ALKALINE PRETREATMENT OF BIOMASS FOR ETHANOL PRODUCTION AND UNDERSTANDING THE FACTORS INFLUENCING THE CELLULOSE HYDROLYSIS

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ALKALINE PRETREATMENT OF BIOMASS FOR ETHANOL PRODUCTION AND UNDERSTANDING THE FACTORS INFLUENCING THE CELLULOSE HYDROLYSIS

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ALKALINE PRETREATMENT OF BIOMASS FOR ETHANOL PRODUCTION AND UNDERSTANDING THE FACTORS INFLUENCING THE CELLULOSE HYDROLYSIS

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VITA

Rajesh Gupta, son of Dinesh Chandra Gupta and Basanti Gupta, was born on July 16, 1973, in Kanpur, India. He graduated from Indian Institute of Technology, Roorkee (Formerly known as “University of Roorkee”) in 1996 with the degree of “Bachelor of Engineering”. He joined the petrochemical company “Reliance Industries Ltd. (RIL), India” as a process engineer. After working eight years in RIL, he joined the Auburn University in August 2004 to pursue the “Doctor of Philosophy” in Chemical Engineering. He married Archana Gupta, daughter of Lt. Gopi Kishan Gupta and Ram Kumari Gupta , on March 01, 2000. They are blessed with a son “Arya”, who was born on June 29, 2002.

DISSERTATION ABSTRACT

ALKALINE PRETREATMENT OF BIOMASS FOR ETHANOL PRODUCTION AND UNDERSTANDING THE FACTORS INFLUENCING THE CELLULOSE HYDROLYSIS

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Alkaline pretreatments were investigated in connection with bioconversion of lignocellulosic biomass into ethanol. Corn stover and two different batches of hybrid poplar (High Lignin (HL) and Low Lignin (LL)) were the primary substrates of this study. Two different alkaline reagents, aqueous ammonia and dilute NaOH, were used in pretreatment of biomass for delignification and enhancement of digestibility.

Two pretreatment processes with aqueous ammonia were used: Soaking in aqueous ammonia (SAA) and Ammonia recycle percolation (ARP). SAA is a batch process whereas ARP is a flow-through semi-batch process. Hybrid poplar is found to be more recalcitrant than the corn stover because of higher lignin content. More than 70%, and 90% of hemicellulose were retained in solids after ARP and SAA pretreatment process, respectively. Presence of hemicellulose is a significant resistance for cellulose hydrolysis. Additional external xylanase supplementation has significantly enhanced

enzymatic hydrolysis of treated hybrid poplar. Overall sugar yields of 90%/60% and 90%/77% were obtained from LL/HL hybrid poplar after ARP and SAA treatments respectively. Modification in SAA process was done by adding H_2O_2 at lower temperature. Different H_2O_2 feeding strategy and temperature profiles in treatment were attempted in the modified SAA treatment with HL hybrid poplar. Above 60% of delignification was attained for hybrid poplar by stepwise increase of temperature (60°C for 4hrs and then 120°C for rest of the treatment). Glucan digestibility of 86% was achieved from the HL hybrid poplar treated under these conditions. NaOH was used as an additional pretreatment reagent (without and with H_2O_2) because of its alkalinity much higher than ammonia. Maximum overall sugar yield obtained from HL hybrid poplar was 80% with 5%NaOH + 5% H_2O_2 at 80°C.

To understand the reaction resistances other than the lignin and hemicellulose, the mechanism of cellulase reaction was investigated using pure cellulosic substrates including Avicel, filter paper, α-cellulose, cotton and NCC (Non-crystalline cellulose). NCC is highly amorphous and has Degree of Polymerization (DP) of 100-150. It was found that exo-glucanase (Exo-G) also contributes in the generation of COS. Initial hydrolysis rate of cellulose is mainly controlled by endo-glucanase (Endo-G) whose activity is strongly influenced by the crystallinity. DP of NCC affects the reactivity of Exo-G and its terminal hydrolysis rate. Surface characteristics of substrate such as adsorptivity and surface area affect the initial hydrolysis rate but DP and crystallinity of cellulosic substrate determine maximum conversion. On the basis of the unique properties of NCC, an analytical procedure was developed that can simultaneously measure relative activities of Endo-G and Exo-G in different cellulases using NCC.

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I. INTRODUCTION

For achieving the goal of replacing 20% of fossil fuel by renewable fuel by 2017, different options for renewable energy sources have been discussed in the past and production of ethanol from lignocellulosic feedstock has emerged as one of the best choices. The main reason for this is the abundance of varied lignocellulosic biomass (agriculture residue, perennial crops, woody substance and municipal solid waste) which can be used for production of bioethanol. Among all of these, poplar was chosen as potential feedstock for cellulosic ethanol because of different reasons (D'Aquino et al., 2007; DOE/SC-0095, 2006).

Poplar is a short rotation woody crop which gives higher yield per acre than agriculture residue like corn stover. Per acre yield of poplar is 3-6 tons. The seasonal variation in properties of poplar is much less than other feedstocks (Ladisch et al., 2007). Extensive research is going on for the genetic modification of poplar so that it can be adapted as per the requirement for an ideal energy crop. Poplar is the first tree whose full genome sequence is known and it is a good candidate for genetic engineering work because of its compact genome i.e. 500 million bases, which is only 2% of pine's genome (Wright and Tuskan, 1997, Tuskan et al.,2006). It is found that poplar genetic parameters are favorable for reducing lignin content and increasing cellulose content and specific gravity (Dinus et al., 2001). Owing to these reasons, poplar was selected as official

feedstock in CAFI-II project for studying its conversion characteristics with different pretreatment strategies (Ladisch et al., 2007).

Pretreatment is an important and first step in the bioconversion process of biomass to ethanol. The purpose of pretreatment is to enhance the enzymatic hydrolysis of biomass. Pretreatment alters physical (increase in surface area, adsorption capacity, cellulase accessibility to cellulose) and chemical (change in carbohydrate/lignin content, crystallinity, cellulose DP) properties of biomass and improves its reactivity. In past, different modes of pretreatment have been attempted such as physical (mechanical communition, irradiation), physio-chemical (steam explosion, SO_2/CO_2 catalyzed steam explosion, ammonia fiber explosion), chemical (acid treatment, alkaline treatment, oxidative delignification, organosolv pretreatment, hot water treatment/ auto-hydrolysis, ozonolysis) and biological pretreatment (by use of lignin degrading organisms) (Cadoche and Lopez, 1989; Fan et al., 1982; MacMillan et al.,1994; Dale et al., 1996 ; Bjerre et al., 1996 ; Torget et al., 1992; Karr and Holtzapple, 2000 ; McGinnis et al.1983 ; Vidal and Molinier, 1988).

After the pretreatment, biomass goes through the enzymatic hydrolysis for conversion of polysaccharides into monomer sugars such as glucose, xylose etc. Cellulose and hemicellulose degrading enzymes, called cellulase and xylanase, are used in enzymatic digestion of pretreated biomass. Subsequent conversion of these monomer sugars into ethanol is carried out in the fermentation process by use of different microorganisms. Pretreated biomass can be directly converted to ethanol by using the process called simultaneous saccharification and fermentation (SSF) (Ghosh et al., 1984).

Two batches of hybrid poplar with varying composition (denoted as High Lignin (HL) hybrid poplar and Low Lignin (LL) hybrid poplar) and corn stover were used as main feedstocks in this study. The corn stover has a very different carbohydrate composition than the other two hybrid poplar feedstocks used in this study. The performance of these feedstocks with varying physical and chemical properties, have been compared in the alkaline pretreatment. This comparison helped in understanding the role of different physical, structural and compositional properties of biomass on the overall sugar yield.

The criteria for comparison were optimum process conditions, delignification, hemicellulose retention, hydrolysis of pretreated biomass and effect of xylanase addition on the pretreated biomass. Retention of hemicellulose in the solids is very important for the high sugar yields. If hemicellulose is degraded and removed to the pretreatment liquor, recovery of sugars from liquid stream is very difficult. The liquid stream contains a lot of other lignin and sugar degradation byproducts which are inhibitory to enzymes and microorganisms. If most of the hemicellulose sugars are retained in the solid, the processing of liquid stream can be eliminated and that would improve the economy of the conversion process. The pretreated biomass with high hemicellulose content can be hydrolyzed with external xylanase supplementation in enzymatic hydrolysis and very high sugar yield can be obtained. Same approach was tested in this study.

Alkaline reagents such as aqueous ammonia and dilute sodium hydroxide, which are very efficient in delignification of biomass while retaining the most of the hemicellulose, were employed in this study. Because commercial cellulase also contains

the hemicellulase activity, hemicellulose and cellulose both can be hydrolyzed to monomer sugars by cellulase. This resulted in the much higher yield of sugars with pretreated solids than the acid pretreatment methods. Acid pretreatment improves the digestibility of biomass by degradation of hemicellulose which lowers the sugar yield and inhibits the fermentation process.

Two processes with ammonia reagent, called ammonia recycle percolation (ARP) and soaking in aqueous ammonia (SAA), were studied for hybrid poplar. ARP is a flow through process and it is operated at high temperature (150°C-200°C) and short residence time (10-30min). SAA pretreatment of biomass is carried out in a batch reactor. Typically low temperature and long reaction time (6-24hrs) are used in the SAA process. These approaches have already been investigated by researchers in our lab for different feedstocks such corn stover (Iyer et al., 1996; Kim and Lee, 1996; Kim et al., 2003; Kim et al, 2005), hardwood (Yoon et al., 1995) and pulp mill sludge (Kim et al., 2000). Ammonia is very effective in swelling and delignification of biomass. It has very high selectivity for the reactions with lignin over the carbohydrate. Ammonia can also be recovered and reused because of its high volatility. The operating conditions in the ARP and SAA processes were optimized for HL hybrid poplar initially and then investigation was focused near that optimum operating condition. The difference in the performance of HL and LL hybrid poplar was assessed by using the same operating conditions for both the feedstocks. The effect of external xylanase and pectinase addition on enzymatic hydrolysis of ammonia treated hybrid poplar with cellulase was studied.

H_2O_2 addition under alkaline condition helps in additional lignin removal by oxidative action on lignin. In oxidative delignification, C-C bonds are also broken in addition to the aryl ether linkages (Alen et al., 2000). Addition of H_2O_2 was attempted with 15% ammonia in modified SAA pretreatment for improving hydrolysis of biomass at low pretreatment severity.

In order to achieve higher delignification of biomass, NaOH treatment was tried by use of batch reactor. NaOH is a well known pulping reagent (Sjostrom et al.,1981). It has been used without and with additives for biomass delignification and enhancement of digestibility. (Fan et al., 1981; Koullas et al., 1993, Carr et al., 1984; Gould et al., 1984) . NaOH reagent is also recoverable and its recovery process is commercially used in the pulping industry. In this study, dilute NaOH has been used for pretreatment of corn stover, HL hybrid poplar and LL hybrid poplar. The criteria of pretreatment performance were extent of delignification, retention of hemicellulose and improvement in enzymatic hydrolysis (without and with external xylanase addition).

Process modification of NaOH treatment was done by addition of 5% H_2O_2 with NaOH in the pretreatment. This modification was attempted with an intention to improve the delignification and retention of hemicellulose.

In the pretreatment study, it was realized that in addition to the physical barrier of lignin and hemicellulose, reactivity of cellulose is also dependent upon the crystallinity and degree of polymerization (DP) of cellulose. Different studies suggest the role of crystallinity and DP in the cellulose hydrolysis by cellulase, but the exact cellulase mechanism involving the individual role of different enzymatic components and substrate

features has not been established yet (Zhang et al, 2004; Reese et al, 1977;Gan et al., 2003).

Cellulase is a mixture of mainly three different functional protein groups: exo-glucanase (Exo-G), endo-glucanase (Endo-G) and β-glucosidase (β-G). Cellulose is a polymer of glucose. Cellulose molecules are joined together by inter- and intra-molecular hydrogen bonds and this makes it a highly crystalline insoluble substrate. End-G attacks on the accessible amorphous region of cellulose and breaks down the cellulose molecules into the smaller DP chains. Exo-G reacts with the cellulose from the chain ends in a processive manner and produces cellobiose. β-G converts cellobiose into the glucose which is the end product of cellulose hydrolysis. Thus synergetic action of these three functional proteins in cellulase hydrolyzes the cellulose into the glucose (Lynd et al., 2002). It is found that 100% conversion is not achieved even with pure cellulosic substrates though very high enzyme loadings are used (Yang et al.,2006). Different reasons and mechanisms are suggested for this phenomenon (Gan et al., 2003; Ramos et al,1993; Walker et al.,1991; Holtzapple et al., 1990; Converse et al.,1988) but still uncertainty and difference in opinion prevails.

In this study, Non-crystalline cellulose (NCC), a product of our lab, was chosen as the main substrate for the study of cellulase mechanism. Outstanding properties of NCC such as highly amorphous molecular structure, low DP, hydrophilic nature and abnormally high initial hydrolysis rate, make it a very special substrate. These properties of NCC were utilized in the study of cellulase mechanism. Other pure cellulosic

substrates, such as avicel, cotton, α-cellulose and filter paper, were also employed for studying the reaction behavior.

Activity measurement of cellulase and its enzymatic components is an important task in the optimization process of cellulase production. The activity of Endo-G and Exo-G is typically measured with two different substrates CMC and Avicel respectively. A procedure was devised in this study where one single substrate NCC was used to measure both the activities in one experiment.

Objectives:

The following main objectives are undertaken in this study:

- To develop a pretreatment concept where all the hemicellulose sugars could be retained in the solids and the processing of pretreatment liquid could be totally eliminated.
- To understand the role of compositional and structural features of lignocellulosic feedstock in the alkaline pretreatment processes with ammonia and NaOH.
- To improve the enzymatic conversion of recalcitrant hybrid poplar into the monomer sugar by use of processes with ammonia (ARP and SAA) and NaOH.
- To explore the new possibilities for modified pretreatment method using the alkaline medium and additive H_2O_2. Goals are higher delignification and more

hemicellulose retention in the solids at lower pretreatment severity than used in ammonia or NaOH pretreatment.

- To study the effect of external xylanase addition in the enzymatic hydrolysis of ammonia and NaOH treated hybrid poplar and corn stover.
- To study the cellulase mechanism using pure cellulosic substrates.

II. LITERATURE REVIEW

Need for cellulosic ethanol

80% of the world's total energy demand is met by oil, coal and gas, termed as fossil fuels. After combustion, these fossil fuels generate carbon dioxide as the main product which is identified as a potential green house gas (GHG) (Goldemberg et al., 2007).

It has been estimated by Campbell and Laherrere in 1998 that worldwide crude oil production would start decreasing after 2010 and in 2050, annual global production capacity would decline to 5 billion barrels from 25 billion barrels at that time (Campbell et al., 1998). The increasing demand for crude oil, declining oil reserve and other environmental issues have led to the greater awareness for the renewable energy resources which constitute as little as 13% of total current energy consumption. Among the various renewable energy options, biofuels derived from biomass have emerged as the front runner. The main reasons for this are the feedstock availability, high energy quality and less environmental impact (Sun et al., 2002).

In the 2006 State of the Union address, President Bush emphasized the need for technological breakthrough in alternative fuels and announced the American competitiveness initiative to increase the R&D investments in biofuels research. The focus of this investment would be in direction of linking the revolutionary biofuel

technologies with the advances in biological, physical, computational and engineering sciences. This will lead to development of an efficient, economic and sustainable biofuel industry. The US imports approximately 60% of the crude oil it consumes. DOE has identified the goal to make the biofuels cost competitive. The target is to produce 7.5 billion gallons/year by 2012 and to displace the 30% of US current gasoline use by 2030. 60 billion gallons/year of ethanol is required to fulfill this ambition. In 2004, 3.4billion gallons of ethanol were produced by using 11% of total corn grain harvested in the US (DOE/SC-0095, 2006).

In January 2007, President Bush announced the goal of replacing the 20% of fossil fuel by the renewable fuel by 2017 and this amounts to 35 billion gallons/year which is 5 times the target of 7.5 billion gallons/year production for 2012. The production capacity of the starch-based ethanol industry is not capable of fulfilling the demand of bioethanol. The current production capacity of ethanol in US is 5-6 billion gallons/yr and based upon the projected agriculture yield of corn, the maximum 12-15 billion gallons/year of corn based ethanol can be produced by 2017. Still these figures are way behind the goal. Cellulosic ethanol is one of the main options for filling this gap and industrialization of cellulosic ethanol is an important thrust area for realizing this goal (Kemppainen et al., 2005; D'Aquino et al., 2007; DOE/SC-0095, 2006).

Energy security, economic security, sustainability and environmental benefits are the main drivers for the cellulosic ethanol industry. Today ethanol is blended with the gasoline mainly in 10:90 ethanol: gasoline ratio which is called E-90. E-85 is the ethanol and gasoline blend with 85% ethanol. E-90 can be used in all automobiles without any

modification but the use of E-85 requires the modification of vehicle engines. According to Farrel et al, corn based ethanol can reduce the green house emission by only 18% because of high energy inputs, but because of modest energy inputs, cellulosic ethanol is capable of reducing the green house gas emission by 88%. It was demonstrated through life cycle assessment of ethanol production from corn stover that E-85 (85% ethanol blend with gasoline) can reduce petroleum consumption by 95% per kilometer compared to conventional gasoline. According to a study by National Renewable Energy Laboratory (NREL) and US Department of Agriculture (USDA) in 2005, the US has enough cellulosic biomass for ethanol production which can replace 60% of the total oil used (Kemppainen et al., 2005; D'Aquino et al., 2007; Wu et al., 2006; Holdren et al., 2007).

Understanding the feedstocks for the cellulosic ethanol

The main constituents of biomass are three polymeric substances: cellulose, hemicellulose and lignin. Cellulose microfibrils are the main building blocks for cell walls. Hemicellulose helps in binding the cellulose fibers together and lignin provides the protective covering to the cellulose-hemicellulose matrix. Lignin has a very important role in providing strength to the biomass structure and prevents the degradation of other cellular components. The lignocellulosic biomass, which can be used as feedstock for ethanol production, can be classified into various categories as follows:

*Woody substances***:** The source of wood substance can be forest or agriculture land. One of the main characteristics of wood is a higher content of lignin. As the height and

strength of tree are higher than agricultural crops, they require more lignin to support its growth. Most of the knowledge available about the cellular structure of biomass is through the analysis of wood. All the wood species are broadly classified into two categories: softwood and hardwood. The seeds of hardwood trees are covered in flowers while the seeds of softwood trees are produced in cones.

Agricultural residue: This encompasses the residue obtained from the annual crops such as corn, wheat, rice and other crops grown for food and fiber. The residue consists mainly of stems and leaves of the plant. Examples are corn stover, wheat straw etc.

Perennial crops: This includes grasses and fast growing tree especially for bioenergy production. Examples are switch grass, Miscanthus etc.

*Municipal solid and other industrial waste***:** The composition of these feedstocks varies greatly according to the source. One of the main problems in utilization of these feedstocks is the logistics involved in the collection of raw materials from diverse sources to the main biofuel production facilities (DOE/SC-0095, 2006).

Prerequisites for energy crops and related issues

The main objective of most of the agriculture crops grown these days is to fulfill the need for food, feed and fibers. There are two main requirements for any crop to be used as an energy crop. First, It should have desirable cell wall properties with the criterion of giving high carbohydrate hydrolysis rate and second, high biomass productivity per acre of land. Based on these criteria, switch grass, poplar wood etc. have been identified as a few major energy crops. Optimizing the crop characteristics as per

need of biofuel production is one of the main objectives for research agencies. “To obtain maximum usable organic carbon per acre in an environmentally and economically sustainable way” is the goal of the DOE biomass program (DOE/SC-0095, 2006).

Genome sequencing is an important step in this goal that can provide the information about the link between genomics and carbon to cellulose and lignin biosynthesis. This knowledge can be used to engineer the faster-growing and easily convertible feedstocks for biofuels. The genome sequence of poplar is completely known (Tuskan et al., 2004) which will help in domestication of the tree according to needs of an energy crop. Identification and integration of favorable domestication genes into the energy crop is the next step after genome sequencing. Enhanced breeding and testing of the crop for its desired traits is important for new biomass crops development. In this direction of research, the primary goal of breeding is to identify useful genetic variations for different traits of interest such as disease resistance, drought tolerance, low lignin/carbohydrate ratio etc. Increasing the rate of CO_2 fixation through photosynthesis is another goal of scientists working with feedstock optimization. Plants regulate their water loss through relative opening of stomata, the openings in the leaves. Water loss relative to photosynthesis rate varies greatly from plant to plant. Understanding this relation will help in reducing the water requirement, which is the main input for plant growth. The tolerance level of plants for different environmental factors (such as drought, salt, cold etc.) is also a big determinant for an effective energy crop and its regulation is very important for the adaptability of plants (DOE/SC-0095, 2006).

It had been reported in 1999 by the molecular biology researchers from North Carolina State University, Raleigh, that they had successfully grown the engineered poplar tree with 50% less lignin and more cellulose than the conventional poplar tree by use of genetic engineering. They are also working for reduction in the crystallinity of cellulose fibers in the tree by manipulating the genes that control the biosynthesis of cellulose (Service et al., 2007).

Perennial crops such as switch grass and Miscanthus are being considered as good choices for energy crops. A few advantages of perennial crops over annual crops are lower fertilizer requirements, easy cultivation, high water-use efficiency, absence of disease, reduced soil erosion, high annual rate of photosynthesis, and enhanced wild life habitat (Adler et al.,2006). With switch grass, a maximum of 10 tons per acre cultivation can be achieved, which can be translated into 1000 gallons of ethanol per acre. Researchers are trying to increase the yield to 20 dry tons per acre per year with adequate rainfall and good soil conditions. Switch grass yield is also affected by the harvesting time. As harvesting time is delayed from fall to spring, the biomass yield was decreased by approximately 40% but ash and moisture content also decreased. Low ash is desirable for higher conversion and lower waste disposal problems. Low moisture would lead to safe storage of biomass (Adler et al.,2006).

There has always been debate about the sustainability of biofuels because of the competition for the land needed to grow energy crop with the agricultural land required for food and fiber. Recently, the concept of a low input high diversity (LIHD) mixture of native grass perennials provided very useful information about the use of abandoned and

agriculturally degraded land. High diversity grassland provides a much higher net energy gain per hectare basis than the normal single crop, such as switch grass, which uses agricultural land. Biofuels produced from monoculture, though better than fossil fuels, do not help much in reducing atmospheric carbon. Atmospheric CO_2 sequestration by LIHD crops was 30 times higher than the plots planted with single species. LIHD crops also increase the soil organic carbon (SOC) which is in contrast from the single crop scenario where SOC decreases. The cultivation of mixed species would also avoid the potential instability of monoculture, and promote biodiversity (Tilman et al., 2006).

Sustainability of environment is one of the biggest challenges for crop maintenance. The loss of SOC by stover removal has been associated with a lower yield of crop (Tilman et al., 2006). Microbial communities present in the soil are responsible for maintaining soil quality. Proper knowledge of these microbes is crucial in maintaining the soil quality to address the sustainability issue.

Logistics for feedstock collection and distribution is another challenge for the biofuel industry. Feedstock handling is an important aspect to deal with when designing the cellulosic bioethanol plant. Feedstock storage conditions such as moisture, temperature, air circulation etc. can affect the conversion.

Anatomy of biomass

The knowledge of biomass anatomy and cellular organization is only developed through the analysis of the wood obtained from trees. Outer bark in trees is composed of dead cells, while cells in inner bark (phloem) are alive. Xylem can be divided into two

sections, sapwood and heartwood. Sapwood is physiologically active and a living part of the wood. The cells in this region contribute in food storage and transportation of nutrients from roots to different parts of tree. Heartwood is darker in color, lower in moisture and higher in density. The cells in heartwood do not have any physiological function and are mainly responsible for structural support of the tree. The dark color of the heartwood is because of secretion of resinous organic and phenolic compounds that help in protecting the tree against the degradation by microbes. One annual growth ring is composed of earlywood and latewood. During the transition from earlywood to latewood, cell diameter becomes smaller while the cell wall becomes thicker. The maximum diameter of earlywood tracheids (cells) is 32μm while minimum diameter of latewood tracheids is 7μm. Thick wall latewood tracheids provide the mechanical strength, while earlywood tracheids conduct water and minerals within the tree (Alen et al., 2000; Fengel and Wegener, 1984; Sjostrom et al., 1981; Stamm et al., 1964; Fujita et al., 2001).

All trees can be classified into two broad categories: Softwood and Harwood. Anatomical differences between these two types of wood are explained here:

Softwood (Gymnosperm / coniferous or evergreen species):

The structure of softwood is simpler than hardwood and contains 90-95% tracheids cells which are long & slender cells with tapered edges and have axis along the length of the stem. The length of these fibers is 2.5-7mm which serve for conduction of nutrients. They are normally 100 times longer than their diameter. The cross section of these tracheids is rectangular or circular with an average width of 33 μm (26 μm lumen and 7μm wall thickness). There are cell groups which extend in the radial direction and

these are called the wood ray. Some softwood also contains radial oriented tracheids accompanying the ray parenchyma cells. Radially oriented parenchyma cells are call rays which store and transport nutrients. Epithelial cells are the secreting elements which surround the resin canals. These canals are the vertical or radial cavities within the softwood tissue. Resin ducts which are continuous tubes extending in fiber direction but as they are clogged with resin, don't contribute in any transport.

Communication between fibers takes place through bordered pits which are circular openings in the adjacent wall. These pits appear as chains of elongated beads along the fiber wall. The numbers of pits are 50-300 per fiber and they are mainly concentrated in the radial faces. Pit membrane pore size is approx 1mm or greater but this size depends upon the type and part of wood. Conducting cells and supporting cells are dead cells containing cavities which are filled with water or air. In softwood, tracheids are the living cells which also transfer the nutrients (Alen et al., 2000; Fengel and Wegener, 1984; Sjostrom et al., 1981; Stamm et al., 1964; Fujita et al., 2001).

Hardwood (Angiosperm / deciduous species):

The basic tissue for strength in hardwood is libriform fiber or fiber tracheids. The fibers of hardwood are smaller than the softwood. Conducting vessels are distributed within these fibers. These vessels contain large lumina with a length from few centimeters to few meters. In some of the woods, these vessels contain the film like growths known as tyloses. These tyloses reduce the movement of gases and liquids through the structure under pressure permeability conditions. Wood that contains tyloses is hard to treat with any chemical. Hardwood contains larger amounts of radial and

longitudinal parenchyma cells which are short with stubby ends. Wood rays in hardwood are made up of a more complex array of ray cells and take a larger proportion of wood volume than the softwood. Diffusion through ray cells is very effective and it has been shown that because of ray cells, diffusion in radial direction is more effective than in the tangential direction. Bordered pits in hardwood are much smaller than in the softwood and permeation/diffusion through these pits is believed to be considerably lower than the softwood (Alen et al., 2000; Fengel and Wegener, 1984; Sjostrom et al., 1981; Stamm et al., 1964; Fujita et al., 2001).

Defense mechanism of biomass

For invention of the effective pretreatment strategy, proper understanding of structure of biomass is very important. Reactivity of a reagent with the cell wall components is mainly affected by various defense strategies adopted by the nature for preventing any type of degradation of the lignocellulosic biomass. Following defense strategies in biomass helps to protect it against the reagents or microbes (Gershenzon et al., 1998):

- A waxy outer layer called cuticle and a secondary layer of protective tissue called periderm are the primary defense barriers for any type of microbial attack to the plant. These layers also prevent the water loss from the living tree.

- Secondary metabolites, which happened to be toxic for the herbivores, do not affect the plants adversely. These metabolites are classified in three categories :
  * Terpenes, which are lipids synthesized from acetyl CoA or other glycolysis intermediates, serve as antiherbivore defense compounds in plants. Terpenes can further be classified into monoterpenes (C-10 compounds found in leaves, flower & resin duct and act as a toxic agent for insects), sesquiterpenes (C-15 compounds found in pigment glands and responsible for significant resistance to the insects), Diterpenes (C-20 compounds found in resin canals are toxins and feeding deterrents to the herbivores), triterpenes (C-30 compounds found in plasma membrane as sterol and control the permeability of the membrane. It is found in citrus fruit which acts as a powerful deterrent to insect) and polyterpenes (n(C-5) compounds mainly found in rubber acts as a herbivore).
  * Phenolic compounds, from shikimic acid or malonic acid pathway, are found in heartwood which gives dark color to heartwood and resist the decay of wood. These compounds are also the precursor of lignin which is the second most abundant organic substance after the cellulose. Lignin plays an important role in protecting the cellulose and hemicellulose in the cell wall by forming a hydrophobic covering around the carbohydrate networks. Another class of phenolic compounds are flavonoids which can further be classified into anthocyanins (aromatic compounds for attracting

the animals which help in pollination and seed dispersion), flavones/ flavonols (found in flower and leaves and protect the plants against the ultraviolet radiation) and Isoflavonoids (known for its insecticidal action).

* Nitrogen containing compounds such as alkaloids synthesized from amino acid, also, serves as anti-herbivore.

Effective hydrolysis of carbohydrates present in the cell wall by the cellulase requires the accessibility of the enzyme through these defensive barriers. Pretreatment of biomass is meant to remove these barriers by use of a reagent which should not destroy the carbohydrate but has high selectivity of the reaction towards the defensive compounds. Thus the main challenge in the pretreatment process is to design the regents and process conditions in such a way that cellulosic and hemicellulosic part in plant cell wall remains intact, but the structure of lignin and hemicellulose matrix is altered so that this matrix is no longer defensive towards the enzymatic attack. This structural disturbance can be made with, or without, the removal of lignin and hemicellulose. For designing the effective pretreatment process conditions, it becomes a prerequisite to understand the cell wall structure and the component distribution in the best possible manner.

Microstructure of biomass

Even though wood is highly porous with void volume of 80-85%, permeability is still very low because of the lack of intercommunication between the voids. Cell walls account for 95% of wood material and are made of the thin primary wall and a thicker

secondary wall. The primary wall serves as the transition between the intercellular substance called middle lamella and the secondary wall (Stamm et al., 1964; Allen et al., 2000).

After the cell differentiation, a very thin layer of plastic primary wall encases the protoplasm in each cell. During the thickening of the cell wall, the secondary wall is initiated. The amount of cell wall thickening depends upon the time of the year (early wood or latewood). The secondary wall, especially S2 layer, is largely responsible for the physical properties of wood (Stamm et al., 1964; Allen et al., 2000).

Primary cell wall: In the primary cell wall, cellulose microfibrils are embedded in a tightly hydrated, amorphous matrix made of hemicellulose and pectin. Pectin acts as a hydrophilic filler between the cellulose microfibril and can be defined as a gel phase in which the cellulose and hemicellulose network is embedded. Pectin is the most easily extractable polymer in the cell wall. Their association with cellulose is different from hemicellulose. The buckled form of polygalactouronates in pectin provides the possibility for strong cohesion with cellulose with the involvement of cation forming non-covalent gel. The primary wall also contains a lot of water. Primary walls of mature wood fibers are highly lignified with considerable hemicellulose and 20-25% of cellulose. Generally, the hemicellulose content decreases from primary wall end to lumen of the cell (Cosgrove et al., 1998).

Middle lamella: This layer contains 80% lignin and 20% hemicellulose with practically no cellulose. At early stage of growth, the middle lamella is mainly composed of pectic substance, but eventually it becomes highly lignified (Stamm et al., 1964).

Secondary wall: This layer consists almost entirely of cellulose with a little amount of lignin and hemicellulose (22% of lignin and the rest is polysaccharide). The secondary wall can further be divided into three layers: S1, S2 and S3. S1 and S2 contain the highest content of cellulose as well as the highest amount of uronic acid, whereas S3 is rich in L-arabino-D-glucurono-D-xylans. The S2 layer is much thicker than the S1 and S3 layer and forms bulk of the cell wall substance. Each layer is built of a series of cellulose sheets. Cellulose fibers of individual sheets are aligned in a, more or less, parallel orientation; but direction varies within different sheets. The S1 layer is made of more than one sub-layer and has inclined fibril wrapping at right angles to each other in alternate layers. The S2 layer is laminated but not crossed and is deposited nearly parallel to the fiber axis. The S3 layer is less definite in structure. The cellulose molecule in S3 layers are deposited in a flat helix with respect to the fiber axis. The molecular structure of cellulose in cotton and wood is very similar. Cotton fibers are formed independently and thus contain no intercellular substance (Clarke et al., 1997, Cowling et al., 1975; Stamm et al., 1964).

As the secondary wall volume is much higher than the middle lamella, the fraction of total lignin present in secondary cell wall would be much higher than the total lignin present in middle lamella. The highest fraction of total lignin in wood is present in the S2 layer (40-60%), then in the middle lamella (20-30%) and very less in S1 and S3 layer of secondary wall (Saka et al., 2001).

In each layer of the secondary wall, cellulose and other cell wall constituents are aggregated into long slender bundles called microfibrils. The smallest cellulosic strand,

with an average width of 3.5nm in a mature cell wall, is termed as elementary fibril. These fibrils, in turn, are organized into the strands known as microfibril which is 5-30nm wide. Cellulose DP of higher plants lies in the range of 7,000-14,000 in the secondary wall and approx. 500-6,000 in the primary wall. These microfibrils consist of a "crystalline core" (cellulose) and a "paracrystalline sheath" (composed of hemicellulose and lignin in wood and mainly cellulose in cotton) (Richmond et al., 1991).

Lignin is formed in the cell wall in the later part of the biosynthesis process. As lignin forms in the wall, it replaces the water from the matrix and forms the hydrophobic meshwork that binds tightly to carbohydrate and reduces the digestibility of cellulose in plant material by an enzyme or a microorganism (Cosgrove et al., 1998). Distribution of lignin in cell walls can be made visible by UV microspocopy. Guaiacyl and syringyl components of lignin have different absorption maxima for UV light which corresponds to 280nm and 270nm, respectively. This helps in finding the syringyl / guaiacyl ratio in different cell wall layers. Relative composition of lignin changes in different cell wall layer. It is found that syringly content increases with the increase of wall thickness of the fiber and the vessels in hardwood. This is related to availability of oxygen during the lignification process. Concentration of the hydroxyl group is found much higher in the secondary wall than in the Middle lamella (Fegnel and Wegener, 1984).

Inter-organization of cellulose, hemicellulose and lignin:

In general, cellulose works as the natural framework in wood cell wall and exists in the form of cellulose microfibrils. Hemicellulose is the matrix substance present

between microfibrils. Lignin, on the other hand, is the encrusting substance binding the wood cell together and giving rigidity to the cell wall. An analogy of cell wall has been given with reinforced concrete where cellulose microfibrils act as steel rods and the embedding matrix of hemicellulose as binder (Saka et al., 2001; Richmond et al., 1991). Around the core of cellulose fibrils, paracrystalline regions of cellulose are thought to exist which are associated with hemicellulose and lignin. Lignin encases them and binds them into the rigid structure of the wood cell wall. At molecular level, chemical interaction between lignin and carbohydrates forms lignin-carbohydrate complex (LCC). LCC is considered to be a compatibilizer like substance localized at the interface between hydrophobic macromolecules of lignin and hydrophilic carbohydrates. There are some conflicting views about the presence of individual components in the cellulose-hemicellulose-lignin matrix. According to Fig.II-1, there is no direct interaction between cellulose and lignin but references are available where the interaction of lignin and hemicellulose with the amorphous region of cellulose is mentioned (Cowling et al., 1975).

Different models for supramolecular structure of the cell wall have been proposed by different researchers. Most of the models have one point in common and that is the close association of cellulose and hemicellulose as well as of lignin and hemicellulose. Hemicellulose association with the cellulose is chemical as well as physical in nature and occurs mainly in the paracrystalline region of cellulose (Fegnel and Wegener, 1984).

Biosynthesis of cell wall polysaccharides

Terminal complex (TC) which represents the cellulose-synthesizing enzyme complex is found in the cell membrane. The size of the microfibril seems strongly dependent upon the configuration of the TC. Green algae produce the highly crystalline cellulose due to their linear TC with long rectangular shapes. Certain algae and higher plants are associated with the small hexagonal TC producing smaller microfibril, which is called rosette. The method for assembling of rosette is different from that of TC in green algae. TC seems to be assembled in the plasma membrane by transportation of subunits via the golgi complex while rosette seems to be preassembled in the golgi apparatus and then transferred to the plasma membrane. Microfibrils in higher plants are different from many algae in two manners:

1. The lateral dimension of microfibril is much smaller and lies between 3-4 nm.
2. Crystallite size is much smaller and separated by an amorphous or paracrystalline region.

Polymerization of cellulose molecules occurs in the cell membrane and association of cellulose and hemicellulose occurs during the fibrillogenesis in periplasm (outside the cell membrane). This close association might control the crystallinity of cellulose and provide the microfibril a mobility which allows a spontaneous orientation of microfibril related to the preexisting cell wall layer. One possibility for high crystallinity of cellulose in algal system might be the absence of close association of cellulose and hemicellulose matrix. In higher plant system, the hemicellulose molecule might play a role as internal plasticizer and twisting agents for microfibril. This

immediate association of hemicellulose to cellulose molecule gives a helicoidal texture to the cell wall. From these observations, it can be hypothesized that hemicellulose content can also be related to the crystallinity of the cellulose (Haigler et al., 1991).

Subsequent microscopic and crystallographic studies demonstrated the different widths and crystallite sizes of microfibril in different cellulose producing microorganisms. There are two hypothesis of polymerization and crystallization of cellulose microfibril. In one of these, it is proposed that the glucan chain crystallization occurs spontaneous outside the cell wall, and in the other, it is mentioned that crystallization of microfibril is controlled by the cellular enzyme system or can be directly synthesized in the cell. In vitro synthesis and crystallization of cellulose in the thermodynamically favored form of cellulose II supports the idea of cellular control of microfibril formation. Because if spontaneous crystallization occurs outside the cell wall, cellulose should form the form II polymorph while in nature most of the cellulose is found as cellulose I (Haigler et al., 1991).

The proof has been found with the *A.xylinum* that the polymerization and crystallization is a consecutive but coupled process. Microfibril substructure and crystallite size in cellulose depends upon the size and shape of the terminal complex in cell wall. The process of cell directed self assembly of glucan chains outside the cell is responsible for a particular size and crystallinity of cellulose. The size and shape of cellulose microfibril seems to be regulated by the size and geometry of terminal complex in the plasma membrane. It is found in some organisms that the ordered packing of terminal complex regulates the cell wall arrangement of microfibrils. There is a gap

between the polymerization and extrusion of glucan chains by the enzyme present in the terminal complex and crystallization outside the membrane. Coincident extrusion and binding of hemicellulose also regulate the size and crystallization. Wide distribution of polymerization and crystallinity in the cellulose polymer suggest the varied configuration of terminal complex. The mechanism of polymerization and protein structure in the terminal complex somehow controls the crystallinity. The crystallinity and degree of polymerization of cellulose synthesized by cell directed self-assembly can be varied for better adaptation to different environments (Haigler et al., 1991).

Lignification of cell wall

Lignin precursors are synthesized and stored in Golgi vesicles and endoplasmic reticulum in the form of vesicles. These vesicles are transported and fused to the plasma membrane of the lignifying cell. The lignification of a plant cell is affected by the mineral elements, physiologically active compounds such as auxin and other genetic factors.

It is believed that lignification of the cellulose/hemicellulose framework occurs at the final phase of the differentiating process of the secondary xylem cell. Different studies have confirmed that the initial lignification starts in the cell corner when the surface enlargement of cell is finished and just before secondary wall layer, S1, starts thickening. Lignin deposition proceeds further in intercellular layers of middle lamella the (ML) and the primary wall (P). Lignification of ML and P continues during the differentiation of secondary wall into two layers, S1 and S2, till the formation of layer S3. Initial lignification of the secondary wall occurs slowly but proceeds very rapidly after

the completion of S3. These findings indicate that the lignification of different cell walls takes place after considerable delay to the synthesis of cellulose and hemicellulose (Fengel and Wegener, 1984).

Cellulose

In the cell walls of wood, cellulose acts as the natural framework in the form of cellulose microfibrils. It is present in the highest concentration in the biomass. Cellulose is the polymer of glucose monomer bonded with β-(1→4)-glycosidic linkages. In the process of polymerization of two glucose monomers, one water molecule is eliminated. 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 (Richmond et al., 1991; Clarke et al., 1991). Every anhydroglucose unit forms a chain configuration and cellobiose (polymer of two glucose monomers) is the repeating unit of cellulose chain. The hydroxyl group and glycosidic bonds are the main functional group in cellulose which determines the chemical reactivity of cellulose (Fan et al., 1987). Polymeric chains of cellulose are linear and are joined together by intermolecular hydrogen bonds. A highly ordered crystalline macro-molecular structure of cellulose is formed because of intermolecular and intra-molecular hydrogen bonds. The diameter of microfibrils was assumed to be 10-30 nm. These microfibrils are found to contain the smaller unit called elementary fibril whose diameter is 4nm-5nm. These elementary fibrils contain approximately 100 cellulose chains (Fig. II-1) (Fengel and Wegener, 1984; Zhang et al.,2004; Fan et al., 1987**)**. Two types of cellulose molecular models have been proposed. The first is straight microfibril with alternate crystalline and

paracrystalline region. The second model suggests that the cellulose molecules exist in a folded chain lattice formed as a ribbon which in turn is wound in a tight lattice. In another model by Rowland and Roberts, elementary fibril contains a highly ordered crystalline region with readily accessible slightly disordered surfaces. The paracrystalline regions are assumed to be associated with the hemicellulose (Cowling et al., 1975; Rowland et al., 1972). There are two arguments about the orientation of cellulose chain the crystal: parallel and anti-parallel. Parallel chain configuration of cellulose is more thermodynamically favored and has the strongest arrangement of hydrogen bonds (Richmond et al., 1991; Alen et al., 2000).

The glucopyranose ring and β-glycosidic bonds lie in the ab plane or 110 face. The ac plane or 11'0 plane contains the edges of the rings. Data obtained from the X-ray crystallography shows that glucose residues are arranged parallel in the bc or 200 plane and they are interconnected by extensive h-bonding but that there is very weak hydrogen bonding across the 200 plane. This indicates towards the hydrophobic nature of the 200 plane surface and these sheets seem only connected with the van der waal forces. That might be the reason for production of the rectangular microfibril with plane 110 and 11'0 exposed in the cellulose producing organism (Kuga et al., 1991; Zhang et al., 2004).

The dimensions of unit cell and inclination angle between different planes vary in four different polymorphs of cellulose: Cellulose I, II, III, IV (Fengel and Wegener, 1984). Native cellulose found in wood is termed as Cellulose I. A slight variation in cellulose I structure was observed and two different forms have been designated as I_α and I_β. I_α form is predominant in algal/ bacterial cellulose while I_β is mainly found in higher plant

cellulose (Kuga et al., 1991). Cellulose II is formed after extensive washing of NaOH swelled cellulose. The transition in crystalline structure from cellulose I→ II occurs because of the dissolution of cellulose in NaOH and reorganization. Cellulose III and Cellulose IV can be obtained by Cellulose I and Cellulose II by various thermochemical treatments (Fengel and Wegener, 1984). Cellulose VI is explained as a disorganized form of cellulose I with remarkably low microfibril width and crystallite size. The presence of cellulose IV was observed in the primary cell wall (Kuga et al., 1991).

Hemicellulose

Hemicellulose is an important carbohydrate fraction after cellulose in wood. It is bound to the cellulose and lignin component by covalent and non-covalent bonds in the cell wall (Saha et al., 2003). Hemicellulose generally is soluble in alkaline while cellulose is not. Hemicellulose can be classified as xylans, heteroxylans, galactomannan etc. based upon the composition and intra-structural bonding. The composition of these hemicellulosic fractions varies from one wood species to another. The optical rotation is an important property of all carbohydrates and is mostly used for their characterization. Hardwood hemicellulose mostly contains xylans while softwood hemicellulose contains glucomannan. Some softwood such as larchwood contain appreciable amount of arabinogalactan while its content in other softwood is less than 1% (Alen et al., 2000). Arabinogalactan is partly or totally water soluble. Grasses generally contain 20-40% arabinoxylan which is arabino-4-O-methylglucurono-xylan with a varying ratio of arabinan and xylan (Clarke et al., 1997).

Xylan

Pure deacetylated xylan is able to crystallize in the form of hexagonal platelets with various layers of about 5 nm in thickness. There are six monomer units in one hexagonal space unit. Because of side groups of acetyl or arabinose or uronic acid, a strict molecular order of xylan is not possible A trigonal unit has been detected by X-ray diffraction for xylan monohydrate, and with an increase in moisture, increase in cell size can be observed (Fengel and Wegener, 1984).

Hardwood Xylan

Hardwood hemicellulose contains mostly xylans (Saha et al.,2003) and is laced with groups of 4-O-methylglucuronic acid (Me-GcluU) with an (1-2)-glycosidic linkage with xylose units. Many –OH groups at C2 and C3 of xylose units are substituted with an O-acetyl group. On average in hardwood, molar ratio of xyl: Me-GluU is 10:1 and Xyl: acetyl is 1:0.5. Hardwood xylan has two or three branching points with very short chains linked at C3 of the backbone. The average DP of the xylan backbone varies from 100-200. The DP of hardwood xylan is higher than softwood xylan. Minor amounts of rhamnose and galactouronic acid are associated with the main chain of hardwood xylan (Clarke et al., 1997). Studies have shown that the reducing ends of xylans consist of a combination of xylose and galacturonic acid with the sequence β-D-Xylp-1-4- β-D-Xylp-1-3- α-L-Rhap-1-2- α-D-GalpU-1-4- β-D-Xyl. When xylose is removed from the reducing end, galacturonic acid makes it more stable toward alkali degradation (Fengel and Wegener, 1984).

Softwood Xylan

Softwood xylan differs from hardwood xylan by the lack of an acetyl group and by the presence of arabinofuranose units linked by α-(1-3)-glycosidic bonds to the xylan backbone. Softwood xylan has higher proportion of 4-O-methylglucuronic acid and the ratio of Xyl : Me-GluU is 5:1. The ratio of Xyl : Arab is 6-10:1. Higher molecular weight xylans contain increased numbers of arabinose units and more branching points. Arabinofuranose units are easterified with p-cumaric acid and ferulic acid (Fengel and Wegener, 1984).

Mannan

Wood mannans are correctly referred as glucomannans, and mannan's backbone consists of mannose and glucose units. The ratio of mannose and glucose unit is about 1.5-2:1 in hardwood mannan. The DP of glucomannan in hardwood is about 60-70 and they are only 3-5%. Softwood contains about 20-25% glucomannan and ratio of mannose to glucose units is 3:1. Acetyl groups and galactose groups are attached to glucose/mannose and mannose groups respectively. On average, one acetyl group per 3-4 hexose units is present. There is evidence that parts of the galactoglucomannans are linked to lignin (Alen et al., 2000).

Reactive functional groups in carbohydrates

The main functional groups in the carbohydrate part of biomass are reducing end groups in polysaccharide chains, glycosidic linkages and hydroxyl groups. Reducing end

groups are hemiacetal in nature and found partially converted to open chain aldehyde function in solution. The accessibility of reducing ends in crystalline cellulose for any reagent is slightly lower than that of the amorphous cellulose. Glycosidic linkages are acetal in nature and can be hydrolyzed under acidic, alkaline and oxidative conditions. Acid hydrolysis of glycosidic bonds requires less severe condition than alkaline hydrolysis. Each anhydrous sugar unit in a polysaccharide chain contains one primary hydroxyl and two secondary hydroxyl groups. Hydroxyl group at number 2 position is most acidic in nature because an activating effect of the anomeric center. The hydroxyl group goes through oxidation and is converted into an aldehyde and keto group. In cellulose, the reactivity of these groups is again constrained by the accessibility of the reagent due to its crystalline nature (Lai et al., 2001).

Lignin

Lignin can be defined as a polyphenolic material arising primarily from enzymatic dehydrogenative polymerization of three phenyl-propanoid (p-hydroxycinnamyl alcohols) units: trans-coniferyl alcohol, trans-sinapyl alcohol and trans-p-coumaryl alcohol. 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. In consideration of its polymeric properties, lignin can be defined as a thermoplastic high molecular-mass material which serves the dual purpose of acting as a binder between wood cells and imparting rigidity to the cell walls (Alen et al., 2000; Wayman et al., 1990). Another purpose of lignification in a plant cell wall is to make it more resistant to

microbial and chemical attack (Himmel et al., 2007).The typical content of lignin in the wood lies in the range of 18-33, and it is the second most abundant polymeric organic substance after cellulose (Alen et al., 2000).

Lignin has a different molecular structure in softwood, hardwood and grass. Normal softwood lignin is derived from the guaiacyl units which mainly originate from trans-coniferyl alcohol (90%) with the remainder consist of trans-p-coumaryl alcohol. Hardwood lignin can be termed as "guaiacyl-syringyl lignin" which is composed of trans -coniferyl alcohols and trans-sinapyl alcohols units in varying ratio (about 50% trans-coniferyl alcohol and 50% trans-sinapyl alcohol). Grass lignin is also classified as “guaiacyl-syringyl lignin”, although it additionally contain significant amounts of structural elements derived from trans-p-coumaryl alcohol and some aromatic residues (Sakakibara et al., 2001).

Different building blocks as described above are joined together by ether bonds (C-O-C), carbon-carbon bonds (C-C) or ester bonds (C-O-O-C). Two thirds of the linkages are ether type linkages between the phenolic ring and one of the side chain carbon at a different position (β-O-4, α-O-4, γ-O-4). The second most prevalent linkages are carbon carbon linkages such as linkage between two phenolic rings, between side chain carbon and phenolic ring or between two side chain carbon. The ester type of linkages is typically less than 10%. The main functional groups found in the lignin are phenolic hydroxyl, aliphatic hydrolxyl, methoxyl and carbonyl. Hardwood contains more of the methoxyl group than softwood. The elemental mass ratio of C:H:O for softwood and hardwood lignin are 64:6:30 and 59:6:35. The DP of softwood lignin is higher than

that of hardwood lignin. The polydispersity of lignin is also very high in comparison to cellulose. Isolated lignin shows its maximum solubility in solvents including dioxane, acetone, methyl cellosolve (ethylene glycol monomethyl ether), tetrahydrofuran, dimethyl formaldehyde (DMF) and dimethyl sulfoxide (DMSO) (Alen et al., 2000).

Lignin Carbohydrate Complex (LCC)

Studies have suggested a close and strong association between hemicellulose and lignin. This interaction is physical as well as chemical. This close interaction between lignin and hemicellulose is termed lignin carbohydrate complex (LCC). LCC is mainly used to describe the complexes formed as a result of covalent linkages. These linkages are mainly benzyl ether type, benzyl ester type and phenyl glycoside type. Softwood contains all type of linkages while in hardwood mainly ester bonds and glycoside linkages are prevalent. Hemicellulose side groups such as L-arabinose, D-galactose and 4-O-methyl-D-glucuronic acid as well as main chain end groups of xylan and glucomannan form linkages with lignin functional groups. All the ether and ester linkages are mainly formed at the α-carbon location in phenyl propane units. Glycosidic linkages are formed between the reducing end group of hemicellulose chain and the phenolic hydroxyl group. Ether linkages in LCC are much more stable than ester or any other type of linkages. The molecular weight of these complexes ranges from 600 to 15,000 (Alen et al., 2000; Fengel & Wegener, 1984).

Two models have been suggested to explain the frequency of the linkages between lignin and hemicellulose. In first model, the coiled hemicellulose chain in

repeatedly connected with the large lignin particles (Fengel et al., 1976) and in the second model, small single lignin particles or particle aggregates are joined only once with the hemicellulose chain (Kosikova et al., 1978).

Pretreatment

The enzyme (Amylase) requirement for the hydrolysis of starch is much smaller than the requirement of cellulase for cellulose hydrolysis. This increases the cost of cellulose conversion to the monomer glucose drastically. Though starch and cellulose are both the polymers of glucose, different linkages between the monomers lead to widely different molecular structures. Starch being amorphous, partially soluble water is more receptive to the enzyme and has much less mass transfer resistance for enzyme accessibility to the reactive site. Contrary to this, the high crystallinity and insoluble characteristics of cellulose build up additional mass transfer resistance for the cellulase accessibility. All the resistances for cellulase accessibility in the biomass have been presented schematically in Fig. II-2**.** Physical resistance is because of a very low specific surface area and the hydrophobic nature of the biomass. These are the primary hurdles for the enzyme adsorption to the solid. After enzyme adsorption, enzyme accessibility is hindered by the presence of protective sheath of lignin and hemicellulose matrix.The pretreatment of lignocellulosic feedstock is a vital step in the biomass to ethanol conversion process. The purpose of the pretreatment is to remove all the resistance in cellulase accessibility to cellulose and to improve the hydrolysis of cellulose (Mosier et al., 2005).

The effectiveness of the pretreatment is judged by following criterion:

- Improvement in the enzymatic hydrolysis of cellulose and hemicellulose.
- Minimization of the degradation and loss of carbohydrate, thus maximization the carbohydrate yield.
- Minimization of the formation of inhibitor and toxins for the subsequent enzymatic hydrolysis and fermentation step
- Energy requirement and cost of pretreatment

Different pretreatment strategies include the following:

Physical Pretreatment

1. *Mechanical comminution:* This includes different modes of milling (dry, wet and vibratory milling), chipping and grinding to reduce the size and increase the surface area of the biomass (Millet et al.,1976, Caufield et al., 1974; Sintsyn et al., 1991). These methods are believed to reduce the crystallinity as well. It is not certain that the benefits derived from the milling are due to a reduction in crystallinity or an increase in surface area (Chang et al., 2000). Typically these mechanical operations are energy intensive, costly and time consuming (Caufield et al., 1974).
2. *Irradiation:* Electron beam or other high energy irradiation and microwave heating are the main methods in this class. These methods are very slow, ineffective and very expensive (Chang et al., 1981; Fan et al., 1982).

Physio-Chemical Pretreatment

1. *Steam explosion*: This is one of most the widely used method for the pretreatment of biomass. In this method, biomass is treated with high pressure steam (200-450 psig), and then the pressure is released immediately (MacMillan et al.,1994; Grous et al., 1986). The temperature range in the steam explosion pretreatment is 150°C to 270°C, and treatment time is a few minutes. The results of steam explosion are affected by temperature, residence time, particle size and moisture content (Duff et al., 1996). This method comes under the physio-chemical category because decompression causes the physical disturbances in the biomass and organic acids released during the stream treatment cause the autohydrolysis of the hemicellulose/lignin. This method causes considerable hemicellulose degradation with little lignin removal. Enzymatic hydrolysis of biomass is improved but sugar yield from this pretreatment is low due to hemicellulose degradation. One of the other limitations of steam explosion is the generation of inhibitory compounds for further hydrolysis and fermentation steps (MacMillan et al.,1994).
2. *SO_2 or CO_2 Catalyzed Steam explosion:* The addition of SO_2 and CO_2 during steam explosion treatment can further improve the enzymatic hydrolysis of biomass by making the pretreatment environment more acidic. SO_2 forms sulfuric acid and CO_2 forms the carbonic acid. The limitation of these methods is the lower yield of hemicellulose sugars.

3. *Ammonia fiber explosion (AFEX):* In the AFEX process, the biomass is soaked in anhydrous ammonia at a high temperature and pressure for some time and then pressure is released suddenly. The physical disruption due to decompression and alkaline hydrolysis improves the enzymatic hydrolysis of the biomass. AFEX treatment does not solubilize much of the hemicellulose and results in higher sugar yield than acid catalyzed steam explosion. AFEX was found to be very effective for the pretreatment of low lignin substrate (agricultural residue, herbaceous crops and grasses) but does not work well with high lignin feedstock (woody biomass) (Dale et al., 1996; Moniruzzaman et al., 1997; Foster et al., 2001).

Chemical Pretreatment

1. *Acid pretreatment*: Different acidic reagents such as dilute sulfuric acid, dilute nitric acid, dilute hydrochloric acid, dilute phosphoric acid and peracetic acid have been used for the pretreatment process. Dilute acid pretreatment using sulfuric acid is one of the most commonly used methods to improve the cellulose hydrolysis. There are two approaches for dilute acid pretreatment: high temperature (more than 160°C) continuous flow reactor for low solid loading and low temperature batch process for high solids loading. One of the main advantages of the dilute acid pretreatment is that it is effective with most of the lignocellulosic feedstocks. Xylose yield from dilute acid pretreatment is higher than steam explosion but lower than AFEX and other alkaline treatment processes.

Performing the dilute acid pretreatment with a percolation reactor can give a xylose yield of more than 90% of the theoretical maximum value. The cost of dilute acid treatment is higher than the physiochemical treatment. Because of the corrosive nature of the reagent, reactor and maintenance cost is high. And other disadvantage of this method is the neutralization of pH after the pretreatment for next step of enzymatic hydrolysis (Grohmann et al., 1985; Torget et al., 1992; Nguyen et al., 2000).

2. *Alkaline pretreatment*: The main reagents used for alkali pretreatment are sodium hydroxide, ammonia, ethylene diamine (Detroy et al., 1981) and calcium hydroxide. A strong sodium hydroxide solution is widely used in the soda pulping and kraft pulping processes for the delignification of wood. Dilute NaOH treatment was shown to cause swelling, removal of lignin and hemicellulose, increase in surface area and decrease in the degree of polymerization (Fan et al. 1982). Saponification of ester bonds in hemicellulose and the peeling mechanism of glycosyl residue in hemicellulose/cellulose lead to drastic loss of sugars in the pretreatment (Lai et al., 2001) with NaOH treatment. NaOH treatment was found to be effective in increasing the hydrolysis rate of hardwood and agriculture residue with low lignin content (Bjerre et al., 1996). Softwood with very high lignin content could not be effectively treated with dilute NaOH (Millet et al., 1976). NaOH treatment causes significant amount of hemicellulose solubilization (Sjostrom et al., 1981) which depends upon the choice of substrate and operating conditions.

Lime treatment using calcium hydroxide was also used by Playne et al. and Kar et al. Considering the cost of the chemical, calcium hydroxide stands as a good choice among the basic reagents. Holtzapple et al. used long treatment time (a few days) and low temperature for treating the corn stover and poplar. Similar to other alkaline pretreatment, calcium hydroxide causes a large amount of delignification and increases the hydrolysis rate (Playne et al., 1984; Karr et al., 2000; Chang et al., 2000; Chang et al., 2001).

Aqueous ammonia was very effective in enhancing the delignification and hydrolysis of corn stover and switch grass (Iyer et al., 1996). Two processes, based on aqueous ammonia, ammonia recycle percolation (ARP) and soaking in aqueous ammonia (SAA), were used by Kim et. al. to improve the hydrolysis of corn stover close to the quantitative maximum The ARP process also enhanced the enzymatic digestibility of poplar wood (Yoon et al., 1995). Desirable characteristics of ammonia are its high selectivity for lignin reaction, high retention of hemicellulose, low cost and the possibility of recycling due to its volatile nature (Kim et. al., 2003).

3. *Oxidative delignification:* Hydogen peroxide is widely used as an oxidizing reagent for the bleaching of wood. Azzam et al. used the H_2O_2 to pretreat the cane bagasse and achieved 50% delignification. This improved the enzymatic hydrolysis of cellulose to 95% but caused a significant amount of hemicellulose solubilization. H_2O_2 was used with sulfuric acid, ammonia and water for the treatment of oak by percolation process by Kim et al. and showed considerable

improvement in enzymatic hydrolysis. Enzymatic digestibility of agriculture residue such as wheat straw, corn stover, corn cobs and oak shavings was greatly enhanced by use of alkaline peroxide treatment (Azzam et al., 1989; Glould et al.1984; Bjerre et al., 1996; Kim et. al., 2001).

4. *Oraganosolv pretreatment:* Various organic solvents (Methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol) have been used to dissolve the lignin from the biomass. The organosolv process is very helpful in increasing the enzymatic hydrolysis due to delignification, but these solvents are costly and require high pressure equipment. Recycling of the solvent is important for reduction of cost. Removal of the solvent from the pretreated biomass is a crucial step as the solvent can be inhibitory in the enzymatic hydrolysis and fermentation reactor. The effect of other acidic and alkali catalysts in the organosolv process has also been studied and found that hemicellulose solubilization increases due to these catalyst. These catalysts help in lowering the temperature of the pretreatment (Chum et al., 1988; Thring et al., 1990).
5. *Hot water treatment/Autohydrolysis:* Reactions in hot water treatment are very similar to steam explosion. The hydrolysis of hemicellulose due to water ionization at high temperatures causes solubilization of hemicellulose sugars and cleavage of the acetyl group. Acetic acid catalyzes the autohydrolysis reaction. Other acedic moieties released as a result of lignin (various phenolic acids) and hemicellulose degradation (formic acid, glucuronic acid etc.) accelerate the reaction. These structural alterations increase the accessibility and hydrolysis of

cellulose. (McGinnis et al.1983; Timell et al. 1967, Fernandez-Bolonas et al. 1999, Garrote et al., 2002)

6. *Ozonolysis:* Pretreatment of biomass by ozone has a few desirable characteristics such as high selectivity for lignin removal with slight effect on hemicellulose part, low treatment temperature/pressure and the absence of fermentation inhibitors after pretreatment. Ozonolysis of the biomass leads to a drastic improvement in enzymatic hydrolysis but due to large amount of ozone required, this pretreatment process is expensive (Vidal and Molinier, 1988). This pretreatment process has been tried on different feedstocks such as wheat straw, bagasse, cotton straw and poplar sawdust (Ben-Ghedalia and Miron, 1981; Neely et al., 1984; Ben-Ghedalia and Shefet, 1983; Vidal and Molinier, 1988)

Biological Pretreatment

This category of pretreatment employs mainly lignin solubilizing microorganisms such as white-rot and soft-rot fungi (Schurz et al., 1978). White-rot fungi are one of the most effective organism for the biological pretreatment of biomass (Fan et al., 1987). These fungi cause considerable loss of cellulose in the pretreatment. A cellulase deficient mutant of *Sporotrichum pulverulentum* was developed to prevent cellulose loss (Ander and Eriksson 1977). These organisms produce extracellular lignin degrading enzymes such as lignin peroxidases during secondary metabolism as a result of carbon and nitrogen limitation (Boominathan and Reddy, 1992; Kirk and Farrel, 1987). Biological pretreatments have very low energy requirements due to the mild conditions of

pretreatment. However, these treatment processes have certain drawbacks such as long treatment time and a lower enzymatic hydrolysis rate after pretreatment in comparison to the chemical treatments (Hatakka et al., 1983).

Hydrolysis of biomass by cellulase components

The structure and configuration of the cell wall in the biomass is designed by nature in such a manner that decomposition of cellulose and hemicellulose is very difficult by action of an individual enzyme. The diverse architecture of the plant cell wall necessitates a variety of enzymes for its degradation (Lynd et al.,2005). The efficient degradation of biomass polysaccharides requires synergistic action of different proteins meant to cleave specific substrates present in the lignocellulosic biomass. Cellulase is the complex mixture of diverse enzymes which work synergistically and degrade the cellulose/hemicellulose.

Hemicellulose in agriculture residues and hardwood is mainly composed of a xylan backbone with the side chains of other polysaccharides. Xylanase removes the covering of this heteroxylan from the cellulose microfibrils by catalyzing the hydrolysis of the xylan backbone. Heteroxylan becomes debranched by the action of other auxiliary enzymes present and then xylanase acts on the xylan chain (Dekker et al., 1985).

After the exposure of cellulose molecules to enzymatic attack, Endo-glucanase (Endo-G) preferably acts on the amorphous part of the cellulose chain (Clarke et al, 1997.) and produces shorter DP cellulose chains. The molecular weight of Endo-G from fungi is in the range of 30-55 KDa. Bacterial Endo-G are little larger. Most of the Endo-glucanases

are glycosylated. Glycosylation is thought to protect the enzyme from proteolytic attack and improves the thermal stability of protein. It also helps in the adsorption of Endo-G into the insoluble cellulose (Langsford et al., 1987; Merivuori et al., 1985; Olden et al., 1985; Chanzy et al., 1984).

Exo-glucanase acts on the cellulose chain from the reducing or non-reducing end progressively and releases cellobiose as the main product. Cellobiohydrolases are larger than the endoglucanase with an average size of 41-85 KDa and are also glycosylated (Clarke et al., 1997).

β-glucosidases hydrolyze the cellobiose and cello-oligosaccharides from the non-reducing ends and form glucose as the end product of cellulose hydrolysis. It also helps in reducing the competitive inhibition of cellobiose (Howell et al.1975). β-glucosidase is the largest of all three cellulolytic enzyme, has moleculer weight from 41-170 KDa and is mostly in the form of di or multimeric form. Like endo-glucanase and exo-glucanase, most of the β-glucosidases are also glycosylated. β-glucosidase has been detected as an intracellular as well as extra-cellular enzyme in fungi like *T.reesei* (Selby et al., 1965; Umile et al., 1986) .

Five endo-glucanases (EG-1 to EG-5), two exo-glucanases (CBH-1 and CBH-2), β-glucosidases and several hemicellulases have been identified by 2-D electrophoresis in the *T.reesei* cellulase complex (Vinzant et al., 2001). Out of these proteins, CBH-1, CBH-2 and EG-1 are three main proteins in *T.reesei* cellulase with the respective compositions of 60%, 20% and 12% (Goyal et al., 1991).

Xylanases are known to catalyze the hydrolysis reaction of β-(1-4) bonds between xylose residue in the xylan chain including heteroxylan and xylo-oligosaccharides (XOS). Affinity as well as reactivity of XOS with xylanase reduces with a decrease in the DP. Few xylanases are very specific towards the xylan molecules, although some are able to hydrolyze the cellulose molecules as well. Reactivity of cellulose molecules with the xylanases is very low in comparison to the xylan, but still, a few xylanases can bind with the cellulose (Fournier et al., 1985; Hall et al., 1989). Acetylation of the xylan molecule reduces the reactivity of xylanase, and product of the reaction of heteroxylan with xylanase would be low DP but larger substituted XOS (Wood et al., 1986). Some xylanases, however can only attack the heteroxylan chain near the substituted site (Frederick et al., 1985). The overall reactivity of xylanase can also be affected by presence of auxiliary debranching enzymes like ά-glucuronidase, ά-arabinofuranosidase, acetylxylan esterase, or ferulic acid esterase. Both fungal and bacterial xylanases have a molecular weight in the range of 10-85 KDa and all of them are single-subunit proteins (Clarke et al.,1997). Xylanases below 30 KDa are mainly basic protein and above 30KDa are acidic proteins with few exceptions (Wong et al., 1988). Similar to β-glucosidases, β-xylosidases catalyze the hydrolysis of xylo-oligosaccharides and xylobiose. The reactivity of these enzymes increases with a decrease in the DP of oligosaccharides (Matuso et al., 1984; Doorslaer et al., 1985). β-xylosidases are much larger proteins than the xylanases and are usually acidic (Clarke et al., 1997).

One of the common features in most of the cellulases and xylanases is the glycosylation with O-link and N-link carbohydrate chain. These glucan associated with

the enzymes are found to contain high amounts of mannose residue (Gum et al., 1976; Salovuori et al., 1987). Glycosylation of cellulolytic and xylanolytic provides the protection from protease action (Langsford et al., 1987), helps in the secretion of enzyme from cell (Willick et al., 1985; Kubicek et al., 1987) and helps in adsorption of enzyme to the insoluble substrate (Rabinovitch et al., 1982). Mannan residue in the glycan chain has affinity for the crystalline cellulose (Chanzy et al., 1982), and protein containing the mannan rich glycan chain has been suggested to have functional role in the binding affinity of protein to the insoluble cellulose (Chanzy et al., 1984).

On the basis of homology, 18 out of 46 distinct families of β-glycosidases comprise the cellulolytic or xylanolytic enzymes. Members of these families have high similarities in amino acid sequence with some differences. For example, family 6 which comprises the endo-glucanase as well as cellobiohydrolase has two subtypes. The catalytic site in these two subtypes is conserved. But in subtype-2, few amino acids form an extended surface loop in the enzyme which prevents this protein from acting as endo-glucanase as in subtype 1. This protein acts as cellobiohydrolase (Gilkes et al., 1991).

Most of the cellulases and xylanases consist two domains known as the cellulose binding domain (CBD) and the catalytic domain. In absence of CBD, the activity of cellulase toward the insoluble cellulose decreases appreciably, but its activity towards the soluble substrate like CMC was not impaired. CBD enhances the ability of the enzyme to bind to insoluble cellulose and hence increase adsorption. CBD consists of a flat hydrophilic side containing a number of conserved amino acids, and three of which are main amino acids. These three amino acids are two Tyr and Gln which are responsible for

tight binding of the CBD with the cellulose molecule. CBM plays an important role in the initiation and progressive action of exo-glucanase on cellulose chain. CBM also helps to separate the cellulose chain fragments from the crystalline bundle and enhances the hydrolysis rate (Clarke et al., 1997; Lynd et al., 2002).

Length of linker peptide which connects the catalytic domain and CBD also affects the catalytic activity of the enzyme. The linker sequences in the cellulases are thought to provide the flexibility to the enzyme molecule during the CBD binding and helps in breaking the hydrogen bonding network in cellulose. Apart from this, the linker region is also thought to provide the stability to the fungal cellulases (Clarke et al., 1997).

Multienzyme cellulase system

Fungal cellulases contain multiple endo-glucanases and exo-glucanases which are different proteins with different molecular weights and structures. This indicates the possibility for the need of multiple endo-glucanases and exo-glucanases to hydrolyze the insoluble cellulose effectively. This looks plausible from the perspective of the stereochemistry of cellulose. Alternate up and down orientation of β (1-4) glucosidic bonds in glucan chain can possibly necessitate the requirement of two types of endo-glucanases and two types of exo-glucanases. Each type would have the substrate specificity for one of β (1-4) glucosidic bond (either up or down). However, microorganisms, producing multiple cellulolytic enzymes at one time, encode for only one of each type of enzyme. So the origin of the multiplicity of enzymes lies at the stage of transcription, as distinct m-RNA directed products for each enzyme type have been

found to exist. One reason for so many enzymatic components might be proteolytic cleavage of the parent protein by extra cellular proteases secreted by the organism which gives rise of many different types of proteins. Another theory explains this multiplicity by non-specific adsorption of components from culture media by a cellulolytic enzyme, as variation in the molecular weight has been observed in the case of EG1 produced by *T.reesei* using different culture media. Similarly, different enzymatic components of same functional protein also differ only by the amount of glycosylation (Clarke et al., 1997).

Synergy in Cellulase

Collective activity of the cellulase enzyme mixture is greater than the sum of the individual components. This phenomenon is known as synergy. Synergistic action of different cellulase components depends upon the ratio of different enzymes in the cellulase and the nature of cellulose. The degree of synergism is very high for highly crystalline cellulose, low for amorphous cellulose and virtually absent with a soluble substrate like CMC. Synergism between CBH-1 and CBH-2 for avicel hydrolysis is explained on the basis of the need of different enzymes for different stereospecific substrates at the reducing and the non-reducing ends of the cellulose chain. Sometimes, purified CBH is not able to hydrolyze the crystalline cellulose, but the addition of endo-glucanase can help in the hydrolysis. On the other hand, addition of CBH doesn't help in the hydrolysis of cellulose by CBH. Physical evidence from electron microscope studies suggests that Endo-G causes swelling followed by erosion of microfibril layers

inside the fibers, while the action of CBH produces deep cracks to lumen structure of cotton fiber (Clarke et al., 1997).

Similar to the cellulolytic enzyme, synergistic action was also found in the xylanases. Different components of xylanase work synergistically with each other, and this synergy can be defined as homeosynergy or heterosynergy. Homeosynergy is the synergy between enzymes working on the main xylan chain or the side chain. Heterosynergy is caused by synergistic action between two enzymes, one working in the side chain and other working in the main chain. In both cases, action of one enzyme paves the way for another enzyme by removing any steric hinderance caused by the side chain group. The role of heterosynergy in the case of xylanolytic enzymes is crucial because of the complex side chain structure of hemicellulose. This has been proved by studies in which the acetyl xylan was found to be effectively hydrolyzed by the mixture of xylanase and β-xylosidase only in the presence of acetylxylan esterase (Clarke et al., 1997; Lynd et al., 2002).

Catalytic mechanism of cellulase

The catalytic mechanisms of the different cellulase enzymes differ based on whether they retain or invert the anomeric configuration of the glycosyl residue. These two types of glycosidases are called retaining type or inverting type β-glycosidases. All cellulolytic and xylanolytic enzymes are classified into various families based on their structure and base pair sequence homology. The enzymes that belong to one particular family either follow the retaining type or the inverting type (Clarke et al., 1997).

In both types of mechanism, one amino acid residue works as a general acid and the other residue works as a general base. The inverting type enzyme follows a single step mechanism in which the general base residue deprotonates the nucleophilic water molecule and the general acid residue protonates the departing glycosidic oxygen. The reaction is thought to proceed via the oxycarbonium ion like transition state which is partly stabilized by the deprotonated general acid group. Retaining type glycosidases form a covalent-glycosyl intermediate in which the anomeric configuration of the intermediate is opposite to that of the substrate. This mechanism also involves the oxicarbonium type of transition state (Clarke et al., 1997).

The participation of acidic amino acid residues is required in both types of mechanism and evidence suggests a role of Aspartyl and Glutamyl residues in cellulolytic and xylanolytic enzymes. Bell-shaped pH dependence of activity also supports the theory of presence of both, an acidic residue which is protonated and a stabilizing anion-nucleophile which is deprotonated (Clarke et al., 1997).

Different strategies for biomass to ethanol conversion process

Saccharification of pretreated biomass is carried out by the cellulase enzyme in the enzymatic hydrolysis step. Sugars produced from the hydrolysis of biomass are Glucose, Galactose, Mannose (C-6 Sugars) and Xylose, Arabinose (C-5 Sugars). Glucose and Xylose are the main C-6 and C-5 sugars respectively. Different level of process integration is shown in Fig. II-3.

When the saccharification and fermentation of biomass is performed in separate reactors, it is called separate hydrolysis and fermentation (SHF). In this type of configuration, a mixture of glucose and xylose is produced in the enzymatic hydrolysis. This first enters into the C-6 sugar fermentation reactor where glucose is converted into ethanol, and unconverted xylose sugar stream proceeds to the C-5 sugar fermentation reactor. In both the fermentation reactors, different organisms are used and require different operating temperatures and pH (Hamelinck et al., 2005).

In the simultaneous saccharification and fermentation (SSF) process, the two steps of enzymatic hydrolysis of biomass and glucose fermentation are combined and performed in one reactor. This is possible because the operating conditions in both type of reaction are comparable. The SSF was first introduced in 1977 and offers several advantages over the SHF process. The advantages of SSF are avoidance of product (Glucose) inhibition for cellulase, low cellulase requirement, higher ethanol yields and the elimination of a separate reactor for hydrolysis which reduces the potential for microbial contamination (Ghosh et al., 1984, Grohmann et al., 1993). Left over stream from the SSF reactor contains xylose sugars which can be further converted into ethanol in a separate reactor (Lynd et al., 1996).

Simultaneous saccharification and co-fermentation (SSCF) is one step ahead of SSF where xylose fermentation is also carried out in the same reactor with biomass hydrolysis and glucose fermentation. Here the glucose and xylose sugars are simultaneously converted to ethanol by a single microorganism. This method has significant advantages over the SSF. As xylose constitutes a major portion of the total

polysaccharides, its efficient conversion is very important for a high yield of ethanol on the basis of a dry biomass. A low concentration of xylose in the SSCF reaction facilitates the xylan to xylose conversion by eliminating the end product (xylose) inhibition of xylanase components of cellulase (Clarke et al., 1997). This helps to increase total hydrolysis rate of biomass and ethanol yield.

Consolidated BioProcessing (CBP) is a concept where all the steps, enzyme production, enzymatic hydrolysis and fermentation, are carried out in one reactor with the use of single microorganism or group of microorganisms operating at the same conditions. The microorganism community used in the reactor produces enzymes required for all the steps in the biomass to ethanol process (Lynd et al.,1996). It was projected that CBP can reduce the cost of biological conversion by more than 4 times that of SCCF. Two strategies can be adopted for engineering the microorganisms for this purpose. First, naturally occurring cellulolytic microorganism can be engineered for expressing the enzymes required for ethanol production. Second, ethanol producing organisms can be improved genetically to express the cellulase system (Lynd et al., 2005).

Microorganisms used in the bioethanol process

As described earlier, the fermentation of sugars obtained from biomass is the final step in the bioprocess for ethanol production. The criteria for an efficient organism in the ethanol production process are high ethanol productivity, reduction of growth factors, high product and inhibitors tolerance.

Yeast *Saccharomyces cerevisiae* is used commercially to produce the ethanol from starch and sucrose. The wild type strain of *S.cerevisiae* can only ferment the glucose derived from the hydrolysis of lignocellulosic feedstock and it is commonly used in the SSF process. *S.cerevisiae* can produce ethanol with a maximum concentration of 18% in the fermentation broth (Lin et al., 2005). This microorganism utilizes the glucose by the Embden-Meyerhof Pathway (EM). The strains of *Saccharomyces* species have been developed to consume the xylose as well. Yeast *Pichia stipitis* can naturally ferment the glucose, xylose, galactose and mannose (Jeffries et al., 2004; Sedlak et al., 2004). Other yeast species which can produce ethanol are *K.fragilis, K. marxianus, Candida utilis* and *Pachysolen tannophilus* (Lin et al., 2005).

The bacteria *Zymomonas* use the Entner-Doudoroff (ED) pathway to consume the glucose for ethanol production. Because of the low ATP requirement in the ED pathway than in the EM pathway, more glucose is consumed and a higher yield of ethanol is obtained from *Zymomonas* (Sprenger et al., 1996).

Zymomonas mobilis is a gram-negative bacteria which gives 5-10% more ethanol per fermented glucose than the yeast. *Zymomonas mobilis* can only ferment glucose, fructose and sucrose and not well suited for all the biomass because it can not consume galactose/mannose. Because of this limitation, *Z.mobilis* is not appropriate for softwood substrates. This microorganism utilizes the homoethanol pathway and tolerates up to 120 g/l ethanol. NREL engineered the *Zymomonas mobilis* for xylose and other pentose sugar utilization which can tolerate up to 10% of ethanol concentration (Service et al., 2007).

Successful generation of the *E.Coli* strain for ethanol production was one of first successful application of metabolic engineering. *E.coli* can consume variety of sugars and does not require the complex growth media but has very narrow operable range of pH(6-8) and is less sturdy than yeast. *E.coli* (KO11) was reengineered for consumption of pentose sugar to produce ethanol by Ingram et al. KO11 could convert 90-95% sugars in biomass into ethanol but has very low tolerance of ethanol (4-6.5%) (Ingram et al., 1987; Service et al., 2007; Lin et al., 2005). *E.coli* has a higher optimal temperature than other known strains of bacteria.

In addition to E.coli, Ingram et al. have also worked with gram negative bacteria *K.oxytoca* and *E.chrysanthemi* for genetic transformation. *K.oxytoca* can grow in a low pH of 5 and a low temperature of 35°C. This organism can also consume cellobiose and cellotriose in addition to the hexoses and pentoses. 90% of the total fermentation products by Strain M5A1 of *K.oxytoca* is ethanol (Dien et al., 2003).

The sensitivity of different strains for various inhibitors is also a big concern in the fermentation process. *Z. mobilis* is very sensitive to acetic acid (Dien et al., 2003). For direct conversion of cellulose to ethanol in CBP scheme, different microorganism are used such as *Clostridium thermocellum, Monilia sp., Neurospora crassa, Neurospora sp., Zygosaccharomyces rouxii, Aspergillus sp.,Trichoderma viride, Paecilomyces sp.*. The fermentation of cellulose by these organisms is rather slow and results in lower ethanol yield. The organisms also produce several byproducts such as acetic acid and lactic acid which work as inhibitors (Lin et al., 2005).

Alkaline degradation of biomass

Hydrolysable ether linkages in lignin and glycosidic bonds in polysaccharides are the main sites for alkaline catalyzed degradation of wood. Whether it is the pulping process or pretreatment for ethanol production, carbohydrate degradation is always undesirable and the selectivity of degradation towards lignin is favorable. The reaction of hemicellulose with alkali also causes a alkali requirement because of the neutralization effect of released acids.

Endwise degradation or peeling reaction in the carbohydrates under alkaline conditions is the dominant reaction. It starts from the reducing ends. The peeling reaction of cellulose depolymerizes one glycosyl residue which is converted into some acidic moiety (isosaccharinic acid or lactic acid), and the remaining chain again goes through same type of reaction until a stable end is formed consisting of an acidic group. The main acids are metasaccharinic acid and 2-C methylglyceric acid (Johansson et al., 1974).

The extent of endwise degradation is controlled by two competing reactions, peeling and stopping. The comparative rate of these reactions depends upon the nature of the substrate, the process conditions and the reagent.

In cellulose, aside from chemical stopping, the physical stopping reaction is also prevalent due to the inaccessibility of the degrading end. The reason is the crystalline supra-molecular structure of cellulose. Glucan loss, as a result of the peeling reaction, depends upon the average length of the accessible segment located at the amorphous-crystalline region. Below a particular NaOH concentration (< 2 M NaOH), a number of peeled off glucose units are independent of the reaction temperature and alkaline

concentration. The value of peeled off glycosyl units is approximately 68 per reducing end for the native cellulose and 40 for mercerized cellulose (Haas et al., 1967).

Cleavage of glycosidic linkages also occurs during the alkaline degradation. The reactivity of different type of sugars for glycosidic cleavage is different and follows the order: glucuronic acid>arabinose>xylose>glucose>mannose>galactose. This trend is completely different from acidic hydrolysis. The activation energy of the gycosidic bond cleavage (36-38 Kcal/mol) is higher than that of the peeling reaction (21.2-24.6 Kcal/mol). This indicates that at a higher temperature, less cleavage but more peeling reaction would occur. In the temperature range of 150-170°C, the peeling reaction in cellulose is 10^7 times faster than the alkaline hydrolysis of glycosidic linkage. Alkaline degradation through the glycosidic bond cleavage would have a more pronounced effect on DP and pulp viscosity than the peeling degradation. The activation energy for alkaline degradation of cellulose in wood is even higher than for cotton cellulose (43Kcal/mol). Glycosidic cleavage reaction in mercerized cellulose is 70% higher than native cellulose because of higher accessibility (Lai et al, 2001).

Many physical and chemical changes occur in hemicellulose during alkaline degradation such as swelling, dissolution, saponification, reprecipitation, peeling and glycosidic cleavage reactions. As indicated in the order of reactivity above, the side chain of galactoglucomannans is fairly resistant to alkaline degradation. Contrary to the reactivity trend, glucomannans are more reactive than xylans. The rate of the peeling reaction in hemicellulose can be considerably different depending upon its structure. The presence of the 4-O-methylglucuronic acid group substituted at the C-2 position of xylan

end group retard the peeling process. But at moderate to high temperatures, the degradation of this stable group occurs by β-elimination process due to the presence of carboxylic group (Lai et al, 2001).

During the alkaline degradation of lignin, the cleavage of α and β aryl ether linkages are the main reactions. α-ether linkages are easily cleaved under the mild alkaline conditions in lignin but these linkages in lignin carbohydrate complex are stable under same conditions. The number of β-ether cleavage is considerably higher in kraft pulping (0.1M NaOH + O.015 Na2S) than soda pulping (0.1M NaOH). Alkaline cleavage is shown to be proportional to the alkali concentration in certain range (0.1M-1M) and then levels off. The reactivity of syringyl units is very high with NaOH for β-ether cleavage. The reactivity of β-ether linkages toward alkaline degradation greatly depends upon the R group located at the γ position in phenolic units. C-C bond cleavage occurs strictly in the β-γ linkage yield to formaldehyde and enol ether (Lai et al, 2001).

FIGURES:

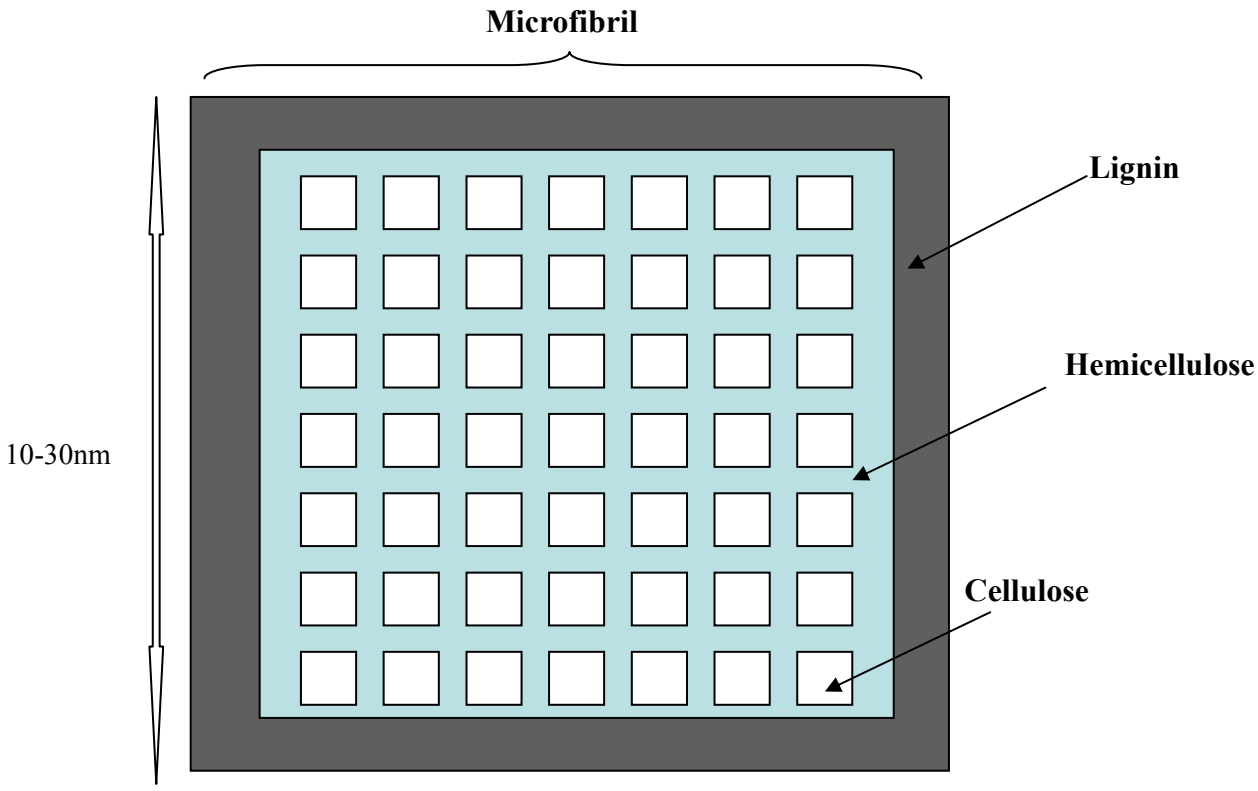


Fig. II-1. Schematic presentation of Cellulose microfibril (Zhang et al.,2004)

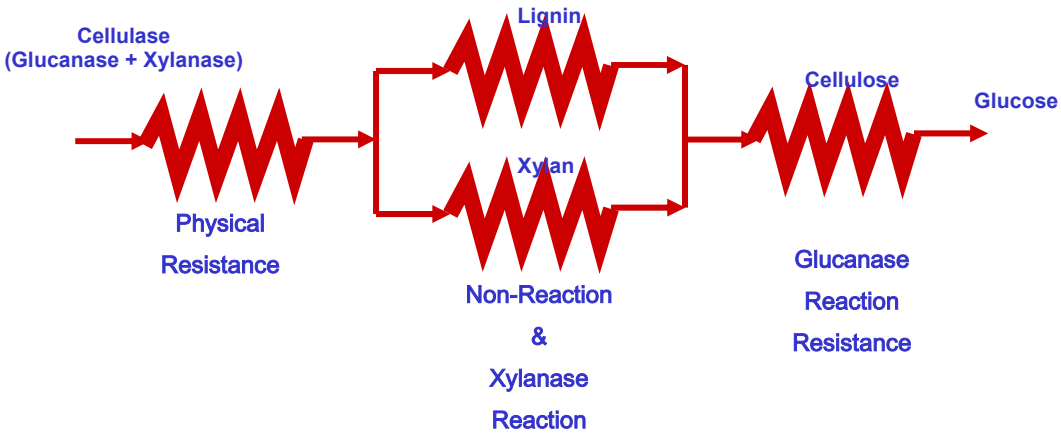
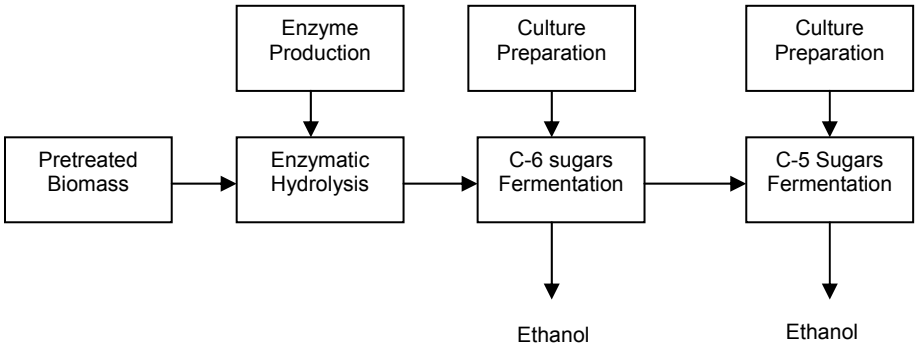
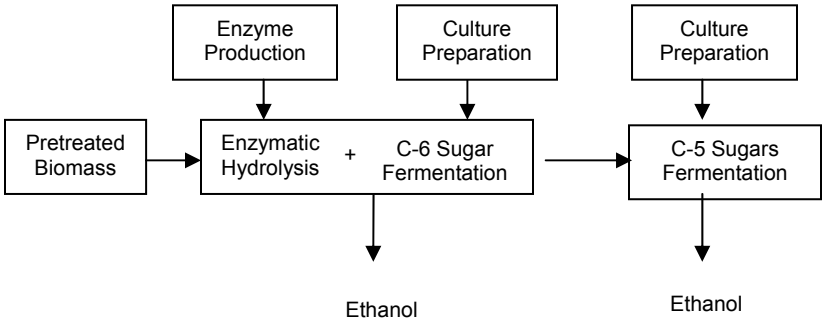


Fig. II-2. Resistances for the cellulose hydrolysis

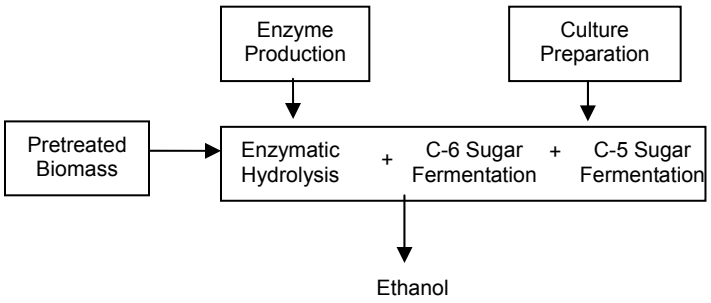
Separate Hydrolysis and Fermentation (SHF)



Simultaneous Saccharification and Fermentation (SSF)



Simultaneous Saccharification and Co-Fermentation (SSCF)



Consolidated Bio-Processing CBP)

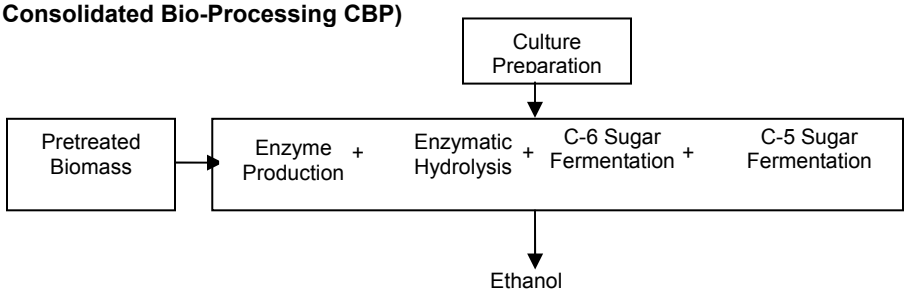


Fig. II-3. Process strategies for Bioethanol Production (Hamelinck et al., 2005)

III. SUBSTRATE DEPENDENCY AND EFFECT OF XYLANASE SUPPLEMENTATION ON ENZYMATIC HYDROLYSIS OF AMMONIA TREATED BIOMASS

ABSTRACT:

Pretreatment based on aqueous ammonia was investigated under two different modes of operation: soaking in aqueous ammonia (SAA) and ammonia recycle percolation (ARP). These processes were applied to three different feedstocks with varied composition: corn stover, high lignin (HL), and low lignin (LL) hybrid poplars. One of the important features of ammonia-based pretreatment is that most of the hemicellulose is retained after treatment, which simplifies the overall bioconversion process and enhances the conversion efficiency. The pretreatment processes were optimized for these feedstocks taking carbohydrate retention as well as sugar yield in consideration. The data indicate that hybrid poplar is more difficult to treat than corn stover, thus requires more severe conditions. On the other hand, hybrid poplar has a beneficial property that it retains most of hemicellulose after pretreatment. In order to enhance the digestibility of ammonia treated poplars, xylanase was supplemented during enzymatic hydrolysis. Due to high retention of hemicellulose in treated hybrid poplar, xylanase supplementation significantly improved xylan as well as glucan digestibility. Of the three feedstocks, best results and highest improvement by xylanase addition was observed with LL hybrid

poplar showing 90% of overall sugar yield.

Keywords: Hybrid poplar; corn stover; xylanase; aqueous ammonia; pretreatment

INTRODUCTION:

Various pretreatment technologies are being used to improve the digestibility of carbohydrates in biomass by making cellulosic part more accessible by enzymes (Zhang et al., 2004). Various pretreatment technologies are being investigated to achieve this goal. Among the major findings of recent pretreatment research is that a given pretreatment reagent exhibits selectivity towards a certain type of reaction attacking specific chemical bonds in lignin-hemicellulose-cellulose matrix. Pretreatment methods operated at low pH including steam explosion (Fernandez-Bolanos et al., 1999; Schwald et al., 1988) , hot water treatment(Allen et al., 2001; Garrote et al., 2002; Vaquez et al., 2001), controlled pH treatment & dilute acid treatment(Burns et al., 1989; Jacobsen et al., 2000) remove substantial amount of hemicellulose. On the other hand, pretreatment methods applying high pH such as AFEX (Dale et al., 1986; Dale et al., 1996; Foster et al., 2001), lime pretreatment (Chang et al., 1997; Chang et al., 2000) and ARP (Iyer et al., 1996; Kim et al., 1996) show little effect on hemicellulose, but high interaction with lignin. In low pH pretreatments, the liquid contains hemicellulose sugars and other degradation byproducts. These byproducts are known to be inhibitory in subsequent bioconversion process. The examples of the inhibitory compounds are phenolics derived from lignin degradation, furan derivatives (HMF and furfural) from sugar decomposition, and aliphatic acids (Palmqvist et al., 2000). Sugar-lignin condensation reactions in pretreatment liquid

further reduces the sugar yield (Yang et al., 2004; Kim et al., 2001; Qian et al., 2004; Negro et al., 2003; Chua et al., 1979). Glucose/xylose co-fermenting microorganisms were found to be very sensitive to these inhibitory chemicals (Du Preez et al., 1994; Hahn-Hagerdal et al., 1994). Various methods have been used to detoxify these toxins (Palmqvist et al., 2000), but it bears substantial additional cost. From this viewpoint, hemicellulose retention is a positive feature in pretreatment because it eliminates the need of converting sugars in pretreatment liquid (Mosier et al.,2005).

In most of the pretreatment studies, process conditions of pretreatment are optimized in terms of glucan digestibility. One of the factors that limit the digestibility of pretreated biomass is insufficient xylanase activity in "cellulase" (Wood et al., 1986) . This is particularly true for substrates with high xylan content. According to Berlin et al., the hydrolysis rate of different organosolv treated hardwood did not show any correlation with the filter paper activity of different enzyme preparations, yet showed significant correlation with the endogenous xylanase activity (Berlin et al., 2006). Covalent and non-covalent association of xylan with cellulose and lignin is an essential factor for holding the structural integrity of cell wall (Thomson et al., 1993). Removal of xylan either by pretreatment or by xylanase would enhance enzymatic hydrolysis of cellulose. Xylanase not only degrade xylan but also assist in delignification as observed in bio-bleaching of pulp (Saha et al., 2003).

In Fig.III-1, lignin vs xylan content of various biomass feedstocks is shown (www.eere.energy.gov). The data indicate that there is an inverse relationship between lignin and xylan content of biomass. These feedstocks were divided into three different

categories: region 1, 2 & 3 represent the feedstocks with high xylan & low lignin, low xylan & high lignin and moderate xylan & moderate lignin respectively. In this study, feedstocks representing the three regions were selected; corn stover, HL hybrid poplar, LL hybrid poplar. Recent work in our laboratory has proven that SAA and ARP are very efficient for delignification of corn stover retaining high amount of hemicellulose (Kim et al., 2005; Kim et al., 2003; Kim et al., 2001).

The objective of this study is to apply pretreatments based on aqueous ammonia on feedstocks of varying physical and structural properties and seek insights on how the pretreatment effects are influenced by definable parameters. The optimum conditions were defined for different feedstocks and the investigation was focused on the pretreatment region near optimum condition. Special attention was paid to the role of xylan and its removal by external xylanase in the enzymatic hydrolysis of biomass.

MATERIALS AND METHODS:

Substrates and Reagents:

Corn stover was supplied by NREL. It was ground, screened, and the fraction collected between 9 and 35mesh was used in all experiments. Two different batches of hybrid poplar chips (1/4") were supplied by NREL. Moisture content of both the hybrid poplar batches was approximately 50%. Avicel PH-101 was purchased from the Sigma (Cat. No.11365 and Lot No. 1094627). Ammonium hydroxide of 30 wt % was purchased from Fisher-Scientific In all experiment, 15% ammonium hydroxide solution was used.

Enzymes:

Cellulase enzyme (Spezyme-CP) and Xylanase (Multifect xylanase) were kind gifts of Genencor International Inc. (Paulo Alto, CA). Two different batches of Spezyme-CP were used in this study: Spezyme CP-A (lot no. 301-00348-257) and Spezyme CP-B (lot no. 301-04075-054). The nominal filter paper activities for Spezyme CP-A and Spezyme CP-B were 31.2FPU/ml (determined by NREL) and 59FPU/ml (determined by Genencor) respectively. Spezyme CP-B was used in ARP treated samples and Spezyme CP-A was used in SAA treated samples. The protein content of Multifect xylanase was 42mg of protein/ml. β-Glucosidase was purchased from Sigma (Novozyme 188 from Novo Inc., Sigma No. C-6150 and Lot No. 11K1088). The specified activity as determined in our lab was 750CBU/ml.

Experimental set up and operation of ARP:

The ARP experimental system employs a packed-bed column reactor through which the pretreatment liquid flows. Details of reactor set up are described elsewhere (Kim et al., 2003). ARP experiments were conducted under the following optimum set of conditions chosen for each feedstocks:

Corn stover: The temperature of 170°C was used with 20 minutes of reaction time (10 minutes with ammonia solution followed by 10 minutes with DI water) and 5ml/min of liquid flow rate. The overall solid-to- liquid (15% ammonia) ratio was 1:3.33 (w/w). The conditions for corn stover were chosen on the basis of our previous study (Kim et al., 2003).

LL and HL hybrid poplar: Substrate was first soaked overnight in 15% ammonia solution with solid to liquid ratio of 1:3.67 (w/w) before ARP experiment. Temperature of reactor containing the soaked biomass was raised to 185°C and DI water was passed for 27.5 min with 2 ml/min flow rate. The temperature of the reactor during the reaction remained constant. Unless stated otherwise, all the ARP pretreatment experiments were conducted under these conditions**.**

It was found that overnight soaking of HL hybrid poplar prior to ARP treatment does not affect the hydrolysis of treated biomass much. So in other experiments, where effect of different process variable in ARP process was studied (Fig.III-5(a) to Fig. III-6(c)), HL hybrid poplar was soaked for only 10min before starting the pretreatment**.** For determination of optimum conditions for HL hybrid poplar, the upper limit of temperature was set at 195°C, above which severe decomposition of carbohydrates were observed and the system pressure rose to 450 psig. These conditions were applied for both HL and LL hybrid poplar feedstocks.

Experimental set up and operation of SAA:

Batch reactors were used for the SAA pretreatment. For low temperature operation up to 80°C, biomass was soaked with 15% ammonia in screw capped laboratory glass bottle and kept in an oven. For runs above 80°C, stainless steel reactors (1.375”IDx6”L) were used**.** For corn stover, 60°C temperature was used with 12hrs soaking time and 1:8 solid to liquid ratio. For HL and LL hybrid Poplar, the temperature of 150°C was employed with 24hrs soaking time and 1:10 solid to liquid ratio. Apart

from high pressure in the reactor, there can be increase in the liquid volume upto 10% (based upon water) of initial volume due to rise in temperature to 150°C. Owing to this reason, reactor should only be filled maximum upto 75% of total reactor volume.

Enzymatic digestibility:

Enzymatic digestibility tests were done with 1.0 %w/w glucan loading. Thus total amount of solids in the reactor varied according to the glucan content in biomass. The reaction of enzymatic digestibility was carried out in 250ml Erlenmeyer flask with total liquid volume of 100ml. This test was carried out according to the NREL chemical analysis and test (CAT): LAP No. 009 (NREL,1996) (www.eere.energy.gov/biomass/analytical_procedures.html). Maximum expected variation in digestibility values is within 3% of the reported values.

Cellulase loading of 15FPU/g glucan and β-glucosidase loading of 30CBU/g glucan are used in all of the enzymatic digestibility experiments. Unless noted otherwise, xylanase loading was 31.5mg protein/g glucan in the hydrolysis experiments with xylanase supplementation. The glucan or xylan digestibility was defined as the percentage of theoretical glucose or xylan released after 72 h of incubation with enzyme.

Analytical procedures:

Composition analysis of the treated and untreated biomass was done according to the NREL Laboratory Analytical Procedure: “Preparation of samples for compositional analysis” and “Determination of structural carbohydrates and lignin in biomass”

(eere.energy.gov/biomass/analytical_procedures.html). The moisture content in biomass was measured by an infrared moisture balance (Denver Instrument, IR-30). Sugar contents in compositional analysis and enzymatic digestibility were determined by HPLC using a Bio-Rad Aminex HPX-87P. Sugar analysis was carried out in duplicate for each sample and average values are reported.

Crystallinity index:

Crystallinity of treated & untreated hybrid poplar feedstock were measured by X-ray diffractometer (Rigaku DMAX). Cu-Kα radiation was generated at 40kV and 40mA. Samples were scanned from 2θ = 10°-40° with 0.01° increment. The following formula has been used for calculation of crystallinity index of samples (Cao et al., 2005):

$CrI = (I_{002} - I_{am}/I_{002}) \times 100$,

where, I_{002} is peak intensity corresponding to 002 lattice plane of cellulose molecule observed at 2θ equal to 22.5° , and I_{am} (at 2θ =19°) is peak intensity corresponding to amorphous cellulose.

SEM

Microscope pictures were taken using the Field Emission Scanning Electron Microscope (JEOL JSM-7000F) with magnification of 300 and 350 for corn stover and hybrid poplar, respectively.

RESULTS AND DISCUSSION

Observation of feedstocks

Fig. III-2 shows the composition of corn stover, LL hybrid poplar and HL hybrid poplar. Moving from Corn stover to HL hybrid poplar, lignin content is increasing and xylan content is decreasing. Among these three feedstocks, LL hybrid poplar is highest in carbohydrates.

Fig. III-3 shows the SEM pictures of the three feedstocks. The surface of HL hybrid poplar and LL hybrid poplar look very much similar but surface of corn stover shows more open structure, which would allow more adsorption capacity for pretreatment reagent into the solid hence higher reactivity. Diffusivity of pretreatment reagent in agriculture residues was found to be much higher than the hardwood (Kim et al., 2002). The surface characteristics of biomass are one of the important factors affecting the global reactivity of pretreatment reagent with biomass.

Effect of ARP pretreatment

Xylan and lignin removal by the ARP treatment are shown in Fig. III-4(a) & (b). The glucan digestibility of ARP treated feedstocks is presented in Fig. III-4(d). The optimum conditions described earlier were applied in these tests. The extent of delignification and xylan removal in corn stover is much higher than hybrid poplar. With corn stover, more than 50% xylan is lost to liquid. The glucan digestibility of ARP treated corn stover with enzyme loading of 15FPU cellulase and 30CBU b-glucosidase reached 90%. Xylan removal in LL and HL hybrid poplar were 22% and 33%, recpectively. The

glucan digestibilities of ARP treated LL and HL hybrid poplar were 66% and 49%, respectively.

Effects of ARP process variables on hybrid poplar

The effect of reaction time on the performance of ARP pretreatment with the HL hybrid poplar is shown in Fig. III-5(b). Lignin removal of hybrid poplar increases with reaction time. Xylan removal, however, was only slightly affected by ARP reaction time. After a certain point the xylan content remained constant indicating that there is a portion of xylan that is resilient and difficult to remove. The 72-hr glucan and xylan digestibilities correlate more closely with xylan removal than lignin removal (Fig. III-6 (b)).

These results are in line with the findings of Kim et al. that aqueous ammonia is highly effective in delignifying lignocellulosic biomass (Kim et al., 2003). Reactivity of ammonia with hemicellulose appears to decline after removal of certain fraction of hemicellulose from biomass. The reasons for this are found in previous studies concerning mechanistic studies. Alkaline degradation of carbohydrate during the Kraft pulping occurs from the reducing end and the reaction of alkali with the carbohydrate chain stops when reducing end (-OH) is replaced by alkali stable end (-COONa) (Stenius et al., 2000). The same mechanism may apply to ammonia treatment. It has also been reported that the reducing ends of xylans consist of combination of xylose and galacturonic acid with the sequence of β-D-Xylp-1-4- β-D-Xylp-1-3- α-L-Rhap-1-2- α-D-GalpU-1-4- β-D-Xyl. When xylose is removed from the reducing end, galacturonic acid

makes the xylan molecule more stable toward alkali degradation. (Ericson et al., 1977; Andersson et al., 1978). Vian et al. and Reis et al. have provided evidences for tight association of glucuronoxylans with cellulose microfibril (Vian et al., 1986; Reis et al.,1991). Mora et al. have proven that there is a strong interaction and retention of heteroxylans with cellulose microfibril even after a strong alkaline treatment such as Kraft pulping (Mora et al., 1986). These findings suggest that the hemicellulose bound with the cellulose microfibril is difficult to remove. Among the three feedstocks, xylan loss during the ARP pretreatment is found to be inversely proportional to the cellulose content in the biomass. One plausible explanation for this is that the hemicellulose-cellulose linkage makes the hemicellulose stable, thus making it less amenable for ammonia induced degradation. Corn stover having less cellulose content possesses fewer cellulose-hemicellulose linkages than poplar. This may explain that corn stover loses more xylan than hybrid poplar during ARP pretreatment.

Sugars in ARP liquid exist only in oligomer form, i.e., xylo-oligosaccharides (XOS). After acid hydrolysis of the ARP liquor, large amount of xylose monomer appears. It proves that hemicellulose sugars in the ARP liquor is either in the form of xylo-oligosaccharides (XOS) or lignin carbohydrate complex (LCC). Hemicellulose removal in ARP is proportional to the extent of lignin removal. The reason for this is unclear at this time.

Fig. III-5(c) and III-6(c) show the effect of ammonia flow rate on the composition and digestibility. It is reaffirmed here that the digestibility correlates better with xylan removal than lignin removal, especially for hybrid poplar. This also indicates that

delignification alone is not sufficient to attain high digestibility and that xylan removal is also required. Effects of ARP temperature on pretreatment of HL hybrid poplar are shown in Fig. III-5(a) and III-6(a). Delignification or xylan removal was not significantly affected by the ARP temperature, staying relatively constant over the range of 170°C – 195°C. However, there was a significant effect on glucan digestibility increasing from 27% to 52% as the temperature was raised from 170°C to 195°C.

This indicates that accessibility of enzymes to cellulose can be increased without significant change in composition. It is most likely due to breakage of certain bonds and reconfiguration of components in lignin-hemicellulose matrix. Possibility exists that the ARP can be operated in such a way that digestibility is enhanced without further loss of carbohydrates. This claim may be limited to highly recalcitrant substrates such as hybrid poplar. Retention of carbohydrate in the solid is a desirable trait in pretreatment technology and hybrid poplar appears to be a substrate well-suited for this purpose within the context of ARP.

Effect of process variables on crystallinity

As indicated in Fig. III-7(a) & (b), crystallinity of HL hybrid poplar increases after the ARP pretreatment. These results are in accordance with the findings of Kim et al. that total content of crystalline cellulose increases due to removal of amorphous part (Lignin and Hemicellulose), and that contributes to increase of Crystallinity (Kim et al., 2003). Cao et al. also observed that crystallinity of treated pulp increases due to removal of amorphous part by enzymatic action (Cao et al., 2005). Fig. III-7(a) shows that the

crystallinity increases with reaction time since removal of lignin and hemicellulose also increases. Further increase of ARP temperature from 175°C to 195°C caused slight decrease in Crystallinity (Fig. III-7(b)). In this case, the decrease of crystallinity is due to the structural change in cellulose rather than composition. With ARP operated below 185°C, the basic structure of cellulose is not altered regardless of reaction time, only removal of hemicellulose and lignin occurs.

Effect of xylanase supplementation on the ARP treated biomass

The effects of xylanase supplementation on glucan /xylan digestibility with ARP treated feedstocks are summarized in Fig. III-8 & Fig. III-9. In all cases, digestibility increased significantly after xylanase supplementation. The increase was highest with LL hybrid poplar and lowest with corn stover. Glucan and xylan digestibility of treated LL hybrid poplar approached 100% after xylanase supplementation. In Fig. III-10, the digestibilities of pure cellulose (Avicel) with and without xylanase addition are shown. The fact that there is no difference between them proves that the xylanase enzyme does not have any cellulase activity. This further proves that xylanase addition increases the accessibility of cellulase enzymes to cellulose chains by removing the hemicellulose barrier.

ARP of corn stover removes 70% of lignin and 50% of hemicellulose. Therefore, a large part of the hindrance for cellulase action has been removed and high digestibility is attained without xylanase addition. On the other hand, ARP of LL hybrid poplar removes only 32% of lignin and 25% of xylan, consequently glucan digestibility of only

65% is obtained. When supplemented with xylanase, a drastic improvement of the digestibility was seen with LL hybrid poplar reaching 97%. It has been reported that cellulase availability to cellulose is reduced due to unproductive binding of cellulase to lignin (Yang et. al., 2004; Lu et al., 2002). Hydrophobic interaction of surfactants with lignin reduces the unproductive binding of cellulase with lignin. It decreases the effect of hydrophobic environment caused by lignin, thus increasing the access of water and cellulase molecule to cellulose (Eriksson et. al., 2002). Despite these evidences, lignin may not be the overriding factor in cellulase action. Increase of digestibility to near quantitative level under the presence of high amount of lignin as seen here with LL hybrid poplar indicate that high level of lignin removal is not a necessary condition to achieve high digestibility. In the case of LL poplar, the main barrier to hydrolysis appears to be hemicellulose rather than lignin. This is not to say that lignin is not a major factor controlling digestibility. Lignin needs to be removed, but only to a certain extent, to achieve accessibility to carbohydrates. Kanda et al. reported that xylan molecule also binds with endo-glucanase active-site, and the Km value of endo-glucanase with xylan is greater than with cellulose by factor of 1.6 but Vmax value for xylan is only 18% than for cellulose substrate (Kanda et. al., 1976). This suggests the xylan acts as a competitive inhibitor to endo-glucanase.

ARP of HL hybrid poplar removes 40% lignin and 32% of xylan, which is higher than LL hybrid poplar. Yet the lignin content in the ARP treated HL hybrid poplar is higher than treated LL hybrid poplar (Fig. III-4(c)). With similar amount xylanase supplementation, high level of digestibility was not achieved with treated HL hybrid

poplar. In the case of HL hybrid poplar, lignin is still a deciding factor for enzyme accessibility to carbohydrate. Glucan digestibility increases in proportion with amount of xylanase addition for both corn stover and LL hybrid poplar. For HL hybrid poplar, the effect of xylanase addition is significant only with higher xylanase loading (Fig. III-11(a)).Unproductive binding with lignin may be the reason because HL hybrid poplar contain higher amount of residual lignin. Lower xylan digestibility of HL hybrid poplar in comparison to LL hybrid poplar with xylanase supplementation (Fig. III-11 (b)) also supports this.

SAA pretreatment

Our previous work on the SAA using corn stover provided satisfactory digestibility. Corn stover treated by SAA under moderate process conditions (60°C, 1:8 S:L ratio, and 12 hrs), gave approximately 90% glucan digestibility. In the case of hybrid poplar, higher severity conditions (150^{o}C, 24 hrs) were required to attain the acceptable level of digestibility. Even at the temperature as high as 150°C, glucan digestibility of SAA treated LL hybrid poplar and HL hybrid poplar was only 73% and 60%, respectively, with 15FPU cellulase loading.

Composition and digestibility of the SAA treated feedstocks are summarized in Fig. III-12 (a)-(c). SAA of corn stover removes 65% of lignin, yet retains 85% of xylan in solid. In case of hybrid poplar, negligible amount of xylan is lost to liquid and delignification is much less than corn stover. Effectiveness of pretreatment is assessed with the two criteria, first, the retention of carbohydrates in solid, and second, the

enzymatic digestibility of pretreated biomass. According to first criterion, SAA is quite effective, retaining above 90% of xylan in hybrid poplar. The enzymatic digestibility of SAA treated hybrid poplar, however, is not in acceptable range. With xylanase supplementation, the digestibility rose to 94% and 86% for LL and HL hybrid poplar respectively (Fig. III-13), meeting the second criterion as well. As is the case with the ARP, the glucan/xylan digestibilities with xylanase addition are higher for LL hybrid poplar than HL hybrid poplar.

CONCLUSION:

In ARP pretreatment of hybrid poplar, delignification is increased with the treatment severity but xylan removal occurs only to a certain extent. Increase of temperature from 175^{o}C to 195^{o}C in ARP does not affect xylan /lignin removal in hybrid poplar, yet the glucan digestibility of treated solid is increased. This is due to increase of cellulase accessibility to cellulose created by breakage of certain bonds and reconfiguration of the components in hemicellulose-lignin matrix. Crystallinity index of hybrid poplar increases after the ARP treatment. It is primarily due to removal of amorphous components i.e. hemicellulose and lignin.

Xylanase supplementation in enzymatic hydrolysis is effective for the substrates with high xylan content. Xylanase supplementation not only increases the xylan digestibility of treated biomass but also the glucan digestibility. Xylanase reduces the hindrance caused by resilient hemicellulose layer on the cellulose microfibril, thus improving cellulase accessibility to the cellulosic part.

Addition of external xylanase improves the digestibility of ARP treated hybrid poplar. The increase of digestibility is higher with LL hybrid poplar than with HL hybrid poplar. Higher amount of residual lignin in HL hybrid poplar is a plausible cause of it. Lignin is the primary barrier in enzymatic hydrolysis of biomass. A certain degree of lignin removal is, therefore, necessary to facilitate enzyme access to carbohydrates in treated biomass.

In SAA treatment, higher amount of hemicellulose is retained than in ARP because of lower severity of SAA. The SAA is more effective for LL hybrid poplar than HL hybrid poplar.

Performance of ARP and SAA treatments are summarized in Tables III-1 and III-2. In both ARP and SAA, LL hybrid poplar showed the best results. The overall sugar yield from LL hybrid poplar reached 90% (56g of fermentable sugar/100g of feed stock). It was achieved with xylanase supplementation. High retention of carbohydrate in the solid after pretreatment and added xylanase activity in enzymatic hydrolysis are two contributing factors for high overall sugar yield. High reactivity of aqueous ammonia with lignin over carbohydrate is the primary reason for retention of hemicellulose in ARP and SAA.

FIGURES AND TABLES

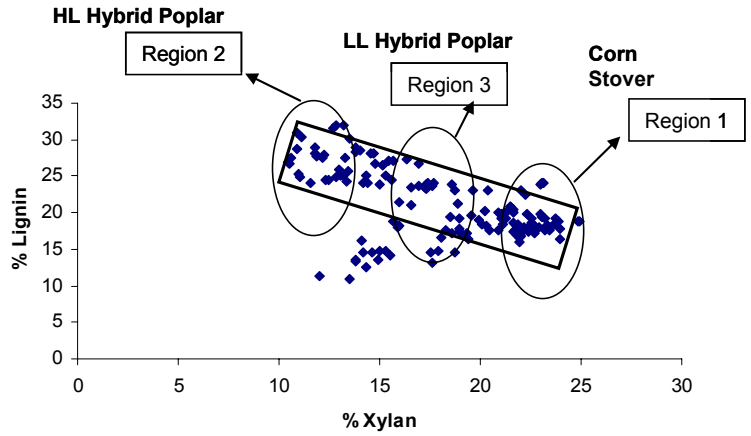


Fig. III-1. Relation between lignin and xylan content in different biomass
(Courtesy: US-DOE website)
http://www.eere.energy.gov/biomass/progs/search1.cgi

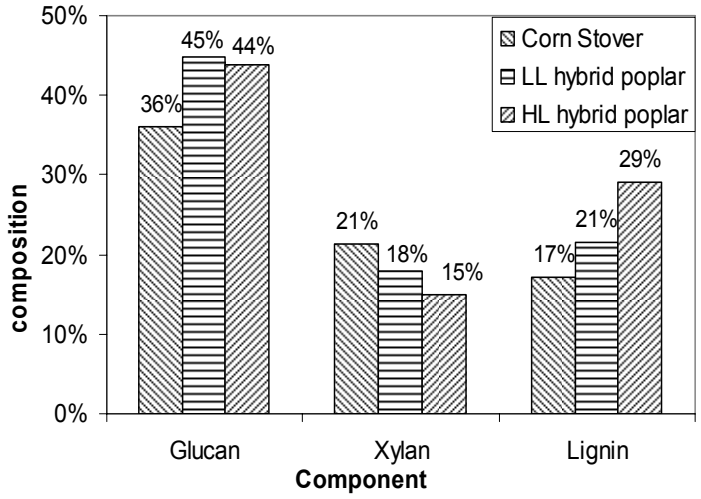


Fig. III-2. Comparison of feedstock composition

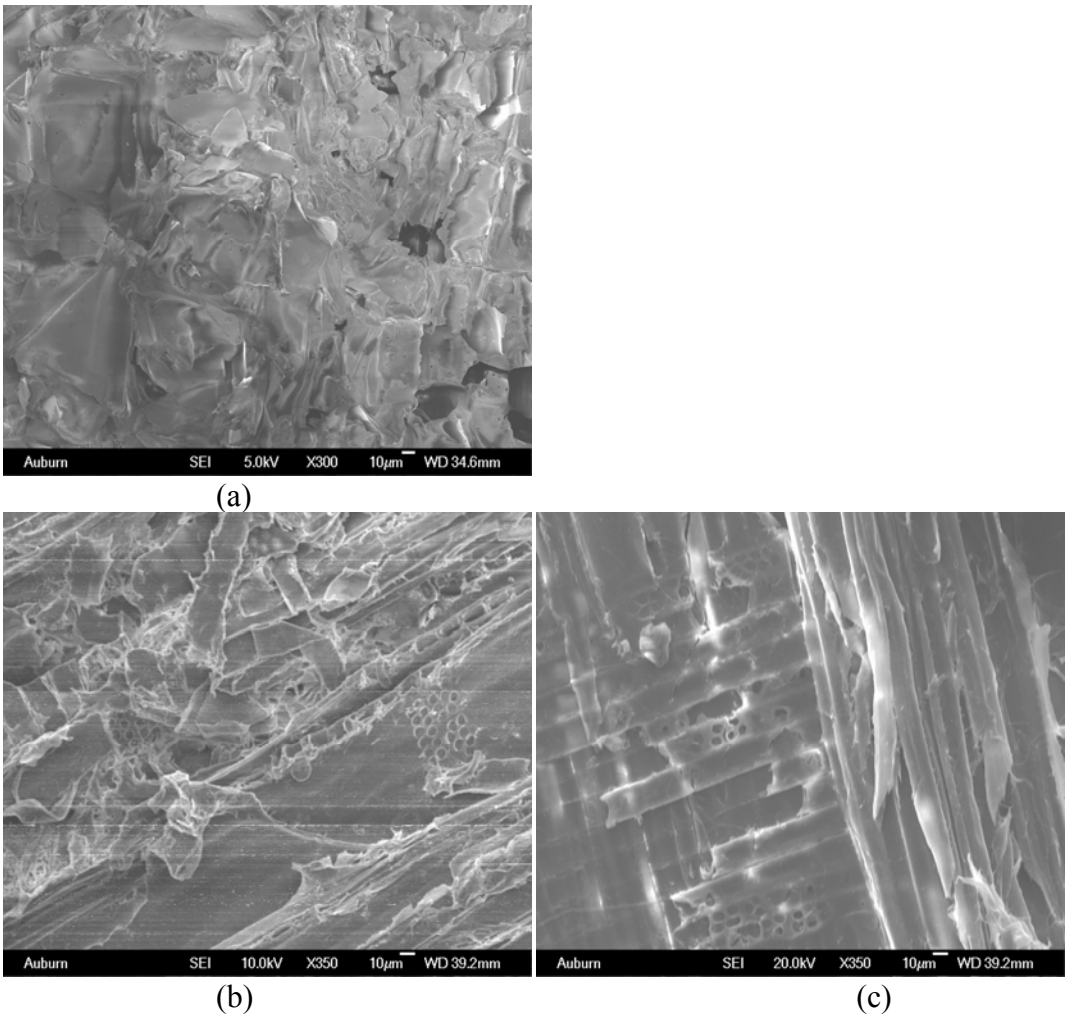


Fig.III-3. SEM pictures of feedstocks (a) Corn stover (b) HL hybrid poplar (c) LL Hybrid poplar

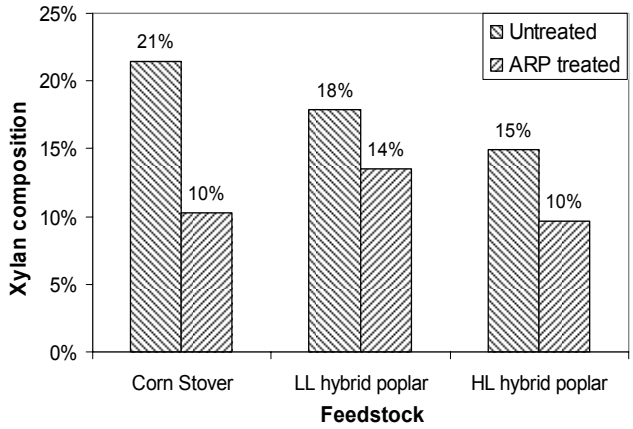


Fig. III-4(a). Xylan removal in ARP pretreatment

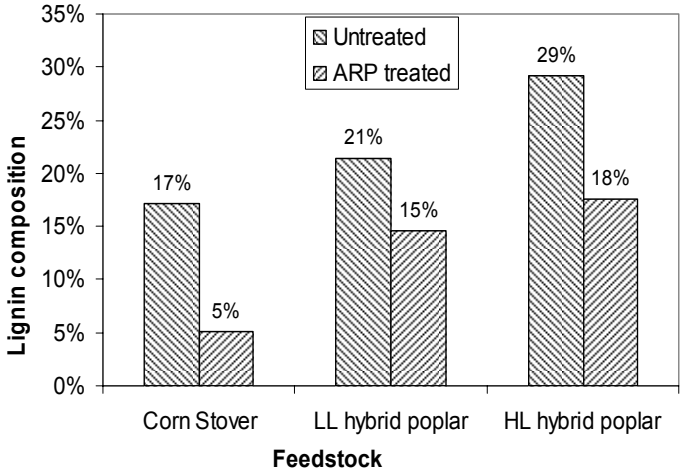


Fig. III-4(b). Lignin removal in ARP pretreatment

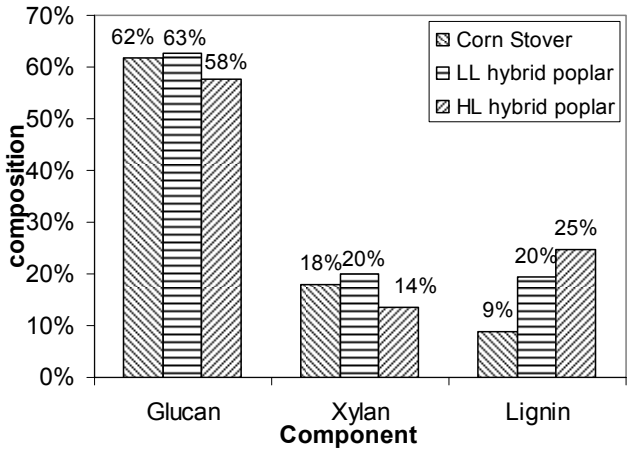


Fig. III-4 (c). Composition of sugars and lignin in ARP treated biomass

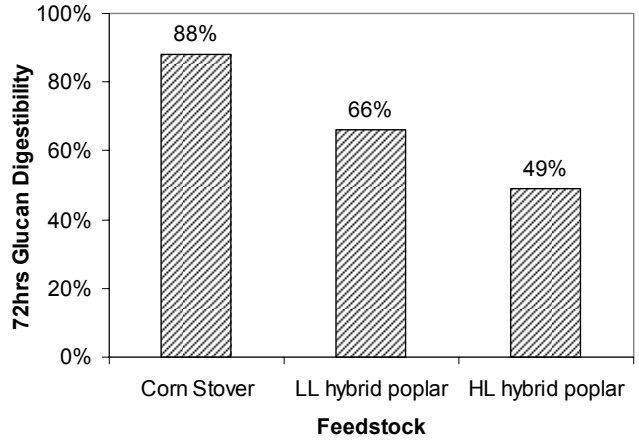


Fig. III-4(d). Glucan digestibility of ARP treated feedstocks

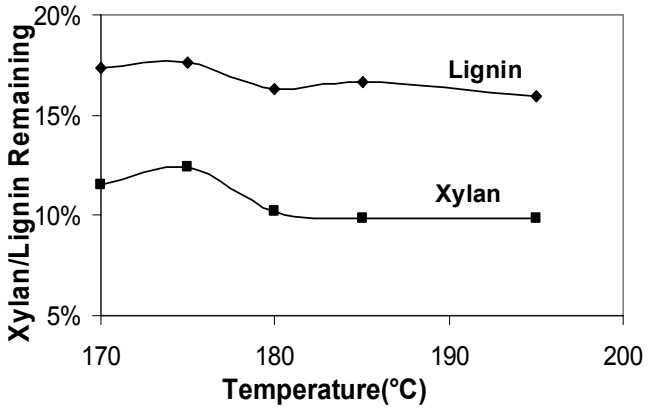


Fig. III-5(a). Effect of Temperature on the component removal of HL hybrid poplar during ARP pretreatment

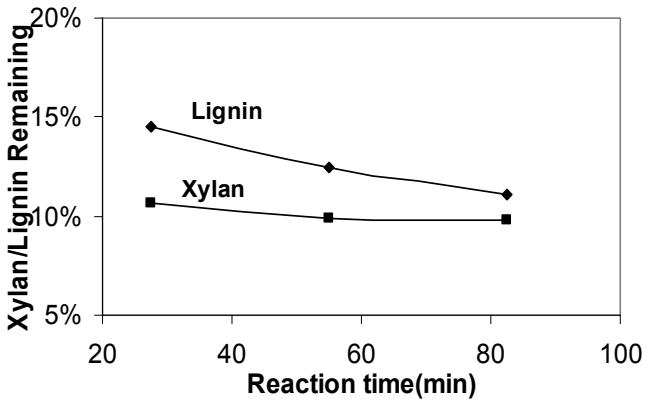


Fig. III-5(b). Effect of reaction time on component removal of HL hybrid poplar during ARP Pretreatment

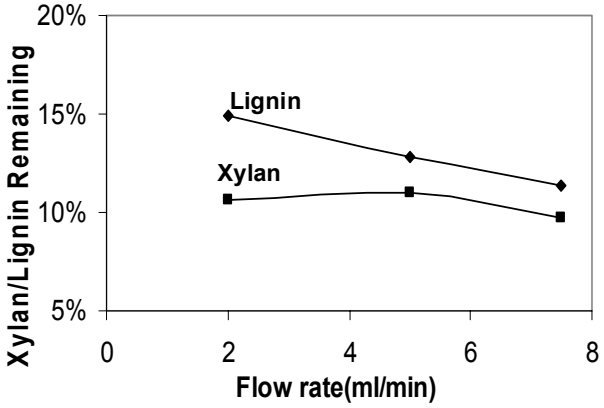


Fig. III-5(c). Effect of liquid flow rate on component removal of HL hybrid poplar during ARP pretreatment

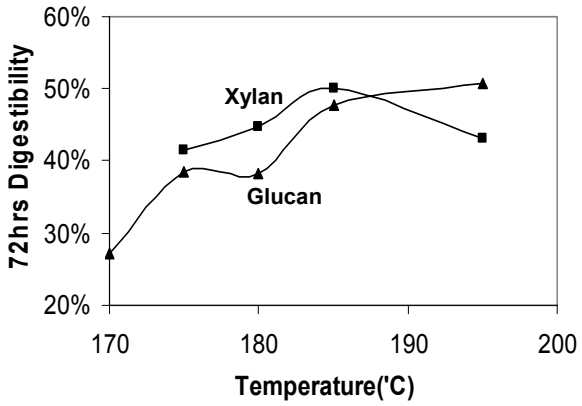


Fig. III-6(a). Effect of pretreatment temperature on digestibility of ARP treated HL hybrid Poplar

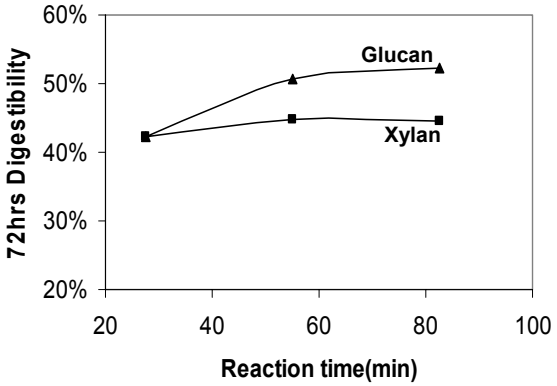


Fig. III-6(b). Effect of pretreatment time on digestibility of ARP treated HL hybrid poplar

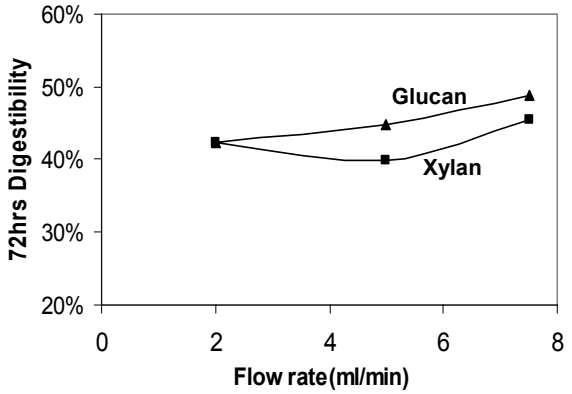


Fig. III-6(c). Effect of liquid flow rate during pretreatment on digestibility of ARP treated HL hybrid poplar

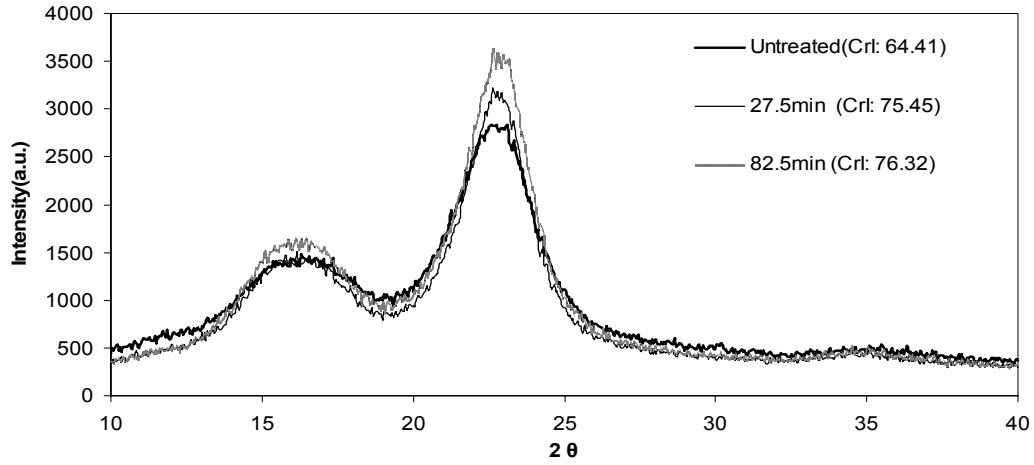


Fig. III-7(a). XRD plots of ARP treated HL hybrid poplar samples treated with various reaction time

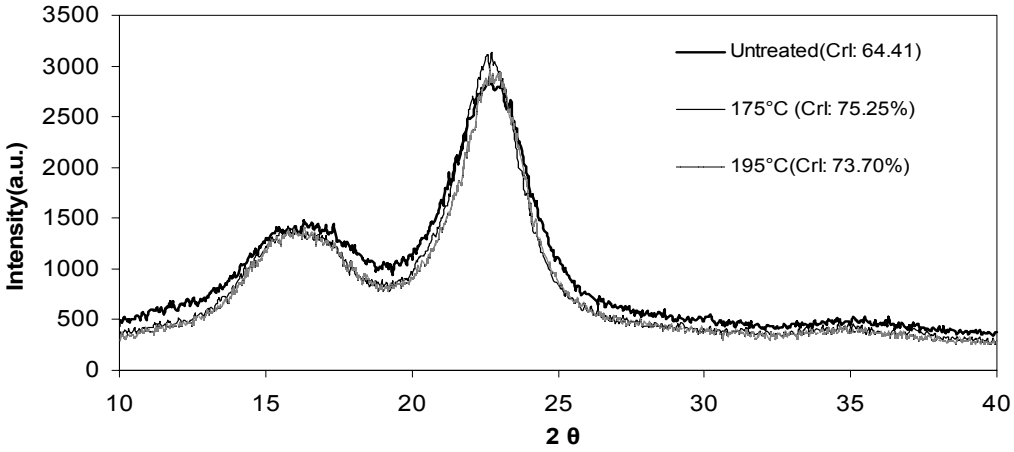
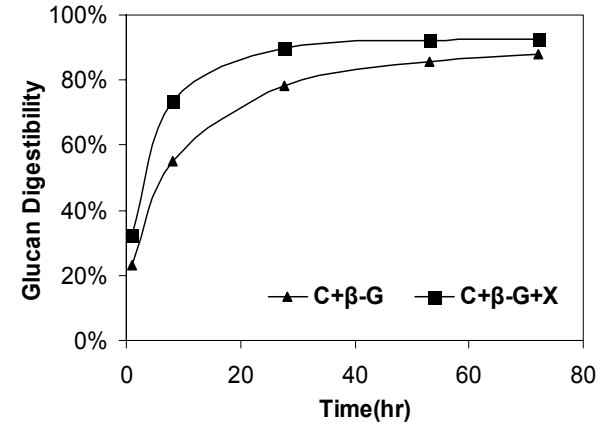
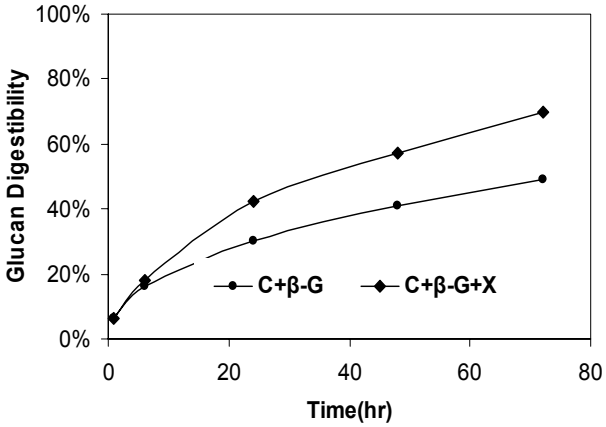


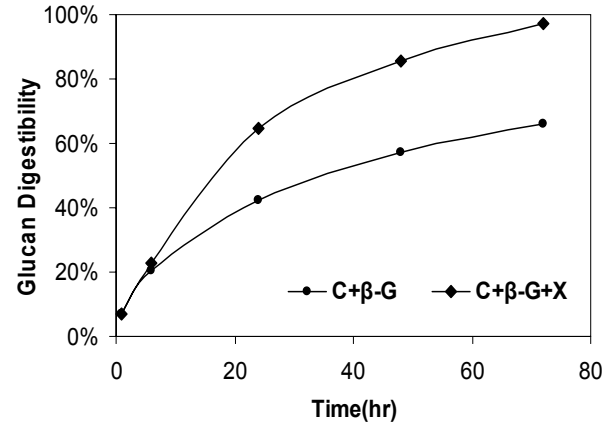
Fig. III-7(b). XRD plots of ARP treated HL hybrid poplar samples treated at different temperature.



(a)

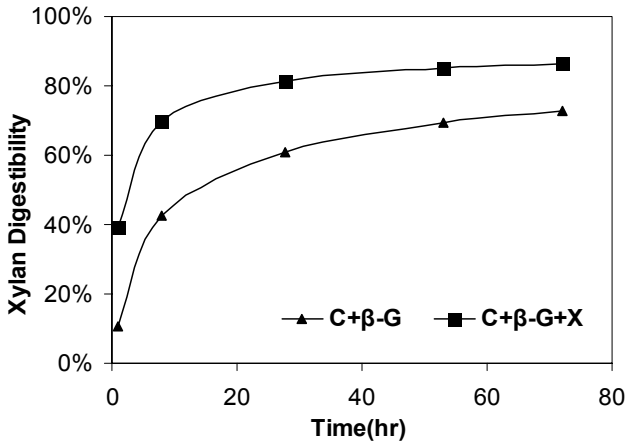


(b)

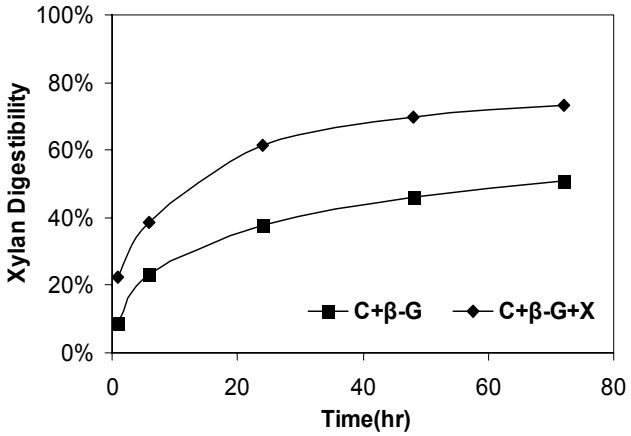


(c)

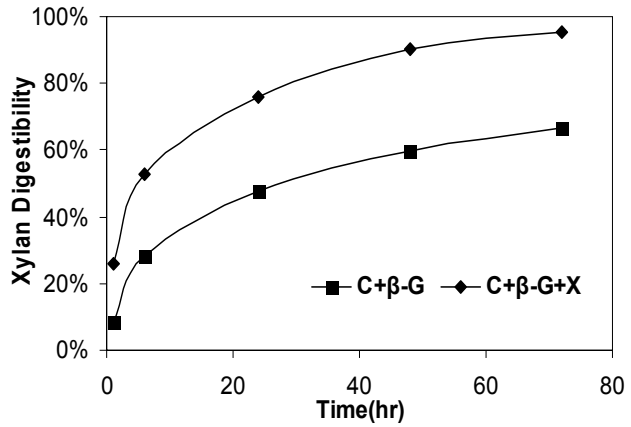
Fig. III-8. Effect of xylanase addition on Glucan digestibility of ARP treated biomass
(a) Corn Stover (b) HL hybrid poplar (c) LL hybrid poplar
(C+β-G: Cellulase + β-glucosidase, C+ β-G+X: Cellulase + β-glucosidase+Xylanase)



(a)



(b)



(c)

Fig. III-9. Effect of xylanase addition on Xylan digestibility of ARP treated biomass
(a) Corn Stover (b) HL hybrid poplar (c) LL hybrid poplar
(C+β-G: Cellulase + β-glucosidase, C+ β-G+X: Cellulase + β-glucosidase+Xylanase)

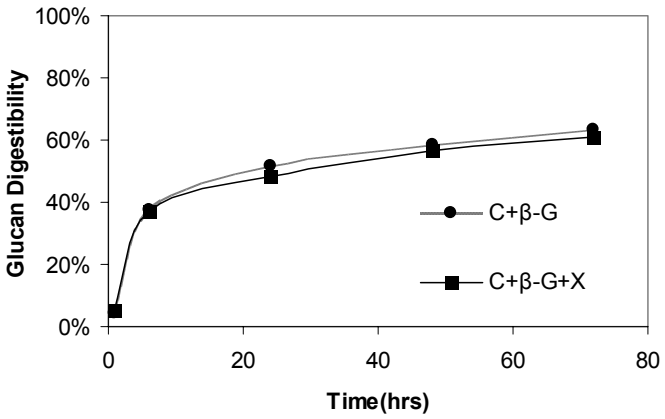


Fig. III-10. Effect of xylanase addition on digestibility of Avicel
(C+β-G: Cellulase + β-glucosidase, C+ β-G+X: Cellulase + β-glucosidase+Xylanase)

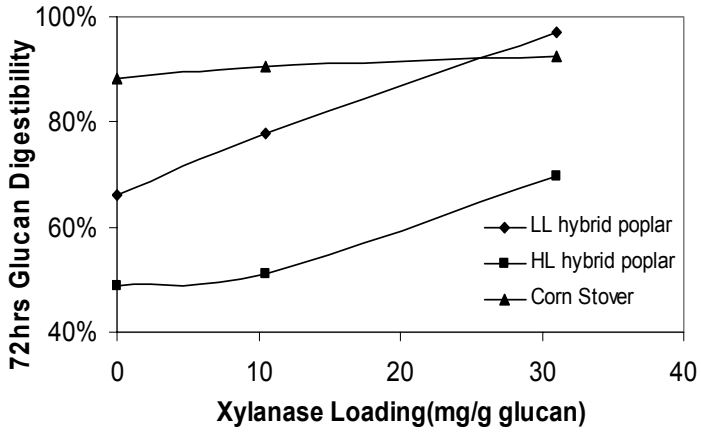


Fig. III-11(a). Glucan Digestibility of ARP treated feedstocks with different Xylanase loading

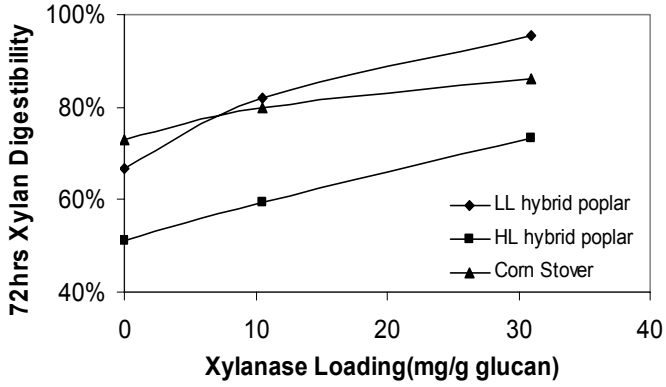


Fig. III-11(b). Xylan Digestibility of ARP treated feedstocks with different Xylanase loading

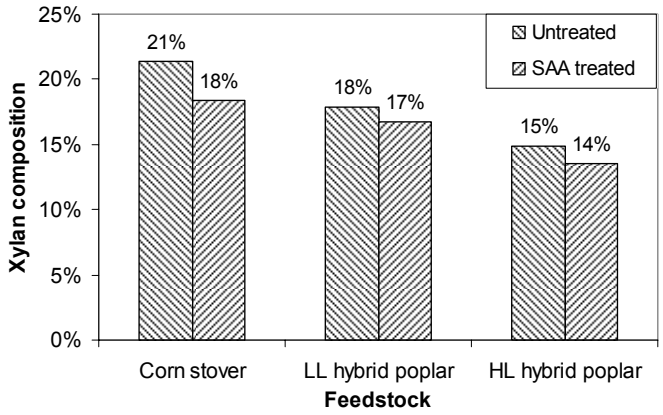


Fig. III-12(a). Xylan removal in SAA treatment

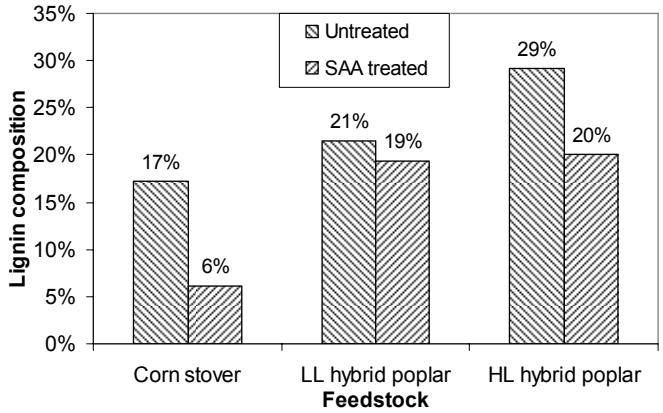


Fig. III-12(b). Lignin removal in SAA treatment

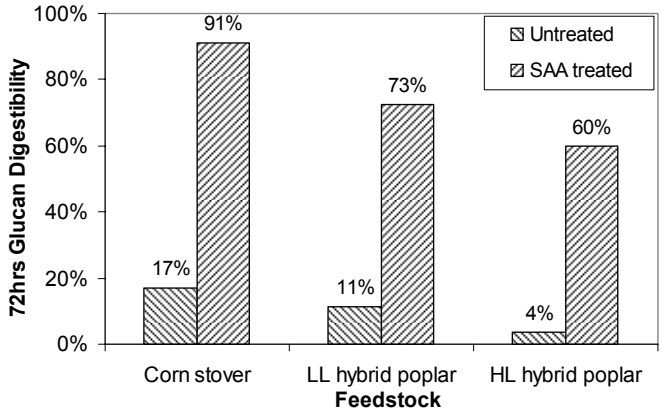
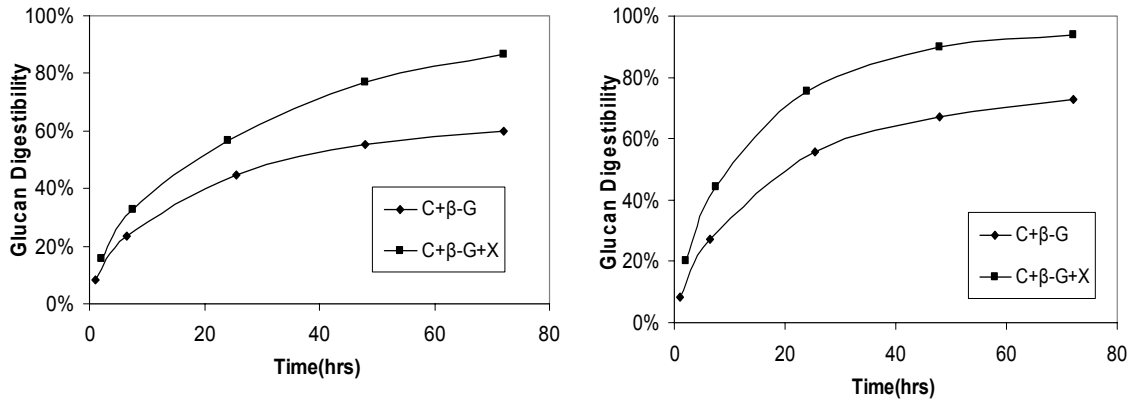
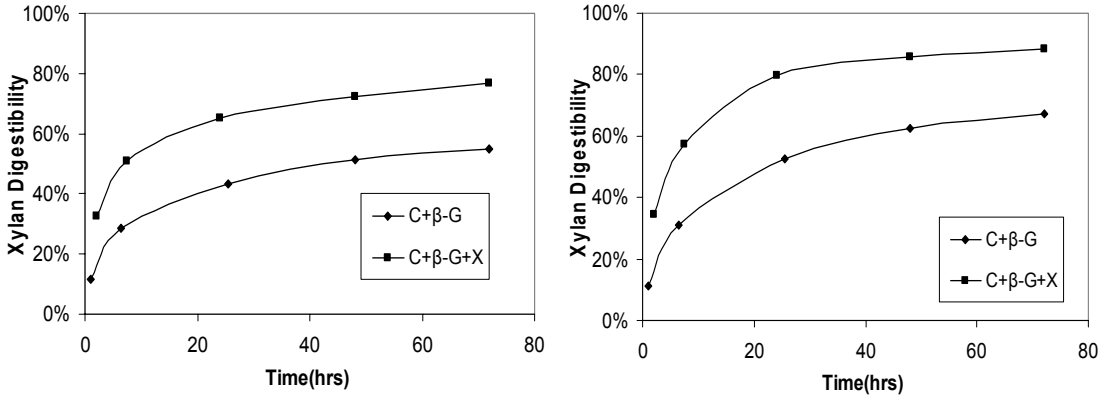


Fig. III-12(c). Glucan digestibility of untreated and SAA treated biomass with SC-A



(a) (b)

Fig. III-13. Effect of xylanase addition on Glucan digestibility of SAA treated biomass (a) HL hybrid Poplar (b) LL hybrid poplar
(C+β-G: Cellulase + β-glucosidase, C+ β-G+X: Cellulase + β-glucosidase+Xylanase)



(a) (b)

Fig. III-14. Effect of xylanase addition on Glucan digestibility of SAA treated biomass (a) HL hybrid Poplar (b) LL hybrid poplar
(C+β-G: Cellulase + β-glucosidase, C+ β-G+X: Cellulase + β-glucosidase+Xylanase)

		Corn Stover		LL hybrid poplar		HL hybrid poplar	
Feed Stock	Glucan	36.1		44.91		43.8	
	Xylan	21.4		17.85		14.9	
After ARP treatment	Glucan	35.6		44.5		40.84	
	Xylan	10.3		13.5		9.61	
	Sugar Yield	**79.83%**		**92.42%**		**85.95%**	
		Without Xylanase	With Xylanase	Without Xylanase	With Xylanase	Without Xylanase	With Xylanase
After Enzymatic Hydrolysis	Glucan	31.38	32.88	29.42	43.23	19.96	28.45
	Xylan	7.51	8.89	8.99	12.90	4.91	7.05
	Sugar Yield	**84.72%**	**91.00%**	**66.24%**	**96.77%**	**49.30%**	**70.35%**
Total Sugars(g)/100g biomass		**38.89**	**41.77**	**38.42**	**56.13**	**24.87**	**35.49**
Overall Sugar Yield		**67.63%**	**72.64%**	**61.21%**	**89.43%**	**42.37%**	**60.47%**

Table.III-1. Summary of sugar yields in ARP and subsequent enzymatic hydrolysis

		Corn Stover		LL hybrid poplar		HL hybrid poplar	
Feed Stock	Glucan	36.1		44.91		43.8	
	Xylan	21.4		17.85		14.9	
After SAA treatment	Glucan	35.3		44.9		40.52	
	Xylan	18.4		16.7		13.57	
	Sugar Yield	**93.39%**		**98.15%**		**92.15%**	
		Without Xylanase	With Xylanase	Without Xylanase	With Xylanase	Without Xylanase	With Xylanase
After Enzymatic Hydrolysis	Glucan	32.19	33.50	32.62	42.12	24.30	35.03
	Xylan	14.83	16.93	11.22	14.78	7.43	10.44
	Sugar Yield	**87.57%**	**93.90%**	**71.16%**	**92.37%**	**58.65%**	**84.07%**
Total Sugars(g)/100g biomass		**47.02**	**50.42**	**43.84**	**56.90**	**31.72**	**45.47**
Overall Sugar Yield		**81.78%**	**87.69%**	**69.85%**	**90.66%**	**54.04%**	**77.47%**

Table III-2. Summary of sugar yields in SAA and subsequent enzymatic hydrolysis

IV. PRETREATMENT OF HL HYBRID POPLAR BY AQUEOUS AMMONIA

ABSTRACT

HL Hybrid poplar was treated with aqueous ammonia by three different methods: ammonia recycle percolation (ARP), soaking in aqueous ammonia (SAA), and a modified version of SAA (supplementation with H_2O_2). ARP treatment of hybrid poplar removed large amount of lignin, yet the enzymatic digestibility of treated solids did not exceed 50%. The hybrid poplar feedstock, used in this study, contained very high amount of lignin. The residual lignin after the treatment was still a major hurdle for cellulase accessibility to carbohydrates. A large amount of hemicellulose was retained after the ARP treatment, which is also a hindrance to cellulose hydrolysis. Enzymatic hydrolysis of ARP treated hybrid poplar was studied, using the combination of cellulase with xylanase and pectinase, to understand the role of each enzyme and to assess the total amount of enzyme (protein loading) required for effective hydrolysis. The effect of xylanase addition was much more significant than pectinase addition. Addition of xylanase improved the digestibility of not only xylan but also glucan. Supplementation of 31.5 mg xylanase with 15 FPU of cellulase/g-glucan improved the digestibility of ARP treated hybrid poplar from 50% to 86%. Addition of H_2O_2 into the SAA enhanced delignification due to its oxidative degradation of lignin. Several different H_2O_2 feeding schemes and different temperature profiles were attempted in operation of SAA to

investigate the oxidative effects of H_2O_2 on degradation of lignin and carbohydrates in hybrid poplar. More than 60% of lignin in hybrid poplar was removed with stepwise increase of temperature (60°C for 4hrs and then 120°C for rest of treatment time). Significant degradation of carbohydrate was also observed under this condition.

Key words: Pretreatment, Hybrid poplar, Hydrogen peroxide, Ammonia, Xylanase, Pectinase

INTRODUCTION

Poplar is one of the attractive energy crops. The projected per acre biomass yield of poplar is about 10 tons/year, which is higher than agriculture residues or perennial herbaceous crops such as corn stover or switch grass. Seasonal property variation of poplar is much less than that of other feedstocks (Ladisch et al., 2007). Extensive research is being carried out on the genetic modification of poplar in order to improve the conversion characteristics so that it can be adapted as an ideal energy crop. Poplar is the first tree whose full genome sequence has been identified. It is a good target for genetic improvement because of its compact genome of 500 million bases, which is only 2% of pine (Wright and Tuskan, 1997, Tuskan et al.,2006). Poplar genetic parameters are favorable for reduction of lignin and increase of cellulose and specific gravity (Dinus et al., 2001). A recent study reported that a genetically engineered poplar tree has 50% less lignin and more cellulose than a conventional one. The same study also reported that work is under way to reduce the crystallinity of the cellulose fibers by manipulation of the genes which control the biosynthesis of cellulose (Service et al.,

2007). With this understanding, poplar was selected as the primary feedstock in the CAFI-2 project, the overall objective of which is to investigate the conversion characteristics with different pretreatment strategies (Ladisch et al., 2007).

Ammonia is widely used in various pretreatment processes including ARP (Kim et. al., 2003), AFEX (Alizadeh et al., 2005) and SAA (Kim et al., 2005). Under normal pretreatment conditions, ammonia in aqueous solution reacts primarily with lignin showing little effects on carbohydrates in biomass (Kim et al., 2005; Gupta et. al., 2007). Aqueous ammonia has been investigated extensively for the pretreatment of various feedstocks in our laboratory. The ammonia–based processes have been proven to be highly effective in improving the glucan digestibility, in some cases, approaching the theoretical maximum for low lignin feedstocks such as corn stover. Higher retention of hemicellulose was observed in corn stover with SAA than with ARP because of the low operating temperature in the SAA. In our previous study of SAA and ARP, we found that feedstocks with moderate lignin content (young hybrid poplar with 21% lignin, for example) retain high amount of hemicellulose. The treated biomass is easily digested when the cellulose is supplemented with xylanase. The sugar yield from the enzymatic hydrolysis exceeded 90%. Retention of hemicellulose is a unique feature in ammonia-based pretreatment. It can also become a desirable pretreatment strategy because the hemicellulose sugars are recovered enzymatically. The bioconversion process thus becomes simpler, not having to deal with detoxification of the pretreatment fluid in subsequent bioconversion, a process step yet to be established and considered costly (Gupta et. al., 2007).

Substrates with very high lignin content such as mature hybrid poplar tree (the main feedstock of this study with 29% lignin content) are not easily digested even after treatment by aqueous ammonia because of insufficient delignification (Gupta et. al., 2007). The first part in this study was devoted for optimization of ARP treatment of hybrid poplar. The optimally treated hybrid poplar was further characterized for the enzymatic reactivity using different enzyme combinations and loadings. The purpose was to assess the amount of total enzyme required for effective hydrolysis.

In order to effectively pretreat hybrid poplar containing high lignin content, high severity reaction condition is required (Gupta et. al., 2007), which leads to high processing cost. Hydrogen peroxide has been used as a supplementary pretreatment reagent in combination with alkaline as well as acidic media (Kim et. al., 2001; Kim et. al., 1996; Kim et. al., 2003; Kim et. al., 2000). It became of our interest to use H_2O_2 as a means to lower the severity of the SAA and yet attain high enough delignification. In alkaline conditions, hydrogen peroxide reacts nucleophilically with electron-deficient carbonyl and conjugated carbonyl structures. In neutral to acidic media, hydrogen peroxide reacts with electron rich aromatic and olefinic structures via electrophilic pathways (Kadla et al., 2001). Alkaline degradation of lignin causes the cleavage of aryl ether linkages. Hydrogen peroxide under alkaline condition induces breakage of carbon-carbon linkages in lignin along with the cleavage of the aryl ether bond, resulting oxidative delignification (Alen et al., 2000). Oxidative breakage of lignin also causes demethoxylation, ring cleavage, and reduction in the phenolic hydroxyl group (Northey et al., 2001).

Hydrogen peroxide is widely used in pulping processes as a bleaching reagent. It reacts with the chromophoric components in residual lignin after the pulping at low temperature but does not cause appreciable degradation of lignin. Additional degradation of lignin by oxidative action of alkaline H_2O_2 is possible at a relatively higher temperature and longer reaction time (Suchy et. al., 2001). High temperature with H_2O_2 is undesirable because of its decomposition into molecular oxygen and other free radicals. These free radicals react with lignin but are also very reactive with carbohydrates. Decomposition of H_2O_2 at high temperature increases the selectivity of reaction towards carbohydrate degradation (Xiang et. al., 2000). Rapid decomposition of H_2O_2 also reduces availability of peroxide for lignin reaction. Previous studies indicate that at a low alkali charge, synergistic action of oxygen and H_2O_2 can improve the selectivity toward delignification (Suchy et. al., 2001). The second part of this investigation was undertaken to see if supplementation of H_2O_2 to the SAA can improve the extent of delignification as well as the selectivity of reaction towards the lignin over carbohydrates. Various reaction conditions and H_2O_2 addition strategies were attempted to understand how they affect delignification and carbohydrate degradation.

MATERIALS AND METHODS

Substrate, Reagent and Enzymes

HL hybrid poplar was supplied by NREL in the form of small chips with a particle size less than 0.25". The moisture content of the received hybrid poplar was approximately 50%. It was stored at -20°C until use. In all the pretreatment experiments,

feedstock was used as received without additional drying or grinding. Ammonium hydroxide solution of 30% (weight basis) was purchased from Fisher-scientific, diluted to 15% in all experiments.

Enzymes cellulase (Spezyme CP), xylanase (Multifect), and pectinase were supplied by Genencor-Danisco (Paulo Alto, CA). According to the supplier, the activity of Spezyme CP was 59 FPU/ml, and the protein content of Multifect xylanase was 42 mg of protein/ml. β-Glucosidase was purchased from Sigma (Novozyme 188, Sigma, C-6150). Its activity, as determined in our laboratory, was 750 CBU/ml.

Experimental setup and operation of ARP

The schematics of laboratory ARP experimental setup is shown in Fig.IV-1 (Kim et. al., 2003). The ARP employs a flow through column reactor. The reagent solution is pumped through the reactor packed with biomass. The reactor temperature is controlled by a GC forced-air convection oven. The reactor system is back-pressured by nitrogen (300-425 psig) to prevent flash evaporation. The reactor was made out of SS-316 with dimension of 0.9" ID, 10" length and 101.9 cm^3 internal volume. The substrate loading was 15 dry grams in ARP experiments which occupied approximately 80% of the reactor volume. The rest of the volume was filled with perforated Teflon rods. ARP experiments for the hybrid poplar were carried under the conditions of: 170-195°C, 2-7.5 ml/minutes flow rate, and 27.5 – 82.5 minutes of reaction time.

Experimental setup and operation for SAA and modified SAA

In SAA experiments, flanged batch reactors were used. The reactors were made out of SS-316 to the dimension of 1.375" ID x 6" L. In the modified SAA, a nozzle was connected to the top of flange for syringe feeding of H_2O_2 solution. For SAA treatment, biomass was soaked with 15% ammonia solution and placed in a convection oven set at desired reaction temperature without agitation (Kim et. al, 2005). In modified SAA treatment, 2-5% H_2O_2 was added to the reactor. In all SAA and modified SAA experiments, 24 hrs of total reaction time and 1:10 of solid: liquid ratio was applied. Apart from high pressure in the reactor, there can be increase in the liquid volume upto 5-10% (based upon water) of initial volume due to high reaction temperature. Owing to this reason, reactor should only be filled maximum upto 75% of total reactor volume.

Enzymatic digestibility

Enzymatic digestibility tests were carried out according to the NREL - Laboratory Analytical Procedure (LAP) No. 009 (www.eere.energy.gov/biomass/analytical_procedures.html). One gram of glucan loading/100ml of total reactant volume was applied. The total solid loading in the reactor varied according to the glucan content in the biomass. The enzymatic reactions were carried out in 250ml Erlenmeyer flasks. Reaction conditions were: 50°C, pH 4.8 (maintained by 0.05M sodium citrate buffer), 150 rpm (Shaker/Incubator: NBS, Innova-4080). The digestibility was defined as the percentage glucose released after 72 h of incubation on the basis of theoretical maximum. Cellulase loading of 15 FPU/g glucan

and β-glucosidase loading of 30CBU/g glucan were used. Unless noted otherwise, xylanase loading of 31.5mg/g glucan was added to cellulase where applicable. Maximum expected variation in digestibility values is within 3% of the reported values.

Simultaneous saccharification and fermentation (SSF) and Simultaneous saccharification and co-fermentation (SSCF)

Both SSF and SSCF experiments were performed as per the procedure described in the NREL LAP-008. The microorganism, *Saccharomyces cerevisiae*, ATCC® 200062 (also known as NREL-D5A) was used in the SSF experiments. The growth media for D5A was YP medium, a mixture of 1% yeast extract (Sigma Cat. No. Y-0500) and 2% peptone (Sigma Cat. No. P-6588). The conditions employed in SSF experiments were 4.8 pH (0.05M citrate buffer), 38°C, and 150 rpm. Recombinant *E. coli* ATCC® 55124 (KO-11) was used in the SSCF experiments. LB medium (Sigma, L-3152) containing 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L chloroamphenicol was used for the growth of KO-11. The SSCF was carried out without pH control.

Analytical procedures

Prior to enzymatic digestibility of biomass, composition analysis was done as per the NREL LAP: "Preparation of samples for compositional analysis" and "Determination of structural carbohydrates and lignin in biomass" (www.eere.energy.gov/biomass/analytical_procedures.html). The moisture content in biomass was measured by Infrared Moisture Balance (Denver Instrument, IR-30). Sugar

concentration for compositional analysis and enzymatic digestibility was determined by HPLC using a Bio-Rad Aminex HPX-87P column. Sugar analysis was carried out in duplicate for each sample and average values are reported.

Diffusive Reflectance FT-IR (DRIFT) and Surface Characterization

FTIR analysis of untreated and ARP treated hybrid poplar samples were performed at the "Biomass conversion research laboratory (BCRL)" of Professor Bruce Dale, Michigan State University using Perkin Elmer FT-IR System 2000 with Diffusive Reflection Accessory. The spectra were measured using 32 scan of powdered samples without dilution, triangular apodization, 4 cm^{-1} resolution, and an interval of 1 cm^{-1}. Surface characterization of biomass samples (Table.IV-1) was done at Micromeritics Analytical Services, Norcross, GA. The BET surface area was measure by ASAP 2420 instrument by krypton gas adsorption. Hg analysis was performed by Micromeritics Analytical Services using AutoPore IV 9520 instrument.

RESULTS AND DISCUSSION

ARP treatment on Hybrid Poplar:

The ARP pretreatment of hybrid poplar was previously investigated in our laboratory (Gupta et al., 2007), summary of which is presented here. ARP treatment at very high temperature of 195°C, 27.5min reaction time, and 2ml/min liquid flow rate attained 50% delignification. The digestibility of treated solids was approximately 50% with cellulase loading of 15 FPU/ g glucan. Higher lignin removal up to 62% was

achieved when higher flow rate (7.5 ml/min) of ammonia solution or a longer reaction time of 82.5min. was applied. Despite higher delignification, glucan digestibility of these samples did not improve staying at 48% - 53% range. The residual lignin of ARP treated hybrid poplar appeared to be an obstacle for the cellulase accessibility to the carbohydrate part of the biomass. A large fraction of xylan was retained in the solid after ARP. Even after ARP of very severe conditions (185°C, 82.5min reaction time and 2ml/min flow rate), xylan content did not go below 9.8%. Increase of ammonia concentration to 21% in the ARP did not show significant effect on lignin or hemicellulose removal. The glucan digestibility rose slightly to 54% (data not shown). Kim et. al (2003) reported that ARP on corn stover showed selective reaction towards lignin and did not remove hemicellulose beyond a certain point (Kim et. al., 2003). Retention of hemicellulose after pretreatment is a desirable feature associated with alkaline pretreatment since it simplifies the overall bioconversion process. The "cellulase" enzymes possess xylanase activity as well as glucanase activity. The hemicellulose (primarily xylan) left in the treated biomass can be hydrolyzed by "cellulase" along with cellulose. In acidic or neutral pretreatment, the hemicellulose sugars are solubilized into pretreatment effluent. Recovery and bioconversion of the hemicellulose sugars from the pretreatment fluid is a costly process involving detoxification. This step can be eliminated if aqueous ammonia is used as the pretreatment reagent since hemicellulose remain as solid and biologically converted via SSCF without additional cost.

The fraction of lignin in hybrid poplar remaining after ARP treatment is resistant to degradation by aqueous ammonia. According to Kim et. al. (2003), the ammonia resistant fraction of lignin in corn stover after treatment was much lower than that of hybrid poplar, and did not pose much problem for enzymatic digestion of cellulose. This indicates that it is not just the amount of lignin but the structural features of the lignin in hybrid poplar that affects the accessibility and reactivity of the cellulase enzyme. Even with xylanase supplementation into cellulase, the enzymatic hydrolysis of optimally treated ARP hybrid poplar attained only 70% of glucan digestibility (Table IV-2). This supports the possibility of unproductive binding of enzyme with the residual lignin as suggested by Yang et al. (2004).

Characterization of untreated and ARP treated Hybrid Poplar

Table IV-1 shows the changes in the surface properties of the hybrid poplar as a result of ARP treatment. The BET surface area of the treated poplar is 4~5 times higher than the untreated one. There is a slight increase in porosity after the ARP treatment. Apparent density of the treated biomass also decreased reflecting the increase in the porosity.

Enzymatic hydrolysis results of ARP treated hybrid poplar indicates that the residual lignin impedes the enzymatic hydrolysis by blocking the access of cellulase as well as unproductive binding with the cellulase enzyme. To understand the nature of changes occurring in lignin as a result of ARP, FTIR spectra of untreated and treated hybrid poplar were examined focusing on the peaks corresponding to the bonds in the

lignin region. As shown in Fig IV-2, appreciable reduction in the peak intensity was observed at 1026 cm^{-1} and 1726 cm^{-1}. These peaks correspond to the stretching of the unconjugated carbonyl group (C=O). Reduction in intensity at these locations indicates that carbonyl groups are one of the main reaction sites in degradation of lignin in ARP pretreatment. Another noticeable drop in peak intensity was observed near 1260-1270 cm^{-1}. This corresponds to the guaiacyl (G) ring breathing with C-O stretching. There is not appreciable change in the 1300-1330 cm^{-1} region that represents the syringyl (S) ring breathing (Sakakibara et. al., 2001). This shows that the guaiacyl type of lignin is more readily hydrolysable than the syringyl type. This is in line with the findings of Davidson et. al. (2005) that a low S/G ratio gives better hydrolysis rate of biomass with dilute acid treatment. This further indicates that hydrolysis of guaiacyl lignin is comparatively easier than syringyl lignin.

Hydrolysis of ARP treated hybrid poplar by mixtures of enzyme:

Table IV-2 shows the glucan and xylan digestibilities for optimally treated hybrid poplar by ARP. The data were obtained from experiments using different enzyme combinations and loadings. Addition of external xylanase in the enzymatic hydrolysis of ARP treated hybrid poplar significantly improved the digestibility of glucan as well as xylan (Gupta et. al., 2007). The role of xylanase was to remove the layer of hemicellulose, thus increasing the accessibility of cellulase enzyme to glucan. With addition of 10.5mg xylanase/g glucan, the glucan digestibility increased slightly from 48.8% to 51.2%., whereas with addition of 31.5 mg xylanase/g glucan, the increase was to 70%.

Supplementation of pectinase showed much less effect on the digestibility of ARP treated hybrid poplar than xylanase. Pectinase loading as high as 41mg/g glucan was required to observe discernible difference in the glucan digestibility (48.8% to 55.3%). It appears that the pectic substances in the cell wall matrix of hybrid poplar are not major hurdle for cellulose hydrolysis.

Overall sugar yield data are presented in Fig. IV-3. The overall yield is defined as the sum of sugars obtained from pretreatment liquor and enzymatic hydrolysis of the pretreated solids divided by total carbohydrates in untreated biomass. The overall sugar yields are plotted against the total enzyme loading (expressed as total mg protein) since the digestibility tests were done using mixtures of different enzymes, e.g., cellulase, β-glucosidase and xylanase. With use of mixed enzymes, maximum overall yield of 89% observed from ARP treated hybrid poplar. It occurred with 161 mg protein/g glucan, the highest mixed enzyme loading. The trend of total sugar in this graph indicated that sugar yields steadily increase with total protein (enzyme) loading for ARP treated hybrid poplar. The same did not hold true with other pretreatments where the yield vs. total protein profile shows quick rise up to a certain point and leveling off afterwards (Rajeev et. al., 2006). This showed that the properties of cellulose in hybrid poplar are not altered by ARP treatment.

It is also seen that the total sugar yield as well as the individual sugar yields (xylose and glucose) are mainly dependent upon the total protein loading although different combinations of cellulase and xylanase were used. It is well known that certain types of surfactants improve the enzymatic digestibility of biomass by reducing the

unproductive binding of enzyme with lignin (Eriksson et. al., 2002). Rajeev et al. (2008) observed that the digestibility of ARP treated corn stover improved substantially when a surfactant was added in the enzymatic hydrolysis. However, the same test did not show much increase in digestibility for corn stover treated by other pretreatment technologies. These findings collectively indicate that the unproductive binding of protein occurs with certain type or state of lignin, and it is one of the main factors affecting the efficiency of cellulase enzymes.

Fermentation of ARP treated Hybrid Poplar

SSF and SSCF experiments were performed on the ARP treated hybrid poplar. The experiments were carried out to verify the effects of inhibitory compounds released from the hydrolysis of ARP treated hybrid poplar on the fermentation. These experiments were done with 3% glucan loading (or 5.2% solid loading in this case). As shown is Fig. IV-4a, 64% ethanol yield was obtained after 72-h of SSF (based on the theoretical maximum from the glucan in treated biomass). The enzyme loadings were: 15 FPU (Cellulase), 30 CBU (β-glucosidase), and 31.5 mg of xylanase per gram of glucan. Ethanol yield increased further when the experiment was carried out beyond 72h. In the SSF experiment, xylose accumulated in the liquid because of inability of *S. cerevisiae* to consume xylose (Fig. IV-4b). In the SSCF, however, both glucose and xylose were consumed by the recombinant *E.coli*, and the ethanol yield increased to 97% based upon the glucan content in the biomass. This yield is equivalent to 78% of theoretical maximum based on the total sugar (Glucan +Xylan) content in the ARP treated hybrid

poplar. Enzymatic digestibility of ARP treated hybrid poplar with similar enzyme loadings produced only 70% of total sugars present in substrate. This proves the well-known benefit of SSF/SSCF that low monomeric sugar level indeed eliminates the inhibition effects and improves the enzymatic digestibility.

SAA and Modified SAA treatment on Hybrid Poplar

SAA treatment on hybrid poplar with high lignin content is not effective giving poor digestibility even when it was operated at high temperature of 150°C (Gupta et al., 2006). Addition of H_2O_2 to 15% ammonia at lower temperature was then attempted as a modified SAA treatment. The intent was to investigate the combined effect of H_2O_2 and aqueous ammonia on delignification. Different schemes of H_2O_2 addition are shown in Table IV-3, all of which were done at 120°C. Addition of H_2O_2 improved the delignification and digestibility to a certain extent. In scheme 3 in which H_2O_2 was added after 4 hrs of the starting point, delignification and the digestibility were lower than the scheme 2 where H_2O_2 was added at the beginning of the treatment with 15% NH_3. It appears that H_2O_2 decomposes rapidly when it is added at high temperature, thus lowering delignification.

Table IV-4 shows the effect of temperature on the modified SAA treatment. In these experiments, 5% H_2O_2 was added to 15% NH_3 at the beginning of the pretreatment. The delignification and glucan digestibility of hybrid poplar treated at 60°C in the presence of H_2O_2 was higher than that treated with 120°C without the H_2O_2. This proved that addition of H_2O_2 has significant effect on SAA treatment. However, as the

temperature was raised from 60°C to 120°C, there was only a slight increase in delignification and digestibility because of unstable nature of H_2O_2 at high temperature. Nonetheless, high temperature is still required for the aqueous ammonia to function effectively as a delignifing reagent in the SAA. In an attempt to resolve this issue, a step change of temperature was tried as shown in Table IV-5. First hybrid poplar was soaked in 15% ammonia + 5% H_2O_2 for 4hrs at 60°C, then temperature was raised to a higher level for 20hrs. Under these conditions, removal of lignin and xylan was found to be directly proportional to temperature. Significant difference in glucan digestibility was noticed between 80°C and 120°C (Table IV-5). With this strategy, approximately 60% of delignification was achieved at 120°C, but half of xylan and 16% of glucan were also removed from solid. Glucan digestibility of poplar treated under these conditions increased to 68.5% with cellulase alone and 82.6% with xylanase supplementation. In order to improve the retention of carbohydrates, a lower concentration of H_2O_2 was used under similar experimental conditions (Table IV-5). When H_2O_2 concentration was lowered to 2%, about 50% of delignification was achieved with increased retention of xylan (77.5%) and glucan (96%) (Table IV-6). Due to lower delignification at 2% H_2O_2, however, the digestibility was also decreased.

In an effort to increase carbohydrate protection and to improve delignification, H_2O_2 was added intermittently as shown in scheme 5. This attempt was only half successful that xylan retention was improved but delignification decreased. It appears that effective oxidative degradation of lignin requires certain level of H_2O_2 concentration. It was then thought that perhaps slow adsorption of H_2O_2 in biomass and fast

decomposition of H_2O_2 might be the reason for inefficient delignification. This was further explored in scheme 7 where biomass was first soaked with H_2O_2 and then a 30% ammonia solution was added in such a manner that the final concentration of ammonia became 15%. However, no improvement in delignification was found in scheme 7 compared to scheme 6 in which the run was made without soaking of H_2O_2.

Table IV-8 shows the fate of xylan in a modified SAA treatment. For all of schemes shown in the table, xylose was always present as xylo-oligomers in the pretreatment liquor. In the absence of H_2O_2, the degradation of carbohydrates was insignificant. A reason for this may be that most of ammonia is reacting with lignin, leaving a small fraction of it to interact with carbohydrate. Addition of H_2O_2 in SAA treatment caused some degradation of xylan at elevated temperature of 120°C. At 60°C and 80°C, however, degradation of carbohydrates was not evident (data not shown). Degradation of carbohydrates, when it occurs, was found to be proportional to the extent of delignification. In scheme 4 of Table IV-8, more than 40% of xylan and 15% of glucan (of the total present in untreated biomass) was decomposed or unaccounted for in the liquid. Negligible amount of glucan was found in the liquid while substantial amount of xylan was detected as xylo-oligomers. These xylo-oligomers were generated as a result of glycosidic cleavage in hemicellulose chains. This was not the case with cellulose because of its crystalline structure. The absence of monomeric sugars in the pretreatment liquor indicates that ammonia in the presence of H_2O_2 may cause the endwise degradation of glucan or xylan and produce the acidic entities (Lai et. al., 2001). These results collectively indicate that in treatment of biomass with aqueous ammonia and H_2O_2 at

high temperature, sugar decomposition becomes significant because of the endwise degradation of the glucan and xylan chains, whereas, glycosidic cleavage of the chain occurs at low temperatures.

Enzymatic hydrolysis of treated hybrid poplar with modified SAA

Supplementation of xylanase to cellulase improved the glucan digestibility of treated hybrid poplar by 10-15% (Table IV-3 to Table IV-7). This increase is primarily due to enhanced removal of hemicellulose. Although hemicellulose itself is a substrate to xylanase, it is also a factor hindering the cellulase access to cellulose. This increase is much lower than what was reported by Gupta et al. (2007), in which they studied a different batch of hybrid poplar with low lignin content. Hemicellulose digestibility was also low for treated high lignin hybrid poplar even with xylanase supplementation. The residual lignin after pretreatment appears to affect not only the cellulase but also the xylanase action by unproductive binding with the enzymes (Yang et. al., 2004).

Xylo-oligosaccharides (XOS) and xylose are present in the liquid during the enzymatic hydrolysis of treated poplar. Fig. IV-5 shows the profiles of XOS during the enzymatic hydrolysis of hybrid poplar treated under various modes of modified SAA. In all cases, except scheme 4, total XOS was in the range of 2-3% of total xylan. With scheme 4, however, the profile of XOS was quite different from other cases, where its level went up to 10%, then decreased to 6%. The generation of large amount of XOS in scheme 4 suggests that the H_2O_2 under alkaline conditions strongly promote breaking of

β-1-4 bonds in hemicellulose. This is a desirable feature because enzymatic removal of XOS from treated biomass further improves the accessibility of cellulase to cellulose.

CONCLUSIONS

ARP pretreatment was optimized specifically for mature poplar feedstock which contained high amount of lignin. Although more than 60% of lignin was removed by ARP, the digestibility was relatively low not exceeding 50%. The residual lignin was the main factor limiting access of cellulase enzyme to carbohydrates. Unproductive binding of the enzyme to lignin was another factor lowering the efficiency of the cellulase enzyme. FTIR analysis proved that removal of gauaiacyl type lignin is more prominent than syringyl type lignin in the ARP treatment. ARP treatment increased the BET surface area of hybrid poplar by factor of 4-5. Supplementation of the xylanase in cellulase improved the enzymatic digestibility of ARP treated poplar from 49% to 70%. Pectinase supplementation was not effective in the hydrolysis of treated hybrid poplar. SSF of treated hybrid poplar gave ethanol yield of 64% of the theoretical maximum based on glucan while the yield from SSCF was 78% of theoretical maximum based on total sugars (Glucan + Xylan).

Addition of H_2O_2 into the SAA treatment (modified SAA) raised the level of delignification and digestibility of hybrid poplar. The effectiveness of the modified SAA was highly sensitive to the operating temperature. High temperature favors delignifcation by aqueous ammonia, but causes rapid decomposition of H_2O_2. A slight improvement in delignification and enzymatic digestibility was observed when the temperature was

increased from 60°C to 120°C. Step change of temperature (60°C for first 4 hrs for effective oxidative reaction and 120°C for rest 20 hrs for delignification) improved delignification to above 60%, but decomposition of carbohydrates occurred. Further optimization in operation scheme and process conditions is necessary to properly assess the effectiveness of hydrogen peroxide addition in the pretreatment of high lignin substrate by aqueous ammonia.

FIGURES AND TABLES

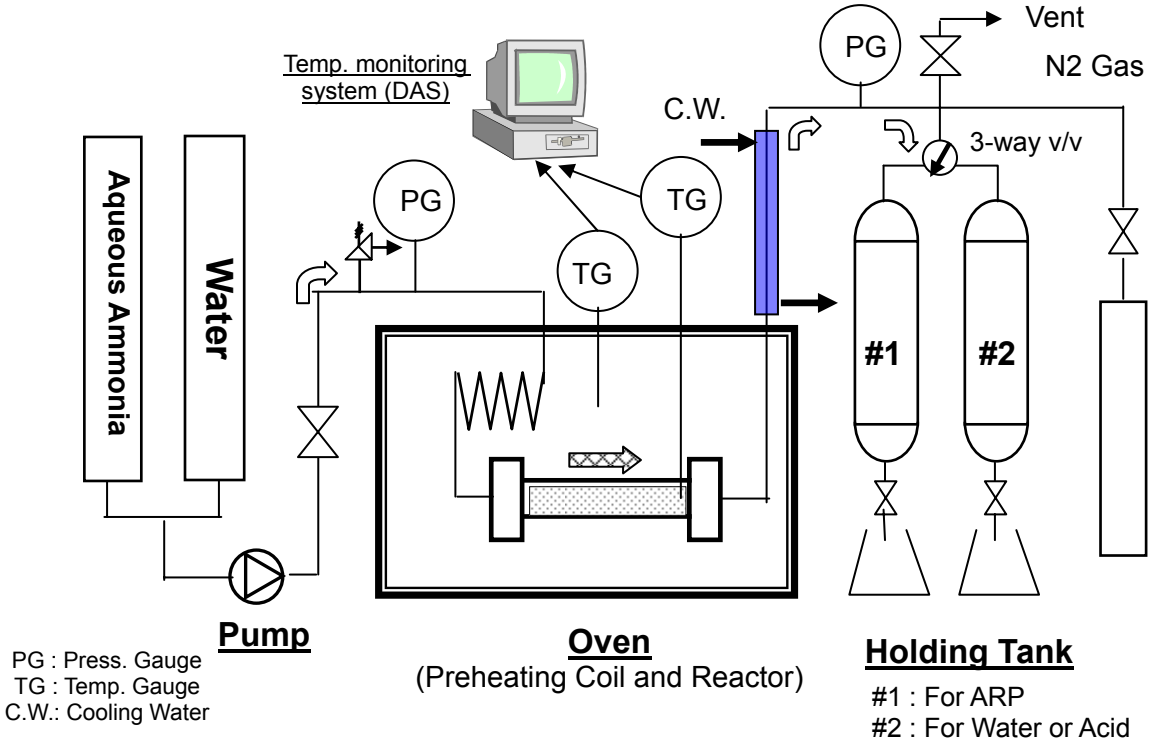


Fig. IV-1. Schematic diagram for ARP reactor set up (Kim et. al., 2003)

		Untreated	ARP treated
Total intrusion volume	ml/g	1.6952	2.372
Total pore Area	m²/g	11.684	15.268
Average pore diameter	µm	0.5804	0.6214
Apparent density	g/ml	1.4192	1.3949
Porosity	%	70.6378	76.7896
BET Surface Area	m²/g	0.6509	3.147

Table IV-1. Surface properties of untreated and ARP treated hybrid poplar

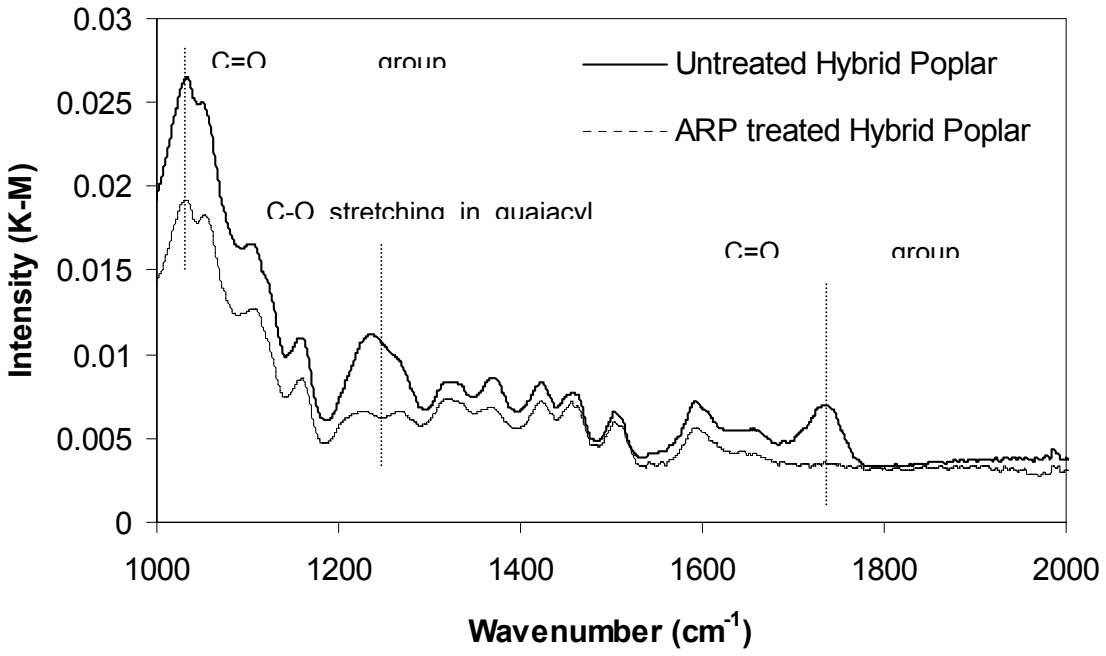


Fig.IV-2. FTIR spectra of untreated and ARP treated hybrid poplar

Enzyme Loading					Digestibility	
Cellulase (FPU)	Beta-G (CBU)	Xylanase (mg)	Pectinase (mg)	Total Protein (mg)	Glucan (%)	Xylan (%)
15	0	0	0	31.27	40.29%	46.61%
15	0	31.5	0	62.77	61.47%	71.92%
15	30	0	0	36.27	48.88%	51.08%
15	30	10.5	0	46.77	51.17%	59.34%
15	30	31.5	0	67.77	69.65%	73.35%
60	30	0	0	130.08	84.14%	68.78%
60	30	31.5	0	161.58	89.06%	81.96%
5	30	31.5	0	46.92	41.04%	59.33%
10	30	31.5	0	57.35	56.22%	70.05%
15	30	0	16.4	52.67	53.11%	59.92%
15	30	0	41	77.27	55.28%	62.85%

Table IV-2. Digestibility of ARP treated hybrid poplar with different enzymes combinations and loading.

Note: ARP treatment conditions for hybrid poplar: 185°C-2ml/min-27.5min -15% NH_3

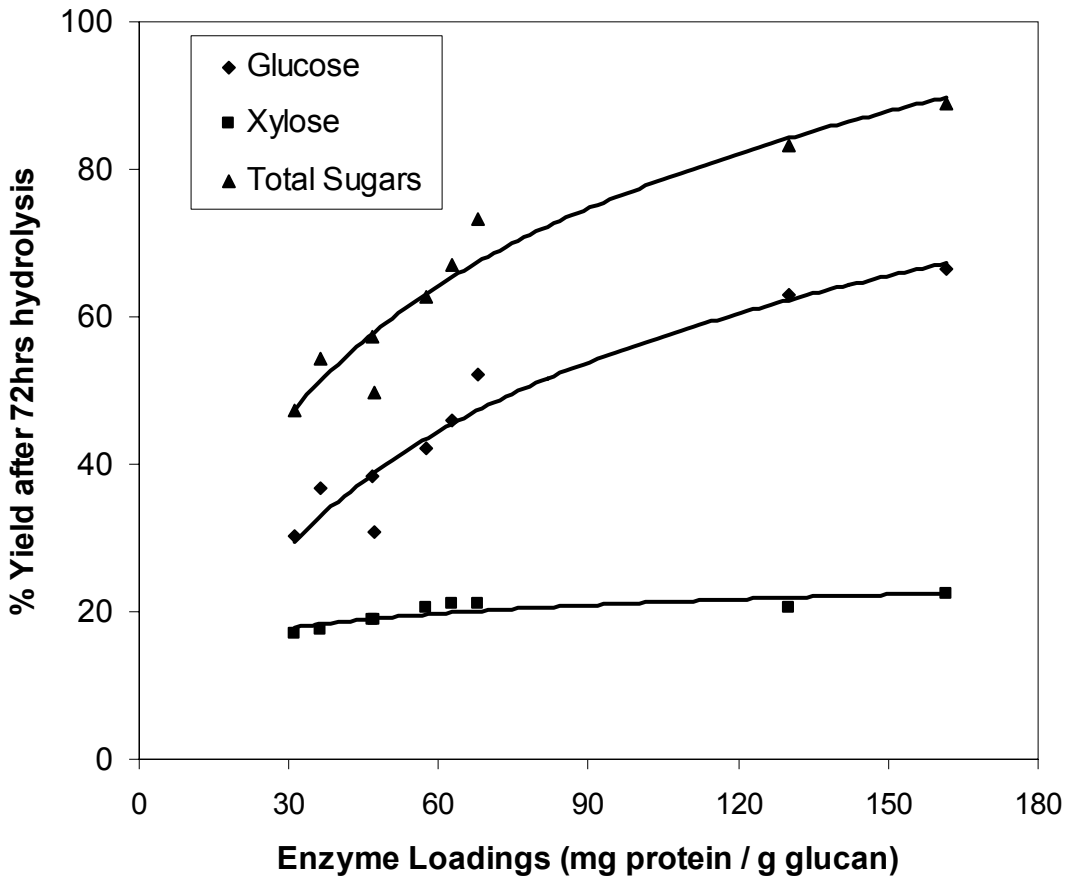
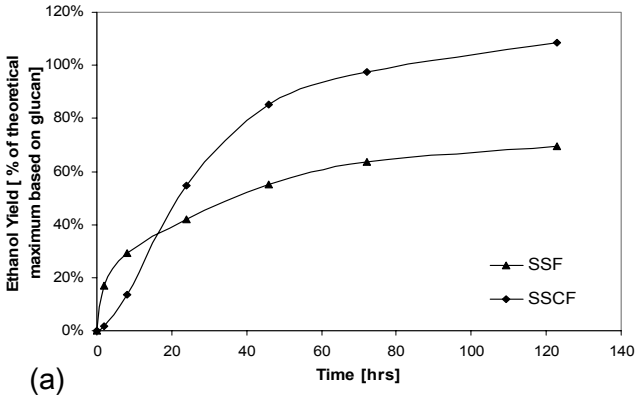
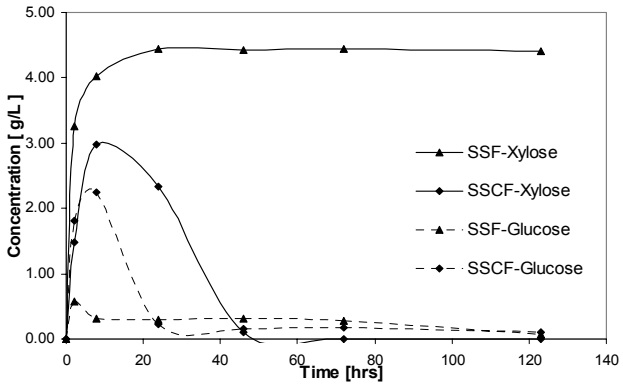


Fig. IV-3. Sugar yield with ARP treated hybrid poplar as a function of protein loadings

Note:
1. ARP treatment conditions for hybrid poplar: 185°C-2ml/min-27.5min -15% NH_3
2. Enzymes used for the hydrolysis are Cellulase, β-glucosidase and Xylanase
3. ARP treatment conditions for hybrid poplar: 185°C-2ml/min-27.5min – 15% NH_3



(a)



(b)

Fig.IV-4. a. Ethanol yield from SSF and SSCF of ARP treated hybrid poplar
b. Profile of Glucose and Xylose sugars in SSF and SSCF of ARP treated hybrid poplar

Note: 1. Enzyme loading: (15FPU (Cellulase)+30CBU (β-glucosidase) +31.5 mg of xylanase) per gram of glucan
2. Solid loading : Equivalent to 3 gram glucan / 100ml
3. ARP treatment conditions for hybrid poplar: 185°C-2ml/min-27.5min -15% NH3

	Composition			Digestibiltiy			
				Without Xylanase		With Xylanase	
	Glucan	Xylan	Lignin	Glucan	Xylan	Glucan	Xylan
Untreated	43.8%	14.9%	29.1%	2.6%	1.7%	3.0%	1.6%
Scheme 1	43.5%	14.6%	24.5%	21.8%	36.2%	38.0%	58.3%
Scheme 2	43.2%	12.3%	18.4%	37.9%	50.8%	53.6%	72.0%
Scheme 3	43.4%	12.6%	23.1%	30.9%	44.2%	40.9%	62.5%

Table IV-3. Effect of H_2O_2 addition in SAA process at 120°C with following schemes:

Scheme 1: 120°C(0-24hrs)-without H_2O_2 addition

Scheme 2: 120°C(0-24hrs)-5% H_2O_2 (added at 0hrs with 15%NH_3 solution)

Scheme 3: 120°C(0-24 hrs)-5% H_2O_2 (added after 4hrs of adding 15% NH_3 solution)

Note:
1. Other pretreatment conditions: Reaction time (24 hrs); Solid/ Liquid ratio (1:10)
2. Enzyme Loading: Without Xylanase: 15FPU (Cellulase)+30CBU (β-glucosidase) / g glucan
With Xylanase: 15FPU (Cellulase)+30CBU (β-glucosidase) +31.5 mg of xylanase / g glucan

Temperature	Composition			Digestibiltiy			
				Without Xylanase		With Xylanase	
	Glucan	Xylan	Lignin	Glucan	Xylan	Glucan	Xylan
Untreated	43.8%	14.9%	29.1%	2.6%	1.7%	3.0%	1.6%
60°C	43.3%	14.1%	21.6%	26.5%	33.3%	34.0%	48.5%
80°C	42.3%	12.1%	19.6%	32.2%	36.8%	38.8%	60.9%
120°C	43.2%	12.3%	18.4%	37.9%	50.8%	53.6%	72.0%

Table IV-4. Effect of temperature in modified SAA process

Note:

1. Other pretreatment conditions: Reaction time (24 hrs); Solid/ Liquid ratio (1:10); H_2O_2 Conc. (5%)
2. Enzyme Loading: Without Xylanase: 15FPU (Cellulase)+30CBU (β-glucosidase) / g glucan
   With Xylanase: 15FPU (Cellulase)+30CBU (β-glucosidase) +31.5 mg of xylanase / g glucan

Temperature	Composition			Digestibiltiy			
				Without Xylanase		With Xylanase	
	Glucan	Xylan	Lignin	Glucan	Xylan	Glucan	Xylan
60°C	43.3%	14.1%	21.6%	26.5%	33.3%	34.0%	48.5%
80°C	43.8%	12.2%	18.2%	45.8%	53.1%	58.0%	74.0%
120°C	36.7%	7.1%	12.0%	68.5%	65.8%	82.6%	85.6%

Table IV-5. Effect of temperature in modified SAA process

Note: 1. Other pretreatment conditions: Reaction time (24 hrs); Solid/ Liquid ratio (1:10); H_2O_2 Conc. (5%)
2. Temperature was kept at 60°C in first 4hrs of reaction and then raised to the shown value of in table for rest 20hrs.
3. Enzyme Loading: Without Xylanase: 15FPU (Cellulase)+30CBU (β-glucosidase) / g glucan
With Xylanase : 15FPU (Cellulase)+30CBU (β-glucosidase) +31.5 mg of xylanase / g glucan

H_2O_2 Conc.	Composition			Digestibiltiy			
				Without Xylanase		With Xylanase	
	Glucan	Xylan	Lignin	Glucan	Xylan	Glucan	Xylan
5%	36.7%	7.1%	12.0%	68.5%	65.8%	82.6%	85.6%
2%	42.0%	11.5%	15.0%	44.9%	54.2%	64.5%	79.5%

Table IV-6. Effect of H_2O_2 concentration in modified SAA process

Note: 1. Other pretreatment conditions: Reaction time (24 hrs); Solid/ Liquid ratio (1:10); Temperature (120°C)
2. Temperature was kept at 60°C in first 4hrs of reaction and then raised to 120°C in table for rest 20hrs.
3. Enzyme Loading: Without Xylanase: 15FPU (Cellulase)+30CBU (β-glucosidase) / g glucan
With Xylanase: 15FPU (Cellulase)+30CBU (β-glucosidase) +31.5 mg of xylanase / g glucan

	Composition			Digestibiltiy			
				Without Xylanase		With Xylanase	
	Glucan	Xylan	Lignin	Glucan	Xylan	Glucan	Xylan
Scheme 4	36.7%	7.1%	12.0%	68.5%	65.8%	82.6%	85.6%
Scheme 5	37.6%	10.4%	16.4%	53.4%	53.0%	73.3%	75.2%
Scheme 6	42.0%	11.5%	15.0%	44.9%	54.2%	64.5%	79.5%
Scheme 7	40.3%	12.7%	18.5%	40.6%	46.4%	52.7%	64.5%

Table IV-7. Effect of different H_2O_2 addition schemes in modified SAA process as described below:

Scheme 4: 60°C(0-4hrs)-120°C(4-24hrs)-5% H_2O_2 (added at 0hrs with 15%NH3 solution)

Scheme 5: 60°C(0-8hrs)-120°C(8-24hrs)-Intermittent addition of H_2O_2 (1.25% each times at 0,2,4 &6hrs)

Scheme 6: 60°C(0-4hrs)-120°C(4-24hrs)-2% H_2O_2 (added at 0hrs with 15%NH3 solution)

Scheme 7: 60°C(0hrs)-120°C(4hrs)-2% H_2O_2 soaking prior to adding NH_3(0hrs)

Note:

1. Other pretreatment conditions: Reaction time (24 hrs); Solid/ Liquid ratio (1:10)
2. Enzyme Loading: Without Xylanase: 15FPU (Cellulase)+30CBU (β-glucosidase) / g glucan
   With Xylanase: 15FPU (Cellulase)+30CBU (β-glucosidase) +31.5 mg of xylanase / g glucan

	Xylan		
	Solid	Liquid	Solid+Liquid
Untreated	14.85%	0	14.85%
Scheme 1	14.59%	0.30%	14.89%
Scheme 2	12.33%	1.25%	13.58%
Scheme 3	12.57%	1.21%	13.79%
Scheme 4	7.06%	1.46%	8.52%
Scheme 5	10.38%	2.53%	12.91%
Scheme 6	11.51%	1.71%	13.22%
Scheme 7	12.74%	2.06%	14.80%

Table IV-8. Distribution of xylan in solid and liquid after the pretreatment with different schemes

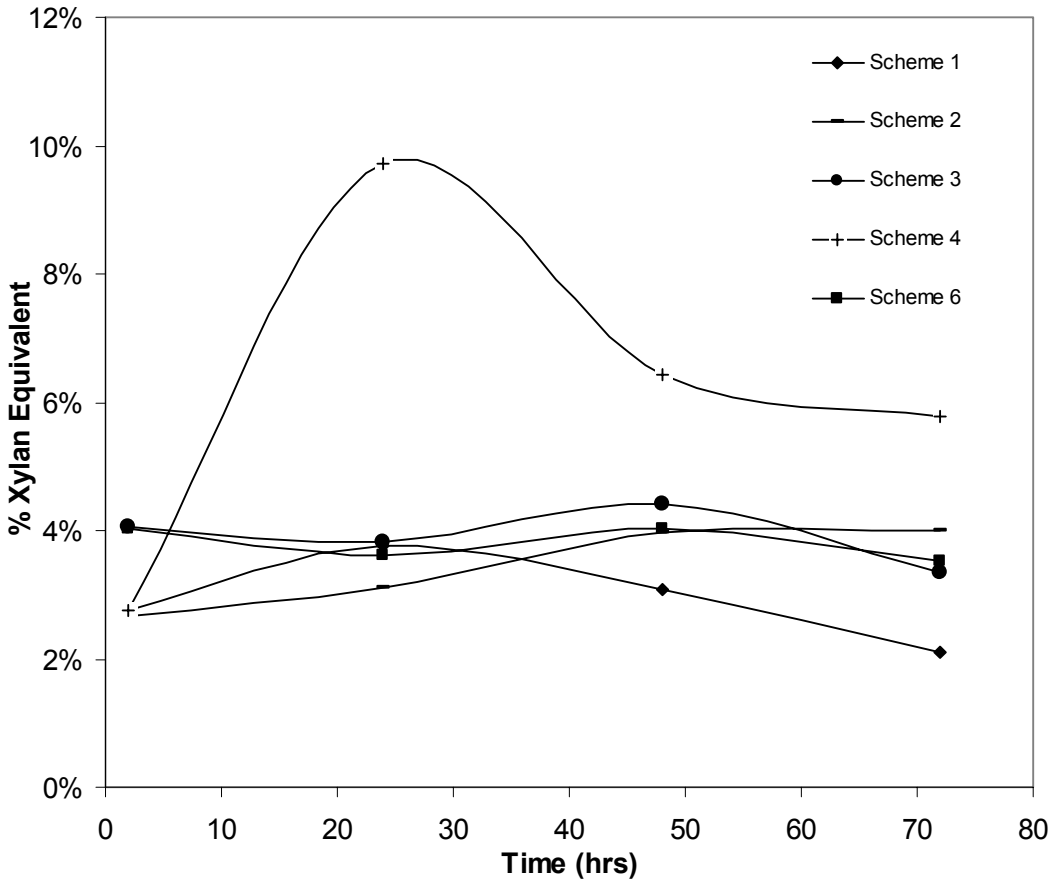


Fig IV-5. Profile of XOS in the enzymatic hydrolysis of treat hybrid poplar with different schemes of modified SAA

V. PRETREATMENT OF CORN STOVER AND HYBRID POPLAR BY SODIUM HYDROXIDE

ABSTRACT

NaOH and its derivatives are used as pulping reagents, wherein the spent NaOH is recovered in salt form and reused. In this study, low concentrations of NaOH (approx. 1-5%) were used for pretreatment of corn stover and two batches of hybrid poplar denoted as high lignin (HL) poplar and low lignin (LL) poplar. It was done with the understanding that NaOH can be recovered. In alkaline pretreatments, it is technically feasible to retain large amount of carbohydrates while removing a large fraction of lignin from the biomass. Lignin removal is the primary reason for the enhancement of enzymatic digestibility. Retention of hemicellulose after pretreatment gives a significant economic benefit since it eliminates the need for detoxifying hemicellulose sugars. The main objective of this study was to see if NaOH treatment can achieve that goal. In this study, the optimum process conditions were identified for each of the feedstocks on the basis of carbohydrate retention and digestibility of the treated solid. Corn stover, after being treated with NaOH under moderate conditions, attains near quantitative glucan digestibility. On the other hand, hybrid poplar required treatment with much higher severity, in terms of temperature and NaOH concentration, in order to attain acceptable level of digestibility. Supplementation of hydrogen peroxide in the pretreatment significantly raises

delignification and digestibility of hybrid poplar. It was also helpful in retaining the carbohydrates in the treated solids. Supplementation of xylanase to the cellulase during the enzymatic digestion of treated hybrid poplar has shown a profound increase in the digestibility of glucan as well as xylan.

INTRODUCTION:

The economical and environmental benefits that can be obtained by using cellulosic ethanol as an alternative to fossil fuel have led to extensive research in bioconversion of lignocellulosic feedstocks into ethanol. Pretreatment of biomass is an essential step for increasing the access of enzyme to cellulose and subsequently converting cellulosic fraction to sugars. Lignin, a major hurdle for cellulase access to carbohydrate fraction in biomass, also binds with the celluloytic and xylolytic protein fraction in cellulase and thus reduces the effective availability of enzymes to carbohydrates (Himmel et al., 2007; Yang et al.,2004). Alkaline reagents like ammonia and calcium hydroxide are found to be selective in reacting with lignin with mild effect on carbohydrate. Dilute acid treatment selectively removes the hemicellulose. Both modes of reactions have few common features which are essential in any pretreatment process i.e. an increase in internal surface area, porosity, and water retention in biomass thus increasing enzyme accessibility to the carbohydrate part of biomass (Mosier et al., 2005). But one factor which makes alkaline treatment more attractive is the carbohydrate retention in solids after pretreatment thus avoiding the processing of a contaminated liquid stream from pretreatment (Kim et al, 2005; Gupta et al., 2007).

NaOH has extensively been used in soda and kraft pulping for delignification of wood (Sjostrom et al.,1981). NaOH, both alone (Fan et al.,1981; Koullas et al., 1993) and with additives(Carr et al., 1984; Gould et al., 1984), has been employed to treat the biomass to increase its enzymatic hydrolysis. NaOH is known for its ability to delignify the lignocellulosic feedstock (Carr et al, 1984; Detroy et al., 1981). In alkaline pulping, lignin degradation occurs due to the breakage of aryl ether linkages which constitute approximately 50-70% of total linkages. Diaryl ethers and carbon-carbon bonds are relatively stable. Hydroxyl ions catalyze the cleavage of ether linkages in the lignin and thus liberate the soluble sodium phenolates in the liquid. The breakage of these bonds increases the hydrophilicity of lignin (Alen et al., 2000). High alkalinity of the NaOH reagent causes solvation of hydroxyl groups in carbohydrates and creates the swelling effect in sugar residue (Fengel and Wegener, 1984). This further increases the diffusivity of the reagent through the capillaries. These factors combined would help to increase the effective adsorption of the enzyme molecule in NaOH treated biomass, thus increasing the accessibility and digestibility of the carbohydrate fraction in the biomass (Carr et al, 1984; Detroy et al, 1981).

During the NaOH pretreatment of biomass, degradation of carbohydrates is a big problem which affects the sugar yield in pretreated solids. The enzymatic hydrolysis of carbohydrates carried away to pretreatment liquor is very difficult due to the presence of inhibitors and cost associated with the neutralization. NaOH promotes the carbohydrate degradation by a peeling mechanism in which one sugar residue is removed from the reducing end and this peeling reaction stops when the reducing end is converted to an

alkali stable carboxyl group. Both the peeling and stopping reaction are competitive reactions and the comparative rate of these reactions depends upon the nature of substrate, process conditions and reagent. This points toward the possibility to optimize the process parameters in such a way that the loss of polysaccharides is minimized and the hydrolysis rate is maximized. The rate of peeling reaction in hemicellulose depends upon its constituents. Glucomannans, the main hemicellulose in softwoods, are more susceptible to carbohydrate peeling the xylans, the main hemicellulose in hardwood and agriculture residue (Sjostrom et al.,1981). In this work, hybrid poplar and corn stover are employed for NaOH treatment and, as xylan is the prominent hemicellulose component in these feedstock, the possibility of minimizing the carbohydrate loss is greater.

The peeling reaction of carbohydrates can be stopped if the reducing end group can be oxidized to the alkali stable carboxyl group or reduced to hydroxyl group. Different reducing chemicals such as sodium borohydride or sodium dithionite and oxidizing agents such as anthroquinone derivatives, polysulfide, ozone, and hydrogen peroxide can be used to stabilize the carbohydrate chain ends (Sjostrom et al., 1981; Alen et al., 2000).

The function of hydrogen peroxide in the lignin degradation reaction of microorganisms has been discussed elsewhere (Gould et al., 1984). Hydrogen peroxide is also used in the bleaching of pulp. Gould et al used the alkaline hydrogen peroxide to enhance the delignification of different biomasses and obtained favorable results, but he did not discuss the effect of hydrogen peroxide in carbohydrate retention. The addition of hydrogen peroxide in the NaOH treatment leads to the oxidative delignification which

causes less environmental impact than normal soda pulping. Oxidative delignification causes the breakage of carbon-carbon linkages in lignin along with the cleavage of the aryl ether bonds. Oxidative action of H_2O_2 under alkaline conditions oxidizes the carbohydrate reducing ends which are resistant to the peeling reaction (Alen et al., 2000). In this study, NaOH reagent was used for pretreatment of corn stover and two batches of hybrid poplar, High lignin (HL) hybrid poplar and Low lignin (LL) hybrid poplar. The effect of the addition of hydrogen peroxide to NaOH treatment was also studied. The composition and physical characteristics of these feedstocks are very different. Our previous work with the ammonia pretreatment reagent found that carbohydrate retention strongly depends on the feedstock composition (Gupta et al., 2007). This study will help to understand the effects of different variables on biomass degradation reactions using the strong alkali media without and with oxidative conditions.

MATERIAL AND METHODS:

Substrate and Reagent:

Corn stover was supplied by NREL. It was ground and screened. The fraction collected between 9 and 35mesh was used in all experiments. Both batches of hybrid poplar were supplied by NREL in the form of small chips with a particle size of less than 0.25". Moisture content of both hybrid poplar batches was approximately 50%. Hybrid poplar was used for pretreatment as received.

Enzymes:

Cellulase enzyme (GC 220) and Xylanase (Multifect xylanase) were obtained from Genencor International Inc (Paulo Alto, CA). The reported filter paper activity of GC 220 was 90 FPU/ml, and protein content of Multifect xylanase was 42mg of protein/ml. β-Glucosidase was purchased from Sigma (Novozyme 188 from Novo Inc., Sigma catalog no. C-6150 and lot no. 11K1088) with activity of 750CBU/ml (measured in our lab).

Experimental procedure for NaOH treatment:

NaOH treatment of corn stover and hybrid poplar was done in batch process. Screw -capped laboratory glass bottles were used for lower temperature operation (maximum temperature: 60°C) and stainless steel reactors (ID: 1.375" and Length: 6") were used for higher temperature. Biomass was soaked in specified concentration of NaOH with a S:L ratio of 1:10 and kept in convection oven for pretreatment. If not stated otherwise, treatment time was 24hrs. Apart from high pressure in the reactor, there can be increase in the liquid volume upto 5-10% (based upon water) of initial volume due to high reaction temperature. Owing to this reason, reactor should only be filled maximum upto 75% of total reactor volume.

Enzymatic digestibility:

All the experiments for enzymatic digestibility of treated and untreated biomass were done with 1g glucan loading/100ml of total reactant volume. Thus the total amount

of solids in the reactor varied according to the glucan content in biomass. The reaction of enzymatic digestibility was carried out in 250ml Erlenmeyer flasks, and the total volume of reactant in these flasks was 100ml. Enzymatic digestibility reaction was carried out according to NREL LAP number 009 (www.eere.energy.gov./biomass/analytical_procedures.html). Maximum expected variation in digestibility values is within 3% of the reported values.

The digestibility was defined as the percentage of theoretical glucose released after 72 hrs of incubation with enzyme. Cellulase loading of 15FPU/g glucan and β-glucosidase loading of 30CBU/g glucan were used in all of the enzymatic digestibility experiments. Unless noted otherwise, xylanase loading was 31.5mg protein/g glucan.

Analytical procedures:

Composition analysis of the treated and untreated biomass was performed according to the NREL Laboratory analytical procedure (LAP): "Preparation of samples for compositional analysis" and "Determination of structural carbohydrates and lignin in biomass" (www.eere.energy.gov./biomass/analytical_procedures.html). Sugar analysis was carried out in duplicate for each sample and average values are reported. The moisture content in biomass was measured by an infrared moisture balance (Denver Instrument, IR-30). Sugar concentration during compositional analysis and enzymatic digestibility were determined by HPLC using the Bio-Rad Aminex HPX-87P column.

Scanning electron microscopy

Microscope pictures presented in this paper were taken using the Field Emission Scanning Electron Microscope (JEOL JSM-7000F) with a magnification of 300 and 350 for corn stover and hybrid poplar respectively.

RESULTS AND DISCUSSION

NaOH treatment of Corn stover

The SEM pictures of untreated and treated corn stover are shown in Fig.V-1. The difference in the surface of ammonia treated corn stover and NaOH treated corn stover is very clear. The surface of the NaOH treated sample a looks little darker and more deformed than the ammonia treated sample. Both the treated samples go through approximately same amount of delignification during the pretreatment and give equally high glucan digestibility. But the hemicellulose loss is higher in the NaOH treatment. The reactivity of ammonia towards hemicellulose in corn stover at low temperature is very less but still it is very effective in delignification (Kim et al, 2005). The reactivity of NaOH with hemicellulose is higher than that of ammonia as is evident from this study.

Fig V-2 shows the effect of NaOH concentration on the removal of carbohydrate and lignin. At very low 1% NaOH concentration, corn stover goes through approximately 65% of delignification and after the reaction, digestibility goes high to 82% as indicated in Fig.V-4. As concentration is increased, delignification and hemicellulose loss is also increased which proportionally helps in improvement of solid digestibility. The noticeable point here is that approximately 10% of the glucan is lost from the solid in

treatment even at very low concentrations of NaOH (Fig. V-2). This shows that the minimum loss of glucan in NaOH treatment is unavoidable. One possibility for this is that the lost glucan during pretreatment was a part of hemicellulose not of cellulose. Hemicellulose reactivity with NaOH is much higher than cellulose as hemicellulose has amorphous characteristics which lead to high accessibility of reagent molecule to the reactive site (Lai et al, 2001). It is already indicated that hexosan from hemicellulose during the alkaline treatment can be removed very easily (Gassan et al).

Fig.V-3 shows the distribution of glucan and xylan in solid and liquid stream after the NaOH treatment. It can be observed that there is some degradation of glucose in the liquid side as some of the glucan is unaccountable. Xylan looks stable at a low concentration of NaOH. As the NaOH concentration is increased, glucan and xylan both are degraded in the liquid (Data not shown).

Xylan digestibilities of NaOH treated corn stover are not very good and a maximum 71% digestibility has been achieved. As shown in Fig.V-5, an appreciable amount of xylo-oligosaccharides (XOS) are also present in liquid. These XOS were quantified by peak area in the HPLC chromatograph using peak area of xylose standard as reference. It was found that it accounted for 10-15% of total xylan present originally in the solids. These XOS are not converted to xylose monomer due to low β-xylosidase activity in the cellulase.

In order to prevent the glucan loss from the corn stover in the NaOH treatment, mild conditions (25°C-1% NaOH concentration) were tried. It was found that the there was less removal of glucan and xylan from solids under these conditions and most of the

carbohydrates were retained (Fig.V-6). The effect of xylanase supplementation on the enzymatic digestibility of corn stover treated with 25°C-1% NaOH concentration is shown in Fig.V-7. Glucan and xylan hydrolysis rates have been significantly improved to 83% and 76% respectively as a result of xylanase supplementation.

As shown in Fig. V-7 (a), the addition of 5% H_2O_2 in treatment of corn stover with a 25°C-1% NaOH concentration did not help in further delignification and removal of carbohydrates was also reduced. It was observed that the corn stover treated with H_2O_2 and NaOH at low temperature was very bright in appearance and was floating in the liquid after the pretreatment. It has also been indicated in other studies that under the alkaline conditions at low temperatures, relatively stable H_2O_2 only reacts with the chromophoric compounds in lignin and gives the bleaching effect. It does not help in degradation of the lignin network (Suchy et al., 2001). It seems the reaction of H_2O_2 with chromophoric compounds under alkaline conditions makes biomass more hydrophobic. This also explains why the biomass digestibility reduced after the H_2O_2 addition in NaOH treatment as evident from Fig. V-7(b).

Table.V-1 shows that approximately 74% of the total carbohydrates present in the untreated corn stover could be converted into the sugar monomers (sugars present in the pretreatment liquor are not added in this yield) after the mild NaOH treatment and subsequent enzymatic digestion with xylanase supplementation.

NaOH treatment of hybrid poplar:

Fig.V-8 to Fig.V-11. show the effect of treatment temperature and NaOH concentration on the composition and solid digestibility of NaOH treated hybrid poplar. An increase in treatment temperature increases the delignification as well as hemicellulose loss from the solids. Many physical and chemical changes occur in hemicellulose during the NaOH treatment such as swelling, dissolution, saponification, reprecipitation, peeling and glycosidic cleavage reactions. The rate of these competing reactions is mainly dependent upon the composition of hemicellulose (Lai et al, 2001). As observed here (Fig.V-8 and Fig. V-9) with hybrid poplar, hemicellulose degradation in NaOH treatment is primarily affected by the NaOH concentration. For a particular NaOH concentration, hemicellulose loss increases with increase in temperature but it is more sensitive to NaOH concentration. It is known that in the peeling mechanism of xylan, the rate of stopping reaction decreases, as temperature goes high (Lai et al, 2001). In general with hybrid poplar, hemicellulose loss is more affected by the NaOH concentration than the temperature in the pretreatment.

On the contrary, the delignification of hybrid poplar is not much affected by NaOH concentration at the lower temperature of 60°C and 80°C. But at higher temperature of 120°C, lignin removal from hybrid poplar is proportional to the NaOH concentration. To obtain a high degree of lignin removal, both, high temperature as well as high NaOH concentration, is required. In both the hybrid poplar samples, maximum delignification (>75%) is achieved at 120°C and 5%NaOH which helped in increasing the glucan digestibility of biomass to more than 95% (Fig. V-11 (c) and (f)).

At 1.5% NaOH concentration, the glucan loss in the LL hybrid poplar is constant but in HL hybrid poplar it steadily increases with an increase in the temperature. It can be concluded for HL hybrid poplar based on Fig.V-8, that appreciable glucan loss occurs at even the low temperature of 60°C. When temperature is increased further, glucan degradation increases though not proportionally with temperature. Below a particular concentration in alkaline degradation, minimum glucan loss occurs that is independent of temperature (Vuorinen et al.,1982). During the peeling reaction of cellulose in alkaline degradation, physical accessibility is a major factor in glucan degradation (Haas et al., 1967). The physical accessibility of the reagent to the reactive reducing end of cellulose is a function of cellulose crystallinity and surface area, which can vary by biomasses. As the temperature is increased, both, the physical accessibility of cellulose as well as the rate of peeling reaction, are increased which leads to more glucan loss.

As seen in Fig. V-9 (c) & (f), the rate of glucan degradation in pretreatment is not much affected by the NaOH concentration at the high temperature of 120°C. While at the low temperature of 60°C, glucan degradation decreases as NaOH concentration increases. This observation holds true for both the hybrid poplars. This observation confirms that the rate of the peeling reaction becomes constant after a particular concentration but rate of stopping reaction increases as NaOH concentration increases (Lai et al, 2001). This would lead to less degradation of glucan at higher NaOH concentrations. These observations and previous findings suggest that operating at a low temperature and a high NaOH concentration would be advantageous for a high yield of sugars.

This pattern of treated hybrid poplar digestibility and composition indicates that the enzymatic hydrolysis primarily depends upon the delignification which is only possible at very high temperature and NaOH concentration. One point worth noting is that at low temperatures, xylan digestibility of treated hybrid poplar is higher than glucan digestibility but as temperature increases to 120°C, glucan digestibility supersedes xylan digestibility (Fig. V-10). The reason for this is the generation of high DP XOS during the enzymatic digestion of treated solids; these are not converted to xylose because of less β-xylosidase activity in the cellulase. When external xylanase is supplemented in the enzymatic hydrolysis of treated hybrid poplar as shown in Fig.V-12, these XOS are converted to xylose monomers and xylan digestibility of the same solid treated at 120°C surpasses glucan digestibility.

The test of external xylanase supplementation was only performed on the hybrid poplar treated with low concentration of NaOH. Xylanase supplementation is more effective in increasing glucan hydrolysis of the treated solid, which contains higher amount of hemicellulose, and the retention of hemicellulose was higher in the hybrid poplar treated with a low concentration of NaOH. The maximum glucan digestibilities of the NaOH treated HL and LL hybrid poplar samples were 71.6 and 79.6% with xylanase supplementation (Fig. V-12). This shows that the residual lignin in these NaOH treated hybrid poplar samples is still a large obstacle in cellulase accessibility to carbohydrates.

Effect of H_2O_2 addition in the NaOH treatment of hybrid poplar

H_2O_2 was added to increase the delignification of hybrid poplar in NaOH treatment at lower temperatures. 5% H_2O_2 was added with 1.5% and 5% of NaOH at 80°C. Fig.V-13 shows the effect of H_2O_2 addition on NaOH treatment of hybrid poplar. H_2O_2 addition increases the delignification of HL hybrid poplar appreciably in both the concentrations of 1.5% and 5% NaOH. At 5% NaOH concentration, the addition of H_2O_2 addition helps in reducing hemicellulose loss from the solid.

In general, carbohydrate hydrolysis of NaOH treated hybrid poplar increased when H_2O_2 was added in the treatment process (Fig.V-14). The main reason for this increase was higher lignin removal caused by oxidative degradation in presence of H_2O_2. The increase in glucan digestibility was particularly very high for the HL hybrid poplar treated with 5%NaOH+5%H_2O_2. HL hybrid poplar has abnormally high amounts of lignin which imparts the biomass a very high recalcitrance for biological or enzymatic degradation (Himmel et al., 2007). In our previous study with ammonia treatment, it was not possible to attain a high degree of delignification in HL hybrid poplar even at the very high temperature of 190°C (Gupta et al., 2007). Without H_2O_2, at 120°C and 5% NaOH concentration, 80% of delignification was achieved but at the expense 50% of xylan removal from the HL hybrid poplar. When treated with 5%NaOH+5%H_2O_2, approximately 60% delignification was achieved in HL hybrid poplar at the very low temperature of 80°C and only 35% of xylan was lost to the liquid stream. Under the alkaline conditions, only aryl ether bonds are broken in the lignin while ester and C-C bond are very stable. When H_2O_2 is added in NaOH treatment, oxidative delignification

causes the cleavage of C-C as well and that is the reason for higher lignin removal (Lai et al, 2001.). The extent of lignin removal in LL hybrid poplar was much less than in the HL hybrid poplar in NaOH treatment both with and without H_2O_2 addition. As is evident from Fig. V-14, the digestibility of HL and LL hybrid poplar improved significantly because of the addition of H_2O_2.

As shown in Fig.V-15, NaOH/H_2O_2 treated hybrid poplar produces XOS during its enzymatic hydrolysis, while NaOH treated hybrid poplar does not. The reason for this might be the high rate of alkaline cleavage of glycosidic bonds in the presence of H_2O_2. When treatment is done only with the NaOH, the loss of hemicellulose sugar molecules is primarily because the peeling mechanism occurs through the reducing end and the rate of hydrolytic cleavage of glycosidic bond is much less. The enzyme hydrolyzes the glycosidic bonds in the hemicellulosic part of NaOH treated hybrid poplar, but the hydrolyzed segment is not solublized because of larger chain length and extensive cross linking. However in NaOH/H_2O_2 treated hybrid poplar, the length of the hydrolyzed segments of chain in hemicellulose is smaller because of more glycosidic cleavage in treatment, and these chains solublize as the XOS after the enzymatic action. These XOS are generated at the very beginning of the reaction and not consumed further during the reaction due to low β-xylosidase activity in cellulase. One of the reasons for high cellulose hydrolysis might also be the removal of these XOS. Once these XOS are removed, the cellulase accessibility to the cellulosic part would also increase. As both, the removal of these XOS and delignification, is higher in case of HL hybrid poplar, cellulose

digestibility of NaOH/H_2O_2 treated HL hybrid poplar is higher than that of treated LL hybrid poplar.

This indicates that by the introduction of H_2O_2 in the NaOH treatment, the rate of peeling reaction can be reduced because as an oxidizing agent, H_2O_2 helps in the formation of alkali stable reducing ends by converting aldehyde group into the acidic entity. On top of that, the presence of an oxidizing agent also increases the rate of hydrolytic cleavage. Cleavage of glycosidic bonds does not enhance the loss of hemicellulose in the pretreatment process but improves the possibility of solubilization XOS in the enzymatic hydrolysis process. This further helps in increasing the cellulase accessibility to cellulose and improves the enzymatic hydrolysis rate.

TableV-2 shows the amount of NaOH unaccounted for after the pretreatment process. This amount was calculated by the titration of pretreatment liquor. There are two possible reasons for this loss. First, the amount was consumed in the reaction with biomass components and second, it was used in neutralization of acidic components released from the solids during the pretreatment. When H_2O_2 is not added in the treatment, approximately 30% NaOH is unaccounted for. This increases substantially when H_2O_2 is added with NaOH in the treatment. Regardless of these reasons for NaOH loss, it can be concluded that the reactivity of NaOH was increased in the presence of H_2O_2. As we have already observed, the loss of hemicellulose was reduced and the rate of delignification was increased due to the addition of H_2O_2. This points out that the selectivity of alkaline degradation reaction towards the lignin was increased substantially. It is already known that the extent of lignin removal under oxidative conditions is much higher than under

normal alkaline conditions (Gould et al., 1984). Gould et al. have also indicated that the in the alkaline peroxide reaction, lignin is the major site of chemical attack (Gould et al., 1984).

The increase in delignification of hybrid poplar because of H_2O_2 addition is more pronounced at higher NaOH concentration (Fig.V-13). The mechanism of the alkaline peroxide reaction was explained by Gould et al., who showed that pH in the reaction plays an important role in the reactivity of the NaOH/H_2O_2 mixture (Gould et al., 1985). As a result of hemicellulose degradation in the treatment, acetic acid and uronic acid are produced which reduce the alkalinity of the reagent. A high NaOH concentration would be required to maintain a high pH for proper reactivity of H_2O_2 with lignin.

Effect of Xylanase supplementation with H_2O_2/NaOH treated hybrid poplar

As indicated by Fig.V-16, there is a significant increase in the glucan/xylan digestibility of the H_2O_2/NaOH treated hybrid poplar in comparison to that of the only NaOH treated hybrid poplar. Increase in glucan digestibility is more pronounced in the solids treated with 5% NaOH. In all cases, the xylan digestibility is more than 90%. One of reasons for this increase in the xylan digestibility is the fast conversion of XOS present in the liquid to the xylose monomer. In the case of HL hybrid poplar, both glucan/xylan digestibilities approach the theoretical maximum. Glucan/xylan digestibilities of LL hybrid poplar are also more than 92%. High lignin was identified as the main hurdle for the good digestibility of HL hybrid poplar in our previous study with the ammonia treatment (Gupta et al., 2007). A high degree of lignin removal in the pulping process is

always associated with a large amount of sugar loss due to reaction with NaOH (Sjostrom et al., 1981). Similarly in this study, high amount of delignification was only achieved at the high temperature of 120°C and 5% NaOH which increased its digestibility to 96% (Fig. V-11). However, treatment involving high temperature and high concentration also caused a great loss of hemicellulose which affected the overall yield as shown in Table V-3. But the use of 5%H_2O_2 with 5% NaOH in the treatment reduced the pretreatment temperature, increased the hemicellulose retention, and increased the delignification thus enhancing the hydrolysis of the carbohydrates and the overall yield of sugars.

CONCLUSION:

NaOH was very effective in the delignification and improvement of cellulose hydrolysis in corn stover under very mild treatment conditions. 94% glucan digestibility can be attained from corn stover treated with 1.5% NaOH at 60°C for 24 hrs. Carbohydrate yield of corn stover can be improved by xylanase supplementation in the enzymatic hydrolysis of treated corn stover by cellulase. Both batches of hybrid poplar required much harsher conditions in the treatment than the corn stover. High amount of lignin removal (80%) was only achieved at high temperature (120°C) and high NaOH concentration (5%). This increased the enzymatic hydrolysis of glucan above 95% in the hybrid poplar. Because of the high severity used in the treatment, hemicellulose degradation was excessive which caused a low overall sugar yield. The addition of 5% H_2O_2 in the NaOH treatment led to a high overall sugar yield at a lower temperature. The treatment of hybrid poplar with 5% NaOH+5%H_2O_2 resulted in more retention of

carbohydrates in the solids, greater delignification, and a higher hydrolysis rate. Xylanase supplementation in the enzymatic hydrolysis of NaOH/ H_2O_2 treated hybrid poplar created a significant improvement in glucan/xylan digestibilities. In the case of HL hybrid poplar, digestibilities were very close to theoretical maximum value and for LL hybrid poplar, digestibilities were more than 92%. A higher retention of hemicellulose in solids and a high solid hydrolysis rate contributed to the increase of the overall sugar yield to approximately 80% for the both HL and LL hybrid poplar.

FIGURES AND TABLES:

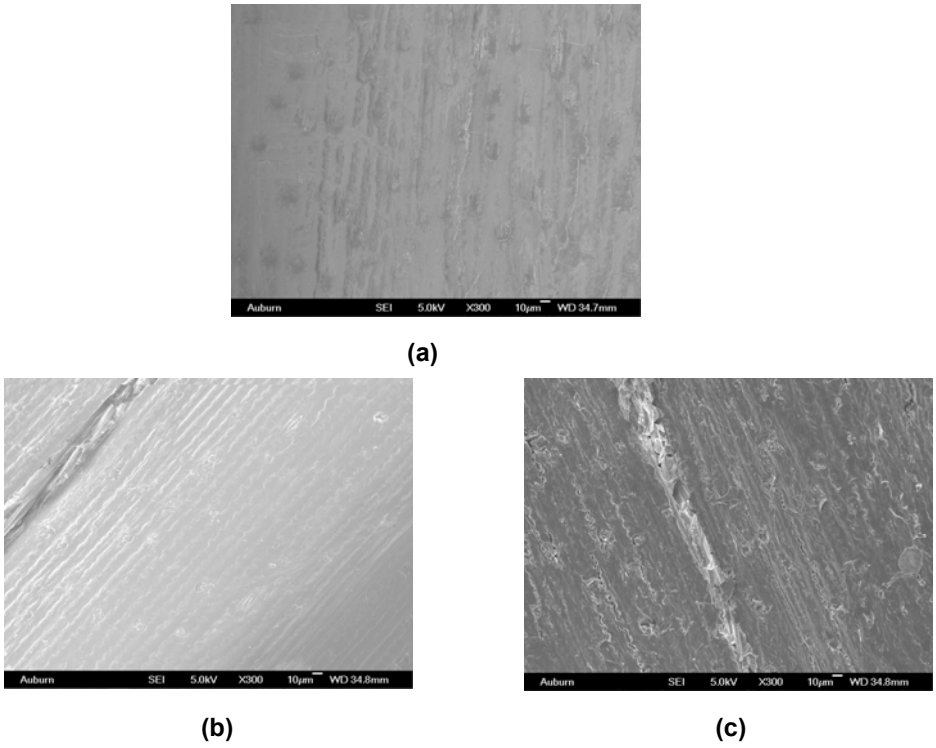


Fig.V-1. SEM Pictures of corn stover (a) Untreated (b) Treated with 15% ammonia (c) Treated with 1.5% NaOH

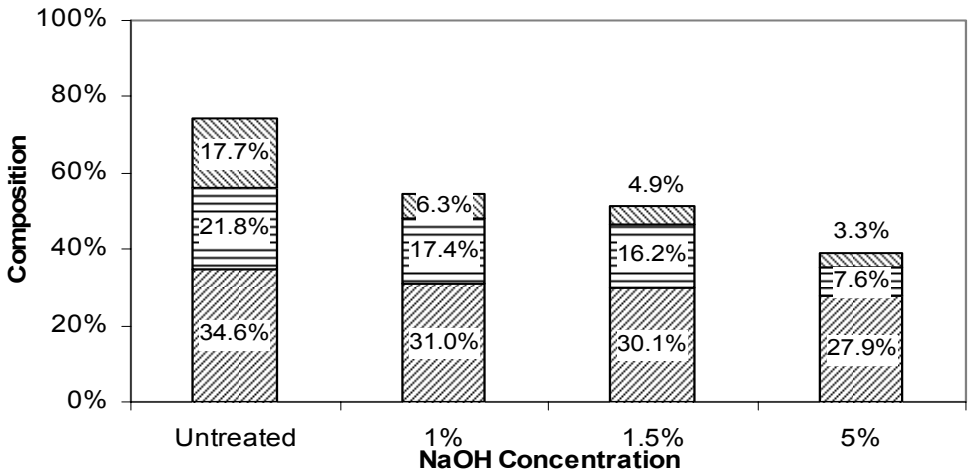


Fig.V-2. Effect of NaOH concentration on the composition of treated corn stover

Glucan Xylan Lignin

(All compositions are based on untreated dry corn stover)

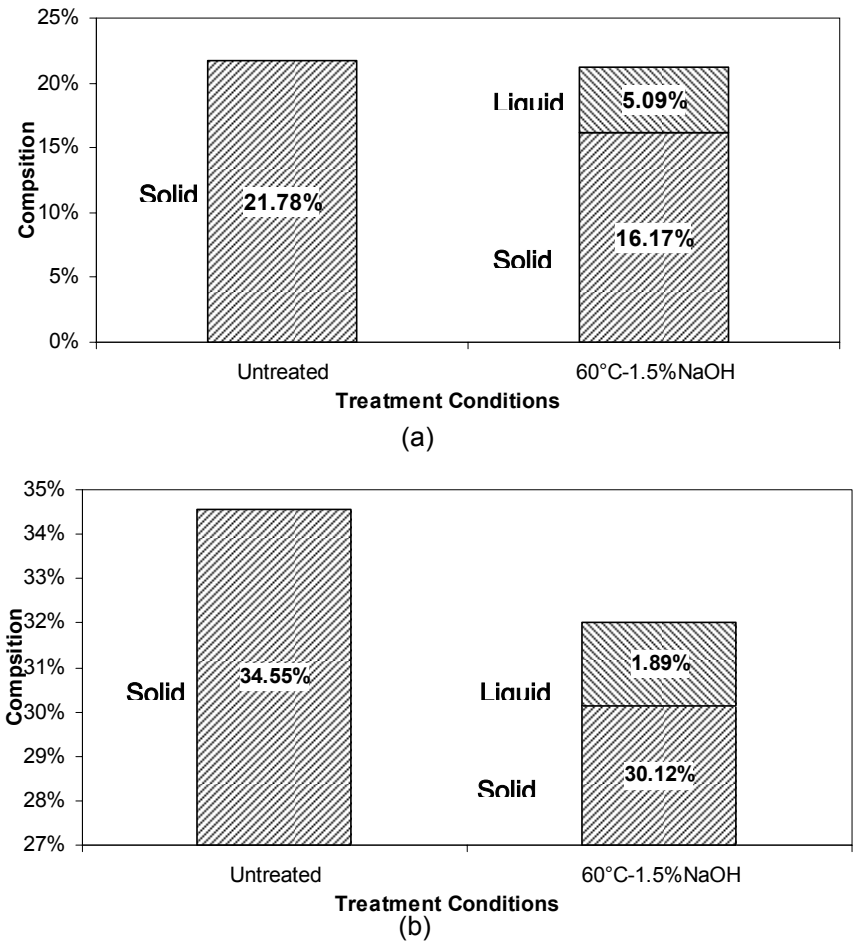


Fig.V-3. Distribution of sugars in solid and liquid stream after NaOH pretreatment
(a) Xylan (b) Glucan
(All compositions are based on untreated dry corn stover)

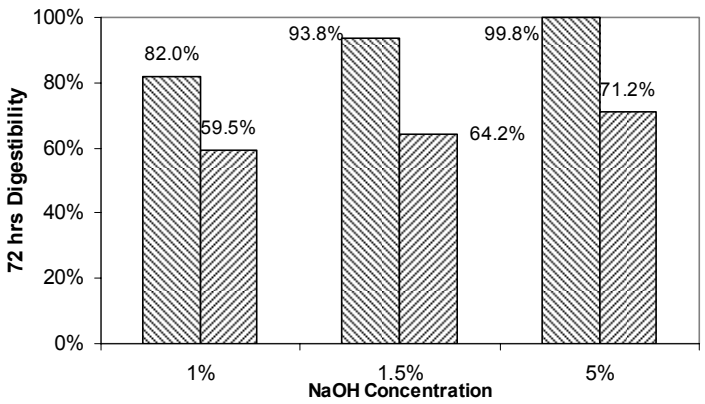


Fig.V-4. Effect of NaOH concentration Glucan and Xylan digestibility of treated corn stover

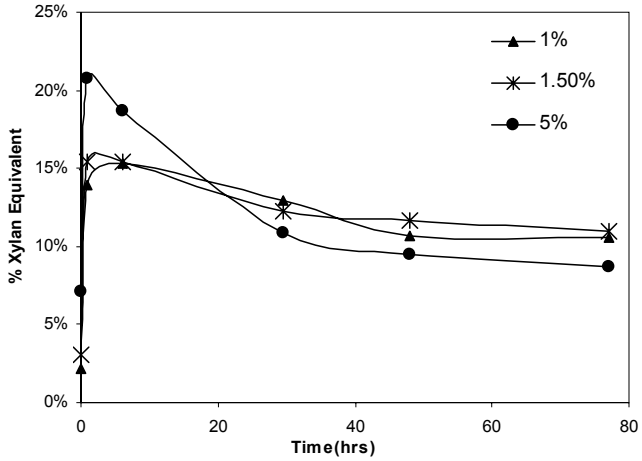


Fig.V-5. Profile of XOS in Enzymatic digestibility of corn stover treated with different Concentration of NaOH

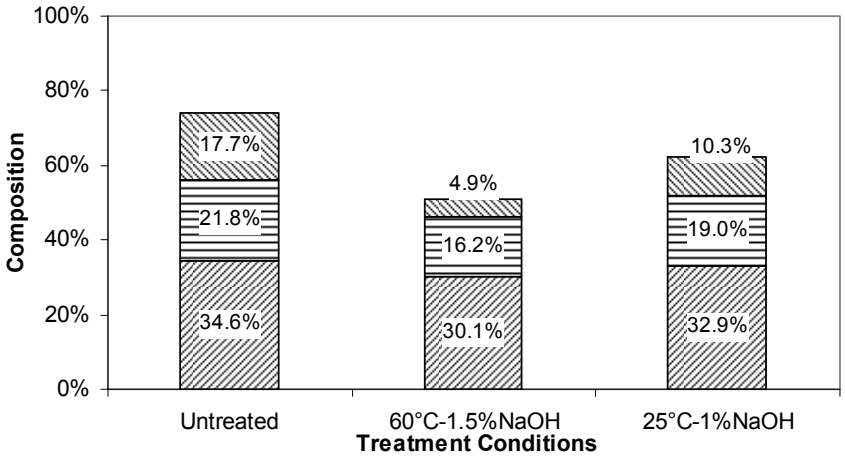


Fig.V-6. Composition change in corn stover treated with different conditions

Glucan Xylan Lignin

(All compositions are based on untreated dry corn stover)

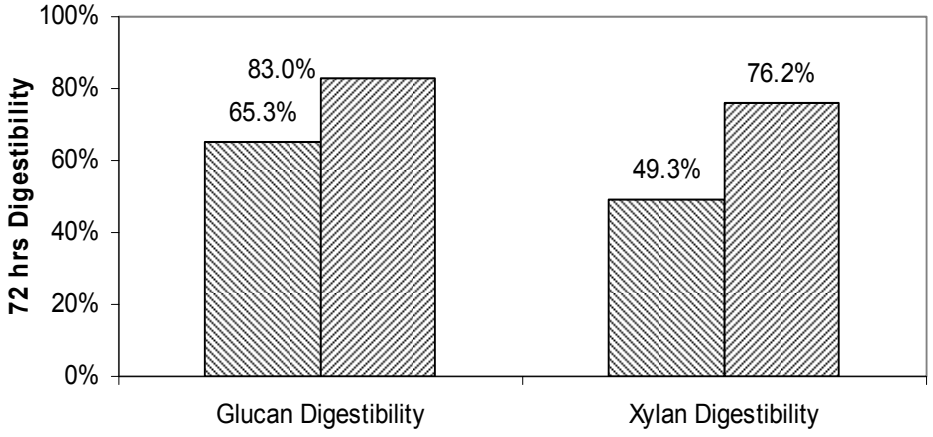


Fig.V-7. Effect of Xylanase supplementation on digestibility of NaOH treated corn stover at (25°C-1% NaOH).

Without xylanase With xylanase

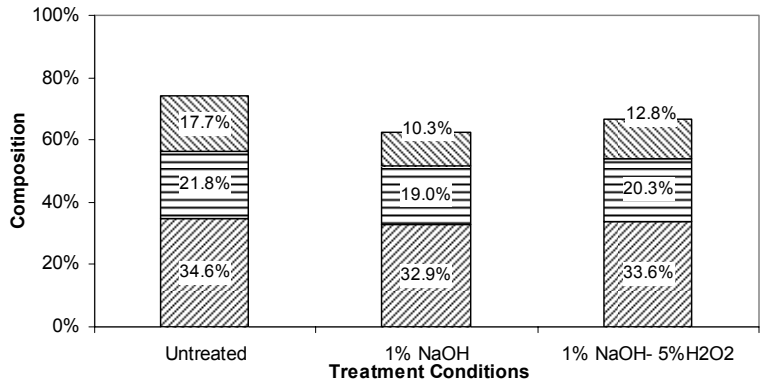


Fig.V-7a. Effect of H_2O_2 addition on corn stover composition in NaOH treatment

Glucan Xylan Lignin

Note: 1. All compositions are based on untreated dry corn stover
2. Other pretreatment conditions:
Temperature: 25°C; Reaction time:24hrs

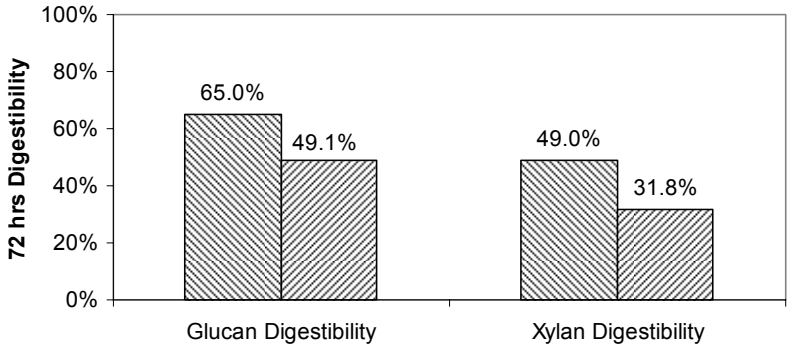


Fig.V-7b. Effect of 5% H2O2 addition on corn stover composition in NaOH treatment

With out H_2O_2 With H_2O_2

Note: 1. Treatment Conditions:
Temperature: 25°C; Reaction time: 24hrs;NaOH concentration: 1%

	60°C-1.5%NaOH	25°C-1%NaOH	
	w/o xylanase	w/o xylanase	w xylanase
Glucan	81.74%	62.22%	79.05%
Xylan	47.69%	42.92%	66.37%
Total Sugar Yiled	68.58%	54.76%	74.15%

Table V-1: Overall sugar yield from corn stover with NaOH treatment

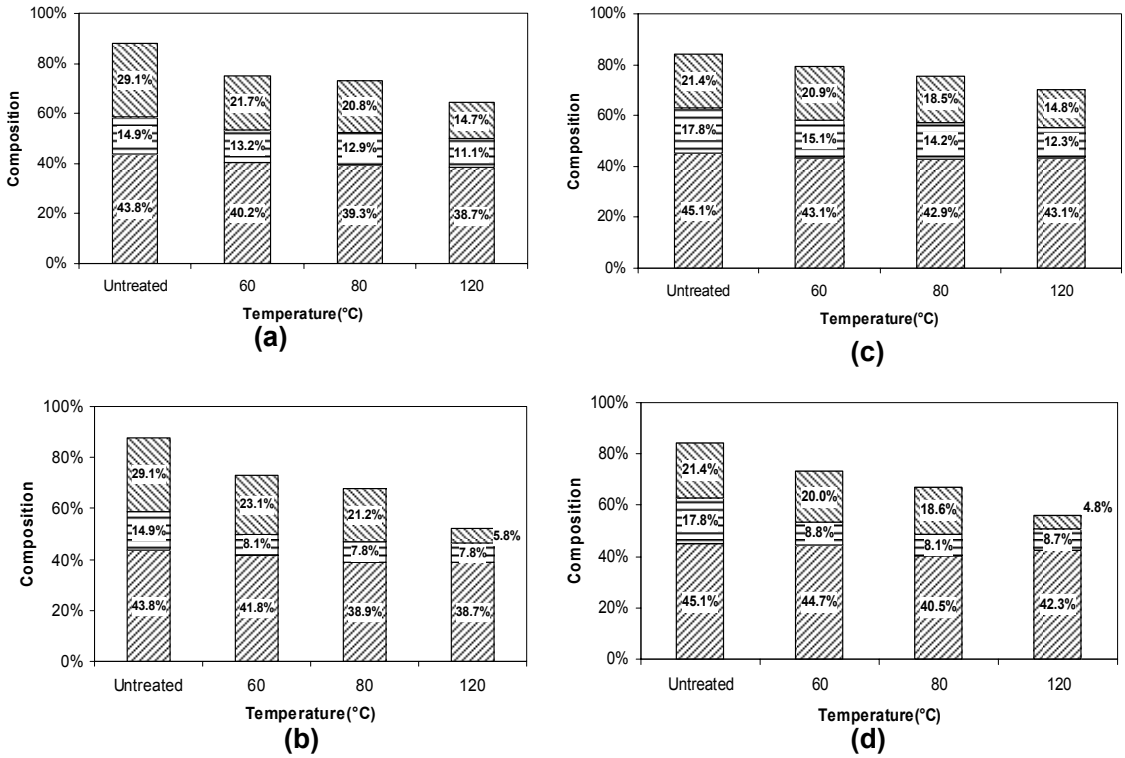


Fig.V-8. Effect of temperature at different concentration on the composition of treated HL hybrid poplar (a) 1.5% (b) 5% ;LL hybrid poplar (c) 1.5% (d) 5%

Glucan Xylan Lignin

(All compositions are based on untreated dry feedstock)

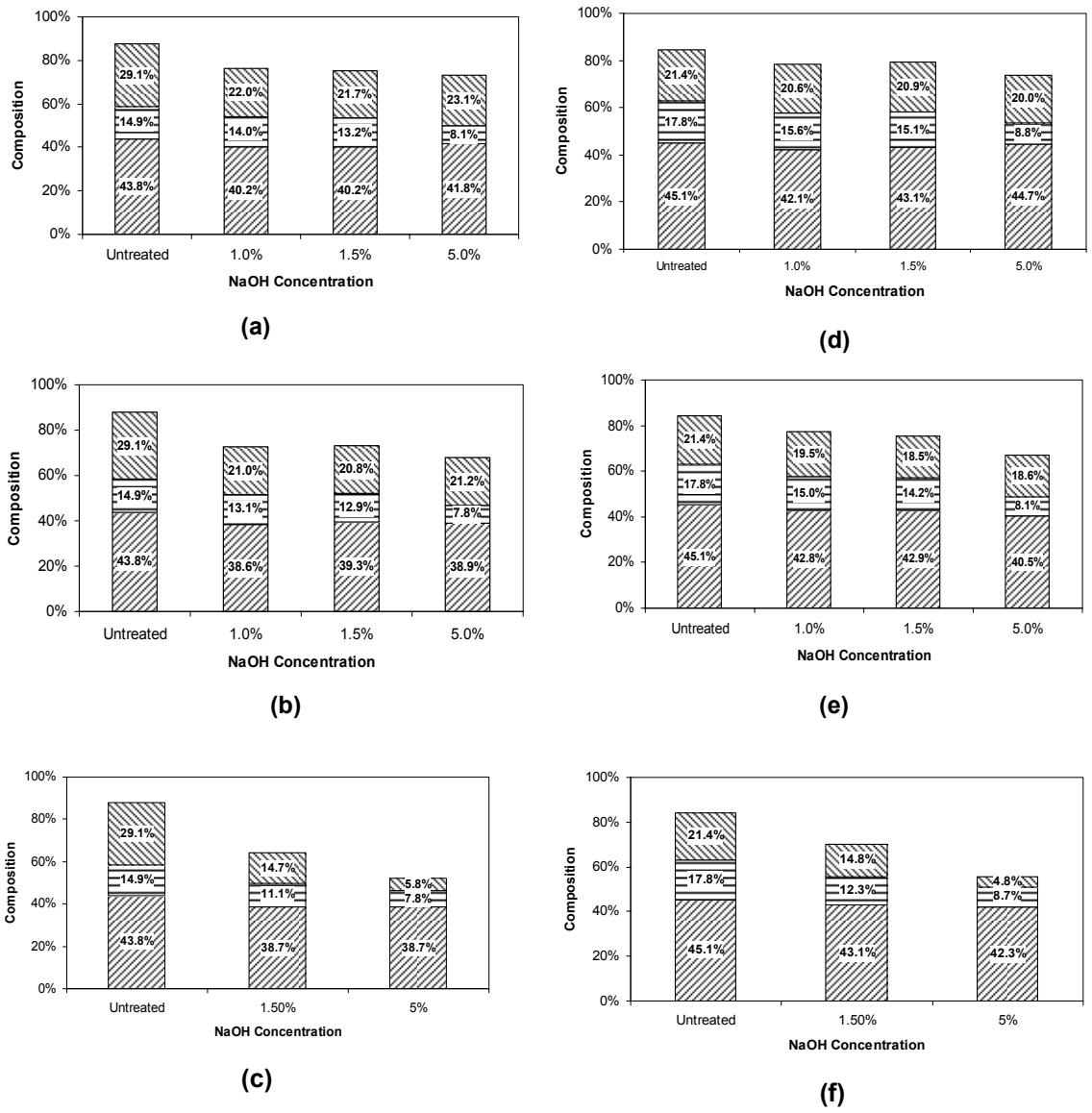


Fig.V-9. Effect of concentration at different temperature on the composition of treated HL hybrid poplar at (a) 60°C, (b) 80°C, (c) 120°C;
LL hybrid poplar at (d) 60°C, (e) 80°C, (f) 120°C

Glucan xylan Lignin

(All compositions are based on untreated dry feedstock)

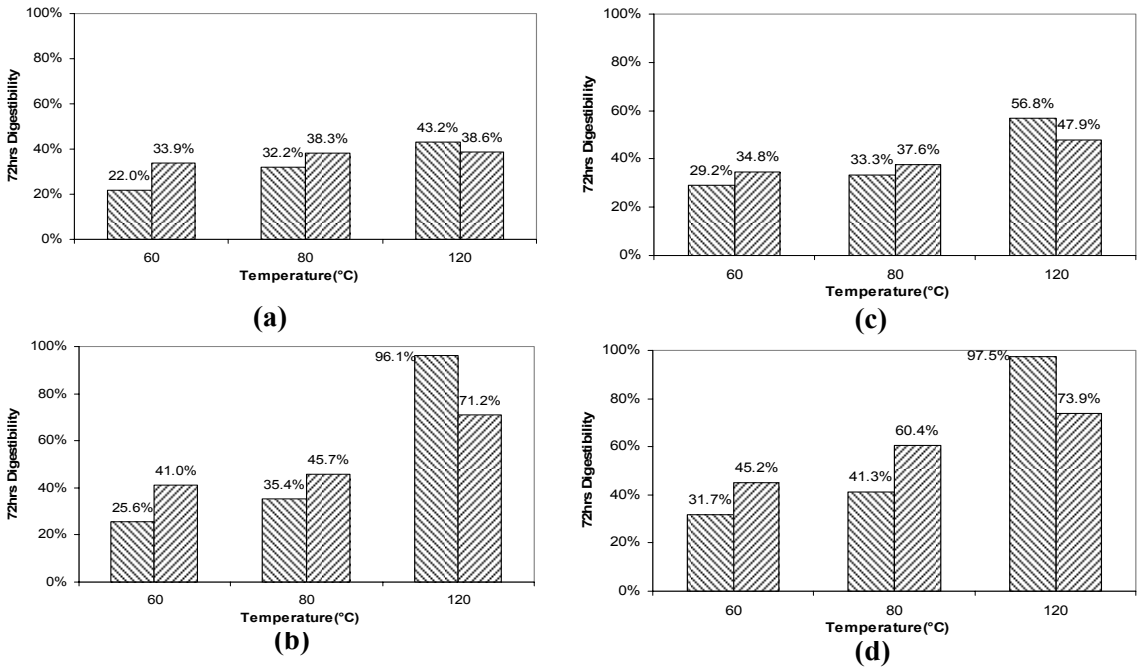


Fig.V-10. Effect of temperature at different concentration on the digestibility of treated HL hybrid poplar (a) 1.5% (b) 5%; LL hybrid poplar (c) 1.5% (d) 5%

Glucan digestibility Xylan digestibility

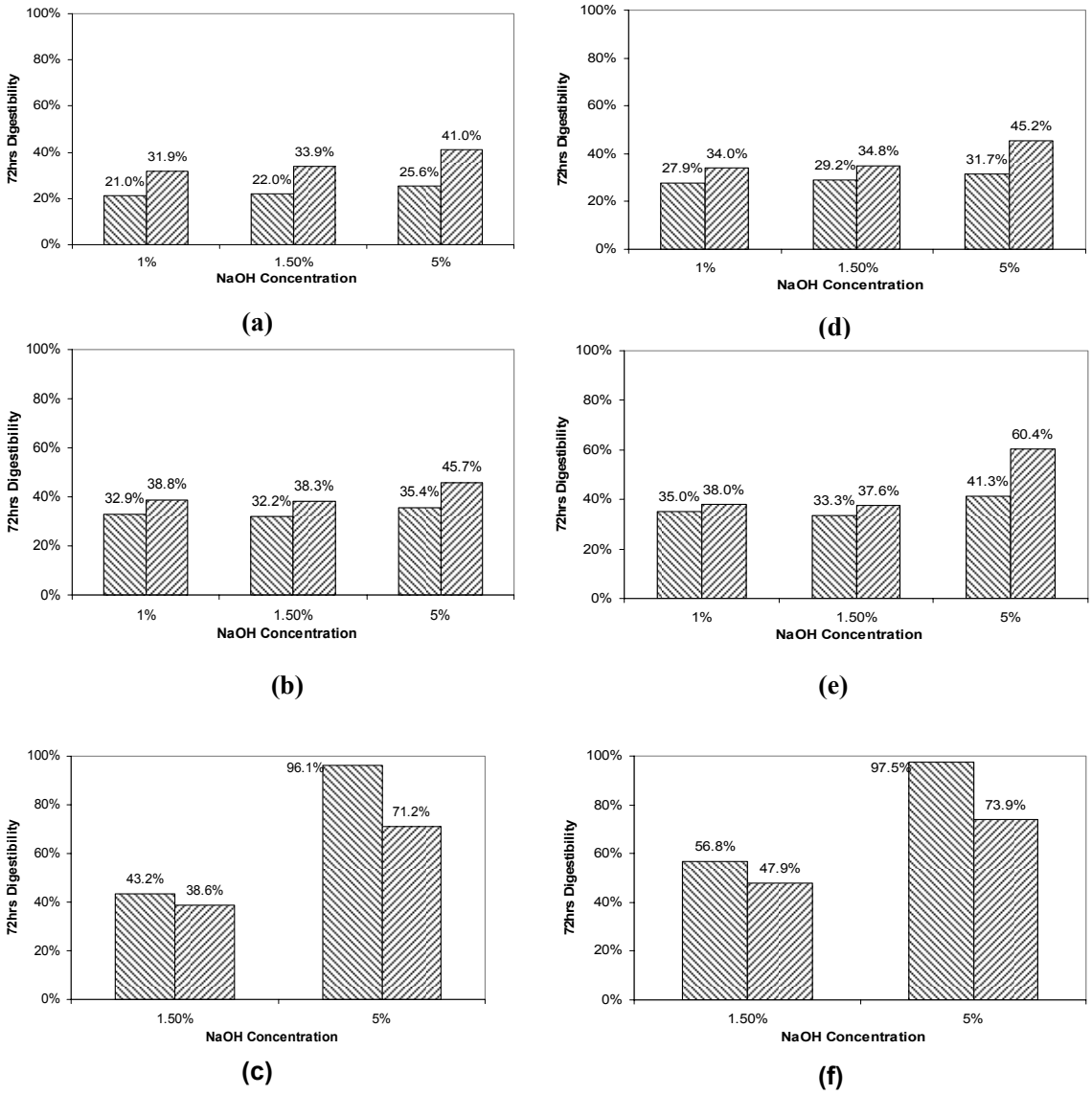


Fig.V-11. Effect of NaOH concentration at different temperature on the digestibility of treated:
HL hybrid poplar at (a) 60°C, (b) 80°C, (c) 120°C
LL hybrid poplar at (d) 60°C, (e) 80°C, (f) 120°C
Glucan Xylan

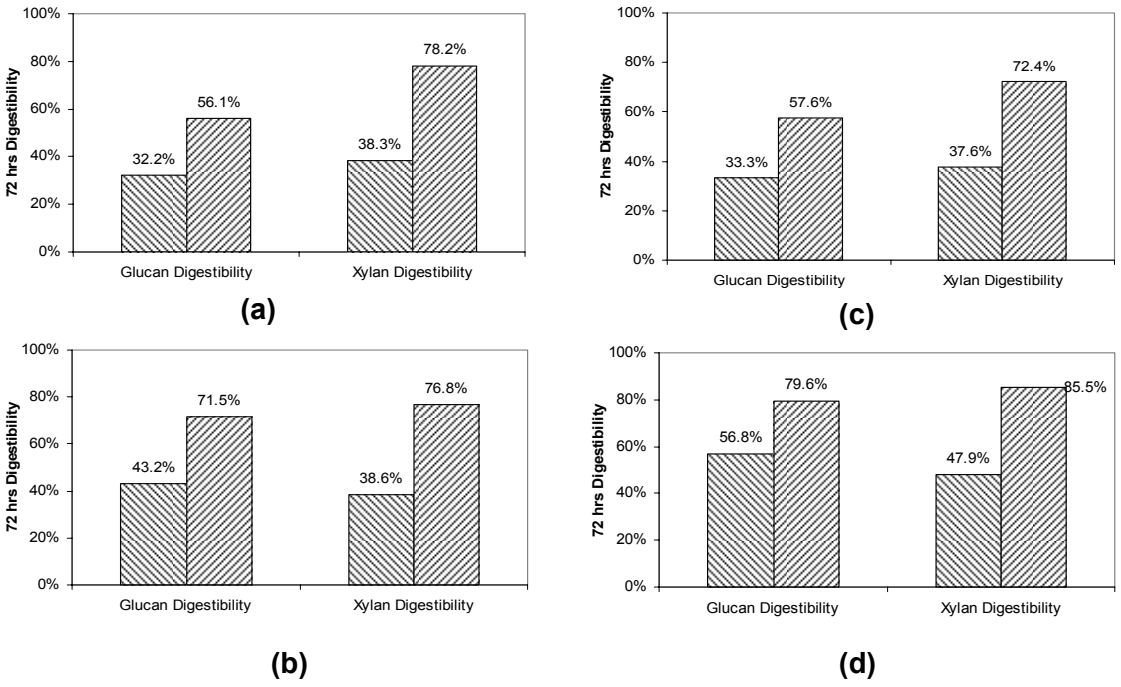


Fig.V-12. Effect of xylanase supplementation on digestibility of NaOH (1.5%) treated hybrid poplar
HL hybrid poplar (a) 80°C (b) 120°C ;LL hybrid poplar (c) 80°C (d) 120°C

without xylanase with xylanase

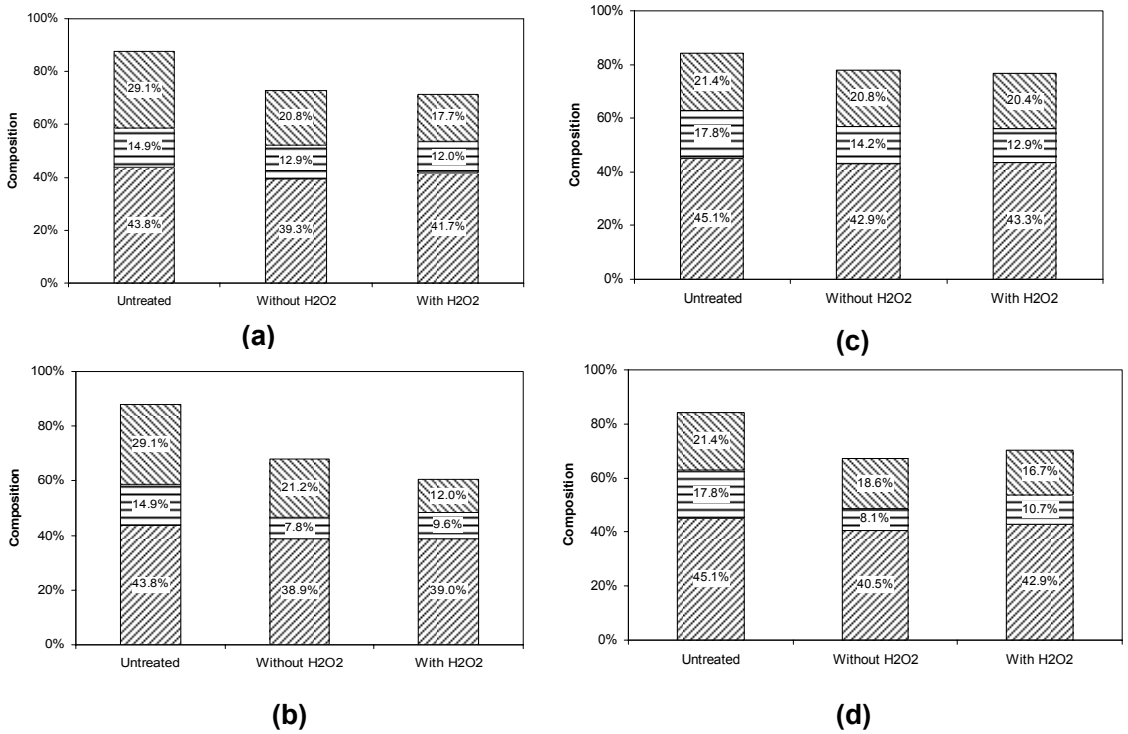


Fig.V-13. Effect of 5% H_2O_2 addition in NaOH treatment (80°C) at different NaOH concentration on composition of hybrid poplar
HL hybrid poplar at (a) 1.5% (b) 5%; LL hybrid poplar (c) 1.5% (d) 5%
Glucan Xylan Lignin

(All compositions are based on untreated dry feedstock)

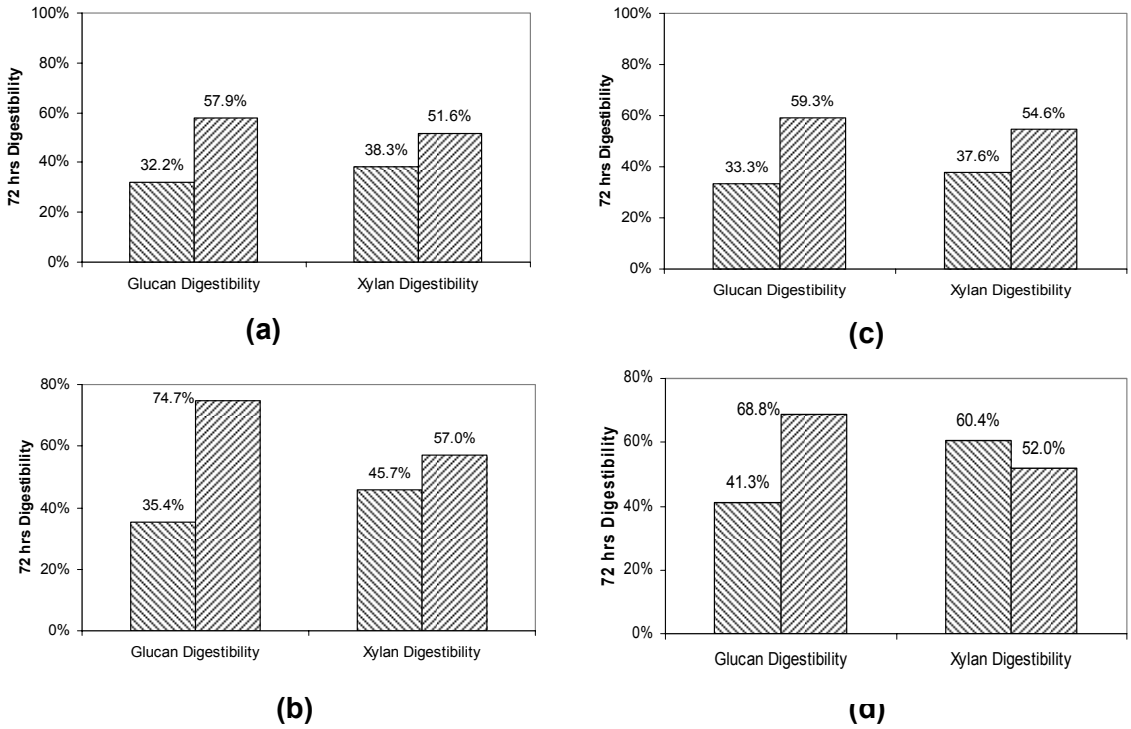


Fig.V-14. Effect of 5% H2O2 addition in NaOH treatment (80°C) at different NaOH concentration on digestibility of hybrid poplar
HL hybrid poplar (a) 1.5% (b) 5% ;LL hybrid poplar (c) 1.5% (d) 5%

Without H_2O_2 With H_2O_2

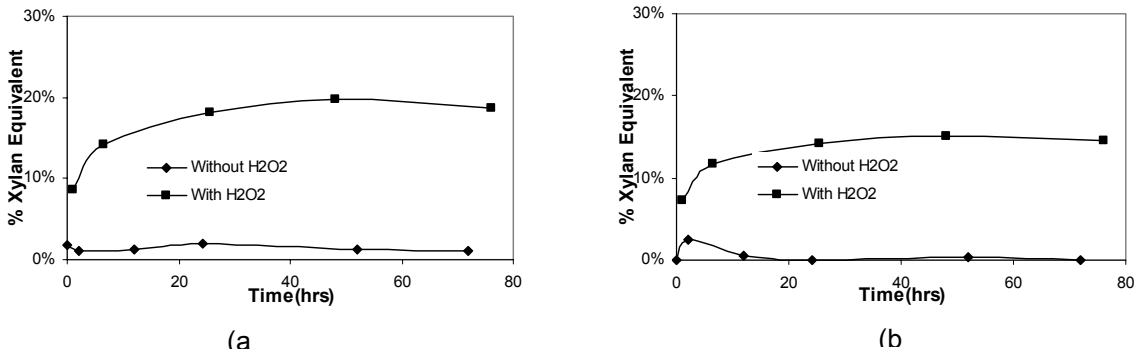


Fig.V-15. Profile of XOS during enzymatic hydrolysis of NaOH treated hybrid poplar
(a) HL hybrid poplar (b) LL hybrid poplar

	Corn stover	High Lignin Poplar	Low Lignin Poplar
Without H_2O_2	0.742	0.733	0.839
With 5% H_2O_2	1.512	1.201	1.352

Table V-2. Amount of NaOH unaccounted in liquid after NaOH treatment

* All values have unit of g NaOH / 10g of untreated biomass

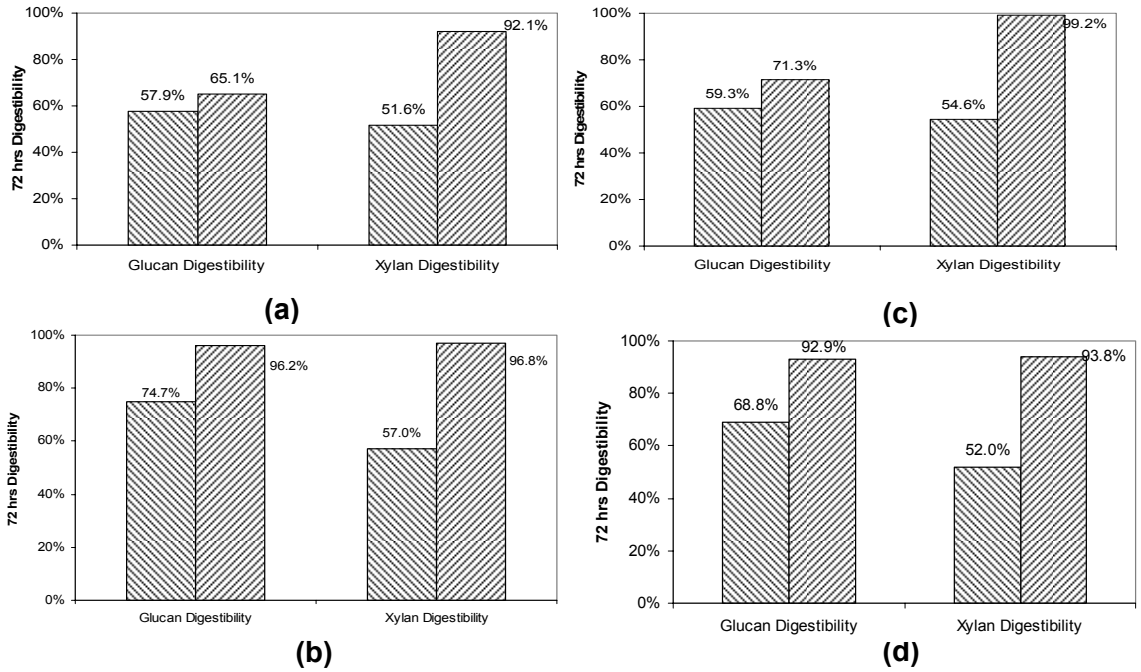


Fig.V-16. Effect of xylanase supplementation on digestibility of NaOH (80°C) 5% H2O2 treated hybrid poplar at different NaOH concentration
HL hybrid poplar (a) 1.5% (b) 5%; LL hybrid poplar (c) 1.5% (d) 5%

without xylanase with xylanase

		120°C-5%NaOH	80°C-5%NaOH-5% H2O2	
		w/o Xylanase	w/o Xylanase	w Xylanase
High Lignin Poplar	Glucan	84.92%	66.45%	85.53%
	Xylan	37.26%	36.83%	62.54%
	Total Sugar	72.85%	58.95%	79.71%
Low Lignin Poplar	Glucan	91.36%	65.43%	88.35%
	Xylan	36.19%	31.23%	56.33%
	Total Sugar	75.75%	55.75%	79.28%

Table V-3. Overall sugar yield for hybrid poplar with different treatment conditions

VI. STUDY OF THE CELLULASE MECHANISM USING PURE CELLULOSIC SUBSTRATES

ABSTRACT

Various forms of pure cellulosic substrates were utilized to study the reaction mechanism in cellulase-cellulose reaction. The substrates employed were micro-crystalline cellulose (Avicel), α-cellulose, filter paper, cotton, and non-crystalline cellulose (NCC). These substrates were first characterized with respect to degree of polymerization (DP) and crystallinity. NCC is a product of our laboratory. It is a highly amorphous cellulose with a crystallinity index of less than 10%. When hydrolyzed with cellulase, NCC produces a significant amount of cello-oligosaccharides (COS) as reaction intermediates, along with glucose and cellobiose. COS were categorized into two separate fractions: Low DP cello-oligosaccharides (LD-COS) and high DP cello-oligosaccharides (HD-COS). LD-COS, from DP 1-7, were detected by HPLC whereas HD-COS were estimated only after secondary hydrolysis of liquid. LD-COS are not detected in the hydrolysis of crystalline substrate. The average DP of NCC can be varied adjusting the process conditions in its preparation. NCCs with various DP were used as the substrate to study the dependence of enzyme action on DP. The substrate DP was found to affect the hydrolytic action of Exoglucanase as well as the maximum hydrolysis rate. The surface properties of the substrate play a significant role in the determination of

the adsorption rate of enzyme and the initial hydrolysis. However, the extent of maximum achievable conversion of cellulose depends primarily on its inherent characteristics such as DP and crystallinity.

INTRODUCTION

A high conversion yield in enzymatic hydrolysis reaction of biomass by cellulase is the prerequisite for making any cellulosic ethanol process a feasible one. Cellulase accessibility to the cellulosic part of biomass is the most important factor for high digestibility. The presence of other structural components (lignin and hemicellulose) and the physical characteristics of biomass (surface area, pore size distribution etc.) are the main factors that affect the cellulase accessibility to the cellulosic part in the biomass (Mosier et al., 2005). Apart from lignocellulosic biomass, reactivity of pure cellulosic substrate with cellulase is also not satisfactory and requires high enzyme loading for attaining an acceptable hydrolysis rate (Kim et al., 2003). This will contribute to a very high enzyme cost in the bioethanol production process. Generally the hydrolysis rate of cellulose decreases with time as the reaction proceeds (Yang et al., 2006). This indicates that cellulase accessibility or reactivity with the cellulose chain is somehow reduced. Though the literature offers a large number of possible explanations for this phenomenon, the exact mechanism involving the combined role of different enzymatic components and substrate features has not yet been established. Changes in substrate properties (such as Crystallinity index, DP, surface area or external and internal diffusion resistance), hydrolysis product (cellobiose, glucose) inhibition, substrate inhibition, enzyme

inactivation, and interference by irreversibly attached proteins on the substrate are a few of main reasons illustrated in the literature (Reese et al, 1977; Gan et al., 2004; Ramos et al,1993; Walker et al., 1991;Holtzapple et al.,1990; Converse et al.,1988; Eriksson et al., 2002).

In most of the studies done to understand the cellulase-cellulose reaction mechanism, typically a single substrate with crystalline property was used (Zhang et al.,2006). The BET surface area of crystalline substrates is lower than non-crystalline substrates, hence cellulase accessibility. Cellulase accessibility is governed by mass transfer resistances and effective cellulase adsorption area in cellulose. On the other hand, cellulase reactivity is controlled by the chemical characteristics of cellulose (Zhang et al., 2004). In order to understand the effect of intrinsic chemical features such as crystallinity and DP on the reactivity of various cellulolytic components, the physical barrier for cellulase accessibility must be overcome. NCC was chosen in this study with the objective to minimize these physical barriers and maximize the cellulase accessibility to the reaction site. Because of their varied characteristics, other cellulosic substrates (Avicel, Filter paper, α-cellulose and cotton) used in this study were very useful in understanding the effects of these chemical features. The substrates have been characterized in terms of their comparative DP and crystallinity. Subsequently, enzymatic action on these substrates has been studied.

In previous studies, investigation of cellulase mechanism was done by monitoring the formation of glucose and cellobiose (Eriksson et al.2002, Valjamae et al., 1998) or measuring the reducing ends by colorimetric method (Gan et al, 2003) or measuring the

weight loss of substrate (Valjamae et al., 1998) as methods to calculate the extent of saccharification. These monitored parameters can give an indirect indication about the action of individual proteins present in the cellulase but do not provide direct information about their action. Monitoring of cello-oligosaccharides (COS), which is an intermediate hydrolysis product in the reaction, has provided an additional tool to interpret the action of different functional enzymes present in cellulase.

In order to predict the action of an individual functional protein in cellulase, separation of these individual components was required. Subsequent reaction of these components, such as Endo-glucanase (Endo-G), Exo-glucanase (exo-G), with the cellulosic substrate provided the information about the action of that particular protein (Suurnakki et al., 2000; Valjamae et al., 1998). However, the result of individual enzyme reactions can be very much different from the result with cellulase mixture in which different supplementary non-hydrolytic proteins are also present during the reaction. Synergistic action of different cellulolytic proteins has been widely proposed in literature and these proteins can be hydrolytic or non-hydrolytic (Wood et al., 1975; Lynd et al., 2002). Instead of separating the cellulase components for reaction mechanism study, this study proposes that it would be better to categorize all proteins into different functional groups, use the whole cellulase mixture for reaction, and then hypothesize the action of one functional protein group based upon the its product profile. This approach was adopted in this study with the use of different cellulosic substrate.

Endo-G is known to react with the amorphous region of the cellulose, generating new reducing ends and producing the COS (Lynd et al., 2002). The profile of reducing

ends in solids and COS in liquid indicated the action of Endo-G. Exo-G action has been studied by profile of glucose and cellobiose. The available information on the structural features of cellulase enzyme has been correlated with the hydrolysis results.

MATERIALS AND METHODS

Substrates

Avicel PH101, which contains 96.5% of cellulose, was purchased from the Sigma (Cat. No.11365 and Lot No. 1094627). α-cellulose containing 76.4% of cellulose & 22.2% of xylan was also purchased from Sigma (Cat. No. C-8002 and Lot No. 114k0244). Whatman No.1 filter paper, whose cellulose content was more than 99%, was used in this study. Cotton sheets were provided by the Buckeye Technologies Inc. Before doing any experiment on cotton, it was blended and converted into fluffy uniform substrate.

Non-Crystalline Cellulose (NCC), which is produced by acid hydrolysis of cotton, is a product of our lab (US Provisional Patent Application No.: # 60/762,439). NCC is a low DP and highly amorphous substrate. The difference between cotton and NCC is shown in fig.VI-1. The BET surface area of cotton and NCC was measured by Micromeritics Analytical Services, Atlanta with ASAP 2420 instrument using krypton gas adsorption. The moisture content of NCC was in the range of 75-85%. If not stated otherwise, wet NCC was always used for hydrolysis.

Enzymes

Cellulase enzyme (Spezyme CP) was obtained from Genencor International Inc. (Paulo Alto, CA). The reported filter paper activity of cellulase was 59FPU/ml. β-Glucosidase, with an activity of 750CBU/ml (measured in our lab), was purchased from Sigma (Novozyme 188 from Novo Inc., Sigma cata. no. C-6150 and lot no. 11K1088).

Enzymatic digestibility

All enzymatic hydrolysis experiments were done with 1g glucan loading/100ml of total reactant volume. These tests were carried out in 250ml Erlenmeyer flasks with the total reactant volume of 100ml following the NREL LAP number 009 (www.eere.energy.gov./biomass/analytical_procedures.html). Maximum expected variation in digestibility values is within 3% of the reported values.

Conditions of the enzymatic reaction were as follows: 50°C, pH 4.8 (0.05M sodium citrate buffer was used to maintain pH), shaking of flask with 150rpm (Shaker/Incubator: New Brunswick Scientific, Model Innova 4080). Soluble sugars (glucose, cellobiose and Cello-oligosaccharides) released in enzymatic hydrolysis were converted into the percentage glucan equivalent of initial glucan loading by using the appropriate conversion factor.

Analytical procedures

The concentration of sugars in liquid after enzymatic digestibility was determined by HPLC using a Bio-Rad Aminex HPX-87P column. As shown in Fig.VI-2, along with

glucose & cellobiose, some cello-oligosaccharides (COS) were generated from the enzymatic hydrolysis of NCC as observed in the HPLC chromatograph. These COS were called Low DP COS (LD-COS). After doing secondary hydrolysis of this sugar solution, it was found that total amount of sugars present in the solution was much higher than the sum of glucose, cellobiose and LD-COS. This unaccounted sugar (not detected in the HPLC chromatograph) was called the High DP cello-oligosaccharides (HD-COS).

Production of COS and its quantification

COS were produced in our lab by acid hydrolysis of cotton whose HPLC chromatograph is shown in Fig.VI-3. Seven distinct peaks can be seen in the chromatograph. When quantified, using glucose as standard, it was found that approximately 50% of the sugars in COS solution were not detected in HPLC. Apparently these unaccounted sugars were HD-COS with DP more than 7.

Crystallinity index

The Crystallinity of all the substrates was measured using X-ray diffractometer (Rigaku DMAX) with Cu-Kα radiation generated at 40kV and 40mA. Samples were scanned from 2θ = 10°-40° with 0.01° increments. The following formula was used for calculating the crystallinity index of samples (Cao et al., 2005):

CrI = $(I_{002} - I_{am}/I_{002}) \times 100$; where I_{002} is peak intensity corresponding to 002 lattice plane of cellulose molecule, which is observed at 2θ equal to 22.5°, and I_{am} (at 2θ =19°) is the peak intensity corresponding to amorphous cellulose.

Scanning electron microscopy

Microscope images presented in this paper were taken using a Field Emission Scanning Electron Microscope (JEOL JSM-7000F) with a magnification of 10,000.

Determination of relative DP of Substrates by DNS reagent

50 mg of each substrate on dry basis was mixed with 3ml of DNS reagent in glass- capped tubes. DNS reagent was prepared as per procedure described in NREL LAP-006 (eere.energy.gov./biomass/analytical_procedures.html). These mixture tubes along with the tube containing only 50mg of glucose and 3ml of DNS reagent were heated for 5min in boiling water and then immediately cooled down by transferring the tubes into ice cold water. The content of the tubes were centrifuged and 0.2ml of supernatant was taken from each tube for the measuring absorbance at 540nm. Referring to Table VI-1, the comparative DP of three substrates was calculated using glucose as reference. Following the finding of Sengupta et al., it was assumed that the absorbance of cellulose with DNS reagent which contains same number of reducing ends as of glucose, would be much higher than that of glucose (Sengupta et al., 2000). The factor for calculating the cellulose absorbance from the glucose absorbance was taken into account while calculating DP from absorbance. These DP values are not absolute valves but can be used for comparison among these substrates. As it is already known that the DP of filter paper and cotton are much higher than that of α-cellulose (Stamm et. al., 1964*),* It can be concluded that cellulose DP in these substrates would be in following order:

Cotton> Filter Paper> α-cellulose> Avicel > NCC

Determination of solid absorbance and DP in NCC hydrolysis

For calculating the absorbance of solid at any particular time of NCC hydrolysis, 0.2ml of the sample was taken from uniform suspension of hydrolysis liquor in each of the two centrifuge tubes. Before taking the sample, the flask was well stirred and it was ascertained that the sample withdrawn from the flask actually represented the solid concentration of the reaction mixture. 3ml of DNS reagent was added in first sample and second sample was centrifuged, supernatant was separated in another tube, and then 3ml of DNS reagent was added in the separated supernatant. Both tubes were kept in hot water at boiling temperature for exactly 5min. and then immediately cooled by placing the tubes in ice cold water. Glucose standards solutions (0.2ml) of different concentration were also reacted with DNS using the same conditions as the samples. The content both sample tubes were centrifuged and then the absorbance of 0.2ml of supernatant was measured. The difference in the absorbance value of first and second tube represented the solid absorbance, as shown in Fig VI-12.

DP values of solid samples, as shown in Fig.VI-12 were calculated using the absorbance of glucose standards as reference. Using the information in the literature (Sengupta et al, 2000), the factor for converting glucose absorbance into cellulose absorbance (this depends upon the DP of cellulose) was taken into account while calculating the DP of the solid from its absorbance value with DNS.

RESULTS AND DISCUSSION

Comparison between Crystalline and Non-Crystalline Cellulose

The difference between cotton and NCC is shown in Fig.VI-1. SEM images indicate that the surface of NCC looks more open and deformed than the crystalline cotton. The BET surface area of NCC is approximately double that of the cotton. NCC is able to retain more than 400% of moisture by its weight which is evidence of its hydrophilic nature. Fig.VI-2(b) shows that the crystallinity index of NCC is very low (8%). The initial hydrolysis rate of NCC with cellulase enzyme was more than 10 times that of the cotton. The high initial enzymatic reactivity of NCC can be attributed to various factors such as high surface area, more open structure, low crystallinity, the hydrophilic nature of the substrate, and low DP. These factors would contribute to very high cellulase accessibility to the reactive sites in cellulose chain.

Zhang et. al. produced the phosphoric acid swollen cellulose (PASC) which shows similar characteristics as NCC in terms of DP, crystallinity, surface area and very high initial hydrolysis rate (Zhang et al, 2005). The cellulase accessibility, as determined by the actual adsorption of the protein, in PASC was 20 times higher than the Avicel (Zhang et al, 2004). Similarity in substrate properties suggests that the cellulase accessibility of NCC would also be very high like PASC.

Low cellulase accessibility to cellulose reactive sites and high crystallinity are the two main reasons for the sluggish hydrolysis of crystalline cellulose (Mosier et al.2005.). Swelling of the crystalline cellulose can help to increase the cellulase accessibility by increasing the intermolecular distance in cellulose bundle. Swelling of cellulose

molecules depends upon number of available free hydroxyl groups which do not participate in inter- or intra- chain hydrogen bonds. Water adsorption in cellulose is a chemisorption process with a negative value of enthalpy and entropy change. Extensive adsorption of water in the molecules of cellulose is only possible when the hydrogen bonding pattern within the cellulose bundle is disturbed and free hydroxyl groups are available for making hydrogen bonds with water molecules. Crystalline cellulose has a hydrophobic nature because free hydroxyl groups are less available. (Fengel & Wegener, 1984). In NCC, there is extensive water swelling because of the disturbance of hydrogen bonding as a result of acid hydrolysis. Water molecules accommodated between the cellulose chains increase the intermolecular distance between the chains. This helps in rapid hydrolysis of NCC in two ways: First, by increasing the accessibility of enzyme between the cellulose chain, and second, by making the glycosidic bonds more hydrophilic. As indicated by Divne et al, lots of water molecules are present on the inside surface of the CBH-1 active site tunnel surface (Divne et al.,1998). Large numbers of water mediated hydrogen bonds are formed amongst cellulose chain and binding subsites inside the active site tunnel in CBH-1. These water molecules help in the sliding of the chain during the hydrolysis which makes the progressive action of CBH-1 possible. In the absence of water molecule the binding of substrate would be weak due the absence of water mediated hydrogen bonds (Becker et al., 2001). These observations show that even though the reaction between cellulase and cellulose is heterogeneous, a hydrophilic environment in the active site is very important for efficient hydrolysis reaction. Because

NCC is highly amorphous and hydrophilic, the cellulase accessibility and hydrolytic action are both very fast.

Cellobiose and LD-COS, both are substrate for β-glucosidase

The profile of various sugars produced during the enzymatic hydrolysis of NCC at two enzyme loadings is presented in Fig. VI-5. With high enzyme loading, cellobiose level substantially increases due to high activity of Exo-G on NCC and then decreases to a very low level due to β-glucosidase activity. But at low enzyme loading, the cellobiose level keeps increasing because of insufficient β-glucosidase activity. In both the cases, the LD-COS profile is exactly the same as of cellobiose and when excess amount of external β-glucosidase is added into the reaction mixture, both, cellobiose and LD-COS, are hydrolyzed to glucose. This proves that LD-COS is also the substrate for β-glucosidase in addition to the cellobiose.

HD-COS does not react with any of cellulolytic components

Referring to Fig.VI-5, the profile of HD-COS is very much different from that of LD-COS. HD-COS is mainly generated in the early stage of hydrolysis reaction and is not consumed afterwards, even after the addition of external β-glucosidase. This indicates that HD-COS is not consumed by any of cellulolytic components present in cellulase produced from *T. ressei*. It has already known that CBH from *T.ressei* does not have activity towards the soluble substrates (Nummi et al,1983). Trends here indicate that β-glucosidase is also not reacting with the HD-COS.

Profile of various sugars in hydrolysis of crystalline substrates

The hydrolysis profiles of different cellulosic substrates with cellulase are shown in Fig.VI-6. Unlike the NCC, LD-COS is not detected during the hydrolysis of crystalline cellulose. Highest glucan digestibility is obtained with α-cellulose and lowest is with the avicel. Generation of HD-COS is also highest in the case of α-cellulose. The generation of HD-COS is approximately similar for other three substrates.

The profile of cellobiose generation in filter paper hydrolysis is noteworthy where cellobiose level keeps increasing till external β-glucosidase is not added. While in other substrates, it attains the maxima and then keeps decreasing till it reaches the minimum. Fig. VI-7 shows the cellobiose profile in filter paper digestibility with different cellulase loadings and Fig.VI-8 presents the trend how cellobiose value at 72hrs changes with the change in enzyme loading in case of different substrates. In NCC and avicel reactions, the cellobiose value at 72hrs decreases as enzyme loading increases. When using filter paper, this trend is completely different where cellobiose level goes up when enzyme loading is increased from 3.5FPU to 7.5FPU and then declines as loading is increases to 15FPU. Even at a high enzyme loading of 15FPU, cellobiose level is highest for filter paper. Cellobiose value in enzymatic hydrolysis of cellulose depends upon comparative generation by Exo-G and consumption by β-glycosidase. Fig.VI-8 indicates towards the fact that effective β-glucosidase activity in the liquid is lower in the filter paper hydrolysis test though same enzyme has been used with all the substrates. The varying adsorption capacity of different substrates towards β-glucosidase may be a reason for this (Fujishima et al.1989). The adsorption capacity of β-glucosidase in filter paper might

be higher than the other substrates which leads to less residual activity of β-glucosidase in the liquid and more accumulation of cellobiose.

Another interesting point in Fig. VI-6(c) is the increase in hydrolysis rate of cotton and filter paper after the addition of external β-glycosidase. The reason for this might be the increased activity of Exo-G as a result of the elimination of the inhibitory effect of cellobiose. This increase is not observed with α-cellulose and avicel. This indicates toward a difference in some intrinsic characteristic of these substrates such as DP which is much higher in cotton/filter paper than in α-cellulose/avicel.

Effect of enzyme loading in hydrolysis of crystalline cellulose and NCC

Fig.VI-9 shows the change in hydrolysis rate of different substrates with a change in the enzyme loading. The reactivity of NCC does not change much even if the enzyme loading is increased from 3.5FPU to 15FPU. This indicates that it is possible to achieve a very high hydrolysis rate using very low enzyme loading with NCC. Substrate characteristics determine the adsorption and accessibility of the enzyme to the reaction site. Previous studies have already indicated that the lower hydrolysis rate of cellulose in comparison to starch is because of substrate characteristics not the type of bond (Zhang et al, 2004). The high reactivity of NCC supports this theory as it achieves 60% digestibility with the very low enzyme loading of 3.5FPU. But this digestibility does not increase proportionally with enzyme loading which indicates that the maximum digestibility of NCC is limited by some particular trait of the substrate. With crystalline substrates, substantial increase in the hydrolysis rate is observed with an increase in enzyme loading.

This increase is highest with filter paper and lowest with avicel. At high enzyme loading of 15FPU, filter paper digestibility value surpassed the NCC hydrolysis. As we analyzed previously with β-glycosidase, the surface properties of filter paper might be conducive for protein adsorption in general and that would lead to more cellulase accessibility to the substrate.

Effect of substrate DP on NCC hydrolysis

To study the effect of substrate DP on its enzymatic hydrolysis, NCC substrates with varying DP were prepared by giving each a different reaction time during its preparation. As the reaction time for NCC preparation increases, the product DP would decrease. These different DP NCC substrates have been hydrolyzed with two enzyme loadings: One at the very low enzyme loading of 0.005ml/g glucan and another at the high enzyme loading of 0.15ml/g glucan.

As the DP of all the NCC substrates is very low (~100), at very low enzyme loading it can be assumed that the number of cellulose chains are much higher than required for an Exo-G reaction. Under this assumption, Exo-G action would not be affected by new chain ends created by Endo-G reaction with NCC. This indicates that there is no synergy between Endo-G and Exo-G action when very low enzyme loadings are used with NCC. Based on this assumption, the amount of G1+G2 (Glucose + Cellobiose) would indicate the action of Exo-G and the change in the solid side reducing end would suggest the extent of Endo-G action on NCC.

Hydrolysis profiles of difference NCC substrates with low enzyme loading is shown in Fig.VI-11. The reaction time for NCC preparation is indicated in the figure with the hydrolysis profile of different sugars. Initial hydrolysis rates are similar but overall hydrolysis rates are different for different DP substrates. For lower DP NCC, the overall hydrolysis rate is lower.

High enzyme loading is used in Fig.VI-12 for the hydrolysis of the same NCC substrates as in the earlier experiment. Unlike the results with the low enzyme loading, the difference in the hydrolysis rate for different DP substrates was observed from beginning of the reaction and the hydrolysis rate is lower for low DP NCC substrate.

Change in Absorbance and DP with hydrolysis time

Fig.VI-12 shows the change in solid absorbance with a DNS reagent as enzymatic hydrolysis reaction of NCC proceeds. A drastic rise in the solid absorbance was observed immediately after the addition of the enzyme. This indicates that the reducing ends in the solid increase sharply as soon as enzyme is added to the substrate. Endo-G is responsible for producing the new reducing ends by cleaving the cellulose chains (Lynd et al., 2002), and it is known that Endo-G works on the amorphous part of cellulose. NCC, being highly amorphous, prompts a very fast Endo-G reaction in the beginning of hydrolysis. After the early part of reaction, reducing ends did not increase. Absorbance of solids becomes constant, and after a certain time, it decreases. Fig.VI-14 shows the relation between the reaction time for making NCC and the increase in absorbance of NCC with DNS in the initial phase of the hydrolysis reaction with cellulase. It can be interpreted

from this graph that as average DP of NCC goes down, the reactivity of Endo-G increases. The reason for this is the higher availability of cellulose chains for Endo-G action in low DP NCC.

A profile of DP change of substrate in the NCC-Cellulase reaction is shown in Fig. VI-13. DP of un-reacted substrates is shown as the points before 0 hrs in the graph. It is evident from the Fig.VI-13 that NCC made with less reaction time has highest the DP. DP values of NCC substrates made with 15.5, 20.0 and 26.0 min reaction time have approximate DP values of 140, 120 and 80 respectively. As cellulase is added to the substrate, the DP value of NCC goes down to a range of 40-50 within practically no time and after that the DP of NCC remains in the same range as the reaction proceeds. The same behavior of the DP profile was shown by Zhang et al with PASC hydrolysis by cellulase (Zhang et al., 2005). Interestingly, this value of DP at 0hrs after enzyme addition does not depend on the DP of the substrate. As Endo-G causes the reduction of DP, this indicates towards the fact that Endo-G reactivity with cellulose chain is negligible when the DP of the substrate goes below a particular value. From these trends and previous studies (Zhang et al., 2005), it appears that this value of DP lies somewhere in the range of 30-60.

Endo-G generates the HD-COS

It is evident from Fig.VI-15 that HD-COS generation in NCC hydrolysis reaction is proportional to the reaction time for NCC preparation. HD-COS is generated mainly at the initial phase of NCC-cellulase reaction (Fig.VI-5&6). The co-incidence of high level

of HD-COS generation with a sharp increase in solid side reducing ends as well as a sharp decrease of NCC DP indicates that Endo-G reaction with cellulose chains is responsible for all these concurring events. When Endo-G reacts with a particular cellulose chain in NCC, it cleaves the chain into two fragments and if the DP of one fragment is lower that a particular value, it dissolves into the liquid as HD-COS. Now the question is why the quantity of HD-COS is much smaller in the hydrolysis of crystalline substrate than in the hydrolysis of NCC. Two factors are responsible for this. The first is high crystallinity thus less Endo-G action and the second is extensive patterned hydrogen bonding within the chains in crystalline substrates, which decreases the solubility of HD-COS. It has also been reported in an other study that cellodextrin with DP of more than 4 are formed as a result of hydrolysis by Endo-G and these are associated with a solid phase in case of crystalline cellulose but get solublized in the liquid when cellulose is amorphous. (Stalbrand et al., 1998).

From the above evidence, it is clear that the Endo-G reactivity on NCC is negligible after the initial phase of reaction. Two plausible reasons can be given for this. The first is low DP of the substrate and the second is the inactivation of the protein. As the average DP of NCC decreases as a result of Endo-G action, the extent of Endo-G reaction also decreases and reactivity becomes negligible as the DP of all the chains goes below a certain value. Endo-G found less effective towards the lower DP bacterial cellulose even though the crystallinity of substrates are the same (Valjamae et al, 1999). Other studies have mentioned about the inactivation of enzymes during the course of hydrolysis (Gan et al,2003; Wang et al, 2005). Quantitatively the activity of Endo-G in

cellulase from *T reseei* is several folds lower than that of Exo-G (Kleywegt et al 1997; Lynd et al,2002). Very low enzyme loading in the beginning and further inactivation of protein in the reaction might lead to negligible activity of Endo-G after the initial phase of hydrolysis reaction.

Exo-G is responsible for LD-COS generation

One of the main observation in all the NCC hydrolysis experiments (Fig.VI-5, VI-10, VI-11 and VI-16) is that the profile of LD-COS in the reaction is similar to that of cellobiose. This is possible when the enzyme responsible for generation and consumption of LD-COS is same as of cellobiose. It is already known that β-glucosidase consumes both cellobiose and LD-COS (Lynd et al., 2002). As Exo-G is responsible for the generation of cellobiose, LD-COS ought to be produced as a result of Exo-G action on the cellulose chains.

It is known that once CBH is bound to the cellulose chain end with the help of its CBD, 10 glycosyl residues bind with 10 binding subsites inside the active site tunnel (Divne et al., 1998). After that the cellulose chain is hydrolyzed with the release of one cellobiose molecule. After the release of cellobiose, the chain slides forward into the tunnel and again two more residues take the position of the vacant binding sites in the CBH tunnel. Similarly, hydrolysis of the cellulose chain progresses until the end of the chain. It is known that as the DP of cello-oligosaccharides goes up, the Km value of CBH goes down and the rate of reaction increases (Koivula et al.,2002). This means that CBH would have much less affinity for and low reactivity to a lower DP cellulose chain. The

mechanism described here for cellulose chain hydrolysis by CBH indicates that the binding of the 10 glucosyl residues of the cellulose chain in the active site is the first and most important step for the hydrolysis reaction.

Based on the results in this study and the available information in the literature about Exo-G action described above, a hypothesis is proposed here that LD-COS is produced due to Exo-G action on cellulose chain. While working on a particular cellulose chain, Exo-G produces cellobiose as a hydrolysis product, the chain further moves into the active site tunnel and two more glycosyl residues bind into the two vacant subsites. This process continues till the DP of the cellulose chain goes below a particular value and all the subsites are not bound to the glycosyl residue of the chain. At this point, the reactivity of Exo-G becomes very low and that low DP chain (with DP below 7-8) is detached from the active site tunnel and dissolves into the liquid. These LD-COS are not visible in the hydrolysis of a crystalline substrate because of the extensive hydrogen bonding which prevent the solubilization of the remaining segment of chain.

Why hydrolysis rate of lower DP NCC is less

It is evident from Fig.VI-10 & Fig. VI-11 that the Glucose (G1) + Cellobiose (G2) produced from the hydrolysis of high DP NCC (prepared with low reaction time) is more. G1+G2 is produced due to the Exo-G activity on NCC which seems to be higher for a high DP NCC substrate. The polydispersity index (Mw/Mn) of NCC would be expected to be very high because of the method employed for NCC production. Lower DP NCC will have a greater number of smaller cellulose chains. If the cellulose chain length in

NCC is smaller than a certain value, its affinity for Exo-G would be very low. These very low DP cellulose chain in NCC remain un-reacted until the very end and causes a lower hydrolysis rate for lower DP NCC. In the case of low enzyme loading, the initial hydrolysis rate is same because initially the cellulose chains are in excess of what is required for reacting with Exo-G molecules. This is not the case for high enzyme loading and the difference in hydrolysis rate of different DP NCC substrates can be observed from the very beginning.

Comparison of hydrolysis results of crystalline and non-crystalline substrates

Like NCC, a low affinity of Exo-G for lower DP cellulase chains can be a reason for a drop in the hydrolysis rate as time elapses. Crystalline cellulose can retain more of the smaller DP chains in solid than NCC because of extensive hydrogen bonding (Stalbrand et al., 1998). Due to the inactivity of these smaller chains towards any of the enzymatic components, the smaller DP chains remain un-hydrolyzed and decrease the effective hydrolysis rate of the substrate. That is the reason why after β-glucosidase addition, the hydrolysis rate of filter paper and cotton increases while in case of α-cellulase and avicel, it remains unchanged (Fig.VI-6(c)). After addition of β-glucosidase, cellobiose is converted to glucose and in absence of the inhibitory effect of cellobiose, the enhanced activity of Exo-G causes further hydrolysis of the high DP cotton/filter paper but not the lower DP α-cellulase / avicel. It is shown in one study that the hydrolysis rate of CBH-1 treated BMCC is lower than the untreated BMCC (Valjamae et al., 1999). Treatment of BMCC with CBH-1 would reduce the effective DP and increase the

polydispersity index of the cellulose. This again supports the theory that the very low DP glucan chains in the solids can contribute to the reduction of effective hydrolysis rate of the cellulose.

Effect of physical properties on NCC hydrolysis

In order to investigate the effect of other physical properties (diffusion and other mass transfer resistance on the surface and inside the particle) of NCC on hydrolysis rate, three samples of NCC (two samples of dry NCC and one wet NCC sample) with very different physical characteristics were employed in enzymatic hydrolysis. One of the two dry samples was dried in 45°C oven and the other was dried in lyophilizer. The lyophilized (freeze dried) NCC was very brittle and produced very soft, uniform, porous powder after crushing. On the other hand, the oven dry NCC sample was very hard and difficult to crush. The bulk density of oven dry sample was much higher than the lyophilized NCC sample. The moisture content of the dry NCC samples was approx. 5% and wet NCC sample was more than 80%. It is obvious that the mass transfer resistance in the oven dried sample would be higher than the lyophilized or the wet NCC sample.

The results of enzymatic hydrolysis with dry and wet NCC sample are shown in Fig.V-16. The initial hydrolysis rate of lyophilized and wet sample was much higher than the oven dried sample but final hydrolysis rate was approximately equal in all of the NCC samples. Even the generation of HD-COS was approximately equal in all NCC samples, though initial rate of HD-COS generation was lower for over dried NCC. This shows that the physical barriers affect the cellulase accessibility during the initial phase. Certain

studies indicate that the drop in the cellulose hydrolysis rate with time is because of the change in the surface and other physical properties (Gan et al., 2003), but this does not seem to be the case here. However the change is surface properties due to enzymatic action is one of the reasons for the high hydrolysis rate of oven dried NCC in the later phase of the reaction. This led to the same overall conversion for oven dried NCC as other two forms of NCC samples. Studies have shown that enzymatic hydrolysis increases the roughness of the cellulose surface, and the structure becomes more receptive for protein adsorption. The surface area of pure cellulosic substance also increases due to enzymatic reaction (Wang et al., 2005; Park et al., 2007). This indicates that although initial mass transfer resistance was higher with the oven dried NCC which led to a lower initial hydrolysis rate, in the later phase it attained the same conversion as of wet or lyophilized NCC because of the structural changes made by cellulase action. This points out toward the fact that the inherent chemical characteristics (predominantly DP and crystallinity) of NCC are controlling the final hydrolysis rate not the physical properties.

CONCLUSION

Based on the study in this paper, the mechanism for the action of different functional proteins in NCC hydrolysis by cellulase has been proposed in Fig.VI-17. Endo-G works very fast on the highly amorphous cellulose and brings the average DP of NCC down into the range of 30-60 in the very beginning of reaction. After that, activity of Endo-G on NCC becomes negligible. Endo-G action on NCC produces the insoluble

lower DP NCC and soluble HD-COS. HD-COS is not visible in the HPLC chromatograph and this indicates that the DP of HD-COS is higher than 7. Generation of HD-COS is also observed in the hydrolysis of crystalline substrate but the quantity is much less in comparison to NCC. This is because there is less Endo-G action on crystalline cellulose. The amount of HD-COS produced among various crystalline substrates is highest for the substrate with lowest crystallinity.

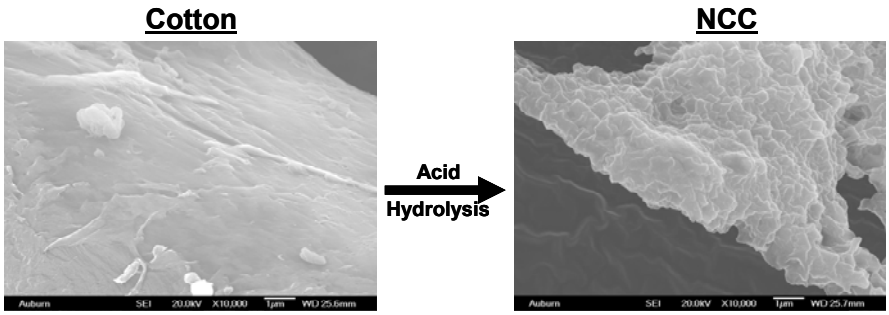
The COS with a DP of less than 7 are visible in the HPLC chromatograph and these are termed LD-COS. LD-COS solublizes in the liquid phase once the binding affinity of the cellulose chain to Exo-G drops very low due to low DP. These LD-COS are not detected in crystalline substrates because of extensive hydrogen bonding among the molecules. Lower DP NCC is less reactive towards the Exo-G because the binding affinity of the low DP cellulose chain is less.

Once cellobiose and LD-COS are solubilized in the liquid by Exo-G action, β-glucosidase reacts with them and produces glucose. HD-COS are not reactive toward any of the cellulolytic protein and remain unconsumed until the end.

For a substrate like NCC which is highly amorphous, very little Endo-G activity is required to hydrolyze the big chain into smaller DP chains. In that case, the overall hydrolysis rate of a substrate depends upon the Exo-G activity in the cellulase. For crystalline substrates, enzyme accessibility is the limiting factor for the action of all the cellulase components. That might necessitate higher activity of Endo-G, as the probability of reaction would depend upon the number of enzyme molecules.

The surface characteristics of a substrate can be a deciding factor in the initial hydrolysis of cellulose, but the maximum hydrolysis rate depends upon inherent chemical characteristics of cellulose like DP and crystallinity. Crystallinity determines the initial reactivity of cellulose because of high Endo-G reactivity with the amorphous region. DP decides the final hydrolysis rate because of low Exo-G reactivity with the low DP cellulose chain.

FIGURES AND TABLES:



BET Surface Area (m^2/g)	**:**	**1.232**	**2.311**
DP	**:**	**>2000**	**100-150**
Initial hydrolysis rate (% glucan conversion/hr) Enzyme loading: 3FPU/g glucan	**:**	**3.4**	**37**

Fig.VI-1. Comparison between cotton and NCC

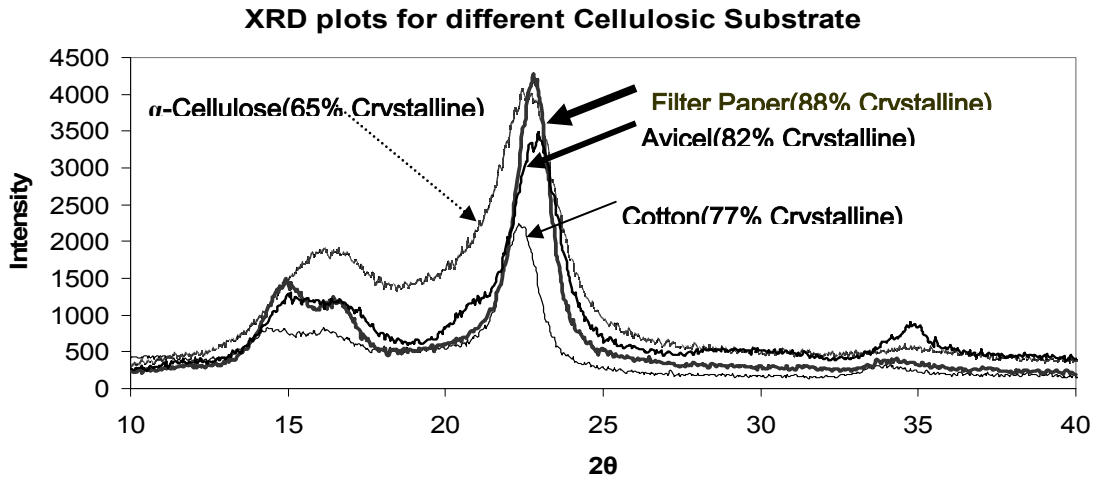


Fig.VI-2(a). Comparison of XRD plots for different pure cellulosic substrate

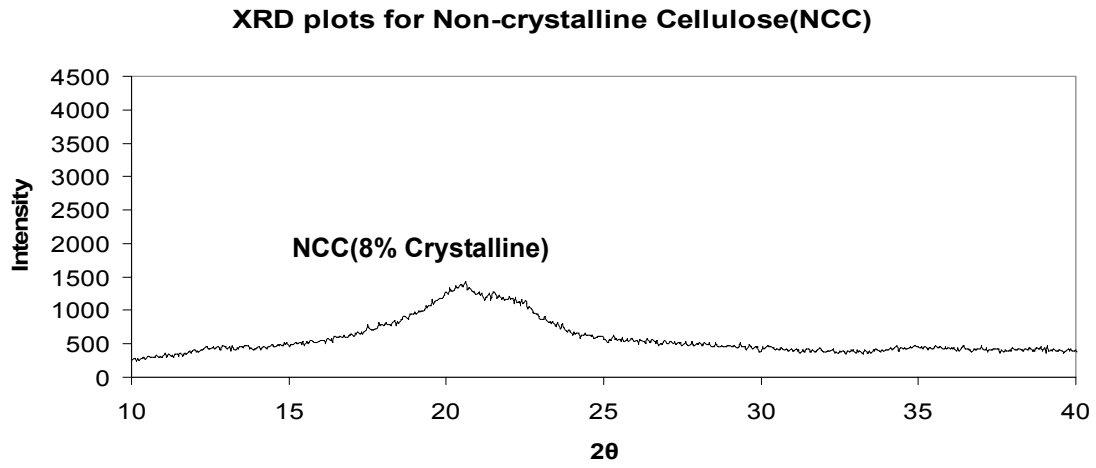


Fig.VI-2(b). XRD plot for NCC

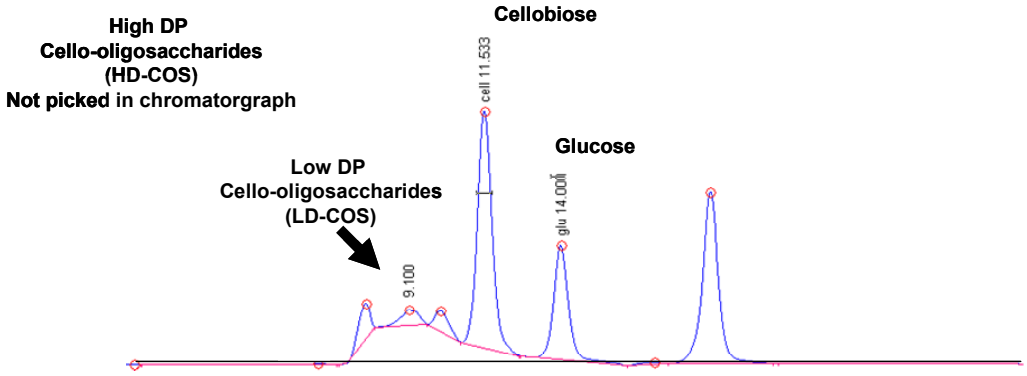


Fig.VI-3. HPLC chromatograph of sugar solution after 12 hrs enzymatic hydrolysis of NCC with enzyme loading of 0.005ml/g glucan.

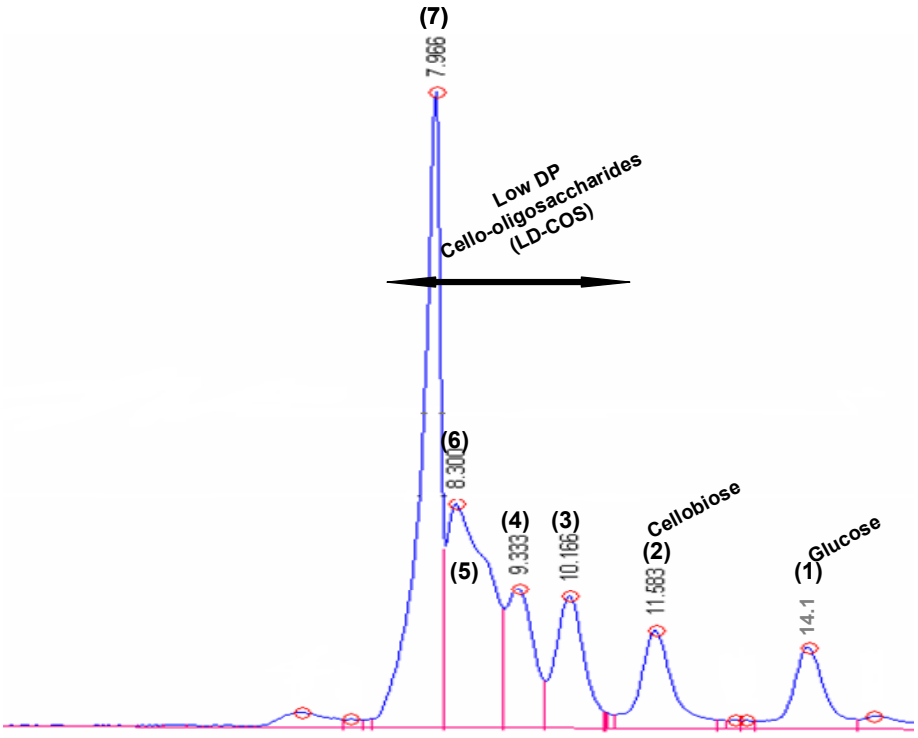


Fig VI-4. HPLC chromatograph of Cello-oligosaccharide solution

	Absorbance*		gmol of reducing ends	Average mol.wt	Average DP
	5min	20min			
NCC	6.420	13.140	2.03E-06	24607.9	152
Avicel	4.390	11.225	1.39E-06	35988.2	222
alpha cell	1.250	1.795	3.95E-07	126426.8	780

Table VI-1. Determination of DP of different cellulosic substrate

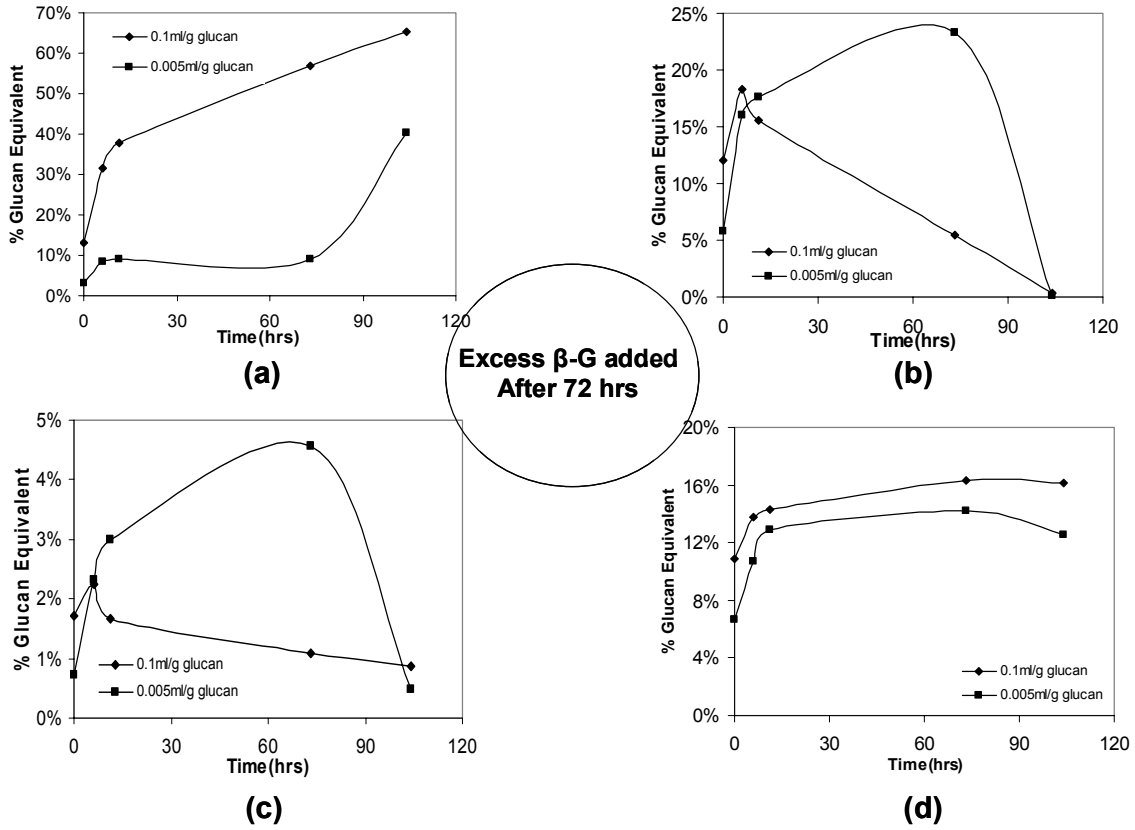


Fig. VI-5. Results of enzymatic hydrolysis of NCC with two enzyme loading
(a) Glucose (b) Cellobiose (c) LD-COS (d) HD-COS

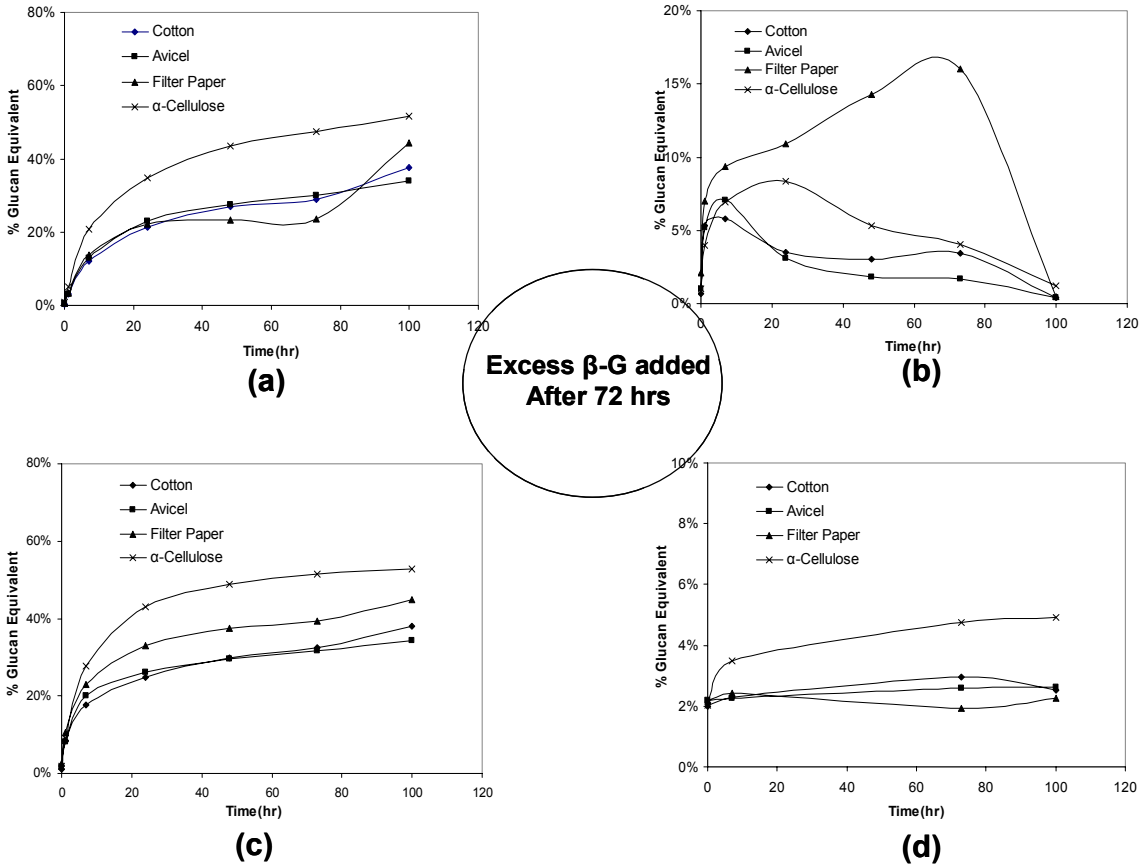


Fig. VI-6. Profiles of different sugars in enzymatic hydrolysis of crystalline cellulosic substrates with enzyme loading of 0.1ml/ g glucan.
(a) Glucose (b) Cellobiose (c) Glucose+Cellobiose (d) HD-COS

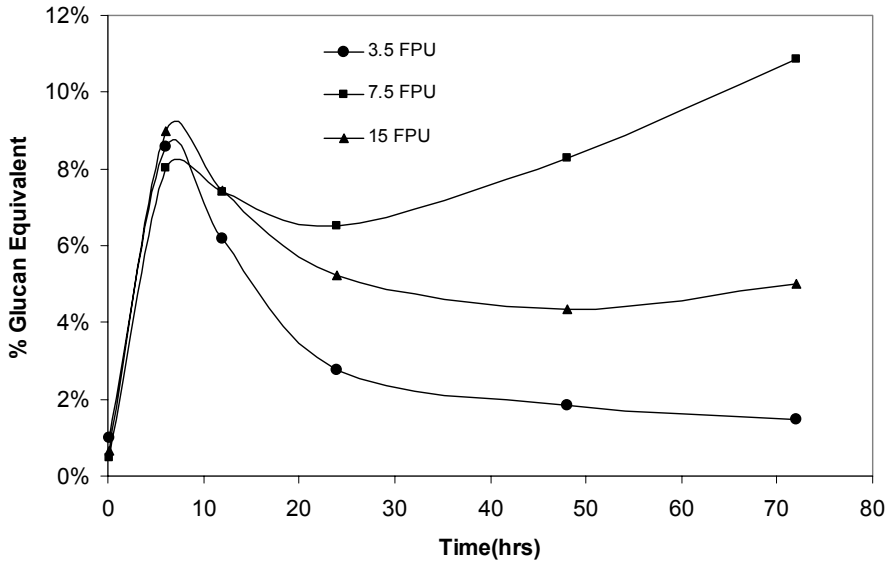


Fig. V-7. Profile of cellobiose production during enzymatic hydrolysis of filter paper with different cellulase loading

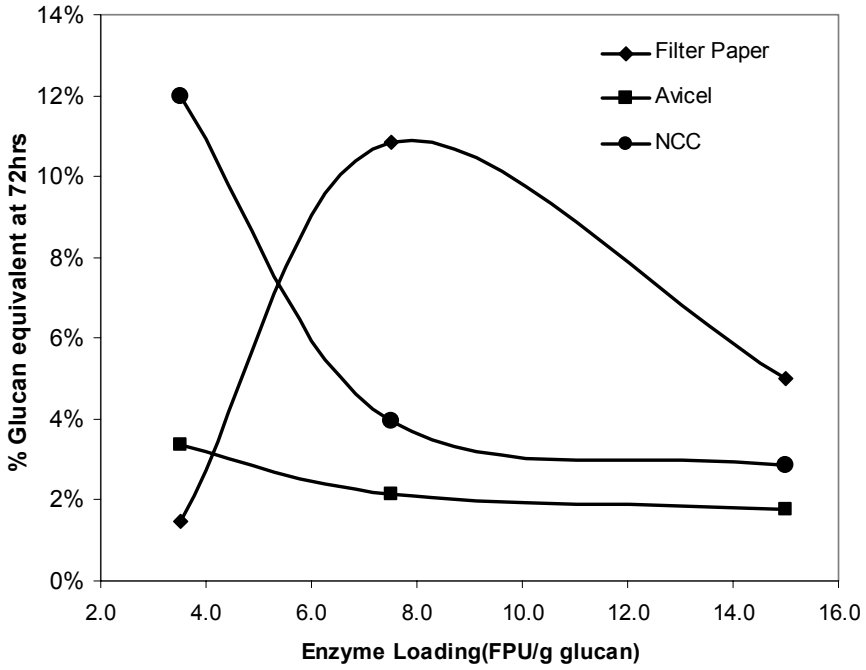


Fig.V-8. Change in cellobiose value at the 72hrs with the change in cellulase loading using different substrates

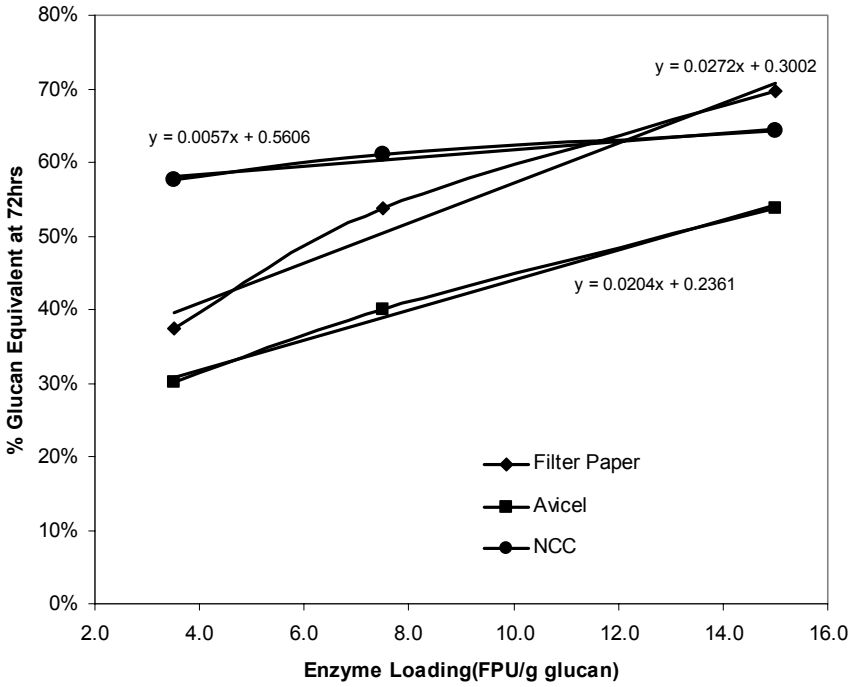


Fig.VI-9. Change in glucan digestibility with the change in cellulase loading using different substrates

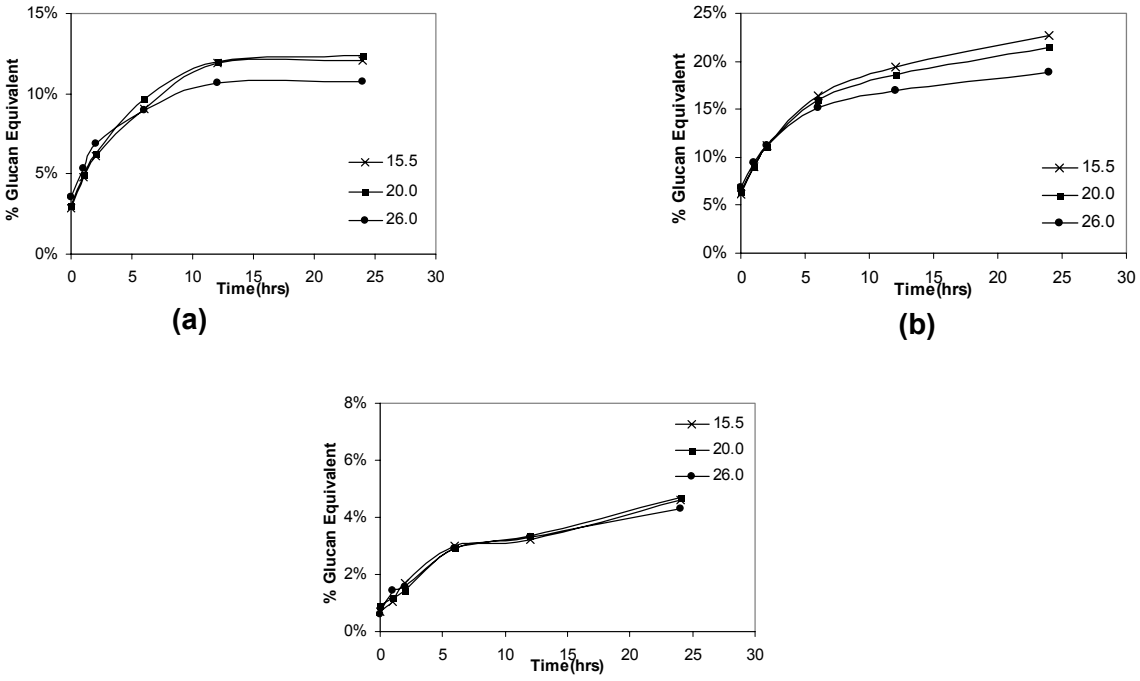


Fig.VI-10. Profile of different sugars in hydrolysis of different DP NCC substrates with low enzyme loading of 0.005ml/g glucan.
(a) Glucose (b) Cellobiose (c) LD-COS

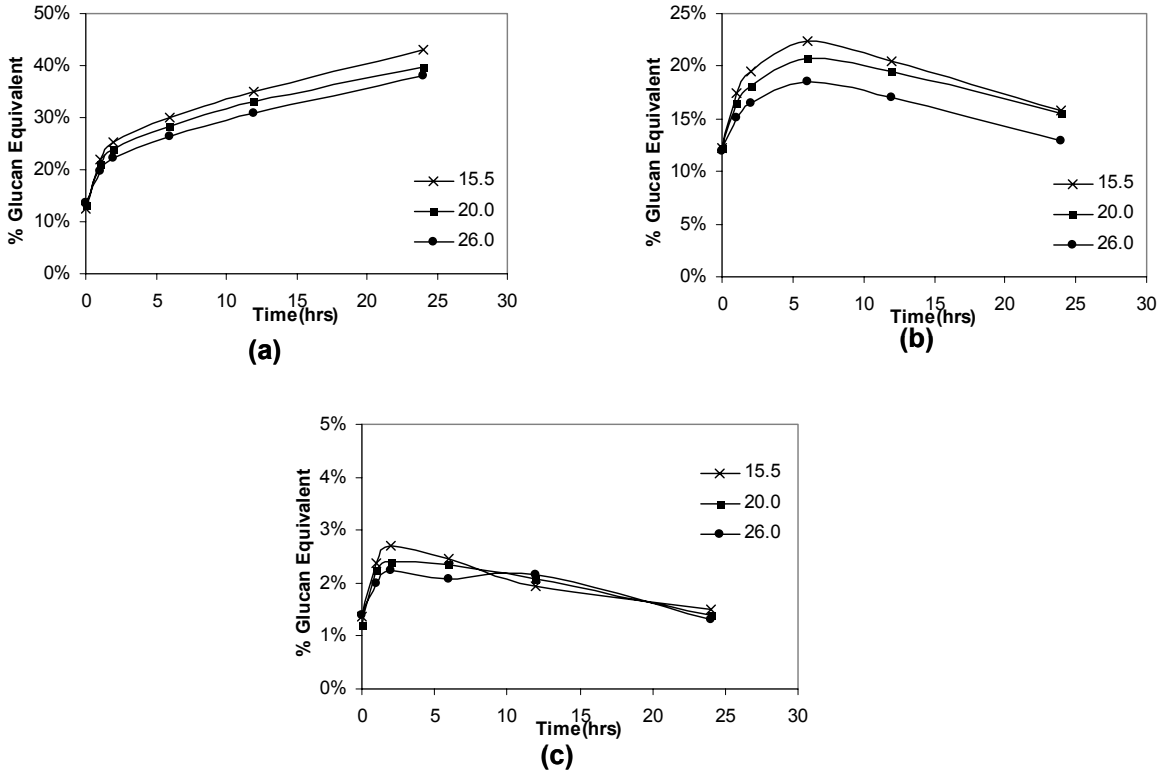


Fig.VI-11. Profile of different sugars in hydrolysis of different DP NCC substrates with high enzyme loading of 0.1ml/g glucan.
(a) Glucose (b) Cellobiose (c) LD-COS

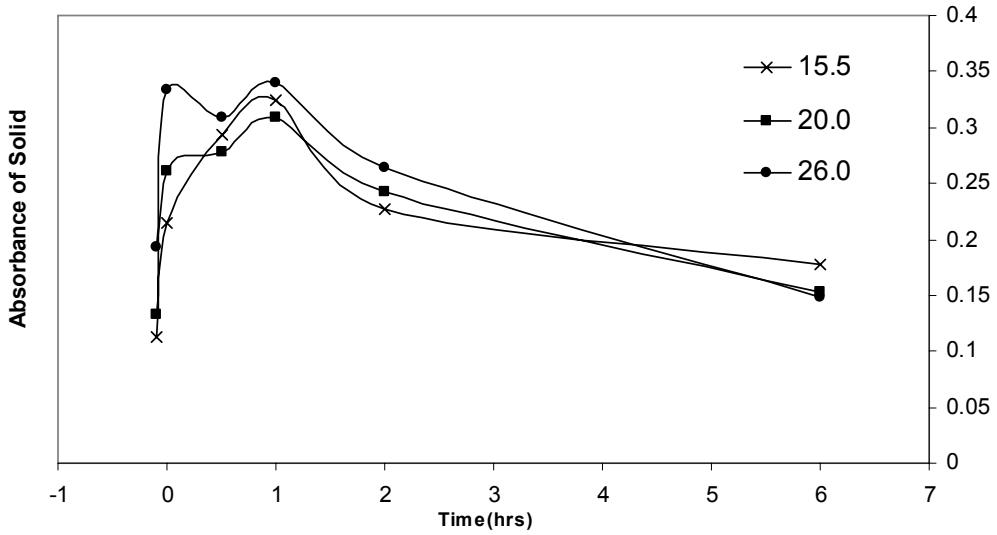


Fig.VI-12. Profile for solid absorbance with DNS in enzymatic hydrolysis reaction of NCC. (Cellulase loading: 0.005ml/g glucan)

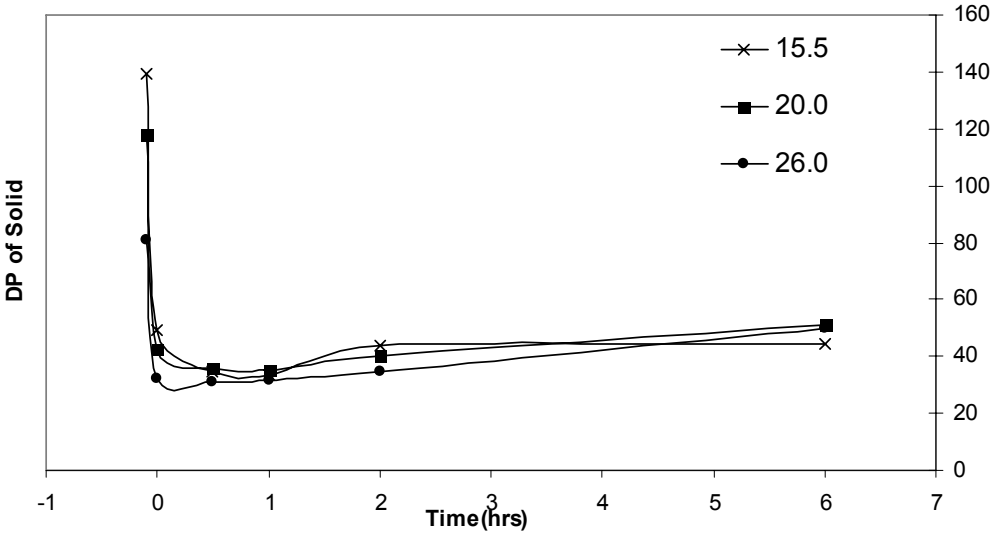


Fig.VI-13. Profile for solid DP in enzymatic hydrolysis reaction of NCC. (Cellulase loading: 0.005ml/g glucan)

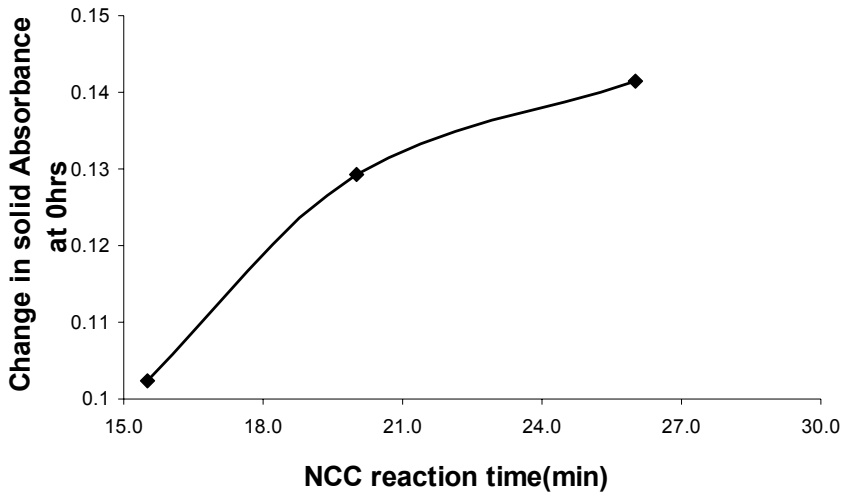


Fig.VI-14. Relation between increase in solid absorbance after cellulase addition and reaction time in NCC preparation.

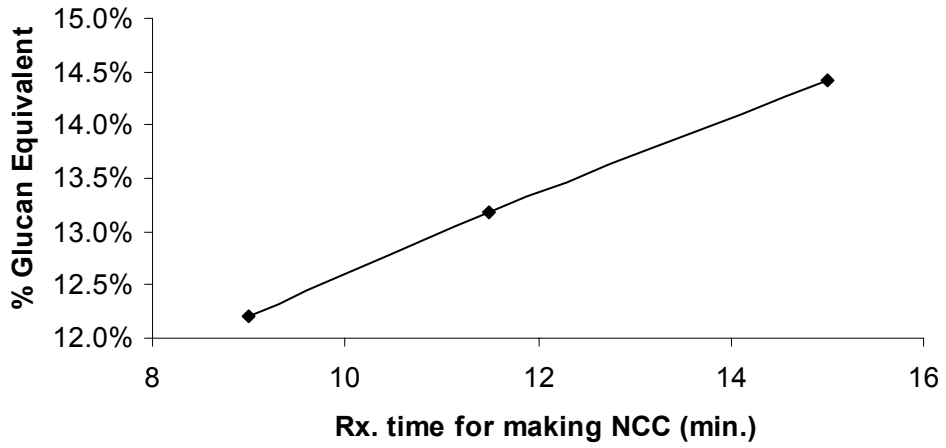


Fig.VI-15. Relation between HD-COS produced in NCC hydrolysis and reaction time in NCC preparation.

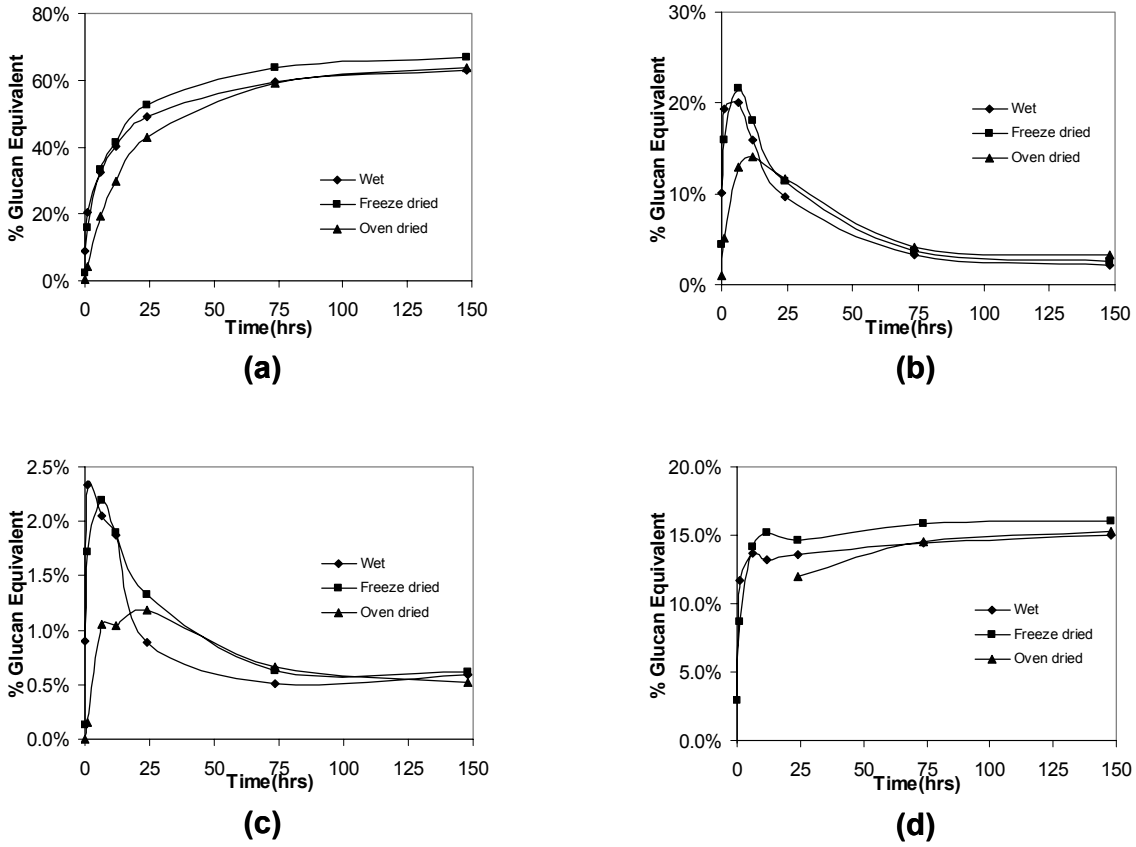


Fig.VI-16. Profile of various sugars in hydrolysis reaction of different forms of NCC (a) Glucose (b) Cellobiose (c) LD-COS (d) HD-COS.

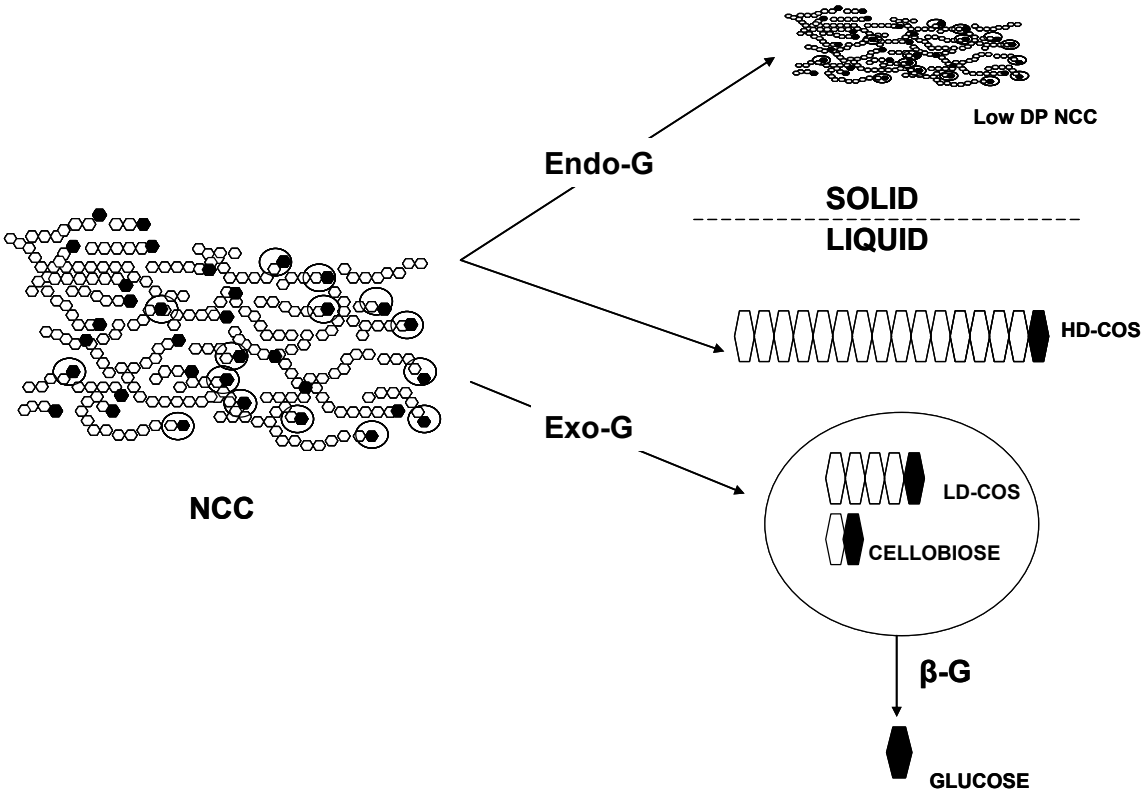


Fig.VI-17. Schematic presentation of proposed action of Endo-G and Exo-G

VII. MEASUREMENT OF ENDO-GLUCANASE AND EXO-GLUCANASE ACTITVITY IN CELLULASES WITH NON-CRYSTALLINE CELLULOSE

ABSTRACT

Non-Crystalline Cellulose (NCC) is used to measure the relative activities of Endo-G and Exo-G in different cellulase preparations. The absence of synergism between Endo-glucanase (Endo-G) and Exo-glucanase (Exo-G) in cellulase is a prerequisite for simultaneous measurement of both the activities with one substrate. X-ray crystallography has confirmed that NCC has a highly amorphous character with less than 10% crystallinity. The highly amorphous nature and low DP of NCC substrate justify the assumption of "no-synergism" between Endo-G and Exo-G in NCC-cellulase reaction at low enzyme loadings. Under these conditions, product generation (shorter cellulose chains by Endo-G and soluble glucose + cellobiose by Exo-G) would only be a function of enzyme concentration. The procedure proposed in this work is convenient and less time-consuming because a single substrate (NCC) is used and only one experiment is required to determine the relative activities of both Endo-G and Exo-G in cellulase. In this study, both HPLC and colorimetric methods were used to quantify the soluble sugars and the reducing end groups in the insoluble NCC, respectively. Initial rate data for the release of G1+G2 represent the Exo-G activity, and the change in the reducing-ends in solid represents the Endo-G activity. Three cellulase preparations were measured for

Endo-G and Exo-G activities using the proposed method and results were found to be in reasonable agreement with the conventional method.

Keywords: cellulase, activity, NCC, Exo- glucanase, Endo-glucanase

INTRODUCTION

The cost of enzymes (cellulase, xylanase etc.) for the hydrolysis of lignocellulosic feedstock is one of the major limiting factors for the economic feasibility of cellulosic ethanol production (Lynd et al., 2005). The enzyme cost can be minimized by two approaches: first, by minimizing the loading of cellulase for biomass hydrolysis which can be achieved by pretreating the biomass effectively and second, by reducing the production cost of cellulase. Several studies have indicated that the composition and quantity of enzymes used in the hydrolysis of pretreated biomass is a very strong function of the compositional and structural features of the substrate (Gupta et. al.,2007; Berlin et al., 2006). Thus, the substrates pretreated with different methods require different composition and quantities of biomass hydrolyzing enzymes such as cellulase, xylanase, pectinase and others. Considering this, optimization of enzyme composition for biomass hydrolysis is a big challenge in enzyme production. Activity measurement of the enzyme is one of most time consuming task in this optimization process. The method for measuring enzyme activity needs to be accurate and less time taking.

Cellulase is an extra-cellular enzyme produced by different cellulose degrading microorganisms. It is a complex mixture of different proteins that degrades the lignocellulosic biomass. The cellulase activity is the result of synergetic action of

primarily three functional protein components: Exo-glucanase (EC 3.2.1.91), Endo-glucanase(3.2.1.4) & beta-glucosidase(3.2.1.21) (Clarke et al., 1997). The most often used method for cellulase activity measurement is the filter paper unit (FPU) method. The activity of Endo-G, Exo-G, and β-G in cellulase preparation can be measured separately using CMC, Avicel and cellobiose as respective substrates (Zhang et al., 2006). Most of these assays use the DNS reagent to quantify the reducing ends in the liquid, and an increase in reducing ends gives the estimation of enzyme activity.

Repeatability of these assays has always been questionable because of a few drawbacks associated with DNS reagent (Coward-Kelly et. al., 2003). The reactivity of reducing end groups with DNS reagent varies considerably with change in the DP of cello-dextrin. The change in reducing end reactivity with DNS diminishes as the DP of cello-dextrin increases (Sengupta et al, 2000). Different reactivity of DNS with varying DP of cello-dextrin may contribute to a very large error when color produced by DNS reaction with mixture of cello-dextrin (produced as a result of enzymatic reaction on any substrate) is converted back into the reducing end concentration with the help of the glucose standard curve. If two cellulase preparations with similar FPU activity but different ratios of Endo-G and Exo-G react with insoluble cellulose, they produce the different number of reducing ends but still, when reacted with DNS reagent after enzymatic reaction, both might possibly give similar color. Berlin et al found that the FPU activity is not directly proportional to CMCase or Avicelase activity but is always proportional to the protein number (Berlin et. al., 2006). This suggests two possibilities: either there is some additional functional protein responsible for the hydrolysis of

insoluble cellulose or CMCase is not the true indication of Endo-G activity. So the kinetics of Endo-G action might be different with CMC than insoluble cellulose. In either case, using only insoluble cellulose for Endo-G activity determination would provide the correct picture, if it could be done.

A novel method of Endo-G and Exo-G activity determination is presented here which addresses these problems effectively. This method can be used effectively to find the relative activities of Endo-G and Exo-G in different cellulase preparations. In this method, a new substrate called Non-crystalline cellulose (NCC), a product of our laboratory, is used. NCC, which is insoluble in water, is produced by acid hydrolysis of crystalline cellulose. DNS reagent is also employed in this method for measurement of the reducing ends but that would not cause error in reducing end quantification. Here the reactivity of the reducing ends in the NCC with DNS for different cases would be approximately the same due to high DP of cello-dextrin in the solids (Sengupta et al, 2000). Crystallinity index of NCC is found less than 10% by X-ray diffraction method. NCC has been found to be excellent choice for the determination of activity due to the following reasons:

- It is highly amorphous which is a favorable condition for Endo-G activity
- It is a very low DP substrate with DP ranging from 100-150. This condition is favorable for Exo-G activity.
- The BET surface area of NCC is more than double that of Avicel which further helps to increase its reactivity.
- Initial enzymatic hydrolysis rate of NCC is more than 10 times higher than the crystalline cellulose.

- Both (Endo-G and Exo-G) activities can be measured in only one experiment by using NCC as single substrate. This method would consume less time and expenses.

The difference in the structure of crystalline cellulose and NCC in depicted based upon the characteristics known about these substrates (Gupta et. al., 2008) and presented in Fig.VII-1. Endo-G preferably attacks on the amorphous region of cellulose and Exo-G attacks on the chain ends (Lynd et. al, 2002.). It is evident from Fig. VII-1 that in NCC, the substrates required for both the enzyme components are available in excess, when very low enzyme loadings are used and the reaction is carried out for short duration. Under these circumstances, the reaction would be independent of the substrate concentration (Fig. VII-1), a prerequisite for enzyme activity measurement.

The action of Endo-G hydrolyzes the NCC into smaller DP glucan chains, and action of Exo-G produces the cellobiose which is further hydrolyzed into glucose by the action of β-glucosidase. When Exo-G acts on a glucan chain, one cellobiose molecule is produced which becomes soluble in the liquid, and there is no net change in the total chain end groups in the insoluble NCC. That way, any increase in chain end groups in the insoluble part of NCC is caused by the action of Endo-G. Because of low enzyme loading, it is appropriate to assume that the increase in the chain ends due to Endo-G reaction with NCC would not affect the reactivity of Exo-G with NCC. The reason for this is the presence of excess number of cellulose chains for Exo-G reaction. This shows that there is no synergism between Exo-G and Endo-G action on NCC as opposed to the action on crystalline cellulose. This condition of “No synergism” between these two components is

very important when both the activities are measured with single substrate. For crystalline and high DP substrates such as cotton or alpha cellulose, the synergism between these two components is very high (Clarke et. al., 1997). As the crystallinity and DP of NCC is very low and very low enzyme loadings are being used in these experiments, the assumption of no synergism between these two components is a valid one. Under these assumptions, the increase in the chain end would give the estimation of Endo-G activity, and the rate of production of Cellobiose + Glucose would give the estimation of Exo-G activity (Fig. VII-2).

MATERIALS AND METHODS

NCC and other substrates

NCC was prepared by acid hydrolysis of cotton using the method described in the patent application (US Provisional Patent Application No.: # 60/762,439). Cotton was provided by Buckeye Technologies Inc, in the sheet form which had been ground before further processing. Other substrates for different activity measurement such as filter paper (Whatman No.1), CMC, Avicel, beechwood xylan and cellobiose were purchased from Fisher Scientific.

Enzymes

Three different cellulase preparations were provided by Genencor International Inc. (Palo Alto, CA) that were used here for comparison of the enzymatic activity. These enzyme were GC 220 and two different lots of spezyme CP which were addressed here as

SC-A (Lot No. 301-00348-257) & SC-B (Lot No. 301-04075-054). The reported filter paper activity of SC-A, SC-B and GC 220 was 31.2 FPU/ml (Calculated at NREL), 59 FPU/ml and 90 FPU/ml (Provided by Genencor). As provided by Genencor, the protein content of SC-A, SC-B and GC-220 was 106, 123 and 184 mg/ml respectively.

Activity measurement

CMCase activities in cellulase were measured using the protocol developed by Ghosh et al (Ghosh et. al., 1987). Enzymatic reactions were performed in a capped glass tubes and tubes were incubated in a water heater with shaker. DNS reagent was prepared as per the method described in NREL Laboratory analytical procedure (LAP) no.6 (www.eere.energy.gov/biomass/analytical_procedures.html).

Beechwood xylan (Sigma Catalog No.: X4252) was used as a substrate for xylanase activity measurement. Xylo-oligosaccharides (XOS), produced from the hydrolysis of xylan with very low enzyme loading, were taken as a measure for xylanase activity in cellulase. HPLC with a Bio-Rad Aminex HPX-87P column was used for quantification of the XOS. The relative activity of xylanase in different cellulases was calculated by comparing the slope of the graph between XOS produced and enzyme dilution (Fig. VII-6). Similarly relative activity of Exo-G was measured with Avicel as a substrate. It was calculated by comparing the slope of the graph between Glucan equivalent of G1+G2 and enzyme dilution at very low enzyme loading.

In the *initial slope method* for activity measurement, enzyme-substrate reaction was carried out for only one hour with very low enzyme loading to avoid inhibition of the

product. Substrate loading in this method was equivalent to 1g glucan/100 ml of reaction volume. Enzymatic reaction was carried out in 250 ml Erlenmeyer flasks. Slope of hydrolysis plot was taken as an estimate of the enzymatic activity. Sugars concentration after enzymatic reaction was determined by HPLC using a Bio-Rad Aminex HPX-87P column.

Procedure For Proposed Method

Step 1:

- NCC contains approx. 80% moisture and has paste like appearance. In order to disperse the NCC in the buffer solution evenly, sonication of NCC and buffer mixture should be done for making 2% uniform suspension. 0.05M citrate buffer has been used in this procedure. In order to ensure the uniformity, suspension should be sieved from a 60mesh sieve.
- Multiple enzyme dilutions (at least two) should be prepared separately for all the cellulase preparations that need to be compared for its activities. Buffer should be used to make the enzyme dilutions.

  (*Note:* Initially when the enzyme activity is not known, a wide range of enzyme dilutions are required to get the estimate of operating range where the enzyme-substrate reaction should satisfy the criterion of excess substrate with respect to Endo-G and Exo-G (Fig.VII-1). As explained in the introduction, this criterion requires that the trend of enzyme dilution used vs. sugar released after hydrolysis (for Exo-G action) and trend of enzyme dilution vs. total reducing ends in the

solid after hydrolysis (for Endo-G action) be straight lines. Enzymes with higher activity will have higher dilution factors. Reducing ends in solids are proportional to the absorbance of solid with DNS reagent at 540 nm. When the intent is to find out the relative activity of Endo-G in different cellulases, the slope of the enzyme dilution vs. solid absorbance (with DNS reagent) graph can be compared for different enzymes.)

- As described in Fig.VII-3, 0.5 ml of diluted enzyme solution and 0.5ml of 2% NCC suspension should be incubated in a 5-10 ml closed capped test tube at 50°C for 30min. The procedure should be carried out for all the enzyme dilutions along with the DNS blank (1ml buffer only), substrate blank (0.5ml of 2% NCC suspension + 0.5ml of buffer) and enzyme blanks (0.5ml of diluted enzyme + 0.5ml buffer) in one experiment simultaneously.

  (*Note*: Any type of difference in the reaction conditions among these test tubes should be minimized to improve the accuracy of the procedure)

Step 2:

- Hydrolysis should be stopped by boiling all the test tubes for 5 min. Add 2 ml of ice cold DI water in all the test tubes.

Step 3:

- Mix the content of test tube uniformly and remove one sample to measure the sugar concentration in the liquid by HPLC.

- Take two more samples from this uniform suspension (each having exact volume of 0.5 ml): one in a 5-10ml closed cap test tube and other in 1.5 ml micro-centrifuge tube.
- Centrifuge the sample in micro-centrifuge tube and remove all of the supernatant very carefully, leaving the solid dense NCC in the bottom. Put the supernatant in another 5-10 ml closed cap test tube.

Step 4:

- Add 3ml of DNS reagent in these two closed cap test tubes (the first containing 0.5 ml suspension and the second containing only supernatant). Boil both test tubes simultaneously for 5 min, and then put these test tubes in ice cold water to cool them down immediately.
- Centrifuge both the test tubes and measure the absorbance of the supernatant from both test tubes. Absorbance of first tube would give the value corresponding to Solid + Liquid and absorbance of second tube would give the value corresponding to Liquid.
- The total amount of Glucose and Cellobiose released is calculated by measuring the concentration of respective sugars from HPLC (Bio-Rad Aminex HPX-87P column) and then multiplying the concentration with the volume (3ml) after dilution in step 2. Glucan equivalent of G1+G2 is calculated by using the formula 0.9*Glucose + 0.95*Cellobiose.

% Hydrolysis is defined as follows:

$$\% \text{ Hydrolysis} = \frac{\text{Glucan Equivalent (G1+G2)}}{\text{Total Glucan in Substrate}} \times 100$$

A sample calculation is shown in Table VII-1. The values in the column corresponding to dilution zero show the value of substrate blank minus DNS blank. The values in the other columns show corresponding value of that sample minus the enzyme blank for that dilution. The slope of the graph between the solid absorbance and the enzyme dilution would give the estimation of Endo-G activity, and the slope of the graph between the glucan equivalent of G1+G2 and the enzyme dilution would give the estimation of Exo-G activity.

In order to minimize the error in the assay, following precautions should be taken:

- Reactions for all the enzyme dilutions corresponding to different cellulases (which are being compared) in addition to all the enzyme blanks and the substrate blank must be carried out side by side in one experiment and any sort of difference in process conditions should be avoided
- Owing to the fact that Endo-G reactivity with the amorphous cellulose is very fast, the enzyme dosing in the experiment should be extremely low.
- As the reaction volume is very low, evaporation loss during enzymatic reaction or DNS-sugar reaction can cause error. This loss should be totally eliminated.

- In step 3, when liquid is separated from solid after the centrifugation, proper care must be taken to avoid any solid particles in the liquid. Liquid from the centrifuge tube should be separated with the use of a 0.2 ml pipette tip. When NCC is used as a substrate, a compact cake of solid is formed in the bottom of centrifuge tube after the centrifugation of the liquid-solid mixture, and it is very easy to remove the liquid from top of that. This is not the case with other cellulosic substrates like avicel or α-cellulose. Accuracy is strongly affected if part of solid goes to the liquid side during the separation.

RESULTS

As shown in Fig.VII-4, the trend of solid absorbance vs. enzyme loading is a straight line for very low enzyme dilution, but as the enzyme loading is increased to a slightly higher value, solid absorbance does not increase as fast and ultimately that trend would approach a maximum value. If the enzyme loading is increased further, the absorbance value of solids with DNS even goes down after attaining a maximum due to the consumption of cellulose chains by Exo-G. To calculate the relative activity, one needs to operate in the region where this trend is a straight line. As shown in Fig.VII-4 (b), slope of the trend line of these graphs would give an estimate of Endo-G activity. The same experiment was conducted using a higher substrate loading of 5% NCC suspension but with the same enzyme loadings. The increase in solid absorbance was approximately the same as with a 2% NCC suspension when extremely low enzyme loadings were used (data not shown). This indicated that the assumption of excess substrate in the

reaction is correct in this straight line region with extremely low enzyme loading.

Fig.VII-5 (a) and (b) shows the trend between the glucan equivalent of glucose + cellobiose released vs the enzyme dilution. For very low enzyme loading with NCC, the value of soluble sugar released is very low, and as enzyme loading is increased above a certain value, a significant improvement in NCC hydrolysis can be observed. This indicates that as a result of the interaction of NCC and Exo-G, a certain degree of enzyme inactivation might take place, and the effective availability of the enzyme to the reducing ends would be negligible. Hence, it is important to use the enzyme loading over that minimum value. As indicated by Fig. VII-5(a), the trend of hydrolysis rate and enzyme loading is almost a straight line after the first value. The slope of these lines gives an estimate of Exo-G activity as shown in Fig. VII-5(b).

Table VII-2 summarizes the relative activity of all the enzyme components in given cellulase preparations measured by different methods in our lab. Activity measured by the proposed method is the mean value of different runs done with these enzymes. The relative activities of cellulase components measured by conventional and proposed methods are not exactly the same but follow similar trends.

FIGURES AND TABLES

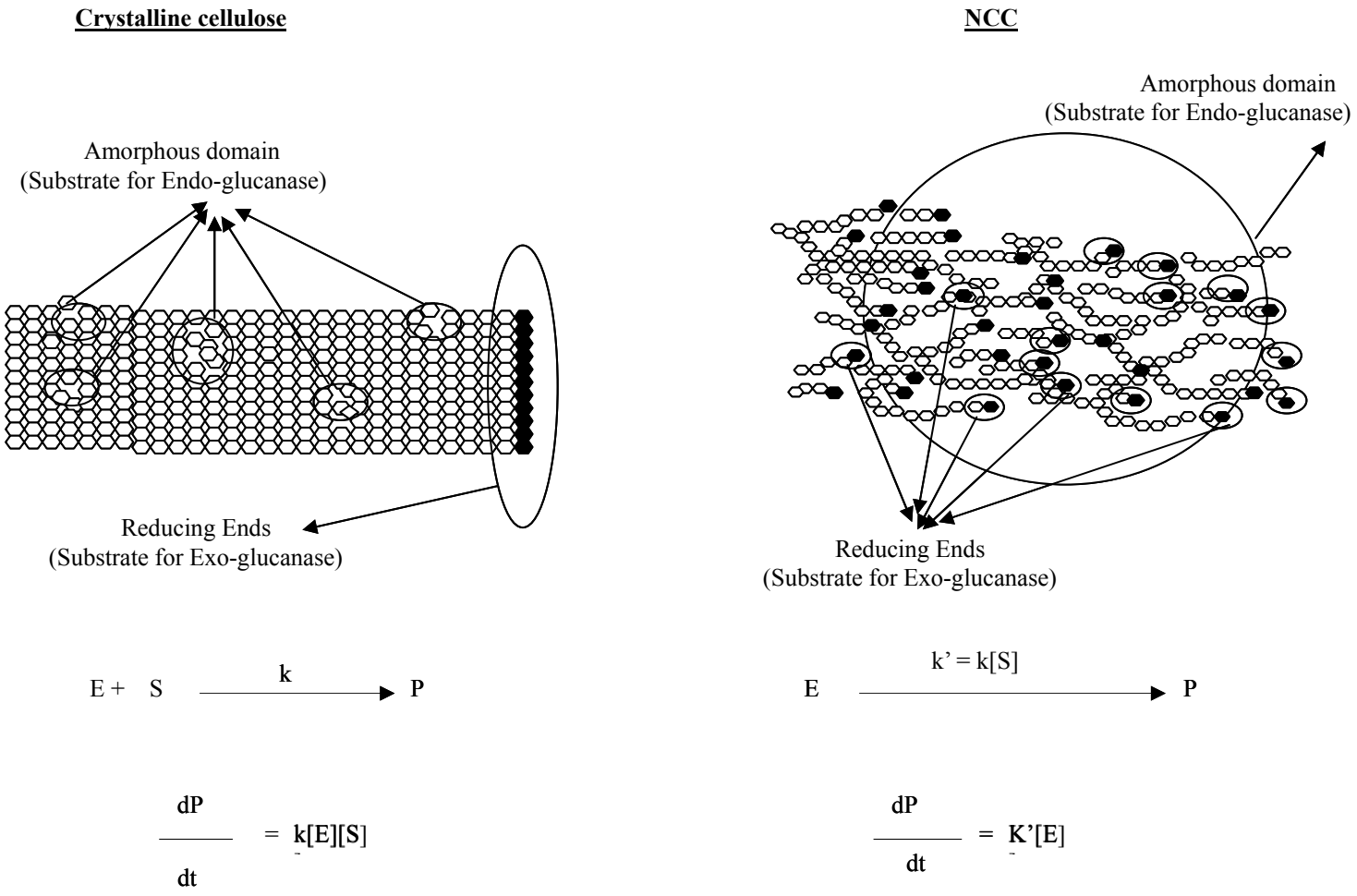


Fig.VII-1. Schematic diagram of crystalline cellulose and NCC

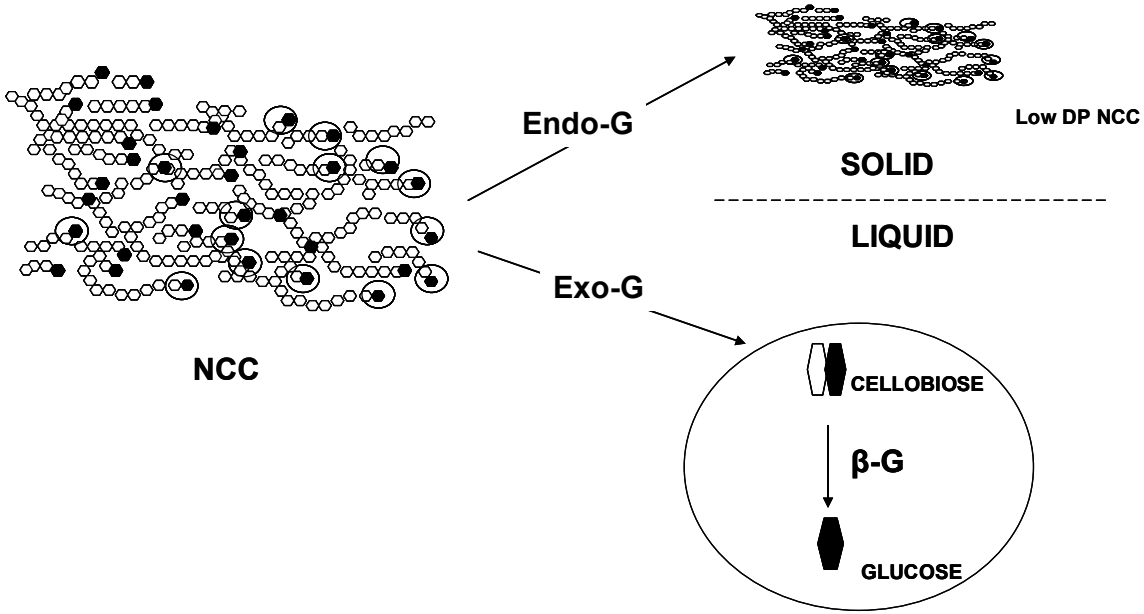


Fig.VII-2. Schematics of NCC reaction with different enzymatic components

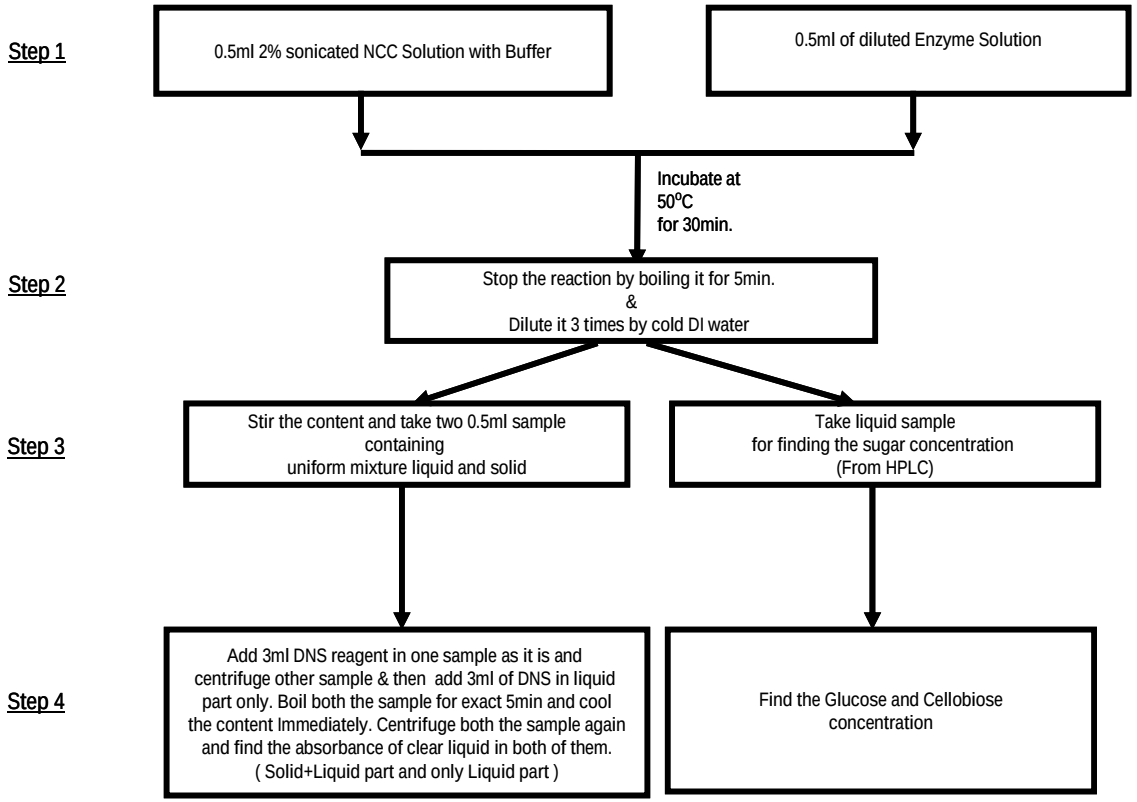
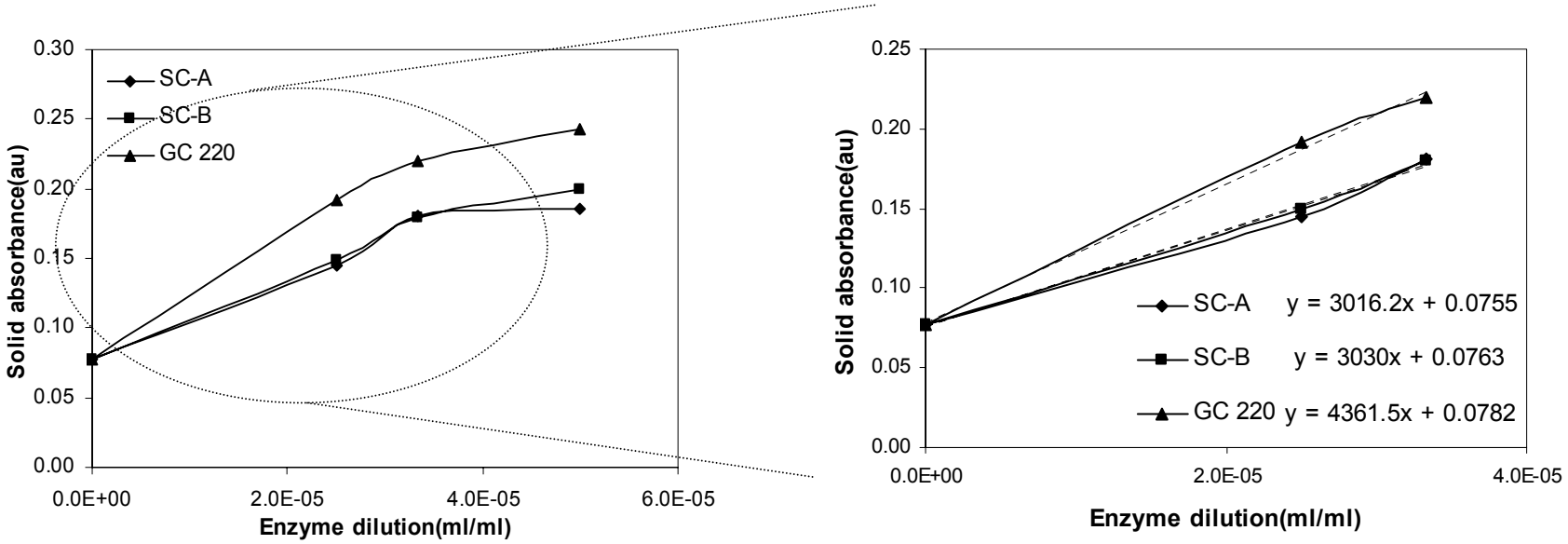


Fig.VII-3. Proposed procedure for relative activity measurement of Endo-G and Exo-G

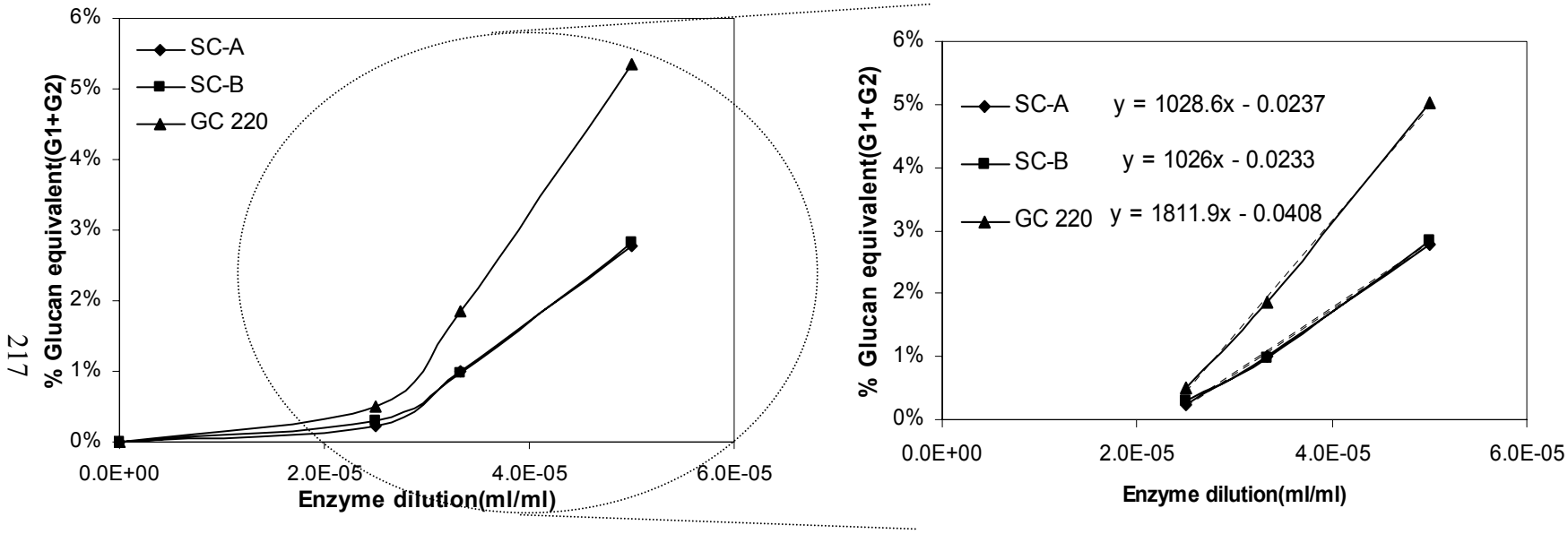
		SC-A				SC-B				GC 220			
Enzyme loading	Dilution (ml of enzyme/ml)	0.0	2.5E-05	3.3E-05	5.0E-05	0.0	2.5E-05	3.3E-05	5.0E-05	0.0	2.5E-05	3.3E-05	5.0E-05
	(ml of enzyme/g of substrate)	0.000	0.001	0.002	0.003	0.000	0.001	0.002	0.003	0.000	0.001	0.002	0.003
Absosrbance (au)	Solid+Liquid	0.205	0.287	0.330	0.385	0.205	0.308	0.341	0.430	0.205	0.391	0.421	0.516
	Liquid	0.128	0.142	0.150	0.200	0.128	0.159	0.161	0.230	0.128	0.199	0.201	0.273
	Solid	0.077	0.145	0.181	0.188	0.077	0.149	0.180	0.200	0.077	0.192	0.220	0.243
% Hydrolysis	% Glucan Equivalent(G1+G2)	0.00%	0.23%	1.01%	2.79%	0.00%	0.30%	0.98%	2.83%	0.00%	0.51%	1.86%	5.01%

Table VII-1. Sample Calculation for activity measurement



(a) (b)

Fig.VII-4. Trend of solid absorbance vs. enzyme dilution



(a) (b)

Fig.VII-5. Trend of Glucan Equivalent of G1+G2 vs. enzyme dilution

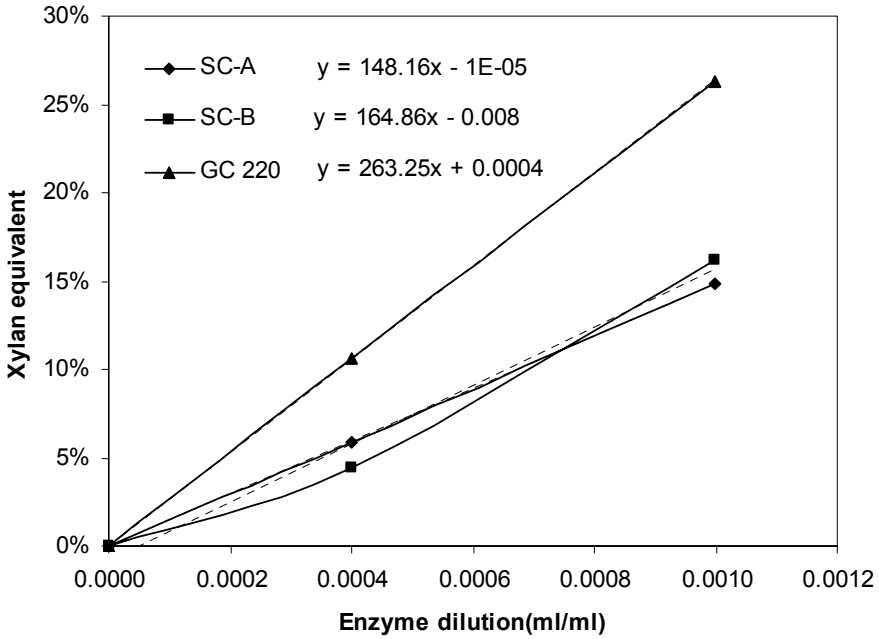


Fig. VII-6. Trend of XOS generation vs enzyme dilution

Enzyme Component	Substrate	Method	Relative Activity		
			SC-A	SC-B	GC 220
Endo-G	NCC	Proposed	1	0.86	1.46
	CMC	Ghose et al.	1	1.07	1.51
Exo-G	NCC	Proposed	1	1.08	1.77
	NCC	Initial Slope	1	1.02	1.60
	Avicel	As in M&M*	1	1.12	1.48
β-Glucosidase	Cellobiose	Initial Slope	1	0.69	2.31
Xylanase	Xylan	As in M&M*	1	1.11	1.78
Overall	Filter Paper	Initial Slope	1	0.89	1.31

Table VII-2. Cellulase activities measured with different methods
(* Materials and Methods)

VIII. CONCLUSION AND FUTURE WORK

This study showed the potential applicability of the alkaline reagents (Aqueous ammonia and NaOH) for the pretreatment of various lignocellulosic feedstocks. Due to the high selectivity for delignification and mild effect on the carbohydrates, alkaline reagents tend to retain large amount of carbohydrates in the solid after pretreatment which can easily be hydrolyzed into monomer sugars by the use of cellulase and xylanase. Because very less amount of sugars are removed from the solid in pretreatment, processing of pretreatment liquor is not required for sugar recovery. For these reasons, cellulosic ethanol production using alkaline pretreatment offers a number of benefits: lower energy requirement, higher fermentation efficiency, less environmental impact and maximum utilization of biomass.

The following points are suggested for the future study aimed at improving the process for cellulosic ethanol production based on alkaline pretreatment:

1. *Use of metal complexes in alkaline treatment of biomass in presence of H_2O_2:*

   It is observed in this study that the stability of H_2O_2 in oxidative delignification under alkaline condition is very important. At higher temperatures with aqueous ammonia, H_2O_2 caused significant carbohydrate decomposition. It is known that the some metal (manganese or molybdenum)

complexes improve the stability of H_2O_2 and helps in the reduction of carbohydrate degradation (Suchy et. al., 2001). Further investigation is required to study the effect of metal complexes in the treatment of different substrate with H_2O_2 under the alkaline condition.

2. *Characterization of liquid stream for sugar and lignin degradation byproducts*

Various lignin and sugar degradation components present in pretreatment liquor can be characterized by using different HPLC columns coupled with mass spectrometer and photodiode array (Chambliss et. al., 2007). Identification and tracking the release of these byproducts would provide the information of basic mechanism in the pretreatment reaction.

Characterization of solublized lignin in alkaline pretreatment would also be useful for understanding the reaction pattern. For this task, alkali soluble lignin in pretreatment should be precipitated by reducing the pH and then be dried by means of lyophilizer. The dry lignin sample can be characterized by different methods such as TGA (Thermo-gravimetric Analysis), DSC (Differential scanning calorimeter), FTIR and Gel permeation chromatography (for molecular weight).

3. *Use of surfactant in the enzymatic hydrolysis of ammonia treated biomass*

Literature information is abundant that the addition of surfactant increases the enzymatic hydrolysis of biomass (Eriksson et. al., 2002), especially ammonia treated biomass (Rajeev et. al., 2008). Investigation on the effect of

surfactant addition during the enzymatic hydrolysis of ammonia treated biomass would be helpful in reduction of enzyme loading

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