogen bonding capacity with the phenolic compounds. Their observation is in agreement with ours. The extraction efficiency of phenolic compounds with [OMIM][BF_4] was 64.94% (Sample 1) while only a 36.55% extraction

efficiency was observed for the same sample using [OMIM][PF_6]. [OMIM][BF_4] and [OMIM][PF_6] have the same cation $[OMIM]^+$ but the anion $[BF_4]^-$ resulted in higher affinity as compared to $[PF_6]^-$ for the extraction of phenolic compounds. Hou et al. (2013) reported the following order of anions for the extraction of phenolic compounds from oil using imidazolium-based ionic liquids: $[Cl]^- > [Br]^- > [BF_4]^- > [PF_6]^-$, with [BMIM]Cl extracting 90% of the phenolic compounds. They reported that the effect of anions on the extraction of phenolic compounds was more prominent than that of cations.

Extractibility of phenolic compounds was affected by the type of anion present in the ionic liquid. However, the length of the alkyl chain also plays an important role in the extractibility of phenolic compounds by ionic liquids. It was demonstrated that a longer alkyl chain of the cation present in the ionic liquid translates to a better partitioning of the phenolic compounds into the ionic liquid as longer alkyl chains boost their hydrophilic interactions (Archana et al., 2016). We observed the same effect of alkyl chain length on the extractability of phenolic compounds from dilute ammonia pretreated energy cane bagasse. [OMIM] cation with an eight carbon alkyl chain showed a higher extraction efficiency for the phenolic compounds as compared to [BMIM] cation containing a four carbon alkyl chain. Nosrati et al. (2011) compared phenol removal from waste water using room temperature ionic liquids and found that [HMIM][BF_4] yielded better results as compared to [BMIM][BF_4]. Since the anion was the same, they concluded that a longer alkyl chain in the cation increased the extractability of phenols by imidazolium-based ionic liquids. Effective extraction and recovery of lignin-derived phenolic compounds is of great interest. They are valuable chemicals with great potential in various industries (Tejado et al., 2007). Vanillin has several applications in the food and cosmetic industry (Tejado et al., 2007). Currently, vanillin from lignin oxidation accounts for 18% of its worldwide production (Araújo et al., 2010). Phenol formaldehyde (the most common used adhesive in the plywood industry) as a petroleum-based product can be substituted by lignin-phenol formaldehyde from renewable sources (Zhang et al., 2013).

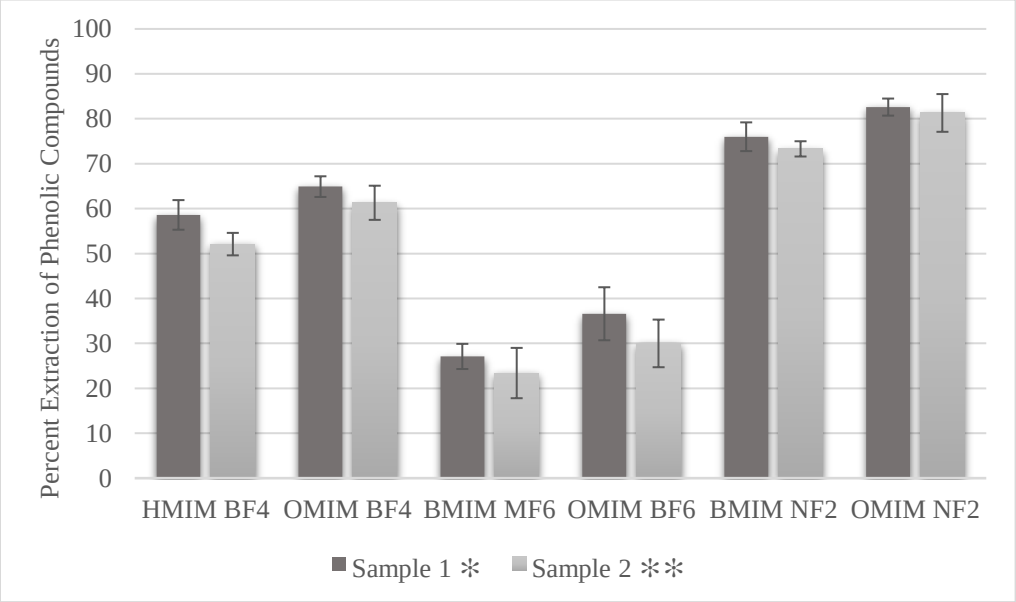

Figure 4. 1. Percent Extraction of Phenolic Compounds by Imidazolium-Based Ionic Liquids from Hydrolysates.
Sample 1*: Hydrolysate from enzymatic hydrolysate of liquid ammonia pretreated energy cane bagasse, unwashed, hydrolyzed with 12.10% w/w CTec2, 1.90% w/w HTec2, 4.89% w/w Tween® 80, 0 IU/g laccase.
Sample 2**: Hydrolysate from enzymatic hydrolysate of liquid ammonia pretreated energy cane bagasse, unwashed, hydrolyzed with 19.53% w/w CTec2, 8.30% w/w HTec2, 22.34% w/w Tween® 80, 11.10 IU/g laccase.

They can also be utilized in phenol-formaldehyde resins (Tejado et al., 2007). P-cumaric acid, one of the main components of lignin, has many applications in the food, pharmaceutical and cosmetic industries. Sugarcane bagasse has 1.76% P-cumaric acid (dry base weight) which can be used to decrease low density lipoprotein (LDL) peroxidation, as an antimicrobial and in the production of aromatic chemicals such as 4-vinylphenols (Zhao et al., 2011).

4.3.5. Extraction of organic acids

All the ionic liquids in our study failed to extract formic and acetic acids (Table 4.3). The pH of the hydrolysate samples was around 5 which is slightly above the Pk_a of the targeted organic acids. Reduction of the pH in the hydrolysate to 3 by the addition of hydrochloric acid did not improve the extractability of

the organic acids (data not shown). Our results are in agreement with those published by Matsumoto et al. (2004).They reported a poor extractability of organic acids, including acetic acid, from fermentation broths using imidazolium-based ionic liquids. They observed that the extractability of the organic acids was affected by the hydrophobicity of ionic liquids with an order of extractability reported as follow: [Omim] $[PF_6]^-$ < [Bmim]$[PF_6]^-$ < [Hmim]$[PF_6]^-$. McFarlane et al. (2005) investigated the effect of nine different hydrophobic ionic liquids in the extraction of polar water pollutants including organic acids. In agreement with our observations, it was reported that acetic acid did not partition into the ionic liquid phase regardless of the extraction criteria. Klasson et al. (2004) investigated the effect of ionic liquids including [BMIM] $[PF_6]$, [BMIM] $[NF_2]$ and [OMIM] $[NF_2]$ on the extraction of acetic, lactic and succinic acids from fermentation broths. They reported the same failure of imidazolium-based ionic liquids to extract the organic acids regardless of the pH of the solution. The only promising results (with a distribution coefficient of 60) were observed with a sulfonate-anion ionic liquid (trihexyltetradecylphosphonium methanesulfonate) for the extraction of succinic acid. However, diluents such as nonanol and trioctylamine were used in order to improve the performance of the ionic liquid due to its high viscosity.

Organic acids need to be in their un-dissociated form in order to pose an inhibitory effect on microbial growth. Formic acid with a pK_a value of 3.75 as compared to acetic acid which has a pK_a value of 4.76 has the strongest inhibitory effect on microbial growth (Jönsson et al., 2013).The high polarity of formic acid and acetic acid prevents them from properly partitioning into hydrophobic ionic liquids; however, they might partition well into hydrophilic ionic liquids. Lopez and Hestekin (2015) were able to effectively improve the extractability of organic acids from the water phase into hydrophilic ionic liquids1-Ethyl-3-methylimidazolium trifluoromethanesulfonate ([EMIM][OTf]) and 1-butyl-3-methylimidazolium acetate ([BMIM][OAc]) using electrodialysis. They reported that ionic liquids showed different affinity towards

organic acids and that this difference was prominently dictated by their types of anions. Similarly, Li et al. (2009) was able to improve the extraction of butyric acid and its salts by using 1-ethyl-3-methylimidazolium-triflouromethansulfonate assisted with electrodialysis.

The alkyl chain of an ionic liquid affects its distribution ratio. It is shown that ionic liquids with higher distribution ratios are better solvents for the extraction of organic compounds. Furthermore, hydrophilicity of the compound in relation to the distribution ratio of the ionic liquid has an important role in the extractability of organic compounds (Swatloski et al., 2002). Lateef et al. (2012) were able to extract lactic acid from wine using 1-Butyl-3-methylimidazolium bromide ([HMIM][Br]) at 80 °C after 30 min of mixing at 800 rpm. A recovery yield of 36% was achieved using diethyl ether. Oliveira et al. (2012) compared the extractability of lactic acid, malic acid and succinic acid using phosphonium-based ionic liquids ($[P_{66614}][Cl]$, $[P_{66614}][Dec]$, $[P_{66614}][Phos]$). They observed that the anion had a significant effect on the extractability of the organic acids. As for the first two ionic liquids with chlorine and decanoate anions ($[P_{66614}][Cl]$ and $[P_{66614}][Dec]$), succinic acid showed the highest partition coefficient due to its less hydrophilicity and tendency to leave water coupled with its smaller molecular size. However, lactic acid extraction was improved only when the anion of the ionic liquid was phosphinate. They suggested that this was due to the hydrogen bonding of phosphinate anion with the pendant hydroxyl group of lactic acid. The highest organic acids recovery observed was 73% at optimum conditions. Although our set of ionic liquids failed to extract organic acids, there are many applications for recovered organic acids. For example, acetic acid can be used in the production of vinylacetate polymer or ethylacetate as a green solvent (Sauer et al., 2008). Recent studies have demonstrated that the non-toxicity and high energy density of formic acid makes it a great feed for fuel cells (Liu et al., 2015).

Table 4. 3. Percent extraction of formic acid and acetic acid by imidazolium-based ionic liquids from dilute ammonia pretreated energy cane bagasse hydrolysates.						
Organic Acids	[HMIM][BF_4]	[OMIM][BF_4]	[BMIM][PF_6]	[OMIM][PF_6]	[BMIM][NF_2]	[OMIM][NF_2]
Formic Acid (Percent Extraction)						
Enzyme Hydrolysate Sample 1*	4.10±0.64	2.60±0.10	0.58±0.22	0	5.48±1.03	4.06±0.52
Enzyme Hydrolysate Sample 2**	4.30±0.60	2.66±0.40	0	0	5.27±0.81	4.51±0.94
Acetic Acid (Percent Extraction)						
Enzyme Hydrolysate Sample 1*	6.73±0.14	2.54±0.61	0	0	6.81±0.64	5.39±0.60
Enzyme Hydrolysate Sample 2**	5.81±0.63	3.44±0.19	0	0	5.02±0.17	5.18±0.54
Sample 1*: Hydrolysate from enzymatic hydrolysate of liquid ammonia pretreated energy cane bagasse, unwashed, hydrolyzed with 12.10% w/w CTec2, 1.90% w/w HTec2, 4.89% w/w Tween® 80, 0 IU/g laccase. Sample 2**: Hydrolysate from enzymatic hydrolysate of liquid ammonia pretreated energy cane bagasse, unwashed, hydrolyzed with 19.53% w/w CTec2, 8.30% w/w HTec2, 22.34% w/w Tween® 80, 11.10 IU/g laccase.						

4.3.6. Extraction of furfural and 5-HMF

Furfurals partitioned into ionic liquids at relatively lower amounts as compared to phenolic compounds (Figures 4.2 and 4.3). [OMIM][NF_2] was the best ionic liquid for extracting furfural from the aqueous phase. Extraction efficiencies of 43.09% (Sample 1) and 47.44% (Sample 2) were observed with [OMIM][NF_2]. The next effective furfural extraction was achieved with [OMIM][BF_4] with observed percent extractions of 45.46% (Sample 1) and 42.80% (Sample 2). [BMIM][PF_6] showed the lowest furfural extractability at 21.32% (Sample 1) and 19.47% (Sample 2). 5-HMF showed a relatively higher extractability as compared to furfural (Figure 4.3). Just like with furfural, [OMIM][NF_2] and [OMIM][BF_4] worked best in extracting 5-HMF. 5-HMF percent extractability observed with

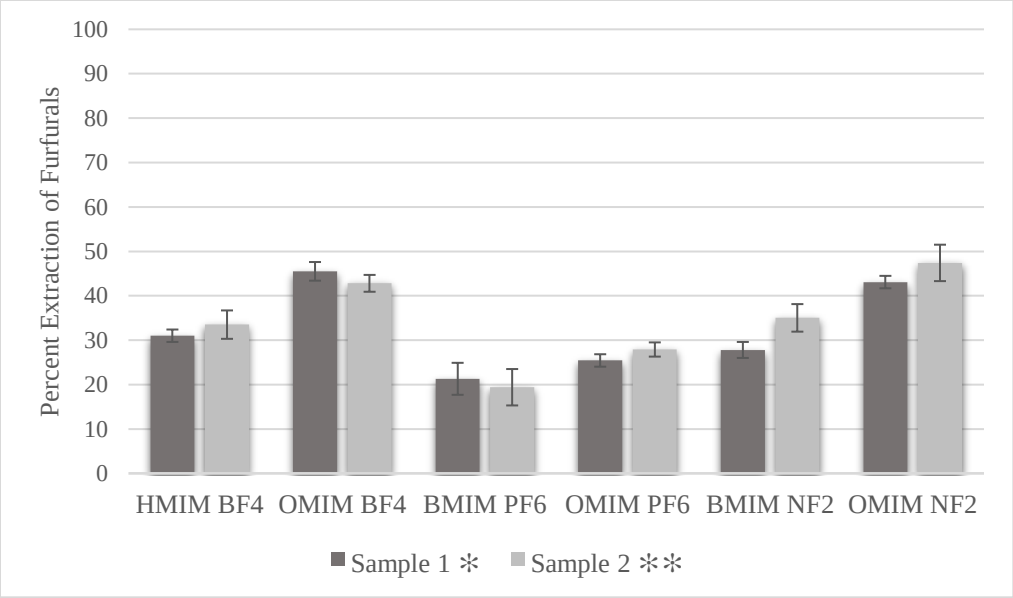

Figure 4. 2. Percent Extraction of Furfural by Imidazolium-Based Ionic Liquids from Hydrolysates.
Sample 1*: Hydrolysate from enzymatic hydrolysate of liquid ammonia pretreated energy cane bagasse, unwashed, hydrolyzed with 12.10% w/w CTec2, 1.90% w/w HTec2, 4.89% w/w Tween® 80, 0 IU/g laccase.
Sample 2**: Hydrolysate from enzymatic hydrolysate of liquid ammonia pretreated energy cane bagasse, unwashed, hydrolyzed with 19.53% w/w CTec2, 8.30% w/w HTec2, 22.34% w/w Tween® 80, 11.10 IU/g laccase.

[OMIM][NF_2] was 56.48% (Sample 1) and 58.25% (Sample 2). [OMIM][BF_4] also resulted in 51.14% (Sample 1) and 51.80% (Sample 2) 5-HMF extractability, whereas with [BMIM][PF6] only 25.70% (Sample1) and 24.40% (Sample 2) of total 5-HMF were extracted. Our results indicated that the type of anion as well as the cation and its alkyl chain are important in partitioning furfural and 5-HMF into the ionic liquids. Limited information exists on the extraction of furans by ionic liquids. Pei et al. (2008) investigated the effect of [BMIM][PF_6], [HMIM][PF_6] and [OMIM][PF_6] in extracting pure furfural and 5-HMF from an aqueous solution. They reported that [HMIM][PF_6] worked best at a mixing ratio of 5:1 (aqueous solution to water) resulting in 76% furfural and 83% 5-HMF extractions. Extractions improved in the presence of sodium chloride or sodium sulfate due to the competition over hydration. It was suggested that the presence of an extra alkyl group in 5-HMF added to the hydrophobicity of the molecule and subsequently increased its tendency to partition into the hydrophobic ionic liquids. In our study, lower

percent extractions were observed for both furfural and 5-HMF with imidazolium-based ionic liquids. Furfural is among the top 30 value added products that can be sustainably produced from biomass (Bozell and Petersen, 2010). It can be used as the building block of other valuable products such as furfuryl alcohol which holds almost 75% of the total furfural market in the USA (Peleteiro et al., 2016). According to the US Department of Energy, 5-HMF is one of the most important chemicals that can be obtained from biomass in the presence of proper catalysts (Zakrzewska et al., 2010). It is the building block of many value-added chemicals including 2,5-dimethylfuran, 2,5-dimethyltetrahydrofuran, and 2,5-furandicarboxylic acid which are used in various polymer applications (Van Nguyen et al., 2016).

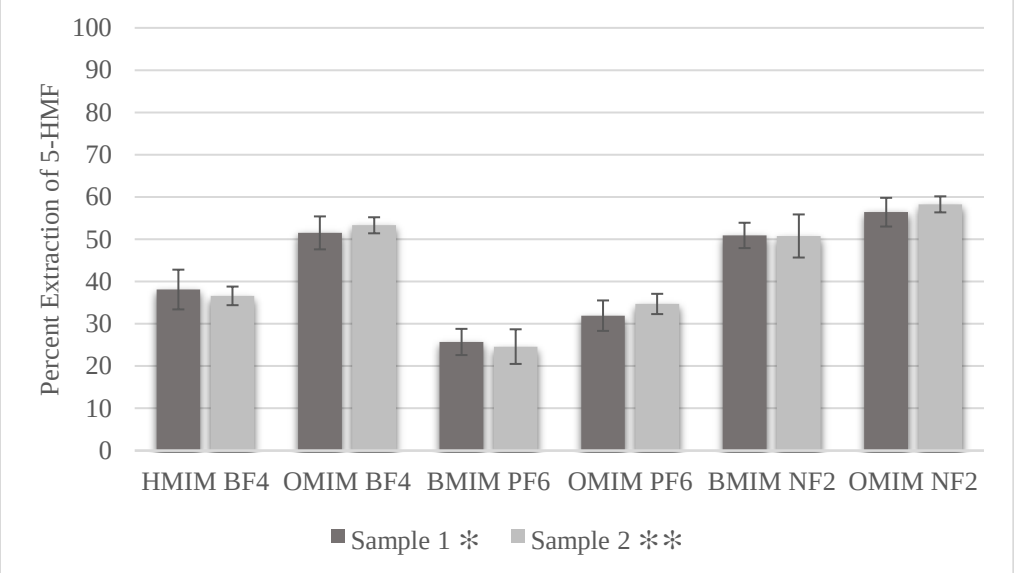

Figure 4. 3. Percent Extraction of 5-HMF by Imidazolium-Based Ionic Liquids from Hydrolysates.
Sample 1*: Hydrolysate from enzymatic hydrolysate of liquid ammonia pretreated energy cane bagasse, unwashed, hydrolyzed with 12.10% w/w CTec2, 1.90% w/w HTec2, 4.89% w/w Tween® 80, 0 IU/g laccase.
Sample 2**: Hydrolysate from enzymatic hydrolysate of liquid ammonia pretreated energy cane bagasse, unwashed, hydrolyzed with 19.53% w/w CTec2, 8.30% w/w HTec2, 22.34% w/w Tween® 80, 11.10 IU/g laccase.

4.3.7. Regeneration of ionic liquids

[OMIM][BF_4], [HMIM][BF_4], [BMIM] [NF_2], and [OMIM][NF_2] extracted the most phenolic compounds, furfurals and 5-HMF from hydrolysates and were further assessed for their regeneration

(Table 4.4). Regeneration of ionic liquids for organic acids were not conducted due to poor extractability as reported above. No detectable sugar losses were observed for any of these four ionic liquids after regeneration. All the ionic liquids maintained at least 91% of their extraction efficiencies after the first regeneration for the extraction of phenolic compounds, furfural or 5-HMF. [OMIM][NF_2] showed a 94% reusability for the extraction of phenolic compounds after the first regeneration (second use) in Sample 1 and Sample 2. However, extraction efficiencies decreased to almost 81% in Sample 1 and to 83% in Sample 2 after the second regeneration (third use). [BMIM][NF_2] showed 92% reusability for the extraction of phenolic compounds after the first regeneration in both Sample 1 and Sample 2, and extraction efficiencies dropped to 70% and 72% after the second regeneration in Sample 1 and Sample 2, respectively. Ionic liquids containing the [BF_4] anion retained 91% extractability for phenolic compounds after the first regeneration in both Sample 1 and Sample 2. After the second regeneration, extraction of phenolic compounds decreased to 76% for [HMIM][BF_4] and to 77% for [OMIM][BF_4]. Archana et al. (2016) used similar regeneration methods for the recovery of Cyanex – 923 when extracting phenolic compounds and recommended no more than two regenerations. In their study, extraction efficiencies dropped from 96% (first regeneration) to 95% (second regeneration) to a final 79% after a third regeneration of Cyanex – 923. The lower percent extractions observed in our study can be attributed to the differences in the extraction conditions used, such as pH and temperature (Fan et al., 2008). There is a wide variety of phenolic compounds with different Pk_a values in the hydrolysate, and the type and chemical composition of the phenolic compounds play a major role in their extractability by ionic liquids (Hanai et al., 1997).

Table 4. 4. Percent extraction of sugars and non-sugar compounds from enzymatic hydrolysates of top four imidazolium-based ionic liquids after regeneration.

Compound	Hydrolysate Samples	[HMIM][BF_4]			[OMIM][BF_4]			[BMIM][NF_2]			[OMMIM][NF_2]		
		First Use	Second Use	Third Use	First Use	Second Use	Third Use	First Use	Second Use	Third Use	First Use	Second Use	Third Use
Sugars (glucose, xylose)	Sample 1*	2.18±0.71	ND^	ND	0.5±0.20	ND	ND	3.17±0.40	ND	ND	1.91±0.20	ND	ND
	Sample 2**	2.60±0.32	ND	ND	0.8±0.10	ND	ND	3.80±0.70	ND	ND	1.83±0.10	ND	ND
Total Phenolic Compounds	Sample 1*	58.61±3.31	55.19±0.71	45.07±2.60	64.94±2.33	63.64±2.20	49.88±1.90	76.14±3.22	72.88±1.81	53.56±3.28	82.62±1.87	77.59±2.04	67.11±2.33
	Sample 2**	52.11±2.55	48.61±0.63	40.10±1.91	61.31±3.82	59.50±1.87	46.64±2.21	73.33±1.74	70.45±2.60	54.22±2.71	81.30±4.20	77.12±1.31	67.64±2.40
Furfurals	Sample 1*	31.00±1.81	28.40±2.64	20.20±0.83	45.46±2.10	41.41±3.16	31.20±2.25	27.77±2.84	26.10±0.53	20.00±1.40	43.09±1.33	40.67±2.03	30.57±1.40
	Sample 2**	33.54±3.21	30.87±2.60	23.11±1.10	42.80±1.91	39.30±2.83	29.55±1.70	35.00±3.14	32.94±1.10	24.97±0.91	47.44±4.42	44.54±0.93	33.38±2.17
5-HMF	Sample 1*	38.11±4.70	34.72±2.09	27.17±0.74	51.14±3.94	48.55±1.60	36.21±2.31	50.07±3.37	48.85±2.70	36.60±1.07	56.48±3.34	53.60±0.67	41.24±0.09
	Sample 2**	36.68±2.25	34.08±1.80	26.60±0.55	51.82±2.94	49.20±3.31	37.84±1.40	50.71±5.11	49.21±2.20	38.54±0.42	58.25±2.61	54.87±2.63	42.04±1.21

Sample 1*: Hydrolysate from enzymatic hydrolysate of liquid ammonia pretreated energy cane bagasse, unwashed, hydrolyzed with 12.10% w/w CTec2, 1.90% w/w HTec2, 4.89% w/w Tween® 80, 0 IU/g laccase.
Sample 2**: Hydrolysate from enzymatic hydrolysate of liquid ammonia pretreated energy cane bagasse, unwashed, hydrolyzed with 19.53% w/w CTec2, 8.30% w/w HTec2, 22.34% w/w Tween® 80, 11.10 IU/g laccase.
ND None detected.

First regeneration caused a slight decrease (3% to 9%) in the extractability of furans (furfural and 5-HMF) by [OMIM][BF_4], [HMIM][BF_4], [BMIM] [NF_2], and [OMIM][NF_2] regardless of the hydrolysate sample used (Sample 1 or 2). After the second regeneration, a 28% decrease in the extractability of furans by [BMIM][NF_2] was observed. This decrease in extractability was also observed in the other ionic liquids (29% for [OMIM][NF_2], 31% to 35% for [HMIM][BF_4] and 31% for [OMIM][BF_4]). Overall, first regeneration of these four ionic liquids caused a slight decrease of less than 10% in the extractability of phenolic compounds and furans. However, up to a maximum of 35% decrease in extraction efficiency was observed after the second regeneration. Our results indicate that no more than two regenerations are recommended for [OMIM][BF_4], [HMIM][BF_4], [BMIM][NF_2], and [OMIM][NF_2] when extracting phenolic compounds and furans from dilute ammonia pretreated energy cane bagasse enzymatic hydrolysates.

4.4. Conclusion

Pretreatment of lignocellulosic biomass can result in the generation of by-products (i.e., furans, organic acids, phenolics) which might have inhibitory effects on enzyme activity and microbial growth. Application of an effective strategy for their removal would greatly improve enzymatic hydrolysis and fermentation yields of pretreated biomass. Furthermore, recovery and purification of these by-products can become available as building blocks for the production of value- added chemicals.

Imidazolium-based ionic liquids ([OMIM][PF_6], [BMIM][PF_6], [OMIM][BF_4], [HMIM][PF_4], [OMIM][NF_2], and [BMIM][NF_2]) were evaluated for their effectiveness during liquid-liquid extraction of non-sugar compounds from enzymatically hydrolyzed dilute ammonia pretreated energy cane bagasse hydrolysates. Organic acids (acetic acid and formic acid) failed to partition into any of the imidazolium-based ionic liquids. However, [OMIM][BF_4], [HMIM][BF_4], [BMIM] [NF_2], and [OMIM][NF_2] were effective in extracting phenolic compounds, furfurals and 5-HMF. The most non-sugar compounds were extracted

by [OMIM][NF_2] at 82.62% phenolic compounds, 47.44% furfural and 58.25% 5-HMF. This can be attributed to the type of anion as well as the alkyl chain of the cation present in the ionic liquid. Less than 4% sugar losses were observed with all six ionic liquids. No more than two regenerations of [OMIM][BF_4], [HMIM][BF_4], [BMIM][NF_2], and [OMIM][NF_2] are recommended for the extractability of phenolic compounds, furfural and 5-HMF from dilute ammonia pretreated energy cane bagasse enzymatic hydrolysates.

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CHAPTER 5

SUMMARY AND FUTURE WORK

The rationale behind this work was to effectively convert the cellulose polymers from energy cane bagasse to a clean hydrolysate of fermentable sugars to be used as potential feedstock for the production of green fuels and chemicals. Lignocellulosic material is mainly composed of the polymeric sugars cellulose (glucan) and hemicellulose (xylan). Lignin is made up of aromatic compounds and it is responsible for providing the plant structural support and acts as the glue that holds the cellulose and the hemicellulose together. During pretreatment, the lignin is removed, the hemicellulose is solubilized and cellulose crystallinity is reduced. All these changes make the cellulose more accessible to hydrolyzing enzymes. From the pretreatments available today, ammonia-based pretreatments have been shown to target lignin removal while generating less inhibitory or non-sugar compounds as compared to other popular pretreatment methods such as dilute acid pretreatment. Lignocellulosic materials respond differently to a pretreatment method due to their unique chemical composition in terms of the amount of cellulose, hemicellulose and lignin available. The crystallinity index of each biomass material requires a tailored pretreatment that would ensure cellulose digestibility by enzymes and at the same time the minimum generation of inhibitory or non-sugar compounds (i.e. phenolic compounds, organic acids, furan derivatives).

Energy cane bagasse (variety HO 02-113) was the lignocellulosic material used in this study with a chemical composition of 40.14 ±0.16% glucan, 24.23 ± 0.51% xylan, 2.76 ± 0.04% arabinan, 24.41 ± 0.37% lignin, 3.76 ± 0.62 extractives, and 4.70 ± 0.04% ash (dry basis). Energy cane contains higher fiber yield per acre and it is more cold tolerant, disease and drought tolerant than sugarcane. These characteristics make energy cane a great candidate for the sustainable production of bio-fuels and bio-chemicals. Energy cane bagasse was pretreated with ammonium hydroxide and the pretreatment

parameters such as temperature (160-220 °C), residence time (30-60 min) and biomass to ammonium hydroxide ratio (1:0-0.5, w/w) were optimized for maximum cellulose digestibility or glucose yield. Response Surface Methodology (RSM) was the computational tool used to find the most effective parameters and their interactions on the response (glucose yield) as well as the optimum value for each parameter. A total of 20 pretreatment experiments were carried out in duplicate. All pretreatments caused considerable loss of solids, mostly attributed to lignin removal. Recovered solids varied from 55.50% to 74.01% depending on the severity of the pretreatment conditions. Temperature had the most significant effect (correlation, -0.88) on solid loss (p<0.001) followed by ammonium hydroxide concentration (correlation, 0.23). Lignin removal was the most important factor affecting cellulose digestibility as it provides a physical barrier for enzymes. Lignin removal ranged from 35.95% to 66.85%. Hemicellulose losses ranged from 27.94% to 95.79% with temperature being the only and most effective variable. Lignin removal and hemicellulose solubilization also had a positive high correlation meaning that they were simultaneously removed due to the selectivity of ammonium hydroxide towards lignin. A linear model was significant (p<0.001) for xylan loss (R^2=0.86) with temperature being the only variable posing a significant effect on the response at a 0.05 significance level. Increasing pretreatment temperatures from 160 to 220 °C resulted in xylan losses in the range of 27.94% to 95.79%. Only a small amount of glucan (4.49% to 12.95%) was lost during pretreatment. A quadratic model was fitted (p<0.001) for glucan loss, with ammonium hydroxide concentration being the dominant variable followed by temperature. Temperature was found to be the most effective variable on glucose yield followed by ammonium hydroxide concentration and residence time. At lower temperatures (160 °C), increasing the residence time had a positive effect on the response; while at high temperatures (above 190 °C), increasing the residence time above 41 min caused a reduction in glucose yield probably due to the degradation of sugar polymers and subsequent generation of inhibitory compounds. A quadratic model was fitted on the

experimental data (R^2 = 0.97) and the optimum points for the variables within the experimental design were predicted to be 208 °C, 36 min and a biomass to ammonium hydroxide to water ratio of 1:0.4:20, respectively. Interaction effect of all the pretreatment variables (temperature, residence time and ammonium hydroxide to biomass ratio) was significant on the glucose yield from energy cane bagasse. A cellulose digestibility of 75.62% and a glucose yield of 30.76 g/ 100 g of energy cane bagasse was predicted by our model and confirmed experimentally. The quadratic model was found to be reliable for the prediction of glucose yield within the design space.

In addition to the use of an appropriate pretreatment, the combined use of accessory enzymes and/or surfactants as well as washing of the pretreated material can further improve enzymatic hydrolysis yields. Among the available accessory enzymes, xylanase has shown a high degree of synergy with cellulase. It also can hydrolyze the residual hemicellulose and expose more of the cellulose to the hydrolyzing enzymes. Moreover, xylo-olygomers generated from the partial breakdown of xylan with known inhibitory effects on enzymes, can be further hydrolyzed into their xylose monomers. Laccase is another enzyme that is mostly studied for the purpose of biological pretreatment due to its lignin oxidizing properties. However, there is great potential in the simultaneous use of laccase with cellulase to investigate the outcome of their interaction on cellulose digestibility. The addition of laccase during enzymatic hydrolysis can help improve cellulose digestibility through the oxidation of lignin as well as lignin-derived products such as phenolic compounds to compounds with less inhibitory effect. Tween 80, a non-ionic surfactant, can prevent enzyme degradation induced by heat and shear stress by forming protective micelles. Moreover, Tween 80 reduces the unproductive binding of lignin to enzymes. Surfactants can also modify the structure of the biomass by creating micropores on its surface thus expanding the surface area of the substrate. Surfactants can also improve the adsorption of enzymes to the enzyme-substrate interface by reducing the interfacial tension between enzyme and substrate and by increasing substrate

hydrophilicity. Washing is a simple an effective strategy to remove inhibitory or non-sugar compounds generated from the degradation of cellulose, hemicellulose and lignin during pretreatment. Using RSM, a total of 30 experiments were performed in duplicate to investigate the interaction effect of xylanase (HTec2, 0-15% g/g glucan), laccase (0-15 IU/g biomass) and Tween 80 (0-15% g/g glucan) along with cellulase (CTec2, 1.5-20% g/g glucan) on the cellulose digestibility of washed and unwashed optimized ammonium hydroxide pretreated energy cane bagasse. Our results showed that while the washed material yielded a higher cellulose digestibly, the percent range of cellulose digestibility (as a result of the addition of accessory enzymes and Tween 80) was broader for the unwashed material (47.70% to 83.90%) as compared to the washed material (82.20% to 94.20%). This suggests that washing and the removal of inhibitory or non-sugar compounds significantly improved the digestibility of cellulose. Furthermore, the addition of accessory enzymes (lacasse, xylanase) can be more effective in improving biomass digestibility in the unwashed material as compared to the washed material. Cellulase had the highest correlation with cellulose digestibility of unwashed material (0.8) and washed material (0.6), indicating a close to linear relationship. Despite the fact that our pretreatment resulted in xylan losses of 84.14% (with most harsh pretreatment conditions), the addition of xylanase (HTec2) was found to be effective in improving cellulose digestibility for unwashed biomass. This indicated that regardless of the xylan content, the addition of xylanase improved cellulose digestibility due to their synergistic effect which involves increasing the porosity of biomass and decreasing the unproductive binding of non-sugar compounds (such as lignin-derived compounds) to cellulase. At 1.5% CTec2 loading (g/g glucan, dry basis), addition of HTec2 from 0 to 15% (g/g glucan, dry basis) improved cellulose digestibility of the unwashed biomass by 101.90%; whereas, for the washed biomass only a 14% increase was observed. At 20% CTec2 loading, increasing HTec2 loading from 0 to 15% (g/100g glucan, dry basis) resulted in a

10.90% decrease in cellulose digestibility for the unwashed samples. This indicated that by increasing the loading of accessory enzymes the synergistic effect shifts to co-hydrolysis.

Laccase had a significant effect on the response for washed and unwashed biomass. At lower cellulase loading (1.5% CTec2 g/g glucan), increasing the concentration of laccase from 0 to 50 IU/ g biomass caused a 13% reduction in cellulose digestibility in unwashed biomass. This negative effect was attributed to the inhibitory effect of laccase on β-glucosidase activity. This negative effect was emphasized in the unwashed substrate due to the higher concentration of inhibitory or non-sugar compounds. At higher cellulase loadings (20% CTec2 g/g glucan), the addition of laccase resulted in a slight improvement in glucose yields. The slight increase can be attributed to the oxidation of lignin-derived compounds and phenolic compounds by laccase. This explains the increased yields observed in the unwashed substrate (9.1%) as compared to the washed substrate (1.2%) as there are more lignin-derived compounds in the unwashed samples that can be oxidized and degraded by laccase. Also, a higher cellulase loading translates into a higher concentration of β-glucosidase in the hydrolysate; therefore, cellulose digestibility is less affected by the non-productive binding of laccase to β-glucosidase. In addition to lignin removal and oxidation of phenolic compounds, modification of the lignin surface with laccase could reduce non-productive binding of lignin to cellulase. Laccase treatment can also increase cellulose accessibility by micropores formation through the oxidization of the aromatic rings present in the lignin.

Tween 80 had a significant effect on the response and improved cellulose digestibility in washed and unwashed substrate having a more significant effect on the unwashed substrate. Tween 80 can cause a steric repulsion between lignin and cellulase which would prevent non-productive binding of lignin to cellulase. Tween 80 also interacts with the enzyme and forms a micelle through which both enzyme activity and stability are preserved. This is especially important in unwashed biomass where the concentration of non-sugar compounds is high. Our results indicated that combinations of high

concentrations of Tween 80 (15% w/w) and high CTec2 loadings (20% w/w) worked best in improving cellulose digestibility. The opposite was observed with the addition of low concentrations of Tween 80 and high CTec2 loadings. At 20% CTec2 loading (highest cellulase loading), increasing the concentration of Tween 80 from 3.80% to 11.30% resulted in an increase in cellulose digestibility by 36.20% and 7.80% for unwashed and washed biomass, respectively. Interestingly, the same increase in Tween 80 concentration at 1.5% CTec2 loading (lowest cellulase loading) resulted in a 25.03% and 1.20% decrease in cellulose digestibility in unwashed and washed material, respectively. At any constant concentration of Tween 80, increasing cellulase loadings improved cellulose digestibility. This improvement was more prominent at 15% Tween 80 where increasing the cellulase loading from 1.5% to 20% resulted in a 128% and 15% increase in cellulose digestibility in unwashed and washed biomass, respectively. A quadratic model was significant (p-value<0.0001) for unwashed and washed ammonium hydroxide pretreated energy cane bagasse. All models passed the lack of fit test. High determination coefficients were observed for unwashed ($R^2 = 0.99$) and for washed ($R^2 = 0.99$) pretreated biomass. Validity of the predicted response was confirmed by conducting actual experiments at the assigned values for each variable. All the experimental results fell within the 95% confidence interval of the model predicted values. Highest cellulose digestibility values observed with minimum loading of enzymes and Tween® 80 were 68% and 86% for unwashed and washed materials, respectively. Maximum cellulose digestibility values observed with no constraints set for the variables within the range of experimental design were 84.30% and 97.10% for unwashed and washed materials, respectively.

Three different groups of non-sugar compounds (organic acids, furans and phenolic compounds) can be generated during pretreatment and are known to pose inhibitory effects on enzyme activity during enzymatic hydrolysis and on microbial growth during fermentation. Several detoxification strategies have been developed to remove and/or recover these compounds from hydrolysates to improve downstream

processes. These non-sugar compounds, if recovered, can also serve as building blocks of many other value-added products. Due to the varied nature of these compounds, multiple detoxification strategies are needed in order to reach a targeted level of purity in the enzymatic hydrolysate. Ionic liquids have unique advantages over conventional solvents for the purpose of extraction of non-sugar compounds due to their non-toxicity, non-flammability, low vapor pressure, recyclability, and thermal and shear stability. A set of six different hydrophobic imidazolium-based ionic liquids with three different anions and cations of different alkyl chain lengths (1-Methyl-3-octylimidazolium hexafluorophosphate [OMIM][PF6], 1-Butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF6], 1-Methyl-3-octylimidazolium tetrafluoroborate [OMIM][BF4], 1-hexyl-3-methylimidazolium tetrafluoroborate [HMIM][PF4], 1-Methyl-3-octylimidazolium bis (trifluoromethylsulfonyl) imide [OMIM][NF2], and 1-Butyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide [BMIM][NF2]) were evaluated for their effectiveness to remove furfural, 5-hydroxymethyl-2-furaldehyde (5-HMF), formic acid, acetic acid, and phenolic compounds with minimal sugar losses from ammonium hydroxide pretreated energy cane bagasse hydrolysates. Enzymatic hydrolysates from unwashed ammonium hydroxide pretreated energy cane bagasse were used in the detoxification studies as they contained higher concentrations of non-sugar compounds as compared to enzymatic hydrolysates from washed pretreated biomass. Sugar losses were minimum (less than 3.8% with [BMIM][NF2] followed by 2.6% with [HMIM][BF4] and 1.9% with [BMIM][PF6]) and were only observed when the mixing time (hydrolysate and ionic liquid) exceeded 10 min. The solubility of sugars in the ionic liquids matched the strength of their anion. Phenolic compounds were effectively extracted by all the ionic liquids in our study. Extractions ranged from 23.40% to 82.60% following the order: [OMIM][NF_2] > [BMIM][NF_2] > [OMIM][BF4] > [HMIM][BF4] > [OMIM][PF6] > [BMIM][PF6]. Acidic conditions of the hydrolysates (pH 5) favored the extractability of phenolic compounds as their Pk_a values were higher than 7 and they showed best extractability in their un-

dissociated form. Our results showed that the anion of the ionic liquids was the dominant factor in the extraction of phenolic compounds followed by the length of their alkyl chains. In the case of the same anion, ionic liquids with longer alkyl chains performed more effectively in extracting phenolic compounds. Furfural partitioned into ionic liquids at relatively lower amounts as compared to phenolic compounds. Compared to phenolic compounds, furans were extracted at lower rates. 5-HMF showed better extractability as compared to furfural. This is believed to be due to the presence of an extra methyl group which adds to the hydrophobicity of the compound. [OMIM][NF2] with a percent extraction of 47.40% was the most effective ionic liquid in the extraction of furfural followed by [OMIM][BF4] with a percent extraction of 45.50%. The same two ionic liquids worked best in extracting 5-HMF with 58.25% and 53.30% extraction efficiencies, respectively. [BMIM][PF6] yielded the lowest extractability for both furfural (19.40%) and 5-HMF (24.40%). Our results indicated that the type of anion as well as the cation and its alkyl chain played a crucial role in partitioning furfural and 5-HMF into the ionic liquids. All six ionic liquids failed to extract most organic acids. The pH of the hydrolysate and mixing time had no effect on improving the extractability of these acids. The highest extraction of formic acid was achieved with [OMIM][NF2] (4.51%) and the lowest with [OMIM][PF6] (0%); and for acetic acid, the highest extraction was achieved with [BMIM][NF2] (6.81%) and the lowest with [OMIM][PF6] (0%).

Regeneration studies for [OMIM][BF_4], [HMIM][BF_4], [BMIM] [NF_2], and [OMIM][NF_2] were carried out because out of the six ionic liquids, these four performed best at extracting the phenolic compounds, furfural and 5-HMF from ammonium hydroxide pretreated energy cane bagasse hydrolysates. No detectable sugar losses were observed for any of these four ionic liquids after regeneration. The four ionic liquids maintained extraction efficiencies of at least 91% after their first regeneration. However, their extraction efficiencies dropped after their second regeneration. In the case of phenolic compounds, a second regeneration caused a 23-30% drop in their extraction efficiency while for furans that number

decreased to 24-35%. No more than two regenerations is recommended for [OMIM][BF_4], [HMIM][BF_4], [BMIM] [NF_2], and [OMIM][NF_2].

The effect of particle size and solid loading during pretreatment should be further investigated as it would save on water and energy consumption. Also, the use of accessory enzymes such as peroxidases and pectinases, and the use of non-ionic surfactants (other than Tween 80) during enzymatic hydrolysis should be evaluated and their effect on carbohydrate digestibility assessed. The use of ionic liquids for the recovery of non-sugar compounds from hydrolysates (detoxification process) is an interesting area of research as the tune-ability of ionic liquids allows for targeted designs of ionic liquids with desired solvent properties. However, more research is needed in order to understand the underlying mechanisms for the extraction and recovery of non-sugar compounds using ionic liquids. This would help in designing ionic liquids with an effective and broader extraction spectrum as well as developing feasible recovery methods that would allow for clean hydrolysates of fermentable sugars to be used as potential building blocks for green fuels and chemicals. Moreover, finding methods for selective separation and purification of extractants (non-sugar compounds) from the ionic liquid should be further studied as they too are building blocks for the production of value added products.

APPENDIX A. SUPPLEMENTARY DATA FOR LIQUID AMMONIA OPTIMIZATION OF ENERGY CANE BAGASSE

Table A.1. Analysis of variance (ANOVA) for response surface quadratic model for the effect of pretreatment variables on recovered solids.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	
Model	495.11	9	55.01	36.42	< 0.0001	significant
A-Temperature	393.77	1	393.77	260.67	< 0.0001	
B-Time	0.22	1	0.22	0.15	0.7110	
C-Ammonium Hydroxide	27.07	1	27.07	17.92	< 0.0017	
AB	1.76	1	1.76	1.16	0.3060	
AC	26.83	1	26.83	17.76	0.0018	
BC	3.24	1	3.24	2.14	0.1739	
A^2	4.81	1	4.81	3.18	0.1047	
B^2	25.86	1	25.86	17.12	0.0020	
C^2	8.89	1	8.89	5.88	0.0357	
Residual	15.11	10	1.51			
Lack of Fit	7.38	5	1.48	0.95	0.5195	not significant
Pure Error	7.73	5	1.55			
Std. dev.	1.23		R-squared	0.9704		
Mean	64.50		Adj R-squared	0.9437		
Coefficient of Variation%	1.91		Pred R-squared	0.8474		
PRESS	77.85		Adeq precision	21.213		

Table A.2. Analysis of variance (ANOVA) for response surface 2FI model for the effect of pretreatment variables on lignin removal.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	
Model	1210.73	6	201.79	21.77	< 0.0001	significant
A-Temperature	482.08	1	482.08	52.00	< 0.0001	
B-Time	150.35	1	150.35	16.22	0.0014	
C-Ammonium Hydroxide	373.90	1	373.90	40.33	< 0.0001	
AB	12.00	1	12.00	1.30	0.2757	
AC	106.58	1	106.58	11.50	0.0048	
BC	85.81	1	85.81	9.26	0.0094	
Residual	120.51	13	9.27			
Lack of Fit	105.54	8	13.19	4.40	0.0596	not significant
Pure Error	14.97	5	2.99			
Std. dev.	3.04		R-squared	0.9095		
Mean	49.69		Adj R-squared	0.8677		
Coefficient of Variation%	6.13		Pred R-squared	0.6405		
PRESS	478.64		Adeq precision	17.970		

Table A.3. Analysis of variance (ANOVA) for response surface quadratic model for the effect of pretreatment variables on glucan loss.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	
Model	73.74	9	8.19	138.09	< 0.0001	significant
A-Temperature	1.76	1	1.76	29.59	0.0003	
B-Time	0.11	1	0.11	1.8	0.2089	
C-Ammonium Hydroxide	31.72	1	31.72	534.65	< 0.0001	
AB	9.16	1	9.16	154.37	< 0.0001	
AC	15.62	1	15.62	263.32	< 0.0001	
BC	13.36	1	13.36	225.24	< 0.0001	
A^2	0.048	1	0.048	0.81	0.3903	
B^2	0.86	1	0.86	14.54	0.0034	
C^2	0.90	1	0.90	15.22	0.0030	
Residual	0.59	10	0.059			
Lack of Fit	0.31	5	0.063	1.13	0.4494	not significant
Pure Error	0.28	5	0.063			
Std. dev.	0.24		R-squared	0.9920		
Mean	9.37		Adj R-squared	0.9848		
Coefficient of Variation%	2.50		Pred R-squared	0.9609		
PRESS	2.91		Adeq precision	50.871		

Table A.4. Analysis of variance (ANOVA) for response surface quadratic model for the effect of pretreatment variables on xylose yield g/ 100 g (dry weight) of untreated biomass.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value Prob > F	
Model	92.20	9	10.24	23.49	< 0.0001	significant
A-Temperature	60.73	1	60.73	139.28	< 0.0001	
B-Time	1.36	1	1.39	3.20	0.1040	
C-Ammonium Hydroxide	2.86	1	2.86	6.55	0.0284	
AB	6.66	1	6.66	15.28	0.0029	
AC	1.05	1	1.05	2.41	0.1516	
BC	0.45	1	0.45	1.03	0.3330	
A^2	10.44	1	10.44	23.95	0.0006	
B^2	0.67	1	0.67	1.53	0.2450	
C^2	10.44	1	10.44	23.95	0.0006	
Residual	4.36	10	0.44			
Lack of Fit	2.17	5	0.43	0.99	0.5031	not significant
Pure Error	2.19	5	0.44			
Std. dev.	0.66		R-squared	0.9548		
Mean	5.07		Adj R-squared	0.9142		
Coefficient of Variation%	13.02		Pred R-squared	0.7955		
PRESS	19.75		Adeq precision	15.679		

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