Ethanol from Lignocellulosic Biomass: Deacetylation, Pretreatment, and Enzymatic Hydrolysis

by

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Abstract

Chemical pretreatment methods applicable for switchgrass and corn stover were investigated to improve enzymatic hydrolysis and fermentation yields. Various pretreatment strategies were studied including acidic, alkaline and their combination.

Dilute acid pretreatment hydrolyses hemicellulose from biomass producing liquid rich in hemicellulose sugars and solid containing cellulose and lignin. Degradation products of biomass significantly inhibit the enzymatic hydrolysis reaction reducing the glucan digestibility by 63%, and xylan digestibility by 90% when the enzymatic hydrolysis was carried out with a 50/50 mixture of buffer and the pretreatment liquid. When the same mixture was subjected to simultaneous saccharification and fermentation (SSF), there was no ethanol production. Glucose, cellobiose and acetate were identified as strong inhibitors to cellulase activity; each showing 10%+ reduction of activity at less than 1 g/L. Fermentation under the SSF scheme can eliminate the inhibitory effects of sugars by concurrent consumption of the sugars in the broth. The concentration of acetate in the pretreatment liquid was found to be 13 g/L, which makes it the most potent inhibitor in the system.

To overcome the inhibitory effects of acetic acid present in dilute acid prehydrolysate, a two-stage processing of alkaline deacetylation followed by dilute acid pretreatment was investigated. The main advantage of this scheme is that the acetate in the feedstock is removed early in the process and does not interfere with the dilute acid pretreatment. Deacetylation with

5% Na_2CO_3 at 30ºC for 6 h followed by pretreatment with 1% H_2SO_4 at 130ºC for 12 h reduced the final acetyl concentration in hydrolysate by 73%. This improved the enzymatic hydrolysis yield of corn stover by 30% over dilute acid pretreatment alone.

In contrast to acids, alkaline reagents selectively remove lignin from biomass. This method has an advantage over acid pretreatment that it does not require detoxification of hydrolysate from the pretreatment process. Three different alkaline reagents were studied, namely, sodium hydroxide, ammonia and sodium carbonate in a pretreatment process named Soaking in Aqueous Alkali (SAAL). The SAAL pretreatment applying each of the three alkaline reagents attained above 75% glucan digestibility for switchgrass. For corn stover, the digestibility was significantly higher at 85%. Each reagent has its own merits: Ammonia is easily recoverable; NaOH is a strong base, thus requiring low dosage; Na_2CO_3 is a weak alkali, but much less expensive than NaOH and easier to recover. Sodium carbonate was further studied as a pretreatment reagent for herbaceous feedstocks.

The SAAL process using Na_2CO_3 was effective in delignification. It removed 50-70% of the lignin from switchgrass and from corn stover. Switchgrass is a more recalcitrant substrate than corn stover due to its high lignin content. It required higher severity pretreatment conditions. SAAL pretreatment on switchgrass applied under the conditions of 15% Na_2CO_3 at 90ºC for 24 h gave a glucan digestibility of 71% and xylan digestibility of 49% in the enzymatic hydrolysis using 30 mg total enzyme (cellulase + β glucosidase)/g glucan. Enzymatic digestibility of corn stover treated with SAAL under the conditions 15% Na_2CO_3 at 60ºC for 24h were 84% for glucan and 67% for xylan. Supplementation of external xylanase enzyme applied at the level of 10 mg/g glucan improved the glucan and xylan digestibility of switchgrass to 76%

and 64% and of corn stover to 86.5% and 78% respectively. Because of the low yield observed with switchgrass, high severity pretreatment conditions were explored. SAAL pretreatment at 150ºC improved switchgrass glucan digestibility to 81%. Under these conditions, however, 50% of xylan was lost from the biomass.

Sodium carbonate percolation (SCP) treatment was therefore investigated in an attempt to improve the enzymatic hydrolysis of switchgrass and to reduce the loss of hemicellulose during pretreatment. The SCP treatment applied under 15% Na_2CO_3 at 160ºC for 20 minutes achieved a glucan and xylan digestibility of 76% and 57% while reducing the xylan loss by half to 25% in comparison to SAAL. Xylanase supplementation further improved the digestibility to 86% and 78% for glucan and xylan respectively.

SEM images of treated biomass have shown a highly disrupted and porous surface. The BET surface area was also increased, which collectively provide a ground for increase in cellulose accessibility. However, the data were insufficient to deduce quantitative correlation the digestibility and surface area. FTIR of SCP treated lignin has shown a pattern comparable to that of NaOH treated (Kraft) lignin indicating that the mechanism of delignification is similar for the two reagents. Higher severity of the Kraft pulping process released larger fraction of the complex lignin, which is indicated by slower decomposition profile in TGA compared to SCP treated lignin.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

PL	Pretreatment liquor
10PT	10% tolerance
SAAL	Soaking in Aqueous Alkali
SCP	Sodium Carbonate Percolation
CS	Corn stover
SG	Switchgrass
DP	Degree of Polymerization
SSF	Simultaneous Saccharification and Fermentation
SSCF	Simultaneous Saccharification and Cofermentation

Chapter I Introduction

I.1 Motivation

Limited availability of fossil fuels and concerns with greenhouse gas emissions have motivated years of research on renewable sources of energy. Currently, nearly all renewable sources of energy such as hydroelectric, solar, wind, tidal, geothermal target conversion to electrical energy [1]. Since liquid fuels make up a large share of the energy demand, a replacement for these is essential. Ethanol from biomass is among the most promising replacements for non-renewable fossil fuels.

Starch and sugars (first generation feedstocks) have been investigated as the main feedstocks for ethanol production to this point. These substrates were available in limited quantities and could not be used as a complete replacement for petroleum based liquid fuels [2]. Second generation biofuels rely on lignocellulosic biomass as the feedstock for biofuel production. The focus of this study was placed on the use of these cellulosic feedstocks and their efficient conversion to sugars for ethanol production. Lignocellulosic feedstocks are highly recalcitrant against biological conversion. A substantial preprocessing is required to make it amenable to saccharification and fermentation.

The highly complex structure of biomass cell wall acts as a barrier to enzymatic hydrolysis and subsequent fermentation. Cellulose, hemicellulose and lignin form the main components of this matrix. Dilute acid pretreatment and hydrothermal pretreatments improve cellulose accessibility by partial removal of the hemicellulose fraction in biomass [3]. Hemicellulose hydrolysates generated from dilute-acid pretreatment contain toxins that strongly inhibit the biological reactions. The concentration of these toxins and inhibitors must be reduced to utilize the sugars in the hydrolysate effectively. To effectively carry out this task, the nature of the toxins and their specific action on the enzyme must be delineated. In alkaline pretreatments, the main reaction is delignification. Thus, most of the carbohydrate is retained in the solid. The bioconversion process can be carried out under a clean environment without the interference of inhibitors. In this dissertation, pretreatment methods using low-cost alkaline reagents were investigated with the following specific objectives:

I.2 Objectives

- To study the inhibitory effect of dilute acid pretreatment hydrolysate on enzymatic hydrolysis and identify the main inhibitory components.
- To study the effects of individual inhibitors on the enzymatic hydrolysis and to determine the tolerance of the enzyme against individual components.
- To seek methods of alleviating the problems concerning inhibitory effects of the pretreatment liquor.
- To investigate the feasibility of selectively delignifying feedstocks.

- To investigate the use of Na_2CO_3 as a pretreatment reagent for switchgrass and corn stover.
- To characterize physical properties of biomass after pretreatment using crystallinity, bond structures, surface area, surface morphology, and decomposition profiles.

Chapter II Literature Review

II.1 Need for biofuels

II.1.1 Environmental concerns

Conventional fossil fuels release carbon dioxide, NO_x, methane, and other such pollutants that are the main culprits of the greenhouse gas effect. Since petroleum is the most important source of energy available today and its major use is in transportation, suitable renewable replacements for transportation fuels are required [1]. Ethanol from biomass is among the most promising replacements for non-renewable fossil fuels in the transportation sector. Ethanol –gasoline mixtures are increasingly being used as alternative motor fuels as these can reduce air pollution by decreasing the amount of particulates and NO_x emissions [2]. Burning of ethanol as a fuel releases carbon dioxide which is used by biomass during its growth cycle. Thus there is no net increase in the amount of carbon dioxide released [2, 4].When we burn fossil fuels, CO_2 that was sequestered by plants millions of years ago is released, thus it is considered as a net increase in greenhouse gas (GHG) emissions [5]. Depletion of crude oil reserves is a concern as the demand for crude oil is increasing every year. Crude oil consumption in US has doubled in the last 30 years. Since the fossil fuel reserve is limited, our dependence on these fuels must be reduced [4, 6].

At present, US imports more than 70% of its crude oil from the Middle East. US dependence on foreign oil must be reduced to maintain its energy security. The US is taking initiatives to reduce its dependence on foreign oil [4, 7]. In one of these initiatives, the United States government approved the Energy Independence and Security Act of 2007, which mandates the production of 21 billion gallons of advanced biofuels by 2022, of which 16 billion gallons must be derived from lignocellulosic feedstocks [1, 4, 8].

Production of ethanol from biomass is an effective way to reduce crude oil consumption and environmental pollution [2, 5, 8].

II.1.2 Benefits of using biomass

- Using biomass for fuel generation reduces our dependence on fossil fuels, which are a fast depleting, limited resource.
- Biofuels release the same amount of carbon dioxide that was fixed by the plant during its growth. Thus they are carbon neutral. Burning of fossil fuels releases carbon dioxide that has been naturally sequestered over millions of years [9].
- Biofuels are a potential replacement to liquid transportation fuels that are currently imported into the country. Thus use of biomass for liquid fuel production can potentially reduce dependence on foreign oil [4, 5].
- Second generation biofuel plants use agricultural wastes, or dedicated energy crops that are not grown on agricultural land, or municipal solid wastes and biomass waste streams from chemical plants such as the pulp and paper industry [10, 11]. Thus, these have the potential to reduce dependence on agricultural grains.

II.2 Biomass: types and composition

II.2.1 Types of feedstock

Various types of lignocellulosic feedstocks are being used for ethanol production. First generation biofuels were produced from starches [12] (corn, wheat, etc) or sugars (sugar cane, energy cane, etc). These are easily digested by enzymes and converted to ethanol using micro-organisms such as *Saccharomyces cerevisiae* or *Escherichia coli.* The biomass yield per acre of these feedstocks is low and the price of the raw materials is increasing significantly due to their high demand. Second generation biofuels use lignocellulosic biomass such as agricultural residues, wood and herbaceous crops, and municipal solid wastes and paper mill sludge wastes. These 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 [13-15].

Woody biomass: These are of two types 1) Softwoods (pine) & 2) Hardwoods (poplar). Hardwoods have a lower level of lignin than softwoods. Softwoods have longer structural and conducting fibers while the hardwoods have shorter fibers The DP of softwood lignin is higher than that of hardwood lignin. The composition of lignin differs between the two types of woody biomass. Softwood lignin is derived from guaiacyl units which mainly originate from trans-coniferyl alcohol and ρ-coumaryl alcohol [16]. Hardwood lignin is composed of guaiacyl, syringyl and ρ-coumaryl alcohols [17]. Figure II-1 shows guaiacyl, syringyl and ρ-coumaryl alcohols and Table II-1 shows the average ratio of the three alcohols in hardwood and softwood species Hardwood lignin also contains more methoxyl groups than softwood. Softwood species generally show higher recalcitrance toward external chemical and enzymatic attack compared to the hardwood species of wood.

Agricultural Residues: These are the residues left over in the field after grains are harvested, for example, corn stover, wheat straw, and rice straw. These raw materials can be obtained from existing farmland. This biomass is traditionally left in the field or burned. Additional infrastructure is required for collection and baling of these residues [18]. These feedstocks are easier to convert compared to the woody biomass probably due to their lower lignin content.

Energy crops: Switch grass, hybrid poplars, energy cane, etc are crops that are grown for the sole purpose of using them for energy production [19]. 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 industry, that show higher conversions to ethanol. Aspen wood and maize feedstocks with low lignin content, that are easily pretreated at mild treatment conditions were reported by Sticklen et al., Hu et al., and Halpin et al. [20-22]. This is unlike the agricultural residues where the main objective is high yield of a safe food crop for the food industry [11].

Wastes: Municipal solid wastes and waste sludge from pulp and paper mills, recycled paper. etc are the main sources of this type of biomass. These contain 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 too require a pretreatment step before enzymatic hydrolysis not unlike any of the other feedstocks [23].

II.2.2 Biomass composition

Composition of biomass varies greatly with type of feedstock [14] as well as its species [24, 25], and the growth conditions, soil, climate, and fertilizers used [26]. The composition also varies with time of harvest [27], the length of time that the crop was growing in the fields [28], etc. Young plants have a higher ash content and lower lignin content than more mature plants [26, 28]. Table II-2 shows the average composition of various biomass species [14].

In general the three main components of lignocellulosic biomass are cellulose, hemicellulose and lignin.

Cellulose: Cellulose is a polymer of D-glucose units linked by β-1,4-glycosidic linkages [29]. Cellulose in a plant contains some amorphous regions and some crystalline regions [30]. The average crystallinity (measured using Segal method [31]) of cellulose in biomass ranges from 50% for corn stover to 80% for cotton [30]. Cellulose chains are bundled together in a microfibril. Each microfibril is bound by inter and intramolecular hydrogen bonds. O'Sullivan has summarized detailed model for the cellulose structure [32, 33]. Cellulose DP of higher plants lies in the range of 7,000-14,000 in the secondary wall and approx. 500-6000 in the primary wall. Data obtained from X-ray crystallography shows that glucose residues are arranged parallel in the 200 plane and they are interconnected by extensive H-bonding. Each 200 plane surface are connected with Van der Waals forces.

Hemicellulose: Hemicellulose is a complex, branched polymer containing various monomer sugars: glucose, galactose, mannose, xylose and arabinose, and sugar acids [34-36]. Softwood hemicelluloses have high mannose content while hardwoods agricultural and herbaceous hemicelluloses contain mainly xylose. The hemicellulose chains are shorter than cellulose (DP

100-200), are amorphous in nature and branched with short chains of sugars forming a matrix with the lignin, shielding cellulose from external chemical or biological attack [33]. They are the most thermal and chemical sensitive amongst the three components of biomass. At high temperatures (160-180°C) and also at mild acidic conditions, hemicellulose is the first to be hydrolyzed hence it is easy to recover as well as quick to degrade [37-39]. Acetic acid is present on the xylan backbone in a molar ratio of 3 – 6 depending on biomass composition. [40]

Lignin: Lignin is a complex, amorphous 3 dimensional polymer made of phenylpropane units. The polymer is made up of 3 different monolignols: P-coumaryl, coniferyl and sinapyl alcohol (Figure II-1) which are linked together via β-*O*-4, α-*O*-4, β-5, β-1, 5-5, 4-*O*-5 and β–β linkages [33, 41, 42]. The various ratios of the different monomers in different plant species is listed in Table II-1. It can be hydrolyzed with alkaline reagents such as NaOH, $Ca(OH)_2$, and NH_3 [43-47]. Alkyl ether linkages, are the most prevalent in the lignin structure, the second most prevalent linkages are alkyl-alkyl (C-C). Ten percent of these linkages are ester linkages. Detailed studies on the degradation kinetics of lignin in Kraft pulp mill were first discussed by Gierer in 1980 [42, 48, 49]. Table II-2 is a compilation of the composition of different types of biomass [14, 46, 50-53]. The DP of the lignin molecule is in the range of 450-500. The structural units are highly cross linked which leads to an amorphous structure. Lignin forms a hydrophobic sheath to make it more resistant to microbial and chemical attack.

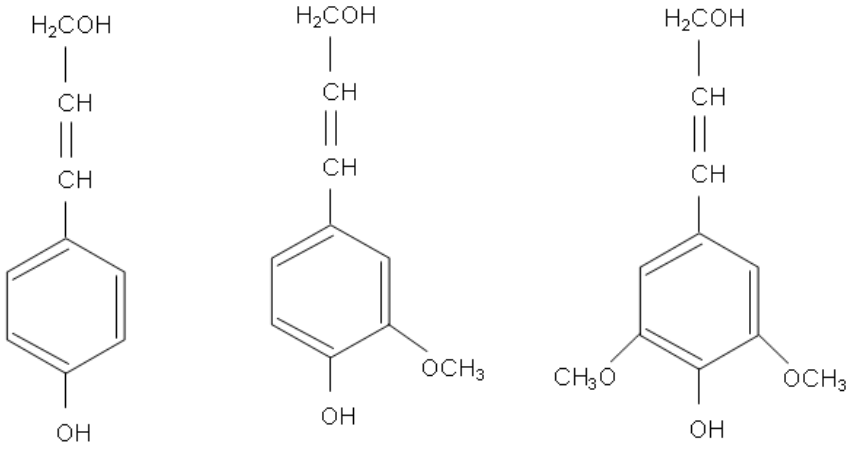


Figure II-1. Three lignin monomers (a) ρ-coumaryl / hydroxyphenyl (H); (b) coniferyl / guaiacyl (G); and (c) sinapyl / syringyl alcohol (S).

Table II-1. Ratio of the lignin monomers in plant species.

	H / G / S ratio (%)
Softwood	(2-18) / (82-98) / trace
Hardwood	0 / (22-66) / (44-86)
Herbaceous plants	(5-26) / (27-54) / (44-67)

The ratio of the three lignin monomers H: (hydroxyphenyl) ρ-coumaryl alcohol, G: (guaiacyl) coniferyl alcohol and S: (sinapyl) syringyl alcohol as found in lignin of softwood, hardwood and herbaceous plant species

Ash: In addition to the three main components, the biomass contains minor components such as extractives, and ash. Water soluble and ethanol soluble extractives are present in native biomass at 5-20% by weight. These include chlorophyll, waxes, terpenes and proteins that form an important part of the plant defenses against microbial decomposition. Waxes help prevent excessive loss of water from the living plants. Biomass such as sugarcane molasses contains non structural sugars such as sucrose, glucose or fructose that are included in extractives [54]. The

inorganic material present in the biomass is measured as the ash content. It is expressed as the percentage of solids remaining after dry oxidation of the biomass at 600ºC. At this temperature, the organic material namely, cellulose, hemicellulose, lignin, extractives and proteins decompose. Ash content is anywhere between 0.5 – 11% of the dry weight of biomass [28].

Table II-2. Composition of different biomass feedstocks.

	wt % of dry biomass		
Feedstock	Cellulose	Hemicellulose	Lignin
Hardwood	40-55	20-40	18-25
Soft wood	45-50	25-35	25-35
Grasses	25-40	35-50	17-30
Straws	30-40	20-30	15-25
Waste paper	60-70	10-20	5-10
MSW	8-15	ND[+]	24-29

Numbers are %wt on the basis of dry biomass. [+]ND= not determined.

II.2.3 Need for pretreatment

The complex nature of biomass which is cellulose microfibrils covered with a lignin-hemicellulose matrix, stabilizes it against external chemical and microbial attack. In addition to the chemical structure, physical characteristics such as biomass crystallinity, high degree of polymerization and low accessible surface area are also barriers to enzymatic attack. Thus enzymatic digestibility of untreated biomass is generally very low, in the range of 2 to 12%. To increase this digestibility, Sticklen et al., have reported work on genetically modified plants which would be easier to hydrolyze [20]. For regular plants (non-genetically modified), an

external pretreatment which would break down one or more of these barriers is a very important step in the bioethanol production process.

In general any pretreatment must have the following attributes:

- Efficient utilization of sugars from cellulose and hemicellulose in the process with minimal pretreatment degradation losses.
- Efficient enzymatic hydrolysis and fermentation to achieve maximum ethanol yield from cellulose as well as hemicellulose sugars at low enzyme cost
- Integrated process to minimize use of energy since biofuel production from biomass is an energy intensive process, and to reduce water consumption.
- Efficient use of lignin or other byproducts, since hemicellulose can be used for production of value added products rather than ethanol.

Pretreatment affects many characteristics of the plant material that impede digestion including cellulose crystallinity, lignin content, acetyl linkages, and the complex hemicellulose-lignin shield that surrounds cellulose in the plant cell wall. For industrial applications, a pretreatment must be effective economical, safe, environmentally acceptable and easy to use [55].

II.3 Pretreatment effects and its types

II.3.1 Effects of pretreatment

All pretreatments cause one or more of these changes in biomass:

- Size reduction

- Degradation of one or more of the main components of biomass - cellulose, hemicellulose, or lignin
- Increase in surface area and porosity of biomass
- Change in crystallinity and DP of cellulose

These effects in turn increase the enzymatic digestibility of treated biomass. Although each pretreatment method has a different effect on the biomass, most methods show improved enzymatic hydrolysis rates compared to untreated sample. This emphasizes our lack of thorough understanding of the complex nature of biomass.

Figure II-2 is a schematic representation of the three main components of biomass and the effect of pretreatment on these components [3]. In addition to physical disruption, there is a chemical breakdown of the components of biomass – cellulose, hemicellulose and lignin [3].

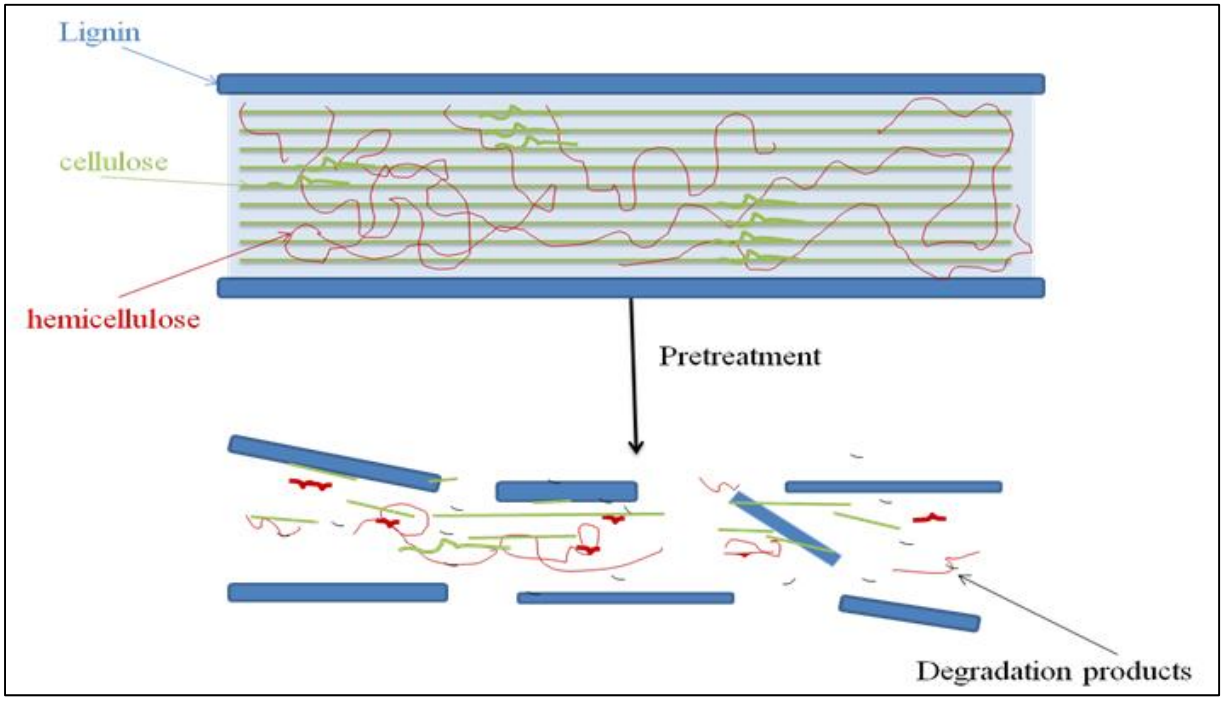


Figure II-2. Effect of pretreatment.
(Modified from figure by Mosier et al., [3])

II.3.2 Types of pretreatment

II.3.2.1 Physical

The high degree of polymerization (DP), crystallinity and low accessible surface area are important factors affecting the low enzymatic hydrolysis yield of untreated biomass. Physical treatments are aimed at size reduction and mechanical decrystallization. Some physical pretreatments such as ball milling or irradiation also change one or more structural features of the biomass. However, physical pretreatment methods are limited in their effectiveness with respect to increasing enzymatic digestibility and are often expensive.

- **Milling**: Milling reduces particle size and crystallinity and increases surface area and bulk density [56]. Increasing bulk density is an important advantage as it allows the use of high substrate concentrations. This helps in reducing reactor volume and thus the capital cost. But milling is highly energy intensive which makes it prohibitive for use as a pretreatment reagent at a large scale. Use of high temperature helps in increasing yield of reducing sugars after pretreatment. Different types of milling known to show good results are ball milling, two-roll milling, hammer milling, etc [56-58]. Prolonged milling reduces cellulase enzyme accessibility to the cellulose due to the collapse of pore structure and agglomeration of particles. Simultaneous milling and Saccharification for lignocellulosics has been reported to give near quantitative sugar yields at low enzyme loadings [59, 60]. Although this process seems to give high sugar yields, the amount of energy used makes it a highly expensive pretreatment method.
- **High energy radiation**: Radiation treatments break down the lignin-cellulose complex in vitro for the biomass. This significantly improves acidic or enzymatic hydrolysis of lignocellulosic biomass. Microwave or γ-radiation is typically used for pretreatment. γ-

irradiation alone ruptures macromolecules in cellulose fibers in vacuum and in air. Other effects of radiation are a decrease in the degree of polymerization and an increase in the concentration of functional groups and in the tendency to undergo hydrolysis. Numerous long lived free radicals are also formed [61]. Irradiation in presence of catalysts such as inorganic salts, acids or alkali improves the effect of radiation pretreatment on the samples [62, 63]. The Issue of safety prohibits the use of radiation pretreatment at a large scale [62].

- **Pyrolysis:** Pyrolysis involves temperatures as high as 200°C. Biomass is heated at desired temperature in a gaseous atmosphere. The type of gas used affects the result of pretreatment greatly. There could be depolymerization, dehydration, and oxidation reactions in presence of air, while in presence of inert atmosphere dehydration and oxidation reactions may not occur thus reducing by-product formation [64]. Pyrolysis is typically used for production of biochar, charcoal or for gaseous fuels such as syngas [65, 66]. Recently it has also been used for conversion of biomass to bio-oil or bio-crude [67-69]. Pyrolysis shows higher yields when coupled with other pretreatment methods and reagents and catalysts like acids (CO_2, SO_2), sulfuric acid, etc [14, 69].

II.3.2.2 Chemical

Chemical pretreatments are most widely studied. These generally break down specific components of the biomass and increase the cellulase enzyme accessibility to cellulose. A wide variety of chemical agents have been reported for use in pretreatment of biomass such as non-aqueous cellulose solvents, sodium hydroxide, aqueous ammonia, calcium hydroxide, carbonates and other inorganic salts, alkaline hydrogen peroxide, SO_2, CO_2, sulfuric acid, hydrochloric acid, acetic acid, and solvents such as ethanol and methanol in the presence of acid or alkaline

catalyst. These can broadly be classified into three categories: Acidic, Neutral, and Alkaline. In addition to these chemicals, ionic liquids and other solvent extraction methods are also being studied as pretreatment agents by Dadi et al [70].

- **Acidic pretreatment**: Dilute acid (DA) pretreatment uses various acids such as sulfuric acid, hydrochloric acid or nitric acid at low concentrations (0.5-1.5%) and at temperatures of around 160ºC [37, 47, 71]. One of the main advantages of this pretreatment is that it has been shown to be effective with most of the lignocellulosic feedstocks. The main effect of dilute acid treatment is hydrolysis of hemicellulose in biomass. Lignin and cellulose do not hydrolyze at the pretreatment conditions. Removal of 75 – 90% hemicellulose increases the subsequent enzymatic hydrolysis rates significantly. Another effect is of DA pretreatment is a decrease in the DP of cellulose. Cellulose crystallinity is not affected [14, 52, 53, 71, 72]. Since hemicellulose sugars can be used for fermentation by some organisms that convert them to ethanol, the liquid stream rich in hemicellulose is added back to the bioreactor (Figure II-3). This requires detoxification of the liquid stream as in addition to sugars, this stream also contains a number of compounds inhibitory to the enzymes and micro-organism. Some studies suggest having a separate bioreactor for the pentose-rich liquid stream, which would also require a detoxification step [73]. Thus the process requires high capital investment with respect to a corrosion resistant reactor system, detoxification of liquid hydrolysate, and neutralization of pretreated solids is required before the enzymatic hydrolysis step. In high severity pretreatments, lignin may break down and recondense and precipitate.

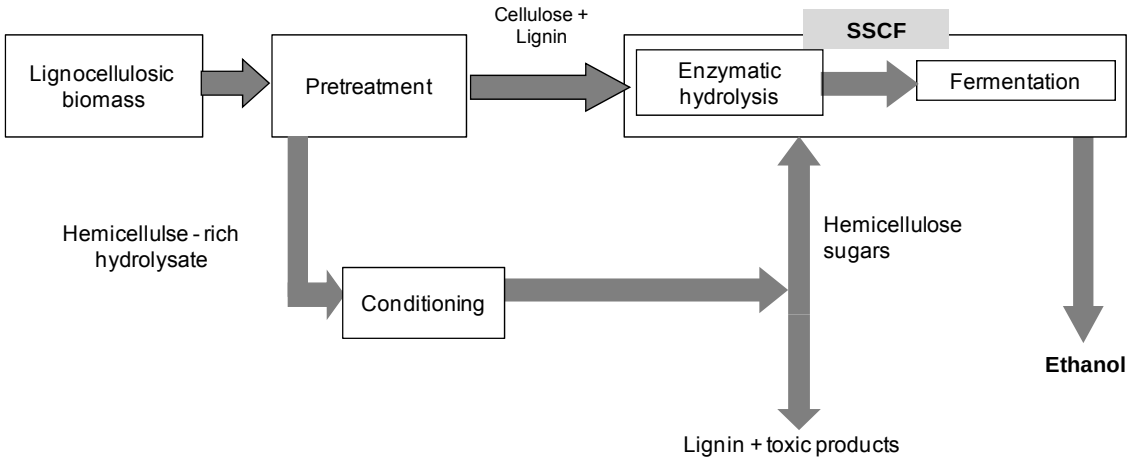


Figure II-3. Process diagram for dilute acid pretreatment of biomass for ethanol production.

- **Neutral pretreatment**: This type of pretreatment uses water as the pretreatment reagent as it is an environmentally benign, non toxic and inexpensive reagent, it forms a great reaction medium. Liquid hot water (LHW) pretreatment is also known as autohydrolysis as in this pretreatment, water at a high temperature ionizes and reacts with the biomass and releases some acetic acid, reduces pH and further catalyzes hemicellulose degradation not unlike the dilute acid pretreatment process. To keep the water in the liquid state at high temperatures, the pretreatment is carried out at a high pressure. In optimized LHW pretreatment process, some of the lignin and hemicellulose is hydrolyzed, thus increasing the enzymatic hydrolysis of remaining solid [39, 62, 74-77].
- **Alkaline Pretreatment:** The main effect of all alkaline reagents is delignification and swelling of biomass. The swelling and delignification together increase the surface area and makes biomass more accessible to enzymes and microorganisms. At high concentrations of alkali, peeling and hydrolytic cleavage of carbohydrates can occur resulting in loss of sugar to carbon dioxide or even carbon. Dilute alkali are used extensively for pretreatment of biomass. NaOH has been used for a long time in the pulping industry for delignification of

wood [49]. Lime, NaOH, and Ammonia (aq.) are the main reagents studied for pretreatment [43, 44, 46]. Sodium hydroxide and lime are stronger reagents as compared to ammonia. NaOH is known to cause the peeling and random cleavage reactions in polysaccharides causing a significant loss of sugars [32]. Ammonia being a milder alkali helps by delignifying biomass while easily retaining most of the hemicellulose in the solid fraction of biomass. Ammonia pretreatment shows high enzymatic hydrolysis yields with agricultural residues such as corn stover, wheat straw or even switchgrass species that have a low lignin content as compared to hardwoods and softwoods. For lignocellulosic feedstocks with higher lignin content which are more recalcitrant, NaOH is a better delignifying reagent [46]. The main disadvantage of these alkaline regents is that they are expensive. Calcium hydroxide (lime) is considered as the best choice for an alkaline regent as it is the most inexpensive among the most commonly used alkaline reagents. Recovery of the reagent is an important part of an alkaline pretreatment process without which process economics may become prohibitive. Since high amount of xylan is retained in the solid, an enzyme cocktail which contains high xylanase activity is more beneficial than the one containing cellulase alone. Alkaline pretreatments have a few advantages over other chemical pretreatments of biomass to form ethanol [3]

- They have a high selectivity for reaction with lignin over carbohydrates.
- The liquid hydrolysate stream has less sugars, thus can be eliminated from bioprocess before the fermentation step, unlike acidic pretreatments.
- The fermentation efficiency is high because of the absence of toxins and inhibitors, which are taken out with the liquid stream.

- Cellulose as well as hemicellulose sugars are retained in solid and can be used in the fermentation

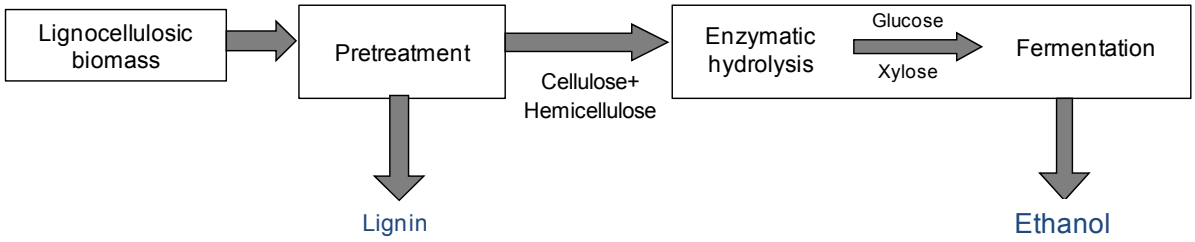


Figure II-4. Process diagram for alkaline pretreatment of biomass for ethanol production.

II.3.2.3 Solvent extraction methods

Various organic solvents selectively remove one of the components of biomass. These are thus very good for fractionation of biomass into its components cellulose, hemicellulose and lignin. Concentrated sulfuric and phosphoric acid are known to completely dissolve cellulose, which can be reprecipitated using ethanol or methanol. The DP of sulfuric acid treated cellulose falls drastically and the crystallinity also decreases substantially [71].

Ionic liquid pretreatment is gaining interest in recent years [70]. This pretreatment selectively dissolves carbohydrates, which are reprecipitated easily by adding an anti-solvent such as water or an organic solvent (e.g., ethanol, methanol). The main problem with these solvent extraction methods is that the cost of the solvent or the ionic liquid is too high to be practical at a large scale.

II.3.2.4 Physicochemical pretreatments

These methods are a combination of physical and chemical methods. Pretreatments consisting of high pressure steaming followed by de-pressurization (explosion), with or without additional

catalysts fall into this category. The high pressurization and depressurization pulverizes the biomass and thus increases the surface area more than simple chemical pretreatments.

- **Steam explosion**: The difference between steam treatment (auto hydrolysis) and steam explosion is that in steam explosion pretreatment, there is a rapid depressurization which shatters the physical structure of biomass. Steam explosion treatment causes hydrolysis of a majority of the hemicellulose same as the acid and neutral pretreatment methods. It also releases degradation products toxic to microorganisms and enzymes. Some of the cleaved lignin precipitates in the acidic environment. This method has been proven to work for most lignocellulosic biomass. High glucan yield after enzymatic hydrolysis has been achieved with corn stover, wheat straw, hybrid poplar and other feedstocks. Xylose is recovered in the hemicellulose stream in a similar manner as acid and neutral pretreatments [75, 78].
- **Acid catalyzed steam explosion**: In this method, sulfuric acid or SO_2 is impregnated into the biomass before steam explosion [79]. Adding an acidic catalyst improves the enzymatic hydrolysis of the treated biomass significantly. The main advantage of acid (or SO_2) catalyzed steam pretreatment is that it can effectively pretreat even the highly recalcitrant substrates like softwoods and high lignin containing hardwoods [79, 80]. Sulfur dioxide release must be contained in an industrial setting since it is highly hazardous and environmental laws regarding SO_2 release into the atmosphere are very stringent. This increases the cost of the pretreatment, in addition to the expensive high pressure acid-resistant reactors.
- **Ammonia fiber explosion (AFEX):** This method involves liquid ammonia and steam explosion, and is one of the leading pretreatment processes studied by Teymouri et al., Zhong et al., Balan et al., [45, 81, 82]. Ammonia Fiber Explosion (AFEX) treats biomass using

concentrated ammonia under pressure and then suddenly releases the pressure. This causes ammonia to be released and recovered, and biomass to be disrupted. The process does not contain a liquid stream and it does not remove either hemicellulose or lignin. In spite of this, it significantly improves the enzymatic hydrolysis of various lignocellulosic feedstocks such as corn stover, wheat straw, rice straw, bagasse, sweet sorghum etc. This process requires that ammonia be recycled for environmental reasons and also uses high pressure equipment.

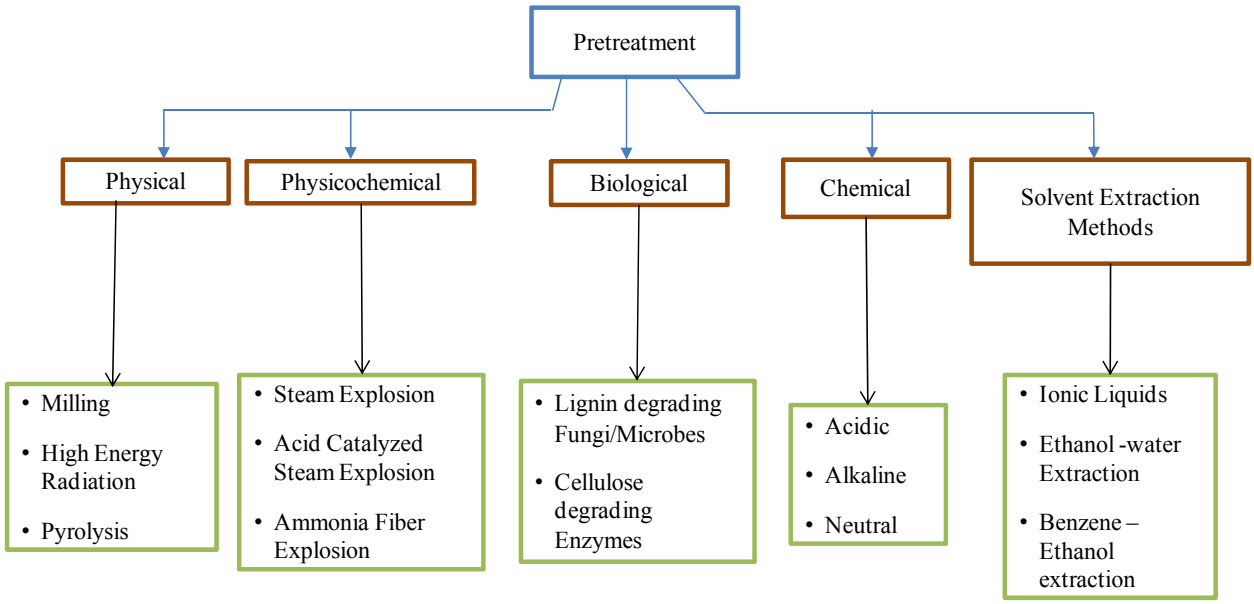


Figure II-5. Types of pretreatment.

II.3.2.5 Biological pretreatment

Biological pretreatment uses lignin or cellulose degrading enzymes and microorganisms or fungi such as the white rot fungi for pretreatment [32]. One advantage of biological treatment is that the enzymes and microbes are highly specific, rarely produce any inhibitors or toxins and effectively degrade lignin. But they are very slow with reaction times reaching anywhere between 5 days to a month as compared to chemical or physical means where reaction times range from 10 or 20 min to about 24 h for most pretreatments.

II.4 Alkaline reagent recovery methods

II.4.1 Ammonia:

Ammonia recovery process involves a solid liquid separation step after pretreatment, followed by evaporation of the ammonia. Process for the Soaking in Aqueous Ammonia (SAA) process is depicted in Figure II-6, modified from that reported by Furcht et al [56]. As another method, ammonia can be recovered by flashing followed by steam stripping of the pretreated slurry. The resulting ammonia vapor can either be recompressed or quenched using cooling and chilled water [83]. The solid pretreated biomass can be sent to a bioreactor for enzymatic digestion and microbial fermentation.

II.4.2 Lime:

Lime ($Ca(OH)_2$) is recovered by neutralizing the effluent from lime pretreatment of biomass using CO_2 to form $CaCO_3$. A lime kiln is used for regenerating lime from the calcium carbonate [84-86]. The Lime kiln for recovery is an established process in the pulp and paper industry [87]. Using this process, anywhere between 20-80% of the lime [84, 86] can be recovered, depending on the biomass and pretreatment conditions.

II.4.3 *Sodium hydroxide*:

As shown in Figure II-7, sodium hydroxide can be recovered using the process that is used in the Kraft pulping industry. After pretreatment, the lignin rich liquid stream (black liquor) is sent to an evaporator for concentration and then to a recovery boiler at 1100ºC where lignin is burnt for recovering energy for the process. The smelt left behind is mainly sodium carbonate. When causticized with lime, sodium carbonate solution (green liquor) is converted to sodium hydroxide (while liquor), which is recycled. The spent lime (calcium carbonate) is recovered in the lime kiln at 1200°C [87].

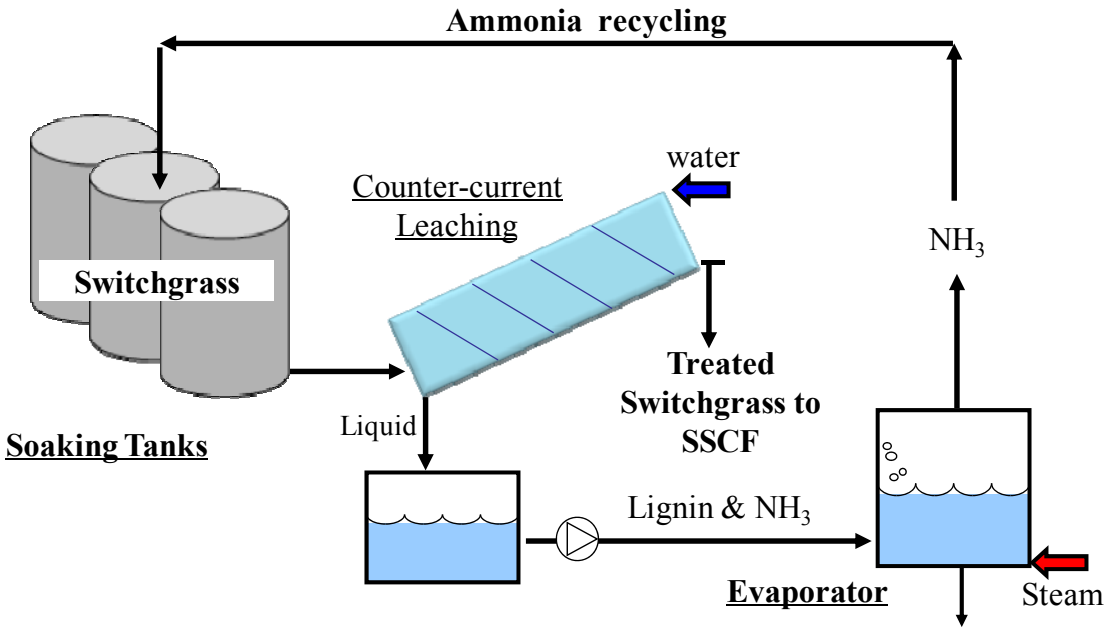


Figure II-6. Soaking in aqueous ammonia (SAA).
Recovery process based on [56]

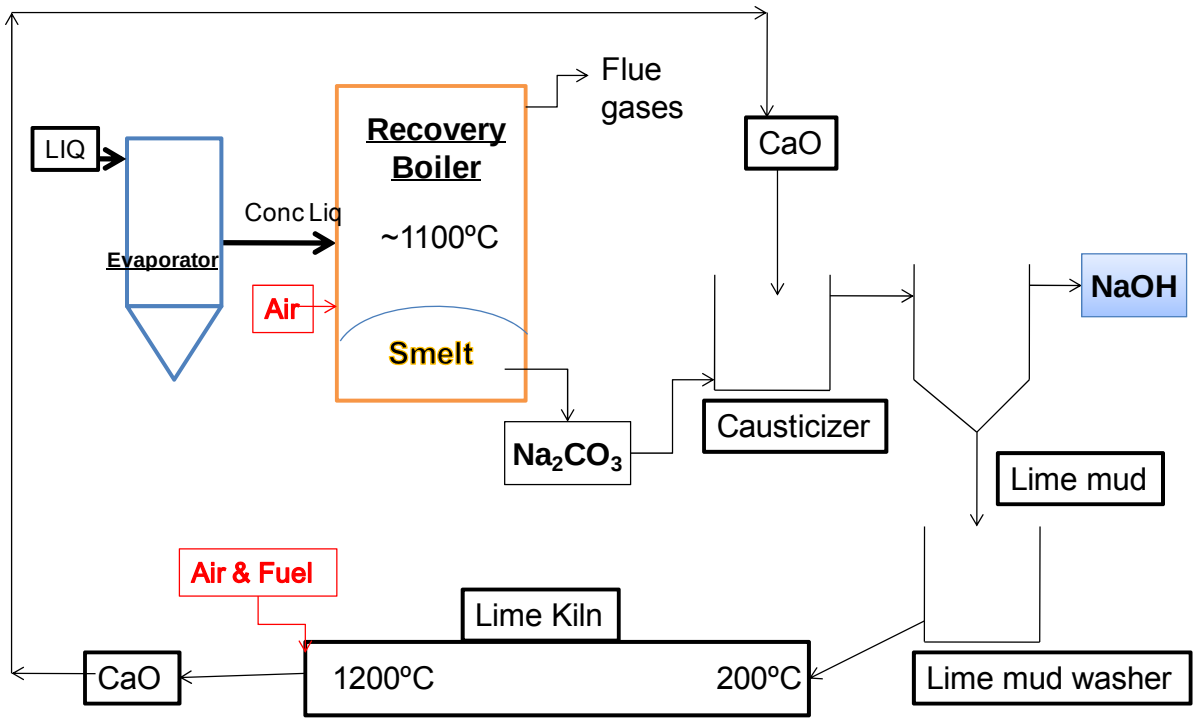


Figure II-7. Recovery process for NaOH.
Based on [87]

II.5 Degradation of carbohydrates after pretreatment

Chemical pretreatments are specific in their effects on lignocellulosic biomass. After pretreatment, one or more of the components break down. Various reaction mechanisms have been proposed for biomass hydrolysis using alkaline, acidic and neutral/steam pretreatments.

II.5.1 Acidic degradation

In acidic or neutral pretreatments, around 50 to 80% of the hemicellulose hydrolyses into the liquid stream. For effective utilization of these sugars, this liquid stream must be added to the

bioprocess (Figure II-3). Under high temperature acidic conditions, the hydrolyzed hemicellulose oligomers and monomers further degrade into a number of other products including acids and aldehydes (Figure II-8). Pentoses degrade to furfural while hexoses form hydroxymethylfurfural (HMF). Acetic acid is an initial product from the hydrolysis of hemicellulose, and lignin which are acetylated to some extent in the biomass [88, 89]. HMF under high temperature and acidic conditions is converted to levulinic and formic acid. Cellulose and lignin also degrade to a small extent during dilute acid pretreatment as is seen in Figure II-8.

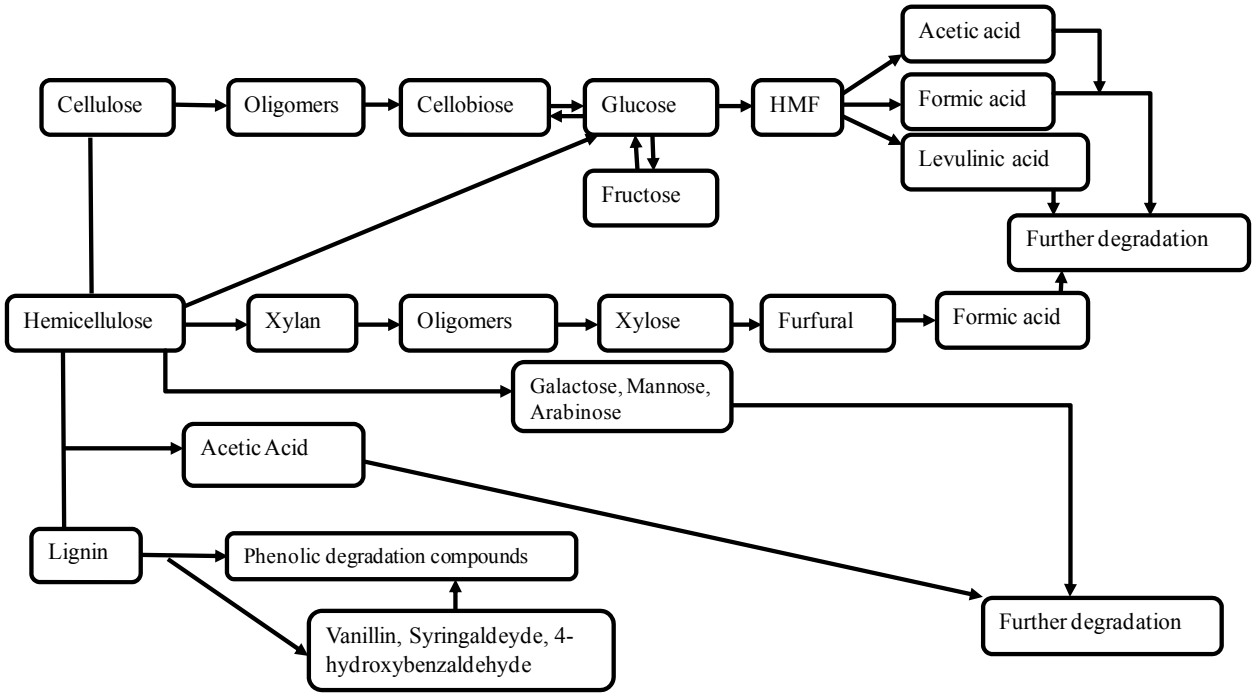


Figure II-8. Degradation products from acid pretreatment.
Adapted from various sources [29, 38, 88-90]

II.5.2 Alkaline degradation

Cellulose & Hemicellulose: Alkaline degradation of hemicelluloses causes swelling, dissolution, saponification, reprecipitation, peeling and glycosidic cleavage. Under strong alkaline conditions

carbohydrates undergo a “peeling” reaction. Classical peeling causes stepwise shortening of the polymer at the reducing end. The single glycosyl residue released and is converted to an acidic moiety. The new polymer generated contains another reducing end which reacts with alkali to further shorten the chain length by one unit. This reaction goes on until a stable end group such as an acidic group is formed. “Random cleavage” of glycosidic linkages also occurs in alkaline conditions to a smaller extent. At high temperatures (150ºC) the peeling reaction is faster than the alkaline hydrolysis of the glycosidic linkages [91-93].

II.6 Degradation of lignin after pretreatment

II.6.1 Alkaline degradation

Degradation of lignin under alkaline pulping conditions has been well established by Gierer, Chakar et al., [42, 87]. Lignin is a complex three dimensional polymer of phenylpropane units, covalently linked to cellulose and hemicellulose forming a cross-linked matrix [94]. It is considered as one of the main factors for recalcitrance of biomass to enzymatic hydrolysis [51]. It acts as an obstacle for accessibility of cellulose by enzymes and slowing down absorption of enzyme to the biomass surface [95]. The lignin polymer is made up of three different monolignols: p-coumaryl (hydroxyphenyl), coniferyl (guaiacyl) and sinapyl (syringyl) alcohols which are linked together via β-*O*-4, α-*O*-4, β-5, β-1, 5-5, 4-*O*-5 and β–β linkages between the aryl and aliphatic components of the monomers [33, 41, 42]. Many studies in degradation kinetics of lignin during alkaline pulping processes have been published in literature [42, 49, 87, 96]. Each of them states that α and β aryl ether linkages constitute 70% of the total linkages in

lignin and are most easily disrupted in alkaline conditions [42, 96], diaryl ether, diaryl and C-C linkages are relatively stable. Syringyl lignin units are more susceptible to hydrothermal degradation than guaiacyl lignin units (refer to Figure II-1) [89].

The degradation of α and β-aryl-ether bonds in phenolic units follow a first order kinetics with respect to the starting structure, provided the phenolic hydroxyl group in the substrate is completely ionized and pH exceeds 11. The reaction is independent of hydrosulfide ions. These reactions form 20-30% of the degradation reactions in lignin [42]. β-aryl-ether bonds of non-phenolic units follow first order kinetics with respect to OH- ions as well as the reacting species, these reactions are relatively easily cleaved under strong alkaline conditions. These bonds together constitute 65-70% of lignin present in biomass [42].

II.6.2 Acidic degradation

Lignin degrades under acidic pretreatment conditions into a large variety of phenolic, aromatic and aldehydic monomers and oligomers. Low molecular weight phenolic compounds have a strong inhibitory effect on enzymes and micro organisms. Vanillin, syringaldehyde and hydroxybenzaldehyde are some of the inhibitory compounds released under acidic hydrolysis. Lignin is soluble to a small extent in acidic medium.

II.7 Effect of degradation compounds on microorganisms and enzyme

II.7.1 Acetic acid

Acetic acid inhibits production of cell mass. Acetic acid toxicity is pH dependent. Organic acids inhibit the growth and metabolism of *E coli*. This in turn reduces yield, titer, and productivity. Acetate concentrations as low as 0.5g/L have shown to inhibit cell growth by 50%. Weak acid in undissociated form, permeates the cell membrane and goes into the cytoplasm. Once inside the cytoplasm, they dissociate into an anion and proton. This reduces the internal pH of the cell, giving an acidified cytoplasm. The acidic cytoplasm inhibits synthesis of macromolecules such as amino acids and DNA, thus inhibit growth [97-99]. Formic acid is more toxic due to its extraordinarily high permeability through the membrane, but is found at low concentrations in the dilute acid pretreatment liquor. The anions accumulate inside the cell and affect the cell turgor pressure.

II.7.2 HMF and Furfural

Furfural is a key inhibitor in the hydrolysate because it is toxic by itself and also acts synergistically with other inhibitors. Furfural has shown a significant effect on multiple glycolytic enzymes that are essential to central metabolism of the cell and fermentative enzyme function; HMF shows similar effects on microorganisms. DNA treated with furans leads to single-strand breaks at sequence sites with three or more adenine or thymine bases [99]. Palmqvist et al reported that a longer lag phase was seen during growth because of the toxic effect of furans [90].

II.7.3 Phenolics

Phenolic compounds from lignin degradation cause a loss of integrity of biological membranes and other hydrophobic targets. This affects their ability to serve as selective membranes and enzyme matrices, inhibiting cell growth as well as sugar assimilation. Aromatic acids cause partial membrane leakage while aromatic aldehydes cause no significant damage. Vanillin, a phenolic aldehyde also causes partial disruption of potassium gradients in *E. coli*. Membrane destabilization is experienced by 29% of the population after treatment with vanillin [99].

II.8 Detoxification methods

II.8.1 Biological detoxification methods

Enzymes such as laccase, peroxidase, and acetyl xylan esterase can be used for removing acetic acid and phenolics from the pretreatment liquor. The reaction time is fairly short, at 1 h and up to 90% of the toxic compounds are removed. Mutant strains of *S cerevisiae* can assimilate acetic acid and furfural. Using a large concentration of inoculum in the fermentation broth reduces the concentration of furfural and HMF due to assimilation. Microorganisms can also be adapted to tolerate large concentrations of inhibitors. This method is known to work well with *S cerevisiae* since it can also metabolize furfural. Some bacterial cultures can also be adapted to the inhibitors present in pretreatment liquor (PL)[100].

II.8.2 Physical

Methods such as vacuum evaporation/roto-evaporation remove volatile inhibitors such as acetic acid and furfural from dilute acid pretreatment liquor [101]. Another method for removing volatiles from the hydrolysate is passing steam through hydrolysate to bring temperature up to 90ºC. Phenolics from lignin degradation (non-volatile components) are usually more inhibitory to enzyme and microorganism than the volatiles such as furfural. Since these were not eliminated using the evaporation methods, these must be combined with other methods such as charcoal adsorption. Using steam stripping method, under the high temperature and acidic conditions a significant loss of sugars is also reported [99, 102].

Cheng et al, reported more than 90% acetic acid removal with less than 5% loss of sugars using electrodialysis (membrane separation). Use of membranes tends to be more expensive than other methods of detoxification [103]. Ion exchange resins have reported 80% decrease of total phenolics, almost all levulinic acid, formic acid, 70% of furfural and HMF. Anionic resins work better than cation or non-charged ion exchange resins. The main disadvantage of using this method is the high expense [104]. About 10% of the sugars also degrade using the ion exchange treatment.

Treatment of the hydrolysate with solvents can also reduce specific inhibitors from hydrolysate. Organic solvents such as ethyl acetate can reduce the phenolics content from the hydrolysate. Remove phenolics/lignin from solid or liquid (eg. ethyl acetate) [101]. Diethyl ether removes 56% acetic acid and almost all of furfural, vanillin, 4-hydroxybenzioc acid [102].

II.8.3 Chemical

II.8.3.1 Overliming and other alkali

Overliming is one of the most common methods of detoxification. The liquid is treated with lime (CaO) until the pH reaches 10. This is followed by the addition of acid to reduce the pH to 5 and separation of the precipitated solids. This method is effective in reducing phenolic content, ketones and furans; its effect on acetic acid removal show conflicting results. This method is reported to remove some part of the sugars as well. Handling of gypsum and calcium oxalate precipitates formed during this process is a problem with this method [73, 101]. The solids must be removed using centrifugation prior to fermentation.

Other alkaline reagents such as NH_4OH, NaOH, $Ca(OH)_2$ can show similar results with the hydrolysate. When ammonia is used, it helps with detoxification, any remaining ammonia can act as a nutrient for microorganisms during the fermentation step. Calcium hydroxide precipitates the toxic compounds more easily than other alkali. Treatment with reducing agent sodium sulfite in addition to overliming was most efficient. This method removes ketones, aldehydes and volatiles.

II.8.3.2 Activated charcoal and diatomaceous earth

Activated charcoal and diatomaceous earth are the least expensive materials that help detoxify hydrolysate. These methods can remove almost all phenolics, and some furans. Acetic acid is not affected. Diatomaceous earth is cheaper than activated charcoal, but has limited effectiveness [101].

II.9 Enzymatic hydrolysis

Enzyme complexes cellulase, β-glucosidase, and hemicellulases are used to break down cellulose and hemicellulose components of biomass [32, 36, 105]. The use of enzymes is necessary as these are highly specific biocatalysts which can potentially give near quantitative yields of products without further degradation or any-product formation. However, the structure of lignocellulosic biomass is very complex and needs to be broken down to a certain extent for the enzymes to be able to access cellulose chains and depolymerize them [95, 105]. Thus enzymes are used in combination with pretreatment of biomass to give sugars which are further converted into ethanol by various micro organisms.

In addition to using external enzymes or microbes for degradation of biomass, investigations are in effect on making genetically modified plants which are easily degraded [20]. Synthetic lignin compounds are introduced in the plant cell wall, which in mild pretreatment conditions would readily degrade. The formation of syringyl lignin units can also be suppressed so that the plant contains easily degradable lignin [105]. This research is still in its nascent stages and hasn't been proven at large scales.

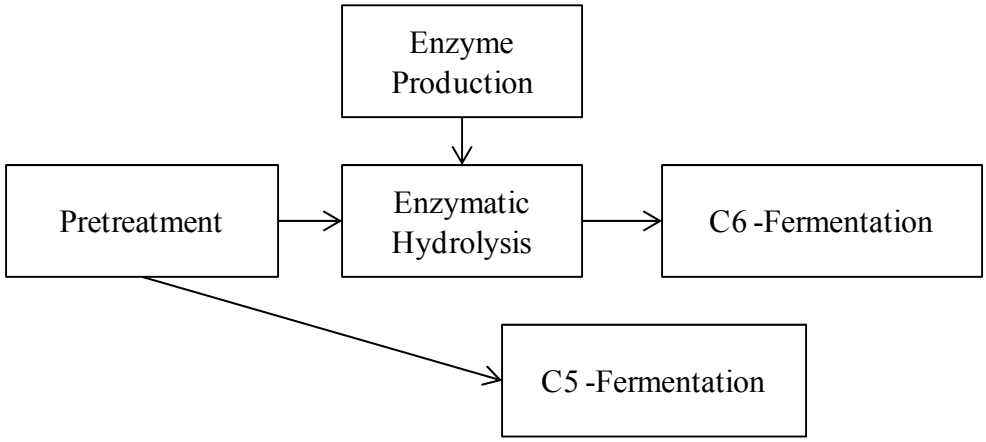
II.10 Enzymatic hydrolysis and fermentation modes

Various modes of hydrolysis and fermentation are shown in Figure II-9. Saccharification or hydrolysis of pretreated biomass is carried out using enzymes in the enzymatic hydrolysis reactor. Here cellulase enzyme complex converts cellulose to glucose and hemicellulose to

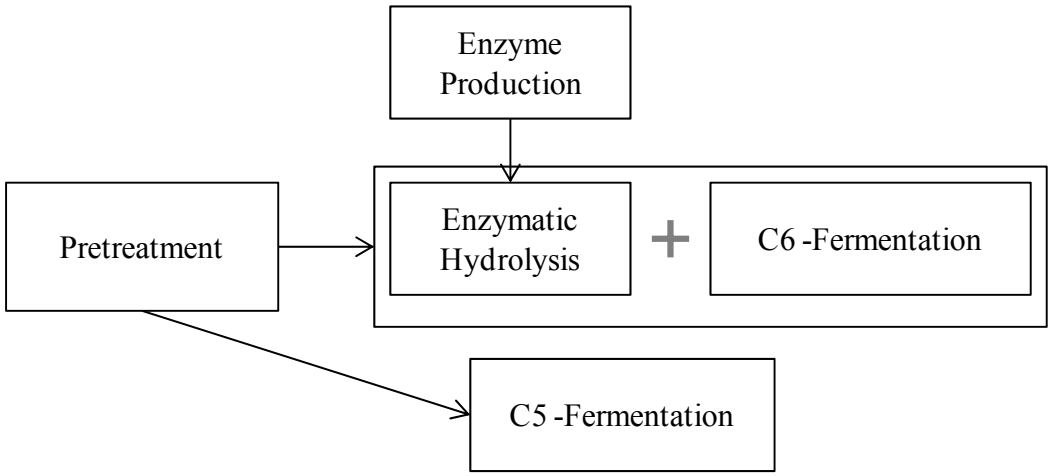
glucose, galactose, mannose (C-6 sugars), and xylose and arabinose (C-5 sugars). The hemicellulose from hardwood and herbaceous plants contains a majority of xylose which is a 5 carbon sugar and thus, the hemicellulose rich stream is called the C-5 rich stream even though small amounts of C-6 sugars are present. The sugars are further converted, using microorganisms, to ethanol. When cellulase production, hydrolysis and C-5 and C-6 fermentation are all performed as separate steps, the process is called separate hydrolysis and fermentation (SHF) (Figure II-9 a). In this mode, each step is performed at conditions optimized to give high products in each stage. The C-6 and C-5 sugars are also fermented separately with different organisms and operating conditions that can best convert the respective sugars. In the simultaneous saccharification and fermentation (SSF) process, enzymatic hydrolysis and fermentation of C-6 sugars is combined in one step (Figure II-9 b). The main advantage of this method is that glucose, which is an inhibitor to cellulase activity, is converted to ethanol using the microorganism as soon as it is formed. This keeps the C-6 sugar concentration in the hydrolysis step low, and thus improves the cellulase activity. Other advantages of the SSF process include a low enzyme requirement and high ethanol yields and reducing of the potential for external contaminations while transferring the hydrolysate into fermentors [106, 107]. The left over stream from the SSF reactor contains xylose sugars which can be further converted into ethanol in a separate reactor [108]. In simultaneous saccharification and co-fermentation (SSCF) scheme, enzymatic hydrolysis, fermentation of C-6 sugars and that of C-5 sugars are all performed in a single reactor (Figure II-9 c). Thus glucose and xylose are simultaneously converted by a single microorganism. The main advantage of this process is - a low concentration of xylose in the reaction which eliminates the xylose inhibition of the xylanase components of the enzyme complex. This helps increase total hydrolysis of biomass and ethanol

yield [109, 110]. consolidated bioprocessing (CBP) is a new process scheme, where the steps of enzyme production, enzymatic hydrolysis, and fermentation are carried out in one reactor with a single microorganism or group of microorganisms operating at the same conditions [111, 112] (Figure II-9 d).

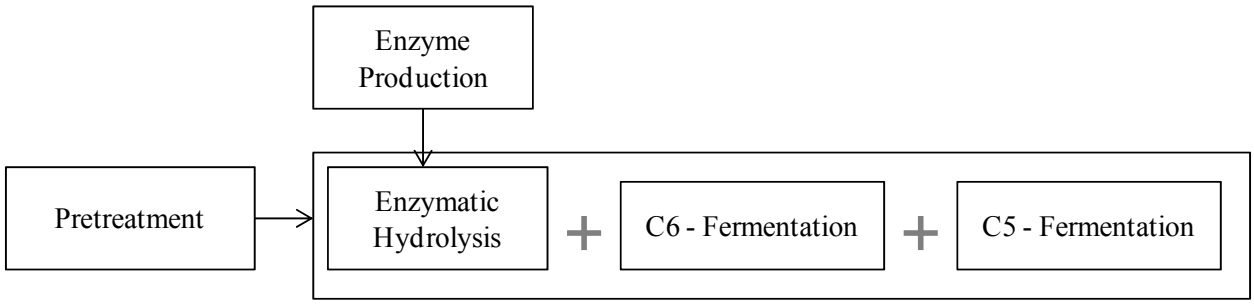
(a) Separate Hydrolysis and Fermentation:



(b) Simultaneous Saccharification and Fermentation:



(c) Simultaneous Saccharification and Cofermentation:



(d) Consolidated Bioprocessing:

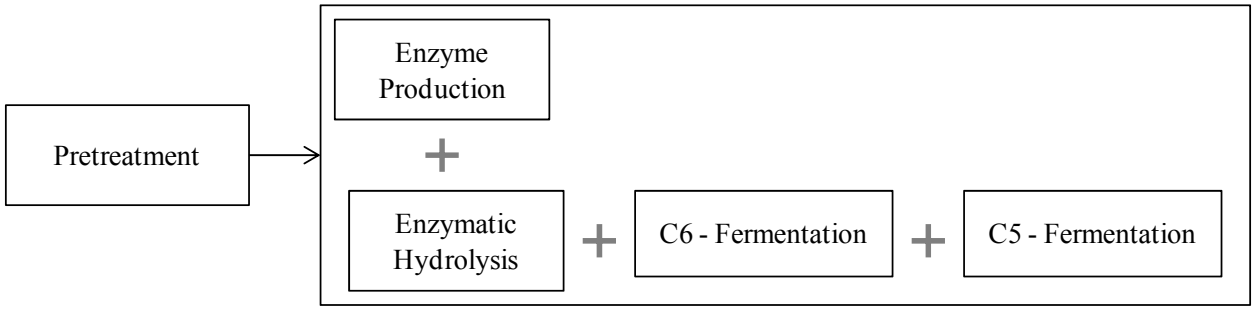


Figure II-9. Various Modes of hydrolysis and fermentation (a) Separate Hydrolysis and Fermentation; (b) Simultaneous Saccharification and Fermentation; (c) Simultaneous Saccharification and Cofermentation; and (d) Consolidated Bioprocessing.

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Chapter III Inhibition Effects of Dilute-Acid Prehydrolysate of Corn Stover on Enzymatic Hydrolysis of Solka Floc

III.1 Abstract

Dilute-acid pretreatment liquor (PL) produced at NREL through a continuous screw-driven reactor was analyzed for sugars and other potential inhibitory components. Their inhibitory effects on enzymatic hydrolysis of Solka Floc (pure cellulosic substrate) were investigated. When the PL was mixed into the enzymatic hydrolysis reactor at 1:1 volume ratio, the glucan and xylan digestibility decreased by 63% and 90%, respectively. The tolerance level of the enzyme for each inhibitor was determined. Of the identified degradation components, acetic acid was found to be the strongest inhibitor for cellulase activity, as it decreased the glucan yield by 10% at 1 g/L. Among the sugars, cellobiose and glucose were found to be strong inhibitors to glucan hydrolysis, whereas xylose is a strong inhibitor to xylan hydrolysis. Xylo-oligomers inhibit xylan digestibility more strongly than the glucan digestibility. Inhibition by the PL was higher than that of the simulated mixture of the identifiable components. This indicates that some of the unidentified degradation components, originated mostly from lignin, are potent inhibitors to the cellulase enzyme. When the PL was added to a simultaneous saccharification and co-fermentation (SSCF) using *E coli* KO11, the bioprocess was severely inhibited showing no ethanol formation or cell growth.

III.2 Introduction

Hemicellulose and lignin cover and protect the cellulose from degradation by enzymes and chemicals [1]. Dilute acid pretreatment is one of the most promising methods of pretreatment. In this method, a large part of hemicellulose is hydrolyzed into the liquid hydrolysate stream [2-4]. Cellulose and most of the lignin remain in the solid after dilute acid treatment. In addition to sugars, the hydrolysate also contains degradation products in the form of phenolics, acids, and aldehydes. The sugars from hemicellulose (15-30% of original dry biomass [5, 6]) can be converted to ethanol by co-fermenting organisms which can use hexose sugars as well as pentose sugars for fermentation [7-10]. To effectively utilize the hemicellulose sugars, detoxification of the hemicellulose stream (pretreatment liquor) is necessary before it is introduced into the bioreactor.

The effects of the pretreatment liquor (PL) on various microorganisms used in bioconversion processes have been investigated extensively [1, 11-19]. Literature information is rather scant with regard to the extent of inhibition of the PL on the enzymatic hydrolysis reaction [20-22]. The composition and concentration of the degradation products in PL varies with the type of feedstock, the chemistry, and the pretreatment process parameters such as temperature, time, pressure, pH, redox conditions, and presence of catalysts [1, 12]. Figure II-8 is a schematic representation of degradation products formed during dilute acid hydrolysis of biomass, discussed in Section II-5 and 6. The overall bioconversion process involves enzymatic hydrolysis and microbial conversion. The most common mode of bioconversion is simultaneous

saccharification and fermentation (SSF), or co-fermentation (SSCF) when conversion of hexoses and pentoses is applied. In either case, the mixture of PL and the pretreated solid substrate is to be processed by enzyme and consequently by microorganism. In the case of SSF or SSCF, the inhibitory effects of toxins are compounded as they deter the enzymatic hydrolysis reaction as well as the microbial activity.

The effects of dilute acid pretreatment hydrolysate on fermentation are well-documented [11-13, 16, 23, 24]. At the present time, however, it is unclear to what extent they affect the cellulase enzyme reactions. This investigation was undertaken to identify the inhibitory components existing in the dilute acid pretreatment liquor and to assess their effects on enzymatic hydrolysis. Solka Floc was chosen as the substrate in order to eliminate the effects of extraneous components in biomass focusing more on the carbohydrates, the main target of the cellulase enzyme. The upper limit to which these inhibitors can be tolerated by the enzymes, an index of inhibition, was among the main items of interest.

III.3 Materials and methods

III.3.1 Substrates

Solka Floc (International Fiber Corporation, Urbana, OH, Cat. No. U064072) was used as the cellulose substrate. Its composition was determined to be 77.3% glucan and 22.8% xylan. Birchwood xylan was obtained from Sigma Aldrich (Cat. No. 038K0751). It contained >99.9% xylan. Avicel PH-101 was obtained from Fluka (Cat. No. 1344705), containing 97.7% glucan. Other chemicals used in this work were laboratory grade chemicals purchased from Sigma Aldrich. Corn Stover and Pretreated Corn Stover (PCS) were provided by the National Renewable Energy Laboratory (NREL, Golden, CO).

III.3.2 Enzymes

Cellulase enzyme, Genencor Spezyme CP (Lot No. 301-00348-257), was a kind gift from Genecor/Danisco, Palo Alto, CA. The activity of Spezyme CP, as determined by NREL, was 59 FPU/mL. Activity of β-glucosidase (Novozyme 188 from Novo Inc, Batch No 018K0735) was 750 CBU/mL. Multifect Xylanase (protein content 42 mg/mL) was a kind gift of Genecor/Danisco, Palo Alto, CA.

III.3.3 Microorganism

The microorganism used for the Simultaneous Saccharification and Co-fermentation (SSCF) experiments was recombinant *Escherichia coli*, ATCC-55124 (KO11). This organism was grown

in LB medium (Sigma, L-3152), which contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L chloroamphenicol.

III.3.4 Analytical methods

Sugars in liquid sample were determined by HPLC equipped with a refractive index detector and using a Biorad-HPX-87P column. The BioRad-HPX-87H column was used for measurement of organic acids, ethanol, and furans. Solid substrates were analyzed for carbohydrates and ash using NREL standard biomass analytical procedures NREL-TP-510-42618 to 42622. Moisture content of biomass was measured by an infrared moisture balance (Denver Instruments IR-30). Cellulase and β-glucosidase activities were measured using NREL-TP-510-42628 using filter paper and cellobiose as substrates respectively.

III.3.5 Quantification of sugars and oligomers in liquid

All liquid samples from enzymatic hydrolysis and SSCF were first analyzed for composition using HPLC: HPX-87H column for aldehydes and acids and HPX-87P column for monomeric sugars. Oligomeric sugar concentration was obtained by secondary hydrolysis of the liquid according to the NREL standard biomass analytical procedure NREL-TP-510-42623. The secondary hydrolysis was performed with 4% sulfuric acid, at 121˚C for 1 h. The lignin degradation products vanillin and syringaldehyde were measured using GC/MS (GC: Agilent 7890A; MS: Agilent 5975). DB-1701 (30 m x 250 μm x 0.25 μm) column and the NIST mass spectral library were used in the GC.

III.3.6 Preparation of Hydrolysate, Oligomers and Soluble lignin

Corn stover pretreated by dilute-acid was obtained from NREL. The pretreated corn stover (PCS) was squeezed and vacuum filtered to obtain dilute acid hydrolysate.

Xylo-oligomer solution used in this study was obtained by treating pure Birchwood xylan at 121ºC for 25 min to get an oligomer solution. This condition was selected since it gave the minimum amount of degradation products while solubilizing most of the solid xylan. Also, as an alternate method, pure Birchwood xylan was treated with Multifect Xylanase **(**enzyme loading 20 mg/g xylan) for a fixed time (30 min) as shown by Zhu [25]. After the set time, the liquid solution was placed in a boiling water bath for 10 min to ensure that the enzyme activity stopped. The resulting solutions had a mixture of xylose and xylo-oligomers, but no lignin or its degradation products.

Water soluble lignin solution was prepared by extracting lignin from the PL using ethyl acetate. The solvent was evaporated and the solid remaining behind was dissolved in water at desired concentrations. Insoluble lignin was extracted from the PCS solids using sodium hydroxide and then precipitated from the alkaline solution by adding acid.

III.3.7 Enzymatic hydrolysis

The enzymatic digestibility of substrate was determined according to the NREL standard biomass analytical procedure NREL-TP-510-42621. Screw capped 250 mL Erlenmeyer flasks were used as the hydrolysis reactors. Solka Floc was used as the pure cellulose substrate in most

of the experiments. The solid loading applied was such that the glucan content was 1% (w/v) in the reactor. Enzyme loading used was 15 FPU and 30 CBU/g glucan. Antibiotics tetracycline and cyclohexamide were added into the reactor to maintain sterile conditions. The enzymatic digestibility tests were carried out at 50ºC, and pH 4.8 using 0.05 M sodium citrate buffer in an incubator shaker (New Brunswick Scientific, Innova-4080) agitated at 150 rpm. Desired concentration of inhibitors was added to the reaction mixture. When the inhibitor was acidic, the solution was first treated with calcium carbonate to increase the pH to 4.8. The pH of the enzymatic hydrolysis mixture was tested before and after the experiment to make sure that it remained constant. The total reaction volume was kept constant at 100 mL irrespective of whether inhibitors were added to the mixture. Hydrolysate samples were taken at 6, 12, 24, 48, and 72 h, and analyzed for glucose, xylose, and cellobiose. Total released glucose and cellobiose after 72 h of hydrolysis were used to calculate the enzymatic digestibility:

$$\text{Glucan Digestibility (\%)} = \frac{\text{Glucose released (g)} + 1.053 \times \text{Cellobiose released (g)}}{1.111 \times \text{Glucan added (g)}} \times 100 \qquad \text{Eq. III-1}$$

The xylan digestibility was also determined in a similar manner. For xylan digestibility, hydration factor of 1.136 instead of 1.111 was used in the above equation.

III.3.8 Simultaneous saccharification and co-fermentation (SSCF)

The procedure for the fermentation test was based on the NREL standard analytical procedures (NREL/TP-510-42630**).** Erlenmeyer flasks (250 mL) were used as the bioreactors. They were operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 37°C and 150 rpm with 100 mL working volume. Substrate loading was 3% (w/v). Dilute acid pretreatment liquor

was added to one set of reactors for comparison. The samples were sterilized by autoclaving (121°C for 30 min). SSCF of sample was carried out at 37°C and pH 6 (0.05 M sodium phosphate buffer). The cellulase enzyme loading was 15 FPU Spezyme CP supplemented with 30 CBU Novozyme-188/g-glucan. The ethanol yield was calculated as follows:

$$\text{Theoretical maximum ethanol yield } (\%) = \frac{\text{Ethanol produced (g)in reactor}}{\text{Initial sugar added (g)in reactor} \times 0.511} \times 100 \quad \text{Eq. III-2}$$

Sugar is interpreted as glucose plus xylose in SSCF.

III.3.9 Tolerance of enzyme to specific inhibitors (10% Tolerance)

Spezyme CP was also tested for tolerance by different inhibitor compounds identified in this study. The inhibitor to be tested was added at different concentrations in the range of 1 to 10 g/L in the enzymatic reaction mixture as explained earlier, and the digestibility tests were run in parallel with one control flask. The "*10% Tolerance"* (10PT) for an inhibitor was defined as the concentration of the inhibitor at which the glucan digestibility decreases by 10% compared to the control.

III.4 Results and Discussion

III.4.1 Composition of hydrolysate

The composition of dilute acid pretreatment liquor is shown in Table III-1. The main sugar component in the hydrolysate is xylose, the main ingredient in hemicelluloses, which is easily hydrolyzed by dilute-acid [1]. HMF and furfural were also present in the liquor indicating that

there is degradation of the released sugars. Among the acids, acetic acid was the predominant component. The liquor also contains a large amount of unidentified compounds as seen in the HPLC and GC chromatograms in Figure III-1. Most of the unidentified components are believed to be from lignin and sugar degradation, as discussed in Sections II-5 and 6. The lignin degradation products identified and tested in this study were vanillin and syringaldehyde as these are known to significantly inhibit the enzymes and microorganisms [5, 11].

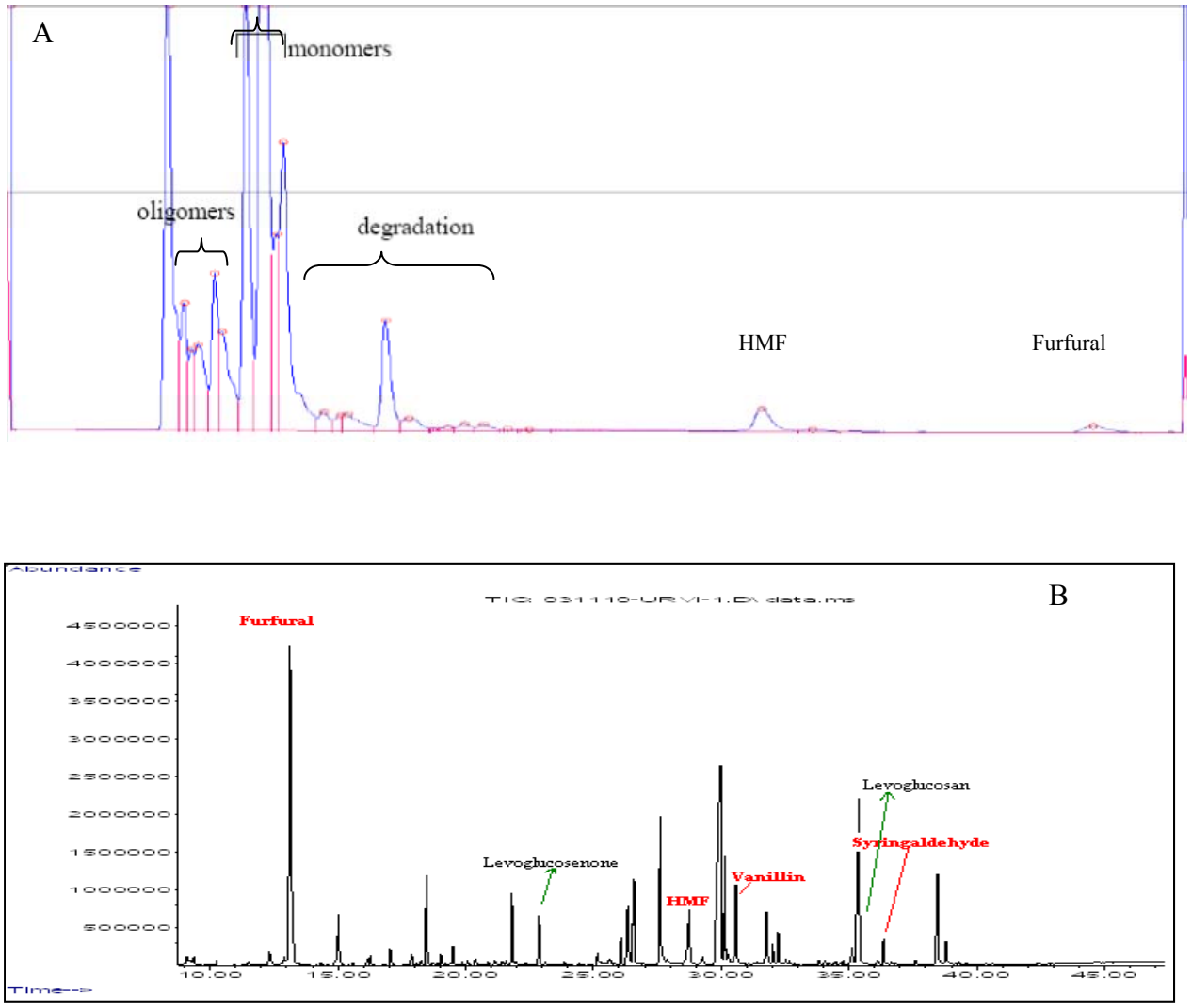

Figure III-1. (a) HPLC chromatogram and (b) GC/MS for dilute acid hydrolysate.

Table III-1. Composition of pretreatment liquid.

Component	Concentration (g/L)
Cellobiose	2.45
Glucose	21.80
Xylose	74.08
Galactose	12.35
Arabinose	11.48
Mannose	2.55
Oligomers	
Xylo-oligomers	17.39
Gluco-oligomers (DP>2)	2.82
Identifiable Degradation products	
From Carbohydrates	
HMF	2.00
Furfural	1.14
Acetate	13.23
Formate	3.73
From Lignin	μg/L
Vanillin	22.65
Syringaldehyde	386.14

III.4.2 Fermentation test (SSCF)

In the SSCF carried out with addition of 50/50 mixture of PL and buffer, there was no ethanol formation (Figure III-2). The sugars accumulated throughout the fermentation, whereas in the control run, the sugar levels remained near zero throughout, which indicates that the organism is unable to take up any of the sugars during fermentation. This further indicates that the organism did not grow due to toxic effects of the hydrolysate, although the enzyme released sugars from cellulose. Obviously the inhibitory effects of the PL are more acute on the microbial process (both fermentation and cell growth) than the enzymatic hydrolysis reaction. Since the effects of the pretreatment hydrolysate on fermentation are well understood, the focus of this study was placed on the enzymatic hydrolysis.

III.4.3 Enzymatic hydrolysis of the pretreatment liquor

The glucan and xylan digestibility profiles with and without addition of PL are shown Figure III-3. It is clearly seen that cellulase activity is significantly reduced by the presence of dilute acid pretreatment liquor. Since the liquor was neutralized before hydrolysis, pH was not the cause of this drop. With 50% (v/v) mixture of PL and buffer solution, the 72 h digestibility of glucan in Solka Floc was reduced by 63% in comparison to the control run (no PL addition). The decrease in digestibility for the xylan fraction of the Solka Floc was even greater at 93%. This may be due to the fact that Spezyme CP has low xylanase activity and that the xylan in Solka Floc is a resilient fraction left behind during the manufacturing process.

The same tests were repeated applying different levels of enzyme loading. The results are shown in Figure III-4. The increased enzymatic loading has a positive effect on the digestibility, which

is well-known [4]. A point to be noted here is that increase of enzyme loading tends to decrease the inhibition effects as the per-cent difference in glucan digestibility between the two runs reduced as the enzyme loading was increased. Moderate enzyme loading of 15 FPU/g glucan was used in most of the experiments in this study for comparison purpose, as this level has been adopted in many of the previous investigations [26].

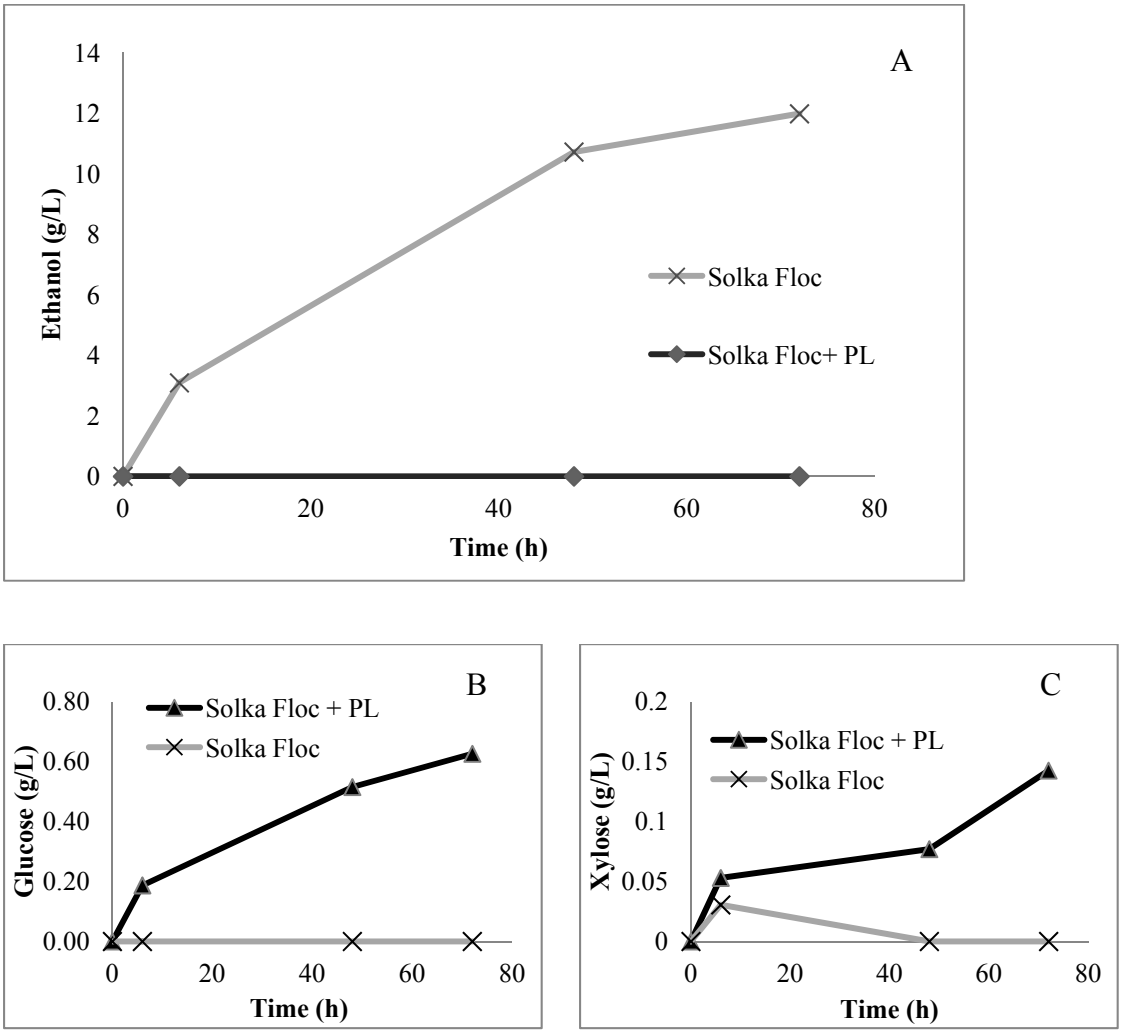

Figure III-2. Effect of dilute acid hydrolysate (PL) on fermentation of Solka Floc.
(a) Ethanol, (b) Glucose and (c) Xylose Profile
Enzyme loading: 15FPU/g glucan, 30CBU/g glucan; Substrate: Solka Floc 1% glucan loading; Inhibitor: Dilute acid hydrolysate (PL) 50 % (vol.); Micro Organism: *E. coli* KO11

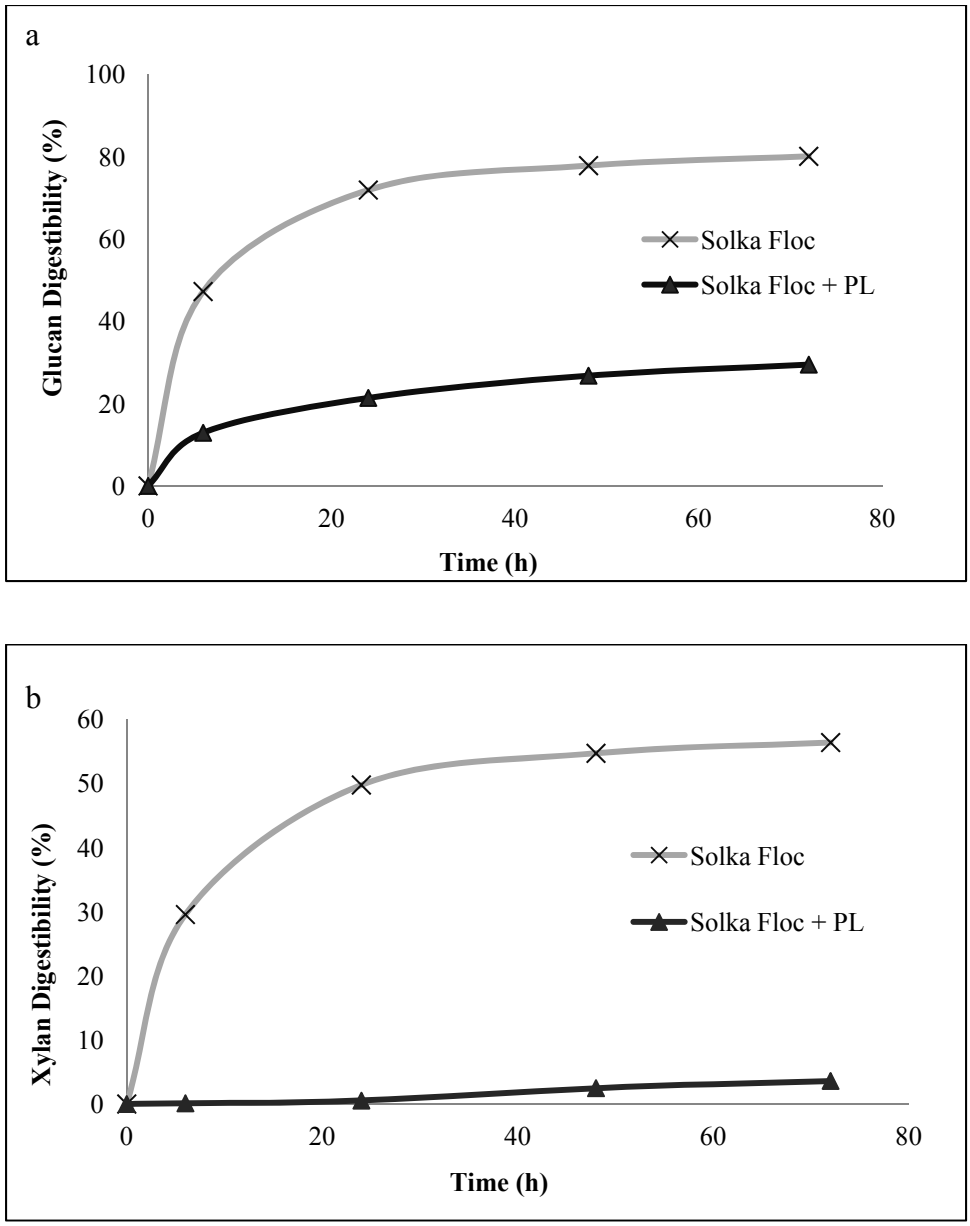

Figure III-3. (a) Glucan and (b) xylan digestibility of Solka Flock with and without PL.
Enzyme loading: 15FPU/g glucan, 30CBU/g glucan
Substrate: Solka Floc 1% glucan loading; Inhibitor: Dilute acid hydrolysate (PL) 50 % (vol.).

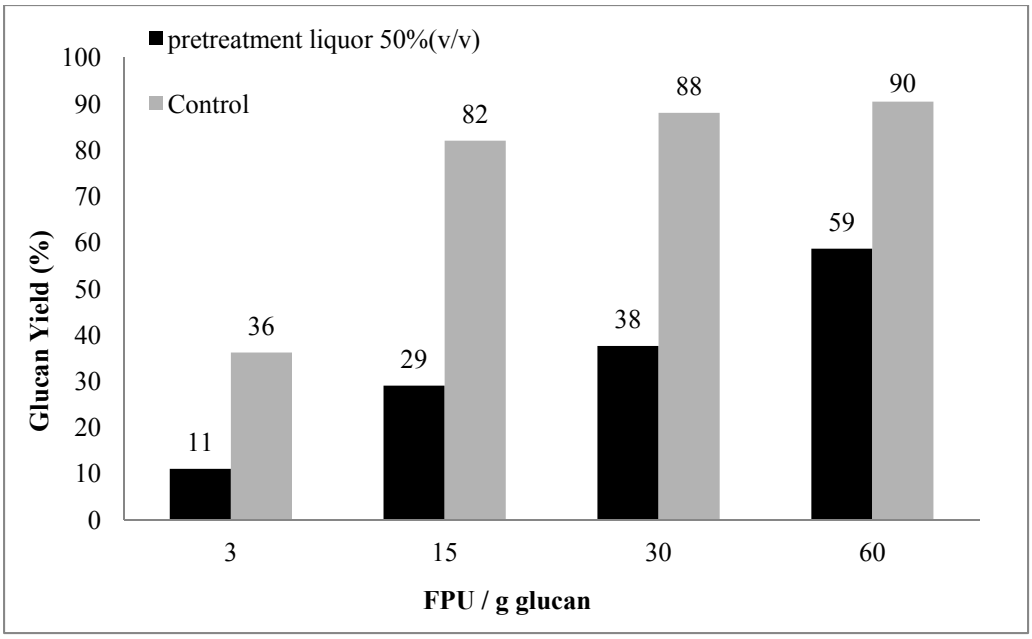

Figure III-4. Effect of enzyme loading on inhibition.
Enzyme added: Spezyme CP: Novozyme 188 ratio 1 FPU: 2 CBU
Substrate: Solka Floc 1% glucan loading
Inhibitor: Dilute acid hydrolysate (PL) 50 % (vol.).

Table III-2. 10% Tolerance (10PT) of cellulase for various inhibitors.

	Inhibitor concentration (10%Tolerance) (g/L)	% inhibition of cellulase activity (Spezyme CP)	Inhibitor concentration (10%Tolerance) (g/L)	% inhibition of xylanase activity (Spezyme CP)
Glucose	**1**	16	2	11
Cellobiose	**1**	21	1	21
Xylose	40	8	1	43
HMF	5	11	5	14
Furfural	10	9	10	9
Acetate	**1**	10	1	12
Formate	3.5	10	3.5	11
Vanillin	4	11	4	9
Syringaldehyde	5	9	5	10

III.4.4 Effects of sugar degradation products

It is well-known that HMF and furfural are toxic to the microorganisms [16, 17, 27-29]. Various levels of HMF and furfural were applied to the enzymatic hydrolysis tests to assess their inhibitory effects. The author finds that they show significant inhibition, but only at concentrations above 5 g/L (Table III-2 and Figure III-5). In the pretreatment hydrolysate used in this work, the concentrations of HMF and furfural found were much lower at 2 g/L and 1.14 g/L respectively. The test data show that inhibition on cellulase activity by HMF and furfural at these concentrations is insignificant (Figure III-5). It is important to note that the concentrations of HMF and furfural shown in Figure III-6 is half the actual concentration in the pretreatment liquor since it was diluted at 1:1 with buffer before it was subjected to hydrolysis test. Although the

inhibitory effects of these compounds on the growth of microorganisms and enzyme activity are well recognized [18, 19], at the level existing in the pretreatment liquor, the inhibition on cellulase activity by each individual components is less than 5%. The maximum HMF tolerated by the enzyme such that the cellulase activity decreased by 10% is to be termed as "10% tolerance", abbreviated by 10PT in this paper. The 10% tolerance for HMF is approximately 5 g/L, since at this concentration of HMF the glucan and xylan hydrolysis yields decreased by 11% and 14%, respectively. The same for furfural was determined to be 10 g/L where both glucan and xylan yields decreased by 9% (Figure III-5 and Table III-2). .

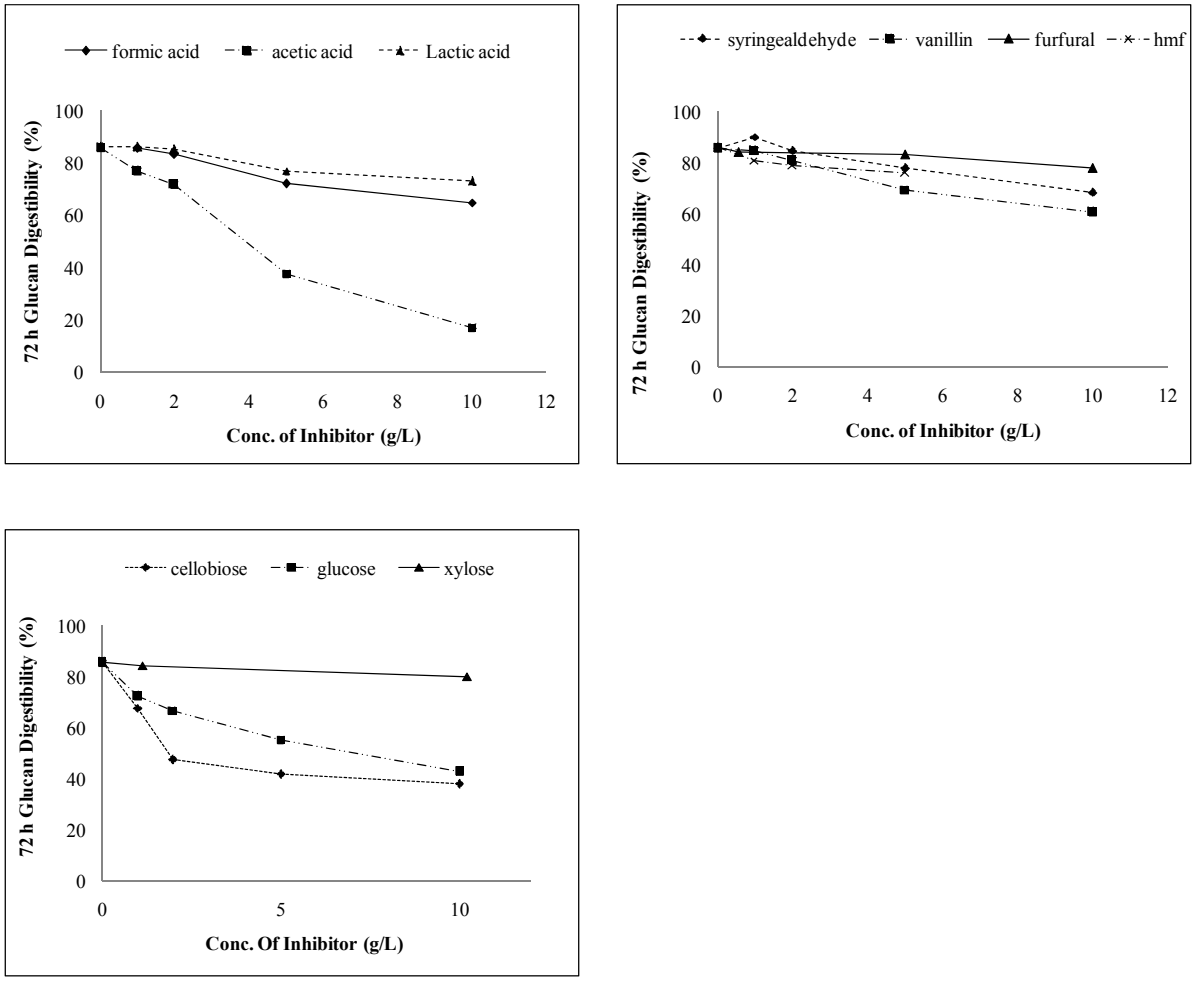

Figure III-5. Glucan digestibility of Solka Floc in presence of pure component inhibitors.

III.4.5 Effect of acids

In the PL, acetate is of the highest concentration at 13 g/L (Table III-1). Acetate is generated from hemicellulose-lignin composite structure which contains acetyl groups. It may also be formed from further degradation of HMF and furfural in acidic conditions [12, 30, 31]. Formate and levulinate are produced by degradation of HMF and furfural. The inhibitory effect of the acetate is found to be the highest amongst the three acids of acetate, formate and levulinate (levulinic acid data not shown). The extent of specific inhibition (g/L basis) of the acids is in the order of: acetate > formate > levulinate. The global inhibition is by far the highest for acetate since its concentration is also the highest in the PL. The author has determined the 10PT against the acetic acid to be 1 g/L. With 5g/L concentration in the PL, acetate becomes indeed a very strong inhibitor to enzymatic hydrolysis. It appears to play a major role by reducing glucan hydrolysis yield by more than 50% (Figure III-6). Formate is a weaker inhibitor than acetate, but with the concentration higher than 5 g/L in the PL, it decreased the glucan hydrolysis yield by about 20%. Levulinic acid is also known to be inhibitory to the enzyme and microorganisms [12, 22], but its concentration in the hydrolysate was much lower than the other two acids. The 10PT for acetic acid was 1 g/L, at which the glucan and xylan yields decreased by 10% and 12%. The 10PT for formate was estimated to be 3.5 g/L and at this concentration the glucan digestibility was reduced by 10% and xylan digestibility by 11% (Table III-2 and Figure III-5).

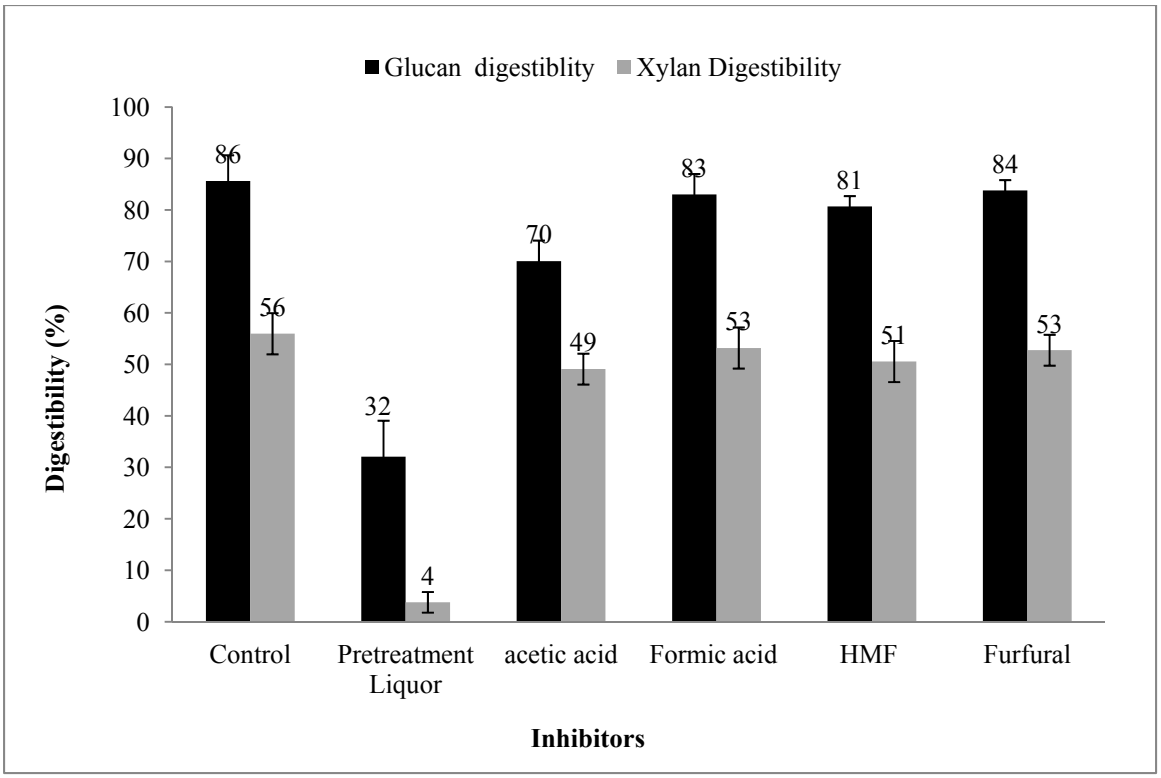

Figure III-6. Comparison of effect of individual components furfural, HMF, acetic acid and pretreatment liquor.
Enzyme loading: 15FPU/g glucan, 30CBU/g glucan; Substrate: Solka Floc 1% glucan loading; Inhibitors: Dilute acid hydrolysate 50 % (vol.); Furfural: 0.57 g/L; HMF: 1 g/L; acetic acid: 6.62 g/L, Formic acid: 1.9 g/L.

III.4.6 Effects of soluble lignin and lignin degradation products

The PL contains various lignin degradation products and water soluble lignin. Syringaldehyde and vanillin are among the identified components. The concentration of these two components in the PL was found to be less than 1 g/L. At this level, their inhibition on glucan or xylan digestibility was insignificant (<1 %, <5% respectively). The 10PT for syringaldehyde was estimated to be 5 g/L, as the yield for glucan and xylan decreased by 9% and 19%, respectively, and at 4 g/L, by 10% and 9% with vanillin. The PL also contains large number unidentified phenolic compounds with wide range of MW [5, 11] which originate from lignin. Some of these

are breakdown products of solid lignin whose MW became low enough to be water soluble. Although not individually identified, the lignin breakdown products are believed to impose strong inhibition to cellulase. The soluble lignin in the PL was extracted using ethyl acetate and then dissolved in water to desired concentrations. This water-soluble lignin was mixed with pure substrates (filter paper and cellobiose) to determine the reduction in cellulase and β-glucosidase activities in presence of the lignin (Figure III-7). At a concentration of 3 g/L water-soluble lignin decreased the cellulase activity by approximately 10%.

The lignin remaining in solid biomass after pretreatment is known to bind with cellulase enzyme, a phenomenon known as "nonproductive binding" [32, 33]. In this mode of action, the enzyme is irreversibly bound, essentially deactivating the enzyme. This is different from inhibition, which is a reversible interference to the enzyme reaction. It has been reported that lignin also forms a complex with cellulase enzyme (lignin-cellulase complex) [32, 34]. The 10PT for water insoluble lignin tolerated by the enzymes was determined to be 5 g/L where the cellulase activity decreased by more than 15%. To remove this inhibition, surfactants and other long chain compounds are used. Yang et al., found that surfactants adsorb to lignin and thus prevent nonproductive binding of enzymes to lignin [33]. Errikson et al., proposed that surfactants form a mycelial structure around lignin by hydrophobic interactions and block the lignin molecules from adsorbing to the enzyme [32].

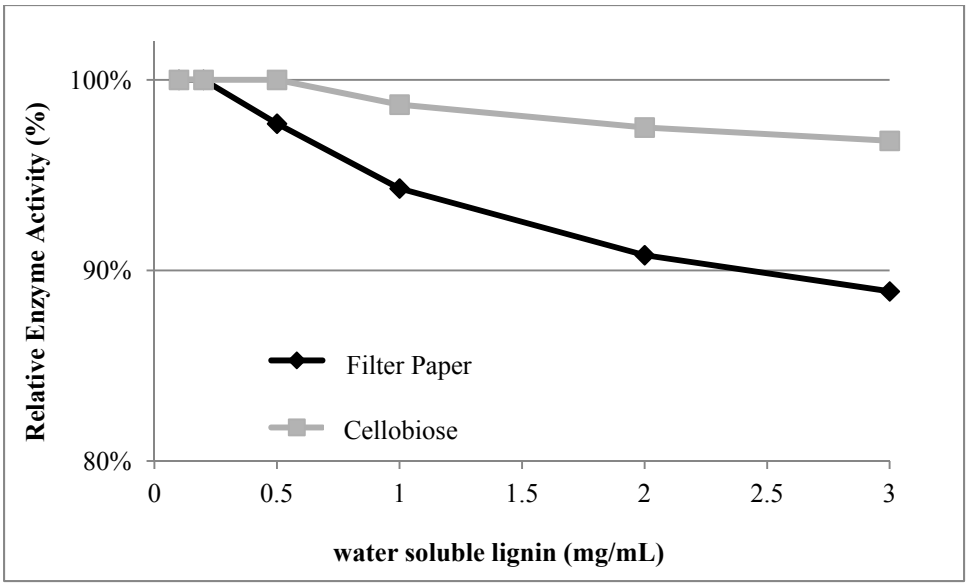

Figure III-7. Effect of water-soluble lignin on enzyme activity.
Substrates: Filter paper (FPU/ml activity) and Cellobiose (CBU/ml).

III.4.7 Effect of sugars

Inhibition experiments were carried out for sugars found in the PL, namely, glucose, cellobiose, xylose, and xylose oligomers. The experiments were carried out for each individual sugar. The sugars were added to the hydrolysis reactor to such that the concentration is the same as those in the 50/50 hydrolysate-buffer mixture. The results summarized in Figure III-8, show the 72 h digestibility with and without addition of individual sugar components. The 72 h digestibility of glucan in Solka Floc was affected strongly by cellobiose and glucose. On the other hand, the digestibility of xylan was strongly affected by xylose. Here, it is also important to note that the degree of inhibition by three sugars combined (glucose + cellobiose + xylose) is far less than the addition of the inhibition by each component. This reaffirms the established concept that the cellulose enzymes are inhibited by the reaction intermediates (cellobiose) as well as the end-products (glucose, xylose) [35, 36]. The product inhibition is specific to the enzyme; a product

inhibits mainly the enzyme by which it was produced. The data in Figure III-8 also show that the inhibition can also go the other way; although the degree of inhibition is much lower, xylose inhibits glucan hydrolysis and vice versa. It appears that the various non-cellulolytic enzymes existing in "cellulase" are affected by the sugars released from hemicellulose, inhibiting the hydrolysis of hemicellulose fraction (a barrier impeding accessibility of the enzyme to cellulose), then eventually the glucan hydrolysis. This concurs with findings of previous research along these lines that glucose and cellobiose are the major inhibitors to cellulase and β-glucosidase, whereas xylose is a minor inhibitor [37-40]. According to Xiao et al., glucose inhibits Exo-glucanase as well as β-glucosidase. Xylose, mannose, and galactose also have a significant inhibitory effect, but only on the initial cellulase activity [41]. This subject was further investigated to see if hydrolysis of pure glucan is inhibited by xylose, which is not a product of glucanase. The experimental results are shown in Figure III-9, where the profiles of Avicel hydrolysis with and without xylose addition are compared. An interesting point here is that although Avicel does not contain any xylan (97.7% glucan), glucan hydrolysis was inhibited by xylose, but only at the early phase of the reaction up to 24 h. The final 72 h digestibility was not affected significantly. Even though xylose is not a natural substrate to cellulase, it seems to interfere with glucan hydrolysis perhaps acting as a competitive inhibitor to active sites of certain cellulase enzyme component(s). The extent of inhibition then gradually diminishes as glucose, the main inhibitor, is accumulated, which is in agreement with Liao et al., [42].

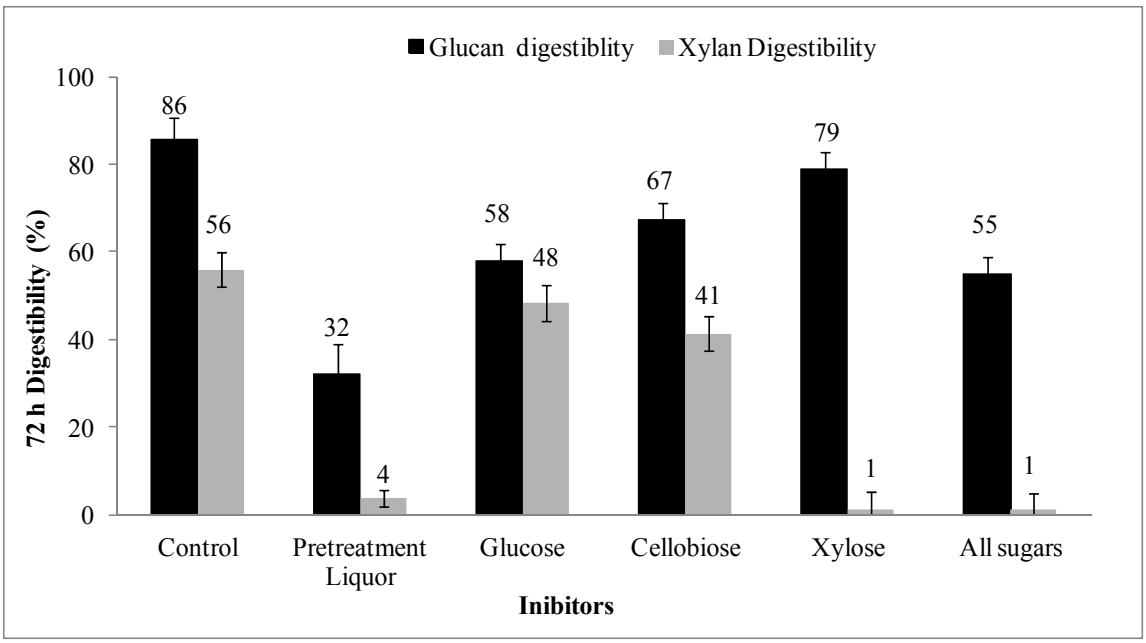

Figure III-8. Comparison of effect of individual components main sugars: glucose, xylose and cellobiose and sugar mixture.
Enzyme loading: 15FPU/g glucan, 30CBU/g glucan ; Substrate: Solka Floc 1% glucan loading
Inhibitors: Glucose 11 g/L, Xylose 37 g/L, Cellobiose 1.23 g/L, All sugars as found in 50 mL dilute acid hydrolysate

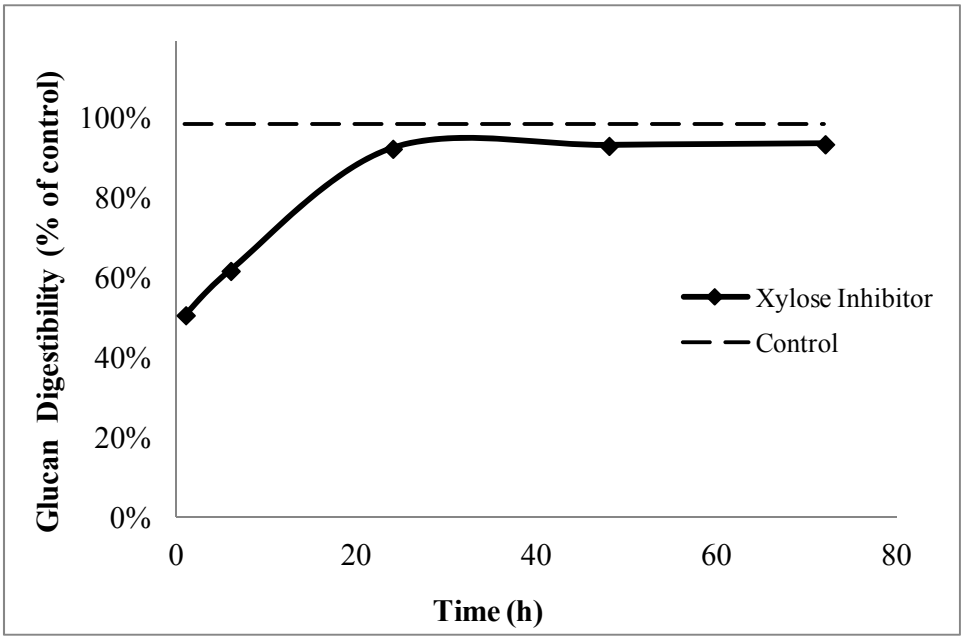

Figure III-9. Xylose inhibition over time plotted as % of control.
Xylose decreases initial glucan yield by around 50% while final yield is almost the same as control. Avicel 97.7% cellulose + 10 g/L xylose.

Inhibition by xylo-oligomers is shown in Figure III-10. The data indicate that the oligomers severely inhibit hydrolysis of xylan, but only slightly the hydrolysis of glucan at the concentration seen in the PL. It was also found that oligomers did not disappear after 72 h of enzymatic hydrolysis. This indicates that the cellulase enzyme does not hydrolyze the oligomers effectively. Addition of external xylanase (Multifect Xylanase, Genencor-Danisco) increased conversion of xylo-oligomers, but at the end of 72 h there were still about 20% oligomers were left unhydrolyzed. As significant as the inhibition by sugars to the cellulase enzyme is, this issue can be resolved by adoption of SSF or SSCF bioprocess scheme [10, 43].

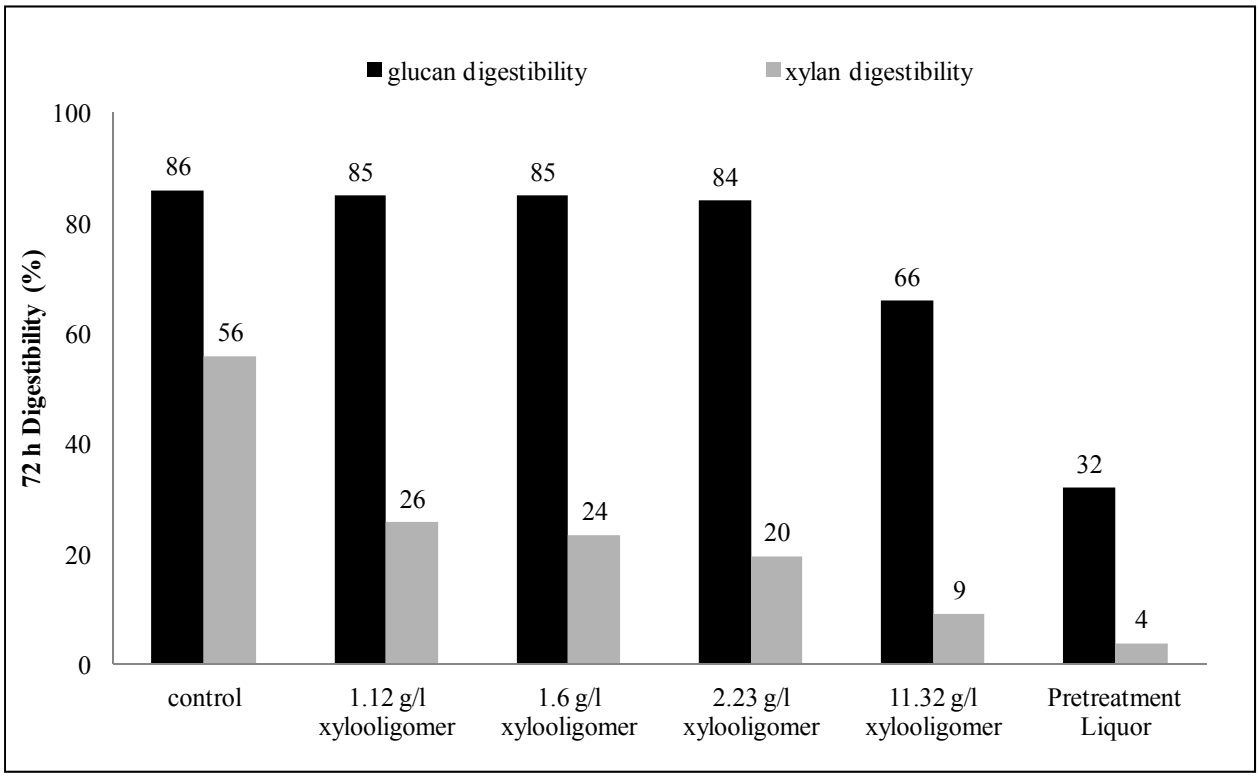

Figure III-10. Effect of xylo-oligomers on the glucan and xylan digestibility of Solka Floc. Substrate: Solka Floc 1% glucan loading; Inhibitor: xylo-oligomers

III.4.8 Combined effects

The overall inhibition effects by groups of known inhibitors on glucan and xylan digestibility are summarized in Figure III-11. Inhibition by each group and by all the known inhibitor groups combined were investigated to assess the overall inhibition effects and to see if there is any interaction between the different groups of inhibitors. With all of the known inhibitors combined, the cellulase activity decreased by 42% (86% down to 50%). An important point here is that the overall inhibition on glucan hydrolysis by PL (63%) is much higher than that by mixture of all known inhibitors combined. This indicates that the unidentified inhibitors, mostly phenolic compounds generated by lignin degradation, also act as major inhibitors comparable to any of the known inhibitor groups as suggested by Palmqvist et al., [15]. The inhibition effects by each group are not necessarily additive. The inhibitor groups interact with each other as the combined inhibitory effects were found to be different from addition of the individual inhibition. The sugar group decreased the glucan digestibility by 33% and xylan digestibility by 98%, which was more than the decrease seen by any of the inhibitor groups. The reason for this is unclear. Perhaps the sugars had an interaction, increasing the overall inhibitory effect. Similarly, adding all degradation products together showed a higher inhibition as compared to any one compound alone. The inhibition mechanism for these inhibitors is poorly understood at this time. Various reaction schemes have been proposed for describing inhibition. Some models assume competitive inhibition for certain type of inhibitors while some assume non-competitive inhibition schemes [22, 44, 45]. In these studies however the inhibition (interference) and deactivation (irreversible loss of activity) were not distinguished. This investigation was restricted to the gross effects of inhibitors and deactivators that exist in the PL of corn stover on the overall terminal digestibility.

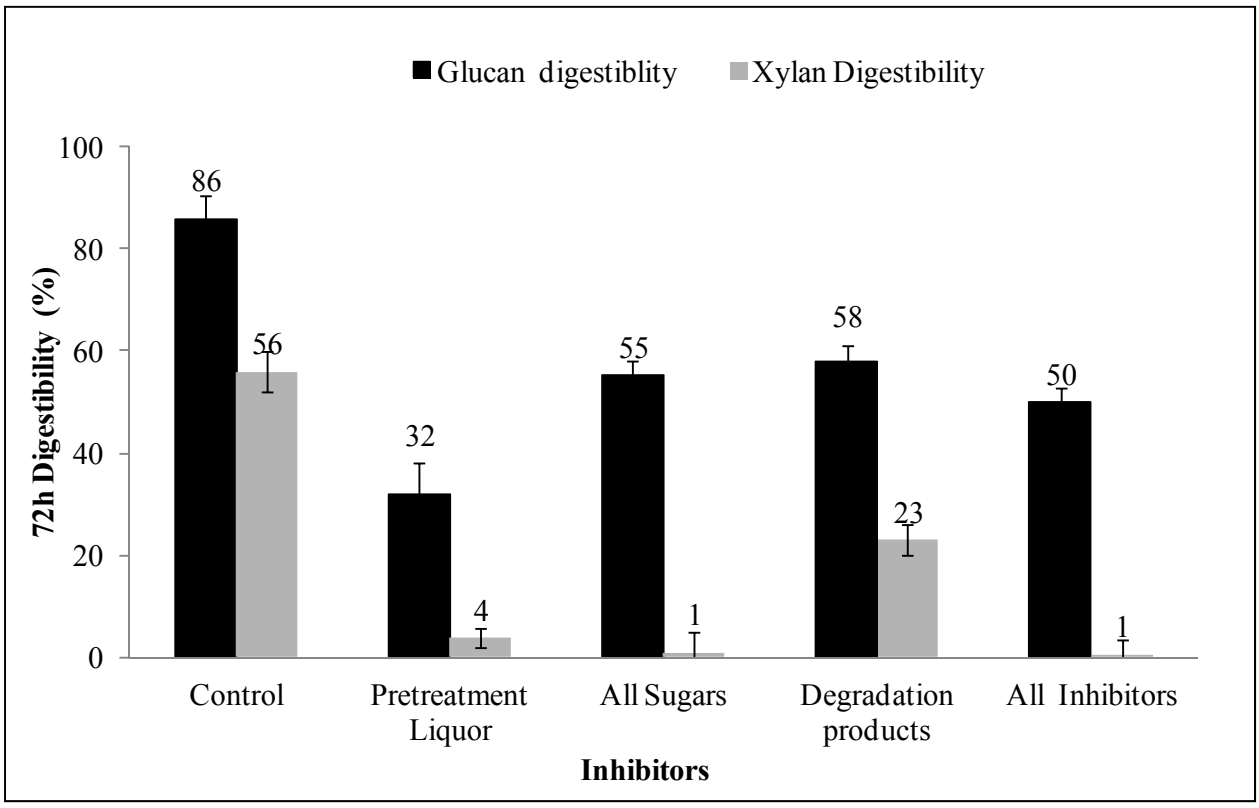

Figure III-11. Comparison of effect of inhibitor groups: sugars, degradation products and all inhibitors.
Enzyme loading: 15FPU/g glucan, 30CBU/g glucan; Substrate: Solka Floc 1% glucan loading
Inhibitors: All inhibitors = all compounds from Table 1 as found in 50 mL dilute acid hydrolysate; Degradation products = carbohydrate degradation products (acids and furans) from Table 1.

III.5 Conclusion

Potential inhibitors existing in the dilute-acid pretreatment liquor (PL) of corn stover were identified and their inhibition effects on enzymatic hydrolysis of Solka Floc were investigated. The inhibitors were grouped into sugar/lignin degradation products, sugars, and unknowns. The relative inhibition effects of each group were determined using 72 h enzymatic hydrolysis yield of glucan and xylan as the index. Inhibition by the dilute-acid PL was found to be higher than

that by simulated mixture of individual compounds. The inhibition effects by individual groups are not additive. Of the individual inhibitors studied, cellobiose, glucose, and acetate were identified as the major inhibitors to glucan digestibility. The 10%-tolerance level (10PT) of these inhibitors was in the vicinity of 1 g/L. The unidentified inhibitors also play a significant role in the overall inhibition. Xylose and xylo-oligomers inhibit xylanase activity and initial glucanase activity in Spezyme CP. For all the known inhibitors existing in the PL, at the given concentrations, the order of inhibitory strength is: Phenolic Lignin Derivatives > Sugars > Organic Acids > Furans.

III.6 References

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Chapter IV Deacetylation by Sodium Carbonate to Improve Enzymatic Hydrolysis of Dilute Acid Pretreated Corn Stover

IV.1 Abstract

Hydrolysate from dilute acid pretreatment of biomass contains various products of biomass degradation that are inhibitory to enzymes and microorganisms used in the bioconversion of sugars to ethanol. One of the main inhibitory compounds is acetic acid which is released from the lignin-hemicellulose matrix in the biomass. Acetic acid at 1 g/L inhibits cellulase activity by 10% and significantly increases the inhibition at higher concentrations. In most pretreatment liquors, the acetic acid concentration is between 3-10 g/L, which inhibits the cellulase activity by 20-60%. In this study, mild alkaline treatment using sodium carbonate was used as an initial deacetylation step to remove some of the acetyl content from native corn stover with the aim of reducing the final acetic acid concentration in the dilute acid pretreatment hydrolysate. 5-10% Na_2CO_3 was used at 30-60ºC for the deacetylation step and 0.5-1% H_2SO_4 at 60-130ºC were used as the pretreatment step conditions. Using this two-step process (deacetylation followed by dilute acid pretreatment) the final acetyl concentration in hydrolysate was reduced by 50-80% keeping hemicellulose loss in the range of 2-12%. This improved the enzymatic hydrolysis yields of the pretreated corn stover solids by 30% as compared with dilute acid pretreatment alone. Sodium carbonate was chosen as the deacetylation reagent since it is a mild alkali that can be

used without a significant loss in carbohydrates, and is an inexpensive reagent, and easily recovered using a part of the well-established Kraft recovery process.

IV.2 Introduction

The cell wall of corn stover primarily contains cellulose (C), hemicellulose (H) (composed of mainly xylan), lignin (L) and small amounts of other components such as ash and extractives. Acetyl groups form linkages between lignin and hemicellulose and are present on the xylan backbone in a molar ratio of 3 – 6 depending on the biomass composition. Acetates are esterified on the xylan backbone at positions O2 or O3 [1]. Use of cellulosic biomass for production of ethanol and other bioproducts requires three main steps: pretreatment, enzymatic hydrolysis and fermentation. The choice of a pretreatment method is important since it affects the enzymatic hydrolysis and fermentation yields, and the economics of the entire process [2, 3]. Dilute acid pretreatment is one of the most common pretreatment methods. The process involves hydrolysis of hemicellulose from biomass, leaving cellulose and lignin in the solid (Figure II-B) [4-6]. The washed solids and sugars from the pretreatment liquor are further converted to ethanol using enzymes and microorganisms. Pretreatment using a high solid concentration followed by high solids enzymatic hydrolysis can give high product concentrations and reduce capital costs in downstream processing [7-9]. Although high product concentrations can be achieved using high solids enzymatic hydrolysis of washed solids, at an industrial scale, the use of solid and liquid together as whole slurry without washing is simple and beneficial in process economics. This heterogeneous slurry presents a challenge to enzymatic hydrolysis since lower yields are

obtained in presence of hydrolysate. This behavior is more pronounced at higher solid levels since the background sugars and degradation compounds increase proportionately at these loadings [4].

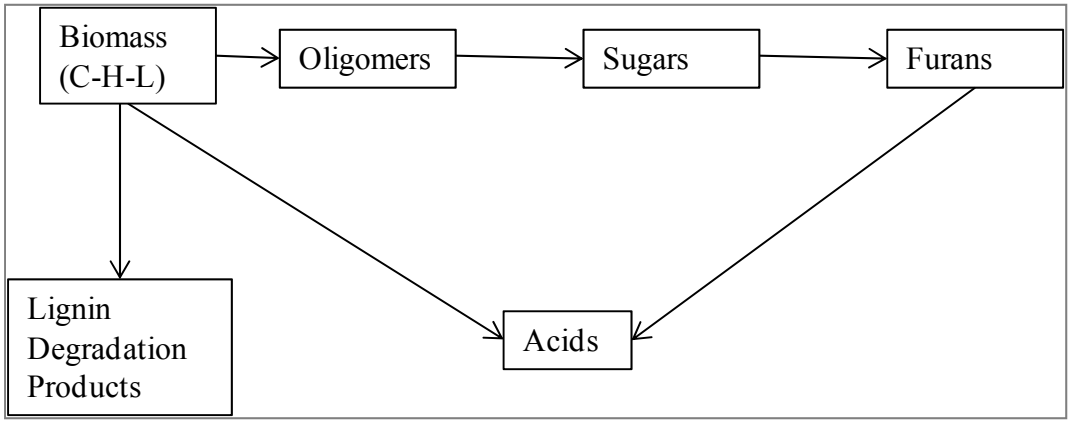


Figure IV-1. Simplified biomass degradation scheme.
(Refer to Figure II-8 for a detailed schematic for the degradation process.)

In Section III.4, it was seen that pretreatment, along with hydrolyzing hemicellulose, releases biomass degradation products such as furans, organic acids and phenolic compounds (Figure IV-18) [10]. These compounds severely inhibit the cellulase and β-glucosidase enzyme activity [10-13]. The concentration and distribution of these soluble inhibitors largely depends on the type and severity of pretreatment, the composition of biomass, and the concentration of solids applied during the pretreatment and the enzymatic hydrolysis processes. Acetate was the most potent inhibitor with a low 10PT (10% tolerance of enzyme) coupled with high concentration in the pretreatment liquor [10]. It has a significant impact on enzymatic saccharification [10, 11, 14] and Chen et al showed that removal of 75% of acetyl groups prior to pretreatment improves enzymatic digestibility significantly [26] [15]. Moreover, acetic acid also inhibits the

fermentation process interfering with the microbial cell physiology. In presence of the acid, microbial cell membrane becomes fluid due to proton imbalance which in turn, affects cell metabolism and growth. This gives a low growth yield, low fermentation rate, an increased lag phase, and inhibits essential enzyme activity due to the acidified cell cytoplasm [16, 17]. Thus, fermentation in presence of high acetate levels significantly reduces the ethanol yields.

Detoxification methods such as overliming, ion exchange treatment and/or activated charcoal treatments can reduce the concentration of acids, furans and phenolics and improve bioconversion efficiency [18]. Some of these methods involve the use of expensive membranes and ion exchange columns [19, 20]. Less expensive methods such as overliming and adsorption on charcoal show limited benefits and work best in combination with other detoxification treatments. High gypsum from the overliming detoxification method creates additional solid handling problems [21-25]. These detoxification methods require separation of solid and liquid, followed by treatment of liquid and thorough washing of solids. The clean inhibitor-free solid and liquid streams are then re-combined into a single bioreactor and converted to ethanol. These steps add costs and complications to the biomass-to-ethanol process. One finds significant economic benefit in performing treatment and fermentation of the whole slurry (solid liquid mixture) in a single step.

The focus of this study is to explore the feasibility of a two stage pretreatment process wherein the first stage is an alkaline deacetylation, to selectively remove acetyl groups from biomass followed by a second stage of dilute acid pretreatment and hence reduce the toxicity of the dilute acid pretreatment liquor. This method can reduce acetic acid concentrations to the level tolerated by the bioprocess and eliminate separate handling of solid and liquid stream. Literature

information is scarce in reducing the acid content of biomass prior to DA pretreatment. Chen et al and Garrote et al reported deacetylation using NaOH at 60-80ºC [15, 26]. Kim et al. observed solubilization of acetic acid along with lignin during lime pretreatment [27]. In this study, sodium carbonate was used as the deacetylation reagent since it is a mild alkali, which at low severity conditions does not hydrolyze sugars from the biomass. Another important point is that it is a low-cost alkali and is easy to recover than sodium relying on a part of the established recovery system in the Kraft pulping process. Details on the recovery process are discussed Sections V.2 and VI.2.

IV.3 Materials and methods

IV.3.1 Feedstock

Feedstock used in this study was corn stover, provided by the National Renewable Energy Laboratory (NREL, Golden, CO). It was stored in dry conditions at room temperature with a moisture content <10% (wt.). Its composition as obtained using standard NREL procedures (NREL-TP-510-42618 to 42622) was 34% glucan, 22% xylan, 6% other sugars, 18% lignin, 4% acetyl, 6% ash. Solka Floc (International Fiber Corporation, Urbana, OH, Cat. No. U064072) was used as the pure cellulose substrate. Its composition was determined to be 77.3% glucan and 22.8% xylan.

Cellulase enzyme (Spezyme CP) and Multifect Xylanase were a gift from Genencor-Danisco (Paulo Alto, CA) and β-Glucosidase was purchased from Sigma (Novozyme 188, Sigma, C-

6150). Activities of Cellulase and β-Glucosidase were 59FPU/ml and 600 CBU/ml respectively. Xylanase activity was 42 mg protein/mL.

IV.3.2 Dilute acid pretreatment

Corn stover was treated using dilute acid pretreatment in a batch reactor. A flanged batch reactor made of SS-316 (1.375" ID x 6" L) was used as the pretreatment reactor. In all experiments, 10 dry grams of corn stover was soaked with 0.5-1.5% H_2SO_4 solution. The reactor was then placed in an oven at desired treatment conditions. Liquid to solid ratio was varied from 5:1 to 10:1, temperature from 60-180ºC and time from 0.3-12 h.

IV.3.3 Deacetylation

Acetic acid from biomass can be extracted either by using acid or alkali. Chen et al al, were able to remove > 75% of acetic acid from corn stover using an NaOH solution at 60-80ºC [26]. Deacetylation in this study was performed as a batch treatment using 2-20% sodium carbonate solutions in screw capped glass bottle reactors. In all experiments, 20 dry grams of corn stover was soaked with specified concentration of Na_2CO_3 solution. The reactor was then placed in a convection oven at desired treatment conditions. The liquid to solid ratio was varied from 5:1 to 10:1, temperature from 30-90ºC and the treatment time from 3 to 24 h.

IV.3.4 Analytical methods

IV.3.4.1 Composition of solid and liquid

For complete composition analysis, the deacetylated or dilute acid treated solid/liquid mixture was separated using 25 μm filter papers to make sure the composition of the solids was not affected by presence of the dissolved sugars and degradation products.

Sugars in the liquid sample were determined in an HPLC equipped with an RI detector using a Biorad-HPX-87P column. The BioRad-HPX-87H column was used for measurement of organic acids and furans. Oligomers were quantified based on the NREL protocol NREL-TP-510-42623. Secondary hydrolysis was performed on the extracted liquid at 4% acid concentration, 121ºC for 60 min. The liquid after secondary hydrolysis was again analyzed for sugars in HPLC. The difference in monomer concentration between liquid before and after secondary hydrolysis was taken as the oligomer concentration.

The solids were analyzed for carbohydrates, lignin and ash using standard NREL protocols (NREL-TP-510-42618 to 42622). The composition of the treated samples was reported here on the basis of the original, taking the weight loss after pretreatment into account. Moisture was calculated using an infrared moisture balance (Denver Instrument, IR 30). All analysis was carried out in duplicate with a standard variance of +/- 0.05.

IV.3.4.2 Enzymatic Hydrolysis

Though low solids enzymatic digestions on clean solids generally display promising results, a process that requires exhaustive washing of pretreated slurry is not likely to be commercially viable. To demonstrate effectiveness of the deacetylation and dilute acid pretreatment process in

a commercially relevant manner, whole slurry enzymatic saccharification was performed in this study, unless otherwise specified.

The solid/liquid mixture (unwashed solids) after dilute acid pretreatment was neutralized using NaOH till pH was close to 4.8. If enzymatic hydrolysis of washed solids was desired, no NaOH was necessary; the pretreated solids were washed with cold DI water, until pH reached close to 5.

The enzymatic digestibility of the neutralized slurry was determined based on the NREL standard biomass analytical procedure NREL-TP-510-42621. Screw capped 250 mL Erlenmeyer flasks were used as the hydrolysis reactors. The insoluble solids loading in the enzymatic hydrolysis process was between 1% and 7% (w/v). Enzyme loading used was 10 FPU and 30 CBU/g glucan. Antibiotics tetracycline and cyclohexamide were added into the reactor to maintain sterile conditions. The enzymatic digestibility tests were carried out at 50ºC, and pH 4.8 using 0.05 M sodium citrate buffer in an incubator shaker (New Brunswick Scientific, Innova-4080) agitated at 150 rpm. The pH was checked before and after hydrolysis to make sure it remained constant and the chemicals used in the preparation of solids did not affect the results.

HPLC sample preparation consists of collecting a small amount of biomass slurry, centrifuging to separate the free liquid, and diluting with deionized water. Total amounts of released glucose and cellobiose after 0, 6, 24, 48 and 72 h of hydrolysis were used to calculate the enzymatic digestibility in Eq. III-1. Glucose in the sample at time 0 h was subtracted from the rest of the samples. The xylan digestibility was determined in a similar manner, with a hydration factor of 1.136 instead of 1.111 in the above equation.

IV.4 Results and Discussion

IV.4.1 Dilute acid pretreatment and its effect on acetyl content

This study focuses on developing the methods and understanding the impacts of deacetylation of corn stover on dilute acid pretreatment and enzymatic hydrolysis. For comparison, dilute acid pretreatment was performed in batch reactors, for deacetylated and non-deacetylated biomass at the same pretreatment conditions.

Dilute acid pretreatment of untreated corn stover at 1% H_2SO_4, L:S of 7:1 for 6 h at 130°C showed 79% glucan digestibility using 10 FPU/ g glucan Spezyme CP. Composition of the pretreated biomass solid and liquid are discussed in Figure IV-10 and IV-11. The acetic acid content in the liquid hydrolysate was found to be 2.87g/L (Figure II-8), a level far above the 10PT. From our earlier study, acetic acid at concentrations of 1g/L inhibits cellulase activity by 10%. In a repeated test, it was confirmed that 2.87 g/L acetic acid inhibits glucan digestibility by 29% [10]. Generation of acetic acid depends on the severity of the dilute acid pretreatment. For example, Schell et al., show that dilute acid pretreatment at 0.5% acid 150°C for 20 min and 45% solid loading produces 4.9 g/L acetic acid, and the same pretreatment at higher severity conditions of 160°C, 2% acid for 5 min produced 11.8 g/l acetic acid [28].

IV.4.2 Washed vs. unwashed pretreated biomass

Dilute acid pretreatment yields a slurry with pH of 1 to 3 depending on the pretreatment severity. Corn stover pretreated with 1% H_2SO_4 at 130ºC for 6 h was divided into two equal parts. One part was washed with DI water until neutral (washed solids). The other was treated with 1M NaOH to bring the pH to 5 (unwashed solids). Both parts were hydrolyzed using a mixture of

cellulase and β-glucosidase at 15 FPU + 30 CBU /g glucan to show the difference in conversion between washed and unwashed material Figure IV-2 indicates a 25% reduction in the final glucose yield when unwashed solids were used compared to washed solids. Improved enzymatic digestibility upon washing indicates that at least some of the inhibitors in the pretreated solids are water soluble.

Figure IV-3 reaffirms the effect of pretreatment liquor (PL) on enzymatic hydrolysis (Details in Section III.4). Cellulase activity is significantly reduced in the presence of dilute acid pretreatment liquor. With 50% (v/v) mixture of PL and buffer solution, the 72 h digestibility of glucan in Solka Floc was reduced by 63% in comparison to the control run (no PL addition). Although inhibition in enzymatic hydrolysis can be eliminated by washing the treated biomass, it is not a favorable choice in process design because of costs associated with it.

The main reason for poor hydrolysis yield of unwashed samples is the biomass degradation products released during pretreatment. The unwashed samples also contain a high concentration of background sugars. Glucose, cellobiose and xylooligomers reduce cellulase activity significantly by product inhibition [34]. This is proven in Figure IV-4 which shows the enzymatic hydrolysis of pure cellulose with inhibitory compounds from dilute acid pretreatment liquor as discussed in Section III.4. The Figure IV-4 shows the main individual components that affect enzymatic digestion. It was found that glucose, cellobiose and acetic acid were the most potent inhibitors, with 10PT of <1g/L. Their actual concentrations in the slurry were higher, thus inhibiting cellulase significantly. The effect of inhibition by sugars can be reduced if Simultaneous Saccharification and Cofermentation (SSCF) process is used for fermentation, wherein the sugar concentrations remain near zero as the SSCF proceeds under sugar-limited

condition. Thus acetic acid remains as the most potent inhibitor. In spite of this high inhibitory effect and its high concentrations, very little work has been reported on reducing the acetic acid content in biomass prior to pretreatment. If acetic acid is removed early in the process, it would help reduce the toxicity of the dilute acid pretreatment liquor.

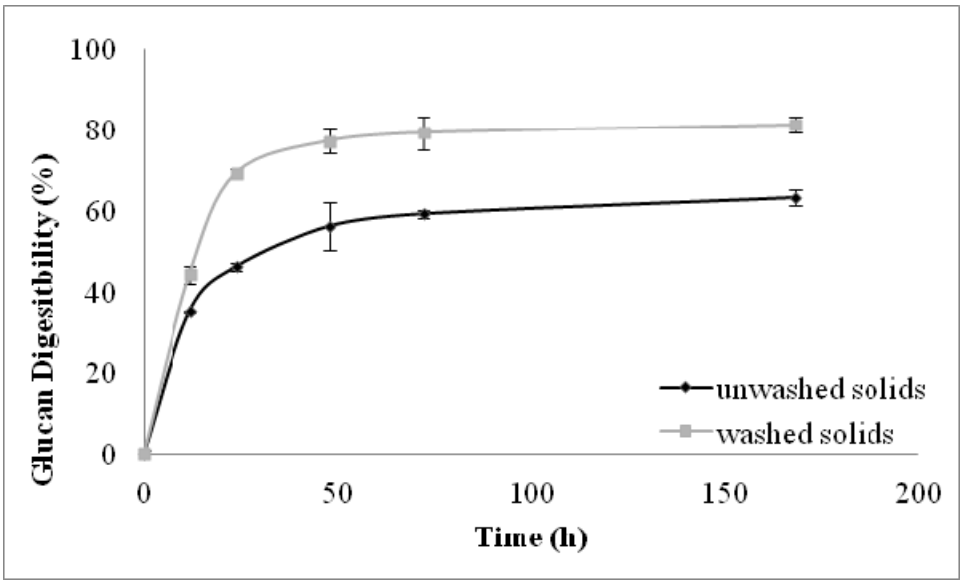


Figure IV-2. Effect of washing on enzymatic hydrolysis of dilute acid pretreated corn stover. Pretreatment conditions: 1% H_2SO_4, 130°C, 4 h.; Substrate loading: 1% glucan; Enzyme loading: 15FPU + 30 CBU/g glucan.

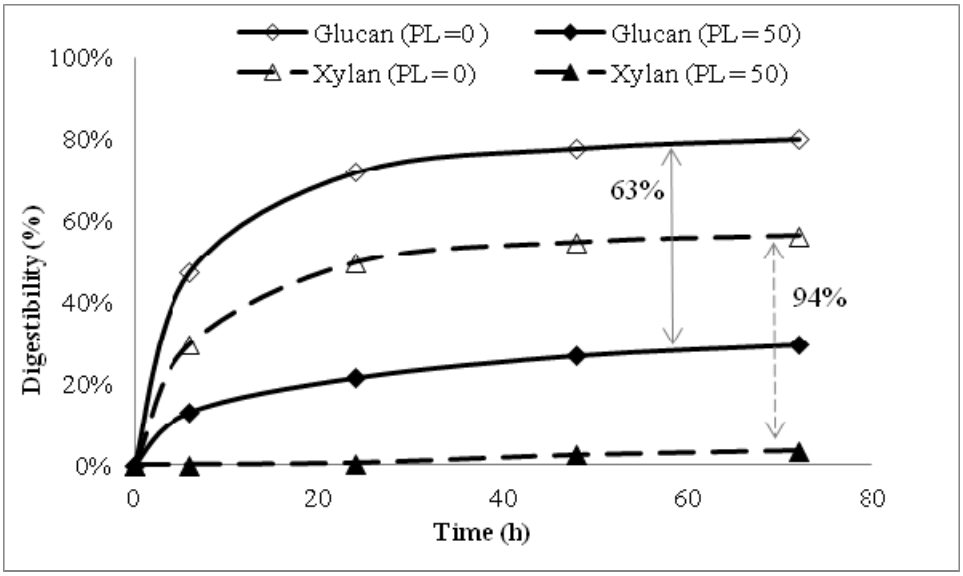


Figure IV-3. Effect of dilute acid pretreatment liquor on glucan and xylan digestibility. Enzyme loading: 15FPU/g glucan, 30CBU/g glucan; Substrate: Solka Floc 1% glucan loading Inhibitor: Dilute acid hydrolysate (PL) 50 % (vol.).

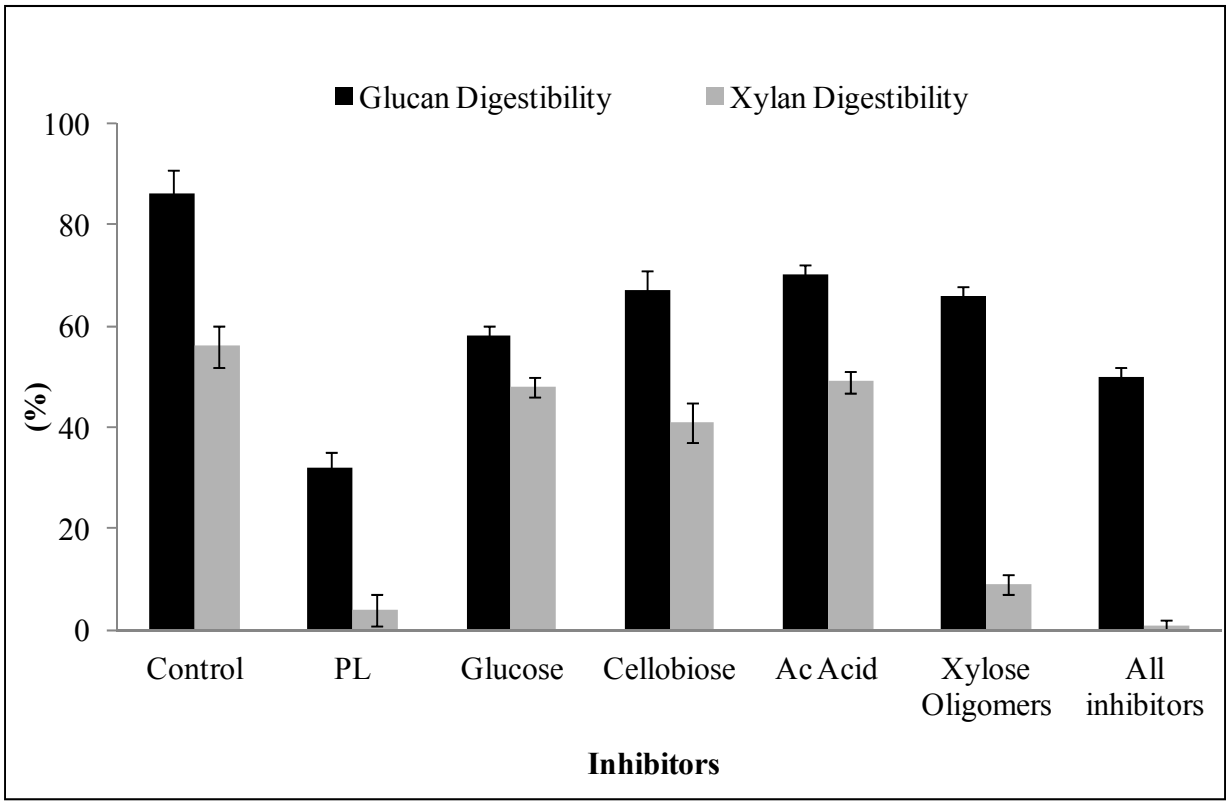


Figure IV-4. Effect of individual components from pretreatment liquor on digestibility. Enzymatic digestibility: 15FPU + 30CBU /g glucan; 1% glucan loading Solka Floc; Inhibitors: Dilute acid hydrolysate (PL) 50 % (vol.), all inhibitors added in the same concentration as found in the PL

IV.4.3 Deacetylation treatment conditions

Figure IV-5 (b) shows a process diagram of the proposed two step pretreatment process. First step is an alkaline deacetylation, carried out in batch mode under a mild condition using sodium carbonate. The second step is a dilute acid pretreatment using 1% H_2SO_4, at 130°C for 12 h. Alkaline deacetylation was done covering the conditions of: 2-20% Na_2CO_3, at 5:1 to 10:1 L: S ratio and 30-90°C for 0.5 - 24 h. The conditions were optimized to minimize acetic acid from the biomass and the loss of hemicellulose. In reference to Figure IV-5b, the acetic acid concentration in stream 2 in b is to be minimized while keeping hemicellulose loss in the stream 1 to a minimum. Hemicellulose loss was calculated based on the solid composition of original untreated biomass.

IV.4.3.1 Effect of temperature and time

Deacetylation of corn stover at 15% Na_2CO_3 at 10:1 L: S ratio for 24 h, at varying temperatures was studied. The results are shown in Figure IV-6. The composition data indicate that hemicellulose loss in alkali treatment increases rapidly with temperature. Hemicellulose loss was discernible even at temperature as low as 60°C. Since at this stage, carbohydrate loss is not acceptable, deacetylation temperature of 30°C was chosen. Among the main components of biomass cell wall, hemicellulose is the first component to be released and the most sensitive to temperature at neutral conditions [29]. At acidic and alkaline conditions, the sensitivity to temperature is more pronounced as seen here, and also supported by Hendriks [29]. Acetate from the xylan backbone was also removed under these conditions (data not shown). The main criteria determining the temperature of the deacetylation step was minimizing of the hemicellulose loss.

The effects of treatment time on the deacetylation are summarized in Figure IV-7, in which corn stover was deacetylated using 15% sodium carbonate at room temperature and varying treatment times from 30 min to 24 h. The data indicate that as the time of deacetylation treatment increases, hemicellulose loss and deacetylation of biomass both increase. At 6 h, 6% of hemicellulose was lost from biomass.

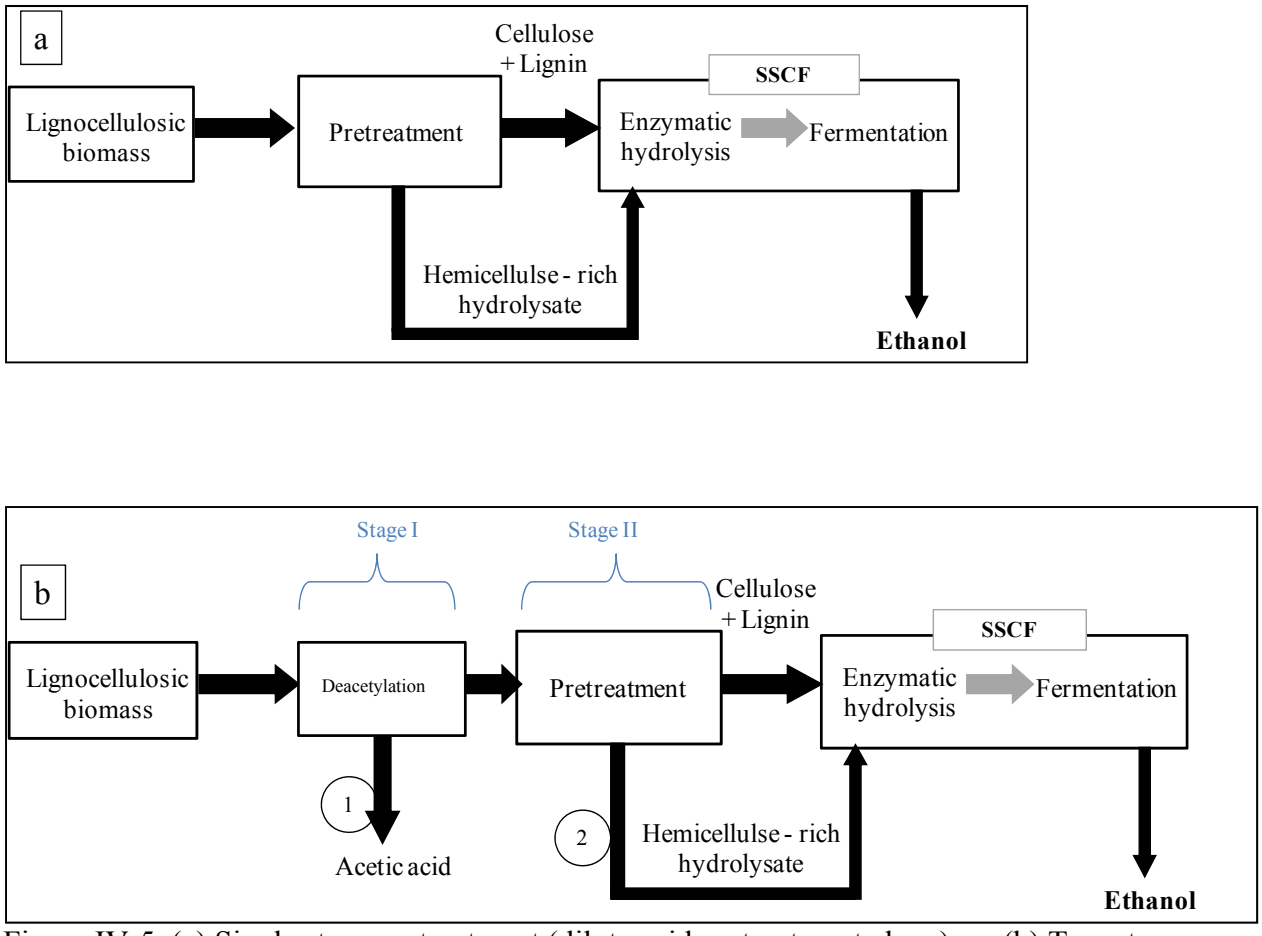


Figure IV-5. (a) Single stage pretreatment (dilute acid pretreatment alone) vs. (b) Two stage pretreatment (Deacetylation followed by dilute acid pretreatment) of corn stover.

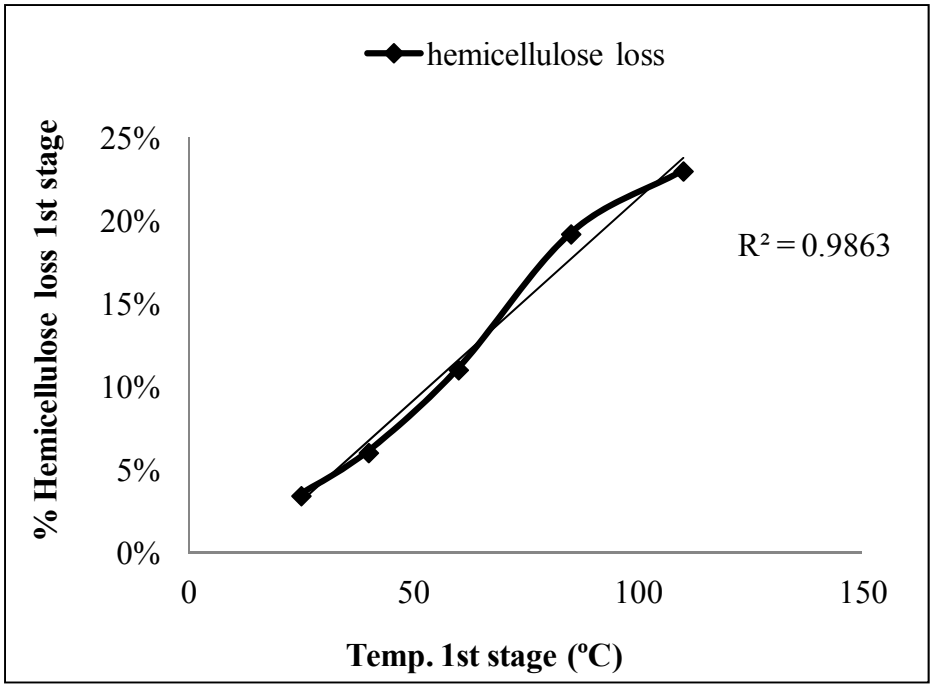


Figure IV-6. Effect of temperature on deacetylation of biomass.
Deacetylation conditions: 5% Na_2CO_3, 24h, 10:1 L: S ratio

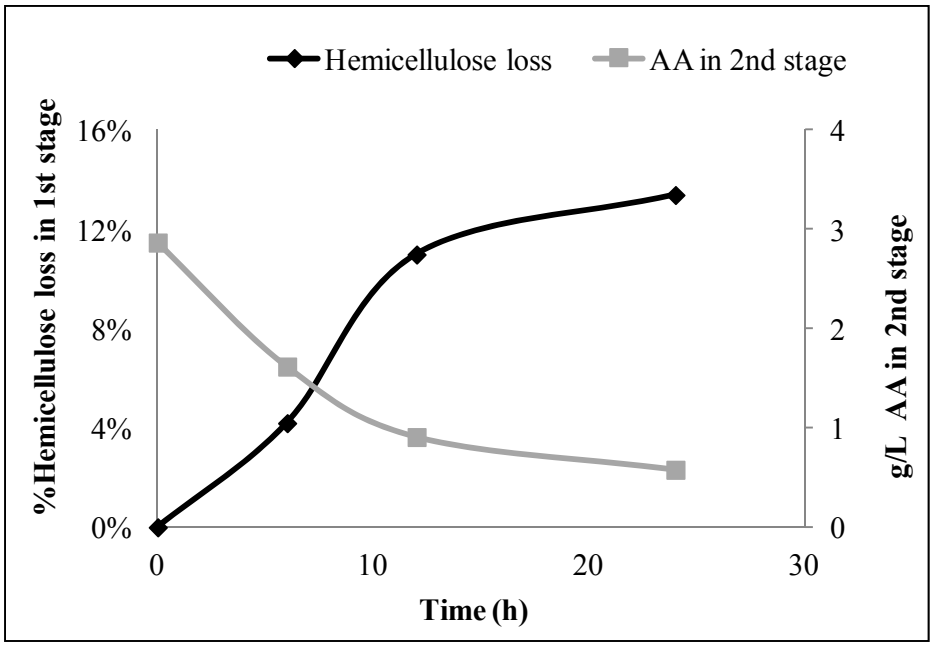


Figure IV-7. Effect of time on deacetylation of biomass.
Deacetylation conditions: 15% Na_2CO_3, 30°C, 10:1 L: S ratio.

The alkali-deacetylated biomass was further treated using dilute acid pretreatment with 1% H_2SO_4, at 130°C for 12 h. When the alkali extracted biomass was further treated with dilute acid, the acetic acid content in the hydrolysate was reduced from 2.87 to 1.62 g/L, a 44% reduction; when the deacetylation time was changed to 24 h hemicellulose loss increased to 10% and the acetic acid released in the 2nd stage was reduced by 82% in comparison to dilute acid treatment without any deacetylation.

IV.4.3.2 Effect of sodium carbonate concentration and liquid to solid ratio

Figure IV-8 shows the effects of sodium carbonate concentration on deacetylation. Beyond 5% Na_2CO_3, the hemicellulose loss was above 4% and the acetic acid concentration in the second stage stayed relatively constant. Under the conditions of 5% Na_2CO_3, for 24 h, 30ºC, 10:1 L: S ratio, 4% hemicellulose was lost. When deacetylation was followed by dilute acid pretreatment at 1% H_2SO_4 at 130ºC for 12 h, the pretreatment liquor (stream 2 in Figure IV-5 b) contained 0.81 g/L acetic acid, a 72% reduction from the control.

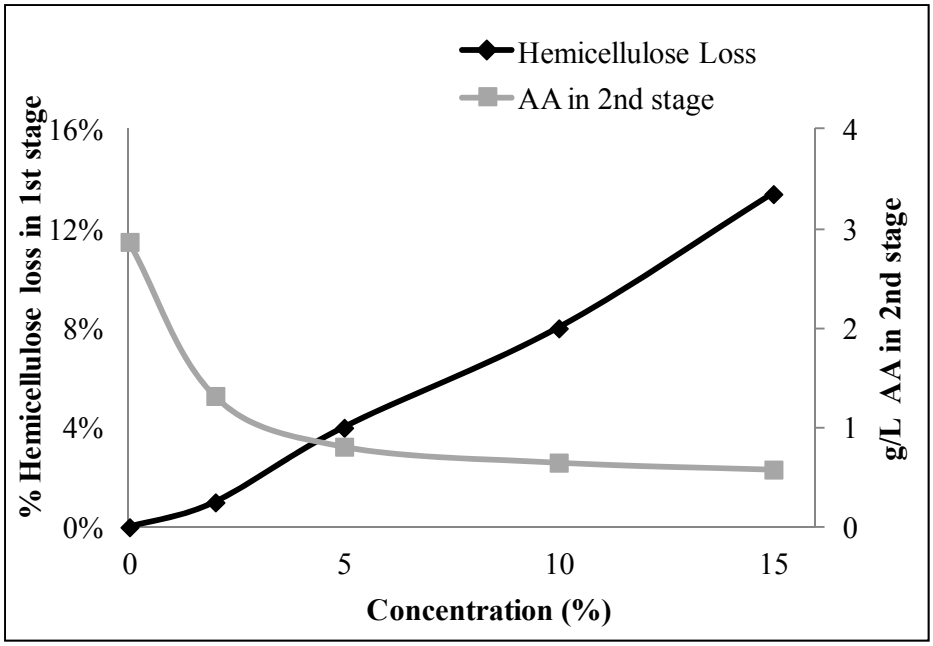


Figure IV-8. Effect of sodium carbonate on deacetylation of biomass. Deacetylation conditions: 24 h, 30 °C, 10:1 L: S ratio.

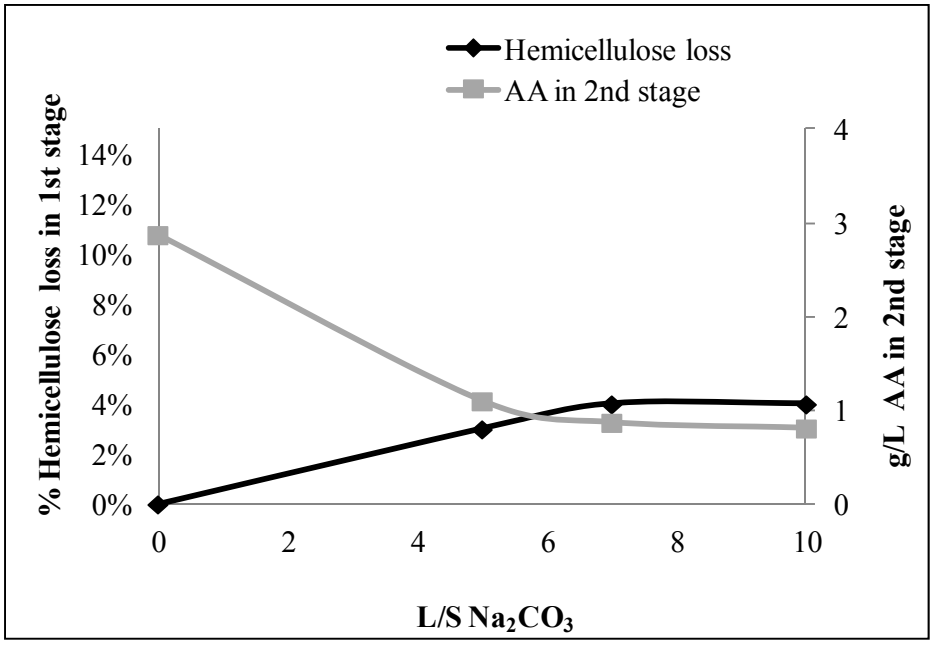


Figure IV-9. Effect of liquid: solid ratio on deacetylation of biomass. Deacetylation conditions: 5%, 24 h, 30 °C, 10:1 L: S ratio.

The effects of L/S ration in deacetylation are shown in Figure IV-9, where it was varied from 5:1 to 10:1. For deacetylation conditions of 5% sodium carbonate, at 30ºC for 24 h, the optimum L: S ratio was about 7:1, beyond which the acetic acid level in the 2nd stage did not change significantly and hemicellulose loss remained below 4%.

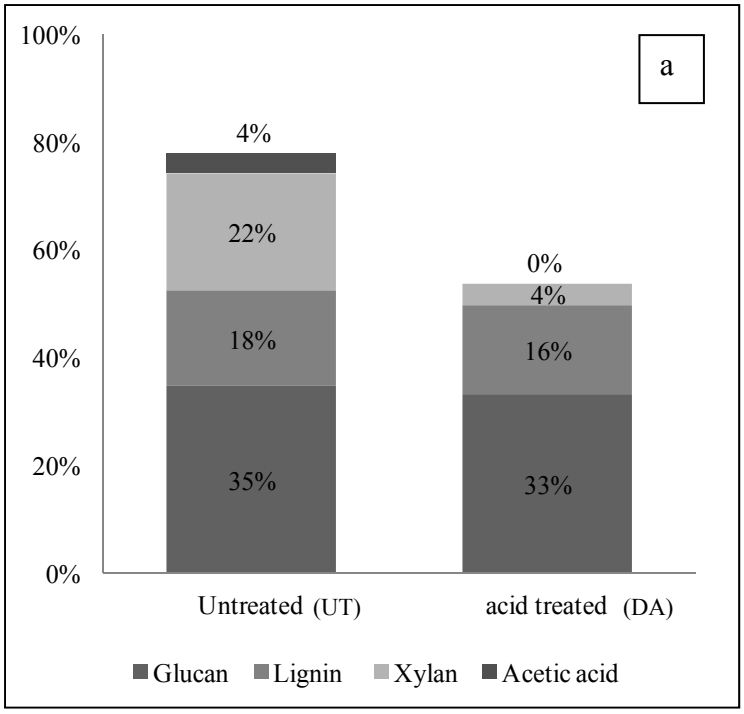
Thus the optimum deacetylation conditions were taken to be 5% sodium carbonate, at 7:1 L: S ratio, at room temperature for 12 h. At these conditions, the hemicellulose loss after the extraction (stage I) was 3.6% and the acetic acid in the 2nd stage was reduced by 73% to 0.8 g/L compared to the control.

IV.4.4 Composition analysis

For deacetylation of corn stover, 20 g of biomass was treated with 140 mL of 5% Na_2CO_3 at room temperature, giving a reagent to biomass ratio of 0.35 g Na_2CO_3/g biomass. Deacetylation was followed by dilute acid pretreatment (Stage II of Figure IV-5 b) with 1% H_2SO_4 at 130°C for 12 h. During the deacetylation process (stage I of Figure IV-5 (b)), approximately 20% of the initial corn stover mass was extracted due to the removal of extractives, acetic acid and some portion of lignin. For comparison, biomass was treated only with dilute acid at the same pretreatment conditions without any deacetylation, washed and dried in a similar manner (Figure IV-5 a). The composition data of corn stover before and after treatments are summarized in Figure IV-10 a-b: dilute acid pretreated only (DA), deacetylation only (D), deacetylation + dilute acid pretreatment (DDA), and untreated. The acetate content of the deacetylated corn stover (D) was about one third of the control (DA corn stover). The deacetylated corn stover also had slightly lower lignin content over untreated corn stover. The composition figure shows only the

main components, and all compositions are based on that of the untreated corn stover and reflect the weight lost during various treatments.

Hydrolysis using 1% solid loading was performed with washed pretreated solid to delineate the isolated effect of the alkaline deacetylation and that of lignin loss seen in Figure IV-10 b. for this purpose, the enzymatic hydrolysis was performed using untreated corn stover (UT), deacetylated corn stover (D), deacetylated + dilute acid pretreated corn stover (DDA) and only dilute acid pretreated corn stover (DA) at 1% glucan loading, The solids contained no free sugars since they were washed thoroughly before the test. The glucan digestibility of the UT, D, DDA, and DA samples were 7%, 24%, 87.6% and 84% respectively. This shows that deacetylation by itself does not have a significant effect on improving the digestibility; slight improvement in digestibility of deacetylated corn stover over the untreated indicating a small effect of the lost lignin. The main purpose of deacetylation it to remove the toxicity of the liquid generated by dilute acid pretreatment.



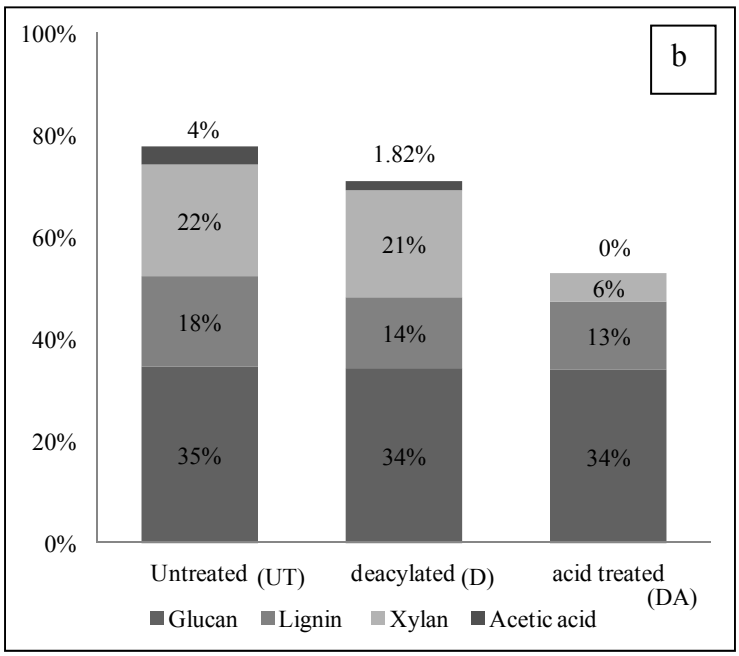


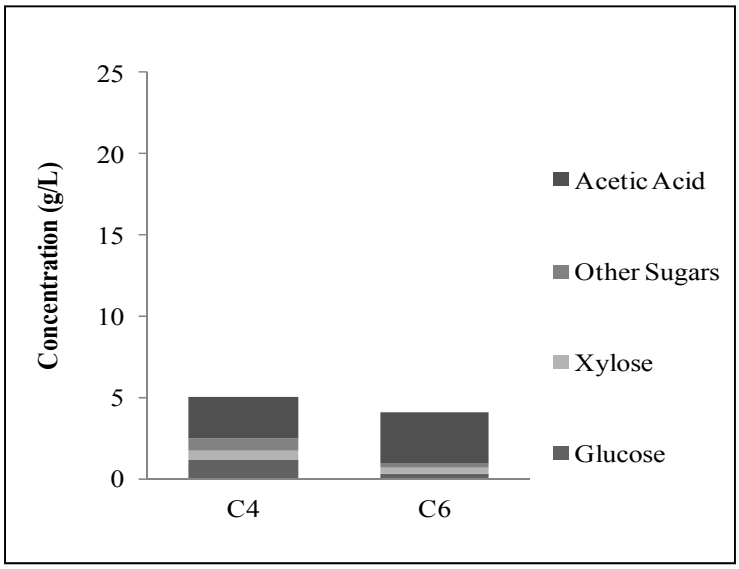
Figure IV-10. Solid analysis.
DA Pretreatment: 1% H_2SO_4 at 130°C for 12 h
Deacetylation condition in (b): 5% Na_2CO_3, 7:1, 30ºC

IV.4.5 Liquid analysis

The objective of the two-step pretreatment was to remove acetyl content in the biomass in the first stage using alkaline deacetylation, while minimizing the sugar loss. Figure IV-11 a shows the liquid concentration from alkaline deacetylation of corn stover. The data show that deacetylation is indeed achieved without losing the sugars as intended choosing the said treatment conditions.

The second step of dilute acid pretreatment solubilizes hemicellulose and acetate from the biomass. Biomass that was deacetylated prior to dilute acid pretreatment are denoted here as DDA sample and the biomass that was only dilute acid treated is denoted as DA sample. Figure IV-11 b shows the composition of liquid from dilute acid pretreatment of deacetylated corn stover (DDA) in comparison with only dilute aid pretreated corn stover (DA).

For DDA samples, the acetic acid content of the liquid reduced by more than 70% when compared with DA sample. The glucan hydrolyzed by deacetylated and dilute acid treated (DDA) samples was halved when compared to that of only dilute acid pretreatment (DA). This also reduced further decomposition of the glucan, since HMF concentration of DDA samples reduced by 90% compared to DA sample. The furfural concentration in DA as well as DDA samples was in the range of 0.5-1.0 g/ L.



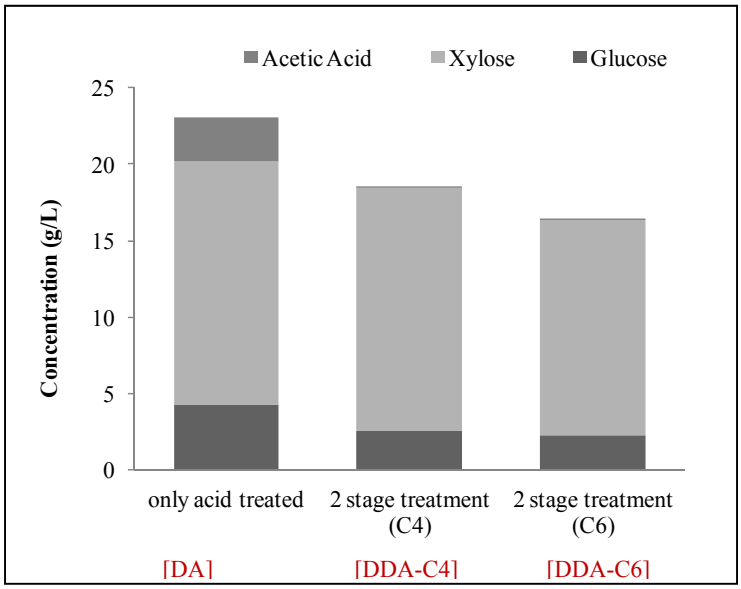


Figure IV-11. Liquid analysis.
DDA-C4: liquid from deacetylation treatment : 5% Na_2CO_3, 5:1, 30ºC.
DDA-C4: liquid from deacetylation treatment: 5% Na_2CO_3, 7:1, 30ºC.

IV.4.6 Enzymatic hydrolysis

To further demonstrate the effectiveness of the optimized deacetylation process, whole slurry enzymatic hydrolysis was performed for acid pretreated corn stover, and compared with deacetylated acid pretreated corn stover applying solid loadings of 1% to 7% (wt.). Enzyme was added on the basis of the glucan content of the insoluble solids at 10 FPU+30 CBU/ g glucan. The slurry after pretreatment is acidic; hence NaOH was added to increase the pH to close to 4.8 which is the optimum for cellulase enzyme activity. Figure IV-12 shows the comparison in terminal glucan digestibility for deacetylated and non-deacetylated samples. At low solid loading (1% solids), the effect of alkaline deacetylation is very low, showing only a 4% increase, which is not statistically significant. As the solid loading was increased, the effect of alkaline deacetylation became more prominent. At 7% solids, the improvement was by 30% over that of the acid-only pretreated sample. An important point to be noted here is that the terminal digestibility decreases as solid loading increases. The glucan digestibility decreased from 82% to 45% for acid-only treated samples as the solid loading was increased from 1% to 7%. The decrease was 85% to 63% for deacetylated + acid treated samples.

The decrease in digestibility for high solid loading is well-documented [35, 36]. The reduced glucose yield with whole slurry high solid loading can be explained by the higher concentration of degradation compounds present in the pretreatment liquor which inhibit the cellulase enzyme activity.. Presence of high background xylose monomer and oligomers significantly affect the xylanase activity reducing the conversion of the remaining xylan [30]. Xylan and xylooligomers are known to inhibit the cellulase activity significantly [31, 32]. Adsorption, diffusion and mass transfer related barriers also affect the high solids enzymatic hydrolysis [33]. DDA samples

contained lower concentration of acetate inhibitor in the slurry, which provides an explanation for the 30% increase in enzymatic digestibility observed in this test.

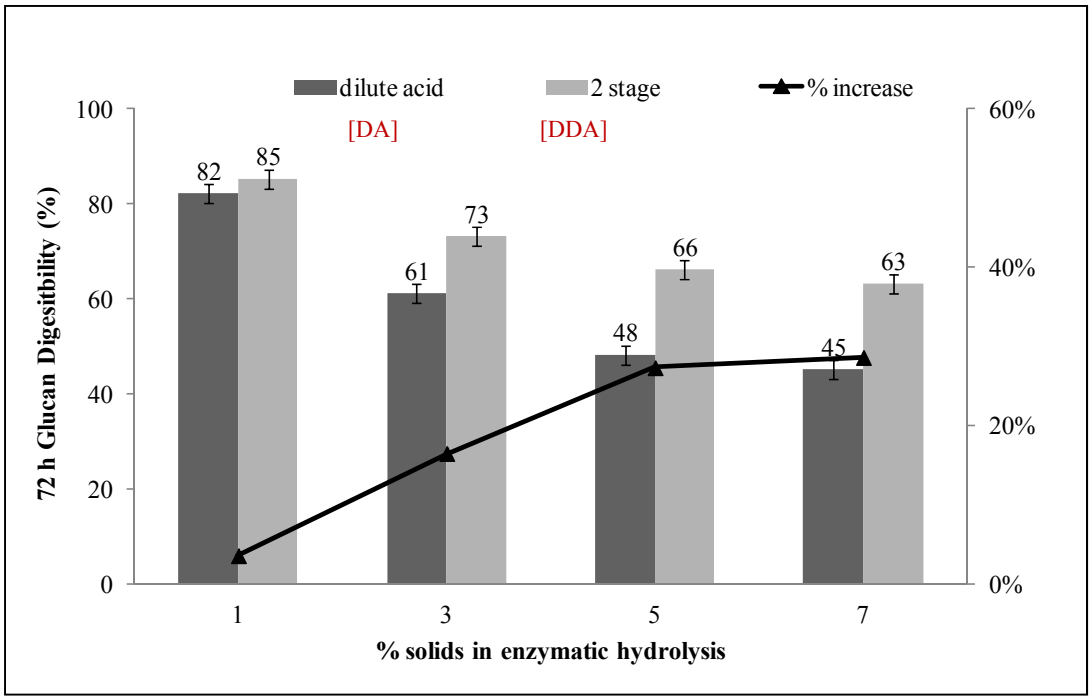


Figure IV-12. Whole slurry enzymatic hydrolysis of acid treated corn stover and deacetylated + dilute acid treated corn stover.
Deacetylation condition: 5% Na_2CO_3, 7:1, 30ºC
DA Pretreatment: 1% H_2SO_4 at 130°C for 12 h; slurry neutralized with NaOH until pH 5
Enzymatic hydrolysis: 10FPU+30CBU/g glucan, whole slurry hydrolysis.

Although this process uses an inexpensive, easy-to-recover mild alkali, it does add to the process cost. Thorough economic evaluation would be required to better understand the impact of deacetylation over other methods of reducing hydrolysate toxicity. This study was undertaken as a proof of concept; hence, the deacetylation and pretreatment both were performed using batch reactors. Additional work using continuous, or semi batch reactor models will be useful in improving yields and potentially reducing the water usage in both steps.

IV.5 Conclusions

Alkaline deacetylation of corn stover was effective in improving the whole-slurry enzymatic hydrolysis of dilute acid pretreated corn stover. Dilute acid pretreatment liquor inhibits the cellulase enzyme activity by hindering action of the degradation compounds released during pretreatment. Among the individual degradation products found in the liquor, acetic acid was identified as the most potent inhibitor, which reduces the cellulase activity by 20-60%. Alkaline deacetylation of corn stover at room temperature using 5% Na_2CO_3, removed more than 70% of acetate from hemicellulose, while keeping xylan loss to 3 – 4 % of the initial content. Deacetylation improved the xylose to acetyl ratio from 5.5 to 11.5. Deacetylated biomass also showed a lower solubilization of glucan in the dilute acid pretreatment liquor. This led to a 90% reduction in HMF which indicates a higher glucan recovery in the overall bioconversion process. Alkaline deacetylated biomass improved enzymatic hydrolysis yields by 30% under process relevant high-solids, whole slurry conditions.

IV.6 References

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Chapter V Comparison of Alkaline Reagents in Pretreatment of Herbaceous Biomass Under SAAL Treatment Scheme

V.1 Abstract

Sodium hydroxide, aqueous ammonia, and sodium carbonate were investigated as pretreatment reagents for corn stover switchgrass. The pretreatment scheme used was Soaking in Aqueous Alkali (SAAL) which is a batch process. The effects of reagent concentration, size of substrate, temperature and time of treatment on enzymatic hydrolysis were investigated to determine optimum conditions. The effect of pretreatment varied significantly depending on the type of alkaline reagent. Switchgrass has higher lignin content therefore requires pretreatment with higher severity than corn stover. Each of the alkaline reagents has its own merits in their ability to selectively remove lignin, the non-digestible part of the biomass. Ammonia is easily recoverable; NaOH is a strong base, and a proven delignifying reagent; and Na_2CO_3 is a weaker alkali, but much less expensive than NaOH. Various concentrations of the reagent were used for the treatment. Small amount of supplementary reagents such as hydrogen peroxide, sodium sulfide and surfactants were added where applicable to improve the yields on enzymatic hydrolysis. It was found that the alkaline treatments applied in this work removed 45-75% of lignin, improving glucan digestibility of switchgrass and corn stover to above 70% and 80% respectively. Representative treated substrates were further studied for surface structure and

crystallinity index by SEM, BET and XRD. The crystallinity surface porosity and specific surface area all increased after alkaline pretreatment.

V.2 Introduction

Chemical pretreatments break down specific components of biomass (hemicellulose or lignin) and make the cellulose more accessible [1, 2]. In Chapter III, the effects of dilute acid pretreatment on enzymatic hydrolysis and fermentation are presented. Pretreatments that use alkaline reagents have notable advantages over other chemical pretreatments. Alkaline pretreatments offer high selectivity for reaction with lignin over carbohydrates, thus conserving most of the carbohydrates in solid. In this type of pretreatment, sugar stream is not produced; therefore, it eliminates post treatment of the pretreatment liquid. The absence of toxins and inhibitors leads to high fermentation efficiency [3-5]. Various alkaline reagents such as calcium hydroxide (lime) [6, 7], sodium hydroxide [8, 9], and ammonia [10-12], have been studied for pretreatment of biomass. The recovery processes for these reagents is discussed in detail in Section II. 6

In alkaline pulping with sodium hydroxide, lignin degradation occurs due to the breakdown of aryl-ether linkages which constitute about 50-70% of total linkages in biomass. Aryl-aryl linkages and C-C linkages are relatively stable and more difficult to hydrolyze [13]. The α-aryl-ether linkages in lignin are readily cleaved under alkaline conditions, and the reaction is controlled primarily by the bond structure, not sensitive to [OH-] as long as the pH is greater

than 11 [13]. The β-aryl-ether linkages show a first order kinetics with respect to the bond structure and hydroxyl ions and are independent of [HS-]. Thus cheaper alkaline reagents, which can increase the pH to higher than 11, may be explored for pretreatment of biomass. Sodium carbonate is one such reagent; it is 4-6 times cheaper than sodium hydroxide [14] and easier to recover than NaOH. As shown in Figure V-1 only a part of the sodium hydroxide recovery process used in the Kraft pulping industry is required for the recovery of sodium carbonate.

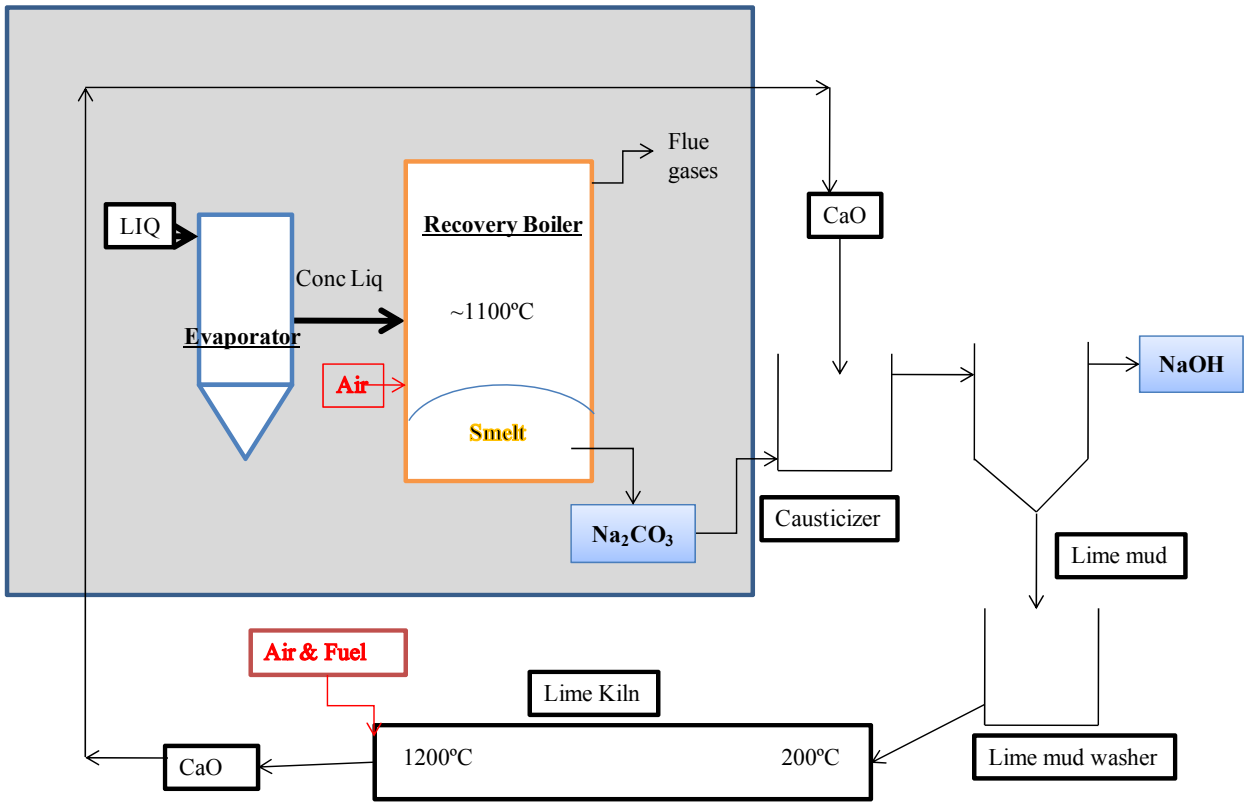


Figure V-1. Recovery process for Na_2CO_3 and NaOH.
Sodium carbonate recovery (shaded portion) is part of the sodium hydroxide recovery (full figure).

After solid liquid separation, the lignin rich liquid stream is concentrated and sent to a recovery boiler where lignin is burned at 1100°C. Here, sodium carbonate is left behind as smelt, which can be recycled [15]. Sodium carbonate has not been extensively studied for pretreatment of herbaceous biomass. Since it is a cheaper reagent than sodium hydroxide and easier to recover, it was investigated in this work along with two other reagents, aqueous ammonia, and sodium hydroxide. A batch pretreatment process, Soaking in Aqueous Alkali (SAAL), was employed in the experiments. The pretreatment conditions for SAAL pretreatment were optimized in consideration of two opposing factors: enzymatic hydrolysis yield and loss of carbohydrate to the liquid stream. Process variables considered for optimization include pretreatment reagent, reagent concentration, time and temperature of pretreatment, liquid to solid ratio, and size of substrate, with two substrates switchgrass and corn stover.

V.3 Materials and methods

V.3.1 Substrates

Switchgrass (SG1) was locally grown at Auburn University, (Department of Agriculture, Agronomy and Soils). Corn stover (CS) was obtained from National Renewable Energy Laboratory (NREL, Colorado). Biomass was stored at room temperature and size reduced to (–) 20 mesh size prior to pretreatment. Moisture content of the feedstocks as received was between 8-10 wt%. Figure V-3 shows the composition of switchgrass and corn stover raw materials. The selected batch of switchgrass SG1 contained unexpectedly high lignin content (27%) compared to other feedstocks (18-20%).

V.3.2 Reagents

All pure reagents including pretreatment reagents Ammonium Hydroxide, Sodium Hydroxide and Sodium Carbonate, hydrogen peroxide, and high purity standards for experimental analysis were purchased from Sigma Aldrich.

V.3.3 Enzymes

Cellulase enzyme, Genencor Spezyme CP (Lot No. 301-00348-257), was a kind gift from Genecor/Danisco, Palo Alto, CA. The activity of Spezyme CP, as determined by NREL, was 59 FPU/mL. Activity of β-glucosidase (Novozyme 188 from Novo Inc., Sigma Cat. No. C-6150) was 750 CBU/mL.

V.3.4 Pretreatment: Experimental set up for Soaking in Aqueous Alkali (SAAL)

SAAL is a batch operation in which one of the three alkaline reagents aqueous ammonia, sodium hydroxide or sodium carbonate was used for each of the pretreatments. For pretreatment temperatures up to 90°C, biomass was soaked with alkali in a screw-capped glass bottle and kept in the oven for 6-24 h. For higher temperatures, steel reactors (2.5”ID × 15” L) were used. A head volume of 25% was left in the reactors, and they were weighed before and after pretreatment to ensure no leakage.

V.3.5 Analytical methods

Sugars were determined by HPLC using a BioRad-HPX-87P column. A BioRad-HPX-87H column was used for measurement of organic acids and degradation products. A refractive index detector was used in the HPLC.

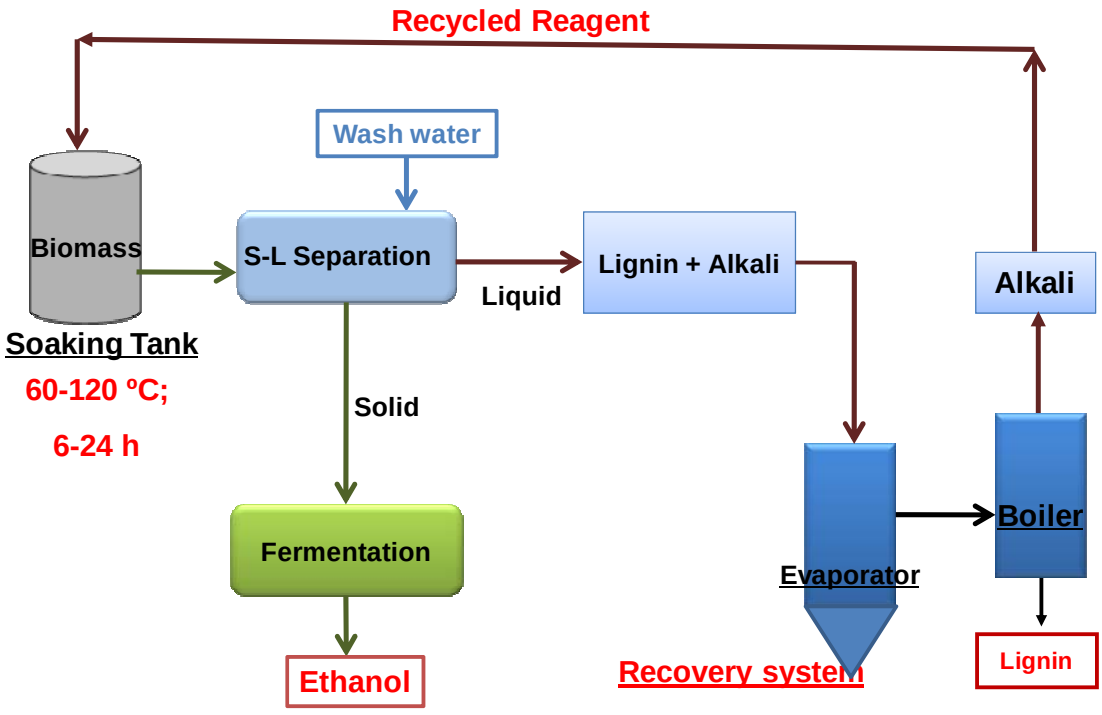


Figure V-2. Soaking in Aqueous Alkali (SAAL).

V.3.6 Composition analysis for Solid samples

Treated and untreated biomass solids were analyzed for sugars, lignin and ash using standard NREL Protocols (NREL-TP-510-42618 to 42622). Composition of treated samples was reported here on the basis of the original, taking the weight loss after pretreatment into account. Moisture

was calculated using an infrared moisture balance (Denver Instrument, IR 30). All analysis was carried out in duplicate with a standard variance of +/- 0.05.

V.3.7 Composition analysis for Liquid samples

Liquid from the pretreatment of biomass was neutralized with concentrated H_2SO_4 and then analyzed for sugars, oligomers and degradation products in the HPLC following the standard NREL protocol NREL-TP-510-42623. Monomeric sugars, acids and degradation products were measured directly in the HPLC. Secondary hydrolysis of the liquid using 4% sulfuric acid at 121°C for 1 h was performed to obtain the concentration of oligomers in the liquid.

V.3.8 Enzymatic hydrolysis

The enzymatic digestibility of substrate was determined according to the NREL Chemical Analysis and Testing Standard Procedure (NREL-TP-510-42621). Screw-capped 250 mL Erlenmeyer flasks were used as the hydrolysis reactors. Enzyme level added for all experiments was 15 FPU and 30 CBU/g glucan. Antibiotics tetracycline and cyclohexamide were added into the reactor to maintain sterile condition. The enzymatic digestibility tests were carried out at 1% glucan loading (w/v), 50ºC, and pH 4.8 using 0.05 M sodium citrate buffer in an incubator shaker (New Brunswick Scientific, Innova-4080) agitated at 150 rpm. Hydrolysate samples were taken at 6, 12, 24, 48, and 72 h, and analyzed for glucose, xylose, and cellobiose. Total released glucose and cellobiose after 72 h of hydrolysis were used to calculate the enzymatic digestibility using Eq. III-1. The xylan digestibility was also determined in a similar manner. For xylan

digestibility, a hydration factor of 1.136 was used in the equation. Multifect Xylanase was added to a few of the experiments to demonstrate its effect on enzymatic hydrolysis yields.

V.3.9 Scanning Electron Microscopy (SEM)

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

V.3.10 Crystallinity index

Crystallinity of treated and untreated biomass was measured using an X-ray Diffractometer (Rigaku Miniflex). Cu-Kα radiation was generated at 30kV and 15mA. Samples were scanned from 2θ=10 º to 30º with a step size of 0.01, scan rate 1/min. The cellulose crystallinity index (*CI*) in the switchgrass was determined by

$$CrI = \frac{(I_{002} - I_{AM})}{I_{002}} \qquad \text{Eq. V-1}$$

where I_{002} is the peak intensity corresponding to (*002*) lattice plane of cellulose molecule, and I_{AM} is the peak intensity observed at 2θ equal to 18°. I_{002} represents both crystalline and amorphous cellulose while I_{AM} represents amorphous cellulose [16].

V.3.11 BET surface area

The method of Brunauer, Emmett, and Teller (BET) was used to determine the specific surface area of the biomass. Raw and pretreated switchgrass samples were analyzed for the BET surface area by multi-point analysis method at Micromeritics Analytical Services (Norcross, GA) using krypton as the adsorptive gas in an AutoPore IV 9520.

V.4 Results and Discussion

V.4.1 Composition of solids

Composition of the substrates is shown in Figure V-3. The lignin value of switchgrass used in this study was high compared to corn stover and also when compared with other varieties of switchgrass. It is a highly recalcitrant substrate and requires a more severe pretreatment to achieve high sugar recovery [17, 18]. The high lignin is probably due to the fact that the plant was grown for a long period time before harvesting [18, 19]. Other factors such as location, harvest time, climate, type of soil, fertilizer, also affect the composition of biomass [19-21]. Many of these factors affect the pretreatment and enzymatic hydrolysis yields [22].

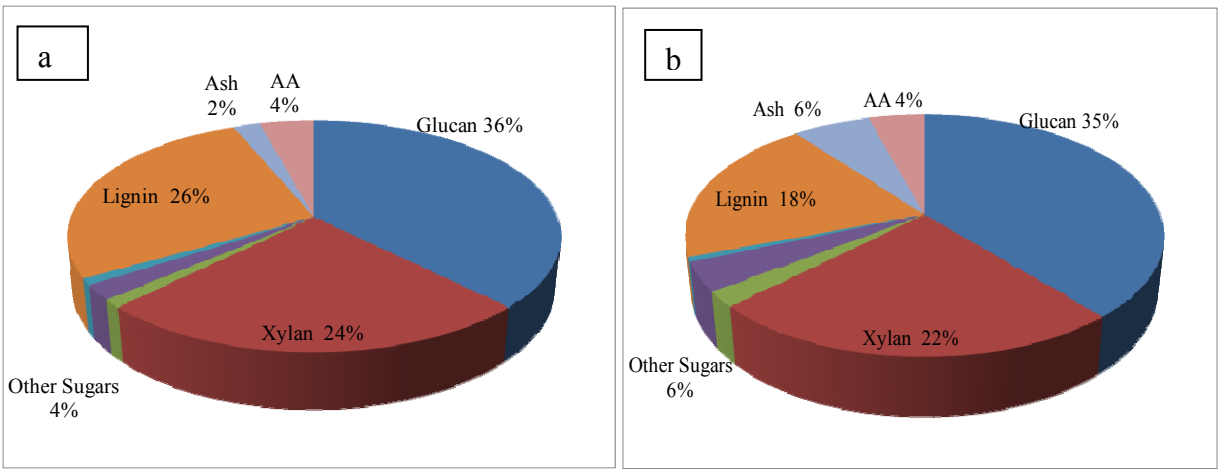


Figure V-3. Composition of (a) switchgrass and (b) corn stover.

V.4.2 Effect of reagent concentration

Three alkaline reagents were tested for pretreatment of switchgrass using the SAAL pretreatment process. Pretreatment conditions applied were 60°C, 24 h and liquid to solid ratio of 10:1 in a batch reactor. Sodium hydroxide is the strongest alkali amongst the three reagents; therefore, lower concentration of it is required to effectively treat the biomass. Treatment with sodium hydroxide, sodium carbonate and ammonia resulted enzymatic hydrolysis yields of 88%, 67% and 77% respectively. For both NaOH and Na_2CO_3, the glucan digestibility increased with reagent concentration up to a certain point and then leveled off. As seen in Figure V-4 and Figure V-5, 2% NaOH solution and 10-15% Na_2CO_3 solution were level-off points for these two reagnets. Hence, these concentrations were chosen in further experiments. Soaking in aqueous ammonia (SAA) has been used in the past for various substrates from which it was shown that, aqueous ammonia solution less than 15% is not effective [10, 11]. Two levels at 15% and 30% were tested to verify the effect of ammonia concentration. The observed digestibilies were 41% and 77% for 15%, 30% NH_3, respectively (Figure V-6). The amount of reagent required for

switchgrass is in the order NaOH < Na_2CO_3 < NH_3. The same order also holds true for corn stover, but the concentrations of reagent required are lower than that required for switchgrass. With pretreated corn stover, glucan digestibilities of 83%, 84% and 85% were achieved with 1% NaOH, 5% Na_2CO_3 and 15% NH_3, respectively.

With sodium carbonate SAAL done at 60°C, 24 h, 15% Na_2CO_3, the digestibility of switchgrass was 67%. With use of sodium hydroxide and ammonia in SAAL treatments (done under the same conditions), the digestibilities were 88% at 10% NaOH and 78% at 30% NH_3. In an attempt to reduce the regent to cost, further SAAL experiments were carried out applying temperatures above 60^{o}C, extended treatment time, and addition of supplementary reagents.

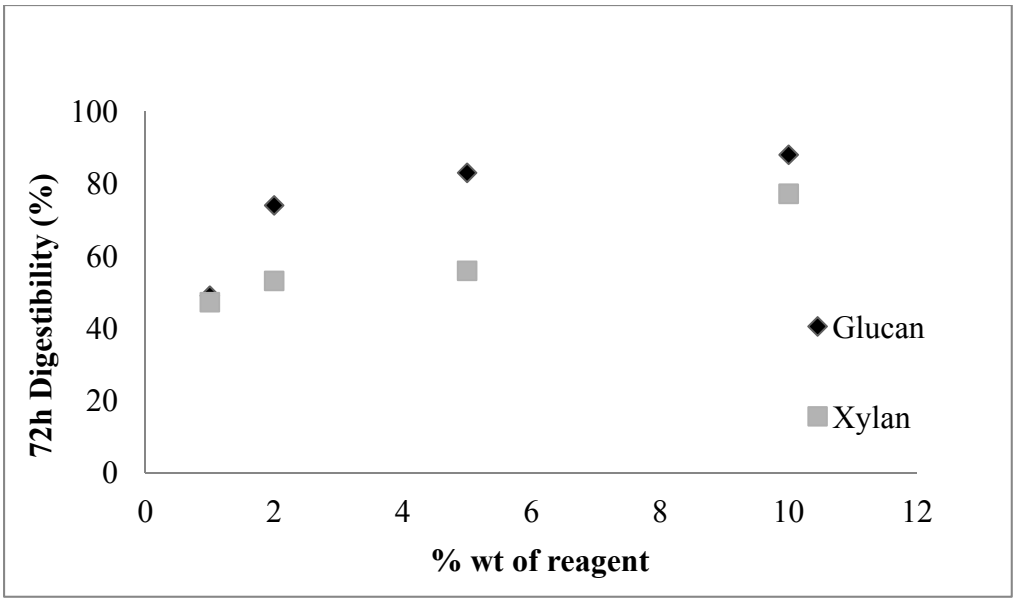


Figure V-4. Effect of reagent: NaOH.
SAAL Pretreatment conditions: 60°C, 24 h, L:S of 10:1

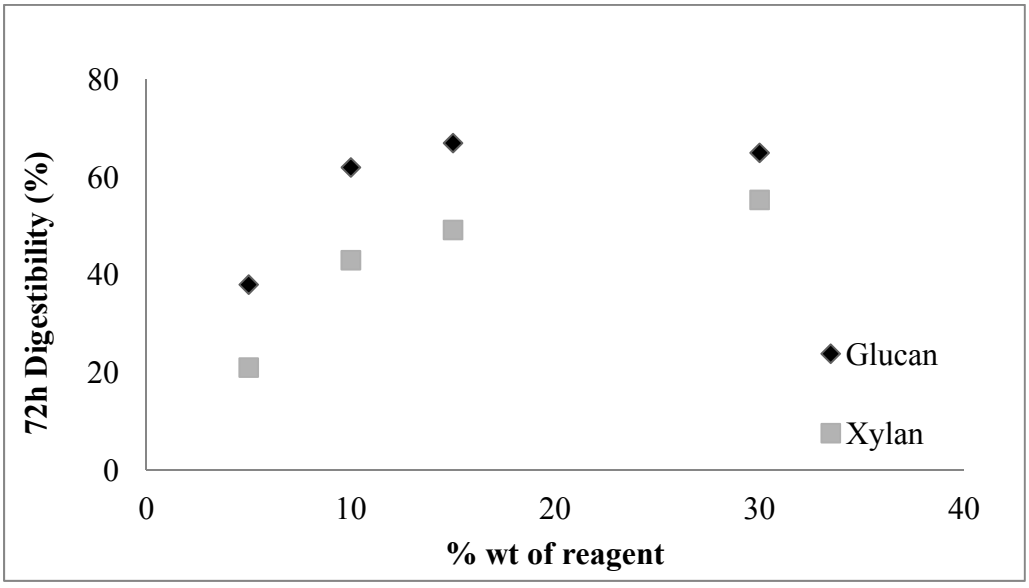


Figure V-5. Effect of reagent: Na_2CO_3.
SAAL Pretreatment conditions: 60°C, 24 h, L:S of 10:1

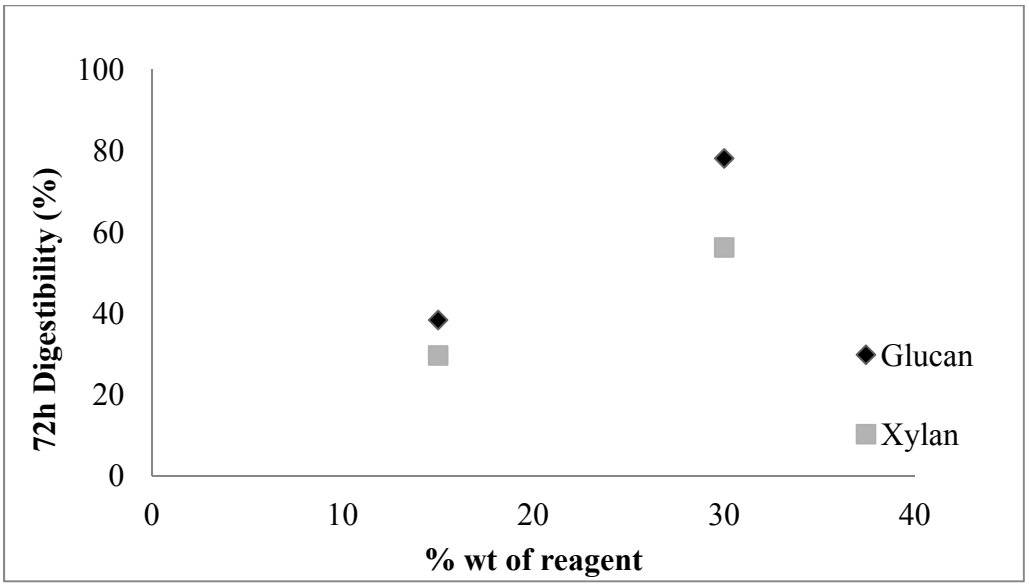


Figure V-6. Effect of reagent: NH_3.
SAAL Pretreatment conditions: 60°C, 24 h, L:S of 10:1

V.4.3 Effect of temperature

High lignin switchgrass requires higher severity pretreatment conditions. The effect of increasing pretreatment temperature on glucan digestibility of switchgrass was investigated. For each of the reagents, higher temperatures resulted in higher glucan digestibility (Figure V-7). Xylan digestibility also increased (data not shown). At 120°C, 1% sodium hydroxide treated sample shows 77% glucan digestibility in comparison to 50% at 60°C. The gain in sugar yield between the two temperatures is 50-100% (Figure V-7).

The undesired side-effect of increasing the temperature of pretreatment is loss of sugars. Hemicellulose is known to be more sensitive to temperature than cellulose [23], which is

reaffirmed by the data shown in Figure V-8. At high temperature, hemicelluloses is first hydrolyzed to monomers and eventually converted to degradation products [24]. This poses a problem in alkaline pretreatment where the sugar loss to liquid stream must be minimized [25]. Figure V-8 confirms that after 90 °C, 50% hemicellulose was lost after pretreatment with 15% ammonia (aq.) at 10:1 L:S and 24 h.

V.4.4 Time of pretreatment

Effect of pretreatment time on the digestibility of switchgrass is shown in Figure V-9. The figure shows that at 6 h pretreatment of switchgrass was incomplete, while beyond 24 h there was no substantial increase in glucan digestibility. Thus, for 1% NaOH pretreatment at 60°C and 10:1 L:S loading, 24 h was taken as the optimum time of pretreatment. It is noted that for higher temperatures, the optimum pretreatment time is lower as hemicellulose loss would then become significant [26] (Chapter VII).

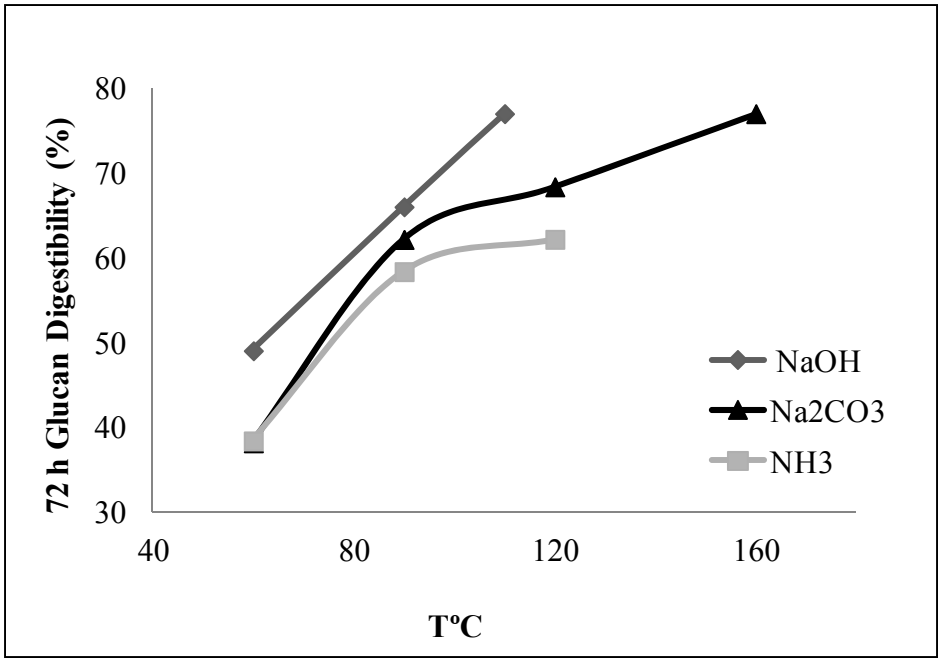


Figure V-7. Effect of temperature on glucan digestibility.
Pretreatment conditions: 24 h 10:1 L:S and 1% NaOH, 15% NH3 (aq.) or 10 % Na2CO3.

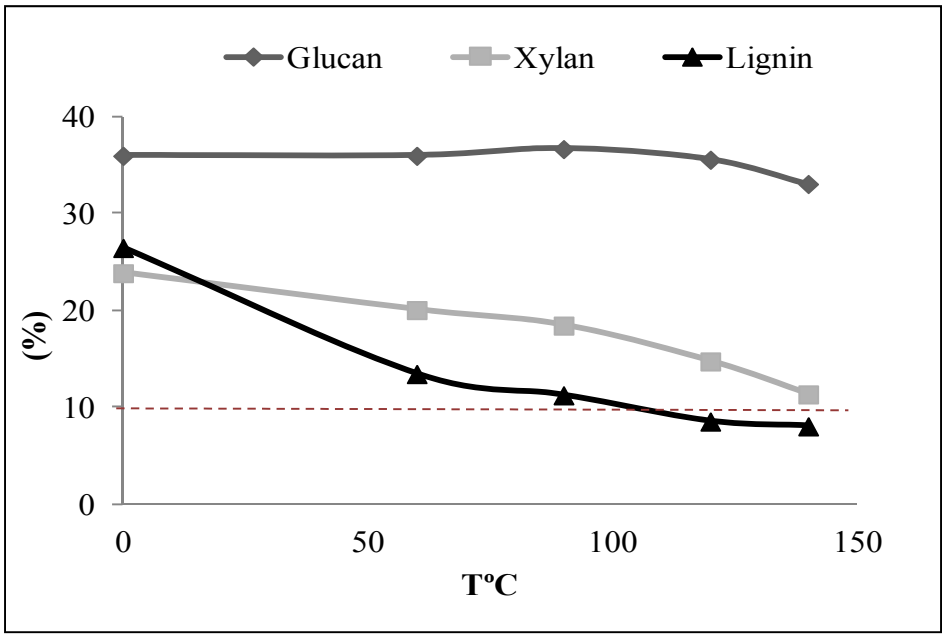


Figure V-8. Change in composition of treated substrate with Temperature.
Pretreatment conditions: 15% sodium carbonate (aq.) at 10:1 L:S and 24 h.

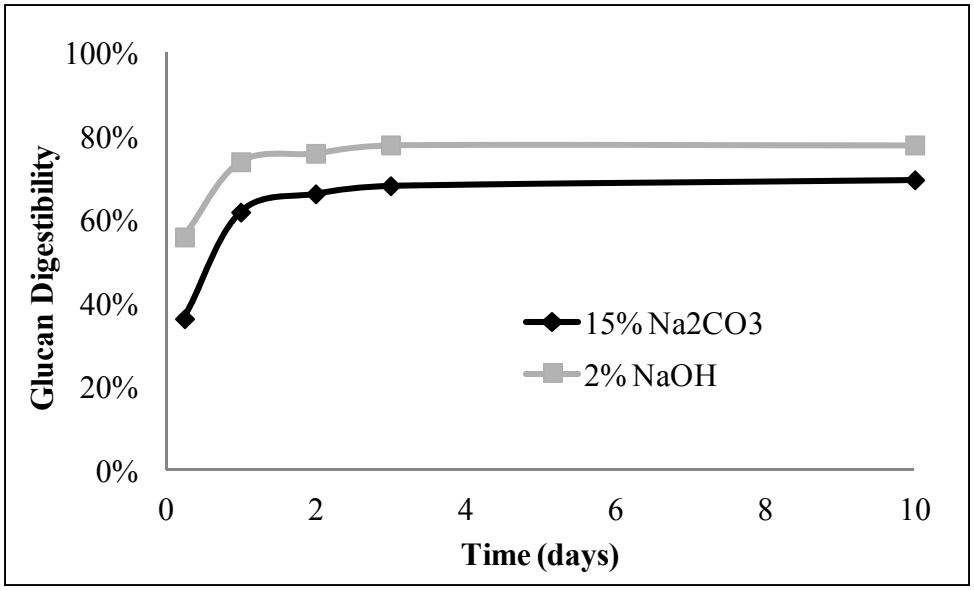


Figure V-9. Effect of time of pretreatment.
Pretreatment conditions: (red) 2% NaOH, 60°C, 10:1 L: S; (blue) 15% Na_2CO_3, 60°C, 10:1 L: S.

V.4.5 Effect pretreatment on composition of biomass

Table V-1 shows the composition of switchgrass after treatment under various pretreatment conditions. In most of the pretreatments, cellulose was well preserved while high amount of lignin was removed. Hemicellulose loss increased with increase in pretreatment severity. With highly recalcitrant substrate such as switchgrass, lignin and hemicellulose tend to be tightly entangled in a complex matrix and a certain degree of hemicellulose loss is inevitable.

Results of composition and enzymatic hydrolysis of pretreated corn stover are summarized in Table V-2. With corn stover, higher delignification was attained at lower pretreatment severity than switchgrass. This means that corn stover requires lower reagent strength, lower temperature, and shorter time than switchgrass.

Among the three reagents, ammonia and sodium carbonate are weaker alkali; thus less delignification is achieved than that with sodium hydroxide. It was also observed that higher delignification using sodium hydroxide accompanied higher hemicellulose loss. Sodium carbonate and ammonia treatments of switchgrass conserved more carbohydrates in the solids. The selectivity of lignin removal/xylan loss was reduced at high temperature for all reagents. At a given temperature, the selectivity was found to be in the order NH_3> Na_2CO_3> NaOH (Table V-1).

Table V-3 shows a snapshot of comparison among three reagents with respect to the effectiveness of pretreatment, pretreatment conditions and cost and corrosiveness of the reagents. Since sodium carbonate is much less expensive and comparable in its pretreatment performance as sodium hydroxide, furthur investigation on its use was pursued.

Table V-1. Comparison of optimum conditions for each pretreatment: Switchgrass.

	Switchgrass	**Ammonia**			**Sodium Hydroxide**		**Sodium Carbonate**		
	Untreated	15%-60C	15%-90C	30%-60C	1%-60C	1%-110	5%-60	15%-60C	5%-150
Glucan	36%	35.9%	35.6%	35.8%	34.3%	32.2%	34.5%	35.6%	33.4%
Xylan	24%	18.6%	17.4%	16.0%	18.2%	8.2%	19.4%	20.0%	14.9%
Galactan	1%	0.0%	0.6%	0.0%	0.0%	0.0%	0.3%	0.0%	0.0%
Arabinan	3%	1.8%	2.6%	2.2%	1.2%	2.1%	2.2%	2.2%	1.9%
Mannan	1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Total Lignin	26%	17.3%	15.5%	7.0%	12.4%	7.0%	17.4%	16.8%	14.1%
Ash	2%	1.4%	0.7%	0.9%	1.4%	0.9%	1.8%	1.2%	1.9%
Glucan Digestibility	5.32%	35.9%	53.7%	78.1%	42.5%	77.0%	41.0%	67.0%	78.0%
Xylan Digestibility	2.40%	39.4%	52.4%	56.2%	44.2%	59.0%	22.0%	49.2%	56.0%

Table V-2. Comparison of optimum conditions for each pretreatment: Corn Stover.

Composition	**Corn stover**	**Sodium Carbonate**				**Sodium Hydroxide**	
	Untreated	**5%-60**	**10%-60**	**15%-60**	**30%-60**	**1%-60**	**1%-90**
Glucan	34.6%	34.1%	33.5%	32.1%	32.0%	35.2%	32.5%
Xylan	21.8%	19.4%	18.7%	18.7%	16.7%	20.4%	18.9%
Galactan	1.9%	2.4%	1.6%	0.0%	0.0%	1.4%	0.9%
Arabinan	3.5%	3.6%	2.5%	1.1%	2.1%	2.0%	1.3%
Mannan	0.6%	0.0%	0.4%	0.2%	0.0%	0.4%	0.3%
Total Lignin	17.8%	13.7%	12.1%	11.6%	7.0%	13.7%	8.3%
Ash	5.6%	0.0%	3.2%	2.1%	2.0%	4.3%	1.6%
Glucan Digestibility	12.0%	69.0%	77.8%	82.9%	91.2%	75.3%	83.3%
Xylan Digestibility	4.2%	50.3%	51.2%	67.1%	66.4%	45.1%	65.1%

Table V-3. Comparison among three reagents.

	NH_3	**NaOH**	**Na_2CO_3**
Relative Cost (/T)	0.5	1	0.16-0.25
Pretreatment Process			
Pressure	High	Low	Low
Temperature	High	Low	High
Concentration	15%-30%	1-5%	5-15%
Recovery process	Easy, high pressure equipment required.	Kraft Process, expensive	Partial Kraft process, less expensive
Corrosiveness	Low	High	Medium

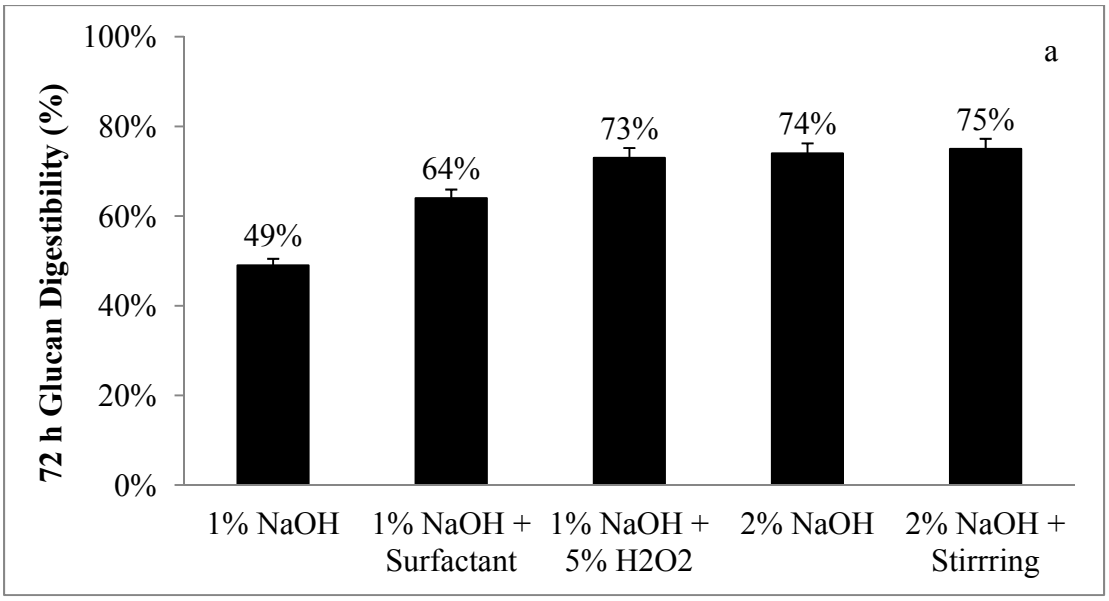
V.4.6 Effect of additives on pretreatment of switchgrass

Pretreatment of switchgrass under 1% sodium hydroxide at 60ºC 10:1, 24 h gives 49% glucan digestibility. When 5% H_2O_2 was added to the pretreatment, the digestibility improved to 73%. Improvement with hydrogen peroxide has been investigated for oxidative delignification by Gupta et al, Kim et al. [9, 27]. Hydrogen peroxide aids in cleavage of the relatively stable C-C linkages in the lignin, thus improving digestibility. Biermann and Gupta et al suggest that at low temperature, hydrogen peroxide protects carbohydrates by oxidizing the reducing end group on the polysaccharide [9, 18]. Effect of the surfactants on enzymatic hydrolysis has been investigated by Qing et al., and Alkasrawi et al [28, 29]. The surfactants form a micelle structure around free lignin and prevent unproductive binding of lignin with cellulase enzyme improving cellulose conversion [29, 30]. Surfactant can also improve delignification during alkaline pretreatment by impeding lignin recondensation onto the biomass surface following a similar mechanism. To verify this, Tween-80 was added in the pretreatment run in which the conditions

were: 1% sodium hydroxide at 60ºC 10:1, for 24 h. Addition of 1mg Tween-80/100 mg substrate increased the glucan digestibility 49% to 64%. The positive effect of surfactant is reaffirmed in this test.

Agitation (at 250 rpm) during pretreatment did not show any effect on the digestibility, probably because under high liquid: solid ratio tested in Figure V-10, the viscosity of the pretreatment mixture is low, keeping biomass uniformly wet with the reagent solution.

The effect of the addition of a nonvolatile sulfur compound, sodium sulfide (Na_2S) was investigated. The results are shown in Figure V-10. Sulfur compounds enhance 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 [13]. The sulfur induced pretreatment method with use of sodium carbonate (green liquor pretreatment) has been promoted by Yongcan et al. [31] . In this work, pretreatment experiment was done supplementing 1% Na_2S to the SAAL treatment run with 5% sodium carbonate at 60ºC 10:1, 24 h. With addition of Na_2S, the glucan and xylan digestibility improved from 69% to 81%, and 49% to 69%, respectively.



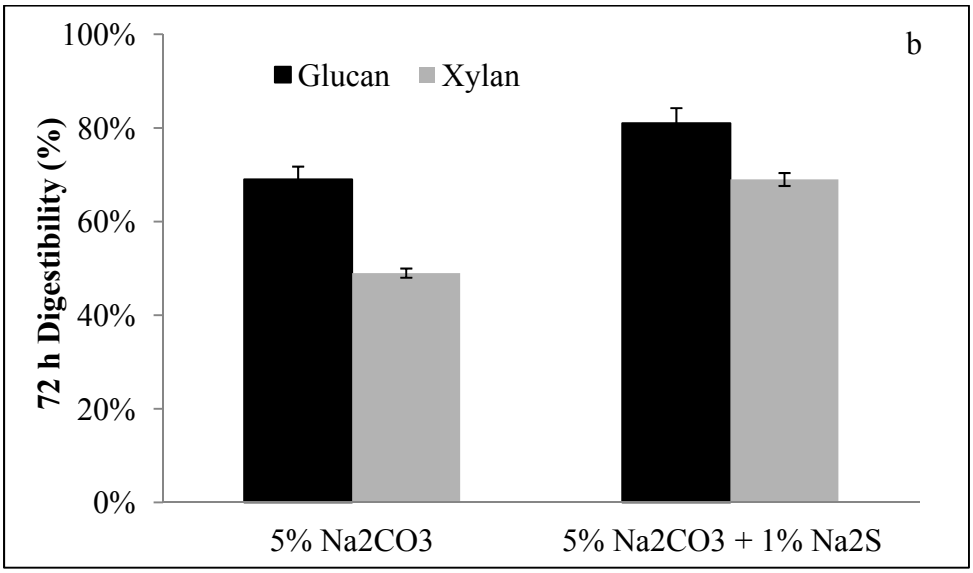


Figure V-10. Effect of various additives on alkaline pretreatment.
(a) SAAL Pretreatment conditions: 60°C, 24 h, L:S of 10:1, 1% NaOH with and without additives; (5%H_2O_2 or 1mg Tween-80/100mg biomass), 2% NaOH with and without agitation (agitation at 250rpm)
(b) SAAL Pretreatment conditions: 60°C, 24 h, L:S of 10:1, 15% Na_2CO_3 with and without 1%Na_2S

V.4.7 Solid characterization

V.4.7.1 XRD

Crystallinity index (CrI) of treated samples measured using the Segal method [16] increased after all the pretreatments. From Figure V-11 it is observed that there was no change in the XRD pattern for any of the pretreatments when compared to the untreated sample. The average crystallinity index increases because all the pretreatments tested here hydrolyze a major portion of lignin and some hemicellulose from the biomass Table V-1. Since these are amorphous components of the biomass, the average crystallinity of treated biomass is higher than that of the untreated raw material. The XRD pattern remains unchanged with respect to number and positions of the peaks; this indicates that there was no change in the crystalline structure of cellulose [32].

Table V-4. Crystallinity index of treated samples.

Sample	**CrI**
Untreated	53.13
NH_3	63.56
NaOH	72.02
Na_2CO_3	61.86

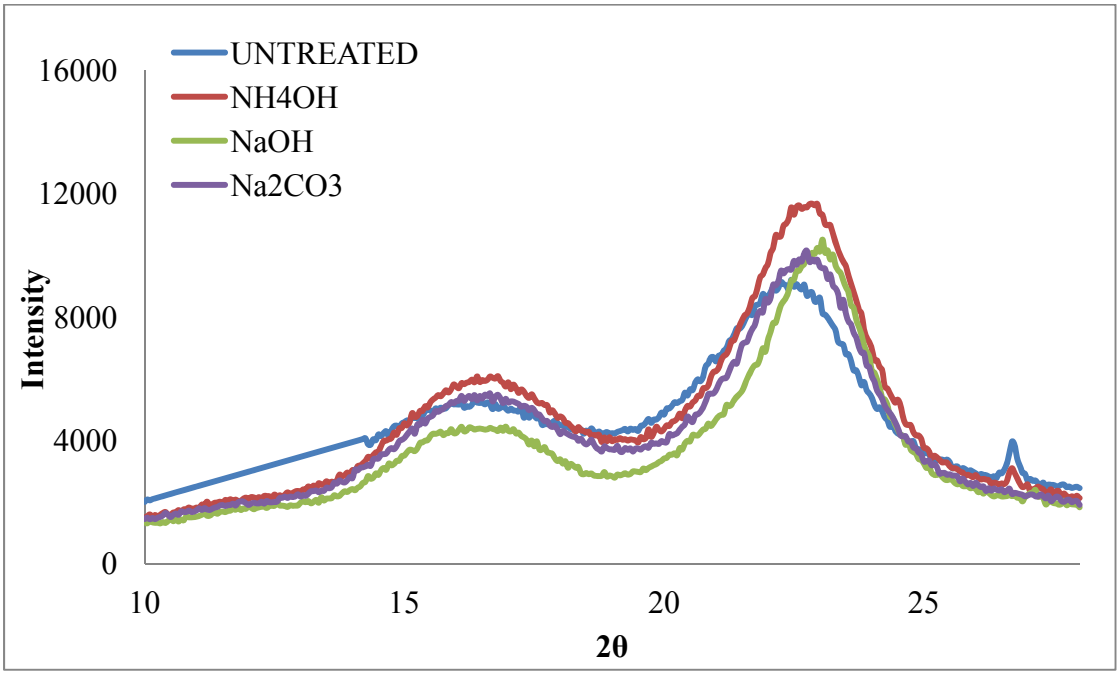


Figure V-11. XRD Spectra treated and untreated sample
Pretreatment conditions: 1% NaOH, 15% NH_3, and 5% Na_2CO_3 at 110ºC 10:1 L:S ratio, 24 h.

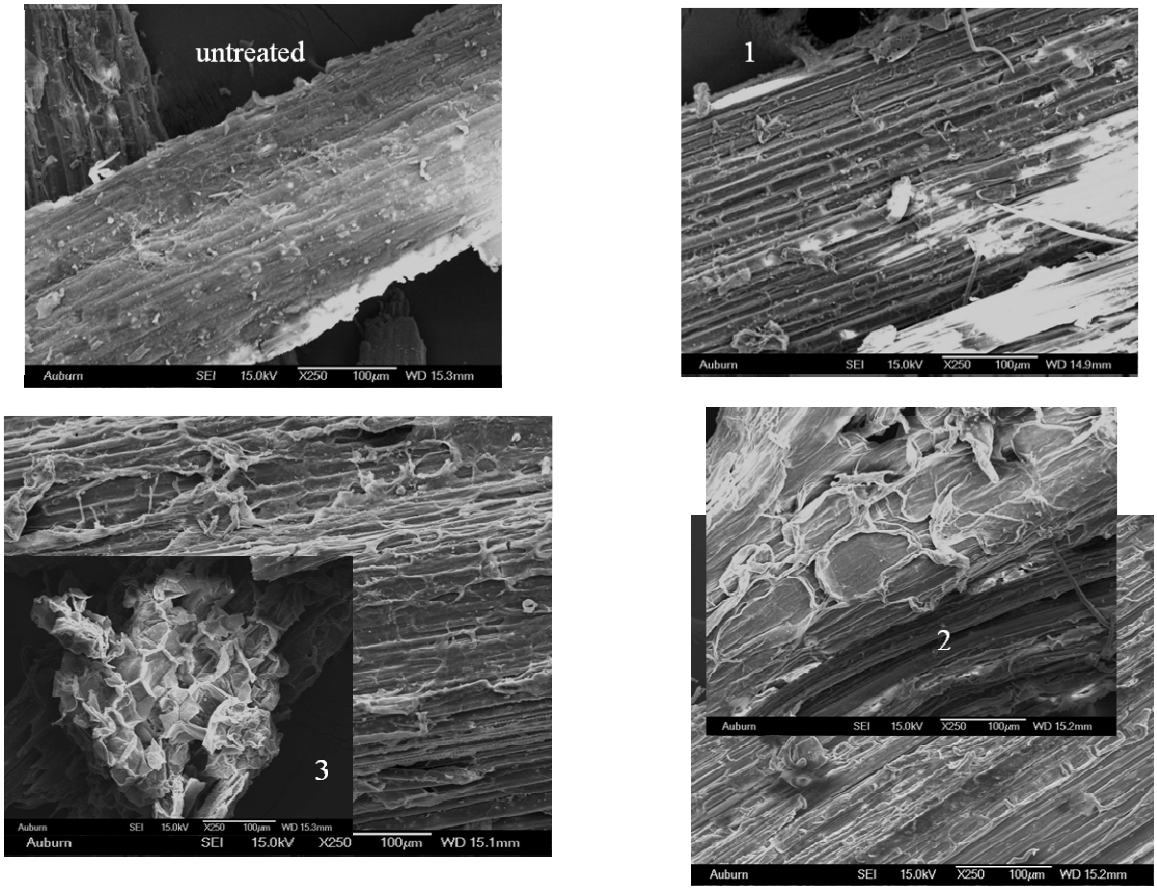


Figure V-12. Scanning Electron Microscope (SEM) images.
Untreated, 1= NH_3 treated, 2= NaOH treated, 3= Na_2CO_3 treated switchgrass (all scales 100 μm).
Pretreatment conditions: 1% NaOH, 15% NH_3, and 5% Na_2CO_3 at 110ºC 10:1 L:S ratio, 24 h.

V.4.7.2 Scanning Electron Microscope (SEM) Imaging

SEM images of each of the treated samples are shown in Figure V-12 in comparison with that of the untreated raw material. Surface structure shows high level of disruption after pretreatment. Pretreatment with NaOH and Na_2CO_3 shows a higher disruption than that with NH_3.

V.4.7.3 BET surface area

The BET surface area increases for all pretreatments. For sodium hydroxide treated samples, the surface area increased 3 times that of the untreated sample. The ammonia treated sample showed a 2 fold increase, but the sodium carbonate samples showed only a 1.2 times increase. The reason for the difference amongst the three reagents is not known at this time. Even though the BET surface area increased only 1.2-3 times, the digestibility increased by a factor of 10 or more after pretreatment. This implies that the surface area is not the only factor affecting digestibility. This phenomenon was also reported by Burns et al [33].

V.5 Conclusion

The three alkaline reagents (sodium hydroxide, sodium carbonate and ammonia) meet the basic requirement as a pretreatment reagent: selective delignification to enhance the enzymatic digestibility to acceptable level while conserving carbohydrates. Above 70% glucan digestibility was obtained with each of the three reagents with the enzymes 15 FPU/g glucan Spezyme CP and 30 CBU/g glucan Novozyme 188 for switchgrass. Each reagent has its own merits: ammonia is easily recoverable, sodium hydroxide is a strong base and a proven delignifying reagent, and sodium carbonate is a weaker alkali but is 4-6 times less expensive than sodium hydroxide and easy to recover. For corn stover, the yields were much higher than switchgrass primarily due to low lignin content. The enzymatic hydrolysis yield increased with increase in treatment temperature up to 110°C, after which, the hemicellulose loss increased to the extent that the overall sugar recovery yield decreased. Additives such as hydrogen peroxide, sodium sulfide and

surfactant during pretreatment significantly improved enzymatic hydrolysis yields. Crystallinity index (by XRD), surface roughness (morphology as seen in SEM), and BET surface area increased after pretreatment as amorphous parts of biomass were reduced. Since sodium carbonate is much cheaper and almost equally effective in pretreatment of switchgrass and corn stover as sodium hydroxide, further investigation on its use is discussed in the next two chapters (Chapter VI and VII).

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Chapter VI Sodium Carbonate as a Pretreatment Reagent for Herbaceous Feedstocks

VI.1 Abstract

Sodium carbonate is a low-cost and recoverable alkaline reagent. Pretreatment of switchgrass and corn stover using sodium carbonate was investigated for enhanced enzymatic hydrolysis of the native carbohydrates in the biomass into sugars. Pretreatments were done soaking the biomass in the aqueous solution of the alkali. This method was effective in delignifying the biomass removing 50-70% of the total lignin. With this treatment, the enzymatic digestibility was improved to more than 70% with switchgrass and 85% with corn stover with moderate enzyme loading of 15FPU and 30 CBU/g-glucan. The digestibility was further improved when the cellulase enzyme (Spezyme CP) was supplemented with external xylanase. The process conditions were optimized for the two different substrates in terms of temperature, reaction time, and solid-liquid ratio determined under criteria of attaining high enzymatic digestion as well as high carbohydrate retention. SEM images and XRD were taken for the treated and untreated substrates to study the effects of the pretreatment on the physical properties. With the pretreatment, the surface area and porosity as well as the crystallinity of the biomass increased. These increases were due to the fact that amorphous part of biomass (lignin and hemicellulose) was removed during pretreatment. The basic crystalline structure of the cellulose was not altered by the pretreatment.

VI.2 Introduction

Alkaline pretreatment is one of the major process options being investigated for bioconversion of lignocellulosic biomass. The main feature of it is that it delignifies biomass and makes it amenable to enzymatic hydrolysis and fermentation to value-added bioproducts [1-3]. Pretreatment using alkaline reagents induces chemical interactions with biomass quite different from those of neutral or acid pretreatment in that they react primarily with lignin, which is a byproduct in the cellulosic-to-ethanol process, and remove it from biomass rather than hemicellulose. With alkaline pretreatment, hemicellulose is retained in the treated solid, which is then hydrolyzed to sugars by "cellulase" enzyme which also carries hemicellulase enzymes. Since the treated biomass is free of pretreatment liquid, it eliminates the extra step of detoxifying liquid hydrolysate which is generally required in the neutral or dilute-acid pretreatment process [4-8].

Alkaline reagents such as sodium hydroxide, lime, ammonia are known to swell the biomass cell wall and readily attack aryl ether linkages in lignin. The structure and degradation of lignin in alkaline conditions has been studied extensively, mostly in relation with the Kraft pulping process [9, 10]. Lignin is a three-dimensional polymer of phenyl-propane units with various types of linkages such as ether, aryl, alkyl, and their combinations [11, 12]. Many of these bonds are attacked during alkaline pulping with NaOH, which removes 70-90% of lignin from biomass [9, 13]. Under treatment conditions milder than those used in the Kraft pulping process, lower degree of delignification is obtained [14]. For pretreatment of biomass, delignification at the

level obtained in the Kraft pulping process is not required; hence use of less amounts alkali or milder alkaline reagents is desirable. In the lignin structure, alkyl-aryl linkages form 50% - 70% of the total linkages [9]. The alkaline degradation of about half of these bonds follows first order kinetics with respect to the lignin alone and does not depend on the [OH^-] concentration, as long as alkaline conditions are maintained [10].The rest follow first order kinetics with respect to [OH^-] [9, 10]. Aryl-aryl linkages and C-C linkages are relatively stable and more difficult to hydrolyze. Using mild alkaline reagents protects he carbohydrate since the characteristic peeling reaction due to strong alkali (NaOH) is prevented [15].

It is important to identify novel and inexpensive or recoverable reagents suitable for alkaline pretreatment. Sodium carbonate is one such reagent; its cost is about 20% that of sodium hydroxide [16], and it is easier to recover than NaOH. The proposed recovery process for sodium carbonate is shown in Figure V-1, which uses a part of the established NaOH recovery process used in the Kraft pulping mills [13]. As shown in Figure V-1, recovery of sodium carbonate is much simpler than the sodium hydroxide recovery process used in the Kraft pulping. After the solid-liquid separation, the lignin rich liquid stream is concentrated and sent to a recovery boiler where lignin is burnt at 1100°C. The sodium carbonate is left behind as smelt, which is recycled [13, 17]. Similar studies on softwood pretreatment with green liquor have been reported for repurposing of Kraft mills [18-20] where a mixture of NaOH, Na_2CO_3 and Na_2S is used as the pretreatment reagent [19, 20].

Switchgrass is a perennial grass, native to North America. It grows well on marginal, non agricultural land [21] and can tolerate water and nutrient scarcities [22]. Switchgrass gives high biomass yield, 6-9 dry-ton per acre per year, and the fertilizer requirement is only one third that

of corn [21]. It also has relatively high cellulose content [23]. For these reasons, it is considered as one of the most promising energy crops for production of ethanol and other value-added products.

Other than use of green liquor as the pretreatment reagent, which contains a mixture of Na_2CO_3, Na_2S and NaOH [17, 18], very little literature information is available on using sodium carbonate as a sole reagent for pretreatment of herbaceous feedstock. This investigation was therefore focused on evaluating sodium carbonate as a pretreatment reagent for herbaceous feedstocks switchgrass and corn stover.

VI.3 Materials and methods

Switchgrass was grown in a test plot in Agronomy and Soil Department at Auburn University [24]. The feedstock was first washed to remove soil and sand impurities with warm (45ºC) water three times and air dried. The clean switchgrass was milled to size <20 mesh before further experimentation. Moisture content of the clean switchgrass was 10%. Na_2CO_3 (99.5% pure) and all standard reagents were purchased from VWR.

Cellulase enzyme (Spezyme CP) and Multifect Xylanase were a gift from Genencor-Danisco (Paulo Alto, CA) and β-Glucosidase was purchased from Sigma (Novozyme 188, Sigma, C-6150). Activities of Cellulase and β-Glucosidase were 59FPU/ml and 600 CBU/ml respectively. Xylanase was 42 mg protein/ml

VI.3.1 Pretreatment

Soaking in Aqueous Alkali (SAAL) Pretreatment is a batch pretreatment method. The projected process scheme is shown in Figure V-2 Screw-capped glass bottles were used as the reactor for pretreatment temperatures less than 90ºC; and flanged SS-316 tubular reactors with the dimension of 6"Lx1.375" ID were used for pretreatment temperature higher than 90ºC. In all experiments, 10 dry grams of switchgrass was soaked with specified concentration of Na_2CO_3 solution. The reactor was then placed in an oven at desired pretreatment conditions. Liquid to solid ratio was varied from 5:1 to 10:1, temperature from 60-120ºC and time from 6 to 24 h when short pretreatment time was desired or from 1-7 days when long pretreatment time was desired.

VI.3.2 Analytical methods

Composition of solids and liquids was performed according to Section V.3.7, and the enzymatic digestibility of substrates was determined according to section V.3.8.

VI.3.3 Physical characterization

SEM imaging, X-Ray Diffraction measurement and BET Surface area measurement were performed using methods described in Sections V.3.9 through 11.

VI.4 Results and discussion

VI.4.1 Properties of the feedstock

Composition of the feedstocks used in this work is shown in Table VI-1. Corn stover contains 62% carbohydrates and 18% lignin while switchgrass contains 67% carbohydrates and 27% lignin. The lignin content in switchgrass and that of other feedstocks available from literature are compiled in Figure VI-1. This comparison shows that the batch of switchgrass used in this work has lignin content that is closer to that of hardwood species (hybrid poplar) than herbaceous species, which is much higher than other species of switchgrass. The composition of biomass within a species varies widely with the age of plant, storage time, harvest time (fall vs. spring), genotype (upland vs. lowland) [26], location of the cultivar (northern vs. southern), quality of soil, fertilizers used, seasonal changes, and precipitation[27-29]. The high lignin content in this batch of switchgrass was probably due to the fact that the plant was growing in the field for approximately 10 years before it was harvested. The composition of biomass used strongly affects its treatability. It is generally observed that feedstocks with high lignin are more recalcitrant compared to the ones with low lignin [28]. High recalcitrance in feedstocks requires pretreatment with more severe conditions to attain acceptable sugar yields upon enzymatic hydrolysis. Other factors influencing enzymatic hydrolysis yields are cellulose accessibility, surface area, porosity and crystallinity [30, 31].

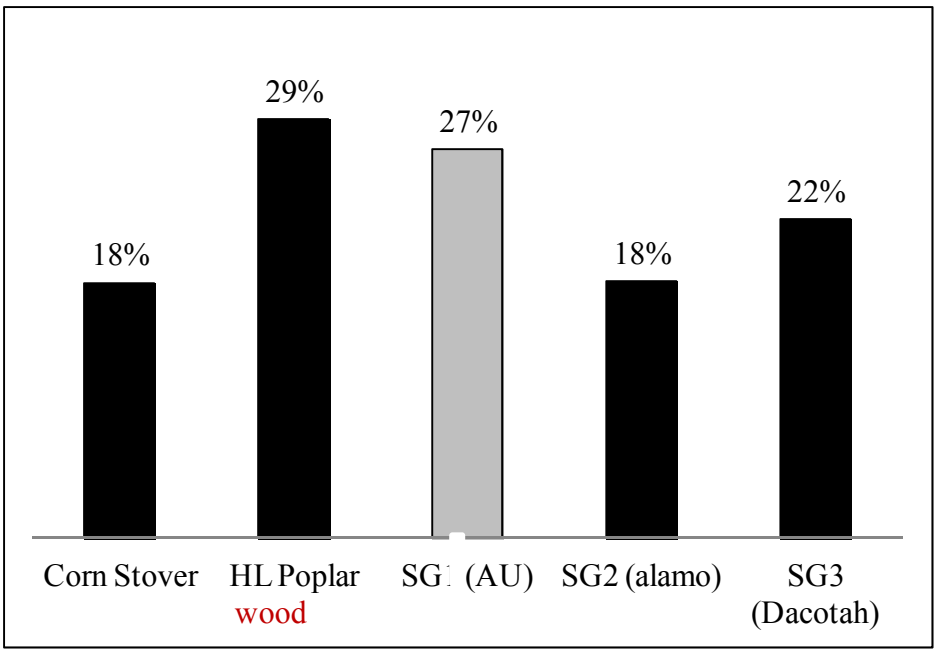


Figure VI-1. Lignin content of various feedstocks.

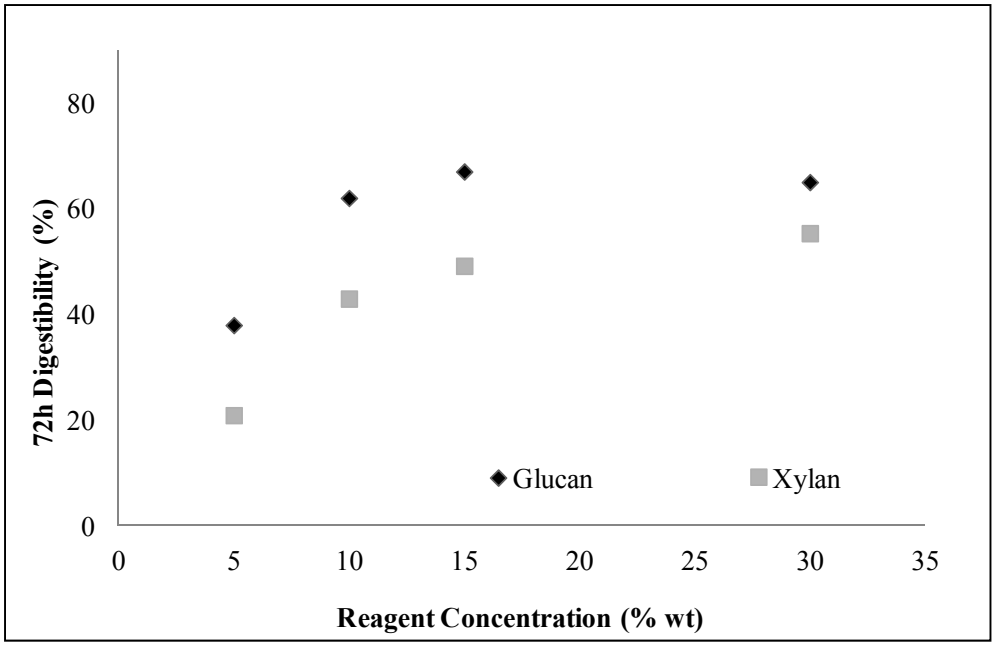
Table VI-1. Composition of feedstocks.

Component	**Switchgrass**	**Corn stover**
Glucan	37%	34%
Xylan	25%	22%
Other sugars	5%	6%
Lignin	27%	18%
Ash	2%	6%
Others	4%	14%

VI.4.2 SAAL pretreatment

VI.4.2.1 Effect of sodium carbonate on switchgrass composition

Switchgrass was treated with varying concentrations of sodium carbonate to assess its effect on biomass. Biomass was soaked in 2 - 30% sodium carbonate solution at 10:1 solid to liquid ratio at 60ºC for 24 h in a batch reactor. Figure VI-2a shows the change in concentration of the main components (glucan, xylan and lignin) after pretreatment. The glucan was well preserved and xylan loss was less than 20% while the lignin was reduced by up to 45%. Selective delignification demonstrated here is a desirable feature in an alkaline pretreatment. Figure VI-2 b shows the results of enzymatic hydrolysis of the treated substrates measured at 15FPU and 30 CBU/g glucan enzyme loading. The glucose yield as high as 70% was achieved after SAAL treatment using 15% sodium carbonate solution. Further improvement of the pretreatment was sought to increase the glucose yield to above 70% level by optimizing the process conditions.



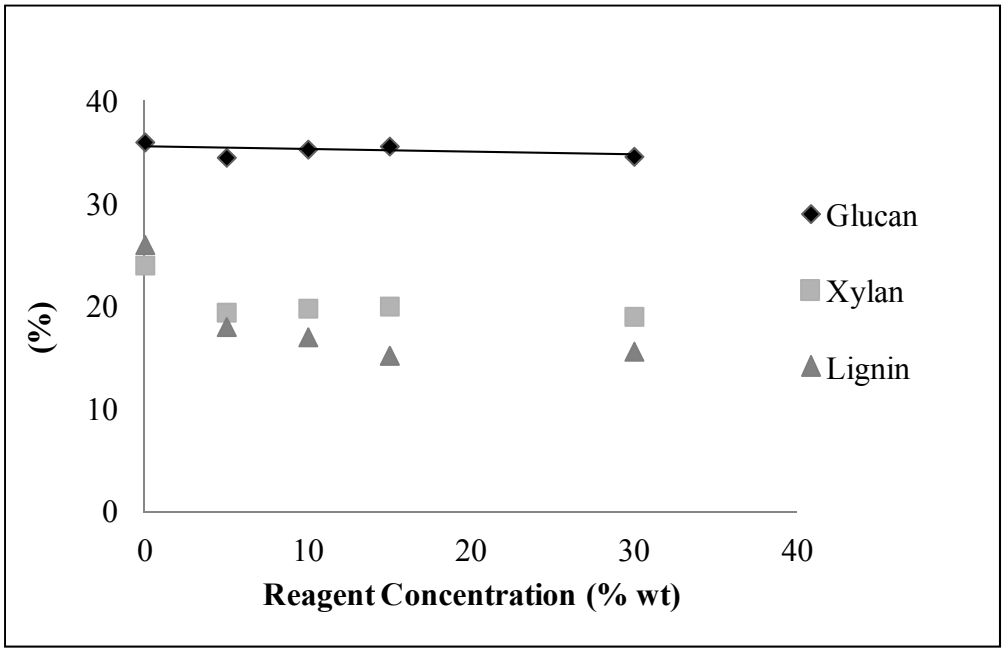
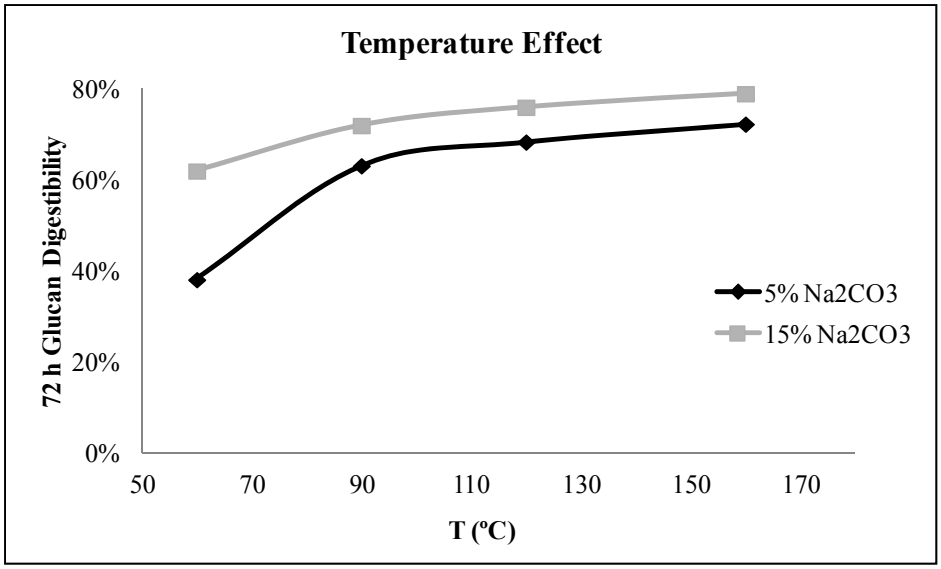


Figure VI-2. (a) Effect of reagent concentration on enzymatic digestibility and (b) Effect of reagent concentration on biomass composition.
Pretreatment Conditions: S/L ratio 1:10, 60ºC, 24 h
Enzyme Loading: 15 FPU, 30 CBU/g glucan
Substrate Loading: 1% glucan.

VI.4.2.2 Effect of temperature and time on switchgrass composition

Increase in the treatment severity was achieved by increasing the time and temperature of the pretreatment. Figure VI-3 and 4 show the effect of pretreatment conditions on composition of the biomass and the sugar released after enzymatic hydrolysis. Pretreatment time beyond 24 h did not affect either the composition or the enzymatic digestibility substantially when pretreatment temperature used was 60ºC. This means that the biomass is well soaked with alkali and the reaction reached equilibrium after 24 h. On the other hand, temperature of pretreatment had a significant effect on the biomass. The glucan yield of pretreated solids more than doubled to 80% and the xylan yield increased three times to close to 60% at a high temperature of 150ºC. Composition of the biomass after high temperature pretreatments is seen in Figure VI-3b. significant glucan losses are seen beyond 120ºC. Xylan losses increase linearly with temperature and at 150ºC, more than 50% of hemicellulose was lost to the liquid stream. Since this was not acceptable, pretreatment temperature of 110ºC was chosen as the maximum. At this temperature, delignification was 63% and hemicellulose loss was about 35%.

It is observed in this substrate, the selectivity of lignin removal over hemicellulose was lower than expected. The xylan and lignin seem to be more tightly matrixed with each other; which makes it that much harder to selectively remove lignin without hurting the xylan content of biomass.



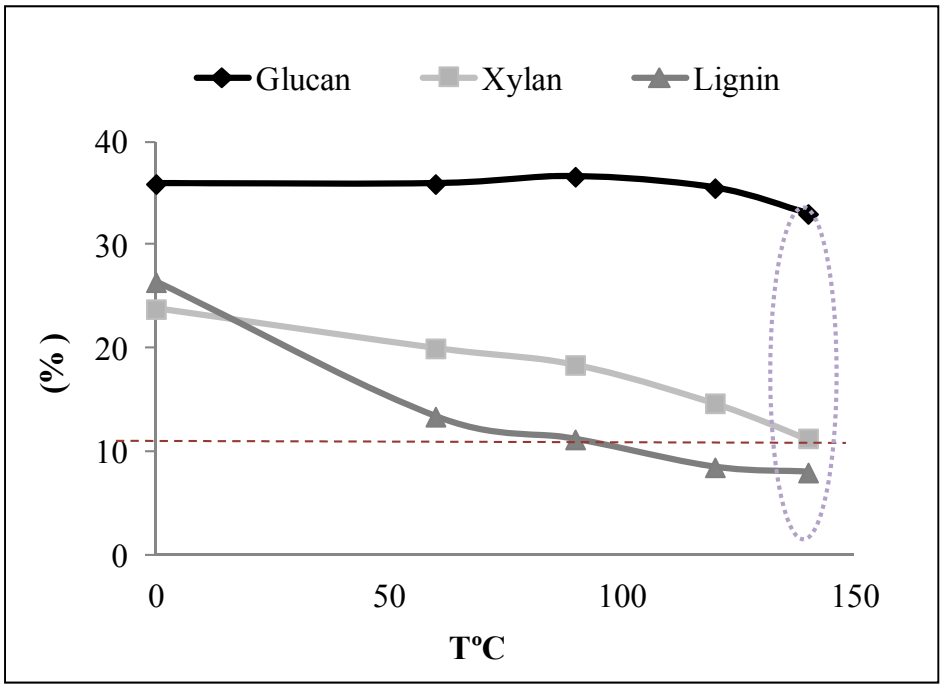
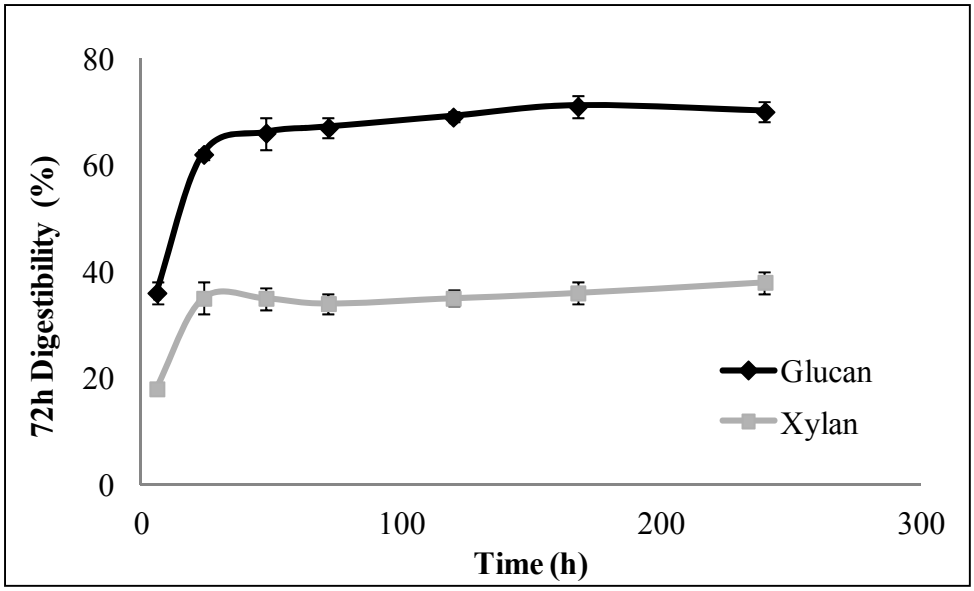


Figure VI-3. (a) Effect of pretreatment temperature on enzymatic digestibility and (b) on composition of biomass.
Pretreatment Conditions: S/L ratio 1:10, 15% Na_2CO_3, 24 h
Enzyme Loading: 15 FPU, 30 CBU/g glucan
Substrate Loading: 1% glucan



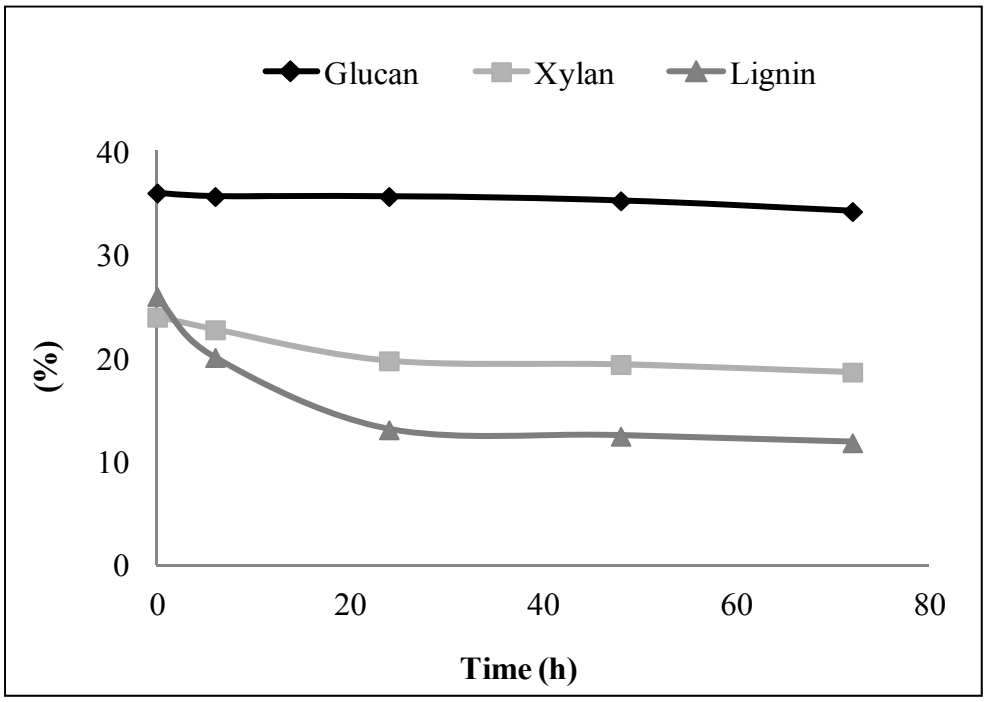


Figure VI-4. (a) Effect of pretreatment time on enzymatic digestibility and (b) on composition of biomass.
Pretreatment Conditions: S/L ratio 1:10, 15% Na_2CO_3, 60ºC
Enzyme Loading: 15 FPU, 30 CBU/g glucan
Substrate Loading: 1% glucan.

VI.4.3 Comparison with corn stover

In comparison with switchgrass, corn stover is an easier substrate since it starts out with much lower lignin. Severity of pretreatment required for the same glucan yields was found to be much lower. For example Figure VI-5 shows the performance of switchgrass and corn stover at the same pretreatment conditions of 5% Na_2CO_3, 60ºC for 24 h. The glucan and xylan digestibility for switchgrass are 38% and 21% while those for corn stover are 69%and 53% respectively.

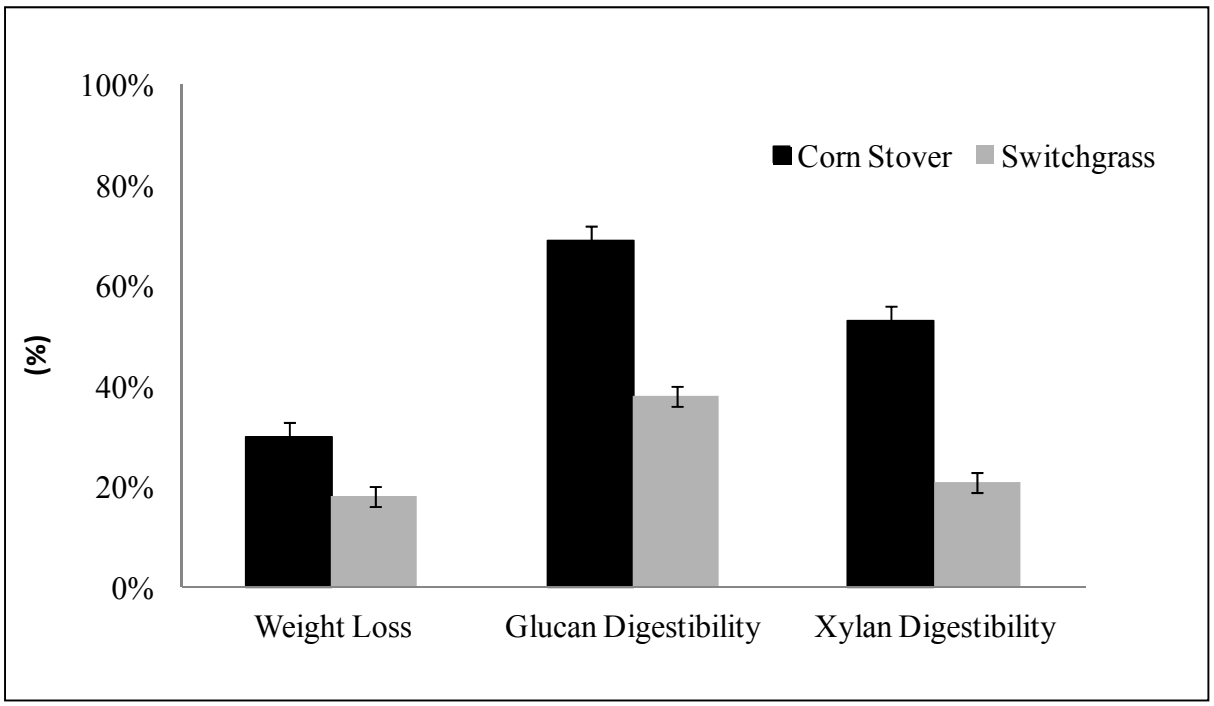
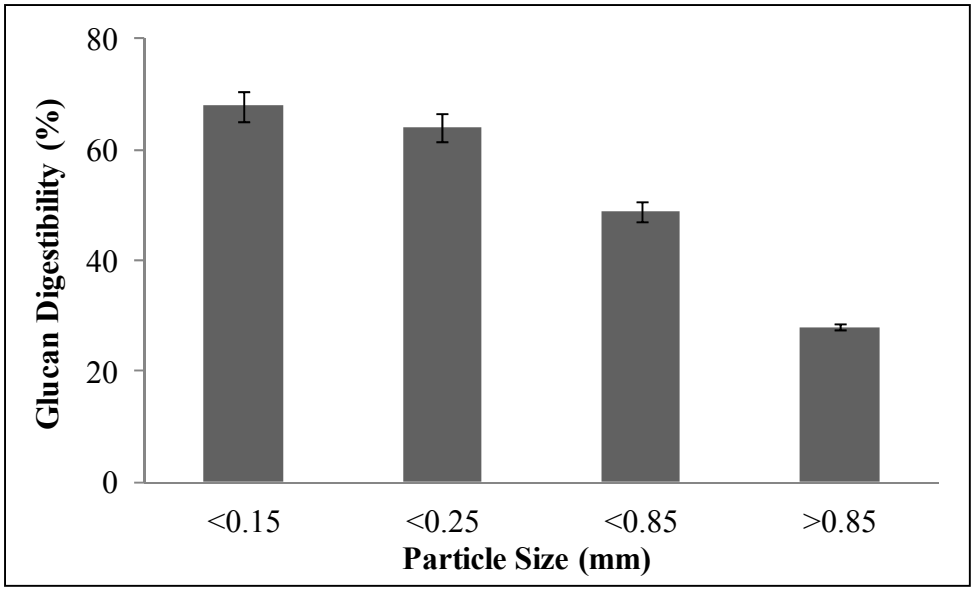


Figure VI-5. Effect of feedstock on pretreatment conditions.
Substrates: Corn Stover > Switchgrass
Pretreatment Conditions: 10:1, 60ºC, 24 h, 5% Na_2CO_3
Enzymatic hydrolysis: 15 FPU, 30 CBU/g glucan.

Overall higher glucan and xylan digestibility were achieved with corn stover at all pretreatment conditions. The trends with respect to alkali concentration, temperature and time were the same (data not shown), other than the fact that conditions milder than those for switchgrass gave high sugar yields. The sodium carbonate loading had an optimum at 5% above which improvement

was not significant. Higher temperatures gave higher enzymatic digestibility but lower sugar accountability due to loss of hemicellulose. With corn stover, glucan was also hydrolyzed at high temperatures, which was not seen in switchgrass at the same conditions. Pretreatment conditions were optimized for highest carbohydrate retention and highest enzymatic digestibility yields. Using this, the optimum pretreatment conditions for corn stover were 10% Na_2CO_3, 60ºC, 24 h giving 85% glucan yield and 67% xylan yield at 15 FPU and 30 CBU/g glucan enzyme loadings.

When a different species of switchgrass was used, which had lower lignin, the pretreatment results were closer to corn stover. The difference in performance of feedstocks with varying recalcitrance has been investigated by Gupta and Lee [32]. It would be important to choose the right substrate, harvest time, and cultivation conditions in order to attain high bioconversion efficiency. The CAFI team has conducted a comprehensive investigation on pretreatment of different species of switchgrass as well as different species of hybrid poplar. Their results concur with the findings of this study that the pretreatment efficiency varies substantially with the subspecies of biomass within a given feedstock [27, 28, 33]. It is not only the species of the feedstock that influences the bioconversion efficiency. Moreover, cultivation and harvest practices also play a significant role in the conversion path.



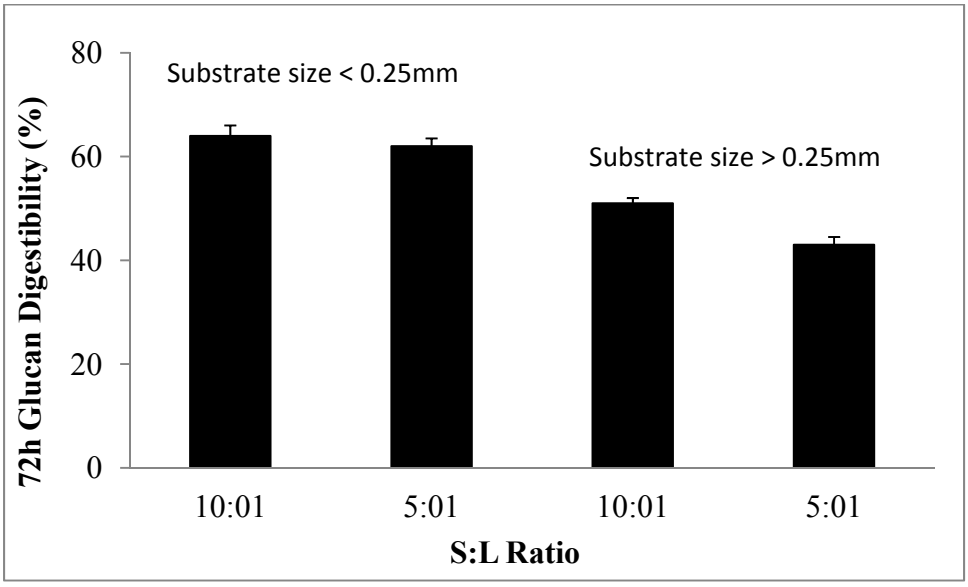


Figure VI-6. (a) Effect of particle size on digestibility and (b) Relation between particle size and S/L ratio.
Pretreatment Conditions: 5% Na_2CO_3, 60ºC, 24h
Enzyme Loading: 15 FPU, 30 CBU/g glucan
Substrate Loading: 1% glucan.

VI.4.4 Effect of substrate size

The raw material used in the first part of the study had particle size <0.85 mm. Size reduction is highly energy intensive. The effect of particle size on pretreatment was investigated. Switchgrass was ground and sieved into 4 different fractions: -100mesh (<0.15 mm), -60mesh (<0.25 mm), -20 mesh (<0.85 mm), unground (>.85 mm). The solids were treated with 10% Na_2CO_3, 10:1 L: S ratio, at 60ºC for 24 h. The 72 h glucan digestibility generally increased with smaller particle size (Figure VI-6 a). Pretreatment of unground samples showed 72 h glucan digestibility of only 28%. With smaller particles (<0.85mm) the digestibility rose to almost 50%, and with 0.25mm particles it reached 65%. This is probably due to high available external surface area of finely ground substrate and that the enzyme molecules have easier access to glucans of inner region of the particles. Grinding biomass to smaller than 0.25 mm did not show further increase in glucan digestibility. Other factors such as improving cellulose accessibility by pretreatment, change in crystallinity of cellulose, composition of treated and untreated biomass, etc play an important role in improving glucan yields. Also, size reduction of particles is also energy intensive and needs to be optimized. Zhu et al have studied the energy requirements in size reduction and indicated that size reduction is to be done in two stages (before as well as after pretreatment), and the strategy must be optimized with regard to the extent of size reduction in each stage [34].

VI.4.5 Liquid : Solid ratio

Two batches of biomass, one ground to 0.85 mm and the other 0.25 mm were pretreated identically at 60ºC, 10% Na_2CO_3 and 24 h. Different L:S were used while keeping the strength of solution constant. Between samples pretreated with L:S ratio of 10:1 and 8:1, enzymatic

hydrolysis yield did not show much of a difference, but with L:S of 5:1, substantial difference was seen for larger particles (0.85 mm). Interestingly, for the small particles (0.25 mm), L:S ratio of 5:1 gave the same sugar yield as the sample treated with 10:1 (Figure VI-6 b). This happens because, for effective pretreatment by the SAAL process, liquid should enter all the pores of biomass. If the amount of liquid is decreased this was not possible. Decreasing the particle size reduces the bulk density of solid and less amount of liquid is required for effective swelling of biomass in SAAL.

This shows us that size reduction helps in reducing the amount of reagent used during pretreatment of biomass. Thus the cost of size reduction and cost of reagent and its recovery are two variables to be optimized concurrently for minimizing overall cost of pretreatment.

VI.4.6 Enzymatic hydrolysis with xylanase supplementation

As established earlier, alkaline pretreated biomass removes lignin from the biomass leaving most of the cellulose and hemicellulose behind. This leaves a high amount of xylan in the solids. Xylan is known to inhibit cellulose digestibility by acting as a barrier between enzyme and cellulose [33, 35, 36]. Although cellulase enzyme cocktail used has various activites including xylanase activity [37]; these were not able to convert the carbohydrates efficiently. Multifect Xylanase was added to the enzyme mixture to improve the xylan yields. Since the supplementation improved conversion of xylans, glucan digestibility also increased because of the removal of the xylan barrier and improving cellulose accessibility [33]. Thus by adding 15 mg xylanase/g glucan to the original enzyme mixture, glucan and xylan digestibility increased to 92% and 88% for corn stover. For the more recalcitrant substrate switchgrass, xylan and glucan

digestibility improved to 86 and 75% respectively. Another interesting result was that when the total mg protein was the same, digestibility improved by 36% and 10% respectively (to 75% and 65%) as is shown in Figure VI-7 for switchgrass. Mixed cocktail improved the enzymatic digestibility yields more than cellulase enzyme alone at the same total protein loadings.

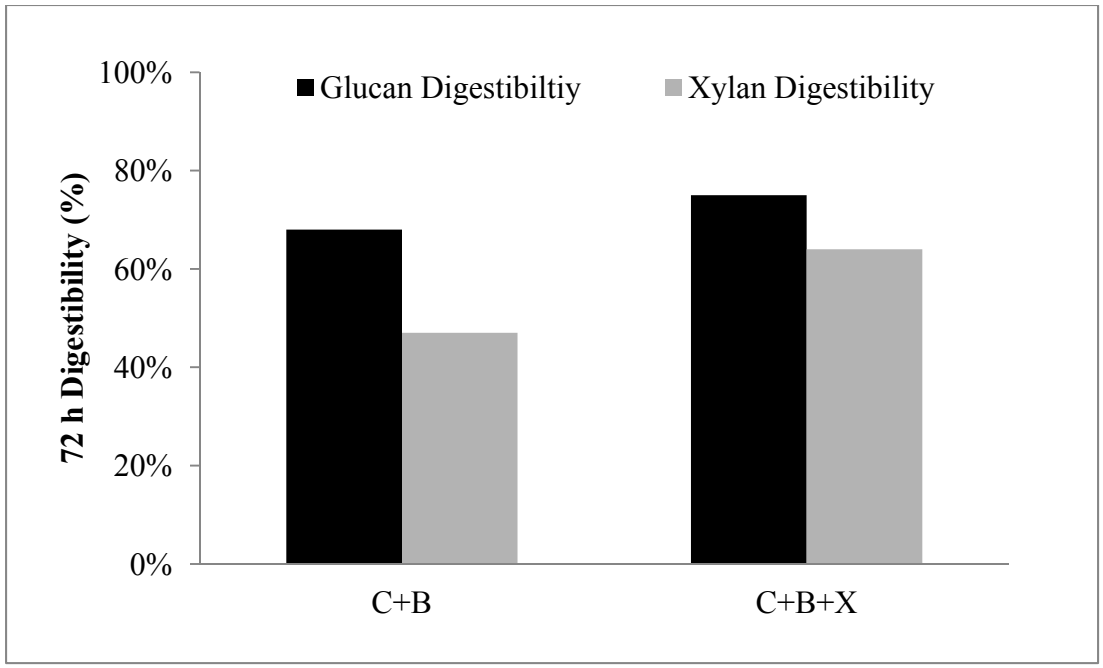


Figure VI-7. Xylanase supplementation.
Enzyme Loadings: cellulase + β-glucosidase 30 mg total proteins/g glucan; vs. cellulase + β-glucosidase + xylanase 30 mg total proteins/g glucan
SAAL Pretreatment Conditions: 90C; 24h; 10:1; 10% Na_2CO_3.

VI.4.7 Physical changes in biomass after pretreatment

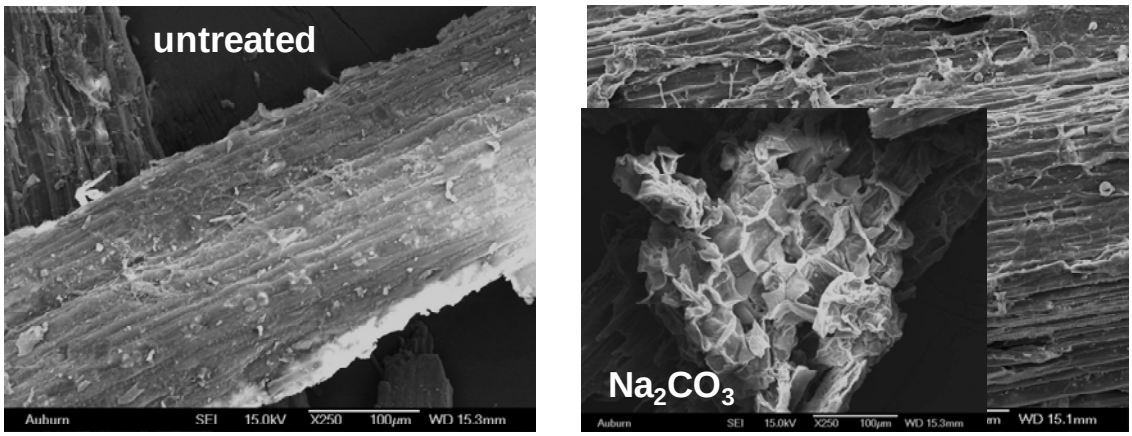
The average crystallinity index can be calculated the Segal method [25]. Crystallinity index (CrI) of treated samples increased after all the pretreatments. From Figure VI-8, there was no change in the XRD pattern for any of the pretreatments as compared to the untreated sample. The

average crystallinity index increases from 53.13 % for untreated biomass to 61.86% for biomass treated with Na_2CO_3. This is because all the pretreatments tested here remove certain amounts of lignin and hemicellulose from the biomass. Since these are amorphous components of the biomass, the average crystallinity of treated biomass is higher than that of the untreated raw material. No change in XRD pattern tells us that there was no change in the cellulose crystalline structure. If there was any change in the cellulose, it would show up as a slightly different pattern with peaks of different cellulose forms [38, 39]. Crystallite size of the crystalline cellulose remained the same after pretreatment as deduced from the peak width at the I_{002} plane. No significant correlation was seen between average crystallinity index and enzymatic hydrolysis of treated biomass (data not shown), as is also seen by other researchers [35].

SEM image of treated sample is shown in Figure VI-8 in comparison with that of the untreated raw material. Surface structure shows high level of disruption after pretreatment. This shows an improved accessibility of cellulose after pretreatment. This is one of the important effects of most pretreatments [40]. The improved accessibility translates to higher enzymatic digestibility of the treated substrate.

BET method measures the total surface area of substrate using gas adsorption. The method is able to measure micro as well as macro pores in a given sample. BET surface area increases slightly when compared with untreated sample. Even though the BET Surface area increased about 1.5 to 2 times, the digestibility increased substantially after pretreatment. This implies that the surface area is not the main factor affecting digestibility. It is the property of the surface that controls the enzymatic reaction rather than the gross surface area. It has been postulated that if

the increase in surface area is due to increase in micro pores, enzyme may still not be able to access the cellulose and further chemical disruption of the biomass may be necessary.



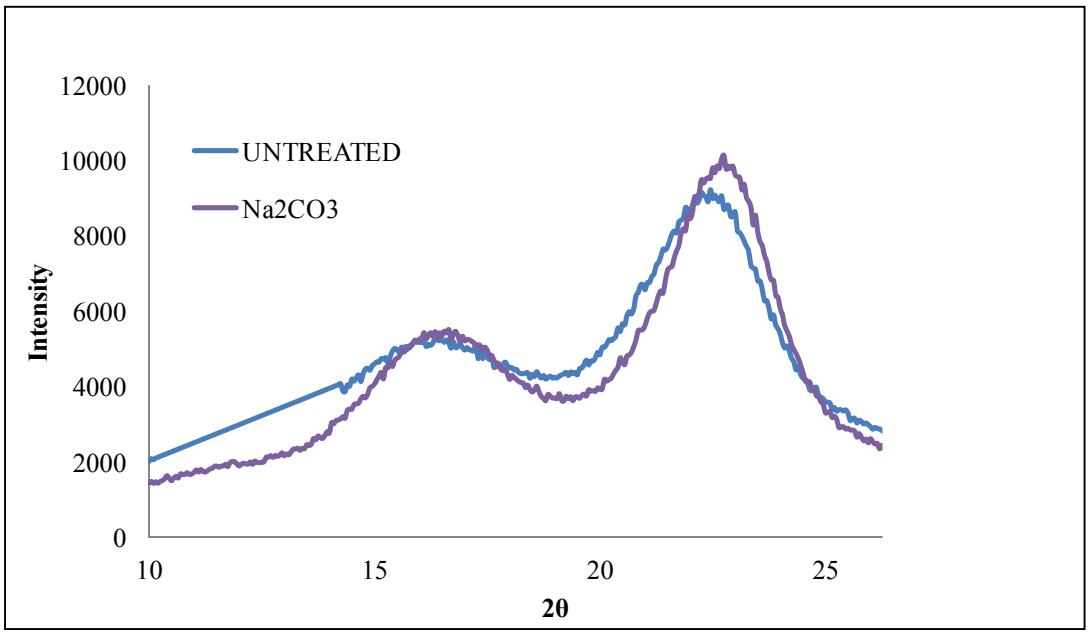


Figure VI-8. Effect of pretreatment on biomass characteristics (a) SEM, (b) X-Ray Diffraction.

VI.5 Conclusion

Switchgrass and corn stover were pretreated by aqueous solution of sodium carbonate under various conditions. Switchgrass was found to be more recalcitrant than corn stover mainly due to its high lignin content. More severe pretreatment conditions were required for efficient pretreatment of the switchgrass. Selectivity of delignification over loss of hemicellulose with switchgrass is lower than that with corn stover. With enzyme loading of 15 FPU/g-glucan, pretreated switchgrass has shown glucan digestibility of above 70%, whereas the pretreated corn stover has shown above 80% of glucan digestibility. When external xylanase enzyme was added to the enzyme mixture matching the protein loading of cellulase (Spezyme CP) alone, the xylan as well as glucan digestibility increased by 10% and 36%. Physical properties of biomass changed substantially with pretreatment, the porosity and BET surface area and the crystallinity index all increased significantly after pretreatment. However, no clear correlation was found between the digestibility and the physical properties.

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Chapter VII Sodium Carbonate Percolation (SCP) Pretreatment of Switchgrass

VII.1 Abstract

Pretreatment of a recalcitrant variety of switchgrass by sodium carbonate was investigated using a fixed-bed flow-through (percolation) reactor, termed as sodium carbonate percolation (SCP). Alkaline reagents react primarily with lignin conserving the carbohydrates in the solid. This offers an economic benefit since the need for detoxification of liquid stream is eliminated in the bioconversion stage. The SCP treatment on the switchgrass was done covering the conditions of 110 - 180ºC and 10 - 30 minutes of reaction time. The SCP treatment removed 65% - 75% of lignin and 10% - 35% of hemicellulose from the biomass, while leaving most of the cellulose intact. The substrates treated under the optimum conditions yielded 80% of glucan digestibility and 55% xylan digestibility, with use of 30 mg of total enzyme (cellulase + β-glucosidase)/g-glucan. Xylanase supplementation improved the glucan digestibility to 85% and the xylan digestibility to 78% Biomass undergoes physical and chemical changes after pretreatment. The results of enzymatic hydrolysis of biomass were correlated with pretreatment severity factor, weight loss, and percentage delignification. SEM images have shown that pretreatment increases surface area and porosity of the substrate. XRD analysis of biomass indicated an increase in average crystallinity of biomass because of removal of amorphous substance in biomass, but no significant changes were seen in the cellulose crystalline structure. FTIR showed changes in the

syringyl/guaiacyl ratios of the remaining lignin and changes in the bond structure of remaining biomass after pretreatment. TGA of the separated lignin was compared with that of a commercial lignin to study the decomposition profiles.

VII.2 Introduction

Switchgrass is one of the promising energy crops being considered for biological conversion to fuels, because of its high biomass yield and low water and nutritional requirements [1, 2]. It grows well on fallow or marginal lands giving 6-9 tons/acre/year of biomass with a high cellulosic content [3]. This cellulose can easily be converted to sugars and further to ethanol using enzymes and micro organisms. Pretreatment is an essential step required before the bioprocess to improve cellulose accessibility and digestibility of the biomass. Lignin is considered as a byproduct of the biomass to ethanol process. There is a distinct advantage of taking lignin out early in the conversion process, before the biological step. During high temperature and/or acidic conditions, lignin degrades into oligomers and its degradation compounds which inhibit enzymatic and microbial activity [4-6]. Lignin remaining in the solid is also a major barrier which reduces cellulose accessibility [7, 8]. Alkaline reagents can be used for delignification and hence pretreatment of biomass for improving its conversion to sugars and ethanol [9-14]. These reagents cleave the aryl-ether and alkyl-aryl linkages in the lignin present in the plant cell wall thereby dissolving it [15, 16].

Various feedstocks have different treatability at the same pretreatment conditions due to their varied recalcitrance or resistance to digestion. In general it is known that softwood species are the most difficult to treat and require severe pretreatment conditions. Similarly corn stover which is an agricultural residue is one of the easier feedstocks to treat, requiring milder pretreatment conditions. It is also found that within the same species of feedstock, cultivars from different areas, and climates can behave quite differently with respect to their treatability. Factors such as age of plant before harvest, harvest time (fall vs. spring), genotype (upland vs. lowland) [1], location of the cultivar (northern vs. southern), quality of soil, fertilizers used, seasonal changes, and precipitation [17-21] play a role in biomass composition and its ease of conversion [18, 22].

In earlier section, sodium carbonate was studied as a potential alkaline reagent for pretreatment. Sodium carbonate is 4-6 times cheaper than NaOH, it is less volatile than ammonia, hence exerts lower process pressure, and most importantly it is easier to recover than NaOH or lime [23]. Strong alkaline conditions can degrade cellulose by the characteristic peeling reaction or enolization [24, 25]. Use of mild alkali may suppress some of the sugar degradation reactions. The process scheme used in earlier section was soaking in aq. alkali (SAAL). Sodium carbonate showed very good pretreatment capability with corn stover, and one species of switchgrass, but with AU switchgrass the digestibility at moderate SAAL conditions was found to be low due to its high lignin content. To improve enzymatic digestibility of this batch of recalcitrant switchgrass, higher severity conditions were applied. However, using high temperatures adversely affected the hemicellulose retention of the biomass. Hemicellulose from biomass is randomly cleaved by peeling reaction, thus dissolved at temperatures above 120ºC in the SAAL process.

The current study was focused on reducing the hemicellulose losses in sodium carbonate pretreatment of switchgrass. A different type of reactor system known as Sodium carbonate percolation (SCP) was used for this purpose. In this system, biomass is packed in a reactor and the fresh reagent is continuously passed through it. There are a couple of advantages to this system over a batch reactor. Since fresh reagent pushes the reacted liquid out of the reactor continuously, the hydrolyzed components are taken out with it. This prevents lignin re-deposition on the biomass surface. The semi-continuous process can be operated at high temperature for a short time, which reduces hemicellulose losses. This type of process has been previously investigated using ammonia in ammonia recycled percolation (ARP) for various feedstocks [10, 26-28]. The physical and chemical factors affecting enzymatic hydrolysis were also investigated in this work.

VII.3 Materials and methods

Switchgrass was provided by Dr. Bransby, Auburn University from the test plot at the Department of Agronomy and Soil. Biomass was first washed with warm 45°C water to remove soil and sand impurities and dried in air. Clean switchgrass was milled to < 20 mesh size before further experimentation. Moisture content of washed and dried switchgrass was approximately 8.5 to 9.3%. Na_2CO_3 and other standard reagents were purchased from VWR.

Cellulase enzyme used was Spezyme CP with an activity of 59 FPU/ml or 125 mg total protein/ml. Xylanase enzyme used was Multifect Xylanase with 42 mg total protein/mL. These

two enzymes were a kind gift from Genencor-Danisco (Paulo Alto, CA). Cellulase enzyme used here was supplemented with β-Glucosidase purchased from Sigma (Novozyme 188, C-6150) with an activity of 600 CBU/mL.

VII.3.1 Sodium Carbonate Percolation (SCP)

In this method the biomass was packed in steel reactor (SS-316) and reagent pumped through it (Figure VII-1). The reactor 0.9" ID x 10" long was made of SS-316. Reactor temperatures were controlled using a GC convection oven. The system was kept at high (300-425 psig) back pressure with nitrogen gas to prevent flash evaporation. 15 g substrate was added in the reactor, which allowed for 25% empty head volume. The rest of the volume was filled with perforated Teflon rods. Sodium carbonate percolation treatment of switchgrass was studied at various pretreatment conditions from 100-200°C for 10-30 min at various reagent concentrations, flow rates and pressures. After pretreatment, the temperature and pressure was reduced and liquid collected for analysis. Pretreated solids were washed with water in the system till pH was neutral and stored in the refrigerator in wet condition until further analysis.

VII.3.2 Obtaining lignin

The liquid effluent collected from the alkaline pretreatment was acidified using concentrated sulfuric acid, till pH was less than 1. Care was taken to contain the foam formed due to the release of CO_2 from the reaction. In acidic conditions, the dissolved lignin precipitated, and the solids were separated using filtration, lyophilized and stored for further analysis.

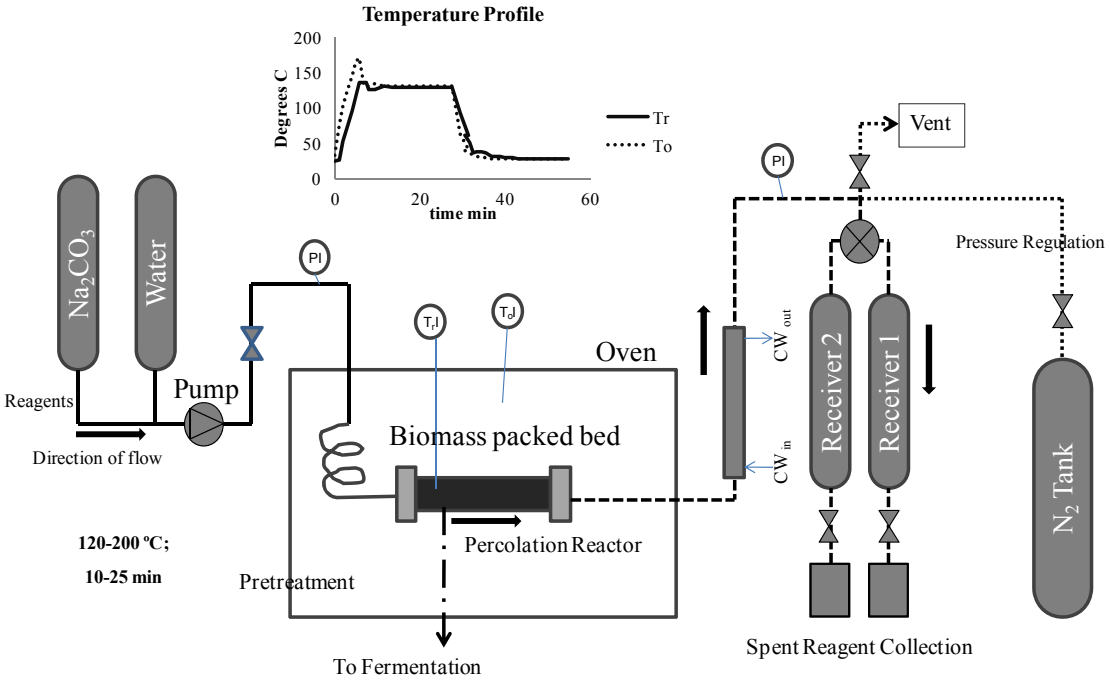


Figure VII-1. Sodium carbonate percolation pretreatment and typical temperature profile.

VII.3.3 Analytical methods

Composition of solids and liquids was performed according to Section V.3.7, and the enzymatic digestibility of substrates was determined according to Section V.3.8.

VII.3.4 Physical characterizations

VII.3.4.1 Scanning Electron Microscopy (SEM)

The treated and untreated biomass samples were studied under a scanning electron microscope to visualize the effect of pretreatment on biomass. The samples were placed on an adhesive carbon

tape on an aluminum stub and sputter coated with gold. Surface morphology of the samples was studied using a Field Emission Scanning Electron Microscope (JEOL JSM-7000F).

VII.3.4.2Crystallinity Index

Crystallinity of pretreated and original biomass was measured using an X-ray Diffractometer (Rigaku Miniflex). Cu-Kα radiation was generated at 30kV and 15mA. Samples were scanned from 2θ = 5º to 30º with a step size of 0.01, and a scan rate 1/min. The cellulose crystallinity index (CrI) in biomass was determined using the following equation proposed by Segal [29].

$$CrI = \frac{(I_{002} - I_{AM})}{I_{002}} \qquad \text{Eq. VII-1}$$

Where I_{002} is the peak intensity corresponding to (*002*) lattice plane of cellulose molecule, and I_{AM} is the peak intensity observed at 2θ = 18°. I_{002} represents both crystalline and amorphous cellulose while I_{AM} represents amorphous cellulose.

VII.3.4.3BET surface area measurement

The method of Brunauer, Emmett, and Teller (BET) was used to determine the specific surface area of the biomass. Raw and pretreated switchgrass samples were analyzed for the BET surface area by multi-point analysis method at Micromeritics Analytical Services (Norcross, GA) using krypton as the adsorptive gas in an AutoPore IV 9520.

VII.3.4.4Fourier transform infrared Analysis (FTIR)

A Nicolet IR100 FTIR spectrometer for measurements in the 4000-400cm^{-1} range was used to analyze pretreated biomass and precipitated lignin samples. The equipment used a TGS.PE detector and a silicon beam splitter with a resolution of 1 cm. The samples to be tested were first

lyophilized to remove all moisture. 1-25mg of sample was mixed with 100 mg of KBr to prepare a sample disc. The spectrum of a pure KBr disc of similar thickness was subtracted from the spectrum of each individual sample.

VII.3.4.5 Thermogravimetric analysis (TGA)

TGA was performed using a Thermo-gravimetric analyzer (Model-Q5000IR, TA instruments, New Castle, Delaware). Analysis was carried out under N_2 atmosphere, to avoid combustion of the samples and applying a 10°C/minute temperature rise. Temperature range used for the analysis was 50 to 750ºC, at which temperature it was safely assumed that the residue was only C and all the H and O was liberated.

VII.4 Results and Discussion

VII.4.1 Properties of biomass

In our earlier investigation, it was found that the switchgrass used in this study was highly recalcitrant (Section VI.4). The biomass contained 37% glucan, 25% xylan, 5% other sugars, 27% lignin, and 2% ash. When compared with other feedstocks, at the same pretreatment conditions, corn stover gave significantly higher sugar yields than switchgrass. The main reason for this was thought to be the high lignin content of the particular batch of switchgrass used here, which was aged in the field for 10 years before harvest (Figure VI-1). Pretreatment using the soaking in aqueous ammonia process at moderate temperatures gave low carbohydrate yield after enzymatic hydrolysis. Very high temperature and strong alkaline conditions caused a high loss of

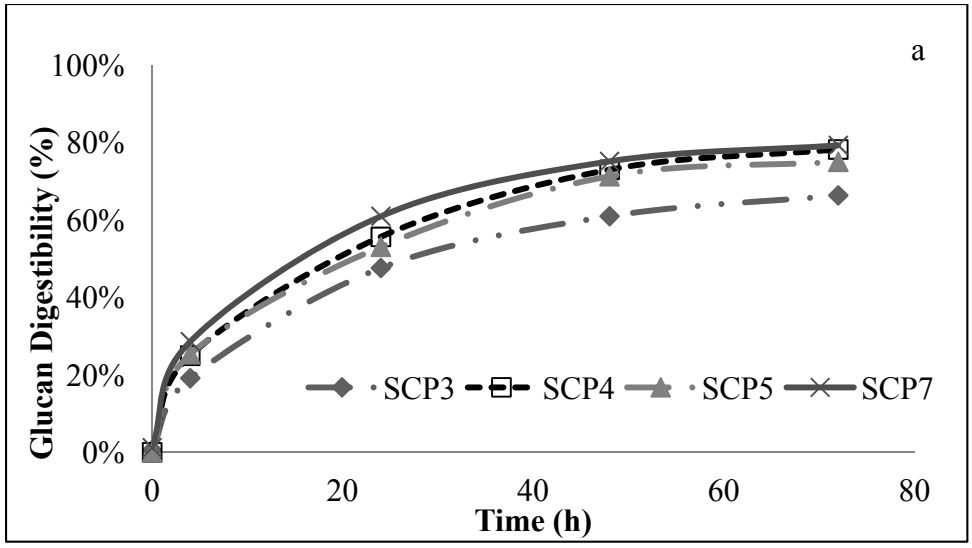
hemicellulose (up to 50% at 140ºC for 24 h with 15% Na_2CO_3). A high severity- short time pretreatment discussed in this work proved to be a better choice for such a feedstock.

Table VII-1. Pretreatment conditions for Figure VII-2.

S No	Pretreatment Conditions	T (°C)	t (min)	Flow rate (mL /min)
SCP3	15% Na_2CO_3	110	20	5
SCP4	15% Na_2CO_3	180	10	5
SCP5	15% Na_2CO_3	160	15	5
SCP7	15% Na_2CO_3	170	20	5

VII.4.2 Effect of SCP pretreatment

On the basis of our previous investigation, 10-15% solution of $Na_2CO_3$was used as pretreatment reagent in SCP pretreatment of switchgrass. Liquid reagent was passed through a packed bed of biomass at 110-200°C with Na_2CO_3 flow rate of 2-5 mL/min for 5 to 20 min. The effect of different pretreatment conditions on the enzymatic hydrolysis and on the composition of biomass are summarized in Figure VII-2 a and b. Using a moderate enzyme loading of 15 FPU/g glucan cellulase and 30 CBU/g glucan β-glucosidase enzymes (27 mg total protein), up to 80% glucan digestibility was achieved with the SCP pretreatment of switchgrass. The SCP process removed 50% - 75% lignin and 50-60% of the ash from the biomass. Xylan losses during pretreatment ranged from 5% to 25% of original and glucan loss was less than 10% depending on the severity of pretreatment. Delignification under alkaline conditions was rapid and within 10 min of pretreatment, high %- delignification was achieved. The total solid mass reduced by 40 – 60%.



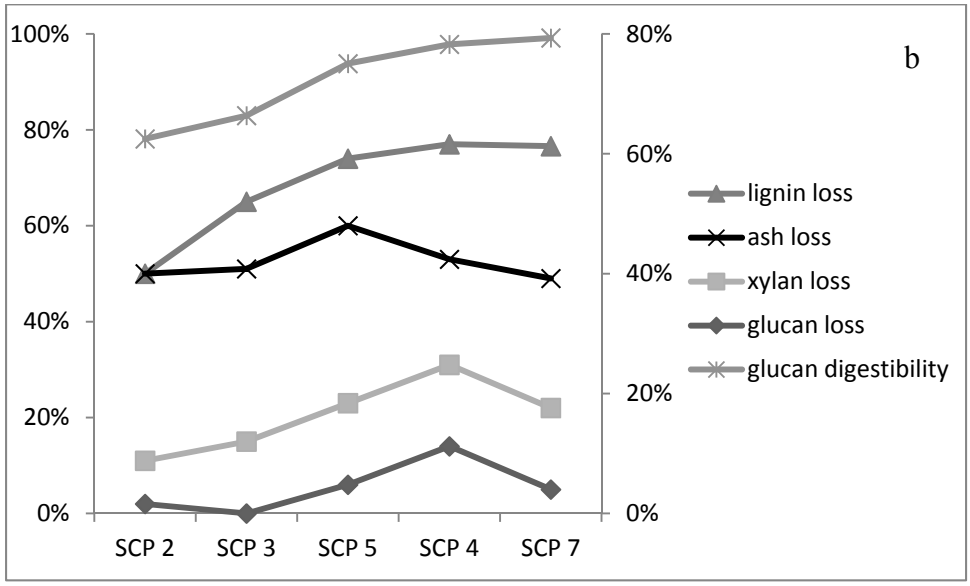


Figure VII-2. Effect of SCP pretreatment on (a) enzymatic digestibility and (b) composition of biomass.
Pretreatment conditions in Table 1; Enzymatic Hydrolysis: 1%glucan loading, 15 FPU + 30 CBU/ g glucan enzyme loading.

VII.4.3 Effect of pretreatment parameters

VII.4.3.1 Effect of sodium carbonate concentration

Two levels of sodium carbonate concentration were applied in SCP treatments of switchgrass, 10% and 15% Na_2CO_3. Figure VII-3 summarizes the effect of sodium carbonate concentration on enzymatic hydrolysis. The results indicate that at higher Na_2CO_3 concentration higher enzymatic hydrolysis yields were achieved, but sugar losses also increased. Xylan loss was more significant when high temperature accompanied high sodium carbonate concentrations. In the Figure VII-3, closed circles represent data taken at 10% Na_2CO_3 and open circles represent data at 15% Na_2CO_3. Triangles represent % xylan lost, squares represent % delignification and circles represent the terminal (72 h) glucan digestibility.

At mild temperature of 110ºC the sugar losses were almost the same at 10% and 15% Na_2CO_3 concentration. Extent of delignification was not affected significantly by increase in alkalinity, indicating that major portion of the aryl ether linkages were cleaved at 10% Na_2CO_3 concentration, the remaining lignin may be more recalcitrant and difficult to hydrolyze. The glucan digestibility of treated sample was between 60% and 80%, with 15 FPU/g glucan enzyme loading.

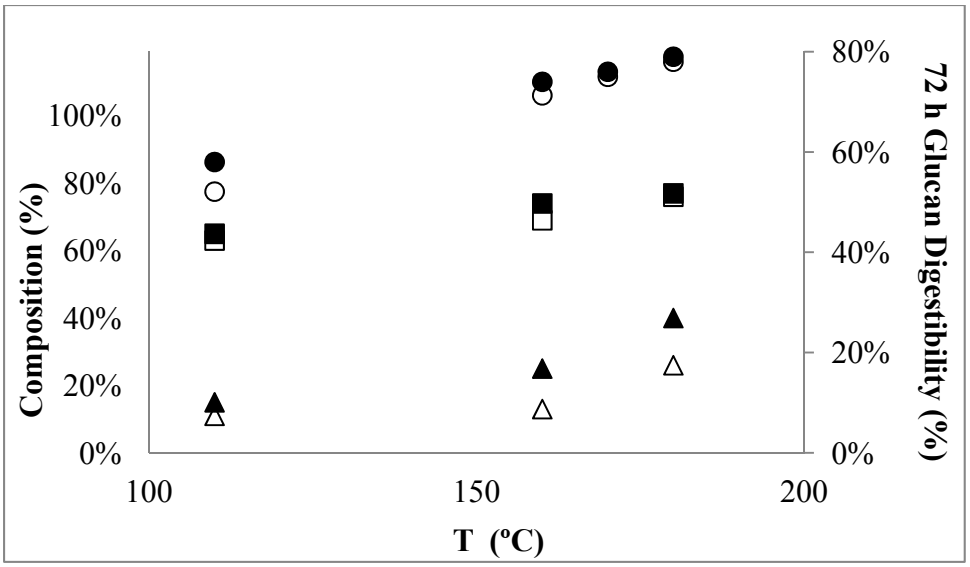


Figure VII-3. Effect of sodium carbonate concentration on enzymatic digestibility at different temperatures.
Circles = 72h Glucan Digestibility, Squares = % Delignification, Triangles = % Xylan lost;
Solid shapes = 15% Na2CO3, Open shapes = 10% Na2CO3
Pretreatment conditions: 5mL/min, 20 min
Enzyme loading 15 FPU + 20CBU / g glucan; Substrate loading: 1% glucan.

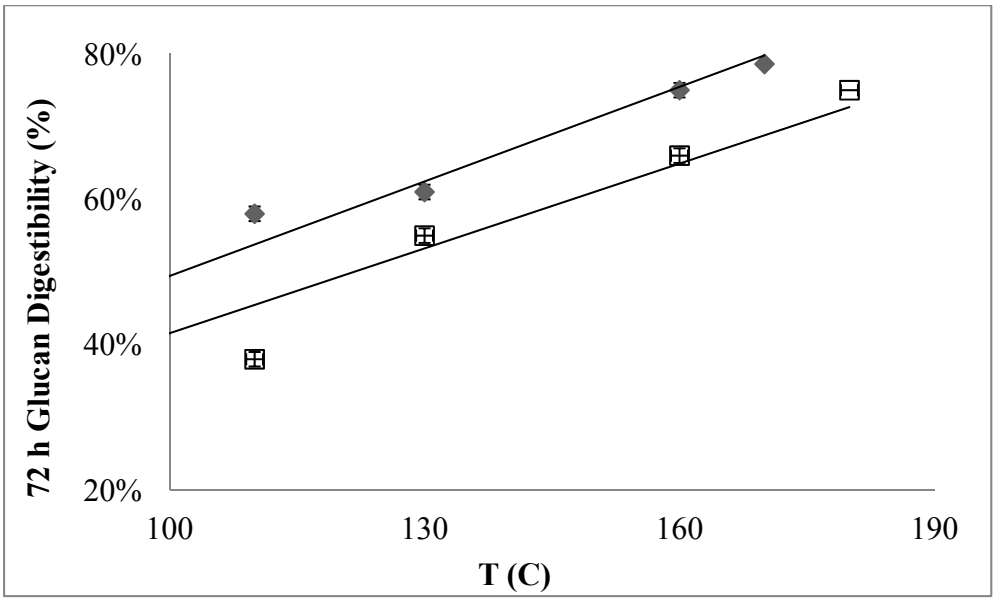
VII.4.3.2 Temperature and time of pretreatment

Figure VII-4 shows the effect of pretreatment temperature on enzymatic digestibility at two different pretreatment times. The results indicate that enzymatic digestibility increases with temperature in general, this phenomenon has been reported by various researchers [30, 31]. The composition of biomass treated at different temperatures shows that xylan losses increased almost linearly with temperature. Xylan is the main component of hemicellulose and the second most abundant sugar in switchgrass. The effect of percolation reactor system is evident in this study. Here, high temperature pretreatment was employed for short durations (10-30 min) as compared to batch process studied earlier (24-72 h). Since the delignification reaction was rapid and in an alkaline medium, high lignin removal was seen within the short durations and

hemicellulose losses were minimized. 40% of lignin was hydrolyzed within 10 min of pretreatment at 110°C and at 180°C delignification reached 70% and 77% in 10 and 20 min of pretreatment time, respectively. Xylan loss was 10% at 110ºC and 25% at 170ºC for 20 min pretreatment time. At a pretreatment temperature of 190ºC for 15 min using 15% Na_2CO_3, 45% xylan and 15% glucan were lost on the basis of original biomass composition. When compared with the batch pretreatment process soaking in aq. Alkali (SAAL) at same temperatures the xylan losses were much higher and close to 50% for pretreatment at 140°C and above. Optimum pretreatment conditions were selected for further study which gave the maximum enzymatic digestibility at high carbohydrate retention. Severe pretreatment conditions that show xylan losses above 30% and glucan losses above 10% were not preferred. The ideal pretreatment temperature range chosen was 150-170ºC for further experiments.

The sugars lost from the biomass solid were found in the SCP effluent mainly in the form of oligomers. The total sugar accountability was 92% for glucan and 81% for xylan, on the basis of biomass when these were included.

The sugars found in the effluent can be utilized after neutralization and separation of lignin from the liquid. In earlier studies, it was found that the conversion of these oligomers using enzymes gives a low yield [32] and the oligomers also significantly inhibit cellulase enzyme activity [4, 33, 34].



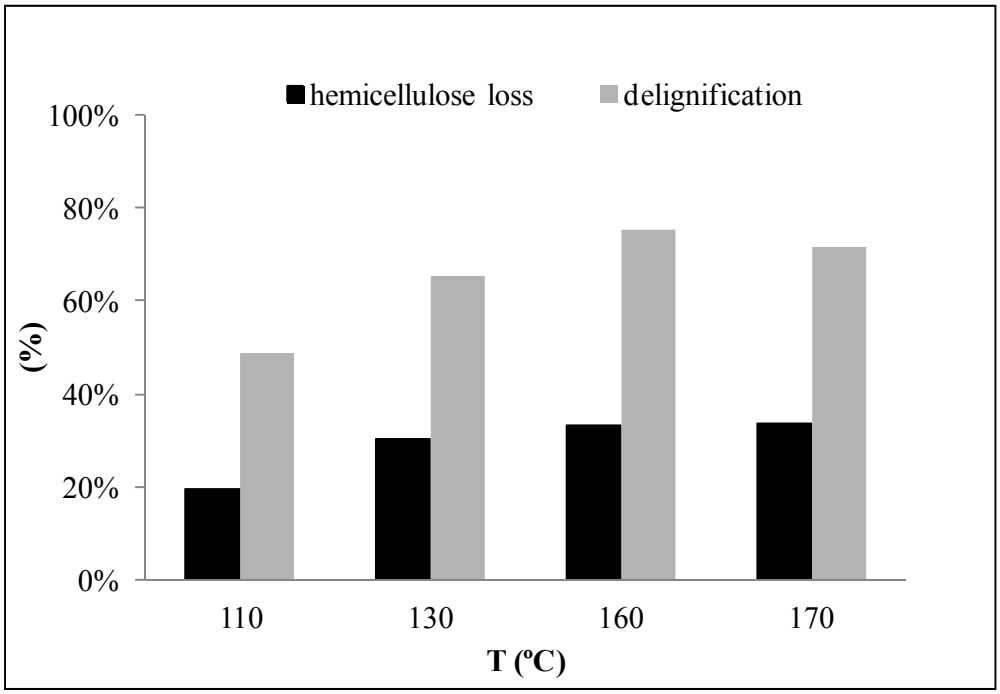


Figure VII-4. Effect of pretreatment temperature of pretreatment on (a) Enzymatic hydrolysis and (b) biomass composition.
In (a) Diamonds = 20 min reaction time; Squares = 10 min reaction time; in (b) data is for 20 min reaction time ; Pretreatment conditions: 15%Na_2CO_3, 5mL/min; Enzymatic Hydrolysis: 1%glucan loading, 15FPU + 30CBU/ g glucan enzyme loading.

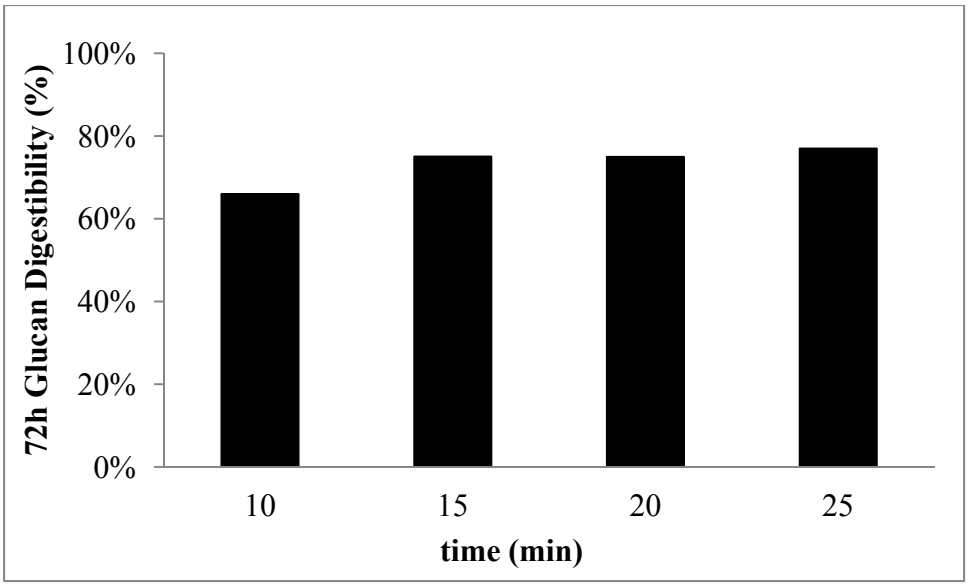


Figure VII-5 . Effect of pretreatment time on enzymatic hydrolysis.
Pretreatment conditions: 15% Na_2CO_3, 5mL/min, 160°C; Enzymatic Hydrolysis: 1%glucan loading, 15FPU + 30CBU/ g glucan enzyme loading.

During the alkaline pretreatment stage, pretreatment conditions can be optimized for maximum sugar retention, to avoid treatment costs related to effluent treatment. Alternative pathways for utilizing solubilized sugars have been proposed in literature such as hydrothermal carbonization, which gives a high energy solid fuel product from the dissolved sugars [35].

VII.4.4 Effect of enzyme supplementation:

The cellulase enzyme, Spezyme CP used in this study is an enzyme complex with various components including β-glucosidase, xylanase and pectinase activities [36]. The β-glucosidase activity in the enzyme alone is not enough to efficiently convert the cellobiose, hence it was externally added using Novozyme 188 at a level of 2 CBU/FPU in all experiments. Alkaline treated biomass generally contains a higher amount of residual xylan in the pretreated solids when compared with other pretreatments [30, 35, 37, 38]. Although Spezyme CP has some xylanase activity, it was not enough to efficiently convert the xylan remaining in pretreated biomass. Xylan is also known to act as a barrier to enzymatic hydrolysis [39], hence conversion of these sugars also helps improve glucan digestibility results. As is seen in Figure VII-6, SCP treated biomass was hydrolyzed using 30 mg mixture of cellulase and β-glucosidase enzyme to give 76% glucan digestibility and 57% xylan digestibility. When 30 mg cellulase enzyme alone was used, the glucan and xylan digestibility were 71% and 47% respectively (not shown in the figure). This shows that Spezyme CP needs supplemental β-glucosidase for efficient conversion. When the mixture was supplemented with 30 mg Multifect Xylanase, the glucan digestibility jumped to 86% and xylan digestibility was 78%. To keep a low enzyme dosage, lower amounts of the enzyme cocktail was added to give a total enzyme loading of 30 mg total protein/g glucan (13.0 mg cellulase + 3.9 mg β-glucosidase, 13.0 mg xylanase). This enzyme dose also showed a significant improvement over adding 30 mg cellulase enzyme alone (82% with 30 mg CBX vs. 71% with 30 mg C alone)

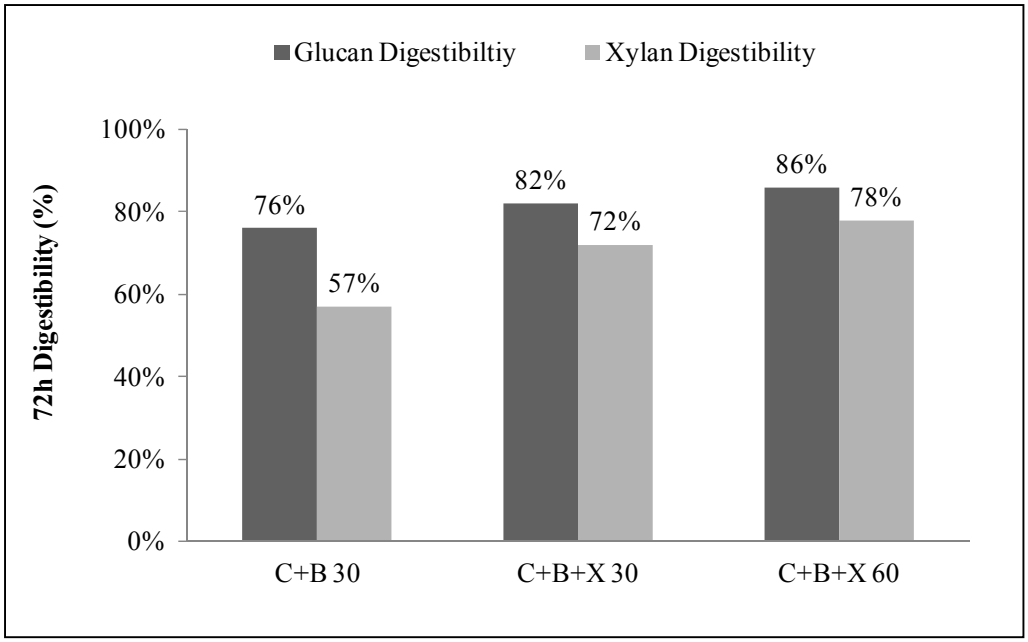


Figure VII-6. Effect of enzyme supplementation.
Pretreatment conditions: 15% Na_2CO_3, 5mL/min, 160°C; Enzymatic Hydrolysis: 1%glucan loading, 15FPU + 30CBU/ g glucan enzyme loading.

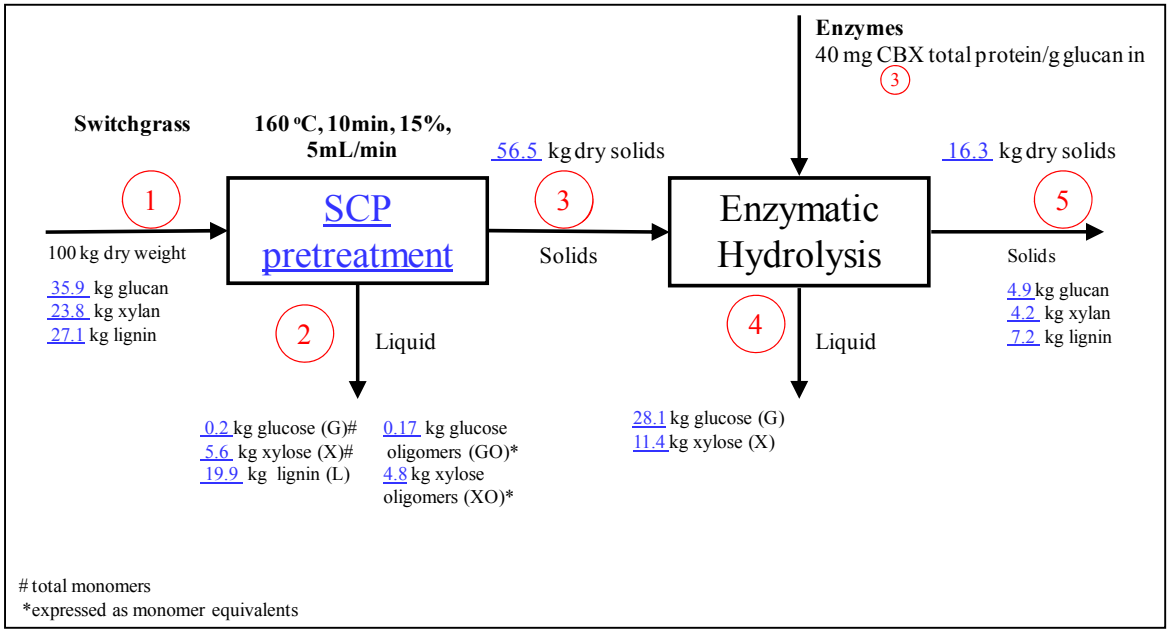


Figure VII-7. Mass balance on pretreatment and hydrolysis.
Pretreatment conditions: 15% Na_2CO_3, 5mL/min, 160°C; Enzymatic Hydrolysis: 1%glucan loading; 40mg total protein/ g glucan enzyme loading (17.4 mg cellulase + 5.2 mg β-glucosidase, 17.4 mg xylanase)

Figure VII-7 shows an overall mass balance for the pretreatment and enzymatic hydrolysis process using SCP pretreatment conditions of 160°C, 10 min, 15% Na_2CO_3, 5mL/min and enzymatic hydrolysis with an enzyme mixture of cellulase, β-glucosidase and xylanase at 40 mg total protein/g glucan enzyme loading.

VII.4.5 Main factor affecting digestibility

Pretreatment brings about many different changes in the biomass, physical as well as chemical, which together improve the enzymatic hydrolysis. It was of interest to find out which factors were the main causes for the improvement in carbohydrate yields.

Enzymatic digestibility of biomass was correlated with the weight loss, delignification and xylan loss seen after pretreatment and the pretreatment severity factor in Figure VII-8 a-d.

The data indicate that the % delignification of biomass was most closely related with the enzymatic digestion with an R^2 value of 0.93. Lignin is present in plant cell walls mainly as a structural support to the plants and as a protection around the cellulose and hemicellulose. It forms a hydrophobic sheath around the carbohydrates and acts as a barrier to enzymatic and microbial attack [40]. Thus, removal of this barrier substantially improves the enzymatic digestibility. This is one of the main advantages of alkaline pretreatments such as the SCP. Once solubilized, lignin can be used for energy generation in the process.

Weight loss of the biomass is also a good indicator of the pretreatment effect. In this study, weight loss correlated well with the enzymatic digestibility, giving an almost linear graph with R^2 value of 0.9. Thus weight loss may be used as a qualitative indicator of the effectiveness of

pretreatment in processes such as SCP which rely on solubilization of either lignin or hemicellulose components from the biomass. This result must be interpreted with caution as there are many pretreatment methods in literature including AFEX pretreatment and physical pretreatment methods, which do not show a significant loss in weight of biomass but do improve the enzymatic hydrolysis significantly [38, 41, 42]. This difference in pretreatment effects highlights the fact that details of the structural complexity of biomass cell walls at the molecular level still remain unclear.

Some portion of the xylan from biomass was also hydrolyzed during SCP pretreatment. Although xylan loss seemed to be less related with improved digestibility (Figure VII-8 c), there was a definite upwards trend. Among the three main components of the biomass cell wall – cellulose hemicellulose and lignin – hemicellulose is the most sensitive to temperature and chemicals. Xylan can be extracted very well in an acid or alkaline environment, and the solubility of xylose monomer is high [43]. Hemicellulose serves as a connection between lignin and cellulose fibers, giving structural rigidity to the cellulose-hemicellulose-lignin matrix. Loss of any hemicellulose therefore does help improve enzymatic digestibility by increasing cellulose accessibility. Since the C-H-L matrix is highly cross linked, some amount of hemicellulose losses along with lignin may be inevitable [4]. Alkaline treatments are used for removing lignin and preserving most of the hemicellulose in the solid. The data shows that though hemicellulose removal does improve digestibility to a certain extent, effect of lignin removal was significantly higher. Comparison between lignin free and hemicellulose free biomass by Kim et al, showed higher enzymatic digestibility with the lignin free samples [7]. In addition, if the hemicellulose barrier was removed during the enzymatic hydrolysis process using enzymes, these sugars can be

further used for ethanol production in a cleaner fermentation process [4] compared to acid pretreatment.

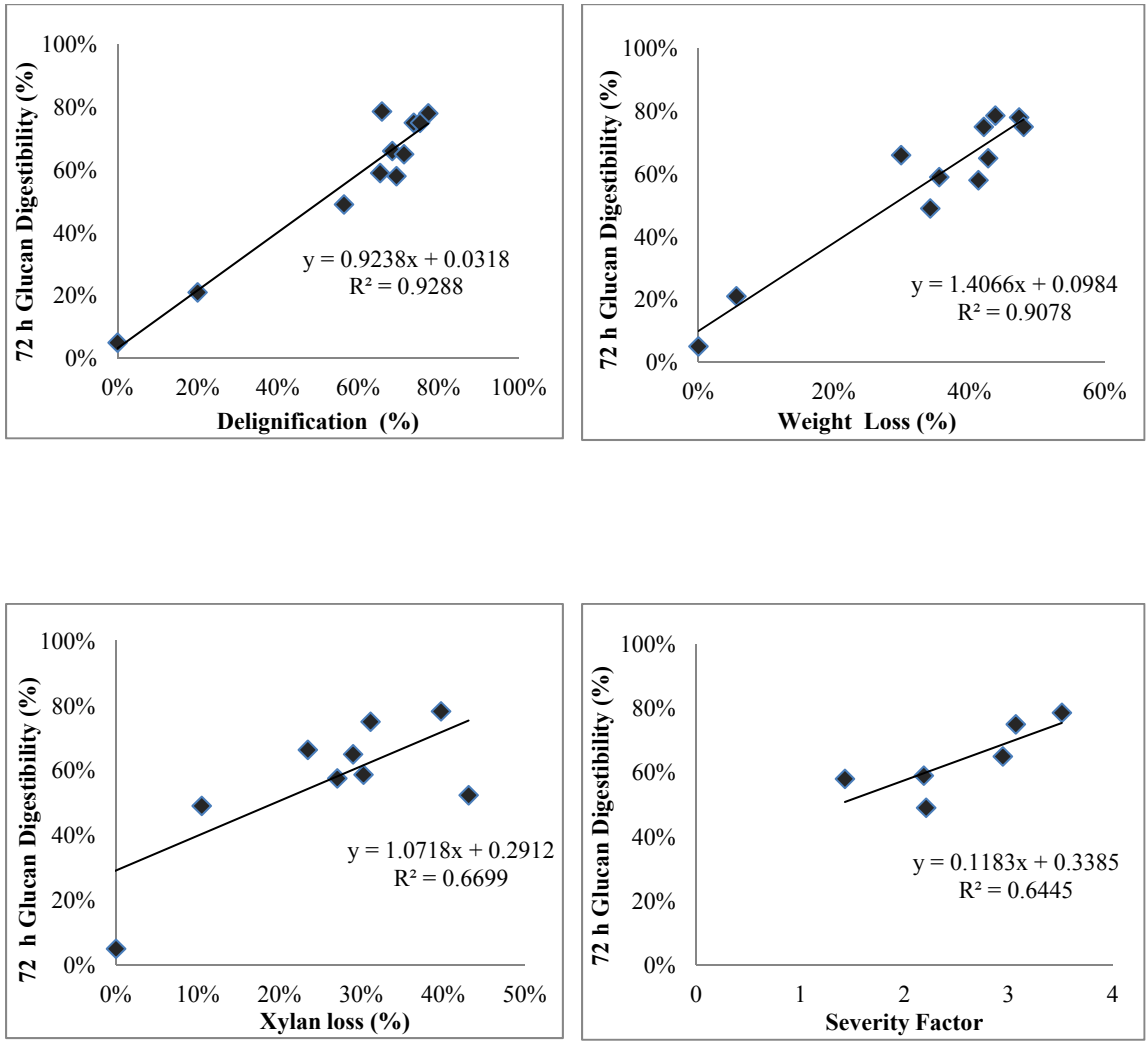


Figure VII-8. Factors affecting enzymatic hydrolysis.
Effect of (a) delignification, (b) weight loss, (c) xylan loss, (d) pretreatment severity factor.

Pretreatment severity factor was calculated using the equation:

$$\log R_0 = t.e^{\left(\frac{(T-100)}{14.75}\right)} \quad \text{Eq. VII-2}$$

This equation is generally used to quantity severity of pretreatment conditions when the pH is close to neutral. When the pretreatment involves acids or alkali, a combined severity factor is defined as

$$\log R = |\log R_0 - pH| \quad \text{Eq. VII-3}$$

In this work, we compared the digestibility and severity factor for pretreatment conditions where the Na_2CO_3 concentration was kept constant. Therefore, the effect of pH would be the same for those pretreatments. Hence the Figure VII-8 d was plotted using Eq VII-2, which indicated that though severity of pretreatment shares a close trend with enzymatic digestibility, it alone could not be used as a predictor of enzymatic hydrolysis of SCP pretreated switchgrass.

VII.4.6 Physical characteristics

SEM images of the treated samples (Figure VII-9) show a significant change in surface morphology. The initial cohesive structure in Figure VII-9 (a) of untreated biomass is disrupted as shown in Figure VII-9 b. These images indicate higher surface area and higher porosity of biomass after pretreatment. This further indicates higher cellulose accessibility which helps improve enzymatic digestibility. The same result was reported with the SAAL pretreatment process in Figure VI-8. The surface of SCP treated biomass shows a higher disruption compared to SAAL pretreated samples.

Specific surface area of pretreated material, as measured by the BET method showed a 2 fold increase from that of the untreated biomass while the enzymatic hydrolysis increased by a higher degree. BET method estimates all the available surface area on biomass, but some of it may not be available to the enzyme. Most of the area is in pores too small for the enzyme to enter [44] hence the total specific surface area proves to be a poor indicator of enzymatic hydrolysis yields.

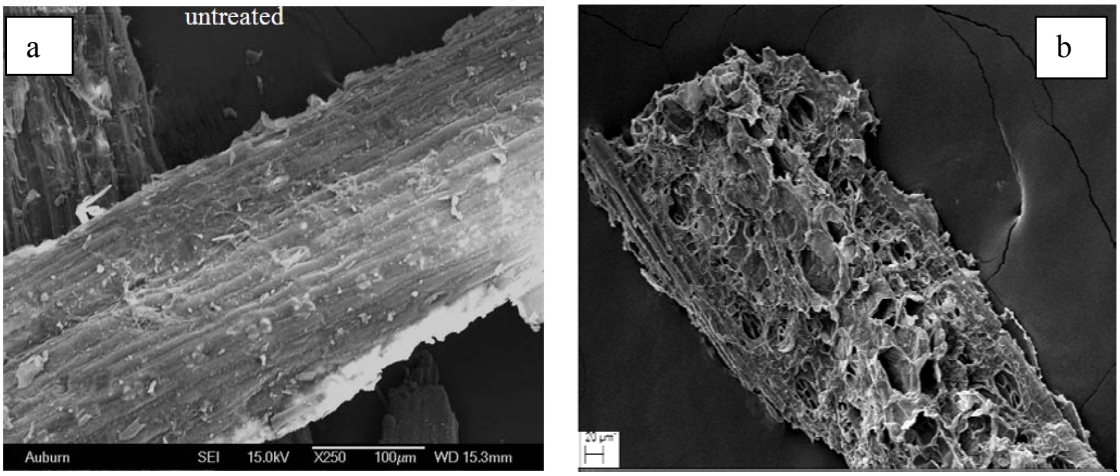


Figure VII-9. SEM images of (a) untreated biomass, (b) SCP treated biomass.
Pretreatment conditions: 15% Na_2CO_3, 5mL/min, 160°C; Enzymatic Hydrolysis: 1%glucan loading, 15FPU + 30CBU/ g glucan enzyme loading.

Other factors such as cellulose crystallinity, porosity, mean pore diameter can affect enzymatic digestibility [45]. X-ray diffraction patterns were taken and the average crystallinity index of treated and untreated biomass was calculated using Eq. V-1. The width of the cellulose crystallinity peak I_{002} indicates the average crystallite size of crystalline cellulose. The 'full width at half height' is used to measure crystallite size [46]. McMillan found that the reduction of the crystallinity of cellulose improves enzymatic digestion [45]. However, in this study, the

crystallinity index of biomass found to increase after pretreatment. This happens because the pretreatment removes lignin from biomass which is an amorphous component, thus increasing the average crystallinity of biomass. According to the Figure VII-10, the full with at half height is the same for treated as well as untreated sample, proving that the crystalline structure of cellulose was not altered by the pretreatment. The shape of the particular XRD pattern also shows that the cellulose structure is not changed by pretreatment [47]. Additional studies have been made along these lines which collectively indicate that the crystallinity of cellulose is merely one of the factors affecting enzymatic hydrolysis [48]. Laureano-Perez et al., found that CrI does not affect the terminal (72 h) glucan digestibility significantly although it may affect the initial hydrolysis rates [49].

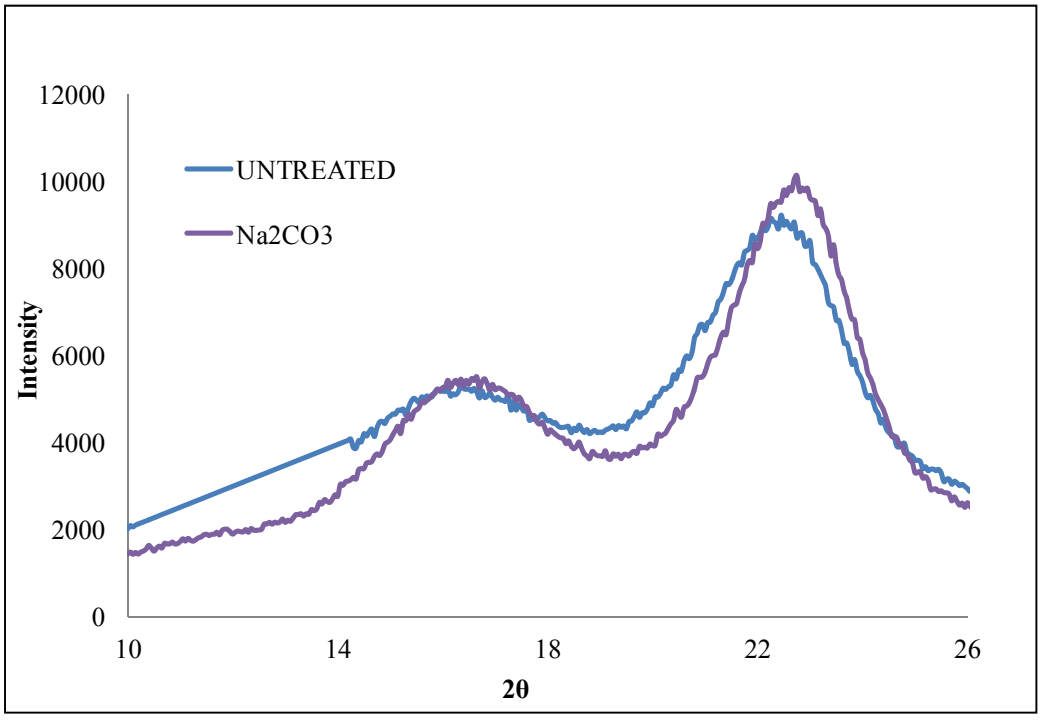


Figure VII-10. X-ray diffraction pattern of treated and untreated biomass.
Pretreatment conditions: 15% Na_2CO_3, 5mL/min, 160°C; Enzymatic Hydrolysis: 1%glucan loading, 15FPU + 30CBU/ g glucan enzyme loading.

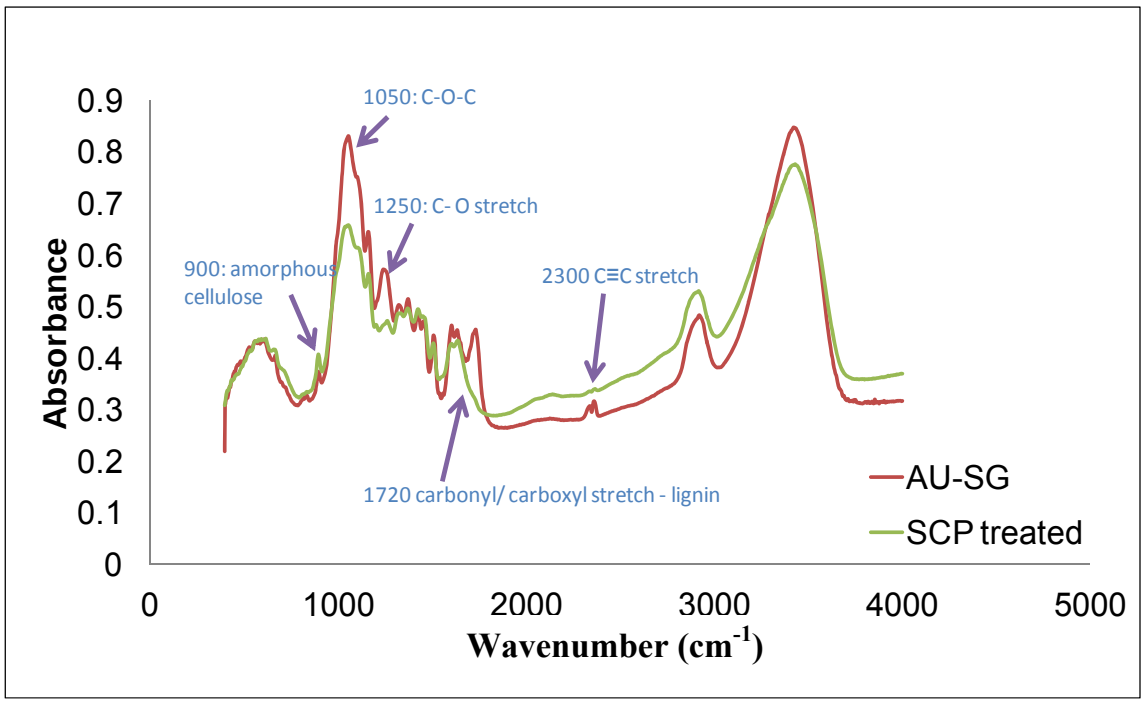


Figure VII-11. FTIR of SCP treated biomass.
Pretreatment conditions: 15% Na_2CO_3, 5mL/min, 160°C; Enzymatic Hydrolysis: 1%glucan loading, 15FPU + 30CBU/ g glucan enzyme loading.

FTIR of treated and untreated biomass was compared to see the specific bond changes after pretreatment (Figure VII-11). A slight increase in the pyranose ring vibration or the amorphous cellulose peak (900) and a marked reduction in the peak associated with C-O-C bond or the cellulose peaks (1050, 1150) show the depolymerization of the biomass polymer cellulose [49, 50]. Very little change in the amorphous cellulose peak (900) reaffirms that cellulose crystallinity was not significantly disrupted during SCP pretreatment. The following changes were seen with regard to lignin : C-O stretch (1240-1306) or guaiacyl ring breathing shows a lower peak in treated sample indicating lignin loss [51]. Peaks at 1405-1430 and 1585-1700 are related to aromatic ring vibrations and the peak at 1720 is related to carbonyl stretch, which is clearly reduced after treatment, confirming delignification effect of SCP pretreatment.

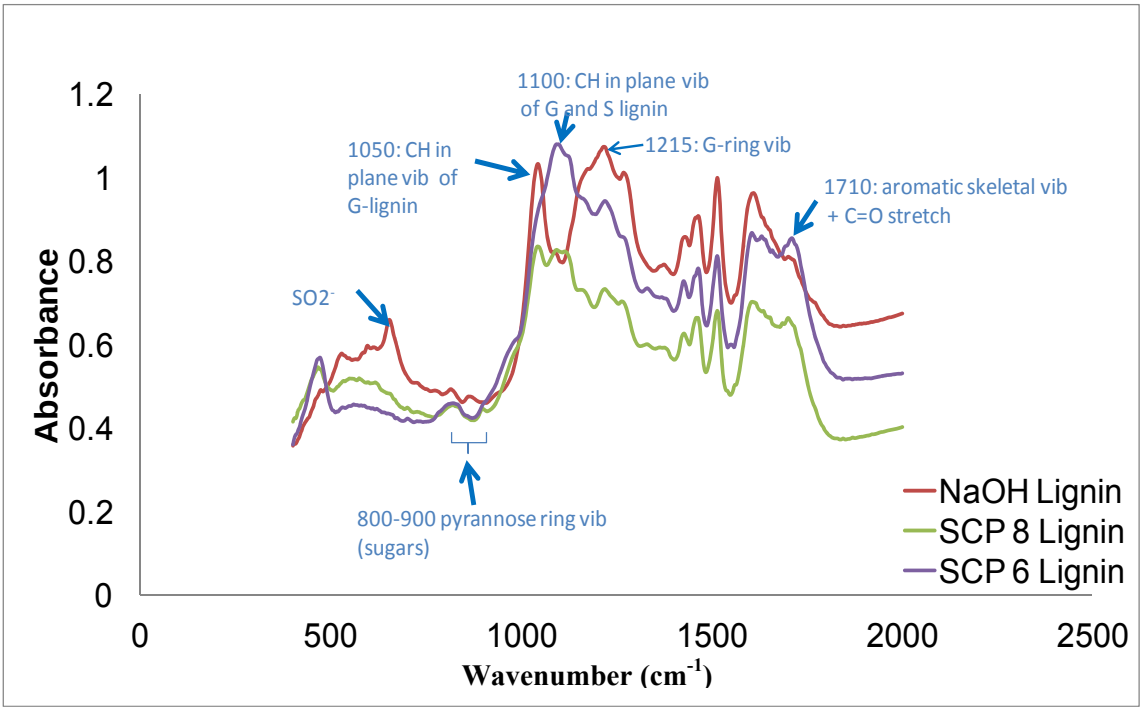


Figure VII-12. FTIR of SCP treated lignin.
Pretreatment conditions: SCP6= 15% Na2CO3, 5mL/min, 160°C and SCP8 = 15% Na2CO3, 5mL/min, 140°C; Enzymatic Hydrolysis: 1% glucan loading, 15FPU + 30CBU/ g glucan. enzyme loading; Lignin was precipitated using 72% H_2SO_4 till pH1 and separated using filter paper.

VII.4.7 Lignin characterization

Effluent from SCP pretreatment was collected and acidified to precipitate the dissolved lignin and the solids were used for further analysis using FTIR and TGA.

Figure VII-12 shows a comparison between lignin extracted from SCP pretreatment and commercial Kraft lignin. SCP-6 was from pretreatment with 15%Na_2CO_3, 160°C for 20 min, and SCP-8 was from pretreatment with 15% Na_2CO_3 at 140°C for 20 min. More lignin was removed from SCP-6 than from SCP-8 pretreatment. The three FTIR patterns were very similar to each other, which indicates that the mechanism of pretreatment using NaOH and Na_2CO_3 are similar.

There are some differences between the commercial lignin and SCP lignins. A sharp peak of sulfate ion (650 nm) is present in the Kraft lignin which was expected because the Kraft process uses Na_2S which reacts with the lignin [50, 52]. FTIR of SCP lignins shows peaks associated with the syringyl and guaiacyl ring vibrations (1100 nm), but the Kraft lignin shows peak corresponding to guaicyl ring alone [51, 53]. This is also expected since the Kraft process uses softwood which contains no syringyl units in the lignin while the SCP process used switchgrass which contains both type of lignin monomers. Aromatic skeletal vibrations associated with lignin at 1700 cm-1are present in all three graphs. Another important difference is the presence of pyranose ring vibrations (900 nm) in the SCP lignins which are absent in the Kraft lignin. These vibrations indicate presence of sugars in the SCP lignins [50], which indicates that the soluble lignin form a complex with the soluble sugars in the pretreatment liquid resulting in a lignin-carbohydrate complex (LCC) [54]. Commercial Kraft lignin is generally treated to remove traces of sugars from the final product, thus no sugar-peaks are seen at 900 cm-1in the FTIR.

VII.4.8 Thermogravimetric analysis of lignin

Among cellulose, hemicellulose and lignin found in biomass, lignin has the most complex structure containing a number of aromatic rings and branched structures. Its degradation profile is more complex than that of the other two polymers and it degrades over a wide temperature range. Lignin separated using different methods, shows different characteristics due to the difference in composition. The Figure VII-13 shows the rate of weight loss pattern for lignin obtained from SCP pretreatment and the commercial lignin. The inset picture shows the weight loss of the 3 lignins with respect to temperature. Residual moisture in the lignin samples

evaporates between 85-150°C. All three lignin polymers decompose between 200-450°C; the maximum rate of decomposition for all three samples was seen at temperature of ~290°C. This shows that the SCP and Kraft lignins were comparable in the degradation pattern. NaOH lignin decomposed over a broader range of temperature than the SCP lignin as shown by the broader peak in Figure VII-13. This indicates a more complex structure prevails in the commercial lignin. The Kraft process undergoes higher severity condition than the SCP process. This explains the more complex nature of NaOH lignin since the process removes a large portion of the lignin including some part of the more recalcitrant lignin from biomass under the severe treatment conditions. The SCP pretreatment is a low severity process that removes only the easily removable fraction of the switchgrass lignin leaving the recalcitrant portion of the lignin behind in the pretreated solids. TGA analysis of SCP lignin along with higher pyranose ring vibrations in FTIR implies the presence of residual sugars in the SCP lignin, which were not present in the commercial lignin.

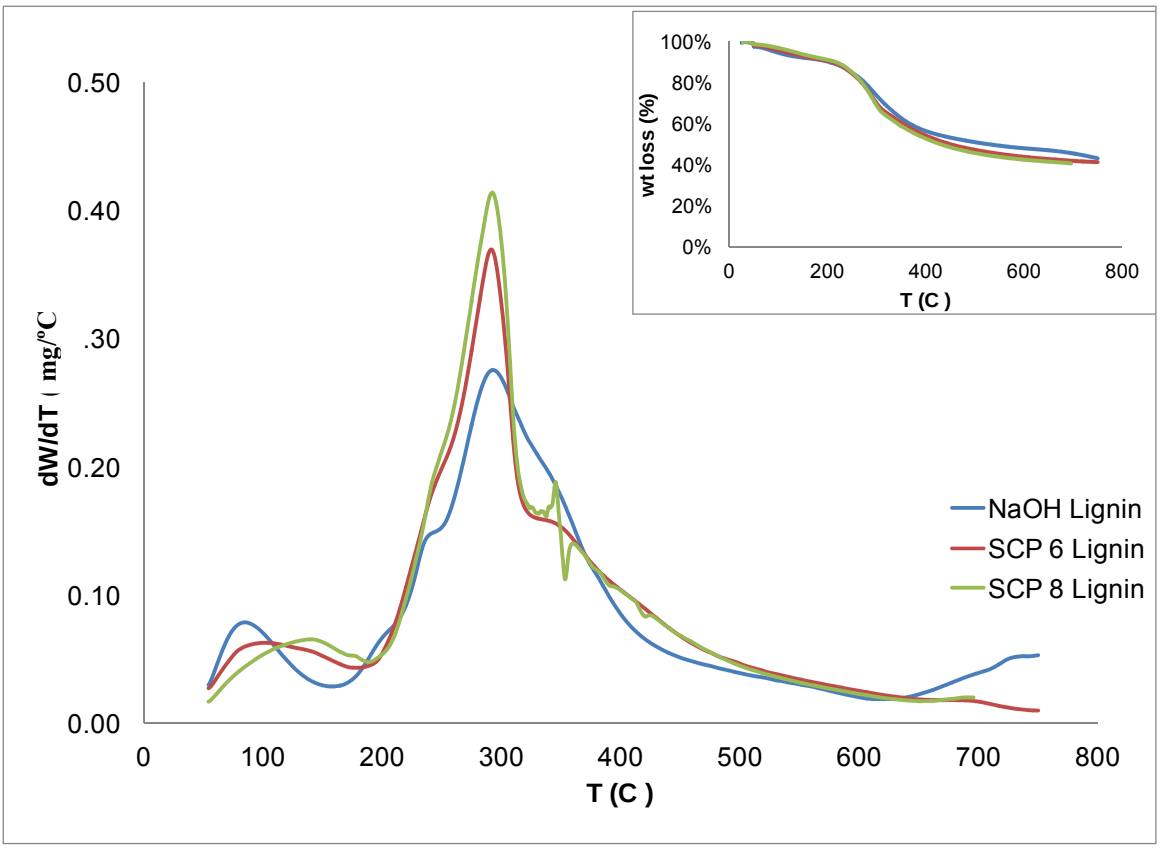


Figure VII-13. Thermogravimetric analysis of lignins from SCP pretreatment. Pretreatment conditions: SCP6= 15% Na_2CO_3, 5mL/min, 160°C and SCP8 = 15% Na_2CO_3, 5mL/min, 140°C; Enzymatic Hydrolysis: 1% glucan loading, 15FPU + 30CBU/ g glucan enzyme loading; Lignin was precipitated using 72% H_2SO_4 till pH1 and separated using filter paper.

VII.5 Conclusion

Switchgrass was treated with sodium carbonate using a flow through reactor system. The percolation system performed better than the batch system since it allows high severity-short residence time pretreatments and continuous removal of the dissolved products.

The SCP pretreatment of the recalcitrant variety of switchgrass used in this study showed an enzymatic hydrolysis yield of 80% with moderate cellulase enzyme loadings. Xylanase supplementation improved the glucan digestibility to 85% and the xylan digestibility to 78% after SCP pretreatment operated under optimum conditions (60°C for 20 min, using 15% sodium carbonate at 5 mL/min). The total sugar accountability was 92% for glucan and 81% for xylan indicating that the carbohydrate decomposition is less than 10% in the SCP treatment. The most important factor affecting enzymatic hydrolysis is the extent of delignification. A certain degree of xylan loss is inevitable due to the highly cross-linked nature of lignin and hemicellulose existing in the cell wall. Temperature adversely affected the carbohydrate retention, but short pretreatment times reduce hemicellulose loss during SCP pretreatment. Other factors affecting enzymatic hydrolysis are surface area, crystallinity index, porosity, all of which increase after pretreatment. Increase of surface area and porosity enhanced the accessibility of cellulose, thus contributed to improved digestibility. Increase of crystallinity index is the result of decrease of the amorphous fraction of biomass. The crystallinity does not directly affect the enzymatic hydrolysis. FTIR studies on lignin extracted from SCP pretreatment effluent showed that the bond structure of SCP lignin is very similar to that of commercial Kraft lignin, indicating that the mechanism of delignification is similar in both cases. TGA showed the same decomposition temperatures for SCP and Kraft lignin. The peak for Kraft lignin decomposition is broader indicating that the Kraft process is more extensive and able to remove the recalcitrant portion of the lignin as well as the easy fraction of lignin in biomass.

VII.6 References

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Chapter VIII Summary and Future work

The first part of this study identified potential inhibitors existing in the dilute-acid pretreatment liquor (PL) of corn stover on enzymatic hydrolysis. Among the identified individual inhibitors, acetate was identified as a strong inhibitor and alkaline deacylation (with Na_2CO_3) as a method of reducing the acetic acid concentration in the PL was proposed. Inhibition by the dilute-acid PL was found to be higher than that by simulated mixture of individual compounds indicating that the unidentified inhibitors, thought to be phenolics from lignin degradation also play a significant role in the overall inhibition. The second part of this study focused on the use of alkaline reagents (NaOH, Na_2CO_3 and aq. NH_3) in the pretreatment of biomass. Due to the high selectivity of the reagents for lignin removal, alkaline pretreatments retain a large amount of carbohydrates in the solids and treatment of the liquid stream is not necessary for sugar recovery. In alkaline pretreatment, large amount of reagents are required and they are more expensive than acids. The cost of reagent recovery must be accounted for in the economic evaluations of the pretreatment process. Sodium carbonate proved to be an effective pretreatment reagent with corn stover. With high lignin feedstock such as switchgrass, high severity pretreatments using a flow through reactor system was proven effective.

On the basis of this work, the following topics are suggested for future study aimed at further understanding and improvement of the cellulose-to-ethanol process.

VIII.1 Future work

VIII.1.1 Use of Na_2S supplementation in alkaline pretreatment

The proposed method uses a non-volatile sulfur compound (sodium sulfide, Na_2S) as an additive to an alkaline treatment method. Sulfur considerably increases the cleavage of alpha ether linkages in the lignin polymer. When sodium sulfide is supplemented to the Na_2CO_3 alkali solution, the sulfur forms covalent-bonds with the released lignin and helps in lignin condensation. The method can be used with sodium carbonate (green liquor pretreatment) or with ammonia. Improved reaction selectivity (lignin removal/hemicellulose removal) was seen as a result of small amount of Na_2S supplementation leading to a significant improvement in digestibility (Section V.4.6). In the cellulosic to ethanol process, the byproduct lignin is used for energy generation. The lignin-rich effluent from the pretreatment process is burnt in the recovery where Na_2CO_3 and Na_2S can both be recycled, as a part of the Kraft recovery process [1, 2]. Small amount of sulfur lost is replenished by adding sodium sulfate before the recovery boiler as the makeup chemical, since it is much cheaper than Na_2S. The sodium sulfate is converted to sodium sulfide under the reducing environment at the base of the recovery boiler [1].

VIII.1.2 Reduced hydrolysis yields in presence of phenolic compounds in PL and at high solids content

Phenolic content is present in the dilute acid pretreatment liquor in small concentrations, measured at 2-5 g/L [3]. These compounds are mainly products of lignin degradation under

dilute acid pretreatment conditions. Compounds from sugar degradation, such as levoglucosan, which is the dehydration product of glucose, were also present in the PL in small concentrations. Phenolics play a significant role in inhibition of micro organisms. Low molecular weight soluble phenolic oligomers are the most inhibitory. The effect on enzymatic hydrolysis by phenolic monomers from lignin has been studied by a Ximenes et al, Palmqvist et al., and Tejirian et al, [3-5]. The main detoxification methods proposed in literature use the enzyme laccase and/or expensive ion exchange resins to remove 80% of the phenolic compounds from the PL. Overliming method can remove up to 30% of the phenolic content but also reduces the sugar content by 10% [4, 6]. An economical and effective method of selectively reducing the phenolic content in the PL is not available. Future work on studying these phenolic compounds and their effects on enzyme activity followed by effective detoxification methods would be useful in improving enzymatic hydrolysis yields. This dissertation also touched upon the reduced enzymatic hydrolysis yields seen when high solids content and unwashed pretreated slurry were used in the hydrolysis step. This phenomenon has not been satisfactorily explained or solved. Further investigation on the reasons for the reduced yields and using additives such as cPAM and surfactants to improve the enzymatic hydrolysis yield at high solids content will be a valuable addition to the current knowledge of the phenomenon.

VIII.1.3 Economic evaluation

This work gives us an insight on the effects of various pretreatment schemes on biomass:

- dilute acid pretreatment

- dilute acid pretreatment followed by detoxification of hydrolysate
- deacylation followed by dilute acid pretreatment
- alkaline pretreatment

The choice of feedstock species as well as cultivation and harvest practices play an important role in the effectiveness of the pretreatment process. A thorough techno-economic evaluation of the various schemes can direct the course of future research towards reducing the cost of ethanol derived from lignocellulosic biomass.

VIII.2 References

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