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LIGNOCELLULOSIC SYRUP PRODUCTION FROM ENERGY CANE BAGASSE AND FUMARIC ACID FERMENTATION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Nutrition and Food Sciences

by
Fang Deng
B.S. South China University of Technology, 2013
August 2017

Dedicated to my parents

Ms. Jiamin Zhang and Mr. Shang Deng

for their endless love, support and encouragement through all these years

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ABSTRACT

Lignocellulosic syrup from dilute ammonia pretreated energy cane bagasse is a promising fermentable sugar feedstock that can improve the logistics associated with long-distance transportation, long-time storage, and year-round supply of lignocellulosic biomass to processing industries. However, non-sugar compounds such as organic acids, furans and phenolic compounds formed during pretreatment can have negative effects on downstream processes such as fermentation.

This dissertation focused on addressing challenges associated with the bioconversion of energy cane bagasse to syrup and its fermentation to fumaric acid. The first goal (Chapter 2) was to evaluate the removal of non-sugar compounds with minimal sugar losses from dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate using the cationic flocculants polyethylenimine (PEI) and poly-diallyldimethylammonium chloride (pDADMAC). Optimum conditions for both PEI and pDADMAC were 15 g/L dose at unadjusted hydrolysate pH of 4.5. At these conditions, PEI outperformed pDADMAC by having greater adsorption efficiencies towards non-sugar compounds with minimal sugar losses. PEI removed 43% organic acids, 100% furans and 73% total phenolic compounds with less than 10% total fermentable sugar losses. However, recycling of PEI and recovery of adsorbed non-sugar compounds are not recommended for more than two cycles.

The second goal (Chapter 3) was to optimize activated carbon (AC, powdered and granular) detoxification of dilute ammonia pretreated energy cane bagasse hydrolysate via Response Surface Methodology. Optimum conditions for powdered AC were 9.21% (w/w) dose, at pH 1.96 for 10 min. Optimum conditions for granular AC were 12.64% (w/w) dose, at pH 1.91 for 51.60 min. At

optimum conditions, approximately 73% organic acids, 95% furans and 99% total phenolic compounds were removed by AC treatment with less than 10% fermentable sugar losses.

The last goal (Chapter 4) evaluated the use of AC treated lignocellulosic syrup as the carbon source for fumaric acid fermentation by *Rhizopus oryzae* ATCC® 20344™. Fumaric acid is a building block chemical that can be potentially manufactured using renewable lignocellulosic biomass and can serve as the raw material in the production of polymer resins, plasticizers, esters, and inks. Fumaric acid production was 34.20 g/L, with a yield of 0.43 g/g and a productivity of 0.24 g/L/h.

CHAPTER 1
INTRODUCTION

1.1. Bioconversion of Lignocellulosic Biomass

Energy demand is increasing every year due to the growth of world population and economy (Nanda et al., 2014). As the currently predominant and readily available energy source, fossil fuels have boosted industrialization and economic growth over the years. However, the adverse effects of fossil fuels such as greenhouse gas emission, air pollution and global climate change have raised concerns regarding their economic and environmental sustainability, and have shifted the attention to renewable energy resources such as wind, solar, nuclear, and bioenergy (Nanda et al., 2015). Among these renewable energy resources, bioenergy from lignocellulosic biomass is one of the most promising options having minimal impact on food and water resources, land-use and the ecosystem (Chung et al., 2013). Lignocellulosic biomass can also be used in the production of bio-hydrogen, microbial lipids and other platform chemicals such as lactic acid, fumaric acid, levulinic acid, and succinic acid, offering environmentally friendly alternatives to the traditional petroleum-based products (Chen et al., 2010; Girisuta et al., 2007; Liang et al., 2012; Maas et al., 2006; Pattra et al., 2008; Xu et al., 2010). The U.S. Department of Agriculture reported that the land resource in the U.S. is sufficient to produce over 1.3 billion tons of dry biomass annually, enough to sustain large-scale bioenergy and biorefinery industries by mid-21st century, while still meeting the demand for forestry, food and fiber products (Perlack et al., 2005). Crop residues (i.e., sugarcane bagasse, energy cane bagasse, sorghum bagasse, corn stover, rice straw, wheat straw), hardwood (i.e., black locust, poplar, oak, plum), softwood (i.e., cedar, pine, spruce), herbaceous biomass (i.e., switchgrass, Bermuda grass), and municipal solid wastes are some examples of lignocellulosic biomass to be used in the production of biofuels and bio-chemicals.

1.2. Energy Cane

Energy cane, a hybrid of commercial and wild sugarcanes, is one of the most attractive energy crops and lignocellulosic biomass, bred specifically for high fiber content (Kim & Day, 2011). Energy cane is cold tolerant and requires less fertilizer and water input than sugarcane (Sierra et al., 2008). Energy cane Ho 02-113 is a non-commercial variety developed by the U. S. Department of Agriculture – Agricultural Research Service, Houma, LA and Louisiana State University Agricultural Center Sugar Research Station, St. Gabriel, LA. It has an average fiber content of 261 g/Kg (Dry basis) and yield of 125.3 Mg/ha (Hale et al., 2013); whereas LCP 85-384, the most widely grown sugarcane variety in Louisiana, only has 117 g/Kg (Dry basis) fiber content and 59.2 Mg/ha yield (Gravois et al., 2009).

Lignocellulosic biomass is not readily available for bioconversion into bio-products due to its highly resistant nature (Laureano-Perez et al., 2005). Lignocellulose is mainly composed of cellulose (35-50% dry biomass), hemicellulose (20-35% dry biomass) and lignin (15-20% dry biomass) (Fig. 1.1). A cellulose chain consists of glucose units linked together by β-1,4 glycosidic bonds. Cellulose chains are bound together by hydrogen bonds and van der Waals forces, which make cellulose a highly crystalized polymer (Aita & Kim, 2010). Due to its crystalline structure, cellulose is water insoluble and highly resistant to chemical and biological degradation (Mood et al., 2013). Hemicellulose, a heterogeneous branched polymer of pentose, hexose and urgonic acids, is relatively amorphous with low molecular weight (Mood et al., 2013; Lee et al., 2009). The major component of hemicellulose is xylan, a polysaccharide with backbone chains of 1, 4-linked β-D-xylopyranose units (Menon & Rao, 2012). However, the hemicellulose layer with its covalent linkage to lignin and non-covalent interaction with cellulose limits enzyme accessibility (Agbor et al., 2011). Lignin is a highly branched aromatic polymer that mainly consists of ether linked

phenylpropanoid precursors including syringyl, guaiacyl and p-hydroxy phenol (Demirbas, 2008). Lignin matrix binds to cellulose and hemicellulose layers, giving both rigidity and resistance to the lignocellulosic structure (Mood et al., 2013). The association and complexity of the polysaccharides-lignin complex make enzymatic accessibility a challenge, which is the main obstacle in the bioconversion of fermentable sugars (Aita & Kim, 2010; Lee et al., 2009).

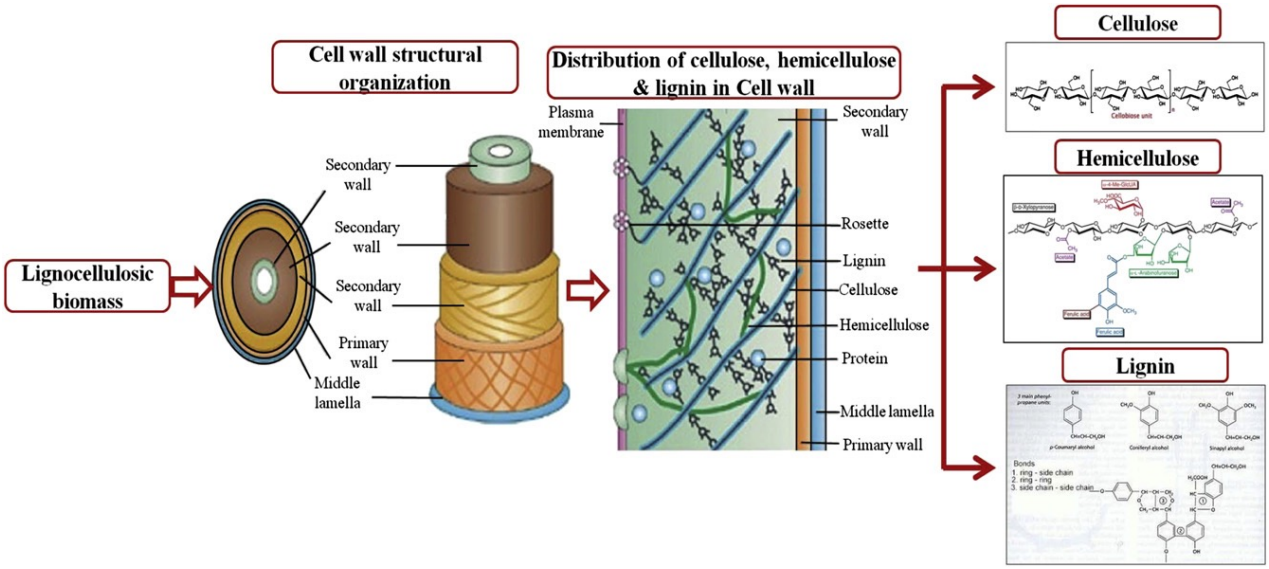

Figure 1.1 Structure and chemical composition of lignocellulosic biomass (Menon & Rao, 2012).

1.3. Pretreatment

Pretreatment prior to enzymatic hydrolysis is necessary to decrease the crystallinity of cellulose, reduce the lignin content, increase biomass porosity, and soften the hemicellulose-lignin shield that surrounds the cellulose (Kumar et al., 2009) (Fig. 1.2). The overall target of pretreatment is to provide enzymes better accessibility to the polymeric sugars, thus enhancing the bio-digestibility of biomass (Behera et al., 2014). A successful pretreatment should meet the following requirements: improve enzymatic accessibility with minimal fermentable sugars losses, reduce the formation of non-sugar compounds (i.e., organic acids, furan derivatives, phenolic compounds) and be cost-effective (Sun & Cheng, 2002). Numerous pretreatment methods have been reported, including physical, chemical, physicochemical, biological, or a combination of

these methods. Each type of pretreatment method has its own advantages and disadvantages (Laureano-Perez et al., 2005).

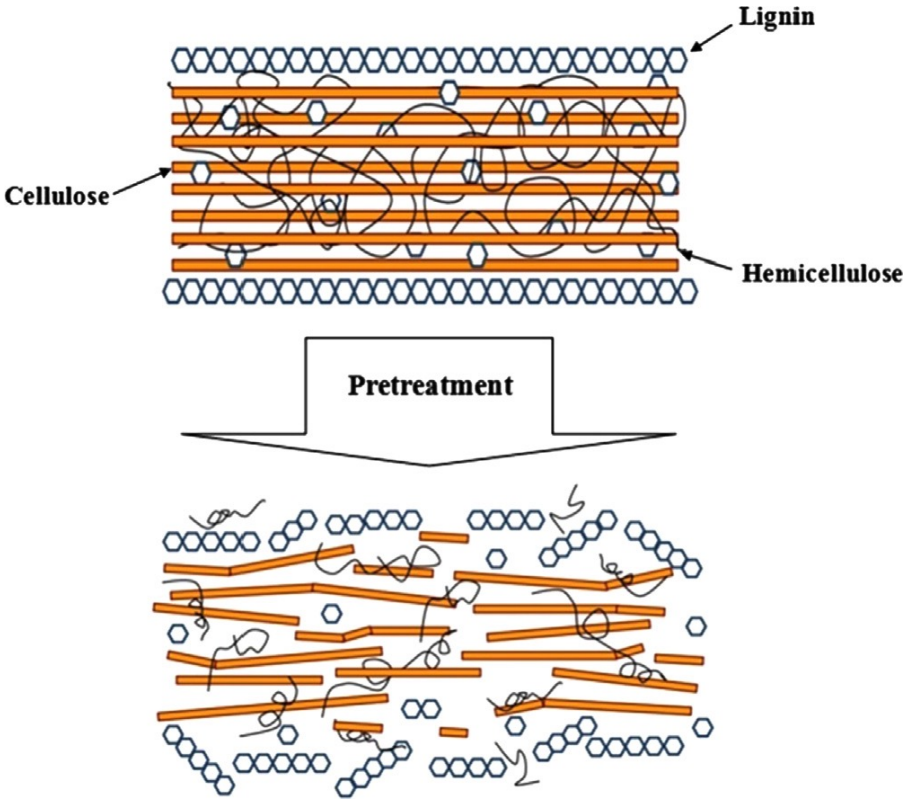

Figure 1.2 Effect of pretreatment on lignocellulosic materials (Mood et al., 2013).

1.3.1. Physical Pretreatments

Physical processes such as milling, grinding, extrusion, chipping, and irradiation can be used to increase the surface area of biomass and decrease the degree of polymerization of cellulose, which can improve enzymatic digestibility of lignocellulosic biomass (Harmsen et al., 2010). However, high energy consumption associated with these methods make them less economical and practical (Behera et al., 2014). Extrusion can result in both physical and chemical modifications of lignocellulosic materials (Karunanithy & Muthukumarappan, 2010). Irradiation (i.e., microwave, gamma-ray, ultrasonic waves) provides enough energy to breakdown the hydrogen bonds in the crystalline structure of cellulose, thus dramatically enhancing the efficiency of enzymatic hydrolysis (Bochek, 2003; Imai et al., 2004).

1.3.2. Chemical Pretreatments

1.3.2.1. Acid Pretreatment

Acid pretreatment by sulfuric acid or hydrochloric acid is one of the most widely applied techniques for lignocellulosic biomass pretreatment (Mood et al., 2013). Acid pretreatment can be conducted under either concentrated acid at mild temperatures or diluted acid at high temperatures (Taherzadeh & Karimi, 2008). Although acid pretreatment has been proven to be effective in enhancing enzymatic digestibility, it has some drawbacks that include the generation of non-sugar compounds, corrosiveness to equipment, toxicity, and high maintenance costs (Romero et al., 2010; Sun & Cheng, 2002).

1.3.2.2. Alkaline Pretreatment

Alkaline pretreatments increase enzymatic accessibility by increasing surface porosity and by decreasing the degree of polymerization as result of saponification of the ester bonds that crosslink the hemicellulose, cellulose and lignin (Tarkow & Feist, 1969). Sodium hydroxide pretreatments have been extensively investigated and reported to be effective in multiple lignocellulosic materials such as hardwood, softwood, wheat straw, and corn stalk (Bjerre et al., 1996; Chosdu et al., 1993; Mirahmadi et al., 2010; Zhao et al., 2008). Dilute ammonia pretreatment has been previously reported to be highly effective in delignifying and improving enzymatic digestibility with reduced formation of non-sugar compounds (Aita et al., 2011; Chen et al., 2012; Oladi & Aita, 2017). Unlike acid pretreatment, mild alkaline pretreatments can be achieved at lower temperatures, which result in reduced solubilization of hemicellulose and the formation of non-sugar compounds (Jönsson & Martín, 2016). The major disadvantage of alkaline pretreatment is the generation of large amount of irrecoverable salts (Zheng et al., 2009).

1.3.2.3. Ionic Liquid (IL) Pretreatment

Ionic liquids (ILs) are organic salts that remain in the liquid form below 100°C or even at room temperature (Tan et al., 2011). ILs can be used in the pretreatment of lignocellulosic biomass due to their ability to dissolve carbohydrates and lignin with minimal generation of non-sugar compounds (Dadi et al., 2007). Energy cane bagasse pretreated with [EMIM][OAc] (5% (w/w)) at 120 °C for 30 min resulted in 32% lignin removal and improved cellulose digestibility from 5.5% (untreated biomass) to 87% (Qiu et al., 2012). Drawbacks include high operational and recovery costs (Fu & Mazza, 2011).

1.3.2.4. Ozonolysis

Ozone gas pretreatment can degrade lignin and hemicellulose and increase the biodegradability of cellulose. Ozonolysis has been shown to be effective in various lignocellulosic materials such as wheat straw, pine, cotton straw, and poplar sawdust (Sun & Cheng, 2002). As a strong oxidant, ozone gas has a high solubility and availability in water. Advantages include high efficiency in lignin removal, reduced formation of non-sugar compounds, and mild temperature and pressure requirements (Vidal & Molinier, 1988). Binder et al. (1980) reported that 200 mg ozone/g wheat straw was required to achieve a 60% lignin removal (Binder et al., 1980). A major drawback is the high cost and use of ozone gas at industrial scale.

1.3.3. Physicochemical Pretreatments

1.3.3.1. Steam Explosion

Steam explosion exposes biomass to high temperature (160–260 °C) and pressure (0.69–4.83 MPa) for a short period of time followed by a rapid pressure release. High temperature and pressure treatment result in lignin and hemicellulose degradation (Zhang et al., 2017). Acid catalysts such as sulfuric acid (H_2SO_4) or carbon dioxide (CO_2) can be introduced during steam

explosion pretreatment to achieve a higher removal of lignin and hemicellulose and improve enzymatic digestibility (Morjanoff & Gray, 1987). However, high temperature and the addition of acids can lead to an increase in the formation of non-sugar compounds, a major downside of steam explosion pretreatment (Mackie et al., 1985). Despite such disadvantages, steam explosion is one the very few fully commercialized pretreatment technologies due to its lower capital investment and higher energy efficiency as compared to other pretreatment processes such as dilute acid pretreatment and ammonia fiber explosion (Baral & Shah, 2017).

1.3.3.2. Ammonia Fiber Explosion (AFEX)

AFEX is an ammonia based steam explosion pretreatment. However, unlike steam explosion, AFEX only requires moderate temperatures (60–120°C) thus less non-sugar compounds are generated (Kumar et al., 2009; Sun & Cheng, 2002). AFEX is effective in most biomass materials except for those with high lignin content such as aspen chips (25% lignin) (McMillan, 1994). A drawback is the efficient ammonia recovery and processing costs (Holtzapple et al., 1992).

1.3.4. Biological Pretreatments

Lignocellulosic biomass pretreatment can be achieved through biological pathways using microorganisms such as white-, brown- and soft-rot fungi that are able to delignify and improve enzymatic digestibility (Sun & Cheng, 2002). Brown-rot fungi only breaks down cellulose; whereas, white-rot fungi can break down both cellulose and lignin (Fan et al., 1987). In biological pretreatment, process parameters including fungi species, biomass particle size, biomass moisture content, pretreatment time and temperature must be carefully optimized and controlled to achieve an ideal enzymatic hydrolysis yield (Mood et al., 2013). Unlike other pretreatments, microbial pretreatment does not require chemicals, which makes it an environmentally friendly and cost

effective alternative to lignocellulose pretreatment (Rouches et al., 2016). Additionally, biological pretreatment with white-rot fungi can be combined with wet storage, an efficient way to not only preserve the cellulose, but also improve enzymatic digestibility by selectively removing the lignin (Cui et al., 2012).

1.4. Enzymatic Hydrolysis

Hydrolysis is the processing step that comes after pretreatment and converts the polymeric sugars into their monomeric forms (mostly glucose and xylose). Several hydrolysis methods have been studied throughout the years. Compared to acid or alkaline hydrolysis, enzymatic hydrolysis is the preferred method due to its effectiveness, moderate requirement of pH (4-5) and temperature (45-55°C), and non-corrosive properties (Sun & Cheng, 2002). Enzymes used for lignocellulose hydrolysis can be produced by both bacteria such as *Clostridium cellulovorans* and fungi such as *Trichoderma reesei* and *Aspergillus niger* (Talebnia et al., 2010).

Cellulase is a class of enzymes that attacks β-1,4 glycosidic bonds, and breaks down cellulose into glucose. Commercially available cellulase is a mixture of three different enzymes, namely endoglucanase, exoglucanase and β-glucosidase. Endoglucanase attacks the low crystallinity region of cellulose, which releases free chain-ends. The free chain-ends are then cleaved by exoglucanase releasing cellobiose. β-glucosidase hydrolyzes cellobiose into the final product, glucose (Sun & Cheng, 2002; Talebnia et al., 2010).

Addition of accessory enzymes such as xylanase, feruloyl esterase, pectinase, and laccase can further improve cellulose digestibility (Talebnia et al., 2010). Xylanase, one of the most commonly used accessory enzymes, generally consists of endoxylanase and β-xylosidase. Hemicellulose is first cleaved by endoxylanase releasing xylooligosaccharides, which are then broken down into xylose by β-xylosidase (Juturu & Wu, 2012). The hydrolysis of hemicellulose

improves cellulase accessibility to cellulose. Additionally, xylanase can boost cellulose digestibility by hydrolyzing xylooligosaccharides, which are known to inhibit cellulase. Moreover, a synergistic effect (higher sugar yield) has been reported with combined use of cellulase and xylanase (Duff & Murray, 1996; Juturu & Wu, 2012; Song et al., 2016).

1.5. Syrup Production

Lignocellulosic hydrolysates are extremely susceptible to microbial deterioration due to their high water and sugar content (Eggleston et al., 2015). Syrup production can significantly reduce the water activity, control microbial growth and preserve the sugars in the hydrolysates. According to Eggleston et al. (2013), an 80°Bx syrup inhibited the growth of most microorganisms found in sorghum juice. Similar observations were reported by Bruemmer & Bowers (1977) when yeast growth was significantly hindered in a 70°Bx orange juice syrup. Under vacuum conditions, hydrolysates can be concentrated into syrups with minimal entrainment and sugar degradation. Therefore, concentration of hydrolysates into a stable syrup is critical and beneficial for long-distance transportation, long-time storage and year-round supply to bio-based fuels and chemicals manufacturing plants (Andrzejewski et al., 2013).

1.6. Generation of Non-Sugar Compounds

1.6.1. Inhibitory Effect of Non-Sugar Compounds

Hydrolysates that result from the pretreatment of lignocellulosic biomass may contain by-products other than sugars, including organic acids (i.e., acetic acid, formic acid, levulinic acid), furans (i.e., furfural, 5-hydroxymethylfurfural (HMF)) and phenolic compounds (Fig. 1.3). These non-sugar compounds can negatively affect downstream processes such as enzymatic hydrolysis and fermentation. The nature and concentration of the generated non-sugar compounds are directly related to pretreatment conditions and biomass composition (Jönsson & Martín, 2016).

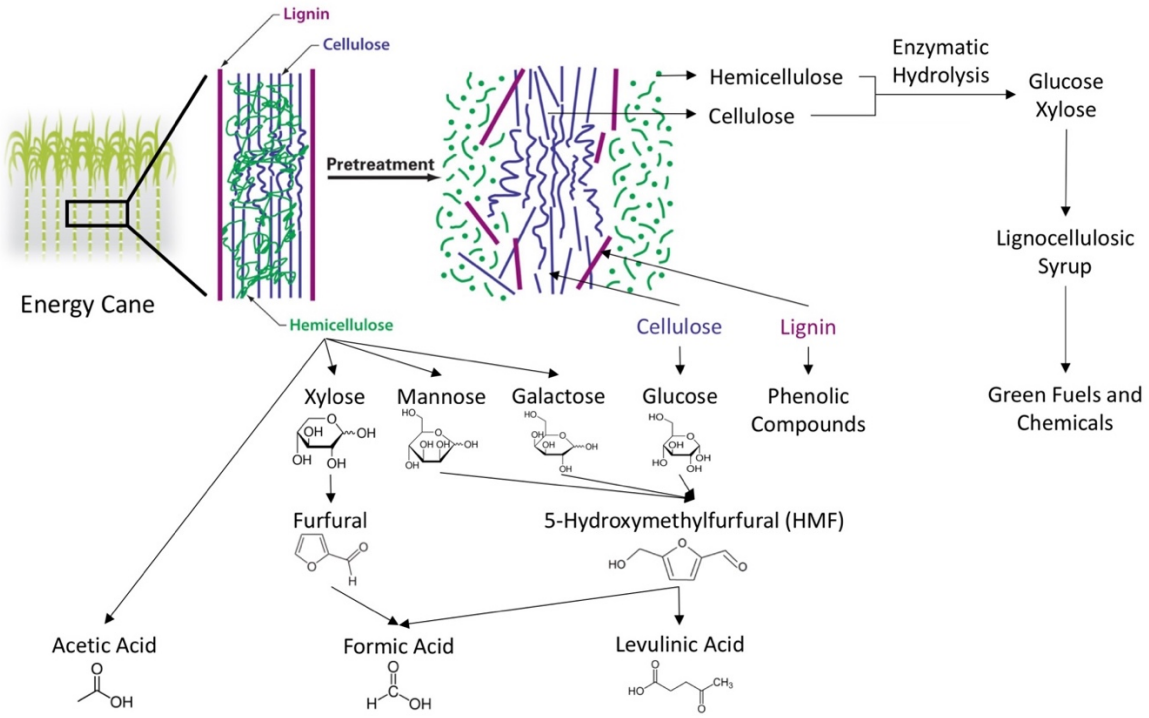

Figure 1.3 Formation of non-sugar compounds from pretreated lignocellulosic biomass (Palmqvist & Hahn-Hägerdal, 2000b).

Organic acids production is promoted when high temperatures and long residence times are applied during pretreatment (Carter et al., 2011b; Jönsson & Martín, 2016). Acetic acid is a degradation product of hemicellulose. Formic acid is formed when furfural and HMF are degraded, while levulinic acid is formed by HMF degradation (Palmqvist & Hahn-Hägerdal, 2000a). Organic acids are liposoluble in their un-dissociated forms and can pass through plasma membranes causing a drop in their intracellular pH. The accumulated organic acids can be neutralized by ATPase; however, this results in the decrease of ATP available for hydrolysis, a reaction that provides energy for cell growth (Palmqvist & Hahn-Hägerdal, 2000b). Toxicity of organic acids towards microorganisms can differ. Formic acid and acetic acid have relatively stronger inhibitory effects on microbial growth compared to levulinic acid, citric acid and succinic acid (Jönsson & Martín, 2016). Concentrations of such organic acids exceeding 100 mM have been observed to interfere with cell growth in *Saccharomyces cerevisiae* (Larsson et al., 1999).

Furans including furfural and HMF are formed from the degradation of pentose and hexose sugars, respectively. Furfural is metabolized by microorganisms such as *S. cerevisiae* and reduced to furfuryl alcohol with the excretion of acetaldehyde and pyruvate, which results in a longer lag-phase and inactivation of cells replication (Palmqvist et al., 1999). Similarly, HMF can be metabolized by microorganisms and converted to 5-hydroxymethyl furfuryl alcohol (Taherzadeh et al., 2000). However, the conversion of HMF has been found to be slower than furfural, which results in an even longer lag-phase (Palmqvist & Hahn-Hägerdal, 2000b). Inhibitory effects of furans can be noticeable at concentrations exceeding 1 g/L (Carter et al., 2011a; Klinke et al., 2004).

Phenolic compounds such as 4-hydroxybenzoic acid, vanillin and catechol are formed from the thermal degradation and oxidation of lignin (Palmqvist & Hahn-Hägerdal, 2000b). Generally, each individual aromatic carboxylic acid exists at a much lower concentration in lignocellulosic hydrolysates as compared to organic acids. However, the toxicity of aromatic carboxylic acids to microorganisms is stronger than organic acids, which makes phenolic compounds a group of strong inhibitors (Jönsson & Martín, 2016). Phenolic compounds with low molecular weights have strong inhibitory effects during fermentation. Ando et al. (1986) reported that 4-hydroxybenzoic acid at 1g/L reduced ethanol yields from *S. cerevisiae* by 30%; whereas, vanillic acid had no adverse effect at the same concentration. Phenolic compounds also interfere with the activity of enzymes during enzymatic hydrolysis by binding to enzymes (cellulase and β-glucosidase) and by preventing them from attaching to cellulose (Ximenes et al., 2011).

1.6.2. Value-added Products

Non-sugar compounds (i.e., formic acid, acetic acid, levulinic acid, HMF, furfural) generated during pretreatment can be separated and recovered as platform chemicals for numerous

products. Formic acid (USD 500 million market size) is a colorless liquid with major applications in silage and animal feed preservation, textiles, formate salts, food preservatives, rubber chemicals, catalysts, and plasticizers (Hietala et al., 2000). Acetic acid (USD 9 billion market size) is used directly as a condiment, and in the pickling of vegetables and other foods. The largest industrial application for acetic acid is in the production of vinyl acetate monomers, closely followed by acetic anhydride and ester production (Le Berre et al., 2000). Levulinic acid (USD 19 million market size) has diverse applications as a plasticizer. Several derivative chemicals can also be produced from levulinic acid through chemical processes including γ-valerolactone (GVL), 5-aminolevulinic acid and diphenolic acid. GVL is another crucial platform chemical that can be used as a gasoline additive, 5-aminolevulinic acid is a biological herbicide and diphenolic acid is a potential replacement for bisphenol, which is used in the production of resins and plastics (Choi et al., 2015). HMF can be converted to levulinic acid, dimethylfuran, 2,5-furandicarboxylic acid, and dihydroxymethylfuran, which are building blocks in the manufacture of alternative fuels, polymers, foams, and polyesters (Rosatella et al., 2011). HMF can be produced from the dehydration of C6 sugars and a bio-based production method has been commercialized (Choi et al., 2015). Furfural (USD 1200 million market size) has an increasing market size estimated to be over 200,000 t per year due to its attractive thermosetting properties, physical strength and corrosion resistance as well as diverse functionalities (Cai et al., 2014). Furfural can be further converted to various other chemicals including furfuryl alcohol or 2-methylfuran through hydrogenation and to maleic acid or furoic acid through oxidation (Choi et al., 2015). Furfuryl alcohol and 2-methylfuran can be potentially used in the production of alternative fuels. Maleic acid and furoic acid are industrial materials used in the production of multiple chemicals and can be used as food preservatives.

1.7. Detoxification

Non-sugar compounds present in the hydrolysate are concentrated during syrup production and can negatively affect downstream processes. Removing these non-sugar compounds with minimal fermentable sugar losses can result in better fermentability when compared to untreated hydrolysates (Canilha et al., 2012; Kamal et al., 2011; Margeot et al., 2009). Therefore, a detoxification process is necessary to improve syrup quality and purity. Furthermore, the separated non-sugar compounds can be recovered and used as building blocks in the production of value-added chemicals (Carter et al., 2011a). A variety of effective detoxification methods have been studied over the years, which can be categorized as physical, physicochemical and biological methods (Palmqvist & Hahn-Hägerdal, 2000a).

1.7.1. Physical Methods

Evaporation is one of the most widely used physical detoxification methods. Evaporation under vacuum conditions can selectively remove the volatile portion of the non-sugar compounds such as acetic acid, furfural and vanillin without significant sugar losses (Palmqvist & Hahn-Hägerdal, 2000a). However, the non-volatile fraction of non-sugar compounds such as phenolic compounds have stronger inhibitory effects on fermentation processes as compared to the volatile compounds and are concentrated during evaporation (Palmqvist et al., 1996). Therefore, evaporation is often combined with other detoxification methods for the removal of all inhibitory compounds (Villarreal et al., 2006).

1.7.2. Physicochemical Methods

1.7.2.1. Organic Solvent Extraction

Polar organic solvents such as ethyl acetate have strong capabilities in removing acetic acid, furfural and low molecular weight phenolic compounds such as 4-hydroxybenzoic acid and

vanillin (Mussatto & Roberto, 2004). The solvents can be recycled to reduce operational cost. However, a major drawback of solvent extraction is high cost due to large amounts of organic solvents required and the relatively long extraction times needed (Cantarella et al., 2004; Palmqvist & Hahn-Hägerdal, 2000a). Cantarella et al. (2004) pointed out that although ethyl acetate extraction alone on steam-exploded poplar wood enzymatic hydrolysate significantly removed phenolic compounds, the actual improvement in the final ethanol fermentation yield was found to be minimal.

1.7.2.2. Ion Exchange Resin

Ion exchange resin detoxification has been reported to be effective in removing non-sugar compounds from hydrolysates and improving fermentability (Canilha et al., 2004; Chandel et al., 2007; Villarreal et al., 2006). The major concerns with ion exchange resin detoxification are high pressure, long process times, and degradation of sensitive biological products, which result in high operational and maintenance costs and difficulties in scale-up despite the reusability of the resins (Canilha et al., 2012). Ion exchange resin detoxification carried out in a column is more efficient, convenient and flexible than a batch process. Factors such as the charge of resins, hydrolysate pH and flow rate can significantly affect the detoxification efficiency (Nilvebrant et al., 2001). Villarreal et al. (2006) reported that eucalyptus (hemicellulose) acid hydrolysate at pH 5.5 passing through an anion exchange resin column at 10 mL/min flow rate was able to remove 96.2% HMF, 96.1% furfural, 100% acetic acid, and 87.7% phenolic compounds without noticeable xylose losses, which improved xylitol fermentation yields by 71%.

1.7.2.3. Overliming

Overliming is an alkaline treatment that increases the hydrolysate pH to 9-10 using calcium hydroxide, sodium hydroxide or calcium oxide and then readjusts the pH with sulfuric acid. The

mechanism behind overliming is precipitation and instability of some non-sugar compounds at high pH (Palmqvist & Hahn-Hägerdal, 2000a). However, overliming has some disadvantages such as high sugar losses, waste generation and complex filtration requirements (Canilha et al., 2013). Mussatto & Roberto (2004) reported that overliming of olive tree pruning residue acid hydrolysate with calcium hydroxide removed up to 52% acetic acid, 79% furans and 69% phenolic compounds with an undesirable 47% total sugar loss. Heat can be applied during overliming to achieve higher removal of non-sugar compounds with the sacrifice of increased sugar losses (Horváth et al., 2005).

1.7.2.4. Activated Carbon

Activated carbon (AC) is one of the most widely applied methods for removing non-sugar compounds from enzymatic and acid hydrolysates due to its low cost, high capacity of adsorption and ease of use (Kamal et al., 2011). AC dose, surface area, pH, agitation, contact time, and temperature are the major factors that affect the performance of the detoxification process. The effectiveness of detoxification also depends on the type of lignocellulose hydrolysate and conditions of pretreatment and hydrolysis used (Mussatto & Roberto, 2004). AC dose and external surface area affect the total adsorption capacity towards adsorbates, pH influences the charge properties of both adsorbate and adsorbent, and adequate contact time between AC and hydrolysate is essential to achieve the AC equilibrium with the non-sugar compounds (Liu et al., 2010; Mussatto & Roberto, 2004). Among all these factors, agitation and temperature have been proven to be statistically insignificant to responses including acetic acid removal, furfural removal and total sugar loss (Mateo et al., 2013). The increase in agitations from 100-200 rpm and the raise in temperature from 25-35°C had no significant improvement in non-sugar compounds removal and total sugar loss (Mateo et al., 2013).

AC adsorption demonstrated higher efficiency in furans and phenolic compounds removal when compared to acetic acid removal. Lee et al. (2011) reported that in acid treated woody hydrolysate, AC treatment at 5% (w/w) AC dose for 0.5 h removed 32.4% acetic acid, 72.4% formic acid and nearly 100% HMF and furfural. Mateo et al. (2013) observed that AC detoxification at 8% (w/w) dose removed 97% furfural and 81% total phenolic compounds, but only 45% acetic acid was removed from olive tree pruning residue acid hydrolysate. AC treatment parameters need to be carefully controlled, otherwise the loss of fermentable sugars reach up to 20-30% (Lee et al., 2011; Wang & Chen, 2011).

1.7.2.5. Flocculation

Flocculation is a process usually applied to enhance solid-liquid separations. Various mechanisms (i.e., ionic strength, hydrophobic interactions) can take place depending on the characteristics of flocculants (i.e., charge density, chain length, branch structure) and the properties of components in the targeted solutions (i.e., charge, particle size) (Carter et al., 2011a). Polyethylenimine (PEI) and poly-diallyldimethylammonium chloride (pDADMAC) are two water soluble, high molecular weight and high charge density cationic polyelectrolytes. Both PEI and pDADMAC have been shown to be effective in separating not only solids from solutions, but also dissolved components, such as humic and fulvic acids from groundwater (Matilainen et al., 2010). Moreover, these polyelectrolytes have been used in the detoxification of soft wood slurries and hydrolysates with promising results in removing organic acids and furans (Carter et al., 2011b; Yasarla & Ramarao, 2012). Organic acids are adsorbed by a polyamine group following weak acid neutralization, and the generated complex is then removed by microfiltration process (Cannella et al., 2014; Carter et al., 2011a). Furans can be adsorbed by the primary and secondary amine functionalities following the Mannich reaction as demonstrated in Reaction (1) (Carter et al.,

2011a). Addition of water or decreasing the pH can drive the equilibrium to the left and reverse the reaction. Similarly, weak acid or weak base neutralization can be reversible if the pH is properly adjusted. Therefore, flocculants can be potentially recycled and the adsorbed non-sugar compounds can be recovered as value-added products (Cannella et al., 2014; Carter et al., 2011a). The performance of flocculants highly depends on the following factors: (I) flocculation treatment conditions (i.e., flocculant dose, hydrolysate pH); (II) pretreatment and hydrolysis methods used; (III) stage of detoxification (i.e., pre-hydrolysis or post-hydrolysis); (IV) properties of the hydrolysate (i.e., charge density, charge distribution, turbidity); (V) interferences from non-inhibitory compounds in hydrolysates, such as suspended particles, sugars, enzymes, and ions (Cannella et al., 2014; Gurram et al., 2011).

$$R_2NH + 2C_5H_4O_2 \leftrightarrows R_2NCC_4H_3OC_5H_3O_2 + H_2O \quad (1)$$

1.7.3. Biological Methods

Biological detoxification uses specific microorganisms or enzymes to reduce the amount of non-sugar compounds present in the hydrolysate (Canilha et al., 2013). It is considered an environmentally friendly method due to the minimal chemical involvement, low waste generation and reduced energy consumption. However, biological detoxification generally requires longer process times as compared to physicochemical methods (Moreno et al., 2012). Laccases are multicopper-containing phenoloxidases that catalyze the oxidation of phenols and aromatic thiols. Laccase detoxification aims specifically at removing monoaromatic phenolic compounds, thus improving fermentation productivity (Jönsson et al., 1998). Microorganisms such as *Issatchenkia occidentalis* and *Issatchenkia orienalis* have been used in the detoxification of sugarcane bagasse acid hydrolysates that resulted in the complete removal of acetic acid and furfural and doubled xylitol fermentation productivity (Hou-Rui et al., 2009).

1.8. Fumaric Acid Fermentation

1.8.1. Fumaric Acid Properties and Applications

Fumaric acid is a dicarboxylic acid with a USD 700 million market size. Fumaric acid has been selected by the U.S. Department of Energy as one of the top 12 chemical building blocks that can potentially be produced from lignocellulosic biomass (Liu et al., 2015). The largest use for fumaric acid is in the synthesis of different types of resin, including paper resins, unsaturated polyester resins, and alkyd resins (Engel et al., 2011). Fumaric acid is used as an acidulant in food products, such as tortillas, doughs, desserts, fruit juice, and wine, and it can be added to animal feeds to improve weight gain and lower methane emissions (Mondala, 2015; McGinn et al., 2004). It can be used as the chemical intermediate for the synthesis of maleic acid, succinic acid and malic acid (Mondala, 2015). The ester derivates of fumaric acid also have wide applications in multiple industries. For example, monoethyl fumarate and dimethyl fumarate can serve as mold inhibitor and in the medical treatment to psoriasis (Moharregh-Khiabani et al., 2009).

1.8.2. Current Production Methods

Fumaric acid is currently produced via the catalytic isomerization of petroleum-derived maleic acid, having actual yields of up to 90% (Zhang et al., 2013). Although the petroleum-based chemical method has a high production yield, economic and environmental sustainability concerns as well as consumers' preference for bio-based products are encouraging the use of bio-based manufacturing processes using renewable feedstocks. Fumaric acid can be biologically produced as an intermediate compound in the tricarboxylic acid (TCA) cycle (Fig. 1.4). Fumaric acid production has been shown to occur mostly in filamentous Mucoralean fungi, particularly those belonging to the *Rhizopus* genus. Glucose is the preferred carbon source due to its direct biochemical pathway towards fumaric acid production. Various substrates have been used in the

production of fumaric acid by *Rhizopus oryzae* (Table 1.1) but no information is available on the use of lignocellulosic syrup as potential feedstock.

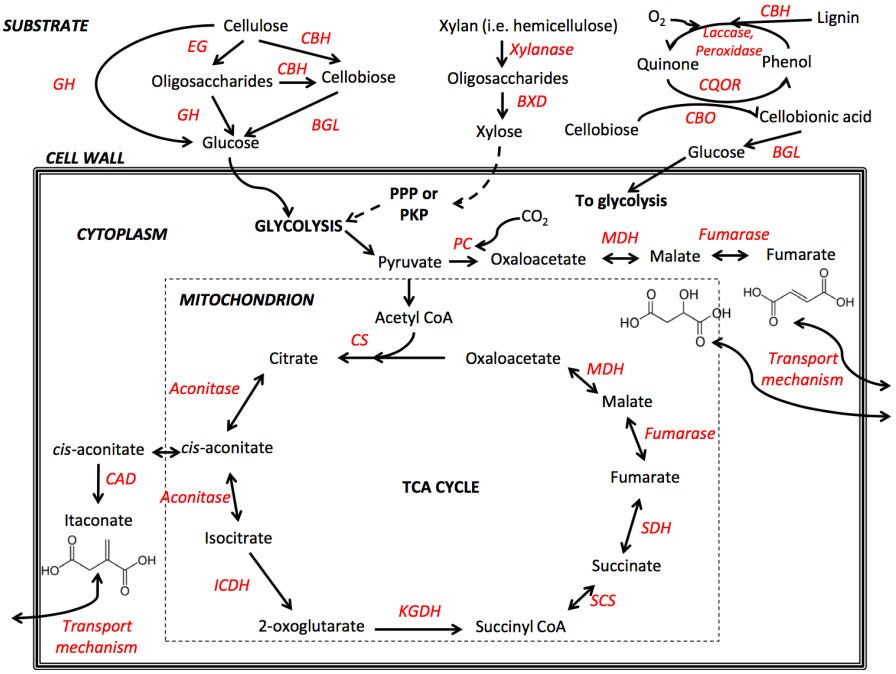

Figure 1.4 Biochemical pathways of the bioconversion of fumaric acid from lignocellulose by filamentous fungi (Mondala, 2015).

Table 1.1 Fumaric acid production by *Rhizopus oryzae*.

Fermenter	Substrate	Production (g/L)	Yield (g/g)	Productivity (g/L/h)	Reference
Shake flask	Glucose	50.2	0.72	0.34	Zhang et al., 2015
Stirred tank	Glucose	56.2	0.54	0.7	Fu et al., 2010a
Shake flask	Glucose	40.3	0.51	0.56	Ding et al., 2011
Shake flask	Corn straw	27.8	0.35	0.33	Xu et al., 2010
Stirred tank	Dairy manure	31	0.31	0.32	Liao et al., 2008
Shake flask	Eucalyptus wood	9.84	0.44	0.43	Rodríguez-López et al., 2012
Stirred tank	Glucose	42.5	0.56	0.51	Fu et al., 2009
Airlift loop reactor	Glucose	37.4	0.75	0.81	Du et al., 1997
Rotary biofilm contactor	Glucose	85	0.91	4.25	Cao et al., 1996
Bubble column	Glucose	37.2	0.53	1.03	Zhou et al., 2002
Shake flask	Glucose	56.2	0.54	0.7	Fu et al., 2010b
Stirred tank	Glucose	41.1	0.48	0.37	Huang et al., 2010
Stirred tank	Glucose	32.1	0.45	0.32	Kang et al., 2010

1.8.3. Fermentation Process

Generally, fumaric acid fermentation consists of two steps: (1) seed culture and (2) acid production. Seed culture medium provides adequate nutrients for the fast accumulation of cells. Harvested cells are then transferred to an acid fermentation medium, which is nitrogen limited for maximum fumaric acid production. During acid production, neutralizing agents such as calcium carbonate ($CaCO_3$) are added to the medium to eliminate end-product inhibition by neutralizing the fumaric acid. Fumarate salt is acidified and filtrated at elevated temperatures (80°C) to release the fumaric acid (Mondala, 2015; Xu et al., 2010; Zhang et al., 2015). Fungal morphology control is crucial for fermentation by filamentous fungi as to their complex morphologies (e.g. large clumps) may lead to high broth viscosity, poor agitation and insufficient dissolved oxygen levels (Zhang et al., 2015). Uniform small pellets are favored for optimum agitation, culture rheology and oxygen transfer in fumaric acid fermentation by *Rhizopus oryzae*, (Liao et al., 2007).

Glucose is the preferred carbon source for the genus *Rhizopus*. Xylose utilization from lignocellulosic hydrolysates has been found to be a less favorable carbon source than glucose due to the differences in their metabolic pathways as shown in Fig. 1.4. It was reported that using xylose as the sole carbon source by *Rhizopus arrhizus* resulted in a reduced fumaric acid yield (0.23 g per g xylose consumed) as compared to glucose (up to 0.60 g/g) (Kautola & Linko, 1989; Moresi et al., 1992). However, Liu et al. (2015) reported that *Rhizopus arrhizus RH 7-13-9#* yielded 45.31 g/L fumaric acid in a high xylose concentration medium. Another solution to better utilize the xylose in the hydrolysate is to use it in the seed culture medium. Xu et al. (2010) reported that using corn straw acid hydrolysate (30 g/L xylose) as seed culture and enzymatic hydrolysate from corn straw (80 g/L glucose) as acid production medium resulted in up to 27.8 g/L fumaric acid production. Nitrogen source can also significantly influence fungal growth and fumaric acid

production. It has been reported that complex nitrogen sources such as peptone, yeast extract and soybean flour are best for fugal growth and biomass accumulation; whereas, pure or inorganic nitrogen sources such urea and potassium nitrate are better for fumaric acid production (Carta et al., 1999). The ratio of carbon source (C) to nitrogen source (N) is another crucial factor that can significantly affect fungal growth and fumaric acid fermentation productivity. A high C/N ratio (up to 400 (w/w)) is generally preferred since the limitation of nitrogen supply favors fumaric acid production due to increase fumarase (the key enzyme that catalyzes the dehydration reaction of malic acid to fumaric acid) activity under nitrogen limited conditions (Ding et al., 2011; Xu et al., 2010). However, reduced fumaric acid yields have been reported at N-free conditions (Zhang et al., 2013).

Neutralizing agents are added to the acid production media to control the media pH and to provide CO_2 as a source for oxaloacetate, an intermediate product in fumaric acid production (Mondala, 2015; Zhang et al., 2013). Calcium carbonate, sodium carbonate and sodium bicarbonate are commonly used as neutralizing agents. However, sodium salts are less favored due to the inhibitory effect of their ions. Xu et al. (2012) reported that the high concentrations of Na^+ in the hydrolysate favored the production of by-products such as malic acid and ethanol as compared to calcium carbonate.

1.9. Goals of This Study

1. Evaluate the effect of flocculants on the removal of non-sugar compounds from dilute ammonia pretreated energy cane bagasse hydrolysates. Optimize process parameters (flocculant type, dose, and hydrolysate pH) for maximum removal of non-sugar compounds and minimum fermentable sugars losses. Investigate flocculant recyclability and recovery of non-sugar compounds.

2. Optimize activated carbon (AC) detoxification process using Response Surface Methodology for maximum non-sugar compounds removal and minimum fermentable sugars losses from dilute ammonia pretreated energy cane bagasse hydrolysates.

3. Produce lignocellulosic syrup from AC treated energy cane bagasse enzymatic hydrolysate. Optimize fermentation parameters (fungal morphology and medium composition) for maximum fumaric acid yields by *Rhizopus oryzae* ATCC® 20344™.

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CHAPTER 2
DETOXIFICATION OF DILUTE AMMONIA PRETREATED ENERGY CANE BAGASSE ENZYMATIC HYDROLYSATE BY SOLUBLE POLYELECTROLYTE FLOCCULANTS

2.1. Introduction

Lignocellulose is a highly sustainable and renewable natural resource that can be used in the production of syrup, as a stable, long-term storage feedstock, for the processing of hydrogen, microbial lipids, biodegradable plastics, and other value-added chemicals (Canilha et al., 2012; Duarte et al., 2010; Liang et al., 2012; Pattra et al., 2008). Lignocellulose is made up of highly crystalline cellulose, amorphous hemicellulose and branched lignin. Such complex structure makes lignocellulosic materials highly resistant to chemical and biological degradation (Laureano-Perez et al., 2005). Energy cane, a hybrid of commercial and wild sugar canes, is one of the most attractive lignocellulosic materials due to its higher fiber content, cold resistance, lower water input and fertilizer requirement as compared to sugarcane (Kim & Day, 2011). A year-round supply of lignocellulosic biomass to biorefineries is challenging due to the seasonality of the crops and their high water and sugar content, which make them highly susceptible to microbial degradation. Syrup production from the concentration of sugars from hydrolysates can significantly reduce water activity, control microbial growth and preserve the sugars thus providing solutions to challenges associated with long-distance transportation, long-time storage, and biomass supply to bio-based fuels and chemicals manufacturing plants (Eggleston et al., 2015).

Pretreatment prior to enzymatic hydrolysis is necessary to reduce the lignin content and soften the hemicellulose-lignin shield that surrounds the cellulose (Laureano-Perez et al., 2005). Dilute ammonia pretreatment is one of the pretreatments that is effective in delignifying and improving enzymatic digestibility (Aita et al., 2011). Post pretreatment, hydrolysis converts

polysaccharides into fermentable monomeric sugars. Compared to acid or alkaline hydrolysis, enzymatic hydrolysis is the preferred method due to its effectiveness, moderate pH and temperature requirements, and non-corrosive properties (Sun & Cheng, 2002). Regardless of the positive outcomes that result from pretreatment and hydrolysis processes, harsh processing conditions can promote the formation of non-sugar compounds (i.e., organic acids, furans, phenolic compounds) from lignocellulosic materials. Organic acids (i.e., acetic acid, levulinic acid, formic acid) result from the degradation of hemicellulose. Furans including furfural and 5-hydroxymethylfurfural (HMF) are formed from the degradation of pentose and hexose sugars, respectively. Degradation of lignin produces various phenolic compounds such as 4-hydroxybenzoic acid, vanillin and catechol. These non-sugar compounds interfere with downstream processes (i.e. fermentation) due to their inhibitory effect on microbial growth (Palmqvist & Hahn-Hägerdal, 2000). Organic acids inhibit cell growth and reduce fermentation yields (Palmqvist & Hahn-Hägerdal, 2000). Furfural and HMF inactivate cell replication which results in longer lag-phases (Larsson et al., 1999). Phenolic compounds interfere with cell growth as well as the activity of cellulase and β-glucosidase (Ximenes et al., 2011). It has been widely agreed that improved fermentation yields were achieved by removing these non-sugar compounds from hydrolysates (Kamal et al., 2011; Lee et al., 2011; Villarreal et al., 2006). On the other hand, these non-sugar compounds can serve as platform chemicals to various products in multiple industries. For example, acetic acid is used as a condiment in food and animal feed industries and serves as the raw material in the manufacture of polymers (Le Berre et al., 2000). HMF and furfural are widely used in the production of alternative fuels, polymers, foams, and polyesters (Choi et al., 2015; Rosatella et al., 2011). Therefore, the strategy for producing lignocellulosic syrups should

be designed for not only the removal of the non-sugar compounds from the hydrolysates, but their recovery as potential value-added products (Cannella et al., 2014).

Physical, physicochemical and biological detoxification methods have been studied over the years including evaporation, flocculation, overliming, adsorption with activated carbon, ion exchange resins and laccases (Lee et al., 2011; Mateo et al., 2013; Moreno et al., 2012; Villarreal et al., 2006). The key to detoxification during syrup production is separating the non-sugar compounds from the hydrolysate without a significant loss of fermentable sugars. Flocculation is a process known to enhance solid-liquid separations with the potential of flocculant recyclability (Cannella et al., 2014; Carter et al., 2011a). Flocculants can be inorganic salts (i.e., alum, ferric chloride) or organic polyelectrolytes (polyamines, chitosan) with various mechanisms or mode of action (i.e., ionic strength, hydrophobic interactions) depending on the characteristics of the flocculants (i.e., charge density, chain length and structure) and the properties of the targeted solution (i.e., charge, particle size) (Carter et al., 2011a; Matilainen et al., 2010). Polyethylenimine (PEI) and poly-diallyldimethylammonium chloride (pDADMAC) are water soluble cationic polyelectrolytes with relatively low molecular weights and high charge densities. PEI and pDADMAC have been reported to be effective in separating not only solids, but also dissolved components in solutions, such as precipitation of humic and fulvic acids from groundwater, and have also shown promising results in the detoxification of lignocellulosic biomass (Ponderosa pine slurry, maple wood hydrolysate) (Cannella et al., 2014; Carter et al., 2011b; Matilainen et al., 2010).

This study investigated the effectiveness of flocculation treatment in the removal of organic acids, phenolic compounds, furfural, and HMF from dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate for syrup production. PEI and pDADMAC were evaluated at

different doses and pH to determine the optimum flocculation parameters for removing non-sugar compounds while minimizing fermentable sugars losses. The recovery of the non-sugar compounds and the recyclability of flocculants were also evaluated.

2.2. Materials and Methods

2.2.1. Energy Cane Biomass

Non-commercial energy cane variety Ho 02-113 was harvested (stalks and leaves) at the Sugar Research Station in St. Gabriel, LA. Energy cane samples were passed through a roller press (Farrel Corporation, Ansonia, CT) three times. The solid fraction after juice extraction (referred to as bagasse) was dried in a 45°C oven for 24 h to a final moisture content of 5%, then finely milled (Wiley Mill, Swedesboro, NJ) and sieved (2 mm mesh). Milled bagasse was stored at -20°C until further use.

2.2.2. Dilute Ammonia Pretreatment

Finely milled and dried energy cane bagasse was pre-mixed with ammonium hydroxide (28% v/v solution, Fisher Scientific, Pittsburgh, PA) and water at a ratio of 1: 0.5: 8. The mixture was then transferred to a 4 L reactor (Parker Autoclave Engineers, Erie, PA) where it was heated to 160°C at100 rpm for 1 h (Aita et al., 2011). Post pretreatment, the slurry was pressed to remove any excess liquid. The collected solid fraction was dried in a 45°C oven until a final moisture content of 5% and then stored at 4°C. The chemical composition of untreated and pretreated energy cane bagasse was analyzed following National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedures (LAP TP-510-42618, 42619, 42620, 42621, 42622).

2.2.3. Enzymatic Hydrolysis

Commercially available enzymes Cellic® CTec2 (cellulase) and HTec2 (xylanase) were supplied by Novozymes (Novozymes, Franklinton, NC). Cellulase activities of CTec2 (132

FPU/mL) and HTec2 (56 FPU/mL) were measured using No. 1 filter paper (Whatman, Maidstone, UK) following NREL LAP-510-42628. β-glucosidase activities of CTec2 (3230 IU/mL) and HTec2 (16.52 IU/mL) were determined using the method documented by Ghose (1987). Xylanase activities of CTec2 (16294 IU/mL) and HTec2 (23301 IU/mL) were tested using the method provided by Bailey et al. (1992). Enzymatic hydrolysis studies were carried out at a 5% (w/w) bagasse loading followed by the addition of 25% (w/w) g/g glucan CTec2, 5% (w/w) g/g glucan HTec2 and 0.05M citric buffer. The buffer was used to ensure the pH of the hydrolysate remained at 5, which is the optimum pH for CTec2 and HTec2. Samples were incubated at 55°C for 72 h at 200 rpm. Post hydrolysis, the solid fraction was removed by filtration using a 0.2 μm filter (VWR, Radnor, PA) and the hydrolysate was kept at -20°C. Hydrolysate samples were analyzed for total phenolic compounds and for sugars and non-sugar compounds (organic acids, HMF and furfural) before detoxification by flocculants as described below.

2.2.4. Flocculation

PEI (50% w/v, avg. MW 60,000) was purchased from Fisher Scientific (Pittsburgh, PA) and pDADMAC (35% w/w, avg. MW 100,000) was obtained from Sigma-Aldrich (St. Louis, MO). Preliminary experiments and published information have indicated that flocculation with PEI or pDADMAC were not affected by contact time, agitation or temperature. Therefore, flocculation parameters of 30 min contact time, 150 rpm agitation and 22°C were selected based on published information (Cannella et al., 2014; Carter et al., 2011a). Flocculants were separately added to hydrolysate samples at various doses (5, 15, 30 g/L) and the mixture agitated at 150 rpm for 30 min at 22°C. The effect of hydrolysate pH on flocculation was also investigated by adjusting the hydrolysate pH to 2.0, 4.5 (unadjusted) or 8.0 using sulfuric acid or sodium hydroxide as needed. Post flocculation, flocculant polymers were separated by filtration using a 10,000 kDa molecular

weight cutoff (MWCO) centrifugal filter (EMD Millipore, Billerica, MA) at 14,000 × g for 30 min. The permeate was analyzed for total phenolic compounds and for sugars and non-sugar compounds (organic acids, furfural and HMF) as described below.

2.2.5. PEI Recycling

PEI was selected for the recycling studies as it was more efficient than pDADMAC in the removal of non-sugar compounds from the hydrolysate. The initial concentration of HMF and furfural in the dilute ammonia pretreated energy cane bagasse hydrolysate prior to detoxification by PEI was low (0.04 g/L furfural and 0.06 g/L HMF). Therefore, in this section, the concentration of furfural and HMF in the hydrolysate was adjusted to 2 g/L (the average concentration for organic acids and total phenolic compounds) by adding pure chemicals to the hydrolysate to accurately evaluate the adsorption efficiency of PEI, and the recovery of furans over the recycling process. HPLC-grade HMF and furfural were purchased from Sigma-Aldrich (St. Louis, MO). Flocculant recycling was performed by mixing PEI with the hydrolysate at 150 rpm and at optimum conditions of 15 g/L for 30 min at unadjusted pH 4.5. Post flocculation, PEI was separated by centrifugation using a 10,000 kDa MWCO filter (14,000 × g, 30 min). The collected flocculant was then stripped from the non-sugar compounds by adding 0.05M H_2SO_4 solution for 10 min followed by filtration using a 10,000 kDa MWCO filter (14,000 × g, 30 min). The permeate after each centrifugation was analyzed for total phenolic compounds and for sugars and non-sugar compounds as described below. The acid washed PEI was regenerated for 10 min with 0.1M NaOH solution. The regenerated PEI was collected by centrifugation using a 10,000 kDa MWCO filter (14,000 × g, 30 min) (Cannella et al., 2014; Carter et al., 2011a). PEI was only recycled five times since percent removal of non-sugar compounds decreased by more than 50% after the fifth cycle.

2.2.6. Analytical Methods

Sugars (cellobiose, glucose, xylose, arabinose, mannose) were analyzed by HPLC (Agilent 1200 Series) with a BioRad Aminex HPX-P87P (P), lead form, 300 × 7.8 mm (ID), 9 µm column at 80°C and a differential Refractive Index Detector (G1362A Agilent). The eluent was HPLC water set at a flow rate of 0.8 mL/min with a 20µL injection volume. Formic acid, acetic acid, levulinic acid, HMF, and furfural were analyzed by HPLC (Agilent 1100 Series) with a Diode Array Detector (G1315B Agilent). The column was a Shimadzu VP-ODS (Shimadzu, Kyoto, Japan), 250 mm x 4.6 mm (ID), controlled at 40°C. Mobile phase was 0.005 N H_2SO_4 (pH 2.5) set at a flow rate of 0.35 mL/min with a 10 µL injection volume. Total phenolic compounds were analyzed by measuring UV absorbance at 280 nm (Somers & Ziemelis, 1985). All chemicals were HPLC-grade and purchased from Sigma-Aldrich (St. Louis, MO).

2.2.7. Statistical Analysis

Statistical significance was detected by analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test at a 95% confidence level using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA).

2.3. Results and Discussion

2.3.1. Chemical Composition of Untreated and Dilute Ammonia Pretreated Energy Cane Bagasse

Untreated energy cane bagasse contained 40.26±0.34% glucan, 19.81±0.57% xylan, 28.74±0.24% lignin, 1.87±0.23% arabinan, 5.45±0.53% extractives, and 3.87±0.77% ash. These results are comparable to those reported by others (Aita et al., 2011; Oladi & Aita, 2017; Qiu et al., 2012). It is known that complete removal of lignin from lignocellulosic biomass is extremely challenging and unnecessary (Kim et al., 2003). After dilute ammonia pretreatment, 44% of lignin and 17% xylan were removed, with more than 90% glucan being retained.

2.3.2. Chemical Composition of Dilute Ammonia Pretreated Energy Cane Bagasse Enzymatic Hydrolysate

The concentrations of fermentable sugars, organic acids, furans, and total phenolic compounds in the hydrolysate are summarized in Table 2.1. Only glucose and xylose were detected in the hydrolysate at 15.74 g/L glucose and 9.69 g/L xylose after 72 h enzymatic hydrolysis. As for the non-sugar compounds, the highest concentration of organic acids was observed with formic acid (3.57 g/L), followed by acetic acid (2.38 g/L) and levulinic acid (1.31 g/L). The concentration of total phenolic compounds averaged 0.51 g/L. Furans including furfural (0.04 g/L) and HMF (0.06 g/L) were also detected. However, their concentrations were much lower than the ones observed for organic acids and total phenolic compounds.

Organic acids including formic acid, acetic acid and levulinic acid can inhibit cell growth (Carter et al., 2011b; Jönsson & Martín, 2016). Furans including furfural and HMF can be metabolized by microorganisms with the release of acetaldehyde and pyruvate, which leads to longer lag-phase and the inactivation of cell replication (Palmqvist et al., 1999). Phenolic compounds interfere with cell growth and enzyme efficiency (Behera et al., 2014). Inhibitory effect of these non-sugar compounds can be noticeable in downstream fermentations when concentrations of formic acid, acetic acid and levulinic acid are >100 mM and phenolic compounds are > 1g/L (Larsson et al., 1999; Palmqvist & Hahn-Hägerdal, 2000). Biomass pretreatment and hydrolysis conditions can significantly alter the composition of the hydrolysate. The milder conditions used with dilute ammonia pretreatment generated fewer amounts of non-sugar compounds than those reported for steam explosion and acid pretreatment methods (Behera et al., 2014; Jönsson & Martín, 2016). Chandel at al. (2007) reported that sugarcane bagasse treated with dilute hydrochloric acid achieved a maximum sugar yield of 30.29 g/L alongside the formation of non-sugar compounds including acetic acid (5.45 g/L), furans (1.89g/L) and total phenolic

compounds (2.75 g/L). Mateo et al. (2013) observed that in olive tree residue acid-treated hydrolysate, 25.23 g/L of total fermentable sugars along with1.67 g/L acetic acid, 0.22 g/L HMF, and 3.76 g/L total phenolic compounds were detected.

Table 2.1 Chemical composition of dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate.

	Chemical Compound	Concentration (g/L)
Sugars	Glucose	15.74±0.56
	Xylose	9.69±0.24
Non-sugar Compounds	Formic Acid	3.57±0.13
	Acetic Acid	2.38±0.06
	Levulinic Acid	1.31±0.06
	Furfural	0.04±0.00
	HMF	0.06±0.01
	Total Phenolic Compounds	0.51±0.03

2.3.3. Flocculants Dose Effect on Non-Sugar Compounds Removal

The hydrolysate pH was kept at unadjusted 4.5 to investigate the effect of flocculant dose on the removal of non-sugar compounds. The slight drop in pH observed post hydrolysis can be attributed to the release of trapped organic acids formed during pretreatment. The percent removal of non-sugar compounds at different doses of PEI or pDADMAC treatment are summarized in Fig. 2.1. Furfural and HMF were not detected after PEI or pDADMAC treatment at all doses. As for organic acids and total phenolic compounds, the percent removal was greater when increased doses of either flocculant were applied. When increasing PEI doses from 5 to 15 g/L, significant ($p < 0.05$) increases were observed in the removal of non-sugar compounds (Fig. 2.1). Formic acid removal (Fig. 2.1A) increased from 19.9% to 41.7%, acetic acid removal (Fig. 2.1B) increased from 12.8% to 36.4%, levulinic acid removal (Fig. 2.1C) increased from 24.4% to 49.8%, and total phenolic compounds removal (Fig. 2.1D) increased from 37.7% to 73.2%. A similar trend was observed with pDADMAC as doses were increased from 5 to 15 g/L (Fig.2.1). Formic acid removal (Fig. 2.1A) increased from 15.4% to 32.1%, acetic acid removal (Fig. 2.1B) increased

from 7.9% to 25.9%, levulinic acid removal (Fig. 2.1C) increased from 20.1% to 41.5%, and total phenolic compounds removal (Fig. 2.1D) increased from 34.2% to 68.8%. However, when increasing the flocculant dose from 15 to 30 g/L, no significant ($p > 0.05$) improvements were observed in the removal of formic acid and acetic acid with PEI or pDADMAC.

Addition of cationic flocculants to hydrolysates can cause oppositely charged compounds to be adsorbed by the flocculants through bridging, charge neutralization or the patching mechanism (Gregory, 1987). Electrostatic interaction, which is the main driving force behind flocculation, increases with increasing concentrations of highly charged flocculants (Zhou & Franks, 2006). Thus, a positive correlation between the removal of non-sugar compounds and flocculant dose can be expected. Carter et al. (2011b) reported that increasing PEI dose from 2.8 to 5.6 g/L in dilute acid pretreated Ponderosa pine hydrolysate significantly improved the removal of furfural from 43% to 84%, and HMF removal from 35% to 67%. Additionally, the average removal of acetic acid, furfural and HMF from spruce acid hydrolysate increased from 43% to 80% when PEI dose was increased from 15 to 30 g/L (Cannella et al., 2014). In dilute acid pretreated pine wood sawdust slurry, the addition of 26 g/L PEI removed 9% acetic acid, 54% HMF and 71% furfural (Gurram et al., 2011). Saeed et al. (2012) observed that the addition of 0.4 g/L pDADMAC to the pre-hydrolysis liquor of kraft-based dissolving pulp process removed 14% acetic acid and 40% furfural.

PEI significantly ($p < 0.05$) outperformed pDADMAC by 30% (formic acid removal), 41% (acetic acid removal) and 20% (levulinic acid removal) at a flocculant dose of 15g/L. No significant ($p > 0.05$) differences were observed with the removal of total phenolic compounds. The reduced adsorption efficiency of pDADMAC as compared to PEI can be attributed to the ammonium cation functional group and chloride anion present in pDADMAC. The functional

group of pDADMAC has a higher affinity towards chloride ion rather than organic acids ions (Carter et al., 2011a). In addition, the reduced molecular weight of PEI (avg. MW 60,000) as compared to pDADMAC (avg. MW 100,000) may favored the adsorption of non-sugar compounds as lower molecular weight cationic polyelectrolytes have greater adsorption efficiencies towards compounds of opposite charges (Oveissi et al., 2016; Yasarla & Ramarao, 2012).

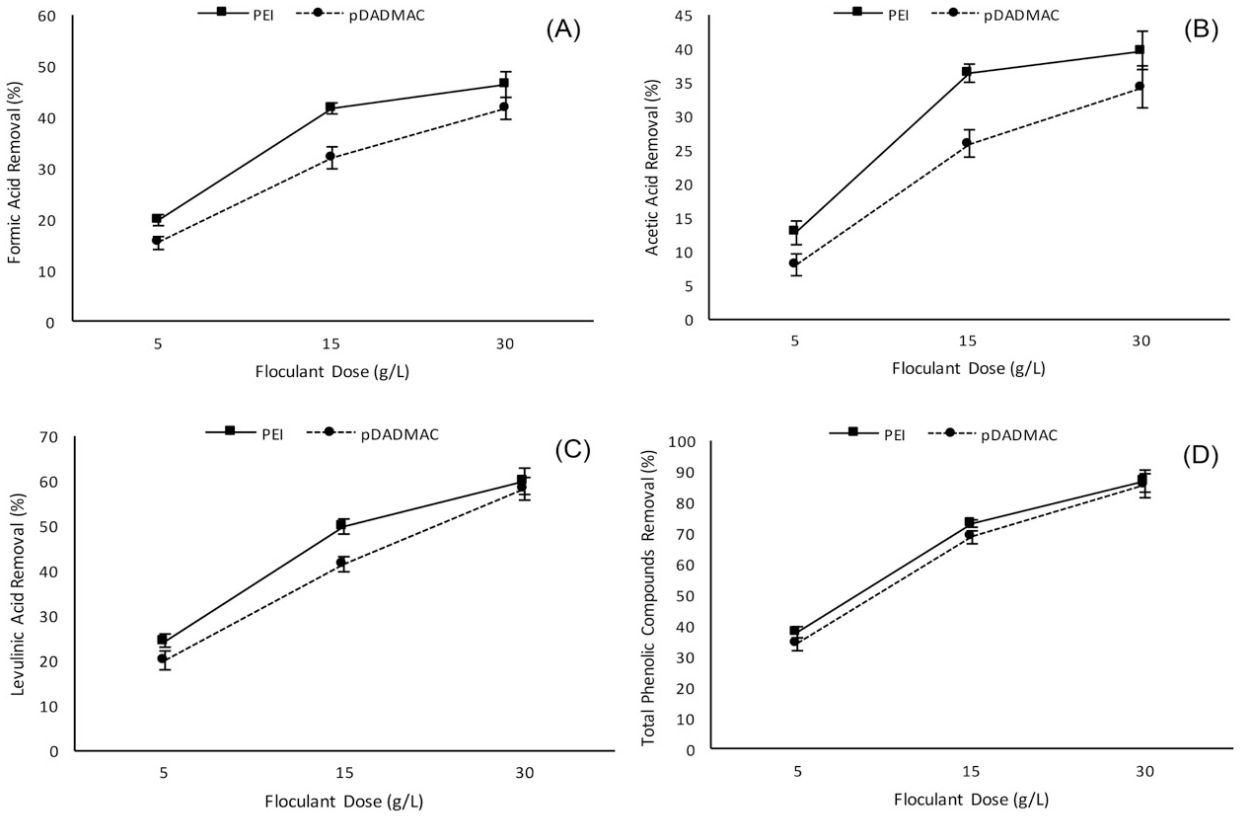

Figure 2.1 Effect of flocculant dose on the removal of non-sugar compounds from dilute ammonia pretreated energy cane bagasse hydrolysate. (A) formic acid, (B) acetic acid, (C) levulinic acid, and (D) total phenolic compounds.

Flocculant adsorption efficiency towards non-sugar compounds is also affected by the nature of the adsorbate. Carter et al. (2011) reported up to 89% acetic acid removal from a synthetic aqueous solution (to simulate the composition of hydrolysate) at a PEI dose of 1 molar equivalent,

which is much higher than the observed 39.7% acetic acid removal by PEI from dilute ammonia pretreated enzymatic hydrolysate. Similarly, Cannella et al. (2014) reported that at 15 g/L PEI, 46% acetic acid, 35% HMF and 49% furfural were removed from an aqueous solution, whereas in acid pretreated spruce enzymatic hydrolysate, only 6% acetic acid, 10% HMF and 20% furfural were removed. Unlike the above mentioned aqueous solutions, enzymatic hydrolysates are complex solutions characterized by the presence of oppositely charged constituents such as suspended solids, enzymes, ions, lignin fractions, and oligomers that can compete with the non-sugar compounds for available binding sites on the cationic flocculants (Carter et al., 2011b; Yasarla & Ramarao, 2012). Cannella et al. (2014) observed that lignocellulose fractions including klason lignin, phosphoric acid swollen cellulose and Avicell® reduced PEI adsorption efficiencies on acetic acid, HMF and furfural. It was also reported that removing the suspended solids from acid pretreated Ponderosa pine enzymatic hydrolysate by filtration (prior to PEI treatment) through a 0.2 μm or 10 kDa MWCO filter improved furfural removal by 6.8 and 8.6 times, respectively, at 1 molar equivalent PEI dose (Carter et al., 2011b).

2.3.4. Flocculants Dose Effect on Sugar Losses

Dose effect of PEI or pDADMAC on sugar losses are presented in Fig. 2.2. At 5 g/L flocculant dose, PEI resulted in 1.1% glucose loss and 7.8% xylose loss; whereas, pDADMAC observed sugar losses were 2% glucose and 9.8% xylose. However, at higher doses, sugar losses became more severe. PEI at 15 g/L removed 5.3% and 9.7% of glucose and xylose respectively; whereas, pDADMAC treatment resulted in 6.3% glucose and 12.7% xylose losses. At 30 g/L, 11-17% sugar losses were observed after PEI or pDADMAC treatment. pDADMAC resulted in significantly ($p < 0.05$) higher glucose losses at all doses tested, and significantly ($p < 0.05$) higher xylose losses at 15 g/L dose as compared to PEI. Wide ranges of sugar losses have been reported

depending on the nature of the target solution and flocculation conditions (Cannella et al., 2014; Carter et al., 2011a; Saeed et al., 2012; Yasarla & Ramarao, 2012). It was reported that no significant sugar losses were observed in an aqueous solution of pure glucose and xylose with up to 11.3 g/L PEI (Carter et al., 2011a). However, in a more complex matrix such as a biomass slurry or hydrolysate, sugar losses increased after flocculation. Cannella et al. (2014) reported a 2% glucose loss after 15 g/L PEI treatment in acid pretreated spruce slurry. In hot water pretreated maple chip hydrolysate, 22% and 37% total sugar losses were observed after treatment with 0.05 g/L PEI or pDADMAC, respectively (Yasarla & Ramarao, 2012). Saeed et al. (2012) pointed out that 0.5 g/L pDADMAC treatment in the kraft-based dissolving pulp process pre-hydrolysis liquor resulted in 6.5% monomeric and 25% oligomeric sugars losses.

It is known that sugars are physically trapped or mechanically entangled within the flocculant complex during flocculation (Liu et al., 2011; Ström et al., 1985). The increased sugar losses observed at a higher flocculant dose in the hydrolysate can be attributed to the greater amount of flocculant complex being formed as result of flocculant concentration and complexity of hydrolysates. In synthetic aqueous solutions, insignificant sugar losses are expected due to the absence of charged constituents (i.e., suspended solids, enzymes, ions, lignin fractions, oligomers) and minimal flocculant complex formation; whereas, in complex matrices such as biomass hydrolysates or slurries, higher sugar losses are observed. Differences in sugar losses induced by PEI or pDADMAC can also be attributed to the different chemical and physical properties of the flocculant complex (i.e., particle size, surface area, and porosity) (Yasarla & Ramarao, 2012).

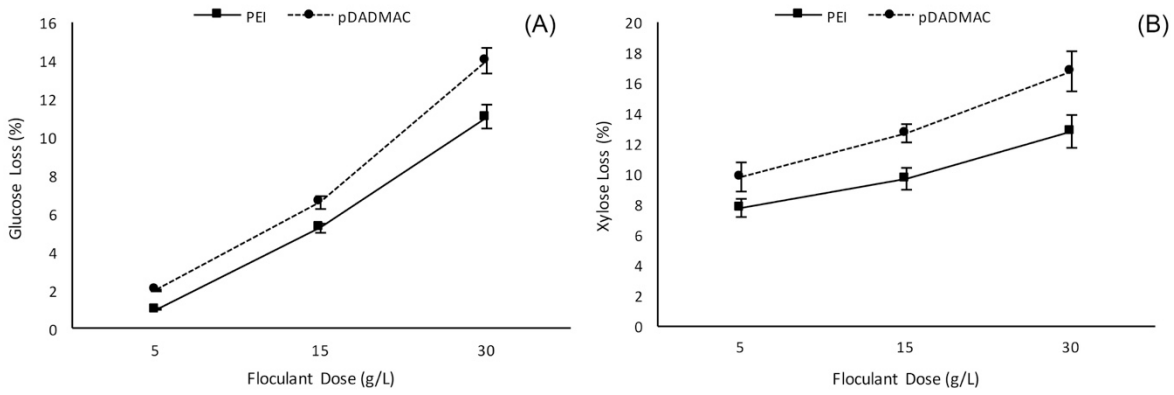

Figure 2.2 Effect of flocculant dose on sugar losses from dilute ammonia pretreated energy cane bagasse hydrolysate.
(A) glucose loss, (B) xylose loss.

2.3.5. Hydrolysate pH Effect on Non-Sugar Compounds Removal

The flocculant dose of 15 g/L was selected for further analysis to evaluate the effect of hydrolysate pH (2.0, 4.0, 8.0) on the removal of non-sugar compounds by PEI or pDADMAC from dilute ammonia pretreated energy cane bagasse hydrolysate. HMF and furfural were not detected at all tested pH conditions after treatment with PEI or pDADMAC (Fig. 2.3). The removal of organic acids and total phenolic compounds was significantly ($p < 0.05$) higher at unadjusted pH 4.5 than pH 2.0 or pH 8.0 for both PEI or pDADMAC. At pH 2.0, 10.7% formic acid, 12.5% acetic acid, 16.5% levulinic acid, and 30.8% total phenolic compounds were removed by PEI. A decrease in percent removal by 74%, 67%, 66%, and 58% was observed for formic acid, levulinic acid, acetic acid, and total phenolic compounds, respectively, as compared to unadjusted pH 4.5. At pH 8, only 6.4% formic acid, 7.6% acetic acid, 10.5% levulinic acid, and 28.4% total phenolic compounds were removed by PEI. The observed values correspond to a decrease in percent removal by 85%, 79%, 77%, and 61% for formic acid, acetic acid, levulinic acid, and total phenolic compounds, respectively, as compared to unadjusted pH 4.5. Similar trends were observed with pDADMAC when hydrolysate pH was adjusted to 2.0 or 8.0. Overall, PEI outperformed

pDADMAC in the removal of non-sugar compounds in the range of 5% to 41% under various pH conditions at 15 g/L dose.

Cater et al. (2011a) found that in a 5 g/L acetic acid aqueous solution at unadjusted pH 3.4, 1 molar equivalent PEI removed 90% acetic acid while no significant amounts of acetic acid were removed at pH 7.0 (adjusted with NaOH). Yasarla and Ramarao (2012) observed that in hot water-extracted maple chips hydrolysate, the addition of PEI or pDADMAC at pH 3.5 significantly decreased the turbidity and zeroed the zeta potential of the hydrolysate, an indication of a strong flocculation effect. However, when the hydrolysate of the pH was adjusted to 8.0, no significant decrease in turbidity was observed and the zeta potential of the hydrolysate remained negative. Cannella et al. (2014) pointed out that in a synthetic aqueous solution, the percent removal of acetic acid, HMF and furfural by 15 g/L PEI decreased by 89%, 72% and 53%, respectively, when the pH of the solution was reduced from 4.8 to 2.0. The charge density of pDADMAC is not affected by changes in pH (Wandrey et al., 1999; Yasarla & Ramarao, 2012). However, the charge density of PEI decreases as the pH of the solution increases, thus interfering with its ability to neutralize the negatively charged compounds and particles present in the hydrolysate (Yasarla & Ramarao, 2012). The reduced organic acid and phenolic compounds removal observed at adjusted pH 2.0 and 8.0 can be attributed to the addition of competitive ions during pH adjustment of the hydrolysate samples. At pH 8.0, the addition of NaOH introduced OH^- to the hydrolysate, which competed with the non-sugar compounds such as acetate anions and reduced the efficiency of both cationic polymers, PEI and pDADMAC (Carter et al., 2011a). At pH 2.0, the reduced adsorption efficiency observed with PEI and pDADMAC can be attributed to competition from sulfate anions, which were introduced by the addition of sulfuric acid when adjusting the pH of hydrolysate samples. It has been reported that the addition of 160 mM sodium chloride to 5 g/L acetic acid

aqueous solution reduced acetic acid removal by 1 molar equivalent of PEI from 89% to 27%. However, the addition of 160 mM sodium sulfate resulted in an even stronger inhibitory effect against PEI as acetic acid removal was reduced from 89% to only 4% under the same conditions (Carter et al., 2011a).

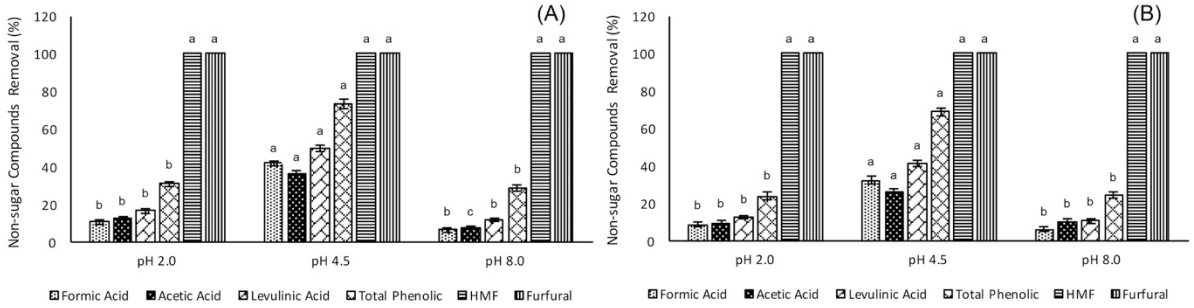

Figure 2.3 Effect of hydrolysate pH on the removal of non-sugar compounds from dilute ammonia treated energy cane bagasse hydrolysate by flocculation at 15 g/L flocculant dose.
(A) PEI and (B) pDADMAC
Significant ($p < 0.05$) differences in percent non-sugar compounds removals at different pH are indicated by letters.

2.3.6. Hydrolysate pH Effect on Sugar Losses

Sugar losses at different pH after PEI or pDADMAC treatment are shown in Fig. 2.4. There were no significant ($p > 0.05$) differences observed in sugar losses at pH 2.0, 4.5 or 8.0 by both flocculants. PEI treatments resulted in 5.3% glucose and 9.7% xylose losses; whereas, 6.6% glucose and 12.7% xylose losses were observed for pDADMAC regardless of the hydrolysate pH. Reduced sugar loss is considered one of the advantages of flocculation (Cannella et al., 2014; Carter et al., 2011b). Cater et al. (2011a) reported no glucose or xylose losses in aqueous solutions adjusted to pH 6.7 or 5.2 when 1 molar equivalent dose of PEI was used. Similar observations were reported in acid pretreated spruce slurry adjusted to pH 2.0 or spruce enzymatic hydrolysate adjusted to pH 4.8 after treatment with 15 g/L PEI (Cannella et al., 2014). However, others have reported significant sugar losses after treatment with flocculants (Duarte et al., 2010; Saeed et al.,

2012; Yasarla & Ramarao, 2012). Yasarla and Ramarao (2012) reported 34% glucose and 20% xylose losses with 0.05 g/L PEI, and 45% glucose and 36% xylose losses with 0.047 g/L pDADMAC in hot water extracted maple chips hydrolysate adjusted to pH 3.5. Another report pointed out that 0.047 g/L pDADMAC treatment of hot water pretreated maple chips extract adjusted to pH 3.2 resulted in only 3% total fermentable sugar losses (Duarte et al., 2010). Saeed et al. (2012) reported 7% monomeric and 25% oligomeric sugar losses with 0.5 g/L pDADMAC in pulp pre-hydrolysis liquor adjusted to pH 7.0. The inconsistency of the results observed and reported in literature is also an indication that the sugars may not be chemically adsorbed by flocculants, but rather physically trapped in a flocculant complex, a mechanism which is similar to the one proposed for activated carbon detoxification (Lee et al., 2011).

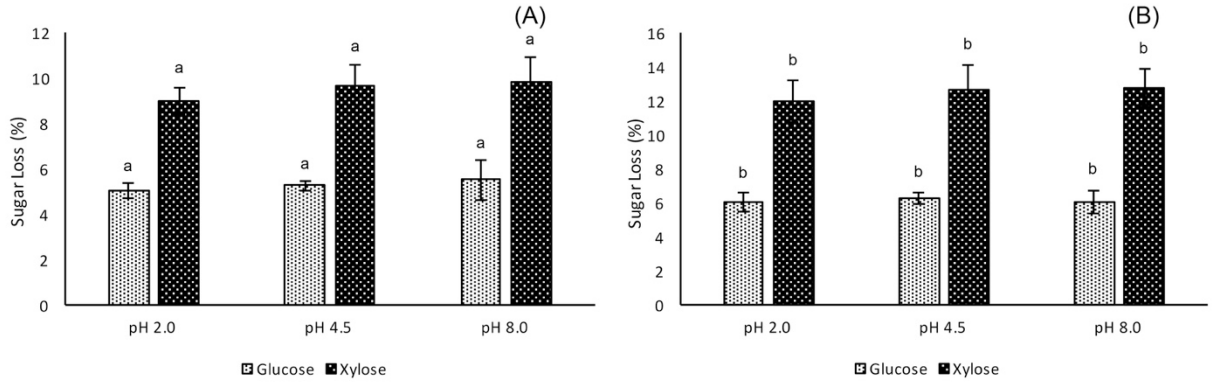

Figure 2.4 Effect of hydrolysate pH on sugar losses from dilute ammonia treated energy cane bagasse hydrolysate by flocculation at 15 g/L flocculant dose.
(A) PEI and (B) pDADMAC
Significant ($p < 0.05$) differences in percent non-sugar compounds removals at different pH are indicated by letters.

2.3.7. PEI Recycling and Recovery of Non-Sugar Compounds from PEI

Flocculant recycling and non-sugar compounds recovery studies were conducted with PEI as it outperformed pDADMAC in achieving maximum non-sugar compounds removal with minimal sugar losses. The proposed mechanism of PEI adsorption towards furans is described by

the Mannich reaction (Reaction 1), and for organic acids and phenolic compounds (weak acids) can be described by the acid/base neutralization reaction (Reaction 2) as shown below (Carter et al., 2011a).

Mannich Reaction

$R_2NH + 2C_5H_4O_2 \leftrightarrows R_2NCC_4H_3OC_5H_3O_2 + H_2O$ Reaction 1

Acid/Base Neutralization Reaction

$R–NH–R' + CH3OOH \leftrightarrows RN^{+}H_2R' + CH3OO^{-}$ Reaction 2

Both reactions can be reversed by the addition of water or dilute acids, thus providing opportunities to desorb and recover the adsorbed non-sugar compounds from the PEI polymer (Carter et al., 2011a). Recycling studies with PEI were conducted up to five times (Fig. 2.5). Additional recycling would have resulted in percent removal of non-sugar compounds of less than 50%. No significant ($p > 0.05$) changes in total fermentable sugar loss (< 10%) were observed after each cycle of reuse and regeneration. In the first three cycles of PEI reuse and regeneration, percent removal of organic acids significantly ($p > 0.05$) decreased from 40% (first cycle) to 16% (second cycle) and to 3% (third cycle). The percent removal of total phenolic compounds was significantly ($p > 0.05$) reduced from 73% (first cycle) to 23% (second cycle) and to 7% (third cycle). The observed percent removal for HMF decreased from 86% (first cycle) to 83% (second cycle) and to 71% (third cycle). The percent removal for furfural decreased from 90% (first cycle) to 86% (second cycle) and to 78% (third cycle). Extending PEI reuse and regeneration to the fourth and fifth cycle resulted in no significant ($p > 0.05$) changes observed in the percent removal of organic acids and total phenolic compounds (less than 10%). Percent removal of HMF significantly ($p > 0.05$) decreased to 44% and to 31%, and percent removal of furfural decreased to 50% and to 33%, after the fourth and fifth cycle, respectively. Promising results have been reported with PEI in

synthetic aqueous solutions where the removal of acetic acid decreased from 60% (first cycle) to 22% (fifth cycle), and the percent removal of HMF and furfural remained at 40% over the five cycles (Cannella et al., 2014).

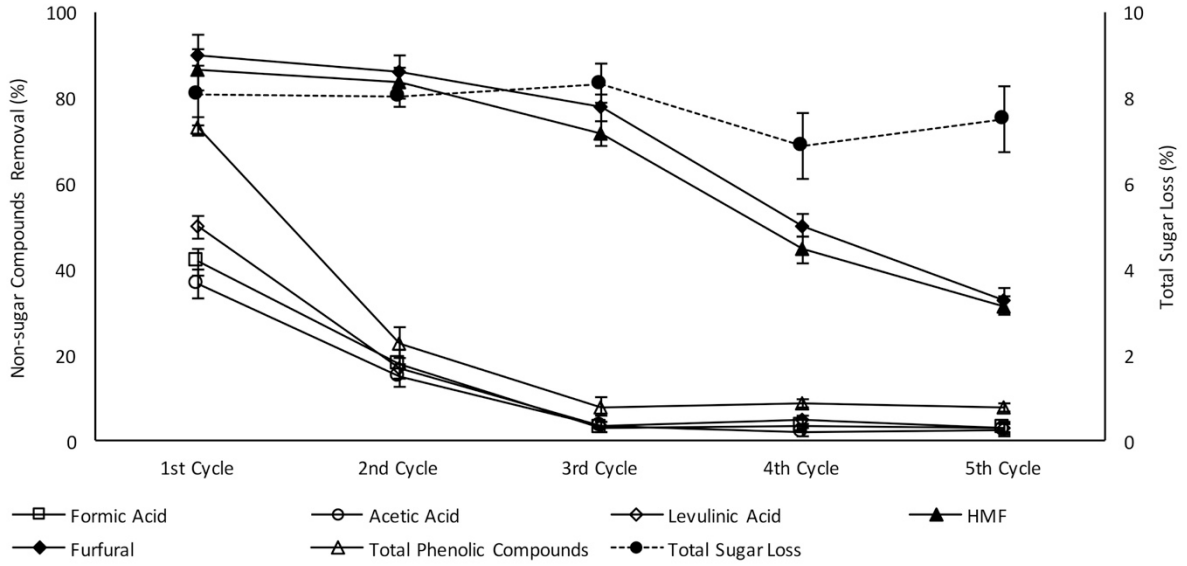

Figure 2.5 Effect of recycling PEI on the removal of non-sugar compounds and total sugar loss from dilute ammonia pretreated energy cane bagasse hydrolysate.

During the PEI recycling process, adsorbed non-sugar compounds were desorbed from the polymer by stripping it with 0.05M sulfuric acid and the non-sugar compounds recovered from the permeate by centrifugation (Fig. 2.6). The recovery of non-sugar compounds from PEI decreased after each cycle, and the percent recovery of organic acids and total phenolic compounds were much lower than that of HMF and furfural. The observed percent recovery for both HMF and furfural from PEI was similar and it decreased from 90% (first cycle) to 20% (fifth cycle), a 10-20% decrease after each cycle. Percent recoveries observed for organic acids were 40% (first cycle), 17% (second cycle) and to 4% (third cycle). Recovery rates of approximately 2% were observed at the fourth and fifth cycles, which were not statistically significant ($p > 0.05$). A similar trend to that of organic acids was observed with the percent recoveries of total phenolic compounds.

Percent recoveries for total phenolic compounds were reduced significantly from 53% (first cycle) to 19% (second cycle) and to 8% (third cycle). Recovery of total phenolic compounds from PEI at the fourth and fifth cycles was less than 2%. Cater et al. (2011a) reported recoveries of 20% furfural and 35% HMF when PEI was stripped with pure water, and recoveries of 81% furfural and 97% HMF when sulfuric acid (pH 2.0) was used as the stripping agent. Cannella et al. (2014) pointed out that in an aqueous solution of non-sugar compounds, 90% acetic acid, HMF and furfural were recovered from PEI with 0.36% w/v sulfuric acid after five reuse cycles.

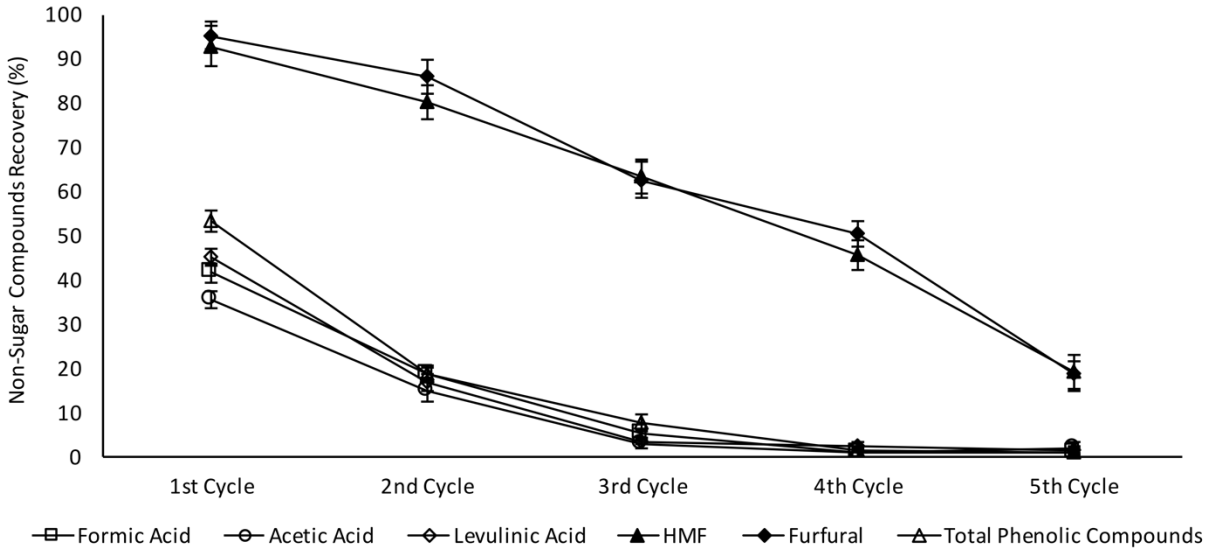

Figure 2.6 Recovery of non-sugar compounds from PEI after each reuse and regeneration cycle of PEI.

It was noticed that the percent removal and recovery of organic acids and phenolic compounds (weak acids) from PEI decreased more significantly ($p < 0.05$) as compared to HMF and furfural after each reuse and regeneration cycle. Organic acids and phenolic compounds are more difficult to be stripped from the PEI complex due to their stronger ionic bonds with PEI as compared to the covalent bonds between furans and PEI, which is reflected in the reduced percent recoveries observed (Carter et al., 2011a). Furthermore, the lower percent recoveries of non-sugar compounds observed from lignocellulosic hydrolysates as compared to those in synthetic aqueous

solutions are attributed to the presence of suspended solids, enzymes, ions, lignin fractions, and oligomers which can bind to the PEI polymer through bridging, charge neutralization, or electrostatic patch mechanisms and reduce its adsorption capacity during recycling. The reduced and incomplete recovery of non-sugar compounds from PEI after each cycle also explains the reduced recyclability of PEI (Cannella et al., 2014). Therefore, more advanced recycling and regeneration techniques are necessary to efficiently reuse PEI for the recovery of non-sugar compounds from complex environments such as lignocellulosic hydrolysates.

2.4. Conclusions

Flocculants can have the potential to remove non-sugar compounds (i.e., organic acids, furan derivatives, phenolic compounds) generated or released during pretreatment and enzymatic hydrolysis of lignocellulosic biomass with reduced sugar losses. The generated and released non-sugar compounds can have negative effects during downstream processes (i.e., separation, fermentation). However, flocculant type and dose, chemical composition of biomass hydrolysate, and hydrolysate pH play important roles in the removal of these compounds from hydrolysates. A 15 g/L flocculant dose and unadjusted hydrolysate pH of 4.5 were selected as optimum conditions for both PEI and pDADMAC. At optimum conditions, PEI outperformed pDADMAC in removing organic acids (43%), total phenolic compounds (73%) and furans (100%) with less than 10% sugar (glucose and xylose) losses from dilute ammonia pretreated energy cane bagasse hydrolysate. These non-sugar compounds can be recovered and serve as building blocks for various chemicals. However, recycling of PEI should be limited to one cycle when recovering organic acids and total phenolic compounds and to two cycles when recovering furan derivatives due to the complex nature of the hydrolysate as compared to an aqueous solution. PEI recycling had no effect on sugar losses (<10%).

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CHAPTER 3
OPTIMIZATION OF ACTIVATED CARBON DETOXIFICATION OF DILUTE AMMONIA PRETREATED ENERGY CANE BAGASSE ENZYMATIC HYDROLYSATE BY RESPONSE SURFACE METHODOLOGY

3.1. Introduction

Lignocellulose is a promising renewable resource that can be used in the production of fermentable sugars-enriched syrups, a feedstock with potential use in multiple processes to produce fuels, bio-hydrogen, microbial lipids, and other chemicals (Canilha et al., 2012; Liang et al., 2012; Pattra et al., 2008; Whitfield et al., 2012). Lignocellulose is a complex structure of cellulose, hemicellulose and lignin, which is highly resistant to chemical and biological degradation. Energy cane, a hybrid of commercial and wild sugarcanes, is one of the most attractive sources of lignocellulosic material, and has a higher fiber content and biomass yield than regular sugarcane (Bischoff et al., 2008; Kim & Day, 2011; Sierra et al., 2008).

Lignocellulose materials must be converted into monomeric fermentable sugars to produce sugars-enriched syrups. This process generally consists of two main steps, pretreatment and hydrolysis. Pretreatment is a process which involves increasing the surface area of the material, partially removing the hemicellulose and/or lignin, and decreasing the crystallinity of the cellulose while minimizing the losses of sugars (Canilha et al., 2012). The overall target of pretreatment is to allow enzymes better accessibility to the polymeric sugars. Generally, pretreatments are categorized into four categories: physical, physiochemical, chemical, or biological. It has been previously demonstrated that dilute ammonia pretreatment significantly removed lignin and improved cellulose and hemicellulose digestibility (Aita et al., 2011). After pretreatment, the next step is to convert polysaccharides into fermentable monomeric sugars. Acid hydrolysis and

enzymatic hydrolysis are well developed methods used for achieving this goal. Compared to acid hydrolysis, enzymatic hydrolysis is the preferred method due to its effectiveness, moderate requirement of pH and temperature, and non-corrosive properties (Moe et al., 2012; Sun & Cheng, 2002).

A flexible, year-round supply of lignocellulosic biomass to biorefineries can be extremely challenging due to the seasonality of the crops and harvest times. Lignocellulosic hydrolysates are extremely susceptible to microbial deterioration because of their high water and sugar content (Eggleston et al., 2015). Syrup which results from the concentration of sugars from hydrolysates can significantly reduce water activity, control microbial growth and preserve the sugars. According to Eggleston et al. (2013), an 80°Brix syrup inhibited the growth of most microorganisms found in sorghum juice. Under vacuum conditions, hydrolysates can be concentrated into syrups with minimal entrainment and sugar degradation. Therefore, concentration of hydrolysates into a stable syrup is critical and beneficial for long-distance transportation, long-time storage and year-round supply to bio-based fuels and chemicals manufacturing plants. However, non-sugar compounds such as organic acids (i.e., acetic acid, formic acid, levulinic acid), furaldehydes (i.e., furfural, 5-hydroxymethylfurfural (HMF)) and phenolic compounds can be formed during pretreatment and released during enzymatic hydrolysis, thus altering the final quality and purity of lignocellulosic syrups. Acetic acid is a degradation product of hemicellulose (Palmqvist & Hahn-Hägerdal, 2000). Furfural and HMF are formed from the degradation of pentose and hexose sugars, respectively. Formic acid is formed when furfural and HMF are degraded, while levulinic acid is formed by HMF degradation. Thermal degradation and oxidation of lignin result in the release of phenolic compounds such as 4-hydroxybenzoic acid, vanillin and catechol. The concentration of these non-sugar compounds during syrup production

can negatively affect downstream processes such as fermentation (Palmqvist & Hahn-Hägerdal, 2000). Organic acids inhibit cell growth and reduce fermentation yields (Palmqvist & Hahn-Hägerdal, 2000). Furfural and HMF cause inactivation of cell replication which results in a longer lag-phase (Larsson et al., 1999). Phenolic compounds can damage the integrity of the biological membrane, which interferes with cells growth and enzyme efficiency (Palmqvist & Hahn-Hägerdal, 2000). Nevertheless, these non-sugar compounds can be recovered and be used as platform chemicals in the production of value-added products for numerous industries (Carter et al., 2011). For example, acetic acid is widely used in the food industries and in the production of vinyl acetate monomers, acetic anhydride and ester (Le Berre et al., 2000). HMF can be converted to levulinic acid, dimethylfuran, 2,5-furandicarboxylic acid, and dihydroxymethylfuran, which are building blocks for the manufacture of alternative fuels, polymers, foams, and polyesters (Rosatella et al., 2011).

Detoxification aims at removing and/or recovering non-sugar compounds with minimal sugars losses (Margeot et al., 2009; Canilha et al., 2012; Kamal et al., 2011). Several detoxification methods have been reported for removing non-sugar compounds such as evaporation, activated carbon adsorption, use of ion exchange resins, laccases and peroxidases, as well as liming and overliming processes (Kamal et al., 2011; Mateo et al., 2013; Moreno et al., 2012; Villarreal et al., 2006). Among these methods, activated carbon is one of the most widely applied methods for removing inhibitors from enzymatic and acid hydrolysates, due to its low cost, high capacity of adsorption, and ease of use (Kamal et al., 2011). Activated carbon type, dose, hydrolysate pH, and contact time are the major factors that affect activated carbon adsorption efficiencies (Mateo et al., 2013; Mussatto & Roberto, 2004). The effectiveness of detoxification also depends on the type of

lignocellulose hydrolysate, conditions of pretreatment and enzymatic hydrolysis (Mussatto & Roberto, 2004).

Response surface methodology (RSM) is one of the most popular statistical modeling methods used to obtain optimal process conditions. RSM offers several advantages such as the capability of obtaining large amounts of information from reduced number of experiments, and investigating the interaction effects among the independent variables (Baş & Boyacı, 2007). Central composite design (CCD) is an experimental design that is commonly used in RSM. Compared to multilevel factorial design, CCD requires much fewer experiments without sacrificing critical information (Khataee et al., 2010). CCD consists of fractional factorial design with center points for replication and a group of axial points.

This study investigated the removal of organic acids, furaldehydes and total phenolic compounds from dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate for syrup production by activated carbon adsorption. RSM was used to optimize the process parameters such as activated carbon type, dose, contact time, and hydrolysate pH for maximum non-sugar compounds removal while minimizing fermentable sugars losses.

3.2. Material and Methods

3.2.1. Biomass

A non-commercial variety of energy cane (Ho 02-113) was collected from the Sugar Research Station in St. Gabriel, LA. Energy cane juice was extracted by passing the whole stalk, leaves and tops through a roller press (Farrel Corporation, Ansonia, CT) three times. Energy cane bagasse, the solid portion left behind after juice extraction, was stored at -20°C until further use.

3.2.2. Dilute Ammonia Pretreatment

Energy cane bagasse was dried in a 45°C oven for 24 h to a moisture content of 5%, finely milled in a Wiley mill (Swedesboro, NJ) and sieved with a 2 mm mesh sieve prior to pretreatment. Milled bagasse was then stored at 4°C until further use. Pretreatment was conducted in a 4 L reactor (Parker Autoclave Engineers, Erie, PA) by mixing energy cane bagasse, ammonium hydroxide (28% v/v solution, Fisher Scientific, Pittsburgh, PA) and water at a ratio of 1: 0.5: 8. The reactor was heated to 160°C for 1 h, and then cooled down to 50°C (Aita et al., 2011). Post pretreatment, all the bagasse was carefully collected from the reactor and pressed to remove excess liquid. Pretreated bagasse was dried in a 45°C oven for 24 h to a moisture content of approximately 5% and stored at 4°C. Compositional analysis was conducted for both untreated and pretreated bagasse following standard Laboratory Analytical Procedures (LAP TP-510-42618, 42619, 42620, 42621, 42622) documented by the National Renewable Energy Laboratory (NREL).

3.2.3. Enzymatic Hydrolysis

Cellic® CTec2 and HTec2 are commercially available enzymes supplied by Novozymes (Franklinton, NC). Cellic® CTec2 contains cellulase, β-glucosidase and hemicellulase. Cellic® HTec2 is an endoxylanase complex with cellulase that converts hemicellulose to fermentable sugars. Cellulase activity was measured using No. 1 filter paper (Whatman, Maidstone, UK) as described by NREL's LAP-510-42628. β-glucosidase activity was determined by the Ghose method using cellobiose as substrate (Ghose, 1987). Xylanase activity was analyzed using the method described by Bailey et al. (1992). CTec2 enzyme activities were 132 FPU/mL cellulase, 3230 IU/mL β-glucosidase and 16294 IU/mL xylanase. HTec2 enzyme activities were 56 FPU/mL cellulase, 16.52 IU/mL β-glucosidase and 23301 IU/mL xylanase. CTec2 was added at 25% (w/w) g/g glucan and HTec2 was added at 5% (w/w) g/g glucan to a bagasse loading of 5% (w/w). Citric

acid buffer (0.05M) was used to ensure the pH of the hydrolysate was kept at optimum 5.0. The mixture was incubated at 50°C for 72 h at 200 rpm. Post hydrolysis, solids were separated by passing the hydrolysate thru a 0.2 μm filter (VWR, Radnor, PA). The hydrolysate was stored in a -20°C freezer until further analysis.

3.2.4. Activated Carbon Detoxification and Experimental Design

Powdered AC (Norit® CN1 (Cabot Corporation, Alpharetta, GA)) with a surface area of 1400 m^2/g and granular AC (DARCO® 12x40 (Cabot Corporation, Alpharetta, GA)) with a surface area of 650 m^2/g were evaluated. Enzymatic hydrolysates from dilute ammonia pretreated energy cane bagasse were mixed with powdered AC (Table 3.1) or granular AC (Table 3.2) at different conditions (AC dose, hydrolysate pH and contact time). All AC treatments were agitated at 200 rpm and incubated at 22°C. Preliminary experiments on contact time indicated that contact time was not a significant factor in the adsorption efficiency of powdered AC, thus the contact time for powdered AC treatment was fixed at 10 min, which was the adequate time to achieve maximum non-sugar compounds removal. AC was removed from hydrolysates by filtration using 0.2 μm syringe filters (VWR, Radnor, PA). Sugars (glucose and xylose) and non-sugar compounds (formic acid, acetic acid, levulinic acid, furfural, HMF, and total phenolic compounds) present in the hydrolysates were analyzed before and after each AC treatment. Percent acetic acid removal was selected for model fitting and optimization because it has been reported as the most difficult compound to be removed by AC treatment among all non-sugar compounds found in lignocellulosic hydrolysates (Lee et al., 2011; Mateo et al., 2013).

A CCD was employed to investigate the effect of parameters (AC dose, hydrolysate pH and contact time) on the responses (acetic acid removal and total sugar loss). The experiments were designed and data was analyzed by Design-Expert 10.0.3.1 (State Ease Inc., Minneapolis,

MN). The levels of each independent variable are presented in Table 3.1 for powdered AC and in Table 3.2 for granular AC. A full second-order polynomial function (Equation (1)) was fitted to the collected data.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

Where Y is the response variable, x_i and x_j are the independent variables, β_0 is the constant coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, ε is the random error, and n is the number of independent variables.

The significance of the fitted model for each coefficient and model adequacy was analyzed by analysis of variance (ANOVA). Response surfaces were constructed by the software to evaluate the effect of independent variables on the response and to determine the optimal parameters.

Table 3.1 Coded levels of powdered AC treatment condition variables in the CCD.

Variable	Unit	Coding	Coded Levels				
			−α*	-1	0	1	+α*
AC dose	% (w/w)	A	6.2	7.0	9.0	11.0	11.8
pH	-	B	1.3	1.5	2.0	2.5	2.7

*To maintain the rotatability, $\alpha = [2^k]^{1/4}$, where k is the number of independent variables.

Table 3.2 Coded levels of granular AC treatment condition variables in the CCD.

Variable	Unit	Coding	Coded Levels				
			−α*	-1	0	1	+α*
AC dose	% (w/w)	A	9.6	11.0	13.0	15.0	16.4
pH	-	B	1.2	1.5	2.0	2.5	2.8
Contact Time	Minutes	C	6.4	20.0	40.0	60.0	73.6

*To maintain the rotatability, $\alpha = [2^k]^{1/4}$, where k is the number of independent variables.

3.2.5. Analytical Methods

Sugars were analyzed by high performance liquid chromatography (HPLC) (Agilent 1200 Series) with a BioRad Aminex HPX-P87P (P), lead form 300 × 7.8 mm (ID), 9 µm column at 80°C and a differential Refractive Index Detector (G1362A Agilent) (Aita et al., 2011). The eluent used was HPLC water set at a flow rate of 0.8 mL/min and 20µL injection volume. Organic acids

(formic acid, acetic acid and levulinic acid), furfural, and HMF were analyzed by HPLC (Agilent 1100 Series) with a Diode Array Detector (G1315B Agilent) at 210nm. The column used was a Shimadzu VP-ODS thermally controlled at 40°C. The mobile phase was 0.005 N sulfuric acid, with a flow rate of 0.35 mL/min, and 10 µL injection volume. Total phenolic compounds were analyzed by measuring UV absorbance at 280 nm (Somers & Ziemelis, 1985). All chemicals were HPLC-grade and were purchased from Sigma-Aldrich (St. Louis, MO).

3.3. Results and Discussion

3.3.1. Chemical Composition of Untreated and Dilute Ammonia Pretreated Energy Cane Bagasse

The chemical composition of untreated and dilute ammonia pretreated energy cane bagasse is presented in Table 3.3. Untreated energy cane bagasse contained 40.26% glucan, 19.81% xylan, 28.74% lignin, and 11.19% others (i.e., ash, proteins, mannan). Complete removal of lignin is unnecessary (Kim et al., 2003). A 55% lignin removal resulted in up to 87% cellulose conversion from dilute ammonia treated energy cane bagasse (Aita et al., 2011).

Table 3.3 Chemical composition of untreated and dilute ammonia pretreated energy cane bagasse.

Biomass Component (g/100g dry biomass)	Untreated Bagasse	Dilute Ammonia Pretreated Bagasse
Ash	3.87±0.77	2.74±0.28
Extractives	5.45±0.53	4.36±0.60
Lignin	28.74±0.24	15.96±0.25
Glucan	40.26±0.34	37.61±0.16
Xylan	19.81±0.57	16.53±0.15
Arabinan	1.87±0.23	1.96±0.01
Mannan	ND*	ND*
Total	100.00	78.48±0.93

*ND: None detected.

After dilute ammonia pretreatment, a 22.52% total dry weight loss was observed mainly due to delignification and the solubilization of hemicellulose (Moreno et al., 2012). Approximately, 44% lignin and 17% xylan were removed in dilute ammonia pretreated energy cane bagasse. More

than 90% glucan was retained after dilute ammonia pretreatment. These results are in agreement with those reported by others (Aita et al., 2011; Oladi & Aita, 2017; Qiu et al., 2012).

3.3.2. Chemical Composition of Enzymatic Hydrolysate

The concentrations of fermentable sugars, organic acids, furfurals, and total phenolic compounds in the hydrolysate post enzymatic hydrolysis are summarized in Table 3.4. A concentration of 25.43 g/L total fermentable sugars was achieved after 72 h. Glucose was the predominant sugar (15.74 g/L) detected, while xylose concentrations were much lower (9.69 g/L). The highest concentration of organic acids resulted from formic acid (3.57 g/L), followed by acetic acid (2.38 g/L) and levulinic acid (1.31 g/L). Furaldehydes including furfural (0.04 g/L) and HMF (0.06 g/L) and total phenolic compounds (0.51 g/L) were detected in the hydrolysate. Dilute ammonia pretreatment followed by enzymatic hydrolysis yielded comparable amounts of fermentable sugars with fewer amounts of non-sugar compounds as compared to those reported for acid hydrolysis pretreated materials due to its milder treatment conditions and less hemicellulose solubilization (Behera et al., 2014; Jönsson & Martín, 2016). Chandel et al. (2007) reported that sugarcane treated with dilute hydrochloric acid reached a maximum sugar yield of 30.29 g/L and resulted in the generation of non-sugar compounds such as acetic acid (5.45 g/L), furaldehydes (1.89g/L) and total phenolic compounds (2.75 g/L). Mateo et al. (2013) observed the release of 25.23 g/L fermentable sugars and the formation of 1.67 g/L acetic acid, 0.22 g/L HMF and 3.76 g/L total phenolic compounds in olive tree residue acid hydrolysates. Organic acids can inhibit cell growth while furfural and HMF can be metabolized by microorganisms resulting in longer lag-phases (Palmqvist & Hahn-Hägerdal, 2000). Phenolic compounds disrupt biological membranes thus decreasing cell growth and enzyme efficiency (Behera et al., 2014). Inhibitory effect of these non-sugar compounds can be noticeable during fermentation when concentrations

of aliphatic carboxylic acids are > 100 mM and phenolic compounds are > 1g/L (Palmqvist & Hahn-Hägerdal, 2000). It has been widely reported that fermentation yields improved when non-sugar compounds were partially or totally removed from the hydrolysates (Kamal et al., 2011; Lee et al., 2011; Villarreal et al., 2006). Kamal et al. (2011) reported that AC detoxification of sago trunk acid hydrolysate resulted in more than 58% furfural and 78% phenolic compounds removal, thus improving xylitol fermentation yields by more than 100%. Similarly, Lee et al. (2011) observed a 79% increase in ethanol yields from woody acid hydrolysate post AC detoxification.

Table 3.4 Chemical composition of dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate.

	Chemical Compound	Concentration (g/L)
Sugars	Glucose	15.74±0.56
	Xylose	9.69±0.24
Non-sugar Compounds	Formic Acid	3.57±0.13
	Acetic Acid	2.38±0.06
	Levulinic Acid	1.31±0.06
	Furfural	0.04±0.00
	HMF	0.06±0.01
	Total Phenolic Compounds	0.51±0.03

3.3.3. Powdered Activated Carbon Treatment

Preliminary experiments showed that AC adsorption efficiency towards acetic acid was the lowest among all non-sugar components evaluated, which agree with published results (Lee et al., 2011; Mateo et al., 2013; Villarreal et al., 2006). Therefore, model fitting and parameter optimization were conducted based on the percent acetic acid removal.

3.3.3.1. Acetic Acid Removal by Powdered AC Treatment

Acetic acid removal (%) and total sugar loss (%) after each powdered AC treatment are summarized in Table 3.5. Significant acetic acid removal (32.60% - 42.77%) was observed for all treatment conditions. Based on the experimental data of percent acetic acid removal, a quadratic model was fitted and a second-order polynomial equation was built.

Table 3.5 Acetic acid removal (%) and total sugar loss (%) after powdered AC treatment.

Powdered AC Treatment Runs	Factor A: AC Dose (%)	Factor B: pH	Response A: Acetic Acid Removal (%)	Response B: Total sugar Loss (%)
1	9	1.3	32.60	8.70
2	11	1.5	40.45	11.65
3	9	2	39.56	9.90
4	11	2.5	37.40	12.07
5	9	2	38.83	9.60
6	9	2.7	33.34	11.23
7	9	2	39.78	10.20
8	6.2	2	33.66	7.01
9	11.8	2	42.77	12.20
10	7	1.5	31.43	6.85
11	9	2	40.06	10.30
12	9	2	39.78	9.60
13	7	2.5	32.62	8.80

Coefficients were estimated and analysis of variance (ANOVA) was performed to test the significance of linear, quadratic and interactive effects using Design-Expert 10.0.3.1. Lack-of-fit tests, R-squared, adjusted R-squared, and predicted R-squared were evaluated to ensure the model adequacy and goodness of fit. The obtained quadratic equation and corresponding ANOVA table are shown in equation (2) and Table 3.6, respectively. The fitted model was highly significant with a P-value < 0.0001, which indicated that both AC dose and pH had a significant impact on the response of percent acetic acid removal with significant quadratic and interactive effects (P-value < 0.01). P-value (0.28) of lack-of-fit test was greater than 0.05, which indicated the proposed statistical model fitted adequately. The determination coefficient (R-squared) was 0.9881, which suggested a strong correlation between the actual and predicted values as shown in Fig. 3.1. In addition, the adjusted determination coefficient (Adj R-squared) of 0.9797 was in reasonable agreement with the predicted determination coefficient (Pred R-squared) of 0.9434. The difference between Adj R-squared and Pred R-squared was less than 0.2, which also confirmed the adequacy

of the fitted model. The coefficient of variation (C.V. %) was low (1.46%), which implied the collected experiment data was precise and reliable.

Table 3.6 Analysis of variance (ANOVA) table for the model built for acetic acid removal (%).

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Value	p-value Prob > F	
Model	172.00	5	34.40	116.60	< 0.0001	significant
A-Ac Dose	88.99	1	88.99	301.61	< 0.0001*	
B-pH	0.083	1	0.083	0.28	0.6124	
AB	4.50	1	4.50	15.24	0.0059*	
A^2	3.63	1	3.63	12.30	0.0099*	
B^2	77.86	1	77.86	263.89	< 0.0001*	
Residual	2.07	7	0.30			
Lack of Fit	1.19	3	0.40	1.82	0.2831	not significant
Pure Error	0.87	4	0.22			
Corrected Total	174.07	12				
Std. Dev.	0.54		R-squared	0.9881		
Mean	37.10		Adj R-Squared	0.9797		
C.V. %*	1.46		Pred R-Squared	0.9434		

*Coefficient of variation.

$$\text{Acetic acid removal (\%)} = -62.24 + 7.04A + 62.87B - 1.06AB - 0.18A^2 - 13.38B^2 \quad (2)$$

Where, A: AC Dose (% w/w), B: pH

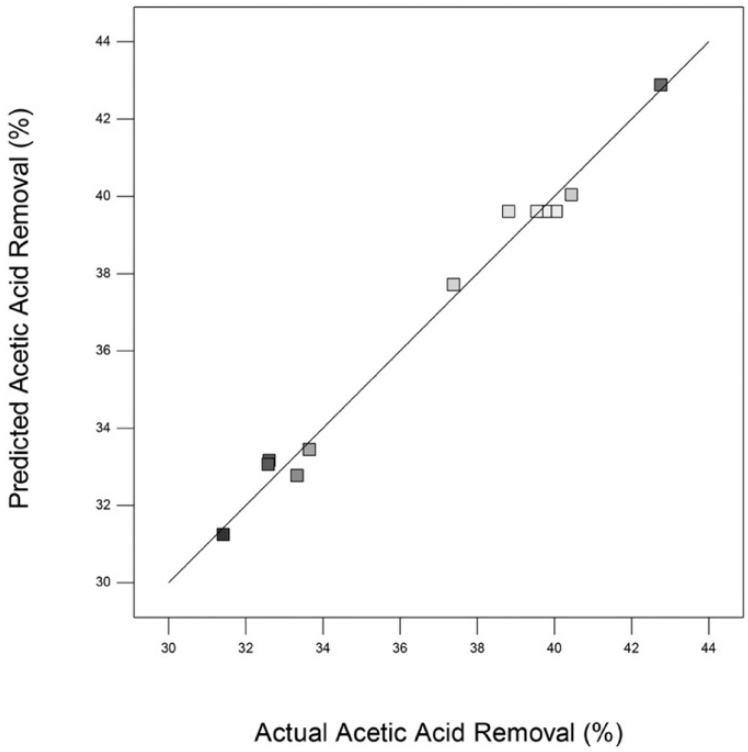

Figure 3.1 Predicted versus actual percent acetic acid removal by powdered AC treatment.

Based on the constructed quadratic model, three-dimensional response surface was plotted to investigate the effects of AC dose and hydrolysate pH on the response of acetic acid removal as shown in Fig. 3.2. A positive correlation between powdered AC dose and percent acetic acid removal was determined. At pH 2.5, acetic acid removal increased from 32.62% to 37.40% as the AC dose increased from 7% to 9%; whereas, at pH 1.5, acetic acid removal increased from 31.43% to 40.45% with a similar AC dose increase. Similar observations have been reported by Lee et al. (2011) where percent acetic acid removal improved from 16.2% to 39.4% after 30 min treatment as the AC dose increased from 1% to 10% (w/w) in acid treated woody hydrolysates. Mateo et al. (2013) found that in acid pretreated olive tree pruning residue hydrolysates, acetic acid removal increased from 15% to 45% when AC dose was increased from 2% to 8%.

pH had a strong quadratic effect on the percent removal of acetic acid (Fig. 3.2). At the AC dose of 11% (w/w), the highest percent acetic acid removal (42%) was observed at around pH 2.

When pH was adjusted to 1.5 or 2.5, the acetic acid removal declined to approximately 40% and 37%, respectively. Hydrolysate pH can strongly affect the AC adsorption efficiency as it changes properties of both adsorbent and adsorbate. An acidic environment (particularly when pH < pKa of the adsorbate) favors weak acids adsorption by AC as the non-ionized state of weak acids is more readily removed by AC adsorption when compared to the anion state at higher pH (Mussatto & Roberto, 2004). A decrease in pH value increases the concentration of non-ionized acetic acid (pKa = 4.76), thus resulting in an increase in acetic acid removal. Villarreal et al. (2006) reported that up to 32.2% of acetic acid was removed at pH 1.8, while only 10.6% was removed at pH 5.5 after 60 min at 5% (w/w) AC dose adsorption treatment in acid pretreated eucalyptus hydrolysates. However, when the pH was lowered to close to 1, the AC adsorption efficiency decreased as well, most likely due to the adsorption of protons on the basic sites on the AC, which decreases the interactions between these sites and acetic acid (Liu et al., 2010).

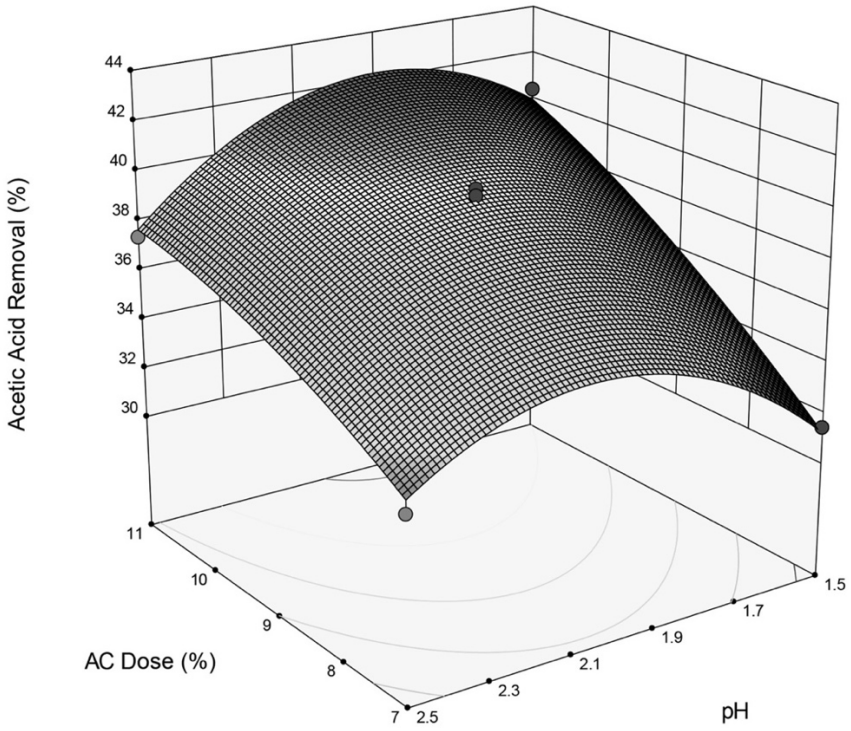

Figure 3.2 Response surface plot of the effect of AC dose and hydrolysate pH on acetic acid removal (%) after powdered AC treatment.

3.3.3.2. Total Sugar Loss by Powdered AC Treatment

A linear model was built for the response of total sugar loss as shown in equation (3). Based on the ANOVA results (Table 3.7), the fitted model ($R^2 = 0.9762$) was highly significant (P-value< 0.0001), which indicated that both AC dose and pH had a significant impact on the response (percent total sugar loss). Model adequacy was evaluated based on the same criteria as previously discussed.

Total Sugar Loss (%) = – 8.63 + 1.72A + 4.9B – 0.38AB (3)

Where, A: AC Dose (% w/w), B: pH

Table 3.7 Analysis of variance (ANOVA) table for the model built for total sugar loss (%).

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Value	p-value Prob > F	
Model	34.67	3	11.56	123.17	< 0.0001	significant
A-AC Dose	29.69	1	29.69	316.49	< 0.0001	
B-pH	4.40	1	4.40	46.85	< 0.0001	
AB	0.58	1	0.58	6.18	0.0347	
Residual	0.84	9	0.094			
Lack of Fit	0.42	5	0.083	0.78	0.6129	not significant
Pure Error	0.43	4	0.11			
Corrected Total	35.51	12				
Std. Dev.	0.31		R-squared	0.9762		
Mean	9.85		Adj R-Squared	0.9683		
C.V. %*	3.11		Pred R-Squared	0.9487		

The relationship between the response percent total sugar loss and variables AC dose and hydrolysate pH is shown in Fig. 3.3. As observed with percent acetic acid removal, total sugar loss had a positive correlation to AC dose. Total sugar loss increased from 6.85% to 11.65% when AC dose increased from 7% to 11% at pH 1.5. While at pH 2.5, total sugar loss increased from 8.80%

to 12.07% with the same AC dose increase. Villarreal et al. (2006) pointed out that 1% (w/w) AC treatment for 30 min resulted in 0% xylose loss at pH 5.5; however, when increasing the AC dose to 5% (w/w), xylose loss increased to 5.6%. Lee et al. (2011) reported that 1% (w/w) AC dose treatment for 30 min removed 2% of total fermentable sugars, but when the AC dose was increased to 10%, total sugar loss sharply increased to 30% in acid treated woody hydrolysates. Hydrolysate pH also affected total sugar loss. A reduced total sugar loss (8.7%) was observed at pH 1.3 as compared to the total sugar loss (11.2%) at pH 2.7 when the AC dose was fixed at 9% (w/w). Comparable results were reported in olive tree pruning residue acid hydrolysates, where removal of fermentable sugars increased from 20% to 26% as the hydrolysate pH was increased from 2 to 6 with a fixed AC dose at 5% (w/w) (Mateo et al., 2013).

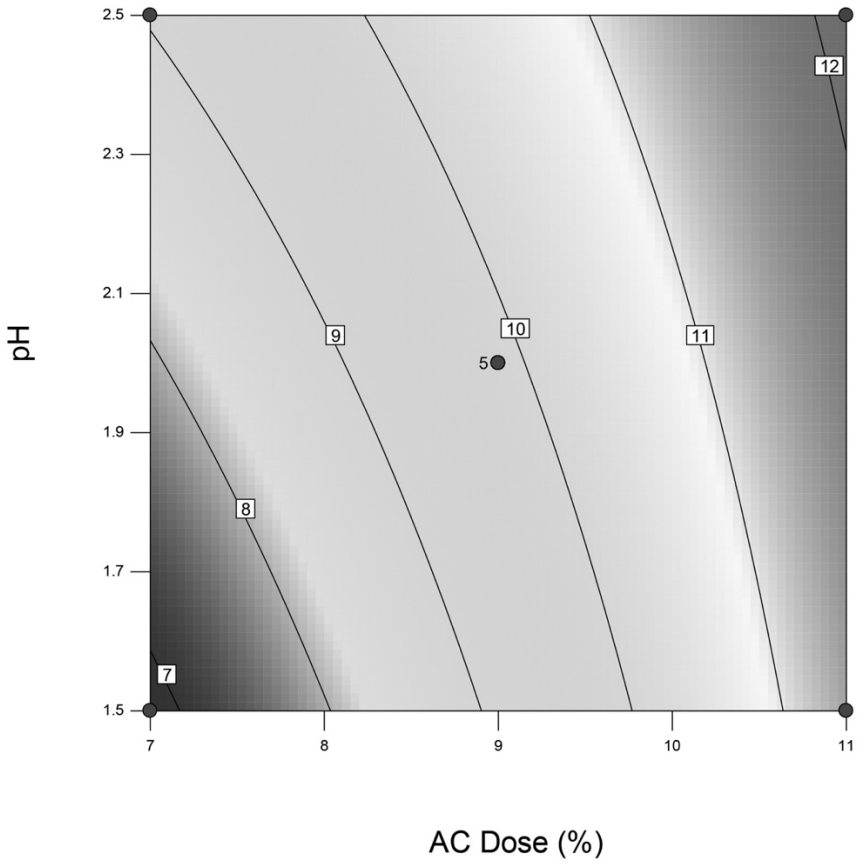

Figure 3.3 Contour plot of the effect of AC dose and hydrolysate pH on total sugar loss (%) after powdered AC treatment.

3.3.3.3. Optimization and Experimental Validation of Powdered AC Treatment

Optimum processing parameters in terms of AC dose and hydrolysate pH were estimated using Design-Expert 10.0.3.1 software. Parameters optimization was conducted by maximizing percent acetic acid removal, while limiting the total fermentable sugar loss to 10%. Powdered AC treatment of 9.21% AC dose and pH 1.96 predicted a 39.93% acetic acid removal with a 95% prediction interval of 38.53% to 41.34%. The experimental value for acetic acid removal (40.33%) fell within the 95% confidence interval of the predicted value, which indicated that this fitted model was reliable within the range of the experimental design space.

3.3.4. Granular Activated Carbon Treatment

3.3.4.1. Acetic Acid Removal after Granular AC Treatment

The acetic acid removal (%) and total sugar loss (%) after each granular AC treatment is summarized in Table 3.8. Significant acetic acid removal was noticed after granular AC treatment and ranged from 28.02% to 40.34%. A second-order quadratic model fitted for acetic acid removal (%) was significant (P value < 0.0001) with $R^2 = 0.9780$ (Table 3.9 and equation (4)).

$$\text{Acetic acid removal (\%)} = -96.15 + 8.13A - 63.67B + 0.45C - 0.87AB + 0.006AC - 0.07BC - 0.21A^2 - 12.80B^2 - 0.003C^2 \quad (4)$$

Where, A: AC Dose (% w/w), B: pH, C: Contact time (min)

Table 3.8 Acetic acid removal (%) and total sugar loss (%) after granular AC treatment.

Powdered AC Treatment Runs	Factor A: AC Dose (%)	Factor B: pH	Factor C: Contact Time (min)	Response A: Acetic Acid Removal (%)	Response B: Total sugar Loss (%)
1	13	2	73.6	39.08	12.47
2	13	2	40	37.34	9.10
3	13	2	40	38.06	9.15
4	15	2.5	20	30.81	11.98
5	9.6	2	40	31.03	5.22
6	11	1.5	60	33.59	7.89
7	11	2.5	20	29.39	6.32
8	13	1.2	40	29.59	9.52

Table 3.8 continued

Powdered AC Treatment Runs	Factor A: AC Dose (%)	Factor B: pH	Factor C: Contact Time (min)	Response A: Acetic Acid Removal (%)	Response B: Total sugar Loss (%)
9	15	2.5	60	34.68	13.27
10	11	2.5	60	31.38	7.98
11	13	2	40	38.34	10.22
12	11	1.5	20	28.02	5.71
13	16.4	2	40	40.34	14.32
14	13	2	40	37.64	10.04
15	13	2	6.4	29.16	7.08
16	15	1	60	39.52	14.44
17	15	1.5	20	33.74	12.33
18	13	2	40	38.02	9.75
19	13	2.8	40	28.46	9.65
20	13	2	40	39.06	10.02

Table 3.9 ANOVA table for the model of acetic acid removal (%) after granular AC treatment.

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Value	p-value Prob > F	
Model	344.75	9	38.31	49.46	< 0.0001	significant
A-Ac Dose	75.10	1	75.10	96.97	< 0.0001	
B-pH	8.09	1	8.09	10.45	0.0090	
C-Time	84.07	1	84.07	108.55	< 0.0001	
AB	6.03	1	6.03	7.78	0.0191	
AC	0.54	1	0.54	0.70	0.4222	
BC	3.76	1	3.76	4.86	0.0521	
A^2	10.27	1	10.27	13.26	0.0045	
B^2	147.48	1	147.48	190.43	< 0.0001	
C^2	28.14	1	28.14	36.33	0.0001	
Residual	7.74	10	0.77			
Lack of Fit	5.98	5	1.20	3.39	0.1033	not significant
Pure Error	1.76	5	0.35			
Corrected Total	352.49	19				
Std. Dev.	0.88		R-squared	0.9780		
Mean	34.36		Adj R-Squared	0.9583		
C.V. %*	2.56		Pred R-Squared	0.8624		

The response percent acetic acid removal against variables AC dose and pH is shown in Fig. 3.4 (A). A positive correlation between the response percent acetic acid removal and AC dose was observed, while pH had a quadratic effect on the percent removal of acetic acid. The highest percent acetic acid removal (40%) was observed at the highest AC dose (15% (w/w)) and pH around 2.0. Acetic acid removal decreased to 38% and 34% as pH was adjusted to 1.5 and 2.5 respectively. These results are comparable to those observed with powdered AC. The effects of AC dose and contact time on the response of percent acetic acid removal are shown in Fig. 3.4 (B). A positive correlation was determined between the response percent acetic acid removal and contact time. At the AC dose of 15% (w/w), acetic acid removal increased from 35% to 41% as the contact time increased from 20 to 60 min. At the AC dose of 11% (w/w), acetic acid removal increased from 32% to 36% as the contact time increased from 20 to 60 min.

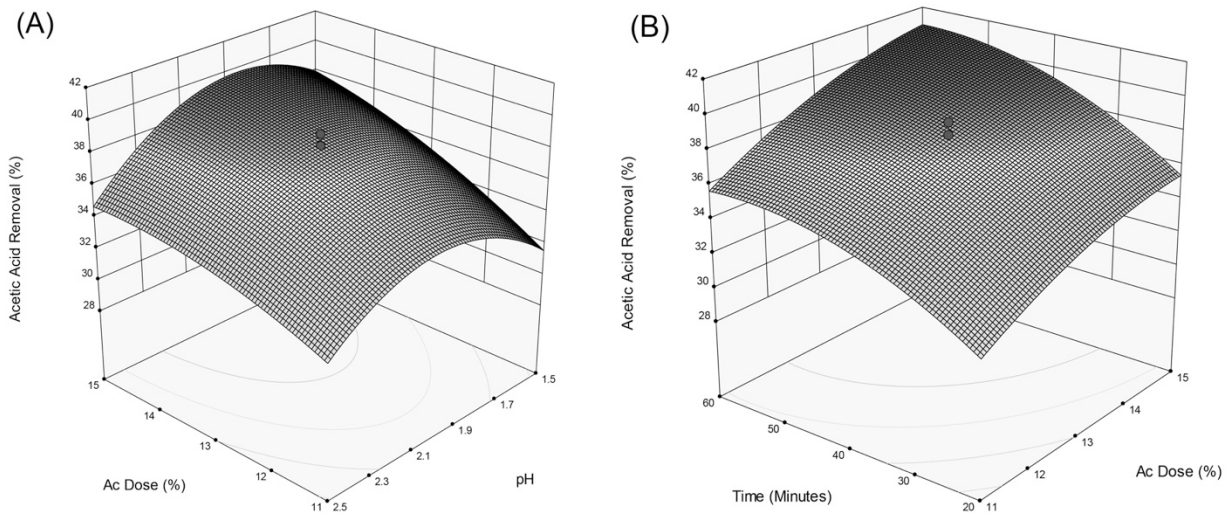

Figure 3.4 Response surface plots of the effect of AC dose, hydrolysate pH and contact time on acetic acid removal (%) after granular AC treatment.
(A) Effect of AC dose and hydrolysate pH on the response of percent acetic acid removal.
(B) Effect of AC dose and contact time on the response of percent acetic acid removal.

Adequate contact time between granular AC and hydrolysate is essential to achieve the AC equilibrium with the non-sugar compounds (Mussatto & Roberto, 2004). Lee et al. (2011) reported

that in acid treated woody hydrolysate, when increasing contact time from 10 to 30 min at 2.5% (w/w) AC dose, formic acid removal increased from 39% to 49%, acetic acid removal increased from 22% to 25%, and HMF removal increased from 70% to 96%. Granular AC required longer contact times (60 min) to achieve the maximum acetic acid removal (40%) as compared to powdered AC (10 min). It was also reported that coarse AC required greater contact times as compared to finely ground AC to approach similar removal of organic contaminants (polychlorinated biphenyl and polycyclic aromatic hydrocarbons) in marine sediments at the same treatment conditions (Lebo et al., 2003; Zimmerman et al., 2005).

3.3.4.2. Total Sugar Loss after Granular AC Treatment

A linear model (P-value < 0.0001) with AC dose and contact time was fitted for the response of total sugar loss (%) with $R^2 = 0.9676$. ANOVA is summarized in Table 3.10. Total sugar loss had a linear positive relationship with both granular AC dose and contact time based on the contour plot as shown in Fig. 3.5. When contact time was fixed at 60 min, total sugar loss increased from 7.98% to 13.27% as granular AC dose increased from 11% to 15% (w/w). Similarly, total sugar loss increased from 6.32% to 7.98% as contact time increased from 20 to 60 min when AC dose was held at 11% (w/w). Wang and Chen (2011) indicated that 7.5% (w/v) activated charcoal treatment of enzymatic hydrolysate from steam-exploded corn stover resulted in 20% total fermentable sugar losses. Carvalheiro et al. (2005) reported a 13% total sugar loss in brewery's spent grain hydrolysate after 1 h granular AC treatment at 10% (w/v) dose. Unlike powdered AC, hydrolysate pH had no significant effect on total fermentable sugar loss. Lee et al. (2011) reported that sugars are not chemically adsorbed by AC, but rather being physically entrapped within the AC particles, which may be able to explain the difference in performance observed by granular and powdered AC.

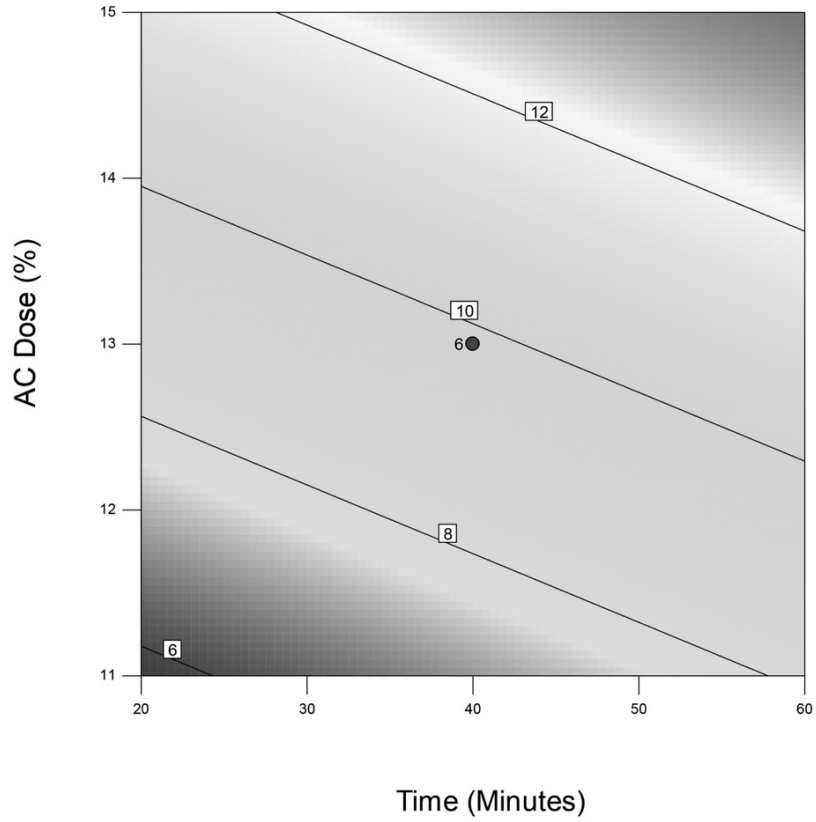

Figure 3.5 Contour plot of the effect of AC dose and contact time on total sugar loss (%) after granular AC treatment.

Table 3.10 ANOVA table for the model of total sugar loss (%) after granular AC treatment.

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Value	p-value Prob > F	
Model	133.26	2	66.63	254.22	< 0.0001	significant
A-AC Dose	113.76	1	113.76	434.04	< 0.0001	
C-Time	19.50	1	19.50	74.41	< 0.0001	
Residual	4.46	17	0.26			
Lack of Fit	3.31	12	0.28	1.20	0.4492	not significant
Pure Error	1.15	5	0.23			
Corrected Total	137.72	19				
Std. Dev.	0.51		R-squared	0.9676		
Mean	9.82		Adj R-Squared	0.9638		
C.V. %*	5.21		Pred R-Squared	0.9520		

3.3.4.3. Optimization and Experimental Validation of Granular AC Treatment

Optimization of granular AC treatment was conducted based on the same criteria as powdered AC. The parameter combination of 12.64% (w/w) AC dose, pH 1.91 and 51.60 min contact time was predicted to yield a percent acetic acid removal of 38.65% with a 95% prediction interval from 36.54% to 40.76%, while limiting the total sugar loss to 10%. The experimental acetic acid removal (37.98%) fell within the 95% confidence interval of the predicted value, which indicated that this model is trustworthy within the range of the designed space.

3.3.5. Comparison of Powdered and Granular Activated Carbon Treatments

Percent removal of non-sugar compounds and total fermentable sugar losses at optimized conditions for powder and granular AC are summarized in Table 3.11. Powdered AC treatment removed 78% formic acid, 40% acetic acid, 100% levulinic acid, 89% HMF, 100% furfural, and 99% total phenolic compounds. Granular AC treatment removed 76% formic acid, 38% acetic acid, 97% levulinic acid, 90% HMF, 100% furfural, and 98% total phenolic compounds. Formic acid, levulinic acid, furfural, HMF, and phenolic compounds were more readily to be removed by AC treatment compared to acetic acid. Lee et al. (2011) reported that in acid treated woody hydrolysates, treatment at 5% (w/w) AC dose for 0.5 h removed 72.4% formic acid and nearly 100% HMF and furfural, while acetic acid removal was only 32.3%. Mateo et al. (2013) observed similar results when comparing the total phenolic compounds removal to acetic acid removal by AC treatment. AC treatment at 8% (w/w) dose resulted in the removal of 97% furfural, 81% total phenolic compounds and only 45% acetic acid in hydrolysates from olive tree pruning residue. When the total sugar loss was limited to 10%, similar non-sugar compounds removal was achieved by both powdered and granular AC at their optimum conditions. However, granular AC required higher dosage (12.64%) and longer contact times (51.60 min) compared to powdered AC (9.21%

AC dose for 10 min). This is due to the reduced surface area of granular AC (650 m^2/g) compared to powered AC (1400 m^2/g). Lebo et al. (2003) reported that finely ground AC outperformed coarse AC by up to 110% in removing organic contaminants from their aqueous solutions at the same treatment conditions. Ou et al. (2007) observed that powdered AC had 33% higher adsorption capacity over granular AC towards ferulic acid in the purification of alkaline hydrolysate from sugarcane bagasse.

Table 3.11 Effect of powdered and granular AC treatments at optimized conditions.

		Powdered AC	Granular AC
Optimized Conditions	AC Dose	9.21%	12.64%
	pH	1.96	1.91
	Contact Time	10 min	51.60 min
Formic Acid Removal (%)		77.68±0.51	75.68±0.92
Acetic Acid Removal (%)		40.33±0.59	37.98±0.88
Levulinic Acid Removal (%)		100.00±0.00	96.54±0.80
HMF Removal (%)		89.33±0.38	90.43±0.85
Furfural Removal (%)		100.00±0.00	100.00±0.00
Phenolic Compounds Removal (%)		98.87±0.83	97.93±0.78
Total Sugar Loss (%)		9.65±0.32	9.78±0.51

3.4. Conclusions

Non-sugar compounds generated from dilute ammonia pretreatment of energy cane bagasse can be successfully removed by both powdered and granular AC treatments with minimal fermentable sugar loss. Variables including AC dose, hydrolysate pH and contact time were optimized for maximum percent acetic acid removal and minimal total sugar loss by RSM. Optimum conditions for powdered AC were 9.21% (w/w) AC dose, pH 1.96 and 10 min contact time. Optimum conditions for granular AC were 12.64% (w/w) AC dose, pH 1.91 and 51.60 min contact time. At optimum conditions, powdered and granular AC removed 40% acetic acid, 75% formic acid, 100% levulinic acid, 90% HMF, 100% furfural, and 98% total phenolic compounds with less than 10% total sugar losses. Particle size of AC significantly affected AC adsorption

efficiency. Powdered AC required less dose and a shorter contact time to achieve maximum non-sugar compounds removal compared to granular AC. Therefore, powdered AC adsorption is a promising alternative for the removal of non-sugar compounds with minimal fermentable sugar loss from dilute ammonia pretreated energy cane bagasse hydrolysates for producing high purity lignocellulosic syrups and the potential recovery of these non-sugar compounds as value-added products.

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CHAPTER 4
FERMENTATION OF FUMARIC ACID FROM PURIFIED LIGNOCELLULOSIC SYRUP BY *RHIZOPUS ORYZAE* ATCC® 20344™

4.1. Introduction

Fumaric acid, a dicarboxylic acid, has been identified by the U.S. Department of Energy as one of the "top 12" building-block chemicals that can be potentially manufactured using renewable lignocellulosic biomass (Liu et al., 2015). Fumaric acid can serve as an acidulant in food and animal feeds, and as the raw material in the production of polymer resins, plasticizers, esters, and inks (Engel et al., 2011; Zhang et al., 2015). It is also a chemical intermediate in the synthesis of malic acid, succinic acid and aspartic acid, which are widely used as food additives and as precursors to polymers and resins (Choi et al., 2015). Monoethyl fumarate and dimethyl fumarate are the ester derivatives of fumaric acid and can be used as mold inhibitors and in the medical treatment of psoriasis (Moharregh-Khiabani et al., 2009).

Fumaric acid is currently produced via the isomerization of petroleum-derived maleic acid with a yield of up to 90% (Zhang et al., 2013). However, limitation on petroleum resources, economic and environmental sustainability concerns, as well as consumer preference for bio-based products have advocated the use of bio-based manufacturing methods for renewable feedstocks in the production of fumaric acid (Roa Engel et al., 2008). Fumaric acid is biologically synthesized as an intermediate compound in the tricarboxylic acid cycle. *Rhizopus oryzae* is one of the most commonly used microorganisms in the fermentation of fumaric acid due to high yields and low by-product formation (Mondala, 2015; Zhang et al., 2013).

The major challenges in fumaric acid fermentation are the control of fungal morphology and medium composition. In submerged fermentation, filamentous fungi can grow into different morphologies, which can be affected by medium composition, inoculum size, pH, and medium

additives (Zhang et al., 2015; Zhou et al., 2000). Small pellets are the preferred morphology for fermentation to achieve optimum agitation, culture rheology and oxygen transfer (Liao et al., 2007). Generally, high fumaric acid fermentation yields can be achieved through a two-stage fermentation, seed culture stage and acid production stage. During the seed culture stage, adequate nutrients are provided to promote fungal growth; whereas, in the acid production stage, fungal growth is limited by a reduced supply of nitrogen to favor the overproduction of fumaric acid (Roa Engel et al., 2008; Xu et al., 2010; Zhang et al., 2013). Therefore, the carbon to nitrogen (C/N) ratio (w/w) in the acid production medium is critical for achieving high fumaric acid production. Additionally, in the acid production medium, the addition of a neutralizing agent (i.e., calcium carbonate) is important for maintaining an optimum pH, removing any inhibitory end products, and providing the required supply of carbon dioxide to produce fumaric acid (Xu et al., 2012).

Pure glucose is the most favorable carbon source for fumaric acid fermentation by filamentous fungi. However, using pure sugars as carbon source significantly increases production costs and sacrifices potential food supplies (Mondala, 2015). Thus, efforts have been made to achieve ideal fermentation yields using sugar and nutrient-enriched materials including sugar molasses, corn starch and potato flour to lower production costs (Moresi et al., 1992; Moresi et al., 1991; Petruccioli et al., 1996). Lignocellulosic biomass (i.e., energy cane bagasse, corn straw, wood chips) is one of the most abundant and readily available renewable resources that can also be used as a carbon source in the production of fumaric acid (Carta et al., 1999; Liao et al., 2008; Rodríguez-López et al., 2012; Xu et al., 2010). However, harsh pretreatment and hydrolysis conditions needed to access the lignocellulosic sugars (mostly glucose and xylose) result not only in the release of fermentable sugars but the generation of non-sugar compounds such as organic acids, furans and phenolic compounds, which can inhibit cell growth and fermentation yields

(Palmqvist & Hahn-Hägerdal, 2000b). Another challenge in using lignocellulosic biomass as the carbon source is the utilization of xylose. Xylose is not readily fermented like glucose by *Rhizopus oryzae* due to its indirect metabolism pathway (Mondala, 2015). As a result, reduced fumaric acid yields have been observed. Liao et al. (2008) pointed out that using acid pretreated dairy manure hydrolysates containing 20 g/L glucose and 4 g/L xylose resulted in a yield of only 0.15 g/g fumaric acid by *R. oryzae*. Xu et al. (2010) achieved 0.35 g/g fumaric acid by *R. oryzae* using acid pretreated corn straw enzymatic hydrolysate (31.2 g/L glucose and 1.8 g/L xylose) as a carbon source. However, fumaric acid yields ranging from 0.45 to 0.91 g/g by *R. oryzae* have been observed when using synthetic medium containing pure glucose as the carbon source (Cao et al., 1996; Ding et al., 2011; Kang et al., 2010).

Improved strategies have been developed to enhance the utilization of lignocellulosic biomass for fumaric acid fermentation. Rodríguez-López et al. (2012) reported that after removing non-sugar compounds (acetic acid, furfural and 5-hydroxymethylfurfural (HMF)) from Eucalyptus wood hydrolysate using ion-exchange resins, fumaric acid yields increased from 0.35 to 0.44 g/g. Liu et al (2015) observed that *Rhizopus arrhizus RH 7-13-9#,* a new strain derived from *R. arrhizus RH 7-13,* increased fumaric acid production from 28.5 g/L to 45.3 g/L, using xylose as the sole carbon source.

This study evaluated fumaric acid fermentation yields by *R. oryzae* ATCC® 20344™ using activated carbon purified lignocellulosic syrup, a syrup mostly of glucose and xylose derived from dilute ammonia pretreated energy cane bagasse.

4.2. Materials and Methods

4.2.1. Lignocellulosic Syrup Preparation

Non-commercial energy cane variety Ho 02-113 was harvested from the Sugar Research Station in St. Gabriel, LA. Energy cane (with stalks and leaves) was passed through a roller press (Farrel Corporation, Ansonia, CT) three times. Bagasse collected after juice extraction was dried in a 45°C oven for 24 h to a final moisture content of 5%, finely milled (Wiley Mill, Swedesboro, NJ) and sieved with a 2 mm mesh sieve. Pretreatment was carried out in a 4 L reactor (Parker Autoclave Engineers, Erie, PA) with finely milled and sieved energy cane bagasse, ammonium hydroxide (28% v/v solution, Fisher Scientific, Pittsburgh, PA) and water at a ratio of 1: 0.5: 8 at 160°C for 1 h. Post pretreatment, bagasse was dried at 45°C for 24 h to a moisture content of approximately 5%. Chemical composition of both untreated and pretreated bagasse was analyzed following National Renewable Energy Laboratory Analytical Procedures (LAP TP-510-42618, 42619, 42620, 42621, 42622). Enzymatic hydrolysis was conducted using commercially available enzymes Cellic® CTec2 (cellulase) and HTec2 (xylanase) provided by Novozymes (Novozymes, Franklinton, NC). Hydrolysis were conducted at 5% (w/w) dilute ammonia pretreated bagasse loading, and enzymes added at 25% (w/w) g/g glucan CTec2 and 5% (w/w) g/g glucan HTec2. The mixture (pH 5.0) was incubated at 50°C for 72 h at 200 rpm. The solid fraction was removed by filtration using a 0.2 μm filter (VWR, Radnor, PA). Collected hydrolysate samples were subjected to powdered activated carbon (AC) (Cabot Corporation, Alpharetta, GA) adsorption at optimum conditions (9.2% (w/w) dose, pH 2, 200 rpm, 22°C) for the removal of non-sugar compounds (organic acids, furans and total phenolic compounds) from hydrolysates. Powdered AC purified dilute ammonia pretreated energy cane bagasse hydrolysate was then concentrated to 65°Bx under vacuum at 70°C using a rotary vacuum evaporator (Cole-Parmer, Vernon Hills, IL).

The lignocellulosic syrup was stored at 4°C and diluted accordingly prior to fumaric acid fermentation studies. Sugars (glucose and xylose) and non-sugar compounds (acetic acid, formic acid, levulinic acid, furfural, HMF, total phenolic compounds) present in the hydrolysate, powdered AC treated hydrolysate and purified syrup were analyzed using the analytical methods described below.

4.2.2. Microorganism and Media

Rhizopus oryzae Spores

Rhizopus oryzae ATCC® 20344™ was cultured on potato dextrose agar (PDA) (Sigma-Aldrich, St. Louis, MO) plates for 6 days at 30°C. Spores were collected by washing the surface of the PDA plates with sterile distilled water. Spores were stored at 4°C and their concentration determined using a hemocytometer.

Seed Culture Medium

Unless otherwise specified, all chemicals (purity > 99%) used in the media were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). Seed culture medium was prepared with 30 g/L glucose, 2.0 g/L urea (added post sterilization) or yeast extract, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.11 g/L $ZnSO_4 \cdot 7H_2O$, and 0.0088 g/L $FeSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to 3.0, 4.0 or 5.0 with 3N HCl to investigate the effect of the two nitrogen sources (urea or yeast extract) and pH on fungal morphology and biomass accumulation. All seed culture experiments were performed in 250 ml baffled flasks each flask containing 50 ml seed culture medium. Silicone sponge closures were used to ensure sufficient oxygen transfer during fermentation. All flasks were sterilized at 121°C for 20 min. A concentrated urea solution was sterilized by 0.2 μm filtration and aseptically transferred to the sterilized medium to a final concentration of 2 g/L. A 1 ml spore suspension (10^6 spores/mL) was then inoculated into each

flask and all flasks incubated at 35°C for 24 h at 200 rpm. Optimum *Rhizopus oryzae* ATCC® 20344™ seed conditions (pH and nitrogen source) were selected and used during the fumaric acid production studies.

Acid Production Medium

Acid production medium was used to evaluate the effect of C(glucose)/N(urea) ratio on fumaric acid production yield. The media contained 0, 0.2, 0.4, or 0.8 g/L urea (added post sterilization), 80 g/L glucose, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.11 g/L $ZnSO_4 \cdot 7H_2O$, 0.0088 g/L $FeSO_4 \cdot 7H_2O$, and 50g/L $CaCO_3$. Calcium carbonate ($CaCO_3$) was added as the neutralizing agent and the medium kept at pH 5.5. Fumaric acid fermentations were carried out in 250 ml baffled flasks with silicone sponge closures each containing 50 ml of acid production medium. All flasks were sterilized at 121°C for 20 min. A concentrated urea solution was sterilized by 0.2 µm filtration, diluted accordingly and aseptically transferred into each flask. Each flask was inoculated with 10% (v/v) *Rhizopus oryzae* ATCC® 20344™ seeds and incubated at 35°C for 96 h at 200 rpm. *Rhizopus oryzae* ATCC® 20344™ seeds selected for fumaric acid production studies were uniform compact pellets grown in a seed culture medium adjusted to pH 3 and containing 2.0 g/L urea as the nitrogen source. Flasks were sampled every 24 h and analyzed for glucose residue and fumaric acid concentration by high performance liquid chromatography (HPLC) as described below.

4.2.3. Lignocellulosic Syrup as a Carbon Source for Seed Culture and Acid Production Media

Lignocellulosic syrup was investigated as an alternative carbon source in seed culture and acid production media. Seed culture and acid production media optimum conditions were chosen based on the results obtained from previous sections. Syrup-enriched seed culture medium was prepared with lignocellulosic syrup diluted to 30 g/L total sugars (20 g/L glucose and 10 g/L

xylose), 2 g/L urea, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.11 g/L $ZnSO_4 \cdot 7H_2O$, 0.0088 g/L $FeSO_4 \cdot 7H_2O$ and the medium pH adjusted to 3.0. Syrup-enriched acid production medium was prepared with lignocellulosic syrup diluted to 80 g/L total sugars (53 g/L glucose and 27 g/L xylose), 0.2 g/L urea, 0.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.11 g/L $ZnSO_4 \cdot 7H_2O$, 0.0088 g/L $FeSO_4 \cdot 7H_2O$, 50 g/L $CaCO_3$ and the medium pH maintained at 5.5. Control seed culture medium (20 g/L glucose and 10 g/L xylose) and control acid production medium (53 g/L glucose and 27 g/L xylose) were prepared using HPLC grade sugars (purity > 99%) as a carbon source rather than the diluted lignocellulosic syrup. Source and concentrations for nitrogen (urea), C (glucose and xylose)/N (urea) ratio, mineral ions, and neutralizing agent (calcium carbonate) remained the same. All seed culture media and acid production media (syrup-enriched and controls) experiments were carried out in 250 ml baffled flasks each flask containing 50 ml seed culture medium or acid production medium (syrup-enriched or controls). All flasks were sterilized at 121°C for 20 min followed by the addition of a filtered sterilized urea solution to a final concentration of 2.0 g/L in the seed culture medium and 0.2 g/L in the acid production medium. A 1 ml spore suspension (10^6 spores/mL) was inoculated into each seed culture media flask (syrup-enriched and control) and incubated at 35°C for 24 h at 200 rpm. Seeds from each seed culture media flask were inoculated at 10% (v/v) to either syrup-enriched or control acid production media and incubated at 35°C for 144 h at 200 rpm (Table 4.1).

Table 4.1 Summary of seed culture and acid production media composition.

Test	Carbon Sources	
	Seed Culture Medium	Acid Production Medium
A	Lignocellulosic Syrup	Lignocellulosic Syrup
B	HPLC Grade Sugar	Lignocellulosic Syrup
C	HPLC Grade Sugar	HPLC Grade Sugar

4.2.4. Analytical Methods

Fumaric acid can form calcium fumarate in the presence of calcium carbonate, the neutralizing agent present in the acid production medium. To release the fumaric acid from calcium carbonate, the acid production medium was acidified with 3N hydrochloric acid at 80°C post fermentation. The acidified medium was then diluted accordingly and filtered for HPLC analysis. Enzymatic hydrolysate and lignocellulosic syrup were analyzed for formic acid, acetic acid, levulinic acid, furfural, and HMF by HPLC. Organic acids were analyzed using a HPLC (Agilent 1100 Series) equipped with a Diode Array Detector (G1315B Agilent) at 210 nm and a Shimadzu VP-ODS column thermally controlled at 40°C. The mobile phase was 0.005 N sulfuric acid with a flow rate set at 0.35 mL/min. Total phenolic compounds in the hydrolysate and lignocellulosic syrup were analyzed by measuring the UV absorbance at 280 nm (Somers & Ziemelis, 1985).

Sugars (glucose and xylose) from the hydrolysate, lignocellulosic syrup and acid production medium post fermentation were quantified by HPLC (Agilent 1200 Series) equipped with a BioRad Aminex HPX-P87P (P) column set at 80°C and a differential Refractive Index Detector (G1362A Agilent). Sample volume was set at 20 µl with HPLC water used as the mobile phase at 0.8 mL/min.

Fungal biomass was determined by filtering the seed culture and acid production media (post fermentation) through a Whatman® filter paper No. 1. The collected mycelia clump or pellets were rinsed twice with distilled water (for seed culture medium) or with 3N HCl (for acid production medium) and dried in an oven at 100°C until constant weight was achieved (Liao et al., 2007; Singh et al., 2012).

4.2.5. Calculations and Statistical Analysis

Fumaric acid yield (g/g) was calculated as the amount of acid produced (g) divided by the amount of substrate consumed (g). The volumetric productivity (g/L/h) is expressed as grams of fumaric acid synthesized per liter per hour. Statistical significance was detected by analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test at a 95% confidence level using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA).

4.3. Results and Discussion

4.3.1. Chemical Composition of Purified Lignocellulosic Syrup from Energy Cane Bagasse

The chemical composition for enzymatic hydrolysate, powdered AC treated enzymatic hydrolysate and lignocellulosic syrup from dilute ammonia pretreated energy cane bagasse are summarized in Table 4.2. Concentration of sugars in the hydrolysate were 17.41 g/L glucose and 10.25 g/L xylose. Non-sugar compounds present in the hydrolysate were 2.81 g/L formic acid, 1.74 g/L acetic acid, 0.97 g/L levulinic acid, 0.02 g/L HMF, 0.05 g/L furfural, and 0.61 g/L total phenolic compounds prior to treatment with activated carbon. The presence of non-sugar compounds in the hydrolysate can significantly inhibit fermentation (Palmqvist & Hahn-Hägerdal, 2000a). Organic acids can pass through plasma membranes and reduce the intracellular pH, thus inactivating cell replication. Furans are metabolized by microorganisms with the excretion of acetaldehyde and pyruvate, which extend the lag-phase. Phenolic compounds disrupt biological membranes thus limiting cell growth and enzymatic activities (Behera et al., 2014; Palmqvist & Hahn-Hägerdal, 2000b). It has been reported that the negative effect of these non-sugar compounds during fermentation can be significant when concentrations of formic acid, acetic acid or levulinic acid are greater than 0.2 M and phenolic compounds exceed 1g/L (Larsson et al., 1999; Palmqvist & Hahn-Hägerdal, 2000b).

Table 4.2 Chemical composition of untreated and powdered AC treated dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate and lignocellulosic syrup.

	Untreated Enzymatic Hydrolysate	Powdered AC Treated Hydrolysate	Lignocellulosic Syrup
Glucose (g/L)	17.41±0.56	16.59±0.51	407.55±8.33
Xylose (g/L)	10.25±0.24	8.96±0.32	204.34±5.82
Formic Acid (g/L)	2.81±0.15	0.64±0.03	N.D.
Acetic Acid (g/L)	1.74±0.13	1.02±0.07	0.92±0.11
Levulinic Acid (g/L)	0.97±0.09	N.D.	N.D.
HMF (g/L)	0.02±0.00	N.D.	N.D.
Furfural (g/L)	0.05±0.01	N.D.	N.D.
Total Phenolic Compounds (g/L)	0.61±0.04	0.01±0.00	N.D.
Ash (% w/w)	< 0.5	< 0.5	8.81

N.D. None Detected.

Powdered AC treatment of dilute ammonia pretreated hydrolysates resulted in the removal of 78% formic acid, 40% acetic acid, and more than 90% levulinic acid, HMF, furfural, and phenolic compounds with only 8% total fermentable sugar losses. Similar observations have been reported elsewhere (Kamal et al., 2011; Lee et al., 2011; Mateo et al., 2013). Lee et al. (2011) pointed out that powdered AC treatment of woody acid hydrolysates resulted in the removal of 42% formic acid, 14% acetic acid, 96% HMF, and 93% furfural with 9% total fermentable sugar losses, and an observed 79% increase in ethanol yields by *Thermoanaerobacterium saccharolyticum*. Kamal et al. (2011) reported that by removing 58% furfural and 78% total phenolic compounds with 6% fermentable sugar losses from 2.5% (w/v) AC treated sago trunk acid hydrolysates improved xylitol fermentation yields by 154% as compared to controls.

Lignocellulosic syrup produced by evaporating powdered AC treated dilute ammonia pretreated energy cane bagasse hydrolysates at 70°C under vacuum to a final 65°Bx contained 407.55 g/L glucose, 204.34 g/L xylose and 0.92 g/L acetic acid. In addition, 8.81% ash content was detected in the lignocellulosic syrup. The presence of ash may be problematic in downstream biorefinery processes. Ash can deactivate catalysts, and cause blockage and erosion issues to the

equipment during thermochemical processes (Ahlgren et al., 2008; Jenkins et al., 1998). Thus, the ash present in the lignocellulosic syrup may need to be reduced. Electrodialysis or ion exchange resin are example of processes that can serve this purpose (EI Khattabi et al. 1996).

4.3.2. Effect of Seed Culture Media Conditions on Fungal Morphologies

The effect of seed culture media pH (3.0, 4.0, 5.0) and nitrogen sources (urea, yeast extract) on the morphology of *Rhizopus oryzae* ATCC® 20344™ is shown in Fig. 4.1. Small uniformly dispersed pellets over filamentous or large clumps are the favored morphology for *Rhizopus oryzae* ATCC® 20344™ during fumaric acid fermentation for better agitation, culture rheology, oxygen transfer efficiency, and fumaric acid separation (Liao et al., 2007). Both, the nitrogen source and the initial medium pH play important roles on fungal morphology. Generally, inorganic nitrogen sources such as urea, potassium nitrate and ammonium sulfate promote the formation of compact smooth pellets, while the use of complex organic nitrogen sources such as peptone, yeast extract, potato dextrose broth, and soybean flour result in filamentous forms (Papagianni, 2004). At pH 3.0, compact ($d \approx 1$ mm), uniformly distributed and spherical pellets were formed when using urea (2.0 g/L) as the nitrogen source; whereas, non-uniform pellets ($d = 0.5\sim4$ mm) were formed when yeast extract at 2.0 g/L was used as the nitrogen source in the seed culture medium (Fig. 4.1A, B). Non-uniform pellets and large aggregated mycelium clumps were also observed in both 2.0 g/L urea or yeast extract seed culture medium adjusted to pH 4.0 and 5.0 (Fig. 4.1C, D, E, F). Similar results have been reported by Zhang et al. (2015) where seed culture medium (pH 3.2) containing 2.0 g/L urea or yeast extract as a nitrogen source produced uniform pellets by *R. oryzae*; whereas, non-uniform pellets with large aggregated mycelia clumps were formed when the media was adjusted to pH 4.2-6 containing the same concentration of urea or yeast extract. Zhou et al. (2000) also reported that when using 2.0 g/L urea as a nitrogen source, small, uniformly dispersed and

smooth pellets (d ≈ 1 mm) were formed in the seed culture media adjusted to pH 2.60 – 3.36; whereas, the less preferred filamentous form was observed at pH 3.93 – 5.59 with 2.0 g/L urea. Du et al. (2003) observed compact smooth pellets of *Rhizopus chinesis* when grown in a medium using 2.0 g/L inorganic ammonium sulfate as a nitrogen source, while entangled filaments were observed when rich organic yeast extract was used at 2.0 g/L.

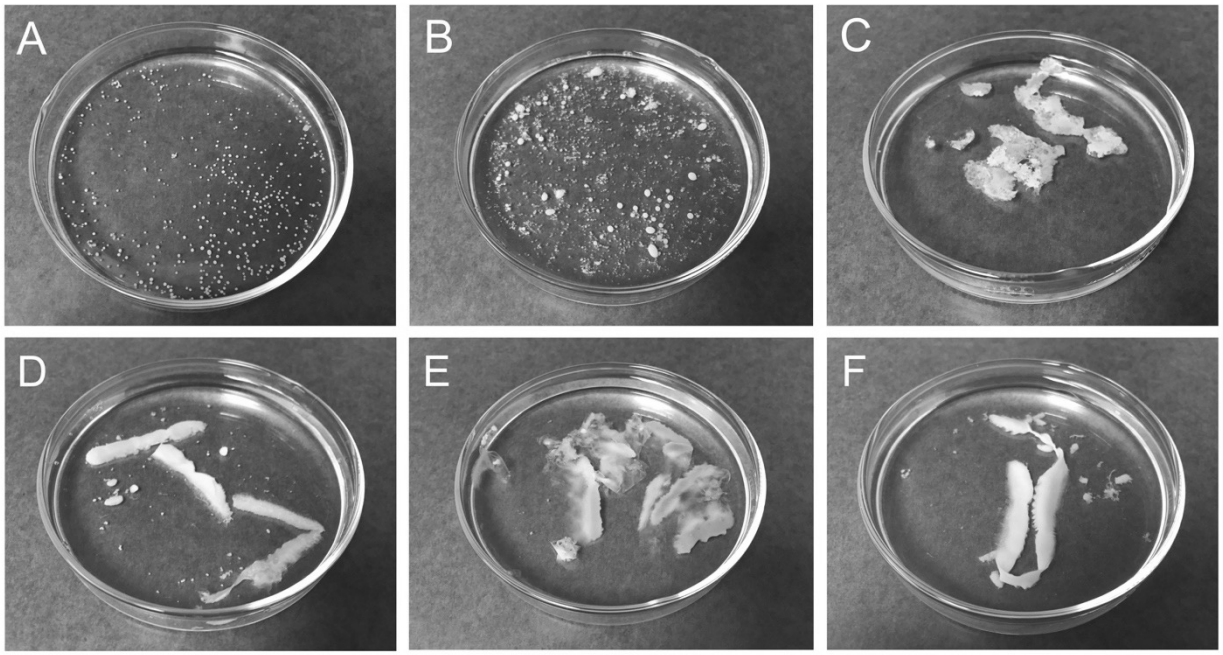

Figure 4.1 Morphologies of *Rhizopus oryzae* ATCC® 20344™ cells grown under different seed culture media conditions (nitrogen source and medium pH). (A) urea at pH 3.0, (B) yeast extract at pH 3.0, (C) urea at pH 4.0, (D) yeast extract at 4.0, (E) urea at pH 5.0, and (F) yeast extract at 5.0.

Dry weight of fungal biomass grown in seed culture media under various conditions is summarized in Fig. 4.2. No significant ($p > 0.05$) differences in fungal biomass weight were observed when *Rhizopus oryzae* ATCC® 20344™ cells were grown in seed culture media adjusted to pH 3.0 – 5.0. However, nitrogen sources can affect fugal biomass accumulation (Carta et al., 1999; Zhang et al., 2015). Approximately, 1.7 g/L biomass was accumulated when using 2 g/L yeast extract as the nitrogen source at pH 3.0, which was significantly ($p < 0.05$) greater than 1.2

g/L biomass observed with urea at pH 3.0. Xu et al. (2010) reported 2 g/L *R. oryzae* biomass in seed culture medium adjusted to pH 4.0 and containing 2 g/L of urea. In general, nutrient rich organic nitrogen sources promote cell growth; whereas, defined nitrogen sources benefit fumaric acid production (Carta et al., 1999). Liao et al. (2008) pointed out that using 1 g/L urea as a nitrogen source in acid production medium (pH > 5) resulted in the accumulation of 1.9 g/L biomass and in the production of 7.4 g/L fumaric acid; whereas, using nutrient-rich soybean peptone at the same nitrogen concentration produced 4.51 g/L biomass but only 2.47 g/L fumaric acid by *R. oryzae*. It has also been reported that *Rhizopus formosa* in acid production medium (pH 6.5) containing 4.4 g/L urea as a nitrogen source resulted in 4 g/L fungal biomass accumulation; whereas, 17 g/L fungal biomass was observed when the same amount of yeast extract was used as the nitrogen source (Carta et al., 1999).

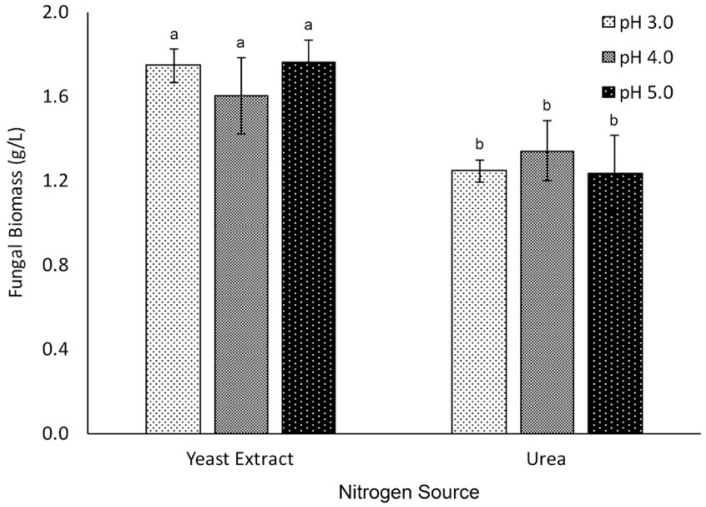

Figure 4.2 Fungal biomass accumulation under different seed culture media conditions (nitrogen source and media pH).
Yeast extract or urea concentration in seed culture medium was 2 g/L. Significant differences at $p < 0.05$ levels are indicated by different letters.

4.3.3. Effect of C/N Ratio on Fumaric Acid Fermentation

Fumaric acid production is enhanced by exposing cells to a limited nitrogen supply during cell growth (Mondala, 2015; Zhang et al., 2013). Therefore, the C/N ratio in the acid production

medium is crucial during fumaric acid fermentation and a high C/N ratio of 120–250 (w/w) is favored (Yang et al., 2011). In this study, urea concentrations of 0, 0.2, 0.4, and 0.8 g/L corresponding to C/N ratios of N-free, 400, 200, and 100, respectively, were investigated using pure glucose as the carbon source. The effect of C/N ratio on fumaric acid production and fungal biomass accumulation are summarized in Table 4.3. Although some fumaric acid production yields have been reported without the addition of a nitrogen source, it was observed that fungal cells lost their productivity during fermentation (Yang et al., 2011; Zhou et al., 2000). Liao et al. (2008) also reported that no significant fungal growth or fumaric acid production was detected without the supply of nitrogen during the acid production stage. In this study, *R. oryzae* was unable to grow and produce significant amounts of fumaric acid under N-free conditions. Highest fumaric acid production (39.05 g/L fumaric acid, 0.49 g/g yield, 0.41 g/L/h productivity) was observed at 400 C/N ratio (0.2g/L urea) after 96 h, which was significantly ($p < 0.05$) higher than those observed with lower C/N ratios (200 and 100). When increasing the nitrogen concentration in the medium to 0.8 g/L with a 100 C/N ratio, the highest fungal biomass accumulation (13.24 g/L) was observed but fumaric acid concentration decreased to 11.25 g/L with a yield of 0.15 g/g and a productivity of 0.23 g/L/h. Glucose was consumed within 48 h at 100 C/N ratio (Fig. 4.3). However, at higher C/N ratios (200 and 400), glucose was completely consumed after 96 h. Ding et al. (2011) reported that when the C/N ratio in the acid production media was adjusted from 40 to 800, the fumaric acid production increased from 14.4 to 40.3 g/L and fumaric acid yields increased from 0.18 to 0.51 g/g, while biomass accumulation decreased from 12.8 to 4.2 g/L with glucose consumption rates reduced from 2.2 to 1.1 g/L/h. Similar observations were made by Carta et al. (1999) in the production of fumaric acid by *R. formosa* using potassium nitrate as a nitrogen source. Fumaric

acid production increased from 16 to 21 g/L as the C/N ratio was changed from 21 to 168, but the concentration rapidly decreased to 14 g/L when the C/N ratio was adjusted to 189.

Table 4.3 Effect of C/N ratio on fumaric acid production and fungal biomass accumulation.

Urea Concentration (g/L)	C/N Ratio	Fumaric Acid Concentration (g/L)	Fungal Biomass Concentration (g/L)	Fermentation Time (h)	Fumaric Acid Yield (g/g)	Fumaric Acid Productivity (g/L/h)
0	N free	2.18±1.99^{d}	0.89±0.08^{c}	96	0.10±0.01^{c}	0.02±0.02^{c}
0.2	400	39.05±1.19^{a}	4.58±0.72^{b}	96	0.49±0.01^{a}	0.41±0.01^{a}
0.4	200	23.82±1.12^{b}	4.89±0.81^{b}	96	0.30±0.01^{b}	0.25±0.01^{b}
0.8	100	11.25±1.79^{c}	13.24±1.27^{a}	48	0.14±0.02^{c}	0.23±0.04^{b}

Significant differences at $p < 0.05$ levels are indicated by different letters.

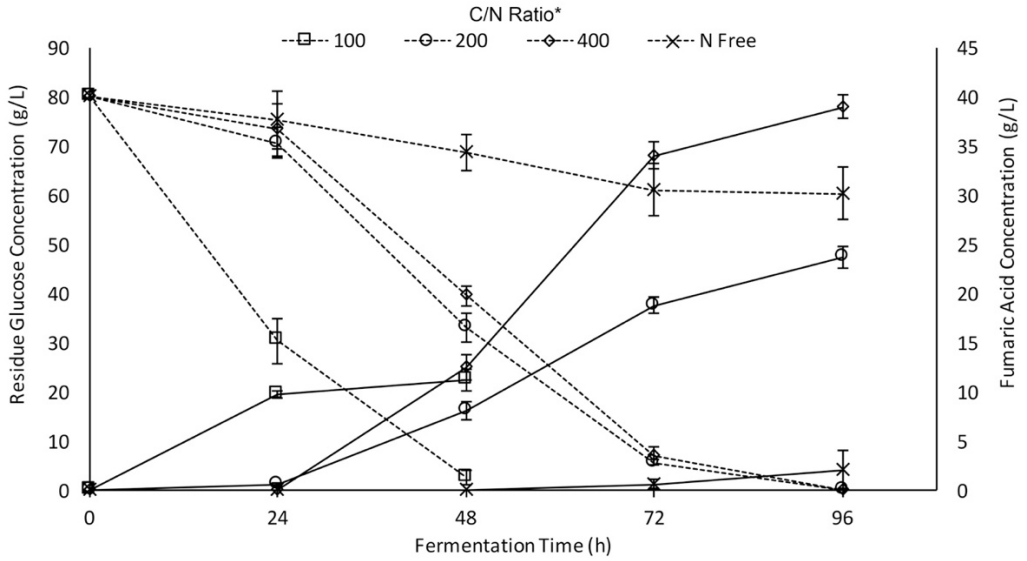

Figure 4.3 Fumaric acid fermentation kinetics at different C/N ratios. Fumaric acid concentration is depicted in solid lines, residue glucose concentration is depicted in dash lines.
*Acid production medium at C/N ratios of N free, 400, 200, 100 containing 0, 0.2, 0.4, 0.8 g/L urea, respectively.

4.3.4. Purified Lignocellulosic Syrup as Carbon Source in the Seed Culture Medium

Fungal cell morphologies in the syrup-enriched seed culture medium were slightly larger and uniform pellets (d = 2 – 4 mm) as compared to cells (d ≈ 1 mm) grown in glucose only enriched seed culture medium (Fig. 4.4). It has been reported that carbon source has no significant influence on pellet formation, but it can have a large impact on biomass accumulation (Liao et al., 2007). When using syrup as the carbon source in the seed culture medium (test A), 2.02 g/L fungal

biomass was accumulated, which was significantly ($p < 0.05$) higher than 1.22 g/L observed in the control medium containing pure glucose and xylose (test B). Liao et al. (2007) pointed out that using potato dextrose broth (PDB) as the carbon source resulted in a 72% increase in *R. oryzae* biomass, as compared to the medium with pure glucose due to the rich nutrients found in PDB such as vitamins and minerals. In the syrup-enriched seed culture medium, diluted lignocellulosic syrup provided additional minerals (0.14 g/L potassium, 0.02 g/L magnesium, 0.06 g/L calcium) which can be beneficial during fungal growth. Thus, the increased biomass concentration observed with syrup-enriched seed culture medium.

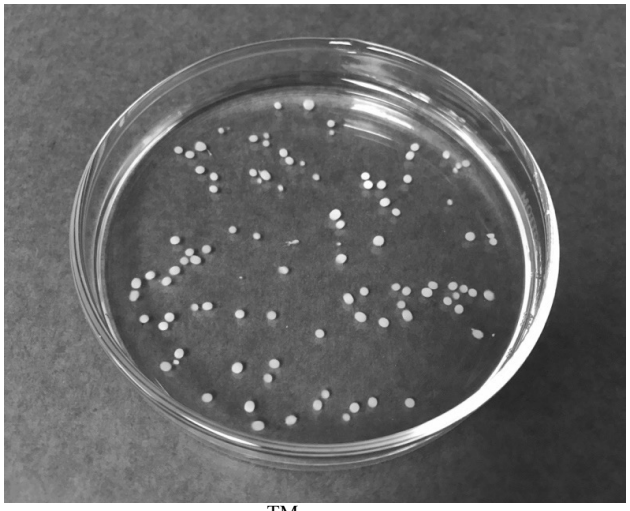

Figure 4.4 *Rhizopus oryzae* ATCC® 20344™ cells grown on seed culture medium containing lignocellulosic syrup as the carbon source.

Significant ($p < 0.05$) sugars consumption was observed within the first 24 h in test A and 48 h in test B (Fig. 4.5). The reduced lag phase observed in test A can be attributed to the beneficial nutrients (i.e., potassium, magnesium, calcium) present in the lignocellulosic syrup. Liao et al. (2008) reported that minerals such as 0.15 g/L potassium, 0.03 g/L magnesium and 0.11 g/L calcium in liquid manure were essential nutrients for the growth and metabolism of *R. oryzae*. Zhang et al. (2015) observed that soybean meal hydrolysate added to the seed culture medium

provided additional nutrients including proteins and minerals, which reduced the lag phase by up to 15 h as compared to the seed culture medium containing only glucose.

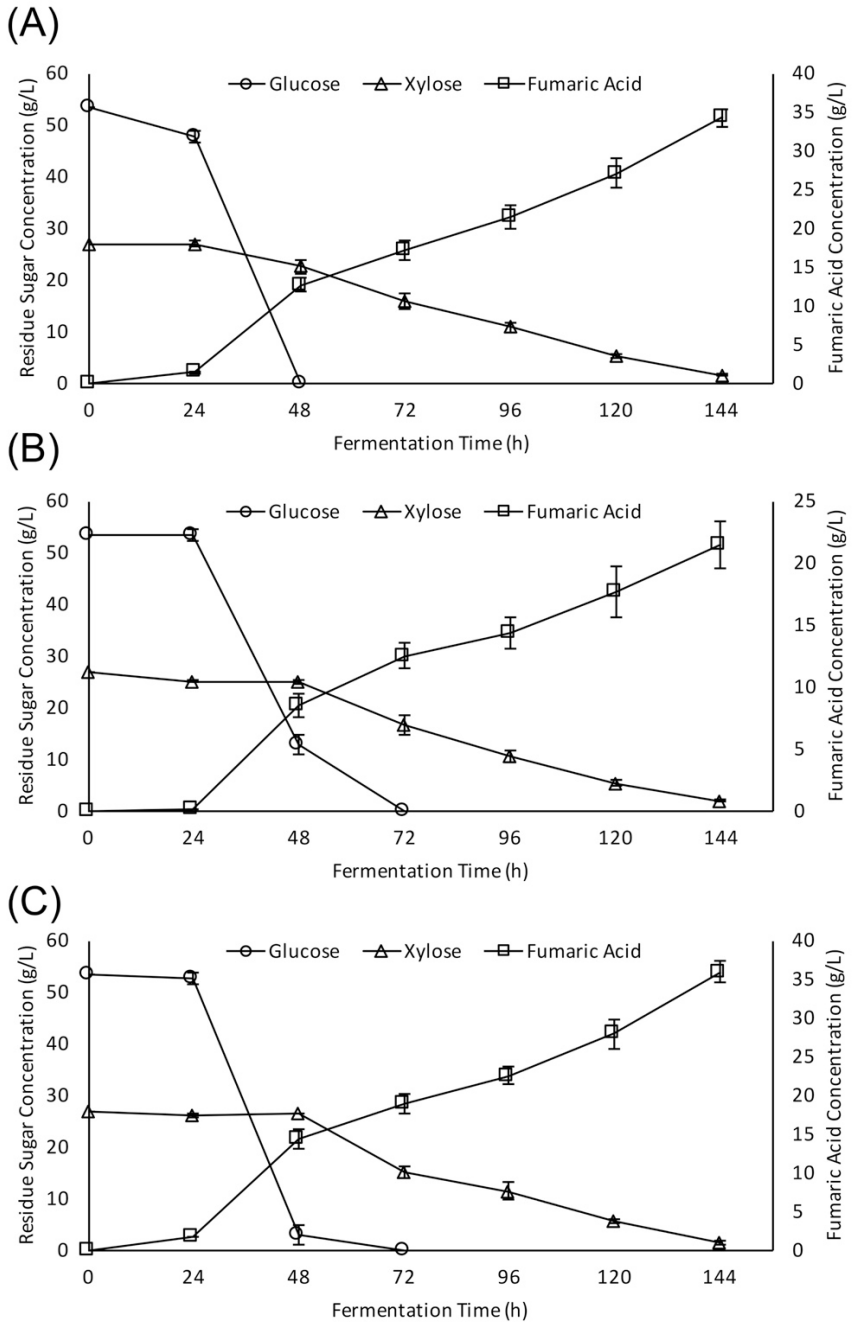

Figure 4.5 Fumaric acid production and sugars consumption by *Rhizopus oryzae* ATCC® 20344™ from different carbon sources. (A) lignocellulosic syrup as the carbon source in seed culture and acid production media; (B) pure glucose and xylose as the carbon source in seed culture media and lignocellulosic syrup in acid production medium; (C) pure glucose and xylose as the carbon source in seed culture and acid production media.

4.3.5. Purified Lignocellulosic Syrup as Carbon Souce in Fumaric Acid Fermentation

Purified lignocellulosic syrup was diluted to 80 g/L total fermentable sugars (53.3 g/L glucose and 26.7 g/L xylose) and evaluated as a carbon source for fumaric acid production at optimum 400 C/N ratio and 0.2 g/L urea (Table 4.4). A fumaric acid concentration of 34.20 g/L and a yield of 0.43 g/g were observed in the syrup-enriched acid production medium (test A), while 35.96 g/L fumaric acid and 0.45 g/g yield were observed with pure sugars (glucose and xylose) as control (test C). Efforts have been made to utilize lignocellulosic materials as the carbon source for fumaric acid fermentation by the *Rhizopus* genus fungi. Rodríguez-López et al. (2012) observed a 9.84 g/L fumaric acid production and a yield of 0.44 g/g by *R. arrhizus* using hot water pretreated Eucalyptus wood enzymatic hydrolysate. Liao et al. (2008) reported a 31 g/L fumaric acid production and a yield of 0.29 g/g by *R. oryzae* in a nitrogen-rich dairy manure enzymatic hydrolysate. Xu et al. (2010) showed that using concentrated corn straw enzymatic hydrolysate resulted in 27.8 g/L fumaric acid production and 0.33 g/g yield by *R. oryzae*.

Statistical analysis indicated no significant ($p < 0.05$) differences in the fumaric acid concentrations and yields between syrup-enriched medium (test A) and pure sugars medium as control (test C). An indication that the non-sugar compounds that remained in the syrup-enriched medium (0.12 g/L acetic acid) had no inhibitory effects on fumaric acid fermentation. Similarly, Maas et al. (2006) reported that 1 g/L acetic acid in wheat straw hydrolysate had no negative effect on lactic acid fermentation by *R. oryzae* compared to the control medium with pure sugars. Liao et al. (2008) also indicated that the fumaric acid yield (0.34 g/g) by *R. oryzae* using alkaline pretreated manure fiber enzymatic hydrolysate as fermentation medium were similar to the yield (0.28 g/g) observed from the glucose control.

Table 4.4 Fumaric acid fermentation yields and biomass concentrations from different carbon sources.

Carbon Source* (Test)	Fungal Biomass Concentration Seed Culture Medium (g/L)	Fungal Biomass Concentration Acid Production Medium (g/L) **	Fumaric Acid Concentration (g/L)	Fumaric Acid Yield (g/g)	Fumaric Acid Productivity (g/L/h)
A	2.02±0.21[a]	10.52±1.19[a]	34.20±1.24[a]	0.43±0.02[a]	0.24±0.01[a]
B	1.22±0.06[b]	3.03±0.43[b]	21.43±1.93[b]	0.27±0.02[b]	0.15±0.01[b]
C	1.21±0.05[b]	4.47±0.68[b]	35.96±1.07[a]	0.45±0.02[a]	0.25±0.01[a]

Significant differences at $p < 0.05$ levels are indicated by the different letters.
*(A) lignocellulosic syrup as the carbon source in seed culture and acid production media; (B) pure glucose and xylose as the carbon source in seed culture media and lignocellulosic syrup in acid production medium; (C) pure glucose and xylose as the carbon source in seed culture and acid production media.
** Biomass concentrations after 144 h fermentation by *Rhizopus oryzae* ATCC® 20344™.

Xylose and glucose in the acid production media were consumed after 144 h incubation; whereas, glucose (as the sole carbon source) was completely utilized within 96 h. Fumaric acid productivity significantly ($p < 0.05$) decreased from 0.41 g/L/h achieved in glucose only acid production medium to 0.24 g/L/h in syrup-enriched acid production medium (test A) and 0.25 g/L/h in control acid production medium (test C). The reduced conversion rate of xylose to fumaric acid as compared to glucose has been reported elsewhere. Maas et al. (2006) reported that 40 g/L xylose was completely fermented to lactic acid by *R. oryzae* after 90 h fermentation, while 40 g/L glucose was digested within 40 h under the same culture conditions. Kautola & Linko (1989) reported a fumaric acid productivity of 0.071 g/L/h by *R. arrhizus* using xylose as the sole carbon source and of 1.02 g/L/h when the carbon source was glucose (Xu et al., 2012).

A diauxic growth pattern was observed when both glucose and xylose were present in the acid production medium, with glucose being consumed first followed by xylose (Fig. 4.5). In the syrup-enriched acid production medium (test A), xylose digestion began after all glucose had been consumed (48 h). In the control acid production medium (test C), glucose was depleted after 72 h followed by the digestion of xylose. A similar fermentation pattern was observed in lactic acid

production by *R. oryzae* when using alkaline pretreated wheat straw hydrolysate as the fermentation medium containing 20 g/L glucose and 10 g/L xylose (Maas et al., 2006). The reduced conversion rates and diauxic growth profiles observed are due to the indirect metabolic pathway of xylose compared to glucose (Liao et al. 2008). The intermediate pyruvate can be readily produced by the glycolysis of glucose in fungal cells, while additional conversion steps through the pentose phosphate pathway or phosphoketolase reaction pathway are required for xylose utilization (Maas et al., 2008; Mondala, 2015).

4.4. Conclusions

Lignocellulosic syrup from dilute ammonia pretreated energy cane bagasse can be used as a novel substrate in the fermentation of fumaric acid by *Rhizopus oryzae* ATCC® 20344™. Optimum seed culture medium conditions for *Rhizopus oryzae* ATCC® 20344™ were adding 2.0 g/L urea as the nitrogen source and adjusting the medium to pH 3.0, which allowed for the formation of preferred compact ($d \approx 1$ mm), smooth and uniformly dispersed pellets. Optimum acid production medium required 0.2 g/L urea as the nitrogen source with C/N ratio of 400 for achieving maximum fumaric acid production yields. Under optimum conditions, 53 g/L glucose and 27 g/L xylose in the lignocellulosic syrup enriched medium were depleted after 144 h fermentation, and resulted in a fumaric acid production of 34.20 g/L, a yield of 0.43 g/g and a productivity of 0.24 g/L/h. These results agreed with those observed from media containing pure sugars as carbon source. Thus, lignocellulosic syrup, a renewable feedstock from dilute ammonia pretreated energy cane bagasse, has the potential to substitute pure sugars as the carbon source for fumaric acid production by *Rhizopus oryzae* ATCC® 20344™.

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CHAPTER 5
SUMMARY AND FUTURE WORK

5.1. Summary

Lignocellulosic materials are promising renewable resources in the production of green fuels and chemicals. Pretreatment and hydrolysis are required to convert the polymeric sugars cellulose and hemicellulose, present in lignocellulosic materials, into fermentable monomeric sugars glucose and xylose. These sugars can be concentrated into a syrup to improve the logistics associated with long-distance transportation, long-time storage, and year-round supply of lignocellulosic biomass to processing industries. However, harsh biomass pretreatment conditions generate undesired non-sugar compounds including organic acids, furans and phenolic compounds, which can be inhibitory to downstream processes such as enzymatic hydrolysis and fermentation. It is widely agreed that the partial or complete removal of these non-sugar compounds result in improved fermentation yields. Furthermore, these non-sugar compounds can serve as building blocks to various chemicals in the food, pharmaceutical and polymer industries. Detoxification strategies can be used to not only remove these non-sugar compounds from hydrolysates to improve downstream fermentation yields, but also to recover them as potential value-added products.

This study evaluated the detoxification of dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate using flocculants or activated carbon (AC) to achieve maximum removal and/or recovery of the non-sugar compounds as value-added products with minimal fermentable sugar losses. The detoxified hydrolysate was concentrated under vacuum to produce a lignocellulosic syrup which was then evaluated as the carbon source in fumaric acid production by

Rhizopus oryzae ATCC® 20344™. Fumaric acid production via fermentation is an environmental sustainable alternative to petroleum-based fumaric acid production.

Energy cane (non-commercial variety Ho 02-113) bagasse had a chemical composition of 40.26±0.34% glucan (cellulose), 19.81±0.57% xylan (hemicellulose), 1.87±0.23% arabinan, 28.74±0.24% lignin, 5.45±0.53% extractives, and 3.87±0.77% ash (dry basis). Dilute ammonia pretreatment was used to partially remove the lignin, reduce the crystallinity of the cellulose, and increase the biomass surface area to allow enzymes better access to the polymeric sugars. Post dilute ammonia pretreatment, 44% lignin and 17% xylan were removed and more than 90% glucan was retained. Enzymatic hydrolysis was carried out using commercially available enzymes, Cellic® CTec2 and HTec2. Dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate had a chemical composition of 15.74 g/L glucose, 9.69 g/L xylose, 3.57 g/L formic acid, 2.38 g/L acetic acid, 1.31 g/L levulinic acid, 0.04 g/L furfural, 0.06 g/L 5-hydroxymethylfurfural (HMF), and 0.51 g/L total phenolic compounds. This hydrolysate was used for the detoxification studies presented in Chapter 2 and Chapter 3.

Chapter 2 investigated the effect of two cationic flocculants (polyethylenimine (PEI) and poly-diallyldimethylammonium chloride (pDADMAC)) on the removal of non-sugar compounds (organic acids, furan derivative, and total phenolic compounds) from dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate with minimal sugar losses (glucose and xylose). Parameters including flocculant dose and hydrolysate pH were studied. Optimum conditions for both PEI and pDADMAC were 15 g/L dose and at an unadjusted hydrolysate pH of 4.5. At these conditions, PEI removed 41.7% formic acid, 36.4% acetic acid, 49.8% levulinic acid, 73.2% total phenolic compounds, and 100% HMF and furfural with less than 10% total fermentable sugar losses; whereas, pDADMAC resulted in reduced non-sugar compounds removal and increased

fermentable sugar losses as compared to PEI. PEI recycling and recovery of the adsorbed non-sugar compounds were evaluated. More than 80% of the adsorbed HMF and furfural was recovered after two cycles. However, only 20% of the adsorbed organic acids and total phenolic compounds were recovered. Sugar losses of less than 10% were observed throughout the recycling process.

Chapter 3 investigated the effect of powdered and granular AC on the removal of non-sugar compounds from dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate. Response Surface Methodology was used to optimize process parameters such as AC dose, hydrolysate pH and contact time. Optimum conditions for powdered AC were 9.21% (w/w) AC dose, hydrolysate pH 1.96 and 10 min contact time. However, a higher AC dose and a longer contact time were required by granular AC to achieve comparable results to those observed with powdered AC. Optimum conditions for granular AC were 12.64% (w/w) AC dose, hydrolysate pH 1.91 and 51.60 min contact time. At optimum conditions, powdered and granular AC removed 40% acetic acid, 75% formic acid, 100% levulinic acid, 90% HMF, 100% furfural, and 98% total phenolic compounds with less than 10% total sugar losses. Powdered AC was selected over granular AC, PEI and pDACMAC as the detoxification method in the production of lignocellulosic syrup from dilute ammonia pretreated energy cane bagasse enzymatic hydrolysate. Lignocellulosic syrup at a final 65°Bx contained 407.55 g/L glucose, 204.34 g/L xylose, 0.92 g/L acetic acid, and 8.81% ash.

Chapter 4 evaluated the production of fumaric acid by *Rhizopus oryzae* ATCC® 20344™ from Powdered AC treated lignocellulosic syrup as the only carbon source. The effect of nitrogen source and initial pH in the seed culture medium on cell morphology were investigated. The C/N ratio in the acid production medium was also optimized for maximum fumaric acid production. Optimum seed culture medium conditions (pH of 3.0, 2.0 g/L urea) produced compact, smooth

and uniform pellets. A C/N ratio of 400 and 0.2 g/L urea were the optimum conditions in the acid production medium for maximum fumaric acid production. A fumaric acid production of 34.20 g/L (from xylose and glucose), with a yield of 0.43 g/g and a productivity of 0.24 g/L/h were observed at optimum conditions. The results showed the potential of using lignocellulosic syrup as a green alternative for fumaric acid production by *Rhizopus oryzae* ATCC® 20344™.

5.2. Future Work

Detoxification of lignocellulosic hydrolysates prior to fermentation is a must to prevent enzymes and microbial inhibition by non-sugar compounds (i.e., organic acids, phenolic compounds, furan derivatives). However, multiple detoxification methods are needed if seeking to remove and/or recover all non-sugar compounds due to the individual characteristics of these compounds. Another approach is to use genetic engineering or strain selection methods to increase the tolerance of microorganisms towards the inhibitory effects of these non-sugar compounds. The ash content present in the lignocellulosic syrup can be reduced by technologies such as electrodialysis and ion-exchange resins. Recycle and regeneration of the detoxification chemicals as well as the recovery of the separated non-sugar compounds as potential value-added products can offset the additional cost introduced by detoxification. However, recovery of the adsorbed non-sugar compounds from activated carbon remains a challenge. Organic solvent extraction could be applied to AC regeneration and recovery of AC adsorbed non-sugar compounds.

Powdered AC treated lignocellulosic syrup was successfully used as the carbon source in fumaric acid production by *Rhizopus oryzae* ATCC® 20344™. Different sugar feeding strategies such as fed-batch fermentation can be further evaluated to increase fumaric acid production yields. The use of enhanced equipment such as a rotary biofilm contactor, an airlift loop reactor, or a bubble column should be evaluated as ways to improve agitation and oxygen transfer efficiency

during fermentation. Strategies such as CO_2 addition, two-stage dissolved oxygen control and multi-stage seed culture methods could be investigated as ways to boost fumaric acid production.

VITA

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