AN ABSTRACT OF THE DISSERTATION OF

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Title: Science and Efficacy of Mild Sodium Hydroxide Treatments in Enzyme-Based Wheat Straw-to-Glucose Processing

Abstract approved:_____

Michael H. Penner

The work described in this dissertation focused on chemistry related to the use of aqueous sodium hydroxide as a treatment in the processing of wheat straw. A major emphasis was the comprehensive evaluation of straw component partitioning due to sodium hydroxide (NaOH) processing. This was evaluated over a range of NaOH concentrations (0-10%, w/v), all at 50°C, 5 h treatment period, and 3% solid loading. Solid and liquid phases resulting from NaOH treatments were evaluated. Total solids recovered in the NaOH-treated solid phase ranged from 47.4-88.0%. Overall carbohydrate recovery in the combined solid and liquid phases was negatively correlated with the alkali concentration of the treatment liquor. The glucan content of the NaOH-treated solid phase ranged from 37.2-67.4%. Glucan recovery in the solid phase was relatively high in all cases, the minimum value being ~98%. Increasing amounts of xylan partitioned into the liquid phase as sodium hydroxide concentrations increased – it ranged from 31-83% of the xylan being recovered in the soluble phase. Carbohydrate analyses of the pretreated liquor revealed that the majority of carbohydrate loss from the solid fraction could be recovered in the liquid phase in form of oligomers and monomers due to alkaline degradation. The interconversion of

glucose, fructose, and mannose under the alkaline condition played an important role in the presence of those sugars. Increase in NaOH concentration contributed to increase in amount of cellulose-derived and hemicellulose-derived oligomers in the pretreated liquor. All oligomers except fructooligomers in NaOH pretreated liquor were higher than those found in water extraction at 50°C, 5 h. Total carbohydrate recovery from the solid and liquid fractions was as high as 99% for glucose and glucan in 5% NaOH treatment and 80-95% for xylose and xylan in 1-10% NaOH treatment. The presence of NaOH as extraction reagent dramatically induced lignin and ash removal from the pretreated solid with up to 63% acid insoluble lignin (AIL) and 87% ash extraction. Solid fractions resulting from NaOH pretreatments (up to 5% NaOH) were tested for their susceptibility to enzymatic saccharification using cellulase and cellulase/xylanase enzyme preparations. The cellulase/xylanase enzyme preparation was found to be more effective at cellulose saccharification than the cellulase enzyme preparation alone. Maximum glucose yield, which corresponded to the 5% NaOH treatment, was 82% over the standard 48 h saccharification period. Extended saccharifications times to 120 h showed that the conversion yield approached 90%. Sequential treatments of the straw (*i.e.* initial alkali treatment – first enzyme saccharification – second alkali treatment - second enzyme treatment) revealed the NaOH treatment has the potential to render essentially all (~99%) of the straw glucan susceptible to enzyme saccharification. This suggests that the layered molecular arrangement of cellulose, hemicellulose, and lignin in the cell wall impacts biomass recalcitrance and glucan conversion yield.

The other major focus of this dissertation research was the characterization of alkali neutralization, which occurs during the aqueous alkali processing of wheat straw. The approach taken was to evaluate the time course of alkali uptake and to determine the underlying nature of alkali uptake. The knowledge generated from this study is useful for understanding the nature of the alkali-induced chemistry that is at the heart of alkali processing of

agricultural byproducts, foods, and forest products. Alkali uptake/acid generation measurements were monitored for wheat straw suspensions at pH 11 and 30˚C. The first phase of alkali uptake corresponded to the 30-second time period over which the pH of the wheat straw suspension was adjusted from its original pH (~6.6) to pH 11. Alkali neutralization during this period was attributed to the instantaneous ionization of solvent accessible Brønstad acids. Following pH adjustment to 11.0, the time course of subsequent alkali uptake was recorded. The time course appeared biphasic. The early phase, which corresponded to the relatively rapid uptake of alkali, was evident during the first 24 hours. The later phase, which was characterized by the relatively slow uptake of alkali, was maintained for the length of the study (up to 96 hours). Alkali uptake during the early phase of the time course appears to be determined by the rate of hydrolysis of readily accessible esters – primarily acetic acid esters (acetyl groups). Alkali uptake during the later phase of the time course appears to be impacted by the rate of alkali penetration into the straw and the rate of production of alkali-induced acid degradation products. The uptake of alkali in the pH adjustment phase was ~ 120 μEq per gram wheat straw, the uptake of alkali in the early phase of time course was ~ 1,064 μEq per gram wheat straw, and the rate of uptake in the later phase of the time course 6.10 μEq per gram wheat straw per hour. Amount of acetyl groups, ferulic acid, and *p*-coumaric acid generated during 96-h pretreatment revealed that they are major esters being hydrolyzed under the studied condition. Combined, these ester-derived acids contributed up to ~ 28% of overall alkali uptake. In addition, alkaline degradation products quantified in this study showed additional ~ 28% contribution to the overall alkali uptake.

Science and Efficacy of Mild Sodium Hydroxide Treatments in Enzyme-Based Wheat Straw-to-Glucose Processing

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Supaporn Sophonputtanaphoca, Author

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CONTRIBUTION OF AUTHORS

Dr. Michael Penner was involved with writing of the manuscripts, and data interpretation. Tiraporn Junyusen assisted with pretreatment, compositional analysis of pretreated wheat straw, all HPLC analysis of Chapter 2, protein analysis of wheat straw, and HPLC analysis of Chapter 3. Dr. Christine Kelly shared her discussion opinion and provided instrumental use (bioreactor) in Chapter 3.

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1 GENERAL INTRODUCTION

Global energy demand has rapidly increased over the past century due to the growth of human population and prosperity of industrial processes (Sun and Cheng, 2002; Talebnia et al., 2010) while fossil fuels are still served as the major energy source throughout the world (Talebnia et al., 2010; Yang and Wyman, 2007). The concerns about the depletion of petroleum supplies, the increase in oil prices, and the impact of green house gases on environment caused by fossil fuels lead to a number of research in the area of renewable energy platforms over the past few decades (Kumar et al., 2009; Zheng and Zhang, 2009). Among alternative renewable energy resources derived from plant biomass, lignocellulosic biomass represents promising and competitive to starch and sugar crops due to the abundance, non-food feedstock, and sustainability toward bioconversion into liquid transportation fuels such as bioethanol on a large-scale production (Alvira et al., 2010; Zheng and Zhang, 2009; Yang and Wyman, 2008; Jørgensen et al., 2007). In addition to biofuels, lignocellulosic biomass has gained more interest in research involving conversion low value products, such as agricultural waste, food processing waste, to a variety of value-added products based on biorefinery concept. More information on biorefinery, can be found in a review article on a perspective of biorefinery concept including recent advances on treatment and analysis of lignocellulosic biomass written by FrizPatrick et al. (2010).

Recalcitrance of Plant Cell Walls

As much as its attractive qualification to be renewable resources, lignocellulosic conversion to other chemicals is still hindered by the complexity of cell walls of biomass (Kumar et al., 2009). Biomass recalcitrance refers to the ability of plants and plant materials to resist microbial and enzymes attack (Himmel and Picataggio, 2008), insect predation, water loss and effect of extreme

temperature (Xu, 2010). Plants have various defense systems including structure, organization of vascular tissue, molecular structure and composition of cell walls. For example, in grasses, the outer layer of the anatomy called epidermis is the first defense line that exposes to environment. Epidermis is composed of thick-walled cells and cells that secrete waxes and oils to coat the outer layer of the plant tissue (Himmel and Picataggio, 2008). Other hydrophobic substances such as cutin and suberin are also parts of the coating materials on the surface of terrestrial plants. The latter substance is found in underground part of plants (Xu, 2008). Composition of cell walls plays an important role in biomass recalcitrance. There are three major constituents in plant cell walls: cellulose, hemicellulose, and lignin. Cellulose, the most abundant natural polymer, is made of linear homopolymers of glucose (Figure 1.1) whereas hemicellulose comprises of branched, heterogeneous sugar polymers. Lignin is composed of amorphous polymers of phenylpropane units with three precursors aromatic alcohols: *p*-coumaryl, coniferyl, and sinapyl alcohols (Buranov and Mazza, 2008). There are two types of plant cell walls, primary and secondary. Primary cell walls occur when cells are developing and expanding. Some cell types, such as parenchyma cells, only have primary cell wall. These walls are typically non-lignified. Secondary cell walls are formed on the primary wall after the cells are fully enlarged and when the cells are matured the entire wall, both primary and secondary cell walls, is typically lignified. The middle lamella is the interfacial layer between adjacent cells is also typically lignified. Cell wall polysaccharides in primary and secondary cell walls are categorized into two phases, fibrillar and matrix (Harris and Stone, 2008).

In grasses, cellulose is the only component in the fibrillar phase of both primary and secondary cell walls while the matrix phases of both cell walls comprise of various types of non-cellulosic polysaccharides (Harris and Stone, 2008). In the vegetative parts of grasses, glucuronoarabinoxylan (GAX) is the major non-cellulosic polysaccharide in both primary and lignified secondary

walls (Harris et al., 2008; Vogel, 2008). As showed in Figure 1.2, GAX has a linear backbone chain of (1→4)-β-D-xylose residues substituted by single α-L-arabinose residues primarily at the O-3 position and less frequently substituted by single α-D- glucuronic acid residues or its 4-O-methyl derivative mainly at the O-2 position of the xylose units (Carpita and Gibeaut, 1993; Carpita, 1996; Harris and Stone, 2008; Vogel, 2008). The substitution of arabinose and glucuronic acid to the xylan backbone is in a non-repeating fashion (Vogel, 2008). The number of side groups along the xylan chain varies with only 10% or less of the xylose units having appendant units (Carpita and Gibeaut, 1993).

In addition to single arabinose and glucuronic acid residues, several oligosaccharide side chains containing L-arabinose, D-xylose, D-galactose, and L-galactose also occur in GAXs (Harris and Stone, 2008). Esterification of ferulic acid, low amounts of *p*-coumaric acid, and sometimes sinapic acid, occur at the O-5 position of the arabinose side groups in both single and oligomer forms (Harris and Stone, 2008). This esterification to the polysaccharides occurs in the matrix phase of the primary cell walls and in the secondary cell walls. The presence of these hydroxycinnamic acids is one of the unique characters for cell walls of family Poaceae (grasses). The matrix phase of the secondary cell walls contains most of the lignin (up to 20%) compared to that of in the primary cell walls (Vogel, 2008). Besides phenolic acid esterification, acetylation (by ester linkage) of GAX by acetyl groups at the O-2 and O-3 positions of the xylosyl units of the xylan backbone could account for 50-70% of the substitutions (Carpita, 1996; Chesson et al., 1983) or 1-2% of the dry weight of grasses (Puls and Schuseil, 1993).

As mentioned above about unique characteristic of grass cell walls with the presence of hydroxycinnamic acids, the predominant hydroxycinnamic acids are ferulic acid and *p*-coumaric acid. They exist in form of esterified and etherified to polysaccharides and lignin, cross-linked through ester-ether bridges in case of ferulate (major cross-linking) between lignin and polysaccharides

(Vogel, 2008; Sun et al., 2002; Iiyama et al., 1994), and unbound or free forms (Vogel, 2008; Iiyama et al., 1994). In case of ester-ether cross-linking in ferulic acid, the carboxylic group serves as esterification site to polysaccharide while the hydroxyl group on the aromatic ring provides etherification site to lignin (Pan et al., 1998). In contrast, *p*-coumaric acid does not involve in ester-ether bridges between arabinoxylan and lignin as ferulic acid does (Hatfield et al., 1999). The majority of *p*-coumaric acid is esterified to lignin or polysaccharides in all growth stages of wheat straw whereas approximately equal amount of ferulic acid is found in form of esterification and etherification (Pan et al., 1998). Pan and coworkers (1998) reported that wheat straw contained 0.48% ferulic acid and 0.42% *p*-coumaric acid, and 56% of total ferulic acid was esterified while the other 44% was etherified. Unlike ferulic acid, more than 80% of *p*-coumaric was in esterified form. This agreed with findings from Sun et al. (2002), i.e., more than 65% of *p*-coumaric acid of wheat straw was in esterified form with lignin while approximately 68% of ferulic acid was in ether linkages with lignin. Esterification of these phenolic acids was primarily to lignin and hemicellulose (Lawther et al., 1996). Release of phenolic acid esters into aqueous fraction occurs when lignocellulosic materials are subjected to alkaline treatments that lead to solubilization of lignin and hemicellulose as well as hydrolysis of ester bonds (Lawther et al., 1996).

Alkaline Pretreatment

In bioconversion of cellulose in lignocellulosic biomass to glucose, lignin and hemicelluloses act as barriers that limit the enzyme accessibility to cellulose (Mosier et al., 2005; Yang and Wyman, 2008). To overcome the biomass recalcitrance, lignocellulosic biomass must be subjected to pretreatment process(es) to reduce cellulose crystallinity and to deconstruct lignin and hemicelluloses barriers that limit the enzyme accessibility to cellulose (Mosier et al., 2005; Yang and Wyman, 2008). Pretreatment methods can be categorized into

at least 5 major groups: physical, physicochemical, chemical, biological, electrical, or a combination of above (Kumar et al., 2009). Several chemical pretreatment methods have been developed involving the use of alkali, acids, oxidizing agents, and solvents (Kumar et al., 2009; Johnson and Elander, 2008). Each pretreatment method works based on its own particular chemical reactions toward lignocellulosic materials and provides different characteristics of pretreated biomass (Mosier et al. 2005). To be considered as an effective pretreatment, there are requirements as following: improvement in sugar production; less in carbohydrate loss due to severity of the process; less in degradation byproducts which can be inhibitors in the subsequent processes; and cost-effective (Sun and Cheng, 2002). Among those pretreatment processes, alkaline pretreatment shows several advantages. For example, the process can be carried out at wide range of temperatures and pressures from room temperature up to 180°C (Kumar et al., 2009; Mosier et al., 2005). In addition, it generates less degradation products that can cause inhibitory effect to the down stream processes and many salts derived from alkali reagents can be recovered and/or regenerated (Kumar et al., 2009). The general effects of alkaline pretreatment are: (1) removal of lignin and hemicelluloses, (2) alteration of lignin structure, (3) swelling fibers, (4) increase in surface area (5) reduction in cellulose crystalinity, (6) depolymerization of cellulose (Johnson and Elander, 2008), and (6) preservation of the majority of cellulose in solid fraction (Gupta and Lee, 2010a; Chen et al. 2009). Such characteristics make lignocellulsic biomass more accessible to be rendered by cellulolytic and xylanolytic enzymes for fermentable monosaccharide production.

Several alkali reagents have been used for pretreatment with different kinds of lignocelluloses, for example, hydroxide-providing reagents (e.g., sodium, calcium, ammonium, potassium) (McIntosh and Vancov, 2011; Xu et al., 2010b; Gupta and Lee, 2010b; Chang and Holtzapple, 2000) and carbonate-providing reagents (e.g., sodium) (Pederson et al., 2010). They can be used as a sole reagent, or with other additives such as oxidizing agent, e.g., hydrogen peroxide

(Gupta and Lee, 2010a; Gupta and Lee, 2010b) or combined alkali (Xu and Cheng, 2011; Zhang et al., 2011), or in sequential pretreatment with acid (Pederson et al., 2010), or with a mechanical process, e.g., radio frequency-based dielectric (RF) heating (Hu et al., 2008), microwave-assisted heating (Hu and Wen, 2008). Summary of alkaline pretreatment methods and enzymatic conversion yields from literatures is listed in Table 1.

Among alkali reagents, sodium hydroxide (NaOH) has been intensively studied the most (Kumar et al., 2009; Mosier et al., 2005). The properties of NaOH-treated biomass provide benefits for several field areas including biorefinery approaches, pulping processes, animal feed, and biogas production (Talebnia et al., 2010; Zanuttini et al., 1998; Moharrery, 2007; Pavlostathis and Gossett, 1985a) due to its major powerful effects on delignification, hemicellulose removal, decrease in crystallinity and degree of polymerization of cellulose, and swelling fiber (Hsu, 1996). Enzymatic digestibility improvement was greatly enhanced by the decrease in lignin, hemicellulose, and cellulose crystallinity, and the increase in water retention of the solid fraction, porosity, and internal surface area (Sun and Cheng, 2002). NaOH treatment has been applied to a variety of biomass including woods (hardwoods – e.g. hybrid poplar, and softwoods – e.g. Douglas fir) (Gupta and Lee, 2010a; Pan et al., 2004), herbaceous crops (e.g. corn stover, grasses, straws) (Chen et al., 2009; Kumar and Murthy, 2011; Pedersen et al., 2011). These feedstocks contained variable amount of lignin from low (grasses, straws) to high (softwood, hardwood). However, NaOH works more efficiently in term of improvement of degradability of lignocelluloses in low lignin substrates than in high lignin plants (Sun and Cheng, 2002).

Alkali Consumption during Alkaline Pretreatment of Lignocellulosic Materials

Considering mode of action of alkali reagents on the cell wall components, cleavages of ester and ether linkages catalyzed by hydroxide ions result in solubilization of lignin and hemicellulose (Sun et al., 2002), thus reduce lignin

and hemicellulose in pretreated solid (Gupta and Lee, 2010a). Saponification (hydrolysis of ester bonds) and ionization of functional groups, i.e., carboxyl and hydroxyl, in carbohydrates and lignin (Xu and Cheng, 2011) under alkali conditions with pH greater than pK_a of those functional groups, liberate hydrogen ions into the system. These hydrogen ions are neutralized by hydroxide ions derived from the alkali reagent causing reduction of alkalinity over time (Figure 1.3). This effect is known as "alkali consumption" or "buffer capacity" of biomass. Buffer capacity of biomass impairs effectiveness of the pretreatment due to the difficulty in maintaining a given pH during pretreatment and more alkali load is required in order to achieve a given condition. This directly affects cost of chemical-based pretreatment, as the reagent load is a major factor of the cost in pretreatment processes.

Alkaline Degradation of Carbohydrates

Another source of alkali consumption of biomass is acids generated from alkaline degradation of carbohydrates and lignin during the pretreatment (Pavlostathis and Gossett, 1985a). Alkali reagent, for example, NaOH causes solvation of hydroxyl groups in carbohydrates, which consequently make the biomass swollen and create porous structure to facilitate enzyme adsorption and digestibility (Gupta and Lee, 2010a). NaOH solubilizes carbohydrates, mainly hemicellulose, due to low reactivity of cellulose to alkali and high crystallinity of cellulose, partial degradation of carbohydrates is inevitable (Gupta and Lee, 2010a; Gupta and Lee, 2010b). Alkaline treatment of carbohydrates results in various degradation products via several reaction mechanisms depending on several factors, e.g., types of constituent sugars, concentration of the carbohydrates, type of alkali, degree of alkalinity, and treatment conditions (temperature and residence time) (Lai, 2001). Three main categories of alkaline degradation could be assigned to reducing sugars, non-reduction sugars, and oligosaccharides/polysaccharides.

When reducing sugars are treated with dilute alkali solution, they undergo a series of reversible and irreversible reactions to yield several types of degradation products. The reversible reactions include ionization, mutarotation, enolization, and isomerization (Clarke et al., 1997). For example, alkaline degradation of D-glucose is initiated by ionization at the anomeric carbon to generate an anion form. Mutarotation of the anomeric carbon gives D-mannose as a product. Enolization and isomerization of the sugar anion produce enediol anion intermediates and D-fructose (Clarke et al., 1997; Sartori et al., 2003). This interconversion of reducing sugars is known as *Lobry de Bruyn-van Ekenstein rearrangement* (de Bruijn et al., 1987; Nevell, 1985; Clarke et al., 1997; Sartori et al., 2003). A series of the irreversible reactions begins with β-elimination of a hydroxyl group at the β-position of a reducing sugar resulting in the formation of an α-dicarbonyl compound (Clarke et al., 1997; Sartori et al., 2003). This compound is highly reactive under alkaline conditions leading to several degradation reactions to take place including benzilic acid rearrangement, α-dicarbonyl cleavage, aldol condensation, and retro-aldol condensation (Clarke et al., 1997). Degradation products generated from those reactions are various carboxylic acids, e.g., saccharinic acid, isosaccharinic acid, metasaccharinic acid, 2-deoxy-pentonic acid, 3,4-dihydroxy-butyric acid, glycolic acid, lactic acid, and formic acid, etc. (Clarke et al., 1997; Sjöström, 1991; Knill and Kennedy, 2003).

As for a heterogeneous system in the presence of insoluble carbohydrates in alkali solution, degradation is limited by accessibility of the alkali (Lai, 2001). Endwise peeling plays a major role in degradation of oligosaccharides and polysaccharides. Peeling reaction takes place at reducing end groups of oligosaccharides and polysaccharides under alkaline conditions (Lai, 2001; Sartori et al., 2003). For cellulose, as showed in Figure 1.4, reducing end groups undergo enolization to form anion species of enediol intermediates. The enediol intermediates can further involve in different reactions to generate various acid degradation products. β-elimination of the reducing end groups from the

cellulose chain shortens the polymer by one glucose unit (Lai, 2001; Sartori et al., 2003; Peng et al., 2010; Knill and Kennedy, 2003). This is so-called a stepwise peeling reaction or classical peeling reaction (Sartori et al., 2003). Benzilic acid rearrangement of the peeled group generates isosaccharinic acid. If the peeled group undergoes the C3 and C4 linked cleavage followed by benzilic acid rearrangement, the end product is lactic acid (Lai, 2001). The fragmentation of the acids to yield lower molecular weight acids such as glycolic acid and formic acid is also common for the alkaline degradation of cellulose at elevated temperatures (Sjöström, 1991). Alternatively, the enediol intermediates can undergo different reaction pathways (Figure 1.4). The cleavage of the C4 – C5 linkage results in 2-C methylglyceric acid. The β-elimination of the C3 hydroxyl group leads to the formation of metasaccharinic acid (Lai, 2001).

At elevated temperatures (150-180°C), the endwise peeling of the existing reducing ends occurs rapidly as primary peeling process. In addition, the random cleavage of the glycosidic linkages generates new reducing end groups (Lai, 2001; Nevell, 1985) and results in depolymerization of cellulose. These new reducing end groups are further subjected to endwise peeling repeatedly. This alkaline scission causes considerable degradation compared to the primary peeling process (Lai, 2001; Knill and Kennedy, 2003) due to the fact that cellulose has high DP (Lai, 2001) up to 16000 (Lui and Sun, 2010). The weight loss due to the alkaline hydrolysis of the glycosic linkages and the peeling is much higher at higher temperatures than at lower temperatures (Knill and Kennedy, 2003). Alkaline treatment of amorphous hydrocellulose at 60-80°C with 1 M NaOH leads to alkaline scission of the glycosidic bonds as happened at elevated temperatures (Gentile et al., 1987). However, the degradation of cellulose does not continue until all cellulose degraded completely (Nevell, 1985). Stopping reaction occurs when the end groups of the polymer chain are stabilizing acids instead of reducing end groups. Saccharinic acids and other stabilizing acids interrupt the peeling process (Sartori et al., 2003; Knill and Kennedy, 2003).

Alkaline degradation of xylan proceeds by endwise peeling of the reducing end groups as well as the degradation reaction of cellulose (Lai, 2001). However, xylan is much more susceptible to alkali than cellulose. Degradation products of xylan include xyloisosaccharinic acid, 2-hydroxybutanoic acid, 3-deoxypentonic acid (Sjöström, 1991). The degradation of xylan is terminated by the formation of xylometasaccharinic acid that acts as stabilizing end group of the xylan chain (Lai, 2001).

The predominant hemicellulose in grasses is glucuronoarabinoxylan, GAX (Puls and Schuseil, 1993; Vogel, 2008). GAX consists of β-1,4-glycosidic linkages of xylose as backbone with substitution branches of arabinose, glucuronic acid, and acetyl groups on the xylan chain (Figure 1.2). The glucuronic moiety is substituted by a methyl group at C4 position. The peeling reaction is impeded when it reaches the xylan terminal that is substituted by 4-O-methyl-glucuronic acid at its C2 position (Lai, 2001). However, effect is less pronounced at elevated temperatures.

The presence of oligosaccharides is known to the alkaline treatment of polysaccharides with β-1,4-glycosidic linkages (Gupta and Lee, 2010b; Pedersen et al., 2010; McIntosh and Vancov, 2011). Endwise peeling of the polysaccharides and random cleavages of cellulose and hemicellulose result in the formation of soluble oligosaccharides. Reducing ends of oligosaccharides undergo endwise peeling as that of polysaccharides resulting in stepwise shortening by one carbohydrate unit. The reaction continued proceeding along the length of the oligomer until yielding the final product such as glucose in case of cellooligomer degradation (Sartori et al., 2003; Peng et al., 2010). Sartori et al. (2003) investigated kinetics of alkaline degradation of glucose, cellooligomers (DP = 2-5), xylose, xylooligomers (DP = 2-4) in strong NaOH (18%, w/w) at 20-50°C. The rates of degradation of cellooligomers and xylooligomers were higher than that of their monomeric constituents. The degradation rates of increased as the DP increased (cellotriose > cellobiose > glucose). The same trend was followed in

cased of xylooligosaccharides. These findings were corresponded with the study by Peng et al. (2010) who studied alkaline degradation of cellooligomers with degree of polymerization (DP) 3 to 5 in 19% NaOH solution. The reactivity of disaccharides are in order as followed; xylobiose > cellobiose > mannobiose (Lai, 2001). Degradation of cellbiose yields glucose and a non-glucose degradation product by endwise peeling reaction. Glucose, as a byproduct of the degradation, further participates in other reactions under an alkali condition such as *Lobry de Bruyn-van Ekenstein rearrangement*, which is reversible interconversion between glucose, fructose, and mannose. Bonn et al., (1985) found that alkaline degradation of cellobiose in NaOH solutions followed first order reaction. The degradation of 1% cellobiose in 0.01 N NaOH at 66 and 85°C resulted in 40-57% cellobiose loss and yielded 1-2% fructose and 10% glucose after 24 min of incubation. Higher temperature and higher alkalinity caused more degradation. Other degradation products at higher alkalinity (0.1 N NaOH, 70°C) included methylglyoxal, glyceraldehyde, formic acid, acetic acid, lactic acid, glycolic acids, saccharinic acids. In addition, none or trace amount of hydroxymethylfurfural (HMF) and furfural were detected and were accounted for reduced in pH during the alkaline treatment. The other pathway of isomerization of cellbiose is reversible intermediate states of cellobiose-cellobiulose-glucosylmannose (Bonn et al., 1985).

Alkali-resistant ability among monosaccharides and disaccharides depends on type and structure of the sugars. Sucrose as a non-reducing sugar is the most resistant to alkaline degradation compared to reducing sugars (Clarke et al., 1997; Yang and Montgomery, 1996). The alkali degradation by OH^- attack takes place at the reducing end of a sugar molecule. Having no reducing end in sucrose makes it more stable in alkali and the alkaline degradation does not occur by hydrolysis of the glycosidic bond yielding the invert sugars as it proceeds in acid-catalyzed hydrolysis (Clarke et al., 1997). The degradation followed S_NiCB mechanism, which is a slow and rate-determining mechanism.

Oxyanions derived from 1'-OH or 3'-OH of fructose moiety substitutes C-1 of glucose subunit without breaking the glycosidic bond generating 1- or 3-*O*-β-D-glucopyranosyl-D-fructose, which is further degraded rapidly (Clarke et al., 1997; Yang and Montgomery, 1996). However, the cleavage of the glycosidic linkage occurs at elevated temperature. Yang and Montgomery (1996) reported that the breakdown of fructofuranosyl linkage was found at 130-140°C when sucrose was incubated in 0.1 M $Ca(OH)_2$ resulting in the release of fructose and glucose. These reducing sugars are further degraded to acids such as lactic acid, pentonic acids, 2-*C*-methylglyceric acid, 3-deoxy-ribo-hexonic acid, etc. At higher temperatures, 200-250°C, lactic production increased where the yield of 3-deoxy-ribo-hexonic acid was comparable in all temperatures tested.

Studies on Alkali Consumption

In the past, alkali consumption or buffer capacity of biomass has been studied in area of animal feedstuffs, such as straws and grasses (Jasaitis et al., 1987; Giger-Reverdin et al., 2002; Moharrery, 2007), and biogas production (Pavlostathis and Gossett, 1985a; Pavlostathis and Gossett, 1985b; Banks and Humphreys, 1998) since alkaline treatment of those substrates is required in order to improve digestibility. In addition, alkali remaining after the pretreatment, which is associated with the pretreated solid, is useful for anaerobic digestion in biogas production as pH controller (Pavlostathis and Gossett, 1985b). Results from such studies have shown that straw-based feedstocks, such as wheat straw, have buffering capacity in both the acid and base directions (Jasaitis et al., 1987; Giger-Reverdin et al., 2002). This property is important with respect to the pretreatment of biomass, such as in biofuel production, because such pretreatments are typically acid- or alkali-based. More alkali reagent needs to be added into the reaction mixture in order to keep the same alkalinity level throughout the treatment (Pavlostathis and Gossett, 1985a). The amount of alkali load required depends on types of biomass and condition of

the alkali treatment (Pavlostathis and Gossett, 1985a; Zanuttini et al., 1998). Pavlostathis and Gossett (1985a) reported that the maximum alkali consumption of wheat straw in 30 days at room temperature was 0.055 g NaOH/g dry matter. The release of functional groups, e.g., acyl and carboxyl groups, into the liquid phase after the alkali treatment corresponded to the alkali consumption. At slightly higher of temperature (50˚C) in pulping of cottonwood, alkali consumption increased as amount of alkali load increased and was also dependent of treatment time. Deacetylation of the cottonwood was accounted for the major portion of the alkali consumption as acetic acid released when acetyl groups were hydrolyzed (Zanuttini et al., 1998). Likewise, different plants or fraction of plants required different amount of alkali to raise the pH of the biomass suspension to a desired pH (Moharrery, 2007; Giger-Reverdin et al., 2002; Jasaitis et al., 1987). For example, amount of NaOH required to raise pH of these following biomass suspensions: orchard grass, beet pulp, wheat straw, barley straw, and wheat bran, in water to pH 9 at room temperature were 0.25, 0.27, 0.06, 0.10, and 0.24 meq/g biomass, respectively (Jasaitis et al., 1987).

Recently, alkali consumption has become a concern in alkali pretreatment of lignocellulosic biomass for enzymatic conversion to yield fermentable sugars. The decrease in pH of biomass suspension in alkali solution after pretreatment has been investigated. Pedersen et al. (2010) reported pH difference before and after treatment of wheat straw at 100-140˚C, 10 min with NaOH (pH 13.0 to pH 10.6; pH 10.0 to pH 6.4; pH 7.0 to pH 6.3) and $NaCO_3$ (pH 11.3 to pH 10.3). The authors stated that the change in pH might have had impact on carbohydrate released during the pretreatment and following saccharification. In 2011, Pedersen et al. showed that at higher pH of wheat straw treatment between pH 10-13, higher sugar conversion yields obtained from pretreated wheat straw. In lime ($Ca(OH)_2$) pretreatment, the decrease in pH due to alkali consumption was compensated by higher $Ca(OH)_2$ loading than its solubility at a pretreatment

condition. Due to the fact that solubility of $Ca(OH)_2$ in water is poor and the higher temperature, the lower solubility. Loading excess amount of solid lime, allows it gradually dissolve into the solution as readily available hydroxide ions are consumed by biomass. This helps maintain pH at a certain value throughout the pretreatment (Xu and Cheng, 2011). Kim and Holzapple (2005) reported specific lime consumption by corn stover treated under non-oxidative condition (N_2 gas supplied) and oxidative condition (air supplied) at 55°C, 4 weeks, to be 0.058 and 0.073 g $Ca(OH)_2$/g biomass, respectively (equivalent to 1.56 mmol OH^-/g biomass and 1.97 mmol OH^-/g biomass).

In this dissertation, we investigated chemistry of alkali pretreatment on herbaceous biomass using NaOH as alkali reagent due to its complete solubility in water and wheat straw as representative of herbaceous biomass. As stated earlier about advantages of NaOH pretreatment over herbaceous crops and wheat straw is abundant and has low cost. This dissertation encompasses two main topics regarding chemistry of alkali pretreatment and alkali buffer capacity of biomass. In the first study, we comprehensively investigated partitioning of macrocomponents of wheat straw in solid and liquid fractions after NaOH pretreatment at 50°C to gain more understanding in the mechanism of NaOH extraction of carbohydrates, lignin, and ash and the role of NaOH treatment on reactivity of glucan in the pretreated solid. The goal of this study is to elaborate the relationship between reduction of biomass recalcitrance, with respect to its structural feature and molecular arrangement, and improvement in enzymatic deconstruction of glucan in lignocellulosic biomass. In the second study, role of buffer capacity of wheat straw was intensively studied using NaOH pretreatment at a relatively mild condition of pH 11.0, 30°C. The experiments were designed to reveal alkali consumption (buffer capacity) with respect to pretreatment time. The goal of this study is to attain the basis of alkali consumption and buffer component generation in order to estimate amount of alkali required to maintain

a certain alkaline pH at a relatively low temperature and their relationship on enzymatic hydrolysis of pretreated wheat straw.

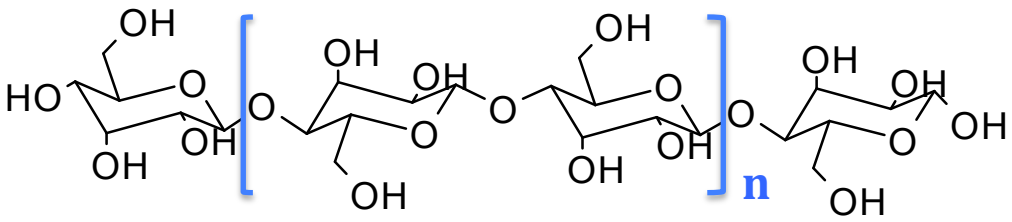

Figure 1.1. Cellulose chain comprises of β-1,4-linked glucose molecules

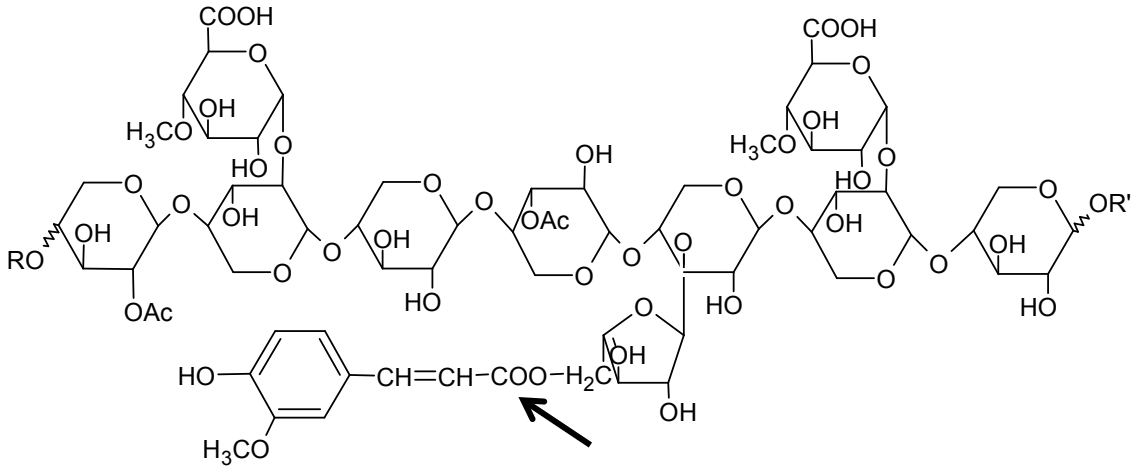

Figure 1.2. Glucuronoarabinoxylan (GAX) (Adapted from Puls and Schuseil, 1993). Ac = acetyl groups. Arrow indicates esterified ferulic acid to arabinose side chain of GAX. Note that *p*-coumaric acid also esterifies to arabinose side chain as well as ferulic acid (picture not showed, see Appendix C for chemical structure of *p*-coumaric acid).

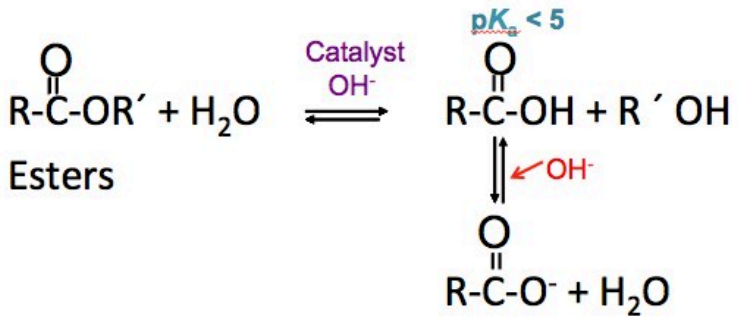

Figure 1.3. Chemical reaction of ester hydrolysis using hydroxide ions (OH^-) as a catalyst to generate carboxyl-containing organic acids as a proton donating groups that consumed hydroxide ions derived from NaOH causing the decrease in pH.

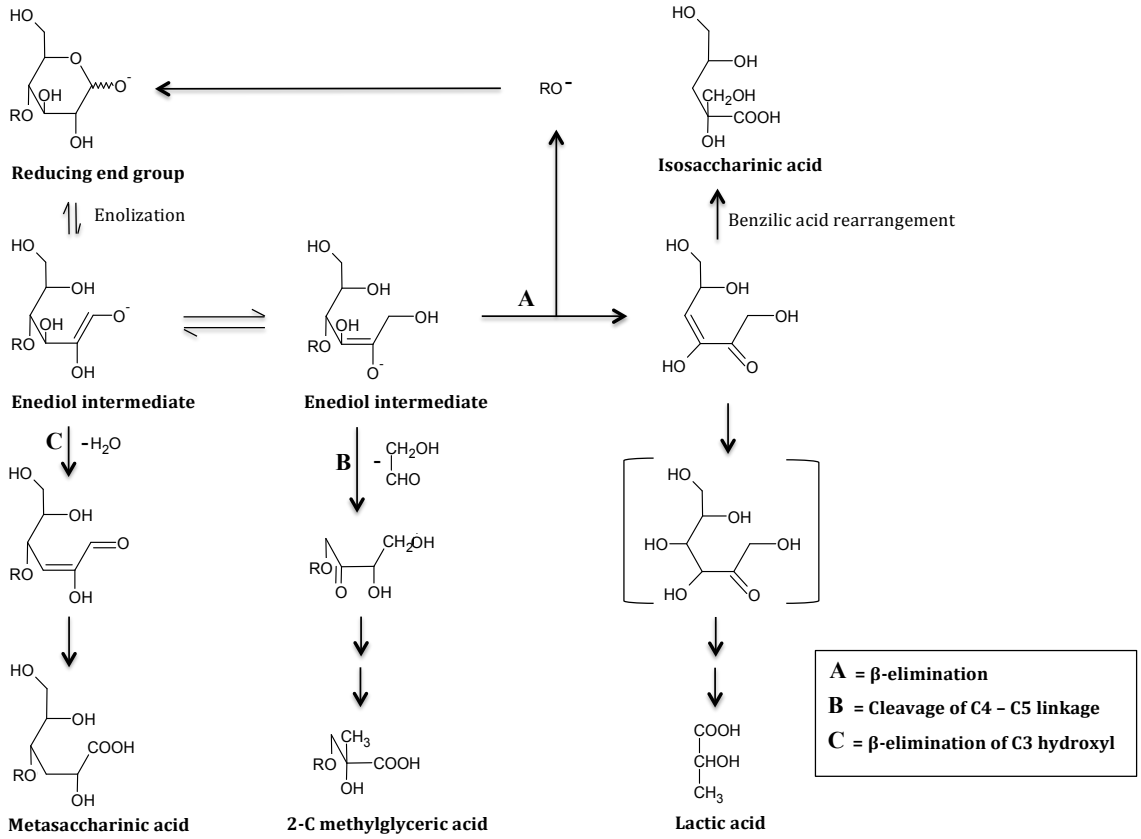

Figure 1.4. Alkaline degradation of cellulose by endwise peeling process (Adapted from Lai, 2001).

Table 1.1 Summary of alkaline pretreatment and saccharification of lignocellulosic biomass from literatures.

Source of alkali	Alkali loading, and/or additives	Feedstock	Solid loading	Pretreatment condition (time, temp., pH)	Enzyme loading, and/or additives	Time to max. yield	Max. cellulose conversion yield	Ref.
NaOH	0-15% (w/w) straw basis; Control: untreated straw	Wheat straw	12.5% (w/v) (liquid to solid ratio = 8:1 (w/w)	*Hot treatment*: 120°C, 30 min; *Cold treatment*: 20°C and 36°C, 4 h *Post pretreatment process*: Washed with water, air dried	Crude cellulase preparation from *Fusarium oxysporum*, F3; no enzyme loading info available	5 h	*Hot treatment*: 2% NaOH = 25%; 4% NaOH = 39%; 6% NaOH = 57%; 8% NaOH = 69%; 10% NaOH = 75% 15% NaOH = 100% *Cold treatment*: 15% NaOH, 36°C = 98%	Koullas et al. (1993)
NaOH Na_2CO_3	NaOH: initial pH = 13 (OH^- = 0.1 M, 0.4% NaOH, [OH-] = 2 mmol/g biomass); final pH = 10.6 Na_2CO_3: initial pH = 13, final pH = 10.3 Two step PT: HCl+NaOH H_2SO_4+NaOH	Wheat straw	5%	Autoclave at 140°C, 10 min *Post pretreatment process*: filtered while still hot, cooled down to 5°C, no washing indicated	1 g PT-solid loading; 100 time dilution of PT-liquid loading. Celluclast 1.5L = 0.6 FPU/g biomass, Novozyme 188 = 0.7 CBU/g biomass	24 h	*One step PT*: NaOH → 55% cellulose conversion obtained from PT-solid; 20% conversion of PT-liquid *Two step PT*: HCl+NaOH → 41% cellulose conversion obtained from PT-solid; 28% conversion obtained from PT-liquid	Pedersen et al. (2010)
NaOH	0.5, 1, 2% (w/v)	Sorghum straw (rotary mill grinding to pass 1.5 mm screen)	10% (w/v)	60°C, 120°C (autoclave; 30, 60, 90 min *Post pretreatment process*: filtered and washed to neutral; stored at -20°C	Solid loading = 5% (w/v)	72 h		McIntosh and Vancov (2010)

Table 1.1. Summary of alkaline pretreatment and saccharification of lignocellulosic biomass from literatures (Continued).

Source of alkali	Alkali loading, and/or additives	Feedstock	Solid loading	Pretreatment condition (time, temp., pH)	Enzyme loading, and/or additives	Time to max. yield	Max. cellulose conversion yield	Ref.
NaOH	*Type of PT:* 0.1 g NaOH/ g biomass; NaOH+Radio frequency-based dielectric (RF) heating;	Switchgrass (particle size < 2mm using a knife mill)	16.6-50%	Presoaked at room temp. 2 h followed by 90°C, 60 min; alkali-soak only without heating *Post pretreatment process*: filtered after cooled at room temperature, washed	Celluclast 1.5L = 12 FPU/g biomass (15-20 FPU/g glucan), Novozyme 188 = 21 U/g biomass	72 h	Opt. 20% solid loading; OH^- = 2.5 mmol/g biomass NaOH heating: 41% conversion NaOH+RF: 53% conversion NaOH w/o heating: 32% 72% conversion	Hu et al. (2008)
	NaOH loading: 0.05-0.3 g NaOH/g biomass w/ RF heating (*Opt. 0.25 g NaOH/ g biomass* → OH^- =6.25 mmol/g biomass	*Particle size:* <0.25-2.0 mm *(Opt. 0.25 mm)*	20%	As above and with heating	As above	72 h	68%	
NaOH	2% conc.→ OH^- = 4 mmol/g biomass	Corn stover (particle size <2 mm using a hammer mill)	12.5%	120°C, 30 min *Post pretreatment process*: Washed to neutral, dried at 50°C	Dry cellulase koji produced by *T. reesei* and dry cellobiase koji produced by *A. niger*; Loading 2 FPU:1 CBU; 8% solid loading (20 FPU/g substrate)	48 h	63%	Chen et al. (2009)
NaOH	0.75-2% (w/v)	Wheat straw (< 1.5 mm)	10%	60°C, 121°C; 30, 60, 90 min *Post pretreatment process*: Filtered and washed to neutral	Cocktail of cellulases and xylanase (NS50013, NS50010, NS50030); 5% solid loading	72 h	Pretreatment at 2% NaOH,121°C, 30 min → reducing sugar = 850 mg/g biomass	McIntosh and Vancov (2011)

Table 1.1. Summary of alkaline pretreatment and saccharification of lignocellulosic biomass from literatures (Continued).

Source of alkali	Alkali loading, and/or additives	Feedstock	Solid loading	Pretreatment condition (time, temp., pH)	Enzyme loading, solid loading, and/or additives	Time to max. yield	Max. cellulose conversion yield	Ref.
NaOH	1%	Straws: Perennial rye grass, tall fescue, bent grass	10%	180°C; 15 min *Post pretreatment process*: Filtered and washed to neutral	Accellerase 1500: 60 FPU/g glucan	120 h	Glucose production ~80%	Kumar and Murthy (2011)
NaOH	0.05-0.3 g/g biomass	Switchgrass (particle size 425-850 µm)	10%	Presoaked at 20°C, 2h *Microwave-assisted*: 70-190°C, 30-120 sec *Conventional heating*: 190°C, 5 min *Post pretreatment process*: Washed to neutral and freeze dried	Celluclast 1.5L = 12 FPU/g biomass (15-20 FPU/g glucan), Novozyme 188 = 21 U/g biomass 1% solid loading	120 h	- Xylose and glucose yields from microwave-assisted = 58.5% (53% higher than conventional heating) - Xylose and glucose yields from microwave-assisted followed by hydrolysis = 90% - Yield from 190°C, 5% solid load, 30 min treatment = 99%	Hu and Wen (2008)
NaOH	0.5-3% (w/v)	Coastal Bermuda grass (< 2 mm)	10%	Autoclave at 121°C; 15-90 min *Post pretreatment process*: Filtered and washed to neutral	Cellulase (NS50013): 40 FPU/g biomass; β-glucosidase (NS50010): 70 CBU/g biomass	72 h	Reducing sugars → 1% NaOH, 30 min = 77%	Wang et al. (2011

Table 1.1. Summary of alkaline pretreatment and saccharification of lignocellulosic biomass from literatures (Continued).

Source of alkali	Alkali loading, and/or additives	Feedstock	Solid loading	Pretreatment condition (time, temp., pH)	Enzyme loading, solid loading, and/or additives	Time to max. yield	Max. Cellulose conversion yield	Ref.
NaOH	2% (w/v)	Wheat straw, triticale straw, barley straw, triticale hay, barley hay, millet hay, barley hay, sweet sorghum hay (<1 mm)	10%	Autoclave at 121°C; 60 min *Post pretreatment process*: Filtered and washed to neutral	Different combinations of Cellulase, β-glucosidase, xylanase: Celluclast 1.5 L (40, 60 FPU/g glucan), Novozyme 188 (1.1.75 v/v, Spezyme CP, Multifect (1.1.75 v/v)	72 h	Wheat straw = 79-85%; other straws = 50-80%; other hays = 67-100%	Chen et al. (2007)
NaOH	1% and 5% NaOH conc. 5% NaOH + 5% H_2O_2	Switchgrass (fractions retaining within 10-20 mesh)	Solid/liquid: 1/10 (10%)	60°C, 85°C; 24 h *Post pretreatment process*: Filtered to separate liquid and solid; Liquid → compositional analysis, lignin separation and characterization; Solid →compositional analysis, enzymatic hydrolysis	Speczyme CP = 15 FPU/g/g glucan, Novozyme 188 = 30 CBU/g glucan	72 h	1% NaOH - 60°C = 42.5% 5% NaOH - 85°C = 70.0% 5% NaOH + 5% H_2O_2 - 85°C = 92.4%	Gupta and Lee (2010a)
NaOH	1%, 5%, 10% (w/w)	Corn stover (3 mm particle size)	10%	Autoclave at 120°C; 1 h *Post pretreatment process*: Filtered and washed to neutral	Celluclast 1.5L and Novozyme 188: 25 FPU/g biomass 5% solid loading	72 h	Reducing sugar yields: 1% NaOH = 65.6%; 5% NaOH = 72.8%; 10% NaOH = 79.4%	Varga et al. (2002)
	0.5% (w/w)	As above	10%	100°C; autoclave at 120°C; 30, 60, and 90 min *Post pretreatment process*: Filtered and washed to neutral	As above	48 h	90 min treatment: 100°C → Reducing sugar yields = 71.6% 120°C → Reducing sugar yields = 83.3%	

Table 1.1. Summary of alkaline pretreatment and saccharification of lignocellulosic biomass from literatures (Continued).

Source of alkali	Alkali loading, and/or additives	Feedstock	Solid loading	Pretreatment condition (time, temp., pH)	Enzyme loading, and/or additives	Time to max. yield	Max. Cellulose conversion yield	Ref.
NaOH	pH 10, 11.5, and 13	Wheat straw (particle size = 700-1000 µm)	2% (w/w)	100, 120, and 140°C; 5 and 10 min *Post pretreatment process*: Filtered to separate solid and liquid	Cellic C-Tec: 10-200 mg protein/g dry matter	24 h	Pretreatment at pH 13, 140°C, 10 min → Glucose yields = 65-70% Xylose yield = 100% (Yields from hydrolysis of pretreated liquid and solid fractions)	Pedersen et al. (2011)
NaOH	2% (w/v)	Wheat straw (average particle size = 40 mesh)	10%	120°C; 60 min *Post pretreatment process*: Filtered and washed to neutral	Cellulase from *Trichoderma reesei* (20 FPU/g cellulose), Cellobiase – Novozyme 188 (40 CBU/g cellulose); 2% solid loading	48 h	Reducing sugar yield after 6 h of hydrolysis = 90%; yield after 48 h of hydrolysis = 100%	Qi et al. (2011)
NaOH	1%	Wheat straw (1-2 cm particle size, washed and air dried)	12.5%	*Conventional heating*: boiling; 15 min to 2 h *Microwave-assisted heating*: 300, 500, and 700 W; 15 min to 2 h *Post pretreatment process*: Filtered and washed to neutral, dried at 65°C and cut to 10-20 mesh particle size for enzymatic hydrolysis	Cellulase from *Trichoderma reesei* (10.6 FPU/g biomass, Cellobiase (3.8 CBU/g biomass); 5% solid loading	72 h	*Conventional heating (60 min)* – reducing sugar yield after 72 h hydrolysis = 40.2 g/L; Microwave-assisted heating (700 W, 25 min) – yield after 72 h = 42.9 g/L (with higher conversion rate)	Zhu et al. (2006)

Table 1.1. Summary of alkaline pretreatment and saccharification of lignocellulosic biomass from literatures (Continued).

Source of alkali	Alkali loading, and/or additives	Feedstock	Solid loading	Pretreatment condition (time, temp., pH)	Enzyme loading, and/or additives	Time to max. yield	Max. Cellulose conversion yield	Ref.
NaOH	0.5%, 1%, and 2% (w/v)	Switchgrass (cultivar Performer, dried at 50°C, 72 h and milled to pass through 2 mm screen)	10%	Autoclave at 121°C, 0.25-1 h; water bath at 50°C, 1-48 h; room temperature at 21°C, 1-96 h *Post pretreatment process*: Filtered and washed to neutral	Novozyme preparation: Cellulase from *Trichoderma reesei* (35 FPU/g biomass, Cellobiase from *Aspergillus niger* (61.5 CBU/g biomass); ~3% solid loading	72 h	Pretreatment at 21°C, 96 h, 2% NaOH – glucose yield = 74.0% Pretreatment at 50°C, 12 h, 2% NaOH – glucose yield = 74.4% - xylose yield = 62.8% Pretreatment at 121°C, 0.5 h – glucose yield = 78.5%	Xu et al. (2010a)
NaOH + CaO	NaOH = 0.25%, 0.5%, 1% (w/v) CaO = 0, 0.05, 0.1 g/g raw biomass	Corn stover (AgVenture Variety R9534VBW, air dried and milled to pass through 1 mm screen)	10%	Room temperature at 21°C, 3, 6, and 9 h *Post pretreatment process*: Filtered and washed to neutral	Cellic CTec (cellulases) = 40 FPU/g biomass, Cellic HTec (endoxylanase) = 75 FXU/g biomass ~3% solid loading	72 h	Pretreatment 6 h with 0.05 g NaOH/g biomass and 0.05 g CaO/g biomass – Glucan conversion yield = 82.4% Xylan conversion yield = 62.9%	Zhang et al. (2011)

Table 1.1. Summary of alkaline pretreatment and saccharification of lignocellulosic biomass from literatures (Continued).

Source of alkali	Alkali loading, and/or additives	Feedstock	Solid loading	Pretreatment condition (time, temp., pH)	Enzyme loading, and/or additives	Time to max. yield	Max. Cellulose conversion yield	Ref.
NaOH	1% (w/v)	Swithcgrass (dried at 50°C, 72 h); Coastal Bermuda grass (air dried), milled to pass through 2 mm screen)	10%	50°C, 12 h *Post pretreatment process*: Filtered and washed to neutral	(1) Cellulase (NS50013) = 0-40 FPU/g biomass + β-glucosidase (NS50010) = 0-50 CBU/g biomass; (2) Cellic CTec (cellulases) = 0-40 FPU/g biomass + Cellic HTec (endoxylanase) = 0-75 FXU/g biomass	72 h	Switchgrass → At optimal enzyme combination (1) – reducing sugar yield = 431.4 mg/g biomass; enzyme combination (2) = 399.1 mg/g biomass Coastal Bermuda grass → At optimum for enzyme combination (1) – reducing sugar yield = 396.0 mg/g biomass; enzyme combination (2) = 379.5 mg/g biomass	Xu et al. (2011)
NaOH; **NaOH + $Ca(OH)_2$**	NaOH = 0.05, 0.10, 0.20 g/g biomass $Ca(OH)_2$ = 0-0.10 g/g biomass	Switchgrass (cultivar Performer, dried at 50°C, 72 h and milled to pass through 2 mm screen)	5%	Ambient temperature, 3, 6, and 9 h; Varying time of NaOH addition *Post pretreatment process*: Filtered and washed with 100, 200 mL water/g biomass	Novozyme preparation: Cellulase from *Trichoderma reesei* (35 FPU/g biomass, Cellobiase from *Aspergillus niger* (61.5 CBU/g biomass); ~3% solid loading	72 h	Optimal pretreatment condition → 0.1 g NaOH/g biomass: glucan conversion = 48.1%, xylan conversion = 38.9% → 0.2 NaOH/g biomass: glucan conversion = 69.5%, xylan conversion = 54.8% → 0.1 NaOH/g biomass + 0.02 g $Ca(OH)_2$/g biomass: glucan conversion = 59.4%, xylan conversion = 57.3%	Xu and Cheng (2011)

Table 1.1. Summary of alkaline pretreatment and saccharification of lignocellulosic biomass from literatures (Continued).

Source of alkali	Alkali loading, and/or additives	Feedstock	Solid loading	Pretreatment condition (time, temp., pH)	Enzyme loading, solid loading, and/or additives	Time to max. yield	Max. Cellulose conversion yield	Ref.
NaOH	1%, 5% NaOH conc. 1.5% NaOH + 5% H_2O_2 5% NaOH + 5% H_2O_2	Corn stover (fractions retaining within 9-35 mesh)	Solid/liquid: 1/10 (10%)	25°C; 60°C, 80°C, 120°C; 24 h *Post pretreatment process*: Filtered to separate liquid and solid; Liquid → compositional analysis, lignin separation and characterization; Solid →compositional analysis, enzymatic hydrolysis	Speczyme CP = 15 FPU/g/g glucan, Novozyme 188 = 30 CBU/g glucan	72 h	Corn stover: 1% NaOH - 25°C = 65.3% 1% NaOH - 60°C = 82.0% 1.5% NaOH - 60°C = 93.8% 5% NaOH - 60°C = 99.8% 5% NaOH + 5% H_2O_2 - 85°C = 92.4% 1% NaOH + 5% H_2O_2 - 25°C = 49.1%	Gupta and Lee (2010b)
NH_3+H_2O_2 NH_3	15% NH_3, 15% NH_3 + 5% H_2O_2	Switchgrass	Solid/liquid: 1/10 (10%)	60°C, 80°C, 120°C; 24 h; two step temperature: 0-4 h, 4-24	Speczyme CP = 15 FPU/g/g glucan, Novozyme 188 = 30 CBU/g glucan	72 h	36.9-96.4%	Gupta and Lee (2010a)

Table 1.1. Summary of alkaline pretreatment and saccharification of lignocellulosic biomass from literatures (Continued).

Source of alkali	Alkali loading, and/or additives	Feedstock	Solid loading	Pretreatment condition (time, temp., pH)	Enzyme loading, solid loading, and/or additives	Time to max. yield	Max. cellulose conversion yield	Ref.
$Ca(OH)_2$	0.4 g $Ca(OH)_2$/g biomass → OH^- = 10.8 mmol/g biomass	Corn stover (particle size <2 mm using a hammer mill)	10%	120°C, 4 h (non-oxidative condition)	Dry cellulase koji produced by *T. reesei* and dry cellobiase koji produced by *A. niger*; Loading 2 FPU:1 CBU; 8% solid loading (20 FPU/g substrate)	48 h	45% cellulose conversion	Chen et al. (2009)
$Ca(OH)_2$	0.1 g/g biomass	Switchgrass (dried at 50°C, 72 h); Coastal Bermuda grass (air dried), milled to pass through 2 mm screen)	10%	100°C, 15 min *Post pretreatment process*: Filtered and washed to neutral	(1) Cellulase (NS50013) = 0-40 FPU/g biomass + β-glucosidase (NS50010) = 0-50 CBU/g biomass; (2) Cellic CTec (cellulases) = 0-40 FPU/g biomass + Cellic HTec (endoxylanase) = 0-75 FXU/g biomass	72 h	*Switchgrass* → At optimal enzyme combination (1) – reducing sugar yield = 433.0 mg/g biomass; enzyme combination (2) = 412.3 mg/g biomass *Coastal Bermuda grass* → At optimum for enzyme combination (1) – reducing sugar yield = 426.4 mg/g biomass; enzyme combination (2) = 429.7 mg/g biomass	Xu et al. (2010b)
$Ca(OH)_2$	0.02-0.20 g/g biomass	Coastal Bermuda grass (air dried, pass through 2 mm sieve)	10%	21°C (34 h), 50°C (6 h) , 80°C (3 h), 100°C (15 min), 121°C (15 min) *Post pretreatment process*: Filtered and washed with 200 mL d.i. water/0.1 g lime used	Cellulase (NS50013) = 40 FPU/g biomass; β-glucosidase (NS50010) = 70 CBU/g biomass	72 h	Lime loading = 0.1 g/g biomass; 100°C (15 min) → glucan conversion = 87.4%; xylan conversion = 67.5%	Wang and Cheng (2011)

Table 1.1. Summary of alkaline pretreatment and saccharification of lignocellulosic biomass from literatures (Continued).

Source of alkali	Alkali loading, and/or additives	Feedstock	Solid loading	Pretreatment condition (time, temp., pH)	Enzyme loading, solid loading, and/or additives	Time to max. yield	Max. cellulose conversion yield	Ref.
NH_3+H_2O_2 NH_3	15% NH_3, 15% NH_3 + 5% H_2O_2	Switchgrass	Solid/liquid: 1/10 (10%)	60°C, 80°C, 120°C; 24 h; two step temperature: 0-4 h, 4-24	Speczyme CP = 15 FPU/g/g glucan, Novozyme 188 = 30 CBU/g glucan	72 h	36.9-96.4%	Gupta and Lee (2010a)

2 SODIUM HYDROXIDE-INDUCED PARTITIONING OF WHEAT STRAW COMPONENTS AND CORRESPONDING EXTENT OF CELLULOSE SACCHARIFICATION

Supaporn Sophonputtanaphoca, Tiraporn Junyusen, and Michael H. Penner

SODIUM HYDROXIDE-INDUCED PARTITIONING OF WHEAT STRAW COMPONENTS AND CORRESPONDING EXTENT OF CELLULOSE SACCHARIFICATION

2.1 ABSTRACT

Comprehensive study in components partitioning in sodium hydroxide (NaOH) pretreated wheat straw was investigated over a range of NaOH concentrations (0-10%, w/v), all at 50°C, 5-h treatment period, and 3% solid loading. Solid and liquid phases resulting from NaOH treatments were evaluated. Total solids recovered in the NaOH-treated solid phase ranged from 47.4-88.0%. Overall carbohydrate recovery in the combined solid and liquid phases was negatively correlated with the alkali concentration of the treatment liquor. The glucan content of the NaOH-treated solid phase ranged from 37.2-67.4%. Glucan recovery in the solid phase was relatively high in all cases, the minimum value being ~98%. Increasing amounts of xylan partitioned into the liquid phase as sodium hydroxide concentrations increased – it ranged from 31-83% of the xylan being recovered in the soluble phase. Carbohydrate analyses of the pretreated liquor revealed that the majority of carbohydrate loss from the solid fraction could be recovered in the liquid phase in form of oligomers and monomers due to alkaline degradation. The interconversion of glucose, fructose, and mannose under the alkaline condition played an important role in the presence of those sugars. Increase in NaOH concentration contributed to increase in amount of cellulose-derived and hemicellulose-derived oligomers in the pretreated liquor. All oligomers except fructooligomers in NaOH pretreated liquor were higher than those found in water extraction at 50°C, 5 h. Total carbohydrate recovery from the solid and liquid fractions was as high as 99% for glucose and glucan in 5% NaOH treatment and 80-95% for xylose and xylan in 1-10% NaOH treatment. The presence of NaOH as extraction reagent dramatically induced lignin and ash removal from the pretreated solid with up to 63% acid insoluble lignin (AIL) and

87% ash extraction. Solid fractions resulting from NaOH pretreatments (up to 5% NaOH) were tested for their susceptibility to enzymatic saccharification using cellulase and cellulase/xylanase enzyme preparations. The cellulase/xylanase enzyme preparation was found to be more effective at cellulose saccharification than the cellulase enzyme preparation alone. Maximum glucose yield, which corresponded to the 5% NaOH treatment, was 82% over the standard 48 h saccharification period. Extended saccharifications times to 120 h showed that the conversion yield approached 90%. Sequential treatments of the straw (*i.e.* initial alkali treatment – first enzyme saccharification – second alkali treatment - second enzyme treatment) revealed the NaOH treatment has the potential to render essentially all (~99%) of the straw glucan susceptible to enzyme saccharification. This suggests that the layered molecular arrangement of cellulose, hemicellulose, and lignin in the cell wall impacts biomass recalcitrance and glucan conversion yield.

2.2 INTRODUCTION

Among alternative renewable energy resources, lignocellulosic biomass represents promising and competitive to starch and sugar crops due to the abundant and sustainability toward bioconversion into liquid transportation fuels such as bioethanol on a large-scale production (Alvira et al., 2010; Zhen and Zhang, 2009; Yang and Wyman, 2008; Jørgensen et al., 2007). However, conversion of cellulose from lignocellulosic biomass to glucose, which is subsequently fermented to fuels or products, is impeded by the complexity (physicochemical, structural, molecular arrangement, and compositional factors) of the cell wall of biomass itself (Kumar et al., 2009). To overcome the biomass recalcitrance, lignocellulosic biomass must be subjected to pretreatment process(es) to reduce cellulose crystallinity and to deconstruct lignin and hemicelluloses barriers that limit the enzyme accessibility to cellulose (Mosier et al., 2005; Yang and Wyman, 2008). Moreover, lignin also causes non-productive adsorption to cellulolytic enzymes resulting in less availability of active enzymes and low glucose production (Qi et al., 2011; Berlin et al., 2005; Xu et al., 2008).

Sodium hydroxide (NaOH) is a powerful reagent with potential for application in lignocellulosic biomass processing. Several positive effects include swelling of biomass, increase in internal surface area, decrease in crystallinity and the degree of polymerization, disruption of lignin-carbohydrate linkages, alteration of lignin structure, and removal of lignin (Sun and Cheng, 2002). The degree of hemicellulose removal differs among types of biomass as well as pretreatment conditions (Qi et al., 2011). Since cellulose has lower reactivity to alkaline reagent, the majority of cellulose is preserved in the pretreated solid (Gupta and Lee, 2010a & b; Chen et al. 2009). Such characteristics make potential carbohydrates in pretreated substrates more accessible to be rendered by cellulolytic and xylanolytic enzymes for production of fermentable monosaccharides. Despite the effectiveness in pretreatment of lignocellulosic

biomass, NaOH was considered as an expensive chemical and difficult to recover reagent compared to other alkaline reagent such as calcium hydroxide (lime) (Chang et al., 1997). However, the use of NaOH in biomass-to-ethanol processes and pulping industry has recently become interesting since processes for NaOH recovery and reuse now look promising (Gupta and Lee, 2010a). It has been reported that efficiency of NaOH treatment was more pronounce in lignocellulosic materials with low lignin content such as hardwood and herbaceous biomass (e.g., grasses, straws) (Sun and Cheng, 2002; Qi et al., 2011).

Recent studies in NaOH pretreatment for enzymatic hydrolysis to produce sugars showed that several kinds of herbaceous biomass has been investigated, e.g., wheat straw (Pedersen et al., 2010; Pedersen et al., 2011; McIntosh and Vancov, 2011; Qi et al., 2011; Zhu et al., 2006), corn stover (Gupta and Lee, 2010a; Chen et al., 2009; Zhang et al., 2011), switchgrass (Gupta and Lee, 2010b; Xu et al., 2010; Xu et al., 2011; Xu and Cheng, 2011; Hu and Wen, 2008; Hu et al., 2008), and other straws or grasses (Cheng et al., 2010; Wang et al., 2010; Kumar and Murthy, 2011; Xu et al., 2011). However, there has been little information on intensive study of macrocomponent partitioning in pretreated biomass and pretreated liquor relative to effect of NaOH extraction of macrocomponents and alkaline degradation of carbohydrates relative to enzymatic degradation of cellulose. In this study, wheat straw was chosen as substrate for pretreatment and enzymatic hydrolysis due to its abundant and low cost. Wheat straw can be grown under a wide range environment throughout the world as it is the world's most widely cultivated crop (Talebnia et al., 2010). North America has been ranked as the third largest wheat production after Asia and Europe (Kim and Dale, 2004). The estimation of total wheat straw supply in the US by the Energy Information Administration (EIA) in 2006 was approximately 2.9 million dry tons/year (secondary after corn stover production) with the cost of biomass at $30-40/dry ton (Haq and Easterly, 2006). During the past five years, the use of wheat straw in pretreatment processes and enzymatic conversion as a potential

feedstock for 2nd generation bioethanol has been gaining interest not only in the US (Kristensen et al., 2008; Chen et al., 2007; Saha and cotta, 2007; Saha and cotta, 2006) but also in other parts of the world, especially in Europe (Fu et al., 2010; Pedersen et al., 2011; Maache-Rezzoug et al., 2011; Pedersen et al., 2010; Kaparaju et al., 2009; Kootstra et al., 2009; Kabel et al., 2007; Qi et al., 2011; Qi et al., 2010; Zhu et al., 2006; Sun and Chen, 2008; McIntosh and Vancov, 2011; Guragain et al., 2011). From those studies, the glucan digestibility ranged from 50-95% depending on pretreatment methods and enzymatic hydrolysis conditions.

In this work, we comprehensively investigated partitioning of macrocomponents of wheat straw in solid and liquid fractions after NaOH pretreatment at 50°C to gain more understanding in the mechanism of NaOH extraction of carbohydrates, lignin, and ash and the role of NaOH treatment on enzymatic hydrolysis of glucan. Temperature at 50°C was chosen as it is the same condition for the standard method of enzymatic saccharification provided by the National Renewable Energy Laboratory (NREL) that the substrate for enzymatic hydrolysis must be brought to 50°C prior to digestion. Effect of xylanase supplementation to cellulolytic enzymes and extended saccharification were investigated. We first established three-step sequential pretreatment (alkali-enzyme-alkali pretreatment) followed by enzymatic saccharification to clarify common phenomenon of incomplete hydrolysis of glucan in NaOH pretreated biomass (regardless pretreatment severity and hydrolysis condition). So far there has been no clear explanation of why enzymatic hydrolysis is failed to unlock glucan in the remaining residue for further sugar production to obtain a 100% theoretical yield. These experiments were designed to help elaborate the relationship between reduction of biomass recalcitrance, with respect to its structural feature and molecular arrangement, and improvement in enzymatic deconstruction of glucan in lignocellulosic biomass

2.3 MATERIALS AND METHODS

Feedstock

Mature above-ground biomass of winter wheat straw was harvested Hyslop Research Farm of Oregon State University, Corvallis, Oregon. The vegetative parts of straw (stems and leaves) were collected and air-dried before milling to pass a 2 mm sieve with a knife mill (Retsch). The milled straw was kept in a glass jar with a screw cap lid at room temperature and used for all experiments.

Reagents and Enzymes

All chemicals used for pretreatment, saccharification, and analytical procedures were reagent grades purchased from Sigma-Aldrich (Sigma Chemical Co., USA), Mallinckrodt (Mallinckrodt Baker, Inc., NJ, and EMD (USA). Sugar standards (Sigma) were HPLC grade. Glucose oxidase/peroxidase kit (GOPOD-FORMAT, K-GLUC) was purchased from Megazyme (Ireland). Commercial cellulase preparation (Accellerase 1500) and xylanase preparation (Accellerase XY) were gifts from Genencor (USA). Filter paper activities of both enzyme preparations were measured according to NREL method (Adney and Baker, 1996). Filter paper of Accellerase 1500 was found to be 57 FPU/mL. β-glucosidase activity of Accellerase 1500 was measured against *p*-nitrophenyl-β-D-glucopyranoside as described by Wood (1988). The β-glucosidase activity was found to be 699 *p*NPGU/mL. Accellerase XY did not have filter paper activity (< 0.37 FPU/mL). Xylanase activity of both enzyme preparations was determined toward birchwood xylan as described by Bailey et al. (1992). Accellerase XY contained 27,478 Units/mL of xylanase activity while Accellerase 1500 had low xylanase activity (701 Units/mL). Invertase activity of Accellerase 1500 was tested by incubating the enzyme with 0.5 mg/mL sucrose solution in 50 mM Na-

citrate buffer, pH 4.8 at 50°C for 24 h. It showed that this enzyme preparation does not have invertase activity.

Invertase (I4504, EC 3.2.1.26, Sigma) was used to confirm the presence of sucrose in pretreated liquor. Activity and dosage of invertase preparation were used as provided by Sigma. Cellulase activity of the invertase preparation was tested by incubating the enzyme with 0.25 mg/mL cellobiose solution in 50 mM Na-citrate buffer, pH 4.8 at 50°C for 24 h.

Compositional Analyses of Wheat Straw

All analytical methods for determination of wheat straw composition followed the laboratory analytical procedures (LAP) prepared by National Renewable Energy Laboratory (NREL). Moisture content and total solid was determined in triplicate followed NREL protocol (Sluiter et al., 2008a) by drying approximately 1 g (weighed to the nearest 0.1 mg) of the straw at 105°C to obtain a constant weight (5 h). The total solid content from the moisture determination was referred as oven dry weight. After cooling the sample in a desiccator to room temperature, the sample in an aluminum container was weighed to the nearest 0.1 mg. Determination of structural carbohydrates and lignin was conducted with extractives free wheat straw to avoid interference of extractives on true values of structural carbohydrates and lignin. Extractives-free wheat straw was prepared by extraction of the original wheat straw in Soxhlet apparati with water followed by 95% ethanol for 24 h of each solvent extraction (Sluiter et al., 2005a). Air-dried extractives-free solid was subjected to compositional analysis by two-step hydrolysis (Sluiter et al., 2008b). Neutral sugars of hydrolyzate were measured by HPLC method. Glucose was also measured by glucose oxidase/peroxidase as described below in order to comparable with glucan conversion by saccharification in subsequent process. Water and ethanol extractives in the liquid phase were dried and quantitatively determined by gravimetric method. Sugars (monomers and cellooligomers) in the liquid phase were qualitatively and

quantitatively determined as described below in "Carbohydrate Analyses of Water-Soluble Extractives." Determination of ash was done by ashing native wheat straw in a furnace at 575°C for 24 h (Sluiter et al., 2005b). All experiments were done in triplicate.

Screening Pretreatment Conditions

Wheat straw was treated with NaOH solutions with 3% solid loading for all experiments. NaOH concentrations (w/v) used for the screening pretreatment conditions ranged from 0.5% to 5%. To initiate the pretreatment, equivalent of 0.3 g oven dry weight of wheat straw was mixed with 10 mL of 50°C NaOH solution (being warmed in a water bath at 50°C) in a 20-mL screw-cap top scintillation vial. For the control treatment, 50°C double distilled water was added to the wheat straw instead of a NaOH solution. Then the reaction mixture was kept in the water bath at 50°C for 0.5, 1, 2, and 5 h with 15 min intervals of manual agitation.

After the predetermined time elapsed, the slurry was immediately transferred into a glass gooch crucible (Pyrex, 10-15 μm pore size). The pretreated solid and liquor were quickly separated using vacuum filtration. The pretreated solid was then washed with 50 mL of hot double distilled water followed by dilute acid wash with 10 mL of 0.01 mM HCl (ambient temperature solution) and then washed again with 50 mL of approximately 50°C double distilled water. Washing with hot water was applied to prevent lignin coalescence on the surface of the pretreated solid upon temperature decreasing. The pH of the final washed water was about 6-7 as measured by a pH paper.

Total Solids in Pretreated Biomass

The washed solids in the gooch crucible were subjected to drying in a vacuum oven at 40°C for 24 h. After cooling down to room temperature in a desiccators, dried pretreated solid was then weighed to the nearest 0.1 mg for the

determination of total solid recovery and removal by comparison with the starting weight of the biomass before the pretreatment (0.3 g).

Sodium Hydroxide Pretreatment

Pretreatment for subsequential analyses and saccharification were done with 0, 1, 5, and 10% (w/v) NaOH for 5 h at 50°C. Pretreatment procedure was carried out as described in "Screening Pretreatment Conditions." Flow chart in Figure 2.1 showed steps of the experimental procedures.

Washed pretreated solid was prepared in three batches. One was for determination of total solid recovery (see "Total Solids in Pretreated Biomass") and determination of ash. The other one was for compositional analysis in pretreated solid. After determination of total solids, dried pretreated residue was subjected to ash determination by ashing in a furnace at 575°C for 24 h. The third batch of the washed pretreated solid was for saccharification. Washed solid was subjected to saccharification without further drying.

As shown in Figure 2.1, pretreated liquor was collected after filtering to separate solid and liquid phase of the slurry. Known volume of the liquor was neutralized by 2 N HCl to approximately pH 7 determined by a pH meter. Amount of acid used was recorded for dilution factor and ionic strength of the neutralized liquor. The neutralized liquor was stored in a refrigerator for further carbohydrate analyses.

Carbohydrate Analyses of Pretreated Liquor

The carbohydrate analyses were divided into four parts: monosaccharides; cellooligomers and sucrose; hemicellulose-derived oligomers; fructooligomers (Figure 2.2). Monosaccharides (glucose, xylose, galactose, arabinose, and mannose) were determined directly by HPLC method without further treatment.

Cellooligomers and Sucrose

Cellulase treatment was conducted for quantification of cellooligomers and sucrose. Cellooligomers were determined from differences of glucose before and after the enzyme treatment of the pretreated liquor. Since cellulase has specific hydrolysis properties for β-1,4-linkage of polymeric glucose, glucose liberated from this treatment was particularly obtained from cellooligomers not sucrose. Three mL of neutralized pretreated liquor was mixed with 3 mL of 0.1 M Na-citrate buffer, pH 4.8, 60 μL of 2% sodium azide, and excess cellulase, 100 uL. The reaction mixture was incubated in a rotary incubator at 50°C for 24 h. One mL aliquot was collected and subjected to glucose measurement by HPLC. Amount of additional glucose generated after the enzyme treatment was converted to dehydro form (polymeric form) by applying a correction factor, 0.9, and reported as cellooligomers (DP ≥ 2). After cellulase treatment, sucrose was determined as the remaining peak previously co-eluted with cellobiose on the HPLC chromatogram. To confirm the presence of sucrose, 1 mL aliquot of cellulase treated, pretreated liquor was incubated with 0.1 mL of 60 Units/mL invertase at 50°C for 24 h. The disappearance of the peak of interest on the HPLC chromatogram and the production of glucose equivalents were determined by HPLC method.

Hemicellulose-Derived Oligomers

Dilute acid treatment (4% H_2SO_4) of the pretreated liquor was carried out for quantification of oligomers derived from hemicelluloses followed the method ofet al. (2006). Ten mL of the cool neutralized pretreated liquor was transferred to a 100-mL autoclavable, Teflon-lined, screw-capped bottle. Then the liquor was adjusted to yield final concentration of H_2SO_4 equal to 4% (w/w) by adding 348 μL of 72% (w/w) H_2SO_4 while constantly swirling the sample during the acid addition to minimize sugar degradation upon coming in contact with the strong acid. Appropriate concentrations of mixed sugar recovery standards (glucose, fructose, xylose, galactose, arabinose, and mannose) were prepared in the same manner of the mixture of pretreated liquor and acid. The concentration of each

sugar standard was as close as that found in the sample. All samples were autoclaved at 121°C for 1 h (the 1-h time period started once the autoclave had reached 121°C). Following the hydrolysis treatment, the sample bottles were transferred to an ice bath for rapid cooling. Once the samples were cooled to room temperature, they were transferred to 50-mL flasks and $CaCO_3$ solid was added to raise the pH to about 6 (determined by pH paper). The neutralized samples were then set aside for 1 h at room temperature to allow $CaSO_4$ precipitation to develop and settle at the bottom of the flasks. Supernatant of each sample was collected for sugar determination by HPLC. All experiments for carbohydrate analyses were done in duplicate.

Fructooligomers

Determination of fructose equivalents derived from fructooligomers in pretreated liquor was done as described by Nguyen et al. (2008). Ten mL of neutralized pretreated liquor was diluted to 25 mL in a volumetric flask with appropriate amount of 19% (w/w) H_2SO_4 solution to yield 1% (w/w) H_2SO_4. Molar concentration of acid for dilution was standardized by 0.1 N NaOH to the phenolphthalein end point. The mixture was transfer to a 100-mL autoclavable, Teflon-lined, screw-capped bottle and was incubated in a water bath at 100°C for 1 h. The residence time started once the bottle was placed in the water bath. Once the incubation was over, the bottle was put in an ice bath for rapid cooling to room temperature. The hydrolyzate was neutralized with $CaCO_3$ solid as described above. Fructose was determined by HPLC. Fructose equivalents as fructooligomers was determined from additional fructose generated after the acid treatment and converted to polymeric form by applying correction factor, 0.9. The experiment was done in duplicate.

Carbohydrate Analyses of Water-Soluble Extractives

Aliquot of water–soluble extractives of native wheat straw obtained from Soxhlet method was divided into two portions for carbohydrate analyses. One

portion was for monosaccharides and sucrose analysis by HPLC. The other portion was for cellooligosaccharide analysis by cellulase treatment as described in "Carbohydrate Analyses of Pretreated Liquor." All experiments were done in duplicate.

Alkaline Degradation of Sugars

Sugar solutions (1 mg/mL of glucose, xylose, cellobiose, and sucrose) in distilled water were prepared separately. Prior to initiate the reaction, 3 mL of each sugar solution in a 20-mL screw cap-top scintillation vial was brought up to 50°C in a water bath. Zero time of the reaction started by adding 3 mL of 2% NaOH (w/v) to the sugar mixture to yield the final sugar concentration of 0.05% (w/v) in 1% NaOH solution. After mixing well, the reaction mixture was allowed to incubate in the water bath at 50°C up until different predetermined times over the period of 5 h with manually mixing every 15 min. To quench the reaction, a sample vial was removed from the water bath and neutralized with 2 M HCl. Note that the acid was added while the vial was constantly swirling to minimize contact between the sugars and the strong acid. Then the sample vial was put in an ice bath immediately. All sample vials were kept cool in the ice bath and subjected to neutral sugar analysis by the HPLC method as described below. Sugar recovery was calculated based on the amount of the individual sugar at interval times compared to that of at the zero time. The experiment was done in duplicate.

High-Performance Liquid Chromatography (HPLC) Analyses

Prior to HPLC analyses, all samples were filtered through 0.2-μm Acrodisc© syringe filters (Pall, USA) into autosampler vials. The analyses were carried out on a Shimadzu HPLC system (Model: Prominence UFLC) equipped with Aminex HPX-87P column (300×7.8 mm, Bio-Rad, USA), deashing guard column (Bio-Rad, USA), and an evaporative light scattering detector (ELSD;

Model: ELSD-LTII). Chromatographic separation was achieved with isocratic elution at column temperature of 85°C, 20 μL injection volume, Milli-Q grade water mobile phase with flow rate of 0.6 mL/min, and running time of 35 min. Data analysis of the chromatograms were done with LCsolution Software (Shimadzu).

Enzymatic Saccharification

The saccharification procedure was slightly adapted from NREL protocol (Selig et al., 2008). Washed pretreated solid was transferred into a pre-weighed and tarred 20-mL screw-cap top scintillation vial. The substrate loading was varied depending upon the total solid recovery due to NaOH concentration used during the pretreatment (e.g. 1.5% solid loading in enzymatic saccharification for 5% NaOH pretreated sample). Five mL of 0.1 M sodium citrate buffer, pH 4.8, 0.1 mL of 2% sodium azide (antimicrobial reagent), was added to the pretreated solid. Water was then added to the reaction mixture to obtain the total weight of 10 g by assuming all substrate and reagents occupied volume 1 mL per 1 g. The weight of the reaction mixture was then recorded for calculation of total volume. The suspension was kept in a refrigerator until saccharification the following day. The mixture was then equilibrated to 50°C using a water bath. To initiate the reaction, cellulase and/or xylanase preparations (30 FPU/g glucan of Accellerase 1500, 2,750 Units/g glucan of Accellerase XY) were added into the mixture and mixed well. The vial of the reaction mixture was then placed in a rotary incubator and incubated at 50°C. One mL aliquot was collected at predetermined time and immediately filtered through a 0.45 μm PTFE syringe filter. The supernatant was kept frozen (-20°C) until analyzed. Predetermined times for this study were 0, 1, 2, 4, 8, 12, 18, 24, and 48 h. Glucose production was quantified by glucose oxidase/peroxidase assay.

For zero hour of saccharification, denatured enzymes were required to avoid rapid hydrolysis occurred upon the addition of enzymes. Appropriate

amount of enzymes was added into 5 mL of 0.1 N Na-citrate buffer, pH 4.8 in a 20-mL screw-cap top scintillation vial and boiled for 10 min. After cooling down to room temperature, the denatured enzyme solution was added to the pretreated solid, water, and antimicrobial reagent mixture to the total weight of approximately 10 g. The "zero hour" mixture was treated along with other reaction mixtures by equilibrated to 50°C prior to saccharification. Sample collection and analysis were done as other samples collected at predetermined times. All experiments were done in duplicate.

Extended Enzymatic Saccharification

The extended saccharification experiment (up to 120 h) was carried out with 5% NaOH pretreated wheat straw with xylanase supplementation to cellulase (same enzyme loadings as above). The detail of procedure was followed "Enzymatic Saccharification." The experiment was done in duplicate.

Three-Step Sequential Pretreatment (Alkali-Enzyme-Alkali Pretreatment)

The 120-hour-enzymatic-hydrolyzed sample from the extended saccharification experiment was used in this study. All samples were centrifuged at 12,00 rpm, 10 min, in a refrigerated centrifuge to separate solid and liquid. The supernatant was decanted. The solid was then washed with double distilled water four times by centrifuging before subjected to the second NaOH pretreatment. The washed residue was re-suspended with 3 mL of double distilled water and 3 mL of 10% NaOH to yield the final concentration of 5% NaOH (w/v). The sample was incubated at 50°C for 5 h in a water bath followed by washing with double distilled water/dilute HCl/distilled water (as described in "Screening Pretreatment Conditions") until the final decanted liquid was at neutral pH (determined by pH paper). The treated residue was then re-suspended in double distilled water. For each treatment, the suspension was divided into two groups: one for compositional analyses, one for enzymatic

hydrolysis. For the compositional analysis, 3 mL of suspension was added into a 20-mL scintillation vial and was dried at 40°C in a vacuum oven until a constant weight obtained. Then the dried residue was subjected to compositional analyses as described earlier in "Compositional Analyses of Wheat Straw."

For enzymatic saccharification, 3 mL of suspension, 3 mL of 0.1 M Na-citrate buffer, pH 4.8, 60 μL 2% Na-azide was mixed in a 20-mL scintillation vial and kept in a refrigerator until saccharification. Enzyme blank was prepared with the same manner as that of the sample except using 3 mL double distilled water instead of the residue suspension. The hydrolysis protocol was followed as described earlier in "Enzymatic Saccharification" except enzyme loadings. Excessive cellulase and xylanase were used (50 μL Accellerase 1500 and 95 μL Accellerase XY). All samples were incubated at 50°C in a rotator incubator for another 48 h. The supernatant was collected by filtering through a syringe filter and kept frozen (-20°C) until analyzed for glucose by glucose oxidase/peroxidase assay. The experiment was done in duplicate.

Glucose Oxidase/Peroxidase Assay

Reagent, buffer, and enzymes were prepared according to Megazyme protocol without modification. A slight modification was applied to the analysis method as followed. Frozen samples derived from saccharification experiments were thawed to allow them to reach room temperature. After thoroughly mixing, 33 μL of appropriate diluted sample was transfer into a test tube and 1.00 mL glucose oxidase/peroxidase reagent was added to start the reaction. Reagent blank was done using double distilled water to replace glucose or sample. Glucose standard solutions were prepared by diluting 1.0 mg/mL glucose in water to 0.1-0.8 mg/mL. All assays, reagent blank, and standards were incubated at 40°C for 20 min. Color development was measured by a spectrophotometer (UV160U, Shimadzu) at 510 nm against water as reference. Standard curve

generated from the standard solutions was used to calculate amount of glucose in the assays. The glucose assay was done duplicate.

Glucose produced during enzymatic saccharification was calculated for glucan conversion after subtraction glucose in zero hour mixture because enzyme preparations contained trace amount of glucose. Glucose was then converted to anhydrosugar as a polymeric form of glucan by multiplying absolute amount of glucose with a covertion factor of 0.9. The calculation of glucan conversion was as followed:

$$\%\ Glucan\ conversion = \frac{g\ of\ glucan\ digested}{g\ of\ glucan\ added}\ x100$$

2.4 RESULTS AND DISCUSSION

Composition of Wheat Straw

Composition of structural carbohydrates, lignin, ash, extractives, and sugars including carbohydrate oligomers in water extractives of the native wheat straw are summarized in Table 2.1a and Table 2.1b. Wheat straw that used as substrate for the compositional analyses has been undergone exhaustive extraction by sequential water and 95% ethanol for 24 h each step prior to the analyses. The reasons for extraction prior to the compositional assay are explained below. The values in the table are expressed in term of mass percent of the components relative to mass of original wheat straw. Major polysaccharides of wheat straw were glucan (33.7%) and xylan (21.1%). Most of lignin in wheat straw was acid insoluble lignin (AIL, 13.8%). The majority of extractives were in water extract (14.5%) compared to 3.04% in ethanol extract. That suggests that most of extractives in wheat straw are hydrophilic compounds. Inorganic materials are represented as ash, which was accounted for 6.57% (total ash) of the native wheat straw, where 70% of inorganic matters were water extractable and 30% was non-extractable by both water and ethanol and remained in the solid fraction. Dry matters of non-structural carbohydrates ranged up to 0.82% of the original biomass (Table 2.1b), which attributed to 5.66% of water extractives. The three predominant sugars were fructose, glucose, and sucrose. Others were present in trace amount while no mannose was found. Water extractives also contained oligomers. Approximately 25% of water extractives were neutral carbohydrates. This was comparable to 25-32% of total water-soluble carbohydrates in switchgrass (Chen et al., 2010) but was considerably low compared to 30-57% of those in corn stover (Chen et al., 2007).

Screening Pretreatment Conditions

Residence pretreatment times and NaOH concentrations were optimized based on the maximum total solid removal under the alkaline condition at 50°C

since the majority of the cell wall compositions removed is hemicelluloses, lignin, and ash whereas most of glucan is preserved in the solid fraction. The preliminary study for NaOH pretreatment of wheat straw was carried out by treating wheat straw with 0-5% NaOH concentration at 50°C with 3% solid loading for 0.5-5 h. Temperature used in this study was selected since it was the same temperature for saccharification in the subsequent process. The pretreated solid in the reaction mixture must be brought to 50°C prior to start the saccharification by adding enzymes. In addition, a mild condition in term of low temperature was suitable for monitoring changes in partitioning of macrocomponents due to the alkaline treatment. A more severe condition such elevated temperatures may obscure the changes in term of rate and extent of generation or degradation of macrocomponents, for example, degradation of sugars so that it was difficult to see if there were sugars present in the pretreated liquor before they degraded.

Total solid recovery was used as a factor for selecting pretreatment residence time and NaOH concentration based on the fact that the majority of solid removal by NaOH was hemicelluloses and lignin while glucan was preserved in the solid fraction (Wang et al., 2010; Gupta and Lee, 2010b). Figure 2.3 shows a 3D plot of solid recovery versus pretreatment time and NaOH concentrations. Increase in residence time and alkalinity favored the removal of total solids from wheat straw. This finding corresponded with others (McIntosh and Vancov, 2011; Chen et al., 2007). Distilled water was used as a control and was able to remove 12% solid after 5 h. By comparison, extraction of water-soluble extractives by Soxhlet method for 24 h was able to remove 14.5% total solids (Table 2.1b). This indicates that 83% of water-soluble compounds in wheat straw were extractable within 5 h at temperature as low as 50°C and 17% of water-extractable compounds required higher temperature and longer time to be extracted.

The presence of NaOH enhanced the extractability up to 48% when 5% NaOH was used to extract for 5 h. The residence time of 5 hours and 0%, 1%, 5 %, and 10% NaOH concentrations were then chosen for further study in partitioning of macrocomponents and reactivity of glucan to enzymatic saccharification.

Sodium Hydroxide Pretreatment

The residence time of 5 hours and 5 % NaOH concentration were selected for this study. For comparison, 1% and 10% NaOH were carried out along with a control treatment (0% NaOH) by using distilled water for pretreatment. Doubling in NaOH concentration from 5% to 10% was used to compare the extractability of hemicelluoses and lignin. Total solid recovery in all pretreatment conditions shows in Figure 2.4. Total solid recovery ranged from 47.4% to 88.0%. Increase in concentrations of NaOH promoted total solid loss from the residue. The lowest total solid recovery was found in 10% NaOH treatment while the highest total solid recovery was found in 0% NaOH treatment.

Using 1% NaOH increased 23% solid removal of wheat straw compared to water extraction at the same condition. However, the increase in solid removal gradually level off when higher concentrations of NaOH were used (14% and 4% increase for 5% NaOH and 10% NaOH treatments, respectively). That suggests the limitation in total solid extraction with NaOH under the time and temperature in this study. The total solid removals of wheat straw in this study were comparable with other reports with respect to alkalinity levels (McIntosh and Vancov, 2011; Chen et al., 2007).

Effect of water and NaOH treatment on chemical composition of wheat straw is summarized in Table 2.2 and percent recovery of macrocomponents partitioning in the solid fraction shows in Figure 2.5 for structural glycans. Treatment of wheat straw with water (0% NaOH) at 50°C, 5 h did not significantly affect amount of glucan compared to that of untreated biomass. However, there were variations in all measured glycans (Figure 2.5) compared to

true values determined from extractives free biomass according to the standard protocol provided by NREL. The sources of the bias were partial extractives that still associated to the water-pretreated solid (Sluiter et al., 2008b). Extractable carbohydrates in feedstock can obscure the accuracy measurement of structural carbohydrates especially cellulose content since cellulose is non-soluble in extracting solvents while glucan derived from non-structural carbohydrates is (Thammasouk et al., 1997). However, we did not see the increase in values of measured glycan except galactan, i.e., probably because most of water-soluble glycans were removed during the pretreatment leaving mostly hydrophobic compounds (e.g., chlorophyll, waxes, etc.) intact to the residue. These compounds impede penetration of sulfuric acid during the hydrolysis of biomass for structural determination making it difficult to release some polymeric carbohydrates (Sluiter et al., 2005a).

Effect of pretreatment on chemical composition was more pronounced with the addition of NaOH as extracting reagent. As concentrations of NaOH in the treatment increased, distribution of glucan in pretreated solid increased up to 67% whereas hemicellulose-derived glycans decreased substantially (Table 2.2). The increase in glucan in pretreated solid as NaOH concentrations increased has been reported in NaOH treatment of wheat straw and other herbaceous crops (Qi et al., 2011; Chen et al., 2007; Chen et al., 2009). As showed in Figure 2.5, high recovery of glucan (95-98%) was achieved when concentration of NaOH up to 10% was used. Structural glucan (or cellulose) is more resistant to alkali due to its low reactivity to alkali and high crystallinity of cellulose (Gupta and Lee, 2010b). As a result, the majority of glucan was preserved in the solid fraction. Slightly loss of glucan from the solid fraction after NaOH treatment seems unavoidable as agreed with other findings (Gupta and Lee, 2010a, b; Pedersen et al., 2010). Gupta and Lee (2010a) pointed out that small amount of extracted glucan from corn stover was probably derived from hemicellulose-containing glucans - minor components in grasses (Vogel, 2008). Hemicellulose, however,

has greater reactivity to NaOH than that of glucan based on the fact that hemicellulose has branched and amorphous structure which allows more penetration of the reagent for the reaction (Gupta and Lee, 2010a). Consequently, solubilization of hemicellulose occurs as a common effect of NaOH pretreatment and the degrees of hemicellulose removal vary depending on severity of pretreatment and types of biomass (Gupta and Lee, 2010a; Pedersen et al., 2010; McIntosh and Vancov, 2011). The highest carbohydrate removal was found in xylan, the major hemicellulose in grasses, 31%, 69%, and 83% of xylan were removed when treated wheat straw with 1%, 5%, and 10% NaOH, respectively. Note that the most difference in xylan removal (38%) was detected when the concentration of NaOH increased from 1% to 5% and only 14% more removal was achieved when doubling concentration of NaOH from 5% to 10%. The same trend was found in partitioning of arabinan in the pretreated solid. This may be due to the fact that glucuronoarabinoxylans (GAXs), which consist of xylan backbone and substituted arabinose and glucuronic acid, are the predominant hemicellulose in grasses (Harris and Stone, 2008).

Lignin and ash recovery in the solid fractions of NaOH treated wheat straw are illustrated in Figure 6. Both AIL and ASL recovery in 0% NaOH (water) treated wheat straw exceeded 100%. Inaccuracy in determination of lignin can be caused by partial extractives partitioned in water-extracted wheat straw. Besides interference in structural carbohydrate determination, extractives also play a major role in accuracy of lignin determination. The increase in amount of "measured" lignin in water-treated wheat straw with partial endogenous extractives was consistent with the findings of Thammasouk et al. (1997) when switchgrass, tall fescue, and corn stover were used as substrates. Outer layer of wheat straw is lined with hydrophobic components such as waxes and silica resulting in low wettability (Jiang et al., 2009). Consequently, hydrophobic compounds in extractives impede penetration of sulfuric acid during the hydrolysis of biomass to release polymeric carbohydrates during the

compositional analyses (Sluiter, 2005a). Furthermore, aromatic-containing polymers in extractives, such as waxes (Peng et al., 2010; Bargel et al., 2006) and cutin (Xu, 2010), might act as lignin-like compounds. Condensation of incomplete hydrolyzed carbohydrates (Sluiter et al., 2005a) and possibly those hydrophobic materials with AIL remained in the solid fraction resulting in false AIL value when measured by gravimetric method. The determination of ASL was done by means of UV-vis spectroscopy. Hydrolyzates obtained from the two-step hydrolysis were measured absorbance at 320 nm. Overestimation in ASL measurement of water-treated wheat straw was probably caused by the interference of absorbance measurement from the lignin-liked compounds in the extractives that become soluble under the acidic condition of the analyses. However, we did not attempt to determine those lignin-liked compounds or their sources that responsible for the false AIL and ASL in this study.

From Figure 2.6, lignin recovery (both AIL and ASL) in 1% NaOH treated solid dramatically dropped from 100% recovery in the original biomass when 54% and 67% of AIL and ASL, respectively, were partitioned in pretreated solid. As NaOH concentrations increased to 5% and 10%, slightly additional lignin was removed from the solid. Considering the use of 5% and 10% NaOH, only a few more percentages of both AIL and ASL were extracted even though the concentration of NaOH was double. Obviously, stronger alkalinity was not enough to remove more than 65% of AIL at the same temperature and residence pretreatment time. McIntosh and Vancov (2011) reported that temperature was main factor in delignification of wheat straw by NaOH treatment. Thus, those 65% delignification by NaOH was likely from saponification of ester-linked compounds as ester linkages are more susceptible to alkali compared to ether linkages. The pretreatment condition in this study may not sufficient to extract ether-linked components in wheat straw that require a harsher condition to extract. Sun et al. (2002) used 1 M NaOH, 25°C, 18 h to extract esterified lignin and 4 M NaOH, 170°C, 2 h to extract etherified lignin in wheat straw.

As for extraction of ash, water extraction at 50°C, 5 h was able to remove inorganic materials or ash up to about 40% (Figure 2.6). Using NaOH contributed to higher ash removal (78-87%). The predominant mineral in grasses is silicon, which accounted for 65-70% of total minerals (Xu, 2010) and NaOH can solubilize silica (Jiang et al., 2009) and depolymerize cutin (Xu, 2010).

Carbohydrate Analyses of Pretreated Liquor

Monomers and oligomers in pretreated liquor are summarized in Table 2.3. All monomers in neutralized pretreated liquor (no neutralization required for 0% NaOH or water extract) were measured directly by HPLC method. Glucose oxidase/peroxidase couple-reaction was not appropriate for glucose measurement in pretreated liquor due to high salt concentration generated during the neutralization of pretreated liquor and of acid-treated pretreated liquor in oligomer quantification. The high ionic strength caused precipitation of the enzymes in some cases resulting in underestimate glucose content. Both glucose oxidase (Voet et al., 1981) and horseradish peroxidase (Bauduin et al., 2006) are sensitive to ionic strength due to the change in electrostatic interactions. Furthermore, brown color of the liquid sample interfered the colorimetric assay. Thus, free glucose and glucose derived from hydrolysis of cellooligomers were determined by HPLC method. Quantification of cellooligomers was done hydrolysis of pretreated liquor with excessive cellulase because of the enzyme-substrate specificity. Sucrose was determined after cellulase treatment of pretreated liquor as well due to the co-elution of sucrose with cellobiose under the HPLC method used in this study. The cellulase preparation, Accellerase 1500, had no invertase activity when incubated with standard sucrose solution. The presence of sucrose in all extracts was confirmed by hydrolysis with an invertase preparation (see Materials and Methods for details). Fructooligomers were quantified by relatively mild acid hydrolysis while

the standard method at more severe condition was use to determine other oligomers (see Figure 2.2).

Almost all sugars and oligomers were detected in water extract (0% NaOH) obtained from the treatment at 50°C, 5 h. Predominant water-extractable carbohydrates were glucose, fructose, and fructooligomers (0.87%, 1.19%, and 0.15%, respectively). Amount of these three carbohydrates were greater (43% for glucose, 45% for fructose, and 67% for fructooligomers) compared to those found in water extractives obtained from fractionation of wheat straw with a Soxhlet apparatus for 24 h (see Table 2.1b). Water extractives was boiling constantly for a prolonged time under the reflux condition of extraction with Soxhlet apparatus. However, fructose is susceptible to degradation by heat. Our previous work showed that 10% of standard fructose solution in water was lost after incubation at 100°C for 6 h (Nguyen et al., 2009). The caution of fructose degradation was applicable to fractionation water extractives from herbaceous biomass using Soxhlet apparatus. Nevertheless, other non-structural carbohydrates were extracted more with the use of Soxhlet apparatus for extended periods.

Interestingly, sucrose as a water-extractable marker was not found in the water extract of wheat straw obtained from the condition at 50°C, 5 h (Table 2.3) but was detected in water extractives obtained from the Soxhlet fractionation (see Table 2.1b). However, fructans, which are found in grasses and contain sucrose as a constituent in the fructosyl-linkages (Ritsema and Smeekensy, 2003), were found in both water extracts. In addition, sucrose was found in all extracts obtained from NaOH treatment (Table 2.3). This raised a question if sucrose in wheat straw is present as free sucrose or conjugate to other constituents in cell wall matrix and will liberate as free sucrose after fractionation with water for an extended period of time or after extraction with a reagent such as NaOH at a lower temperature and shorter time. Sucrose is much more resistant to alkaline degradation in comparison to the invert sugar. The

alkali degradation by OH^- attack takes place at the reducing end of a sugar molecule. Having no reducing end in sucrose makes it more stable in alkali and the alkaline degradation does not occur by hydrolysis of the glycosidic bond yielding the invert sugars as it proceeds in acid-catalyzed hydrolysis (Clarke et al., 1997). We tested the susceptible of sucrose solution in 1% NaOH at 50°C, 5 h and found that approximately 100% of sucrose was recovered (data not showed). We have tried to find source of sucrose by treating water extract obtained from 50°C, 5 h condition with 1% NaOH at the same extraction condition but still did not find sucrose. However, sucrose was detected at 0.14 and 0.12 mass percent when using 1% NaOH to treat water-extracted residue (solid fraction after extraction with water at 50°C, 5 h) and extractives-free residue (from exhaustive extraction in Soxhlet apparatus with water and subsequently 95% ethanol for 24 h of each extraction), respectively. The presence of sucrose even in extractives-free wheat straw prepared under the standard condition for determination of structural carbohydrates might suggest that partial sucrose in wheat straw exists in non-water or non-ethanol extractable form and probably associate with other cell wall components. However, we did not investigate the specific source of sucrose.

From Table 2.3, low amount of glucose was present in 1% NaOH and 5% NaOH pretreated liquor compared to glucose present in water extract at the same pretreatment condition. Trace amount of xylose was detected in 1% NaOH pretreated liquor while galactose, arabinose, and mannose were absence in all NaOH pretreated liquor. Loss of monosaccharides was caused by alkaline degradation. Gupta and Lee (2010b) reported that no monosaccharides were found in pretreatment liquid of switchgrass treated with 5% NaOH at 85°C for 24 h. That was probably a harsher condition (higher temperature, longer pretreatment time) than the conditions used in this study. The presence of monosaccharides in pretreated liquor probably came from a few possible sources: remaining free monosaccharides after alkaline degradation; primary end

products of endwise peeling reaction at reducing ends of disaccharides; final product of stepwise peeling of oligosaccharides (Lai, 1991; Sartori et al., 2003; Knill and Kennedy, 2003; Peng et al., 2010); and interconversion of monosaccharides (reducing sugars) via *Lobry de Bruyn-van Ekenstein rearrangement* as primary action of reducing sugars, e.g., glucose, fructose, mannose, in dilute alkali (de Bruijn et al., 1987; Nevell, 1985). The latter assumption may be applied to the presence of fructose and/or glucose in 1% NaOH pretreated liquor since glucose, fructose, and mannose can reversibly undergo mutarotation and isomerization among themselves before being degraded to acids in subsequent reactions (Clarke et al., 1997). The degradation reaction of monomeric reducing sugars to various degradation products begins with elimination of the hydroxyl group at β-position resulting in the anionic intermediate, which subsequently forms α-dicarbonyl compound, an alkaline-unstable compound, that undergoes to several other degradation reactions (Sartori et al., 2003).

Our preliminary study on degradation of sugar standards in 1% NaOH (0.25 M NaOH), 50°C, 5 h, revealed the evidence of the occurrence of those three sugars, as shown in Figure 2.8, for fructose and mannose formation in the degradation of a glucose standard (Figure 2.8a) and from the interconversion of glucose, where glucose generated from the peeling reaction of a cellobiose standard (Figure 2.8b), respectively. Amount of glucose remaining after the alkali treatment was determined by HPLC method and confirmed by GOPOD assay (data not showed for the GOPOD assay). As shown in Figure 2.8a, approximately 28% of original glucose was lost in the first 15 min of the incubation. At the same time, however, 82% of the lost glucose existed as fructose and mannose, which are still fermentable sugars, resulted from *Lobry de Bruyn-van Ekenstein rearrangement*. This implies that the rates of the interconversion to other sugars were faster than that of the degradation of glucose to other compounds besides neutral sugars. de Bruijin et al. (1987) revealed that the rate

constants of the isomerization of sugars among glucose, fructose, mannose, and psicose, in 0.01 M KOH, 78°C, were higher than those of individual sugars degraded to acid compounds. The formation of fructose and mannose in alkaline degradation of glucose from this study consented to the finding reported by de Bruijin et al. (1987). Likewise, Bonn et al. (1985) has reported the generation of glucose and fructose from alkaline degradation of cellobiose.

Here we reported the equivalent recovery of fructose and mannose together since they were co-eluted, especially at low amount for either of them, when separated by the HPLC method used in this study. The sign of co-elution was that the retention time shifted to a value between that of fructose and mannose with respect to amount of each sugar. Recovery of glucose reduced over the time of incubation where as the recovery of fructose and mannose increased to the maximum (32%) at 30 min to 1 h of incubation. Thereafter, the decrease in fructose + mannose recovery became more significant until only 7.5% recovery was measured after 5 h. To prove that the exist of fructose + mannose at the end of the 5-h incubation was probably from the isomerization of the sugars, the water extract obtained from the extraction wheat straw with distilled water at 50°C, 5 h, was treated in 1% NaOH at 50°C, 5 h. The result showed that the only sugar retained in the treated solution was glucose (5% of the initial amount) where all fructose was degraded. This supports the assumption that the presence of fructose in 1% NaOH pretreated liquor was derived from the interconverison of the reducing sugars and the source of glucose itself probably came from both the water-soluble glucose and the peeling reaction of carbohydrates (discussed below).

Cellooligomers and hemicellulose-derived oligomers were present at larger amount in comparison to their monomer constituents (Table 2.3). Increase in alkali concentrations resulted in more oligomers liberated into the pretreated liquor. This was consistent with other findings in pretreatment of wheat straw with NaOH (McIntosh and Vancov, 2011). The occurrence of these oligomers in

this study were agreeable with those in literatures in case of NaOH treatment of switchgrass (Gupta and Lee, 2010b) and wheat straw (Pedersen et al., 2010; McIntosh and Vancov, 2011). Classical peeling reaction is a stepwise peeling starting at a reducing end of a polymer resulting in a shortening polymer by one carbohydrate moiety (Sartori et al., 2003). In addition to endwise peeling from reducing ends of polymers, random cleavage in hemicellulose and cellulose chains leads to solubilization of the polysaccharides and exist as oligomers in the liquid phase (Lai, 1991; Knill and Kennedy, 2003). Then the oligomers undergo further degradation reactions. Sartori et al. (2003) investigated kinetics of alkaline degradation of glucose, cellooligomers (DP = 2-5), xylose, xylooligomers (DP = 2-4) in strong NaOH (18%, w/w) at 20-50°C. They revealed that degradation rates of cellooligomers and xylooligomers were higher than that of their monomeric constituents and the degradation rate of cellotriose > cellobiose > glucose. The same trend was followed in case of xylooligomers. Alkaline degradation reaction of oligomers occurred via the classical stepwise peeling reaction at the reducing end as mentioned above by shortening one sugar unit from the oligomeric chain. The reaction continued proceeding along the length of the oligomer until yielding the final product such as glucose in case of cellooligomer degradation. As for the reducing ends, benzilic acid rearrangement and oxidation generate saccharinic acid and lactone. The formation of saccharinic acid stops the degradation reaction (Sartori et al., 2003). This corresponded with the study by Peng et al. (2010) who studied alkaline degradation of cellooligomers with degree of polymerization (DP) 3 to 5 in 19% NaOH solution. They found glucose and oligomers with lower DP. In this study, however, we did not attempt to determine DP of oligomers as well as other degradation products besides sugars generated from the alkaline degradation of wheat straw. The content of oligomers present in Table 2.3 was calculated from additional monomeric sugars in anhydrous forms (polymeric forms) obtained after acid hydrolysis of pretreated liquor.

The presence of cellobiose that co-eluted with sucrose on the HPLC chromatogram was likely generated from alkaline degradation of oligomers with higher DP since all cellobiose standard (0.5 mg/mL) was all degraded within 5 h in 1% NaOH solution (0.25 M NaOH) at 50°C (Figure 2.7). The rate of cellobiose degradation appeared higher than those of xylose and glucose although primary degradation products, in case of the cellobiose and glucose degradation, were present in form of the other sugars (Figure 2.8b). The rate of the peeling reaction to yield primary products of glucose and a degradation product was fast; thus, glucose was present in the reaction mixture. Then fructose and mannose formation from glucose via the interconvesion occurred as happened in the degradation of glucose as the initial substrate but differed in the rates and extent of recovery contributed by amount of the substrate. Bonn et al. (1985) showed that the rate constants of cellobiose degradation to yield glucose and that of the interconversion between glucose and fructose, and vice versa, were much higher than the rate constants of those sugars degraded to acids, when cellobiose was treated with 0.1 N NaOH at 60-80°C. The faster conversion and degradation reactions were found at elevated temperatures and higher alkalinity. In our study, 64% of cellobiose was lost inn the first 30 min while 11% and 4% of the recovery of glucose and fructose + mannose (relative to the initial amount of cellobiose), repectively, were detected. The time courses of the cellobiose recovery and the generation of glucose and fructose somewhat agreed to the findings reported by Bonn et al. (1985) despite the difference in the treatment conditions. The similarity of higher rate of cellobiose degradation compared to that of glucose degradation was also reported in the study by Sartori et al. (2003) as mentioned earlier. Peng and co-workers (2010) disclosed the presence of arabinose in NaOH treatment of cellotriose. However, no arabinose was detected in any liquor obtained from the NaOH treatment of wheat straw in our study. Perhaps the content was too low for the sensitivity of the detection or none

existed. Content of total carbohydrates in pretreated liquor ranging from approximately 3% to 19% as illustrated in Table 2.3.

Table 2.4 summarizes total carbohydrate recovery in both solid and liquid fractions after pretreatment. High glucose and glucan recovery (≥ 97%) was achieved even at as high as 10% NaOH treatment. However, the recovery of xylose and xylan, as a major hemicellulose, significantly decreased as concentrations of NaOH increased. Only 85% was recovered while the rest 15% was degraded in 5% NaOH treatment. Since this pretreatment condition was being emphasized in saccharification in subsequent process due to high glucan recovery after the pretreatment and high glucan conversion after enzymatic hydrolysis (discuss later), low xylan and xylose recovery may become a factor to consider in case of xylan conversion. Potential carbohydrates and theoretical ethanol yields of NaOH treated wheat straw from C6 and C5 sugars regardless the sources of carbohydrates either solid or liquid fractions are summarized in Table 2.5. Mass percent of total carbohydrates ranged from 57 to 63% with potential carbohydrates from C6 higher than those from C5 sugars. Total potential carbohydrates trended to reduce as the alkalinity levels increased, especially at 10% NaOH treatment. Theoretical ethanol yields mirrored the content of potential carbohydrates. Approximately, 63 to 66 gallons of ethanol/dry ton of biomass were estimated from C6 sugars, which accounted for 95 to 97% of theoretical ethanol yield of the original straw. Estimated theoretical ethanol yields from C5 sugars were 36 to 44 gallons/dry ton of biomass, which accounted for 83 to 97% of that of the original straw.

Enzymatic Saccharification

Enzymatic saccharification of NaOH pretreated wheat straw was first tested on xylanase supplement with 5% NaOH treated biomass. This pretreatment condition was selected based on high recovery of glucose equivalents (99%) and other carbohydrates while treatment at 10% NaOH

significantly lost more carbohydrate (see Table 2.4 & 2.5). In addition, high removal of lignin and xylan in the solid fraction was achieved as those components act as physical barriers to cellulase accessibility; thus, reduce hydrolysis efficiency (Jeoh et al., 2007; Sun and Cheng, 2002; Mosier et al., 2005). Washed solid was subjected to saccharification without further drying since the accessibility of cellulase to cellulose will decrease due to reduction in surface area and pore volume, thereby causing negative impact on the conversion yield (Jeoh et al., 2007; Boussaid and Saddler, 1999). Enzymatic hydrolysis was carried out with 1.5% substrate loading, 30 FPU/g glucan of cellulase loading (Accellerase 1500), and with and without 2,750 U/g glucan of xylanase supplement (Accellerase XY). The dosage of xylanase was followed the recommendation of Genencor for Accellerase XY. Accellerase 1500 has low activity of xylanase toward birchwood xylan (701 Units/mL) compared to xylanase activity of Accellerase XY (27,478 Units/mL). Conversely, Accellerase XY does not possess filter paper activity (< 0.37 FPU/mL).

Figure 2.9 shows glucan conversion profiles of the saccharification of 5% NaOH treated wheat straw with and without xylanase supplement over the period of 48 h. The glucan degradation profiles of both treatments followed the general enzyme-catalyzed hydrolysis of cellulose with a rapid initial rate followed by a slow rate approaching a constant rate until no substrate available (Ncibi, 2010). Although the positive effect of xylanase was not observed in the first two hours of the hydrolysis, the increase in glucan conversion was significant thereafter. Accellerase 1500 contains sufficient β-glucosidase activity (699 *p*NPGU/mL) for catalyzing hydrolysis of cellobiose to glucose. Adequate β-glucosidase is important to cellulose degradation by cellulase since accumulated cellobiose can become substrate inhibition to cellulase (Chen et al., 2007). The cellulase loading in this experiment was also accounted for 367 *p*NPGU/g glucan of β-glucosidase, which was considered excessive as recommended dose by the NREL's standard protocol is 64 *p*NPGU/g glucan (Selig et al., 2008). In addition to

data provided by Genencor on efficiency of Accellerase 1500, the need of additional β-glucosidase supplement to Accellerase 1500 in catalyzing hydrolysis of lime pretreated wheat straw had been investigated and no significant increase in glucan conversion between with and without additional β-glucosidase was observed (authors, unpublished data). The improvement of glucan conversion in xylanase supplement treatment was greater by 7% compared to that of non-xylanase supplement at 48 h of incubation. The assumption was made that the positive effect on cellulolytic hydrolysis was solely due to the xylanase addition. The maximum glucan conversion yield in the xylanase supplement was 82% versus 75% in the absence of additional xylanase. The increase in the conversion was low but significant. This may be because at the beginning of saccharification, the substrate was full of readily accessible cellulose and amorphous regions of cellulose resulting from the NaOH pretreatment. These characteristics of biomass have high reactivity to cellulase to adsorb and catalyze hydrolysis. Over the time, the amorphous regions depleted, the catalytic hydrolysis in crystalline regions is slower. The crystalline region of cellulose fibers that tightly packed will be slowly dispersed or swelling by the carbohydrate-binding molecule cellulase in order to increase internal surface area for the cellulose fibers to interact with cellulase. This initial stage of cellulolytic degradation is called 'amorphogenesis' (Arantes and Saddler, 2010). Boussaid and Saddler (1999) revealed that at the initial hydrolysis of a heterogenous reaction system, filter paper and endoglucanase activity was strongly partitioned to the insoluble residue of Douglas fir kraft pulp whereas xylanase and β-glucosidase activity was more associated to the soluble fraction. Readily hydrolysable amorphous hemicellulose was possibly explained the action of xylanase in the soluble phase. However, as less amorphous hemicellulose, i.e., more recalcitrance portion retained in the insoluble phase over the extended period of saccharification, the majority of xylanase became associated to the insoluble substrate instead (Boussaid and Saddler, 1999). Xylanase helps remove xylan coating and cross-linkages to cellulose; thus provide

more accessible area of cellulose to cellulase (Kumar and Wyman, 2009a; Moraïs et al., 2010). This suggests that 31% xylan retention in the substrate affected the glucan conversion yield in the absence of additional xylanase although the cellulase preparation itself contains low xylanase activity. Kumar and Wyman (2009a) pointed out that xylan content in the substrate had an indirect role in improvement of glucan conversion when xylanase supplement was applied. Increase in glucose yields had linear relationship to xylose liberated although the extents of glucan conversion were different among the leading pretreatment technologies. Another possible explanation to lower glucan conversion in the absence of xylanase supplementation is that the low xylanase activity in the cellulase preparation may result in incomplete hydrolysis of xylan and/or xylooligomers to xylose. Kumar and Wyman (2009b) revealed that enzymatic hydrolysis of lignocellulosic materials with commercial enzymes containing low activity of xylanase could lead to accumulation of substantial amount of xylooligomers in the reaction mixture. Unfavorably, it has been reported that xylooligomers can act as a strong inhibitor to cellulase causing a reduction of initial hydrolysis rate and maximum glucose production (Kumar and Wyman, 2009a; Kumar and Wyman, 2009b; Qing et al., 2010). The adverse impact of xylooligomers on efficiency of glucan hydrolysis by cellulase was even more than inhibitory effect of cellobiose and glucose to cellulase (Qing et al., 2010). Positive effect of xylanase supplementation on glucan digestibility has been reported in literatures for NaOH pretreated wheat straw (McIntosh and Vancov, 2011) and corn stover (Gupta and Lee, 2010a), as well as other substrates derived from other pretreatment technologies (Kumar and Wyman, 2009a; Kumar and Wyman, 2009c; García-Aparicio et al., 2007). Hence, cellulase supplemented with xylanase at the same dosage was applied to all further saccharification of NaOH treated wheat straw.

For comparison, effect of all NaOH pretreatment conditions on glucan reactivity of pretreated wheat straw was illustrated in Figure 2.10. Time courses

of saccharification over 48 h with addition of the constant dosages of cellulase and xylanase were applied to all substrates. Rates and extents of NaOH pretreated substrates outstandingly surpassed those of water-treated and untreated substrates. Profiles, rates, and extents of glucan digestibility of water-treated (0% NaOH) and untreated wheat straw were not significantly different. Only 19% conversion yields were attained, i.e., at least 48% lower that those obtained from the NaOH pretreated substrates. In NaOH pretreated substrates, rates and extents of glucan conversion were proportional to the alkalinity of the pretreatment condition. The maximum conversion yields after saccharification for 48 h were 67%, 82%, and 89% for 1%, 5%, and 10% NaOH pretreatment, respectively. The range of glucan digestibility yields in this study was comparable with other findings for NaOH treatment of herbaceous biomass (Pedersen et al., 2010; McIntosh and Vancov, 2011; Gupta and Lee, 2010a & 2010b; Chen et al., 2009; Wang et al., 2010; Chen et al., 2007). The highest pretreatment severity (10% NaOH) resulted in 7% more glucan digestibility compared to that of 5% NaOH treatment. On the other hand, the conversion yield at 48 h of 5% NaOH treated wheat straw was 15% greater than that of 1% NaOH treatment. The variation in enhancing glucan conversion by pretreatment was likely due to differences in degree of positive effect causing by alkalinity levels in NaOH pretreatment, i.e., removal of physical barriers such as lignin and hemicellulose, as well as alteration other structural features such as swelling, increase in internal surface area, decrease in degree of polymerization, decrease in crystallinity, and disruption of lignin structure (Sun and Cheng, 2002).

Extended Enzymatic Saccharification

As showed in Figure 2.8, glucan conversion profiles of all NaOH treated wheat straw obviously did not reach the maximum attainable yields. Consequently, extended saccharification experiment up to 120 h was demonstrated in 5% NaOH treated substrate in order to obtain the maximum

attainable yield under the same enzyme loadings. Profile of glucan conversion is showed in Figure 2.11. The conversion yields leveled off after 96 h with the final yield about 88% conversion at 120 h. This raised questions whether constant final yields during 96-120 h were resulted from limiting accessible areas for cellulose to cellulase in the substrate at the final phase of extensive hydrolysis. Here, we tested the latter hypothesis on the accessibility of the enzyme to glucan.

Considering ultrastructure of mature cell walls in wheat straw, cellulose exists in bundles of microfibrils coating and cross-linking in layers of hemicellulose and lignin in primary and secondary cell walls like other higher plants. Although wheat is a non-woody biomass, secondary cell walls of wheat straw consist of three layers (S1, S2, and S3) as those found in wood. However, orientation of fibrils in each layer differs from that found in spruce. For example, orientation of fibrils in thick S1 layer is cross helix, i.e., making it difficult for defibration of wheat straw in pulping process (Xu, 2010). In addition, cellulose crystallinity of wheat straw is low and differs slightly in different parts of wheat straw (Liu et al., 2005) (Note that the wheat straw used in this study was the mixture of the entire above-ground vegetative parts). Kristensen and co-workers (2008) revealed that the major obstacle for enzymatic attack on cellulose is hemicellulose and lignin that physically block cellulose. Thus, the remaining 10% of non-hydrolyzed glucan was probably locked up inside layers of hemicellulose and lignin that cannot be removed by either the pretreatment condition used in this study due to strong covalent bond such as ether bond, or they were unexposed to the alkaline reagent, or to enzymatic hydrolysis in the case of xylan.

Three-Step Sequential Pretreatment (Alkali-Enzyme-Alkali Pretreatment) and Enzymatic Hydrolysis

To prove the hypothesis that inaccessible glucan is blocked inside lignin, a set of sequential pretreatment method was developed. Wheat straw was first treated with 5% NaOH to removal partial lignin and hemicellulose while 97%

glucan was preserved in the solid. Then enzymatic hydrolysis with cellulase and xylanase was applied to remove accessible major carbohydrates for an extended period of time (120 h). We hypothesized that the steady hydrolysis yields after 72 h of the glucan conversion (~90% conversion) is hindered by next layers of lignin and hemicellulose that are unexposed to alkali reagent during the pretreatment due to the blockage by cellulose, which is resistant to alkali. These unexposed lignin and hemicellulose becomes exposed to environment after the removal of glucan by the enzymatic hydrolysis. These lignin and hemicellulose shield the next layer of unhydrolyzed glucan as they did to the biomass before the alkali pretreatment; thus, enzymatic hydrolysis is not able to produce more glucose from unaccessible glucan. If this hypothesis is true, that means another NaOH treatment at the same condition can remove those lignin and hemicellulose resulting in additional glucose production in the subsequent enzymatic hydrolysis.

Our result from the three-step sequential pretreatment showed that after sequential treatment, approximately 10% more glucan conversion (Total conversion = 99%) was obtained when this residue was subjected to the second round of the enzymatic hydrolysis. This evidence supports the hypothesis above that accessibility of cellulose plays an important role in enzymatic conversion of cellulose to glucose. In addition, the majority of lignin and hemicellulose, partitioning in the pretreated solid obtained from the first round of the NaOH pretreatment, were not removed not because of the inadequate pretreatment severity but because of being shielded by cellulose. This finding also revealed one of the true reasons of why 100% glucan conversion and slow conversion rate in the final phase of alkali pretreated biomass obtained from a single-step pretreatment, regardless the severity of pretreatment conditions, alkali reagents, and types of herbaceous biomass, was difficult to achieve even at high enzyme loadings, sources of enzyme preparations, and/or extended saccharification period as reported in many literatures (McIntosh and Vancov, 2011; Hu an Wen,

2008; Wang et al., 2010; Xu et al., 2010; Kumar and Murthy, 2011; Pedersen et al., 2011). Although it may not be considered a cost-effective pretreatment for the three-step sequential pretreatment, structural features and molecular arrangement of the macrocomponents in cell walls of biomass are important factors to consider in biomass conversion point of view.

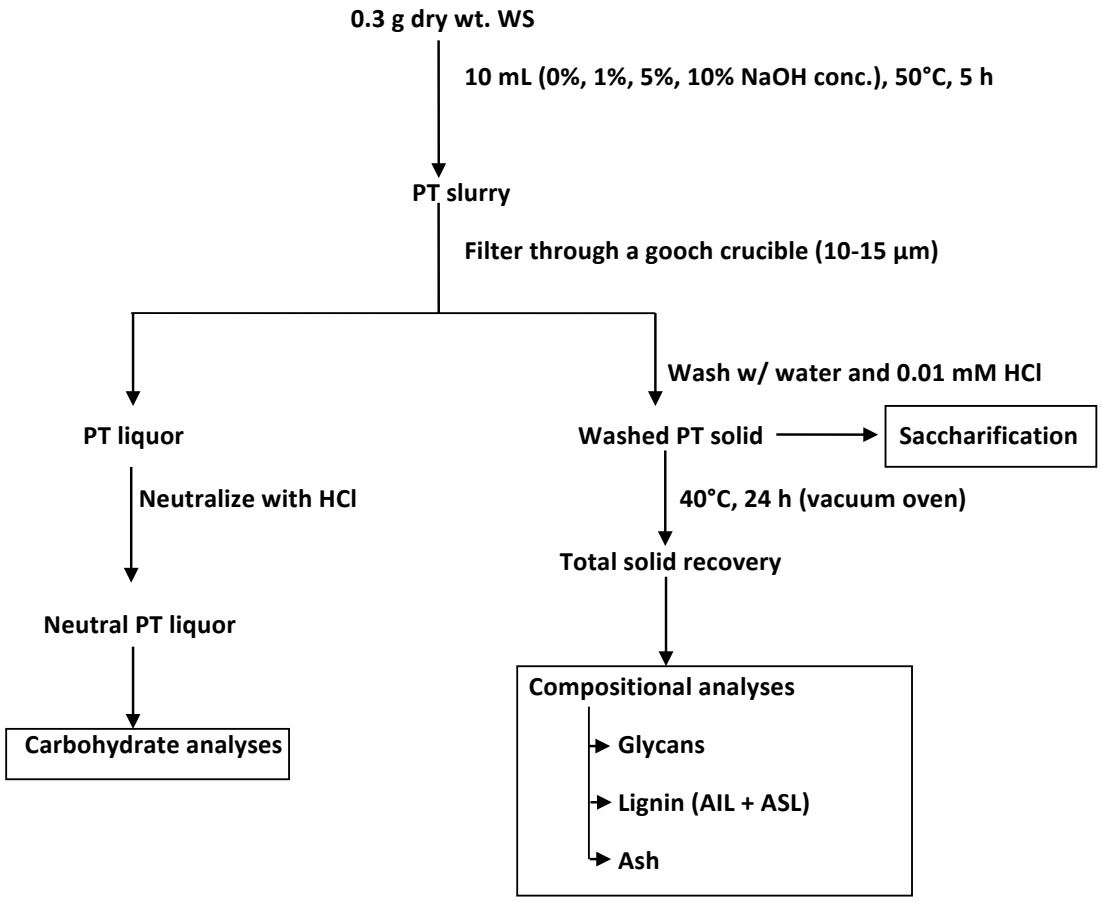

Figure 2.1. Flow chart of sodium hydroxide pretreatment of wheat straw at 50°C, 5 h, with 3% solid loading and post-pretreatment processes for further analyses. Note that control experiment (0% NaOH) was carried out with distilled water; thereby no neutralized was not required. Abbreviation: WS = Wheat straw; PT = Pretreated; AIL = Acid insoluble lignin; ASL = Acid soluble lignin

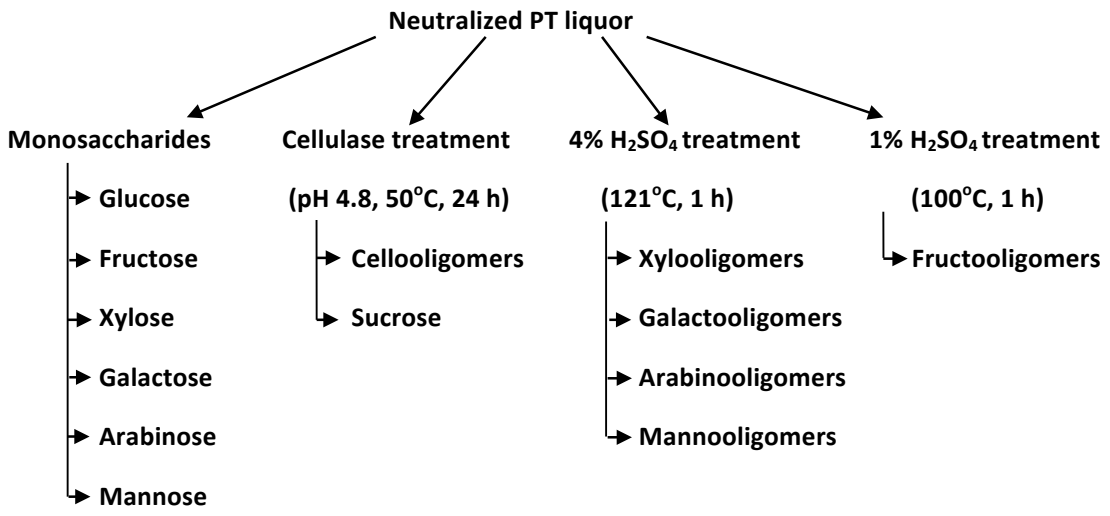

Figure 2.2. Flow chart of four different carbohydrate analyses of neutralized pretreated liquor obtained from sodium hydroxide pretreatment of wheat straw at 50°C, 5 h, with 3% solid loading.

Table 2.1a. Mass percent of structural carbohydrates, lignin, ash, and extractives of wheat straw. Values in parentheses represent ±1 standard deviation.

Component	% oven dry weight
Glucan	33.7 (0.45)
Xylan	21.1 (0.10)
Galactan	1.09 (0.12)
Arabinan	2.83 (0.05)
Mannan	0.35 (0.09)
Acid insoluble lignin (AIL)	13.8 (0.02)
Acid soluble lignin (ASL)	0.81 (0.02)
Total lignin	14.6
Total ash	6.57 (0.07)
Extractives[a]	17.54
Ash in extractives[b]	12.92
Total mass[c]	**93.17**

[a]Total extractives in water and ethanol extracts.
[b]Mass percent of ash in extractives = 4.62% (See Table 1b)
[c]Total mass = mass closure of carbohydrates, lignin, ash, and extractives (taking into account of ash in extractives)

Table 2.1b. Mass percent of extractives in water and ethanol fractions and non-structural carbohydrates in water extractives extracted by Soxhlet method for 24 h. Values in parentheses represent ±1 standard deviation.

Component	% oven dry weight	% of water extractives dry weight
Extractives		
Water extractives	14.5 (0.28)	
Ethanol extractives	3.04 (1.22)	
Total extractives	17.54	
Ash in water extractives	4.62 (0.28)	
Ash in ethanol extractives	0.00 (0.00)	
Ash in extractives free wheat straw	1.95 (0.04)	
Total ash in extractives	6.57	
Non-Structural Carbohydrates in Water Extractives		
Sucrose	0.27 (0.02)	1.86
Glucose	0.61 (0.09)	4.21
Fructose	0.82 (0.11)	5.66
Xylose	0.09 (0.07)	0.62
Galactose	0.01 (0.02)	0.07
Arabinose	0.06 (0.02)	0.41
Mannose	nd*	-
Cellooligomers[a]	0.48 (0.21)	3.31
Xylooligomers[b]	0.08 (0.05)	0.55
Galactooligomers[b]	0.61 (0.07)	4.21
Arabinooligomers[b]	0.42 (0.13)	2.90
Mannooligomers[b]	0.09 (0.01)	0.62
Fructooligomers[c]	0.09 (0.01)	0.62
Total	**3.62**	**25.0**

*nd = not detected

[a]Cellooligomers (DP ≥ 3) are calculated from polymeric form of additional glucose produced after cellulase treatment of water extractives. Other carbohydrate oligomers were not determined.

[b]Hemicellulose-derived oligomers were determined from polymeric forms of additional monosaccharides produced after 4% H_2SO_4treatment of neutralized pretreated liquor at 121°C, 1 h. Correction factor of sugar degradation during acid hydrolysis was applied.

[c]Fructooligomers were measured as fructose equivalents of polymeric form of additional fructose generated after 1% H_2SO_4treatment of neutralized pretreated

liquor at 100°C, 1 h. Half the weight percent of sucrose in water extractives was taken into account. Correction factor (5.28%) of fructose degradation at the hydrolysis condition was applied.

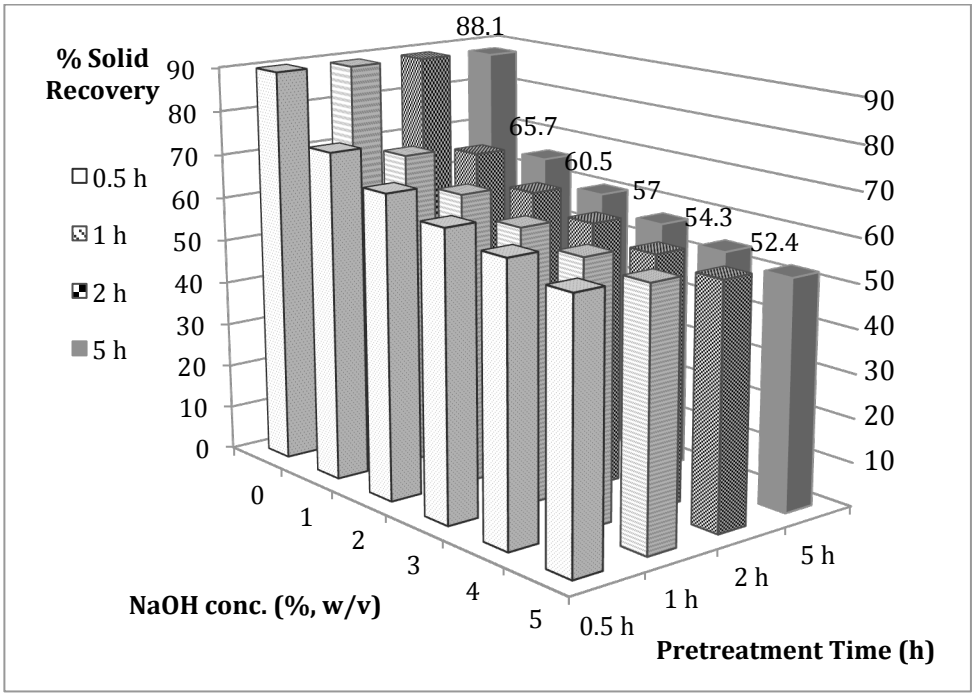

Figure 2.3. Effect of pretreatment time and NaOH concentrations on total solid recovery of wheat straw when pretreated at 50°C with 3% solid loading

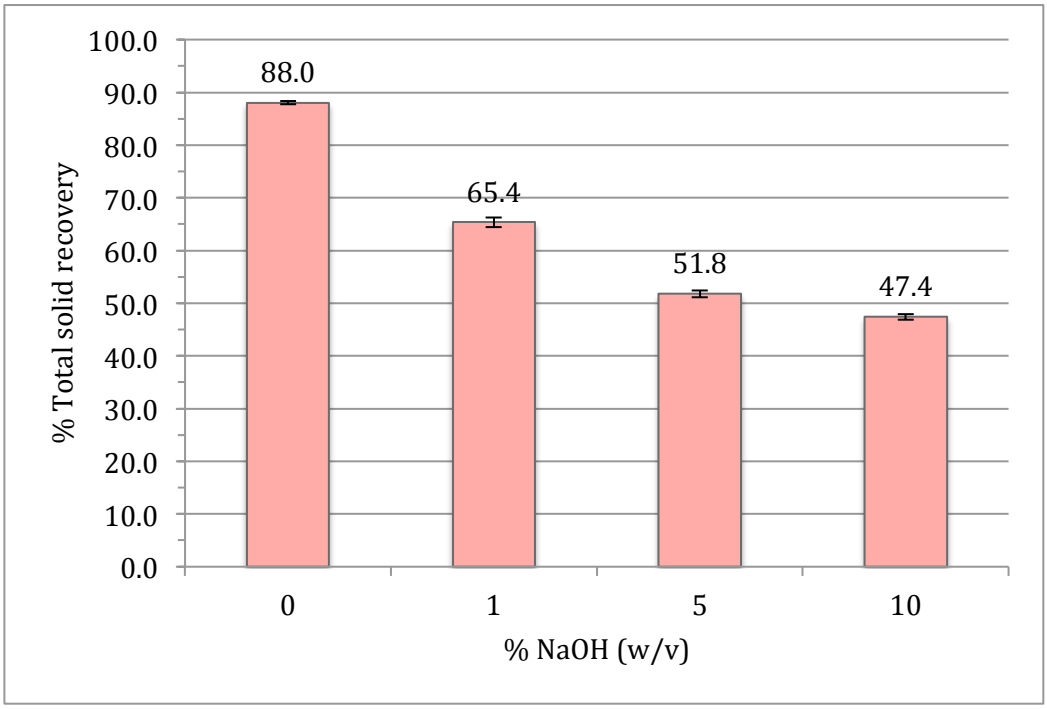

Figure 2.4. Total solid partitioning in solid fraction of NaOH pretreated wheat straw (Pretreatment condition: 50°C, 5 h, 3% solid loading). Percent recovery relative to oven dry weight of original biomass before pretreatment. Distilled water (0% NaOH) was used as control. Error bars represent ±1 standard deviation.

Table 2.2. Chemical composition of NaOH treated wheat straw (solid fraction). All values expressed in percent mass of oven dry weight of pretreated biomass. Pretreatment conditions: 0%, 1%, 5%, 10% NaOH; 3% solid loading; 50°C; 5 h. Distilled water was used as control (0% NaOH). Numbers in parentheses show ±1 standard deviation.

	Treatment			
	0% NaOH	1% NaOH	5% NaOH	10% NaOH
Glucan	37.2 (0.86)	50.6 (0.84)	63.0 (0.27)	67.4 (0.90)
Xylan	22.5 (0.70)	22.4 (0.35)	12.5 (0.18)	7.76 (0.10)
Galactan	1.34 (0.11)	1.16 (0.07)	1.02 (0.06)	1.10 (0.09)
Arabinan	3.13 (0.11)	3.39 (0.07)	2.45 (0.07)	2.06 (0.05)
Mannan	0.39 (0.04)	0.50 (0.03)	0.58 (0.04)	0.64 (0.08)
Total glycans	64.6	78.1	79.6	79.0
AIL	18.1 (0.08)	11.4 (0.06)	10.7 (0.19)	10.8 (0.12)
ASL	1.28 (0.05)	0.84 (0.03)	0.92 (0.05)	0.97 (0.05)
Total lignin	19.4	12.2	11.6	11.8
Ash	4.30 (0.05)	2.25 (0.11)	2.11 (0.09)	1.83 (0.22)

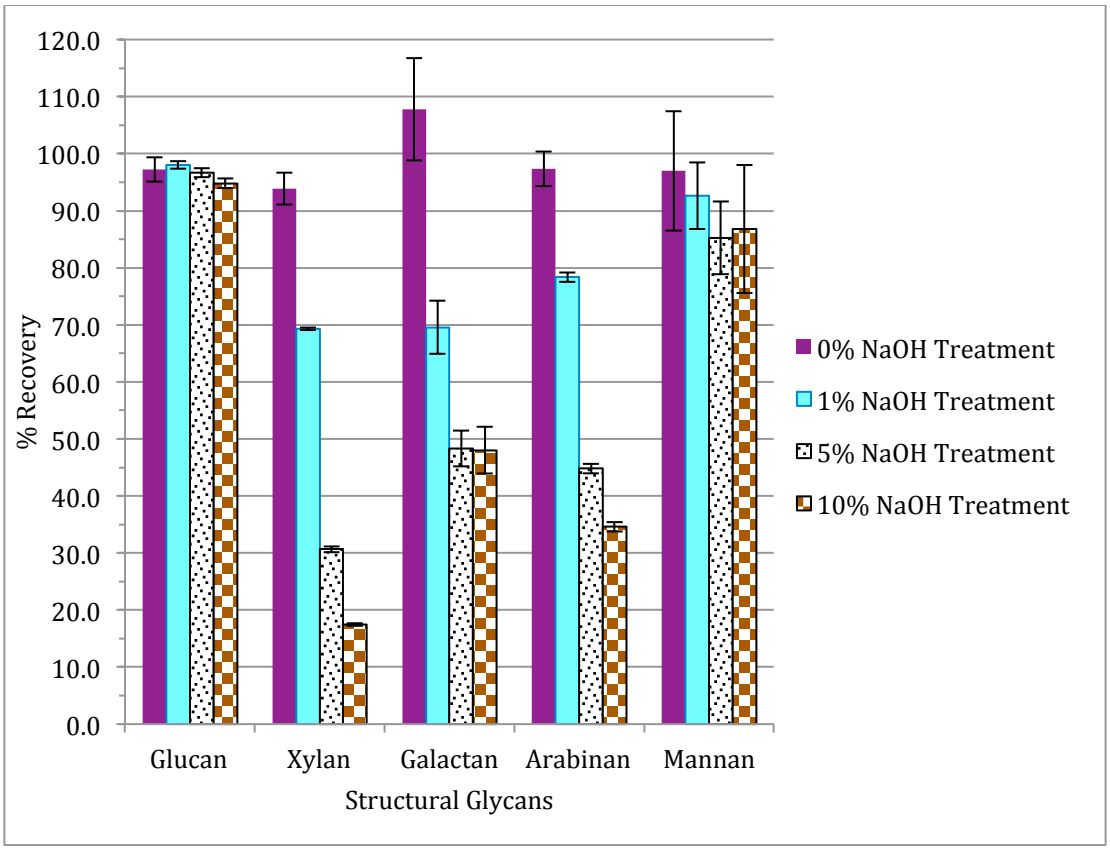

Figure 2.5. Effect of NaOH pretreatment on structural glycans partitioning in solid fraction of wheat straw after pretreatment (50°C, 5 h, 3% solid loading). Percent recovery relative to oven dry weight of a component in original biomass before pretreatment. Error bars represent ±1 standard deviation.

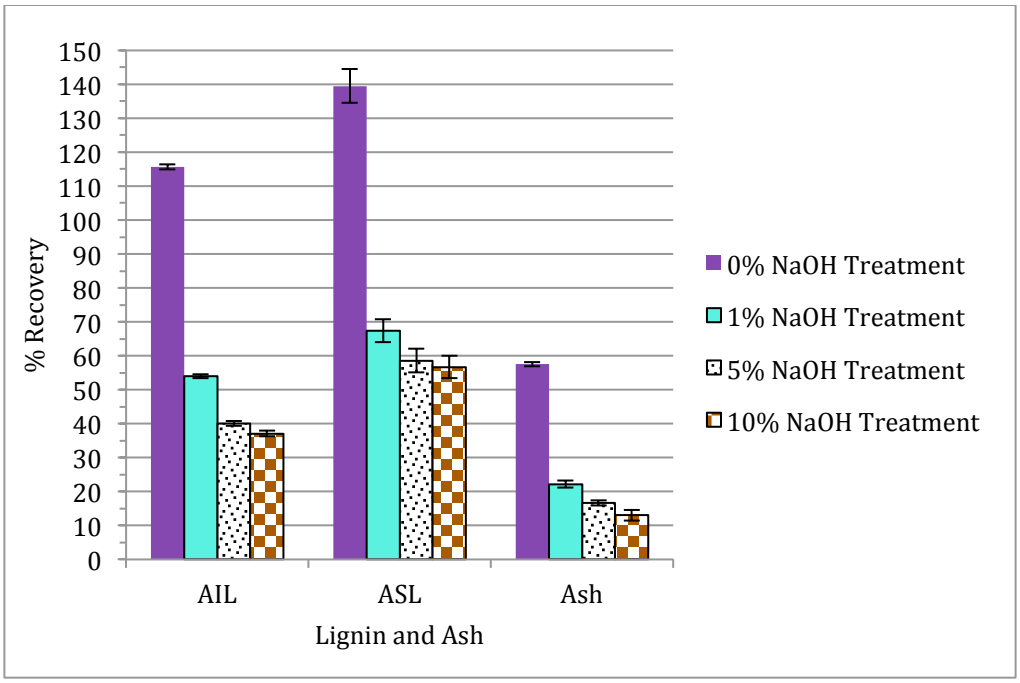

Figure 2.6. Effect of NaOH pretreatment on lignin and ash partitioning in solid fraction of wheat straw after pretreatment (50°C, 5 h, 3% solid loading). Percent recovery relative to dry weight of a component in original biomass. Error bars represent ±1 standard deviation.

Table 2.3. Mass percent of carbohydrates (based on oven dry weight of original biomass) in pretreated liquor of NaOH pretreatment of wheat straw (50°C, 5 h, 3% solid loading). The number in the parenthesis shows ±1 standard deviation.

	Pretreated liquor			
	0% NaOH	1% NaOH	5% NaOH	10% NaOH
Glucose	0.87 (0.02)	0.10 (0.03)	0.06 (0.01)	nd*
Fructose	1.19 (0.01)	0.12 (0.01)	nd	nd
Xylose	0.07 (0.002)	0.01 (0.003)	nd	nd
Galactose	0.03 (0.003)	nd	nd	nd
Arabinose	0.04 (0.01)	nd	nd	nd
Mannose	nd	nd	nd	nd
Sucrose	nd[a]	0.49[b] (0.03)	0.49[b] (0.02)	0.56[b] (0.01)
Fructooligomers[c]	0.15 (0.02)	0.09 (0.03)	nd	nd
Cellooligomers[d]	0.25 (0.03)	0.84 (0.08)	1.77 (0.61)	1.80 (0.81)
Xylooligomers[e]	0.09 (0.00)	5.64 (0.08)	11.7 (0.09)	13.4 (0.22)
Galactooligomers[e]	0.12 (0.01)	0.52 (0.02)	0.76 (0.02)	0.88 (0.05)
Arabinooligomers[e]	0.15 (0.01)	1.36 (0.01)	2.10 (0.03)	2.37 (0.00)
Mannooligomers[e]	nd	0.05 (0.00)	0.09 (0.01)	0.14 (0.02)
Total carbohydrates	2.96	9.22	16.97	19.15

nd*= not detected

nd[a] = Not detected by direct measurement from the pretreated liquor without further treatment with enzymes or acid.

[b]Sucrose was measured after cellulase treatment of neutralized pretreated liquor to remove cellobiose that co-eluted with sucrose by HPLC method. Note that

Accellerase 1500 does not have invertase activity. The presence of sucrose was confirmed by invertase treatment (See Materials and Methods).

[c]Fructooligomers were measured as fructose equivalents of polymeric form of additional fructose generated after 1% H_2SO_4treatment of neutralized pretreated liquor at 100^oC, 1 h. Half the weight percent of sucrose in pretreated liquor was taken into account. Correction factor (5.28%) of fructose degradation at the hydrolysis condition was applied. Note: Fructooligomers obtained from acid hydrolysis of raw native wheat straw = 0.34%

[d]Cellooligomers (DP ≥ 2) were determined from polymeric form of additional glucose produced after cellulase treatment of pretreated liquor.

[e]Hemicellulose-derived oligomers were determined from polymeric forms of additional monosaccharides produced after 4% H_2SO_4treatment of neutralized pretreated liquor at 121^oC, 1 h. Correction factor of sugar degradation during acid hydrolysis was applied.

Table 2.4. Total recovery of major carbohydrates in solid and liquid fractions of NaOH treated wheat straw (Pretreatment condition: 50°C, 5 h, 3% solid loading). Values calculated from mass percent of individual carbohydrate compared to that of original wheat straw.

	Treatment			
	0% NaOH	1% NaOH	5% NaOH	10% NaOH
Glucose and glucan	97.3	97.8	99.1	97.0
Xylose and xylan	93.8	95.4	85.4	80.3
Galactose and galactan	77.7	74.8	75.3	82.0
Arabinose and arabinan	89.0	108.1	101.8	101.1
Mannose and mannan	78.0	85.7	88.7	100.8

Table 2.5. Mass percent of potential polymeric sugars in solid and liquid fractions of NaOH pretreated wheat straw and their theoretical ethanol yields[a] (gallons/dry ton[b] of biomass).

	Treatment				
	Untreated	0% NaOH	1% NaOH	5% NaOH	10% NaOH
Potential polymeric C6 sugars*	38.0	36.7	36.4	36.6	36.2
Potential polymeric C5 sugars*	24.6	22.9	23.9	21.5	20.4
Total potential polymeric sugars	62.6	59.6	60.3	58.1	56.6
Theoretical ethanol yields from C6 sugars	65.6	63.4	62.8	63.2	62.5
Theoretical ethanol yields from C5 sugars	43.5	40.6	42.2	38.2	36.2
Total theoretical ethanol yields	109	104	105	101	98.7

[a]Calculation based on mass percentage of sugars in polymeric forms (http://www1.eere.energy.gov/biomass/ethanol_yield_calculator.html).

[b]1 ton = 1,000 kg.

*Monomeric sugars in the liquid fractions were converted into polymeric forms using correction factor of 0.9 for C6 sugars and 0.88 for C5 sugars.

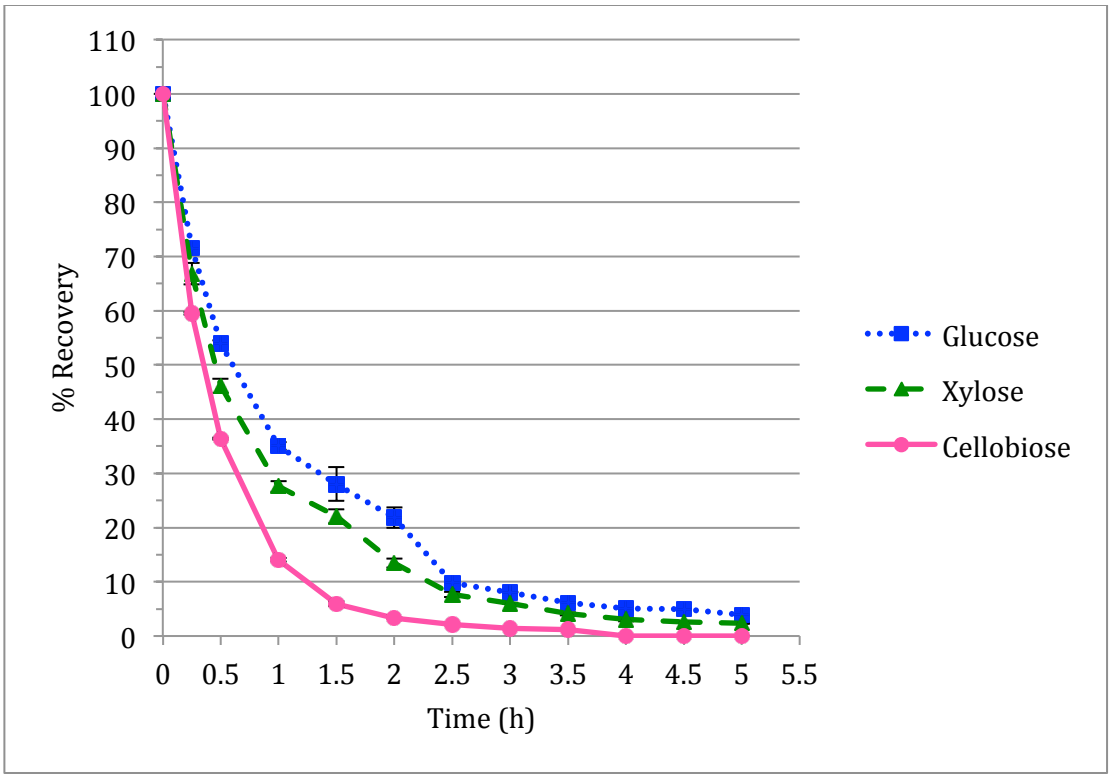

Figure 2.7. Time courses of recovery of monosaccharides and disaccharides in 1% NaOH (w/v) at 50°C. Initial amount of individual sugar = 3.0 mg (0.05%, w/v). Error bars represent ±1 standard deviation.

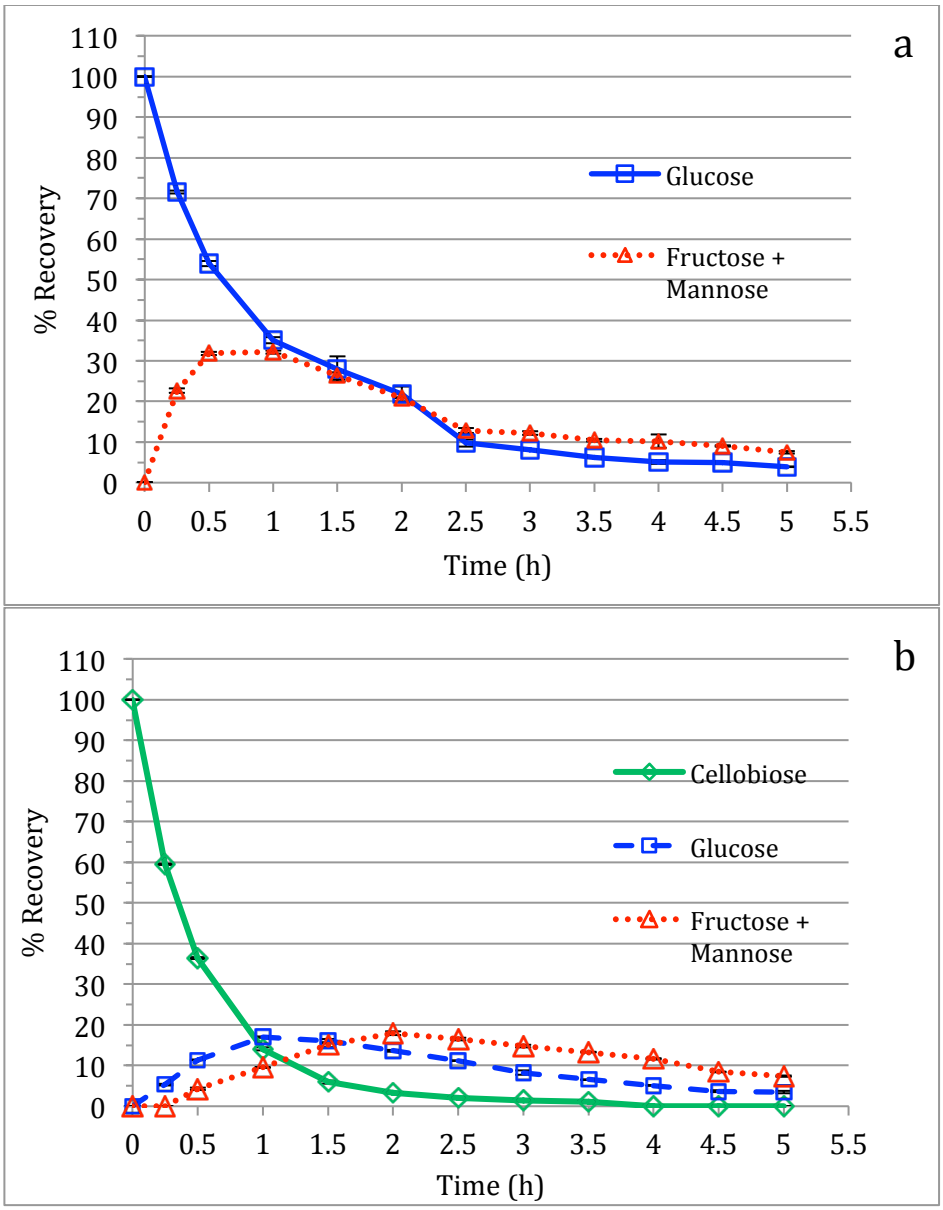

Figure 2.8. Time courses of (a) recovery of glucose and formation of fructose via *Lobry de Bruyn-van Ekenstein rearrangement* in 1% NaOH (w/v) at 50°C. Initial amount of glucose = 3.0 mg (0.05%, w/v); (b) recovery of cellobiose and formation of glucose via peeling reaction and intercoversion of glucose, fructose, and mannose via *Lobry de Bruyn-van Ekenstein rearrangement* in 1% NaOH (w/v) at 50°C. Initial amount of cellobiose = 3.0 mg (0.05%, w/v). Note that the relative recovery of fructose + mannose was calculated based on the initial amount of glucose in case of glucose degradation and relative recovery of glucose, fructose + mannose was calculated based on the initial amount of cellobiose. Error bars represent ±1 standard deviation.

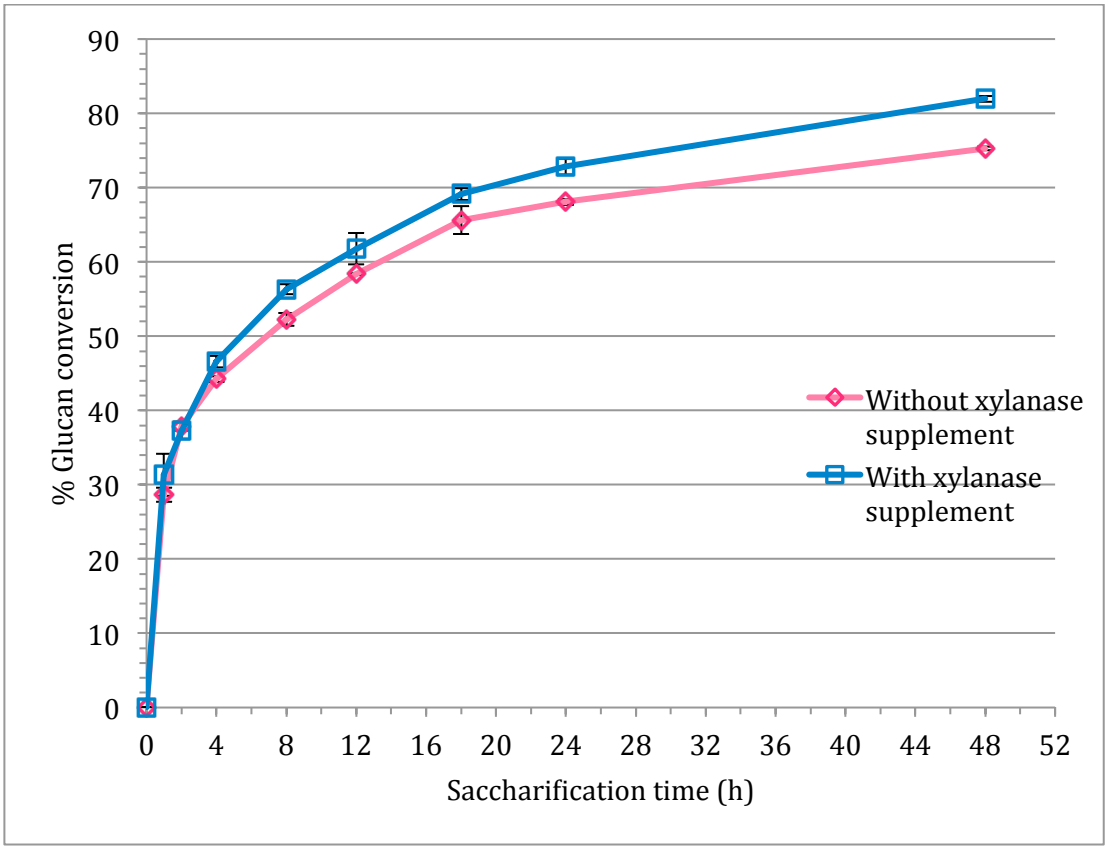

Figure 2.9. Effect of xylanase supplementation on glucan conversion of enzymatic hydrolysis of 5% NaOH treated wheat straw. Pretreatment condition: 50°C, 5 h. Saccharification condition: cellulase loading = 30 FPU/g glucan; xylanase loading = 2,750 Units/g glucan; pH 4.8; 50°C. Error bars represent ±1 standard deviation.

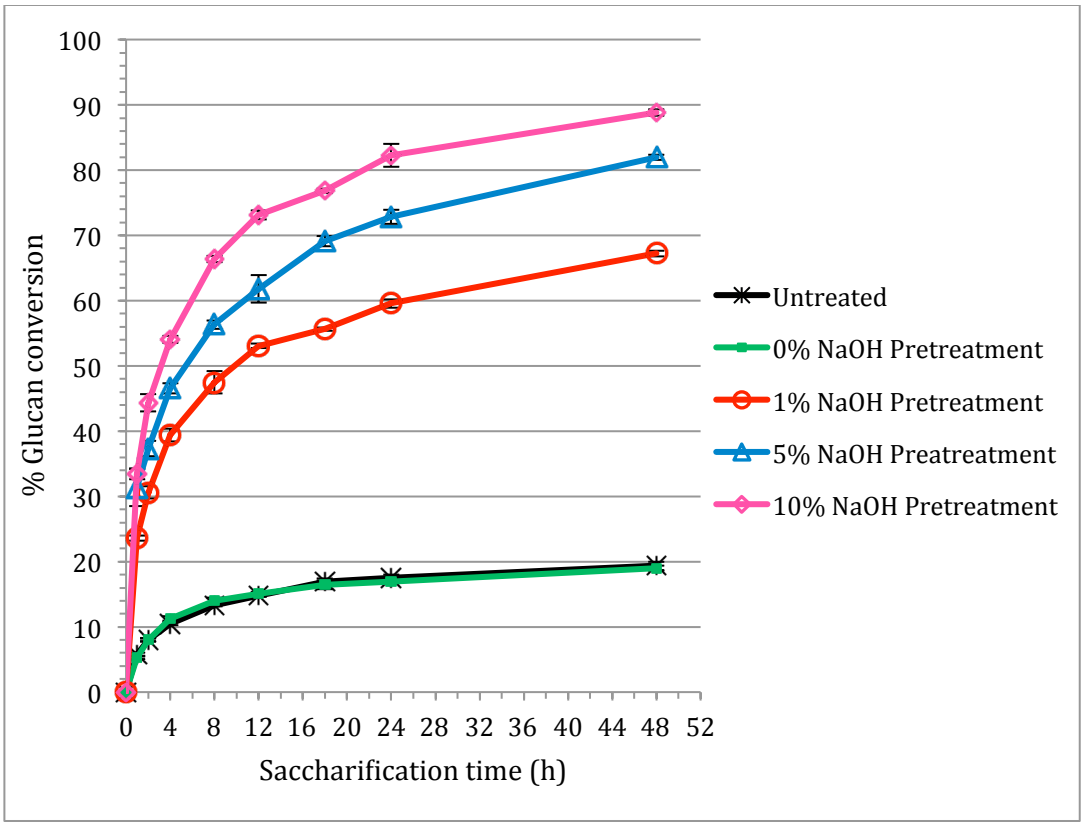

Figure 2.10. Comparison of glucan conversion of enzymatic hydrolysis of untreated (original wheat straw with extractives) and NaOH treated wheat straw (0% NaOH = distilled water treatment was used as control). Pretreatment condition: 50°C, 5 h. Saccharification condition: cellulase loading = 30 FPU/g glucan; xylanase loading = 2,750 Units/g glucan; pH 4.8; 50°C. Error bars represent ±1 standard deviation.

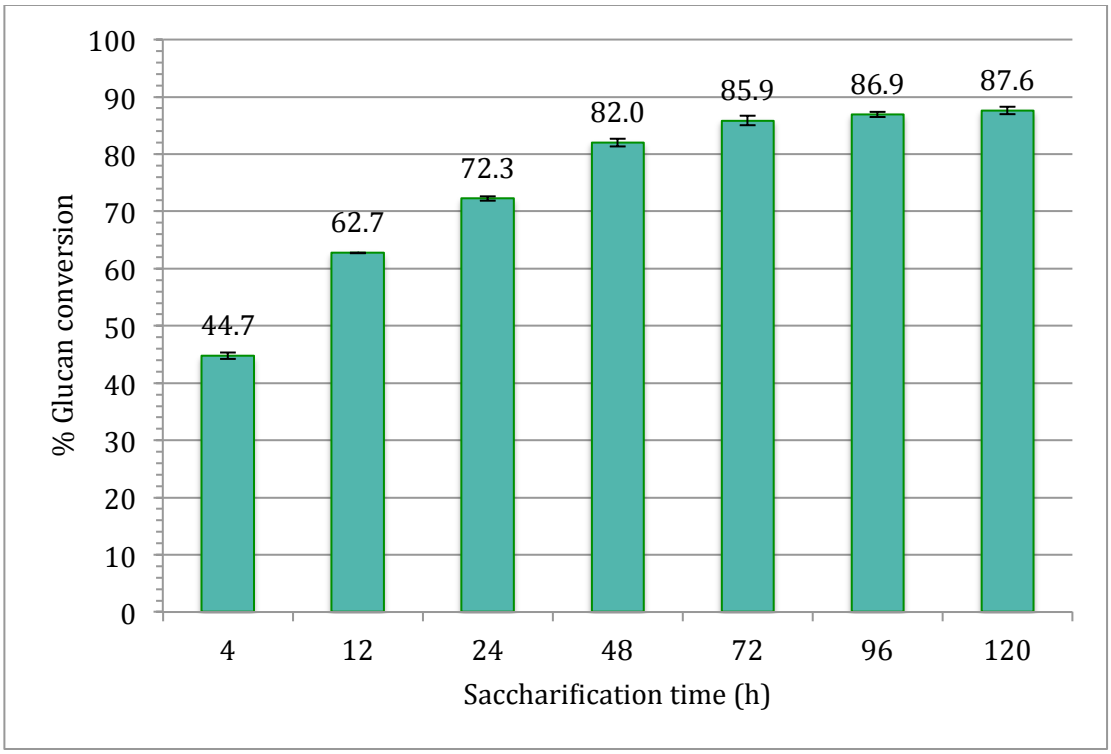

Figure 2.11. Glucan conversion of enzymatic hydrolysis of 5% NaOH pretreated wheat straw obtained from extended saccharification experiment. Saccharification condition: cellulase loading = 30 FPU/g glucan; xylanase loading = 2,750 Units/g glucan; pH 4.8; 50°C. Error bars represent ±1 standard deviation.

3 CHARACTERIZATION OF ALKALI UPTAKE DURING HIGH pH PROCESSING OF WHEAT STRAW

Supaporn Sophonputtanaphoca, Tiraporn Junyusen, Christine Kelly, and Michael H. Penner

CHARACTERIZATION OF ALKALI UPTAKE DURING HIGH pH PROCESSING OF WHEAT STRAW

3.1 ABSTRACT

This study focuses on characterization of alkali neutralization, which occurs during the aqueous alkali processing of wheat straw. The approach taken was to evaluate the time course of alkali uptake and to determine the underlying nature of alkali uptake. The knowledge generated from this study is useful for understanding the nature of the alkali-induced chemistry that is at the heart of alkali processing of agricultural byproducts, foods, and forest products. Alkali uptake/acid generation measurements were monitored for wheat straw suspensions at pH 11 and 30˚C. The first phase of alkali uptake corresponded to the 30-second time period over which the pH of the wheat straw suspension was adjusted from its original pH (~6.6) to pH 11. Alkali neutralization during this period was attributed to the instantaneous ionization of solvent accessible Brønstad acids. Following pH adjustment to 11.0, the time course of subsequent alkali uptake was recorded. The time course appeared biphasic. The early phase, which corresponded to the relatively rapid uptake of alkali, was evident during the first 24 hours. The later phase, which was characterized by the relatively slow uptake of alkali, was maintained for the length of the study (up to 96 hours). Alkali uptake during the early phase of the time course appears to be determined by the rate of hydrolysis of readily accessible esters – primarily acetic acid esters (acetyl groups). Alkali uptake during the later phase of the time course appears to be impacted by the rate of alkali penetration into the straw and the rate of production of alkali-induced acid degradation products. The uptake of alkali in the pH adjustment phase was ~ 120 μEq per gram wheat straw, the uptake of alkali in the early phase of time course was ~ 1,064 μEq per gram wheat straw, and the rate of uptake in the later phase of the time course 6.10 μEq per gram wheat straw per hour. Amount of acetyl groups, ferulic acid, and *p*-coumaric acid generated during 96-h pretreatment revealed that they are major esters being

hydrolyzed under the studied condition. Combined, these ester-derived acids contributed up to ~ 28% of overall alkali uptake. In addition, alkaline degradation products quantified in this study showed additional ~ 28% contribution to the overall alkali uptake.

3.2 INTRODUCTION

Alkaline pretreatment is one of the pretreatment methods to help unlock complex cell wall structure of lignocellulosic biomass to facilitate enzyme accessibility to cellulose in order to obtain high yields of cellulose conversion into fermentable sugar (Mosier et al., 2005; Yang and Wyman, 2008; Kumar et al., 2009). Among alkali reagents, sodium hydroxide (NaOH) has been intensively studied the most (Kumar et al., 2009; Mosier et al., 2005). NaOH treatment has been used in several approaches such as biorefinery approaches, pulping processes, animal feed, and biogas production (Talebnia et al., 2010; Zanuttini et al., 1998; Moharrery, 2007; Pavlostathis and Gossett, 1985a) due to its major powerful effects on delignification, hemicellulose removal, decrease in crystallinity and degree of polymerization of cellulose, and swelling fiber. NaOH works more efficiently in term of improvement of degradability of lignocelluloses in low lignin substrates such as grasses and straws than in high lignin plants such as softwood and hardwood (Sun and Cheng, 2002). Cleavages of ester and ether linkages catalyzed by hydroxide ions result in solubilization of lignin and hemicellulose (Sun et al., 2002), thus reduce lignin and hemicellulose partition in pretreated solid (Gupta and Lee, 2010a).

Acetylation (by ester linkage) of glucuronoarabinoxylan (GAX), major hemicellulose in grasses, by acetyl groups at the O-2 and O-3 positions of the xylosyl units of the xylan backbone could account for 50-70% of the substitutions (Carpita, 1996; Chesson et al., 1983) or 1-2% of the dry weight of grasses (Puls and Schuseil, 1993). The presence of hydroxycinnamic acids, ferulic acid and *p*-coumaric acid as predominant hydroxycinnamic acids, is one of the unique characters for cell walls of family Poaceae (grasses) including wheat (*Triticum aestivum*). Both hydroxycinnamic acids exist in form of esterified and etherified covalently to polysaccharides and lignin, cross-linked through ester-ether bridges in case of ferulate (major cross-linking) between lignin and polysaccharides

(Vogel, 2008; Sun et al., 2002; Iiyama et al., 1994), and unbound or free forms (Vogel, 2008; Iiyama et al., 1994). In case of ester-ether cross-linking in ferulic acid, the carboxylic group serves as esterification site to polysaccharide while the hydroxyl group on the aromatic ring provides etherification site to lignin (Pan et al., 1998). In contrast, *p*-coumaric acid does not involve in ester-ether bridges between arabinoxylan and lignin as ferulic acid does (Hatfield et al., 1999) since most of esterified *p*-coumaric acid in lignin is not covalently linked to other polymers (Grabber et al., 2004). The majority of *p*-coumaric acid is esterified to lignin at γ-position of phenylpropanoid sidechains of syringyl units (Grabber et al., 2004) or polysaccharides in all growth stages of wheat straw whereas approximately equal amount of ferulic acid is found in form of esterification and etherification (Pan et al., 1998). Pan and coworkers (1998) reported that wheat straw contained 0.48% ferulic acid and 0.42% *p*-coumaric acid (total esters and ethers), and 56% of total ferulic acid was esterified while the other 44% was etherified. Unlike ferulic acid, more than 80% of *p*-coumaric was in esterified form with lignin. This agreed with findings from Sun et al. (2002), i.e., more than 65% of *p*-coumaric acid of wheat straw was in esterified form with organosolv lignin (extracted with ethanol/water) while approximately 68% of ferulic acid was in ether linkages with organosolv lignin. Esterification of these phenolic acids was primarily to lignin and hemicellulose (Lawther et al., 1996). Release of phenolic acid esters into aqueous fraction occurs when lignocellulosic materials are subjected to alkaline treatments leads to solubilization of lignin and hemicellulose (Lawther et al., 1996; Grabber et al., 2004). With respect to acetylation on xylan backbone of GAX, ester-ether bridges between hemicellulose and lignin by ferulic acid, esterified *p*-coumaric acid, other esterified phenolic acids, and unbound phenolic acids on lignin structures (Vogel, 2008; Sun et al., 2002), alkaline treatment at room temperature breaks down ester bonds of these acids (Grabber et al., 2004) and causes ionization of their functional groups (carboxyl and hydroxyl) in carbohydrates and lignin (Xu and Cheng, 2011).

Dissociation of protons derived from these esters and ionization of their functional groups neutralize hydroxide ions derived from the alkali and contribute to alkali consumption during the pretreatment.

Another source of buffer capacity of biomass is acids generated from alkaline degradation of carbohydrates and lignin during the pretreatment (Pavlostathis and Gossett, 1985a). Endwise peeling reaction from reducing ends of oligosaccharides and polysaccharides and degradation of reducing sugars through isomerization, enolization, followed by a series of reactions, e.g., β-elimination, benzilic acid rearrangement, α-dicarbonyl cleavage, aldol condensation, and retro-aldol condensation (Clarke et al., 1997). Alkaline treatment of carbohydrates results in various degradation products via several reaction mechanisms depending on several factors, e.g., types of constituent sugars, concentration of the carbohydrates, type of alkali, degree of alkalinity, and treatment conditions (temperature and residence time) (Lai, 2001). Neutralization of hydroxide ions in the alkali medium by dissociated protons from those acids impairs the effect of the pretreatment since the alkalinity decreases. Consequently, more alkali is required in order to achieve a given pH condition, which in turn increases the cost of a chemical-based pretreatment, as the reagent load is a major factor of the cost in pretreatment processes.

The concept of alkali consumption by biomass has been studied in alkali pretreatment of lignocellulosic biomass for animal feedstuffs such as straws and grasses (Jasaitis et al., 1987; Giger-Reverdin et al., 2002; Moharrery, 2007), biogas production (Pavlostathis and Gossett, 1985a; Pavlostathis and Gossett, 1985b; Banks and Humphreys, 1998), and pulping processes (Zanuttini et al., 1998). Results from such studies have shown that straw-based feedstocks, such as wheat straw, have buffering capacity in both the acid and base directions (Jasaitis et al., 1987; Giger-Reverdin et al., 2002). More alkali reagent needs to be added into the reaction mixture in order to keep the same alkalinity level

throughout the treatment (Pavlostathis and Gossett, 1985a). The amount of alkali load required depends on types of biomass and condition of the alkali treatment (Pavlostathis and Gossett, 1985a; Zanuttini et al., 1998). Pavlostathis and Gossett (1985a) reported that the maximum alkali consumption of wheat straw in 30 days at room temperature was 0.055 g NaOH/g dry matter. The release of functional groups, e.g., acyl and carboxyl groups, into the liquid phase after the alkali treatment corresponded to the alkali consumption. Zanuttini et al. (1998) found that deacetylation of the cottonwood at 50˚C was accounted for the majority of the alkali consumption as acetic acid released when acetyl groups were hydrolyzed. Different plants (e.g. grasses, legumes, etc.) or fraction of plants (e.g. vegetative part, grain, etc.) required different amount of alkali to raise the pH of the biomass suspension to a desired pH (Moharrery, 2007; Giger-Reverdin et al., 2002; Jasaitis et al., 1987).

Recently, alkali consumption has become a concern in alkali pretreatment of lignocellulosic biomass for enzymatic conversion to yield fermentable sugars. In lime ($Ca(OH)_2$) pretreatment, the decrease in pH due to alkali consumption was compensated by excess $Ca(OH)_2$ loading than its solubility at a pretreatment condition since solubility of $Ca(OH)_2$ in water is poor and the solubility decreases as the temperature increases. Excess amount of solid lime, allows it gradually dissolve into the solution as readily available hydroxide ions are consumed by biomass. This helps maintain pH at a certain value throughout the pretreatment (Xu and Cheng, 2011). However, this is not applicable to NaOH treatment to hold a constant pH all the time. The decrease in pH of biomass suspension in alkali solution after pretreatment has been investigated. Pedersen et al. (2010) reported the decrease in pH after pretreatment of wheat straw at 100-140˚C, 10 min with NaOH, for example, pH 13.0 to pH 10.6 and pH 10.0 to pH 6.4.

In this study, we have developed a method to study alkali uptake (buffer capacity) of wheat straw using a pH-STAT method to maintain a constant pH at a

given temperature. The objective is to characterize the alkali neutralization properties of wheat straw, a relevant, representative herbaceous feedstock. Experiments were done under relatively mild conditions (pH 11, 30°C) to slow the progress of alkali uptake and reduce the complexity of the associated chemistry. Sodium hydroxide was used as the alkaline salt due to its current applications in industry and its relatively simple solubility properties compared to the analogous calcium salts. Relationship between rates and extent of alkali consumption and buffer component generation derived from predominant free and esterified compounds in wheat straw cell walls as well as selected major alkaline degradation products of carbohydrates were determined. Time courses of buffer component production as constituents of lignin and hemicellulose were revealed to gain more understanding in alkali uptake and its relation to structural and chemical composition of cell walls under low-temperature and high-pH processing of wheat straw.

3.3 MATERIALS AND METHODS

3.3.1 Buffer Capacity of Wheat Straw in the Closed-System Reactor

Feedstock

Mature aboveground biomass of winter wheat straw was harvested from Hyslop Research Farm of Oregon State University, Corvallis, Oregon. The vegetative parts of straw (stems and leaves) were collected and air-dried before milling with a knife mill to pass a 20-mesh sieve (particle size < 0.841 mm). The milled straw was kept in a glass jar with a screw cap lid at room temperature and used for all experiments.

Chemicals

All chemicals were reagent grades purchased from Sigma-Aldrich (Sigma Chemical Co., USA), Mallinckrodt (Mallinckrodt Baker, Inc., NJ, and EMD (USA). Sugar standards (Sigma) were HPLC grade. Nitrogen gas (standard grade) was purchased from Industrial Wielding (Corvallis, OR). Sodium phosphate, dibasic mixed with potassium phosphate, monobasic (pH 7.00 at 25°C) and ammonium chloride (pH 10.01 at 25°C) buffer solutions (Ricca, Maryland) were used as standard buffers for calibrating pH electrode and pH meter.

Reagents and Enzymes

All chemicals used for pretreatment, saccharification, and analytical procedures were reagent grades purchased from Sigma-Aldrich (Sigma Chemical Co., USA), Mallinckrodt (Mallinckrodt Baker, Inc., NJ, and EMD (USA). Sugar standards (Sigma) were HPLC grade. Glucose oxidase/peroxidase kit (GOPOD-FORMAT, K-GLUC) was purchased from Megazyme (Ireland). Commercial cellulase preparation, Accellerase 1500, was a gift from Genencor (USA). Filter paper activity of the enzyme was measured according to NREL method (Adney

and Baker, 1996). Filter paper of Accellerase 1500 was found to be 57 FPU/mL. β-glucosidase activity of Accellerase 1500 was measured against *p*-nitrophenyl-β-D-glucopyranoside as described by Wood (1988). The β-glucosidase activity was found to be 699 *p*NPGU/mL. The other commercial cellulase preparation, Accellerase 1000 (Genencor) had measured filter paper activity of 44.5 FPU/mL. Commercial β-glucosidase preparation, NS50010, was a gift from Novozymes (USA). The β-glucosidase activity was measured as stated above and was found to be 541 *p*NPGU/mL.

Chemical Composition of Wheat Straw

All analytical methods for determination of wheat straw composition were followed the laboratory analytical procedures (LAP) prepared by National Renewable Energy Laboratory (NREL). Structural carbohydrates, lignin, and ash were used as reported by Masrungson (2006). Determination of moisture content and total solid was followed NREL protocol (Sluiter et al., 2008a). Determination of extractives was followed Sluiter et al. (2005a). Proteins were analyzed using Kjeldahl method (Hames et al., 2005). All experiments were done in triplicate.

Determination of Free Acetic Acid and Acetyl Groups in Different Fractions of Wheat Straw

Free acetic acid in wheat straw was determined from two preparations of water extracts: extraction at 30°C, 30 min, under N_2 and Soxhlet extraction, 24 h. Water extract at 30°C was prepared from incubation 2% (w/w) wheat straw in 30°C distilled water at 30°C for 30 min under constant N_2 bubbling. Nitrogen gas was used to prevent the interference of atmospheric CO_2 to the pH value under the stirring condition. A thermo-regulating hotplate stirrer (VWR) was used for temperature control with a stirring speed of 120 rpm. After incubation, the suspension was filtered through a filter paper Whatman No.1 followed by 2.5μm glass fiber filter. The filtrate was collected for free acetic acid and acetyl group

determination. Soxhlet water extraction was prepared from extraction 4 g wheat straw with distilled water in a Soxhlet apparatus for 24 h (Sluiter et al., 2005a). Determination of acetyl groups in the liquid fractions was done by dilute acid treatment (4% H_2SO_4, 121°C, 1 h). Additional amount of acetic acid generated after the dilute acid treatment was converted to amount of acetyl groups per dry matter of wheat straw. Acetic quantification was done by HPLC method (See "Acetyl Groups in Pretreated Liquor"). Amount of acetyl content in the solid fractions was carried out by two-step acid hydrolysis as described by Sluiter et al. (2008b). The solid fractions included raw wheat straw, water-extracted wheat straw (30°C, 30 min, under N_2), Soxhlet water-extracted wheat straw (Soxhlet water extraction, 24 h), wax-free wheat straw (Soxhlet extraction by toluene/ethanol, 2:1, 6 h), and extractives-free wheat straw (Soxhlet water extraction followed by ethanol extraction, 24 h each step, Sluiter et al., 2005a). The sample preparation for wax-free wheat straw was stated in the following sections.

Determination of Total Free and Esterified Phenolic Acids in Wheat Straw

Method for determination of free and esterified forms of phenolic acids by alkaline treatment was adapted from Sun et al. (2002). The determination was carried out in four different fractions of wheat straw, i.e., raw wheat straw, extractives-free wheat straw wax-free wheat straw, and organosolv lignin. Extractives-free was prepared extraction with distilled water followed by 95% ethanol using Soxhlet apparatus according to the standard analytical procedure as described by NREL (Sluiter et al., 2005a). Wax-free wheat straw was prepared by extraction 4 g raw wheat straw (previously dried at 55°C, 16 h) with 180 mL toluene-ethanol (2:1, v/v) using Soxhlet apparatus for 6 h (Sun et al., 2002) as showed in Figure 3.1. The wax-free residue was air dried prior to use. To determine extractives of toluene-ethanol extract, 30 mL of the extracted liquid was dried at 40°C in a vacuum oven until constant weight attained. Wax-free

residue was used as substrate for organosolv lignin preparation. Organosolv lignin was isolated using ethanol/water (60/40, v/v) under acid catalyst (0.2 M HCl) at 75°C, 3 h (see Appendix A, Figure A-1, for details).

Alkaline extraction of free and esterified phenolic acids was achieved by treating raw wheat straw, extractives-free wheat straw wax-free wheat straw, and organosolv lignin with 1 M NaOH at 25°C for 18 h as showed in Figure 3.2. After neutralization and filtering, the supernatant was subjected to determination of phenolic acids by HPLC analysis.

Time course of Alkali Uptake

The method for determination of buffer capacity of wheat straw was developed using a pH-STAT titration method in a closed system. A relatively mild condition, pH 11.0, 30°C was used to investigate time courses of alkali uptake (buffer capacity development) to maintain the given pH by wheat straw. A bioreactor (BioFlo 110, New Brunswick, USA), equipped with a 3 L water jacket reactor, was used as closed system for studying buffer capacity of wheat straw. The water jacket surrounded the reactor tank acts as temperature control unit. Measurement of pH values was accomplished using a combination pH electrode (405-DAS-SC-K8S, 200 mm length, Mettler Toledo) that was connected to the bioreactor's operating system. The electrode was stored in 3 M KCl when not used and before calibration to equilibrate the diffusion rate of cations and anions between internal filling solution, i.e., 3 M KCl, of the reference electrode and the storage solution. Junction potential can develop if there is difference in rate of the ion diffusion between those two medium causing errors in pH measurement. pH monitoring, NaOH solution addition, and temperature control, were automatically controlled by the bioreactor as setting to maintain a constant pH 11.0 at 30°C. Before carrying out the experiment, the pH electrode was calibrated using the two buffer solutions (pH values at 30°C = 6.99 and 9.95, respectively). The buffers were warmed to 30°C as the same temperature of the reaction

mixture to compensate for temperature effect on pH values according to Nernst Equation.

To obtain time course of alkali uptake by wheat straw at 30°C, 30 g oven dry weight of wheat straw and distilled water to yield 2% (w/w) suspension was loaded into the reactor. The straw suspension was thoroughly mixed and brought to 30°C under N_2 gas bubbling (flow rate = 2 LPM) and agitation (200 rpm). The total equilibration time was 30 min. N_2 gas was applied in order to repel dissolved CO_2 in the distilled water and to prevent atmospheric CO_2 diffusing into the reactor through the exhaust condenser (a sterile filter was put at the outer end of the exhaust condenser to prevent contamination). Dissolving of atmospheric CO_2 in water generates carbonic acid that can interfere with the pH values resulting in overestimation of alkali uptake. The straw suspension was then quickly titrated, with standardized 1 M NaOH, to pH 11.0 – which marked "zero time" of the time course of alkali uptake. "Buffer capacity of pH adjustment phase" was defined as initial equivalents of OH^- needed to bring biomass suspension to pH to 11.0 at 30°C . This was determined as the difference in the number of equivalents or millimoles of OH^- required to raise the biomass suspension to pH 11.0 and the number of equivalents required to bring the pH of the same amount of distilled water to pH 11.0. The suspension was then maintained at 30°C and the pH was maintained at pH 11.0 by automatically inputting the standardized base. Each incremental addition of base (required to maintain the pH at 11.0) was noted with respect to time and quantity of addition. Amount of NaOH added was observed by the decrease in level of the solution in a 50-mL burette (the top of the burette was covered with a needle-punched layer of parafilm to prevent vacuum developing, and evaporation). The number of equivalents of OH^- required to maintain the pH at 11.0 was designated as practical buffer capacity. The experiment was done in triplicate with the control experiment carried out with distilled water. The experiment was done in triplicate.

Determination of Buffer Components

To determine buffer components in the aqueous solution and subsequent enzymatic saccharification following the alkali treatment, 10 mL of suspension was withdrawn at predetermined times through a port on the headplate using a slightly cut-tip 10-mL disposable pipette. Note that the sampling port equipped with the bioreactor was inappropriate for sampling in this case since wheat straw particles easily clogged the sampling vessel preventing to obtain homogeneous sample (solid: liquid ratio = 1: 1). The sample was transferred to a 20-mL scintillation vial containing 10 mL of 0.1 M sodium citrate buffer, pH 4.7 to quench the reaction by reducing pH and temperature of the suspension. The sample was then divided into two portions: 5 mL for determination of buffer components, and 10 mL for enzymatic saccharification. To obtain supernatant sample for buffer component analysis, 5 mL suspension was filtered through a 0.45 μm PTFE syringe filter into a microcentrifuge tube. This sample was kept frozen at -20°C until analysis by HPLC method for aliphatic and phenolic acid determination. As for the 10 mL wheat straw suspension, 150 μL of 2% (w/v) sodium azide was added as antimicrobial reagent. This sample was stored in refrigerator for enzymatic saccharification. The experiment was done in duplicate.

HPLC Analyses

Buffer Components: Aliphatic Acids

The analysis was carried out as reported in Determination of sugars, byproducts, and degradation productions in liquid fraction process samples provided by NREL (Sluiter et al., 2006). Prior to HPLC analyses, all samples and standards were filtered through 0.2-μm Acrodisc© syringe filters (Pall, USA) into autosampler vials. Organic acids (buffer components) generated during alkaline treatment of wheat straw were determined using Shimadzu HPLC system (Model:

Prominence UFLC) equipped with an Aminex® HPX-87H column (300×7.8 mm, BIO-RAD, USA), a cation-H guard column (cat# 125-0129, BIO-RAD, USA), and a reflective index detector (Model: RID-10A). Chromatographic separation was achieved with isocratic elution at column temperature of 65°C, 50 μL injection volume, 0.01 N sulfuric acid in HPLC grade water (Milli-Q water) as mobile phase with the flow rate of 0.6 mL/min, and run time of 50 min. A series of possible alkaline degradation products of carbohydrates and organic acids generated from the alkaline reactions such as saponification were used for the identification and quantification of the acids. The commercially available organic acids are acetic acid, lactic acid, formic acid, and glycolic acid. Data analysis of the chromatograms for all samples was performed with LCsolution Software (Shimadzu) by comparing retention time of the peak of interest with the standards. The experiment was done in duplicate.

Buffer Components: Phenolic Acids

Sample preparation was done as described in determination of aliphatic acids. HPLC system and condition were carried as above except mobile phase and detector. The separation was achieved using 10% acetonitrile in 0.01 N sulfuric acid with isocratic system and the chromatogram was detected by a photodiode array detector (PDA detector, Model: SPD-M20A) at 310 nm and 320 nm *p*-coumaric acid, ferulic acid, respectively. Appropriate concentrations of phenolic acid standards (*p*-coumaric acid and ferulic acid) were prepared in Milli-Q water. Note that extended period of stirring by magnetic stirring at room temperature in a sealed container was required to allow the phenolic acids dissolved completely. The data analysis was performed as described above. Adsorption spectra of individual phenolic acid at UV-visible range were used to confirm the identification of phenolic acids. The experiment was done duplicate.

Acetyl Groups in Pretreated Liquor

Amount of acetyl groups hydrolyzed was determined from amount

of acetic acid production using acetic acid as standard. A correction factor represent the ratio of molecular weight of acetic acid to that of acetyl group (43/60) was applied to convert mass of acetic acid in solution to mass of acetyl groups as present in the original straw (Kaar et al., 1991).

3.3.2 Estimation of Acetyl Groups Remaining in the Pretreated Solid

Estimation of acetyl groups remaining in the pretreated solid is a means to quantify actual amount of acetyl groups released from the ester-bound acetyl in the wheat straw by base-catalyzed hydrolysis. The experiment was carried out in a simple system mimicking the condition of the bioreactor. Ten aliquot of suspension of 2% (w/w) wheat straw in distilled water (distilled water has previously been purged with N_2 gas) was put in a cap-sealed, 20-mL scintillation vial. After equilibrating at 30°C, 30 min, in a rotating incubator, the suspension was quickly brought to pH 11 by adding NaOH solution and marked as zero time. N_2 gas was purged into the headspace before sealing the vial with a screw cap. The reaction mixture was incubated on a rotator at 30°C up to 96 h and pH of the reaction mixture was kept at pH 11 by adding the NaOH solution periodically. Samples were taken at time intervals during that period. Then the pretreated solid was collected and washed to neutral with distilled water before subjecting to dry at 40°C, 24 h in a vacuum oven. The amount of acetyl content in dried, pretreated solid was carried out by two-step acid hydrolysis as described by Sluiter et al. (2008b). Acetic acid was determined by HPLC method and measured as acetyl groups as described above in “HPLC Analysis of Aliphatic Acids” and “Acetyl Groups in Pretreated Liquor”, respectively. The experiment was done in duplicate.

The difference in total acetyl groups in water-extracted wheat straw plus water-extracted liquid (extracted at 30°C, 30 min, under N_2 purging) and acetyl groups remaining after the alkaline treatment was amount of acetyl groups

hydrolyzed. Note that the estimation of amount of acetyl groups hydrolyzed was a minimum estimation compared to the actual amount obtained the real closed-system of the bioreactor. The difference in equivalents of total acetic acid (μmol/g wheat straw) detected in the pretreated liquor and acetyl groups remaining in the pretreated solid revealed a maximum estimation of acetic acid generated from alkaline degradation of carbohydrates.

3.3.3 Buffer Capacity of the pH-Adjustment Phase of Wheat Straw Water-Soluble Phase

To determine alkali uptake at zero time contributed by buffer components in the aqueous phase of wheat straw water extract, the water extract was prepared as described in "Determination of Free Acetic Acid and Acetyl Groups in Different Fractions of Wheat Straw". Before titration of the filtrate, 50 mL filtrate was equilibrated at 30°C for 10 min under constant N_2 bubbling and stirring condition. Titration was achieved by adding 0.4 M NaOH to pH 11.0 at 30°C. Amount of NaOH used was recorded and reported as equivalent of OH^- uptake per gram biomass. Distilled water was used as control. The definition of buffer capacity of the pH-adjustment phase was designated as described above.

3.3.4 Buffer Capacity of Ester Models

The experiment was performed in bench-scale using a pH meter (Corning Pinnacle 540) equipped with a pH electrode with automatic temperature compensation (Mettler Toledo, InLab® Expert Pro). Heating system was carried out using a hotplate stirrer (VWR) with adjustable stirring speed.

Cellulose Acetate

Cellulose acetate, a heterogeneous acetylated polysaccharide, was chosen as ester model to represent a heterogeneous hydrolysis system as occurred with

lignocellulosic biomass. The experiment was categorized into two groups: hydrolysis without swelling the polysaccharide, and hydrolysis after swelling the polysaccharide. To obtain time course of alkali uptake of cellulose acetate (180955, Aldrich; 39.8% (w/w) acetyl content), 0.2090 g (acetyl content equivalent to that of 4% wheat straw) was suspended in 100 mL distilled water and brought to 30°C using a magnetic hotplate stirrer. The suspension was equilibrated at this temperature under N_2 gas bubbling for 30 min before starting the titration. N_2 gas bubbling was applied constantly thereafter. The suspension was then quickly titrated, with standardized 0.4 M NaOH, to pH 11.0 to mark the zero time. Amount of NaOH added to maintain pH 11.0 at 30°C and time of addition was recorded throughout the experiment. For swelling cellulose acetate condition, equal amount of cellulose acetate was dissolved in acetone and then washed with distilled water by centrifuging before resuspending in 100 mL distilled water for the titration. Cellulose acetate was swollen after resuspension in water. The titration to obtain time course of alkali uptake was done in the same manner of the non-swelling condition.

Sucrose Octaacetate

Sucrose octaacetate was used as dimer model. Amount of sucrose octaacetate (0.0679 g) for preparing 100 mL of 1 mM solution (amount of acetyl groups equivalent to that of 2% wheat straw) was dissolve in 2 mL of acetone. Then the solution was mixed with 98 mL of 30°C distilled water to yield the final solution of 1 mM sucrose octaacetate. Nitrogen gas was constantly bubbling into the solution to prevent the interference of atmospheric CO_2 to the pH value under stirring condition. The solution was equilibrated under nitrogen bubbling for 10 min at 30°C before quickly brought to pH 11.0 by adding 0.4 M NaOH and kept constant at pH 11.0. Amount of NaOH used to hold the constant pH was recorded over the entire period of incubation for determination buffer capacity development. Half a mL aliquot of the reaction mixture was removed periodically

and transferred into a microcentrifuge tube containing 0.5 mL of cold 20 mM sodium phosphate monobasic buffer, pH 4.7 to neutralize and lower temperature of the reaction mixture. Samples were kept frozen until acetate production analysis by HPLC method. For control experiment, 1 mM sucrose solution and distilled water were used to replace sucrose octaacetate. The experiment was done in duplicate.

3.4 RESULTS AND DISCUSSION

Chemical composition of wheat straw used in this study is presented in Table 3.1. The time course for hydroxide neutralization/acid generation in a 2% (w/v) wheat straw suspension at pH 11 is depicted in Figure 3.2a. The time course appears biphasic; the "early phase" of relatively rapid alkali uptake gives way to a slower nearly linear "late phase" after ~ 24 h. The late phase was maintained throughout the experimental period (up to ~96 h). The rate of acid generation in the earliest part of the time course, up to 4 h, is approximately 20-fold faster than the rate of acid generation in the "late phase" (based on slopes presented in Figure 3.2b; initial velocity slope, 124 ± 8.1 μEq H^+/g wheat straw (WS); late phase slope, 6.1 ± 1.3 μEq H^+/g/h).

The time-course data may be used to estimate the alkali load required to process wheat straw under the given conditions. The amount of alkali required to bring the WS suspension to pH 11.0, based on the relatively rapid addition of alkali (~ 30 sec; this phase is not depicted in the presented time course), was 120 ± 10 μEq OH^-/g WS. Maintaining the WS suspension at pH 11 over the 24 h period following the initial pH adjustment (*i.e.* following the rapid phase of alkali uptake) required an additional 1,064 ± 10 μEq OH^-/g WS. The prolonged relatively slow linear phase of alkali uptake, most easily visualized beyond 24 h, demonstrates the need for continuous alkaline addition in order to maintain the pH during prolonged processing. The rate of alkali uptake during this later period was 6.1 ± 1.3 μEq OH^-/g WS/h. Combined, the data suggest an alkali load of 1,184 μEq OH^-/g WS is required over the first 24 h to maintain pH 11 (calculated as alkali required to raise initial pH, plus alkali consumed in the relatively fast phase of acid generation) and the subsequent addition of ~146 μEq OH^-/g WS/day thereafter (valid up to 4 days in the present study).

These results are in general agreement with those of Pavlostathis & Gossett (1985a), which noted prolonged, up to 30 days, alkali uptake by wheat

straw soaked in alkali solutions. They reported 24-hour alkali uptakes of 347 and 892 μEq OH^-/g WS for aqueous alkali suspensions with starting pH values of 12 and pH 13, respectively. In the present study, we report 24-hour alkali uptakes of ~ 1,064 μEq OH^-/g WS for suspensions maintained at pH 11. The value reported in this study, upon first consideration, seems particularly high based on the expectation that exposure of the straw to increasingly higher pH values will correspond to greater alkali uptakes due, at least in part, to the alkali-induced generation of acids from the carbohydrate fraction of the straw (Clarke et al., 1997; Lai, 2001). However, in the previous study alkali uptake was determined by back titration of the alkali solution to pH 8.3, whereas in the present study alkali uptake was determined by monitoring alkali input to maintain the pH of the suspension at 11.0. Hence, the present study accounts for the ionization of Brønstand acids with pK_a values in the alkaline region, whereas the prior study does not. The phenolic hydroxyls of lignin are an example of such acids (Lundquist & Parkås, 2011). The alkali neutralizing capacity of straw is underestimated if these acids are not fully accounted for.

Although we speak of two phases, "early" and "late", for the presented time course, the production of acid and consequential alkali neutralization is more appropriately, although still simplistically, viewed as occurring in three phases. The first phase (here referred to as the "pH-adjustment phase"), which is not depicted in the time course Figure 3.2, is the nearly instantaneous liberation of hydrogen ions, presumably from solvent-accessible Brønstad acids, as the pH is raised from that of the original wheat straw suspension (2% wheat straw suspension in water, pH ~ 6.6) to the target pH of the treatment (pH 11 in the present case). In general, these acids are expected to have pK_a values ranging from approximately that of the initial pH of the aqueous suspension to that approximating the final treatment pH value. This suggests that carboxyl groups, with typical pK_a values in the range of 4 to 5, are not the major contributors to this buffer capacity. Buffer capacity associated with this initial phase of pH

adjustment can be calculated as the amount of base required to raise the pH of the 2% biomass suspension to pH 11 minus the amount of base required to raise an equivalent amount of water to the same pH. Reasonable estimates of alkali uptake in this initial phase require the pH adjustment be done as rapidly as possible to minimize acid generation associated with the second phase of buffer capacity development. In the present study, initial phase pH adjustments took approximately 30 seconds. Results from these experiments indicate the buffer capacity associated with the pH-adjustment phase corresponded to ~ 120 μEq OH^-/g WS. This corresponds to approximately one-tenth the hydrogen ion generated in the suspension over the 24-hour period following its adjustment to pH 11. If one considers the source of these protons to be ionizable phenolic groups, then this would represent ~ 120 μmol of solvent accessible, ionizable, phenolic groups per gram wheat straw – based on the assumption the phenolic hydroxyls have pK_a values of ~ 9 (Erdemgil et al., 2007) and, thus, are fully ionized in going from the initial pH of ~6.6 to pH 11. To our knowledge, there is no data with which to compare the presented value for the instantaneous neutralizing capacity of wheat straw. However, related experiments addressing the acid content of different wheat straw lignin preparations have been published (Scalbert et al., 1986; Scalbert and Monties, 1986). Therein, wheat straw-derived "milled lignin" and "enzyme lignin" preparations contained 0.70 and 0.46 mEq "very weak acid" per gram lignin preparation. Combined, these lignin preparations accounted for approximately 65% of the total acid insoluble lignin originally present in straw and, assuming all such acids are solvent accessible, correspond to ~ 45 % of the instantaneous buffer capacity reported in this study. The other high pK_a acids contributing to instantaneous alkali neutralization are likely associated with the unaccounted for acid insoluble lignin, the acid soluble lignin fraction, and non-lignin components. Separate experiments evaluating the instantaneous alkali neutralizing capacity of the water-soluble components of wheat straw (see "Methods") revealed that approximately 60% of that observed

for the whole wheat straw suspension could be accounted for by these water-soluble components. This is important in that it suggests that at least some of the lignin-associated Brønstad acids are not solvent accessible during the initial pH adjustment. It is possible that ester hydrolysis could contribute to this phase of alkali uptake. However, this is unlikely based on our finding (data not shown) that the free acetate concentration of the liquid phase of an aqueous suspension of wheat straw was essentially equivalent to that of the suspension immediately after raising its pH to 11 (*i.e.* the time-point corresponding instantaneous alkali neutralizing capacity). If significant acetyl hydrolysis had occurred, acetyl esters being the most prevalent esters in this feedstock, then acetate levels would have been noticeably higher following pH adjustment to 11.

Rates of hydroxide ion neutralization during alkali processing may be influenced by at least three potentially rate determining phenomena. The first is ionization of solvent exposed Brønstad acids with appropriate pK_a values, which is herein considered to occur the instant the acidic functional group is exposed to solvent (as discussed in the previous paragraph). The second is the result of (hydroxide-catalyzed) reactions that generate new Brønstad acids; ester hydrolysis (Hatfield et al, 2009) and sugar degradation (Clarke et al., 1997; Lai, 2001) are expected to be major contributors in this category. The third phenomenon is related, indirectly, to the alkali-induced swelling of the straw; where swelling increases the straw's solvent accessible surface area and, hence, provides greater exposure of previously non-solvent accessible Brønstad acids and alkali-labile functional groups (*e.g.* esters).

The second phase of alkali neutralization begins immediately after pH adjustment of the wheat straw suspension to 11.0. Under the pH/temperature conditions used in this study, this second phase covers the first 24 hours post pH adjustment (analogous to the "early phase" of the time courses in Figures 3.2b). Alkali neutralization in this phase may be viewed as resulting from (a) hydroxide-induced reactions, other than simple proton dissociation, which generate solvent-

accessible Brønstad acids with pK_a values ≤ ~ 12 and (b) swelling-related phenomena that generate such acids. Not included in this category is the ionization of those Brønstad acids that are solvent accessible in the original aqueous wheat straw suspension. Those acids are presumed ionized during the adjustment of the suspension to pH (*i.e.* they constitute the majority of the alkali neutralizing capacity during the pH adjustment phase). A major contributor to alkali neutralization during the first 24 hours post pH adjustment is ester hydrolysis. Ester hydrolysis will result in at least one ionizable proton, that from the carboxylic acid. The hydrolysis-generated hydroxyls will not typically be ionized because in most cases they are aliphatic hydroxyls with pK_a values >13.

Quantitative values for the predominant free esters in wheat straw, acetic, ferulic, and *p*-coumaric, are presented in Tables 3.2 & 3.3. The alkali neutralizing capacity associated with the hydrolysis of these esters can be substantial. The hydrolytic susceptibility of straw-associated, solvent-accessible, acyl esters can be considered in light of rates of hydrolysis of representative ester compounds under the same reaction conditions. Sucrose octaacetate and acetone-swollen cellulose acetate were evaluated in the present study. Pseudo-first order kinetic analyses provided half-life values for the hydrolysis of acetyl groups of sucrose octaacetate (combined data for primary and secondary esters) and acetone-swollen cellulose acetate of approximately 19 min and 30 h, respectively. The value for sucrose octaacetate may be viewed as an approximate upper limit for the rate at which analogous functional groups of wheat straw (*i.e.*, secondary acetyl groups esterified to either the 2 or 3 position of xylopyranosyl units of xylan chains) may be hydrolyzed. Cellulose acetate is representative of acetylated insoluble polymers for which the acetyl groups are likely to be at least somewhat hindered – as is likely the case for some esters of wheat straw. For comparison, the rate of acetyl hydrolysis over the first 8 h of incubation corresponded to a half-life of approximately 17 h when monitored by acetyl groups remaining in the straw residue and 7.5 h if monitored by acetic acid appearance in the soluble

phase. These calculations are simplistic in the sense that the first-order plots area based on the entire population of acetyl groups being equivalent with respect to reactivity. The 17 h value represents a maximum half-life due to the difficulty of keeping the pH at 11.0 during those experiments; the 7.5 h value represents a minimum half-life due to the possible production of acetic acid from non-acetyl sources (as discussed below).

Figure 3.3 depicts 96-hour time courses for acetate appearance in the soluble phase and acetyl hydrolysis based on determination of acetyl groups remaining in the solid phase. The most obvious aspect of the time courses is that they, like that for alkali neutralization, appear biphasic. It can be seen that the two profiles are similar. The curves differ in that the pH was strictly controlled in the curve representing acetate appearance, but only the soluble phase could be sampled in these experiments and acetate content of the soluble phase is a measure of total acetic acid generated – not just acetic acid generated as a result of acetyl hydrolysis. In contrast, pH control of reaction mixtures for the experiments determining acetyl hydrolysis, measured as acetyl groups remaining in the solid phase, was not automated and thus could not be strictly maintained; in the case of these experiments the pH drifted below the target of pH 11.0 between manual pH adjustments (see methods). Periods when the pH drifted below 11 would, in theory, result in a somewhat reduced rate of hydrolysis since the reaction is presumed to be first order with respect to both ester and hydroxide ion concentrations (Kirby, 1972). Consequently, these values are to be considered approximate minimum values for these reaction conditions. The legends to the time courses of Figure 3.3 indicate actual amounts of acetyl groups generated per gram straw and the percent of the straw's original time-zero acetyl groups that have been hydrolyzed. The latter is important because it clearly shows that the amount of acetic acid generated in the soluble phase is more than can be attributed to the number of acetyl groups in the starting material. It is to

be kept in mind that soluble acetic acid in the starting material, i.e. water-soluble acetic acid, was accounted for in these calculations.

First-order reaction plots of acetyl hydrolysis, Figure 3.4a &b, provide insights into the nature of the populations of acetyl groups in lignocellulosic materials. Both plots, one based on acetate appearance in the soluble phase and the other based on acetyl disappearance from the solid phase, appear linear for the first 6-8 h and then transition into a second phase consistent with the rate of hydrolysis slowing to a greater degree than predicted by first-order kinetics. The data are consistent with their being a multiple populations of acetyl groups; at a minimum one population is more reactive than a second. Considering the data of Figure 3.4b, the break point (~8 h) corresponds to approximately 30% of the acetyl groups being in the more reactive population. A plausible explanation is that this population represents acetyl groups initially exposed to solvent. The remainders of the acetyl groups, those hydrolyzed in the slow phase, represent acetyl groups hindered from hydrolyzing solvent at the initiation of alkali processing. As the alkali penetrates the straw, the latter acetyl groups become susceptible to alkali degradation. Hence, the slow phase may be viewed as being dictated by the rate of alkali penetration into the straw. An alternative explanation is that all acetyl groups are equally exposed to solvent, at the beginning of the alkali processing, but the two populations differ with respect to inherent reactivity. This alternative explanation is less convincing since the nature of the majority of the acetyl linkages in wheat straw are similar, acetylation being primarily limited to the 2 or 3 position of the xylopyranosyl residue of glucuronoarabinoxylans (Carpita, 1996; Chesson et al., 1983; Puls and Schuseil, 1993).

Time courses depicting the solubilization of ferulic acid (FA) and *p*-coumaric acid (PCA) during alkali treatment are presented in Figure 3.5a &b. The mild alkali conditions used in this study are expected to solubilize only those solvent exposed PCA and FA that are solely ester-linked, not those involved in

intermolecular associations via ether or carbon-carbon bonds (Pan et al, 1998). The percentages of theoretically possible FA and PCA solubilized, 36% and 14% at 96 h, respectively, are considerably lower than that for acetic acid (~84% at 96 h based on acetyl groups remaining in the solid phase). The time courses for hydrolysis of the different esters may provide insight into the nature of their respective environments. The production of free FA was approximately linear (with possibly a slight trend toward increased rates of FA ester hydrolysis with time) over the course of the experiment. This is noteworthy in that the extent of hydrolysis (~40%) was sufficient to correspond to a decrease in rate if FA ester hydrolysis obeyed first-order kinetics (as appeared to be the case with a population of acetyl esters). The apparent zero-order kinetics for FA solubilization suggests new solvent-accessible FA is generated over time. The time-course for FA ester hydrolysis is consistent with the concept that the rate of hydrolysis is largely dependent on the rate at which FA esters are exposed to the hydrolyzing solvent, *i.e.* the rate of alkali-penetration into the straw and the associated "swelling". In such a scenario, alkali penetration into the straw, and the subsequent straw swelling, results in solvent access to previously non-exposed FA esters; these newly exposed esters are then hydrolyzed to produce free FA.

Production of PCA was essentially linear over the 96-hour processing period (Figure 3.5a) and the rate of free PCA generation was very similar to that of FA production. When considered from the perspective of percent of theoretically available PCA hydrolyzed, the yields of free PCA were approximately half that of FA (the total amount of esterified FA in straw being approximately 58% of the amount of esterified PCA). The time course of PCA production provides limited information, other than that the rate of hydrolysis of PCA esters is relatively slow. Little further information can be attained from the plot because of the low extent of conversion over this time period (only ~15% of theoretically available PCA was hydrolyzed). The low extent of conversion makes

interpretation of kinetic plots untenable. Nonetheless, the results are not inconsistent with the concept that the rate of PCA ester hydrolysis is dictated by alkali penetration into the straw – as proposed above for FA ester hydrolysis and the slow phase of acetic acid ester hydrolysis.

The notion that alkali penetration/swelling of straw has a major effect on rates of non-solvent-accessible ester hydrolysis can be considered with respect to the following reaction scheme:

(1) $E_{SI} \rightarrow E_{SA}$ (relatively slow)

(2) $E_{SA} + OH^- \rightarrow IA^- + AL$ (relatively fast)

Where:

E_{SI} = solvent inaccessible ester

E_{SA} = solvent accessible ester

IA^- = corresponding ionized acid (carboxylate form)

AL = corresponding alcohol

Reaction (1) reflects the alkali-induced swelling of straw, which can be viewed as a time dependent increase in surface area. The increase in surface area in turn corresponds to an increase in solvent accessible esters. Reaction (2) reflects the hydrolysis of solvent exposed esters. In the simplest case, reaction (1) would be much slower than reaction (2), and therefore rates of hydrolysis of all esters would reflect the rate constant governing reaction (1).

In the present study we calculated second-order rate constants from the linear production of FA and PCA. A second-order rate constant was also calculated for the linear slow phase of acetic acid production (24 – 96 hour). These second-order rate constants were calculated based on average ester concentrations for the reaction period considered. This approach accounts for

the different concentrations of FA, PCA, and acetic acid esters in wheat straw. The values are as follows: FA, 0.001 $L \bullet mol^{-1} \bullet sec^{-1}$; PCA, 0.0004 $L \bullet mol^{-1} \bullet sec^{-1}$; and slow phase acetic acid production, 0.004 $L \bullet mol^{-1} \bullet sec^{-1}$. If the simplest case of the reaction scheme above applied to the present phenomena, then the rate constants for all three esters would be equal, *i.e.* the applicable rate constant would reflect the swelling of the straw and not the inherent reactivity of the different esters. This was obviously not the case. Reasonable explanations for the differences in the rate constants include (a) the esters are not evenly distributed throughout the straw and (b) the kinetics does not simply reflect the alkali-induced swelling. With respect to "(a)", it is easy to rationalize that the different esters will have unique distributions because of the nature of the esters. Acetic acid and FA esters are primarily associated with the hemicellulose component of the cell wall, whereas PCA esters are primarily associated with the lignin (Grabber et al., 2004; Pan et al., 1998; Sun et al., 2002; Hatfield et al., 1999). That fact, along with the relative hydrophobicity of the acyl groups themselves, suggests the acetic acid esters would be in relatively hydrophilic environments and PCA esters in relatively hydrophobic environments. The determined rate constants for FA, PCA, and acetic acid esters, considered relative to one another, reflect the environments the ester are likely to be found provided one makes the plausible assumption that aqueous alkali penetration is favored in the hydrophilic portions of the straw. With respect to "(b)" above, it is clear that the kinetics of swelling and ester hydrolysis are complex and dependent on multiple elementary events – so the impact of steps other than the actual hydrolytic event cannot be ruled out (*e.g.* mass transfer issues). It is to be kept in mind that (a) and (b) above are not mutually exclusive.

Throughout this discussion we have treated alkali penetration/swelling as leading to subsequent ester hydrolysis. However, it is important to consider that swelling itself is likely dependent, or at least enhanced, by ester hydrolysis (Zanuttini and Marzocchi, 1997). The hydroxycinnamic acid esters (*i.e.* FA and

PCA esters) expected to contribute in the largest way to this phenomena are those involved in crosslinking cell wall macromolecules. (The hydrolysis of such esters, i.e. those involved in crosslinking, would not be detected in this study because hydrolysis of the ester does not result in free hydroxycinnamic acid.) This co-dependency of swelling and ester hydrolysis leads to a cyclic phenomena: swelling begets ester hydrolysis, which begets more swelling, etc.

Summing the ester-linked acetic acid, PCA and FA gives an approximation of the amount of buffering capacity that can be attributed to these esters during the time frame of the experiment. Clearly, acetic acid is the predominant ester. Even if we account for PCA being esterified to lignin, and that one mole of PCA ester yields two protons, it is still only 2% of that observed for acetic acid (Table 3.4). There is considerable evidence that a portion of the hydroxycinnamic acids serve to cross-link plant cell wall polymers, particularly FA (Iiyama et al., 1994; Grabber et al., 2004; Vogel, 2008; Sun et al., 2002). Hence, hydrolysis of these esters will not yield a soluble FA and would not have been detected in this study. This study focused only on those that were esterified.

It is found that the other source of acetic acid generation besides acetyl ester hydrolysis during the course of the reaction is alkaline degradation of carbohydrates. Generation of carboxylic acids from alkaline degradation of carbohydrates is well established (Clarke et al., 1997; Lai, 2001). In the present study the alkaline treatment was relatively mild, so it was somewhat surprising the amount of acid generated. Alkaline degradation products determined in this study include acetic acid, formic acid, glycolic acid, and lactic acid (Table 3.5). Combined, they contributed ~28% of alkali uptake at 96 h. These acids, along with the yet unreacted esters, are expected to be sources of base neutralization well into the alkali treatment (at 96 hours and beyond).

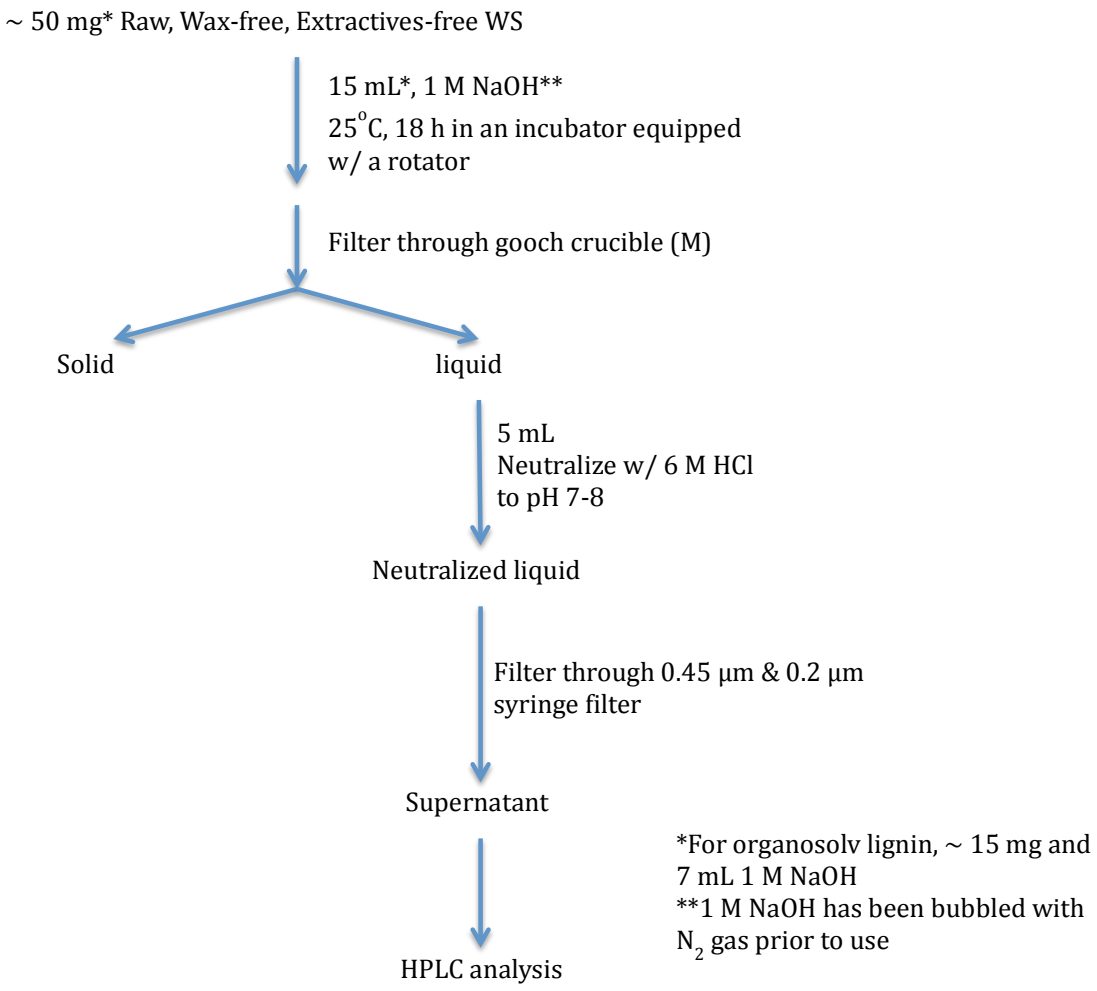

Figure 3.1. Determination of total esterified phenolic acids in wheat straw preparations and organosolv lignin derived from wheat straw by mild alkaline treatment.

Table 3.1 Mass percent of chemical composition of wheat straw. Values represent average values obtained from three replicates.

Component	% on oven dry weight
Cellulose[a]	34.1
Xylan[a]	18.7
Arabinan[a]	1.21
Acid insoluble lignin[b]	16.7
Acid soluble lignin[a]	1.62
Ash[a]	7.24
Extractives[b]	16.2
Proteins[b]	2.36

[a]Masrungson (2006). Master's Degree Thesis. Oregon State University.
[b]Measured in this study

Table 3.2. Acetic acid and acetyl content with standard deviation determined by two-step acid hydrolysis of different wheat straw fractions.

Fraction	Acetic acid (μEq/g WS)	%acetic acid (by wt. WS)	%acetyl group equivalent (by wt. WS)
Raw wheat straw	530 ± 16.8	3.19 ± 0.10	2.28 ± 0.07
Water-extracted wheat straw[a] (30°C, 30 min)	491 ± 10.2	2.95 ± 0.06	2.11 ± 0.04
Water extract (30°C, 30 min)	60.8 ± 1.70	0.32± 0.01	0.23 ± 0.01[b]
Dilute acid treated water extract	4.47 ± 0.92[c]	0.03 ± 0.01	0.02 ± 0.004

[a]Values based on dry wt. of original biomass
[b]If determined free acetic acid as acetyl groups
[c]Different amount after dilute acid treatment (4% H_2SO_4, 121°C, 1 h)

Table 3.2. Acetic acid and acetyl content with standard deviation determined by two-step acid hydrolysis of different wheat straw fractions (Continued).

Fraction	Acetic acid (μEq/g WS)	%acetic acid (by wt. WS)	%acetyl group equivalent (by wt. WS)
Soxhlet water-extracted wheat straw (solid)[d]	441 ± 3.86	2.65 ± 0.02	1.90 ± 0.02
Extractives-free wheat straw[e]	417 ± 10.8	2.51 ± 0.07	1.79 ± 0.05
Wax-free wheat straw[f]	447 ± 11.1	2.68 ± 0.07	1.92 ± 0.05
Soxhlet water extract (liquid)[d]	80.0 ± 5.85	0.48 ± 0.04	0.34 ± 0.03
Dilute acid treated water extract	nd*	nd	nd
Soxhlet ethanol extract[g]	4.73 ± 0.18	0.03 ± 0.001	0.02 ± 0.001

*nd = Not detected additional acetic acid after dilute acid treatment (4% H_2SO_4, 121°C, 1 h)
[d]Water extraction by Soxhlet method for 24 h
[e]Water extraction followed by ethanol extraction by Soxhlet method for 24 h of each step
[f]Toluene/ethanol (2:1, v/v) extraction by Soxhlet method for 6 h
[g]Ethanol extraction by Soxhlet method for 24 h

Table 3.3. Total free and esterified *p*-coumaric acid and ferulic acid content in wheat straw determined by alkaline hydrolysis (1 M NaOH, 25°C, 18 h) and values comparison between this study and those from literatures of different wheat straw fractions.

Fraction	% oven dry wt. original WS	
	p-Coumaric acid	Ferulic acid
Raw wheat straw	0.41 ± 0.01	0.24 ± 0.005
Wax-free wheat straw	0.37 ± 0.01	0.22 ± 0.01
Extractives-free wheat straw	0.35 ± 0.004	0.23 ± 0.003
Organosolv lignin	0.03 ± 0.0004 1.33 ± 0.02*	$0.004 \pm 4 \times 10^{-5}$ 0.20 ± 0.002*

*by wt. of organosolv lignin

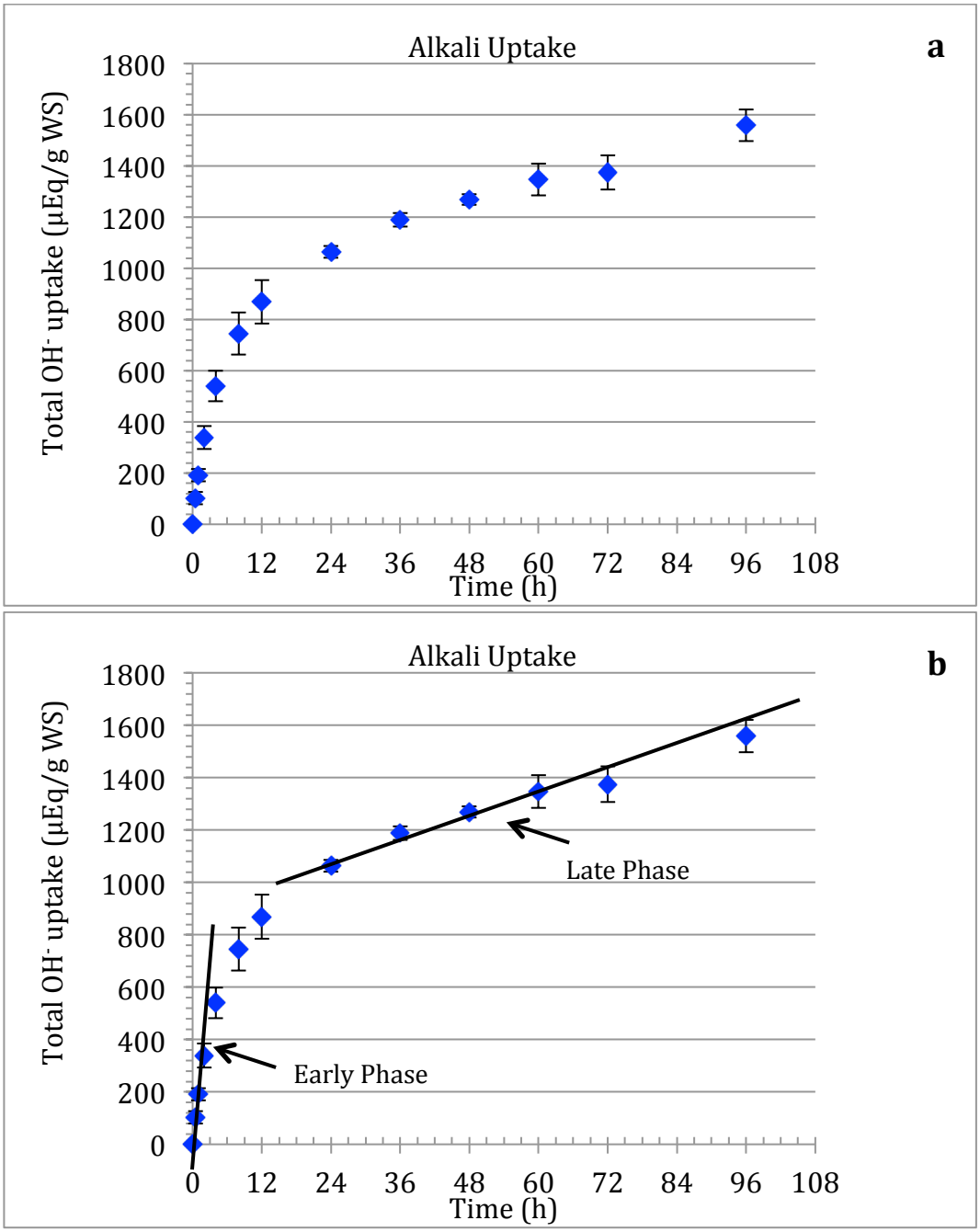

Figure 3.2. Time courses of alkali uptake (a). Different phases in time course of alkali uptake (b) by 2% wheat straw at pH 11.0, 30°C in a closed-system bioreactor. Error bars represent ± 1 standard deviations.

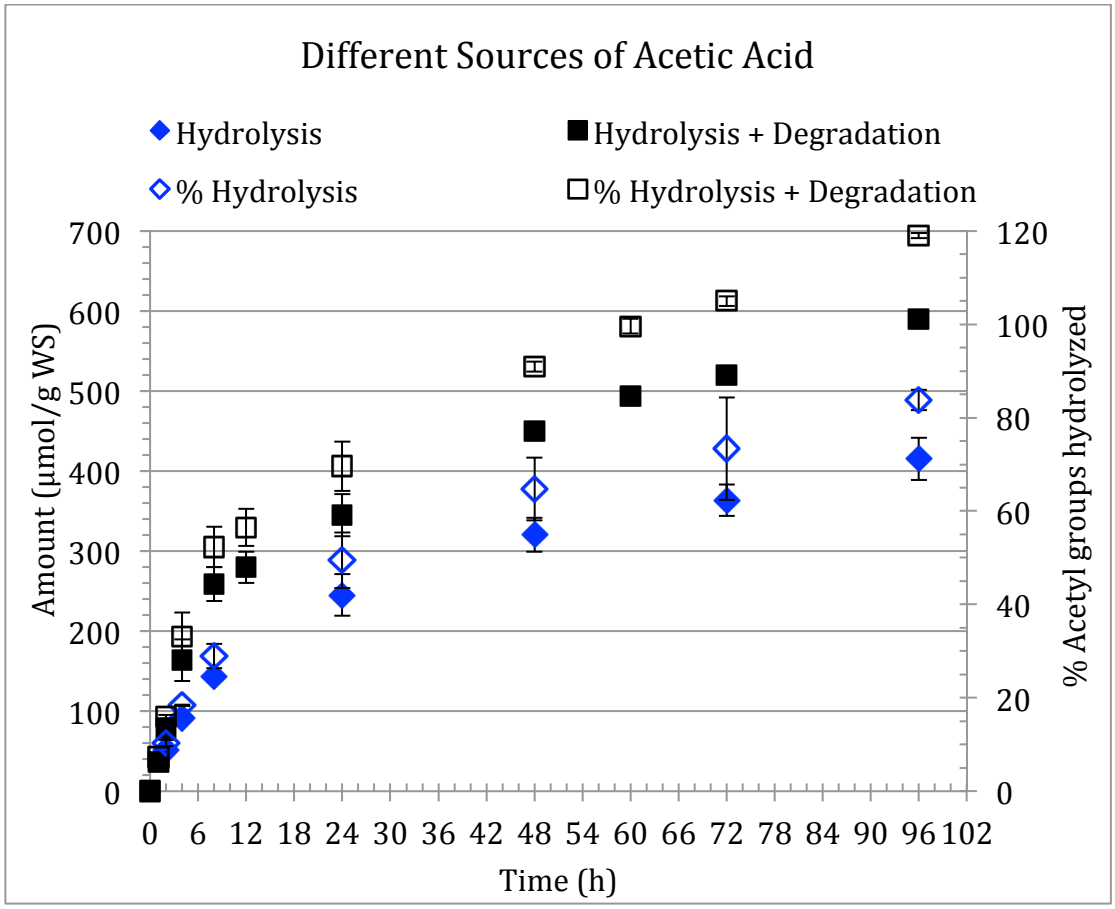

Figure 3.3. Comparison of time courses of acetic acid production from hydrolysis of acetyl groups (determined from acetyl groups remaining in the solid fraction) and hydrolysis + alkaline degradation (total acetic acid in the liquid fraction) by 2% wheat straw at pH 11.0, 30°C in a closed-system bioreactor. Solid-filled data points represent absolute amount of acetic acid; blank-filled data points represent percent theoretical of acetyl groups hydrolyzed. Error bars represent ± 1 standard deviations.

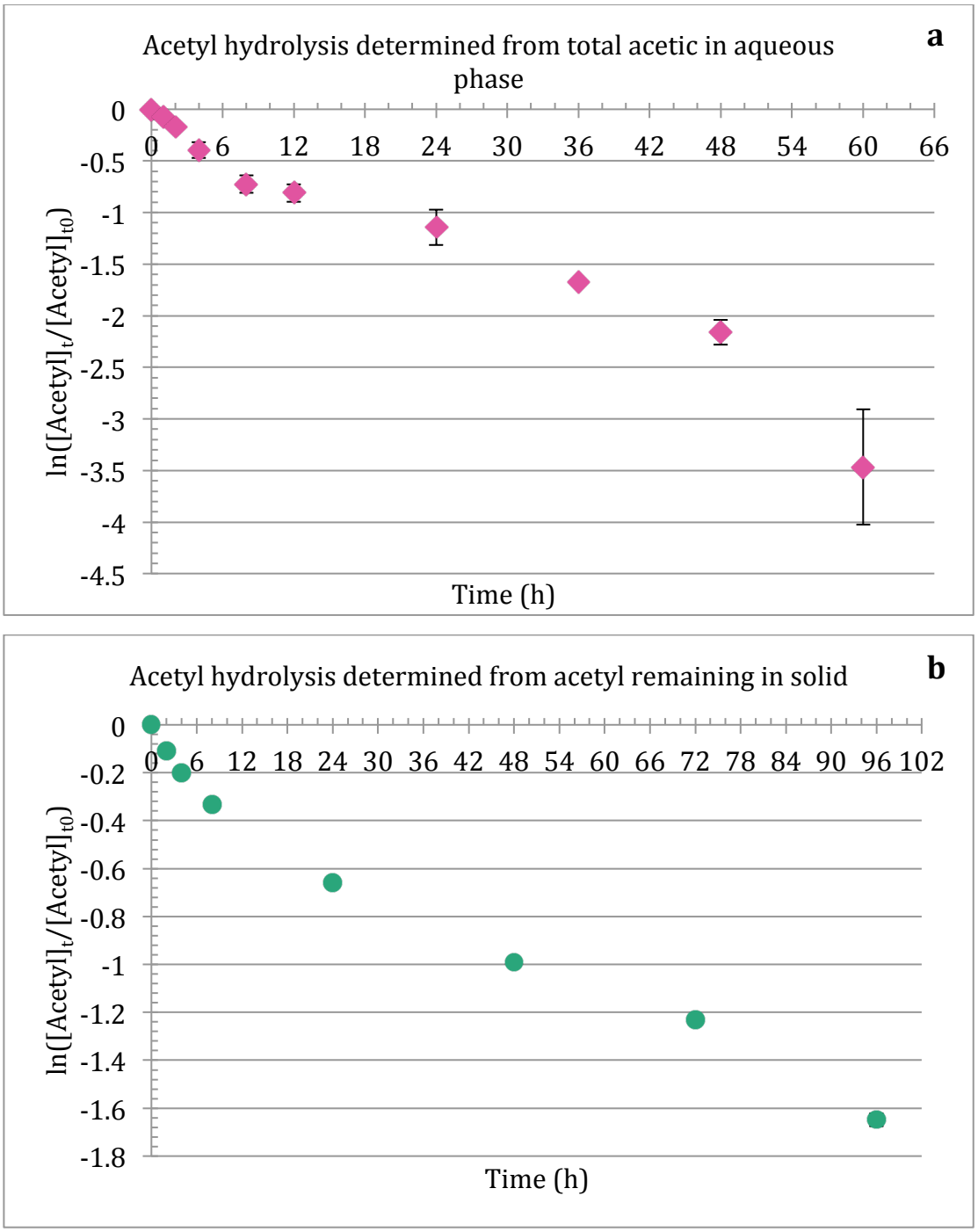

Figure 3.4. First-order reaction plots of acetyl groups hydrolysis by 2% wheat straw at pH 11.0, 30°C in a closed-system bioreactor. Acetyl hydrolysis determined from total acetic acid in aqueous phase (a). Acetyl hydrolysis determined from acetyl groups remaining in pretreated solid (b). [Acetyl] at starting time (t = 0 h) = 9.84 mM (equivalent to acetyl content in 2% raw wheat straw. Error bars represent ± 1 standard deviations.

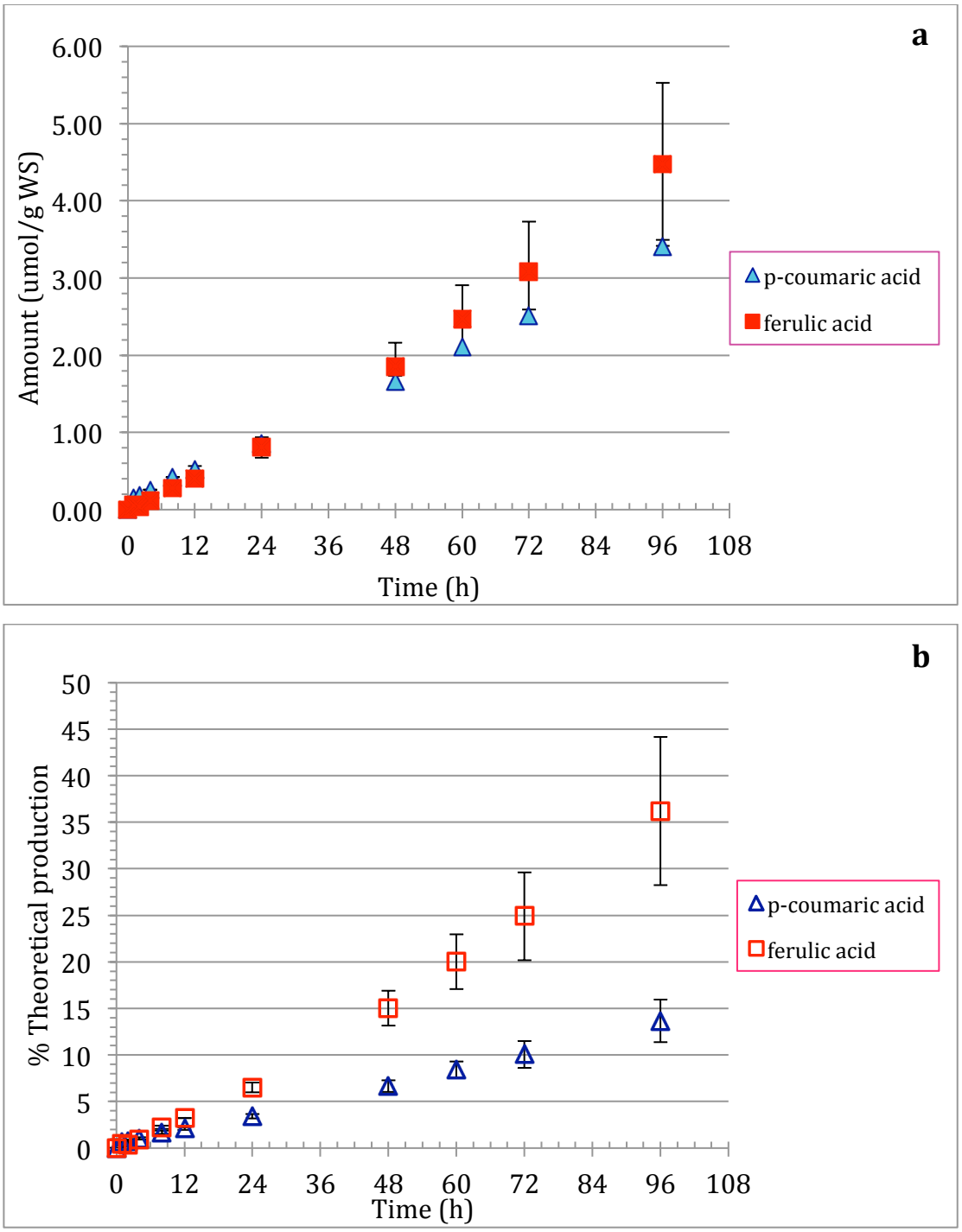

Figure 3.5. Comparison of time courses of two predominant esterified phenolic acid production in absolute amount (a); in percent theoretical production (b) by 2% wheat straw at pH 11.0, 30°C in a closed-system bioreactor. Error bars represent ± 1 standard deviations.

Table 3.4. Percent contribution of free and esterified buffer components in the liquid fraction at 96 h to alkali uptake by 2% wheat straw at pH 11.0, 30°C.

Buffer components	% of OH^- equivalent uptake
Acetic acid*	26.6
p-Coumaric acid	0.25 (if yielding 1:1 of H^+:OH^-) 0.50 (if yielding 2:1 of H^+:OH^-)
Ferulic acid	0.32 (if yielding 1:1 of H^+:OH^-) 0.64 (if yielding 2:1 of H^+:OH^-)
Total % of OH^- equivalent uptake contributed by acid derived from esterified-compounds	27.2 – 27.7

*Acetic acid derived from hydrolysis of acetyl groups excluding acetic acid derived from alkaline degradation.

Table 3.5. Probable alkaline degradation products in the liquid fraction at 96 h and their contribution to alkali uptake by 2% wheat straw at pH 11.0, 30°C.

Degradation product	Pretreated liquor at 96 h, pH 11.0, 30°C		% of OH^- equivalent uptake
	μmol/g WS	% by wt. WS	
Formic acid (MW = 46.03)	169 ± 47.6	0.78 ± 0.22	10.8
Acetic acid* (MW = 60.05)	174 ± 10.8	1.04 ± 0.07	11.2
Glycolic acid (MW = 76.05)	10.2 ± 1.99	0.08 ± 0.02	0.65
Lactic acid (MW = 90.08)	81.2 ± 0.004	0.73 ± 0.004	5.21
Total % of OH^- equivalent uptake contributed by degradation products			27.9

*Only acetic acid derived from acetic acid derived from alkaline degradation.

Note: Total of OH^- equivalent uptake contributed by acids determined in this study = 55.1-55.6%

4 GENERAL CONCLUSION

Alkaline pretreatment of wheat straw as reported in the two studies of this dissertation gave informative in point of view of science and effectiveness of how alkaline conditions affects the recalcitrance of complex, integrity, and resistant-destructed cell walls in order to enhance the digestibility of cell wall for sugar platform production. Enzymatic conversion barriers – lignin and hemicelluloses – were effectively reduced from the biomass after pretreatment with 5% NaOH at 50°C, 5 h and gave high glucan conversion yield up to approximately 90% after extended saccharification period. The condition of 5% NaOH at 50°C, 5 h has been selected as optimum condition for alkaline processing of wheat straw with the minimum carbohydrate loss due to alkaline degradation of carbohydrates partition in the solid fraction. Nevertheless, it has been known that alkaline pretreatment of lignocellulosic materials inevitably results in alkali consumption due to several chemical reactions catalyzed by hydroxide ions. The acids generated from the reactions neutralize hydroxide ions and lower the alkalinity of the system, thereby impair the effectiveness of the pretreatment. Characterization of alkali uptake during wheat straw processing at high pH and low temperature (low temperature was chosen to avoid complexity of chemical reactions) disclosed interesting insight that complexity and molecular arrangement of cell walls play an important role in structural and chemical alteration of cell wall with respect to pretreatment time. Surface accessibility enhances the removal of acetyl groups and phenolic compounds, as they are predominant esters in hemicellulose and lignin. Rapidly removal in the initial phase of alkaline pretreatment indicates amount of solvent accessible of those compounds. The prolong treatment suggests that swelling and distribution of esters in biomass matrix are the major factors in solvent accessibility to cleave the ester linkages and liberate those hydrolyzed esters into solution. In addition, alkaline degradation products are also considered as additional factor to alkali

uptake after extended pretreatment period used in this study although the condition was relatively mild. Accessibility and alkaline degradation might be possible causes that acid generation at later period did not obey first-order reaction as it showed with hydrolysis of ester models. The knowledge generated from this study is useful for understanding the nature of the alkali-induced chemistry that is at the heart of alkali processing of agricultural byproducts, foods, and forest products.

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APPENDICES

A. ADDITIONAL INFORMATION

- Wheat straw used in Chapter 2 was harvested in August 2008
- Wheat straw used in Chapter 3 was harvested in August 2004
- All chemical structures (based on SciFinder's database) were drawn using Chemsketch program

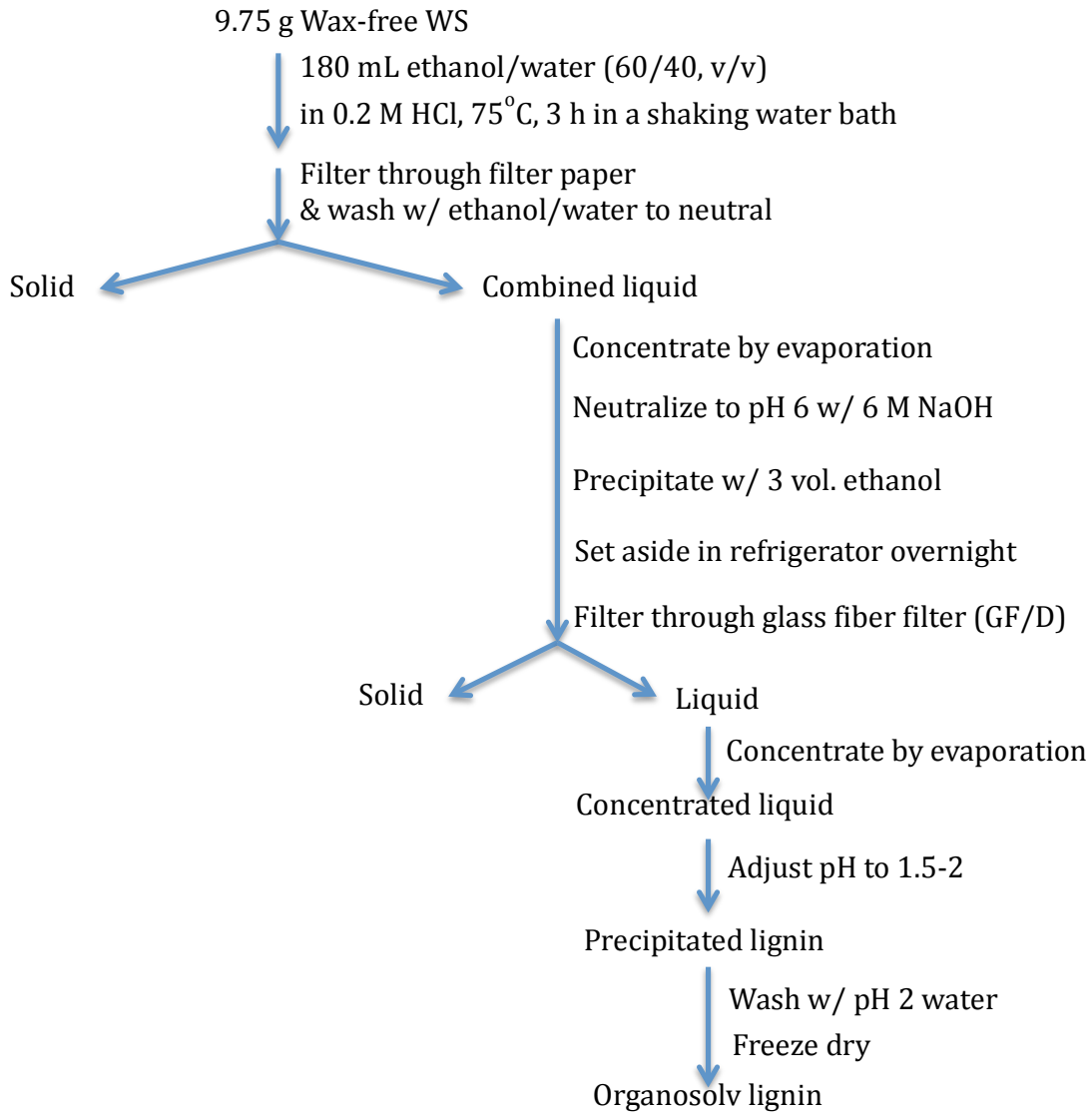


Figure A-1. Preparation of organolsolv lignin from wax-free wheat straw.

Table A-1. Mass percent of extractives with standard deviation in different fractions obtained from wheat straw.

Fraction	% Extractives
Water extract (30°C, 30 min, under N_2)	4.57 ± 0.16
Water extract (Soxhlet method, 24 h)	14.1 ± 1.40
Ethanol extract (following water extract; Soxhlet method, 24 h)	2.14 ± 0.11
Toluene/Ethanol extract (2:1, v/v; Soxhlet method, 6 h)	4.68 ± 0.02

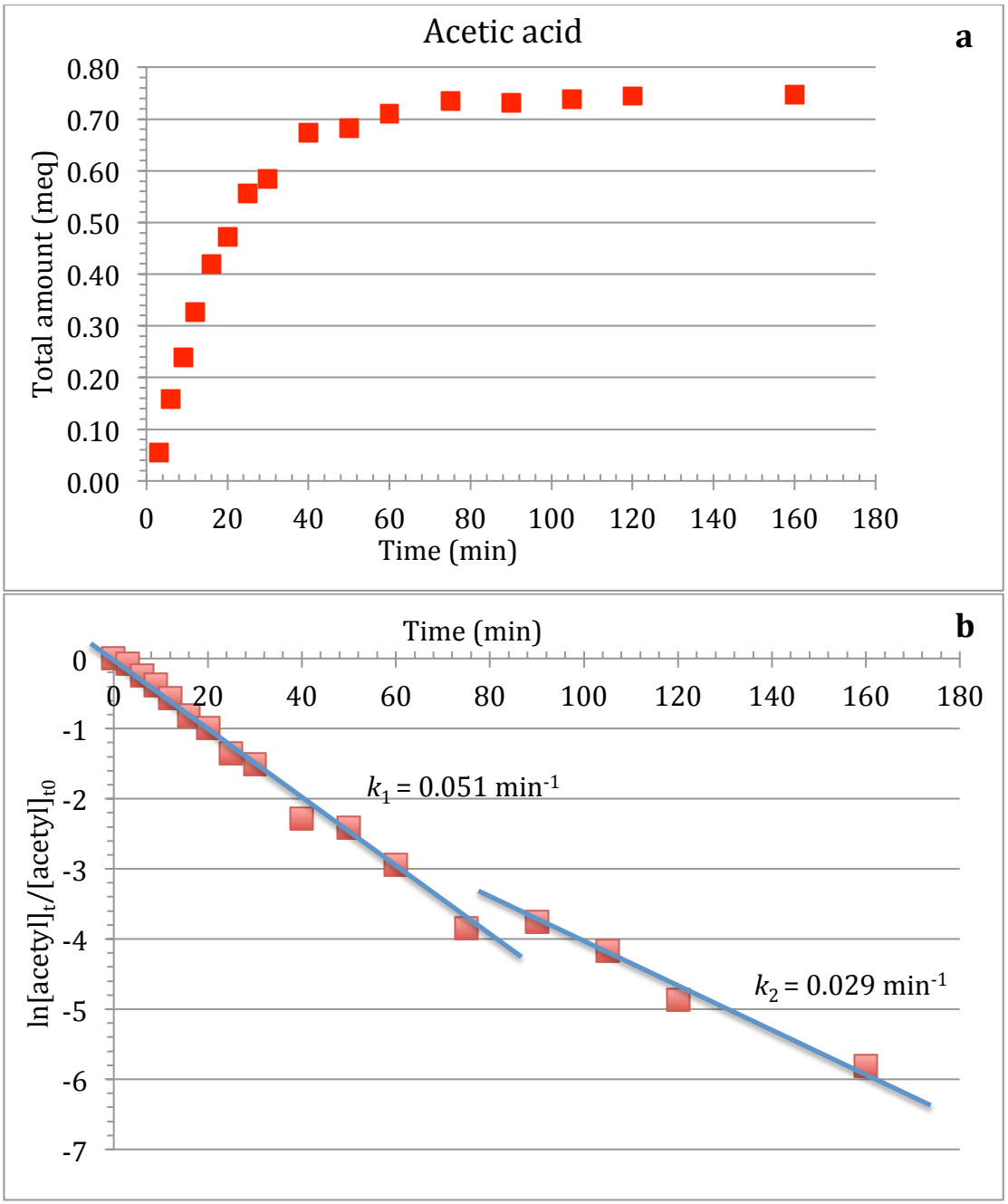


Figure A-2. (a) Comparison of relationship between time course of acetic acid production of 100 mL, 1 mM sucrose octaacetate at pH 11.0, 30°C (amount of acetyl groups equivalent to that of 2% wheat straw). (b) First-order reaction plot of deacetylation of acetyl groups in sucrose octaacetate at pH 11.00, 30°C. The first 75 min, the reaction rate was dominated by secondary type of acetyl groups; thereafter, the reaction rate was dominated by primary type of acetyl groups. Overall rate constant = 0.036 min^{-1}.

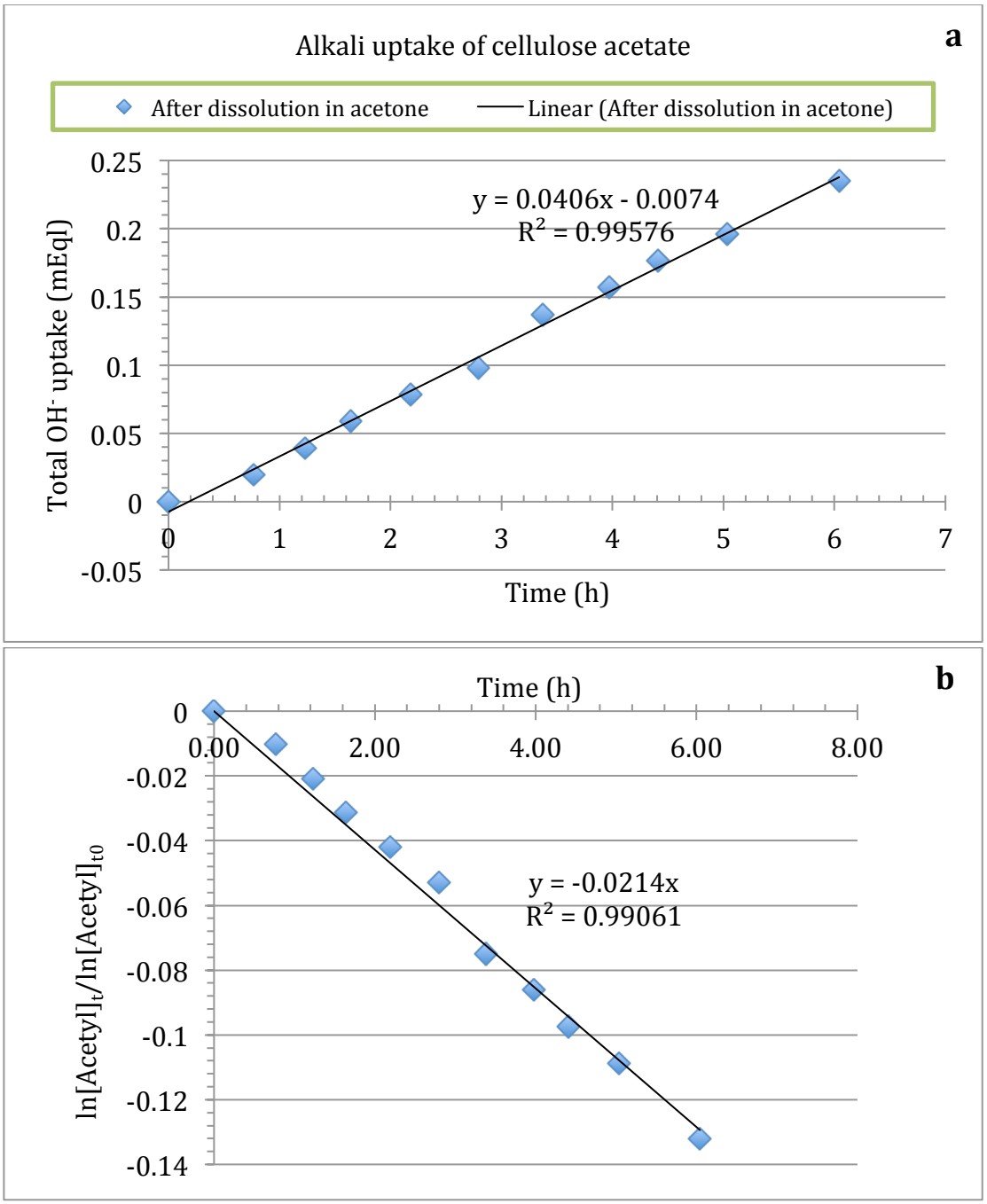


Figure A-3. (a) Effect of swelling on cellulose acetate in acetone on alkali uptake. Amount of acetyl groups in cellulose acetate equivalent to that of 4% wheat straw at pH 11.00, 30°C. (b) First-order reaction plot of hydrolysis of acetyl groups in swollen cellulose acetate at pH 11.00, 30°C. Amount of acetyl groups in cellulose acetate equivalent to that of 4% wheat straw.

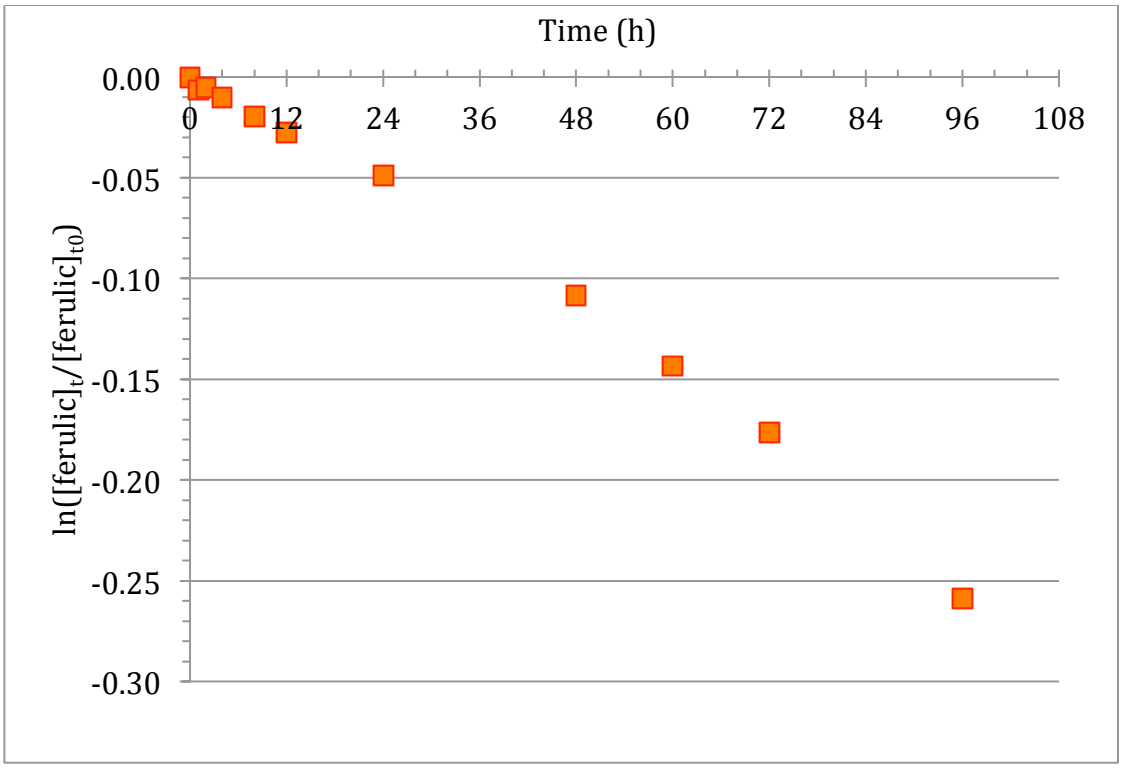


Figure A-4. First-order reaction plots of ferulic acid ester hydrolysis during alkaline processing of 2% wheat straw at pH 11, 30°C.

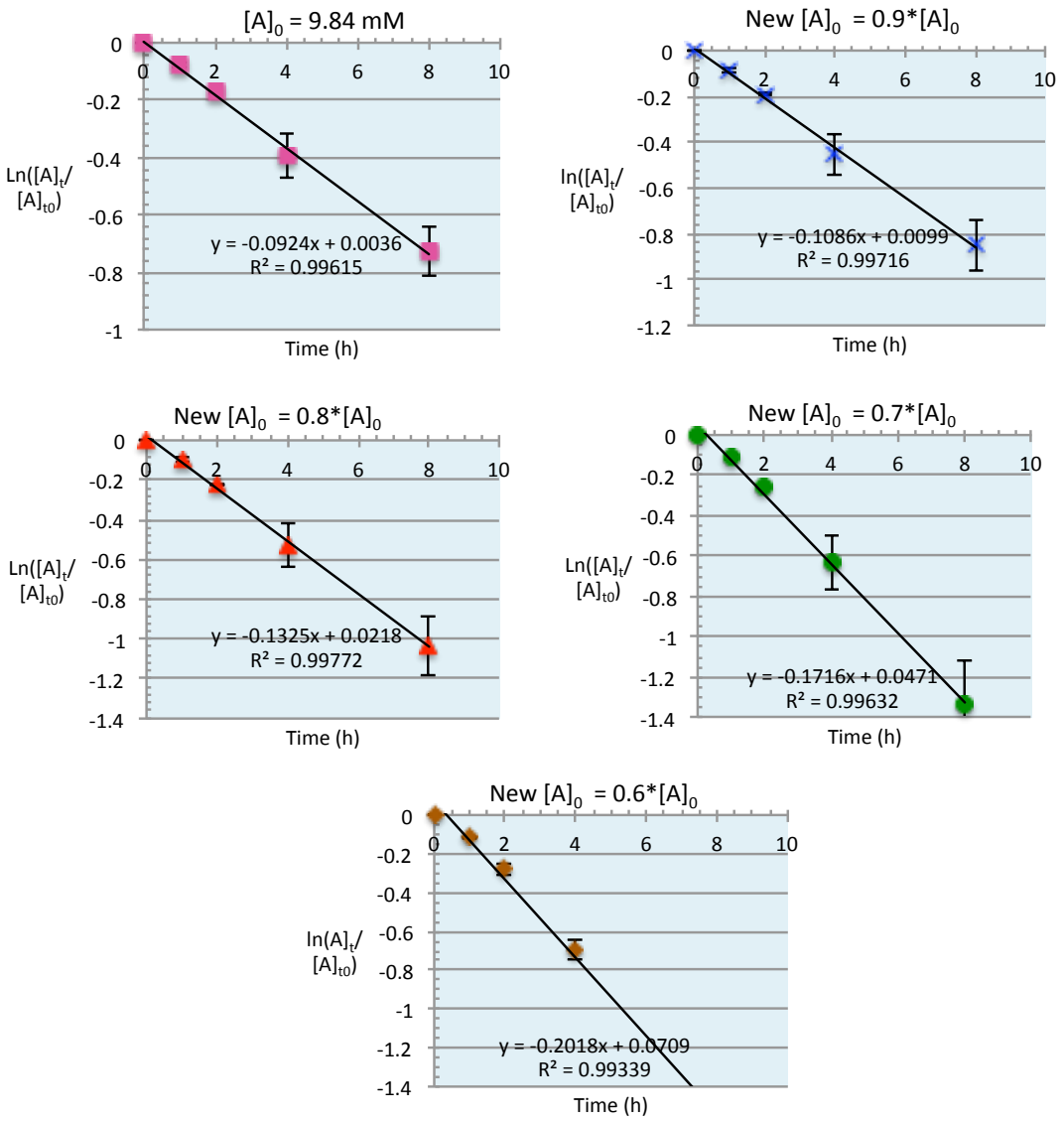


Figure A-5. Different first-order reaction plots of acetyl ester hydrolysis with respect to estimated various amount of acetyl groups as starting substrate in wheat straw. $[A]_o$ refers to molar concentration of acetyl groups at time = 0.

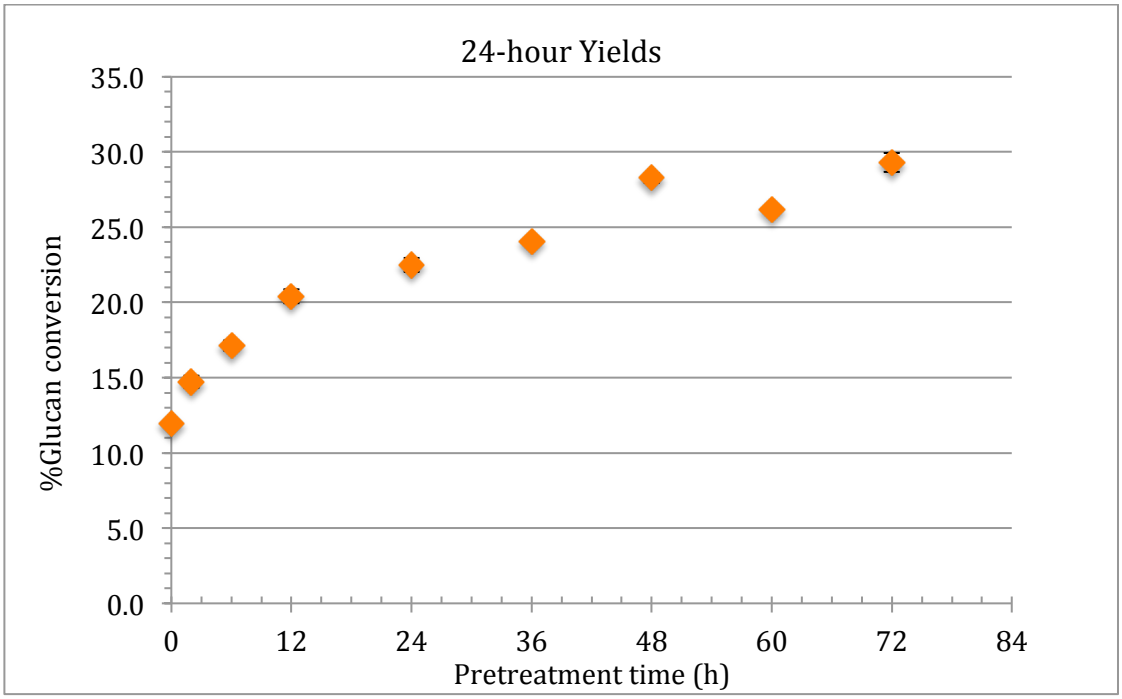


Figure A-6. Glucan conversion yields at 24 h of enzymatic saccharification of pretreated Wheat straw obtained from alkali buffer capacity experiment (pretreatment condition: pH 11.0, 30°C). Enzyme loading: Accellerase 1500 = 60 FPU/g glucan, containing β-glucosidase = 686 pNPGU/g glucan). Error bar represents ± 1 standard deviation.

Note: The digestibility test for pretreated wheat straw at pH 11.0, 30°C for 0-96 h under N_2 condition showed low glucan conversion only up to approximately 30% after saccharification with the cellulase preparation. Low glucan conversion may be caused by inhibitors, e.g., lignin, degradation products, etc., in the reaction aliquot since, after the alkaline pretreatment, the neutralization was achieved without separating the pretreated liquor. These inhibitors affect cellulase performance.

B. pH ELECTRODE CALIBRATION AND MAINTAINING

pH is a measurement of hydrogen ion concentration, $[H^+]$. In 1909, Soren Peter Lauritz Sorensen introduced the definition of pH as "the negative logarithm of the hydrogen ion concentration:

$$pH = -\log [H^+]$$

pH meters are a special type of millivolt meters with some added features. They convert electrode potential into pH units. Measurement of pH can be affected by temperature. It can change dissociation constants of the ions in the solution (or change hydrogen ion activity) can affect glass electrode potential (glass electrode resistance will change due to the change in solution temperature. As the temperature *increases*, the resistance across the glass bulb *decreases*. According to Nernst Equation as presented below, a pH value of a solution can change depending on temperature of the same solution being measured.

Nernst Equation

$$E = E_0 + 2.303 \frac{RT}{NF} \log A$$

Where:

E = measured electrode potential

E_0 = standard electrode potential

R = universal gas constant

F = Faraday constant

T = absolute temperature (K^o)

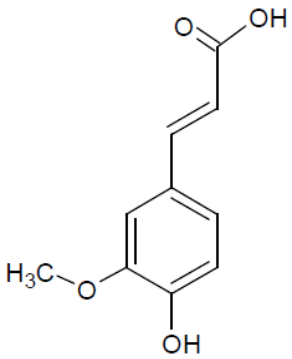
N = number of charges on the ion

A = activity of the ion being measured

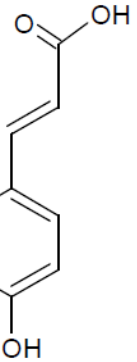
During calibration, pH meter will adjust pH electrode output potential according to the slope of Nurnst equation that varies depending on temperature of the sample solution. Temperature compensator can compensate for the change in slope with temperature. The slope will be adjusted to compensate for the inability of the measuring electrode to accurately produce its output signal. This can be achieved with a combination pH electrode with a built in temperature sensor or a separate temperature sensor. With the automatic temperature compensation (ATC), The meter automatically adjusts the slope to be correct in accordance with the Nernst equation at the measured temperature during calibration. ATC relies on the accurate measurement of the temperature of the solutions. This can be achieved by using an electrode with a built in temperature sensor or a separate temperature sensor. However, if an electrode does not have a temperature sensor, the compensation during calibration is done manually. Buffer solutions for the calibration have to be brought to the same temperature of the sample so that the meter adjusts the slope according to the actual temperature of the sample. Note that pH values of the buffer solutions for setting up the meter have to follow actual pH of that buffer at that temperature. Normally, commercial standard buffers have pH values at different temperatures on the label.

To maintain pH electrode function, short-term storage pH electrode between use is important. Commercial pH electrode storage solutions or 3 M KCl are good for this job. Since the inner filling solution of the electrode contains 3 M KCl, using the same chemical concentration for the outside glass bulb of the electrode prevents the chance of loosing ions from the inner solution to the outside solution. This keeps balance of the potential. Using deionized water as storage solution causes the leak of the ions from the inner solution of the electrode and creates potential resulting in error in pH measurement.

C. CHEMICAL STRUCTURES OF PHENOLIC ACIDS



Ferulic acid



p-coumaric acid

Figure C-1. Chemical structures of phenolic acids determined in this study.

D. pK_a OF PHENOLIC ACIDS AND PROTEIN SIDE CHAINS

Table D-1. pK_a values for phenolic acids in water at 25°C.

Compound	pK_a number	pK_a values
Gallic acid	1	4.24
	2	8.27
	3	9.23
Protocatechuic acid	1	4.38
	2	8.74
	3	10.67
p-Hydroxybenzoic acid	1	4.38
	2	8.97
Vanillic acid	1	4.31
	2	8.81
Syringic acid	1	4.20
	2	9.00
p-Coumaric acid	1	4.39
	2	8.37
Ferulic acid	1	4.56
	2	8.65

Erdemgil et al. (2007): Talanta. 72: 489-496.

Table D-2. pK_a values of the ionizable groups of proteins side chains in peptide.

Functional Group	pK_a	Reference
Asp (β-COOH)	3.9	Grimsley et al. (2009)
C terminus (α-COOH)	3.7	Thurlkill et al. (2006)
Glu (γ-COOH)	4.3	
His	6.5	
Cys (-SH)	8.6	
Tyr	9.8	
Lys (ε-NH_3^+)	10.4	
N terminus (α-amino, α-NH_3^+)	8.0	
Arg (-NH-C-NH_2)	12.3	Pace et al. (2009)

Grimsley et al. (2009): Protein Science. 18: 247-251.
Pace et al. (2009): Journal of Biological Chemistry. 284(20): 13285-13289.
Thurlkill et al. (2006): Protein Science. 15: 1214-1218.

Table D-3. pK_a values of glucose and uronic acid.

Compound	pK_a	Reference
Glucose (hydroxyl group)	12.3	Bunton et al. (1978)
Uronic acid (carboxyl group)	2.93	Wang et al. (1991)

Bunton et al. (1978): The Journal of Organic Chemistry. 43(10): 1925-1929.
Wang et al. (1991): Biochemistry Journal. 278: 689-695.