ASSESSMENT OF OPTIONS FOR THE INTEGRATION OF FOOD AND FUEL PRODUCTION IN CELLULOSIC ETHANOL REFINING

By

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ABSTRACT

ASSESSMENT OF OPTIONS FOR THE INTEGRATION OF FOOD AND FUEL PRODUCTION IN CELLULOSIC ETHANOL REFINING

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Reducing petroleum consumption is one of the primary challenges of the United States and the world in the 21st century. Biofuels are seen as a primary alternative, yet concerns of decreasing food production due to increased demand for land remain. This research proposes two technologies to integrate biofuel production with animal feeds and lessen this demand: using AFEX treated biomass as a fiber source for ruminants and extracting leaf protein as a protein source. The purpose of this study is to investigate the viability of these two technologies for both economic value and increasing the productivity of land.

Experimental results indicate that an early harvest of switchgrass, which would be required for protein production, requires milder pretreatment condition and has higher yields than late harvest biomass. Ammonia-based extraction was successful in removing approximately 40% of the protein from switchgrass. However, AFEX did not increase extraction yields, and resulting sugar yields decreased after extraction. After hydrolysis, nearly all of the protein was soluble, but ultrafiltration could only concentrate 30-45% of the protein. AFEX increased the digestibility of fiber in multiple feedstocks and increased the crude protein content to levels comparable to common forages. The digestibility of pretreated

late harvest switchgrass is comparable to high quality forages, while the energy in corn stover is approximately 85% of the value of corn grain.

From these experimental results, two models were created to determine the potential of these technologies. The first, an economic and material model, suggests that animal feed integration with ethanol production can displace the equivalent of 2900-4800 L gasoline per ha of land removed from feed use compared to 1600 L/ha if no feed integration is performed. Likewise, the profitability of the land increases to $150-$380/ha for integrated animal feed scenarios compared to $35/ha for ethanol production. The second model considers the total land use in the United States and estimates the potential market for biofuels, AFEX-treated feeds, and protein extracts. These two technologies increase the amount of biofuel that can be produced by 42 GL of ethanol on cropland currently used for animal feed or ethanol production without decreasing animal feed production. Approximately 85 Tg of AFEX-treated feeds and 52 Tg of biomass for protein extraction are consumed in this scenario.

Thus, this study suggests further research into integrating animal feed production with biofuel production should be pursued. Emphasis should be applied to readying AFEX-treated feeds for commercialization, primarily through animal feeding trials. For protein extraction, future research should be focused primarily on using the remaining fiber for ethanol production.

DEDICATION

To my parents, Carl and Barbara Bals, for all of their love and support

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KEY TO SYMBOLS AND ABBREVIATIONS

AFEX – Ammonia Fiber Expansion

CDF – Cumulative Distribution Function

CIR – Cave-in-Rock

CP – Crude Protein

DDGS – Dried Distillers Grains and Solubles

DG – Distillers Grains

DGS – Distillers Grains and Solubles

DMI – Dry Matter Intake

FPU – Filter Paper Unit

HPLC – High Performance Liquid Chromatography

LPC – Leaf Protein Concentrate

MWCO – Molecular Weight Cutoff

NDF – Neutral Detergent Fiber

NEL – Net Energy Available for Lactation at 3X Maintenance

NOx – Nitrous Oxides

NPN – Non Protein Nitrogen

NRC – National Resource Council

NREL – National Renewable Energy Laboratory

PMSF – Phenylmethanesulphonyl Fluoride

pNPGU – p-nitrophenyl-β-D-galactopyranoside Unit

RBPC – Regional Biomass Processing Center

SDS – Sodium Dodecyl Sulfate

SEM – Standard Error of the Mean

TDN – Total Digestible Nutrients

TED – Total Energy Density

TMP – Transmembrane Pressure

UF - Ultrafiltration

VFA – Volatile Fatty Acids

CHAPTER 1 : INTRODUCTION

One of the primary challenges facing the United States and the world in the 21st century is the transformation from fossil fuel to renewable energy. Of primary interest is reducing petroleum consumption. Approximately 40% of the United States' total energy consumption is petroleum, the single largest source of energy consumed (see Figure 1.1). Of this energy, 70% is used for transportation, primarily gasoline (45%) and diesel (20%) fuel. Furthermore, transportation is the least diversified end use of energy in the United States, with over 95% of transportation energy supplied by petroleum [1]. Thus, for petroleum displacement with renewable energy, careful attention should be focused on displacing these transportation fuels.

Numerous reasons – including political, economic, and environmental – have been cited by the media, scientists, private industry, and politicians to move away from petroleum based transportation fuels. The four primary arguments against petroleum fuels are summarized below:

- As a fossil fuel, petroleum is a major pollutant. Besides producing greenhouse gases, gasoline and diesel engines produce pollutants such as particulate matter, NOx, and ozone, thereby concentrating pollutants within cities [2]. In addition, the risk remains of severe ecological disasters due to oil spills such as the Exxon-Valdez spill of 1989.

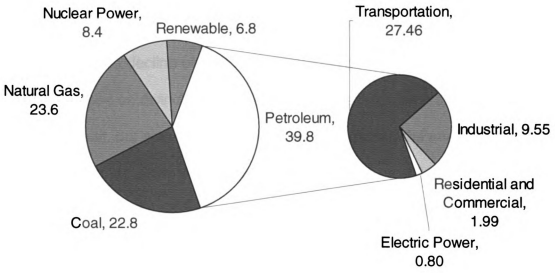

Figure 1.1 : United States primary energy consumption by source (left) and petroleum use by sector (right) in 2007. All numbers are in quadrillion BTUs. [1]

- The price of oil is highly volatile, increasing from $61/bbl in April 2007 to $137/bbl in July 2008, before rapidly falling to $40/bbl by December 2008 [1]. These price shocks can have a dramatic effect on the economy, and thus should be avoided.

- The difficulty of avoiding these price shocks is increased due to the political instability of several oil-producing nations. The Middle East produces 30% of the world's oil, and has been a source of political unrest for decades. In addition, the United States' need to import oil has often

been cited as a confounding factor in its international relations, hampering the US' foreign policy goals [3].

- As a nonrenewable resource, it is uncertain how much recoverable petroleum remains in the world. In 1956, M. King Hubbert correctly predicted the decline of United States oil production beginning around 1970 based on dwindling supplies. Similar assessments of world supply are difficult, but some analysts claim we are nearing the peak of world oil production [4]. Even if large quantities of petroleum still exist, it is in areas that are harder to extract from (such as shale oil), thereby leading to increased costs and likely increased environmental impact.

Despite the clear need to move away from petroleum based transportation fuels, two factors are limiting. The cost of alternatives tends to be higher than gasoline or diesel fuel, thus limiting their growth. Also of importance is the "chicken and egg" conundrum. It is difficult to provide the infrastructure for alternative fuels before there are consumers, and it is difficult to find consumers before there is an infrastructure. One solution to this problem is to use an alternative fuel that is compatible with existing infrastructure. Biofuels such as ethanol are a prime example of this approach. Ethanol can be blended in small quantities with gasoline and used in all cars. In addition, "flex fuel" cars have been designed to run on both gasoline and ethanol at little extra cost, allowing the potential demand for ethanol to grow before the infrastructure is in place. Because of this

innate advantage, ethanol is the leading renewable source of transportation fuel in the United States, with 9.2 billion gallons produced in 2008 [5].

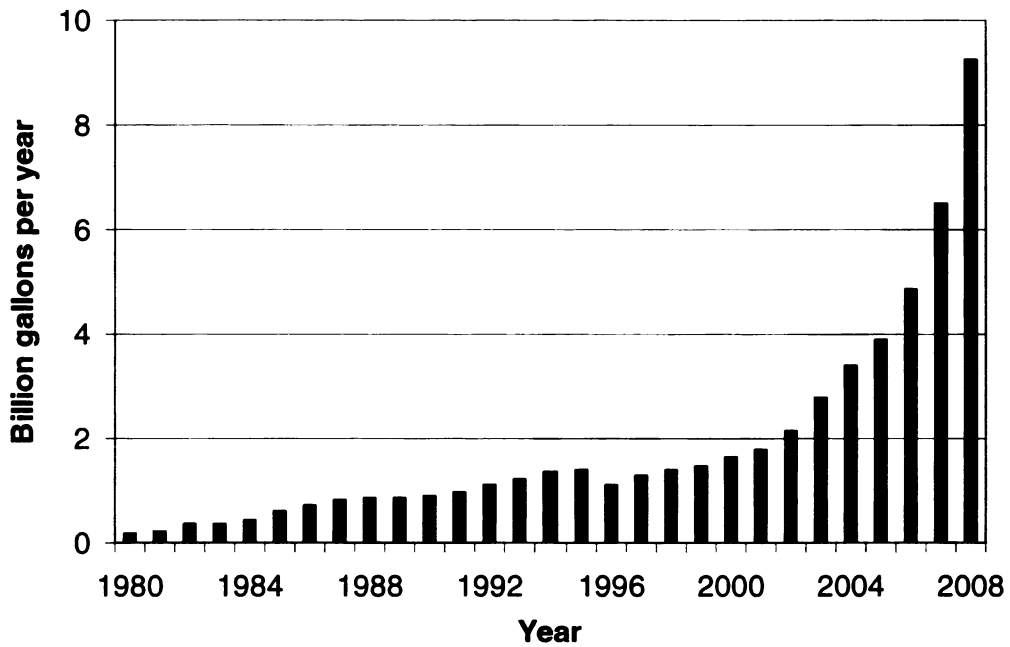

Figure 1.2 : United States ethanol production, 1980-2008. [5]

The vast majority of ethanol produced in the United States is from corn starch. However, there is a limit to the amount of ethanol that can be produced from corn due to limited farmland and the high demand of that same farmland for feed purposes. While this limit is a source of debate, one valuable estimate is approximately 15 billion gallons per year (approximately 10% of US gasoline demand). This value is the maximum starch based ethanol mandated in the 2007 Energy Independence and Security Act, as shown in Figure 1.3 [6]. Cellulosic ethanol is seen as a long-term solution. By obtaining ethanol from fibrous material rather than starch, several additional feedstocks become

available for bioenergy, including agricultural wastes, municipal solid waste, forests and wood based residues, and grasslands. Making cellulosic ethanol commercially viable has been a major focus of research, both academic and industrial, over the past 5 years.

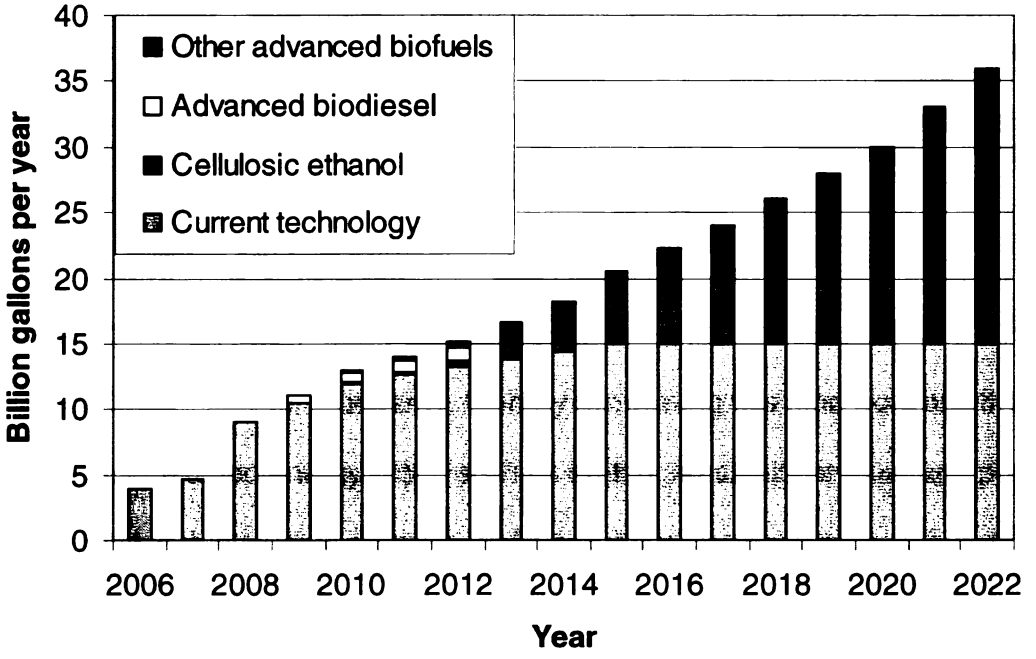

Figure 1.3 : Renewable fuel standard requirements in the United States as implemented in the Energy Independence and Security Act of 2007. Current technology is dominated by corn-based ethanol, yet the mandate limits its production to 15 billion gallons per year (bgpy). By 2022, the single largest source of renewable fuel required is cellulosic ethanol (16 bgpy).

While cellulosic ethanol can enhance the amount of biofuels available for use compared to corn grain, there is no consensus on how much total biofuel energy is available. Agricultural residues, such as corn stover, rice straw, and wheat straw, are particularly useful, as they require no additional land for use. However, low yields per acre, competing uses such as animal bedding, and the

need to leave some material on the farm to prevent erosion and improve soil quality means these sources are limited. Graham et al. [7] claim that nearly 200 million Mg of corn stover is produced in the United States, but only 58 million Mg is harvestable for biomass use. Gallagher et al. [8] give a higher number for collectable stover at 98 million Mg. Accepting this higher number as valid and assuming 80 gallons per Mg, corn stover alone can only increase the total ethanol produced in the US by approximately 50%. Other residues can increase this number, but it is clear that residues alone cannot account for all liquid fuel demand.

Thus, dedicated energy crops will likely be required. These crops are defined as those produced solely for bioenergy, thereby eliminating this land from all other uses. Most dedicated energy crops such as switchgrass, miscanthus, and poplar are perennial, achieve rapid growth with little water or fertilizer input, achieve high yields per acre, and require little pesticide or herbicide application. These crops can often be grown on marginal land unsuitable for traditional crops, although yields are generally lower than in prime soil. In addition, switchgrass and other grasses can be grown in land enrolled in the Conservation Reserve Program, which in 2008 was over 13 million hectares [9]. Again, yields would likely be lower than in prime farmland.

While there are benefits to large-scale cellulosic ethanol production, concern regarding a large-scale bioenergy economy remains due to the effect it would

have on current agricultural practices. Walsh et al. [10] studied the effect of biofuel production on overall agricultural economics. They concluded that if 171 million tons of perennial crops could be produced, traditional crop prices would rise by 9-14%. These price increases occur naturally due to the increased demand for land in a bioenergy future. Other researchers believe further impacts could be caused by indirect land use change. Searchinger et al. [11], for example, reported a 50% increase in greenhouse gas emissions for cellulosic biofuel compared to gasoline. This is due to land use change; diverting fallow land to cropland results in the loss of the benefits of these fallow lands. If other croplands are diverted to biofuels, then it is expected that other fallow lands or forests would be cleared for food/feed use. If these analyses are correct, then this further limits the amount of land available to biofuels, particularly if the benefits of fallow land are perceived as outweighing the benefits of reduced petroleum consumption.

1.1 Justification

While cellulosic ethanol offers greater variety in terms of potential sources for biofuels, the question of its viability still remains. Can cellulosic ethanol replace a significant portion of petroleum use without impacting food production? Due to the encroachment of cellulosic feedstocks on farmland, would large-scale production of ethanol be a positive impact on the world? As stated previously, several analyses claim that large-scale bioenergy production is unsustainable due to this reason, but such studies are based on the current agricultural

landscape. Rather than accepting this premise, this study envisions a future where cellulosic feedstocks and food production are compatible with each other.

Such a future would require integrating food production with biofuel production. This is already performed in the corn ethanol industry, as distiller's grains, the byproduct of corn processing, are sold as animal feed, partially offsetting the loss of farmland for feed purposes. Similar integration may be required for cellulosic facilities as well. In fact, integrating animal feed operations with cellulosic ethanol may help to reduce or eliminate several of the potential hurdles for cellulosic biofuel commercialization.

At 714 g starch per kilogram corn grain [12] and 151 bushels of corn per acre [13], approximately 6.77 metric tons (Mg) of digestible carbohydrates per hectare can be produced from corn. While dedicated energy crops are a new concept and their potential is currently unknown, it is expected that they would be able to produce 10-20 Mg of dry biomass per ha [10; 14]. This relates to the equivalent of 5.5-11 Mg of carbohydrates per hectare if all cell wall carbohydrates are available for energy consumption [15]. By the same token, switchgrass harvested in early summer may have up to 10-15% protein content, although the biomass yields during spring or summer are approximately 33-50% of the total harvest [16; 17]. Thus, it is possible to produce 0.5-1.5 Mg of protein per hectare with switchgrass, which compares favorably with soybean at approximately 1.1 Mg of protein per hectare [13; 15].

Thus, it is conceivable that a hectare of switchgrass could produce as much animal feed as a hectare of corn or soy while simultaneously providing biofuel. Thus, land for biofuels can partially displace land for feed with little or no impact on food production. This increases the amount of land available for cellulosic ethanol, increasing the potential amount of petroleum that can be displaced. In addition, these animal feeds would be a large source of revenue for a refinery, and potentially quite profitable as well. This can help to reduce the economic risk of early refineries, as there is a second revenue stream, and reduce the impact of volatile market prices of both inputs (feedstock) and output (ethanol, which would compete with volatile gasoline prices). Carolan et al. [18] estimate that animal feed coproducts can reduce the cost of ethanol by approximately 9-20 cents per gallon ethanol for a fibrous energy feed, while Greene [19] estimates 11 cents per gallon for a protein feed.

1.2 Project Description

This project seeks to build a foundation upon which animal feed and biofuel integration can be pursued. As there is currently little interest in such research, this foundation needs to be built in order to determine which approaches have the greatest potential. Of primary interest is using dedicated feedstocks such as switchgrass in mature refineries. Other feedstocks are also tested as needed.

Different options are available to integrate food and fuel production. Two primary approaches are considered – treating the fibrous matter with ammonia fiber expansion (AFEX) pretreatment as a feed for ruminants, and separating the protein from the fiber to displace soybean meal. Different methods and feedstocks are available for these options, and both must be integrated with biofuel production.

As stated previously, these options are meant to build a foundation for future study. Multiple integration options are compared using two different models in order to assess their value in reducing competition between food and fuel. Thus, the most promising options can be more fully studied and implemented. The main objectives and limitations of this research are summarized below.

1.3 Objectives

- Identify key opportunities for integration of animal feed with cellulosic ethanol
- Analyze integration options to determine optimal conditions and approach
- Obtain mass balances around all components for each option on a consistent basis
- Compare all options using current models to predict economic and material impact
- Identify key knowledge gaps for future research

1.4 Limitations

- Limited to feedstocks available at the time of study. Due to the high degree of variation among feedstocks, results may change with different compositions. Sensitivity analyses within the models mitigate this limitation.
- Limited in scale of experiment. No animal feeding trials were possible. *In vitro* rumen digestion experiments were used instead for AFEX-treated feeds.
- Limited in depth of experimentation. The goal is to identify key areas for further study, and so not all avenues of research were explored due to time and material constraints.
- Limited to current models and assumptions. Due to lack of information, cost estimates can vary greatly for biofuel or animal feed production. Sensitivity analyses will mitigate this to some extent.
- Limited to economic and material outputs rather than environmental impacts. This is related directly to the justification of the project.

CHAPTER 2 : LITERATURE REVIEW

2.1 Ethanol Production

Bioethanol can be produced from various carbohydrates, including simple sugars, starch, and cellulose. While both simple sugars and starch based ethanol are currently commercially viable, cellulosic ethanol is still an unproven technology. There are currently two different approaches being considered for commercialization: the biochemical and the thermochemical platform [20]. In the thermochemical platform, the plant material is burned with limited oxygen, producing syngas (carbon monoxide and hydrogen), which is then converted into ethanol using either catalysts or microbial fermentation. Alternatively, the biomass can be burned in the absence of oxygen, called pyrolysis, which produces a liquid product that can be upgraded to various biofuels. Since the thermochemical platform uses all materials within the biomass – including cellulose, lignin, lipids, acids, and protein – there is little opportunity for coproducing animal feeds. Thus, this research focuses solely on the biochemical platform. In this approach, the complex carbohydrates are broken down into component sugars using enzymes, and then these sugars are fermented into ethanol.

The primary drawback of the biochemical platform is the recalcitrance of cell wall materials. Cell walls are composed of a dense lignocellulosic structure containing cellulose nanofibers and hemicellulose linkages, and surrounded by

hydrophobic lignin. Because of this dense structure, cellulases and hemicellulases are unable to effectively break down the carbohydrates, resulting in poor sugar yields [21]. Thus, a pretreatment step is necessary prior to enzymatic hydrolysis. This process, generally performed at elevated temperatures and with either an acid or base catalyst, reduces or eliminates the barriers to enzymatic hydrolysis and dramatically improves the sugar yields.

A summary of leading pretreatments is shown in Table 2.1. Dilute acid hydrolyzes hemicellulose, leaving a highly digestible solid residue composed primarily of cellulose and lignin. However, this process also produces harmful sugar degradation compounds such as furfural, which can inhibit hydrolysis and fermentation. Thus, a potentially costly detoxification step is necessary. Hot water pretreatment reduces the inhibitory compounds formed by controlling the pH, but does not solubilize as much hemicellulose as dilute acid pretreatment. Steam explosion reduces the water use and may optionally be performed with acid catalysts. Due to the low water use, there is no liquid separation after pretreatment, and so both cellulose and hemicellulose must be hydrolyzed in the same reactor. Alkaline pretreatments using strong hydroxides or ammonia have also been used. These pretreatments tend to reduce the inhibitors formed and sugars degraded, but do not solubilize as much hemicellulose.

Of these, ammonia fiber expansion (AFEX) is the focus of this study. Highly concentrated ammonia is added to biomass at a moderate temperature and

pressure, and allowed to reside for 5-30 minutes. The pressure is then released, rapidly evaporating ~90% of the ammonia. This process results in partial solubilization of hemicellulose, removal of lignin to the surface of the biomass, and partial decrystallization of cellulose [22]. AFEX, like steam explosion, is a dry-to-dry process, and so all compounds remain in the solid form with no separate liquid phase. Little sugar degradation occurs relative to acidic treatments, leading to the potential for high sugar recovery. Various byproducts are also formed primarily from the reactions between ammonia and various linkages between the hemicellulose and lignin, primarily acetamide. These products may inhibit enzymatic hydrolysis and fermentation, or alternatively may benefit downstream processes by providing a valuable nitrogen source.

Table 2.1 : Five leading pretreatment technologies and their operating conditions for treating corn stover. All information was obtained from Wyman et al. [23] except for steam explosion, which was obtained from Bura et al. [24].

Pretreatment	Catalyst	Temp (C)	L:S ratio	Time (min)
Dilute Acid	H_2SO_4	160	4:1	20
Steam Explosion	SO_2	190	Not given	5
Controlled pH	None	190	6:1	15
AFEX	NH_3	90	0.6:1	5
Lime	$Ca(OH)_2$	55	5:1	4 weeks

AFEX is a strong pretreatment candidate for feed co-products for several reasons. The moderate temperatures during the AFEX reaction are less likely to degrade valuable protein. The lack of inhibitory compounds, which may also be toxic for animal feed purposes, is also an asset. As AFEX is a dry-to-dry process, only one process stream is present after pretreatment, allowing for a separate fractionation process dedicated to co-product recovery. Finally, AFEX

can be performed at a relatively low capital and operating expense, allowing for pretreatment and co-product facilities separate from a biorefinery [25].

This latter idea, hereafter named regional biomass processing centers (RBPCs), is a novel approach to cellulosic refining [18]. These centers would have smaller capacities than a full-scale biorefinery (between 100-1000 tons per day), and so several RBPCs would service one refinery. By separating the pretreatment and co-product formation from the rest of biomass processing, the capital costs per ton of material of these operations will increase due to lower economies of scale. However, there are several advantages to RBPCs that may outweigh the additional cost:

- Transportation and logistics of biomass collection is streamlined, as the refinery need not contract with hundreds of farmers and arrange for transport of material. Instead, the refinery will contract with a manageable number of RBPCs, who in turn will contract with a smaller number of farmers.

- Feed co-products remain in the rural setting, potentially decreasing the transportation time required. Furthermore, as large feeding operations may already be centrally located around farms, the RBPC may be co-located with the animal operation to eliminate the transportation, storage, and drying requirements.

- The risk associated with ethanol production decreases for multiple reasons. The direct capital cost to the biorefinery decreases, causing a lower barrier to entry. In addition, RBPCs could be built and begin producing animal feeds prior to construction of a biorefinery, thus spreading the overall capital costs required for the entire system over a longer period of time.

- RBPCs incentivize farmers to produce cellulosic materials by increasing the potential markets for the biomass. In addition, these RBPCs may be partly owned by the farmers, thereby retaining more wealth from ethanol and co-product production in the rural community.

2.2 Food vs Fuel

The primary concern regarding large-scale cellulosic ethanol is the amount of land required to produce the material. Due to the high use of petroleum fuels in the United States, over 2 billion Mg of biomass would be required to displace gasoline use. Few researchers suggest that this amount of material is available. One prominent study from the USDA and DOE proposed that 1.2 billion Mg of biomass could be sustainably grown and removed every year within the United States. This includes corn grain (79 Tg), agricultural residue (388 Tg), forest resources (334 Tg), and dedicated energy crops (342 Tg), as well as 96 Tg of other assorted materials such as animal manure [26]. Somewhat optimistic scenarios were applied to obtain these numbers, including high rates of removal

for agricultural residues, large increases in crop productivity, and land use change. For the land use change, 60 million acres were assumed to be available for dedicated energy crops, of which 25 million acres would be obtained from active cropland. Although the study claims that this approach is sustainable, no mention of the effect on food production is given. Presumably, the increase in grain and bean yields would offset this loss of land.

Table 2.2 : Literature values for potential biofuel production within the United States

Reference	EtOH[a] (bgpy)	Notes
Lynd, 1996 [27]	51	Crop residues, CRP land, mature technology
McLaughlin, 2002 [28]	14	Switchgrass only
Kadam, 2003 [29]	9	Corn stover and wheat straw only
Kim, 2004 [30]	17	Waste crop and crop residues only
Greene, 2004 [19]	140	Land use change, mature technology
USDA/ DOE, 2005 [26]	109	Forest and cropland, land use change
Campbell, 2008 [31]	24	Abandoned crop and pasture land only
Lal, 2008 [32]	65	100% of crop residue, manure, and MSW

[a] bgpy – billion gallons per year. If total biomass is given in the reference instead, this value assumes 80 gallons per dry ton of biomass.

Most other studies have tended to look at near term possibilities instead, which provide far less potential bioenergy. A summary of potential ethanol production from these studies is listed in Table 2.2. In most of these studies, bioenergy can only produce a small fraction of the transportation fuel currently in use. These studies do not look at land use change and are thus limited in scope. The USDA study also included no land use change as a sensitivity as well as little improvement in yields, and obtained results comparable to the other studies presented. Those studies that allow for significant land use change show the

potential for over 100 billion gallons of ethanol per year, representing over 50% of current US gasoline demand.

The concern over land use change, however, is significant. Searchinger et al. [11] proposed that changes in land use would cause biofuels to increase greenhouse gas emissions relative to gasoline use. This study uses economic models to predict the effect biofuels would have on the global agricultural market. In these models, additional land must be converted to agriculture in order to meet the new demand for food and fiber, and the carbon cost of converting excess land to cropland offsets the benefits of renewable fuel. In addition to the environmental concerns, predictions that food costs will rise due to biofuels are also common. An economic study by Oak Ridge National Laboratory on a county-by-county basis throughout the United States suggests that food prices would rise by 9-14% if 55 million Mg of biomass is sold for biofuels [10]. A considerable expansion beyond 55 Tg could increase prices even further. If these studies are correct, it is clear that considerable effort must be made to reduce the impact on land use change if biofuels are to be a large-scale industry.

Current land use is dominated by the production of animal feeds, particularly in the United States. Table 2.3 displays the major allocation of rangeland and cropland within the United States. The two primary crops on cropland are corn and soy, which represent 21% and 16% of total US cropland, respectively. Corn is primarily used as an energy feed due to its high starch content, while soy acts

as a protein supplement. These two crops are the primary components of nonruminant diets, particularly swine and poultry. Ruminants, primarily beef and dairy cattle, also consume a great deal of fibrous forages in addition to corn and soy, which are produced on both rangeland and cropland. A large portion of corn in the United States is devoted to ethanol production as well.

Table 2.3 : Total farmland in the United States and their primary usage. [9] Land in the Conservation Reserve Program may fall into other categories, particularly idle land.

Land	MM acres	% total	Land	MM acres	% total
Cropland	406.4	44.1	Rangeland	408.8	44.3
Grazing	35.8	3.9	Wood pasture	28.7	3.1
Idle/failed	61.0	6.6	CRP land*	38.5	4.2
Corn	86.2	9.3	Other	78.2	8.5
Soy	63.9	6.9			
Wheat	50.9	5.5			
Other	108.6	11.8	Total	922.1	

Thus, in order to reduce the conversion of native grasslands or forests to croplands in a biofuel economy, it will be necessary to adapt current cropland or rangeland to biofuel use while maintaining current agricultural output. This research focuses on increasing land use efficiency, or the amount of feed or biofuel production produced on an acre of land.

2.3 Ammoniation of Feeds

Forages, defined as feedstuffs high in fiber such as hay and silage, are a common source of energy for ruminant animals such as dairy and beef cattle. These forages are digested in the rumen, where microbes ferment the fiber into volatile fatty acids (VFA), namely acetate, propionate, and butyrate. The acids

are then absorbed through the wall of the rumen and metabolized by the cattle. While forages are generally less expensive than grain, ranging from $50-$150 per dry ton depending on the quality, their use has declined in modern livestock farming due to relatively slow growth and poor utilization of fiber compared to starch. Despite this, a minimal amount of forage is generally included in the diet. For example, the NRC recommends at least 25% of a lactating dairy cow's diet should be composed of NDF (neutral detergent fiber, or the sum of cellulose, hemicellulose, and lignin) [33].

The digestibility of forage material is one of its most important qualities, and thus a major factor in its price. Digestible forages include alfalfa and orchardgrass hay. These feedstocks tend to have a high leaf to stem ratio, low NDF concentration, and very little lignin. These characteristics are common for immature material, although harvesting early in the growing season tends to lower overall yields. Thus, chemical treatment, particularly alkaline treatment, of poor digestible forages has been developed to increase the range of acceptable feeds in livestock production. These treatments, as with AFEX, tend to cleave ester bonds between cellulose and lignin, allowing for improved rates of digestion while leaving most of the fiber intact.

Particular interest has been on ammonia or urea treatments, as these nitrogen containing compounds can increase the crude protein content in the biomass as well as improving fiber digestibility [34]. In general, anhydrous ammonia is

pumped under a tarp containing bales of hay, at a rate of 3-4 g ammonia per 100 g dry biomass and allowed to stand at ambient temperatures for 6-8 weeks. This process tends to increase NDF digestibility by 100-150 g/kg NDF and crude protein by 50-100 g/kg biomass, as seen in Table 2.4. Ammoniation also improves ruminant uptake and helps to eliminate spoilage.

Table 2.4 : Improvements in fiber digestibility (NDF) and crude protein (CP) content due to ammonia treatment for five types of biomass.

	NDF Unt. (g/kg NDF)	NDF Treat. (g/kg NDF)	CP Unt. (g/kg BM)	CP Treat (g/kg BM)	Ref.
Wheat Straw	384	509	46	110	[35]
Bermudagrass	407	584	66	171	[36]
Cotton	394	489	78	160	[37]
Sorghum	528	674	58	106	[38]
Rice Straw	419	540	n/a	n/a	[34]

While ammoniation does have commercial value, it is limited due to the modest increase in digestibility as well as the relatively high costs associated with the process. The ammonia used in this process is not recoverable, requiring 30-40 g/kg forage. In addition, the tarp or plastic covering may also be included in the costs. Likewise, the increase in digestibility is not likely to make the treated forages comparable to high quality feeds. Orchardgrass silage, for example, can have fiber digestibility of 720 g/kg, higher than all treated values shown in Table 2.4 [39]. Thus, ammoniation is used primarily during the winter, when fresh forages are not available, or in countries such as China where land is limited.

Using more extreme ammonia treatments such as AFEX to further improve digestibility has been considered but is currently not in practice. One animal

feeding trial has been performed using AFEX treated rice straw at 7% of the total diet in lactating dairy cows [40]. A greater proportion of the total diet was NDF, with AFEX treated rice straw displacing high protein alfalfa. Here, total dry matter intake did not significantly change, but milk production increased by 3% ($p = 0.02$). Because the level of inclusion of treated rice straw was low, it is difficult to draw concrete conclusions on the effectiveness of AFEX treatment relative to the conventional ammonia treatments. Perdok and Leng fed rice straw treated at elevated temperatures and relatively long residence time (24-72h) at higher concentrations of total diet, but this led to hyperexcitability in the cows [41]. It is likely that this is due to the production of 4-methylimidazole from reducing sugars reacting at elevated temperatures and residence time. Weiss et al., however, did not see signs of hyperexcitability in sheep when directly fed 4-methylimidazole at high levels [42]. The exact mechanism of hyperexcitability due to ammonia treated feeds is currently unknown.

2.4 Leaf Protein Concentrate

The concept of using leaf proteins as a protein source has been investigated since the 1940s [43]. The amino acid profile of leaf protein is fairly consistent across a wide variety of species, and compares favorably to other sources of protein such as corn and soy. Leaf protein concentrates (LPC) also tend to be highly digestible, and their overall biological value is superior to soy proteins. The major setbacks to leaf protein concentrates are poor color and flavor and the possibility of Maillard reactions and lipid oxidation [44].

Due to the presence of fiber, which is indigestible for nonruminants such as swine and poultry, it is necessary to first remove these proteins from the leaf. One such method is mechanically pressing a protein-rich juice from fresh forages, while another is to extract the proteins using water or a solvent. This second option can be performed on either fresh or dried forages, allowing more versatility, but may require higher downstream costs due to a potentially less concentrated protein stream.

Investigation into mechanical pressing of leaf proteins has been ongoing for several decades [44; 45]. In brief, the plant is first macerated before undergoing a dewatering step, most likely using a screw press. Emphasis is focused on the percentage of cells ruptured and insuring maximum solubility and filtration of proteins [46]. Protein from the extracted juice is precipitated and dried, either through steam injection or acid precipitation. The remaining whey can be either recycled to the incoming biomass or evaporated and combined with the fiber-rich press cake.

Attempts have been made to commercialize leaf protein production using mechanical pressing. One of these attempts was an alfalfa dehydration facility modified to produce a protein product known as Pro-Xan [47]. This commercial scale demonstration of leaf protein technology created a 56% protein product, which accounted for 35% of the protein from alfalfa. An economic analysis of the

process indicated that profitability could be obtained depending upon the length of operation of the facility, which depended heavily upon the growing season of alfalfa, as well as the value of the deproteinated fiber. Furthermore, it was found that adding ammonia prior to pressing the fiber improved the quality of the final protein product, and that recycling a portion of the deproteinated juice improved overall protein yields.

Commercial leaf protein production was also attempted in New Zealand, which also used alfalfa as a feedstock [48]. This approach also used mechanical pressing to release the protein. However, after the first pressing, a disc mill was used to further disrupt the fiber, and a second pressing was performed. This resulted in higher protein extraction yields, obtaining up to 70% of the available protein. However, this production facility ceased operations due to difficulties in accessing the export market [49]. A third commercial facility, Desialis, is currently operational in France, and specializes in aquaculture feed and other specialty protein sources.

In addition to mechanical pressing, solid-liquid extraction has been considered for the production of LPC. There have been no reported studies of extracting proteins from switchgrass, although several other types of biomass have been considered for production of protein concentrates. Dilute solutions of a strong alkali such as sodium hydroxide are generally used, with the pH between 8 and 12. Extractions generally range from 30 to 60 minutes at 10:1 or higher liquid to

solid ratio. Protein yields varied considerably depending upon the types of biomass, generally resulting in high yields of protein from grains and moderate to low yields from leaf proteins. Betschart and Kinsella [50] were able to extract 35% of protein from soybean leaves, for example, while Fernandez et al. [51] obtained 41% from Atriplex leaves. In general, it appears that simple extractions are not sufficient to obtain complete protein recovery from leafy biomass.

Table 2.5 : Yields of leaf protein concentrates from solid/liquid extraction obtained from different studies

Biomass	Solvent	L/S Ratio	Extracted	Ref.
Sunflower seed	0.5M KOH	20:1	66%	[52]
Rice Bran	Water	10:1	12%	[53]
Tobacco	pH=7 buffer	10:1	1.6%[a]	[54]
Soybean	NaOH pH=8	30:1	71%	[55]
Atriplex Leaf	pH=10 buffer	5:1	41%	[51]
Soy Leaf	Tris pH=7.4	13:1	35%	[50]

[a] Given as percent total biomass (protein content of leaves not given)

Few of these studies speculate on the economic attractiveness of this method. Cost estimates of the commercial Pro-Xan facility indicated that this process was economical, but the economics depended greatly on the length of the operating season and the yield obtained. Due to the need to extract material shortly after harvesting in the mechanical pressing method, such considerations are extremely important. A diversity of crops, such as cover crops and alfalfa, can extend the operating season significantly, as can extracting protein from dried hay. Also of interest is the value of the remaining fiber. The Pro-Xan report speculates a selling price of $82/ton (in 1977 dollars), compared to $51/ton for untreated alfalfa [47]. However, much of alfalfa's value lies in its protein, and so

it is uncertain whether this value is correct. Other studies suggest biofuel production as a viable secondary market [56], yet this too has not been proven.

While leaf protein extraction is a known technology, very little research has been performed on integrating extraction with fiber processing. De la Rosa [57] and Urribarri [58] found increases in protein yields from coastal bermudagrass and dwarf elephant grass, respectively, when undergoing ammonia pretreatment prior to extraction. By disrupting the lignocellulosic structure of the biomass, proteins appear to more easily diffuse out of the biomass and into solution. Over 50% of the protein in AFEX treated dwarf elephant grass, for example, was extracted using calcium hydroxide compared to 11% for untreated samples [58]. The feed value of these protein concentrates, relative to untreated samples, was not tested in either publication.

CHAPTER 3 : ENZYMATIC HYDROLYSIS OF SWITCHGRASS

3.1 Introduction

Switchgrass (*Panicum vergatum*) is perhaps the most commonly cited dedicated feedstock for cellulosic ethanol production. As one of the leading feedstocks, several different treatments have been investigated for its use in pretreating switchgrass [59]. However, due to the variability in switchgrass type and harvest dates, these results are not comparable to each other. Few of these publications give details of harvest time and switchgrass type. Of particular interest is a previous study using AFEX pretreatment [60]. Here, approximately 90% of the glucose and 70% of the xylose were released using relatively modest pretreatment conditions. An optimum was found for both ammonia and water loadings in terms of maximum sugar yields, while xylose yields continued to increase with rising temperature. While the harvest date of switchgrass is not mentioned, it is likely that the material was immature.

If protein extraction for animal feed purposes is to be considered for a biorefinery, multiple harvest dates must be used. Protein concentration decreases rapidly between the summer and autumn, with generally less than 3% protein by October. It is thus unlikely that protein extraction would be practiced on this mature material. Switchgrass harvested early in the season will also have lower fiber concentration, thus limiting the potential sugar production. However, early

harvest material also has less lignin, and thus the fiber is likely more susceptible to enzymatic attack.

In addition to the amount of recoverable sugar and protein within the switchgrass, overall yield per acre also is affected by harvest date. In general, there will be less switchgrass harvested in the summer compared to one harvest later in the season, although multiple harvests may produce as much material as a single harvest [17; 61; 62]. Multiple harvests also remove more minerals and nitrogen from the field, and thus require more fertilization. These considerations are also important if protein recovery is included in a biorefinery, and so must be included in any analysis.

Several different varieties of switchgrass have been created for use as bioenergy. These varieties can generally be categorized into two classes: lowland and upland switchgrass. Lowland varieties are more acclimated to wetter environments, and generally have coarser stems and grow taller than upland varieties. Different varieties may also react to pretreatment in different manners as well.

Thus, further research is necessary on the pretreatment and hydrolysis of switchgrass before any conclusions can be made regarding a protein recovery system as part of the biorefinery concept. A comparative study between two harvest dates – July and October – and two switchgrass varieties grown in

different locations – Cave-in-Rock (upland) grown in East Lansing, MI, and Alamo (lowland) grown in Auburn, AL – has been undertaken. This study aims to address the differences between pretreatments at different harvest dates and locations and the resulting sugar yields. Differences in pretreatment conditions and enzyme requirements are considered as these factors are important in evaluating the overall costs of ethanol production.

The objectives of this chapter are as follows:

- Obtain optimization parameters for AFEX and hydrolysis for the different varieties and harvest dates of switchgrass
- Determine the sugar yields obtainable for early and late harvest switchgrass
- Analyze the differences in AFEX and hydrolysis conditions between different varieties and harvest dates of switchgrass

3.2 Materials and Methods

3.2.1 Feedstock

Two varieties of switchgrass were used for this study. Alamo (a lowland variety) switchgrass, grown at Auburn University (Auburn, AL), was harvested in mid-July and mid-October of 2005. Cave-in-Rock (CIR) switchgrass (an upland variety) was grown at Michigan State University (East Lansing, MI) and harvested in early July and mid-October of 2008. All varieties were milled using a Fitzpatrick JT-6 Homoloid hammer mill (Continental Process Systems, Westmont, IL) to a mesh

size of 2mm. Samples were dried to less than 10% moisture and stored at 2°C until use.

3.2.2 AFEX Conditions Experiment

For screening AFEX conditions, pretreatment was performed in a 22mL reactor. Switchgrass was premixed with water at the desired loading and 3g dry weight was added to the reactor before being sealed shut. Air was removed from the reactor using a vacuum. Anhydrous ammonia was preheated to a desired pressure in the ammonia loading vessel, and the biomass preheated to the desired temperature. Both the ammonia pressure and biomass temperature were chosen in order to reach a specified temperature once the ammonia was added to the biomass. The heat of mixing between ammonia and water raises the temperature beyond the preheated values, and a precise final temperature is therefore difficult to obtain. Instead, a range of preheated values was used to obtain a range of temperatures, and the final temperature of the biomass was recorded and used. Pressure was released at the end of the desired residence time by turning a ball valve. After the reaction, the biomass was removed and allowed to dry in a fume hood overnight. Based on previous studies [63], it was assumed that no net change in the biomass weight occurred during pretreatment.

AFEX conditions ranged from 0.4-2 g ammonia / g dry biomass, 0.4-2 g water / g dry biomass, and 5-30 minute residence time. In addition, the temperature range was generally between 80-200°C. At least 45 total AFEX conditions were

chosen for each type of switchgrass tested. The "corner points" of ammonia and water, representing the maximum and minimum values in the scope of this experiment, were specifically chosen at a moderate temperature and pressure. In addition, a near center point (1 g ammonia/g dry biomass, 1 g water/g dry biomass, 15 minute residence time, moderate temperature) was replicated multiple times. For the remaining data points, the experimental conditions were randomly assigned.

Enzymatic hydrolysis was performed at 3% dry biomass loading and 15 mL total volume. A 0.05 M citrate buffer was used to keep the pH constant at ~5.0. Tetracycline and cycloheximide were added to prevent microbial and fungal growth. Accelerase 1000 (Genencor, batch# 1600844643) was used as the cellulase and loaded at 5mg Accelerase/g dry biomass (equivalent to 3.2 FPU/g biomass). Hydrolysates were incubated at 50°C and rotated at 200rpm for 72 hours. After the incubation period, enzymes were deactivated by heating samples to 99°C. Monomeric glucose and xylose concentration was determined through HPLC using a Bio-Rad (Richmond, CA) Aminex HPX-87P carbohydrate analysis column. Degassed HPLC water with a flow rate of 0.6 mL/min was used as the mobile phase, while the temperature in the column was kept constant at 85°C.

A reduced linear model based on the total monomeric glucose and xylose released during enzymatic hydrolysis was used to analyze the results using

Minitab 15 as the statistical software package. Ammonia loading, water loading, residence time, and the final reaction temperature were used as parameters as well as all interaction effects. Any parameter or interaction term that did not have a significant effect ($p<0.05$) was eliminated. The final model was used to analyze the response of total sugar yield to each pretreatment parameter as well as to estimate the optimal AFEX conditions for each switchgrass sample.

3.2.3 Enzyme Addition Experiment

For all subsequent experiments in this paper, AFEX was performed at the conditions determined from the above experiments. These estimated optimal conditions are listed in Table 3.3. The same treatment method was used except that AFEX was performed in a 1.5 L reactor rather than a 22 mL reactor. Between 80-150 g dry switchgrass was used for each batch. The amount of switchgrass depended on the ammonia loading, as a practical limitation of the ammonia loading vessel was 160 g. Multiple batches of AFEX treatment were performed, and no significant differences ($p<0.05$) were observed in sugar released through enzymatic hydrolysis between batches. All batches were then combined before proceeding with further experiments.

Four commercial enzymatic mixtures were used in these experiments: Accelerase 1000, the β-glucosidase Novozyme 188 (Novozymes, Batch#058K1144), Multifect Xylanase (Genencor, Batch#4900805391), and Multifect Pectinase (Genencor, Batch#4010833580). Enzyme loading varied

between 5-20 mg/g biomass for Accelerase and 0-10 mg/g biomass for the other enzyme mixtures. Enzyme loadings were based off previous experiments, in which more cellobiohydralase (found primarily in Accelerase) is required than xylanases in corn stover hydrolysis. Likewise, very low yields were obtained when no cellobiohydrolase was included, which was not the case for the xylanases [64].

A total of 48 hydrolysis experiments were run for each type of switchgrass, representing 25 different enzyme combinations determined using the Box-Behnken method [65]. Hydrolysis was performed in the manner stated above. Results were analyzed with Minitab 15 using response surface methodology to determine the importance of each type of enzyme in releasing sugars.

3.2.4 Rate Determination

For the rate experiments, the enzyme loading for each biomass was used as determined from the previous experiment and listed in Table 3.5. Hydrolysis was performed in duplicate in 250 mL flasks with a working volume of 100 mL. All other conditions were as stated previously. Samples of 1 mL were taken at 0, 3, 6, 10, 24, 72, and 168 hours. Cut pipette tips were used in order to sample both solids and liquid from the flasks, thereby preventing bias in later hydrolysis time periods due to changing the solid loading.

3.2.5 Composition Analysis

Switchgrass cell wall composition was determined based upon the standard method described by NREL [66]. For total carbohydrate analysis, unextracted switchgrass was hydrolyzed in 72% sulfuric acid at 30°C for 1h, followed by 1h hydrolysis in 4% sulfuric acid at 121°C. The resulting hydrolysate was filtered, and the remaining solids were gravimetrically analyzed to determine acid-insoluble lignin. Total sugars released within the hydrolysate were analyzed using a Biorad Aminex 87H column with a constant flow rate of 0.6mL/min using 5mM sulfuric acid and a temperature of 65°C. Ash content was gravimetrically determined by combusting at 575°C for 16 h. Total extractives were determined using an accelerated solvent extractor with water followed by ethanol as the solvent at 1500 psi. A portion of the water extract was analyzed via HPLC for soluble sugars.

3.3 Results

3.3.1 Compositional Analysis

Table 3.1 shows the composition of the four harvests of switchgrass. In general, later harvest materials contain more glucan, xylan, and lignin than early harvest. Of particular importance is the lignin, which increases from 16.9% to 22.6% between the July and October harvest of CIR switchgrass. The amount of lignin present has been linked to poor hydrolysis yields, particularly as AFEX does not completely remove lignin. However, increased glucan and xylan content shows the potential for later harvests to achieve higher overall sugar yields. Both xylan

and lignin showed a general trend of increasing with increasing maturity, as the July harvest of the Michigan CIR switchgrass contained the least amount of both compounds between all harvests, while the October harvest of CIR contained the most of each. The CIR switchgrass, grown in Michigan, experienced a shorter growing season than the Alamo harvests, and thus the July harvest of CIR is less mature than Alamo, while the October harvest of CIR is more mature than Alamo, as the northern variety tends to senesce earlier in the year.

Table 3.1 : Composition analysis for the July and October harvests of Cave-in-Rock (CIR) and Alamo switchgrass

	CIR – July		CIR – October		Alamo – July		Alamo – Oct.	
	%	sem[a]	%	sem	%	sem	%	sem
Glucan[b]	30.4	1.6	30.5	0.3	29.7	2.2	33.2	0.5
Xylan	15.8	0.7	19.5	0.2	17	1.1	18.2	0.1
Arabinan	2.6	0.2	2.4	0.1	2.2	0.2	2.3	0.1
Lignin[c]	16.9	0.4	22.6	0.7	18.9	0.2	21.4	0.3
Solubles[d]	26.0	0.6	15.8	0.1	18.1	0.2	15.0	0.2
Ash	5.0	0.06	3.9	0.02	2.5	0.03	2.0	0.01
Closure	96.7	1.9	94.7	0.8	88.4	2.5	92.1	0.6

[a] Standard error of the mean
[b] Includes both glucan and soluble monomeric and oligomeric papers.
[c] Acid insoluble (Klason) lignin
[d] Includes both water and ethanol solubles

As expected, solubles and ash decreased for later harvests, as the grass senesces at the end of the growing season and mobilizes those compounds for storage in the root system. CIR switchgrass also tended to contain more solubles and ash than the Alamo switchgrass. Overall mass balance closure ranged from 88% to 97%. Remaining material may include acid-soluble lignin, galactan and other minor sugars, and insoluble protein. Acid soluble lignin in particular can range from 2-6% of the total composition [67; 68]. AFEX does not

remove any material from the biomass, so this acid soluble lignin may still impact hydrolysis yields.

3.3.2 AFEX Conditions

The reduced linear model for each of the four switchgrass samples is seen in Table 3.2. In all cases, the models gave a reasonable approximation of the results, with R^2 values above 75%. Each model showed different main and interaction effects, indicating that both the harvest date and either the ecotype or location are important in determining pretreatment parameters. Based on these models, conditions that obtained maximum sugar yield for each type of switchgrass are shown in Table 3.2. As the optimal point is often outside the range of conditions tested, the maximum of each condition is used instead. The ranges of conditions studied are considered to be the practical limits of AFEX treatment of forages.

The range of glucose and xylose yields is shown in Figure 3.1. Xylose yields are within the same range for all harvests of switchgrass, with most samples between 40 to 100 g/kg switchgrass depending upon the conditions used. The maximum yields for the October harvests were slightly higher than the July harvests for xylose. The CIR July harvest had the least variability among xylose yields, as most AFEX conditions produced xylose yields between 70 and 100 g/kg switchgrass. The amount of glucose released tended to be higher for CIR switchgrass harvested in July relative to all other harvests, and tended to be

lower for the October CIR harvest. Furthermore, different AFEX conditions did not greatly affect glucose yields for this harvest, with most samples between 120 and 170 g/kg biomass. In comparison, Alamo October harvest had samples between 130-220 g/kg switchgrass. The July harvest of CIR also behaved dramatically different in terms of glucose yields between various AFEX conditions. Both harvests of Alamo switchgrass behaved similarly and showed similar yields. However, since the October harvest of Alamo switchgrass has significantly higher glucan and xylan content than the July harvest, the July harvest has a greater conversion of fiber than the October harvest. Thus, like the CIR switchgrass, the earlier harvest was more digestible, although not to the same extent as the CIR material.

Both xylan and glucan hydrolysis were affected in a similar manner for most AFEX conditions. AFEX conditions that produced high glucose yields also provided strong xylose yields. This correlation was particularly high in the October harvests, with a correlation coefficient of 0.88 and 0.94 for CIR and Alamo harvests, respectively. However, there was more variability in the July harvests. In particular, two different conditions for the Alamo harvest and one for the CIR harvest resulted in high glucose yields, but low xylose yields. Both of these points were at high treatment temperatures, suggesting sugar degradation or competitive reactions as a possible explanation. This may be general xylan degradation or the formation of other inhibitors that specifically reduce hemicellulase activity.

Table 3.2 : Reduced linear model for modeling monomeric sugar release with respect to four AFEX pretreatment parameters

	Model Parameters[a]			
	CIR – July	CIR – Oct	Alamo – July	Alamo - Oct
Constant	489.6	-376.1	-602.6	-177.0
Am[b]	-	88.90	262.4	140.5
Wa[c]	-52.95	110.6	-194.0	34.55
Re[d]	-	9.237	7.282	12.07
Te[e]	-1.572	6.168	9.086	1.624
Am*Am	-27.44	-	-40.13	-17.35
Am*Wa	-	-43.22	-	-16.01
Am*Re	-	-	-	-1.466
Am*Te	0.6324	-	-0.8142	-
Wa*Wa	-	-	-	-
Wa*Re	-	-3.377	-	-
Wa*Te	0.5298	-	1.423	-
Re*Re	-0.0612	-0.1178	-	-0.0796
Re*Te	0.0302	-	-0.0417	-0.0356
Te*Te	-0.0033	-0.0234	-0.0289	-
R^2	80.0%	79.9%	83.6%	94.1%

[a] Model predicts total monomeric sugars (glucose + xylose) in g/kg biomass. Terms with a p value less than 0.05 were dropped and the model reanalyzed.
[b] Ammonia loading measured in g/g biomass
[c] Water loading measured in g/g biomass
[d] Residence time measured in minutes
[e] Temperature measured in C

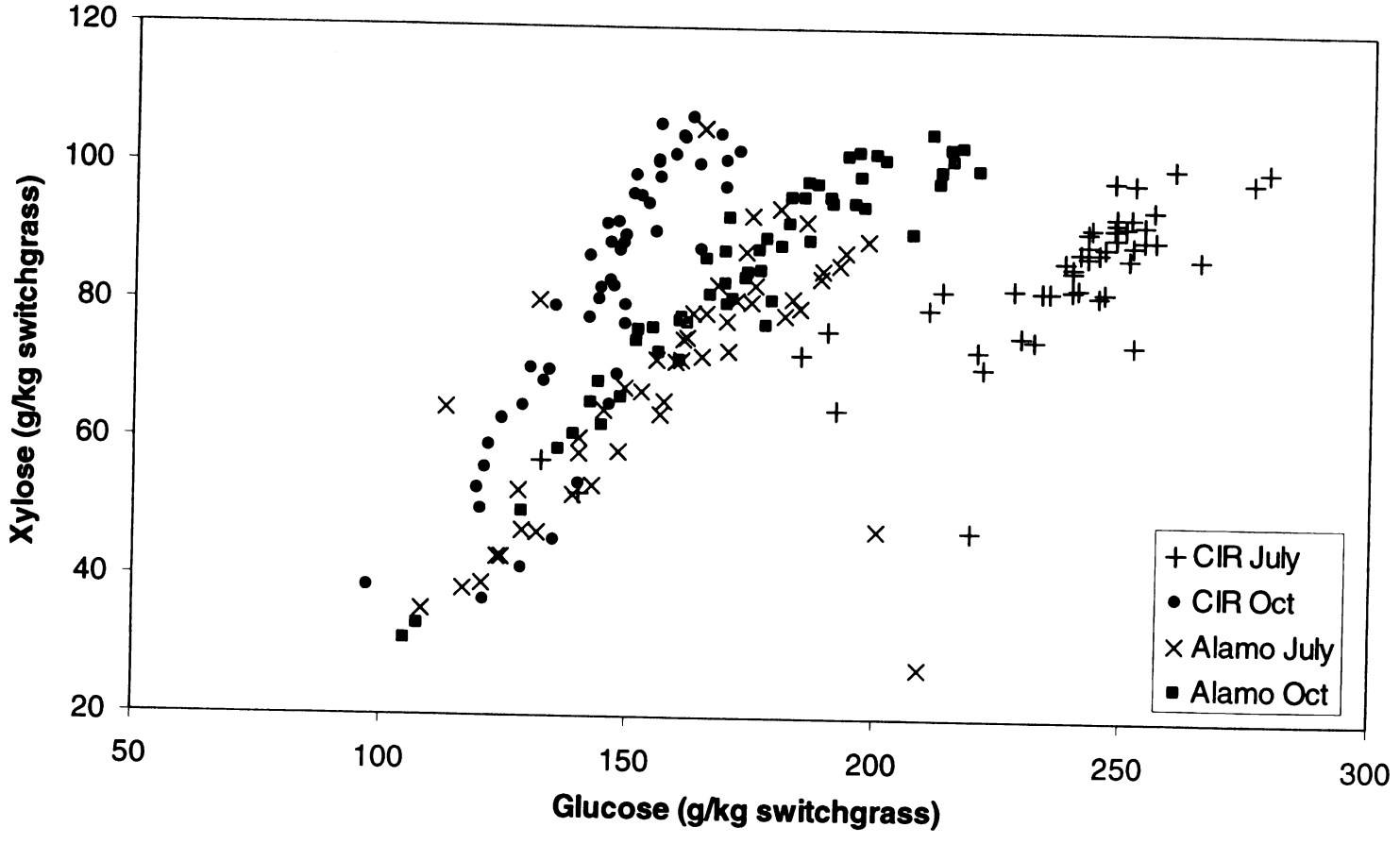

Figure 3.1 : Glucose and xylose yields at varying pretreatment conditions. Each individual data point represents yields after 72 hours of enzymatic hydrolysis for a specific AFEX pretreatment. Pretreatment conditions were varied as stated in the text. For all data points, hydrolysis was performed at 3% solid loading, 50°C, 200 rpm rotation, and 5 mg Accelerase/g dry biomass.

In general, the October harvests required more severe conditions (higher temperature, more ammonia, and/or longer residence times) than the July harvests, as seen in Table 3.3. In particular, the maximum ammonia loading required for both Cave-in-Rock and Alamo switchgrass was higher for October harvests than July. For CIR switchgrass, the differences are also pronounced in terms of temperature and residence time, with the later harvest requiring a higher temperature and longer residence time. Both harvests of Alamo switchgrass had similar pretreatment conditions other than ammonia loading. This further suggests that biomass maturity, which is a function of both harvest date and location, provides the greatest impact on ethanol production.

Table 3.3 : Optimal AFEX conditions within the parameters tested in this study

	CIR – July	CIR – Oct	Alamo – July	Alamo – Oct
Ammonia (g/g BM)	0.9	2.0	1.6	2.0
Water (g/g BM)	0.4	0.4	2.0	2.0
Res. Time (min)	20	30	30	25
Temperature (°C)	80	130	160	150

The CIR July harvest was the switchgrass sample most responsive to pretreatment and hydrolysis in this study, producing over 350 g sugar / kg biomass at optimal conditions. Here, increasing temperature had an adverse effect on sugar yields, although high ammonia, water, and residence times reduced this negative effect. The high temperatures may be degrading soluble sugars and creating inhibitory products, which are offset by improved digestibility at more severe conditions. Ammonia requirements were moderate, with yields peaking at 0.9 g ammonia / g biomass. There was also minimal water requirement. However, more water was needed at temperatures greater than

100°C. At low temperatures, residence time does not play a major role in total sugars released, but increased residence time greatly improves sugar yields at high temperature.

For CIR switchgrass harvested in October, few interaction terms were present, with only water and ammonia as well as water and residence time having interactive effects. In both cases, the interaction reduced total sugar yields. Due to the strong positive effect of residence time on sugar yield, increased ammonia and residence time are offset with decreased water content. High ammonia loading and residence time are desired compared to a low water content. There is no interaction term with temperature. However, optimal sugar yields are obtained at 130°C, a relatively low temperature for recalcitrant biomass.

The July harvest of Alamo switchgrass, like CIR, was more digestible than the October harvest, with total sugars released approaching 300 g/kg biomass. Here, temperature plays a dominant role, with interaction effects with ammonia, water, and residence time. A negative interaction effect is seen for both ammonia and residence time interacting with temperature. However, the coefficients for these negative interactions are relatively small, and so a temperature of 160°C achieves the highest yields. At this temperature, residence time does not make a large impact on yields, although the highest residence time is desired. For both water and ammonia content, the maximum amount gives the greatest yields at T=160°C.

The highest correlation was seen with the Alamo switchgrass harvested in October, as over 90% of the data can be explained by the model. Here, interaction effects are present for both ammonia/water, ammonia/residence time, and residence time/temperature. Increasing water tends to increase sugar yields, but this effect is very small at high ammonia loadings. Likewise, increasing residence time has a smaller effect at high ammonia loadings and residence times. At high temperatures and ammonia loadings, a moderate residence time provides the optimal conditions.

3.3.3 Enzyme Conditions

The response of different enzyme loadings is seen in Table 3.4. The model fit the data well for three harvests, although the correlation coefficient for the October Alamo harvest was fairly low (65%). In general, higher enzyme loadings led to greater sugar production, as expected. However, due to the high costs of enzymes, high enzyme loadings are unlikely to provide maximum economic benefit. As the actual cost of enzymes is unknown, the economic optimal enzyme loading is currently unknown and will likely change with future research into enzyme combinations and production. Instead, the maximum sugar yields produced using at most 15 mg enzyme / kg biomass was used to determine optimal enzyme loadings. The relative amounts of each enzyme that provide these maximum sugar yields are listed in Table 3.5.

Table 3.4 : Reduced linear model for modeling monomeric sugar release with respect to enzyme addition

	Model Parameters[a]			
	CIR – July	CIR – Oct	Alamo – July	Alamo - Oct
Constant	392.69	315.60	344.48	450.49
Ac[b]	3.986	2.606	3.727	-0.634
No[c]	6.299	-3.721	-2.990	6.809
Xy[d]	13.108	20.302	5.013	27.018
Pe[e]	15.435	10.184	4.154	11.199
Ac*Ac	-	-	-	-
Ac*No	-	-	-	-
Ac*Xy	-0.706	-	-	-1.458
Ac*Pe	-	-	-	-
No*No	-	-	-	-
No*Xy	-	1.350	1.414	-
No*Pe	-0.697	-	-	-1.369
Xy*Xy	-	-2.446	-	-
Xy*Pe	-	-	-	-2.221
Pe*Pe	-0.599	-0.561	-	-
R^2	81.34%	76.47%	81.50%	64.87%

[a] Model predicts total monomeric sugars (glucose + xylose) measured in g/kg dry switchgrass. Terms with a p value less than 0.05 were dropped and the model reanalyzed.
[b] Accelerase 1000 loading, measured in mg/g switchgrass
[c] Novozyme 188, measured in mg/g switchgrass
[d] Multifect Xylanase, measured in mg/g switchgrass
[e] Multifect Pectinase, measured in mg/g switchgrass

Despite different conditions providing varying amounts of cellulase and hemicellulase, there was also a reasonably strong correlation between glucose and xylose yields for all harvests studied, as seen in Figure 3.2. Correlation coefficients ranged from 0.65 for the October harvest of Alamo switchgrass to 0.76 for the July harvest of CIR switchgrass. This suggests a degree of synergy between glucan and xylan hydrolysis regardless of the enzyme used. Given how closely glucan and xylan polymers are intertwined with each other in the cell wall, this is not an unexpected result. Furthermore, each of the enzyme complexes

contain activities on several different compounds [69], and so the cellulase mixtures also contain hemicellulase activity and vice versa. Increased breakdown of cellulose in cellulase-rich enzyme conditions may be increasing accessibility to the xylan, and vice versa in hemicellulase-rich enzyme conditions, thus explaining the strong correlation.

Table 3.5 : Optimal enzyme loadings for enzymatic hydrolysis of four harvests of switchgrass

	CIR – July	CIR – Oct	Alamo – July	Alamo - Oct
Accelerase[a]	5.0	6.4	5.0	5.0
Novozyme[a]	0	0	0	0
M Xylanase[a]	5.0	3.6	5.0	5.0
M Pectinase[a]	5.0	5.0	5.0	5.0
Pred. Sugars[b]	523	411	409	557
Act. Sugars[c]	521	410	410	445

[a]All values are in mg enzyme per g dry switchgrass
[b]Monomeric sugars released (g/kg switchgrass) after 72h of hydrolysis as predicted by the model in Table 4.
[c]Actual monomeric sugars released (g/kg switchgrass) after 72h of hydrolysis.

The harvest time and cultivar/location also affected the ratio of glucose and xylose released. The CIR switchgrass harvested in July produced significantly more glucose than the other three harvests, yet released similar amounts of xylose. In contrast, the Alamo October switchgrass produced substantially more xylose than either the CIR October or Alamo July switchgrass, despite a similar range of glucose released.

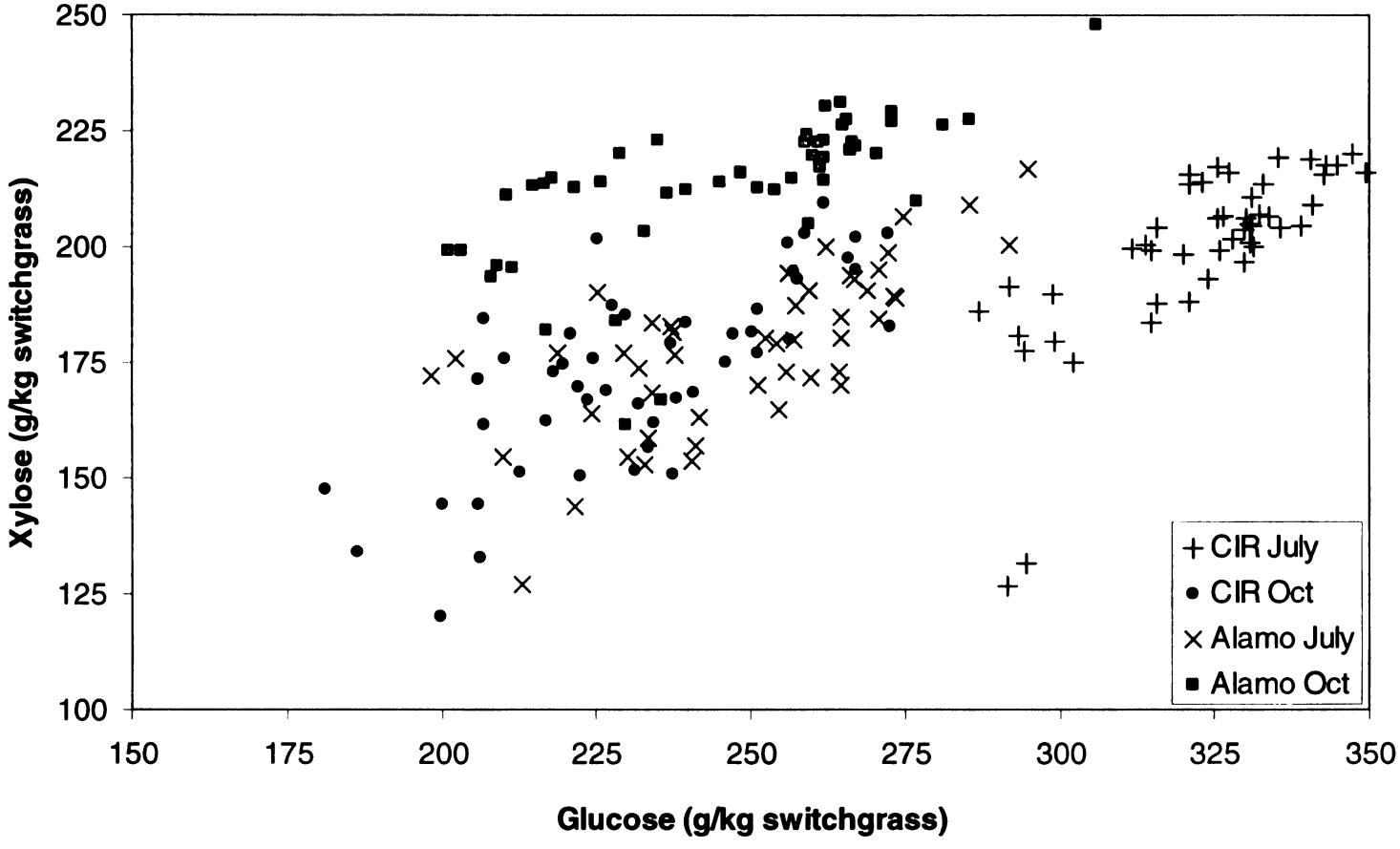

Figure 3.2: Glucose and xylose yields at varying enzyme loadings. All data points within the same harvest were performed at the same AFEX pretreatment conditions (listed in Table 3), and each data point represents a separate combination of enzymes. Hydrolysis was performed at 3% solid loading, 50°C, and 200 rpm rotation, with samples collected after 72 hours.

Sugar yields exceeding 500g/kg biomass were seen at relatively low (<20 g/kg biomass) enzyme dosage for the Alamo October and CIR July harvests, suggesting strong potential as cellulosic feedstocks. Some synergistic effects appear to be occurring, as glucose yields increase significantly in the presence of pectinase and xylanase when Accelerase is held constant (data not shown). Thus, while Accelerase is only a third of the total enzyme loading, both glucose and xylose are being effectively released. While yields increased for the CIR October harvest compared to no additional enzymes, the overall conversion remains low. The Alamo July harvest was also lower than expected. During AFEX pretreatment for this material, the temperature rapidly rose to the desired set point, yet decreased to 90-110°C throughout the reaction in all runs. This large drop in temperature was not seen in the other switchgrass harvests, and thus the low conversions seen here is likely due to the non-optimal AFEX conditions.

Harvest date and cultivar/location do not appear to affect the composition of the enzyme complex required to break down the biomass, despite the different compositions of the biomass. With the exception of the October harvest of CIR switchgrass, all switchgrass harvests obtained optimal yields using an equal blend of Accelerase, Multifect Pectinase, and Multifect Xylanase. For the CIR October harvest, slightly more pectinase is needed than xylanase. Strong responses to the hemicellulases suggest that the AFEX pretreatment is not completely separating the cellulose from the surrounding cell wall material in any

substrate. Although small amounts of cellobiose were seen in hydrolysates without Novozyme 188, its presence did not have a positive impact on sugar yields. Multifect Pectinase also contains strong β-glucosidase activity [69], likely eliminating the need for Novozyme 188. Increasing Accelerase beyond 5g/kg produced only modest increases in sugar yields, which are unlikely to be economically competitive.

3.3.4 Rate of Hydrolysis

All harvests of switchgrass showed a rapid response to enzyme addition, as seen in Figure 3.3. As expected, both glucose and xylose released during hydrolysis rose rapidly within the first 24 hours, with a slow release afterwards. Interestingly, xylose was released faster than glucose for all samples except the July harvest of CIR switchgrass. The initial rate (defined as sugar release within the first 3 hours) was between 35-45 g/kg/h for xylose compared to 25-30 g/kg/h for glucose. In addition, glucose released after 168 hours was 25% higher than 24 hours for all harvests except for the CIR July harvest (which was 16% higher). Xylose at 168 hours compared to 24 hours was only 8-14% higher for all harvests. The xylan appears to be readily accessible to enzymes after AFEX pretreatment relative to cellulose and responds rapidly to enzymatic attack with high xylanase addition.

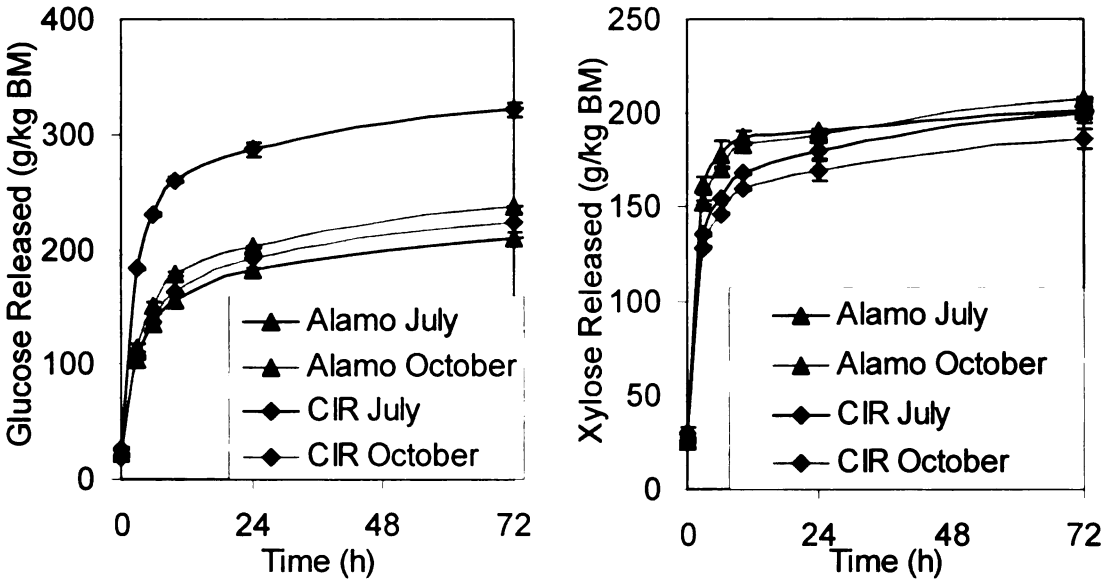

Figure 3.3 : Rate of hydrolysis. Glucose (left) and xylose (right) released during enzymatic hydrolysis between 3 and 168 hours of residence time. Pretreatments were performed at the conditions listed in Table 3.3 and enzyme addition as listed in Table 3.5. Hydrolysis was performed at 3% solid loading, 50°C, and 200rpm rotation. Results are the average of duplicate samples with error bars representing the high and low values.

With the exception of the CIR July harvest, the trends for glucose released were similar in all harvests tested. These three harvests saw a similar initial rate, with the primary differences appearing between 3-24 hours of hydrolysis. In comparison, the CIR July harvest showed a very rapid initial release of glucose, with nearly all glucose released within 24 hours. It is clear that AFEX pretreatment is very effective in opening up cellulose to enzymatic attack in this harvest relative to the other three harvests, likely due to its being the most immature sample. Further research is needed to determine specifically what factors influence this immediate glucose release.

The harvest location or type appears to have a greater effect on xylose released than harvest date. The Alamo harvests showed a significantly higher initial rate (42-45 g/kg/h) of xylose release than the CIR harvests (34-35 g/kg/h). In addition, a greater proportion of the total xylose release was seen within the first 24 hours for the Alamo harvests compared to CIR harvests.

3.4 Discussion

As expected, substantial differences are present between both the harvest date and either the location or ecotype of switchgrass. These differences are seen in the response to both pretreatment and hydrolysis conditions. In general, early harvest switchgrass requires milder pretreatment conditions and lower enzyme requirements. The CIR switchgrass has greater variability in total sugars released between the two harvests than the Alamo switchgrass. Optimization of both pretreatment and enzyme conditions greatly improved both glucose and xylose yields. All harvests showed a rapid initial response to enzymatic hydrolysis and, with the exception of the CIR July harvest, acceptable fermentation of both glucose and xylose.

AFEX pretreatment is a complex process involving several physical and chemical interactions with the biomass. Thus, it is not surprising that the different compositions of each type of switchgrass react differently to changing AFEX conditions. In general, a balance is needed between disrupting the lignocellulosic structure and reducing or eliminating competing reactions that

form degradation products or inhibitory compounds. Early harvest materials have more soluble sugars, which are more likely to degrade then polymeric compounds. Furthermore, an important reason for the effectiveness of AFEX appears to be that large amounts of lignin and hemicellulose oligomers are dissolved and brought to the surface, thereby creating a network of pores in the treated biomass (unpublished data). The July harvest of CIR switchgrass contained the least hemicellulose and lignin, and requiring fewer reactions to solubilize and remove this material.

In particular, high temperatures are generally not desired due to the increase in competing reactions. In all four harvests, the highest temperatures did not give the highest yields, suggesting that higher temperatures do not provide additional access to cell wall polymers. High temperatures primarily affect xylose yields, particularly for the October harvest of Cave-in-Rock switchgrass. Above 160°C, xylose yields drop from between 65-105 g/kg biomass to 35-65 g/kg biomass, depending on the other conditions.

The different responses to harvest date and location/ecotype have large implications for biomass refining. Harvesting early in the season provides lower costs for pretreatment and higher potential ethanol yields, which may help offset the costs of a second harvest. With the potential additional revenue from co-products such as proteins, the benefit for early harvests appears strong. However, any pretreatment facility will likely be designed to satisfy both harvests,

and so reductions in capital costs may not actually occur. In addition, if yields from the stand decrease over time due to multiple harvests [62], a multiple harvest scenario may not be an economically viable for agronomic reasons.

Also of concern is the fact that different harvest locations or different types vary in response to pretreatment and hydrolysis. The Cave-in-Rock switchgrass, grown in Michigan, begins growing later in the season and senesces earlier than the Alamo switchgrass grown in Alabama. As such, while the harvest dates were similar, the relative maturities of the two varieties were different. As the CIR switchgrass was more digestible in July and less digestible in October than the Alamo material, the different relative maturities appear to have more effect on digestibility than the types of cultivars. It remains to be seen if different cultivars harvested in the same region and same season react differently to pretreatment and hydrolysis parameters. Thus, harvest practices in the northern latitudes may be adjusted to avoid the highly recalcitrant late harvest material.

3.5 Conclusions

Fermentable sugars are effectively released from switchgrass using AFEX pretreatment and enzymatic hydrolysis. Relatively mild pretreatment conditions result in high ethanol yields for early harvest material, while more severe conditions are necessary for later harvests. A mixture of enzymes, including xylanase and pectinase, are required to release the greatest amounts of sugar. Xylan was digested faster than glucan for all types of biomass. The upland

Cave-in-Rock switchgrass from Michigan harvested in July showed the greatest response to pretreatment and hydrolysis, while harvesting in October showed the least response. All hydrolysates were fermentable, although the July CIR harvested material showed poor xylose utilization.

Harvest date and location/ecotype have a substantial impact on AFEX pretreatment conditions and sugar released, although this is not a major factor in enzyme requirements. Early harvest switchgrass was generally more digestible and required less severe AFEX conditions than the later harvests. For the northern CIR switchgrass used in this study, large differences were seen in pretreatment conditions and response between the two harvests, while this difference was muted in the southern Alamo switchgrass. The known effect of maturity suggests that the harvest location is the dominant response, as the northern climate has a shorter growing season than the southern climate. Further research is required to separate the effects of harvest location and ecotype.

These findings have major implications for the possibilities of co-producing protein from early harvest grasses. It is clear that pretreatment operations must be flexible to both the needs of early and late harvest material. This requires flexibility in sizing the equipment, electricity and other operating costs, and ammonia requirements. The impacts of pretreatment conditions on the economics of the process are discussed in further detail in Chapter 7.

CHAPTER 4 : PROTEIN EXTRACTION

4.1 Introduction

As described in Chapter 2, there are two primary methods to extract protein from leafy materials: an aqueous extraction using an alkaline solution and mechanical pressing of wet material. Both options are considered here, although little research could be performed on the mechanical pressing option. There were two primary limitations to mechanical pressing research. Since it requires fresh grass, it could only be performed at the proper time in the growing season. More importantly, a proper mill and screw press was considered impractical for laboratory scale studies, limiting the control over the rate and input of the material. Thus, most of the research was conducted on alkaline extraction.

The interaction of AFEX pretreatment and subsequent hydrolysis with protein extraction is of particular interest. Due to the presence of ammonia in AFEX pretreatment, it was chosen as the alkaline medium for protein extraction. If a bleed stream is required in the ammonia recycle process, adding this bleed stream to a post-AFEX extraction would be an efficient use of materials. If the extraction is prior to AFEX, then any residual ammonia remaining on the biomass would offset the amount required during AFEX pretreatment. The effect of extraction on sugar yields is also important.

The goals of this chapter are as follows:

- Optimize alkaline extraction on dry switchgrass using aqueous ammonia

- Investigate options for concentrating the protein following extraction
- Integrate AFEX pretreatment and subsequent hydrolysis with protein extraction and determine its impacts
- Determine key gaps in commercializing alkaline extraction and concentration relative to traditional methods

4.2 Materials and Methods

4.2.1 Feedstock

The feedstock used in this experiment was Alamo switchgrass obtained from Auburn University and harvested on May 22, 2005, unless otherwise specified. The moisture content of the material was approximately 9%. All other grasses used in this study were obtained from Michigan State University farms and dried at 50°C prior to use. All material was ground to less than 2 mm prior to experiments. Composition of the biomass was determined as described in Chapter 3.

4.2.2 Pretreatment

The AFEX pretreatment was performed in a 300 mL stainless steel pressure vessel. Water was mixed with the switchgrass to increase the moisture content to 80% dry weight basis. Glass spheres were added to minimize void space, thereby reducing the amount of ammonia in the gaseous state. The lid was bolted shut, and a sample cylinder loaded with 1 (+/-0.04) g NH_3 per g dry biomass, allowing the ammonia to be charged into the vessel. The reactor was

heated using a 400W Parr heating mantle, and allowed to stand at 100°C (+/- 1°C) for five minutes. The pressure was explosively released by rapidly turning the exhaust valve. The treated samples were removed and were placed in a fume hood overnight to remove residual ammonia.

4.2.3 Hydrolysis

The enzymatic hydrolysis procedure used is described in Chapter 3. The enzyme loading used for this chapter is 15 FPU Spezyme CP per g glucan and 64 pNPGU Novozyme 188 per g glucan. A solid loading of 10% was used for all experiments in this chapter.

4.2.4 Protein Extractions

Screening for optimal protein extraction conditions was done using a Dionex (Sunnyvale, CA) ASE 200 Accelerated Solvent Extractor. Extractions were performed at 1500 psi, which reduces the required residence time from 30 to 3 minutes. Extractions were done using 11:1 (w/w) liquid/solid ratio and two separate extractions per sample. For experiments involving varying the pH, hydrochloric acid was used to reduce the pH. The pH of the solution was measured after the extraction was complete. Once the optimal extraction conditions were obtained, all further extractions were performed in flasks for 30 minutes with a 10:1 liquid/solid ratio while continuously stirred. After the extraction was complete, the solids and liquids were separated using filtration.

Solid cakes were washed with distilled water at approximately 10:1 liquid/solid ratio to insure that all soluble material is removed.

Due to the presence of ammonia nitrogen, both during the AFEX pretreatment and subsequent extractions, it is impossible to use standard nitrogen analysis methods (the Kjehldahl or Dumas methods) to measure total protein content. Instead, protein concentration was measured using a Pierce (Rockford, IL) bicinchoninic acid colorimetric assay kit using bovine serum albumin (BSA) as a standard. To reduce the effects of interfering agents such as ammonium salts, lignin components, and glucose, the proteins were first precipitated and resolubilized [70]. A 100 μL 0.15% sodium deoxycholate was added to 100 μL protein solution and allowed to sit for 15 minutes. 200 μL of 15% trichloroacetic acid solution was added, and allowed to sit at 2°C overnight. The mixture was centrifuged at 13000 RPM for 10 minutes, and the resulting pellet washed with acetone. The pellet was resolubilized in a buffer solution containing 0.1M Tris, 2.5M urea, and 4% sodium dodecyl sulfate (SDS). Known concentrations of protein extracts were used to calibrate the protein recovery of this method.

In order to obtain more accurate numbers for the mass balance shown in Figure 4.6, a full amino acid analysis of the extracts and solid residues was undertaken. Samples were hydrolyzed in concentrated acid to their component amino acids and measured using HPLC analysis. All derivitization and HPLC analysis was

performed at the Macromolecular Structure Facility in the Department of Biochemistry at Michigan State University.

In the experiments involving multiple feedstocks and precipitation studies, 0.1M sodium hydroxide was used as the extraction medium on untreated samples. Because no ammonia was used for treatment, nitrogen analysis was used to analyze the protein removed. A Skalar Primacs SN Total Nitrogen Analyzer was used to obtain the nitrogen concentration in all samples as described in Chapter 5.

4.2.5 Protein concentration

Ultrafiltration was performed using a Sartorius Sartoflow 200 crossflow ultrafiltration system equipped with a Tandem 1081 peristaltic pump. This system allows can create a constant crossflow rate or a constant transmembrane pressure (TMP) by varying the speed of the pump. Additional control could be created by restricting the flow between the pump and the filter. Transmembrane pressure was measured as the difference between atmospheric pressure (the permeate) and the average pressure of the inlet and retentate. The permeate flux was determined gravimetrically as an average over 10 minutes. A 50 cm^2, 10 kDa molecular weight cutoff (MWCO), polyethersulfone Pellicon Biomax 10 cartridge was used as the filter for all experiments. The concentration factor was calculated using the following equation:

$$CF = \frac{M_I}{M_I - M_P} \tag{4.1}$$

where M_I is the mass of the initial extract and M_P is the mass of the permeate.

Precipitation was performed in 50 mL centrifuge tubes using either 6 M H_2SO_4 or 100% ethanol. Acid was added dropwise until the desired pH was obtained with a tolerance of pH 0.1. Ethanol was added until the final concentration of ethanol was 70%. After precipitation, samples were centrifuged at 4400 RPM for 10 minutes. The pellet was washed in acetone and recentrifuged before being allowed to dry. Protein determination was performed using total nitrogen analysis on the solids while polyphenolic concentration was determined using the remaining liquid.

4.2.6 Polyphenolics Concentration

Total polyphenolics was measured using the modified Prussian blue method [71]. For each sample, 100 µL 0.016 M potassium ferricyanide and 100 µL 0.02 M iron chloride was added to 10 µL extract and 100 µL distilled water and immediately vortexed. After 15 minutes, 500 µL solution of 17% phosphoric acid and 0.2% gum arabic is added to stabilize the color. The absorbance was read at 700 nm. Gallic acid was used as the standard. Because of the potential variety of polyphenolics present in solutions and the uncertain composition of these compounds, a precise measurement of polyphenolics is not possible. Thus, all values are measured and reported as µM of gallic acid equivalent.

4.3 Results

4.3.1 Composition Analysis

The composition of the switchgrass used in this study is shown in Table 4.1. Not included in this composition is minor sugars and acid soluble lignin. The amount of protein present was lower than reported in literature for other strains of switchgrass [17]. Switchgrass grown as a biomass energy crop and harvested early in the growing season would likely have protein contents near 10%, and thus might be more suitable for integrated protein and sugar processing. The amount of fiber present is lower than switchgrass harvested at a later date (see Chapter 3), which seems to suggest lower sugar yields would also result from using an earlier cut. However, early cut switchgrass is less recalcitrant than that harvested in the fall as shown in Chapter 3, and thus the lower cellulose and hemicellulose content may not be a significant factor. The low amount of lignin is a promising sign, as this implies less interference with hydrolysis as well as fewer harmful degradation products that could inhibit sugar production or otherwise be present in the protein product.

Table 4.1 : Composition of switchgrass used in this study. All values given as g/100g dry matter.

Component	% Value
Glucan	29.8
Soluble glucan	3.4
Xylan	16.4
Arabinan	3.5
Protein	7.3
Al Lignin[a]	10.8
Lipids	7.3
Water solubles	21.5
Ash	4.8
Total[b]	101.4

[a] Al – Acid insoluble (klason) lignin
[b] A portion of the ash and protein may be double-counted in the water solubles. Acid soluble lignin and minor sugars such as galactan are not counted.

4.3.2 Extraction optimization

Figure 4.1 shows the effect of the temperature of the extraction on the overall protein and mass yields. Protein yields increased significantly from 25°C to 40°C, but further increases in temperature did not result in major improvements in protein yield. It is likely that most if not all of the proteins present in the switchgrass are in their natural state, and so the mild temperatures should not unfold the proteins or significantly affect their solubility.

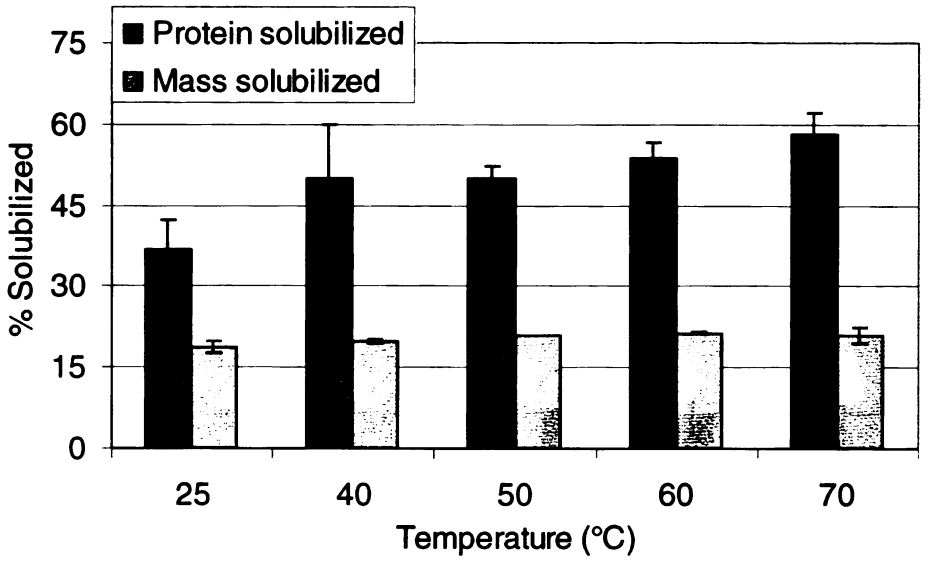

Figure 4.1 : Effect of extraction temperature on protein yields. All extractions were performed in duplicate with 3% ammonia at pH = 10.5. The results are combined after two separate extractions using 11:1 liquid/solid ratio and 3 minute residence time under 1500 psi pressure.

The effect of ammonia concentration on extraction yields is seen in Figure 4.2. It is clear that the presence of hydroxide is necessary to achieve strong yields, as the yield without ammonia was approximately 65% of those that were performed in ammonia. Protein yield remains constant from 1-3% NH_4+, but then begins to drop off. This is most likely due to "salting out" the protein, as the increase in salt concentration decreases the amount of water available to solubilize the protein. It is also possible that greater hydrolysis of the peptide chains is occurring at higher salt concentrations, as these smaller peptide chains would then be lost during the TCA precipitation. However, lower salt concentrations are still more desirable, due to both the lower cost of solvent as well as the relative ease of concentration larger protein fragments compared to small peptide chains. There does not appear to be any salting in effect, likely because 1% salt solution is

already a sufficient concentration to solubilize the protein. The total mass solubilized was unaffected by salt concentration, as expected.

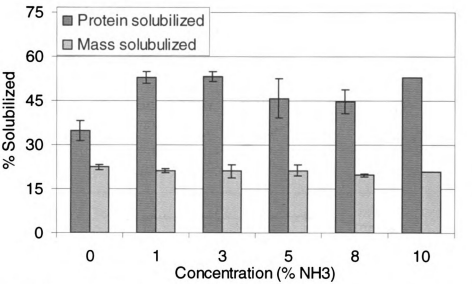

Figure 4.2 : Effect of ammonia concentration on protein yields. All extractions were performed in duplicate at 50°C and at pH = 10.5 except for 0% concentration, which was performed using distilled water at 50°C. The results are combined after two separate extractions using 11:1 liquid/solid ratio and 3 minute residence time under 1500 psi pressure.

The most significant factor in determining protein yields is the pH of the system, as seen in Figure 4.3. The amount of protein extracted increased dramatically from a pH of 8 to 10.5 before leveling off. Similar trends have been seen in other types of biomass [45; 50; 52]. Most proteins have an acidic isoelectric point, the pH at which the protein will have no net charge and therefore be the least soluble in a polar medium. Thus, increasing the pH should increase protein solubility, as demonstrated here. The most alkaline solution also produced a significant drop in the total mass solubilized, a potentially useful characteristic. If there is less biomass in solution, it should be easier to purify the proteins. In addition, the

biomass lost during extraction likely includes hemicellulose that could be hydrolyzed into sugars for ethanol production. Further increases in pH would require a stronger base than ammonia and might degrade the protein, and thus were not considered.

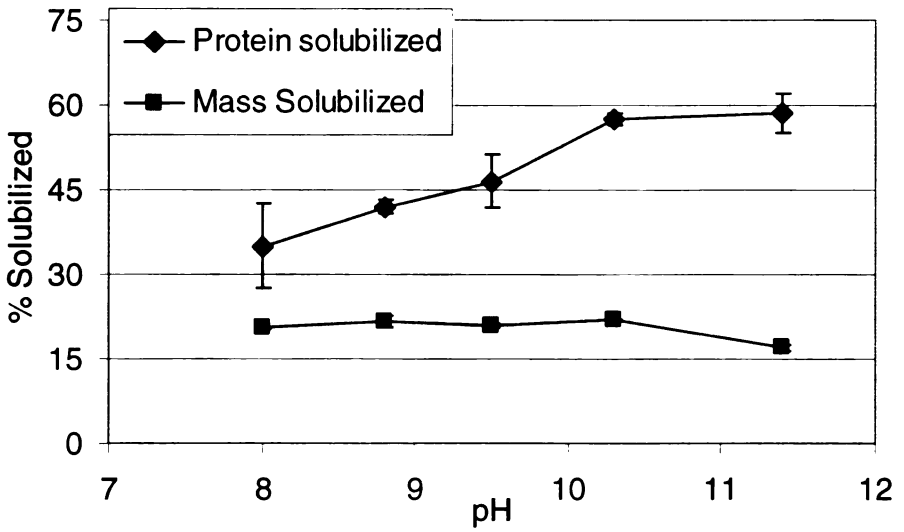

Figure 4.3 : Effect of extraction pH on protein yields. All extractions were performed in duplicate with 3% ammonia and at 50°C. The results were combined after two separate extractions using 11:1 liquid/solid ratio and 3 minute residence time under 1500 psi pressure.

As seen in Figure 4.4, attempts were made to improve yields by the addition of the nonionic surfactant Tween 80, the ionic surfactant SDS, and β-mercaptoethanol, a reducing agent. No significant improvements were found by the addition of either surfactant or reducing agent for the untreated switchgrass. However, adding β-mercaptoethanol and Tween 80 to AFEX treated grass did increase protein removal. This would seem to suggest that the AFEX process affects the proteins in some manner. This effect might be through the creation of sulfur-sulfur bonds, which would then be cleaved by β-mercaptoethanol, or by

proteins unfolding and exposing hydrophobic sites, which can be resolubilized with surfactants. The total mass solubilized also increased with the addition of surfactants, most likely due to interactions between the surfactants and hydrophobic portions of the biomass.

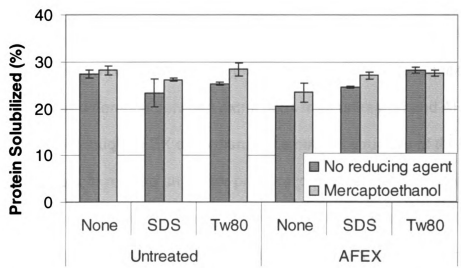

Figure 4.4 : Effect of reducing agents on protein yields for untreated and AFEX treated samples. All extractions were performed in duplicate with 3% ammonia, 25°C, and at pH = 10.5. The results are combined after two separate extractions using 11:1 liquid/solid ratio and 30 minute residence time at atmospheric pressure. Both the ionic sodium dodecyl sulfate (SDS) and the nonionic Tween 80 (Tw80) surfactants were tested, both with and without the addition of β-mercaptoethanol.

Thus, optimal extraction conditions for switchgrass are approximately 3% aqueous ammonia at a pH of 10 and temperature of 40-50°C. These conditions are in line with those seen for protein extraction of other types of biomass, and are the conditions used for all subsequent experiments reported here. Total protein yields are approximately 40%.

4.3.3 Integration

To determine whether AFEX pretreatment affects the types of proteins recovered, the composition of the individual amino acids was determined, as seen in Figure 4.5. Both the untreated and AFEX treated samples were extracted at the optimal ammonia conditions without adding surfactant or reducing agent. Although the amino acid profile for the proteins solubilized during extraction compared to the total protein from switchgrass is quite different, there is very little difference between extractions from untreated and AFEX treated grass. Although AFEX does disrupt the cellular structure of the biomass, it does not appear to release any other proteins to be available for extraction. Therefore, it appears that the primary hindrance to protein extraction does not appear to be the disruption of cell walls, but rather the solubility of the proteins themselves.

Thus, it appears that AFEX has little impact on the amount of protein removed from the biomass. However, there may be an impact on both subsequent processes such as enzymatic hydrolysis and fermentation as well as the quality of the protein. AFEX acts to remove lignin to the surface of the biomass, and is also partially broken down into smaller molecular weights. As lignin is partially soluble in alkaline media, some of these polyphenolic lignin compounds may also be removed during protein extraction. In addition, these compounds may be bound to the protein, potentially rendering them indigestible. A Prussian blue analysis of the extract revealed that polyphenolic compounds are twice as high in

the AFEX extract as in untreated switchgrass extract, confirming the potential problems of this AFEX treatment prior to extraction.

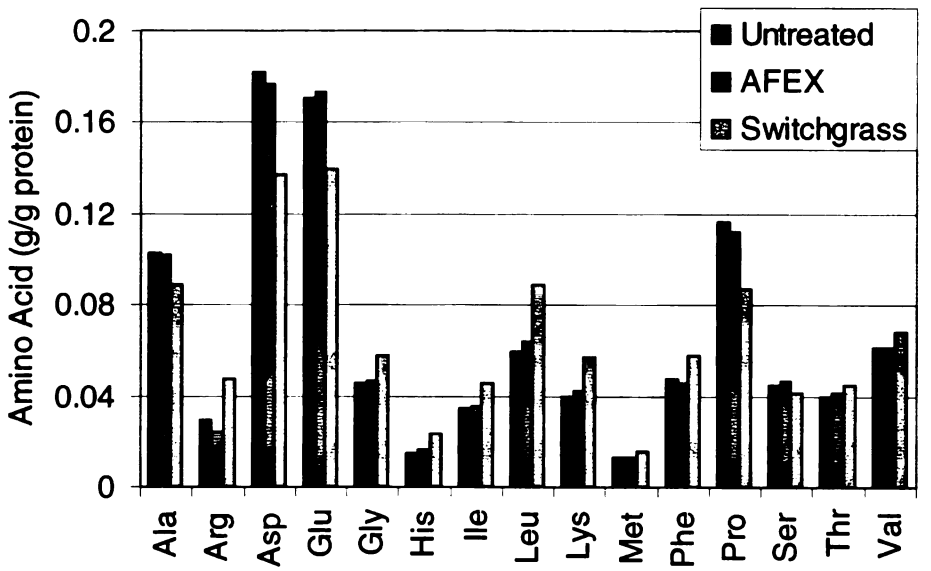

Figure 4.5 : Amino acid profiles for untreated protein extract, AFEX treated protein extract, and the native switchgrass protein. Asx and Glx are a combination of aspartic acid and asparagine and glutamic acid and glutamine, respectively. Cysteine and tryptophan were not detected due to their instability during acid hydrolysis.

Several potential scenarios for integrated sugar and protein recovery were studied, with the overall sugar released presented in Table 3.2. Extracting protein prior to AFEX decreased sugar yields significantly, likely due in part to removing soluble sugars and oligomeric hemicellulose from the biomass. Removing these compounds may also impact the effectiveness of pretreatment and may require different pretreatment conditions. However, this was not studied. An initial extract may also be changing the amount of type of inhibitors produced during AFEX. If a second extraction is performed after AFEX and prior to hydrolysis, an additional 40 g/kg glucose is produced during hydrolysis,

although the xylose yield remains low. After the proteins are concentrated, the remaining liquid can be used as a hydrolysate media, thus recovering the soluble sugars. In a separate experiment, an additional 20 g/kg glucose is seen in the hydrolysate when the protein extract prior to AFEX is used as the hydrolysate medium. Extracting protein after AFEX only showed a slight decrease in hydrolysis yields. Again, this decrease is likely due to removing soluble carbohydrates, although this is partially offset by removing hydrolysis inhibitors.

Table 4.2 : Amount of monomeric glucose, monomeric xylose, and protein solubilized during each operation in potential processes

		Amount released in each step (g/kg dry biomass)			
Process[a]	Component	Hydrolysate	Extraction 1	Extraction 2[b]	Total
EAH	Glucose[c]	165 ± 8			165
	Xylose[c]	82.7 ± 6.1			82.7
	Protein	19.4 ± 6.2	22.4 ± 3.7	n/a	41.8
AEH	Glucose	223 ± 8			223
	Xylose	104 ± 3			104
	Protein	22.9 ± 6.5	20.2 ± 5.3	n/a	43.1
AHE	Glucose	254 ± 10			254
	Xylose	113 ± 6			113
	Protein	33.9 ± 4.0	18.9 ± 3.8	n/a	42.8
EAEH	Glucose	208 ± 20			208
	Xylose	79.7 ± 7.8			79.7
	Protein	24.1 ± 0.6	22.4 ± 3.7	10.6 ± 1.2	57.1
EAHE	Glucose	165 ± 8			165
	Xylose	82.8 ± 6.1			82.8
	Protein	19.4 ± 6.2	22.4 ± 3.7	1.4 ± 1.0	43.4

[a] Sequence of operations performed for each process: A – AFEX treatment, E – alkaline extraction, H – enzymatic hydrolysis. For example, Process EAH is protein extraction followed by AFEX treatment and then hydrolysis.
[b] For process EAEH and EAHE, this column represents the protein recovered in the second alkaline extraction operation.
[c] Monomeric glucose and xylose were only measured in the hydrolysate.

The overall mass balance for integrated sugar and protein with extraction prior to hydrolysis is seen in Figure 4.6. Final yields were 240 g glucose, 85.4 g xylose, and 80.7 g protein per kg dry biomass. Sugar recovery was approximately 74% of theoretical values, indicating further improvements in sugar recovery can be made. Approximately 40% of the protein was found in the extract and 60% in the hydrolysate, demonstrating that protein must be recovered from both streams in order to be economical. The insoluble biomass was washed after hydrolysis to insure all soluble components were recovered, and thus this may have acted as a second extraction to remove any remaining proteins bound to insoluble portions of the biomass. Total protein yield is approximately 87% of the total, taking into account both the switchgrass protein and the enzymes used in hydrolysis. However, very little insoluble protein remained in the biomass, thus suggesting that the remaining protein was broken down and lost at some point during the process.

Approximately 40% of the biomass is solubilized during the initial protein extraction step. It may be possible to utilize this soluble fraction of the biomass after the proteins have been removed. The protein might be concentrated and removed through ultrafiltration or heat precipitation, while the remaining solution undergoes further processing.

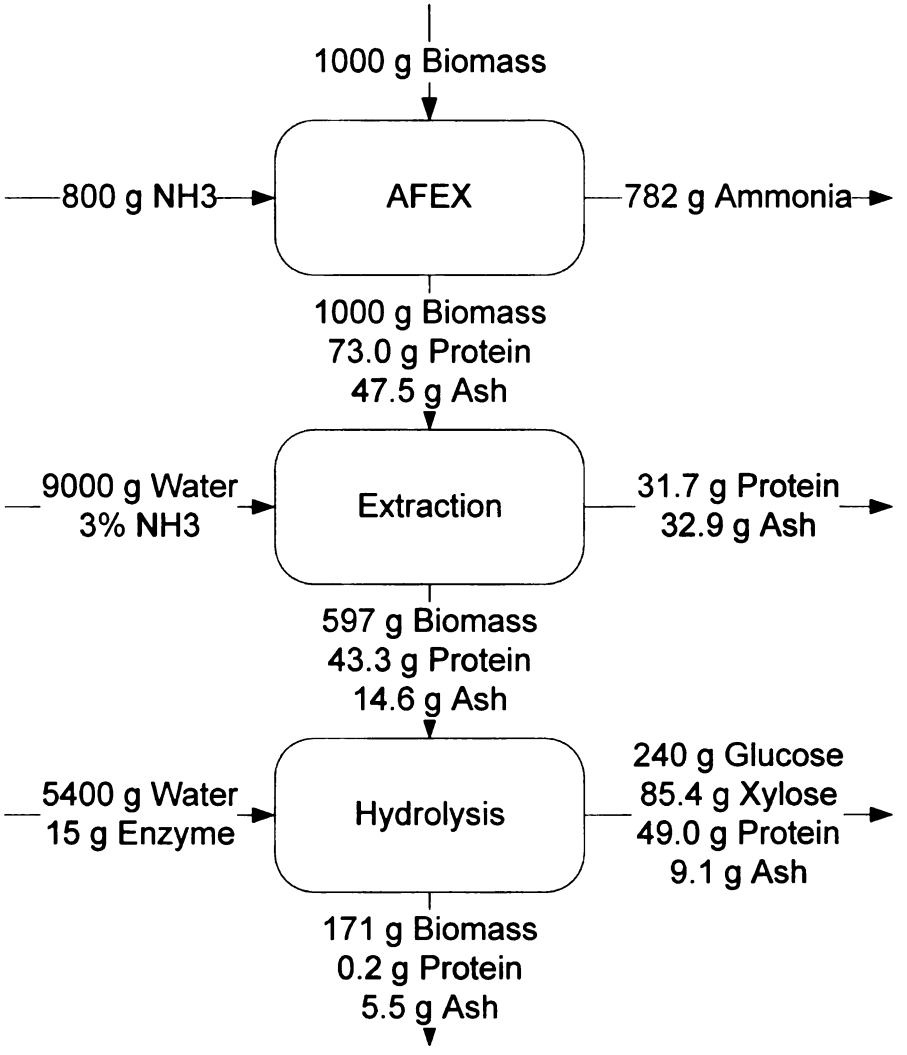

Figure 4.6 : Mass balance for switchgrass extraction. Water is not included in the balance, as the biomass was washed and allowed to dry between each step to insure complete solid/liquid separation. In the hydrolysis stage, the enzyme entering is included as protein exiting.

Most of the ash was removed from the biomass during the first extraction step. It is important to remove this ash, as the final insoluble residue would likely be burned to provide heat and power for the refinery. The ash content in switchgrass, particularly sodium and potassium, has been shown to cause problems with slagging in coal/biomass co-firing power plants [72]. The

remaining biomass contains only 3% ash, and thus should reduce this risk in heat and power generation. It remains to be seen if the ash in the extraction step can be separated and returned to the land. The fact that most of the ash is removed during one unit operation should help keep the costs of any ash processing step low, as only one stream needs to be treated.

Approximately 17% of the biomass remains insoluble throughout this process. There is virtually no protein or ash still present in this residue, which is mostly composed of unhydrolyzed fiber and insoluble lignin. This material would likely be burned for heat and power generation in the refinery, thus reducing natural gas or coal requirements.

The amount of insoluble material remaining is less than that of the previous scenario, indicating that less heat and power can be produced. Although less ash is present, there is still a great deal of protein remaining. Thus, due primarily to the higher protein yields, an extraction prior to hydrolysis is likely to be the best option despite the slightly lower sugar yields.

4.3.4 Ultrafiltration

The first method of protein concentration tested was ultrafiltration. Here, a 10 kDa membrane was used for protein recovery. A minimum transmembrane pressure (TMP) of 15 psi is required to effectively concentrate the protein, as seen in Figure 4.7. Such a pressure differential is not uncommon in ultrafiltration

systems, as a system to recover proteins from cheese whey uses a TMP of 22 psi [73]. The permeate flux was fairly low, however, peaking at 5 g/s/m^2 at a TMP of 30 psi, as seen in Figure 4.8. In comparison, cheese whey UF had a flux of 14 g/s/m^2. A high crossflow rate (50 mL/min) is needed to achieve this value. This likely speaks to significant fouling of the membrane, as a higher crossflow rate tends to remove materials deposited on the surface.

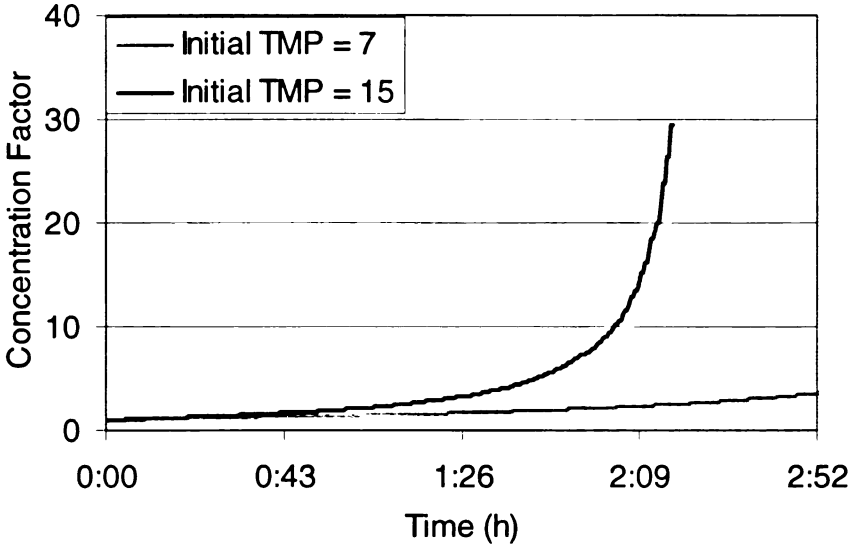

Figure 4.7 : Concentration profile for ultrafiltration of 100 mL switchgrass extract using a 50 cm^2 10 kda molecular weight cutoff membrane. Both runs were performed at a constant 30 mL/min crossflow rate and the transmembrane pressure (TMP) allowed to increase as necessary.

After 3 hours, no difference is seen in the permeate flux between 30 and 50 mL/min. This suggests that the fouling occurring during filtration is not limited to the surface of the membrane, as higher flow rates are unable to remove the fouling material. Deposits within the pores of the membranes are not as easily removed and decreases the permeate flux beyond the level seen for surface deposition. The decrease in flux is fairly rapid followed by a steady flux,

suggesting that fouling will not be a significant issue in protein recovery. Washing the membranes in 0.1 M sodium hydroxide was effective at removing all fouling material, and multiple experiments after washing were all consistent, suggesting a reasonably long life time for industrial ultrafiltration membranes.

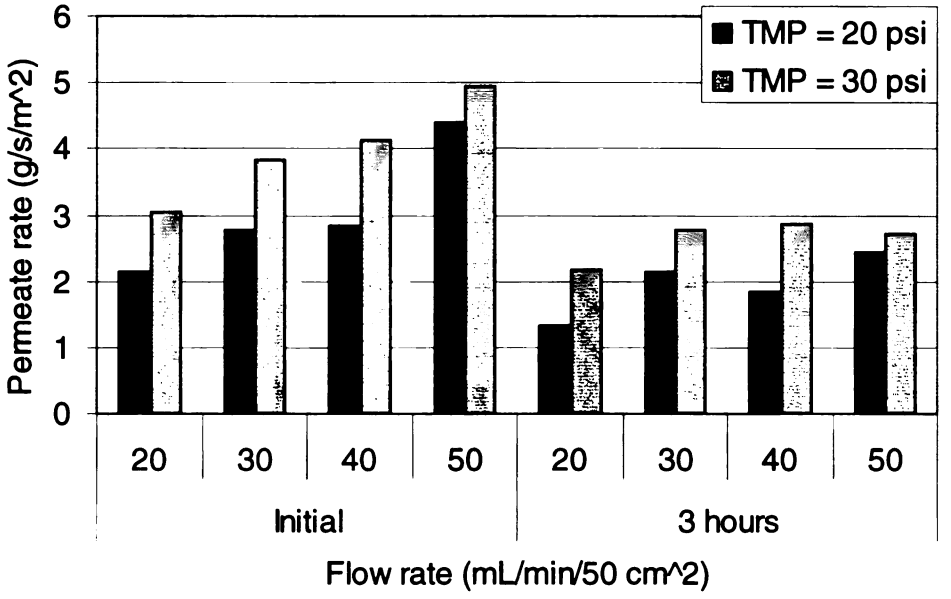

Figure 4.8 : Permeate flux for ultrafiltration of switchgrass extract using a 10 kDa molecular weight cutoff membrane at two different transmembrane pressures (TMP). Permeate rate is determined based on the average rate for 10 minutes, either immediately after starting filtration or after 3 hours of running. The permeate and retentate were recombined with the feed to keep a constant protein concentration in the feed.

Unfortunately, protein recovery was fairly low, as seen in Figure 4.9. This is especially true for the protein released in solution following enzymatic hydrolysis. An SDS-PAGE analysis of the initial solutions and permeate streams confirm that no protein larger than 10 kDa is present in the permeate for any media, indicating that the low recovery is not due to larger than expected pores or damage to the membrane. The primary enzyme mixture used in these experiments (Spezyme

CP) contains few proteases [74], although the long residence time (168 hours) may be sufficient to break down proteins in solution. In addition, the pH of the hydrolysis is ~5, which is near the isoelectric point for many proteins. In other words, larger proteins may remain insoluble at these conditions, leaving only the peptides released when the cell walls were broken down. High protein retention is seen in the protein recovered after a second extraction.

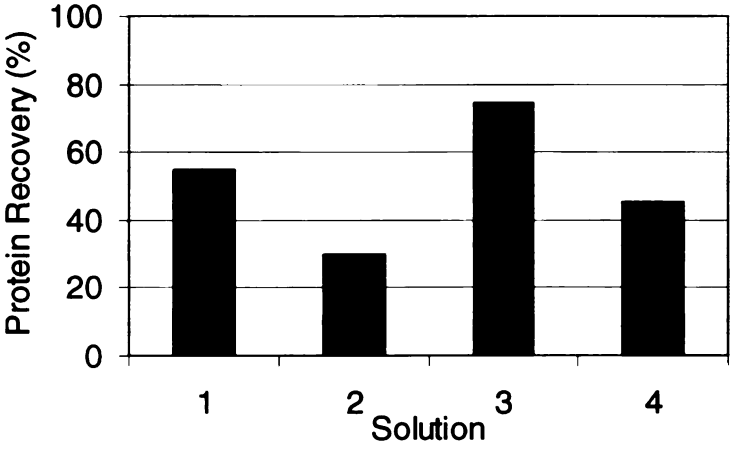

Figure 4.9 : Protein recovery using ultrafiltration for four separate protein solutions: 1 – extraction on AFEX treated switchgrass, 2 – hydrolysate, 3 – extraction on post hydrolysis solid residue, 4 – extraction on untreated switchgrass.

Interestingly, AFEX improved the recovery of proteins compared to untreated extracts. This may indicate that protein is being bound to polyphenolics, which would increase their size and therefore retention. As stated previously, AFEX increases the amount of polyphenolics that is solubilized during extraction. During ultrafiltration, the permeate stream is a light yellow color while the retentate remains dark, suggesting that these phenolics do not pass through the membrane. The large amount of protein or peptides passing through the

membrane suggests that much of the protein within the switchgrass is already degraded prior to extraction. The moderate alkaline and temperature during extraction should not significantly break down the protein, indicating that the degradation happened between cutting the switchgrass and milling it. If the protein is degraded due to cutting and drying the biomass on the field, allowing the grass to release proteases which reduce overall yield, it is not clear whether aqueous extraction is a viable approach.

4.3.5 Precipitation

Attempts were first made to precipitate the protein through heat coagulation, the method used in attempts at commercial leaf protein production in both Colorado and New Zealand. However, very poor precipitation yields were observed. It is possible that the rate of heat increase was too low or the protein too dilute to effectively coagulate the protein. Commercial operations used direct steam injection, a system unavailable for this project, and so the extract had to be heated in an oven. Instead, acid precipitation using 6 M H_2SO_4 was used. In addition, ethanol precipitation was also considered due to the possibility of integrating protein production with an ethanol facility.

Acid precipitation at pH = 3.5 was highly successful at concentrating protein, as seen in Figure 4.10. Between 65-70% of the protein is recoverable using this protein precipitation technique, greater than values for heat coagulation in the literature [47; 75]. By contrast, ethanol precipitation only recovered 45% of the

protein. Furthermore, non-protein materials were also precipitated in greater quantities for this product, leading to a less pure protein product. Ethanol precipitates were only 22% protein, compared to 30-40% protein for acid precipitation. Farmers have an incentive to feed protein products in as high a concentration as possible (i.e., g protein / kg protein supplement), as more non-nutritious material in the feed decreases intake and therefore reduces growth. Interestingly, the addition of phenylmethanesulphonyl fluoride (PMSF), a protease inhibitor, appears to increase protein concentration in the precipitates for acid pretreatment, although this trend is not significant (p=0.10). A slight increase is also seen in the amount of protein precipitated, although this too is not significant. The addition of PMSF had no impact on the amount of protein extracted, as both extracts solubilized 43% of the total protein in the switchgrass.

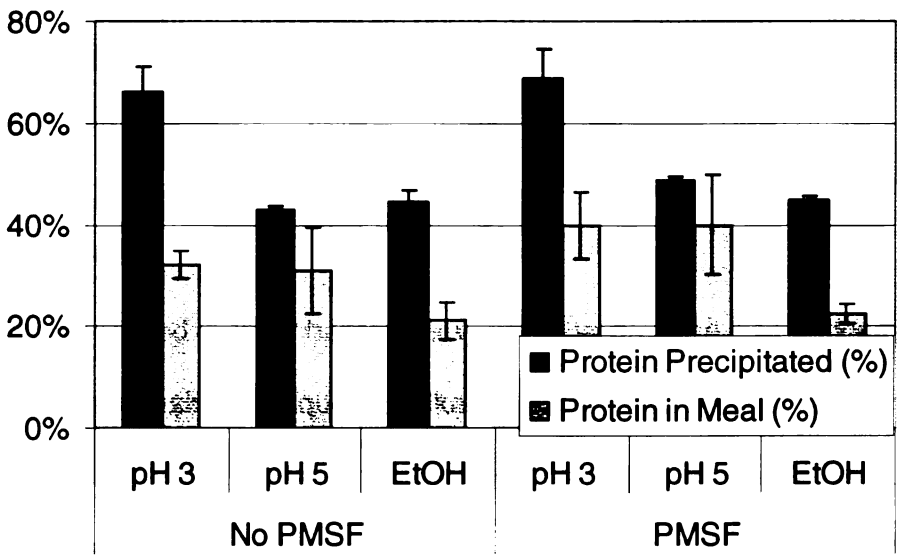

Figure 4.10 : Effect of ethanol or acid precipitation at pH 3.5 and 5 on protein concentration. Untreated switchgrass extracted at 50C using 0.1 M NaOH was used as the extract in this study. PMSF was added at 0.1% concentration during the extraction for half of the experiments. Error bars represent the high and low values of duplicate trials.

Acid precipitation does also precipitate some polyphenolics, as seen in Figure 4.11. No change in polyphenolic concentration is seen using acid precipitation at pH = 5, although as seen in Figure 4.10 above this results in lower precipitation yields. At pH = 3.5, however, approximately 23% of the phenolics is removed with the protein for untreated material.

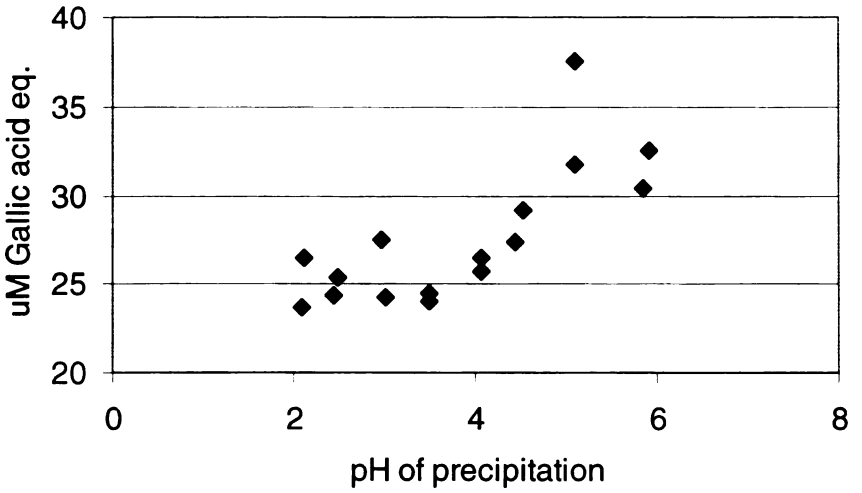

Figure 4.11 : Amount of polyphenolic compounds (in equivalent moles of gallic acid) remaining in the extract after protein precipitation on untreated switchgrass extract.

4.4 Discussion

The results presented in this chapter show both the promise and difficulty of extracting proteins using an aqueous alkaline extraction. Ammonia has been shown to be an effective solvent for removing proteins from the biomass, thus opening up possibilities of integrating with AFEX pretreatment. Up to 40% of the protein could be extracted using dilute solutions of ammonia, consistent with literature values for other species of leaf protein.

The largest issue to be considered is determining whether extraction should be performed before or after AFEX pretreatment. AFEX does not significantly affect protein extraction yields, but does decrease resulting sugar yields and increases the polyphenolics removed in the extract. Although further research is needed to confirm this assumption, it is likely that extraction prior to AFEX is the preferred option. While there is concern regarding sugar loss, no effort was made to improve AFEX treatment on extracted switchgrass. Several options are available here for increasing yields, including AFEX pretreatment conditions, not drying the biomass between extraction and pretreatment, and using the extract as the hydrolysate media to recover soluble sugars. Thus, it is likely that sugar yields when protein is extracted can be increased to levels comparable with switchgrass in which no extraction is performed.

If extraction is performed after AFEX treatment, the polyphenolic concentration may be an issue. If the protein is precipitated, only a portion of these compounds remain with the biomass, yet most appear to remain with the protein if ultrafiltration is used. Further studies on the digestibility of these protein products are needed prior to determining its true impact. One other concern with protein extraction following AFEX treatment is ammonia reactions with soluble sugars. Protein extraction is likely to occur on early-harvest grasses due to higher protein contents, which contain significant quantities of reducing sugars such as sucrose. These sugars can react with ammonia at elevated temperatures to produce degradation compounds such as 4-methylimidizole, which has been linked to

nervous disorders in cattle. If the post-AFEX fiber is to be used as an animal feed, extraction prior to AFEX will insure this compound is not formed during AFEX by removing the soluble sugars.

In addition to a dedicated protein extraction, protein appears to naturally solubilize after enzymatic hydrolysis of the lignocellulosic material. This suggests that protein recovery after hydrolysis and fermentation is a feasible approach as well. If simultaneous saccharification and fermentation is performed, then removing the protein must be performed after fermentation or distillation. In this case, there may be concern with the protein being consumed by the fermentation organism. One method may be to lyse the cells prior to separating the lignin residue from the fermentation media, allowing the protein to return to solution.

Despite the promise of this approach, the low recovery from ultrafiltration remains a concern. Likewise, the dark color of the concentrated protein also suggests high phenolic content. It appears as though the small protein size associated with poor ultrafiltration recovery is due both to the switchgrass itself and the protein breaking down during enzymatic hydrolysis. Inhibiting proteases during hydrolysis may improve the latter result, yet protein yields would still be low. Either smaller molecular weight cutoffs are required for ultrafiltration or the switchgrass must be processed in a manner that does not degrade the protein. Using smaller MWCO membranes would increase the energy requirement of the process and increase the amount of nonprotein compounds in the protein

product, both undesirable results. Further research is clearly required in this area if ultrafiltration is to be an alternative to steam injection.

Aqueous alkaline protein extraction would be an alternative to the mechanical pressing that has been attempted commercially, as discussed in Chapter 2. These two operations have substantial differences that must be taken into account if protein concentrates are to be developed commercially. Mechanical pressing would tend to use less water, leading to reduced downstream costs. However, the power requirement for presses can remain high. Furthermore, decisions must be made between different methods of concentration, including acid precipitation, steam injection, and ultrafiltration. While attempts were made to obtain experimental results from pressing the biomass, no results are presented here. This is due to poor process control with the equipment, as the feed rate of biomass and pressure were highly variable and uncontrollable with the lab-scale equipment used.

Despite the promise of leaf protein extraction, the low protein concentration in the switchgrass used for these experiments may be problematic economically. The protein content in the switchgrass samples used in this study ranged from 5-7% of the total dry weight. Assuming protein recovery of 40%, this amounts to only 20-30 kg protein per Mg of switchgrass. Even an optimistic selling price for the protein product would not recover the cost of the biomass itself, leading to concerns about the economics of the process. This issue is discussed in more

detail in Chapter 7. In addition, alfalfa and other legumes are potential sources of protein as well. An intriguing possibility is to double crop corn and soy rotations, harvesting these grasses or legumes in the spring for their protein product before planting corn or soy. This possibility is considered in Chapter 8.

4.5 Conclusions

These experimental results show that the integrated recovery of sugar and protein from grasses appears to be a feasible approach to a cellulosic biorefinery. Ammonia has been shown to be an effective solvent for removing proteins from the biomass, thus opening up possibilities of integrating with AFEX pretreatment or providing a nitrogen source during fermentation. Additional protein is released during enzymatic hydrolysis; however, sugar yields decrease if an extraction is performed prior to AFEX pretreatment. Multiple methods of concentrating the protein were also investigated. Of these, acid precipitation provides the highest recovery, although precipitation of polyphenolic compounds is also occurring.

The major obstacles for commercializing aqueous protein extraction appear to be in concentrating the protein using ultrafiltration following extraction and the difficulties with integrating the process with AFEX and subsequent hydrolysis and fermentation. Protein degradation, both prior to extraction and during lignocellulosic hydrolysis, is a major concern, lowering protein recovery to 45% following extraction and 30% following hydrolysis. In addition, the tradeoff

between sugar and protein yields for extraction prior to AFEX is a cause for concern. Although using the protein extract as a hydrolysate media replaces some sugar loss, the overall result is declining ethanol yields. Finally, the quality of the protein product, particularly after AFEX treatment, may be an issue. It is clear that polyphenolic compounds are included in the extract and concentrated with the protein. It remains to be seen if these compounds interfere with protein digestibility.

Thus, further research is necessary before alkaline aqueous extraction can be developed commercially. Particular emphasis should be placed on pretreating the fiber remaining after protein extraction. Identifying the reasons behind the poor sugar yields and adapting to these reasons are likely to be vital to the economics of this process, as seen in Chapter 7. If necessary, other pretreatments should be pursued, although AFEX is preferable due to the use of alkaline materials for extraction. The second area of research to be pursued should be the quality of the protein and the impact of AFEX treatment on this quality. This may require animal testing, although experiments with pepsin and trypsin digestibility can be performed as well. Improving ultrafiltration recovery is also important. However, because of the low protein recovered in the untreated extract, other concentration techniques, including steam injection, should be studied as well.

CHAPTER 5 : FIBER RUMEN DIGESTIBILITY

5.1 Introduction

As stated in Chapter 2, ammonia-based treatments have been used to enhance the fiber digestibility of forages for decades. However, the conventional method of ammoniation – low pressure, room temperature, and a residence time of several weeks – does not sufficiently break down the structure of highly indigestible forages, and so even ammonia treated straws are not as attractive as feedstuffs compared to traditional forages such as alfalfa. A more severe treatment such as AFEX may be necessary to allow low quality forages to compete economically. If so, then agricultural residues or dedicated crops that have higher yields than traditional forages may displace traditional forages and possibly some corn grain in cattle diets, thereby allowing more land for biofuels.

Pretreating feedstocks with AFEX technology for animal feed purposes also aligns with the RBPC concept. By removing pretreatment from the biorefinery and placing it at a smaller scale, it can be closer to animal feed operations and therefore reducing transportation costs. Furthermore, it allows two potential revenue streams from the RBPC: pretreated feed and pretreated biomass for ethanol, thus limiting the risk for these centers.

If AFEX-treated feeds are to be used for ruminant feeding, there are several important questions that need to be answered. While animal feeding trials are

required to fully understand their use in feeding operations, it is currently impractical to prepare enough material for such trials due to the size of the AFEX reactors available. Thus, *in vitro* analysis is used to estimate the true fiber digestibility. The objectives of this chapter are as follows:

- Determine which feedstocks are suitable as AFEX-treated animal feed. This includes traditional energy crops such as switchgrass, traditional forages such as alfalfa, and agricultural residues traditionally associated with ammonia treatments. Because research into AFEX-treated animal feeds is so limited, it is important to expand the range of feedstocks beyond switchgrass in this study.

- Determine the effect of pretreatment on both fiber digestion and nitrogen addition. For fiber digestion, several different factors must be considered. These include the rate and extent of digestion, the breakdown of fiber due to pretreatment vs rumen enzymes, and the relative amount of fiber breakdown.

5.2 Materials and Methods

5.2.1 Feedstocks

Eleven different feedstocks were used during this experiment, and are listed in Table 5.1. Three of these forages – corn silage, orchardgrass hay, and alfalfa hay – are defined as traditional forages in this paper, as they are commonly used

for ruminant feeding without treating the forage. The remaining materials are defined as nontraditional forages. Two of these – corn stover harvested in October 2007 from Michigan State University (East Lansing, MI) and Alamo switchgrass harvested in October 2005 from Auburn University (Auburn, AL) – were chosen for further experiments as being representative feedstocks of agricultural residues and dedicated energy crops, respectively.

5.2.2 Treatments

AFEX pretreatment was performed in a 1.5 L stainless steel reactor vessel in a manner described in Chapter 3. As the purpose of this paper is to evaluate the potential for several AFEX-treated forages rather than to compare the forages to each other, different treatment conditions were used for each forage. These conditions were chosen to be as close to optimal values as possible as reported in the literature while accounting for differences in AFEX procedures. These optimal values provide the greatest disruption of cell wall material, allowing for maximum yields of sugars after hydrolysis using commercial cellulases. If no reference was available, conditions were determined based on other forages with fiber that is expected to be similar in recalcitrance to enzymatic breakdown. In general, digestible forages required mild AFEX conditions (lower temperatures, residence times, and ammonia loadings). For these forages, harsher conditions generally do not significantly improve accessibility to enzymatic attack (see Chapter 3). For more indigestible material, relatively harsh AFEX conditions are

required to effectively break down the cell wall structure. AFEX conditions for each biomass are shown in Table 5.1.

To compare AFEX with other treatment methods, corn stover and Alamo switchgrass were ammoniated at room temperature according to Solaiman et al. [35]. Forages were ammoniated by placing 30 g dry weight of samples into plastic bags. Concentrated (30%) ammonia was added at 40 g/kg dry matter, and the total moisture content was adjusted to 300 g/kg dry biomass. Samples were thoroughly mixed and left sealed for 30 days at room temperature. After 30 days, samples were dried to remove residual ammonia. For the purpose of this paper, this treatment is defined as conventional ammoniation.

5.2.3 In Vitro Rumen Digestibility

Neutral detergent fiber (NDF) digestibility was based upon the standard method of Goering and Van Soest [76]. A rumen buffer solution was prepared including peptone, macrominerals, and microminerals, and 40 mL were added to 0.5 g corn stover (or other feedstock). The flasks were placed in a 40°C water bath and 2 mL of a reducing solution containing cysteine, sodium hydroxide, and sodium sulfide was added. Flasks were placed under CO_2 and allowed to reduce prior to inoculating with rumen fluid. Rumen fluid was collected from a fistulated dairy cow at Michigan State University's Dairy Farm approximately 2 h after feeding. The fluid and partially digested fiber from the cow were blended before filtering the fiber from the fluid, and the fluid was kept under CO_2 at all

times to minimize bacterial death. After removing the fiber, 10 mL of rumen fluid was injected into each flask. Samples were kept at 40°C for 48 h, although other time periods were studied. After the desired residence time, 10 mL of neutral detergent solution were added to each vial to stop fiber digestion.

NDF, both before and after *in vitro* digestion, was determined as described by Mertens [77]. Amylase and sodium sulfite were both added to the samples prior to neutral detergent digestion. Samples were boiled for 1h in neutral detergent solution before cool being filtered through crucibles. The remaining fiber was rinsed with water and acetone, and allowed to dry to determine its dry weight. Samples were then ashed, and the remaining ash subtracted from the weight of the sample.

5.2.4 Commercial Enzyme Hydrolysis

Fiber was hydrolyzed using a mixture of commercial enzyme cocktails to simulate cellulosic ethanol production. These experiments used the pretreatment parameters and hydrolysis conditions from the October harvest of Alamo switchgrass as described in Chapter 3. As material was limited for both experiments, not all AFEX conditions originally tested in Chapter 3 were used for ruminant testing.

5.2.5 Nitrogen Analysis

Nitrogen content within the biomass was determined using a Skalar Primacs SN Total Nitrogen Analyzer (Breda, The Netherlands), which uses the Dumas method of combusting all nitrogen to NOx [78]. Approximately 100-300 mg of biomass, depending on the estimated nitrogen content is placed in a crucible and combusted at 1100°C for 6 minutes. The resulting gas is reduced to N_2 and measured through thermal conductivity. A standard curve using EDTA from 0.5-10 mg nitrogen was used to calibrate results.

5.2.6 Statistical Analysis

Only differences between treated and untreated samples were considered in this study, not differences between different forages. Two experiments, the rate of digestion for corn stover and switchgrass and the impact of varying AFEX treatment conditions for switchgrass, were performed in duplicate for each sample or time point. All other nitrogen and NDF values were determined in triplicate. A two-tailed student t-test was used to determine if differences between untreated and treated samples, and all statements of significance were based on a probability level of 0.05.

The rate of digestion for corn stover and late harvest switchgrass was determined for each treatment using the following first order degradation model [79]:

$$NDF = A \cdot e^{-kt} + U \qquad (5.1)$$

where NDF is the amount of undigested NDF remaining, A is the amount of digestible NDF in the forage (g/kg dry matter), k is the rate constant, t is time (h), and U is the amount of indigestible NDF in the forage (g/kg dry matter). Constants were determined using the Gauss-Newton method to minimize the sums of squares for the error.

An analysis of variance was performed for the rate of digestion model. A sum of squares reduction test was used to compare each pair of treatments [80]. In this case, the comparison is between the full model and a reduced model with the following constraints:

$$A_i = A_j \quad (5.2)$$

$$k_i = k_j \quad (5.3)$$

$$U_i = U_j \quad (5.4)$$

where i and j are either no treatment, AFEX treatment, or ammonia treatment. The test statistic is calculated as

$$F_{obs} = \frac{(SSR_{reduced} - SSR_{full})/(df_{full} - df_{reduced})}{MSE_{full}} \quad (5.5)$$

using an F distribution with the numerator degrees of freedom as the difference in degrees of freedom between the full and reduced models and the denominator degrees of freedom as the residual degrees of freedom in the full model. In addition, each pair of parameters was also compared for significance using the same method. No differences between switchgrass and corn stover were tested. All statistical analysis was performed using SAS 9.1 software (Cary, NC).

Table 5.1 : AFEX treatment conditions for eleven potential forages

Name	NH3 g/g BM	Water g/g BM	Time min	Temp °C	Reference[a]
Corn silage	1.0	2.0	15	130	Unpublished data
Alfalfa hay	1.0	0.8	15	130	
Orchardgrass hay	1.0	0.8	15	130	
Rice straw	1.0	0.8	15	140	Balan 2008 [81]
July Cave-in-rock switchgrass	1.0	0.8	15	130	Alizadeh 2005 [60]
Forage sorghum	2.0	1.2	15	140	Unpublished data
Corn stover	1.0	0.6	15	130	Teymouri 2005 [63]
October Alamo switchgrass	1.5	1.0	30	150	
Wheat straw	1.0	0.8	15	140	
Sugarcane bagasse	1.5	1.0	30	150	
Miscanthus	2.0	1.5	30	150	Murnen 2007 [82]

[a]When available, optimal AFEX conditions were chosen from previous references, with time and temperature data adjusted for rapid heating of material.

5.3 Results

5.3.1 Characteristics of AFEX-treated forages

Ammoniation darkened the color of all forages, and the color was darker for AFEX treatment compared to traditional ammoniation for corn stover and Alamo switchgrass. Nitrogen and NDF values for all samples are shown in Table 5.2. AFEX treatment decreased NDF concentration in all samples, ranging from 48-195 g/kg dry matter, with an average of 110 g/kg dry matter. A slight linear trend was observed between untreated initial NDF and NDF loss for AFEX-treated samples ($R^2 = 0.348$, $p = 0.052$). This trend can partially be explained as forages with a higher NDF concentration have more reactive sites for the AFEX treatment to disrupt. The different cell wall compositions among the various forages likely account for these differences as well. Ammoniation of biomass,

particularly AFEX treatment, serves primarily to cleave the ester linkages within lignin and hemicellulose as well as the linkages between lignin and polymeric carbohydrates [83]. Furthermore, research in enzymatic digestion of AFEX-treated corn stover has shown that washing the biomass prior to digestion reduces pentose yields yet increases glucose yields, strongly suggesting that a portion of the hemicellulose is broken down into oligomeric sugars during pretreatment [84]. It should be noted that, as washing is not necessary following AFEX treatment, these oligomeric sugars should still be present in the final product and therefore available to the cattle for digestible energy.

This NDF loss appears to be higher than what can be expected for conventional ammoniation. For example, two references for ammoniation of wheat straw give NDF losses of 63 and 78 g/kg forage, respectively [35; 85], while 82 g/kg was hydrolyzed during AFEX treatment. In addition, NDF loss for Alamo switchgrass was significantly ($p<0.05$) higher for AFEX than conventional ammoniation (136 vs 38 g/kg for switchgrass) in this study, although the greater NDF loss was not significant for corn stover (89 vs 58 g/kg). This data suggests a greater disruption of cell wall material for AFEX-treatment compared to conventional ammoniation, likely due to the higher temperature and ammonia loading during treatment.

Table 5.2 : NDF and nitrogen content of treated and untreated forages

	NDF (g/kg dry biomass)					Nitrogen (g/kg dry biomass)				
	Untr.[a]	S.E.M.	Treat	S.E.M.	Diff.[b]	Untr.[a]	S.E.M.	Treat	S.E.M.	Diff.[b]
Corn silage	481	10.5	399	5.7	82	18.2	0.13	33.9	0.38	15.7
Alfalfa hay	492	11.7	426	5.0	67	26.2	0.24	48.2	0.32	22.0
Orchardgrass hay	678	10.0	611	7.0	105	21.1	0.83	40.1	0.09	18.9
Rice straw	711	2.6	663	13.0	48	6.5	0.09	23.8	0.08	17.3
July Cave-in-rock switchgrass	769	8.1	593	6.2	176	11.0	0.10	25.2	0.25	14.1
Forage sorghum	781	4.4	684	5.3	97	7.1	0.12	16.0	0.46	8.9
Corn stover	806	15.3	717	0.2	89	10.8	0.17	27.4	0.70	16.7
Corn stover[c]			748	19.2	58[d]	10.8	0.17	18.3	0.18	7.5
October Alamo switchgrass	819	11.1	683	7.1	136	3.6	0.02	23.3	0.09	19.7
Alamo switchgrass[c]			781	0.8	38	3.6	0.02	18.4	0.08	14.8
Wheat straw	821	9.7	739	9.9	82	4.1	0.05	19.0	0.22	14.9
Sugarcane bagasse	835	21.0	692	10.0	143	3.6	0.11	19.1	0.12	15.4
Miscanthus	909	7.1	713	22.4	195	2.5	0.06	20.8	0.07	18.3

[a]Untreated samples

[b]Difference between treated and untreated samples

[c]Conventional ammoniation treatments. All other treatments are AFEX treated

[d]Denotes no significance ($p<0.05$) between untreated and treated samples. All other treatments were significantly different from untreated samples.

Ammonia treatment increased the nitrogen concentration for all forages, ranging from 8.9 g/kg dry matter increase for sorghum to 22.0 g/kg increase in alfalfa. Untreated nontraditional forages all had low nitrogen concentration. With the ammonia addition, these samples are more comparable to the untreated traditional forages. Furthermore, nitrogen addition was significantly ($p<0.05$) greater for AFEX treatment compared to ammoniation for both switchgrass and corn stover, with AFEX increasing nitrogen over conventional ammoniation by 4.9 g/kg for switchgrass and 9.1 g/kg for corn stover. Increases in crude protein for AFEX compared to ammoniation were approximately the same as literature values for rice straw [86], with 10.8 g/kg nitrogen increase vs 10.5 g/kg cited. For wheat straw, literature values for ammoniation [35; 85] were slightly higher than AFEX treatment (9.5 and 11 g/kg, respectively, vs 9.3 g/kg reported here). It is believed that most of the additional nitrogen is in the form of acetamide [87].

5.3.2 In vitro digestibility of AFEX-treated feeds

AFEX improved the 48 h in vitro NDF digestibility of most of the forages tested, as seen in Table 5.3. However, AFEX treatment did not increase in vitro digestibility of NDF for four forages: the three conventional forages and the early harvest switchgrass. These forages are already highly digestible, and over 40% of the fiber was digested for the untreated forages. AFEX treatment of Alamo switchgrass and wheat straw increased rumen digestion by 223 and 197 g NDF / kg dry matter respectively, the largest increases among all forages. AFEX treatment increased NDF digestibility of several nontraditional forages to levels

that are greater than untreated traditional forages. AFEX-treated corn stover had the greatest amount of fiber digested (564 g/kg dry matter) of any treated or untreated forage in this study. AFEX treatment of forage sorghum and miscanthus improved NDF digestibility, but the overall amount of fiber digested was still poor compared to other untreated traditional forages. When taking into account both loss due to treatment and microbial digestion, AFEX increases the total fiber loss for all forages.

Also of note is the percentage of fiber digested for AFEX treated samples. Several forages showed an increase of 25-35 percentage points compared to untreated samples. Nearly 80% of the NDF in AFEX-treated corn stover was digested, as well as nearly 70% for wheat straw and 60% for Alamo switchgrass. NDF digestibility of untreated traditional forages ranged from 40-50%, with early harvest switchgrass being the only highly digestible untreated forage. Thus, these treated samples have both more digestible NDF per ton of NDF as well as per ton of dry matter compared to untreated traditional forages.

Table 5.3 : NDF digested after 48 hours of in vitro fermentation for eleven forages

	Digested (g/kg)[a]						Digested (% NDF)[b]			Total Digested (g/kg)[c]			
	Untr.[d]	S.E.M.	Treat	S.E.M.	Diff.[e]	Inc.[f]	Untr.	Treat	Diff.	Treat	S.E.M.	Diff.	Inc
Corn silage	208	23	275	10	67[g]	32	43.4	69.1	25.7	357	11	149	72
Alfalfa hay	211	16	197	11	-14[g]	-7	42.9	46.3	3.4	264	12	53	25
Orchardgrass	349	13	354	13	4[g]	1	51.5	57.9	6.4	459	14	110	31
Rice straw	347	4	507	16	160	46	48.8	76.5	27.8	555	20	208	60
Switchgrass (CIR July)	492	11	483	9	-10[g]	-2	64	81.4	17.4	659	11	167	34
Sorghum	179	6	237	17	59	32	22.9	34.7	11.8	334	18	155	87
Corn stover	370	17	564	9	194	52	45.9	78.7	32.8	653	9	283	76
Corn stover[h]			325	20	-45[g]	-12		47.5	1.6	383	28	13	3
Switchgrass (Alamo Oct)	174	12	397	9	223	128	21.3	58.2	36.9	533	12	359	206
Switchgrass[h]			233	19	59	34		29.8	8.5	271	19	97	56
Wheat straw	315	12	512	14	197	63	38.3	69.3	30.9	594	17	279	89
Sugarcane bagasse	212	22	356	17	143	68	25.4	51.4	26	499	20	287	135
Miscanthus	51	9	132	32	81	159	5.6	18.5	12.9	327	39	276	542

[a]NDF digested during rumen digestion only, given as g NDF / kg dry forage

[b]NDF digested as a percentage of total NDF in the treatment

[c]Total NDF removed for treated samples, including both the amount removed due to AFEX or ammonia treatment and the amount digested during *in vitro* fermentation, given as g NDF / kg dry matter

[d]Untreated samples

[e]Difference between treated and untreated samples

[f]Percent increase in treated sample over untreated sample

[g]Denotes no significant ($p<0.05$) difference between untreated and treated samples. All other treatments were significantly different from untreated samples

[h]Conventional ammonia treatments. All other treatments are AFEX-treated

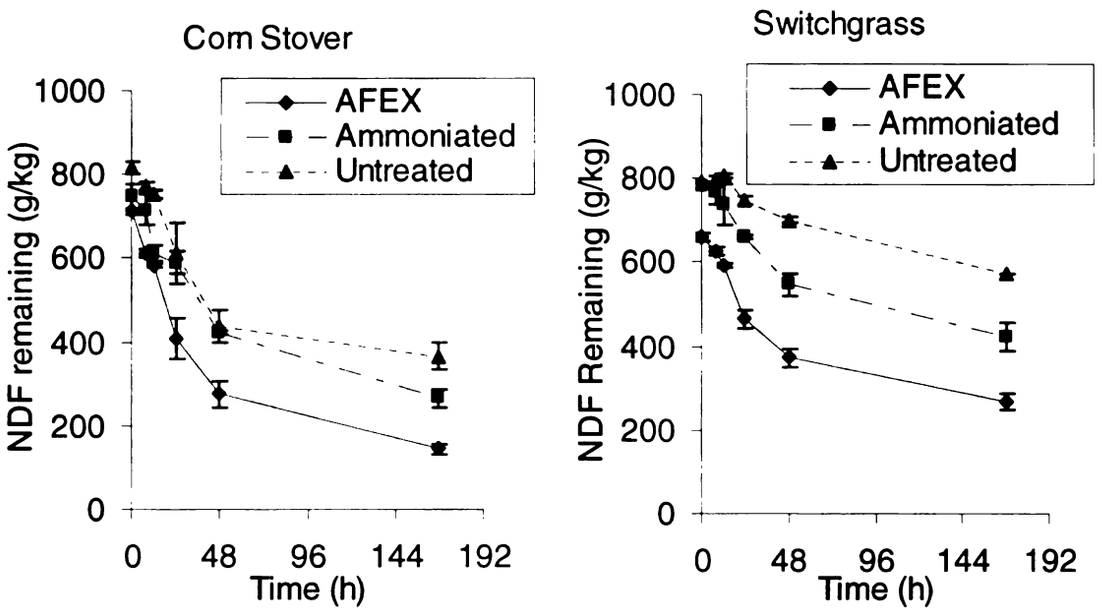

Figure 5.1: Effect of conventional ammoniation and AFEX treatment compared to untreated corn stover (left) and switchgrass (right) on NDF remaining over time during NDF digestion. Error bars represent the high and low values for duplicate samples.

AFEX treatment appears to have a greater effect on both the rate and extent of fiber digestion over both untreated and conventional ammonia-treated samples, as seen in Figure 5.1. The amount of NDF remaining after 168 hours was more than twice as high for untreated switchgrass vs AFEX treated (574 g/kg vs 267 g/kg, $p < 0.01$), and conventional ammoniation (420 g/kg) was slightly higher than AFEX treated grass ($p = 0.06$). AFEX treatment of corn stover resulted in even greater digestion compared to both untreated (145 g/kg vs 365 g/kg, $p = 0.02$) and ammoniated (266 g/kg, $p = 0.04$) samples. A first order degradation model was determined for each treatment, and the parameters are shown in Table 5.4. For corn stover, there was no significant difference between ammonia treatment and no treatment ($p=0.10$). However, AFEX treatment significantly improved the

degradation of NDF compared to either ammonia treatment or untreated samples. For switchgrass, all three treatments were significantly different from each other.

Table 5.4 : Comparison of the rate of fiber removal for corn stover and Alamo switchgrass

		Parameters[a]					
		A	SE[b]	k	SE	C	SE
Corn Stover	AFEX[c]	593[c]	44	0.029[c]	0.005	136[c]	37
	Ammonia[d]	512[c]	48	0.020[c]	0.005	246[c,d]	46
	Untreated[d]	471[c]	45	0.023[c]	0.006	339[d]	41
Alamo Switchgrass (late harvest)	AFEX[c]	427[c]	26	0.026[c]	0.004	257[c]	23
	Ammonia[d]	405[c]	29	0.018[c,d]	0.004	398[d]	29
	Untreated[e]	319[c]	103	0.008[d]	0.005	490[d]	110

[a]Parameters obtained using a least squares nonlinear regression on the rate of NDF removal (Data from Figure 5.1). The regression equation is $NDF = Ae^{-kt} + U$, where NDF is the NDF remaining in the biomass (g/kg dry forage), t is time after inoculation (h), A is the amount of digestible NDF, k is the rate constant, and U is the amount of indigestible NDF.
[b]Approximate Standard Error
[c,d,e]Different letters denote significant differences ($p<0.05$) among the parameters and treatments using the sum of squares reduction test. Significant differences are tested only between treatments and not between forages.

The primary impact of AFEX treatment is to decrease the amount of indigestible NDF. For both corn stover and switchgrass, AFEX has significantly less indigestible fiber than untreated samples, and less indigestible fiber than ammonia treated samples for switchgrass. In addition, AFEX treatment significantly improves the rate of digestion for switchgrass compared to no treatment.

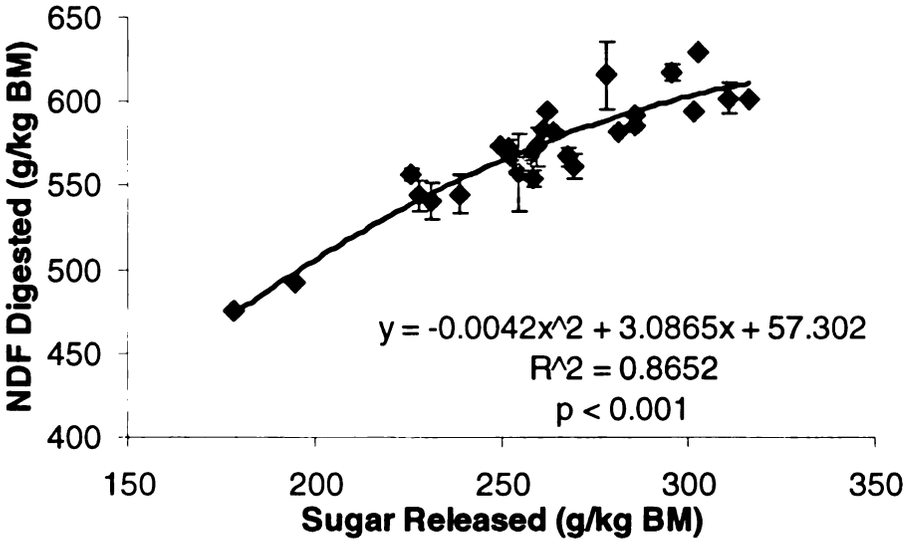

Figure 5.2 : Relationship between monomeric sugars released during enzymatic hydrolysis with commercial cellulases (x-axis) and in vitro NDF digestion at 48h (y-axis) for multiple AFEX conditions of late harvest switchgrass. The line represents a quadratic curve obtained from an ordinary least squares regression. AFEX conditions ranged from 0.4-2.0 g water / g dry biomass, 0.4-2.0 g ammonia / g dry biomass, 5-30 minute residence time, and 80-150°C.

As previously stated, the effectiveness of AFEX pretreatment depends on the conditions present during the reaction. Much literature has been published for optimizing AFEX conditions based upon theoretical ethanol production; these conditions are likely to be optimal for ruminant feed as well. As expected, there is a significant correlation between enzymatic digestion using commercial cellulases and in vitro rumen digestion, as seen in Figure 5.2. AFEX increases the accessibility of cellulose and hemicellulose to enzymatic attack, which should affect fiber digestibility using both methods. The amount of NDF digested during in vitro studies is approximately twice as high as sugar released by commercial cellulases. This is due partly to different conditions, as a low enzyme loading was used and without the complete array of enzymes present in rumen microbes,

and partly to a difference in analyses, as the sugar analysis does not include oligomers or solubilized lignin.

5.4 Discussion

Based upon our results, it appears that AFEX pretreatment is an effective treatment for improving the digestibility of some forages for ruminant feeding. For in vitro digestibility studies, we see a clear improvement in NDF digestibility for both corn stover and late harvest switchgrass compared to ammonia treatment. An increase in nitrogen content was also seen, providing additional non protein nitrogen and therefore additional value as a feed.

Many types of biomass appear to be viable candidates for AFEX-treated animal feeds. In general, early-harvest feedstocks and forages commonly used as feeds are not appropriate for AFEX treatment, as only modest improvements in digestibility are seen. In addition, NDF digestibility of miscanthus remained low (18.5% of NDF) after AFEX treatment, and thus further research is required in order to make it viable for animal feed. Corn stover and late harvest switchgrass are of particular interest due to being commonly cited sources of cellulosic ethanol while simultaneously offering large improvements in crude protein and fiber digestibility [88; 89].

5.4.1 Non Protein Nitrogen

The increase in nitrogen due to ammoniation is also important due to the high protein content of many untreated forages. Balancing protein is an important part of feeding operations, and so similar crude protein values between untreated forages and AFEX-treated feedstocks allow for the displacement of these untreated forages without adding potentially expensive protein supplements. It is believed that most of the nitrogen addition is in the form of acetamide. Several studies have reported that acetamide is digestible to rumen microorganisms, although less so than common non-protein nitrogen supplements such as urea [90]. Furthermore, improved digestibility of fiber requires greater N uptake in order to allow for increased microbial production.

While there are advantages to non-protein nitrogen, primarily the price compared to protein supplements such as soy, there have been concerns regarding non-protein nitrogen (NPN) addition. Rapid urea intake has caused rumen toxicity due to spiking ammonia levels within the rumen. However, a proper transition period to non-protein nitrogen can allow the cattle to have relatively high intake of urea without toxicity problems. Furthermore, studies strongly suggest that, while beef and dairy cattle can be raised solely on NPN, the highest milk producers require true protein for full milk production [91]. However, these studies were performed with urea as the NPN source. Acetamide likely breaks down at a slower rate than urea within the rumen, lessening the potential for ammonia

toxicity. Also, there remains some crude protein in the biomass, as well as any protein in other supplements to the cattle diet.

While it remains to be seen whether the NPN addition is sufficient for cattle diets, it does not seem likely that it will have a detrimental effect. As stated previously, the increased digestibility of the fiber necessitates increased ammonia use, and the likely slow breakdown of acetamide should reduce the risk of rumen toxicity. Furthermore, as seen in chapter 8, it is possible to adjust NPN addition significantly by changing the conditions present during AFEX pretreatment. While this will also likely affect digestibility, conditions may be arrived at to provide optimal nitrogen addition and fiber digestibility improvement.

5.4.2 NDF Digestibility

The greater total NDF in traditional bioenergy feedstocks leads to the potential for higher total energy per ton of feed. Corn stover, for example, contains more digestible fiber than the total fiber in alfalfa or corn silage. Furthermore, all potential feedstocks suggested here have more digestible fiber per kg biomass than any of the three traditional forages tested. Because of the higher amounts of digestible fiber, the diet requires less tonnage of fibrous material. Thus, the value of the fiber in AFEX-treated feedstocks should be higher than that of untreated forages, potentially increasing their selling price. In addition, this raises the possibility of adding more digestible energy into the diet, improving weight gain or milk production.

The improvement in the percentage of NDF digested, rather than the total NDF digested, is also worth noting. The rate of NDF digestion directly corresponds to the rate of passage through the rumen. As NDF breaks down, it becomes less aerated due to less microbial action. This increases the density of the material, allowing it to sink in the rumen and be removed. Increased passage through the rumen allows for increased dietary intake, leading to greater growth rates for beef cattle or milk production for dairy cattle. While this will increase overall feed costs, the cost per kg weight gain or per gal milk will decrease, as the energy for maintenance for the cattle will be the same.

It seems likely that the increased digestibility of AFEX-treated feeds can lead to greater intake of feed, thereby increasing production. A second possibility may be to displace a portion of the grain in cattle diets with AFEX feed. Such an approach has the potential to reduce overall land use, as bioenergy feedstocks either have greater yields per acre than corn or, for agricultural residues, increase the productive biomass per acre for grains. Table 5.5 shows the total digestible nutrients (TDN), net energy available for lactation at 3X maintenance (NEL), and crude protein (CP) for AFEX treated corn stover and switchgrass vs corn grain and several common forages. Whereas switchgrass has numbers comparable to the forages, corn stover is more digestible, although not as digestible as corn grain. These numbers are based on calculations obtained from the composition of the materials and fiber digestibility, and thus only an

approximation. The true values will also depend on currently unknown factors, including the digestibility of the fiber *in vivo*, as well as the digestibility of protein and its binding to lignin or other polyphenolics, as stated in Chapter 4.

Table 5.5 : Total Digestible Nutrients (TDN), Net Energy available for Lactation at 3X maintenance (NEL), and crude protein (CP) content in five traditional feeds and two AFEX feeds

	TDN	NEL	CP
	% DM	Mcal/kg	% DM
Corn grain[a]	88.7	2.01	9.4
Soybean hulls	67.3	1.46	13.9
Corn silage[a]	68.8	1.45	8.8
Orchardgrass hay[a]	63.1	1.37	18.1
Alfalfa hay[a]	58.9	1.27	20.2
AFEX Corn Stover	75.6	1.74	17.2
AFEX Switchgrass	63	1.48	14.6

[a] Values obtained from NRC 2001 [33]

5.4.3 Displacing Corn Grain

AFEX treated corn stover is highly digestible at approximately 85% of the value of corn grain measured using either TDN or NEL. While discussion has previously focused on replacing traditional forages with AFEX treated feeds, it may be possible to replace grains in the diet with corn stover as well. Several recent studies suggest that soybean hulls, a fibrous and highly digestible feedstock, can replace corn or other grains in beef cattle with little detrimental effect [92-94]. In general, these studies report dry matter intake (DMI) of feed as well as dry matter digestibility to be similar between corn grain diets and soybean hull diets, as seen in Table 5.5. Furthermore, soybean hull diets tended to

improve the NDF digestibility of other forages within the diet. This may be due to the easily digestible fiber stimulating the enzymatic response of ruminant microbes, increasing the overall activity of fiber breakdown. These studies also suggest that hulls may improve protein and overall energy utilization compared to corn.

While soybean hulls are more digestible than AFEX treated corn stover, these results suggest corn stover may also be used in a similar manner. AFEX treated corn stover would need to be fed at greater levels than corn grain, although the potential for improved digestibility and energy utilization may reduce this need. This would effectively increase the productivity per acre of corn by nearly 60%, assuming 70% of the stover can be harvested.

While this may be a solution for beef cattle, replacing all of the corn grain in dairy cattle is unlikely to be effective. During ruminant digestion, carbohydrates are converted to volatile fatty acids, which are absorbed by the cattle and converted for use. While multiple VFAs are produced, fiber digestion is high in acetate relative to propionate or butyrate. Starch, meanwhile, produces a greater amount of propionate as well as lactate. These latter two acids are gluconeogenic, and thus can be used by the cow to produce the sugars in milk. Thus, some amount of corn grain will remain in dairy diets in order to optimize milk production.

5.5 Conclusions

Ammonia Fiber Expansion (AFEX) pretreatment caused an improvement in neutral fiber digestibility of multiple feedstocks during in vitro studies. The greatest improvements were observed for moderately indigestible material not commonly used as cattle feed. Of particular interest is corn stover and late-harvest switchgrass, which saw 53% and 128% improvement in 48h digestibility over untreated material and 74% and 70% improvement over ammonia treated samples, respectively. AFEX treatment improved corn stover's total digestible nutrients to a level comparable with highly digestible fiber sources such as soybean hulls, while improving switchgrass nutrient value to levels comparable to traditional forages.

Improvements were seen in both the rate and extent of fiber digestion. Although NDF is lost during the pretreatment, it is expected that much of that fiber is converted to oligomeric sugars, which can still be of nutritional value for the ruminants. Crude protein content also increased, with treated samples being comparable to common ruminant feeds.

This study strongly suggests that treated agricultural residues such as corn stover or dedicated energy crops such as switchgrass can compete with common forages such as alfalfa or orchardgrass for ruminant diets. In addition, corn stover may be competitive with energy crops such as corn grain. Such competition will depend upon the cost of AFEX pretreatment and the true value

of the final feed. Initial information suggests these feeds can be produced at a cost comparable to traditional forages while potentially giving added benefits such as improved intake and milk production.

CHAPTER 6 : SUMMARY OF SWITCHGRASS TREATMENT MODULES

6.1 Introduction

In the previous chapters, several options were considered for integrating food and fuel production cellulosic ethanol refineries. While the general advantages and disadvantages of each option were discussed, they were not comparable on an established metric. In addition, the aqueous alkaline protein extraction studied experimentally could not be compared to the mechanical pressing extractions performed commercially. Such comparisons are required in order to narrow these options to the ones with the greatest potential for economic development and maximum productivity.

The technology behind both potential new technologies of feeds as well as mechanical pressing of protein is fairly well established. Solid/liquid extraction can be easily modeled and has been included in an AFEX biorefinery model [95]. In addition, extensive literature references to the mechanical pressing of green juice and subsequent heat coagulation are also available. For AFEX treated feeds, the only additional processing needed is drying the material. Thus, the economic viability of each approach can be considered when analyzing each option.

These two protein extraction models – aqueous protein extraction and mechanical pressing – are combined with AFEX pretreatment and hydrolysis/fermentation within a biorefinery to obtain the relevant costs and

revenues. These models can be adapted for given scenarios. For example, the fiber produced after protein extraction must be dried and the whey evaporated if the fiber product is to be used for feed, but this operation not necessary if it is used for ethanol production. Each module – extraction, AFEX treatment, and the remaining refinery – can be combined in different ways to produce all possible combinations of feeds and fuel from one specific source of biomass. For each scenario, the parameters in the models must be adjusted to reflect the conditions required.

The objective of this chapter is to introduce the three major components of the cellulosic refining models – aqueous extraction, mechanical pressing, and AFEX and subsequent hydrolysis and fermentation – and determine the effect of individual adjustments for different processing conditions.

6.2 AFEX Pretreatment and Ethanol Production

The AFEX pretreatment module is based on an integrated biorefinery model produced by NREL and later adapted by Dr. Mark Laser at Dartmouth University. This model provides material and energy balances for a cellulosic ethanol biorefinery with AFEX pretreatment using Aspen Plus 2006 software (AspenTech, Burlington MA). After the simulation is successfully completed, an economic analysis tool created in Microsoft Excel uses the material, heat, and work streams to determine the capital and material costs. Details of this model can be found in Aden et al. [96], Sendich et al. [25], and Laser et al. [95]. A

process flow diagram for the major components in the pretreatment block of the simulation model is seen in Figure 6.1.

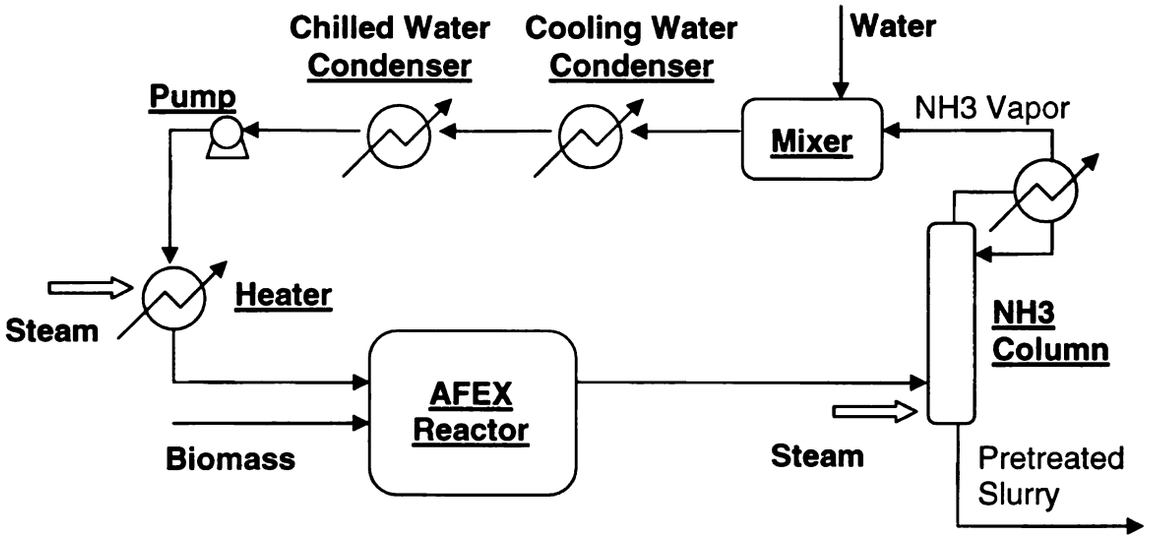

Figure 6.1 : Simplified process flow diagram of AFEX pretreatment and ammonia recovery system.

6.2.1 Full Refinery Analysis

Due to the time consuming nature of the Aspen model, it is impractical to run it for multiple simulations. Thus, a simplified model of the biorefinery was created by determining the trends in costs associated with the refinery at different pretreatment conditions. A general full factorial model was attempted, with three levels for each factor, as seen in Table 6.1. However, the Aspen model did not successfully converge at most individual runs at the high value for water loading and temperature. Thus, these conditions were dropped, and only two levels were used for water and temperature. Thus, a total of twelve Aspen simulations were performed with varying ammonia, water, and temperature conditions, and each simulation was considered at 3 different residence times.

Table 6.1 : Levels used within the general full factorial model of AFEX pretreatment conditions

	Units	Low	Medium	High
Ammonia Loading	g/g dry BM	0.5	1.25	2.0
Water Loading	g/g dry BM	0.5	1.25	(2.0)[a]
Temperature	°C	80	140	(200)[a]
Residence Time[b]	minutes	10	20	30

[a] Levels dropped from factorial design due to errors associated with the Aspen model

[b] Factor dropped from equipment, energy, and material cost analysis due to only affecting one piece of equipment

For details regarding how varying pretreatment conditions impact equipment, material, and energy costs, only the data points at the low residence time were used. The Aspen simulation model is not affected by residence time within the AFEX pretreatment reactor, although residence time is a variable during pretreatment. In order to estimate the effect of residence time, the size of the pretreatment reactor was varied within the accompanying Excel spreadsheet in order to achieve the same flow rate at different residence times. Thus, it was assumed that changing the residence time would have no impact on material streams, energy costs, or other equipment costs. As the reactor accounts for ~10% of the overall equipment costs, this change can be quite significant.

For the purposes of this study, the scale of the biorefinery was assumed to be 850 tons per day. This value is equivalent to 23 million gallons ethanol produced per year, and was chosen as a near term scenario consistent with the size of many current commercial cellulosic ethanol projects. The electricity selling prices

were assumed to be $0.04/kWh. The feedstock was assumed to be corn stover sold at $40/ton. Overall ethanol yield was assumed to be 78 gal/ton biomass. Ethanol selling price is assumed to be $1.70/gal. However, all of these variables are separated out in the final model and can be varied separately.

6.2.2 Effect of Pretreatment Parameters

For AFEX pretreatment conditions, the primary driver of capital costs within a biorefinery is the ammonia loading, as seen in Table 6.2. The water loading during pretreatment is the second most important factor, while temperature only has a mild impact. Overall, changing these three pretreatment conditions can increase or decrease capital costs by several million dollars, indicating its importance. For the biological conversion area, the only piece of equipment with a high variation due to pretreatment conditions is the cooler immediately following ammonia recovery and prior to the fermentation vessel. However, this is because the model assumes a constant amount of water added to the pretreated material, changing the solids loading during hydrolysis and fermentation. This is an unlikely scenario, and so this variation can be discarded.

Most of the variation in capital costs occurred within the pretreatment and utilities areas. For the pretreatment area, the amount of ammonia present has the largest impact on the cost of most pieces of equipment. This is due to the fact that the ammonia recycle process is the dominant process within the pretreatment area. Water content also increases the size of the recovery

system, as more water is vaporized and included in the recycle stream during ammonia stripping. Reaction temperature only has a strong impact on the initial condenser. The two most important pieces of equipment in terms of their share of cost are the AFEX reactor itself and the ammonia stripping column.

Table 6.2 : Impact of ammonia loading, water loading, and temperature on equipment cost within the biorefinery model. Only individual equipment with a coefficient of variation > 5% and average capital cost > $100,000 are shown.

	Average	C.V.[a]	Impact of factors[b]		
			A	W	T
Feedstock Handling Area	$4,157,610	0.00%	0	0	0
Pretreatment Area	$8,845,306	20.7%	+++	++	+
Cooling water condenser	$300,959	58.3%	+	+++	+++
Chilled water condenser	$405,930	49.7%	+++	-	-
AFEX Reactor	$5,034,677	19.6%	+++	++	+
Ammonia stripping column	$2,204,306	16.7%	+++	++	+
Ammonia Day Tank	$613,906	32.8%	+++	++	+
Biological Conversion Area	$1,695,628	1.3%	+	+++	--
Fermentation feed cooler	$26,776	23.2%	+	+++	---
Product Recovery Area	$7,921,171	0.4%	+	++	--
Wastewater Treatment Area	$17,538,525	0.2%	+	+++	--
Storage Area	$722,567	0.2%	+	++	--
Residue Processing Area	$19,731,042	0.0%	0	0	0
Utilities Area	$3,425,759	15.3%	+++	+	+
Cooling Tower System	$455,953	22.3%	+	+++	+++
Cooling Water Pump	$382,232	22.6%	+	+++	+++
Chilled Water Package	$1,424,040	32.9%	+++	-	-
Total Cost	$64,037,608	3.7%	+++	++	+

[a] Coefficient of Variation

[b] Qualitative assessment of the importance of ammonia loading (A), water loading (W), and Temperature (T) on the costs of individual pieces of equipment or area. A + represents an increase in price with an increase in level, a - represents a decrease of price with increase in level, and 0 represents no change, with the number of + or – representing its relative importance.

The remaining variation is in the utilities area, or specifically within the cooling tower system. This is due to changing the duties on cooling water throughout the ammonia recovery system as well as pretreatment. Here, the primary impacts are due to reaction temperature and water loading. More heat is added to the system at higher temperatures, which leads to greater heat removal in the recovery system. Likewise, excess water has a high heat capacity, which also exacerbates the heating duty required. Because the utilities are required for AFEX treatment, the capital costs in this area must be considered for both the full refinery and for AFEX-treated feeds.

The impact of AFEX conditions on heat and work streams is shown in Table 6.3. The most important stream is the steam used for the ammonia stripping column. As expected, the temperature of the pretreatment reactor is the most important variable, as less additional heat is required to remove the ammonia at high temperatures. Interestingly, ammonia loading has only a slight impact, as much of it is removed at the initial flash. Water loading plays a greater role, due both to its high heat capacity as well as increasing the amount of ammonia that can remain soluble in it. The only other steam used during pretreatment is to heat the ammonia before adding it to the biomass. However, this stream is five orders of magnitude lower than the steam used to strip ammonia, and so is not significant to the overall cost of the refinery. The remaining differences in heat streams are due to the cooling duty around the pretreatment and ammonia recovery systems. The changes in cooling duty affects the amount of heat

removed in the cooling tower system, which impacts costs due to the required size of the cooling system, as seen in Table 6.2. As stated previously, water loading and temperature has the greatest effect on cooling duties.

Table 6.3 : Impact of ammonia loading, water loading, and temperature on heat and electricity streams within the biorefinery model. A residence time of 10 minutes was used for this analysis. Only individual equipment with a coefficient of variation > 5% are shown

Heat streams	Average Mcal/Mg	C.V.[a]	Impact of factors[b] A	W	T
Heat removed from feed	100.51	53.8%	+	+++	---
Chilled water condenser	360.89	64.2%	++	-	-
Cooling water condenser	238.17	78.4%	+	++	+++
AFEX Reactor heat removal	313.58	115.8%	++	-	---
Cooling Tower System	675.68	28.6%	+	+++	+++
Steam for ammonia stripping[c]	1469.82	69.6%	+	++	---
Electricity Streams	kWh/Mg		A	W	T
Total Electricity	144.00	25.1%	+++	-	-
Cooling Tower System	4.37	28.6%	+	+++	+++
Chilled Water System	53.40	64.2%	+++	-	-
Makeup water pump	0.02	79.9%	-	+++	-
Recycle NH3 pump	1.48	41.9%	+++	++	+
Hydrolysate Feed Pump	11.76	9.1%	+	++	--
Cooling Water Pump	8.26	28.6%	+	+++	+++
Water Circulation Pump	1.38	7.9%	+	++	+++

[a] Coefficient of Variation

[b] Qualitative assessment of the importance of ammonia loading (A), water loading (W), and Temperature (T) on the costs of individual pieces of equipment or area. A + represents an increase in price with an increase in level, a - represents a decrease of price with increase in level, and the number of + or – representing its relative importance.

[c] Measured as the enthalpy of the steam entering the column

While electricity required for the plant is assumed to be produced from the insoluble residue, it reduces the amount that can be sold to the grid for profit. Several pieces of equipment are affected by changing pretreatment conditions. Temperature and water loadings both have a large role in the cooling tower

electricity requirements, for reasons stated previously. The greatest electricity usage is associated with the chilled water system. This system is required to condense the recycled ammonia to a liquid, preventing the need for a costly compression system. As the ammonia exiting the chilled water condenser must be at a very low temperature, this requires a specialized refrigeration system for the chilled water as opposed to the cooling tower. This system accounts for approximately 50% of the total electricity in the refinery at high ammonia loadings. Because of this, ammonia loading is also the dominant factor for overall electricity costs.

Table 6.4 : Impact of ammonia loading, water loading, and temperature on variable operating costs within the biorefinery model.

	Average cents/gal	C.V.[a]	Impact of factors[b] A	W	T
Makeup Water	0.49	32.1%	++	+	+++
Cooling Tower Chemicals	0.04	28.6%	++	+++	+++
Electricity Credit [c]	14.81	11.3%	---	+	+
Ammonia	2.92	2.74%	-	++	--
Diammonium phosphate	0.35	3.37%	-	++	--
Biomass	51.13	0.04%	-	++	--
Enzymes	16.06	0.04%	-	++	--
Total Operating Costs	60.32	2.8%	+++	-	+

[a] Coefficient of Variation

[b] Qualitative assessment of the importance of ammonia loading (A), water loading (W), and Temperature (T) on the costs of individual pieces of equipment or area. A + represents an increase in price with an increase in level, a - represents a decrease of price with increase in level, and the number of + or – representing its relative importance.

[c] Credit due to selling excess on-site electricity produced to the grid. The qualitative assessment is based off the magnitude of this credit; i.e., high ammonia loadings decrease the size of the credit, which increases the total operating costs.

Changes in electricity usage is the primary effect of pretreatment conditions on the total variable operating costs of the biorefinery, as seen in Table 6.4. In this model, fixed operating costs such as salaries, etc. are determined as a function of the capital cost and so are not considered here. Virtually no change is seen in either the biomass feed or the enzyme requirement, which are the two largest sources of raw material cost. Together, these components represent over $70/Mg biomass of operating cost. Makeup ammonia represents the third largest raw material cost, but pretreatment conditions in this model do not show a large effect on ammonia costs. This issue is explored in further detail below. Makeup water, which is affected primarily by the temperature of the AFEX reactor, does vary significantly, as does the amount of chemicals required in the cooling tower. However, these two components are small relative to the total operating costs.

Thus, the magnitude of the electricity credit provides the largest impact on variable operating costs. Pretreatment conditions have virtually no effect on the amount of insoluble residue remaining after fermentation, and so the changes seen are due to changes in heat and power requirements as seen in Table 6.3. Ammonia has the largest impact in total electricity requirements due to the chilled water system. This is represented in the electricity credit, as an increase in ammonia loading decreases the amount of electricity available to sell back to the grid. As the electricity credit offsets approximately 20% of the total variable operating costs, ammonia loading also has the largest impact on the total variable operating cost.

6.2.3 Makeup Ammonia

As stated in Chapter 5, it is known that some ammonia reacts with the biomass to produce nitrogen-based side products such as acetamide. The Aspen model assumes a constant 10.8 g ammonia reacting per kg biomass regardless of pretreatment conditions. Experiments were carried out to determine the impact of pretreatment conditions on the total ammonia lost due to reactions during pretreatment. The pretreated samples of October harvest CIR switchgrass described in Chapter 3 were used for this analysis. Nitrogen analysis was performed using a Skalar Primacs SN Total Nitrogen Analyzer as described in Chapter 5. It was assumed that the difference in mass of the switchgrass before and after pretreatment was negligible [63]. The amount of ammonia lost due to competing reactions was therefore calculated based on the difference in nitrogen on AFEX treated switchgrass against untreated grass.

All four pretreatment parameters impact the nitrogen increase in AFEX treated biomass, as seen in Table 6.5. In general, increased water content decreases nitrogen addition, as water can also react with acetyl or other groups, thus competing with ammonia. Interestingly, at low temperatures (<100°C), greater ammonia loadings decrease acetamide formation, although the effect is small. At higher temperatures, ammonia loading has a positive impact on ammonia reactivity, as expected. Residence time also increased ammonia reactivity at mid and high levels of water loading. Temperature has the greatest positive impact

at high temperatures, as the two largest nitrogen increases were seen when AFEX was performed at 200°C. Within the range of conditions studied in the Aspen simulation, makeup ammonia can vary between 15 and 25 g / kg biomass.

Table 6.5 : Reduced linear model for the makeup ammonia required. The MESP model does not include the cost of makeup ammonia.

Predictor[a]	N addition (g/kg)	Predictor	N addition (g/kg)
Constant	44.81132	R*W	0.49561
R	-0.34281	T*T	0.00153
T	-0.34917	T*A	0.05169
A	-5.00470		
W	-9.66895		
R-Sq	91.30%		

[a] Predictors are R - residence time, T – temperature, A – ammonia loading, W – water loading. Units are as shown in Table 6.1.

In the pretreatment model, all reacted ammonia is assumed to be in the form of acetamide. Changing the amount of acetamide produced during AFEX pretreatment within the model does affect the capital and energy costs of the biorefinery, although these costs are insignificant compared to the changing cost of makeup ammonia. For example, decreasing the acetamide formation by 50% increases the capital costs in the pretreatment area by less than 0.1%. This increase is due to the slight increase in ammonia that needs to be recovered. However, the additional processing cost due to more ammonia recycled is an order of magnitude lower than the savings in makeup ammonia when acetamide formation is decreased. Further research on the interactions of ammonia and biomass during AFEX pretreatment is required in order to build an accurate model of acetamide formation within the Aspen simulation. A reasonable

approximation of its impact can be made by accounting for the change in makeup ammonia costs without considering changes in the capital or energy costs.

6.2.4 Impact of Biorefinery Size

In the Aspen model, the size of the biorefinery does not impact the variable operating costs nor the overall material balance. As expected, the capital costs per ton of biomass are affected, as economies of scale allow a larger refinery to reduce the cost per ton of biomass. Since the fixed operating costs are determined as a function of capital costs, these too are affected. Thus, for all future purposes, direct capital costs and fixed operating costs will be considered simultaneously and labeled as fixed costs. These costs also include all relevant financial assumptions as well. These fixed costs were plotted against biorefinery size and fitted using a power regression. When plotted against annual Mg biomass, the capital cost was determined to follow a power law with exponent -0.5012.

6.2.5 Simplified Economic Model of the Biorefinery

Using the information above, a simplified economic model of a biorefinery can be produced. For a consistent basis, the revenue is determined as $/Mg biomass. A list of equations used in this model is shown below:

$$P = R_{EtOH} + R_{Elec} - C_{Feed} - C_{Enzyme} - C_{NH3} - C_{Other} - C_{Fixed} \tag{6.1}$$

$$R_{EtOH} = P_{EtOH} \cdot (Y_{glu} \cdot M_{glu} + Y_{xyl} \cdot M_{xyl}) \cdot 0.3348 \tag{6.2}$$

$$R_{Elec} = P_{Elec} \cdot \begin{pmatrix} E_{Cons} + E_A \cdot a + E_T \cdot t \\ + E_{AW} \cdot a \cdot w + E_{AT} \cdot a \cdot t + E_{WT} \cdot w \cdot t \end{pmatrix} \quad (6.3)$$

$$C_{Enzyme} = P_{Enzyme} \cdot E_{loaded} \quad (6.4)$$

$$C_{NH3} = P_{NH3} \cdot N_{Added} \quad (6.5)$$

$$C_{NH3} = P_{NH3} \cdot \begin{pmatrix} N_{Cons} + N_R \cdot r + N_T \cdot t + N_A \cdot a \\ + N_W \cdot w + N_{RW} \cdot r \cdot w + N_{TT} \cdot t^2 + N_{TA} \cdot t \cdot a \end{pmatrix} \quad (6.6)$$

$$C_{Fixed} = \begin{pmatrix} F_{Cons} + F_R \cdot r + F_A \cdot a + F_{RA} \cdot r \cdot a \\ + F_{RW} \cdot r \cdot w + F_{RT} \cdot r \cdot t + F_{AA} \cdot a^2 \\ + F_{AW} \cdot a \cdot w + F_{AT} \cdot a \cdot t \end{pmatrix} \cdot \left(\frac{S}{771.11} \right)^{-0..5013} \quad (6.7)$$

Table 6.6 : List of variables used in the biorefinery portion of the direct land use model

	Explanation	Unit		Explanation	Unit
P	Profit	$/Mg BM	P_{NH3}	Ammonia Price	$/Mg
R_{EtOH}	Ethanol Revenue	$/Mg BM	P_{Elec}	Electricity Price	$/kWh
R_{Elec}	Electricity Revenue	$/Mg BM	P_{Enzyme}	Enzyme Price	$/kg
C_{Feed}	Feed Cost	$/Mg BM	N_{Added}	Ammonia on BM	g/g BM
C_{Enzyme}	Enzyme Cost	$/Mg BM	a	Ammonia loading	g/g BM
C_{NH3}	Ammonia Cost	$/Mg BM	w	Water loading	g/g BM
C_{Other}	Other material cost	$/Mg BM	r	Residence time	min
C_{Fixed}	Fixed Costs	$/Mg BM	t	Temperature	C
P_{EtOH}	Ethanol price	$/gal	S	Biorefinery size	Mg/day
Y_{Glu}	Glucose hydrolysis yield	kg/Mg BM	Y_{Xyl}	Xylose hydrolysis yield	kg/Mg BM
M_{Glu}	Glucose fermentation yield	kg/kg glucose	M_{Xyl}	Xylose fermentation yield	kg/kg xylose

A list of variables, their units, and their explanations is shown in Table 6.6. Constants for eq 6.6 are seen in Table 6.5. Constants for eq 6.3 and 6.7 are shown in Table 6.7. These equations were obtained by combining the information obtained in the above sections. In eq 6.2, the extra constant (0.3348) is the conversion of gallons of ethanol to kg, as the price of ethanol is more intuitive as gallons than kg. Two equations, (6.5 and 6.5) are given for the cost of nitrogen. If the amount of nitrogen addition is known, then eq 6.5 should be used. If it is unknown, it can be estimated with eq 6.6, which uses the study based on October Cave-in-Rock switchgrass as seen above. The constants used to calculate fixed costs were determined at 850 short tons per day, which is 771.11 Mg/day.

Table 6.7 : List of constants in the biorefinery portion of the land use model. Units are such that the final equation will result in $/Mg BM.

Electricity		Fixed Costs	
Constant	Value	Constant	Value
E_{Cons}	-0.1833	F_{Cons}	44.41
E_A	0.0260	F_R	0.0755
E_T	9.63E-5	F_A	1.481
E_{AW}	3.66E-3	F_{RA}	.0571
E_{AT}	-3.00E-5	F_{RW}	0.0343
E_{WT}	-8.20E-5	F_{RT}	2.72E-4
		F_{AA}	-0.3092
		F_{AW}	0.0591
		F_{AT}	6.25E-3

6.2.6 Separated AFEX Economics

For AFEX-treated animal feed, the cost of AFEX must be separated from the rest of the refinery. Fortunately, as AFEX conditions do not significantly impact any

aspect of the refinery other than the utilities, only minor adjustments in the biorefinery model need to be made. Feedstock handling, pretreatment, chilled water package, and the cooling tower were included in the capital cost; the remaining equipment is not required. This remaining equipment was determined to be a constant $36.52/Mg in fixed costs, and so this amount is eliminated from the fixed costs determined in eq 6.7. Likewise, the electricity model was changed to eliminate all electricity produced from the lignin and consumed during the remaining portion of the refinery.

In addition, additional capital and energy costs are required to dry the AFEX-treated feed after processing. For the early harvest material, these costs are accounted for in the protein extraction models shown in Sections 6.3 and 6.4 below. For the late harvest, the cost of the dryer is assumed to be $1 million for a 1000 Mg/day facility, which is approximately 20% of the combined dryer/evaporator cost shown in Section 6.3 and 6.4. This reflects both the fact that no evaporator is required as well as the fact that there is less water to remove in this scenario. Due to the uncertainties in how AFEX treated feed will be fed to the cattle, no post-processing of the AFEX material is included other than drying to 15% moisture. However, additional transportation cost is included to ship the AFEX-treated material back to the farms. This cost is assumed to be identical to the transport cost to the refinery.

6.3 Aqueous Protein Extraction

A separate version of the biorefinery model includes a protein extraction option. This model has two extractions, one immediately before and immediately after AFEX pretreatment. However, such a configuration is not likely due to the information presented in Chapter 4. Thus, the process must be redesigned. Rather than use the Aspen model directly, a separate process flow diagram for the proposed setup was created with economic assumptions based on the Aspen model.

The process flow diagram for pretreatment and protein extraction is shown in Figure 6.2. A crossflow extraction column is used to remove protein at a low liquid/solid ratio prior to performing AFEX. It is assumed that the remaining fiber exits at 30% solids loading, which is fed into the AFEX reactor as is. Since ammonia is used during the extraction process, the ammonia fed into the AFEX reactor is reduced by the amount present in the biomass. The protein is removed from the extract via ultrafiltration, and is then dried and sold. A concentration factor of 30 is used for the ultrafiltration step, as seen from Chapter 4. The remaining liquid enters a stripping column to remove the residual ammonia, which is then recycled. The remaining water is used as a diluent for hydrolysis and fermentation in order to recapture the soluble sugars removed during extraction. If desired, the fermentation broth can undergo a separate filtration after distilling the ethanol to recover protein from the fermentation media. A material balance for this process is shown in Table 6.8.

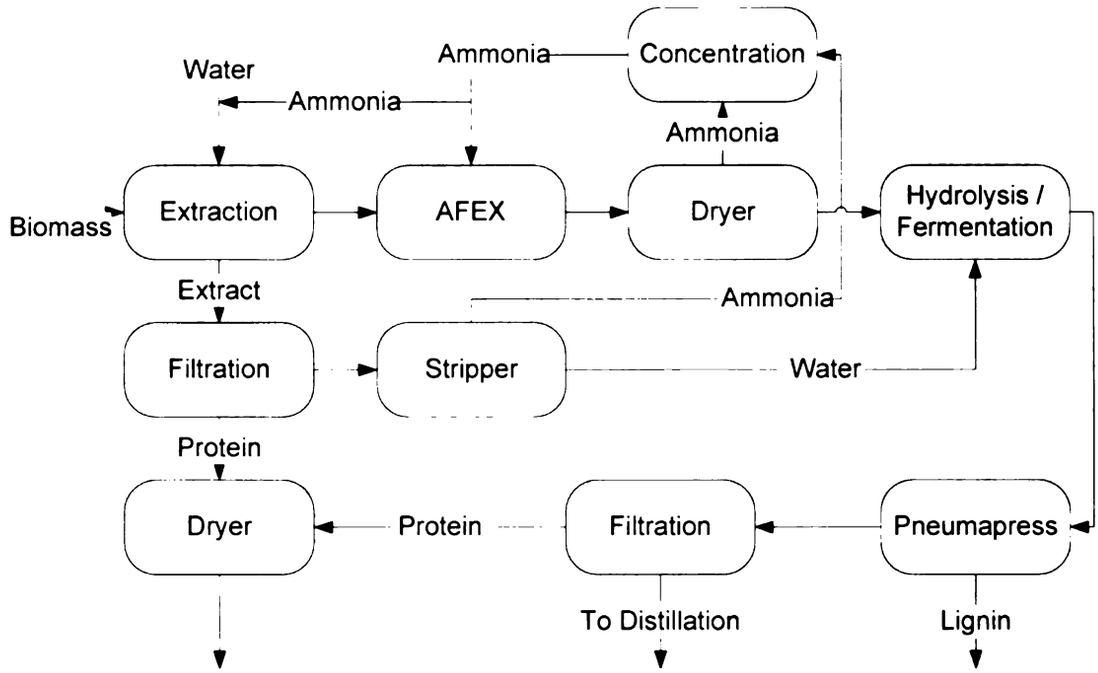

Figure 6.2 : Process flow diagram for aqueous protein extraction

The primary economic drivers of this operation are the capital cost and the operating cost of the ultrafiltration system. For capital costs, the extraction column, filter, dryer, and stripping column must be included. In addition, minor capital costs such as pumps, etc will also be present. No major changes are made to the AFEX process, although pretreatment conditions must be adapted to the high water loading. The ammonia dryer is not affected by the extraction process, although the remaining ammonia recovery system must increase in size to account for the additional ammonia from the stripping column. Operating costs for ultrafiltration modules were assumed to be $0.56/Mg water [97].

Table 6.8 : Example material balance for aqueous protein extraction. Stream labels are shown in Figure 6.2

	Inlet BM	Ext water	To AFEX	To UF	To Dryer	To stripper
Ammonia	0.0	52.0	18.9	33.1	1.1	32.0
Water	110.0	5150.0	1870.0	3280.0	109.0	3171.0
Biomass	1000.0	0.0	872.6	127.4	22.9	104.5
Protein	100.0	0.0	80.9	19.1	11.5	7.6

	To hydrolysis	To AFEX recovery	Hydrolysis/ Ferment.	To UF 2	To Dryer 2
Ammonia	1.6	30.4	1.6	0	0
Water	3105.0	66.0	5129.6	5129.6	331.3
Biomass	104.5	0.0	977.1	n/a	82.8
Protein	7.6	0.0	88.5	103.5	41.4

Other assumptions must be made regarding the costs of the system. Heating costs are relatively low, requiring only steam for the stripping column and the dryer. Drying costs for distillers grains are 0.06 Mg natural gas / Mg water removed, or 3.2 GJ/Mg water [98]. Although lignin would be used the energy source for the dryer, this would decrease the amount of electricity generation in the refinery. It is assumed that electricity generation is 30% efficient, and at $0.05/kWh this amounts to $4.17/GJ of reduced electricity production. For the purposes of this model, this is counted as a cost in the protein model, with the refinery model continuing to produce all electricity. For the stripping column, estimates using Aspen suggest that 0.29 GJ/Mg water is sufficient to remove 95% of the ammonia. Because the water requirement for AFEX is flexible, most of the water from the extraction process can remain in the biomass. Only modest pressure is needed to increase the solids content to 30%. Electricity costs are low, as pumping costs are only a fraction of the total process electricity required in the plant. As a first approximation, it is assumed that total process electricity

costs increase 5% due to the addition of a protein recovery process. Water loss is primarily through drying the protein product. It is assumed that this water is not recovered. Drying the fiber, if necessary, was assumed to require the same amount of steam per Mg water evaporated as the protein dryer. Evaporating the solubles was assumed to be performed using a waste heat evaporator , which would require no additional thermal energy but require $0.75/Mg water evaporated in electrical costs [47].

Protein recovery is also included after hydrolysis and fermentation assuming the AFEX-treated fiber is not used for animal feed. The only additional cost of this process is the added filtration unit and extra dryers. Filtration and drying must be separated if two separate protein products are to be marketed due to the potential for differing quality. As specifications for the capital cost of drying required multiple dryers, this is not explicitly taken into account.

Thus, overall capital and operating costs are listed in Table 6.9. Virtually all of the capital and operating costs are associated with the amount of liquid in the process rather than the solid portion. Not included are the added costs to the ammonia recovery system; these costs are included in the AFEX treatment process. The capital cost for fiber drying and evaporation was estimated from the cost of distiller's grains [98]. All other capital costs were obtained from the integrated biorefinery Aspen model. Minor equipment such as pumps, etc., were assumed to be 10% of the overall capital costs. Overall, the cost for producing

protein in this manner is $22/Mg initial biomass if the fiber is used for ethanol production using the base case assumptions and $48/Mg if the fiber is used for animal feed. The primary difference in costs is due to the high energy cost of drying the fiber after AFEX, which is not required if it is to be used for ethanol production.

Table 6.9 : Variable and operating costs for the aqueous extraction module.

Variable Costs[a]	Cost	Unit	Cost ($/Mg BM)[b]
Filtration 1	0.56	$/Mg water	$1.84
NH3 Stripping	1.21	$/Mg water	$3.84
Filtration 2	0.56	$/Mg water	$2.87
Drying 1 - electricity	0.55	$/Mg water	$0.06
Drying 1 - heat	13.3	$/Mg water	$1.42
Drying 2 - electricity	0.55	$/Mg water	$0.13
Drying 2 - heat	13.3	$/Mg water	$3.18
Other electricity	0.46	$/Mg biomass	$0.46
Lost ammonia	350	$/Mg ammonia	$0.56
Fiber drying	13.3	$/Mg water	$23.13
Evaporator Electricity	0.75	$/Mg water	$3.73

Capital Costs[a]	Cost $MM	TPI $MM	Depreciation[b] $/Mg BM
Extraction column	0.795	2.709	$1.27
Ultrafiltration 1	0.132	0.448	$0.21
Protein Dryer 1	1.949	6.643	$3.11
Ultrafiltration 2	0.142	0.486	$0.23
Protein Dryer 2	2.111	7.195	$3.37
Fiber Drying and evaporator	5.223	17.800	$8.34
Minor Equipment 1	0.288	0.980	$0.46
Minor Equipment 2	0.225	0.768	$0.36

[a] List of operations and equipment. The number 1 on some operations refers to the initial extraction prior to AFEX; the number 2 refers to the filtration of the fermentation media.
[b] Example of costs for the base case scenario, using the material balance shown in Table 6.8.

6.4 Mechanical Pressing

6.4.1 Model choice

Two separate processes for mechanical pressing are established in the literature. The first approach as demonstrated in the Pro-Xan process, uses two twin screw presses to remove the juice following a hammer mill [47]. The second approach, as demonstrated in New Zealand, uses only one screw after a hammer mill, but then passes the fiber residue through a disk mill [48]. After the second milling, a second and third screw press removes the remainder of the juice. In both cases, the protein is removed from the juice through heat coagulation.

The primary difference between these two approaches is a tradeoff between yield and mechanical energy for the mills and presses. The second approach, with the disk mill, has resulted in protein yields greater than 70% compared to 50-60%% for the first approach. However, the disk mill requires approximately twice as much electricity as the hammer mill. As the focus of this study is to maximize production of feed or fuel, the second approach will be used.

Using information from these two sources, an economic and material model can be created for mechanical pressing. There are three primary operations performed in this process: milling the biomass, extracting the juice, and precipitating the protein from the juice. For the purposes of this model, heat coagulation will be used to precipitate the protein, being the most widely accepted practice. A value of 75 g steam/kg water was used for precipitation,

obtained from Enochian et al. [47]. In addition, two assumptions are made regarding the mass balance around the coagulation step. It is assumed that 70% of the soluble protein can be precipitated, and it is assumed that the precipitants are 50% protein. The second assumption agrees with multiple sources [48; 99], while the first assumption is obtained from the New Zealand model [48].

The role of the screw press is to dewater the biomass. Both literature studies considered multiple presses in order to maximize the amount of protein recovered, although both two and three screw presses have been considered. In addition, both studies supply excess water to the biomass. Since presses cannot remove all water, the excess water serves to increase the proportion of water removed during the press, and therefore increase the soluble protein content.

In order to optimize this procedure, a simple model was constructed to determine the number of sequential presses to use and the water content prior to extracting. In this model, it was assumed that the water content in the biomass was reduced to 65% (total weight basis). Likewise, it was assumed that all solubles were well mixed, and so the proportion of solubles removed is equal to the proportion of water removed. The water content of the biomass was varied between 70-90% (total weight basis) and one, two, or three presses were considered. For multiple press scenarios, the water content was increased back to the initial value for subsequent presses. The biomass was assumed to be composed of 20% water

solubles and 80% insolubles based on the composition data obtained in Chapters 3 and 4.

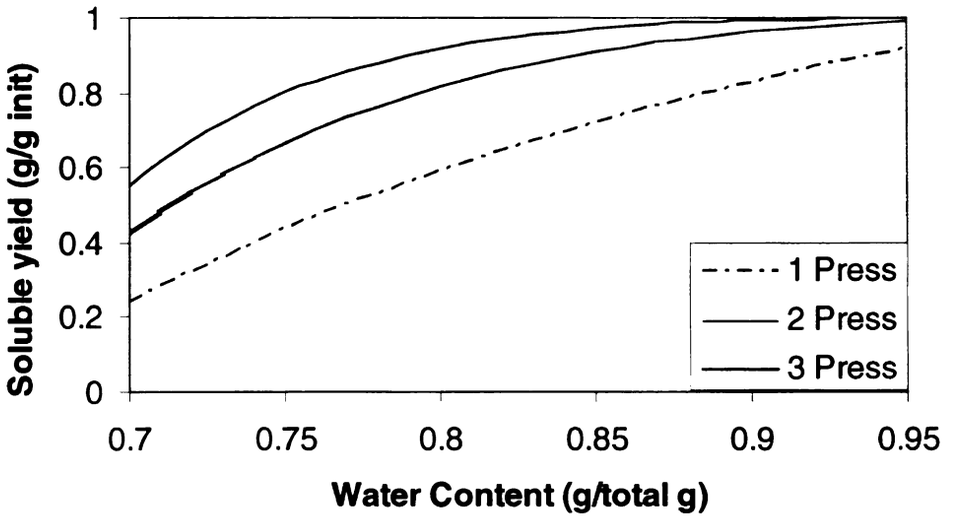

Figure 6.3 : Yield of solubles obtained at different starting water contents (g water / g wet biomass) for a 1, 2, or 3 press system.

The yield of solubles removed by the screw press is shown in Figure 6.3. This does not take into account loss of yield due to incomplete cell disruption or the protein yield after heat coagulation. As expected, yield increases with increasing water content as well as increasing the number of presses. A one-press system does not appear to be economical, as the soluble yield was only 92% when the initial water content of the biomass was an unreasonably high 95% water. In comparison, both of the multipress systems obtained yields in excess of 99%. As yield is a primary driver of the economics of the process, it is unlikely that a one-press system would be desirable.

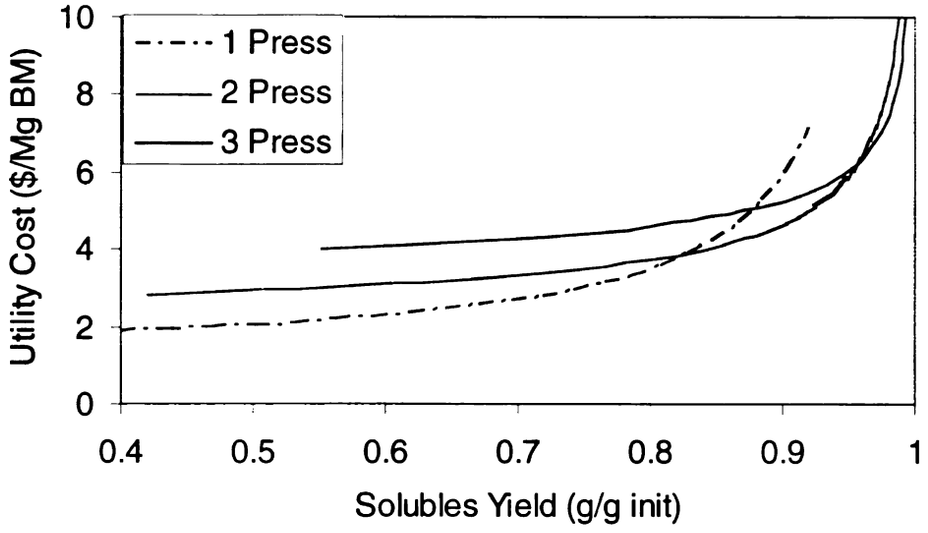

Figure 6.4 : Utility cost (steam for heat coagulation and electricity for the screw press) as a function of solubles yield for the 1, 2, or 3 press system.

In order to determine these tradeoffs, the utility cost of both options was considered. It was assumed that the electricity cost for the screw press is equivalent to 25 kW / dry ton of insoluble residue. One reference fixes a value of 25 kW / dry ton for the last press, where most of the solubles are removed [48]. In addition, a second reference gives a value of 15 kW / initial dry ton [47]. Thus, this value should be reasonable. Steam price was assumed to be $2.40 / 1000 lb. This steam is used to coagulate the juice after pressing. The cost to cool the whey after protein precipitation is not considered, nor is the capital cost of additional presses.

The utility cost relative to solubles release is shown in Figure 6.4. For each press, costs increase as the yield increases due to increased water content, which increases the cost of coagulation. The price trends appear to rise exponentially due to diminishing returns at high water contents, returning only

slight improvements in solubles yield despite adding a large amount of water that must be heated during coagulation. The single press system is the cheapest option for utility costs unless a yield in excess of 82% is desired. However, since high yields are expected and the utility cost is relatively low, this option is unlikely. The three-press system is the cheapest at yields above 95%. If one assumes that the maximum protein product yield is 60% of the solubles (a reasonable value based on McDonald et al.) and a selling price of $360/Mg, a yield of 95% is also the economic optimum.

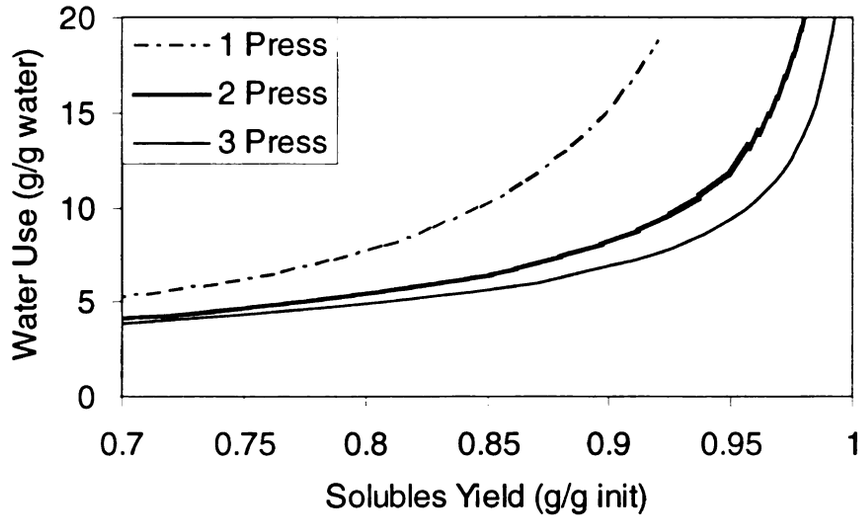

Figure 6.5 : Amount of water required for the process for a 1, 2, or 3 press system.

Since yield and utility cost are the same at this point, other considerations must be taken into account. A three-press system will require the additional capital cost of an extra press, while the two-press system requires more water use. At 95% soluble yield, this amounts to approximately 12 Mg water / Mg biomass for the two press system, while a third press reduces the water requirement to ~9.5 Mg / Mg. In both cases, the water requirement is much greater than that required

for hydrolysis and fermentation, which is likely to be performed between 15-20% solid loading. The excess water is recycled through the process, which increases the concentration of uncoagulated solubles in the protein extraction process. Alternatively, the solubles can be condensed through evaporation. Further information on the impact of concentrated recycles must be obtained before the economic optimum can be determined. For the purposes of this study, however, the two-press option is used in order to save on the additional capital costs. Any adverse effect of increased solubles concentration is not considered.

6.4.2 Mechanical Press model

Thus, a process flow diagram of the mechanical protein extraction process is shown in Figure 6.6. The process consists of both a hammer mill and disk mill to increase cell disruption. The model does not consider the amount of cell disruption from the individual mills, but instead uses the total degree of cell disruption. These two mills are thus treated as one in the process flow diagram. Two presses are used in sequence to remove the juice, which is then combined for direct steam injection. After centrifugation, the protein is dried while the whey is recycled to either the presses or the hydrolysate. An example material balance is shown in Table 6.10.

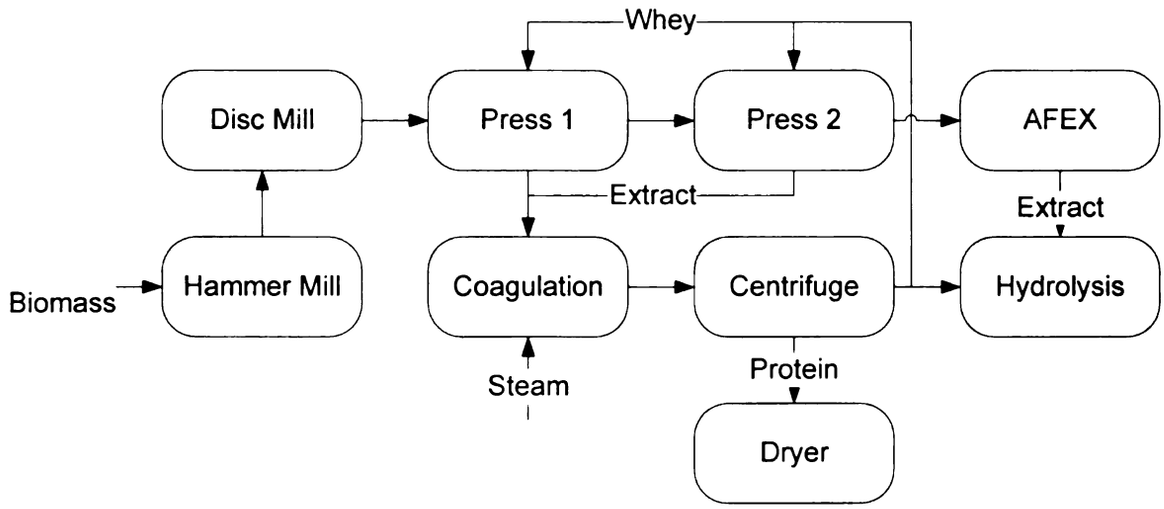

Figure 6.6 : Process flow diagram for mechanical pressing module of the biorefinery. The scenario where the fiber is used for hydrolysis is shown here. If the fiber is used for feed, the remaining whey that would be used for hydrolysis is evaporated and condensed.

For this model, it was assumed that the final moisture content would be 63% after pressing [100]. The solubles are included in the dry biomass, which means the amount of whey recycled impacts the water loading at each press. Because of the presence of the feedback loop, an iterative process was used to obtain the final mass balance in the model. The water content prior to each press was fixed at 85%. The amount of whey added to the hydrolysate was determined by insuring the final ethanol concentration after fermentation would be 6% assuming 90% of the fiber is hydrolyzed. Under these conditions, evaporating a portion of the whey in order to condense solubles was not deemed necessary. If the fiber is to be used as AFEX-treated feed, then the whey that would be used for hydrolysis is evaporated and the solubles returned to the fiber. The biomass was assumed to be reasonably fresh and entered at a moisture content of 50%. If this number increases, it decreases the amount of makeup water required.

Table 6.10 : Example mass balance for the mechanical pressing module of the biorefinery. Example is for 1 Mg biomass; all values are in kg.

	Biomass	Makeup water	Whey to press 1	Cake after press 1	Juice from press 1	Whey to press 2
Water	1000.0	1937.4	4452.4	1281.8	6108.1	5073.7
Insolubles[a]	664.0	0.0	0.0	664.0	0.0	0.0
Protein[b]	56.0	0.0	0.0	9.7	46.3	0.0
Solubles	280.0	0.0	304.1	101.3	482.8	346.5
	Juice from press 2	Cake after press 2	Steam	Protein	Whey	Whey to hydro-lysis
Water	5102.0	1253.5	763.2	432.7	11540.5	2014.4
Insolubles	0.0	664.0	0.0	0.0	0.0	0.0
Protein	7.8	1.9	0.0	54.1	0.0	0.0
Solubles	359.5	88.3	0.0	54.1	788.2	137.6

[a] Includes solubles that were not released due to incomplete cell disruption
[b] Does not include non-coagulating protein

The cost of the process is shown in Table 6.11. The cost of the hammer mill was determined based on the value obtained in Mani et al. [101]. The disk mill was assumed to be twice the cost. Biomass drying and whey evaporation (if not used for ethanol production) were estimated based on distillers grains as stated in Section 6.3. Protein dryers were calculated by the same method used in Section 6.3. The cost of steam was calculated using the heat of vaporization of water at 1 atm (2.257 MJ/kg). This energy was assumed to displace electricity, assuming the efficiency of electricity conversion is 30%. All other capital costs were estimated using Peters et al. [102]. Utility costs were obtained from the two models in the literature [47; 48]. The chemicals mentioned include stabilizers, antifoam agents, and other compounds required to obtain a valuable protein product.

Table 6.11 : Operating and capital costs for the mechanical pressing module.

Operating costs	Required	Unit	Cost ($/Mg BM)[a]
Steam	14.35	kWh	$0.72
Makeup water	1.94	Mg	$0.00
Hammer mill energy	45.00	kWh	$2.25
Disk mill energy	145.00	kWh	$7.25
Press energy	33.20	kWh	$1.66
Centrifuge energy	50.30	kWh	$2.52
Other electricity	10.00	kWh	$0.50
Chemicals			$2.80
	Cost ($MM)	TPI ($MM)	Depreciation ($/Mg)[a]
Screw Press	0.720	2.045	$0.96
Centrifuge	1.000	2.840	$1.33
Dryer	3.028	8.598	$4.03
Disk mill	0.160	0.454	$0.21
Hammer mill	0.080	0.227	$0.11
Steam injector	0.100	0.284	$0.13
Extruder	0.320	0.909	$0.43
Minor Equipment	0.54	1.536	$0.72
Dryer / Evaporator	5.223	14.833	$6.95

[a] Values shown are for the base case scenario with values as shown in Table 6.10.

CHAPTER 7 : DIRECT LAND USE MODEL

7.1 Introduction

In Chapter 6, several models were proposed to calculate the costs associated with AFEX feed and LPC production. Each aspect of the process: pretreatment conditions, pretreatment and feedstock costs, refining costs, and various methods to produce LPCs, were considered separately and adapted from existing models or data. These models must be combined in order to obtain the overall impact of each of these technologies.

Two primary issues are addressed in this chapter. The first is to maximize the economic potential of switchgrass land by considering various technologies. AFEX feeds, cellulosic ethanol, and LPCs are all possible technologies to pursue; however, the economic viability of these technologies is unknown. The second consideration is the productivity of these technologies. Given the concern regarding biofuels overcrowding the food supply, an economically attractive option that requires more acreage for the same amount of food and fuel relative to current technology may not be publicly feasible. Thus, the technologies are considered for the maximum production on the land as well.

Furthermore, given the general lack of research in this field and the immaturity of the cellulosic ethanol industry, the costs and products associated with these technologies are still unknown. While the experimental chapters are intended to

provide a greater understanding of these costs and the material balances, variations between switchgrass types and harvest location are known to produce differences in the response to AFEX treatment. In addition, different LPC technologies are available, as well as differences in various AFEX configurations possible. Naturally, petroleum prices also fluctuate, which affects many of the costs and selling prices used in these models. Because of these differences, sensitivity analyses are vital to fully understanding the potential of these technologies.

Thus, the goals of this chapter are as follows:

- Combine the models from the previous chapter in order to determine the land productivity and economic benefit of different feed and biofuel technologies
- Determine the robustness of each technology through multiple sensitivity analyses
- Identify the most promising technologies for further research

7.2 Method

The economic benefit and land productivity of an acre of switchgrass are determined using the economic models described in Chapter 6. Several options are considered for producing ethanol and/or feed from an acre of switchgrass, and are listed in Table 7.1. These options include all possible scenarios that involve either LPC technology or AFEX treated feeds as well as appropriate

controls. Two potential switchgrass harvests are used: an early July harvest and a late October harvest.

For the economic model, the cost and revenue of each option are determined from the models described in Chapter 6. Agricultural inputs and costs are determined based on yields as well as the number of switchgrass harvests used during the process. Ethanol yields and costs are based on experimental data and the AFEX conditions required for each harvest. Protein yields are based on experimental data and literature, while costs are based on the two types of extraction processes described previously. AFEX feed costs are based on pretreatment costs only, and the selling price of these feeds is determined as the average of the two models seen in Chapter 5. The revenues from ethanol, protein, and AFEX feeds are balanced against the costs, and the overall profit per hectare is determined.

Table 7.1 : Scenarios considered for the direct land use model

	First (July) Harvest	Second (October) Harvest
A	None	Ethanol production
B	None	AFEX Feed
C	Feed (no technology)	Ethanol production
D	Feed (no technology)	AFEX Feed
E	Aqueous protein extract	Ethanol production
F	with fiber as feed	AFEX Feed
G	Mechanical protein extract	Ethanol production
H	with fiber as feed	AFEX Feed
I	Aqueous protein extract	Ethanol production
J	with fiber as ethanol	AFEX Feed
K	Mechanical protein extract	Ethanol production
L	with fiber as ethanol	AFEX Feed

For agricultural production costs, three separate costs are calculated. The first is the price per Mg of biomass produced. This accounts for all of the harvesting costs, which will increase as yield increases due to the need for extra passes, larger equipment, etc. The second is the price per hectare, which accounts for land rent and weed/insect control. The last is the price per harvest, which deals with fertilizing costs. Because an early harvest removes several nutrients from the field, it is assumed that greater fertilizer inputs are required for a two harvest system.

To determine land productivity, each product is compared to the amount of land or petroleum it displaces. For ethanol production, the control is gasoline at a ratio of 0.67 L gasoline/L ethanol based on the energy density of each fuel. For protein feeds, the comparison is the hectares of soy displaced assuming 1.02 Mg protein/ha. For AFEX feeds, an equivalent amount of corn, soy, and grassland is displaced based upon the available TDN, protein, and fiber. For fiber, only feedstocks with long fibers – grass hay, untreated switchgrass, and late harvest October switchgrass – are included. Only long fibers, which comprise about 75% of a ruminants' diet, are balanced in this model. Early harvest switchgrass that has undergone extraction and pretreatment is not included, as the extraction process will reduce the length of these fibers. The values of these three nutrients for each crop are shown in Table 7.2.

In order to compare all scenarios on a constant metric, a value defined as Total Energy Density (TED) is used. This value is defined as the total gasoline potentially displaced by removing one hectare of farmland from animal feeding purposes. For each scenario, the amount of gasoline directly displaced by the hectare of land is added to the potential gasoline displaced from all other animal feed land displaced by the original hectare. It is assumed that all animal feed land displaced is replaced with a single harvest of switchgrass for ethanol. Thus, the equation for total energy displacement is as follows:

$$TED_i = G_i + L_i \cdot G_a \tag{7.1}$$

where TED is the total energy displaced for scenario i, G_i is the gasoline directly displaced (L/ha) for scenario i, L_i is the land (ha/ha) from animal feed displaced by scenario i, and G_a is the gasoline displaced (L/ha) for scenario A.

Table 7.2 : Nutrient values of all feeds produced in this study and references for corn, soy, and grass hay. Values are in Mg/ha for the reference feeds (soy, corn, hay) and Mg/Mg biomass for the feeds produced in the study.

	TDN[a]	Protein	Fiber
Soybean meal	1.73	1.02	
Corn grain	8.92	0.945	
Grass hay	2.38	0.45	2.72
July untreated SG[b]	0.563	0.1	0.739
October AFEX SG	0.63	0.146	0.819
July AFEX SG – MP[c]	0.809	0.056	
July AFEX SG – AQ[d]	0.809	0.097	
Protein product	0.814	0.5	

[a] Total digestible nutrients (see Chapter 8 for a full description)
[b] SG – Switchgrass
[c] AFEX treated fiber from the July harvest after mechanical pressing
[d] AFEX treated fiber from the July harvest after aqueous extraction

In addition, two sensitivity analyses were performed. In the first case, a Monte Carlo simulation was performed by randomly varying several unknown or poorly defined factors. Three levels of each factor are considered and listed in Table 7.3. Protein quality refers to its value in comparison to soybean meal at an equivalent protein concentration. This affects both the selling price of the protein as well as land use. Ammonia, harvesting, and fertilizer costs are varied simultaneously, as all are assumed to be linked to the price of fossil energy. Corn and soy prices are likewise varied simultaneously. Biorefinery size and transport costs are also linked, as the distance to the refinery decreases as the refinery size decreases. All of the switchgrass yields are also varied simultaneously.

The selling price of AFEX-treated feeds is currently unknown. Based upon their composition, digestibility, and cost of corn and soybean meal, an estimate of $140/Mg was obtained. This is similar to the selling price of good quality hay. If the fiber can compete with this hay, then the price should be reasonable [103]. Soybean hulls sell for approximately $80/Mg, and thus represent the low end of the price range. Likewise, leaf protein concentrate value is unknown, and so in this model is tied to soybean meal. The price is determined by the selling price of soybean meal and the quality of the LPC. It is not clear if the quality would be above or below the value of soybean meal. If there's a large amount of indigestible polyphenolics in the concentrate, particularly if bound to the protein, then the quality and therefore selling price will likely be lower than soybean meal.

However, LPCs may contain valuable pigments such as xanthophyll, which can greatly increase the value of the meal [47]. Thus, a wide range of values for the quality of LPCs is used in the sensitivity model.

The costs of producing and harvesting the switchgrass were determined by the only farm-scale study on switchgrass farming economics available [104]. The average cost across all sites studied was considered. To better adapt to the needs of this study, the cost was broken down into three segments. Costs that were constant (cost per hectare) included land rent, seeding, and weed control. It is expected that an early harvest would require greater fertilizer costs due to the first harvest removing nutrients from the soil. Thus, the cost of fertilizer (cost per harvest) is doubled in the two-harvest scenario compared to the single harvest. Finally, it was assumed that harvesting costs were directly comparable to the yield produced, and so make up the final portion (cost per Mg).

The Monte Carlo simulation is used to determine the robustness of each technology using stochastic dominance analysis. This analysis is important in decision making and considers the variability within each process. Both the expected outcome and risk associated with this outcome are taken into account. The variation in land productivity is also considered, although the variation is not as great due to fewer factors impacting material changes. The mean and standard deviation for each approach is shown as well.

Table 7.3 : Variables considered in the sensitivity analyses. The medium values are used for the base case scenario.

Low	Med	High	Unit	Variable	Ref
250	350	500	$/Mg	Ammonia price	[105]
80	160	240	$/Mg	Corn selling price	[106]
180	360	540	$/Mg	Soybean Meal selling price	[106]
1.2	1.7	2.2	$/gal	Ethanol selling price	[107]
80	140	200	$/Mg	AFEX Feed selling price	a
50	100	150	% of soy	LPC Quality	
33	67	100	% of LPC	Post hydrolysis LPC quality	
12	16	20	$/Mg	Agricultural cost per Mg	[104]
110	210	310	$/ha	Agricultural cost per hectare	[104]
27	37	47	$/ha	Agricultural cost per harvest	[104]
5	10	15	$/Mg	Transport cost	
1000	2000	4000	Mg/day	Biorefinery size	
7	9.66	13.71	Mg/ha	Fall only SG yield	[17]
2.23	4.46	6.69	Mg/ha	July yield	[17]
1.67	3.34	5.01	Mg/ha	Fall second cut yield	[17]
50	100	150	g/kg	Protein in July	[17]
65	80	95	%	Degree of Cell Disruption	[48]
30	40	50	%	Aqueous protein yield	b
40	60	80	%	Extraction filtration yield	b
20	40	60	%	Fermentation filtration yield	b
75	100	125	%	Relative protein capex costs[c]	

[a] Estimated based on data in Chapter 5 and feeding value calculator obtained from the University of Wisconsin [108]
[b] Based on results from Chapter 4.
[c] Reflects the uncertainty present in the capital cost of both mechanical pressing and aqueous extraction.

In addition to the Monte Carlo simulation, the impact of each of these variables on the fourteen options is considered. The variables are changed to determine what, if any, conditions are necessary to cause certain options to be superior to others. This is especially important for the multiple protein operations available.

7.3 Results and Discussion

7.3.1 Base Case Simulation

A single October harvest used solely for forage feed (scenario B) displaces more land than any other scenario, as seen in Table 7.4. This is due in part to the higher yield of a single harvest system and the high displacement value of AFEX treated forages. AFEX-treated October switchgrass would be used predominantly as a fiber source, and grass hays have relatively low yields (2.72 Mg/ha of fiber) in this study. While this fiber product also changed the amount of corn and soy displaced, the displacement of grass accounted for 97% of the total land displaced. Because of the high land displacement, this scenario also has the highest potential for total gasoline displacement. The effect of taking one ha out of feed production is equivalent to producing ethanol from switchgrass on 3 ha of land, thus enabling nearly 5000 L of gasoline to be displaced. Likewise, this scenario also produced the largest profit per ha, producing $376/ha, which would likely be split between the farmer and the AFEX processing facility.

In contrast, a single harvest for ethanol production creates a very low profit per ha. The high cost and the relatively small revenue of ethanol production is the primary reason for the low value of this scenario, making it an unlikely option. Likewise, total energy density was also fairly low compared to other scenarios. Because no animal feed is produced on this land, there is no land displacement. This difference between feed and fuel production from the October harvest extends to the multiple harvest scenarios as well. Regardless of how the July

harvest is used, the land displacement is 1.11 ha higher, the total energy density is 1100 L/ha higher, and the profitability is nearly $120/ha higher if the October harvest is used for feed instead of fuel.

Table 7.4 : Direct land displaced, gasoline displaced, and total energy displaced per ha of each scenario produced using the base case assumptions. TED – Total energy displaced.

	Protein (ha)	July Fiber (ha)	October Fiber (ha)	Gasoline disp. (L)	Land disp (ha)	TED (L/ha)
A	0.00	0.00	0.00	1608	0.00	1608
B	0.00	0.00	3.00	0	3.00	4825
C	0.00	1.11	0.00	554	1.11	2343
D	0.00	1.11	1.03	0	2.15	3452
E	0.06	0.45	0.00	554	0.51	1375
F	0.06	0.45	1.03	0	1.54	2484
G	0.24	0.28	0.00	554	0.51	1377
H	0.24	0.28	1.03	0	1.55	2486
I	0.15	0.00	0.00	1453	0.15	1687
J	0.15	0.00	1.03	898	1.18	2796
K	0.24	0.00	0.00	1453	0.24	1833
L	0.24	0.00	1.03	898	1.27	2942

Economic and TED values for the multiple harvest scenarios tend to be between these two scenarios. Scenarios that include using the July harvest as AFEX-treated feed (E-H) provide poor land displacement. This is due to the assumption that the fiber, despite its high digestibility, is not valuable as a displacement for hay. Due to the high yields of corn and soy for energy and protein, respectively, AFEX-treated July switchgrass cannot displace more land than it uses. Combined with protein extraction, only 0.51 ha are displaced per ha of July harvest switchgrass. For aqueous protein extraction, most of this land displacement is in the fiber, as it has a higher protein content due to the lower yields of this process. For mechanical pressing, the land displacement is similar.

In comparison, simply using the July harvest switchgrass as feed without processing the material displaces slightly more than 1 ha of land, as its yield is slightly higher than the expected value for grass hay.

Table 7.5 : Net profit for each operation for all scenarios under the base case assumptions

	Refinery gate ($/ha)	Protein ($/ha)	July Fiber ($/ha)	October Fiber ($/ha)	Ethanol ($/ha)	Total Profit ($/ha)
A	-498.16	0.00	0.00	0.00	533.36	35.20
B	-498.16	0.00	0.00	874.31	0.00	376.15
C	-486.54	0.00	401.40	0.00	183.86	98.72
D	-486.54	0.00	401.40	301.39	0.00	216.25
E	-486.54	-163.63	508.46	0.00	183.86	42.14
F	-486.54	-163.63	508.46	301.39	0.00	159.68
G	-486.54	-61.78	510.19	0.00	183.86	145.72
H	-486.54	-61.78	510.19	301.39	0.00	263.26
I	-486.54	21.22	0.00	0.00	570.92	105.60
J	-486.54	21.22	0.00	301.39	387.06	223.13
K	-486.54	59.31	0.00	0.00	570.92	143.69
L	-486.54	59.31	0.00	301.39	387.06	261.23

Despite the poorer land displacement potential of treating July harvest switchgrass for feed relative to feeding directly to cattle, the economics of these scenarios tend to be better than direct feeding. When mechanical protein pressing is performed, the total profit per ha is $47 greater than if the July harvest is fed directly as feed, although it is $56 less if aqueous protein extraction is performed. This is despite the fact that protein processing is unprofitable in both sets of scenarios. The lack of profitability is due to the high cost of evaporating the solubles and drying the biomass after processing. In addition, the low yields obtained during aqueous extraction also contribute to the high costs. AFEX treatment of the remaining fiber, however, provided additional value

over the untreated fiber, thus leading to improved economics overall. While these results suggest that a scenario in which no extraction process is performed prior to AFEX treatment would further improve the economics, concerns regarding sugar degradation creating neurotoxins (see Chapter 2) may prevent such an approach.

If the July harvest is to be processed, then the scenarios involving using the fiber for ethanol (I-L) have higher energy densities than using the harvest for feed (E-H). Less feed land is displaced, as only 0.17 ha are displaced from aqueous protein refining and 0.24 ha for mechanical refining. However, the July harvest has high ethanol yields, displacing nearly 900 L gasoline per ha. Because of this difference, the overall energy density is higher for July harvests, as the amount of gasoline displaced per ha removed from feed ranges from 1700 – 2900 L/ha compared to 1400 – 2500 L/ha for scenarios with AFEX-feeds in July. In addition, whereas the energy density for aqueous protein extraction and mechanical pressing were nearly identical when the fiber was fed as feed, mechanical pressing displaces an additional 110 L/ha compared to aqueous extraction when the fiber is used as ethanol. Despite the high yields obtained from aqueous extraction, the low quality of the post-fermentation protein limits its value as land displacement, which accounts for the difference in total energy density.

Despite the improvement in total energy density, using the July harvest for ethanol production does not necessarily improve the economics of the process. For mechanical protein extraction, the profit produced per ha is $2 less than if the fiber portion is used for feed. However, if aqueous protein extraction is performed, more profit is produced per acre ($64) for ethanol production than animal feed. In both cases, protein extraction is profitable, as there is no need to evaporate the whey or dry the fiber. However, ethanol production from the July fiber is less profitable than feed production. If ethanol production is performed, there is less difference in the economics between mechanical and aqueous protein extraction, as the post-fermentation protein recovery adds little in cost but greatly increases the revenue of protein recovery.

While the total profit is positive in all scenarios, the opportunity cost in comparison to traditional crops may not be valuable in all cases. Profits for crops can vary widely across seasons and locations. For example, the farmer's profit for traditional crops in North Dakota (one of the states that the switchgrass production costs used in this model is based on) ranged from $180 to $230 per hectare in 2008 [109], but only $50 to $120 per hectare in 2009 [110]. The total profit per ha in this model must be split among both the farmers and the refinery, and thus the farmer sees only a portion of the values shown in Table 7.5. Thus, only a few scenarios would be competitive with traditional crops in 2008, while several could compete in 2009. Farmers would be forced to undergo risk assessment; since switchgrass is perennial, a field of switchgrass would be

planned for several years. Alternatively, switchgrass may only be grown on marginal land rather than prime cropland. In this case, the opportunity cost of growing switchgrass may be less. One approximation of this cost is the Conservation Reserve Program credit, given for land taken out of production for the purpose of growing grasses for environmental purposes. These payments will be $125 per ha in 2010 [111], and thus comparable with the profits produced in this model for both LPC production and AFEX treated feeds.

7.3.2 Monte Carlo Analysis

The mean and standard deviation of the Monte Carlo analysis for economic and material analysis of each condition are shown in Table 7.6. As with the base case simulation, the single harvest animal feed production scenario is the most profitable scenario per hectare of land, and is 34% higher than the second most profitable scenario. Likewise, for all multiple harvest scenarios, using the second harvest for feed production is, on average, more profitable than any scenario where the second harvest is used for fuel production. For scenarios where the October harvest is used for ethanol, mechanically extracting the protein from the July harvest and using the remaining fiber for feed tends is on average the most profitable scenario, while allowing the July switchgrass to be used for feed without any treatment or the fiber to be used for ethanol after protein extraction tends to be less profitable. Scenario E, in which aqueous protein extraction is performed on the July harvest and ethanol is produced from the October harvest,

is the least profitable, showing on average virtually no value. All scenarios had a large standard deviation however, ranging from $203/ha to $596/ha.

Likewise, the average total energy density follows the same patterns as the base case simulation. Again, the single October harvest for ethanol displaces the most gasoline, averaging nearly 5700 L of gasoline per ha of AFEX-treated feed produced. If a July harvest is to be used, either using the untreated material as feed or removing the protein and producing ethanol from the remaining biomass have strong potential for overall gasoline displacement.

Table 7.6 : Mean and standard deviations of both total energy density and profit for the Monte Carlo analysis for each scenario.

	Total Energy Density (L/ha)		Profit ($/ha)	
Scenario	Mean	St. Dev.	Mean	St. Dev.
A	1684	466	18.09	304.58
B	5697	3037	341.94	596.04
C	2649	1622	74.01	203.02
D	4032	2611	179.24	302.44
E	1524	940	2.22	376.75
F	2907	1905	107.45	443.26
G	1526	942	110.52	454.76
H	2910	1907	215.76	515.90
I	1713	782	72.03	319.23
J	3096	1776	177.27	343.30
K	1907	927	119.16	352.17
L	3290	1913	224.39	374.06

For both economic and land displacement analysis, high variability is seen in this analysis. For a closer inspection of this variation, the cumulative distribution function (CDF) of each option is shown in Figure 7.1. As seen from these figures, a single harvest for ethanol production is a very high-risk endeavor, with nearly a 50% chance of being unprofitable. The single harvest for ethanol has a

higher risk associated with it than any other scenario except scenario E, as it has both a higher chance of being unprofitable and greater losses than other scenarios. A two harvest system, with no treatment of the first harvest, decreases this risk, but at the cost of lower profits at the more desirable market conditions. The risk associated with this scenario is also lower than any two harvest scenario with protein extraction. However, scenario K has much greater potential for profitability. If the latter harvest is to be used for ethanol production, these two scenarios, C and K, are likely to be the two strongest depending upon the propensity for risk taking among the producers.

Mechanical protein processing tends to be superior to aqueous extraction across nearly the entire CDF regardless of how the July fibrous fraction is used. If the July harvest is used for ethanol production, aqueous extraction is better in the worst case scenarios (less than 1% probability), although this difference is only ~$10/ha. The benefit of mechanical pressing is greater when the July fiber is used for ethanol, particularly at the more advantageous market conditions. For the top third of the CDF, mechanical pressing on average is $200/ha more profitable than aqueous extraction when the fiber is used as feed, while only $80/ha when the fiber is used for ethanol. This is due to the greater protein recovery for aqueous extraction when integrated with biofuels. Thus, according to this model, it is unlikely that aqueous protein extraction would be pursued. For both aqueous and mechanical protein production, there is less risk associated with using the fiber for ethanol as opposed to feed. However, there is

greater potential for profit with fiber feeding. The choice of these two options is dependent upon the amount of risk that a developer is willing to commit to.

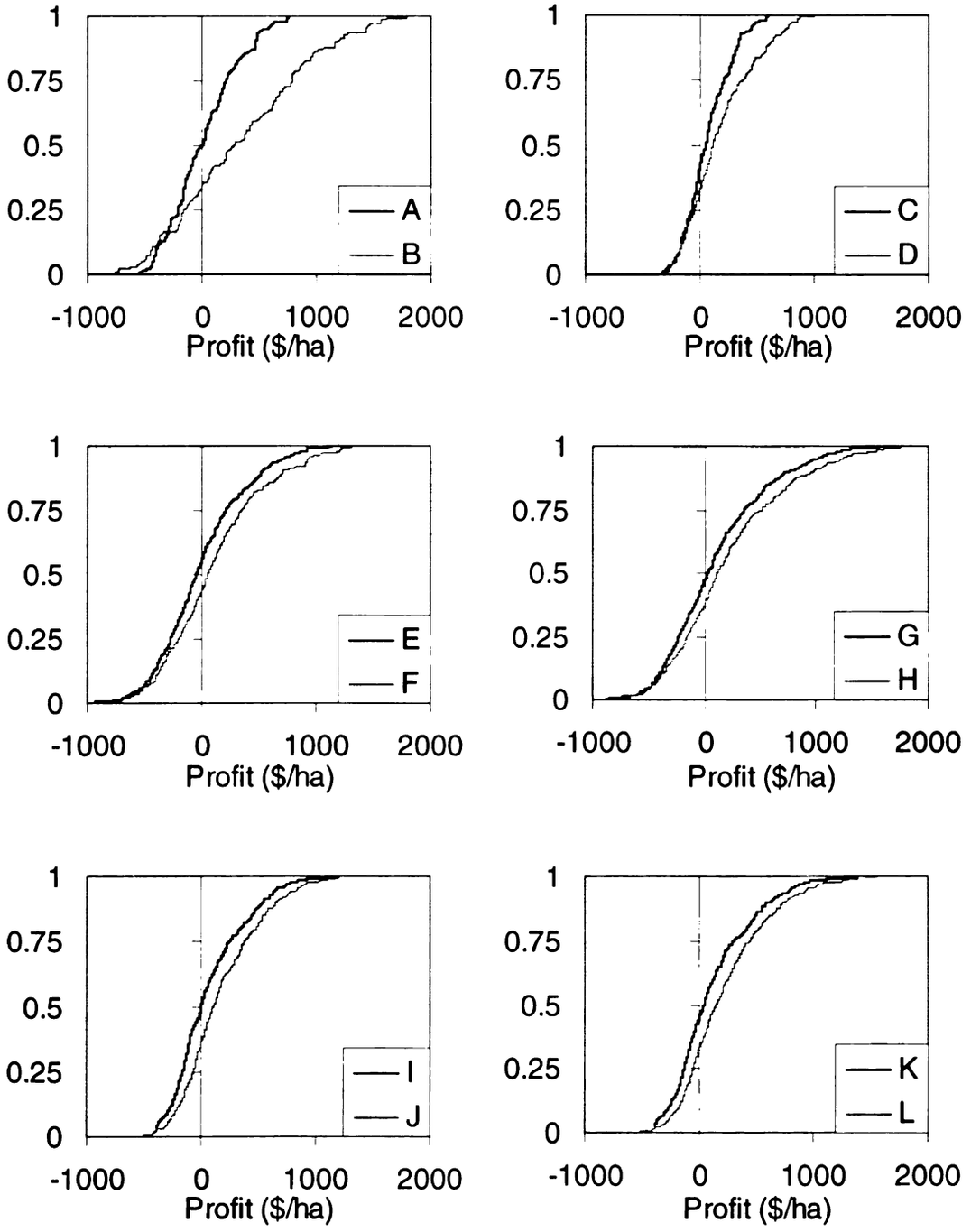

Figure 7.1 : Cumulative distribution functions for profit for all scenarios.

The greatest potential for profit, however, is from the single harvest used as animal feed. This approach has the potential to produce $2000/ha according to this model, and has only a 33% chance of being unprofitable. This is likely due to either low biomass yield or poor selling price of feed. Despite this, it does not stochastically dominate all other scenarios, as several scenarios do not lose as much money at the low end of the spectrum. It should be noted, however, that no new technology is introduced between this scenario and scenario D, where the early harvest is used as untreated feed. In other words, a farmer could potentially choose between a one or two harvest system dependent upon the economic conditions of the year without regard to the capabilities of a refinery or RBPC. Thus, both scenarios are interchangeable on a year-to-year basis given the economic conditions present, further improving the value of these two options relative to all others.

Since economics are unimportant for the land use function, the probability distribution is more discrete and primarily based on farm yields as well as protein extraction yields. Despite this, the preference for a single harvest for feed is also seen in the land use functions, as seen in Figure 7.2. Likewise, Scenario D has stochastic dominance over all other scenarios as well except scenario B. It is clear that, in this model, producing fibrous feed is the strongest way to increase land displacement and therefore increase biofuel production. These are also the only two scenarios that show land displacement greater than 1 ha/ha in the lower 33% probability. This probability range is associated with low

switchgrass yield, and so high yields are vital if co-production of food and fuel is to be performed. However, it should be noted that switchgrass yield is often a factor of location, weather, and soil quality. Thus, a hectare of switchgrass planted in poor cropland would not be competing with the average yield of corn or soy, but rather with lower yields of these crops.

For land use distribution, differences were seen between mechanical and aqueous protein processing when the July fiber was used for ethanol production. Despite the fact that the aqueous production is more profitable, the mechanical press tends to displace more land than the aqueous extraction. This is due primarily to the lower quality of the post-AFEX protein, which decreases its ability to displace soy despite higher yields. When the fiber is used as animal feed, however, there is virtually no difference in land displacement. Any protein that is not extracted remains with the fiber, and thus eventually is used as animal feed.

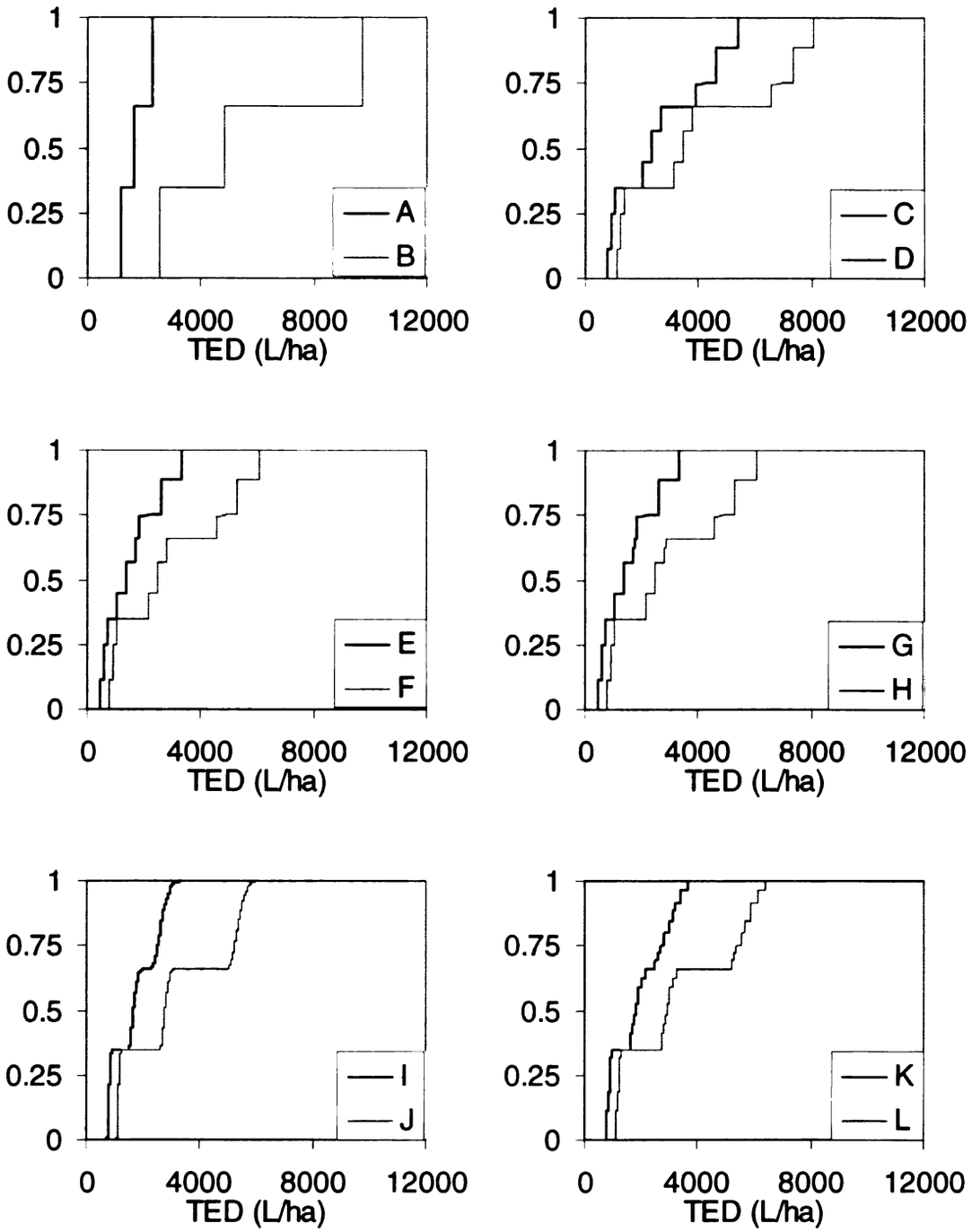

Figure 7.2 : Cumulative distribution functions for the total energy density (TED) of each scenario for the Monte Carlo analysis.

7.3.3 Other Sensitivity Analyses

The impact of each variable on the economics of scenario J is shown in Figure 7.3. This scenario was chosen as representative of all scenarios, as it is the only one in which each variable impacted the economics. All values for each scenario is shown in Table 7.7. The selling prices of the products and co-products, crop yield, and harvest costs, are the dominant influences on the economics of all scenarios. The selling price of either ethanol or AFEX treated fibrous feed has a larger impact on the single harvest scenarios than the multiple harvest, as the single harvest scenarios rely solely on one product. If the selling price of AFEX-treated feed drops from $140/Mg to $80/Mg, for example, the profit from scenario B drops $580/ha, while only dropping $200/ha for the multiple harvest scenarios. Alternatively, crop yields had the largest impact on the multiple harvest scenarios, ranging from $192-$328/ha difference between low and medium crop yields compared to $79-$222/ha for the single harvest system. For the multiple product scenarios such as Scenario J, the selling price of both feed and ethanol are important, as both are produced.

Protein selling price, which depends on the crop price and quality of protein products, is not as important to the overall economics due to the lower overall yields of a protein product. This is true for all scenarios with protein production, although the impact is larger with mechanical pressing due to its higher yields. Despite the uncertainty in the capital cost of the protein recovery options, it has little impact on the overall economics, with less than $10/ha difference for all

scenarios. Biorefinery size and transportation costs have modest impacts on all scenarios. Interestingly, since biorefinery size and transportation cost were varied simultaneously, both the low and high refinery sizes produce less profit than when the size is 2000 Mg/day. This only occurs when AFEX-treated feeds are sold, as they must be returned to the farms. This suggests that, as refinery sizes grow, the RBPC concept is critical if animal feeds are produced as well.

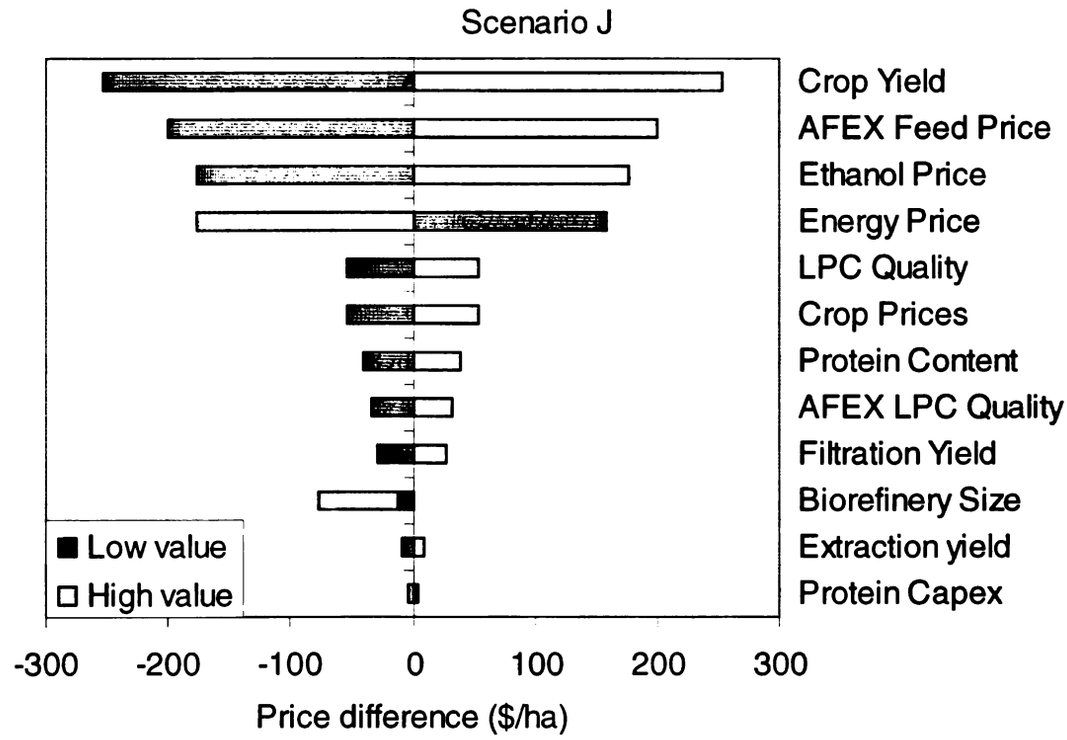

Figure 7.3 : Tornado plot of low and high values for several variables on the economic value of Scenario J. All variable values are seen in Table 7.3. Energy price refers to ammonia price, electricity price, and price per harvest. Crop prices refers to both transportation cost and biorefinery size.

Of particular interest is the impact of the amount of protein recoverable during the filtration process in aqueous protein extraction. For scenario J, the impact of filtration on economics was $29/ha between the low and medium values, compared to $9/ha for protein extraction yields. If the fiber is not used for ethanol

production (scenarios E and F), the impact of filtration drops to only $17/ha due to the very low yields involved with only one extraction. Given the small difference between aqueous extraction and mechanical protein when the fiber is used for ethanol production, improvements in filtration technology or decreasing the breakdown of protein are critical to implementing aqueous extraction. Likewise, the impact of the quality of the post fermentation protein also has a significant impact, changing the economics by $33/ha. If both improved filtration recovery and a high quality of post-AFEX protein can be obtained, then it is possible that aqueous protein extraction can compete with mechanical pressing with improved technology.

This model assumes that the amount of switchgrass harvested in a two-harvest scenario is approximately 20% less than the yield in a single harvest. To test the impact of this assumption, the ratio between the combined yield of the two-harvest system and the single harvest was allowed to vary. The ratio between the yield of the July harvest and October harvest for the two harvest scenario was kept constant. The overall profit per hectare for scenario D, H, and L was compared to scenario B. These three scenarios are the most profitable multiple harvest scenarios.

Table 7.7 : Effect of each variable on the profitability for each scenario. Low and high values are as shown in Table 7.3

Low values	A	B	C	D	E	F	G	H	I	J	K	L
Energy Price[a]	$136	$197	$130	$151	$173	$194	$170	$191	$137	$158	$137	$158
Crop Prices[b]	$0	$0	$0	$0	($342)	($342)	($408)	($408)	($53)	($53)	($87)	($87)
Ethanol Price	($317)	$0	($109)	$0	($109)	$0	($109)	$0	($286)	($177)	($286)	($177)
AFEX Feed Price	$0	($580)	$0	($200)	$0	($200)	$0	($200)	$0	($200)	$0	($200)
LPC Quality	$0	$0	$0	$0	($20)	($20)	($87)	($87)	($53)	($53)	($87)	($87)
AFEX LPC Quality	$0	$0	$0	$0	$0	$0	$0	$0	($33)	($33)	$0	$0
Biorefinery Size[c]	($77)	$72	$2	$54	($4)	$47	($17)	$34	($63)	($12)	($67)	($15)
Protein Content	$0	$0	$0	$0	($21)	($21)	($84)	($84)	($42)	($42)	($82)	($82)
Crop Yield[d]	($78)	($172)	($191)	($250)	($163)	($222)	($215)	($273)	($195)	($253)	($214)	($272)
Extraction yield[e]	$0	$0	$0	$0	($10)	($10)	($33)	($33)	($9)	($9)	($32)	($32)
Filtration Yield[f]	$0	$0	$0	$0	($17)	($17)	$0	$0	($29)	($29)	$0	$0
Protein Capex	$0	$0	$0	$0	$9	$9	$18	$18	$4	$4	$6	$6

High values												
Energy Price	($121)	($243)	($125)	($167)	($207)	($249)	($200)	($242)	($135)	($177)	($134)	($176)
Crop Prices	$0	$0	$0	$0	$342	$342	$408	$408	$53	$53	$87	$87
Ethanol Price	$317	$0	$109	$0	$109	$0	$109	$0	$286	$177	$286	$177
AFEX Feed Price	$0	$580	$0	$200	$0	$200	$0	$200	$0	$200	$0	$200
LPC Quality	$0	$0	$0	$0	$20	$20	$87	$87	$53	$53	$87	$87
AFEX LPC Quality	$0	$0	$0	$0	$0	$0	$0	$0	$33	$33	$0	$0
Biorefinery Size	($10)	($179)	($53)	($112)	($79)	($137)	($69)	($128)	($7)	($66)	($5)	($63)
Protein Content	$0	$0	$0	$0	$21	$21	$88	$88	$39	$39	$85	$85
Crop Yield	$118	$261	$192	$250	$163	$222	$215	$274	$195	$254	$214	$273
Extraction yield	$0	$0	$0	$0	$10	$10	$33	$33	$9	$9	$32	$32
Filtration Yield	$0	$0	$0	$0	$17	$17	$0	$0	$28	$28	$0	$0
Protein Capex	$0	$0	$0	$0	($9)	($9)	($18)	($18)	($4)	($4)	($6)	($6)

[a] Includes ammonia price, electricity price, and agricultural price per harvest

[b] Includes both corn and soy selling prices

[c] Includes both biorefinery size and transport costs

[d] Includes crop yields for both a single harvest and two harvest systems

[e] Includes degree of cell disruption for mechanical pressing and extraction for aqueous extraction

[f] Includes filtration of protein extraction and of the fermentation media

The ratio between these scenarios and scenario B compared to the ratio between two-harvest yields and a single harvest is shown in Figure 7.4. If protein extraction is to be performed, then this option becomes more profitable than a single harvest when the combined harvest is 98% of the yield of a single harvest, regardless of whether the July fiber is used for feed or fuel. Thus, despite the potential for a single harvest devoted to animal feed, more information is required regarding the yields of switchgrass under multi-harvest scenarios. If two harvests produce more biomass than a single harvest, then protein extraction becomes a viable choice under the base case scenario. However, simply allowing the July material to be used as animal feed without treatment would require the multiple harvests to produce approximately 7% more biomass than the single harvest in order to be profitable. Despite the changes in profitability, the total energy displacement still tends to be superior for scenario B. Scenario D required a harvest ratio of 1.13 to obtain higher energy displacement than the single harvest, while scenarios H and L required ratios of 1.58 and 1.34, respectively. Thus, while it may be possible for a two harvest system to be more profitable than the single harvest under the base case scenario, it is unlikely that this approach will be able to displace more petroleum per ha removed from farmland.

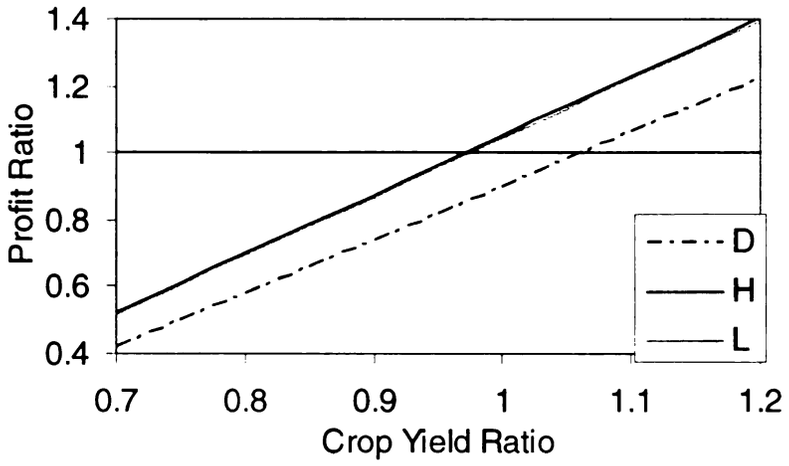

Figure 7.4 : Impact on relative crop yields for a one or two harvest system on the economics of each scenario. The combined crop yield of the two harvest system divided by the yield of the single harvest is the crop yield ratio, while the net profit of scenario D, H, or L divided by the net profit of scenario B is the profit ratio.

7.4 Conclusions

The direct land use model presented in this chapter strongly suggests that a single harvest used solely for animal feed is the optimal use of a hectare of switchgrass land. Under the base case assumptions, this scenario displaced more land and produced a greater profit than any other scenario. Multiple sensitivity analyses confirmed the robustness of this option. While the single harvest for feed scenario showed great variability in response to changing assumptions, it had the lowest possibility of being unprofitable and stochastically dominated all other scenarios except for a two harvest system with no treatment for the July harvest. The yield of switchgrass and the selling price of AFEX-treated fiber were the most important variables in determining the economics of this scenario.

In contrast, a single harvest for ethanol production gave the poorest economic return of all scenarios tested, although it displaced more land than some scenarios. According to the Monte Carlo analysis, there is nearly a 50% chance of this scenario being unprofitable using current information. This result, combined with the benefits of a single harvest for feed as mentioned previously, clearly illustrate the need for dedicated energy crops to diversify beyond bioenergy alone.

For protein extraction, the simulation suggests focusing on mechanical pressing as opposed to aqueous extraction. If the fiber after protein removal is to be used as feed, mechanical pressing is more profitable than aqueous extraction due to obtaining much higher yields. Both protein operations, however, are highly unprofitable due to the need to evaporate the whey after protein concentration. If the fiber is to be used for ethanol production in which no evaporation is required, then there is only a small difference in the profitability of the two protein options. If aqueous extraction is to compete with mechanical protein extraction, research should be focused on improving the quality and recovery of the protein extract rather than the initial protein yield.

CHAPTER 8 : AGGREGRATE LAND USE MODEL

8.1 Introduction

In Chapter 7, the farm scale economics and material benefit were considered. However, this level of analysis does not take into account the potential changes to the overall farm landscape due to changes in animal diets. Likewise, the model does not consider the amount of AFEX-treated feeds or leaf protein concentrate required in the feed marketplace. Thus, it is not clear what the maximum amount of biofuel that can be produced on cropland due to improving feed efficiency. This chapter will explore this impact.

The goal of this model is to determine the maximum ethanol production on cropland harvested for animal feed provided that the land also produces enough animal feed to satisfy current requirements. In addition to animal feed land, current US corn land for ethanol production is also considered, as this land is already producing biofuels as well as animal feed. The model does not take into account the economics of any process involved, nor is geographical location taken into account. Instead, average yields are used for all farmland, and it is assumed that all crops are available for processing or for animal feed regardless of where they are grown. In the large scale, the prices of these commodities will fluctuate if significant portions of the feed market are displaced with new types of feed based on the laws of supply and demand. Because of this, economic modeling would drastically increase the complexity of the model beyond the

scope required here. Instead, it is assumed that the economics will support both AFEX treated feeds and LPC production.

This model serves several primary purposes. Most importantly, it provides a theoretical upper limit to the amount of ethanol produced on current cropland used for feed. This theoretical upper limit is only on the cropland studied, and is thus not a theoretical upper limit on US biofuel production. However, this model can be added to estimates on biofuel production from forest land, idle land, changes in exports, or municipal solid waste. Furthermore, this model is a general estimate of indirect land use change. The second purpose is to determine the size and scope of the two feed technologies used. These two purposes combined can determine what impact the two feed technologies have on the volume of ethanol produced. Third, the model can showcase what the crop distribution would be in a future with the two new feed technologies incorporated.

Table 8.1 : Cropland and yields used for this study [9]

Crop	Size (MM acres)	Yield (Mg/acre)	Yield (Tg)
Corn for feed	40.5	4.07	164.6
Soy	37.8	1.07	40.4
Alfalfa	20.2	3.19	64.6
Other Haylage	41.2	1.71	70.5
Cropland for pasture	35.8		
Corn for ethanol	20.0	4.07	81.6
Total	195.5		421.7

8.2 Method

The land use considered in this model is shown in Table 8.1. This land amounts to 48% of US cropland. Specifically excluded from this land are crops grown for human consumption (primarily wheat), crops grown for export, and idle land. Current animal feed requirements are shown in Table 8.2. These do not include minor livestock such as goats. Table 8.1 does not consider rangeland pasture, specialty protein products such as synthetic amino acids or bone meal, or non-protein nitrogen for ruminants. For the model, the feed requirements were used, with values increased by 10% to account for potential losses. These losses can be due to spoilage, overfeeding (particularly proteins), and other causes.

Due to the scope of the model, various assumptions are made in order to simplify the model for use. Thus, only material balancing is used here, and it is assumed that the economic vitality of the animal feed production is sufficient to produce these changes. The time required to convert farms to these crops and build the refineries and treatment centers is also not considered. Therefore, no attempt is made to predict yields or feed requirements for a specific year in the future, although both are varied as a sensitivity analysis. Finally, location is not considered. It is assumed that all farmland is available for any type of crop and use. Again, sensitivity analyses are used to determine the impact of this assumption.

Table 8.2 : Livestock population and feed requirements in the United States

Type	Animal Class	Herd size[a] 1000	Protein Requirement[b] g/d	Protein Requirement[b] Tg/y	TDN Requirement[b] kg/d	TDN Requirement[b] Tg/y
Dairy	Lactating cow	7997	2750	8.03	11.07	32.31
	Dry cow	1333	1310	0.64	5.14	2.50
	Heifer	4410	1070	1.72	4.59	7.39
	Calves	1877	421	0.29	1.59	1.09
	Total	15617		10.68		43.29
Beef	Lactating cow	15850	1440	8.33	6.00	34.71
	Dry cow	15850	880	5.09	4.41	25.51
	Heifer	5530	900	1.82	4.50	9.08
	Finishing	16800	859	5.27	5.05	30.94
	"Other"	9650	900	3.17	4.50	15.85
	Calves	13023	656	3.12	2.27	10.80
	Bulls	2180	572	0.46	5.30	4.21
	Total	78883		27.25		131.11
Swine	Gestating sow	5127	233	0.44	1.41	2.64
	lactating sow	840	936	0.29	3.98	1.22
	Hogs < 27 kg	21673	194	1.53	0.59	4.67
	Hogs 27-54 kg	15008	350	1.92	1.50	8.22
	Hogs 54-82 kg	12584	401	1.84	1.93	8.87
	Hogs > 82 kg	10847	406	1.61	2.18	8.64
	Total	66079		7.62		34.26
Eggs and Poultry	Layers	340,000	16.8	2.08	0.07	8.18
	Broilers	9075261	1117	10.14	4.00	36.30
	Turkeys	271425	6517	1.77	24.09	6.54
	Total	9686686		13.99		51.02
Nonruminant		9752765		21.62		85.28
Ruminant		94500		37.92		174.39
Total				59.54		259.67

[a] Values obtained from the USDA [9]
[b] Values obtained from Dale et al. [112]

Two primary feed requirements are considered: energy and protein. Energy is measured as total digestible nutrients (TDN), which is defined as 4.4 kcal/g TDN. The energy requirement used is digestible energy. Specific constraints are added to the model to insure ruminant and non-ruminant needs are met. It is assumed that any feedstocks with large amounts of fiber present are unavailable

for non-ruminants, including distiller's grains. Although distiller's grains have been included in limited quantities in non-ruminant diets, this would require separate TDN values compared to ruminants, as they cannot digest the fiber. Since soybean oil is currently used for industrial applications and also grown on this land, oil was also balanced based on the amount produced from soybeans in 2007 [9]. Here, the options available for oil were canola, soy, or, in one sensitivity scenario, corn.

Other major assumptions are as follows:

- Ammonia-based nitrogen is limited to 27% of ruminant protein requirements. This is based on recommended values of 10% for dairy cattle and 33% for beef cattle [113]. While more can be fed, it lowers the performance of the animals. In addition, even if excess true protein is added, excess nitrogen increases the environmental impact of ruminant production, and thus should be avoided.
- Rough fiber is required at 20% of ruminant TDN feed. This is based on the general determination that approximately 25-28% of a ruminant diet should be fiber, of which 75% should be rough fiber [113]. Only alfalfa, cover crops, and AFEX-treated switchgrass are available for rough fiber. AFEX-treated corn stover and DDGS fiber are not considered rough fiber, as they are rapidly degradable and unlikely to be in the form of long fibers.
- At least 60% of non-ruminant protein must be from soy or LPCs in order to satisfy the lysine requirement (approximately 5% of total amino acids)

[113]. Lysine is often the limiting amino acid in non-ruminant diets, and thus is the only amino acid considered.

In addition, several assumptions are made on the farmland available. It is assumed that only 1/3 of the cropland is suitable for growing a cover crop. This applies to all non-forage crops. For corn land without a cover crop, only 40% of stover was allowed to be removed vs 70% for land with a cover crop [29; 114]. In addition, non-forage crops are constrained to the 98.28 million acres, or the amount of corn and soy land currently used for ethanol and feed [9]. Crop rotation was limited to 3 years of corn for every year of either corn or canola.

The model assumes the following crops. The yield and composition of each crop are listed in Table 8.3.

- Corn grain and stover collection: The corn grain can be fed directly to cattle or can be used for ethanol and DG production. The corn stover is AFEX treated and used for either ethanol production or a ruminant feed.
- Alfalfa: Alfalfa can be either fed to cattle as is or used for LPC production. If LPC is produced, the remaining fiber is converted to ethanol. The fiber is not considered for animal feed based on the low digestibility improvements seen for alfalfa in Chapter 7.

- Soybean: The possibility remains that soy is the optimal source for protein and oil production. In addition, soy is needed to insure that corn is not grown continuously on the same land.
- Canola: If soybeans are not produced, this represents a loss of oil for industrial purposes. Canola can produce more oil per acre than soy. Nothing else is produced from canola land other than vegetable oil.
- Switchgrass: Switchgrass can be used for two purposes. The first is as an AFEX treated feed for ruminants, while the second is solely ethanol production. Because of the low protein content present, no early harvest is considered here.
- Cover crops: No additional land is dedicated to cover crops. Instead, 1/3 of corn, soy, and canola land are also used to produce the cover crop. As with alfalfa, the crops can either be fed as is or used for LPC and ethanol production.

In addition to the base case scenario, several alternative scenarios were considered as a sensitivity analysis.

- 0 - Base scenario
- A - All crop yields increase by 25%
- B - Switchgrass yields increase by 25%
- C - No cover crop is produced
- D - Animal feed needs increase 25%
- E - A maximum of 15 billion gallons of corn grain ethanol is produced

- F - Corn oil is produced in addition to ethanol + DDGS
- G - Protein extraction yields increase 25%
- H - Protein extraction yields decrease 50%
- I - A three year rotation is used rather than four years
- J - Non-protein Nitrogen constraint lifted
- K - Non-protein Nitrogen constraint decreases 50%
- L - No LPC is produced; cover crops are used for feed alone
- M - No AFEX treated feeds are produced
- N - Neither AFEX treated feeds nor LPC are produced

Table 8.3 : Yields and nutritional value for all feeds produced in the aggregate land use model.

	Yield Mg/ha	TDN g/g	Protein g/g	NPN[a] g/g	Fiber g/g	Oil g/g	Ref
Corn	10.05	0.887	0.094				[33]
Corn Stover[b]	6.10	0.756	0.042	0.130			Ch 5
Alfalfa	7.90	0.589	0.202		0.396		[33]
Alfalfa LPC[c]		0.214	0.132				
Canola	3.36					0.510	[115]
Switchgrass[b]	13.71	0.630	0.014	0.132	0.819		Ch 5
Cover Crop	5.61	0.631	0.180		0.496		[33]
Cover LPC[c]		0.190	0.117				
Soybean	2.64	0.654	0.386			0.196	[33]
DDGS[d]		0.256	0.096				

[a] Crude protein from ammonia addition (non protein nitrogen)
[b] AFEX-treated feeds
[c] Yield assumes 65% protein recovery and TDN comparable to soybean meal. Values listed are per Mg alfalfa or cover crop, not per Mg LPC.
[d] DDGS – Nutritional values are calculated per Mg corn grain

These scenarios reflect the uncertainty in a future, intensely managed farm scenario. In addition, they reflect the possible limitations of intensive farming.

Cover crops may not be economical to harvest or even available in all areas. Yields for crops can improve with time and technology. This is particularly true for switchgrass, which does not have a history of farming associated with it. Concerns regarding the environmental performance of corn, particularly corn ethanol, also influence the creation of these scenarios. The final three scenarios reflect the importance of the technologies considered in this study.

8.3 Results and Discussion

As stated in the previous chapter, the purpose of this model is to determine the material impact on biofuel production in the United States due to more intensive farming and feed production practices. In addition to the base-case scenario, thirteen additional scenarios were also considered.

The total ethanol produced per year in each of these scenarios is seen in Figure 8.1. In the control scenario, 271 billion liters (GL) per year of ethanol or its equivalent are produced, which is equivalent to approximately 35% of total US gasoline consumption. This value is not the total amount of biofuel that can be produced in the United States, as stated previously. An average of over 3400 liters of ethanol are produced per hectare on land currently devoted to animal feed or ethanol land, which compares favorably to 4200 liters per hectare on current corn ethanol land. This includes 64 GL of starch-based ethanol, slightly higher than the cap given in the EISA 2007 bill as seen in Chapter 1. While this scenario does create more corn land for ethanol production, the presence of a

cover crop means that this land is also producing protein or forage. Furthermore, over 43 million hectares are removed from animal feed production and converted to either switchgrass or corn for ethanol. In other words, the amount of land required to feed animals decreases under this intensive scenario, thereby freeing more land for ethanol production.

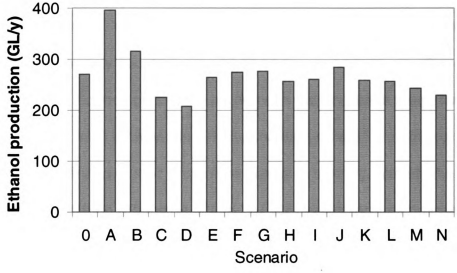

Figure 8.1 : Ethanol production on selected cropland for the base case and 13 scenarios tested.

The first four scenarios produced the largest changes in ethanol production. As expected, increasing crop yields improved ethanol production, in this case to nearly 400 GL per year. This is larger than a 25% change, as animal feed needs remain constant. Eliminating cover crop production and increasing animal feed requirements both have similar impacts, driving the ethanol production down to 210-220 GL/yr. Of the remaining scenarios, lifting the non-protein nitrogen constraint increased production the most, as an extra 13 GL/yr of ethanol are produced. Because AFEX treated switchgrass produces more fiber per ha than

alfalfa or a cover crop, lifting this constraint frees additional land for ethanol production. Both the decrease in protein extraction yields and a three year crop rotation decreased ethanol yields, as both scenarios forced more land into soy acreage.

Of particular emphasis are the last three scenarios. Interestingly, the addition of these two technologies did not cause a great increase in ethanol production, as ethanol production only decreased by 42 GL/yr for scenario N. In other words, adding these two technologies to the market can only increase the potential ethanol produced from farmland by ~18%. Instead, most of the ethanol seen in the base case scenario comes from more intensive farming, namely the addition of cover crops and high producing alfalfa. AFEX-treated forages have a larger impact on improving ethanol yields than LPC production. Compared to scenario N, allowing AFEX treated feeds increases ethanol production by nearly 28 GL/yr compared to only 14 GL/yr for LPC production.

Despite only modest improvements in ethanol production, the two animal feed technologies considered in this study are a large industry in these scenarios, as seen in Figure 8.2. Approximately 85 Tg of AFEX treated forages are produced in nearly all studies. In all cases, the non-protein nitrogen constraint is limiting for this forage. Thus, only scenario J and D (where the constraint is increased 25% due to increased animal feed requirements) increase the size of the industry, while only scenario K (where the constraint is decreased 50%). The amount of

LPC produced varies greatly, however. The base scenario produced approximately 13 Tg of LPC from 52 Tg forage. When animal feed requirements increase, LPC production is more important in order to meet these requirements while still maintaining high ethanol production. Alternatively, LPCs become less important as crop yields increase, or as corn land decreases due to crop rotation limitations. Relaxing the NPN constraint also increases LPC production, mostly due to decreasing the amount of alfalfa and cover crop dedicated to cattle feed. Also of note is scenario H. When low LPC yields are considered, this technology is almost entirely eliminated, indicating the necessity of reaching very high yields associated with a large degree of cell disruption. For the base case scenario, these two industries represent a $17 billion per year industry, assuming an average selling price of $150/Mg AFEX feed and $350/Mg for LPC feed.

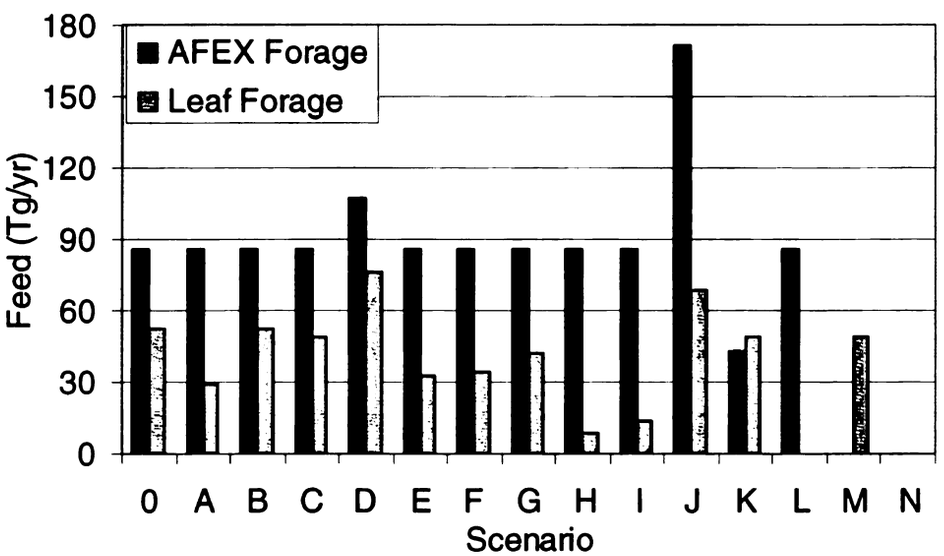

Figure 8.2 : Amount of biomass used for advanced feed technologies. Both AFEX-feeds and forage for leaf protein production are shown.

The makeup of cropland for each scenario is shown in Table 8.4. Corn land is maximized in most scenarios, due to its high overall productivity between grain, stover, and cover crops. It is only when LPC production is eliminated (thus requiring more soy land) or corn grain ethanol is limited (thus eliminating much of the land's value) that corn land is reduced below the maximum constraint. Also of interest is the amount of land present in alfalfa. Alfalfa land tends to be low in most scenarios, and is eliminated at high crop yields due to the amount of cover crops present as well as when the NPN constraint is lifted, as switchgrass produces all fiber needs for ruminants in this case. Eliminating LPC production also nearly eliminates alfalfa, as the cover crop is used solely as cattle feed in these two scenarios. Alfalfa production is high when no cover crop is produced, animal feed requirements increase, or no AFEX feed is used.

Table 8.4 : Land use (in hectares) of the five crops under the scenarios studied.

	Corn	Soy	Canola	Alfalfa	Switchgrass
0	29.8	7.7	2.3	3.9	35.5
A	29.8	9.0	1.0	0.0	39.4
B	29.8	7.7	2.3	3.9	35.5
C	29.8	7.7	2.3	12.4	26.9
D	29.8	7.7	2.3	13.0	26.3
E	28.6	9.5	1.7	2.9	36.5
F	29.8	9.9	0.0	1.7	37.7
G	29.8	7.7	2.3	2.6	36.7
H	25.4	14.0	0.4	0.1	39.2
I	26.5	12.4	0.9	1.1	38.3
J	29.8	7.7	2.3	0.0	39.4
K	29.8	7.7	2.3	7.4	32.0
L	25.4	14.0	0.4	0.1	39.2
M	29.8	7.7	2.3	12.1	27.3
N	25.4	14.0	0.4	8.5	30.8

One interesting metric in these scenarios is the percentage of digestible nutrients in ruminant diets, as seen in Figure 8.3. Here, the base scenario had the most digestible diet, matched only by the high switchgrass yield scenario (this scenario only affected ethanol production and not animal feed makeup) and the high LPC yield scenario. The lowest TDN diet is the scenario in which no advanced feed technologies are produced, as the digestible nutrients in nonruminant diets decrease by five percentage points compared to the base case. This mirrors the discussion in Chapter 5, and indicates that hidden benefits in the efficiency of meat or dairy production may be present in new feeding technologies. If, as this model states, ruminant diets overall are more digestible, then the cattle performance (growth or milk production) would not be as limited by the amount they are able to eat per day. Again, AFEX treated feeds show greater improvements in %TDN compared to LPC production relative to scenario N, which is unsurprising given LPCs tend to displace soy meal, which has the same TDN value as LPC.

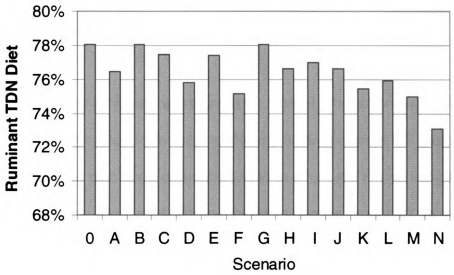

Figure 8.3 : Amount of digestible nutrients in ruminant diets as a percentage of dry matter intake.

8.4 Conclusions

In summary, nearly half of the cropland currently devoted to animal feeding can be diverted to cellulosic ethanol production without impacting feed production. Most of this land is freed by adding and harvesting cover crops to corn, soy, and canola land. However, an additional 43 million hectares can be devoted to ethanol production with the addition of LPCs and AFEX feeds. While these two technologies only result in an 18% increase in potential ethanol production, they may be vital to the economics present. Growing and harvesting a cover crop may not be economical without LPC production, which dramatically reduces the potential for ethanol as seen in Scenario C. Likewise, the potential for high profits from AFEX treated feeds increases the possibility of collecting lignocellulosic material for both feed and ethanol production. Thus, scenarios K-

M do not supply the full picture for the impact of these technologies on ethanol production due to the limitations of this model.

These results clearly indicate the potential for biofuel production within the United States without harming feed production. Nearly 35% of current US gasoline consumption can be displaced on cropland alone while still maintaining the total meat and dairy requirements. Additional biofuels can be produced on rangeland, forest land, cropland used for exports or human use, and idle land. The improvements are primarily due to intensive farming rather than new technologies; however, these technologies may enable the intensive farming to be profitable.

CHAPTER 9 : CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

Based on the experimental results and models presented, AFEX-treated fibrous feeds have strong potential as a method to integrate feed and fuel production. Preliminary experimental results were promising for a wide variety of feedstocks, ranging from a 50 g NDF/kg BM increase in digested fiber for alfalfa to 350 g/kg for late harvest switchgrass. According to the direct land use model, AFEX-treated switchgrass can displace large amounts of animal feed land and is highly profitable relative to biofuel production. These advantages are robust, as the value of this approach tends to remain strong among a wide variation in unknown variables. There is also a large potential market for AFEX-treated feeds, as up to 85 Tg can be annually produced within the United States according to the aggregate land use model compared to only 52 Tg of harvested biomass for protein production.

The nature of AFEX feeds also allows for flexibility in commercializing and marketing the technology. As stated in Chapter 5, certain feedstocks such as early harvest switchgrass or corn stover may be competitive with corn grain, while other feedstocks such as late harvest switchgrass can be competitive with high value forages. Because AFEX treatment improved digestibility in multiple feedstocks, there is potential for commercialization throughout the United States and the world. Furthermore, standalone AFEX treatment facilities can market

their product to ethanol facilities as well, opening multiple markets to commercialization.

Despite the strong potential for commercialization, there is a large knowledge gap for this technology before it is viable. Although the in vitro studies show large improvements in digestibility, the results must be replicated in vivo. Full animal feed trials must be completed in order to determine the actual value of AFEX treated feeds.

Leaf protein concentrates also appear to be an economically viable integration technique, although the amount of land saved due to this technology is less than that of the AFEX-treated fibrous feeds. Using the remaining fiber for ethanol production rather than animal feed eliminates the cost of drying the fiber and evaporating the whey. Furthermore, the productivity of the land increases if the fiber is used as ethanol, as early harvest extracted switchgrass would displace highly productive corn land. Aqueous protein extraction combined with enzymatic hydrolysis of cellulose was able to solubilize virtually all of the protein in switchgrass. However, only 35% of this protein could be concentrated using ultrafiltration. According to the direct land use model, a hectare of switchgrass could conceivably displace 0.24 ha of soybean while still producing a biofuel such as ethanol.

Both yield and economic value were not appreciably higher with alkaline extraction and ultrafiltration than literature values for mechanical pressing and steam injection. This latter method of producing leaf proteins can be commercialized in conjunction with biofuels, although potential for improving the technology of LPC production is still possible. Likewise, it is unlikely that integration with AFEX or other pretreatments will improve protein yield or quality.

9.2 Recommendations for Further Study

Based on the results of this study, further research into animal feed integration with biofuel production is warranted. For AFEX treated feeds, research into potential ammonia-based toxins is required before feeding trials can be performed. Determining the amount of 4-methylimidazole formed during AFEX is necessary to insure the concentration is below levels mentioned in the literature that caused toxicity in cattle. If too much imidazole is produced, then research into reducing these levels, either through performing AFEX at milder conditions or preprocessing the biomass, is required. Likewise, a complete mass balance of all ammonia based compounds produced during AFEX is preferable in case other toxins are present. This research can coincide with fundamental research into the kinetics of ammonia-biomass interactions, and would additionally provide more information into the nutritional value of non-protein nitrogen within the feed.

Another possibility for future research is to determine the fermentation profile for AFEX treated feeds. The relative ratios of volatile fatty acids produced can help

determine the value of these feeds, particularly for lactating dairy cows and fattening steers. In addition, the amount of methane produced can be quantified in these fermentations. This can be particularly important given the potential environmental regulations surrounding feed and fuel production. The quality of the fiber can also be assessed to determine if the AFEX treated feed can produce a fibrous mat required for a healthy rumen.

Finally, feeding trials are required to insure that AFEX treated feeds are healthy and effective, and would also help assess how such feeds could be produced commercially. Important considerations are the particle size of the biomass being used, whether or not pelletization after AFEX is required or desirable, and if the treated fiber needs to be dried for storage. The length of the fibers may be a tradeoff between the ease of transferring biomass within the treatment process and the value to the cattle. AFEX treatment is likely to improve the stability of the fiber, which could reduce or eliminate a costly drying process. Additionally, these trials would improve the economic modeling, as they would provide a better estimate of the costs of the process as well as the potential selling price of the fiber.

For leaf protein concentrates, further research into pretreatment and hydrolysis of extracted biomass is needed to improve sugar yields. Likewise, this research can be expanded to include feedstocks likely to be used for protein production such as alfalfa and viable winter cover crops. Potential fiber processing research

includes re-optimizing pretreatment conditions for the fiber product and determining if glucan yield can be increased to levels comparable with non-extracted biomass. The impact of drying the material after extraction in comparison to pretreating the material at its native pretreatment may also be considered. Separate research into changes in inhibitor formation during AFEX due to a pre-extraction would also be useful for advancing biofuel production.

Further research into aqueous alkaline extraction is required in order to become economically competitive with mechanical protein extraction. The impact of lignin or ammonia-based degradation products on the quality of protein concentrates obtained from a post-AFEX extraction or from the hydrolysate or fermentation media is currently unknown. Simple pepsin and trypsin digestibility tests may provide some information, although small scale animal feed trials may be performed as well. In addition, a comparison between protein recovered after hydrolysis and after fermentation can be performed to determine the effect of microbes on the recovered protein. This approach does not necessarily require an initial protein extraction, and can be used on late harvest materials as well if the microbes can produce enough protein from residual ammonia compounds following AFEX pretreatment.

Finally, research into reducing protein degradation in order to improve ultrafiltration yields may be performed. This may require research into fresh harvests to determine if the proteins are degraded prior to initially drying the

biomass. Likewise, protein degradation during hydrolysis and fermentation must be closely monitored and controlled if possible due to the poor yields in concentrating the hydrolysate media. If it is impossible to keep the protein size above 10 kDa, then research into smaller filtration cartridges may be necessary. Concern here is that the protein content of the final product may be too low. If so, some pre-processing may be required to hydrolyze any polyphenolics or other compounds that were concentrated.

With improved experimental research, the models can be updated to improve their reliability. Likewise, the models themselves can also be refined. Because no pilot scale facility of AFEX pretreatment and particularly ammonia recovery system has been constructed, the costs associated with pretreatment are only estimates. The ammonia recovery system modeled here is a novel approach that does not currently have experimental validation. Replacing this model with a more conventional ammonia recovery system may be more applicable in the near term. Furthermore, improved information on handling for AFEX treated materials and designs for their use as animal feeds can be included as well. This can result in one or multiple models of a regional biomass processing center, which may or may not include protein production. An RBPC model could expand the scope of the land use model, allowing for multiple scales of operation to be considered at once. Likewise, including geographical considerations into both the direct land use model and aggregate model would allow for more accuracy.

These considerations include changing biomass yields and the feasibility of certain operations in different climates.

APPENDIX A: DISTILLER'S GRAIN HYDROLYSIS

A.1 Introduction

While the primary aim of this research was focused on switchgrass and other cellulosic feedstocks, an interesting opportunity for co-producing food and fuels is through distiller's grain fractionation. The dominant process for producing grain ethanol in the United States is the dry-grind process. During this process, distiller's dry grain and solubles (DDGS) is created as a coproduct generally sold as an animal feed. The various forms of distiller's grain have been cited as being ideal for early cellulosic ethanol production due to the ability to integrate into starch-based facilities and the low lignin content of DG [116; 117]. Such a process may also lower the risk of saturating the market for DGs, which would their selling price and thus affecting the viability of the industry. In addition, removing the fiber from DG increases the possibility of using the remaining material as feed in the swine and particularly poultry markets.

Despite the potential of this resource, little effort has been placed in studying enzymatic hydrolysis and fermentation of DG before this study. Furthermore, due to the high quantity of protein present, it is prudent to use mild pretreatments on the grains in order to prevent degradation of this protein. Thus, AFEX is a strong contender for such a first generation refinery. The goal of this chapter, then, is to assess the potential of AFEX for DG hydrolysis.

There are several concerns specific to DG pretreatment and hydrolysis that needs to be addressed. First, the variability of DG, both in types and across various refineries, may result in differences in both yield and conditions. Belyea et al. found significant variations in DDGS composition from one ethanol facility across five years of operation, including both the fiber and starch components [12]. While research into DDGS is common due to its ability to be stored indefinitely and ease of use in handling, a first generation ethanol facility would likely use wet DG or DGS as a feed source in order to eliminate the costly drying operation. The high moisture content of these materials, as well as the different composition of DG vs DGS, may require changes in AFEX conditions and result in different yields.

All forms of DG also have relatively low fiber content compared to traditional lignocellulosic feedstocks. In general, 30-40% of DG consists of carbohydrates, compared to 50-70% for grasses or woody materials [12]. Ethanol production requires high ethanol titers in order to make distillation economically viable. However, since DG fiber content is so low, a higher initial DG solid loading is required in order to achieve high ethanol concentration. This may create mixing issues as well as the traditional problems associated with end product inhibition.

Because DG is a unique lignocellulose source, it may also require unique enzyme mixtures to effectively break down the structure. The hemicellulose in corn grain is a complex arabinoxylan structure, consisting of a xylan backbone

with several branching and crosslinked chains [118]. The xylanases found naturally in enzyme cocktails such as Spezyme CP or Multifect Xylanase may not be sufficient. Research performed cocurrently with this project at the USDA National Center for Agricultural Utilization Research has shown that a combination of pectinase and ferulic esterase are required to effectly break down hemicellulose [69]. In addition, a significant portion of the glucan in DG is starch, which requires amylases to remove. However, these enzymes are known to be inhibitory to cellulase enzymes.

Finally, throughout the pretreatment and hydrolysis, careful attention must be given to the protein content present in the DG. The revenue associated with increased ethanol production per ton of corn due to DG processing will be less than the revenue lost from DG sales. Thus, it is vital that the remaining material in DG, particularly the valuable protein, retain its value throughout the process. This scrutiny will be further discussed in Appendix B.

Thus, the objectives of this chapter are as follows:

- Optimize AFEX conditions for both DDGS and WDG
- Assess the variability of DDGS, both from different vendors and different batches of material
- Determine the effect of high solid loadings
- Investigate cellulase and amylase addition

A.2 Materials and Methods

A.2.1 Material Source

Several sources of DG were used throughout these experiments. Unless otherwise indicated, the DDGS or WDG from Big River Resources, LLC. Two separate batches of DDGS were obtained from Big River, one in 2005 and the second in 2006. Unless otherwise indicated, the 2005 batch was used for these experiments. The WDG used was obtained in 2005 as well.

A.2.2 Biomass Pretreatment

For early experiments focusing on changing AFEX conditions, the AFEX pretreatment process was performed in a 300 mL stainless steel pressure vessel. When not performed at the native moisture content, water was added to DDGS and evenly mixed before loading it into the vessel. Glass spheres were added to minimize void space, thereby reducing the amount of ammonia in the vapor phase within the reactor. The lid was bolted shut, and a separate cylinder loaded with the proper amount of liquid anhydrous ammonia was connected, allowing the ammonia to be charged into the vessel. The reactor was heated using a 400W PARR heating mantle, and allowed to stand at the desired temperature (+/- 1°C) for five minutes. The pressure was explosively released by rapidly turning the exhaust valve. The treated samples were removed and were placed in a fume hood overnight to remove any residual ammonia.

With improved AFEX process designs, high solid loading experiments were performed using rapid heating of biomass. Both the biomass and ammonia were heated prior to the introduction of ammonia into the reaction vessel. In addition, the temperature of external heating mantle is limited to 20°C above the desired temperature in order to reduce burning or charring. After ammonia addition, the biomass rapidly heats to the desired temperature within one minute. The total residence time before the ammonia is released is 15 minutes.

A.2.3 Enzymatic Hydrolysis

The enzymatic hydrolysis procedure is based on that described in Chapter 3. An amount of biomass equal to 0.15 g cellulose was placed in a vial and brought to a total volume of 15 mL with autoclaved water. The solution was buffered to pH 4.8 by 0.75 mL 1M citrate buffer. Spezyme CP (Genencor, Palo Alto, CA) cellulase was loaded at 16.5 FPU/g glucan (31 mg protein/g glucan), and β-glucosidase (Novozyme 188, Bagsvaerd, Denmark) at 56 pNPGU/g glucan. One experiment involved the amylase Stargen (Genencor) added along with the cellulases. All samples were incubated at 50°C with 75 rpm rotation. Samples were collected at various time points after hydrolysis, generally 24 and 72 hours.

A.2.4 Analytical Methods

Sugar analysis was done using a Waters High Performance Liquid Chromatograph (HPLC) system equipped with a Bio-Rad (Richmond, CA) Aminex HPX-87P carbohydrate analysis column. Degassed HPLC water with a

flow rate of 0.6 mL/min was used as the mobile phase, while the temperature in the column was kept constant at 85°C. A Waters 410 Differential Refractometer was used to measure the peaks obtained.

A.3 Results and Discussion

A.3.1 DG Composition

A complete composition analysis on the distiller's grains and wet cake of four different refineries is seen in Table A.1. Mass closure for the wet DG samples were near 100% except for the third sample. In general, there were not large differences in protein, glucan, or xylan content (<10% difference) between the first three refineries, indicating a reasonable consistency among separate feedstocks. The fourth sample shows significant differences, both in the dry matter content as well as the overall composition. This sample was taken after the solubles were added, making it wet DGS rather than wet DG. This may be an acceptable alternative to separating wet cake and solubles, and thus is also a potential feedstock. All four samples of DDGS were also consistent, although sample 1 has a lower protein and higher glucan content than the other samples. Mass closures greater than 100% are due to the presence of protein and ash in the water extracts. As seen in Appendix B, only a small portion of protein is water soluble, and the mass closures would be near 100% if 20-25% protein is soluble, which is in the range reported by various studies.

Table A.1: Composition of four different distiller's grain samples used in this study. Sample 1 was obtained from Big River Resources and is used in all experiments in this chapter. Sample 4 was obtained from the Woodbury, MI plant of US Bioenergy and used for all experiments in Appendix B.

	DDGS			
	1	2	3	4
Water Extractives	23.1	23.0	24.6	21.7
Ether Extractives	11.4	11.9	11.3	10.0
Crude Protein	27.3	31.1	31.7	32.0
Glucan (total)	21.3	19.3	19.4	19.4
Xylan and Arabinan	18.9	17.6	16.7	18.5
Xylan	12.5	11.6	11.1	12.1
Arabinan	6.4	6.0	5.6	6.3
Ash	4.5	4.4	4.3	4.4
Mass Closure	106.5%	107.4%	108.0%	105.9%

	Wet DG			
	1	2	3	4
Water Extractives	8.1	10.4	3.6	20.2
Ether Extractives	8.1	8.2	7.0	11.4
Crude Protein	33.7	36.4	36.0	29.6
Glucan (total)	22.1	20.3	21.1	18.1
Xylan and Arabinan	26.9	23.9	24.2	15.8
Xylan	15.8	14.7	15.3	10.3
Arabinan	11.1	9.1	9.0	5.5
Ash	2.2	2.1	1.9	5.5
Mass Closure	101.1	101.2	93.9	100.5

A.3.2 Optimizing AFEX Conditions for DDGS

The effect of pretreatment temperature on glucose released after enzymatic hydrolysis is seen in Figure A.1. Glucose yields increase sharply from 60°C to 70°C, while further temperature increase has a statistically insignificant ($p<0.05$) effect at 72h. Further temperature increases do appear to slightly increase the rate, as the glucose released after 24 hours was higher at 80°C than 70°C. At 100°C, the grain appeared to be burnt, although this did not affect glucose yields.

In all cases from 70°C to 100°C, greater than 100% of the theoretical glucose yield from cellulose can be obtained after 72 hours of hydrolysis. Thus, there are two possible explanations for obtaining yields in excess of 100%: either the measured cellulose composition is lower than its actual value or there is some starch hydrolysis occurring. It was found that the β-glucosidase complex used in this study, Novozyme 188, has some amylase activity, making it likely that at least a portion of the residual starch in the DDGS is hydrolyzed as well.

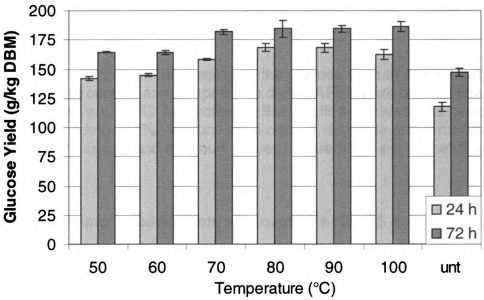

Figure A.1: Effect of temperature on the glucose yield of AFEX treated DDGS. During AFEX, water loading was held constant at 0.13 g/g biomass, and ammonia loading at 1.0 g/g biomass. Enzymatic hydrolysis was performed at a cellulose loading of 1%. All runs were done in duplicate and error bars represent the maximum and minimum values. Untreated (unt) is shown as a control.

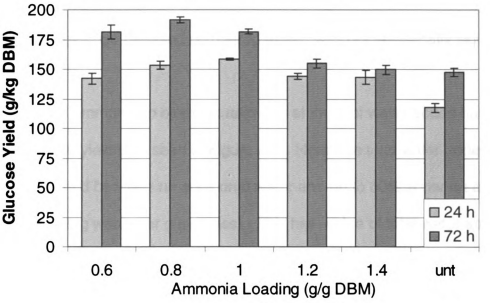

Figure A.2: Effect of ammonia loading on the glucose yield of AFEX treated DDGS. Moisture content and temperature during AFEX were held constant at 0.13 g/g biomass and 70°C, respectively. Enzymatic hydrolysis was performed at a cellulose loading of 1%. All runs were done in duplicate and error bars represent the maximum and minimum values. Untreated (unt) is shown as a control.

Relatively low ammonia loadings are also needed for DDGS hydrolysis, as seen in Figure A.2. There is a slight increase in glucose yield from 0.6 to 0.8 g NH_3/g biomass, and a slight decrease from 0.8 to 1 g NH3 / g biomass. Yields dramatically decline at higher ammonia loadings. It is not immediately clear why high ammonia loadings reduce glucose yields, although it may be due to an increase in competing reactions such as those between ammonia and sugar. These trends are similar to other types of easily digestible biomass, including early harvest switchgrass (see Chapter 3). Approximately 190 g glucose was released after 72 hours at the optimal conditions, representing 81% of the total

glucan (starch + cellulose) conversion. When hydrolysis was extended to 168 h, the increase in glucose released compared to 72h was not statistically significant.

At 70°C and 1:1 ammonia to biomass ratio, the amount of water added had little effect on glucose yields, as seen in Figure A.3. Here, the total water added to the biomass varied between no additional water and up to 60% water on a total weight basis (1.5 g water per g biomass). This high value of total water addition is approximately the moisture content prior to drying the material. No significant effect ($p<0.05$) of water addition is seen for either 24 or 72 hour glucose yields. With little lignin present in DDGS, the primary effect of AFEX on cellulose conversion is likely the swelling and decrystallization of cellulose. The presence of water appears to be irrelevant to these reactions. Despite the similar glucose yields, AFEX treated DDGS at high moisture appeared much darker than low moisture samples. As this did not effect glucose conversion, the darker color is likely due to increased reactions with other components in the biomass, including possibly protein.

While AFEX improved yields over untreated material, high glucose yields were obtained from untreated DDGS as well, at nearly 150 g/kg biomass. This is most likely due to the relatively low lignin and cellulose content as well as the effect of the dry grind ethanol process. The presence of lignin can create inhibitory byproducts during pretreatment such as coumeric acid. In addition, lignin that is not removed to the surface of the biomass prevents enzymes from reaching the

cellulose, and enzymes may also bind to lignin and deactivate, lowering the overall enzymatic activity of the hydrolysis. During the dry grind process, the corn fiber is naturally pretreated, being subjected to milling and elevated temperatures during the steeping, distillation, and drying processes. These act as mild pretreatments, improving sugar yields without undergoing AFEX or other processes required for lignocellulosic biomass.

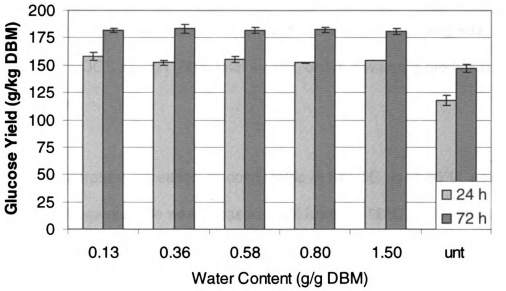

Figure A.3: The effect of water content during AFEX on glucose yields from DDGS. AFEX temperature was held constant at 70C and ammonia loading held constant at 1 g/g biomass. Enzymatic hydrolysis was performed at a cellulose loading of 1%. All runs were done in duplicate and error bars represent the maximum and minimum values.

Optimal AFEX conditions for DDGS were determined to be 70°C, no additional water, and 0.8:1 kg/kg ammonia loading. These are fairly mild compared to optimal conditions for other types of biomass. As untreated material is already digestible, extreme conditions are not needed. Under these conditions,

approximately 190g glucose/kg dry biomass is released after 72 hours. Both the rate and extent of digestion is improved over untreated material.

A.3.3 WDG Optimization

Wet distiller's grain was obtained from the same source as DDGS, and separate experiments were conducted to determine the optimal AFEX conditions. As this material was already at high moisture content, only temperature and ammonia loading were varied. Furthermore, in order to more accurately compare DDGS with wet DG, the effect of reaction temperature and ammonia loading was determined for DDGS at high (60% total weight, or 1.5 g water/ g biomass) moisture content.

Increasing temperature increased glucose yields in wet DG up to 80°C, as seen in Figure A.4. More glucose was released at 72 hours at 90°C than either 80°C or 100°C, but no significant difference was seen at either 24 or 168 hours. As with the dry DDGS, the glucose yield for high moisture DDGS increased between 60°C to 70°C and remained constant at higher temperatures. No reduction in glucose yields at elevated temperatures was seen for either sample in the range of temperatures tested. Lower temperatures are generally required to reduce inhibitor formation or reduce the risk of damaging protein, and so no high temperatures were tested.

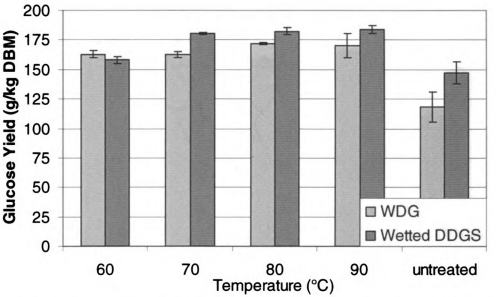

Figure A.4: The effect of AFEX pretreatment temperature on glucose yields for both WDG and wetted DDGS. The water content was 1.5 g/g biomass for both samples, and the ammonia loading was 1.0 g/g biomass. Enzymatic hydrolysis was performed at a cellulose loading of 1%. All runs were done in duplicate and error bars represent the maximum and minimum values.

Interestingly, the amount of ammonia present did not appear to significantly affect glucose yields for either wet DG or high moisture DDGS, as seen in Figure A.4. For DDGS, glucose yields also remained constant through the range shown in the figure, although lower yields were seen at high (1.6 g/g) ammonia loadings. For wet DG, no decline in glucose yields was seen up to 1.2 g/g, although no conditions were tested at higher ammonia loadings. Research into understanding the interactions between ammonia and water on lignocellulosic material is ongoing, so it is not immediately clear why the addition of water allows higher ammonia loading to be used relative to the low water DDGS. This may be due to lowering the concentration of ammonia ions or the pH of the system.

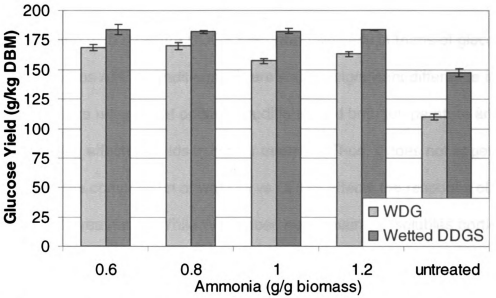

Figure A.5: The effect of AFEX pretreatment ammonia loading on glucose yields for both wet DG and wetted DDGS. The water content was 1.5 g/g biomass for both samples, and the temperature was 80°C. Enzymatic hydrolysis was performed at a cellulose loading of 1%. All runs were done in duplicate and error bars represent the maximum and minimum values.

While there was no significant difference in wet DG glucose yields between 70°C and 80°C at 1:1 g/g ammonia loading, and no difference between 0.8:1 and 1:1 g/g ammonia loading at 80°C, the glucose yield at 70°C and 0.8:1 g/g ammonia loading was lower than these values (145 g/kg biomass). Thus, some interaction between ammonia and temperature is occurring at low severity conditions. Such interactions were also seen for highly digestible switchgrass. As the increased ammonia addition is likely to be more expensive than the increased temperature (see chapter 6), 0.8 g/g ammonia and 80°C was determined to be the optimal conditions for wet DG.

A.3.4 Variability within DGs

Both wet DG and high moisture DDGS performed similarly in terms of glucose released at various AFEX conditions. There was no significant difference in the amount of glucose released at optimal conditions, and both temperature and ammonia loading affected yields in similar manner. Thus, it does not appear that drying DGs or the composition of wet DG vs DDGS affects the response of glucan to AFEX treatment. While WDG does not contain the solubles portion, the glucan content is similar (see Table A.1) to DDGS. Thus, the oligomeric sugars and fine cellulose particles found in the solubles do not appear to be more reactive to enzymatic breakdown than the grain portion.

There are differences in the response to AFEX treatment between wet DG and dry DDGS, however. As the differences are more pronounced between high moisture DDGS and no added moisture DDGS than high moisture DDGS and wet DG, it appears that these differences are due primarily to the additional water present during pretreatment. The water appears to reduce the adverse effect of high ammonia loadings. This may be due to reducing the concentration of ammonia contacting with biomass or to reduce the pH of the system. Yields were slightly lower for wet material than dry, although this difference was not significant.

A comparison was made between the two separate batches of DDGS obtained in different years from Big River Resources. Table A.2 shows the results of

hydrolysis on untreated DDGS. At both 48 and 120 hours, the glucose yields for the new batch of DDGS were about 80% of the values for the old batch of DDGS. Differences within duplicates were in line with previous experiments. No significant differences were seen in the composition of the two batches. However, the second batch was a darker color than the first, which may mean it was subjected to higher temperatures during the dry-grind process, as the darker color is likely due to Maillard reactions with the protein. Such differences in enzymatic response may reduce the likelihood of DGS as a cellulosic ethanol source, as this increases the economic risks of the process.

Table A.2: Differences in glucose yields released after enzymatic hydrolysis of two batches of DDGS obtained from the same dry grind facility.

	48 hour	120 hour
2005 DDGS	138.1	153.1
2006 DDGS	108.0	122.1

A.3.5 Enzyme Addition

Although glucose yields were high throughout all previous experiments, xylose yields were negligible. Multifect Xylanase was added at up to 50% of the cellulase loading. However, no xylose was detected at any level of xylanase loading after 168 hours, meaning less than 20% of the xylan was hydrolyzed. Separate experiments performed at USDA NCAUR (Peoria, IL) determined that Multifect Pectinase and Ferulic Esterase could convert 63% of xylan and 98% of arabinan into monomeric sugars [69].

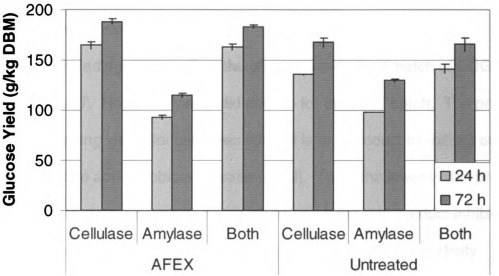

Figure A.6: Effect of amylase on glucose yields for both AFEX treated and untreated DDGS. Cellulase loading was 16.5 FPU/g glucan, while amylase addition was added at 14mg enzyme/g glucan. β-glucosidase was added at 56 pNPGU/g glucan in all cases.

As the dry grind process did not obtain complete conversion to ethanol, DDGS contains some residual starch. Thus, attempts were also made to increase the glucose yields by the addition of amylase. However, as seen in Figure A.6, adding Stargen amylase at 14 mg enzyme per gram of glucan to the cellulase cocktail did not significantly ($p<0.05$) affect the overall glucose yield. As stated previously, a portion of the starch may already be being broken down by the cellulase enzymes, thus reducing the need for additional amylase. Novozyme 188 contains some amylase activity, producing monomeric glucose from starch. Moreover, the amylase is likely inhibiting the cellulase by binding to cellulose sites, thus decreasing cellulose conversion and offsetting any increase in starch hydrolysis.

A.3.6 High Solid Loading

Increased solid loading did not affect the glucose yields 2006 batch of DDGS, as seen in Figure A.7. However, yields did decline for the 2005 batch. The primary cause of decreasing yields for cellulosic ethanol is end product inhibition, or the amount of glucose and cellobiose released [119]. Thus, the lower yields present in the 2006 batch do not cause as much inhibition. If the end-product inhibition issues are not resolved, then this may displace the issue of DG variability. Other factors that may be associated with lower yields, including byproduct inhibitors, competitive binding on lignin, and poor mixing do not appear to decrease yields. This is consistent with the mild pretreatment conditions required for DDGS and its low lignin content. In addition, DDGS solubilizes rapidly even at high solid loadings after enzymes are added. High solid loading hydrolysis of wet DG showed similar results to the 2005 batch of DDGS. At 15% solid loading, yields decreased from 180 g/kg wet DG to 150 g/kg.

At the same solids loading the AFEX treated wet DG gives 34 g/L glucose and 27 g/L xylose as the maximum theoretical concentrations. Enzyme digestion of the AFEX treated WDG at 15% solids loading with the addition of the auxiliary enzymes showed a slight increase (from 68% to 72%) in the glucose yield and approximately 4 times increase in the xylose yield (from 12% to 45%) as compared to the case without addition the xylanase and feruloyl esterase enzymes. The rise in glucan conversion as well as xylan is likely due to

synergistic effects between the different enzymes. As more hemicellulose is hydrolyzed, this likely increases the glucan susceptibility for attack, thereby slightly improving glucose yield as well.

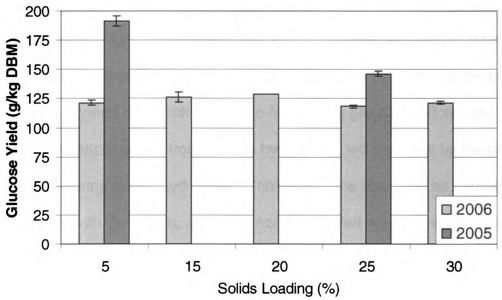

Figure A.7: The effect of solid loading during enzymatic hydrolysis on glucose yields from AFEX treated DDGS. Two separate batches of DDGS were used. In all cases, the optimal treatment of 70°C, 0.8 g ammonia/g biomass, and no additional moisture was used. Samples were collected after 120 hours.

A.4 Conclusions

Our experimental results show that the AFEX process is an effective pretreatment for the enzymatic hydrolysis of distiller grains. High yields of glucose were obtained from relatively mild AFEX conditions for both wet DG and DDGS. Xylose yields were negligible, and require further enzymes to release monomeric pentoses. Sugar yields did vary across different types of distiller grains as well as different batches of grain.

These results suggest that dry grind refineries can improve their overall ethanol yields from corn by hydrolyzing the fiber as well as the starch. The primary impediments to this approach appear to be the low pentose yields and difficulty in achieving high sugar concentrations. Both of these problems can be solved by co-fermenting the DG hydrolysate with the starch hydrolysate. The pentoses will not be fermented, although the lost revenue will be offset by removing the costs of additional enzymes or the difficulty in co-fermenting glucose and xylose [120]. The low sugar concentration from the DG hydrolysate will be offset by the high concentration during starch hydrolysis. Conversely, the fiber may be hydrolyzed simultaneously with the starch, an option not considered here. The third primary issue, variability within DG, is something that must be confronted by individual refineries. It remains to be seen whether the use of DG as a feedstock for ethanol will be an economically attractive option for all or some refineries.

APPENDIX B: DISTILLER'S GRAIN PROTEIN FRACTIONATION

B.1 Introduction

As seen in the previous chapter, distiller's grains show strong potential as a fiber source in first generation cellulosic ethanol facilities. Despite this potential, the remaining protein must retain its value as a protein source. Ethanol from corn fiber and residual starch can increase the overall ethanol yields of an integrated starch-fiber facility by 11%, but the revenue from DG sales approaches 15-25% of the total value of current refineries [98; 121]. One option, discussed in the previous chapter, is simply to sell the remaining fiber, either with or without the solubles added, as a hydrolyzed distiller grain feed.

However, the possibility of fractionating DG for biofuels allows one to consider other methods of adding value to the material, particularly the protein. The protein in DG is limited in value as an animal feed by its low lysine content, and thus it may be possible to fractionate the protein into low and high lysine products. The high lysine material can be used as a feed while the low lysine used for other purposes. If the low lysine product is easily separated from the rest of the grain, it can be used for value added products such as bioplastic precursors. If it is not easily separated, it and the remaining material may be burnt for energy.

Likewise, the high lysine product's value will be dependent upon the other material obtained with it. High amounts of lipid or phosphorous can reduce the value of DG, particularly if their concentrations increase with reduced fiber content. Thus, obtaining a high lysine protein that is relatively pure will likely be of the highest value. As the low lysine protein is primarily zein, which is hydrophobic, aqueous extraction of protein should produce a solubilized protein higher in lysine. This protein would then be concentrated and be relatively free of fiber, lipids, and high levels of ash.

Another method of separating protein is through enzymatic extraction using proteases. As the solubilized protein will be broken down into peptides or amino acids, these peptides are not useful as animal feed, and instead should be used as precursors for value-added products [122]. Thus, in this situation, the protein susceptible to enzymatic attack should be low in lysine.

The primary objective of this study, then, is to determine if fractionation of protein is feasible and, if so, what methods are preferable. The specific objectives are as follows:

- Optimize protein extraction using aqueous or alkaline solvents with particular emphasis on the amount of lysine extracted
- Integrate protein extraction using solvents with AFEX
- Determine the effectiveness of protease extraction on distiller's grain
- Determine the recovery of lysine in all methods tested

B.2 Materials and Methods

B.2.1 Feedstock

For aqueous extraction, fresh wet distiller's grain and solubles (DGS) was generously donated by the US Bioenergy ethanol plant in Woodbury, MI on October 4, 2007. Samples were kept frozen at -20°C until use. The composition of DGS is summarized in Table A.1 in the previous experiment. For protease experiments, the feedstock tested is wheat heavy stillage, obtained from Wheyfeed Ltd. (Nottingham, UK). This stillage contains the residue, both solid and soluble, in a dry grind process immediately after distilling off the ethanol. The dry weight of the stillage was 14.4% of the total weight, with the insoluble portion of the stillage at 10.4% of the total weight while the solubles contributed 4%. Nitrogen analysis gave a total protein content of 37.8 mg protein per g stillage (270 mg protein per g dry weight). The pH of the stillage was 3.5.

B.2.2 Pretreatment

The AFEX pretreatment was performed in a 1.5 L stainless steel pressure vessel preheated to 130°C. Approximately 150 g (dry weight) wet cake was added to the vessel at its native moisture content. The lid was bolted shut and the air removed using a vacuum pump. A cylinder loaded with 0.6 (+/-0.04) g NH_3 per g dry biomass was connected and the ammonia charged into the vessel. The temperature of the wet cake reached 120°C in 5 minutes and decreased to 114°C after 15 minutes. The final pressure inside the reactor was 1.45 MPa. After 15 minutes, the pressure was explosively released. The treated samples

were immediately placed in a sealed bag to minimize exposure to oxygen and thus potentially harmful oxidation reactions.

B.2.3 Aqueous Protein Extractions

Protein extractions were performed in Erlenmeyer flasks. Solutions of sodium hydroxide at the desired concentrations were allowed to preheat to the desired temperature before adding to the flask. An amount of wet cake equivalent to 2.0 g dry biomass was added to each flask, and the preheated sodium hydroxide solutions were added to bring the total weight to 40 g. When β-mercaptoethanol was used, it was added immediately prior to the extraction. Extractions were performed in shake flask incubators set at the desired temperature for 1 hour and shaken at 200 rpm. After extraction, the samples were centrifuged and the supernatant removed. Samples of the supernatant were removed for protein analysis. Pellets were weighed and allowed to dry to determine the total water remaining in the pellet. It was assumed that the concentration of soluble proteins within the water remaining on the pellet was the same as the concentration in the supernatant. The additional soluble protein remaining with the water in the pellets were not included in total protein extracted when integrating with hydrolysis, as this would lead to double-counting the protein with the subsequent operation.

B.2.4 Enzymatic Protein Extraction

All protease extractions and hydrolyses were performed in 50mL Erlenmeyer flasks placed in a shake-flask water bath set at 90 rotations per minute. Unless otherwise stated, the temperature was held constant at 50°C. An amount of stillage equal to 2.0 g dry weight was added, and the pH brought up to 7.5 by the addition of 3.6 mL of 1.0M NaOH. Water was added to bring the mixture to 20g total weight. Although higher concentrations may be used in industry to decrease energy use downstream, this 10% solid loading was deemed to be an acceptably high solid loading while remaining easy to handle and remove samples. Protease was added based on weight, as the operational energy costs in the model were based on weight rather than activity. Unless otherwise stated, protease was added at 0.1% w/w loading, or 1g protease per kg stillage protein. Samples for analysis were taken at 24 hours after the addition of protease.

In certain treatments, the stillage was allowed to incubate for one hour at 70°C prior to the addition of protease in an attempt to extract extra proteins from the insoluble matrix. The stillage was brought to the desired pH to either 7.5 or 12 using 1.0M NaOH and placed in a shake flask incubator. After 1h, the flasks were cooled and the alkaline flasks neutralized with HCl prior to the addition of the protease. Residence time was measured as beginning from the time of protease addition. Samples were then taken immediately after neutralization as well as 24 h after protease addition.

B.2.5 Protein Analysis

Due to the presence of ammonia nitrogen resulting from AFEX pretreatment, it is impossible to use standard nitrogen analysis methods, such as the Kjeldahl or Dumas methods, to measure total protein content in all samples. For three experiments, the complete amino acid profile was determined at the Macromolecular Structure Facility at Michigan State University. Samples were hydrolyzed in either concentrated acid or base and quantified through high pressure liquid chromatography. Both the insoluble residues after processing as well as the liquid extracts were quantified. The amino acids were then summed together to obtain the protein content.

Due to the cost and time associated with this method, it was impractical to perform for all trials. Instead, individual amino acids were measured based on an LC/MS/MS procedure developed at the Mass Spectrometry Facility at Michigan State University [123]. Protein samples were first hydrolyzed into their component amino acids using 6 M HCl at 110°C overnight. Deuterated valine, D-valine-d_8 (Sigma) was added as an internal standard. The resulting hydrolysates were diluted and filtered through a 0.22 μm filter. Samples passed through a Waters Symmetry C18 LC column before entering a Waters Quattro Micro mass spectrometer and quantified using QuanLynx software. The flow rate was 0.3 mL/min and the mobile phase was a gradient of 1 mM perfluoroheptanoic acid and acetonitrile. Multiple reaction monitoring was performed using electrospray ionization in positive mode.

This method allowed the detection and quantitation of eleven amino acids. The nine amino acids not detected were ala, asn, asp, cys, gly, met, ser, thr, and trp. From this data, ratios of individual amino acids were determined among different treatments to determine their variance. It was found that only hydroxide concentration had a large effect on this variance. For example, in the experiment determining the effect on temperature at low concentration (Figure B.1a), the average ratio of proline to glutamic acid in all samples and replicates was 0.518 with a standard deviation of 0.024 and no significant interaction with temperature. In comparison, this average ratio was 0.717 for high (≥0.5M) concentrations of hydroxide and significantly different than the low composition. Thus, a complete amino acid profile was obtained at the Macromolecular Structure Facility as stated previously for one representative experiment at both low and high concentration hydroxide, as well as hydrolysate medium. The ratio of the eleven detected amino acids to the complete amino acid profile was then used to estimate the total protein content in all samples.

For enzymatic digestion, the primary method of protein quantification used was by amino acid analysis [124]. All peptides that were solubilized were assumed to be available for bioplastic precursors. Samples were centrifuged at 13000 RPM for five minutes in order to remove suspended solids, and the liquid portion hydrolyzed into individual amino acids by heating at 110°C in 6M HCl overnight. The acid was removed by evaporating under a vacuum at room temperature and

the samples resolubilized in 0.1N HCl. The amino acids were then derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and separated using a Waters Nova-Pak c18 amino acid column. The amino acid peaks were measured and summed together to obtain the total protein. The protein value obtained was adjusted upward using an amino acid profile averaged from multiple milling companies to estimate the added weight of the undetected amino acids (tryptophan, cystein, methionine). The weight of the protease added was then subtracted from the final amount of protein, as it was assumed that all of the protease added would be present in the liquid phase.

B.2.6 Fiber Hydrolysis

The enzymatic hydrolysis procedure was based upon the procedure described in Chapter 3. Samples were hydrolyzed in Erlenmeyer flasks at 10% solid loading buffered to pH 5.0 by 1M citrate buffer. Spezyme CP (Genencor) cellulase was loaded at 75 FPU/g DGS (13.6 FPU/g glucan), and β-glucosidase (Novozyme 188) at 320 pNPGU/g DGS. All samples were incubated at 50°C with 200 rpm rotation. Sugar concentration after 72 hours was determined using a Waters High Performance Liquid Chromatograph (HPLC) system equipped with a Bio-Rad (Richmond, CA) Aminex HPX-87P carbohydrate analysis column. Degassed HPLC water with a flow rate of 0.6 mL/min was used as the mobile phase, while the temperature in the column was kept constant at 85°C.

In certain treatments, samples were hydrolyzed using aqueous extraction after protein extraction. Solids were separated from the liquid extract through centrifugation and the mass loss determined using the method described in the following paragraph. The remaining solid residue was hydrolyzed at 10% solid loading at 75 FPU/g residue. The extract solution was brought to a pH of 5.0 using 1M citric acid and enzymes loaded at 75 FPU/g DGS solubilized and 320 pNPGU/g DGS solubilized in order to hydrolyze solubilized oligomeric sugars. In addition, half of the samples were rinsed with approximately 10g water per g biomass after extraction and prior to hydrolysis.

B.2.7 Mass Balances

Results are listed either in percent yield or in g/kg biomass. In all cases, the reference value is the original, untreated dry biomass. Thus, the percent yield for protein is the amount of protein released divided by 296 g protein/kg dry untreated biomass. In cases where multiple operations are performed, a mass balance was used to insure all values were compared to untreated rather than treated biomass. After each operation, solid/liquid separation was performed using centrifugation at 4000 rpm for 20 minutes or 8000 rpm for 15 minutes. The solids were weighed after centrifugation and portions dried overnight at 105°C to determine moisture content. This information was used to calculate the mass solubilized at each step, and used to convert protein and sugar values released from later operations to g substrate/kg dry, untreated biomass.

B.2.8 Error Analysis

The extractions testing temperature and alkaline concentration were performed in duplicate, while all other extractions were performed in triplicate. Error bars represent the range for duplicates and the standard deviation for triplicates. For all significance tests, a student t-test was used requiring a probability $p<0.05$ to be significant.

B.3 Results and Discussion

B.3.1 Extraction Optimization

The effect of extraction temperature on protein yields is shown in Figure B.1a. Protein and lysine yields as well as total biomass solubilized increased steadily as temperature increased before leveling off at 70°C. However, protein yields were quite small, as no more than 15% of the proteins were solubilized, a result comparable to literature values. This protein is disproportionately high in lysine, as 31% of the total lysine was extracted at 70°C compared to 13% of the bulk protein. It is likely that zein protein, which is deficient in lysine, is not as soluble as other proteins in DGS, as has been suggested in previous research with corn [125]. Although the solubilized protein is relatively high in lysine, nearly 70% of the total lysine remains within the insoluble residue.

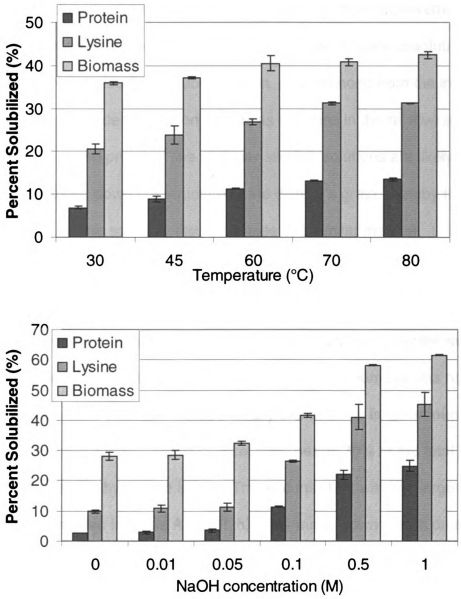

Figure B.1: Effect of extraction temperature and hydroxide concentration on solubility of protein, lysine, and biomass. All extractions were performed at 0.1 M NaOH for the top figure and 60°C for the bottom figure on untreated DGS. Extractions were performed at 20:1 liquid: biomass ratio for 1 h and rotated at 200 RPM.

A much greater effect was seen with the concentration of NaOH used, as given in Figure B.1b. Here, protein yields increased significantly between 0.05 and 0.5 M NaOH, increasing from less than 4% to 22% of the total protein. Further

increases in hydroxide concentration did not result in further improvements in solubility. The increased alkalinity of the solution was effective in solubilizing proteins from the previously insoluble residue, as evidenced from the increased yields compared to water extraction as well as changes in the relative amino acid ratios of the extracted protein. The stronger alkaline solutions are likely more effective in breaking down the structure of the distiller's grain, thereby freeing more proteins. Lysine solubility also increases in a similar manner.

As hydroxide concentration increased it became more difficult to separate the solids and liquids after extraction. A film of insoluble biomass less dense than the liquid appeared at 0.5 and 1.0 M NaOH. This film of biomass was not considered part of the pellet, and thus partially explains the large amount of biomass solubilized during extraction. However, nearly 30% of the total biomass is soluble even at low hydroxide levels. This is consistent with the large amount of water solubles seen in Table A.1, but this may make downstream processing of the protein stream more difficult. If the remaining solubles are not separated from the soluble protein, the protein content within the final product may be too low to provide maximum value as an animal feed.

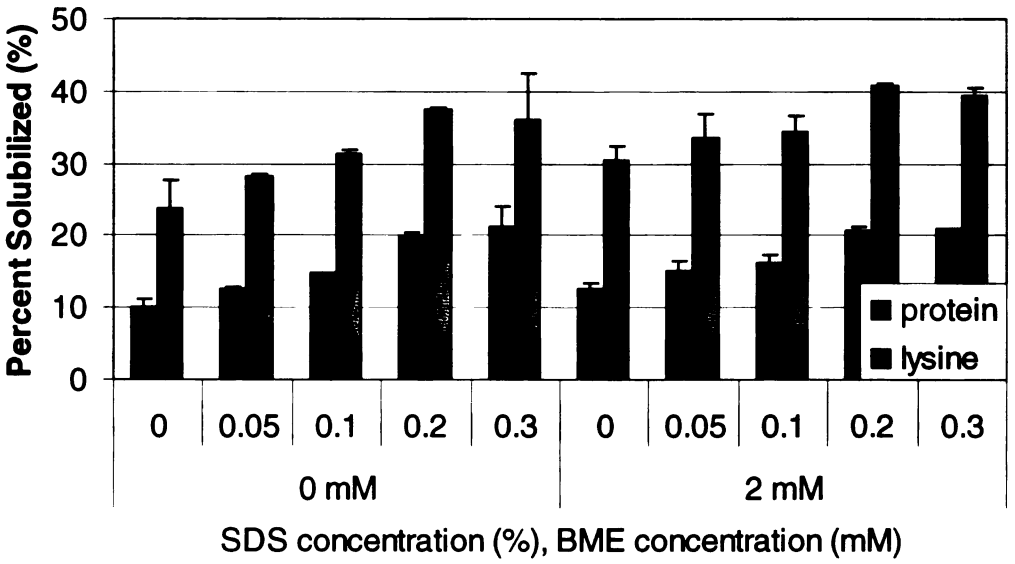

Figure B.2: Effect of adding a surfactant (sodium dodecyl sulfate) and reducing agent (b-mercaptoethanol) during alkaline extraction on protein and lysine yields. All extractions were performed at 70°C using 0.1M NaOH, a 20:1 liquid: biomass ratio for 1 hour, and rotated at 200 RPM on untreated DGS.

The effect of adding a surfactant (sodium dodecyl sulfate, or SDS) and a reducing agent (β-mercaptoethanol) is seen in Figure B.2. The reducing agent, which can cleave sulfur-sulfur bonds, did not significantly affect protein solubility except at an SDS concentration of 0.05%. The concentration of SDS, however, did have a significant role in protein yield, peaking at approximately 0.2% loading (w/v). The extent of the increase, however, was to increase protein solubility to approximately 20%, doubling the amount of protein released compared to no surfactant. The increase in lysine solubility showed similar trends. It is likely that as the corn was processed in the dry grind facility, hydrophobic portions buried within the protein were exposed to the surface, thus limiting their solubility. Furthermore, the SDS may also be increasing oil emulsification and potentially displacing protein from the oil-water interface [126]. Boatright and Hettiarachchy

[127] found that the presence of oils in soy protein isolates reduced their solubility in aqueous solutions.

However, the addition of a surfactant did not affect protein solubility at high hydroxide concentrations and temperatures, as seen in Figure B.3. One possible explanation is that the surfactant is only aiding in solubilizing the protein fragment that is already soluble in strong basic solutions while having no effect on the remaining insoluble protein. Another possibility is that saponification is occurring between the oils present in DGS and the NaOH at high concentrations. A soap-like substance has been observed in highly alkaline extractions when no SDS was added to the extract. This resulting soap may then act as a surfactant, thus eliminating the need for further SDS addition.

Approximately 40% of the protein and 50% of the lysine was extracted at 70°C and 1M NaOH, a significant improvement over other conditions tested. The percentage of protein that is lysine is 4.5% in this extract, compared to 3.5% in the original DGS. Saponification is also likely to be occurring during AFEX, although the addition of SDS still improved protein yields in extraction of AFEX treated grain, as protein solubility increased from 21% to 32% with the addition of the surfactant. Performing AFEX pretreatment prior to extraction improved protein and lysine yields when 0.1M NaOH was the solvent, but not at higher concentrations.

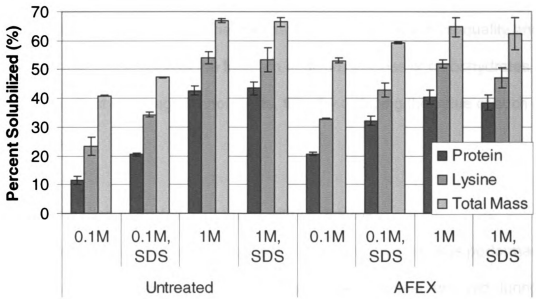

Figure B.3: Comparison of protein, lysine, and biomass solubility using different extraction conditions for untreated and AFEX-treated wet DGS. Either 0.1M or 1.0M sodium hydroxide was used as the solvent, with or without the addition of sodium dodecyl sulfate at 0.2% loading. Extractions were performed at 70°C, 20:1 liquid: biomass ratio for 1 hour, rotated at 200 RPM.

The total mass solubilized also increased as protein solubility increased. The addition of SDS increased the total mass solubilized at 0.1 M NaOH, as an additional 6% of the biomass went into solution in both the untreated and AFEX treated samples. Much of this additional mass is the increase in protein solubility. However, performing AFEX causes an additional 12% solubilization, indicating that compounds other than protein are solubilized due to AFEX pretreatment. At high hydroxide concentrations, the total biomass solubilized is approximately 65%, regardless of whether the grain is treated or a surfactant is added. At these conditions, only 20% of the soluble material is protein. The amount of non-protein biomass solubilized clearly indicates that at least a portion

of the oil and carbohydrate fractions are among those extracted. If the extract is to be used as an animal feed, these extra fractions may affect the quality and therefore the selling price of the feed. In addition, the loss of carbohydrates may adversely affect resulting ethanol yields, thereby reducing the value of such an operation.

B.3.2 Integration of Protein Extraction with Hydrolysis

Overall protein solubility is improved when enzymatic hydrolysis is performed following alkaline extraction, as seen in Figure B.4a. Protein removed during the extracted step was slightly lower than in previous experiments. This is likely due the higher solid loading used (10:1 liquid:solid ratio vs 20:1 for previous experiments), as more water and thus soluble protein is trapped within the biomass. These proteins are later released in either the wash phase or hydrolysis. Approximately 30% of proteins were solubilized for 0.1 M NaOH samples, only a slight improvement over the amount of protein released during hydrolysis alone. This is due to a decrease in the protein present within the hydrolysate, indicating that alkaline extraction at low hydroxide concentrations works primarily on proteins that are already soluble once the fiber matrix is destroyed. In comparison, 0.5 M NaOH was able to solubilize 46% of the total protein, a significant improvement over 24% protein solubilized without extraction. Including a rinse step in between extraction and hydrolysis slightly decreased yields in the three scenarios studied. This may be due to the presence of added salts formed as the alkaline is neutralized, which may be

beneficial for protein solubility during hydrolysis. Furthermore, a noticeable amount of protein is found in the rinse water, which is unlikely to be economically recoverable. Although the addition of SDS improved protein solubilized during extraction at low hydroxide concentrations, the total protein solubilized was approximately equal. Relative amino acid ratios of the total protein solubilized were similar for samples with and without SDS addition (data not shown). Thus, adding a surfactant does not appear to affect total protein solubility when fiber hydrolysis is performed.

In addition, extracting the biomass also decreased glucose yields in the hydrolysate, as seen in Figure B.4b. Due to the large amounts of glucose present in the extract, it is clear that much of the glucan, most likely starch and oligomers, is soluble under alkaline conditions [128]. This is especially true at higher concentrations of hydroxide, perhaps due to the greater disruption of the structure of DGS or to increased solubility of starch. At 0.5 M hydroxide, 42% of the glucan and 47% of the xylan was removed during the protein extraction according to acid hydrolysis of the resulting pellet. Thus, while protein removal improved the percent glucan conversion over unextracted samples (68% vs 55% of available glucan present), glucose yields were significantly lower. Adding cellulase enzymes to the extract media released additional glucose, particularly for the 0.5 M hydroxide extract. Despite this additional glucose, overall yields were significantly lower than unextracted material for 0.5 M hydroxide, while only

one case at 0.1M hydroxide saw slightly improved glucose yields. Thus, it is unlikely that extraction is opening up more glucan to enzymatic attack.

From these data, it appears that extraction prior to enzymatic hydrolysis is not an effective method of DGS fractionation. The protein extracted is separated into two different streams, as is the sugar released. This could potentially lead to additional downstream costs due to lower final ethanol concentrations. Although it is possible that the extract could be used as hydrolysate media after removing the protein in order to recombine the two sugar streams, this would require neutralizing the hydroxide. Furthermore, such a process would be unlikely for extracts with surfactants due to their adverse effects on the enzymes. In addition, a tradeoff is seen between sugar and proteins solubilized, as stronger alkaline conditions lead to greater protein content yet lower sugar yields. Furthermore, protein yields remain below 50% despite their improved lysine content, thus making any additional value achieved from this process unlikely.

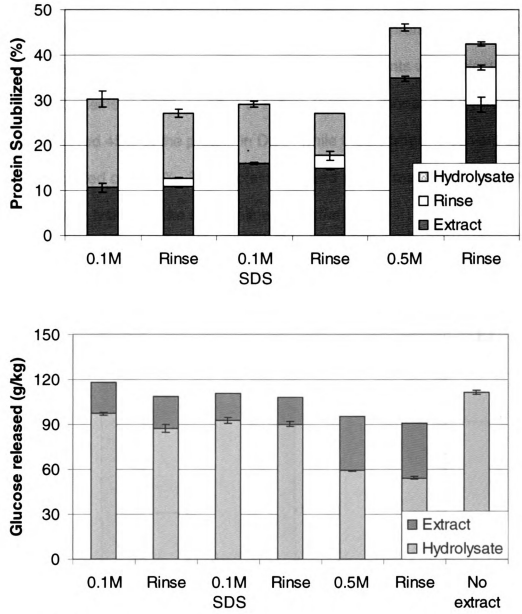

Figure B.4: Protein (top) and glucose (bottom) yields for integrated extraction and hydrolysis. Alkaline extractions were performed at 70°C, 10:1 liquid: biomass ratio for 1 hour and rotated at 200 RPM prior to AFEX pretreatment and enzymatic hydrolysis of fiber. Three extraction conditions – 0.1M NaOH with and without SDS addition and 0.5M NaOH with no SDS addition – were tested. For each condition, one set of samples (labeled as Washed) was rinsed at 10:1 liquid: biomass ratio following extraction to remove residual hydroxide and surfactant. Glucose released in the extract and hydrolysate was determined 72h after the addition of cellulase enzymes to the media. The enzymatic hydrolysate from AFEX-treated DGS without a previous alkaline extraction is also shown as a control.

B.3.3 Integration with Biosolvent Extraction

Attempts were made to use various biosolvents to extract protein simultaneously with enzymatic hydrolysis of cellulose. Hydrophobic solvents were effective at solubilizing proteins at 50°C, as seen in Figure B.5. D-Limonene, a hydrophobic solvent, removed 45% of the protein in DGS, while the hydrophilic solvent ethyl lactate solubilized only 30%. The protein removed was primarily zein protein and therefore low in lysine, unlike the alkaline extractions.

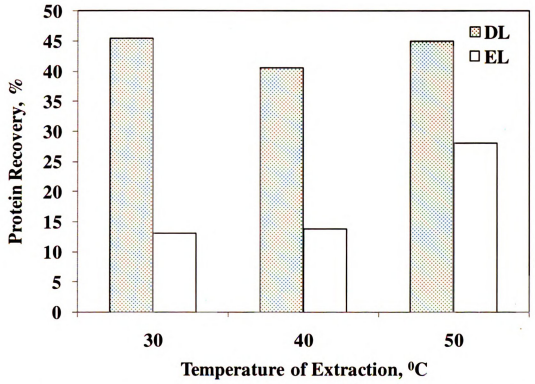

Figure B.5: Protein recovery as a function of temperature for a hydrophilic (Ethyl Lactate, EL) and a hydrophobic (D-Limonene, DL) solvents. Protein recovery was calculated based on the initial protein content in DGS, i.e., 30 % (dry basis)

Performing hydrophobic solvent extraction in conjunction with enzymatic hydrolysis did not harm hydrolysis yields, as seen in Figure B.6. Yields were

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lower than the control (no solvent added) for limonene, but similar for the methyl esters. In addition, when only cellulase was added, methyl ester addition improved sugar yields. Overall glucose yield was low, however, potentially due to end product inhibition. As the solvents formed a separate layer, the DGS loading was 33%.

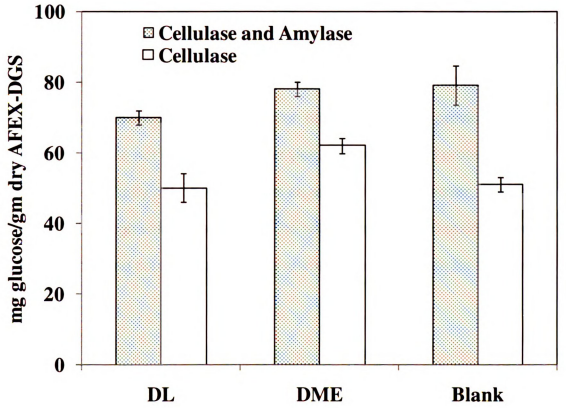

Figure B.6: Effect of the enzyme amylase on the enzymatic saccharification of cellulosic materials of DGS present in different solutions. The solvents used are D-Limonene (DL), distilled methyl esters (DME) and buffer (blank). Time = 120 h, temperature = 50 ^{0}C, AFEX-DGS : buffer : biosolvent = 1 : 2 : 1. The data points represent the mean experimental value and the error bars represent standard deviation.

Protein solubilization was low, with negligible protein in the solvent phase for the methyl ester. It is likely that mass transfer issues are preventing the protein from

reaching the solvent. As the enzymes break down the cellulosic structure, large portions of the DGS are solubilized leading to a separate aqueous layer. This layer prevents the hydrophobic proteins from interacting with the solvent, limiting its solubility. The insoluble DGS, meanwhile, is denser than water, further insuring no interaction with the less dense methyl ester. While the hydrolysis is performed under vigorous rotation, no emulsification was seen during the reaction.

Thus, integrating biosolvents with enzymatic hydrolysis does not appear to be a viable option. However, protein extraction prior to hydrolysis provides 45% protein yield while not decreasing sugar yields. The removed protein is low in lysine, and thus may not be valuable as an animal feed. However, it could be used for other high value processes. The remaining insoluble residue would be high in lysine, and could be used as an animal feed rather than for heat and power.

B.3.4 Composition of Insoluble Residue

One potential option to fractionate the protein is simply to separate the solubles from insoluble material after hydrolysis and fermentation of the fiber. A complete composition analysis of the insoluble fraction of DGS after fiber hydrolysis compared to the initial composition is shown in Table B.1. Here, the protein fraction increases dramatically, to 56% of the total product. The lysine content also increased, although roughly at the same level as the protein increase. Thus,

the relative lysine content is the same as before. Due to the high concentration of protein and no loss of lysine, this insoluble residue may be a more valuable animal feed than extracted solubles. Further research on the digestibility of both protein and specifically lysine within hydrolyzed DG is ongoing.

Table B.1: Composition of distiller's grains used in this study. The first column represents the original wet distiller's grain and solubles while the second column represents the insoluble residue after enzymatic hydrolysis of fiber.

Component (%)	Untreated	Hydrolyzed
Protein	29.6	56.2
Lysine	1.05	1.80
Glucan	18.1	7.0
Xylan	10.3	5.0
Arabinan	5.5	2.1
Water Extracts	20.2	8.1
Ether Extracts	11.4	13.6
Ash	5.5	1.6
Phosphorous	1.09	0.23
Total	100.5	93.6

Carbohydrate content decreased due to hydrolysis, although the total glucose yields (120 g/kg biomass) are well below expected (Kim et al., 2008b) for the loss of fiber. This is likely due to incomplete hydrolysis of the fiber creating oligomers in the resulting hydrolysate. In addition, natural variance between DG at separate refineries may account for the lower response to enzymatic hydrolysis. Mass closure on the insoluble residue did not reach 100%, and the remaining material is possibly fiber not broken down during acid hydrolysis. Xylan and arabinan in particular do not appear in the amounts expected due to their low enymatic digestibility. A more complete set of enzymes may be able to further break down these oligomers, thereby increasing ethanol yields. The lack of fiber,

while decreasing the value as an energy source in ruminants, may serve to improve ileal amino acid digestibility in swine [129].

Ash content in the insoluble residue also significantly decreased, including phosphorous, as most of the ash was soluble during hydrolysis. It is unclear whether a lower composition of ash and especially phosphorus will improve or detract from the value of the DGS in nonruminant diets. Phosphorus in DG is highly digestible compared to that in cereal grains, and thus is of great value [130]. However, high phosphorus levels may lead to toxicity. Furthermore, phosphorus and calcium requirements are closely linked, even though DG are relatively deficient in calcium. Carefully managing the amount of solubles that are returned to the insoluble residue prior to feeding may be necessary to balance the mineral requirements for livestock.

Although an oil layer appeared in the hydrolysate, a significant amount of lipids remain in the insoluble material. This may cause problems with swine diets, as high fat DDGS has been linked to soft bellies in swine, generally an undesirable property [131]. Extraction of corn oil, either prior to starch conversion to ethanol or prior to cellulose conversion, may be necessary in order to keep lipid content low as well as providing a second co-product.

B.3.5 Protease Extraction

Figure B.7 shows the rate of protein solubility for both high (1.0%) and low (0.1%) protease loadings up through 168 hours of protein digestion. Data points were fitted to a logarithmic curve, and high protease loading had a significant ($p<0.05$) increase in the rate of protein solubility. Low protease loading approximately doubled the amount of protein in solution after 24 hours, but very little additional protein was released after this point. Higher protease loadings showed both an improved rate and extent of protein solubility, continuing to release protein throughout the experiment. The reduced rate of protein solubility, particularly for the low protease loading, is likely due to protease degradation over time, either due to protein unfolding or the protease attacking itself.

Gel electrophoresis suggests the latter explanation, as no band is seen for the protease after 24 hours (data not shown). Approximately 18% of the total protein is available as soluble protein prior to protease addition, demonstrating the importance of using whole stillage as the substrate rather than solely the insoluble material. Furthermore, more protein is initially soluble in the stillage for wheat compared to corn [132], indicating that wheat based dry mills may be better suited to this technology than corn.

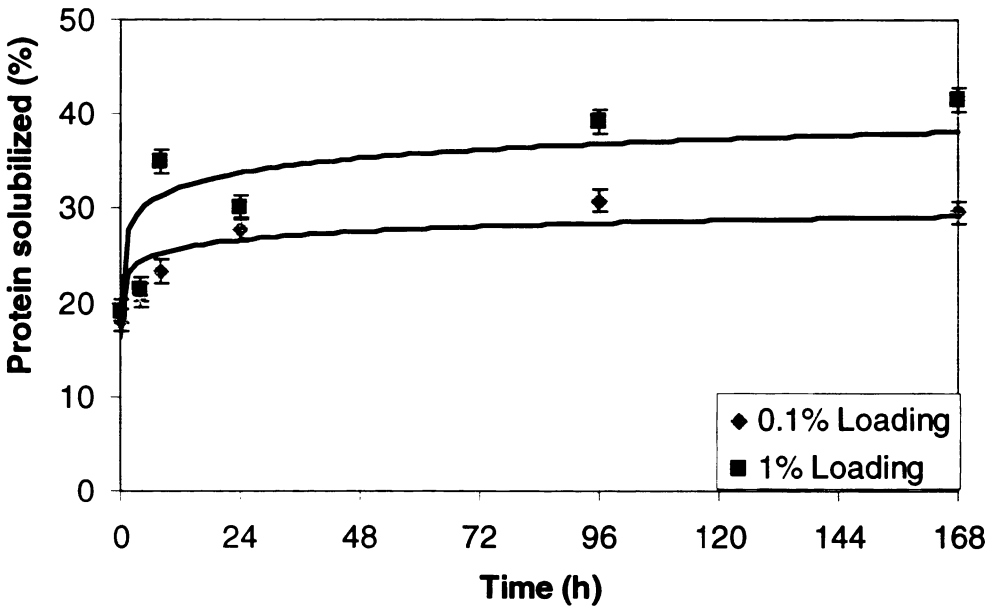

Figure B.7: Rate of protein removal during protease digestion for both low (0.1% w/w) and high (1.0% w/w) loading of protease. Lines represent best logarithmic fit. The amount at 0h represents the initial soluble protein within the whole stillage. Extractions were performed at 50°C, a pH of 7.5, and 90 rpm.

Attempts to extract the proteins prior to protease addition are seen in Figure B.8. The high temperatures used were necessary to obtain a significant extract. As expected, the initial amount of protein in solution increased when a prior extraction was performed, although little difference is seen between the neutral and basic extracts. This led to a subsequent increase in the final protein concentration in solution after protease addition. Despite the increase in protein concentration, virtually all of it is still broken down into smaller peptides (data not shown). Thus, it is likely that the number of active sites within the insoluble matrix is the limiting factor in the release and breakdown of the proteins, rather than the activity of the proteases themselves.

It is interesting to note that, despite the lack of initial increase in protein removal in alkaline extraction, the final result after the addition of proteases did continue to increase the protein removal. Thus, the alkaline conditions may also be opening up more sites for the protease to attack.

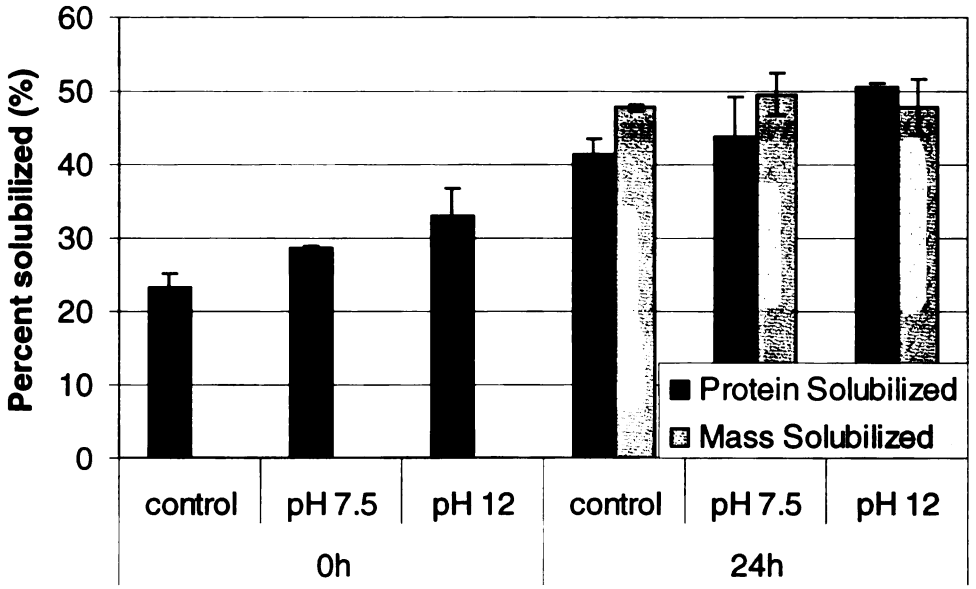

Figure B.8: Effect of extractions prior to protease addition. The control is protease digestion without extraction. Extractions were performed at two different pH levels at 70°C for 1h. The alkaline extract was neutralized prior to protease addition. Here, time at 0h represents the beginning of protease digestion.

Due to the slight improvement seen with alkaline extraction, the alkaline protease Protex 6L was also tested. In addition, Protex 51P was tested due to its exopeptidase activity, as well as a combination of 51P and 14L. Protex 6L digestions were performed at pH 10, the optimal level suggested by the manufacturer, whereas 14L and 51P digestions were performed at pH 7.5. As seen in Figure B.9, Protex 6L showed a significant improvement over the other

two proteases. This could be due to the increased solubility at alkaline conditions. The exopeptidase enzymes released fewer proteins than either of the other two enzymes tested. This is most likely due to a decrease in active sites, as the ends of the proteins are embedded within the insoluble matrix or have been modified during the original dry grind process. The combination of 14L and 51P were only slightly lower than 14L alone, despite the decrease in 14L loading. This indicates that there may be some synergistic activity between the two types of protease, indicating other protein combinations may also improve protein removal with lower overall loadings.

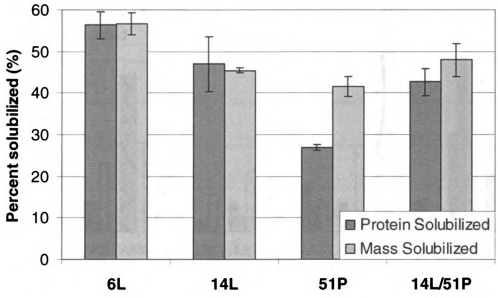

Figure B.9: The effect of different proteases on protein removal. The enzymes used are Protex 6L, Protex 14L, and Protex 51P. Digestions were performed for 24h at 50°C and 90rpm. Protease was loaded at 0.1% (w/w) and the pH was kept constant at 7.5 for Protex 14L and 51P and 10 for Protex 6L.

Figure B.10 shows the effect of Protex 6L protease digestion on the amino acid profiles of the stillage. Both the profile of the whole stillage as well as the soluble

portion are shown for comparison. In general, the protease digestion served to moderate the amino acid profile, increasing the relative ratios of amino acids that were not originally soluble in stillage. However, relative amounts of both aspartic acid/asparagine and lysine increased more than expected, while glycine and histidine decreased more than expected. Proteases attack at specific sites within a protein, and thus are not likely to attack all proteins equally. Thus, a protease cocktail may help digest specific proteins or peptide sequences that Protex 6L alone is unable to attack, potentially increasing the yield of soluble amino acids.

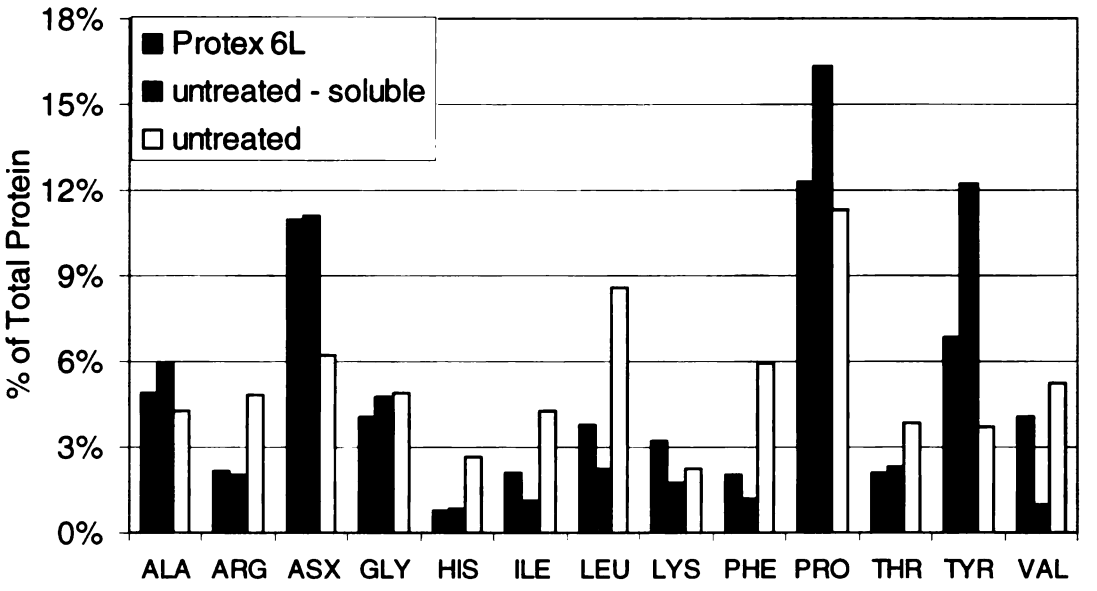

Figure B.10: Amino acid profiles for proteins released by Protex 6L, soluble proteins in wheat stillage, and total proteins in wheat stillage. Met, Cys, and Trp were not detected due to being destroyed during acid hydrolysis.

B.4 Conclusions

Extraction of proteins from wet distiller's grains using alkaline solvents in combination with pretreatment and enzymatic hydrolysis of cellulose does not appear to be an effective method of enhancing the protein quality of distiller's

grains. Only 32% of the proteins and 43% of the lysine was extracted using 0.1 M NaOH. In addition, this separated the glucose into two separate streams, adding to downstream costs. In comparison, if enzymatic hydrolysis is performed without alkaline extraction, 45% of protein is solubilized. Extraction of hydrolyzed material resulted in an additional 30% of protein extracted, to bring the total amount of soluble protein to 62%.

Protease extraction of wheat stillage using 0.1% Protex 6L yielded a maximum of 57% of protein in soluble form. However, approximately 32% of these proteins were already soluble prior to protease addition, indicating the importance of using the whole stillage as a feedstock rather than solely the solid distiller's grains. These soluble proteins as well as those extracted were effectively broken down into smaller peptides as well. With 0.1% protease loading, digestion was essentially complete after 24 hours. Improvements in protein solubilization were also seen with an alkaline extraction prior to protease addition for proteases active in neutral conditions. Increased protease addition primarily improves the extent of hydrolysis, rather than the rate. In addition, a cocktail of different alkaline proteases may further improve yields and therefore reduce carbon emissions.

Simultaneous aqueous phase enzymatic saccharification of cellulosic materials and hydrophobic biosolvent phase extraction of proteins was not successful. This is likely due to mass transfer issues, as little contact was present between

the hydrophobic solvent and the proteins. Thus, bio-solvents were unable to recover additional proteins from DGS at the current conditions. A separate extraction and hydrolysis, however, was successful in removing proteins and hydrolyzing the fiber, although the protein was low in lysine.

Thus, it remains necessary to recover proteins in the insoluble, solid portion of the biomass, as an enhanced distiller's grain product. Added value is obtained from removing fat, fiber, low-lysine protein, and water solubles from the product rather than removing the high-lysine protein. This product is low in ash and fiber and high in protein, potentially increasing its value as an animal feed to nonruminants. Corn oil may be removed prior to the dry grind process and sold as a separate product or alternatively removed using a hydrophobic solvent. Glucan can be removed easily by enzymatic hydrolysis, although hemicellulose requires a more complete set of enzymes if using a mild pretreatment such as AFEX. While a cocktail of enzymes has been shown to be effective in releasing pentoses, it remains to be seen whether the added cost of the enzymes would make this approach economically viable. The protein remaining in solution after hydrolysis and fermentation can then be dried and returned to the insoluble portion, similar to what is currently performed in dry grind ethanol facilities.

If the soluble portion of the hydrolysate is not recombined with the insoluble portion, it may still be possible for the remaining proteins to have some value. It has been suggested that protein in distiller's grains be used as a feedstock for

bioplastics [133]. This would require breaking the proteins down into their component amino acids, probably using proteases. However, these proteases are costly and cannot completely solubilize all protein in DGS. Thus, one option would be to simply break down those proteins in solution after hydrolysis. Less protease would be needed, thereby reducing costs. In addition, it may also be possible to separate out the lysine after protease hydrolysis and return it to the DGS to improve its value as an animal feed.

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