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Evaluation of Alkaline- and Fungal-Assisted Wet Storage of Energycane Bagasse

Jing Cao
Louisiana State University and Agricultural and Mechanical College

EVALUATION OF ALKALINE- AND FUNGAL-ASSISTED WET STORAGE OF ENERGYCANE BAGASSE

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ABSTRACT

Lignocellulosic biomass is a promising renewable resource for the production of biofuels and biochemicals. Energycane is considered a lignocellulosic biomass characterized by its high fiber content and cold tolerance. It can be planted on marginal land and does not need to compete with the food supply. Wet storage in combination with sodium hydroxide (NaOH) or white-rot fungi was applied to energycane bagasse to preserve the lignocellulosic polymeric sugars (cellulose and hemicellulose) during short-term storage (60 days) and to make them accessible for conversion into biofuels and biochemicals.

Alkaline-assisted wet storage was successful in preserving the biomass by minimizing microbial degradation, increasing lignin degradation, preventing cellulose degradation, and enhancing sugar digestibility. Four sodium hydroxide loadings (0, 5, 7.5, and 10 g NaOH/kg dry matter) at two moisture contents (45% and 75%) were applied to energycane bagasse. Higher loadings of sodium hydroxide and lower moisture content resulted in less cellulose degradation and greater lignin degradation. Higher moisture content (75%) resulted in higher sugar digestibility. Sodium hydroxide loading of 10% wt. at a moisture content of 45% was the optimal condition that preserved the most glucan (40%), degraded the most lignin (67%) and hemicellulose (48%), and resulted in 69% cellulose digestibility and 43% hemicellulose digestibility during the 60 days storage of energycane bagasse.

Fungal-assisted wet storage using white-rot fungus, *Ceriporiopsis subvermispora*, was also successful in preserving the biomass by inhibiting microbial growth, increasing lignin degradation, preventing cellulose degradation, and enhancing sugar digestibility. At 75% moisture, fungal-assisted storage of energycane bagasse resulted in 44% lignin degradation and 2% cellulose loss as compared to 14% and 31% from untreated samples, respectively. The majority

of lignin degradation occurred after 10 days, and no significant difference ($p > 0.05$) was observed in fungal treated samples after 50 days. Cellulose digestibility (67%) and hemicellulose digestibility (34%) of white-rot fungus treated samples were higher than untreated samples (38% and 20%, respectively).

This study indicated that sodium hydroxide and white-rot fungal assisted wet storage were efficient in preserving glucan, removing lignin and increasing sugar digestibility of energycane bagasse.

CHAPTER 1
INTRODUCTION

1.1 Lignocellulosic biomass

1.1.1 Lignocellulosic biomass and energycane

Lignocellulosic biomass, namely agricultural residues and energy crops, have been drawing much attention as candidate feedstocks for the production of biofuels and biochemicals (Limayem and Ricke, 2012). It is widely available from agricultural (e.g., sugarcane, energycane, switchgrass, sweet sorghum), forestry (e.g., hard and soft wood), municipal, and other sources (Kim et al., 2010). The ideal energy crop should have high yield and biomass composition with the least contaminants, require low energy and nutrients for production, and have low production cost (McKendry, 2002).

Energycane is a desired energy crop and a lignocellulosic resource. Energycane L 79-1002 (a non-commercial variety) is a hybrid of the cross between the female parent, cultivar CP 52-68, and the male parent, Tainan (a clone of *Saccharum spontaneum*) developed by the Louisiana State University Agricultural Center Sugar Research Station in St. Gabriel, LA in collaboration with the U. S. Department of Agriculture-Agricultural Research Service (USDA-ARS) in Houma, LA (Bischoff et al., 2008). The average energycane tonnage is 100 t/ha/year, which is much higher than that of sugarcane and sweet sorghum (Kim and Day, 2011). Unlike sugarcane and sweet sorghum, energycane has higher fiber content and can be harvested during colder seasons due to its cold tolerance attributes (Kim and Day, 2011). L 79-1002 has an average fiber content of 257 g/kg (dry basis) and cane yield of 83.3 Mg/ha (dry basis) as compared to L 99-233, the predominant commercial variety of sugarcane grown in Louisiana,

with a fiber content of 132 g/kg (dry basis) and cane yield of 80.1 Mg/ha (dry basis) (Bischoff et al., 2008; Gravois et al., 2009). Additionally, energycane can be planted on marginal land and does not need to compete with the food supply (Shields and Boopathy, 2011). Once juice is pressed from energycane, the fibrous residue left behind is called energycane bagasse. Bagasse or lignocellulosic biomass can be processed in a number of ways to yield value added products and has been at the forefront of sustainable research.

Carbohydrates and lignin make up a major portion of lignocellulosic biomass (Fig. 1.1). Carbohydrates, including cellulose and hemicellulose, are bound in the matrix of the biomass, and lignin is a complex phenolic polymer that works as the cement for the cross-linkage of cellulose and hemicellulose to form a rigid three-dimensional lignocellulosic structure (Zheng et al., 2014). Cellulose is a linear polysaccharide polymer of cellobiose made up of D-glucose subunits linked by β- (1→4) glycosidic bonds. The cellulose strains are bundled together through hydrogen bonding to form cellulose fibrils (Rubin, 2008). Cellulose contains crystalline and amorphous parts, and the higher degree of crystallinity, the more difficult the degradation of cellulose becomes. Hemicellulose is a more amorphous, branched polymer composed mostly of pentoses (xylose and arabinose units), hexoses (mannose, glucose and galactose) and sugar acids. It connects the lignin and cellulose and renders the cellulose-hemicellulose-lignin matrix extremely rigid and cohesive (Zheng et al., 2014; Barakat et al., 2015; Qiu and Aita, 2013). Lignin is an amorphous heteropolymer consisting of three different phenylpropane units (p-coumaryl, coniferyl and sinapyl alcohol). In general, lignin is the primary barrier that resists biomass from enzymatic hydrolysis and by-product conversion (Nakagame et al., 2011; Sawatdeenarunat et al., 2015).

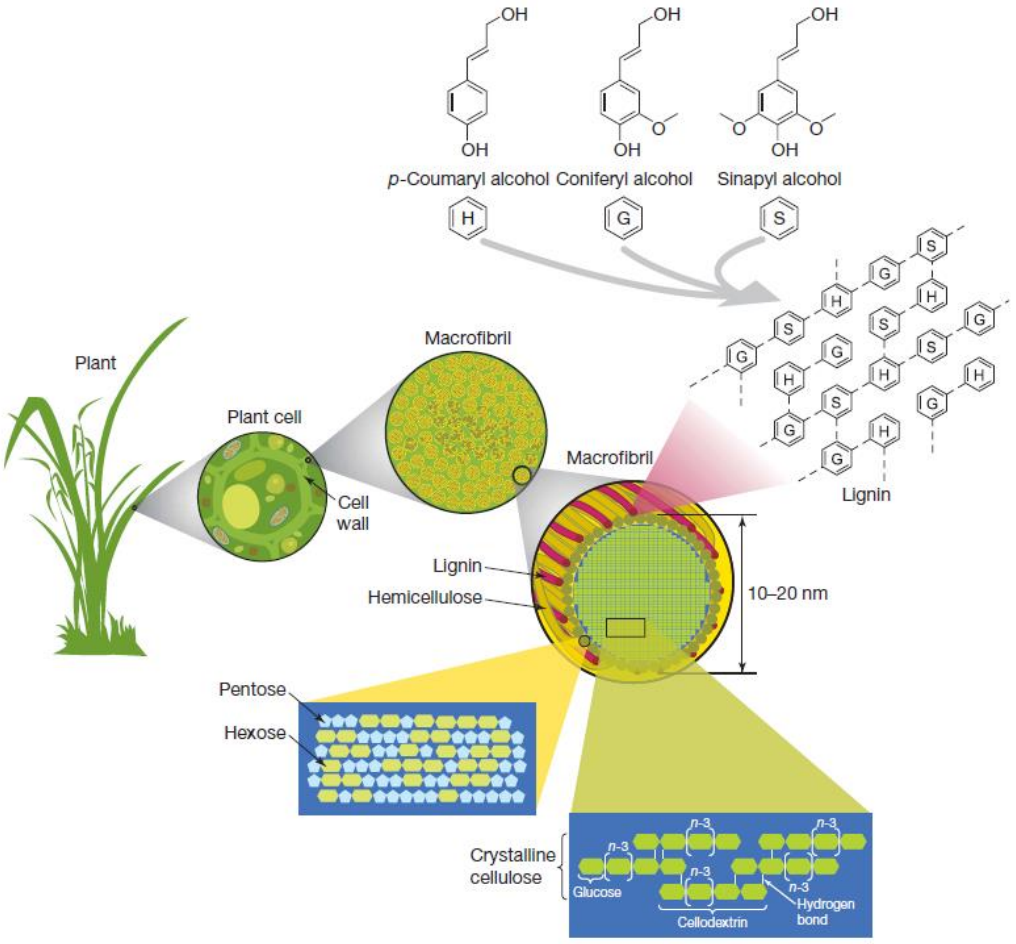

Fig. 1. 1 Structure of lignocellulose (Rubin, 2008).

1.1.2 Biomass storage and pretreatment

Green fuels and chemicals obtained from lignocellulosic resources require processing of large volumes of biomass to match the fuel and energy output of fossil fuels. Since the agricultural crops that result in lignocellulosic residues are typically harvested only at certain times of the year, large volumes of biomass would need to be stored prior to processing in a biorefinery. Storage of biomass on-field (i.e., at the site where harvested) can result in biodegradation of fibers, and thus loss of energy content, and possible spoilage if stored without treatment of the fibers. Certain microbes and chemicals (acids, alkali) can be added during

storage immediately following harvest at a moisture content that is at least 45% (wet storage), to help retard microbial activity and promote enzymatic conversion of plant carbohydrates into fermentable sugars (Thompson et al., 2005; Cui et al., 2012). Biomass after wet storage is more uniform and more digestible than dry stored lignocellulosic biomass (Richard, 2010; Shinners et al., 2007).

The breaking down of the polymeric sugars to their monomeric counterparts for subsequent fermentation to biofuels and by-products is typically done by adding a pretreatment step to the biorefinery process. However, biomass storage and pretreatment can be conducted concurrently and cost effectively to provide a homogeneous, delignified biomass for the biorefinery. Liu et al. (2013) screened the micro-structure and crystallinity of wet stored corn stover and found that the biomass maintained its flexible and porous structure as compared to dry stored biomass, which led to high permeability of biomass and efficiency during pretreatment and hydrolysis. Pretreatment can further decompose the lignin and hemicellulose, disrupt hydrogen bonds in crystalline cellulose, and make the surface area of biomass accessible to enzymes (Kumar et al., 2009; Hendriks and Zeeman, 2009; Haghighi Mood et al., 2013). A schematic representation of the effect of pretreatment on lignocellulosic biomass is presented in Fig. 1.2. Several pretreatment methods have been investigated and can be categorized as physical (e.g., milling), chemical (e.g., dilute acid, alkali, phenol, organic solvent) or a combination of both (e.g., steam explosion), and biological (e.g., brown and white-rot fungi) processes (Singh et al., 2014; Taherzadeh and Karimi, 2008; Ohgren et al., 2007; Yat et al., 2008; Zheng et al., 2009; Wang et al, 2013; Zhang et al., 2016). Chemical pretreatment with dilute acid can be suitable for a wide range of feedstocks and results in high sugar yields with relatively low cost. However, this process does not remove lignin efficiently, results in the corrosion of process equipment, and

requires the neutralization of acidic pre-hydrolysates before fermentation (Singh et al. 2014; Rodriguez et al., 2016). Alkali-based pretreatments using anhydrous ammonia (NH_3), aqueous ammonia (NH_4OH), or sodium hydroxide (NaOH) with or without heat and/or pressure have shown great success in the delignification of lingocellulosic biomass (Aita et al., 2009). Biological pretreatments using fungi can be an attractive option due to their low energy demands and selective degradation of lignin and hemicellulose, but they can be time-consuming (Shirkavand et al., 2016).

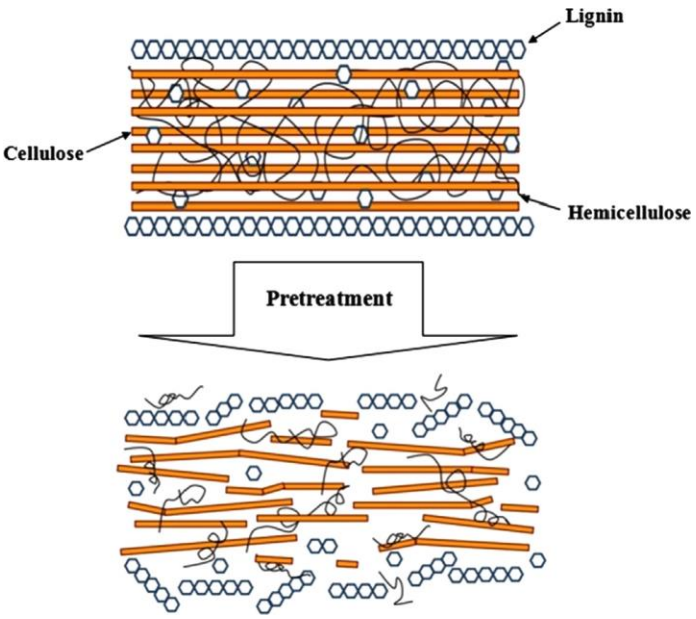

Fig. 1. 2 Schematic pretreatment of lignocellulosic material (Haghighi-Mood et al., 2013)

1.2 Biomass storage and sodium hydroxide pretreatment

Sodium hydroxide can disrupt the carbohydrate-lignin matrix by removing the hemicellulose, lignin and amorphous cellulose, and by hydrolysing uronic and acetic acid esters (Jackson, 1977; Li et al., 2010; Wu et al., 2011; Xu et al., 2015; Li et al., 2016). Meanwhile, the

ester bonds found among cellulose fibers, hemicellulose and lignin are sensitive to alkali treatment resulting in the swelling of cellulose fibers; thus, increasing the accessibility of enzymes to carbohydrates (Whistler and Teng, 1970; Feist et al., 1970; Sun et al., 1995). The hydrolysis of acetyl (ester) groups is a nucleophilic substitution reaction where a nucleophile (OH^- or H_2O) attacks the electron deficient carbon atom of the carbonyl group (Carey, 2003). The mechanism of hydrolysis of acetyl groups in alkaline medium is shown in Fig. 1.3. Due to the strong nucleophilicity, hydroxide ions can attack weakly electrophilic carbonyl carbon atom of acetyl groups spontaneously and form a tetrahedral intermediate. This intermediate then collapses with the loss of the leaving group (OX^-). At last, the leaving group functions as a base and deprotonates the acetic acid (Patil, 2012). The removal of uronic acid substitutes and acetyl groups reduces steric hindrance of hydrolytic enzymes and increases cellulosic digestibility (Wan et al., 2011).

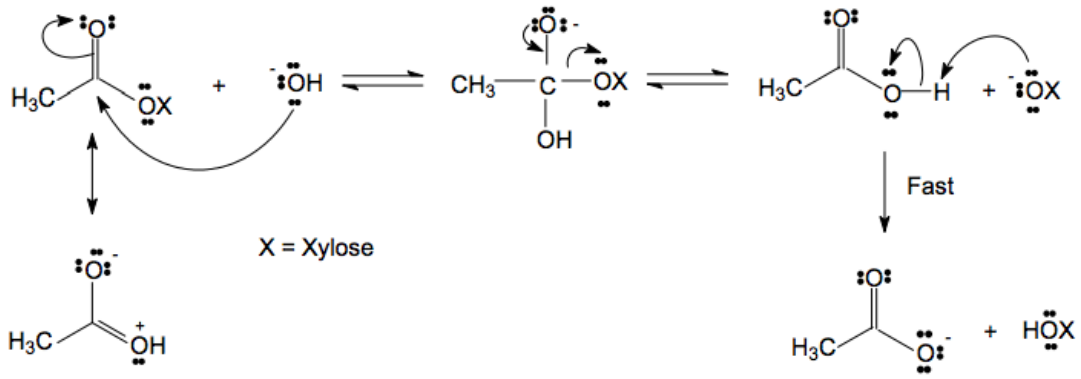

Fig. 1. 3 Mechanism of hydrolysis of acetyl groups in alkaline medium (Patil, 2012).

Sodium hydroxide pretreatment has a strong ability for removing lignin and hemicellulose thus enhancing cellulose digestibility. Guo et al. (2013) indicated that sodium

hydroxide pretreatment preserved more cellulose (98%), degraded more hemicellulose (68%) and removed more lignin (77%) in corn straw than when pretreated with steam explosion (82%, 55% and 33%, respectively). Zhao et al. (2008) reported that sodium hydroxide pretreatment resulted in higher cellulose conversion (19%) on Crofton weed at 110℃ with a NaOH loading of 10% (w/w) and liquid-to-solid ratio of 6:1 for 120 min as compared to sulfuric acid pretreatment (5%) at 120℃ with 3% (w/v) sulfuric acid for 120 min. Compared to other alkali, sodium hydroxide may lead to more desirable results due to its high reaction rate and effectiveness for improving enzymatic digestibility (Xu, 2010). Sun et al. (1995) also found that sodium hydroxide pretreatment on wheat straw resulted in higher hemicellulose (50%) and lignin (21%) degradation than that of potassium pretreatment under similar pretreatment conditions of 1.5% (w/v) alkali at 20℃ for 6 h. Furthermore, sodium hydroxide pretreatment of different biomass may lead to different results. Park and Kim (2012) reported that pretreatment with sodium hydroxide resulted in different cellulose, hemicellulose and lignin degradation yields in Eucalyptus residues, *Larix leptolepis* and *Pinus rigida*. Li et al. (2014) reported that different chemical compositions after sodium hydroxide pretreatment were found in different parts (bamboo green, timber and yellow) of bamboo due to the different characteristics of biomass, such as density, hardness, ash and extractives.

Sodium hydroxide pretreatment is often applied under severe conditions such as high temperature and concentration to achieve high efficiency. Gupta and Lee (2010) found that sodium hydroxide can improve the glucan digestibility of corn stover to 94% under mild conditions with 1.5% (w/v) NaOH at 60℃ for 24 h and also increase the glucan digestibility of hybrid poplar to 95% under more severe condition with 5% (w/v) NaOH at 120℃ for 24 h. Xu (2010) reported the highest yield of total reducing sugars of 453.4 mg/g at 50℃ for 12 h with

1.0% (w/v) NaOH in switchgrass. Wang et al. (2010) indicated that sodium hydroxide pretreatment achieved the highest sugar yield of about 71% at 120℃ for 15 min with 0.75% (w/v) NaOH for coastal Bermuda grass. Similarly, sodium hydroxide pretreatment in combination with heating resulted in short pretreatment time and high level of delignification (66%) and cellulose conversion (61%) in cotton stalk treated with 2% (w/v) NaOH for 90 min at 121 °C/15 psi (Silverstein et al., 2007). Pretreatment by soaking in sodium hydroxide with a concentration of 4% for 24 h supplemented with 30 min microwave irradiations (720 W, 180℃) removed 65% of the lignin and preserved 74% of the cellulose in paddy straw (Kaur and Phutela, 2016).

Sodium hydroxide pretreatment under intense conditions results in high energy cost and safety issues (Xu, 2010). Sodium hydroxide soaking at ambient temperature can address this issue to some extent, even though longer reaction times are needed (hours or days) to achieve satisfactory results. Sun et al. (1995) reported a 60% hemicellulose degradation and 80% lignin degradation after corn straw was soaked in 1.5% (w/v) NaOH for 144 h at 20 °C. Wan et al. (2011) pretreated soybean straw with sodium hydroxide (4–40 g NaOH/100gdry straw) at ambient conditions for 24 h and achieved a glucose yield of 65% and xylan removal of up to 47%. Cui et al. (2012) reported glucose yields of 45-55% and xylose yields of 40-45% for corn stover stored with a 50 g NaOH/kg dry biomass at a moisture content of 45% for 90 days. Soaking rice straw with 1% (w/v) NaOH solution at room temperature (18-28°C) for 72 h removed 49% of the lignin, retained 87% of the cellulose and 79% of the hemicellulose, while improving both cellulose and hemicellulose digestibility to 73% and 62%, respectively, as compared to untreated samples (17% and 15%, respectively) (Ai et al., 2014).

1.3 Biomass storage and white-rot fungi pretreatment

Pretreatment with white-rot fungi is considered a promising biological pretreatment because of its low energy input, simple culturing and inoculation techniques, and mild environmental conditions (Sun and Cheng, 2002). *Ceriporiopsis subvermispora* can effectively increase lignin degradation and improve sugar yields during enzymatic hydrolysis. It has been applied to various biomass materials such as rubberwood, corn stover and wood chips (Nazarpour et al., 2013; Wan and Li, 2010a; Giles et al., 2015).

Generally, white-rot fungi degrade both polysaccharides and lignin by producing extracellular oxidative enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). White-rot fungi cleave β-O-4 lignin model structures, the most common non-phenolic lignin structures in wood lignin, by mainly secreting LiP and then releasing benzylic fragments (Fig. 1. 4). However, unlike most white-rot fungi which are LiP-dependent, the fungus *Ceriporiopsis subvermispora* can degrade non-phenolic lignin structures without producing LiP (Srebotnik et al., 1994). A similar mechanism to the one-electron oxidation mechanism of LiP-producing fungi has been found in *C. subvermispora*, indicating that this fungus might use a cryptic LiP and some other one-electron oxidants to catalyze the degradation of lignin (Srebotnick et al., 1997). A MnP-dependent lipid peroxidation (extracellular lipid peroxidation) mechanism has been proposed. Jenson et al. (1996) indicated that *C. subvermispora* cleaved the nonphenolic β-O-4 lignin model compounds in a mimic system with MnP, Mn (II) and unsaturated lipid *in vitro*, which means that the fungus is MnP-dependent. Unsaturated fatty acids, such as free 9,12-octadecadienoic, 9-octadecenoic, 11-octadecenoic, hexadecanoic, and octadecanoic acids, were produced by *C. subvermispora* on wood meal cultures and disappeared rapidly with increasing organic hydroperoxides after prolonged cultivation, an indication that

unsaturated fatty acids might be consumed by extracellular lipid peroxidation (Enoki et al., 1999). The extracellular lipid peroxidation system allows *C. subvermispora* to degrade lignin leaving the cellulose backbone untouched. Besides the major β-O-4 structure, another substructure called β-5 linkage substructure can be found in some softwood lignin and it can also be degraded by *C. subvermispora* (Daina et al., 2002). However, enzymes like MnP and Lac cannot permeate a sound cell wall due to their high molecular weights. Thus, at a site far from enzymes, lignin degradation must be catalyzed by low molecular weight compounds. A variety of different agents, such as peroxyl radical, hydroperoxyl radical and chelated Mn (III), have been proposed. Peroxyl (ROO·) and hydroperoxyl (·OOH) radicals can be generated from hydroperoxide compounds and might work as reactive oxygen agents involved in wood decay (Kapich et al., 1999; Hammel et al., 2002). Chelated Mn (III) is activated by ligninolytic enzymes and works as a strong generator of free acyl radicals from lipids and lipid hydroperoxides in lignin biodegradation (Watanabe et al., 2000). Furthermore, other enzymes may also be involved in lignin degradation. Fackler et al. (2006) found no analytical correlation between peroxidases excreted by *C. subvermispora* and lignin degradation of spruce wood shavings, indicating that other systems might be involved. Years later, Hori et al. (2014) found that several manganese peroxidases and an aryl alcohol oxidase, both associated with lignin degradation, were produced by *C. subvermispora* after three days of incubation on aspen wood. Besides, a sequential production of glycoside hydrolase, which relates to cellulose and xylan degradation, provides a mechanism consistent with selective ligninolysis by *C. subvermispora* (Hori et al., 2014).

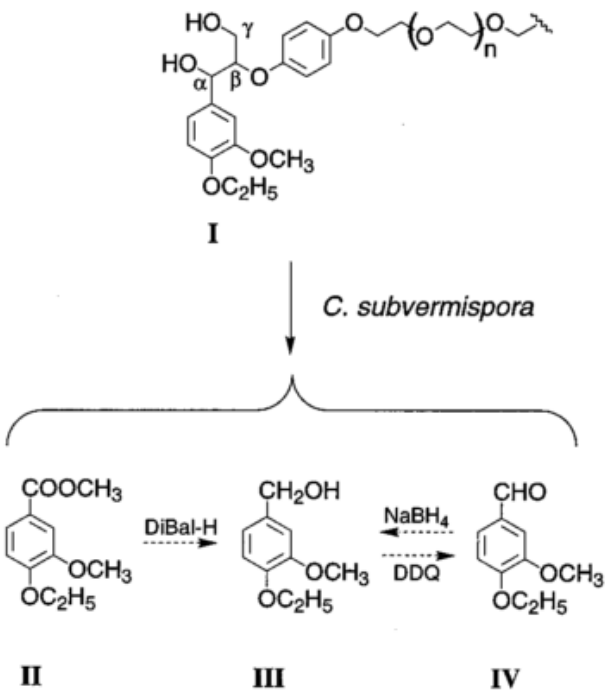

Fig. 1. 4 Benzylic fragments obtained when β-O-4-linked lignin structures are oxidized by *C. subvermispora* cultures (Jensen et al., 1996). Dotted arrows indicate chemical oxidoreductions that were performed to confirm product identifications. DiBal-H, diisobutylaluminum; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone.

In addition to biomass pretreatment, white-rot fungi has been widely applied to biopulping, forage upgrading, and bioremediation of soil and wastewater. *C. subvermispora* has been used on biochemical pulping to increase unbleached pulp brightness (Bajpai et al., 2004; Mosai et al., 1999). *C. subvermispora* can improve the nutritive value (cellulose, hemicellulose, crude protein, etc.), gas production and methane production of agricultural by-products with high lignin content (e.g., rice straw, oil palm frond, sugarcane bagasse) for ruminants (Tuyen et al., 2013; Okano et al., 2006). White-rot fungus, *Trametes versicolor*, can remove mostly common emerging contaminants (e.g., bisphenol A, estrone) and reduce estrogenic activity in wastewater treatment plant effluent (Shreve et al., 2016).

Different factors like particle size, inoculum size, temperature, and aeration can have a modest impact in delignification (Wan and Li, 2012). Fungal pretreatment of some biomass materials have been enhanced when combined with other pretreatments or fungi, or the extracted fungal enzymes were used directly on the substrates. *C. subvermispora* in combination with liquid hot water pretreatment at 170℃ for 3 min improved fungal degradation, lignin removal and glucose yield of soybean straw (Wan and Li, 2011). Fungal treatment followed by microwave hydrothermolyisis at 180℃ for 20 min increased sugar and ethanol yields (Sasaki et al., 2011). Co-cultivation of the *Coprinus comatus* with *Trichoderma reesei* on corn stover resulted in an improvement in delignification (Ma and Ruan, 2015). Crude ligninolytic enzyme extracts containing LiP, MnP and Lac from the white-rot fungus *Phanerochaete chrysosporium* have been applied to sugarcane bagasse for less than 36 h at 25-29℃, prior to pulping treatment, resulting in higher pulp yield, lower energy consumption, and higher pulp tensile index and brightness as compared to pretreatment using only *C. subvermispora* for two weeks at 27℃ and at 70% relative humidity (Ramos et al., 2004).

1.4 Goal of this study

The goal of this study was to assess the effect of wet storage in combination with alkaline (Chapter 2) or fungal (Chapter 3) pretreatment on the delignification and cellulose and hemicellulose digestibility of energycane bagasse.

CHAPTER 2
EVALUATION OF ALKALINE ASSISTED WET STORAGE OF ENERGYCANE BAGASSE

2.1 Introduction

Lignocellulosic biomass is a promising renewable resource for the production of biofuels and biochemicals (Limayem and Ricke, 2012). It is widely available from agricultural (e.g., sugarcane, energycane, switchgrass, sweet sorghum), forestry (hard and soft wood), municipal, and other resources (Kim et al., 2010). Carbohydrates and lignin make up a major portion of lignocellulosic biomass. Energycane, non-commercial variety L79-1002, is a hybrid of commercial and wild sugarcanes, which has higher fiber content and cold tolerance attributes (Bischoff et al., 2008; Kim and Day, 2011). Furthermore, energycane can be planted on marginal land and does not need to compete with the food supply (Shields and Boopathy, 2011). Once juice is pressed from energycane, the fibrous residue left behind is called energycane bagasse. Bagasse or lignocellulosic biomass can be processed in a number of ways to yield value added products and has been at the forefront of sustainable research.

Carbohydrates, including cellulose and hemicellulose, are bound in the matrix of the biomass, and lignin is a complex phenolic polymer that works as the cement for the cross-linkage of cellulose and hemicellulose to form a rigid three-dimensional lignocellulosic structure (Zheng et al., 2014). In general, lignin is the primary barrier that resists biomass from enzymatic hydrolysis and by-product conversion (Nakagame et al., 2011; Sawatdeenarunat et al., 2015). Pretreatment is a necessary step before enzymatic hydrolysis of any lignocellulosic material because this process can further decompose the lignin and hemicellulose, disrupt hydrogen bonds

in crystalline cellulose, and increase the accessible surface area of the biomass (Kumar et al., 2009; Haghighi Mood et al., 2013). Compared to other pretreatments, sodium hydroxide pretreatment has a strong ability to remove lignin and hemicellulose and enhance enzyme digestibility. Sodium hydroxide can disrupt the carbohydrate-lignin matrix by removing the hemicellulose, lignin and amorphous cellulose, and by hydrolysing uronic and acetic acid esters (Jackson, 1977; Sambusiti et al., 2013; Michalska et al., 2015).

Sodium hydroxide pretreatment is applied under severe conditions such as high temperature and concentration to achieve high efficiency. However, sodium hydroxide pretreatment under intense conditions results in high energy cost and safety issues (Xu, 2010). Chemicals such as alkali compounds can be added during storage immediately following harvest at a moisture content that is at least 45% (wet storage), to help retard microbial activity and promote enzymatic conversion of plant carbohydrates into fermentable sugars (Thompson et al., 2005; Cui et al., 2012). In the previous work, soaking rice straw with 1% (w/v) NaOH solution at room temperature (18-28°C) for 72 h removed 49% of the lignin, retained 87% of the cellulose and 79% of the hemicellulose, while improving both cellulose and hemicellulose digestibility to 73% and 62%, respectively, as compared to untreated samples (17% and 15%, respectively) (Ai et al., 2014). Therefore, sodium hydroxide soaking or sodium hydroxide assisted wet storage at ambient temperature can soften the biomass during storage, reduce energy and chemical costs often involved with severe pretreatment conditions, and improve sugar digestibility(Xu, 2010).

The aim of this study was to assess the effect of wet storage in combination with sodium hydroxide pretreatment on the delignification, carbohydrate preservation and sugar yield of energycane bagasse.

2.2 Methods

2.2.1 Biomass

Energycane (non-commercial variety L79-1002) was harvested at Louisiana State University Agricultural Center Sugar Research Station in St. Gabriel, LA. Tops, leaves and stalks of energycane were retained for milling and the juice was extracted by pressing the stalks through a roller press (Farrel Company, Ansonia, CT) three times. The remaining fibers or bagasse were stored at -20℃ for future use. Particle size of biomass averaged ¼ in.

2.2.2 Sodium hydoxide pretreatment and wet storage

Energycane bagasse was dried to below 10% moisture content in a 40-45℃ oven prior to use. A 20% wt. sodium hydroxide (NaOH, Fisher Scientific, Fair Lawn, NJ), solution and energycane bagasse were mixed to obtain a final loading of 0, 50, 75, and 100 g NaOH/kg dry energycane bagasse, with moisture contents of 45% and 75%. Samples (20 g) were placed in reagent bottles (United Scientific Supplies, Inc., Waukegan, IL) after mixing and incubated at 30℃ in an incubator (Amerex Instruments Inc., Lafayette, CA) for 60 days. Control samples (untreated) consisted of energycane bagasse at 45% and 75% moisture content without the addition of sodium hydroxide. Subsamples were taken at 6 h, 5, 10, 20, 30, 40, 50, and 60 days for analysis of pH, dry matter loss, chemical composition, and enzymatic hydrolysis. All tests were carried out in duplicate.

2.2.3 Chemical composition of energycane bagasse

All sodium hydroxide treated and control samples were analyzed for glucan, xylan, arabinan, mannan, lignin, ethanol extractives, and ash content by following the National Renewable Energy Laboratory (NREL)'s laboratory analytical procedures (LAPs) TP-510-42618, 42619, 42620, 42621, and 42622. NREL reference material (8491 sugarcane bagasse) was also analyzed as an internal sample to ensure the accuracy of the procedures.

2.2.4 Enzymatic hydrolysis

Sodium hydroxide treated bagasse and control samples were hydrolyzed using a combination of commercially available enzymes, Cellic CTec2 and Cellic HTec2 (Novozymes North America, Inc., Franklinton, NC). Cellic CTec2 is a cellulase complex enzyme (cellulases and β–glucosidases); whereas, Cellic HTec2 is mainly an endoxylanase with high specificity toward soluble hemicellulose. CTec2 was used at 32 CPU/g glucan and HTec2 was added at the recommended ratio of 9:1 (CTec2:HTec2). Enzymatic hydrolysis was conducted as indicated by NREL's LAP TP-510-43629. Briefly, 2.5g (dry basis) of sodium hydroxide treated or untreated energycane bagasse were mixed with 1.25 g citrate buffer (0.1 M stock solution, pH 4.8) into an Erlenmeyer flasks and water was added to each sample to a final weight of 50 g. The pH of each sample was adjusted to 4.8 with concentrated hydrochloric acid. All flasks were sterilized at 121℃ for 30 min and then cooled to 30℃. Enzymes were added and the samples were incubated at 50℃ in a shaker incubator (Amerex Instruments Inc., Lafayette, CA) at 200 rpm for 24, 48 and 72 h. All subsamples (duplicate) were boiled for 10 min to inactivate the enzymes prior to high performance liquid chromatography (HPLC) analysis.

2.2.5 Chemical analysis of hydrolyzed samples

All collected subsamples were centrifuged (5000 rpm) for 10 min, filtered (0.2 μm, Syringe Filters, Environmental Express, Inc., Mt. Pleasant, SC) and diluted to 10^{-1} concentration, accordingly. Sugars (cellobiose, glucose, xylose, and arabinose) were analyzed by HPLC (Agilent 1200 Series) with a BioRad Aminex HPX-87P (P), lead form, 300×7.8 mm (ID), 9 μm column and a differential refractive index detector (G1362A Agilent). Percent theoretical cellulose and hemicellulose yields were calculated using the equations provided by NREL's LAP TP-510-42630 as described below.

$$\%\ Theoretical\ Cellulose\ Yield = \frac{[Glucose] + 1.053[Cellobiose]}{1.111 f [Biomass]} \times 100\%$$

$$\%\ Theoretical\ Hemicellulose\ Yield = \frac{0.9[xylose] + [Arabinose]0.9}{1.136 f [Biomass]} \times 100\%$$

where, $[Glucose]$ is the residual glucose concentration (g/L), $[Cellobiose]$ is the residual cellobiose concentration (g/L), 1.053 is the multiplication factor that converts cellobiose to equivalent glucose, 1.111 is the factor that converts cellulose to equivalent glucose, $[Biomass]$ is the dry biomass concentration at the beginning of the enzymatic hydrolysis (g/L), f is the cellulose or hemicellulose fraction in dry biomass (g/g), $[xylose]$ is the residual xylose concentration (g/L), $[Arabinose]$ is the residual arabinose concentration (g/L), 1.136 is the factor that converts hemicellulose to equivalent xylose.

3.2.6 Statistical analysis

SAS 9.4 software (SAS Institute Inc., Cary, NC, USA) was used for analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test at a 95% confidence level (α = 0.05) on the chemical composition and enzymatic hydrolysis data.

2.3 Results and Discussion

2.3.1 Moisture and pH condition

Moisture content of energycane bagasse remained stable at 45% and 75%, respectively, during the 60 days storage ensuring a constant moisture condition throughout the study. The change of pH during storage is shown in Fig. 2. 1a, b. The initial time was set as 6 h, instead of 0 h, because results did not differ between time 0 h and 6 h. The initial pH for the energycane bagasse with no addition of sodium hydroxide was 7 and slightly decreased to around 6 after 30 days and remained at this level for the remaining of the study. However, this pH was not acidic enough to control microbial growth. In order to prevent microbial growth, the pH should be kept below 4. The addition of 5-10% wt. NaOH raised the initial pH to 10.5, and the pH remained above 8 during the 60 days wet storage regardless of the moisture content (Fig. 2. 1a, b). Similar observations were made by Cui et al. (2012) when the pH of sodium hydroxide pretreated corn stover decreased from 10-11 to a level of 8-9 during 90 days wet storage. Digman et al. (2010) conducted a research on switchgrass and reed canarygrass pretreated with calcium hydroxide and found that a relatively dramatic drop of pH happened after 5 days, which may result from the release of acetate during the solubilization of lignin and hemicellulose.

2.3.2 Effect of sodium hydroxide during wet storage on biomass composition

The chemical composition of untreated energycane bagasse was 43.2% (g/100g dry biomass) glucan (cellulose), 21.5% xylan (hemicellulose), 30.3% lignin and 5.0% other (e.g., arabinan, ash, proteins)(Table 2. 1). Similar results have been reported by Aita et al. (2011), and Qiu and Aita (2013). Wet storage at lower moisture content (45%) had slightly higher cellulose (0.6-0.8%) and hemicellulose content (0.2-3.7%) with lower lignin remaining (0.2-1.3%), but no significant difference ($p > 0.05$) was found. However, the accumulation of each part led to

significant ($p < 0.05$) lower dry matter loss (0.9-4.2%). Cui et al. (2012) reported that higher moisture contents (60% and 75%) resulted in higher glucan and xylan degradation and higher dry matter loss compared to lower moisture content (45%) in corn stover. Therefore, moisture content of 45% may be more economical and suitable for wet storage of energycane bagasse with sodium hydroxide pretreatment.

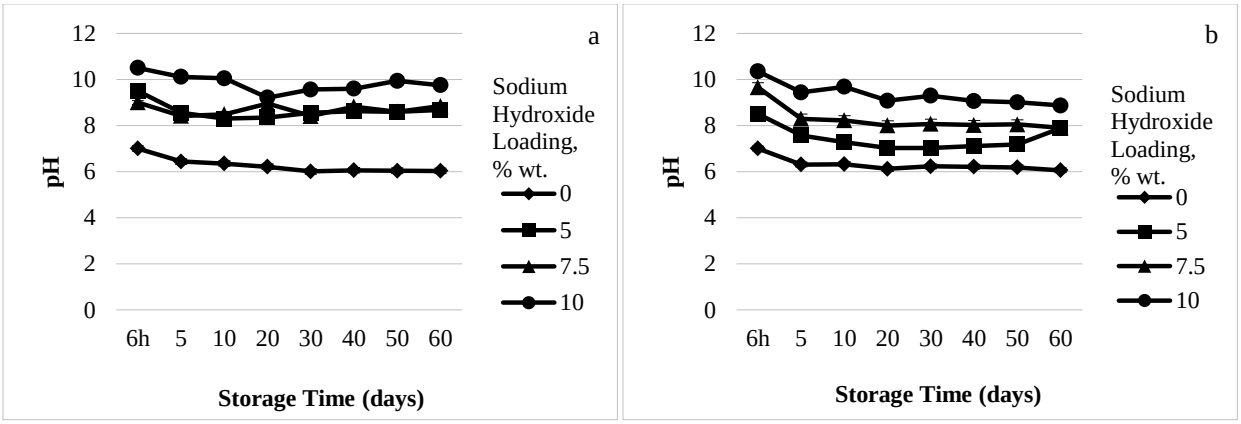

Fig. 2. 1 pH of energycane bagasse with (a) 45% and (b) 75% moisture content during 60 days wet storage.

After 60 days wet storage, the higher loading of sodium hydroxide, the less degradation of cellulose and higher delignification, which resulted in less amount of solids remaining.

Table 2. 1 Chemical composition analysis of untreated and pretreated energycane bagasse after 60 days wet storage.

	Total Lignin	Glucan	Xylan	Arabinan	Solids Remaining
	Percent (g/100 g dry biomass)				
Untreated	30.27±0.01^{a}	43.21±0.01^{a}	21.52±0.01^{a}	1.09±0.12^{a}	100.00±1.01^{a}
0% NaOH*,45%**	27.40±0.16^{b}	31.16±0.05^{f}	15.92±0.32^{b}	1.37±0.12^{a}	79.49±1.20^{b}
0%NaOH,75%	27.63±0.25^{b}	30.36±0.21^{f}	12.32±0.65^{de}	1.37±0.28^{a}	75.31±1.42^{c}
5%NaOH,45%	19.33±0.46^{c}	33.45±0.23^{e}	14.81±0.32^{bc}	1.39±0.54^{a}	72.59±0.48^{d}
5%NaOH,75%	20.60±0.08^{c}	32.79±0.14^{e}	13.00±0.41^{de}	1.44±0.23^{a}	71.39±0.65^{e}

(Table 2. 1 continued)

7.5%NaOH,45%	9.87±0.32^{de}	38.43±0.09^{cd}	13.59±0.09^{cd}	1.43±0.44^{a}	66.89±0.75^{f}
7.5%NaOH,75%	10.10±0.12^{de}	37.68±0.32^{d}	12.46±0.18^{de}	1.45±0.19^{a}	65.24±1.23^{f}
10%NaOH,45%	9.01±0.07^{e}	40.11±0.47^{b}	12.32±0.21^{de}	1.15±0.21^{a}	66.44±0.91^{f}
10%NaOH,75%	10.53±0.16^{d}	39.38±0.15^{bc}	12.04±0.05^{e}	1.43±0.32^{a}	66.95±0.52^{g}

Notes: *= % wt.; **= moisture content.
Each value represents mean ± SD of 2 replicates in each treatment. In the same column, significant differences at $p < 0.05$ levels are indicated by the different letters.

Lignin degradation was nearly 33% in energycane bagasse samples containing 5% wt. of NaOH, and significantly ($p < 0.05$) increased to 60% when sodium hydroxide concentration was increased to 7.5% wt. and 10% wt. Guo et al. (2013) also reported that higher sodium hydroxide concentrations led to higher lignin degradation rate and the influence of sodium hydroxide concentration on lignin degradation is even bigger than soaking time. As indicated in Fig. 2. 2a, b, a dramatic lignin degradation occurred after 10 days. This process took longer than the 5 days reported by Cui et al. (2012) in corn stover, which may be due to the higher lignin content and the difference in the structure of lignin in energycane bagasse. No significant difference ($p > 0.05$) was found after 40 days storage with 10% wt. NaOH regardless of the moisture content.

Glucan degradation happened gradually throughout the 60 days storage (Fig. 2. 3a, b). This degradation was 28.8% in energycane bagasse samples without the addition of sodium hydroxide and 21.0% in samples containing 5% wt. NaOH. However, glucan degradation significantly ($p < 0.05$) decreased to 10.4% in energycane bagasse samples containing 7.5% wt. NaOH and to 8.4% in samples with 10% wt. NaOH. The higher concentration of sodium hydroxide prevented cellulose degradation by preventing the growth of anaerobic fermentative bacteria (Cui et al., 2012). Xylan degradation is shown in Fig. 2. 4a, b. Almost half the amount of xylan was removed during the 60 days wet storage. However, no significant difference ($p >$

0.05) was observed between the samples with different loadings of sodium hydroxide and moisture content. It should be noticed that xylan degradation can be related to lignin degradation due to the lignin-xylan association (Barakat et al., 2015). The loss of xylan or hemicellulose may also attribute to deacetylation. FT-IR analysis has shown the removal of acetyl groups in hemicellulose from sodium hydroxide pretreated biomass (Xu et al., 2015; Li et al., 2016). Similarly, Guo et al. (2013) indicated that sodium hydroxide pretreatment preserved the most cellulose (98%) and degraded more than half amount of hemicellulose (68%) in corn straw.

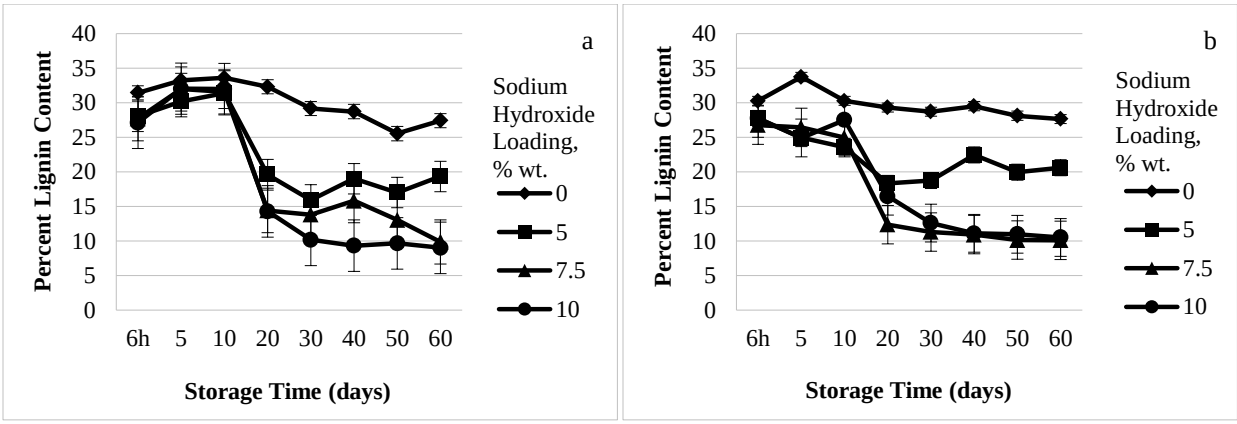

Fig. 2. 2 Lignin content of energycane bagasse with (a) 45% and (b) 75% moisture content during 60 days wet storage.

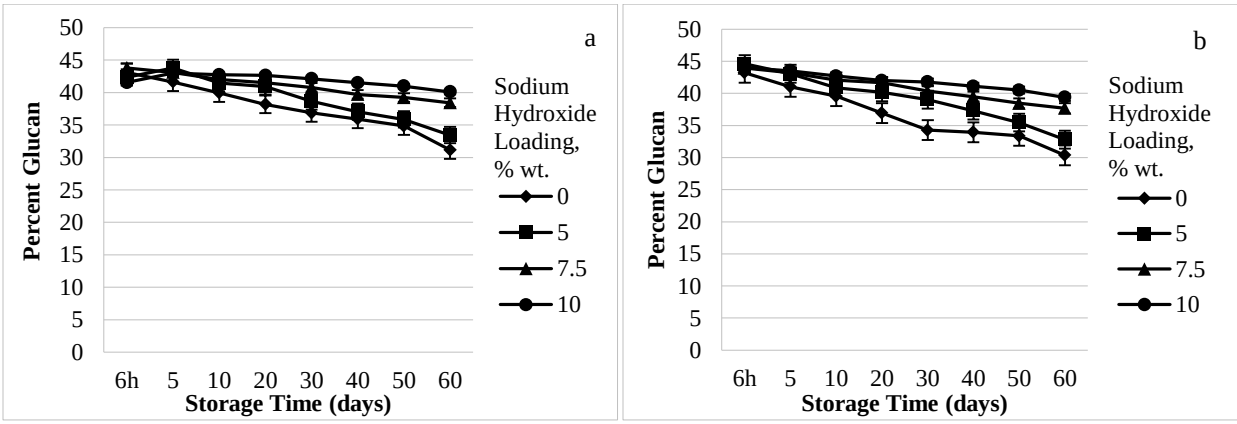

Fig. 2. 3 Glucan content of energycane bagasse with (a) 45% and (b) 75% moisture content during 60 days wet storage.

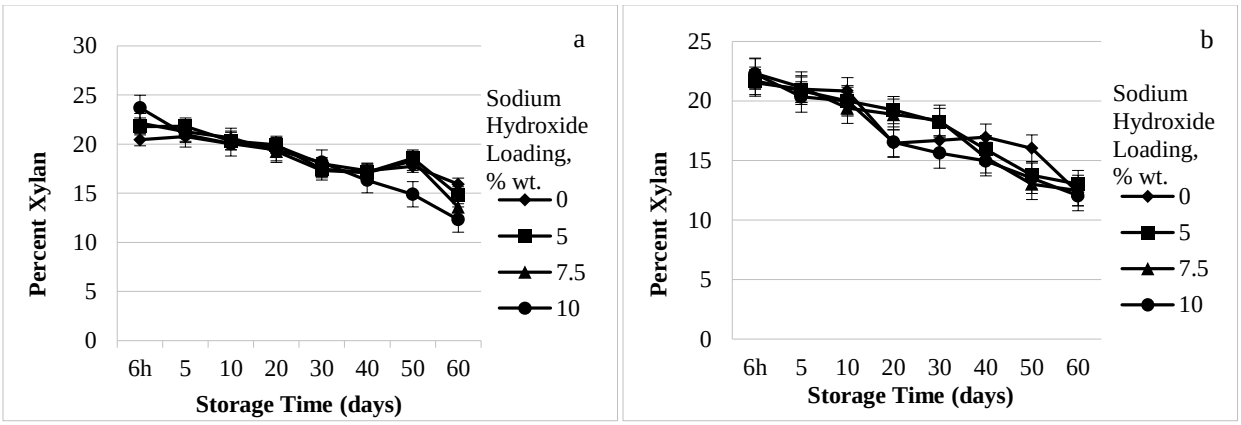

Fig. 2. 4 Xylan content of energycane bagasse with (a) 45% and (b) 75% moisture content during 60 days wet storage.

Overall dry matter loss ranged from 27.4-34.8% after 60 days of wet storage with addition of sodium hydroxide, which was significantly ($p < 0.05$) higher than the observed 20.5-24.7% in the control samples (Fig. 2. 5a, b). The observed results were much higher than those reported with corn stover (3.9-21.8%), indicating that different types of biomass influenced the effect of wet storage (Shinners et al., 2007; Cui et al., 2012). Similarly, a dramatic increase in dry matter loss was found after 10 days of storage, which are in agreement with the results observed with lignin degradation. Sodium hydroxide loading strongly affected the chemical

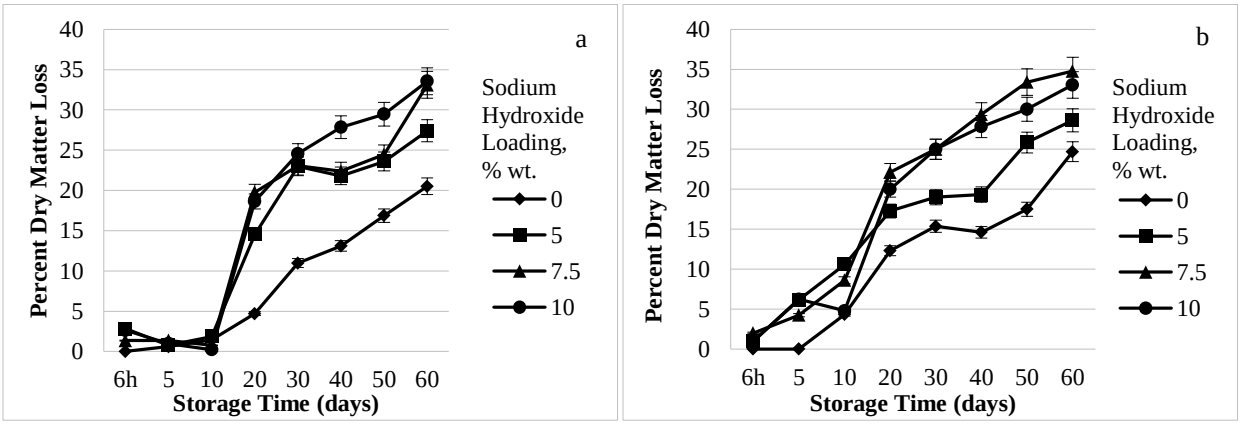

Fig. 2. 5 Dry matter loss of energycane bagasse with (a) 45% and (b) 75% moisture content during 60 days wet storage.

composition of energycane bagasse during wet storage, and sodium hydroxide loading of 10% wt. at a moisture content of 45% might be a preferred treatment for energycane bagasse storage.

2.3.3 Effect of sodium hydroxide pretreatment during wet storage on enzymatic digestibility of energycane bagasse

Sodium hydroxide-assisted wet storage improved cellulose digestibility and hemicellulose digestibility of energycane bagasse over the 60 days storage period (Fig. 2. 6a, b; 2. 7a, b). Sodium hydroxide loading positively enhanced the percent theoretical cellulose digestibility and hemicellulose digestibility as compared to controls, especially in samples with sodium hydroxide loadings of 7.5% wt. and 10% wt. For the samples with initial moisture content of 45%, untreated energycane bagasse resulted in 33.5% cellulose digestibility and 18.2% hemicellulose digestibility over the 60 days wet storage. Treated samples with 5% wt. NaOH resulted in significant ($p < 0.05$) higher cellulose digestibility (44.7%) and hemicellulose digestibility (29.9%) as compared to controls. The highest cellulose digestibility and hemicellulose digestibility observed were 69.2% and 42.7%, respectively, with the addition of 10% wt. NaOH, which were significantly ($p < 0.05$) higher than the control. It is worth mentioning that percent sugar digestibility obtained for bagasse samples with 7.5% wt. NaOH loading were very similar to those containing 10% wt. NaOH at 45% moisture content. At 7.5% wt. NaOH a cellulose digestibility of 62.7% and a hemicellulose digestibility of 38.7% was observed, followed by 69.2% and 42.7% at 10% NaOH, respectively. The highest digestibility (77.2% cellulose digestibility and 45.0% hemicellulose digestibility) in samples with moisture content of 75% was significantly ($p < 0.05$) higher than the ones obtained from samples with moisture content of 45%. However, a similar pattern was observed in which the higher the sodium hydroxide loading the greater the digestibility regardless of the moisture content used in

this study. For the samples with initial moisture content of 75%, untreated energycane bagasse resulted in 37.9% cellulose digestibility and 20.2% hemicellulose digestibility over the 60 days wet storage. Treated samples with 5% wt. NaOH resulted in significantly ($p < 0.05$) higher cellulose digestibility (46.9%) and hemicellulose digestibility (24.2%) as compared to controls. The highest cellulose digestibility and hemicellulose digestibility observed were 77.2% and 45.0%, respectively, with the addition of 10% wt. NaOH, which were significantly ($p < 0.05$) higher than controls. Also, the results observed for samples containing 7.5% wt. NaOH loading were close to those containing 10% wt. NaOH at 75% moisture content. At 7.5% wt. NaOH a cellulose digestibility of 74.3% and a hemicellulose digestibility of 42.6% was observed, followed by 77.2% and 45.0% at 10% NaOH, respectively. Wan et al. (2011) pretreated soybean straw with sodium hydroxide (4–40 g NaOH/100gdry straw) at ambient conditions for 24 h and achieved a glucose yield of 65% and xylan removal of up to 47%. Similarly, Cui et al. (2012) reported glucose yields of 45-55% and xylose yields of 40-45% for corn stover stored with a 50 g NaOH/kg dry matter at a moisture content of 45% for 90 days.

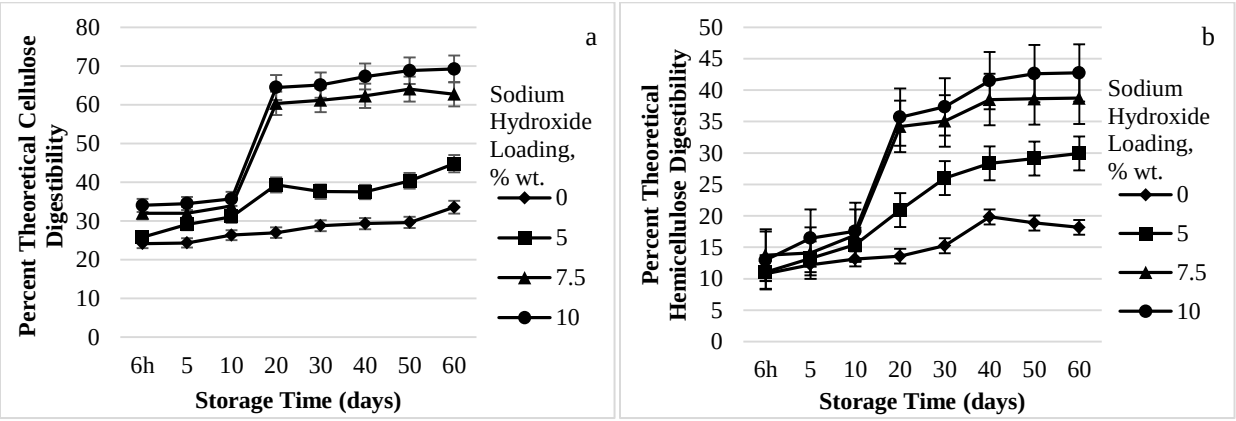

Fig. 2. 6 Percent theoretical (a) cellulose digestibility and (b) hemicellulose digestibility of energycane bagasse with 45% moisture content during 60 days wet storage.

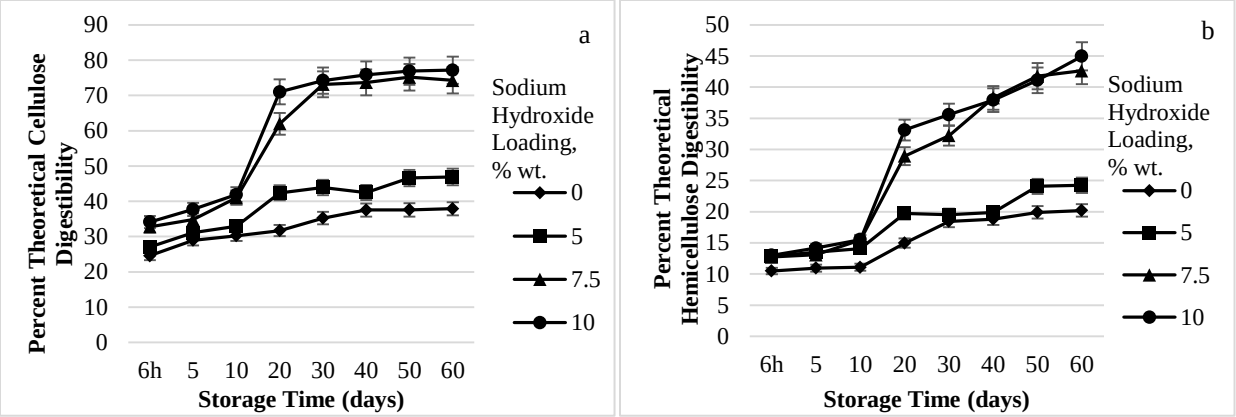

Fig. 2. 7 Percent theoretical (a) cellulose digestibility and (b) hemicellulose digestibility of energycane bagasse with 75% moisture content during 60 days wet storage.

The main effect of sodium hydroxide pretreatment in lignocellulosic biomass is delignification by breaking the ester bonds cross-linking lignin and xylan, thus increasing the porosity of biomass and the internal surface of cellulose (Sun and Cheng, 2002; Mosier et al., 2005). Therefore, enzymes have better access to the surface of biomass to break down cellulose and hemicellulose into their main monomeric forms glucose and xylose, respectively. This explains the significant increase ($p < 0.05$) observed in sugar digestibility after 10 days storage with sodium hydroxide in this study, which might be caused by the dramatic lignin degradation observed and as described above. Furthermore, no significant difference ($p > 0.05$) in cellulose digestibility was observed in energycane samples stored with sodium hydroxide after 40 days at 45% or 75% moisture.

2.4 Conclusions

Wet storage assisted with sodium hydroxide significantly ($p < 0.05$) improved sugar digestibility by mostly preserving the glucan and removing the lignin. Higher loading of sodium hydroxide and lower moisture content resulted in less cellulose degradation and greater lignin

degradation. Cellulose decreased by 7.2% and 8.9% in 10% wt. NaOH treated samples with 45% and 75% moisture content, respectively, as compared to 27.8% and 29.7% from untreated samples over the 60 days wet storage. Lignin degradation of 33% was observed with 5% wt. NaOH. However, it significantly ($p < 0.05$) increased to at least 60% with the addition of 7.5% wt. or 10% wt. NaOH within 10 days of storage. Cellulose digestibilities (62.7-77.2%) and hemicellulose digestibilities (38.7-45.0%) in samples with over 7.5% wt. NaOH loading were significantly ($p < 0.05$) higher than controls (33.5-37.9% and 18.2-20.2%, respectively). No significant difference ($p > 0.05$) in cellulose digestibility was observed in hydrogen peroxide treated energycane bagasse samples stored after 40 days at 45% or 75% moisture. Almost half the amount of xylan was degraded during wet storage. However, no significant difference ($p > 0.05$) was observed between samples stored with different loadings of sodium hydroxide and moisture content.

This study have shown that sodium hydroxide loading of 10% wt. at a moisture content of 45% preserved the most glucan (40.1%), degraded the most lignin (66.8%) and xylan (48.0%), and resulted in high cellulose digestibility (69.2%) and hemicellulose digestibility (42.7) for energycane bagasse during short term wet storage.

CHAPTER 3
EVALUATION OF FUNGAL ASSISTED WET STORAGE OF ENERGYCANE BAGASSE

3.1 Introduction

Energycane L79-1002 is a hybrid of the cross between the female parent, cultivar CP 52-68, and the male parent, Tainan, a clone of *Saccharum spontaneum* (Bischoff et al., 2008). Compared to sugarcane and sweet sorghum, energycane has higher fiber content and can be harvested during colder temperatures due to its cold tolerance attributes (Kim and Day, 2011). After harvesting, juice is pressed from energycane and the fibrous residue left behind is called bagasse. Bagasse or lignocellulosic biomass is a promising material than can be processed in many ways to yield value added products.

Since the agricultural crops that result in lignocellulosic residues are typically harvested only at certain times of the year, large volumes of biomass would need to be stored prior to processing in a biorefinery. Storage of biomass on-field (i.e., at the site where harvested) can result in biodegradation of fibers, and thus loss of energy content, and possible spoilage if stored without treatment of the fibers. Proper storage will ensure the quality of biomass, improve the efficiency of pretreatment and further hydrolysis (Liu et al., 2013). Certain microbes can be added during storage immediately following harvest at a moisture content that is at least 45% (wet storage), to help retard microbial activity and increase the susceptibility of biomass to enzymatic hydrolysis (Thompson et al., 2005; Cui et al., 2012).

Lignocellulosic biomass is primarily made of carbohydrates and lignin. Lignin works as a wall that surrounds the cellulose and links it to the hemicellulose thus making it difficult for

enzymes to access the polymeric carbohydrates (Zheng et al., 2014). Therefore, removing lignin is a crucial task for pretreatment prior to enzymatic hydrolysis for the conversion of lignocellulosic biomass into fuels and chemicals. Biological pretreatments using white-rot fungi can be an attractive option due to their low energy demands, selective degradation of lignin and hemicellulose, and production of value added by-products (Lopez-Abelairas et al., 2012; Shirkavand et al., 2016). White-rot fungi can produce enzymes such as manganese peroxidase (MnP), laccase (Lac) and lignin peroxidase (Lip) in order to degrade the lignin (Isroi et al., 2011). *Ceriporiopsis subvermispora* only produces MnP and laccase. Since the lack of a complete cellulolytic enzyme complex, *C. subvermispora* is an excellent selective white-rot fungus that will primarily target the lignin (Wan and Li, 2012).Researchers have shown that white-rot fungi can significantly remove lignin and improve sugar yields in biomass. Lopez-Abelairas et al. (2012) found that the sugar yield of wheat straw pretreated with white-rot fungus *Irpex lacteus* increased from 33 to 54%. Deswai et al. (2013) observed that white-rot fungal pretreatment improved the amenability of wheat bran and sugarcane bagasse for enzymatic hydrolysis. Nazarpour et al. (2013) indicated that rubberwood treated with *Ceriporiopsis subvermispora* resulted in increased sugar yields of almost 30% after 90 days storage. In addition to biomass pretreatment, white-rot fungi has been widely applied to biopulping, forage upgrading, and bioremediation of soil and wastewater (Okano et al., 2006; Wan and Li, 2012).

The aim of this study was to assess the effect of wet storage in combination with fungal pretreatment on the delignification and cellulose and hemicellulose digestibility of energycane bagasse during 60 days.

3.2 Methods

3.2.1 Biomass

Energycane (non-commercial variety L79-1002) was harvested at Louisiana State University Agricultural Center Sugar Research Station in St. Gabriel, LA. Tops, leaves and stalks of energycane remained during milling and the juice was extracted by pressing the biomass through a roller press (Farrel Company, Ansonia, CT) three times. The remaining fibers or bagasse were stored at -20℃ for future use. Particle size of biomass averaged ¼ in.

3.2.2 Fungal pretreatment and wet storage

The white-rot fungus *C. subvermispora* (ATCC 96608) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The fungus was revived on 2% (w/v) malt extract agar (MEA) at 28℃ for five days. Ten discs (each 10 mm in diameter) from the MEA plates were transferred to 2% (w/v) malt extract liquid medium in a 500 ml cotton-plugged Erlenmeyer flask and incubated at 28℃ (Amerex Instruments Inc., Lafayette, CA) for seven days. The energycane bagasse was dried in an oven overnight at 40-45℃ to a final moisture of 10%. Twenty grams of energycane bagasse (dry basis) were mixed with deionized water to obtain an optimal moisture content of 75% and autoclaved in 500 ml Erlenmeyer flasks at 121℃ for 15 min. After cooling to room temperature, all the flasks were inoculated with 4 ml of liquid fungal culture and sealed with cotton plugs and aluminium foil to retain the moisture content throughout the study. Energycane bagasse mixed with *C. subvermispora* was incubated at 28℃ (Amerex Instruments Inc., Lafayette, CA) for up to 60 days. Subsamples were taken at 5, 10, 20, 30, 40, 50, and 60 days for analysis of pH, dry matter loss, chemical composition, and enzymatic

hydrolysis. Control samples consisted of energycane bagasse without the addition of *C. subvermispora*. All tests were carried out in duplicate.

3.2.3 Chemical composition of energycane bagasse

All fungal-treated and control samples were analyzed for glucan, xylan, arabinan, mannan, lignin, ethanol extractives, and ash content by following the National Renewable Energy Laboratory (NREL)'s laboratory analytical procedures (LAPs) TP-510-42618, 42619, 42620, 42621, and 42622. NREL reference material (8491 sugarcane bagasse) was also analyzed as an internal sample to ensure the accuracy of the procedures.

3.2.4 Enzymatic hydrolysis

White-rot fungus treated and control samples were hydrolyzed using a combination of commercially available enzymes, Cellic CTec2 and Cellic HTec2 (Novozymes North America, Inc., Franklinton, NC). Cellic CTec2 is a cellulase complex enzyme (cellulases and β–glucosidases); whereas, Cellic HTec2 is mainly an endoxylanase with high specificity toward soluble hemicellulose. CTec2 was used at 32 CPU/g glucan and HTec2 was added at the recommended ratio of 9:1 (CTec2:HTec2). Enzymatic hydrolysis was conducted as indicated by NREL's LAP TP-510-43629. Briefly, 2.5g (dry basis) of fungal-treated or untreated energycane bagasse were mixed with 1.25 g citrate buffer (0.1 M stock solution, pH 4.8) into an Erlenmeyer flask and water was added to each sample to a final weight of 50g. The pH of each sample was adjusted to 4.8 with concentrated hydrochloric acid. All flasks were sterilized at 121℃ for 30 min and then cooled to 30℃. Enzymes were added and the samples were incubated at 50℃ in a shaker incubator (Amerex Instruments Inc., Lafayette, CA) at 200 rpm for 24, 48 and 72 h. All

subsamples (duplicate) were boiled for 10 min to inactivate the enzymes prior to high performance liquid chromatography (HPLC) analysis.

3.2.5 Chemical analysis of hydrolyzed samples

All collected subsamples were centrifuged (5000 rpm) for 10 min, filtered (0.2 μm, Syringe Filters, Environmental Express, Inc., Mt. Pleasant, SC) and diluted to 10^{-1} concentration, accordingly. Sugars (cellobiose, glucose, xylose, and arabinose) were analyzed by HPLC (Agilent 1200 Series) with a BioRad Aminex HPX-87P (P), lead form, 300×7.8 mm (ID), 9 μm column and a differential refractive index detector (G1362A Agilent). Percent theoretical cellulose and hemicellulose yields were calculated using the equations provided by NREL's LAP TP-510-42630 as described below.

$$\%\ Theoretical\ Cellulose\ Yield = \frac{[Glucose] + 1.053[Cellobiose]}{1.111f[Biomass]} \times 100\%$$

$$\%\ Theoretical\ Hemicellulose\ Yield = \frac{0.9[xylose] + [Arabinose]0.9}{1.136f[Biomass]} \times 100\%$$

where, $[Glucose]$ is the residual glucose concentration (g/L), $[Cellobiose]$ is the residual cellobiose concentration (g/L), 1.053 is the multiplication factor that converts cellobiose to equivalent glucose, 1.111 is the factor that converts cellulose to equivalent glucose, $[Biomass]$ is the dry biomass concentration at the beginning of the enzymatic hydrolysis (g/L), f is the cellulose or hemicellulose fraction in dry biomass (g/g), $[xylose]$ is the residual xylose concentration (g/L), $[Arabinose]$ is the residual arabinose concentration (g/L), 1.136 is the factor that converts hemicellulose to equivalent xylose.

3.2.6 Statistical analysis

SAS 9.4 software (SAS Institute Inc., Cary, NC, USA) was used for analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test at a 95% confidence level ($\alpha = 0.05$) on the chemical composition and enzymatic hydrolysis data.

3.3 Results and Discussion

3.3.1 Moisture, pH and temperature condition

Moisture content of fungal-treated and untreated (control) energycane bagasse remained stable at 75% during the 60 days wet storage. No significant difference ($p > 0.05$) was found between the moisture contents of treated and control samples. In order to achieve the highest delignification with minimal loss of cellulose and hemicellulose, bagasse samples inoculated with *C. subvermispora* were store at 75% moisture. Optimal moisture content for *C. subvermispora* is around 60-75% as previously reported (Wan and Li, 2010b; Cui et al., 2012). The change in pH during wet storage is shown in Fig. 3. 1. The initial pH of energycane bagasse was 7.0 and in control samples the pH gradually decreased to around 6.1 after 30 days and remained at this level for the remaining 30 days of the study. This pH, however, was not acidic enough to control microbial growth. In fungal-treated samples, as the population of *C. subvermispora* started to increase a rapid drop in the pH from 7.0 to 4.0 was observed, which is in the optimal pH range (4-5) for *C. subvermispora* (Reid, 1989). Similar trends have been observed in wheat straw with other white-rot fungi (*Pycnoporus cinnabarinus*, *Cyathus stercoreus*, *Dichomitus squalens*) (Agosin et al., 1985).

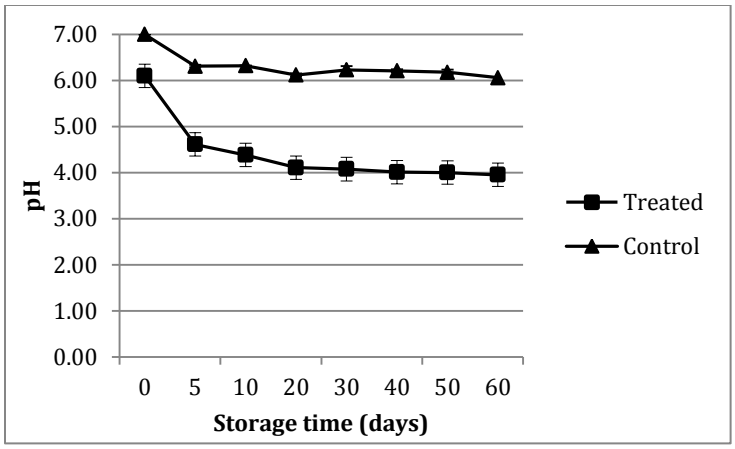

Fig. 3. 1 pH of energycane bagasse during 60 days wet storage.

3.3.2 Effect of *C. subvermispora* during wet storage on biomass composition

The chemical composition of untreated energycane bagasse before wet storage was 44.1% (g/100g dry biomass) glucan (cellulose), 22.1% xylan (hemicellulose), 30.2% lignin, and 5.0% other (e.g., arabinan, ash, proteins)(Table 3. 1). Previous studies have reported similar results on the chemical composition of energycane bagasse (Aita et al., 2011; Qiu and Aita, 2013). Without any fungal treatment, energycane bagasse with 75% moisture content lost 31.1% of cellulose and 44.3% of hemicellulose, and only 13.6% of lignin, which led to 23.3% dry matter loss after 60 days wet storage. Inoculating and storing energycane bagasse with *C. subvermispora* preserved almost all of the glucan (43.4%), removed nearly half the amount of xylan (11.7%) and significantly ($p < 0.05$) improved delignification (44.0%) after 60 days wet storage. Cui et al. (2012) applied *C. subvermispora* to corn stover and found that inoculating this fungus resulted in 37.7% lignin degradation and only 10.7% cellulose degradation during 90 days wet storage. Ash and extractives increased after 60 days wet storage, regardless of the treatment with or without *C. subvermispora.*

Table 3. 1 Chemical composition analysis of control and fungal treated energycane bagasse after 60 days wet storage.

	Ash	Extractives	Total Lignin	Glucan	Xylan	Arabinan	Solids Remaining
Percent (g/100 g dry biomass)							
Control - 0 day	2.31±0.15[a]	1.88±0.41[a]	30.15±1.01[a]	44.06±0.53[a]	22.13±0.14[a]	1.11±0.10[b]	100.02±1.13[a]
Control - 60 day	4.02±0.04[b]	2.57±0.23[b]	26.05±0.45[b]	30.36±1.02[b]	12.32±0.33[b]	1.37±0.08[a]	76.69±2.75[b]
Fungal Treated - 60 days	5.18±0.01[c]	2.57±0.35[b]	16.87±1.16[c]	43.39±3.68[a]	11.73±1.65[b]	1.07±0.11[b]	85.72±4.87[c]

Note: Each value represents mean ± SD of 2 replicates in each treatment. In the same column, significant differences at $p < 0.05$ levels are indicated by the different letters.

Lignin degradation of energycane bagasse is shown in Fig. 3. 2. *C. subvermispora* significantly ($p < 0.05$) removed 44.2% of lignin in energycane bagasse, which is 30.6% more than that observed with control samples. It should be noticed that the majority of lignin degradation occurred after 10 days storage, and no significant difference ($p > 0.05$) was observed in fungal treated sample after 50 days storage. Cui et al. (2012) also found that most of the delignificantion (35.8%) in corn stover occurred during the first 35 days and only marginal lignin degradation appeared thereafter. This might be due to the higher lignin content and difference in the structure of lignin in energycane bagasse.

Glucan content of fungal-treated energycane bagasse slightly decreased throughout the 60 days wet storage (Fig. 3. 3). Glucan degradation was around 30% in the control samples. Only 1.5% glucan degradation was observed in samples with *C. subvermispora*, which is significantly difference ($p < 0.05$) from control. The growth of the white-rot fungus prevented glucan degradation by inhibiting the growth of anaerobic fermentative bacteria naturally found in the biomass. As indicated in Fig. 3. 4, a gradual xylan degradation was observed in both control and fungal-treated samples. Almost half the amount of xylan was removed by the end of the 60 days

wet storage. No significant difference ($p > 0.05$) in xylan removal was found between control and fungal-treated bagasse. Xylan degradation can be attributed to lignin removal and the crosslinking of the lignin-xylan structure (Barakat et al., 2015).

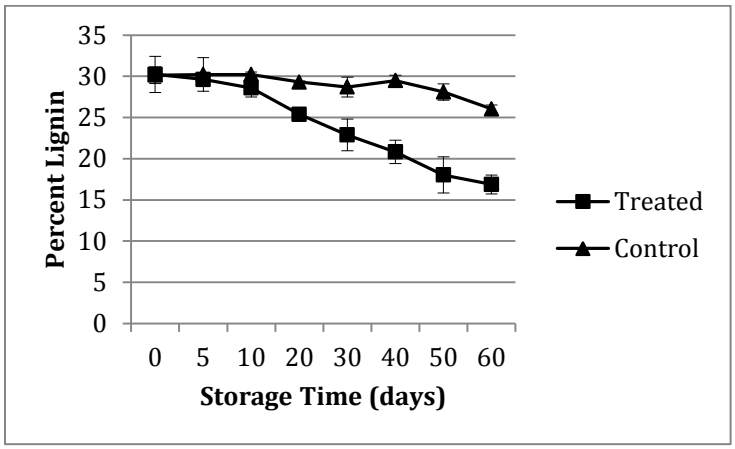

Fig. 3. 2 Lignin content of energycane bagasse during 60 days wet storage.

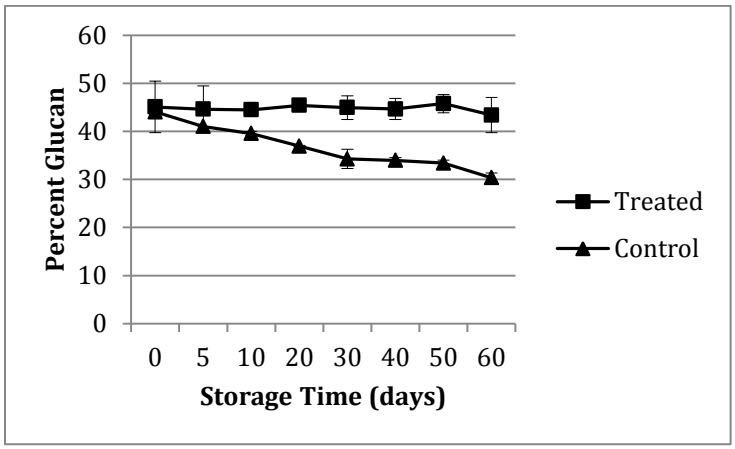

Fig. 3. 3 Glucan content of energycane bagasse during 60 days wet storage.

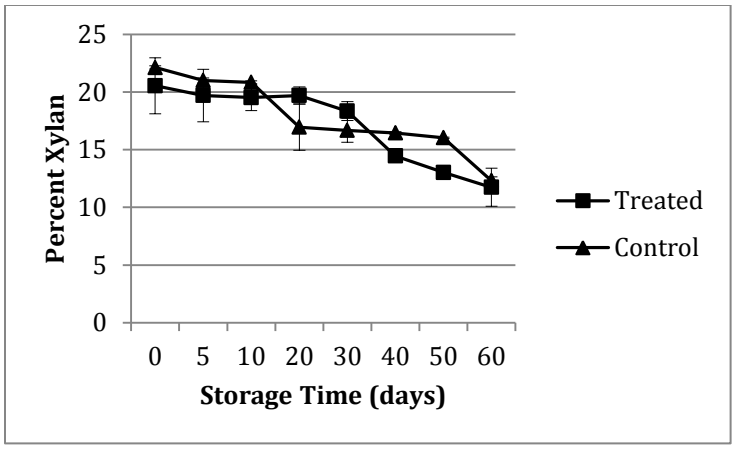

Fig. 3. 4 Xylan content of energycane bagasse during 60 days wet storage.

Overall dry matter loss of fungal-treated biomass was 14.3% after 60 days wet storage, which was significantly ($p < 0.05$) lower than that observed with controls (23.3%)(Fig. 3. 5). Cui et al. (2012) reported 17.6% dry matter loss after 60 days wet storage and 20.0% dry mater loss after 90 days wet storage in corn stover, demonstrating that different types of biomass might influence the effect of wet storage (Shinners et al., 2007). The white-rot fungus *C. subvermispora* significantly ($p < 0.05$) changed the chemical composition of energycane bagasse by selectively degrading the lignin and preserving the glucan during the 60 days wet storage.

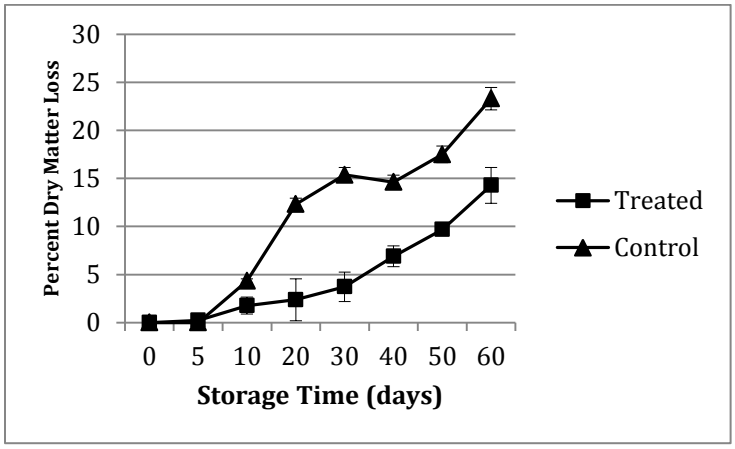

Fig. 3. 5 Dry matter loss of energycane bagasse during 60 days wet storage.

3.3.3 Effect of *C. subvermispora* pretreatment during wet storage on enzymatic digestibility of energycane bagasse

Fungal-assisted wet storage improved cellulose and hemicellulose digestibility of energycane bagasse during the 60 days storage (Fig 3. 6a, b). Control samples resulted in 36.9% cellulose digestibility and 19.2% hemicellulose digestibility over the 60 days wet storage. Fungal-treated bagasse resulted in the highest cellulose digestibility and hemicellulose digestibility of 66.5% and 34.3% at day 50, respectively. A significant increase in cellulose and hemicellulose digestibility began at day 5-10. After day 50, sugar digestibility started to decrease. A similar pattern was observed by Cui et al. (2012) where glucose yield of fungal-treated corn stover was 64.5% at day 35 and decreased to 56.2% at day 90. This is possibly due to cellulose degradation and slow changing in lignin content. Wan and Li (2010a) found that both cultivation conditions and the type of lignocellulosic biomass influenced the degree of selective delignification. Wan and Li (2010a) reported 39.2% lignin removal and 67% glucose yield after 42 days storage of corn stover with *C. subvermispora*. Wan and Li (2011) also reported that combining liquid hot water pretreatment (at 170℃ for 3 min) prior to fungal treatment could efficiently improve fungal degradation of soybean straw, resulting in 36.7% lignin removal and 64.3% glucose yield.

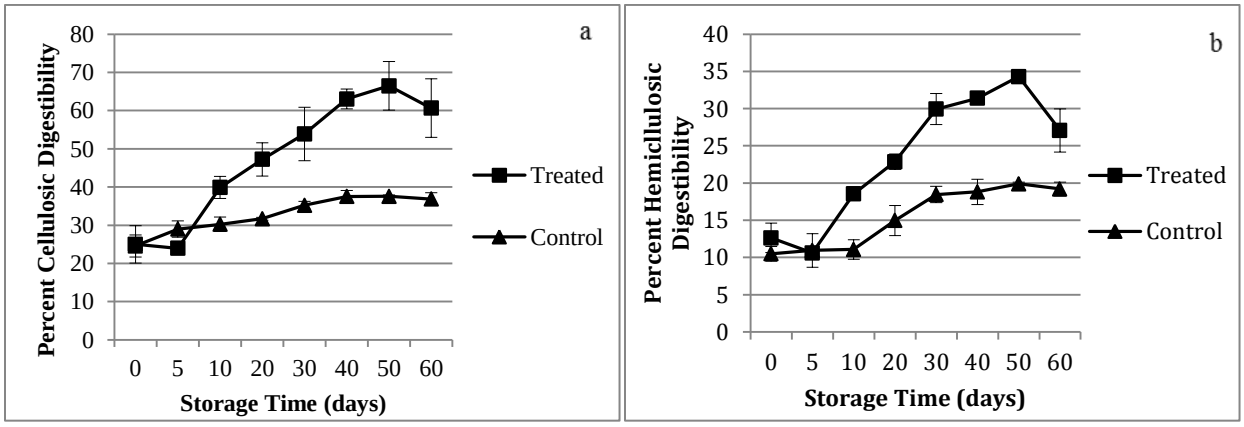

Fig. 3. 6 Percent theoretical (a) cellulose digestibility and (b) hemicellulose digestibility of energycane bagasse during 60 days wet storage.

3.4 Conclusions

Wet storage assisted with *C. subvermispora* significantly ($p < 0.05$) improved sugar digestibility by selectively removing lignin and by mostly preserving the glucan. With samples stored at a moisture content of 75%, a 44.2% lignin removal and 1.5% cellulose loss was observed in fungal-treated bagasse as compared to 13.6% and 31.1% from control samples, respectively, over the 60 days wet storage. The majority of lignin degradation occurred after 10 days, and no significant difference ($p > 0.05$) in lignin removal was observed in fungal treated sample after 50 days. Almost half the amount of xylan was removed by the end of the 60 days wet storage. Cellulose digestibility (66.5%) and hemicellulose digestibility (34.3%) in fungal-treated samples were higher than those observed in controls (37.6% and 19.9%, respectively).

This study indicated that fungal-assisted wet storage of energycane bagasse was efficient in removing the lignin, preserving the glucan and increasing cellulose digestibility.

CHAPTER 4
SUMMARY AND FUTURE WORK

Energycane is a prospective renewable energy resource for the production of biofuels and bioproducts. Since the agricultural crops that result in lignocellulosic residues are typically harvested only at certain times of the year, large volumes of biomass would need to be stored prior to processing in a biorefinery. In order to avoid biodegradation of fibers, loss of energy content, and possible spoilage, certain chemicals (e.g., alkali) and microbes (e.g., white-rot fungi) can be added during storage immediately following harvest at a moisture content that is at least 45% (wet storage), to help inhibit microbial activity, maintain biomass quality and promote enzymatic conversion of plant carbohydrates into fermentable sugars.

Wet storage assisted with sodium hydroxide significantly ($p < 0.05$) improved sugar digestibility by removing lignin and by mostly preserving the glucan. Higher loadings of sodium hydroxide and lower moisture content resulted in less cellulose degradation and greater lignin degradation. A 10% wt. NaOH loading at a moisture content of 45% resulted in a nearly 60% lignin degradation. A glucan loss of 7.2% as compared to 27.8% from control was observed during the 60 days wet storage. Higher cellulose digestibilities (62.7-77.2%) and hemicellulose digestibilities (38.7-45.0%) were observed in samples containing 7.5% wt. or 10% wt. NaOH at 75% moisture than controls (33.5-37.9% and 18.2-20.2%, respectively) after 10 days storage. However, sodium hydroxide loading of 10% wt. at a moisture content of 45% preserved the most glucan (40.1%), degraded the most lignin (66.8%) and xylan (48.0%), and resulted in high cellulose digestibility (69.2%) and hemicellulose digestibility (42.7) during storage of energycane bagasse.

Wet storage assisted with *C. subvermispora* significantly ($p < 0.05$) improved sugar digestibility by selectively removing lignin and by mostly preserving the glucan. Fungal-assisted storage of energycane bagasse resulted in 44.2% lignin degradation and 1.5% cellulose loss as compared to 13.6% and 31.1% from untreated samples, respectively, at a moisture content of 75%. The majority of lignin degradation occurred after 10 days, and no significant difference ($p > 0.05$) in lignin removal was observed in fungal treated samples after 50 days. Cellulose digestibility (66.5%) and hemicellulose digestibility (34.3%) of white-rot fungus treated samples were higher than untreated samples (37.6% and 19.9%, respectively). This study indicated that fungal-assisted wet storage of energycane bagasse was efficient in removing lignin (44.0%), preserving cellulose (43.4%) and resulted in high cellulose digestibility (66.5%).

Further work will focus on evaluating the combined effect of sodium hydroxide with other alkali such as lime and dilute ammonia during wet storage of energycane bagasse and crops alike (e.g, sugarcane, sweet sorghum) to lower chemical loading and reduce storage time. Shorter storage times could be applied to biomass since no significant difference in cellulose digestibility was observed after 50 days.

Co-cultivation of *C. subvermispora* with other fungi such as *Coprinus comatus* and *Trichoderma reesei* or with combined pretreatments (e.g., microwave hydrothermolysis, liquid hot water) could be used on biomass during storage to improve sugar yields. The use of enzyme extracts from *C. subversmipora* and other fungi during biomass storage should be also evaluated. Fermentation of stored-treated biomass should be carried out and ethanol yields evaluated.

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VITA

Jing Cao was born in China in 1989. She received her Bachelor of Engineering degree in Food Science and Engineering in July 2012 from Tianjin University, China. Jing Cao started her Master of Science program in the Department of Food Science at Louisiana State University Agricultural Center in August 2013. In May 2016, she anticipates graduating with her master degree in Food Science with a concentration in biomass conversion and storage.