EFFECT OF SWITCHGRASS MATURITY ON ETHANOL PRODUCTION VIA SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

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Abstract:

Switchgrass has been selected as a substrate to produce ethanol. One method of producing ethanol from switchgrass is through pretreatment followed by saccharification and fermentation. The harvest date of switchgrass could impact the production of ethanol due to changes in plant chemical composition. Kanlow switchgrass was harvested in July, August, September, October, and November in Stillwater, OK in 2008. The switchgrass was comminuted, analyzed for chemical composition, pretreated by hydrothermolysis, and converted to ethanol via simultaneous saccharification and fermentation (SSF). The objectives were to determine changes in structural carbohydrate and lignin contents in switchgrass over a typical harvest season and to determine the effect of switchgrass maturity on the production of ethanol via SSF.

Structural carbohydrate and lignin contents increased throughout the harvest period. Extractives content decreased throughout the harvest period. The amount of switchgrass dissolved during hydrothermolysis decreased after September. Ethanol concentration via SSF was highest for the August harvest, followed by July, October, September, and November harvest dates. Initial fermentation rates decreased throughout the harvest period. Ethanol yield in terms of liters per ton of switchgrass was highest for the October harvest, followed by the November, August, July, and September harvests. Much of the increase in structural carbohydrate content over the harvest period was due to a decrease in extractives content, rather than addition of new structural carbohydrates. Increasing lignin content through the harvest period had a negative effect on fermentation rates and yields. The lignin content after pretreatment did not appear to correlate to fermentation rates and yields as did the lignin content of untreated switchgrass. The decreased amount of switchgrass dissolved during hydrothermolysis at the end of the harvest period had a positive effect on ethanol yields. Ethanol yield in terms of liters per ton of switchgrass for July, August, October, and November harvest dates were not significantly different; a significantly lower yield was obtained for the September harvest date.

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ABBREVIATIONS AND ACRONYMS

ADF	acid detergent fiber
ADL	acid detergent lignin
AFEX	ammonia fiber expansion
C5	five-carbon
C6	six-carbon
HMF	5-hydroxymethylfurfural
LAP	lab analytical procedure
NDF	neutral detergent fiber
NREL	National Renewable Energy Laboratory
OD	optical density
SAA	soaking in aqueous ammonia
SHF	separate hydrolysis and fermentation
SSCF	simultaneous saccharification and co-fermentation
SSF	simultaneous saccharification and fermentation
YP	yeast extract and peptone

CHAPTER I

INTRODUCTION

Ethanol is used as a fuel and in fuel blends, such as E10 gasoline. Corn has been the largest substrate used to make ethanol in the United States (McLaughlin and Walsh, 1998; USDA, 2010). The starch in corn can be easily converted to simple sugars for fermentation by yeast. This fermentation produces ethanol. However, corn is a high input crop due to the amount of fertilizer used as well as the amount of fuel used in farm equipment. Corn is also used as food for both humans and animals. As an alternative to high-value grains such as corn, other plant matter such as stalks and leaves can be used to produce ethanol. Plant cell walls also contain sugars that can be converted to ethanol via yeast fermentation.

Plant cell walls are composed of a matrix of three materials: cellulose, hemicellulose, and lignin. Cellulose is a chain of six-carbon (C6) sugars, meaning there are six carbon atoms in the sugar molecule. C6 sugars in monomer form are readily fermentable by yeast. Hemicellulose is a chain of both five-carbon (C5) sugars and C6 sugars. C5 sugars can also be converted to ethanol, but not as easily as C6 sugars (Mosier et al., 2005). Lignin is a phenolic molecule that is not fermentable to ethanol (Casler and Boe, 2003), but may be burned for heat (Mosier et al., 2005). Obtaining monomer sugars for fermentation is difficult due to the matrix formed by cellulose, hemicellulose, and lignin. Pretreatment methods have been developed to disrupt the

matrix structure and separate cellulose, hemicellulose, and lignin prior to the saccharification of cellulose and hemicellulose (Alvira et al., 2010; Mosier et al., 2005).

Once the matrix structure of the plant cell wall is disrupted, cellulose and hemicellulose can be converted to monomer sugars for fermentation by yeast. Enzymes from fungi are used to cleave the bonds that connect individual sugar molecules together in a chain, which is called saccharification. Yeast produces ethanol when supplied with C6 sugars in the absence of oxygen. If both the saccharification and fermentation steps are combined into a single process, it is called simultaneous saccharification and fermentation (SSF). SSF is advantageous over performing each step separately because enzymes can be inhibited by the sugars they release from cellulose and hemicellulose, but yeast consume the sugars fast enough to prevent this from occurring in the combined process (Suryawati et al., 2008; Teugjas and Valjamae, 2013). Further, there is less chance of sugar being consumed by contaminant organisms in SSF as opposed to separate hydrolysis and fermentation steps. Finally, capital costs are reduced with SSF because fewer tanks are needed.

All plants could be used to produce ethanol through pretreatment, saccharification, and fermentation, but plants that have low inputs are seen as advantageous. Switchgrass has been identified as a bioenergy crop to be used for producing ethanol (McLaughlin and Kszos, 2005). Optimizing all parameters of the process of converting switchgrass to ethanol will improve its economic viability. One parameter to optimize is the harvest window of switchgrass used for ethanol production. As an example, wheat grain is harvested once the plant is mature and the seed is ripe. The wheat is too wet for storage if the harvest is early. Quality and yield of wheat decrease if the harvest is late. The decision of when to harvest switchgrass needs to include factors affecting ethanol production, not just maximizing the mass of switchgrass harvested per area of land. Switchgrass grows through the summer and senesces as the plant matures in late summer and fall. Since ethanol is produced from cell wall carbohydrates, it will be useful to

know how the composition of cell walls change as switchgrass matures. Ethanol yields may best be calculated in terms of the amount of ethanol produced per area of land. Determining when these maximum ethanol yields can be obtained will provide an optimum switchgrass harvest window for ethanol production. Thus, ethanol production via SSF of switchgrass harvested throughout the maturing process was explored in this study. Switchgrass samples harvested from late summer through fall were analyzed for structural carbohydrate and lignin content. Further, the samples underwent a hydrothermolysis pretreatment process and were analyzed again for structural carbohydrate and lignin content. Finally, ethanol was produced from the pretreated samples via SSF.

CHAPTER II

OBJECTIVES

The objectives of this research are to:

1. Determine changes in structural carbohydrate and lignin contents in switchgrass over a typical harvest season.

2. Determine the effect of switchgrass maturity on the production of ethanol via a simultaneous saccharification and fermentation process.

CHAPTER III

REVIEW OF LITERATURE

3.1 Switchgrass Characteristics

Switchgrass (*Panicum virgatum* L.) is a perennial, warm season C4 grass native to the United States (Porter, 1985). It grows over most of the United States, as well as part of Mexico and Canada (McLaughlin and Walsh, 1998). It is drought resistant and suitable for marginal soils (Casler and Boe, 2003). There are two morphological strains of switchgrass: lowland ecotypes and upland ecotypes (Porter, 1985). Lowland ecotypes are tall, vigorous, course-stemmed, adapted to wet conditions, and light green (Lemus et al., 2002; Porter, 1985). Upland ecotypes are short, rhizomatous, relatively fine-stemmed, adapted to drier conditions, and blue-green (Lemus et al., 2002; Porter, 1985). Lowland ecotypes have higher yield potentials than upland ecotypes (Adler et al., 2006). There are four populations based on ecotype and latitude of origin: southern lowland with germplasm originating from southern and central Texas, northern lowland with germplasm originating from Oklahoma, southern upland with germplasm originating from Oklahoma, and northern upland with germplasm originating from the Central Great Plains (Sanderson et al., 1996). Northern populations flower earlier than southern populations as flowering is related to the latitude of origin since switchgrass is sensitive to photoperiod (Lemus et al., 2002). Switchgrass is suited as a bioenergy crop because it produces high yields compared

with other herbaceous species, it requires less energy to manage because it is perennial, it can grow in poor soils that are not producing cash crops, and farmers are already familiar with growing and harvesting grasses (McLaughlin and Kszos, 2005).

3.2 Switchgrass Yields

The amount of switchgrass harvested will directly affect the amount of ethanol that can be produced. Variety, location, time of harvest, and number of harvests in a season all affect switchgrass yields.

3.2.1 Comparison of Varieties

Research shows that the best variety of switchgrass will be dependent upon the location of farms. Sladden et al. (1991) compared lowland ecotype varieties Alamo and Kanlow and upland ecotype varieties Blackwell, Cave-in-Rock, Kansas Native, Pathfinder, Summer, and Trailblazer in Shorter, Alabama in 1989 and 1990. Stands were planted in 1988. Switchgrass was cut twice per year (a two-cut management) at a 5 cm stubble height with the harvest after anthesis when it was assumed that little further yield increases would occur. Anthesis is the flowering stage of a plant. The lowland ecotype varieties were harvested about a month after the upland ecotype varieties both years. Both lowland ecotype varieties yielded more biomass than the upland ecotype varieties each year, and Alamo yielded higher than Kanlow both years. The upland varieties did not differ significantly in yield (Sladden et al., 1991). Upland ecotypes are not adapted to the climate in Alabama, whereas lowland ecotypes are suited for Alabama and should have higher yields.

Lemus et al. (2002) studied 20 switchgrass varieties near Chariton, Iowa from 1998 to 2001. Plots were planted in 1997. Varieties compared were Alamo, Blackwell, Caddo, Carthage, Cave-in-Rock, Forestburg, Kanlow, Pathfinder, Shawnee, Shelter, Sunburst, Trailblazer, IA-GT, IA-LM, NL93-2CH, NU94-2CH, SU92-ISO, SU94-2CH, HDMD-C3, and HYLD-C3. Plots

were harvested on November 13, 1998, September 30, 1999, and October 15, 2001 with a cutting height of 7.5 cm. The harvest for the 2000 crop was delayed until early January 2001 and was used for composition, but was not included for yield data. Comparison of varieties only showed the average yield of the three years, as no cultivar by year interaction was observed. The average yield for all varieties was 9.0 Mg/ha. Kanlow had the highest yield at 13.1 Mg/ha, which was significantly higher than all other varieties except Alamo (12.1 Mg/ha). Alamo yielded significantly more biomass than most other varieties, except Kanlow, NU94-2CH (11.2 Mg/ha), and HDMD-C3 (10.5 Mg/ha). NU94-2CH and HDMD-C3 are both upland ecotype varieties. The lowland cultivars Alamo and Kanlow yielded the most biomass, but the winters were mild for Iowa during these years (Lemus et al., 2002). Lowland switchgrass cultivars are not suited for cold winters. Kanlow and Pangburn, both lowland cultivars, failed to survive the first winter after planting in Pennsylvania in 1968 (Berg, 1971).

McLaughlin and Kszos (2005) compared 9 switchgrass cultivars planted in 1992 at 18 sites across 13 states: Virginia, West Virginia, Tennessee, Kentucky, North Carolina, Georgia, Alabama, Texas, Arkansas, Louisiana, North Dakota, South Dakota, and Iowa. The best commercial varieties in terms of yield after 10 yr of production were Alamo in the deep South, Alamo and Kanlow at mid-latitudes, and Cave-in-Rock, Trailblazer, and Sunburst for northern latitudes (McLaughlin and Kszos, 2005). Casler and Boe (2003) compared six upland ecotypes of switchgrass at two locations: Brookings, South Dakota and Arlington, Wisconsin. The cultivars Cave-in-Rock, Dacotah, Forestburg, Shawnee, Sunburst, and Trailblazer were harvested by a single cutting in August, September, and November from 1998 to 2001 after a 1997 planting. Shawnee ranked first in yield for 5 of the 8 year-location combinations. Location of origin had an effect as some cultivars did better at one location or the other. Cave-in-Rock originates from Illinois and performed better in Wisconsin, whereas Trailblazer originates from Nebraska and performed better in South Dakota (Casler and Boe, 2003).

3.2.2 Harvest Date Effect on Yield for Single-cut Management

Switchgrass should be fully grown for a single-cut management because an earlier harvest would greatly reduce yields. Lockert (1974) harvested Summer variety switchgrass at Brookings, South Dakota on June 17 at the vegetative growth stage, July 8 at late jointing, and August 12 when grass was 100% headed (any part of the panicle exposed above the flag leaf). Yields were 1.5 Mg/ha in June, 6.7 Mg/ha in July, and 9.6 Mg/ha in August (Lockert, 1974). McLaughlin and Kszos (2005) stated in a review that switchgrass yields were maximized when harvested by mid-September. Harvest after late September reduced yields by up to 20%. After these initial losses, further loss over the winter appeared minimal and the switchgrass often benefited from conserved nutrients (McLaughlin and Kszos, 2005). Sanderson et al. (1999) found that in Dallas, Texas in 1993 and Stephenville, Texas in 1993 and 1995, a November harvest date yielded less than either a September or October harvest date. In 1994, yields at both locations were higher in September than October and November (Sanderson et al., 1999). However, some research obtained maximum yields after September. In Stephenville, Texas in 1996, September harvest yielded lower than October and November harvests (Sanderson et al., 1999). Casler and Boe (2003) harvested six upland ecotypes from 1998 to 2001 in August, September, and October at Brookings, SD and Arlington, WI. Delaying harvest in 1998 lowered yields. This trend gradually changed through 1999 and 2000 to an increase in yield with delayed harvest in 2001. Stands were planted in 1997 (Casler and Boe, 2003). Sanderson et al. (1999) planted stands in 1992 and harvested from 1993 to 1996. Similar to Casler and Boe (2003), yields were also better for earlier harvests in the first few years after establishment and switched to better yields for delayed harvest in the last year of the study.

Researchers have also investigated delaying switchgrass harvest until the following spring, leaving stands in the field over the winter. Adler et al. (2006) found Pennsylvania switchgrass yields were greater when harvested in the fall (between mid-October and mid-

November) versus waiting until the following spring (between early April and early May). Spring yields were lower due to increased field residue from cutting and bailing as well as from ash content reduction. Including residue, spring biomass yield was 11% lower than fall biomass yield. Snowfall over the winter also affected yields at Rock Springs, Pennsylvania. In the winter of 2001-2002, snowfall was 56 cm and yields were similar between fall and spring. The following two winters each had about 153 cm of snow and average yield decreased almost 40% over the winter (Adler et al., 2006). Makaju et al. (2013) harvested a mature Kanlow switchgrass stand at Stillwater, Oklahoma once a month from November to March for switchgrass grown in 2007, 2008, and 2009. A significant decrease in yields for the 2007 and 2009 crops was observed as harvest was delayed, but no decrease in yield was observed for the 2008 crop. No consistent association between rainfall and decrease in yield was found (Makaju et al., 2013).

3.2.3 Harvest Date Effect on Yield for Multi-cut Management

McLaughlin and Kszos (2005) stated that for a harvest management with two cuts per year, the first cutting needed to be in July or later to sustain high yields in future years. Further, yields were maximized when the final cutting was by mid-September. However, harvesting after the first frost has the benefit of maximizing carbon and energy translocation to the root system (McLaughlin and Kszos, 2005).

Lockert (1974) harvested switchgrass every 14 and 28 days after initial harvest through September 9 in 1971 in South Dakota using three different initial harvest dates and two cutting heights: 6.4 and 25.4 cm. For an initial harvest date of June 17 at the vegetative growth stage, the 14-day harvest interval yielded less biomass than the 28-day harvest interval. No effect was observed between 14 and 28-day harvest intervals when the initial harvest date was delayed to either July 8 at the late jointing stage or August 12 when grass was 100% headed. Total season yields generally increased as initial harvest was delayed. The one exception to this trend was at a

cutting height of 25.4 cm, where the initial harvest date of June 17 with a 28-day harvest interval yielded higher for the season than the initial harvest date of July 8 for either the 14 or 28-day harvest interval. Regardless of cutting height or harvest frequency, the initial harvest date of August 12 yielded the highest for the season. Yields were higher with a 6.4 cm cutting height than a 25.4 cm cutting height (Lockert, 1974).

3.2.4 Single vs. Multi-cut Harvests

Sanderson et al. (1999) found in Stephenville, Texas, that Alamo switchgrass under a single-cut management yielded more biomass than a two, three, or four-cut management every year from 1993 through 1996. This was regardless of whether the final cut was in September, October, or November. In Dallas, Texas, Alamo switchgrass harvested in 1994 also yielded more biomass under a single-cut management. In Dallas in 1995, there was not a significant difference between single and two-cut managements, which both yielded higher than three and four-cut managements. In Dallas in 1993 and 1996, the single-cut yielded the lowest (Sanderson et al., 1999). However, in Dallas in 1993, 1995, and 1996, no yield exceeded 8 Mg/ha. Yields for Dallas in 1994 and all years in Stephenville were between 10 and 21 Mg/ha for the single-cut management (Sanderson et al., 1999). McLaughlin and Kszos (2005) reported the best yield in a year was with a two-cut management in Alabama with lowland variety Alamo switchgrass. The upland variety Cave-in-Rock yielded higher than lowland varieties in some years in the Southeast under two-cut management (McLaughlin and Kszos, 2005). In Pennsylvania, four-cut management eliminated stands the following year for Caddo, Summer, and Pathfinder varieties cut in June, July, August, and September 1969 (Berg, 1971). It is difficult for switchgrass to recover from multi-cut harvests due to the location of its growing points. Growing points for switchgrass are rapidly extended above cutting height and are removed upon harvest (Porter, 1985). Lockert (1974) observed minimal regrowth once the stem growing points were removed. Frequent harvests have reduced the yield and persistence of switchgrass (Porter, 1985). These

studies have shown that exceeding two harvests in a season is not beneficial for switchgrass. Two-cut management may only work in southern regions with long growing seasons. Single-cut management would use less fuel and put less wear on equipment each season than two-cut management.

3.2.5 Harvest Date Effect on Stand Health

An August harvest in Wisconsin and South Dakota reduced stand density over time compared with September and October harvests (Casler and Boe, 2003). Adler et al. (2006) observed similar results in Pennsylvania. Sanderson et al. (1999) reported a reduction of yield in May following a September harvest compared with an October or November harvest of Alamo switchgrass in Texas. One reason for this observation may be due to nutrient loss over time. Adler et al. (2006) states a mid-August harvest would remove twice as much nitrogen and higher amounts of other minerals compared to a fall harvest in Pennsylvania. Another reason is a reduction in carbohydrate reserves. Switchgrass depends on carbohydrate reserves in the stem base for regrowth and survival (Porter, 1985). Switchgrass loses growing points and leaves during harvest, which causes cut shoots to die and reduces photosynthetic area, thus reducing carbohydrate reserves (Porter, 1985). Also, regrowth after harvest can consume carbohydrate reserves without adequate time to replace them during the remainder of the growing season (Porter, 1985). Casler and Boe (2003) observed little regrowth for September and no regrowth for October harvests, indicating retention of carbohydrate reserves.

3.3 Switchgrass Storage

Ethanol production facilities would operate year-round to improve economic viability. However, it is not feasible to harvest switchgrass year-round, so storage of harvested switchgrass is necessary. Switchgrass can be harvested and stored in bales, just as hay is traditionally stored.

It is also possible to 'store' switchgrass in the field over the winter, delaying the harvest until following spring. Losses could occur from either scenario, reducing ethanol yields.

3.3.1 Storage Moisture Content

Adler et al. (2006) stated excessive moisture content can lead to microbial degradation of soluble and storage carbohydrates and self-ignition of switchgrass. Standard storage moisture content of hay is 15 to 18% (w/w) (Adler et al., 2006). Lewandowski and Kicherer (1997) reported storage moisture content of switchgrass should be less than 23% (w/w). Sanderson et al. (1997) baled switchgrass at 19% and 11% without any temperature rise over ambient temperature, which indicates there was neither microbial respiration nor spoilage.

3.3.2 Harvest Date Effect on Moisture Content, Residue, and Storage

Switchgrass moisture content is well above the storage moisture range until late in the fall. Moisture content decreased between July 25 at early heading and August 19 at anthesis in Wisconsin for upland ecotypes Blackwell and Pathfinder from 74% (w/w) to 67% (w/w) in 1983 and from 67.5% (w/w) to 60% (w/w) in 1984 (Porter, 1985). Moisture content decreases as above ground switchgrass tissue dies during senescence in the fall. Ravindranath et al. (2009) harvested upland switchgrass ecotypes Blackwell and Cave-in-Rock in Oklahoma and Arkansas each month from July to December. Moisture content declined for both varieties in Oklahoma from about 50% (w/w) in July to about 9% (w/w) in December. In Arkansas, the moisture content increased from July to September for Cave-in-Rock and decreased for Blackwell and Cave-in-Rock from about 50% and 55% (w/w), respectively, in September to about 9% (w/w) in December for both varieties (Ravindranath et al., 2009). Switchgrass can be allowed to dry between cutting and baling if the moisture content is too high at the time of cutting. However, if switchgrass becomes too dry and brittle at the time of cutting, losses during baling can occur. In Pennsylvania, Adler et al. (2006) found residue left behind after baling increased from 21% (db)

in November to 45% (db) in April for switchgrass left standing through the winter. The moisture content of switchgrass fell from 35% (w/w) in November to 7% (w/w) in April (Adler et al., 2006). Sanderson et al. (1997) measured switchgrass residue after baling at 1.8% to 4.4% (db) for three different October cuttings and 6.0% (db) for a November cutting in Stephenville, Texas over a three year span. Residues increased in these studies for later harvests.

Further losses of biomass can occur after switchgrass is baled. Switchgrass stored in bales left outside on sod for 12 months at Stephenville, Texas lost 5.6% (db) of bale mass for an October 1993 cutting and 6.0% (db) of bale mass for a November 1994 cutting (Sanderson et al., 1997). Sanderson et al. (1997) observed reduced bale losses when bales were stored either outside on gravel or inside a building compared with outside storage on sod. The depths of the visibly weathered layer of bales after 12 months were 12 and 13 cm for outside storage on gravel and sod, respectively (Sanderson et al., 1997). Although the depths of the weathered layers were similar, bales stored on sod had a large rotted area on the bottom while bales stored on gravel did not (Sanderson et al., 1997). Other data have shown much smaller losses during storage of switchgrass bales. Wiselogel et al. (1996) measured the composition of Alamo switchgrass bales after 6 months of storage in Stephenville, Texas for a grazed stand cut in October 1991 and an ungrazed stand cut in August 1992. Structural carbohydrate losses varied little between the inside and outside layers of bales. In 1991, inside and outside bale layers lost 5.6 and 5.8% (db) of glucan, respectively, and 5.6 and 6.0% (db) of xylan, respectively (Wiselogel et al., 1996). In 1992, inside and outside bale layers lost 1.2 and 2.5% (db) of glucan, respectively; while very slight increases in xylan were measured (Wiselogel et al., 1996). The only significant reduction in structural carbohydrates was for xylan from the 1991 cutting, which fell from 24.9 to 23.4% (db) on an extractives free basis for the outer layers of the bales (Wiselogel et al., 1996). The extractives content also significantly decreased for the 1991 cutting from 17.0 to 9.3% (db) for the inner layers and 6.5% (db) for the outer layers of the bales, however, extractives content only

decreased from 14.2 to 12.4% (db) for the outer layers of bales from the 1992 cutting (Wiselogel et al., 1996). The larger loss in extractives for the 1991 bales corresponds to a thicker weathered layer. Wiselogel et al. (1996) found the visibly weathered layer depth of the bales after 6 months to be 19 cm for the 1991 cutting and 8 cm for the 1992 cutting. Although the harvest date for the 1992 cutting was in August, the 1991 cutting in October may have contained less mature switchgrass due to grazing; combined with different harvest years, it is not possible to conclude an effect from maturity on storage. The preservation of structural carbohydrates during storage indicates that switchgrass can be effectively stored after harvest with little loss of potential ethanol production.

3.4 Composition of Switchgrass

Plant cell walls contain cellulose and hemicellulose, and some contain lignin (Mosier et al., 2005). Cellulose microfibrils have hydrogen bonds to hemicellulose, forming the structural backbone to the cell wall (Mosier et al., 2005). Cellulose is further protected by lignin (Mosier et al., 2005). Cellulose is a polymer of glucose molecules arranged in tightly packed, crystalline structures (Mosier et al., 2005). These structures are water insoluble and resistant to depolymerization. Hemicelllulose is a branched polymer of glucose or xylose, substituted with glucose, xylose, galactose, arabinose, mannose, fructose, glucuronic acid, or acetyl groups of ferulate (Mosier et al., 2005).

3.4.1 Compositional Analysis Methods

3.4.1.1 Forage Fiber Analysis

Goering and van Soest (1970) developed a procedure to determine neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL). NDF is a measure of cell wall material that is left after removal of constituents that are soluble and available as nutrients. NDF is obtained by boiling ground biomass in a neutral detergent solution for 1 h, followed by

filtering and rinsing solids first with hot water and then with acetone. ADF is cellulose, lignin, cutin, and acid insoluble ash consisting mainly of silica. ADF is obtained after ground biomass is boiled in sulfuric acid solution for 1 h, followed by filtration and rinsing with hot water. An estimate of hemicellulose is given by subtracting ADF from NDF, although it includes some proteins attached to cell walls. ADL is lignin, cutin, and acid insoluble ash. ADL is obtained by pouring 72% sulfuric acid solution over the ADF sample at room temperature and stirring at regular intervals for 3 h, followed by filtration and rinsing with hot water. Cellulose is calculated by subtracting ADL from ADF. The ADL sample is then heated in a muffle furnace at 500 to 550°C for 3 h. Lignin, including cutin, is calculated by subtracting the ash from ADL. There is a permanganate lignin method in the procedure that does not include cutin, which is a large fraction in some seed hulls. However, the amount of cutin is not important in common forages (Goering and Van Soest, 1970).

The permanganate lignin method removes lignin from ADF, leaving cellulose and ash in the remaining solid material (Goering and Van Soest, 1970). This method can be used instead of determining ADL, which uses 72% sulfuric acid to remove cellulose from ADF. In the permanganate lignin method, potassium permanganate and a lignin buffer solution are added to the ADF sample at room temperature and stirred at regular intervals for 1 h. The solution is removed by filtration and the residue is washed with a demineralizing solution until residue is white, followed by subsequent ethanol and acetone washes. Permanganate lignin is calculated by the weight loss from ADF. The residue is heated in a muffle furnace at 500°C for 3 h. Cellulose is calculated by subtracting the ash weight from the residue weight (Goering and Van Soest, 1970). Cellulose and hemicellulose tend to be overestimated and lignin underestimated from this forage fiber analysis procedure (Dien et al., 2006; Wiselogel et al., 1996).

3.4.1.2 Determination of Structural Carbohydrates and Lignin in Biomass

Sluiter et al. (2004) developed a procedure for the National Renewable Energy Laboratory titled "Determination of Structural Carbohydrates and Lignin in Biomass." Extracted biomass is analyzed for lignin and structural polymers of the sugars glucose, xylose, galactose, arabinose, and mannose. Extracted biomass has undergone water and ethanol extraction to remove non-structural components such as sucrose, nitrates, nitrites, protein, ash, chlorophyll, and waxes. A two-stage acid hydrolysis (72% and then 4% sulfuric acid) separates the sugars from the extracted biomass and hydrolyzes them into monomers for analysis by high performance liquid chromatography (HPLC). Lignin is separated into both an acid soluble and an acid insoluble fraction. The acid soluble lignin is analyzed by UV-Vis spectroscopy. The acid insoluble fraction is found by burning the remaining material in a muffle furnace at 575°C. Total lignin is the sum of the acid soluble and acid insoluble lignin. The acetyl content can also be measured if necessary via analysis of the liquid fraction on HPLC (Sluiter et al., 2004).

3.4.2 Harvest Date Effect on Composition

Porter (1985) observed an increase in NDF, ADF, ADL, and cellulose from July 25 at early heading to August 19 at anthesis in 1983 and 1984 in Wisconsin for upland varieties Pathfinder and Blackwell. Switchgrass was also separated and analyzed by section: lower stem, upper stem, and leaves. Between early heading and anthesis, the percentage of leaf component decreased, the percentage of lower stem increased, and the percentage of upper stem did not change. In the lower stem, cellulose did not change between harvest times. In the upper stem, cellulose increased from early heading to anthesis in 1984, but not in 1983. For leaf tissue, NDF, ADF, ADL, and cellulose did not change between harvest times. NDF and ADF increased from early heading to anthesis for both upper and lower stem components. ADL did not increase significantly during the same interval for either upper or lower stem components (Porter, 1985).

Bals et al. (2010) harvested lowland variety Alamo at Auburn, Alabama in July and October 2005 and upland variety Cave-in-Rock at East Lansing, Michigan in July and October 2008, though it was not clear if it was under a single or double-cut management. Larger differences in composition were seen between harvests for Cave-in-Rock than for Alamo. Glucan content increased from 30.6 to 33.6% (db), xylan content increased from 19.4 to 25.3% (db), lignin increased from 10.4 to 16.7% (db), and total extracts decreased from 26.0 to 15.8% (db) for Cave-in-Rock from July to October. Alamo switchgrass from July to October had glucan content increase from 32.6 to 32.9% (db), xylan content increase from 22.8 to 23.0% (db), lignin content increase from 15.4 to 17.2% (db), and total extracts decrease from 18.1 to 15.0% (db). All changes listed were significantly different except glucan and xylan contents for Alamo (Bals et al., 2010).

Lemus et al. (2002) harvested 20 different switchgrass varieties in Iowa in November 13, 1998, September 30, 1999, and early January 2001. They found cell wall components increase in later harvests, which they attributed to the loss of more easily degraded plant components. Cellulose content was significantly different for each harvest date: September 1999 (34.0%), November 1998 (36.1%), and January 2001 (41.1%). The November and January harvests had more hemicellulose than the September harvest. ADL was different for all three harvest dates: 6.2% for September 1999, 7.0% for November 1998, and 5.7% for January 2001(Lemus et al., 2002).

Kim et al. (2011) also observed an increase in structural carbohydrates with delayed harvest. Alamo switchgrass was harvested in November 2007 and December 2006 at Ardmore, Oklahoma from two different plots. Glucan content increased from 29.9 to 32.1% (db), xylan content increased from 20.5 to 21.6% (db), lignin content increased from 18.8 to 19.5% (db), and water extractable sugar content decreased from 9.6 to 6.9% (db) from November to December, though harvests were in different years and from different plots (Kim et al., 2011). The

composition of two northern upland varieties were also compared, with Shawnee switchgrass harvested in December 2006 in Stillwater, Oklahoma and Dacotah switchgrass harvested in May 2008 in Pierre, South Dakota. Composition was affected more by harvest date than by variety, year, or location. The May harvest from Dacotah switchgrass left standing over the winter had very low water extractable sugar content at 0.8% (db). Glucan content was 35.3% (db), xylan content was 22.5% (db), and lignin content was 22.6% (db) for the May harvest (Kim et al., 2011).

Adler et al. (2006) found glucan and xylan concentrations increased significantly from October to April for Cave-in-Rock switchgrass left in the field over winter at Rock Springs, Pennsylvania in both 2002-2003 and 2003-2004. Soluble carbohydrates decreased significantly from fall to spring, 3.6 to 0.4% (db). Soluble components probably leached out over the winter. Storage polysaccharides, which are starches in switchgrass, also decreased significantly from fall to spring, 0.9 to 0.3% (db). Starch loss was likely due to seeds falling off over the winter. Klason lignin increased from fall to spring (Adler et al., 2006). Makaju et al. (2013) harvested Kanlow switchgrass in Stillwater, Oklahoma once a month from May through March of the following year for three growing seasons, 2007-2009. NDF, ADF, and ADL all increased significantly during the growing season from May to October for each year. However, NDF, ADF, and ADL did not change significantly from November to March for all three years, except for ADF for the 2007 crop, which increased significantly (Makaju et al., 2013).

McLaughlin and Kszos (2005) found ash content was reduced by delaying harvest until after the first frost, although yield was also reduced. Adler et al. (2006) observed a 30% reduction in ash content from a fall to spring harvest. Ash content reduction was due to element loss from leaching in the winter (Adler et al., 2006). However, Makaju et al. (2013) found that ash content did not change significantly from November to March for crops grown in 2007, 2008, and 2009.

3.4.3 Comparison of Composition of Switchgrass Varieties

Sladden et al. (1991) in Shorter, Alabama in 1990 found lowland ecotype varieties Kanlow and Alamo had significantly more cellulose than upland ecotype varieties Blackwell, Cave-in-Rock, Kansas Native, Pathfinder, Summer, and Trailblazer for initial harvest after anthesis. Kanlow and Alamo were harvested on July 10 and September 21 in a two-cut system. The other varieties were harvested June 6 and August 14 in a two-cut system. No significant difference in cellulose content was found between all varieties for the second cutting. In the first cutting, there were no significant differences in hemicellulose contents among all varieties. In the second cutting, Kansas Native was significantly higher and Summer was significantly lower than the other varieties in hemicellulose content. The lowland ecotype varieties had higher permanganate lignin than all upland ecotype varieties except Summer for the initial harvest. No difference in lignin was found for the second harvest (Sladden et al., 1991).

Lemus et al. (2002) harvested 20 different varieties in Iowa in November 13, 1998, September 30, 1999, and early January 2001. The results were averaged for each variety. No significant difference in cellulose was observed between varieties. NU942 had the highest hemicellulose content (33.5%), which was significantly higher than 13 other varieties. NU942 was followed by Alamo and Kanlow in hemicellulose content (both at 32.8%), which was only significantly higher than 4 other varieties. NL932 and NU942 had significantly lower ADL (5.3 and 5.4%, respectively) than all other varieties except Alamo (5.7%). Alamo had the lowest ash content (5.2%), which was significantly lower than all other varieties except Kanlow (5.4%). The ash content of Kanlow was significantly lower than 15 other varieties (Lemus et al., 2002).

3.4.4 Effect of Fertilizer on Switchgrass Composition

In Wisconsin, Porter (1985) reported that nitrogen fertilizer increased switchgrass yield (Mg/ha) when harvested on July 25 at early heading. Fertilizing with nitrogen also increased

NDF, ADF, and cellulose concentrations. However, the cellulose concentration of fertilized switchgrass only increased at early heading, not at anthesis. Also, ADF increased to a greater extent on July 25 at the early heading stage than on August 19 at anthesis stage. Nitrogen fertilization increased ADL in 1983, but not in 1984. Nitrogen fertilizer increased upper and lower stem percentage, but decreased leaf percentage (Porter, 1985).

3.5 Pretreatment

Lignocellulosic material needs to be pretreated prior to enzymatic hydrolysis due to the crystalline structure of cellulose and the seal of lignin (Mosier et al., 2005). Without a pretreatment step, sugar yields from subsequent enzymatic hydrolysis are low (Kim et al., 2011; Mosier et al., 2005). Pretreatment of lignocellulose disrupts cell wall structure and provides enzymes access to cellulose and hemicellulose (Mosier et al., 2005). Pretreatment methods include comminution, extrusion, alkali, concentrated acid, dilute acid, ozonolysis, organosolv, ionic liquids, aprotic solvents, metal complexes, ammonia fiber expansion (AFEX), soaking in aqueous ammonia (SAA), wet oxidation, microwave, ultrasound, carbon dioxide explosion, steam explosion, and hydrothermolysis (Alvira et al., 2010; Kim et al., 2011; Mosier et al., 2005). The effects of the pretreatment vary depending on the method used. The pretreatment method will affect downstream processing steps for conversion of biomass to ethanol (Alvira et al., 2010). The pretreatment step will also affect the economic viability of the process, as it is considered one of the most expensive steps (Mosier et al., 2005). Hydrothermolysis has been considered one of the leading pretreatment methods, especially for grasses (Alvira et al., 2010; Kim et al., 2011; Mosier et al., 2005). Hydrothermolysis produced the second highest glucose and highest xylose contents in a comparison of AFEX, SAA, lime, dilute sulfuric acid, and hydrothermolysis pretreatment technologies using switchgrass (Kim et al., 2011).

Hydrothermolysis is a liquid hot water pretreatment where water is added to lignocellulosic material and heated under pressure so the water remains in the liquid state (Mosier et al., 2005). Hydrothermolysis depolymerizes and dissolves some lignin, dissolves most of the hemicellulose, and increases digestibility of cellulose by enzymes (Alvira et al., 2010). The amount of biomass dissolved ranges from 40 to 60% (Mosier et al., 2005). Temperatures for hydrothermolysis range from 140 to 240°C for a duration of 10 to 30 min (Alvira et al., 2010; Mosier et al., 2005; Suryawati et al., 2009; Yu et al., 2008). The combination of temperature and time affect the severity of the pretreatment, and can be calculated by the severity equation, $R_O = t \times e^{[(T-100)/14.75]}$, where t is time in min and T is temperature in °C (Overend and Chornet, 1987). The logarithm of R_O is typically reported (Yu et al., 2008). During pretreatment, acetic acid and other organic acids are formed by O-acetyl and uronic acid substitutions from hemicellulose. These acids help to catalyze the formation and removal of oligosaccharides (Mosier et al., 2005). If conditions are too severe, sugars will degrade into aldehyde compounds that can inhibit fermentation organisms; hexoses will degrade to 5-hydroxymethylfurfural (HMF) and pentoses will degrade to furfural (Mosier et al., 2005). Maintaining pH between 4 and 7 retains hemicellulose as oligomers and minimizes formation of monomers, which reduces sugar degradation to fermentation inhibitors (Alvira et al., 2010; Mosier et al., 2005). If the severity factor is too low, the pretreatment will be incomplete and the digestibility of cellulose will be impeded. A severity factor of $\log(R_O) = 3.65$ for switchgrass resulted in much lower ethanol production during SSF than higher severity factors (Suryawati et al., 2009). Glucose yields from rice straw dropped below 80% (db) for severity factors of $\log(R_O) = 3.35$ and less (Yu et al., 2008). Suryawati et al. (2009) optimized milled switchgrass hydrothermolysis pretreatment with the conditions 200°C for 10 min ($\log(R_O) = 3.94$) to balance maximizing ethanol from cellulose fermentation, dissolving hemicellulose, retaining hemicellulose as oligomers, and minimizing formation of inhibitors. Yu et al. (2008) optimized hydrothermolysis pretreatment of rice straw

harvested in November 2006 in Japan and determined the optimum conditions were 180°C for 30 min ($\log(R_O) = 3.83$). Rice straw pretreated at 200°C for 10 min had a slightly higher severity factor ($\log(R_O) = 3.94$) than conditions at 180°C for 30 min ($\log(R_O) = 3.83$); and both pretreatment conditions produced nearly the same glucose yield from hydrolysis at two different enzyme loadings, 10 FPU Acremonium/g substrate and 40 FPU Acremonium/g substrate. However, 180°C for 30 min produced lower inhibitor concentrations (Yu et al., 2008).

Hydrothermolysis allows for separation of solids enriched in cellulose from the liquid fraction rich in hemicellulose through filtration (Alvira et al., 2010). Suryawati et al. (2008) reported increasing glucan content of from 36.6 to 56.6% (db) and decreasing xylan content from 21.0 to 2.4% (db) in the solid fraction of switchgrass by hydrothermolysis at 200°C for 10 min with 10% (w/w) solids loading. Similarly, Faga et al. (2010) increased glucan content from 34.2 to 53.2% (db) and decreased xylan content from 23.3 to 2.6% (db) in the solid fraction of switchgrass by hydrothermolysis at 200°C for 10 min with 10% (w/w) solids loading. Yu et al. (2008) increased glucan content from 36.4 to 53.0% (db) and decreased xylan content from 19.2 to 2.8% (db) in the solid fraction of rice straw by hydrothermolysis at 180°C for 30 min with 9% solids loading. The liquid fraction is referred to as prehydrolyzate (Suryawati et al., 2008). A portion of the biomass will dissolve into the prehydrolyzate during hydrothermolysis. Suryawati et al. (2008) dissolved approximately 43.9% (db) and Faga et al. (2010) dissolved approximately 37.7% (db) of switchgrass into the prehydrolyzate. Suryawati et al. (2008) reported 4.6% (db) glucan and 28.0% (db) xylan of switchgrass were recovered in the prehydrolyzate.

While the prehydrolyzate contains sugars, it also can contain inhibitors to fermentation. In vivo tests showed that acetic acid affects both glycolysis enzymes and NADH dehydrogenase in the yeast *Saccharomyces cerevisiae* (Zhao et al., 2008). Ethanol production was inhibited by 50% for *S. cerevisiae* in the presence of acetic acid at two different concentrations and pH values: 4.3 g/L acetic acid at pH 5.5, and 1.4 g/L acetic acid at pH 4.5 (Olsson and Hahn-Hagerdal,

1996). However, Delgenes et al. (1996) found the strain *S. cerevisiae* CBS 1200 at pH 5.6 produced 99% of ethanol as the control in the presence of 5 g/L acetic acid and 73% of the control at 10 g/L acetic acid. Undissociated weak acids such as acetic acid can diffuse across the plasma membrane in microorganisms and dissociate in the cytosol, lowering the cytosolic pH (Palmqvist and Hahn-Hagerdal, 2000). More of the undissociated forms of weak acids are present at lower pH values. Thus, acetic acid inhibition of ethanol production via yeast fermentation increases as the pH decreases. Ethanol production for *S. cerevisiae* CBS 1200 was reduced to 57% in the presence of 0.5 g/L furfural and 29% in the presence of 1 g/L HMF (Delgenes et al., 1996). *S. cerevisiae* CBS 1200 was more sensitive to furfural than other strains of *S. cerevisiae* (Delgenes et al., 1996).

3.6 Hydrolysis and Fermentation

After pretreatment, the cellulose and hemicellulose can be hydrolyzed by enzymes into monomeric sugars for fermentation by microorganisms. Separate hydrolysis and fermentation (SHF) is the two-step approach where enzymes are first added and allowed time to generate sugar monomers before the fermentation step. Simultaneous saccharification and fermentation (SSF) combines enzymes and microorganisms to both hydrolyze oligomers and ferment sugar monomers in the same step. Simultaneous saccharification and co-fermentation (SSCF) is an SSF of both cellulose and hemicellulose together (Mosier et al., 2005).

Both hydrolysis rates of glucan and final glucose yields were found to be lower when switchgrass was left in the field and harvested the following spring. Kim et al (2011) reported 1 h glucose yields of May 2008 harvested Dacotah switchgrass to be half that of December 2006 harvested Shawnee and Alamo switchgrass after ammonia fiber expansion (AFEX), dilute acid, and hydrothermolysis pretreatments, despite higher glucan content in the Dacotah switchgrass. The difference in glucose yields between harvest times decreased with time of hydrolysis.

Dacotah 168 h glucose yields were 5 to 20% less than Shawnee and Alamo for five different pretreatments. The extent to which glucose yields for Dacotah switchgrass were lower was greatest for ammonia pretreatments. Shawnee and Dacotah switchgrass were subjected to the same conditions for AFEX pretreatment, which differed from conditions for Alamo switchgrass. Glucose yields, however, were similar for Shawnee and Alamo, with a much lower yield for Dacotah. This suggests that harvest date may have a large effect on glucose yields after AFEX pretreatment, but the effect could be confounded by switchgrass variety. Alamo and Shawnee switchgrass were pretreated at the same conditions for soaking in aqueous ammonia (SAA) pretreatment, with different conditions for Dacotah switchgrass. Conditions varied among ammonia pretreatments because optimum conditions were chosen. Glucose yields for SAA followed the same pattern as AFEX, with similar yields for Alamo and Shawnee versus a lower yield for Dacotah. The lower glucose yield for SAA may be due to different pretreatment conditions, unknown effects from different switchgrass varieties, or it could also be an indicator of reduced sugar yields for ammonia pretreatments at later harvest dates. Conditions were the same across switchgrass cultivars for dilute acid, hydrothermolysis, and lime pretreatments. Glucose yields for Dacotah switchgrass were closer to glucose yields for Alamo and Shawnee switchgrass for these three pretreatments, but they were still the lowest yields among the three cultivars. The effect of harvest date may be confounded by switchgrass variety in this study (Kim et al., 2011). Adler et al. (2006) found in vitro gas production rate, an indication of SSF yields, decreased 25% when harvest was delayed from fall to spring for Cave-in-Rock switchgrass at Rock Springs, Pennsylvania from fall of 2002 to spring of 2005. There was no significant harvest season by year interaction (Adler et al., 2006).

Bals et al. (2010) compared separate hydrolysis and cofermentation after AFEX pretreatment of July and October harvests of Alamo switchgrass at Auburn, Alabama in 2005 and Cave-in-Rock switchgrass at East Lansing, Michigan in 2008. It was not clear if the harvests

were under a one-cut or two-cut management. AFEX pretreatment conditions were optimized for all four harvests, resulting in different optimal pretreatment conditions for each harvest. Enzyme loadings were then optimized for switchgrass pretreated under the optimal conditions for each harvest, comparing varying amounts of Accellerase, β-glucosidase Novozyme 188, Multifect Xylanase, and Multifect Pectinase. Optimal amounts were a combination of Accellerase, Multifect Xylanase, and Multifect Pectinase, each loaded at 5 mg enzyme/g dry switchgrass for both Alamo harvests and the July Cave-in-Rock harvest. The optimal amounts for the October Cave-in-Rock harvest varied from these amounts by an increase in Accellerase to 6.4 mg enzyme/g dry switchgrass and a decrease in Multifect Xylanase to 3.6 mg enzyme/g dry switchgrass. Glucose and xylose yields from hydrolysis were higher for July harvest (32.1 and 20.0 % (db) switchgrass, respectively) than October harvest (22.3 and 18.7 % (db)) for Cave-in-Rock switchgrass, while October harvest (23.7 and 20.8 % (db)) yielded more glucose and xylose than July harvest (21.0 and 20.1 % (db)) for Alamo switchgrass. July harvest of Cave-in-Rock switchgrass yielded much more glucose than the other harvests. Sugar yields were determined by g sugar/kg switchgrass, but it was not clear if the yields were in terms of untreated or pretreated switchgrass. Cofermentation of glucose and xylose by *Saccharomyces cerevisiae* 424A, a genetically modified yeast that can ferment xylose in addition to glucose, was conducted for switchgrass from each harvest after pretreatment under optimal conditions with optimal enzyme loadings. Solids loading was 20%, except for October Alamo switchgrass, which was at 10% solids loading. Ethanol was still increasing when fermentations were stopped at 96 hr, when glucose was consumed, but xylose was still being utilized. Ethanol yield for Cave-in-Rock at 96 h was higher for July harvest at 34 g/L than October harvest at 30 g/L, and also contained more residual xylose which was still being consumed. When comparing varieties at July harvests, Cave-in-Rock also had a higher ethanol yield than Alamo, which produced 30 g/L. Comparison of ethanol yield of October Alamo switchgrass to the other ethanol yields is difficult due to a different solids loading in the October Alamo fermentation (Bals et al., 2010).

3.7 Conclusion

A single-cut management will likely be used across much of the United States for switchgrass grown for ethanol production. Timing of the harvest will likely focus on maximizing long term yield; minimizing a combination of stand loss from earlier harvests and residue loss from later harvests.

There is lack of a detailed carbohydrate composition of switchgrass throughout likely harvest periods from the end of the growing season through senescence. Further, there is a lack of switchgrass hydrolysis and fermentation data throughout this period. A comparison of multiple harvest dates within the same harvest season for a single variety of switchgrass grown at the same location will aid in defining a harvest window for switchgrass used for ethanol production.

CHAPTER IV

MATERIALS AND METHODS

4.1 Harvest and Sample Preparation

Switchgrass (*Panicum virgatum*, var. Kanlow) was used for all experiments. Kanlow is a lowland cultivar. The switchgrass was from a mature, ten-year old stand planted in 1998 in an Easpur loam soil. The switchgrass received no application of fertilizer, nutrients, or pesticides both during and three years prior to the harvest for this study. It was grown at an Oklahoma State University research field near Stillwater, OK. Weather data for the switchgrass plot is provided in the Appendix. Additional data for the switchgrass can be found in Makaju et al. (2013), as both that study and this study used the same switchgrass plot.

Switchgrass was harvested near the 22nd of July, August, September, October, and November of 2008, dependent of weather. The first freeze of the fall occurred in mid-November, and the November sample was harvested after this freeze. Thus the effects of a freeze on switchgrass could be analyzed. Normally, anthesis of lowland switchgrass occurs in August in Oklahoma. Senescence typically begins at the end of August in the leave blades and is completed by November (Yanqi Wu, personal communication). A single-harvest management was used. Each harvest was the first cutting of the season; different plants within the same plot were harvested each time. There were six samples collected from the plot each month. The plot was divided into six zones, with a sample taken from each zone. The samples were combined into a

single bulk sample for each month. The bulk samples were dried for a week at 50°C, after which the moisture content was 5 ± 1% for all samples. After drying, each bulk sample was ground from a bundle of whole stalks to particles that could pass through a 2 mm sieve. A Thomas-Wiley Laboratory Mill, model 4 (Arthur H. Thomas Company, Philadelphia, PA, U.S.A.), was used for grinding the switchgrass, which was then stored at room temperature in a plastic zip-loc bag.

4.2 Percent Solids Determination

The dry solids content of the switchgrass was determined after grinding, after extraction, after pretreatment, and before fermentation. The National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedure (LAP), "Standard Test Method for Moisture, Total Solids, and Total Dissolved Solids in Biomass Slurry and Liquid Process Samples" was used to determine the dry solids content (Ehrman, 1994). Samples and weighing tins were heated in an oven (Lab-Line Instruments, Inc., Melrose Park, IL, U.S.A.) at 105 ± 5°C. The dried samples and weighing tins were cooled inside a vacuum desiccator. The mass of the samples and weighing tins were measured using an analytical balance (P-314, Denver Instruments, Bohemia, NY, U.S.A.) to the nearest 0.1 mg.

4.3 Pretreatment

A hydrothermolysis pretreatment was used to disrupt the lignin structure and dissolve hemicellulose, making the cellulose available for enzymatic hydrolysis. A Parr reactor (Series 4520, Parr Instrument Company, Moline, IL, U.S.A.) was used to conduct the pretreatment. A mass of 60 g of dry, ground switchgrass and 540 g deionized water (resistivity < 18 MΩ/cm) were mixed at 500 rpm and heated from approximately 20°C to 200°C. The temperature was maintained at 200 ± 2°C for 10 min. The severity factor was $\log(R_O) = 3.94$. These conditions were chosen to optimize sugar recovery and ethanol yield, while keeping inhibitor production low

(Suryawati et al., 2009). The switchgrass and water slurry was cooled to 40°C using an ice water bath before opening the gas tight container; thus losses of volatile compounds were minimized.

The liquid (also known as prehydrolyzate) and solid portions of the slurry were then separated via vacuum filtration using a Whatman #5 filter (Whatman Schleicher & Schuell, Maldstone, England) The mass of solids remaining on the filter and the mass of prehydrolyzate were determined. The prehydrolyzate was measured for pH and then stored at 4°C. The solids were washed four times with 500 mL of 60 to 63°C deionized water. The rinse water was removed by vacuum filtration and its pH was measured after cooling to room temperature. After the fourth rinse, the solids were kept under vacuum long enough to remove most of the water. A sample consisting of approximately 5 g of wet solids was taken to determine dry solids content. Washed solids were stored at 4°C.

A mass balance was attained by measuring the mass of the solid and liquid material both before and after pretreatment. The following were measured:

mass of the switchgrass loaded into the Parr reactor = m_{gl}

percent dry solids of switchgrass before pretreatment = $\%Solids_{gl}$

mass of water loaded into the pretreatment cell = m_w

mass of the prehydrolyzate = m_p

mass of the wet solids remaining on filter after filtration, but before rinsing = m_{wsi}

mass of wet solids after rinsing = m_{wsf}

percent dry solids of wet solids after rinsing = $\%Solids_{wsf}$

The mass recovered after the pretreatment process was calculated using the following equation:

$$\%recovered = \frac{m_{gl} + m_w}{m_{wsi} + m_p} \quad (1)$$

The amount of grass dissolved into the water during the pretreatment process was calculated by the following equation:

$$\%\,\text{dissolved} = 1 - \frac{\text{m}_{\text{wsf}} * (\%\text{Solids}_{\text{wsf}}/100)}{(m_{gl} * \%Solids_{gl}) * \%recovered} \quad (2)$$

4.4 Compositional Analysis

Samples of each month's switchgrass harvest were analyzed for structural carbohydrates and lignin. This analysis was performed both before and after hydrothermolysis pretreatment. The samples had to undergo either an extraction or pretreatment process before constituents could be determined. The extraction procedure followed was the NREL LAP titled "Determination of Extractives in Biomass" (Sluiter et al., 2007). Structural carbohydrates were then determined using the NREL LAP titled "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al., 2004). Figure 4.1 shows an overview of the analysis of the switchgrass.

Dried, ground switchgrass underwent a water extraction followed by an ethanol extraction. The extractions were performed using an Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA, U.S.A.) Both the water and ethanol extractions used the following method: 1,500 psi, 100°C, 5 min heat time, 7 min static time, 150% flush volume, 120 sec purge time, and 3 static cycles. Ethanol was allowed to evaporate from the ethanol extracts in a fume hood. A sample of the water extracts was pulled for sugar content analysis. The water was evaporated in a 40°C oven from the water extracts.

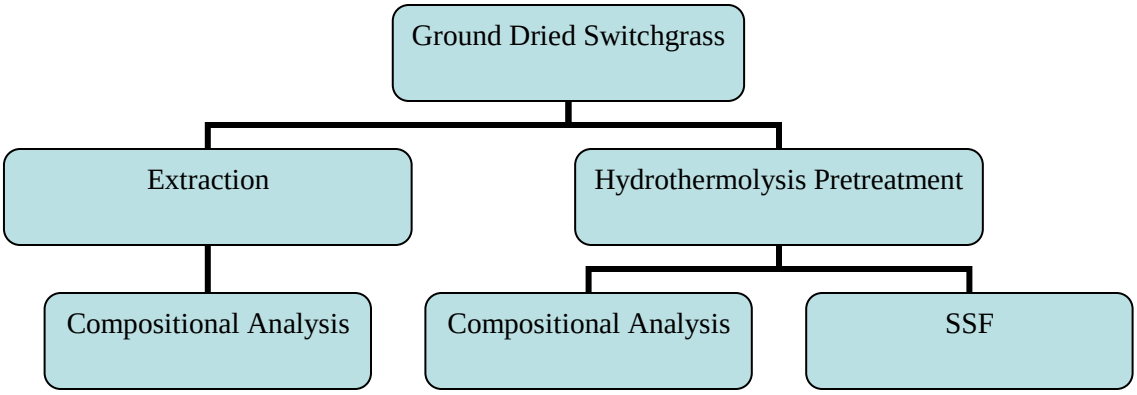

Figure 4.1 Overview of Analyses. Compositional Analysis was performed on ground switchgrass both before and after pretreatment. An extraction was required before compositional analysis for untreated switchgrass. Pretreated switchgrass was then processed by simultaneous saccharification and fermentation (SSF).

The extracts were calculated as a percent of switchgrass on a dry weight basis:

$$\%Extractives = \frac{mass_{extractives}}{mass_{grass} * \%Solids} \quad (3)$$

Glucose and sucrose contents of the water extractives were determined with a High Performance Liquid Chromatograph (HPLC) (Agilent 1100 Series, Santa Clara, CA, U.S.A.). A Biorad Aminex HPX-87P sugar column at 85°C with a deionized water mobile phase at a 0.6 mL/min flow rate was used for carbohydrate separation. A refractive index detector (RID) (Agilent 1100 Series) was used for quantification of compounds.

Switchgrass was analyzed for lignin and structural carbohydrates after either extraction or pretreatment. A two-stage acid hydrolysis with 72% sulfuric acid at 30°C for 60 min and 4% sulfuric acid at 121°C for 60 min was used to disrupt lignin and hydrolyze structural carbohydrates. Each acid and grass suspension was vacuum filtered using a filter crucible. Acid insoluble lignin (AIL) was determined from the dry mass of the acid insoluble residue (AIR) and the ash in the solids. The following equation gives the percent of AIL on an extractives free basis:

$$\%AIL_{ExtractivesFree} = \frac{m_{AIR} - m_{ash}}{m_{drygrass}} * 100 \quad (4)$$

where $m_{drygrass}$ is the dry mass of the switchgrass acid hydrolyzed.

Dry mass was measured after heating in an oven for 24 h and cooling in a vacuum desiccator. Ash mass was measured after heating in an Isotemp Programmable Muffle Furnace (Fisher Scientific, Dubuque, IA, U.S.A.) using the following program: increase temperature to 105°C, hold at 105°C for 12 min, increase temperature to 250°C at a rate of 10°C/min, hold at 250°C for 30 min, increase temperature to 575°C at a rate of 20°C/min, hold at 575°C for 180 min, allow

temperature to decrease to 105°C. Acid soluble lignin (ASL) was determined by measuring the absorbance of the filtrate with a UV-Vis spectrophotometer (Cary 50 Bio UV-Visible Spectrophotometer, Varian, Palo Alto, CA, U.S.A.) at a wavelength of 205 nm using a quartz cuvette (Thammasouk et al., 1997). Acid soluble lignin on an extractives free basis was calculated with the following equation:

$$\%ASL_{ExtractivesFree} = \frac{UVabsorbance * Volume_{Filtrate} * Dilution}{\varepsilon * m_{drygrass}} * 100 \quad (5)$$

where ε is the absorptivity of biomass at a specific wavelength (110 L/g cm) and $m_{drygrass}$ is the dry mass of the switchgrass acid hydrolyzed. (Thammasouk et al., 1997).

Total lignin content on an extractives free basis is the sum of %AIL and %ASL. Acid filtrate was neutralized with calcium carbonate and filtered through a 0.2 μm filter. Structural carbohydrate contents were determined from neutralized filtrate by HPLC analysis using the same method as for extractive sugar content. Samples were analyzed for cellobiose, glucose, xylose, galactose, arabinose, and mannose.

Lignin and structural carbohydrates were calculated on an as received basis to account for the mass removed by extraction. The following equations were used:

$$\%AIL_{As\,Received} = \%AIL_{ExtractivesFree} * \left(1 - \frac{\%Extractives}{100}\right) \quad (6)$$

$$\%ASL_{As\,Received} = \%ASL_{ExtractivesFree} * \left(1 - \frac{\%Extractives}{100}\right) \quad (7)$$

Acid insoluble lignin, acid soluble lignin, and structural carbohydrates were calculated as a percent of switchgrass on a dry weight basis.

4.5 Simultaneous Saccharification and Fermentation

Pretreated, rinsed switchgrass was converted to ethanol through a SSF process. The NREL LAP "SSF Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation" was followed with some modifications (Dowe and McMillan, 2001). Two SSF experiments were conducted, one with all five harvest dates and one using only July, September, and November harvest dates.

4.5.1 Switchgrass Preparation

Switchgrass pretreated using the hydrothermolysis pretreatment described in section 4.3 was used in the SSFs. The solids from two batch hydrothermolysis pretreatments of each harvest date were combined and mixed. After compositional analysis, the combined pretreated switchgrass solids were used in the SSF. The liquid fraction from hydrothermolysis pretreatment was not added to the SSF. The SSF of July, September, and November switchgrass used different pretreated batches than the SSF of all five harvest dates. The moisture content of the pretreated switchgrass was determined one day before use in the SSF.

4.5.2 Yeast Preparation

The yeast strain *Saccharomyces cerevisiae* D_5A was used for the fermentation. The yeast was stored in a refrigerator at 4°C on an agar slant consisting of 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 22 g/L dextrose monohydrate, and 22 g/L agar. Aseptic procedure was used to inoculate liquid medium with yeast from the slants. The liquid medium consisted of 10 g/L yeast extract, 20 g/L peptone, and 50 g/L glucose. The medium was filter sterilized through a 0.22 μm bottle-top filter. A volume of 100 mL of liquid medium was poured into a 250 mL baffled flask and inoculated with a loop of cells. The flask was covered with a Bugstopper (Whatman Inc., Florham Park, NJ, U.S.A.), which allows aerobic conditions while maintaining a monoculture through aseptic gas transfer. The flask was placed in a Max Q 4450 incubated

orbital shaker (Thermo Scientific, Dubuque, IA, U.S.A.) at 37°C and 250 RPM. A higher rotational speed was used for the aerobic growth of the yeast culture than the anaerobic SSF to ensure adequate oxygen to the yeast culture, whereas the SSF only need to be well-mixed.

A second flask was filled with 90 mL of liquid medium and inoculated with 10 mL of well-mixed volume from the first flask. This was done 16 h after the first flask was inoculated. The yeast were in the exponential growth phase after that period of incubation. The second flask was covered with a Bugstopper and incubated in an orbital shaker at 37°C at 250 RPM. The optical density (OD) of the second flask was measured to determine whether adequate cell growth had occurred. The optical density was measured by a UV-Vis spectrophotometer (Cary 50 Bio UV-Visible Spectrophotometer, Varian, Palo Alto, CA, U.S.A.) at 600 nm. The absorbance measurement was multiplied by the dilution factor, which is the total volume divided by the sample volume. This product is the optical density of the yeast culture.

The volume of culture needed to supply the amount of cells necessary for the SSF was calculated based on the yeast culture OD, desired starting OD of the SSF, the volume of the SSF, and the number of flasks to be used for the SSF.

$$Volume_{inoculum} = \frac{Volume_{SSF} \times OD_{SSF,initial}}{OD_{inoculum}} \times \left(number_{SSF\_flasks} + 1\right) \quad (8)$$

Adding one to the number of flasks creates a slightly larger working volume. This is advantageous as it is difficult to pipette the final droplets remaining in a container; and the entire volume would be required without adding one to the number of flasks. The initial OD was 0.5 for each SSF. There were 18 flasks for the SSF of all five harvest dates and 7 flasks for the SSF of July, September, November harvest dates.

The volume of inoculum that is calculated was then withdrawn via pipette from the well-mixed inoculum flask using sterile technique. The volume was split between two 50 mL sterile

centrifuge tubes. The inoculum was centrifuged at 3,750 RPM for 6 min with a Sorvall Legend RT centrifuge (Kendro, Asheville, NC, U.S.A.). Then the supernatant was decanted. The cells were resuspended in DI water to wash away residual sugar. The cell suspension was centrifuged at 3,750 RPM for 6 min. The supernatant was decanted and the cells were resuspended in DI water. The volume used to suspend the cells was the number of flasks used plus one, in mL. Since 18 flasks were used, the cells were suspended in 19 mL. This allows for each flask to receive one mL of cell suspension with one mL excess.

4.5.3 Enzyme

A commercial enzyme, Accellerase 1500 (Genencor, Palo Alto, CA, U.S.A.), was used to saccharify the switchgrass cellulose into monomers for the SSF of all five harvest dates. Another commercial enzyme, Fibrilase (Iogen, Ottawa, Canada) was used for saccharification in the SSF of only July, September, and November harvest dates. The activity of the enzyme was measured in filter paper units (FPU) using NREL LAP "Measurement of Cellulase Activities" (Adney and Baker, 1996).

4.5.4 Loading Quantities

Switchgrass comprised 8% of the SSF on a dry mass basis. SSFs are typically loaded by glucan content, rather than dry mass. However, in keeping with the objective, a comparison of ethanol production among different harvest dates based on a dry mass basis is more suitable than a comparison based on glucan content. Ethanol production can be compared between different harvest months based on yield per dry mass unit. This yield can be directly correlated to the yield of dry mass of switchgrass per area of land. Combining the two yields allows a comparison of harvest dates based on ethanol per area of land. Each SSF consisted of 100 g of material loaded into a 250 mL baffled flask. Wet, pretreated switchgrass was loaded based on its moisture

content to total 8 g dry grass. DI water was also added on a mass basis according to the following equation:

mass wet grass + 5 g citrate buffer at pH 4.5 + 10 g 10X YP media + 1 g yeast + 1 g enzyme + DI water = 100 g

The flasks containing wet switchgrass and DI water were capped with Bugstoppers. Then the mass of each flask was measured and recorded. The flasks and a container of DI water were sterilized at 121°C for 1 h by an autoclave. The flasks were dried and allowed to cool. The mass of each flask was measured again. The difference in mass was attributed to evaporation of water in the autoclave. The sterilized DI water was added aseptically to replace the evaporated water. A 1.0 M citrate buffer solution was filter sterilized. A 10X yeast extract and peptone (YP) nutrient solution was prepared with 100 g/L yeast extract and 200 g/L peptone and filter sterilized. Citrate buffer was at 50 mM, yeast extract at 10 g/L, and peptone at 20 g/L for fermentations. Volumes of 5 mL of 1.0 M citrate buffer at pH 4.5, 10 mL of 10X concentrated YP medium, 1mL of 100X concentrated yeast solution, and 1 mL of enzyme were also added aseptically. The time of the SSF started once the enzyme was added. The yeast was added second to last and the enzyme was added last. The SSF of all five harvest dates using Accellerase enzyme contained 9 FPU/g glucan. The SSF of July, September, and November harvest dates using Fibrilase enzyme contained 14 FPU/g glucan.

4.5.5 SSF Conditions

Flasks were placed into a C25 Incubator Shaker (New Brunswick Scientific, Edison, NJ, U.S.A.) after initial samples were taken. The temperature was held at 37°C and the shaker speed was at 130 RPM. Anaerobic conditions were maintained using a one-way air valve and rubber stopper to cap each flask. Each rubber stopper contained a hole in the center. A one-way air valve was inserted into this hole. Gases were allowed out of the flask by the one-way air valve,

which prevented excessive pressure from building up. No gases were allowed into the flask, which maintained the anaerobic condition and prevented contamination.

4.5.6 Sampling

Each flask was sampled aseptically at 0, 6, 24, 48, 72, 96, 120, 144, and 168 h after enzyme addition. Flasks were transferred to a freshly sterilized biosafety cabinet. The one-way air valve was removed, the mouth of the flask flamed, the flask was swirled to ensure a well-mixed slurry, and a sterile pipette tip used to remove 1.5 mL of sample. The samples were put into 2 mL microcentrifuge tubes and centrifuged at 13,000 RPM for 10 min by an accuSpin Micro microcentrifuge (Fisher Scientific, Hampton, NH, U.S.A.). The supernatant was filtered through a 0.45 µm nylon filter into an HPLC vial. The pH of the flasks was measured after the last sample at 168 h. The pH was measured using a pH probe (ORION 310 pH meter, Thermo Electron Corporation, Beverly, MA, USA; VWR symphony probe, West Chester, PA, USA).

4.5.7 Analysis

The filtered SSF samples were analyzed by HPLC to determine the concentrations of cellobiose, glucose, xylose, xylitol, succinic acid, glycerol, ethanol, and acetic acid. The mobile phase was 0.01 M H_2SO_4. An Aminex HPX-87H column was used for separation of compounds. RID was used to quantify the compounds. External standards were used for the calibration. There were three SSF flasks set up for each harvest date of switchgrass, as well as the control, for the SSF of all five harvest dates. There were two SSF flasks set up for each harvest date and only one control flask for the SSF of July, September, and November harvests. Tukey's test was used to separate means between harvest dates at a 95% confidence interval with SAS Release 9.3 (SAS, Cary, NC, U.S.A.).

CHAPTER V

RESULTS AND DISCUSSION

5.1 Composition of Switchgrass through Harvest Season

5.1.1 Extractives Content

The percentage of total mass extracted decreased throughout the harvest interval, from 13.8% (db) in July to 5.3% (db) in November. Figure 5.1 shows the extractives as percent dry mass. The amount extracted decreased over the harvest interval for both the water and ethanol extractions. Ethanol extractives include chlorophyll, waxes, and other minor constituents (Ruiz et al., 2007). Water extractives include inorganic material, non-structural carbohydrates, and nitrogenous material (Ruiz et al., 2007). Water extractives decreased faster after September.

Water extractives were analyzed for sugar content by HPLC. Both sucrose and glucose were detected. Extractable sucrose content of switchgrass ranged from 2.43% (db) in August to 0.09% (db) in November. Extractable glucose content ranged from 1.29% (db) in September to 0.18% (db) in November. A reduction in the amount of sugar extracted was observed after September, as shown in Figure 5.2. Adler et al. (2006) and Lemus et al. (2002) also observed that soluble and storage sugars declined as plants aged. Sugars are not produced by photosynthesis as above ground switchgrass tissue dies during senescence. Extractable glucose and sucrose peaked near 3.5% (db) in August and September.

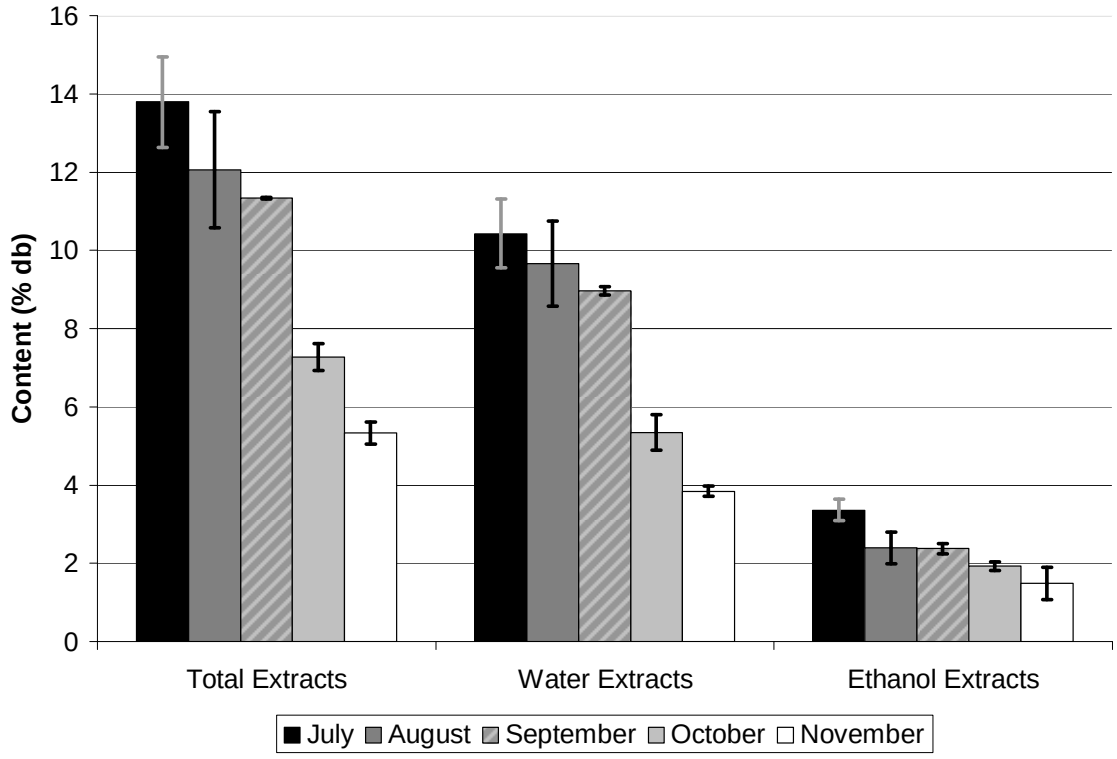

Figure 5.1 Water and ethanol extractive content in switchgrass harvested during different months.

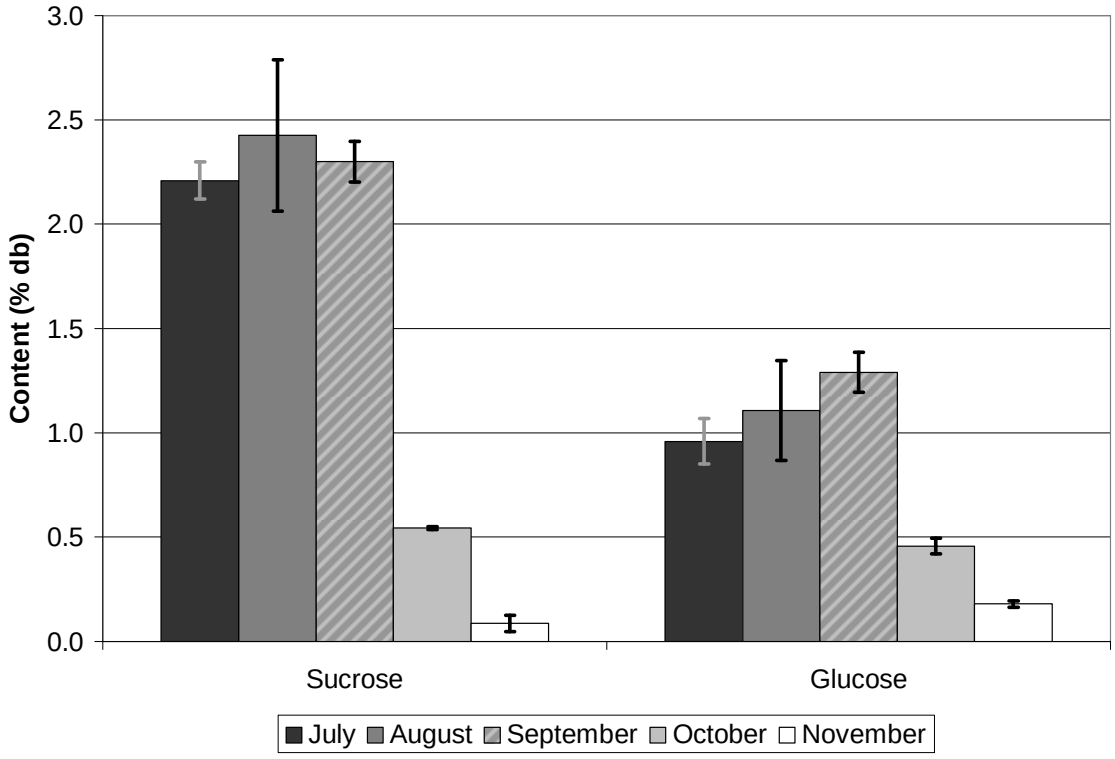

Figure 5.2 Sugar content removed by water extraction in switchgrass harvested during different months.

5.1.2 Structural Carbohydrate Content

Structural carbohydrates were analyzed after extraction by water and ethanol. Contents are expressed as a percentage of switchgrass dry mass before the removal of extracts. Glucan content increased over the harvest period; values for each month from July to November were 36.5, 37.1, 37.7, 39.7, and 41.4% (db), respectively. Figure 5.3 shows changes in structural carbohydrate content over the harvest period. Makaju et al. (2013) found a similar increase in cellulose content over the same period for the same switchgrass stand; cellulose contents were 41, 42, 42, 44, and 44% (db) from July to November, respectively. The cellulose content measured by the method used by Makaju et al. (2013) tends to overestimate structural glucan content by 2 to 4% (Wolfrum et al., 2009). Bals et al. (2010) measured a smaller increase in glucan content of Alamo switchgrass over a similar period in Auburn, AL in 2005, with 32.6% (db) for July and 32.9% (db) for October. However, the switchgrass in Auburn may not have senesced as much by October as the Kanlow grass in Stillwater, OK due to differences in climate and latitude. Bals et al. (2010) did not observe a large decline in extractives in October as was the case in this study. Porter (1985) observed a 4% (db) cellulose increase from July to August in Wisconsin for two upland cultivars, more than the 0.6% (db) glucan content increase observed in this study for the lowland cultivar Kanlow. Xylan content held steady from July through September at approximately 22.5% (db), increased in October to 26.1% (db), and then decreased to 24.8% (db) in November (Figure 5.3). In Iowa, Lemus et al. (2002) observed an increase in cellulose and hemicellulose of 2.1 and 3.6%, respectively, for average values of both upland and lowland cultivars from September to November, although harvests were from different years. These results from Lemus et al (2002) correspond to glucan and xylan content increases of 3.7 and 2.1% (db), respectively, from September to November for this study. Dien et al. (2006) measured a 3.9 and 2.8% (db) increase in glucan and xylan content, respectively, for upland Cave-in-Rock switchgrass from anthesis to post frost growth stages in 2003 at Mead, Nebraska.

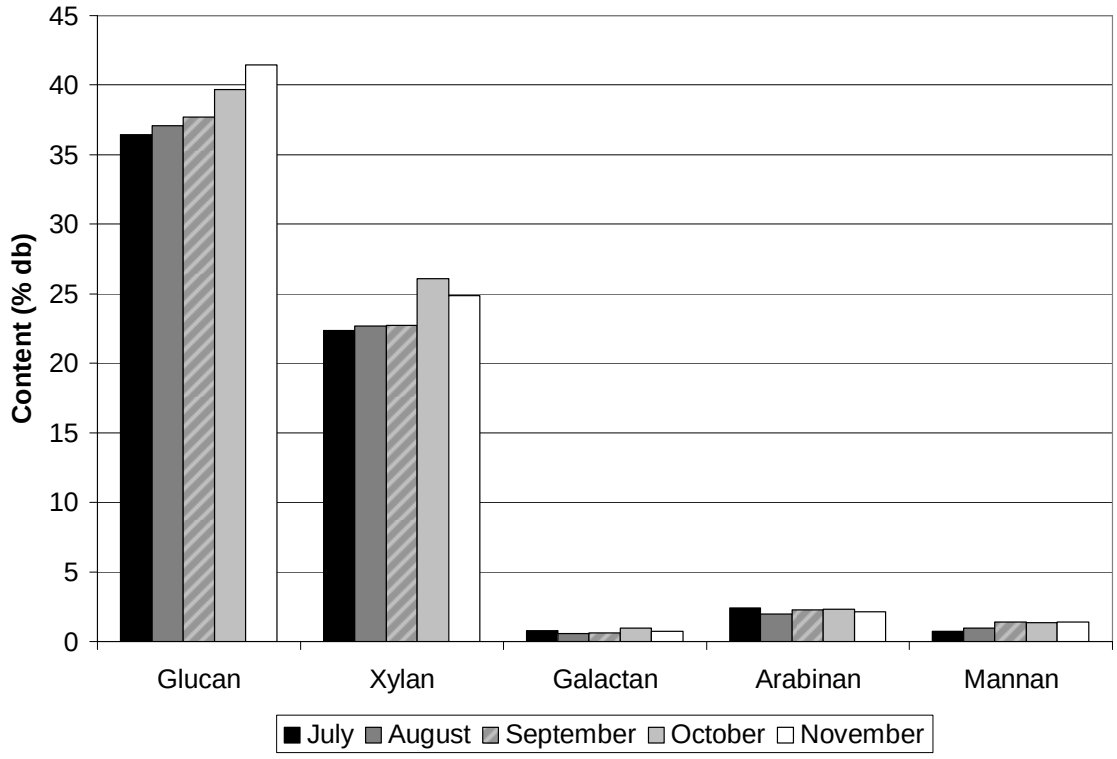

Figure 5.3 Structural carbohydrate content in switchgrass harvested during different months.

Dien et al. (2013) measured changes in glucan and xylan content from anthesis to post frost growth stages of Cave-in-Rock to be -0.7 and 1.9% (db), respectively, and Kanlow N1 to be 2.2 and 0.7% (db), respectively, at Mead, Nebraska. These growth stages correspond to August and November harvests in this study where glucan and xylan content increased by 4.3 and 1.4% (db), respectively. Arabinan content ranged from 2.0 to 2.4% (db) and mannan content ranged from 0.7 to 1.4% (db) (Figure 5.3). Galactan content was less than 1.0% (db) for all months (Figure 5.3).

Glucan content was more constant over the harvest period on an extractives free basis than on a whole plant basis, as shown in Figure 5.4. This indicates that most of the increase in glucan content over the harvest period for total plant composition is from the declining extractives content rather than additional structural carbohydrates. If there are insignificant gains in structural carbohydrates over the harvest period, then the same mass of glucan can be harvested at any time. Further, it is not necessary to preserve the soluble sugars present in the extractives because they would be degraded in almost all types of pretreatments. It is unlikely to be cost effective to extract these sugars for fermentation.

5.1.3 Lignin Content

Lignin was analyzed in switchgrass after extraction by water and ethanol. Lignin content is expressed as a percent of switchgrass before extraction. Lignin content increased from July at 17.8% (db) until September at 20.5% (db), after which a very slight increase to 20.8% (db) in November was observed. Figure 5.5 shows the lignin content of switchgrass over the harvest interval. The acid insoluble portion of lignin was the major lignin component and followed the same pattern as the total lignin, ranging from 15.2 to 18.8% (db) from July to November. However, the acid soluble portion of lignin decreased from 2.6 to 2.0% (db) from July to November.

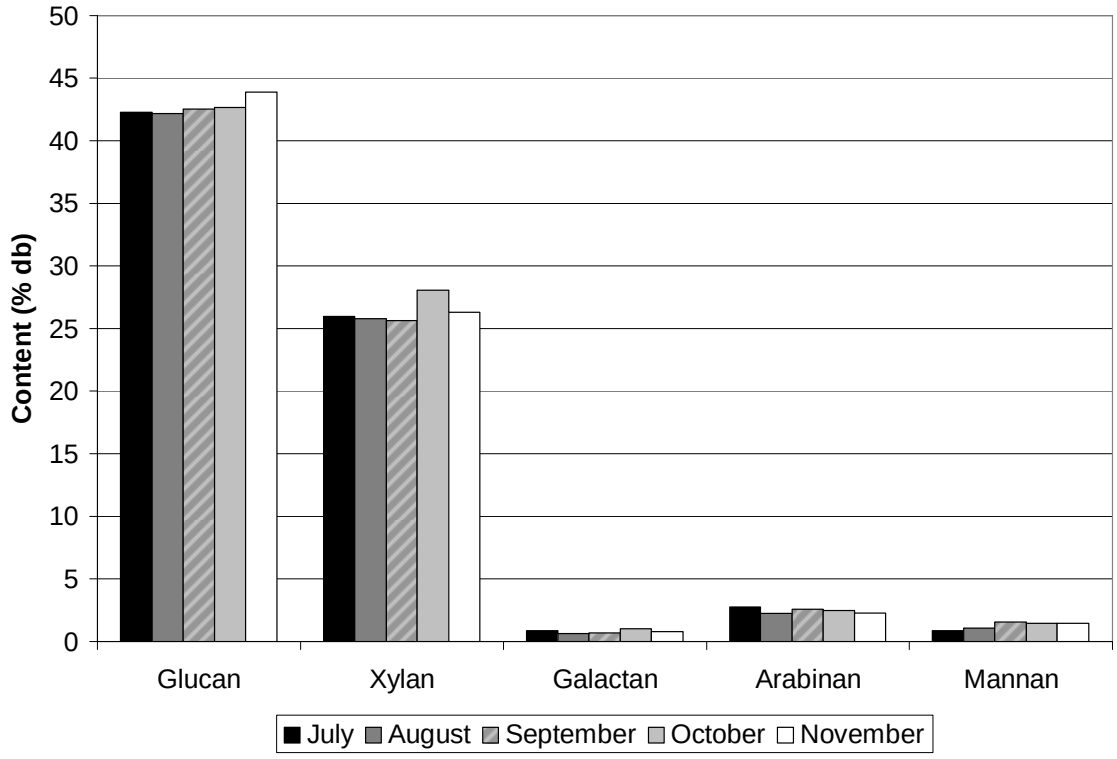

Figure 5.4 Structural carbohydrate content on extractives free basis in switchgrass harvested during different months.

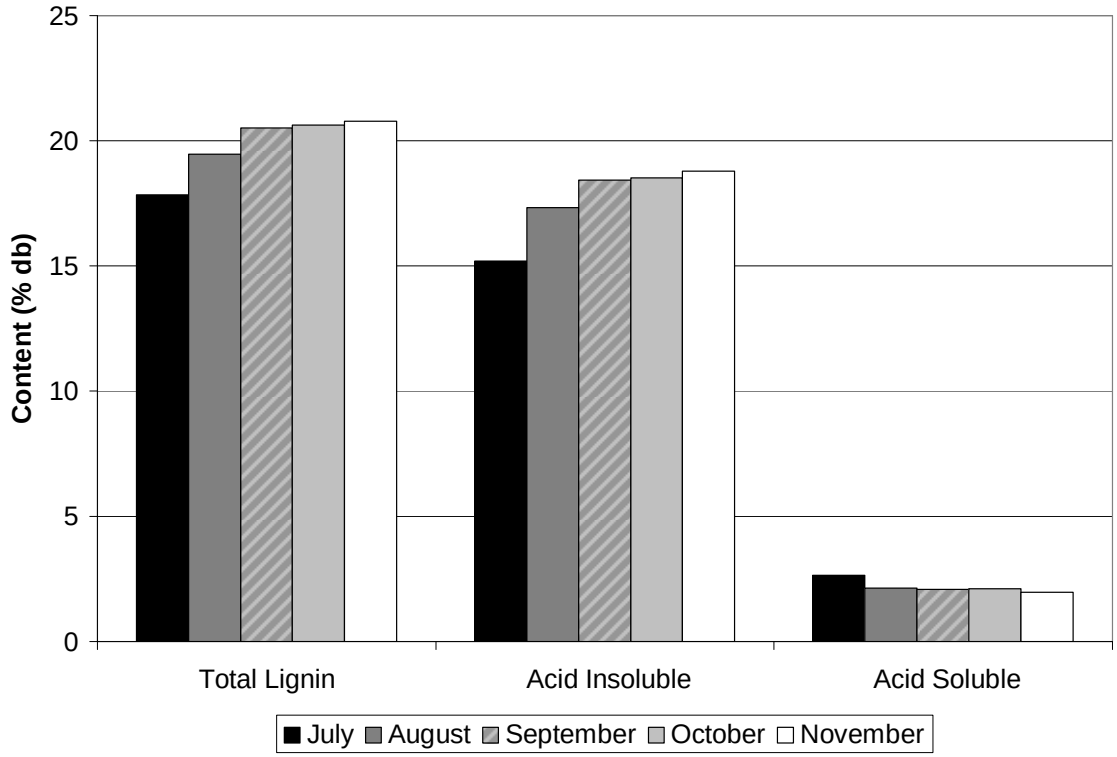

Figure 5.5 Lignin content in switchgrass harvested during different months. AIL is acid insoluble lignin and ASL is acid soluble lignin.

Increases in lignin will decrease access to structural carbohydrates. Depending on the effectiveness and cost of pretreatment, an earlier harvest may be advantageous to avoid increased lignin content.

5.2 Effect of Switchgrass Maturity on Simultaneous Saccharification and Fermentation Yields

5.2.1 Composition of Switchgrass after Pretreatment

Switchgrass was pretreated by hydrothermolysis to disrupt lignin structure so that enzymes could access cellulose during SSF. The percent of switchgrass dissolved by pretreatment begins to decline in October. The decrease in the percent dissolved is similar to the decrease in the percent extracted from July to November, shown in Figure 5.6.

The effects of the pretreatment on the composition of switchgrass were analyzed. The sample used to determine the dry matter content of switchgrass analyzed by acid hydrolysis was lost for the November harvest sample. The average of the other four harvest date samples was used to estimate the dry matter content of the November sample. The standard deviation for the dry matter content of the samples from the other four harvest dates was 0.157%; therefore, it can be assumed that the error introduced is very small from this factor.

Lignin contents of pretreated switchgrass are shown in Figure 5.7. Lignin content increased in switchgrass solids after pretreatment by hydrothermolysis for all harvest dates (Figures 5.5 and 5.7). Lignin mostly remained in the solid fraction, while other components were dissolved into the liquid fraction. The resultant mass loss from dissolved components makes lignin a larger constituent in the remaining solids. After pretreatment, the lignin content profile over the harvest season changed. The highest lignin content of pretreated switchgrass at 34.6% (db) occurred with an August harvest date (Figure 5.7). The October and November harvest dates had the highest lignin content before pretreatment at 20.6 and 20.8% (db) (Figure 5.5), but the

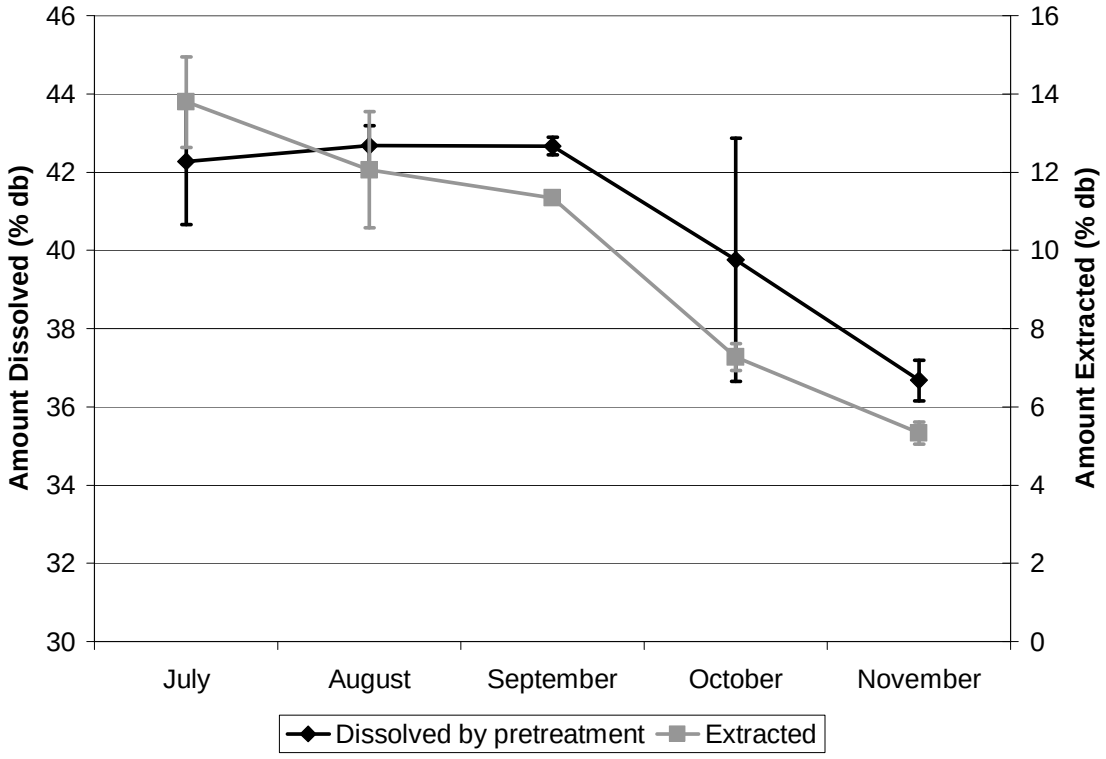

Figure 5.6 Amounts of switchgrass harvested during different months dissolved by hydrothermolysis pretreatment and removed by extraction.

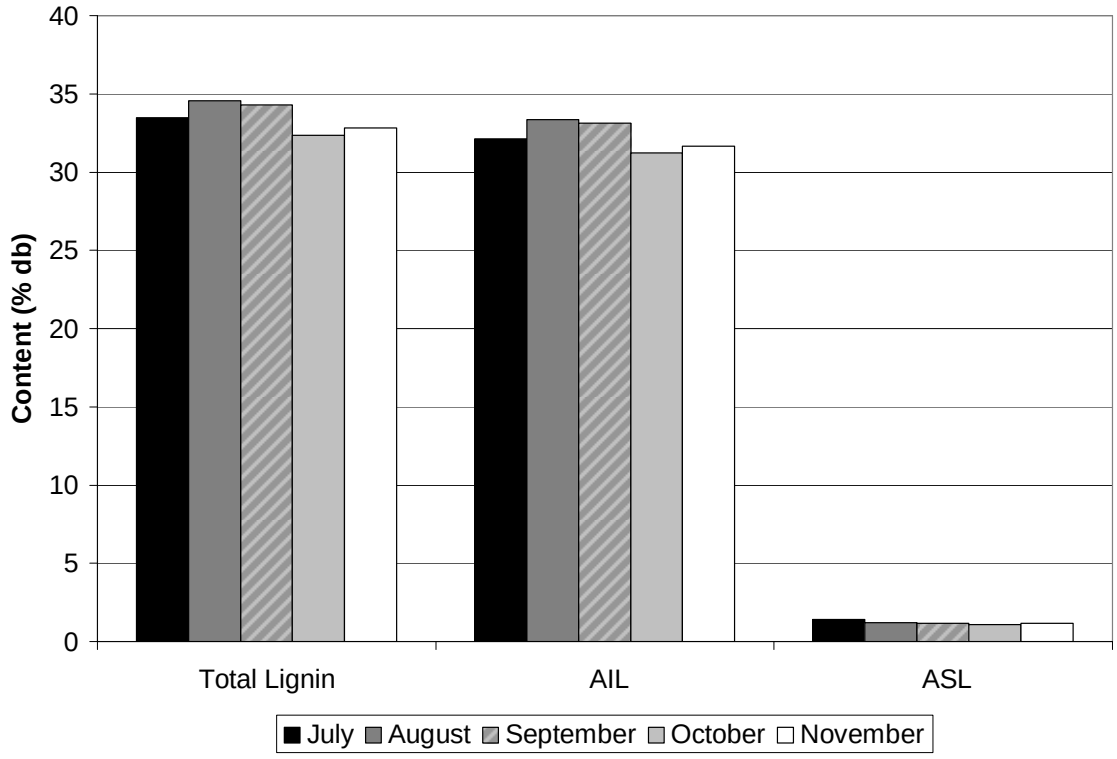

Figure 5.7 Lignin content of switchgrass harvested during different months after hydrothermolysis pretreatment. AIL is acid insoluble lignin and ASL is acid soluble lignin.

lowest lignin content after pretreatment at 32.3 and 32.8% (db) (Figure 5.7). Acid insoluble lignin content also increased in the solid fraction after pretreatment for all harvest dates, however, acid soluble lignin decreased for all harvest dates.

Glucan content increased in switchgrass solids after hydrothermolysis pretreatment. Figure 5.8 shows the structural carbohydrate content of the pretreated switchgrass. Glucan content ranged from 37.1 to 41.4% (db) before pretreatment (Figure 5.3) and 56.3 to 59.8% (db) after pretreatment (Figure 5.8). This increase is due to the preservation of glucan in the solid fraction and the removal of other components from switchgrass to the liquid fraction. Xylan content was greatly reduced in the solid fraction after pretreatment. Xylan ranged from 22.7 to 26.5% (db) before pretreatment (Figure 5.3) and 2.0 to 2.7% (db) after pretreatment (Figure 5.8). Nearly all galactan, arabinan, and mannan were removed from the solid fraction during pretreatment. For August harvested switchgrass, 0.6% (db) galactan and 0.4% (db) mannan were detected in the solid fraction after pretreatment (Figure 5.8). No galactan, arabinan, or mannan were detected in pretreated switchgrass solids for any other harvest date.

Figure 5.9 shows the preservation of structural carbohydrates in the solid fraction. For glucan, 87.0 to 92.6% (db) was preserved in the solids after pretreatment. A small amount of glucan was dissolved into the liquid fraction. Only 5.1 to 6.8% (db) xylan was preserved in the solid fraction after pretreatment. Most of the xylan was dissolved into the liquid fraction.

Figure 5.10 shows the concentrations of sugars in the liquid fraction after hydrothermolysis pretreatment of switchgrass and subsequent acid hydrolysis. The acid hydrolysis was performed to convert sugar polymers to monomers. The amount of glucose in the liquid fraction increased from 3.2 to 3.6 g/L from July to September harvests, and then declined to 2.0 g/L for the November harvest. Xylose increased from 11.0 to 14.7 g/L from July to

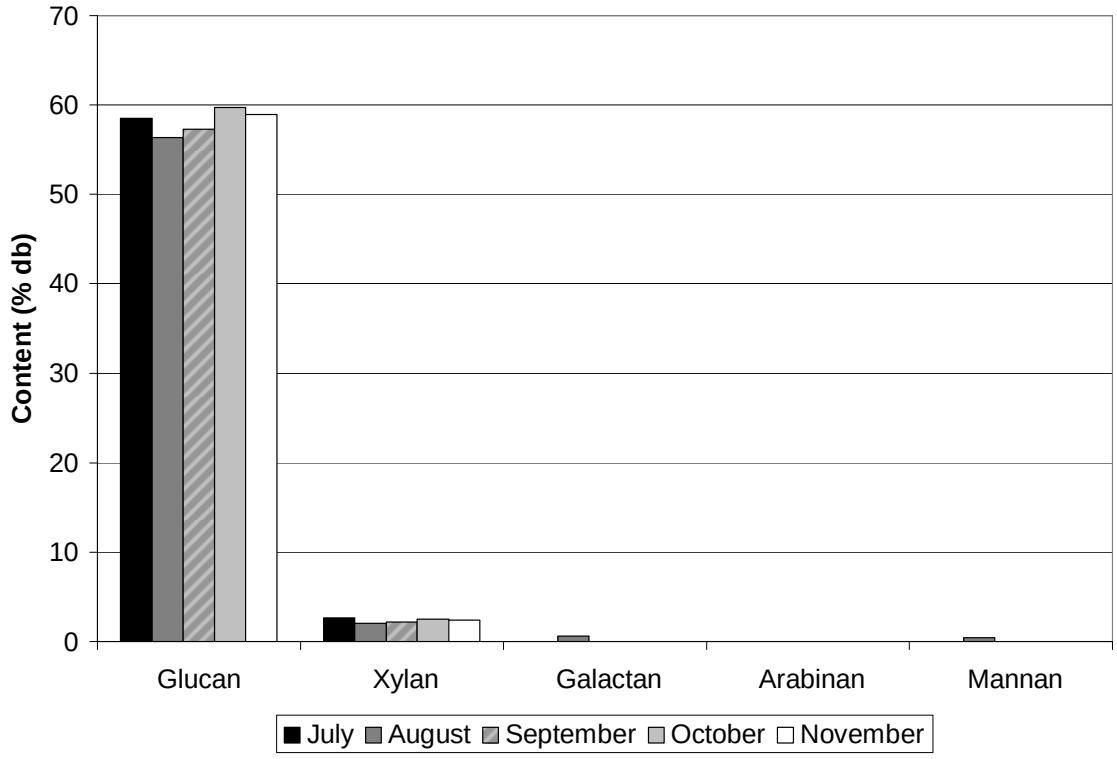

Figure 5.8 Structural carbohydrate content of switchgrass harvested during different months after hydrothermolysis pretreatment.

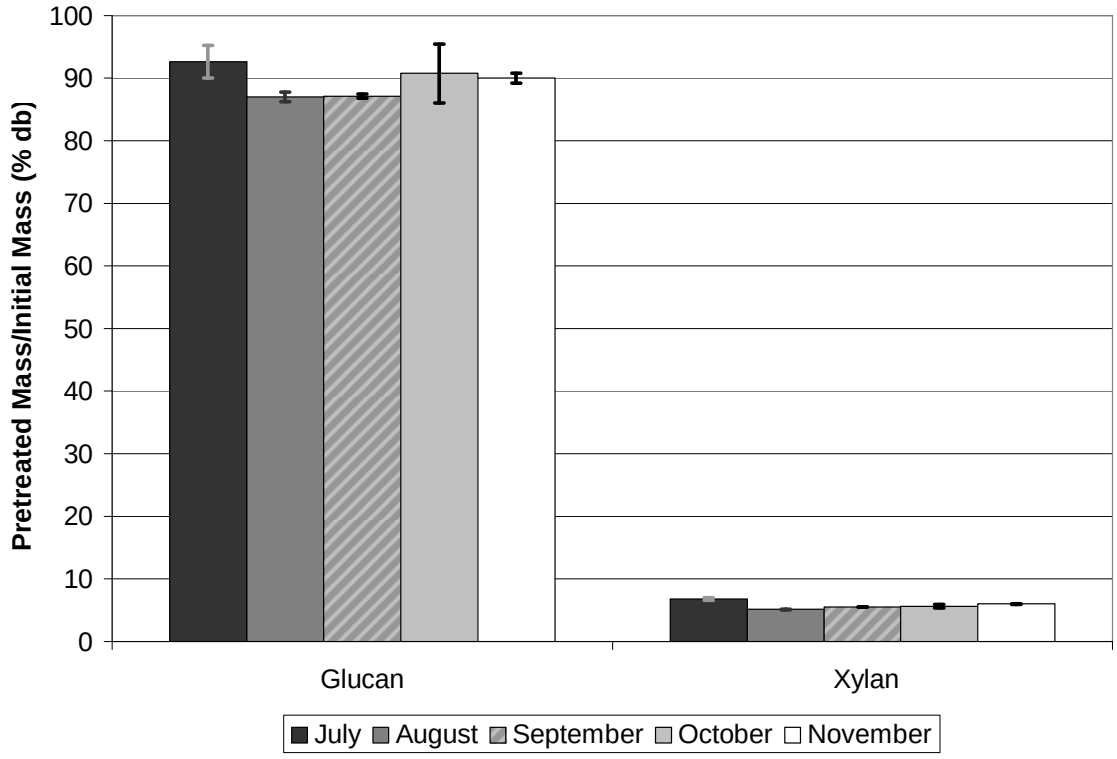

Figure 5.9 Preservation of structural carbohydrates in solid fraction of switchgrass harvested during different months after hydrothermolysis pretreatment.

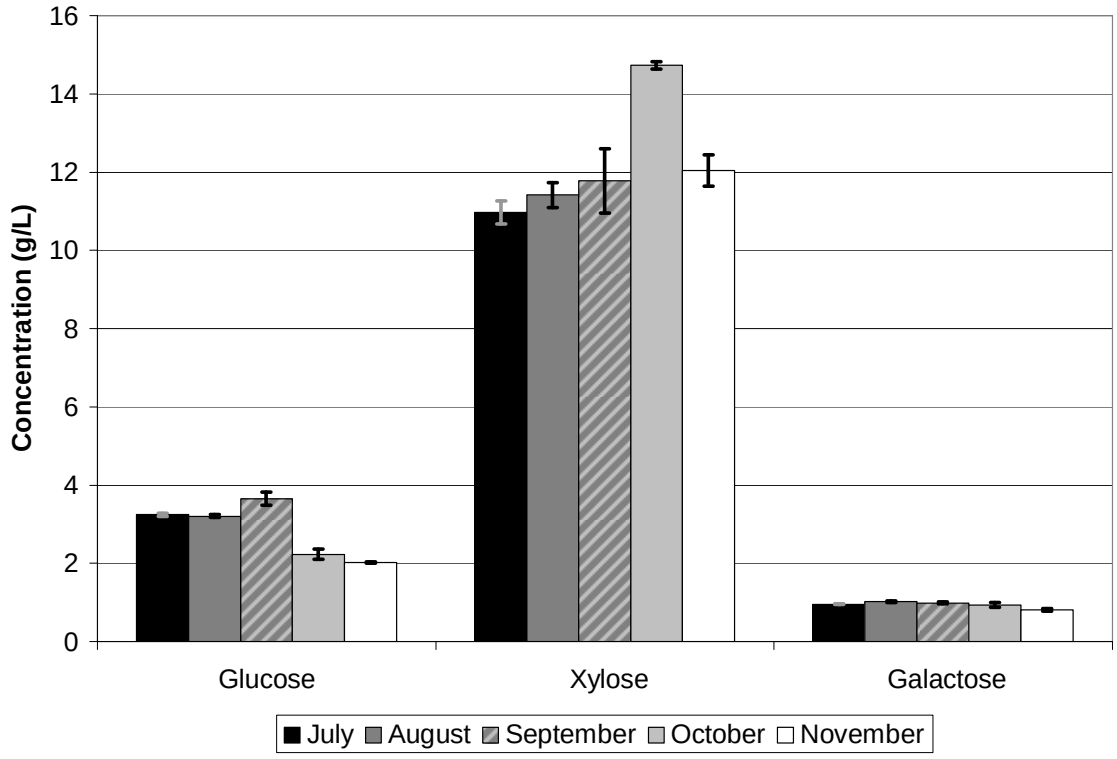

Figure 5.10 Concentration of sugars in liquid fraction after pretreatment of switchgrass harvested during different months.

October harvests, and then declined to 12.0 g/L for the November harvest. Galactose ranged from 0.8 to 1.0 g/L.

Figure 5.11 shows the percent of structural carbohydrates dissolved into the liquid fraction during hydrothermolysis pretreatment. The amount of glucan dissolved into the liquid fraction was between 4.4 and 8.7 % (db). The amount of xylan dissolved into the liquid fraction was higher, between 42.6 and 49.7% (db). The xylan content recovered in the liquid fraction was higher than other switchgrass pretreated under the same conditions. Suryawati et al. (2008) found 4.6% (db) of glucan and 28.0% (db) of xylan in the liquid fraction.

The liquid fraction after pretreatment by hydrothermolysis contained inhibitors. A small amount of glucose was degraded to hydroxymethylfurfural (HMF). Some of the xylose was degraded to furfural during hydrothermolysis. Figure 5.12 shows the inhibitor concentrations formed by pretreatment over the harvest period. Both HMF and furfural increased from July to August and then decreased through November. HMF ranged from 0.2 g/L in November to 0.7 g/L in August. Furfural ranged from 2.7 g/L in November to 3.5 g/L in August. Acetic acid was between 2.6 and 2.8 g/L for all harvest dates.

Suryawati et al. (2009) and Suryawati et al. (2008) found similar acetic acid concentrations at 3.4 and 3.7 g/L, respectively, similar HMF concentrations at 0.3 and 0.2 g/L, respectively, and lower furfural concentrations at 0.8 and 0.9 g/L, respectively, for the liquid fraction of switchgrass pretreated by hydrothermolysis at 200°C for 10 min. However, Yu et al. (2008) found a similar furfural concentration at 2.8 g/L and a similar HMF concentration at 0.4 g/L for the liquid fraction of rice straw harvested in November 2006 in Japan and pretreated by hydrothermolysis at 200°C for 10 min.

The furfural and HMF produced can be metabolized by *S. cerevisiae*, but will cause a lag phase in the fermentation (Palmqvist and Hahn-Hagerdal, 2000). The 2.8 g/L acetic acid

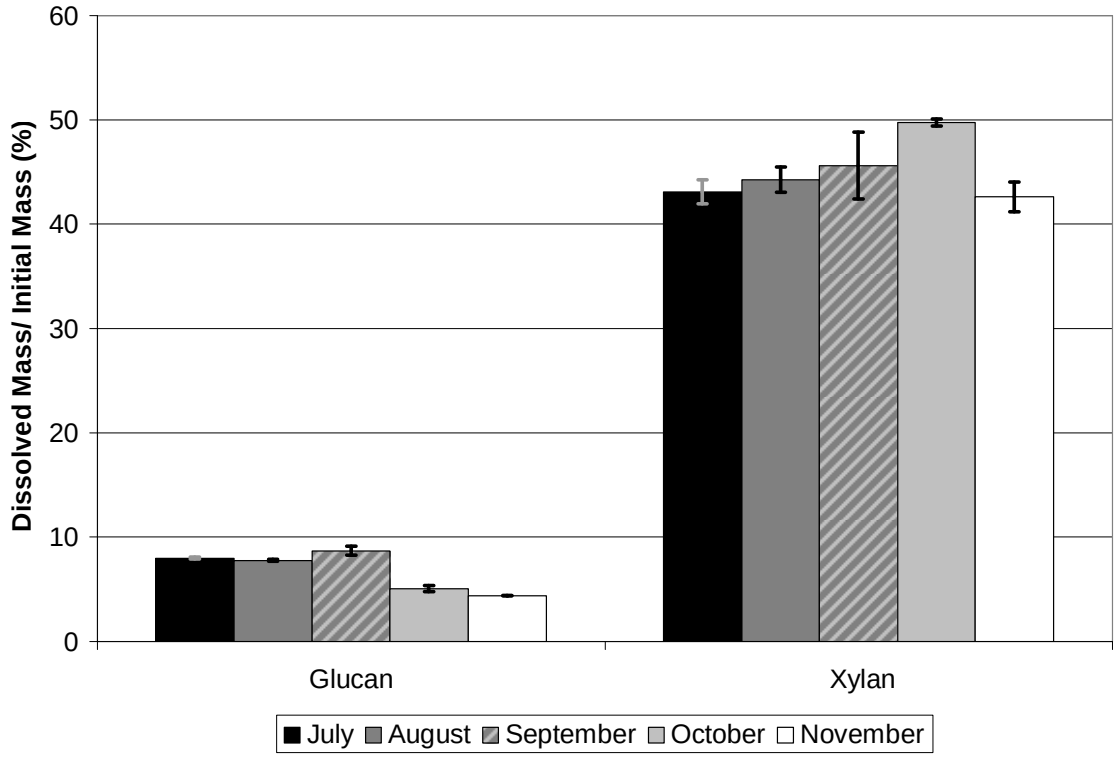

Figure 5.11 Structural carbohydrate content dissolved into liquid fraction by hydrothermolysis pretreatment of switchgrass harvested during different months.

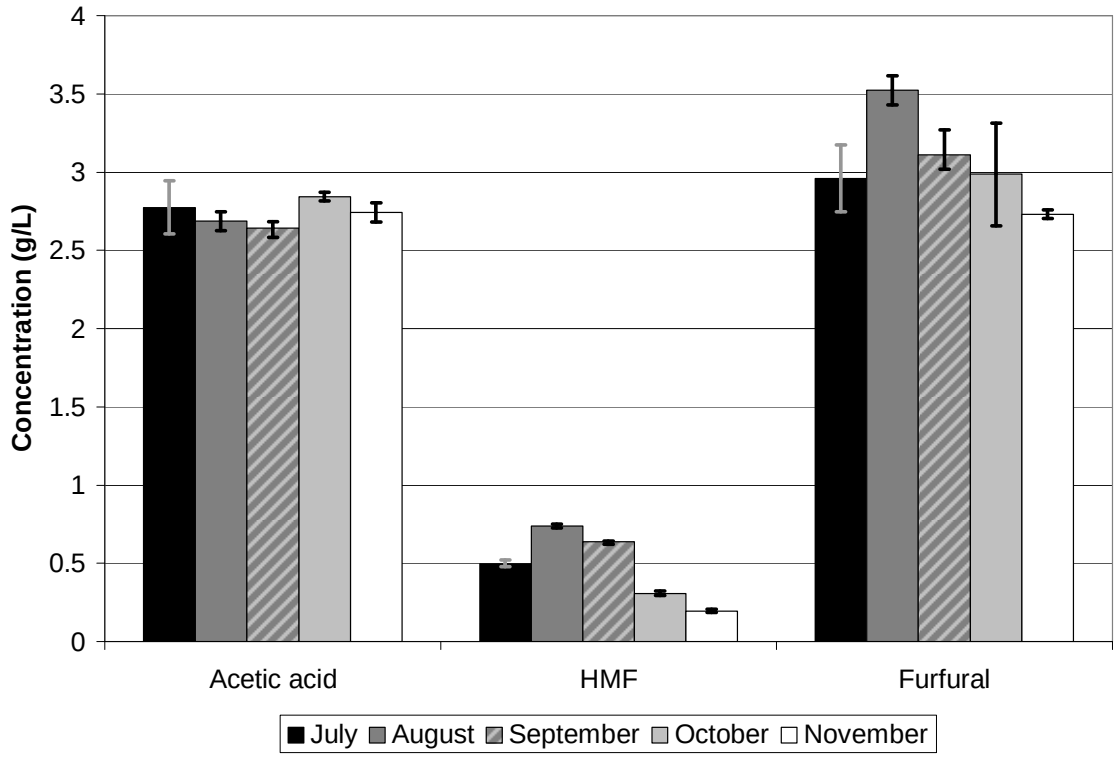

Figure 5.12 Inhibitors in liquid fraction after hydrothermolysis pretreatment of switchgrass harvested during different months.

produced in these pretreatments can be expected to cause less than 50% inhibition for ethanol production by *S. cerevisiae* at pH 5.5 (Olsson and Hahn-Hagerdal, 1996). However, fermentation at pH 5.5 of the liquid fraction after hydrothermolysis of November harvested switchgrass with glucose added to 20 g/L using *S. cerevisiae* D5A did not produce ethanol (data not shown).

5.2.2 Simultaneous Saccharification and Fermentation

Initial fermentation rates (0 to 24 h) slowed as switchgrass aged. Initial fermentation rates were calculated by dividing the 24 h ethanol concentration by 24 h. July harvested switchgrass had the highest initial fermentation rate of 0.470 g ethanol/L/h, with declining rates for switchgrass harvested from each month through November, when a rate of 0.370 g ethanol/L/h was observed. These rates appear to be related to the lignin content of switchgrass before pretreatment rather than the lignin content after pretreatment. Lignin increased in switchgrass throughout the harvest period, as shown in Figure 5.5. However, lignin content of switchgrass after pretreatment was lowest for October and November, shown in Figure 5.7. The disruption of lignin likely varied between harvest dates because the same pretreatment process was used on varying lignin contents. A harsher pretreatment could be used for increased lignin content, but increased degradation of structural carbohydrates may occur. Pretreatment conditions for ammonia fiber expansion and soaking in aqueous ammonia have been adjusted for harvest date and ecotype (Bals et al., 2010; Kim et al., 2011). Faster rates of fermentation will allow for smaller or fewer fermentation vessels to be used to produce the same amount of ethanol, thus reducing production cost.

Ethanol production appeared to have stopped by 144 h for July and August harvested switchgrass, but production was continuing slowly for September, October, and November harvested switchgrass until 168 h. This is likely due to a less disrupted lignin complex for the latter months of harvest, which slowed enzyme accessibility to structural carbohydrates. Ethanol

concentrations by harvest times from highest to lowest were August at 18.0 g/L, July at 17.8 g/L, October at 17.5 g/L, September at 16.5 g/L, and November at 16.4 g/L. Figure 5.13 shows the ethanol production for the SSF over time. Ethanol concentrations for July and August harvest dates were significantly higher than September and November harvest dates ($p<0.05$). October ethanol concentration was not significantly different from any other harvest date ($p>0.05$). These ethanol concentrations were higher than those measured after 96 h for SSF using *S. cerevisiae* YR400 at 10% solids loading of Kanlow N1 switchgrass harvested at anthesis and post frost maturity stages, 13.8 and 13.2 g/L respectively, which correspond to August and November harvests for this study (Dien et al., 2013).

Ethanol yields were low after 168 h, between 61.4 and 70.1% of maximum theoretical yields based on glucan in pretreated solids. This is likely due to a low enzyme loading of 9 FPU/g glucan. Figure 5.14 shows the percent theoretical yield throughout the fermentation. SSF of August harvested switchgrass produced the highest percentage of maximum theoretical yield. Pessani et al. (2011) found the optimum enzyme loading to be 58 FPU Accellerase 1500/g glucan for SSF at 45°C with *K. marxianus* IMB3 of post-frost November harvested Kanlow switchgrass from the same stand as this study after pretreatment by hydrothermolysis at 200°C for 10 min.

An SSF experiment using a greater enzyme loading of 14 FPU/g glucan was done in duplicate with pretreated switchgrass from July, September, and November and produced 91.7, 87.8, and 85.9% of theoretical yield of the pretreated solids, respectively. Fibrilase was used for the enzyme instead of Accellerase 1500. This experiment shows results with a good theoretical yield and supports data from the SSF using Accellerase 1500 enzyme at a lower loading. The initial fermentation rate in this experiment was also highest for July harvested switchgrass, as shown in Figure 5.15. Ethanol concentrations of 24.4 g/L from July, 23.5 g/L from November, and 22.3 g/L from September harvested switchgrass were not significantly different ($p>0.05$), however, statistical significance between data requires more separation for duplicate analysis than

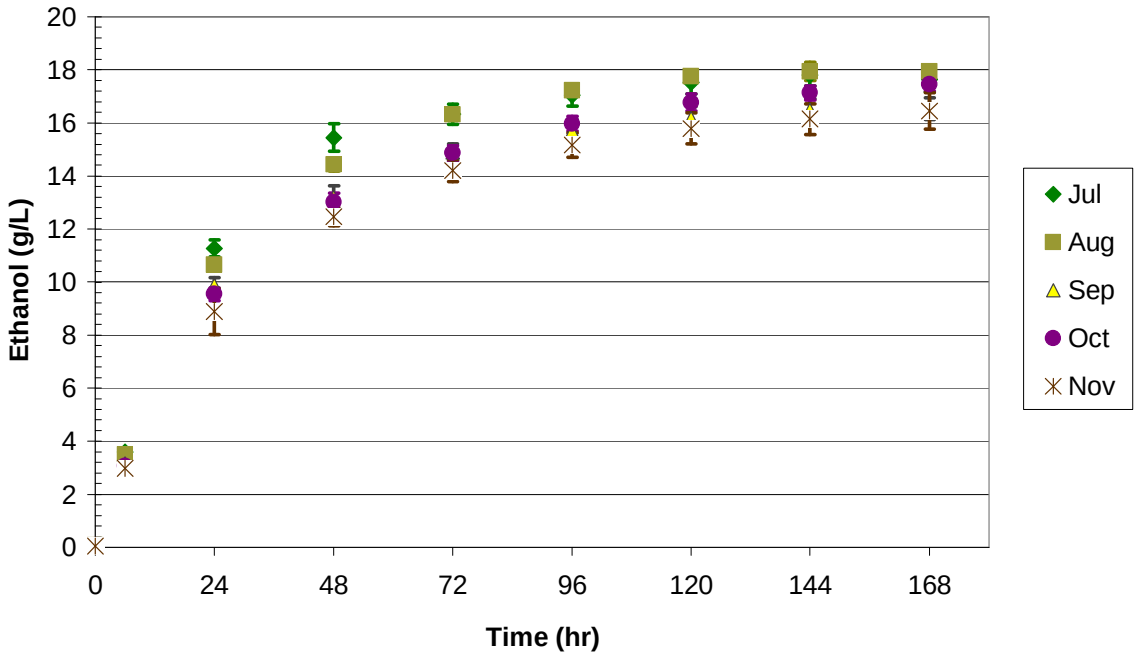

Figure 5.13 Ethanol production of SSF using Accellerase enzyme at 9 FPU/g glucan.

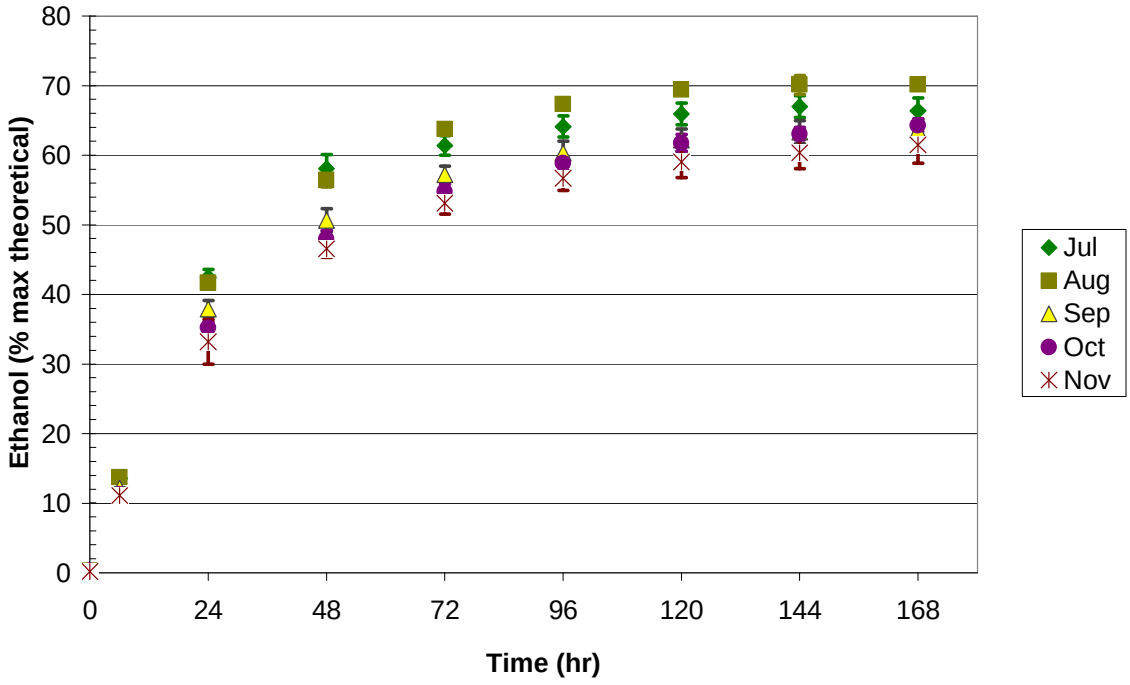

Figure 5.14 Theoretical ethanol yield of SSF using Accellerase enzyme at 9 FPU/g glucan.

Theoretical yield was calculated from glucan content of pretreated switchgrass in each SSF.

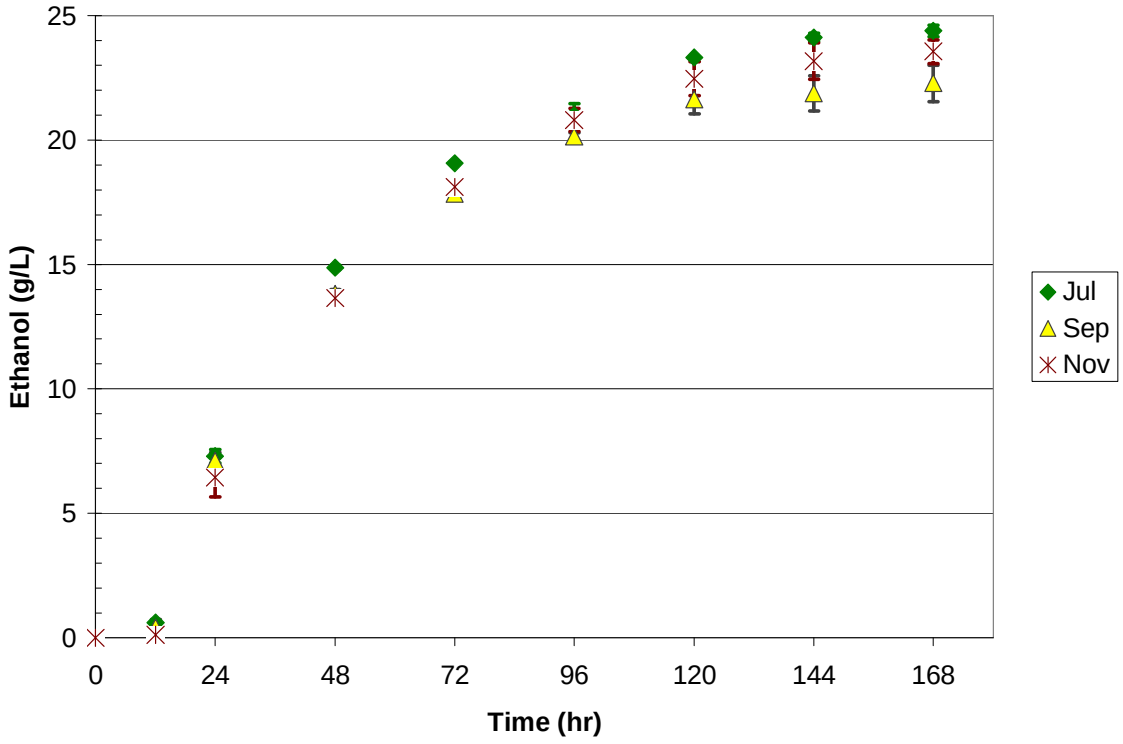

Figure 5.15 Ethanol production of SSF using Fibrilase enzyme at 14 FPU/g glucan.

triplicate analysis. Pessani et al. (2011) observed nearly the same percent theoretical ethanol yield, about 87%, as this experiment for an SSF using 58 FPU Accellerase 1500/g glucan and *S. cerevisiae* D_5A at 37°C.

There was a lag time for ethanol production during the SSF using Fibrilase at a higher enzyme loading. Ethanol production was 3.0 g/L after 6 h for the SSF of all harvest dates, where less than 0.6 g/L ethanol was produced after 12 h for the SSF with higher enzyme loading. It should be noted that the SSF with higher enzyme loading was sampled at 0, 12, and 24 h, whereas the SSF of all harvest dates with a lower enzyme loading was sampled at 0, 6, and 24 h. The glucose concentration peaked higher than 2.5 g/L at 12 h for the SSF with higher enzyme loading during the lag time, while the glucose peak was less than 1.0 g/L at 6 h for the SSF of switchgrass from all harvest dates. This indicates that metabolic growth stage of the yeast caused the lag time for ethanol production during the SSF with higher enzyme loading. Yeast was likely not in the exponential phase when it was used for inoculation.

Glucose concentrations were less than 1 g/L throughout SSF with the lower enzyme loading, indicating continuous conversion of glucose to ethanol. Figure 5.16 shows the glucose concentration during SSF over time. Acetic acid production was similar for the first 72 h of SSF, but concentrations diverged by 168 h. Acetic acid production ranged from 0.9 to 1.4 g/L, with declining concentrations for later harvest dates. Figure 5.17 shows acetic acid production for the SSF. Xylitol production ranged from 1.25 to 1.39 g/L. Succinic acid production ranged from 1.64 to 1.75 g/L. Glycerol production ranged from 0.59 to 0.65 g/L.

Ethanol yield in terms of liters of ethanol per metric ton of untreated, dry switchgrass provides useful data to determine the best harvest date. An October harvest date yielded the highest among harvest dates with 167 L ethanol/Mg switchgrass. July, August, and November harvest dates provided slightly lower yields that were within 4 L ethanol/Mg switchgrass of the

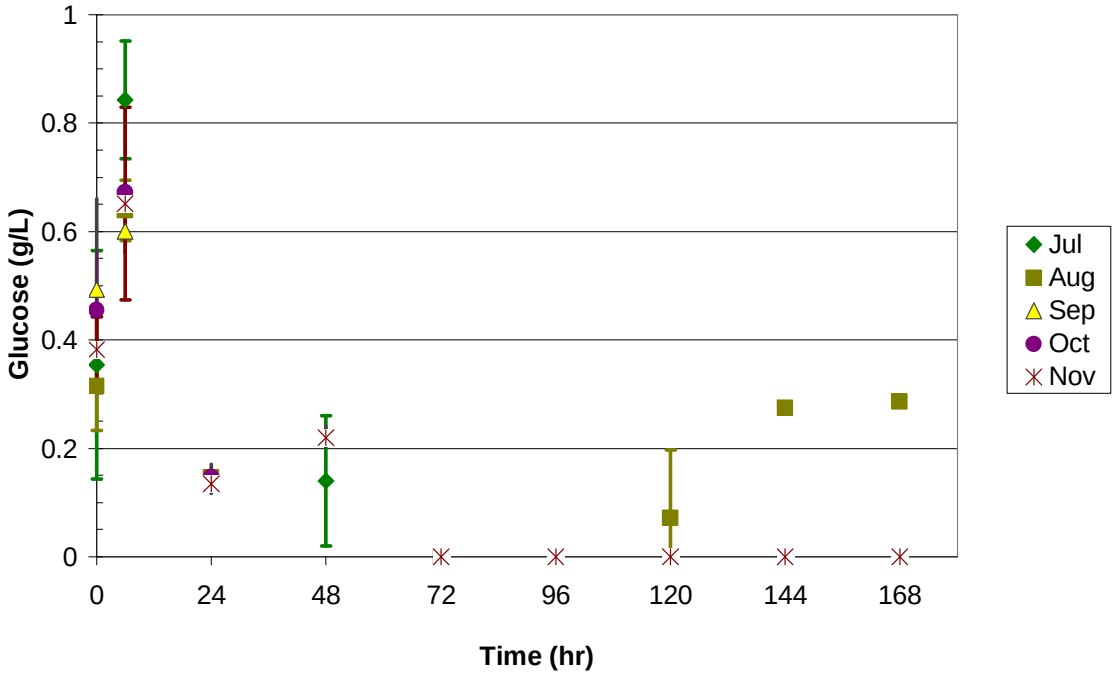

Figure 5.16 Glucose concentration of SSF using Accellerase enzyme at 9 FPU/g glucan.

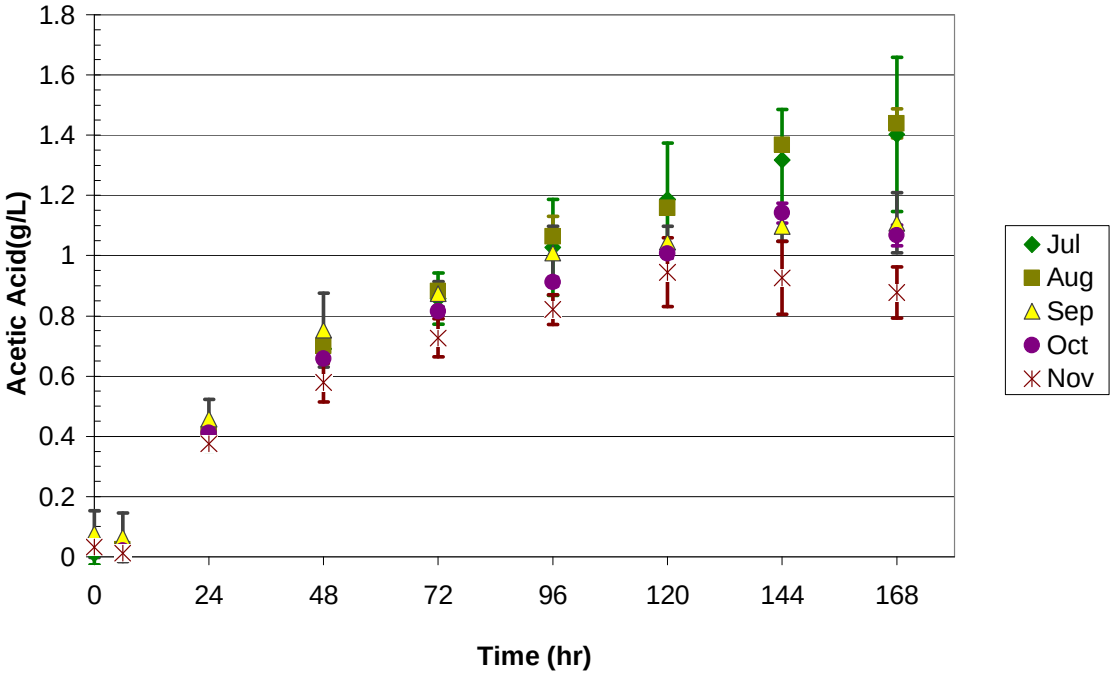

Figure 5.17 Acetic acid production of SSF using Accellerase enzyme at 9 FPU/g glucan.

August harvest date. The September harvest date had a considerably lower yield at 150 L ethanol/Mg switchgrass. July, August, October, and November harvest dates yielded significantly more L ethanol/Mg switchgrass than the September harvest date ($p<0.05$). Table 5.1.A illustrates how yields were similar over the harvest period, except for September, and how those yields correspond to both the theoretical maximum yield and the percent of theoretical maximum yield obtained based on glucan content of untreated switchgrass. Aside from September, these yields were close considering how the earlier harvest dates generally produced higher ethanol concentrations. It appears July, August, October, and November harvest dates had such similar ethanol yields per metric ton of untreated switchgrass due to two factors. First, the amount of switchgrass dissolved into the liquid fraction during hydrothermolysis varied, as shown in Figure 5.6. Less switchgrass was dissolved during hydrothermolysis for October and November harvests, leaving more switchgrass solids for fermentation. Further, the amount of glucan dissolved into the liquid fraction was less for October and November harvests (Figure 5.11). Second, lignin contents of switchgrass were lower for July and August harvests, (Figure 5.5), which likely increased ethanol yields for the early months. The September harvest benefited from neither the effect of lower dissolved solids during pretreatment nor the effect of lower lignin content, which accounts for the decreased yield of ethanol per ton of untreated switchgrass.

Ethanol yield based upon the SSF using Fibrilase at a higher enzyme loading for July, September, and November harvested switchgrass indicates larger differences between harvest dates. Table 5.1.B shows these results. For this experiment, a November harvest yielded 15 L ethanol/Mg switchgrass more than a July harvest. The September harvest yielded the lowest as it did in the experiment of all five harvest dates, 11 L ethanol/Mg switchgrass lower than the July harvest. The November harvest yielded significantly more ethanol than the September harvest ($p<0.05$). The July harvest was not significantly different from either the September or November harvests ($p>0.05$). The larger difference in ethanol yield observed in this experiment

Table 5.1 Comparison of ethanol yield between harvest dates based on SSF with (A) Accellerase 1500 and lower enzyme loading and (B) Fibrilase and higher enzyme loading.

Ethanol yields with different letters are significantly different ($p<0.05$). Means were separated by Tukey's test. Theoretical ethanol yield is based on glucan content in untreated switchgrass.

A

Harvest Date	Ethanol yield (L/Mg)	Theoretical ethanol yield (L/Mg)	% Theoretical Yield
July	163 a	262	62.0
August	164 a	267	61.3
September	150 b	271	55.4
October	167 a	286	58.3
November	165 a	298	55.3

B

Harvest Date	Ethanol yield (L/Mg)	Theoretical ethanol yield (L/Mg)	% Theoretical Yield
July	223 a, b	262	85.1
September	212 b	271	78.1
November	238 a	298	79.9

between July and November harvests than in the experiment with all five harvest dates may be due to several reasons. First, variation within ground switchgrass samples may account for the difference, as the two SSF experiments used different batches of pretreated switchgrass. The glucan content for pretreated November switchgrass was 1.4% (db) higher for the SSF with increased enzyme loading than the SSF with all fives harvest dates. The glucan content for the pretreated July switchgrass was the same for both SSF experiments. The glucan content for the pretreated September switchgrass was 1.4% (db) lower for the SSF with increased enzyme loading than the SSF with all five harvest dates. Second, the increased enzyme loading may have released more glucose during SSF from the November switchgrass relative to the July switchgrass than was released with the lower enzyme loading. Third, the Fibrilase enzyme used in the SSF of increased enzyme loading may have functioned better on the November switchgrass than the Accellerase 1500 enzyme used in the SSF of all harvest dates due to differences between enzyme cocktails, such as varying amounts of endoglucanases, exoglucanases, and β-glucanases. SSF with Fibrilase at 14 FPU/g glucan from this study produced nearly the same percent of theoretical ethanol yield as SSF with Accellerase 1500 at 58 FPU/g glucan using the same switchgrass and pretreatment method (Pessani et al., 2011). Dien et al. (2013) found ethanol yields via SSF of 193 and 184 L/Mg Kanlow N1 switchgrass harvested at anthesis and post frost, respectively, at Mead, Nebraska. These maturity stages correspond to August and November harvest dates for this study. The decrease in ethanol yield from switchgrass observed by Dien et al. (2013) at later maturity was not observed in this study for either SSF experiment (Table 5.1). The SSF with Fibrilase had higher ethanol yields (Table 5.1.B) than those obtained using a glucose and xylose fermenting strain of *S. cerevisiae* (Dien et al., 2013).

The SSF experiment with Accellerase 1500 found a nearly constant ethanol yield per ton of untreated switchgrass for July, August, October, and November (Table 5.1A), which would allow for a wide harvest window from the standpoint of ethanol production. The other SSF

experiment with Fibrilase found a larger increase in ethanol yield by delaying harvest until late in the fall (Table 5.1B). More weight can be given to the SSF with Accellerase 1500 because the experiment was performed in triplicate, while the SSF with Fibrilase was performed in duplicate. This data needs to be combined with multiple factors to determine the best harvest date. Two of the more important factors are the yield in terms of tons of switchgrass per hectare and the harvest date effect on stand persistence. A 10% decrease in the mass of switchgrass harvested over the harvest period would more than offset the higher ethanol yields of October and November from the two SSF experiments, and allow more ethanol to be produced from a July harvest. The percent difference from the lowest ethanol yield in Tables 5.2.A and 5.2.B shows how much higher the yield was for each month than the lowest yield obtained from September harvested switchgrass. The percent difference in ethanol yields between harvest dates reveals how much the mass yields of switchgrass need to differ in order to offset the ethanol yield to produce the same amount of ethanol per area of land. For example, in Table 5.2.A, a July harvest yielded 8.3% more volume of ethanol per mass of switchgrass than a September harvest, so the September dry mass yield would need to be 8.3% higher than the July yield to obtain the same volume of ethanol.

An estimate of revenue was calculated (Table 5.2) based on the ethanol yields obtained from these experiments for 350,000 tons (318,000 Mg) of switchgrass, the annual estimated mass of switchgrass needed for a 25,000,000 gal/yr (95,000,000 L/yr) ethanol plant (http://www.abengoabioenergy.com/web/en/2g_hugoton_project/, accessed 4-23-14). A price of $2.00/gal ethanol ($0.53/L ethanol) was used for the calculation; the price was estimated as a future baseline price using a 10-year chart for ethanol on the Chicago Board of Trade (http://www.nasdaq.com/markets/ethanol.aspx?timeframe=10y, accessed 4-23-14). This calculation does not take into consideration cost factors such as transportation cost, which is likely higher for earlier harvests due to higher extractives content.

Table 5.2 Comparison of revenue between harvest dates based on SSF with (A) Accellerase 1500 and lower enzyme loading and (B) Fibrilase and higher enzyme loading.

A

Harvest Date	% difference from lowest ethanol yield	difference in production of L ethanol/318,000 Mg switchgrass	revenue difference at $0.53/L ethanol
July	8.3	3,977,000	$2,102,000
August	8.9	4,276,000	$2,259,000
September	0.0	0	$0
October	10.9	5,212,000	$2,754,000
November	9.8	4,678,000	$2,472,000

B

Harvest Date	% difference from lowest ethanol yield	difference in production of L ethanol/318,000 Mg switchgrass	revenue difference at $0.53/L ethanol
July	5.3	3,561,000	$1,882,000
September	0.0	0	$0
November	12.4	8,342,000	$4,408,000

CHAPTER VI

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

Much of the increase in structural carbohydrate content over the harvest period was due to a decrease in extractives content, rather than addition of new structural carbohydrates. Increasing lignin content through the harvest period had a negative effect on fermentation rates and yields. The lignin content after pretreatment did not appear to correlate to fermentation rates and yields as did the lignin content of untreated switchgrass. The decreased amount of switchgrass dissolved during hydrothermolysis at the end of the harvest period had a positive effect on ethanol yields. Ethanol yield in terms of liters per ton of switchgrass for July, August, October, and November harvest dates were not significantly different; a significantly lower yield was obtained for the September harvest date.

6.2 Recommended Future Work

Repetition of this study for different years, switchgrass varieties, and locations is recommended. The data obtained from such research will aid to create a harvest guide across the United States for switchgrass to be used in ethanol production via fermentation. Further, collecting switchgrass yield data in terms of mass per unit of land area for each repetition of this study is recommended. Obtaining yield data will allow for a better optimization of the harvest

period for producing ethanol from switchgrass.

Optimization of the pretreatments, such as hydrothermolysis, at different harvest dates should improve ethanol yields. Later harvest dates from this study had lower inhibitor production during hydrothermolysis and lower % theoretical ethanol yields during SSF than earlier harvest dates. It may be possible to achieve higher % theoretical ethanol yields during SSF for later harvest dates. Slight adjustments to the severity factor, R_0, by varying temperature by a few degrees and time by a few minutes can provide this optimization. The results of such a study may help to better optimize a harvest period for switchgrass.

REFERENCES

Adler, P. R., M. A. Sanderson, A. A. Boateng, P. I. Weimer, and H.-J. G. Jung. 2006. Biomass yield and biofuel quality of switchgrass harvested in fall or spring. *Agronomy Journal* 98:1518-1525.

Adney, B., and J. Baker. 1996. Measurement of Cellulase Activities. National Renewable Energy Laboratory.

Alvira, P., E. Tomas-Pejo, M. Ballesteros, and M. J. Negro. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology* 101:4851-4861.

Bals, B., C. Rogers, M. Jin, V. Balan, and B. Dale. 2010. Evaluation of ammonia fibre expansion (AFEX) pretreatment for enzymatic hydrolysis of switchgrass harvested in different seasons and locations. *Biotechnology for Biofuels* 3(1):11.

Berg, C. C. 1971. Forage Yield of Switchgrass (Panicum virgatum) in Pennsylvania. *Agronomy Journal* 63(September-October):785-786.

Casler, M. D., and A. R. Boe. 2003. Cultivar X environment interactions in switchgrass. *Crop Science* 43(6):2226-2233.

Delgenes, J. P., R. Moletta, and J. M. Navarro. 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae. *Enzyme and Microbial Technology* 19:220-225.

Dien, B. S., H.-J. G. Jung, K. P. Vogel, M. D. Casler, J. F. S. Lamb, L. Iten, R. B. Mitchell, and G. Sarath. 2006. Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass. *Biomass and Bioenergy* 30:880-891.

Dien, B. S., P. J. O'Bryan, R. E. Hector, L. B. Iten, R. B. Mitchell, N. Qureshi, G. Sarath, K. P. Vogel, and M. A. Cotta. 2013. Conversion of switchgrass to ethanol using dilute ammonium hydroxide pretreatment: influence of ecotype and harvest maturity. *Environmental Technology* 34(13-14):1837-1848.

Dowe, N., and J. McMillan. 2001. SSF Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation. National Renewable Energy Laboratory.

Ehrman, T. 1994. Standard Test Method for Moisture, Total Solids, and Total Dissolved Solids in Biomass Slurry and Liquid Process Samples. National Renewable Energy Laboratory.

Faga, B. A., M. R. Wilkins, and I. M. Banat. 2010. Ethanol production through simultaneous saccharification and fermentation of switchgrass using Saccharomyces cerevisiae D5A and thermotolerant Kluyveromyces marxianus IMB strains. *Bioresource Technology* 101:2273-2279.

Goering, H. K., and P. J. Van Soest. 1970. Forage Fiber Analysis. Agricultural Research Service, U.S. Department of Agriculture.

Kim, Y., N. S. Mosier, M. R. Ladisch, V. R. Pallapolu, Y. Y. Lee, R. Garlock, V. Balan, B. E. Dale, B. S. Donohoe, T. B. Vinzant, R. T. Elander, M. Falls, R. Sierra, M. T. Holtzapple, J. Shi, M. A. Ebrik, T. Redmond, B. Yang, C. E. Wyman, and R. E. Warner. 2011. Comparative study on enzymatic digestibility of switchgrass varieties and harvests processed by leading pretreatment technologies. *Bioresource Technology* 102:11089-11096.

Lemus, R., E. C. Brummer, K. J. Moore, N. E. Molstad, C. L. Burras, and M. F. Barker. 2002. Biomass yield and quality of 20 switchgrass populations in southern Iowa, USA. *Biomass and Bioenergy* 23:433-442.

Lewandowski, I., and A. Kicherer. 1997. Combustion quality of biomass: practical relevance and experiments to modify the biomass quality of Miscanthus x giganteus. *European Journal of Agronomy* 6:163-177.

Lockert, R. A. 1974. Influence of growth stage, stubble height, and cutting frequency on the carbohydrate reserves and yield of switchgrass. In *Thesis (M.S.)--South Dakota State University*.

Makaju, S. O., Y. Q. Wu, H. Zhang, V. G. Kakani, C. M. Taliaferro, and M. P. Anderson. 2013. Switchgrass winter yield, year-round elemental concentrations, and associated soil nutrients in a zero input environment. *Agronomy Journal* 105(2):463-470.

McLaughlin, S. B., and L. A. Kszos. 2005. Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. *Biomass and Bioenergy* 28:515-535.

McLaughlin, S. B., and M. E. Walsh. 1998. Evaluating environmental consequences of producing herbaceous crops for bioenergy. *Biomass and Bioenergy* 14(4):317-324.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple, and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96:673-686.

Olsson, L., and B. Hahn-Hagerdal. 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme and Microbial Technology* 18(312-331).

Overend, R. P., and E. Chornet. 1987. Fractionation of lignocellulosics by steam-aqueous pretreatments. *Philosophical Transactions of the Royal Society of London. Series A, Mathematical and Physical Sciences* 321(1561):523-536.

Palmqvist, E., and B. Hahn-Hagerdal. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource Technology* 74:25-33.

Pessani, N. K., H. K. Atiyeh, M. R. Wilkins, D. D. Bellmer, and I. M. Banat. 2011. Simultaneous saccharification and fermentation of Kanlow switchgrass by thermotolerant *Kluyveromyces marxianus* IMB3: The effect of enzyme loading, temperature and higher solid loadings. *Bioresource Technology* 102:10618-10624.

Porter, P. A. 1985. The effect of nitrogen, mefluidide, and maturity on switchgrass (Panicum virgatum L.) forage quality and yield. In *Thesis (M.S.)--University of Wisconsin--Madison*.

Ravindranath, S. V., N. Uppugundla, J. O. Lay, E. C. Clausen, M. Wilkins, R. G. Ingraham, C. West, Y. Wu, and D. J. Carrier. 2009. Policosanol, α-Tocopherol, and Moisture Content as a Function of Timing of Harvest of Switchgrass (Panicum virgatum L.). *Journal of Agricultural and Food Chemistry* 57(9):3500-3505.

Ruiz, R., C. Scarlata, J. Sluiter, and D. Templeton. 2007. Determination of Extractives in Biomass. National Renewable Energy Laboratory.

Sanderson, M. A., R. P. Egg, and A. E. Wiselogel. 1997. Biomass losses during harvest and storage of switchgrass. *Biomass and Bioenergy* 12(2):107-114.

Sanderson, M. A., J. C. Read, and R. L. Reed. 1999. Harvest management of switchgrass for biomass feedstock and forage production. *Agronomy Journal* 91(1):5-10.

Sanderson, M. A., R. L. Reed, S. B. McLaughlin, S. D. Wullschleger, B. V. Conger, D. J. Parrish, D. D. Wolf, C. Taliaferro, A. A. Hopkins, W. R. Ocumpaugh, M. A. Hussey, J. C. Read, and C. R. Tischler. 1996. Switchgrass as a sustainable bioenergy crop. *Bioresource Technology* 56:83-93.

Sladden, S. E., D. I. Bransby, and G. E. Aiken. 1991. Biomass yield, composition and production costs for eight switchgrass varieties in Alabama. *Biomass and Bioenergy* 1(2):119-122.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, and D. Templeton. 2004. Determination of Structural Carbohydrates and Lignin in Biomass. National Renewable Energy Laboratory.

Sluiter, A., R. Ruiz, C. Scarlata, J. Sluiter, and D. Templeton. 2007. Determination of Extractives in Biomass. National Renewable Energy Laboratory.

Suryawati, L., M. R. Wilkins, D. D. Bellmer, R. L. Huhnke, N. O. Maness, and I. M. Banat. 2008. Simultaneous saccharification and fermentation of Kanlow switchgrass pretreated by hydrothermolysis using Kluyveromyces marxianus IMB4. *Biotechnology and Bioengineering* 101(5):894-902.

Suryawati, L., M. R. Wilkins, D. D. Bellmer, R. L. Huhnke, N. O. Maness, and I. M. Banat. 2009. Effect of hydrothermolysis process conditions on pretreated switchgrass composition and ethanol yield by SSF with Kluyveromyces marxianus IMB4. 540-545.

Teugjas, H., and P. Valjamae. 2013. Product inhibition of cellulases studied with 14C-labeled cellulose substrates. *Biotechnology for Biofuels* 6(104):1-14.

Thammasouk, K., D. Tandjo, and M. H. Penner. 1997. Influence of extractives on the analysis of herbaceous biomass. *Journal of Agricultural and Food Chemistry* 45(2):437-443.

USDA. 2010. USDA Biofuels Strategic Production Report. USDA.

Wiselogel, A. E., F. A. Agblevor, D. K. Johnson, S. Deutch, J. A. Fennell, and M. A. Sanderson. 1996. Compositional changes during storage of large round switchgrass bales. *Bioresource Technology* 56:103-109.

Wolfrum, E. J., A. J. Lorenz, and N. deLeon. 2009. Correlating detergent fiber analysis and dietary fiber analysis data for corn stover collected by NIRS. *Cellulose* 16(4):577-585.

Yu, G., S. Yano, H. Inoue, S. Inoue, T. Endo, and S. Sawayama. 2008. Pretreatment of Rice Straw by a Hot-Compressed Water Process for Enzymatic Hydrolysis. *Applied Biochemistry and Biotechnology*.

Zhao, J., Z. Wang, M. Wang, Q. He, and H. Zhang. 2008. The inhibition of *Saccharomyces cerevisiae* cells by acetic acid quantified by electrochemistry and fluorescence. *Bioelectrochemistry* 72:117-121.

APPPENDICES

Table A.1 Monthly average of the mean daily temperature at Stillwater, OK, from 2008 compared with 30-yr average (1971-2000).

——— air temperature, ℃ ———

Month	2008	30-yr mean
January	3.1	1.4
February	3.7	4.4
March	10.2	9.6
April	14.3	14.9
May	20.6	20.1
June	25.5	25
July	27.9	27.9
August	26.3	27.4
September	21.1	22.7
October	15.2	16.3
November	9.3	9.3
December	3.1	3.6

Source: www.mesonet.org/index.php/weather/station_monthly_ summaries and http://ggweather.com/normals/OK71.htm.

Adapted from Makaju et al. (2013).

Table A.2 Monthly total precipitation at Stillwater, OK, from 2008 compared with 30-yr average (1971-2000).

	——— precipitation, cm ———	
Month	2008	30-yr mean
January	1.4	3.3
February	6.6	4.1
March	10.5	8.2
April	14.6	8.8
May	16.2	13.7
June	12.5	11
July	12.7	6.8
August	3.4	7.7
September	4.2	10.5
October	5.3	8.2
November	6.5	6.5
December	2.3	4.4

Source: www.mesonet.org/index.php/weather/monthly_rainfall_table /stil and http://ggweather.com/normals/OK71.htm.

Adapted from Makaju et al. (2013).

Table A.3 Monthly total solar radiation at Stillwater, OK, from 2008.

solar radiation, MJ m–2

Month	2008
January	9.58
February	11.38
March	15.69
April	19.96
May	23.14
June	23.19
July	23.67
August	18.73
September	16.86
October	14.16
November	11.58
December	8.21

Source: www.mesonet.org/index.php/weather/station_ monthly_summaries.

Adapted from Makaju et al. (2013).

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