Conversion of Lignocellulosic Biomass into Monomeric Sugars and Levulinic Acid: Alkaline Pretreatment, Enzymatic Hydrolysis, and Decrystallization

by

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ABSTRACT

The technical feasibility of applying uniform pretreatment conditions across the different switchgrass varieties to achieve similar sugar yields was investigated. Four different types of switchgrass species including, Shawnee, Alamo-I and Alamo-II, and Dacotah were investigated using aqueous ammonia as a pretreatment reagent. A majority of the pretreatment experiments were carried out using Soaking in Aqueous Ammonia (SAA) method; while a selected few pretreatment experiments with Dacotah were carried out using Ammonia Recycle Percolation (ARP) method. SAA is a batch process, whereas ARP is a flow-through semi-batch process. The pretreated solids were subjected to enzymatic hydrolysis using 15 FPU/g-glucan of Spezyme-CP and 30 CBU/g-glucan of Novozymes-188. Hydrolysis results show that with the similar pretreatment conditions (90°C, 15% NH_4OH, liquid to solid ratio of 9, and 24 hr), Alamo-I, Alamo-II, and Shawnee resulted in substantially higher glucan/xylan (as high as 19%/13%) digestibility than the Dacotah. The optimum pretreatment conditions for Shawnee, Alamo-I, and Alamo-II were: 90°C, 15% NH_4OH, liquid to solid ratio of 9, and 24 hr. Increase in SAA pretreatment severity and applying ARP pretreatment increased the glucan/xylan digestibility of Dacotah, but also caused significant loss of carbohydrate content during pretreatment.

The majority of results available in the literature for pretreated solids report high saccharification yields using uneconomically high enzyme loadings. Therefore, the goal of this

work was to create a defined enzyme mixture that results in high yields of six and five carbon sugars from the pretreated switchgrass with the least amount of enzyme loading. The enzymatic hydrolysis experiments were carried out by applying various mixtures and levels of commercial enzymes including, Spezyme-CP (cellulase), Novozyme-188 (β-glucosidase), Multifect Xylanase (xylanase), and Multifect Pectinase (pectinase) with the solids produced from SAA pretreated Dacotah. Addition of β-glucosidase increased the glucan and xylan digestibilities with all levels of enzyme loading throughout the hydrolysis. Glucan and xylan digestibilities were rapidly increased approximately up to 25 mg protein/g-glucan with and without addition of β-glucosidase, after which the increase was gradual. The increase in the total protein loading of cellulase and β-glucosidase from 25 to 32 mg protein/g-glucan only increased the glucan/xylan digestibility by 4%/3%, whereas by supplementing same amount of xylanase and pectinase increased the glucan/xylan digestibility by 21%/22% and 10%/19%, respectively. Addition of β-glucosidase decreased the cellobiose, glucose and xylose oligomers. Supplementation of xylanase/pectinase only reduced xylose oligomer at 72 hr hydrolysis period.

Use of surface active additives is expected to reduce the enzyme dosage in the bioconversion processes by 20-40%, a significant economic benefit. Therefore, in this work to evaluate the effect of additives on enzymatic hydrolysis, different water soluble polymers were studied including, non-ionic (polyvinyl alcohol, polyvinyl pyrrolidone, and polyethylene glycol) and ionic (cationic polyacrylamide and polyethylene imine) polymers. In addition, to evaluate the effect of presence of lignin, various feedstocks including, Solka Floc and hardwood pulp (lignin free substrates), dilute sulfuric acid pretreated corn stover, dilute sulfuric acid pretreated switchgrass, aqueous ammonia pretreated switchgrass (lignin containing substrates) were used. The enzymatic hydrolysis experiments were carried out by applying 5 FPU/ g-glucan of C Tec 2.

Hydrolysis results indicate that with the supplementation of 10 mg polymer/g-substrate, glucan/xylan digestibilities were increased by 10-30% depending on type of the substrate and polymer. The non-ionic polymer (PVP, PVA and PEG) addition resulted in significantly higher hydrolysis yields than the ionic polymers (PEI and C-PAM) for all substrates. The main reasons for improvement in digestibility with polymer addition are improvement in cellulase activity and reduction in unproductive binding of cellulase to lignin.

Sulfur containing reagents are highly reactive with lignin, thus can serve as delignification enhancers. Therefore, the effect of adding small amount of sodium sulfide in alkaline pretreatment of Dacotah switchgrass and palm residue was investigated. The alkaline reagents tested were either recoverable ones (ammonia, sodium carbonate) or inexpensive one (lime). Pretreatments were primarily carried out in batch mode under low severity conditions: 90-120°C, 12-24 hr treatment time, 0-15% alkali, 0-3% sodium sulfide, and liquid to solid ratio of 9. The enzymatic hydrolysis experiments were carried out by applying 5 FPU/ g-glucan of C Tec 2. Addition of sodium sulfide did not affect the carbohydrate content, but increased the delignification by 14-42%. Addition of sodium sulfide substantially increased the digestibility for all the alkali treatments: 15-45% in glucan digestibility and 10-40% in xylan digestibility depending on type of substrate and pretreatment severity.

Levulinic acid (LA) is an ideal platform chemical that can be utilized to produce a number of bio-chemicals. However, the majority of the results reported in the literature are about LA production from monomeric sugars/lignocellulosics using dilute sulfuric concentrations (1-5% (w/v)), low substrate loadings (1-5% (w/v)), and at high temperatures (160-220^{o}C). Thus, this work mainly focused on evaluating the technical feasibility of production of LA using high substrate loadings (10-20%), moderate acid concentrations (10-25%), and at low temperatures

(80-140^{o}C). LA production from different soluble sugars (glucose, fructose, sucrose, galactose, and mannose), lignocellulosic substrates (Solka Floc, corn stover, pretreated corn stover, pine, and municipal solid waste), and starch was studied. Study shows that it is technically possible to obtain high LA yields with moderate acid concentrations and at low temperatures from different substrates. All the substrates irrespective of their characteristics produced 60% or more LA molar yields with 20% H_2SO_4, 12% initial solids, and at 120^{o}C. Molar yields obtained from different substrates are comparable or better than the literature reported yields on the basis of g-H_2SO_4/g-substrate. The primary difference between lignocellulosic substrates and soluble sugars is initial LA production rates. The initial LA production rates of lignocellulosic substrates are slower than the soluble sugars. Among the soluble sugars, fructose produced the highest LA yield (70.5%) and among the lignocellulosic substrates it was with Solka Floc (64.5%), whereas starch yielded 68% LA.

Decrystallization of cellulose is an exothermic process because the hydrogen bonds are broken and the bond energy is released. Therefore, the temperature is expected to increase if there is decrystallization. Thus, this study focused on experimental verification on detecting decrystallization of cellulose by tracing the temperature in a system where pure crystalline cellulose is treated with sulfuric acid. Different substrates were used including, pure cellulose (Avicel PH 101), sulfuric acid treated pure cellulose (decrystallized), cellobiose, and glucose, to differentiate the extent of energy release by two different types of bonds. Decrystallization of cellulose occurs at room temperature with sulfuric acid up to 55-72%. Avicel crystallinity decreased substantially with increase in the acid concentration at room temperature and the decrease in crystallinity was from 67.9% to 4.2%. A sharp increase in temperature between Avicel-acid and Avicel-water could be observed within initial few seconds (15-25 seconds) with sulfuric acid up to 55-72%. However, the increase in temperature due to heat release was not entirely from decrystallization (hydrogen bond energy); a part of heat also came from hydrolysis of decrystallized cellulose to low DP cellulose and/or glucose. The heat releasing patterns and the crystallinity indices of 5 min and 30 min acid treated samples corroborate the fact that the higher the crystallinity, the higher the heat release. Initial enzymatic hydrolysis rates of 65-72% acid treated Avicel at room temperature were sharply increased, while rates were relatively sharper with 60% acid treated Avicel than with 55% acid treated and untreated Avicel. Data proves that decrystallization occurs at acid concentrations below 55%, if proper temperature is chosen. Avicel treated with 50% H_2SO_4 and at 90^{o}C for 5 min released heat significantly and the loss in crystallinity was by 9%.

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List of Abbreviations

AFEX	Ammonia Fiber Expansion
ARP	Ammonia Recycle Percolation
ASG	Aqueous Ammonia treated switchgrass
BET	Brunauer Emmett Teller
CAFI	Consortium for Applied Fundamentals and Innovation
CIR	Cave-in-Rock
CBU	Cellobiase Units
CPAM	Cationic Poly Acrylamide
CS	Corn Stover
DOE	Department of Energy
DP	Degree of Polymerization
DSG	Dacotah Switchgrass
FA	Formic Acid
FPU	Filter Paper Units
GC	Gas Chromatography
HMF	Hydroxy Methyl Furfural
HP	Hardwood Pulp

HPLC	High Pressure Liquid Chromatography
LA	Levulinic Acid
MSW	Municipal Solid Waste
MW	Molecular Weight
N	Novozyme-188
NREL	National Renewable Energy Laboratory
OSX	Oat Spelt Xylan
P	Multifect Pectinase
PCS	Dilute sulfuric acid treated corn stover
PEG	Poly Ethylene Glycol
PEI	Poly Ethylene Imine
PSG	Dilute sulfuric acid treated switchgrass
PVA	Poly Vinyl Alcohol
PVP	Poly Vinyl Pyrrolidone
S	Spezyme CP
SAA	Soaking in Aqueous Ammonia
SEM	Scanning Electron Microscopy
SF	Solka Floc
SRY	Sugar Recovery Yield
X	Multifect Xylanase

Introduction

Building a sustainable world requires substantial reduction in dependency of fossil reserves and lowering the amount of pollution that is being generated. Production of biofuels and biochemicals from lignocellulosic biomass, the most abundant organic chemical on the surface of the earth, is an area in which these two goals can be achieved simultaneously. Practically viable and economic conversion of lignocellulosic biomass into biofuels and value added biochemicals is an important challenge that requires the effective utilization of sugars and lignin and integrated processing (Werpy et al., 2004). The biofuels and biochemicals can offer significant environmental, economic, and strategic advantages (Lynd et al., 2008).

Monomeric sugars glucose and xylose are building blocks for the biofuels and many value added biochemicals. Polymeric cellulose and hemicelluloses present in the lignocellulosic can be converted into their respective monomeric sugars through pretreatment route followed by enzymatic hydrolysis (Mosier et al., 2005; Wyman et al., 2005; Wyman et al., 2011). Pretreatment is applied to disrupt the cell wall structure of the lignocellulosic biomass in order for enzymes to access cellulose and hemicelluloses effectively during the enzymatic hydrolysis. Various factors influence the effectiveness of the pretreatment including, chemical, feedstock, temperature, and time, etc. Over the years different pretreatment methods have been attempted including, physical (ball milling, steam), physico-chemical (SO_2-steam, AFEX), chemical (acid, alkaline, hot water), and biological (Hsu, 1996; Schell et al., 1998; Zheng et al., 1998; Boussaid et al., 2000; Mosier et al., 2005; Mohan et al., 2006). However, recent years a lot of emphasis has been put on the alkaline pretreatment. In the alkaline pretreatment, it is technically feasible to retain most of the carbohydrates, while removing a large fraction of lignin in the biomass and the enhancement in hydrolysis yields is primarily caused by lignin removal. In addition, retention of hemicellulose after the pretreatment gives a significant economic benefit since it eliminates the need for detoxification of hemicellulose sugars. Some of the proven alkaline pretreatment reagents are: lime, sodium hydroxide, aqueous ammonia, and sodium carbonate (Kaar and Holtzapple, 2000; Kim et al., 2003; Kim and Lee, 2005; Mosier et al., 2005; Gupta and Lee, 2009; Gupta and Lee, 2010; Yang et al., 2012). Ammonia, being one of the milder alkali reagents, has advantage over other alkaline reagents due to ease in recovery because of its high volatility, cost effectiveness compared to sodium hydroxide, and non-corrosiveness.

After evaluating several potential herbaceous crops, the US Department of Energy (DOE) chose switchgrass as one of the promising species for bioenergy production because of its potential for high yields, wide range of distribution, and beneficial environmental characteristics (McLaughlin et al., 1999; Lewandowski et al., 2003). Because of these reasons, switchgrass has been selected as a feedstock in Consortium for Applied Fundamentals and Innovation (CAFI-III) project for studying its conversion characteristics with different pretreatment technologies (Wyman et al., 2011). Different factors affect the switchgrass composition and yield including, plantation and harvest time, genotype (lowland vs. upland), ecotype (southern vs. northern), precipitation, fertilizer, type of storage, and other environmental conditions (Sanderson et al., 1999; Casler et al., 2004; Jefferson and McCaughey, 2012).

Four different switchgrass (Alamo-I, Alamo-II, Dacotah and Shawnee) and two different ammonia based pretreatment methods (SAA, ARP) were used in the first part of this dissertation. The objectives of this study were to examine how the seasonal harvest time (Fall vs. Spring) and cultivars (upland vs. lowland) of switchgrass affected the biochemical conversion of switchgrass processed by aqueous ammonia pretreatment, and to evaluate the technical feasibility of uniform pretreatment conditions across the different switchgrass varieties to achieve similar sugar yields. A majority of the pretreatment experiments were carried out using SAA method; while a selected few pretreatment experiments with Dacotah were carried out using ARP method. SAA experiments were carried out at 60-160^{o}C and long reaction time (1-48 hr) in a batch reactor, whereas ARP experiments were carried out at high temperature 150-190°C and short residence time (10-20 minutes) in a flow-through reactor. The pretreated solids were subjected to enzymatic hydrolysis using cellulase (15 FPU/g-glucan of Spezyme-CP) and β-glucosidase (30 CBU/g-glucan of Novozymes-188). The performance of these feedstocks after subjecting to aqueous ammonia pretreatment and enzymatic hydrolysis has been compared. This comparison helped in understanding the role of harvest time and compositional properties of switchgrass on the overall sugar yield.

After the pretreatment, still many challenges limit the efficiency of enzymatic hydrolysis process of few substrates (in the present study Dacotah switchgrass), challenges including, several substrate characteristics and a number of enzyme factors (Chang and Holtzapple, 2000; Kim and Holtzapple, 2006; Chandra et al., 2009; Kim et al., 2011; Garlock et al., 2011; Wyman et al., 2011). Therefore, to overcome these challenges, increase in pretreatment severity and large amounts of hydrolytic enzymes have been employed, but both the alternatives reported to be resulting in high processing cost (Himmel et al., 2007; Tao et al., 2011). Thus, in the present study, three different methods were attempted to improve enzymatic hydrolysis efficiency of Dacotah switchgrass: applying a defined enzyme mixture that results in high yields of six and five carbon sugars from the SAA pretreated switchgrass with the least amount of enzyme loading, addition of small amounts of water-soluble polymers during enzymatic hydrolysis, and addition of small amount of sodium sulfide during alkaline pretreatment.

The majority of data available for pretreated solids report high saccharification yields using uneconomically high enzyme loadings (Himmel et al., 2007). Non-cellulosic components in the pretreated biomass, particularly hemicelluloses and lignin, exert significant restraints on cellulose hydrolysis (Berlin et al. 2005b; Borjesson et al., 2007a; 2007b). In addition, oligomers produced from hemicelluloses inhibit the activity of cellulase enzymes (Qing and Wyman, 2011). One way to improve the efficiency of enzymatic hydrolysis by minimizing aforementioned inhibitions is to adjust the enzyme formulation (Berlin et al., 2005a). Thus, the second part of this dissertation focused on evaluating the effects of the enzyme mixture on SAA pretreated Dacotah switchgrass. Different factors were evaluated including, identification of a suitable β-glucosidase level to a fixed cellulase loading, application of high cellulase loading with and without supplementation of β-glucosidase, supplementation of xylanase/pectinase. In addition to improving the glucan and xylan digestibilities, emphasis was also put on cellobiose and oligomers of glucose/xylose present at the initial and end of the hydrolysis.

The use of additives has been shown to enhance the enzymatic hydrolysis of lignocellulosics (Kaar and Holtzapple, 1998; Eriksson et al., 2002; Yang and Wyman, 2006). Although the use of additives adds additional cost to biofuel or biochemical production process, clear benefits have been shown. Benefits are reduction in unproductive binding to lignin that can make effective use of enzymes, and decrease in enzyme loading (Borjesson et al., 2007a; 2007b).

So far most of the additives studied have been surfactants (Kaar and Holtzapple, 1998; Eriksson et al., 2002; Yang and Wyman, 2006). In recent years, the focus has been shifted to other additives such as proteins (bovine serum albumin) (Yang and Wyman, 2006), polymers such as polyethylene glycol (PEG) (Borjesson et al., 2007a; 2007b; Kristensen et al., 2007; Ouyang et al., 2009), polyvinyl alcohol (PVA) (Hata, 2011), cationic polyacrylamide (Mora et al., 2011). However, the majority of reported results using polymer addition are about PEG. Therefore, third part of this dissertation focused on the effect of addition of different water-soluble polymers (polyvinyl alcohol, polyvinyl pyrrolidone, polyethylene glycol, cationic polyacrylamide, and polyethylene imine) on enzymatic hydrolysis of SAA treated Dacotah switchgrass. In addition, effect of polymer with and without lignin was also evaluated using various lignin containing substrates (aqueous ammonia pretreated Dacotah switchgrass and dilute sulfuric acid pretreated corn stover/switchgrass) and lignin free substrates (Solka Floc and hardwood pulp).

Addition of small amounts of sodium sulfide to the alkaline treatment is known to reduce the carbohydrate loss and improve the delignification (Gierer, 1986). Sulfur based compounds increase the cleavage of phenolic β-ether linkages and carbon-carbon linkages. They also cause fragmentation of lignin and condensation of the lignin fragments (Gierer, 1986). Sodium carbonate pretreatment with the addition of sodium sulfide (green liquor pretreatment) has been studied by several authors (Gu et al., 2012; Yang et al., 2012; Gu et al., 2013). However, the sodium sulfide addition to other alkaline reagents such as aqueous ammonia and lime has not been studied so far. Therefore, in the fourth part of this dissertation, effect of the addition of a small amount of sodium sulfide to alkaline pretreatment of biomass was studied. Two different feedstocks were used including, Dacotah switchgrass and palm residue. The alkaline reagents tested were either recoverable ones (ammonia and sodium carbonate) or inexpensive one (lime).

In recent years attention has been growing on the utilization of soluble sugars and lignocellulosic biomass to produce value added biochemicals. Levulinic acid is one such value-added chemical. LA is produced through acid-catalyzed dehydration of hexose sugars. It is an ideal platform chemical that can be utilized to produce a number of chemicals (Rackemann and Doherty, 2011). However, the majority of the results reported in the literature are about LA production from monomeric sugars/lignocellulosics using dilute sulfuric concentrations (1-5% (w/v)), low substrate loadings (1-5% (w/v)), and at high temperatures (160-220^{o}C) (Hayes et al., 2006; Girisuta et al., 2006; 2007; Galletti et al., 2012). Farone and Cuzens (2000) invented a process in which LA is produced from paper at high substrate loadings (20-40%). However, their process used multiple steps to process such high substrate concentrations. Thus, the fifth part of this dissertation focused on using high substrate loadings (10-20%), moderate acid concentrations (10-25%) and at low temperatures (80-140^{o}C) in a single step. Different soluble sugars (glucose, fructose, sucrose, galactose, and mannose), lignocellulosic substrates (Avicel, corn stover, pretreated corn stover, pine, and municipal solid waste), and starch were studied.

The degree of cellulose crystallinity is a major factor affecting the enzymatic or acid hydrolysis of cellulose (Ioelovich, 2012). The reduction in cellulose crystallinity improves the cellulose hydrolysis by cellulase, especially the initial rate (Laureano-Perez et al., 2005). Pretreatments alter the cellulose crystalline structures by disrupting inter/intra hydrogen bonding of cellulose chains. Decrystallization is an exothermic process because the hydrogen bonds are broken, and the bond energy is released. Therefore, the temperature is expected to increase if there is decrystallization. Although some researchers reported cellulose decrystallization followed by solubilization with different acid concentrations and temperatures (Camacho et al., 1996; Ioelovich, 2012), but to our knowledge so far nobody has reported cellulose decrystallization by heat releasing pattern and relating that with crystallinity. Therefore, this work focused on detection of sulfuric acid induced decrystallization by instantaneous tracing of the temperature change caused by breakage of hydrogen bonding.

1.1. Objectives

- Study the technical feasibility of uniform pretreatment conditions across the different switchgrass varieties to achieve similar sugar yields
- Create a defined enzyme mixture that results in high yields of six and five carbon sugar from the pretreated switchgrass with the least amount of enzyme loading
- Evaluate the effect of addition of water-soluble polymers during enzymatic hydrolysis of lignocellulosic biomass
- Study the effect of adding a small amount of sodium sulfide in alkaline pretreatment of lignocellulosic biomass
- Evaluate the production of levulinic acid from soluble sugars and lignocellulosic substrates using moderate sulfuric acid concentrations, high substrate loadings, and at low temperatures in a single step
- Understand the fundamental aspects of decrystallization of cellulose

2. Literature Review

2.1. Biofuels and bio-chemicals

The world is faced with dwindling fossil reserves, and the most heavily used resources, petroleum, has the lowest supplies that are nearing a point of reduced production. Building a sustainable world requires a substantial reduction in dependency of fossil reserves and lowering the amount of pollution that is generated. While renewable-power technologies, such as wind (Kaldellis and Zafirakis, 2011) and solar (Chu and Meisen, 2011), may have major roles in the future for the production of electricity, provision must still be made for the supply of industrial chemicals and fuels that are currently produced predominately from petroleum. Production of biofuels and biochemicals from lignocellulosic biomass, the most abundant organic chemical on the surface of the earth, is an area in which these goals can be achieved simultaneously. Practically viable and economic conversion of lignocellulosic biomass into biofuels and other biochemicals is an important challenge that requires integrated processing and the effective utilization of sugars and lignin (Werpy et al., 2004; Wettstein et al., 2012). Biofuels and biochemicals can offer significant environmental, economic, and strategic advantages when implemented properly (Lynd et al., 2008).

Monomeric sugars glucose and xylose are building blocks for biofuels and many value added biochemicals (Figure 2.1). Polymeric cellulose and hemicelluloses present in lignocellulosic biomass can be converted into their respective monomeric sugars through pretreatment followed by enzymatic hydrolysis (Figure 2.2) (Mosier et al., 2005; Kim et al., 2011; Wyman et al., 2011). Biofuels by definition are fuels that are generated from biological material, a concept that has recently been narrowed down to renewable sources of carbon. Ethanol is one of the best known biofuels in the Americas, although other biofuels, mostly biodiesel, are commonly used in other parts of the world such as Europe, Asia, and increasingly in Brazil. Both ethanol and biodiesel are considered as first-generation biofuels, although other types of biofuels like cellulosic algae based ethanol are emerging, which could be characterized as second and third-generation biofuels (Lee and Lavoie, 2013). First generation biofuels were produced from starches (corn, wheat, etc.) or sugars (sugar cane, energy cane, etc.) (Wheals et al. 1999; Erdei et al., 2010; Goldemberg and Guardabassi, 2010; Dias et al., 2011). These are easily digested by enzymes, and converted to ethanol using microorganisms such as *Saccharomyces cerevisiae* or *Escherichia coli.* Second generation biofuels use lignocellulosic biomass such as agricultural residues, wood, herbaceous crops, municipal solid wastes and paper mill sludge wastes. The production of second generation biofuels does not compete against farming for agricultural area, avoids the “food or fuel” conflict (Lavigne and Powers, 2007; Ajanovic, 2011). Biofuels and biochemicals can offer significant environmental, economic, and strategic advantages when implemented properly (Lynd et al., 2008). Second generation feedstocks have distinct advantages over the first generation feedstocks since they have a much higher biomass yield per acre, and make use of wastes from various sources (Lavigne and Powers, 2007; Blanco-Canqui, 2010; Keshwani and Cheng, 2009). The most accepted definition for third-generation biofuels is fuels that are produced from algal biomass, which has a very distinctive growth yield as compared with starch and lignocellulosic biomass. Production of biofuels from algae usually relies on the lipid content of the microorganisms (Lee and Lavoie, 2013).

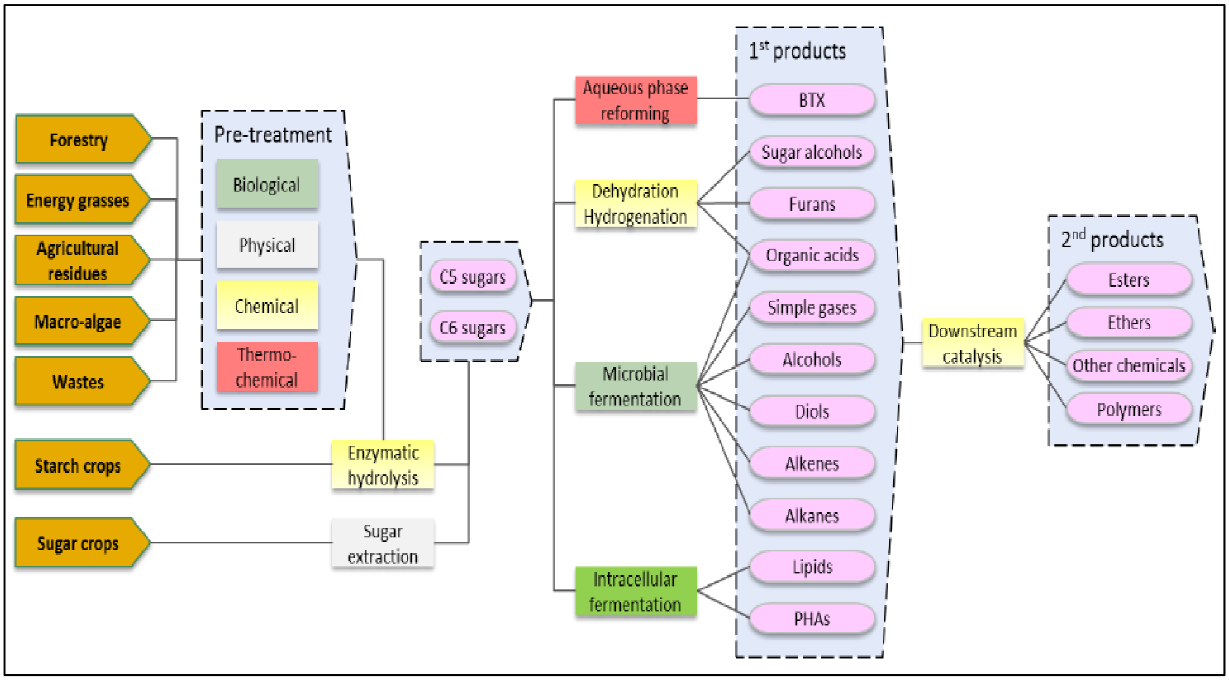

Figure 2.1: Different biofuel and biochemical pathways from biomass (Taylor et al., 2015).

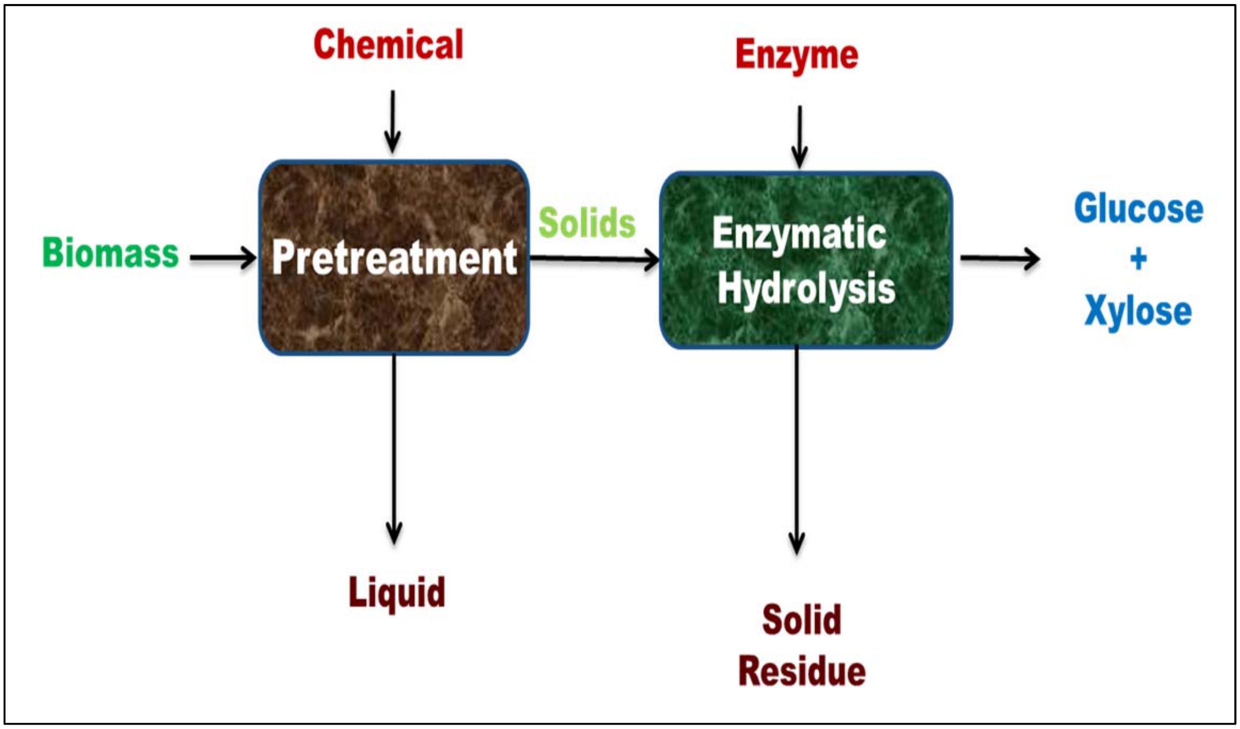

Figure 2.2: Conversion biomass to monomeric sugars

2.2. Lignocellulosic biomass and its composition

Lignocellulosic biomass is an abundant and diverse renewable resource and is the major constituent of most plant life. Examples of lignocellulosic biomass are agricultural residues, herbaceous crops, wood crops, municipal solid wastes and paper mill sludge wastes. Examples of agricultural residues include corn stover, wheat straw, and rice straw; herbaceous crops include switchgrass, Miscanthus, eastern gama grass, giant reed, maize, reed canary grass, sorghums (Lemus and Parrish, 2009). These feedstocks are easier to convert into fuels and chemicals compared to the woody biomass due to their lower lignin content. Herbaceous crops are grown for the sole purpose of using them for energy production (Adler et al., 2009). These are fast growing crops, which require minimum nutrients and can be cultivated on fallow, non-agricultural lands. Dedicated energy crops are an attractive solution to the high demand of lignocellulosics as they can also be genetically modified to obtain better quality of raw material for the biofuels and biochemical industry that show higher conversions to ethanol and bio-based chemicals.

Woody crops (softwoods and hardwoods) proven to be more recalcitrant than herbaceous biomass (Gupta and Lee, 2010). Softwoods include redwood, pine, and Douglas fir, while examples of hardwoods are maple, oak, elm, and aspen. Among woody crops, softwood species generally show higher recalcitrance toward external chemical and enzymatic attack compared to the hardwood species of wood because of the lignin level (Alkasrawi et al., 2003; Mohsenzadeh et al., 2012). Softwoods have longer structural and conducting fibers while the hardwoods have shorter fibers. The degree polymerization (DP) of softwood lignin is higher than that of hardwood lignin. Softwood lignin is derived from guaiacyl units which mainly originate from transconiferyl alcohol and ρ-coumaryl alcohol. Hardwood lignin is composed of guaiacyl, syringyl and ρ-coumaryl alcohols.

Municipal solid wastes, waste sludge from pulp and paper mills, recycled paper contain a high percentage of cellulose and low lignin by weight and hence are very good substrates for biofuel production. However, since they contain many inorganic additives inherent to their source, they also require a pretreatment step before enzymatic hydrolysis not unlike any of the other feedstocks (Holtzapple et al., 1992).

Lignocellulosic biomass is composed of cellulose, hemicellulose, lignin, extractives, proteins, inorganics, and silicon dioxide. The main components of the woody and non-woody feedstocks consist mainly of cellulose, hemicellulose, and lignin. All three components can be used to produce different value-added products depending on their end use (Zahedifar, 1996; Lejeune and Deprez, 2010). Table 2.1 shows the percentage major components present in different feedstocks (Burvall, 1997).

Table 2.1: Composition of different feedstocks

Feedstock	**Dry biomass (wt. %)**		
	Cellulose	**Hemicellulose**	**Lignin**
Grasses	25-40	35-50	17-30
Straws	30-40	20-30	15-25
Hardwood	40-55	20-40	18-25
Softwood	45-50	25-35	25-35
MSW*	35-50	10-25	15-30
Waste Paper	60-70	10-20	5-10

* Determined in our laboratory

2.2.1. Cellulose

Cellulose molecule is a linear, crystalline, homo-polymer with a repeating unit of D-glucose formed together by β-glycosidic linkages (Goldstein, 1981). Cellulose is the main constituent of lignocellulosic biomass, approximately 30-45% of dry substance and forms the structural framework of the cell walls. The β-glycosidic linkages in cellulose form linear chains that are highly stable and resistant to chemical attack because the hydrogen bonding between cellulose chains makes it highly crystalline. The free hydroxyl groups in the cellulose are likely to be involved in a number of intra and inter molecular hydrogen bonds, which may give rise to various ordered crystalline arrangements (Lehninger et al., 2004). Thus, bundles of cellulose molecules are aggregated together to form micro-fibrils- diameter of an individual micro-fibril is around 2-5 nm (Zhang et al., 2007), via orderly hydrogen bonds and Van der Waal's forces leading to highly ordered (crystalline) regions alternate with less ordered (amorphous) regions, resulting in low accessibility to enzymes (Lejeune and Deprez, 2010).

2.2.2. Hemicellulose

Hemicellulose is a highly complex, branched polymer made up of different monomeric sugars attached through different linkages. Hemicellulose can be classified as xylans, mannans, galactans, etc. Based upon the composition and intra-structural bonding, and these groups can exist separately as single components or in combination (Applegarth and Dutton, 1965; Roelofsen, 1959; Jayme and Tio, 1968). It contains five-carbon sugars (usually D-xylose and L-arabinose), six-carbon sugars (D-galactose, D-glucose, and D-mannose), acetic acid, and uronic acid. Hemicellulose is bound to the cellulose and the lignin component by covalent and non-covalent bonds in the cell wall (Saha, 2003). Agricultural residues, dedicated energy crops, hard wood and sugar cane bagasse hemicellulose mostly contain xylan, while softwood hemicellulose contains xylan and mannan. Xylan is a complex polysaccharide composed of a backbone of β-1, 4-linked xylopyranosyl residues that, depending on the plant source, can be variably substituted by the side chains of arabinosyl, glucuronosyl, methylglucuronosyl, acetyl, feruloyl and p-coumaroyl residues. The mannan can be classified into four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomannan (Petkowicz et al., 2001). Each of these polysaccharides contains mannose or a combination of glucose and mannose residues (Liepman et al., 2007).

2.2.3. Lignin

Lignin is a major non-carbohydrate in wood and other native plant material that encrusts the cell walls and cements the cells together. It is amorphous in nature and plays a critical role in giving structural rigidity to hold plant fibers together. The carbohydrate polymers are tightly bound to the lignin by hydrogen bond and covalent bonds (Fan et al., 1982). It is essentially a random, three-dimensional network polymer comprised of variously linked phenyl propane units (Sjöström, 1993). It is structurally similar to phenolic polymer (Feldman et al., 2002). The lignin in plant cell walls must be separated from carbohydrates during biomass conversion to open the protective lignin structure. Therefore, a pretreatment is necessary in order to improve the digestibility of the biomass for a subsequent enzymatic treatment. Most of the current industrial processes have lignin residue which is combusted for energy since it has a much higher heating value (HHV): 5062 cal/g on the oven dry basis (Domalski et al., 1987).

The competitiveness of the carbohydrate dependent biofuel and biochemical process can be enhanced by creating a market for the lignin. Lignin can be used as a precursor for dimethyl sulfoxide, vanilla, phenol, and ethylene (Eckert et al., 2007; Lora and Glasser, 2002; Reddy and Yang, 2005). High-quality lignin has been demonstrated to work as a substitute for polymeric materials, such as resins, surfactants, and dispersants. The National Renewable Energy Laboratory (NREL) has investigated the potential of converting lignin into a hydrocarbon that can be used as a high-octane automobile fuel additive (Montague, 2003). Lignin can also be used as a carbon source for carbon fibers (Feldman et al., 2002; Kadla et al., 2002). The use of lignin-based carbon fiber composite materials could dramatically reduce fuel consumption by lowering the overall weight of the vehicle, and a study in 2002 showed that using 10% lignin can produce enough carbon fiber to replace half of the steel used in domestic passenger vehicles (Leitten et al., 2002).

2.3. Pretreatment

For cellulase enzymes to be able to effectively access the cellulose, cell wall structure needs to be disrupted by external means, namely, pretreatment. Various pretreatment methods have been employed (Mosier et al., 2005). Pretreatment often results in a change of composition, alteration of physical properties including cellulose crystallinity. The economic and environmental concerns, however, limit the applicability of some of known methods. Most pretreatments are done through physical, physico-chemical, biological, thermo-chemical, solvent methods. Figure 2.3 is a schematic representation of cell wall of biomass and how it is affected during the pretreatment.

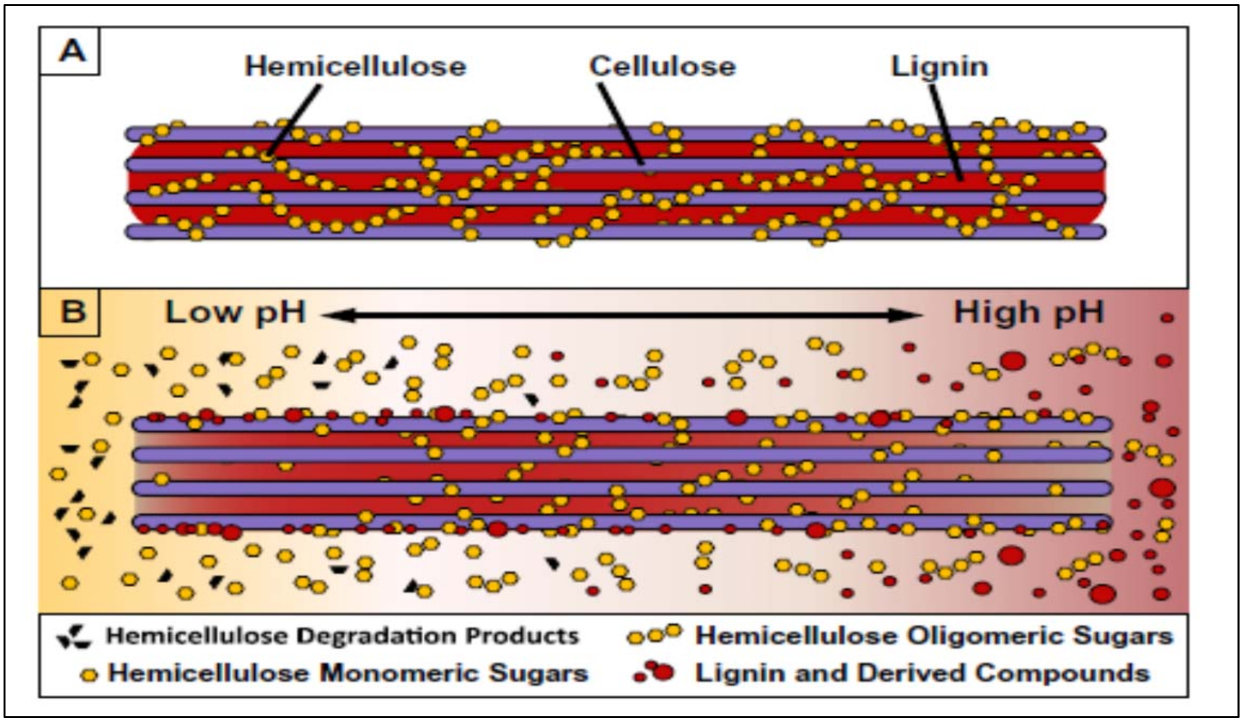

Figure 2.3: Cell wall model showing the general effect of pH on the solubilization of hemicellulose and lignin. (A) Untreated cell wall and (B) cell wall during pretreatment (Garlock et al., 2011)

2.3.1. Physical pretreatment

Physical pretreatment can be calcified into mechanical pretreatment and non-mechanical pretreatment. Mechanical pretreatment includes different modes of milling (ball, two-roll, compression, dry, wet and vibratory milling), chipping, and grinding to reduce the particle size and increase the surface area of the biomass (Caufield et al., 1974; Matsumura et al., 1977; Puri, 1984; Hsu, 1996). Chang and Holtzapple (2000) reported that reduction in crystallinity with ball milling. Mechanical pretreatment is energy-intensive and capital-intensive, and hence unattractive on a commercial scale.

The non-mechanical pretreatment options include irradiation, pyrolysis, steaming, and microwave treatment (Mandels et al., 1974; Beardmore et al., 1980; Mohan et al., 2006). These methods also require considerable energy and have a high operating cost.

2.3.2. Physico-chemical pretreatment

Physico-chemical pretreatment include steam, SO_2-steam, H_2SO_4-steam, CO_2-steam, and Ammonia Fiber Expansion (AFEX). In steam explosion the feedstock is subjected to high steam pressure (usually at 200-50 psig) and high temperatures (usually 150-270ºC) for retention times of 20 seconds-10 minutes, followed by suddenly discharging the product to atmospheric pressure (Boussaid et al., 2000; Yang et al., 2002; Pan et al., 2004). Steam treatment, causes hydrolysis of acetyl groups of hemicellulose which release acetic acid, and therefore cause the auto-hydrolysis of hemicellulose. Lignin phenolic groups content increases after pretreatment and lignin condensation is observed indicate lignin structure is changed (Shevchenko et al., 2001). Depending on the different lignocellulosic material, especially for softwood, and modifying pretreatment efficiency, by adding SO_2, CO_2 and sulfuric acid during steam explosion treatment can facilitate the degradation of biomass and improve the enzymatic digestibility of biomass (Schell et al., 1998; Zheng et al., 1998; Robinson et al., 2002; Tucker et al., 2003; Varga et al., 2004; Shahbazi, et al., 2005). SO_2 forms sulfuric acid and CO_2 forms carbonic acid, which therefore make the pretreatment environment more acidic.

Ammonia Fiber Expansion (AFEX) process was developed by Bruce Dale et al. in the early 1980's, where biomass is soaked with liquid anhydrous ammonia at temperatures (60^{o}C-160^{o}C) and at pressures (250-1000 psi) from 5 to 30 min (Dale and Moreira, 1982; Teymouri et al., 2005; Garlock et al., 2011). Then, the pressure is released suddenly (explosion) resulting in a physical action on the substrate, thus facilitating its disintegration of the biomass. AFEX decreases the crystallinity of cellulose (Gollapalli et al., 2002), retains most of hemicellulose since there is little water present in the process, disrupts the hemicellulose-lignin links by ammonolysis, and modifies the structure of lignin, therefore reducing non-productive binding between cellulase and lignin.

2.3.3. Biological pretreatment

Biological pretreatment uses lignin degrading microorganisms to improve substrate accessibility to cellulase enzymes (Kirk, 1975; Sindhu et al., 2016). Although lignin is proven to be resistant to microbial degradation, and therefore plays an important function in a plant's natural defense, some microorganisms, particularly white-rot fungi and soft-rot fungi, can release the necessary enzymes, such as lignin peroxidases during secondary metabolism, to break lignin down. White-rot fungi are one of the most promising types of organisms for the biological pretreatment (Kirk, 1975). Biological pretreatments require low energy input because of the mild conditions of pretreatment, but its low rate in comparison to the chemical treatments has prevented its usage in commercial scale plants.

2.3.4. Thermo-chemical pretreatment

Different thermo-chemical treatments have been used over the past few decades for removing lignin or hemicellulose, changing the cellulose crystalline structure, increasing the pore size and surface area. Thermo-chemical pretreatments include acid, alkaline, organosolv, hot water, oxidizing reagent, ionic liquid and supercritical fluids etc.

2.3.4.1. Acid pretreatment

Acid pretreatment has been in use for a long time because of its high pretreatment efficiency with most feedstocks (Schell et al., 1998). Some of the acids used in pretreatment are usually sulfuric acid, hydrochloric acid, phosphoric acid. Recently, other acids such as formic acid, acetic acid were used for their pretreatment ability (Jian et al., 2009; Sindhu et al., 2010; Xu et al., 2010; Marzialetti et al., 2011). Usually, sulfuric acid is used because of its low cost. In diluted form (0.1 - 0.7% sulfuric acid as a catalyst), it generally performs at relatively high temperatures (100-240°C) for a few seconds to 60 minutes depending on temperature. Generally, the dilute acid pretreatment process solubilizes the hemicellulose component of biomass into liquid stream, while leaving most of the cellulose fraction intact in the solid part. Figure 2.4 shows the schematic representation of acid pretreatment of biomass for monomeric sugar production. The disadvantages of the dilute acid process include high investment in equipment associated with acid corrosion, and high inhibitor content which makes fermentation very difficult. Since diluted acid pretreatment at high temperature produces large quantities of degradation byproducts and undesirable inhibitory compounds, the concentrated acid pretreatment is employed at temperatures which could be as low as 30^{o}C at near theoretical yields with little degradation. Arkenol and Masada resource group plan to use this process in their bio-ethanol plants (http://www.arkenol.com). The hydrogen bonding between cellulose chains is disrupted during the concentrated acid pretreatment, and cellulose becomes completely amorphous. The amorphous cellulose is extremely susceptible to hydrolysis. Thus, dilution with water at modest temperatures provides complete and rapid hydrolysis to glucose, with little degradation. Minimizing the use of sulfuric acid and recycling the acid cost-effectively are critical factors in the economic feasibility of this process.

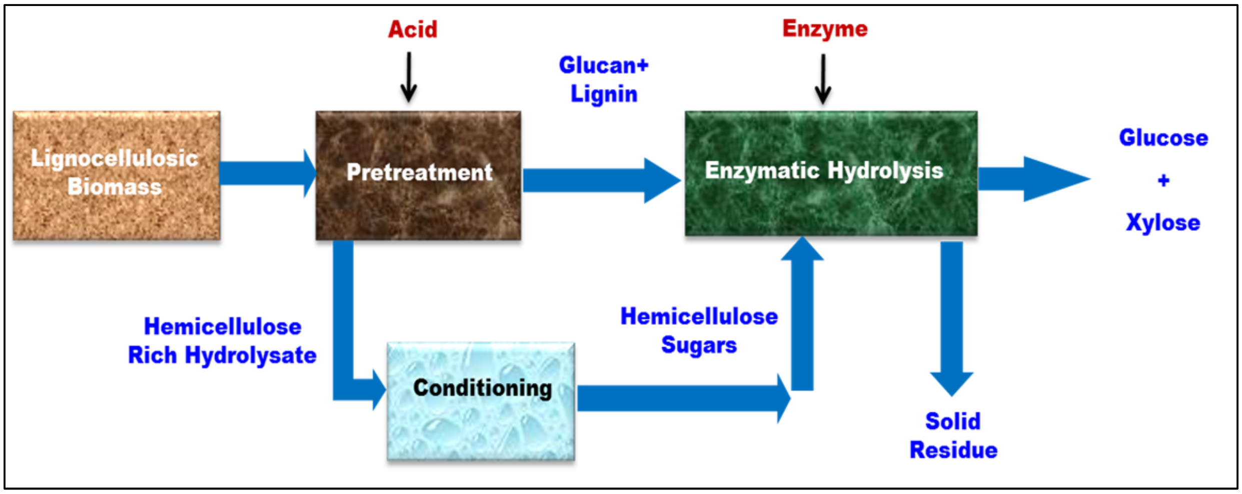

Figure 2.4: Lignocellulosic biomass to monomeric sugars through acid pretreatment

2.3.4.2. Alkaline pretreatment

In alkaline pretreatment reagents such as calcium hydroxide, sodium hydroxide and ammonia have been employed for many years (Iyer et al., 1996; Kim et al., 2003; Kim and Lee, 2005; Gupta and Lee, 2009; Pallapolu et al., 2011; Wyman et al.; 2011). Alkaline pretreatment causes a swelling effect on the biomass, which is an intra-crystalline swelling action penetrating both the amorphous and crystalline structure of cellulose and resulting in irreversible change in the structure of cellulose. This is accompanied by an inter-crystalline swelling action with water, which is a reversible change of dehydration of the substrate and has an effect only on the amorphous phase. During alkaline pretreatment, the lignin is degraded predominantly by cleavage of lignin-hemicellulose bonds, and delignification brings about changes in the structure of cellulose, whose DP and crystallinity decrease and accessible surface area increases, thus making the biomass more susceptible to enzymatic hydrolysis. Around 10% alkali is consumed by acetyl and the carbonyl content of biomass during the pretreatment, which is a significant economic factor. Figure 2.5 shows the schematic representation of alkaline pretreatment of biomass for monomeric sugars.

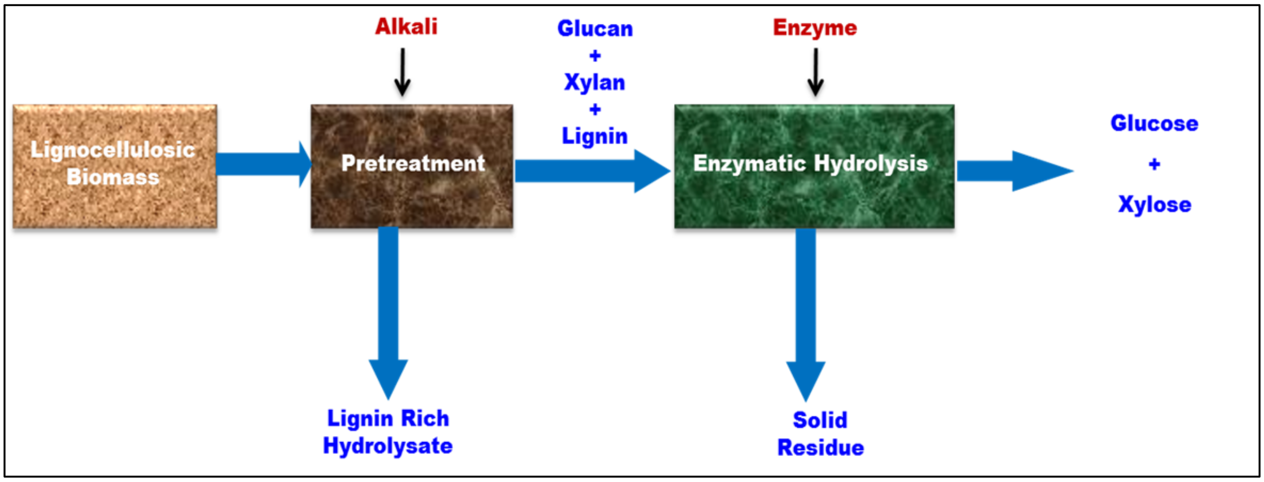

Figure 2.5: Lignocellulosic biomass to monomeric sugars through alkaline pretreatment

Among the alkaline reagents, calcium hydroxide seems a good choice considering the cost of the chemical, process temperatures of 25-100^{o}C for 1 day to 2 weeks, but it is difficult to recover (Hsu, 1996). The cost of sodium hydroxide is relatively high, but the recovery has been well documented in the pulp industry for a long time (Biermann, 1996).

Aqueous ammonia has also been used as a good choice for pretreatment, due to its relatively low cost, about one-fourth the cost of sulfuric acid on a molar basis, and its high volatility which makes it easy to recover and reuse. Also, ammonia is not severely corrosive, unlike sulfuric acid and hydrochloric acid. The ammonolysis cleaves the C-O-C bonds in lignin and the ether and ester bonds in the lignin-carbohydrate complex. Two processes, based on aqueous ammonia, ammonia recycle percolation (ARP) and soaking in aqueous ammonia (SAA), were used to improve the enzymatic digestibility of corn stover and hybrid poplar wood close to the quantitative maximum (Iyer, et al., 1996; Kim et al., 2003; Kim and Lee, 2005; Gupta and Lee, 2009; Pallapolu et al., 2011; Wyman et al.; 2011). Both processes have the high selectivity for lignin reaction and high retention of hemicellulose. In the ARP process, the ammonia is continuously fed and withdrawn from a flow-through (percolation) reactor. The lignin and other extraneous components are cleanly separated from the biomass structure, which prevents recondensation of lignin in the biomass. The SAA is a batch process used at a relatively low severity conditions. Because of low severity conditions, longer treatment time is required. At a typical condition of 15% NH_3OH and 60^oC, which gives a system pressure of about 1 atm (less than 30 psia), a reaction time of several hours to one day is required to achieve an acceptable level of pretreatment effects. Because of low process energy and low equipment cost, the overall processing cost of SAA is substantially lower than ARP. However, within the reaction conditions of temperature ($60\text{-}180^oC$), the upper limit of the ammonia pressure is around 450 psia, which is relatively higher than a normal pulp mill digester pressure. Ammonia works well for feedstocks of low lignin content, especially agricultural residues of annual plants such as corn stover, sugarcane bagasse, and wheat straw, but not for wood species, especially softwood (Iyer et al., 1996).

Lime treatment using calcium hydroxide was used by Playne (1984). Considering the cost of the chemical, calcium hydroxide stands as a good choice among the basic reagents. Similar to other alkaline pretreatment, calcium hydroxide causes a large amount of delignification and increases the hydrolysis rate (Playne, 1984; Kaar and Holtzapple, 2000; Chang et al., 2001; Mosier et al., 2005; Wyman et al., 2011).

Recently, sodium carbonate has attained prominence as an alkaline pretreatment reagent because of its relatively low cost when compared to other alkaline reagents (Yang et al., 2012). It is a mild alkaline reagent, which can delignify biomass while conserving cellulose and hemicellulose in the solid.

2.3.4.3. Water pretreatment

Reactions in liquid hot water (LHW) treatment are very similar to steam explosion (Ladisch et al., 1983; Ladisch et al., 1989; Mosier et al., 2005). The hydrolysis of hemicellulose due to water ionization at high temperatures causes solubilization of hemicellulose sugars and cleavage of the acetyl group. Acetic acid catalyzes the auto-hydrolysis reaction. Acidic moieties released as a result of lignin (various phenolic acids) and hemicellulose degradation (formic acid, glucuronic acid etc.) accelerate the reaction. These structural alterations increase the accessibility and hydrolysis of cellulose (Garrote et al., 2002). Water under high pressure penetrates the biomass for up to 15 min at 200-230^{o}C (Mosier et al., 2005). Like steam explosion, LHW pretreatment keeps most of cellulose and lignin in the solid part after pretreatment and solubilizes most hemicelluloses, low molecular weight lignin decomposed byproduct and extractives into the liquid part. But unlike steam explosion, there is no addition of chemicals in this process, and therefore, the formation of inhibitory components is much lower and the requirement for corrosion-resistant materials is also much lower. In order to minimize hydrolysis to monosaccharides, control of pH during pretreatment is employed by adding of alkali to maintain the pH constant above 5 and below 7. It also appears to improve enzymatic digestibility of cellulose, decrease in cellulose crystallinity, and lower association of cellulose with lignin (Ladisch et al., 1983; Ladisch et al., 1989).

2.3.4.4. Solvent pretreatment

A lot of organic solvents, mainly alcohol, were originally used to extract lignin from wood with high purity in order to study lignin and carbohydrates (Kleinert, 1974). Such solvent based pretreatment is called organosolv pretreatment. Organosolv pretreatment breaks bonds such as α-aryl ether and aryl glycerol-β-aryl ether in the lignin-carbohydrate complex (Kleinert, 1974); bringing significant changes in the lignin structure and decreasing the average molecular weight (Gilarranz et al., 2000). The breakdown products, like solubilized lignin, dissolve in the solvent and can be subsequently used to produce valuable co-products. The organosolv pretreatment is helpful to produce substrates highly susceptible to enzyme and therefore increase the enzymatic digestibility due to delignification (Pan et al., 2004). The acid or base catalyst is added into an aqueous organic solvent to reduce the reaction time and temperature (Thring et al., 1990). Based on organosolv processes, Zhang et al. (2007) developed new cellulose and organic-solvent-based lignocelluloses fractionation (COSLIF) technology to separate lignocellulosic biomass into amorphous cellulose, hemicellulose, and lignin under modest reaction conditions (atmospheric pressure and ~50^{o}C). Recycling of the solvent is important for reduction of cost due to the high cost of solvents. Removal of the solvent from the pretreated biomass is also important since the solvent could be inhibitory to enzymes and microorganisms.

2.3.4.5. Ionic liquid pretreatment

An ionic liquid for pretreatment of cellulosic biomass was developed as a promising green solvent for biomass fractionation (Reichert et al., 2001; Dadi et al., 2006). Ionic liquids are nonflammable and recyclable solvents with very low volatility and high thermal stability. Carbohydrates and lignin can be simultaneously dissolved in ionic liquids with anion activity (e.g. the 1-butyl-3-methylimidazolium cation in the 1-butyl-3-methylimidazolium chloride (BMIMCl) and 1-allyl-3-methylimidazolium cation in the 1-allyl-3-methylimidazolium chloride (AMIMCl)).

2.3.4.6. Oxidizing reagents pretreatment

Delignification can be achieved by a variety of oxidation methods such as treatment by ozone (Garcia-Cubero et al., 2009), sodium chlorite (Hubbell and Ragauskas, 2010), oxygen (Draude et al., 2001), hydrogen peroxide (Sun et al., 2000; Gupta and Lee, 2010), chlorine dioxide (Sannigrahi et al., 2012), Peracetic acid (Yin et al., 2011). Oxidative delignification has primarily been applied for pulping and bleaching. Its application in biomass pretreatment is relatively new. Oxidation increases the positive valance state of a molecule by removal of one or more electrons from an atom or ion. Oxidizing agents, including ozone, hydrogen peroxide, hypochlorite, chlorine, chlorine dioxide, sodium chloride, and peracetic acid have been used for the chemical treatment of biomass (Chapman, 2003). A recent laboratory investigation by Kumar et al. (2013) explored the use of an aqueous solution of acetic acid and sodium chlorite for delignification of biomass and reported that more than 90% delignification was achieved for corn stover and other feedstocks. They achieved this at 70^{o}C for 8 h with successive addition (every two hours) of fresh sodium chlorite and acetic acid. As shown in this investigation, another advantage of acid-chlorite pretreatment is that it removes lignin at temperatures below 100^{o}C.

Experimental evidence exists that acid-chlorite delignification is selective in the removal of lignin with only trace solubilization of glucan and xylan (Ahlgren and Goring, 1971). According to Gierer (1986), however, acid-chlorite primarily acts on lignin in biomass, but it can also affect the polysaccharides. The two plausible mechanisms for cellulose degradation during acid-chlorite delignification are acidic cleavage of the glycosidic bonds and/or oxidative degradation of the polysaccharides. Lewin and Epstein (1962) reported oxidation of cellulose with hypochlorite is non-specific and degradation proceeds most rapidly near neutral pH.

2.3.4.7. Supercritical fluids pretreatment

A supercritical fluid is any substance at a temperature and pressure above its critical point. It behaves as neither a fluid nor a gas, but has some of the properties of each. It can diffuse through solids like a gas, and dissolve materials like a liquid. Supercritical fluids are suitable as a substitute for organic solvents in a range of industrial and laboratory processes. Carbon dioxide, sulfur dioxide and ammonia have been used to treat lignocellulosic biomass. Carbon dioxide becomes more attractive because it is a byproduct of the ethanol fermentation process. Shah et al. (1991) treated hardwood with supercritical carbon dioxide-sulfur dioxide mixtures to make acetone, butanol, and ethanol (ABE). Zheng et al. (1998) used supercritical carbon dioxide to treat Avicel, recycled paper mix, sugar cane bagasse and the repulping waste of recycled paper, and then suddenly released the carbon dioxide pressure to increase the accessible surface area of the cellulosic substrate to enzymatic hydrolysis. However, this technology raises serious questions about equipment costs due to high pressure.

2.4. Effect of additives during pretreatment

Addition of small amounts of sodium sulfide to the alkaline reagent is known to reduce carbohydrate losses and improve delignification (Gierer, 1986). Sulfur based compounds increase the cleavage of phenolic β-ether linkages and carbon-carbon linkages. It also causes fragmentation of lignin and condensation of the lignin fragments with sulfur (Gierer, 1986). The sulfur induced pretreatment method with the use of sodium carbonate (green liquor pretreatment) has been studied by several authors (Gu et al., 2012; Yang et al., 2012; Gu et al., 2013). When sodium sulfide is supplemented to alkali solution, sulfur forms covalent-bonds with the released lignin and helps in lignin condensation.

Surface active reagents have been used during the pretreatment to improve enzymatic hydrolysis. Qing et al. (2010) reported the addition of surfactant increase the delignification, which results in less non-productive binding of the enzyme to lignin and also reduce obstacles to cellulase action (Yang and Wyman, 2006; Kristensen et al., 2007). Ji and Lee (2013) reported improvement in the cellulose and hemicellulose digestibility with the addition of cationic polyelectrolyte during nano-shear hybrid NaOH pretreatment of corn stover. According to them lignin formed globular complex with cationic polyelectrolytes and re-deposited on the surface or in the micro fibril matrix of pretreated corn stover. Cell wall layers experienced significant morphological changes, accompanied by a decrease in retaining lignin and fibrillated cellulose, eventually contributing to less inhibitory of lignin and greater accessibility of polysaccharides to enzymes. Qing et al. (2010) and Ji and Lee (2013) reported increase in cellulose and hemicellulose conversion is due to effect of surface active agent on delignification and lignin redistribution.

2.5. Enzymatic hydrolysis

The enzymatic hydrolysis or saccharification or digestibility of six and five carbon polymeric sugars into their respective monomeric sugars is a complex and slow process because of the physical nature of the substrate. The factors that affect the enzymatic hydrolysis of lignocellulosic biomass include substrate characteristics, enzyme property, and reaction conditions (temperature, pH, etc.). Delignification due to pretreatment causes biomass swelling and modification of the lignin structure, therefore an increase the internal surface area and median pore volume (Fan et al., 1982), which leads to a reduction of irreversible adsorption of enzyme on lignin (Ooshima et al., 1990) and an increase of enzyme accessibility to cellulose

(Mooney et al., 1998; Meunier-Goddik and Penner, 1999). Only 20-65% of the lignin needs to be removed to increase the susceptibility of enzymatic degradation depending on the sources of cellulose.

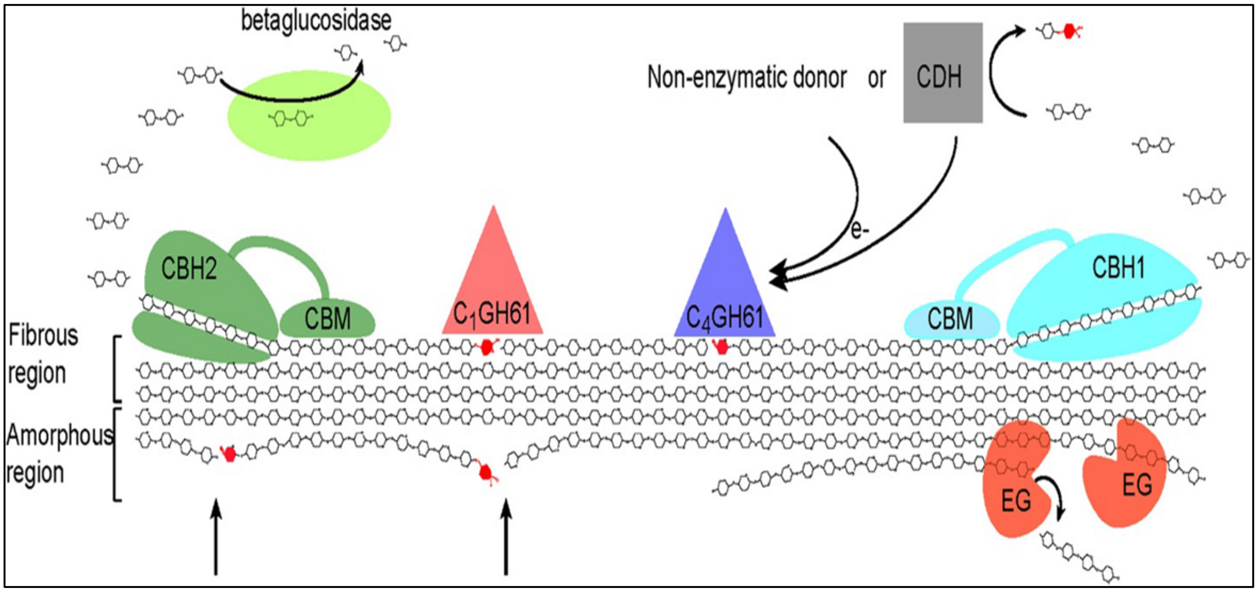

Figure 2.6: Mechanism of enzymatic hydrolysis of cellulose by cellulase (Horn et al., 2012)
EG - endoglucanase; CBH - cellobiohydrolase; CDH - cellobiose-dehydrogenase; CBM - carbohydrate-binding module; C_1GH61 and C_4GH61- lytic polysaccharide monooxygenases
Note: Red color molecules are oxidized sugars

Cellulase enzymes from *Trichoderma reesei* are composed of: cellobiohydrolases, endoglucanase, cellobiose-dehydrogenase, lytic polysaccharide monooxygenases, and β-glucosidases (Horn et al., 2012). The mechanism of enzymatic hydrolysis of cellulose by cellulase is depicted in Figure 2.6. Endoglucanases (EGs) act preferentially at amorphous cellulose regions, fragment cellulose chains into reducing and non-reducing. CBHs degrade cellulose, including crystalline cellulose, from either the reducing (CBH I) or non-reducing (CBH II) ends, to generate primarily small oligosaccharides and cellobiose. β-glucosidase hydrolyzes small oligosaccharides and cellobiose to glucose. It was found that enzyme preparations containing only endoglucanases have little effect on native cellulose. On the other hand, those containing both endo and exocellulases will cause significant degradation of native cellulose. Thus, the endo and exocellulases appear to work in a synergistic or cooperative manner on native high molecular- size cellulose. At high concentrations, cellobiose inhibits CBH activity. Thus β-glucosidase, which converts cellobiose into glucose, is often required for optimal cellulose performance in conditions where cellobiose accumulates. *Trichoderma reesei* produces β-glucosidases at low levels compared to other fungi such as *Aspergillus* species (Reczey et al., 1998). Thus, T*richoderma reesei* cellulase preparations, supplemented with *Aspergillus* β-glucosidase, are used most often for cellulose saccharification on an industrial scale. In addition, β-glucosidases of *Trichoderma reesei* are subject to product (glucose) inhibition (Chen et al., 1992), while those of *Aspergillus* species are more glucose tolerant (Yan and Lin, 1997; Gunata and Vallier, 1999). The discovery of lytic polysaccharide monooxygenases may represent a revolution in enzymatic biomass processing (Horn et al., 2012; Forsberg et al., 2014). According to Horn et al. (2012), many cellulolytic enzyme systems have multiple EG and/or CBH that may act on various parts of the substrate including, different crystal faces or parts differing in terms of crystallinity and accessibility. Figure 2.6 shows a C1 and a C4 oxidizing GH61 which would generate non-oxidizing ends for CBH 2 and CBH 1, respectively. Note that the combined action C1 and C4 oxidizing enzymes may produce native cello-oligosaccharides from the middle of the cellulose chain. The possible consequence of GH61 action is illustrated in the lower left part of Figure 2.6, where new attaching points for CBHs are indicated by allows. CDH may provide GH61s with electrons, it must be noted that not all organisms have genes encoding for both of these enzyme families in their genome.

2.5.1. Effect of enzyme mixture on hydrolysis

Enzymatic hydrolysis of the pretreated lignocellulose to monomeric sugars primarily uses cellulase enzyme complex derived from filamentous fungi, particularly Trichoderma reesei (Nidetzky et al., 1994). Such an enzyme complex contains high levels of endo-glucanase activity and exo-glucanase activity, together with lower levels of other enzyme activities such as β-glucosidase, β-xylosidase, hemicellulases and pectinase, etc. (Kabel et al., 2006; Dien et al., 2008). These lower level enzyme activities are called “background” activities. Attempts to improve the hydrolytic efficiency of such enzyme complexes have traditionally been focused on their component cellulases because cellulose is the major polysaccharide component in lignocellulose (Berlin et al., 2005a; 2005b). However, other components in pretreated biomass, particularly hemicellulose and lignin, exert significant restraints on cellulose hydrolysis (Borjesson et al., 2007a; 2007b). For example, in one mechanism, lignin seems to reduce hydrolytic performance is by binding to enzyme components unproductively (Borjesson et al., 2007a; 2007b). Similarly, it is possible that oligosaccharides produced from hemicelluloses inhibit the activity of cellulase enzymes (Qing and Wyman, 2011; Pallapolu et al., 2011). Consequently, enzyme mixtures with similar cellulase activity may show differences in performance on lignocellulose if they differ in levels of “background” activities (Berlin et al., 2005a; 2005b). However, literature, information on saccharification yields for pretreated lignocellulose collectively indicates that the without ‘‘background’’ activities cellulase loading required to attain acceptable sugar yields is so high that it becomes a major cost item in the overall bioconversion process (Merino and Cherry, 2007; Sun and Cheng, 2004). One way to compensate deficiencies in these ‘‘background’’ activities is the supplementation of external enzymes with appropriate activities to cellulase.

2.6. Additives during enzymatic hydrolysis

Many challenges still limit the hydrolysis process, including several substrate characteristics and a number of enzyme factors (Chang and Holtzapple, 2000; Kim and Holtzapple, 2006; Chandra et al., 2009). To overcome these challenges, pretreatment and large amounts of hydrolytic enzymes were employed, but this resulted in unfavorably high processing cost (Himmel et al., 2007). Many studies have therefore been aimed at lowering the inhibition of enzymatic hydrolysis and improving the efficiency of commercially available enzymes to promote the conversion of lignocellulosics (Himmel et al., 2007; Ouyang et al., 2009).

The use of surface active additives has been shown to enhance the enzymatic hydrolysis of lignocellulosics (Kaar and Holtzapple, 1998; Eriksson et al., 2002; Yang and Wyman, 2006; Kristensen et al., 2007). Although the use of additives adds additional cost to bioethanol production process, clear benefits have been shown. Benefits including reduction in unproductive binding to lignin will make more effective use of the added enzymes or decrease the enzyme loading (Borjesson et al., 2007a, 2007b). So far most of the additives studied have been surfactants (Kaar and Holtzapple, 1998; Eriksson et al., 2002; Yang and Wyman, 2006). In recent years, however, other additives have been studied such as proteins (bovine serum albumin) (Yang and Wyman, 2006), polymers such as polyethylene glycol (PEG) (Borjesson et al., 2007a; 2007b; Kristensen et al., 2007; Ouyang et al., 2009), polyvinyl alcohol (PVA) (Hata, 2011), cationic polyacrylamide (Mora et al., 2011).

Ouyang et al. (2009) reported that addition of PEG during the enzymatic hydrolysis increased the conversions of Avicel by 92% and the improvement is increase in cellulose activity and stability of the enzyme. On the other hand, Borjesson et al. (2007a) reported that PEG addition only has effect on substrates containing lignin. The adsorption of PEG on lignocellulosics is due to hydrophobic and hydrogen bonding interactions between PEG and lignin (Borjesson et al., 2007a; 2007b). Interaction between PEG and lignin is also highly temperature dependent; a large increase in PEG binding as well as the PEG effect on steam pretreated spruce conversion with increase in temperature was reported. There are also strong indications that the presence of lignin in the hydrolysis acts as an inducer of thermal denaturation of the enzymes when performed at temperatures above 40^{o}C.

Hata (2011) proposed new method for processing of biomass with additives. In this method PVA is mixed with tetra sodium borate, which crosslinks PVA and converts it into a viscous, non-newtonian fluid. When crosslinked PVA mixed with biomass it facilitates swelling and hydration, forming viscous gel like material. This processed material is thinned through the addition of water whereby hydrolytic enzymes are mixed into the material and rapid hydrolysis into monomeric sugars.

2.7. Levulinic acid

Levulinic acid (LA) is a short chain fatty acid having a ketone carbonyl group and an acidic carboxyl group. It is a versatile new platform chemical with numerous potential uses, including, textile dye, antifreeze, animal feed, coating material, solvent, food flavoring agent, pharmaceutical compounds and resin (Rackemann and Doherty, 2011). The main method for production of high purity LA in use today uses the petrochemical conversion route from maleic anhydride (Moens et al., 2002) or hydrolysis of furfuryl alcohol (Timokhin et al., 1999). Alternatively, LA can be produced through acid-catalyzed reaction of six carbon sugars (Figure 2.7) (Rackemann and Doherty, 2011). Many materials such as glucose, sucrose, fructose and lignocellulosic biomass materials including wood, starch, cane sugar, grain sorghum and agricultural wastes have been used to produce LA (Rackemann and Doherty, 2011). Lignocellulosic biomass provides an abundant and relatively low cost resource that can be utilized to produce LA.

The key in improving the yield of the LA production from biomass lies in separating the intermediate products at each step of the reaction pathway and reduce the side reactions (Ghorpade and Hanna, 1999; Hayes et al., 2006; Holladay et al., 2007; Rackemann and Doherty, 2011). New technologies, including the use of biphasic systems and/or continuous extraction of products, microwave irradiation, ionic liquids, and the development of selective catalysts would provide the necessary step change for the optimization of key reactions.

The majority LA production with sugars, cellulose and lignocellulosics has been conducted with mineral acid catalysts (Hayes et al., 2006; Girisuta et al., 2006; 2007). It was found the order of effectiveness of dilute acids on the production of LA from sucrose was as follows HBr > HCl > H2SO4 (Cha and Hanna, 2002). Biphasic fluorine based technology facilitates the separation of catalyst and reagents from the product (Gladysz et al., 2002). Among the mineral acid catalysts sulfuric acid is found to be cost effective.

Heterogeneous catalysts represent a viable alternative to homogeneous catalysts due to their ease in handling, reduced corrosion and relatively low cost if the catalyst is easily recycled (Fukuoka et al., 2006).

The solvent based LA production has an advantage over water based LA production in that solvents can be used to alter the properties of the reaction mixture to promote more selective reactions and due to their higher dielectric constant, allow reactions to occur at lower temperatures. Ionic liquids are an important class of chemicals that can act as both a solvent and catalyst (Moreau et al., 2006; Horvath, 2008).

Supercritical fluids are another class of solvents with interesting features that can be used to enable more selective reactions and efficient separation of products (Boisen et al., 2009). Supercritical fluids exhibit both acidic and basic properties.

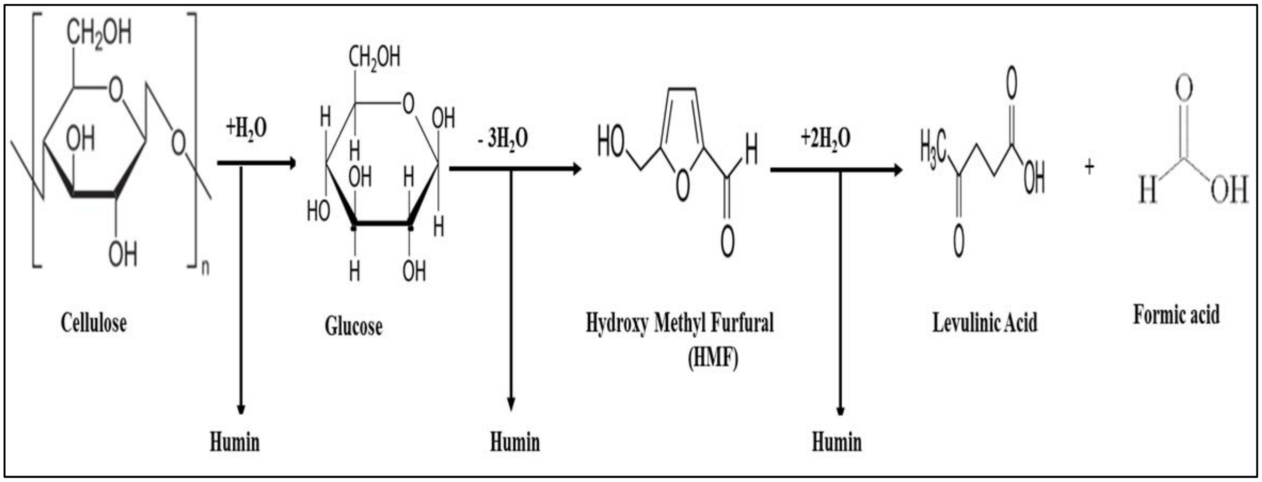

Figure 2.7: Mechanism of production of levulinic acid from cellulose

2.8. Decrystallization of cellulose

Monomeric sugars are produced by two different types of conversion methods: enzymatic hydrolysis and sulfuric acid catalyzed hydrolysis, the former being the predominant topic in recent research efforts. The main resistance of enzymatic or acid hydrolysis of cellulose exists in the rigid crystalline structure which is held by tightly arranged hydrogen bonds. The acid hydrolysis process to produce sugars from lignocellulosic biomass has a long history (Freudenberg, 1930; Seaman, 1945). It has been attempted under two drastically different conditions: dilute acid-high temperature and concentrated acid-low temperature conditions. The cellulose hydrolysis under the high concentrated acid-low temperature was verified by Xiang et al. (2003). They showed that α-cellulose when treated with a varying level of H_2SO_4, an abrupt change in physical structure (fibrous to gelatinous) took place at about 65% H_2SO_4, abrupt in the sense that small change of input (acid concentration from 60% to 65%) caused a drastic change of output. In dilute-acid hydrolysis, whether decrystallization occurs, if so, how it affects the hydrolysis reaction is unclear. The prevailing notion is as follows. Under low temperature and acid condition, the cellulose structure stays in stable crystalline form. Therefore, the reaction mode is endwise hydrolysis. The glucose then becomes the main sugar product, and its yield becomes low. When temperature and/or acid concentration is raised to a certain level, the cellulose structure becomes unstable by cleavage of hydrogen bonding (decrystallization) (Camacho et al., 1996; Ioelovich and Larina, 1999; Ioelovich, 2012). Once the crystalline structure of the cellulose is disrupted, acid molecules can penetrate into the inner layers of the cellulose chains resulting rapid hydrolysis and high yield of glucose. Decrystallization of cellulose is an exothermic process because the hydrogen bonds are broken and the bond energy is released. Therefore, the temperature is expected to increase if there is decrystallization.

3. Effect of Aqueous Ammonia Pretreatment on Different Switchgrass Species

3.1. Abstract

Technical feasibility of uniform pretreatment conditions across different switchgrass varieties to achieve similar sugar yields was investigated. Four different types of switchgrass species, Shawnee, Alamo-I and Alamo-II, and Dacotah were investigated using aqueous ammonia as a pretreatment reagent. A majority of pretreatment experiments was carried out using Soaking in Aqueous Ammonia (SAA) method, while selected few pretreatment experiments with Dacotah were carried out using Ammonia Recycle Percolation (ARP) method. SAA experiments were carried out at 60-160^{o}C and long reaction time (1-48 hr) in a batch reactor, whereas ARP experiments were operated at high temperature 150-190°C and short residence time (10-20 minutes) in a flow-through reactor. Pretreated solids were subjected to enzymatic hydrolysis using cellulase (15 FPU/g-glucan of Spezyme-CP) and β-glucosidase (30 CBU/g-glucan of Novozymes-188). Hydrolysis results show that with the similar pretreatment conditions (90°C, 15% NH_4OH, liquid to solid ratio of 9, and 24 hr), Alamo-I, Alamo-II, and Shawnee resulted substantially higher glucan/xylan (as high as 19%/13%) digestibility than the Dacotah. The optimum pretreatment conditions for Shawnee, Alamo-I, and Alamo-II were: 90°C, 15% NH_4OH, liquid to solid ratio of 9, and 24 hr. Increase in pretreatment severity increased the glucan/xylan digestibility of Dacotah, but the overall sugar recovery yield was decreased due to the loss of sugars during the pretreatment. This study showed that among the

four switchgrass species, Dacotah is highly recalcitrant. ARP pretreatment improved the digestibility of Dacotah, but also caused significant loss of carbohydrate content during pretreatment. SEM showed swelling of surface with SAA pretreated Dacotah and significant disruption in surface with ARP pretreated Dacotah. BET showed increase in surface area with SAA and ARP pretreatments.

3.2. Introduction

Switchgrass is an abundant, fast growing, warm-season grass that occurs naturally from Canada to Central America (Lemus and Parrish, 2009; Jefferson and McCaughey, 2012). Switchgrass is a self-seeding crop, after plantation, its stand can survive for ten years or longer, and is adapted to a wide range of soil conditions, tolerant to drought, requires relatively modest levels of chemical fertilizers (Sanderson and Wolf, 1995; Lemus and Parrish, 2009). Polymeric cellulose and hemicelluloses present in switchgrass can be converted into monomeric sugars through pretreatment followed by enzymatic hydrolysis (Mosier et al., 2005; Wyman et al., 2011). After evaluating several potential herbaceous crops, the US Department of Energy chose switchgrass as one of the promising species for bioenergy production because of its potential for high yields, wide range of distribution, and beneficial environmental characteristics (McLaughlin et al., 1999; Lewandowski et al., 2003). Factors affecting switchgrass composition are plantation and harvest time, genotype (lowland vs. upland), ecotype (southern vs. northern), precipitation, fertilizer, type of storage, and other environmental conditions (Sanderson et al., 1999; Casler et al., 2004; Jefferson and McCaughey, 2012). Harvest time is one of the important factors that affect not only biomass composition, but also yield. Delaying harvest time is known to reduce switchgrass soluble carbohydrate concentrations, while increasing cell-wall carbohydrates, and lignin contents as switchgrass is matured during the growing season (Casler and Boe, 2003; Adler et al., 2006). Lignocellulose content generally increases with latitude in upland-northern types (Cassida et al., 2005; Jefferson and McCaughey, 2012).

For cellulase enzymes to be able to effectively access the cellulose, cell wall structure needs to be disrupted by external means, namely pretreatment. To remove one or more of these barriers, various pretreatment methods have been employed (Mosier et al., 2005; Wyman et al., 2011). Pretreatment often results in a change of composition, alteration of physical properties including cellulose crystallinity, surface area. Over the years different pretreatment methods have been attempted including, physical, physico-chemical, chemical (acid, alkaline, and hot water), and biological (Schell et al., 1998; Zheng et al., 1998; Boussaid et al., 2000; Mosier et al., 2005; Mohan et al., 2006; Wyman et al., 2011). However, recently a lot of emphasis has been put on the alkaline pretreatment. In alkaline pretreatments, it is technically feasible to retain most of carbohydrates, while removing a large fraction of lignin in the biomass. The enhancement of digestibility is caused primarily by lignin removal. Retention of hemicellulose after pretreatment gives a significant economic benefit since it eliminates the need of detoxifying hemicellulose sugars. Lime, sodium hydroxide, aqueous ammonia, and sodium carbonate have been used as alkaline pretreatment reagents (Kaar and Holtzapple, 2000; Kim and Lee, 2005; Gupta and Lee, 2009; Yang et al., 2012). Ammonia, being a milder alkali, has advantages over other alkaline reagents due to ease in recovery because of its high volatility, relatively inexpensive compared to sodium hydroxide, and non-corrosiveness. There have been some publications related to switchgrass pretreatment using different treatment methods (Esteghlalian et al., 1997; Li et al., 2010). However, to our knowledge nobody has reported any work related to effects of harvest time, ecotype, genotype, and cultivars on biochemical conversion of switchgrass processed by alkaline pretreatment, and applicability of uniform pretreatment conditions across the different switchgrass varieties to achieve similar sugar yields. Thus, objectives of this study were to examine the effects of harvest time, cultivars, ecotype, and genotype on biochemical conversion of switchgrass processed by aqueous ammonia pretreatment, and to evaluate the technical feasibility of uniform pretreatment conditions across the different switchgrass varieties to achieve similar sugar yields.

3.3. Materials and methods

3.3.1. Feedstock, Enzymes, and reagents

Switchgrass varieties (Alamo-I, Alamo-II, Dacotah, and Shawnee) were provided by Ceres, Inc (Thousand Oaks, CA). Small bales of each switchgrass type were stored in a building after harvest until they were dried to less than 10% moisture at 50°C and knife-or ball-milled to 2-6 mm size. Spezyme-CP (cellulase) was provided by Genencor International, Inc. (Rochester, NY), and its activity was measured to be 59 FPU/mL. The β-glucosidase (Novozyme-188, Cat. No. C-6150) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and its activity was measured to be 600 CBU/mL. The total protein numbers of Spezyme CP and β-glucosidase were measured by Nitrogen assay and found to be 82 mg protein/mL and 67 mg protein/mL, respectively. All other reagents and chemicals, unless otherwise noted were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.3.2. Extraction of water soluble sugars

Each switchgrass type was soaked in hot deionized (DI) water (80-100ºC) with liquid to solid ratio of 10 for 5 min to remove free soluble sugars. The slurry was vacuum filtered to remove the drainable liquid. The soluble sugar removal procedure was repeated three times, and dried in 45°C oven. The drained liquid was further analyzed for soluble sugars by NREL LAP - Determination of Extractives in Biomass (Sluiter et al., 2008). Water extractable free sugars are shown in Table 3.2. Prior to the pretreatment, dried switchgrass samples were further ground and milled through a 40 mesh screen to provide uniform particle size between the switchgrass types.

3.3.3. Pretreatment methods

Soaking in Aqueous Ammonia (SAA) pretreatment

All the pretreatment experiments were carried out in a stainless steel batch reactor (1.375”ID x 6”L). A 10 g dry soluble sugar free switchgrass was kept in a Gas Chromatography (GC) forced-air convection oven. The heating time to reach the target temperature was about 20 min, and that was not included in the stated reaction time. After pretreatment, the reactor was immediately removed from the oven and quenched to room temperature in a water bath. The cooled slurry was vacuum filtered immediately through a filter paper (Whatman®, Grade 802 Fluted, size 32.0 cm). The vacuum filtered wet solids underwent washing with 1000 mL of cold tap water per approximately 10 g dry solids. Washing was continued until pH reaches approximately 7. Washed solids from filter paper were carefully collected to calculate solid remaining after the pretreatment. The moisture content of the cold-washed solids was measured. Washed solids were not subjected to further drying and kept at 4°C. All pretreatment runs were made in duplicates. SAA process is shown in Figure 3.1 (Kothari and Lee, 2012).

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Ammonia Recycle Percolation (ARP) pretreatment

The ARP employs a flow through column reactor. The reagent solution is pumped through the reactor packed with biomass. The reactor temperature is controlled by a GC forced-air convection oven. The reactor system is back-pressured by nitrogen (300-425 psig) to prevent flash evaporation. The reactor was made out of SS-316 with dimension of 0.9” ID, 10” length. The substrate was loaded such way that it occupies 80% of the reactor volume. The rest of the volume was filled with perforated Teflon rods. The schematic representation of the laboratory ARP process is shown in Figure 3.2 (Kim et. al., 2003).

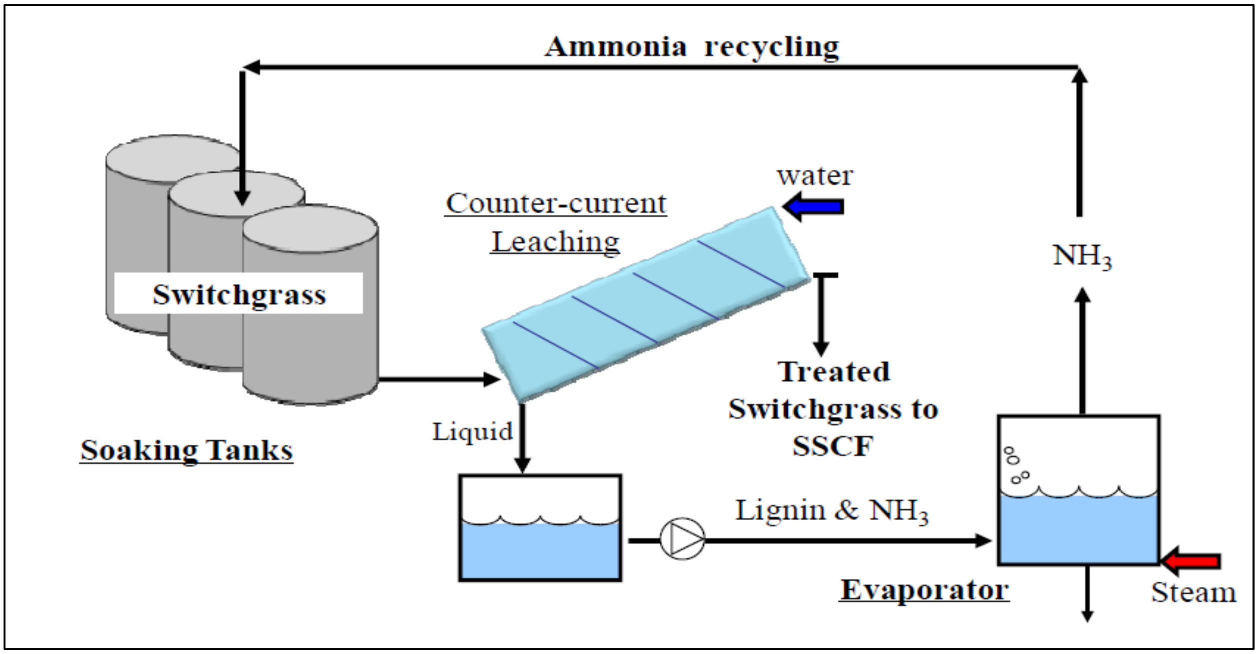

Figure 3.1: Schematic representation of SAA pretreatment

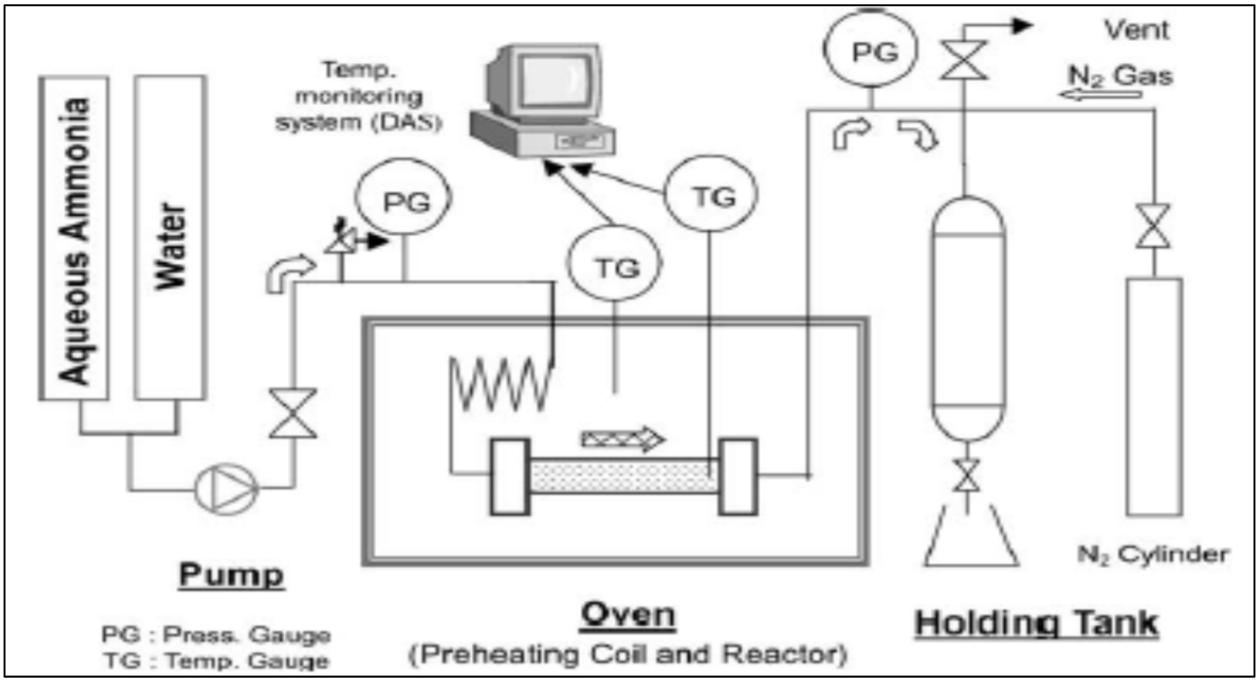

Figure 3.2: Schematic representation of ARP pretreatment

3.3.4. Compositional analysis

Composition of untreated and pretreated switchgrass solids were determined by following National Renewable Energy Laboratory (NREL) LAP standard analytical procedures (Sluiter et al., 2006). Analyzed components were: glucan, xylan, galactan, arabinan, lignin, ash, and acetyl. The liquid fraction of the pretreated switchgrass was analyzed for soluble mono-and oligosaccharides content by following LAP 014 (Sluiter et al., 2005). Sugars were analyzed by HPLC as described in the HPLC Analysis section. All measurements were made in duplicates.

3.3.5. Enzymatic hydrolysis

Enzymatic digestibility of the pretreated solids was determined by following NREL standard protocol (Brown and Torget, 2005). The pretreated switchgrass equivalent to 1 g glucan was transferred into a 250 mL Erlenmeyer flask, and an aliquot of 100 mL 0.05M, pH 4.8 sodium citrate buffer was added to give 1% glucan slurry. Two antibiotics, tetracycline (0.4 mL) and cyclohexamide (0.3 mL) were added to the mixture to prevent microbial activity during the hydrolysis. The enzymes added were cellulase (15 FPU/g-glucan of Spezyme-CP) and β-glucosidase (30 CBU/g-glucan of Novozyme-188), (equivalent to 27 mg protein/g glucan). Hydrolysis experiments were carried out at 50°C and 150 RPM. Samples were taken periodically. Sugars were determined by HPLC using a HPX-87P column. The BioRad-HPX-87H column was used for measurement of organic acids. A refractive index detector was used in the HPLC. Glucan, xylan digestibilities and sugar recovery yield were calculated following equations:

$$\text{Glucan digestibility (\%)} = \frac{\text{Total release of Glucose (g)} \times 0.90}{\text{Initial Glucan loading (g)}} \times 100$$

$$\text{Xylan digestibility (\%)} = \frac{\text{Total release of Xylose (g)} \times 0.88}{\text{Initial Xylan loading (g)}} \times 100$$

$$\text{SRY (\%)} = \frac{\text{Gln after Treat (g)} \times \text{Gln Dig [\%]} + \text{Xln after Treat (g)} \times \text{Xln Dig [\%]}}{\text{(Glucan + Xylan)[g] in untreated biomass}} \times 100$$

SRY: Sugar Recovery Yield, Gln: Glucan, Xln: Xylan, Dig: Digestibility, Treat: Treatment

3.3.6. Physical characterization

Scanning Electron Microscopy (SEM)

SEM Images were then taken using Field Emission Scanning Electron Microscope (JEOL JSM-7000F). Samples were placed on adhesive carbon tape on an aluminum stub and sputter coated with gold.

***Brunauer Emmett Teller* (*BET*) surface area**

The BET surface area was measured by ASAP 2420 instrument by krypton gas adsorption. Hg analysis was performed by Micromeritics Analytical Services using AutoPore IV 9520 instrument.

3.4. Results and discussion

Four different switchgrass varieties, which include two different batches of southern low land type (Alamo-I and Alamo-II) with thick stems, and two different varieties of Northern upland type (Dacotah and Shawnee) with thin stems, were used for this study. Alamo-I & II, and Shawnee were planted in summer and harvested in late fall after the plants were allowed to stand over 5- 18 months, while Dacotah was planted in late Fall, and harvested in late spring after the plant was allowed to stand over approximately 8 years. Different factors affecting switchgrass yield and composition are shown in Table 3.1.

Table 3.1: Factors affecting the switchgrass yield and composition

	Alamo-I	**Alamo-II**	**Dacotah**	**Shawnee**
Plant date	June, 2005	June, 2007	December, 1999	June, 2005
Harvest date	December, 2006	November, 2007	May, 2008	December, 2006
Ecotype	Southern Lowland		Northern Upland	
Morphology	Thick stems		Thin stems	
Latitude of Origin	29°N		46°N	38°N
Harvest Location	Ardmore, OK 34°N (Elev. 870 ft)		Pierre, SD 44°N (Elev. 1420 ft)	Stillwater, OK 36°N (Elev. 960 ft)

3.4.1. Removal of water extractable soluble sugars

Switchgrass contains soluble components which are readily extractable in aqueous phase. These extractives include such as sucrose, glucose, fructose, xylose, and soluble lignin. Chen et al. (2010) reported that the pre-extraction step is required to improve the accuracy of measurement of major components (glucan, xylan, and lignin) in lignocellulose. If water soluble components are not removed from the raw switchgrass, they can interfere with comparisons among the switchgrass types. Particularly, lignin can be overestimated in the raw feedstock due to condensation/precipitation of extractives during the analysis of lignin (Browning, 1967). The water soluble sugars can also overestimate enzymatic digestibility efficiencies of the structural sugars. Thus switchgrass was pre-washed using the procedure mentioned in Section 3.3.2.

Analysis of the water-extractable sugars indicates that most of the sugars extracted are mainly sucrose, glucose, and fructose. Comparisons between the switchgrass samples showed that Dacotah contains <1% (by dry weight) soluble sugars, whereas other varieties contain more than 7% (by dry weight). It should be noted that Alamo-I & II, and Shawnee were planted in summer, and harvested in late fall after the plants were allowed to stand 5-18 months, while Dacotah was planted in late Fall, and harvested in late spring after the plant was allowed to stand over approximately 8 years. The increase in structural sugars and lignin contents in switchgrass harvested in the spring compared to the fall harvest can be attributed to leaching of soluble components such as sugars, protein, and organic acids over the winter (Adler et al., 2006). Not only do the non-structural sugars interfere with measurement of structural sugars and other components, they can be degraded easily during the pretreatment. Therefore, it is desirable to remove and recover non-structural sugars, before pretreatment.

Table 3.2: Water extractable soluble sugars

	Alamo-I	**Alamo-II**	**Shawnee**	**Dacotah**
Glucose (%)	1.85	1.60	2.46	0.25
Sucrose (%)	5.30	4.53	2.92	0.18
Fructose (%)	1.48	1.34	1.95	0.17
Total Soluble Sugars	8.63	7.47	7.33	0.60

3.4.2. Understanding the factors that influence composition of untreated switchgrass

The solid compositions of the pre-washed switchgrass varieties are summarized in Table 3.3. Components analyzed were: glucan, xylan, galactan, arabinan, lignin, ash, and acetyl. It should be noted that the materials were pre-washed with hot DI water to remove the extractives, and the contents of all other components were measured using the extractives-free switchgrass. The values were adjusted to be based on the weight of the unextracted, initial dry material.

No statistically significant difference was found between Alamo-I and Shawnee, despite the fact they are different ecotypes, and of different morphology. However, when Dacotah was compared to the other varieties, the difference was obvious: Dacotah which is the same upland variety as the Shawnee contains more glucan and lignin (Figure 3.3). Total structural sugars present in different switchgrass varieties in the range of 53-62%. The difference in glucan and lignin contents between Dacotah and other varieties was 3-6% and 3-4%, respectively (Figure 3.3). It appears that the difference is due to delay in harvesting rather than plant date or ecotype. Adler et al. (2006) reported that delayed harvest increases structural sugar and lignin contents, while reducing soluble sugars and ash contents in switchgrass. Bals et al. (2010) suggested that the switchgrass mobilizes solubles and ash for storage in root system, thereby causing the reduced solubles and ash contents for later harvests.

Table 3.3: Solid composition of the untreated switchgrass varieties

	Alamo - I	**Alamo - II**	**Shawnee**	**Dacotah**
Glucan (%)	29.9	32.1	30.9	35.3
Xylan (%)	18.1	18.5	17.9	20.1
Galactan (%)	2.4	2.1	2.4	3.1
Arabinan (%)	3.4	2.7	3.1	3.4
Total Structural Sugars (%)	**53.8**	**55.4**	**54.3**	**61.9**
Water Extractable Soluble Sugars (%)	**8.6**	**7.5**	**7.3**	**0.6**
Lignin (%)	18.8	19.5	19.7	22.6
Ash (%)	3.9	4.3	4.2	3.3
Acetyl (%)	2.2	2.8	2.9	3.6
Mass closure (%)	**87.3**	**89.0**	**88.4**	**92.2**

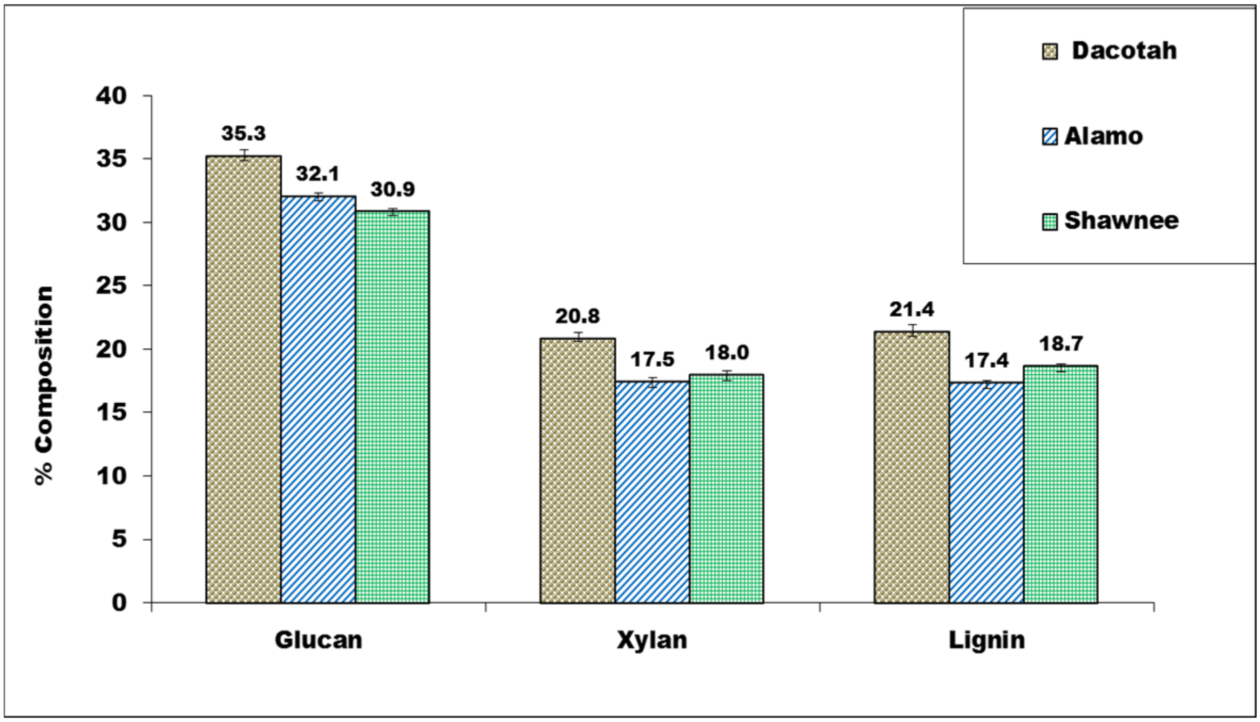

Figure 3.3: Comparison of glucan, xylan, and lignin contents in different switchgrass species

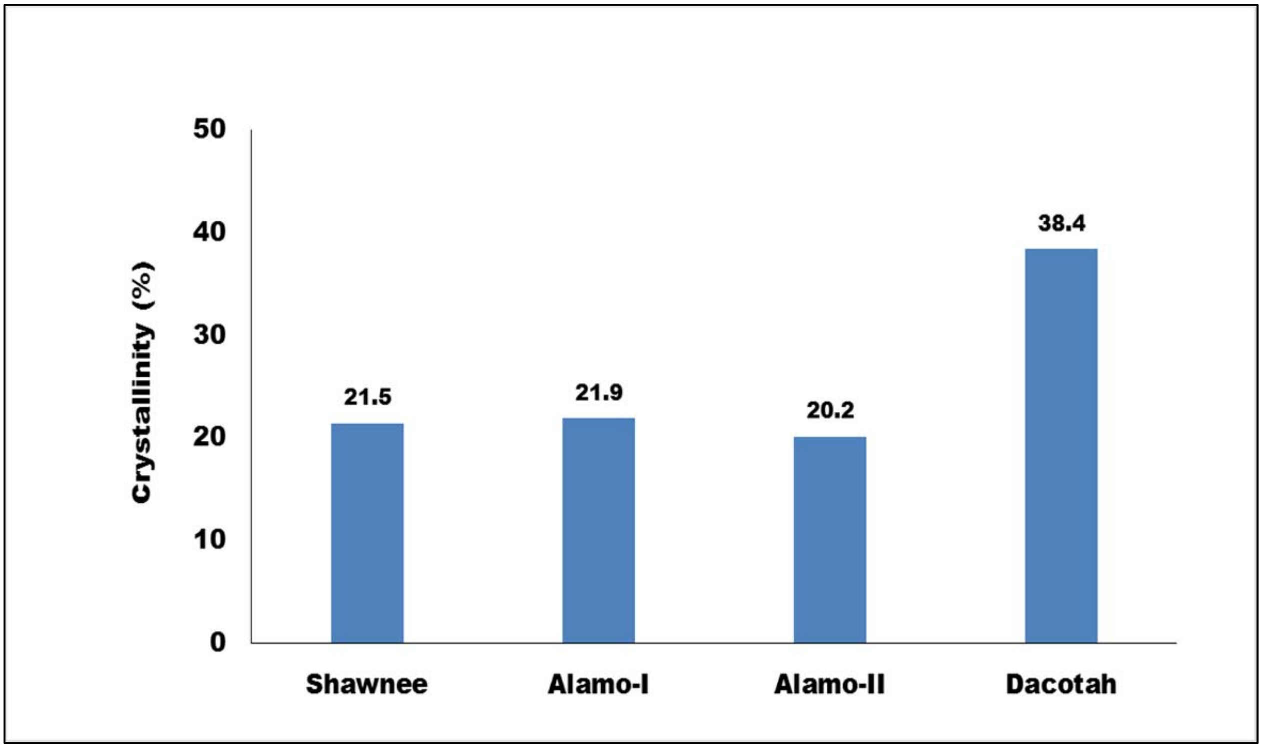

Figure 3.4: Crystallinity index of untreated switchgrass species

3.4.3. Effect of aqueous ammonia pretreatment on different switchgrass varieties

Ammonia proved to be very effective in swelling and delignification of biomass (Yoon et al., 1995; Iyer et al., 1996; Kim et al., 2003; Kim and Lee, 2005). Aqueous ammonia as a pretreatment reagent has been investigated by researchers in our lab for different feedstocks such as corn stover (Kim et al., 2003; Kim and Lee, 2005), hardwood (Yoon et al., 1995), and hybrid poplar (Gupta et al., 2009). Ammonia pretreatment proved to be effective for the substrates containing low lignin, and the switchgrass is one such low lignin containing substrate. Thus in this study the effects of aqueous ammonia pretreatment on solid composition and enzymatic hydrolysis of different switchgrass species were studied. As a part of this study it was also evaluated that the technical feasibility of uniform pretreatment conditions across the different switchgrass varieties to achieve similar sugar recovery yields. The different switchgrass species subjected to aqueous ammonia pretreatment were: Shawnee, Alamo-I, Alamo-II, and Dacotah. Pretreatment experiments with Shawnee, Alamo were carried out using mild SAA method. SAA pretreatment is considered mild because it is primarily operated at low temperature (up to 90^oC) and longer treatment time (12-48 hr) in a batch reactor. A majority of SAA experiments with Dacotah were carried out at low temperature and longer treatment time; while a selected few experiments carried out at high temperature (160^oC) and shorter treatment time (1 hr). The reason for operating SAA pretreatment at high temperature was due to exhibition of recalcitrance of Dacotah at low temperature. In addition, Dacotah was also subjected to ARP pretreatment, which is carried at high temperature and shorter treatment time in a flow through reactor. The reason for subjecting Dacotah ARP treatment was that it proved to be effective with high recalcitrant substrates (Gupta and Lee, 2008). It should be noted that prior to pretreatment all samples were hot water washed to remove soluble sugars. SAA and ARP pretreatment experiments were carried out as described in Methods section. Pretreated solids were enzymatically hydrolyzed following a standard enzymatic hydrolysis protocol as mentioned Methods section. For all the experiments 15 FPU/g-glucan of Spezyme-CP (cellulase) and 30 CBU/g-glucan of Novozyme-188 (β-glucosidase) enzyme loading was applied.

3.4.3.1. Solid composition and enzymatic hydrolysis of SAA pretreated switchgrass varieties

Untreated switchgrass varieties did not exhibit any significant differences in sugar yields. Without pretreatment sugar yields were only 13-16% for glucose and 4-7% for xylose (Figure 3.5, Figure 3.6, Figure 3.7, and Figure 3.8). Pretreatment improved enzymatic hydrolysis yields of all switchgrass verities. First Shawnee was subjected to SAA pretreatment using mild temperatures (60-90^{o}C) and long treatment time (12-48 hr). Different factors were considered before choosing temperature in 60-90^{o}C range. SAA pretreatment proved to be very effective for corn stover below 100^{o}C in delignification and carbohydrate retention while achieving high glucan/xylan digestibilities (Kim and Lee, 2005). Corn stover and Shawnee contain approximately (18%) similar amounts of lignin. Another reason for keeping temperature bellow 100^{o}C was to prevent hemicellulose loss, which is prone to degradation at higher temperatures (Hendriks and Zeeman, 2009). The solid composition and enzymatic digestibilities of untreated and SAA pretreated Shawnee are shown in Table 3.4 and Figure 3.5. Pretreated compositions are shown on the basis of % total initial dry switchgrass before pretreatment to reflect the fate of each component during the pretreatment. Solid composition data show substantial delignification and carbohydrate retention with all the pretreatment conditions. SAA pretreatment of Shawnee at 60^{o}C, 24 hr, solid to liquid ratio of 9, and 15% NH_4OH achieved 37% delignification and 98% glucan/ 78% xylan retention (Table 3.4). Solids obtained at this pretreatment condition resulted in 62% glucan and 48% xylan digestibilities, respectively. Increase in temperature from 60^{o}C to 70^{o}C by keeping other pretreatment variables constant has not affected the carbohydrate and lignin contents but increased the glucan and xylan digestibilities by 7% and 10%, respectively. However when the temperature increased from 60^{o}C to 90^{o}C by keeping other pretreatment variables constant increased the delignification substantially, and caused only slight loss of carbohydrate content (Table 3.4). With this pretreatment condition 97% glucan/65% xylan retention, and 56% delignification, was achieved. Solids obtained at this pretreatment condition resulted in 80% glucan and 69% xylan digestibilities, respectively. Increase in treatment time from 24 hr to 48 hr, neither affected the Shawnee composition nor glucan/xylan digestibilities. Increase in solid to liquid ratio (from 7 to 9) has a marginal effect on delignification and carbohydrate content. However solid to liquid ratio has a significant effect on enzymatic hydrolysis, glucan and xylan digestibilities were improved by 8% and 5%, respectively.

Having obtained 80% glucan digestibility with SAA pretreated Shawnee, which is considered substantial, SAA pretreatment was further applied to Alamo I, Alamo II and Dacotah using similar pretreatment conditions. The solid composition of untreated and SAA pretreated Alamo-I and Alamo-II are shown in Table 3.5. Results show that Alamo-I, Alamo-II, exhibited identical behavior to Shawnee in carbohydrate retention and delignification after the pretreatment. The optimum pretreatment condition found for Alamo-I and Alamo-II are 90°C, 15% NH_4OH, liquid to solid ratio of 9, and 24 hr, which is same as Shawnee. Optimally pretreated Alamo-I and Alamo-II glucan/xylan digestibilities were 82.9%/65.0% and 83.3%/66.4%, respectively (Figure 3.6 and Figure 3.7). However the optimum pretreatment condition of Shawnee and Alamo is not effective enough for Dacotah to achieve high hydrolysis yields (Table 3.6 and Figure 3.8). Enzymatic hydrolysis results show that with the similar pretreatment conditions (90°C, 15% NH_4OH, liquid to solid ratio of 9, and 24 hr), Alamo-I, Alamo-II, and Shawnee resulted substantially higher glucan/xylan (as high as 19%/13%) digestibility than the Dacotah (Figure 3.5, Figure 3.6, Figure 3.7, and Figure 3.8). The primary reason for Dacotah recalcitrance could be due to age of the plant. Dacotah plant has stood for more than 8 years, whereas Alamo and Shawnee plans stood for less than 2 years. With the age glucan, xylan and lignin contents of Dacotah were increased. With increase in glucan content its crystallinity has also increased (Figure 3.4). Crystallinity plays a major role in enzymatic hydrolysis of biomass, i.e., the higher the Crystallinity the lower the amount of cellulose that can be digested (Agarwal et al., 2013). Although the higher glucan content in Dacotah slightly increases its potential biofuel yield, that potential is more difficult to realize due to its higher recalcitrance. Bals et al. (2010) reported higher sugar yields from early harvests than from later harvests of switchgrass with AFEX pretreatment. As suggested by Bals et al. (2010) lower lignin content may be one of the reasons for the greater sugar yields observed in the early harvests of the switchgrass. Another factor would be that the drying of the switchgrass over winter may negatively affect the recalcitrance of the cellulose. Switchgrass plots over the winter dry out and become more brittle as the plots undergo dry and cold weather (Adler et al., 2006). Pretreatment efficiency and enzyme accessibility of switchgrass might have decreased due to collapse of plant cell walls from the drying over the winter. The difference in enzymatic digestibility between the fall harvested/lowland Alamo and fall harvested/upland Shawnee was much less than the difference found between the Shawnee and Dacotah which are the same upland types with different harvest seasons. The enzymatic hydrolysis results suggest that not only compositions, but also reactivity of switchgrass to pretreatment and enzymatic hydrolysis is strongly dependent of harvest season and time, later harvests are more difficult to hydrolyze than early harvests.

Table 3.4: Solid composition of untreated and SAA pretreated Shawnee

	Untreated Shawnee (%)	**Pretreated Shawnee**				
		60^{o}C, L/S: 9, 24 hr (%)	**60^{o}C, L/S: 9, 48 hr (%)**	**70^{o}C, L/S: 9, 24 hr (%)**	**90^{o}C, L/S: 6, 24 hr (%)**	**90^{o}C, L/S: 9, 24 hr (%)**
Glucan	30.90	30.32	30.14	30.17	30.23	30.10
Xylan	18.00	13.46	13.29	12.83	12.49	11.90
Galactan	3.40	2.40	2.42	2.31	2.21	1.94
Arabinan	3.10	2.51	2.65	2.03	1.45	1.24
Mannan	1.01	0.70	0.83	0.65	0.57	0.55
Lignin	18.70	11.81	12.00	11.67	11.40	10.54
Ash	4.20	2.38	2.56	2.27	2.29	2.14
Acetyl	2.90	1.42	1.35	1.30	1.11	0.94
Mass Closure	**82.11**	**65.00**	**65.24**	**63.23**	**61.75**	**59.35**

Note: 15% NH_4OH used for all the experiments; pretreated compositions are shown on the basis of % total initial dry switchgrass before pretreatment to reflect the fate of each component during the pretreatment

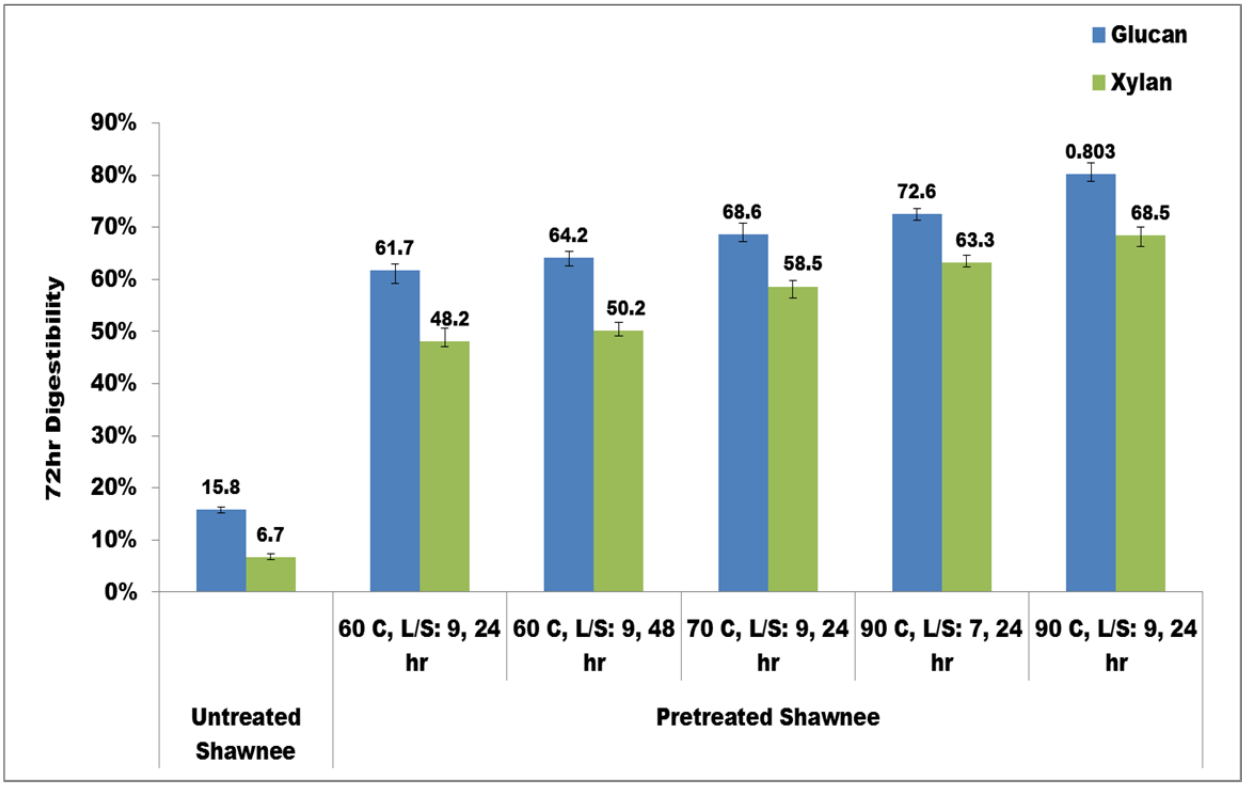

Figure 3.5: 72 hr glucan/xylan digestibility of untreated and SAA treated Shawnee
Enzyme Loading: Spezyme CP (20.84 mg of protein /g-glucan) + Novozyme – 188 (6.25 mg of protein/g-glucan)

Table 3.5: Solid composition of untreated and SAA pretreated Alamo-I and Alamo-II

	Untreated Alamo -I (%)	**Pretreated Alamo-I**					**Untreated Alamo -II (%)**	**Pretreated Alamo-II**	
		60^{o}C, L/S: 6, 24 hr (%)	**90^{o}C, L/S: 6, 24 hr (%)**	**60^{o}C, L/S: 9, 24 hr (%)**	**90^{o}C, L/S: 9, 24 hr (%)**	**90^{o}C, L/S: 9, 12 hr (%)**		**60^{o}C, L/S: 9, 24 hr (%)**	**90^{o}C, L/S: 9, 24 hr (%)**
Glucan	32.13	31.67	31.32	31.52	30.90	30.15	31.57	30.40	30.21
Xylan	17.47	14.96	13.39	14.34	11.15	11.74	18.94	14.15	12.34
Galactan	3.62	2.73	2.05	2.65	2.15	2.45	2.94	1.63	1.47
Arabinan	4.20	2.98	2.33	2.65	1.20	1.24	3.54	1.91	1.12
Mannan	1.33	0.83	0.32	0.84	0.00	0.45	0.00	0.00	0.00
Lignin	17.40	11.56	10.18	11.36	9.80	9.89	18.32	11.05	9.92
Ash	3.54	2.71	2.65	2.65	2.13	2.26	3.36	2.21	1.94
Acetyl	2.43	1.51	1.38	1.45	1.16	1.14	2.53	1.32	1.09
Mass Closure	**82.12**	**68.95**	**63.62**	**67.66**	**58.69**	**59.31**	**81.70**	**62.67**	**58.09**

Note: 15% NH_4OH used for all the experiments; pretreated compositions are shown on the basis of % total initial dry switchgrass before pretreatment to reflect the fate of each component during the pretreatment

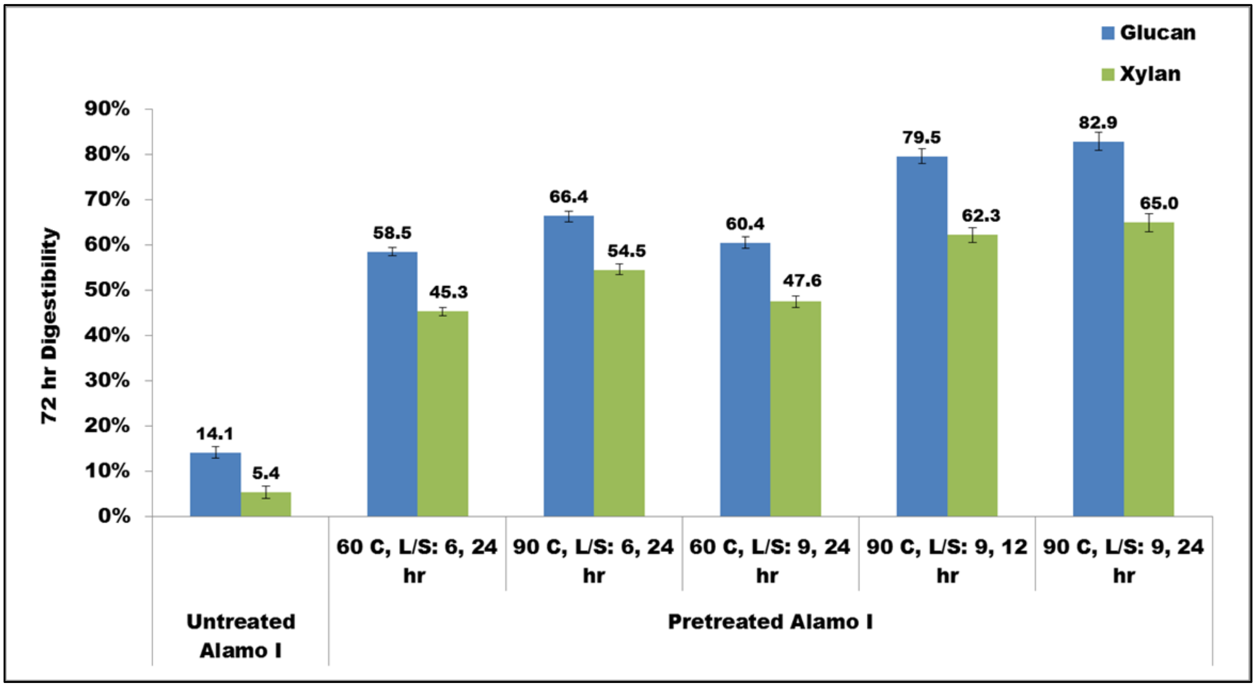

Figure 3.6: 72 hr glucan/xylan digestibility of untreated and SAA treated Alamo-I
Enzyme Loading: Spezyme CP (20.84 mg of protein /g-glucan) + Novozyme – 188 (6.25 mg of protein /g-glucan)

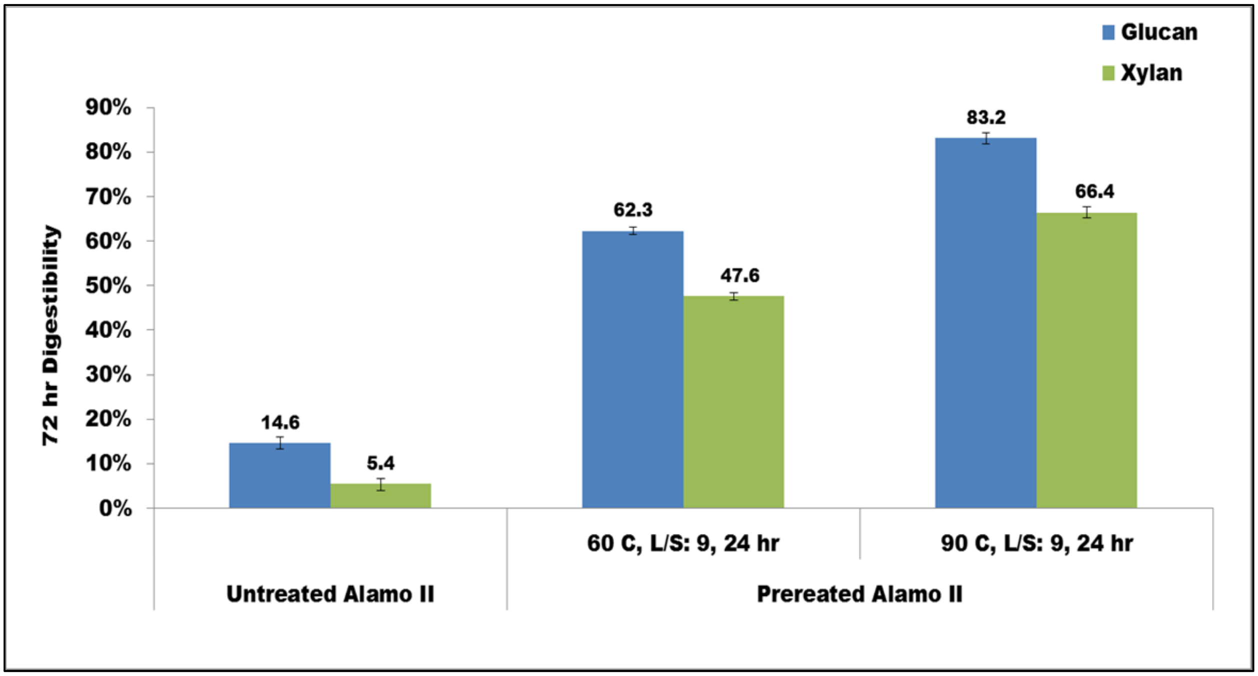

Figure 3.7: 72 hr glucan/xylan digestibility of untreated and SAA treated Alamo-II
Enzyme Loading: Spezyme CP (20.84 mg of protein /g-glucan) + Novozyme – 188 (6.25 mg of protein /g-glucan)

Table 3.6: Solid composition of untreated and SAA pretreated Dacotah

	Untreated (%)	**7% NH_4OH, 90°C, 24 hr**			**L/S: 9, 90°C, 24 hr**			**L/S: 9, 15% NH_4OH, 24 hr**			**L/S: 9, 90°C, 15% NH_4OH**			**L/S: 9, 160°C, 15% NH_4OH**
		L/S: 4 (%)	**L/S: 6 (%)**	**L/S: 9 (%)**	**NH_4OH (%)**			**60°C (%)**	**90°C (%)**	**110°C (%)**	**3 hr (%)**	**15 hr (%)**	**24 hr (%)**	**1 hr (%)**
					7%	**15%**	**28%**							
Glucan	35.30	34.85	34.43	34.20	34.20	34.14	34.33	34.94	34.14	33.91	34.90	34.8	34.14	34.46
Xylan	20.84	17.93	17.27	16.69	16.69	16.23	16.03	17.50	16.23	14.10	18.15	17.66	16.23	13.63
Galactan	3.11	2.67	2.76	2.35	2.35	2.64	2.88	2.79	2.64	2.45	2.85	2.45	2.64	2.32
Arabinan	3.19	2.43	2.31	1.81	1.81	1.51	1.49	2.47	1.51	1.56	2.52	2.32	1.51	1.49
Lignin	21.40	14.75	13.90	13.68	13.68	12.93	11.14	14.51	12.93	10.89	14.38	13.36	12.93	8.58
Ash	3.90	2.53	2.58	2.37	2.37	1.95	2.05	2.98	1.95	2.24	2.61	2.20	1.95	1.76
Acetyl	2.73	1.03	1.31	1.05	1.05	0.93	1.01	1.35	0.93	0.81	1.36	1.25	0.93	0.89
Mass Closure*	**90.47**	**75.78**	**72.88**	**68.90**	**68.90**	**66.11**	**65.93**	**74.36**	**66.11**	**63.70**	**75.77**	**71.04**	**66.11**	**63.13**

Note: Mass closure is excluding extractives; pretreated compositions are shown on the basis of % total initial dry switchgrass before pretreatment to reflect the fate of each component during the pretreatment

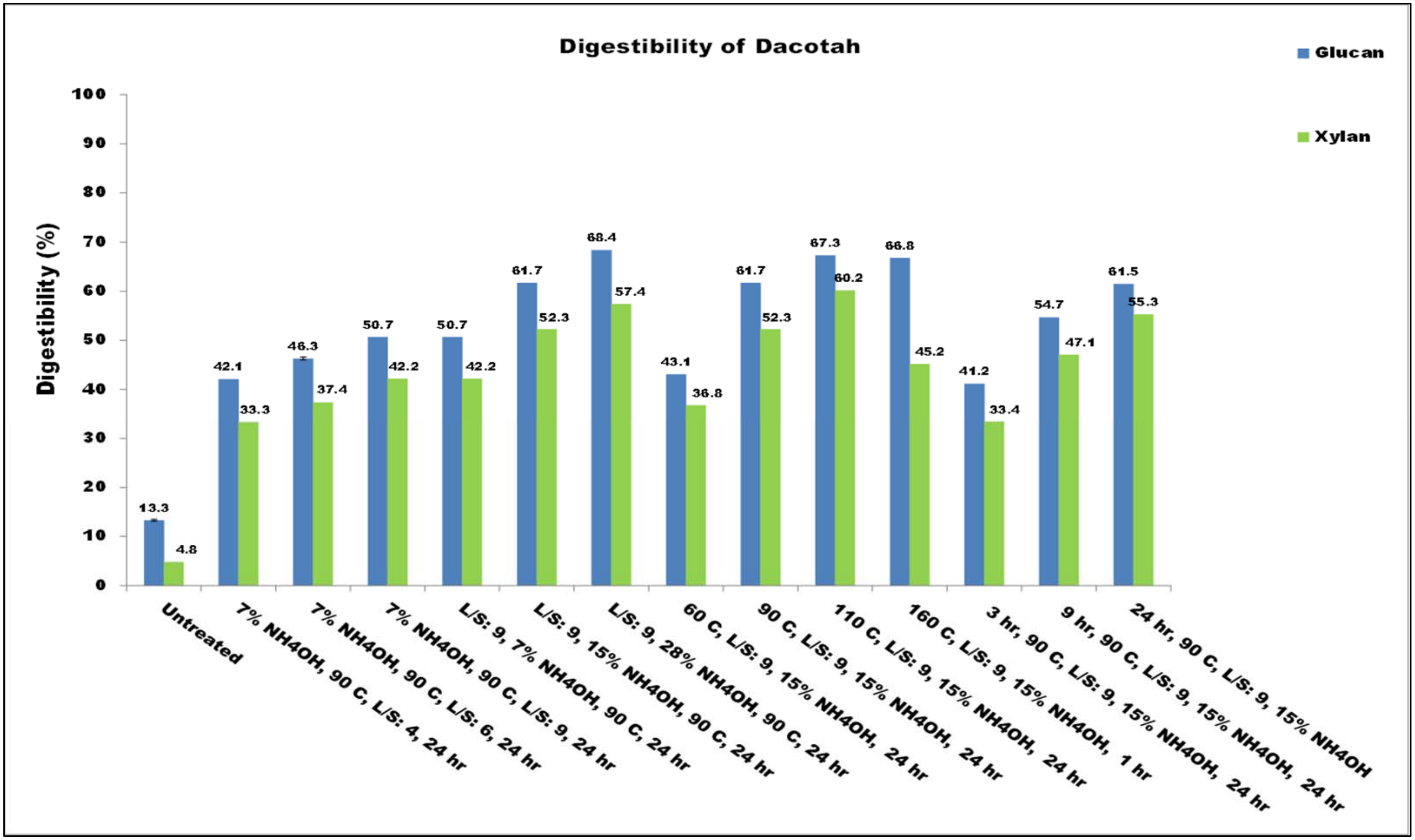

Figure 3.8: 72 hr glucan/xylan digestibility of untreated and SAA treated Dacotah
Enzyme Loading: Spezyme CP (20.84 mg of protein /g-glucan) +Novozyme–188 (6.25 mg of protein /g-glucan)

3.4.3.2. Effect of pretreatment variables on solid composition and enzymatic digestibility of Dacotah switchgrass

As Dacotah proved to be highly recalcitrant at mild pretreatment conditions, it was decided to increase the pretreatment severity. To increase the pretreatment severity experiments were carried out by changing temperature, treatment time, and aqueous ammonia concentration one at a time while keeping other conditions constant. For all the samples SAA pretreatment was used. The main factors considered, while selecting the pretreatment conditions were, overall sugar recovery, increase in delignification, and minimization of carbohydrate loss. The pretreated compositions are shown on the basis of % total initial dry switchgrass before pretreatment to reflect the fate of each component during the pretreatment. The washed pretreated samples were subjected to enzymatic saccharification using 15 FPU/g-glucan of cellulase (Spezyme-CP) and 30 CBU/g-glucan of β-glucosidase (Novozyme-188) (equivalent to 27 mg protein/g glucan).

Effect of temperature

To study the effect of temperature on Dacotah switchgrass composition and enzymatic digestibility, pretreatment experiments were carried out at four different temperatures (60^{o}C, 90^{o}C, 110^{o}C, 130^{o}C) while keeping treatment time (24 hr), ammonia concentration (15%), liquid/ solid ratio (9) constant. Effect of temperature SAA pretreatment on solid composition and enzymatic hydrolysis are shown in Figure 3.9 and 3.10. The results indicate that the substantial delignification was achieved even at lowest temperature 60^{o}C (57%). Results show that increasing the temperature from the 60^{o}C to 90^{o}C increased the delignification substantially, after that no significant delignification was observed (Figure 3.9). On the other hand, increasing the temperature has resulted in a significant xylan loss, but glucan content was not affected. Hemicelluloses are known to be more sensitive to temperature than cellulose (Hendriks and

Zeeman, 2009). Hanim et al. (2012) reported that xylan is sensitive to temperature without any chemical addition. According to their report when the temperature increased from 170°C to 200°C, approximately 50% loss of xylan was observed. This tendency was in agreement with other reports in the extraction of hemicelluloses (Qiao et al., 2009; Cheng et al., 2011).

Increasing pretreatment temperature in general resulted in higher glucan and xylan digestibilities (Figure 3.10). Results show that when the temperature increased from 60°C to 90°C, glucan and xylan digestibilities increase by 18.4% and 15.5%, respectively. Further increase in the temperature from 90°C to 130°C has increased the glucan/xylan digestibilities, but it was not as high as compared to increase in temperature from 60°C to 90°C. It was possibly due to less amount of delignification achieved when the temperature increased from 90°C to 130°C. The amount of delignification achieved fairly correlate with glucan digestibility, but not with xylan digestibility. At 130°C substantial xylan was lost, but loss of xylan did not have any significant effect on xylan digestibility, which is contrary to what happens in acid and hot water pretreatments. Dilute acid and liquid hot water pretreatments cause substantial loss in hemicellulose content, but higher digestibilities are achieved during the enzymatic hydrolysis (Wyman et al., 2011).

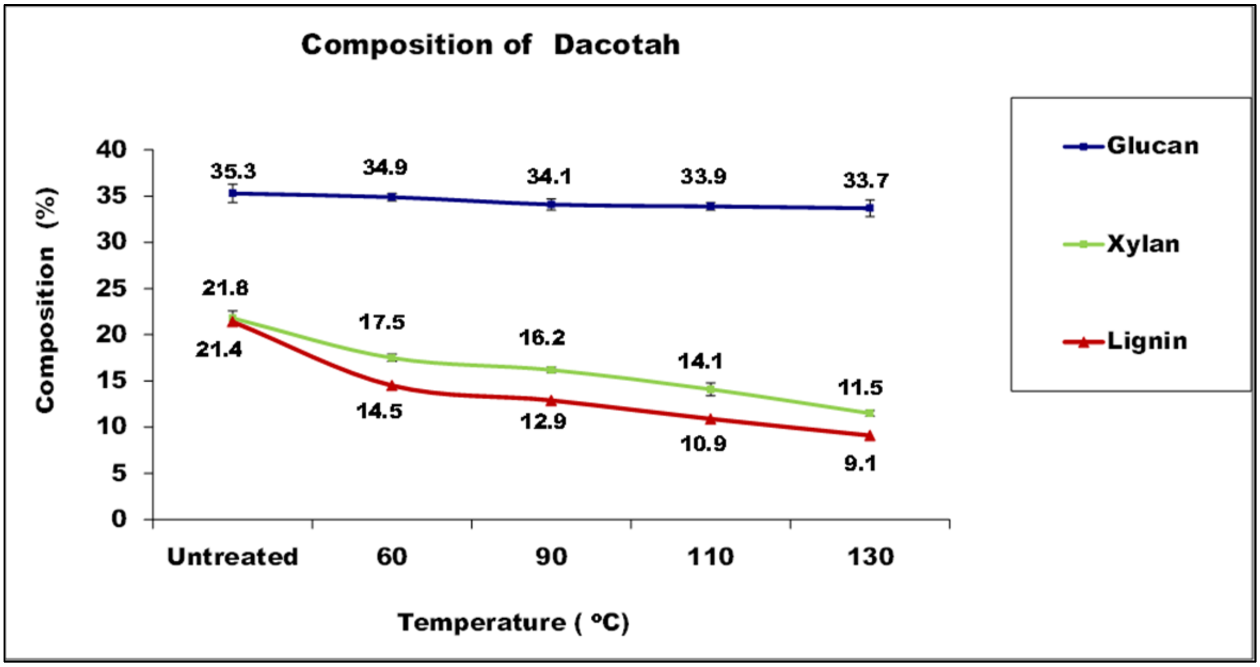

Figure 3.9: Effect of temperature on composition of Dacotah
Other pretreatment conditions: 24 hr, 15% NH_4OH, liquid/ solid ratio of 9

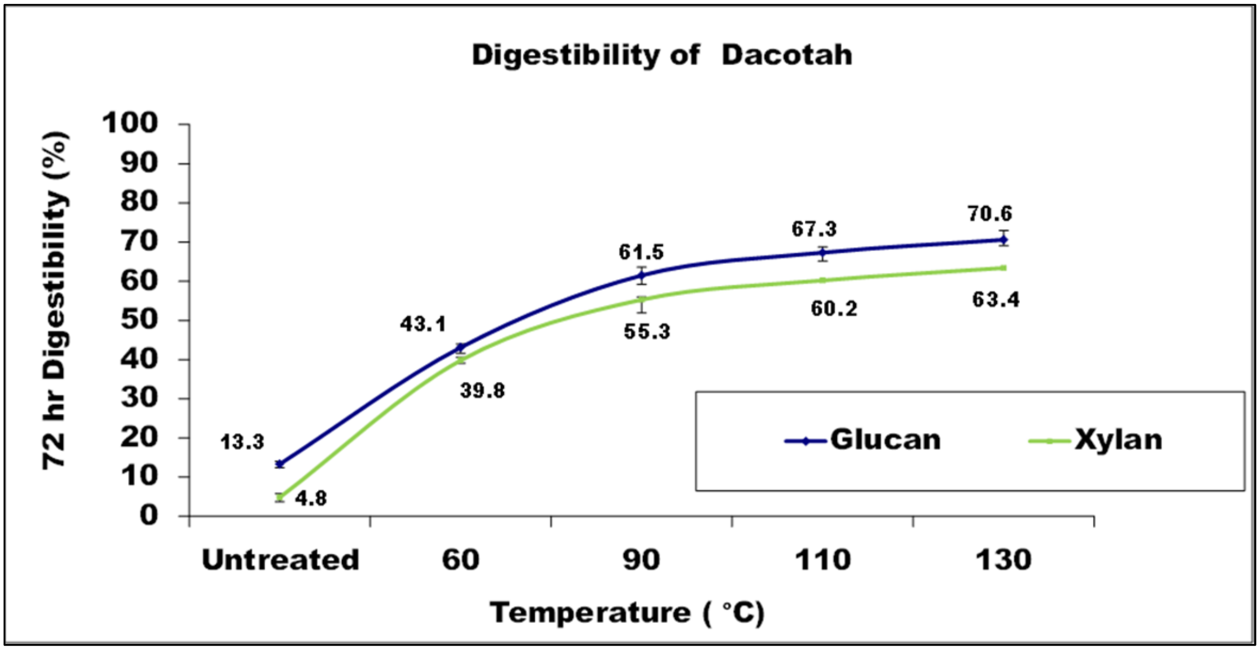

Figure 3.10: Effect of temperature on enzymatic digestibility of Dacotah
Enzyme Loading: Spezyme CP (20.84 mg of protein /g-glucan) + Novozyme – 188 (6.25 mg of protein /g-glucan)

Effect of ammonia concentration

To study the effect of ammonia concentration on composition and enzymatic digestibility of Dacotah switchgrass, experiments were carried out using different ammonia concentrations (7, 15, 28 (wt.%)) while keeping treatment time (24 hr), temperature (90°C), liquid/ solid ratio (9) constant. The solid composition and enzymatic hydrolysis yields of untreated and SAA treated Dacotah switchgrass are shown in Figure 3.11 and Figure 3.12, respectively. The solid composition indicates that the increasing ammonia concentration did not affect the carbohydrate content, but increased the delignification. When the ammonia concentration increased from 7% to 15%, an increase in delignification (by 14.9%) was observed, which resulted an increase in glucan/ xylan digestibilities by 10.8%/11.1%, respectively. Although, increasing the ammonia concentration to 28% increased the delignification by 7.5%, but it did not show any effect on glucan/xylan content. Increase in delignification clearly improved the glucan/xylan digestibilities.

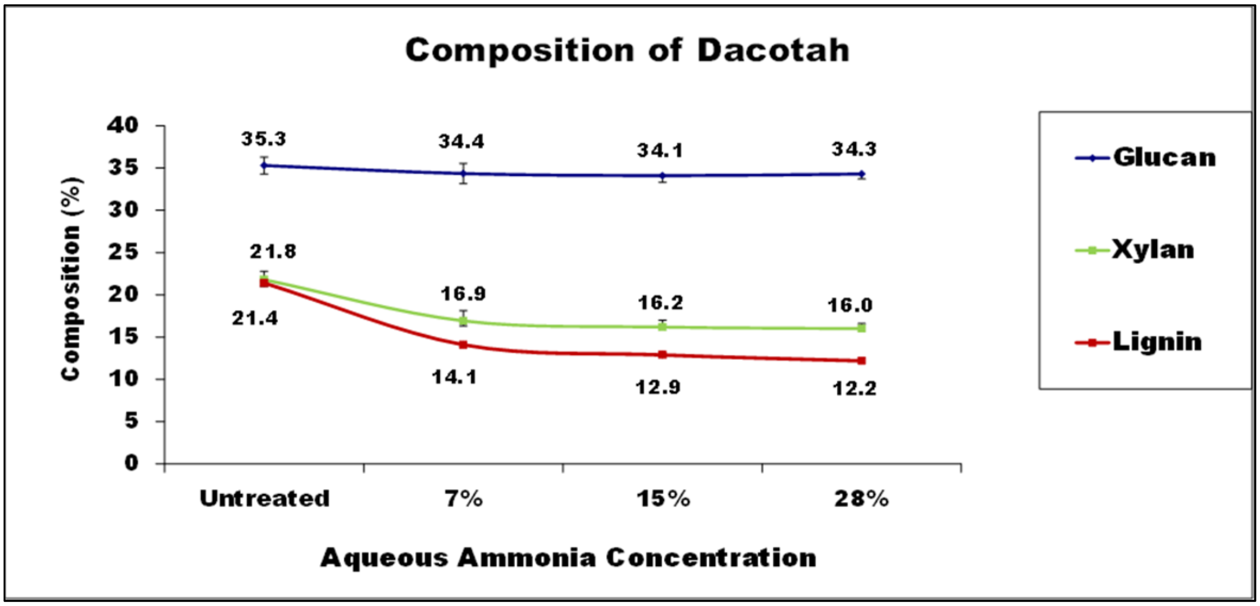

Figure 3.11: Effect of ammonia concentration on composition of Dacotah
Other pretreatment conditions: 24 hr, 90°C, liquid/ solid ratio of 9

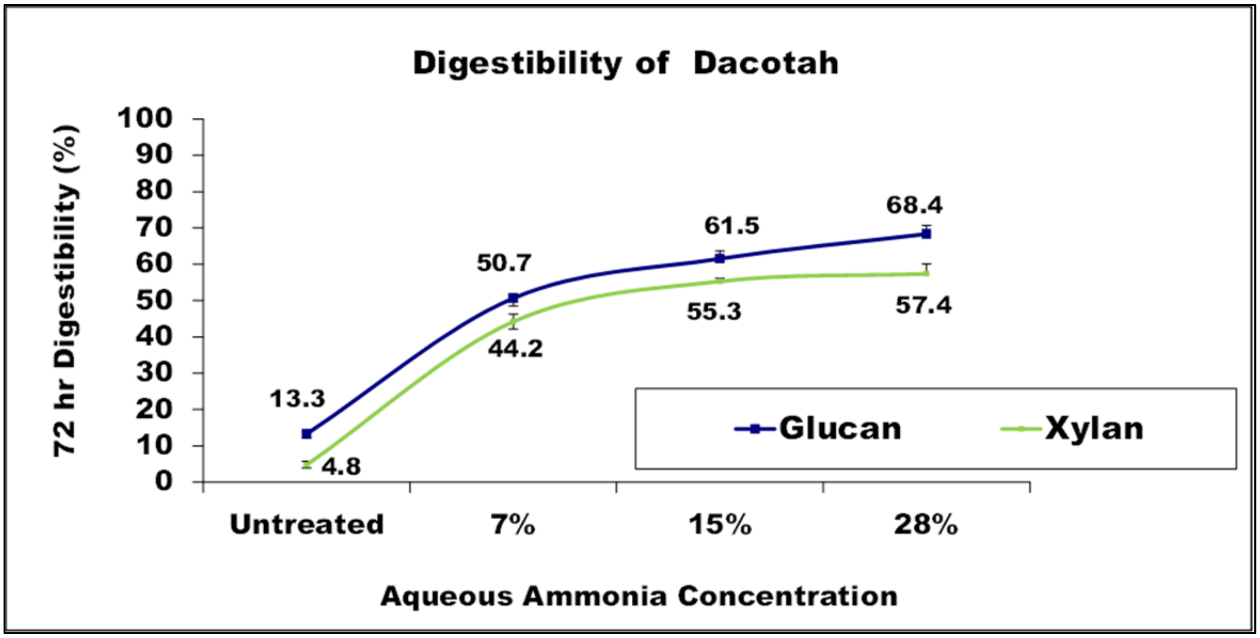

Figure 3.12: Effect of ammonia concentration on enzymatic digestibility of Dacotah
Enzyme Loading: Spezyme CP (20.84 mg of protein /g-glucan) + Novozyme – 188 (6.25 mg of protein /g-glucan)

Effect of pretreatment time

To understand the effect of pretreatment time on composition and enzymatic digestibility of Dacotah switchgrass, experiments were carried out at different pretreatment times (3 hr, 15 hr, 24 hr), while keeping the ammonia concentration (15 wt. %), temperature ($90^{o}C$), and liquid/ solid ratio (9) constant. The composition and enzymatic hydrolysis yields of untreated and SAA treated Dacotah switchgrass are shown Figure 3.13 and Figure 3.14, respectively. Results indicate that in 3 hr treatment time 30.4% delignification, 98.9% glucan and 83.0% xylan retention were achieved. Although delignification caused the increase in the digestibilities, but the increase was not very high, only 41.2% glucan digestibility and 36.4% xylan digestibility were achieved. Increase in the treatment time from 3 hr to 15 hr did not cause any significant carbohydrate loss, but an increase in delignification by 7.2% was attained. Increase in delignification has a positive effect on increasing glucan (by 17.5%) and xylan (by 15.7%) digestibilities, but substantial amount of glucan and xylan still remain unhydrolysed. Increase in treatment time to 24 hr slightly increased the delignification (by 2%), but 6.4% xylan loss was observed. Glucan and xylan digestibilities were marginally improved from 15 hr to 24 hr, which is not surprising considering the low amount delignification.

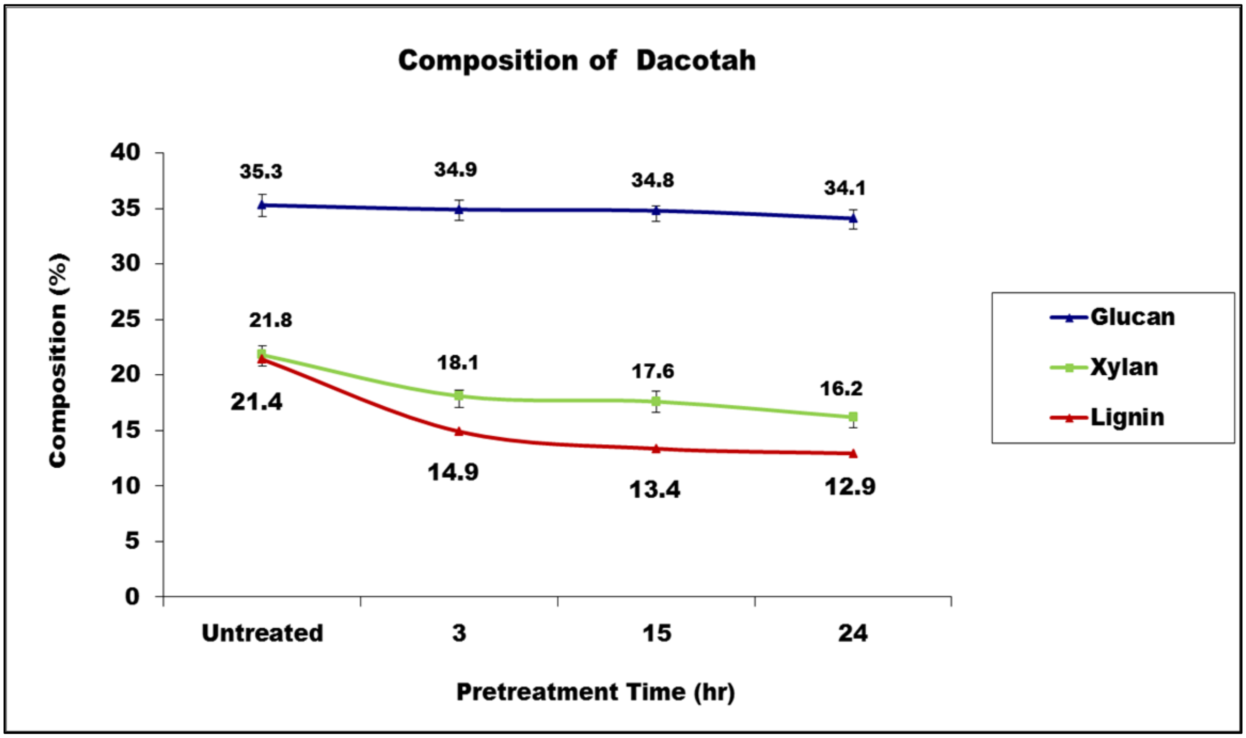

Figure 3.13: Effect of pretreatment time on composition of Dacotah
Other pretreatment conditions: 90^{o}C, 15% NH_4OH, liquid/ solid ratio of 9

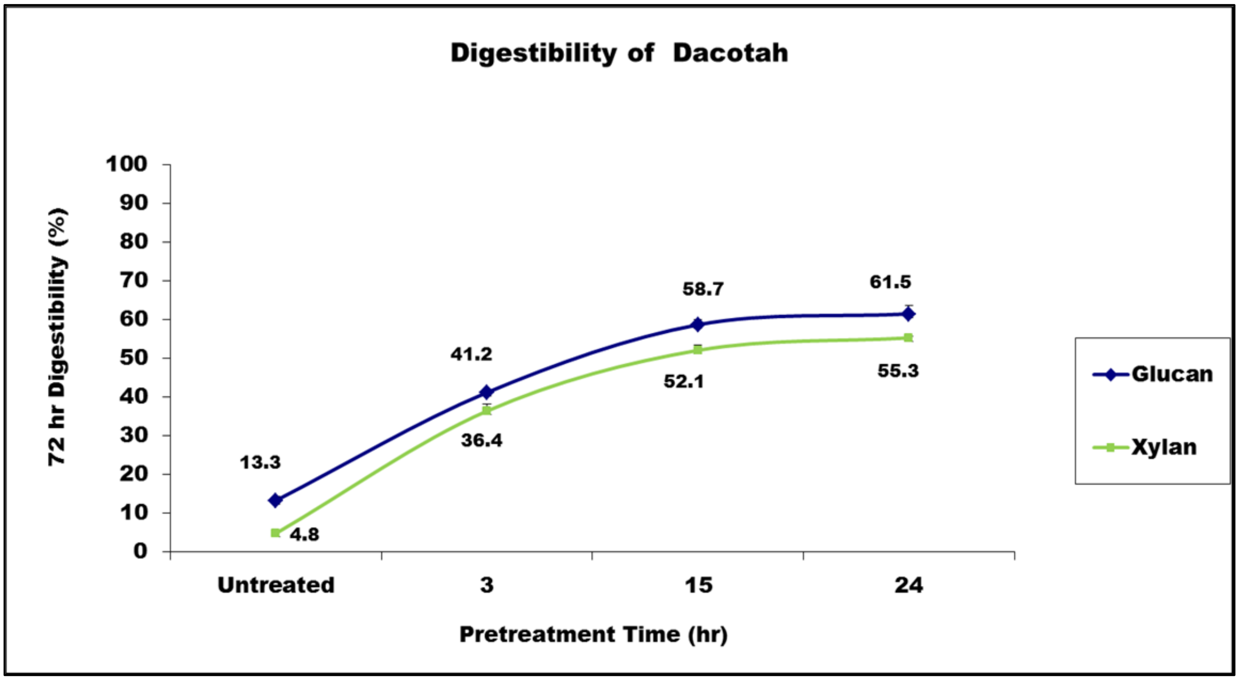

Figure 3.14: Effect of pretreatment time on enzymatic digestibility of Dacotah
Enzyme Loading: Spezyme CP (20.84 mg of protein /g-glucan) + Novozyme - 188(6.25 mg of protein /g-glucan)

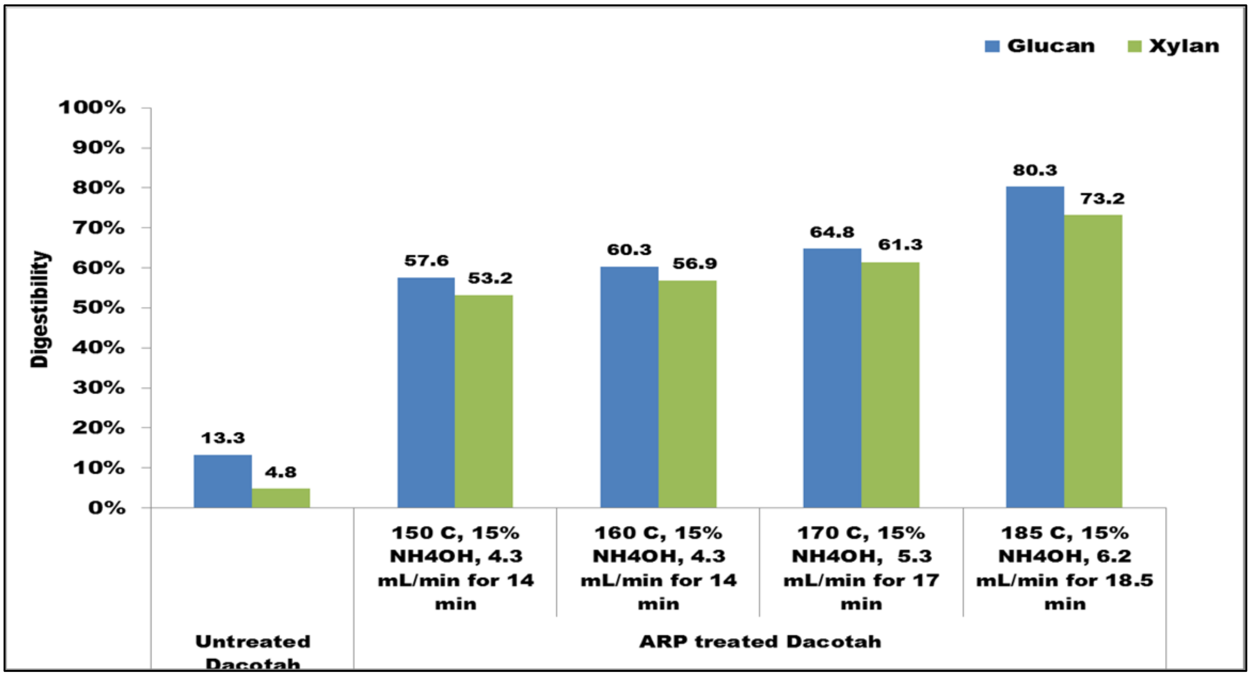

Figure 3.15: 72 hr glucan/xylan digestibility untreated and ARP treated Dacotah
Enzyme Loading: Spezyme CP (20.84 mg of protein /g-glucan) + Novozyme - 188(6.25 mg of protein /g-glucan)

3.4.3.3. Effect of ARP pretreatment on solid composition and enzymatic digestibility of Dacotah switchgrass

The ARP pretreatment proved to be very effective in delignifying highly recalcitrant hybrid poplar and subsequently yielding better enzymatic hydrolysis yields (Gupta et al., 2009). Dacotah switchgrass is proven to be highly recalcitrant even after severe SAA treatment because of insufficient delignification, due to this reason Dacotah switchgrass was subjected to ARP pretreatment. The ARP pretreatment method employs a packed-bed column reactor through which the pretreatment liquid flows. ARP treatment applied to Dacotah switchgrass at 150°C, 14 min treatment time, and 4.3 mL/min liquid flow rate achieved 40% delignification. Composition data of ARP treated Dacotah is shown in Table 3.7. The glucan and xylan digestibilities of treated solids were 57.6% and 53.2, respectively, with 15 FPU/g-glucan Spezyme-CP and 30 CBU/g-glucan Novozyme-188 loading. A very low amount of delignification and corresponding increasing hydrolysis yields were attained when the treatment temperature increased from 150°C to 160°C. However, higher delignification (61%) was achieved when overall pretreatment severity was increased instead of just temperature (185°C, 15% NH_4OH, and 7.2 mL/min for 19.5 min). Although higher pretreatment severity resulted in higher glucan (83.3%) and xylan (77.2%) digestibilities (Figure 3.15), significant amount of carbohydrate loss was also occurred (Table 3.7).

Table 3.7: Solid composition of untreated and ARP treated Dacotah

	Untreated	**Pretreated**			
		150°C, 15% NH_4OH, 4.3 mL/min for 14 min	**160°C, 15% NH_4OH, 4.3 mL/min for 14 min**	**170°C, 15% NH_4OH, 5.3 mL/min for 17 min**	**185°C, 15% NH_4OH, 7.2 mL/min for 19.5 min**
Glucan	35.30	34.93	34.76	34.37	32.12
Xylan	20.84	14.85	14.25	13.38	11.05
Galactan	3.11	2.45	2.57	2.36	1.94
Arabinan	3.19	2.05	1.87	2.36	1.56
Lignin	21.40	12.94	12.06	10.85	8.35
Ash	3.90	2.71	2.31	2.08	2.32
Acetyl	2.73	1.64	1.52	1.32	1.05
Mass Closure	**90.47**	**71.57**	**69.34**	**67.16**	**59.71**

Note: Mass closure is excluding extractives; pretreated compositions are shown on the basis of % total initial dry switchgrass before pretreatment to reflect the fate of each component during the pretreatment

3.4.4. Physical characterization of untreated, SAA and ARP treated Dacotah switchgrass

Untreated, SAA and ARP treated Dacotah samples were analyzed for their physical characteristics using Scanning Electron Microscope (SEM), Brunauer Emmett Teller (BET).

3.4.4.1. SEM

The SEM images of untreated, SAA and ARP treated Dacotah switchgrass are shown in Figure 3.12. Surface structure shows high level of disruption with pretreatment. However, ARP pretreatment shows a higher disruption than that with SAA. SEM images strongly correlates with enzymatic hydrolysis yields, i.e., higher the pretreatment severity higher the disruption of biomass structure, hence higher the enzymatic hydrolysis yields with ARP.

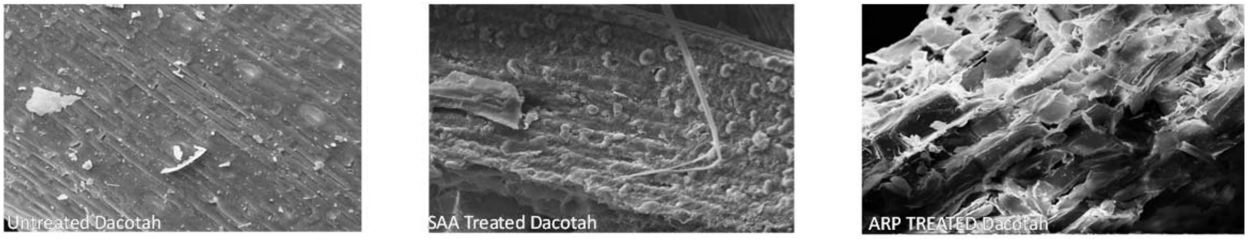

Figure 3.16: SEM images of SAA and ARP treated Dacotah

3.4.4.2. BET surface area

Table 3.8 shows the changes in the surface properties of the hybrid poplar as a result of ARP treatment. The BET surface area of the treated switchgrass is 4~5 times higher than the untreated one. Surface area also strongly correlates with the enzymatic hydrolysis yields with pretreatment severity, i.e., higher the pretreatment severity higher the disruption of biomass structure, hence higher the surface area, therefore higher enzymatic hydrolysis yields with ARP.

Table 3.8: BET surface area of untreated, SAA treated, ARP treated Dacotah

	Untreated (m^2/g)	**SAA treated (m^2/g)**	**ARP treated (m^2/g)**
BET Surface Area	0.6398	2.3601	2.6304

3.5. Conclusions

Aqueous ammonia as pretreatment reagent proved to be effective in delignification of switchgrass while retaining the majority the carbohydrates. Different switchgrass verities released monomeric sugars efficiently at a significantly increased rate and yield with aqueous ammonia pretreatment. Hydrolysis results show that with the similar pretreatment conditions (90°C, 15% NH_4OH, liquid to solid ratio of 9, and 24 hr), Alamo-I, Alamo-II, and Shawnee resulted substantially higher glucan/xylan (by 19%/13%) digestibilities than Dacotah. Enzymatic saccharification yield response was strongly correlated with harvest season, plant standing duration of the switchgrass, even when soluble sugars were excluded. The sugar yields are more dependent on harvest time and plant standing duration than eco/genotypes. Harvest time was a more important factor than the ecological or morphological type of the switchgrass in determining the quality of the switchgrass feedstock for the production of monomeric sugars. Increase in SAA pretreatment severity has not significantly improved the sugar recovery yield. ARP treatment results in higher delignification and higher hydrolysis yields than SAA, but significant amount of carbohydrate loss also occurs. SEM and BET surface area strongly correlates the pretreatment severity and enzymatic hydrolysis yields, i.e., the higher the pretreatment severity the higher the enzymatic hydrolysis yields.

4. Effect of Enzyme Mixture on Enzymatic Saccharification of SAA Pretreated Dacotah Switchgrass

4.1. Abstract

The goal of this work was to create a defined enzyme mixture that results in high yields of six and five carbon sugars from the SAA pretreated Dacotah switchgrass with the least amount of enzyme loading. Pretreated solids were subjected to enzymatic hydrolysis using various mixtures of commercial enzymes including, Spezyme-CP, Novozyme-188, Multifect Xylanase and Pectinase. The measured reaction products were glucose, xylose, cellobiose, oligomers of glucose and xylose. To identify the optimum enzyme ratio, different CBU/FPU (Novozyme-188/Spezyme-CP) ratios were applied, and the corresponding glucan/xylan digestibilities were determined. The optimum ratio based on CBU/FPU for the pretreated Dacotah was found to be 2. It was also evaluated whether the β-glucosidase (Novozyme-188) addition offers any advantage over simply increasing the dose of cellulase (Spezyme-CP). Supplementation of β-glucosidase increased the glucan and xylan digestibilities at all levels of enzyme loading throughout the hydrolysis. Glucan and xylan digestibilities increased rapidly with and without supplementation of β-glucosidase approximately up to 25 mg protein/g-glucan, after which the increase was leveled off. Up on increasing the enzyme loading based on total protein of cellulase and β-glucosidase from 25 to 32 mg protein/g-glucan, only 4%/3% increase in glucan/xylan digestibility was observed, whereas by supplementation of same amount of xylanase (Multifect Xylanase) and pectinase (Multifect Pectinase) increased glucan/xylan

digestibility by 21%/22% and 10%/19%, respectively. Addition of β-glucosidase decreased the cellobiose, glucose and xylose oligomers. Supplementation of xylanase/pectinase has a significant effect on reducing xylose oligomer but not on glucose oligomers at 72 hr hydrolysis period.

4. 2. Introduction

Hydrolysis of the pretreated lignocellulose to monomeric sugars primarily uses cellulase complex derived from filamentous fungi, particularly *Trichoderma reesei* (Nidetzky et al., 1994). Such an enzyme complex contains high levels of endo-glucanase and exo-glucanase activities, together with the lower levels of other enzyme activities such as β-glucosidase, β-xylosidase, xylanase and pectinase, etc. (Kabel et al., 2006; Dien et al., 2008). These lower level enzyme activities are called “background” activities. Enzyme mixtures with similar cellulase activity may show differences in performance on lignocellulose if they differ in levels of “background” activities (Berlin et al., 2005; 2007). For example, as a result of lower levels of β-glucosidase activity in cellulase complex, cellobiose accumulates to the levels that cause significant product inhibition during cellulose hydrolysis (Holtzapple et al., 1990; Berlin et al., 2007). Gupta and Lee (2008) reported inhibition on cellulose hydrolysis by high and low degree polymerization (DP) glucose oligomers produced from glucan, and suggested a suitable level of β-glucosidase can completely hydrolyze low (DP) glucose oligomers. Cellobiose and glucose/xylose oligomers are reaction intermediates produced by the combined actions of cellulases and their background activities.

Attempts to improve the hydrolytic efficiency of enzyme complexes have traditionally been focused on their component cellulases, because cellulose is the major polysaccharide component in lignocellulose (Berlin et al., 2005a; 2005b). Biomass characteristics can also have significant effect on enzymatic hydrolysis performance. Untreated biomass shows significant differences in their cellulosic and non-cellulosic components. Further differences in these components are introduced according to the pretreatment technology employed. However, non-cellulosic component in pretreated biomass, particularly hemicellulose and lignin, induce significant restraints on cellulose hydrolysis (Borjesson et al., 2007a; 2007b). For example, in one mechanism, lignin seems to reduce hydrolytic performance by binding to enzyme components unproductively (Borjesson et al., 2007a; 2007b). Although several studies reported inhibition of cellulase activity by lignin, glucose/xylose, and cellobiose (Holtzapple et al., 1990; Borjesson et al., 2007a; 2007b), but only limited information is available on the effect of oligomers on enzymatic hydrolysis process (Gupta and Lee, 2008; Kumar and Wyman, 2009a; Qing and Wyman, 2011). Most of studies are about xylose oligomers generated from dilute acid treated solids (Qing and Wyman, 2011). Xylose oligomers are known to strongly inhibit cellulase activity (Kumar and Wyman, 2009a; Qing and Wyman, 2011). But the possibility of presence of xylose oligomers is more with alkaline treated solids than dilute acid treated ones as the dilute acid pretreatment retains only 5% of xylan, whereas alkaline pretreatment retains more than 60% of xylan (Kim et al., 2011). One way to improve the efficiency of enzymatic hydrolysis by minimizing aforementioned inhibitions is to adjust the enzyme formulation. In addition, Dacotah switchgrass proved to be highly recalcitrant even after increasing the pretreatment severity. Thus, this work primarily focused on identifying the factors that influence sugar release from SAA pretreated Dacotah switchgrass during enzymatic hydrolysis with mixed enzymes. The different factors including, suitable β-glucosidase loading to cellulase, increase in cellulase loading with and without addition of β-glucosidase, supplementation of xylanase and pectinase were studied.

4.3. Materials and methods

4.3.1. Enzymes

Spezyme-CP (cellulase, 82 mg protein/mL, 59 FPU/mL), Multifect Xylanase (27 mg protein/mL), Multifect Pectinase (42 mg protein/mL) were provided by Genencor International, Inc. as part of CAFI project. Novozyme-188 (β-glucosidase, 67 mg protein/mL, 600 CBU/mL) and was purchased from Sigma-Aldrich (St. Louis, MO). The protein concentration of each enzyme was measured using Trichloroacetic acid precipitation and was reported by Genencor (Spezyme CP, Multifect Xylanase, and Multifect Pectinase) and Michigan State University (Novozyme 188) as a part of CAFI project. Primary and background activities of these enzymes are shown in Table 4.1.

4.3.2. Substrate preparation

Before the pretreatment, Dacotah switchgrass was milled to pass through a 40 mesh mechanical sieve. The pretreatment experiments were carried out in the stainless steel batch reactors with ammonia hydroxide as a pretreatment reagent. The pretreatment conditions used were: 15% aqueous ammonia, 160^{o}C, 60 min, liquid to solid ratio of 9. After the pretreatment, the samples were washed several times with cold water to remove soluble components and ammonia. The washed pretreated DSG was stored in sealed plastic bags at 4^{o}C before enzymatic hydrolysis. Pretreated solids were analyzed for the composition using the NREL Standard procedures (Sluiter et al., 2006). The amounts of glucan, xylan, and lignin present in the pretreated DSG were: 34.36%, 13.63%, and 8.58%.

4.3.3. Enzymatic hydrolysis

Hydrolysis experiments were carried out in 250 mL Erlenmeyer flasks at 1% glucan loading and a total working volume of 100 mL. All samples were adjusted to a pH of 4.8 using 0.05 M sodium citrate buffer solution. To prevent fungal and bacterial contamination during enzymatic hydrolysis, cycloheximide and tetracycline were loaded at a final concentration of 30 µg/mL and 40 µg/mL, respectively. Sugars were determined by HPLC using a HPX-87P column. A refractive index detector was used in the HPLC.

4.3.4. Determination of oligomers

To measure the oligomer content produced during enzymatic hydrolysis, similar enzymatic hydrolysis conditions were applied in separate flasks, and the hydrolysate samples were collected at 6 hr and 72 hr. Oligomer content was determined using different enzyme combination by keeping total protein number constant. The hydrolysate samples were centrifuged to separate undigested solids and insoluble lignin from the liquid. The solid free liquid was analyzed for monomeric carbohydrates as specified in the previous section. Secondary hydrolysis with 4% acid was used to hydrolyze oligomers (Sluiter et al., 2005).

$$\text{Glu Oligomer}\,(\%) = \frac{\text{Glu after 4\% acid hydrolysis }(\%) - \text{Glu before 4\% acid hydrolysis }(\%)}{1.11}$$

$$\text{Xyl Oligomer}\,(\%) = \frac{\text{Xyl after 4\% acid hydrolysis }(\%) - \text{Xyl before 4\% acid hydrolysis }(\%)}{1.136}$$

Glu: Glucose, Xyl: Xylose

4.4. Results and discussion

The protein concentrations, primary and background activities of the commercial enzymes used in this study are shown in Table 4.1. All the enzymes demonstrated substantial differences in their protein content and major and background activities. Spezyme-CP, which is commonly used cellulase enzyme, displayed the highest protein concentration and cellulase activity and it has also demonstrated significant amount of activity. Novozyme-188 (β-glucosidase), Multifect Xylanase (xylanase), and Multifect Pectinase (pectinase) have shown significant amounts of background β-xylosidase activity. Spezyme-CP and Multifect Pectinase also have shown significant background β-glucosidase activity.

Table 4.1: Primary and back ground enzyme activities of different commercial enzymes used

Enzyme	**Spezyme-CP**	**Novozyme-188**	**Multifect Xylanase**	**Multifect Pectinase**
Activities in Units/mL				
Total Protein mg/mL	82±5	67±5	42±5	27±5
Cellulase (FPU)	58.2	8.5	0.77	4.18
β-glucosidase (CBU)	128	665	35.9	345.8
Xylanase (OSX)	2622	123	25203	1164
β-xylosidase	7.3	16.6	22.6	186.2

Note: Background activities are referred from Dien et al. (2008)
OSX: Oat Spelt Xylan; CBU: Cellobiase Units; FPU: Filter Paper Units

4.4.1. Effect of β-glucosidase addition and cellulase loading on glucan and xylan digestibilities

It is well documented that cellulase complexes are typically low in β-glucosidase activity. As a result of low level of β-glucosidase activity in cellulase complex, cellobiose accumulates to the level that causes significant product inhibition during cellulose hydrolysis (Berlin et al., 2007). Addition of external β-glucosidase is often applied to reduce cellobiose inhibition. Over the years to study the enzymatic hydrolysis performance of different substrates CBU/FPU ratio of 2 was used (Mosier at al., 2005, Gupta and Lee, 2008). FPU and CBU are specific activity units of commercial enzymes Spezyme-CP (cellulase) and Novozyme-188 (β-glucosidase); however no effort was made to evaluate the substrate specific effect of CBU/FPU ratio. Thus as a part of enzyme formulation study, first a suitable level of β-glucosidase to cellulase for aqueous ammonia treated switchgrass was identified. Different levels of commercial Novozyme-188 (β-glucosidase) were added to a fixed level of Spezyme-CP (cellulase). Novozyme-188 and Spezyme-CP were added based on their activity units CBU and FPU. Different CBU/FPU ratios used for this study were: 0, 1, 2, 3, and 4. SAA pretreated Dacotah switchgrass was used as a substrate. Figure 4.1 shows the effect of different levels of β-glucosidase supplementation to a fixed cellulase loading on enzymatic hydrolysis of Dacotah switchgrass. Results indicate that β-glucosidase addition to a fixed cellulase loading is only effective up to CBU/FPU ratio of 2. Based on these results for the reaming of enzyme formulation study CBU/FPU was fixed at 2 wherever β-glucosidase has been used.

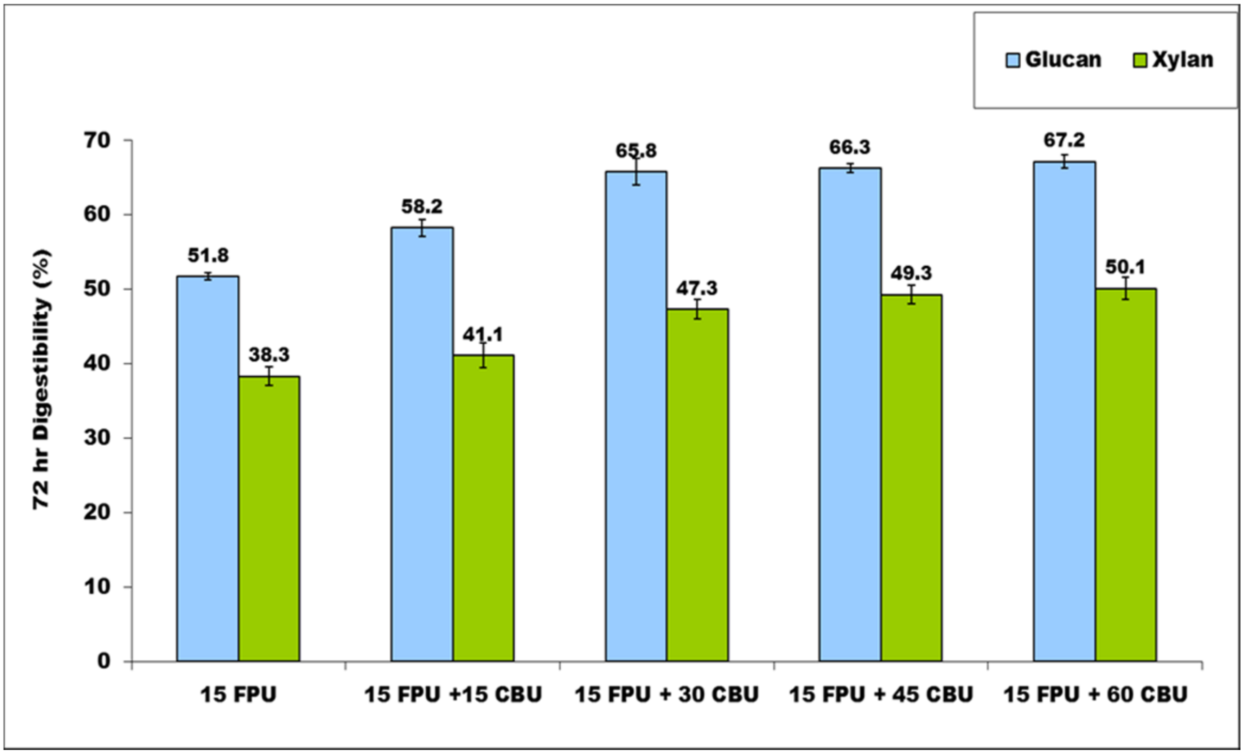

Figure 4.1: Effect of different levels of β-glucosidase supplementation to a fixed cellulase loading on enzymatic hydrolysis of Dacotah switchgrass

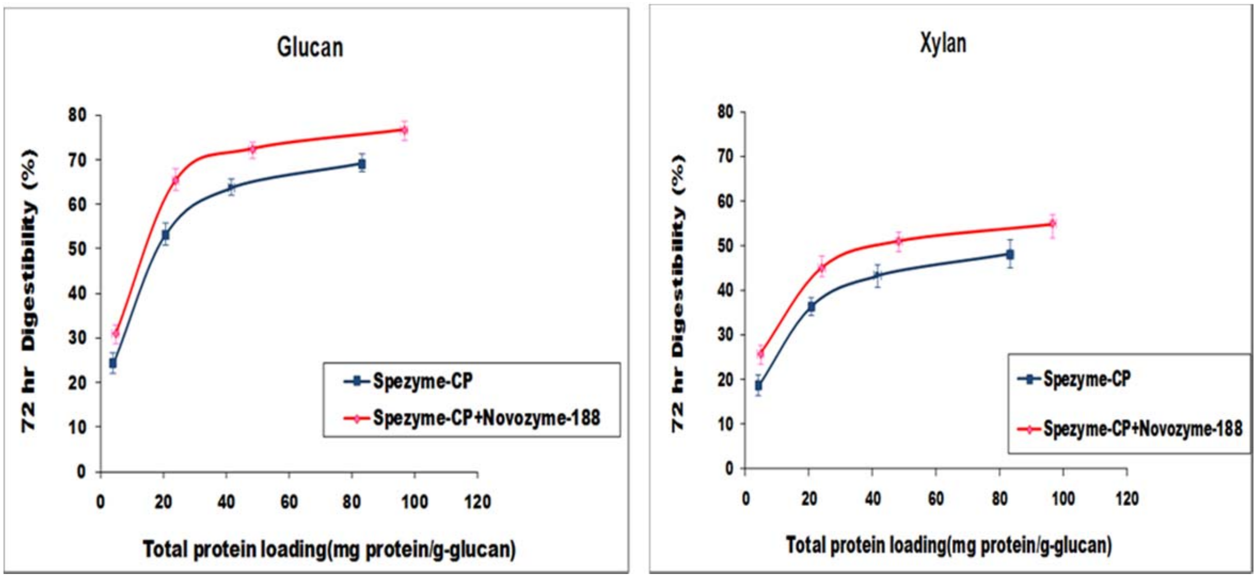

Figure 4.2: Effect of cellulase loading on digestibility with and without addition of β-glucosidase Enzyme loading: With β-glucosidase (Spezyme-CP/Novozyme-188): 4.87, 24.15, 48.4 and 96.8 mg protein/g-glucan; without β-glucosidase (Spezyme-CP only): 4.2, 20.8, 41.7 and 83.4 mg protein/g-glucan; Glucan and xylan digestibilities are based on untreated switchgrass

After identifying a suitable β-glucosidase level, it was evaluated whether addition of β-glucosidase offers any advantage over simply increasing the cellulase dose. To this effect enzyme loading was applied as total combined protein of Spezyme-CP and Novozyme-188, and Spezyme-CP. Four different enzyme loadings based on their protein numbers were used and the enzyme loadings were abbreviated to Low (10 mg protein/g-glucan), Moderate (25 mg protein/g-glucan), High (50 mg protein/g-glucan), and Very High (80 mg protein/g-glucan). The 72 hr digestibility was plotted against the combined protein of Spezyme-CP and Novozyme-188 vs Spezyme-CP (Figure 4.2). The 72 hr glucan digestibility increased sharply up to Moderate enzyme loading, after that the response was gradual increase throughout. Addition of β-glucosidase increased the glucan and xylan digestibilities at all levels of enzyme loading throughout the hydrolysis. At Low level enzyme loading hydrolyzed 30% of glucan and 25% of xylan at 72 hr, upon increasing total enzyme loading to Moderate level glucan and xylan digestibilities increased by 35% and 20%, respectively. While increase in enzyme loading, from Moderate to High level, has improved glucan and xylan digestibilities by 7% and 6%, respectively. However, the increase in enzyme loading from High to Very High has no significant effect on enzymatic digestibilities. The reasons for the not increasing digestibility can be attributed to combination of factors. One factor could be that Moderate enzyme loading may be sufficient enough to convert all the hydrolysable glucan and xylan. Another factor could be that background xylanase and β-xylosidase activities in cellulase and β-glucosidase are perhaps not sufficient enough as both have significant effect on reducing xylose oligomers that have inhibitory effect on cellulase activity (Qing et al., 2010). Another factor could be formation high DP oligomers that cannot be hydrolyzed even at high enzyme loading (Gupta and Lee, 2008). The increase in glucan digestibility is not surprising with β-glucosidase addition as it is expected reduce cellobiose inhibition to cellulase, but the increase in xylan digestibility is interesting considering the fact that β-glucosidase does not have any direct impact on glucan digestibility. The reason for the increase in xylan digestibility can be attributed to the background xylanase and β-xylosidase activities present in the β-glucosidase, especially at High and Very High enzyme loadings proportional xylanase and β-xylosidase activities could be even higher.

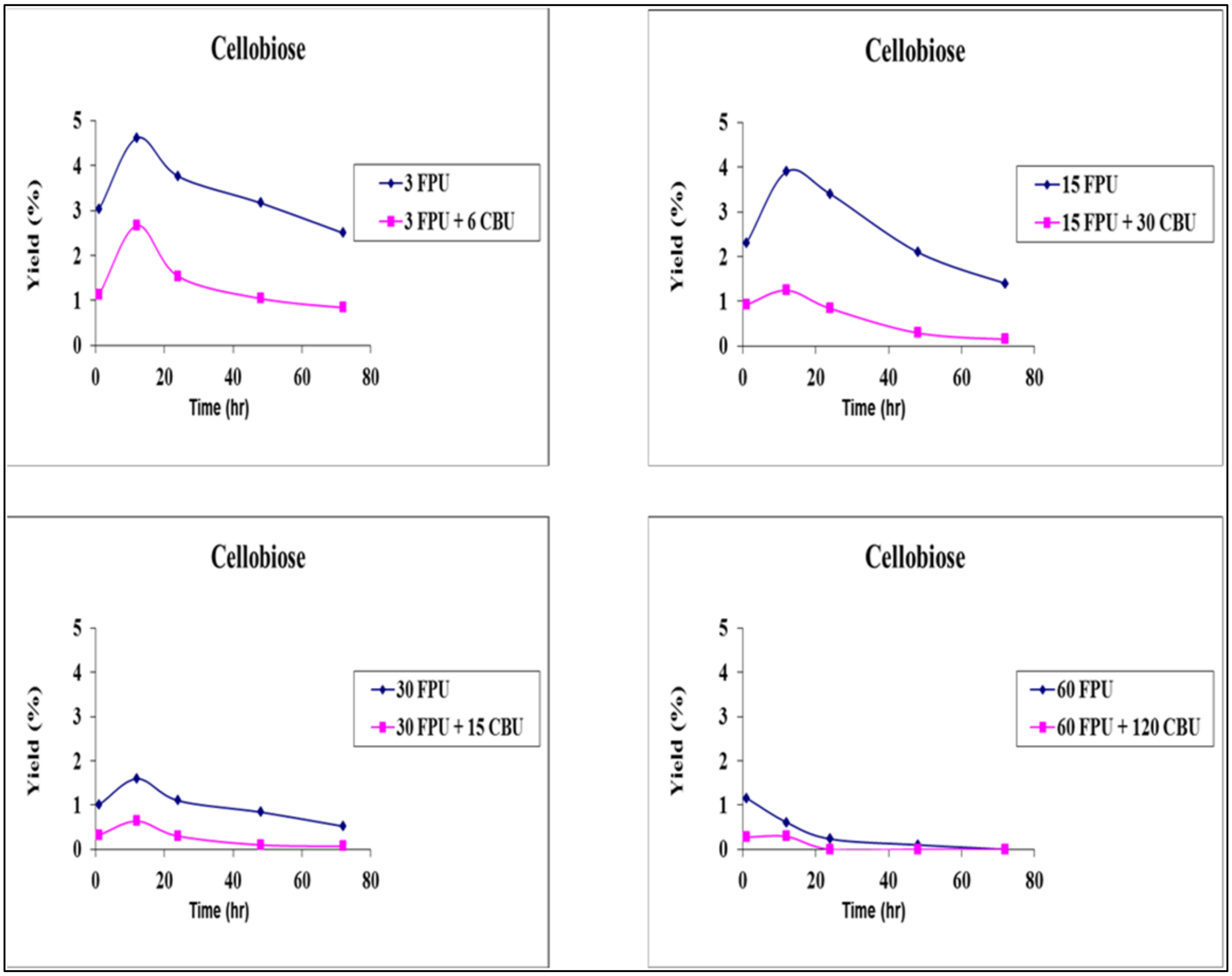

Figure 4.3: Effect of cellulase loading on cellobiose with and without addition of β-glucosidase Enzyme loading: With β-glucosidase (Spezyme-CP/Novozyme-188): 4.87, 24.15, 48.4 and 96.8 mg protein/g-glucan; without β-glucosidase (Spezyme-CP only): 4.2, 20.8, 41.7 and 83.4 mg protein/g-glucan

4.4.2. Effect of cellulase loading and β-glucosidase addition on cellobiose and oligomers

Lignocelluloses produce a significant amount of glucose oligomers as reaction intermediates, along with glucose and cellobiose during enzymatic hydrolysis. Glucose oligomers can be categorized into two separate fractions: Low DP glucose oligomers and high DP glucose oligomers. Low DP glucose oligomers, from DP 1-7, can be detected by HPLC whereas high DP glucose oligomers can be estimated only after secondary hydrolysis of liquid (Gupta and Lee, 2010). In addition to glucose, cellobiose and glucose oligomers, lignocelluloses also produce significant amount of xylose oligomers, along with xylose and xylobiose. Kumar and Wyman (2009a) reported inhibition of cellulase by xylobiose and xylose oligomers. Although several studies reported inhibition of cellulase activity by monomeric sugars and cellobiose (Holtzapple et al., 1990), only limited information is available on the effect of glucose and xylose oligomers. Thus a part of this study is also about effect of cellulase loading with and without β-glucosidase addition on reaction intermediates including, cellobiose, glucose oligomers (DP>2), and xylose oligomers (DP≥2). Cellobiose and oligomers were quantified at four different levels of Spezyme-CP (3, 15, 30, and 60 FPU/g-glucan) and Novozyme-188 (6, 30, 60, and 120 CBU/g-glucan). Addition of β-glucosidase reduced the cellobiose at all levels of enzyme loading, although effect has been significant with Low and Moderate enzyme loadings (Figure 4.3). Significant levels of cellobiose were produced at initial hydrolysis periods with all levels of cellulase without β-glucosidase; however the levels produced were higher with Low and Moderate loadings than High and Very High loadings (Figure 4.3). Reason for the reduction in cellobiose levels with High and Very High loadings could be attributed to the background β-glucosidase activity present in the Spezyme-CP. At high cellulase loadings the level of background β-glucosidase activity present is also high, which is sufficient enough to hydrolyze cellobiose without adding β-glucosidase. However, the reduction in cellobiose did not have a signficant effect on increasing glucan digestibility at High and Very High enzyme loadings (Figure 4.2).

Presence of glucose and xylose oligomers could be one of the reasons for not obtaining high glucan and xylan digestibilities with and without β-glucosidase addition (Figure 4.2). At the early hydrolysis (6 hr) substantial amounts of glucose oligomers (as high as 16.9%) were formed at all levels of cellulase loading and at the end of the hydrolysis (72 hr) still substantial amounts of glucose oligomers were left unhydrolysed at all levels of cellulase (Figure 4.4). A majority of the unhydrolysed glucose oligomers could be high DP oligomers. According to Gupta and Lee (2010), unhydrolysed glucose oligomers are generated at early phase of hydrolysis, and are not hydrolyzed even at high enzyme loadings. Addition of β-glucosidase reduced the glucose oligomers at 6 hr and 72 hr hydrolysis periods for all enzyme loadings and the reduced oligomers were possibly low DP glucose oligomers. Gupta and Lee (2010) reported that addition of sufficient amount of external β-glucosidase can hydrolyze low DP glucose oligomers.

At the early hydrolysis (6 hr) substantial amount of xylose oligomers (as high as 30.1%) were formed at all levels of cellulase loading (Figure 4.5) and at the end of the hydrolysis (72 hr) still substantial amounts of xylose oligomers left unhydrolysed at all cellulase loadings. The formation of high percentage of unhydrolysable xylose oligomers could be attributed to insufficient background xylanase and β-xylosidase activities of Spezyme-CP and Novozyme-188. Though xylanase and β-xylosidase activities were high enough to form high amounts of xylose oligomers, but not sufficient to hydrolyze them to xylose. Several reports suggested the strong inhibition of cellulase by xylose oligomers (Kumar and Wyman, 2009a; Qing et al., 2010, Qing and Wyman, 2011; Jabbour et al., 2013). Qing et al. (2010) reported that inhibition of xylose oligomers to cellulase is far greater than the glucose/xylose or cellobiose. Addition of β-glucosidase reduced the xylose oligomers at 6 hr and 72 hr hydrolysis for all enzyme loadings. Though β-glucosidase has no direct role in reducing xylose oligomers, but the reason for reduction in xylose oligomers could be that the presence of significant level of β-xylosidase activity in Novozyme-188.

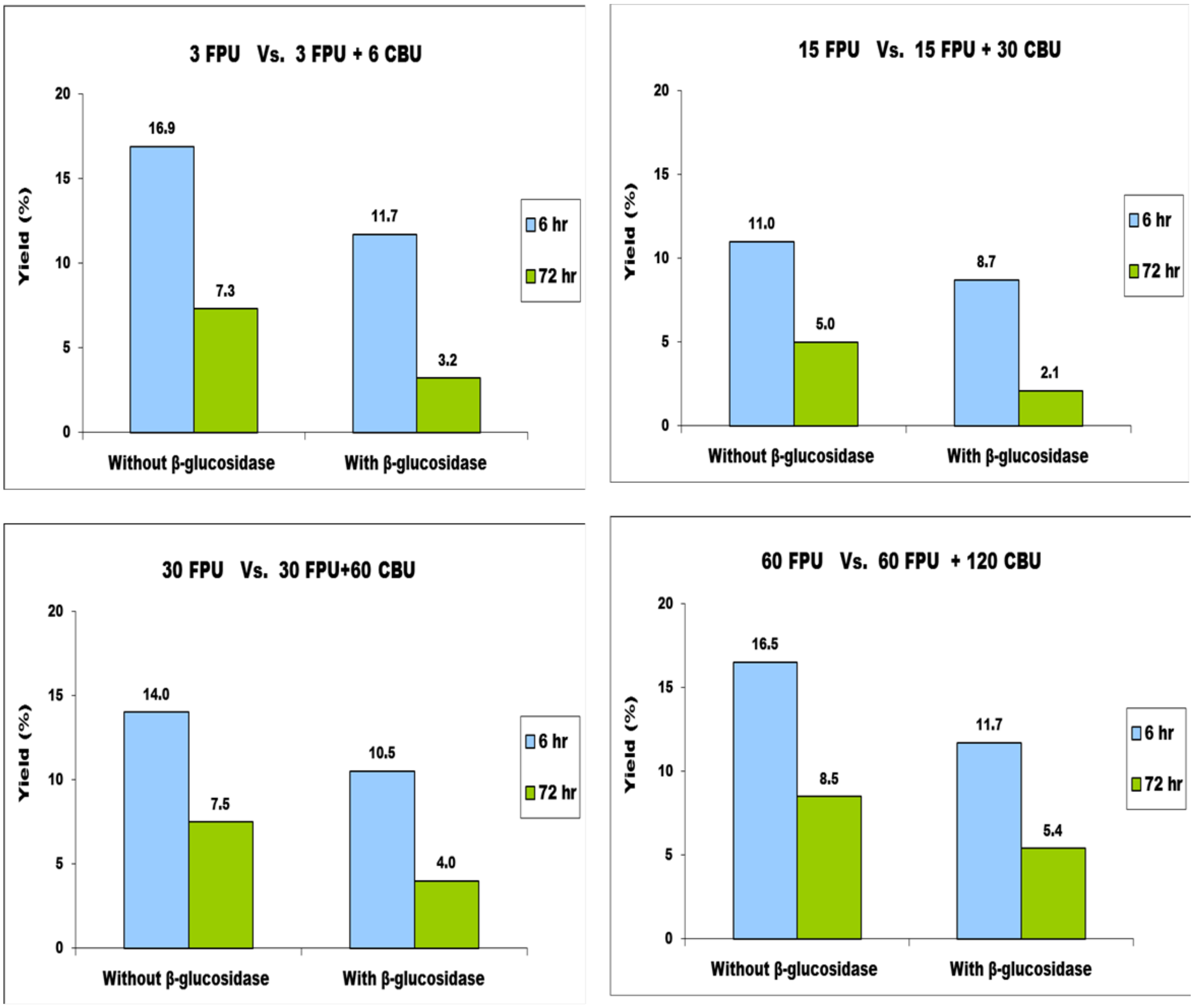

Figure 4.4: Effect of cellulase loading on glucose oligomers (DP>2) with and without addition of β-glucosidase; Enzyme loading: With β-glucosidase (Spezyme-CP/Novozyme-188): 4.87, 24.15, 48.4 and 96.8 mg protein/g-glucan; Without β-glucosidase (Spezyme-CP only): 4.2, 20.8, 41.7 and 83.4 mg protein/g-glucan

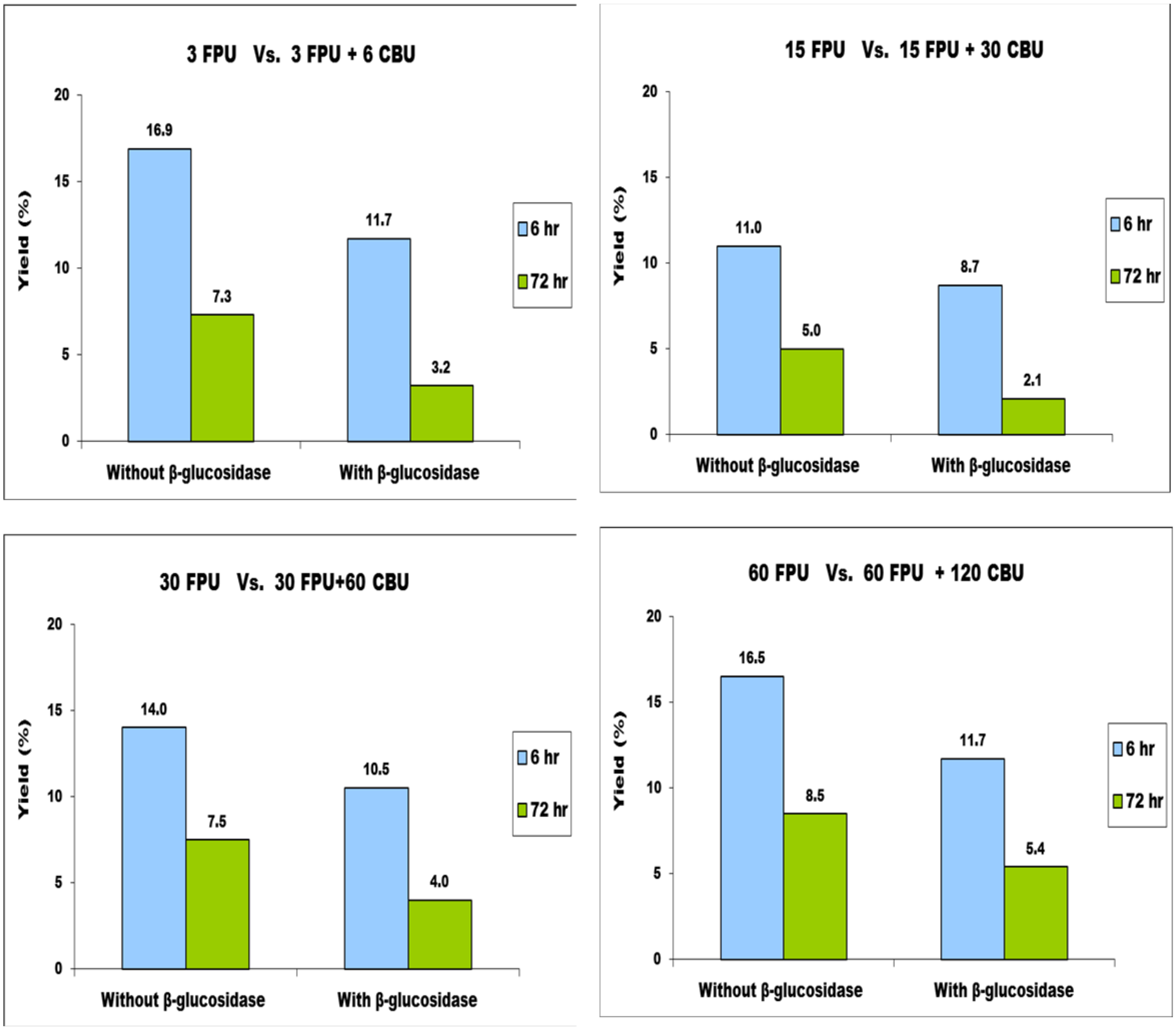

Figure 4.5: Effect of cellulase loading on xylose oligomers (DP≥2) with and without addition of β-glucosidase; Enzyme loading: With β-glucosidase (Spezyme-CP/Novozyme-188): 4.87, 24.15, 48.4 and 96.8 mg protein/g-glucan; Without β-glucosidase (Spezyme-CP only): 4.2, 20.8, 41.7 and 83.4 mg protein/g-glucan

4.4.3. Effect of xylanase and pectinase supplementation on glucan and xylan digestibilities

Hemicellulose and possibly pectin, is thought to restrict the access of cellulases to cellulose in pretreated lignocellulose. As a part of this study we have examined the possibility of further improvements in hydrolysis of ammonia pretreated Dacotah Switchgrass by supplementing hemicellulase and pectinase by using commercially available Multifect Xylanase and Multifect Pectinase. Hydrolysis experiments were carried out with different cellulase loadings (Spezyme-CP) in the absence and presence of β-glucosidase (Novozyme-188), xylanase (Multifect Xylanase), and pectinase (Multifect Pectinase) (Figure 4.6, 4.7, 4.8, and 4.9).

Glucan and xylan digestibilities increased sharply with the increase in enzyme loading up to 25 mg protein/g-glucan (Figure 4.2). Cellulase alone hydrolyzed 62.5% of glucan and 38.4% of xylan at 25 mg protein /g-glucan and supplementation of β-glucosidase by keeping protein number constant at 25 mg protein /g-glucan increased the glucan and xylan digestibilities by 4.3% and 7.2%. The increase in cellulase from 25 to 32 mg protein/g-glucan resulted in only marginal improvement in hydrolysis yields (3.5% glucan and 3.2% xylan) and similar trend could be observed with β-glucosidase supplementation at the same protein number. However, instead of adding cellulase or cellulase and β-glucosidase, a 7 mg protein/g-glucan of xylanase was supplemented, which significantly enhanced the glucan and xylan digestibilities of Dacotah switchgrass by 21% and 22%, respectively (Figure 4.6 and Figure 4.7). The increase in xylan digestibility was expected as xylanases are known to break 1, 4 - β bonds in xylan chain and xylose oligomers (Gupta and Lee, 2009). Xylanases do not have any direct role in glucan hydrolysis except that their minor background cellulase activities can slightly influence cellulose hydrolysis (Hall et al., 1989). A substantial improvement in glucan digestibility with xylanase was perhaps due to combination of factors. One factor is reduction of xylose oligomers with xylanase supplementation, which are known to inhibit cellulase activity strongly (Qing et al., 2010), therefore less inhibition on cellulase activity and more accessible cellulase to cellulose (Figure 4.11). Another factor is that more than 50% of xylan left unhydrolysed with cellulase and β-glucosidase, and hemicelluloses are known to restrict the access of cellulolytic enzymes by coating cellulose fibers (Berlin et al., 2005a; 2005b). A major portion of unhydrolysed xylan was hydrolyzed when xylanase was supplemented; therefore more cellulose fibers are accessible to cellulase. These two factors may have worked together to improve the glucan digestibility. When β-glucosidase portion was replaced with xylanase, glucan digestibility was decreased by 5.8%, and conversely xylan digestibility was further increased by 7.9%. For the same protein loading, a combination of Spezyme-CP, Novozyme-188 and Multifect Xylanase results in higher glucan digestibility than Spezyme-CP and Novozyme-188 (Figure 4.10). For the same protein loading, a combination of Spezyme-CP and Multifect Xylanase results in higher xylan digestibility than Spezyme-CP, Novozyme-188 and Multifect Xylanase (Figure 4.11).

Pectinase supplementation improved glucan and xylan digestibilities by 10%/19.1% (Figure 4.8 and Figure 4.9). Glucan digestibility with pectinase is significantly lower than that of with xylanase at different enzyme loadings (Figure 4.10). However, xylanase and pectinase have identical effect in improving xylan digestibility (Figure 4.11). The improvement in xylan digestibility could be due to presence of high level of background activity in Multifect Pectinase.

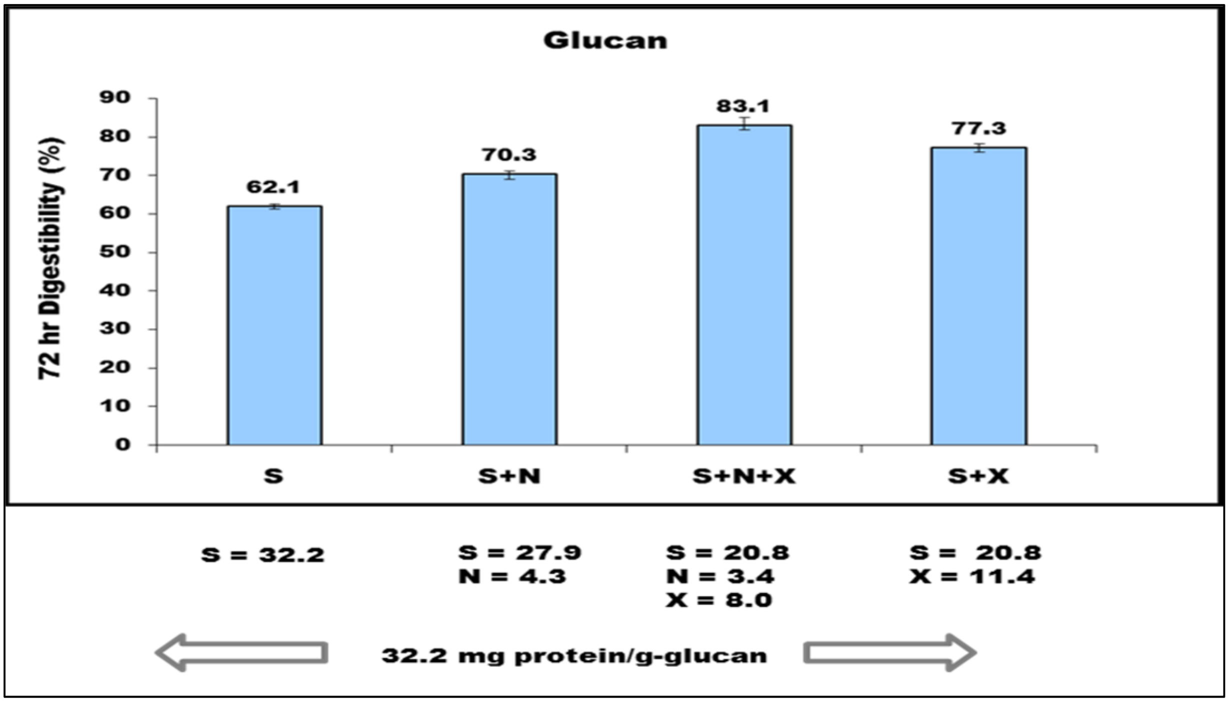

Figure 4.6: Effect of xylanase supplementation on glucan digestibility
S: Spezyme-CP, N: Novozyme-188, X: Multifect Xylanase

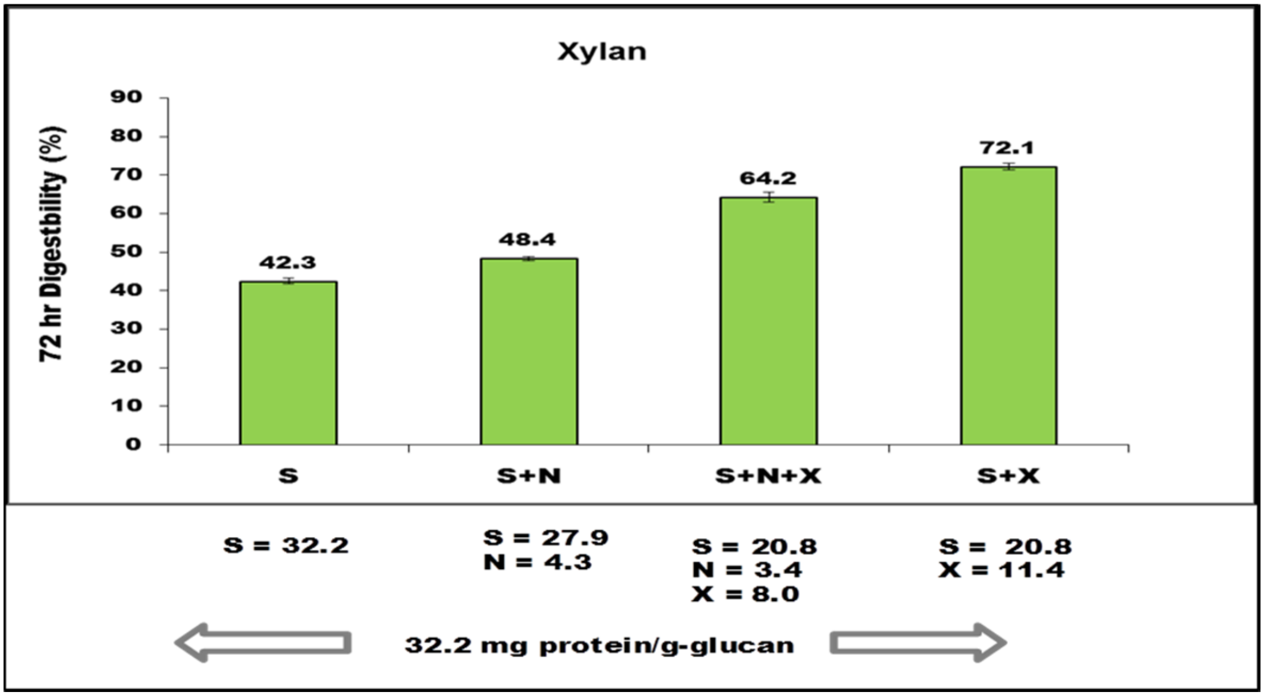

Figure 4.7: Effect of xylanase supplementation on xylan digestibility
S: Spezyme-CP, N: Novozyme-188, X: Multifect Xylanase

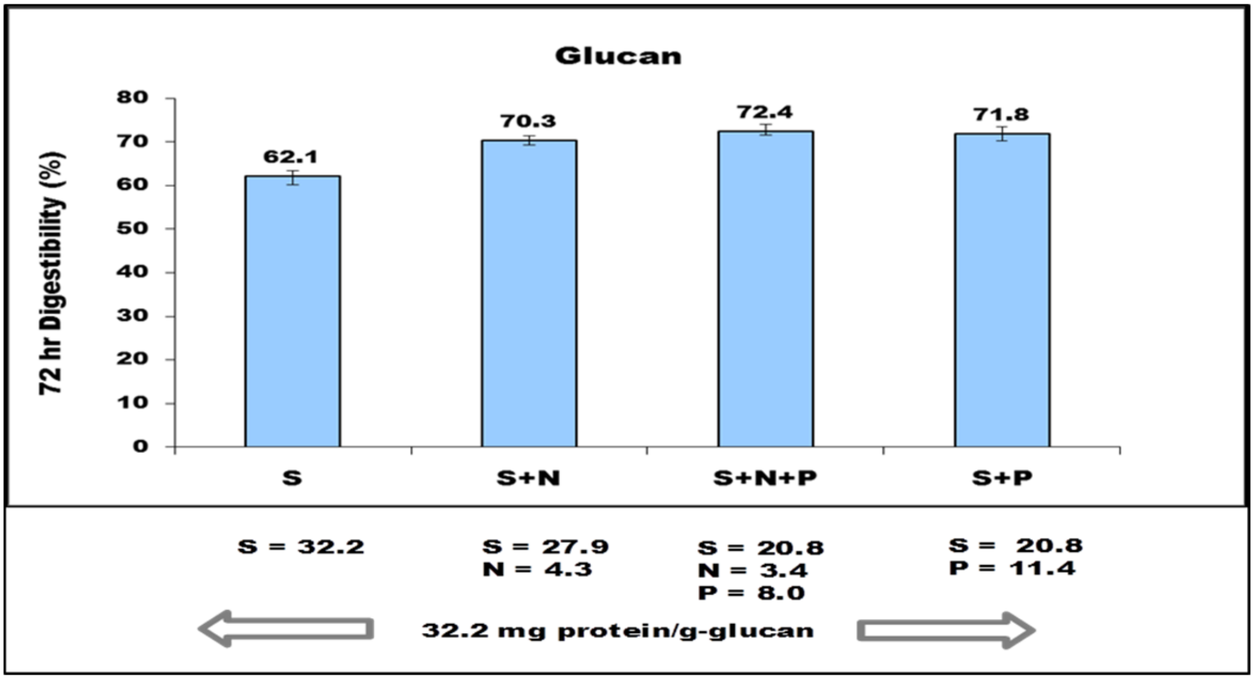

Figure 4.8: Effect of pectinase supplementation on glucan digestibility
S: Spezyme-CP, N: Novozyme-188, P: Multifect Pectinase

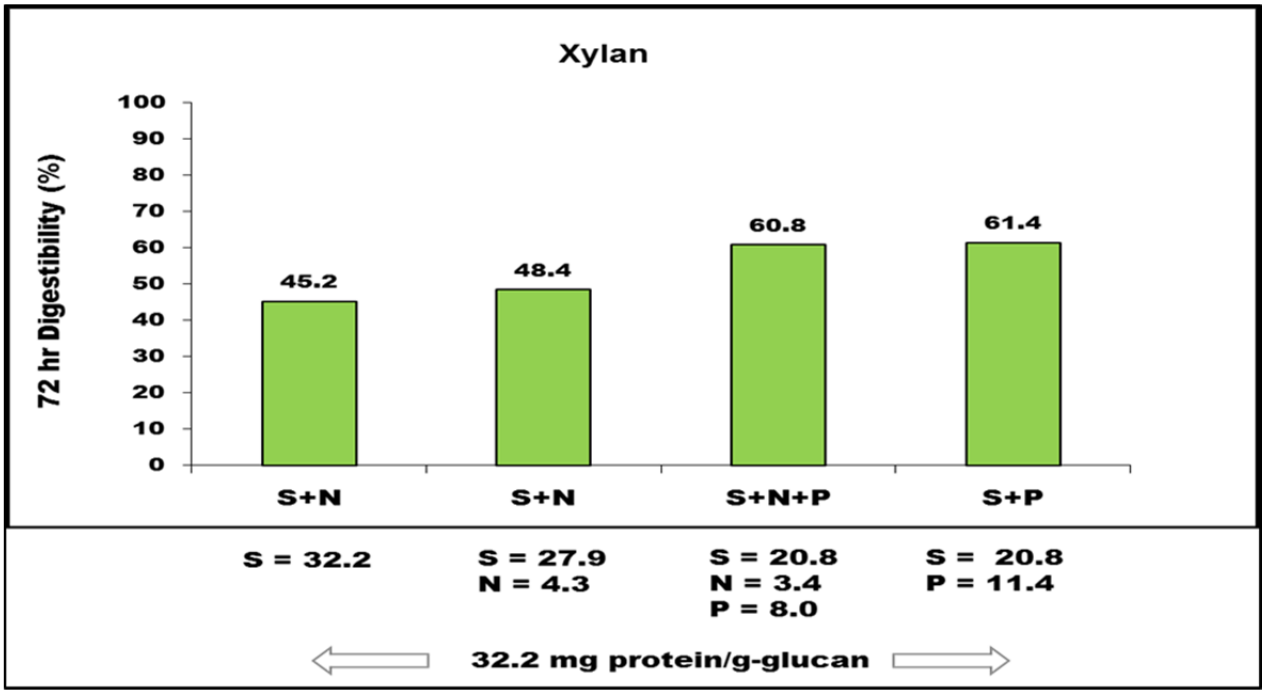

Figure 4.9: Effect of pectinase supplementation on xylan digestibility
S: Spezyme-CP, N: Novozyme-188, P: Multifect Pectinase

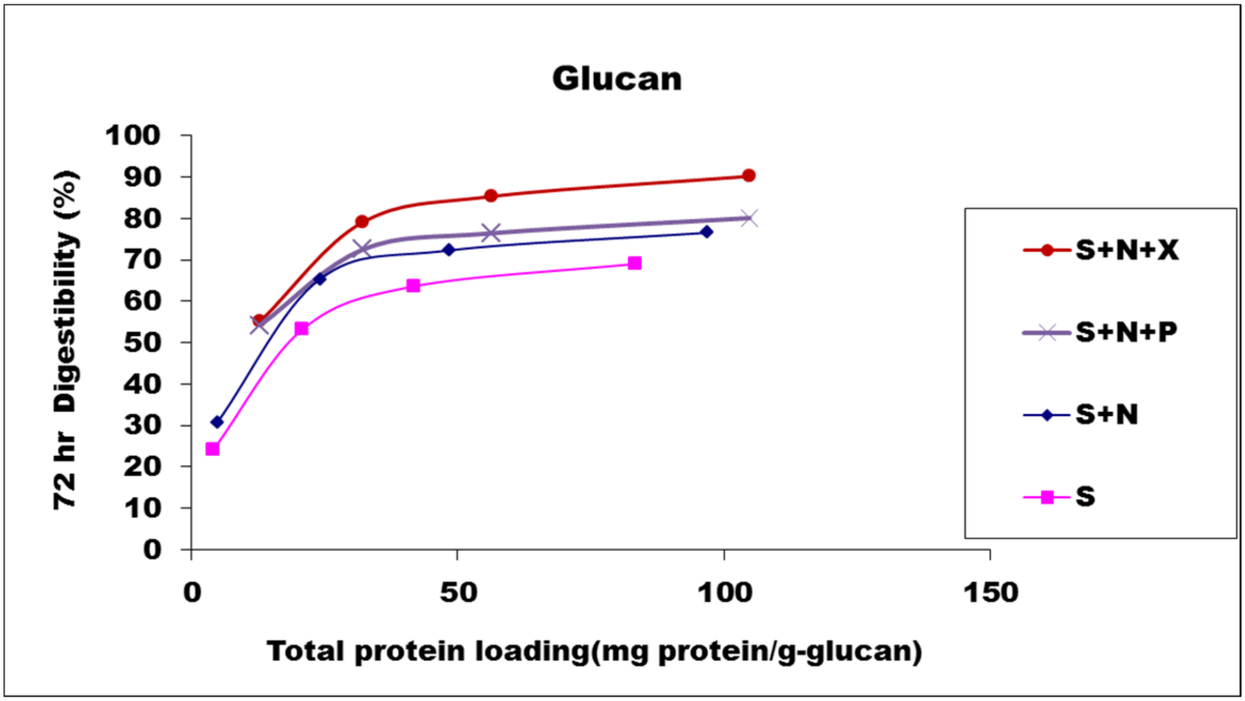

Figure 4.10: Comparison of glucan digestibility with different enzyme combinations
S: Spezyme-CP, N: Novozyme-188, X: Multifect Xylanase; P: Multifect Pectinase

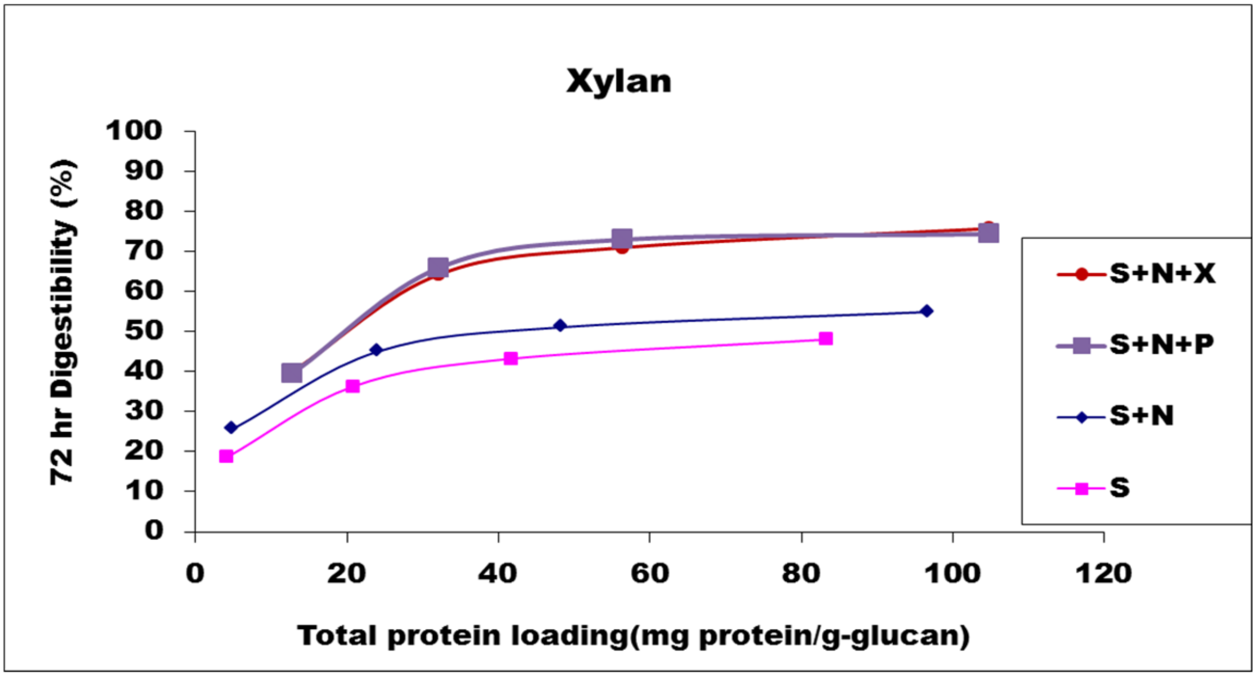

Figure 4.11: Comparison of xylan digestibility with different enzyme combinations
S: Spezyme-CP, N: Novozyme-188, X: Multifect Xylanase; P: Multifect Pectinase

4.4.4. Effect xylanase and pectinase supplementation on oligomers

Substantial amounts of glucose and xylose oligomers left unhydrolysed even after applying high cellulase loadings and β-glucosidase. Therefore, we have decided to study the effect of xylanase and pectinase on glucose and xylose oligomers. Supplementation of xylanase and pectinase has a significant effect on reduction of xylose oligomers at the end of the hydrolysis (72 hr) (Figure 4.13 and 4.15). However, xylanase and pectinase supplementation did not show any significant effect on glucose oligomers (Figure 4.12 and Figure 14). We believe that reduction in xylose oligomers is one of the reasons for increasing substantial amount of xylan digestibility. Kumar and Wyman (2009a) strongly suggested that xylose oligomers inhibit cellulase activity, and that both glucose and xylose release could be significantly enhanced by supplementation with xylanase. Our data concurs with their suggestion. However, decrease in xylose oligomers with pectinase is interesting, considering the fact that pectinase does not have any direct role on xylan hydrolysis. One reason for decrease in xylose oligomers with pectinase could be due to its high background β-xylosidase activity that is known to have a strong effect in reducing xylose oligomers even stronger than xylanase (Qing et al., 2010). Reduction in xylose oligomers has direct and indirect benefits: increasing xylose yields and reduction in cellulase inhibition therefore improvement in glucose yields, respectively.

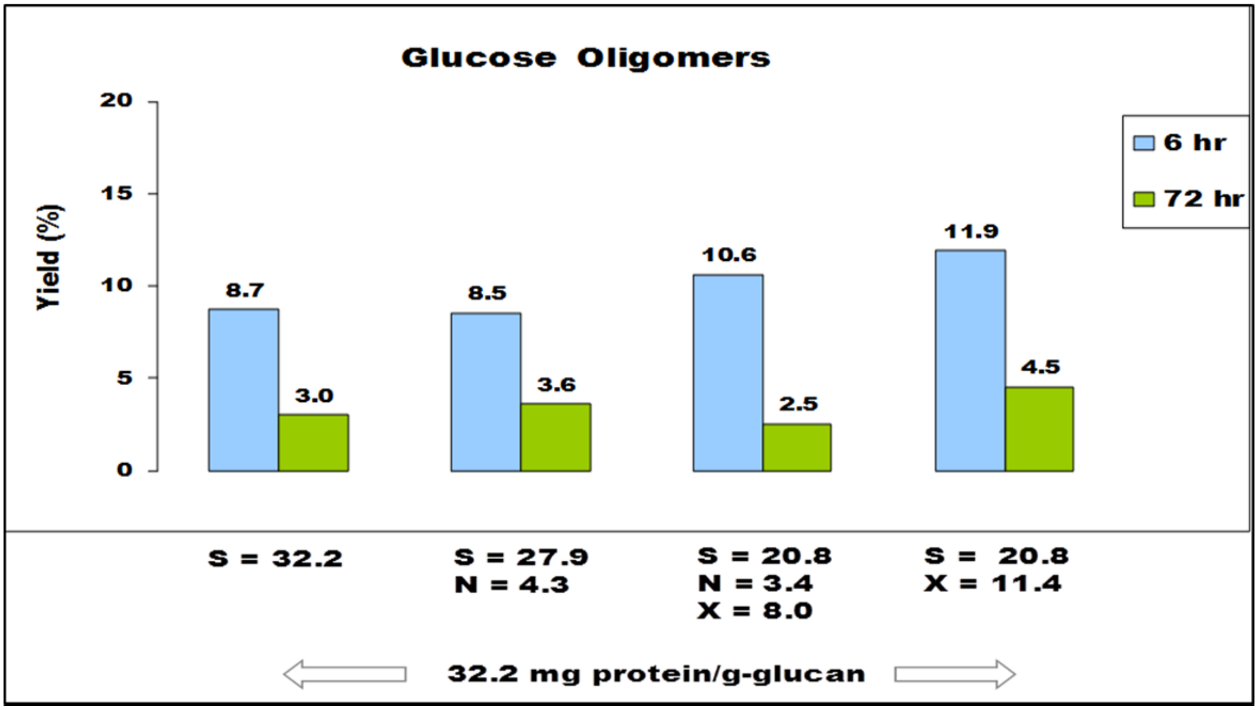

Figure 4.12: Effect of xylanase supplementation on glucose oligomers (DP≥2)
S: Spezyme-CP, N: Novozyme-188, X: Multifect Xylanase

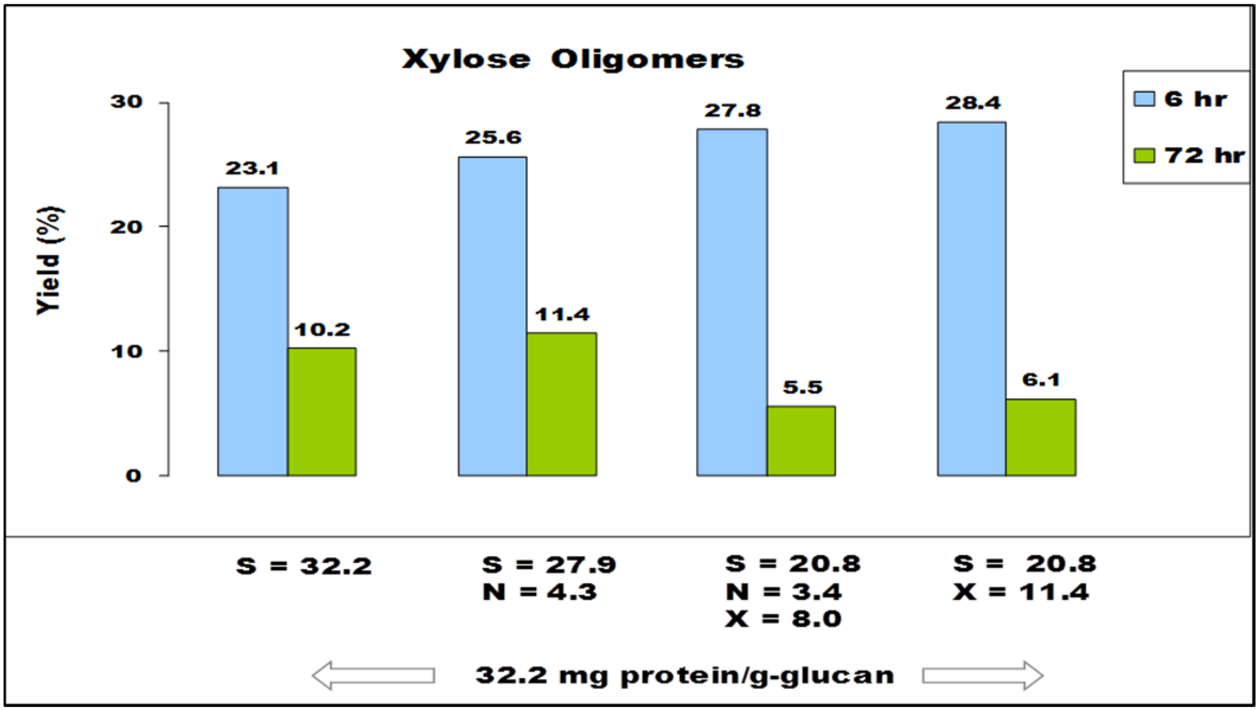

Figure 4.13: Effect of xylanase supplementation on xylose oligomers (DP≥2)
S: Spezyme-CP, N: Novozyme-188, X: Multifect Xylanase

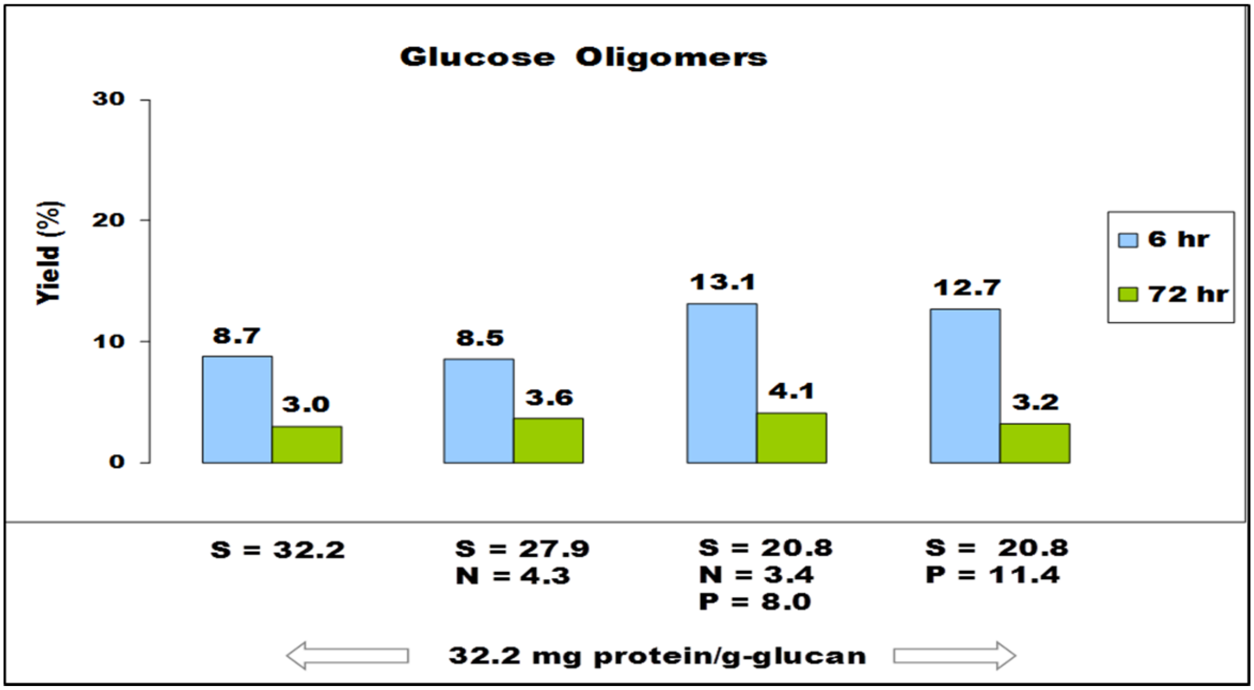

Figure 4.14: Effect of pectinase supplementation on glucose oligomers (DP≥2)
S: Spezyme-CP, N: Novozyme-188, P: Multifect Pectinase

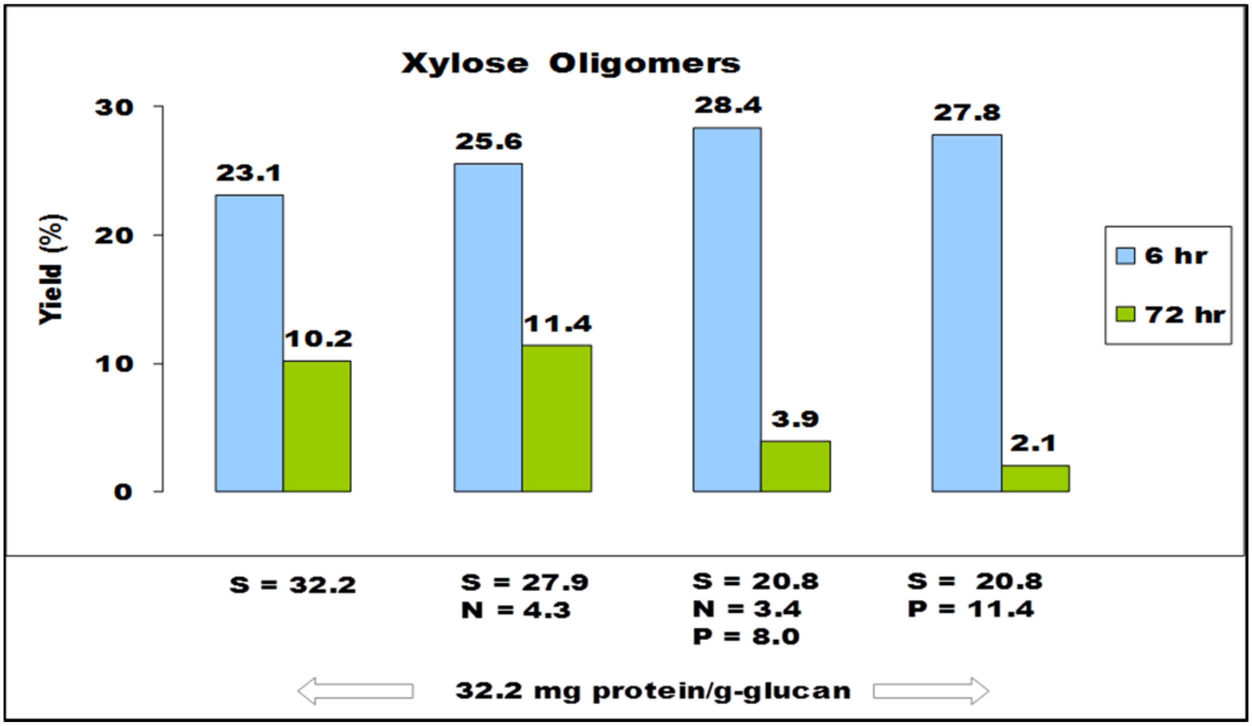

Figure 4.15: Effect of pectinase supplementation on xylose oligomers (DP≥2)
S: Spezyme-CP, N: Novozyme-188, P: Multifect Pectinase

4.5. Conclusions

Mixed enzyme shows significantly higher digestibility than Spezyme-CP alone on the basis of protein loading. Digestibility increases rapidly with different enzyme mixtures approximately up to 25 mg protein/g-glucan, after which the increase was gradual. Addition of different levels of β-glucosidase to a fixed Spezyme-CP level based on their activity units increased the glucan/xylan digestibility significantly up to CBU/FPU ratio of 2. Addition of β-glucosidase causes increase in the glucan and xylan digestibility, and decrease in the cellobiose and oligomers. Addition of xylanase/pectinase improves the digestibilities of xylan and glucan. Supplementation of xylanase/pectinase shows no significant effect on glucose oligomers at the 72 hr hydrolysis, but xylose oligomers are considerably reduced. Reduction in xylose oligomers has direct and indirect benefits: increasing xylose yields and reduction in cellulase inhibition therefore improvement in glucose yields, respectively. For the same protein loading, combination of Spezyme-CP, Novozyme-188, and Multifect Xylanase/Pectinase shows higher glucan digestibility than Spezyme-CP and Novozyme-188. For the same protein loading Spezyme-CP and Multifect Xylanase shows higher xylan digestibility than Spezyme-CP, Novozyme-188, and Multifect Xylanase.

5. Effect of Addition of Water-soluble Polymers During Enzymatic Hydrolysis of Lignocellulosic Biomass

5.1. Abstract

Supplementation of surface active additives during enzymatic saccharification of biomass is known to increase the activity of cellulase. Use of these additives is expected to reduce the enzyme dosage in the bioconversion processes by 20-40%, a significant economic benefit. To evaluate the effect of additives on enzymatic hydrolysis different water-soluble polymers were studied including, polyvinyl alcohol, polyvinyl pyrrolidone, polyethylene glycol, cationic polyacrylamide, and polyethylene imine. In addition, to evaluate the effect of presence of lignin various substrates including, Solka Floc and hardwood pulp (lignin free substrates), dilute sulfuric acid pretreated corn stover/ switchgrass, and aqueous ammonia pretreated switchgrass (lignin containing substrates) were used. Majority of the enzymatic hydrolysis experiments were carried out with 5 FPU/g-glucan C-Tec 2 cellulase enzyme and 5% substrate loading. For a selected few experiments 15 FPU/g-glucan C-Tec 2 was added to determine the effect of enzyme loading on polymer addition. Hydrolysis results indicate that with the addition of 10 mg polymer/g-substrate, glucan digestibilities were increased by 10-25% depending on type of the substrate and polymer. About the same level of increase in xylan digestibility was also observed.

Reasons for improvement in digestibility with polymer addition are improvement in cellulase activity and reduction in unproductive binding of cellulase to lignin. The optimization of polymer loading was also studied. The supplementation effects of polymers on enzymatic hydrolysis of lignin free substrates were compared with pretreated lignocellulosics.

5.2. Introduction

Many challenges still limit the enzymatic hydrolysis process, including several substrate characteristics and a number of enzyme factors (Chang and Holtzapple, 2000; Kim and Holtzapple, 2006; Chandra et al., 2009). To overcome these challenges, increase in pretreatment severity and large amounts of hydrolytic enzymes were employed, but these resulted in unfavorably high processing cost (Himmel et al., 2007). Many studies have therefore been aimed at improving the efficiency of commercially available enzymes to promote the conversion of lignocellulosics (Himmel et al., 2007; Ouyang et al., 2009). The use of surface active additives has been shown to enhance the enzymatic hydrolysis of lignocellulosics (Kaar and Holtzapple, 1998; Eriksson et al., 2002; Yang and Wyman, 2006). Although the use of additives adds additional cost to bioethanol production process, clear benefits have been shown. Benefits including, reduction in unproductive binding to lignin that can make effective use of the added enzymes or decrease the enzyme loading (Borjesson et al., 2007a, 2007b). However, majority of the additives studied have been surfactants (Kaar and Holtzapple, 1998; Eriksson et al., 2002; Yang and Wyman, 2006). In recent years, however, other additives have been studied such as proteins (bovine serum albumin) (Yang and Wyman, 2006), polymers such as polyethylene glycol (PEG) (Borjesson et al., 2007a; 2007b; Kristensen et al., 2007; Ouyang et al., 2009), polyvinyl alcohol (PVA) (Hata, 2011), cationic polyacrylamide (Mora et al., 2011), polyvinyl pyrrolidone (PVP) (Sewalt et al., 1997). Ouyang et al. (2009) reported that addition of PEG during the enzymatic hydrolysis increased the conversion of Avicel by 92% and the reasons for increase in conversion are increase in cellulose activity and stability of enzyme. Hata (2011) proposed a new method for processing of biomass with PVA. In his method PVA is mixed with tetra sodium borate, which crosslinks PVA and converts it into a viscous fluid. The crosslinked

PVA facilitates the swelling and hydration of biomass and forms a viscous gel like material. This processed material is thinned through the addition of water, whereby hydrolytic enzymes are mixed into material and rapid hydrolysis into monomeric sugars. Sewalt et al. (1997) showed reduction in lignin inhibition by addition of different polymers. In their study external lignin was added to the filter paper during enzymatic hydrolysis with PEG and PVP addition. They suggest that pre-established lignin-carbohydrate bond is not a prerequisite for absorption of enzyme on to lignin. Polymer addition effects on enzymatic hydrolysis have been studied exhaustively using PEG (Sewalt et al., 1997; Borjesson et al., 2007a; 2007b; Kristensen et al., 2007; Ouyang et al., 2009), but only limited information available on other water-soluble polymers. Therefore, this work mainly focused on the effects of different commercially available water-soluble polymers on enzymatic hydrolysis of SAA pretreated Dacotah switchgrass. Dacotah was chosen as a feedstock as it proved to be highly recalcitrant even after increasing the pretreatment severity. In addition, effect of polymer addition on presence of lignin was also evaluated using various lignin containing substrates (aqueous ammonia pretreated Dacotah switchgrass and dilute sulfuric acid pretreated corn stover/switchgrass) and lignin free substrates (Solka Floc and hardwood pulp).

5.3. Materials and methods

5.3.1. Feedstocks, enzymes, and polymers

Commercially available Solka Floc was purchased from Fiber Sale & Development Corporation (Urbana, OH). Hardwood pulp (HP) was provided by Eka Chemicals (Atlanta, GA). Dacotah switchgrass was provided by Ceres, Inc (Thousand Oaks, CA). Cellulase Cellic® C-Tec2 (Batch No. VCNI0001) was supplied by Novozymes, North America (Franklinton, NC). It has a specific activity of 119 FPU/mL and protein number of 255.22 ± 4.24 mg protein/mL. Different cationic and non-ionic water-soluble polymers were used. Cationic polyacrylamide (C-PAM) was provided by Eka Chemicals (Atlanta, GA). Polyethylene imine (PEI), polyethylene glycol (PEG), polyvinyl alcohol (PVA), and polyvinyl pyrrolidone (PVP) were purchased from Sigma.

5.3.2. Feedstock preparation and compositional analysis

Corn stover was pretreated using dilute sulfuric acid (1% H_2SO_4, 150^oC, liquid/solids ratio of 9, 30 min). Dacotah switchgrass was pretreated using aqueous ammonia (15% NH_4OH, 90^oC, liquid/solids ratio of 9, 24 hr) and dilute sulfuric acid (1% H_2SO_4, 150^oC, liquid/solids ratio of 9, 60 min). The pretreatment experiments were carried out in a stainless steel batch reactor (1.375"ID x 6"L). A 10 g dry ground feedstock was mixed with the pretreatment reagent, and kept in a GC forced-air convection oven at a set temperature. The heating time to reach the target temperature was about 20 min, and that was not included in the stated reaction time. After pretreatment, the reactor was immediately removed from the oven, and quenched to room temperature in a water bath. The cooled slurry was washed with cold water immediately through a filter paper (Whatman®, Grade 802 Fluted, size 32.0 cm) until pH reaches approximately 7.

Washed solids were not subjected to further drying, and kept at 4°C. All pretreatment runs were made in duplicates. Hardwood pulp was ground using bench top grinder. Composition of Solka Floc, hardwood pulp, and pretreated switchgrass samples were determined by NREL standard analytical procedures (Sluiter et al., 2006). The carbohydrate contents were analyzed by HPLC equipped with Bio-Rad Aminex HPX-87P column.

5.3.3. Preparation of polymer solution

Each polymer was solubilized in buffer to prepare 1% (w/v) solution. 100 mL sodium citrate buffer (0.05 M) was added to 500 mL glass beaker. Before adding polymer, a magnetic stirring bar was placed in buffer and stirring was started. 1 g dry polymer was slowly added into vertex of buffer. Mixing was carried out for 30-45 min depending on polymer.

5.3.4. Enzymatic hydrolysis

Novozymes Celic C-Tec 2 with total specific activity of 119 FPU/mL was used as a cellulase enzyme. All the enzymatic hydrolysis experiments were carried out with 5% (w/v) substrate and 5 and 15 FPU/g-glucan of C-Tec 2. Polymers were added on the basis of 1% (w/v) solution and applied polymer loading was ranged between 0-30 mg/g-solid. The other enzymatic hydrolysis conditions used were 50°C, pH 4.8 (0.05 M sodium citrate buffer), 300 RPM in a Shaker/Incubator (NBS, Innova-4080). Enzymes were added after 2hr of polymer addition and incubation. Hydrolysate samples were periodically taken and analyzed for glucose, xylose using HPLC equipped with Biorad-HPX-87P column and RI detector.

5.3.5. Protein adsorption studies

Adsorption studies were done with and without polymer addition. Enzymes were added after 2 hr of polymer addition and incubation. All adsorption experiments were performed in 5 mL vessels with screw caps, in 0.05 M citric acid buffer, pH 4.8. To avoid the hydrolysis of substrate by the enzyme, the experiments were performed at 4°C at a slow agitation speed of 80 RPM. To measure the enzyme adsorption at 5% substrate concentration (Solka Floc and Corn Stover), 5 FPU/g glucan C Tec 2 (10.71 mg protein/g glucan) was incubated. Samples (0.2 mL) were taken at 0, 10, 20, 30, and 60 during the incubation. Samples were immediately centrifuged to prevent hydrolysis. The supernatant from each sample was collected after centrifugation. The free enzyme content in the supernatant was determined using the Bio-Rad protein assay reagent with BSA as a standard (as per Bio-Rad Laboratories protocol). Absorbance was measured at 595 nm. The amount of bound enzyme was calculated by subtracting the free enzyme from the initial enzyme concentration.

5.4. Results and discussion

The polymer addition effects on enzymatic hydrolysis have been studied exhaustively using PEG, but only limited information available on other water-soluble polymers. Therefore, different commercially available cationic and non-ionic water-soluble polymers were studied in this study. The cationic polymers used were polyethylene imine (PEI) and cationic polyacrylamide (C-PAM), while non-ionic polymers used were polyethylene glycol (PEG), polyvinyl alcohol (PVA), and polyvinyl pyrrolidone (PVP). Dacotah was chosen as a feedstock as it proved to be highly recalcitrant even after increasing the pretreatment severity. In addition, the effect of polymer on presence of lignin was also evaluated using various lignin containing substrates (aqueous ammonia pretreated Dacotah switchgrass and dilute sulfuric acid pretreated corn stover/switchgrass) and lignin free substrates (Solka Floc and hardwood pulp). Substrates with varied characteristics were used to evaluate the effect of polymers on compositional variability during the enzymatic hydrolysis. The composition of different feedstocks is shown in Table 5.1. Solka Floc and hardwood pulp (HP) contain virtually no lignin, but mostly glucan and xylan. Dilute sulfuric acid pretreated corn stover (PCS) and dilute sulfuric acid pretreated Dacotah switchgrass (PSG) predominantly contain glucan, lignin and very little amount of xylan. Aqueous ammonia pretreated Dacotah switchgrass (ASG) contains primarily glucan, xylan, and low lignin content. PCS, PSG, and ASG represent herbaceous crops, while, HP represents a woody crop.

Table 5.1: Solid composition of different feedstocks

	Glucan (%)	Xylan (%)	Lignin (%)
Lignin free substrates			
Solka Floc	76.0	23.5	-
HP	86.5	NM	-
Lignin containing substrates			
PCS	60.5	2.5	26.8
PSG	57.4	3.8	30.1
ASG	51.5	24.6	19.6

5.4.1. Effect of polymer on lignin free substrates

Solka Floc and hardwood pulp are lignin free substrates and contain mostly glucan and xylan. The reason for choosing lignin free substrates was to study effect of polymer addition with the absence of lignin as previous polymer addition studies reported about reduction in unproductive inhibition of lignin (Sewalt et al., 1997).

5.4.1.1. Solka Floc digestibility with polymer addition

The glucan and xylan digestibilities of Solka Floc without and with polymer are shown in Figure 5.1 and Figure 5.2. Glucan and xylan digestibilities achieved without polymer were 58.6% and 44.3%, respectively. However, with the addition of 10 mg polymer/g-solid, the respective digestibilities were increased to as high as 83.5% and 56.3% depending on the type of the polymer. The highest increase in glucan and xylan digestibilities were obtained with PVP by 24.9% and 12.0%, respectively, whereas the lowest increase in glucan and xylan digestibilities were obtained with C-PAM by 7.8% and 4.3%, respectively. The hydrolysis results indicate that all the polymers increased the glucan and xylan digestibilities; however, the increase with non-ionic polymers (PVP, PEG and PVA) is significantly higher than cationic polymers (PEI and C-PAM). The difference in increase in glucan and xylan digestibilities between ionic and non-ionic polymers was 15.3% and 7.1%, respectively. Among the non-ionic polymers PVP resulted in the highest glucan (83.5%) and xylan (56.3%) digestibilities.

Different mechanisms have been proposed for the increase in digestibility with the addition of polymers. Sewalt et al. (1997) showed reduction in lignin inhibition by PEG and PVP addition. In their study external lignin was added to the filter paper during enzymatic hydrolysis and suggested that pre-established lignin-carbohydrate bond is not a prerequisite for adsorption of enzyme on to lignin. According to Borjesson et al. (2007a) ethylene oxide (EO) containing surfactants and polymers, such as PEG, bind to lignin by hydrophobic interaction and hydrogen bonding, and reduce the unproductive binding of enzymes to lignin. While Sewalt et al. (1997) and Borjesson et al. (2007a) proposed mechanisms may be applicable with lignin containing substrates, but may not be applicable with lignin containing substrates. To evaluate that in the current study lignin free Solka Floc was used and the results show substantial improvement in glucan and xylan digestibilities. One possible reason for this was explained by Ouyang et al. (2009) who used lignin-free Avicel. As per their data, addition of PEG during the enzymatic hydrolysis increased the conversion of Avicel by 92%. Ouyang et al. (2009) indicated that the reason for increase in Avicel conversion is due to increase in cellulase activity and stability of enzyme. In addition, as reported by Ouyang et al. (2009), PVA did not increase the glucan digestibility significantly. But in the present study, addition of PVA increased the glucan digestibility substantially like PEG. Our data also concurs with Sewalt et al. (1997), who reported a substantial increase in glucan digestibility with PEG and PVP. Mora et al. (2011)

proposed a different mechanism for cationic polymer C-PAM. According to them both the cellulose and enzyme are negatively charged. Therefore, C-PAM patches to cellulose fiber, and reduces its negative charge and thus promoting the enzyme binding to cellulase. In the current study, C-PAM and PEI may have improved the hydrolysis yields through patching mechanism as both are positively charged polymers.

The enzyme adsorption on to Solka Floc without and with polymer addition was studied in order to understand reasons behind increase in glucan and xylan digestibilities (Data not shown). However, addition of polymers did not show any effect on enzyme adsorption on to Solka Floc. At the end of 30 min hydrolysis period approximately 5 mg protein/g-Solka Floc was adsorbed with and without polymer and amount of the protein adsorbed did not change with the time (at 1 hr, 2 hr, and 3 hr).

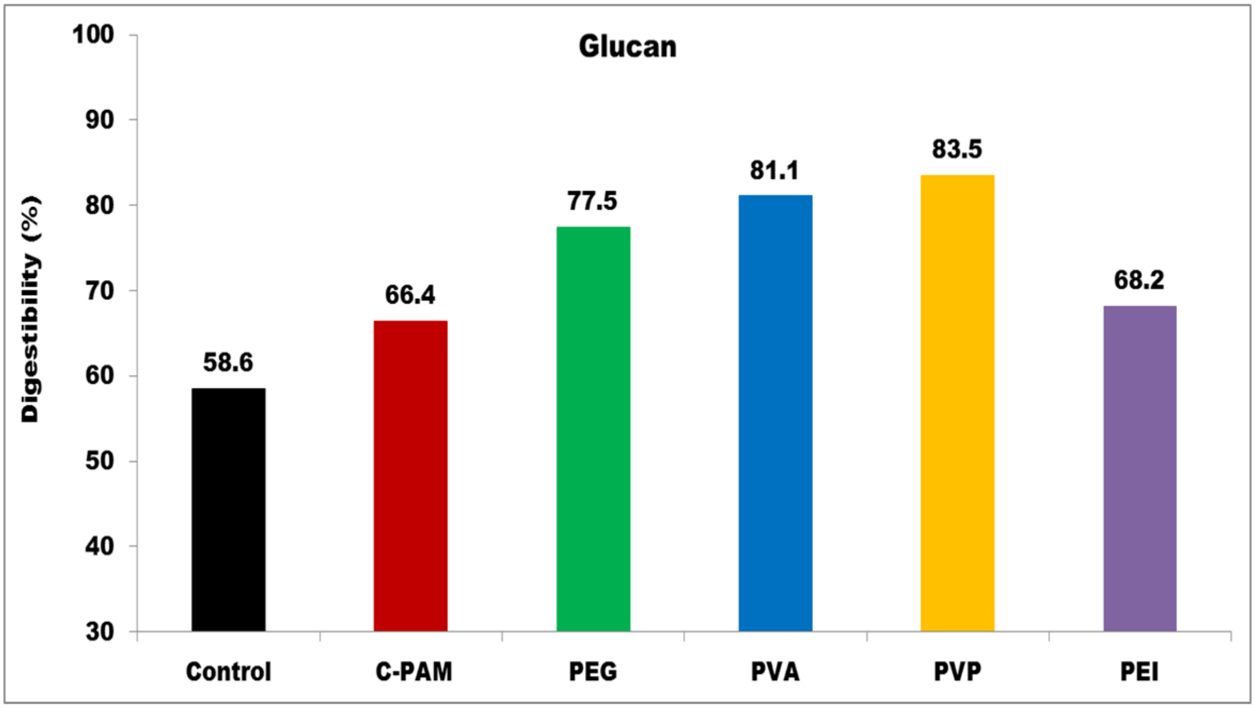

Figure 5.1: 96 hr glucan digestibility of Solka Floc without and with addition of polymers Substrate loading: 5%; Polymer loading: 10 mg/g-substrate; Enzyme loading: 5 FPU/g-glucan of C Tec 2

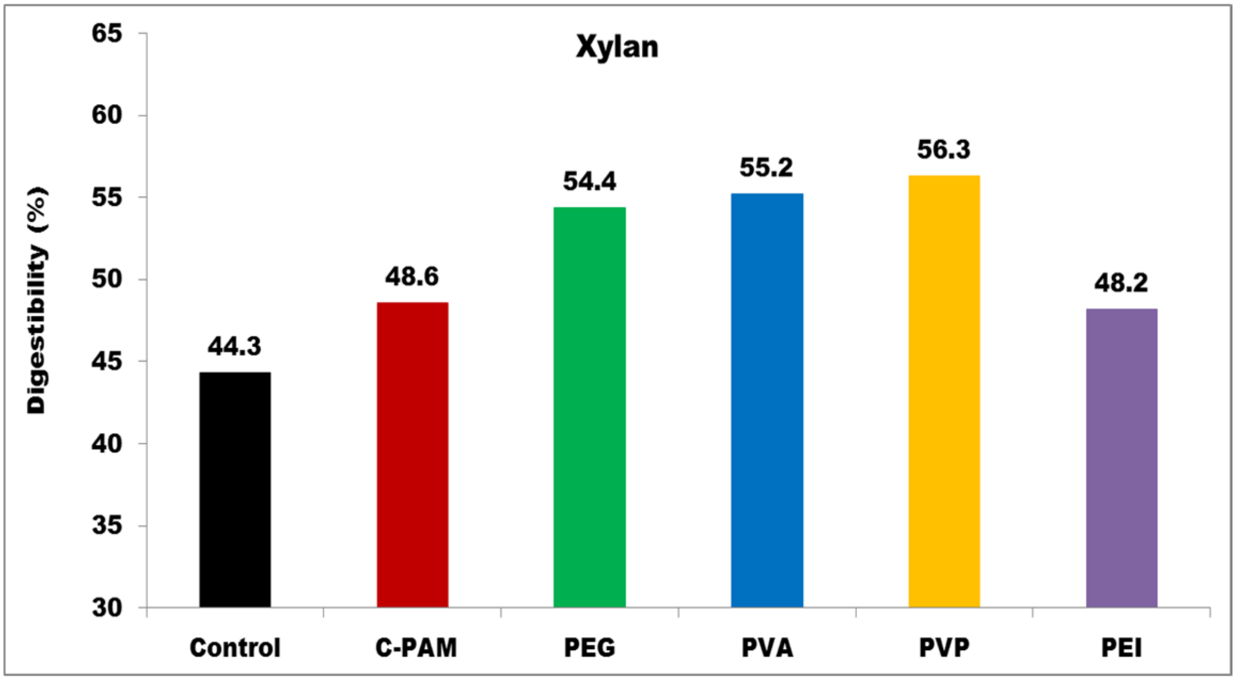

Figure 5.2: 96 hr xylan digestibility of Solka Floc with addition of polymers
Substrate loading: 5%; Polymer loading: 10 mg/g-substrate; Enzyme loading: 5 FPU/g-glucan of C Tec 2

5.4.1.1.1. Effect of different levels of polymer loading on digestibility of Solka Floc

In order to determine the optimum polymer loading different levels of polymer loading were applied. Figure 5.3 and Figure 5.4 show increase in glucan and xylan digestibilities up to 10 mg polymer/g-solid with all the polymers, although the increase was significantly higher with non-ionic polymers.

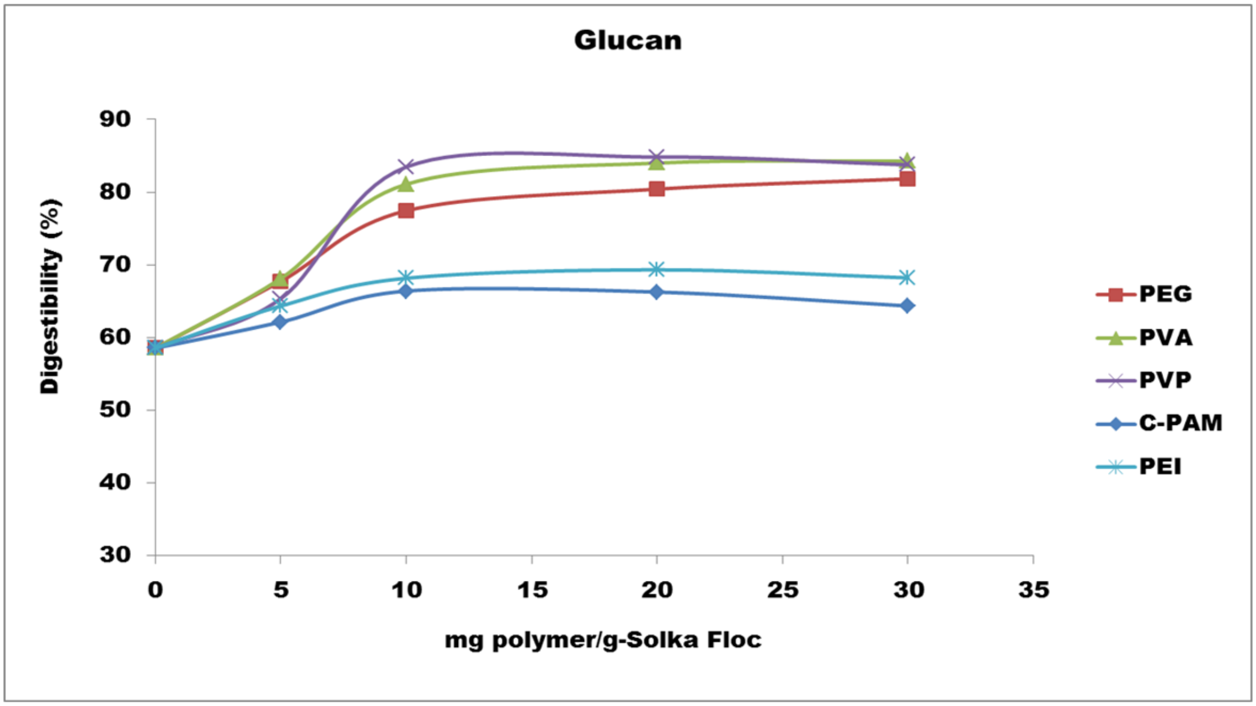

Figure 5.3: Effect of different levels of polymer on 96 hr glucan digestibility
Substrate loading: 5%; Enzyme loading: 5 FPU/g-glucan of C Tec 2

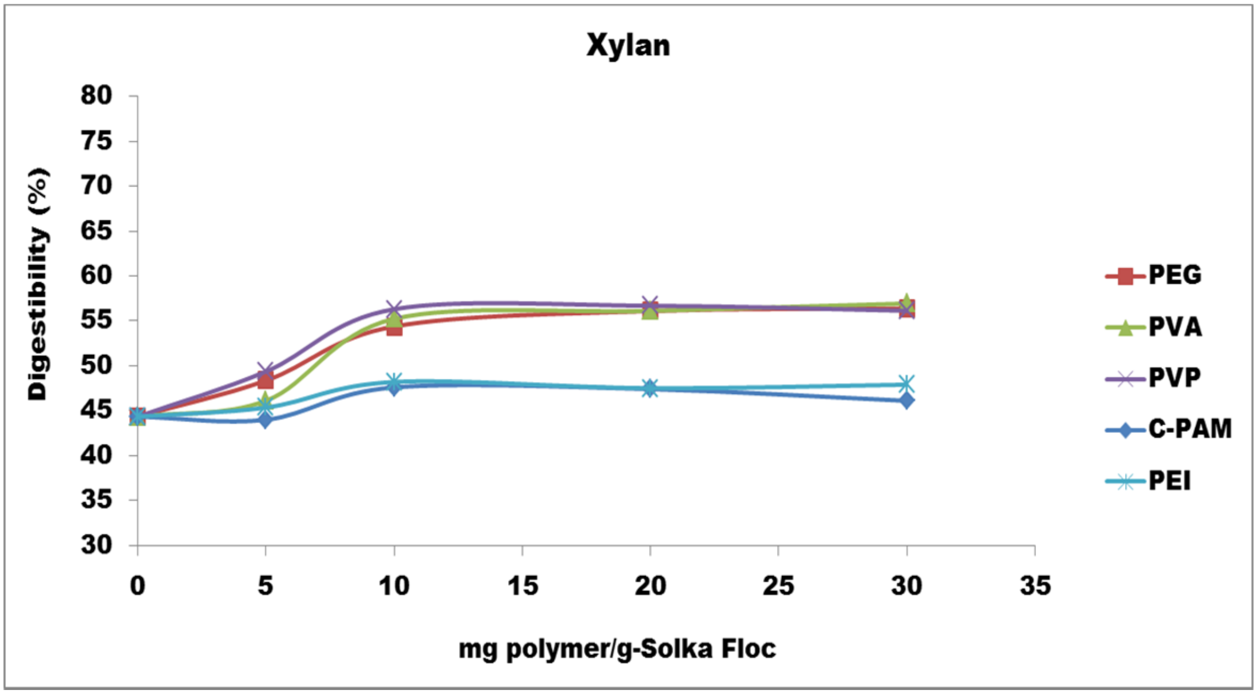

Figure 5.4: Effect of different levels of polymer on 96 hr xylan digestibility
Enzyme loading: 5 FPU/g-glucan of C Tec 2

5.4.1.1.2. Effect of enzyme loading on digestibility of Solka Floc with polymer addition

Polymer addition study was initially started by applying 15 FPU/g-glucan of C-Tec 2 using Solka Floc. The glucan and xylan digestibilities achieved from Solka Floc without adding polymers were 76.6% and 71.2%, respectively. Addition of polymer increased glucan and xylan digestibilities by 1.9%-12.3% and 2.4%-5%, respectively depending on polymer (Figure 5.5 and Figure 5.6). However, reducing the enzyme loading from 15 to 5 FPU/g-glucan, improved the hydrolysis yields substantially. This is interesting considering the fact that enzyme loading was substantially low. One plausible explanation for lack of improvement in digestibility at high enzyme loading: availability of sufficient amount of enzyme after possible loss of enzyme activity, therefore improvement in enzyme activity was not necessary as reported by Ouyang et al. (2009). Contrary to high enzyme loading, 5 FPU has a low enzyme activity that is perhaps not enough to yield high glucan and xylan digestibilities. Therefore, improvement in cellulase activity is beneficial. Ouyang et al. (2009) reported the improvement in cellulase activity with PEG addition and corresponding increase in cellulose conversion with Avicel.

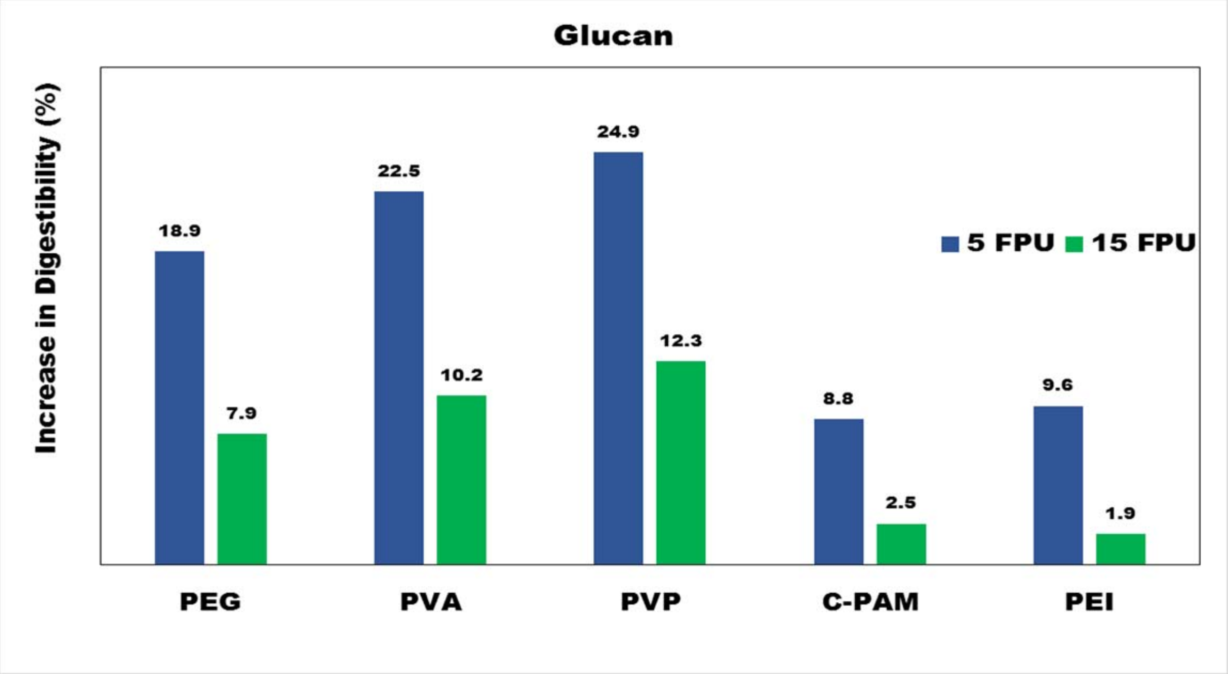

Figure 5.5: Difference in Solka Floc glucan digestibility with different enzyme loadings with polymer addition; Substrate loading: 5%; Polymer loading: 10 mg polymer/g-substrate

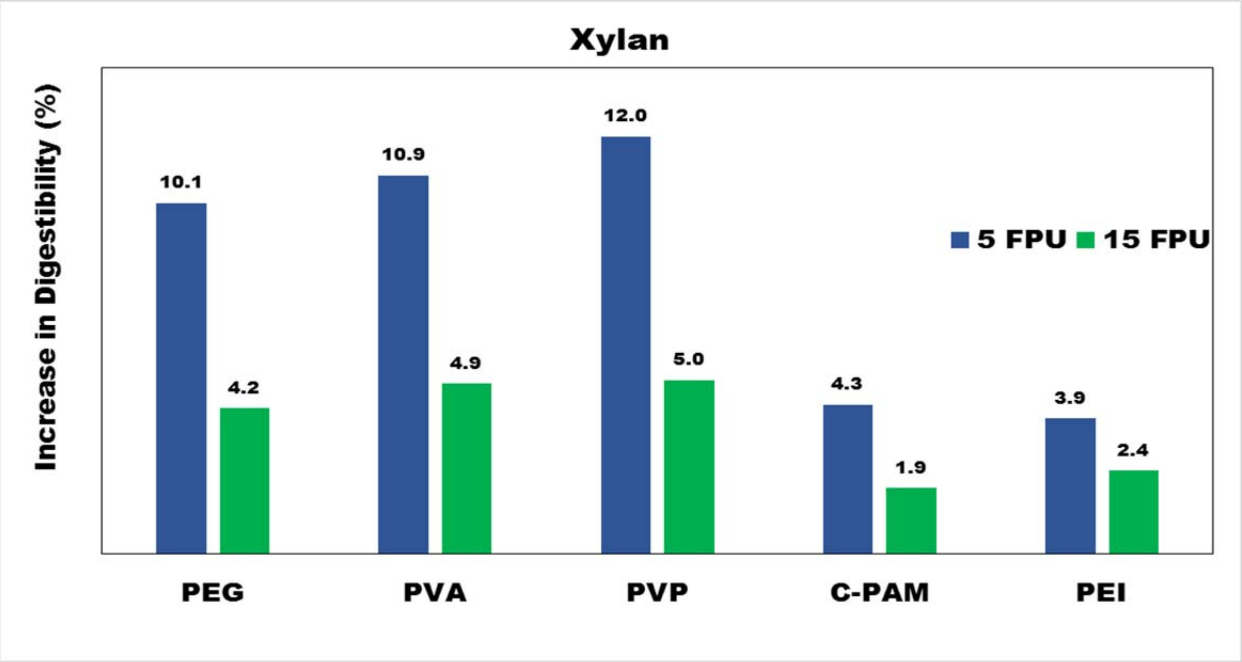

Figure 5.6: Difference in Solka Floc xylan digestibility with different enzyme loadings with polymer addition; Substrate loading: 5%; Polymer loading: 10 mg polymer/g-substrate

5.4.1.2. Hardwood pulp digestibility with polymer addition

Polymer addition effect was also studied on hardwood pulp, which is also a lignin free substrate. Glucan content in HP is 86.5%. Since 10 mg polymer/g-Solka Floc was enough to improve enzymatic hydrolysis, further experiments were carried out using same polymer loading. Glucan digestibility of HP without polymer addition was 33.2%. However, with the polymer addition glucan digestibility was increased to as high as 54.2% (Figure 5.7) depending on polymer. Although the overall increase in glucan digestibility with cationic polymers was significantly lower than the non-ionic polymers; increase is still substantially higher than the control (by 5.6%). Among the non-ionic polymers, PVP yielded highest digestibility (54.2%), whereas among the ionic polymers PEI resulted in highest digestibility (43.7%).

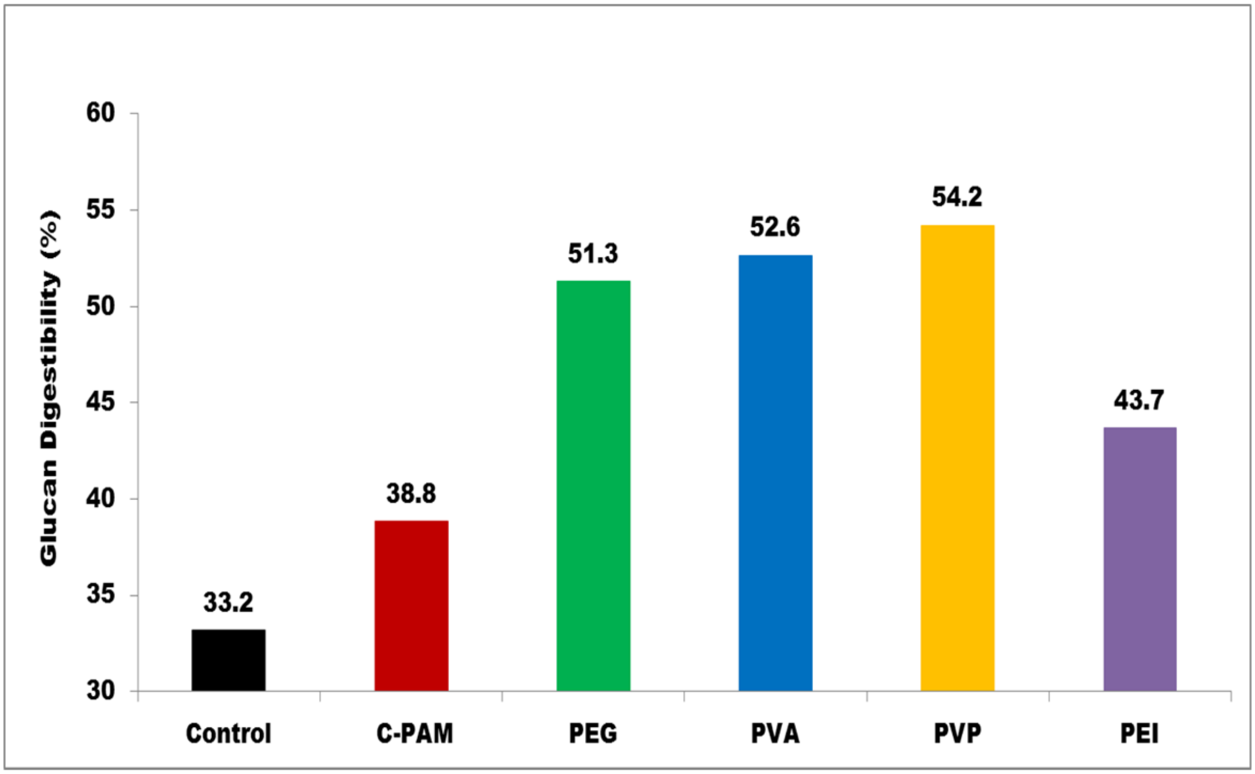

Figure 5.7: 96 hr glucan digestibility of HP without and with addition of polymers
Substrate loading: 5%; Polymer loading: 10 mg polymer/g-substrate; Enzyme loading: 5 FPU/g-glucan of C Tec 2

5.4.2. Effect of polymer addition on lignin containing substrates

Having seen effectiveness of water-soluble polymers on lignin free substrates studies were further carried out with lignin containing substrates to evaluate the presence of lignin. Experiments were first started with SAA pretreated Dacotah switchgrass as one of the goals of this study was to improve monomeric sugar yields from recalcitrant Dacotah switchgrass.

5.4.2.1. Soaking in aqueous ammonia treated Dacotah switchgrass digestibility with polymer addition

Dacotah switchgrass was pretreated following conditions: 15% NH_4OH, 90°C, liquid/solids ratio of 9, 24 hr and pretreated ASG composition is shown in Table 5.1. The glucan and xylan digestibilities of ASG without polymer were found to be 48.6% and 43.2%, respectively (Figure 5.8 and Figure 5.9). However, with the addition of 10 mg polymer/g-ASG, glucan and xylan digestibilities increased by 18.7% and 13.2%, respectively depending on polymer. Among the cationic polymers, C-PAM addition has not caused any improvement in glucan and xylan digestibilities, whereas PEI slightly improved the glucan digestibility (by 2.9%). According to Borjesson et al. (2007a), ethylene oxide (EO) containing polymers such as PEG, bind to lignin by hydrophobic interaction, and reduce the unproductive binding of enzymes to lignin. On the other hand, current study showed the improvement in digestibility of lignin free Solka Floc with the polymer addition. Therefore, mechanism based on unproductive binding of enzyme to lignin is only applicable to lignin containing substrates. In addition, Ouyang et al. (2009) reported a substantial improvement in glucan digestibility of lignin free Avicel by adding PEG. According to their study PEG addition improves the activity and stability of the enzyme. In addition, PVA, PVP and PEI do not contain EO moieties, therefore improvement in the activity and stability of enzyme has played an important role in improving the hydrolysis yields. Adding to that, PVA, PVP, and PEI, perhaps positively affected the enzyme–substrate interactions, which led to improvement in enzymatic hydrolysis. Kaar and Holtzapple (1998) reported that surface active agents improve enzyme–substrate interactions. Later on Eriksson et al. (2002) proved that enzyme-substrate interactions exist with the addition of surface active agents.

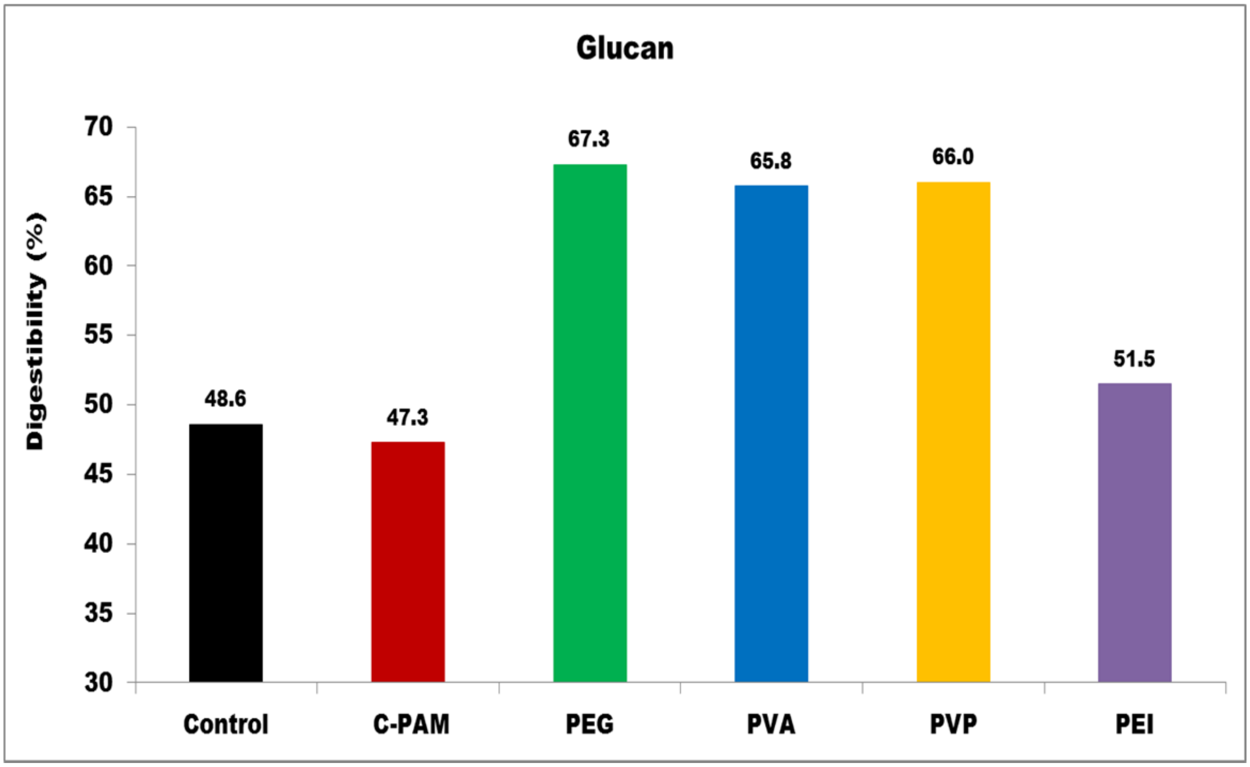

Figure 5.8: 96 hr glucan digestibility of ASG without and with addition of polymers
Substrate loading: 5%; Polymer loading: 10 mg polymer/g-substrate; Enzyme loading: 5 FPU/g-glucan of C Tec 2

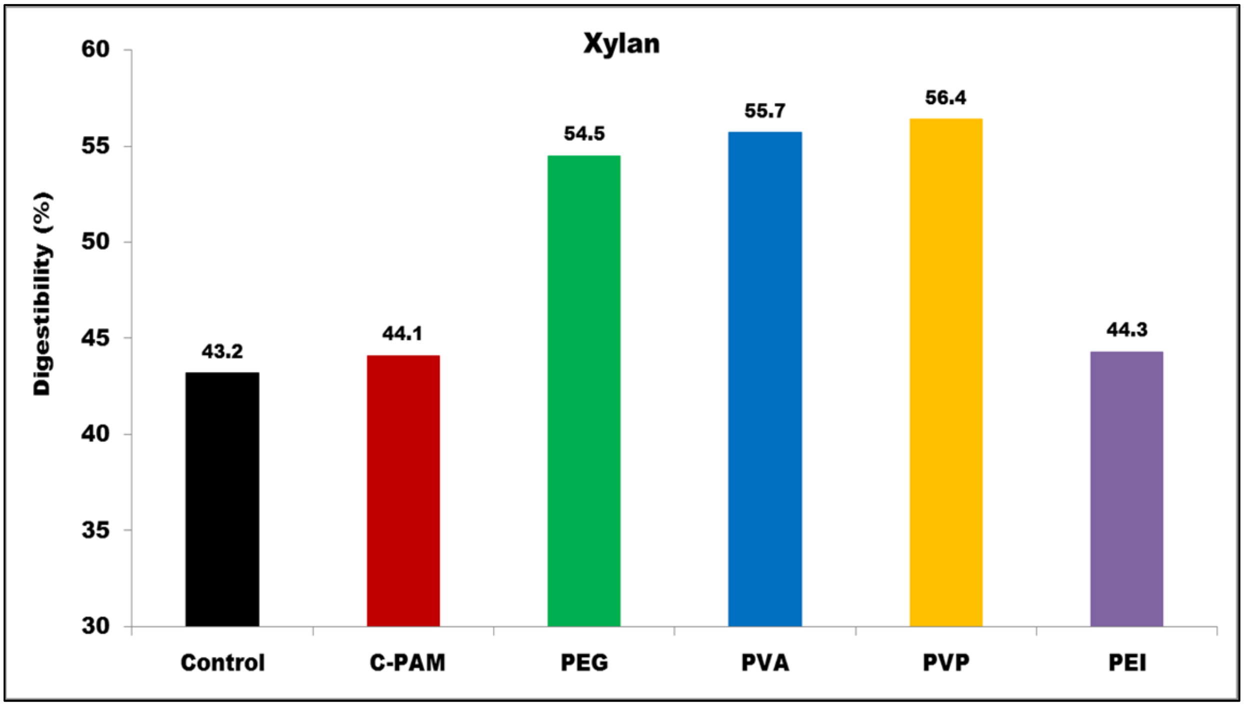

Figure 5.9: 96 hr xylan digestibility of ASG without and with addition of polymers
Substrate loading: 5%; Polymer loading: 10 mg polymer/g-substrate; Enzyme loading: 5 FPU/g-glucan of C Tec 2

5.4.2.2. Dilute sulfuric acid treated Dacotah switchgrass digestibility with polymer addition

As polymer addition during enzymatic hydrolysis proved to be effective for alkaline treated substrate (SAA treated Dacotah), polymer addition studies were carried out with substrates generated under acidic conditions. Therefore, first dilute sulfuric acid treated Dacotah switchgrass was subjected to enzymatic hydrolysis with and without polymer. The pretreated solids were generated using following conditions: 1% H_2SO_4, 150^{o}C, Liquid/solids ratio of 9, 60 min. The amounts of glucan, xylan, and lignin after the pretreatment were found to be 57.4%, 3.8%, and 30.1%, respectively. Glucan digestibility of PSG without and with the addition of polymers is shown in Figure 5.10. Xylose yields were not measured as the xylan content in the PSG was very low. Glucan digestibility achieved without polymer was 42.6%. However, with the addition of 10 mg polymer/g-solid, glucan digestibility was increased to as high as 59.1%

depending on the type of polymer. The highest glucan digestibility with non-ionic polymer was achieved PVP, whereas the lowest was obtained with PEI. Non-ionic polymers have significantly improved the hydrolysis yields of PSG, which is similar with Solka Floc and ASG.

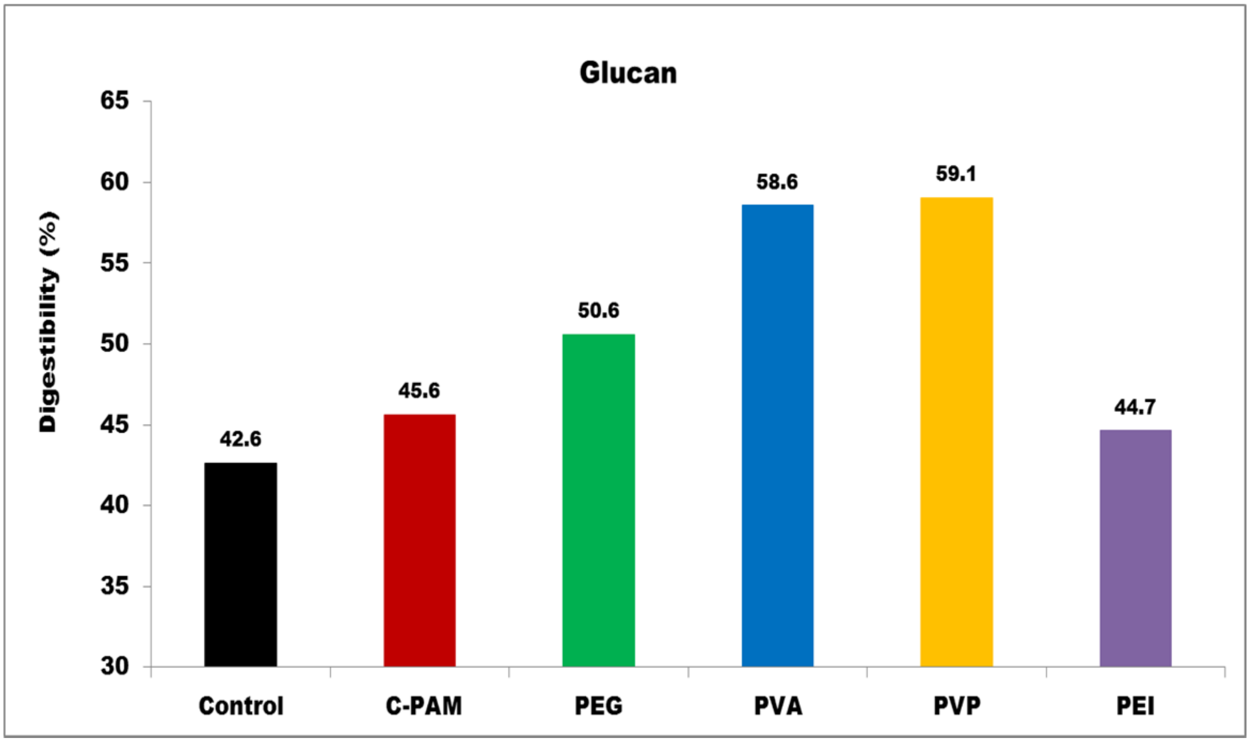

Figure 5.10: 96 hr glucan digestibility of PSG without and with addition of polymers Substrate loading: 5%; Polymer loading: 10 mg polymer/g-substrate; Enzyme loading: 5 FPU/g-glucan of C Tec 2

5.4.2.3. Dilute sulfuric acid treated corn stover digestibility with polymer addition

In addition to PSG, polymer addition studies were carried out using dilute sulfuric treated corn stover. The pretreatment conditions used were: 1% H_2SO_4, 150°C, Liquid to solids ratio of 9, 30 min. PCS composition is shown in Table 5.1. Glucan digestibility is shown in Figure 5.11. Since the xylan presented in the dilute acid pretreated corn stover was very low in quantity, xylan digestibility was not measured. Hydrolysis results show that with the addition of polymer (10 mg/g-solid), glucan digestibility increased from 62.3 to 87.7%, depending on the type of polymer. Non-ionic polymers have a significant effect on increasing hydrolysis yields of PCS. Although, the cationic polymers have increased the digestibility, the increase is very low. Among the non-ionic polymers, PVA and PVP resulted in higher digestibilities than the PEG. Kumar and Wyman (2009b) reported substantial improvement in PCS glucan digestibility with PEG supplementation.

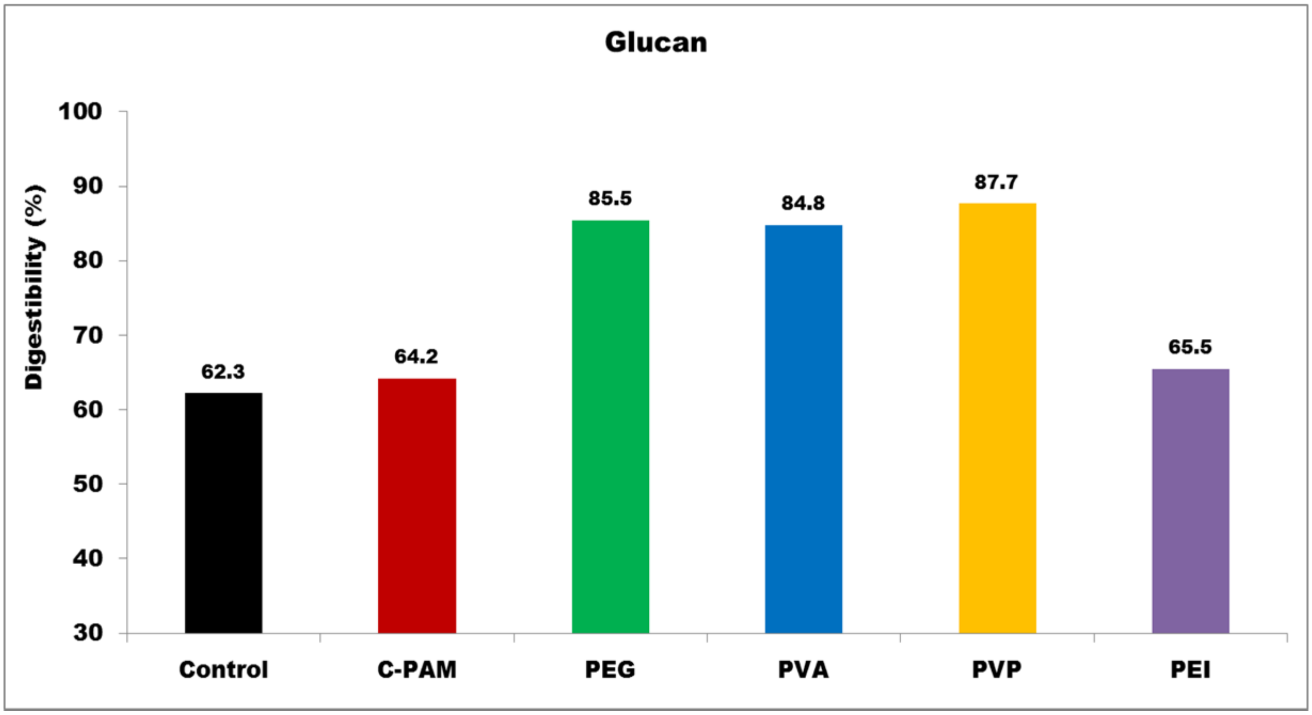

Figure 5.11: 96 hr glucan digestibility of PCS without and with addition of polymers
Substrate loading: 5%; Polymer loading: 10 mg polymer/g-substrate; Enzyme loading: 5 FPU/g-glucan of C Tec 2

5.5. Conclusions

Supplementation of water-soluble polymers has a positive effect on the hydrolysis of lignin-free substrates (Solka Floc and hardwood pulp) and lignin containing substrates (ammonia treated switchgrass, acid treated switchgrass, acid treated corn stover). With 10 mg polymer/g-substrate addition, as high as 25% increase in glucan digestibility and 12% increasing in xylan digestibility were achieved depending on type of the substrate and polymer. The optimum level of polymer loading is 10 mg polymer/g-substrate with all the polymers. Non-ionic polymer (PVP, PVA and PEG) addition resulted in significantly higher hydrolysis yields than ionic polymers (PEI and C-PAM) for all substrates. Non-ionic polymers are effective for both lignin-free and lignocellulosic substrates, whereas ionic polymers are effective only for lignin free substrates to some extent. The main reasons for improvement in digestibility with polymer addition are improvement in cellulase activity and reduction in unproductive binding of cellulase to lignin. Results indicate that 50% of enzyme usage can be reduced with the addition of polymer.

6. Effect of Sodium Sulfide Addition on Alkaline Pretreatment of Lignocellulosic Biomass

6.1. Abstract

Addition of small amounts of sodium sulfide to the alkaline reagent is known to reduce carbohydrate loss and improve delignification. Sulfur containing reagents are highly reactive with lignin, thus can serve as delignification enhancers. Therefore, in this study, effect of adding small amount of sodium sulfide in alkaline pretreatment of Dacotah switchgrass was investigated. A selected few experiments were also carried using palm residue to evaluate the effect of sodium sulfide addition on other lignocellulosics. The alkaline reagents tested were either recoverable ones (ammonia, sodium carbonate) or inexpensive one (lime). Pretreatments were primarily carried out in batch mode under low severity conditions: 90-120°C, 12-24 hr treatment time, 0-15% alkali, 0-3% sodium sulfide, and liquid to solid ratio of 9. A selected few experiments were carried out at 150°C and 180°C to see the effect of temperature on carbohydrate retention and delignification. The pretreated substrates were subjected to enzymatic digestibility tests using 15 FPU/g-glucan of C Tec 2 enzyme. Addition of sodium sulfide did not affect the carbohydrate content, but increased the delignification by 14-42%. Addition of sodium sulfide substantially increased the digestibility for all the alkali treatments: 15-45% in glucan digestibility and 10-40% in xylan digestibility depending on type of substrate and pretreatment severity.

6.1. Introduction

Lignin is a three dimensional polymer of phenyl-propane units with different linkages such as ether, aryl, alkyl, and their combinations (Ralph, 1999). In the lignin structure, alkyl-aryl linkages form about 60% of the total linkages (Gierer, 1986). Many of these bonds are attacked during pulping with NaOH, which removes anywhere from 70-90% lignin from biomass (Gierer, 1986; Biermann, 1996). The alkaline degradation of alkyl-aryl bonds follows first order kinetics with respect to the lignin structure, and does not depend on the $[OH^-]$ concentration, as long as alkaline conditions are maintained (Gierer, 1986). For pretreatment of herbaceous biomass, such a severe delignification is not required; hence use of less amounts alkali or milder alkaline reagents is desirable. Milder alkaline reagents such as ammonia, sodium carbonate and lime have traditionally been used as pretreatment reagents for delignification of biomass to improve enzymatic hydrolysis (Iyer et al., 1996; Kim and Holtzapple, 2006; Garlock et al., 2011; Pallapolu et al., 2011; Yang et al., 2012). Using milder alkaline reagents has the added advantage of carbohydrate preservation since the characteristic peeling reaction due to strong NaOH is prevented. In addition, these reagents are many times cheaper than sodium hydroxide, and easier to recover.

Addition of small amounts of sodium sulfide to the alkaline reagent is known to reduce carbohydrate losses and improve delignification (Gierer. 1986). Sulfur based compounds increase the cleavage of phenolic β-ether linkages and carbon-carbon linkages. It also causes fragmentation of lignin and condensation of the lignin fragments with sulfur (Gierer, 1986). The sulfur induced pretreatment method with the use of sodium carbonate (green liquor pretreatment) has been studied by several authors (Gu et al., 2012; Yang et al., 2012; Gu et al., 2013). When sodium sulfide is supplemented to alkali solution, sulfur forms covalent-bonds with the released lignin and helps in lignin condensation. Therefore, in this study, investigated the effect of adding small amount of sodium sulfide in alkaline pretreatment of Dacotah switchgrass has been investigated. The alkaline reagents tested were either recoverable ones (ammonia, sodium carbonate) or inexpensive one (lime). Pretreatments were applied to Dacotah switchgrass and palm residue.

6.3. Materials and methods

6.3.1. Feedstock, reagents. and enzyme

Dacotah switchgrass and palm residue (PR) were used in all experiments. Ammonium hydroxide solution 30 wt % (Lot. No. 012108), and lime (Lot. No. 926201) were purchased from Fisher-Scientific. Sodium carbonate was purchased from VWR BDH (Cat. No. BDH0284). Sodium sulfide ($Na_2S.9H_2O$) was purchased from Sigma Aldrich (Product No. 208043). Cellulase enzyme Cellic C-Tec 2 was provided by Novozymes with a total specific activity of 119 FPU/mL (Total BCA protein No. of 255 mg/mL, Batch. No. VCNI0001).

6.3.2. Pretreatment experiments

Pretreatment experiments were carried out with addition of sodium sulfide, along with sodium carbonate/aqueous ammonia/lime. Feedstocks used were Dacotah switchgrass and palm residue. Pretreatments were carried out in stainless steel batch rectors under following conditions: 90-180°C, 12-24 hr treatment time, 0-15% alkali, 0-3% sodium sulfide, and L/S of 9. The majority of the experiments primarily carried out in 90-120^{o}C temperature range; however a selected few experiments were carried out at 150^{o}C and 180^{o}C to see the effect of temperature on carbohydrate retention and delignification. Dacotah switchgrass and palm residue were finely ground and passed through 20 and 80 mesh size mechanical sieves. The particles retained between 20 and 80 mesh size was subjected to the pretreatment. After pretreatment, reactors were immediately removed from the oven and quenched to room temperature in a water bath. The cooled slurries from sodium carbonate and ammonia pretreatments were vacuum filtered immediately through a filter paper (Whatman®, Grade 802 Fluted, size 32.0 cm). The vacuum filtered wet solids underwent further cold water-washing. Washing was continued until reaching neutral pH. Lime pretreated samples were first washed with cold water to remove sodium sulfide, after that the slurry was neutralized using 5 N HCl to a pH of approximately 4, then underwent several washing cycles with DI water until reaching neutral pH. Washed solids from filter paper were carefully collected to calculate the solid remaining after the pretreatment. The moisture content of the washed solids was measured. Washed solids were not subjected to further drying, and kept at 4°C. All pretreatment runs were made in duplicates. Pretreated solids after cold water washing were analyzed for their composition using NREL Standard procedures (Sluiter et al., 2006).

6.3.3. Enzymatic hydrolysis

All the enzymatic hydrolysis experiments were carried out with 2% (w/v) substrate and 15 FPU/g-glucan of C-Tec 2. The other enzymatic hydrolysis conditions used were 50°C, pH 4.8 (0.05 M sodium citrate buffer), 150 RPM in a Shaker/Incubator (NBS, Innova-4080). 0.1 mL of a 2% sodium azide solution was added to prevent the growth of organisms during the hydrolysis. Hydrolysate samples are periodically taken, and analyzed for glucose, xylose using HPLC equipped with Biorad-HPX-87P column and RI detector.

6.4. Results and discussion

Addition of small amounts of sodium sulfide to the alkaline reagent is known to reduce carbohydrate losses and improve delignification. Therefore, in this study, effect of adding small amount of sodium sulfide in alkaline pretreatment of Dacotah switchgrass was investigated. Experiments were first started with Dacotah switchgrass as one of the goals of this study was to improve monomeric sugar yields from recalcitrant Dacotah switchgrass. A selected few experiments were carried out using palm residue to evaluate the effect of sodium sulfide addition on other recalcitrant lignocellulosics. The alkaline reagents tested were either recoverable ones (ammonia, sodium carbonate) or inexpensive one (lime). Pretreatments were primarily carried out in batch mode under low severity conditions: 90-120°C, 12-24 hr treatment time, 0-15% alkali, 0-3% sodium sulfide, and liquid to solid ratio of 9. A selected few experiments were carried out at 150^{o}C and 180^{o}C to see the effect of temperature on carbohydrate retention and delignification. The pretreated substrates were subjected to enzymatic digestibility tests using 15 FPU/g-glucan of C Tec 2 enzyme.

6.4.1. Effect of sodium sulfide addition on sodium carbonate pretreatment of Dacotah switchgrass

Dacotah switchgrass was first subjected to green liquor (Na_2CO_3+Na_2S) pretreatment. The pretreatment conditions used were: 1.5 g of sodium carbonate/g-DSG, 0-0.18 g of sodium sulfide/g-DSG, 12 hr treatment time, liquid to solid ratio of 9, 90^{o}C. Figure 6.1 shows the solid composition of untreated and green liquor pretreated Dacotah switchgrass. The amounts of glucan, xylan, and lignin present in the untreated Dacotah were 34.9%, 21.5%, and 22.3%, respectively. Solids generated with and without green liquor pretreatment retained as high as 97-99% of glucan and 73-76% of xylan and a high percentage of delignification was also achieved

(45-69%) depending on amount of sodium sulfide. A significant level (45.3%) of delignification was achieved with sodium carbonate alone (Figure 6.1), however the corresponding glucan (60.1%) and xylan (53.3%) digestibilities were not high enough (Figure 6.2). But addition of 0.12 g of sodium sulfide increased delignification further by 16.5%, which has a significant effect on increasing the glucan and xylan digestibilities by 27.4% and by 19.1%, respectively. A further addition of sodium sulfide by 0.06 g, further improved the delignification and glucan/xylan digestibilities and no apparent loss of carbohydrates was occurred with sodium sulfide addition during the pretreatment. According to Gu et al. (2012), 70% of original polysaccharides were converted to fermentable sugars by applying green liquor pretreatment to corn stover. Gu et al. (2013) also treated rice straw using green liquior, and obtained substantial delignification (39.4%) and excellent glucan (92.2%)/xylan (82.4%) digestibilities. Hydrolysis data suggest that it is possible to achieve high digestibilities from recalcitrant substrates such as Dacotah switchgrass by adding small amount of sodium sulfide with minimal or no loss of carbohydrate content during alkaline pretreatment.

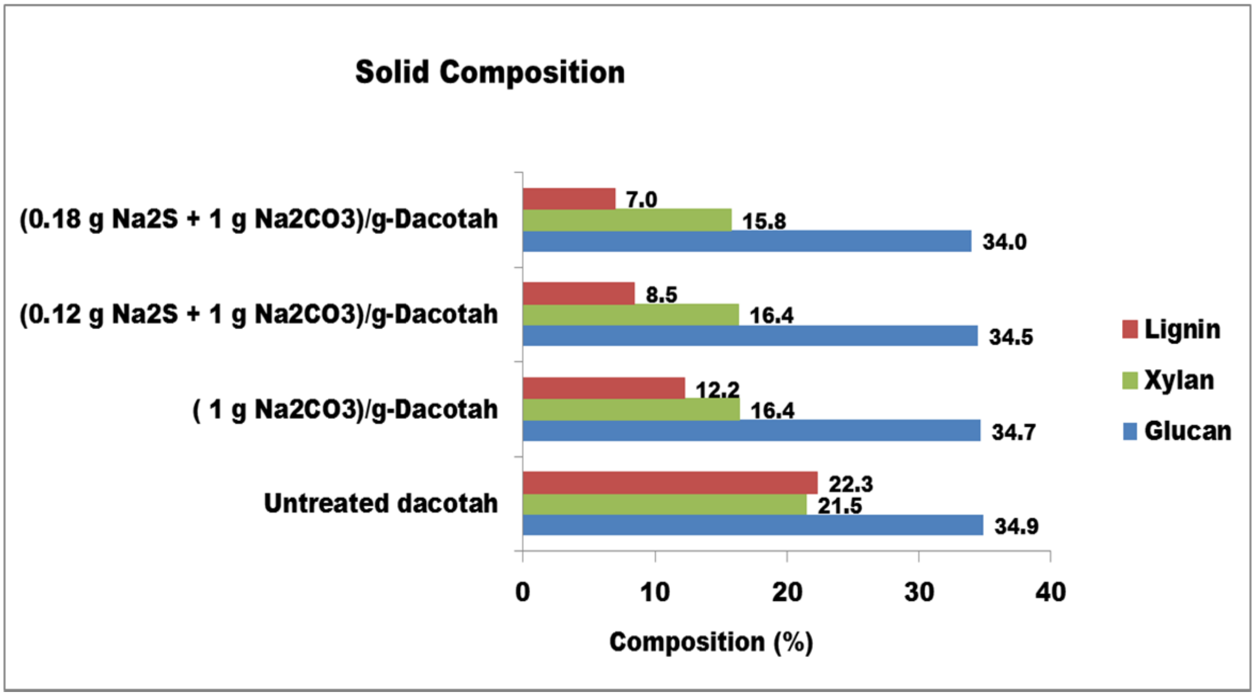

Figure 6.1: Solid composition of untreated, sodium sulfide and sodium carbonate pretreated Dacotah switchgrass; Pretreatment Conditions: 90^{o}C, 12 hr, and liquid to solid ratio of 9

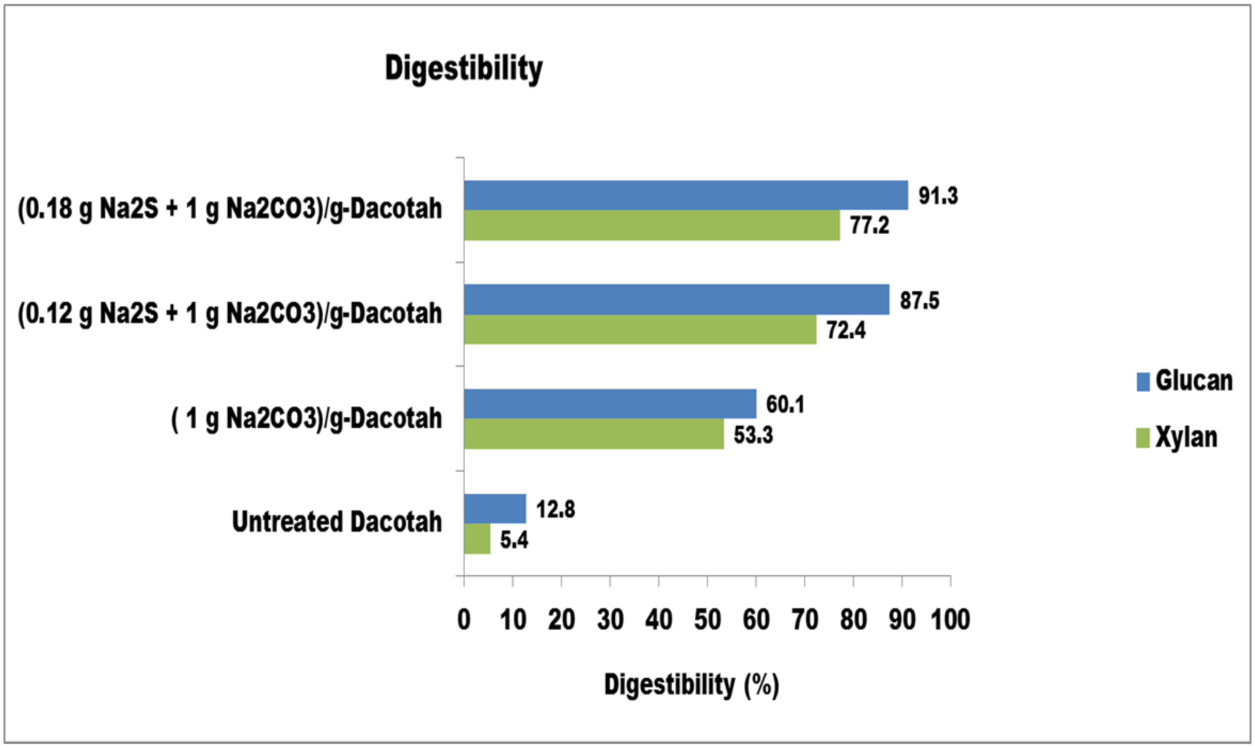

Figure 6.2: Glucan and xylan digestibilities of sodium sulfide and sodium carbonate pretreated Dacotah switchgrass; Enzyme loading: 15 FPU/g-glucan of C-Tec 2

6.4.1.1. Sodium carbonate and sodium sulfide recovery

Recovery of alkali is necessary for alkaline pretreatment to be cost effective. Recovery of NaOH is well an established process in Kraft pulping (Biermann, 1996) (Figure 6.3). Kothari and Lee (2012) took part of the established NaOH recovery process, and proposed a slight modification that can make it cost effective (Figure 6.5). In the original process after solid liquid separation, lignin rich alkali liquid stream goes into an evaporator where it is concentrated. From the evaporator concentrated liquid solution goes into recovery boiler where lignin is burned at 1100^{o}C, and from recovery boiler sodium carbonate and sodium sulfide come out in the form of green liquor. From here green liquor undergoes subsequent recovery steps to from white liquor, which contains sodium sulfide and sodium hydroxide. But for sodium carbonate and sodium sulfide recovery only green liquor is required. Therefore, Kothari and Lee (2012) took initial part of Kraft recovery process, and proposed one change to be more cost effective. In their process instead of adding Na_2S for make up, they proposed to add Na_2SO_4, which is comparatively cheaper than Na_2S (Figure 6.5). Conversion of Na_2SO_4 to Na_2S and other reactions can be seen in Figure 6.4.

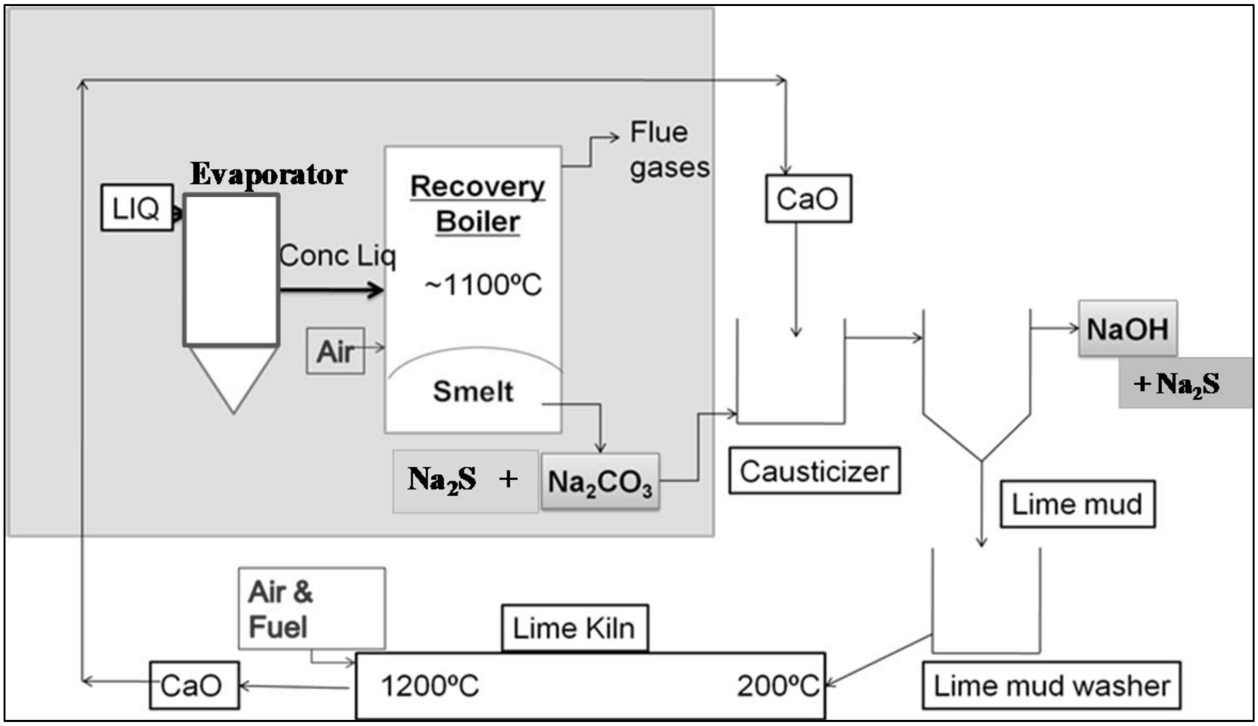

Figure 6.3: NaOH recovery process used in Kraft pulping

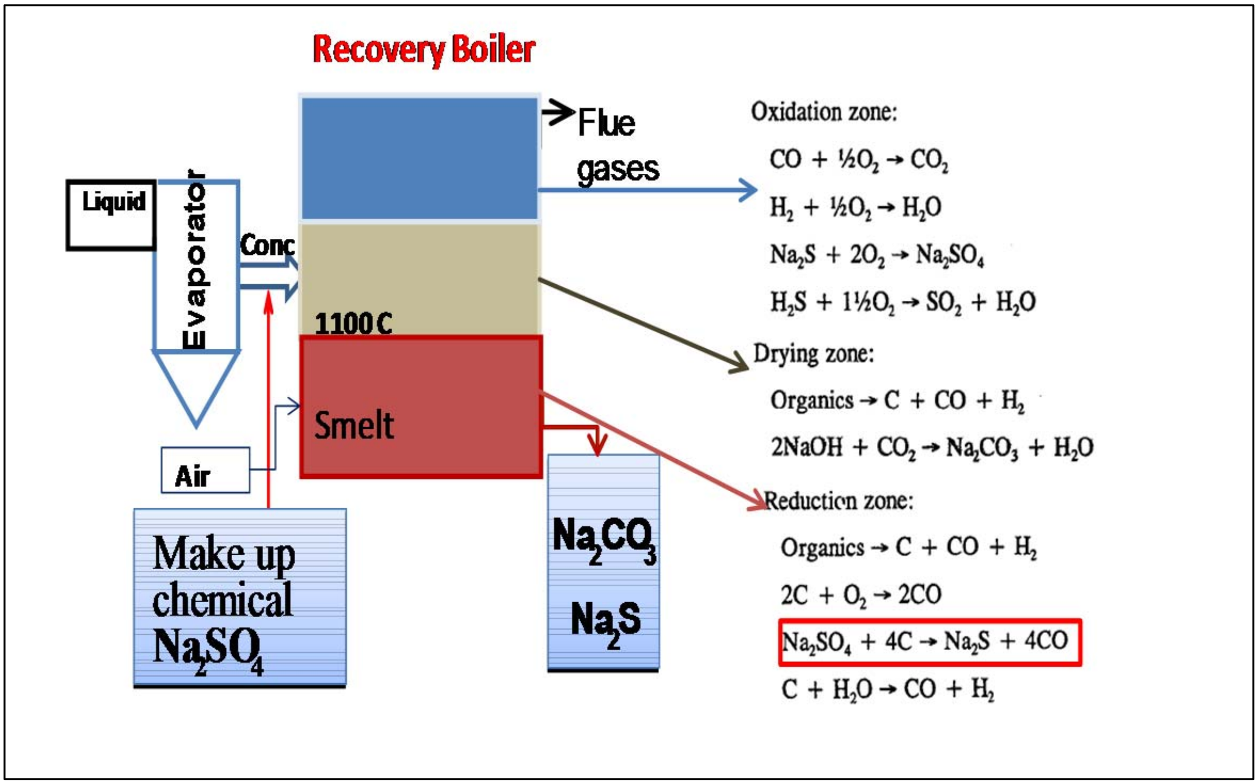

Figure 6.4: Different reactions in recovery boiler

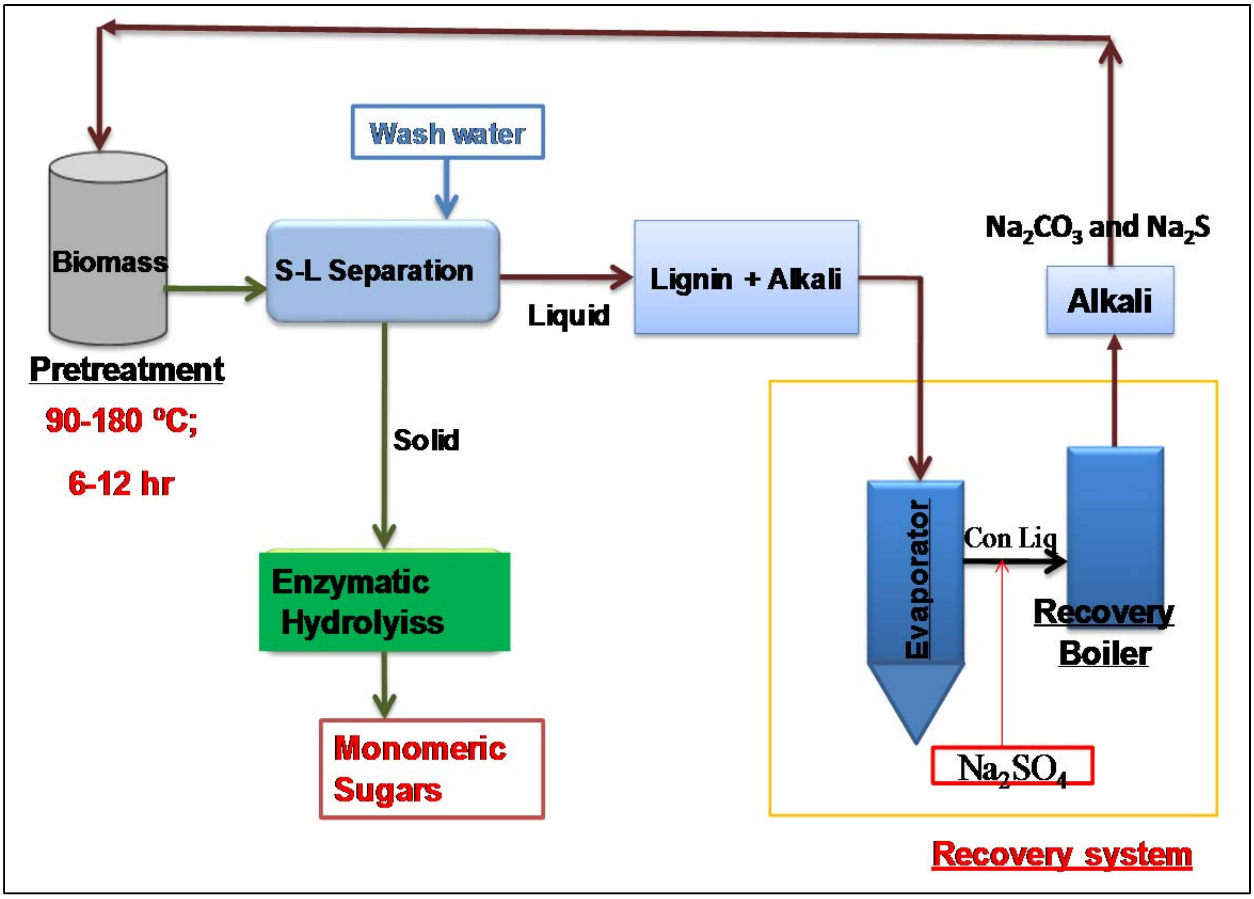

Figure 6.5: Sodium carbonate and sodium sulfide pretreatment and recovery process (Kothari and Lee, 2012)

6.4.2. Effect of sodium sulfide addition on aqueous ammonia pretreatment of Dacotah switchgrass

Having seen positive effect of sodium sulfide addition on sodium carbonate pretreatment of Dacotah switchgrass, we have decided to study effect of sodium sulfide addition on aqueous ammonia pretreatment of Dacotah switchgrass. Pretreatment experiments were carried out using following conditions: 1.3 g of ammonia/g-DSG, 0-0.18 g of sodium sulfide/g-DSG, 12 hr treatment time, liquid to solid ratio of 9, 90^{o}C. Pretreated solids after cold water washing were subjected to enzymatic hydrolysis by applying 2% (w/v) solids and 15 FPU/g-glucan of C-Tec 2. The amounts of glucan, xylan and lignin present in the untreated Dacotah were found to be 34.9%, 21.5%, and 22.3%, respectively. All the pretreatments retained as high as 97-99% of glucan and 78-81% of xylan, and the delignification was achieved by 54-70% depending on amount of sodium sulfide. Data indicate that the addition of sodium sulfide has a significant effect on increasing delignification and improving enzymatic hydrolysis. A significant level of delignification (53.8%) was achieved with aqueous ammonia alone (Figure 6.6), however the corresponding glucan (65.8%) and xylan (60.5%) digestibilities were not high enough (Figure 6.7). But the addition of sodium sulfide increased the delignification further by 17%, which has significantly improved the glucan and xylan digestibilities by 17.4% and 15.7%, respectively.

Solid composition and enzymatic hydrolysis data suggest that sodium sulfide addition to aqueous ammonia pretreatment can be as effective as that of with green liquor pretreatment.

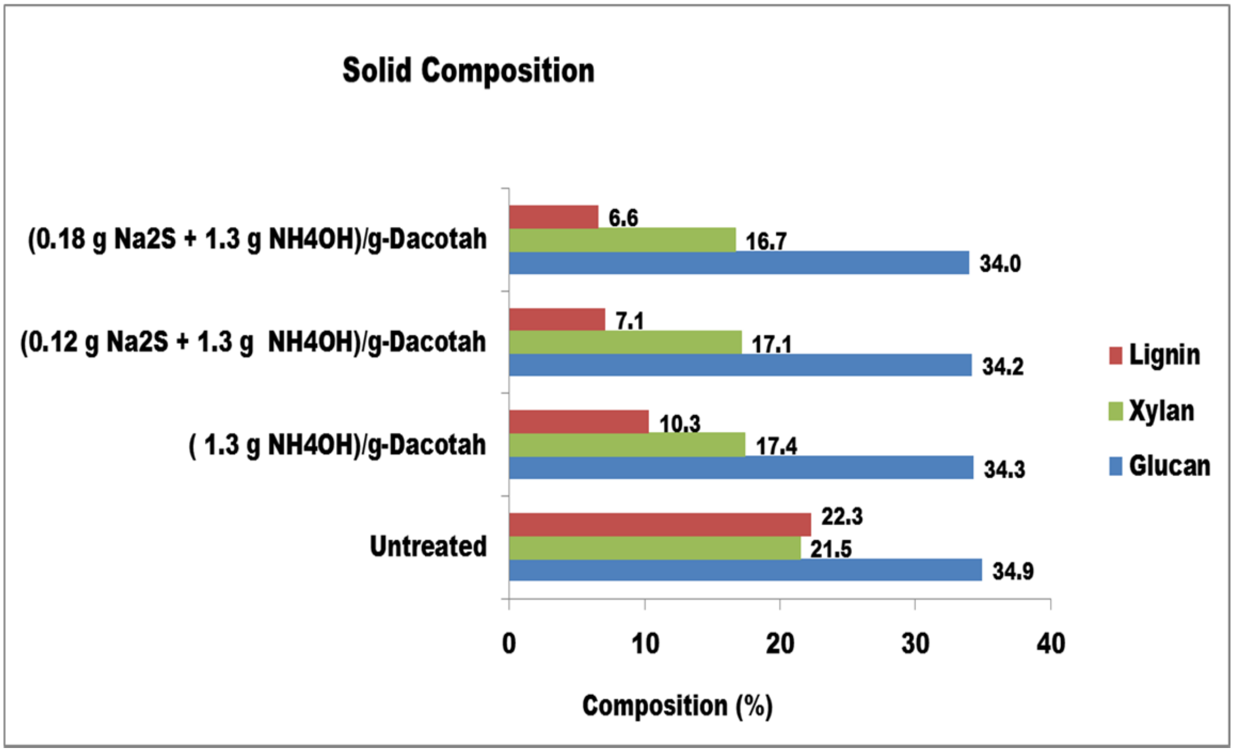

Figure 6.6: Solid composition of untreated, sodium sulfide and aqueous ammonia pretreated Dacotah switchgrass; Pretreatment Conditions: 90°C, 12 hr, and liquid to solid ratio of 9

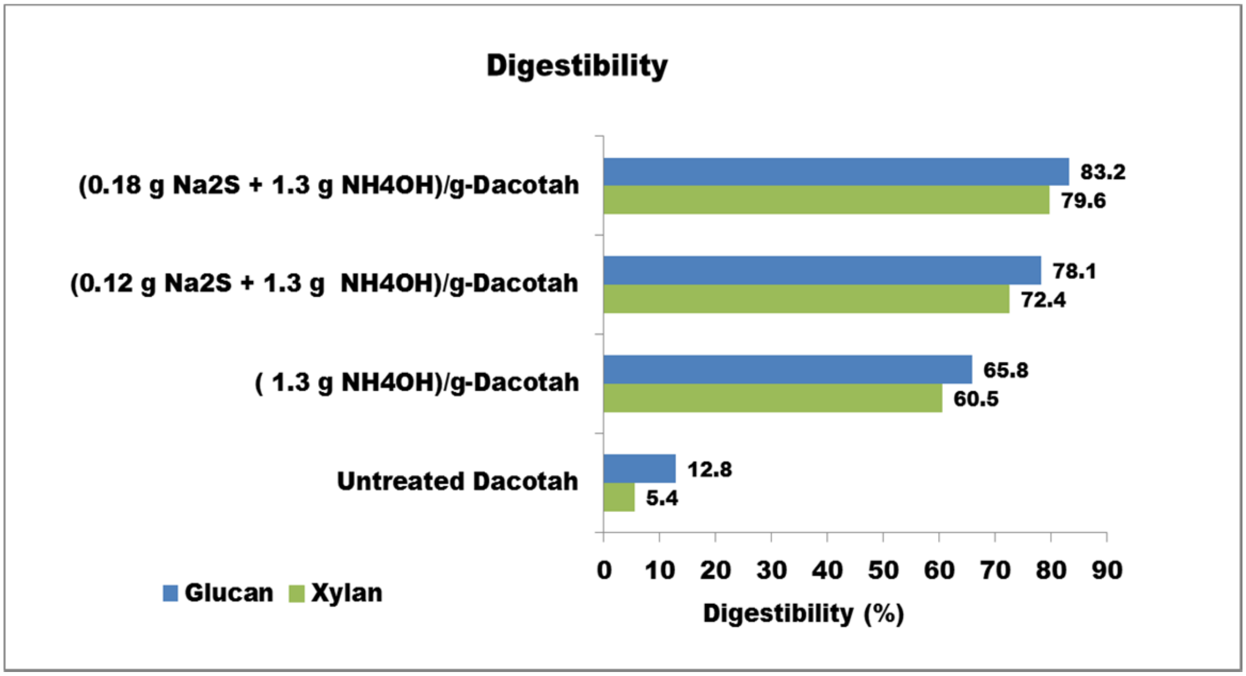

Figure 6.7: Glucan and xylan digestibilities of sodium sulfide and aqueous ammonia pretreated Dacotah switchgrass; Enzyme loading: 15 FPU/g-glucan of C-Tec 2

6.4.3. Effect of sodium sulfide addition on lime pretreatment of Dacotah switchgrass

Lime is another alkaline reagent that has been traditionally used as a pretreatment reagent. Therefore, sodium sulfide addition effects on alkaline treatment were studied using sodium sulfide and lime. The pretreatment conditions used were: 1.5 g of lime/g-DSG, 0-0.18 g of sodium sulfide/g-DSG, 12 hr treatment time, liquid to solid ratio of 9, 90^{o}C. The amount of glucan, xylan, and lignin present in the untreated Dacotah switchgrass were found to be 34.9%, 21.5%, and 22.3%, respectively. Data indicate that after the pretreatment glucan/ xylan were retained by 98-99% and 81-88%, respectively, and the delignification was achieved by 45-59% (Figure 6.8). It was found that with the addition of sodium sulfide delignification was increased by as high as 14% and the increase in delignification has increased the glucan and xylan digestibilities by 16% and 15%, respectively (Figure 6.9). Results indicate that sodium sulfide addition works well with lime pretreatment.

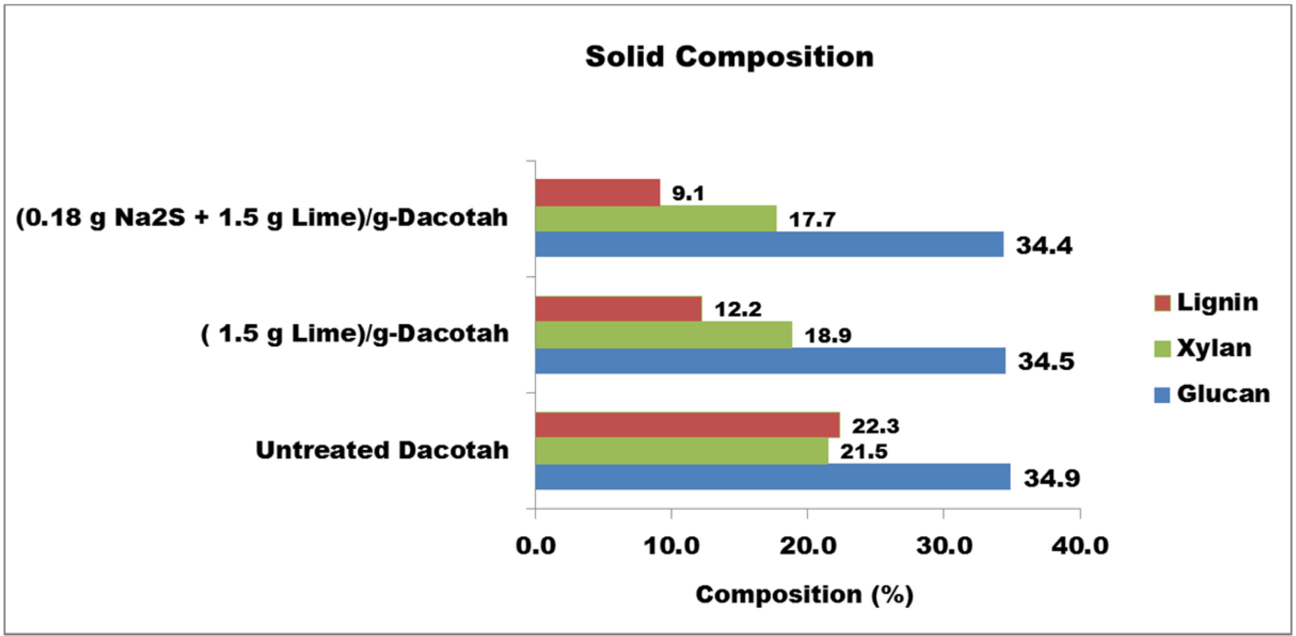

Figure 6.8: Solid composition of untreated, sodium sulfide and lime pretreated Dacotah switchgrass; Pretreatment Conditions: 90^{o}C, 12 hr, and liquid to solid ratio of 9

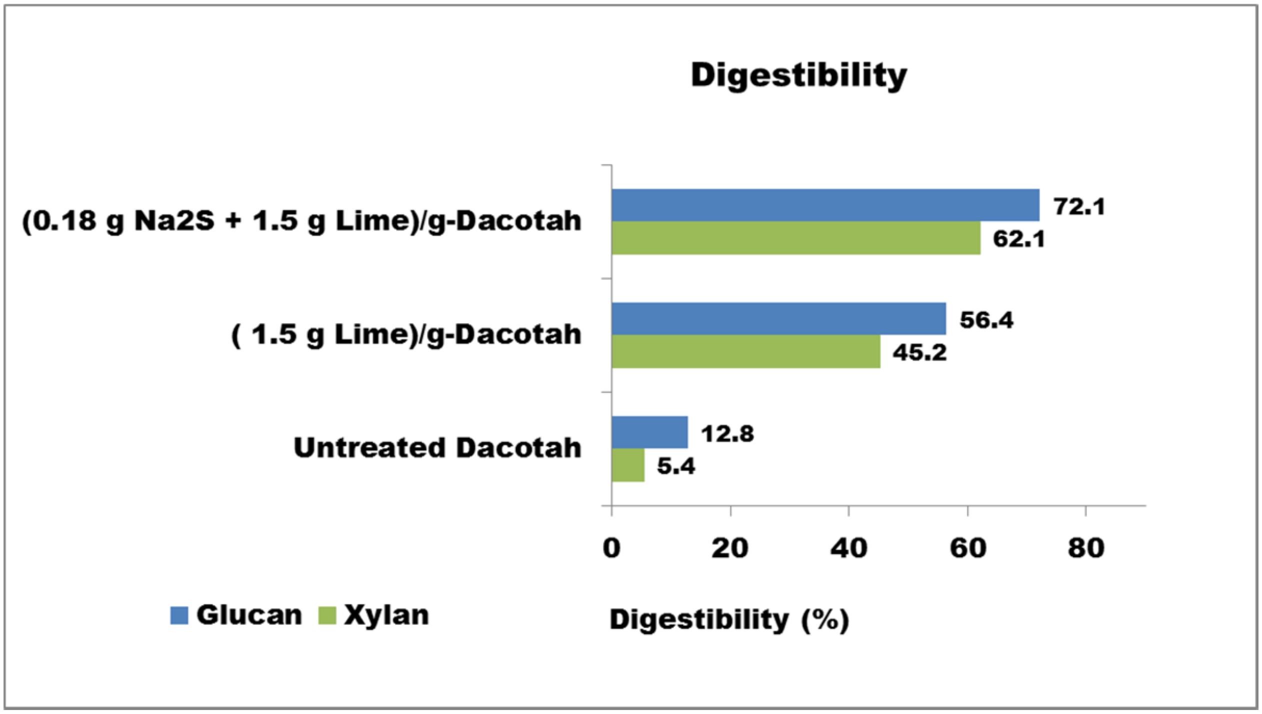

Figure 6.9: Glucan and xylan digestibilities of sodium sulfide and lime pretreated Dacotah switchgrass; Enzyme loading: 15 FPU/g-glucan of C-Tec 2

6.4.4. Effect of sodium sulfide supplementation on sodium carbonate pretreatment of palm residue

Lignocellulosic biomass generated from the oil palm industries includes oil palm trunks, oil palm fronds, empty fruit bunch, and palm pressed fibers, palm shells and palm oil mill effluent. An estimated 60 million tons of palm oil seeds were produced in 2012, out of that only 10% was converted into oil rest went to waste. Palm oil residue contains more than 50% of polymeric sugars, which can be converted into monomeric sugars. However, palm residue contains substantial amount of lignin (>25%), which can be can be recalcitrant to sugar conversion process. Previous sections showed the positive effect of sodium sulfide on alkaline pretreatment of Dacotah switchgrass, therefore, in this section, pretreatment was carried out using palm residue to see the effect of sodium sulfide addition on other recalcitrant lignocellulosics.

The pretreatment conditions used were: 1.5 g of sodium carbonate/g-PR, 0-0.0.18 g of sodium sulfide/g-PR, 12 hr treatment time, liquid to solid ratio of 9, 90°C. Figure 6.10 shows the solid composition of untreated, green liquor pretreated palm residue. The amounts of glucan, xylan, and lignin present in the untreated palm residue were 29.4%, 22.4%, and 25.7%, respectively. Pretreatment experiments carried with and without sodium sulfide addition retained as high as 95-99% of glucan and 63-83% of xylan (Figure 6.10). At the same time high percentage of delignification was also achieved (36-78%) depending on amount of sodium sulfide. Figure 6.10 and Figure 6.11 show that the addition of sodium sulfide has a significant effect on increasing delignification and the corresponding improvement in enzymatic hydrolysis. A significant level (by 36%) of delignification was achieved with sodium carbonate alone (Figure 6.10), but the corresponding improvement in glucan (38.7%) and xylan (34.8%) digestibilities was very low. However, the addition of 0.06 g of sodium sulfide further increased delignification by 9.5%, which has significantly increased the glucan and xylan digestibilities by 19.7% and 20.7%, respectively. A further addition of sodium sulfide by 0.06g, improved the delignification further and glucan/xylan digestibilities. Solid composition and enzymatic hydrolysis data proves that sodium sulfide addition is effective even with high lignin containing substrates.

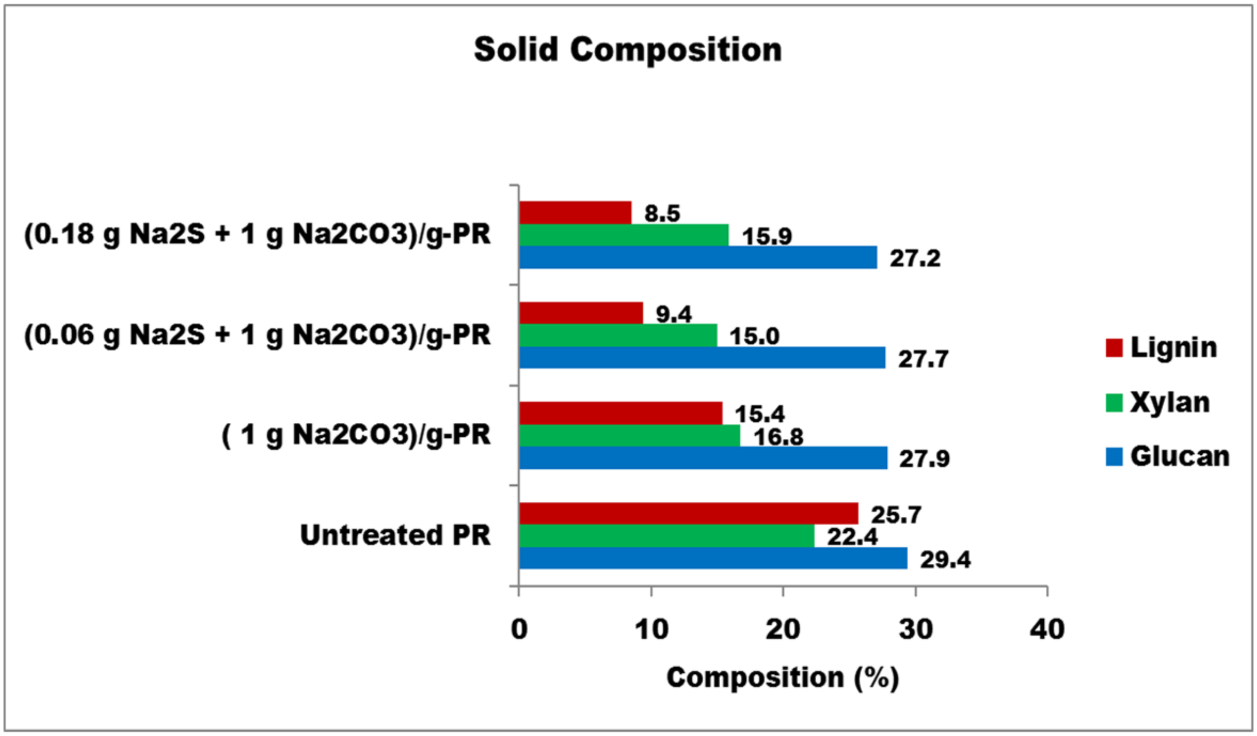

Figure 6.10: Solid composition of untreated, sodium sulfide and sodium carbonate pretreated palm residue; Pretreatment Conditions: 90°C, 12 hr, and liquid to solid ratio of 9

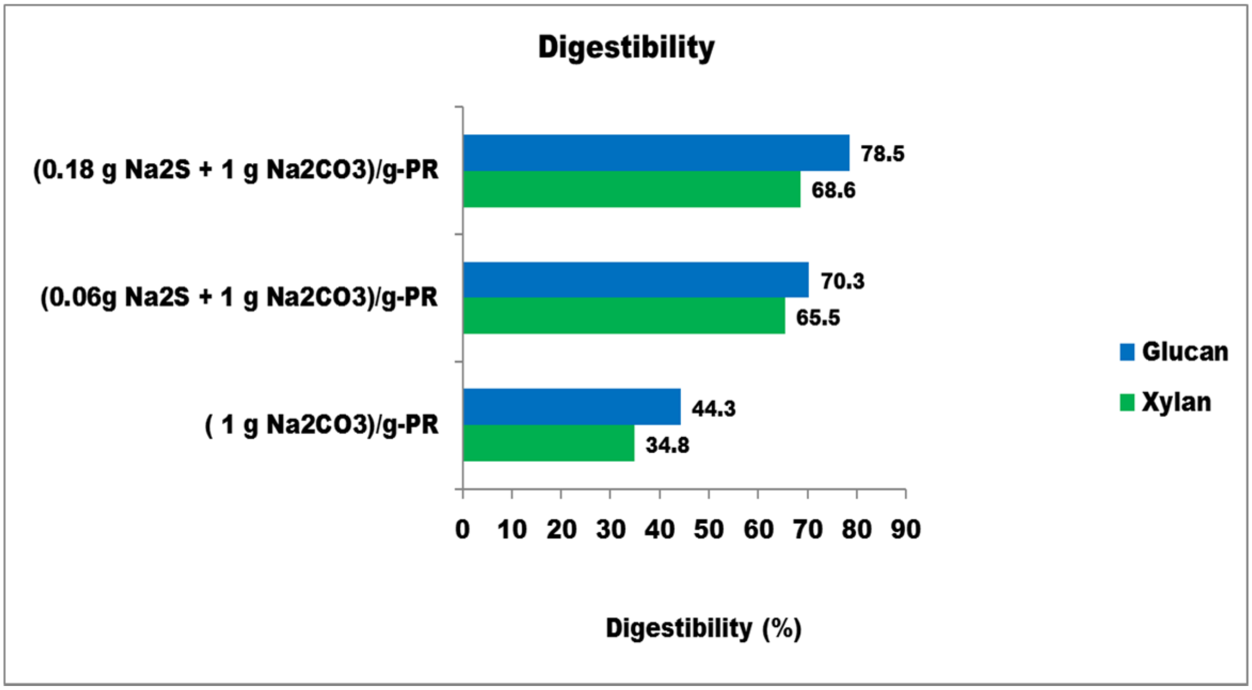

Figure 6.11: Glucan and xylan digestibilities of sodium sulfide and sodium carbonate pretreated palm residue; Enzyme loading: 15 FPU/g-glucan of C-Tec 2

6.4.4.1. Effect of temperature on composition of Na_2S and Na_2CO_3 pretreated palm residue

In order to see the effect of temperature on sodium sulfide supplementation and sodium carbonate pretreatment, experiments were carried out by varying the pretreatment temperature and keeping all the other reaction parameters constant. Pretreatment conditions used were: (0.18 g Na_2S + 1.5 g Na_2CO_3)/g-PR, liquid to solid ratio of 9, 90°C, 120°C, 150°C for 12 hr and 180°C for 6 hr. At 90°C, a significant amount of delignification with marginal loss of carbohydrates was achieved (Figure 6.12). The glucan and xylan digestibilities achieved at 90°C were 70.0% and 62.8 (%), respectively. Increase of treatment temperature from 90°C to 120°C, has no effect on delignification. However substantial increase in glucan and xylan digestibilities was obtained (by 10%/8%) (Figure 6.13).

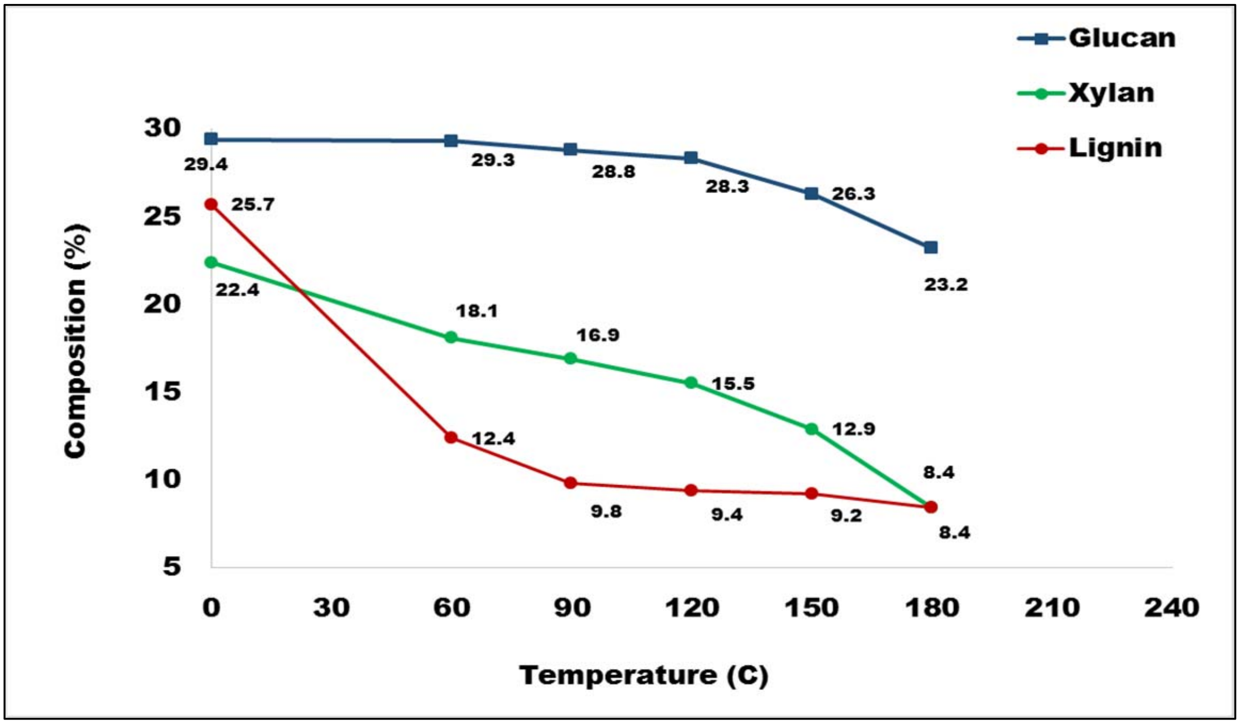

Figure 6.12: Effect of temperature on composition of Na_2S and Na_2CO_3 pretreated palm residue Pretreatment Conditions: (0.18 g Na_2S + 1.5 g Na_2CO_3)/g-PR, L/S-10, 60°C 90°C, 120°C, 150°C-12 hr, 180°C-6 hr

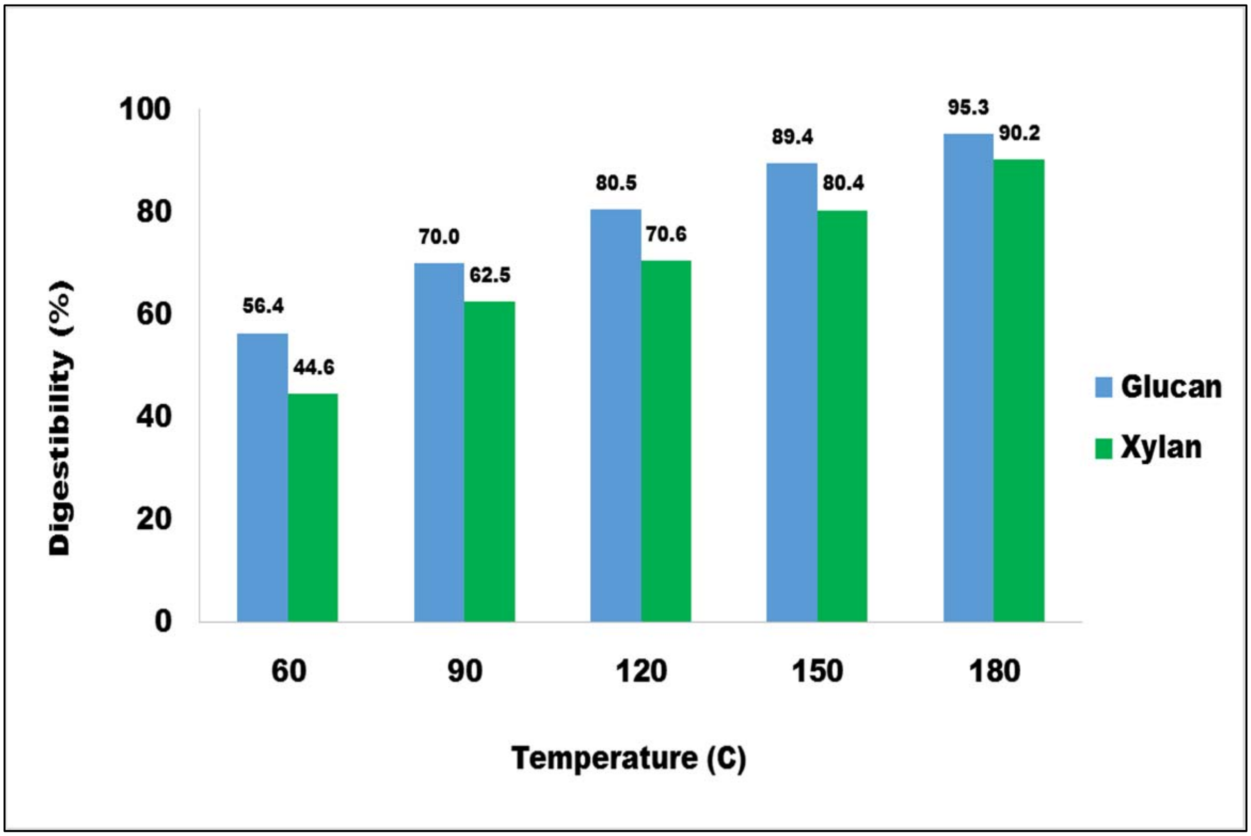

Figure 6.13: Effect of temperature on 72 hr digestibility of Na_2S and Na_2CO_3 pretreated palm residue; Enzyme loading: 15 FPU/g-glucan of C-Tec 2

6.5. Conclusions

Sodium sulfide can be a supplemental pretreatment reagent in alkaline treatment of lignocellulosics. Sodium sulfide addition in alkaline pretreatment increases the delignification, and does not degrade the carbohydrates. Sodium sulfide supplementation in alkaline pretreatment enhanced delignification of Dacotah switchgrass by 14-42% and the order of delignification is Na_2CO_3 > NH_4OH >Lime. Sodium sulfide supplementation in alkaline pretreatment significantly increases glucan and xylan digestibility by 15-31% and 10-21%, respectively. Sodium sulfide supplemented alkaline pretreatment can be effectively carried out at temperatures below $120^{o}C$. Recovery of sodium carbonate and ammonia are relatively easy. Less expensive Na_2SO_4 can be used as a makeup chemical for Na_2S in recovery process.

7. Production of Levulinic Acid from Soluble Sugars and Lignocellulosic Substrates Using Moderate Sulfuric Acid Concentrations and Low Temperatures

7.1. Abstract

The majority of levulinic acid (LA) production studies reported in the literature were carried out using dilute sulfuric concentrations (1-5% (w/v)), low substrate loadings (1-5% (w/v)), and at high temperatures (160-220^{o}C). Thus, this work focused on production of LA using high substrate loadings (10-20%), moderate acid concentrations (10-30%), and at low temperatures (80-130^{o}C). Different lignocellulosic substrates (Solka Floc, corn stover, acid pretreated corn stover, pine, and municipal solid waste) were used to evaluate the effects of substrate characteristics such as cellulose crystallinity, presence of lignin/hemicelluloses/non-organic substances on LA production. In addition, to verify the difference in LA production between soluble sugars and lignocellulosic substrates, various soluble sugars including, glucose, fructose, sucrose, galactose, and mannose were studied. Results show that it is technically possible to produce LA with high solids, moderate acid concentrations, and at low temperatures in a single step. All the soluble and lignocellulosic substrates produced more than 60% LA (molar yield) with 20% H_2SO_4, 12% initial solids, and at 120^{o}C, which is comparable or better than the literature reported yields on the basis of g H_2SO_4/g-substrate. Among the monomeric sugars fructose produced highest LA yield (70%), whereas among the lignocellulosic substrates Solka Floc produced highest LA yield (64%).

7.2. Introduction

Levulinic acid, a member of the gamma-keto acids, can be produced through acid-catalyzed dehydration and hydration of hexose sugars (Rackemann and Doherty, 2011). It is an ideal platform chemical that can be utilized to produce a number of bio-chemicals such as succinic acid, polymers, pharmaceuticals, solvents, plasticizers, and biofuels/oxygenated fuel additives, etc. (Rackemann and Doherty, 2011). The main method for production of high purity LA in use today follows the petrochemical conversion route from maleic anhydride (Moens et al., 2002) or furfuryl alcohol (Timokhin et al., 1999).

Many soluble sugars (glucose, sucrose, and fructose) and lignocellulosics (wood, cane sugar, grain sorghum and agricultural wastes) have been used to produce LA (Fang and Hanna, 2002). The key in improving the yield of the LA production from biomass lies in separating the intermediate products at each step of the reaction pathway, and reduce the side reactions (Ghorpade and Hanna, 1999; Moens et al., 2002; Hayes et al., 2006). However, the majority of the reported studies about LA production from monomeric sugars/lignocellulosics in the literature are using dilute sulfuric concentrations (1-5% (w/v)), low substrate loadings (1-5% (w/v)), and at high temperatures (160-220^{o}C) (Table 7.1). Majority of the reported studies on LA production utilized either high amount of acid per gram substrate (Girisuta et al., 2006; Galletti et al., 2012), and/or a low initial substrate loading (Girisuta et al., 2006; Chang et al., 2007), and/or very high temperatures (Efremov et al., 1997; Chang et al., 2007; Hayes et al., 2006), and/or a complex process (Hayes et al., 2006; Farone and Cuzens, 2000). Hayes et al. (2006) reported more than 70% LA, but their process is very complex. Their process utilizes two different stages. In the first stage HMF is produced at 210-230^{o}C and 17 seconds, whereas in the second stage HMF is converted to LA at 195-215^{o}C and 15 min. Chang et al. (2007) reported

69% LA molar yield using wheat straw at 210^{o}C, but their initial substrate loading was 6%. Farone and Cuzens (2000) invented a process in which LA is produced from paper at high substrate loadings (20-40%). However, they used multiple steps to process such high substrate concentrations. According to their process, in the first step cellulose is decrystallized at low temperature (40-60^{o}C) and high sulfuric acid concentration (10-90%); in the second step decrystallized cellulose is hydrolyzed to monomeric sugars at low temperature (80-100^{o}C) and moderate acid concentration (10-30%); in the third step unhydrolyzed solids are separated from the liquid; in the fourth step make up acid is added to unhydrolyzed solids in such way that acid concentration becomes (10-90%); in the fifth step first three steps are repeated; in the six and final step liquid streams collected from third and fifth step are mixed, and heated at moderate acid concentration (10-30%) and low temperature (80-120^{o}C). Therefore, this work primarily focused on evaluating the technical feasibility of utilization of high substrate loadings, moderate acid concentrations, and low temperatures to produce LA in a single step. Different lignocellulosic substrates (Solka Floc, corn stover, dilute sulfuric acid pretreated corn stover, pine, and municipal solid waste) and polysaccharide starch were used to evaluate the effects of substrate characteristics such as crystallinity, presence of lignin/hemicelluloses/non-organic substances on LA production. In addition, to verify the difference in LA production between soluble sugars and lignocellulosic substrates, various soluble sugars including, glucose, fructose, sucrose, galactose, and mannose were studied.

Table 7.1: Yields and reaction conditions of literature reported LA produced from soluble sugars and lignocellulosic substrates using sulfuric acid

Lignocellulosic Substrates							
Feed Stock	**Cellulose Content (%)**	**g-H_2SO_4/g-glucan**	**Temperature (°C)**	**Reaction Time**	**Initial Solids (%)**	**LA Molar Yield (%)**	**Reference**
Water Hyacinth	26.3	38.02	175	30 min	1	53.0	Girisuta et al., 2007
Paper Sludge	57.1	3.99	200	60 min	7	37.2	Galletti et al., 2012
Tobacco Chops	25.0	4.74	200	60 min	7	20.7	Galletti et al., 2012
Wheat Straw	40.4	1.44	210	38 min	6	69.0	Chang et al., 2007
Paper	25.5	0.40	150	8 hr	30	59.8	Farone and Cuzens, 2000
Pulp Sludge	80.0	0.63	1st stage* 210-230 2nd stage 195-215	17 Seconds 15 min	5-10	>70	Hayes et al., 2006
Aspen Wood	45.7	1.10	200	2 hr	10	47.36	Efremov et al., 1997
Krasnoyarsk Pulp and Paper	99.6	0.50	250	2 hr	10	34.77	Efremov et al., 1997

Soluble Sugars						
	g-H_2SO_4/g-glucose	**Temperature (°C)**	**Reaction Time**	**Initial Solids (%)**	**LA Molar Yield (%)**	**Reference**
Glucose	5.0	140	2 hr	2	60.0	Girisuta et al., 2006
Sucrose	1.5	125	16 hr	6	88.0	Ghorpade and Hana, 1999
Fructose	0.5	140	-	2	74.0	Fachri et al., 2015

*In the first stage HMF is produced

7.3. Materials and methods

7.3.1. Materials

Glucose, fructose, sucrose, galactose, mannose, starch, H_2SO_4 (95-97%), and levulinic acid (98%) were purchased from Sigma-Aldrich, and used as received. Corn stover was pretreated using dilute sulfuric acid (1% H_2SO_4, 150°C, liquid/solid ratio of 9, and 30 min). Solka Floc was purchased from Fiber Sale & Development Corporation (Urbana, OH). Municipal solid waste was received in shredded form from MSW, Florida, and organic material was separated from non-organics as much as possible. Organics in MSW was used as is without any further treatment.

7.3.2. Levulinic acid production

All the experiments were carried out in the acid resistant Nickel-Copper reactors of 5 mL volume. Different soluble 6 carbon sugars (glucose, fructose, sucrose, galactose, and mannose) and lignocellulosic substrates (Corn stover, Solka Floc, sulfuric pretreated corn stover (PCS), pine, and municipal solid waste (MSW)) were studied. The reaction conditions used were: 7-20% of solids, 10-30% sulfuric acid, 80-140°C temperature, and 3-30 hr. Sugars were initially solubilized in the sulfuric acid solution for the experiments carried out using soluble sugars and the resultant solution was subsequently filled into the reactors. With lignocellulosic substrates, first acid and subsequently biomass was added to the reactor. Contents were thoroughly mixed using a glass rod, and allowed for soaking for 4 hours. Subsequently reactors were kept in a GC forced-air convection oven. For each sample point a different reactor was used. Reactors were periodically taken out, and kept under cooling water to allow the reaction mixture to reach room temperature. Subsequently, samples were centrifuged to separate the supernatant from the solids.

The supernatants were analyzed to quantify LA using HPLC, which is equipped with Bio-Rad Aminex HPX-87H ion exchange column (300 mm x 7.8 mm, Bio-Rad Laboratories Inc., Hercules, CA). The column temperature, mobile phase, flow rate were 65°C, 1 N H_2SO_4, and 0.6 mL/min, respectively. LA molar yield is calculated following equation:

$$\text{LA Yield (\%)} = \frac{\text{LA Concentration (g)} \times \text{Carbohydrate MW}}{\text{Initial Carbohydrate Concentration (g)} \times \text{LA MW}} \times 100$$

MW: Molecular weight

Note: Carbohydrate is representation of cellulose, glucose, fructose, sucrose, mannose, galactose, and starch

Table 7.2: Yields and reaction conditions of present study for soluble sugars and lignocellulosic substrates

Lignocellulosic						
Feed Stock	**Cellulose Content (%)**	**g-H_2SO_4/g-glucan**	**Temperature (^oC)**	**Reaction Time (hr)**	**Initial Total Solids (%)**	**LA Molar Yield (%)**
Solka Floc	86.0	1.67	120	15	12	64.2
Corn Stover■	36.1					61.1
PCS	60.5					63.7
Pine	40.1					60.4
MSW*	50.3					60.0
Soluble Sugars						
	g-H_2SO_4/g-glucose		**Temperature (^oC)**	**Reaction Time**	**Initial Total Solids**	**LA Molar Yield (%)**
Glucose	1.67		120	12	12	63.4
Sucrose						66.3
Fructose						70.5
Galactose						60.2
Mannose						62.5

7.4. Results and discussion

As described in Introduction majority of the LA production studies utilized either high amount of acid per gram substrate, and/or a low initial substrate loading, and/or a very high temperature, and/or a complex process. Therefore, we have decided to study the technical feasibility of LA production using high substrate loadings (10-20%), moderate acid concentrations (10-30%), and at low temperatures (80-140^{o}C) in a single step. Different lignocellulosic substrates (Solka Floc, corn stover, dilute sulfuric acid pretreated corn stover, pine, and municipal solid waste) and polysaccharide starch were used to evaluate the effects of substrate characteristics such as crystallinity, presence of non-cellulosic substances (lignin/hemicelluloses/non-organic) on LA production. In addition, to verify the difference in LA production between soluble sugars and lignocellulosic substrates, various soluble sugars including, glucose, fructose, sucrose, galactose, and mannose were studied.

7.4.1. Production of LA from glucose

LA production at low temperatures was first started using 12% glucose, 10-25% H_2SO_4 and 80^{o}C. After 30 hr of reaction time only 5% LA was yielded (Figure 7.1), which is not surprising because at 80^{o}C almost 90% of the glucose was left unconverted (Data not shown). Results indicate that 80^{o}C is not high enough temperature to produce high levels of LA.

Based on very low LA yields obtained at 80^{o}C, further experiments were carried by increasing the temperature to 110^{o}C using 15% and 20% acid concentrations. Increase in reaction temperature to 110^{o}C, increased the LA yields substantially (Figure 7.1 and Figure 7.2). A 9% increase in LA yield was achieved at 110^{o}C with the increase in acid from 15% to 20%. Both the concentrations increased LA yields at a faster rate up to 15 hr of reaction, after which the increase was leveled off, however the conversion of glucose still continued. Increase in glucose conversion without corresponding increase LA yields was possibly due to the formation of humins. After 15 hr of reaction, major portion of glucose was possibly converted into humins. Humin is a black, tarry by product results during the acid catalyzed conversion of glucose to hydroxyl methyl furfural (HMF), and HMF to LA and formic acid (FA).

Different mechanisms have been proposed for the formation of humin. According to Patil et al. (2012), humins are produced from glucose/fructose via HMF through 2, 5 dioxo-6-hydroxyhexanal by aldol addition/condensation. Roman and Winter (2004) reported formation of char through cellulose sulfate at high acid concentration and low temperature. According to them cellulose sulfate formation increases with increase in acid concentration, acid-to-cellulose ratio, and time.

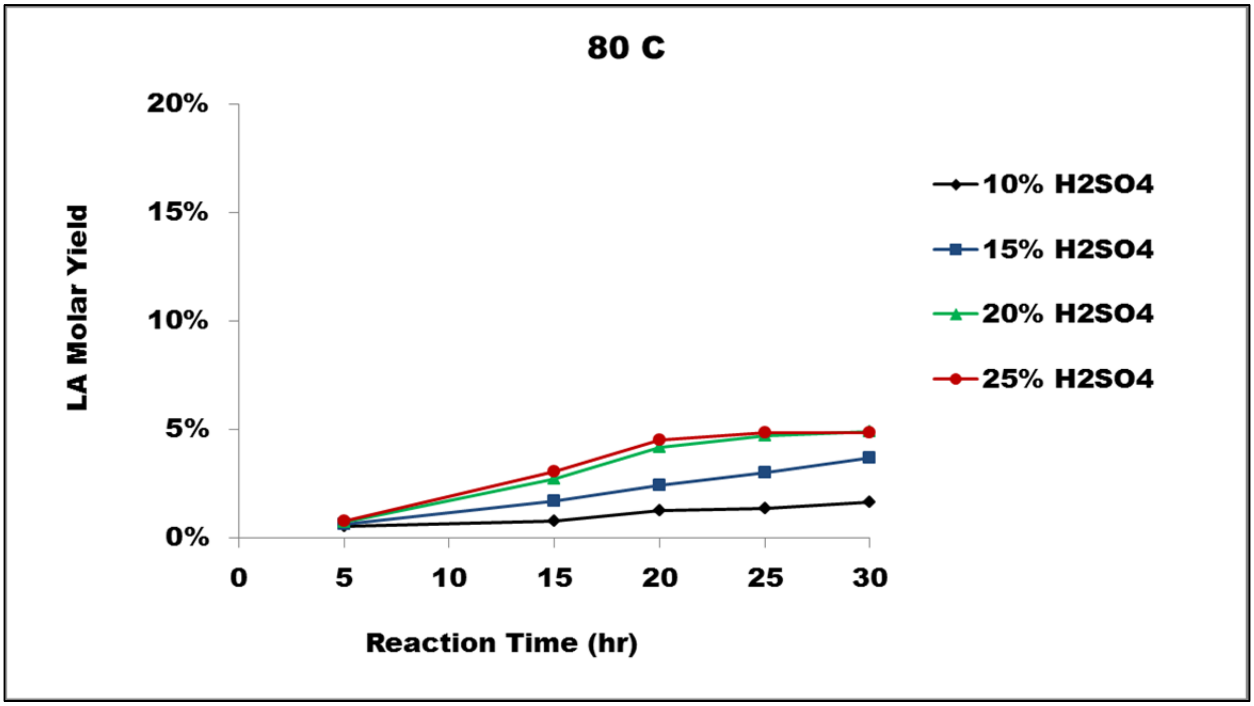

Figure 7.1: LA molar yields and glucose conversion with 12% solids, 10-25% H_2SO_4, and at 80^{o}C

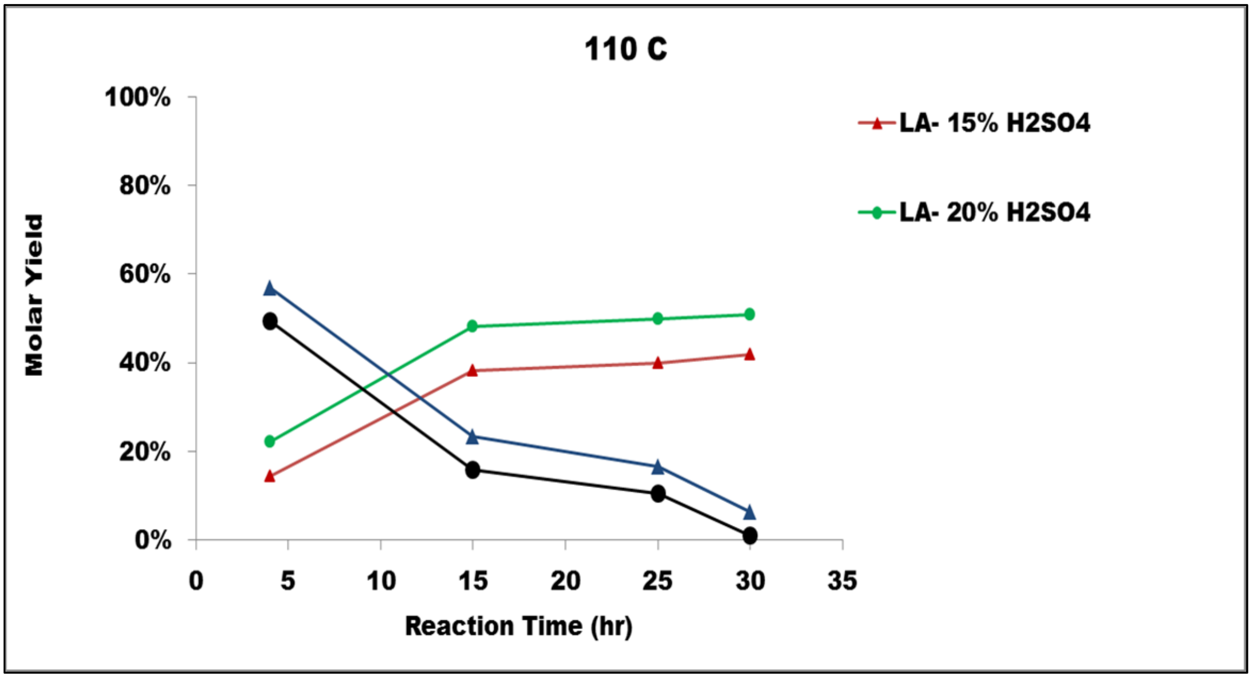

Figure 7.2: LA molar yields and glucose conversion with 12% solids, 15-20% H_2SO_4, and at 110°C

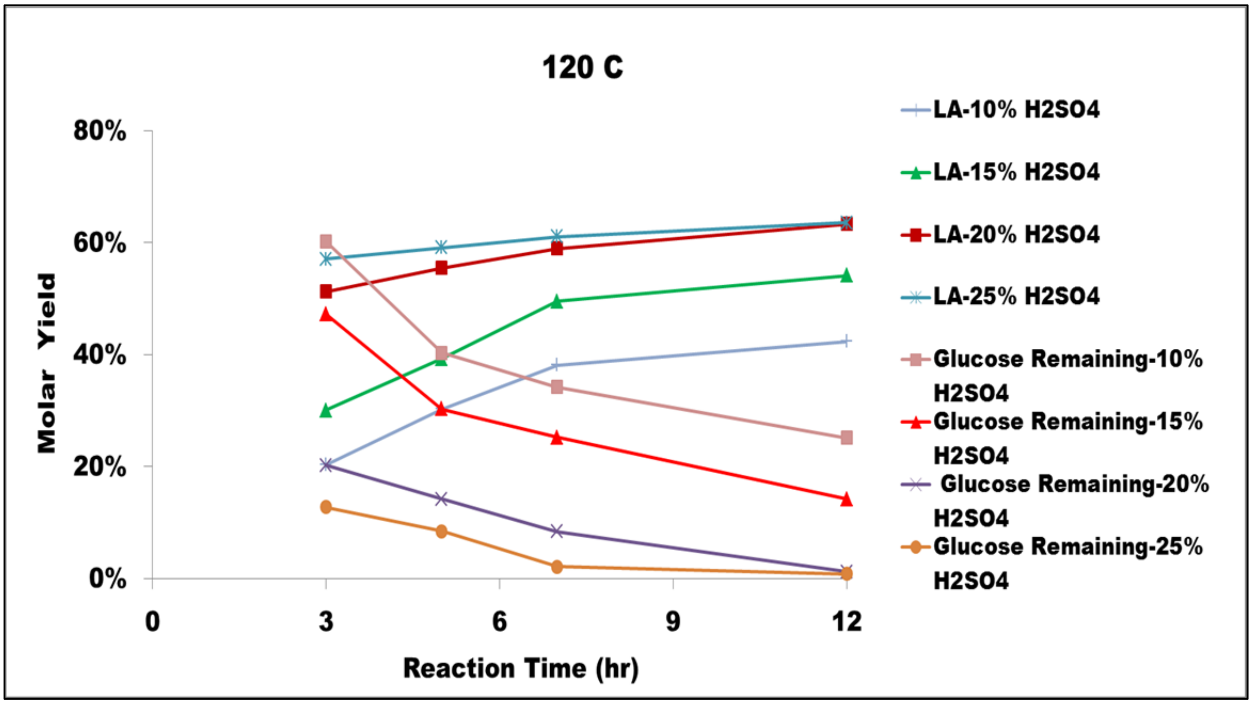

Figure 7.3: LA molar yields and glucose conversion with 12% solids, 10-25% H_2SO_4, and at 120°C

Having seen improvement in LA yields with increasing temperature, further experiments were continued by raising the temperature to 120^{o}C, with 10-25% acid, and 12% glucose. Increase in temperature from 110^{o}C to 120^{o}C, increased in LA yields substantially with 15% (41.7% to 54.1%) and 20% (50.9% to 63.4%) acid concentrations (Figure 7.2 and Figure 7.3). These yields are better than the literature reported yields that were obtained with dilute acid concentrations and at high temperatures (Table 7.1 and Table 7.2). Girisuta et al. (2006) achieved approximately 60% LA yield from 2% glucose with 10% H_2SO_4 and at 140^{o}C, whereas we were able to process 12% glucose and still achieve 63.4% yield. LA initial production rate with 25% acid was faster than 20% acid; however final yields obtained were almost the same (Figure 7.3).

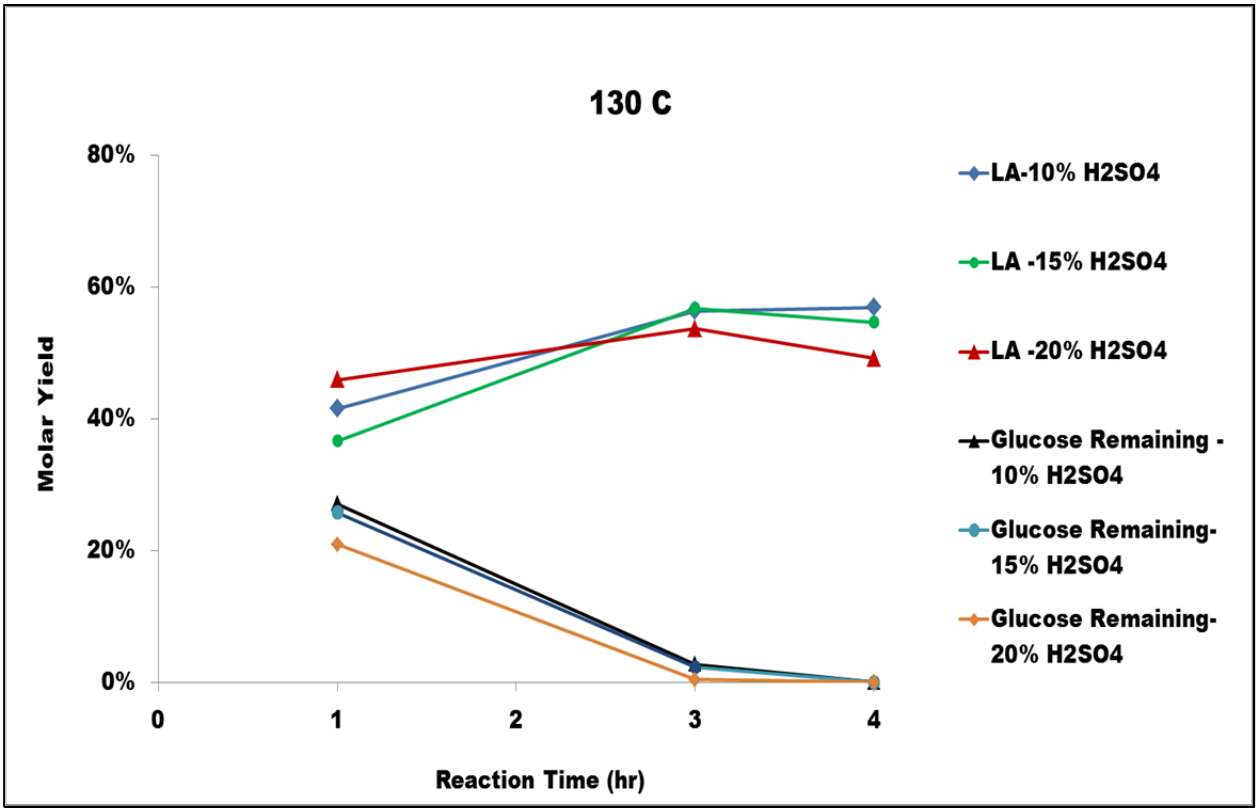

Figure 7.4: LA molar yields and glucose conversion with 12% solids, 15-25% H_2SO_4, and at 130^{o}C

When the reaction temperature increased from 120^{o}C to 130^{o}C, LA molar yield decreased with 20% H_2SO_4 (from 63.6% to 52.6%) and increased with 10% H_2SO_4 (from 42.4% to 56.9%) (Figure 7.3 and Figure 7.4), whereas with 15% H_2SO_4 similar amount of LA yield (approximately 54%) were obtained. However at 130^{o}C all the glucose was consumed, whereas at 120^{o}C still substantial glucose was left unconverted (14.2%). From these results, it can be inferred that the formation of humin is high after reaching certain temperature for a given acid concentration.

7.4.1.1. Effect of glucose concentration on LA production

In an effort to understand the effect of glucose concentration on LA yields, experiments were carried out by applying different glucose loadings (7%, 15%, and 20%), 20% H_2SO_4, and at 120^{o}C (Figure 7.5). The highest LA molar yields achieved with three different glucose concentrations were 64.5%, 57.6%, and 50.3%, respectively. At 7 hr reaction time, 7% glucose concentration yielded 64.5% LA; however when the reaction was continued further LA yield was reduced to 61.8%. This was possibly due to degradation of LA. The reason for degradation could be that the level of acid present per gram glucose was perhaps high. LA yields obtained with 7% and 15% glucose were not statistically very different; however, with increase in glucose concentration to 20%, LA yields were substantially reduced. This was possibly due to low level of acid present per gram of glucose.

To see the temperature effect on glucose concentration (7%, 15%, and 20%), reaction temperature was increased to 140^{o}C (Figure 7.6). The highest LA yields obtained with three different glucose concentrations at 140^{o}C were: 53.3%, 53.3%, and 49.1%, respectively. Results show that 20% glucose concentration at 140°C produced approximately 50% within 1 hr, whereas at 120°C it took 3 hr to produce similar LA yield.

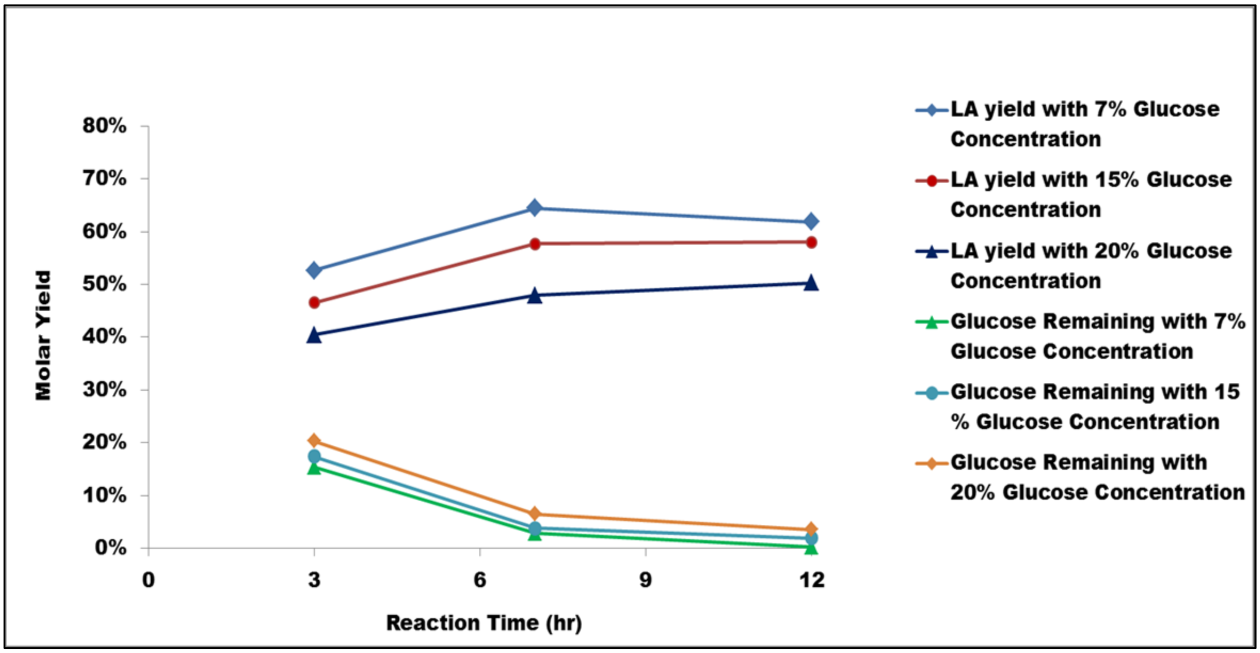

Figure 7.5: LA Molar yields with different glucose concentrations with 20% H_2SO_4 and at 120°C

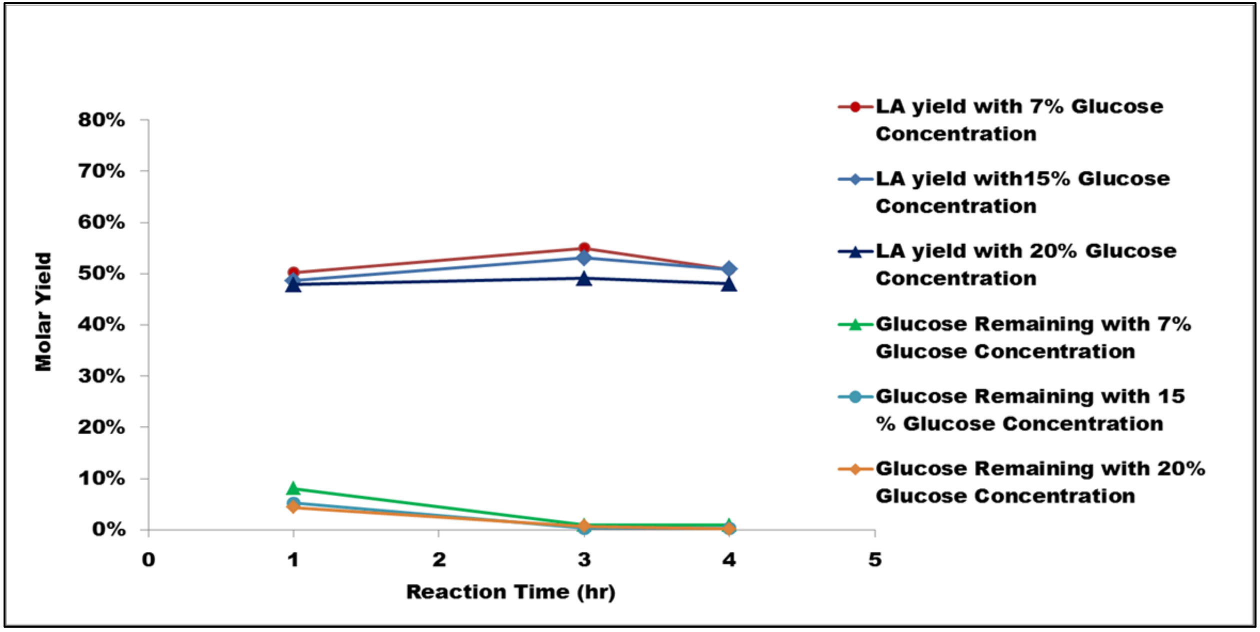

Figure 7.6: Molar yields of LA with different glucose concentrations with 20% H_2SO_4 and at 140°C

7.4.2. Production of LA from different soluble sugars

After seeing the positive effects of moderate acid concentrations and low temperatures on different glucose concentrations in producing LA, we have decided to evaluate the effect of similar conditions on other soluble sugars (Figure 7.7). All the soluble sugars yielded 60% or more LA at 120°C, 20% H_2SO_4, and 12% solids. Within 3 hr of reaction time as high as 50% LA yield, and in 7 hr reaction time 60% or more LA yield was obtained with all the sugars. The highest LA molar yield (70.5%) was achieved with fructose. At end of 3 hr period fructose produced 70% LA yield. Fructose produced relatively high LA yield than other soluble sugars during entire course of reaction. Sucrose resulted in lower yields than fructose but more than glucose, which is understandable as sucrose is the disaccharide of fructose and glucose.

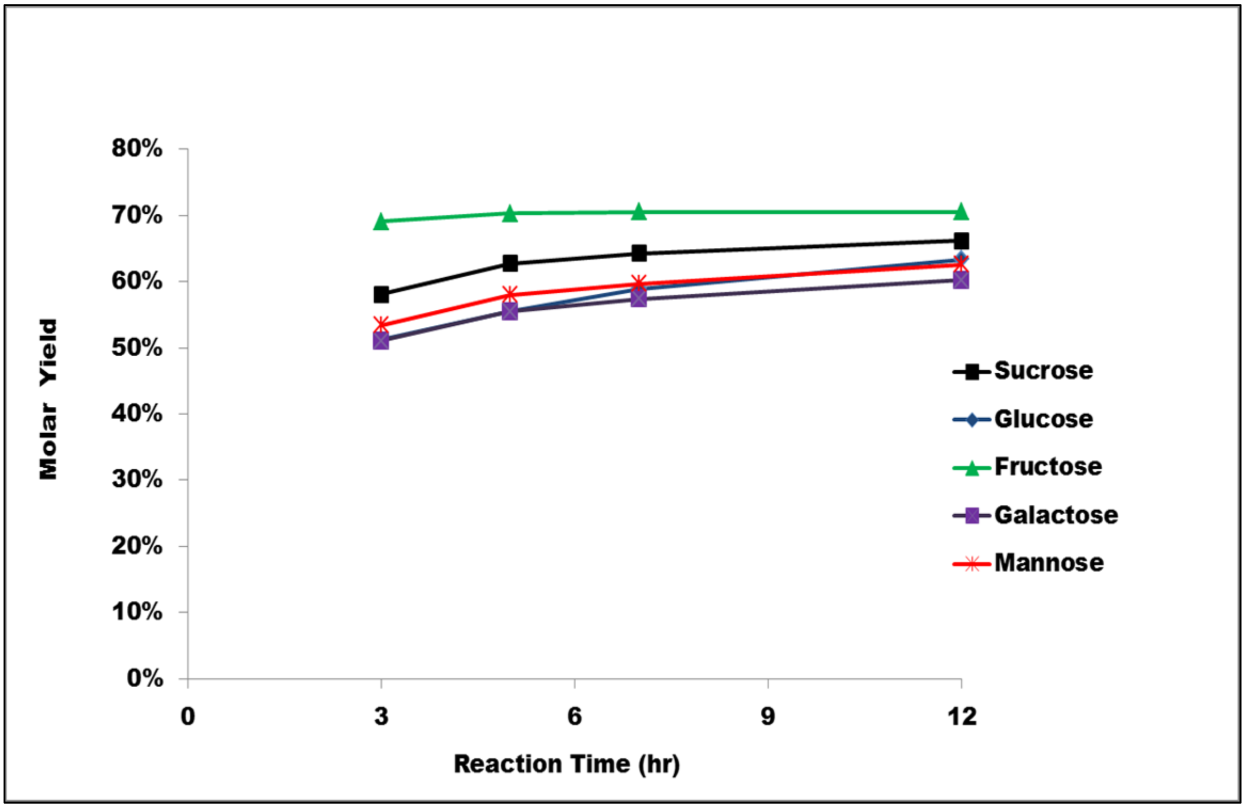

Figure 7.7: LA molar yields obtained from different soluble sugars with 20% H_2SO_4, 12% solids, and at 120°C

7.4.3. Production of LA from lignocellulosic substrates

After evaluating the effects of moderate acid concentrations and low temperatures on LA production from soluble sugars, experiments were carried out with lignocellulosic substrates. As 120°C proved as optimum temperature for production of LA from soluble sugars, we have decided to start experiments with lignocellulosic substrates at the same temperature. The reaction condition used was: 120°C, 20% H_2SO_4, and 12% initial solids. Different lignocellulosic substrates used were: Solka Floc, corn stover, acid pretreated corn stover, pine, and municipal solid waste. Cellulose contents of different lignocellulosics are shown in Table 2. LA molar yields indicate that all the lignocellulosic substrates irrespective of their characters produced 60% or more LA yields with 120°C, 20% H_2SO_4, and 12% initial solids (Figure 7.8). Yields obtained from lignocellulosics are comparable or better than the literature reported yields on the basis of g H_2SO_4/g-substrate (Table 7.1 and Table 7.2). Yields are comparable because in one study Chang et al. (2007) applied 1.44 g H_2SO_4/g-wheat straw at 250°C, and obtained 69% molar yield. In the present study, we were able to obtain as high as 64% molar yield with 1.66 g H_2SO_4/g-Solka Floc and 120°C. However, Chang et al. (2007) were able to process only 6% solids, whereas we could process as high as 12% solids (Table 7.1 and Table 7.2). All the substrates behaved similarly in producing LA acid; and the only exception was Solka Floc initial reaction rate. Solka Floc initial LA production rate is faster than the other lignocellulosic substrates, however, not a difference in LA yield could be observed at the end of the reaction (Figure 7.8). The initial difference in reaction rates was possibly due to the absence of lignin in Solka Floc; therefore cellulose was exposed to acid quickly.

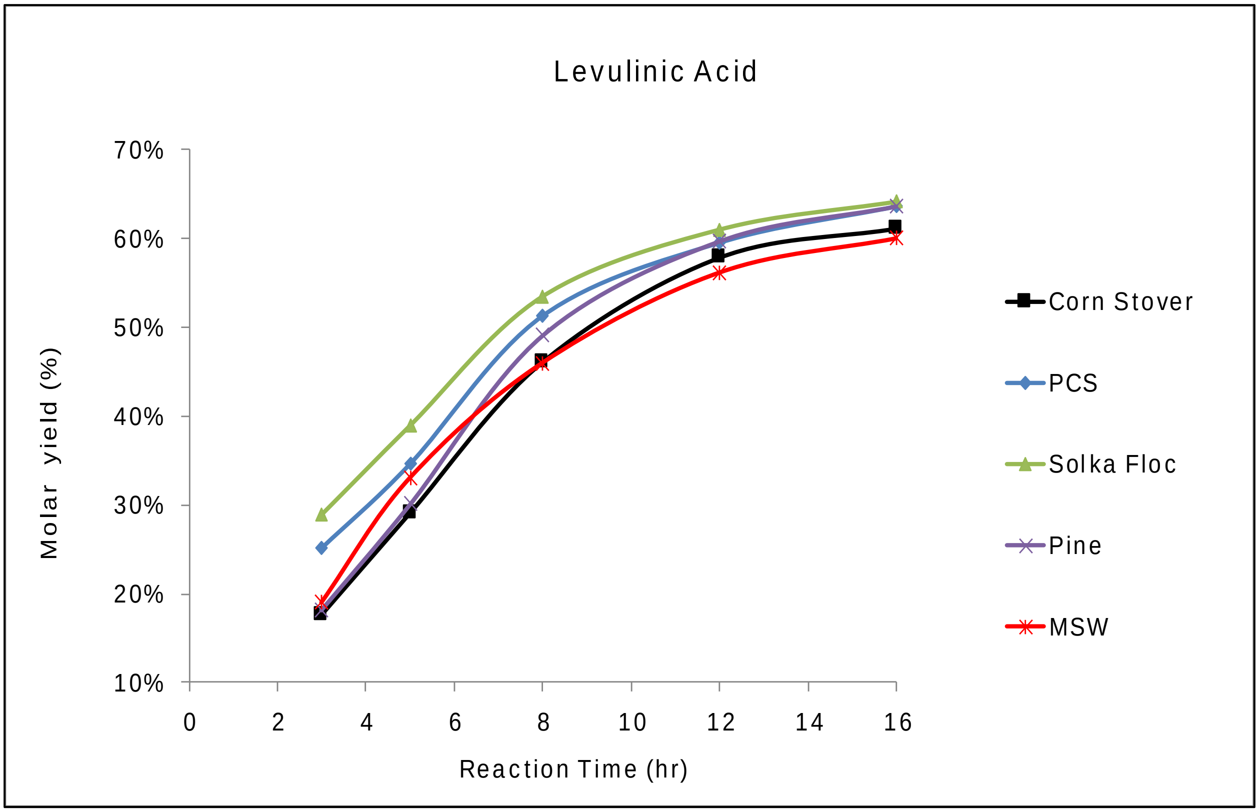

Figure 7.8: LA molar yields obtained from lignocellulosic substrates with 20% H_2SO_4, 12% solids, and at 120°C

Release of high concentrations of monomeric sugars from the softwoods such as pine has been identified as a bottleneck for biorefineries. The difficulty for accessing the cellulose by cellulase has been attributed to high lignin content and crystallinity of cellulose even after severe pretreatment (Söderström et al., 2003; Bansal et al., 2010). On the other hand, municipal solid waste contains very different materials such as metals, glass, textiles, minerals, inerts, and plastics, which adversely affect the enzymes during the enzyme hydrolysis (Jensen et al., 2011). However, no such factors can be hindrance if pine and MSW are used for production of LA. Our present study showed that both pine and MSW can be potential candidates to produce LA. Solka Floc is a lignin free sample and whereas pine is lignin containing sample, however, both the substrates produced similar LA yields. Our current study also indicates that no pretreatment is required to produce high levels of levulinic acid. CS is untreated corn stover; whereas PCS is a dilute sulfuric acid pretreated corn stover that has lost more than 90% of its xylan content during pretreatment. However, both the substrates produced similar levels of levulinic acid.

Data show that glucose and lignocellulosics produced similar levels of levulinic acid, however, one significant difference between glucose and lignocellulosics is initial LA production rate. LA initial production rate of lignocellulosic substrates is comparatively slower than the soluble sugars. For example, it took 8 hr for PCS to produce 50% LA yield with 120^oC, 20% H_2SO_4, and 12% initial solids, whereas for glucose it took less than 3 hr to produce similar levels of LA using same reaction parameters. The reason for slow initial production rates with lignocellulosics is that unavailability of glucose readily; different stages are involved to convert the cellulose in lignocellulosic substrates to glucose. In the first stage, acid reaches the cellulose penetrating through lignin (with lignin containing substrates); in the second stage cellulose decrystallization occurs; in the third stage decrystallized cellulase is converted to oligomers, and in fourth stage oligomers are converted to glucose. Therefore, it takes certain amount of time to generate glucose.

7.4.3.1. Effect of acid concentration and temperature on LA production from pine

Pine was chosen as a representative feedstock to understand the effect of temperature and acid concentration on LA yields from lignocellulosic substrates. Experiments were carried out at 120^oC, 130^oC and 140^oC by applying 10-25% H_2SO_4 and 12% pine. Decrease in acid concentration from 20% to 10%, decreased the LA yield substantially (by 30%) at 120^oC, however 17% of glucose still left unconverted (Figure 7.9). With 20% acid concentration, increase in temperature form 120^oC to 130^oC decreased LA yield of pine from 63.7% to 55.5%

(Figure 7.10). This was possibly due to degradation of LA at higher temperature. The reason for degradation could be that the level of acid present per gram pine was high at 130°C. At 130°C final yields obtained with 15% and 20% acids were almost same. With 10% acid and at 120°C, pine only yielded 35.5% LA. Increase in temperature from 120°C to 130°C, has not increased LA yield to a significant level (by 3%), however a further increase in temperature to 140°C further increased the LA yield by 7% (Figure 7.10 and Figure 7.11). However, at 140°C significant levels of LA yield was reduced with 15% and 20% acids. Data indicates that 120°C is optimum temperature for 20% acid concentration.

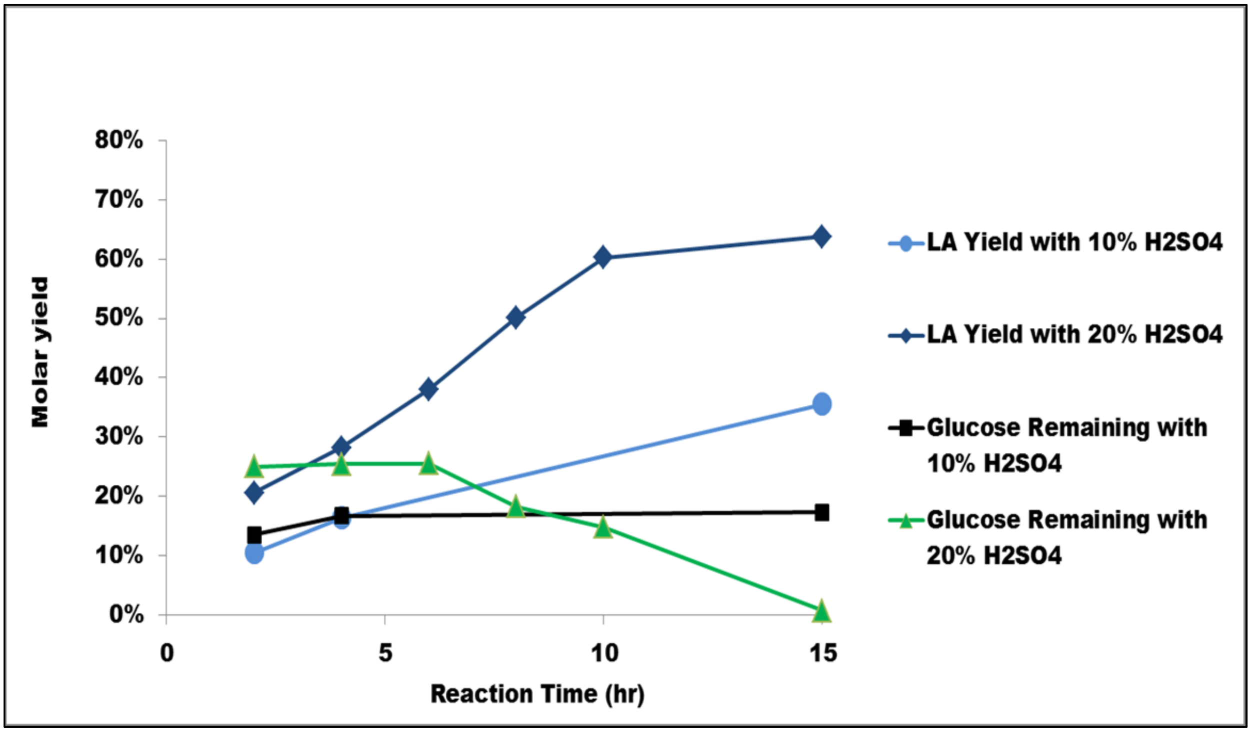

Figure 7.9: LA molar yields obtained from pine with different acid concentrations with 12% solids and at 120°C; Note: Unconverted cellulose is not included in glucose remaining

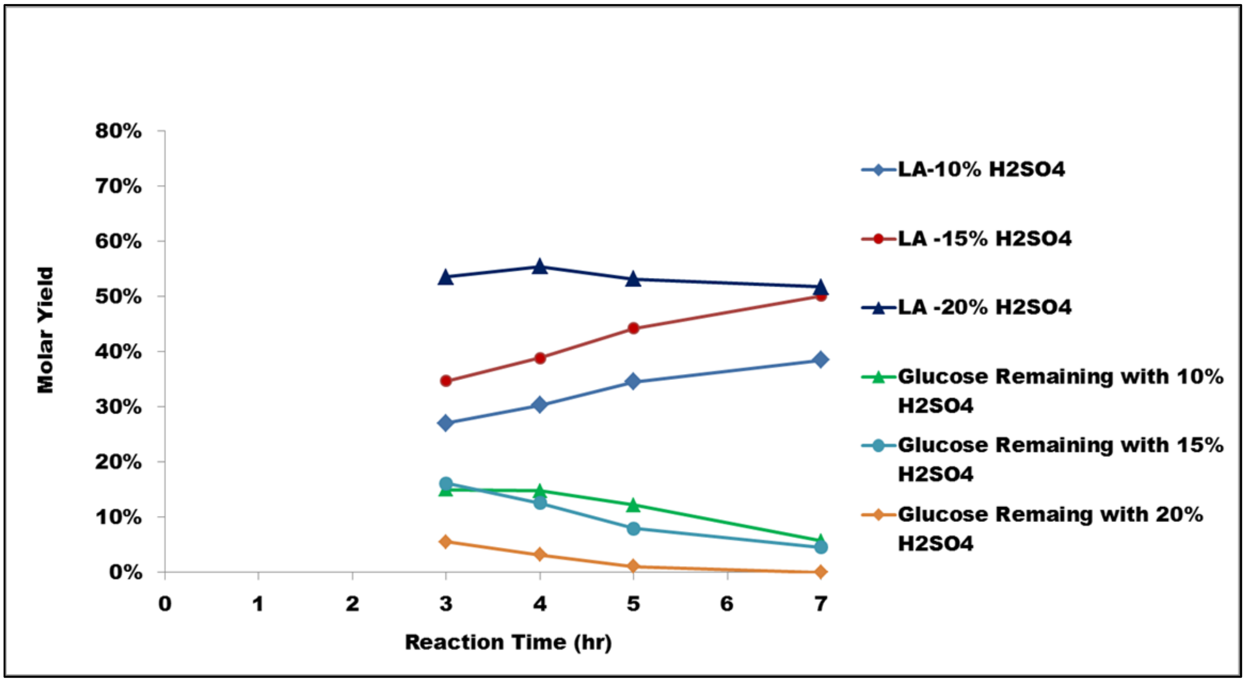

Figure 7.10: LA molar yield obtained from pine with different acid concentration with 12% solids and at 130°C; Note: Unconverted cellulose is not included in glucose remaining

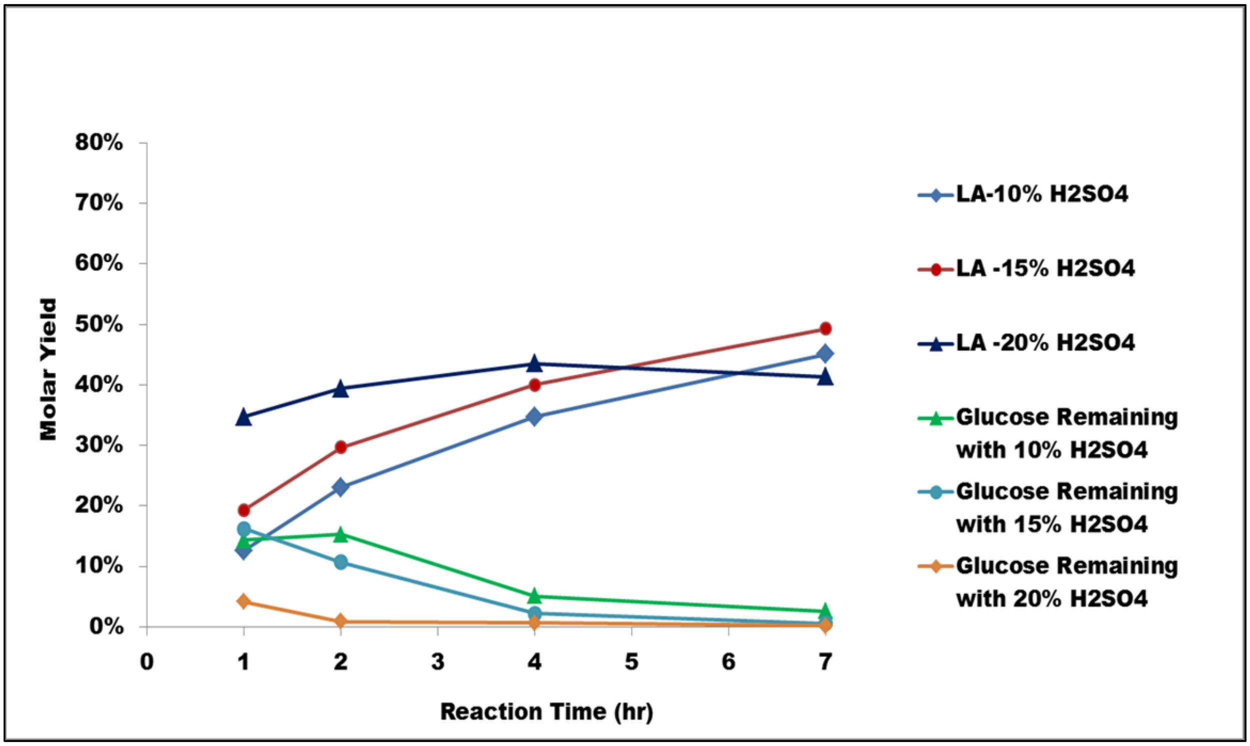

Figure 7.11: LA molar yield obtained from pine with different acid concentration with 12% solids and at 140°C; Note: Unconverted cellulose is not included in glucose remaining

7.4.3.2. Effect of pine concentration on LA production

In an effort to understand the effect of pine concentration on LA yields, experiments were carried out by applying different loadings (7%, 15%, and 20%) at 120^{o}C and 20% H_2SO_4. Results indicate that the LA molar yields reduced with increasing pine concentration; however the reduced yields are still comparable or higher than the literature reported yields that were obtained at fairly low solid concentration (Figure 7.12, Table 7.1 and Table 7.2). Girisuta et al. (2007) reported 53% molar yield with 1% water hyacinth, 10% acid concentration, and 200^{o}C. Galletti et al. (2012) reported 37% molar yield with 7% paper sludge, 12% acid concentration, and 200^{o}C. But in the present study we were able to process as high as 20% solids and achieved as high as 50.3% LA yield. The highest LA molar yields achieved with three different pine concentrations were 62.8%, 57.1%, and 50.3%, respectively.

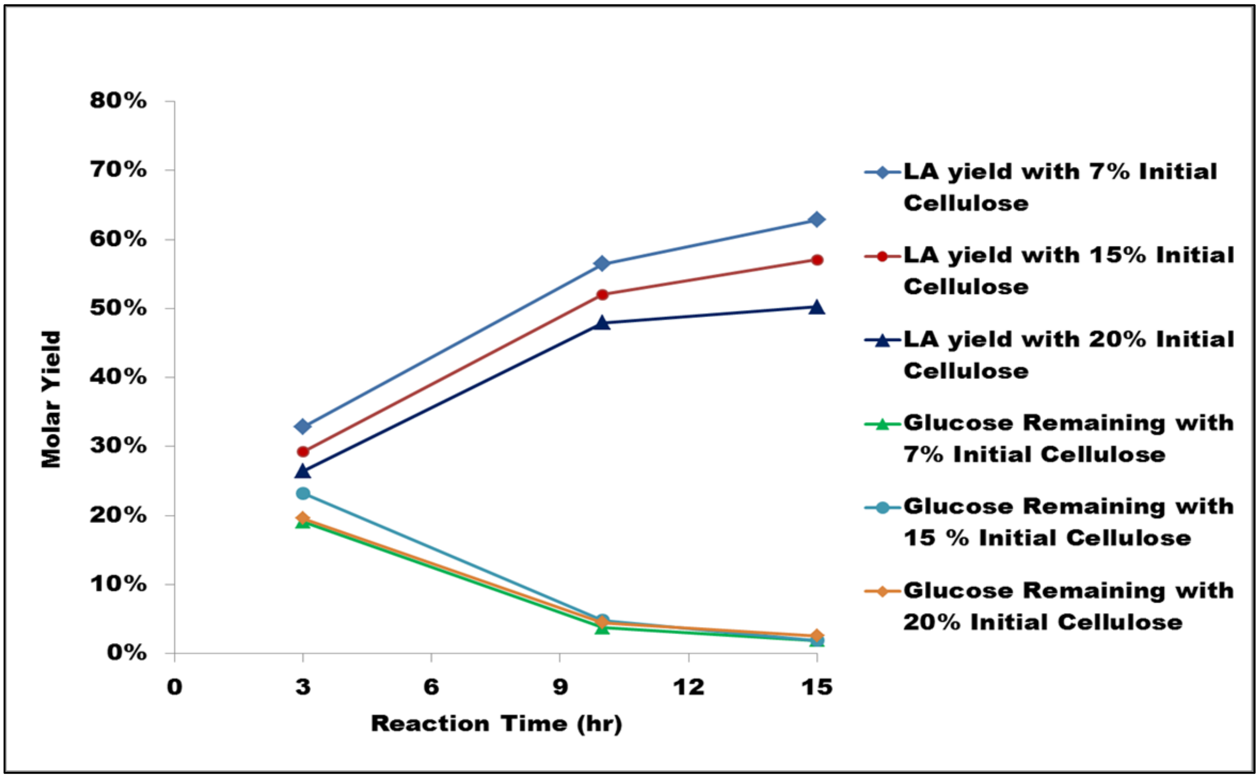

Figure 7.12: LA Molar yields with different pine levels with 20% H_2SO_4 and at 120^{o}C

To see the temperature effect on pine concentration (7%, 15%, and 20%), reaction temperature was further increased to 140°C (Figure 7.13). The highest LA molar yields obtained with three different pine concentrations at 140°C were: 53.7%, 41.5%, and 40.1%, respectively.

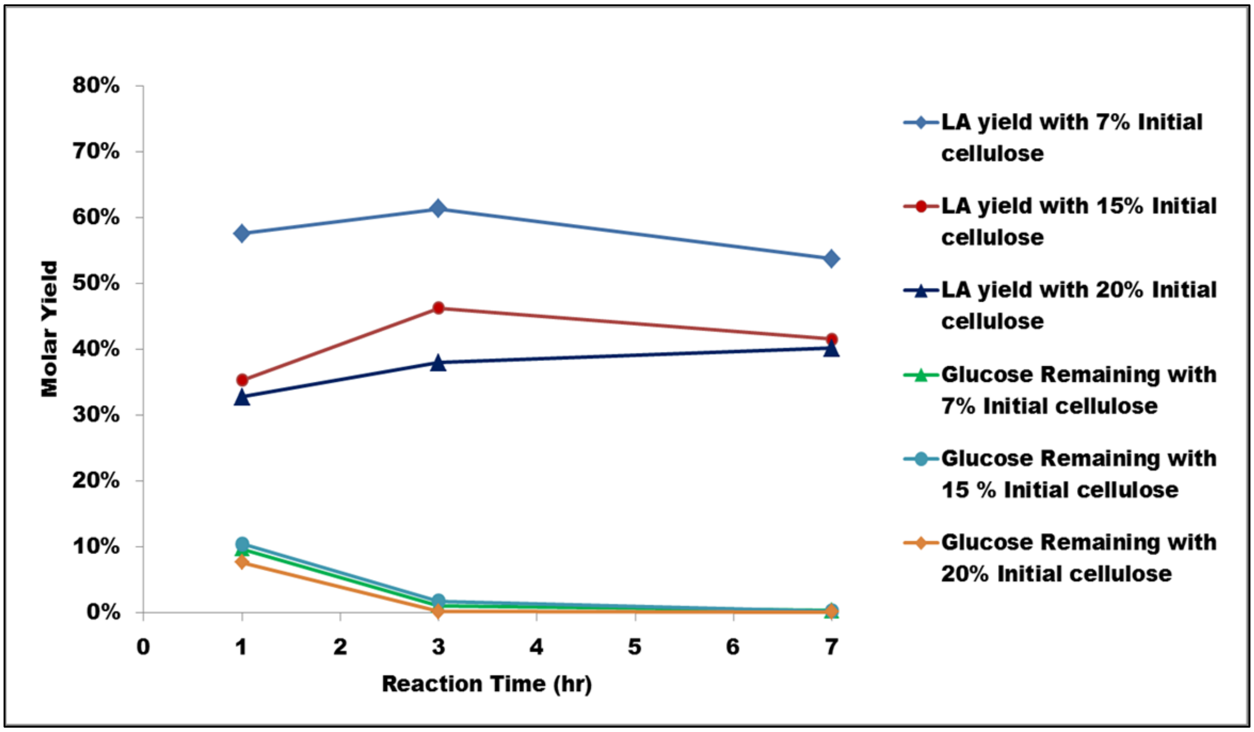

Figure 7.13: LA Molar yields with different pine levels with 20% H_2SO_4 and at 140°C

7.4.4. LA production from starch

After evaluating the effects of moderate acid concentrations and low temperatures on LA production from soluble sugars and lignocellulosics, studies were carried out with starch. Both cellulose and starch are polysaccharides made from glucose. Glucose units in cellulose are linked by 1,4-β-glycodic bonds, whereas glucose units in starch are linked by 1,4-α-glycodic bonds. As 120°C proved as optimum temperature for production of LA lignocellulosics, we have applied the same temperature. The reaction condition used was: 120°C, 20% H_2SO_4, and 12% starch. The highest LA molar yield achieved with starch was 68% (Figure 7.14)

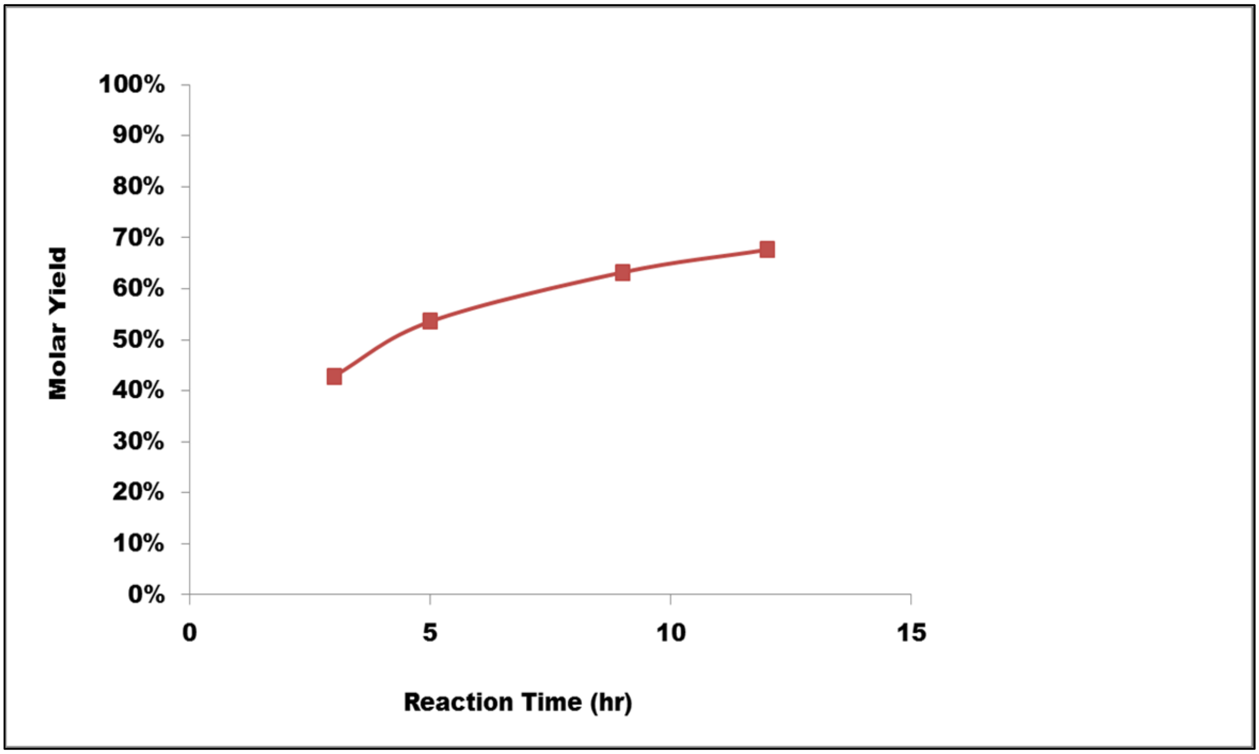

Figure 7.14: LA molar yield from starch with 20% H_2SO_4, 12% solids and at 120°C

7.5. Conclusions

Study shows that it is technically possible to obtain high LA yields with moderate acid concentrations and low temperatures from soluble sugars, lignocellulosics, and starch. Soluble sugars, lignocellulosics, and starch produced 60% or more LA molar yields with 120°C, 20% H_2SO_4, and 12% initial solids. Molar yields obtained from different substrates are comparable or better than the literature reported yields on the basis of g-H_2SO_4/g-substrate. The primary difference between lignocellulosic substrates and soluble sugars is initial LA production rates. Initial LA production rates of lignocellulosic substrates and starch are slower than the soluble sugars. For example, it took 8 hr for pine to produce 50% LA yield with 120°C, 20% H_2SO_4, and 12% initial solids, whereas for soluble sugars it took less than 3 hr to produce similar levels of LA using same reaction parameters. The optimum reaction conditions for the all the substrates were: 120°C, 20% H_2SO_4, 12% initial solids. Among the monomeric sugars, fructose produced highest LA molar yield (70.5%) and among the lignocellulosic substrates it was with Solka Floc (64.5%), whereas starch yielded 68% LA.

8. Fundamental Study on Decrystallization of Cellulose

8.1. Abstract

This study primarily focused on the experimental verification on detecting decrystallization of cellulose (breakage of hydrogen bonding) by tracing the temperature in a system where pure crystalline cellulose is treated with sulfuric acid. The experimental system utilizes two identical reactors operated simultaneously. It was designed to measure the extent of decrystallization by instantaneous detection of temperature change recognizing that decrystallization is an exothermic process. One reactor contains sulfuric acid-substrate, and another one contains deionized water-substrate (acting as a reference cell). Different substrates were used including, pure cellulose (Avicel PH 101), sulfuric acid treated pure cellulose (decrystallized), cellobiose, and glucose, to differentiate the extent of energy release by two different types of bonds: chemical bonds (hydrolysis) that apply for cellobiose and decrystallized cellulose, and the hydrogen bonds (decrystallization) that apply for Avicel. Experiments with high sulfuric acid concentrations (50-72%) were carried out at room temperature, whereas for the experiments with moderate acid concentrations (30-50%) were carried out at 90^{o}C. Results show that decrystallization occurs with sulfuric acid concentrations up to 55-72% at room temperature. In addition, data also indicate that decrystallization occurs at acid concentrations below 55%, if proper temperature is chosen. Crystallinity indices and initial hydrolysis rates confirm the occurrence of decrystallization at low acid concentrations.

8.2. Introduction

The main resistance of acid hydrolysis of cellulose exists in the rigid crystalline structure which is held by tightly arranged hydrogen bonds. The acid hydrolysis process to produce sugars from lignocellulosic biomass has a long history (Freudenberg, 1930; Seaman, 1945). It has been attempted under two drastically different conditions: dilute acid-high temperature and concentrated acid-low temperature conditions. The cellulose hydrolysis under the high concentrated acid-low temperature was verified by Xiang et al. (2003). They showed that α-cellulose when treated with a varying level of H_2SO_4, an abrupt change in physical structure (fibrous form to gelatinous form) took place at about 65% H_2SO_4, abrupt in the sense that small change of input (acid concentration from 60% to 65%) caused a drastic change of output. In dilute-acid hydrolysis, whether decrystallization occurs, if so, how it affects the hydrolysis reaction is unclear. The prevailing notion is as follows. Under low temperature and acid condition, the cellulose structure stays in stable crystalline form. Therefore, the reaction mode is endwise hydrolysis. Glucose then becomes the main sugar product, and its yield becomes low. When temperature and/or acid concentration is raised to a certain level, the cellulose structure becomes unstable by cleavage of hydrogen bonding (decrystallization). Once the crystalline structure of the cellulose is disrupted, acid molecules can penetrate into the inner layers of the cellulose chains resulting rapid hydrolysis and high yield of glucose. Decrystallization is an exothermic process because the hydrogen bonds are broken and the bond energy is released. Therefore, the temperature is expected to increase if there is decrystallization. Although some researcher reported cellulose decrystallization followed by solubilization at different acid concentrations and temperatures (Camacho et al. 1996; Ioelovich and Larina, 1999; Ioelovich, 2012), to the best of our knowledge nobody has so far reported cellulose decrystallization by heat releasing pattern and relating that with crystallinity. Therefore, this work focused on detection of sulfuric acid induced decrystallization by instantaneous tracing of the temperature change caused by breakage of hydrogen bonding.

8.3. Materials and methods

8.3.1. Materials

Microcrystalline cellulose (Avicel PH 101, Sigma Aldrich) was used as reference cellulose substrate. Sulfuric was purchased from VWR. D-cellobiose and D-glucose were purchased from Sigma Aldrich. Teflon coated J-type thermocouples were purchased from Fisher Scientific, used for temperature tracing in the reactors. Inconel tubing (Nickel-Copper, McMaster-Carr) capped with Swagelok fittings were used as acid-resistant reactor. 40 mL screw-cap glass bottles were purchased from the Auburn University chemical store. KitchenAid mixer was purchased from local Walmart.

8.3.2. Acid treated cellulose preparation

Acid treated cellulose was prepared by treating Avicel PH 101 with 72% sulfuric acid at room temperature (23^{o}C). First, Avicel (20 g) was added to 72% sulfuric acid (40 mL) in a 2.5 L glass bowl at room temperature. Then, acid and Avicel was thoroughly mixed with a mixer for 5 min and 30 min. Subsequently, acid treated sample was immediately washed with cold water till the acid is completely removed and filtered using a filter paper. The washed sample was dried overnight in an oven at 45^{o}C. Acid treated cellulose can be called interchangeably as noncrystalline cellulose (NCC) or low-crystalline cellulose (LCC) or amorphous cellulose. For XRD and enzymatic hydrolysis experiments samples were freeze dried instead of oven drying.

8.3.3. Experimental setup for temperature scanning

Two separate reactors were used simultaneously to detect the instantaneous temperature change. One reactor contains sulfuric acid-substrate and another one contains DI water-substrate (a reference cell). Different substrates were treated with acid including, pure cellulose, acid treated cellulose, cellobiose, and glucose. A 40 mL screw-capped glass bottle was used for the experiments carried out at room temperature and high sulfuric acid concentrations (72-50%). For the experiments carried out at 90^{o}C and low to moderate acid concentrations (20-50%) an acid-resistant Nickel-Copper reactor was used. The reactor has dimensions of (1/4" OD, 0.18" ID, 4" L), and Swagelok capped at both ends. Top caps of both the reactors were modified to fit the thermocouples. Caps using Teflon materials were modified such way that when reactors are closed, they appear to be air tight. Inner side of the bottom caps was modified using Teflon to prevent metal corrosion due to the concentrated acid. For the reactions carried out at room temperature, in two separate 40 mL glass bottles, 9 mL of sulfuric acid (72-50%) and 9 mL of DI were added respectively. First, 3 g of substrate on dry basis was added to the glass bottle containing DI water. The water-substrate mixture was mixed using a glass rod. Then, 3 g of substrate on dry basis was added to the glass bottle containing concentrated acid. The acid-substrate mixture was mixed quickly using a glass rod. Two Teflon coated J-type thermocouples were placed immediately into the both bottles containing water-substrate mixture and acid-substrate mixture, respectively. The temperature in both reactors was measured every second using temperature Data Acquisition System. The reaction was carried out to a maximum of 5 min. The solid/liquid ratio in the reactor was kept at 1/3 to maintain uniformly wet condition. For the reactions carried out at 90^{o}C, first 4.5 mL of concentrated acid (30-50%) and 4.5 mL of DI water were added in two separate acid-resistant Nickel-Copper reactors. Then, 1.5 g of substrate on dry basis was added to the both the reactors and mixed using a glass rod. Subsequently, both the reactors were kept in a GC forced-air convection oven and the temperature was set at 90^oC. The temperature in the both reactors was measured every second using Data Acquisition System. The reaction was carried out to a maximum of 10 min.

8.3.4. Crystallinity index

Crystallinity of treated and untreated biomass was measured using an X-ray Diffractometer (Rigaku Miniflex). Cu-Kα radiation was generated at 30 kV and 15 mA. Samples were scanned from $2\theta = 15^o$ to 30^o with a step size of 0.01, scan rate 1/min. The cellulose crystallinity index (CI) was determined by $(I_{22.5} – I_{18})/ I_{22.5}$.

8.3.5. Enzymatic hydrolysis

Enzymatic hydrolysis yields were determined using NREL standard protocol (Brown and Torget, 2005). Hydrolysis experiments were carried out using 125 mL Erlenmeyer flasks. Novozymes Celic C-Tec 2 was used as a cellulase enzyme. It has a total specific activity of 119 FPU/mL. Two different cellulase loadings, 1 FPU/g-glucan and 3 FPU/g-glucan of C-Tec 2 were used to determine the initial hydrolysis rate of substrates generated by treating pure cellulose with different levels of sulfuric acid. Substrate loading applied was based on 2% (w/v). Hydrolysis experiments were carried out at 50°C, pH 4.8 (0.05M sodium citrate buffer), 150 RPM in a Shaker/Incubator (NBS, Innova-4080. Hydrolysate samples were taken periodically and analyzed for glucose in an HPLC equipped with Biorad-HPX-87P column and RI detector.

8.4. Results and discussion

8.4.1. Effect of acid concentration on decrystallization of cellulose at room temperature

Inter-chain and intra-chain hydrogen bonds provide stability to cellulose in the form of β-glycosidic linkages. At high acid concentrations molecules of sulfuric acid are capable to break the hydrogen bonds and penetrate into crystalline domains of the polymer that result in decrystallized cellulose (Camacho et al., 1996; Lee and Harraz, 2005; Ioelovich, 2012). To understand the fundamental aspects of cellulose decrystallization such as breakage of hydrogen bond during acid hydrolysis and resultant heat release, experiments were initially carried out by applying different sulfuric acid concentrations (50-72%) at room temperature. The release of heat due to breakage of inter-chain and intra-chain hydrogen bonds in cellulose was detected by scanning of temperature of acid-Avicel reaction mixture instantaneously. As mentioned in the Experimental Section, simultaneously a water-Avicel test was also carried out to take the relative temperature difference in order to negate the lag in temperature detection by the Teflon coated thermocouple.

Data indicate that the heat release due to decrystallization and/or other probable reactions at room temperature is sufficiently high up to 55% sulfuric acid (Figure 8.1). A sharp rise in temperature difference could be observed within initial few seconds (15-25 seconds) for all the acid concentrations except with 50% sulfuric acid. However, after the initial few seconds increase in temperature was gradually reduced with all the acid concentrations. The sharp rise in temperature could be attributed to sudden release of heat due to breakage of hydrogen bonds in crystalline cellulose. Earlier we hypothesized that the decrystallization of cellulose behaves as a phase-change in that it occurs at a precise point in temperature (critical temperature) for a given acid concentration. However, our current data indicates that the occurrence of decrystallization at infinitely many points instead of a precise point. Shen and Gnanakaran (2009) calculated the stability of cellulose hydrogen bond networks using statistical mechanics. As per their calculations plasticity of hydrogen bonding network in cellulose keeps the overall hydrogen bonding stability persistent over a wide range of temperatures. According to them instead of only one stable pattern, different hydrogen bonding patterns dominate at different temperatures until the disassembly occurs at very high temperatures. They also showed that intra-chain hydrogen bonding is more stable than the inter-chain bonding over a wide range of temperatures.

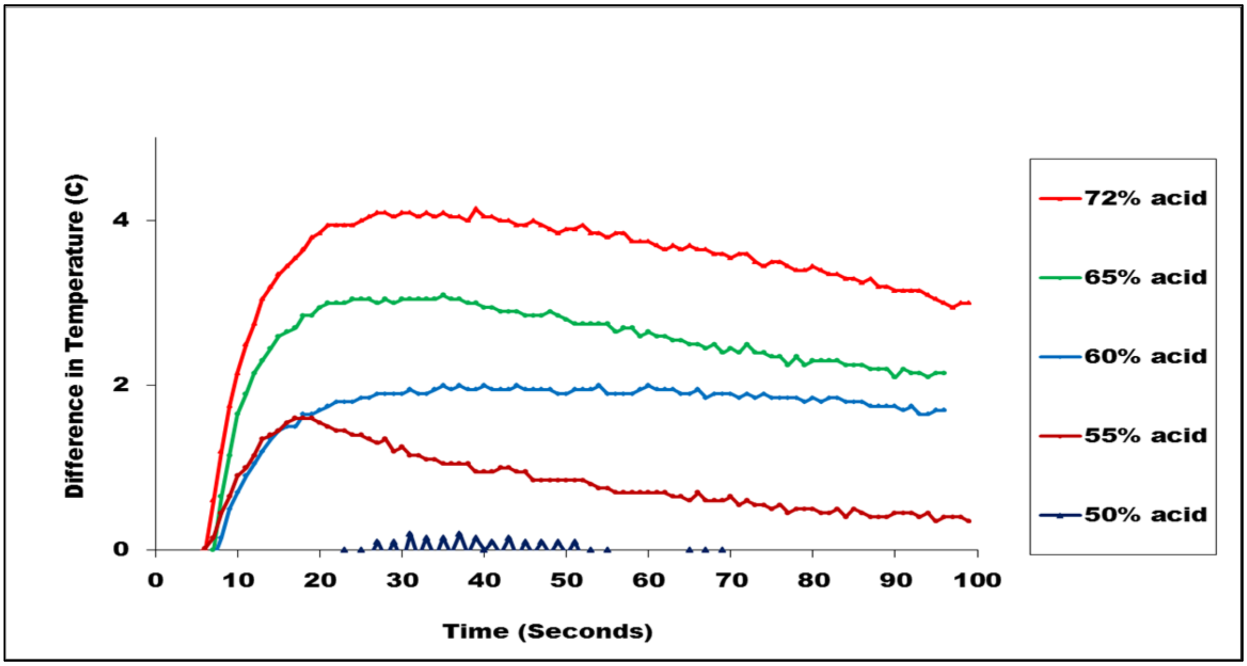

Figure 8.1: Difference in temperature between Sulfuric Acid-Avicel and Water-Avicel at room temperature

The heat release with 72% and 65% acids was much higher than with 60% and 55% acids. According to Camacho et al. (1996), cellulose decrystallization followed by solubilization is effective up to 62% or more acid. They also reported solubilization of cellulose with less than 62% acid, but it was not in significant level. According to their study any concentration less than 62% H_2SO_4 fundamentally serves as a swelling agent. Figure 8.1 also shows no change in temperature difference with 50%. This suggests that no decrystallization occurs with 50% acid at room temperature. Although with high acid concentrations heat release could be observed, but it is not yet clear whether all the released heat was solely due to decrystallization, or decrystallization and formation of cellulose derivatives. To identify that further experiments were carried out.

8.4.2. Treatment of Avicel, acid treated Avicel, cellobiose, and glucose with 72% sulfuric acid

The release of heat during concentrated sulfuric acid treatment of different substrates was studied. The reason for studying different substrates was to differentiate the release of heat from decrystallization and hydrolysis of decrystallized cellulose to low DP cellulose and/or glucose. The different substrates used were: Avicel, acid treated Avicel for 5 and 30 min using 72% sulfuric acid, cellobiose, and glucose. Data indicate that with the addition of 72% acid, untreated Avicel released more heat than acid treated samples, cellobiose, and glucose (Figure 8.2). This is possibly due to presence of more hydrogen bonds in untreated Avicel than acid treated Avicel and cellobiose.

The heat releasing patterns (Figure 8.2) and the crystallinity indices (Figure 8.4) of 5 min and 30 min treated samples are corroborating the fact that the higher the crystallinity, the higher the heat release during the concentrated acid treatment. Another observation from crystallinity data is that the acid treated Avicel for 30 min has the higher crystallinity than the Avicel treated for 5 min. The reason for high crystallinity could be that at the 30 min all the decrystallized cellulose is converted to water soluble low DP oligomers and glucose that are removed during the washing, leaving behind highly crystalline cellulose needles. Roman and Winter (2004) reported the formation of highly crystalline cellulose needles with concentrated acid and longer treatment time. Figure 8.2 shows that treating glucose at room temperature with 72% sulfuric acid has not released any heat. It can be assumed that glucose degradation products were not formed, and therefore no associated heat release.

Roman and Winter (2004) reported formation of cellulose sulfate at high acid concentration (>60%) and low temperature (40°C). According to them cellulose sulfate formation was increased with increase in acid concentration, acid-to-cellulose ratio, and hydrolysis time. However, all of their acid treatments were carried above 1 hr and at 40°C. Our treatment time was 5 min; especially highest amount of heat released was within initial 25 seconds. Within that time frame possibility formation of cellulose sulfate is less, therefore no associated heat release.

From the data it is clear that occurrence of decrystallization and the corresponding heat release at different acid concentrations. However, it can be concluded that all the released heat during acid treatment does not come from decrystalization alone.

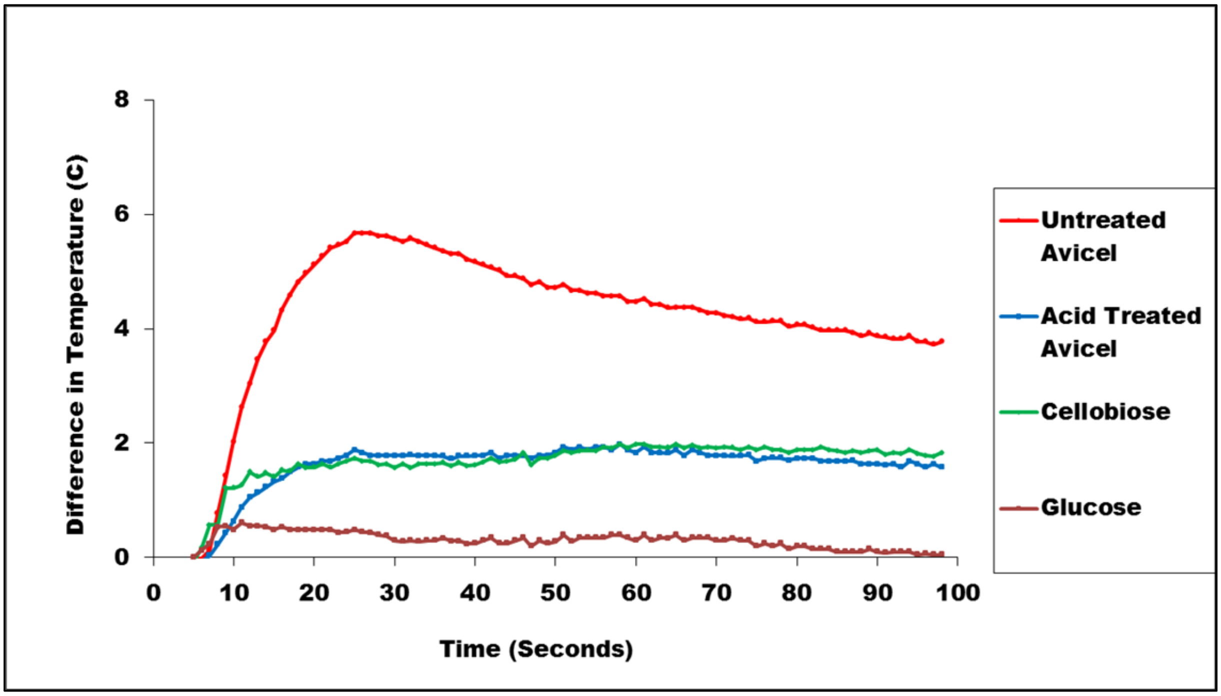

Figure 8.2: Difference in temperature between 72% Sulfuric Acid - different substrates and Water-Avicel at room temperature.

8.4.3. XRD and enzymatic hydrolysis of untreated and acid treated Avicel at room temperature

Crystallinity of cellulose is considered to be an important structural parameter that hinders the enzymatic hydrolysis (Fan et al., 1980; 1981). At high acid concentrations molecules of sulfuric acid are capable to break the hydrogen bonds and convert crystalline cellulose to amorphous and partially crystalline cellulose (Lee and Harraz, 2005). To evaluate the effects of reaction conditions on decrystallization, a selected few samples were further investigated for crystallinity and initial hydrolysis rate by XRD and enzymatic hydrolysis, respectively. Crystallinity indices are shown in Figure 8.3, Figure 8.4, Figure 8.9, Figure 8.14, Figure 8.17, Figure 8.23, and Figure 8.27. Initial enzymatic hydrolysis rates are shown in Figure 8.5, Figure 8.6, Figure 8.10, and Figure 8.11. Avicel crystallinity was decreased substantially with increase in acid concentration at room temperature (Figure 8.3 and Table 8.1). Untreated Avicel has a crystallinity of 67.9%, whereas with acid treatment crystallinity was decreased to as low as 4.2%. These results corroborate the fact that high amount of heat released at 72% acid due to breakage of high percentage of Inter and intra-molecular hydrogen bonds. A sharp decline in crystallinity could be observed for the acid concentrations above 60% (Figure 8.4). Heat releasing patterns (Figure 8.1) and crystallinity indices (Table 8.1) corroborate the occurrence of decrystallization up to 55-72% acid at room temperature.

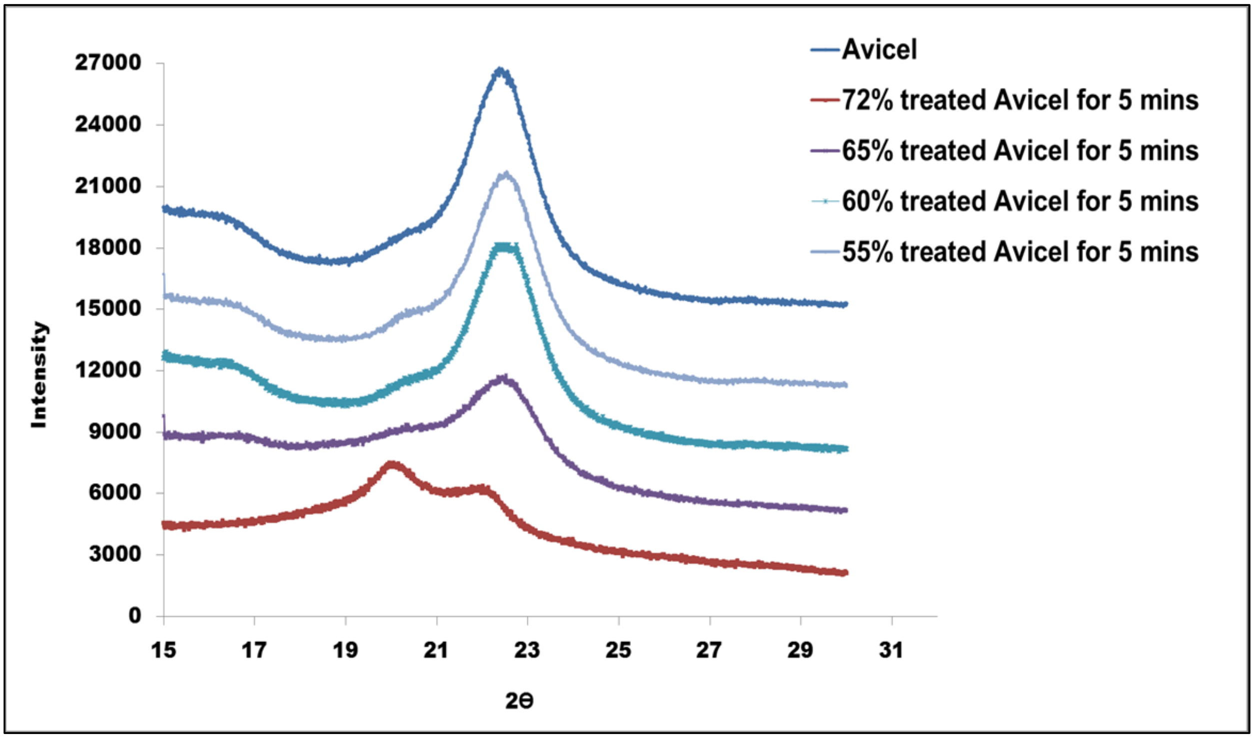

Figure 8.3: X-ray diffractograms of Avicel and sulfuric acid treated Avicel

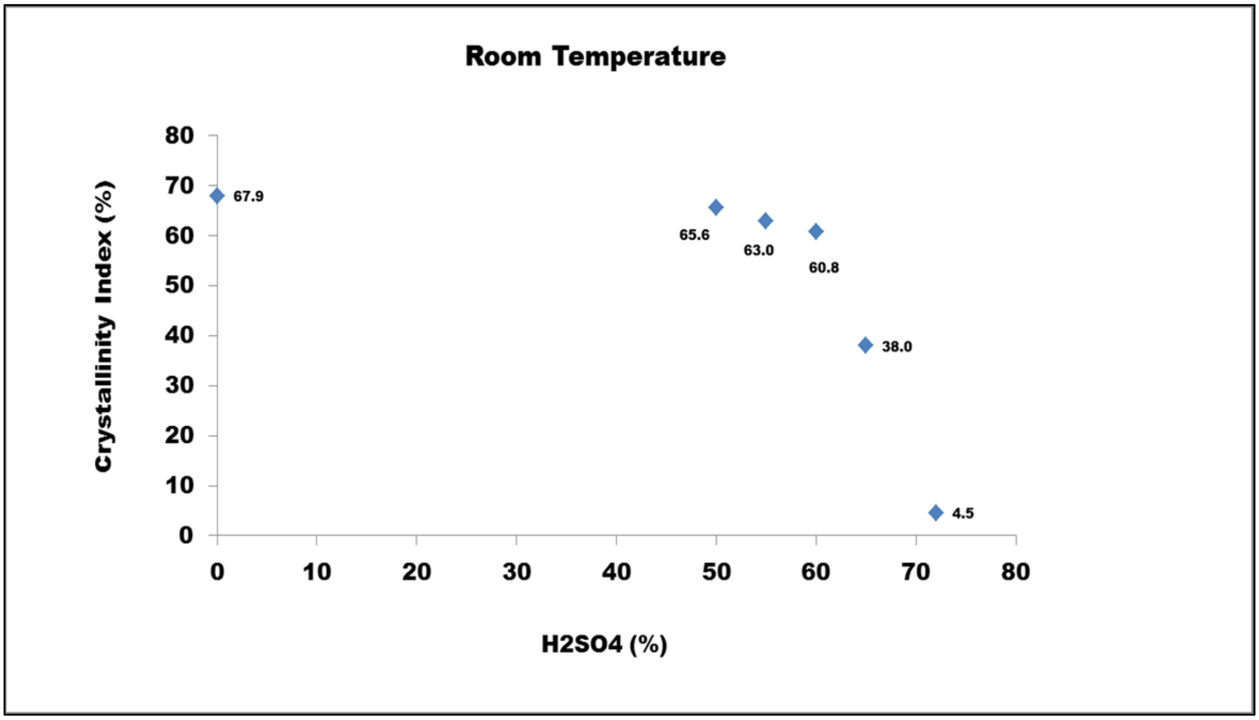

Figure 8.4: Effect of sulfuric acid concentration on crystallinity of Avicel

Table 8.1: Crystallinity index of untreated and sulfuric acid treated Avicel

	Crystallinity Index (%) **$(I_{22.5} - I_{18})/ I_{22.5}$**
Avicel	67.90
72% H_2SO_4 treated Avicel	4.54
65% H_2SO_4 treated Avicel	37.96
60% H_2SO_4 treated Avicel	60.76
55% H_2SO_4 treated Avicel	62.99

Significant differences in initial hydrolysis rates could be observed between 65-72% treated Avicel, 55-60% treated Avicel, and untreated Avicel: a sharp increase with 65-72% treated Avicel, gradual increase with 55-60% treated Avicel and untreated Avicel. The initial hydrolysis rates of 65-72% acid treated Avicel (Figure 8.5 and Figure 8.6) supports the theory proposed by several researchers (Fan et al., 1980; 1981; Zhang and Lynd, 2004) that the highly amorphous cellulose is hydrolyzed much faster than the partially amorphous cellulose. Camacho et al. (1996) reported decrystallization followed by solubilization is effective up to 62% or more acid. Their study also reported solubilization of cellulose with less than 62% acid, but it was not in significant level. According to their study any concentration less than 62% H_2SO_4 fundamentally serves as a swelling agent. This could be the reason for low hydrolysis rates with 55-60% treated Avicel.

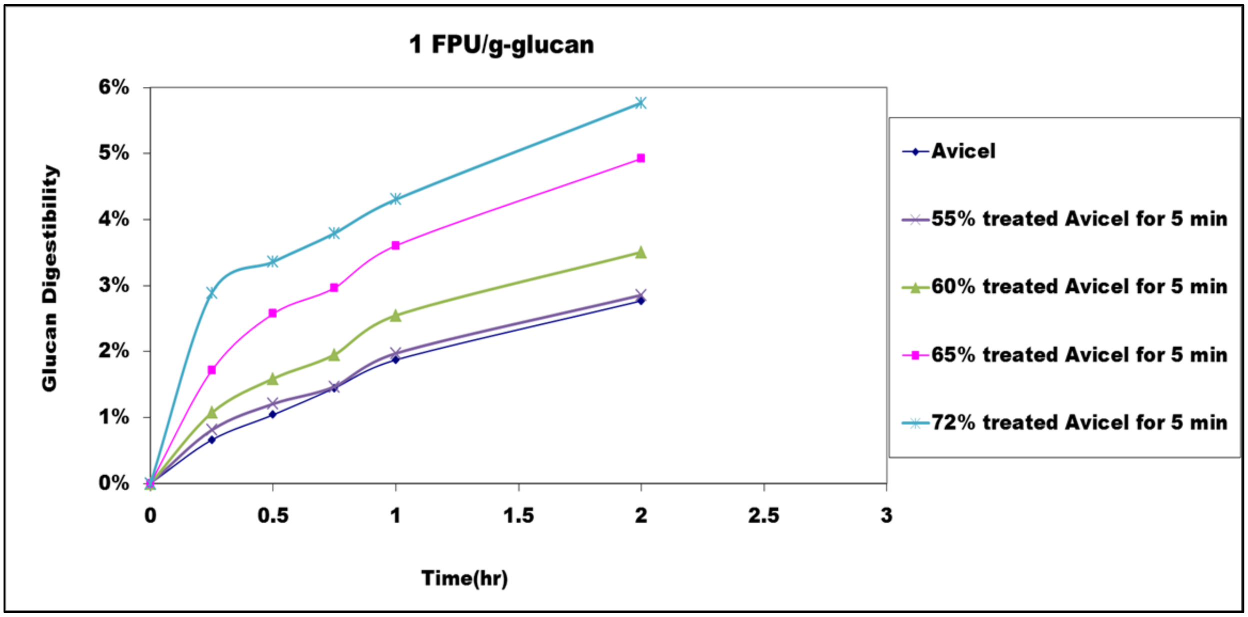

Figure 8.5: Glucan digestibility of Avicel and sulfuric acid treated Avicel with 1 FPU/g-glucan C-Tec 2

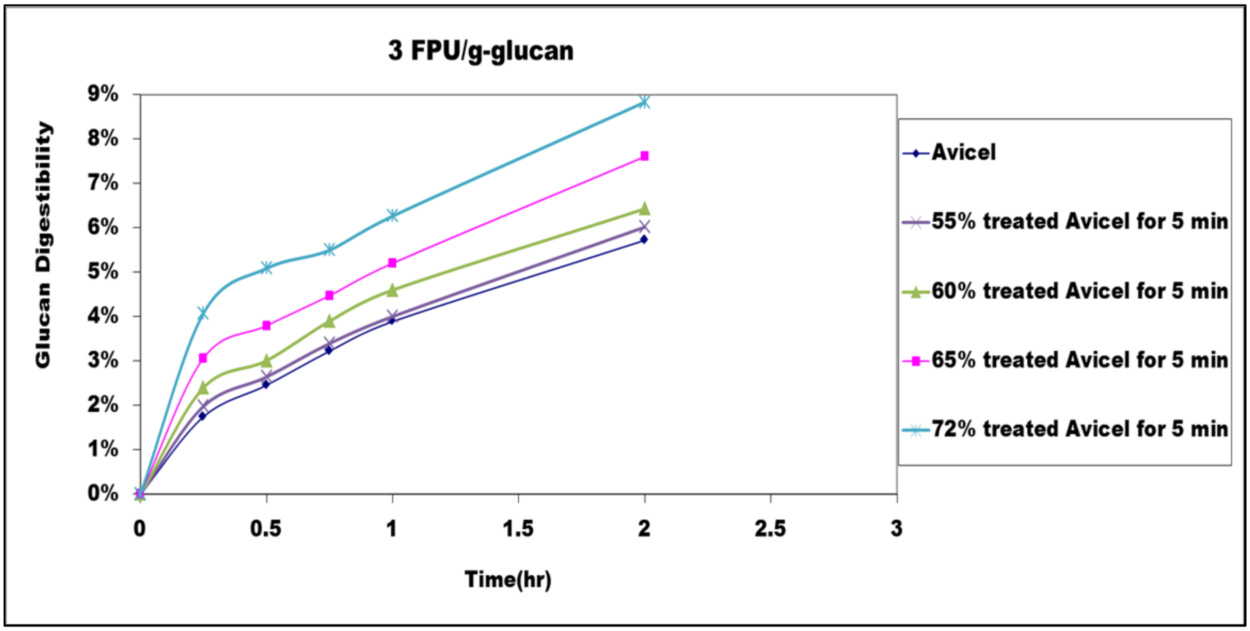

Figure 8.6: Glucan digestibility of Avicel and sulfuric acid treated Avicel with 3 FPU/g-glucan C-Tec 2

8.4.4. Effect of 72% sulfuric acid on decrystallization of Avicel at room temperature

The decrystallization of cellulose with concentrated acid was first started using 72% sulfuric acid as it proved to be optimum concentration for cellulose decrystallization (Lee and Harraz, 2005). Experiments were carried out at room temperature for 5 min. Figure 8.7 shows the temperature-time profile, whereas Figure 8.8 shows difference of temperature of 72% sulfuric acid-Avicel and water-Avicel. Figure 8.8 shows that no change in temperature with the addition of water to Avicel, which was expected, whereas a sharp rise in temperature could be observed with the addition of 72% sulfuric acid to Avicel. The sharp rise in temperature could be attributed to sudden release of heat due to breakage of hydrogen bonds. The rise in temperature of acid-Avicel was gradually reduced after the initial few seconds (Figure 8.7). Earlier we hypothesized that the decrystallization of cellulose behaves as a phase-change in that it occurs at a precise point in temperature (critical temperature) for a given acid concentration. However, our current data indicates that the occurrence of decrystallization at infinitely many points instead of a precise point. Shen and Gnanakaran (2009) calculated the stability of cellulose hydrogen bond networks using statistical mechanics. As per their calculations plasticity of hydrogen bonding network in cellulose keeps the overall hydrogen bonding stability persistent over a wide range of temperatures. According to them instead of only one stable pattern, different hydrogen bonding patterns dominate at different temperatures until the disassembly occurs at very high temperatures. They also showed that intra-chain hydrogen bonding is more stable than the inter-chain bonding over a wide range of temperatures.

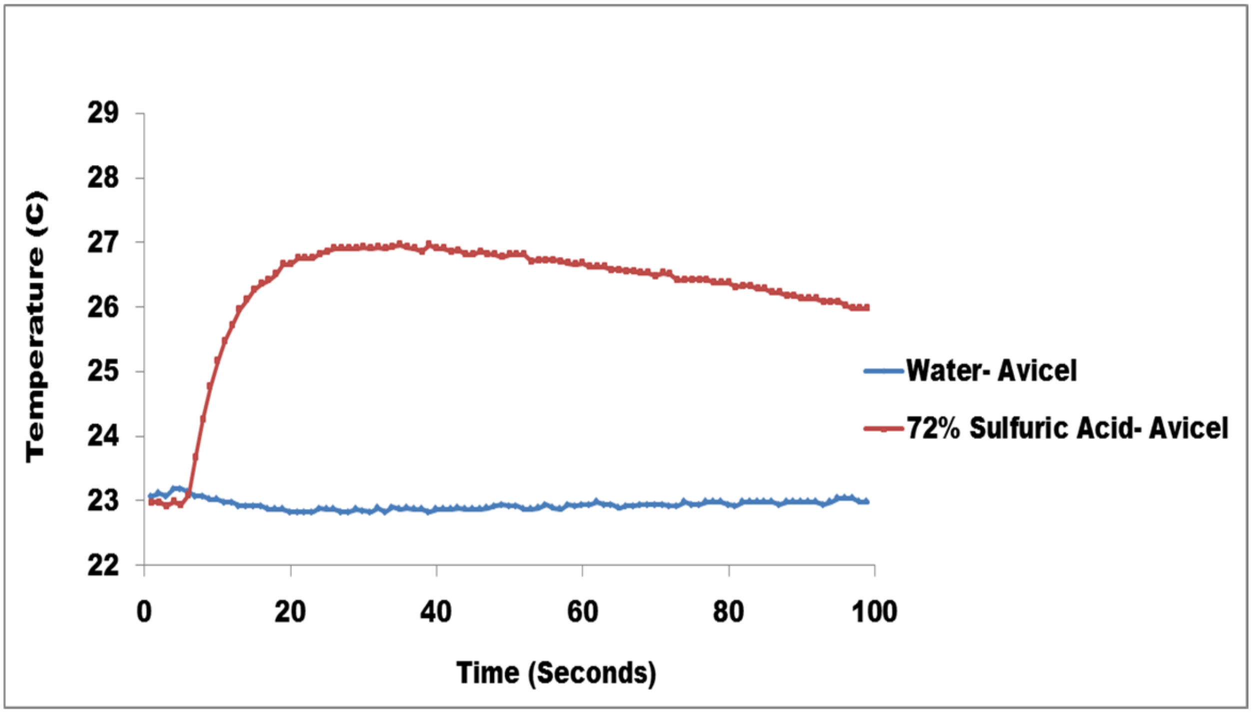

Figure 8.7: Comparative temperature-time profiles of 72% Sulfuric Acid-Avicel and Water-Avicel at room temperature

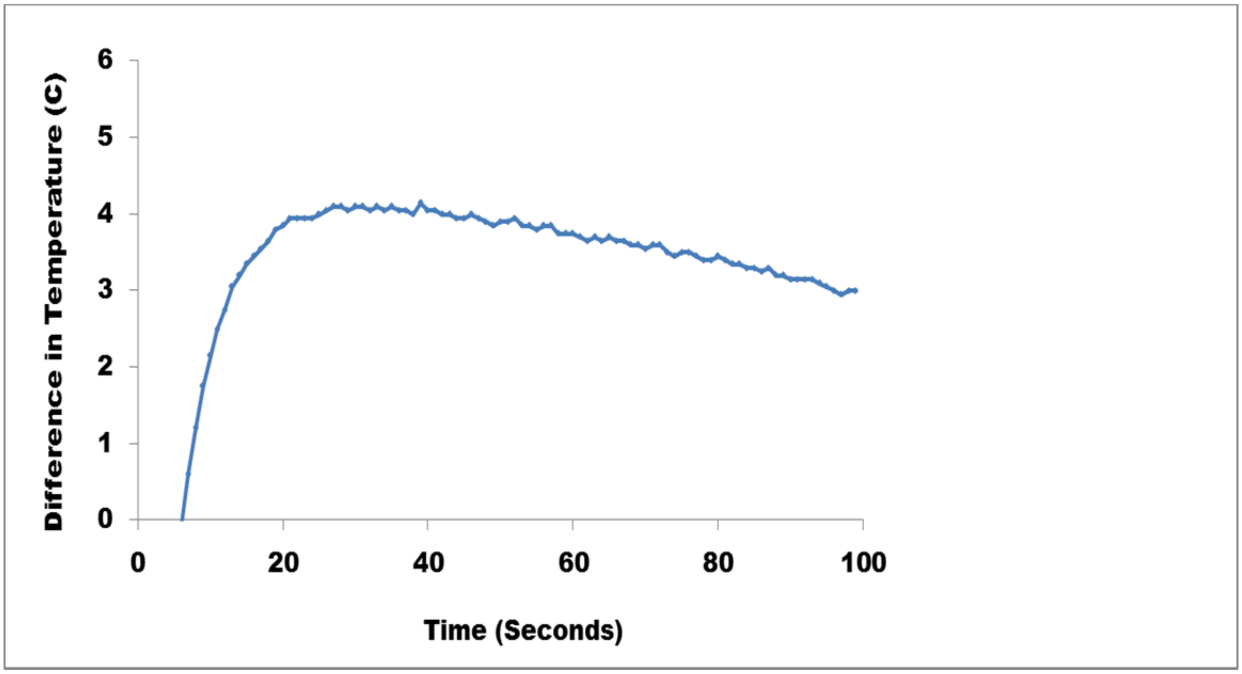

Figure 8.8: Difference in temperature between 72% Sulfuric Acid-Avicel and Water- Avicel at room temperature

8.4.4.1. Effect of treatment time on decrystallization of Avicel at room temperature

To understand the effect of treat time on decrystallization, acid treatment of Avicel was further continued for 30 min. Figure 8.9 shows that 30 min treated Avicel is more crystalline than the 5 min treated Avicel. It could be attributed that at the 30 min all the initial decrystallized cellulose is converted to soluble low DP oligomers and glucose, which are removed during the washing, leaving behind only crystalline cellulose needles. Roman and Winter (2004) reported the formation of highly crystalline cellulose needles with concentrated acid and longer treatment time. However, enzymatic hydrolysis rates (Figure 8.10 and Figure 8.11) have shown opposite behavior to crystallinity (Figure 8.9). One plausible reason for this behavior could be due to formation of low DP crystalline cellulose with 30 min treatment. DP is one the several factors that is known to influence cellulose hydrolysis rate (Puri, 1984).

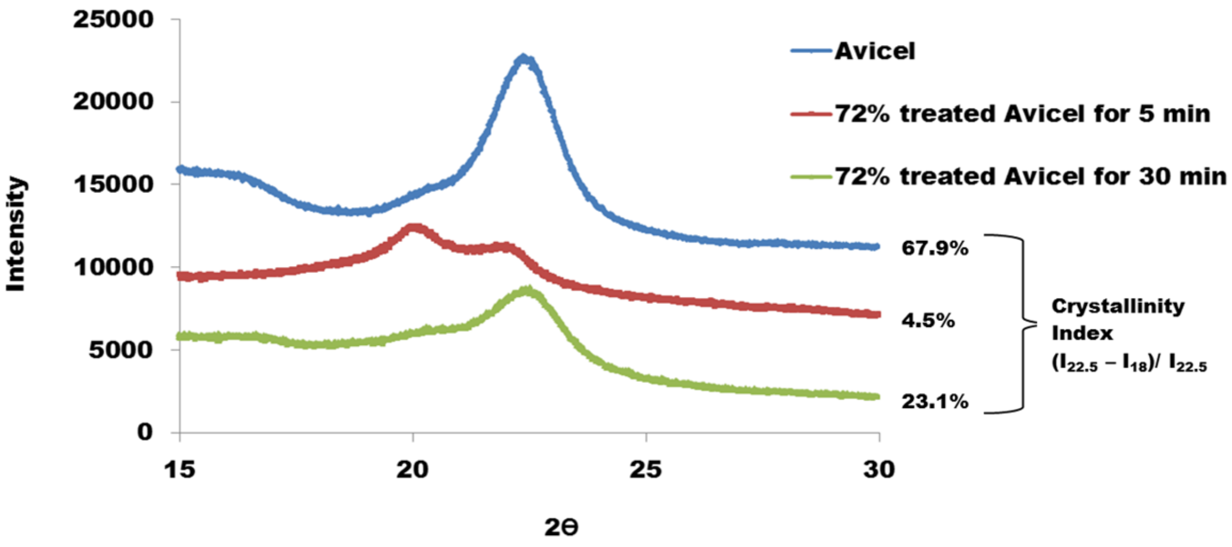

Figure 8.9: X-ray diffractograms of Avicel and 72% sulfuric acid treated Avicel

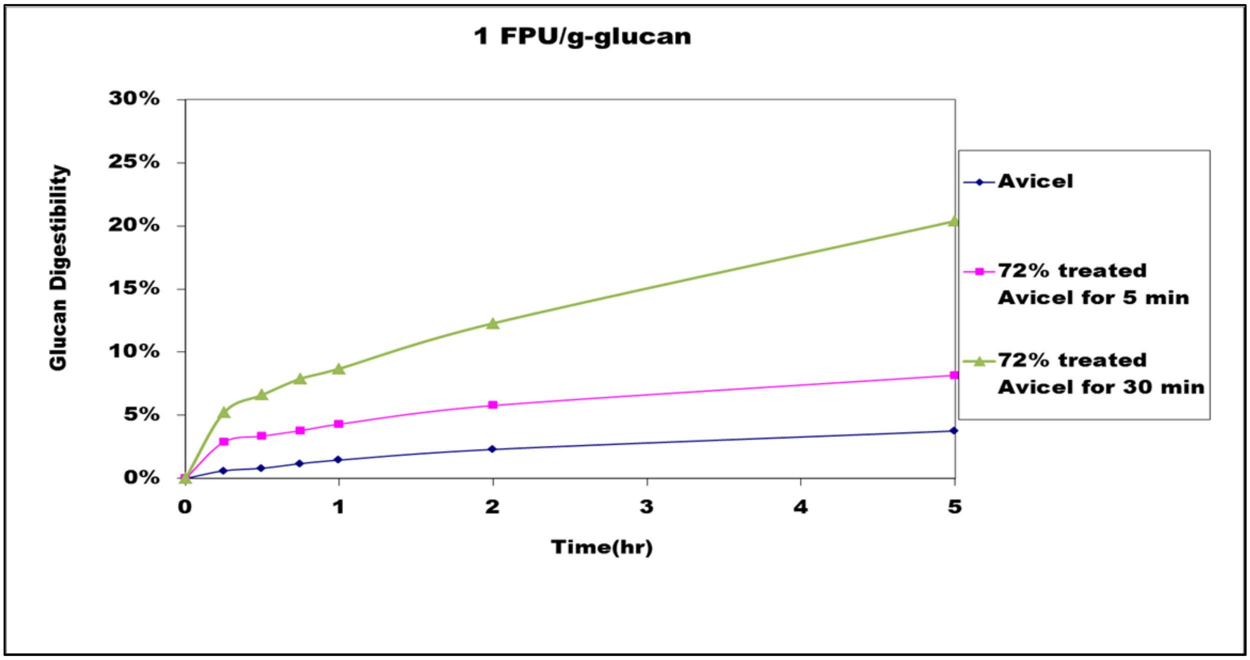

Figure 8.10: Glucan digestibility of Avicel, 72% sulfuric acid treated Avicel for 5 min and 30 min with 1 FPU/g-glucan C-Tec 2

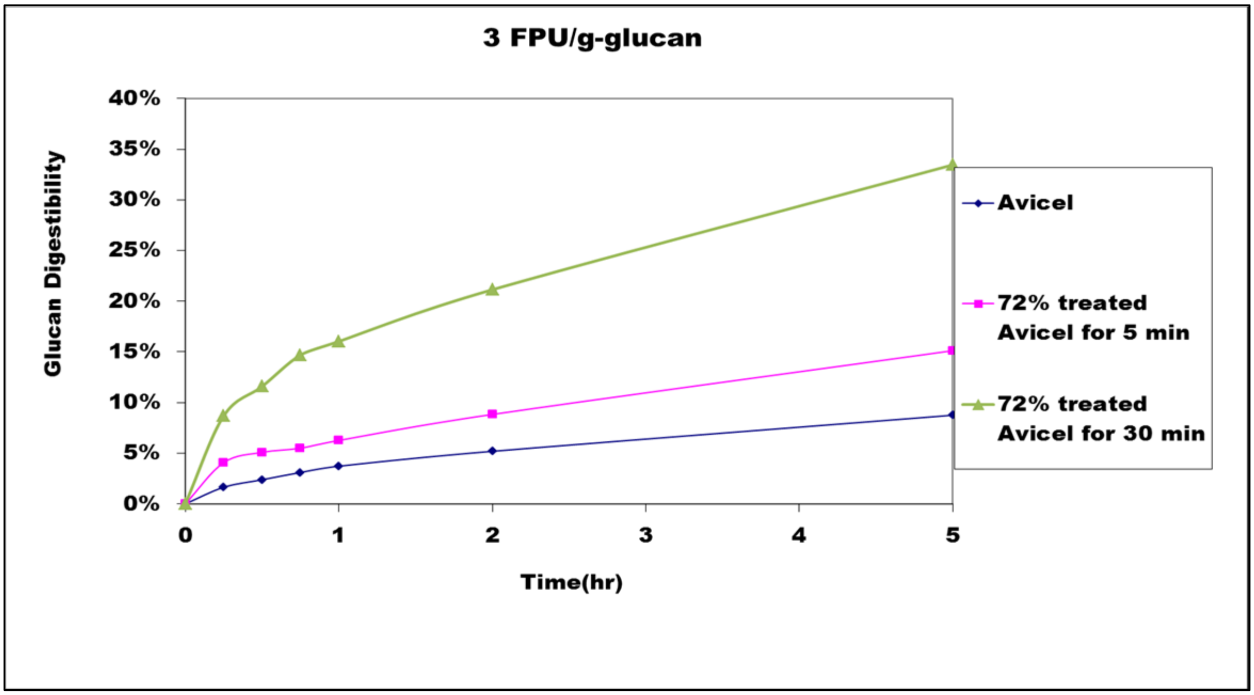

Figure 8.11: Glucan digestibility of Avicel, 72% sulfuric acid treated Avicel for 5 min and 30 min with 3 FPU/g-glucan C-Tec 2

8.4.5. Effect of 65% sulfuric acid on decrystallization of Avicel at room temperature

Figure 8.12 shows the temperature-time profile, whereas Figure 8.13 shows difference of temperature of 65% sulfuric acid-Avicel and water-Avicel. A slightly less amount of heat, but similar heat releasing pattern to 72% was observed with 65% acid (Figure 8.8 and Figure 8.13). Samples treated with 65% acid at room temperature were subjected to XRD and enzymatic hydrolysis. A significant amount of crystallinity (by 44%) was reduced with 65% acid, albeit not to the extent with 72% acid. However, decrease in crystallinity has a significant effect on the initial hydrolysis rate (Figure 8.5 and Figure 8.6).

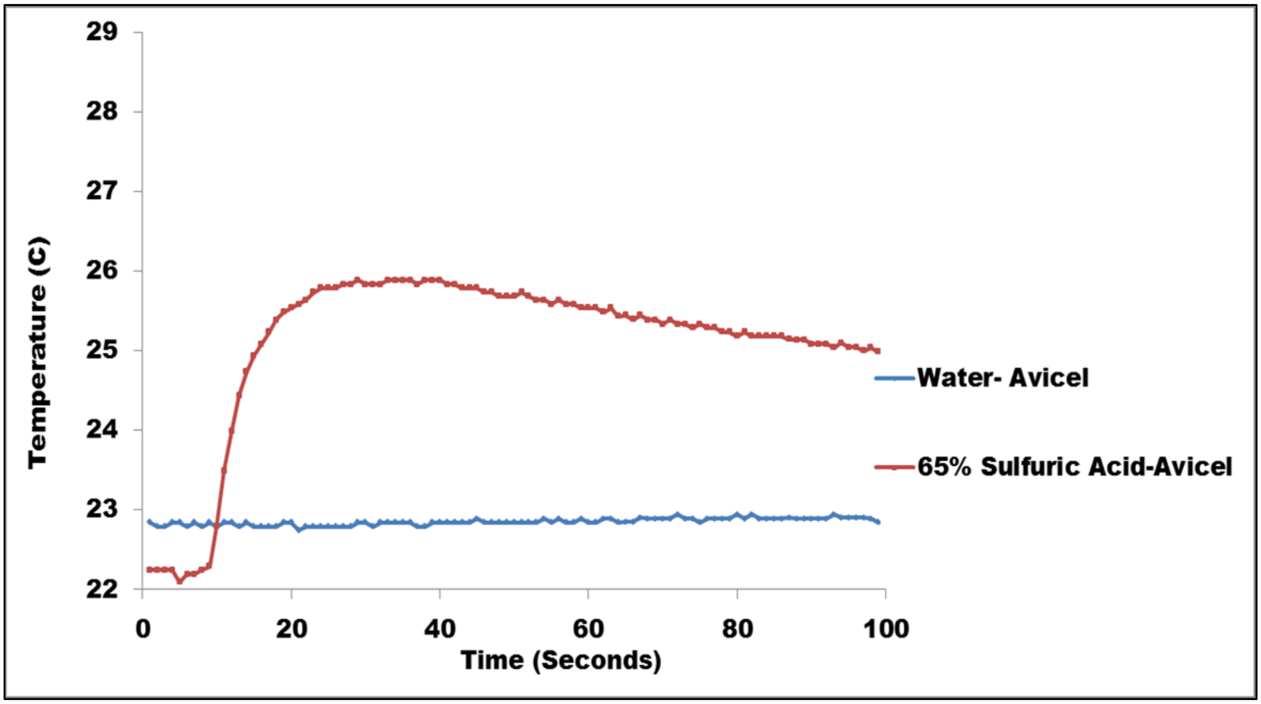

Figure 8.12: Comparative temperature-time profiles of 65% Sulfuric Acid-Avicel and Water-Avicel at room temperature

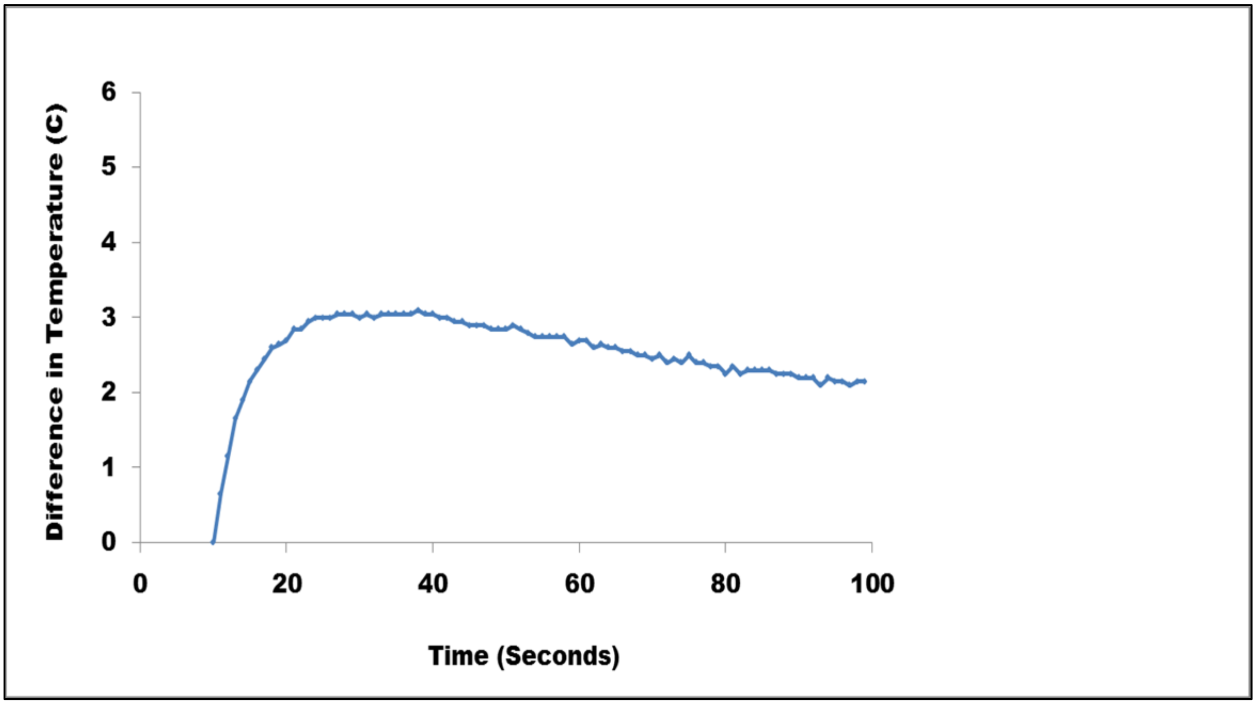

Figure 8.13: Difference in temperature between 65% Sulfuric Acid-Avicel and Water- Avicel at room temperature

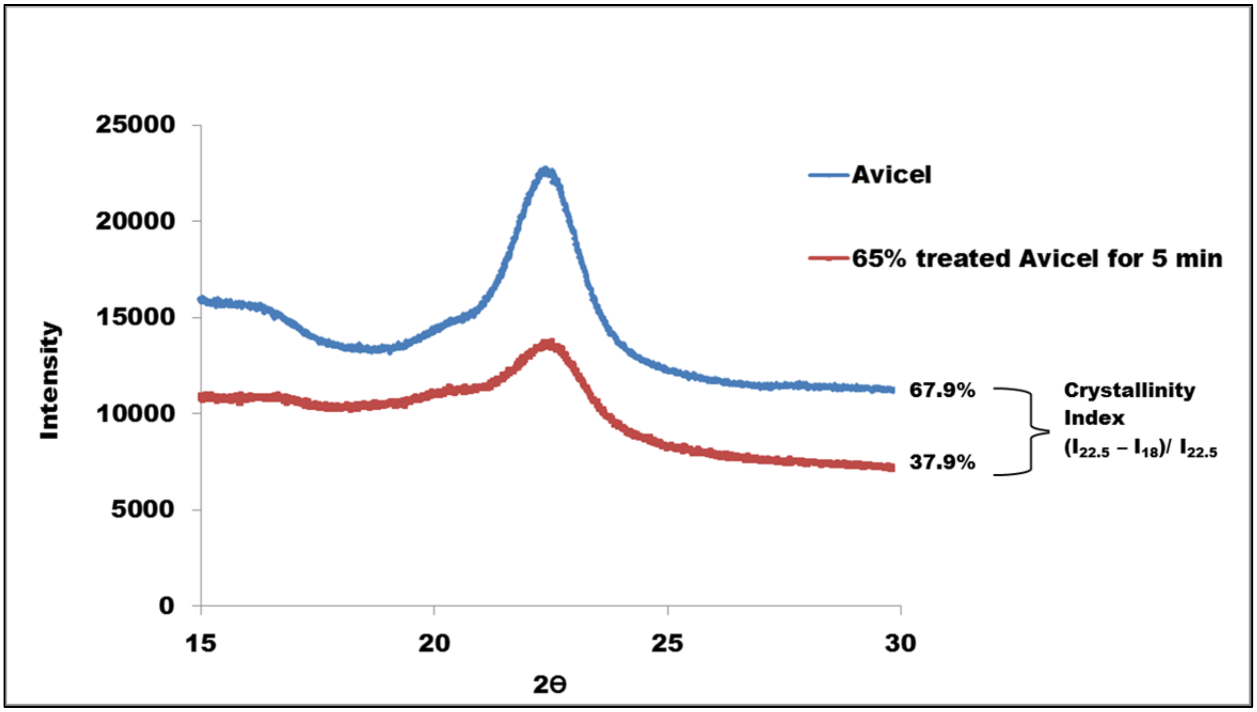

Figure 8.14: X-ray diffractograms of Avicel and 65% sulfuric acid treated Avicel

8.4.6. Effect of 60% sulfuric acid on decrystalization of Avicel at room temperature

Figure 8.15 shows the comparative temperature profiles of 60% acid-Avicel and water-Avicel at room temperature, whereas Figure 8.16 shows the difference in temperature change between 60% sulfuric acid-Avicel and Water-Avicel at room temperature. Although significant amount of heat was released with 60% acid, it was comparatively less than the 65%. According to Camacho et al. (1996) decrystallization followed by solubilization occurs up to 62% or more acid. After that, even though cellulose solubilization occurs, it is not in appreciable range. More than 10% decrease in crystallinity was observed with 60% acid. Crystallinity was further decreased with increase in treatment time to 10 min, by 3% (Figure 8.17).

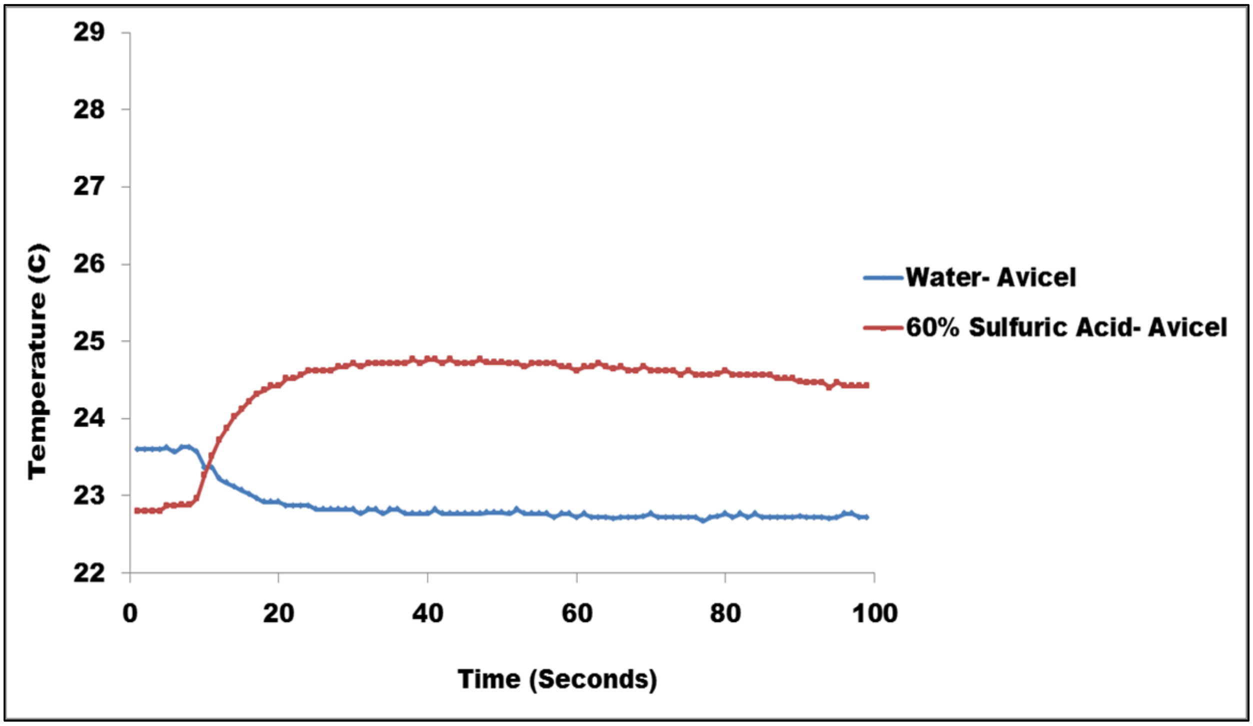

Figure 8.15: Comparative temperature-time profiles of 60% Sulfuric Acid-Avicel and Water-Avicel at room temperature

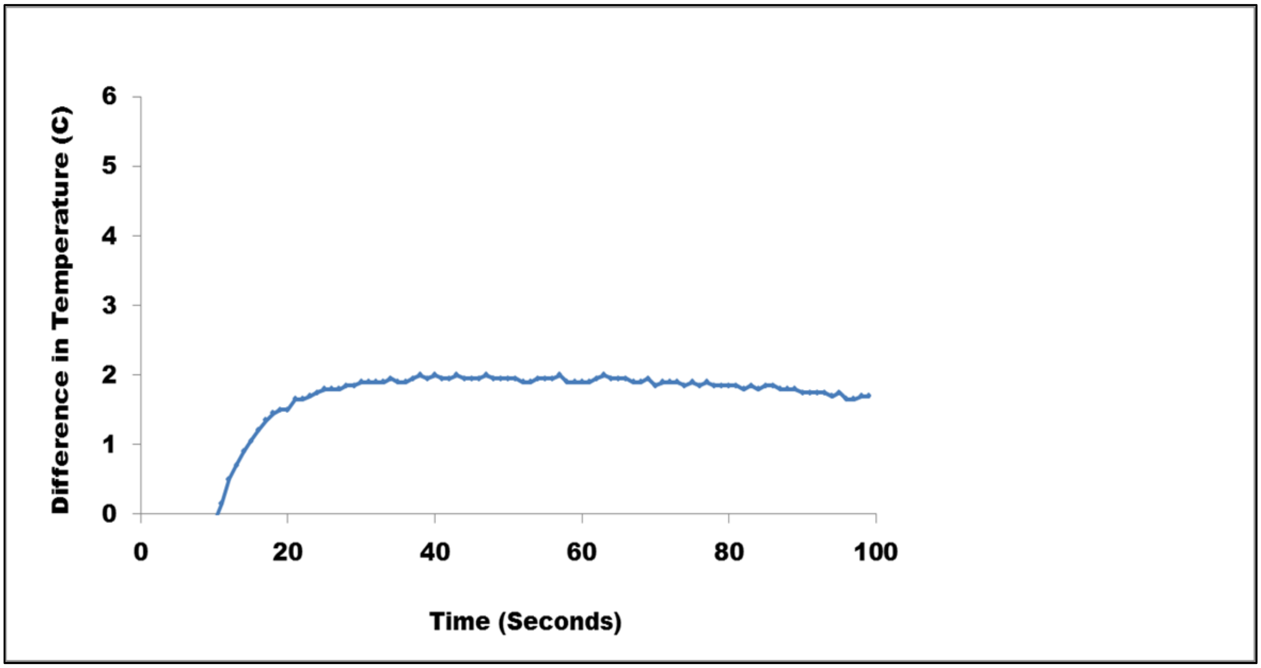

Figure 8.16: Difference in temperature between 60% Sulfuric Acid-Avicel and Water- Avicel at room temperature

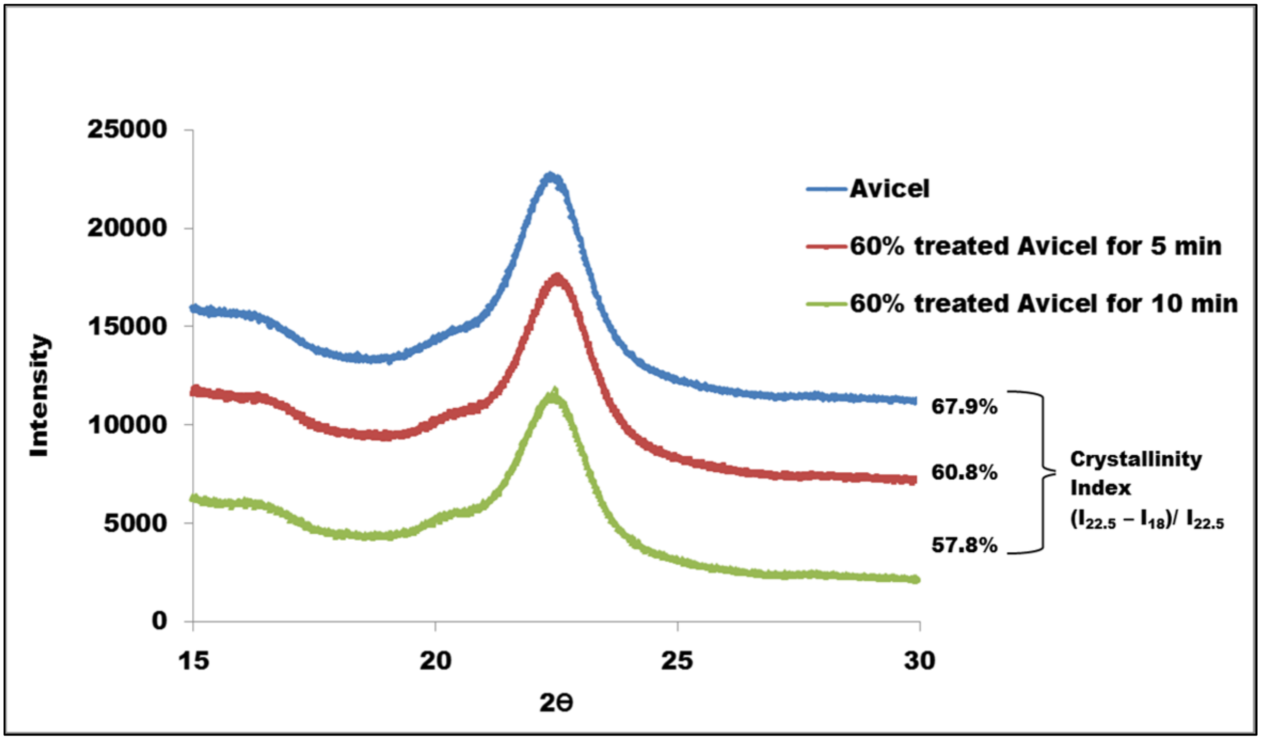

Figure 8.17: X-ray diffractograms of Avicel and 60% sulfuric acid treated Avicel

8.4.7. Effect of 55% sulfuric acid on decrystallization of Avicel at room temperature

At the initial stage, 55% acid treatment has shown identical heat releasing pattern with 60% acid, however with increase in treatment time, rise temperature has reduced sharply (Figure 8.16, Figure 8.17, Figure 8.18 and Figure 8.19). One plausible reason for the decrease in temperature with the increase in time could be that with 55% acid lesser number of hydrogen bonds might have been broken, consequently less heat was produced. Therefore, resultant heat was not high enough to compensate heat loss to surroundings. A 7% loss in crystallinity was observed with the 55% acid treatment (Figure 8.20). It can be concluded that decrystallization of Avicel does occur with 55% sulfuric acid at room temperature breaking the previously held notion that sulfuric acid above 60% is required to decrystallize cellulose.

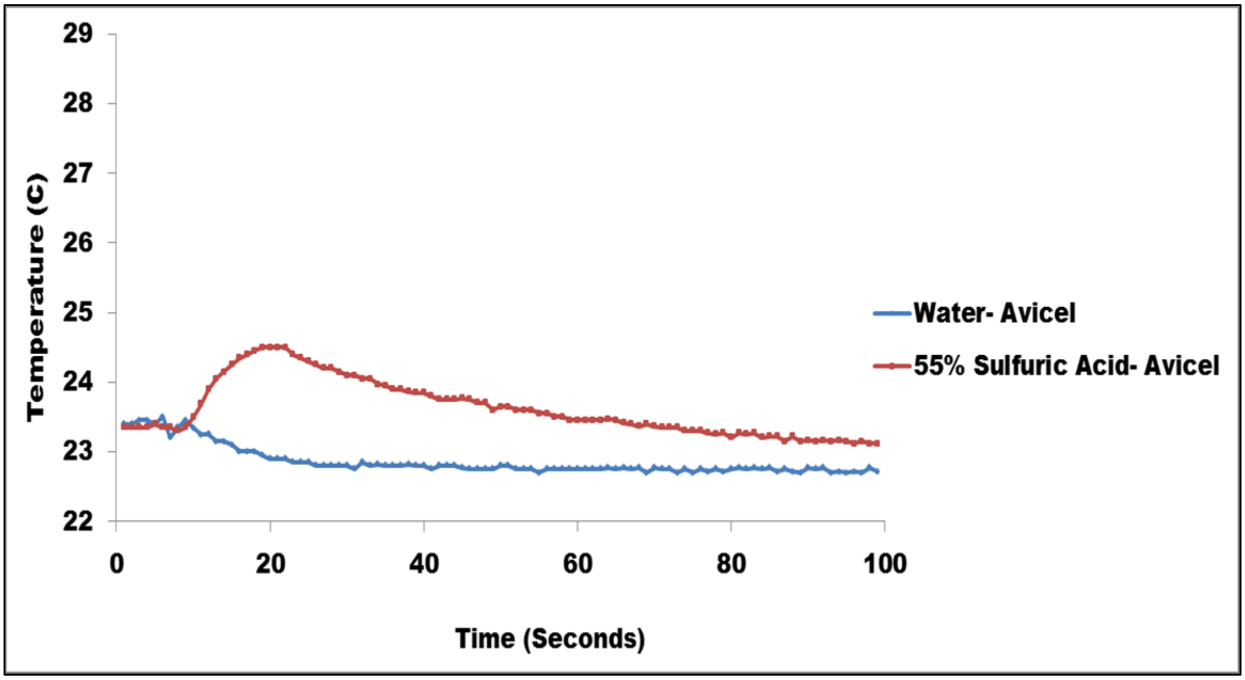

Figure 8.18: Comparative temperature-time profiles of 55% Sulfuric Acid-Avicel and Water-Avicel at room temperature

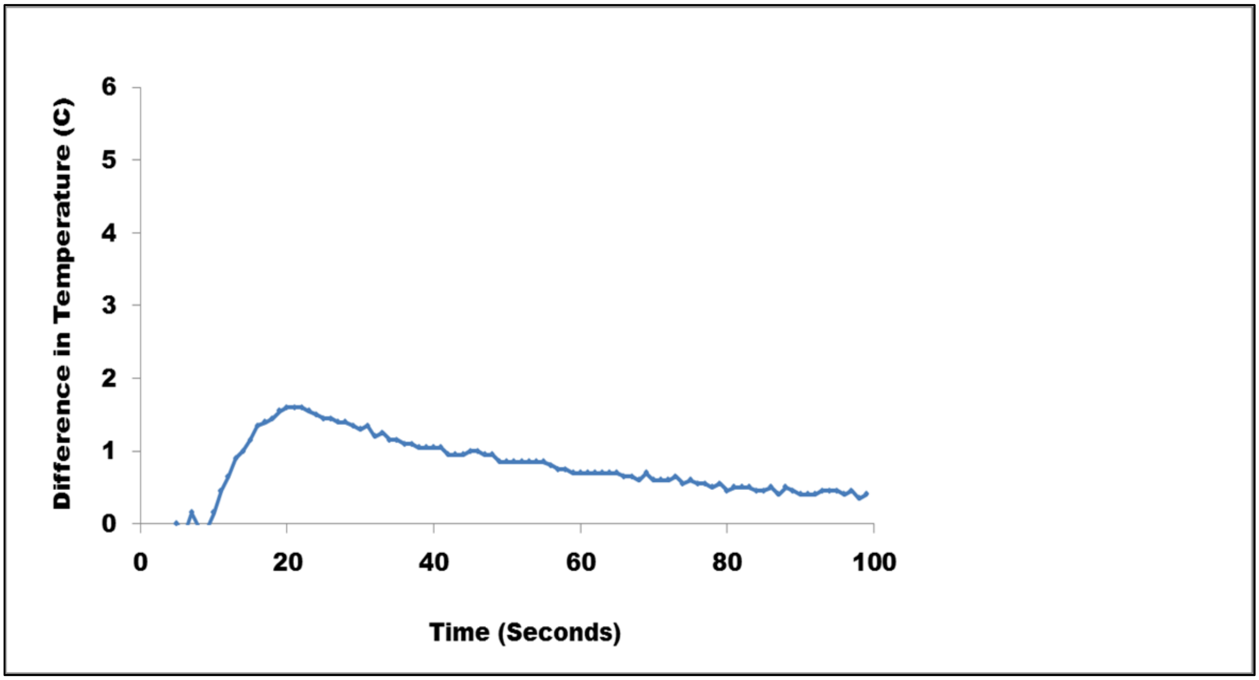

Figure 8.19: Difference in temperature between 55% Sulfuric Acid-Avicel and Water- Avicel at room temperature

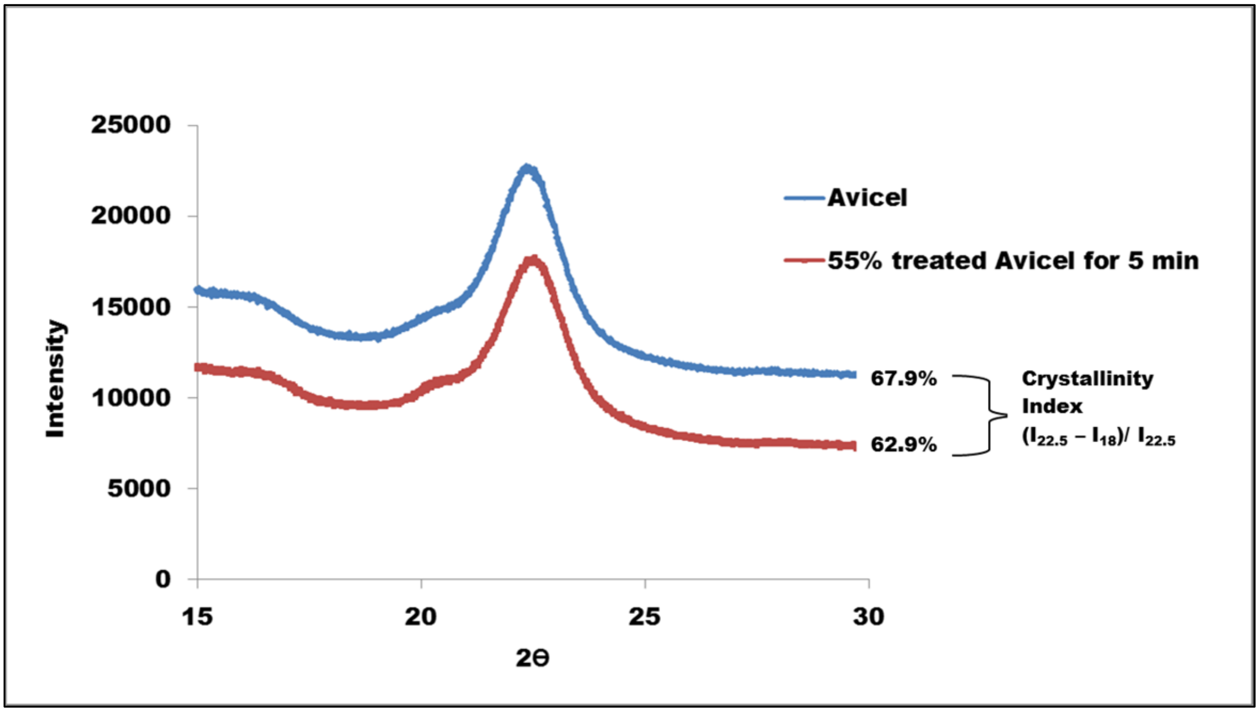

Figure 8.20: X-ray diffractograms of Avicel and 55% sulfuric acid treated Avicel

8.4.8. Effect of 50% sulfuric acid on decrystallization of Avicel at room temperature

At 50% acid virtually no change in temperature was observed due to decrystallization or any other potential reaction (Figure 8.21 and Figure 8.22). XRD graph also indicates not a significant change in crystallinity (Figure 8.23). Based on results obtained at room temperature, further experiments were continued at high temperature (90°C) to find out the occurrence decrystallization with 50% acid and below.

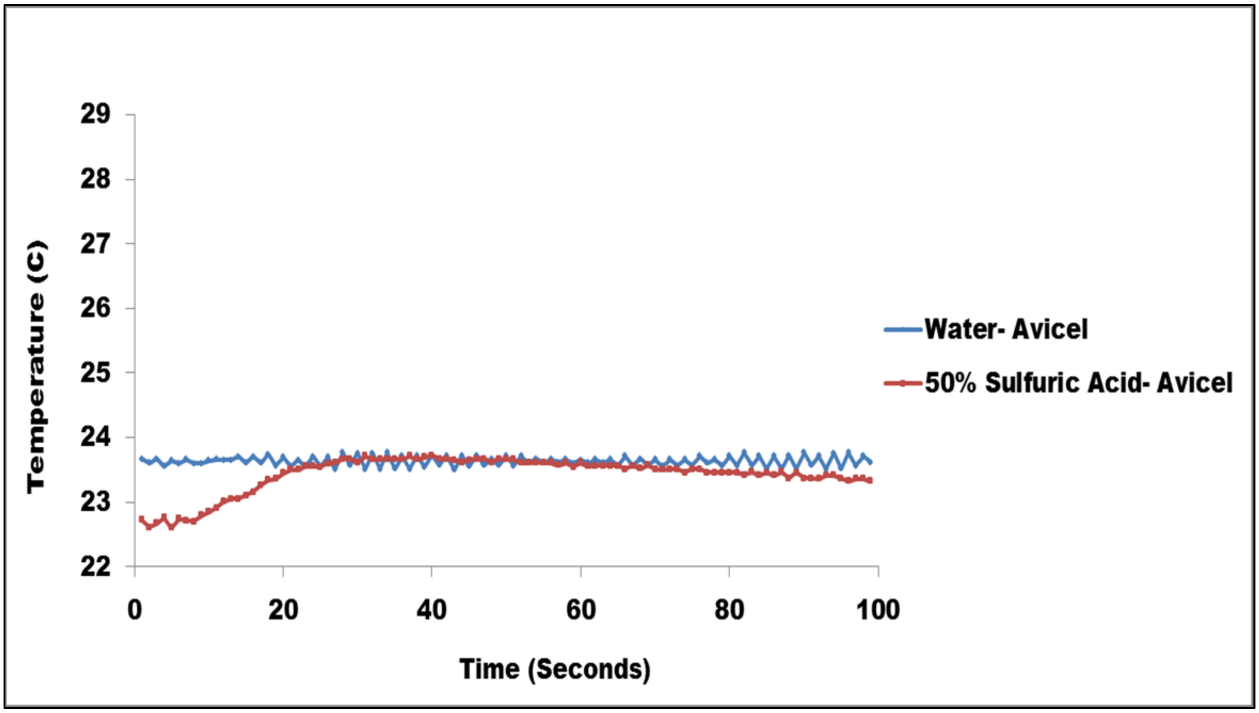

Figure 8.21: Comparative temperature-time profiles of 50% Sulfuric Acid-Avicel and Water-Avicel at room temperature

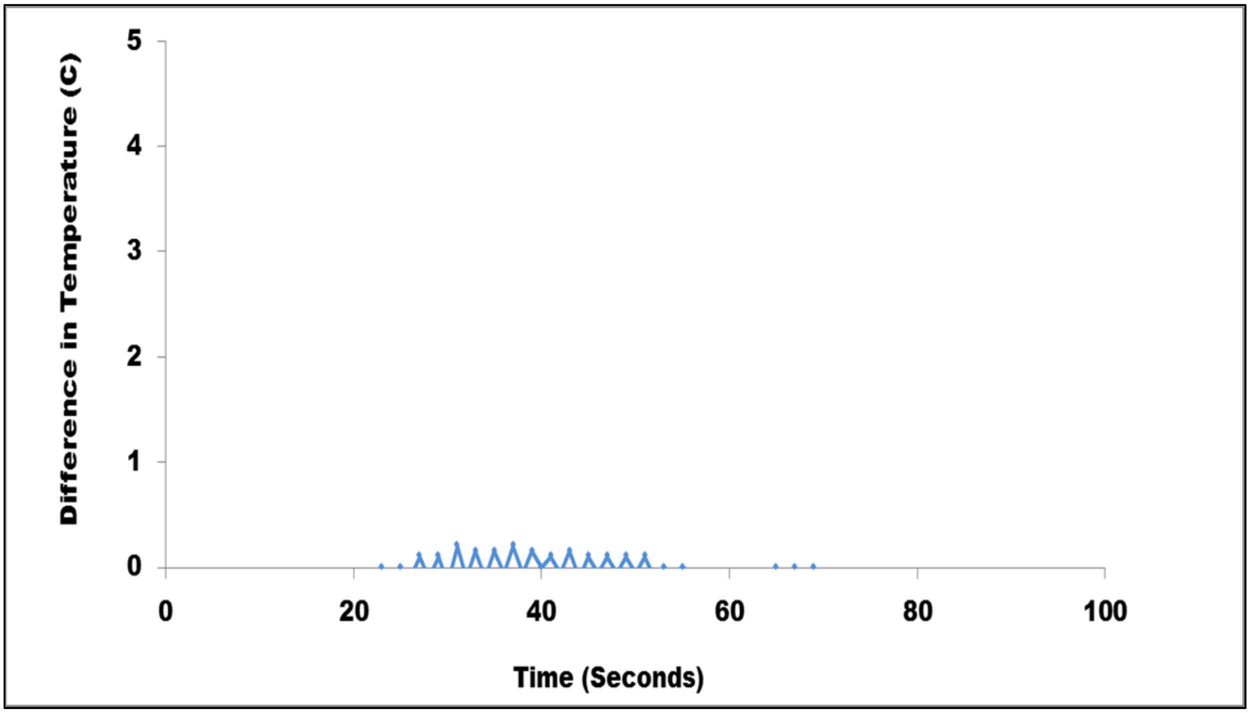

Figure 8.22: Difference in temperature between 50% Sulfuric Acid-Avicel and Water- Avicel at room temperature

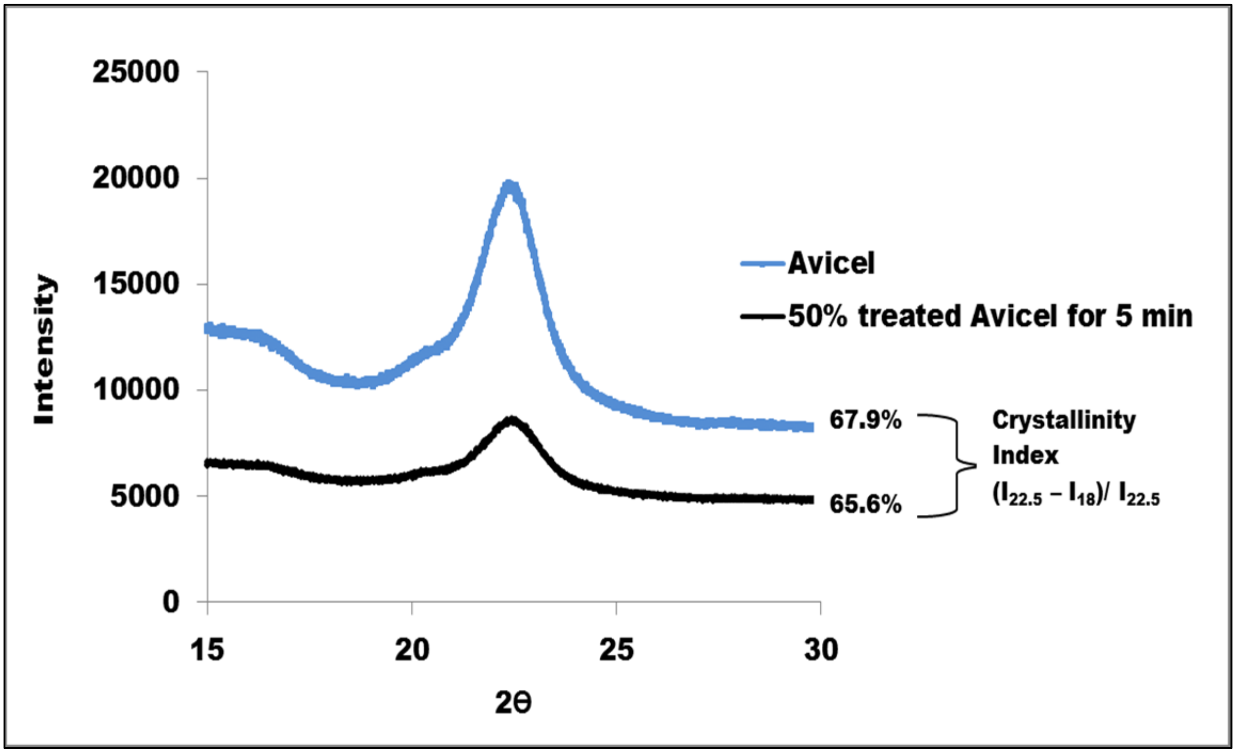

Figure 8.23: X-ray diffractograms of Avicel and 50% sulfuric acid treated Avicel

8.4.9. Effect of acid concentration on decrystallization of cellulose at high temperature

As shown in preceding sections, increase in heat release was only up to 55% acid. Therefore, further experiments were carried out at high temperature (90^{o}C) using acid concentration in the range of 30-50% to see whether there was any decrystallization and corresponding heat release with the acid concentrations below 55%.

8.4.9.1. Effect of 50% sulfuric acid on decrystallization of Avicel at 90^{o}C

The decrystallization studies applying 50% sulfuric acid were carried out using Avicel at 90^{o}C for 5 min and 10 min. Temperature change and crystallinity index data indicate that rise in temperature change and corresponding decrease in crystallinity at 90^{o}C with 50% concentration (Figure 8.24, Figure 8.25, Figure 8.26) at 5 min. Avicel treated with 50% acid and at 90^{o}C has lost 9% of it crystallinity. These results clearly indicate that if the treatment temperature is increased, decrystallization occurs even at acid concentrations below 55%. However, not a significant change in crystallinity can be seen when treatment time increased to 10 min.

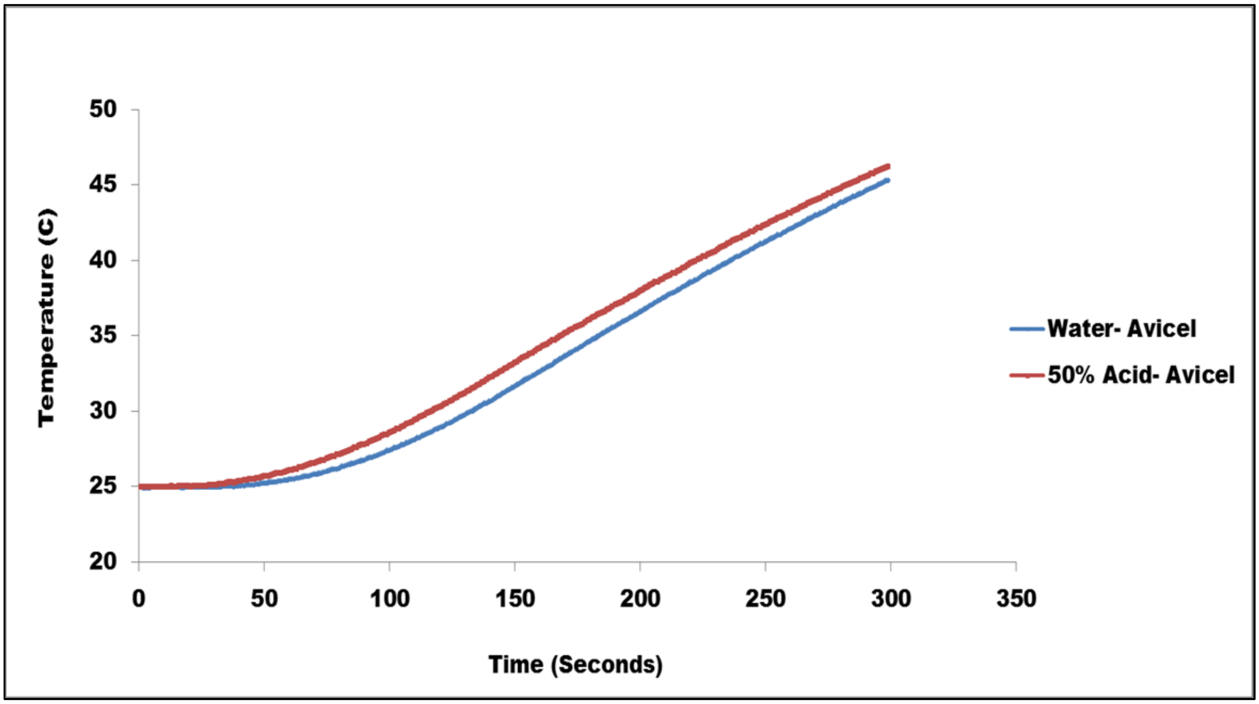

Figure 8.24: Comparative temperature-time profiles of 50% Sulfuric Acid-Avicel and Water-Avicel at 90°C

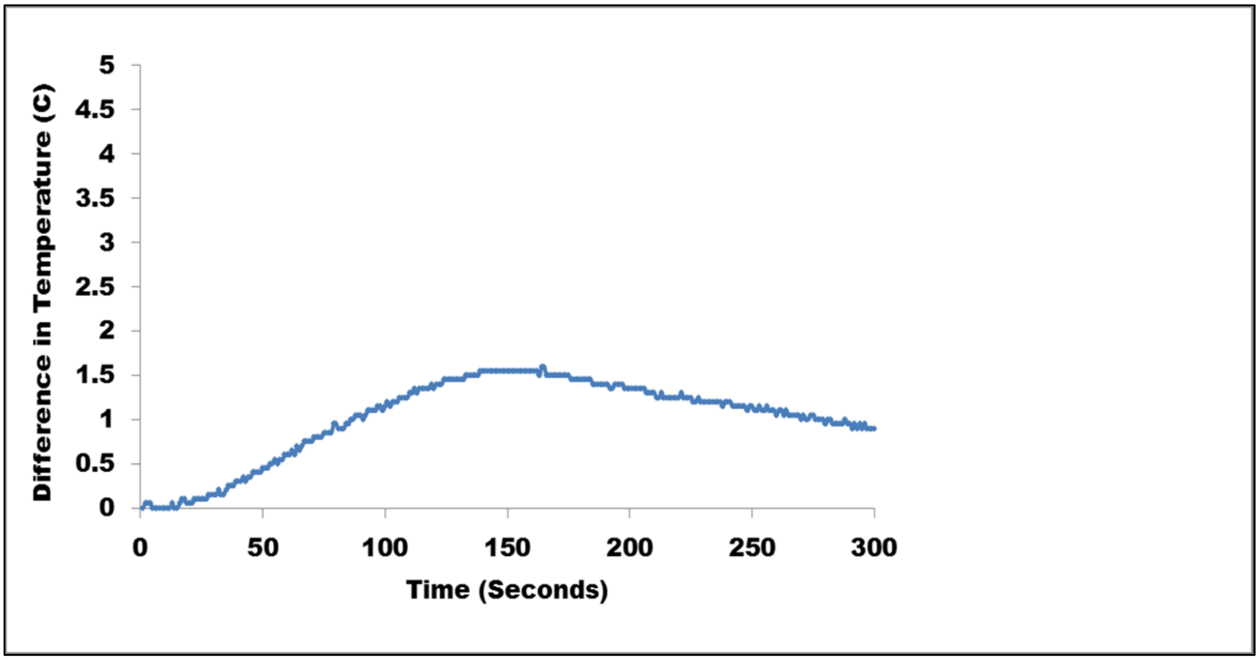

Figure 8.25: Difference in temperature between 50% Sulfuric Acid-Avicel and Water- Avicel at 90°C

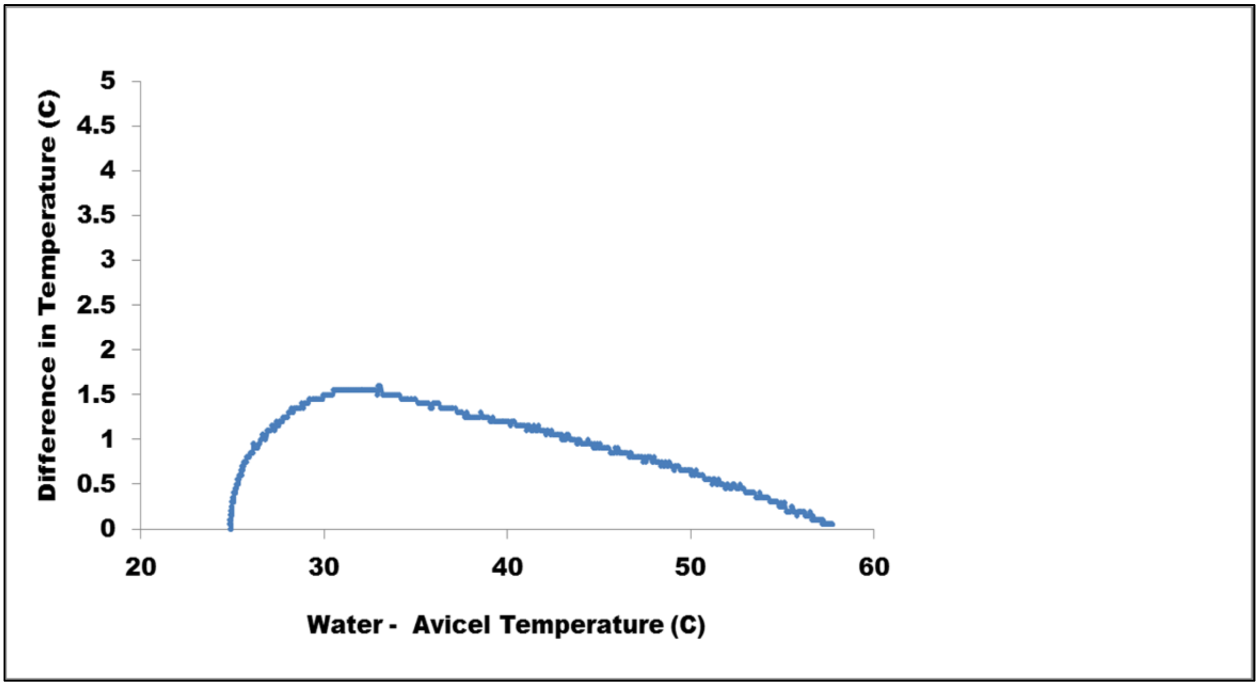

Figure 8.26: Difference in temperature between 50% Sulfuric Acid-Avicel and Water- Avicel verses temperature of water-Avicel at 90^{o}C

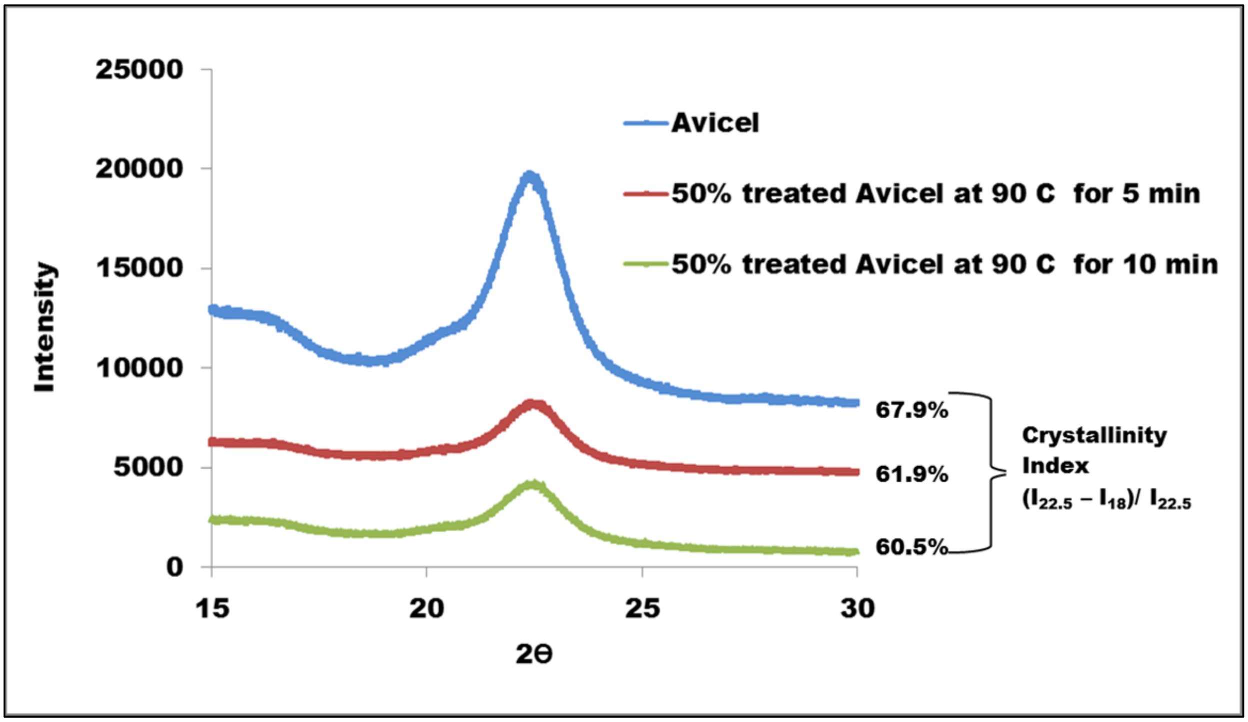

Figure 8.27: X-ray diffractograms of Avicel and 50% sulfuric acid treated Avicel at 90^{o}C

8.4.9.2. Effect of 40% sulfuric acid on decrystallization of Avicel at 90^{o}C

The decrystallization studies applying 40% sulfuric acid were carried out using Avicel at 90^{o}C. Temperature profiles indicate the release of heat with 40% acid and at high temperature (Figure 8.28, Figure 8.29, and Figure 8.30).

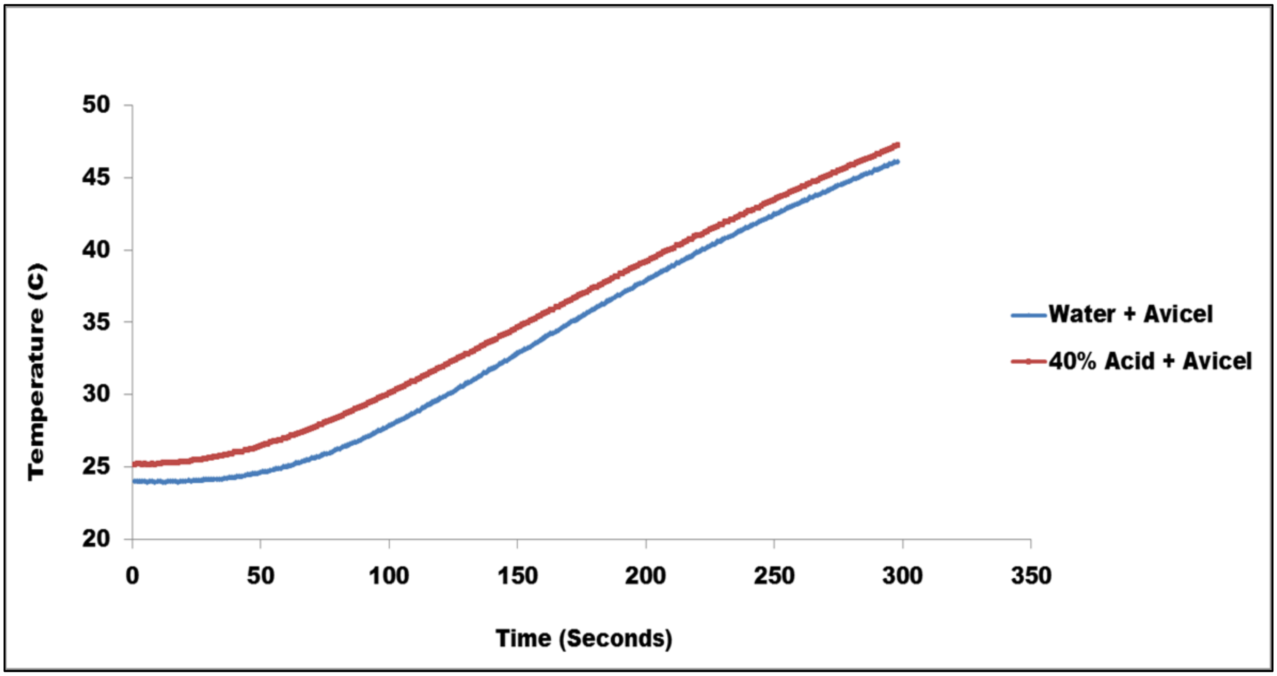

Figure 8.28: Comparative temperature-time profiles of 40% Sulfuric Acid-Avicel and Water-Avicel at 90^{o}C

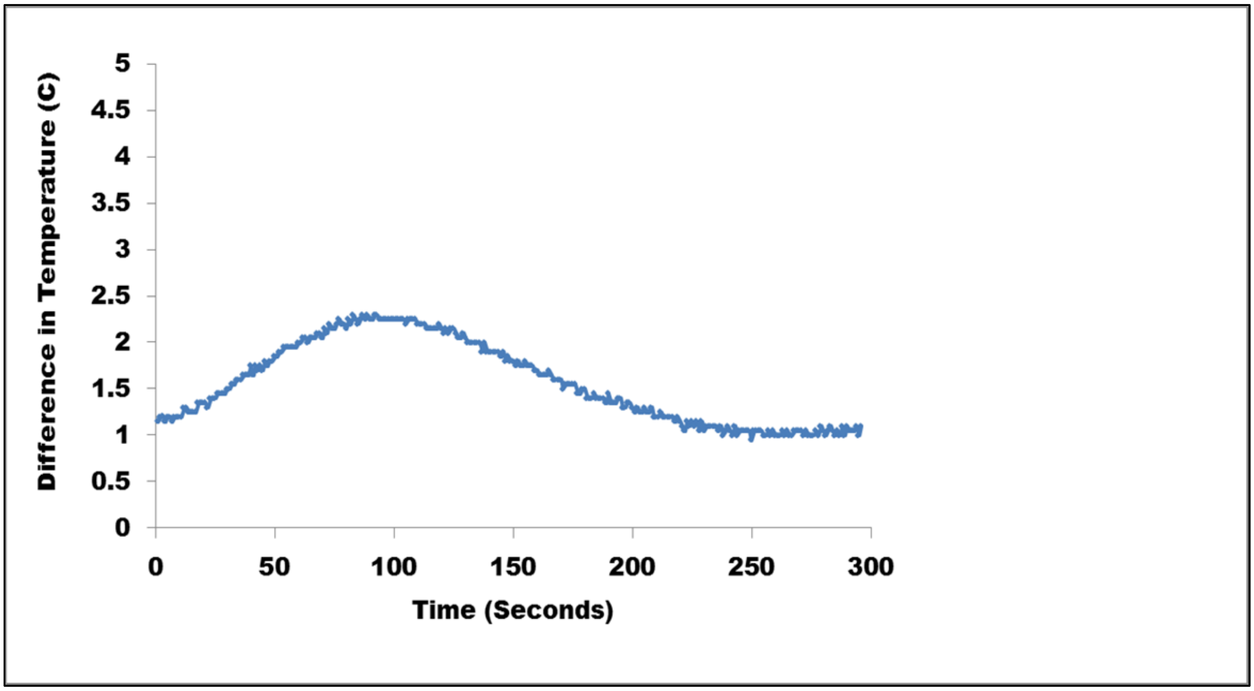

Figure 8.29: Difference in temperature between 40% Sulfuric Acid-Avicel and Water- Avicel at 90°C

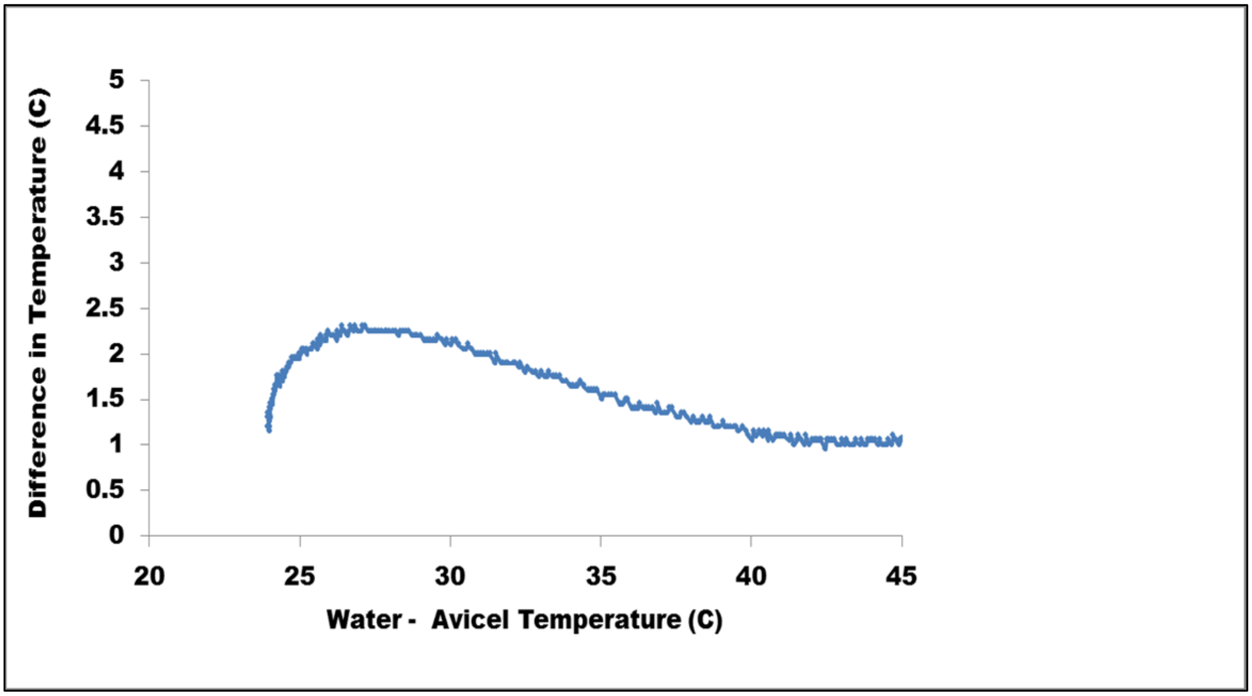

Figure 8.30: Difference in temperature change between 40% Sulfuric Acid-Avicel and Water-Avicel verses temperature of water-Avicel at 90°C

8.4.9.3. Effects of 30% sulfuric acid on decrystallization of Avicel at 90°C

The decrystallization studies applying 30% sulfuric acid were carried out using Avicel at 90°C. Although change in temperature was observed with 30% acid, but it was significantly lower than the 40% acid (Figure 8.31, Figure 8.32, and Figure 8.33). The rise in temperature is possible error range, therefore it is appropriate to carry 30% acid decrystallization studies at the higher temperature.

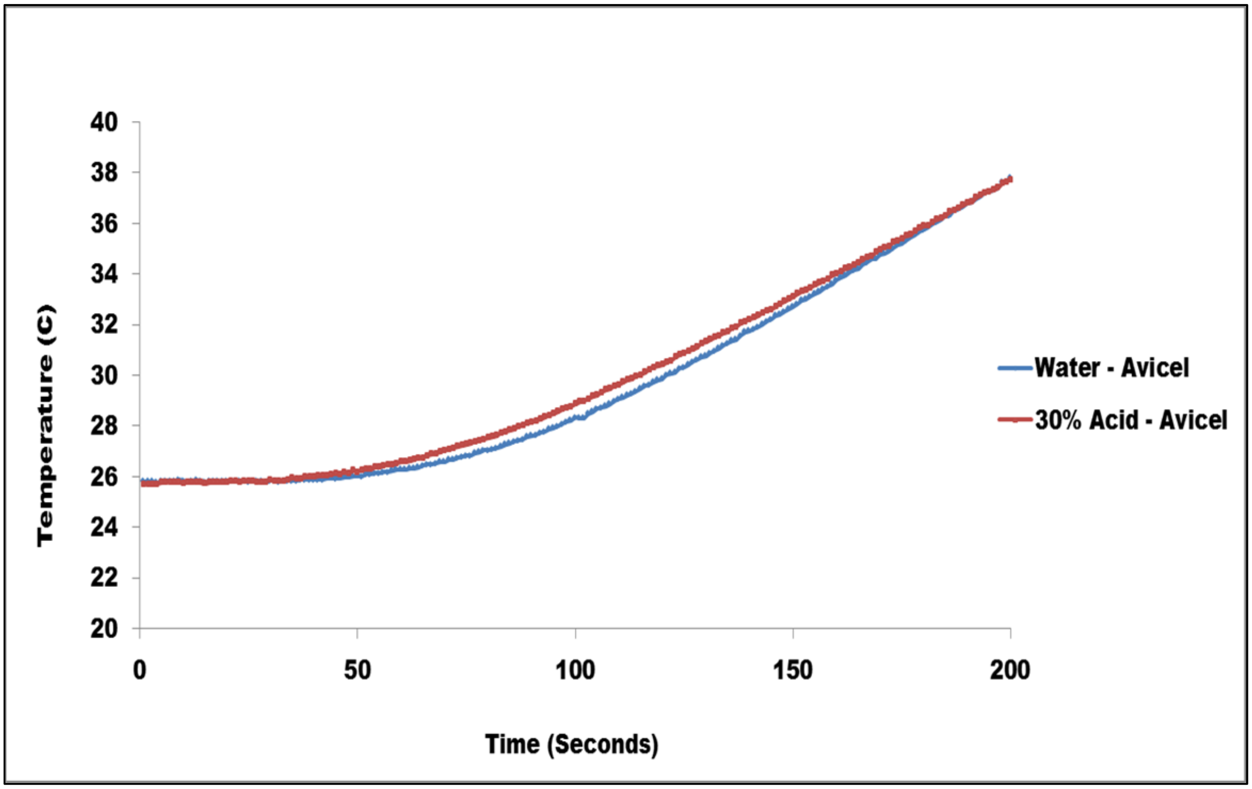

Figure 8.31: Comparative temperature-time profiles of 30% Sulfuric Acid-Avicel and Water-Avicel at 90°C

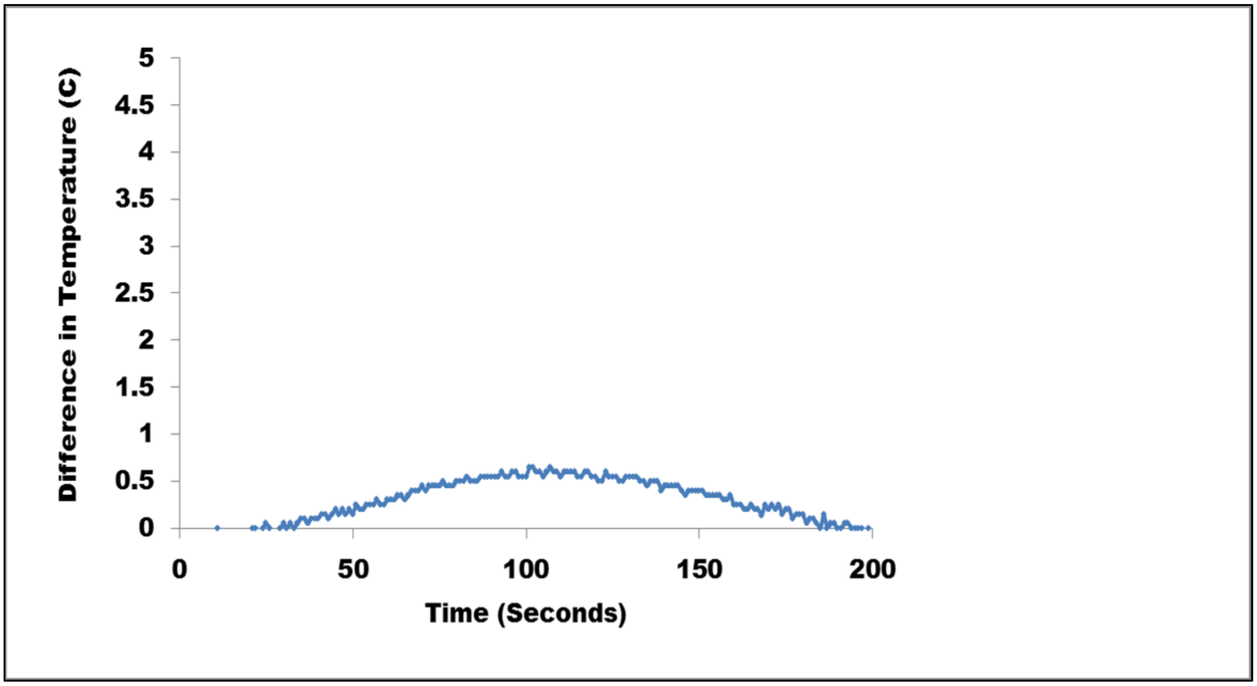

Figure 8.32: Difference in temperature between 30% Sulfuric Acid-Avicel and Water- Avicel at 90°C

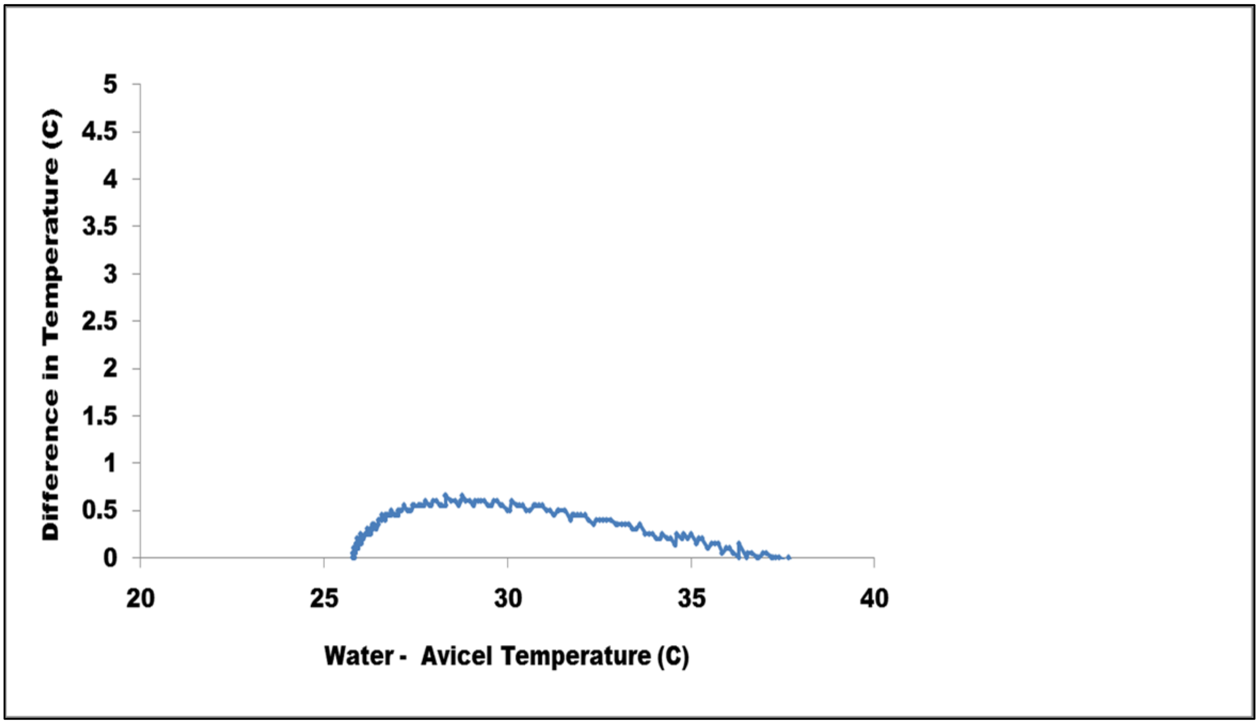

Figure 8.33: Difference in temperature change between 30% Sulfuric Acid-Avicel and Water-Avicel verses temperature of water-Avicel at 90°C

8.5. Conclusions

Decrystallization of cellulose occurs at room temperature with sulfuric acid up to 55-72%. Avicel crystallinity decreased substantially with increase in the acid concentration at room temperature and the decrease in crystallinity was from 67.9% to 4.2%. However, the decrease was substantially high for acid concentration above 65%. A sharp increase in temperature between Avicel-acid and Avicel-water could be observed within initial few seconds (15-25 seconds) with sulfuric acid up to 55-72%. However, temperature increase due to heat release was not entirely from decrystallization (hydrogen bond energy); a part of heat also came from hydrolysis of decrystallized cellulose to low DP cellulose and/or glucose. With the addition of 72% sulfuric acid, untreated Avicel released more heat than the acid treated samples and cellobiose. The heat releasing patterns and the crystallinity indices of 5 min and 30 min acid treated samples corroborate the fact that the higher the crystallinity, the higher the heat release. Initial enzymatic hydrolysis rates of 65-72% acid treated Avicel at room temperature were sharply increased, while rates were relatively sharper with 60% acid treated Avicel than with 55% acid treated and untreated Avicel. Data proves that decrystallization occurs at acid concentrations below 55%, if proper temperature is chosen. Avicel treated with 50% H_2SO_4 and at 90^oC for 5 min released heat significantly and the loss in crystallinity was by 9%.

9. Summary and Future Work

The primary focus of this dissertation was to identify different ways to improve monomeric sugars yields from different switchgrass varieties (Shawnee, Alamo, and Dacotah) using aqueous ammonia as a pretreatment reagent. As a part of this work, technical feasibility of uniform pretreatment conditions across the different switchgrass varieties to achieve similar sugar yields was studied. However, it was proved that applying uniform pretreatment conditions was not possible. Among the different switchgrass verities, Dacotah proved to be highly recalcitrant. After aqueous ammonia pretreatment, still many challenges (crystallinity, high lignin and glucan/xylan content) limit efficiency of the enzymatic hydrolysis of Dacotah. Therefore, to overcome these challenges and improve monomeric sugar yields, three different methods were attempted: applying a defined enzyme mixture that results in high yields of six and five carbon sugars from the pretreated switchgrass with the least amount of enzyme loading, addition of small amounts of water-soluble polymers during enzymatic hydrolysis, and addition of small amount of sodium sulfide during alkaline pretreatment. Mixed enzyme shows significantly higher digestibility than the cellulase alone on the basis of protein loading. Addition of water-soluble polymers shows positive effect on the hydrolysis of lignin free substrate as well as lignin-containing substrates. Sodium sulfide addition in alkaline pretreatment increases the delignification and does not degrade the carbohydrates. Sodium sulfide addition in alkaline pretreatment significantly increases glucan and xylan digestibilities.

Production of levulinic acid from lignocellulosics and soluble sugars by applying moderate acid concentrations and at low temperatures was also studied. Molar yield data indicate that it is technically possible to produce high levulinic acid yields from soluble sugars and lignocellulosic substrates at low temperatures and moderate acid concentrations in a single step.

One of the important factors that influence the monomeric sugar and levulinic acid production rate is crystallinity of cellulose. Therefore, a part of this work also focused on the fundamental understanding of decrystallization of cellulose. Study shows that decrystallization occurs at acid concentrations above 55% at room temperature. Study also shows the occurrence of decrystalization below 55%, if proper temperature is chosen.

The following points are suggested for the future study aimed at improving the conversion of lignocellulosics to monomeric sugars and levulinic acid:

a. Effect of β-xylosidase on xylose oligomers produced during the enzymatic hydrolysis of aqueous ammonia pretreated biomass

Xylose oligomers strongly inhibit cellulase activity (Kumar and Wyman, 2009a; Qing and Wyman, 2011). β-xylosidase proved to be highly effective in hydrolyzing xylose oligomers during the enzymatic hydrolysis of dilute acid treated solids (Qing and Wyman, 2011). However the most of reported studies are about xylose oligomers generated from dilute acid treated solids (Kumar and Wyman, 2009a; Qing and Wyman, 2011). But the possibility of presence of xylose oligomers with the alkaline treated solids is more than the dilute acid treated ones as dilute acid pretreatment retains only 5% of xylan, whereas alkaline pretreatment retains more than 60% of xylan (Pallapolu et al., 2011; Kim et al., 2011).

b. Effect of addition of different combination of polymers on enzymatic hydrolysis of lignocellulosic biomass

Most of the reported polymer addition studies during enzymatic hydrolysis were carried out using one polymer at a time (Borjesson et al., 2007a; 2007b; Hata, 2011; Mora et al., 2011), and the present work also followed the same pattern. However, it is worth studying the effect of addition more than one polymer during enzymatic hydrolysis to evaluate the synergic effect.

c. Techno-economic analysis of different schemes used for improving monomeric sugar yields

The techno-economic analysis can give an insight into different monomeric sugar yield improvement schemes:

- Use of enzyme mix
- Addition of sodium sulfide during pretreatment
- Addition of water soluble polymers during enzymatic hydrolysis

A thorough techno-economic evaluation of the various schemes can direct future research towards reducing the cost of sugars derived from lignocellulosic biomass.

d. Decrystalization studies with low acid concentrations (1-30%) and at higher temperatures (90-200^{o}C)

Earlier it was reported that occurrence of decrystallization with sulfuric acid concentrations above 60%. However, this study suggests the occurrence of decrystallization below 60%, if the right temperature is chosen. In this study, decrystallization was carried out up to 30% acid and at 90^{o}C. Therefore it is worth to study the decrystalization with the acid concentration below 30% and temperatures above 90^{o}C.

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