SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF HYDROTHERMOLYSIS-PRETREATED SWITCHGRASS FOR ETHANOL PRODUCTION

By

LILIS SURYAWATI

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Thesis Approved:

Dr. Mark R. Wilkins

Thesis Adviser

Dr. Danielle D. Bellmer

Dr. Raymond L. Huhnke

Dr. Niels O. Maness

Dr. A. Gordon Emslie

Dean of the Graduate College

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CHAPTER 1

INTRODUCTION

Recently, there has been a global movement toward development of renewable fuels in many parts of the world. Transitioning from fossil to renewable fuel is underway to cope with rising energy costs, environmental pollution, depletion of fossil fuel sources, and national security concerns. In the US, imported oil comprised nearly two thirds of petroleum consumption, mainly in the transportation sector (Wyman et al. 2005). Renewable fuel benefits the nation by reducing dependence on imported oil, reducing air and water pollution, and reducing greenhouse gas emissions (Wyman 1999). More importantly, the Renewable Fuels Association (Anonymous 2005b) states that domestic production of renewable fuel can strengthen agricultural markets, create value added byproducts for farmers, and establish numerous job markets in biorefinery industries, which benefits the US economy.

Ethanol is one form of renewable energy that has received much attention thus far. It is a sustainable energy source and clean-burning fuel, which gives higher thermal efficiency and power than conventional gasoline (Browning et al. 1981). In the US, ethanol has been used in fuel blends such in E10, a mixture of 10% ethanol and 90% gasoline, which has been used as an octane enhancer to improve air quality since the 1980s (Sun and Cheng 2002). Moreover, alternative fuel E85, a blend of 85% ethanol and

15% gasoline is increasingly being used. Flexible fuel vehicles (FFV) allow flexibility in choosing between conventional gasoline and E85. Recently, Congress established a national Renewable Fuels Standard as described in The Energy Policy Act 2005, which expects to nearly double the fuel ethanol market from 4 billion gallons in 2006 to 7.5 billion in 2012 in United States (Anonymous 2005b). Currently, a well-established commercial fuel ethanol industry in the US uses corn and other cereal grains as its feedstock. With the expansion of ethanol production in the nation, concerns are arising about corn demand for biofuels production competing with corn for food production and livestock feed. In order to sustain the continuity of the ethanol industry, other alternative feedstocks that do not compete with food crops need to be utilized. Claasen et al. (1999) estimated that cellulosic biomass accounts for approximately 50% of the biomass in the world. Inexpensive cellulosic biomass sources can supply large scale ethanol production, making ethanol more cost competitive with fossil fuel. Crop residues and perennial grasses are potential cellulosic feedstocks for ethanol production. One such feedstock is switchgrass, a perennial grass native to much of the US and Canada.

The bioconversion of cellulosic biomass to ethanol involves a series of processes. Biomass undergoes pretreatment to alter the structure of biomass and enhance accessibility of cellulose to hydrolysis enzymes (Mosier et al. 2005a). The pretreated slurry is transferred to a simultaneous saccharification and fermentation (SSF) vessel where cellulose hydrolysis catalyzed by enzymes and fermentation of glucose by ethanolgenic microorganisms takes place at the same time. The end product, ethanol, is distilled, purified and used as liquid fuel. The solid residue is processed further to other

products through catalytic conversion, gasification, or combustion, and is used as a source of heat and power.

In the enzymatic cellulosic ethanol process, pretreatment and SSF are two major unit operations that can greatly impact ethanol yield and process cost. SSF was evaluated as contributing over 20% of ethanol production costs (Hinman et al. 1992; Nguyen and Saddler 1991; Vonsivers and Zacchi 1995). Pretreatment and SSF have become the major challenges in cellulosic ethanol conversion technology to date. For the past decade, extensive studies have been done in these areas to improve their performance. In pretreatment, formation of byproducts undesirably inhibits fermentative microorganisms and lowers ethanol yield. There are high production costs associated with the cost of chemicals, chemical neutralization and disposal, and investing in corrosion resistant equipment. Moreover, incompatible temperatures of hydrolysis and fermentation limit SSF (Bollok et al. 2000).

These challenges create a need to improve pretreatment methods and SSF design to minimize formation of inhibitors and achieve faster hydrolysis and greater ethanol yield in less time. One way to improve SSF is to employ thermotolerant yeast. Thermotolerant yeast enables SSF to be carried out at temperatures closer to optimum for enzymatic hydrolysis, which improves saccharification and increases ethanol productivity. At present, little is known about the use of thermotolerant yeast in SSF of switchgrass.

This research proposed using pressurized liquid hot water to pretreat Kanlow switchgrass, followed by SSF of the pretreated switchgrass using thermotolerant yeast to

produce ethanol. Analyses were conducted targeting determination of optimum pretreatment conditions and the effect of SSF temperature on ethanol yield.

CHAPTER 2

OBJECTIVES

The research objectives were:

1. Determine optimum pretreatment temperature and residence time for hydrothermolysis of Kanlow switchgrass, based on ethanol yield from subsequent SSF by *Kluyveromyces marxianus* IMB4.
2. Investigate the effect of fermentation temperature on ethanol yield during SSF of hydrothermolysis-pretreated switchgrass using the thermotolerant yeast *K. marxianus* IMB4

CHAPTER 3

REVIEW OF LITERATURE

3.1 Introduction

Fuel ethanol demand in the United States has continued to increase over the past decade (Anonymous 2005b). Various cellulosic biomass sources such as corn stover, rice straw, sugarcane bagasse, and woody and herbaceous energy crops are untapped, inexpensive resources for ethanol production (Mosier et al. 2005a; Nguyen and Saddler 1991; Wyman 1996). Advancement in cellulosic ethanol technology can supply increasing fuel demand and make commercialization of fuel ethanol production more cost effective in the US (Anonymous 2005a). Research efforts in cellulosic ethanol commercialization have been done using a variety of feedstocks (Mosier et al. 2005a). In light of previous findings of the potential economic benefit and the potential of growing switchgrass in Oklahoma, it is of interest to explore switchgrass utilization for fuel ethanol that can lead to large scale production in the future.

3.2 Switchgrass

Switchgrass (*Panicum virgatum*) is a warm-season, perennial herbaceous crop that is native to North America (McLaughlin and Walsh 1998). Traditionally, switchgrass was mainly used as a forage crop. However, in the last two decades, its usage has

expanded into bioenergy crops. The Bioenergy Feedstock Development Program (BFDP) at Oak Ridge National Laboratory selected switchgrass as a model herbaceous crop for renewable energy development because it has high yield, is suitable for conventional farming practices (Lynd et al. 1991; McLaughlin 1993), and benefits land conservation by preventing soil erosion (McLaughlin et al. 1994). Switchgrass is a C4 species plant, capable of fixing carbon by multiple metabolic pathways with high water use efficiency (Koshi et al. 1982; Moss et al. 1969). Not only is it adapted to water limited soil, but it is also able to thrive in poor and low nutrient soil conditions where food crops cannot grow (McLaughlin 1993). Given the above advantages, switchgrass utilization as an energy crop seems promising. Switchgrass is a versatile energy crop that can be utilized in (1) SSF technology for fuel ethanol production; (2) gasification to produce heat or electricity; or (3) thermochemical conversion to produce various chemicals for synthesis of chemical feedstocks and transportation fuels (McLaughlin et al. 1996).

There are two types of switchgrass, lowland types which are tall, thick-stemmed, and adapted to wet conditions, and upland types which are short, thin-stemmed, rhizomatous, and adapted to drier conditions (Moser and Vogel 1995). Switchgrass begins growing in late April to early May with flowering in early June to early August. More than 90% of dry matter yield is produced from June to August (Gettle et al. 1996; Koshi et al. 1982). Switchgrass reaches full yield only in the third year after planting; it produces a quarter to a third of full yield in the first year, and about two thirds of full yield in the second year. Harvesting can be done once or twice a year for bioenergy production (Bransby 2004). The availability of sugars and lignin content in the biomass affects ethanol yield in SSF. It is desirable to have high cellulose and low lignin content

while minimizing ash and other minerals that cannot be enzymatically hydrolyzed or fermented to ethanol. High cellulose content provides greater source of sugar to be converted to ethanol. On the other hand, low lignin content is preferred because lignin impedes the efficiency of cellulose hydrolysis in SSF (Hsu 1996). Therefore, biomass yield and chemical compositions of cultivars play an essential role in successful biofuel production (Lemus et al. 2002).

Twenty varieties of switchgrass grown in southern Iowa between 1998 and 2001 were evaluated to determine their suitability for biofuel production (Lemus et al. 2002). Two of the twenty cultivars, lowland types Kanlow and Alamo, had the greatest biomass and cell wall yield. Alamo grew up to 178 cm and Kanlow grew to 177 cm when harvested in late autumn in 2000 and 2001. They were shown to have low leaf to stem ratio, which suggests more likelihood to produce better biomass for biofuel production. The stem contained higher fiber content and thus higher cellulose content. Analyses of cell wall components showed high content of cellulose and hemicellulose, but low lignin. Ash level in Alamo and Kanlow were the lowest of the twenty cultivars (Lemus et al. 2002). Various other field trials in the Midwestern and Southern US reported similarly high yields for both varieties (Sanderson et al. 1996; Sladen et al. 1991). The combination of high yield, high cellulose, low lignin, and low ash make them good candidates for biofuel production.

3.3 Switchgrass Composition

The primary constituents of switchgrass are cellulose, hemicellulose, and lignin. On a dry weight basis, switchgrass contains approximately 30-40% cellulose, 20-25% hemicellulose, and 17-22% lignin (Chang et al. 1997; Chung et al. 2005; Wiselogel et al.

1996). The cellulose and hemicellulose fractions represent substantial amounts of energy in switchgrass that can be converted to ethanol. Cellulose, a linear homopolymer of glucose linked by β-(1-4) glycosidic bond, is resistant to chemical attack due to hydrogen bonding between the hydroxyl groups on glucose along cellulose chains (Fan et al. 1982). In contrast to cellulose, hemicellulose is amorphous and easier to hydrolyze to its constituent sugars (Saka 1991). Hemicellulose consists of short, highly branched chains of five-carbon sugars, primarily D-xylose and L-arabinose, six-carbon sugars, such as D-galactose, D-glucose and D-mannose, and uronic acids (Saka 1991).

To obtain glucose monomers that can be readily converted to ethanol, cellulose must be subjected to hydrolysis. Hemicellulose forms a complex network with cellulose, which becomes the structural backbone of the plant's cell wall. These structural carbohydrate polymers are covered by lignin, a complex polymer of phenylpropane units, which are cross-linked to each other with various chemical bonds and are resistant to microbial degradation (Adler 1977). The presence of lignin impedes the efficiency of cellulose hydrolysis as it reduces accessible surface area for cellulase to adsorb onto the substrate and hydrolyze cellulose (Hsu 1996). Minor components of switchgrass include protein, uronic acids, and minerals. These components are of less interest in cellulosic ethanol production because they are not fermentable to ethanol.

3.4 Bioconversion of Cellulosic Biomass to Ethanol

In general, enzymatic conversion of cellulosic biomass to ethanol is comprised of four unit operations: (1) pretreatment, (2) hydrolysis, (3) fermentation, and (4) product separation/purification (Wyman 1999). Figure 3.1 illustrates an enzymatic cellulosic ethanol process. First, cellulosic biomass is passed through a hammer mill or similar mill

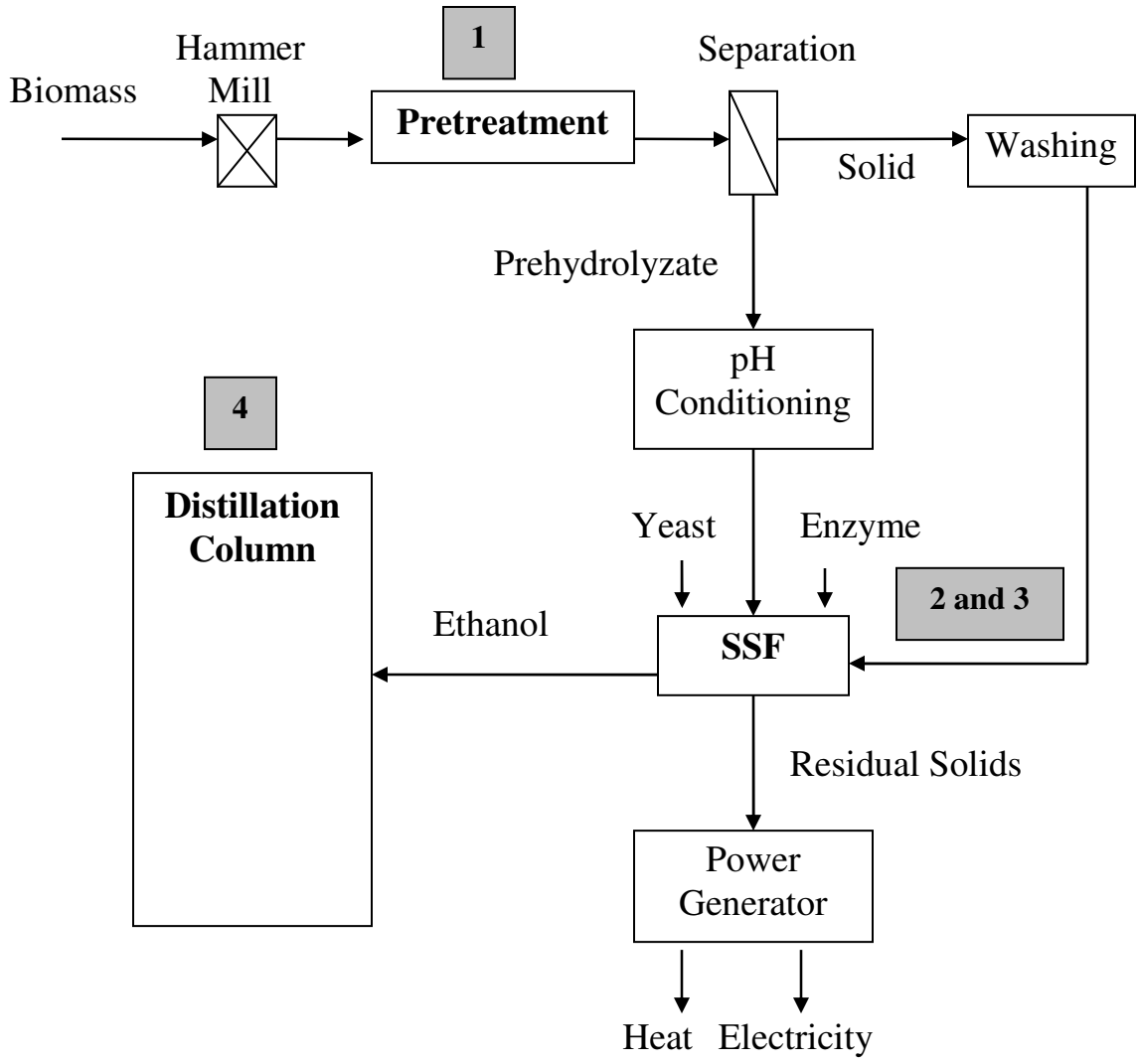

Figure 3.1 Enzymatic Cellulosic Ethanol Process

to reduce its size for pretreatment. Ground biomass is then pretreated with water and/or other chemical additives to prepare it for hydrolysis and fermentation. The pretreated slurry is cooled and passed through a filter to separate the solid and liquid fractions. Pretreatment is followed by washing of solids to remove inhibitors such as furfural and 5-hydroxymethyl furfural (HMF) formed during pretreatment that are harmful for fermentative organisms. The resulting prehydrolyzate is subjected to conditioning to adjust the pH for fermentation.

In the past, hydrolysis was done separately from fermentation. However, with the advancement of fermentation technology over the years, SSF is a more preferred process (Wright 1988). SSF reduces capital equipment cost and increases hydrolysis rate and ethanol yield (Deshpande et al. 1983). Washed solids and prehydrolyzate enter the SSF vessel where cellulase enzyme and microorganisms are added to hydrolyze polysaccharides to monomeric sugars and ferment these sugars to ethanol. The final product, ethanol, is recovered by distillation and purification (Gulati et al. 1996; Ladisch et al. 1984). The residual lignin, unreacted fibers, ash, enzyme, microorganisms, and other components are accumulated in the bottom of the distillation column. When concentrated and burned, they are used as fuel to power the process, or they can be converted to various co-products (Hinman et al. 1992; Wooley et al. 1999; Wyman 1994).

3.4.1 Pretreatment of Cellulosic Biomass

The efficiency of downstream SSF is influenced by the substrate's physical and chemical properties. Previous studies emphasized the importance of pretreatment of cellulosic biomass prior to enzymatic hydrolysis. Pretreatment has been noted as one of

the most expensive and challenging unit operations in cellulosic conversion processes (Lynd et al. 1996; Stone and Lynd 1995). From an economic stand point, this process contributes as much as 30 cents/gallon ethanol produced (Mosier et al. 2005a). The goal of pretreatment is to disrupt lignin, expose amorphous hemicellulose and crystalline cellulose, and increase their accessibility for enzymatic hydrolysis as depicted in Figure 3.2 (Mosier et al. 2005a).

Optimization of pretreatment techniques is crucial for improving efficiency of cellulose hydrolysis and reducing the amount of expensive enzyme needed (Allen et al. 1996; Liu and Wyman 2005; Mok and Antal 1992; Mosier et al. 2005a; Weil et al. 1998). In order to maximize ethanol production, the National Research Council (1999) stated that effective pretreatment must take into consideration high recovery of pentose sugars, minimal formation of degradation products in hydrolyzate which inhibit fermentative organisms, and minimizing the associated energy demand and cost.

3.4.1.1 Pretreatment Techniques

Pretreatments can be categorized as physical, chemical, or biological. Physical pretreatment methods include comminution, irradiation, steam explosion, and hydrothermolysis, whereas chemical pretreatments use acids, bases, and ammonia (Hsu 1996). Researchers have also experimented with solvents as chemical additives. Some of these were cellulose-dissolving solvents such as cadoxen, ferric sodium tartrate, alkaline H_2O_2, ozone, phenol, or ethylene glycol to enhance cellulose hydrolysis (Hamilton et al. 1984; Ladisch et al. 1978; Wood and Saddler 1988). Although these methods were effective, they were too expensive.

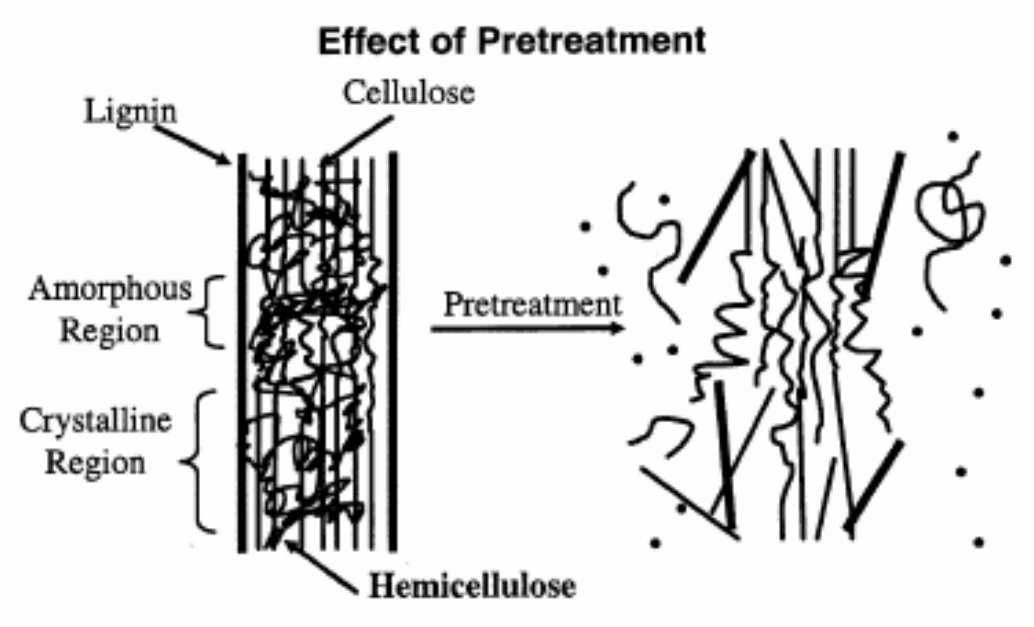

Figure 3.2 Schematic of goals of pretreatment on lignocellulosic material (Adapted from Hsu et al. 1980).

Biological pretreatment using a lignin-solubilizing microorganism is another alternative. Although this method eliminates chemical use and requires low-energy input, it is a slow process (Ghosh and Singh 1993). In more recent literature, Mosier and colleagues reviewed steam explosion, hydrothermolysis, dilute acid, lime, and ammonia as the most promising and cost-effective pretreatment technologies (Mosier et al. 2005a).

In dilute acid pretreatment, biomass is mixed with an aqueous acid mixture at acid concentration ranging from 0.2 to 1.5%. The mixture is heated to temperature above 140°C and held for periods ranging from seconds to minutes. The acid can be added to the biomass mixture by spraying onto the heated solids, direct steam injection, or agitated with the biomass in a reactor (Mosier et al. 2005a). Sulfuric acid is most widely used than other types of acid because it is inexpensive and effectively removing hemicellulose. The removal of hemicellulose resulted in highly digestible cellulose in the pretreated solids (Lee et al. 1999). Chung et al (2005) reported glucan (cellulose) content in dilute acid-pretreated switchgrass with 1.2% H_2SO_4 at 180°C for 0.5 min increased from 32.2% to 81.3%, which corresponded to increased cellulose conversion from approximately 24% to 90% in SSF. More than 80% xylan was recovered in liquid prehydrolyzate as a result of dilute acid pretreatment. To date, dilute acid is one of the most widely developed pretreatment and is used in many experiments due to its high hemicellulose solubilization and high cellulose digestibility. Yet, there are several limitations to this method. High temperature and acidic pretreatment conditions tend to degrade sugars causing formation of toxic products that inhibit fermentative organisms (Palmqvist and Hahn-Hagerdal 2000). This technique also requires corrosion resistant equipment and materials, which

are costly. Furthermore, cost of acid and acid neutralization after the process makes dilute acid treatment less cost effective (Mosier et al. 2005a).

Other pretreatment methods of various cellulosic biomass types also reported similar improvements in glucan content and cellulose conversion (VanWalsum et al. 1996; Weil et al. 1997). Significant lignin removal and increased enzymatic hydrolysis yield can also be achieved using lime pretreatment (Chang et al. 1997; Chang et al. 2001; Kaar and Holtzapple 2000). Lime pretreatment uses calcium hydroxide mixed with water. The lime mixture is sprayed onto biomass material and incubated for several hours to weeks at ambient temperature. Alternatively, increasing incubation temperature can reduce incubation period. Preparation of biomass prior to pretreatment requires particle size of approximately 10mm or less (Mosier et al. 2005a). As a result of lime pretreatment, lignin was removed from biomass, hence increasing reactivity of cellulose. Lime is inexpensive and is recoverable from water as insoluble calcium carbonate by reaction with carbon dioxide. The carbonate can be converted to lime using lime kiln technology (Chang et al. 1998). Despite the benefits, lime pretreatment requires hours to days to be effective.

Another pretreatment option is ammonia fiber explosion (AFEX), in which biomass is treated with liquid ammonia under pressure followed by explosive pressure release at the end of pretreatment. The release of pressure alters the biomass structure by increasing accessible surface area for cellulose hydrolysis. Approximately 5 to15% ammonia solutions at temperatures between 160°C and 180°C for 14 min in a flowthrough reactor was used to delignify biomass and increase enzymatic digestibility of

cellulose. The technology is limited by cost of ammonia and its recovery (Holtzapple et al. 1992).

Physical pretreatment, such as steam explosion and hydrothermolysis, are not catalyzed by chemicals other than water. In steam explosion, high pressure steam was used to heat biomass. The biomass and steam mixture is held for several minutes, followed by rapid explosive decompression. The major effect of steam explosion is hydrolysis of hemicellulose, which increases the accessibility of enzymes to hydrolyze cellulose in the pretreated biomass. Acetic and other acids released during steam explosion hydrolyze hemicellulose. Rapid thermal expansion also alters the physical structure of biomass. Grethlein and Converse (1991) evaluated steam explosion pretreatment of various wood samples. They reported that steam explosion increased the pore volume of the wood as a result of hemicellulose removal. Their analyses showed increased surface area of the pretreated wood, therefore resulting in increased accessibility of cellulose and increased rate of enzymatic hydrolysis. This method however, showed low xylose recovery (Heitz et al. 1991) and produced inhibitory hydrolyzate (Forsberg et al. 1986).

Another physical pretreatment method is hydrothermolysis, which is a pressurized liquid hot water treatment. Hydrothermolysis was reported giving high pentosans recovery (Mok and Antal 1992) and fermentable hydrolyzate (VanWalsum et al. 1996). In hydrothermolysis, biomass is treated with pressurized liquid hot water at temperature above 160°C and held for some period of time ranging from several seconds to minutes. The pressurized water remained in the liquid state at high temperature. In that condition, water has increased level of hydronium ions, thus, water acts as acid catalyst to hydrolyze

biomass (Weil et al. 1997). The amount of liquid water used depends on the reactor design. This method will be described further later in this review.

3.4.1.2 Pretreatment Products: Formation of Inhibitory Compounds

Degradation products such as furfural, 5-hydroxymethyl furfural (HMF), and weak acids (acetic, formic, and levulinic) formed during pretreatment can inhibit fermentative organisms. Figure 3.3 depicts the degradation pathway of sugar polymers during hydrolysis of cellulosic biomass (Palmqvist and Hahn-Hagerdal 2000). Xylose liberated from hydrolysis of hemicellulose is degraded to furfural at high temperature and pressure (Dunlop 1948), whereas glucose released from cellulose hydrolysis is degraded to HMF (Ulbricht et al. 1984). HMF and furfural were shown to inhibit the growth rate of fermentative organisms (Azhar et al. 1981; Boyer et al. 1992; Larsson et al. 1998). and reduce specific ethanol productivities (Palmqvist and Hahn-Hagerdal 2000; Taherzadeh et al. 1998). Further degradation of furfural and HMF formed formic acid (Dunlop 1948; Ulbricht et al. 1984), which decreased the pH of fermentation and interfered with intracellular enzymatic activity of yeast (Pamphuhla and Loureiro-Dias 1990).

In pretreatment, temperature, time, and types of chemical are key parameters influencing formation of fermentation inhibitors in lignocellulose prehydrolyzate (Chum et al. 1990; Overend and Chornet 1987). One study by Larsson et al. (1998) analyzed dilute acid hydrolysis of spruce wood at varying temperatures between 150°C and 240°C at 0.5-4.4% H_2SO_4. They found as pretreatment conditions became more severe, furfural and HMF formation increased. Correspondingly, they observed decreased concentrations of fermentable sugars and hydrolyzate fermentability (Larsson et al. 1998).

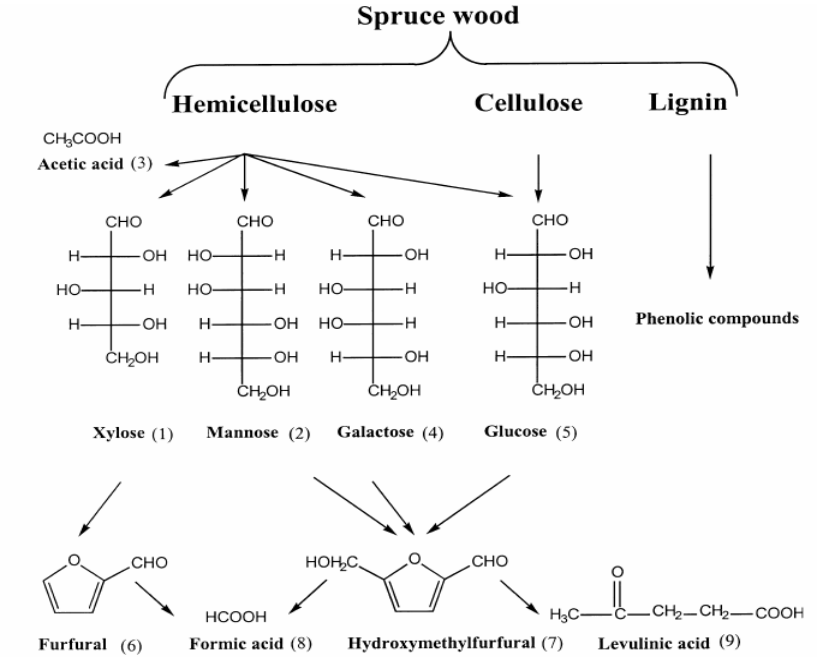

Figure 3.3 Degradation products during hydrolysis of lignocellulosic materials (Adapted from Palmqvist et al. 2000).

Furfural has been shown to reduce specific growth rate of yeast (Azhar et al. 1981; Boyer et al. 1992) and volumetric ethanol productivities (Palmqvist et al. 1999; Taherzadeh et al. 1998). A similar growth inhibition mechanism is also caused by HMF. The presence of both inhibitors was reported completely inhibiting yeast growth (Taherzadeh et al. 1999). In a similar study, Tengborg et al. (1998) reported better hydrolyzate fermentability when SO_2 was used instead of H_2SO_4. Since generation of inhibitory products during pretreatment affects hydrolyzate fermentability, it is vital to select a pretreatment method and design pretreatment parameters that minimize the adverse effect of inhibitory compounds on SSF yield.

3.4.1.3 Hydrothermolysis of Cellulosic Biomass

In hydrothermolysis, cellulosic biomass is treated in hot compressed liquid water (Bobleter et al. 1981; Bobleter et al. 1976; Mok and Antal 1992). Liquid water at elevated temperature has increased levels of hydronium ions, which acts as an acid catalyst and autohydrolyzes the polysaccharides in biomass. Neither acid nor base is added in this technique, thus reducing chemical cost and neutralization, and corrosion of process equipment. Moreover, Allen et al. (1996) reported minimal formation of toxic products during pretreatment. The release of acetic acid during hydrothermolysis is a result of cleavage of hemiacetal linkages (Antal Jr. 1996). Van Walsum et al. (1996) pretreated various lignocellulosic materials at 220°C for 120s at 5MPa. They discovered that liquid hydrolyzate from pretreatment was fermentable to ethanol. This result was supported by three other studies (Lynd et al. 1996; Mosier et al. 2003a; Mosier et al. 2003b). Other research in hydrothermolysis of various feedstocks reported increased hemicellulose and

cellulose solubilization and high hemicellulose recovery (Liu and Wyman 2005; Mok and Antal 1992; Walch et al. 1992; Yang and Wyman 2003).

Three types of hydrothermolysis designs have been studied co-current: counter-current, and flow-through reactor configurations. In co-current design, biomass and water is heated to the desired temperature and held at a fixed residence time followed by cooling (Mosier et al. 2003a; Mosier et al. 2003b; Weil et al. 1998). In counter-current design, biomass and water flow oppositely through the reactor. The flow-through configuration passes hot water over a stationary bed of biomass. The water with dissolved components exits the reactor (Mosier et al. 2005a). Reactor configurations of these different hydrothermolysis modes are depicted in Figure 3.4.

Various researchers have experimented with these different reactor configurations and have developed process parameters to optimize hydrothermolysis method. Weil et al. (1998) used a batch reactor design to treat corn fiber at temperatures between 220 and 260°C. They investigated pH controlled versus uncontrolled hydrothermolysis. Corn fiber was pretreated in a 2 L Parr Reactor and pH was controlled above 4.0 by adding 2.0 M KOH. The initial pH in the uncontrolled pretreatment was 4.0 and decreased to 3.1 at the end of pretreatment. The pH control resulted in decreased degradation of glucose in liquid hydrolyzate as opposed to no pH control. Starch and hemicellulose in corn fiber were dissolved. Cellulose content increased from 21 to 47% as a result of pretreatment compared to 17.5% in untreated corn fiber. Higher pretreatment temperature was harsher as little glucose was obtained from the prehydrolyzate. Enzymatic hydrolysis of pretreated corn fiber resulted in 33 to 84% cellulose conversion to glucose.

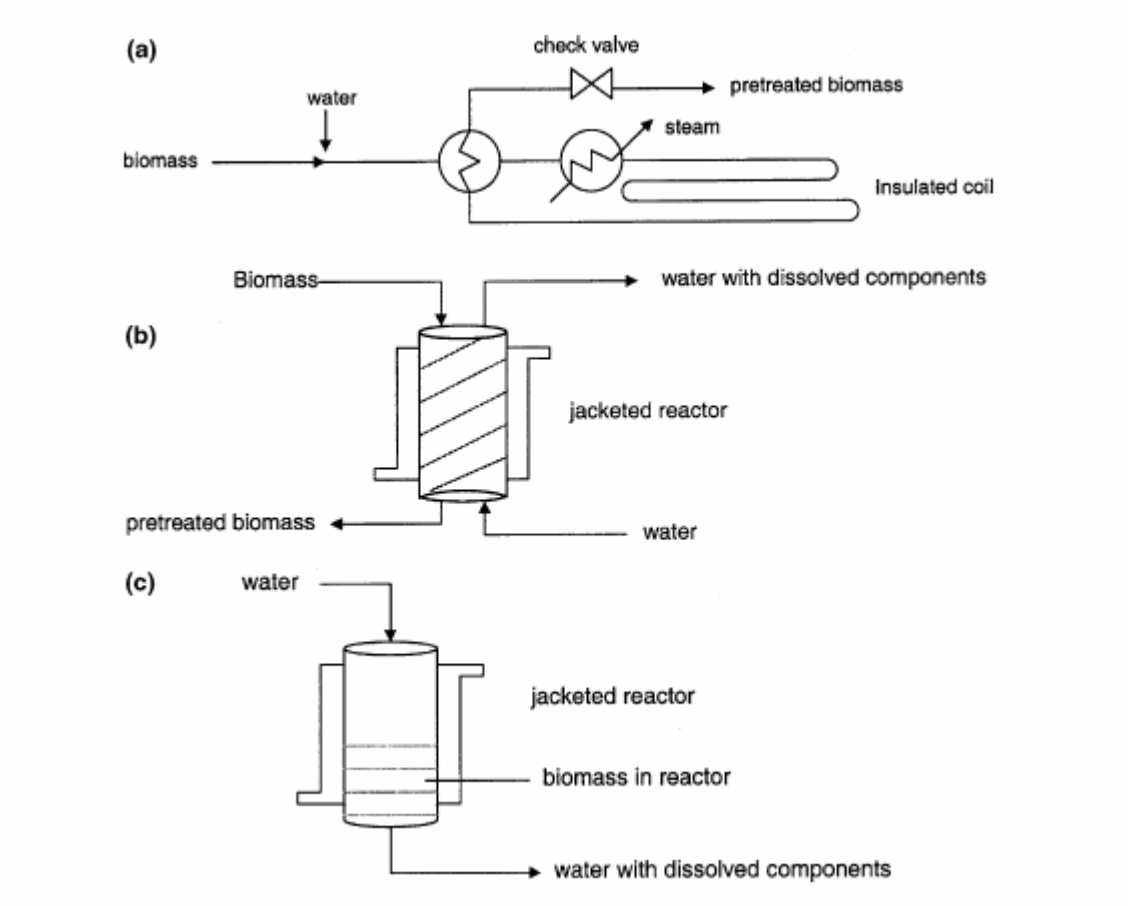

Figure 3.4 Schematic illustration of hydrothermolysis reactor configurations: (a) co-current reactor, (b) counter-current reactor, (c) flow-through reactor (Adapted from Mosier et al. 2005a).

Another study reported a patented pH-controlled hydrothermolysis process for corn fiber, which employed a co-current reactor configuration as depicted in Figure 3.5 (Mosier et al. 2005c). Corn fiber (stream 1) and stillage (stream 2) were mixed in a tank. Heat from slurry exiting the pretreatment coil was recovered to heat the incoming stream in heat exchanger 1 and steam was injected (stream 3) to account for heat loss. Pretreated slurry was separated into solid (stream 4) and liquid (stream 5) fractions by centrifugation. To test industrial application of this system, a pilot study of this configuration was performed. The pretreatment condition applied in the pilot study was based on preliminary laboratory scale batch experiments using capped stainless steel tubes heated in a fluidized sand bath. Optimum pretreatment condition was 160°C for 20 min which resulted in maximum solubilization and minimum degradation of monosaccharide. The pretreatment solubilized 50% of corn fiber and dissolved carbohydrates were 80% oligosaccharides and 20% monosaccharides. Pretreated slurry was hydrolyzed with cellulase at 50°C, followed by fermentation by xylose-fermenting *Saccharomyces. cerevisiae* or ethanolgenic bacteria *Escherichia coli* FBR16. Still bottoms from ethanol distillation of fermentation beer were used to buffer pretreatment slurry above pH 4.0. As a result, they were able to minimize HMF and furfural formation to less than 0.3 and less than 0.5 g/L, respectively, and achieved more than 90% ethanol yield from starch and cellulose (Mosier et al. 2005c).

In another study to simulate a co-current system, Mosier et al. (2005b) experimented with pH controlled hydrothermolysis of corn stover using a laboratory scale, plug-flow coil reactor. The reactor was constructed of 316 stainless steel tubes (1.0 in. OD, 4.5 in. in length, and 45mL internal volume) and was heated in a fluidized

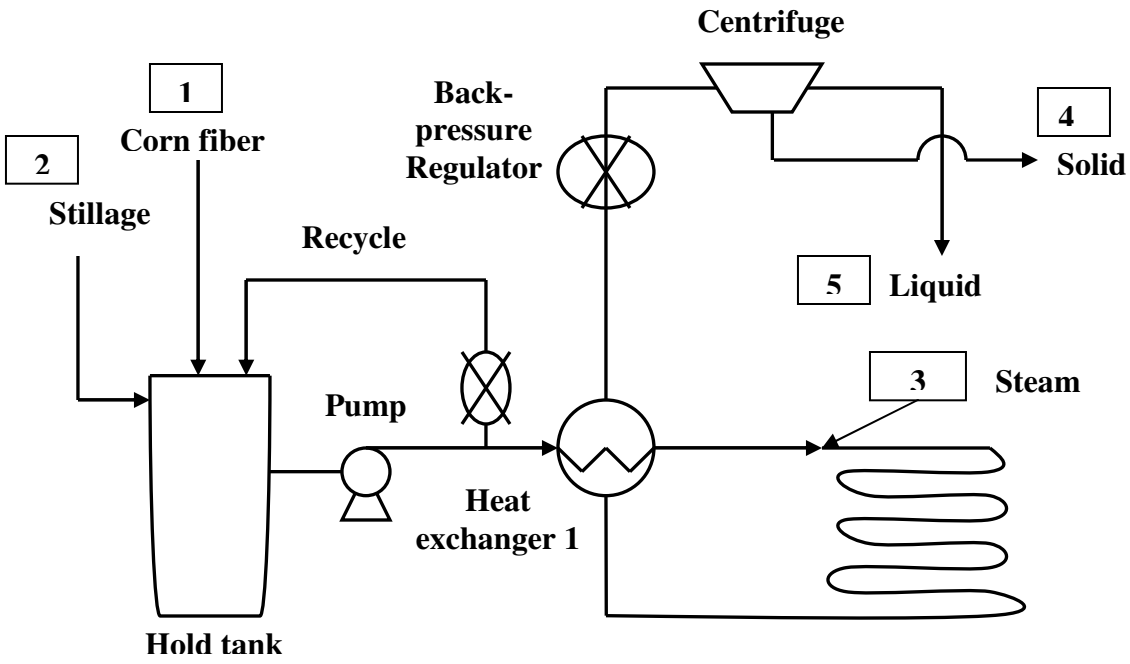

Figure 3.5 Schematic process diagram of pH-controlled hydrothermolysis of corn fiber (Adapted from Mosier et al. 2005c)

sand bath. Corn stover was heated to the desired reaction temperatures between 170 to 200°C and held for 5 to 20 minutes. At the end of pretreatment, the reactor tube was quenched in water until the temperature dropped below 100°C. The optimum pretreatment condition was found to be 190°C for 15 min. After corn stover was pretreated at the optimum conditions, 90% of the cellulose was hydrolyzed to glucose using 15 FPU cellulase/g glucan. Fermentation of hydrolyzate by glucose and xylose fermenting recombinant yeast *S. cerevisiae* 424A (LNH-ST) resulted in 88% theoretical ethanol yield.

Several researchers have used flow-through hydrothermolysis to pretreat cellulosic biomass (Liu and Wyman 2003; Liu and Wyman 2004a; Liu and Wyman 2004b; Liu and Wyman 2005; Mok and Antal 1992). Mok and Antal (1992) pretreated several types of cellulosic biomass for 0 to 15 min at 200 to 230 °C. They observed 40 to 60% biomass solubilization, in which, 4 to 22% cellulose and 35 to 60% of lignin were solubilized. More than 90% of the hemicellulose was recovered as monomers in the liquid hydrolyzate (Mok and Antal 1992). Liu and Wyman (2005) did a comparison study of batch, flow-through (FT), and partial flowthrough (PFP) hydrothermolysis of corn stover. Figure 3.6 illustrates the FT system used in the study. The reactor vessel was a 316 stainless steel tube (25.4 mm OD x 10.7 mm length, and 37.8 mL internal volume).

All three modes of pretreatment were done in the same reactor at 200°C for 24 min. The flow rate was 10 mL/min for FT system. They evaluated two PFP systems, in which PFP1 was run in batch mode for the first 4 min, followed by FT at 10mL/min flow rate for 4 min, and finally switched to batch mode for the rest of pretreatment. PFP2 was

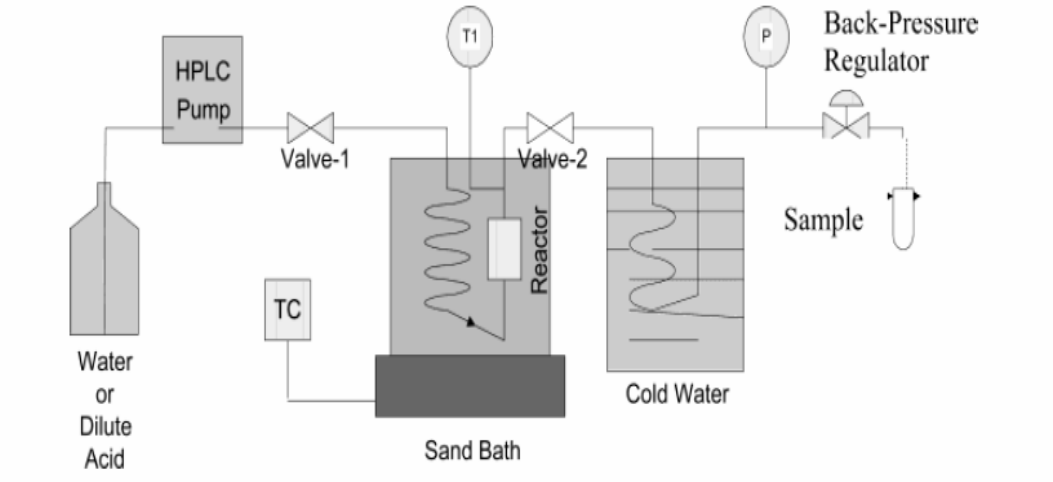

Figure 3.6 Schematic process diagram of flow-through reactor configuration (Adapted from Liu and Wyman 2003).

run at similar mode to PFP1 except at longer FT (8 min). Two batch pretreatments were carried out at 200°C for 20 and 24 min.

Liu and Wyman (2005) reported significant differences in lignin and hemicellulose removal and hemicellulose recovery from all three experiments. Flow-through pretreatment removed the most lignin (60%), followed by partial flow modes (42% and 45%) for PFP1 and PFP2, respectively. The least lignin removal was from batch operation (<12%). The highest xylose yield (96.2%) was achieved in FT operation. Batch mode had the lowest xylose yield of all modes (46.6 and 12.1% for 20 and 24 min, respectively). It was observed that PFP systems removed less lignin and recovered less xylose than FT. Although FT system achieved the greatest hemicellulose recovery and lignin removal, water consumption and energy requirements were high, which made this process uneconomical. On the contrary, PFP mode utilized 40% less water than FT, but still maintained relatively high hemicellulose recovery (94-95%). Thus, PFP appeared to combine the best features of FT and batch modes (Liu and Wyman 2005).

3.4.2 Simultaneous Saccharification and Fermentation

Saccharification of cellulose in biomass can be accomplished via chemical or enzymatic hydrolysis. Chemical hydrolysis uses strong acids or bases resulting in nearly 100% cellulose conversion. However, research showed that this technology is commercially unfeasible due to high chemical cost, corrosion problems, and strict environmental control issues with regard to waste treatment (Wright 1988). Dilute acid hydrolysis using H_2SO_4 or HCl at temperatures between 120 and 200°C was less harsh than strong acids (Grethlein and Converse 1991; Torget and Hsu 1994). However, research showed there were problems with sugar degradation and low ethanol yields

(Wright 1988; Wyman 1994). Enzymatic hydrolysis is a more appealing method compared to acid hydrolysis. Cellulase enzyme exhibits high specificity to catalyze cellulose hydrolysis (Duff and Murray 1996) and the reaction takes place under mild conditions (45-50°C) without sugar degradation (Parisi 1989). Also, since enzymes are proteins, they are biodegradable and environmentally friendly.

Conventionally, enzymatic hydrolysis and fermentation were done separately in a process know as separate hydrolysis and fermentation (SHF). In 1974, researchers at Gulf Oil Company pioneered a SSF process for lignocellulosic conversion, where cellulose hydrolysis and fermentation were done simultaneously in one vessel (Takagi et al. 1977). Since then, SSF has been intensely utilized in ethanol production from various lignocellulosic materials such as woody, herbaceous, and agricultural residues (Chang et al. 2001; Grohmann 1993; Mosier et al. 2005a; Wyman et al. 1992). Efforts to study reactor design and modeling in SSF have been studied as well (Philippidis et al. 1993; South and Lynd 1994). SSF offers advantages in increased hydrolysis rate and ethanol yield by minimizing hydrolysis product inhibition. In SSF, sugar accumulation is minimized as it is rapidly converted into ethanol (Krishna et al. 1999), and the presence of ethanol reduces contamination risk (Nigam and Singh 1995; Philippidis et al. 1993). SSF eliminates the use of separate reactors for saccharification and fermentation, thus reducing equipment capital cost (Deshpande et al. 1983).

Wyman et al. (1992) compared ethanol yield of SSF with SHF from dilute acid pretreated woody and herbaceous feedstocks using the fermentative organism *S. cerevisiae*. They found significantly higher ethanol yield using SSF as a result of minimal cellulase inhibition by sugar. Nonetheless, a drawback to this process is different

temperature requirements for saccharification and fermentation (Chung et al. 2005; Grohmann 1993; Krishna et al. 1999). Cellulose saccharification by cellulase is optimum at 45 to 50°C, whereas fermentation temperature must be maintained between 25 and 35°C to maximize ethanol production and prevent irreversible heat-inactivation of yeast cells (Bollok et al. 2000).

3.4.2.1 Utilization of Thermotolerant Yeast in Simultaneous Saccharification and Fermentation

Over the years, researchers have gained interest in utilizing thermotolerant yeast to overcome the disadvantage of incompatible temperature requirements in SSF. Studies have been done in finding thermotolerant and ethanol tolerant yeast for ethanol production to enhance the efficiency of SSF (Szczodrak and Targonski 1988). Thermotolerant species of *Saccharomyces, Kluyveromyces, and Fabospora* genera were found capable of growing at temperatures above 40°C and fermenting glucose, galactose, and mannose at 40, 43, and 46°C, respectively (Szczodrak and Targonski 1988). In another study, four thermotolerant *S. cerevisiae* yeast strains (VS_1, VS_2, VS_3, and VS_4) were isolated from Thermal Power Plant in India (Kiran Sree et al. 2000). All strains grew on glucose media and produced ethanol at temperatures ranging from 30 to 44°C. Using 150g glucose/L, VS_1 and VS_3 strains grew better at high temperature than the other two strains. The optimum ethanol yield was 66% at 30 and 35°C. Ethanol yield decreased as much as 20% when temperature increased from 30 to 44°C. Despite the decreased ethanol yield, the authors reported all strains still remained viable at high temperature. This could lead to potential improvement for SSF because *S. cerevisiae* typically do not grow and produce ethanol at temperatures above 40°C (Kiran Sree et al. 2000). A more

recent study reported the use of CP11, pentose-utilizing yeast in lignocellulose fermentation (Pasha et al. 2007). The mutant yeast was developed by protoplast fusion of thermotolerant yeast *S. cerevisiae* VS_3 and xylose-utilizing *Candida shehatae*. Acid hydrolysate from pretreated *Prosopis juliflora* wood and enzymatic hydrolysate from hydrolysis of pretreated solid of *P. juliflora* were combined to give a total 84 g/L reducing sugars. Fermentation of the combined hydrolysate using CP11 at 42°C resulted in 32g/L ethanol.

Alternatively, many researchers have been interested in thermotolerant yeast cultures from the *Kluyveromyces* genus as they were reported as being more thermotolerant than *Saccharomyces* species (Hacking et al. 1984; Hughes et al. 1984; Szczodrak and Targonski 1988). Particularly, thermotolerant *K. marxianus* strains have been used in SSF (Ballesteros et al. 1991; Ballesteros et al. 2004; Barron et al. 1997; Bollok et al. 2000; Boyle et al. 1997; Lark et al. 1997). Thermotolerant *K. marxianus* Y.00243 was used in SSF at 42°C using 5% (w/w) steam-pretreated spruce substrate, 37 FPU/g cellulose cellulase loading, and 38 IU/g cellulose β-glucosidase (Bollok et al. 2000). The initial pH of SSF was 5.4 and pH was not controlled. The maximum ethanol concentration (13.9 g/100g substrate) was obtained after 23h. Ethanol yield was 15% lower than SSF with *S. cerevisiae* at 37°C. It was observed that fermentation with *K. marxianus* Y.00243 stopped after 10h. The authors explained this could be caused by inhibition of *K. marxianus* Y.00243 by inhibitory compounds present in the hydrolyzate (Bollok et al. 2000). Ballesteros et al. (2004) used *K. marxianus* CECT 10875, capable of growing and fermenting glucose at 42 to 45°C, to ferment various steam explosion pretreated lignocellulosic biomass. Ethanol yield from SSF ranged between 50 to 72%

theoretical yield from 10% (w/v) substrate loading at 42°C and 15 FPU cellulose/g substrate in 72 to 82h. The maximum ethanol concentrations were 19, 17, 18, 16, and 19 g/l from SSF of poplar, eucalyptus, wheat straw, sweet sorghum bagasse, and *Brassica carinata*, respectively (Ballesteros et al. 2004).

In another study, Krishna et al. (2001) used a thermotolerant yeast *K. fragilis* NCIM 3358 to ferment alkaline H_2O_2 pretreated sugar cane leaves and *Antigonum leptopus* leaves. At 43°C, 10% (w/v) substrate, 10% (v/v) yeast inoculum, and 40 FPU cellulase/g substrate, ethanol concentrations were 2.5% (w/v) in 72h and 2.3% (w/v) in 48h from sugar cane and *A. leptopus* leaves, respectively. In comparison, ethanol concentrations from SSF using *S. cerevisiae* at 40°C were much less, 1.8% (w/v) in 72h and 48 h from sugar cane and *A. leptopus* leaves, respectively. Overall, SSF time was reduced using thermotolerant yeasts. This result is similar to past research which reported conversion >2% w/v ethanol in 4-6 days (Deshpande et al. 1983; Saddler et al. 1982; Spangler and Emert 1986; Takagi et al. 1977).

3.4.2.2 Thermotolerant IMB Yeast

Banat et al. (1992) discovered five novel, thermotolerant, and ethanol producing *K. marxianus* yeast strains: IMB1, IMB2, IMB3, IMB4, and IMB5. The IMB yeast was isolated from an Indian distillery in India. The IMB yeasts grew anaerobically on glucose and lactose media at temperatures up to 50°C (Banat et al. 1992; Banat et al. 1998). This finding showed higher temperature tolerance relative to the previously discussed VS_3, CECT 10875 and *K. fragilis*. IMB2, IMB4, and IMB5 produced 7.2%, 6.8%, 7.0% (w/v) ethanol concentrations at 45°C on 14% glucose media (w/v), which equaled to 95 to 100% theoretical yield. It is possible that IMB strains could achieve greater SSF yield at

higher temperatures than *Saccharomyces* or other *Kluyveromyces* strains. Furthermore, the five thermotolerant *K. marxianus* IMB strains showed ethanol tolerance level to as much as 9.5% (w/v) ethanol concentration when grown at 45°C on glucose containing media. Reduced tolerance levels were observed for IMB2 and IMB4 strains (Banat and Marchant 1995). IMB4 grew on xylose at 45°C and produced 1.2 g/L ethanol. Lignocellulose prehydrolyzate from pretreatment generally contains xylose monomers, which are difficult to ferment to ethanol (Hahn-Hägerdal et al. 1994). Hence, IMB4 may be a good candidate for xylose fermentation.

Previous reviews have reported ethanol production from various substrates using the IMB3 strain. Singh et al. (1998) reported an industrial scale fermentation of 16% w/v sugarcane molasses at 45°C using IMB3. The ethanol yields were 6.0 to 7.2% w/v with the advantage of shorter fermentation time and reduced fermenter cooling compared to fermentation using *S. cerevisiae*. Two published studies used the IMB3 strain to convert barley straw to ethanol in an SSF process at 45°C using commercial cellulase (Barron et al. 1997; Boyle et al. 1997). The barley straw was pretreated with 5M NaOH. Theoretical yields from these studies were not determined, but they were able to produce a maximum of 12 g/L ethanol from 60 g solids/L using barley straw supplemented with nutrient medium and 10.5 g/L ethanol from 60 g solids/L using straw supplemented with whiskey distillery spent wash (Barron et al. 1997; Boyle et al. 1997). There have been no studies in utilization of IMB4 for cellulosic ethanol production reported thus far. Using one or more of IMB strains in an SSF process may allow temperatures closer to ideal for cellulose hydrolysis to be used, thus increasing the hydrolysis rate and reducing SSF time and/or enzyme usage. Conventional SSF utilizing non-thermotolerant yeasts must

maintain temperature between 25 to 35°C by cooling the reactor to prevent heat-inactivation of yeast cells (Banat et al. 1992). SSF using IMB4 may reduce cooling cost and fermentation time, which may offer a more economical process. Considering the advantages mentioned above, it is of interest to investigate the use of IMB4 in SSF of hydrothermolysis-pretreated switchgrass at 45°C.

Chapter 4

HYDROTHERMOLYSIS OF KANLOW SWITCHGRASS IN PREPARATION FOR SSF BY *K. MARXIANUS IMB4*

The following chapter is in a format for submission to a peer-reviewed publication. Miss Lilis Suryawati is the first author and main contributor to the chapter, followed by Dr. Mark R. Wilkins, Miss Suryawati's thesis advisior and primary investigator for the research, Dr. Danielle D. Bellmer, co-PI for the research, Dr. Niels O. Maness and Dr. Raymond L. Huhnke, thesis committee members whom assisted in the design of the research and preparation of the manuscript, and Dr. Ibrahim M. Banat, who provided IMB4 for the study.

4.1 Introduction

Over the past decade, numerous studies have been devoted to bioconversion of cellulosic biomass to fuel ethanol due to increasing interest in development of renewable energy resources (Mosier et al. 2005a). The fuel ethanol industry in the US uses corn and other cereal grains. With the expansion of ethanol production in the nation, concerns are arising about corn demand for biofuels production competing with corn for food production and livestock feed. Cellulosic biomass can provide alternative, inexpensive and abundant natural resources that could sustain the ethanol industry. One of the major challenges in cellulosic ethanol technology is the complexity and expensive cost of

pretreatment processes (Lynd et al. 1996; Wooley et al. 1999). Unlike grains, fermentable sugars in cellulosic biomass are not readily extracted. Most of the sugars in cellulosic biomass are stored as structural carbohydrate polymers such as cellulose and hemicellulose. Cellulose is highly crystalline and resistant to depolymerization (Fan et al. 1982).

In addition, these polysaccharides are protected by lignin, which gives cellulosic biomass its rigid structure and resistance to microbial degradation (Adler 1977). Crystalline cellulose, lignin, and covering of cellulose fiber by hemicellulose hamper the efficiency of cellulose conversion to ethanol (Hsu et al. 1980). Past studies have reported the importance of pretreatment of cellulosic biomass prior to enzymatic hydrolysis to improve efficiency of cellulose hydrolysis and reduce the amount of expensive enzyme (Mosier et al. 2005a; Wyman 1999).

Cellulosic biomass requires physical and/or chemical treatment to disrupt lignin, dissolve hemicellulose, and increase accessibility of cellulose to hydrolysis enzymes (Mosier et al. 2005a). Various pretreatment technologies have been developed including dilute acid, ammonia, steam explosion, and hydrothermolysis (Wyman et al. 2005). Of these, dilute acid is one of the most widely developed and used in many experiments due to its high hemicellulose solubilization and high cellulose digestibility. However, the drawback of this method is formation of toxic products that cause inhibition of fermentative microorganisms. High temperature pretreatment conditions tend to degrade sugars causing formation of furfural and 5-hydroxymethyl furfural (HMF) (Palmqvist and Hahn-Hagerdal 2000). Furthermore, cost of acid and the cost of acid neutralization after the process make dilute acid treatment less cost effective (Mosier et al. 2005a).

To overcome the disadvantages of dilute acid, hydrothermolysis, which is pretreatment using pressurized liquid hot water, can be used alternatively. Pretreatment of cellulosic material by hydrothermolysis has several advantages. Hydrothermolysis could minimize formation of toxic products during pretreatment (Allen et al. 1996). Additionally, neither acid nor base is used in this technique, hence reducing chemical cost and the need for neutralization, and corrosion of process equipment. Many previous hydrothermolysis studies of various feedstocks reported increased hemicellulose and cellulose solubilization and high hemicellulose recovery (Liu and Wyman 2005; Mok and Antal 1992; Mosier et al. 2005a; Walch et al. 1992; Yang and Wyman 2003).

The main objective in this investigation was to determine optimum pretreatment temperature and residence time for hydrothermolysis-pretreated Kanlow switchgrass based on cellulose digestibility. Cellulose digestibility was determined by utilizing the hydrothermolysis-pretreated Kanlow switchgrass in subsequent SSF. A thermotolerant, ethanol producing *K. marxianus*, IMB4 yeast was used as fermenting organism in SSF at 45°C. IMB4 was first isolated from an Indian distillery in India (Banat et al. 1992) and was reported growing at temperatures up to 52°C and demonstrated ethanol tolerance greater than 7.5% (w/v) (Banat and Marchant 1995). By conducting SSF at high temperature using thermotolerant yeast, cellulose hydrolysis can be performed at temperatures closer to optimum cellulose saccharification while maintaining cell viability. Therefore, faster cellulose hydrolysis rate and shorter SSF time can be achieved.

4.2 Materials and Methods

4.2.1 Sample preparation

Kanlow switchgrass was harvested from the Oklahoma State University Research Center and milled with a hammer mill fitted with a 13 mm screen (Model E9506, Bliss Industries, Ponca City, OK). The biomass was stored in a sealed plastic bag and kept refrigerated (4°C) for use in all experimentation. Switchgrass was grounded to 2 mm particle size using Thomas-Wiley mill prior to compositional analysis (Model 4, Arthur H. Thomas Co., Philadelphia, PA). Compositional analysis of switchgrass before pretreatment and fermentation was performed using the National Renewable Energy Laboratory (NREL) procedures LAP-001, 002, and 005 (Sluiter et al. 2004a; Sluiter et al. 2004b; Sluiter et al. 2004c). Absorbance reading of acid soluble lignin (ASL) was taken at 205 nm using a UV-Visible spectrophotometer (Cary 50 Bio, Varian Inc., U.S.A) with high purity quartz cuvettes of pathlength 1 cm (Hellma Cells Inc., Plainview, NY). The suggested 205 nm wavelength (λ) and absorptivity (ε) of switchgrass (110 L/g cm) were chosen based on previous work by Thammasouk (1997) .

Prior to determination of structural carbohydrates and lignin in biomass, a two-step extraction process was performed using an NREL procedure (Ruiz et al. 2005). Automatic extraction by ethanol followed by water was conducted using a Dionex Accelerated Solvent Extractor, ASE® 300 system (Dionex Corporation, Sunnyvale, CA). The operating parameters for both steps were 1500 psi at 100°C, 150% flush volume, 7 min static time, 120 sec purge time, and 3 static cycles. All extractions were done in duplicate in 33 mL extraction cells using 95% ethanol and distilled water for ethanol and water extractions, respectively. Removal of solvents from extractives was done using a

RapidVap® N2 Evaporation System (Labconco Corporation, Kansas City, MS) set at 500 mbar and 40°C until all solvents evaporated. Evaporation took approximately 24h and 48h for ethanol and water, respectively. Extracted switchgrass solids were air dried for at least 24 h prior to use in subsequent analysis of structural carbohydrates and lignin.

4.2.2 Hydrothermolysis

Hydrothermolysis of switchgrass was conducted in a 1-L bench top stirred reactor and pressure vessel (Parr Series 4520, Parr Instrument Company, Moline, IL) equipped with a propeller agitator and a temperature controller as depicted in Figure 4.1. The reactor was filled with 66.52 g switchgrass (90.2% dry matter) and 533.48 g deionized water to achieve a 10% dry matter mixture. Three reaction temperatures (190, 200, and 210°C) and hold times (10, 15, and 20 min) were used to pretreat Kanlow switchgrass. The agitator was set at 150 rpm. The warm up period to reach the desired temperature was between 30 and 40 min. After the desired temperature was reached, the sample was held at temperature for the specified hold time. At the end of pretreatment, the reactor was quickly transferred into an ice bath for cooling. Subsequently, the entire reactor contents were emptied and separated into liquid and solid fractions by vacuum filtration using a Buchner funnel lined with Whatman #5 filter paper (Whatman plc, Brentford, UK).

The reaction mixture was washed repeatedly with deionized water to remove residual glucose and inhibitors until filtrate pH was greater than 4.5. The solid and liquid fractions were stored in sealed glass jars and refrigerated at 4°C until ready for use. Determination and quantification of structural carbohydrates, sugars, byproducts, and

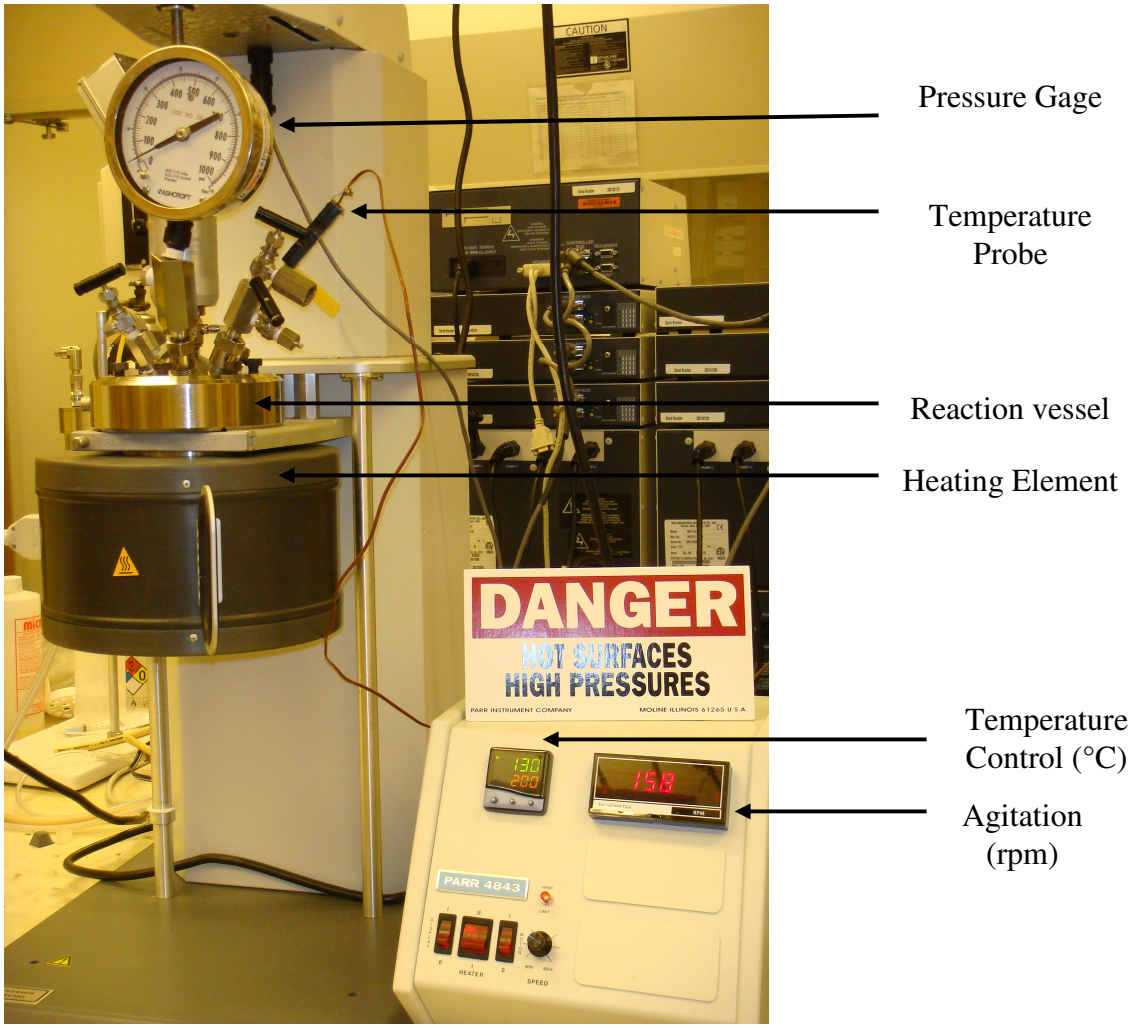

Figure 4.1 Experimental set up of hydrothermolysis of switchgrass conducted in 1-L bench top stirred reactor and pressured vessel (Parr Series 4520).

degradation products in insoluble solids and liquid prehydrolyzate from pretreatment were conducted according to NREL procedures LAP 002 and 014 using HPLC with Aminex HPX-87H (organic acids and furfurals) and HPX-87C (sugars) columns (Bio-Rad, Hercules, Cal., USA) with refractive index detection (1100 Series, Agilent, Santa Clara, Cal., USA) (Sluiter et al. 2004b; Sluiter et al. 2004c).

4.2.3 Microorganism and inoculum preparation

K. marxianus IMB4 was grown on liquid yeast peptone dextrose (YPD) medium containing (g/L): yeast extract 10.0, peptone 20.0, and glucose (dextrose) 50.0. Nutrients were obtained from Fisher Scientific (Pittsburgh, PA, USA). A loopful of IMB4 culture was aseptically transferred into 250 mL baffled culture flasks containing 100 mL of YPD medium covered with an aerobic stopper. The IMB4 inoculum was incubated at 45°C for 18 h while being rotated 130 rpm on a shaker. The cells were collected by centrifugation at 3750 rpm for 7 minutes. The supernatant was decanted and cells were resuspended in distilled water and centrifuged again twice. Finally, cells were resuspended in distilled water to give final OD of 6.0 (Dowe and McMillan 2001).

4.2.4 Enzyme Activity Measurement

Commercial cellulase from Iogen (Fibrilase, Ottawa, Canada) was used for SSF. Cellulase activity was measured using the filter paper standard assay procedure developed by the International Union of Pure and Applied Chemistry (Ghose 1987). Whatman No. 1 filter paper was cut into 1.0 x 6.0 cm strips weighing 50 ± 5 mg. Dinitrosalicylic Acid (DNS) reagent was used to estimate the reducing sugar released from filter paper assay. Stock solutions of 50 mM citrate buffer, pH 4.8, and 10 mg/mL

anhydrous glucose were prepared and stored at refrigerated temperature. Three enzyme dilutions (1:150, 1:175, and 1:200) were prepared using citrate buffer. Dilutions were chosen such that one of the dilution released glucose slightly above and another below 2.0 mg. Four glucose dilutions at concentrations of 6.7, 5.0, 3.3, and 2.0 mg/mL were prepared from the glucose stock using citrate buffer. For the calibration curve, 0.5 mL of each glucose dilution and 1mL of citrate buffer were added into glucose standard test tubes.

Enzyme sample tubes contain 0.5 mL of each enzyme dilution and 1 mL of citrate buffer. Enzyme blanks for each enzyme dilution and spectro zero (buffer blank) were also prepared. All sample tubes were incubated in a water bath set at 50 ± 3°C for 60 min. Filter paper strips were added only to enzyme sample tubes and the strip was completely immerse in the solution. At the end of 60 min, 3 mL of DNS reagent was added to all tubes. All standards, enzyme blanks, enzyme samples and the spectro zero blank were boiled together for exactly 5 min to inactivate the enzyme and quickly transferred into an ice water bath. Twenty mL of distilled water was added to all tubes and mixed thoroughly by inverting the tubes several times. Samples were held for 20 min at room temperature and a 4 mL aliquot was pipetted into disposable polypropylene cuvettes. Color absorbance was taken at 540 nm using a UV-Spectrophotometer (Cary 50 Bio, Varian Inc., U.S.A). Three replicates of filter paper assays were performed and the average cellulase activity was reported as FPU/mL enzyme, with an FPU = 1 μmol glucose released/min.

4.2.5 Simultaneous Saccharification and Fermentation and Analyses

Yeast fermentation medium (YFM) was prepared using deionized water consisting of (g/L): yeast extract, 5.0; KH_2PO_4, 20.0, $(NH_4)_2SO_4$, 20.0; $MgSO_4.7H_2O$, 10.0; and $MnSO_4.H_2O$, 1.0 (Banat et al. 1992). Commercial cellulase with activity of 67 FPU/mL was used for hydrolysis of solid substrate. SSF was conducted in 250 mL baffled flasks sealed with a rubber stopper fitted with a 1-way air valve to maintain an anaerobic environment as shown in Figure 4.2. SSF was conducted according to NREL procedure LAP 008 (Dowe and McMillan 2001) modified as described here. Each fermentation flask contained 10 mL YFM, 5 mL 1M sodium citrate buffer at pH 4.8, washed, pretreated switchgrass to provide 41 g/L glucan, 15 FPU/g glucan of cellulase, and 10 mL yeast inoculum with optical density of 6.0. The initial cell density of IMB4 in each flask was 0.17 g/L. Deionized water was added to bring the total volume to 100mL.

All flasks were incubated at 45°C while being rotated at 130 rpm on a shaker. Aliquots of 4.5mL were taken at 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h and frozen immediately. To account for ethanol produced from sugars present in the commercial cellulase mixture, duplicate fermentations using media with same composition as other SSF flasks, excluding the washed pretreated switchgrass, were included in the experiment. The ethanol concentration produced in the enzyme control was subtracted from the final ethanol concentration produced in SSF. For analyses, samples were thawed and centrifuged at 13,000g for 12 min twice. Supernatant was collected, filtered through a 25mm diameter, 0.2μm syringe filter from Fisher Scientific (Pittsburgh, PA, USA), and analyzed for ethanol using GC packed column (Porapak QS C-5000 AT-Steel, Alltech, Deerfield, IL, USA) with 200°C oven temperature, 30 mL/min H_2 flow, 25 mL/min N_2,

Figure 4.2 Experimental set up of SSF of hydrothermolysis-pretreated switchgrass at 45°C using orbital shaker agitated at 130 rev/min.

400 mL/min air flow and detected by a flame ionization detector at 250°C (Agilent, Palo Alto, CA, USA). Glucose, xylose, cellobiose, and organic acids were analyzed with HPLC on HPX-87H column (Bio-Rad, Sunnyvale, Ca.) with 0.01 N H_2SO_4 as eluent, 0.6 mL/min flow rate at 60°C using refractive index detection (1100 Series, Agilent, Santa Clara, Cal., USA).

The theoretical yield of ethanol production was calculated as follows:

$$\% \text{ Theoretical Yield} = \frac{[\text{EtOHt}] - [\text{EtOHo}]}{0.511 \times (\text{f } [\text{Biomass}] \times 1.11)} \times 100\%$$

where [EtOHt] is the concentration of ethanol at time t, [$EtOH_0$] is the initial ethanol concentration, f is glucan fraction of dry biomass (g/g), [Biomass] is dry biomass concentration at the beginning of fermentation (g/L), 0.511 is the conversion factor for glucose to ethanol, and 1.11 is the conversion factor for glucan to glucose. Combination of three levels of pretreatment temperatures (190°C, 200°C, and 210°C) and hold times (10, 15, 20 min) resulted in nine pretreatment conditions. One additional pretreatment condition at 200°C for 20 min was repeated unintentionally, resulting in a total of ten different pretreated switchgrass substrates used for SSF. Two fermentations per pretreated switchgrass batch were carried out resulting in a total of 20 fermentations.

4.3 Results and Discussion

4.3.1 Switchgrass Composition

Ethanol-water extraction of switchgrass resulted in 12.46% (db) extractives, of which 7.27% were extracted by ethanol and 5.19% were extracted by water. The final composition of native switchgrass was 8.53% moisture and the dry matter was 36.56% glucan, 21.05% xylan, 0.99% galactan, 2.76% arabinan, 0.79% mannan, 16.26% Klason

lignin, 1.96% acid soluble lignin, 12.46% extractives, and 4.95% ash. These results are similar to work reported by Thammasouk et al. (1997). Compositional analysis of non-extracted native switchgrass showed higher percentages of glucan and xylan than extracted samples (data not shown). Additionally, the presence of extractives overestimated the Klason lignin value in unextracted samples (Thammasouk et al. 1997). It was suggested that high lignin value was attributed to condensation of extractives under harsh condition during lignin measurement (Browning 1967). Total glucan in extracted switchgrass was lower because extraction removed non-structural carbohydrates such as low molecular weight sugars that were soluble in ethanol/water (Theander 1991). Extraction therefore impacts subsequent compositional analysis of native switchgrass and provides a more accurate analysis of lignin and structural carbohydrates in switchgrass that are available for conversion to ethanol.

4.3.2 Hydrothermolysis

Glucan composition of washed pretreated solids increased compared to the native switchgrass. Hydrothermolysis pretreatment of switchgrass increased glucan content in the solids 39 to 83 %. Pretreated solids contained 51 to 67% glucan (Figure 4.3) as compared to 36.56% in native switchgrass. At 10 min hold time, glucan contents of insoluble solids pretreated at 190, 200, and 210°C were 57.1, 51.3, and 58.3%, respectively. At 15 min hold time, the glucan contents of insoluble solids pretreated at 190, 200, and 210°C were 57.2, 63.2., and 58.3%, respectively. At 20 min hold time, the glucan contents of insoluble solids pretreated at 190, 200, and 210°C were 64.3, 63.0, and 58.3%, respectively. Glucan content of insoluble solids pretreated at 190°C, 20 min was the greatest and glucan content of insoluble solids pretreated at 200°C, 10 min was the

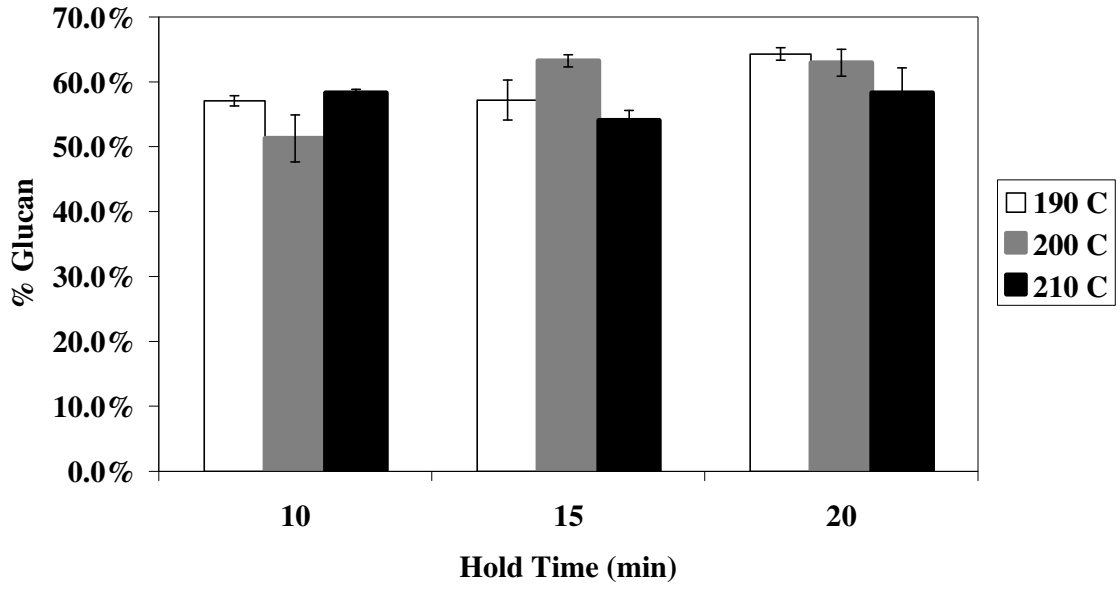

Figure 4.3 Glucan content of washed pretreated switchgrass at various temperatures and hold times by 4% dilute sulfuric acid hydrolysis. Error bars indicate +/- 1 standard deviation. All values are average of two replicates.

least among the pretreated switchgrass samples. Glucan content in the prehydrolyzate was less than 2.0 g/L for all conditions, indicating most of the glucan was preserved as a polymer in the insoluble solids.

Xylan solubilization and recovery as xylose monomers or oligomers in prehydrolyzate decreased with increased pretreatment temperature and hold time as illustrated in Figure 4.4. For pretreatments at 190°C, xylan recovery was 73.1% for 10 min, 66.8% for 15 min, and 47.9% for 20 min. For pretreatments at 200°C, xylan recovery was 23.3% for 10 min, 21.0% for 15 min, and 8.2% for 20 min. For pretreatments at 210°C, xylan recovery was 3.8% for 10 min, 1.5% for 15 min, and 1.8% for 20 min. Xylose monomers recovered in prehydroyzate was shown in Figure 4.5. The greatest xylose concentration was found in in prehydrolyzate from pretreatment at 190°C for 20 min. Xylose recovery as monomers decreased with increasing hold time at 200°C. Xylose concentrations from 210°C pretreatment at all hold times were lower than those at 190 and 200°C. Xylan recovery as monomers increased when hold time increased from 15 to 20 min. Almost all xylan recovered over 90% in 200°C and 210°C samples were monomers.

Greater preservation of xylose as oligomers was observed at 190°C than at 200°C or 210°C (Figures 4.4 and 4.5). For 190°C pretreatments, 87.6%, 77.9%, and 47.3% of xylan recovered were oligomers at 10, 15, and 20 min, respectively. Preservation of glucan and xylan as oligomers has been observed to reduce HMF and furfural formation (Weil et al. 1998). Pretreatment results in greater solubilization of hemicellulose than cellulose during hydrothermolysis because hemicellulose is more amorphous and less stable than cellulose (Mok and Antal 1992). Liquid water at elevated temperature has

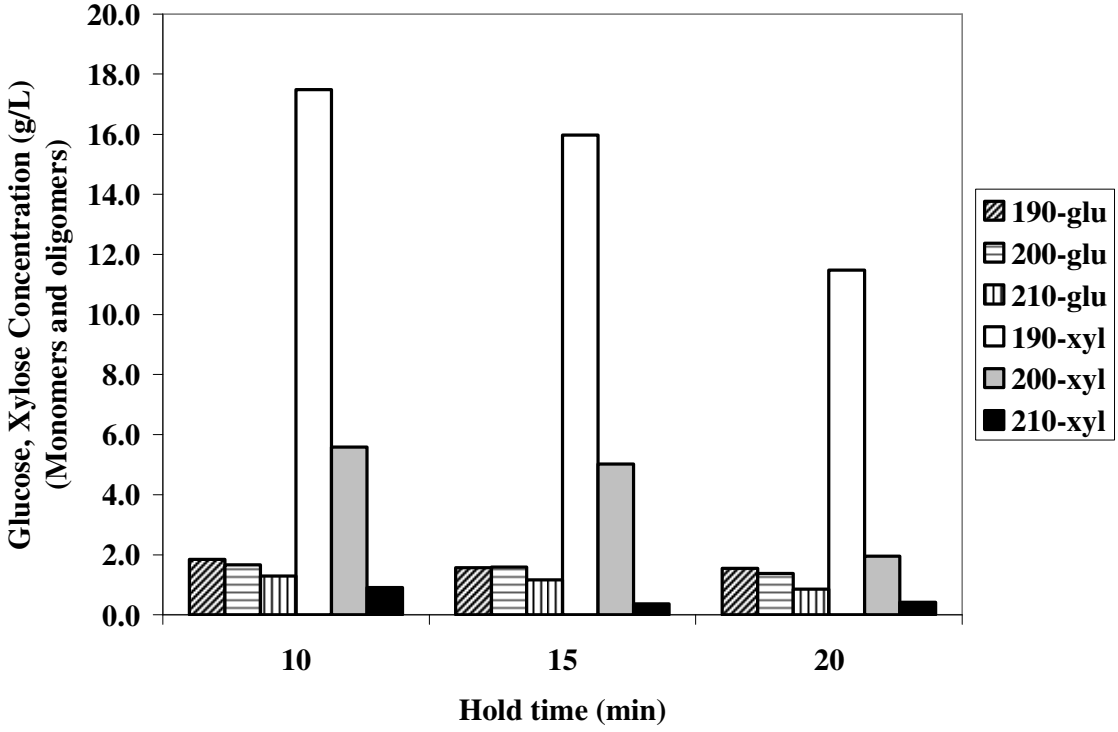

Figure 4.4 Monomer and oligomers xylose and glucose concentrations in prehydrolyzate from hydrothermolysis of switchgrass at various temperature and hold times detected by HPLC without further sample treatment.

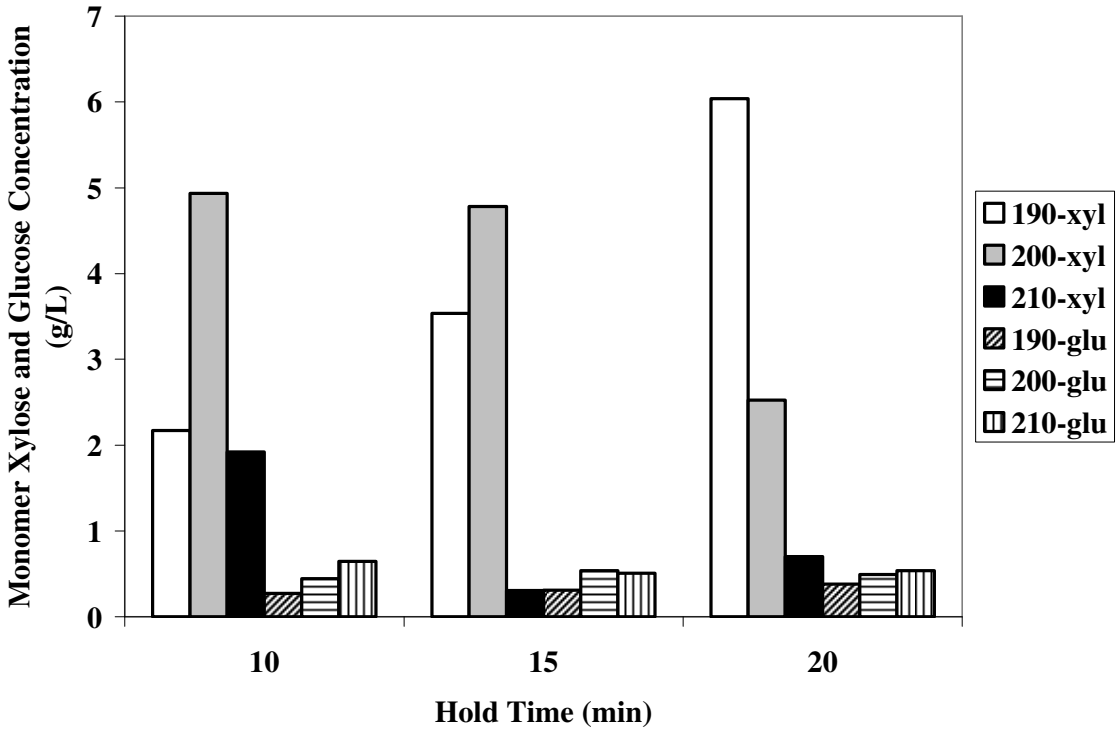

Figure 4.5 Monomer xylose and glucose concentrations in prehydrolyzate from hydrothermolysis of switchgrass at various temperature and hold times detected by HPLC without further sample treatment.

elevated levels of hydronium ions, which acts as an acid and hydrolyzes the polysaccharides in biomass (Antal Jr. 1996).

Less than 1 g/L of the sugar degradation compounds and yeast inhibitors HMF and furfural were measured in prehydrolyzate (Figure 4.6). Xylan measured in insoluble solids and inhibitors formed do not account for all the xylose not recovered in prehydrolyzate. It is likely that xylan not accounted for was volatilized as furfural or degraded into products not identified by HPLC (Allen et al. 2001). Glucose and xylose liberated from hemicellulose and cellulose can be degraded to form 5-hydroxymethylfurfural (HMF) and furfural, respectively, at the temperatures used in this study (Palmqvist and Hahn-Hagerdal 2000). HMF concentration increased with increasing temperature. The greatest concentration was detected at 210°C, 20 min (0.79 g/L) (Figure 4.6). Pretreatment at 190°C resulted in less than 0.15 g/L HMF at all three hold times. Pretreatment at 200°C for 10 and 15 min resulted in similar HMF concentrations (~0.32 g/L), but HMF increased to 0.51 g/L for 20 min.

HMF concentration was greatest among temperatures at 210°C for all three hold times, and HMF increased with increasing hold time in 210°C prehydrolyzates. Furfural concentration was the greatest at 200°C, 20 min. Furfural concentrations in prehydrolyzate from pretreatment at 190°C at all three hold times were less than those found in 200°C and 210°C treatments. Furfural concentration increased as hold time increased at all temperatures, except at 210°C. At 10 and 15 min hold time, furfural concentrations at 210°C were 0.91g/L and 0.86g/L, respectively, which were greater than at 190°C and 200°C. At 210°C furfural concentration decreased as hold time increased.

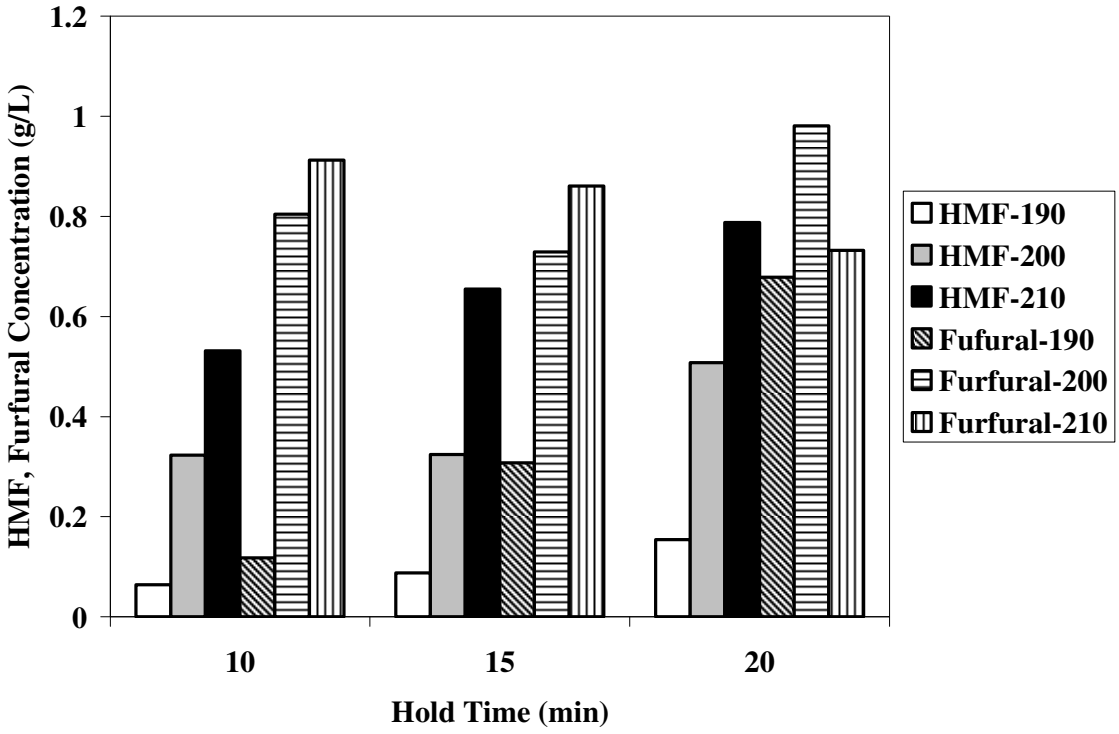

Figure 4.6 Concentration of HMF and furfural in prehydrolyzate from hydrothermolysis of switchgrass at various temperatures and hold times detected by HPLC without further sample treatment.

A lower furfural concentration was observed at 20 min hold time at 210°C than at 200°C. This suggests that furfural could have been further degraded to other compounds not detected by HPLC at more severe pretreatment conditions. Furfural and HMF can be degraded further to formic and levulinic acid during cellulosic hydrolysis (Dunlop 1948; Ulbricht et al. 1984).

Overall, all treatment conditions produced less than 1 g/L HMF and furfural. Additionally, acetic acid formation was observed in prehydrolyzate (Figure 4.7). Higher pretreatment temperature resulted in more acetic acid. Concentration of acetic acid increased from 2.0 to 3.4 g/L as hold time increased from 10 to 20 min at 190°C. At 200°C acetic acid concentration increased from 3.4 to 4.0 g/L when hold time increased from 10 to 15 min. However, increasing hold time to 20 min did not increase concentration of acetic acid. Prehydrolyzate at 210°C showed decreased acetic acid concentration from 10 to 15 min and increased at longer hold time. As much as 5 g/L was measured at 210°C (Figure 4.7). Such conditions are not preferable due to the inhibitory effect of acetic acid to yeast cell growth (Maiorella et al. 1983; Palmqvist and Hahn-Hagerdal 2000).

4.3.3 Simultaneous Saccharification and Fermentation of Hydrothermolysis-pretreated Switchgrass Using IMB4

Figure 4.8 shows glucose and cellobiose concentration over the course of each SSF. Enzymatic hydrolysis of cellulose to glucose first produced a disaccharide, cellobiose, which was then hydrolyzed to a monosaccharide, glucose. Cellobiose remained relatively constant throughout fermentation as it was hydrolyzed to glucose

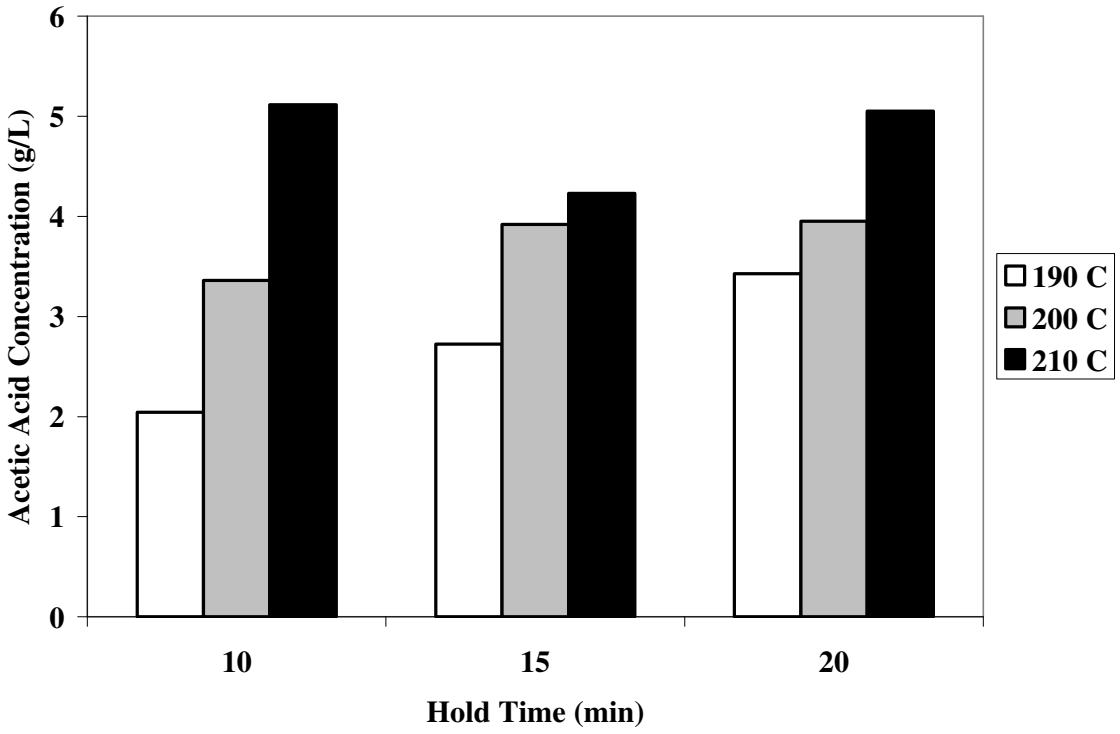

Figure 4.7 Concentration of acetic acid in prehydrolyzate from hydrothermolysis of switchgrass at various temperatures (°C) and hold times detected by HPLC without further sample treatment.

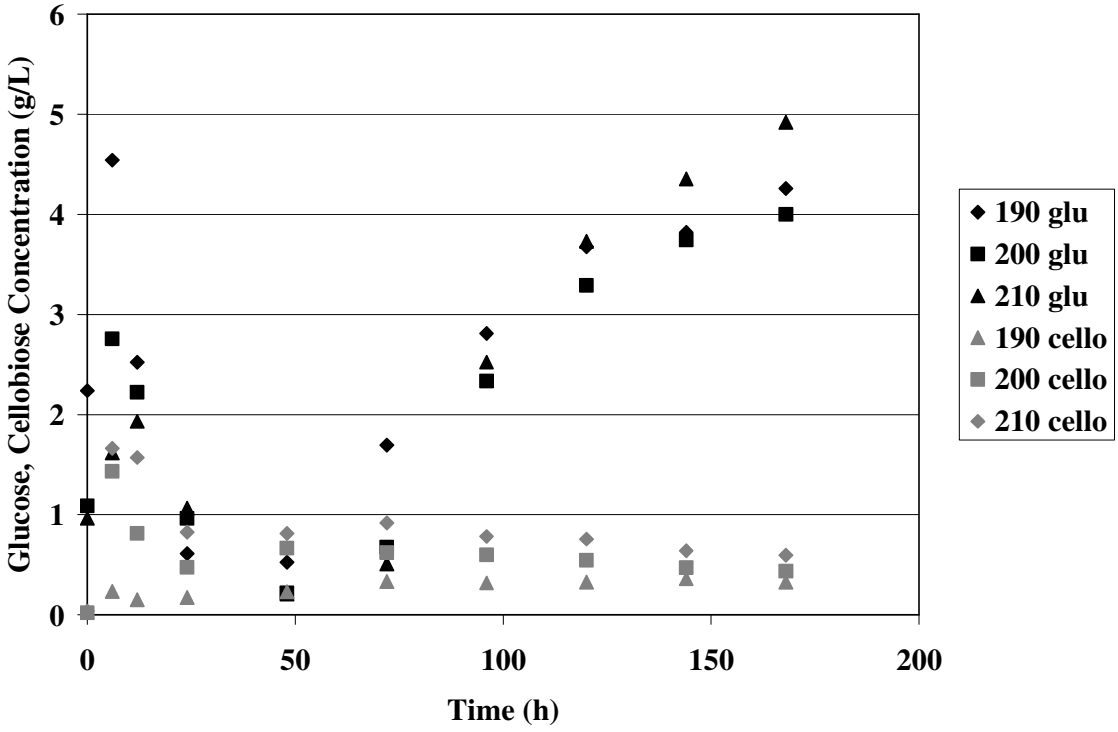

Figure 4.8 Mean residual glucose and cellobiose concentration during 168 h simultaneous saccharfication and fermentation using IMB4 at 45°C with 4.1% glucan loading from nine pretreated substrates and 15 FPU/ g glucan cellulase loading. All values are averages of duplicate samples.

continuously, indicating sufficient beta-glucosidase activity in the cellulase preparation. Glucose from enzymatic hydrolysis was rapidly consumed by IMB4, indicated by a decrease in glucose from 0 to 72h. After 72h, glucose concentration increased while ethanol concentration remained relatively constant, indicating cessation of fermentation.

Figure 4.9 shows the percent theoretical ethanol yield after 72h. After 72h, ethanol concentrations from substrate pretreated at 200°C and 210°C for all three hold times were greater than ethanol concentrations at 190°C. Theoretical ethanol yield was from 29 to 74%. The maximum ethanol concentration that could be theoretically produced was 23.2 g/L (41g glucan/L * 0.568). The greatest theoretical yield observed was 74.2% (17.3 g/L) at 200°C, 10 min, followed by 72.4% (17.1 g/L) at 210°C, 15 min. The lowest theoretical yield observed was 29.1% (6.8 g/L) for pretreated solids at 190°C, 10 min. For SSF of switchgrass pretreated at 190 and 210°C, ethanol yield increased when pretreatment hold time increased from 10 to 15 min, but decreased from 15 to 20 min. For switchgrass pretreated at 200°C, a decrease in yield from 74.2% to 54.1% was observed as hold time increased from 10 to 15 min. Further increase in hold time to 20 min at 200°C resulted in an increase in yield to 60.4%. Greater ethanol yields suggest greater cellulose hydrolysis.

This result was similar to another study from using pH controlled liquid hot water pretreatment of corn stover (Mosier et al. 2005b). They observed an increase in glucose production from cellulose hydrolysis of pretreated corn stover at 190°C as hold time increased from 5 to 15 min, then a decrease in yield at a hold time of 20 min. Similarly, glucose production from cellulose hydrolysis of corn stover pretreated at 200°C increased

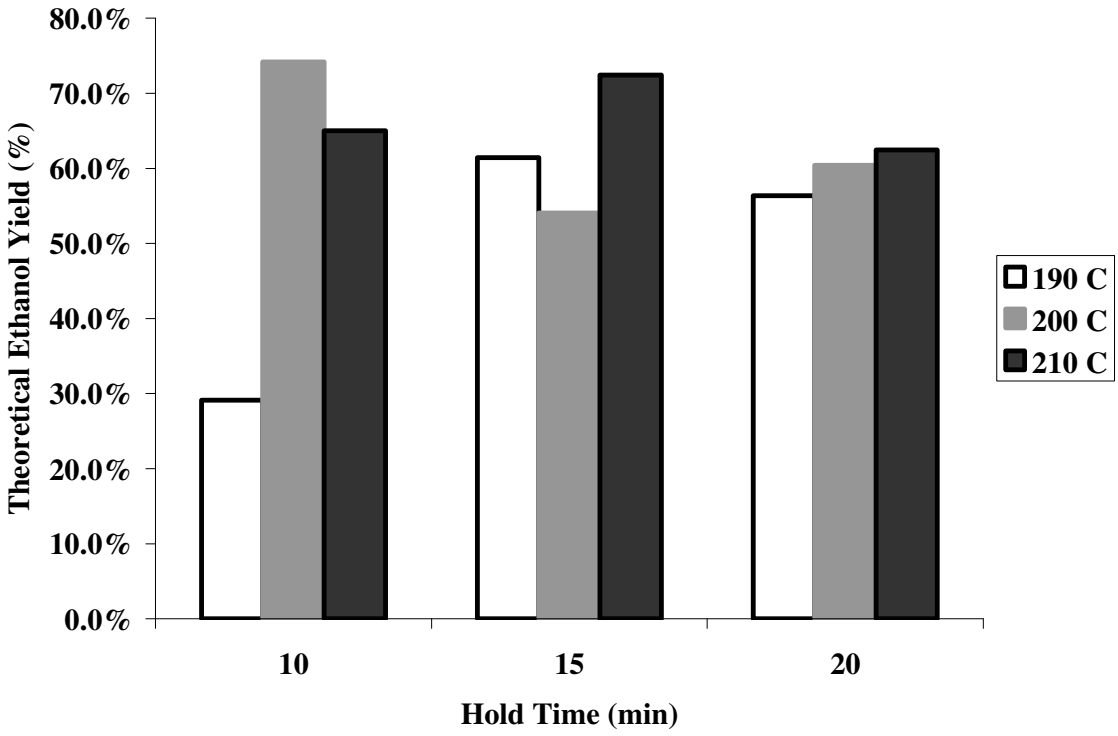

Figure 4.9 Maximum theoretical yield of ethanol of IMB4, after 72 h SSF at 45°C with 4.1% glucan loading and 15 FPU/ g glucan cellulase loading. All values are averages of duplicate samples.

as hold time increased from 5 to 10 min, but hydrolysis decreased sharply at longer hold times.

A study on SSF at 42°C of various pretreated lignocellulosic biomass using thermotolerant *K. marxianus* CECT 10875 reported ethanol yields similar to this study (Ballesteros et al. 2004). Researchers obtained 50 to 72% theoretical ethanol yield from 5% (w/v) steam-explosion pretreated biomass within 72 to 82h. Another study by Krishna et al. (2001) reported ethanol production between 2 to 2.5% (w/v) from SSF at 40°C of 10% (w/v) cellulosic wastes using thermotolerant *Kluyveromyces fragilis* NCIM 3358. Ethanol yields from the experiment were not determined because glucan content in the solid substrate was not provided. *K. marxianus* IMB4 used in this experiment were more thermotolerant than the *Kluyveromyces* strains discussed above. Compared to previous studies discussed above, IMB4 fermentation attained slightly higher yield at higher SSF temperature. Perhaps, this could be explained by better substrate digestibility and faster cellulose hydrolysis at 45°C, consequently, more glucose was fermented to ethanol.

Acetic acid concentrations produced during SSF are shown in Figure 4.10. Acetic acid concentration increased from 0 to 120h and remained relatively constant afterwards. After 72h, acetic acid concentration was found to be between 1 and 2 g/L. The greatest acetic acid concentration was formed during SSF of 210°C, 15 min pretreated substrate. At this point, it was uncertain whether acetic acid produced during SSF actually caused cessation of fermentation after 72h. Further investigation could explain more clearly the inhibitory effect of acetic acid and other fermentation products on IMB4.

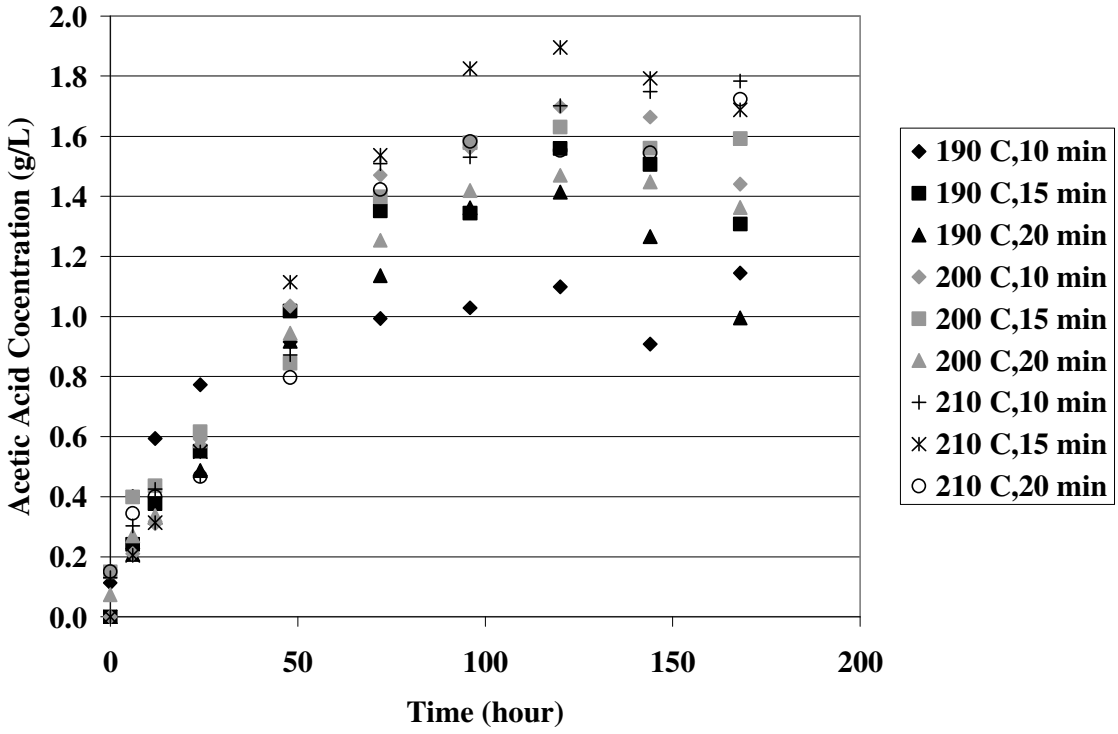

Figure 4.10 Acetic acid concentrations produced by thermotolerant Kluyveromyces marxianus, IMB4 during SSF at 45°C with 4.1 % glucan loading and 15 FPU/ g glucan cellulase loading. All values are averages of duplicate samples.

Based on results from numerous studies on SSF of cellulosic biomass, theoretical yields greater than 70% were considered desirable for selection of pretreatment conditions for future studies with switchgrass. Under this criterion, either 200°C, 10 min or 210°C, 15 min were possible treatment combinations for hydrothermolysis pretreatment of switchgrass. Of these, 200°C, 10 min was selected as the best pretreatment condition to prepare switchgrass for SSF based on lower pretreatment temperature requirements and reduced concentration of toxic compounds such as HMF and furfural as compared to 210, 15 min.

4.4 Conclusions

Switchgrass treated at 190ºC, 10 min had the greatest xylan recovery. Formation of glucose monomers was minimal during pretreatment, as most glucose was retained as cellulose in the solid substrate, reducing glucose degradation to HMF. Pretreatment at higher temperature and longer hold time decreased xylan recovery and increased concentrations of inhibitory compounds such as acetic acid, HMF, and furfural. HMF and furfural concentrations in the prehydrolyzate were less than 1 g/L from all treatments. Based on the ethanol yield from SSF, 200°C for 10 min was selected as the best hydrothermolysis condition for pretreatment of switchgrass. These parameters will be used in further studies to investigate SSF using *K. marxianus* IMB4.

Chapter 5

THE EFFECT OF FERMENTATION TEMPERATURE ON ETHANOL YIELD

The following chapter is in a format for submission to a peer-reviewed publication. Miss Lilis Suryawati is the first author and main contributor to the chapter, followed by Dr. Mark R. Wilkins, Miss Suryawati's thesis advisor and primary investigator for the research, Dr. Danielle D. Bellmer, co-PI for the research, Dr. Niels O. Maness and Dr. Raymond L. Huhnke, thesis committee members who assisted in the design of the research and preparation of the manuscript, and Dr. Ibrahim M. Banat, who provided IMB4 for the study.

5.1 Introduction

Industrial ethanol production in the US has grown vastly over the past decade. The Renewable Fuels Association (2005b) reported US ethanol production has increased from 175 million to 4 billion gallons within the past 15 years. Cellulosic biomass, such as switchgrass represents a potential alternative feedstock to meet increasing demand of fuel ethanol. Switchgrass, a perennial grass that is native to most of the US, was selected as a model energy crop by Department of Energy (Lynd et al. 1991; McLaughlin 1993). One unit operation in biological conversion of cellulosic biomass that has been intensely researched is simultaneous saccharification and fermentation (SSF). It has been documented that SSF reduces contamination risk due to the presence of ethanol and

eliminates the need for separate reactors, thus reducing capital costs (Nigam and Singh 1995; Philippidis et al. 1993).

Additionally, SSF increases the hydrolysis rate by reducing product inhibition as the sugars are rapidly consumed by yeast during SSF (Takagi et al. 1977). SSF is constrained by the different optimum temperatures for saccharification and fermentation (Chung et al. 2005; Grohmann 1993; Krishna et al. 1999). Saccharification by cellulase is optimum at temperature between 40 and 50°C; whereas, fermentation temperature using the most commonly used ethanolgenic yeast *S. cerevisiae* cannot exceed 38°C (Bollok et al. 2000). Above 40°C, the viability of yeast culture diminishes, which consequently affects ethanol yield.

One approach to improve SSF is by using thermotolerant, ethanol producing microorganisms to carry out SSF at higher temperature. Previous literature reported isolation of thermotolerant yeasts for potential use in SSF above 40°C (Banat et al. 1992; Szczodrak and Targonski 1988). Thermotolerant species of *Saccharomyces, Kluyveromyces, and Fabospora* genera have been observed growing at temperatures above 40°C and fermenting six carbon sugars at 40, 43, and 46°C, respectively (Szczodrak and Targonski 1988). Several researchers have experimented with thermotolerant strains of *S. cerevisiae*, *K. marxianus*, and *K. fragilis* for SSF of cellulosic substrate (Ballesteros et al. 1991; Ballesteros et al. 2004; Barron et al. 1997; Bollok et al. 2000; Boyle et al. 1997; Lark et al. 1997). Among these, thermotolerant *K. marxianus* IMB strains isolated by Banat and Marchant (1995) from Indian distillery in India are of particular interest. IMB yeasts were reported capable of growing and producing ethanol at temperatures of up to 50°C, which are more thermotolerant than other yeasts discussed

thus far in the literature. Moreover, in their investigation the authors indicated that the ethanol tolerance of several strains of IMB can reach 9.5% (w/v). Two previous studies used the IMB3 strain to convert barley straw to ethanol in an SSF process at 45°C (Barron et al. 1997; Boyle et al. 1997). Few studies have reported successful ethanol production using thermotolerant yeast at temperature as high as 45°C. Therefore, it is of interest to utilize one of the IMB yeast strain in SSF at 45°C, which is a temperature closer to ideal for cellulose hydrolysis. This may enhance the hydrolysis rate and reduce SSF time and/or enzyme usage. The present study investigated the effect of fermentation temperature on SSF of hydrothermolysis-pretreated switchgrass using IMB4 yeast and compared the results with SSF of the same substrate using ethanolgenic yeast *S. cerevisiae* D_5A. Ethanol yield from SSF using IMB4 at temperature 37, 41 and 45°C were investigated.

5.2 Materials and Methods

5.2.1 Substrate preparation and pretreatment

Sample preparation and pretreatment of Kanlow switchgrass were described in detail previously in Chapter 4. A 10% dry matter mixture of Kanlow switchgrass was subjected to a batch hydrothermolysis pretreatment in a 1-L bench top stirred reactor and pressure vessel (Parr Series 4520, Parr Instrument Company, Moline, IL) at 200°C for 10 min. Pretreatment conditions were selected with regard to lower operating temperature and residence time to minimize formation of inhibitory compounds during pretreatment and achieve greater than 70% theoretical ethanol yield as discussed previously in Chapter 4. Five batch pretreatments were conducted to prepare sufficient substrate for SSF experiments, and then the material was combined. Determination and quantification of

structural carbohydrates, sugars, byproducts, and degradation products in insoluble solids and liquid prehydrolyzate from pretreatment were conducted according to NREL procedures LAP 002 and 014 using HPLC with an Aminex HPX-87H (organic acids, furfurals, and sugars) column (Bio-Rad, Hercules, Cal., USA) with refractive index detection (1100 Series, Agilent, Santa Clara, Cal., USA) (Sluiter et al. 2004b; Sluiter et al. 2004c).

5.2.2 Microorganism and inoculum preparation

K. marxianus IMB4 and *S. cerevisiae* D_5A (ATCC, Manassas, VA, USA) were grown on liquid yeast peptone dextrose (YPD) medium containing (g/L): yeast extract 5.0, peptone 10.0, and glucose (dextrose) 50.0. All nutrients were obtained from Fisher Scientific (Pittsburgh, PA., USA). One loopful of IMB4 or D_5A cells grown on a YPD agar slant was added to 250 mL baffled culture flasks containing 100 mL of YPD medium covered with an aerobic stopper. IMB4 was incubated at 45°C and D_5A was incubated at 37°C for 18 h while being rotated 130 rpm on a shaker (Banat et al. 1992; Dowe and McMillan 2001).

5.2.3 Simultaneous Saccharification and Fermentation and Analyses

Yeast fermentation medium (YFM) was prepared using deionized water consisting of (g/L): yeast extract, 5.0; KH_2PO_4, 20.0, $(NH_4)_2SO_4$, 20.0; $MgSO_4.7H_2O$, 10.0; and $MnSO_4.H_2O$, 1.0 (Banat et al. 1992). Commercial cellulase (Fibrilase, Iogen, Ottawa, Canada) with activity of 62 FPU/mL, as determined by the procedure of Ghose (1987), was used for hydrolysis of solid substrate. SSF was conducted in 250 mL baffled flasks sealed with a rubber stopper fitted with a 1-way air valve to maintain an anaerobic

environment. SSF was conducted according to NREL procedure LAP 008 (Dowe and McMillan 2001) modified as described here. Each fermentation flask contained 10 mL YFM, 5 mL 1M sodium citrate buffer at pH 4.8, washed, pretreated switchgrass to provide 41 g/L glucan, 15 FPU/g glucan of cellulase, and 10 mL yeast inoculum with optical density of 5.0. Deionized water was added to bring the total volume to 100 mL. Initial cell density was 0.14 g/L and 0.2 g/L for IMB4 and D_5A, respectively.

All flasks were incubated at the specified temperature while being rotated 130 rpm on a shaker. Additional SSFs at 45°C using IMB4 were performed to investigate the effect of fermentation nutrients and pH on ethanol yield. In the nutrient experiment, SSF was conducted as before except the nutrient concentration was tripled. In the pH experiment, SSF was also performed as before except 50 mM sodium citrate buffer at pH 5.5 was used. Aliquots of 4.0 mL were taken at 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h and frozen immediately. At the end of fermentation, the pH of all fermentation slurries were recorded. To account for ethanol produced from sugars present in the commercial cellulase mixture, duplicate fermentations using IMB4 and D_5A were done without switchgrass, thus only the enzyme and nutrients provided substrate for the yeast. The ethanol concentration produced in the enzyme control was subtracted from the final ethanol concentration.

For analyses, samples were thawed and centrifuged at 13,000g for 12 min twice. Supernatant was collected, filtered through 0.2μm 13 mm syringe filter from Fisher Scientific (Pittsburgh, Pa., USA), and analyzed for ethanol, organic acids, and sugar residues (cellobiose, glucose, and xylose) by HPLC on an HPX-87H column (Bio-Rad, Sunnyvale, Ca.) with 0.01 N H_2SO_4 as solvent, 0.6 mL/min flow rate at 60°C using

refractive index detection (1100 Series Agilent, Santa Clara, Cal., USA). Previously in Chapter 4, ethanol analysis was done using GC. However, due to inconsistent repeatability of GC results when these experiments were conducted, ethanol analysis was done on HPLC instead.

The percent cellulose conversion or theoretical yield of ethanol production was calculated as follows:

$$\% \text{ Theoretical Yield} = \frac{[\text{EtOHt}] - [\text{EtOHo}]}{0.511 \times (\text{f } [\text{Biomass}] \times 1.11)} \times 100\%$$

where [EtOHt] is the concentration of ethanol at time t, $[EtOH_0]$ is the initial ethanol concentration, f is glucan fraction of dry biomass (g/g), [Biomass] is dry biomass concentration at the beginning of fermentation (g/L), 0.511 conversion factor for glucose to ethanol and 1.11 is the conversion factor for glucan to glucose.

5.2.4 Experimental design and statistical analysis

All treatment conditions were repeated in triplicate. However, one of the replicates from the IMB4 pH 5.5 experiment stopped fermenting sugar before 48h. It was uncertain what caused the problem since there was no indication of yeast inhibition when observing acetic acid results in SSF. Therefore, only two SSF results were obtained for the pH 5.5 experiment. The experiment could not be repeated due to lack of pretreated switchgrass. Mean ethanol yields and specific ethanol productivities of SSFs at different temperatures, nutrient concentration, and fermentation pH were analyzed statistically using Dunnett's comparison to the control at a 95% confidence level ($p<0.05$) with the control being *S. cerevisiae* D_5A (Kuehl 2000).

5.3 Results and Discussions

5.3.1 Hydrothermolysis

The composition of native switchgrass was 8.53% moisture and the dry matter was 36.56% glucan, 21.05% xylan, 0.99% galactan, 2.76% arabinan, 0.79% mannan, 16.26% Klason lignin, 1.96% acid soluble lignin, 12.46% extractives, and 4.95% ash. Approximately 43.9% (dry weight) of switchgrass was solubilized during hydrothermolysis. The average glucan and xylan content available for SSF in all five batch hydrothermolysis-pretreated solids at 200°C for 10 min were 56.6% and 2.39%, respectively. Hydrothermolysis resulted in greater solubilization of hemicellulose than cellulose, 13.2% glucan and 93.7% xylan were solubilized during pretreatment. Figure 5.1 shows concentrations of HMF and furfural detected in prehydrolyzate from hydrothermolysis. Hydrothermolysis at 200°C for 10 min was able to minimize fermentation inhibitors such as HMF and furfural. The prehydrolyzate from five hydrothermolysis batches contained 0.15 to 0.29 g/L HMF and 0.64 to 1.0 g/L furfural. Acetic acid concentration in the prehydrolyzate ranged from 3.38 to 3.90 g/L (Figure 5.2). High acetic acid concentration is undesirable as it can inhibit fermentative microorganisms (Palmqvist and Hahn-Hagerdal 2000).

During hydrothermolysis, acetic acid was generated as a result of cleavage of hemiacetal linkages (Antal Jr. 1996); whereas furfural and HMF were formed from degradation of xylose and glucose monomers. The glucose and xylose monomers found in prehydrolyzate were 0.39 and 4.4 g/L, respectively. Xylan and glucan recovery as

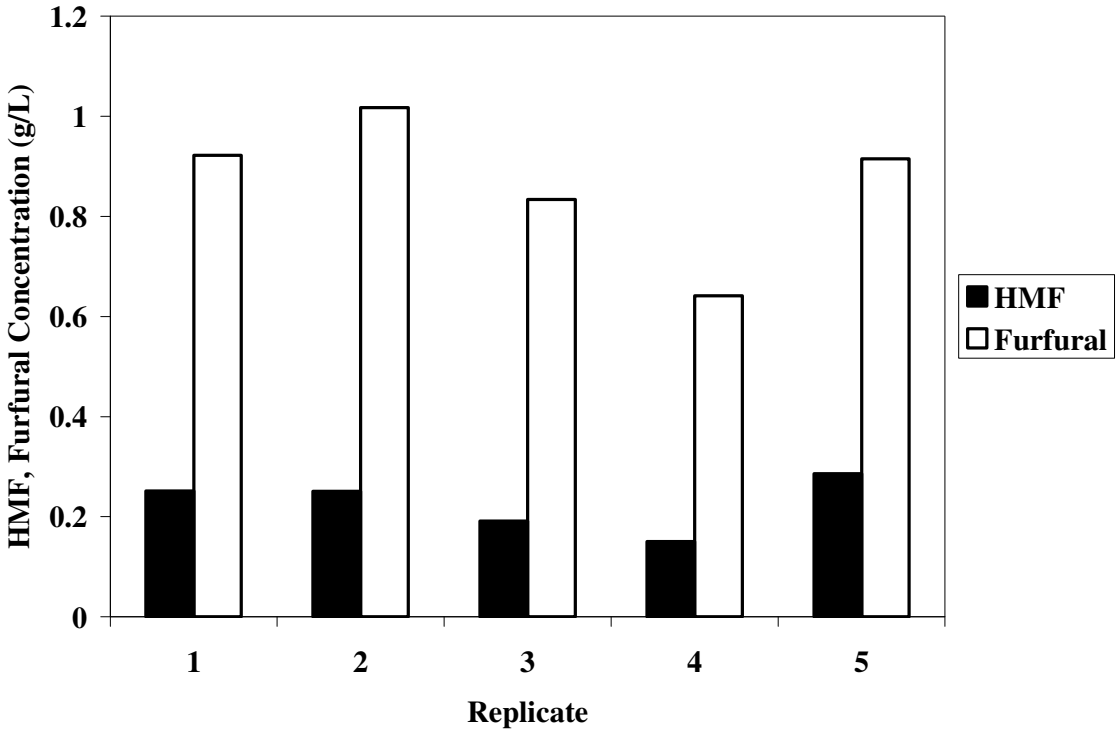

Figure 5.1 HMF and furfural concentration in liquid prehydrolyzate from five hydrothermolysis batches of Kanlow switchgrass at 200°C for 10 min.

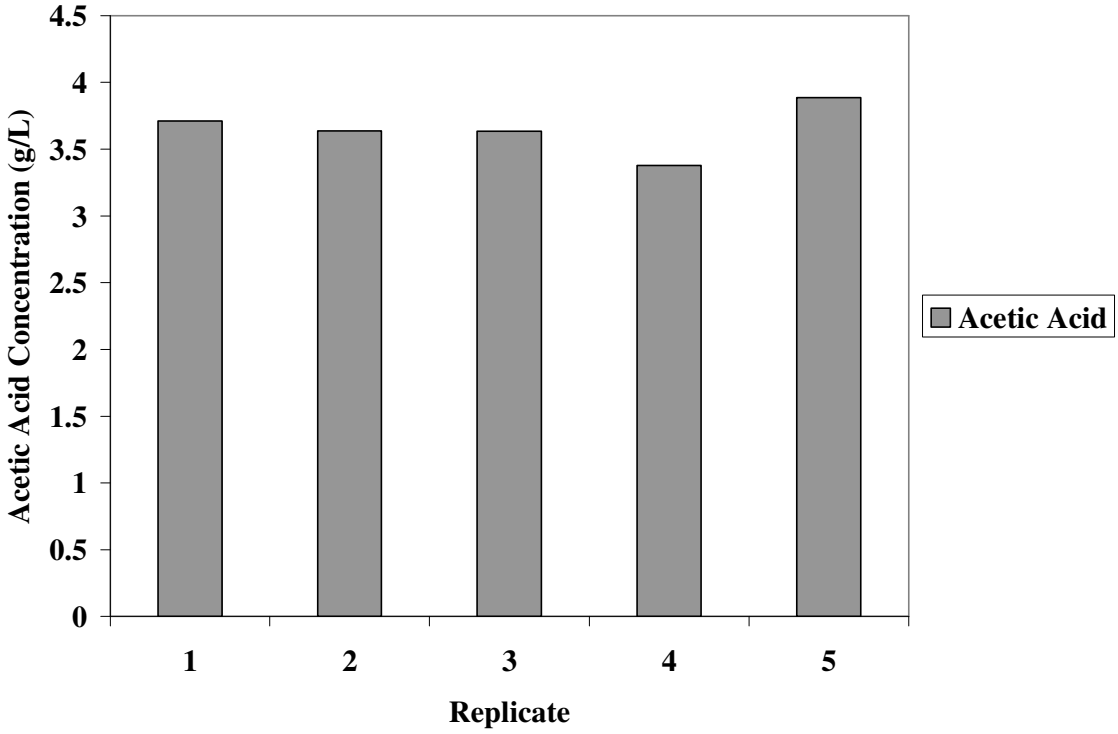

Figure 5.2 Acetic acid concentration in liquid prehydrolyzate from five hydrothermolysis batches of Kanlow switchgrass at 200°C for 10 min.

oligomers and monomers was 28% and 4.7%, respectively. Xylan and glucan recovery as monomers in prehydrolyzate was 15.5% and 8.3%, respectively.

5.3.2 Simultaneous Saccharification and Fermentation

Washed pretreated solids from five hydrothermolysis batches at 200°C for 10 min were combined and mixed thoroughly and used as substrate in SSF using thermotolerant *K. marxianus* IMB4 and *S. cerevisiae* D_5A (control) at 15 FPU/g glucan. Theoretical ethanol yield over a period of 168 h from SSFs at pH 4.8 and 37, 41, and 45°C is illustrated in Figure 5.3. The maximum ethanol concentration that can be produced from 4.1% glucan is 23.2g/L. The maximum ethanol concentrations produced from fermentation of enzyme controls were 1.4 g/L using IMB4 and 1.5 g/L using D_5A. Ethanol concentrations from the enzyme controls were subtracted from ethanol concentrations from SSFs.

Figures 5.4 and 5.5 show glucose and cellobiose concentrations over 168h. From 0 to 72h, ethanol concentrations increased continuously and residual glucose after 72h remained below 2 g/L. Over the course of fermentation, cellobiose concentrations remained relatively constant as it was hydrolyzed to glucose continuously, indicating sufficient beta-glucosidase activity in the medium. Glucose concentrations increased and ethanol concentration remained constant after 72 and 96h for IMB4 at 45 and 41°C, respectively. On the other hand, fermentation continued until 168h in the IMB4, 37°C and control SSFs as shown by relatively low glucose concentrations and increases in ethanol concentration. Based on the Dunnett's test, ethanol yields (% theoretical) were 68.0 at 37°C after 168h, 69.6 at 41°C after 96h, 68.1 45°C after 72h for IMB4 at pH 4.8 , and 77.8 after 168h for the control. Ethanol yields after 24 h at 41°C were greater than

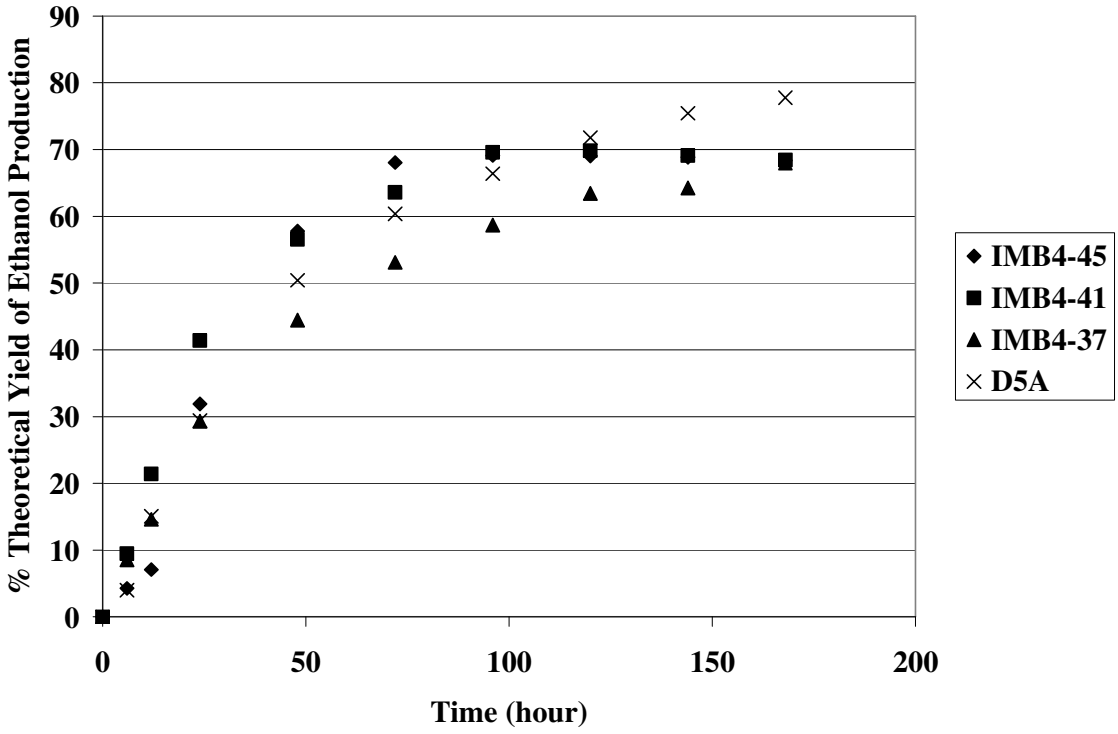

Figure 5.3 Ethanol production during 168 h simultaneous saccharfication and fermentation using thermotolerant *K. marxianus* IMB4 at pH 4.8 and at 37, 41, and 45°C and ethanolgenic *S. cerevisiae* D_5A at 4.1% glucan loading from hydrothermolysis-pretreated switchgrass and 15 FPU/ g glucan cellulase loading. All values are means of three runs.

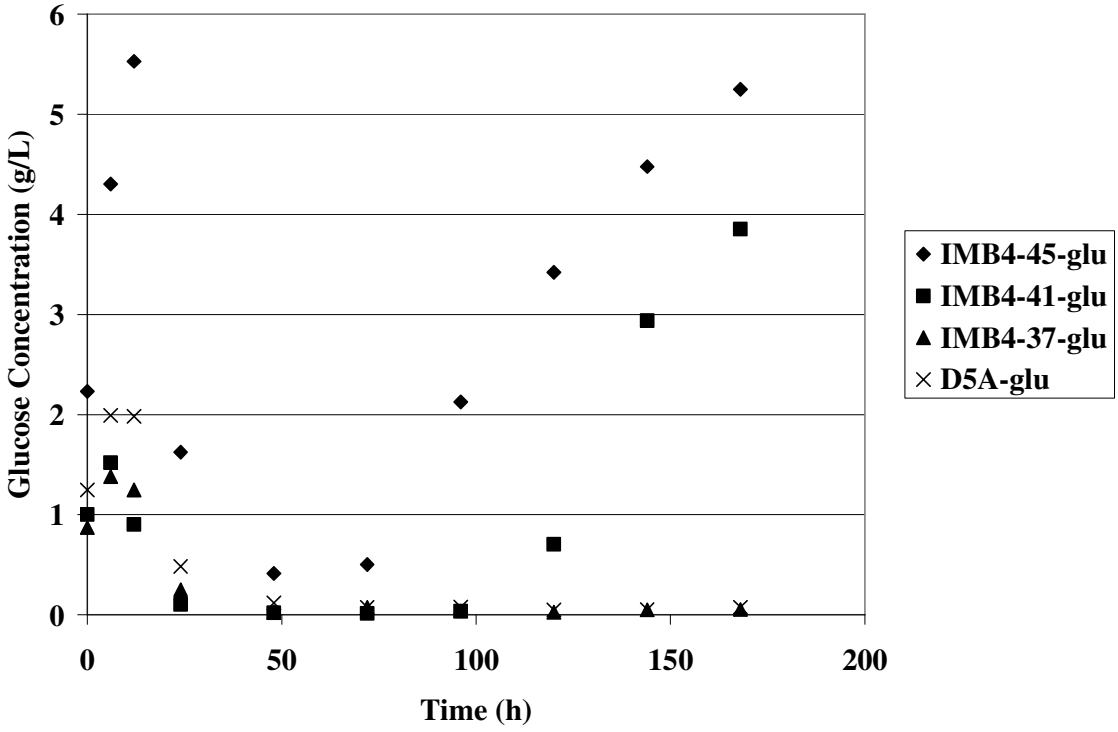

Figure 5.4 Mean glucose concentration during 168 h simultaneous saccharfication and fermentation using thermotolerant *K. marxianus* IMB4 at pH 4.8 and at 37, 41, and 45°C and ethanolgenic *S. cerevisiae* D_5A at 4.1% glucan loading from hydrothermolysis-pretreated switchgrass and 15 FPU/ g glucan cellulase loading. All values are means of three runs.

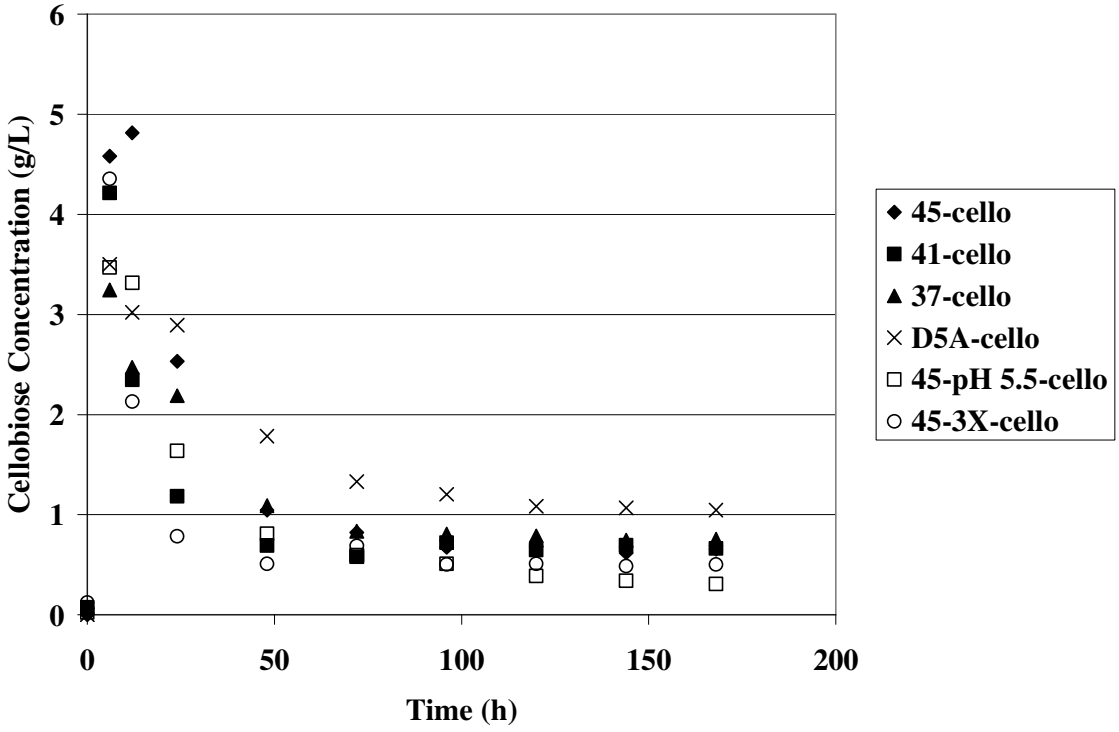

Figure 5.5 Mean cellobiose concentration during 168 h simultaneous saccharfication and fermentation using thermotolerant *K. marxianus* IMB4 at pH 4.8 and at 37, 41, and 45°C, 45°C pH 5.5 and ethanolgenic *S. cerevisiae* D_5A at 4.1% glucan loading from hydrothermolysis-pretreated switchgrass and 15 FPU/ g glucan cellualse loading. Value for IMB4 at 45°C and pH 5.5 was mean of two runs; others are means of three runs.

the control ($p<0.05$), other temperatures were the same as the control ($p>0.05$). Ethanol yields after 48h at 41 and 45°C were greater than the control ($p<0.05$), yield at 37°C was not different than the control ($p>0.05$). Ethanol yield after 72h at 45°C was greater than the control ($p<0.05$), yield at 41°C was not different than the control ($p>0.05$), and yield at 37°C was less than the control ($p<0.05$). Ethanol yield after 96h at 37°C was less than the control, and yields at 41°C and 45°C were not different than the control ($p>0.05$).

During SSF, increasing acetic acid production was observed in all fermentations as shown in Figure 5.6. At the end of fermentation, final pH of all SSFs ranged from 4.36 to 4.51. The greatest acetic acid concentration (2.8g/L) was found in SSF using IMB4 at 41°C, followed by 37°C (1.7 g/L), 45°C (1.2g/L), and the control (0.61 g/L). The final pH at 41°C was 4.36, followed by 37°C (4.4), 45°C (4.5), and the control (4.49). Acetic acid concentrations increased from 0 to 96h in all SSFs. After 96h, acetic acid concentrations decreased at 37°C for both yeast, but not at other temperatures. Build up of acetic acid produced by IMB4 may cause inhibition of IMB4 growth and fermentation. In all SSFs, the final pH was below the pKa of acetic acid (4.76) (Berg et al. 2007). In this case, most of the acetic acid was undissociated, which causes greater inhibition (Pamphuhla and Loureiro-Dias 1990). The combination of greater temperature, ethanol concentration, and decreased pH may have caused the cessation of fermentation by IMB4 after 72h at 45°C and 96h at 41°C. A similar result was observed by Ballesteros et al. (2004) which utilized thermotolerant *K. marxianus* CECT 10875 at 42°C for SSF of various cellulosic biomass. They reported cessation of fermentation between 72 and 82h. They attributed this to metabolic stress caused by low glucose concentration and the presence of ethanol.

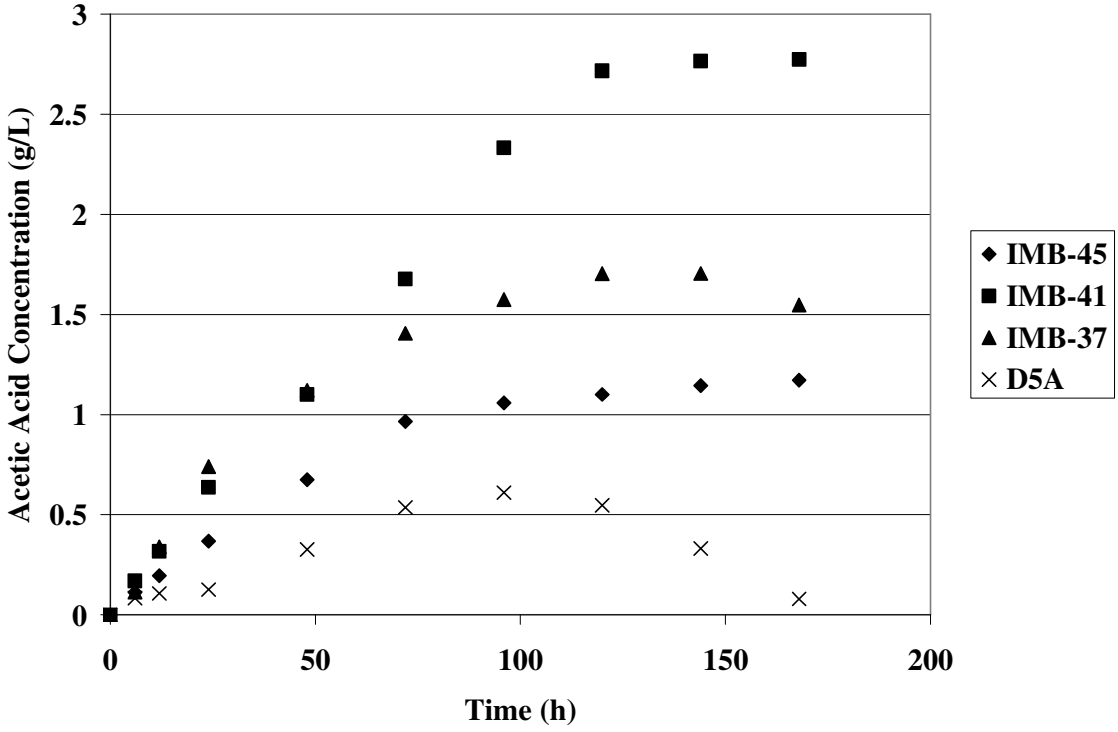

Figure 5.6 Mean acetic acid concentration during 168 h simultaneous saccharfication and fermentation using thermotolerant *K. marxianus* IMB4 at 37, 41, and 45°C at pH 4.8, and ethanolgenic *S. cerevisiae* D_5A, at 4.1% glucan loading from hydrothermolysis-pretreated switchgrass and 15 FPU/ g glucan cellulase loading. Value for IMB4 at 45°C and pH 5.5 was mean two runs; others are means of three runs.

They did not report any discussions pertaining to acetic acid. Another study reported the effect of lignocellulosic degradation compounds on fermentation by *K. marxianus* CECT 10875 (Oliva et al. 2003). Increasing acetic acid concentration inhibited the growth of yeast. At 5 g/L acetic acid, growth was 40% less than the control of no acetic acid. Ten g/L of acetic acid inhibited yeast growth by 53%. However, they reported ethanol production was not affected by acetic acid. At pH 5.5, addition of 5g/L of acetic acid did not affect ethanol yield. However, when SSF pH was reduced to 4.0, the same concentration of acetic acid significantly decreased ethanol production by 80%. Maiorella et al. (1983) reported inhibition of *S. cerevisiae* cell growth by acetic acid due to interference with cell maintenance function leading to membrane disruption. Acetic acid concentrations in the range of 0.5 to 9 g/L inhibited yeast cell growth. At 7.5 g/L acetic acid concentration, cell mass was reduced by 80%.

Due to previous studies showing inhibition of *K. marxianus* fermentation by acetic acid and low pH, SSFs using a greater pH were performed. Banat and Marchant (1995) reported growing IMB4 on glucose and adjusting initial SSF pH to 5.5. This pH may have provided better conditions for growth of IMB4 and therefore could result in improvement of ethanol yield. Maintaining fermentation pH above the pKa of acetic acid (4.76) has been observed to reduce acetate inhibition (Oliva et al. 2003). Another study utilizing a different thermotolerant strain of *K. marxianus* (CECT 10875) in SSF also adjusted initial SSF pH to 5.5 (Ballesteros et al. 2004).

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Ethanol yield at 45°C and pH 5.5 was 78.9% theoretical (Figure 5.7) after 96h. Glucose concentration increased and ethanol concentration was constant after 96h, indication cessation of fermentation (Figure 5.8). Ethanol yield at pH 5.5 and 45°C was greater than the control at 48, 72, 96, and 120h ($p<0.05$). At 96h, ethanol yield of SSF at 45°C and pH 5.5 was greater than that of SSF at 45°C and pH 4.8. The maximum acetic acid concentration produced during SSF at 45°C and pH 5.5 was 0.71g/L, which was 40% lower than that of SSF at 45°C and pH 4.8 as shown in Figure 5.9. The final pH at 45°C and pH 5.5 was 4.79, which was close to the pKa of acetic acid. This result was similar to previous work by Oliva et al. (2003) indicating that ethanol yield is affected by initial SSF pH.

Also, from personal communication with Dr. Ibrahim Banat, it was thought that by having more nutrients available, IMB4 might continue growing and producing ethanol past 72h at 45°C. Therefore, additional SSF experiments at 45°C were also performed to investigate the effect of nutrient concentration on ethanol yield from IMB4 fermentations. In order to examine this, triplicate SSFs using IMB4 at 45°C and pH 4.8 were performed as before, except the nutrient concentration was tripled. Ethanol yield was 56.9% theoretical after 72h, which was less than previously observed (Figure 5.7). As was previously observed with the reduced nutrient concentration, ethanol concentration remained constant and glucose concentration increased after 72h, showing cessation of fermentation (Figure 5.8).

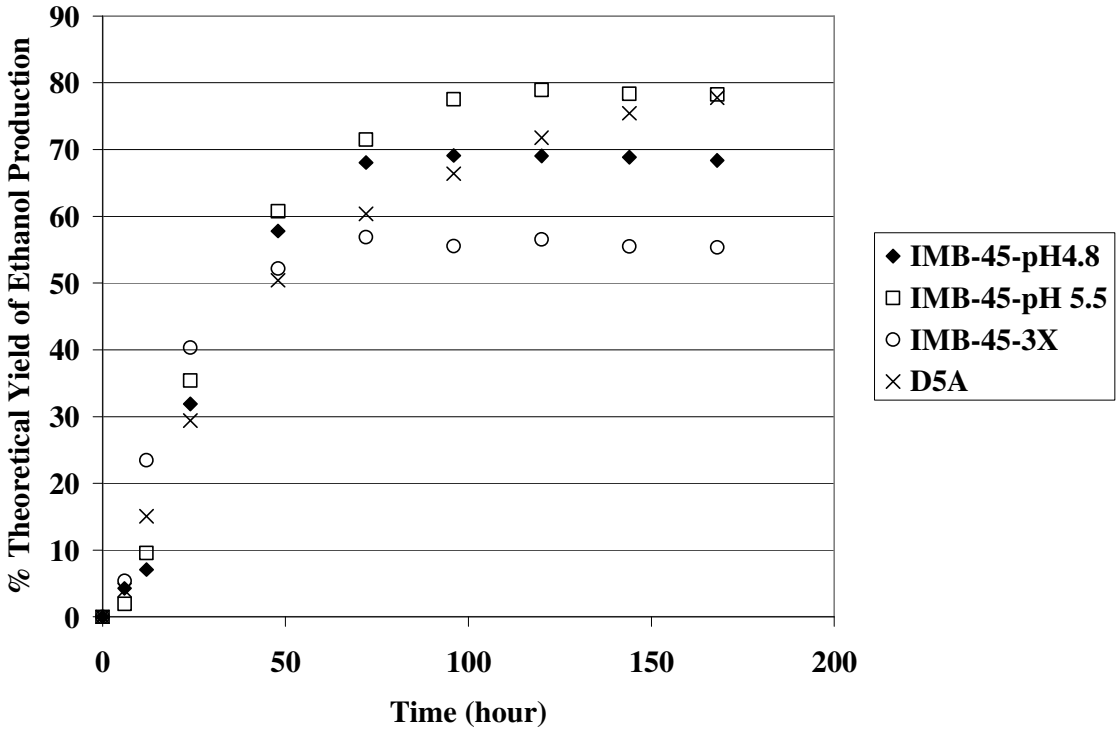

Figure 5.7 Ethanol production during 168 h simultaneous saccharfication and fermentation using thermotolerant *K. marxianus* IMB4 at 45°C and pH 4.8 and 5.5, and tripled nutrient concentration, and ethanolgenic *S. cerevisiae* D_5A at 4.1% glucan loading from hydrothermolysis-pretreated switchgrass and 15 FPU/ g glucan cellulase loading. Value for IMB4 at 45°C pH 5.5 was mean of two runs; others are means of three runs.

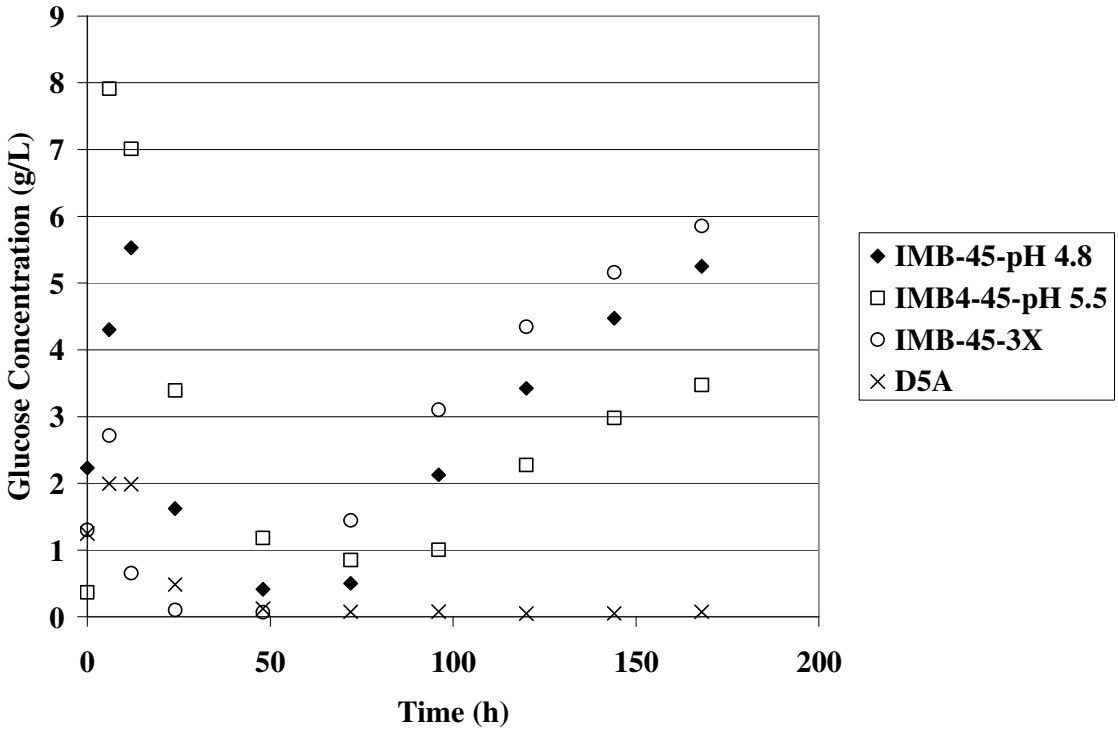

Figure 5.8 Mean glucose concentration during 168 h simultaneous saccharfication and fermentation using thermotolerant *K. marxianus* IMB4 at 45°C and pH 4.8 and 5.5, and tripled nutrient concentration, and ethanolgenic *S. cerevisiae* D_5A at 4.1% glucan loading from hydrothermolysis-pretreated switchgrass and 15 FPU/ g glucan cellulase loading. Value for IMB4 at 45°C and pH 5.5 was mean of two runs; others are means of three runs.

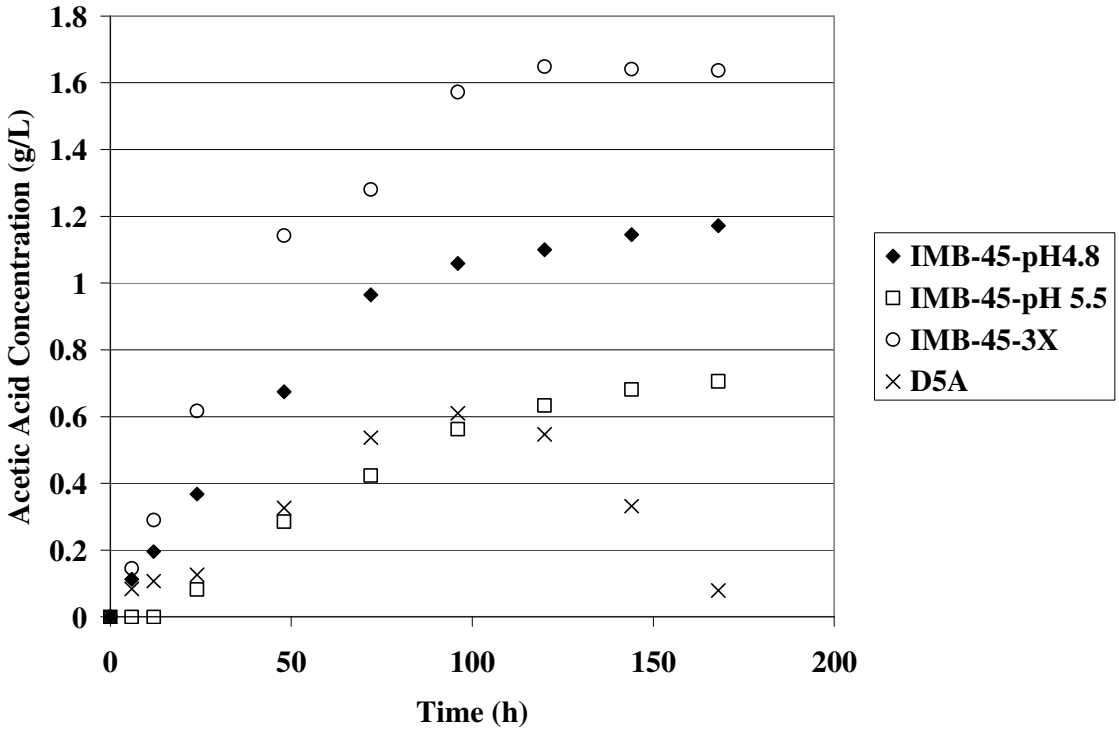

Figure 5.9 Mean acetic acid concentration during 168 h simultaneous saccharfication and fermentation using thermotolerant *K. marxianus* IMB4 at 45°C pH 4.8 and 5.5, and tripled nutrient concentration, and ethanolgenic *S. cerevisiae* D_5A, at 4.1% glucan loading from hydrothermolysis-pretreated switchgrass and 15 FPU/ g glucan cellulase loading. Value for IMB4 at 45°C and pH 5.5 was mean of two runs; others are means of three runs.

Table 5.1 shows specific ethanol productivity after 72h of IMB4 and D_5A strains at various SSF conditions. The time of 72h was chosen since this is when IMB4 at 45°C, pH 4.8 ceased fermentation of glucose. The specific ethanol productivity of IMB4 was greater for all treatments than the control ($p<0.05$). Cellulose hydrolysis rate increased as temperature increased; however, fermentation of glucose by IMB4 at 45°C did not occur as rapidly as the rate of hydrolysis as shown by residual glucose measured during SSF (Figure 5.4). Residual glucose concentrations in SSFs at 37 and 41°C were close to zero, indicating that hydrolysis and fermentation rates were equal.

5.4 Conclusions

Hydrothermolysis of switchgrass at 200°C for 10 min produced minimal concentrations of HMF and furfural. Ethanol fermentation ceased before completion of cellulose hydrolysis using *K. marxianus* IMB4 at 41 and 45°C at initial pH 4.8. When initial pH was increased to 5.5, ethanol yield at 45°C increased and fermentation continued for 24 more hours than at pH 4.8, but glucose fermentation still was not completed. Acetic acid production lowered the pH of fermentation, which contributed to yeast inhibition. Maintaining pH of SSF above the pKa of acetic acid is critical in preventing yeast inhibition in order to obtain greater ethanol yield. Increasing nutrient concentration decreased ethanol yield. Specific ethanol productivity was greater for IMB4 than the control (D_5A).

Table 5.1 Maximum ethanol productions during 168 h simultaneous saccharfication and fermentation using thermotolerant *K. marxianus* IMB4 at various temperatures and ethanolgenic *S. cerevisiae* D_5A at 4.1% glucan loading from hydrothermolysis-pretreated switchgrass and 15 FPU/ g glucan cellulase loading. Value for IMB4 at 45°C and pH 5.5 was mean of two runs; others are means of three runs.

Yeast	Temperature (°C)	Initial SSF pH	Ethanol Concentration at 72h (g/L)[a]	Specific Ethanol Productivity at 72 h (g Ethanol/g cell/h)[b]
IMB4	37	4.8	12.3	1.22
IMB4	41	4.8	14.8	1.46
IMB4	45	4.8	15.8	1.57
IMB4	45	5.5	16.6	1.65
D_5A	37	4.8	14.0	0.97

[a]Ethanol concentration does not include ethanol produced from sugars in enzyme preparation or nutrient medium.

[b]Based on initial cell concentration.

Chapter 6

FUTURE WORK

There are several areas of future research that can be investigated in order to improve current hydrothermolysis and SSF systems. One study that can be done is determining the effect of enzyme loading on ethanol yield using SSF and *K. marxianus* IMB4, which could lead to higher enzymatic hydrolysis and ethanol yield. The cost of enzyme utilized in enzymatic hydrolysis greatly contributes to the production cost of bioconversion of cellulosic biomass to ethanol (Mosier et al. 2005a). Also, investigating the use of different enzyme preparations that may be lower in cost would be of benefit. Additionally, the effect of fermentation byproducts on viability and ethanol yield of IMB4 still requires further investigation. In this laboratory scale study, only washed pretreated switchgrass was used as substrate for SSF. Further study can be performed to look at the utilization of the entire hydrothermolysis slurry, which includes the liquid prehydrolyzate. Prehydrolyzate from hydrothermolysis contained HMF, furfural, acetic acid, and other organic acids which may inhibit fermentative organisms. Therefore, fermentability of the prehydrolyzate fraction requires further investigation. Furthermore, as discussed in Chapter 4, the liquid prehydrolyzate contained sifgnificant amount of xylose that can potentially be converted to ethanol by xylose fermenting organisms. Also, production of other products from xylose, such as xylitol should be explored.

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APPENDIX

Program for Dunnett Test in Chapter 5

```
options ls=74 ps=60;
data IMB24h;
infile "h:\Lilis Thesis\24hethanol.csv" dlm=",";
input strain$ etol24 @@;
cards;
run;
proc glm data=IMB24h; class strain;
model etol24 = strain;
means strain/dunnett ('SC');
run;
data IMB48h;
infile "h:\Lilis Thesis\48hethanol.csv" dlm=",";
input strain$ etol48 @@;
cards;
run;
proc glm data=IMB48h; class strain;
model etol48 = strain;
means strain/dunnett ('SC');
run;
data IMB72h;
infile "h:\Lilis Thesis\72hethanol.csv" dlm=",";
input strain$ etol72 @@;
cards;
run;
proc glm data=IMB72h; class strain;
model etol72 = strain;
means strain/dunnett ('SC');
run;
data IMB96h;
infile "h:\Lilis Thesis\96hethanol.csv" dlm=",";
input strain$ etol96 @@;
cards;
run;
proc glm data=IMB96h; class strain;
model etol96 = strain;
means strain/dunnett ('sc');
```

```
run;
data IMB120h;
infile "h:\Lilis Thesis\120hethanol.csv" dlm=",";
input strain$ etol120 @@;
cards;
run;
proc glm data=IMB120h; class strain;
model etol120 = strain;
means strain/dunnett ('SC');
run;
options ls=74 ps=60;
data prod;
infile "h:\Lilis Thesis\productivity.csv" dlm=",";
input trt$ prd @@;
cards;
run;
proc glm data=prod; class trt;
model prd = trt;
means trt/dunnett ('control');
means trt;
run;
```

Output from SAS Release 9.1 for Dunnett Test program

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	37 41 454.8 455.5 SC

Number of Observations Read	14
Number of Observations Used	14

The GLM Procedure

Dependent Variable: etol24

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	16.40791134	4.10197784	10.32	0.0020
Error	9	3.57644885	0.39738321		

Corrected Total 13 19.98436020

R-Square	Coeff Var	Root MSE	etol24 Mean
0.821038	8.142439	0.630383	7.741947

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	16.40791134	4.10197784	10.32	0.0020

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	16.40791134	4.10197784	10.32	0.0020

The GLM Procedure

Dunnett's t Tests for etol24

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	9
Error Mean Square	0.397383
Critical Value of Dunnett's t	2.95828

Comparisons significant at the 0.05 level are indicated by ***.

strain Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
41 - SC	2.7819	1.2593 4.3045	***
455.5 - SC	1.3909	-0.3115 3.0933	
454.8 - SC	0.5792	-0.9435 2.1018	
37 - SC	-0.0247	-1.5473 1.4979	

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	37 41 454.8 455.5 SC

Number of Observations Read	14
Number of Observations Used	14

The GLM Procedure

Dependent Variable: etol48

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	24.96605708	6.24151427	18.29	0.0002
Error	9	3.07155973	0.34128441		
Corrected Total	13	28.03761681			

R-Square	Coeff Var	Root MSE	etol48 Mean
0.890449	4.701297	0.584196	12.42626

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	24.96605708	6.24151427	18.29	0.0002

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	24.96605708	6.24151427	18.29	0.0002

The GLM Procedure

Dunnett's t Tests for etol48

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	9
Error Mean Square	0.341284
Critical Value of Dunnett's t	2.95828

Comparisons significant at the 0.05 level are indicated by ***.

strain Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
455.5 - SC	2.3996	0.8219	3.9772	***
454.8 - SC	1.7118	0.3007	3.1229	***
41 - SC	1.4263	0.0152	2.8374	***
37 - SC	-1.3873	-2.7983	0.0238	

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	37 41 454.8 455.5 SC

Number of Observations Read	14
Number of Observations Used	14

Dependent Variable: etol72

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	28.80923745	7.20230936	28.74	<.0001
Error	9	2.25563070	0.25062563		
Corrected Total	13	31.06486815			

R-Square	Coeff Var	Root MSE	etol72 Mean
0.927390	3.436441	0.500625	14.56813

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	28.80923745	7.20230936	28.74	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	28.80923745	7.20230936	28.74	<.0001

The GLM Procedure

Dunnett's t Tests for etol72

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	9
Error Mean Square	0.250626
Critical Value of Dunnett's t	2.95828

Comparisons significant at the 0.05 level are indicated by ***.

strain Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
455.5 - SC	2.5844	1.2325	3.9364	***
454.8 - SC	1.7853	0.5761	2.9946	***
41 - SC	0.7485	-0.4607	1.9577	
37 - SC	-1.6807	-2.8899	-0.4714	***

The GLM Procedure

Class Level Information

Class	Levels	Values
strain	5	37 41 454.8 455.5 sc

Number of Observations Read	14
Number of Observations Used	14

Dependent Variable: etol96

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	24.78101936	6.19525484	13.20	0.0008
Error	9	4.22537972	0.46948664		
Corrected Total	13	29.00639908			

R-Square	Coeff Var	Root MSE	etol96 Mean
0.854329	4.364465	0.685191	15.69931

Source	DF	Type I SS	Mean Square	F Value	Pr > F
strain	4	24.78101936	6.19525484	13.20	0.0008

Source	DF	Type III SS	Mean Square	F Value	Pr > F
strain	4	24.78101936	6.19525484	13.20	0.000

The GLM Procedure

Class Level Information

Class	Levels	Values
trt	6	37 3x 41 45 5.5 control

Number of Observations Read	17
Number of Observations Used	17

The GLM Procedure

Dependent Variable: prd

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.83730242	0.16746048	87.95	<.0001
Error	11	0.02094459	0.00190405		
Corrected Total	16	0.85824701			

R-Square	Coeff Var	Root MSE	prd Mean
0.975596	3.240710	0.043635	1.346478

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	5	0.83730242	0.16746048	87.95	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	5	0.83730242	0.16746048	87.95	<.0001

Dunnett's t Tests for prd

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	11
Error Mean Square	0.001904
Critical Value of Dunnett's t	2.95001

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison		Difference Between Means	Simultaneous 95% Confidence Limits		
5.5	- control	0.67416	0.55665	0.79167	***
45	- control	0.59488	0.48978	0.69999	***
41	- control	0.49202	0.38692	0.59712	***
3x	- control	0.33756	0.23245	0.44266	***
37	- control	0.25103	0.14593	0.35614	***

Level of trt	N	prd Mean	prd Std Dev
37	3	1.22252326	0.08805440
3x	3	1.30904555	0.01301461
41	3	1.46351196	0.01524660
45	3	1.56637426	0.03085846
5.5	2	1.64564660	0.03461078
control	3	0.97149049	0.02767091

CURRICULUM VITAE

Lilis Suryawati

Candidate for the Degree of

Master of Science

Thesis: SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF HYDROTHERMOLYSIS-PRETREATED SWITCHGRASS FOR ETHANOL PRODUCTION

Major Field: Biosystems Engineering

Biographical:

Personal Data: Born in Surabaya, East Java, Indonesia, on January 24, 1981. The daughter of Rini Utami and Wong Yan Peter Wongso.

Education:
Graduated with special distinction and received Bachelor of Science in Industrial Engineering from University of Oklahoma, Norman, Oklahoma, in May 2004.
Completed the requirements for the Master of Science in Biosystems Engineering at Oklahoma State University, Stillwater, Oklahoma in December, 2007.

Experience:
Engineering Intern at Yo-Yo's Yogurt Co., Norman, OK, January 2002 to May 2002.
Engineering Intern at CompOne Company, Oklahoma City, OK, January 2004 to May 2004.
Graduate Research Assistant at Oklahoma State University, Stillwater, OK, August 2005 to present.

Professional Memberships: American Society of Agricultural and Biological Engineers, Golden Key International Honor Society, Phi Kappa Phi Engineering Honor Society.

Name: Lilis Suryawati Date of Degree: December, 2007

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF HYDROTHERMOLYSIS-PRETREATED SWITCHGRASS FOR ETHANOL PRODUCTION

Pages in Study: 104 Candidate for the Degree of Master of Science

Major Field: Biosystems Engineering

Scope and Method of Study: The first part of the experiment was pretreatment of Kanlow switchgrass in pressurized liquid hot water, called hydrothermolysis, at various temperatures and residence times. The objective was to determine the optimum pretreatment temperature and residence time which gave high cellulose digestibility. Based on the optimum pretreatment condition obtained in the first experiment, hydrothermolysis-pretreated switchgrass was used in simultaneous saccharification and fermentation (SSF) using thermotolerant yeast *Kluyveromyces marxianus* IMB4 to investigate the effect of fermentation temperature on ethanol yield.

Findings and Conclusions: The best hydrothermolysis condition for pretreatment of switchgrass was 200°C for 10 min. Theoretical ethanol yield from the best pretreatment condition was 74.2%. The concentration of inhibitory compounds such as furfural and HMF in prehydrolyzate was less than 1 g/L from all treatments.

Theoretical ethanol yield from SSF of hydrothermolysis-pretreated switchgrass using *K. marxianus* IMB4 yeast at 45°C and pH 5.5 was 78.9%, which corresponded to 18.3 g/L ethanol concentration after 96h. Enzymatic hydrolysis and fermentation occurred faster at higher temperature using IMB4 compared to *S. cerevisiae* D_5A.

The initial pH of fermentation significantly affected ethanol yield. Acetic acid lowered the pH of fermentation, which caused yeast inhibition.

ADVISER'S APPROVAL: Dr. Mark R. Wilkins