# DEVELOPMENT OF OXIDATIVE LIME PRETREATMENT AND SHOCK TREATMENT TO PRODUCE HIGHLY DIGESTIBLE LIGNOCELLULOSE FOR BIOFUEL AND RUMINANT FEED APPLICATIONS

A Dissertation

by

MATTHEW DAVID FALLS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2011

Major Subject: Chemical Engineering

Development of Oxidative Lime Pretreatment and Shock Treatment to Produce Highly Digestible Lignocellulose for Biofuel and Ruminant Feed Applications

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# ABSTRACT

Development of Oxidative Lime Pretreatment and Shock Treatment to Produce Highly Digestible Lignocellulose for Biofuel and Ruminant Feed Applications. (August 2011)

Matthew David Falls, B.A., Texas A&M University

Chair of Advisory Committee: Dr. Mark T. Holtzapple

At present, the United States generates biofuels (ethanol) from corn grain. Unfortunately, low crop yields and limited growth regions result in limited availability. Furthermore, the use of staple food crops for ethanol production has generated a highly controversial food vs. fuel debate. Because of its high abundance and relatively low cost, lignocellulosic biomass is a promising alternative feedstock for biofuel production; however, structural features of lignocellulose limit accessibility of enzymes or microorganisms. These structural barriers include high lignin content, acetyl groups on hemicellulose, high cellulose crystallinity, cellulose degree of polymerization, and small pore volume. To overcome these barriers, a variety of pretreatment processes (chemical and mechanical) have been developed.

Oxidative-lime pretreatment (OLP) is highly effective at reducing lignin content and removing acetyl groups from hemicellulose. Combining OLP with a mechanical treatment process greatly enhances the enzymatic digestibility of lignocellulose.

Recommended OLP conditions were determined for Dacotah (120 °C, 6.89-bar $O_2$, 240 min) and Alamo (110 °C, 6-89-bar $O_2$, 240 min) switchgrass. Using

recommended conditions, 72-h glucan digestibilities (g glucan hydrolyzed/100 g glucan in raw biomass; 15 filter paper units/g raw glucan) of 85.2 and 88.5 were achieved for Dacotah and Alamo, respectively. Adding ball milling to OLP further enhanced glucan digestibility to 91.1 (Dacotah) and 90.0 (Alamo).

In previous studies, shock treatment achieved promising results, but was often inconsistent. This work refined shock treatment with a focus on using consistent procedures and performance analysis. The combination of OLP and shock treatment enhanced the 72-h glucan digestibility of several promising biomass feedstocks: bagasse (74.0), corn stover (92.0), poplar wood (94.0), sorghum (71.8), and switchgrass (89.0).

Highly digestible lignocellulose can also be used as ruminant animal feed. Shock treatment plus OLP increased the total digestible nutrients ($TDN_N$; g nutrients digested/100 g organic matter) of corn stover from 51.9 (untreated) to 72.6. Adding in pre-washed corn stover solubles to produce a combined feed (17.8% corn stover solubles and 82.2% shock + OLP corn stover) increased $TDN_N$ to 74.9. Mixing in enough solubilized protein to match the crude protein content of corn grain further improved $TDN_N$ to 75.5, only 12.6 less than corn grain.

*To my parents, who have provided me with support and experiences far beyond what is expected. I am forever grateful.*

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# CHAPTER I

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 General Background

Because of population growth, global energy demand is rapidly increasing. In only 10 years (2001–2011), world population grew from 6.1 to 6.9 billion people (Census, 2011). The United Nations predicts that it could exceed 10 billion by 2050 (UN, 2009). Furthermore, expanded industrialization and increased standards of living have significantly increased energy demand (Salameh, 2003).

In the United States, fossil fuels contribute over 80% of annual energy consumption (Energy, 2009), and includes coal (25%), natural gas (30%), and petroleum (45%). Because of limited U.S. petroleum reserves, almost 60% of the consumed petroleum is imported, of which 40% comes from OPEC countries (Energy, 2009). Dependence on foreign oil has led to numerous military conflicts, resulting in economic instability and lost lives.

Heavy petroleum use negatively impacts the environment. Locating, drilling, and establishing piping infrastructures for new petroleum reserves can alter sensitive ecosystems. Also, off-shore drilling and transportation of petroleum have

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This dissertation follows the style of Bioresource Technology.

resulted in several major oil-spills (e.g., Exxon Valdez, BP), which hurt both the environment and the economy. Because fossil fuel combustion generates significant greenhouse gas emissions, global warming is another serious concern.

When considering the limited supply and adverse environmental effects of fossil fuels, it is clear that a significant shift towards alternative energy is necessary. There are numerous potential alternative energy solutions: solar, wind, hydroelectric, and biomass. Biomass has significant potential to dramatically shift U.S. energy production and consumption, but a combination of many energy sources will be necessary to minimize dependence on fossil fuel (Salameh, 2003).

## 1.2 Potential Alternative Energy Solutions

### 1.2.1 Solar

Solar energy sustains life on Earth. Solar energy is produced by nuclear reactions in the Sun, and it is transmitted to Earth as electromagnetic waves. Solar energy is clean and abundant in some regions of the world. It is particularly promising for rural areas that do not have access to a traditional power grid (Foster et al., 2009). Solar energy is primarily captured using photovoltaic cells, which are comprised of semiconducting materials that convert photons to direct current electrical power. The main disadvantages of solar energy include: (1) diffuse fuel source, (2) high installation costs, and (3) lack of economical energy storage (Luque & Hegedus, 2003). Currently, only 0.1% of U.S. energy consumption comes from solar or photovoltaic sources (Energy, 2009).

### 1.2.2 Wind

Wind turbines are one of the cleanest methods to harness energy. Wind turbines convert the kinetic energy in wind to electricity and require no fossil fuels. Wind energy is derived from the uneven heating of the Earth's surface. Improved technology allows the extraction of wind energy to be much more efficient. Unfortunately, wind power is unreliable and unavailable in certain regions. The northwest and northeast regions of the United States are best suited for wind turbines. The construction and material cost of wind farms is substantial, and noise pollution from commercial wind turbines is often the target of public protests and petitions (Nelson, 2009). As of 2009, wind energy accounted for less than 1% of total U.S. energy consumption (Energy, 2009).

### 1.2.3 Hydroelectric

Hydroelectric power is produced by the gravitational force of falling or flowing water. It currently is the second largest contributor of U.S. alternative energy and accounts for almost 3% of total energy consumption (Energy, 2009). Most hydroelectric power is derived from the potential energy of dammed water, which drives a water turbine and generator. A common misconception is that dams are built primarily to generate electricity. In reality, dams are built for many reasons, most notably water management. Hydroelectric generation is often just a positive by-product. Unfortunately, dams have negative issues. Building a dam is virtually irreversible and causes dramatic changes in the environment. Also, although hydroelectric power plants can quickly adjust their generation rate, this rapidly changes the downstream flow, eroding downstream river banks and degrading downstream recreational activities

(Edwards, 2003). Furthermore, although it is generally believed that hydroelectric generation is emission-free, recent discoveries have shown that water stored in dams often becomes silted with vegetation. This vegetation eventually rots, which emits large amounts of methane, a potent greenhouse gas (Delmas et al., 2005; Galy-Lacaux et al., 1999). Finally, hydroelectric power completely depends on the whims of nature. Years of low rainfall can significantly hinder hydroelectric generation (French et al., 1998).

### 1.2.4 Biomass

Biomass is biological material from living, or recently living, organisms and includes food crops, energy crops, agricultural residues, forestry residues, industrial waste, and municipal solid waste. Many conversion technologies are currently used, or being explored. Direct biomass combustion is the most prevalent, accounting for approximately 90% biomass-derived energy. Biomass combustion produces heat that is converted to mechanical power using a steam turbine, which generates electricity. Gasification and pyrolysis are examples of other biomass conversion (Callé, 2007). Hydrolysis and fermentation of biomass can generate liquid transportation fuels (biofuels). Because our current transportation infrastructure heavily relies on liquid fuels, conversion of biomass into liquid biofuels is one of the most promising applications of alternative energy.

## 1.3 First-generation Biofuels

First-generation bio-refineries process raw biomass into a relatively pure carbohydrate feed, which is then fermented into a liquid fuel (e.g., ethanol) (Soetaert & Vandamme, 2009). Oilseeds (e.g., soybeans, rapeseed, palm seeds) can also be

processed into oils that are subsequently converted to biodiesel (Du et al., 2003). Current conversion technologies are discussed below.

### 1.3.1 Biofuels from food crops

Currently, biofuels are predominantly generated from food crops (e.g., corn grain and sugarcane). Brazil, the second-largest biofuel producer after the United States, currently produces about 79% of their ethanol from sugarcane juice, and the remainder from cane molasses (Wilkie et al., 2000). Sucrose is extracted from the sugarcane, and then is fermented to ethanol. *Saccharomyces cerevisiae* is typically the microorganism of choice because it can hydrolyze sucrose to glucose and fructose, which are then fermented to ethanol (Sánchez & Cardona, 2008). This process is relatively simple; unfortunately, sugarcane only grows in semi-tropical locales of the United States.

In the United States, ethanol is almost exclusively produced from corn grain. The corn grain is milled to extract the starch, which is then enzymatically hydrolyzed to glucose. Traditionally, hydrolysis was performed using acids, but it has been replaced by α-amylase enzymes. Amylases are highly specific and perform well under mild reaction conditions. The resulting glucose syrup is fermented using *S. cerevisiae* at temperatures of 30–32 °C with the addition of ammonium sulfate or urea as nitrogen sources (Bothast & Schlicher, 2005). Currently, 37% of U.S. corn grain produced is used to generate biofuels (USDA, 2011b).

### 1.3.2 Primary issues with first-generation biofuel processes

There are numerous disadvantages to using food crops as primary feedstocks in ethanol conversion processes. The most significant is the limited supply of corn. In

2010, almost 12.5 billion bushels of corn were produced, of which 37% was consumed by the fuel ethanol industry (USDA, 2011b). At an estimated corn-to-ethanol yield of 2.75 gallons of ethanol/bushel of corn (Sokhansanj et al., 2010), this produced 827 thousand barrels of ethanol/day. Considering that ethanol has only 2/3 of the energy content of gasoline, this is equivalent to approximately 550 thousand barrels of gasoline/day. Current U.S. gasoline consumption is slightly over 9 million barrels/day (USDoE, 2011), so current ethanol production provides 6% of the energy in gasoline blends. If the entire annual corn supply were devoted to ethanol production, this would increase to 16%. This clearly demonstrates the need to dramatically shift agricultural practices, or change feedstocks.

Corn is a staple food crop largely used for animals, but also for humans. Continued production of corn-based ethanol has generated a highly controversial food vs. fuel debate (Dale, 2008). In the past 10 years, as the percentage of corn devoted towards ethanol fuel has increased (currently 37%), corn consumed for other uses has dramatically decreased: from 60% to 38% for livestock feed; from 14% to 10% for food, alcohol, and industrial use; and from 20% to 14% for exports (Figure 1-1). Furthermore, in the last 10 years, global population has increased by over 800 million people, providing an increased stress on the global supply of staple food crops. This leads to a very important question: Can crop productivity grow fast enough to meet global demand for food, fuel, and animal feed (G. Cassman & Liska, 2007)?

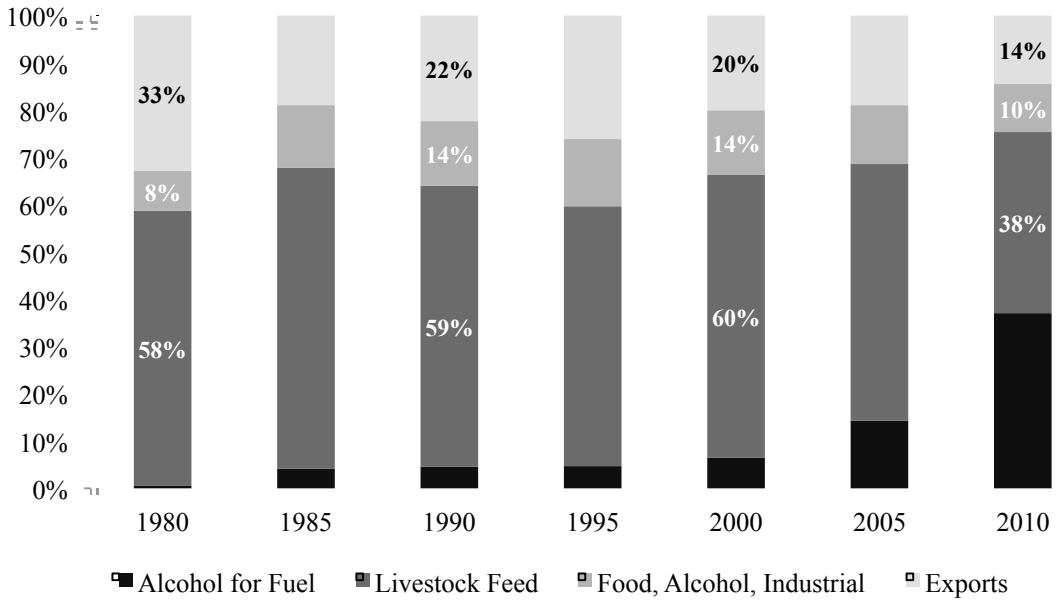


**Figure 1-1.** U.S. corn consumption during the last 30 years (USDA, 2011a).

Another significant result of this rapid increase in demand for food crops used in biofuel production is a dramatic price increase for corn. From 1970 to 2000, the price of corn was relatively stable (around $2–3/bushel), but recently has rapidly climbed to over $7/bushel (Figure 1-2). Other staple food crops have similarly increased in price, resulting in food riots in many developing countries (e.g., Egypt, Haiti, Indonesia, Senegal, and Somalia) (Rosegrant & International Food Policy Research, 2008). By 2050, global demand for food is expected to double, whereas global demand for transportation fuels is expected to increase even more rapidly, necessitating the development of alternative processes (Foley et al., 2005; Gomiero et al., 2010).

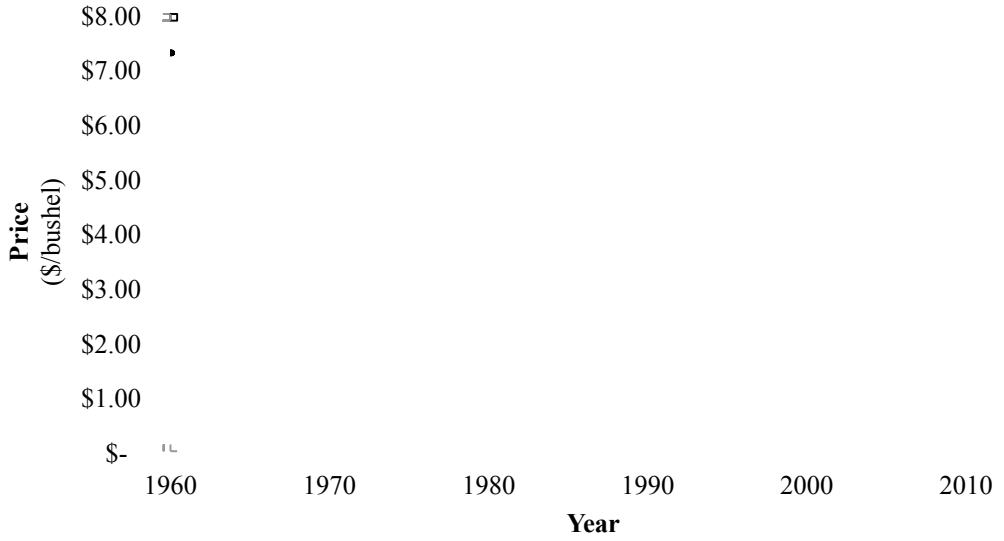


**Figure 1-2.** Historical corn prices by year (USDA, 2011b).

## 1.4 Lignocellulosic Biomass

First-generation biofuel processes are based on free sugars or starch, which are in limited supply. In contrast, lignocellulose is much more abundant. Lignocellulose is a composite of cellulose, hemicellulose, and lignin that structurally supports the plant cell wall. Every lignocellulosic biomass has different ratios of these three key structural components, but overall they represent approximately 90% of the dry weight of most plants. The remainder of the biomass is other polymeric constituents (e.g., starch, pectin, proteins) and low-mass compounds, such as extractives. The three main components are described further below.

### 1.4.1 Cellulose

Cellulose, the world's most abundant biological material, is a linear, unbranched polymer of anhydroglucose. The β-glucose monomer units are joined together with ether linkages between the C1 and C4 positions. These β-1,4 linkages differentiate cellulose from starch, which is polymerized α-glucose. The different linkages between cellulose and starch result in significantly different properties. Starch is easily hydrolyzed by enzymes, whereas cellulose highly resists enzymatic digestion. Generally, native cellulose has a degree of polymerization between 3,500 to 10,000 units, which results in molecular weights between 600,000 and 1,500,000. Hydrogen bonding between the equatorial hydroxyl groups allows cellulose to crystallize. Native cellulose contains both crystalline and amorphous regions. Cellulose accounts for 10–50% of plant cell walls, and is primarily embedded in a matrix of hemicellulose (Holtzapple, 2003a).

### 1.4.2 Hemicellulose

After cellulose, hemicellulose is the world's second most abundant biological material. Hemicellulose polymers are considerably shorter, with a typical degree of polymerization ranging from 50–200. Unlike cellulose, hemicellulose is often Y-branched, and typically has attached side groups. It is made up of three hexose monomers (glucose, galactose, and mannose) and two pentose monomers (xylose and arabinose). Xylan, a specific type of hemicellulose, is characterized by a β-1,4-linked xylose backbone. Acetylation often occurs at the C2 position, and less frequently at the

C3 position. Hemicellulose comprises 20–30% of the cell wall, and forms a matrix around the cellulose fibrils (Holtzapple, 2003b).

### 1.4.3 Lignin

Lignin, the third primary component in lignocellulose, is the world's most abundant non-carbohydrate biological material. The primary roles of lignin are to hold the plant cell wall together and to prevent water loss from plant vascular systems. Similar to cellulose, it highly resists enzymatic digestion. Lignin is a highly branched polymer, which is comprised of three phenylpropylene monomers: *trans*-coniferyl alcohol, *trans*-sinapyl alcohol, and *trans*-*p*-coumaryl alcohol (Figure 1-3). The ratio of these three monomer units differs greatly between plant types. Hardwoods generally contain significant amounts of *trans*-coniferyl and *trans*-sinapyl alcohols, softwoods contain primarily *trans*-coniferyl alcohol, and grasses have relatively equal amounts of each monomer. Within the lignin polymer, there is significant cross-linking between the

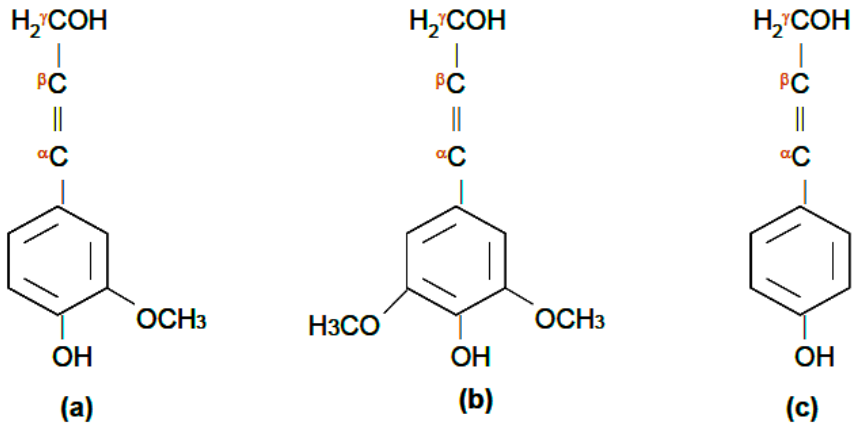


**Figure 1-3.** Lignin monomers: (a) trans-coniferyl alcohol, (b) trans-sinapyl alcohol, (c) trans-p-coumaryl alcohol (Holtzapple, 2003c).

phenylpropylene monomers, as well as covalent bonding with the surrounding hemicellulose. Lignin is primarily amorphous, and comprises about 20% of the plant cell wall (Holtzapple, 2003c).

## 1.5 Development of Second-generation Biofuels

With the previously mentioned concerns regarding ethanol from food crops, considerable research effort has been devoted to developing second-generation biofuels from lignocellulosic sources. The key advantages of lignocellulosic feedstocks are lower costs, greater availability, and wider variety. Lignocellulose includes energy crops, grasses, forestry residues, agricultural residues, industrial waste, and municipal solid waste. There are three main platforms being researched: (1) thermochemical (e.g., syngas), (2) sugar, and (3) carboxylate. The sugar and carboxylate platforms are discussed further below.

### 1.5.1 Simultaneous saccharification and fermentation (SSF)

Sugar platform conversion technologies are characterized by four primary steps: (1) pretreatment to reduce lignocellulose recalcitrance, (2) enzymatic hydrolysis of complex carbohydrates to simple carbohydrates, (3) fermentation of simple sugars to ethanol, and (4) distillation (Rabelo et al., 2009).

Enzymatic hydrolysis is performed using cellulase enzymes, which are divided into three sub-categories, each with a specific role. *Endogluconases* reduce the degree of polymerization of cellulose by hydrolyzing interior bonds, primarily in amorphous regions. *Exogluconases* shorten the cellulose molecules by binding to the polymer ends and releasing cellobiose, a glucose disaccharide. *β-glucosidase* is responsible for

hydrolyzing the cellobiose into two glucose units (Olofsson et al., 2008). The yeast employed for the alcohol fermentation is typically *S. cerevisiae*; however, it can ferment only hexoses. To ferment pentoses, *S. cerevisiae* must be genetically modified (Ho et al., 1998).

When enzymatic hydrolysis and fermentation are performed sequentially, it is designated as separate hydrolysis and fermentation (SHF). In 1976, Gauss et al. patented the idea of performing the two steps simultaneously, which is designated simultaneous saccharification and fermentation (SSF). In 1977, this simultaneous process (Figure 1-4) was successfully demonstrated by Takagi et al. There are several advantages to combining the processes: (1) lower capital costs because separate vessels are not necessary, (2) reduced end-product inhibition by sugars formed in the hydrolysis, (3) inhibitors from pretreatment are metabolized by fermentation microorganisms, and

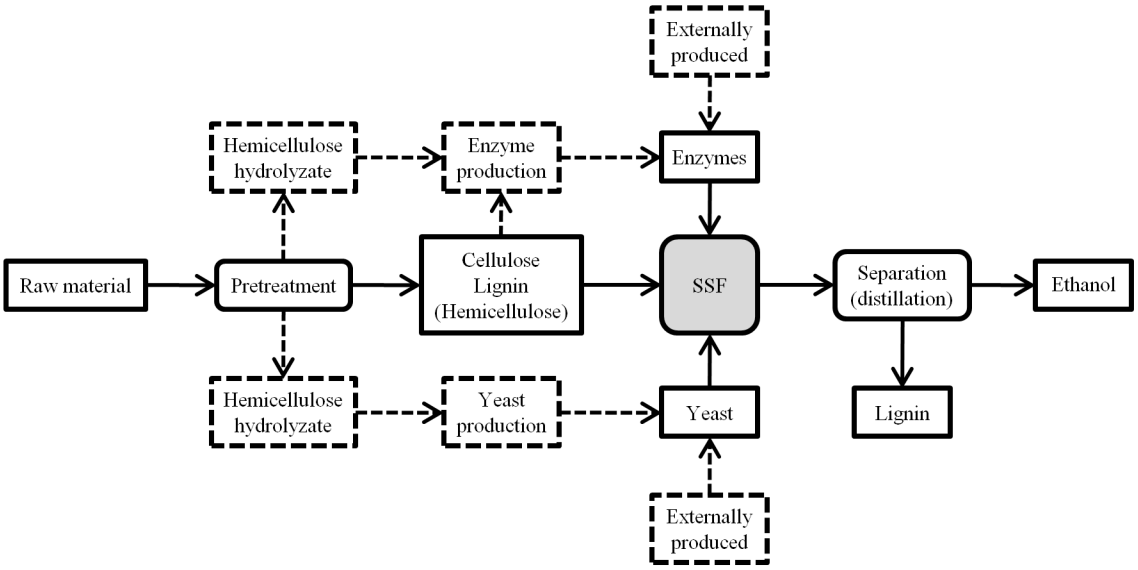


**Figure 1-4.** Schematic representation of an SSF process (Olofsson et al., 2008).

(4) higher ethanol concentrations reduce contamination risks (Öhgren et al., 2007; Olofsson et al., 2008; Wyman et al., 1992). The most significant drawback to using SSF is that the optimum temperatures for enzymes and yeast are different, rendering it impossible to run the simultaneous steps at optimal conditions.

### 1.5.2 MixAlco process

The MixAlco process (Figure 1-5) is one of several carboxylate platform technologies. The MixAlco process is a patented technology that converts any bidegradable material into mixed alcohol fuels (Holtzapple et al., 1999). This method employes a buffered mixed-culture fermentation to convert all non-lignin biomass components to carboxylate salts, which is more energy efficient than thermochemical conversion (Holtzapple & Granda, 2009). Because it integrates enzyme production, substrate hydrolysis, and the mixed-acid fermentation into a single step, it is an example of consolidated bioprocessing (Fu & Holtzapple, 2010).

The overall process is shown in Figure 1-5 and includes the following steps: (1) pretreatment with lime, (2) acid fermentation using a mixed culture, (3) dewatering of the carboxylate salts, (4) thermal conversion of carboxylate salts to ketones, (5) hydrogenation of the ketones to mixed alcohols, and if desired (6) oligomerization of alcohols to hydrocarbons using zeolite catalysts (Pham et al., 2010). For the fermentation process, there are numerous advantages to using a mixed culture over a pure culture. The mixed culture does not require aseptic conditions, which dramatically reduces the cost of fermentation vessels. Also, the mixed culture is robust and can process a large variety of non-sterile feedstocks. Fully optimizing a mixed-culture

fermentation is time consuming, and methods to regulate the carbon-nitrogen ratio and pH are still being developed (Smith & Holtzapple, 2010).

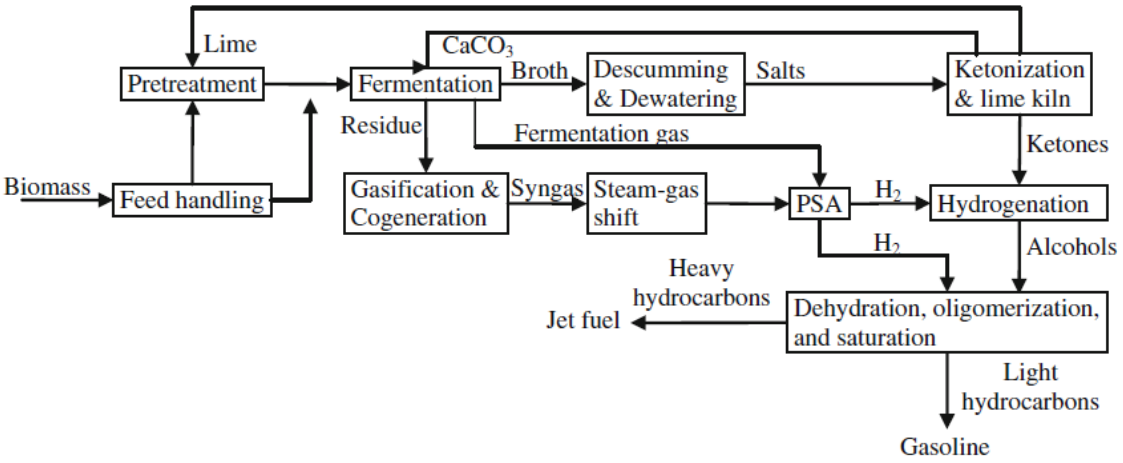


**Figure 1-5.** MixAlco process overview (Pham et al., 2010).

## 1.6 Promising Lignocellulosic Feedstocks

One of the most promising aspects of using lignocellulose to produce liquid biofuels is the large variety of lignocellulosic feedstocks available. Considerable research has been invested in studying the feasibility of using different feedstocks; important considerations are cost, adaptability, yield, and input requirements. Some of the more promising feedstocks are discussed below.

### 1.6.1 Bagasse

Bagasse is a high-volume agricultural crop residue resulting from the sugar and alcohol industries in Brazil, India, Cuba, and China (MartInez et al., 2003). In tropical countries, it is the most abundant lignocellulosic material (Peng et al., 2009). A single

tonne of sugarcane generates about 280 kg of bagasse (Cerqueira et al., 2007). Annually, $5.4 \times 10^8$ dry tonnes of sugarcane are processed throughout the world (Cardona et al., 2010), so about $1.5 \times 10^8$ tonnes of bagasse are available. Currently, about 50% of the generated bagasse is burned in distillery plants as fuel and the remainder is stockpiled (Pandey et al., 2000); thus, there is great interest in developing bagasse as a biofuel feedstock (Adsul et al., 2004). Sugarcane bagasse generally contains 40–45% cellulose, 30–35% hemicellulose, and 20–30% lignin (Peng et al., 2009).

### 1.6.2 Corn stover

Corn stover (*Zea mays*) is the most abundant agricultural residue in the United States, resulting from the harvest of corn grain. Recent estimates indicate that approximately 82 million dry tons per year of corn stover are available (Kadam & McMillan, 2003). It is composed of several components, all with different characteristics. Husks, shanks, silks, and cobs comprise 30% of corn stover mass. The remaining 70% is white stalks, tassels, leaf blades, and leaf sheaths (Hanway, 1963). The cobs, leaves, and husks represent the portion with the highest glucose potential (Crofcheck & Montross, 2004). Corn stover typically has about 35% cellulose, 21% xylan, and 17% lignin (Elander et al., 2009).

### 1.6.3 Switchgrass

Switchgrass (*Panicum virgatum*) is a perennial warm-season prairie grass, and has been chosen by the United States Department of Energy as a model biomass feedstock (McLaughlin et al., 2002). It can be grown in most of the eastern two-thirds of

the United States, as well as Mexico and Canada. It is highly adaptable and drought resistant (Wright & Turhollow, 2010). On marginal lands, it can be grown in very high yields (13.4 Mg/(ha·yr)) with little fertilizer, herbicide, or pesticide input required (Schmer et al., 2006; Walsh et al., 2003).

### 1.6.4 Sorghum

Sorghum (*Sorghum bicolor*) is a hardy, drought-tolerant grass that has several advantages when considered as a potential biofuel feedstock. One advantage is that sorghum is an excellent source of lignocellulose, sugar, and starch. Sorghum is more water and nutrient efficient than sugarcane or corn (Murray, 2008). Nearly 7 million ha is devoted to growing sorghum in the United States (Rooney et al., 2007).

Sorghum has four categories: grain sorghum, sweet sorghum, forage sorghum, and "high-energy" sorghum. Grain sorghum provides starch, useful for current biofuel conversion technologies. Sweet sorghum contains high levels of sugar in the stalk of the plant, which can be converted to biofuels using similar processes to those developed for sugarcane. Forage sorghum is fed to ruminants, such as cattle, so it must have a relatively low lignin content to be readily digested. "High-energy" sorghum is selected for high biomass yields regardless of its ruminant digestibility (McBee et al., 1987). Annual sorghum yields ranging from 20–30 Mg/ha have been demonstrated (Rooney et al., 2007), and these yields required approximately 33% less water than is required for corn (McCollum et al., 2005). The composition of sorghum varies greatly depending on the variety; however, it contains significant amounts of lignocellulose.

### 1.6.5 Poplar wood

Poplars (*Populus)* are versatile and are among the highest yielding trees in temperate regions. Some advantages of poplar feedstocks include very fast growth, easy hybridization, high biomass yield, and strong adaptability to soil and climate. Considerable water demand and high susceptibility to disease are disadvantages to using poplar. Worldwide, there are approximately 80 million hectares of naturally occurring poplars, with an additional 5.3 million hectares of poplar plantations (Bridgewater et al., 2010). Realistic biomass yields from poplar plots range from 10 to 15 Mg/(ha·yr) (Afas et al., 2008). The typical composition of poplar wood is 45% cellulose, 25% hemicellulose, and 20% lignin (McDougall et al., 1993). It typically contains about 55–60% water, which is high for wood (Kauter et al., 2003).

## 1.7 Barriers to Enzymatic Digestion

There are a number of lignocellulose structural features that hinder enzymatic digestion. Understanding these structural features is vital to designing a biomass pretreatment process that minimizes or eliminates as many of these limitations as possible. Unfortunately, the mechanisms by which these features hinder enzymatic digestibility are not fully understood, and are still under considerable debate.

### 1.7.1 High lignin content

Lignin is a key component that hinders biomass digestibility. It closely associates with cellulose microfibrils, blocking access to the carbohydrate fractions of biomass. Research has shown that removing lignin, either by hydrolysis or degradation, swells the biomass, which increases surface and median pore volume (Chang &

Holtzapple, 2000; Zhu et al., 2008). There is no consensus on the extent of delignification required; however, it is generally accepted that reducing lignin content directly correlates with increased enzymatic yields (Lynd, 1996; Taherzadeh & Karimi, 2008).

### 1.7.2 Presence of acetyl groups on hemicellulose

As discussed previously, xylan backbones are typically acetylated ($CH_3COO–$) with approximately 70% of xylan residues containing acetyl groups (Holtzapple, 2003b). Numerous studies have shown that removing acetyl groups from xylan induces swelling, which increases biomass enzymatic digestibility (Chang & Holtzapple, 2000; Mosier et al., 2005; Zhu et al., 2008).

### 1.7.3 High cellulose crystallinity

In lignocellulosic biomass, cellulose has both crystalline and amorphous regions. Crystalline regions resist binding to cellulase, so they are difficult to hydrolyze. In contrast, amorphous regions readily bind to cellulose, so they are easier to hydrolyze (Klyosov et al., 1986). Reducing biomass crystallinity should improve biomass digestibility; however, research shows conflicting results regarding the correlation between crystallinity and enzymatic digestibility (Chang & Holtzapple, 2000; Fan et al., 1980; Puri, 1984; Zhu et al., 2008). The conflicting results could result from non-related factors (drying conditions, substrate preparation) or the inherent error in measuring the cellulose crystallinity in a non-pure substance (e.g., biomass).

### 1.7.4 Degree of polymerization of cellulose

Another cellulose feature that is thought to affect biomass digestibility is the degree of polymerization (DP), which is defined as the number of glucosyl residues per cellulose chain. Cellulose DP determines the number of terminal ends relative to interior β-glucosidic bonds, which are substrates for exo- and endo-acting cellulase enzymes, respectively (Zhang & Lynd, 2004). Exogluconases react with terminal ends, decreasing DP incrementally, whereas endogluconases act on interior portions of the chain, rapidly decreasing DP. Decreased DP leads to cellulose solubulization, which may favor digestibility, although conclusive evidence has not been presented (Irwin et al., 1993; Kruus et al., 1995; Puri, 1984; Reverbel-Leroy et al., 1997).

### 1.7.5 Surface area and pore volume

For cellulase enzymes to hydrolyze cellulose, they must bind to the surface of the substrate. During enzymatic hydrolysis of cellulose, the maximum amount of enzymes that can be adsorbed is a limiting factor for both hydrolysis yields and rates. The amount of enzyme that can adsorb is directly related to the accessibility of active sites on the cellulose substrate (Kumar & Wyman, 2008). A variety of methods are used to measure surface area and pore volume including BET method, X-ray scattering (SAXS), and mercury porosimetry. Furthermore, enzyme accessibility can be estimated by measuring the difference between the total amount of protein initially added and the amount remaining at any time of hydrolysis (Kumar & Wyman, 2008).

## 1.8 Biomass Pretreatment Technologies

Before the biomass can be subject to enzymatic hydrolysis and later fermentation, the barriers to enzymatic digestion necessitate a pretreatment step (Figure 1-6). In any cellulosic ethanol process (sugar or carboxylate platform), this is the first step. Its primary goal is to reduce, or completely remove, hindrances that cause lignocellulose to be recalcitrant. Considerable research efforts have been devoted to biomass pretreatment because it has been estimated to account for almost 20% of the entire process cost, second only to the cost of biomass itself (Aden & Foust, 2009). Some favorable pretreatment characteristics include high glucan recovery, moderate to high hemicellulose recovery, high lignin removal, some cellulose decrystallization, and an increase in pore size or surface area.

There are many pretreatment techniques being explored, most of which are classified as either chemical or physical. The chemical pretreatments can be categorized

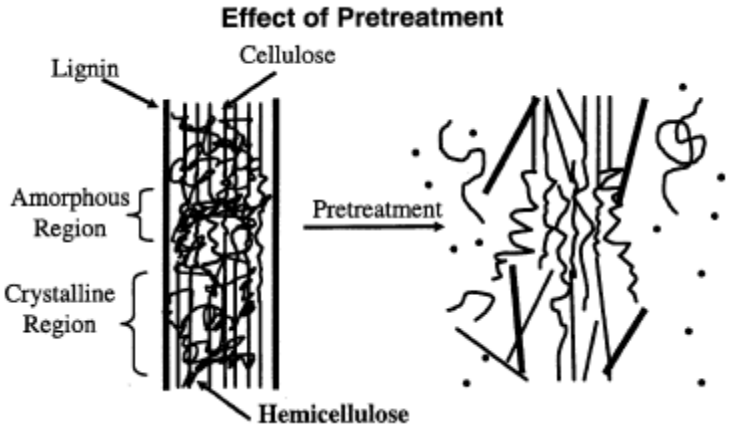


**Figure 1-6.** Goals of pretreatment (Mosier et al., 2005).

into acid (e.g., dilute acid, sulfur dioxide), alkaline (e.g., ammonia fiber expansion, lime, soaking in aqueous ammonia), or neutral (liquid hot water) pretreatments. Some examples of physical pretreatments are ball-milling, two-roll milling, and irradiation. Examples of some of the leading biomass pretreatment methods are described further below.

### 1.8.1 Dilute-acid

Dilute-acid pretreatment is a popular pretreatment choice, and has received the most development. Addition of dilute sulfuric acid to cellulosic materials has been used for years to commercially manufacture furfural (Zeitsch, 2000). In biomass pretreatment, dilute sulfuric acid is mixed with biomass to hydrolyze hemicellulose to xylose and other simple sugars. Degradation of xylose can continue to produce furfural, which can be recovered by distillation. This pretreatment is performed at 140–190 °C, and effectively removes most hemicellulose (Wyman et al., 2005b). The removal of hemicellulose increases the susceptibility of cellulose to enzymatic digestion (Knappert et al., 1981). This pretreatment does not significantly remove lignin, but research suggests that its structure is disrupted thereby increasing cellulose digestibility (Yang & Wyman, 2004).

Dilute-acid pretreatment can be performed as either batch or flow-through. In batch pretreatment, the biomass is soaked in dilute sulfuric acid for at least 4 hours at room temperature, and then is placed in the reaction vessel, which is either heated through the vessel walls or by steam injection. Flow-through pretreatment requires

aqueous acid to be pre-heated, and then injected through a layer of biomass (Lloyd & Wyman, 2005; Schell et al., 2003).

The primary limitations with this pretreatment involve the corrosive nature of the dilute acid, which mandates that all pretreatment vessels be constructed of expensive materials. Furthermore, the low-pH pretreated solids must be neutralized before the sugars proceed to fermentation (Mosier et al., 2005).

### 1.8.2 Liquid hot water

Another common pretreatment technology, termed hydrothermolysis, uses pressure to maintain water in the liquid state at elevated temperature (Bobleter et al., 1976). Research has demonstrated that high-pressure water can penetrate the cell structure of biomass, and solubilize hemicellulose (Hörmeyer et al., 1988; Walch et al., 1992). At temperatures of 200–230 °C and reaction times of less than 15 min, complete removal of hemicellulose can be achieved (Mok & Antal, 1992). Furthermore, 35–60% of the lignin is also removed at these reaction conditions. At these elevated temperatures, the $pK_a$ of pure water is significantly affected, resulting in a pH of nearly 5.0. Also, hot water cleaves hemiacetal linkages and liberates acids in the biomass. In response to these issues, the addition of a base is occasionally required to maintain the pH between 5 and 7. This is termed "pH-controlled liquid hot water pretreatment," and is necessary to minimize cellulose degradation (Weil et al., 1998). Some benefits of liquid hot water pretreatment include: (1) neutralization after pretreatment is not necessary because acid is not added, and (2) size reduction of the incoming biomass is not needed (Kohlmann et al., 1996; Weil et al., 1997).

### 1.8.3 Ammonia fiber expansion

Ammonia fiber expansion (AFEX) is a batch pretreatment where lignocellulosic biomass is exposed to liquid ammonia at 70–200 °C and 6.9–27.6 bar for a desired reaction time (Bals et al., 2010a). Upon completing the pretreatment time, the pressure is suddenly released causing rapid vaporization of the ammonia, which both aids in the recycle of ammonia and further improves digestibility (Dale & Moreira, 1982). AFEX increases enzymatic digestibility of cellulose in several ways: (1) reduces cellulose crystallinity (Gollapalli et al., 2002), (2) deacetylates acetyl linkages (Mitchell et al., 1990), (3) modifies the lignin structure (Martínez et al., 1991), and (4) removes some hemicellulose (Ferrer et al., 2000). This pretreatment process has shown great promise, but the cost of ammonia and ammonia recovery need to be considered (Holtzapple et al., 1992).

### 1.8.4 Lime

Lime pretreatment exposes a mixture of lignocellulosic biomass, calcium hydroxide, and water to different conditions of temperature and pressure for a desired reaction time. Oxidative lime treatment refers to the addition of an oxygen source, which further improves performance (Kim & Holtzapple, 2005). Lime pretreatment has proven to selectively reduce the lignin content of lignocellulosic biomass and remove acetyl groups, while maintaining high carbohydrate yields (Sierra et al., 2009).

## 1.9 Development of Lime Pretreatment

As discussed previously, lime pretreatment is a promising pretreatment technology because of its high selectivity for lignin removal while maintaining high

carbohydrate yields. The use of lime as the alkaline agent is beneficial for a number of reasons (Holtzapple & Davison, 1999):

- Least expensive alkali
- Easy to recover, making it cost-effective and environmentally friendly
- Safe to handle

Lime is also very compatible with oxidants, and research has shown that adding an oxidant during lime pretreatment significantly improves lignin removal (Gierer et al., 2001; Klinke et al., 2002; Yang et al., 2003). Also, during pretreatment, the acetyl groups located on the xylan backbone are removed, which results in improved cellulase access (Pan et al., 2006; Pan et al., 2004). Lime pretreatment has an additional advantage over other alkaline pretreatments. Most alkaline pretreatments achieve significant lignin removal and highly digestible cellulose; however, harsher alkalis result in high cellulose degradation. During lime pretreatment, carbon dioxide resulting from cellulose and hemicellulose degradation reacts with the calcium hydroxide to form calcium carbonate, which forms protective layers over the cellulose and prevents significant degradation (Lopez et al., 2000). Because of this, glucan recovery is extremely high in most cases, often greater than 95%. Furthermore, hemicellulose yields are moderate to good (Falls et al., 2011b; Sierra et al., 2010).

Lime pretreatment has been studied and implemented for a number of applications, but this work focuses primarily on its application in cellulosic biofuel processes. The effectiveness of lime pretreatment has been studied for numerous feedstocks, and over a variety of different temperatures, pressures, and reaction times

(Chang et al., 2001; Falls et al., 2011b; Kaar & Holtzapple, 2000; Kim & Holtzapple, 2005; Sierra, 2010b; Xu et al., 2010). Through these efforts, a very clear division has developed between lime pretreatment methods which can be classified based on reaction times. *Long-term lime pretreatments* are designated as pretreatments lasting several weeks. Generally, the pretreatment conditions are quite mild, with maximum reaction temperatures of 75 °C. For these pretreatments, air is used as the oxidizing agent, but is often not necessary. *Short-term pretreatments* use harsher reaction conditions, and are more effective with oxidative agents (typically oxygen). Temperatures can range up to 180 °C, and reaction times can range from minutes to several hours.

The Holtzapple research group has spent considerable effort determining the recommended lime pretreatment conditions for a variety of feedstocks (Table 1-1). The results show a relatively consistent trend. Feedstocks with lower lignin contents (<22%) favored less harsh temperature and pressure, and increased pretreatment time. Those with higher lignin contents (>22%) responded well to a shorter pretreatment time (2 h), but required more severe temperature and oxygen pressure. Other research laboratories are also exploring lime pretreatment (Rabelo et al., 2009; Saha & Cotta, 2008; Xu et al., 2010).

Another promising application of lime pretreatment, which is explored as part of this dissertation work, is in the generation of highly digestible lignocellulosic animal feed. Lime pretreatment is particularly suited for this application because lime is not toxic, is inexpensive, and pretreatment conditions can be mild (Sierra, 2010b). Results have shown moderate to good increase of *in vitro* digestibility (Chang et al., 1997), as

well as a doubling of *in situ* digestibility (Gandi et al., 1997). Furthermore, lime pretreatment has been shown to effectively hydrolyze protein from animal waste (e.g., chicken feathers and animal hair) (Coward-Kelly et al., 2006a; Coward-Kelly et al., 2006b). To further increase ruminant digestibility, this work will explore adding physical treatment processes in combination with the lime pretreatment.

**Table 1-1.** Recommended lime pretreatment conditions.

| Biomass | Lignin (%) | Time | Temp (°C) | Lime Loading (g $Ca(OH)_2$/g biomass) | Oxygen pressure (bar) |
|---|---|---|---|---|---|
| Pine [a] | 34.1 | 2 h | 140 | Not Reported | 20.7 |
| Poplar Wood [b] | 29.3 | 2 h | 160 | 0.23 | 13.8 |
| Bagasse [c] | 23.7 | 2 h | 130 | Not Reported | 6.9 |
| Sorghum [a] | 22.0 | 2 h | 180 | Not Reported | 6.9 |
| Switchgrass [d] | 21.4 | 4 h | 120 | 0.30 | 6.9 |
| Corn Stover [a] | 20.9 | 4 h | 110 | Not Reported | 6.9 |
| Corn Stover [e] | 20.9 | 4 wk | 55 | 0.07 | 0.21 |

[a] (Sierra, 2010a); [b] (Sierra et al., 2009); [c] (Meysing, 2011); [d] (Falls et al., 2011b); [e] (Kim & Holtzapple, 2005)

## 1.10 Shock Tube Development

This work explored a novel application of a very well-studied apparatus: the shock tube. Traditionally, shock tubes consist of a uniform-cross-section tube that is filled with a low-pressure and a high-pressure gas, separated by a diaphragm. The shock wave is initiated by rupturing the diaphragm. The first shock tube was operated in 1899 to understand gas explosions in mines. Over the past 50 years, shock tubes have primarily been used by kineticists as high-temperature wave reactors. In this application, rate coefficient data for thermal decomposition reactors, oxidation reactors, and even some heterogeneous reactions can be obtained under diffusion-free conditions

(Bhaskaran & Roth, 2002; Hong et al., 2011). A more recent application, the Hydrodyne process, uses a shock tube to tenderize red meat (Long et al., 2007; Solomon et al., 1997) or chicken breasts (Claus et al., 2001; Meek et al., 2000). Similar to the Hydrodyne process, shock tubes can potentially be used to increase lignocellulosic digestibility. Preliminary results by the Holtzapple research group have shown some improvement in biomass digestibility, but have been mostly inconsistent.

### 1.11 Biomass Refining Consortium for Applied Fundamentals and Innovation

A majority of this dissertation work was conducted in cooperation with the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI). One issue that has plagued biomass pretreatment research is inconsistency in reporting methods, analytical techniques, and enzyme loadings. These inconsistencies make it nearly impossible to draw meaningful comparisons between different pretreatment methods. To overcome this, in late 1999, the CAFI group was formed by leaders of biomass pretreatment research. The collaboration originally consisted of five universities and the National Renewable Energy Laboratory (NREL) (Wyman et al., 2005b). The latest iteration included Texas A&M University, Auburn University, Michigan State University, University of California Riverside, Purdue University, NREL, Ceres, Inc., and Genencor, a Danisco Division. The members coordinated the development of consistent analytical methods, cellulase sources, feedstock sources, and reporting methods.

The first collaborative effort (CAFI I) focused on improving the enzymatic digestibility of corn stover (Elander et al., 2009). Glucose and xylose yields were

compared between seven different pretreatment methods. Lime pretreatment showed very competitive yields (Wyman et al., 2005a), and was determined to have the lowest total fixed capital investment (Eggeman & Elander, 2005).

CAFI II compared six different pretreatment methods using a common source of poplar wood. The six pretreatment studied were dilute acid, $SO_2$ steam explosion, controlled pH, ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), and lime. The overall sugar yield for lime was 91.3 g hydrolyzed/100 g in raw biomass, which outperformed every treatment except $SO_2$ steam explosion (Wyman et al., 2009).

The third and final CAFI collaboration investigated improved digestibility resulting from pretreating several varieties of switchgrass. Once again, multiple pretreatment methods were compared, and lime pretreatment was highly competitive. A number of publications were generated from this work, covering topics including surface characterization of pretreated switchgrass (Donohoe et al., 2011), comparative material balances (Garlock et al., 2011), effect of β-glucosidase supplementation (Pallapolu et al., 2011), enzyme formulation (Falls et al., 2011a), and adsorption of cellulase and hemicellulase enzymes on pretreated switchgrass (Shi et al., 2011). This dissertation work contributed to the CAFI III effort.

## 1.12 Research Objectives

The overall purpose of this work was to increase the digestibility of lignocellulosic biomass, thereby increasing its value. This was accomplished using a combination of chemical (e.g., oxidative lime) and physical (e.g., ball milling, shock treatment) pretreatments.

The first two projects explored the use of oxidative-lime pretreatment on two different varieties of switchgrass: Alamo (southern lowland) and Dacotah (northern upland). The goal of this project was two-fold: (1) gain a strong understanding of the oxidative-lime pretreatment process, and (2) determine the recommended pretreatment conditions (temperature, $O_2$ pressure, and time) that produced the most digestible switchgrass. Switchgrass was chosen for this study because it is recognized by the U.S. Department of Energy as a model biomass feedstock. Two different switchgrass varieties were studied because of compositional differences resulting from different ecotypes, morphologies, harvest locations, and harvest dates.

The next project was a collaborative effort between CAFI members, with the objective of comparing enzyme performance on Dacotah switchgrass subjected to leading pretreatment technologies. The effect of combining cellulase, β-glucosidase, and xylanase was also explored. Furthermore, this project explored whether it was valuable to combine chemical and physical pretreatments.

Because of the prohibitive cost associated with ball milling, and its tendency to destroy the physical integrity of the biomass, the objective of the fourth project was to develop a novel physical pretreatment process: shock treatment. The shock treatment project had three primary goals: (1) prove the effectiveness of shock treatment on a variety of promising lignocellulose feedstocks, (2) determine the recommended set of operating conditions for shock treatment, and (3) determine the effect of shock treatment on cellulose crystallinity and cellulose degree of polymerization.

The purpose of the final project was to utilize the knowledge gained on both oxidative-lime pretreatment and shock treatment to enhance the ruminant digestibility of lignocellulose. This project combined oxidative-lime pretreatment and shock treatment, with the goal of achieving an overall ruminant digestibility similar to that of corn grain.

# CHAPTER II

## OXIDATIVE LIME PRETREATMENT OF DACOTAH SWITCHGRASS*

Oxidative lime pretreatment increases the enzymatic digestibility of lignocellulosic biomass primarily by removing lignin. In this study, recommended pretreatment conditions (reaction temperature, oxygen pressure, lime loading, and time) were determined for Dacotah switchgrass. Glucan and xylan overall hydrolysis yields (72-h, 15 FPU/g raw glucan) were measured for 105 different reaction conditions involving three different reactor configurations (very-short term, short term, and long term). The short-term reactor was the most productive. At the recommended pretreatment condition (120 °C, 6.89 bar $O_2$, 240 min), it achieved an overall glucan hydrolysis yield of 85.2 g glucan hydrolyzed/100 g raw glucan and an overall xylan yield of 50.1 g xylan hydrolyzed/100 g raw xylan. At this condition, glucan oligomers (1.80 g glucan recovered/100 g glucan in raw biomass) and xylan oligomers (25.20 g xylan recovered/100 g xylan in raw biomass) were recovered from the pretreatment liquor, which compensate for low glucan and xylan pretreatment yields.

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## 2.1 Introduction

Petroleum is currently responsible for almost 40% of U.S. energy consumption, with renewable energy accounting for only 8%. Because of limited domestic petroleum reserves, approximately 70% of U.S. petroleum consumption is imported (Energy, 2009). Dependence on foreign oil has led to military conflicts and fluctuating oil prices, resulting in economic instability (Yang & Wyman, 2008). Environmental issues (e.g., groundwater contamination, acid deposition, air pollution, and oil spills) and human health effects have increased desire to develop sustainable alternatives (Hubbard, 1991; McLaughlin et al., 2002). Greenhouse gas emissions from burning fossil fuels have been linked to climate change as well.

The current transportation infrastructure is built on liquid fuels, so renewable liquid biofuels are a promising solution. Current commercial biofuel technology uses starch from corn, or sucrose from sugarcane, to produce ethanol fuel. However, limited feedstock availability and feed vs. fuel pressures prevent these processes from producing the necessary quantities to make a meaningful impact (Schmer et al., 2008). Alternatively, lignocellulosic biomass is very abundant and is comprised of many feedstocks: high-yield energy crops, forestry residues, agricultural waste, municipal solid waste, and industrial waste (Lee, 1997; Saha & Cotta, 2008).

The biological conversion of lignocellulosic biomass to ethanol has four primary steps: (A) pretreatment to increase cellulose accessibility and enzymatic reactivity, (B) enzymatic hydrolysis of carbohydrate polymers to free sugars, (C) fermentation of sugars to ethanol, and (D) ethanol recovery (Rabelo et al., 2009). Several factors inhibit

the hydrolysis of cellulose and hemicellulose to fermentable carbohydrates including high lignin content, presence of acetyl groups on hemicellulose, cellulose crystallinity, degree of cellulose polymerization, and limited surface area (Chang & Holtzapple, 2000; McMillan, 1994; Sun & Cheng, 2002). The goal of biomass pretreatment, which is responsible for a large percentage of the overall process cost, is to minimize these barriers through chemical or mechanical processes (O'Dwyer et al., 2007). Common pretreatment methods include alkali (lime, ammonia fiber expansion, soaking in aqueous ammonia), acid (dilute sulfuric acid, sulfur dioxide) and hot water (Sierra et al., 2008). Alkaline pretreatments are highly effective at removing lignin, which improves enzyme effectiveness by increasing cellulose accessibility and eliminating non-productive adsorption sites (Lee & Fan, 1982). It has also been shown that alkaline pretreatments remove acetyl groups from hemicellulose, which lowers steric hindrances of enzymes and improves carbohydrate digestibility (Kong et al., 1992). Advantages of using lime as the alkaline agent include low cost, compatibility with oxidants, ease of recovery, and ease of use (Holtzapple & Davison, 1999).

This work is part of a collaboration with the Consortium for Applied Fundamentals and Innovation (CAFI). The CAFI team is a devoted group of academic and industry partners who observed an important need for consistent research and reporting of pretreatment studies (Mosier et al., 2005; Wyman et al., 2005b). The feedstocks used by the collaboration were harvested, milled, divided, and then distributed to the individual research laboratories. Common enzymes, washing procedures, analytical methods, and reporting guidelines were used. CAFI I and CAFI II

investigated the effect of different pretreatment methods on corn stover (Wyman et al., 2005a) and poplar wood (Wyman et al., 2009), respectively. This work was performed as part of the CAFI 3 project, which focused on increasing the enzymatic digestibility of multiple varieties of switchgrass (*Panicum virgatum*).

The Department of Energy has chosen switchgrass as a model biomass feedstock because of its adaptability, high yields on marginal lands, draught resistance, low nutrient inputs, and high pest resistance (Schmer et al., 2006; Wright & Turhollow, 2010). It is a native, perennial, warm-season prairie grass that can grow in most of the eastern two-thirds of the United States, as well as Mexico and Canada (McLaughlin et al., 2002). Average yields of 13.4 Mg/(ha·yr) have been achieved (Walsh et al., 2003).

In the past, lime pretreatment of several different feedstocks has been studied including sugarcane bagasse (Chang et al., 1998; Rabelo et al., 2009), corn stover (Kaar & Holtzapple, 2000), and poplar wood (Sierra et al., 2009). The goal of this study was to determine the reaction temperature, time, lime loading, and oxygen pressure that produced the most enzymatically digestible lime-pretreated switchgrass. To determine the most effective treatment conditions, pretreatment yield, carbohydrate yield, and enzymatic yield were considered.

## 2.2 Materials and Methods

### 2.2.1 Substrate and enzymes

The feedstock used in this study was the Dacotah variety of switchgrass (*Panicum virgatum*) kindly provided by Ceres, Inc. This variety was planted on December 6, 1999 in Pierre, SD and harvested on March 1, 2008 after the plot stood

over the winter. The bales were stored indoors until shipped to Hazen Research, Inc. (Golden, CO) where they were ground by a hammer mill equipped with a ¼-in screen. The material was then mixed using the cone-and-quartering technique, separated into 5-kg sub-lots and delivered to the Texas A&M laboratory. The composition determined by Ceres, Inc. was 35.0% glucan, 21.8% xylan, 3.5% arabanin, 21.4% lignin, 2.8% acetyl, and 8.1% extractives.

Cellulase was Spezyme CP® (lot 301-04075-054, 82 mg protein/mL, 59 FPU/mL), kindly provided by Genencor International, Inc®. The β-glucosidase was Novozyme 188® (67 mg protein/mL, 600 CBU/mL) and was obtained from Sigma Aldrich (St. Louis, MO). The protein concentration of each enzyme was measured using TCA precipitation.

### 2.2.2 Pretreatment methods

*Very short-term*

The very-short-tem reactions were conducted in a 304 stainless steel pipe reactor (7-in long, 1.25-in ID; Figure 2-1a). One end of the reactor (Figure 2-1a) was sealed with a temperature gauge, and the other sealed by a 1.25-in stainless steel plate. Three fast-heat conduction bands (Tutco 400 W Better Band, 6 in×2 in) wrapped around the reactor produced the desired reaction temperature. The reactor was attached to a sieve shaker (Combustion Engineering Model RX-86), which provided the shaking action. The reactor was loaded with 8 g dry switchgrass, excess calcium hydroxide (1 g $Ca(OH)_2$/g dry biomass), and water (15 g/g dry biomass). Constant-pressure pure oxygen was supplied through ¼-in stainless steel tubing from an oxygen cylinder.

Reaction time did not include the initial heat-up time, which was typically about 5 min. After the desired reaction time, the heating elements and oxygen supply were turned off, the reactor was cooled by blowing compressed air over the exterior, and the reactor contents were transferred to a 1-L plastic centrifuge bottle. The post-pretreatment conditioning procedure was then performed on the resulting slurry.

*Short-term*

Short-term lime pretreatment was conducted in a pair of 304 stainless steel pipe reactors (5-in long, 1.5-in ID) with 1.5-in 304 stainless steel caps (Figure 2-1c). The reactors were sealed using Teflon tape. Reactors were loaded with 8 g dry switchgrass each and excess calcium hydroxide (1 g $Ca(OH)_2$/g dry biomass) and water (15 g/g dry biomass). Constant-pressure pure oxygen was supplied to a manifold through a flexible stainless steel hose attached to an oxygen tank. The reactors were connected to a swing arm to provide constant stirring and placed in a preheated temperature-controlled oven set at the desired reaction temperature. Initial heat-up time of the reaction contents was included in the overall reaction time. Upon completing the desired reaction time, reactors were removed from the oven and immediately placed in an ice bath to quench the reaction. Once cooled, the reactors were opened slowly to relieve pressure, and the contents were transferred to a 1-L plastic centrifuge bottle using distilled water. The reaction contents underwent the post-pretreatment conditioning procedure.

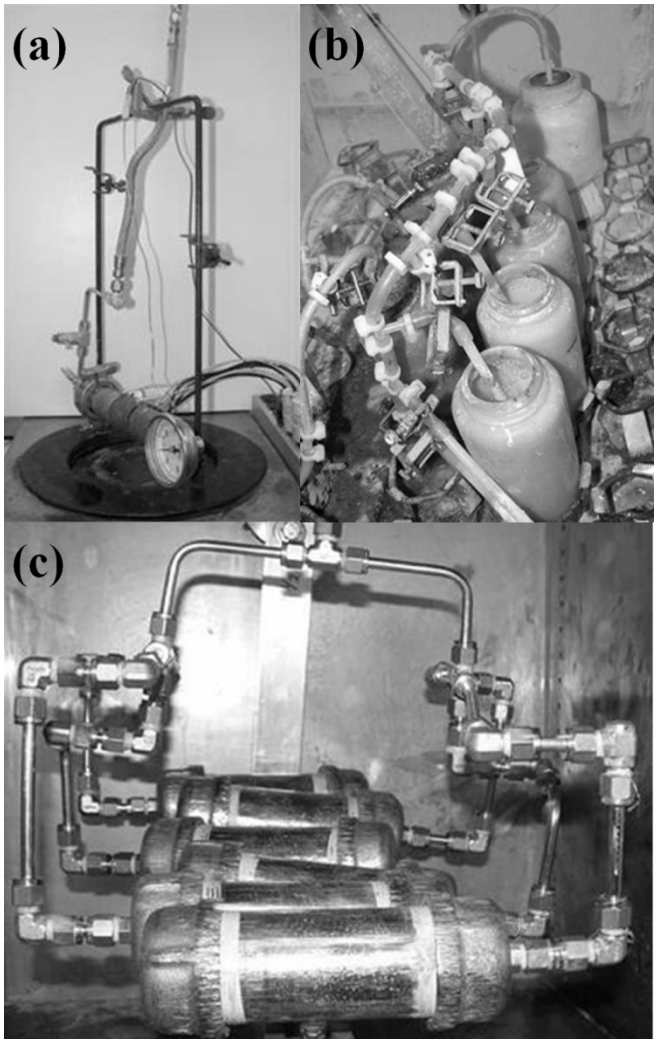


**Figure 2-1.** Pretreatment reactors. (a) very-short-term pretreatment reactor, (b) long-term pretreatment reactor, (c) short-term pretreatment reactor.

*Long-term*

Long-term pretreatment was conducted in plastic 450-mL bottles (Figure 2-1b). The bottles were loaded with 16 g dry switchgrass each and excess calcium hydroxide (1 g $Ca(OH)_2$/g dry biomass). Water was added at a ratio of 15 g/g dry biomass. Compressed air was supplied through a manifold and bubbled into each bottle at 1.01 bar pressure. The bottles were placed in a temperature-controlled oven set at 65ºC. Stirring

was performed manually twice per day using stainless steel spatulas. The water level of each bottle was checked regularly and additional water was added when necessary. Reaction time was 1, 2, 7, 14, and 28 days, after which the post-pretreatment conditioning procedure was performed.

*Post-pretreatment conditioning*

The lime-treated biomass slurry was neutralized using 5-N HCl to a pH of approximately 4.0 to solubilize any residual lime, and then underwent several washings with distilled water until the pH of the slurry rose to approximately 6.0. The final slurry was vacuum filtered and the filtrate was collected for carbohydrate analysis. Moisture content and final solid weight were recorded to obtain pretreatment yield and the solids were stored in the freezer until compositional analysis and enzymatic hydrolysis were performed.

### 2.2.3 Lime consumption

As part of the post-pretreatment conditioning, the lime-treated biomass slurry was neutralized using 5-N HCl. The volume of 5-N HCl required to titrate the solution to an end point of pH 7.0 was recorded and used to calculate the amount of un-reacted excess lime present in the pretreatment slurry. Using this value and the known initial quantity of lime, the amount of lime consumed was calculated.

### 2.2.4 Compositional analysis

Compositional analysis was performed on the raw and pretreated samples. The material was prepared by air drying to a measured moisture content of less than 10%. The composition was analyzed using an NREL acid hydrolysis procedure (Sluiter et al.,

2008b). The sample (0.3 g) was weighed into a glass test tube followed by adding 3 mL of 72 wt % sulfuric acid. The test tubes were placed in a 30 °C water bath and stirred regularly for 1 h. The contents of the test tube were quantitatively transferred to glass autoclave bottles using 84 mL distilled water, capped, sealed, and steam autoclaved for 1 h. Samples were cooled, opened, and filtered through glass filtering crucibles, which were placed in a 105 °C oven to dry. The filtrate was neutralized and then analyzed for carbohydrates using HPLC Analysis (Bio-Rad Aminex HPX-87P column, HPLC-grade water mobile phase, 0.6 mL/min, 80 °C column temperature). The weight of the dried, filtered solids minus their ash weight was recorded and used to calculate lignin content. Ash content was determined by heating samples in a 575 °C furnace until completion. The extractives were determined by extracting the biomass with 95% ethanol for 24 h in a Soxhlet apparatus. The measured compositions for both the raw and pretreated materials were used in the enzymatic hydrolysis loading calculations.

### 2.2.5 Sugar analysis in the pretreatment liquor

Prior to neutralizing the lime-treated biomass slurry, a 10-mL aliquot of pretreatment liquor was obtained using vacuum filtration. The monomeric sugar content of the pretreatment liquor was quantified using HPLC analysis (Bio-Rad Aminex HPX-87P column, HPLC-grade water mobile phase, 0.6 mL/min, 80 °C column temperature).

The oligomeric sugar content of the pretreatment liquor was quantified by subjecting the pretreatment liquor to acid hydrolysis with 4% sulfuric acid using an autoclave at 121 °C for 1 h. HPLC analysis was used to measure the glucose and xylose

concentrations of each sample, which were then recalculated as equivalent glucan and xylan concentrations.

### 2.2.6 Enzymatic hydrolysis

The enzymatic hydrolysis procedure for both glucan and xylan closely followed the enzymatic saccharification procedure provided by NREL (Selig et al., 2008). Hydrolysis samples were prepared in 50-mL plastic centrifuge tubes. Pretreated biomass loading weight was calculated based on moisture content and glucan composition to yield 0.1 g glucan per sample. Sodium citrate buffer (5 mL, 0.1-M, pH 4.8), 0.04 mL tetracycline (10 mg/mL in 70% ethanol), 0.04 mL cycloheximine (10 mg/mL in distilled water), 1 mL of each enzyme dilution (cellulase, β-glucosidase), and an appropriate volume of water were added to bring the total working volume to 10 mL. The enzyme dilutions were calculated on a raw glucan basis using the enzyme activity and a desired enzyme loading. The cellulase enzyme loading was 15 FPU/g raw glucan, and β-glucosidase was loaded in excess at a loading of 60 CBU/g raw glucan. Hydrolysis occurred in a shaking incubator (100 rpm) at 50 °C for 72 h. To quench the hydrolysis, the samples were placed in a 105 °C oven for 5 minutes and then cooled in an ice bath. Samples were stored in a freezer until HPLC analysis. HPLC analysis (Bio-Rad Aminex HPX-87P column, HPLC-grade water mobile phase, 0.6 mL/min, 80 °C column temperature) was used to measure the glucose and xylose concentrations of each sample. These concentrations were then recalculated as equivalent glucan and xylan concentrations to report digestibility yields.

### 2.2.7 Experimental design

The goal of this work was to determine the set of pretreatment conditions (reaction time, lime loading, temperature, $O_2$ pressure) that resulted in the most digestible switchgrass. Table 2-1 shows the full list of conditions. The very-short-term reactions involved a full-factorial experimental design of five temperatures (150, 160, 170, 190, and 200ºC), six reaction times (5, 10, 15, 20, 25, and 30 min), and two $O_2$ pressures (3.45 and 6.89 bar absolute $O_2$). The short-term reactions involved five temperatures (100, 110, 120, 140, and 150ºC), four reaction times (60, 120, 180, and 240 min), and two $O_2$ pressures (3.45 and 6.89 bar absolute $O_2$). The long-term reactions were conducted at 65 °C and 1.01 bar pressure for 1, 2, 7, 14, and 28 days. Lime consumption, pretreatment yields, and overall enzymatic yields were measured. Overall enzymatic yields were obtained using a 72-h enzymatic hydrolysis with a cellulase loading of 15 FPU/g glucan in raw biomass and an excess β-glucosidase loading of 60 CBU/g glucan in raw biomass.

**Table 2-1.** List of pretreatment conditions.

| | Time | Pressure | Temperature |
|---|---|---|---|
| Very-short term | 5, 10, 15, 20, 25, 30 min | 3.45, 6.89 bar $O_2$ | 150, 160, 170, 180, 200 °C |
| Short term | 60, 120, 180, 240 min | 3.45, 6.89 bar $O_2$ | 100, 110, 120, 140, 150 °C |
| Long term | 1, 2, 7, 14, 28 days | 1 .01 bar, bubbling air | 65 °C |

## 2.3 Results and Discussion

### 2.3.1 Lime consumption

Figure 2-2 shows a weak linear correlation between lignin removal and lime consumption for the short-term pretreatment conditions ($R^2$=0.39); however, no correlation was observed for the very-short-term pretreatment conditions ($R^2$=0.01). Lime consumption ranged from 0.07 to 0.42 g lime consumed/g raw biomass (Table 2-2). At the recommended pretreatment condition (120 °C, 6.89 bar $O_2$, 240 min), lime consumption was 0.30 g lime consumed/g raw biomass.

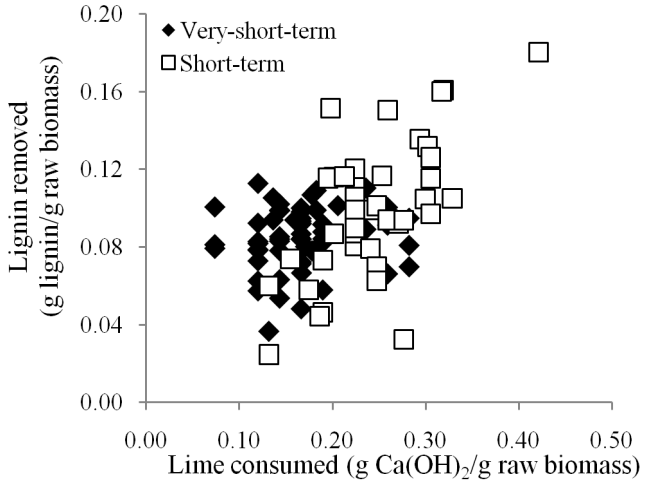


**Figure 2-2.** Relationship between lime consumption and lignin removal for the very-short-term and short-term pretreatment conditions.

### 2.3.2 Sugars recovered from pretreatment liquor

Analysis of the pretreatment liquor revealed the absence of monomeric glucose and xylose; however, small concentrations of glucan oligomers and more substantial concentrations of xylan oligomers were present. Table 2-3 shows the amount of glucan and xylan recovered in the pretreatment liquor for several representative pretreatment

conditions. From the four conditions examined, the highest glucan recovery (g glucan recovered/100 g glucan in raw biomass) was 9.75 (200 °C, 6.89 bar $O_2$, 5 min). Xylan recovery (g xylan recovered/100 g xylan in raw biomass) was significant in three of the four samples: 19.58 (200 °C, 6.89 bar $O_2$, 5 min), 21.76 (110 °C, 6.89 bar $O_2$, 240 min), and 25.20 (120 °C, 6.89 bar $O_2$, 240 min). The high amounts of xylan recovered in the pretreatment liquor compensate for the lower xylan yields shown in the pretreatment yields.

**Table 2-2.** Lime consumption (g $Ca(OH)_2$/g raw biomass) of very-short and short-term lime pretreatments.

| | 3.45 bar $O_2$ | | | | | 6.89 bar $O_2$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Very-short term | 150 °C | 160 °C | 170 °C | 180 °C | 200 °C | 150 °C | 160 °C | 170 °C | 180 °C | 200 °C |
| 5 min | 0.13 | 0.19 | 0.17 | 0.28 | 0.14 | 0.21 | 0.07 | 0.18 | 0.14 | 0.14 |
| 10 min | 0.17 | 0.17 | 0.12 | 0.17 | 0.12 | 0.19 | 0.14 | 0.17 | 0.16 | 0.17 |
| 15 min | 0.12 | 0.14 | 0.12 | 0.19 | 0.07 | 0.26 | 0.17 | 0.17 | 0.18 | 0.19 |
| 20 min | 0.12 | 0.12 | 0.17 | 0.17 | 0.24 | 0.14 | 0.14 | 0.17 | 0.14 | 0.18 |
| 25 min | 0.12 | 0.14 | 0.19 | 0.19 | 0.28 | 0.14 | 0.28 | 0.17 | 0.18 | 0.26 |
| 30 min | 0.12 | 0.07 | 0.26 | 0.17 | 0.17 | 0.12 | 0.19 | 0.14 | 0.17 | 0.24 |
| Short term | 100 °C | 110 °C | 120 °C | 140 °C | 150 °C | 100 °C | 110 °C | 120 °C | 140 °C | 150 °C |
| 60 min | 0.28 | 0.19 | 0.19 | 0.27 | 0.22 | 0.13 | 0.25 | 0.25 | 0.19 | 0.28 |
| 120 min | 0.20 | 0.13 | 0.22 | 0.22 | 0.30 | 0.17 | 0.31 | 0.26 | 0.31 | 0.26 |
| 180 min | 0.24 | 0.15 | 0.20 | 0.22 | 0.33 | 0.24 | 0.22 | 0.21 | 0.32 | 0.20 |
| 240 min | 0.22 | 0.31 | 0.22 | 0.20 | 0.25 | 0.25 | 0.29 | 0.30 | 0.42 | 0.32 |

**Table 2-3.** Sugars recovered from pretreatment liquor.

| Pretreatment conditions | | | Sugars Recovered* | |
|---|---|---|---|---|
| Temperature (°C) | Pressure (bar $O_2$) | Time (min) | Glucan | Xylan |
| 110 | 6.89 | 60 | 2.24 | 4.24 |
| 110 | 6.89 | 240 | 3.42 | 21.76 |
| 120 | 6.89 | 240 | 1.80 | 25.20 |
| 200 | 6.89 | 5 | 9.75 | 19.58 |

*g component recovered/100 g component in raw biomass.

### 2.3.2 Pretreatment yields

When comparing the effectiveness of each pretreatment condition, it is important to consider degradation of three main components present in the biomass (glucan, xylan, and lignin). Pretreatment yields of the solid material were calculated using the following definition:

$$Y_i = \frac{C_i\, Y_t}{C_{i0}} \qquad [2\text{-}1]$$

where

$i$ = component (lignin L, glucan G, xylan X)

$Y_i$ = pretreatment yield of Component $i$ at time $t$ (g residual Component $i$/g Component $i$ in raw biomass)

$C_{i0}$ = Component $i$ content at time zero (g Component $i$ in raw biomass/g raw biomass)

$C_i$ = Component $i$ in time $t$ (g residual Component $i$/g residual biomass)

$Y_t$ = total solids pretreatment yield at time $t$ (g residual biomass/g raw biomass).

The primary goal of lime pretreatment is to achieve low lignin pretreatment yields (i.e., high lignin removal) while maintaining high glucan and xylan pretreatment yields.

*Very-short-term*

The very-short-term pretreatments (Figure 2-3) resulted in very low glucan

degradation. The glucan pretreatment yields (g glucan recovered/100 g glucan in raw biomass) were typically greater than 80. For the 3.45-bar $O_2$ samples, the highest glucan pretreatment yields were 99.2 (5 min, 200 °C), 99.1 (5 min, 150 °C), and 98.0 (5 min, 180 °C). The lowest glucan pretreatment yields were 78.5 (30 min, 160 °C) and 79.9 (25 min, 160 °C), with the remaining glucan pretreatment yields greater than 80. Increased pressure (6.89 bar $O_2$) lowered glucan pretreatment yields with the maximum glucan pretreatment yields being 99.7 (5 min, 180 °C) and 96 (10 min, 180 °C). The remaining glucan pretreatment yields ranged from 80–95.

Xylan pretreatment yields (g xylan recovered/100 g xylan in raw biomass) showed significantly more degradation than glucan. For the low-pressure case (3.45-bar $O_2$), xylan pretreatment yields were as high as 77.6 (5 min, 150 °C), and as low as 49.2 (30 min, 200 °C). The majority of the samples showed a xylan pretreatment yield between 65–75. The highest xylan yields at 6.89-bar $O_2$ were 73.8 (10 min, 160 °C), 72.7 (15 min, 150 °C and 5 min, 160 °C), and 72.6 (5 min, 150 °C). Xylan pretreatment yields were as low as 52.1 (30 min, 200 °C) and 52.7 (5 min, 170 °C); however, the majority were 60–70.

Lignin pretreatment yields (g lignin recovered/100 g lignin in raw biomass) of the 3.45-bar $O_2$ samples were inconsistent. Although the goal of lime pretreatment is to significantly reduce lignin content, in many cases xylan degradation was more significant than lignin degradation. Lignin pretreatment yields ranged from 84.0 (5 min, 150 °C) to 55.4 (25 min, 160 °C). However, the 6.89-bar $O_2$ samples consistently showed lower lignin pretreatment yields than either xylan or glucan pretreatment yields.

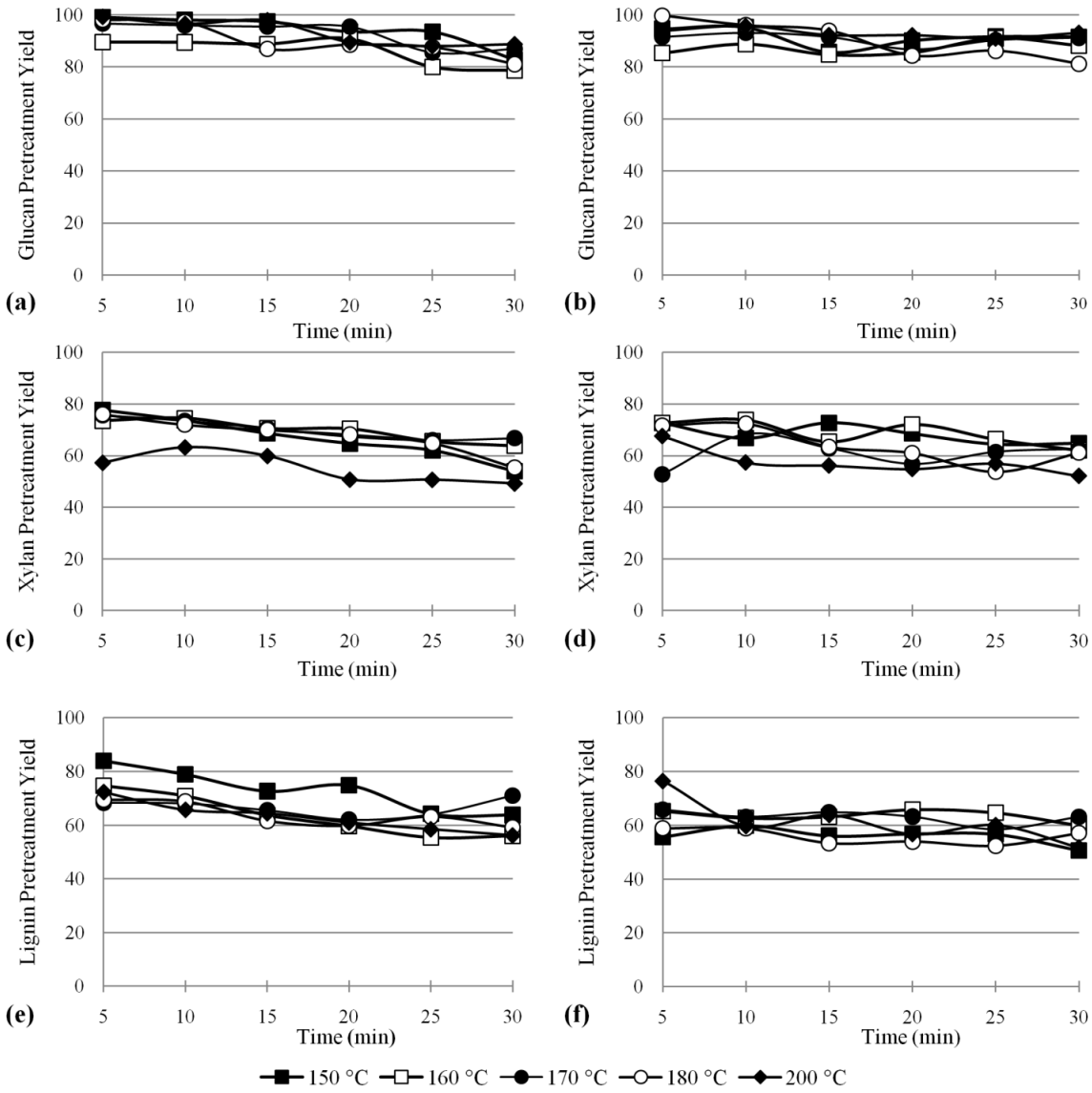


**Figure 2-3.** Very-short-term pretreatment yields. (a) glucan 3.45 bar $O_2$, (b) glucan, 6.89 bar $O_2$, (c) xylan, 3.45 bar $O_2$, (d) xylan, 6.89 bar $O_2$, (e) lignin, 3.45 bar $O_2$, (f) lignin, 6.89 bar $O_2$. [Note: All pretreatment yields are expressed as g component recovered/100 g component in raw biomass.]

Lignin pretreatment yields were observed as low as 50.7 (30 min, 150 °C) and 51.8 (30 min, 200 °C).

For the very-short-term pretreatments, the average glucan pretreatment yields were 91.2 (3.45-bar $O_2$) and 90.5 (6.89-bar $O_2$). Xylan showed a little more degradation with average pretreatment yields of 65.9 (3.45-bar $O_2$) and 63.9 (6.89-bar $O_2$). Average lignin pretreatment yields were 66.0 (3.45-bar $O_2$) and 60.0 (6.89-bar $O_2$), which is similar to xylan.

*Short-term*

Overall, the short-term pretreatments were more successful in selectively degrading lignin while maintaining high glucan and moderate xylan pretreatment yields (Figure 2-4). Glucan pretreatment yields were typically greater than 80, with certain conditions maintaining glucan pretreatment yields of almost 100. For the 3.45-bar $O_2$ case, glucan pretreatment yields were 98.4 (60 min, 120 °C and 60 min, 140 °C) and 98.2 (60 min, 100 °C). With increased reaction time, glucan pretreatment yields fell as low as 74.5 (240 min, 150 °C) and 81.1 (240 min, 140 °C). At 6.89-bar $O_2$, almost all of the glucan (>99) was conserved for the 60-min samples at 100, 120, and 140 °C. Again, with increased reaction time, glucan recovery decreased with pretreatment yields as low as 69.8 (240 min, 150 °C) and 79.8 (240 min, 110 °C).

At 3.45-bar $O_2$, the maximum xylan pretreatment yields were 94.8 (60 min, 100 °C) and 86.6 (60 min, 120 °C). The 150 °C samples showed the lowest xylan pretreatment yields of 57.8 (180 min) and 53.3 (240 min). Compared to the 3.45-bar $O_2$

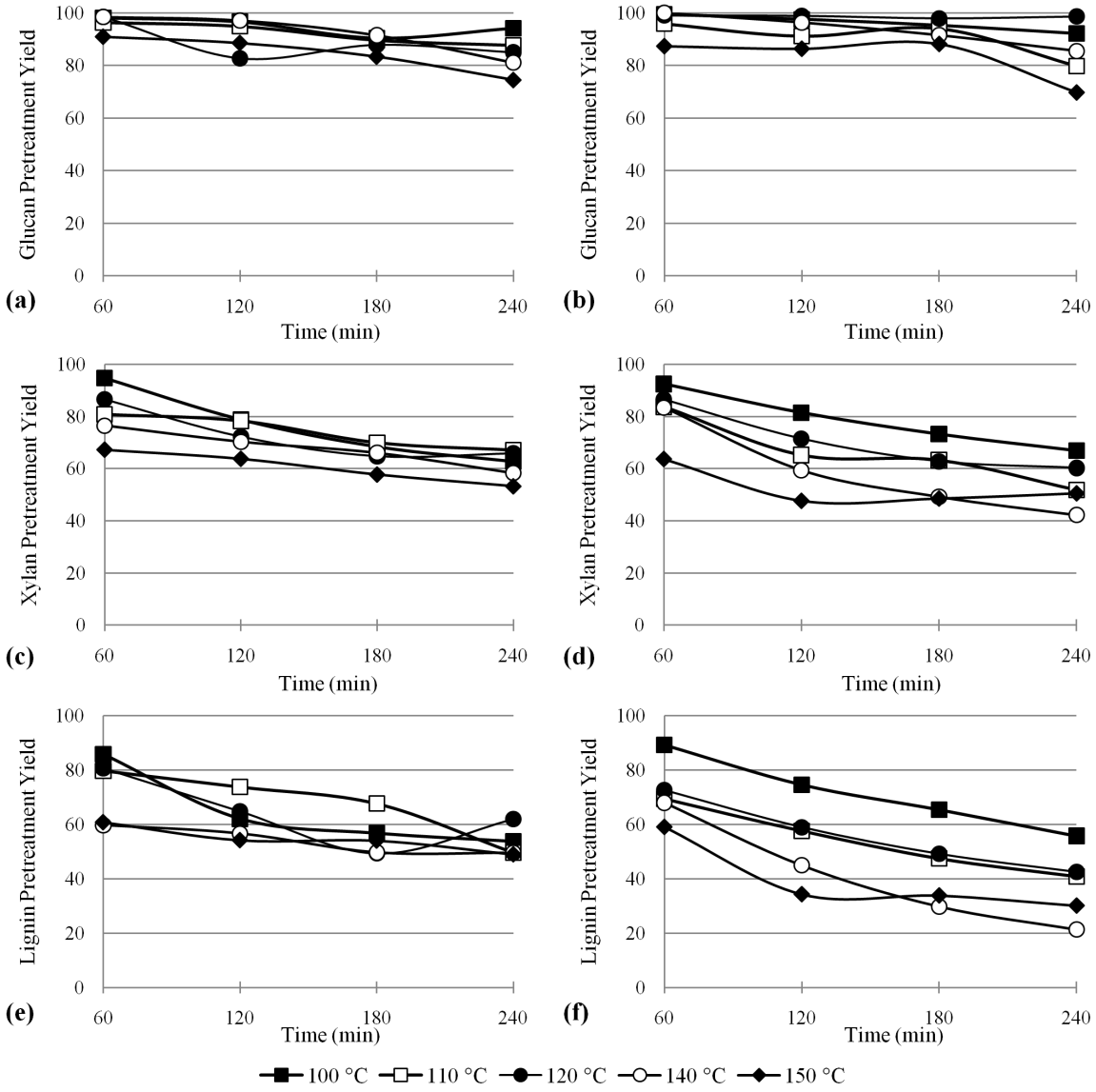


**Figure 2-4.** Short-term pretreatment yields. (a) glucan 3.45 bar $O_2$, (b) glucan, 6.89 bar $O_2$, (c) xylan, 3.45 bar $O_2$, (d) xylan, 6.89 bar $O_2$, (e) lignin, 3.45 bar $O_2$, (f) lignin, 6.89 bar $O_2$. [Note: All pretreatment yields are expressed as g component recovered/100 g component in raw biomass.]

samples, the 6.89-bar $O_2$ samples showed slightly more xylan degradation. The highest xylan pretreatment yields observed were 92.5 (60 min, 100 °C) and 86.7 (60 min, 120 °C), with the lowest being 42.3 (240 min, 140 °C), 47.7 (120 min, 150 °C), and 48.6 (180 min, 150 °C).

The short-term lime pretreatments showed significantly greater lignin degradation than either glucan or xylan degradation. At the lower pressure (3.45-bar $O_2$), lignin pretreatment yields ranged from 85.8 (60 min, 100 °C) to as low as 49.0 (240 min, 150 °C), with the majority in the range of 50–70. Increasing the pressure to 6.89-bar $O_2$ strongly improved the degree of lignin degradation. Lignin pretreatment yields were 21.3 (240 min, 140 °C), 29.8 (180 min, 140 °C), 30.1 (240 min, 150 °C), and 33.9 (180 min, 150 °C). Only a single sample (60 min, 100 °C) showed very slight lignin degradation with a lignin pretreatment yield of 89.2.

For the short-term pretreatments, glucan pretreatment yields decreased with increased severity of conditions (increasing temperature or time). Glucan was typically conserved with average pretreatment yields of 90.4 (3.45-bar $O_2$) and 92.2 (6.45-bar $O_2$). Xylan degradation was slightly more severe with average pretreatment yields of 70.2 (3.45-bar $O_2$) and 65.2 (6.45-bar $O_2$). Lignin degradation was the most severe with average lignin pretreatment yields of 61.0 (3.45-bar $O_2$) and 52.3 (6.45-bar $O_2$). From these averages, it is clear that increasing oxygen pressure significantly improves lignin degradation, with the negative side effect of also removing additional xylan. The data also demonstrate that increasing the severity of conditions (increasing temperature or time) helps improve lignin degradation with only a slight increase in glucan degradation.

*Long-term*

The long-term pretreatment samples all maintained high glucan pretreatment yields (>95). Xylan pretreatment yields were lower and decreased with time. The 1-day pretreatment had a xylan pretreatment yield of 84.5, which decreased to 66.1 for the 28-day pretreatment. Lignin degradation was promising with lignin pretreatment yields starting at 72.9 (1 and 2 days), decreasing to 58.0 after 7 days, and reaching a minimum of 55.0 after 28 days. Table 2-4 shows the complete set of results.

**Table 2-4.** Long-term pretreatment yields.

| Reaction Time (days) | Pretreatment Yields* 65°, Air | | |
|---|---|---|---|
| | Glucan | Xylan | Lignin |
| 1 | 99.9 | 84.5 | 72.9 |
| 2 | 97.3 | 86.8 | 72.9 |
| 7 | 95.9 | 72.6 | 58.0 |
| 14 | 97.0 | 72.1 | 56.8 |
| 28 | 96.3 | 66.1 | 55.0 |

*g component recovered/100 g component in raw biomass

### 2.3.4 Enzymatic yields

The primary goal of this study was to determine the set of pretreatment conditions (reaction time, lime loading, temperature, and pressure) that resulted in the most digestible switchgrass. This study used a 72-h enzymatic hydrolysis with a cellulase loading of 15 FPU/g glucan in raw biomass and an excess loading of β-glucosidase (60 CBU/g glucan in raw biomass). The primary factor in choosing the best-performing pretreatment condition was overall yield of glucan and xylan. Overall yield

($Y_{oi}$) is defined as the amount of glucan or xylan enzymatically hydrolyzed after pretreatment per unit of glucan or xylan in the raw feedstock.

$$Y_{oi} = Y_i \times Y_{ei} \quad [2\text{-}2]$$

where

$i$ = component (glucan G or xylan X)

$Y_{oi}$ = overall yield of Component $i$ (g hydrolyzed Component $i$/g Component $i$ in raw biomass)

$Y_i$ = pretreatment yield of Component $i$ (g residual Component $i$/g Component $i$ in raw biomass)

$Y_{ei}$ = enzymatic yield of Component $i$ (g hydrolyzed Component $i$/g Component $i$ in pretreated biomass).

*Very-short-term*

Overall, the very-short-term pretreatments did not effectively increase glucan overall yield. Results (Figure 2-5) were inconsistent making it difficult to derive any meaningful conclusions from the data.

At 3.45-bar $O_2$, overall glucan yields (g glucan hydrolyzed/100 g glucan in raw biomass) ranged from 26.9–45.0. In general, the most successful temperature was 160 °C, with overall glucan yields of 38.4, 44.8, 44.5, 41.0, and 40.7 for reaction times of 5, 10, 15, 20, 25, and 30 min, respectively. It is apparent that although lignin degradation increases with reaction time, overall pretreatment yield decreases; therefore, there is a delicate balance between reaction time and overall glucan yield. Overall xylan yields (g

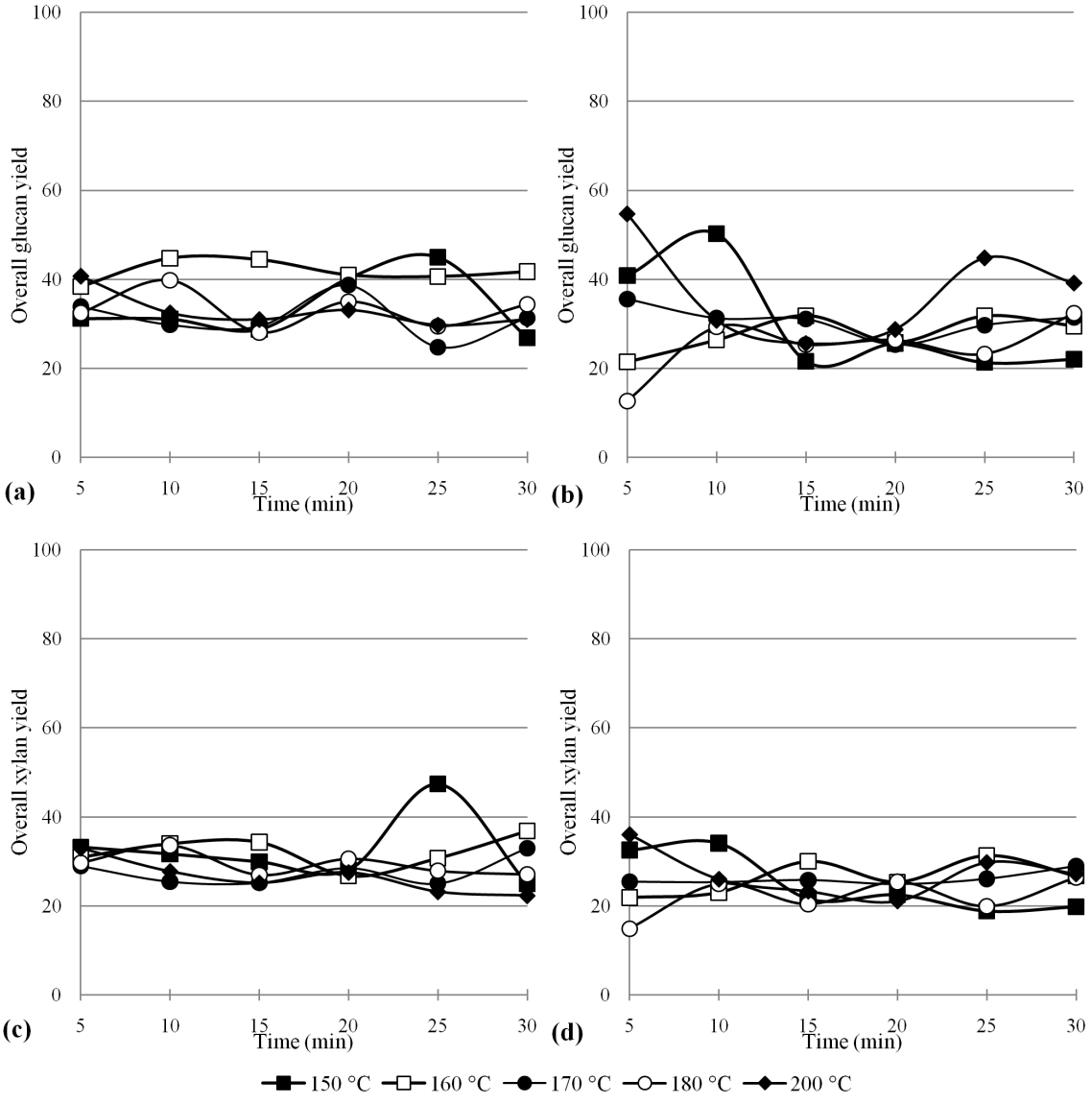


**Figure 2-5.** Overall enzymatic yield results for very-short-term pretreatments. Enzymatic hydrolysis was performed for 72 h with a cellulase enzyme loading of 15 FPU/g glucan in raw biomass. (a) overall glucan yield, 3.45 bar $O_2$, (b) overall glucan yield, 6.89 bar $O_2$, (c) overall xylan yield, 3.45 bar $O_2$, (d) overall xylan yield, 6.89 bar $O_2$. [Note: All overall enzymatic yields are expressed as g component hydrolyzed/100 g raw component.]

xylan hydrolyzed/100 g xylan in raw biomass) were also inconsistent, and were between 22.3 (30 min, 200 °C) and 47.4 (25 min, 150 °C). In most cases, overall xylan yield had similar trends as overall glucan yield.

In the very-short-term reactor, pretreating the switchgrass at 6.89-bar $O_2$ proved slightly more successful, although still inconsistent. Overall glucan yields of 54.7 (5 min, 200 °C) and 50.2 (10 min, 150 °C) were achieved with the highest overall glucan yields obtained at short reaction times. Some pretreatments were highly unsuccessful, with overall glucan yields as low as 12.7 (5 min, 180 °C) and several others below 25 (15 min, 150 °C; 25 min, 150 °C; 30 min, 150 °C; 5 min, 160 °C; 25 min, 180 °C). Although a few high-pressure samples showed improved overall yields compared to the low-pressure samples, most of the high-pressure samples did considerably worse. Overall xylan yields were also quite low, with values ranging from 14.9 (5 min, 180 °C) to 36.0 (5 min, 200 °C).

The average overall glucan yields for the very-short-term reactor were 34.7 (3.45-bar $O_2$) and 30.2 (6.89-bar $O_2$), clearly demonstrating the ineffectiveness of the very-short-term reactor. With the poor performance of the very-short-term reactor, it appears reaction times were too short to obtain a highly digestible substrate.

*Short-term*

Although the very-short-term pretreatment proved unsuccessful at producing highly digestible switchgrass, the short-term pretreatment demonstrated that oxidative lime pretreatment is a promising approach (Figure 2-6).

At the lower pressure (3.45-bar $O_2$), overall glucan yields were moderate and similar to the very-short-term pretreatment. A reaction time of 180 min consistently produced the highest overall glucan yields of 53.9 (100 °C), 49.2 (120 °C), 47.7 (140 °C), and 44.3 (150 °C). Additionally, overall glucan yields improved with time up to 180 min. In all cases except for 100 °C, a reaction time of 240 min led to low pretreatment yields, which negatively affected overall glucan yields. Overall xylan yields were relatively low as well, with a maximum yield of 40.5 (240 min, 140 °C) and a minimum yield of 25.5 (60 min, 150 °C). The majority of the overall xylan yields were 27–37.

In this short-term study, the most promising results occurred at 6.89 bar $O_2$. At 100 °C, overall glucan yields were low. The 60-min sample had an overall glucan yield of 24.0, which improved to 43.9 for the 180- and 240-min samples. Increasing the temperature to 110 °C resulted in overall glucan yields of 43.9 (60 min) to 73.9 (240 min). The most successful temperature of the study was 120 °C, with overall glucan yields of 45.2 (60 min), 62.1 (120 min), 78.5 (180 min), and 85.2 (240 min). The overall glucan yield of 85.2 (6.89-bar $O_2$, 120 °C, 240 min) was the highest yield observed in this study; therefore, this set of conditions was chosen as the recommended oxidative lime pretreatment condition for switchgrass. Increased temperatures (140 °C and 150 °C) had low pretreatment yields, which decreased overall glucan yields. For these temperatures, overall glucan yields were 29.5 (60 min, 140 °C) to 78.8 (120 min, 150 °C). Overall xylan yields also improved at the higher pressure, with several samples having overall xylan yields greater than 40. The recommended pretreatment condition

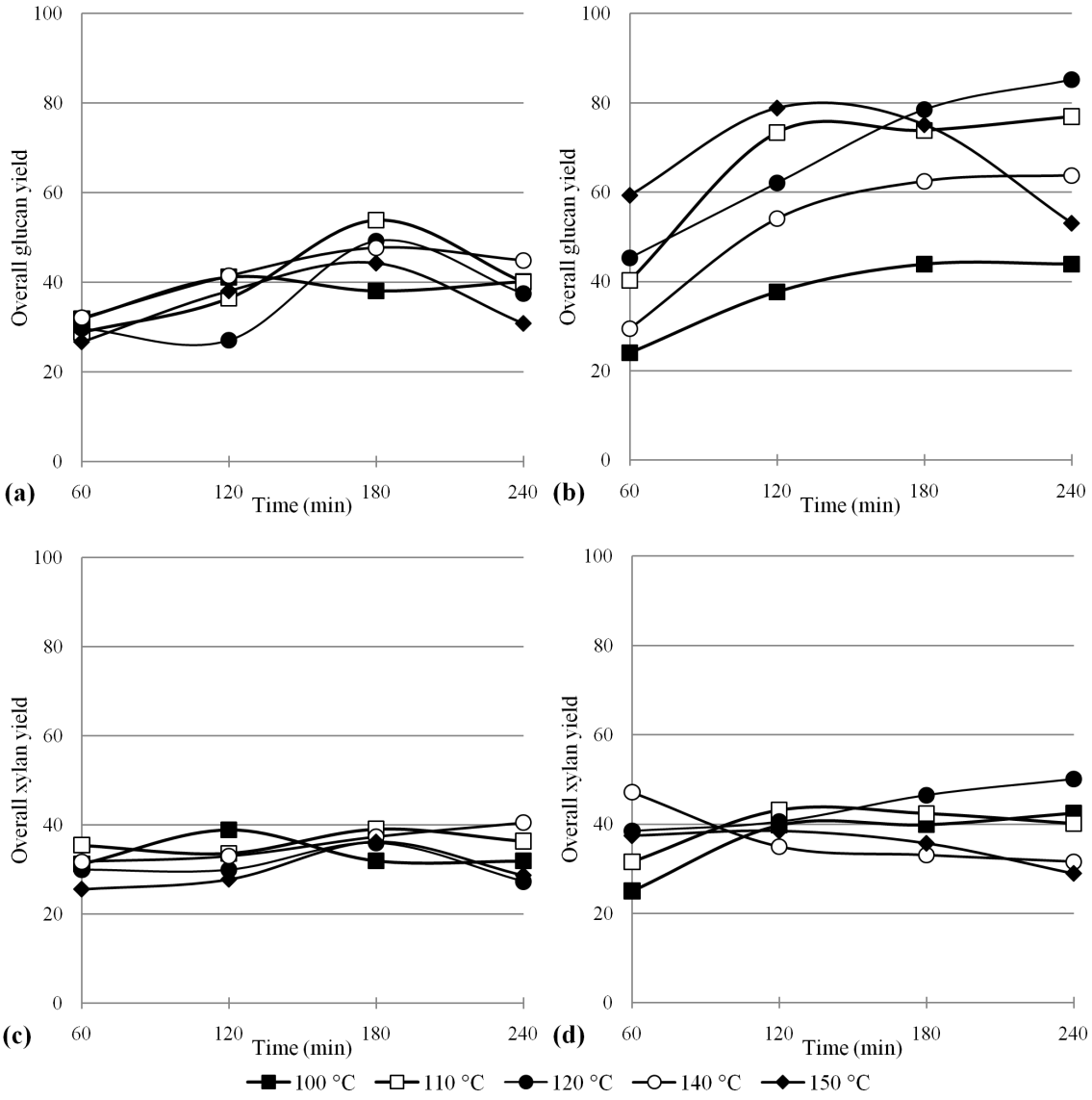


**Figure 2-6.** Overall enzymatic yield results for short-term pretreatments. Enzymatic hydrolysis was performed for 72 h with a cellulase enzyme loading of 15 FPU/g glucan in raw biomass. (a) overall glucan yield, 3.45 bar O2, (b) overall glucan yield, 6.89 bar O2, (c) overall xylan yield, 3.45 bar O2, (d) overall xylan yield, 6.89 bar O2. [Note: All overall enzymatic yields are expressed as g component hydrolyzed/100 g raw component.]

(6.89-bar $O_2$, 120 °C, 240 min) had an overall xylan yield of 50.1, which was also the highest observed.

Particularly at the higher pressure, the average overall glucan yields of the short-term reactor were clearly better than the very-short-term reactor (38.0, 3.45-bar $O_2$ and 58.0, 6.89-bar $O_2$). Average overall xylan yields of the short-term reactor (38.3, 6.89-bar $O_2$) also showed significant improvement over the very-short-term reactor (25.3, 6.89-bar $O_2$).

*Long-term*

The long-term lime pretreatment had similar trends as the shorter pretreatments (Table 2-5). With increased time, overall glucan yield increased from 30.1 (1 day) to 63.9 (14 days). At 28 days, overall glucan yield decreased to 54.5, showing the importance of maintaining a high pretreatment yield. Overall xylan yields showed the same trend, increasing from 29.0 (1 day) to 44.4 (14 days), before decreasing to 37.1 (28 days).

**Table 2-5.** Long-term enzymatic overall yields.

| Reaction Time (days) | Enzymatic Overall Yield* 15 FPU/g raw glucan | |
|---|---|---|
| | Glucan | Xylan |
| 1 | 30.1 | 29.0 |
| 2 | 38.7 | 30.2 |
| 7 | 53.5 | 31.6 |
| 14 | 63.9 | 44.4 |
| 28 | 54.5 | 37.1 |

72 hour hydrolysis, *g component digested/100 g raw component

## 2.4 Conclusions

For Dacotah switchgrass, the recommended oxidative lime pretreatment conditions are 120 °C, 6.89-bar $O_2$, and 240 min. At these conditions, lime consumption was 0.30 g $Ca(OH)_2$/g raw biomass, overall glucan yield was 85.2 g glucan digested/100 g glucan in raw biomass, and overall xylan yield was 50.1 g xylan digested/100 g xylan in raw biomass. Also, significant xylan oligomers (25.20 g xylan recovered/100 g xylan in raw biomass) were recovered in the pretreatment liquor. In general, the short-term reactions performed at 6.89-bar $O_2$ were the only successful results. The long-term reactor achieved moderate results, whereas the very-short-term reactor was not productive.

# CHAPTER III

## OXIDATIVE LIME PRETREATMENT OF ALAMO SWITCHGRASS*

Previous studies have shown that oxidative lime pretreatment is an effective delignification method that improves the enzymatic digestibility of many biomass feedstocks. The purpose of this work is to determine the recommended oxidative lime pretreatment conditions (reaction temperature, time, pressure, and lime loading) for Alamo switchgrass (*Panicum virgatum*). Enzymatic hydrolysis of glucan and xylan was used to determine the performance of the 52 studied pretreatment conditions. The recommended condition (110 °C, 6.89-bar $O_2$, 240 min, 0.248 g $Ca(OH)_2$/g biomass) achieved glucan and xylan overall yields (g sugar hydrolyzed/100 g sugar in raw biomass, 15 FPU/g raw glucan) of 85.9 and 52.2, respectively. In addition, some glucan oligomers (2.6 g glucan recovered/100 g glucan in raw biomass) and significant levels of xylan oligomers (26.0 g xylan recovered/100 g xylan in raw biomass) were recovered from the pretreatment liquor. Combining a decrystallization technique (ball-milling) with oxidative lime pretreatment further improved the overall glucan yield to 90.0 (7 FPU/g raw glucan).

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## 3.1 Introduction

In a recent technoeconomic analysis of a current enzymatic ethanol process, lignocellulose feedstock and biomass pretreatment were the largest contributors to process costs with estimates of 38% and 19%, respectively (Aden & Foust, 2009). To maximize yields from lignocellulosic feedstocks requires highly effective biomass pretreatments.

Currently, ethanol is derived from food crops (e.g., corn, sugarcane). Rather than using food crops for ethanol production, it is advantageous to use lignocellulosic biomass for the following reasons: (1) more abundant, (2) high yields, (3) large variety, (4) and lower cost (Zaldivar et al., 2001). Sources of lignocellulosic biomass include energy crops, agricultural crop residues, and wastes (e.g., industrial, food, and municipal solids) (Lee, 1997; Saha & Cotta, 2008). The main disadvantage of using lignocellulosic biomass is its inherent resistance to enzymatic hydrolysis.

Lignocellulosic biomass is primarily composed of three components: cellulose, hemicellulose, and lignin. Some barriers that limit lignocellulose digestibility include: high lignin content, cellulose crystallinity, high degree of cellulose polymerization, low accessible surface area, small pore volume, and presence of acetyl groups on hemicellulose (Chang & Holtzapple, 2000; McMillan, 1994; Sun & Cheng, 2002). In a lignocellulose-to-ethanol production process, the role of biomass pretreatment is to remove these barriers to generate more digestible biomass.

Many chemical pretreatments have been employed to increase enzymatic digestion of lignocellulose. Previous studies showed that alkaline pretreatments are

highly effective at removing lignin, which improves enzymatic digestibility by increasing cellulose accessibility (Lee & Fan, 1982). Alkaline pretreatments have also demonstrated the ability to significantly remove acetyl groups from hemicellulose, which lowers steric hindrance of enzymes (Kong et al., 1992). This study employed lime ($Ca(OH)_2$) as the alkaline agent because of its low cost, compatibility with oxidants, ease of recovery, and ease of use (Holtzapple & Davison, 1999). Lime pretreatment has previously been studied for corn stover (Kaar & Holtzapple, 2000; Kim & Holtzapple, 2006; Kim & Holtzapple, 2005; O'Dwyer et al., 2007), bagasse (Chang et al., 1998; Rabelo et al., 2009), and poplar wood (Chang et al., 2001; Sierra et al., 2009; Sierra et al., 2010; Wyman et al., 2009).

When choosing a lignocellulosic feedstock, it is important to consider cost, adaptability, yield, and input requirements. The United States Department of Energy has chosen switchgrass (*Panicum virgatum*), a perennial warm-season prairie grass (McLaughlin et al., 2002), as a model biomass feedstock. Switchgrass is highly adaptable and tolerant to draught and poor soils, which allows it to be grown in high yields on marginal lands (Schmer et al., 2006; Wright & Turhollow, 2010). It requires low nutrient inputs and is highly resistant to pests, minimizing fertilizer, herbicide, and pesticide use. Switchgrass can be grown in most of the eastern two-thirds of the United States, as well as Mexico and Canada. Average yields of 13.4 Mg/(ha·yr) have been achieved (McLaughlin et al., 2002; Walsh et al., 2003).

This work was performed in cooperation with the Consortium for Applied Fundamentals and Innovation (CAFI). In late 1999, the CAFI team was formed to

include leaders in biomass pretreatment and hydrolysis. To compare the effectiveness of leading pretreatment technologies, the members observed a need to develop consistent methods (Mosier et al., 2005; Wyman et al., 2005b). The CAFI team employs common feedstocks, shared enzymes, and identical analytical and reporting methods. CAFI 1 and CAFI 2 studied pretreatment of corn stover (Wyman et al., 2005a) and poplar wood (Wyman et al., 2009). This work was performed as part of CAFI 3, which focuses on increasing the enzymatic digestibility of switchgrass.

The primary goal of this work was to determine the effectiveness of oxidative lime pretreatment on Alamo switchgrass, and to recommend the reaction time, pressure, temperature, and lime loading that produces the most enzymatically digestible switchgrass. This recommended condition was determined by considering pretreatment solid yield, pretreatment carbohydrate yield, and enzymatic yield. Furthermore, to examine the difference between each variety of switchgrass, the recommended treatment condition was compared to that obtained for Dacotah switchgrass in a previous study.

## 3.2 Materials and Methods

### 3.2.1 Substrate and enzymes

The primary feedstock used in this study was the Alamo variety of switchgrass (*Panicum virgatum*). This variety is a southern lowland ecotype with thick stems. It was planted in Ardmore, OK on June 11, 2007 and harvested on November 11, 2007. During the growing season, total fertilizer applications were approximately 100.9 kg of nitrogen/hectare and 50.4 kg of phosphorous/hectare. Five small square bales were harvested and shipped from Ardmore, OK to Haven Research, Inc. (Golden, CO).

The second variety used in this study was Dacotah switchgrass. Dacotah is a northern upland switchgrass with thin stem morphology. It was planted in Pierre, SD on December 6, 1999 and harvested on March 1, 2008 after the plot stood over the winter. During the last growth season, no fertilizer or herbicide was utilized. Three small square bales were harvested and shipped from Pierre, SD to Hazen Research, Inc. (Golden, CO).

Once both varieties arrived at Hazen Research, Inc., the bales were shredded and then milled using a hammer mill equipped with a ¼-in screen. While keeping each variety separate, the combined milled materials were homogenized using the cone-and-quartering technique, separated into 5-kg sub-lots, and delivered to the Texas A&M laboratory.

Both Alamo and Dacotah feedstocks were kindly provided by Ceres, Inc. Their respective measured compositions are reported in Table 3-1.

**Table 3-1.** Composition of raw switchgrass.

| Constituent | Dacotah (% dry wt.) | Alamo (% dry wt.) |
|---|---|---|
| Glucan | 35.0 | 33.2 |
| Xylan | 21.8 | 21.0 |
| Lignin | 21.4 | 17.9 |
| Arabanin | 3.5 | 3.2 |
| Sucrose | 1.5 | 4.0 |
| Acetyl | 2.8 | 2.5 |
| Protein | 1.4 | 5.7 |
| Extractives | 8.1 | 10.2 |
| Ash | 3.3 | 3.7 |
| Total | 98.8 | 101.4 |

Cellulase was Spezyme CP® (lot 301-04075-054, 82 mg protein/mL, 59 FPU/mL), which was kindly provided by Genencor®, a Danisco Division. The β-glucosidase was Novozyme 188® (67 mg protein/mL, 600 CBU/mL) and was obtained from Sigma Aldrich (St. Louis, MO).

### 3.2.2 Pretreatment methods

*Substrate preparation*

Prior to pretreatment, the switchgrass was further milled to pass through 40 (ASTM) mesh and pre-washed in 200 g batches. Each batch was mixed with 2 L of 80–90 °C distilled water and allowed to stand 10–15 minutes. The slurry was vacuum filtered using Whatman No. 41 filter paper. The mixing and filtration was performed three times followed by drying the washed solids in a 45 °C oven.

*Short-term*

Short-term lime pretreatment was conducted in a pair of 304 stainless steel pipe reactors (5-in long, 1.5-in ID) with 1.5-in 304 stainless steel caps. The reactors were sealed using Teflon tape. Reactors were loaded with 8 g dry switchgrass each and excess calcium hydroxide (1 g $Ca(OH)_2$/g dry biomass) and water (15 g/g dry biomass). Constant-pressure pure oxygen was supplied to a manifold through a flexible stainless steel hose attached to an oxygen tank. The reactors were connected to a swing arm to provide constant stirring and placed in a preheated temperature-controlled oven set at the desired reaction temperature. Initial heat-up time of the reaction contents was included in the overall reaction time. Upon completing the desired reaction time, reactors were removed from the oven and immediately placed in an ice bath to quench the reaction.

Once cooled, the reactors were opened slowly to relieve pressure, and the contents were transferred to a 1-L plastic centrifuge bottle using distilled water. The reaction contents underwent the post-pretreatment conditioning procedure.

*Long-term*

Long-term pretreatment was conducted in plastic 450-mL bottles. The bottles were loaded with 16 g dry switchgrass each and excess calcium hydroxide (1 g $Ca(OH)_2$/g dry biomass). Water was added at a ratio of 15 g/g dry biomass. Compressed air was supplied through a manifold and bubbled into each bottle at 1.01 bar pressure. The bottles were placed in a temperature-controlled oven set at the reaction temperature of either 55 °C or 65ºC. Stirring was performed manually twice per day using stainless steel spatulas. The water level of each bottle was checked regularly and additional water was added when necessary. Reaction time was 28 days, after which the post-pretreatment conditioning procedure was performed.

*Post-pretreatment conditioning*

The lime-treated biomass slurry was neutralized using 5-N HCl to a pH of approximately 4.0 to solubilize any residual lime, and then underwent several washings with distilled water until the pH of the slurry rose to approximately 6.0. The final slurry was vacuum filtered and the filtrate was collected for carbohydrate analysis. Moisture content and final solid weight were recorded to obtain pretreatment yield and the solids were stored in the freezer until compositional analysis and enzymatic hydrolysis were performed.

*Ball-milling*

The pretreated solids were thoroughly dried (moisture content < 10%) before ball-milling in a 300-mL porcelain jar loaded with 0.375-in zirconia grinding medium. The grinding medium was loaded to fill 50% of the jar volume (approximately 258 g) and biomass was loaded at a ratio of 43 g grinding medium/g dry biomass. The jars were sealed and placed on rollers rotating at 68 rpm for 3 days.

### 3.2.3 Lime consumption

As part of the post-pretreatment conditioning, the lime-treated biomass slurry was neutralized using 5-N HCl. The volume of 5-N HCl required to titrate the solution to an end point of pH 7.0 was recorded and used to calculate the amount of un-reacted excess lime present in the pretreatment slurry. Using this value and the known initial quantity of lime, the amount of lime consumed was calculated.

### 3.2.4 Compositional analysis

Compositional analysis was performed on the raw and pretreated samples. The material was prepared by air drying to a measured moisture content of less than 10%. The composition was analyzed using an NREL acid hydrolysis procedure (Sluiter et al., 2008b). The sample (0.3 g) was weighed into a glass test tube followed by adding 3 mL of 72 wt % sulfuric acid. The test tubes were placed in a 30 °C water bath and stirred regularly for 1 h. The contents of the test tube were quantitatively transferred to glass autoclave bottles using 84 mL distilled water, capped, sealed, and steam autoclaved at 121 °C for 1 h. Samples were cooled, opened, and filtered through glass filtering crucibles, which were placed in a 105 °C oven to dry. The filtrate was neutralized and

then analyzed for carbohydrates using HPLC Analysis (Bio-Rad Aminex HPX-87P column, HPLC-grade water mobile phase, 0.6 mL/min, 80 °C column temperature). The weight of the dried, filtered solids minus their ash weight was recorded and used to calculate lignin content. Ash content was determined by heating samples in a 575 °C furnace until completion. The extractives were determined by extracting the biomass with 95% ethanol for 24 h in a Soxhlet apparatus. The measured compositions for both the raw and pretreated materials were used in the enzymatic hydrolysis loading calculations.

### 3.2.5 Sugar analysis in the pretreatment liquor

Prior to neutralizing the lime-treated biomass slurry, a 10 mL aliquot of pretreatment liquor was obtained using vacuum filtration. The monomeric sugar content of the pretreatment liquor was quantified using HPLC analysis (Bio-Rad Aminex HPX-87P column, HPLC-grade water mobile phase, 0.6 mL/min, 80 °C column temperature).

The oligomeric sugar content of the pretreatment liquor was quantified by subjecting the pretreatment liquor to acid hydrolysis with 4% sulfuric acid using an autoclave at 121 °C for 1 h. HPLC analysis was used to measure the glucose and xylose concentrations of each sample, which were then recalculated as equivalent glucan and xylan concentrations.

### 3.2.6 Enzymatic hydrolysis

The enzymatic hydrolysis procedure for both glucan and xylan closely followed the enzymatic saccharification procedure provided by NREL (Selig et al., 2008). Hydrolysis samples were prepared in 50-mL plastic centrifuge tubes. Pretreated biomass

loading weight was calculated based on moisture content and glucan composition to yield 0.1 g glucan per sample. Sodium citrate buffer (5 mL, 0.1-M, pH 4.8), 0.04 mL tetracycline (10 mg/mL in 70% ethanol), 0.04 mL cycloheximine (10 mg/mL in distilled water), 1 mL of each enzyme dilution (cellulase, β-glucosidase), and an appropriate volume of water were added to bring the total working volume to 10 mL. The enzyme dilutions were calculated on a raw glucan basis using the enzyme activity and a desired enzyme loading. The cellulase enzyme loading was 15 FPU/g raw glucan, and β-glucosidase was loaded in excess at a loading of 60 CBU/g raw glucan. Hydrolysis occurred in a shaking incubator (100 rpm) at 50 °C for 72 h. To quench the hydrolysis, the samples were placed in a 105 °C oven for 5 minutes and then cooled in an ice bath. Samples were stored in a freezer until HPLC analysis. HPLC analysis (Bio-Rad Aminex HPX-87P column, HPLC-grade water mobile phase, 0.6 mL/min, 80 °C column temperature) was used to measure the glucose and xylose concentrations of each sample. These concentrations were then recalculated as equivalent glucan and xylan concentrations to report digestibility yields.

### 3.2.7 Experimental design

The primary goal of this work was to assess the effectiveness of oxidative lime pretreatment in increasing the enzymatic digestibility of Alamo switchgrass. A total of 52 different pretreatments (Table 3-2) were performed using a full-factorial experimental design of five temperatures (100, 110, 120, 140, and 150 °C), three $O_2$ pressures (3.45, 6.89, and 10.3-bar absolute $O_2$), and four reaction times (60, 120, 180, and 240 minutes). Because of the severe conditions, the high-pressure pretreatments (10.3-bar $O_2$) were

only run at 100, 110, and 120 °C. The recommended pretreatment condition (reaction time, lime loading, temperature, and $O_2$ pressure) was determined by considering pretreatment yield, carbohydrate yield, and enzymatic yield. The long-term reactions involving both Alamo and Dacotah switchgrass were conducted at reaction temperatures of 55 and 65 °C, reaction pressure of 1.01 bar $O_2$ pressure, and reaction time of 28 days. Overall enzymatic yields were obtained using a 72-h enzymatic hydrolysis performed in triplicate with a cellulase loading of 15 FPU/g glucan in raw biomass and an excess β-glucosidase loading of 60 CBU/g glucan in raw biomass.

## 3.3 Results and Discussion

### 3.3.1 Lime consumption

Figure 3-1 shows no significant correlation ($R^2$=0.01) between lime consumption and lignin removal for the 3.45 and 10.3-bar $O_2$ pretreatments. A weak linear correlation was observed for the 6.89-bar $O_2$ pretreatments ($R^2$ = 0.37), demonstrating that increased lignin removal consumed more lime. Lime consumption ranged from 0.074 to 0.375 g lime consumed/g raw biomass (Table 3-3). At the recommended pretreatment condition (110 °C, 6.89-bar $O_2$, 240 min), lime consumption was 0.248 g lime consumed/g raw biomass.

**Table 3-2.** Short-term pretreatment conditions.

| Pressure (bar $O_2$) | Time (min) | Temperature (°C) |
|---|---|---|
| 3.45 | 60, 120, 180, 240 | 100, 110, 120, 140, 150 |
| 6.89 | 60, 120, 180, 240 | 100, 110, 120, 140, 150 |
| 10.3 | 60, 120, 180, 240 | 100, 110, 120 |

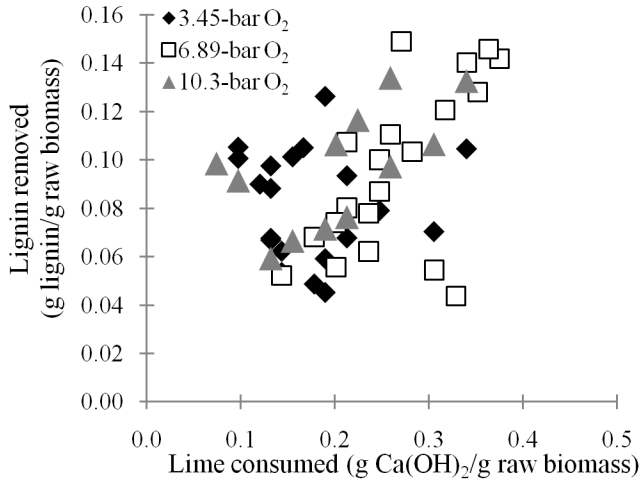


**Figure 3-1.** Relationship between lime consumption and lignin removal.

**Table 3-3.** Lime consumption (g $Ca(OH)_2$/g raw biomass).

| Time | 3.45-bar $O_2$ | | | | |
|---|---|---|---|---|---|
| (min) | 100 °C | 110 °C | 120 °C | 140 °C | 150 °C |
| 60 | 0.18 | 0.19 | 0.14 | 0.19 | 0.21 |
| 120 | 0.14 | 0.13 | 0.13 | 0.12 | 0.17 |
| 180 | 0.31 | 0.25 | 0.21 | 0.10 | 0.15 |
| 240 | 0.13 | 0.13 | 0.34 | 0.10 | 0.19 |

| | 6.89-bar $O_2$ | | | | |
|---|---|---|---|---|---|
| | 100 °C | 110 °C | 120 °C | 140 °C | 150 °C |
| 60 | 0.33 | 0.14 | 0.31 | 0.20 | 0.24 |
| 120 | 0.24 | 0.18 | 0.20 | 0.28 | 0.35 |
| 180 | 0.25 | 0.21 | 0.26 | 0.34 | 0.37 |
| 240 | 0.21 | 0.25 | 0.32 | 0.27 | 0.36 |

| | 10.3-bar $O_2$ | | |
|---|---|---|---|
| | 100 °C | 110 °C | 120 °C |
| 60 | 0.13 | 0.19 | 0.15 |
| 120 | 0.21 | 0.26 | 0.31 |
| 180 | 0.10 | 0.20 | 0.34 |
| 240 | 0.07 | 0.22 | 0.26 |

### 3.3.2 Sugars recovered from pretreatment liquor

When analyzing pretreatment carbohydrate yields, it is important to note that significant amounts of carbohydrates can be solubilized during the oxidative lime pretreatment process. Analysis of the pretreatment liquor revealed very low concentrations of monomeric glucose or xylose; however, glucan and xylan oligomers were present in more moderate concentrations. Table 3-4 shows the concentrations of glucan and xylan oligomers recovered in the pretreatment liquor for several representative pretreatment conditions. Out of the five samples reported, only one condition (150 °C, 3.45-bar $O_2$, 240 min) contained a significant amount of glucan (8.1 g glucan recovered/100 g glucan in raw). Substantial xylan oligomers were recovered in all five samples with xylan recoveries of 23.7 (120 °C, 3.45-bar $O_2$, 240 min), 25.2 (150 °C, 3.45-bar $O_2$, 240 min), 25.7 (140 °C, 6.89-bar $O_2$, 120 min), 26.0 (110 °C, 6.89-bar $O_2$, 240 min), and 27.4 (150 °C, 6.89-bar $O_2$, 240 min) g xylan recovered/100 g xylan in raw.

**Table 3-4.** Sugars recovered from pretreatment liquor.

| Pretreatment conditions | | | Sugars Recovered* | |
|---|---|---|---|---|
| Temperature (°C) | Pressure (bar $O_2$) | Time (min) | Glucan | Xylan |
| 120 | 3.45 | 240 | 3.27 | 23.67 |
| 150 | 3.45 | 240 | 8.11 | 25.15 |
| 110 | 6.89 | 240 | 2.62 | 26.03 |
| 140 | 6.89 | 120 | 5.41 | 25.65 |
| 150 | 6.89 | 240 | 2.44 | 27.42 |

*g component recovered/100 g component in raw biomass

### 3.3.3 Pretreatment yields

The primary goal of oxidative lime pretreatment is to remove lignin, while minimizing glucan and xylan degradation. When comparing pretreatment effectiveness, it is important to consider the degradation of each of these three key components. As pretreatment severity increases, more lignin is removed at the sacrifice of glucan pretreatment yields. This portion of the work focused on finding a balance between glucan recovery and lignin removal. Pretreatment yields of the solid material (Figure 3-2) were calculated using the following definition:

$$Y_i = \frac{C_i Y_t}{C_{i0}} \qquad [3\text{-}1]$$

where

$i$ = component (lignin L, glucan G, xylan X)

$Y_i$ = pretreatment yield of Component $i$ at time $t$ (g residual Component $i$/g Component $i$ in raw biomass)

$C_{i0}$ = Component $i$ content at time zero (g Component $i$ in raw biomass/g raw biomass)

$C_i$ = Component $i$ in time $t$ (g residual Component $i$/g residual biomass)

$Y_t$ = total solids pretreatment yield at time $t$ (g residual biomass/g raw biomass).

*Glucan pretreatment yields*

The pretreatments performed at 3.45-bar $O_2$ were the most successful in maintaining high glucan pretreatment yields (g glucan recovered/100 g glucan in raw

biomass; Figure 3-2). High glucan recoveries of 96.0 (100 °C, 60 min) and 95.6 (110 °C, 60 min) were observed. Increased reaction temperature and time led to the lowest glucan yields of 78.9 (140 °C , 240 min) and 65.1 (150 °C, 240 min), with the remaining glucan pretreatment yields greater than 80.

Increased pressure (6.89-bar $O_2$) had little effect on glucan pretreatment yields. At this pressure, four conditions resulted in glucan pretreatment yields less than 80 with 67.6 (150 °C, 240 min) and 70.8 (140 °C, 240 min) being the lowest observed. The majority of glucan pretreatment yields at this pressure ranged from 80–95 with the maximum being 97.0 (100 °C, 60 min).

At the highest pressure (10.3-bar $O_2$), glucan pretreatment yields began to decline. Of the 12 pretreatments performed, three resulted in glucan pretreatment yields below 80, with 66.2 (100 °C, 240 min) as the minimum. The highest yields observed were 89.7 (100 °C. 120 min) and 88.2 (180 min). By studying the average glucan pretreatment yield for each pressure (86.2, 3.45-bar $O_2$; 86.6, 6.89-bar $O_2$, 81.9, 10.3-bar $O_2$), 6.89-bar $O_2$ is recommended pressure for achieving high glucan recovery.

*Xylan pretreatment yields*

As expected, xylan pretreatment yields (g xylan recovered/100 g xylan in raw biomass; Figure 3-2) were highest in the pretreatments performed at the lowest pressure (3.45-bar $O_2$). At this pressure, very high pretreatment xylan yields of 92.6 (100 °C, 60 min) and 91.9 (110 °C, 60 min) were observed. At more severe reaction temperatures and longer times, xylan pretreatment yields fell to 40.5 (150 °C, 240 min) and 51.0 (150 °C, 180 min).

As was the case with glucan pretreatment yields, increasing the pressure (6.89-bar $O_2$) did not significantly affect xylan pretreatment yields. Maximum yields of 98.1 (100 °C, 60 min) and 95.4 (110 °C, 60 min), and minimum yields of 34.5 (140 °C, 240 min) and 33.1 (150 °C , 240 min) were observed.

Further increasing the pressure to 10.3-bar $O_2$ reduced the number of samples with very high xylan pretreatment yields. The two maximum xylan pretreatment yields observed at this pressure were 83.7 (100 °C, 60 min) and 78.3 (120 °C, 60 min). Similar minimum yields as the 3.45- and 6.89-bar $O_2$ pretreatments were observed with values of 47.6 (150, 180 min) and 37.9 (150, 240 min).

As discussed previously, oxidative lime pretreatment solubilizes a significant portion of the xylan in switchgrass, resulting in lower xylan pretreatment yields. Average xylan pretreatment yields were 67.7, 63.1, and 62.2 for the 3.45-, 6.89-, and 10.3-bar $O_2$ cases, respectively. This showed there was only a slight decline in xylan pretreatment yields because of increased pressure; however, for each pressure there was a large range in xylan pretreatment yields and increased reaction time dramatically reduced xylan pretreatment yields. One particular case (140 °C, 6.89-bar $O_2$) showed a decline of 44.1 percentage points by increasing the reaction time from 60 to 240 min.

*Lignin pretreatment yields*

As stated previously, the primary purpose of oxidative lime pretreatment is to remove lignin, thus low lignin pretreatment yields (g lignin recovered/100 g lignin in raw biomass; Figure 3-2) are desired. In this work, there was a strong positive correlation between lignin removal and increased enzymatic digestibility. The

pretreatments performed at 3.45-bar $O_2$ were the least successful in removing lignin. Low temperatures and short reaction times produced the highest lignin yields of 79.6 (100 °C, 60 min) and 81.7 (110 °C, 60 min). The lowest lignin pretreatment yields observed were 32.2 (150 °C, 240 min) and 45.0 (140 °C, 240 min).

Increasing reaction pressure to 6.89-bar $O_2$ significantly reduced lignin pretreatment yields; however, increased pressure could not compensate for low temperature and short reaction times. High lignin pretreatment yields of 82.6 (100 °C, 60 min) and 77.5 (110 °C, 60 min) were still observed at these mild conditions. Increasing the severity at this pressure did result in the lowest observed lignin pretreatment yields, with four pretreatments achieving lignin pretreatment yields below 23. The lowest lignin pretreatment yields were 18.3 (140 °C, 240 min) and 20.3 (150 °C, 240 min).

At the highest pressure (10.3-bar $O_2$), the maximum lignin pretreatment yields were 73.2 (100 °C, 60 min) and 68.7 (120 °C, 60 min). There were two successful pretreatments that obtained lignin pretreatment yields below 30: 28.3 (120 °C, 180 min) and 27.4 (120 °C, 240 min).

Overall, selected pretreatment conditions could remove lignin. It was clear that reaction times of 180 or 240 minutes were required to significantly remove lignin. The pretreatments performed at the lowest pressure (3.45-bar $O_2$) were the least promising with an average lignin pretreatment yield of 59.5. Increased pressure clearly improved lignin removal with average lignin pretreatment yields of 51.2 (6.89-bar $O_2$) and 50.4 (10.3-bar $O_2$).

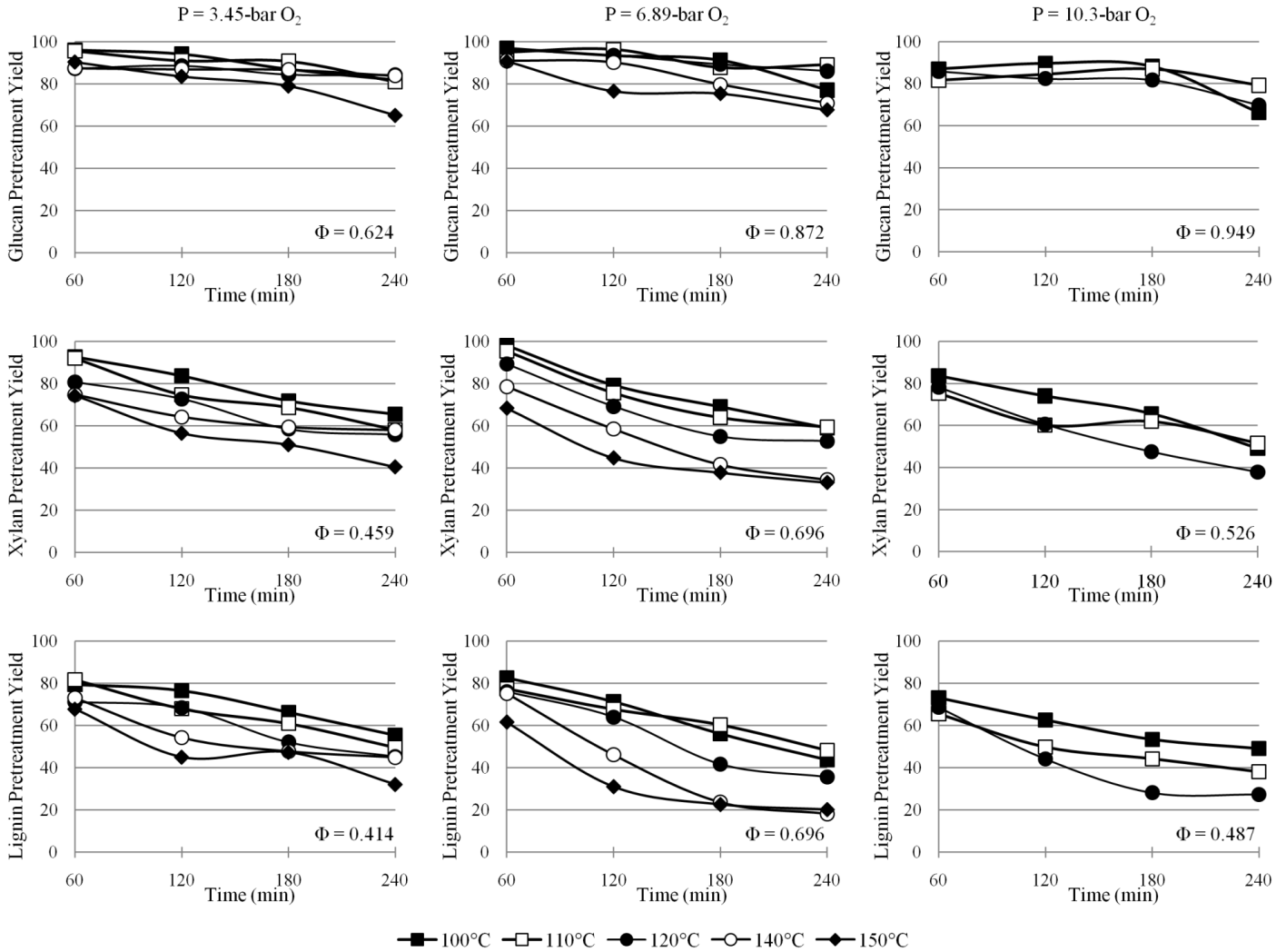


**Figure 3-2**. Short-term pretreatment yields. Φ = Average standard deviation of acid hydrolysis replicates. [Note: All pretreatment yields are expressed as g component recovered/100 g component in raw biomass.]

*Pretreatment yield summary and recommended condition*

In general, oxidative lime pretreatment successfully removed lignin while maintaining high recoveries of glucan. Significant xylan degradation was observed, but it never exceeded lignin degradation, and a large concentration of xylan oligomers can be recovered from the pretreatment liquor.

The recommended conditions for oxidative lime pretreatment of Alamo switchgrass (110 °C, 6.89-bar $O_2$, 240 min) obtained a glucan pretreatment yield of 89.0 g glucan recovered/100 g glucan in raw biomass, xylan pretreatment yield of 59.4 g xylan recovered/100 g xylan in raw biomass, and a lignin pretreatment yield of 48.2 g lignin recovered/100 g lignin in raw biomass. If the glucan and xylan oligomers are recovered from the pretreatment liquor, glucan pretreatment yield improves to 91.6 g glucan recovered/100 g glucan in raw biomass and xylan pretreatment yield improves to 85.4 g xylan recovered/100 g xylan in raw biomass.

### 3.3.4 Enzymatic yields

The primary goal of biomass pretreatment is to increase the enzymatic digestibility of lignocellulosic biomass. When comparing pretreatment performance, it is important to measure the enzymatic digestibility of both glucan and xylan, while considering glucan and xylan pretreatment yields. This study used a 72-h hydrolysis with a cellulase loading of 15 FPU/g raw glucan and an excess β-glucosidase loading of 60 CBU/g raw glucan. When choosing a recommended pretreatment condition (reaction temperature, time, pressure, and lime loading), the determining factor was overall yield of glucan and xylan. Overall yield ($Y_{oi}$) is defined as the amount of glucan or xylan

enzymatically hydrolyzed after pretreatment per unit of glucan or xylan in the raw feedstock.

$$Y_{oi} = Y_i \times Y_{ei} \quad [3\text{-}2]$$

where

$i$ = component (glucan G or xylan X)

$Y_{oi}$ = overall yield of Component $i$ (g hydrolyzed Component $i$/g Component $i$ in raw biomass)

$Y_i$ = pretreatment yield of Component $i$ (g residual Component $i$/g Component $i$ in raw biomass)

$Y_{ei}$ = enzymatic yield of Component $i$ (g hydrolyzed Component $i$/g Component $i$ in pretreated biomass).

Enzymatic hydrolysis results are shown in Figure 3-3. Pretreatments performed at the lowest pressure (3.45-bar $O_2$) were the least successful in producing highly digestible switchgrass. As discussed previously, although glucan recovery after pretreatment was quite high for this set of pretreatments, overall lignin removal was not substantial. With high lignin contents remaining in the pretreated biomass, overall glucan yields were low (g glucan hydrolyzed/100 g glucan in raw biomass) and generally ranged from 55–65. The highest overall glucan yields observed were 66.6 (120 °C, 240 min) and 66.4 (100 °C, 60 min). The worst performing condition had an overall glucan yield of just 46.9 (150 °C, 240 min), well below the average overall glucan yield (58.9) for the pretreatments performed at this pressure. Xylan overall yields

(g xylan hydrolyzed/100 g xylan in raw biomass) were moderate as well, typically in the range of 40–45. The maximum and minimum overall xylan yields were 55.8 (100 °C, 60 min) and 27.6 (150 °C, 240 min), respectively.

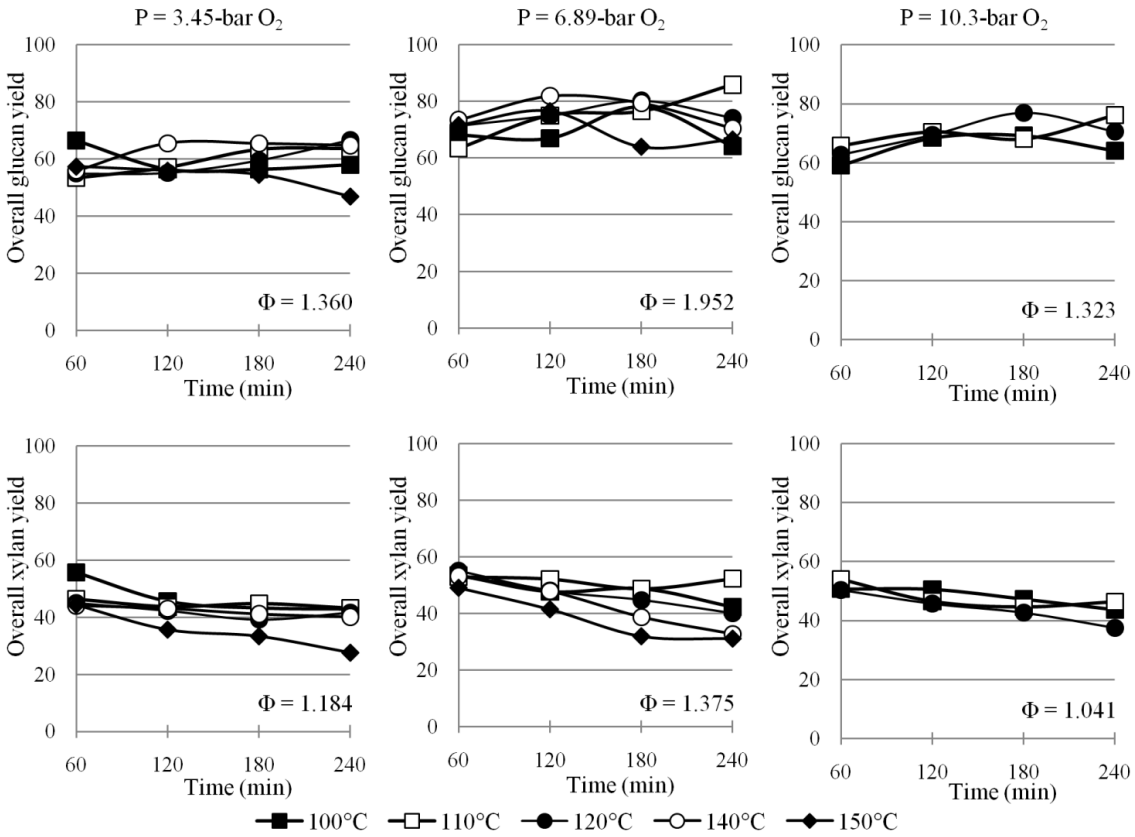


**Figure 3-3.** Overall enzymatic yield results for short-term pretreatments. Enzymatic hydrolysis was performed for 72 h with a cellulase enzyme loading of 15 FPU/g glucan in raw biomass. Φ = Average standard deviation of enzymatic hydrolysis replicates. [Note: All overall enzymatic yields are expressed as g component hydrolyzed/100 g raw component.]

A good balance between glucan recovery and lignin removal was demonstrated in the pretreatments performed at 6.89-bar $O_2$. This balance resulted in a significant

positive shift in overall glucan yields. At this pressure, the recommended pretreatment condition (110 °C, 6.89-bar $O_2$, 240 min) produced an overall glucan yield of 85.9 and an overall xylan yield of 52.2. In terms of overall glucan yield, several other successful pretreatments resulted in high overall glucan yields of 81.9 (140 °C, 120 min), 80.3 (120 °C, 180 min), and 79.5 (140 °C, 180 min). The lowest overall glucan yield was 63.9 (150 °C, 180 min). The average overall glucan yield for pretreatments at this pressure was 73.2, considerably higher than the average observed for the low-pressure (3.45-bar $O_2$) pretreatments. Overall xylan yields were only slightly improved over the 3.45-bar $O_2$ pretreatments, with yields primarily ranging from 40–50. The highest overall xylan yield observed at this pressure was 53.5 (100 °C, 60 min), whereas the lowest was 31.1 (150 °C, 240 min).

At the highest pressure (10.3-bar $O_2$), significant glucan degradation occurred during pretreatment, which reduced overall glucan yields. For the high-pressure pretreatments, the maximum overall glucan yields were 77.0 (120 °C, 180 min) and 76.2 (110 °C, 240 min). The least successful pretreatment at this pressure produced an overall glucan yield of 59.1 (100 °C, 60 min). The average overall glucan yield for the 10.3-bar $O_2$ pretreatments was 68.4, which was between the 3.45- and 6.89-bar $O_2$ conditions. Similar to the other pressures, overall xylan yields were moderate, typically 45–50. The maximum and minimum overall yields observed were 54.0 (110 °C, 60 min) and 37.5 (120 °C, 240 min), respectively.

### 3.3.5 Alamo and Dacotah comparisons

Another purpose of this study was to compare the enzymatic digestibility of lime-pretreated Alamo switchgrass with lime-pretreated Dacotah switchgrass. Alamo switchgrass is a southern lowland variety, whereas Dacotah is a northern upland variety. In terms of morphology, Alamo is thick-stemmed and Dacotah is thin-stemmed. The latitude-of-origin was quite different as well for the two varieties, with Alamo (29°N) being much further south than Dacotah (46°N). The Alamo variety was harvested from Ardmore, OK (34°N), and Dacotah from Pierre, SD (44°N), both close to their latitude-of-origin. Alamo was harvested in late fall of the same year it was planted, whereas the Dacotah stood over the winter before harvesting. The differences in ecotype, morphology, harvest location, and harvest date resulted in compositional differences that altered the recommended conditions of oxidative lime pretreatment.

*Long-term comparison*

The first comparison performed was a long-term lime pretreatment of the two varieties. This pretreatment was conducted at 55 °C over 28 days, with compressed air bubbled into the reaction bottles. Enzymatic digestibility (Figure 3-4) was measured using a 72-h hydrolysis time with a cellulase loading of 15 FPU/g raw glucan and an excess β-glucosidase loading of 60 CBU/g raw glucan.

On a treated glucan basis (g glucan hydrolyzed/100 g pretreated glucan), Alamo was significantly more digestible (82.2) than Dacotah (58.5). However, overall glucan yields (g glucan/100 g glucan in raw), which factor in glucan pretreatment yields, were much more similar. Alamo and Dacotah had overall glucan yields of 60.0 and 53.2,

respectively. Xylan enzymatic yields followed a similar trend. On a treated basis, Alamo was 10.5 percentage points more digestible than Alamo, but only 3.3 percentage points more digestible on an overall basis.

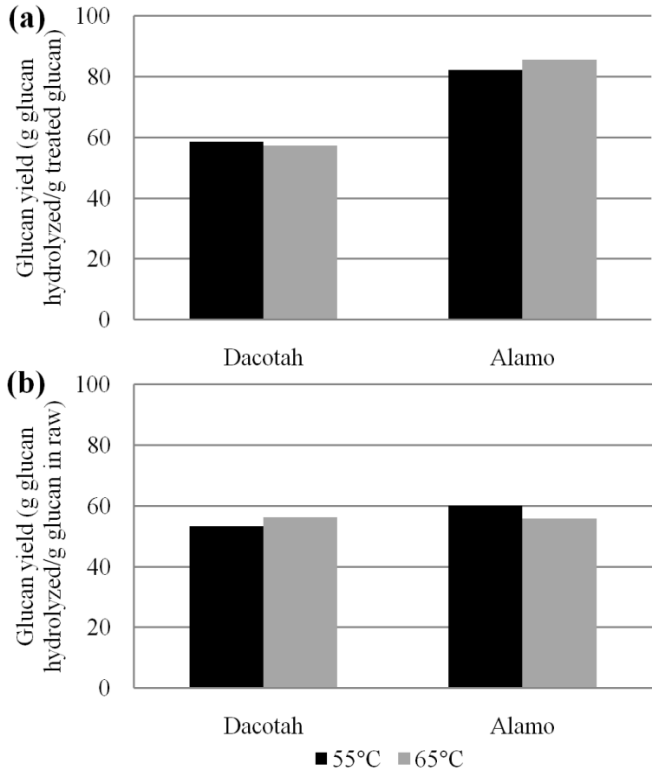


**Figure 3-4.** Enzymatic yield results for long-term lime pretreated Dacotah and Alamo switchgrass. Enzymatic hydrolysis was performed for 72-h with a cellulose enzyme loading of 15 FPU/g glucan in raw biomass. (a) Glucan yields on a treated basis, (b) Glucan yields on a raw basis.

*Recommended pretreatment conditions*

There are key differences in the genotype (lowland vs. upland), ecotype (southern vs. northern), morphology, and harvest dates of the Alamo and Dacotah

samples used in this work. Alamo is a southern lowland switchgrass with thick-stem morphology and a late fall harvest date. Dacotah is a northern upland, thin-stemmed variety that was harvested in the late spring after standing over the winter. Holocellulose content generally increases with latitude for upland varieties, whereas the opposite is true for lowland varieties (Casler et al., 2004; Cassida et al., 2005). The Alamo used in this study was harvested 5° north of its latitude-of-origin, resulting in low cellulose content. Harvest time also probably affected Dacotah's higher cellulose content. It has been observed that harvesting in the spring after the switchgrass stood over the winter decreases mineral concentration but increases lignin and cellulose content (Adler et al., 2006; Casler & Boe, 2003). Although Dacotah had more cellulose content, Alamo had significantly lower lignin. These compositional differences alter how the switchgrass responds to oxidative lime pretreatment.

In a previous study, the recommended pretreatment condition for Dacotah switchgrass was 120 °C, 6.89-bar $O_2$, 240 min (Falls et al., 2011b). After pretreatment at these conditions, Dacotah switchgrass had an overall glucan yield (g glucan hydrolyzed/100 g of glucan in raw biomass) of 85.2 and an overall xylan yield (g xylan hydrolyzed/100 g xylan in raw biomass) of 50.1. This was quite similar to the maximum glucan (85.9) and xylan (52.2) overall yields reported for Alamo in this study. Recommended pretreatment time and pressure were identical for the two varieties; however, the pretreatment temperature was 10 °C less for Alamo. The less severe temperature most likely results from the lower lignin content in the Alamo variety. In general, the Alamo switchgrass was more digestible on a treated basis (g glucan

hydrolyzed/100 g pretreated glucan), but suffered from low pretreatment solids yield. At their respective recommended pretreatment conditions, Dacotah had a pretreatment solids yield of 72.0, whereas Alamo was significantly lower (63.7).

For each variety, another useful metric is to compare how oxidative lime pretreatment selectively removed lignin compared to xylan. Figure 3-5 clearly reveals that oxidative lime pretreatment selectively removed more lignin from Dacotah (1.32 g lignin/g xylan) compared to Alamo (1.16 g lignin/g xylan).

*Ball-milling comparison*

The pretreatment yields demonstrated the effectiveness of oxidative lime pretreatment as a delignification technique, which improved overall sugar yield. Another key barrier to enzymatic digestion of lignocellulose is cellulose crystallinity. Ball-milling is a laboratory decrystallization technique that can be used in conjunction with oxidative lime pretreatment. Although not economical at industrial scale, ball-milling can be used to demonstrate the benefit of combining chemical and mechanical pretreatment methods. By lowering lignin content and cellulose crystallinity, high overall yields can be achieved with reduced enzyme loadings.

Compared to oxidative lime pretreatment alone, adding ball-milling achieved slightly higher overall glucan but at a much lower cellulase loading (Table 3-5). Combining pretreatment techniques to Alamo switchgrass (110 °C, 6.89-bar $O_2$, 240 min, 72-h ball-milling) produced an overall glucan yield of 90.0 at a cellulase loading of 7 FPU/g raw glucan. At the same enzyme loading, Dacotah switchgrass (120 °C, 6.89-

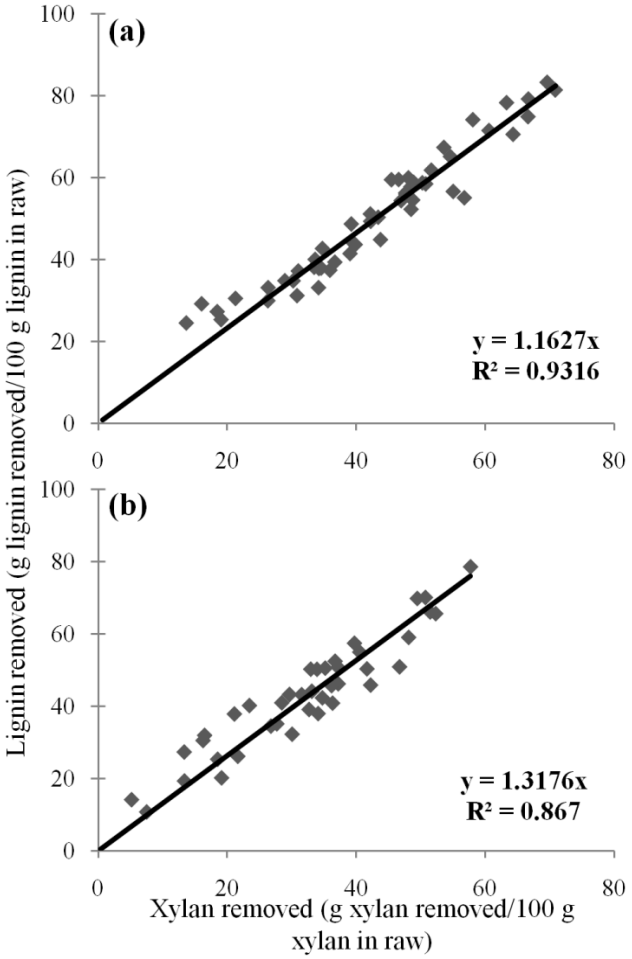


**Figure 3-5.** Selectivity as a function of lignin and lime removal. (a) Short-term lime pretreatment of Alamo switchgrass, (b) Short-term lime pretreatment of Dacotah switchgrass.

bar $O_2$, 240 min, 72-h ball-milling) obtained an overall glucan yield of 91.1. Xylan overall yields were 47.0 and 42.4 for the Alamo and Dacotah varieties, respectively.

Factoring in easily digestible sugars and oligomers recovered from the pretreatment liquor dramatically improves overall yields. Including sugars and oligomers from the pretreatment liquor, Alamo achieved an overall glucan yield of 92.6

and an overall xylan yield of 73.0. Similarly, Dacotah achieved overall glucan and overall xylan yields of 92.9 and 67.6, respectively.

**Table 3-5.** Overall digestibility of oxidative-lime-treated and ball-milled switchgrass.

| | Enzymatic Yields (g component hydrolyzed/100 g component in raw biomass) | | Sugars Recovered from Pretreatment Liquor (g component solubilized/100 g component in raw biomass) | | Overall Digestibility (g component hydrolyzed/100 g component in raw biomass) | |
|---|---|---|---|---|---|---|
| Variety | Glucan | Xylan | Glucan | Xylan | Glucan | Xylan |
| Alamo* | 90.0 | 47.0 | 2.6 | 26.0 | 92.6 | 73.0 |
| Dacotah** | 91.1 | 42.4 | 1.8 | 25.2 | 92.9 | 67.6 |

* 110 °C, 6.89-bar $O_2$, 240 min, 72-h ball-milling, ** 120 °C, 6.89-bar $O_2$, 240 min, 72-h ball-milling, $^{\Phi}$ Includes sugars digested from pretreated solids and oligomeric sugars from pretreatment liquor.

## 3.4 Conclusions

This work demonstrates that oxidative lime pretreatment significantly increases enzymatic digestibility of Alamo switchgrass. At the recommended condition (110 °C, 6.89-bar $O_2$, 240 min), overall glucan and xylan yields (g sugar hydrolyzed/100 g sugar in raw biomass; 15 FPU/g raw glucan) were 88.5 and 78.2, respectively, when sugars and oligomers from the pretreatment liquor are included. With the addition of ball-milling to oxidative lime pretreatment, overall glucan and xylan yields (including sugars and oligomers in the pretreatment liquor) improved to 92.9 and 67.6, respectively, with a lower enzyme loading (7 FPU/g raw glucan). When compared to Dacotah switchgrass, Alamo had lower pretreatment solid yields, but still achieved similar glucan digestibility with a slight decrease in reaction temperature (10 °C).

# CHAPTER IV

## INVESTIGATION OF ENZYME FORMULATION ON PRETREATED SWITCHGRASS*

This work studied the benefits of adding different enzyme cocktails (cellulase, xylanase, β-glucosidase) to pretreated switchgrass. Pretreatment methods included ammonia fiber expansion (AFEX), dilute-acid (DA), liquid hot water (LHW), lime, lime +ball-milling, soaking in aqueous ammonia (SAA), and sulfur dioxide ($SO_2$). The compositions of the pretreated materials were analyzed and showed a strong correlation between initial xylan composition and the benefits of xylanase addition. Adding xylanase improved xylan yields for SAA (+8.4%) and AFEX (+6.3%), and showed negligible improvement (0–2%) for the pretreatments with low xylan content (dilute-acid, $SO_2$). Xylanase addition also improved overall yields with lime + ball-milling and $SO_2$ achieving the highest overall yields from pretreated biomass (98.3% and 93.2%, respectively). Lime + ball-milling obtained an enzymatic yield of 92.3 kg of sugar digested/kg of protein loaded.

*Reprinted with kind permission from Elsevier. Investigation of enzyme formulation on pretreated switchgrass by M. Falls, J. Shi, M. Ebrik, T. Redmond, B. Yang, C. Wyman, R. Garlock, V. Balan, B. Dale, V. Pallapolu, Y. Lee, Y. Kim, N. Mosier, M. Ladisch, B. Hames, S. Thomas, B. Donohoe, T. Vinzant, R. Elander, R. Sierra, M. Holtzapple. Bioresource Technology, In Press. 

## 4.1 Introduction

Because of its high abundance and relatively low cost, lignocellulosic biomass is a promising source of renewable liquid fuels (Klyosov, 1986; Saha & Cotta, 2008). Sources of lignocellulosic biomass include energy crops, agricultural crop residues, industrial waste, and municipal paper waste (Zaldivar et al., 2001). It is composed mainly of cellulose and hemicellulose, which when hydrolyzed provide a source of carbohydrates for ethanol fermentation. However, the hydrolysis of cellulose and hemicellulose is one of the main hurdles to fully realizing the potential of cellulosic ethanol. Some of the key chemical and physical barriers which limit enzymatic hydrolysis include: high lignin content, cellulose crystallinity, degree of cellulose polymerization, low surface area, and presence of acetyl groups on hemicellulose (McMillan, 1994; Sun & Cheng, 2002). The goal of pretreatments, both chemical and physical, is to remove some of these barriers and render the biomass more susceptible to enzymatic digestion.

This study was a collaborative effort between members of the Consortium for Applied Fundamentals and Innovation (CAFI), which was formed to compare different pretreatment technologies using consistent materials and analytical methods (Mosier et al., 2005; Wyman et al., 2005a). The goals of CAFI I and II were to determine optimal conditions for varying pretreatment technologies for corn stover (Wyman et al., 2005b) and poplar wood (Wyman et al., 2009), respectively. This study was part of CAFI III, which focuses on increasing enzymatic digestibility of switchgrass, a promising

bioenergy crop with high biomass yield, moisture efficiency, low nutrient requirement, and stand longevity (Samson & Omielan, 1994). It can grow in many environments, including most regions of the United States (Gould, 1968) and is a promising substrate for ethanol production (Schmer et al., 2006; Wright & Turhollow, 2010). The primary contributors to this study were Auburn University (soaking in aqueous ammonia pretreatment), Michigan State University (ammonia fiber expansion pretreatment), Purdue University (liquid hot water pretreatment), Texas A&M University (lime pretreatment and data analysis), and University of California Riverside (sulfur dioxide and dilute-acid pretreatments).

To determine pretreatment effectiveness and optimum pretreatment conditions, the primary analytical tool utilized by the CAFI team is enzymatic hydrolysis. A significant amount of work has been devoted to studying the effects of cellulase and β-glucosidase on pretreated substrates (Alvira et al., 2010; Cardona et al., 2010; Wyman et al., 2009). With the high cost of feedstock, pretreatment, and enzymes, it is necessary to optimize the enzymatic hydrolysis of both cellulose and hemicellulose (Chandra et al., 2008; Gírio et al., 2010; Gnansounou & Dauriat, 2010; O'Dwyer et al., 2007). The primary goal of this project was to explore the effect of adding a third enzyme, xylanase, to the standard enzyme mixture of cellulase and β-glucosidase. Xylanase is primarily responsible for hydrolyzing hemicellulose by cleaving β-1,4 xylan bonds. Changes in enzymatic digestibility due to xylanase addition were observed by measuring both individual and overall carbohydrate yields. While holding β-glucosidase constant, varying the ratio of cellulase to xylanase achieved an optimal ratio that maximized

overall yields while reducing total enzyme loading. A secondary goal of the project was to study the effect of overall yield in the absence of β-glucosidase, which would determine the need to add β-glucosidase when both cellulase and xylanase were present.

## 4.2 Materials and Methods

### 4.2.1 Substrate and enzymes

The feedstock used in this study was the Dacotah variety of switchgrass (*Panicum virgatum*) kindly provided by Ceres, Inc. This variety was planted on December 6, 1999 in Pierre, SD and harvested on March 1, 2008 after the plot stood over the winter. The bales were stored indoors until shipped to Hazen Research, Inc. (Golden, CO) where they were ground by a hammer mill equipped with a ¼-in screen. The material was then mixed using the cone and quartering technique, separated into 5-kg sub-lots and divided amongst the CAFI members. The composition determined by Ceres, Inc. was 35.0% glucan, 21.8% xylan, 3.5% arabanin, 21.4% lignin, 2.8% acetyl, and 8.1% extractives. Each CAFI laboratory further reduced the particle size to pass through 40 (ASTM) mesh. After reducing the particle size, the switchgrass was washed with hot water. Dry switchgrass (200 g) was mixed with 2 L of 80–90 °C distilled water and allowed to stand 10–15 minutes. The slurry was vacuum filtered using Whatman No. 41 filter paper. The mixing and filtration was performed three times followed by drying the washed solids in a 45 °C oven. The composition of the washed material as measured by Texas A&M University was 37.2% glucan, 23.8% xylan, 2.5% arabanin, and 20.8% lignin.

Cellulase was Spezyme CP® (lot 301-04075-054, 82 mg protein/mL, 59 FPU/mL). Hemicellulase was Multifect xylanase® (lot 301-04021-015, 27 mg protein/mL). Both cellulase and hemicellulase were kindly provided by Genencor International, Inc®. The β-glucosidase was Novozyme 188® (67 mg protein/mL, 600 CBU/mL) and was obtained from Sigma Aldrich (St. Louis, MO). The protein concentration of each enzyme was measured using TCA precipitation and was reported by Genencor (Spezyme CP and Multifect Xylanase) and Michigan State University (Novozyme 188).

### 4.2.2 Pretreatment methods

*Ammonia fiber expansion (AFEX)*

The AFEX pretreatment conditions were chosen to limit hemicellulose degradation. The pretreatment was performed in a 1.5-L stainless steel (#316) Parr reactor. Distilled water was added to the switchgrass at a loading ratio of 2 g $H_2O$/g dry biomass and the slurry was added to the preheated (150 °C) reactor. The reactor was sealed and evacuated using a rotary vacuum pump while ammonia was heated in a separate pressurized vessel. Once heated, the ammonia was added to the reactor at a loading of 1.5 g $NH_3$/g dry biomass. The pretreatment ran for 30 min with a maximum temperature of 155–165 °C, which decreased to a final temperature between 104–119 °C. The reactor was then rapidly vented and the biomass was removed. The biomass was stored overnight in a fume hood to allow evaporation of residual ammonia.

*Dilute sulfuric acid (DA)*

Switchgrass (50 g) was presoaked in 10-g/L dilute sulfuric acid overnight at room temperature with a solid loading of 10 wt %. The pretreatment was performed in a 1-L Parr reactor made of Hasteloy C. Heating was provided by a 4-kW fluidized sand bath with stirring (200 rpm) using two 40-mm-diameter stacked pitched-blade impellers. Pretreatment was run at 140 °C for 40 min, which did not include an additional 2-min heating time. The reactor was quenched in a room-temperature water bath until the temperature dropped to 80 °C. The pretreatment slurry was vacuum filtered through a glass fiber filter with the temperature consistently greater than 60 °C. The resulting solids were washed with room-temperature deionized water until the filtrate pH was greater than 6.0.

*Lime*

Lime pretreatment was conducted in a pair of 304 stainless steel pipe reactors (5-in long, 1.5-in ID) with 1.5-in 304 stainless steel caps. The reactors were sealed using Teflon tape. Reactors were loaded with 8 g dry switchgrass each and excess calcium hydroxide (1 g $Ca(OH)_2$/g dry biomass) and water (15 g/g dry biomass). Constant 6.89-bar pure oxygen was supplied to a manifold through a flexible stainless steel hose attached to an oxygen tank. The reactors were connected to a swing arm to provide constant stirring and placed in a preheated temperature-controlled oven at 120 °C. The reaction time was 4 h after which the reaction was quenched by removing the reactors from the oven and immediately placing them in an ice bath. Once cooled, the reactors were opened slowly to relieve pressure, and the contents were transferred to a 1-L plastic

centrifuge bottle using distilled water. The slurry was neutralized using 5-N HCl to a pH of approximately 4.0, and then underwent several washings with distilled water until the pH of the slurry rose to approximately 6.0. The final slurry was vacuum filtered and the filtrate was collected for carbohydrate analysis. Moisture content and final solid weight were recorded to obtain pretreatment yield and the solids were stored in the freezer until compositional analysis and enzymatic hydrolysis were performed.

*Lime + ball-milling*

Lime pretreatment followed the same procedure as above. The pretreated solids were thoroughly dried (moisture content < 10%) before ball-milling in a 300-mL porcelain jar loaded with 0.375-in zirconia grinding medium. The grinding medium was loaded to fill 50% of the jar volume (approximately 258 g) and biomass was loaded at a ratio of 48 g grinding medium/g dry biomass. The jars were sealed and placed on rollers rotating at 68 rpm for 3 days.

*Liquid hot water (LHW)*

Switchgrass was mixed with deionized water at a loading ratio of 15 wt %. The pretreatment reactor was stainless steel (#316) tubing (1-in OD × 0.083-in wall thickness, 4.5-in length, 45-mL total volume) capped at each end with a 1-in tube end fitting. Sample volume was chosen to be 33.7 mL to allow 25% headspace for liquid expansion. The reaction was run at 200 °C for both 5 min and 10 min. The reactor was heated in a Tecam SBL-1 fluidized sand bath with a heat-up time of 8 min, which was not included in reaction time. Upon completion, the pretreatment was quenched by placing the reactor in water for 10 min.

*Soaking in aqueous ammonia (SAA)*

The SAA pretreatment was performed in a stainless steel batch reactor (1.375-in ID × 6-in long). Switchgrass (10 g) was loaded with 90 mL 15% $NH_4OH$. The reactor was placed in a preheated temperature-controlled oven at 160 °C for 60 min. Heat-up time was 20 min and was not included in the reaction time. The reactor was quenched in a room-temperature water bath. The pretreatment slurry was vacuum filtered and the solids were washed using deionized water until the pH was approximately 6.0.

*Sulfur-dioxide ($SO_2$)*

Moist (approximately 65% moisture) switchgrass was impregnated overnight with 5 wt % gaseous $SO_2$ (>99% pure) at room temperature in a sealed heavy-duty Ziploc bag. The impregnated switchgrass was transferred to a 1-L Hasteloy C Parr reactor and mixed with deionized water to a solid loading of 10 wt % on a dry basis. The reaction was run at 180 °C for 10 min in a 4-kW fluidized sand bath. Stirring was provided by two 40-mm-diameter stacked pitched blade impellers at 200 rpm. Heat-up time was 2 min and was not included in reaction time. The reactor was quenched in a water bath until the reactor temperature dropped to 80 °C. The pretreatment slurry was immediately vacuum filtered while maintaining a temperature greater than 60 °C. The resulting solids were washed with deionized water until filtrate pH was greater than 6.0.

### 4.2.3 Compositional analysis

Compositional analysis was performed on the raw, pre-washed, and pretreated samples. The material was prepared by air drying to a measured moisture content of less than 10%. The composition was analyzed using an NREL acid hydrolysis procedure

(Sluiter et al., 2008b). The sample (0.3 g) was weighed into a glass test tube followed by adding 3 mL of 72 wt % sulfuric acid. The test tubes were placed in a 30 °C water bath and stirred regularly for 1 h. The contents of the test tube were quantitatively transferred to glass autoclave bottles using 84 mL distilled water, capped, sealed, and steam autoclaved for 1 h. Samples were cooled, opened, and filtered through glass filtering crucibles, which were placed in a 105 °C oven to dry. The filtrate was neutralized and then analyzed for carbohydrates using HPLC Analysis (Bio-Rad Aminex HPX-87P column, HPLC grade water mobile phase, 0.6 mL/min, 80–85 °C column temperature). The weight of the dried, filtered solids minus their ash weight was recorded and used to calculate lignin content. Ash content was determined by heating samples in a 575 °C furnace until completion. The extractives were determined by extracting the biomass with 95% ethanol for 24 h in a Sohxlet apparatus. The measured compositions for both the raw and pretreated materials were used in the enzymatic hydrolysis loading calculations.

### 4.2.4 Enzymatic hydrolysis

The enzymatic hydrolysis procedure for both glucan and xylan closely followed the enzymatic saccharification procedure provided by NREL (Selig et al., 2008). Hydrolysis samples were prepared in 50-mL plastic centrifuge tubes. Pretreated biomass loading weight was calculated based on moisture content and glucan composition to yield 0.1 g glucan per sample. Sodium citrate buffer (5 mL, 0.1-M, pH 4.8), 0.04 mL tetracycline (10 mg/mL in 70% ethanol), 0.03 mL cycloheximine (10 mg/mL in distilled water), 1 mL of each enzyme dilution (cellulase, xylanase, β-glucosidase), and an

appropriate volume of water were added to bring the total working volume to 10 mL. The enzyme dilutions were calculated on a raw glucan basis using the enzyme activity and desired enzyme loading. Hydrolysis occurred in a shaking incubator (100 rpm) at 50 °C for 72 h. To quench the hydrolysis, the samples were either placed in a 105 °C oven or in boiling water for 5–10 minutes and then cooled in an ice bath. Samples were stored in a freezer until HPLC analysis. HPLC analysis (Bio-Rad Aminex HPX-87P column, HPLC grade water mobile phase, 0.6 mL/min, 80–85 °C column temperature) was used to measure the glucose and xylose concentrations of each sample. These concentrations were then recalculated as glucan and xylan concentrations to report digestibility yields.

### 4.2.5 Experimental design

Substrate preparation, pretreatments, compositional analysis, and enzymatic hydrolysis were all performed by each individual CAFI laboratory. The compositional analysis and enzymatic hydrolysis results of each pretreatment type were then sent to Texas A&M University. Texas A&M University analyzed carbohydrate yields to determine the most effective enzyme ratio for each pretreatment. For each pretreatment, the experiment measured the enzymatic digestibility in duplicate of 23 different samples (Table 4-1). The 23 samples were comprised of enzyme loadings in two sets: Set A (13.4, 33.4, 78.4, 123.4, and 243.4 mg protein/g raw glucan) and Set B (30.0 mg protein/g raw glucan). In Set A, β-glucosidase was held constant (3.4 mg protein/g raw glucan), to be consistent with previous CAFI research (Wyman et al., 2005b). These enzyme concentrations were chosen to represent enzyme loadings ranging from very low

(economical) to very high (gross excess). Furthermore, little information was available on the effects of cellulase:xylanase loading ratio, so for each of these five total enzyme loadings, four cellulase:xylanase ratios were employed (1:0, 5:1, 2:1, and 1:1). Set B employed three cellulase:xylanase ratios (5:1, 2:1, 1:1), but no β-glucosidase.

**Table 4-1.** Enzyme loadings.

| Sample | Cellulase (mg/g raw glucan) | Xylanase (mg/g raw glucan) | B-glucosidase (mg/g raw glucan) | Total Enzyme (mg/g raw glucan) |
|---|---|---|---|---|
| 1A | 10.0 | 0.0 | 3.4 | 13.4 |
| 2A | 8.3 | 1.7 | 3.4 | 13.4 |
| 3A | 6.7 | 3.3 | 3.4 | 13.4 |
| 4A | 5.0 | 5.0 | 3.4 | 13.4 |
| 5A | 30.0 | 0.0 | 3.4 | 33.4 |
| 6A | 25.0 | 5.0 | 3.4 | 33.4 |
| 7A | 20.0 | 10.0 | 3.4 | 33.4 |
| 8A | 15.0 | 15.0 | 3.4 | 33.4 |
| 9A | 75.0 | 0.0 | 3.4 | 78.4 |
| 10A | 62.5 | 12.5 | 3.4 | 78.4 |
| 11A | 50.0 | 25.0 | 3.4 | 78.4 |
| 12A | 37.5 | 37.5 | 3.4 | 78.4 |
| 13A | 120.0 | 0.0 | 3.4 | 123.4 |
| 14A | 100.0 | 20.0 | 3.4 | 123.4 |
| 15A | 80.0 | 40.0 | 3.4 | 123.4 |
| 16A | 60.0 | 60.0 | 3.4 | 123.4 |
| 17A | 240.0 | 0.0 | 3.4 | 243.4 |
| 18A | 200.0 | 40.0 | 3.4 | 243.4 |
| 19A | 160.0 | 80.0 | 3.4 | 243.4 |
| 20A | 120.0 | 120.0 | 3.4 | 243.4 |
| 21B | 25.0 | 5.0 | 0.0 | 30.0 |
| 22B | 20.0 | 10.0 | 0.0 | 30.0 |
| 23B | 15.0 | 15.0 | 0.0 | 30.0 |

## 4.3 Results and Discussion

### 4.3.1 Composition of pretreated samples

Table 4-2 shows the compositional analysis of the eight pretreated materials plus raw and washed feedstocks. The washing procedure before pretreatment did not greatly affect the composition with just a slight increase (2.2%) in glucan composition. Lime

and SAA pretreatments both reduced lignin content of the washed feedstock, by 7.2% and 12.4%, respectively. There was significant removal of xylan; while, the glucan content of the lime pretreatment significantly increased because of the weight loss after pretreatment. The dilute-acid and $SO_2$ pretreatments both had high levels of xylan reduction, which resulted in a significant increase in glucan content and a slight increase in lignin. The compositions of the AFEX and LHW pretreatments were relatively unchanged from the raw switchgrass composition. (Note: AFEX, LHW 5 min, and LHW 10 min samples were not washed after pretreatment.)

**Table 4-2.** Composition and pretreatment yields. Note: AFEX and LHW were not washed after pretreatment.

| | Glucan (%) | Xylan (%) | Lignin (%) | Other (%) | Pretreatment Yield (g treated biomass/100 g raw biomass) |
|---|---|---|---|---|---|
| Raw | 35.0 | 21.8 | 21.4 | 21.8 | – |
| Washed | 36.5 | 22.7 | 20.7 | 20.1 | 92.6 |
| Lime + ball-mill | 48.6 | 18.7 | 13.5 | 19.2 | 69.2 |
| Lime | 48.6 | 18.7 | 13.5 | 19.2 | 69.2 |
| AFEX | 35.9 | 22.5 | 24.4 | 17.2 | 95.1 |
| SAA | 34.5 | 13.6 | 8.3 | 43.6 | 62 |
| LHW (5 min) | 36.5 | 22.7 | 20.7 | 20.1 | 92.6 |
| LHW (10 min) | 36.5 | 22.7 | 20.7 | 20.1 | 92.6 |
| DA | 50.6 | 7.3 | 28.6 | 13.5 | 60.9 |
| SO2 | 58.7 | 4.5 | 27.6 | 9.2 | 57.3 |

### 4.3.2 Effect of xylanase addition on carbohydrate yields

The primary goal of this study was to determine the optimum ratio of cellulase to xylanase that maximizes overall carbohydrate yield. For each pretreatment, 20 samples

were analyzed which consisted of five total enzyme loadings (13.4, 33.4, 78.4, 123.4, and 243.4 mg protein/g raw glucan) with four different cellulase:xylanase ratios per enzyme loading (1:0, 5:1, 2:1, 1:1). After hydrolysis, the glucan, xylan, and overall yield were calculated based on pretreated compositions using the following definitions:

$$\text{Glucan yield} \equiv \frac{\text{glucan digested}}{\text{initial glucan loaded}} \quad [4\text{-}1]$$

$$\text{Xylan yield} \equiv \frac{\text{xylan digested}}{\text{initial xylan loaded}} \quad [4\text{-}2]$$

$$\text{Overall yield} \equiv \frac{\text{glucan digested} + \text{xylan digested}}{\text{initial glucan loaded} + \text{initial xylan loaded}} \quad [4\text{-}3]$$

Figures 4-1a, 4-1b, and 4-1c show the glucan yield, xylan yield, and overall yield results, respectively. (Note: Only the best performing enzyme loading ratios are shown.) Each pretreatment has a different carbohydrate composition, so the effect of cellulase:xylanase ratio showed a different result for each pretreatment method. In most cases, there was not a significant increase in overall yield once the total enzyme loading was greater than 78.4 mg protein/g raw glucan. All future discussions will focus on an enzyme loading of 78.4 mg protein/g raw glucan. All values are given in relation to a percent increase or decrease over pure Spezyme CP, the control.

For AFEX pretreatment, xylanase addition noticeably improved xylan yield (+6.3%) and glucan yield (+4.6%). Further increasing the xylanase ratio improved xylan yield with increases of 8.1% (2:1) and 9.1% (1:1). At 78.4 mg protein/g raw glucan, the

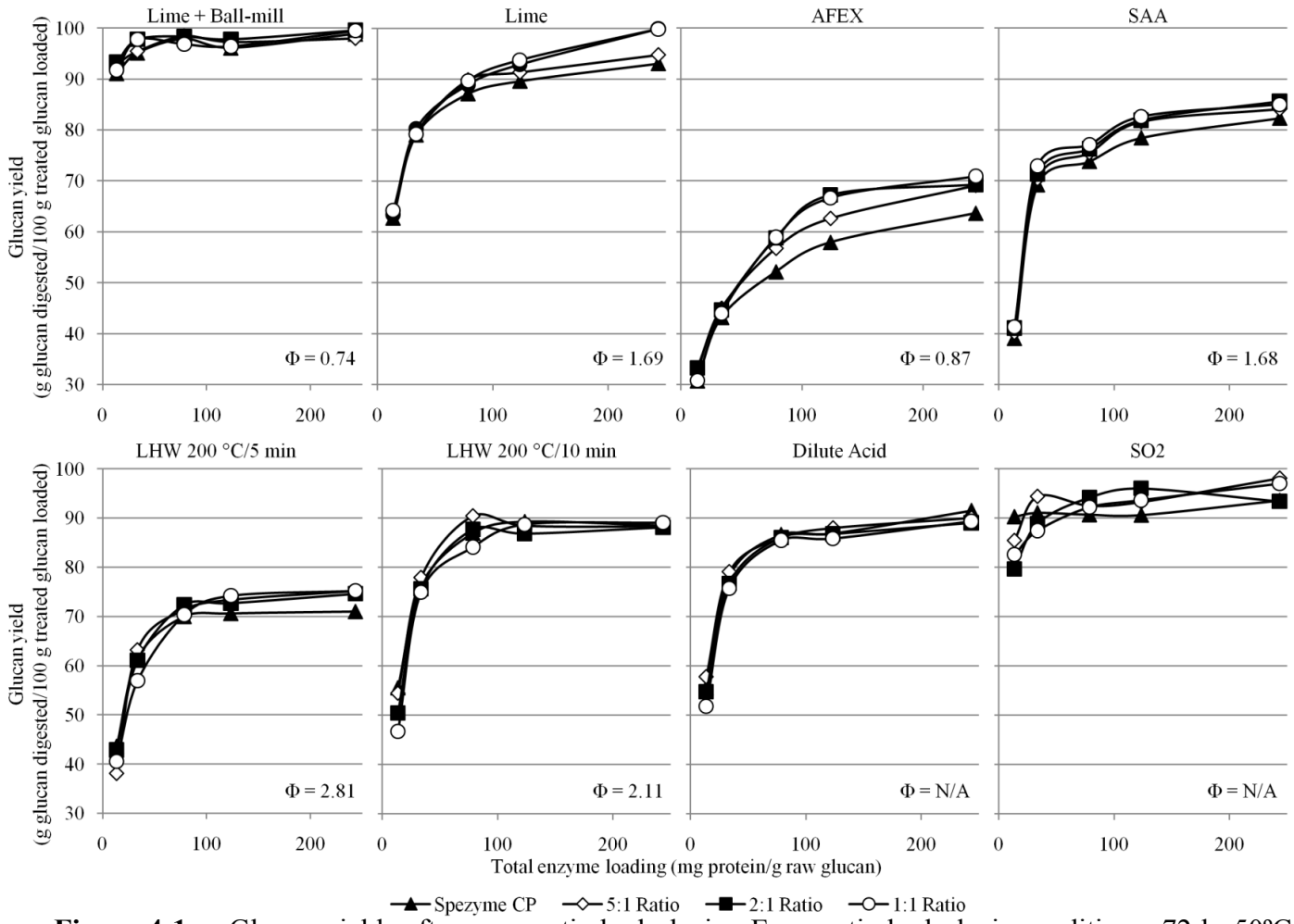


**Figure 4-1a.** Glucan yields after enzymatic hydrolysis. Enzymatic hydrolysis conditions: 72 h, 50ºC, and enzymes (cellulase, xylanase, β-glucosidase) were loaded on a raw glucan basis. Φ = Average standard deviation of enzymatic hydrolysis replicates. Ratio in the figure legend refers to cellulase:xylanase (g:g) loading ratio.

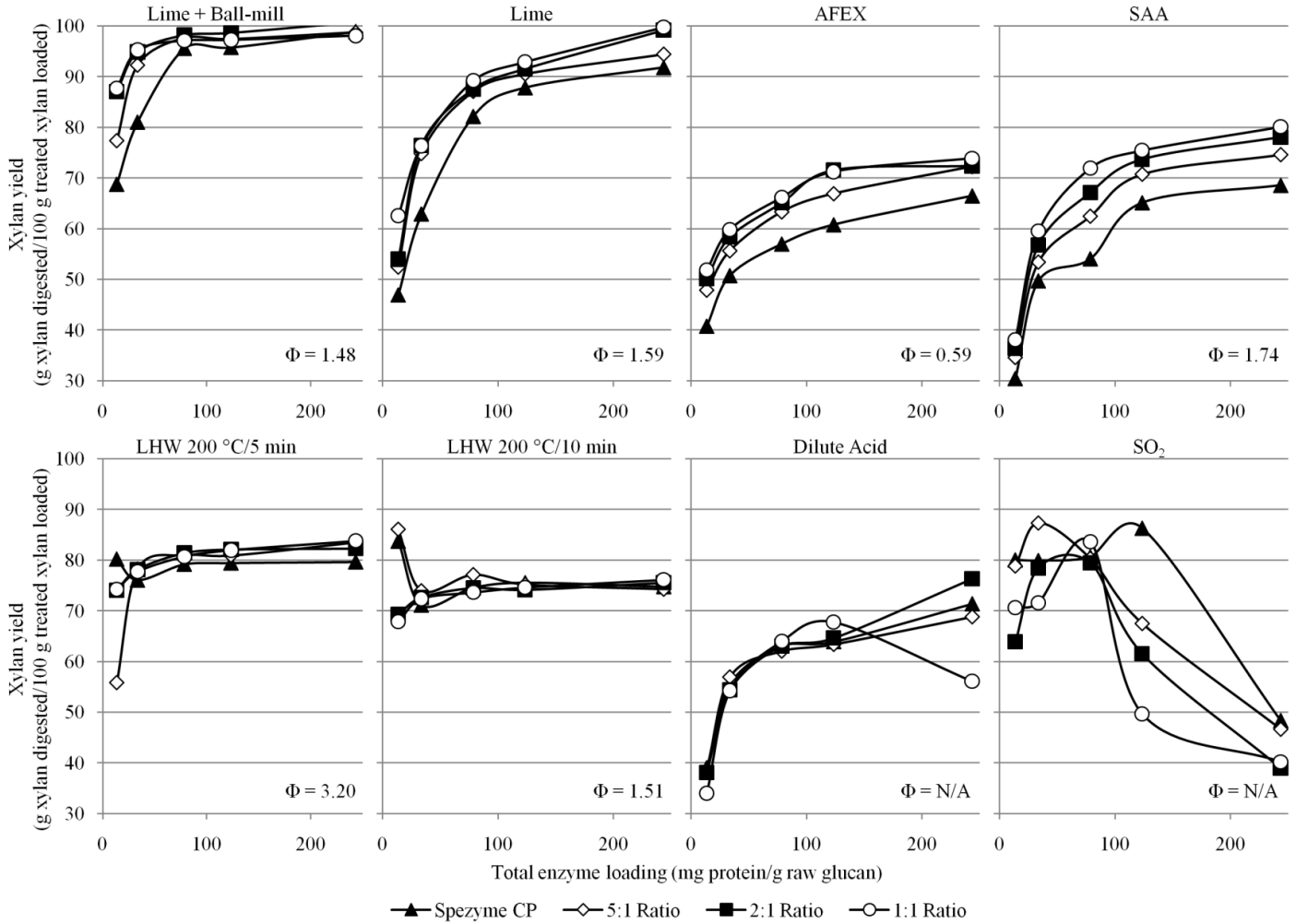


**Figure 4-1b.** Xylan yields after enzymatic hydrolysis. Enzymatic hydrolysis conditions: 72 h, 50ºC, and enzymes (cellulase, xylanase, β-glucosidase) were loaded on a raw glucan basis. Φ = Average standard deviation of enzymatic hydrolysis replicates. Ratio in the figure legend refers to cellulase:xylanase (g:g) loading ratio.

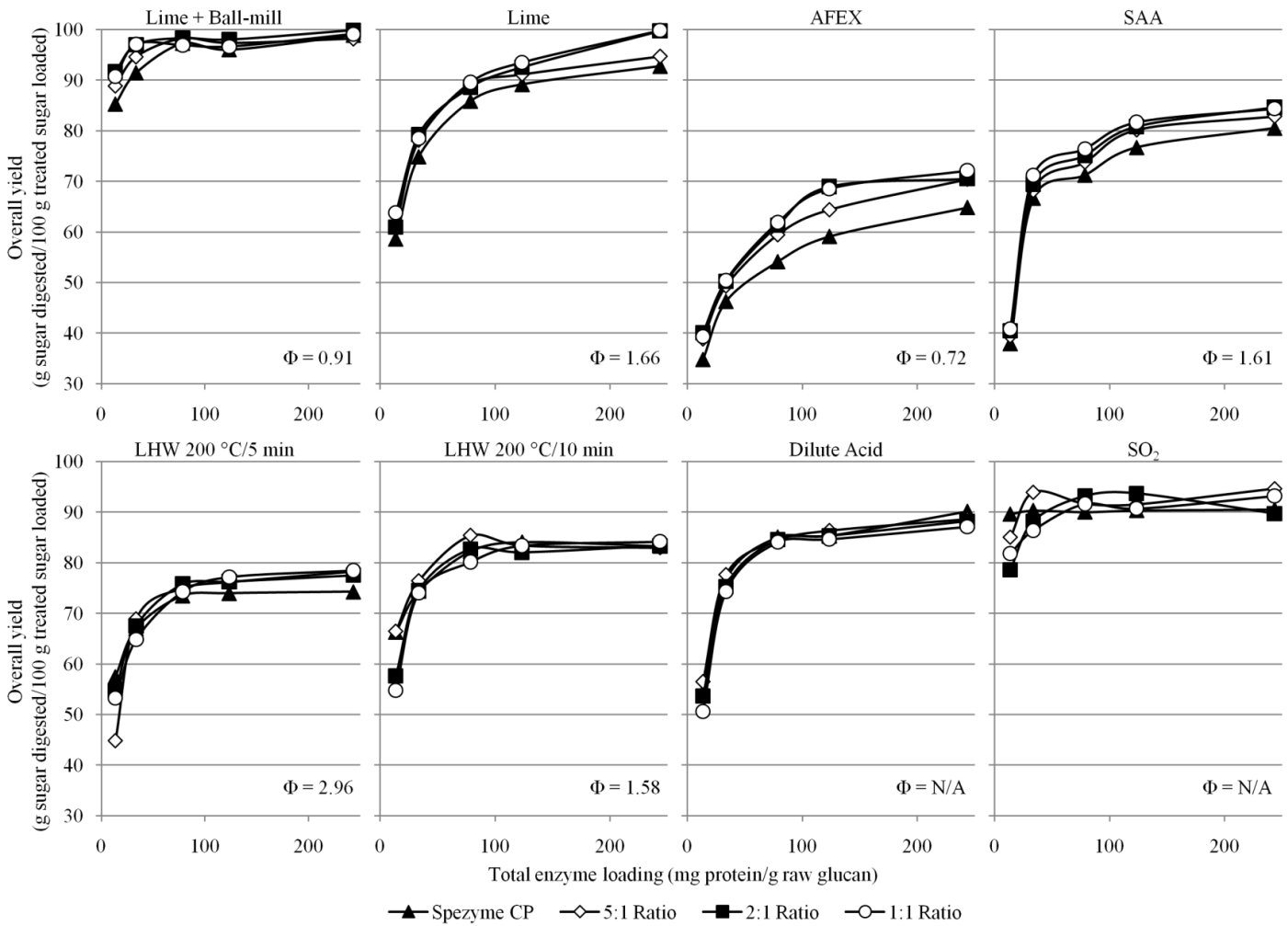


**Figure 4-1c.** Overall carbohydrate (glucan + xylan) yields after enzymatic hydrolysis. Enzymatic hydrolysis conditions: 72 h, 50ºC, and enzymes (cellulase, xylanase, β-glucosidase) were loaded on a raw glucan basis. Φ = Average standard deviation of enzymatic hydrolysis replicates. Ratio in the figure legend refers to cellulase:xylanase (g:g) loading ratio.

overall yield was 61.9% (1:1). [Note: AFEX was the only pretreatment that benefitted from higher enzyme loadings. At 243.4 mg protein/g raw glucan it reached a maximum overall yield of 72.1% at the 1:1 ratio.]

For SAA pretreatment, xylanase addition dramatically increased xylan yield (8.4%). With increased xylanase, xylan digestibility improved 13.1% (2:1) and 17.9% (1:1). At a total protein loading of 78.4 mg/g raw glucan, the maximum glucan yield was 77.1% whereas the maximum xylan yield was 72.0% (1:1). The SAA pretreatment contained only 13.6% xylan in its initial composition. Although xylanase addition significantly increased xylan yield, the increase in overall yield was more moderate, 2.6% (5:1), 3.9% (2:1), and 5.2% (1:1). A maximum overall yield of 76.5% (1:1) was achieved.

Compared to AFEX and SSA pretreatments, lime pretreatment had slightly less benefit from xylanase addition with a 4.9% xylan yield increase (5:1). Increased xylanase addition improved xylan yields by 5.4% (2:1) and 7.1% (1:1). Some increase in glucan yield (2.7%, 1.8%, 2.5%) and overall yield (3.3%, 2.7%, 3.7%) was also observed for the 5:1, 2:1, and 1:1 ratios, respectively. Lime pretreatment obtained a maximum overall yield of 89.6% (1:1).

Ball-milling the lime pretreated sample diminished the benefits of xylanase addition with improved xylan yields of 2.1% (5:1), 2.6% (2:1), and 1.5% (1:1). It outperformed all other pretreatments with a maximum overall yield of 98.3% (2:1).

The 200 °C/5-min LHW pretreatment slightly benefitted from xylanase addition with an increased xylan yield of 1.8% (5:1), 2.2% (2:1), and 1.5% (1:1). The most

promising ratio (2:1) improved glucan yield 2.4% and overall yield 2.3%. At this ratio, the maximum overall yield was 75.8%. For the 200 °C/10-min LHW pretreatment, only the 5:1 ratio increased xylan yield (2.6%). The 2:1 ratio showed negligible improvement and the 1:1 decreased xylan yield (–0.9%). The maximum overall yield was 85.4% (5:1).

The $SO_2$ pretreatment caused xylan yield changes of –0.4% (5:1), –1.2% (2:1), and 2.9% (1:1). The best-performing ratio (2:1) had an overall yield of 93.2%.

For dilute-acid pretreated switchgrass, the effect of xylanase addition was negligible with changes in xylan yields of –1.0% (5:1), 0% (2:1), and 0.9% (1:1). Glucan yield and overall yield decreased with increased xylanase addition. The maximum overall yield (91.2%) was achieved using just Spezyme CP.

Standard deviations were minimal for glucan, xylan, and overall yields of the AFEX, SSA, lime, and lime + ball-milling pretreatments. The yields of the LWH pretreatments produced higher standard deviations, making it difficult to determine if there was an improvement with added xylanase. Unfortunately, standard deviation was not provided for the $SO_2$ and dilute-acid pretreatments.

The optimum enzyme loading ratio was 1:1 cellulase:xylanase for the AFEX, SAA, and lime pretreatments. Lime + ball-mill, 200 °C/5-min LHW, and $SO_2$ pretreatments obtained maximum yields at an optimum ratio of 2:1. The optimum ratios for the 200 °C/10-min LHW and dilute-acid pretreatments were 5:1 and 1:0, respectively. The difference in optimal enzyme loading ratios is highly dependent on the

pretreated composition. AFEX and lime pretreatments, for example, had higher initial xylan compositions than dilute-acid and SO2 and thus favored higher xylanase loadings.

### 4.3.3 Enzymatic yield

The enzymatic yield is defined as the ratio of total carbohydrates digested per unit of protein loaded.

$$\text{Enzymatic yield} \equiv \frac{\text{total glucan} + \text{xylan digested}}{\text{total protein loaded}} \qquad [4\text{-}4]$$

Enzymatic yield is a useful tool to determine the optimal enzyme loading which results in high sugar yields while minimizing the use of costly enzymes. Figure 4-2 shows enzymatic yield as a function of total protein loading. As total protein loading increases, there are diminishing returns in overall yield.

Enzymatic yield can be used to compare the effectiveness of each pretreatment. At the lowest enzyme loading (13.4 mg protein/g raw glucan), Figure 4-2 shows that lime pretreatment has a maximum enzymatic yield of 64.2 g of sugar digested/g protein loaded. When ball-milling is added to the lime pretreatment, the enzymatic yield at the same enzyme loading, significantly increased to 91.3 g of sugar digested/g protein loaded. With knowledge of the cost of enzymes and of the mechanical process, the economic viability of using the mechanical process could be determined.

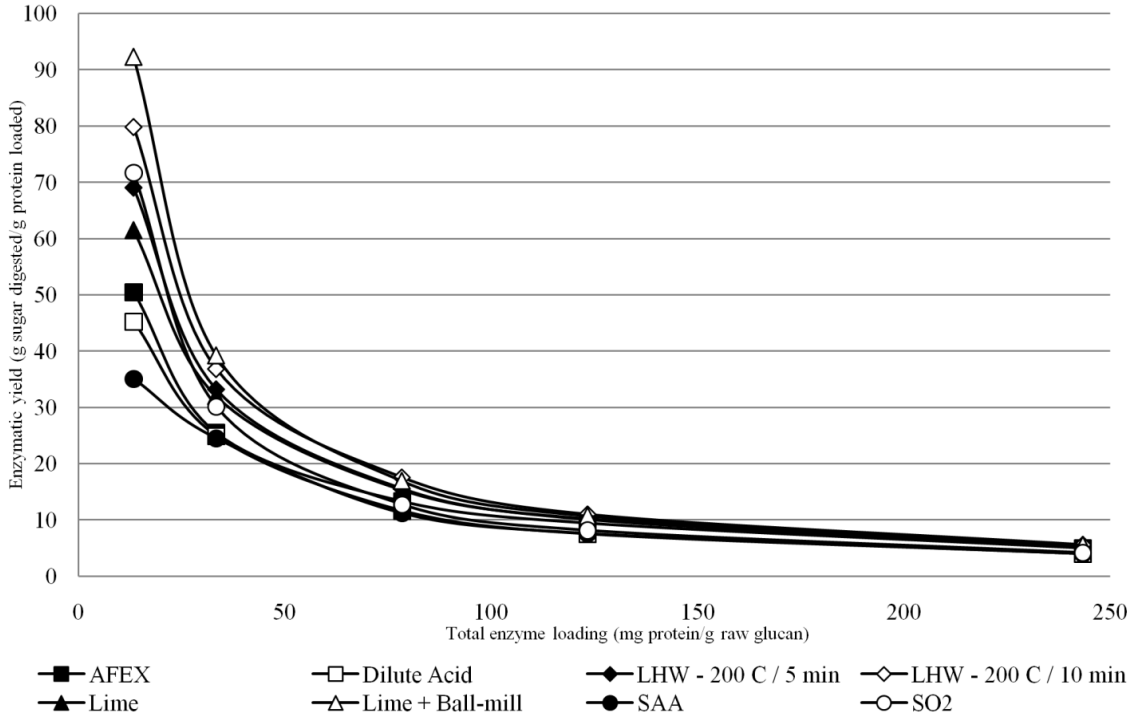


**Figure 4-2.** Enzymatic yield as a function of total protein loading.

Figure 4-3 compares enzymatic yield to overall yield. When designing a hydrolysis system, a typical goal is to achieve a target overall yield. From this plot, a desired overall yield specifies the enzymatic yield, which determines the required amount of enzyme for a desired mass of sugar.

Using lime pretreatment as an example, at a target overall yield of 80%, an enzymatic yield value of 22.6 g sugar digested/g protein is obtained for the Spezyme CP case. When xylanase is added to the enzyme cocktail, at the same target overall yield, the enzymatic yield increases to 27.6 g sugar digested/g protein. In these plots, it is also clear that lime + ball-milled pretreatment and $SO_2$ pretreatment were so effective at

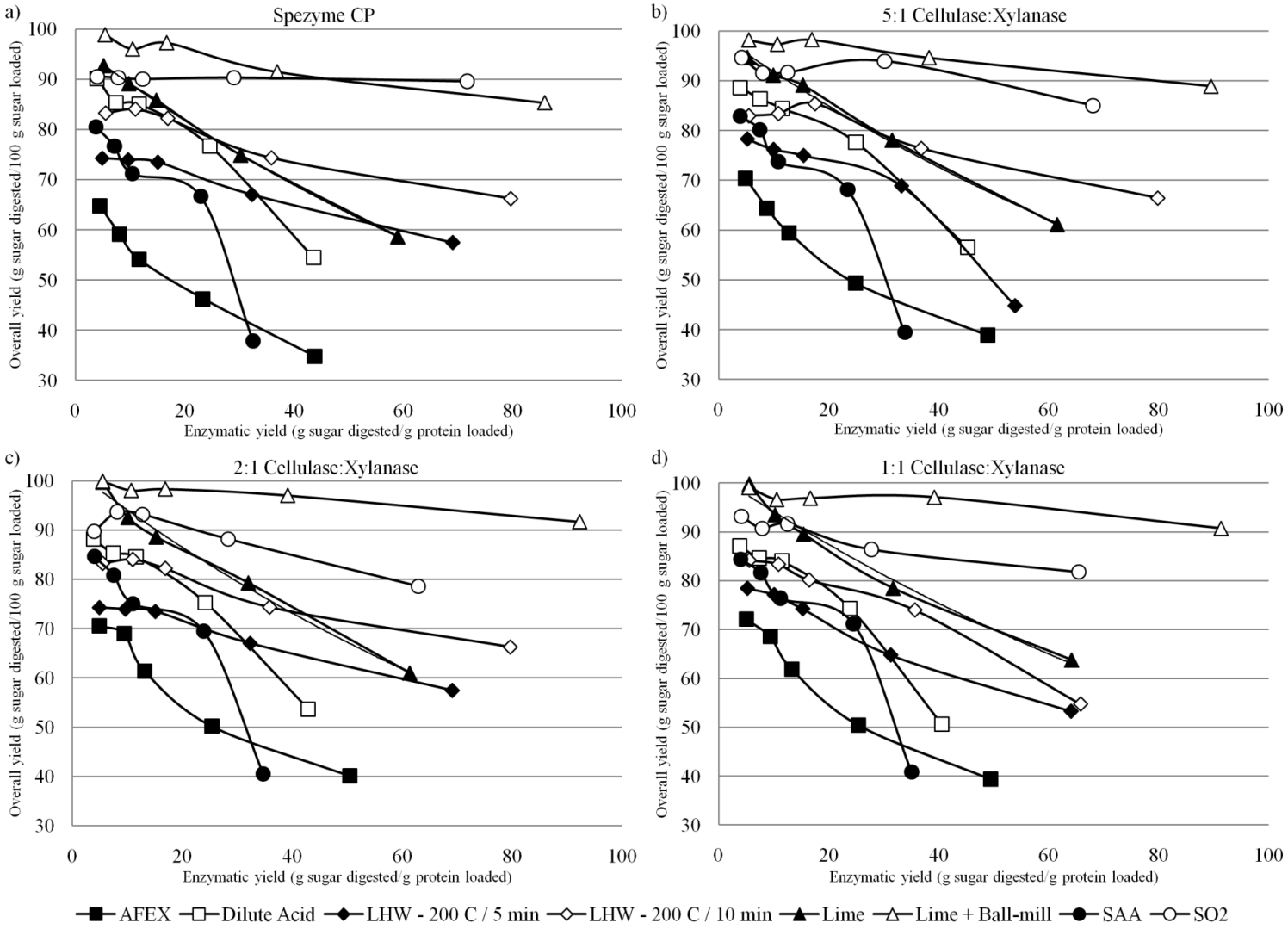


**Figure 4-3.** Enzymatic yield vs. overall yield.

increasing digestibility that regardless of enzymatic yield, they obtain high overall yields.

### 4.3.4 Economic study

According to a recent technoeconomic analysis on an enzymatic ethanol process (Aden & Foust, 2009), the current (2009) minimum ethanol selling price is $2.34/gal with the goal to obtain a minimum ethanol selling price of $1.33/gal ethanol by 2012. To achieve this, the author states that enzyme cost must be significantly reduced, while increasing enzymatic activity and pretreatment effectiveness. The enzymatic and overall yields measured in this study were used to estimate the current state of technology. (Note: One limit to this model is the assumption that the 1% glucan loading used for enzymatic hydrolysis is comparable to a commercially relevant glucan loading.) The current estimated cost of raw biomass is approximately $60/ton ($0.06/kg) with the price decreasing to $46/ton ($0.05/kg) by 2012 (Aden & Foust, 2009). Enzyme cost estimates are not readily available so Figure 4-4 shows cost contributions for a range of enzyme costs. Feedstock cost per liter of ethanol can be calculated as a function of raw biomass cost, pretreatment yield, pretreatment composition, overall yield, theoretical fermentation yield, actual yield, and ethanol density. Enzyme cost per liter of ethanol can be estimated using the cost of enzymes, enzymatic yield, theoretical fermentation yield, actual yield, and ethanol density. Assuming $0.06/kg biomass, $4.41/kg enzyme, 69.2% pretreatment yield, and 90% fermentation yield, a sample calculation using lime + ball-milling follows:

Total sugar composition

$$= \frac{0.486 \text{ kg glucan} \left(\frac{\text{kg glucose}}{0.9 \text{ kg glucan}}\right) + 0.187 \text{ kg xylan} \left(\frac{\text{kg xylose}}{0.88 \text{ kg xylan}}\right)}{\text{kg pretreated biomass}}$$

$$= \frac{0.753 \text{ kg sugar}}{\text{kg pretreated biomass}} \quad [4\text{-}5]$$

Feedstock cost

$$= \frac{\$0.06}{\text{kg raw biomass}} \times \frac{\text{kg raw biomass}}{0.692 \text{ kg pretreated biomass}}$$

$$\times \frac{\text{kg pretreated biomass}}{0.753 \text{ kg sugar}} \times \frac{\text{kg sugar}}{0.917 \text{ kg digested sugar}}$$

$$\times \frac{\text{kg digested sugar}}{0.51 \text{ kg EtOH}} \times \frac{1}{0.9} \times \frac{0.791 \text{ kg EtOH}}{\text{L EtOH}} = \frac{\$0.24}{\text{L EtOH}} \quad [4\text{-}6]$$

$$= \frac{\$0.90}{\text{gal EtOH}}$$

$$\text{Enzyme cost} = \frac{\$4.41}{\text{kg enzyme}} \times \frac{\text{kg enzyme}}{92.3 \text{ kg digested sugar}}$$

$$\times \frac{\text{kg digested sugar}}{0.51 \text{ kg EtOH}} \times \frac{1}{0.9} \times \frac{0.791 \text{ kg EtOH}}{\text{L EtOH}} = \frac{\$0.08}{\text{L EtOH}} \quad [4\text{-}7]$$

$$= \frac{\$0.31}{\text{gal EtOH}}$$

Figure 4-4 shows the calculated feedstock and enzyme costs for each pretreatment at the enzyme loading ratio that minimizes cost assuming $46/ton biomass. Aden and Foust estimate that all other costs (pretreatment, utilities, labor, capital, etc.) should contribute approximately $1.34/gal ethanol (current) or $0.73/gal ethanol (goal). Eggeman and Elander have shown that there is little difference in cost between pretreatment technologies (Eggeman & Elander, 2005). In the study, the most cost-effective pretreatment (as measured by feedstock and enzyme costs alone) was lime + ball-milling with an estimated cost of $2.55/gal ethanol (current) or $1.73/gal ethanol (goal). However, this pretreatment used a costly mechanical process that was not considered in Aden and Foust's estimated pretreatment cost, so further economic analysis is required. This case is included to show the potential benefit of developing an

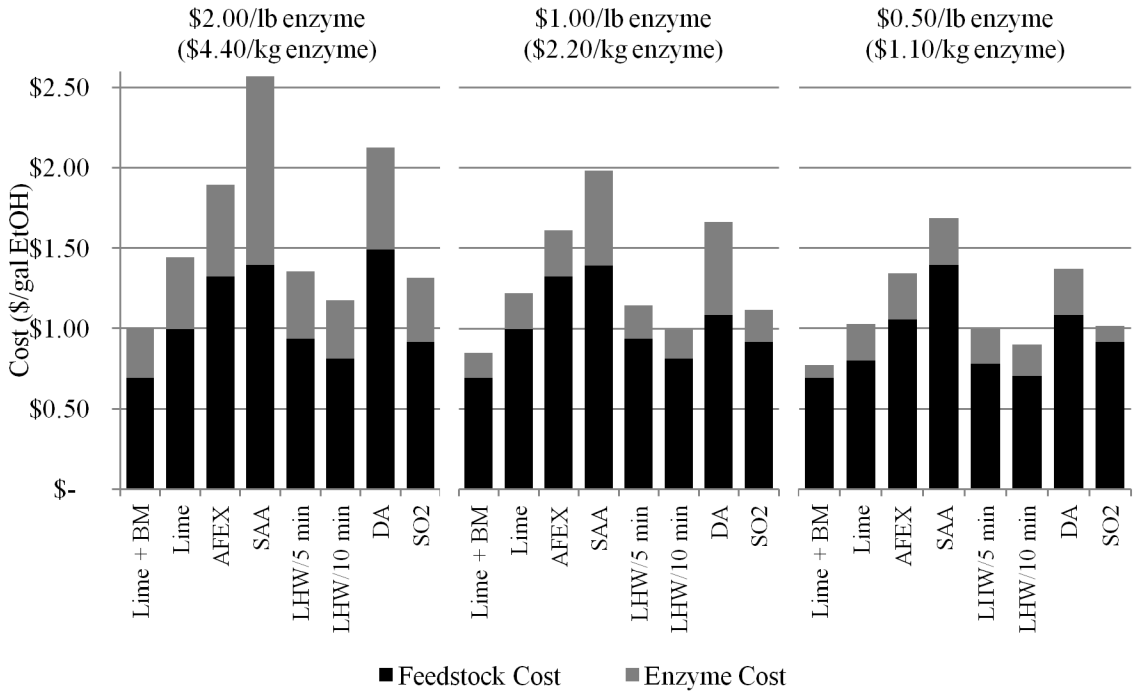


**Figure 4-4.** Enzyme and feedstock cost contributions for three bulk enzyme costs assuming $46/ton biomass.

economically feasible mechanical pretreatment technique. In the case of $SO_2$ treatment, which is similar to the pretreatment considered by Aden and Foust, the estimated cost is $2.94/gal ethanol (current) or $2.05/gal ethanol (goal). (Note: None of these cost estimates include credits for free sugars recovered in washing or pretreatment steps. When these sugars are included, costs will reduce accordingly.)

### 4.3.5 β-glucosidase effectiveness

Another purpose of this study was to determine the effectiveness of β-glucosidase addition when both cellulase and xylanase (5:1, 2:1, and 1:1) are used for hydrolysis. In the absence of β-glucosidase, the total enzyme loading was 30 mg protein/g raw glucan. With β-glucosidase addition, the total enzyme loading was 33.4 mg protein/g raw glucan. After a 72-h hydrolysis, overall carbohydrate yields were compared between the samples loaded with β-glucosidase and those without. The results are shown in Table 4-3.

Lime pretreatment showed the least benefit from adding β-glucosidase and overall yields were relatively unaffected. The opposite effect occurred with the lime + ball-milled pretreated sample. There was a dramatic increase in overall yield when β-glucosidase was added and there was a positive correlation with increased xylanase. The overall yield improved by 7.6% (5:1), 9.7% (2:1), and 10.0% (1:1).

The acidic pretreatments ($SO_2$, dilute-acid) achieved large gains in overall yield with β-glucosidase addition at the 5:1 ratio. Overall yield improved by 10.3% for the $SO_2$ pretreatment and 11.7% for the dilute acid pretreatment. For both pretreatments, the 2:1 and 1:1 samples showed little benefit from β-glucosidase addition.

**Table 4-3**. Comparison of overall yields with the addition of β-glucosidase. Yields reported as g glucan + xylan digested/100 g glucan + xylan loaded.

| | Without β-glucosidase 30 mg protein/g raw glucan | | | With β-glucosidase 34.4 mg protein/g raw glucan | | |
|---|---|---|---|---|---|---|
| | 5:1 | 2:1 | 1:1 | 5:1 | 2:1 | 1:1 |
| Lime + Ball-mill | 87.07 | 87.35 | 87.10 | 94.64 | 97.01 | 97.10 |
| Lime | 78.81 | 81.44 | 78.66 | 78.10 | 79.28 | 78.49 |
| AFEX | 44.96 | 45.72 | 45.59 | 49.33 | 50.24 | 50.42 |
| SAA | 65.04 | 65.49 | 67.29 | 68.16 | 69.46 | 71.18 |
| LHW 5 min | 66.00 | 64.11 | 64.18 | 68.90 | 67.54 | 64.81 |
| LHW 10 min | 73.20 | 70.29 | 66.40 | 76.42 | 74.50 | 74.02 |
| DA | 65.96 | 74.25 | 75.06 | 77.64 | 75.27 | 74.36 |
| $SO_2$ | 83.62 | 88.36 | 85.08 | 93.94 | 88.20 | 86.42 |

Like the lime + ball-milled pretreatment, AFEX showed a positive relationship between β-glucosidase addition and an increased xylanase ratio with yield increases of 4.4% (5:1), 4.5% (2:1), and 4.8% (1:1). This relationship was also seen in the LHW 200 °C/10-min case with improved overall yields of 3.2% (5:1), 4.2% (2:1), and 7.6% (1:1). The LHW 200 °C/5-min and SSA pretreatments had modest increases in overall yield with β-glucosidase addition, which ranged from 3–4%.

## 4.4 Conclusions

In all pretreatment cases, xylanase addition improved xylan yield and in all but the dilute-acid case, overall yields improved as well. Another key observation is that the optimum enzyme mixture depends on the composition of the pretreated material.

Pretreatments with lower xylan composition ($SO_2$, dilute-acid) had less benefit from xylanase addition. Although β-glucosidase typically is a small percentage of the overall enzyme mixture, in most cases it significantly improves overall yields. Enzymatic yield relates the mass of carbohydrates generated by enzymatic hydrolysis per mass of enzyme protein added and typically ranges from 10–90 kg of sugar digested/kg of protein.

# CHAPTER V

## DEVELOPMENT OF SHOCK TREATMENT AS A NOVEL MECHANICAL BIOMASS PRETREATMENT PROCESS

The combination of oxidative lime pretreatment and ball milling significantly improves the enzymatic digestibility of lignocelluloses; however, ball milling is energy intensive and prohibitively expensive at commercial scale. Shock treatment is a novel mechanical pretreatment process that, when combined with oxidative lime pretreatment, greatly increases the digestibility of biomass. This work determined the effectiveness of shock treatment on multiple feedstocks (sugarcane bagasse, corn stover, poplar wood, sorghum, and switchgrass), determined recommended shock treatment conditions for corn stover, and compared cellulose crystallinity and copper number of raw and shock-treated samples. At an enzyme loading of 5 FPU/g raw glucan, the combination of oxidative lime pretreatment (OLP) and shock treatment increased the 72-h glucan digestibility (g glucan digested/100 g glucan treated) of corn stover from 15.3 (untreated) to 84.6 (OLP + shock), an increase of +69.3 over untreated biomass. The other four biomass types showed similar gains in glucan digestibility when compared to the respective untreated biomass: +55.7 (bagasse), +81.6 (poplar wood), +48.2 (sorghum), and +73.1 (switchgrass). Recommended shock treatment conditions show that a single shock at room temperature with never-frozen biomass produces the most digestible corn stover.

## 5.1 Introduction

Dependence on foreign oil has led to fluctuating oil prices, economic instability, and military conflicts (Yang & Wyman, 2008). The United States has limited domestic petroleum production and imports approximately 60% of the net petroleum consumption. Furthermore, petroleum accounts for 40% of U.S. energy consumption (Energy, 2009). Renewable energy currently provides 8% of U.S. energy consumption; research efforts have focused on increasing this percentage. To maintain the current transportation infrastructure, the most desirable alternative energy solution should produce liquid fuels.

At present, the United States generates alternative liquid biofuels (ethanol) from corn grain. Early biofuel efforts adopted this food crop because the primary component (starch) is easily hydrolyzed to glucose, which can then be fermented to ethanol. Unfortunately, low crop yields and limited growth regions result in limited availability (Schmer et al., 2008). Additionally, corn grain is used as staple food for both humans and livestock, resulting in a highly controversial food vs. fuel debate (Dale, 2008; Foley et al., 2005; G. Cassman & Liska, 2007; Pimentel et al., 2009; Rosegrant & International Food Policy Research, 2008). The United States also exports large quantities of corn grain to developing countries to remediate starvation. A more promising approach is to replace corn with lignocellulosic biomass as the feedstock for biofuel production.

Lignocellulosic biomass is highly abundant, relatively inexpensive, and has potential for copious yields (Villas-BÙas et al., 2002). It is comprised of three primary components: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are both

carbohydrate polymers and combined generally comprise 60–80% of the biomass composition. Lignin is a highly cross-linked polymer composed of *p*-hydroxycinnamyl alcohol monomer units (Freudenberg, 1965; Kirk et al., 1977). It is covalently bound to hemicellulose and highly resists biochemical conversion (Holtzapple, 2003c). Lignocellulose can be obtained from a large variety of sources including energy crops (sorghum and energy cane), waste materials (industrial, food, and municipal solids), agricultural residues (corn stover and bagasse), and grasses (switchgrass) (Lee, 1997; Saha & Cotta, 2008).

Typical lignocellulosic ethanol processes have four primary steps: (1) pretreatment to increase cellulose accessibility and enzymatic reactivity, (2) enzymatic hydrolysis of carbohydrate polymers to free sugars, (3) fermentation of sugars to ethanol, and (4) ethanol recovery (Rabelo et al., 2009). A widely studied process is Simultaneous Saccharification and Fermentation (SSF). SSF combines Steps 2 and 3 into the same reactor to minimize product inhibition, capital costs, and contamination (Öhgren et al., 2007; Olofsson et al., 2008; Wyman et al., 1992). Two primary drawbacks to SSF are sterility requirements and different optimal temperatures for yeast and enzymes. A significantly different approach is the MixAlco™ process (Holtzapple et al., 1999), which relies on naturally occurring mixed cultures of bacteria to perform the hydrolysis and fermentation steps in the same vessel. The mixed culture uses a mixed-acid fermentation to generate carboxylic acids from the hydrolyzed free sugars. Downstream processing converts the mixed acid broth to alcohols, gasoline, or a variety

of chemicals (Pham et al., 2010). This process does not require aseptic conditions, and the mixed culture can process a large variety of non-sterile feedstocks.

One constant in most biological processes that convert lignocellulose to biofuels is the necessity for pretreatment (Step 1). Lignocellulose naturally resists enzymatic digestion, and the primary goal of biomass pretreatment is to remove some of the barriers that limit enzymatic digestibility, including high lignin content (Lynd, 1996; Taherzadeh & Karimi, 2008; Zhu et al., 2008), cellulose crystallinity (Fan et al., 1980; Puri, 1984), high degree of cellulose polymerization (Irwin et al., 1993; Kruus et al., 1995), low accessible surface area (Kumar & Wyman, 2008), small pore volume, and presence of acetyl groups on hemicellulose (Mosier et al., 2005). There are numerous pretreatment technologies being explored, each specializing in reducing one or more of these barriers. For example, alkaline pretreatments are highly successful at removing lignin, thereby increasing cellulose accessibility and eliminating non-productive adsorption sites (Lee & Fan, 1982). These pretreatments also completely remove acetyl groups from hemicellulose, which lowers steric hindrances of enzymes (Kong et al., 1992). Because of its low cost, compatibility with oxidants, ease of recovery, and ease of use, a promising alkaline agent is lime (Holtzapple & Davison, 1999).

Physical pretreatments have shown promise in enhancing biomass digestibility. A proven technique is ball milling (Bertran & Dale, 1985; Fan et al., 1980; Fan et al., 1981); however, because of long residence times and excessive energy requirements, it is only a valuable laboratory tool. Acoustic cavitation and hydrodynamic cavitation are

examples of other physical pretreatment processes that have been explored with moderate success (Coward-Kelly, 2002; Jones, 2007).

This work explored the use of a shock tube to increase lignocellulose digestibility. A classic shock tube consists of a uniform-cross-section tube that is filled with a low-pressure and a high-pressure gas, separated by a diaphragm. The shock wave is initiated by rupturing the diaphragm. Shock tubes have traditionally been used by kineticists to study thermal decomposition reactions, oxidation reactions, and even some heterogeneous reactions (Bhaskaran & Roth, 2002; Hong et al., 2011). A more recent use is the Hydrodyne process, a patented process developed to tenderize red meat (Long et al., 2007; Solomon et al., 1997). This has been further expanded to tenderize other types of meat, including chicken breasts (Claus et al., 2001; Meek et al., 2000). The effect of hydrodynamic shock waves on biomass digestibility has not previously been explored.

## 5.2 Materials and Methods

### 5.2.1 Substrate and enzymes

Experiment 1 used bagasse, corn stover, poplar wood, sorghum, and switchgrass. These biomass species were dried, milled, and then stored in large drums until use. Experiment 2 used corn stover from the same batch as Experiment 1. Experiment 3 used commercially available copy paper and commercially available microcrystalline cellulose (CAS 9004-34-6) purchased from Fisher Scientific (Pittsburgh, PA).

Cellulase was Spezyme CP® (lot 301-04075-054, 82 mg protein/mL, 59 FPU/mL), which was kindly provided by Genencor®, a Danisco Division. β-

glucosidase was Novozyme 188® (67 mg protein/mL, 600 CBU/mL) and was obtained from Sigma Aldrich (St. Louis, MO).

### 5.2.2 Pretreatment methods

*Substrate preparation*

For Experiments 1 and 2, the biomass was further dried to approximately 5% moisture then ground in a coffee grinder to a particle size of –20/+80 mesh. For Experiment 3, the copy paper was shredded into 1/2-in × 1/8-in strips using a commercial paper shredder.

*Oxidative lime pretreatment*

Oxidative lime pretreatment (OLP) was conducted in either a 4- or 8-L Parr reactor (Experiment 1 or 2, respectively). The reactor was loaded with equal amounts of dry biomass and calcium hydroxide (loaded in excess), and water (15 g/g dry biomass). Constant-pressure pure oxygen was supplied through a flexible stainless steel hose attached to an oxygen tank. The reactor contents were stirred and heated to the desired reaction temperature. Initial heat-up time of the reaction contents was included in the overall reaction time. Upon completing the desired reaction time, the heating and stirring elements were disabled and the reaction pressure was relieved. The reactor contents were transferred to a 5-L plastic beaker, which was cooled in an ice bath. Once cooled, the reaction contents underwent post-pretreatment conditioning.

*Post-pretreatment conditioning*

The OLP biomass slurry was neutralized using 5-N HCl to a pH of approximately 4.0, and then underwent several washings with distilled water until the

pH of the slurry rose to approximately 6.0. The final slurry was vacuum filtered and the moisture content and final solid weight were recorded to obtain the pretreatment yield. The solids were air-dried until the moisture content reduced to approximately 5%.

*Shock treatment*

The shock tube apparatus (Figure 5-1) consisted of a 20-in steel pipe (4-in Sch. 40) with circular metal flanges (1-in thick; 9.5-in diameter) welded onto each end. Each open end of the flanges is sealed with an additional circular steel flange and a gasket, held by eight 5/8-in bolts at each end.

The upper metal flange had an 11-in-long steel cylinder (3.81-in O.D.) welded onto it. This cylinder was bored to form a cone shape on the inside, with the largest inner diameter (3.56 in) at the bottom portion of the cylinder and the smallest inner diameter (0.88 in) at the top of the cylinder. This cylinder extended 11 in down into the shock tube to help focus the shock waves onto the biomass. A 27.5-in-long barrel (1-in Sch. 40) was welded on to the top of this cone, and the shotgun shell fit inside the open top end. Threading at the top of the barrel allowed the firing mechanism to be securely fastened. The firing mechanism consisted of a spring-loaded firing pin, which struck the top of the shotgun shell to discharge it. When the apparatus was fully sealed and bolted shut, the total interior volume of the shock tube was 3.02 L. The volume below the cone was 2.45 L.


**Figure 5-1.** Shock tube apparatus. (a) Entire apparatus, (b) barrel and cone, (c) shotgun shell loaded in barrel, (d) firing mechanism.

Before shock treatment, biomass samples were weighed in 100-g batches and stored in labeled freezer bags. For frozen samples, the desired amount of water (typically 200 mL) was added to the biomass in the freezer bag, mixed thoroughly, and the sample was stored in the freezer. For non-frozen samples, the 200 mL of water was added and mixed the day of the shock treatment, before being delivered to the shock tube site. Frozen samples were removed from the freezer and allowed to thaw in a 50°C oven for 20 min. When removed, they were placed on ice to be transported to the shock tube site. Non-frozen samples were also transported on ice.

If ambient temperature control was desired, the shock tube was placed in a temperature-controlled water bath (approximate dimensions: 28 in × 17.5 in × 17 in). The upper flange of the shock tube was removed; the biomass sample and the desired amount of water were loaded into the shock tube. The gasket was properly centered on the metal flange, the upper section of the shock tube was re-lowered into position, and the eight nuts and bolts around the flange were tightened. The shotgun shell (Winchester Expert High Velocity 3 1/2-in, 1 3/8-oz steel BB shot) was loaded into the barrel, the firing mechanism was affixed, and the shotgun shell was discharged.

To remove the treated sample, the upper flange was unbolted, and the upper section of the shock tube was lifted away. The shocked material was then gathered and filtered through a sieve to remove the steel shot, plastic wadding, and any other non-biological material. The shocked material was transferred to a freezer bag and transported back to the lab. In the lab, the material was inspected again for shotgun shell remnants, and then air dried for analysis.

*Ball-milling*

The OLP solids were thoroughly dried (moisture content < 5%) before ball milling in a 2-L porcelain jar loaded with 0.375-in-diameter zirconia grinding medium. The grinding medium was loaded to fill 50% of the jar volume (approximately 1806 g) and biomass was loaded at a ratio of 43 g grinding medium/g dry biomass. The jars were sealed and placed on rollers rotating at 68 rpm for 72 h.

*Modified Walseth-treatment*

The purpose of this procedure was to swell the microcrystalline cellulose used in Experiment 3. Cellulose (100 g) was added to a mixture of distilled water (220 mL) and 85% $H_3PO_4$ (4 kg). The solution was stirred until all the cellulose had dissolved to form a viscous mixture. This mixture was slowly added to a large vat of stirred water (6 L) to precipitate the cellulose. The water was changed frequently to prevent a build-up of acid. The precipitated cellulose was filtered using a large, sintered glass filter. The cellulose was then washed with water until the pH of the filtrate equaled the pH of the fresh water. The cellulose was air-dried and then milled to a consistent particle size (–20/+80 mesh).

### 5.2.3 Compositional analysis

Compositional analysis was performed on the raw and pretreated samples. The material was prepared by air drying to a measured moisture content of less than 10%. The composition was analyzed using NREL acid hydrolysis procedure TP-510-42618 (Sluiter et al., 2008b). The sample (0.3 g) was weighed into a glass test tube followed by adding 3 mL of 72 wt % sulfuric acid. The test tubes were placed in a 30°C water bath

and stirred regularly for 1 h. The contents of the test tube were quantitatively transferred to glass autoclave bottles using 84 mL of distilled water, capped, sealed, and steam autoclaved at 121°C for 1 h. Samples were cooled, opened, and filtered through glass filtering crucibles, which were placed in a 105°C oven to dry. The filtrate was neutralized and then analyzed for carbohydrates using HPLC Analysis (Bio-Rad Aminex HPX-87P column, HPLC-grade water mobile phase, 0.6 mL/min, 80°C column temperature). The weight of the dried, filtered solids minus their ash weight was recorded and used to calculate lignin content. Ash content was determined by heating samples in a 575°C furnace until completion. The extractives were determined by extracting the biomass with 95% ethanol for 24 h in a Soxhlet apparatus. The measured compositions for both the raw and pretreated materials were used in the enzymatic hydrolysis loading calculations.

### 5.2.4 Enzymatic hydrolysis

The enzymatic hydrolysis procedure for both glucan and xylan closely followed the enzymatic saccharification procedure (TP-510-42629) provided by NREL (Selig et al., 2008). Hydrolysis samples were prepared in 50-mL plastic centrifuge tubes. The loading weight of pretreated biomass was calculated based on moisture content and glucan composition to yield 0.1 g glucan per sample. Sodium citrate buffer (5 mL, 0.1-M, pH 4.8), 0.04 mL tetracycline (10 mg/mL in 70% ethanol), 0.04 mL cycloheximine (10 mg/mL in distilled water), 1 mL of each enzyme dilution (cellulase, β-glucosidase), and an appropriate volume of water were added to bring the total working volume to 10 mL. Enzyme dilutions were calculated on a raw glucan basis using enzyme activity and

a desired enzyme loading. Hydrolysis occurred in a shaking incubator (100 rpm) at 50°C for the desired hydrolysis time. To quench the hydrolysis, the samples were placed in a 105°C oven for 5 minutes and then cooled in an ice bath. Samples were stored in a freezer until HPLC analysis. HPLC analysis (Bio-Rad Aminex HPX-87P column, HPLC-grade water mobile phase, 0.6 mL/min, 80°C column temperature) was used to measure the glucose and xylose concentrations of each sample. These concentrations were then recalculated as equivalent glucan and xylan concentrations to report digestibility yields.

### 5.2.5 Crystallinity analysis using x-ray diffraction

Cellulose crystallinity was measured using x-ray diffraction as described by Segal et al. (1959). Samples were air dried to less than 10% moisture, and ground to pass through a 40-mesh screen. Data collection was performed by the Texas A&M University Crystal and Molecular Structure Laboratory using a Bruker D8 Advance (Bragg Brentano geometry; CuKa: 40 kV, 40 mA) fitted with LynxEYE detector. The samples were scanned at 2°/min from 2θ = 10° to 26° with a step size of 0.05°. The crystallinity index (CrI) was determined using the following formula:

$$\mathrm{CrI} = \frac{\mathrm{I_{002}} - \mathrm{I_{am}}}{\mathrm{I_{002}}} \times 100 \qquad [5\text{-}1]$$

where $I_{002}$ = maximum intensity of the 002 peak at 2θ = 22.5° and $I_{am}$ = intensity at 2θ = 18.7°.

### 5.2.6 Copper number assay

The copper number method described by Braidy was used to determine the reducing end-groups of cellulose and hemicellulose. This is an empirical approach best suited for comparative purposes.

The alkali solution (Reagent A) consisted of 130 g anhydrous sodium carbonate and 50 g sodium hydrogen carbonate per liter of water. The copper solution (Reagent B) consisted of 100 g copper (II) sulfate pentahydrate per liter of water. Sulfuric acid (140 mL, 93%) and $(NH_4)_2SO_4 \cdot Fe_2(SO_4)_3 \cdot 24\ H_2O$ (100 g) were combined with 1 L of water to make the ferric iron solution (Reagent C). Ceric ammonium sulfate (25.3 g), 93% sulfuric acid (30 mL), and 1 L of distilled water were mixed together to create 0.04-N ceric ammonium sulfate solution (Reagent D). Reagent E was commercially available ferroin indicator (CAS No. 5144-89-8, Ricca Chemical Company, Arlington, TX), and Reagent F was 2-N sulfuric acid.

A solution consisting of 1 part Reagent B and 19 parts Reagent A was added (10 mL) to a culture tube. Dry sample (0.25 g) was added to the culture tube, which was then sealed and heated in a boiling water bath for 3 h. The solution was filtered through a coarse, fritted-glass Gooch crucible with a glass fiber filter bottom. The sample in the crucible was washed with hot 1:1 water:Reagent A, and the filtrate was discarded. One more wash was performed with hot water, and the filtrate was once again discarded. The entrapped copper (I) oxide was dissolved using two 5-mL portions of Reagent C, and the wash was collected in a 100-mL vacuum flask. The sample was then washed with 10 mL of Reagent F, and the filtrate was collected in the same 100-mL vacuum flask.

Ferroin indicator (2–3 drops) was added to the collected wash. Reagent D (1 part) and distilled water (3 parts) were mixed to prepare 0.1-N ceric ammonium sulfate. The collected wash was titrated with the 0.01-N ceric ammonium sulfate until a color change was observed (pale orange to pale green).

The copper number is given by the formula:

$$\text{Copper number} = 0.06354\ \frac{t}{w}$$

where $t$ = volume of 0.01-N ceric ammonium sulfate (mL) and $w$ = dry weight of sample (g). The copper number must be between 0 and 4.5. If a copper number greater than 4.5 was obtained, the procedure was repeated using less initial sample.

## 5.3 Results and Discussion

### 5.3.1 Early design

The shock tube underwent several iterations before its current design. Initially, the shock tube only consisted of the main steel pipe and flanges, lacking both the barrel and the inner cone. Several variables were systematically studied using this preliminary design including shock tube volume, water loading, solid loading, water temperature, and repeated shocks. Bagasse (raw, knife-milled, and unmilled lime-treated) was the substrate used, and enzymatic hydrolysis and crystallinity measurements were used to determine the performance of each condition, with the hope of creating a highly digestible, low-crystallinity biomass.

The first condition tested was the available volume of the shock tube. It was hypothesized that reducing the available volume would result in a more effective pretreatment. The shock tube volume was reduced using two 5-in-long spacers

constructed of 3.5-in-diamater steel pipe. The next set of experiments examined the effect of water and biomass loading on shock tube performance. First, biomass loading was held constant while the water loading was varied from 0 to 3 L. Using the most successful water loading, the biomass loading was then varied from 125 to 200 g. Next, the temperature of the loading water was varied from 0 to 80 °C. Finally, the effect of multiple shocks on the biomass was tested.

Several important conclusions were drawn from this preliminary set of experiments. Reducing the shock tube volume using spacers had no significant effect on either crystallinity or digestibility. In general, water loading and biomass loading had little effect changing the crystallinity of the biomass. Additionally, the temperature of the loading water showed little significance suggesting that moderate temperatures (20 to 40 °C) were adequate. In terms of biomass digestibility, the shock tube samples showed on average a 35% improvement over non-shocked biomass for a 6-h hydrolysis, and 22% increase for the 3-d hydrolysis. This suggested that the benefits of shock treatment are more prominent with initial rate, but also improved the extent of digestion. Although these results (Jones, 2007) were mostly inconclusive, the consistent improvement in biomass digestibility suggested the potential benefits of further exploration.

The next key step in the shock tube development was adding barrel and inner cone. After adding these components, it was necessary to systematically examine if they improved the shock treatment. Once again, the substrate was bagasse (lime-treated) and three variables were explored: water loading, presence of the barrel, and presence of the

inner cone. Results were again inconsistent, but the general consensus was that adding the barrel and inner cone to the shock tube improved shock treatment performance.

After completing this preliminary set of experiments, the shock tube design was finalized and promising results were achieved; however, proving repeatability was necessary. To go forward, a consistent approach to experimental design, procedures, and performance analysis was required.

### 5.3.2 Experiment 1 - Multiple feedstock study

The purpose of this experiment was to prove the consistency and effectiveness of shock treatment using five different biomass feedstocks: bagasse, corn stover, poplar wood, sorghum, and switchgrass. Furthermore, this study compared the difference in digestibility between untreated, oxidative lime pretreated (OLP), OLP + ball-milled, and OLP + shock-treated samples. Table 5-1 shows raw and OLP compositions determined by acid hydrolysis. Table 5-2 shows the conditions used for OLP of each biomass. For each shock, the shock tube was loaded with biomass (100 dry g) and water (2 L). Pretreatment performance was determined using 24- and 72-h enzymatic hydrolysis with cellulase loadings of 5 (Figure 5-2), 15 (Figure 5-3), and 60 (Figure 5-4) FPU/g raw glucan. Every sample employed a β-glucosidase loading of 60 CBU/g raw glucan. Enzymatic hydrolysis was performed in triplicate. The following discussion focuses on glucan digestibility resulting from the low enzyme loading (5 FPU/g raw glucan).

**Table 5-1.** Composition of untreated and oxidative lime-pretreated biomass.

| | Untreated | | | | |
|---|---|---|---|---|---|
| **Constituent** | **Switchgrass** | **Poplar Wood** | **Bagasse** | **Corn Stover** | **Sorghum** |
| **Glucan** | 32.8% | 42.1% | 37.6% | 34.2% | 32.6% |
| **Xylan** | 24.2% | 18.8% | 25.6% | 26.1% | 21.9% |
| **Lignin** | 19.4% | 21.6% | 17.2% | 16.1% | N/M |
| **Ash** | 2.8% | 1.1% | 5.2% | 5.2% | N/M |
| **Extractives** | 5.9% | 3.1% | 4.2% | 13.3% | N/M |
| **Other** | 15.0% | 13.4% | 10.2% | 5.2% | N/M |

| | Oxidative lime pretreated | | | | |
|---|---|---|---|---|---|
| **Constituent** | **Switchgrass** | **Poplar Wood** | **Bagasse** | **Corn Stover** | **Sorghum** |
| **Glucan** | 40.5% | 47.8% | 46.3% | 41.7% | 43.5% |
| **Xylan** | 19.7% | 16.5% | 25.5% | 24.1% | 18.9% |
| **Lignin** | 16.3% | 19.7% | 13.5% | 14.1% | N/M |
| **Ash** | 10.4% | 5.4% | 3.6% | 11.5% | N/M |
| **Extractives** | 0.0% | 0.0% | 0.0% | 0.0% | N/M |
| **Other** | 13.0% | 10.6% | 11.0% | 8.5% | N/M |

**Table 5-2.** Pretreatment conditions and pretreatment solids yield.

| **Biomass** | **Temperature (°C)** | **Pressure (bar)** | **Time (h)** | **Solids Yield (%)** |
|---|---|---|---|---|
| Bagasse | 110 | 6.89 | 2 | 70.61 |
| Corn Stover | 110 | 6.89 | 4 | 73.95 |
| Poplar Wood | 160 | 13.79 | 2 | 74.57 |
| Sorghum | 180 | 6.89 | 2 | 66.96 |
| Switchgrass | 120 | 6.89 | 4 | 67.56 |

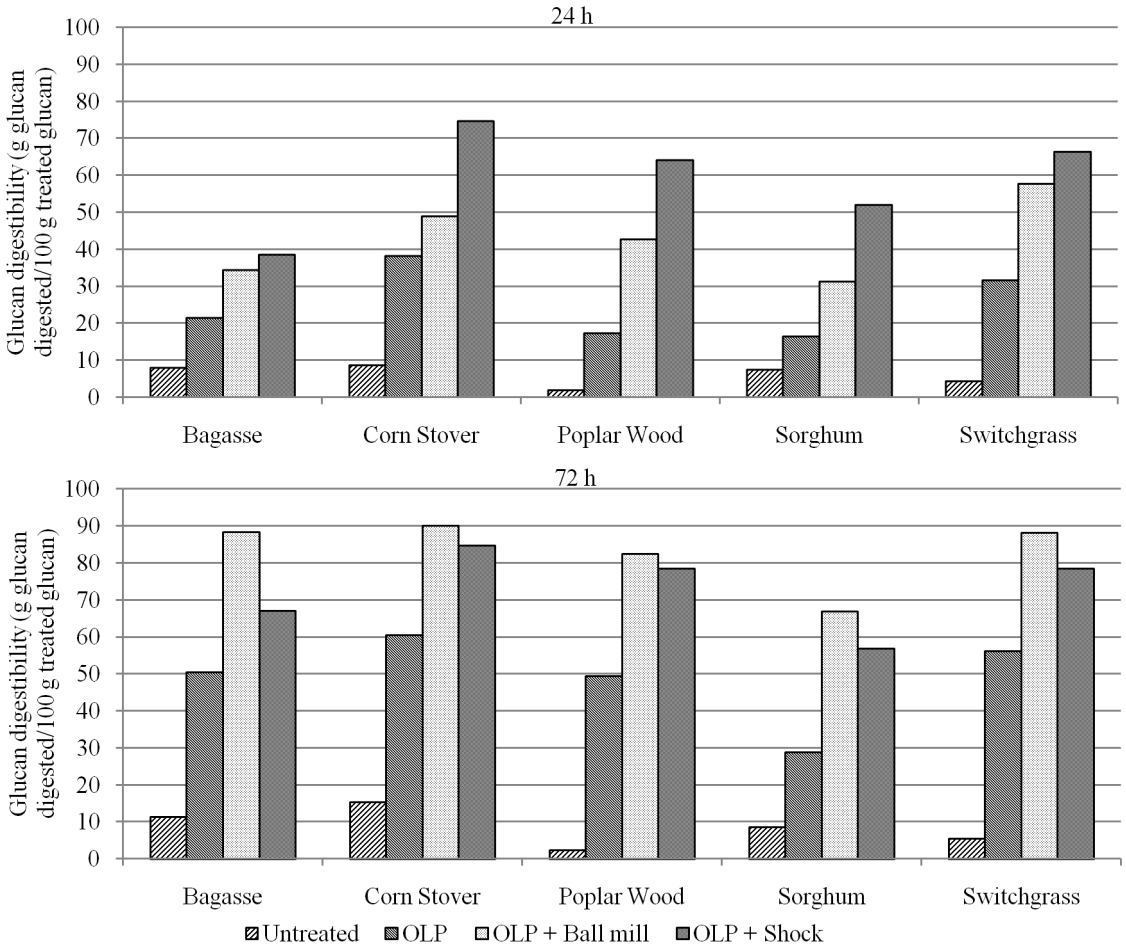


**Figure 5-2.** Glucan digestibility yields for bagasse, corn stover, poplar wood, sorghum, and switchgrass. Enzymatic hydrolysis was performed for 24 and 72 h, with a cellulase loading of 5 FPU/g raw glucan and a β-glucosidase loading of 60 CBU/g raw glucan.

*24-h Enzymatic hydrolysis*

For the 24-h hydrolysis, sugarcane bagasse was the least digestible overall. Untreated bagasse had a glucan digestibility (g glucan digested/100 g treated glucan) of only 7.8 and OLP increased the digestibility to 21.3. Adding ball milling and shock treatment further improved digestibility to 34.2 and 38.4, respectively.

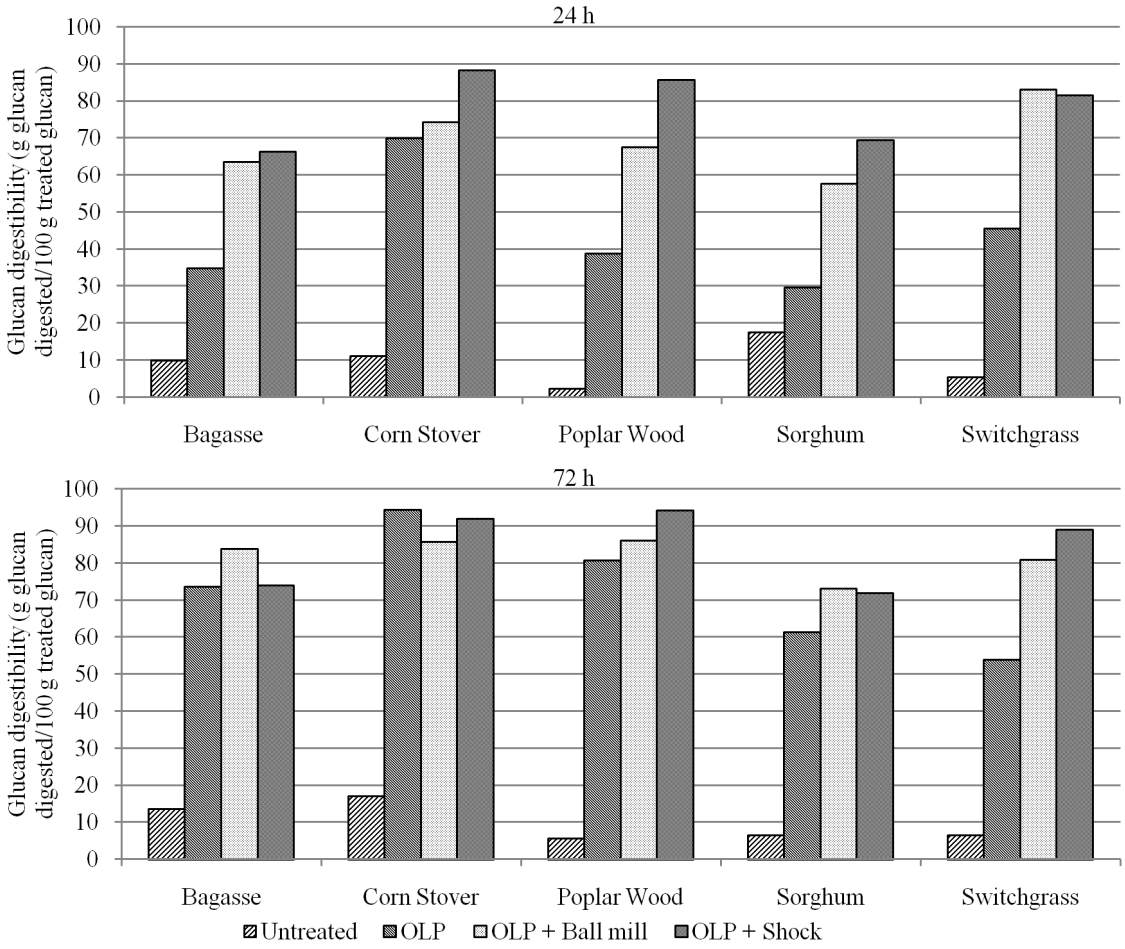


**Figure 5.3.** Glucan digestibility yields for bagasse, corn stover, poplar wood, sorghum, and switchgrass. Enzymatic hydrolysis was performed for 24 and 72 h, with a cellulase loading of 15 FPU/g raw glucan and a β-glucosidase loading of 60 CBU/g raw glucan.

After 24 h, corn stover was the most digestible. Glucan digestibility of OLP corn stover (38.1) was significantly higher than raw corn stover (8.5). Ball milling slightly improved digestibility to 48.9; however, shock treatment resulted in a very significant increase in digestibility (74.6).

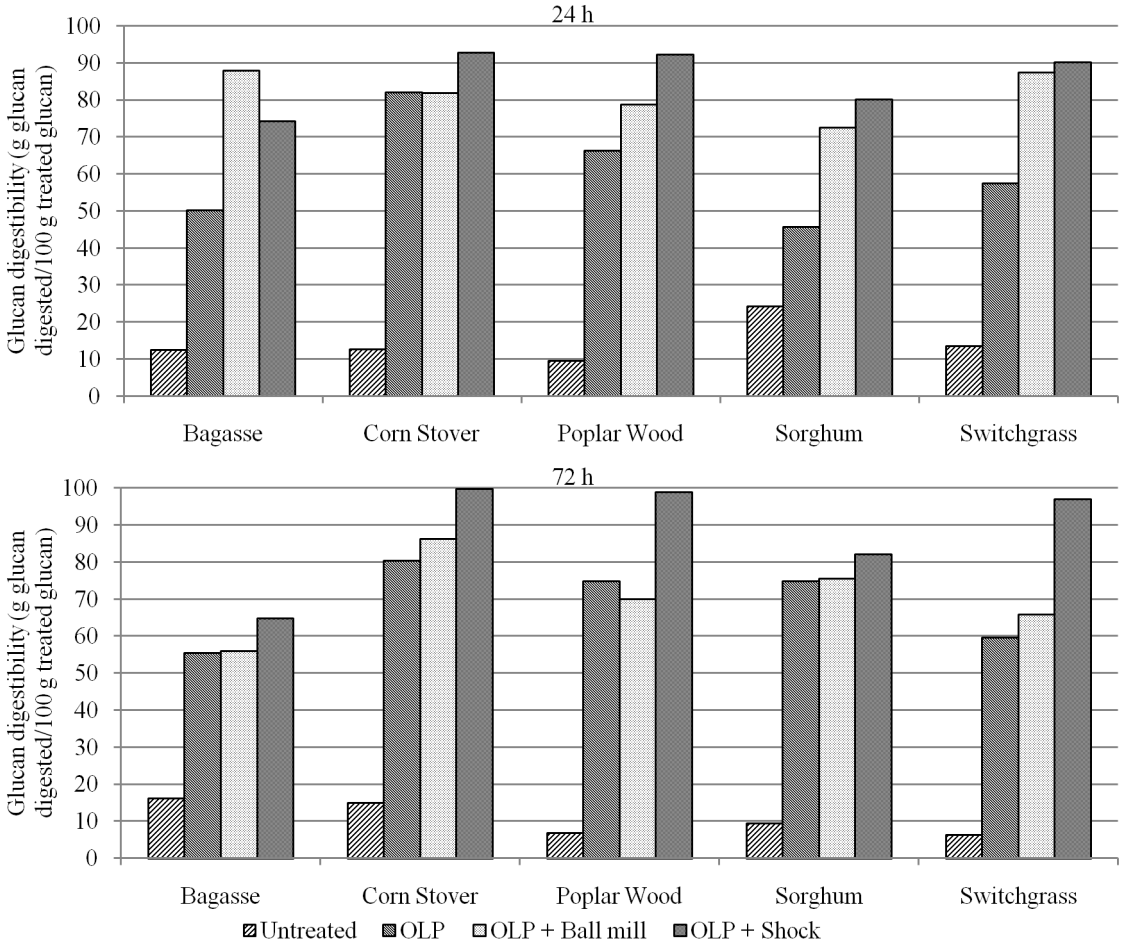


**Figure 5-4.** Glucan digestibility yields for bagasse, corn stover, poplar wood, sorghum, and switchgrass. Enzymatic hydrolysis was performed for 24 and 72 h, with a cellulase loading of 60 FPU/g raw glucan and a β-glucosidase loading of 60 CBU/g raw glucan.

Raw poplar wood proved to be the most resistant to enzymatic conversion (1.7), and OLP yielded only slight improvement (17.1); however, physical pretreatment methods greatly increased conversion. OLP + ball mill and OLP + shock treated poplar wood had glucan digestibilities of 42.6 and 64.1, respectively.

Sorghum showed similar glucan digestibility to bagasse. Glucan digestibilities were 7.3 (untreated), 16.4 (OLP), 31.1 (OLP + ball mill), and 52.0 (OLP + shock).

Switchgrass was moderately digestible. The untreated and OLP samples had glucan digestibilities of 4.1 and 31.6, respectively. Adding ball milling improved the digestibility to 57.6, whereas the adding shock treatment achieved a digestibility of 66.3.

*72-h Enzymatic hydrolysis*

Glucan digestibility (g glucan digested/100 g treated glucan) was significantly higher for the 72-h hydrolysis. Although bagasse was the least digestible after 24 h, it performed moderately well after 72 h. OLP improved digestibility from 11.3 (raw) to 50.5. Ball milling the OLP bagasse significantly improved glucan digestibility to 88.4. The shock-treated OLP bagasse was less, with a glucan digestibility of 67.0.

Once again, corn stover was the most digestible for every treatment classification. Raw corn stover had a digestibility of 15.3, which improved to 60.5 after OLP. Ball milling and shock treatment achieved very high yields of 90.0 and 84.6, respectively.

Poplar wood and switchgrass performed comparably. Poplar wood had glucan digestibilities of 2.2 (raw), 49.4 (OLP), 82.5 (OLP + ball mill), and 78.5 (OLP + shock). Glucan digestibility of switchgrass was 5.4 (raw), 56.2 (OLP), 88.1 (OLP + ball mill), and 78.5 (OLP + shock).

Sorghum showed less significant improvements in glucan digestibility, most likely because of poor OLP performance. Raw sorghum had a digestibility of 8.6, only

improving to 28.7 after OLP. Ball milling and shock treatment improved digestibility to 66.8 and 56.8, respectively.

*Discussion*

The goal of this experiment was to determine whether shock treatment is comparable to ball milling in terms of increasing glucan enzymatic digestibility. In general, this was demonstrated, particularly for the 24-h hydrolysis. For the 24-h hydrolysis, shock treatment produced more digestible biomass than ball milling for every biomass studied. Glucan digestibilities (g glucan hydrolyzed/100 g treated glucan) of OLP + shock samples were greater than OLP + ball mill by 4.2 (bagasse), 8.7 (switchgrass), 20.9 (corn stover, sorghum), and 23 (poplar wood). The 72-h hydrolysis favored ball milling over shock treatment, although only bagasse showed a significant difference in glucan yield (>11 g glucan digested/100 g glucan treated). With a very low enzyme loading (5 FPU/g raw glucan), shock treatment achieved high glucan digestibility (>75) for three of the five biomass species.

This experiment clearly demonstrated that OLP + shock treatment could produce lignocellulose with comparable glucan digestibility as OLP + ball mill. Furthermore, although ball milling achieved higher overall glucan digestibility (72 h), the 24-h hydrolysis results showed that shock treatment significantly improved the rate of glucan digestion.

### 5.3.3 Experiment 2 - Corn stover optimization

After Experiment 1 demonstrated shock treatment could consistently increase glucan digestibility for a variety of biomass species, the purpose of Experiment 2 was to

further define the operating conditions to produce the most digestible biomass. The primary variables studied were particle size, operating temperature, and the effect of multiple shocks (Figure 5-5). Because of its superior performance in Experiment 1, corn stover was chosen as the substrate. Differences in yields (g component hydrolyzed/100 g treated component), both glucan and xylan, were measured using 24-h and 72-h enzymatic hydrolysis with a cellulase loading of 5 FPU/g raw glucan and a β-glucosidase loading of 30 CBU/g raw glucan. Oxidative lime pretreated (OLP) corn stover was used as the control, and all results discussed will be relative to the control.

*Particle size*

A common inconsistency in previous studies was biomass particle size. Milling is an energy intensive and expensive process, so it is necessary to determine whether biomass particle size has a significant effect on shock treatment performance. The raw corn stover was milled to approximately ½-in-long clippings. All of the samples were OLP at this particle size, and then two samples were ground further. One sample was ground to –40/+80 mesh, and the other was ground further (–80). For this trial, the shock tube was placed in a 25°C water bath, and the sample water was also adjusted to 25°C. Shock treatment consisted of a single shock.

In general, there was not a significant difference in digestibility between the different particle sizes; however, all performed better than the control. The control achieved glucan yields of 46.0 (24 h) and 52.8 (72 h). Shock treating the unground sample increased glucan yields to 55.5 (24 h) and 67.3 (72 h). The –40/+80 mesh sample performed slightly worse, with glucan yields of 50.9 (24 h) and 63.2 (72 h).

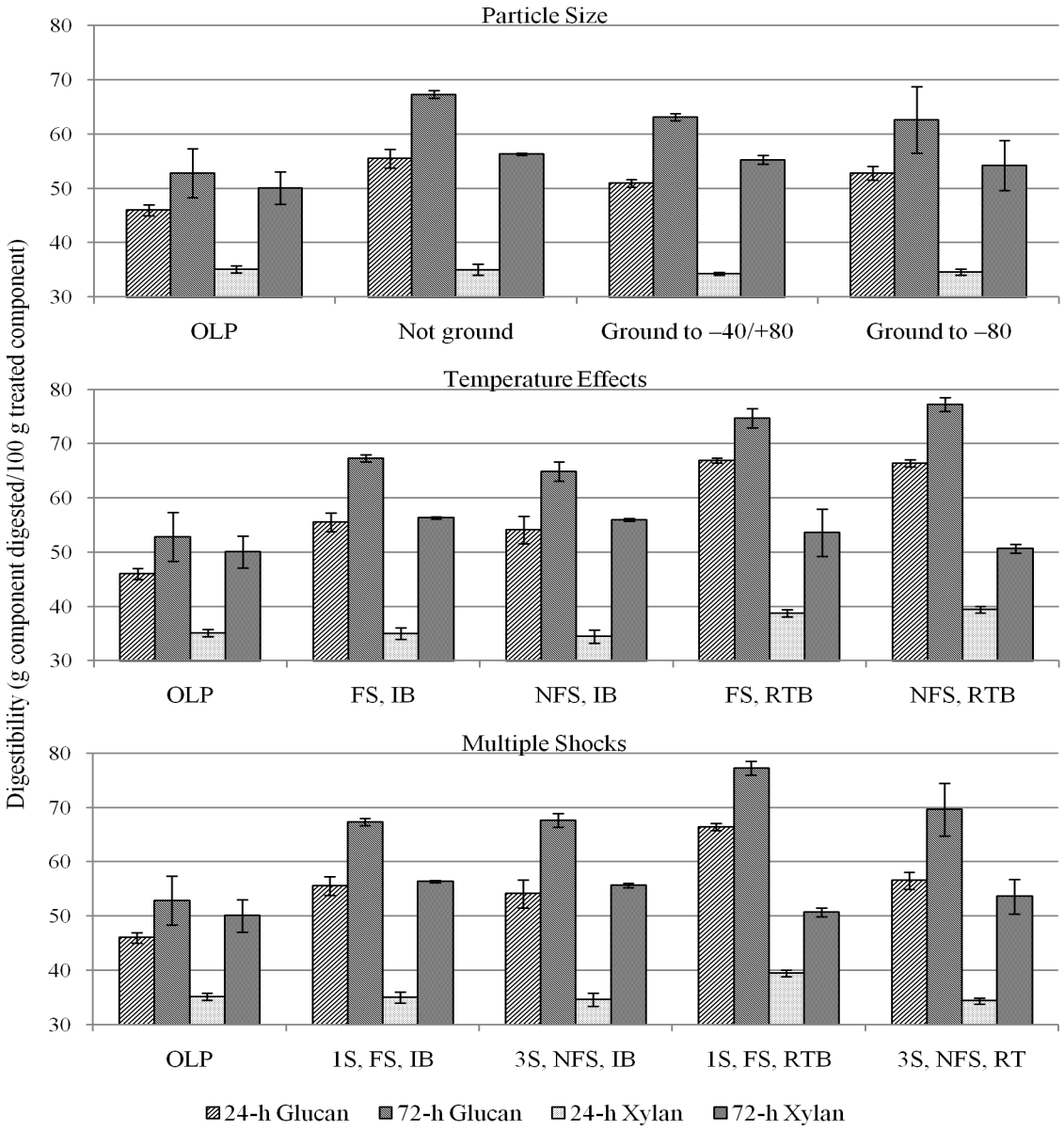


**Figure 5-5.** Glucan and xylan digestibility yields of shock-treated corn stover compared to the control (oxidative lime pretreated (OLP) corn stover) for three variables studied: particle size, temperature effects, and multiple shocks. Enzymatic hydrolysis (24 and 48 h) was performed using a cellulase loading of FPU/g raw glucan and a β-glucosidase loading of 30 CBU/g raw glucan. (OLP = oxidative lime pretreatment, FS = frozen sample, NFS = never frozen sample, IB = ice bath (0 °C), RTB = room-temperature bath (25 °C), 1S = single shock, 3S = three shocks) (Error bars are ± 1σ of the enzymatic hydrolysis replicates.)

There was large standard deviation in the 72-h finely ground sample, but it had a similar 72-h glucan yield (62.6).

Neither shock treatment nor particle size had a significant effect on xylan yields, particularly at 24 h. All four samples achieved a xylan yield of approximately 35.0 (24 h), which increased to 50.0–56.3 for the longer hydrolysis time.

*Temperature effects*

The recommended operating temperature varied greatly from study to study, proving to be one of the largest unknowns in the shock treatment process. The earliest studies showed that shocking room-temperature biomass under moderate operating temperatures (20–40 °C) was satisfactory. After adding the barrel and inner cone, it was hypothesized that freezing the biomass before shock treatment would result in higher performance. This was slightly modified to state that frozen and then slightly thawed biomass performed best. Because freezing the biomass and chilling the shock tube is highly energy intensive, it would be ideal if these processes were not necessary.

Four separate conditions were conducted and compared to the control (OLP corn stover). For the first two, the shock tube was placed in an ice bath, and the sample water was chilled to approximately 0 °C before loading. At this chilled operating temperature, one sample was frozen prior to shock treatment, and the other was simply stored at room-temperature. The other two samples were conducted in a 25 °C water bath, with 25 °C sample water. Once again, one sample was frozen prior to shock treatment, and the other was not.

In terms of glucan yield, both of the samples shocked at 25 °C showed significantly higher glucan yields than those run at the lower temperature. The never-frozen, 25 °C sample increased glucan yields to 66.4 (24-h) and 77.2 (72-h). Likewise, the previously frozen, 25 °C sample improved glucan yields to 66.9 (24-h) and 74.7 (72-h). The two samples performed at the chilled operating temperature still showed significant gains in glucan yield over the control. Xylan yields were mostly unaffected by the operating temperature, or even shock treatment, and ranged from 50.0–56.3 for the 72-h hydrolysis.

These results demonstrated that the shock treatment could effectively be conducted at moderate operating temperatures, and that freezing the biomass prior to shock treatment was not necessary.

*Multiple shocks*

Another reasonable hypothesis was that if a single shock dramatically increased glucan yield, perhaps multiple shocks would further increase digestibility. In the earliest study, this was tested and shown to be false, but with the latest design modifications, it was important to re-evaluate that result. The experiment was designed to compare corn stover that had been shocked a single time, with corn stover that had been shocked three times. For repeated shocks, the biomass was not removed from the shock tube between each shock. Two conditions were examined: (1) 0 °C operating temperature, corn stover that had been frozen, (2) 25 °C operating temperature, corn stover that had not been frozen. These four samples were compared against a control, OLP corn stover.

For the 0 °C operating temperature samples, there was negligible difference in both glucan and xylan yields between the single and multiple shock treatments. The triple shock treatment achieved glucan yields of 55.5 (24-h) and 67.6 (72-h), whereas the single shock treatment achieved glucan yields of 54.0 (24-h) and 67.3 (72-h).

For the 25 °C operating temperature samples, multiple shocks actually reduced glucan yields. Glucan yields for the single shock were 66.4 (24-h) and 77.2 (72-h), compared to 56.5 (24-h) and 69.6 (72-h), for the multiple shock treatment.

These results were consisted with previous results and demonstrated that multiple shocks were not necessary, and repeated shocks could potentially reduce digestibility.

### 5.3.4 Experiment 3 – Crystallinity and degree of polymerization study

The purpose of this experiment was to determine the mechanism by which shock treatment increases biomass digestibility. Because of its heterogeneous nature, it is difficult to perform fundamental studies on lignocellulose. For this reason, microcrystalline cellulose was initially chosen to study the changes in cellulose structure as a result of shock treatment. Untreated and ball-milled microcrystalline cellulose were used as the low- and high-reactivity controls, respectively. Walseth-swollen cellulose was also used in the comparison, as it is also highly reactive. Copy paper (raw, shocked, and ball milled) and corn stover (OLP and OLP + shock) were also studied.

Glucan digestibility (g glucan digested/100 g treated glucan) of shock-treated cellulose was first determined using enzymatic hydrolysis. Shock-treated cellulose and the two controls were all subjected to a 24-h enzymatic hydrolysis with a cellulase loading of 5 FPU/g raw glucan and a β-glucosidase loading of 30 CBU/g raw glucan.

Samples were taken at 6, 12, and 24 h, and the digestibility was measured using HPLC analysis (Figure 5-6). The untreated microcrystalline cellulose demonstrated a very low 24-h glucan digestibility (21.3). Similar glucan digestibilities were observed for shock-treated microcrystalline cellulose: 12.6 (6 h), 17.1 (12 h), and 22.6 (24 h). Ball milling significantly enhanced digestibility with observed glucan digestibilities of 38.7 (6 h), 49.4 (12 h), and 56.5 (24 h). Walseth-swollen cellulose was less digestible than ball-milled cellulose, with a 24-h glucan digestibility of 44.2.

Because shock treatment had negligible effects on the glucan digestibility of microcrystalline cellulose, it was hypothesized that the shock was unable to act on such small particles. To verify this hypothesis, ½-in-long copy paper was studied next. Glucan digestibility was determined for untreated, shock-treated, and ball-milled copy paper. Similar to untreated cellulose, untreated copy paper exhibited a low 24-h glucan digestibility (22.0). Shock treatment only had a slight positive effect on glucan digestibility: 15.5 (6 h), 18.4 (12 h), and 22.8 (24 h). Ball-milled copy paper demonstrated significantly improved glucan digestibilities of 27.5, 34.7, and 39.7 for 6, 12, and 24 h, respectively.

To determine whether shock treatment had an effect on cellulose crystallinity, the crystallinity index (Table 5-3) was determined for microcrystalline cellulose, copy paper, and corn stover samples using the procedure described by Segal et al. (1959). The crystallinity index (CrI) of untreated microcrystalline cellulose was 74.0. The CrI of shock-treated cellulose was slightly higher (80.7), and demonstrated that the shock treatment had negligible effect on the crystallinity of microcrystalline cellulose. Ball

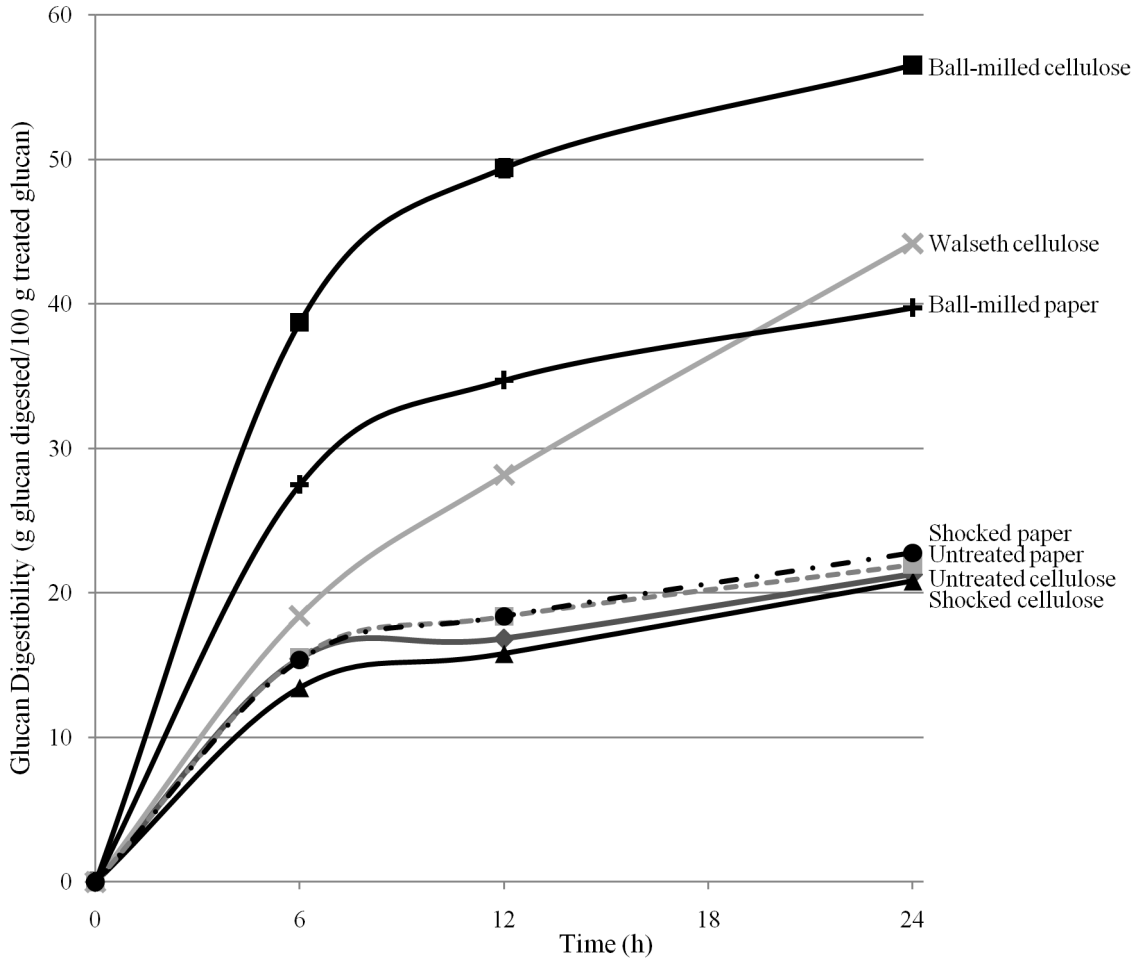


**Figure 5-6.** Glucan digestibility of microcrystalline cellulose and copy paper samples. Enzymatic hydrolysis (6, 12, and 24 h) was performed using a cellulase loading of 5 FPU/g raw glucan and a β-glucosidase loading of 30 CBU/g raw glucan.

milling significantly reduced the CrI to 7.7. Walseth-swollen cellulose had a CrI of 46.1. For copy paper, a similar trend was observed. Untreated copy paper had a CrI of 63.5, whereas the CrI for shock-treated copy paper was 67.5. Ball milling reduced the CrI to 7.8. For corn stover, there was not a significant difference in CrI between OLP corn stover (53.9) and OLP + shock-treated corn stover (58.6).

Another proposed mechanism for improved digestibility after shock treatment is a decrease in cellulose degree of polymerization. This provides additional reducing ends, which can enhance digestibility. For comparative purposes, reducing ends can be determined using the copper number assay. The copper number (Table 5-3) of untreated cellulose was 7.4, which increased to 8.3 after shock treatment. Ball milling had a similar value (8.2), whereas Walseth-swollen cellulose was substantially lower (3.9). The copper number for untreated and shock-treated copy paper was identical (0.7); however, ball milling significantly increased it to 2.4. For corn stover, negligible change was observed between OLP (1.2) and OLP + shock treatment (1.0).

**Table 5-3.** Crystallinity index and copper number of cellulose, paper, and corn stover samples.

| Sample | Crystallinity Index | Copper Number |
|---|---|---|
| Microcrystalline cellulose | | |
| Untreated | 74.0 | 7.4 |
| Shock treated | 80.7 | 8.3 |
| Walseth treated | 46.1 | 3.9 |
| Ball milled | 7.7 | 8.2 |
| Copy paper | | |
| Untreated | 63.5 | 0.7 |
| Shock treated | 67.5 | 0.7 |
| Ball milled | 7.8 | 2.4 |
| Corn stover | | |
| OLP | 53.9 | 1.2 |
| OLP + shock | 58.6 | 1.0 |

(OLP = oxidative lime pretreated)

Shock treatment did not affect microcrystalline cellulose or copy paper, providing negligible changes in enzymatic digestibility, cellulose crystallinity, and

cellulose degree of polymerization. Furthermore, no discernable change in crystallinity or degree of polymerization was observed when comparing OLP and OLP + shock-treated corn stover. It is most likely that shock treatment does not act on cellulose, but rather the complex lignocellulose matrix. Further work is still necessary to determine the mechanism by which shock treatment enhances enzymatic digestibility.

## 5.4 Conclusions

This work demonstrates that combining oxidative lime pretreatment (OLP) with shock treatment significantly increases enzymatic digestibility of lignocellulose. The multiple feedstock study demonstrated improved 72-h glucan digestibilities (g glucan hydrolyzed/100 g treated glucan) for shock-treated bagasse (+55.7), corn stover (+69.3), poplar wood (+81.6), switchgrass (+73.1), and sorghum (+48.2), when compared to each raw variety. With the exception of bagasse, shock treatment performed comparably to ball milling. Recommended shock treatment conditions were determined using OLP corn stover. A single shock at room temperature, with corn stover of ½-in long particle size, achieved glucan digestibilities of 66.4 (24 h) and 77.2 (72 h) using 5 FPU/g raw glucan. The mechanism by which shock treatment enhances digestibility is unknown.

# CHAPTER VI

# DEVELOPMENT OF HIGHLY DIGESTIBLE ANIMAL FEED FROM FORAGE SORGHUM AND CORN STOVER

Oxidative lime pretreatment (OLP) increases lignocellulose digestibility by removing lignin and hemicellulose acetyl content. Adding a mechanical pretreatment process (e.g., ball milling, shock treatment) further improves its digestibility. This study determined the effectiveness of these pretreatments to enhance the ruminant digestibility of lignocellulose. For forage sorghum, the 48-h *in vitro* total digestible nutrients (TDN) were 50, 69, and 77 g nutrients digested/100 g organic matter for raw, short-term OLP, and short-term OLP + ball milling, respectively. For corn stover, the 48-h *in vitro* TDNs were 51.9, 59.7, and 72.6 g nutrients digested/100 g organic matter for raw, OLP, and shock + OLP, respectively. Addition of the extracted corn stover solubles increased TDN to 74.9 g nutrients digested/100 g organic matter.

## 6.1 Introduction

For over 30 years (1973–2005) the price of corn remained relatively stable, with only small fluctuations between $2–$3/bushel. Beginning in 2006 and continuing to the present (2011), the price of corn has steadily escalated to over $7/bushel (USDA, 2011b). As a consequence, the price of pork, poultry, beef, dairy products, and other agricultural commodities has increased as well. These increased food prices have resulted in food riots in many developing countries, including Egypt, Haiti, Indonesia,

and China (Northoff, 2007). Furthermore, global population continues to steadily rise, growing by over 800 million people from 2000 to 2011 (Census, 2011). Global demand for food is expected to double within the next 50 years, necessitating significant growth in agricultural productivity to limit malnutrition and starvation (Fedoroff & Cohen, 1999).

Numerous factors have contributed to increased corn prices; however, it is hard to ignore the tremendous impact of grain-based biofuels (Rosegrant & International Food Policy Research, 2008). Between 2005 and 2010, the U.S. percentage of corn consumption devoted to biofuel production rose from 14% to 37% (USDA, 2011a). This significant increase correlates well with the dramatic increase in the price of corn.

In the United States, livestock feed consumes 38% of the corn produced. Corn grain is heavily used as livestock feed because it primarily consists of non-fiber carbohydrates (NFC): starch, sugar, and soluble fiber. The typical composition of corn grain is approximately 75% NFC, 10% crude protein (CP), 10% neutral detergent fiber (NDF), and the remaining 5% consists of primarily fat and ash (Thornton et al., 1969). This results in a very high TDN (total digestible nutrients), generally over 85% (Chase & Hibberd, 1987).

For ruminants, it is possible to displace corn with lignocellulose, the most abundant organic material on earth (Rajarathnam et al., 1989). Unfortunately, because of its structural features, lignocellulose is highly recalcitrant and requires research to identify methods to increase its digestibility. Although many structural features influence lignocellulose digestibility, Chang and Holtzapple (2000) focused on three;

lignin content hemicellulose acetyl content, and cellulose crystallinity. They demonstrated that lime pretreatment significantly reduces lignin content and completely removes acetyl groups from hemicellulose. Physical pretreatments (e.g., ball milling) are highly effective at lowering cellulose crystallinity (Bertran & Dale, 1985; Puri, 1984). Furthermore, combining lime pretreatment with mechanical pretreatment dramatically improves enzymatic digestibility (Falls & Holtzapple, 2011).

Two potential sources of lignocellulose are energy crops and agricultural residues. Sorghum (*Sorghum bicolor*) is a hardy, draught-tolerant grass that is being developed as an energy crop (McBee et al., 1987). Genetically engineered sorghum hybrids have obtained yields ranging from 20–30 Mg/ha, with 33% less water input compared to corn (McCollum et al., 2005; Rooney et al., 2007). In the United States, corn stover (*Zea mays*) is the most abundant agricultural residue with an availability of approximately 80 million dry tons per year (Kadam & McMillan, 2003).

The purpose of this work was to generate highly digestible lignocellulosic biomass (sorghum and corn stover) to supplement or replace corn grain as ruminant animal feed. To accomplish this, a combination of oxidative lime pretreatment (OLP) and mechanical (ball milling and shock treatment) pretreatments were employed to render the biomass more digestible. To determine the nutritive value of the generated feed, composition and *in vitro* digestibility were determined by university and commercial laboratories.

## 6.2 Materials and Methods

### 6.2.1 Biomass feedstocks

Sorghum was harvested locally in College Station. The sorghum was dried to uniform moisture content (<10%) before being ground to approximately ½-in using a commercially available chipper. Dairy One Forage Testing Laboratory (Ithaca, NY) performed the compositional analysis (Table 6-1). Samples were analyzed for dry matter (DM) (AOAC, 2000; method 930.15), ash (AOAC, 2000; method 942.05), CP (AOAC, 2005; method 990.03), lignin, ADF, NDF (ANKOM A200 Filter Bag Technique with F57 bag), crude fat (AOAC, 2005; method 2003.05), and NFC (AOAC, 1990; method 989.03).

Corn stover was provided by the National Renewable Energy Laboratory (NREL) and was dried to uniform moisture content (<10%) and milled to pass ¼-in round screen. To wash extractives out of the corn stover, de-ionized $H_2O$ was used at a ratio of 10 mL DI $H_2O$ per mL corn stover. Corn stover and DI $H_2O$ were mixed on a rolling bed apparatus for 1 h before centrifugation; the solids were subsequently dried. The supernatant was concentrated using rotary evaporation, and then freeze-dried to a powder using a Labconco Lyph-Lock 6-L freeze dryer system (Model 77530, Labconco Corporation, Kansas City, MO). Cumberland Valley Analytical Services, Inc. (CVAS; Hagerstown, MD) performed the compositional analysis for corn stover samples (Table 6-1). Samples were analyzed for DM (Goering and Van Soest, 1970, and National Forage Testing Association recommendations, 2002), ash (AOAC, 2000; method 942.05), CP (AOAC, 2000; method 990.03), lignin (Goering and Van Soest, 1970),

crude fat (AOAC, 2006; method 2003.05), ADF (AOAC, 2000; method 1973.18), NDF (Van Soest, et al., 1991), and NFC (Dubois, et al., 1956).

In the second trial, two materials were used as controls: cracked corn grain and alfalfa. CVAS performed compositional analysis on these control materials as well (Table 6-1).

**Table 6-1.** Composition of the raw feedstocks.

| Feedstock | % Moisture | Ash (%DM) | CP (%DM) | ADF (%DM) | NDF (%DM) | NFC (%DM) | Lignin (%DM) | Fat (%DM) |
|---|---|---|---|---|---|---|---|---|
| Sorghum | 8.6 | 12.9 | 13.3 | 49.2 | 63.4 | 13.6 | 6.7 | 1.5 |
| Corn stover | 9.5 | 7.9 | 6.5 | 44.5 | 71.2 | 15.5 | 9.6 | 0.9 |
| Corn grain | 14.2 | 1.3 | 8.5 | 4.7 | 11.2 | 75.6 | 2.3 | 3.9 |
| Alfalfa | 7.4 | 9.4 | 15.0 | 35.6 | 44.6 | 30.9 | 8.7 | 1.9 |

(DM = dry matter, CP = crude protein, ADF = acid detergent fiber, NDF = neutral detergent fiber, NFC = non-fiber carbohydrates)

### 6.2.2 Pretreatment methods

*Short-term*

Corn stover was pretreated using short-term oxidative lime pretreatment as described by Sierra et al. (2009) and Falls et al. (2011b). The pretreatment vessel was a 20-L stainless steel batch reactor (Figure 6-1c). Corn stover (500 g), excess calcium hydroxide (250 g), and distilled water (7.5 L) were loaded into the reactor. The reactor was sealed, heated to 110°C, and the stirring mechanism was activated. The reactor was then charged with 6.89-bar pure oxygen, and the reaction proceeded for 3 h. When complete, the heat and stirring were shut off, and the reactor was allowed to cool. Once the reactor was cool enough to handle, it was slowly vented to relieve pressure, and then opened. The pretreated slurry was removed and neutralized to pH 4.0 using 5-N HCl.

The slurry was then vacuum filtered to isolate the pretreated solids. To wash out any residual lime, the pretreated solids were washed with distilled water a minimum of three times, until the pH of the collected wash was equal to that of fresh distilled water. The pretreated corn stover was air dried in metal pans. To prevent microbial growth, the biomass was stirred at least once every 24 h. A portion of the lime-pretreated corn stover was subjected to shock treatment.

Sorghum was also lime pretreated using a similar procedure. Sorghum (8 g, dry basis), lime (5 g), and water (120 mL) were mixed in a 304 stainless steel pipe reactor (1.5-in I.D., 5-in long). The reactor (Figure 6-1d) was sealed and connected to a swing arm located in a temperature-controlled oven. Pure oxygen (6.89 bar) was provided to the reactor through a flexible hose attached directly to an oxygen cylinder. The reaction was performed at 180 ºC for 2 h. Once complete, the reaction was quenched by placing the reactor in an ice-water bath. The reactor was slowly opened to relieve pressure, and the reactor contents were transferred to a 1-L plastic centrifuge bottle. The pretreated slurry received the same neutralization, washing, and drying procedure as the short-term lime pretreated corn stover. A portion of the short-term lime-pretreated sorghum was ball milled.

*Long-term*

Sorghum was pretreated using long-term oxidative lime pretreatment. This pretreatment was performed in a series of 15 packed-bed reactors (Figure 6-1b), made of PVC pipe (1-in Sch. 40, 19-in long). To maintain the desired reaction temperature (55 ºC), the reactors were jacketed with a larger diameter PVC pipe (2-in Sch. 40, 17-in

long) and water was pumped from a temperature-controlled tank. The oxidant employed was compressed air, which was scrubbed of carbon dioxide, preheated to the reaction temperature, and then bubbled through an inlet located at the bottom of each reactor.

Three different lime loadings were studied: 0.1 g lime/g dry sorghum (Reactors 1–5), 0.2 g lime/g dry sorghum (Reactors 6–10), and 0.3 g lime/g dry sorghum (Reactors 11–15). For each reactor, sorghum (80 g, dry basis) and the appropriate amount of lime were thoroughly mixed in a stainless steel tray before being loaded. Water was then added to each reactor until the biomass/lime mixture was completely submerged. The water level was checked daily and additional water was added if necessary. The initial pH was 12.0, and the pretreatment was considered complete when the pH decreased to approximately 7.0. This resulted in a reaction time of 8 d for the 10% lime loading, 22 d for the 20% lime loading, and 34 d for the 30% lime loading. Once complete, the pretreated material was removed from the reactor and thoroughly washed with distilled water to remove any unreacted lime or lignin residue. The material was then air dried to uniform moisture content of less than 10%. Half of each pretreated sample was then ball milled.

*Ball milling*

Ball milling was used to de-crystallize sorghum. Raw or pretreated sorghum (6 dry g) was dried to less than 10% moisture and then transferred into a 300-mL porcelain jar. The porcelain jar was then loaded with 0.375-in-diameter zirconia grinding medium. The grinding medium was loaded to fill 50% of the jar volume (approximately 258 g). The jars were sealed and placed on rollers rotating at 68 rpm for 3 days. Metal sieve

trays and a shaking apparatus were used to isolate the ball-milled sorghum from the grinding media. No corn stover samples were ball milled.

*Shock treatment*

Shock treatment was performed in the shock tube pretreatment apparatus (Figure 6-1a). The shock tube is comprised of a carbon steel tube and carbon steel barrel connected by a 300-lb flange. The bottom tube is a 20-in section of 4-in Sch. 80 pipe, and the top barrel is a 27.5-in section of 1-in Sch. 40 pipe. The 1-in barrel joined the 4-in pipe through an 11-in-long conical section. The conical section has an inner diameter of 0.88 in at the barrel end, which increases to 3.56 in at the tube end. The shock tube was placed in a temperature-controlled water bath (25 ºC), and loaded with 100 g dry corn stover and 2 L water. The barrel section was lowered onto the bottom tube, and the shock tube was sealed. A 12-gauge shotgun shell (Winchester Expert High Velocity 3 1/2-in, 1 3/8-oz steel BB shot) was placed inside the top opening of the barrel and fired by releasing a steel plate firing pin onto the central metal surface of the shell. The flange was unbolted, and the barrel section of the shock tube was lifted away. The shock tube contents were placed in a product container and then filtered to remove lead shot and other shell remnants. The shocked corn stover was then air dried in metal pans to uniform moisture content (<10%).

### 6.2.3 Solubilized protein from chicken feathers

Chicken feathers (provided by Texas A&M Poultry Science Department, College Station, TX) were washed, air-dried, and then completely dried at 105 °C. The dried feathers were ground using a Thomas-Wiley laboratory mill (Arthur H. Thomas

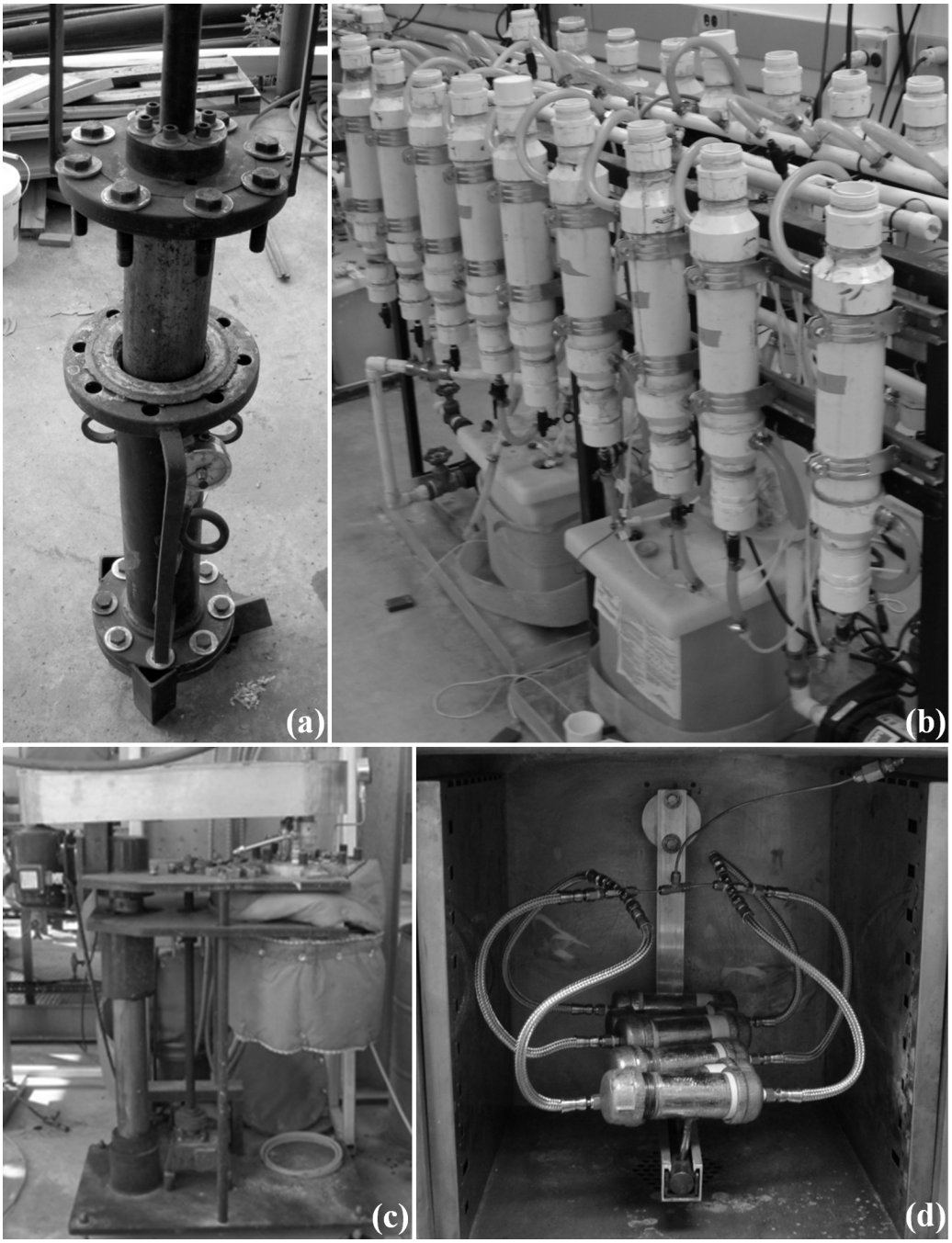


**Figure 6-1.** (a) Shock tube reactor; (b) Long-term-lime pretreatment reactor; (c) 20-L oxidative lime pretreatment reactor; (d) stainless steel pipe reactor used for short-term oxidative lime pretreatment of sorghum.

Company, Philadelphia, PA) and sieved through a 2-mm screen. Lime treatment was performed in a 1-L autoclave reactor with a temperature controller and mixer (1000 rpm). Recommended treatment conditions were used: 100 °C, 300 min, and 0.1 g $Ca(OH)_2$/g dry feather. The treated slurry was centrifuged, and the supernatant was collected as the final product. The solubilized protein solution was frozen until analysis.

### 6.2.4 Total digestible nutrients

Total digestible nutrients (TDN) were used to estimate the value of each prepared sample as an animal feed. TDN was calculated by Dairy One, Inc. and Cumberland Valley Analytical Services, Inc. using compositional analysis results. These laboratories used the Weiss model (Weiss et al., 1992), which calculates TDN based on true digestibility coefficients for available soluble carbohydrates, proteins, fatty acids, and fiber. The equation follows:

$$\begin{aligned} \mathrm{TDN_w} = {} & 0.98 \times (100 - \mathrm{NDFn} - \mathrm{CP} - \mathrm{ash} - \mathrm{EE} + \mathrm{IADFIP}) \\ & + \mathrm{dCP} \times \mathrm{CP} + 2.25 \times (\mathrm{EE} - 1) \\ & + 0.75 \times (\mathrm{NDFn} - \mathrm{lignin}) \times \left[1 - \left(\frac{\mathrm{lignin}}{\mathrm{NDFn}}\right)^{\frac{2}{3}}\right] \\ & - 7 \end{aligned} \qquad [6\text{-}1]$$

$$\mathrm{dCP} = \mathrm{CP} \times \mathrm{e}^{[-0.012 \times \mathrm{ADFIP}]} \qquad [6\text{-}2]$$

$$\mathrm{NDFn} = \mathrm{NDF} - \mathrm{NDFIP} + \mathrm{IADFIP} \qquad [6\text{-}3]$$

where EE = ether extract, ADFIP = average daily feed intake protein, IADFIP = indigestible ADFIP (0.7 × ADFIP for forages), dCP = digestibility of CP, NDFn = NDF adjusted for nitrogen, and NDFIP = NDF-insoluble protein. All values are expressed as percentages of the dry matter.

Texas A&M University Animal Science Department also calculated an adjusted TDN based on measured 48-h neutral detergent fiber digestibilities ($NDFD_{48}$), and used the following equations (Tedeschi et al., 2009):

$$\mathrm{TDN_N} = 0.98 \times [100 - (\mathrm{NDF} - \mathrm{NDIN}) - \mathrm{CP} - \mathrm{EE} - \mathrm{Ash} + \mathrm{dCP} + \mathrm{dEE} + \mathrm{dNDF} - 7] \quad [6\text{-}4]$$

$$\mathrm{dCP} = \left[1 - 0.004 \times \left(\frac{\mathrm{ADIN} \times \mathrm{CP}}{100}\right)\right] \times \mathrm{CP} \quad [6\text{-}5]$$

$$\mathrm{dEE} = 2.25 \times (\mathrm{EE} - 1) \quad [6\text{-}6]$$

$$\mathrm{dNDF} = \mathrm{NDFD_{48}} \times (\mathrm{NDF} - \mathrm{NDIN}) \quad [6\text{-}7]$$

where NDF = neutral detergent fiber, NDIN = neutral detergent insoluble nitrogen, CP = crude protein, dCP = digestible CP, dEE = digestible EE, dNDF is ruminal and intestinal digestible NDF, and ADIN = acid detergent insoluble nitrogen (% of CP). All values, except ADIN, are expressed as percentages of the dry matter.

### 6.2.5 *In vitro* neutral detergent fiber digestibility

Dairy One, Inc. Forage Testing Laboratory analyzed sorghum for 24- and 48-h *in vitro* neutral detergent fiber digestibility (NDFD) using the Ankom Daisy II Filter Bag Technique. Rumen fluid was collected from a total-mixed-ration fed, high-producing

lactating cow. The sorghum samples were incubated in a Van Soest buffer/rumen fluid mixture for 24 and 48 h under anaerobic conditions at 39 °C. The remaining residue was used to determine NDFD.

### 6.2.6 *In vitro* anaerobic fermentation and gas production

Texas A&M University Animal Science Department analyzed the *in vitro* anaerobic fermentation of corn stover using the gas production method described by Tedeschi et al. (2009). The *in vitro* fermentation chamber included an incubator with a multi-plate stirrer, pressure sensors attached to incubation flasks (125-mL Wheaton bottles), an analog-to-digital convertor device, and a PC-compatible computer provided with appropriate software (Pico Technology, Eaton Socon, Cambridgeshire, UK). The pressure inside each flask was automatically recorded every 5 min for 48 h (2,880 data points). Each incubation flask was loaded with feed sample (200 mg), boiled distilled water that had been cooled to room temperature (2 mL), cysteine hydrochloride (14 mL), and filtered mixed ruminal bacteria inocula (4 mL). Recording of the pressure was initiated once the fermentation chamber reached the fermentation temperature (39 °C). Fermentation pH was maintained between 6.8 and 6.9. Once fermentation was complete, 40 mL of neutral detergent solution was added to each bottle, the bottles were crimp sealed, and placed in an autoclave for 60 min at 105°C. The undegraded fiber was filtered using a Whatman 54 filter paper, and NDF was determined gravimetrically.

### 6.2.7 Experimental design

For Trial 1, 10 sorghum samples were analyzed: (1) untreated, (2) ball milled, (3) short-term-lime pretreated, (4) short-term-lime pretreated and ball milled, (5–7) 10%,

20%, and 30% long-term-lime pretreated, and (8–10) 10%, 20%, 30% long-term-lime pretreated and ball milled. Dairy One Forage Laboratory analyzed these samples for composition and 24- and 48-h NDF digestibility.

Trial 2 used shock treatment. Five corn stover samples and two control samples (corn grain and alfalfa) were analyzed: (1) untreated, (2) short-term-lime pretreated, (3) shock pretreated, (4) short-term-lime pretreated and shock pretreated, and (5) shock pretreated and short-term-lime pretreated. Cumberland Valley Analytical Services, Inc. analyzed compositional differences, estimated TDN, and measured 30-h *in vitro* NDF digestibility. Texas A&M University Animal Science Department measured 48-h *in vitro* NDF digestibility and the gas production resulting from the anaerobic fermentations.

## 6.3 Results and Discussion

### 6.3.1 Trial 1 – Sorghum

The purpose of Trial 1 was to increase the ruminant digestibility of forage sorghum using oxidative lime pretreatment (OLP) and ball milling. OLP reduces lignin content and removes acetyl groups from hemicellulose (Chang et al., 1998; Rabelo et al., 2009; Saha & Cotta, 2008; Wyman et al., 2009), whereas ball milling decrystallizes cellulose (Bertran & Dale, 1985).

*Compositional analysis*

Table 6-2 shows the compositional analysis. Because some samples were not fully washed of all unreacted lime, the ash content was high. To compensate for this, all discussion will be on an organic basis (ash free). Furthermore, the following discussion

will focus on three key components: neutral detergent fiber (NDF), non-fiber carbohydrates (NFC), and crude protein (CP).

**Table 6-2.** Compositional analysis of untreated and treated sorghum.

| Sample | Ash (%DM) | CP (%OM) | ADF (%OM) | NDF (%OM) | NFC (%OM) | Lignin (%OM) | Fat (%OM) |
|---|---|---|---|---|---|---|---|
| Raw | 12.9 | 15.3 | 56.5 | 72.8 | 15.6 | 7.7 | 1.7 |
| Ball milled | 11.1 | 10.4 | 45.6 | 72.0 | 20.6 | 10.7 | 0.9 |
| Short-term OLP | 5.7 | 3.1 | 80.5 | 90.1 | 10.8 | 1.5 | 0.6 |
| Short-term OLP + ball mill | 21.2 | 2.7 | 10.2 | 28.6 | 68.5 | 1.8 | 0.4 |
| 10% Long-term OLP | 11.8 | 9.3 | 74.9 | 88.1 | 7.6 | 12.1 | 0.8 |
| 10% Long-term OLP + ball mill | 12.1 | 9.7 | 36.8 | 53.8 | 36.2 | 4.1 | 1.1 |
| 20% Long-term OLP | 26.6 | 9.7 | 74.9 | 83.3 | 9.3 | 11.9 | 0.8 |
| 20% Long-term OLP + ball mill | 30.3 | 10.3 | 12.4 | 19.9 | 69.0 | 3.1 | 0.9 |
| 30% Long-term OLP | 32.2 | 5.5 | 80.7 | 89.5 | 5.9 | 5.2 | 0.7 |
| 30% Long-term OLP + ball mill | 33.1 | 5.2 | 15.3 | 35.0 | 59.4 | 2.8 | 0.5 |

(DM = dry matter, OM = organic matter, OLP = oxidative lime pretreatment, CP = crude protein, ADF = acid detergent fiber, NDF = neutral detergent fiber, NFC = non-fiber carbohydrates)

NDF is comprised of the structural components of the plant cell wall. Typically, it is one of the least digestible components of plant forage. Highly digestible ruminant feeds generally have very little NDF content. For example, corn grain generally has 10% NDF. In contrast, the raw sorghum had 72.8% NDF. After OLP, NDF consistently increased for all conditions. Short-term OLP increased NDF content to 90.1%. The 10%, 20%, and 30% long-term OLPs increased NDF content to 88.1%, 83.3%, and 89.1% respectively. These significant increases are somewhat surprising because lignin, one of the three components in NDF, is removed during lime pretreatment; however, lime pretreatment also degrades other components such as crude protein.

Ball milling generated small particles that introduced significant error in the compositional analysis procedure. For all ball-milled samples, this resulted in

inconsistent and highly unlikely values for both NDF and NFC; however, because ball milling is simply a mechanical process, the chemical composition of the feed should not be affected and can be assumed to be unchanged.

NFC is important because of its high inherent digestibility. The NFC of raw sorghum was quite low (15.61%) when compared to corn grain (76.6%), and even alfalfa (31.4%). Short-term OLP decreased NFC to 10.7%. The long-term OLPs showed similar decreases with the 10%, 20%, and 30% lime loadings resulting in NFC content of 7.6%, 9.2%, and 5.9%, respectively. Traditionally, NFC is a highly digestive component, so any reduction in NFC is considered a negative consequence of pretreatment. For pretreatment to be worthwhile, significant gains need to be shown elsewhere.

Protein is an important component of any diet, and the raw sorghum had a CP content of 15.2%. Unfortunately, protein degradation is another negative aspect of the oxidative lime pretreatment process. The high temperature used in short-term OLP resulted in the harshest protein degradation, with a CP content of only 3.1%. The 10%, 20%, and 30% long-term lime pretreatments had CP contents of 9.3%, 9.7%, and 5.5%, respectively. Although protein degradation is undesired, there are numerous protein supplementation strategies, one of which is described in Section 3.2.

Mineral composition was mostly unaffected by OLP or ball milling (Table 6-3). OLP increased calcium significantly; however, this was primarily caused by unreacted lime resulting from not properly washing the sorghum after pretreatment. OLP did

remove small amounts of phosphorous and magnesium, typically about 0.2% on a dry matter basis. Neither raw nor treated sorghum had measurable quantities of sodium.

In terms of compositional changes, oxidative lime pretreatment of sorghum increases NDF and decreases NFC and CP, all of which would traditionally be considered negative. To better understand the effect of oxidative lime pretreatment on lignocellulosic animal feed, it is necessary to study the digestibility of each component, particularly NDF.

**Table 6-3.** Mineral composition of corn grain, alfalfa, sorghum samples, corn stover samples, solubilized protein, and balanced feeds.

| Sample | Ca (%DM) | P (%DM) | Mg (%DM) | K (%DM) | Na (%DM) |
|---|---|---|---|---|---|
| Corn grain | 0.0 | 0.3 | 0.1 | 0.4 | 0.0 |
| Alfalfa | 1.6 | 0.2 | 0.7 | 2.3 | 0.1 |
| Sorghum | | | | | |
| Raw | 0.5 | 0.5 | 0.4 | 2.9 | 0.0 |
| Ball milled | 0.3 | 0.3 | 0.3 | 2.6 | 0.0 |
| Short-term OLP | 1.2 | 0.1 | 0.1 | 0.1 | 0.0 |
| Short-term OLP + ball mill | 7.5 | 0.2 | 0.3 | 0.0 | 0.0 |
| 10% Long-term OLP | 3.7 | 0.2 | 0.1 | 0.1 | 0.0 |
| 10% Long-term OLP + ball mill | 4.0 | 0.2 | 0.1 | 0.1 | 0.0 |
| 20% Long-term OLP | 10.1 | 0.2 | 0.2 | 0.1 | 0.0 |
| 20% Long-term OLP + ball mill | 11.5 | 0.2 | 0.2 | 0.1 | 0.0 |
| 30% Long-term OLP | 10.5 | 0.2 | 0.2 | 0.0 | 0.0 |
| 30% Long-term OLP + ball mill | 13.2 | 0.2 | 0.2 | 0.0 | 0.0 |
| Corn stover | | | | | |
| Untreated | 0.4 | 0.1 | 0.2 | 1.8 | 0.0 |
| Shock treated | 0.4 | 0.1 | 0.1 | 0.6 | 0.2 |
| OLP | 2.9 | 0.0 | 0.1 | 0.0 | 0.0 |
| OLP + Shock | 1.4 | 0.0 | 0.0 | 0.1 | 0.2 |
| Shock + OLP | 1.3 | 0.0 | 0.0 | 0.1 | 0.0 |
| Solubles | 1.1 | 0.6 | 1.0 | 11.7 | 0.1 |
| Solubilized protein | 3.3 | 0.0 | 0.0 | 0.3 | 1.0 |
| Combined feed | 1.2 | 0.1 | 0.2 | 2.1 | 0.0 |
| Protein-balanced feed | 1.3 | 0.1 | 0.2 | 2.1 | 0.1 |

(DM = dry matter, OLP = oxidative lime pretreatment, Combined feed = 17.8% corn stover solubles and 82.2% shock + OLP corn stover, Protein-balanced feed = 3.3% solubilized protein, 17.2% corn stover solubles, and 79.5% shock + OLP corn stover)

*Neutral detergent fiber digestibility and* in vitro *true digestibility*

Table 6-4 reports the 24- and 48-h neutral detergent fiber digestibility (NDFD) for each sorghum sample. Raw sorghum was used as the control, and had a 24-h NDFD (g NDF digested/100 g NDF fed) of 21 and 48-h NDFD of 29. In general, lime pretreatment and ball milling increased both 24- and 48-h NDFD. Long-term OLP (20%) + ball-milled sorghum was an exception, showing a marked decrease in NDF digestibility (24-h: 7%; 48-h: 33%). Short-term OLP and long-term OLP (30%) sorghum were the most digestible, both digesting 70% of NDF in 48 h.

*In vitro* true digestibility (IVTD) was also determined for each sorghum sample (Table 6-4). All results discussed are 48-h IVTD reported on a % dry matter basis. Raw sorghum had an IVTD of 55, which ball milling increased to 85. Short-term OLP and short-term OLP + ball mill had IVTD values of 74 and 91, respectively. IVTD of long-term OLP improved with increased lime loading. With 10%, 20%, and 30% long-term OLP having values of 54, 71, and 81, respectively. Ball milling further increased IVTD of each long-term samples, with 10%, 20%, and 30% long-term OLP + ball mill having values of 83, 91, and 93.

*Total digestible nutrients*

Total digestible nutrients (TDN) were based on the compositional analysis results and calculated using Equation 6-1. All $TDN_W$ values are reported as g nutrients digested/100 g organic matter fed (Table 6-4). As with NDFD, raw sorghum was used as the control and had a $TDN_W$ of 50. In terms of oxidative lime pretreatment, short-term OLP and 30% long-term OLP (30%) were the most successful, both having $TDN_W$

values of 59. Long-term lime OLP improved with increased lime loading, with 10% long-term OLP and 20% long-term OLP having TDN values of 40 and 48, respectively. Ball-milling improved $TDN_W$ in every case, with short-term OLP + ball mill (77) and 30% long-term OLP + ball mill (78) resulting in the highest $TDN_W$ values observed.

**Table 6-4.** Total digestible nutrients and neutral detergent fiber digestibility of untreated and treated sorghum.

| Sample | TDN (%OM) | 24-h IVTD (%DM) | 48-h IVTD (%DM) | 24-h NDFD (% NDF) | 48-h NDFD (% NDF) |
|---|---|---|---|---|---|
| Raw | 50 | 50 | 55 | 21 | 29 |
| Ball mill | 59 | 70 | 85 | 54 | 76 |
| Short term OLP | 59 | 48 | 74 | 39 | 70 |
| Short-term OLP + ball mill | 77 | 90 | 91 | 57 | 58 |
| 10% Long-term OLP | 40 | 52 | 54 | 38 | 41 |
| 10% Long-term OLP + ball mill | 69 | 71 | 83 | 39 | 63 |
| 20% Long-term OLP | 48 | 66 | 71 | 45 | 53 |
| 20% Long-term OLP + ball mill | 74 | 87 | 91 | 7 | 33 |
| 30% Long-term OLP | 59 | 81 | 82 | 69 | 70 |
| 30% Long-term OLP + ball mill | 78 | 90 | 93 | 56 | 70 |

(OM = organic matter basis, DM = dry matter, OLP = oxidative lime pretreatment, NDF = neutral detergent fiber, IVTD = *in vitro* true digestibility, NDFD = neutral detergent fiber digestibility)

*Discussion*

Ball milling dramatically reduced particle size of the samples, and thus may slip through the pores of the ANKOM F57 bags, and thereby overstate the digestibility. Nonetheless, previous studies have shown that adding ball milling to OLP significantly improves enzymatic digestibility, which should correlate well with ruminant digestibility (Bals et al., 2010b; Falls & Holtzapple, 2011). Even if the rumen digestibilities are high, the samples have no practical use as a feed because the fine particles can readily escape from the rumen before they are digested.

Lessons from Trial 1 can be summarized as follows: (1) use feedstock with low protein content, which prevents its loss in OLP, (2) extensively wash the biomass to remove ash from OLP, and (3) select a mechanical pretreatment that maintains fiber integrity so it is retained in the rumen until digested.

### 6.3.2 Trial 2 – Corn stover

For Trial 2, corn stover was selected as the feedstock because it has a lower protein content (Lesson 1). Further, prior to OLP, the corn stover will be extracted with water to remove soluble protein and other solubles (e.g., free sugars, hemicellulose). After OLP, it will be extensively washed to reduce the ash content of the feed (Lesson 2).

In contrast to ball milling which finely divides the biomass, shock treatment maintains the integrity of the biomass particle (Lesson 3). Shock treatment, when combined with OLP, significantly increased the enzymatic digestibility of lignocellulose, particularly corn stover (Falls et al., 2011c). In this study, corn stover was prepared using OLP alone, shock treatment alone, and combinations OLP + shock and shock + OLP. The corn stover samples were compared to two standards: corn grain and alfalfa. The compositional analysis and digestibility results are discussed here, and are all given on an organic matter basis.

*Compositional analysis*

Similar to Trial 1, compositional analysis was performed to determine changes in composition from pretreatment (Table 6-5). Corn grain had a significantly higher NFC content (76.6%) than both alfalfa (34.1%) and raw corn stover (16.8%), which is why

corn grain is widely used in ruminant diets. Oxidative lime pretreatment of corn stover had a negligible effect on NFC; OLP corn stover had an NFC content of 16.0%. However, shock treatment significantly reduced NFC (6.9%). When combined, the order of pretreatments had little effect on NFC content (10.4%). The effect of shock treatment on NFC is not well understood, and needs to be further explored.

Raw corn stover had significantly higher NDF (77.3%) than alfalfa (49.2%) and corn grain (11.4%). The primary hurdle of implementing lignocellulose in high-quality ruminant feeds is overcoming the high NDF content, which is normally highly indigestible. As with pretreated sorghum in Trial 1, both pretreatment processes significantly increased NDF. OLP alone increased NDF to 81.9%, and shock treatment alone increased NDF to 88.1%. Similar to NFC, when combined, the order of pretreatments had little effect on NDF changes. OLP + shock had similar NDF (87.6%) to shock + OLP (87.1%).

The crude protein content of raw corn stover (7.1%) was only slightly lower than corn grain (8.6%), but considerably lower than alfalfa (16.6%). As discussed previously, a significant drawback to using OLP to generate animal feed is the unavoidable degradation of protein. To some extent, protein can be protected by prewashing the corn stover to recover protein prior to OLP. OLP reduced corn stover CP to 3.2%, whereas shock treatment had negligible effect (6.6%). When combined, OLP + shock and shock + OLP had CP contents of 4.1% and 3.1%, respectively. If OLP is used to produce animal feed, it will be necessary to supplement it with a high-protein source, such as

alfalfa, soybean meal, distillers' grains, or solubilized proteins (Coward-Kelly et al., 2006b).

**Table 6-5**. Compositional analysis of corn grain, alfalfa, corn stover samples, solubilized protein, and balanced feeds.

| Sample | Ash (%DM) | CP (%OM) | ADF (%OM) | NDF (%OM) | NFC (%OM) | Lignin (%OM) | Fat (%OM) |
|---|---|---|---|---|---|---|---|
| Corn grain | 1.3 | 8.6 | 4.8 | 11.4 | 76.6 | 2.4 | 4.0 |
| Alfalfa | 9.4 | 16.6 | 39.3 | 49.2 | 34.1 | 9.6 | 2.1 |
| Corn stover | | | | | | | |
| Untreated | 7.9 | 7.1 | 48.3 | 77.3 | 16.8 | 10.4 | 1.0 |
| Shock treated | 6.6 | 6.6 | 59.9 | 88.1 | 6.9 | 13.5 | 0.4 |
| OLP | 8.7 | 3.2 | 72.6 | 81.9 | 16.0 | 13.4 | 0.7 |
| OLP + shock | 10.3 | 4.1 | 77.2 | 87.6 | 10.4 | 9.1 | 0.6 |
| Shock + OLP | 8.3 | 3.1 | 75.4 | 87.1 | 10.4 | 7.2 | 0.8 |
| Solubles | 31.4 | 28.0 | 1.0 | 1.7 | 69.7 | 0.3 | 1.2 |
| Solubilized protein | 7.0 | 95.9 | 0.4 | 1.0 | 2.9 | 0.2 | 0.6 |
| Combined feed | 12.4 | 6.5 | 65.0 | 75.2 | 18.6 | 6.2 | 0.8 |
| Protein-balanced feed | 12.2 | 9.7 | 62.7 | 72.6 | 18.1 | 6.0 | 0.8 |

(DM = dry matter, OM= organic matter, OLP = oxidative lime pretreated, CP = crude protein, ADF = acid detergent fiber, NDF = neutral detergent fiber, NFC = non-fiber carbohydrates, Combined feed = 17.8% corn stover solubles and 82.2% shock + OLP corn stover, Protein-balanced feed = 3.3% solubilized protein, 17.2% corn stover solubles, and 79.5% shock + OLP corn stover)

Neither OLP nor shock treatment significantly affected the mineral composition of corn stover (Table 6-3). Slight increases of calcium were observed, particularly with OLP alone (2.9% DM), indicating that extensive washing was unable to fully remove all unreacted calcium ions. OLP also removed the majority of potassium, only leaving trace amounts. The corn stover solubles had significant calcium (3.3% DM) and potassium (11.7%) present.

Overall, similar results were observed with OLP and shock pretreated corn stover as with OLP and ball-milled sorghum. NDF increased whereas NFC and CP both decreased. Based on composition alone, OLP and shock treatment negatively affect the

feed value of corn stover; however, digestibility analysis provides a significantly different conclusion.

*48-h Neutral detergent fiber digestibility*

The 48-h NDFD of corn stover samples, corn grain standard, and alfalfa standard was measured using *in vitro* anaerobic fermentation (Figure 6-2). Previous literature has reported that improving forage NDFD increases dry matter intake and milk yield in dairy cows (Oba & Allen, 1999). The corn grain and alfalfa standards had NDFD values (g NDF digested/100 g NDF fed) of 63.2 and 47.9, respectively. The NDFD of raw corn stover (49.3) was similar to alfalfa. OLP alone improved NDFD to 79.0, whereas shock treatment alone reduced NDFD to 43.9. Shock + OLP corn stover (76.0) was slightly less digestible than OLP alone; however, OLP + shock-treated corn stover was the most digestible (79.3).

*Total digestible nutrients*

The TDN of the prepared corn stover samples, corn grain standard, and alfalfa standard were estimated using two methods: (1) Weiss formula (Equation 6-1) using chemical analysis results only ($TDN_W$) and (2) modified Weiss formula (Equation 6-4) that incorporates experimentally measured 48-h NDFD ($TDN_N$). Table 6-6 shows the TDN results derived from both methods on both a dry matter and organic matter basis. In this section, all TDN results discussed are presented as g nutrients digested/100 g organic matter fed.

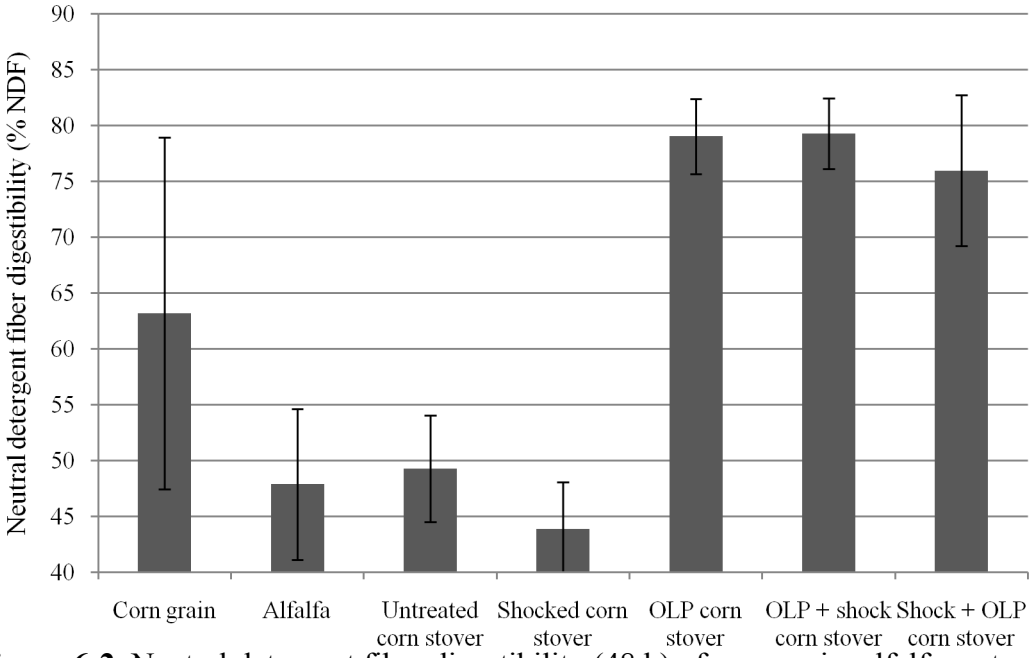


**Figure 6-2.** Neutral detergent fiber digestibility (48 h) of corn grain, alfalfa, untreated corn stover, and treated corn stover samples. NDFD was measured by Texas A&M University Animal Science Department. (Errors bars are ± 1σ of the enzymatic hydrolysis replicates.)

**Table 6-6**. Total digestible nutrients of corn grain, alfalfa, corn stover samples, solubilized protein, and balanced feeds.

| Sample | $TDN_W$ (%DM) | $TDN_W$ (%OM) | $TDN_N$ (%DM) | $TDN_N$ (%OM) |
|---|---|---|---|---|
| Corn grain | 86.0 | 87.1 | 87.0 | 88.1 |
| Alfalfa | 55.7 | 61.5 | 53.9 | 59.4 |
| Corn stover | | | | |
| Untreated | 48.1 | 52.2 | 47.8 | 51.9 |
| Shock treated | 40.9 | 43.8 | 37.5 | 40.2 |
| OLP | 42.7 | 46.7 | 54.5 | 59.7 |
| OLP + Shock | 45.2 | 50.3 | 62.5 | 69.7 |
| Shock + OLP | 49.7 | 54.2 | 66.6 | 72.6 |
| Solubles | 61.0 | 88.9 | NR | NR |
| Solubilized protein | 86.1 | 92.6 | 87.5 | 94.1 |
| Combined feed | 51.7 | 59.0 | 65.6 | 74.9 |
| Protein-balanced feed | 52.8 | 60.2 | 66.3 | 75.5 |

(DM = dry matter basis, OM = organic matter basis, OLP = oxidative lime pretreated, $TDN_W$ = total digestible nutrients calculated using Equation 6-1, $TDN_N$ = total digestible nutrients calculated using Equation 6-4, NR = not reported, Combined feed = 17.8% corn stover solubles and 82.2% shock + OLP corn stover, Protein-balanced feed = 3.3% solubilized protein, 17.2% corn stover solubles, and 79.5% shock + OLP corn stover)

Because of its high NFC content, corn grain had the highest $TDN_W$ (87.1) and $TDN_N$ 88.1. Both methods estimated comparable values for alfalfa (61.5 and 59.4) and corn stover (52.2 and 51.9) for $TDN_W$ and $TDN_N$, respectively. Because of the low NDFD for shocked corn stover, the models resulted in similar values: 43.8 ($TDN_W$) and 40.2 ($TDN_N$).

As discussed previously, OLP, OLP + shock-treated, and shock + OLP all increased NDFD, resulting in significant differences between the two TDN estimation methods. In all three cases, $TDN_N$ was much greater than $TDN_W$ because it accounts for the improved NDFD resulting from the biomass pretreatment methods. $TDN_N$ was 59.7 for OLP corn stover, and 69.7 for OLP + shock-treated corn stover. Of the corn stover samples, shock + OLP demonstrated the highest $TDN_N$ (72.6), a difference of 18.4 from the calculated $TDN_W$ value. These modified TDN values show the effectiveness of the pretreatment processes, and demonstrate that traditional forage empirical models cannot predict the feed value of high-digestibility lignocellulose.

In vitro *gas production*

During the 48-h *in vitro* anaerobic fermentation used to measure NDFD, a pressure sensor was attached to the incubation flask. This pressure sensor measured gas production during fermentation. The sensor recorded the pressure every 5 min for the duration of the fermentation (48 h), resulting in 2880 data points. The resulting gas production plot (Figure 6-3) can be correlated to fermentation rate. Combining $TDN_N$ and gas production, the rate of nutrient digestion can be plotted (Figure 6-4).

From the gas production data, the fractional rate of fermentation (Table 6-7) was determined using the following equation (Tedeschi et al., 2009):

$$V = V_F\{1 - \exp[-kf \times (t - \lambda)]\} \quad [6\text{-}8]$$

where $V$ = cumulative gas volume (mL), $V_F$ = gas volume corresponding to complete matter digestion (asymptote), $kf$ = fractional rate of fermentation ($h^{-1}$), $t$ = time (h), and $\lambda$ = lag time (h).

As expected because of its high NFC content, corn grain had the highest fractional rate of fermentation (0.17/h). Raw corn stover had a low fractional rate (0.04/h), whereas shock + OLP improved the fractional rate (0.13/h).

**Table 6-7.** Fractional rate of fermentation (*kf*).

| Sample | Fractional rate of fermentation (1/h) |
|---|---|
| Corn grain | 0.17 |
| Alfalfa | 0.11 |
| Corn stover | |
| Untreated | 0.05 |
| Shock treated | 0.05 |
| OLP | 0.10 |
| OLP + Shock | 0.08 |
| Shock + OLP | 0.13 |
| Solubles | 0.11 |
| Solubilized protein | 0.07 |

(OLP = oxidative lime pretreated)

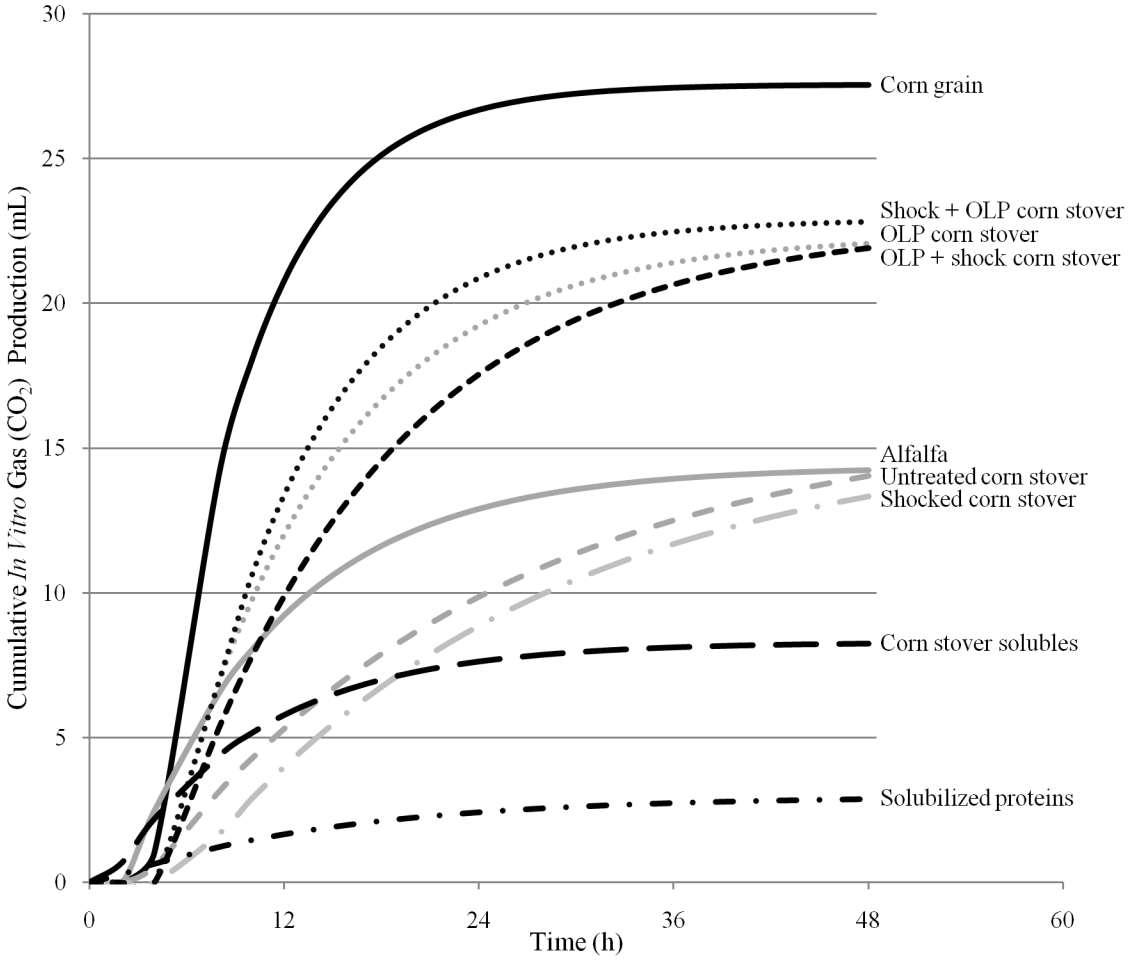


**Figure 6-3.** Gas production (mL) of corn grain, alfalfa, and corn stover samples during *in vitro* anaerobic fermentation.

*Addition of the soluble extractives*

As described in Section 2.1, the raw corn stover was thoroughly washed with hot water to extract soluble components (approximately 14% by dry weight). Table 6-5 shows the composition of the extractives. The extractives had a $TDN_W$ (g nutrients digested/100 g nutrients fed) of 61.0 on a dry matter basis, or 88.9 on an organic matter basis. [Note: NDFD (48 h) was not determined for the extractives, so $TDN_N$ could not

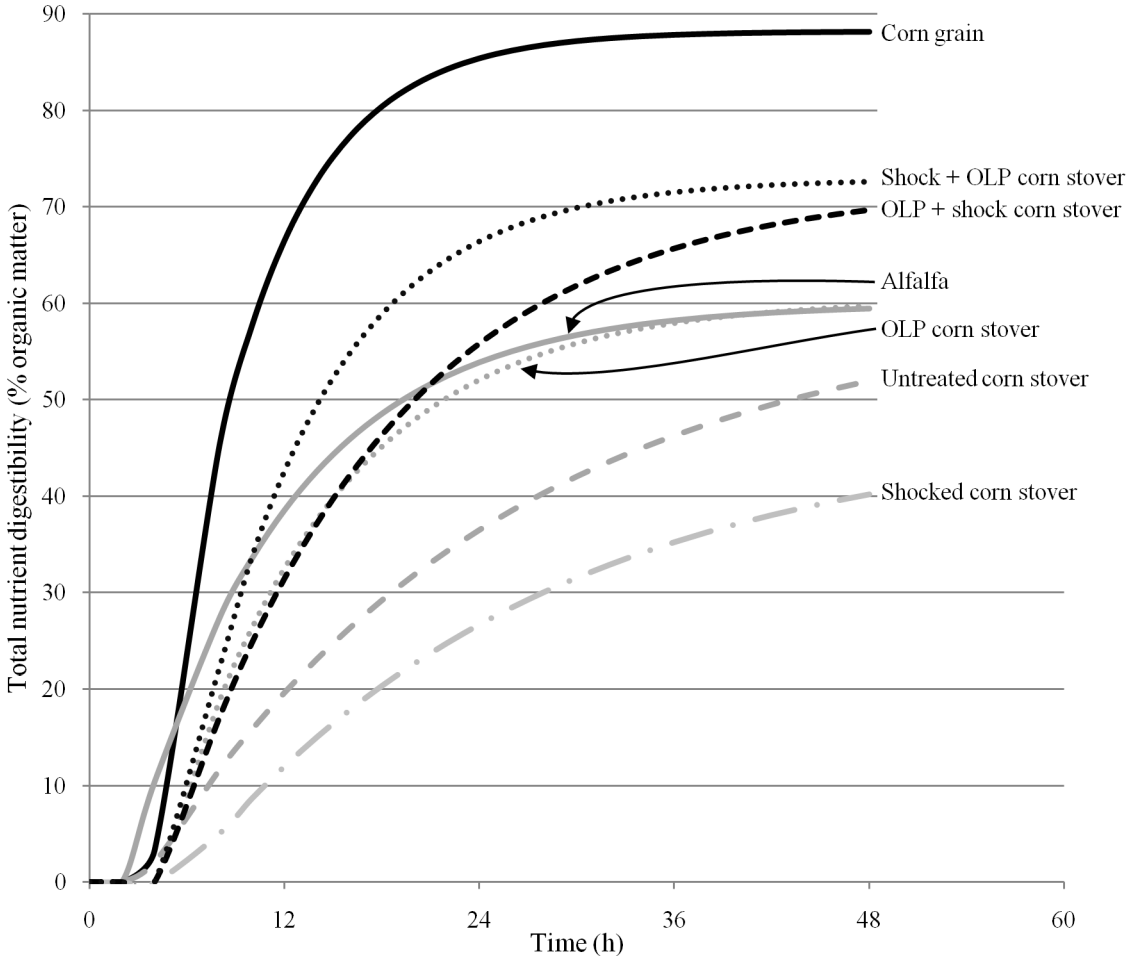


**Figure 6-4.** Total nutrient digestion rate of corn grain, alfalfa, and corn stover samples calculated using *in vitro* gas production and $TDN_N$ on an organic matter basis.

be calculated; however, the NDF content was so low the two TDN methods should produce comparable values.]

Figure 6-5 shows a mass balance for each process step on a dry matter basis. Of untreated corn stover, 14% was soluble and OLP solids yield was 75%. Combining the corn stover sample with the highest $TDN_N$ (shock+ OLP) with extractives is 17.8% extractives and 82.2% shock + OLP.

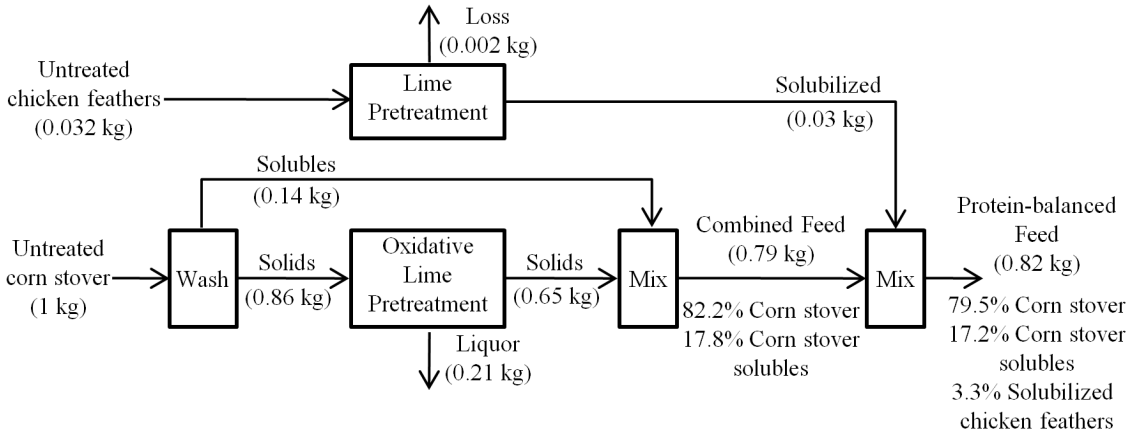


**Figure 6-5.** Mass balance for combining oxidative lime-treated corn stover with pre-washed corn stover soluble extractives and solubilized chicken feathers.

On a dry matter basis, shock + OLP corn stover had a $TDN_N$ of 66.6, and the extractives had a $TDN_W$ of 61.0. Their combined TDN is calculated as follows:

$$\text{TDN of combined feed} = (0.822)(66.6) + (0.178)(61.0) = 65.6$$

This combined TDN (65.6 g nutrients digested/100 g nutrients fed) shows a slightly negative effect from adding the extractives to the treated corn stover, and is considerably lower than corn grain (–20.5). This is because of the high ash content in the extractives material.

On an ash-free basis, the combined TDN can be calculated as follows:

$$\text{TDN of combined feed, ash} - \text{free basis} = \frac{(0.822)(66.6) + (0.178)(61.0)}{(0.822)(1-0.083) + (0.178)(1-0.314)} = 74.9 \qquad [6\text{-}9]$$

This compares more favorably to ash-free corn grain (–12.3).

*Solubilized protein*

Protein degradation is an unavoidable consequence of OLP, necessitating the development of protein supplementation strategies. Coward-Kelly et al. (2006b) used lime treatment to solubilize chicken feathers, resulting in a liquid rich in amino acids and poly-peptides. The primary amino acids present (as determined by high performance liquid chromatography analysis) are glycine + serine (160 g/kg CP), proline (80 g/kg CP), glutamine (50 g/kg CP), leucine (46 g/kg CP), alanine (43 g/kg CP), and valine (42 g/kg CP). One concern with using highly-soluble protein is ammonia production in the rumen; however, solubilized protein from chicken feathers produces similar levels of ammonia as soybean meal or cottonseed meal, and substantially less than urea.

This study determined the macronutrient (Table 6-5) and micronutrient (Table 6-3) composition of solubilized protein from chicken feathers. On an organic matter basis, the solubilized protein was comprised almost solely of crude protein (95.9%), with the second largest constituent being NFC (2.9%). The solubilized protein contained some ash (7% DM), which was primarily calcium (3.3% DM). Because of its low NDF content (1.0% OM), $TDN_W$ and $TDN_N$ were very similar (92.6% OM and 94.1% OM, respectively).

Adding solubilized chicken feathers to the combined feed (0.037 kg solubilized chicken feathers/1 kg combined feed) produces a protein-balanced feed with the same crude protein content of corn grain (Figure 6-5). This feed is comprised of 79.5% shock + OLP corn stover, 17.2% corn stover solubles, and 3.3% solubilized chicken feathers; the resulting $TDN_N$ on an organic basis is 75.5 g nutrients digested/100 g organic matter.

Table 6-5 shows the macronutrient composition of the combined and protein-balanced feeds, and Table 6-3 provides the mineral content of each. Figure 6-6 shows the total nutrient digestion rate of the protein-balanced feed, as well as OLP + shock and combined feed, compared to corn grain and alfalfa standards. Adding solubilized chicken feathers increases $TDN_N$ of the combined feed (+0.7). The balanced feed is slightly less digestible than corn grain (–12.6). Of the 12.6 difference, lignin alone accounts for 6.0, making it difficult to narrow the gap further.

## 6.4 Conclusions

With forage sorghum, OLP improved the NDF digestibility; however, adding ball milling resulted in a particle size that was too small for animal feed applications.

| | A | B | C |
|---|---|---|---|
| Shock + OLP corn stover | 79.5% | 82.2% | 100% |
| Corn stover solubles | 17.2% | 17.8% | 0% |
| Solubilized chicken feathers | 3.3% | 0% | 0% |

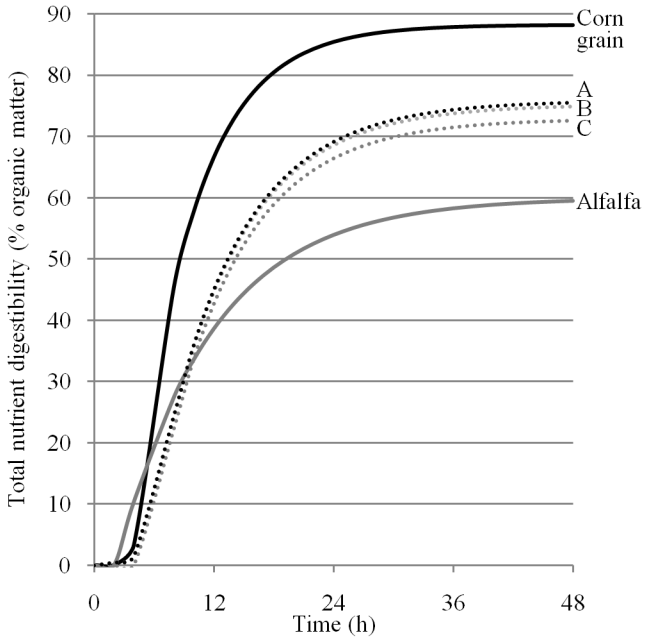


**Figure 6-6.** Total nutrient digestion rate of combined and protein-balanced feeds calculated using *in vitro* gas production and $TDN_N$ on an organic matter basis.

With corn stover, combining OLP with shock treatment improved the 48-h neutral detergent fiber digestibility (NDFD) to 79.0 g NDF digested/100 g NDF fed, compared to 49.3 for raw corn stover. Shock treatment did not further improve NDFD, but did increase total digestible nutrients (TDN). On an organic matter basis, shock + OLP corn stover had a TDN of 72.6, which approached that of corn grain (88.1). When extractives are added, TDN increases to 74.9, which is only 13.2 less than corn grain. When enough solubilized chicken feathers are added to match the protein content of corn grain, TDN increases to 75.5, which is only 12.6 less than corn grain.

# CHAPTER VII

# CONCLUSIONS

The main purpose of this work was to enhance the digestibility of lignocellulosic biomass, essentially generating a high-value energy source from a low-value feedstock. By reducing or eliminating key structural barriers of lignocellulose (e.g., lignin content, acetyl content, or cellulose crystallinity) enzyme or microorganism accessibility can be significantly increased. To accomplish this goal, this study employed a combination of oxidative-lime pretreatment (OLP) and mechanical treatment (e.g., ball milling, shock treatment).

Switchgrass has been chosen as a model biofuel feedstock by the U.S. Department of Energy. To determine the recommended OLP conditions for switchgrass, Dacotah switchgrass, a northland upland variety, was studied using three modes of OLP: very-short term (150–200 °C, 5–30 min, 3.45–6.89-bar $O_2$), short term (100–150 °C, 1–4 h, 3.45–6.89 bar $O_2$), and long term (65 °C, 1–28 d, bubbled air). The short-term OLP was the most successful, and the recommended conditions were 120 °C, 6.89-bar $O_2$, and 120 min. At these conditions, 72-h overall glucan yield (g glucan digested/100 g glucan in raw biomass) was 85.2, and 72-h overall xylan yield (g xylan digested/100 g xylan in raw biomass) was 50.1 (15 FPU/g raw glucan).

To determine the effect that the variety of a biomass species has on OLP, Alamo switchgrass was also studied. Alamo is a southern lowland variety of switchgrass, and

has lower carbohydrate and lignin content than Dacotah. Assuming similar behavior, only short-term OLP was employed for Alamo switchgrass. The recommended conditions for Alamo were similar to Dacotah (110 °C, 6.89-bar $O_2$, and 240 min) and achieved a 72-h overall glucan yield of 88.5 (15 FPU/g raw glucan). The 72-h overall xylan yield was considerably higher (78.2), but also included xylan oligomers recovered from the pretreatment liquor. Adding ball milling to OLP further improved 72-h overall glucan yields to 91.1 and 90.0 for Dacotah and Alamo, respectively. These yields were achieved at a significantly lower enzyme loading (7 FPU/g raw glucan).

Collaborating with the Consortium for Applied Fundamentals and Innovation (CAFI) biomass refining group, several leading biomass pretreatment technologies were compared. Dacotah switchgrass was optimally pretreated using ammonia fiber expansion (AFEX), dilute acid, liquid hot water (LHW), soaking in aqueous ammonia (SAA), sulfur dioxide, OLP, and OLP + ball mill. Each pretreated sample was subjected to a 72-h enzymatic hydrolysis using a variety of total enzyme loadings (13.4–243.4 mg protein/g raw glucan), each consisting of four different cellulase:xylanase loading ratios. This work produced a data set describing the overall glucan and xylan yields for each pretreatment, over a wide variety of enzyme loadings. A useful relationship, enzymatic yield, was defined to determine the optimal enzyme loading which results in high sugar yields while minimizing the use of costly enzymes. For example, OLP had a maximum enzymatic yield (g sugar digested/g protein loaded) of 64.2, which compared favorably to that of AFEX (43.8). Adding ball milling to OLP significantly increased maximum enzymatic yield to 91.3.

Previous work has demonstrated the effectiveness of shock treatment in enhancing the enzymatic digestibility of lignocellulose; however, most results were inconsistent. This study refined the shock treatment procedure using a systematic approach to determine recommended treatment conditions. First, proof of reliability was determined by treating five different biomass feedstocks: bagasse, corn stover, poplar wood, sorghum, and switchgrass. Enzymatic digestibility (24 and 48 h; 5, 15, and 60 FPU/g raw glucan) was compared between untreated, OLP, OLP + ball milled, and OLP + shock-treated samples of each biomass type. For the 24-h hydrolysis (5 FPU/g raw glucan), OLP + shock achieved glucan yields (g glucan hydrolyzed/100 g treated glucan) of 38.4 (bagasse), 74.6 (corn stover), 64.1 (poplar wood), 52.0 (sorghum), and 66.3 (switchgrass); all glucan yields were higher than the respective OLP + ball-milled samples.

Because of the superior performance of corn stover, it was chosen to explore several variables associated with shock treatment: biomass particle size, temperature and state of biomass, and effect of multiple shocks. It was determined that biomass particle size had negligible effect on shock effectiveness, ambient temperature and never-frozen biomass were adequate, and multiple shocks were not necessary.

Preliminary studies explored using OLP and ball milling to generate highly digestible sorghum for ruminant feed applications; however, ball milling results in small particles that are unable to maintain the necessary residence time in the rumen. To remediate this issue, shock treatment + OLP was employed on corn stover. The combined treatments improved total digestible nutrients ($TDN_N$; g nutrients digested/100

g organic matter) from 51.9 (untreated corn stover) to 72.6. Adding the pre-washed soluble content of corn stover to OLP + shocked corn stover increases $TDN_N$ to 74.9. Mixing in solubilized protein from chicken feathers to match the protein content of corn grain further increased $TDN_N$ to 75.5, only 12.6 less than corn grain.

Future work should focus on the following:

- Developing recommended shock treatment conditions for other feedstocks such as sorghum and bagasse.
- Scale-up the shock tube apparatus.
- Fermentation studies using OLP + shock-treated biomass to compare with established enzymatic results.
- Proper feed trial using shock + OLP corn stover.

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# APPENDIX A

## OXIDATIVE LIME PRETREATMENT

Oxidative lime pretreatment is highly effective at reducing lignin content, as well as removing acetyl groups from hemicellulose. The reaction can be performed in a variety of vessels depending on the desired reaction conditions. The oxidative lime pretreatment setups are categorized by reaction time: very-short term (5–30 min), short term (60–240 min), and long term (1–40 days). The start-up procedure for each of these pretreatments is provided here. Post-pretreatment conditioning, determination of lime consumption, and determination of pretreated solids yield are also discussed.

### *Substrate preparation*

1. Determine moisture content of biomass to be pretreated (Appendix D). If grinding is required, dry to a moisture content of <10%.
2. If desired, grind biomass to a consistent particle size. The majority of this work used a particle size of –20/+80.

### *Very-short-term pretreatment procedure*

1. Weigh out 8 g of biomass and 8 g of lime ($Ca(OH)_2$). Mix thoroughly and then transfer into the very-short-term reactor.
2. Slowly add 120 mL of distilled water and then tightly seal the reactor.

3. Place the reactor vessel onto the shaking apparatus (Figure A-1), and then attach the flexible hose attached to the oxygen cylinder.
4. Start the shaking apparatus, open the oxygen line to desired oxygen pressure, and turn on heating element. This starts the reaction time.
5. Monitor the reaction temperature, turning the heating element off/on to maintain desired temperature.
6. Once pretreatment time has elapsed, close the oxygen valve, turn off the shaking apparatus, and turn off the heating element.
7. To speed up the cooling process, blow compressed air over the reactor.
8. Once the reactor has cooled enough to handle with heat-resistant gloves, bleed the pressure line and then very slowly open the reactor.
9. Quantitatively transfer the reactor contents to a 1-L centrifuge bottle using distilled water.
10. Follow the post-pretreatment conditioning procedure.

**Figure A-1**. Very-short-term pretreatment reactor.

***Short-term pretreatment procedure***

1. Weigh out 8 g of biomass and 8 g of lime ($Ca(OH)_2$) for each reactor to be used (Figure A-2). Mix thoroughly and then transfer into the pretreatment reactor (5-in long × 1.5-in I.D. 304 stainless steel).
2. Add 120 mL of water and seal using a 304 stainless steel cap and Teflon tape.
3. Attach the reactor to a holder and load in an oven preheated to the desired reaction temperature.
4. Immediately connect the flexible hose attached to the oxygen cylinder and open the oxygen line to the desired pressure. Start the shaking mechanism (swing arm), which starts the reaction time.
5. When the desired pretreatment time has elapsed, close the oxygen valve, stop shaking, and turn off the oven.
6. Open the oven to start the cooling process. To speed up the cooling process, reactors may be placed in contact with an ice-water bath.
7. Once the reactors have cooled to a safe handling temperature, carefully open the reactors to slowly depressurize the system.
8. Using distilled water, carefully and completely transfer all the reactor contents into a 1-L centrifuge bottle.
9. Follow the post-pretreatment conditioning procedure.

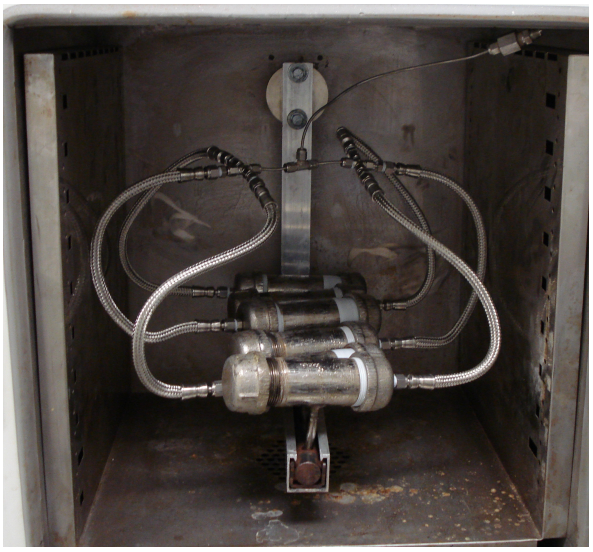

**Figure A-2**. Short-term pretreatment reactor.

***Long-term pretreatment procedure***

1. Fill the water tank (Figure A-3) for the circulating water system. The water level should be nearly full, and this needs to be regularly checked and refilled when necessary.
2. Turn on the centrifugal pump to circulate the water. Check for any leaks in the system and correct as needed.
3. Turn on the temperature controller to heat up the circulating water to the desired temperature.
4. The water tank, centrifugal pump, and temperature controller need to be checked and maintained regularly to ensure the system is operating at steady state.
5. Weigh out 15.0 g of biomass and the desired amount of lime. Mix thoroughly and then transfer into the reactor using a funnel. Add 150 mL of distilled water.

6. Tightly cap the reactor and connect the bubble indicator to measure the gas flow rate.
7. Slowly open the air valve located at the bottom of the reactor to supply air. Adjust the gas flow rate to achieve 2–3 bubbles/second in the bubble indicator apparatus. Regularly check the gas flow rate and adjust as needed.
8. After the pretreatment time has elapsed, remove the reactors and cool to room temperature. Transfer reactor contents to 1-L centrifuge bottles.
9. Follow post-pretreatment conditioning procedure.

**Figure A-3**. Long-term pretreatment setup.

***Post-pretreatment conditioning procedure***

1. Vacuum filter the pretreated slurry using a Buchner funnel and quantitative filter paper.

2. Measure the volume and pH of the filtrate and record these values.
3. Using a spatula and distilled water, transfer the pretreated solids from the Buchner funnel back to the 1-L centrifuge bottle.
4. Add 500 mL of distilled water to the pretreated solids in the centrifuge bottle and thoroughly mix.
5. Slowly add 5-N HCl until the pH reaches 7.0. Record the volume of 5-N HCl required and calculate lime consumption with the following formula:

$$W_{\mathrm{Ca(OH)_2}} = \frac{1\ \mathrm{mol\ Ca(OH)_2}}{2\ \mathrm{mol\ HCl}} \times \frac{N_{\mathrm{HCl}} \cdot (V_{\mathrm{HCl}})}{1000\ \mathrm{mL/L}} \times M_{\mathrm{Ca(OH)_2}}$$

where

$W_{Ca(OH)2}$ = The amount of lime ($Ca(OH)_2$) unreacted (g)

$N_{HCl}$ = Normality of HCl solution (mol $H^+$/L)

$V_{HCl}$ = Volume of HCl required to titrate the biomass slurry (mL)

$M_{Ca(OH)2}$ = Molecular weight of $Ca(OH)_2$, 74.092 g/mol

6. Further titrate the slurry until the pH reaches 4.0. At this point all of the residual lime is solubilized.
7. Vacuum filter the slurry using a Buchner funnel. Transfer the pretreated solids back to the centrifuge bottle using a spatula and distilled water. Add 500 mL distilled water to the bottle and stir for at least 5 minutes.
8. Repeat Step 7 until the pH reaches 6.0. Filter the pretreated slurry one final time.

9. Quantitatively transfer the pretreated solids to a tared weighing dish using a spatula. Record this weight and then determine the moisture content of the pretreated solids (Appendix D).
10. Use the final dry weight (corrected for moisture content) and the initial dry weight (corrected for moisture content) to determine the pretreatment solids yield.

# APPENDIX B

## BALL-MILLING PROCEDURE

Research has demonstrated that cellulose crystallinity is one of the primary hurdles to enzymatic digestion. Ball-milling is a proven laboratory technique to decrystallize biomass without carbohydrate degradation. Ball-milling is often combined with a chemical pretreatment (e.g., oxidative lime pretreatment).

### *Preparation of the sample*

1. Thoroughly dry the biomass sample to moisture content less than 10%.
2. Grind the sample to a consistent particle size, typically –20/+80.

### *Procedure*

1. Determine an appropriate amount of zirconia grinding media (ZGM) to fill approximately 50% of the porcelain jar volume.
2. Record this weight and load the ZGM into the porcelain jar (Figure B-1).
3. Load the prepared biomass sample into the jar at a ratio of 43 g ZGM/g dry biomass.
4. Seal the jar using a rubber gasket and locking lid.
5. Repeat Steps 1–4 for the desired number of jars.
6. Place prepared jars onto the rolling apparatus and allow jars to roll for 72 h.

7. Remove locking lid and transfer ZGM and ball-milled biomass into a metal sieve with a coarse mesh.
8. Using a bottom tray and lid, shake the sieve to separate the ball-milled biomass from the ZGM.
9. Collect the ball-milled biomass.

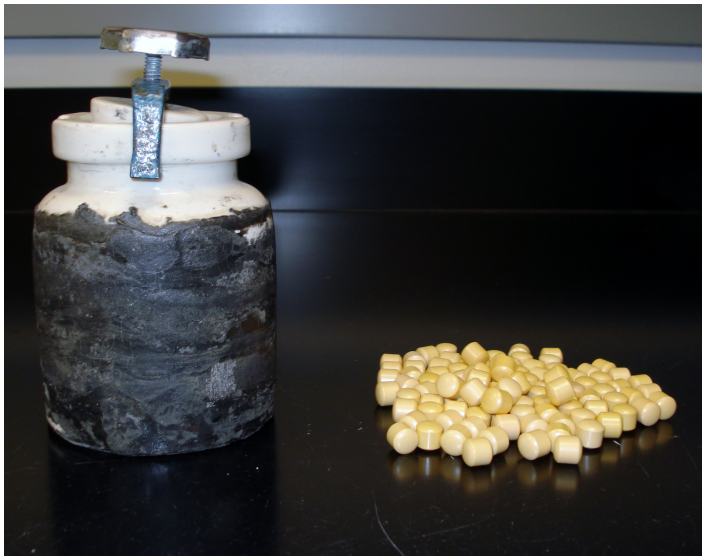

**Figure B-1**. Porcelain jar and zirconia grinding media.

# APPENDIX C

## SHOCK TREATMENT

Shock treatment is a novel mechanical pretreatment method used to further increase the digestibility of biomass, and is used in combination with oxidative lime pretreatment. This work defined the set of operating conditions that resulted in the most enzymatically digestible biomass.

### *Preparation*

1. Weigh out samples in 100 g batches. Place each batch in a labeled freezer bag. Add enough water to thoroughly soak the biomass without any excess.
2. Freeze biomass samples overnight if desired. Thaw for desired amount of time before leaving for the shock tube site.
3. Pack required supplies to take to shock tube site: prepared biomass samples, chest of ice, large graduated cylinder, freezer bags, safety glasses, latex gloves, 4-L plastic buckets, thermometer, shotgun shells, paper towels, and a coarse metal sieve.
4. Once at shock tube site: (1) clean and assemble shock tube (Figure C-1), (2) fill and adjust temperature of the water bath, and (3) lower the shock tube into the water bath using mechanized winch.

***Procedure***

1. Using the graduated cylinder and accounting for the water used to prepare the biomass sample, measure out the volume of water required to bring the total volume to 2 L. This is the sample water.
2. Transfer the prepared biomass sample into the shock tube. Use the sample water to completely transfer all of the biomass.
3. Add any remaining sample water to the shock tube and lower the upper unit of the shock tube into place.
4. Seal the shock tube by tightening the eight bolts using a pneumatic impact wrench.
5. Insert the shotgun shell into the top of the barrel and affix the firing apparatus.
6. Move a safe distance away (behind a steel safety wall) and pull the firing pin, discharging the shotgun shell.
7. Loosen and remove the eight bolts, and lift the upper unit of the shock tube.
8. Transfer the contents of the shock tube into a 4-L bucket, which is to be transferred back to the laboratory.
9. Thoroughly rinse out the shock tube and barrel with water.
10. Repeat Steps 1–9 for additional treatments.
11. Once back at the laboratory, use vacuum filtration or centrifugation to isolate the solid shocked material.
12. Carefully sort through the shocked material to remove any remnants of the shotgun shell or shot.

13. Allow the material to air dry for analysis.


**Figure C-1**. Shock tube apparatus. (a) Entire apparatus, (b) barrel and cone, (c) shotgun shell loaded in barrel, (d) firing mechanism.

# APPENDIX D

## DETERMINATION OF MOISTURE CONTENT IN BIOMASS

For the purpose of consistency, it is vital to perform all biomass procedures and calculations on a dry biomass basis. This procedure is based on the NREL standard procedure "Determination of Total Solids and Moisture in Biomass and Total Dissolved Solids in Liquid Process Samples" (Sluiter et al., 2008a).

### *Procedure*

1. Pre-dry aluminum weighing dishes by placing them in a 105 ± 3 °C drying oven for a minimum of 4 h. Transfer the crucibles to a desiccator until they are cooled to room temperature. Always handle the crucibles with gloved hands or tweezers.
2. Weigh a pre-dried crucible to the nearest 0.1 mg and record this weight as $W_1$.
3. Thoroughly mix the sample, transfer an appropriate amount into the weighing dish, and record the weight of the sample plus weighing dish as $W_2$.
4. Repeat Steps 2 and 3 until all samples are weighed out. Each sample should be analyzed in duplicate, at minimum.
5. Place the samples into a convection oven at 105 ± 3 °C and dry to constant weight. The recommended drying time is 24 h.
6. Transfer the samples from the oven into a desiccator and allow them to cool to room temperature.

7. Record the weight of the dried sample plus weighing dish as $W_3$.

8. Repeat Steps 5–7 until you observe a change of weight ≤ 1%.

***Calculation***

$$\%\ \text{Total Solids}\ (TS) = \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

$$\%\ \text{Moisture Content}\ (MC) = 100 - TS$$

where

$W_1$ = Weight of empty weighing dish

$W_2$ = Weight of wet sample plus weighing dish

$W_3$ = Weight of dry sample plus weighing dish

# APPENDIX E

## DETERMINATION OF ASH CONTENT IN BIOMASS

The purpose of this procedure is to determine the amount of inorganic material present in biomass. This procedure is based on the NREL standard procedure "Determination of Ash in Biomass" (Sluiter et al., 2005a).

### *Sample preparation*

1. Label an appropriate number of 50-mL porcelain ashing crucibles with a porcelain marker, and place them in a muffle furnace at 575 ± 25 °C for a minimum of 4 h.
2. Remove the crucible from the furnace directly into a desiccator. Cool for exactly 1 h.
3. Weigh the crucible to the nearest 0.1 mg and record the weight (*WC*).
4. Determine the moisture content of each sample (Appendix D) immediately prior to weighing the sample.
5. Analyze each sample in duplicate, at minimum.

### *Procedure*

1. Weigh 0.5 to 2.0 g, to the nearest 0.1 mg, of the sample into the tared crucible. Record the sample weight as *WC*.

2. Place the crucibles into the muffle furnace at 575 ± 25 °C for 24 ± 6 h. When handling the crucible, protect the sample from drafts to avoid mechanical loss of sample.
3. Carefully remove the crucible from the furnace directly into a desiccator and cool for exactly 1 h.
4. Weigh the crucibles and ash to the nearest 0.1 mg and record the weight (*WCA*).
5. Repeat Steps 6–8 until a constant weight is achieved.

***Calculation***

$$\% \text{ Ash} = \frac{WCA - WC}{ODW} \times 100$$

where

*WCA* = Weight of the crucible plus ash

*WC* = Weight of the crucible

*ODW* = Dry weight of the sample (correct by moisture)

# APPENDIX F

## EXTRACTIVES IN BIOMASS

This procedure is used to determine the amount of non-structural material present in biomass. It is necessary to remove the non-structural components to prevent interference when measuring carbohydrate and lignin content. This procedure uses a two-step extraction process to remove water-soluble and ethanol-soluble material. It is often sufficient to only perform the ethanol extraction. This procedure is based on the NREL standard procedure "Determination of Extractives in Biomass" (Sluiter et al., 2005b).

### *Preparation*

1. Determine the moisture content of the biomass sample (Appendix D).
2. Dry a boiling flask (500-mL capacity) in a 105 ± 5 °C drying oven for a minimum of 12 h. Transfer glassware straight into a desiccator and cool to room temperature.
3. Weigh the dried boiling flask to the nearest 0.1 mg and record the weight as *WF*.
4. Add 2–10 g of sample to a tared cellulose extraction thimble. Record the oven dry weight to the nearest 0.1 mg as *ODW*. The height of the biomass in the thimble must not exceed the height of the Soxhlet siphon tube.
5. Add 190 ± 5 ml of solvent (HPLC-grade water or 190-proof ethanol) to the dried boiling flask.

6. Assemble the Soxhlet apparatus (heating mantle, boiling flask, Soxhlet tube, and condenser).

***Procedure***

7. Turn on the heating mantles and reflux for 16–24 h.
8. Adjust the heating mantle to provide a minimum of 4–5 siphon cycles per hour for water extraction, and 6–10 siphon cycles per hour for ethanol extraction.
9. Once the desired reflux time is reached, turn off the heating mantles and allow the glassware to cool to room temperature.
10. Remove the thimble and transfer the extracted solids, as quantitatively as possible, onto cellulose filter paper in a Buchner funnel.
11. Wash the solids with approximately 100 mL of fresh solvent (HPLC-grade water of 190-proof ethanol depending on extraction method).
12. Allow the solids to dry using vacuum filtration or air dry.
13. Combine any solvent from the Soxhlet tube with the remaining solvent in the boiling flask.
14. Use a rotary evaporator with a water bath set to 40 ± 5 °C and a vacuum source to remove the solvent. Continue to remove solvent until all visible solvent is gone.
15. Place the flask in a vacuum oven at 40 ± 2 °C for 24 h. Cool to room temperature in a desiccator and then weigh the flask to the nearest 0.1 mg. Record this weight as *WFR*.

***Calculation***

The extractives content is calculated using the following equation:

$$\% \text{ Extractives} = \frac{WFR - WF}{ODW} \times 100$$

where

$WFR$ = Weight of the flask plus residue

$WF$ = Weight of the flask

$ODW$ = Weight of the sample corrected by its moisture content

# APPENDIX G

## DETERMINATION OF STRUCTURAL CARBOHYDRATES AND LIGNIN IN BIOMASS USING ACID HYDROLYSIS

The purpose of this procedure is to quantify the following components of biomass: glucan, xylan, arabanin, and lignin. This procedure is based on the NREL standard procedure "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al., 2008b).

### *Preparation*

1. Using the procedure given in Appendix D, determine the moisture content of the sample. The moisture content must be 10% or less.
2. Grind the biomass until the particle size is in the range –20/+80 mesh.
3. For untreated biomass, the sample must be extractives free (Appendix F). Lime-pretreated samples should already be free of extractives.
4. Dry filtering crucibles (25-mL, medium porosity, Coors #60531) at 105 °C oven for a minimum of 4 h.
5. Transfer filtering crucibles to a desiccator and cool for 1 h. Record their weight to the nearest 0.1 mg as *WC*.
6. Prepare a series of sugar calibration standards. The standards should contain known concentrations of D-cellobiose, D-(+) glucose, D-(+) xylose, and D-(+)

mannose. The range of concentrations is suggested as 0.5, 1.0, 2.0, 5.0, and 10.0 mg/mL.

***Procedure***

1. Weigh 0.3 ± 0.01 g of the sample and transfer it into a labeled 16×100 mm test tube. Record the weight of the sample to the nearest 0.1 mg as *ODW*. Each sample should be run in replicate, triplicates are recommended.
2. Add 3.00 ±0.01 mL of 72% sulfuric acid to each test tube and place the test tubes in a water bath set at 30 ± 3 °C for 1 h. Using a Teflon stir rod, stir the samples every 5 to 10 min without removing them from the water bath.
3. While the samples are incubating, prepare the sugar recovery standard (SRS). This should include every sugar to be analyzed, and their concentrations should be representative of the sugar concentrations in the test sample.
    a. Weigh the required amount of sugar (to the nearest 0.1 mg) and transfer it to a pressure glass bottle. Add 84.0 mL deionized water and 3 mL of 72% sulfuric acid.
    b. Immediately shake vigorously and transfer a 10-mL aliquot into a 50-mL conical centrifuge tube. Neutralize this aliquot using calcium carbonate and label as SRS 1.
4. Once the sample test tubes have incubated for 1 h, remove the tubes from the water bath.

5. Carefully and completely transfer each sample from the test tube to a pressure bottle using 84.00 ± 0.04 mL deionized water. This dilutes the acid to a 4% concentration.
6. Seal the bottles, including the SRS bottle, and place them in an autoclave.
7. Autoclave the samples for 1 h at 121 °C.
8. Allow the samples to slowly cool to room temperature, and then remove their caps.
9. Vacuum filter the autoclaved hydrolysis solution through one of the prepared filtering crucibles.
10. Capture the filtrate in a filtering flask.
11. Transfer a 10-mL aliquot to a labeled conical centrifuge tube. This sample will be used to determine carbohydrate content.
12. Use a minimum of 50 mL of hot deionized water to quantitatively transfer all remaining solids from the pressure bottle into the filtering crucible.
13. Dry the filtering crucible and acid insoluble residue at 105 °C for at least 24 h.
14. Transfer the crucibles containing the dry residue from the oven into a desiccator, cool for 1 h, and then record the weight to the nearest 0.1 mg as *WCR*.
15. Place the crucibles containing the dry residue in a muffle furnace at 575 ± 25 °C for 24 h.
16. Carefully remove the crucible from the furnace and place it into a desiccator to cool for 1 h. Weigh the crucibles and ash to the nearest 0.1 mg. Record this weight as *WCA*.

***Carbohydrate analysis***

1. Use calcium carbonate to neutralize each 10-mL aliquot (including the autoclaved sugar recovery standard, termed SRS 2) to a pH of 5–6.
2. Centrifuge the sample, and pass 1 mL of the decanted liquid through a 0.2-µm syringe filter into a HPLC autosampler vial. Seal and label the vial.
3. Analyze the calibration standards, SRS 1, SRS 2, and samples by HPLC using a Biorad Aminex HPX-87P column equipped with appropriate guard column. Use the following HPLC conditions:

   Injection volume: 20 µL

   Mobile phase: HPLC grade water, 0.2-µm filtered and degassed

   Column temperature: 85 °C

   Detector: Refractive Index

   Run time: 30 min

***Calculations***

Acid insoluble lignin:

$$\%\,AIL = \frac{(WCR - WCA)}{ODW} \times 100$$

where

$\%\,AIL$ = Percentage of acid insoluble lignin

$WCR$ = Weight of crucible plus dry residue

$WCA$ = Weight of crucible plus ash

$ODW$ = Dry weight of the sample

Recovery of SRS:

$$RSRS = \frac{SRS\ 1}{SRS\ 2}$$

where

*RSRS* = Fractional recovery of SRS

*SRS 1* = Concentration of sugar as measured by HPLC before autoclaving

*SRS 2* = Concentration of sugar as measured by HPLC after autoclaving

Percentage of each sugar:

$$\%\ \text{Sugar} = \frac{C_{\text{HPLC}} \times AC \times 87}{RSRS \times ODW \times 10}$$

where

% Sugar = Percent composition of sugar

$C_{\text{HPLC}}$ = Concentration of sugar as given by HPLC (mg/mL)

*AC* = Anhydro correction to calculate the concentration of polymeric sugars from the corresponding concentration of monomeric sugars. This value is 0.9 for glucose and 0.88 for xylose.

*ODW* = Dry weight of the sample

# APPENDIX H

## ENZYMATIC HYDROLYSIS

The purpose of enzymatic hydrolysis is to determine the change in carbohydrate digestibility after biomass pretreatment. Enzymatic hydrolysis was used to compare the digestibility of untreated, oxidative lime pretreated, oxidative lime + ball-mill, and oxidative lime + shock treated biomass. This procedure is based on the NREL standard procedure "Enzymatic Saccharification of Lignocellulosic Biomass" (Selig et al., 2008).

### *Sample and analysis preparation*

1. Ensure that the biomass has been completely neutralized and any residual lime has been washed out. Deviations in the pH significantly affect the enzymatic hydrolysis yields.
2. Determine the moisture content of the samples to be hydrolyzed (Appendix D).
3. Measure carbohydrate content of the samples according to Appendix G.
4. The pretreatment solids yield must be obtained before hydrolysis (Appendix A).
5. If necessary, measure the enzyme activity using NREL standard procedure "Measurement of Cellulase Activities."
6. Calculate the amount of biomass equivalent to 0.1 g of glucan in raw biomass as follows:

$$B = \frac{0.1}{G \times TS}$$

where

$B$ = Biomass to be weighed

$G$ = Glucan fraction in the treated biomass

$TS$ = Solid fraction in the sample (equivalent to 1 minus moisture content)

Calculate the amount of enzyme to be added using the following formula:

$$E_1 = \frac{0.1 \times E}{Y_G \times E_A}$$

where

$E_1$ = Amount of enzyme to be added

$E$ = Enzyme loading (typically 5, 15, or 60 FPU/g glucan in raw biomass)

$E_A$ = Enzyme activity

$Y_G$ = Pretreatment yield of glucan

7. Prepare 1-M citric acid solution by dissolving 210 g of citric acid monohydrate in 1 L of distilled water. Adjust the pH to 4.5 by adding NaOH.

***Procedure***

1. Weigh $B$ g of biomass into a labeled 50-mL conical centrifuge tube.
2. Dilute the 1-M stock citric acid monohydrate solution to 0.1 M.

3. Add sodium citrate buffer (5 mL, 0.1 M, pH 4.8), tetracycline (40 µL, 10 mg/mL in 70% ethanol), cycloheximide (30 µL, 10 mg/mL in distilled water).
4. Calculate the volume of distilled water ($W$) required to obtain a total volume to 10 mL. Assume all components have a specific gravity of 1 mg/mL. Also, calculate the required amount of cellobiase enzyme ($E_2$) to obtain the desired cellobiase loading (typically 60 CBU/g glucan in raw biomass).

$$W = 5 - 0.03 - 0.04 - B - E_1 - E_2$$

5. Measure the pH in the centrifuge tubes and adjust to 4.8 with either a saturated solution of sodium hydroxide or acetic acid as necessary.
6. Tightly cap the tubes and preheat them in a rotary incubator at a speed of 100 rpm and a temperature of 50.0 °C for 1 h.
7. Remove the tubes from the incubators, uncap, and add the enzymes as quickly as possible. Place the tubes back in the incubator, at a minimum angle of 45° to ensure good mixing. Record the time.
8. Once the desired time has elapsed, remove the tubes from the incubator and place them in a temperature controlled oven set at 105 °C for 5 min. This denatures the enzymes.
9. Transfer the tubes to a ice-water bath for 10 min to let them cool.
10. Store samples in the freezer until analysis.

***Analysis***

1. Fully thaw samples if necessary, and then ensure they are well mixed.

2. Centrifuge samples for 10 min at 4000 rpm to separate the solid residue.
3. Pass 1 mL of the decanted liquid through a 0.2-µm syringe filter into an HPLC autosampler vial. Seal and label the vial.
4. Analyze calibration standards and samples by HPLC using a Biorad Aminex HPX-97P column equipped with appropriate guard column. Use the following HPLC conditions:

   Injection volume: 20 µm

   Mobile phase: HPLC grade water, 0.2-µm filtered and degassed

   Column temperature: 85 °C

   Detector: Refractive Index

   Run time: 30 min

***Calculation***

$$\% \text{ Digestion} = \frac{C_{\text{HPLC}} \times AC \times 10}{0.1}$$

where

$C_{\text{HPLC}}$ = Concentration of the sugar as given by HPLC in g/mL

$AC$ = Anhydro correction to calculate the concentration of polymeric sugars from the corresponding concentration of monomeric sugars. This value is 0.9 for glucose and 0.88 for xylose.

The values 10 and 0.1 stand for volume of the sample and grams of glucan added, respectively.

# APPENDIX I

## WALSETH-TREATED CELLULOSE

A widely accepted cellulose decrystallization technique is described by Curtis S. Walseth (1952). Cellulose is swollen in 85% phosphoric acid for a desired length of time, and then rapidly washed with water to minimize degradation. Walseth demonstrated that this swelling significantly reduces crystallinity, enhancing enzymatic digestibility.

### *Procedure*

1. Weigh out the desired amount of microcrystalline cellulose (typically 10–50 g) into a large beaker (500 mL).
2. Add chilled (2 °C) 85% phosphoric acid in sufficient quantity to completely soak the cellulose (approximately 13 mL/g dry cellulose).
3. Store the mixture in the refrigerator at 2 °C for the desired swelling time (2 h was used for this work).
4. Remove the mixture from the refrigerator and slowly add to a vat of ice-cold stirred water (2 L) to precipitate the cellulose.
5. Filter the precipitated cellulose through a large, sintered glass filter.
6. Re-suspend the cellulose in ice-cold water and filter.
7. Repeat Step 6 until the cellulose has been washed four times.
8. Suspend the cellulose in a 1% sodium carbonate solution for 6 h or overnight.

9. Continue washing with room temperature distilled water until the pH of the suspension is the same as that of distilled water.
10. Dry the swollen cellulose under vacuum and then grind in a coffee grinder to break up any clumps.

# APPENDIX J

## DETERMINATION OF CELLULOSE REDUCING ENDS USING COPPER NUMBER ASSAY

A number of procedures have been developed to estimate reducing end-groups in cellulosic materials. A common technique is oxidation of cellulosic material with alkaline copper solutions to obtain the copper number. The copper number is defined as the weight of copper (g) reduced from the cupric to the cuprous state. The specific method used was adapted from Braidy.

### *Reagent Preparation*

The following reagents need to be prepared prior to analysis.

1. Reagent A – Alkali solution (1 L)
    a. Sodium carbonate (anhydrous) 130 g
    b. Sodium hydrogen carbonate 50 g
2. Reagent B – Copper solution (1 L)
    a. Copper (II) sulfate · 5 $H_2O$ 100 g
3. Reagent C – Ferric iron solution (1 L)
    a. $(NH_4)_2SO_4 \cdot Fe_2(SO_4)_3 \cdot 24\ H_2O$ 100 g
    b. 93% Sulfuric acid 140 mL

4. Reagent D – Ceric ammonium sulfate, 0.04 N (1 L)
    a. Ceric ammonium sulfate 25.3 g
    b. 93% sulfuric acid 30 mL
5. Reagent E – Ferroin indicator (100 mL, available commercially)
    a. O-phenanthroline · $H_2O$ 0.1485 g
    b. Ferrous sulfate 0.0695 g
    c. Dissolve in 10 mL of distilled water and then bring to 100 mL with more distilled water.
6. Reagent F – 2-N Sulfuric acid (1 L)
    a. 93% $H_2SO_4$ 105 g

***Procedure***

1. Mix 1 part Reagent B and 19 parts Reagent A. Add 10 mL of this prepared solution to a culture tube.
2. Add 0.25 g of sample with particle size –20/+40.
3. Seal the culture tube and heat in a boiling water bath for 3 h. Mix frequently.
4. Filter the contents of the culture tube through a coarse, fritted glass Gooch crucible with a glass fiber filter at the bottom. Transfer completely by washing the culture tube of any solids that adhere to the side.
5. Wash the sample in the crucible using a hot solution of equal parts distilled water and Reagent A.
6. Wash the sample with hot water and discard the filtrate.

7. The entrapped copper (I) oxide is dissolved using two 5-mL portions of Reagent C. Collect the wash in a 100-mL vacuum flask.
8. Wash the sample with 10 mL of Reagent F and collect the wash in the same 100-mL vacuum flask.
9. Add two or three drops of Reagent E to the collected wash.
10. Prepare 0.01-N ceric ammonium sulfate by mixing 1 part Reagent D to 3 parts distilled water.
11. Titrate the collected wash with the 0.01-N ceric ammonium sulfate until a color change is observed (pale orange to pale green).

***Calculation***

$$\text{Copper number} = 0.06354\ \frac{t}{w}$$

where

$t$ = Volume of 0.01-N ceric ammonium sulfate required (mL)

$w$ = Weight of sample on a dry basis (g)

The copper number must be between 0 and 4.5. If the procedure results in a value greater than 4.5 repeat the procedure using a lower initial sample weight.

# APPENDIX K

## DAIRY ONE, INC. FORAGE ANALYSIS DATA SHEETS

FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

| Sampled | Recvd | Printed | ST | CO |
|---|---|---|---|---|
| | 03/27/08 | 04/09/08 | | |

UNTRT SORGHUM
PRODUCERS COOP. ASSOC.
PO BOX 1112
1800 N TEXAS AVE
BRYAN, TX 77803

ENERGY TABLE - NRC 2001

| | Mcal/Lb | Mcal/Kg |
|---|---|---|
| DE, 1X | 0.99 | 2.19 |
| ME, 1X | 0.80 | 1.76 |
| NEL, 3X | 0.43 | 0.95 |
| NEM, 3X | 0.43 | 0.95 |
| NEG, 3X | 0.19 | 0.41 |
| TDN1X, % | 49 | |

COMMENTS:
1. LAG TIME EQUALS 4.90 HR.
2. THIS SAMPLE WAS TESTED TWICE FOR IDR 24 HR. AND IDR 48 HR. TO CONFIRM THE VALUES LISTED.

| Sample Description | Farm | Code | Sample |
|---|---|---|---|
| SORGHUM SILAGE | | 373D | 12254300 |

Analysis Results

| Components | As Fed | DM |
|---|---|---|
| % Moisture | 8.6 | |
| % Dry Matter | 91.4 | |
| % Crude Protein | 12.2 | 13.3 |
| % Available Protein | 10.3 | 11.3 |
| % ADICP | 1.8 | 2.0 |
| % Adjusted Crude Protein | 11.3 | 12.3 |
| Soluble Protein % CP | | 49 |
| % Acid Detergent Fiber | 45.0 | 49.2 |
| % Neutral Detergent Fiber | 58.0 | 63.4 |
| % Lignin | 6.1 | 6.7 |
| % NFC | 12.4 | 13.6 |
| % Crude Fat | 1.4 | 1.5 |
| % Ash | 11.76 | 12.87 |
| % TDN | 40 | 44 |
| NEL, Mcal/Lb | .34 | .38 |
| NEM, Mcal/Lb | .26 | .29 |
| NEG, Mcal/Lb | .04 | .05 |
| Relative Feed Value | | 74 |
| % Calcium | .47 | .52 |
| % Phosphorus | .44 | .48 |
| % Magnesium | .36 | .40 |
| % Potassium | 2.64 | 2.89 |
| % Sodium | .019 | .020 |
| PPM Iron | 820 | 898 |
| PPM Zinc | 96 | 105 |
| PPM Copper | 9 | 10 |
| PPM Manganese | 87 | 95 |
| PPM Molybdenum | 2.1 | 2.3 |
| % Sulfur | .14 | .15 |
| IVTD 24hr, % of DM | | 50 |
| IVTD 48hr, % of DM | | 55 |
| NDFD 24hr, % of NDF | | 21 |
| NDFD 48hr, % of NDF | | 29 |
| kd, %/hr | | 1.43 |
| Relative Forage Quality | | 43 |
| Milk Lbs./Ton of DM | | 589 |

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FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

| Sampled | Recvd | Printed | ST | CO |
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| | 03/27/08 | 04/09/08 | | |

UNTRT SORGHUM BALL-MILLED
PRODUCERS COOP. ASSOC.
PO BOX 1112
1800 N TEXAS AVE
BRYAN, TX 77803

**ENERGY TABLE - NRC 2001**

| | Mcal/Lb | Mcal/Kg |
|---|---|---|
| DE, 1X | 0.95 | 2.09 |
| ME, 1X | 0.75 | 1.66 |
| NEL, 3X | 0.40 | 0.88 |
| NEM, 3X | 0.40 | 0.87 |
| NEG, 3X | 0.15 | 0.33 |
| TDN1X, % | 47 | |

COMMENTS:
1. KD IS A THEORETICAL VALUE. INTERPRET KD >9 WITH CAUTION. ADJUST AS WARRANTED.
2. LAG TIME EQUALS 4.90 HR.
3. THIS SAMPLE WAS TESTED TWICE FOR LIGNIN, ACID DETERGENT FIBER, IDR 24 HR. AND IDR 48 HR. TO CONFIRM THE VALUES LISTED.

| Sample Description | Farm | Code | Sample |
|---|---|---|---|
| SORGHUM SILAGE | | 373D | 12254250 |

**Analysis Results**

| Components | As Fed | DM |
|---|---|---|
| % Moisture | 8.9 | |
| % Dry Matter | 91.1 | |
| % Crude Protein | 8.4 | 9.2 |
| % Available Protein | 8.2 | 9.0 |
| % ADICP | .2 | .2 |
| % Adjusted Crude Protein | 8.4 | 9.2 |
| Soluble Protein % CP | | 41 |
| % Acid Detergent Fiber | 37.0 | 40.5 |
| % Neutral Detergent Fiber | 58.3 | 64.0 |
| % Lignin | 8.7 | 9.5 |
| % NFC | 16.6 | 18.3 |
| % Crude Fat | .8 | .8 |
| % Ash | 10.14 | 11.13 |
| % TDN | 47 | 52 |
| NEL, Mcal/Lb | .40 | .44 |
| NEM, Mcal/Lb | .38 | .41 |
| NEG, Mcal/Lb | .15 | .17 |
| Relative Feed Value | | 83 |
| % Calcium | .31 | .34 |
| % Phosphorus | .27 | .29 |
| % Magnesium | .25 | .28 |
| % Potassium | 2.40 | 2.64 |
| % Sodium | .019 | .020 |
| PPM Iron | 488 | 535 |
| PPM Zinc | 75 | 83 |
| PPM Copper | 22 | 24 |
| PPM Manganese | 60 | 66 |
| PPM Molybdenum | 1.6 | 1.8 |
| % Sulfur | .11 | .12 |
| IVTD 24hr, % of DM | | 70 |
| IVTD 48hr, % of DM | | 85 |
| NDFD 24hr, % of NDF | | 54 |
| NDFD 48hr, % of NDF | | 76 |
| kd, %/hr | | 9.10 |
| Relative Forage Quality | | 121 |
| Milk Lbs./Ton of DM | | 2,390 |

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FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

| Sampled | Recvd | Printed | ST | CO |
|---|---|---|---|---|
| | 03/27/08 | 04/04/08 | | |

SHORT TERM LIME PRETRT SORGHUM
PRODUCERS COOP. ASSOC.
PO BOX 1112
1800 N TEXAS AVE
BRYAN, TX 77803

**ENERGY TABLE - NRC 2001**

| | Mcal/Lb | Mcal/Kg |
|---|---|---|
| DE, 1X | 1.17 | 2.58 |
| ME, 1X | 0.98 | 2.15 |
| NEL, 3X | 0.55 | 1.21 |
| NEM, 3X | 0.57 | 1.26 |
| NEG, 3X | 0.31 | 0.69 |

TDN1X, % 61

COMMENTS:
1.LAG TIME EQUALS 4.90 HR.
2.THIS SAMPLE WAS TESTED TWICE FOR CRUDE PROTEIN, SOLUBLE PROTEIN, LIGNIN, ACID DETERGENT FIBER, NEUTRAL DETERGENT FIBER, IDR 24 HR., CALCIUM, PHOSPHORUS, MAGNESIUM, POTASSIUM, SODIUM, IRON, ZINC, COPPER, MANGANESE, MOLYBDENUM AND SULFUR TO CONFIRM THE VALU

| Sample Description | Farm | Code | Sample |
|---|---|---|---|
| SORGHUM SILAGE | | 373D | 12254240 |

**Analysis Results**

| Components | As Fed | DM |
|---|---|---|
| % Moisture | 2.3 | |
| % Dry Matter | 97.7 | |
| % Crude Protein | 2.8 | 2.9 |
| % Available Protein | 2.3 | 2.4 |
| % ADICP | .5 | .5 |
| % Adjusted Crude Protein | 2.8 | 2.9 |
| Soluble Protein % CP | | 75 |
| % Acid Detergent Fiber | 74.2 | 75.9 |
| % Neutral Detergent Fiber | 83.0 | 85.0 |
| % Lignin | 1.4 | 1.4 |
| % NFC | 10.0 | 10.2 |
| % Crude Fat | .6 | .6 |
| % Ash | 5.54 | 5.67 |
| % TDN | 63 | 65 |
| NEL, Mcal/Lb | .14 | .15 |
| NEM, Mcal/Lb | .56 | .58 |
| NEG, Mcal/Lb | .31 | .32 |
| Relative Feed Value | | 33 |
| % Calcium | 1.21 | 1.24 |
| % Phosphorus | .08 | .08 |
| % Magnesium | .08 | .08 |
| % Potassium | .09 | .09 |
| % Sodium | .012 | .012 |
| PPM Iron | 566 | 579 |
| PPM Zinc | 66 | 68 |
| PPM Copper | 31 | 32 |
| PPM Manganese | 21 | 21 |
| PPM Molybdenum | .4 | .4 |
| % Sulfur | .01 | .01 |
| | | |
| IVTD 24hr, % of DM | | 48 |
| IVTD 48hr, % of DM | | 74 |
| NDFD 24hr, % of NDF | | 39 |
| NDFD 48hr, % of NDF | | 70 |
| kd, %/hr | | 2.86 |
| Relative Forage Quality | | 104 |
| Milk Lbs./Ton of DM | | 1,977 |

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FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

| Sampled | Recvd | Printed | ST | CO |
|---|---|---|---|---|
| | 03/27/08 | 04/03/08 | | |

SHORT TERM PRETRT SORGHUM BALL-
PRODUCERS COOP. ASSOC.
PO BOX 1112
1800 N TEXAS AVE
BRYAN, TX 77803

**ENERGY TABLE - NRC 2001**

| | Mcal/Lb | Mcal/Kg |
|---|---|---|
| DE, 1X | 1.17 | 2.58 |
| ME, 1X | 0.98 | 2.16 |
| NEL, 3X | 0.55 | 1.21 |
| NEM, 3X | 0.57 | 1.26 |
| NEG, 3X | 0.31 | 0.69 |

TDN1X, % 61

COMMENTS:
1.LAG TIME EQUALS 4.90 HR.
2.THIS SAMPLE WAS TESTED TWICE FOR ACID DETERGENT FIBER AND NEUTRAL DETERGENT FIBER TO CONFIRM THE VALUES LISTED.

| Sample Description | Farm | Code | Sample |
|---|---|---|---|
| MISC HAYLAGE | | 360D | 12254260 |

**Analysis Results**

| Components | As Fed | DM |
|---|---|---|
| % Moisture | 9.5 | |
| % Dry Matter | 90.5 | |
| % Crude Protein | 1.9 | 2.1 |
| % Available Protein | 1.6 | 1.8 |
| % ADICP | .2 | .3 |
| % Adjusted Crude Protein | 1.9 | 2.1 |
| Soluble Protein % CP | | 57 |
| % Acid Detergent Fiber | 7.2 | 8.0 |
| % Neutral Detergent Fiber | 20.3 | 22.5 |
| % Lignin | 1.3 | 1.4 |
| % NFC | 48.9 | 54.0 |
| % Crude Fat | .2 | .3 |
| % Ash | 19.20 | 21.21 |
| % TDN | 55 | 61 |
| NEL, Mcal/Lb | .60 | .66 |
| NEM, Mcal/Lb | .54 | .60 |
| NEG, Mcal/Lb | .31 | .34 |
| Relative Feed Value | | 342 |
| % Calcium | 6.75 | 7.46 |
| % Phosphorus | .17 | .19 |
| % Magnesium | .30 | .33 |
| % Potassium | .02 | .02 |
| % Sodium | .018 | .020 |
| PPM Iron | 562 | 621 |
| PPM Zinc | 97 | 107 |
| PPM Copper | 31 | 34 |
| PPM Manganese | 57 | 64 |
| PPM Molybdenum | .6 | .7 |
| % Sulfur | .01 | .01 |
| IVTD 24hr, % of DM | | 90 |
| IVTD 48hr, % of DM | | 91 |
| NDFD 24hr, % of NDF | | 57 |
| NDFD 48hr, % of NDF | | 58 |
| kd, %/hr | | 4.25 |

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FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

| Sampled | Recvd | Printed | ST | CO |
|---|---|---|---|---|
| | 03/27/08 | 04/07/08 | | |

10% LONG-TERM LIME PRETRT SORGH
PRODUCERS COOP. ASSOC.
PO BOX 1112
1800 N TEXAS AVE
BRYAN, TX 77803

**ENERGY TABLE - NRC 2001**

| | Mcal/Lb | Mcal/Kg |
|---|---|---|
| DE, 1X | 0.71 | 1.58 |
| ME, 1X | 0.52 | 1.14 |
| NEL, 3X | 0.25 | 0.54 |
| NEM, 3X | 0.20 | 0.44 |
| NEG, 3X | 0.00 | 0.00 |

TDN1X, % 37

COMMENTS:
1. LAG TIME EQUALS 4.90 HR.
2. THIS SAMPLE WAS TESTED TWICE FOR ACID DETERGENT INSOLUBLE CRUDE PROTEIN, LIGNIN, ACID DETERGENT FIBER AND NEUTRAL DETERGENT FIBER TO CONFIRM THE VALUES LISTED.

| Sample Description | Farm | Code | Sample |
|---|---|---|---|
| SORGHUM SILAGE | | 373D | 12254310 |

**Analysis Results**

| Components | As Fed | DM |
|---|---|---|
| % Moisture | 6.2 | |
| % Dry Matter | 93.8 | |
| % Crude Protein | 7.7 | 8.2 |
| % Available Protein | 3.2 | 3.4 |
| % ADICP | 4.5 | 4.8 |
| % Adjusted Crude Protein | 4.1 | 4.4 |
| Soluble Protein % CP | | 17 |
| % Acid Detergent Fiber | 62.0 | 66.1 |
| % Neutral Detergent Fiber | 72.8 | 77.7 |
| % Lignin | 10.0 | 10.7 |
| % NFC | 6.3 | 6.7 |
| % Crude Fat | .7 | .7 |
| % Ash | 11.01 | 11.75 |
| % TDN | 33 | 35 |
| NEL, Mcal/Lb | .19 | .20 |
| NEM, Mcal/Lb | .12 | .13 |
| Relative Feed Value | | 45 |
| % Calcium | 3.49 | 3.72 |
| % Phosphorus | .20 | .22 |
| % Magnesium | .13 | .13 |
| % Potassium | .09 | .10 |
| % Sodium | .016 | .017 |
| PPM Iron | 659 | 703 |
| PPM Zinc | 92 | 98 |
| PPM Copper | 9 | 9 |
| PPM Manganese | 55 | 59 |
| PPM Molybdenum | .4 | .5 |
| % Sulfur | .07 | .07 |
| IVTD 24hr, % of DM | | 52 |
| IVTD 48hr, % of DM | | 54 |
| NDFD 24hr, % of NDF | | 38 |
| NDFD 48hr, % of NDF | | 41 |
| kd, %/hr | | 3.28 |
| Relative Forage Quality | | 43 |
| Milk Lbs./Ton of DM | | 463 |

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FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

| Sampled | Recvd | Printed | ST | CO |
|---|---|---|---|---|
| | 03/27/08 | 04/09/08 | | |

10% LONG TERM PRETRT SORGHUM BA
PRODUCERS COOP. ASSOC.
PO BOX 1112
1800 N TEXAS AVE
BRYAN, TX 77803

**ENERGY TABLE - NRC 2001**

| | Mcal/Lb | Mcal/Kg |
|---|---|---|
| DE, 1X | 1.14 | 2.51 |
| ME, 1X | 0.95 | 2.09 |
| NEL, 3X | 0.53 | 1.16 |
| NEM, 3X | 0.55 | 1.21 |
| NEG, 3X | 0.29 | 0.64 |
| TDN1X, % | 58 | |

COMMENTS:
1. LAG TIME EQUALS 4.90 HR.
2. THIS SAMPLE WAS TESTED TWICE FOR IDR 48 HR. TO CONFIRM THE VALUE LISTED.

| Sample Description | Farm | Code | Sample |
|---|---|---|---|
| SORGHUM SILAGE | | 373D | 12254270 |

**Analysis Results**

| Components | As Fed | DM |
|---|---|---|
| % Moisture | 8.5 | |
| % Dry Matter | 91.5 | |
| % Crude Protein | 7.8 | 8.5 |
| % Available Protein | 6.6 | 7.2 |
| % ADICP | 1.2 | 1.4 |
| % Adjusted Crude Protein | 7.8 | 8.5 |
| Soluble Protein % CP | | 22 |
| % Acid Detergent Fiber | 29.6 | 32.3 |
| % Neutral Detergent Fiber | 43.3 | 47.3 |
| % Lignin | 3.3 | 3.6 |
| % NFC | 29.1 | 31.8 |
| % Crude Fat | .9 | 1.0 |
| % Ash | 11.09 | 12.12 |
| % TDN | 56 | 61 |
| NEL, Mcal/Lb | .56 | .61 |
| NEM, Mcal/Lb | .53 | .58 |
| NEG, Mcal/Lb | .29 | .32 |
| Relative Feed Value | | 125 |
| % Calcium | 3.68 | 4.02 |
| % Phosphorus | .22 | .24 |
| % Magnesium | .12 | .14 |
| % Potassium | .08 | .09 |
| % Sodium | .004 | .004 |
| PPM Iron | 757 | 827 |
| PPM Zinc | 109 | 119 |
| PPM Copper | 8 | 8 |
| PPM Manganese | 60 | 66 |
| PPM Molybdenum | .5 | .5 |
| % Sulfur | .07 | .08 |
| IVTD 24hr, % of DM | | 71 |
| IVTD 48hr, % of DM | | 83 |
| NDFD 24hr, % of NDF | | 39 |
| NDFD 48hr, % of NDF | | 63 |
| kd, %/hr | | 3.41 |
| Relative Forage Quality | | 153 |
| Milk Lbs./Ton of DM | | 2,465 |

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FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

| Sampled | Recvd | Printed | ST | CO |
|---|---|---|---|---|
| | 03/27/08 | 04/09/08 | | |

20% LONG-TERM LIME PRETRT SORGH
PRODUCERS COOP. ASSOC.
PO BOX 1112
1800 N TEXAS AVE
BRYAN, TX 77803

**ENERGY TABLE - NRC 2001**

| | Mcal/Lb | Mcal/Kg |
|---|---|---|
| DE, 1X | 0.60 | 1.32 |
| ME, 1X | 0.40 | 0.88 |
| NEL, 3X | 0.17 | 0.37 |
| NEM, 3X | 0.10 | 0.21 |
| NEG, 3X | 0.00 | 0.00 |
| TDN1X, % | 30 | |

COMMENTS:
1. LAG TIME EQUALS 4.90 HR.
2. THIS SAMPLE WAS TESTED TWICE FOR ACID DETERGENT FIBER, NEUTRAL DETERGENT FIBER, ASH AND IDR 48 HR. TO CONFIRM THE VALUES LISTED.

| Sample Description | Farm | Code | Sample |
|---|---|---|---|
| SORGHUM SILAGE | | 373D | 12254320 |

**Analysis Results**

| Components | As Fed | DM |
|---|---|---|
| % Moisture | 7.3 | |
| % Dry Matter | 92.7 | |
| % Crude Protein | 6.6 | 7.1 |
| % Available Protein | 3.3 | 3.6 |
| % ADICP | 3.3 | 3.5 |
| % Adjusted Crude Protein | 4.3 | 4.6 |
| Soluble Protein % CP | | 23 |
| % Acid Detergent Fiber | 51.0 | 55.0 |
| % Neutral Detergent Fiber | 56.7 | 61.2 |
| % Lignin | 8.1 | 8.7 |
| % NFC | 6.3 | 6.8 |
| % Crude Fat | .6 | .6 |
| % Ash | 24.61 | 26.56 |
| % TDN | 33 | 35 |
| NEL, Mcal/Lb | .29 | .31 |
| NEM, Mcal/Lb | .14 | .15 |
| Relative Feed Value | | 70 |
| % Calcium | 9.39 | 10.14 |
| % Phosphorus | .21 | .22 |
| % Magnesium | .18 | .20 |
| % Potassium | .12 | .13 |
| % Sodium | .002 | .002 |
| PPM Iron | 528 | 570 |
| PPM Zinc | 80 | 86 |
| PPM Copper | 8 | 8 |
| PPM Manganese | 53 | 58 |
| PPM Molybdenum | .4 | .4 |
| % Sulfur | .05 | .05 |
| IVTD 24hr, % of DM | | 66 |
| IVTD 48hr, % of DM | | 71 |
| NDFD 24hr, % of NDF | | 45 |
| NDFD 48hr, % of NDF | | 53 |
| kd, %/hr | | 4.81 |
| Relative Forage Quality | | 64 |
| Milk Lbs./Ton of DM | | 545 |

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FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

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|---|---|---|---|---|
| | 03/27/08 | 04/07/08 | | |

20% LONG TERM PRETRT SORGHUM BA
PRODUCERS COOP. ASSOC.
PO BOX 1112
1800 N TEXAS AVE
BRYAN, TX 77803

**ENERGY TABLE - NRC 2001**

| | Mcal/Lb | Mcal/Kg |
|---|---|---|
| DE, 1X | 1.05 | 2.32 |
| ME, 1X | 0.86 | 1.90 |
| NEL, 3X | 0.47 | 1.04 |
| NEM, 3X | 0.48 | 1.06 |
| NEG, 3X | 0.23 | 0.51 |
| TDN1X, % | 53 | |

COMMENTS:
1. LAG TIME EQUALS 4.90 HR.
2. THIS SAMPLE WAS TESTED TWICE FOR CRUDE PROTEIN, SOLUBLE PROTEIN, LIGNIN, ACID DETERGENT FIBER, NEUTRAL DETERGENT FIBER AND ASH TO CONFIRM THE VALUES LISTED.

| Sample Description | Farm | Code | Sample |
|---|---|---|---|
| SORGHUM SILAGE | | 373D | 12254280 |

**Analysis Results**

| Components | As Fed | DM |
|---|---|---|
| % Moisture | 9.5 | |
| % Dry Matter | 90.5 | |
| % Crude Protein | 6.5 | 7.2 |
| % Available Protein | 6.1 | 6.8 |
| % ADICP | .4 | .4 |
| % Adjusted Crude Protein | 6.5 | 7.2 |
| Soluble Protein % CP | | 16 |
| % Acid Detergent Fiber | 7.9 | 8.7 |
| % Neutral Detergent Fiber | 12.6 | 13.9 |
| % Lignin | 2.0 | 2.2 |
| % NFC | 43.7 | 48.3 |
| % Crude Fat | .5 | .6 |
| % Ash | 27.13 | 29.97 |
| % TDN | 47 | 52 |
| NEL, Mcal/Lb | .52 | .57 |
| NEM, Mcal/Lb | .41 | .46 |
| NEG, Mcal/Lb | .19 | .21 |
| Relative Feed Value | | 548 |
| % Calcium | 10.36 | 11.45 |
| % Phosphorus | .22 | .24 |
| % Magnesium | .18 | .20 |
| % Potassium | .11 | .13 |
| % Sodium | .017 | .018 |
| PPM Iron | 578 | 639 |
| PPM Zinc | 94 | 104 |
| PPM Copper | 8 | 8 |
| PPM Manganese | 56 | 62 |
| PPM Molybdenum | .4 | .4 |
| % Sulfur | .07 | .08 |
| | | |
| IVTD 24hr, % of DM | | 87 |
| IVTD 48hr, % of DM | | 91 |
| NDFD 24hr, % of NDF | | 7 |
| NDFD 48hr, % of NDF | | 33 |
| kd, %/hr | | 1.18 |
| Relative Forage Quality | | 351 |
| Milk Lbs./Ton of DM | | 1,939 |

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FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

| Sampled | Recvd | Printed | ST | CO |
|---|---|---|---|---|
| | 03/27/08 | 04/07/08 | | |

30% LONG-TERM LIME PRETRT SORGH
PRODUCERS COOP. ASSOC.
PO BOX 1112
1800 N TEXAS AVE
BRYAN, TX 77803

**ENERGY TABLE - NRC 2001**

| | Mcal/Lb | Mcal/Kg |
|---|---|---|
| DE, 1X | 0.67 | 1.48 |
| ME, 1X | 0.48 | 1.05 |
| NEL, 3X | 0.22 | 0.48 |
| NEM, 3X | 0.16 | 0.36 |
| NEG, 3X | 0.00 | 0.00 |
| TDN1X, % | 35 | |

COMMENTS:
1. LAG TIME EQUALS 4.90 HR.
2. THIS SAMPLE WAS TESTED TWICE FOR ACID DETERGENT FIBER AND NEUTRAL DETERGENT FIBER TO CONFIRM THE VALUES LISTED.

| Sample Description | Farm | Code | Sample |
|---|---|---|---|
| MISC HAYLAGE | | 360D | 12254330 |

**Analysis Results**

| Components | As Fed | DM |
|---|---|---|
| % Moisture | 8.4 | |
| % Dry Matter | 91.6 | |
| % Crude Protein | 3.4 | 3.7 |
| % Available Protein | 1.9 | 2.1 |
| % ADICP | 1.5 | 1.6 |
| % Adjusted Crude Protein | 2.8 | 3.1 |
| Soluble Protein % CP | | 22 |
| % Acid Detergent Fiber | 50.1 | 54.7 |
| % Neutral Detergent Fiber | 55.6 | 60.7 |
| % Lignin | 3.2 | 3.5 |
| % NFC | 3.7 | 4.0 |
| % Crude Fat | .4 | .5 |
| % Ash | 29.48 | 32.19 |
| % TDN | 36 | 40 |
| NEL, Mcal/Lb | .32 | .35 |
| NEM, Mcal/Lb | .21 | .23 |
| Relative Feed Value | | 71 |
| % Calcium | 9.60 | 10.48 |
| % Phosphorus | .15 | .16 |
| % Magnesium | .18 | .19 |
| % Potassium | .03 | .03 |
| % Sodium | <.001 | <.001 |
| PPM Iron | 335 | 366 |
| PPM Zinc | 54 | 59 |
| PPM Copper | 4 | 5 |
| PPM Manganese | 39 | 42 |
| PPM Molybdenum | < 0.1 | < 0.1 |
| % Sulfur | .02 | .02 |
| | | |
| IVTD 24hr, % of DM | | 81 |
| IVTD 48hr, % of DM | | 82 |
| NDFD 24hr, % of NDF | | 69 |
| NDFD 48hr, % of NDF | | 70 |
| kd, %/hr | | 6.07 |

Page 1

FORAGE TESTING LABORATORY
DAIRY ONE, INC.
730 WARREN ROAD
ITHACA, NEW YORK 14850
607-257-1272 (fax 607-257-1350)

| Sampled | Recvd | Printed | ST | CO |
|---|---|---|---|---|
| | 03/27/08 | 04/07/08 | | |

30% LONG TERM PRETRT SORGHUM BA
PRODUCERS COOP. ASSOC.
PO BOX 1112
1800 N TEXAS AVE
BRYAN, TX 77803

**ENERGY TABLE - NRC 2001**

| | Mcal/Lb | Mcal/Kg |
|---|---|---|
| DE, 1X | 0.94 | 2.07 |
| ME, 1X | 0.74 | 1.64 |
| NEL, 3X | 0.39 | 0.87 |
| NEM, 3X | 0.39 | 0.86 |
| NEG, 3X | 0.14 | 0.32 |

TDN1X, % 48

COMMENTS:
1. LAG TIME EQUALS 4.90 HR.
2. THIS SAMPLE WAS TESTED TWICE FOR LIGNIN, ACID DETERGENT FIBER, NEUTRAL DETERGENT FIBER AND ASH TO CONFIRM THE VALUES LISTED.

| Sample Description | Farm | Code | Sample |
|---|---|---|---|
| SORGHUM SILAGE | | 373D | 12254290 |

**Analysis Results**

| Components | As Fed | DM |
|---|---|---|
| % Moisture | 9.8 | |
| % Dry Matter | 90.2 | |
| % Crude Protein | 3.1 | 3.5 |
| % Available Protein | 3.0 | 3.3 |
| % ADICP | .2 | .2 |
| % Adjusted Crude Protein | 3.1 | 3.5 |
| Soluble Protein % CP | | 25 |
| % Acid Detergent Fiber | 9.2 | 10.2 |
| % Neutral Detergent Fiber | 21.2 | 23.4 |
| % Lignin | 1.7 | 1.9 |
| % NFC | 35.8 | 39.7 |
| % Crude Fat | .2 | .3 |
| % Ash | 29.88 | 33.12 |
| % TDN | 47 | 52 |
| NEL, Mcal/Lb | .51 | .56 |
| NEM, Mcal/Lb | .41 | .45 |
| NEG, Mcal/Lb | .19 | .21 |
| Relative Feed Value | | 321 |
| % Calcium | 11.91 | 13.20 |
| % Phosphorus | .18 | .19 |
| % Magnesium | .20 | .22 |
| % Potassium | .04 | .04 |
| % Sodium | .023 | .026 |
| PPM Iron | 425 | 471 |
| PPM Zinc | 70 | 78 |
| PPM Copper | 6 | 7 |
| PPM Manganese | 45 | 50 |
| PPM Molybdenum | .2 | .2 |
| % Sulfur | .03 | .03 |
| IVTD 24hr, % of DM | | 90 |
| IVTD 48hr, % of DM | | 93 |
| NDFD 24hr, % of NDF | | 56 |
| NDFD 48hr, % of NDF | | 70 |
| kd, %/hr | | 5.42 |
| Relative Forage Quality | | 251 |
| Milk Lbs./Ton of DM | | 2,069 |

# APPENDIX L

# CUMBERLAND VALLEY ANALYTICAL LABORATORY FORAGE ANALYSIS DATA SHEETS

**CUMBERLAND VALLEY ANALYTICAL SERVICES, INC.** **February 01, 2011**
**PO Box 669 Maugansville, MD 21767 301-790-1980** **Sample No : 11340054**

**A N A L Y S I S R E S U L T S** **Type: LEGUME FORAGE**

| | | | | | |
|---|---|---|---|---|---|
| **Moisture** | 7.4 | % | ***Minerals*** | | |
| **Dry Matter** | 92.6 | % | Ash | 9.4 | % DM |
| | | | Calcium | 1.56 | % DM |
| ***Proteins*** | | | Phosphorus | 0.21 | % DM |
| Crude Protein | 15.0 | % DM | Magnesium | 0.68 | % DM |
| Adjusted Protein | 15.0 | % DM | Potassium | 2.33 | % DM |
| Soluble Protein | 28.9 | % CP | Sulfur | 0.31 | % DM |
| Ammonia | | | Sodium | 0.109 | % DM |
| ADF Protein (bound protein) | 1.39 | % DM | Iron | 130 | PPM |
| NDF Protein | 1.8 | % DM | Manganese | 25 | PPM |
| Rumen Degr Protein | 64.5 | % CP | Zinc | 12 | PPM |
| Rumen Undgr Protein (Strep. G) | | | Copper | 8 | PPM |
| | | | Selenium | | |
| ***Fibers*** | | | Molybdenum | | |
| Acid Detergent Fiber | 35.6 | % DM | Nitrate Ion | | |
| Neutral Detergent Fiber | 44.6 | % DM | Chloride Ion | 0.94 | % DM |
| Crude Fiber | | | DCAD (Meq/100gdm) | | |
| Lignin | 8.68 | % DM | | | |
| Lignin / NDF Ratio | 19.5 | % NDF | ***Energy / Indexes*** | | |
| Soluble fiber | | | TDN | 55.9 | % DM |
| peNDF | | | Net Energy Lactation | 0.57 | Mcal/lb |
| NDF Digestibility, Invitro | | | Net Energy Maintenance | 0.53 | Mcal/lb |
| 12 hr digestibility | | | Net Energy Gain | 0.28 | Mcal/lb |
| 24 hr digestibility | | | Relative Feed Value (RFV) | 128 | |
| 30 hr digestibility | 39.2 | % NDF | Relative Feed Quality (RFQ) | | |
| 48 hr digestibility | | | Milk/ton | | |
| Indigestible NDF, Invitro 120 HR | | | NFC | 30.9 | % DM |
| NDF Dig. Rate (Kd) | 4.10 | | Enzymatic NSC | | |
| | | | | | |
| ***Non-Fibers, Structure, Utilization*** | | | ***Qualitative*** | | |
| Digestible Dry Matter (fast) | | | pH | | |
| Sugar | 6.3 | % DM | Total VFA | | |
| Starch | 1.8 | % DM | Lactic acid | | |
| --Enzyme Available | | | --Lactic/TVFA | | |
| --Digestibility, 2 hr | | | Acetic acid | | |
| --Digestibility, 7 hr | | | Propionic acid | | |
| Fatty Acids, Total | | | Butyric acid | | |
| Crude Fat | 1.9 | % DM | Isobutyric acid | | |
| Acid hydrolysis fat | | | 1, 2 Propandiol | | |
| CS Processing Score | | | Titratable Acidity (meq NaOH) | | |
| Particle size > 0.75" | | | | | |
| ... 0.31" - 0.75" | | | Mold | | |
| ... < 0.31" | | | Yeast | | |

LISA SLAY
TEXAS A & M UNIVERSITY
KLEBERG CTR, RM 239, 2471 TAMU
COLLEGE STATION TX 77843-2471

Sample : ALFALFA
Farm Name : TERRABON
Received : January 21, 2011
Complete : February 01, 2011
Regression : OH

**CUMBERLAND VALLEY ANALYTICAL SERVICES, INC.** **February 03, 2011**
**PO Box 669 Maugansville, MD 21767 301-790-1980** **Sample No : 11340052**

**A N A L Y S I S R E S U L T S Type: CORN**

| | | | | | |
|---|---|---|---|---|---|
| **Moisture** | 14.2 | % | ***Minerals*** | | |
| **Dry Matter** | 85.8 | % | Ash | 1.3 | % DM |
| | | | Calcium | 0.02 | % DM |
| ***Proteins*** | | | Phosphorus | 0.29 | % DM |
| Crude Protein | 8.5 | % DM | Magnesium | 0.11 | % DM |
| Adjusted Protein | 8.5 | % DM | Potassium | 0.40 | % DM |
| Soluble Protein | 17.1 | % CP | Sulfur | 0.11 | % DM |
| Ammonia | | | Sodium | 0.006 | % DM |
| ADF Protein (bound protein) | 0.44 | % DM | Iron | 31 | PPM |
| NDF Protein | 0.6 | % DM | Manganese | 6 | PPM |
| Rumen Degr Protein | 48.2 | % CP | Zinc | 21 | PPM |
| Rumen Undgr Protein (Strep. G) | | | Copper | 1 | PPM |
| | | | Selenium | | |
| ***Fibers*** | | | Molybdenum | | |
| Acid Detergent Fiber | 4.7 | % DM | Nitrate Ion | | |
| Neutral Detergent Fiber | 11.2 | % DM | Chloride Ion | 0.05 | % DM |
| Crude Fiber | | | DCAD (Meq/100gdm) | | |
| Lignin | 2.32 | % DM | | | |
| Lignin / NDF Ratio | 20.7 | % NDF | ***Energy / Indexes*** | | |
| Soluble fiber | | | TDN | 85.4 | % DM |
| peNDF | | | Net Energy Lactation | 0.90 | Mcal/lb |
| NDF Digestibility, Invitro | | | Net Energy Maintenance | 0.95 | Mcal/lb |
| 12 hr digestibility | | | Net Energy Gain | 0.65 | Mcal/lb |
| 24 hr digestibility | | | Relative Feed Value (RFV) | | |
| 30 hr digestibility | 81.8 | % NDF | Relative Feed Quality (RFQ) | | |
| 48 hr digestibility | | | Milk/ton | | |
| Indigestible NDF, Invitro 120 HR | | | NFC | 75.6 | % DM |
| NDF Dig. Rate (Kd) | 99.99 | | Enzymatic NSC | | |
| | | | | | |
| ***Non-Fibers, Structure, Utilization*** | | | ***Qualitative*** | | |
| Digestible Dry Matter (fast) | | | pH | | |
| Sugar | 3.0 | % DM | Total VFA | | |
| Starch | 71.4 | % DM | Lactic acid | | |
| --Enzyme Available | | | --Lactic/TVFA | | |
| --Digestibility, 2 hr | | | Acetic acid | | |
| --Digestibility, 7 hr | | | Propionic acid | | |
| Fatty Acids, Total | | | Butyric acid | | |
| Crude Fat | 3.9 | % DM | Isobutyric acid | | |
| Acid hydrolysis fat | | | 1, 2 Propandiol | | |
| CS Processing Score | | | Titratable Acidity (meq NaOH) | | |
| Particle size > 0.75" | | | | | |
| ... 0.31" - 0.75" | | | Mold | | |
| ... < 0.31" | | | Yeast | | |

LISA SLAY
TEXAS A & M UNIVERSITY
KLEBERG CTR, RM 239, 2471 TAMU
COLLEGE STATION TX 77843-2471

Sample : CRACKED CORN
Farm Name : TERRABON
Received : January 21, 2011
Complete : February 03, 2011
Regression : OH

**CUMBERLAND VALLEY ANALYTICAL SERVICES, INC.** **February 03, 2011**
**PO Box 669 Maugansville, MD 21767 301-790-1980** **Sample No : 11340048**

**A N A L Y S I S R E S U L T S Type: CORN SILAGE**

| | | | | | |
|---|---|---|---|---|---|
| **Moisture** | 9.5 | % | ***Minerals*** | | |
| **Dry Matter** | 90.5 | % | Ash | 7.9 | % DM |
| | | | Calcium | 0.40 | % DM |
| ***Proteins*** | | | Phosphorus | 0.09 | % DM |
| Crude Protein | 6.5 | % DM | Magnesium | 0.21 | % DM |
| Adjusted Protein | 5.8 | % DM | Potassium | 1.80 | % DM |
| Soluble Protein | 27.5 | % CP | Sulfur | 0.09 | % DM |
| Ammonia | | | Sodium | 0.016 | % DM |
| ADF Protein (bound protein) | 1.45 | % DM | Iron | 505 | PPM |
| NDF Protein | 2.1 | % DM | Manganese | 77 | PPM |
| Rumen Degr Protein | 63.8 | % CP | Zinc | 22 | PPM |
| Rumen Undgr Protein (Strep. G) | | | Copper | 12 | PPM |
| | | | Selenium | | |
| ***Fibers*** | | | Molybdenum | | |
| Acid Detergent Fiber | 44.5 | % DM | Nitrate Ion | | |
| Neutral Detergent Fiber | 71.2 | % DM | Chloride Ion | 0.05 | % DM |
| Crude Fiber | | | DCAD (Meq/100gdm) | | |
| Lignin | 9.55 | % DM | | | |
| Lignin / NDF Ratio | 13.4 | % NDF | ***Energy / Indexes*** | | |
| Soluble fiber | | | TDN | 49.8 | % DM |
| peNDF | | | Net Energy Lactation | 0.50 | Mcal/lb |
| NDF Digestibility, Invitro | | | Net Energy Maintenance | 0.44 | Mcal/lb |
| 12 hr digestibility | | | Net Energy Gain | 0.19 | Mcal/lb |
| 24 hr digestibility | | | Relative Feed Value (RFV) | | |
| 30 hr digestibility | 44.3 | % NDF | Relative Feed Quality (RFQ) | | |
| 48 hr digestibility | | | Milk/ton | | |
| Indigestible NDF, Invitro 120 HR | | | NFC | 15.5 | % DM |
| NDF Dig. Rate (Kd) | 3.34 | | Enzymatic NSC | | |
| | | | | | |
| ***Non-Fibers, Structure, Utilization*** | | | ***Qualitative*** | | |
| Digestible Dry Matter (fast) | | | pH | | |
| Sugar | 2.0 | % DM | Total VFA | | |
| Starch | 3.0 | % DM | Lactic acid | | |
| --Enzyme Available | | | --Lactic/TVFA | | |
| --Digestibility, 2 hr | | | Acetic acid | | |
| --Digestibility, 7 hr | | | Propionic acid | | |
| Fatty Acids, Total | | | Butyric acid | | |
| Crude Fat | 0.9 | % DM | Isobutyric acid | | |
| Acid hydrolysis fat | | | 1, 2 Propandiol | | |
| CS Processing Score | | | Titratable Acidity (meq NaOH) | | |
| Particle size > 0.75" | | | | | |
| ... 0.31" - 0.75" | | | Mold | | |
| ... < 0.31" | | | Yeast | | |

LISA SLAY
TEXAS A & M UNIVERSITY
KLEBERG CTR, RM 239, 2471 TAMU
COLLEGE STATION TX 77843-2471

Sample : CS
Farm Name : TERRABON
Received : January 21, 2011
Complete : February 03, 2011
Regression : OH

**CUMBERLAND VALLEY ANALYTICAL SERVICES, INC.** **February 03, 2011**
**PO Box 669 Maugansville, MD 21767 301-790-1980** **Sample No : 11340053**

**A N A L Y S I S R E S U L T S** **Type: LEGUME FORAGE**

| | | | | | |
|---|---|---|---|---|---|
| **Moisture** | 8.6 | % | ***Minerals*** | | |
| **Dry Matter** | 91.4 | % | Ash | 8.7 | % DM |
| | | | Calcium | 2.85 | % DM |
| ***Proteins*** | | | Phosphorus | 0.02 | % DM |
| Crude Protein | 2.9 | % DM | Magnesium | 0.05 | % DM |
| Adjusted Protein | 2.9 | % DM | Potassium | 0.04 | % DM |
| Soluble Protein | 27.0 | % CP | Sulfur | 0.03 | % DM |
| Ammonia | | | Sodium | 0.017 | % DM |
| ADF Protein (bound protein) | 0.90 | % DM | Iron | 411 | PPM |
| NDF Protein | 1.8 | % DM | Manganese | 21 | PPM |
| Rumen Degr Protein | 63.5 | % CP | Zinc | 23 | PPM |
| Rumen Undgr Protein (Strep. G) | | | Copper | 8 | PPM |
| | | | Selenium | | |
| ***Fibers*** | | | Molybdenum | | |
| Acid Detergent Fiber | 66.3 | % DM | Nitrate Ion | | |
| Neutral Detergent Fiber | 74.8 | % DM | Chloride Ion | 2.43 | % DM |
| Crude Fiber | | | DCAD (Meq/100gdm) | | |
| Lignin | 12.24 | % DM | | | |
| Lignin / NDF Ratio | 16.4 | % NDF | ***Energy / Indexes*** | | |
| Soluble fiber | | | TDN | 43.6 | % DM |
| peNDF | | | Net Energy Lactation | 0.43 | Mcal/lb |
| NDF Digestibility, Invitro | | | Net Energy Maintenance | 0.34 | Mcal/lb |
| 12 hr digestibility | | | Net Energy Gain | 0.09 | Mcal/lb |
| 24 hr digestibility | | | Relative Feed Value (RFV) | 46 | |
| 30 hr digestibility | 75.8 | % NDF | Relative Feed Quality (RFQ) | | |
| 48 hr digestibility | | | Milk/ton | | |
| Indigestible NDF, Invitro 120 HR | | | NFC | 14.6 | % DM |
| NDF Dig. Rate (Kd) | 99.99 | | Enzymatic NSC | | |
| | | | | | |
| ***Non-Fibers, Structure, Utilization*** | | | ***Qualitative*** | | |
| Digestible Dry Matter (fast) | | | pH | | |
| Sugar | 1.3 | % DM | Total VFA | | |
| Starch | 3.4 | % DM | Lactic acid | | |
| --Enzyme Available | | | --Lactic/TVFA | | |
| --Digestibility, 2 hr | | | Acetic acid | | |
| --Digestibility, 7 hr | | | Propionic acid | | |
| Fatty Acids, Total | | | Butyric acid | | |
| Crude Fat | 0.6 | % DM | Isobutyric acid | | |
| Acid hydrolysis fat | | | 1, 2 Propandiol | | |
| CS Processing Score | | | Titratable Acidity (meq NaOH) | | |
| Particle size > 0.75" | | | | | |
| ... 0.31" - 0.75" | | | Mold | | |
| ... < 0.31" | | | Yeast | | |

LISA SLAY
TEXAS A & M UNIVERSITY
KLEBERG CTR, RM 239, 2471 TAMU
COLLEGE STATION TX 77843-2471

Sample : C "E"
Farm Name : TERRABON
Received : January 21, 2011
Complete : February 03, 2011
Regression : OH

**CUMBERLAND VALLEY ANALYTICAL SERVICES, INC.** **February 01, 2011**
**PO Box 669 Maugansville, MD 21767 301-790-1980** **Sample No : 11340049**

**A N A L Y S I S R E S U L T S** **Type: GRASS FORAGE**

| | | | | | |
|---|---|---|---|---|---|
| **Moisture** | 10.0 | % | ***Minerals*** | | |
| **Dry Matter** | 90.0 | % | Ash | 6.6 | % DM |
| | | | Calcium | 0.38 | % DM |
| ***Proteins*** | | | Phosphorus | 0.07 | % DM |
| Crude Protein | 6.2 | % DM | Magnesium | 0.14 | % DM |
| Adjusted Protein | 5.1 | % DM | Potassium | 0.57 | % DM |
| Soluble Protein | 29.2 | % CP | Sulfur | 0.07 | % DM |
| Ammonia | | | Sodium | 0.225 | % DM |
| ADF Protein (bound protein) | 1.66 | % DM | Iron | 4828 | PPM |
| NDF Protein | 1.9 | % DM | Manganese | 92 | PPM |
| Rumen Degr Protein | 64.6 | % CP | Zinc | 35 | PPM |
| Rumen Undgr Protein (Strep. G) | | | Copper | 30 | PPM |
| | | | Selenium | | |
| ***Fibers*** | | | Molybdenum | | |
| Acid Detergent Fiber | 55.9 | % DM | Nitrate Ion | | |
| Neutral Detergent Fiber | 82.3 | % DM | Chloride Ion | 0.06 | % DM |
| Crude Fiber | | | DCAD (Meq/100gdm) | | |
| Lignin | 12.56 | % DM | | | |
| Lignin / NDF Ratio | 15.3 | % NDF | ***Energy / Indexes*** | | |
| Soluble fiber | | | TDN | 42.6 | % DM |
| peNDF | | | Net Energy Lactation | 0.42 | Mcal/lb |
| NDF Digestibility, Invitro | | | Net Energy Maintenance | 0.32 | Mcal/lb |
| 12 hr digestibility | | | Net Energy Gain | 0.08 | Mcal/lb |
| 24 hr digestibility | | | Relative Feed Value (RFV) | 51 | |
| 30 hr digestibility | 42.3 | % NDF | Relative Feed Quality (RFQ) | | |
| 48 hr digestibility | | | Milk/ton | | |
| Indigestible NDF, Invitro 120 HR | | | NFC | 6.4 | % DM |
| NDF Dig. Rate (Kd) | 3.46 | | Enzymatic NSC | | |
| | | | | | |
| ***Non-Fibers, Structure, Utilization*** | | | ***Qualitative*** | | |
| Digestible Dry Matter (fast) | | | pH | | |
| Sugar | 1.6 | % DM | Total VFA | | |
| Starch | 3.4 | % DM | Lactic acid | | |
| --Enzyme Available | | | --Lactic/TVFA | | |
| --Digestibility, 2 hr | | | Acetic acid | | |
| --Digestibility, 7 hr | | | Propionic acid | | |
| Fatty Acids, Total | | | Butyric acid | | |
| Crude Fat | 0.4 | % DM | Isobutyric acid | | |
| Acid hydrolysis fat | | | 1, 2 Propandiol | | |
| CS Processing Score | | | Titratable Acidity (meq NaOH) | | |
| Particle size > 0.75" | | | | | |
| ... 0.31" - 0.75" | | | Mold | | |
| ... < 0.31" | | | Yeast | | |

LISA SLAY
TEXAS A & M UNIVERSITY
KLEBERG CTR, RM 239, 2471 TAMU
COLLEGE STATION TX 77843-2471

Sample : M "E"
Farm Name : TERRABON
Received : January 21, 2011
Complete : February 01, 2011
Regression : OH

**CUMBERLAND VALLEY ANALYTICAL SERVICES, INC.** **February 01, 2011**
**PO Box 669 Maugansville, MD 21767 301-790-1980** **Sample No : 11340051**

**A N A L Y S I S R E S U L T S** **Type: LEGUME FORAGE**

| | | | | | |
|---|---|---|---|---|---|
| **Moisture** | 9.4 | % | ***Minerals*** | | |
| **Dry Matter** | 90.6 | % | Ash | 10.3 | % DM |
| | | | Calcium | 1.44 | % DM |
| ***Proteins*** | | | Phosphorus | 0.03 | % DM |
| Crude Protein | 3.7 | % DM | Magnesium | 0.04 | % DM |
| Adjusted Protein | 2.7 | % DM | Potassium | 0.08 | % DM |
| Soluble Protein | 27.5 | % CP | Sulfur | 0.03 | % DM |
| Ammonia | | | Sodium | 0.202 | % DM |
| ADF Protein (bound protein) | 1.41 | % DM | Iron | 3235 | PPM |
| NDF Protein | 2.4 | % DM | Manganese | 46 | PPM |
| Rumen Degr Protein | 63.7 | % CP | Zinc | 39 | PPM |
| Rumen Undgr Protein (Strep. G) | | | Copper | 19 | PPM |
| | | | Selenium | | |
| ***Fibers*** | | | Molybdenum | | |
| Acid Detergent Fiber | 69.2 | % DM | Nitrate Ion | | |
| Neutral Detergent Fiber | 78.6 | % DM | Chloride Ion | 0.61 | % DM |
| Crude Fiber | | | DCAD (Meq/100gdm) | | |
| Lignin | 8.18 | % DM | | | |
| Lignin / NDF Ratio | 10.4 | % NDF | ***Energy / Indexes*** | | |
| Soluble fiber | | | TDN | 46.6 | % DM |
| peNDF | | | Net Energy Lactation | 0.47 | Mcal/lb |
| NDF Digestibility, Invitro | | | Net Energy Maintenance | 0.38 | Mcal/lb |
| 12 hr digestibility | | | Net Energy Gain | 0.14 | Mcal/lb |
| 24 hr digestibility | | | Relative Feed Value (RFV) | 41 | |
| 30 hr digestibility | 69.0 | % NDF | Relative Feed Quality (RFQ) | | |
| 48 hr digestibility | | | Milk/ton | | |
| Indigestible NDF, Invitro 120 HR | | | NFC | 9.3 | % DM |
| NDF Dig. Rate (Kd) | 6.79 | | Enzymatic NSC | | |
| | | | | | |
| ***Non-Fibers, Structure, Utilization*** | | | ***Qualitative*** | | |
| Digestible Dry Matter (fast) | | | pH | | |
| Sugar | 1.0 | % DM | Total VFA | | |
| Starch | 1.2 | % DM | Lactic acid | | |
| --Enzyme Available | | | --Lactic/TVFA | | |
| --Digestibility, 2 hr | | | Acetic acid | | |
| --Digestibility, 7 hr | | | Propionic acid | | |
| Fatty Acids, Total | | | Butyric acid | | |
| Crude Fat | 0.5 | % DM | Isobutyric acid | | |
| Acid hydrolysis fat | | | 1, 2 Propandiol | | |
| CS Processing Score | | | Titratable Acidity (meq NaOH) | | |
| Particle size > 0.75" | | | | | |
| ... 0.31" - 0.75" | | | Mold | | |
| ... < 0.31" | | | Yeast | | |

LISA SLAY
TEXAS A & M UNIVERSITY
KLEBERG CTR, RM 239, 2471 TAMU
COLLEGE STATION TX 77843-2471

Sample : C & M "E"
Farm Name : TERRABON
Received : January 21, 2011
Complete : February 01, 2011
Regression : OH

**CUMBERLAND VALLEY ANALYTICAL SERVICES, INC.** **February 01, 2011**
**PO Box 669 Maugansville, MD 21767 301-790-1980** **Sample No : 11340050**

**A N A L Y S I S R E S U L T S** **Type: LEGUME FORAGE**

| | | | | | |
|---|---|---|---|---|---|
| **Moisture** | 8.2 | % | ***Minerals*** | | |
| **Dry Matter** | 91.8 | % | Ash | 8.3 | % DM |
| | | | Calcium | 1.26 | % DM |
| ***Proteins*** | | | Phosphorus | 0.03 | % DM |
| Crude Protein | 2.8 | % DM | Magnesium | 0.02 | % DM |
| Adjusted Protein | 2.8 | % DM | Potassium | 0.08 | % DM |
| Soluble Protein | 39.5 | % CP | Sulfur | 0.02 | % DM |
| Ammonia | | | Sodium | 0.026 | % DM |
| ADF Protein (bound protein) | 0.76 | % DM | Iron | 4295 | PPM |
| NDF Protein | 1.3 | % DM | Manganese | 47 | PPM |
| Rumen Degr Protein | 69.8 | % CP | Zinc | 14 | PPM |
| Rumen Undgr Protein (Strep. G) | | | Copper | 25 | PPM |
| | | | Selenium | | |
| ***Fibers*** | | | Molybdenum | | |
| Acid Detergent Fiber | 69.1 | % DM | Nitrate Ion | | |
| Neutral Detergent Fiber | 79.9 | % DM | Chloride Ion | 0.02 | % DM |
| Crude Fiber | | | DCAD (Meq/100gdm) | | |
| Lignin | 6.60 | % DM | | | |
| Lignin / NDF Ratio | 8.3 | % NDF | ***Energy / Indexes*** | | |
| Soluble fiber | | | TDN | 50.6 | % DM |
| peNDF | | | Net Energy Lactation | 0.51 | Mcal/lb |
| NDF Digestibility, Invitro | | | Net Energy Maintenance | 0.45 | Mcal/lb |
| 12 hr digestibility | | | Net Energy Gain | 0.20 | Mcal/lb |
| 24 hr digestibility | | | Relative Feed Value (RFV) | 41 | |
| 30 hr digestibility | 85.9 | % NDF | Relative Feed Quality (RFQ) | | |
| 48 hr digestibility | | | Milk/ton | | |
| Indigestible NDF, Invitro 120 HR | | | NFC | 9.5 | % DM |
| NDF Dig. Rate (Kd) | 99.99 | | Enzymatic NSC | | |
| | | | | | |
| ***Non-Fibers, Structure, Utilization*** | | | ***Qualitative*** | | |
| Digestible Dry Matter (fast) | | | pH | | |
| Sugar | 1.3 | % DM | Total VFA | | |
| Starch | 1.2 | % DM | Lactic acid | | |
| --Enzyme Available | | | --Lactic/TVFA | | |
| --Digestibility, 2 hr | | | Acetic acid | | |
| --Digestibility, 7 hr | | | Propionic acid | | |
| Fatty Acids, Total | | | Butyric acid | | |
| Crude Fat | 0.7 | % DM | Isobutyric acid | | |
| Acid hydrolysis fat | | | 1, 2 Propandiol | | |
| CS Processing Score | | | Titratable Acidity (meq NaOH) | | |
| Particle size > 0.75" | | | | | |
| ... 0.31" - 0.75" | | | Mold | | |
| ... < 0.31" | | | Yeast | | |

LISA SLAY
TEXAS A & M UNIVERSITY
KLEBERG CTR, RM 239, 2471 TAMU
COLLEGE STATION TX 77843-2471

Sample : M & C "E"
Farm Name : TERRABON
Received : January 21, 2011
Complete : February 01, 2011
Regression : OH

**CUMBERLAND VALLEY ANALYTICAL SERVICES, INC.** **April 27, 2011**
**PO Box 669 Maugansville, MD 21767 301-790-1980** **Sample No : 11669084**

**A N A L Y S I S R E S U L T S Type: BYPRODUCT**

| | | | | | |
|---|---|---|---|---|---|
| **Moisture** | 10.3 | % | ***Minerals*** | | |
| **Dry Matter** | 89.7 | % | Ash | 7.0 | % DM |
| | | | Calcium | 3.32 | % DM |
| ***Proteins*** | | | Phosphorus | 0.00 | % DM |
| Crude Protein | 89.2 | % DM | Magnesium | 0.02 | % DM |
| Adjusted Protein | 89.2 | % DM | Potassium | 0.34 | % DM |
| Soluble Protein | 99.9 | % CP | Sulfur | 1.62 | % DM |
| Ammonia | | | Sodium | 0.988 | % DM |
| ADF Protein (bound protein) | 0.22 | % DM | Iron | 20 | PPM |
| NDF Protein | 0.4 | % DM | Manganese | 3 | PPM |
| Rumen Degr Protein | | | Zinc | 26 | PPM |
| Rumen Undgr Protein (Strep. G) | | | Copper | 3 | PPM |
| | | | Selenium | | |
| ***Fibers*** | | | Molybdenum | | |
| Acid Detergent Fiber | 0.4 | % DM | Nitrate Ion | | |
| Neutral Detergent Fiber | 0.9 | % DM | Chloride Ion | 0.90 | % DM |
| Crude Fiber | | | DCAD (Meq/100gdm) | | |
| Lignin | 0.14 | % DM | | | |
| Lignin / NDF Ratio | 15.8 | % NDF | ***Energy / Indexes*** | | |
| Soluble fiber | | | TDN | 85.8 | % DM |
| peNDF | | | Net Energy Lactation | 0.90 | Mcal/lb |
| NDF Digestibility, Invitro | | | Net Energy Maintenance | 0.96 | Mcal/lb |
| 12 hr digestibility | | | Net Energy Gain | 0.65 | Mcal/lb |
| 24 hr digestibility | | | Relative Feed Value (RFV) | | |
| 30 hr digestibility | | | Relative Feed Quality (RFQ) | | |
| 48 hr digestibility | | | Milk/ton | | |
| Indigestible NDF, Invitro 120 HR | | | NFC | 2.7 | % DM |
| NDF Dig. Rate (Kd) | | | Enzymatic NSC | | |
| | | | | | |
| ***Non-Fibers, Structure, Utilization*** | | | ***Qualitative*** | | |
| Digestible Dry Matter (fast) | | | pH | | |
| Sugar | 0.1 | % DM | Total VFA | | |
| Starch | 0.2 | % DM | Lactic acid | | |
| --Enzyme Available | | | --Lactic/TVFA | | |
| --Digestibility, 2 hr | | | Acetic acid | | |
| --Digestibility, 7 hr | | | Propionic acid | | |
| Fatty Acids, Total | | | Butyric acid | | |
| Crude Fat | 0.6 | % DM | Isobutyric acid | | |
| Acid hydrolysis fat | | | 1, 2 Propandiol | | |
| CS Processing Score | | | Titratable Acidity (meq NaOH) | | |
| Particle size > 0.75" | | | | | |
| ... 0.31" - 0.75" | | | Mold | | |
| ... < 0.31" | | | Yeast | | |

LUIS TEDESCHI
TEXAS A & M UNIVERSITY
230 KLEGERG CTR, 2471 TAMU
COLLEGE STATION TX 77843

Sample : 1 - SOL. PROTEIN
Farm Name : WILEY/TEDESCHI
Received : April 15, 2011
Complete : April 27, 2011
Regression : OH

**CUMBERLAND VALLEY ANALYTICAL SERVICES, INC.** **April 27, 2011**
**PO Box 669 Maugansville, MD 21767 301-790-1980** **Sample No : 11669085**

**A N A L Y S I S R E S U L T S Type: BYPRODUCT**

| | | | | | |
|---|---|---|---|---|---|
| **Moisture** | 15.2 | % | ***Minerals*** | | |
| **Dry Matter** | 84.8 | % | Ash | 31.4 | % DM |
| | | | Calcium | 1.12 | % DM |
| ***Proteins*** | | | Phosphorus | 0.59 | % DM |
| Crude Protein | 19.2 | % DM | Magnesium | 1.00 | % DM |
| Adjusted Protein | 19.2 | % DM | Potassium | 11.65 | % DM |
| Soluble Protein | 92.6 | % CP | Sulfur | 0.33 | % DM |
| Ammonia | | | Sodium | 0.124 | % DM |
| ADF Protein (bound protein) | 0.27 | % DM | Iron | 282 | PPM |
| NDF Protein | 0.4 | % DM | Manganese | 271 | PPM |
| Rumen Degr Protein | | | Zinc | 58 | PPM |
| Rumen Undgr Protein (Strep. G) | | | Copper | 30 | PPM |
| | | | Selenium | | |
| ***Fibers*** | | | Molybdenum | | |
| Acid Detergent Fiber | 0.7 | % DM | Nitrate Ion | | |
| Neutral Detergent Fiber | 1.2 | % DM | Chloride Ion | 0.30 | % DM |
| Crude Fiber | | | DCAD (Meq/100gdm) | | |
| Lignin | 0.24 | % DM | | | |
| Lignin / NDF Ratio | 19.5 | % NDF | ***Energy / Indexes*** | | |
| Soluble fiber | | | TDN | 61.0 | % DM |
| peNDF | | | Net Energy Lactation | 0.63 | Mcal/lb |
| NDF Digestibility, Invitro | | | Net Energy Maintenance | 0.61 | Mcal/lb |
| 12 hr digestibility | | | Net Energy Gain | 0.35 | Mcal/lb |
| 24 hr digestibility | | | Relative Feed Value (RFV) | | |
| 30 hr digestibility | | | Relative Feed Quality (RFQ) | | |
| 48 hr digestibility | | | Milk/ton | | |
| Indigestible NDF, Invitro 120 HR | | | NFC | 47.8 | % DM |
| NDF Dig. Rate (Kd) | | | Enzymatic NSC | | |
| | | | | | |
| ***Non-Fibers, Structure, Utilization*** | | | ***Qualitative*** | | |
| Digestible Dry Matter (fast) | | | pH | | |
| Sugar | 3.3 | % DM | Total VFA | | |
| Starch | 0.8 | % DM | Lactic acid | | |
| --Enzyme Available | | | --Lactic/TVFA | | |
| --Digestibility, 2 hr | | | Acetic acid | | |
| --Digestibility, 7 hr | | | Propionic acid | | |
| Fatty Acids, Total | | | Butyric acid | | |
| Crude Fat | 0.8 | % DM | Isobutyric acid | | |
| Acid hydrolysis fat | | | 1, 2 Propandiol | | |
| CS Processing Score | | | Titratable Acidity (meq NaOH) | | |
| Particle size > 0.75" | | | | | |
| ... 0.31" - 0.75" | | | Mold | | |
| ... < 0.31" | | | Yeast | | |

LUIS TEDESCHI
TEXAS A & M UNIVERSITY
230 KLEGERG CTR, 2471 TAMU
COLLEGE STATION TX 77843

Sample : 2 - EXTRACTIVES
Farm Name : WILEY/TEDESCHI
Received : April 15, 2011
Complete : April 27, 2011
Regression : OH

# VITA


Name: Matthew David Falls

Address: Care of: Dr. Mark Holtzapple
Texas A&M University
Dept. of Chemical Engineering
232 Jack Brown Building (MS 3122)
College Station, TX 77843

Email Address: mattdf23@gmail.com


Education: B.A., Chemistry, Texas A&M University, 2005
Ph.D., Chemical Engineering, Texas A&M University, 2011

Professional Interests: Biofuel development; biomass pretreatment; increasing enzymatic digestibility; lignocellulosic animal feed

Personal Interests: Tennis; waterskiing; snow skiing; playing guitar; traveling.

Publications:

**Falls, M**., Sierra-Ramirez, R., Holtzapple, M. 2011. Oxidative lime pretreatment of Dacotah switchgrass. Applied Biochemistry and Biotechnology, **In Press.**

**Falls, M**., Holtzapple, M. 2011. Oxidative lime pretreatment of Alamo switchgrass. Applied Biochemistry and Biotechnology, **In Press.**

**Falls, M**., Shi, J., Ebrik, M.A., Redmond, T., Yang, B., Wyman, C.E., Garlock, R., Balan, V., Dale, B.E., Ramesh Pallapolu, V., Lee, Y.Y., Kim, Y., Mosier, N.S., Ladisch, M.R., Hames, B., Thomas, S., Donohoe, B.S., Vinzant, T.B., Elander, R.T., Warner, R.E., Sierra-Ramirez, R., Holtzapple, M.T. 2011. Investigation of enzyme formulation on pretreated switchgrass. Bioresource Technology, **In Press.**