EVALUATION OF DIFFERENT AGRICULTURAL BIOMASS FOR BIOETHANOL PRODUCTION

By

SUNIL BANSAL

B.Sc. (Medical), Panjab University, Chandigarh, 2003

A THESIS

Submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2010

Approved by:

Major Professor
Praveen V. Vadlani

Abstract

In our study, five different bioenergy crops: wheat straw (*Triticum aestivum*), forage sorghum stover (*sorghum bicolor*), switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus giganteus*) and sweet sorghum baggase (*Sorghum bicolor*) were evaluated for bio-ethanol production at 20% (w/v) initial substrate concentration under separate hydrolysis and fermentation (SHF) process. The substrates were ground to pass through 600μm mesh size and treated with 2% (w/v) NaOH at 121°C for 30 minutes. The washed and neutralized pretreated residues were subjected to saccharification using cellulase and β-glucosidase enzymes (ratio 1:1.25) at concentrations of 25 filter paper unit (fpu)/g and 31.25fpu/g, respectively, in pH 5.0 citrate buffer in an orbital incubator shaker at 150 rpm for 72 h. The hydrolysate obtained was centrifuged and supernatant was collected for fermentation. Fermentation was performed in shake flasks using *Saccharomyces cerevisiae* at 10% (w/v) inoculum concentration at 100 rpm for 24 h.

Alkali treatment was effective in delignification of all the biomass feedstocks. The highest percent removal on raw biomass basis was attained for sorghum stover BMR-DP (81.3%, w/w) followed by miscanthus (79.9%, w/w), sorghum stover BMR-RL (69.2 %, w/w), wheat straw (68.0 %, w/w), switchgrass (66.0%, w/w), and sorghum baggase (65.4%, w/w). Glucan saccharification varied from 56.4-72.6 % (w/w) corresponding to a glucose levels of 0.45-0.34 g/g of dry substrate. Highest saccharification was observed for wheat straw while lowest was observed for miscanthus after 48 hours of hydrolysis. A maximum final ethanol concentration of 4.3% (w/v) was observed for wheat straw followed by sorghum baggase (4.2%), sorghum RL-BMR (3.6%), miscanthus (3.4%), sorghum DP-BMR (3.4%), and switchgrass (3.2%). From our studies, it is evident that high substrate concentration used for enzymatic hydrolysis was able to provide high final ethanol

concentration. The lignin content and its arrangement in different biomass feedstocks may have affected saccharification and subsequent ethanol production.

Bulk density and flowability are the two major key parameters that should be addressed to reduce processing cost of biomass for bioethanol production. Pelleting of biomass can increase the bulk density, thereby reducing the handling and transportation costs. In addition to above study, I analyzed the changes in chemical composition due to pelletization and pretreatment, and its effect on ethanol production by comparing unpelleted and pelleted biomass ethanol production efficiency. Wheat straw and big bluestem pelleted and unpelleted biomass were compared for their ethanol production efficiency.

Pelleted and unpelleted wheat straw (*Triticum aestivum*) and bigblue stem (*Andropogon gerardii Vitman*) at a substrate concentration of 10% (w/v) were subjected to 2% NaOH treatment at 121^0C for 30 min and the resulting residues were analyzed for changes in chemical composition. Saccharification of residue was done at substrate concentration of 12% (w/v) for 48 h. The sugars obtained were fermented to ethanol using *Saccharomyces cerevisiae.* Pelletization did not significantly affect the chemical composition of biomass in terms of glucan, xylan and lignin content. Delignification of pelleted biomass was greater than unpelleted biomass. Pelletization did not influence final ethanol production for both substrates.

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Acknowledgements

I wish to express my sincere appreciation to my advisor, Dr Praveen V Vadlani for his encouragement, support and valuable guidance during my MS work. His vision and direction had helped shape the present work from its inception to completion.

I am immensely grateful to Dr Subramanyam Bhadriraju and Dr Scott Staggenborg for agreeing to be a part of my advisory committee and providing their valuable comments and suggestion to improve my research and manuscript and helping in the statistical analysis.

I would also like to thank Dr Leland Mckinney and his graduate student Johnny Wilson for providing me the pelleted biomass.

I have been very lucky to have very supportive lab members, Anand, Achira, Khushal, Yixing who all helped me a lot in numerous ways like using software, operating HPLC and providing constant encouragement and moral support during my work.

I would like to thank USDA-DOE for providing funding for my research and studies at the Kansas State University. I am also grateful to Department of Grain Science and Industry for giving me opportunity to be a part of their esteemed department and providing the facilities for research work.

Finally I thank my parents for allowing me to come so far to fulfill my dreams and giving me every opportunity to satiate my quest for knowledge.

CHAPTER 1- Introduction

The current dependence on oil for energy and for production of numerous chemicals and materials and concomitant climate change caused by fossil fuels has put tremendous focus on finding alternative renewable sources for the production of fuels and chemicals. In this respect, biomass will be the major contributor in the future supply of energy and chemicals (Herrera, 2006; Ragauskas et al., 2006). Bioethanol can be produced from domestic cellulosic biomass resources such as herbaceous and woody plants, agricultural and forestry residues, and a large portion of municipal solid waste and industrial waste streams (Demirbas, 2005). The billion ton study of Perlack et al., (2005) estimated that total amount of available biomass that can be sustainably removed from agricultural land is 194 million dry tons annually. In the future, through a combination of technology changes (e.g. higher crop yield and improved residue collection technology), adoption of no-till cultivation and changes in land use to accommodate large scale production of perennial crops, this amount can increase fivefold in next 35-40 years to reach nearly 1 billion dry tons of biomass annually. This amount is sufficient to produce cellulosic ethanol production goals to 21 billion gallons annually by 2020. Biomass conversion will also decouple the production of food and bioenergy thereby increasing the value of world's production from agriculture and forestry, contributing favorably to the reduction of CO_2 emission, and ensuring a more stable supply of energy (Larsen et al., 2008).

Ethanol production from lignocellulosics involves three major steps (Fig1.1) (1) size reduction and pretreatment of biomass to remove lignin and make cellulose and hemicelluloses more accessible to the enzymes, (2) enzymatic hydrolysis of pretreated biomass to produce sugars, and (3) fermentation of sugars by pentose and hexose-fermenting microorganism to

ethanol. The lignin and residue generated can be used to produce other chemicals or burn to generate heat and electricity.

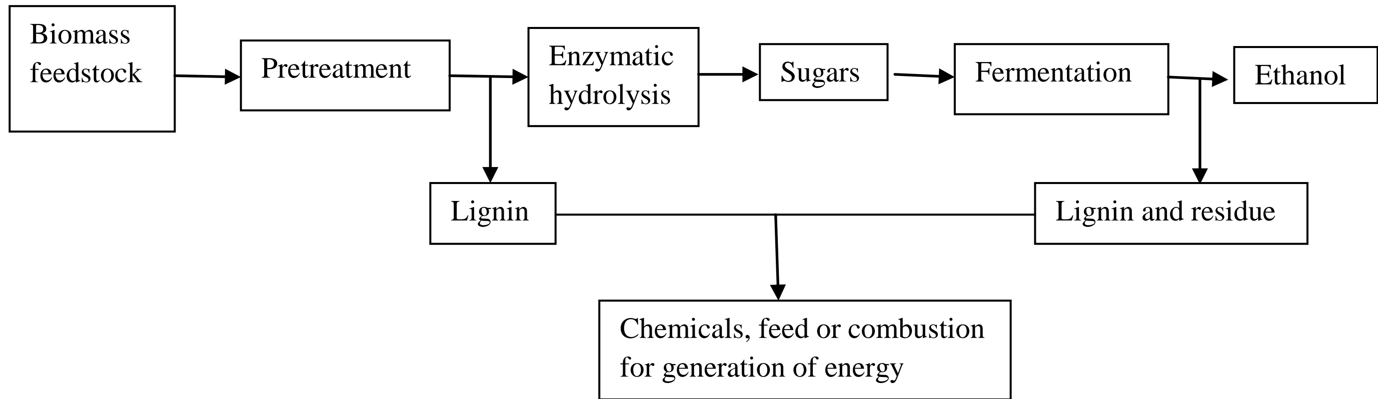


Fig1.1. Flowchart of the process for ethanol production from lignocellulosics

With advancements in the pretreatment processes and development of efficient genetically modified microbial strains with high volumetric productivity for bioethanol production from lignocellulosics, the commercialization of the process seems to be feasible in the near future. Several established chemical companies are now seriously visualizing and evaluating biorefinery concepts based on renewable resources (Bansal et al., 2010). However, a commercial lignocellulosic ethanol based plant can be feasible only if it is flexible enough to utilize a number of different raw materials without much change in the annual productivity, as a particular biomass may not be available in a particular area throughout the year. The amount and type of sugars that can be produced and the conditions required to achieve the optimum production during pretreatment and enzymatic treatment are largely dependent on the chemical composition and structure of biomass materials (Zheng et al., 2007). This requires analyzing

different biomass feedstock in terms of their chemical and structural composition and its effects on the sugars production during hydrolysis and subsequent fermentation to ethanol.

Low-cost ethanol production from lignocellulosic materials such as straw, corn stover, bagasse, wood and wood residues are still limited by a number of factors: price and performance of enzymes, efficient fermentation of all sugars (pentoses and hexoses), pretreatment costs and ability to handle lignocellulosic materials at high-solid concentrations (Jorgensen et al., 2007). Increasing ethanol titer in the fermentation broth is crucially important for cost reduction of cellulosic ethanol due to the great energy demand of ethanol distillation (Galbe et al., 2007; Larsen et al., 2008). Production of fermentable sugars from biomass materials is an important step for biobased chemicals and biofuels production. For lignocellulosic materials, sugars are primarily derived from hemicellulose and cellulose components. High glucose concentration after hydrolysis is preferable for the fermentation process to achieve high ethanol titer. This can be achieved by using high solids concentration (20-30% w/w) during the hydrolysis step. The use of very high gravity (VHG) fermentations (mashes with more than 27% (w/w) dissolved solids) improves plant productivity and process economics by reducing capital, energy, distillation and labour costs (Bayrock and Ingeldew., 2001). The high solids loading will also contribute to the reduction of water use for ethanol production (Gerbens-Leenesetal. 2009).

High solid loading means higher lignin content in the reaction mixture, which in turn leads to greater enzyme inhibition and ultimately lower cellulose hydrolysis. Lignin limits the rate of enzymatic hydrolysis by acting as a physical barrier, preventing the digestible parts of the substrate to be hydrolyzed (Chang and Holtzapple, 2000). Besides, lignin appears to reduce cellulose hydrolysis by non-productively binding cellulolytic enzymes (Esteghlalian et al., 2001).

This necessitates the choice of efficient pretreatment method that can effectively remove lignin from lignocellulosics. Efficient pretreatment of the biomass improves cellulose accessibility to enzymes by removing hemicellulose, lignin and/or reducing crystallinity of cellulose. The alkali pretreatment along with steam explosion is very well known in effectively removing lignin and its degradation products, thereby exposing the cellulose and hemicellulose components to the enzymes (Sun and Chang, 2003). Alkaline treatment has the benefits of less sugar degradation and many of the caustic salts can be recovered and/or regenerated ((Mcdonald et al., 1983; Elshaefi et al., 1991). Alkaline pretreatment also removes acetyl and various uronic acid substitutions on hemicellulose that reduce the accessibility of hemicellulose and cellulose to enzymes (Chang and Holtzapple, 2000). The extent of delignification varies depending on the biomass type. Therefore, it is important to study the effect of pretreatment on the biomass feedstocks in terms of its compositional changes and subsequent enzymatic hydrolysis.

In cellulosic ethanol process, 35-50% of the total production cost is contributed by the biomass feedstock cost (Foust et al., 2007), which, in turn, is affected by a number of factors such as biomass species, yield, location, climate, local economy and the systems used to harvest, collect, pre-process, transport and handle the material (Perlack et al., 2006). Logistics associated with moving the biomass from land to conversion facility can account for 50-75% of the feedstock cost (Hess et al., 2006). Bulk density and flowability are two major key parameters that must be addressed to reduce these costs. Increasing biomass bulk density and converting it to a standardized bulk flowable form near feedstock source can contribute toward reducing these costs (Hess et al., 2007).

Grinding biomass is one way to increase the bulk density as compared to baled biomass (Wright et al., 2006); However grinding adversely affects biomass flowability and hinders its

proper unloading, transport and storage in a biorefinery. Additional conveyer systems will be needed to overcome flowability issues and this makes grinding a costly option (Hess et al, 2006). Baling biomass is expensive and the bales have low bulk densities and tend to be difficult to handle in large scale. Bulfk density can be increased by pelleting but pelleting requires higher energy requirements than grinding because steam is needed to gelatinize starch. There is a possibility to produce pellets with high moisture levels without the use of steam. It can increase the bulk density of biomass to the levels of corn grain so that same handling equipment can be utilized without any additional cost. The bulk density of biomass pellet is 4–10 times that of as raw biomass (Karwandy, 2007). Loose biomass in its raw form has bulk density in the range of 64-96kg/m^3, which can be increased to 128-160kg/m^3 when biomass is ground to 300 microns. Chopping and pelleting of biomass can results in high bulk density of pellets in the range of 320-480kg/m^3. Ground and pelletized biomass flows like cereal grains and can use the existing well-developed handling infrastructure for grains (Cushman et al., 2003).

Pelleting of biomass is usually accomplished by applying heat and pressure to bind the particles. Additionally, a lot of heat is generated during the pelleting process, which can affect the biomass chemical and physical properties. These changes in turn may affect enzymatic hydrolysis and fermentation during ethanol production. Ethanol production from pelleted materials should be analyzed to determine if pellets produce levels of ethanol comparable with current feedstock pre-processing methods. If not, then may pellets require development of new processing technologies.

The thesis has two parts. In the first part, the objectives were:

To compare ethanol production efficiency of alkali pretreated wheat straw (*Triticum aestivum*), forage sorghum stover (*sorghum bicolor*), switchgrass (*Panicum virgatum*),

miscanthus (*Miscanthus giganteus*) and sweet sorghum baggase (*Sorghum bicolor*) using a sequential high solid saccharification and fermentation process. In the second part, the effectiveness of pelleting wheat straw and big bluestem (*Andropogon gerardii Vitman*) on ethanol production efficiency was examined. The Thesis chapters 2 through 5 describe the two objectives. Chapter 2,3, 4 deals with pretreatment, enzymatic hydrolysis of pretreated material at high solid levels, fermentation of enzymatic hydrolysate, respectively.. Chapter 5 deals with the effect of pelleting on the ethanol production. References cited in chapter 1 through 5 are listed in pages 48-53.

CHAPTER 2- Alkali pretreatment of different biomass feedstocks

Introduction

Lignocellulose is the primary building block of plant cell walls. Plant biomass is mainly composed of cellulose, hemicellulose, and lignin, along with smaller amounts of pectin, protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll, and waxes), and ash (Jorgensen et al., 2007). The composition of these constituents can vary from one plant species to another. For example, hardwood has greater amounts of cellulose, whereas wheat straw and leaves have more hemicelluloses (Table 2.1.).

Table 2.1. The cellulose, hemicellulose and lignin contents in some common agricultural residues and wastes

Lignocellulosic material	Cellulose (%)	Hemicellulose(%)	Lignin(%)
Hardwood stem	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nutshells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	50	30	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
News paper	40-55	25-40	18-30
Waste papers from chemical pulp	60-70	10-20	5-10
Coastal Bermuda grass	25	35.7	6.4
Switchgrass	45	31.4	12

Source: Sun and Cheng (2003)

In addition, the ratios between various constituents within a single plant vary with age, stages of growth, and other conditions (Perez et al., 2002). The secondary cell wall of plants contains cellulose microfibrils and hemicelluloses, which are embedded in the lignin matrix. (Fig 2.1). The lignin is resistant to microbial degradation and protects cellulose and hemicellulose by the microorganisms present in the nature.

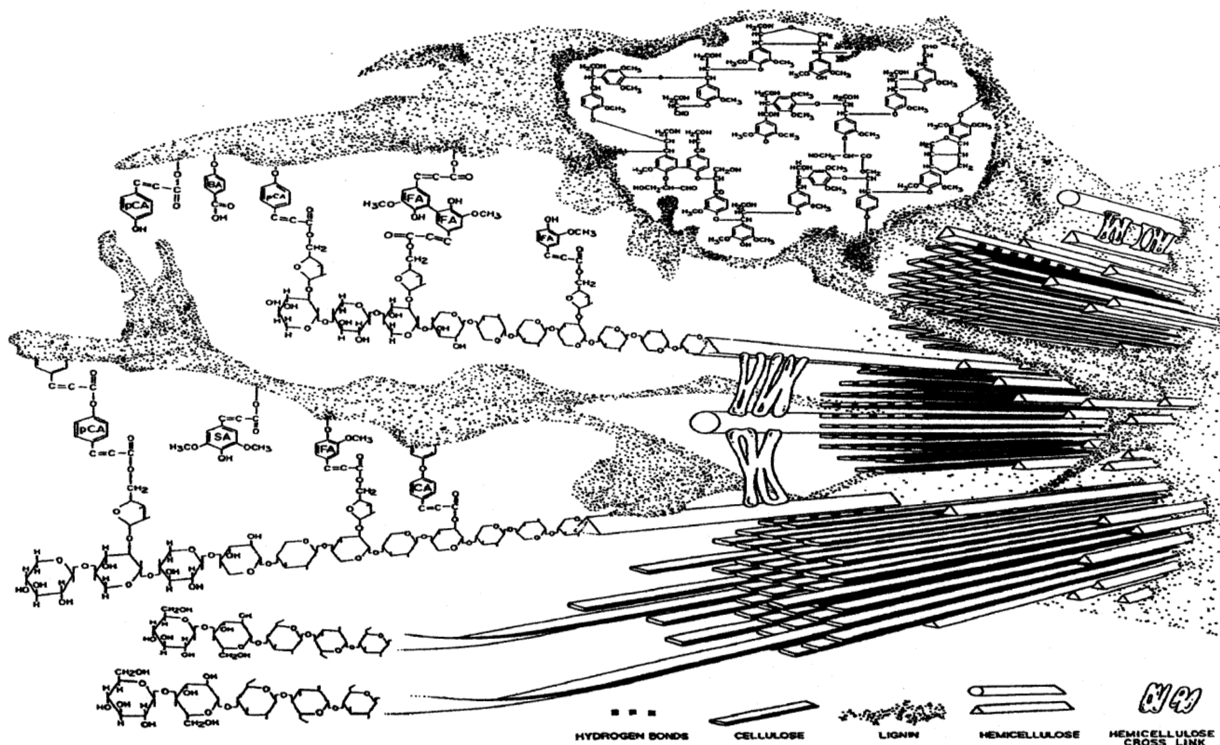


Fig2.1. Grass secondary cell wall (CW) structure. Components are arranged so that the cellulose microfibrils and hemicelluloses chains are embedded in lignin. Specific linkages and components of non-core lignin are shown for a generalized grass secondary CW. Non-core lignin components include p-coumaric acid, ferulic acid, p-hydroxybenzoic acid, sinapic acid and cinnamic acid.

Source: http://digital.library.okstate.edu/OAS/oas_image_files/v72/p51_56Fig1.jpg

Cellulose is the main structural constituent in plant cell walls and is found in an organized fibrous structure. This linear polymer consists of D-glucose subunits linked to each

other by β-(1,4)-glycosidic bonds. Cellobiose is the repeat unit established through this linkage, and it constitutes cellulose chains (Fig 2.2.).

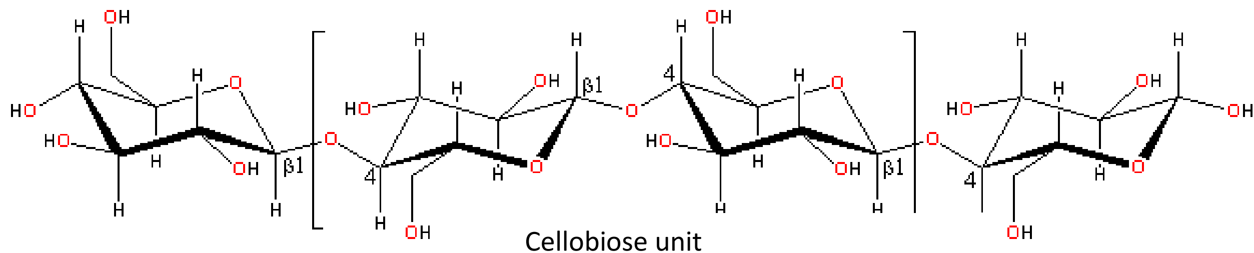


Fig 2.2 Chemical structure of cellulose

Source (http://www.chemistry.oregonstate.edu/courses/ch130/latestnews/hycell.gif)

The long-chain cellulose polymers are linked together by hydrogen and Van der Waals bonds, which cause the cellulose to be packed as microfibrils. Hemicelluloses and lignin cover the microfibrils. Cellulose in biomass is present in both crystalline and amorphous forms. Crystalline cellulose comprises the major portion of cellulose, whereas a small percentage of unorganized cellulose chains form amorphous cellulose. Cellulose is more susceptible to enzymatic degradation in its amorphous form (Beguin and Aubert, 1994). Unlike cellulose, hemicellulose has branches with short lateral chains consisting of different sugars. These monosaccharides include pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids (e.g., 4-*o*-methylglucuronic, D-glucuronic, and D-galactouronic acids). The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by β-(1,4)-glycosidic bonds and occasionally β-(1,3)-glycosidic bonds (Kuhad et al., 1997).

Lignin is a complex, large molecular structure containing cross-linked polymers of phenolic monomers. It is present in the primary cell wall, imparting structural support, impermeability,

and resistance against microbial attack (Perez et al., 2002). Three phenyl propionic alcohols exist as monomers of lignin: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (*p*-hydroxyphenyl propanol), and sinapyl alcohol (syringyl alcohol). Alkyl−aryl, alkyl−alkyl, and aryl−aryl ether bonds link these phenolic monomers together. In general, herbaceous plants such as grasses have the lowest contents of lignin, whereas softwoods have the highest lignin contents (Jorgensen et al., 2007).

Chemical composition of lignocellulosic feedstocks is a key factor affecting efficiency of biofuel production during conversion processes (Hamelinck et al., 2005; Hames et al., 2003). The structural and chemical composition of lignocellulosic feedstocks is highly variable because of genetic and environmental influences and their interactions.

The digestibility of cellulose present in lignocellulosic biomass is hindered by many physical, structural and compositional factors (Kumar et al., 2009). The presence of lignin and hemicellulose makes the accessibility of cellulase enzymes to cellulose more difficult, thus reducing the efficiency of the hydrolysis process. Pretreatment is required to alter the size and structure of the biomass, as well as its chemical composition, so that the hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved rapidly and with greater yields. Chang and Holtzapple (2000) reported correlations between enzymatic digestibility and three structural factors: lignin content, crystallinity, and acetyl content. Their results indicate that an effective lignocellulose treatment process should remove all of the acetyl groups and reduce the lignin content to about 10% in the treated biomass. Pretreatment process, by removal of lignin and hemicellulose, reduction of cellulose crystallinity, and increase of porosity, can significantly enhance enzymatic hydrolysis lignocellulosic by eliminating non-productive adsorption sites and by increasing access of enzymes to cellulose and hemicellulose (Kumar et al., 2009).

Alkali pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies (Mosier et al., 2005). Compared with acid processes, alkaline processes cause less sugar degradation, and many of the caustic salts can be recovered and/or regenerated (Mcdonald et al., 1983; Elshaefi et al., 1991). Kong et al. (1992) reported that alkali remove acetyl groups from hemicellulose (mainly xylan), thereby reducing the steric hindrance of hydrolytic enzymes and greatly enhancing carbohydrate digestibility. They concluded that the sugar yield in enzymatic hydrolysis is directly associated with acetyl group content. Alkaline pretreatment is basically a delignification process, in which a significant amount of hemicellulose is solubilized. The mechanism of action is believed to be saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components, for example, lignin and other hemicelluloses (Zheng et al., 2009). Sodium, potassium, calcium, and ammonium hydroxides are suitable alkaline pretreatment agents. Of these four, sodium hydroxide has been studied the most (Soto et al., 1994). Dilute NaOH treatment of lignocellulosic materials has been found to cause swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Fan et al., 1987). The digestibility of NaOH-treated hardwood was reported to increase from 14% to 55% with a decrease of lignin content from 24-55% to 20%. Dilute NaOH pretreatment was also found to be effective for the hydrolysis of straws with relatively low lignin contents of 10-18% (Bjerre et al., 1996). Hu et al., (2007) found that alkali loading of 0.2 g NaOH/g biomass was optimum for maximum lignin removal from switchgrass in a radio frequency based dielectric heating assisted NaOH treatment at 90°C, and leveled off in the range of 0.2 to 0.3 g NaOH/g biomass. Wang et al., (2010) also found that delignification of coastal bermuda grass increased

upto 2% NaOH pretreatment at 121^0C for 30 minutes and levels off thereafter. Hence 2% (w/v) NaOH at 121^0C was taken as the optimized conditions for pretreatment and for comparing its effect on all the biomass feedstocks in the present study.

There are very few previous studies on the effect of alkaline pretreatment on different biomass feedstocks on the extent of delignification and compositional changes. This study was therefore undertaken to evaluate the effect of alkali pretreatment on the chemical composition of biomass feedstocks especially on lignin which is a major hindrance for the enzymatic hydrolysis of cellulose and hemicellulose. This study will also help to determine whether major process parameter changes will be needed for enzymatic hydrolysis, while using different biomass feedstocks interchangeably in a commercial plant.

Material and Methods

Feedstocks

Two varieties of forage sorghum (*Sorghum bicolor*) known as Brown Mid Rib (BMR) sorghum grown in Riley county (RL) and Doniphan county (DP), Kansas; switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus giganteus*) and sorghum baggase (*Sorghum bicolor*) were obtained from research plots in 2007-2008 managed by the Department of Agronomy, Kansas State University. The two sorghum varieties were kept separate during the study to see the effect of location on the ethanol production. Additionally, wheat straw was obtained from local fields in Manhattan, Kansas. All the biomass samples were chopped to small pieces and transported to laboratory in small gunny bags or paper bags. The chopped samples were ground in a hammer mill (Eliminator Hammermill Bliss Ind. Inc., Ponca, OK) to a small particle size capable of passing through 600μm mesh size.

Alkali Pretreatment

Ground biomass (30 g) of each crop was placed in a 1000ml Erlenmeyer flask and 300 ml of 2% (w/v) NaOH solution was added to it. The flask was cotton plugged and autoclaved at 121°C for 30 min. The material obtained after treatment was dark in color which was then filtered through muslin cloth and washed under running distilled water until no color was visible in the wash water. The neutralized residue was pressed manually to remove excess water and used for the enzymatic hydrolysis. A small portion of the treated biomass was dried in the oven at 70°C for 24 h and ground to fine particle size in a Laboratory Mill (3303, Perten Instruments, springfield, IL) for the compositional analysis studies (see below). All the experiments were replicated three times during 2009-2010.

Compositional analysis

The moisture content of the biomass feedstocks was determined by the Infrared Moisture analyser, (Model IR35, Denver Instruments, Germany). The carbohydrate content and lignin of each biomass was determined by a two-step quantitative hydrolysis according to NREL/TP-510-42618 (www.nrel.gov/biomass/pdfs/42618). In a 125 ml Erlenmeyer flask, 0.3g dried, ground sample was taken and hydrolyzed by adding 3 ml of 72% sulphuric acid and agitated for 1h. This was followed by a second hydrolysis at 121°C in 4% (w/w) sulphuric acid (adjusted by diluting the above mixture with 84ml water) for 1 hour in an autoclave. The hydrolysate was then filtered through Whatman filter paper No. 1 to remove solids from the liquid. The filtrate was neutralized by slowly adding calcium carbonate while shaking the flasks. Each sample was then centrifuged to remove calcium carbonate to get a clear liquid. The liquid is further filtered through 0.45µm RC membranes into the HPLC vials and placed in the autosampler tray (Prominence, SIL-20AC)

and maintained at 4°C. Sugars were quantified by the binary HPLC system (Shimadzu Scientific Instruments, Columbia, MD) using the Refractive Index (RI) detector (RID-10A) and Phenomenex RPM monosaccharide column (300 x 7.8 mm, Phenomenex, USA). Deionised water was collected from the Milli Q (Direct Q, Millipore Inc, Billerica, Massachusetts), degassed using ultrasonicator (FS 60, Fisher Scientific, Pittsburgh, PA) and was used as mobile phase. The column oven (Prominence CTD-20A) was maintained at 80°C, RID at 65°C and the mobile phase was pumped at a flow rate of 0.6 ml/ min through the binary pump (Prominence LC-20AB). The glucan and xylan content was obtained by multiplying glucose and xylose content with a factor of 0.9 and 0.88 as correction for the water molecule that is added during hydrolysis of glucan and xylan respectively. The acid insoluble residue (AIR) retained on the filter paper was removed with spatula and put in a pre-weighed porcelain crucible. The crucible was dried in a hot air oven (Fischer Scientific) at 105°C for 24 h. The crucibles containing dried AIR were weighed again and kept in a muffle furnace (Fischer Scientific, US) at 575°C for 24 h. The crucibles with ash were removed from the furnace into a dessicator and weighed accurately after cooling for 30 minutes. Percentage acid insoluble lignin (%AIL) was calculated using the following formula:

$$\%\ \text{AIL} = \frac{\left(\text{Weight}_{\text{crucible plus AIR}} - \text{Weight}_{\text{crucible}}\right) - \left(\text{Weight}_{\text{crucible plus ash}} - \text{Weight}_{\text{crucible}}\right)}{\text{oven dry weight of sample}}$$

Percent delignification was calculated by using the formula:

$$\%\ \text{delignification} = \frac{\text{Total lignin in raw biomass} - \text{Total lignin in treated biomass}}{\text{Total lignin in raw biomass}}\ \text{x}\ 100$$

Data Analysis

All the experiments were done in triplicates. Levene's test was performed to test the homogeneity of variances using (SAS 2002-2003). The variance was found to not differ significantly hence there was no need to transform the data and different treatments were compared using one-way ANOVA, and differences among means were separated using Ryan-Einot-Gabriel-Welsch test (REGWQ). All the difference were considered significant at the $\alpha = 0.05$ level.

Results and Discussion

Composition

The glucan and xylan contents were in the range of 38.8-41.7% (w/w) and 16.8-22.3% (w/w), respectively (Table 2.3). The highest glucan was found in wheat straw and lowest in sorghum baggase, while the highest xylan was observed for switchgrass and lowest for sorghum (BMR-DP). The glucan content did not vary significantly between the biomass sources, while differences were found in the xylan content of different biomass types. Two sorghum varieties were found to have different xylan contents. The difference observed in chemical composition might be due to the genetic and environmental factors. The acid insoluble lignin varied significantly from 9.0-18.3 % (w/w). Forage sorghum varieties BMR-RL and BMR-DP showed relatively very low lignin content as compared to other biomass feedstocks. As the lignin removal is the primary purpose of pretreatment, hence these varieties might prove to be better in terms of less severe pretreatment conditions and hence lower overall costs than other feedstocks. Wheat straw was found to have highest lignin content. Compositions of various herbaceous biomass feedstocks from a number of sources was summarized and mean value is reported by

Lee et al. (2007) and presented in Table 2.2. Our results compared well with the previous results. The mean values for different sources from around the world varied slightly, which is expected. Also the hemicellulose content is lower in our results because xylan reported in our study includes only xylose sugars whereas in the table values for xylose and other sugars, were reported in the hemicellulose component.

Table 2.2 Composition of herbaceous feedstocks (Source: Lee et al., 2007)

Biomass feedstock	Cellulose % (w/w)	Hemicellulose% (w/w)	Lignin % (w/w)[a]
Wheat straw	38	29	15
Miscanthus	43	24	19
Switchgrass	37	29	19
Forage sorghum	34	17	16
Sweet sorghum	23	14	11

All the values are mean values obtained from a number of sources.
*a = Lignin is total lignin (acid soluble lignin + acid insoluble lignin)

Effect of pretreatment on composition

Alkali treatment caused a relative increase in glucan and xylan content of all the biomass samples as a result of removal of lignin. (Table 2.4). Hemicellulose solubilization displayed an obvious difference from cellulose degradation during sodium hydroxide pretreatment of all biomass types, primarily because hemicellulose is more vulnerable by chemical pretreatment than cellulose (Schmidt and Thomsen, 1998). The results indicate that lignin removal and xylan degradation account for the major parts of total solid loss during sodium hydroxide pretreatment. The pretreated feedstocks did not vary significantly in terms of glucan, xylan and lignin content. The difference observed in xylan and lignin contents between untreated biomass types disappeared after treatment due to removal of xylan and lignin to different extents. Highest

delignification was observed for the sorghum BMR-DP (81.3% w/w) followed by the miscanthus (79.9% w/w), while the lowest was observed for the sorghum baggase (65.4% w/w). Our results are comparable to the results obtained by Wang et al. (2010) observed 82.1% (w/w) of delignification coastal Bermuda grass (CBG) pretreated at 10% substrate level using 2% NaOH 121°C for 30 min and our results showed similar levels of delignifiation. The extent of delignification varied significantly among feedstocks. It may be due to the difference in structural arrangement and chemical bonding of these components among different biomass types. The effectiveness of alkali pretreatment varies with the substrate and treatment conditions. In general, alkali pretreatment is more effective on hardwood, herbaceous crops, and agricultural residues with low lignin content than on softwood with high lignin content (Bjerre et al., 1996).

Conclusions

The alkali pretreatment of the biomass feedstocks were done at 10% (w/v) substrate concentration with 2% NaOH solution at 121°C. It can be concluded that glucan and xylan content became similar after pretreatment hence different biomasses can be easily interchanged for the enzymatic hydrolysis step without much modification in terms of initial substrate concentration. However, lignin content of feedstocks differed significantly for the tested biomass types. Hence pretreatment conditions need to be adjusted accordingly, particularly for low lignin containing biomass types such as sorghum BMR in which case less severe conditions can be applied to get the same delignification thereby reducing the cost of pretreatment. Alkali treatment was found to effectively remove lignin from all the biomass types. Because lignin impedes enzymatic hydrolysis;removal of lignin through pretreatment allowed access to the use of high substrate concentration needed for enzymatic hydrolysis. Detailed study of structural

changes at the microscopic levels using Scanning electron microscopy (SEM) and X-Ray diffraction can help understanding the difference in the delignification of different feedstocks.

Table2.3 Composition of raw biomass feedstocks

Biomass Type	Components (%, w/w)		
	Glucan[a]	Xylan	Acid insoluble lignin
Wheat straw	41.7 ± 1.3	22.3 ± 0.6a	18.3 ± 0.5a
Miscanthus	39.7 ± 0.5	21.9 ± 0.6a	17.2 ± 0.3ab
Switchgrass	40.2 ± 1.3	22.3 ± 0.74a	16.3 ± 0.8ab
Sorghum brown mid rib (BMR-RL)	40.1 ± 0.1	19.9 ± 0.4b	10.4 ± 0.2c
Sorghum brown mid rib (BMR-DP)	39.7 ± 0.6	16.8 ± 0.2c	9.0 ± 0.4c
Sorghum baggase	38.8 ± 1.5	18.9 ± 0.1b	15.6 ± 0.7b

Each mean is based on three replications
Means with in the column followed by different letters are significantly different ($p < 0.005$; REGWQ)
[a] The glucan content was not significantly different among the biomass materials ($F= 0.90$; $df = 5,12$; $P = 0.51$; one way ANOVA)

Table 2.4 Effect of alkali treatment on the composition of different biomass feedstocks

Biomass Type	Components (%, w/w)			
	Glucan[a]	Xylan[b]	Acid insoluble lignin[c]	delignification
Wheat straw	61.4 ± 2.4	23.8 ± 4.0	3.5 ± 0.2	68.0 ± 0.8bc
Miscanthus	60.5 ± 3.1	24.1 ± 2.1	3.5 ± 0.4	79.9 ± 1.6a
Switchgrass	58.2 ± 1.0	22.5 ± 0.6	3.2 ± 0.3	65.9 ± 1.1dc
Sorghum brown mid rib (BMR-RL)	58.9 ± 3.5	26.7 ± 2.6	3.0 ± 1.4	69.2 ± 0.7b
Sorghum brown mid rib (BMR-DP)	57.7 ± 2.8	24.6 ± 0.4	2.9 ± 1.1	81.3 ± 1.0a
Sorghum baggase	59.9 ± 3.1	24.8 ± 1.3	2.3 ± 0.1	65.4 ± 0.5d

Each mean is based on three replications
Means with in a column followed by different letters are significantly different ($p < 0.005$; REGWQ)
[a] The glucan content was not significantly different among the biomass materials (F= 0.77; df = 5,12; P = 0.58, one way ANOVA)
[b] The xylan content was not significantly different among the biomass materials (F= 0.1.01; df = 5,12; P = 0.45; one way ANOVA)
[c] The acid insoluble lignin content was not significantly different among the biomass materials (F= 1.0; df = 5,12; P = 0.46; one way ANOVA)

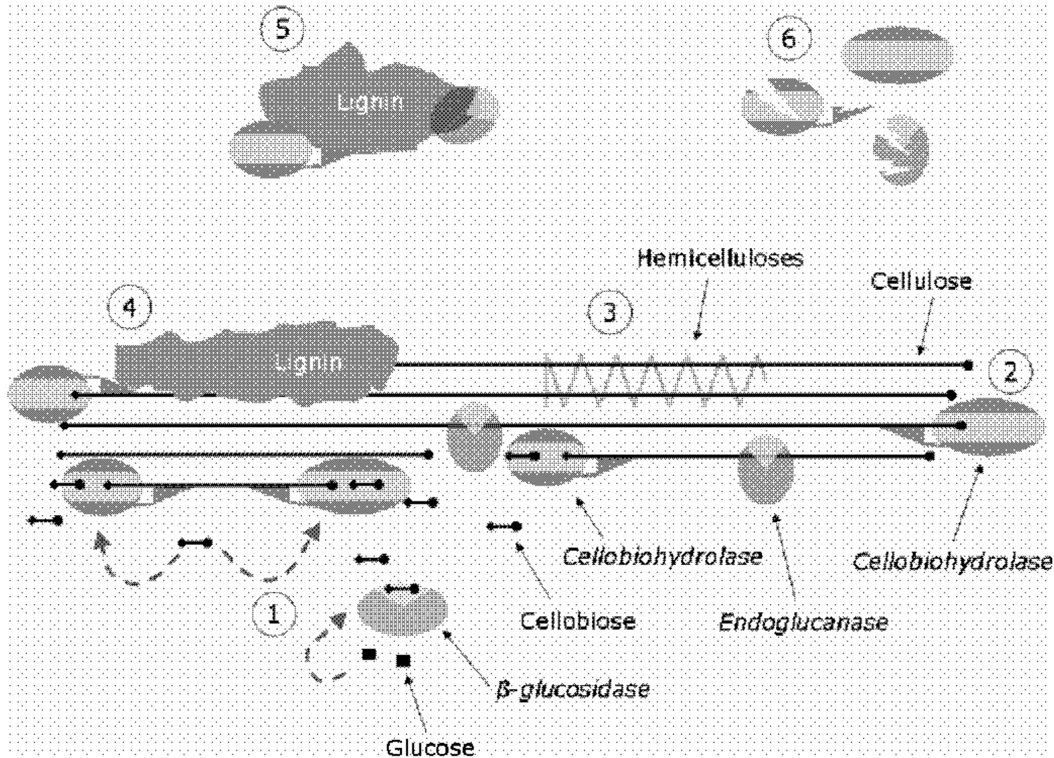
CHAPTER 3- Enzymatic hydrolysis of alkali pretreated biomass feedstocks residues using cellulase enzyme complex

Introduction

Cellulose is the most abundant organic material on earth. It has huge potential to serve as a renewable source of energy and carbon to meet the burgeoning demand for fuels and chemicals. One of the chief roadblocks for its utilization is the recalcitrance of lignocellulosic biomass toward hydrolysis to yield constituent sugars, a primary requirement for processing them further to desired products (Himmel et al., 2007). In nature, what is one of the most important process for carbon recycling, is carried out by some fungi and bacteria. Cellulolytic fungi produce a host of hydrolases, collectively referred to as cellulase enzymes complex, which acts in tandem to progressively breakdown cellulose. Bacteria have complex cellulase systems called polycellulosomes organelles that are exposed on the bacterial cell surface and aid in efficient uptake of the released glucose by preventing loss by diffusion (Zhang and Lynd, 2004)

The widely accepted mechanism for enzymatic hydrolysis involves synergistic actions by endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91),and β-glucosidase (EC 3.2.1.21). Endoglucanase hydrolyze accessible intramolecular β-1-4-glucosidic bonds of cellulose chains randomly to produce new chain ends; exoglucanase progressively cleaves cellulose chains at the ends to release soluble cellobiose or glucose; and β-glucosidase hydrolyze cellobiose to glucose. All these enzymes work synergistically to hydrolyze cellulose by creating new accessible sites for each other removing obstacles and relieving product inhibition (Eriksson et al., 2002; Valjamae et al., 2003).

Enzymatic hydrolysis of lignocellulosic is confronted by a number of obstacles that diminish the enzyme performance as shown in Fig 3.1.



Source: Jorgensen et al. (2007)

Fig 3.1 Simplistic overview of factors affecting the enzymatic hydrolysis of cellulose (symbolized by straight lines). are: 1. Product inhibition of β-glucosidases and cellobiohydrolases by glucose and cellobiose, respectively; 2. Unproductive binding of cellobiohydrolases onto a cellulose chain. Due to the processivity of cellobiohydrolases and their strong binding of cellulose chain in their catalytic core, obstacles can make the enzyme halt and become unproductively bound. 3 and 4. hemicellulose and lignin associated with or covering the microfibrils prevent the cellulases from accessing the cellulose surface 5. Enzymes (both cellulase and hemicellulases) can be unspecifically adsorbed onto lignin particles or surfaces 6. Denaturation or loss of enzyme activity due to mechanical shear, proteolytic activity or low thermostabilty

For almost any application, high sugar concentrations after the hydrolysis are preferable for the fermentation process. This will increase the product concentration and facilitate the downstream processing and product recovery. However, the use of high substrate concentrations increases the level of product inhibition, which results in lower performance of

the enzymes. The presence of lignin, which shields the cellulose chains and adsorbs the enzymes, is also a major obstacle for efficient hydrolysis. Thus effective pretreatment has to be applied to remove lignin. Tengborg et al., (2001) found that washing the pretreated material results in faster conversion of cellulose due to the removal of inhibitors. The present study employed the alkali pretreatment and washing of pretreated material to remove inhibitors so that high substrate concentrations upto 20% (w/v) can be used to get high sugar concentrations. Performing hydrolysis process at high initial substrate concentrations above 10-15% has also been technically difficult, particularly at laboratory scale due to initial high viscosity, which makes the mixing difficult and results in enhanced mass transfer limitations (Mohagegi et al., 1992; Fan et al., 2003; Fan and Lynd, 2007). In our study, high speed shaking (150 rpm) is employed to properly mix the enzyme and substrate and attain higher conversion efficiency.

Material and methods

Enzymes

The enzymes used, NS50013 (cellulase complex) and NS50010 (β-glucosidase) were obtained from the Novozymes North America Inc. (Franklinton, NC) and were a part of their complete biomass hydrolysis kit. They were stored at 4^{0}C in plastic bottles, in laboratory refrigerator. The enzyme activity of cellulase complex was calculated in term of filter paper units (fpu) and that of β-glucosidase in terms of Endoglucanase units (egu) using standard IUPAC method described by Ghose (1987). One fpu is defined as the amount of enzyme that releases 1.0 µmol of reducing sugar equivalents in 1 min from Whatman No. 1 filter paper strips at 50°C and pH 4.8. One egu is defined as the amount of enzyme which releases 1.0 µmol of glucose units from cellobiose per min under the assay conditions. The activities of cellulase

complex and β-glucosidase were found to be 75fpu/ml and 250egu/ml, respectively. All colorimetric observations were recorded using the multiprocessor-based UV-Vis spectrophotometer (UV-1650 PC Shimadzu Scientific Instruments).

Enzymatic hydrolysis

Enzymatic hydrolysis was performed in 250ml screw cap conical plastic flasks. The moist pretreated residues were analyzed for their moisture content using Infrared moisture analyzer (Denver Infrared Moisture Analyser, IR35, Denver Instruments, Germany). The amount of moist pretreated residue (triplicate per sample) containing 10g of dry pretreated biomass was calculated using moisture data and added to each flask. The substrate concentration for the enzymatic hydrolysis was adjusted to 20% (w/w) using citrate buffer (50mM, pH 4.8). Sodium azide (0.3%, w/v) was added to the hydrolysis mixture to prevent microbial growth. Enzymes were added at a concentration of 25 fpu/g and 31.3 egu/g of dry pretreated biomass, respectively. The flasks were incubated at 50°C and 150 rpm in a controlled environment incubator shaker (Model Innova 2025, New Brunswick Scientific, Edison, NJ). Samples were collected at regular intervals of 24h for a period of 72h and analyzed for sugars. Enzyme and substrate blanks were run in the hydrolysis step and analyzed for sugars. They were found to contain no detectable sugars at all the time intervals.

Data analysis

Saccharification efficiency was calculated as:

$$\text{Percent Saccharification (glucan or xylan basis)}$$

$$= \frac{\text{Amount of glucose or xylose produced from the given amount of biomass}}{\text{Amount of glucan or xylan present in same amount of treated biomass}} \times f \times 100$$

(The factor, f of 0.9 was used for glucose and 0.88 for xylose)

All the experiments were done in triplicates. Levene's test was performed to test the homogeneity of variances using (SAS 2002-2003). The variance was found to not differ significantly hence there was no need to transform the data and different treatments were compared using one-way ANOVA, and differences among means were separated using Ryan-Einot-Gabriel-Welsch test (REGWQ). All the difference were considered significant at the $\alpha = 0.05$ level.

Results and discussion

Saccharification of different biomass feedstocks

The saccharification efficiency of different pretreated biomass feedstocks as a result of hydrolysis by cellulase enzyme complex at high solids loading of 20% (w/v) is shown in table 3.1. The saccharification increased significantly from 24 h up to 48 h and then remained constant at 72 h in all the feedstocks (Fig. 3.2). Hence, 48 h can be taken as the optimum time for the enzymatic hydrolysis of all the biomass types. Our results are in line with results of Brijwani et al., (2010) who reported a sharp increase in saccharification of 1.0% (w/v) NaOH treated rice straw upto 48 h of hydrolysis after which the saccharification increased very slowly to reach a final level of 0.3 g sugars per gram of dry substrate. Percent saccharification varied significantly among all the biomass sources at all the time intervals taken. Glucan saccharification varied from 56.4-72.6 % (w/w) which corresponds to glucose levels of 0.45-0.34 g/g of dry substrate. At 48 h, the highest saccharification was observed in case of wheat straw and the lowest for the miscanthus. Lu et al., (2010) reported around 55.9% cellulose conversion for steam exploded and washed corn stover at 20% (w/w) solids loading in 48 h. Similar trends were observed for the

xylan saccharification with an increase up to 48 h and thereafter a slight decrease at 72 h (Fig. 3.3). Xylan saccharification ranged from 48.3-71.0% (w/w) with maximum in case of wheat straw and minimum in case of miscanthus. The glucan (63.2 %) and xylan saccharification (53.3 %) observed in first 24 h were higher than previously reported results of Jorgensen et al., (2007) where they observed only 32% and 49% (w/w) cellulose and xylose conversion for the steam pretreated wheat straw in a counter current flow reactor in 24 h at 20% (w/w) solids loading. Kaar and Holtzapple, (2000) reported 60% and 47% cellulose and xylose conversion for lime pretreated corn stover in 72 h of hydrolysis at enzyme loadings of 25fpu/g dry biomass. As the lignin content was similar in all the pretreated biomass sources, the difference observed in the saccharification may reflect differences in qualities of lignin, cellulose and hemicellulose and their structural arrangement in the treated biomass, which might have affected cellulase enzyme binding and its action on the various biomass types. Esteghlalian et al. (2001) reported that non productive binding of cellulase to lignin is influenced by the nature of the substrate.

The saccharification was not complete in any of the biomass. It may be due to either β-glucosidase inhibition by glucose or cellobiose or due to relative increase in the lignin content of the biomass as a result of hydrolysis of cellulose and hemicelluloses in the pretreated residue. Kaya et al. (2000) reported that cellulase has a higher binding affinity toward lignin than toward carbohydrates. The β-glucosidases from typical cellulase-producing microorganisms are to some extent also inhibited by glucose (K_i of most β-glucosidase is 1-14 mmol L^{-1} glucose) (Decker et al., 2000; Yun et al., 2001). This results in accumulation of cellobiose, which is a potent inhibitor of cellobiohydrolases (Holtzapple et al., 1990; Tolan et al., 1999). Kristensen et al. (2009) reported that neither the lignin content nor the hemicelluloses derived inhibitors affected the sugar yield in high solid saccharification; rather it was the inhibition of enzyme due to adsorption

by hydrolysis products that appear to be the main cause of the decreasing yields. Oberoi et al. (2010) reported the deposition of lignin globules on the surface of cellulose microfibrils by scanning electron microscopy, after acid pretreatment which resulted in low amount of saccharification of rice straw. If different biomass feedstocks have to be used interchangeably in a commercial conversion facility, change in the process conditions like enzyme dosage, substrate concentration, pretreatment method etc. have to be considered to obtain identical sugar yield and subsequently the similar ethanol concentration and productivity. Further studies involving the integrated use of techniques like X-Ray Diffraction (XRD), fourier transformation infrared (FTIR) spectroscopy and microscopic imaging (scanning electron microscopy, transmission electron microscopy), mass spectrophotometery (MS) may help in understanding the mechanism of enzyme action on the different biomass at the physical and chemical structural levels by revealing important facts about enzyme interaction with the lignin and carbohydrates during the reaction. For example FTIR and MS can provide useful information about the transient chemical species formed during the hydrolysis while XRD and imaging can help elaborate structural changes in cellulose, hemicelluloses and lignin polymers at different stages of hydrolysis.

Conclusions

Five different biomass materials after alkali pretreatment were evaluated for their saccharification potential using commercial cellulase at high substrate loading of 20% (w/v) with high speed mixing. All the pretreated biomass materials were hydrolyzed efficiently to yield a high saccharification efficiency and sugar concentration in the hydrolysate. The observed differences in saccharification efficiency for the different materials tested may be attributed to

the quantity and quality of lignin and its degradation products. Because lignin differs in terms of its constituent monomers and their arrangement with carbohydrates within each biomass material, the rate of enzyme activity and hydrolysis rates may have varied during saccharification. The study confirmed that under identical conditions differences in saccharification among different biomass materials is unavoidable. If different biomass materials have to be used interchangeably in a commercial conversion facility, change in the process conditions like enzyme dosage, substrate concentration and pretreatment method among others should be considered or altered to obtain identical sugar and ethanol yields. Further studies involving the integrated use of techniques like X-Ray Diffraction (XRD), fourier transformation infrared (FTIR) spectroscopy and microscopic imaging (scanning electron microscopy, transmission electron microscopy), mass spectrophotometery (MS) may help in understanding the mechanism of enzyme action on the different biomasses at the physical and chemical structural levels by revealing important facts about enzyme interaction with the lignin and carbohydrates during the reaction. For example FTIR and MS can provide useful information about the transient chemical species formed during the hydrolysis while XRD and imaging can help elaborate structural changes in cellulose, hemicelluloses and lignin polymers at different stages of hydrolysis.

Table 3.1 Comparison of enzymatic hydrolysis of different biomass feedstocks

Biomass	Saccharification (% w/w), glucan basis			Saccharification (% w/w), xylan basis		
	24h	48h	72h	24h	48h	72h
Wheat straw	63.2 ± 1.7a	72.64 ± 1.0a	71.2 ± 0.6a	53.3 ± 0.8a	71.0 ± 0.7a	69.5 ± 0.5a
Miscanthus	35.7 ± 1.3e	56.4 ± 0.3c	55.2 ± 0.3c	34.4 ± 1.2cd	48.3 ± 1.2c	47.4 ± 0.7c
Switchgrass	46.0 ± 0.5c	56.9 ± 0.9c	56.0 ± 1.4c	37.6 ± 0.1c	48.7 ± 1.5c	48.1 ± 1.3c
Sorghum brown mid rib (BMR-RL)	42.4 ± 0.1cd	62.5 ± 0.2b	61.7 ± 0.3b	33.2 ± 0.2d	57.0 ± 0.3b	56.0 ± 0.3b
Sorghum brown mid rib (BMR-DP)	40.2 ± 1.5d	60.6 ± 1.1b	59.9 ± 1.1b	35.0 ± 0.2dc	50.2 ± 0.2c	49.1 ± 0.4c
Sorghum baggase	56.8 ± 0.3b	71.1 ± 0.6a	70.0 ± 0.1a	49.2 ± 1.6b	67.6 ± 2.3a	66.8 ± 2.5a

Each mean is based on three replications
Means with in a column followed by different letters are significantly different ($p < 0.005$; REGWQ; one way analysis of variance)

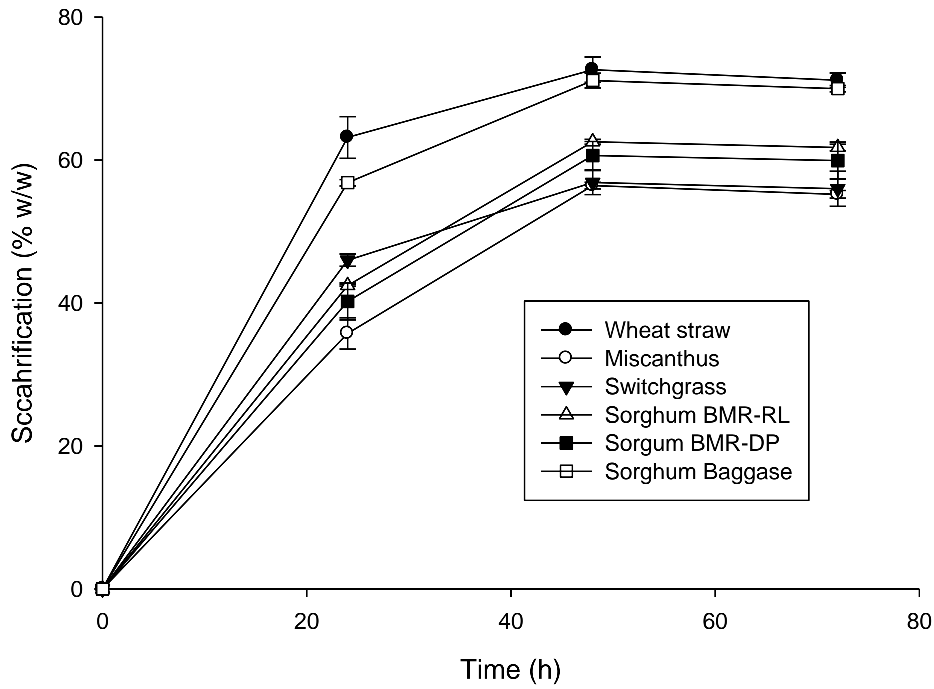


Fig 3.2 Glucan saccharification of biomass feedstocks at different time intervals

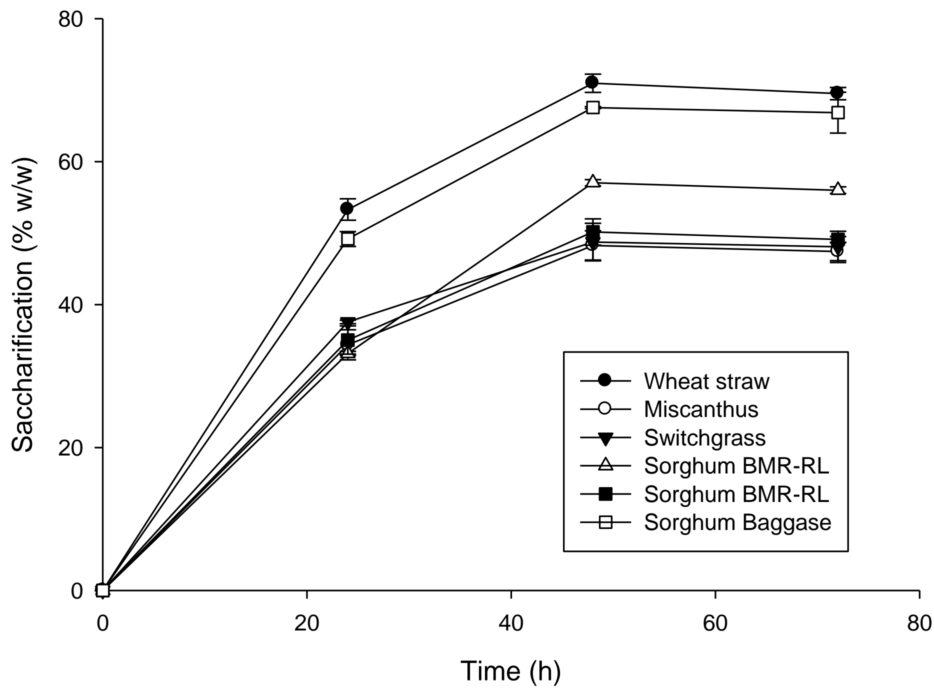
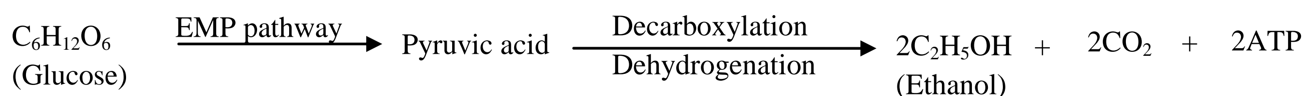


Fig 3.3 Xylan saccharification of biomass feedstocks at different time intervals

CHAPTER 4- Fermentation of sugars derived by enzymatic hydrolysis of pretreated biomass feedstocks

Introduction

After the sugars have been released by the action of enzymes, the next step in the bioethanol production process is the fermentation of sugars to ethanol. Several bacteria, yeast and fungi have been reportedly used for the ethanol production. Historically, the most commonly used microbe has been yeast; and among the yeasts, *Saccharomyces cerevisiae*, which can produce ethanol at high concentration of 18% (w/v) in the fermentation broth, is the preferred one for most ethanol fermentation. This yeast can grow both on simple sugars, such as glucose, and on the disaccharide sucrose. As with many microorganisms, *S. cerevisiae* metabolizes glucose by the Embden–Meyerhof Parnas (EMP) pathway. For every mole of glucose utilized two moles of ethanol are produced with a theoretical ethanol yield of 0.51g/g of glucose. The overall reaction can be summarized as:



Contrary to sucrose and starch-based ethanol production, lignocellulose-based production is a mixed-sugar fermentation in the presence of inhibiting compounds, low molecular weight organic acids, furan derivatives, phenolics and inorganic compounds, released and formed during pretreatment and/or hydrolysis of the raw material (Larsson et al., 2000). In ethanolic yeast fermentation, *in-situ* biological detoxification occurs when carbonyl compounds such as furans and phenolics, are reduced to the corresponding alcohols (Martin and

Jonsson., 2001; Horvath et al., 2003), which are less inhibitory to yeast (Taherzadeh et al., 2000). Due to its wide use in ethanol industry, robustness to tolerate inhibitory compounds and ethanol toxicity, *Sacccharomyces cerevisiae* Ferm Pro^{TM} (Danville, KY) was used in the present study to minimize the effect of different types of compounds released during the pretreatment and enzymatic hydrolysis. However, lignocellulosic raw materials, in particular hardwood and agricultural raw materials, can contain 5–20% (or more) of the pentose sugars xylose and arabinose, which are not fermented to ethanol by the *Saccharomyces cerevisiae*. Hydrolysates from lignocellulosic materials are generally low in nutrients and nitrogen. Pretreated wheat straw contains only around 0.4% total nitrogen on a dry weight basis, whereas mashes used in the traditional fuel ethanol production contains ten times more nitrogen (Jones and Ingledew, 1994; Linde et al., 2008). Wheat straw might contain sufficient inorganic salts and trace metals to support the yeast, but during the pretreatment process some of these may be extracted (Larsen et al., 2008). Considering the low availability of nutrients, the hydrolysate produced after enzymatic saccharification was supplemented with the extra nutrients like yeast extract and ammonium sulphate to maintain proper C:N ratio and supply sufficient minerals and cofactors for fermentation.

In bioethanol production, the commercial feasibility of the process is an important factor. Very high solid content (30% and above) is widely used in fuel ethanol industry based on starch (Bayrock and Ingledew, 2001). Operation with high substrate loading will increase the product concentration and facilitate the downstream process and product recovery, which may have a significant effect on capital cost and operating cost due to reduced energy expenditure for distillation and decreases the number of operations to reach the same ethanol output. A report on Energy Efficiency and Renewable Energy shows that about 10% overall operation cost may be

reduced when the solid level was increased from 20% to 30% (Schell, 2005). Several studies also have demonstrated that the ethanol concentration should reach about 4% to 5% (w/v) to make the process economically feasible. It is reported that the energy demand reduced by about two-thirds by increasing the initial ethanol concentration from 1% to 5% in a single distillation unit for final concentration of 94.5% (w/w) (Zacchi and Axelsson, 1989). In the present study, 20% solids were used in the enzymatic hydrolysis step to attain a final ethanol concentration between 4-5% (w/v). Also the effect of biomass composition and lignin concentration on the final ethanol concentration was evaluated.

Material and Methods

Yeast and Inoculum preparation

Active dry yeast strain *Saccharomyces cerevisiae* (FermPro™) was obtained from bioethanol production plant located in Scandia, Kansas. It was stored under refrigerated conditions (4°C). For inoculum preparation, 2g of dry yeast was added to 250 ml of sterilized Yeast Peptone Dextrose (YPD) broth in an Erlenmeyer flask. The flask was incubated at 32°C for 24 hours after which the cell count of the broth was approximately10^9 cells/ml.

Fermentation of enzymatic hydrolysate

After the enzymatic hydrolysis, the hydrolysate was centrifuged at 15000 rpm for 15 min in a high speed refrigerated centrifuge (Sorvall RCB2, GMI Inc. Ramsey, Minnesota) to remove solids from the liquid stream. Fermentation was performed in 150ml polycarbonate screw-capped flasks containing 50 ml of the enzymatic hydrolysate supplemented with 0.5% (w/v) yeast extract and 0.3% (w/v) ammonium sulphate. The yeast was added at the rate of 10% (v/v).

The flasks were capped and incubated at 32 ± 2°C for 15 h. After the fermentation, samples were collected and centrifuged in 1.00 ml eppendorf tubes at 16001 g units for 10 min at 4°C. The supernatant was analyzed for the sugar and ethanol concentrations. All the experiments were performed in triplicates.

Data analysis

Ethanol yield ($Y_{p/s}$) is calculated as:

$$Y_{p/s} = \frac{\text{Amount of ethanol formed in grams}}{\text{Amount of glucose consumed in grams}}$$

The maximum theoretical yield of ethanol from glucose is 0.51g/g. The productivity is calculated by ethanol in grams present per litre divided by the time taken for the fermentation.

All the experiments were done in triplicates. Levene's test was performed to test the homogeneity of variances using (SAS 2002-2003). The variance was found to not differ significantly hence there was no need to transform the data and different treatments were compared using one-way ANOVA, and differences among means were separated using Ryan-Einot-Gabriel-Welsch test (REGWQ). All the difference were considered significant at the α = 0.05 level.

Results and Discussion

Ethanol production efficiency of different biomass feedstocks

Table 4.1 shows the results of fermentation of enzymatic hydrolysate of pretreated biomass feedstocks. All the glucose present initially was consumed within 15 hours. However

the xylose present in the fermentation broth remained unutilized as the yeast *S. cerevisiae* does not possess the ability to ferment pentose sugars. Ethanol concentration in the range of 3.3-4.3 (%, w/v) was obtained, which is of commercial significance (Zacchi and Axelsson, 1989). Maximum ethanol was observed for the wheat straw followed by sorghum baggase, sorghum BMR, switchgrass and lowest for miscanthus. Our results are better than Maas et al. (2008) who obtained a final ethanol concentration of 21.4 g/l after 53 hours of incubation in an SSF process, using yeast *S. cerevisae* and a commercial enzyme mix GC 220, from lime pretreated wheat straw at a substrate concentration of 35% (w/v). Ohgren et al., (2006) reported a final ethanol concentration of 28 g/l from steam pretreated corn stover at 12% (w/w) substrate concentration in SSF process, using commercial enzymes and genetically engineered strain of *S. cerevisiae* TMB3400 for xylose utilization, in 96 h. Jorgensen et al., (2008) reported a highest ethanol concentration of 32.6 g/l by the fermentation of wheat straw hydrolysate containing yeast extract, corn steep liquor and magnesium sulphate, obtained from saccharification of steam pretreated and washed wheat straw residue at a substrate concentration of 30% dry matter/weight. The ethanol concentration in final fermentation broth varied significantly for the different feedstocks as a consequence of varied amounts of glucose released during the saccharification, which was fermented to ethanol efficiently without any difference in the ethanol yield. Productivity also varied similar to ethanol concentration as the fermentation was completed in the same time for all the biomass types.

Conclusions

Limited studies were conducted in the past to compare the ethanol production efficiency of different feedstocks. The study undertaken showed that alkali pretreatment was successful in delignification of biomass to an extent that is supposed to be non-inhibitory to the cellulase when

solid loadings of 10% or less are used for saccharification. However, when the saccharification was conducted at 20% (w/v) solids loading, it was observed that the cellulose conversion was not complete and leveled off to different extents in different biomass materials. This may be due to several reasons such as: (1) relative increase in the amount of lignin and sugars with time which resulted in non-competitive and competitive inhibition of the cellulase complex (2) The quality of lignin and its degradation products which affected the enzymes to different extents and Degradation of enzyme with time. All these causes needs to be verified using refined techniques such as XRD, FTIR, imaging etc. to better understand the mechanism of enzyme action and inhibition and to better design the hydrolysis process for each biomass separately. One of the benefits of high solid loadings was achievement of comparatively higher glucose concentrations which resulted in higher final ethanol concentrations. The ethanol concentration of 4% or above is considered to be of commercial significance. In that respect only wheat straw and sorghum baggase fulfilled the criteria under this procedure. Other biomass materials were close but conditions for them require certain modifications to get the higher sugars which are possible with detailed knowledge of enzyme substrate interactions during hydrolysis.

Table 4.1 Ethanol production at shake flask level for the different biomass feedstocks

Biomass	Initial glucose concentration (% w/v)	Ethanol concentration (% w/v)	Yield (g/g)[a]	Productivity ($gl^{-1}h^{-1}$)
Wheat straw	8.7 ± 0.0a	4.3 ± 0.0a	0.492 ± 0.0	2.8 ± 0.0a
Miscanthus	6.9 ± 0.1c	3.4 ± 0.0cd	0.489 ± 0.0	2.2 ± 0.0cd
Switchgrass	6.6 ± 0.1c	3.2 ± 0.0d	0.492 ± 0.0	2.1 ± 0.0d
Sorghum brown mid rib (BMR-RL)	7.5 ± 0.1b	3.6 ± 0.1b	0.488 ± 0.0	2.4 ± 0.0b
Sorghum brown mid rib (BMR-DP)	7.0 ± 0.2c	3.4 ± 0.1bc	0.492 ± 0.0	2.3 ± 0.1bc
Sorghum baggase	8.5 ± 0.2a	4.2 ± 0.1a	0.494 ± 0.0	2.8 ± 0.1a

Each mean is based on three replications
Means with in a column followed by different letters are significantly different ($p < 0.005$; REGWQ t-test)
[a] The yield was not significantly different among the biomass materials ($F= 1.2$; $df = 5,12$; $P = 0.90$; one way analysis of variance)

CHAPTER 5- Comparison of ethanol production efficiency of pelleted and unpelleted biomass feedstocks

Introduction

Agricultural biomass will be available in abundance in United States as determined by the Billion Ton study of Perlack et al. (2005), to meet the goal of 30% of energy derived from the renewable sources by 2030. However the low bulk density of agricultural biomass compared to grains is a limitation for their economical use as bioenergy feedstock due to high costs of handling and transportation. For cellulosic ethanol process, 35-50% of the total production cost is contributed by the biomass feedstock cost (Foust et al., 2007), which in turn is affected by a number of factors such as biomass species, yield, location, climate, local economy and the systems used to harvest, collect, preprocess, transport and handle the material (Perlack et al., 2006). Logistics associated with the moving the biomass from land to conversion facility can account for 50-75% of the feedstock cost (Hess et al., 2006). Bulk density and flowability are the two major key parameters that has to be addressed to reduce the processing cost. Increasing biomass bulk density and converting it to a standardized bulk flowable form near feedstock source can contribute toward reducing these costs (Hess et al., 2007).

Grinding biomass is one way to increase the bulk density as compared to baled biomass (Wright et al., 2006); however, the poor flowability of ground biomass due to its physical properties, hinders its proper unloading, transport and storage in a biorefinery. Additional conveyer systems will be needed to transport the ground biomass (Hess et al, 2006), which will add to the overall cost. Baling biomass is expensive and the bales have low bulk densities and are difficult to handle at large scale. Pelleting is another method to increase bulk density; however; it

needs higher energy requirements than grinding due to the use of steam to gelatinize starch. Recent advances now enable to produce pellets with high moisture levels without the use of steam. It can increase the bulk density of biomass to the levels of corn grain so that same handling equipment can be utilized without any additional costs. The bulk density of biomass pellet is 4–10 times that of as received biomass (Karwandy, 2007). Loose biomass in its raw form has bulk density in the range of 4–6 lb/ft^3, which can be increased to 8–10 lb/ft^3 when biomass is ground to 1/8inch. Chopping and pelleting of biomass can results in high bulk density of pellets in the range of 20–30 lb/ft^3. Ground and/or pelletized biomass flows like cereal grains and can use the existing well-developed handling infrastructure for grains (Cushman et al., (2003).

Pelleting of biomass is usually accomplished by applying heat and pressure to bind the particles and also heat generated during the extrusion process can affect the chemical and physical properties of biomass. These changes in turn may affect the biomass deconstruction, and consequently the enzymatic hydrolysis and fermentation during the ethanol production. Present study was undertaken to analyze the changes in chemical composition due to pelletization and pretreatment, and its effect on ethanol production by comparing unpelleted and pelleted biomass ethanol production efficiency.

Material and Methods

Pelleted and unpelleted biomass

Wheat straw and big bluestem bales were collected from Research farms of Department of Agronomy, Kansas State University, Manhattan, Kansas and taken to a farm located east of Manhattan. Each bale was loaded separately into a tub grinder. Large cardboard totes were

placed under the spout to collect the product. The tub grinder was flushed after each forage type to prevent contamination. Bulk densities were measured for each forage type after grinding and they are as follows: wheat-47.7 kg/m^3, and big bluestem-46.6 kg/m^3.The tub grinder was powered by a diesel engine and each bale was powdered in approximately 20 seconds. The boxes were then transported to the Bioprocessing and Industrial Value Added Program (BIVAP) facility located on Kimball Avenue in Manhattan, Kansas. Forages were subjected to further particle size reduction through a Schutte Buffalo Hammermill Model 18-7-300. Screen sizes of 1/8" were used. Forages were pelleted at K-State Pilot Feed Mill located in Shellenberger Hall using a Master Model Series 200 California Pellet Mill and were ran through a ¼" x 1 ¼" die. Pelleting runs consisted of 10.3 kg ground forage and 1.04 kg water, which total to a weight of 11.34 kg for each run. Due to compression of biomass, the temperature of the pellets coming out of die rose to 70-75°C approximately.

Compositional analysis

The unpelleted biomass and pellets were ground to fine particle size using laboratory mill and analyzed for the carbohydrates and lignin by standard NREL laboratory analytical procedures (NREL/TP-510-42618) for "Determination of structural carbohydrates and lignin in biomass" by two stage acid hydrolysis and HPLC analysis as described in chapter 2.

Alkaline pretreatment, enzymatic saccharification and fermentation

For alkali pretreatment, 25 g of either pelleted or unpelleted biomass was placed in 1000ml Erlenmeyer flask and 250 ml of 2% (w/v) NaOH solution was added to it. The flask was cotton plugged and autoclaved at 121^{0}C for 30 min. Pretreated residue was obtained in the same way as described in Chapter 2. For the enzymatic saccharification, 12% (w/v) solids loading was

used. The saccharification was performed in the 250 ml Erlenmeyer flasks with a working volume of 50 ml. The pH was set at 4.8 using 50 mM citrate buffer. Sodium azide was added at a concentration of 0.3% (w/v) to avoid contamination. The flasks were kept at 50°C in an incubator shaker at a speed of 150 rpm for 48 h. One ml samples were drawn at an interval of 24 h. The samples were centrifuged at 10000 rpm for 10 min at 4°C in refrigerated centrifuge (Eppendorf centrifuge 5415R, Hauppauge, NY) and clear supernatant obtained was used for the analysis of sugars by HPLC as described by Oberoi et al. (2010). The saccharification efficiency was calculated as:

Factor 1.1 is used to accommodate addition of a water molecule during hydrolysis

The fermentation was performed in 125 ml polycarbonate screw capped flasks with a working volume of 50 ml. The slurry obtained after enzymatic hydrolysate was centrifuged to separate the solid and liquid fractions. The liquid part containing sugars were supplemented with the yeast extract (0.5% (w/v)) and ammonium sulphate (0.3%, w/v). The flasks were capped and incubated at 32 ± 2°C for 15 h after which the samples were collected and centrifuged in 1ml Eppendorf tubes at 10000 rpm for 10 min at 4°C. The supernatant obtained was analyzed for the sugars and ethanol using HPLC.

Data analysis

All the experiments were done in triplicates. Levene's test was performed to test the homogeneity of variances using (SAS 2002-2003). The variance was found to not differ

significantly hence there was no need to transform the data and different treatments were compared using one-way ANOVA, and differences among means were separated using Ryan-Einot-Gabriel-Welsch test (REGWQ). All the difference were considered significant at the $\alpha = 0.05$ level.

Results and Discussion

Effect of pretreatment on composition of PWS and UPWS and PBBS and UPBBS

The results of compositional analysis and effect of alkali treatment on the composition of pelleted and unpelleted wheat straw and big blue stem are presented in Table 5.1. There was no significant difference observed in the chemical composition in terms of glucan and xylan content of the untreated pelleted and unpelleted biomass, which suggests that the biomass quality is unaltered due to pelleting process. After the alkali treatment, the glucan content of pelleted wheat straw and bigblue stem was higher than the unpelleted biomass. The difference in relative increase in glucan content of pelleted wheat straw and big blue stem biomass over unpelleted were 8.5% (w/w) and 5.3% (w/w), respectively. This can be directly related to higher delignification of pelleted biomass than the unpelleted biomass (Table 5.1). The delignification of big blue stem pellets (88.2%, w/w) was higher than unpelleted biomass (83.0%, w/w). Similar case was found for wheat straw although the difference in the delignification was not statistically significant. There was no difference between the xylan content of pelleted and unpelleted biomass feedstocks. The difference in xylan increase was 1.26% (w/w) and 2.17% (w/w), respectively, for wheat straw and big blue stem pellets over unpelleted feedstock. This may be

due to higher removal of xylan from the wheat straw and big blue stem pellets as compared to the unpelleted mass, which compensated for delignification differences.

Saccharification of PWS and UPWS

Table 5.2 summarizes the saccharification results for the pelleted wheat straw (PWS) and unpelleted wheat straw (UPWS). Glucan saccharification of alkali treated UPWS and PWS reached 86.9% (w/w) and 80.9% (w/w) in first 24 h, respectively, and then increased very slowly to reach 89.6% and 81.5% in the next 24 h. Glucan saccharification of UPWS was significantly higher than PWS, though the delignification trends were opposite. This might be due to difference in lignin byproducts after pretreatment and arrangement of insoluble lignin with the cellulose and hemicelluloses. The trend of xylan saccharification was different than glucan saccharification. During the first 24 h, xylan hydrolysis of UPWS reached 61.9% (w/w) which was much higher than the PWS. In the next 24 h, the xylan saccharification decreased negligibly. In the PWS, the xylan saccharification started slowly as only 42.2% (w/w) xylan was hydrolysed in first 24 h as compared to 61.9% in case of UPWS, but reached 66.1% (w/w) in 48 h similar to the levels attained in unpelleted feedstock. The reason for slow hydrolysis might be the different arrangement of lignin and xylan after pretreatment, which might have affected the binding of xylanase and cellulase to their respective substrates differently.

Saccharification of PBBS and UPBBS

The results of saccharification of alkali treated unpelleted big blue stem (UPBBS) and pelleted big blue stem (PBBS) are presented in table 5.3. The Glucan and xylan saccharification of PBBS was found to be lower than the UPBBS after 48 h of hydrolysis. Trends were almost similar to the wheat straw, expect xylan saccharification of PBBS and UPBBS was identical after

24 h of hydrolysis. This might again be due to quality of lignin and its degradation product which affect the binding and action of cellulase and xylanases.

Ethanol production efficiency of PWS and UPWS

The fermentation parameters of enzymatic hydrolysate of pretreated PWS and UPWS are presented in the Table 5.4. The results showed that pelletization of biomass did not affect their bioethanol production efficiency. All the glucose produced during the saccharification was fermented very efficiently without any significant difference in the yield. However, all the xylose remained unutilized as strain of *S. cerevisiae* used cannot ferment xylose. The ethanol concentration of UPWS and PWS was 2.6% (w/v) with an ethanol yield of 0.22 g/g. These results showed that there was no inhibition of yeast by the lignin byproducts present in the enzymatic hydrolysate.

Ethanol production efficiency of PBBS and UPBBS

Table 5.5 summarizes the ethanol production parameters of enzymatic hydrolysate of pretreated PWS and UPWS. It was clear that pelletization of biomass did not affect bioethanol production efficiency. No residual glucose was detected in the broth after 15h of fermentation without any significant difference in the yield. However, all the xylose remained in the broth, as strain of *S. cerevisiae* used was not able to ferment it. The ethanol concentration of UPBBS and PBBS was found to be 3.1% (w/v) and 2.8% (w/v) with yields of 0.26 g/g of treated UPBBS and 0.23g/g of treated PBBS, respectively.

Conclusions

Separate hydrolysis and fermentation of NaOH treated pelleted and unpelleted wheat straw and big bluestem was carried out to compare their ethanol production efficiency at shake flask level. It was found that pelletization did not have any negative or positive effect on the ethanol production efficiency. Pelletization had no significant effect on the chemical composition of biomass in terms of glucan, xylan and lignin. Alkali pretreatment resulted in higher relative glucan increase in case of pelleted biomass as compared to unpelleted biomass which can be directly attributed to higher delignification of pelleted biomass. However, unequal loss of xylan during alkali treatment, the xylan content of treated unpelleted and pelleted biomass did not differ much. The delignification was better for big blue stem pellets as compared to loose biomass while pelletization of wheat straw did not affect delignification by NaOH. Pelletization showed a negative effect on cellulose hydrolysis of both biomass types, though delignification of pelleted biomass was more as compared to unpelleted biomass. Hence it can be concluded that quantity of lignin alone is not the only factor affecting enzymatic hydrolysis. There could be other factors, which affect the arrangement of carbohydrates and lignin after pretreatment that are responsible for the difference in hydrolysis rates and overall saccharification efficiency. Pelletization did not have any effect on the fermentation of enzymatic hydrolysate of wheat straw and big bluestem, which directly indicate that the concentration of lignin byproducts and sugar produced were within the tolerable limits of the yeast.

Overall it can be said that pelletization of biomass has great potential to be one of the prominent method for the handling of biomass and its transportation as it does not affect any of the ethanol production parameters. It can be beneficial to cut down the cost of ethanol production if pellets can be produced with reduced energy than grinding biomass.

Table 5.1 Effect of alkali treatment on composition of PWS and UPWS

Biomass	Components (% w/v)						
	Glucan		Xylan		Acid insoluble lignin		
	Untreated[a]	Treated	Untreated[b]	Treated[c]	Untreated[d]	Treated[e]	Delignification[f]
Wheat straw	39.6 ± 2.3	55.1 ± 0.7b	24.0 ± 2.4	28.6 ± 1.0	17.2 ± 0.8	5.1 ± 0.4	83.7 ± 1.2
Wheat straw pellets	38.4 ± 1.5	60.4 ± 0.5a	22.9 ± 1.3	27.8 ± 1.9	15.7 ± 1.0	5.1 ± 0.2	86.3 ± 1.2

Each mean is based on three replications, Means with in a column followed by different letters are significantly different ($p < 0.005$; REGWQ t-test)

[a] The glucan content was not significantly different for untreated PWS and UPWS ($F= 0.68$; df = 1,4; $P = 0.46$)

[b]$F=0.44$; df = 1,4; $P = 0.54$, [c]$F= 0.4$; df = 1,4; $P =0.55$, [d]$F=3.9$; df = 1,4; $P =0.12$, [e]$F= 0.02$; df = 1,4; $P =0.88$

[f]$F=7.2$; df = 1,4; $P =0.05$; One way analysis of variance

Table 5.2 Effect of alkali treatment on composition of PBBS and UPBBS

Biomass	Components (% w/v)						
	Glucan		Xylan		Acid insoluble lignin		
	Untreated[a]	Treated	Untreated[b]	Treated[c]	Untreated[d]	Treated	Delignification
Big bluestem	44.8 ± 1.1	65.7 ± 0.2b	27.1 ± 0.9	30.5 ± 0.5	19.4 ± 0.1	6.4 ± 0.3a	83.0 ± 1.2b
Big bluestem pellets	42.5 ± 1.0	67.6 ± 0.5a	26.5 ± 0.5	30.5 ± 0.2	18.1 ± 0.7	5.2 ± 0.1b	88.2 ± 0.8a

Each mean is based on three replications, Means with in a column followed by different letters are significantly different ($p < 0.005$; REGWQ t-test)

[a] The glucan content was not significantly different for untreated PBBS and UPBBS ($F=2.2$; df = 1,4; $P =0.21$)

[b]$F=0.3$; df = 1,4; $P =0.61$)

[c]$F= 0.0$; df = 1,4; $P =0.96$, [d]$F=2.9$; df = 1,4; $P =0.16$; one way analysis of variance.

Table 5.3 Saccharification of pretreated PWS and UPWS

Biomass	Saccharification (%) Glucan basis		Saccharification (%) Xylan basis	
	24h	48h	24h[a]	48h[b]
Wheat straw	86.9 ± 1.7a	89.6 ± 1.3a	61.9 ± 4.2	60.2 ± 0.9
Wheat straw pellets	80.9 ± 2.5b	81.5 ± 2.1b	42.2 ± 4.7	66.1 ± 4.0

Each mean is based on three replications,
Means with in a column followed by different letters are significantly different ($p < 0.005$; REGWQ t-test)
[a] The saccharification xylan basis (24 h) was not significantly different for PWS and UPWS ($F=1.1$; df = 1,4; $P=0.35$; one way analysis of variance)
[b] The saccharification xylan basis (48 h) was not significantly different for PWS and UPWS ($F=6.18$; df = 1,4; $P=0.07$, one way analysis of variance)

Table 5.4 Saccharification of pretreated PBBS and UPBBS

Biomass	Saccharification (%) Glucan basis		Saccharification (%) Xylan basis	
	24h[a]	48h	24h[b]	48h
Big bluestem	77.8 ± 1.7	78.2 ± 1.5a	64.4 ± 1.5	65.3 ± 0.6a
Big bluestem pellets	70.6 ± 2.4	69.0 ± 1.8b	61.6 ± 0.9	60.1 ± 0.5b

Each mean is based on three replications,
Means with in a column followed by different letters are significantly different ($p < 0.005$; REGWQ t-test)
[a] The saccharification glucan basis (24 h) was not significantly different for PBBS and UPBBS $F=5.9$; df = 1,4; $P=0.07$;
[b] The saccharification xylan basis (24 h) was not significantly different for PBBS and UBBS ($F=2.17$; df = 1,4; $P=0.18$, one way analysis of variance)

Table 5.5 Ethanol production from PWS and UPWS

Biomass	Initial glucose concentration (% w/v)[a]	Ethanol concentration (% w/v)[b]	Yield (g/g)[c]	Productivity ($gl^{-1}h^{-1}$)[d]
Wheat straw	5.4 ± 0.6	2.6 ± 0.2	0.48 ± 0.02	1.7 ± 0.1
Wheat straw pellets	5.5 ± 0.2	2.6 ± 0.2	0.48 ± 0.01	1.8 ± 0.1

Each mean is based on three replications, Means with in a column followed by different letters are significantly different (p < 0.005; REGWQ t-test)

[a] Initial glucose concentration was not significantly different for PWS and UPWS (F=0.0.02; df = 1,4; P =0.89; one way analysis of variance)

[b]F=0.0; df = 1,4; P =0.96,.

[c]F= 0.2; df = 1,4; P =0.65,

[d]F=0; df = 1,4; P=0.97 one way analysis of variance

Table 5.6 Ethanol production from PBBS and UPBBS

Biomass	Initial glucose concentration (% w/v)[a]	Ethanol concentration (% w/v)[b]	Yield (g/g)[c]	Productivity ($gl^{-1}h^{-1}$)[d]
Big bluestem	6.1 ± 0.1	3.1 ± 0.1	0.497 ± 0.01	2.03 ± 0.1
Big bluestem pellets	5.7 ± 0.2	2.8 ± 0.2	0.49 ± 0.01	1.9 ± 0.1

Each mean is based on three replications, Means with in a column followed by different letters are significantly different (p < 0.005; REGWQ t-test)

[a] Initial glucose concentration was not significantly different for PBBS and UPBBS (F=2.6; df = 1,4; P =0.21; one way analysis of variance)

[b]F=1.7; df = 1,4; P =0.61

[c]F= 0.1; df = 1,4; P =0.96

[d]F= 1.8; df = 1,4; P =0.25 one way analysis of variance

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