EVALUATION OF PRETREATMENT OPTIONS FOR ENZYMATIC HYDROLYSIS AND FERMENTABLE SUGAR PRODUCTION FROM SWEET SORGHUM

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Abstract: Sweet sorghum (*Sorghum Bicolor* L.) is a promising crop for the production of biofuels due to its high carbohydrate yields, low water requirements, and tolerance to imperfect soil conditions. Numerous options are available for the pretreatment of lignocellulosic biomass. Several pretreatment processes were investigated in order to maximize the overall carbohydrate recovery from sorghum, including mechanical pulping, extrusion, and alkali soaking. In the first part of this study, whole sweet sorghum was pretreated with Thermomechanical pulping (TMP) and Refiner Mechanical Pulping (RMP), which are common technologies in the pulp and paper industry. Enzymatic hydrolysis of TMP pretreated sorghum at 6% (w/v) solid loading resulted in the highest glucose and xylose yields of 60.8 and 12.1% respectively, when cellulase and xylanase loadings of 1.0 mL/g glucan and xylan were used. Using the same enzyme loading, enzymatic hydrolysis of RMP pretreated sorghum resulted in glucose and xylose yields of 41.3 and 10% respectively. In the second part of the study, sweet sorghum bagasse was pretreated using a twin-screw extruder at 80 and 110°C with and without the presence of 1.4 M NaOH. Among all the extrusion treatments that were tested, sorghum bagasse pretreated at 110°C in the presence of alkali resulted in the highest glucose and xylose yields of 62.7 and 46% respectively. Glucose and xylose yields were mostly unaltered with the change in extrusion temperature in the absence of alkali. However, the presence of an alkali improved the glucose and xylose yields significantly. In the treatments with alkali soaking alone, enzymatic hydrolysis of sorghum bagasse that was soaked in alkali at 40% (w/v) solid loading using 2.8, 3.5 and 4.2 M NaOH at several different conditions resulted in glucose yields ranging from 88 to 99%. Glucose yields were high with treatments containing low alkali concentration coupled with high soaking time. Xylose yields up to 78% were obtained with sorghum bagasse that was soaked in 3.5 M NaOH at 90°C for 1h as well as those that were soaked at room temperature. Furthermore, the alkali soaking was conducted at solid state, with 40% solids, and no washing steps required.

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CHAPTER I

INTRODUCTION

1.1 Background

Production of biofuels from plant materials has been a topic of interest around the world as it would lead to the reduction of greenhouse gas emissions, thereby resulting in a cleaner environment (von Blottnitz & Curran, 2007). Use of ethanol as an alternative automobile fuel has been widely studied (Hill et al., 2006; von Blottnitz & Curran, 2007). Use of renewable feedstocks such as corn and sugarcane for first generation biofuels has been well researched and is fully commercialized in the United States, Brazil and a few other countries. In order to meet the 36 billion gallon renewable fuel requirement by 2022 that was set by the Energy Independence and Security Act, use of lignocellulosic feedstocks such as energy crops, and agricultural wastes for the production of biofuels is necessary.

Biological production of ethanol from lignocellulosic materials involves three main steps: (1) pretreatment of biomass (2) saccharification of structural carbohydrates

in to fermentable sugars through hydrolysis and (3) fermentation of sugars to ethanol or other alcohols. To be able to access the structural carbohydrates (cellulose and hemicellulose) in biomass, a pretreatment is necessary to either partially or completely remove the hemicellulose and/or lignin fractions depending on the type of pretreatment (Fang, 2013; Mosier et al., 2005). An ideal pretreatment process would alter the crystallinity of cellulose, solubilize the hemicellulose fraction, remove lignin and increase the surface area of biomass, which would increase the accessibility for the enzymes during the hydrolysis process (Alvira et al., 2010; Fang, 2013; Mosier et al., 2005). However, pretreatment processes are energy intensive and are prone to generate some inhibitory compounds such as furfural and 5-hydroxylmethylfurfural (HMF) that can inhibit the growth of yeast in the fermentation process (Alvira et al., 2010; Fang, 2013; Mosier et al., 2005).

Sweet sorghum is a high photosynthetic efficiency energy crop and is capable of growing in nutrient poor soils (Billa et al., 1997; Rooney et al., 2007). Sorghum, unlike other crops, has a very promising future as a feedstock as it contains starch in the grains, soluble sugars in the juice fraction, and the bagasse is rich in cellulose and hemicellulose (Rooney et al., 2007; Sipos et al., 2009). Furthermore, the stalks of sorghum are reported to have about 6% starch on a dry basis, which can also be used in an enzymatic hydrolysis process (Billa et al., 1997; Rooney et al., 2007; Sipos et al., 2009). Traditionally sorghum juice has been used for the production of edible syrup, biofuels and other value added products. However, the structural carbohydrates and starch can also be used to produce fermentable sugars.

Various pretreatment methods such as dilute acid pretreatment (Zhang et al., 2011), steam explosion pretreatment (Shen et al., 2011), alkali pretreatment (Wu et al., 2011), mechanical pulping (Gonzalez et al., 2011) and extrusion pretreatment (Heredia-Olea et al., 2013) have been tested on sweet sorghum. Most of the research is focused on using the bagasse, juice or grain portion of sorghum separately for biofuel production. However, there is no reported literature on a process where the whole sorghum plant was used in an enzymatic hydrolysis process to hydrolyze the cellulose, starch and hemicellulose fractions.

1.2 Objectives

The overall objective of this study was to utilize the whole sorghum plant in an enzymatic hydrolysis process in order to produce fermentable sugars. The specific objectives of the study were as follows:

1. Evaluate the use of Thermomechanical Pulping (TMP) and Refiner Mechanical Pulping (RMP) as pretreatment options for whole sweet sorghum
2. Determine the effect of enzyme loading on enzymatic hydrolysis of TMP and RMP pretreated sweet sorghum at 6 and 12% (w/v) solid loadings
3. Evaluate the use of a twin-screw extruder as a pretreatment option for sweet sorghum bagasse at two different temperatures, with and without the presence of an alkali.
4. Evaluate the effect of alkali concentration and soaking conditions on the enzymatic hydrolysis of sweet sorghum bagasse

CHAPTER II

LITERATURE REVIEW

2.1 Biomass Feedstocks

Lignocellulosic feedstocks are primarily composed of cellulose, hemicellulose and lignin. Cellulose is a polymer of D-glucose linked via β-1, 4 glycosidic bonds, whereas hemicellulose is a heteropolymer of D-glucose, D-Xylose, D-galactose, D-arabinose and D-mannose linked via β-1, 4 glycosidic bonds. Lignin is a polymer of phenylpropenoid units and is a major recalcitrant in the lignocellulosic ethanol production process (Alvira et al., 2010; Fang, 2013; Pandey et al., 2011; Sun & Cheng, 2002). The amount of each fraction depends on the type of feedstock that is used and also varies within the cultivars (Alvira et al., 2010; Fang, 2013; Pandey et al., 2011; Sun & Cheng, 2002). Some of the biomass feedstocks that are currently being used in bioenergy research are discussed below.

2.1.1 Corn

Corn (*Zea mays* L.), or maize is the third largest cereal crop produced in the world with an average yield of 5 tons/ha (Cheng, 2010). Starch is the predominant component

of corn, which accounts for 64 to 78% of total mass on a dry basis. The production technology for starch to ethanol is well established and fully commercialized in the United States. Corn stover that remains in the field after the harvest contain stalks, leaves, cobs and husks that contain about 35 to 40% cellulose, 17-35% hemicellulose and 7 to 18% lignin (Cheng., 2009). These can be used in an enzymatic hydrolysis and fermentation process to increase the overall ethanol yields. Several companies in the United States have commercialized ethanol production from corn stover

2.1.2 Wheat

Wheat is another cereal crop that can be cultivated in a wide range of climates. The major component of wheat is starch, which accounts for 77% of the total biomass on a dry basis (Cheng, 2009). Besides the starch portion, wheat straw is composed of 33 to 50% cellulose, 24 to 36% hemicellulose and 9 to 17% lignin on a dry basis (Cheng, 2009). Wheat straw could be further used as a feedstock to convert the structural carbohydrates into ethanol in an enzymatic hydrolysis and fermentation process. Production of ethanol from wheat straw has been commercialized in several countries around the world such as Canada, Sweden and Spain (Yang et al., 2013).

2.1.3 Woody Biomass

Woody biomass can be classified into two categories: softwoods and hardwoods. Softwoods, commonly referred to as evergreens, are the dominant source of lignocellulosic biomass in the northern hemisphere. Spruce and pine are some examples of softwoods. Some species of pine are known to tolerate alkaline soils, and frigid and arid environments (Cheng, 2009). Softwoods are composed of 40 to 50% cellulose, 15 to

20% hemicellulose and 20 to 25% lignin on a dry basis (Cheng, 2009). These crops are currently being investigated for their use as a feedstock in a commercial scale ethanol manufacturing process.

Hardwoods are angiosperms, and are commonly referred to as flowering plants with broad leaves. Oak, poplar and willow are some examples of hardwood. Among the crops mentioned, poplar is of great interest as it could be used as a short rotation crop due to its capacity for fast growth. It also has low irrigation requirements and is able to grow in various types of soil at neutral pH (Cheng, 2009). The hardwoods are composed of 40 to 50% cellulose, 11 to 20% hemicellulose and 27 to 30% lignin on a dry basis (Cheng, 2009). A few companies in the USA have commercialized the use of hardwoods as a feedstock in an ethanol manufacturing process (Anonymous, 2016).

2.1.4 Switchgrass

Switchgrass (*Panicum virgatum* L.) is a warm season, high-yielding C4 perennial crop that has a calorific value comparable to wood (Yang et al., 2013). Switchgrass is highly adaptable to a wide geographical range and various soil types. Based on the plant characteristics and habitat preferences, switchgrass is classified into upland and lowland ecotypes. Upland ecotypes such as *Cave-in-rock* and *Trailblazer* are shorter, thinner and are adapted to drier conditions. Lowland ecotypes such as *Kanlow and Alamo* are tall, thick stemmed and are adapted to wetter conditions (Yang et al., 2013; Cheng, 2009). Switchgrass is composed of 28 to 37% cellulose, 25 to 30% hemicellulose and 15 to 20% lignin on a dry basis (Cheng, 2009). These structural carbohydrates can be used for producing fermentable sugars using an enzymatic hydrolysis process. A few companies

in the U.S. have commercialized the industrial scale production of ethanol from switchgrass (Anonymous, 2016).

2.1.5 Miscanthus

Miscanthus *(Miscanthus spp.)* is a warm season C4 perennial grass that has a wide range of climatic adaptability. Unlike switchgrass, some of the Miscanthus plants do not produce viable seeds and hence alternative methods such as plant division, stem cuttings, rhizome cuttings etc. need to be used (Cheng, 2009). Miscanthus contains 38 to 43% cellulose, 18 to 27% hemicellulose and 22 to 25% lignin on a dry basis and can be used for the production of ethanol via enzymatic hydrolysis and fermentation..

2.1.6 Sugarcane

Sugarcane is a perennial grass grown in the tropics and subtropics primarily for the production of sugar and molasses. Sugarcane requires 60 cm of annual rain and a maturity period from 8 to 24 months (Cheng, 2009). Sugarcane stalks are rich in soluble sugars such as sucrose, glucose and fructose and their content varies based on the cultivar. On a wet basis, sugarcane consists of 15% total sugars and 12% lignocellulose. Several sugarcane-based ethanol plants are operating in countries like Brazil, India, and Thailand. The extracted soluble sugars can be easily fermented to produce ethanol or other specialty chemicals. After the sugars from the stalks are extracted, the bagasse can be further used as a feedstock for bioenergy production as it contains up to 43% cellulose, 25% hemicellulose and 23% lignin (Dias et al., 2012).

2.1.7 Sorghum

Sorghum (*Sorghum bicolor*) is a fast growing C4 plant that has high productivity, high resistance to drought conditions and requires less water compared to corn (Yang et al., 2013). Sorghum has a wide range of uses such as production of sugar, syrup, fuel and roofing applications (Cheng, 2009). On a wet basis, sorghum stalks consist of about 10-15% soluble sugars. These soluble sugars can be easily extracted and fermented much like sugarcane. The grain portion is rich in starch and can be hydrolyzed to glucose using similar technology that is used for corn. The structural carbohydrate content varies with the type of cultivar. The sorghum bagasse contains up to 35 to 45% glucan, 25 to 35% hemicellulose and 18 to 25% lignin on a dry basis (Rooney et al., 2007; Zhang et al., 2011). The shorter growing cycle and low water requirements make sorghum a very favorable crop for bioenergy production.

Several types of sorghum such as sweet sorghum, grain sorghum, silage sorghum, forage sorghum and biomass sorghum have been developed based on their end use. Silage and forage sorghum are used as animal feed while other varieties are used for bio-based applications. Sweet sorghum accumulates higher levels of soluble sugars in the stalks and can be used as an alternative sugar source in areas where sugarcane production is not possible (Rooney et al., 2007). Several studies have shown that the stalks of sweet sorghum also contain up to 10% starch. The presence of starch, soluble carbohydrates and structural carbohydrates makes sorghum a unique feedstock for bioenergy production.

2.2 Pretreatment of Biomass

As shown in Figure 2.1, in most lignocellulosic feedstocks, the availability of cellulose and hemicellulose is restricted due to the rigid lignin protection that surrounds the cellulose micro fibrils. This is why an effective pretreatment is required to liberate cellulose from the lignin-hemicellulose seal and increase the accessibility of cellulose and/or hemicellulose to enzymes (Alvira et al., 2010; Mosier et al., 2005; Sohail Toor et al., 2013; Sun & Cheng, 2002). Several studies have been reported that show the poor enzymatic digestibility of unpretreated biomass where glucose yields of about 30% were obtained (Cao et al., 2012; Gonzalez et al., 2011; Zhang et al., 2011). Numerous pretreatment techniques have been developed in the past decades toward improving the enzymatic digestibility of biomass.

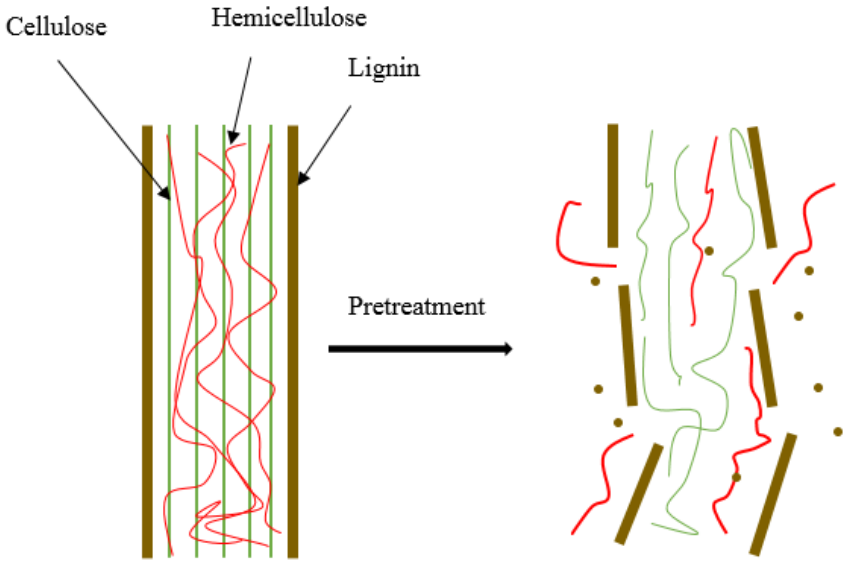

Figure 2.1 Effect of pretreatment on lignocellulosic structure of biomass. Adapted from Mosier et al. (2005).

2.2.1 Chemical Pretreatments

Acid Pretreatment

Acid pretreatments use acids as catalysts to solubilize the hemicellulosic fraction of biomass, thereby increasing the accessibility of cellulose to enzymes (Alvira et al., 2010; Fang, 2013; Mosier et al., 2005). Acid pretreatment processes are classified into two groups: (1) treatments with concentrated acids and (2) treatments with dilute acids. Use of concentrated acids has been shown to produce inhibitory compounds such as furfural and HMF through degradation of structural carbohydrates, which hinder ethanol production during the subsequent fermentation process (Alvira et al., 2010; Fang, 2013; Mosier et al., 2005). Moreover, the use of concentrated acids may result in corrosion of equipment, which makes it less attractive.

Use of dilute acids for pretreatment of a wide range of lignocellulosic materials has been widely studied and has been considered as a candidate for large scale bioethanol production (Alvira et al., 2010; Fang, 2013; Mosier et al., 2005; Pandey et al., 2011). Dilute acid pretreatment can be performed at high temperature over a short period of time or at lower temperature for longer retention time. Besides solubilizing the hemicellulose fraction, dilute acid pretreatment can also convert solubilized hemicellulose to fermentable sugars thereby reducing the need for addition of hemicellulases during the enzymatic hydrolysis process (Alvira et al., 2010; Fang, 2013; Mosier et al., 2005; Pandey et al., 2011). Acids such as H_2SO_4, HCl, H_3PO_4 and HNO_3 have been tested for their use in acid hydrolysis and all were found to be effective. Hydrolysis yields up to 90% have been reported with biomass that was treated with dilute acids (Banerji et al.,

2013; Cao et al., 2012; Xu et al., 2011). Although dilute acids seem to perform better compared to concentrated acids, there is still inhibitor formation and for this reason organic acids are being tested. Use of fumaric, malic and acetic acids have been tested and were shown to produce lower inhibitory compounds compared to H_2SO_4 pretreatments (Fang, 2013; Pandey et al., 2011).

Ozonolysis

Use of the oxidizing property of ozone to reduce the lignin content of biomass and increase the digestibility of treated material is the main goal of ozonolysis pretreatment (Fang, 2013; Mosier et al., 2005; Pandey et al., 2011; Sun & Cheng, 2002). Ozone is highly reactive towards compounds incorporating conjugate double bonds and functional groups with high electron densities. As lignin has a large number of double bonds, it is most likely to be oxidized in the ozonation process, where soluble compounds such as formic and acetic acid are produced (Fang, 2013; Mosier et al., 2005; Pandey et al., 2011; Sun & Cheng, 2002). One of the main advantages of ozone pretreatment is that the reactions occur at ambient temperature and pressure. One major drawback of ozonolysis is the large amount of ozone required, which makes it an expensive and less applicable process (Fang, 2013; Mosier et al., 2005; Pandey et al., 2011; Sun & Cheng, 2002).

Ionic Liquids (ILs) Pretreatment

The capability of ionic liquids to break the extensive hydrogen bonding networks in polysaccharides is the basic principle of ILs pretreatment (Fang, 2013; Pandey et al., 2011; Sun & Cheng, 2002). ILs are salts that are typically comprised of large organic cations and small inorganic anions that exist as liquids at low temperatures (Fang, 2013;

Pandey et al., 2011; Sun & Cheng, 2002). ILs can dissolve large amounts of cellulose at mild conditions and are easy to recover to their initial purity (Fang, 2013; Pandey et al., 2011; Sun & Cheng, 2002). ILs are often referred to as green solvents and have several advantages such as biodegradability, low toxicity, low hydrophobicity, enhanced electrochemical stability, high reaction rates and non-flammable properties (Fang, 2013; Mosier et al., 2005; Pandey et al., 2011; Sun & Cheng, 2002). The mechanism of cellulose dissolution involves the O_2 and H_2 atoms of cellulose hydroxyl groups in the formation of electron donor-electron acceptor complexes which interact with ILs. ILs react with the cellulose, which results in the opening of H_2 bonds between molecular chains of cellulose (Fang, 2013).

Some of the ILs used in the pretreatment process include 1-n-butyl-3-methylimidazolium chloride (BMIMCI), 1-allyl-3-methylimidazolium chloride (AMIMCI), and benzyldimethyl (tetradecyl) ammonium chloride (BDTACI). The presence of water in biomass decreases the dissolution efficiency of ILs, thus making it a suitable process for biomass that has low moisture content such as woody biomass (Fang, 2013; Mosier et al., 2005; Sun & Cheng, 2002). However, the cost of ILs and their regeneration requirement are some of the aspects that prevent ionic liquid pretreatment from being performed on a large scale.

Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose (SPORL)

The SPORL process is based on the sulfite process in which woodchips are pretreated in an aqueous sulfite solution at 160-180 °C and pH 2 to 4 for 30 min (Alvira et al., 2010; Fang, 2013; Pandey et al., 2011; Sun & Cheng, 2002). The woodchips are

then processed using a disk mill to generate fibrous material for subsequent saccharification. The degree of dissolution of hemicellulose, degradation of cellulose, sulfonation and condensation of lignin are increased as reaction time and temperature are increased (Alvira et al., 2010; Fang, 2013; Pandey et al., 2011; Sun & Cheng, 2002). A significant amount of hemicellulose degradation and removal is noticed during the pretreatment process. The strong recalcitrance of woody biomass is reduced in the process through the combined effects of dissolution of hemicelluloses, depolymerization of cellulose, partial delignification, partial sulfonation of lignin, and the increased surface area achieved with disk milling (Alvira et al., 2010; Fang, 2013; Pandey et al., 2011; Sun & Cheng, 2002). Recent studies have shown that minimal amounts of inhibitory compounds such as furfural and HMF were produced compared to other pretreatments.

Uncatalyzed Steam Explosion Pretreatment

Uncatalyzed steam pretreatment or liquid hot water pretreatment is a process in which the biomass is subjected to pressurized steam for a period of time ranging from seconds to several minutes and then suddenly depressurized. This results in an explosive decompression of biomass materials that facilitates the disruption of lignocellulosic structure and enhances the accessibility of biomass contents (Alvira et al., 2010; Fang, 2013; Pandey et al., 2011; Sun & Cheng, 2002). This pretreatment combines the mechanical forces and chemical effects due to the hydrolysis (autohydrolyis) of acetyl groups present in hemicellulose. The acetyl groups lead to the formation of acetic acid along with water that acts as an acid at higher temperatures, and results in the solubilization of hemicellulose and partial delignification (Alvira et al., 2010; Fang, 2013; Pandey et al., 2011; Sun & Cheng, 2002).

Uncatalyzed steam pretreatment has several benefits compared to other pretreatment technologies such as lower environmental impact, less hazardous processing chemicals, and higher sugar recovery. This pretreatment technology has been studied on a wide range of feedstocks including woody biomass, grasses, and bagasse. However, there are also certain disadvantages in the steam explosion process such as formation of acetic acid, HMF and furfural during the autohydrolyis process, which inhibit the growth of microbes during the fermentation process (Alvira et al., 2010; Fang, 2013; Gao et al., 2013; Pandey et al., 2011).

Alkali Pretreatment

Use of alkalis such as NaOH, KOH, and $Ca(OH)_2$ at mild temperatures with residence times ranging from minutes to days is the basis of alkali pretreatment. In a typical alkali pretreatment, lignocellulosic biomass is soaked in NaOH or KOH at a solid loading of 5 to 15% (w/v) db. The temperature and alkali concentrations are altered based on the type of feedstock and the degree of severity needed. Use of alkali pretreatment has several advantages such as low operating temperatures and its lower degradation effect on cellulose and hemicellulose compared to other pretreatment processes (Fang, 2013; Pandey et al., 2011). It is also described to cause less sugar degradation than acid pretreatment. Alkali pretreatment causes swelling, increases the internal surface of cellulose and decreases the polymerization and crystallinity, which also induces lignin structure disruption (Fang, 2013; Lynd et al., 2002; Mosier et al., 2005; Pandey et al., 2011; Sun & Cheng, 2002). Lime or calcium hydroxide that is used in pretreatment removes acetyl groups from hemicellulose, reducing steric hindrance of enzymes, and enhances cellulose digestibility (Mosier et al., 2005).

Although alkali pretreatment has some advantages over other pretreatment processes, there are some challenges such as the neutralization of pretreated material and a washing step that could result in the loss of sugars (Fang, 2013; Mosier et al., 2005).

Addition of oxidizing agents such as O_2/H_2O_2 to alkaline pretreatments has been shown to improve the performance of the pretreatment process by favoring the lignin removal. Furthermore, no furfural or HMF were detected in the hydrolyzate obtained from alkaline peroxide pretreatments, making it an ideal process for simultaneous saccharification and fermentation (Fang, 2013; Mosier et al., 2005; Pandey et al., 2011; Sun & Cheng, 2002).

In a study by Kim and Holtzapple (2005), corn stover was pretreated with calcium hydroxide at a loading of 0.5 g/g db corn stover at temperatures ranging from 25 to 55°C, in oxidative and non-oxidative environments. It was found that the temperature of 55°C and pretreatment time of 4 weeks with aeration was the optimum condition that resulted in 87.5% of the initial lignin removal during the pretreatment process. Enzymatic hydrolysis of the same pretreated solids at 10 g/L glucan concentration resulted in 91.3% glucan conversion (Kim and Holtzapple, 2005). The authors used a pretreatment time of 4 weeks, which may not be practical on a commercial scale.

In another study, sorghum was pretreated using four different NaOH concentrations, two different temperatures and three residence times at a solid loading of 10% (w/v) (McIntosh and Vancov, 2010). It was found that the glucan conversion increased with an increase in all the factors that were tested. Using a pretreatment temperature of 121°C, NaOH concentration of 2% (w/v) and residence time of 90 min,

about 95% saccharification efficiency and 75% lignin removal was achieved. Moreover, a 4.3-fold increase in total sugar yield was noticed with pretreatment at 121°C for 90 min at 2% NaOH compared to pretreatment at 60°C for 90 min (McIntosh & Vancov 2010). Although the authors achieved high yields, a solid loading of 10% was used during the pretreatment process. This requires a solid separation and washing and/or neutralization steps, which makes the process complex. Also, the washing process requires large amounts of water.

A pressurized refiner combined with alkali treatment was used for pretreatment of spruce in a study conducted by Zhao et al. (2008). The refining was performed using a refiner gap size of 0.2 mm at 166.2°C and 90 psig. The refined spruce was then pretreated with various concentrations of NaOH or NaOH/urea at various temperatures for a period of 24 h. It was found that increasing the concentration of alkali increased the glucose yield from 15 to 65%. It was also found that when refined spruce pulp without a chemical pretreatment was used a glucose theoretical maximum yield less than 15% was achieved (Zhao et al., 2008). The authors achieved relatively low hydrolysis yields and used a long alkali pretreatment time, making this process less applicable on an industrial scale.

Wu et al. (2011) used low temperature alkali pretreatment for enhancing the enzymatic digestibility of sweet sorghum bagasse. In their study, four concentrations of NaOH, three solid loadings, two temperatures and four soaking times were evaluated. It was found that increasing the alkali concentration and reaction times favored the lignin removal and also resulted in an improvement in glucan saccharification. It was also noticed that increasing the soaking time beyond 60 min at room temperature did not change the lignin removal significantly. With an alkali concentration of 2.5 M and a

soaking time of 30 min, 90% of glucan conversion was achieved. When pretreatment time was increased to 120 min, the glucan saccharification reached 90% with all alkali concentrations that were tested (Wu et al., 2011). The authors achieved great hydrolysis yields in this study while using low pretreatment times. However, the solid loadings during the pretreatment were from 5 to 15% (w/v). The pretreatment process required an intensive washing and a neutralization step prior to the enzymatic hydrolysis process. This requires large amounts of water, which makes the process less attractive on an industrial scale.

In a study by Umagiliyage et al. (2015), sweet sorghum bagasse was used in a laboratory scale alkali pretreatment optimization study for improving enzymatic digestibility. Several different alkali concentrations, pretreatment times and solid loadings were used as variables to evaluate the pretreatment conditions. The pretreatment temperature was set at 100°C. It was found that using an alkali concentration of 2% (w/v) and a reaction time of 2.3 h, 50% lignin removal and 93% biomass conversions were achieved with a cellulase loading of 0.24 mL/g db sorghum and 0.25 mL/g db sorghum (Umagiliyage et al., 2015). The authors in this study performed the alkali soaking experiments at low solid loadings up to 10% (w/v). This requires a washing and/or neutralization step to remove or neutralize the alkali and other inhibitory compounds present in biomass.

Noori & Karimi (2016) used Elmwood as a feedstock in an alkali pretreatment process using 8% (w/v) NaOH at various temperatures. The authors achieved the highest glucose yield with Elmwood that was pretreated at 0°C for 2h. The authors reported that addition of tween-20 to the enzymatic hydrolysis process improved the glucose yields

significantly. This study did not focus on the enzymatic hydrolysis of the hemicellulose fraction, which could have improved the overall sugar recovery from Elmwood. Also, the authors performed the alkali soaking pretreatment at a solid loading of 10% (w/v), which required a separation, washing and a neutralization step.

Alkali soaking pretreatment seems to be a good option for pretreatment of biomass. The studies have shown very small or no inhibitor formation while achieving very high glucan conversions. However, all the studies that were reported so far performed alkali pretreatment at a submerged state, which requires a large amount of water and acid to neutralize the prehydrolyzate. This makes the pretreatment process expensive and less environmentally friendly. No studies were reported on performing the alkali pretreatment process at a solid state. This could eliminate the concerns with excessive water requirements and may reduce the number of unit operations required in the pretreatment process.

2.2.2 Physical Pretreatment

Mechanical Comminution

The objective of mechanical pretreatment is to reduce particle size and crystallinity of lignocellulosic materials and to increase the surface area of biomass to be accessible to the enzymes in the subsequent process of hydrolysis (Alvira et al., 2010). The process involves some combination of chipping, grinding or milling depending on the type of biomass and the particle size of the material (Sun and Cheng, 2002). Numerous milling processes such as ball milling, two-roll milling, hammer milling, colloid milling and vibro energy milling can be used to improve the enzymatic hydrolysis

of lignocellulosic materials. However, the energy requirements for these processes are high, making them economically challenging (Sun and Cheng, 2002).

Extrusion

In extrusion pretreatment, the biomass is passed through an extruder and in the process it is subjected to heating, mixing and shearing, which results in physical and chemical modification of the biomass (Alvira et al., 2010; Pandey et al., 2011). The barrel temperature and screw speed are the main factors that are involved in the disruption of lignocellulosic structure, causing defibrillation and shortening of fibers thereby increasing the accessibility of carbohydrates to enzymatic hydrolysis (Karunanithy & Muthukumarappan, 2010a; Karunanithy & Muthukumarappan, 2011d). Extrusion pretreatment has excellent adaptability to many process modifications such as addition of alkali, application of high pressure and expansion treatment using steam or other solvents, making it a potential pretreatment technique for lignocellulosic material.

Extrusion as a pretreatment technique has been used in several studies with different kinds of biomass and high glucan conversions have been obtained (Heredia-Olea et al., 2013; Karunanithy & Muthukumarappan, 2011c; Liu et al., 2013). In a study conducted by Karunanithy and Muthukumarappan (2011b), prairie cord grass was soaked in 0.5 to 2.5% alkali solution and subsequently extruded in a single screw extruder at various temperatures and barrel speeds. It was found that the 114°C barrel temperature, 122 rpm screw speed, and 1.7% alkali solution resulted in maximum glucose, xylose and overall sugar yields of 86.8, 54.5 and 82%, respectively (Karunanithy & Muthukumarappan, 2011b).

In another study, Karunanithy & Muthukumarappan (2010b) studied the effect of extruder temperature and screw speed on the enzymatic digestibility of corn stover. Using a single screw extruder, a total of five screw speeds ranging from 25 to 125 rpm and five barrel temperatures from 25 to 125°C were evaluated. The glucose and xylose yields increased as the barrel temperature increased from 25 to 100°C. However, the difference in the yield values was not significant. A further increase in temperature to 125°C resulted in a significant increase in glucose and xylose yields. The authors concluded that the best conditions for the pretreatment of corn stover were 125°C and a screw speed of 75 rpm with a cellulase to β-glucosidase ratio of 1:4, as it resulted in the highest glucose and xylose recoveries (Karunanithy and Muthukumarappan, 2010).

In a similar study, extruder parameters were optimized for pretreatment of switchgrass by Karunanithy and Muthukumarappan (2011d). Switchgrass was extruded using a single screw extruder (Brabender Plasti-corder Extruder Model PL2000, Hackensack, NJ) at several moisture content levels (10-50% wb) and particle sizes (2-10 mm) over a range of barrel temperatures (45-225°C) and screw speeds (20-200 rpm). The results showed that particle size, barrel temperature and screw speed had a significant effect on sugar yields, and the optimum pretreatment conditions were found to be barrel temperature of 176°C, screw speed of 155 rpm, moisture content of 20% wb and a particle size of 8mm, which resulted in glucose and xylose yields of 41.4 and 62.2% respectively (Muthukumarappan and Karunanithy, 2011d). This study did not achieve efficient hydrolysis of glucan and xylan, which, could have been due to the lack of an alkali or an acid to help decrease the recalcitrant property of biomass.

Lamsal et al. (2010) pretreated soybean hulls and wheat bran at three different temperatures and three screw speeds using a twin screw extruder. The extrusion was also performed in combination with NaOH, urea, and thiourea. The results showed that extrusion performed at 110°C/222 rpm and 150°C/420 rpm resulted in the highest sugar yield. It was also found that alkali extrusion of the wheat bran or soy hulls did not show a significant improvement in the sugar yields (Lamsal et al., 2010).

In a similar study conducted by Liu et al. (2013), corn stover was used for pretreatment with alkali and a twin-screw extruder to enhance the enzymatic digestibility and fermentable sugar production. It was found that after the extrusion process at 99°C, alkali loading of 0.06 g/g biomass and 1 h heat preservation time, a 71% removal of lignin was achieved. Enzymatic hydrolysis at 2% solids resulted in glucan and xylan conversions of 83 and 89% respectively (Liu et al., 2013). In order to establish an economically feasible ethanol production process, enzymatic hydrolysis and fermentation should be performed at higher solid loadings. The authors did not perform the enzymatic hydrolysis at higher solid loadings.

Choi & Oh (2012) studied a twin screw extrusion process for dilute acid pretreatment of rape straw. The temperature was varied from 150 to 170°C and the dilute acid feeding rate was varied from 4.5 to 6.9 mL/min. The pretreated solids were then subjected to enzymatic hydrolysis at 1% (w/v) solid loading. It was found that at higher temperatures, diminished xylose yields were noticed, which was attributed to the formation of degradations products. It was also found that using lower acid concentration and higher temperature during pretreatment resulted in the best glucose and xylose yields (Choi & Oh., 2012). This study was conducted at 1% (w/v) solid loading which was too

low. The authors could have performed enzymatic hydrolysis at a higher solid loading to determine the enzymatic digestibility of extrusion pretreated rape straw.

Heredia-Olea et al. (2015) evaluated the effects of extrusion pretreatment parameters on enzymatic hydrolysis and fermentation of sweet sorghum bagasse. Sorghum was extruded using a twin-screw extruder at several temperatures, screw speeds and moisture contents. It was found that sorghum extruded at a temperature of 100°C and 200 rpm resulted in 70% total sugar yields in an enzymatic hydrolysis process performed at 20% solids. The authors did not use any chemicals such as acid or alkali to assist the pretreatment process. This was the only study found relating to the use of sweet sorghum bagasse in an extrusion process.

Mechanical Pulping

Thermomechanical pulping (TMP) is mainly used in the manufacture of mechanical printing papers such as newsprints and uncoated or coated magazine papers (Illikainen, 2008). TMP is an existing and practiced technology in the paper and pulp industry, and can be potentially used for the pretreatment of lignocellulosic biomass for ethanol production. Use of such existing and well known technologies for bioethanol production has several advantages such as availability of equipment manufacturers, experienced operators and lower risks when estimating capital investments (Gonzalez et al., 2012; Gonzalez et al., 2011). TMP consists of two steps: steaming and refining. Refining is considered the heart of the TMP process as it is responsible for disruption of biomass. The operational principle of the refiner is that the biomass is fed between two parallel discs, and at least one of the discs is rotating (Illikainen, 2008). As shown in

Figure 2.2, the patterned refiner segments transfer the rotational energy to the pulp and in the process biomass is broken down due to the compressive and shearing forces (Illikainen, 2008).

Several parameters such as refiner disk gap, temperature and holding time can be varied to test the degree of severity on lignocellulosic materials. Chemical pretreatments such as alkaline or ozone pretreatment can be combined with TMP to boost the pulp characteristics that result in a more efficient enzymatic hydrolysis process (Gonzalez et al., 2012; Gonzalez et al., 2011).

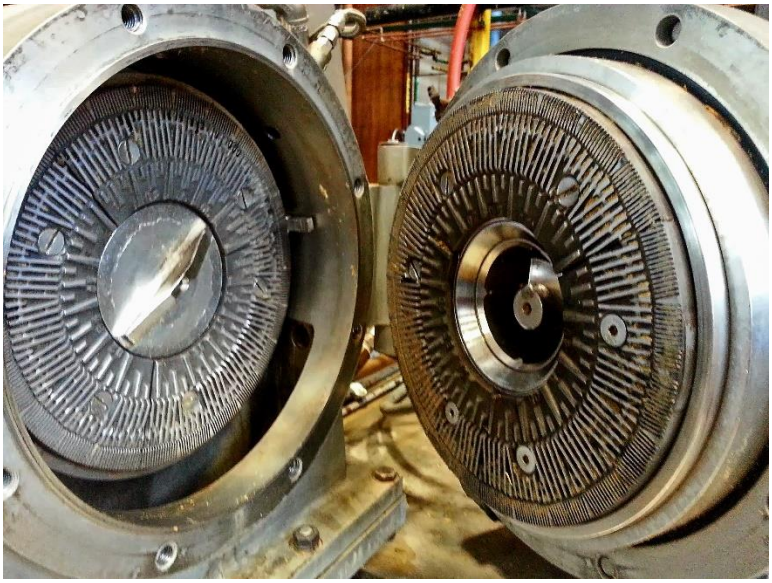

Figure 2.2 Refining discs inside a Thermomechanical Refiner. Courtesy of USDA Forest Products Lab, Madison, Wisconsin.

Wang et al. (2009) used aspen and wood chips in a sulfite pretreatment at 180 °C for 30 min followed by refining using a laboratory 8-inch disk refiner with a refiner gap size of 0.25 mm. Using a cellulase loading of 20 FPU and 30 CBU β-glucosidase, a cellulose conversion of 96% was achieved (Wang et al., 2009). In a similar study

conducted by Zhu et al. (2009), spruce and red pine were pretreated using sulfite pretreatment with 8-10% bisulfite and 1.8-3.7% sulfuric acid at 180°C for 30 min. The sulfite pretreated wood chips were then subjected to mechanical size reduction using laboratory 8 and 12-inch disk refiners with a refiner gap size of 0.25 mm. With a cellulase loading of 20 FPU and 30 CBU β-glucosidase, 93% glucose and 76% xylose yields were achieved (Zhu et al., 2009).

In another study by Jin et al. (2010), mixed hard wood chips were subjected to pretreatment at 160°C using a mixture of sodium carbonate and sodium sulfide with a sulfidity of 25%. The washed pretreated material was then refined with two passes using a Bauer 148-2 disk refiner with the disk gaps at 0.25 mm and 0.05 mm. Using a cellulase loading of 40 FPU/g substrate, a glucan recovery of 87% and xylan recovery of 67% were achieved (Jin et al., 2010). The authors used an energy intensive green liquor pretreatment process followed by a two-step refining process. This could increase the energy costs and make the overall process less feasible on an industrial scale.

Koo et al. (2011) used mixed hardwood chips as a feedstock to find a strategy to reduce enzyme dosage by application of oxygen delignification and mechanical refining. The hardwood chips were pretreated at 160°C at three different green liquor concentrations comprised of 75% (w/w) sodium carbonate and 25% sodium sulfide. A portion of pretreated hardwood chips was delignified using oxygen with 5% NaOH at 110°C for 1 h and refined three times using a disk refiner with a disk gap size of 0.13 mm. The refined hardwood chips were then used in a PFI mill at several speeds ranging from 2000 to 8000 rpm to further improve their digestibility. The authors achieved up to 50% lignin removal with a delignification step using oxygen. The enzymatic hydrolysis

of substrates that were additionally refined using a PFI mill at higher rotation speeds resulted in significantly higher glucan conversions and required lower enzyme loadings (Koo et al., 2011) compared to those without the additional PFI milling step. In this study, biomass was refined three times before an additional PFI milling process to achieve high glucan conversions. However, this may not be an economically feasible option.

In another study conducted by Chen et al. (2014), corn stover was pretreated using 0.1 M NaOH at 8% solids (w/v) at 80°C for 2 h. The alkali pretreated corn stover was then refined using a 36-inch Andritz pilot scale refiner at a refiner rotational speed of 1200 rpm. It was found that enzymatic hydrolysis performed at 15% (w/v) solid loading and 26 mg/g of cellulase resulted in a glucose yield of 84%. It was also found that increasing the energy consumption of refiner from 128 to 468 KWh/ODMT improved the glucose and xylose yields by 13 and 19% respectively (Chen et al., 2014). The study however did not focus on increasing the xylan conversion efficiencies, which could have improved the overall sugar yields.

Jones et al. (2014) studied several types of mechanical refining for their improvements in enzymatic digestibility of pretreated hardwood. Hardwood chips were treated with sodium carbonate liquor at 190°C for 5.5 min. and then refined using various refining technologies such as PFI refiner, a pilot scale TMP unit and an industrial scale refiner. The enzymatic hydrolysis performed at 5% solids (w/v) did not show a significant difference in total sugar yield obtained with pilot scale and industrial scale refiners (Jones et al., 2014). However, the study utilized a two-step pretreatment process in which the biomass was treated at higher temperature followed by milling. This process

required high energy inputs and may not be economically viable.

Batalha et al. (2015) used sugarcane bagasse in a hydrothermolysis pretreatment process at various temperatures and pretreatment times. The pretreated bagasse was refined using a refiner with a disc opening of 0.13 mm followed by PFI milling. With all the pretreatment conditions that were tested, it was found that inclusion of a PFI milling process after the refining step resulted in an improvement in the enzymatic hydrolysis yields (Batalha et al., 2015). However, the improvement in hydrolysis yields was less than 10%. This shows that the PFI milling process may not be necessary as it resulted in a small increase in hydrolysis yield.

Only one study has been performed on the use of TMP as a pretreatment option for enzymatic hydrolysis of sweet sorghum. Gonzalez et al. (2011) tested corn stover, wheat straw and sorghum bagasse for their enzymatic digestibility after pretreatment with TMP. Several process conditions such as refiner gap size, residence time, steaming temperature, and soaking in acetic acid were tested. Glucose yields and xylose yields of 63 and 57%, respectively, were obtained with sweet sorghum bagasse that was pretreated at 170°C (Gonzalez et al., 2011). The authors also reported that decreasing the refiner gap size from 0.15 mm to 0.05 mm increased the hydrolysis yield from 47.2 to 56.8% when wheat straw was used as a substrate. Moreover, soaking wheat straw in acetic acid before TMP at 170°C showed an increase in overall carbohydrate yields from 51 to 66% (Gonzalez et al., 2011). The hydrolysis process did not achieve high glucose and xylose yields due to the low severity of the TMP process.

Mechanical pulping seems to be an effective pretreatment option for various types of lignocellulosic biomass. Inclusion of an oxygen delignification step resulted in a substantial loss of lignin and improved glucan and xylan conversions. The addition of a presoaking step using an acid or an alkali seems to be a very effective strategy for achieving high enzymatic hydrolysis efficiencies. TMP is an established technology, and has several advantages such as the availability of equipment manufacturers and well experienced operators. It also has lower risks in terms of capital investments compared to the current pretreatment technologies that are being tested. Although mechanical pulping has been studied in depth on several different feedstocks, it is not so for sweet sorghum.

2.3 Enzymatic Hydrolysis of Biomass

Enzymatic hydrolysis of pretreated biomass involves the biochemical reactions carried out by highly specific enzymes such as cellulase that convert cellulose to glucose and hemicellulase that convert hemicellulose to pentoses and hexoses (Lynd et al., 2002; Pandey et al., 2011; Sun & Cheng, 2002). Enzymatic hydrolysis has low utility cost due to the milder conditions used compared to alkaline or acid hydrolysis procedures and does not have corrosion issues (Pandey et al., 2011; Sun & Cheng, 2002). Cellulases are produced by both bacteria and fungi and they can be aerobic or anaerobic, mesophilic or thermophilic. Some of the examples of bacterial species that produce cellulases are *Clostridium, Cellulomonas, Bacillus, Thermomonospora* etc. (Lynd et al., 2002; Sun & Cheng, 2002). Although such bacteria can produce cellulases, they are incapable of producing high enzyme titres (Lynd et al., 2002; Sun & Cheng, 2002). Fungal species such as *Trichoderma, Aspergillus, Sclerotium* are capable of producing high titers of cellulases and are widely studied.

Cellulases are a mixture of several enzymes and at least three major groups of cellulases are involved in the hydrolysis process: (a) endoglucanases which attack the amorphous form of cellulose creating free chain ends (b) exoglucanases which degrade the non-reducing ends of oligosaccharides to release cellobiose and (c) cellobiase or β-glucanase that hydrolyze cellobiose to D-glucose (Lynd et al., 2002; Sun & Cheng, 2002). In addition to the three major classes of cellulases, there are also several enzymes that attack hemicellulose, such as xylanase, galactomannase, acetylesterase, and glucomannase (Lynd et al., 2002; Sun & Cheng, 2002). Several factors such as the type of substrate, cellulase activity, and reaction conditions (such as pH and temperature) affect the enzymatic hydrolysis process. Increasing the enzyme dosage usually results in an increase in cellulose or xylan conversion; however, it also results in an increase in the cost of the overall process.

Enzyme loadings ranging from 2 to 70 FPU cellulase/ g glucan and xylanase loading ranging from 5 to 100 IU/g xylan have been reported. However, the amount of enzyme required varies with the type of pretreatment that is used. The enzyme requirement also varies with the brand of the enzyme that is used as each manufacturer has their own combination of enzyme cocktails. This is one reason to test the effect of enzyme loading on the enzymatic digestibility of pretreated biomass.

2.4 Other Pretreatment Methods For Sorghum

Corredor et al. (2009) evaluated forage sorghum as a feedstock for fermentable sugar production. Forage sorghum was subjected to enzymatic hydrolysis using amylases to remove starch and the obtained cake was dried and further pretreated with 2% H_2SO_4

followed by modified steam explosion. The authors obtained 72% hexose and 94% pentose yields after the enzymatic hydrolysis process (Corredor et al., 2009). In another study (Sipos et al., 2009), steam pretreated sweet sorghum bagasse was used as a feedstock for ethanol production. The authors achieved up to 92 % cellulose conversion of the pretreated material when loaded with cellulase at 20 FPU/g and 20 IU/g β-glucosidase (Sipos et al., 2009).

Fresh sorghum stems that were pretreated using H_2SO_3-steam were used as a feedstock for a simultaneous saccharification and fermentation process (SSF) (Yu et al., 2010). With an acid dosage of 0.25g/g at 100°C for 120 min, 44.5 g/L ethanol was obtained after 48 h of SSF of unwashed slurry, which corresponded to 110% theoretical yield (Yu et al., 2010). In another study by Salvi et al. (2010), sorghum bagasse was pretreated using dilute ammonia at 160°C and enzymatic hydrolysis and ethanol fermentation effectiveness were evaluated. The authors reported 44% lignin and 35% hemicellulose removal during the pretreatment process (Salvi et al., 2010). The same research group performed the enzymatic hydrolysis of dilute ammonia pretreated sorghum bagasse at 10% (w/v) solid loading and achieved up to 84% glucose yields (Salvi et al., 2010). This study used high temperatures during the pretreatment process with H_2SO_3, which resulted in the formation of inhibitors. The formation of inhibitors is not favorable as they inhibit the fermentation process.

In a study conducted by Zhang et al. (2011), the effects of four different pretreatments on enzymatic hydrolysis of sorghum bagasse were evaluated. Sweet sorghum bagasse was pretreated using ILs, steam explosion, lime and dilute acid. It was found that steam explosion resulted in the greatest glucan conversion compared to other

treatments resulting in 70% glucose theoretical yield compared to 55, 45 and 40% with dilute acid, lime and ILs respectively. Furthermore, enzymatic hydrolysis of untreated bagasse resulted in glucan conversion of only 27% (Zhang et al., 2011). This study did not achieve high glucan conversions with all the pretreatments that were tested.

In a similar study conducted by Cao et al. (2012), sorghum bagasse was subjected to pretreatment using (1) dilute NaOH solution autoclaving pretreatment, (2) high concentration NaOH solution immersing pretreatment, (3) dilute NaOH solution autoclaving and H_2O_2 immersing pretreatment, (4) alkaline peroxide pretreatment and (5) autoclaving pretreatment. It was found that the highest cellulose conversion of 74.3% was obtained with dilute NaOH solution autoclaving and H_2O_2 immersing pretreatment, and treatments 1-4 resulted in cellulose conversions from 62 to 70%. However, autoclave pretreatment resulted in only 11.78% cellulose yield, which was similar to the cellulose yields that were obtained with controls which were not pretreated (Cao et al., 2012). A novel concept is presented in a study performed by Rohowsky et al. (2013), in which the juice is separated from sorghum stems and the bagasse was subjected to hydrothermolysis pretreatment. The juice was later added to the pretreated solids and enzymatic hydrolysis was performed. At a solid loading of 7.5%, a cellulose conversion of 74% was achieved and addition of juice did not affect the hydrolysis (Rohowsky et al., 2013). This is a good finding since the substrate concentration could greatly affect the enzymatic hydrolysis of cellulases but seemed unaltered in this process. This could be due to the presence of yeasts that are simultaneously converting sugars to ethanol, thereby alleviating the stress caused due to the presence of high concentration of sugars (Lynd et al., 2002; Rohowsky et al., 2013).

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CHAPTER III

EVALUATION OF THERMOMECHANICAL PULPING AND REFINER MECHANICAL PULPING AS PRETREATMENT OPTIONS FOR WHOLE SWEET SORGHUM

3.1 Abstract

Sweet sorghum (*Sorghum bicolor* L.) was chopped to two different particle sizes (18 ± 3.2 mm and 35 ± 17.2 mm) and then subjected to Refiner Mechanical Pulping (RMP) and Thermomechanical Pulping (TMP) treatments. For RMP treatments, the refiner gap was varied in order to achieve different levels of shear stress. For TMP pretreatment, sorghum was steamed to 160°C for 10 min and was then refined using a pressurized refiner. Enzymatic hydrolysis of RMP and TMP pretreated sorghum showed that sorghum that was pretreated with TMP resulted in the highest glucose yields. Within the RMP pretreatments, sorghum that was refined twice (RG3G2: first pass with a gap size of 76.2 μ and second pass with a gap size of 50.8 μ) resulted in the highest glucose yields among all the RMP pretreatments that were tested. Addition of a preheating step prior to the enzymatic step did not improve the glucose %MTY values. Increasing

the α-amylase and amyloglucosidase loading from 0.1 to 0.5 mL improved the glucose %MTY values by about 11% in RG3G2 pretreated sorghum and 61% in TMP pretreated sorghum. Increasing the cellulase and xylanase loadings from 0.5 to 1.0 mL/g significantly improved the glucose and xylose yields with both TMP and RMP pretreated sorghum at 6% solid loading. The highest glucose MTY of 60.8 % was obtained with TMP pretreated sorghum at 6% solid loding at an enzyme loading of 1.0 mL/g (60 FPU/mL). With RMP pretreated sorghum, the highest glucose and xylose yields obtained were 45.7 and 9.3% respectively. Increasing the solid loading from 6 to 12% showed similar glucose and xylose yields with both RMP and TMP pretreated sorghum.

3.2 Introduction

Lignocellulosic biomass is an abundant and renewable resource for the production of bio-based chemicals such as biofuels and bioplastics. Lignocellulosic biomass is composed of structural carbohydrates such as cellulose and hemicellulose that can be enzymatically hydrolyzed to produce fermentable sugars (Taherzadeh & Karimi, 2007). However, the presence of lignin interferes with the access of structural carbohydrates and also inhibits the enzymatic hydrolysis process (Alvira et al., 2010; Fang, 2013). A pretreatment process is used to alter the lignocellulosic structure of biomass in order to increase the accessibility of cellulose and hemicellulose for the enzymatic hydrolysis process (Alvira et al., 2010; Mosier et al., 2005; Wu et al., 2010). Several pretreatment technologies such as steam explosion (Shen et al., 2011; Sohail Toor et al., 2013), dilute acid pretreatment (Banerji et al., 2013), alkali pretreatment (McIntosh & Vancov, 2010; Sun et al., 2014), ionic liquids (Eckard et al., 2012) and extrusion (Liu et al., 2013) have been tested with a wide range of feedstocks. Sweet sorghum is a C4 photosynthetic

species that is well adapted to drought conditions, has low water requirements and can produce very high yields up to 35 Mg/ha (db) (Li et al., 2010; Rooney et al., 2007). Sorghum consists of three sources of carbohydrates: (1) soluble carbohydrates in the stalks (2) storage carbohydrates in the form of starch in the grain and (3) structural carbohydrates in the stalks and leaves, which makes it a versatile crop for a bio-refinery operation. Sorghum as a feedstock has been used in several studies (Cao et al., 2012; Chen et al., 2012; Heredia-Olea et al., 2013; Li et al., 2010; Sambusiti et al., 2013; Wu et al., 2011) and the effect of several pretreatments has been widely studied.

Mechanical pulping is an established technology for fiber development in the paper industry and the same process could be applied as a potential pretreatment option for biofuel operations. Use of such technologies is advantageous due to the availability of manufacturers, experienced operators and the capability to combine pulping with other pretreatments such as alkaline hydrolysis and oxygen delignification (Gonzalez et al., 2012; Gonzalez et al., 2011). Several studies have been performed on the use of mechanical pulping as a pretreatment option (Gonzalez et al., 2011; Liu et al., 2011; Xu & Springfield, 2001; Zhao et al., 2004), however sweet sorghum was only used in one study (Gonzalez et al., 2011) , which was focused on using the bagasse portion of the sorghum. There have been no studies involving the enzymatic hydrolysis of the whole sorghum plant pretreated with a mechanical pulping process.

In the current study, whole sweet sorghum (grain, stem and leaves) was subjected to pretreatment using two types of pulping methods: (a) Thermomechanical pulping (TMP) with an included steaming step and (b) Refiner Mechanical Pulping (RMP) that operates without steam. The refiner gap sizes were varied to increase the intensity of

pretreatment. The pretreated sorghum was then evaluated during enzymatic hydrolysis with various process conditions.

3.3 Materials and Methods

3.3.1 Sample Preparation

Sweet sorghum (*Sorghum bicolor* L.) was harvested from the Oklahoma State University agronomy station and the stalks were chopped in a bowl chopper (K64, Seydelmann, Stuggart, Germany) into fine (18 ± 3.2 mm) and coarse particle sizes (35 ± 17.2 mm). The chopped sweet sorghum was frozen until further use.

3.3.2 Pretreatment of Sorghum

Sweet sorghum was subjected to mechanical pulping using Refiner Mechanical Pulping (RMP) and Thermomechanical Pulping (TMP) machines located at the USDA Forest Products Laboratory in Madison, Wisconsin. In the RMP process, sorghum was fed through the hopper and disk gaps of 2 (2/1000 inch i.e. 50.8 µm) and 3 (3/1000 inch i.e. 76.2 µm) were used to attain different levels of shear stress on the sorghum. Various pretreatment parameters that were tested are listed in the Table 3.1. During the process of RMP pretreatment, a liquid stream containing soluble sugars was generated. A sample of the liquid portion was taken and analyzed for carbohydrate content and for further mass balance calculations. In the TMP process, the sorghum was preheated with steam for 10 min and then fed into a pressurized refiner (12-inch Andritz Sprout-Bauer Pressurized Refiner, St. Louis Park, MN) at 160°C and a pressure of 30 psi. The disc gap was fixed at 127 µm. The pretreated samples were collected and frozen until further use.

Table 3.1 Process parameters used during the RMP and TMP pretreatment of sorghum.

Treatment	Sorghum Particle Type	Refiner Gap Size (µm)	Temperature ° C, Pressure (Psi)
RG3	Coarse	76.2	NA
RFG3	Fine	76.2	NA
RWG3	Coarse	76.2	NA
RFG2	Fine	50.8	NA
RG3G2	Coarse	76.2, 50.8	NA
TMP	Coarse	127	160, 30

RG3 = Sorghum chopped to a coarse particle size and pretreated with RMP process with refiner gap size of 3

RFG3 = Sorghum chopped to a fine particle size and pretreated with RMP process with refiner gap size of 3

RWG3 = Sorghum pretreated with RMP process with refiner gap size of 3 with an added washing step with water

RFG2 = Sorghum chopped to a fine particle size and pretreated with RMP process with refiner gap size of 2

RG3G2 = Sorghum pretreated with RMP process with refiner gap size of 3 and reprocessed with gap size of 2

3.3.3 Compositional and Particle Size Analysis

A sample of sorghum was dried at 85°C for 1 h to kill the enzymes present in the biomass and further dried at 60°C for 12 h to remove the moisture. A portion (about 30 g) of RG3G2 and TMP sorghum was used to determine the particle size distribution. The particle size distribution of the milled sorghum was determined using a sieve shaker, and is shown in Figure 3.1. A total of six 8” sieve (VWR, USA) sizes were selected to give a broad spectrum of particle size ranging from 1.4 mm to 0.075 mm (75µ).

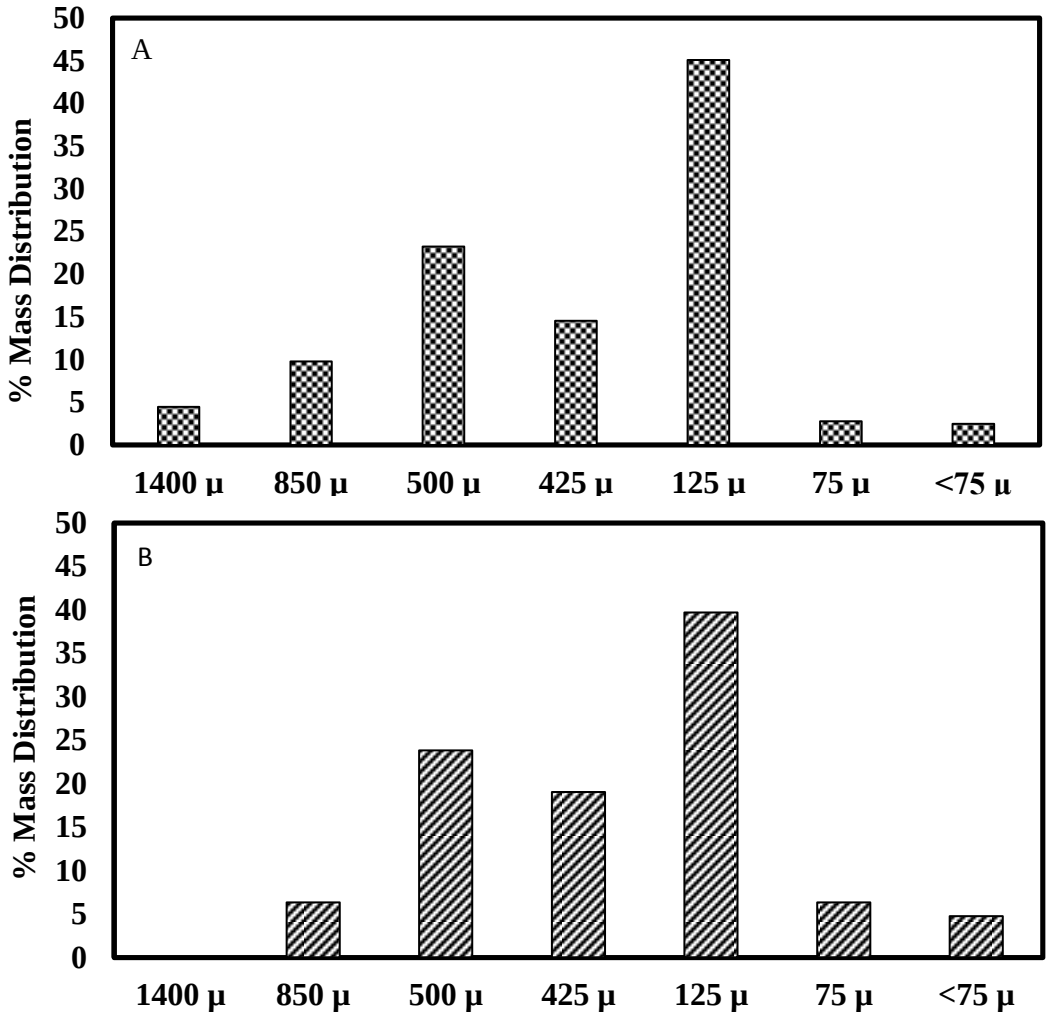

Figure 3.1 Particle size distribution of pretreated sorghum obtained after pretreatment (A) RG3G2 and (B) TMP.

It was found that with RG3G2 sorghum about 45% of sorghum had a particle size between 0.42 and 0.075 mm, and 23% of the particles had a particle size between 0.85 and 0.42 mm. Less than 5% of the milled sorghum had a particle size of 1.4 mm or more (Figure 3.1). With TMP pretreated sorghum, about 40% of sorghum had particle size between 0.42 and 0.075 mm, and 23% of the particles had a particle size between 0.85 and 0.42 mm. Less than 10% of the milled sorghum had a particle size of 0.85 mm or

more (Figure 3.1).

The dried sorghum was further ground using a coffee grinder and used to determine the extractives and structural carbohydrates using NREL procedures. Prior to determination of structural carbohydrates and lignin in the biomass, a two-step extraction process was performed using a procedure described by Sluiter et al. (2005). Automatic extraction by ethanol followed by water was conducted using an ASE® 300 system (Dionex Corporation, Sunnyvale, CA, USA). The operating parameters for both steps were 1500 psi at 100°C, 150% flush volume, 7 min static time, 2 min purge time, and four static cycles. All extractions were conducted in triplicate in 33 mL extraction cells using 95% ethanol and distilled water for ethanol and water extractions, respectively. A portion (1 mL) of ethanol extract was dried over N_2 gas in a glass vial and 1 mL of deionized (DI) water was added to the vial to dissolve the carbohydrates. The contents of the vial were mixed using a vortex mixer and centrifuged at 15000 rpm for 10 min. The supernatant was separated using a 0.2μ nylon filter and analyzed on HPLC as described below. Extracted sorghum solids were air dried for at least 24 h prior to use in subsequent analysis of structural carbohydrates and lignin as described in Sluiter et al. (2004). Absorbance for acid soluble lignin was measured at 205 nm using a UV–Vis spectrophotometer (Beckman DU 520, Beckman Coulter, Inc., Pasadena, CA). The 205 nm wavelength was chosen based on work done by Thammasouk et al. (1997).

3.3.4 Enzymatic Hydrolysis of Pretreated Sorghum

Effect of Enzyme Loading

Pretreated sorghum was used for conducting enzymatic hydrolysis experiments

using NREL procedures. Preliminary experiments were conducted in order to determine the effect of pretreatment on enzymatic hydrolysis using cellulase (Celluclast® 1.5L with an activity of 60 Filter Paper Units/mL or FPU/mL) mixture with and without xylanase (Pentopan® Mono BG with an activity of 500 Fungal Xylanase Units/mL or FXU/mL) supplementation. The activity of cellulase enzyme was measured using a cellulose filter paper assay as described in Adney and Baker (2008). In order to determine the effect of enzyme loading on enzymatic hydrolysis, two types of pretreated solids (TMP and RG3G2) were chosen as substrates. The experiments were conducted at 6 and 12% solids (w/v) and three enzyme loadings, 0.5, 0.7 and 1.0 mL/ g glucan and xylan (Celluclast® 1.5L and Pentopan® Mono BG loading) were tested. These enzyme loadings were equivalent to 0.18, 0.28 and 0.4 mL cellulase/g db sorghum and 0.14, 0.22 and 0.31 mL xylanase/g db sorghum. Furthermore, three levels of α-amylase (0.5, 0.7 and 1 mL/6 g db sorghum) (Fungamyl® 800L with an activity of 634 International Units/mL or IU/mL) and three levels of amyloglucosidase (0.5, 0.7 and 1 mL/6 g db sorghum) (AMG 300L™ with an activity of 300 IU/mL) were added to break down the starch that was present in sorghum.

All enzymatic hydrolysis experiments were conducted in triplicate in 250 mL baffled flasks sealed with a rubber stopper fitted with a one-way air valve (Check valve, Fischer Scientific, Pittsburgh, PA) to avoid contamination by microbes. Each hydrolysis flask contained 10 mL 0.5 M sodium citrate buffer at a pH of 5.5 and 6% solids (w/v). To all the hydrolysis flasks, 100 μL of 0.1% sodium azide was added to prevent contamination by microbes. The total mass of each flask was 100 g. All experiments were conducted in triplicate.

Control flasks containing the same contents as experimental flasks excluding enzymes were maintained to account for the sugars that are diffused during the hydrolysis process. All flasks were incubated at 55°C, 150 rpm in an incubating orbital shaker (C25 incubator shaker, New Brunswik Scientific, Edison, NJ). Samples (1.5 mL each) were collected at 0, 6, 12, 24, 48 and 96 h. The samples were then centrifuged at 15000 rpm for 10 min and the supernatant was filtered through a 0.2µ nylon filter. The filtrate was stored in a freezer until further analysis.

Yield Calculations

Maximum theoretical yield was calculated using the following equation

$$\%\ glucose\ MTY = \frac{glucose\ concentration\ measured}{(fg\ [biomass]*1.11)} X100$$

Where *fg* is the glucan fraction of dry biomass, [biomass] is dry biomass concentration, and 1.11 is the conversion factor for glucan to glucose.

$$\%\ xylose\ MTY = \frac{xylose\ concentration\ measured}{(fx\ [biomass]*1.13)} X100$$

Where *fx* is the xylan fraction of dry biomass, [biomass] is dry biomass concentration, and 1.13 is the conversion factor for xylan to xylose.

Percentage total sugar extracted %TSE is calculated by accounting the total sugar concentration that was measured in the flasks

$$\%TSE = \frac{total\ sugar\ cocentration\ detected}{Total\ theoretical\ sugar\ concentration} X100$$

$$Total\ theoretical\ sugar\ concentration\ = (fg\ [biomass] * 1.11) + (fx\ [biomass] * 1.11) + (fgs\ [biomass] * 1.11) + (s\ [biomass])$$

Where *fgs* is the glucose that can be obtained from the starch portion and *s* is the soluble carbohydrate fraction in the dry biomass.

Effect of Preheating on Enzymatic Hydrolysis of Sorghum

For the effect of preheating experiments, TMP and RMP G3G2 solids were used as substrate. In order to maximize the starch hydrolysis, α-amylase (0.1 mL) was added to the experimental flasks and heated to 85°C for 2 h. The flasks were then cooled to 55°C and cellulase (0.5 mL/g glucan), xylanase (0.5mL/g xylan), and amyloglucosidase (0.1 mL) were added. The effect of increased α-amylase and amyloglucosidase addition was also tested. For these experiments, the same procedure as described above was followed but α-amylase and amyloglucosidase loadings were increased from 0.1 to 0.5 mL respectively. All experiments were conducted in triplicate.

3.3.5 Starch Analysis

A portion of enzymatically hydrolyzed sorghum was used for the analysis of starch. A sorghum sample (approximately 5 g) was dried at 105°C for 16 h to remove the moisture. The dried sample was then ground using a coffee grinder. The starch analysis was performed according to AACC method 76-13.01, using a total starch assay kit (Megazyme, Ireland).

3.3.6 HPLC Analysis

For quantification of cellobiose, glucose, xylose, sucrose, fructose, galactose,

mannose and arabinose from the sorghum compositional analyses, an Aminex HPX-87P column (Bio-Rad, Sunnyvale, CA, USA) was used at 80°C with DI water as eluent flowing at 0.6 mL min^{-1}. For the analysis of inhibitors in the hydrolyzate, an Aminex HPX-87H column (Bio-Rad, Sunnyvale, CA, USA) was used at 60°C with 0.01 N H_2SO_4 as eluent flowing at 0.6 mL min-1. For both columns, a refractive index detector (1100 series, Agilent, Santa Clara, CA) was used.

3.3.7 Statistical Analysis

An analysis of variance (ANOVA) was determined using the GLM procedure of SAS Release 9.3 (Cary, NC, USA). A Duncan's test was used to determine the statistical significance of the structural carbohydrates present in sorghum (glucan, xylan etc.) and enzymatic hydrolysis results (glucose %MTY, xylose %MTY and %TSE). Significance was tested at $\alpha = 0.05$.

3.4 Results and Discussion

3.4.1 Compositional Analysis of TMP and RMP Pretreated Sorghum

The soluble carbohydrate composition in untreated sorghum was quantified as 21.7%. Table 3.2 represents the soluble carbohydrate content of sorghum before and after pretreatment with TMP and RMP processes. A partial removal of soluble carbohydrates was noticed with all the pretreatment methods that were tested.

With the TMP pretreatment, the solids were preheated to 160°C for 15 min before they were sent to the pressurized refiner. The RMP process did not use a preheating step and thus no sugar degradation was noticed. However, during the refining process partial extraction of soluble carbohydrates was noticed. In the RMP process, the liquid fraction

was collected and the analysis showed that up to 25% of the initial soluble carbohydrates were present in the liquid fraction. These soluble carbohydrates can be used in the fermentation process as a medium and hence do not decrease the overall sugar yield of a pretreatment and hydrolysis process. Due to the high temperatures involved in the TMP pretreatment process, the liquid portion could not be recovered. The soluble sugars in the TMP process could have been converted to furfural or 4-hydroxy methyl furfural.

Table 3.2 Soluble carbohydrate composition of sorghum obtained after RMP and TMP processes (n=3).

Treatment	**Soluble Carbohydrate (% db[a]) (avg ± sd)[1]**
RFG3[b]	17.5 ± 0.7
RWG3[c]	19.0 ± 4.2
RG3G2[d]	16.1 ± 0.5
TMP	15.5 ± 1.5
No pretreatment	21.7 ± 2.4

[a] Dry basis

[b] Sorghum chopped to a fine particle size and pretreated with RMP process with refiner gap size of 3 (76.2 μm)

[c] Sorghum chopped to a coarse particle size and pretreated with RMP process with refiner gap size of 3 (76.2 μm) with an added washing step with water

[d] Sorghum chopped to a coarse particle size and pretreated with RMP process with refiner gap size of 3 (76.2 μm) and reprocessed with gap size of 2 (50.8 μm)

[1] Average ± standard deviation

The composition of sweet sorghum before and after various pretreatment processes is shown in Table 3.3. The untreated sorghum contained 38.8% glucan, 23.5% xylan, 2.6% starch and 18.9% lignin. There was no significant alteration of the lignocellulosic content during the process of pretreatment, as is evident from the

compositional analysis data. Among all the different RMP treatments that were tested, the glucan and xylan content was not significantly altered, which could be due to a lack of sufficient shear stress from the refiner. Moreover, the sorghum was not soaked in a solvent such as dilute acid or an alkali and hence the contents of sorghum were not altered. Several studies have shown that the pretreatment of various types of biomass using TMP and RMP processes resulted in increased fibrils, decreased crystallinity of cellulose and partial removal of lignin and xylan (Gonzalez et al., 2011; Han et al., 2015; Phillips et al., 2013; Wu et al., 2011; Zhao et al., 2004). However, these studies were performed using a combination of TMP and RMP pretreatments with an additional chemical treatment step. In the current study, no additional chemicals were added to sorghum and hence did not show a significant difference in lignin or xylan removal. This showed the poor efficiency of the pretreatment process in altering the lignocellulosic structure.

Table 3.3 Compositional analysis of pretreated and untreated sorghum (n=3).

Treatment	**Glucan (%db[a]) (avg ± sd)[1]**	**Xylan (% db[a]) (avg ± sd)**	**Starch (%db[a]) (avg ± sd)**	**Lignin (%db[a]) (avg ± sd)**
RFG3[b]	42.0 ± 0.8	24.5 ± 0.3	2.4 ± 0.3	18.8 ± 0.5
RG3G2[d]	40.5 ± 1.2	22.6 ± 1.6	2.3 ± 0.1	17.4 ± 0.1
TMP	39.7 ± 1.3	25.9 ± 0.3	1.9 ± 0.1	19.7 ± 0.1
No pretreatment	38.7 ± 1.1	23.5 ± 1.4	2.6 ± 0.1	18.8 ± 0.5

[a] Dry basis

[b] Sorghum chopped to a fine particle size and pretreated with RMP process with refiner gap size of 3 (76.2 µm)

[c] Sorghum chopped to a coarse particle size and pretreated with RMP process with refiner gap size of 3 (76.2 µm) with an added washing step with water

[d] Sorghum chopped to a coarse particle size and pretreated with RMP process with refiner gap size of 3 (76.2 µm) and reprocessed with gap size of 2 (50.8 µm)

[1] Average ± standard deviation

3.4.2 Effect of Pretreatment on Enzymatic Hydrolysis

Experiments were conducted with sorghum that was pretreated with various TMP and RMP treatments at 6% solid loading and an enzyme loading of 0.5 mL cellulase/g glucan, 0.5 mL xylanase/g xylan, and 0.1 ml α-amylase and amyloglucosidase. Figure 3.1 shows the glucose Maximum Theoretical Yield (%MTY) obtained from various pretreatments. As shown in Figure 3.2, the highest glucan hydrolysis was achieved with TMP pretreated sorghum, followed by RG3G2 pretreated sorghum. About a 2-fold increase in maximum theoretical yield (% MTY) was achieved with enzymatic hydrolysis of TMP pretreated solids compared to untreated sorghum. Within the RMP pretreatments, RG3 resulted in a significantly lower glucose %MTY ($p>0.05$) after 48 h compared to all other RMP treatments that were tested. ($p>0.05$). Except for RG3 pretreated sorghum, all RMP treatments and the untreated sorghum resulted in similar glucose %MTY after 48 h.

Table 3.4 shows the various yields that were obtained with enzymatic hydrolysis of sorghum pretreated using several techniques. The highest xylose yield of 9.5% was obtained with hydrolysis of the TMP pretreated sample, which was significantly greater ($p<0.05$) than all other treatments that were tested. The lowest xylose yield of 4.5% was obtained with enzymatic hydrolysis of the RG3 and RFG3 solids, which were significantly lower than the xylose %MTY values obtained with RG3G2 and RMPWG3 pretreated solids ($p<0.05$).

The xylose yields with all treatments were lower than 10% (Table 3.4), which showed a very poor hydrolysis efficiency. This was likely due to the lower availability of xylan to the enzyme mixture, which could be attributed to the recalcitrant nature of lignin that prevented the enzyme from coming into contact with the xylan portion (Alvira et al.,

2010; Gao et al., 2014; Gao et al., 2013; Wu et al., 2010). Moreover, cellulose is crystalline in nature and is known to attach with xylan. The higher crystallinity of cellulose along with the presence of lignin could have resulted in the lower enzymatic hydrolysis efficiency of xylan (Cao et al., 2012; Lynd et al., 2002; Persson et al., 2007; Vena et al., 2013; Yesuf & Liang, 2013).

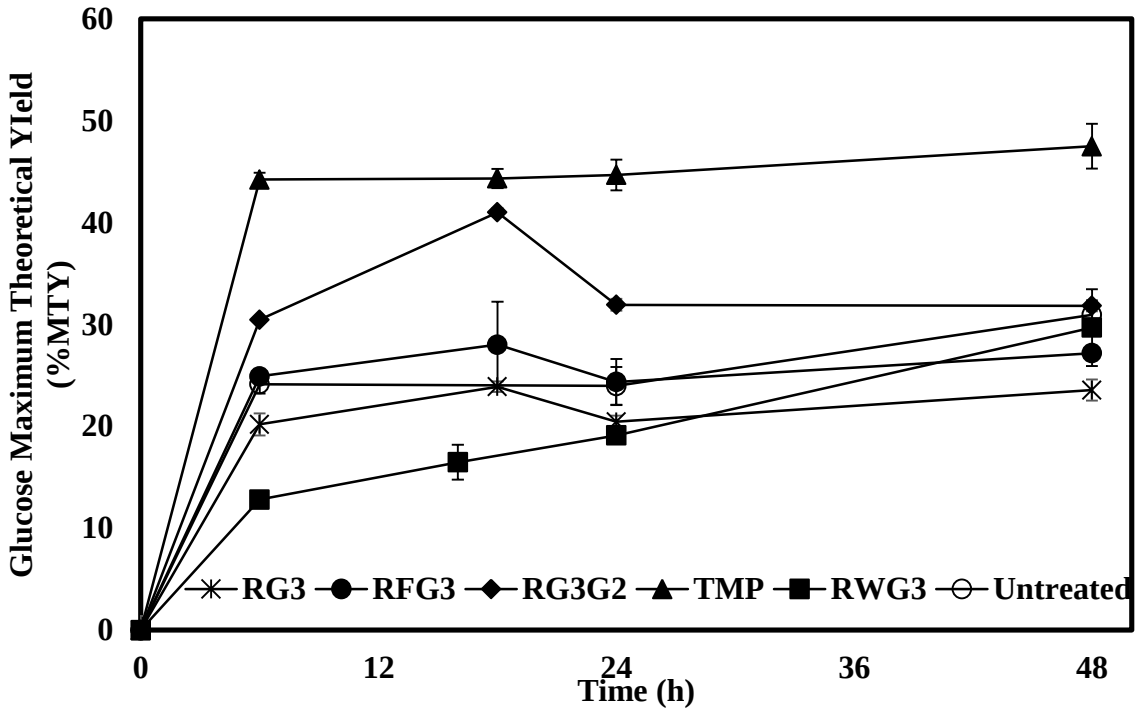

Figure 3.2 Percentage Glucose Maximum Theoretical Yield (%MTY) obtained with various pretreatments. All flasks were loaded with 0.5 mL/g glucan of cellulase (30 FPU/g glucan), 0.5 mL/g xylan of xylanse (250 FXU/g xylan), 0.1 mL of α-amylase (10.5 IU/g db sorghum) and amyloglucosidase (5 IU/g db sorghum). Error bars represent standard deviation (n=3).

The Total Sugar Extracted values take into account the carbohydrates that were extracted during the process of pretreatment, enzymatic hydrolysis and the simultaneous diffusion process. Figure 3.3 illustrates the Total Sugars Extracted (%TSE) during the

process of pretreatment and the subsequent enzymatic hydrolysis process. Figure 3.4 represents the total sugars extracted during the process of RG3G2, followed by enzymatic hydrolysis at 6% solid loading. The highest TSE of 57.3 % was obtained with enzymatic hydrolysis of RG3G2 pretreated sorghum, which was significantly higher than all other treatments that were tested (Table 3.4).

Table 3.4 Effect of pretreatment on enzymatic hydrolysis of sorghum at 6% solid loading, an enzyme loading of 0.5 mL cellulase/g glucan (30 FPU/g glucan), 0.5 mL xylanase/g xylan (250 FXU/g xylan), 0.1 mL/g db sorghum α-amylase (10.5 IU/g db sorghum) and 0.1 mL/6 g db sorghum amyloglucosidase (5 IU/g db sorghum). Values reported were obtained after 48 h of enzymatic hydrolysis (n=3).

Treatment	**% MTY Glucose (avg ± sd)[1]**	**% MTY Xylose (avg ± sd)**	**%TSE (avg ± sd)**
RG3[1]	23.5 ± 1.0^{d}	4.4 ± 0.1^{d}	21.3 ± 1.0^{d}
RWG3[2]	29.7 ± 0.4^{b}	7.3 ± 0.4^{b}	38.4 ± 1.0^{b}
RFG3[3]	27.2 ± 0.0^{c}	4.5 ± 0.2^{d}	25.4 ± 1.4^{c}
RG3G2[4]	31.9 ± 0.5^{b}	6.8 ± 0.5^{b}	57.3 ± 0.5^{a}
TMP	47.5 ± 2.2^{a}	9.5 ± 0.8^{a}	37.8 ± 1.8^{b}
Untreated	30.9 ± 1.3^{b}	5.5 ± 0.6^{c}	38.6 ± 1.5^{b}

[1] Pretreated with RMP process with refiner gap size of 3 (76.2 μm)

[2] Pretreated with RMP process with refiner gap size of 3 (76.2 μm) with an added washing step with water

[3] Sorghum chopped to a fine particle size and pretreated with RMP process with refiner gap size of 3 (76.2 μm)

[4] Sorghum pretreated with RMP process with refiner gap size of 3 (76.2 μm) and reprocessed with gap size of 2 (50.8 μm)

[1] Average ± standard deviation

Values with the same letters within a column are not significantly different

Analysis of sorghum after the enzymatic hydrolysis process revealed that the

starch content decreased significantly showing the hydrolysis to glucose. The final starch content left after the enzymatic hydrolysis process was found to be 0.6 % (db) with TMP and RG3G2 pretreated sorghum. About 60-70% reduction in starch content was observed with both TMP and RMP pretreatments.

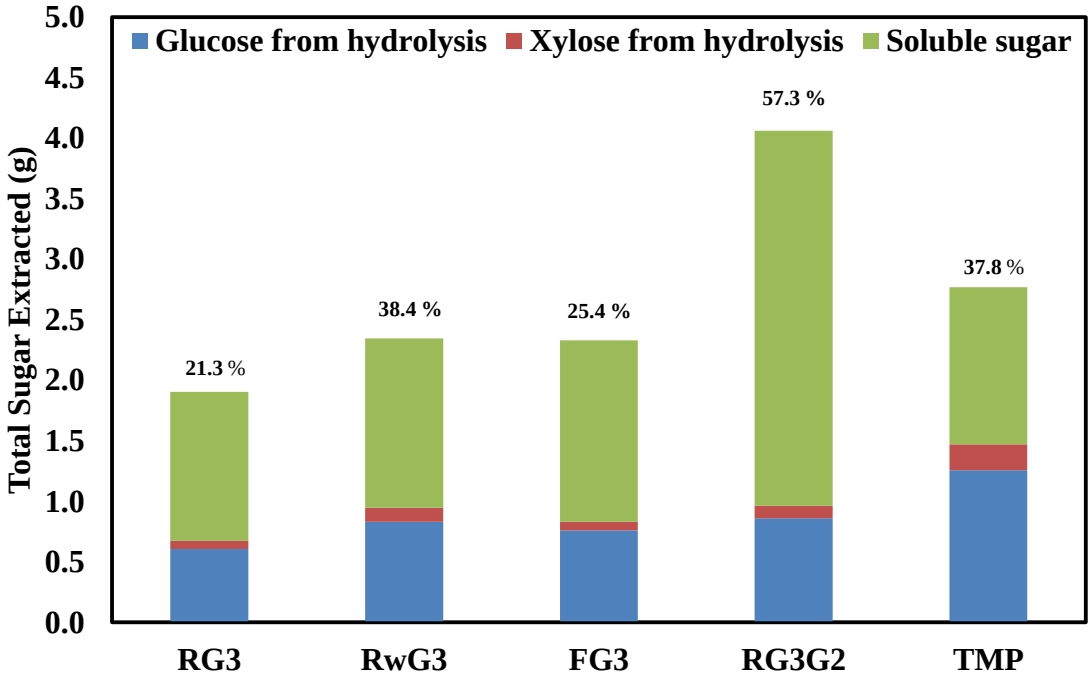

Figure 3.3 Total Sugars Extracted with various RMP and TMP pretreatments with enzymatic hydrolysis of sorghum at 6% solid loading, an enzyme loading of 0.5 mL cellulase/g glucan (30 FPU/g glucan), 0.5 mL xylanase/g xylan (250 FXU/g xylan), 0.1 mL α-amylase (10.5 IU/g db sorghum) and amyloglucosidase (5 IU/g db sorghum). Values on top of the bars represent the %TSE values obtained for each treatment. Values reported were obtained after 48 h of enzymatic hydrolysis (n=3).

During the process of enzymatic hydrolysis, a portion of the soluble carbohydrates was also extracted due to the presence of the concentration gradient in the

medium. The diffusion process resulted in soluble carbohydrate extraction yields from 49 to 80% with the RMP treatments that were tested, with RG3G2 treatment resulting in the highest soluble carbohydrate extraction of 80%, which was significantly higher ($p<0.05$) compared to all other treatments that were tested. Extraction of soluble sugars could be further improved by increasing the temperature to 70°C. However, this could affect the performance of the enzymatic hydrolysis process by degradation of the enzyme mixture.

Figure 3.5 shows the total sugars extracted with TMP pretreatment and the subsequent enzymatic hydrolysis at 6% solid loading. Although TMP pretreated sorghum showed an improved hydrolysis efficiency compared to RMP pretreated solids, the loss of soluble sugars during the pretreatment process lowered the % TSE values of TMP. RMP pretreatment resulted in a partial extraction of sorghum sugars due to the shear that was generated from the process equipment. This extracted sugar was collected and accounted for in the % TSE value and thus exceeded the % TSE values of TMP. The TMP process not only degraded the soluble sugars present in the sorghum, but also did not generate the liquid stream containing soluble sugars, unlike the RMP pretreatments. This decreased the overall efficiency of the TMP pretreatment process.

Since RG3G2 pretreated solids resulted in the best %MTY and %TSE values among the RMP treatments that were tested, they were chosen to perform further experiments from this point forward. The RG3G2 pretreated sorghum will be labeled as RMP pretreated sorghum from this point forward.

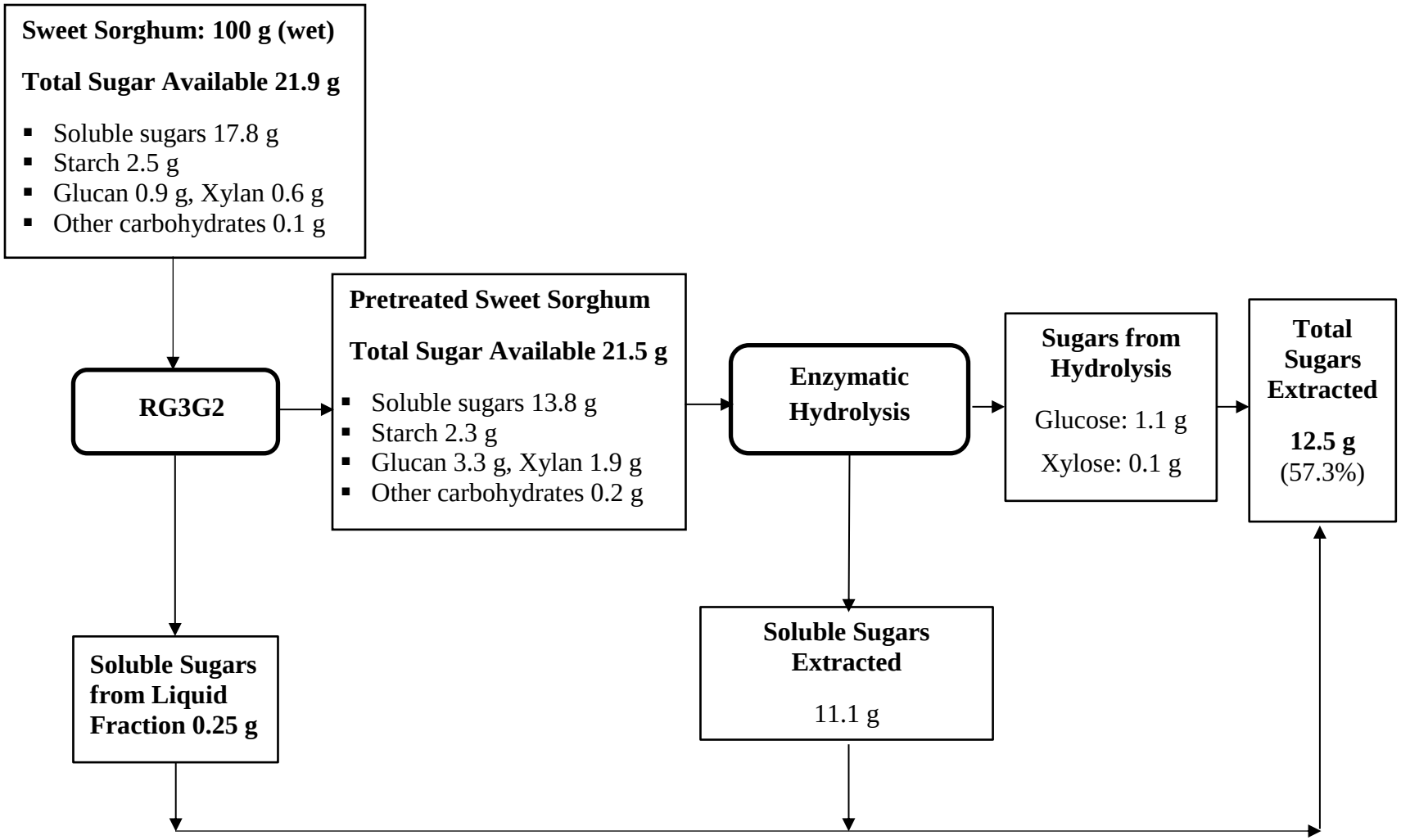

Figure 3.4 Total sugars extracted with RG3G2 pretreatment and the subsequent enzymatic hydrolysis at 6% solid loading.

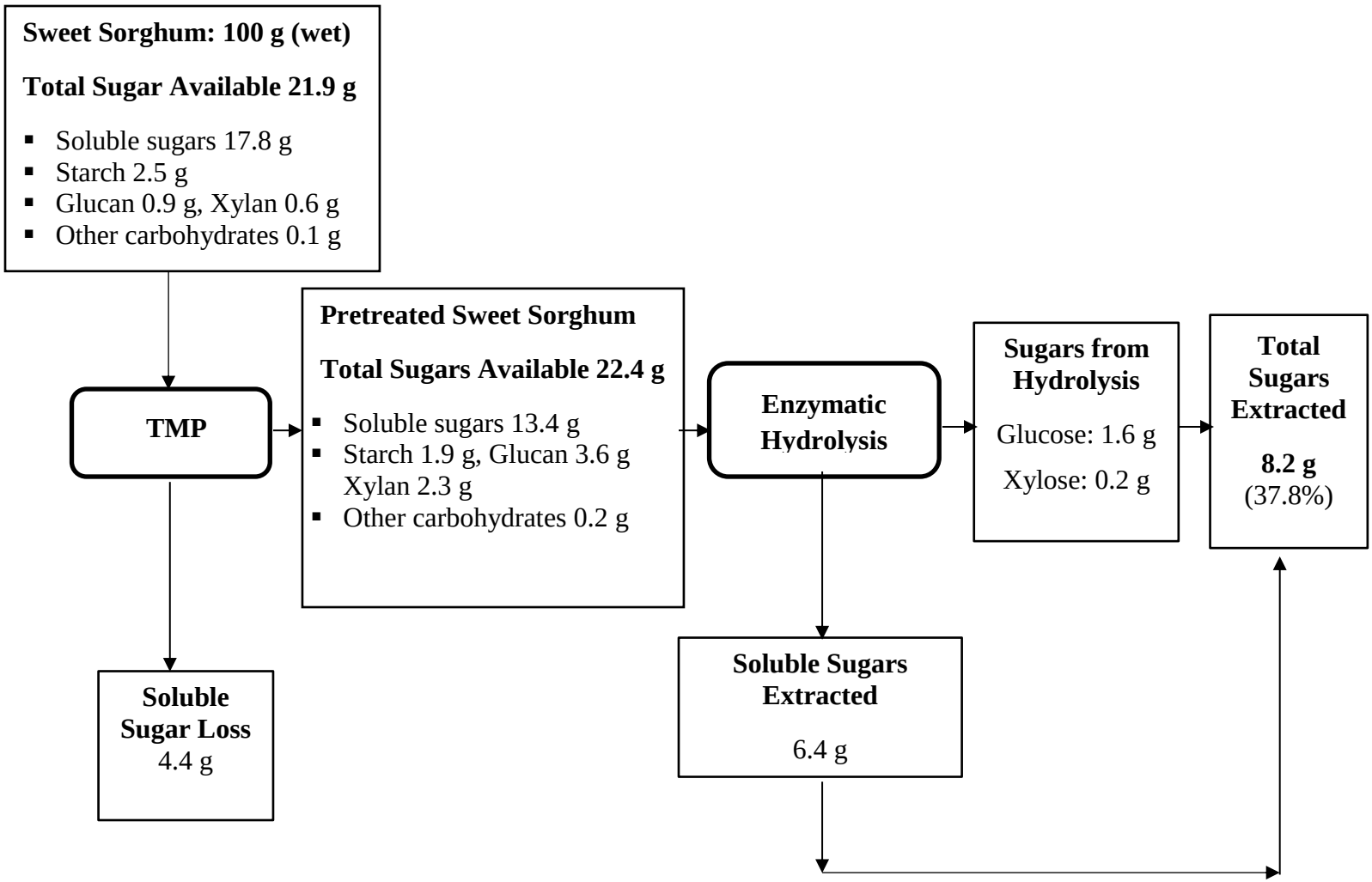

Figure 3.5 Total sugars extracted with TMP pretreatment and the subsequent enzymatic hydrolysis at 6% solid loading.

3.4.3 Effect of Preheating on Enzymatic Hydrolysis of RMP and TMP Pretreated Sorghum

Enzymatic hydrolysis of starch requires temperatures above 70°C in order for α-amylase enzyme to break down starch into oligosaccharides. For this reason, an additional preheating step was evaluated, where sorghum was preheated to 80°C for two hours, following the addition of α-amylase in order to assist the hydrolysis process. After the medium cooled down to 55°C, amyloglucosidase was then added to break down the oligosaccharides to glucose molecules. It was also hypothesized that the breakdown of starch would improve the hydrolysis of glucan and xylan that was present in sorghum. A graph showing the effect of preheating on glucose %MTY that was obtained with an α-amylase and amyloglucosidase loading of 0.5 mL is shown in Figure 3.6. In experiments with a preheating step, the glucose %MTY decreased with TMP pretreatments throughout the enzymatic hydrolysis process. This was also true in case of RMP pretreated sorghum until 24 h. However after 48 h, the glucose %MTY values obtained with and without a preheating step resulted in similar values. The reason for this phenomenon is not clear.

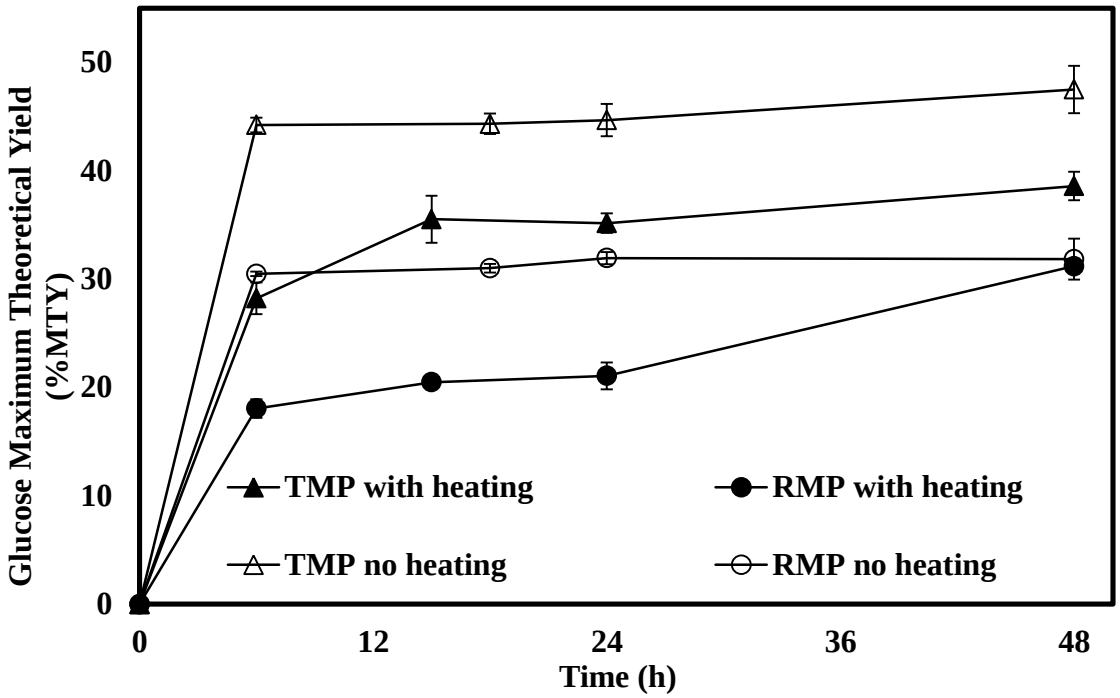

Figure 3.6 Effect of preheating on %MTY of glucose with RMP and TMP preheated sorghum at a solid loading of 6%, 0.5 mL α-amylase (38.6 IU/g db sorghum) and 0.5 mL amyloglucosidase (50 IU/g db sorghum) loading. All the flasks contained a cellulase loading of 0.5 mL/g glucan (30 FPU/g glucan) and a xylanase loading of 0.5 mL/g xylan (250 FXU/g xylan). Error bars represent standard deviation (n=3).

Table 3.5 shows the effect of preheating and increased α-amylase and amyloglucosidase loading on various yield parameters after 48 h. Increasing the α-amylase and amyloglucosidase loading improved glucan hydrolysis with all pretreatments that were tested. The greatest glucan hydrolysis was observed with TMP pretreated sorghum at 0.5 mL α-amylase and amyloglucosidase loading without preheating step as evident from the glucose %MTY (Table 3.5).

Using RMP pretreated sorghum and 0.5 mL α-amylase and amyloglucosidase

loading, a maximum % TSE value of 57.3% was achieved after 48 h, without the added preheating step, which was significantly higher compared to all other treatments tested. In the case of TMP pretreated sorghum, preheating did not affect the %TSE values with both enzyme loadings that were tested. The highest %TSE value of 37.7 was obtained after 48 h which was similar to %TSE that was obtained without the preheating step (Table 3.5). Addition of the preheating step did not improve the glucose yields and therefore further experiments were performed without a preheating step.

Table 3.5 Effect of preheating and increased α-amylase and amyloglucosidase loading on various yield parameters after 48 h with various pretreatments (n=3).

	0.1 mL each of α-amylase (10.5 IU/g db sorghum) and amyloglucosidase (5 IU/g db sorghum)			**0.5 mL each of α-amylase (52.5 IU/g db sorghum) and amyloglucosidase (25 IU/g db sorghum)**		
Treatment	Glucose %MTY **(avg ± sd)**	Xylose %MTY **(avg ± sd)**	%TSE **(avg ± sd)**	Glucose %MTY **(avg ± sd)**	Xylose %MTY **(avg ± sd)**	%TSE **(avg ± sd)**
With Preheating						
RG3G2	25.6 ± 0.8	5.0 ± 0.4	42.7 ± 1.2	31.2 ± 0.3	6.2 ± 0.5	50.8 ± 0.2
TMP	32.5 ± 1.0	8.4 ± 0.3	36.6 ± 0.5	38.6 ± 1.3	9.0 ± 1.0	37.7 ± 0.9
Untreated	26.4 ± 2.0	4.7 ± 0.8	36.0 ± 0.5	32.5 ± 0.6	4.6 ± 0.6	37.1 ± 0.2
Without Preheating						
RG3G2	28.6 ± 1.0	4.1 ± 0.5	45.2 ± 1.5	31.8 ± 0.5	6.8 ± 0.5	57.3 ± 0.2
TMP	29.5 ± 1.0	10.9 ± 0.4	35.6 ± 0.5	47.5 ± 2.2	9.5 ± 0.8	37.8 ± 1.8
Untreated	23.6 ± 0.3	4.5 ± 0.1	35.9 ± 0.4	23.9 ± 1.3	4.0 ± 0.6	36.0 ± 0.5

3.4.4 Effect of Enzyme Loading on RMP and TMP Pretreated Sorghum

The effect of enzyme loading was tested on the TMP and RG3G2 pretreated samples. A graph representing the effect of enzyme loading on the glucose %MTY values is shown in Figure 3.7. For the sake of convenience, experiments conducted at the three enzyme loadings using TMP and RG3G2 sorghum will be labeled as pretreatment type in capital letter followed by enzyme loading value (For example: RMP 0.5 denotes RG3G2 at 0.5 mL/g enzyme loading). With all enzyme loadings that were tested, the glucose concentrations reached their maximum at 6 h and stayed similar until 48 h. After 6 h, hydrolyzed glucose concentrations reached 10.4 g/L with RMP 1.0, which was equivalent to 38.9 % MTY and was significantly higher than RMP 0.5 and RMP 0.7 treatments ($p<0.05$). After 48 hours, the highest RMP treatment remained RMP 1.0, having 41% MTY. This was significantly higher than the RMP 0.5 at that time, but was not significantly different from the RMP 0.7 treatment.

After 48 h, the greatest glucose %MTY was obtained with TMP 1.0 which was significantly higher ($p<0.05$) compared to the other two enzyme levels that were tested. TMP 1.0 treatment achieved 60% glucan hydrolysis after 48 h, which was significantly higher ($p<0.05$) than the other two enzyme levels that were tested (Figure 3.7). TMP 0.5 resulted in the lowest glucan hydrolysis of 47.5%, which was due to the lower availability of enzyme to the cellulose fibers. Analysis of sorghum left after the enzymatic hydrolysis showed that the starch content in TMP and RMP pretreated sorghum was about 0.5%. This showed that about 70% starch was hydrolyzed during the enzymatic hydrolysis process.

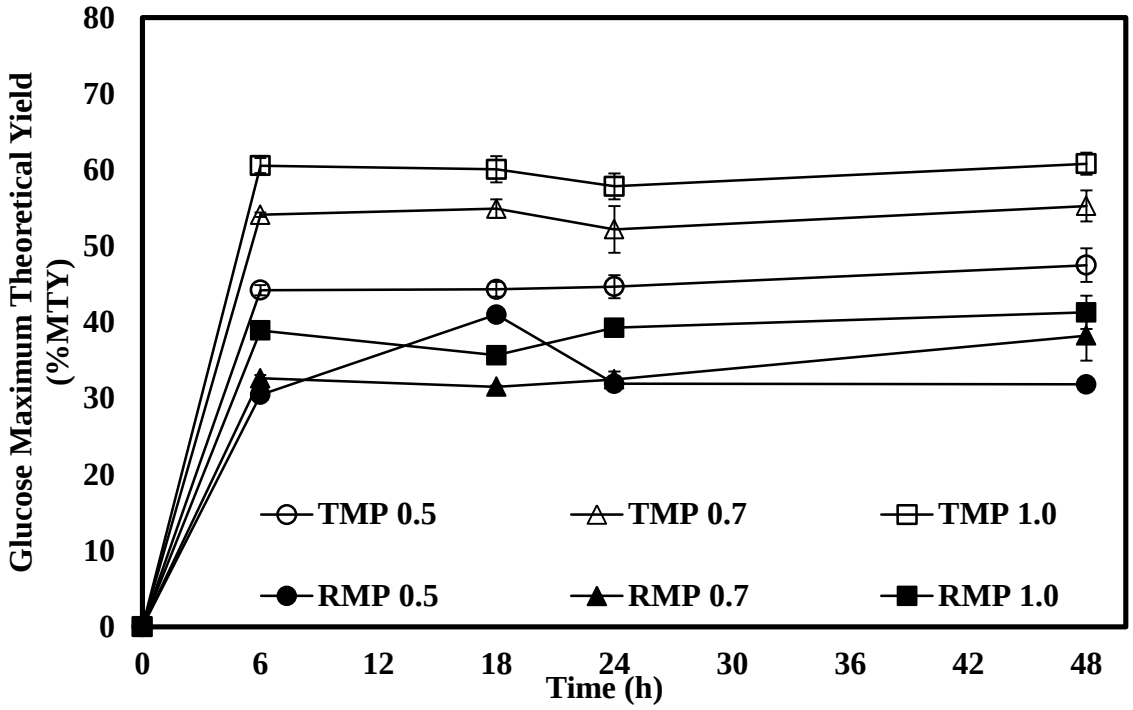

Figure 3.7 Percentage Glucose Maximum Theoretical Yield (% MTY) obtained with enzymatic hydrolysis of RMP and TMP pretreated sorghum at 6% solid loading and three different enzyme loadings of cellulase (mL/g glucan), xylanase (mL/g xylan), α-amylase and amyloglucosidase (mL): 0.5, 0.7 and 1.0. Activity of cellulase: 60 FPU/mL; activity of xylanase: 500 FXU/mL; activity of α-amylase: 634 IU/mL; acticity of amyloglucosidase: 300 IU/mL. Error bars represent standard deviation (n=3).

Table 3.6 shows the effect of enzyme loading on yield paramaters obtained after 48 h of enzymatic hydrolysis. With all the treatments that were tested, xylan hydrolysis showed an increasing trend with an increase in enzyme loadings. TMP resulted in the best xylan hydrolysis with all the enzyme loadings that were tested. However, with all the treatments that were tested, xylose yields did not reach more than 15%. This was due to the low pretreatment efficiency of the RMP and TMP processes to remove lignin and disrupt the lignocellulosic barrier. However adding solvents such as dilute acid to the

pretreatment process could have improved the xylan hydrolysis (Cao et al., 2012; Gonzalez et al., 2011; Sambusiti et al., 2013; Zhang et al., 2011).

Table 3.6 Effect of enzyme loading on yield parameters obtained with hydrolysis of RMP and TMP pretreated sorghum at 6% solids. All values are obtained after 48 h (n=3).

Pretreatment	Enzyme loading[1]	% MTY Glucose (avg ± sd)[2]	% MTY Xylose (avg ± sd)
RMP	0.5	31.8 ± 0.5^{e}	6.8 ± 0.5^{d}
	0.7	38.2 ± 3.3^{d}	8.7 ± 0.3^{c}
	1.0	41.3 ± 2.1^{d}	9.9 ± 0.2^{b}
TMP	0.5	47.5 ± 2.2^{c}	$9.5 \pm 0.8^{b,c}$
	0.7	55.3 ± 2.0^{b}	11.6 ± 0.6^{a}
	1.0	60.8 ± 1.5^{a}	12.1 ± 0.2^{a}

Activity of cellulase: 60 FPU/mL; activity of xylanase: 500 FXU/mL; activity of α-amylase: 634 IU/mL; acticity of amyloglucosidase: 300 IU/mL.

[1] Enzyme loading = mL/g glucan of cellulase, mL/g xylan of xylanse, mL α-amylase and amyloglucosidase.

[2] Average ± standard deviation

3.4.5 Effect of Enzyme Loading on RMP and TMP Pretreated Sorghum at 12% Solids

In order to evaluate the effect of increased solids content, enzyme loading was also studied at 12% solid loading. A graph representing the effect of enzyme loading on RMP and TMP pretreated sorghum at 12% solid loading is shown in Figure 3.8. After 12 h, the highest glucose yield of 43.3% MTY was achieved with TMP 1.0, which was significantly higher than the other two enzyme levels of enzymatic hydrolysis. Among the RMP pretreated samples at 12 h, the highest glucose yield of 37.3% MTY was achieved with RMP 1.0, which was significantly higher than the other enzyme levels that

were tested. However, there was no significant difference between glucose concentrations that were obtained with RMP 0.5 and RMP 0.7 treatments (P>0.05).

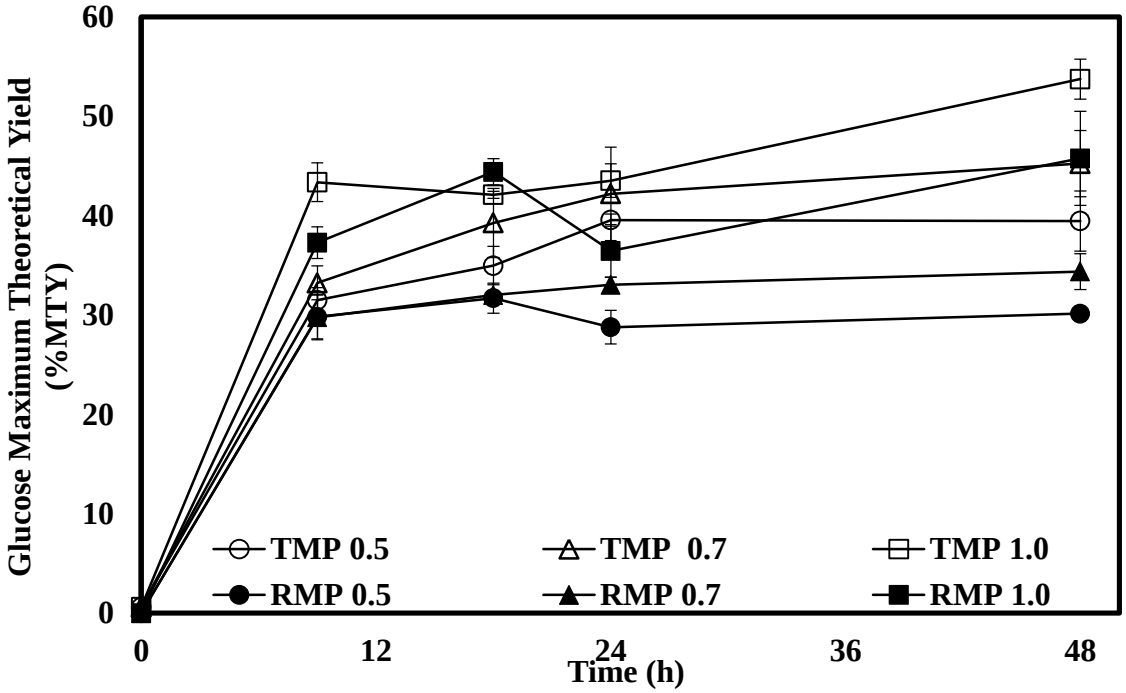

Figure 3.8 Percentage Glucose Maximum Theoretical Yield (% MTY) obtained with enzymatic hydrolysis of RMP and TMP pretreated sorghum at 12% solid loading and three different enzyme loadings of cellulase (mL/g glucan), xylanase (mL/g xylan), α-amylase and amyloglucosidase (mL):) 0.5, 0.7 and 1.0. Activity of cellulase: 60 FPU/mL; activity of xylanase: 500 FXU/mL; activity of α-amylase: 634 IU/mL; activity of amyloglucosidase: 300 IU/mL. Error bars represent standard deviation (n=3).

Table 3.7 shows the effect of enzyme loading on glucose and xylose %MTY obtained after 48 h with TMP and RMP pretreatments. As shown in Table 3.7, After 48 h, the greatest % glucose MTY of 53.8% was achieved with TMP 1.0 treatment, which was significantly higher compared to the other two enzyme levels that were tested (P<0.05).

Table 3.7 Effect of enzyme loading on yield parameters obtained with hydrolysis of RMP and TMP pretreated sorghum at 12% solid loading after 48 hours (n=3).

Pretreatment	Enzyme loading[1]	% MTY Glucose (avg ± sd)	% MTY Xylose (avg ± sd)
RMP	0.5	30.1 ± 0.4^{e}	5.7 ± 1.2^{d}
	0.7	34.3 ± 1.8^{d}	7.3 ± 0.3^{d}
	1.0	45.7 ± 4.7^{d}	9.3 ± 0.4^{c}
TMP	0.5	39.5 ± 2.2^{c}	11.4 ± 1.3^{b}
	0.7	45.3 ± 3.3^{b}	12.1 ± 0.7^{b}
	1.0	53.8 ± 2.0^{a}	18.0 ± 1.2^{a}

[1] Enzyme loading = mL/g glucan of cellulase, mL/g xylan of xylanse, mL α-amylase and amyloglucosidase. Activity of cellulase: 60 FPU/mL; activity of xylanase: 500 FXU/mL; activity of α-amylase: 634 IU/mL; acticity of amyloglucosidase: 300 IU/mL.

Addition of Xylanase to the RMP pretreated solids resulted in the hydrolysis of the xylan fraction with all the three enzyme levels that were tested. After 48 h of hydrolysis, the final xylose yields reached 9.3% with RMP 1.0, which was the greatest among the three enzyme levels that were tested with RMP pretreated sorghum (Table 3.7). After 48 h, the greatest xylose yield of 18% was noticed in TMP 1.0, which was significantly higher compared to the all other treatments that were tested. However, there was no significant difference in xylose yields that were obtained with TMP 0.5 and TMP 0.7. The low xylan hydrolysis in the current study could have been due to the low severity of the TMP pretreatment and the presence of lignin in sorghum. Moreover, the high crystallinity of the cellulose fraction could have reduced the access of xylan to the enzyme mixture, thereby preventing the hydrolysis of xylan to xylose (Gonzalez et al., 2011; Xu & Springfield, 2001; Zhao et al., 2004).

3.4.6 Discussion

In the current study, enzymatic hydrolysis of RMP and TMP pretreated sorghum resulted in glucose MTY values of 41.3 and 60.8 % respectively after 48 h. The highest glucose %MTY values were obtained with experiments that were conducted at the highest enzyme loadings. The TMP pretreated sorghum had better fiber development than the RMP pretreated sorghum and resulted in about 32% higher glucose yield. The results in the current study are similar to those obtained by Gonzalez et al. (2011). In that study, sweet sorghum bagasse was pretreated using TMP and the effect of enzyme loading was evaluated at 5% (w/v) solid loading. In this study, sweet sorghum was subjected to TMP pretreatment at two temperatures and the effect of enzyme loading was tested. The authors achieved 46 and 63% glucan hydrolysis with sorghum that was pretreated at 160 and 170°C, respectively at an enzyme loading of 20 FPU/g db sorghum. However, the authors achieved higher glucan hydrolysis with corn stover that was also tested. Moreover, soaking wheat straw in acetic acid and combining it with the TMP process significantly improved glucan hydrolysis (Gonzalez et al., 2011). The glucose and xylose yields in the current study could be improved by combining the RMP and TMP pretreatments with a chemical pretreatment such as a dilute acid or a dilute alkali pretreatment, which would result in a decreased crystallinity of cellulose, partial removal of xylose and lignin (Cao et al., 2012; Gonzalez et al., 2011; Sambusiti et al., 2013; Zhang et al., 2011).

The effect of solid loading on the glucan hydrolysis is shown in Figures 3.7 and 3.8. Increasing the solid loading from 6 to 12% with TMP pretreated solids, showed a similar hydrolysis rate throughout the process (Figures 3.7 and 3.8). With all the three

enzyme loadings and pretreated solids that were tested, the highest glucose %MTY was obtained at 6% solid loading. This is due to the better mixing efficiency that is seen at lower solid loadings. However, the difference in the yields was very small. In the case of RMP pretreated sorghum, the xylose %MTY values showed a similar decreasing trend with an increase in solid loading from 6 to 12%. With TMP pretreated sorghum, there was an improved xylose %MTY with an increase in solid loading (Tables 3.6 and 3.7). The reason for this phenomenon is not clear. It could be due to the variability in the sample itself. However the Xylose MTY values obtained at 12% solid loading were still less than 20%.

With the increased solid loadings, the soluble sugar yields decreased when solid loading was increased from 6 to 12%. With an increase in solid loading from 6 to 12%, there was a 20 to 25% reduction in soluble sugar extracted with both RMP and TMP pretreated sorghum. This resulted in an overall decrease in %TSE values. The mass transfer limitations in combination with the lack of concentration gradient would be the reason for the lower recovery of sugars from sorghum.

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CHAPTER IV

EFFECT OF ALKALI SOAKING, EXTRUSION AND ALKALI EXTRUSION OF SWEET SORGHUM BAGASSE ON ENZYMATIC HYDROLYSIS AND FERMENTABLE SUGAR PRODUCTION

4.1 Abstract

The use of extrusion, alkali soaking, and alkali extrusion were evaluated as pretreatment options for recovery of carbohydrates from sweet sorghum. Sweet sorghum (*Sorghum bicolor* L.) was chopped using a Seydelman bowl chopper to a particle size of 35 ± 17 mm and was then extracted at 70°C using water at a liquid to solid ratio of 4:1 to remove the soluble sugars present in sorghum. The bagasse was then dried and milled using a Fitzmill comminutor fitted with a 0.8 mm screen. The milled sorghum was then pretreated in a twin-screw extruder at two temperatures (80 and 110°C) with and without the presence of alkali (1.4 M NaOH). It was found that alkali extrusion pretreated sorghum resulted in the best glucose and xylose yields of 62 and 47%, respectively. The temperature of extrusion did not affect glucose yields significantly when sorghum was extruded without alkali. Extrusion without alkali resulted in the lowest glucose and xylose yields, with extrusion at 80°C being the pretreatment condition that resulted in the

lowest glucose and xylose yields of 45 and 11% respectively. Sorghum that was soaked in 1.4 M NaOH and not extruded resulted in glucose and xylose yields that were significantly higher than those that were obtained with extrusion alone. To evaluate the effect of alkali soaking as a pretreatment, 3 levels of alkali (2.7, 3.5 and 4.2 M) were tested at 3 soaking conditions (25°C for 12 h, 55°C for 6 h and 90°C for 1 h). A solid loading of 40% (w/v) was used for the alkali soaking pretreatment. It was found that when temperatures of 55 or 90°C were used, partial lignin removal was noticed. Enzymatic hydrolysis of alkali soaked sorghum resulted in glucose yields over 88% with all the treatments that were tested. The highest xylose yields were noticed with sorghum that was soaked in 3.5 M NaOH at 25°C for 12 h. The study demonstrated that alkali pretreatment can be performed at a solid state instead of the conventional submerged state and still yield great enzymatic hydrolysis results. Furthermore, no washing step was used in this study, which simplifies the overall process.

4.2 Introduction

The exploitation of lignocellulosic feedstocks for their conversion to liquid fuels and chemicals has been a topic of interest around the world. However, the enzymatic conversion of structural carbohydrates in lignocellulosic biomass to fermentable carbohydrates is challenging as these structural carbohydrates are closely associated with lignin (Liu et al., 2013). Moreover, the crystallinity of cellulose, accessible surface area, and degree of polymerization also affect the enzymatic hydrolysis process (Alvira et al., 2010; Liu et al., 2013). Several studies have been performed to evaluate pretreatment processes that could overcome the challenges associated with enzymatic conversion of structural carbohydrates present in the biomass. Technologies such as dilute acid

pretreatment (Banerji et al., 2013), hot water pretreatment (Shen et al., 2012; Shen et al., 2011), ammonia fiber explosion (AFEX) (Li et al., 2010) and ionic liquid pretreatment(da Silva et al., 2013) have been studied in depth and some of them have been commercialized. Extrusion pretreatment is a physicochemical pretreatment method in which biomass is subjected to heat, compression and shear forces that lead to physical disruption and chemical modification of biomass (Liu et al., 2013; Wu et al., 2011). Several studies have been reported on the use of extrusion technology as a pretreatment option. Karunanithy and Muthukumarappan (2011a, 2011b) used a single screw extruder for the pretreatment of switchgrass and prairie cord grass. In another study Lamsal et al., (2010) used a twin screw extruder for the pretreatment of soy hulls. Very few studies have been reported on the use of extrusion involving sweet sorghum as a feedstock. In a study conducted by Heredia-Olea et al. (2013), sorghum bagasse was extruded at two different temperatures (50 and 100°C), two moisture contents (30 and 50%) and two different screw speeds (100 and 200 rpm). The pretreated sorghum bagasse was then subjected to enzymatic hydrolysis and the hydrolysates were fermented to ethanol via fermentation. The authors reported 70% conversion of structural carbohydrates to fermentable sugars (Heredia-Olea et al., 2013). Several studies have also been reported on the use of alkali as a pretreatment aid during the extrusion process (Kang et al., 2013; Karunanithy & Muthukumarappan, 2011a; Liu et al., 2013). However, there have been no studies reported on the alkali extrusion of sweet sorghum.

This study focuses on the effect of extrusion and alkaline extrusion on the enzymatic digestibility of sweet sorghum. In addition, the effect of alkali soaking alone on the enzymatic digestibility of sorghum was investigated.

4.3 Materials and Methods

4.3.1 Raw Material

Sweet sorghum (*Sorghum bicolor* L.) was harvested from the Oklahoma State University agronomy station and the stalks were chopped in a Seydelman bowl chopper (K64, Seydelmann, Stuggart, Germany). The chopped sorghum was then subjected to a three stage batch diffusion process in which sorghum was soaked in water at a L/S ratio of 4:1 at 70°C for 15 min. The liquid portion was then separated from the solids using a cheese cloth. This diffusion process was repeated 2 more times. The diffused solids were then dried at 85°C for 1 h to kill the enzymes present in sorghum and further dried at 60°C for 15 h to remove the moisture.

The dried sorghum was then milled using a Fitzmill Comminutor (Model No. DAS06,. The Fitzpatrick Company, USA) with a 0.8 mm screen. The particle size distribution of the milled sorghum was determined using a sieve shaker, and is shown in Figure 4.1. A total of seven 8" sieve (VWR, USA) sizes were selected to give a broad spectrum of particle size ranging from 0.85 mm to 0.01 mm (pan). It was found that about 41% of the milled sorghum had particle size between 0.42 and 0.15 mm, and 27% of the particles had a particle size between 0.5 and 0.21 mm. Less than 5% of the milled sorghum had a particle size of 0.5 mm or more (Figure 4.1). The milled sorghum was placed in freezer bags and stored in the freezer until further use.

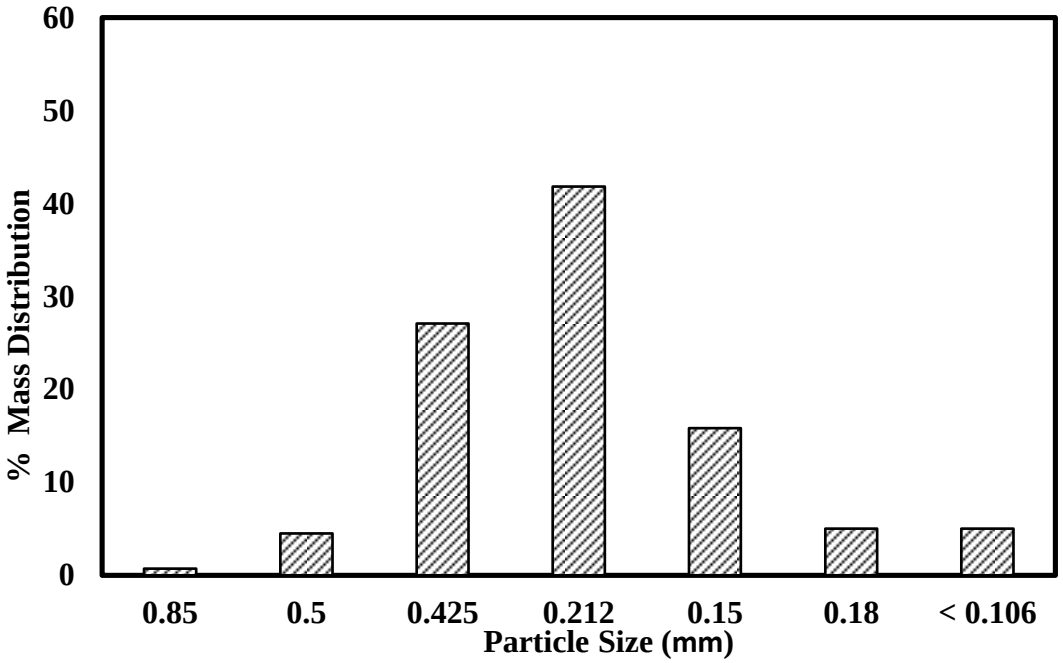

Figure 4.1 Particle size distribution of milled sorghum obtained after sieving.

4.3.2 Extrusion Pretreatment of Sweet Sorghum

Extrusion pretreatment of sorghum bagasse was conducted at the Extrusion Laboratory in the Grain Science Department at Kansas State University, Manhattan, Kansas. A laboratory scale twin-screw extruder (Micro-18, American Leistritz, Somerville, NJ) with a six-head configuration, screw diameter of 18mm, L/D ratio of 30:1 and 6.3 mm circular die opening was used for extrusion pretreatment of sorghum. The screw configuration used for the extrusion process is shown in Figure 4.2. Extrusion was performed at a screw speed of 875 RPM, and a feeder screw speed rate of 1.1 Kg/h. A 2X2 factorial design was used to evaluate the effect of extrusion and alkali extrusion pretreatment on enzymatic hydrolysis of sorghum. Two temperatures (80 and 110°C) at two alkali concentrations (0 and 4% g/g db NaOH) were evaluated. Thus a total of 4 different pretreatment conditions were tested. The concentration of alkali based on 4%

g/g db sorghum was 1.3 M. Prior to extrusion, sorghum was dried (as described previously) and then reconstituted with water to adjust the solid content to 40% (w/v). For alkali extrusion experiments, the amount of water required to adjust the sorghum's moisture content to 60 % was measured. To the measured water, an appropriate amount of NaOH was added and mixed well to get alkali solutions that were equivalent to 1.3 M. The alkali solution was then added to the dried sorghum and allowed to sit for 12 h at room temperature prior to extrusion. After pretreatment, sorghum was placed in freezer bags and stored in the freezer until further use.

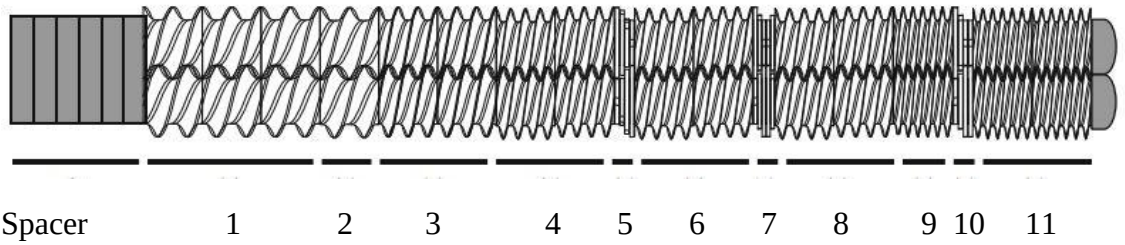

Figure 4.2 Screw configuration and barrel temperature profiles for laboratory scale extruder used in the experiments. Screw Profile: Spacer (6x5 mm); Die opening 6.3 mm. 1=FSE, 30-90; 2=FSE, 30-30; 3=FSE, 20-60; 4=FSE, 15-60; 5=FKB, 4-16-30; 6=FSE, 15-60; 7=FKB, 5-16-45; 8=FSE, 15-60; 9=FSE, 10-30; 10=FKB, 5-16-45; 11=FSE, 10-60. FSE= Forward Conveying Screw Element; FKB= Forward Kneading Block. Numbers on screw elements FSE: Pitch (mm) - Element length (mm), numbers on kneading blocks FKB: Number of disks - Total block length (mm) - Staggering angle of disks.

4.3.3 Alkali Soaking Pretreatment

Sorghum bagasse was used as a raw material to perform the effect of alkali soaking experiments. The amount of water required to adjust the sweet sorghum's moisture content to 60 % was measured. To the measured DI water, an appropriate amount of NaOH was added and mixed well to final alkali concentrations of 2.7, 3.5 and 4.2 M which were equivalent to alkali loading of 8, 10 and 12 % g/g db sorghum respectively. Sorghum that was previously oven dried and milled was then mixed with

these alkali solutions. Three different storage conditions (25°C for 12 h, 55°C for 6 h and 90°C for 1 h) were evaluated for the three alkali loadings that were used. The mixtures were placed in freezer bags and stored at three different conditions: (a) room temperature for 12 h, (b) 55°C for 6 h and (c) 90°C for 1 h. The pretreated material was then stored in the freezer and the composition was determined using the procedures described below.

4.3.4 Compositional Analysis of Pretreated Sorghum

The pretreated sorghum was dried at 104°C to remove the moisture and was further used to determine the extractives and structural carbohydrates using NREL procedures. About 0.3 g of sorghum was used to determine the amount of extractives. A two-step extraction process was performed using an NREL procedure (Sluiter et al., 2005) prior to determination of structural carbohydrates and lignin in biomass. Automatic extraction by ethanol followed by water was conducted using an ASE® 300 system (Dionex Corporation, Sunnyvale, CA, USA). The operating parameters for both steps were 1500 psi at 100°C, 150% flush volume, 7 min static time, 2 min purge time, and four static cycles. All extractions were conducted in triplicate in 33 mL extraction cells using 95% ethanol and distilled water for ethanol and water extractions, respectively. Extracted sorghum solids were air dried for at least 24 h prior to use in the subsequent analysis of structural carbohydrates and lignin. For sorghum that was pretreated using an alkali, a modified procedure was used in which a portion of sorghum was washed with DI water until the pH of wash water reached 7. The washed sorghum was then dried and used for the determination of extractives as described above.

For all the pretreated samples, analysis of structural carbohydrates was performed

using a two-step acid hydrolysis procedure as described in Sluiter et al. (2004). Absorbance for acid soluble lignin was taken at 205 nm using a UV–Vis spectrophotometer (Beckman DU 520, Beckman Coulter, Inc., Pasadena, CA). The 205 nm wavelength was chosen based on work done by Thammasouk et al. (1997). A portion of the acid hydrolyzate was then neutralized using $CaCO_3$, filtered using a 0.2µ nylon filter and analyzed for sugars using HPLC.

4.3.5 Enzymatic Hydrolysis of Pretreated Sorghum

Enzymatic Hydrolysis of Sorghum Extruded at 110°C

Sweet sorghum that was extruded at 110°C with and without alkali was used for determining the effect of enzyme loading on enzymatic hydrolysis using NREL procedures. Three different levels of cellulase (Accelerase® 1500 with a measured cellulase activity of 50 FPU/mL) and xylanase (Accelerase® 1500 XC with an activity of 3150 Acid Birchwood Xylanase Units /mL or AXU/mL) were used to determine the effect of enzyme loading on enzymatic hydrolysis. Cellulase levels used were 0.23, 0.34 and 0.45 mL/g db sorghum and xylanase levels used were 0.14, 0.22 and 0.31 mL/g db sorghum. Cellulase activity was measured using a cellulose filter paper assay as described in Adney & Baker (2008). All enzymatic hydrolysis experiments were conducted in triplicate in 250 mL baffled flasks sealed with a rubber stopper fitted with a one-way air valve (Check valve, Fischer Scientific, Pittsburgh, PA) to avoid contamination by microbes. Each hydrolysis flask contained 10 mL of 0.5 M sodium citrate buffer at a pH of 5.0 and 6% solids (w/v). For sorghum that was extruded with 4% alkali, the pH was adjusted with 5 M HCl. To all the hydrolysis flasks, 100 µL of 0.1%

sodium azide was added to prevent contamination by microbes. The total mass of the flask was 100 g. Control flasks that contained the same contents as experimental flasks excluding enzymes were maintained to account for the sugars that are diffused during the hydrolysis process. All flasks were incubated at 55°C, 150 rpm on an incubating orbital shaker (C25 incubator shaker, New Brunswik Scientific, Edison, NJ). Samples (1.5 mL each) were collected at 0, 6, 12, 24, 48 and 96 h. The samples were then centrifuged at 15000 rpm for 10 min and the supernatant was filtered through a 0.2 µ nylon filter. The filtrate was stored in a freezer until further analysis using an HPLC.

Enzymatic Hydrolysis of Sorghum Extruded at 80°C

Enzyme loading that resulted in the highest glucose and xylose yields was chosen to be used in enzymatic hydrolysis of sorghum that was extruded at 80°C. All the enzymatic hydrolysis experiments were performed as described above except the enzyme loading was set at 0.45 mL cellulase/g db sorghum (22.5 FPU/g db sorghum) and 0.31 mL xylanase/g db sorghum (976.5 AXU/g db sorghum). Samples were collected at 0, 6, 10, 24, 48, 72 and 96 h. The samples were then centrifuged at 15000 rpm for 10 min and the supernatant was filtered through a 0.2 µ nylon filter. The filtrate was stored in a freezer until further analysis using an HPLC.

Enzymatic Hydrolysis of Alkali Soaked Sorghum

Sorghum that was soaked with alkali at various concentrations and temperatures was used to perform enzymatic hydrolysis at 55°C, a solid loading of 6 % (w/v) and an enzyme loading of 0.45 mL cellulase/g db sorghum and 0.31 mL xylanase /g db sorghum was used. The experiments were performed as described previously. Since NaOH was

used in the pretreatment process, 5 N H_2SO_4 was used to adjust the pH of the flasks to 5.0. Samples were collected at 0, 6, 12, 24, 48 and 96 h. The samples were then centrifuged at 15000 rpm for 10 min and the supernatant was filtered through a 0.2 µ nylon filter. The filtrate was stored in a freezer until further analysis using an HPLC.

Yield Calculations

Maximum theoretical yield (MTY) was calculated using the following equation

$$\%\ glucose\ MTY = \frac{glucose\ concentration\ measured}{(fg\ [biomass] * 1.111)} X100 \quad \text{.....Eqn 4.1}$$

Where *fg* is glucan fraction of dry biomass (g/g), [biomass] is dry biomass concentration (g/L), and 1.111 is the conversion factor for glucan to glucose.

$$\%\ xylose\ MTY = \frac{xylose\ concentration\ measured}{(fx\ [biomass] * 1.13)} X100 \quad \text{.....Eqn 4.2}$$

Where *fx* is xylan fraction of dry biomass (g/g), [biomass] is dry biomass concentration (g/L), and 1.13 is the conversion factor for xylan to xylose.

4.3.6 HPLC Analysis

For quantification of samples of cellobiose, glucose, xylose, sucrose, fructose, galactose, mannose and arabinose from the sorghum compositional analyses, an Aminex HPX-87P column (Bio-Rad, Sunnyvale, CA, USA) was used at 80°C with DI water as eluent flowing at 0.6 mL min^{-1}. For the analysis of inhibitors in the hydrolyzate, an Aminex HPX-87H column (Bio-Rad, Sunnyvale, CA, USA) was used at 60°C with 0.01 N H_2SO_4 as eluent flowing at 0.6 mL min-1. For both columns, a refractive index detector

(1100 series, Agilent, Santa Clara, CA) was used.

4.3.7 Statistical Analysis

An analysis of variance (ANOVA) was determined using the GLM procedure of SAS Release 9.3 (Cary, NC, USA). A Duncan's test was used to determine the statistical significance of the structural carbohydrates present in sorghum (glucan, xylan etc.) and enzymatic hydrolysis results (glucose %MTY, xylose %MTY and %TSE). Significance level was tested at $\alpha = 0.05$.

4.4 Results and Discussion

4.4.1 Compositional Analysis of Extrusion Pretreated Sorghum

Table 4.1 shows the composition of sorghum obtained after various pretreatment conditions. It was found that the composition of sorghum was not altered significantly when it was extruded at 80 or 110°C. All the structural carbohydrates were intact and did not suffer any losses due to extrusion. However, in the presence of an alkali, extrusion at 110°C resulted in a minor loss of the xylan portion. However, the losses were not significant. Sorghum that was soaked in alkali for 12 h also had similar composition compared to all other treatments. Due to the high feed rate and screw speeds that were used in the current extrusion process, there was not enough shear generated to change the composition of sorghum. Also, analysis of extruded and alkali extruded sorghum did not show any formation of inhibitory compounds such as HMF and furfural, which was evident from the HPLC analysis of the hydrolyzate obtained after 48 h. This is of great importance as their presence would inhibit the growth of microorganisms in the subsequent stage of fermentation.

Table 4.1 Composition of sorghum after several different pretreatment processes (n=3).

Treatment	Glucan (% db) (avg ± sd)[1]	Xylan (%db) (avg ± sd)	Lignin (%db) (avg ± sd)
Extrusion, 110°C	37.8 ± 1.9[a]	24.5 ± 0.3[b]	17.1 ± 0.8[c]
Extrusion, 80°C	39.7 ± 2.6[a]	23.5 ± 0.8[b]	19.6 ± 1.1[c]
Alkali Extrusion 110°C	40.8 ± 1.6[a]	23.2 ± 2.5[b]	16.9 ± 0.7[c]
Alkali Extrusion 80°C	40.1 ± 1.9[a]	25.3 ± 0.2[b]	18.1 ± 1.4[c]
1.4 M NaOH soaked 25°C	38.4 ± 1.4[a]	25.5 ± 0.6[b]	16.9 ± 0.7[c]
Untreated	40.5 ± 0.2[a]	24.2 ± 0.2[b]	17.0 ± 0.2[c]

Values in a column followed by the same letter are not significantly different.

[1]Average ± standard deviation

4.4.2 Enzymatic Hydrolysis of Sorghum Extruded With and Without Alkali

Figure 4.3 shows the glucose %MTY values obtained with enzymatic hydrolysis of sorghum that was extruded with and without alkali at a cellulase loading of 0.45 mL/g db sorghum and xylanase loading of 0.31 mL/g db sorghum. After 12 h, about 11.2 g/L of glucose that was equivalent to glucose MTY of 42.3% was obtained with sorghum that was extruded at 80°C and was significantly lower compared to the glucose MTY values that were obtained with enzymatic hydrolysis of sorghum that was extruded with alkali. However, there was no significant difference in glucose %MTY values that were obtained with sorghum that was extruded at 80 and 110°C. At 12 h, the greatest glucose %MTY was obtained with enzymatic hydrolysis of sorghum that was extruded at 110°C

with alkali.

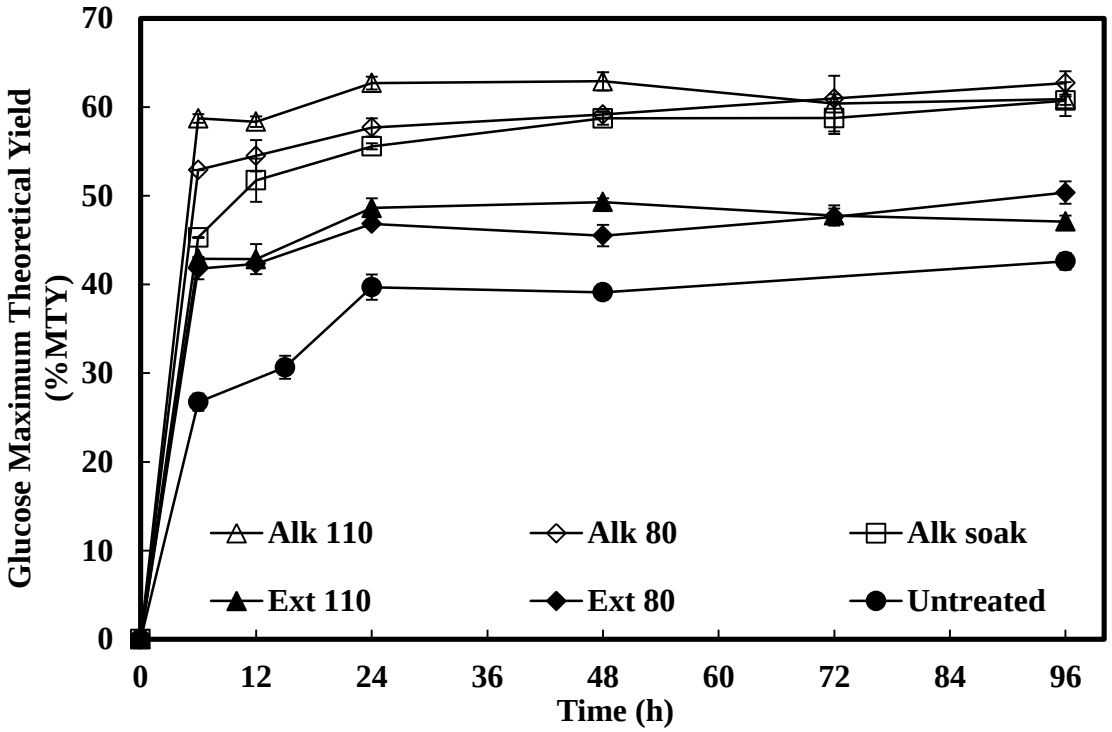

Figure 4.3 Percentage glucose Maximum Theoretical Yield (%MTY) obtained with enzymatic hydrolysis of sorghum extruded with and without alkali at 80°C and 100°C compared to untreated sorghum. Solid loading was 6% solids, cellulase loading was 0.45 mL/g db sorghum (22.5 FPU/g db sorghum) and xylanase loading was 0.31 mL/g db sorghum (976.5 AXU/mL). Ext represents the sorghum that was extruded without alkali, Alk represents the sorghum that was extruded with alkali, and Alk soak represents sorghum that was soaked in alkali but not extruded. Error bars represent standard deviation (n=3).

At 48 h, the highest glucose MTY was obtained with enzymatic hydrolysis of sorghum that was extruded with alkali at 110°C, which was significantly higher compared to all other treatments that were tested ($p<0.05$). Enzymatic hydrolysis of untreated sorghum which was used as control, resulted in a glucose MTY of 42.6% after

96 h, which was significantly lower compared to all the treatments that were tested. The glucose %MTY obtained with sorghum that was extruded without alkali at 80°C was significantly lower compared to all other extrusion treatments that were tested ($p>0.05$). After 48 h, with all the treatments that were tested, the glucose %MTY values remained similar until 96 h. Sorghum that was soaked in 4% NaOH and not extruded was also used as a control to compare the glucose %MTY values. It was found that the alkali soaked sorghum resulted in higher yields than those that were obtained with extruded sorghum without alkali and similar yields to the alkali plus extrusion treatments and (Figure 4.3).

Figure 4.4 represents the xylose %MTY obtained with enzymatic hydrolysis of sorghum that was extruded with and without alkali at a cellulase loading of 0.45 mL/g db sorghum and xylanase loading of 0.31 mL/g db sorghum. After 12 h of enzymatic hydrolysis, the highest xylose concentration of 6.1 g/L that was equivalent to a xylose MTY of 38.8 % was achieved with sorghum that was extruded at 110°C with alkali and it was significantly higher ($p<0.05$) than all other treatments that were tested. At 48 h, a xylose MTY of 47.4% was obtained with enzymatic hydrolysis of sorghum that was extruded with alkali at 110°C which was significantly higher compared to all other treatments that were tested ($p<0.05$).

Enzymatic hydrolysis of sorghum that was soaked in 1.4 M NaOH but not extruded resulted in a xylose yield of 37% after 48 h, which was significantly higher ($p<0.05$) than those that were obtained with enzymatic hydrolysis of extruded sorghum without alkali. However, alkali extrusion samples resulted in significantly higher xylose compared to just alkali soaked sorghum. Sorghum that was extruded without alkali resulted in xylose MTY values less than 20%. Furthermore, enzymatic hydrolysis of

untreated sorghum resulted in xylose yields similar to those that were obtained with sorghum that was extruded at 110°C (Figure 4.4).

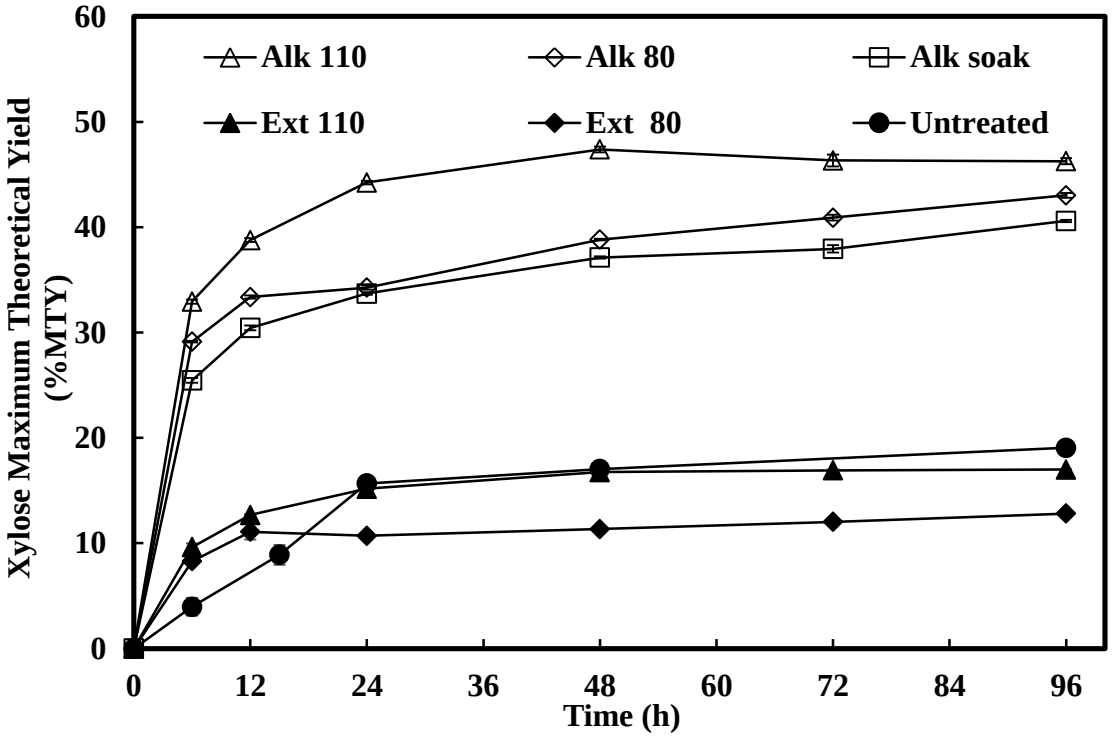

Figure 4.4 Percentage xylose Maximum Theoretical Yield (%MTY) obtained with enzymatic hydrolysis of sorghum extruded with and without alkali compared to untreated sorghum at 6% solids with cellulase loading of 0.45 mL/g db sorghum (22.5 FPU/g db sorghum) and xylanase loading of 0.31 mL/g db sorghum (976.5 AXU/g db sorghum). Ext represents the sorghum that was extruded without alkali, Alk followed by a number represents sorghum that was extruded with alkali, and Alk soak represents sorghum soaked in 4% NaOH but not extruded. Error bars represent standard deviation (n=3).

4.4.3 Effect of Enzyme Loading on Sorghum Extruded at 110°C

For the sake of convenience, the three different enzyme loadings were labeled X, Y and Z (X=cellulase 0.23, xylanase 0.14; Y=cellulase 0.34 and xylanase 0.22 and Z= cellulase 0.45, xylanase 0.31, with all enzyme levels in units of mL/g db sorghum).

Figure 4.5 shows the glucose %MTY values obtained with the enzymatic hydrolysis of sorghum extruded at 110°C at three different enzyme levels. After 12 h, a glucose MTY of 40.4 % was obtained with the lowest enzyme level, X. That was the lowest among the three enzyme levels tested, but there was no significant difference between the glucose %MTY obtained with enzyme loadings of X, Y and Z ($p<0.05$).

At 48 h, a glucose concentration of 13.3 g/L, which was equivalent to about 49% MTY, was obtained with both enzyme loadings of Y and Z and they were significantly higher ($p<0.05$) compared to the glucose %MTY obtained with the lowest enzyme loading of X. However, there was no significant difference between the glucose yields that were obtained with enzyme loadings Y and Z ($p<0.05$).

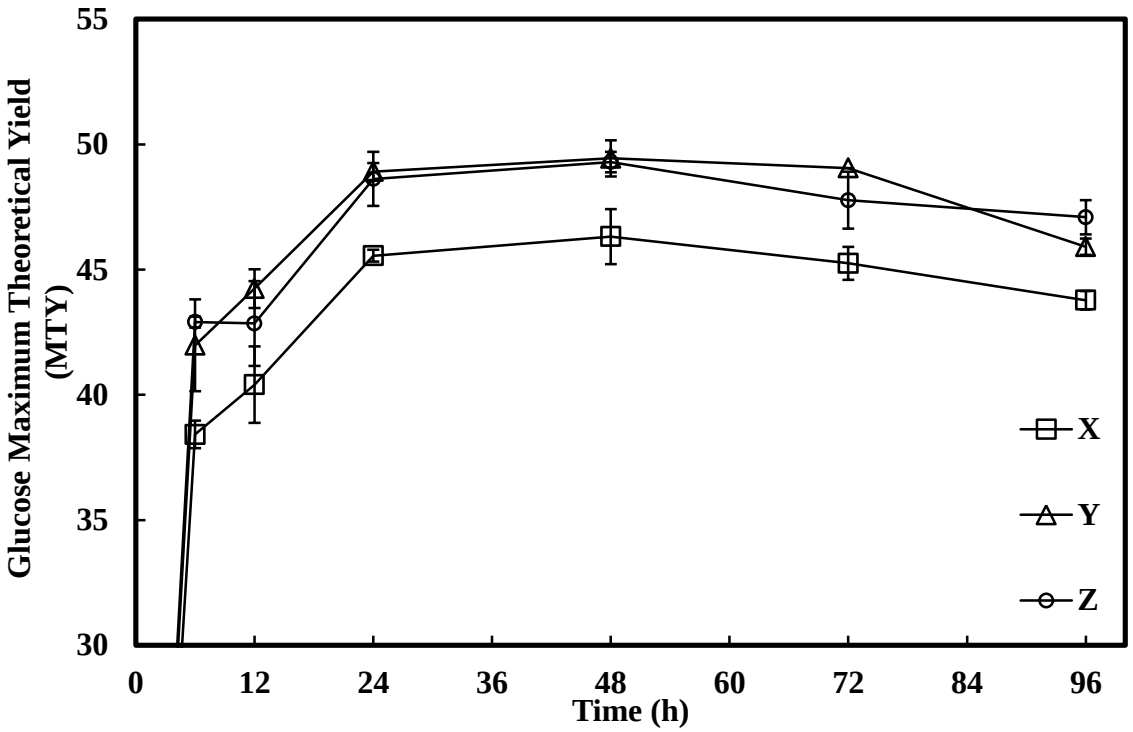

Figure 4.5 Percentage Glucose Maximum Theoretical yield (%MTY) obtained with enzymatic hydrolysis of extrusion pretreated sorghum at 110°C, 6% solid loading and three different cellulase and xylanase loadings of ; X= 0.23 and 0.14 mL (11.5 FPU and 441 AXU) (□), Y= 0.34 and 0.22 mL (17 FPU and 693 AXU) (Δ) and Z= 0.45 and 0.31 mL (22.5 FPU and 976.5 AXU) (○). All enzyme loadings were on a per gram db sorghum basis. Error bars represent standard deviation (n=3).

Figure 4.6 represents the xylose %MTY with the enzymatic hydrolysis of sorghum extruded at 110°C with three different enzyme loadings X, Y and Z. After 12 h of enzymatic hydrolysis, the highest xylose concentration of 2.1 g/L that was equivalent to 12.7 %MTY was achieved with an enzyme loading of Z, which was significantly higher ($p<0.05$) than the xylose %MTY that was obtained with sorghum that was added with enzyme loading of X. However, the xylose %MTY obtained with enzyme loadings of Y and Z were similar (Figure 4.6**)**.

After 48 h, xylose yields of 17% were obtained with both the enzyme loadings of Y and Z, and they were significantly higher ($p<0.05$) than the xylose yield of 13.5 % obtained with an enzyme loading of X. However, there was no significant difference in the xylose %MTY that was obtained with enzyme loadings of Y and Z. The xylose %MTY values remained similar after 48 h with all the enzyme levels that were tested.

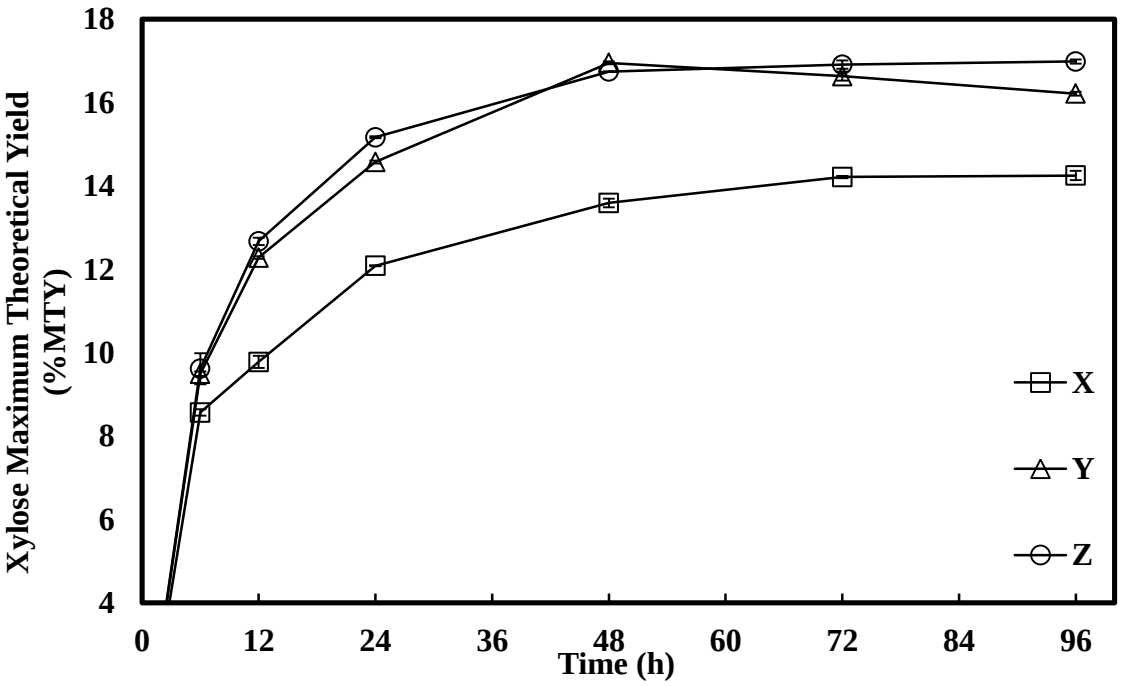

Figure 4.6 Percentage Xylose Maximum Theoretical yield (%MTY) obtained with enzymatic hydrolysis of Extrusion pretreated sorghum at 110°C, 6% solid loading and three different cellulase and xylanase loadings of ; X= 0.23 and 0.14 mL (11.5 FPU and 441 AXU) (□), Y= 0.34 and 0.22 mL (17 FPU and 693 AXU) (Δ) and Z= 0.45 and 0.31 mL (22.5 FPU and 976.5 AXU) (○). All enzyme loadings were on a per gram db sorghum basis. Error bars represent standard deviation (n=3).

4.4.4 Effect of Enzyme Loading on Alkali Soaked Sorghum Extruded at 110°C

Figure 4.7 represents the glucose %MTY values obtained with the enzymatic hydrolysis of alkali extruded sorghum at 110°C with three different enzyme loadings X, Y and Z (mL/g db sorghum). After 12 h, about 16.2 g/L of glucose that was equivalent to 58.5% glucose MTY was obtained with all enzyme levels that were tested.

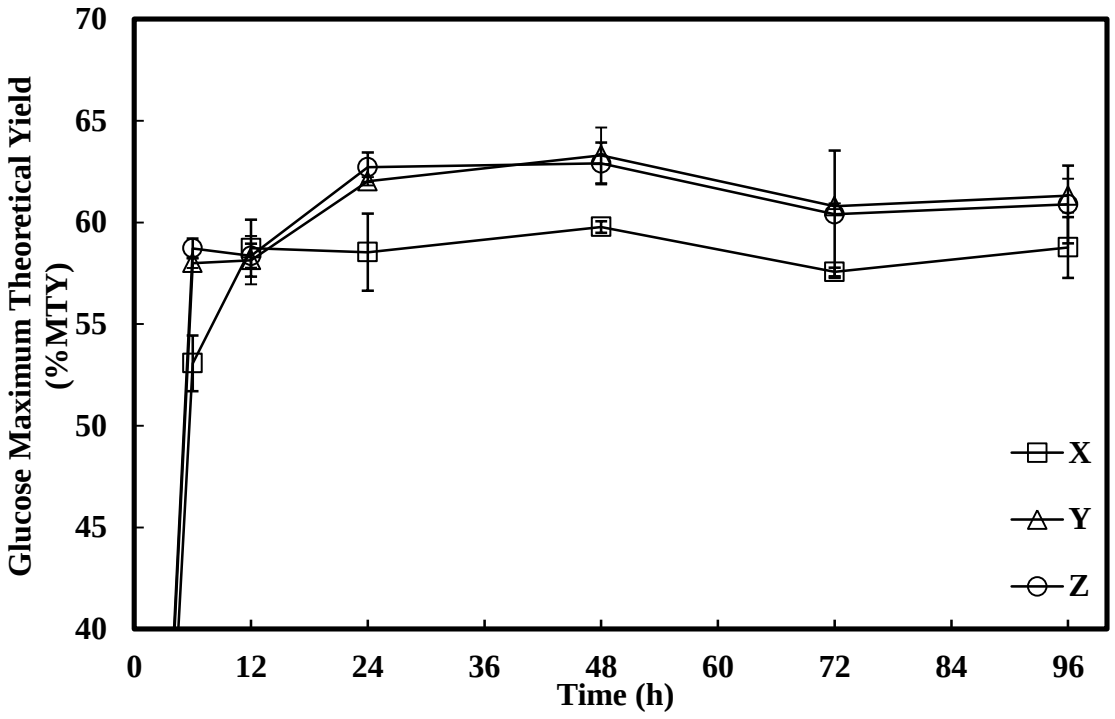

Figure 4.7 Percentage Glucose Maximum Theoretical yield (%MTY) obtained with enzymatic hydrolysis of alkali soaked sorghum extruded at 110°C, 6% solid loading and three different cellulase and xylanase loadings of ; X= 0.23 and 0.14 mL (11.5 FPU and 441 AXU) (□), Y= 0.34 and 0.22 mL (17 FPU and 693 AXU) (Δ) and Z= 0.45 and 0.31 mL (22.5 FPU and 976.5 AXU) (○).All enzyme loadings were on a per gram db sorghum basis. Error bars represent standard deviation (n=3).

After 48 h, a glucose MTY of 63% was obtained with enzyme loadings Y and Z,

which were significantly higher ($p<0.05$) than the glucose MTY that was obtained with enzyme loading X. However, the yields obtained with enzyme loadings of Y and Z were similar ($p>0.05$). After 48 h, the glucose % MTY values remained the same until 96 h.

Figure 4.8 represents the xylose %MTY with the enzymatic hydrolysis of alkali soaked sorghum extruded at 110°C with three different enzyme loadings of X, Y and Z. After 12 h, the highest xylose concentration of 6.1 g/L that was equivalent to 38.8 xylose %MTY was achieved with an enzyme loading of Z and was significantly higher compared to X. However, the xylose %MTY obtained with enzyme loadings Y and Z were similar ($p>0.05$).

After 48 h, a xylose MTY of 47.4% was achieved with an enzyme loading of Z, which was significantly higher compared to xylose yields obtained with enzyme loadings X and Y. Enzyme loading X resulted in a xylose MTY of 42.4% which was the lowest compared to all other enzyme levels that were tested. The xylose %MTY values obtained after 48 h showed that increasing the enzyme loading from X to Z resulted in about 12% improvement in xylose yields.

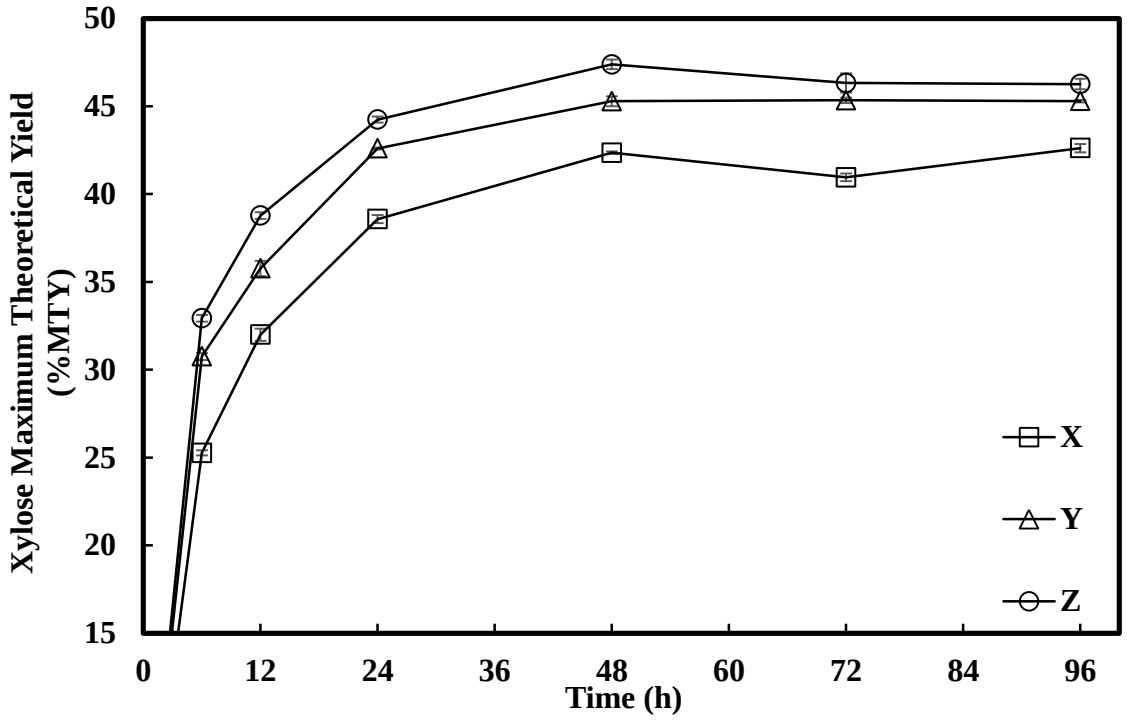

Figure 4.8 Percentage Xylose Maximum Theoretical Yield (%MTY) obtained with enzymatic hydrolysis of alkali soaked sorghum extruded at 110°C, 6% solid loading and three different cellulase and xylanase loadings of ; X= 0.23 and 0.14 mL (11.5 FPU and 441 AXU) (□), Y= 0.34 and 0.22 mL (17 FPU and 693 AXU) (Δ) and Z= 0.45 and 0.31 mL (22.5 FPU and 976.5 AXU) (○).All enzyme loadings were on a per gram db sorghum basis. Error bars represent standard deviation (n=3).

4.4.5 Composition of Alkali Soaked Sorghum Pretreated at Various Conditions

Sweet sorghum bagasse that was previously dried and milled was soaked in three different alkali concentrations (2.7, 3.5 and 4.2 M NaOH) at three different soaking conditions (25°C for 12 h, 55°C for 6 h and 90°C for 1 h) for a total of 9 different treatments. All the soaking experiments were conducted at a solid loading of 40% (w/v). Table 4.2 shows the composition of sorghum before and after pretreatment with alkali at

various conditions. It was found that the glucan content of all alkali soaked sorghum was significantly lower than the control. In general, the alkali treatments resulted in about 35% glucan, whereas the untreated sample had about 40% glucan. None of the treatments were significantly different from each other. The lowest glucan was noticed with sorghum that was treated at 55°C with an alkali concentration of 4.2 M.

The addition of alkali and soaking at room temperature did not show a decrease in lignin content compared to the untreated control. However, increasing the pretreatment temperature to 55 or 90°C resulted in a loss of lignin up to 15%. This is of great importance as the lignin interferes with the enzymatic hydrolysis process. Xylan content did not change significantly compared to the control with all the alkali concentrations and pretreatment conditions that were tested. It was also noticed that the pretreatment of sorghum with alkali at all conditions did not form any inhibitory compounds such as HMF and Furfural. However, about 0.3 to 0.6 g/L of acetic acid and 0.2 to 0.4 g/L of formic acid were detected with all the alkali soaking treatments that were tested. The concentration of these compounds is likely too low to inhibit the growth of microorganisms during the fermentation step. Moreover it has been reported that small amounts of acetic acid and formic acid in fermentation media actually resulted in increased ethanol production (Almeida et al., 2007; Palmqvist & Hahn-Hägerdal, 2000).

Table 4.2 Composition of sorghum after soaking in alkali at various conditions (n=3).

NaOH conc. (M)	Temperature (°C), Soaking time (h)	Glucan (% db) (avg ± sd)[2]	Xylan (%db) (avg ± sd)	Lignin (%db) (avg ± sd)
2.7	25, 12	35.6 ± 1.9[b]	25.4 ± 2.5[a]	17.2 ± 1.5[a,b]
	55, 6	36.0 ± 1.1[b]	25.3 ± 2.7[a]	14.2 ± 0.3[c]
	90,1	35.4 ± 0.9[b]	25.4 ± 2.3[a]	14.5 ± 0.0[c]
3.5	25, 12	35.7 ± 1.9[b]	23.8 ± 0.8[a]	17.0 ± 0.9[a,b]
	55, 6	36.0 ± 1.4[b]	25.3 ± 1.1[a]	17.4 ± 0.1[a,b]
	90,1	35.4 ± 2.3[b]	25.4 ± 1.0[a]	14.5 ± 1.4[a,b]
4.2	25, 12	35.5 ± 1.1[b]	25.4 ± 2.0[a]	18.4 ± 1.3[a]
	55, 6	33.4 ± 1.0[b]	25.6 ± 3.3[a]	15.6 ± 0.1[b,c]
	90,1	35.0 ± 1.9[b]	25.2 ± 2.4[a]	15.6 ± 1.6[b,c]
1.4	25, 12	38.4 ± 1.4[b]	25.5 ± 0.6[a]	16.9 ± 0.7[a,b]
NT[1]	NA	40.5 ± 0.2[a]	24.2 ± 0.2[a]	17.0 ± 0.2[a,b]

[1]= no pretreatment

[2] Average ± standard deviation

Values in the columns with the same letters are not significantly different

4.4.6 Effect of Alkali Concentration on Enzymatic Hydrolysis of Sorghum Pretreated at Room Temperature

Sweet sorghum that was soaked at room temperature for 12 h in three different concentrations of NaOH (2.7, 3.5 and 4.2 M) was used for enzymatic hydrolysis experiments. Figure 4.9 represents the glucose %MTY obtained with sorghum treated with different concentrations of NaOH. After 12 h, a glucose MTY of 87.6 % was obtained with the enzymatic hydrolysis of sorghum pretreated with 4.2 M NaOH, which was higher than the two lower concentrations of NaOH used; however, the differences were not significant ($p>0.05$).

At 48 h, a glucose MTY of 97.2 % was obtained with enzymatic hydrolysis of sorghum pretreated with 3.5 M NaOH, which was significantly higher compared to glucose %MTY that was obtained with the sorghum pretreated with 2.7 M NaOH. However, there was no significant difference between the glucose %MTY values that were obtained with 3.5 and 4.5 M NaOH pretreatments at 48 h. With all the treatments that were tested, glucose concentrations reached their maximum after 48 h and stayed at similar until levels 96 h. After 96 h, no significant difference in glucose MTY values was observed between all the NaOH levels that were tested.

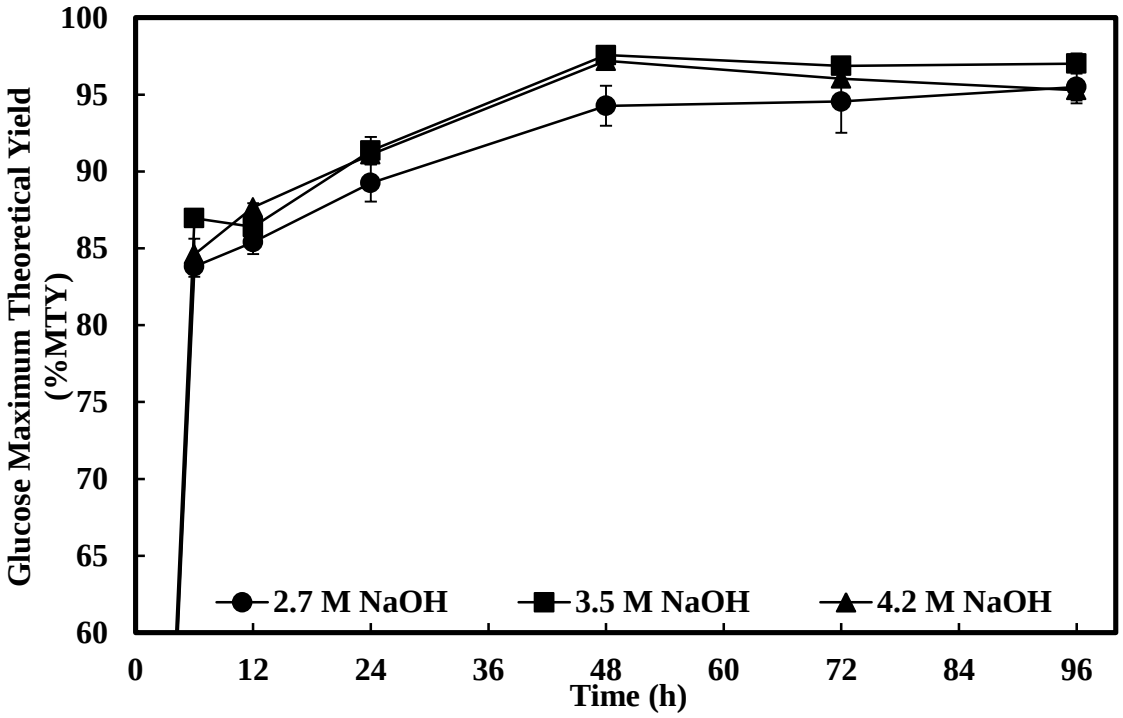

Figure 4.9 Glucose Maximum Theoretical Yield (%MTY) obtained with enzymatic hydrolysis of sorghum pretreated with various concentrations of NaOH at room temperature for 12 h, 6% solid loading, cellulase loading of 0.45 mL/g db sorghum (22.5 FPU/g db sorghum) and xylanase loading of 0.31 mL/g db sorghum (976.5 AXU/g db sorghum). Error bars represent standard deviation (n=3).

Figure 4.10 represents the xylose %MTY obtained with sorghum treated with different concentrations of NaOH at room temperature for 12 h. As shown in Figure 4.10, increasing the alkali concentration during the pretreatment resulted in an increased xylose production during the enzymatic hydrolysis. After 12 h, 61.4 % xylose MTY was obtained with enzymatic hydrolysis of sorghum that was pretreated with 4.2 M NaOH, and was significantly higher ($p<0.05$) compared to the other two treatments that were tested. Sorghum that was soaked in 2.7 M NaOH resulted in the lowest % xylose MTY of 54.4, which was significantly lower than the other treatments ($p>0.05$).

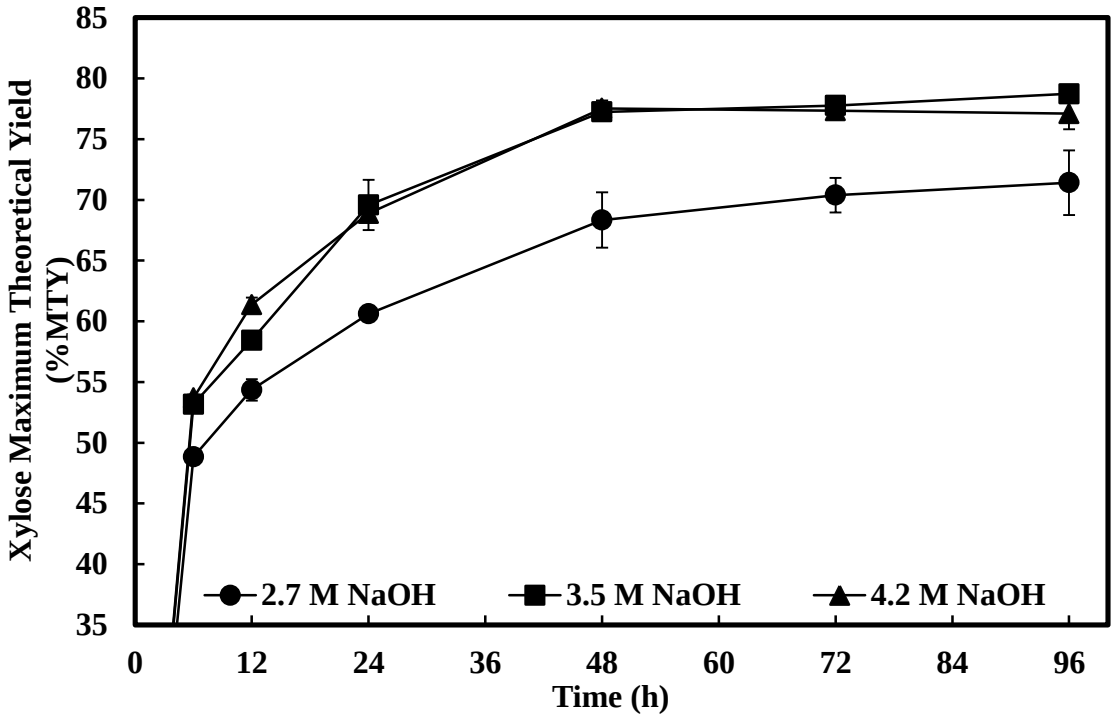

Figure 4.10 Xylose Maximum Theoretical Yield (%MTY) obtained with enzymatic hydrolysis of sorghum pretreated with various concentrations of NaOH at room temperature for 12 h, 6% solid loading, cellulase loading of 0.45 mL/g db sorghum (22.5 FPU/g db sorghum) and xylanase loading of 0.31 mL/g db sorghum (976.5 AXU/g db sorghum). Error bars represent standard deviation (n=3).

After 48 h, the xylose concentrations reached their maximum and stayed similar until 96 h. At 48 h, a xylose MTY of 68.3 % was obtained with enzymatic hydrolysis of sorghum pretreated with 2.7 M NaOH, which was significantly lower than the other two treatments that were tested. However, there was no significant difference in xylose % MTY that was obtained with enzymatic hydrolysis of sorghum that was pretreated with 3.5 and 4.2 M NaOH.

4.4.7 Effect of Alkali Concentration on Enzymatic Hydrolysis of Sorghum Pretreated at 55°C

Sweet sorghum that was soaked at 55°C for 6 h in three different concentrations of NaOH (2.7, 3.5 and 4.2 M) was used for enzymatic hydrolysis experiments. Figure 4.11 represents the glucose %MTY obtained with enzymatic hydrolysis of sorghum pretreated at 55°C at three different concentrations. After 12 h, a glucose MTY of 91.8% was obtained with sorghum that was soaked in 4.2 M NaOH, which was significantly higher than the other two alkali concentrations that were tested. However, the glucose %MTY values obtained from sorghum pretreated with 2.7 and 3.5 M NaOH were similar ($p>0.05$).

At 48 h a glucose MTY of 95.8 % was obtained with sorghum that was pretreated with 4.2 M NaOH, which was significantly higher compared to the other two alkali concentrations that were tested. Sorghum pretreated with 2.7 M NaOH resulted in a glucose MTY of 85.7% after 48 h, which was the lowest compared to the other two alkali concentrations. With all the treatments, the glucose %MTY values did not show a significant increase from 48 h until 96 h (Figure 4.11).

Figure 4.12 represents the xylose %MTY obtained with enzymatic hydrolysis of sorghum pretreated at 55°C at three different concentrations. Increasing the alkali concentration from 2.7 to 4.2 M improved the xylose concentrations. After 12 h, the highest xylose MTY of 62 % was achieved with sorghum that was pretreated with 4.2 M NaOH and was significantly higher compared to the other two alkali concentrations that were tested.

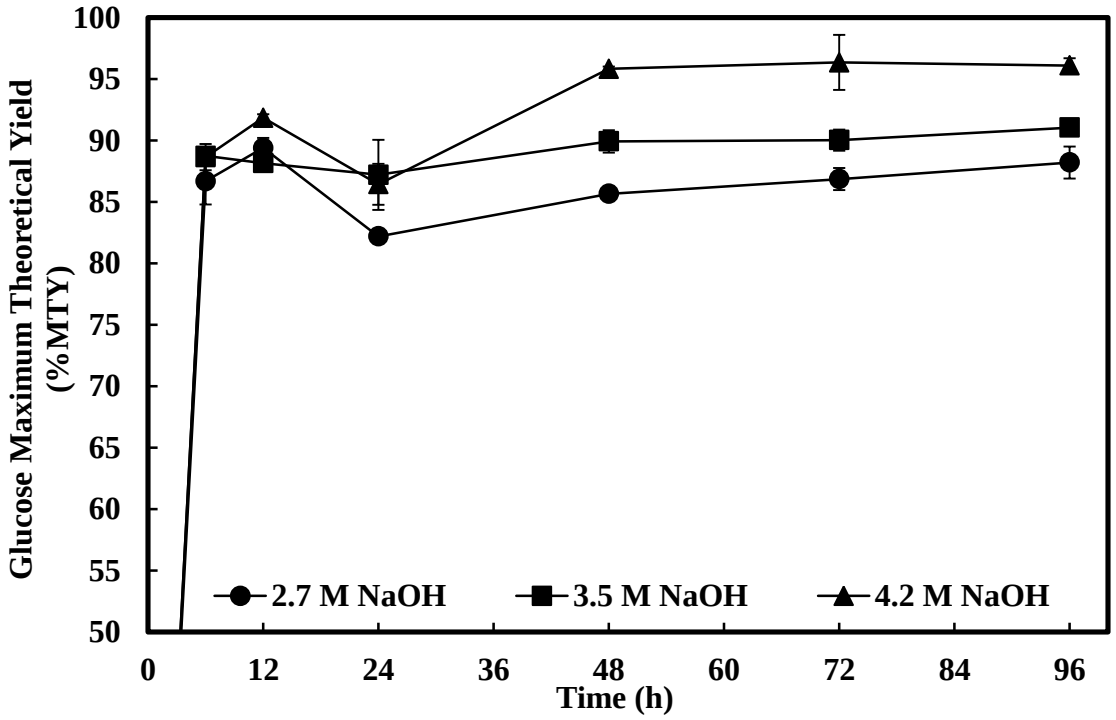

Figure 4.11 Glucose Maximum Theoretical Yield (%MTY) obtained with enzymatic hydrolysis of sorghum pretreated at 55°C for 6 h with various concentrations of NaOH at 6% solid loading, cellulase loading of 0.45 mL/g db sorghum (22.5 FPU/g db sorghum) and xylanase loading of 0.31 mL/g db sorghum (976.5 AXU/g db sorghum). Error bars represent standard deviation (n=3).

After 48 h, the xylose MTY values that were obtained from enzymatic hydrolysis of sorghum pretreatment with 2.7, 3.5 and 4.2 M NaOH were 51.5, 63.5 and 65.8 %, respectively (Figure 4.12), which were significantly different from each other ($p<0.05$). Xylose concentrations showed a further increase until 96 h with all three treatments. The final xylose MTY of about 69% was achieved with sorghum that was pretreated with 3.5 and 4.2 M NaOH, which were significantly higher compared to the xylose MTY that was obtained with sorghum that was pretreated with 2.7 M NaOH. However, there was no

significant difference in xylose yields at 96h with sorghum pretreated with 3.5 and 4.5 M NaOH.

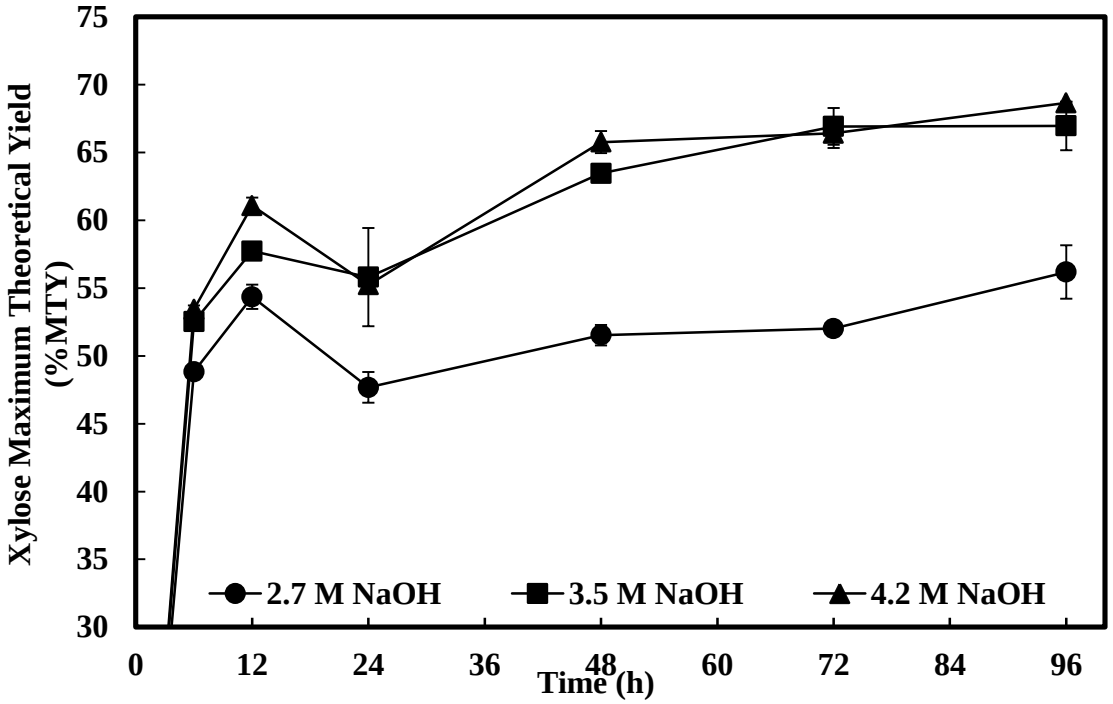

Figure 4.12 Xylose Percentage Maximum Theoretical Yields (%MTY) obtained with enzymatic hydrolysis of sorghum pretreated at 55°C for 6 h with various concentrations of NaOH at 6% solid loading, cellulase loading of 0.45 mL/g db sorghum (22.5 FPU/g db sorghum) and xylanase loading of 0.31 mL/g db sorghum (976.5 AXU/g db sorghum). Error bars represent standard deviation (n=3).

4.4.8 Effect of Alkali Concentration on Enzymatic Hydrolysis of Sorghum Pretreated at 90°C

Figure 4.13 represents the % glucose MTY obtained with enzymatic hydrolysis of sorghum pretreated at 90°C for 1 h at three different NaOH concentrations. After 12 h of enzymatic hydrolysis, with an increase in alkali concentration from 2.7 to 4.2 M, the

glucose concentration significantly increased from 18.8 to 22.4 g/L. This was equivalent to an increase in glucose MTY from 79.5 to 96.1%. At 48 h, a glucose MTY of 98% was observed with enzymatic hydrolysis of sorghum that was pretreated with 4.2 M NaOH, which was significantly higher than the other two alkali levels that were tested. At 48 h, a glucose MTY of 87.1% was obtained with sorghum that was soaked in 2.7 M NaOH, which was significantly lower than the other two alkali concentrations.

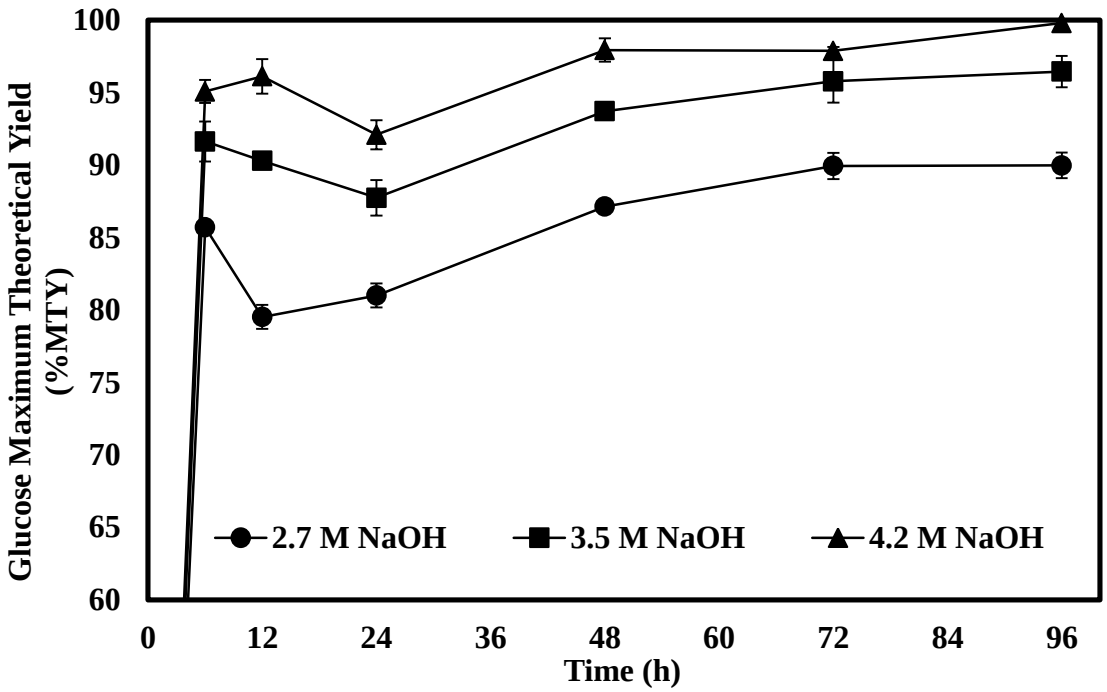

Figure 4.13 Glucose Percentage Maximum Theoretical Yield (%MTY) obtained with enzymatic hydrolysis of sorghum pretreated at 90°C for 1 h with various concentrations of NaOH at 6 % solid loading, cellulase loading of 0.45 mL/g db sorghum (22.5 FPU/g db sorghum) and xylanase loading of 0.31 mL/g db sorghum (976.5 AXU/g db sorghum). Error bars represent standard deviation (n=3).

Figure 4.14 shows the xylose %MTY obtained with enzymatic hydrolysis of sorghum treated at 90°C using various NaOH concentrations. Increase in the alkali

concentration during the pretreatment process improved the xylan conversion to xylose during the enzymatic hydrolysis process. At 12 h, a xylose concentration of 11.5 g/L that was equivalent to xylose MTY of 67.7 % was achieved with sorghum that was pretreated with 3.5 and 4.2 M NaOH, which were significantly higher compared to the xylose %MTY that was obtained with enzymatic hydrolysis of 2.5 M NaOH pretreated sorghum. However, no significant difference in xylose %MTY values was found with the enzymatic hydrolysis of sorghum that was pretreated with 3.5 and 4.2 M NaOH ($p>0.05$).

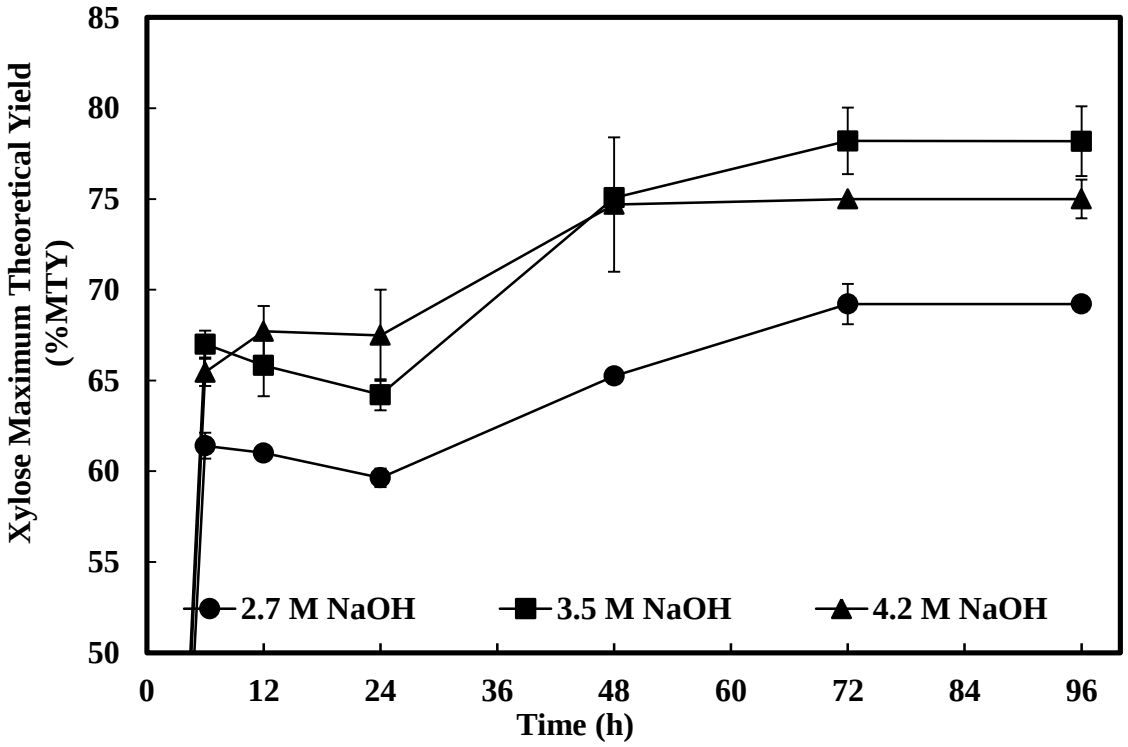

Figure 4.14 Xylose Maximum Theoretical Yield (%MTY) obtained with enzymatic hydrolysis of sorghum pretreated at 90°C for 1 h with various concentrations of NaOH at 6% solid loading, cellulase loading of 0.45 mL/g db sorghum (22.5 FPU/g db sorghum) and xylanase loading of 0.31 mL/g db sorghum (976.5 AXU/g db sorghum). Error bars represent standard deviation (n=3).

After 48 h, a xylose MTY of about 75 % (Figure 4.14) was achieved with enzymatic hydrolysis of sorghum pretreated with 3.5 and 4.2 M NaOH, which was significantly higher ($p<0.05$) compared to the % xylose that was obtained with hydrolysis of sorghum pretreated with 2.7 M NaOH. The xylose concentrations remained at similar levels between 48 h and 96 h.

4.4.9 Discussion

In the current study, sweet sorghum was extruded at two different temperatures with and without the addition of alkali in order to determine the enzymatic digestibility of sorghum bagasse. It was found that extrusion temperature had no significant effect on enzymatic hydrolysis of sweet sorghum when alkali was not used. The high feed rate and screw speeds that were used in the extrusion process that resulted in a lower shear generation, could have resulted in the poor enzymatic hydrolysis of extruded sorghum. The results from this study are similar to those obtained by Heredia-Olea et al. (2013) in which sorghum bagasse was pretreated using a twin screw extruder at various temperatures and screw speeds. The authors found that increasing the rpm to 400 or 500 resulted in sugar recovery to 52.2 and 58.6%, respectively (Heredia-Olea et al., 2013). The residence time in the current study was less than 10 seconds, which resulted in a lower heat transfer to the sorghum particles, thereby decreasing the severity of pretreatment on lignocellulosic structure of sweet sorghum.

In another study conducted by Da Silva et al., (2013) sugar cane bagasse was pretreated using a twin screw extruder at temperatures from 80 to 180 °C in the presence of ionic liquids. The bagasse was extruded twice to increase the severity of the

pretreatment. The authors found that enzymatic hydrolysis of extruded bagasse at 80 and 120 °C resulted in glucose yields of 42.3 and 39.9% and xylose yields of 68.9 and 63.2%, respectively (Da Silva et al., 2013). The results in the current study were also similar to the study conducted by Muthukumarappan and Gibbons (2012), in which pine wood chips were pretreated in a single screw extruder. It was found that enzymatic hydrolysis of pine chips that were extruded at 100°C resulted in glucose and xylose yields of 47 and 37%, respectively. In another study conducted by Karunanithy and Muthukumarappan (2011d), switchgrass was pretreated in a single screw extruder at 90°C with a moisture content of 40%, and that resulted in a glucose yield of 33.5% and xylose yield of 23.8%. In a study by McIntosh & Vancov (2010), sorghum was pretreated with alkali at various concentrations and temperatures. The authors found that inclusion of alkali in the pretreatment improved the total carbohydrate recovery by 5.6 fold with sorghum pretreated at 121°C and 4.3 fold at 60°C compared to controls that did not have any alkali.

Table 4.3 is a summary of the %MTY values obtained in the current study with enzymatic hydrolysis of sorghum that was pretreated with extrusion at 80°C and 110°C with and without alkali, along with the untreated control and the control that was only soaked in alkali and not extruded. It was found that soaking the sorghum in alkali resulted in a significant improvement in glucose and xylose yields, compared to untreated sorghum and sorghum that was extruded at 80 and 110°C without alkali. The increase in glucose and xylose yields in the current study could be attributed to the partial lignin removal and swelling of biomass which was indeed due to the presence of NaOH (Cao et al., 2012; Kang et al., 2013; Karunanithy & Muthukumarappan, 2011b).

Moreover, the glucose %MTY obtained after 96 h with alkali soaked un-extruded sorghum was similar to yields that were obtained with alkali extruded sorghum. This is of great importance since extrusion is an energy intensive process. A simple alkali soaking step could reduce the cost of the pretreatment process. However, the xylose yields obtained with alkali soaked sorghum without extrusion were significantly lower compared to those that were obtained with alkali extruded sorghum (Table 4.3). To further investigate the effect of alkali soaking as a pretreatment for sorghum, a study was performed to evaluate the effect of alkali concentrations and soaking conditions on the enzymatic digestibility of sorghum.

Table 4.3 Effect of pretreatment on %MTY of glucose and xylose obtained with enzymatic hydrolysis of sorghum after 96 h (n=3).

Treatment	**Temperature (°C)**	**Glucose %MTY (avg ± sd)[2]**	**Xylose %MTY (avg ± sd)**
Extrusion	80	50.3 ± 1.26[c]	12.8 ± 0.00[f]
	110	47.1 ± 0.69[b]	17.0 ± 0.05[e]
Alkali Extrusion	80	62.7 ± 1.31[a]	43.0 ± 0.23[b]
	110	60.9 ± 1.91[a]	46.3 ± 0.29[a]
Alkali Soaked[1]	25	60.7 ± 0.47[a]	40.6 ± 0.11[c]
Untreated	NA	42.6 ± 0.93[d]	19.4 ± 0.08[d]

1 = sorghum soaked in 1.3 M NaOH for 12 h

Values within columns that have the same letter are not significantly different

[2] Average ± standard deviation

Table 4.4 is a summary of the glucose and xylose MTY obtained at 96 h with enzymatic hydrolysis of sorghum pretreated at various temperatures and alkali concentrations. In general, increasing the alkali concentration resulted in an increase in % glucose MTY values, although at room temperature, there wasn't much of a concentration effect. This is similar to the results that were obtained in other reported studies. In a study conducted by Wu et al., (2011), sorghum bagasse was pretreated at various alkali concentrations from (0.5 to 2.5 M NaOH), three solid loadings (5, 10 and 15% w/v) and two temperatures (25 and 50°C). The authors found that increasing the alkali concentration increased the glucose yields significantly. It was also found that increasing the pretreatment time from 30 to 120 min showed an improvement in glucan saccharification at the higher concentration of alkali. The authors achieved glucose yields from 80 to 95% (Wu et al., 2011).

In another study conducted by Noori et al., (2016), elm wood was pretreated using 2M NaOH at 0 and 25°C for 2h. It was found that increasing the treatment times significantly improved the glucose yields at each temperature that was tested. A highest glucose MTY of 80% was achieved with elmwood that was treated for 2 h using 2M NaOH (Noori et al., 2016).

In the current study it was found that at an alkali concentration of 2.7 M, an increase in pretreatment temperature coupled with a decrease in pretreatment time resulted in a decrease in glucose % MTY (Table 4.4). This suggests that at lower alkali concentrations, treatment time is more important than temperature. The effect of alkali concentration on xylose MTY values did not appear to be as linear as it was for glucose. At each pretreatment condition, the lower alkali concentration (2.7 M) resulted in

significantly lower xylose yields than the other two concentrations, and the higher two alkali concentrations were often similar to each other. This suggest that a minimum alkali concentration is required in order to achieve maximum xylan hydrolysis. It was also found that at each alkali concentration that was tested, the pretreatment at 55°C for 6 h, resulted in the lowest xylose MTY values, suggesting that both temperature and soaking time are equally important when it comes to xylan hydrolysis.

Table 4.4 Glucose and xylose yields obtained with various alkali concentrations and treatment temperatures after 96 h (n=3).

NaOH conc. (M)	**Temperature (°C), Soaking time (h)**	**% Glucose MTY (avg ± sd)**	**% Xylose MTY (avg ± sd)**
2.7	25, 12	$95.5 \pm 2.73^{c,d}$	71.4 ± 2.66^{d}
	55, 6	88.2 ± 1.31^{e}	56.2 ± 1.97^{g}
	90,1	89.9 ± 0.90^{e}	69.0 ± 0.01^{e}
3.5	25, 12	97.0 ± 0.66^{c}	78.7 ± 0.56^{a}
	55, 6	98.5 ± 0.86^{b}	66.9 ± 1.79^{f}
	90,1	96.5 ± 1.08^{c}	$78.2 \pm 1.92^{a,b}$
4.2	25, 12	95.3 ± 0.67^{d}	77.1 ± 1.3^{b}
	55, 6	96.1 ± 0.59^{c}	68.7 ± 0.08^{f}
	90,1	99.8 ± 0.27^{a}	75.0 ± 1.07^{c}

Values in the column that have same letters are not significantly different.

The highest xylose MTY of 78.7% was obtained at an alkali concentration of 3.5

M that was pretreated at room temperature for 12 h. It was also found that increasing the concentration beyond 3.5 M did not show a significant increase in xylose yields irrespective of pretreatment temperature.

The results from the current study show that the use of alkali at mild conditions could lead to high conversion efficiencies without the use of expensive equipment such as an extruder or a high pressure reactor. The small particle size could have also been a factor in creating the high glucan conversion efficiencies in the current study. The effect of alkali could have been facilitated by the smaller particle size.

Also, this study shows that biomass could be soaked at very high solid loadings (40% w/v) and still achieve glucose yields up to 99%. This is of great importance since the findings so far in literature (Karunanithy & Muthukumarappan, 2011a; McIntosh & Vancov, 2010; Noori & Karimi, 2016; Sambusiti et al., 2013) utilize low solid loadings (up to 15%) which require a series of washing steps in order to remove the alkali. This could further lead to a loss of carbohydrates that would be formed during the pretreatment step. The process used in the current study did not have a washing step but a neutralization step, with a small amount of a concentrated acid (up to 1.5 mL 5N H_2SO_4). This also eliminates the possible loss of carbohydrates during the additional washing step that would otherwise be required with the traditional alkali pretreatment.

Moreover, there was no furfural or 5-hydroxymethyl furfural formation during any of the conditions that were investigated (Appendix C2). This is of great value as the presence of these inhibitors would inhibit the microbial growth. Overall this study shows a cost effective yet an efficient way of pretreating sorghum to achieve very high

conversion efficiencies.

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CHAPTER V

CONCLUSIONS

5.1 Effect of TMP and RMP on Enzymatic Hydrolysis of Sorghum

- Pretreatment of sorghum using TMP and RMP did not show a significant change in the lignocellulosic composition of sorghum.
- Supplementation of cellulase with xylanase improved the glucose yield during the enzymatic hydrolysis of RMP pretreated sorghum.
- The highest %MTY values for glucose and xylose were obtained with TMP and RG3G2 pretreated sorghum. RG3G2 resulted in the highest TSE values of 57.3%.
- Addition of a preheating step prior to the enzymatic step did not improve the glucose %MTY values.
- Without using a preheating step, increasing the α-amylase and amyloglucosidase loading from 0.1 to 0.5 mL improved the glucose %MTY values by about 11% in case of RG3G2 pretreated sorghum and 61% in case of TMP pretreated sorghum.

- Increasing the cellulase and xylanase loadings from 0.5 to 1.0 mL/g significantly improved the glucose and xylose yields with both TMP and RMP pretreated sorghum at 6% solid loading. The highest glucose MTY of 60.8 % was obtained with TMP pretreated sorghum with an enzyme loading of 1.0 mL/g. Increasing the solid loading from 6 to 12% showed similar glucose and xylose yields.

5.2 Effect of Twin-screw Extrusion on Enzymatic Hydrolysis of Sorghum

- Sorghum bagasse that was pretreated using a twin-screw extruder with or without alkali did not show significant changes in glucan or xylan content.
- The highest glucose and xylose yields of 62 and 47%, respectively, were obtained after 48 h with sorghum that was extruded at 110°C with alkali using cellulase and xylanase loadings of 0.45 and 0.31 mL/g db sorghum, respectively.
- Extrusion without alkali resulted in the lowest glucose and xylose yields, with extrusion at 80°C being the pretreatment condition that resulted in the lowest glucose and xylose yields of 45 and 11% respectively.
- Sorghum that was soaked in 1.3 M NaOH and not extruded resulted in glucose and xylose yields that were significantly higher than those obtained with extrusion pretreated sorghum without alkali. However, alkali extruded sorghum resulted in the greatest glucose and xylose yields compared to all treatments that were tested.
- Decreasing the cellulase loading from 0.45 to 0.23 mL/g db sorghum did not affect the glucose %MTY using sorghum that was extruded at 110°C. However decreasing the xylanase loading resulted in significantly lower xylose %MTY values.
- Temperature of the extruder did not affect the glucose yields significantly when

sorghum was extruded without alkali. However, the inclusion of alkali resulted in a significant increase in glucose and xylose yields when the temperature was increased from 80 to 110°C.

5.3 Effect of Alkali Soaking on Enzymatic Hydrolysis of Sorghum

- Soaking of sorghum at three different concentrations of alkali (2.7, 3.5 and 4.7 M NaOH) showed a significant decrease in glucan content compared to untreated sorghum. Lignin content was significantly altered when temperatures of 55 and 90°C were used.
- Using a cellulase loading of 0.45 mL/g db sorghum (22.5 FPU/g db sorghum), all alkali treatments resulted in glucose yields over 88%. The soaking time of pretreatment seemed to have a very important role in converting glucan and xylan to glucose and xylose.
- The study demonstrated that alkali pretreatment can be performed at a solid state instead of the conventional submerged state and still yield great enzymatic hydrolysis results. Furthermore, no washing step was used in this study, which simplifies the overall process.

CHAPTER VI

FUTURE WORK

- Alkali soaking resulted in the best results in the whole study. However, a much more detailed study is needed to further understand the effect of soaking time at each temperature that was tested. Furthermore, the effect of decreased enzyme loading could be tested to further decrease the cost of the overall process.
- Alkali soaking at 55°C resulted in glucan conversions over 90% showing that severe pretreatment conditions may not be necessary to achieve higher glucan conversion efficiencies. This also can lead to a consolidated process in which the pretreatment and enzymatic hydrolysis process can be performed in a single reactor. Furthermore, using a Simultaneous Saccharification and Fermentation methodology, the challenges with substrate inhibition can be decreased. This will eliminate the use of extra equipment and could significantly decrease the cost of the overall process.
- Test the effect of alkali soaking pretreatment at even higher solid loadings. Forty percent worked very well, so higher loading may be possible.
- Determine the effect of the alkali soaking on fresh sorghum.
- Perform a techno-economic evaluation of the pretreatment options that were tested in the current study.

APPENDIX A

A.1. Measurement of Cellulase Activity For Accellerase 1500

Enzyme dilutions that were tested: 0.00875, 0.0075, 0.005, 0.00375 and 0.0025

$$Activity\ of\ enzyme\ \left(\frac{FPU}{mL}\right) = \frac{0.37}{enzyme\ dilution\ that\ released\ 2mg\ glucose}$$

From logarithmic plot the dilution that released 2 mg of glucose was 0.0045

$$Enzyme\ activity = \frac{0.37}{0.0073} = 50.6\frac{FPU}{mL}$$

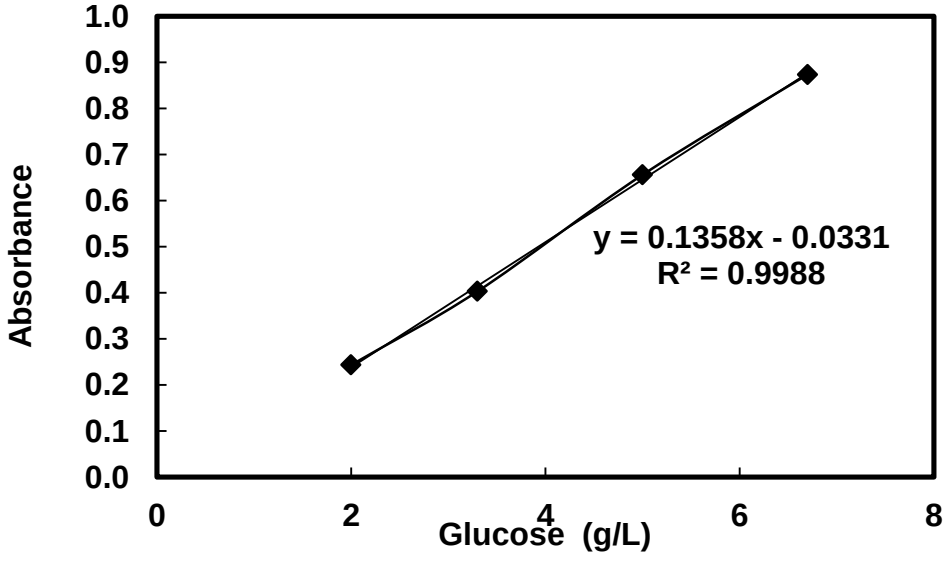

Fig. A.1 Glucose standard curve.

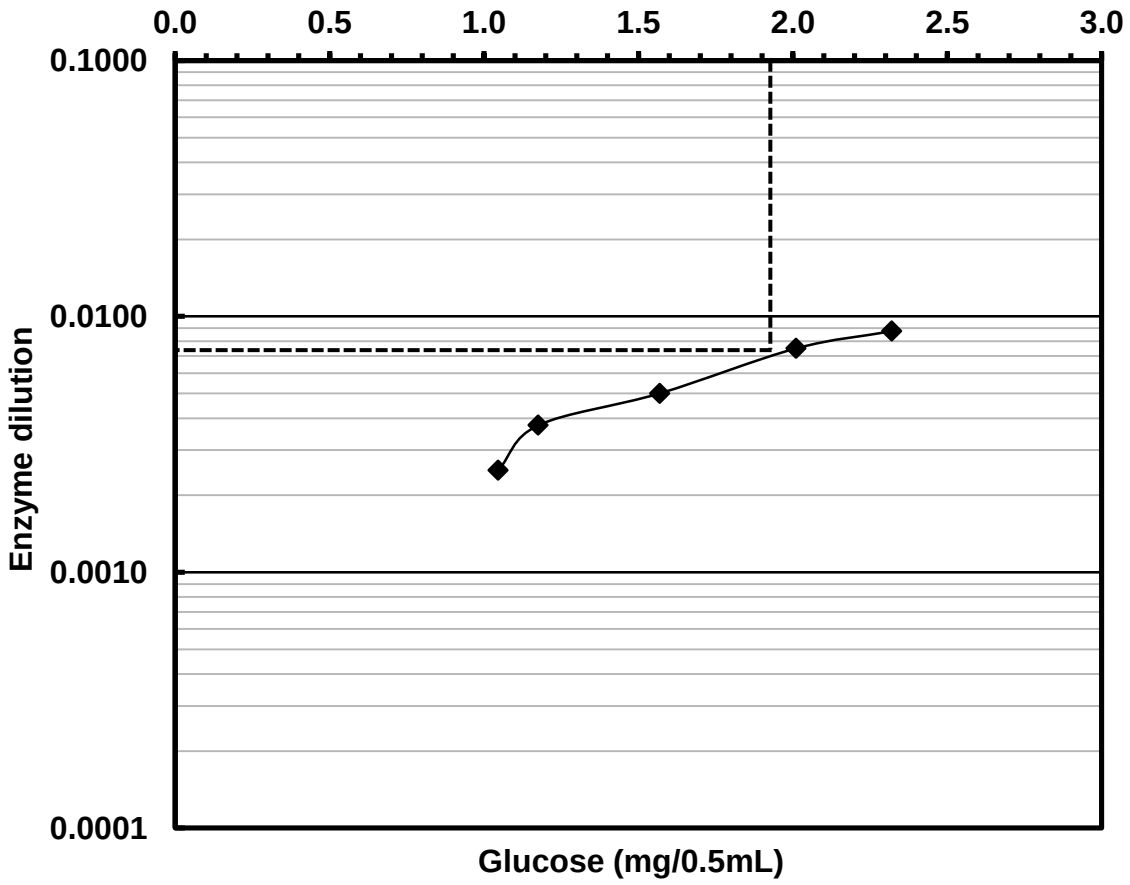

Fig. A.2 Logarithmic plot of glucose concentrations obtained with different enzyme dilutions.

A.2 Sample Calculations Involved in Acid Hydrolysis Test

Determination of Owen dry weight (ODW):

$$ODW = \frac{mass\ of\ air\ dry\ sample\ x\%\ total\ solids}{100}$$

$$\%\ total\ solids: \left[1 - \frac{A - B}{A - C}\right] * 100$$

Where: Mass of air dry sample is the mass of air dry solids added in to the pressure tubes = 0.3001 g

A is the mass of sample of air dry pretreated solids and aluminum pan = 1.9606 g

B is the mass of oven dry pretreated grass and aluminum pan = 1.9453 g

C is the mass of aluminum pan = 1.2682 g

$$\% \, total \; solids = \left[1 - \frac{1.9453 - 1.2682}{1.9606 - 1.5069}\right] * 100 = 97.79\%$$

$$ODW = \; 0.3001 \left(\frac{97.79}{100}\right) = 0.2935g$$

Mass of crucible: 33.3787 g

Mass of crucible and Acid insoluble residue (AIR): 33.4285 g

Mass of crucible and Ash: 33.3823 g

$$\%AIR = \left(\frac{mass \; of \; crucibles \; plus \; AIR - mass \; of \; crucible}{ODW}\right) * 100 \quad = \left(\frac{33.4285g - 33.3823g}{0.2935}\right) * 100$$

$$\%AIR \; = 16.97\%$$

%Acid insoluble Lignin = % AIL

$$\%AIL =$$

$$[((mass \; of \; crucibles \; plus \; AIR - mass \; of \; crucible) -$$

$$(mass \; of \; crucibles \; plus \; ash - mass \; of \; crucible))/ODW] * 100$$

$$= \left[\frac{[(33.4285 \; g - 33.3787g) - (33.3823 \; g - 33.3787g)]}{0.2935g}\right] * 100$$

$$\%AIL \; = 15.74\%$$

% Acid soluble lignin (%ASL):

$$\% ASL = \frac{UVabs * volume\ of\ filtrate * dilution}{\varepsilon * ODW} * 100$$

Where:

UV_{abs} is the average UV-Vis absorbance for sample at 205 nm

Volume of filtrate is 87 mL

$$Dilution = \frac{volume\ of\ sample + volume\ of\ diluting\ solvent}{volume\ of\ sample}$$

$$= \frac{(300\ ml + 3000 ml)}{(300\ ml)} = 11$$

ε is the absorptivity of biomass at specific wavelength = 40

$$\% ASL = \frac{0.462 * 0.087 * 11}{40 * 0.2935} * 100 = 3.77\%$$

$$\% lignin\ on\ extractives\ free\ basis = \%AIL + \%ASL = 15.74\% + 3.77\% = 19.51\%$$

Calculations Involved with HPLC determined sugars:

$$\% CVS\ recovery = \frac{conc.detected\ by\ HPLC}{known\ conc.of\ standard} * 100$$

Taking glucose in to consideration, conc. of glucose in sugar recovery standards (SRS) = 10.002 g L^{-1}

Conc. of glucose determined by HPLC = 9.21 g L^{-1}

$$\% CVS\ recovery = \left(\frac{9.21}{10}\right) * 100 = 92.2\%$$

HPLC determined conc. of glucose from acid hydrolysis sample = 1.389 g L^{-1}

$$Corrected\ concentration\ of\ sugars = \ HPLC\ determined\ conc * \%CVS\ recovery$$

$$= \frac{1.3890}{\left(\frac{92.2}{100}\right)} \ = \frac{1.5066g}{L}$$

Concentration of polymeric sugars prior to hydrolysis

$$C\ anhydro = (Cx) * \ anhydro\ correction + cellobiose\ conc$$

Anhydro correction is 0.9 for C5 sugars and 0.88 for C6 sugars.

$$C\ anhydro = \left(1.5066 * \frac{162}{180}\right) + 0.0177 \ = \frac{1.3737g}{L}\ glucan$$

$$\%S\ ext\ free \ = (C\ anhydro * \ Volume\ of\ filtrate) * \frac{100}{ODW}$$

%S ext free is the % sugars on extractives free basis.

For glucan:

$$\%S\ ext\ free = \frac{[1.3787*0.087*100]}{0.2935} = 40.7\%$$

A.3 Sample Calculations Involved in Enzymatic Hydrolysis

% Solids in RMP G3G2 pretreated sorghum = 24.6, which is determined as mentioned in sample calculations in pretreatments section.

Total mass inside the flask=100 g

Desired solid loading (%w/v) = 6

Glucan dry wt = 40.5% as obtained from acid hydrolysis test.

Glucan present in 6% solids (g) = $\frac{40.5}{100} * 6 = 2.3\ g$

Xylan present in 6% solids (g) = $= \frac{22.6}{100} * 6 = 1.4\ g$

$$Sorghum\ needed = \frac{\%\ solids}{\%total\ solids\ in\ sorghum} = \frac{6}{\frac{24.6}{100}}$$

$$Sorghum\ needed = 24.39g$$

Sorghum added to the flask: 24.3 g

Desired cellulase loading: 1.0 mL/g glucan; Activity of Celluclast 1.5 L =60 FPU/mL

$$Cellulase\ added = \ Actual\ glucan\ loaded * enzyme\ loading\ per\ gram\ glucan$$

$$= 2.3 * 1.0 = 2.3\ ml$$

Desired xylanase loading: 1.0 mL/g xylan

Xylanase added: 1.4 mL

0.5 M citrate buffer added = 10 mL, Sodium azide added = 0.1 mL

$Water\ to\ be\ added \quad = 100 - (\ sorghum\ added - cellulase\ added - xylanase\ added - citrate\ buffer\ added - sodium\ azide\ added)$.

$$Water\ added = 100 - (24.3g - 2.3mL - 1.4mL - 10mL - 0.1mL) = 61.9g$$

Theoretical yield of glucose:

$$= \left(\left(\%\frac{Glucan}{100}\right) * (Actual\ sample\ loaded\ ) * \left[\frac{\frac{\%total\ solids}{100}}{\frac{total\ mass}{1000}}\right] * 1.111\right)$$

$$= \left(\left(\frac{40.5}{100}\right) * \left(24.3g * \left[\frac{\frac{24.6}{100}}{\frac{100g}{1000}}\right] * 1.111\right)\right)$$

=26.8 g/ L

Theoretical yield of xylose:

$$= \left(\left(\%\frac{Xylan}{100}\right) * (Actual\ sample\ loaded\ ) * \left[\frac{\frac{\%total\ solids}{100}}{\frac{total\ mass}{1000}}\right] * 1.3\right)$$

$$=\left(\left(\frac{22.6}{100}\right) * \left(24.3g * \left[\frac{\frac{24.6}{100}}{\frac{100g}{1000}}\right] * 1.13\right)\right)$$

=15.2 g/ L

Glucose Maximum Theoretical Yield (%MTY)

$$\%MTY = Glucose\ obtained\ (g/L)/Theoretical\ yield\ of\ glucose(g/L)$$

Glucose obtained (g/L) = Glucose from flasks-glucose from controls

= 21.7-13.1=8.6 g/L

Glucose % MTY=$\frac{8.69}{26.9} * 100$=31.9%

Xylose Maximum Theoretical Yield (%MTY)

$$\%MTY = Xylose\ obtained\ (g/L)/Theoretical\ yield\ of\ Xylose(g/L)$$

Xylose obtained (g/L) = Xylose from flasks-Xylose from controls

= 1.02-0=1.02 g/L

Xylose % MTY=$\frac{1.02}{15.2} * 100$=6.7%%

.

APPENDIX B

B1. Effect of Xylanase Addition on Enzymatic Hydrolysis

Pretreated sorghum was used for conducting enzymatic hydrolysis experiments using NREL procedures. Preliminary experiments were conducted in order to determine the effect of pretreatment on enzymatic hydrolysis using cellulase (Celluclast® 1.5L with an activity of 60 Filter Paper Units/mL or FPU/mL) mixture with and without xylanase (Pentopan® Mono BG with an activity of 500 Fungal Xylanase Units/mL or FXU/mL) supplementation. The activity of cellulase enzyme was measured using a cellulose filter paper assay as described in Adney and Baker (2008). A cellulase loading of 0.5 mL/g glucan (30 FPU/g glucan) and a xylanase loading of 0.5 mL/g xylan (300 FXU/g xylan) was used. Furthermore, 0.1 mL of α-amylase (10.5 IU/g db sorghum) and 0.1 mL of amyloglucosidase (5 IU/g db sorghum) were added to break down the starch that is present in sorghum. All enzymatic hydrolysis experiments were conducted in triplicate in 250 mL baffled flasks sealed with a rubber stopper fitted with a one-way air valve (Check valve, Fischer Scientific, Pittsburgh, PA) to avoid contamination by microbes. Each hydrolysis flask contained 10 mL 0.5 M sodium citrate buffer at a pH of 5.5 and 6% solids (w/v). To all the hydrolysis flasks, 100 μL of 0.1% sodium azide was added to prevent contamination by microbes. The total mass of each flask was 100 g.

Control flasks containing the same contents as experimental flasks excluding enzymes were maintained to account for the sugars that are diffused during the hydrolysis process. All flasks were incubated at 55°C, 150 rpm in an incubating orbital shaker (C25 incubator shaker, New Brunswik Scientific, Edison, NJ). Samples (1.5 mL each) were collected at 0, 6, 12, 24, 48 and 96 h. The samples were then centrifuged at 15000 rpm for 10 min and the supernatant was filtered through a 0.2μ nylon filter. The filtrate was stored in a freezer until further analysis.

B2. Results

Preliminary experiments were conducted to determine the enzymatic digestibility of sorghum at 6% solid loading using cellulase and an α-amylase/amyloglucosidase mixture. The results were then compared to experiments that were performed with the supplementation of xylanase. Table B.1 shows the glucose and xylose obtained with enzymatic hydrolysis of various RMP and TMP pretreatments.

In all pretreatments that were tested, without the xylanase addition, no xylose was obtained. With the addition of xylanase, the glucose yields increased by 2.4 and 2.3 fold with the RWG3 and RG3 pretreated sorghum respectively, which could have been due to the partial hydrolysis of xylan that resulted in the increased availability of glucan for enzymatic hydrolysis. However, with RG3G2 and TMP pretreatments, similar glucose concentrations were obtained with and without the xylanase addition.

Table B.1 Effect of xylanase supplementation on glucose yields obtained after 48 h (n=3) with the enzymatic hydrolysis of sorghum at 6% solid loading, an enzyme loading of 0.5 mL cellulase/g glucan (30 FPU/mL), 0.5 mL xylanase/g xylan (250 FXU/g xylan), 0.1 mL α-amylase (10.5 IU/g sorghum) and 0.1 mL amyloglucosidase (5 IU/g db sorghum).

Treatment	**α-Amylase, Amyloglucosidase, Cellulase and no Xylanase added (avg ± sd)**	**α-Amylase, Amyloglucosidase, Cellulase and Xylanase added (avg ± sd)**	
	Maximum glucose (g/L)	Maximum glucose (g/L)	Maximum Xylose (g/L)
RG3[a]	2.5 ± 0.3	5.9 ± 0.1	0.7 ± 0.1
RFG3[b]	4.8 ± 0.7	5.7 ± 0.7	0.8 ± 0.1
RWG3[c]	3.7 ± 0.3	8.4 ± 0.7	1.2 ± 0.1
RG3G2[d]	7.1 ± 1.5	6.8 ± 1.1	0.7 ± 0.1
TMP	12.4 ± 0.5	11.8 ± 0.3	1.9 ± 0.1
No pretreatment	6.3 ± 1.0	6.1 ± 0.4	1.1 ± 0.0

[a] Sorghum chopped to coarse particle size and pretreated with RMP process with refiner gap size of 3 (76.2 μm)

[b] Sorghum chopped to a fine particle size and pretreated with RMP process with refiner gap size of 3 (76.2 μm)

[c] Sorghum chopped to a fine particle size and pretreated with RMP process with refiner gap size of 3 (76.2 μm) with an added washing step with water

[d] Sorghum chopped to a coarse particle size and pretreated with RMP process with refiner gap size of 3 (76.2 μm) and reprocessed with gap size of 2 (50.8 μm)

TMP pretreatment resulted in the formation of the highest xylose concentrations (Table. B.1) compared to all other treatments that were tested. Within the refiner, the material is chopped and the cell walls are separated due to the continuous rotation of refiners, resulting in the formation of fibrous material that has lower crystallinity and

higher swelling (Hodgson and Berg, 1988; Illikainen, 2008). This could have helped in achieving higher enzymatic hydrolysis efficiencies with TMP. The results showed that addition of xylanase not only increased the xylan hydrolysis but also facilitated the glucan hydrolysis in some cases, since cellulose and hemicellulose are linked together in a matrix.

B.3 References

Hodgson, K.T., Berg, J.C. 1988. Dynamic wettability properties of single wood pulp fibers and their relationship to absorbency. *Wood Fiber Sci*, **20**(1), 3-17

Illikainen, M. 2008. Mechanisms of Thermomechanical Pulp Refining. in: *Department of Process and Environmental Engineering*, Vol. PhD, University of Oulu. Oulu

APPENDIX C

C.1 Effect of Pretreatment on Enzymatic Hydrolysis of Galactan, Arabinan and Mannan

Addition of cellulase and xylanase to pretreated sorghum resulted in the hydrolysis of galactan, arabinan and mannan portion with all pretreatments except for. TMP and RMP pretreatmented sorghum This could be due to the low severity of the pretreatment process. However with extrusion, alkali extrusion and alkali soaking experiments, these polysaccharides were hydrolyzed. The concentration of galactose, arabinose and mannose formed during the enzymatic hydrolysis of sorghum pretreated with several methods is shown in Table C.1.

C.2 Effect of Pretreatment on Inhibitor Formation

The hydrolyzate obtained after the enzymatic hydrolysis process was used to measure the concentration of inhibitory compounds such as acetic acid, furfural and HMF using HPLC. Table C.2 shows the concentration of inhibitory compounds obtained with several different pretreatments. With all the treatments that were tested the concentration of furfural and HMF did not exceed more than 0.05 g/L. Lactic acid, concentrations did not exceed 1.1 g/L with all the treatments that were tested. Acetic acid and formic acid concentrations were less than 0.7 g/L with all the treatments.

Table C.1 Galactose, arabinose and mannose concentrations obtained with various pretreatments after 48 h of enzymatic hydrolysis (n=3).

Treatment	Galactose (g/L)	Arabinose & Mannose (g/L)
RG3G2	ND	ND
TMP	ND	ND
EXT 80°C	0.11	0.8
EXT 110°C	0.17	1.1
Alk EXT 80°C	0.17	1.6
Alk EXT 110°C	0.16	1.5
2.7 M RT 12 h	0.25	2.3
3.5 M RT 12 h	0.22	2.1
4.2 M RT 12 h	0.22	2.0
2.7 M 55°C 6 h	0.21	2.0
3.5 M 55°C 6 h	0.22	2.1
4.2 M 55°C 6 h	0.22	2.0
2.7 M 90°C 1 h	0.16	2.3
3.5 M 90°C 1 h	0.16	2.3
4.2 M 90°C 1 h	0.15	2.0

*EXT represents extrusion and ALK EXT represents alkali extrusion. RT= room temperature, ND=Not detected

Table C.2 Inhibitory compounds formed with various pretreatment methods (n=2).

Treatment	Lactic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Levulinic acid (g/L)	HMF (g/L)	Furfural (g/L)
RG3G2	ND	ND	ND	ND	ND	ND
TMP	0.5	0.25	ND	ND	ND	ND
EXT 80°C	ND	ND	ND	0.1	ND	ND
EXT 110°C	ND	ND	ND	0.3	ND	ND
Alk EXT 80°C	ND	ND	ND	0.4	ND	ND
Alk EXT 110°C	ND	ND	ND	1.7	ND	ND
2.7 M RT 12 h	0.6	0.18	0.30	2.6	0.04	0.02
3.5 M RT 12 h	0.8	0.18	0.36	2.3	0.04	0.04
4.2 M RT 12 h	0.5	0.14	0.25	1.5	ND	0.05
2.7 M 55°C 6 h	0.7	ND	0.60	2.0	ND	0.03
3.5 M 55°C 6 h	1.1	0.23	0.37	2.1	ND	0.04
4.2 M 55°C 6 h	1.1	0.25	0.44	2.3	ND	0.07
2.7 M 90°C 1 h	1.1	0.24	0.40	2.4	ND	0.05
3.5 M 90°C 1 h	1.6	0.31	0.61	2.6	ND	0.07
4.2 M 90°C 1 h	1.7	0.34	0.52	2.4	ND	0.09

*EXT represents extrusion and ALK EXT represents alkali extrusion. RT= room temperature, ND=Not detected.

C.3 SAS Program

SAS Program Code For Determining The Effect of Pretreatment on %MTY of Glucose

Several different TMP RMP Pretreatments were coded 1 through 6 for convenience.

1=RG3, 2=RFG3, 3=RFG2, 4=RWG3; 5=RG3G2 and 6=TMP. %MTY glucose was labeled as mtyg.

```
dm 'clear output';

DATA set c;

INPUT trt REP$ mtyg; DATALINES;

1       a       21.082

1       b       19.701

2       a       25.453

2       b       25.958

3       a       20.587

4       a       31.597

4       b       31.838

5       a       32.534

5       b       29.967

6       a       45.482

6       b       49.838

RUN;

PROC PRINT DATA=setc; RUN;

PROC GLM DATA=setc; CLASS trt rep;

MODEL mtyg = trt rep;

MEANS trt/lsd duncan;

RUN;
```

The SAS System

The GLM Procedure

Class Level Information

Class Levels Values 6

trt 1 2 3 4 5 6

REP

2 a b

Number of Observations Read 12

Number of Observations Used 12

The SAS System

The GLM Procedure

Dependent Variable: mtyg

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	1008.509940	168.084990	56.43	0.0002
Error	5				
		14.894072			
		2.978814			
Corrected Total	11				
		1023.404013			

R-Square	Coeff Var	Root MSE	mtyg Mean
0.985447	5.826216	1.725924	29.62342

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Trt	5	1008.172930	201.634586	67.69	0.0001
REP	1	0.337010	0.337010	0.11	0.7503

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	5	1008.172930	201.634586	67.69	0.0001

REP	1	0.337010	0.337010	0.11	0.7503

The SAS System

The GLM Procedure t Tests (LSD) for mtyg

Note: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha 0.05

Error Degrees of Freedom 5

Error Mean Square 2.978814

Critical Value of t 2.57058

Least Significant Difference 4.4366

Means with the same letter

are not significantly different.

t Grouping	Mean	N	trt
A	47.656	2	6
B	31.718	2	4
B			
B	31.116	2	5
C	25.706	2	2
D	21.155	2	3
D			
D	20.392	2	1

The GLM Procedure Duncan's Multiple Range Test for mtyg

Note: This test controls the Type I comparison wise error rate, not the experiment wise error rate.

Alpha 0.05

Error Degrees of Freedom 5

Error Mean Square 2.978814

Number of Means	2	4	6
	3	5	
Critical Range	4.437	4.575	4.633
	4.654	4.655	

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	trt
A	47.656	2	6
B	31.718	2	4
B			
B	31.116	2	5
C	25.706	2	2
D	21.155	2	3
D			
D	20.392	2	1

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