ABSTRACT

PANDEY, PANKAJ. Combined Alkaline Pretreatment of Wheat Straw for Fermentable Sugar Production: Optimization through Response Surface Methodology. (Under the direction of Dr. Jay. J. Cheng.)

Combined alkaline pretreatment of wheat straw was investigated in this study to obtain the optimum total sugar yield by optimizing pretreatment conditions through Response Surface Methodology (RSM). The effect of four processing variables, 0.05-0.15 g loading of sodium hydroxide (NaOH) per gram raw biomass, 0.025-0.075 g loading of lime (CaO) per gram raw biomass, residence time (RT) of 3-9 h and particle size (PS) of 2-25 mm has been investigated for optimum reducing sugars yield (%). From Analysis Of Variance (ANOVA) it was revealed that all the four variables NaOH, CaO and RT and PS had significant impact on total reducing sugars yield (%) with NaOH being the most significant of all with a p-value of 0.0018. Highest total reducing sugars yield (%) for 2 mm, 13.5 mm and 25 mm observed was 82.78, 80.55 and 82.48 (%), respectively. It was found that for 9 h residence time, 2 mm particle yielded maximum reducing sugars with 88.83% of glucan and 82.73% of xylan conversion. For 3 h residence time in spite of high solid recovery for 25 mm substrate it did not translate into high sugar conversion because of ineffective pretreatment conditions. The optimized total reducing sugars yield obtained using JMP software (SAS,Cary) was 84.61% for the combination of (0.106 g/g NaOH, 0.075 g/g CaO, 9 h RT, 2 mm PS).

Combined Alkaline Pretreatment of Wheat Straw for Fermentable Sugar Production: Optimization through Response Surface Methodology

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BIOGRAPHY

Pankaj hails from a city of Faridabad, a national capital region to New Delhi, capital of India. Although he was not born there yet he cherishes the time he lived in the city which includes most of his adulthood. He did his senior schooling at Kendriya Vidyalaya Andrews Ganj, New Delhi. Given his inquisitiveness in area of biological science, he went on to pursue his under graduation in Bio-Technology at DCR University of Science and Technology and completed in year 2009 with a distinction. Thereafter he undertook a project training of 6 months in Noida ,India and subsequently to further enrich his knowledge he later moved down to the US to explore his new life as graduate student. In spring 2011 he started his master's program at North Carolina State University.

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CHAPTER ONE

CONVERSION OF LIGNOCELLULOSIC BIOMASS INTO BIO-ETHANOL: A REVIEW

1.1 INTRODUCTION

The consumption of energy has risen tremendously with increase in population and industrial advancements of various developing countries. Currently fossil fuels such as oil, coal and natural gas are the main source of energy for transportation and other industrial sectors. Due to the upward trend in energy demand, it has become incumbent upon the researchers to explore other energy options which are primarily sustainable in nature. For its oil needs the US has to import oils from oil producing countries, which themselves are said to be facing depletion in their crude oil reserves, causing instability in oil markets and threatening energy security of the country (Wyman, 1994; Sun and Cheng, 2002).

Lignocellulosic biomass includes energy crops, agricultural residues, herbaceous biomass such as switchgrass, miscanthus and woody biomass such as softwood and hardwood trees. Lignocellulosic biomass have certain advantages over fossil fuels - they are renewable in nature, do not contribute significant increase in carbon dioxide (CO_2) emission levels as they require CO_2 for their own growth, thus lower green house gas emissions, reduce air pollution and make a country to be energy independent (Wyman, 1994).

Currently the main resources for the production of bio-ethanol are based on sugar and starch materials such as sugarcane and corn-grains. A potential alternative to these first generation biofuels resources is a lignocellulosic biomass. As the demand for food goes up the use of

food grains for ethanol brings up the moral issue of “food vs fuel” supplies. This problem makes them less popular to be used for the production of biofuels and may also lead to fluctuation in their prices as a feedstock (Talebnia et al., 2010). Thus, to produce biofuels on regular basis it is important to understand the complexities lying with the structure of lignocellulosic biomass.

1.2 LIGNOCELLULOSIC BIOMASS

Lignocellulosic biomass is made of three main components: cellulose, hemicellulose and lignin. Cellulose is the most abundant compound present in the plant cell wall. Generally it constitutes 35-50% of total polymers present in the structure. It is a linear polymer of β-1,4 D-glucose units forming long fibrils that are joined together tightly by intramolecular hydrogen bonds which is very difficult to break (Darvall et al.1980). Cellulose exists in two forms as crystalline and amorphous, in which the former state is more well organized than the latter one (Hendriks and Zeeman, 2009).

Generally hemicellulose constitutes about 20-35% of total structural components of the biomass. It consists of heteropolymers such as five carbon sugars called pentoses and six carbon sugars called hexoses, and sugar acids. Pentose sugars present are mainly D-xylose and L- arabinose. Six carbon sugars present are D-mannose, D-glucose and D-galactose. The main fraction of hemicellulose is xylan in all agricultural residues and herbaceous plants whereas it is glucomannan for softwoods (Fengel and Wegener, 1984). Hemicellulose acts as a strong linker between cellulose fibers and lignin rendering rigidity to the whole structure

(Laureano et al., 2005). It is lower in molecular weight than cellulose and is easily hydrolysable to sugars (Fengel and Wegener, 1984).

Lignin is a third major component present in the lignocellulosic structure after cellulose and hemicellulose. Present in the plant cell wall and is made of amorphous heteropolymer having three phenyl propane units as p-coumaryl, coniferyl and sinapyl alcohol held together with different linkages. The main characteristics of lignin in plants are conferring them a strength against degradation are that they are optically inactive and indissoluble in water. Some of its general but important functions in the plants are to provide them a strong structural support to grow vertically, protection against microbial attack, combat oxidative stress & impermeability (Fengel and Wegner, 1984).

1.3 BARRIERS TO CONVERSION OF LIGNOCELLULOSIC BIOMASS INTO ETHANOL

1.3.1 Cellulose crystallinity

The cellulose component forms elementary fibrils of 36 glucan chains which gives it a crystalline characteristic. The crystalline nature of cellulose acts as a major barrier to enzymes to get access to cellulosic sugars present in the biomass making the hydrolysis of the carbohydrates to be very difficult.

1.3.2 Presence of lignin barrier

Lignin inhibits the rate of enzymatic hydrolysis in two ways. Firstly, by acting as a physical barrier thus prevents the hydrolysis of the digestible parts. Secondly, by binding the

cellulolytic enzymes, thus enhancing the non-productive bindings of enzymes with the substrate (Alvira et al., 2010).

1.3.3 Hemicelluloses content

When hemicellulosic matrix surrounding the cellulose gets dissolved, an increase in the accessibility of cellulose with increased pore size of the substrate is obtained (Chandra et al., 2007). Number of acetyl group in hemicelluloses interferes with the breakdown of polysaccharide chain because of its linkage with lignin, creating barrier for hydrolysis (Chang and Holtzapple, 2000).

1.3.4 Porosity

Chandra et al., (2007) found that the pore size of the lignocellulosic substrate to the size of enzymes is the main impeding factor during the enzymatic hydrolysis. In case of much larger internal surface area than the outer/external area leads to the trapping of cellulases in the inside area, which is mostly the case with lignocellulosic biomass (Alvira et al., 2010).

1.4 PRETREATMENT

To overcome the above problems related with lignocellulsic biomass the insertion of pretreatment process is a necessary step for the conversion of the biomass into biofuels. Conversion of lignocellulosic biomass to bio-ethanol requires four unit operations- pretreatment, enzymatic hydrolysis, fermentation and distillation of which the pretreatment step is of utmost importance. The tightly bound structure of lignocellulosic biomass yields it a natural resistance to breaking down into its constituents, so in order to open up the strong

lignocellulosic matrix for enzymes to have a better access of the sugars, pretreatment is generally performed (Wyman, 1994). Pretreatment is an essential step for making cellulose sugars available by dissolving barriers such as cellulose crystallinity, resistance of cell wall and lignin present in the biomass (Wyman et al., 2005). The objectives of doing pretreatment are following: (1) improving the ability to form sugars or to produce them during enzymatic hydrolysis; (2) minimizing the degradation or loss of carbohydrate; (3) reducing the formation of by-products inhibitory to subsequent hydrolysis and fermentation (Sun and Cheng, 2002). Pretreatment technology is yet not fully matured and involves a high cost for its operation (Lynd, 1996). Different pretreatment categories used for converting lignocellulosic biomasses to fermentable sugars are physical, physico-chemical, chemical, and biological processes.

1.5 PRETREATMENT TECHNOLOGIES FOR LIGNOCELLULOSIC BIOMASS

1.5.1 PHYSICAL PRETREATMENT

1.5.1.1 Mechanical comminution

This type of pretreatment is usually employed to reduce the crystallinity of cellulose and particle size of lignocellulosic biomass that would result into an increased specific surface area and reduction in degree of polymerization. Chipping, grinding and milling sometimes can be applied in combination to each other based on final particle size required such as 10-30 mm after chipping and 0.2-2 mm of after milling or grinding of biomass can be achieved (Sun and Cheng, 2002). Based on the type of biomass, type of milling and its duration, hydrolysis yield was found to be increased by 5-25% with reduction in technical digestion

time from 23 to 59% (Hendriks and Zeeman, 2009). Advantage of using this method could be the absence of inhibitors such as furfural and hydroxymethyl furfural (HMF) but due to its high energy demands and rise in energy prices this method is still not economically viable (Hendricks and Zeeman, 2009).

1.5.1.2 Pyrolysis

Lignocellulosic materials when heated above 300 $^{\circ}$C, then cellulose present in them quickly disintegrates into gaseous products and residual char (Kilzer and Broido, 1965). (Fan et al., 1987) observed that doing mild acid hydrolysis i.e. 1N H_2SO_4, 97 $^{\circ}$C, 2.5 h of residual from pyrolysis pretreatment converts 80-85% of cellulose into glucose with a yield of more than 50%. The drawback of this method is its cost ineffectiveness and bad consequences on the environment caused by burning of wood contributing in some sense to global warming.

1.5.2 PHYSICOCHEMICAL PRETREATMENT

1.5.2.1 Steam explosion

Steam explosion is the most extensively used and studied physico-chemical pretreatment for all lignocellulosic biomass (McMillan, 1994). The main goal of this method is to cause the solublization of hemicelluloses for better accessibility of cellulose during hydrolysis and to avoid production of inhibitors that interfere with hydrolysis and fermentation process (Hendriks and Zeeman, 2009).

In this process already chipped biomass is put into a vessel and supplied with high pressure steam at temperature and pressure conditions ranging from 160 $^{\circ}$C-240 $^{\circ}$C and 0.7-4.8 MPa

respectively. The interaction of steam with biomass however can vary from a several seconds to few minutes which when released swiftly causes hydrolysis of hemicellulose. The consequence of this sudden decompression results into increase in the digestibility of the cellulose as most of the hemicellulose gets solubilize in liquid phase due to the formation of acetic acid from acetyl groups present in its structure and other acids being produced during the process. Together with all these reactions lignin also observes alterations in its structure due to high temperature conditions (Agbor et al., 2011).

Four effective parameters that determine the impact of steam explosion pretreatments are residence time, temperature, particle size and moisture content (Duff and Murray, 1996). It was found that either high temperature with short residence time (270 $^{\circ}$C, for 1 min) or lower temperature with larger residence time (190 $^{\circ}$C,10 min) both give optimal solubilization of hemicellulose where second type offers an extra advantage that it does not allow the formation of inhibitory compounds (Wright, 1988). The efficiency of the steam explosion method can be increased greatly by the addition of H_2SO_4, SO_2 and CO_2. It helps in obtaining higher hemicellulose sugars, high efficiency during hydrolysis and finally inhibiting the formation of inhibitory compounds (Sun and Cheng, 2002).

There are several advantages involved with this method such as low or less energy input and chemical loading, reduced recycling cost and maintaining the sugar quality. Some disadvantages associated with this method are a risk of precipitation and condensation of soluble lignin components due to incomplete disintegration of lignin linked matrix. Also

sometimes it may annihilate a part of xylan and forms toxic compounds at high temperature. Can easily digest herbaceous and hardwood biomass but not softwoods (Agbor et al., 2011).

1.5.2.2 Ammonia fibre/ freeze explosion (AFEX)

Ammonia fibre / freeze explosion- In this process anhydrous liquid ammonia is reacted with biomass at a loading ratio about 1:1 to 1:2 (1-2 kg of ammonia/ kg of dry biomass) between a temperature range from 60 $^{\circ}$C to 100 $^{\circ}$C for 10-60 min and pressure above 3 MPa (Agbor et al., 2011). After some time the pressure is swiftly released causing a sudden drop in temperature and expansion of ammonia gas resulting into the swelling and disruption in the structure of biomass together with partial decrystallization of cellulose which leads to an increase in accessible surface area (Alvira et al., 2010). Crystal structure of cellulose changes from cellulose I to cellulose III which impact the cellulase accessibility at both micro and macro levels of cellulose (Mosier et al., 2005). Little degradation or removal of lignin and hemicellulose was observed during the pretreatment. However, cellulose was reported yielding high amount of glucose even at low enzyme loading during hydrolysis (Dale et al., 1996). An alteration or modifications in lignin structure hinders it to cause any disruption to enzymatic digestion of cellulose (Martinez et al., 1991). Many lignocellulosic materials are already used for this technology, including alfafa, wheat straw, wheat chaff, barley straw, corn stover and rice straw, municipal solid waste, switchgrass. However, biomass having high lignin content has been found to be ineffective against AFEX pretreatment (Sun and Cheng, 2002). Nevertheless it is seen that this method is moderately efficient for hardwoods but not useful for softwoods (McMillan, 1994). Pretreatment of bermudagrass (approx. 5%

lignin) and bagasse (15% lignin) using AFEX yielded over 90% hydrolysis of cellulose and hemicelluloses (Holtzapple et al., 1991).

1.5.2.3 Liquid hot water pretreatment

In this process a pressure is applied in order to keep water in a liquid state at elevated temperatures (Bobleter, 1994; Walch et al., 1992; Allen et al., 1996). The main purpose of the pretreatment is to do the solubilization of hemicellulose for better accessibility of cellulose and impeding the formation of inhibitory compounds by maintaining the pH between 4-7, which restricts sugar formation to oligomeric form, thus preventing the formation of monomeric sugars and ultimately the production of toxic substances (Kohlmann et al., 1995; Weil et al., 1997). When the biomass was given this pretreatment for 15 minutes at temperatures around 200-230°C, between 40-60% of total biomass was dissolved with 4-22% of the cellulose, 35-60% of the lignin and all hemicellulose being removed (Mok and Antal, 1992; Mok and Antal, 1994). A significant difference between liquid hot water and steam pretreatment is observed in terms of amount and concentration of solubilized products. Amount of solubilized products is higher for liquid hot water treatment whereas concentration is lower compared to steam pretreatment (Bobleter, 1994). The cleaving of O-acetyl groups and uronic acid from hemicellulose can be beneficial as well as hostile to liquid hot water pretreatment because these acids do both removal and production of oligosaccharides which may give rise to the formation of aldehydes such as furfural and HMF which are the inhibitors of microbial fermentation (Palmqvist and Hahn-Hagerdal, 2000a).

There are mainly three types of reactors being used for liquid hot water pretreatment which are Co-current, countercurrent and flow through. Co-current pretreatment involves heating slurry of biomass and water to required temperatures for a controlled residence time. In Countercurrent pretreatment design the lignocellulosic biomass and water move in opposite directions. Flow through pretreatment involves passing of hot water over stationary bed of lignocellulosic biomass causing hydrolysis and dissolving of components and then carrying them out of reactor (Mosier et al., 2005).

The impact of this pretreatment step can be realized during enzymatic hydrolysis in the form of higher enzymatic digestibility of pretreated biomass. Some of the herbaceous biomasses such as corn stover (Mosier et al., 2005), sugarcane bagasse (Laser et al., 2002) had enhanced enzymatic digestibility after this pretreatment. Yang and Wyman (2004) discovered that it was the flow through system not the batch system that solubilized more hemicellulose and lignin at same severity factors. Furthermore, when external acids were added to the system, higher hemicellulose and lignin removal was observed than for the batch system.

1.5.3 CHEMICAL PRETREATMENT

1.5.3.1 Alkali pretreatment

The interaction of bases with the lignocellulosic biomass supports its use for alkaline pretreatment. But it greatly depends upon the proportion of lignin present in the biomass material (McMillan, 1994). Solvation and saponification of ester linkages between the polymers is the first reaction that takes place during alkaline pretreatment (Sun and Cheng, 2002) which generally increase the digestibility of cellulose. Lignocellulosic material when

treated with dilute NaOH would result into swelling of biomass thus providing larger internal surface area, causing reduction in degree of polymerization and cellulose crystallinity followed by the breaking of the lignin structure and its linkages with other polymers (Fan et al.,1987). It was shown that alkaline pretreatment causes much less degradation of sugar than acid pretreatments and was more effective against herbaceous biomass in comparison to woody biomass (Kumar et al., 2009a). During alkaline pretreatment, biomass generally absorbs some of the alkali itself, so for reactions it is always a leftover alkali that reacts with the biomass (Gossett et al., 1982). When the lignin content was decreased from 24% to 20% there was an increase in the digestibility of biomass from 14% to 55% when pretreated with dilute NaOH. But in case of softwood having more than 26% lignin in its structure, dilute NaOH addition had no effect (Millet et al., 1976). Another way of reducing lignin content was proposed by Iyer et al. (1996), who described the use of ammonia in a process called as ammonia recycled percolation in which at 170 $^{\circ}$C, using ammonia concentration between 2.5-20% with time of 1 h the delignification of lignin in corn cobs and switchgrass was observed to be 60-80% and 65-85% respectively.

1.5.3.2 Lime pretreatment

Alkaline pretreatments can be effective at lower temperatures and pressures in comparison to other pretreatment processes. However, at ambient temperature residence time is much longer for alkali pretreatments (Mosier et al., 2005). Lime pretreatment is accounted to improve the digestibility of lignocellulosic biomass (Chang et al., 1998). The mechanism of the pretreatment is the solubilization of hemicellulose and lignin using Ca $(OH)_2$ as an alkali

reagent (Chang et al., 1997). They also observed when switchgrass was pretreated with lime at temperature 120 °C for 2 h approximately 26% of xylan and around 29-33% of lignin was solubilized. The interaction of lime with biomass causes a breakup of the acetyl and lignin linkages causing openings in the structure of the material (Chang and Holtzapple, 2000). With the removal of lignin, digestibility of cellulose improved and unproductive adsorption reactions during enzymatic hydrolysis was much reduced (Kim and Holtzapple, 2006). Lime pretreatment has already been used for wheat straw at 85 °C for 3 h (Chang et al. 1998), poplar wood at 150 °C for 6 h at 14 atm oxygen (Chang et al. 2001), switchgrass at 100 °C for 2h (Chang et al., 1997) and corn stover at 100 °C for 13 h (Karr and Holtzapple, 1998; Karr and Holtzapple, 2000).

Advantages of using lime pretreatment are that it is cheap in cost and is much safer to use than NaOH and KOH pretreatments, can be easily collected in the form of calcium carbonate from water when reacted with CO_2 and then can undergo lime kiln technology to be recovered as lime again (Mosier et al., 2005).

1.5.3.3 Acid pretreatment

Dilute acid pretreatment has drawn a lot of attention both for its feasible use in place of enzymatic hydrolysis and as a pretreatment step for lignocellulosic biomass to increase its digestibility (Lee et al., 1999, Nguyen, 2000). Some concentrated acids such as H_2SO_4 and HCl already being used for the pretreatment of lignocellulosic biomass, but they are not favoured as they are toxic, unsafe and corrosive in nature demanding high specifications of reactors. One further drawback is the need to recover the concentrated acids after

saccharifcation, which is an important aspect for any economical process (Sun and Cheng, 2002).

The main purpose of dilute acid pretreatment generally is to solubilize hemicelluloses and enhance digestibility of cellulose. In this process, first the biomass is dissolved in water and then heated to the required temperature in a stainless steel reactor to which preheated dilute sulfuric acid with concentrations less than 4wt. % is added to start the pretreatment process (Esteghalian et al., 1997; Torget et al., 1990). The pretreatment temperature for this process is somewhere between 140°C-215°C, with residence time from few seconds to some minutes. The reaction starts only when acid is added into the reactor. A two stage dilute acid pretreatment was proposed to improve biomass digestibility and sugar recovery (Nguyen, 2000). In first stage at low severity i.e. low acid concentration and low temperature hemicelluloses hydrolyze and in second stage remaining portion of biomass mainly cellulose gets converted to monomeric sugars (Nguyen, 2000). Saha et al. 2005 observed when wheat straw was treated with 0.75% v/v of H_2SO_4 for 1 h at 121°C the saccharification yield rose to as high as 74%. In another experiment when olive tree biomass was pretreated with 1.4% H_2SO_4 at 210°C hydrolysis yield up to 76.5% was observed (Cara et al., 2008). Other than sulfuric acid other acids reported to be used for pretreatment of lignocellulosic biomass are nitric acid (Brink, 1993, 1994), phosphoric acid (Israilides et al., 1978) and hydrochloric acid (Israilides et al., 1978; Goldstein et al., 1983).

A remarkable feature of using dilute acid pretreatment is its ability to promote high reaction rates and increase the saccharification yield of cellulose and hemicellulose at different

severity level of pretreatment (Agbor et al., 2011). Despite of achieving high sugar yield with low loadings of acid, the cost of the reactor to be used and recovery of various products make it very expensive process (Mosier et al., 2005).

1.5.4 BIOLOGICAL PRETREATMENT

In this pretreatment process, microorganisms such as brown-, white-, and soft-rot fungi are used for the degradation of lignin and hemicellulose. The most effective of all different biological pretreatments is the one which performs the degradation of lignin by using white-rot fungi which acts through the secretion of lignin-degrading enzymes such as peroxidases and laccases (Kumar et al., 2009a). Hattaka (1983) studied the pretreatment of wheat straw using 19 white-rot fungi and found that 35% of the straw was converted to reducing sugars by *Pleurotus ostreatus* in duration of five weeks which was slightly higher in comparison to 12% conversion of the untreated straw. Several white- rot fungi have shown high delignification efficiency in various lignocellulosic biomasses. List of fungi includes- *Phanerochaete chrysosporium, ceriporia lacerate, cyathus stecolerus,Ceriporiopsis subvermispora, Pycnoporus cinnarbarinus and Pleurotus ostreaus* (Alvira et al., 2010). Some general advantages with such processes are, low capital investment, low energy requirements, no use of any chemicals and the conditions used are mild. Main drawback of the process is its low hydrolysis rate when compared to other discussed technologies. To make the process to be cost effective with improvement in hydrolysis rate more and more no. of basidiomycetes fungi are needed to be studied to do the efficient and quick delignification of plants and grasses.

1.6 OBJECTIVES

Wheat is the second largest crop being produced around the world. The straw is usually left on the field, removed or burned after the grains are taken off the field. The burning of straw has been challenged as it causes serious threat to health of human beings by releasing poisonous gases in the environment. So to avoid such harmful impacts there has been great interest to utilize them to produce different value added products of which bioethanol is of much interest. To reduce the resistance of the biomass alkaline pretreatment has been investigated. Specific objectives of the study were- 1) To investigate the impact of all the four variables- sodium hydroxide, lime, residence time and particle size on the subsequent enzymatic hydrolysis. 2) To obtain the best pretreatment conditions for optimized total reducing sugars yield (%) and delignification (%) of biomass using JMP (SAS, Cary).

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CHAPTER TWO

COMBINED ALKALINE PRETREATMENT OF WHEAT STRAW FOR FERMENTABLE SUGAR PRODUCTION: OPTIMIZATION THROUGH RESPONSE SURFACE METHODOLOGY

ABSTRACT

Lignocellulosic biomass has shown a tremendous potential to be utilized for the production of biofuels. The resistance of the biomass can be overcome by breaking the ester bonds cross linking lignin with hemicelluloses matrix. In this study Response Surface Methodology based on central composite design was used to optimize combined alkaline pretreatment of wheat straw for maximum sugar conversion with respect to sodium hydroxide (NaOH) concentration (0.05-0.015 g/g of raw biomass), lime (CaO) concentration (0.025-0.075 g/g of raw biomass), residence time (RT) (3-9 h) and particle size (PS) (2-25 mm). From Analysis Of Variance it was revealed that of all processing variables considered for the study of total reducing sugars yield, only linear effects of NaOH, CaO and residence time (RT) and particle size (PS) were statistically significant at 95%. The optimized total reducing sugars yield obtained using JMP (SAS, Cary) was 84.61% for the combination of (0.106 g/g NaOH, 0.075 g/g CaO, 9 h RT, 2 mm PS).

2.1 INTRODUCTION

Bio-ethanol has a unique characteristic of being the cleanest (CO_2 neutral) renewable biofuel that can be produced from plethora of organic materials present in nature. Currently corn is the main source of bio-ethanol in the US whereas sugarcane is a major feedstock in Brazil (Limayema & Ricke, 2012). However, with little opportunity available for any further improvement in corn based biofuel production technology and with a moral issue of fuel versus feed cropping up there has been a great interest in exploration of other feedstocks that are renewable in nature. Lignocellulosic biomass is mainly made of cellulose, hemicelluloses and lignin; together they form a recalcitrant structure that makes it rather difficult for enzymes or microbes to get the access of the surface of the biomass. This tightly bound structure necessitates the step of pretreatment with the objectives of increasing porosity, solubilizing lignin and degrading hemicelluloses to increase the susceptibility of biomass to subsequent step of enzymatic hydrolysis.

Wheat is the most widely grown crop around the world, for its ability to adapt in a wide range of environmental conditions. The estimated production of wheat in year 2008 was predicted to be 650×10^{12} g (Atwell,2001), considering residue to crop ratio of 1:3 total of 850×10^{12} g of wheat straw can be obtained from it which when properly processed can yield about 238×10^{9} L of bioethanol. An upward trend in the production of ethanol has been observed with 52×10^{9} L being produced in 2012 in comparison to year 2000 when 6.17×10^{9} L were produced (renewable fuels association, 2012).

Thus far, several pretreatment processes have been tested on various biomass such as ammonia fiber explosion (AFEX), ammonia recycle percolation (ARP), dilute acid, lime,

steam explosion, and organosolv (OS) (Alvira et al., 2010; Talebnia et al., 2010; Wyman et al., 2005). Sodium hydroxide pretreatment has been shown to be an effective alkaline reagent at mild temperature (Xu et al., 2010; Wang et al., 2010). The main mechanism of alkali based pretreatment is to do the delignification of biomass through breaking of ester bonds cross linking lignin and xylan thus obtaining solid fractions rich in cellulose and hemicelluloses (Sun and Cheng, 2002).

The pretreatment of biomass with the combination of sodium hydroxide supplemented with lime at ambient temperature has been reported to be a cost effective than that of sodium hydroxide alone (Xu and Cheng, 2010; Zhang et al., 2011). The characteristics of lime make it suitable to be used as a supplementary agent for the reaction mixture. Lime is a poor solute so its dissolves gradually in the solution, this helps in maintaining high pH during the reaction required by the biomass. The calcium divalent ions of lime formed after its dissociation help in forming linkages between the biomass thus reduces higher solid loss. Finally, lime is inexpensive as well.

The main objective of this study was to assess the combined effects of all four variables sodium hydroxide and lime loading, residence time and particle size on total sugar yield of wheat straw by using response surface methodology (RSM). RSM is a statistical technique that is used for modeling and optimization of multiple variables, which allows a proper experimental design to obtain and solve multivariate equations simultaneously (Ferreira et al., 2009). The main advantage of RSM is that it allows the evaluation of multiple factors and their interactions that can be done with reduced number of experimental trials.

2.2 MATERIALS AND METHOD

2.2.1 Biomass preparation

Post-grain harvested wheat straw (*Triticum aestivum)* used in this study (Pioneer 26R12) was sourced from Kinston, North Carolina. The wheat straw was oven dried at 50°C for 72 h, ground in a Thomas Wiley Laboratory Mill (Model No. 3) and sieved to obtain two particle sizes: 2 mm and 13.5 mm. The third particle size of approximately 25mm in size was obtained by cutting the biomass with scissors. All three fractions were separately stored in plastic bags at ambient temperature for further use. The chemical composition on dry basis for all three particles is shown in Table 2.1.

2.2.2 Protocol of wheat straw combined alkali pretreatment

All pretreatment experiments were carried out at 21°C (room temperature). The pretreatment of wheat straw was performed in serum bottles with 3 g of dry biomass, 30 mL of deionized (DI) water, and desired amount of NaOH and CaO properly mixed to obtain a solid loading of 1:10. After pretreatment, the solid fraction of biomass was recovered by filtering through a Fisherband P8 qualitative filter paper placed in a porcelain Buchner filter funnel and washed with 300 mL of DI water to get rid of any excess alkali or dissolved / inhibitory products that might be unfavorable to the subsequent step of enzymatic hydrolysis. About 1g (dry basis) of the biomass was dried at 40°C in a Fisher Scientific Isotemp vacuum oven to a constant weight for composition analysis, and the rest was stored in a plastic bag at 4°C for enzymatic hydrolysis. Total amount of polysaccharides (glucan and xylan) recovered after pretreatment was calculated using the following equation:

$$\%\ Conversion\ = \left(\frac{C_{PT-WS}}{C_{WS}}\right) \times 100 \quad (1)$$

where C_{WS} is the concentration of polysaccharide in wheat straw, C_{PT-WS} is the concentration of polysaccharide present after the pretreatment of wheat straw, which was calculated as the multiplication of estimated sugar concentration (g/g) with recovered solids after pretreatment. The % delignification by was calculated using the following equation:

$$\%\ Delignification\ = \left(\frac{L_{WS} - L_{PT-WS}}{L_{WS}}\right) \times 100 \quad (2)$$

where L_{WS} is the concentration of lignin in the raw biomass and L_{PT-WS} is the concentration of lignin in pretreated biomass, which was calculated as the multiplication of estimated lignin concentration (g/g) with recovered solids after the pretreatment.

2.2.3 Enzymatic hydrolysis

The enzymatic hydrolysis of pretreated biomass was performed by suspending 0.5 g of pretreated biomass (on a dry basis) in 50 mM sodium citrate buffer (pH 4.8) to reach a final volume of 15 mL, placed in a 50 mL plastic tubes in a controlled environment reciprocal shaking bath (model C76, New Brunswick Scientific, Edison, NJ) and incubated at 50°C for 72 h with an agitation of 150rpm. Cellic CTec2 (enzyme complex of aggressive cellulases, high level of β-glucosidase, and hemicellulase) and Cellic HTec2 (endoxylanase with cellulase background) donated by Novozymes North America, Inc. (Franklinton, NC) with reported densities of 1.203 and 1.238 g / mL, respectively, were used in this study.

Table 2.1 Components of Wheat Straw Particles in % (w/w) dry basis

Particle Size	Glucan	Xylan	Arabinan	Galactan	Lignin	Ash
2 mm	42.89 ± 1.34	20.35 ± 1.86	2.47 ± 1.11	0.50 ± 1.32	22.62 ± 3.45	1.23 ± 0.1
13.5mm	42.50 ± 1.7	20.90 ± 1.96	2.31 ± 0.89	0.3 ± 0.3	23.03 ± 3.54	0.6 ± 0.1
25 mm	42.29 ± 2.2	20.19 ±1.67	2.53 ± 0.2	0.4 ± 0.2	24.58 ± 3.22	1.0 ± 0.3

Excessive dosage of CTec 2 and HTec 2 of 40 and 6% (g of enzyme/g of dry biomass) respectively, were employed to make the final solid loading to be 3.3% (w/v) for hydrolysis. Excessive loading of the enzymes were used to avoid the impact of enzyme limitation on comparison of different pretreatment conditions. Sodium azide (0.3% w/v), an antimicrobial agent, was added to the reaction mixture to arrest any kind of microbial growth during hydrolysis. After the 72 h hydrolysis step, the hydrolyzate was centrifuged at 4000rpm for 15 min and the supernatant was collected in 15mL plastic storage tubes and stored at -20°C for later step of sugar analysis.

2.2.4 Analytical methods

Determination of total solids, ash, structural carbohydrates, and lignin in both raw and pretreated biomass was determined using Laboratory Analytical Procedures (LAP) designed by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2005a,b, 2008). Total reducing sugars in the hydrolyzate were measured by the DNS (3,5-dinitrosalicylic acid) method (Miller, 1959; Ghose, 1987). High performance liquid chromatography (HPLC) (Dionex UltiMate 3000, Dionex Corporation, Sunnyvale, CA) was used for the determination of main monomeric sugars such as glucose and xylose present in the hydrolyzates. The HPLC system was equipped with a Bio-Rad Aminex HPX-87H column (300 X 7.8 mm), a Bio-Rad Micro-Guard column, a thermostatted autosampler, an isocratic analytical pump, and a refractive index detector. The analysis was performed at 65°C with 50 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. A high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE−PAD) (Dionex ICS-5000,

Dionex Corporation, Sunnyvale, CA) was used for measuring all monomeric sugars (glucose, xylose, galactose and arabinose) that were present in both the untreated and pretreated biomass. The HPAE−PAD system was equipped with a CarboPac PA1 (100 µ equiv per 4 × 250 mm) analytical column operated at 18°C with 0.018 M potassium hydroxide as the mobile phase at a flow rate of 0.9 mL / min, a CarboPac PA1 guard column (4 × 50 mm), a thermostatted autosampler, and a quaternary pump.

2.2.5 Designing of experiments

The design used for this study was a Central Composite Face experimental Design (CCD) of Response Surface Methodology (RSM). A three-level-four-factor RSM was studied with varied NaOH loading of 0.05-0.15 g/g of raw biomass and lime loadings of 0.025-.075 g/g of raw biomass, residence times of 3-9 h and particle size of 2-25 mm. A total of 26 experiments with (2^4) factorial points, 2 × 4 axial points formed a CCD with 2 center points was used as suggested by the statistical software used JMP 10 (SAS, Cary). The range and the centre point were chosen after preliminary trials. All 26 experimental combinations were run in random order to minimize the effects of unexpected variability in observed responses due to extraneous factors. The levels have been coded as -1, 0 and +1. The coded values are shown in Table 2.2.

Table 2.2 Various factors considered with their different levels.

Factors	Coded	Coded Variable levels		
		-1	**0**	**1**
NaOH (g / g)	X_1	0.05	0.1	0.15
CaO (g / g)	X_2	0.025	0.05	0.075
Residence Time (h)	X_3	3	6	9
Particle size (mm)	X_4	2	13.5	25

2.3 RESULTS AND DISCUSSION

2.3.1 Solid recovery

Solid recovery of a pretreatment process is a good indicator of the potential amount of sugars that can be obtained in the subsequent step of hydrolysis. Any process with high solid loss represents hostile condition for the retention of sugars. Certain extent of solid loss with alkaline pretreatment is always observed as a result of solubilization of hemicelluloses and lignin. In this study, three different particle sizes in the order of 2mm, 13.5 mm and 25 mm exhibited the solid recoveries ranging from 80.60-89.43%, 84.01-91.32% and 93.22-94.38% respectively as shown in Table 2.10. Of all four variables only particle size and NaOH had significant effect ($p<0.05$) on recovery of biomass. For 25 mm particle although the solid recovery was consistently high but surprisingly it did not translate into high sugar yield mostly for the residence time of 3 h with mild loadings of NaOH and CaO which made us to

hypothesize that no effective pretreatment occurred that could deconstruct the structure of 25 mm particle size to yield a better sugar yield.

2.3.2 Model development

The experimental data were used to determine the regression coefficients of the second order polynomial equation using JMP 10 (SAS, Cary, NC) software and the following model that describes the reducing sugar yield in terms of actual parameters was obtained.

$$\text{Total Sugar} = 79.4636 + 3.6889 \times \left[\frac{(\text{NaOH}-1)}{0.5}\right] + 2.7556 \times \left[\frac{(\text{CaO}-0.05)}{0.025}\right] + 2.7828 \times \left[\frac{(\text{RT}-6)}{3}\right] - 2.1711 \times \left[\frac{(\text{PS}-13.5)}{11.5}\right] + \left[\frac{(\text{NaOH}-1)}{0.5}\right] \times \left[\left[\frac{(\text{Cao}-0.05)}{0.025}\right] \times -1.8006\right] + \left[\frac{(\text{NaOH}-1)}{0.5}\right] \times \left[\left[\frac{(\text{RT}-6)}{3}\right] \times -0.4431\right] + \left[\frac{(\text{CaO}-0.05)}{0.025}\right] \times \left[\left[\frac{(\text{RT}-6)}{3}\right] \times -0.7581\right] + \left[\frac{(\text{NaOH}-1)}{0.5}\right] \times \left[\left[\frac{(\text{PS}-13.5)}{11.5}\right] \times 0.8156\right] + \left[\frac{(\text{CaO}-0.05)}{0.025}\right] \times \left[\left[\frac{(\text{PS}-13.5)}{11.5}\right] \times 0.8581\right] + \left[\frac{(\text{RT}-6)}{3}\right] \times \left[\left[\frac{(\text{PS}-13.5)}{11.5}\right] \times -0.4188\right] + \left[\frac{(\text{NaOH}-1)}{0.5}\right] \times \left[\left[\frac{(\text{NaOH}-1)}{0.5}\right] \times -2.9264\right] + \left[\frac{(\text{CaO}-0.05)}{0.025}\right] \times \left[\left[\frac{(\text{CaO}-0.05)}{0.025}\right] \times 0.8236\right] + \left[\frac{(\text{RT}-6)}{3}\right] \times \left[\left[\frac{(\text{RT}-6)}{3}\right] \times -1.1314\right] + \left[\frac{(\text{PS}-13.5)}{11.5}\right] \times \left[\left[\frac{(\text{PS}-13.5)}{11.5}\right] \times -0.7164\right] \quad (3)$$

Where NaOH, CaO, RT, PS are concentration of NaOH loading and lime loading, residence time and particle size, respectively. A model with a p-value lower than 0.05 is considered to be significant suggesting that there is only 5% chance that a model p-value could occur because of noise. The model for total sugar yield (%) was significant as the p-value was

0.017 less than 0.05. This low p-value for the model tells that the developed model equation adequately explains the response. In the study all four variables were significant ($p<0.05$) with NaOH being the most significant followed by CaO, RT, and PS. However, no interaction effects between different variables were significant ($p>0.05$).

To determine the adequacy of the model the lack of fit test was conducted which was insignificant with a p-value of 0.0901. On the other hand the coefficient of determination represented as R^2 which represents the correlation between the observed and predicted value had a moderate value of 83%, shown in figure 2.1. Based on all statistical analysis it can be said of the developed model that it represents a good relationship between the variables and the response. Moreover, the effect of each pretreatment variable in the given range can well explain their effect on total sugar yield.

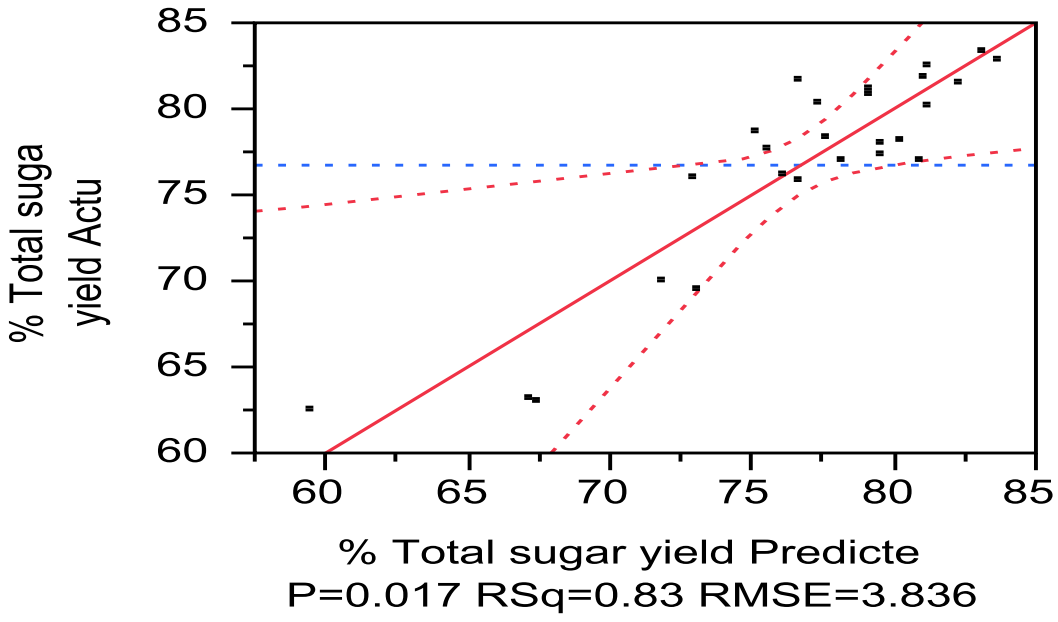

Figure 2.1 Predicted vs Actual total sugar yield (%)

2.3.3 Effects of processing variables on total reducing sugars yield

Four different processing variables (NaOH concentration, CaO concentration, residence time and particle size) were used to study their effects on total reducing sugars yield (%) which was analyzed through RSM. From the ANOVA table (Table 2.4-2.6) it was observed that linear effects of NaOH, CaO and RT and PS were significant ($p < 0.05$) with NaOH being the most significant variable with a p-value of 0.0019. All the factors had positive coefficient which shows that with the increase in their respective values there will be a corresponding linear increase in the total reducing sugars yield (%) except for the coefficient of PS which was negatively related to total sugar yield. The highest total reducing sugars yield (expressed in mg/g of raw biomass) was 82.78% obtained for the combination of 0.15 and 0.025 g of NaOH and CaO per gram raw biomass, respectively, residence time of 9 h and particle size of 2 mm. For 25 mm particle, 3 h holding time was found to be too small to be effective as in spite of having high solid recoveries of more than 93%, a low total reducing sugars yield of 69.55% was observed for (0.15 g/g NaOH, 0.025 g/g CaO, 3 h) and 62.53% of total reducing sugars was observed for (0.05 g/g NaOH, 0.025 g/g CaO, 3 h) which made us to hypothesize that 3 h was not enough to pretreat the 25 mm particle effectively. However, 9 h pretreatment with high alkali concentrations gave the maximum total reducing sugars yield of 82.48% was obtained that corroborates that residence time of 3h for 25 mm was not enough to increase the susceptibility of the particle. On the other hand for 2 mm particle with high alkali concentration maximum sugar yield for 3 h RT obtained was 80.84% (0.15 g/g NaOH, 0.075 g/g CaO, 3 h) and for 13.5 mm size 77.64% of sugar was obtained. For sugar yield model all the quadratic and interaction terms were insignificant ($p>0.05$) suggesting the quadratic

response surface may not be the best fitting for the data. It seems there is potentially some other interactions were present, probably more than two factor interactions which are not recognized by the model or maybe the levels for the variables were too conservative. The combined effect of increase in NaOH and CaO loading g/g raw biomass was insignificant ($p>0.05$) and was negatively correlated to the increase in sugar yield. Across all particle sizes glucan recovery (%) was found in the range of 69.12% to 91.59%, highest being observed for 13.5 mm substrate (0.1 g/g NaOH, 0.050 g/g CaO, 6 h). Xylan being more vulnerable to pretreatment than glucan had higher loss during the process; lowest xylan conversion (%) of 47.28% was obtained for (0.05 g/g NaOH, 0.025 g/g CaO, 3 h and 25 mm) which shows that lowest level of alkalis was enough to break the xylan into xylose whereas for highest chemical loadings, highest xylan conversion (%) of 83.10% was obtained for (0.015 g/g NaOH, 0.075 g/g CaO, 9 h, 25 mm). This high recovery of xylan in high NaOH loading can be explained from the synergistic effect of CaO forming linkage within the biomass (Xu et al., 2011). The respective glucan and xylan recovery(%) for different combinations has been presented in Table 2.3. This result shows that low level of alkali is not enough even to facilitate the digestibility of the carbohydrates present in the biomass. Using JMP (SAS, Cary, NC) the optimized total sugar yield obtained was 84.61% for the combination of (0.106 g/g NaOH, 0.075 g/g CaO, 9 h RT, 2 mm PS) for which the surface plot is obtained in Figure 2.2.

Table 2.3 Glucan and Xylan conversion (%) in hydrolysate for CCD matrix design

Run	X_1:NaOH	X_2:Lime	X_3:RT	X_4:Particle size	Glucan (%)	Xylan (%)
1	0	0	+1	0	88.83	73.74
2	0	+1	0	0	91.59	72.93
3	-1	-1	+1	+1	69.78	55.47
4	-1	+1	+1	-1	85.54	73.88
5	+1	-1	-1	-1	86.89	72.80
6	-1	0	0	0	79.12	66.34
7	+1	-1	-1	+1	70.18	56.09
8	+1	-1	+1	-1	88.83	82.73
9	+1	0	0	0	90.34	73.10
10	-1	-1	-1	-1	69.12	49.94
11	+1	+1	+1	+1	90.14	83.10
12	0	0	0	0	85.19	70.96
13	0	0	0	+1	88.44	74.62
14	-1	+1	-1	+1	74.45	61.37
15	+1	+1	+1	-1	89.69	70.84
16	-1	+1	-1	-1	78.12	65.31
17	0	0	0	0	81.88	68.21
18	0	0	0	-1	83.90	85.46

Table 2.3 Continued

19	0	0	-1	0	85.06	72.65
20	-1	-1	-1	+1	69.77	47.28
21	+1	+1	-1	+1	85.98	77.54
22	+1	+1	-1	-1	86.50	71.54
23	-1	+1	+1	+1	76.60	68.69
24	0	-1	0	0	83.17	69.94
25	+1	-1	+1	+1	85.07	76.71
26	-1	-1	+1	-1	80.91	70.40

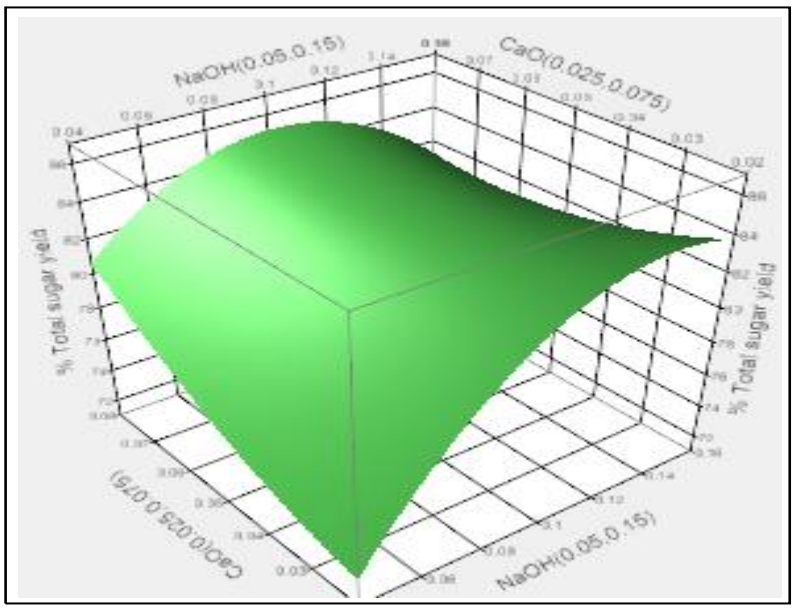

Figure 2.2 Response surface plot for optimized total reducing sugars yield (%).

Table 2.4 ANOVA for selected CCD for total reducing sugars yield (%)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	14	769.82	54.99	3.7361	0.0170
Error	11	161.90	14.72		
C.Total	25	931.72			

Table 2.5 Summary of Fit for total reducing sugars yield (%)

R- Square	82.62
R Square Adj	60.51
Root MSE	3.84
Mean of Response	76.73

Table 2.6 Lack of Fit for total reducing sugars yield (%)

Source	DF	sum of squares	Mean Square	F ratio	Pr > F
Lack of Fit	10	161.68	16.17	74.2327	0.0901
Pure Error	1	0.22	0.22		
Total Error	11	161.90			

2.3.4 Delignification

Lignin is one of the important constituents present in cell wall of plants, which not only provides protection against microorganisms but also serves as a structural support to plant and being responsible for the internal transportation of water and nutrients. (Buranov and Mazza, 2008*).* However, in order to make it easier for enzymes during hydrolysis to get better access of substrate surface it is imperative to breakdown the protective sheath of lignin surrounding carbohydrates. In this study, for three different particle sizes different ranges of lignin reduction was observed which was in the order for 2,13.5 and 25 mm was 21.95-39.00%, 18.88-31.02% and 8.11-22.02%, respectively, shown in Table 2.10. Linear effects of particle size and NaOH had significant ($p<0.05$) effect on total lignin reduction of the biomass whereas no higher degree terms and interaction term were significant ($p>0.05$). The maximum lignin reduction was observed for 2 mm particle for the condition of 0.15 and 0.075 g/g of raw biomass of NaOH and CaO, respectively, and RT of 9 h. NaOH pretreatment always results in high solid loss during the process. But adding lime to NaOH explains the reduction in the removal of lignin from the biomass. The divalent ions of calcium ions are believed to form a linkage with lignin during pretreatment causing a remarkable reduction in lignin removal (Sundin and Hartler, 2000; Xu and Cheng, 2011). However, the low lignin reduction across different pretreatment conditions had no remarkable impact on total reducing sugars yield. The probable reason for that can be explained as until all the chemical bonds stiffening the biomass is broken; there will be no resistance as such is offered by the disrupted structure to the hydrolytic enzymes during hydrolysis (Xu et al., 2010). The optimized delignification of biomass (%) obtained using the

software was 41.08 shown in Figure 2.3 for the combination (0.15 g/g NaOH, 0.025 g/g CaO, 7.44 h, 2 mm). Results of ANOVA for delignification (%) presented in table 2.7-2.9.

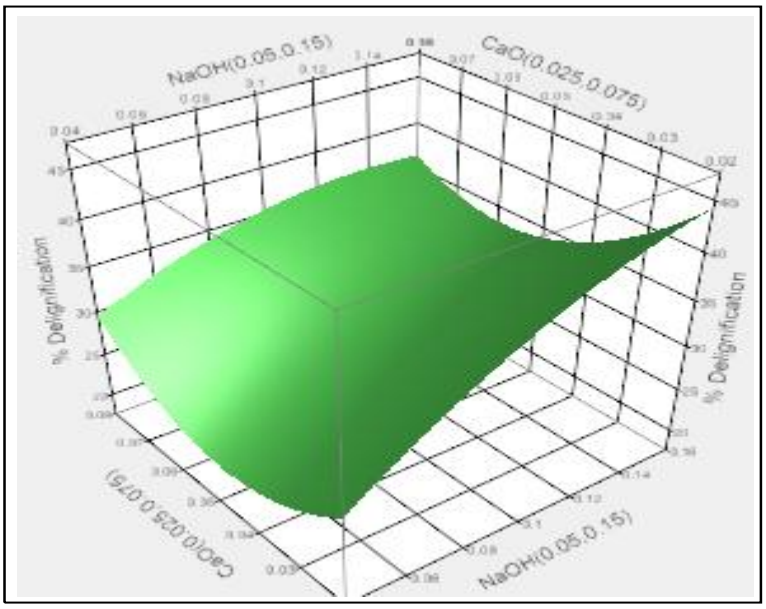

Figure 2.3 Response surface plot for optimized delignification (%)

Table 2.7 ANOVA for selected CCD for delignification (%)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	14	1870.02	133.57	6.8327	0.0014
Error	11	215.04	19.55		
C.Total	25	2085.06			

Table 2.8 Summary of Fit for Delignification (%)

R-Square	89.69
RSquare Adj	76.56
Root MSE	4.42
Mean of response	22.54

Table 2.9 Lack of Fit for delignification (%)

Residual	DF	sum of squares	Mean Square	F value	Pr > F
Lack of Fit	10	211.20	21.12	5.5052	0.3210
Pure Error	1	3.84	3.84		
Total Error	11	215.04			

Table 2.10 Central composite design matrix for solid recovery, delignification and total reducing sugars yield in (%)

Run	X_1: NaOH	X_2: CaO	X_3: RT	X_4: PS	NaOH (g g^{-1})	Lime (g g^{-1})	RT (h)	PS (mm)	Solid Recovery (%)	Delignification (%)		Total Sugar Yield (%)	
										Actual	Predicted	Actual	Predicted
1	0	0	+1	0	0.1	0.05	9	13.5	86.81	26.84	24.40	80.24	81.11
2	0	+1	0	0	0.1	0.075	6	13.5	86.97	30.50	29.20	80.38	77.32
3	-1	-1	+1	+1	0.05	0.025	9	25	93.45	8.29	8.96	62.98	67.36
4	-1	+1	+1	-1	0.05	0.075	9	2	89.53	23.84	27.39	81.87	81.02
5	+1	-1	-1	-1	0.15	0.025	3	2	80.60	35.93	34.00	80.38	83.04
6	-1	0	0	0	0.05	0.05	6	13.5	91.32	18.88	21.08	76.07	72.85
7	+1	-1	-1	+1	0.15	0.025	3	25	93.60	14.36	16.20	69.55	72.98
8	+1	-1	+1	-1	0.15	0.025	9	2	81.80	35.68	40.20	82.78	83.60
9	+1	0	0	0	0.15	0.05	6	13.5	84.40	30.32	27.56	78.22	80.23
10	-1	-1	-1	-1	0.05	0.025	3	2	87.81	24.05	23.71	63.23	67.09

Table 2.10 continued

11	+1	+1	+1	+1	0.15	0.075	9	25	94.29	8.11	13.84	82.48	81.20
12	0	0	0	0	0.1	0.05	6	13.5	85.17	23.82	26.04	77.97	79.46
13	0	0	0	1	0.1	0.05	6	25	93.22	9.84	13.00	81.72	76.58
14	-1	+1	-1	+1	0.05	0.075	3	25	93.78	10.03	10.90	70.07	71.82
15	+1	+1	+1	-1	0.15	0.075	9	2	81.47	39.00	34.78	81.50	82.28
16	-1	+1	-1	-1	0.05	0.075	3	2	88.45	24.86	23.18	76.21	76.00
17	0	0	0	0	0.1	0.05	6	13.5	88.61	26.59	26.04	77.31	79.46
18	0	0	0	-1	0.1	0.05	6	2	83.30	33.73	30.01	76.99	80.92
19	0	0	-1	0	0.1	0.05	3	13.5	91.00	19.31	21.18	77.64	75.55
20	-1	-1	-1	+1	0.05	0.025	3	25	94.38	11.65	10.62	62.53	59.48
21	+1	+1	-1	+1	0.15	0.075	3	25	93.60	14.03	9.69	76.94	78.12
22	+1	+1	-1	-1	0.15	0.075	3	2	87.96	21.95	26.67	80.84	79.03

Table 2.10 continued

23	-1	+1	+1	+1	0.05	0.075	9	25	93.52	14.50	11.17	75.89	76.68
24	0	-1	0	0	0.1	0.025	6	13.5	84.01	31.02	31.76	78.40	77.53
25	+1	-1	+1	+1	0.15	0.025	9	25	92.47	22.02	18.44	81.15	79.09
26	-1	-1	+1	-1	0.05	0.025	9	2	87.25	26.92	26.00	78.59	75.14

2.4 CONCLUSIONS

The conversion of carbohydrates present in three different particle sizes of wheat straw into reducing sugars through combined alkali pretreatment was presented in this study. The results demonstrated that wheat straw is a potential feedstock for the end goal of bio-ethanol production with the highest sugar yield obtained was 84.61% of total sugars present in raw biomass. Our results indicate that content of lignin in biomass after the pretreatment doesn't seem to have a direct correlation with sugar yield which can be attributed to the cross linking of calcium ions with lignin during the process. However, as long as the chemical bonds stiffening the structure are disrupted thus far lignin does not interfere with enzymatic hydrolysis.

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