GREEN PROCESSING OF NAPIER GRASS FOR GENERATION OF BIOFUEL AND BIOBASED PRODUCTS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MOLECULAR BIOSCIENCES AND BIOENGINEERING

DECEMBER 2012

By

Devin T. Takara

Dissertation Committee:

Samir Kumar Khanal, Chairperson
Andrew G. Hashimoto
Jon-Paul Bingham
Soojin Jun
Richard M. Ogoshi
John F. Yanagida

Keywords: Napier grass, green processing, biofuel, biobased products, biorefinery

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my advisor, Dr. Samir Kumar Khanal, for affording me an excellent opportunity to learn and grow, both as a person and as an aspiring researcher in the field of bioenergy and sustainability. Thank you for your uncompromising generosity, mentorship, and friendship. I would also like to express my thanks to Dr. Jon-Paul Bingham for his endless support and for taking much time to teach me the art of HPLC analysis, Dr. Richard M. Ogoshi for his tremendous help in the field and unrivaled agronomic expertise, Dr. John F. Yanagida for his inexhaustible patience and wisdom in tutoring me with economics among other topics, Dr. Soojin Jun for his many years of encouragement and advice, and Dr. Andrew G. Hashimoto for his invaluable knowledge and for serving as one of my earliest academic mentors at UHM. I have had the distinct privilege of knowing many of you for a number of years, and cannot fully express in words the depth of my gratitude.

Thank you to my amazing labmates and my academic family, for the wonderful times and shared experiences. I have thoroughly enjoyed our many laughs, and always found myself eager to work alongside friends each day. Our fruitful discussions of research have proven to be priceless, and I am extremely fortunate to have had the chance to meet all of you. Best wishes in your future research, wherever they may take you.

To my mom, dad, Renee, family and friends, thank you for your inexhaustible patience, relentless support, and for treading ever so lightly across eggshells at times. Words on a page cannot justly encapsulate the sincerity of my appreciation, and the many contributions and silent sacrifices that you have made in allowing me to pursue my dreams of higher education. Thank you for the many hours of "talk story time" and for helping to keep me grounded and the important things (in life) in perspective.

Last but certainly not least, thank to you Mieko for sticking by my side through this character-defining endeavor. I am truly grateful to have had your company on the late research nights, as well as your understanding when I occupied more time at the laboratory than at home. Thank you for humoring my often lengthy discussions of fantastical research ideas which ended up being less fantastic (and successful) in practice, and above of all, thank you for your love and patience.

ABSTRACT

Napier grass, *Pennisetum purpureum*, is a high yielding perennial C-4 grass that has been naturalized in Hawai‘i and resembles the former staple crop of the state, sugarcane. Because of its high moisture content, Napier grass presents a unique and relatively unexplored opportunity for fractionation into solid and liquid components via green processing. The resulting clean, solid fibers can serve as a substrate for (advanced) biofuel production, while the nutrient-rich liquids (juice) can serve as a supplemental additive for diverse microbial co-products generation. The recalcitrant lignocellulosic fibers of Napier grass contain structural carbohydrates which require pretreatment and enzymatic saccharification to release monomeric sugars for fermentation into biofuels. In this study, the effects of dilute acid pretreatment on structural carbohydrate release of Napier grass were investigated for the first time. The optimal conditions for green processed Napier grass were determined to be 5% (w/w) sulfuric acid, 120°C, 45 minutes; producing near theoretical xylose yields and ~85% of the glucose from hemicellulose and cellulose, respectively. Preliminary trials of high value co-product generation were successful in cultivating protein-rich fungal biomass, *Rhizopus oligosporus*, on crude Napier grass juice. In determining the applicability of green processing for future biorefineries, an important and often overlooked consideration of the incoming feedstock is age. As Napier grass matures, significant changes may occur in its biochemical composition, subsequently affecting fractionation and biofuel and co-product generation. The composition of Napier grass was examined for the ages of 2, 4, 6, and 8 months old. Ash and lignin constituents, in particular, were found to be dependent on age and both constituents increased with feedstock age. Changes in the juice characteristics were also found to correlate with Napier grass maturation. Overall, the compositional data of Napier grass at different stages of growth represented the first of its kind for bioenergy crops grown in the (sub)tropical climate of Hawai‘i, which has gained considerable attention for the development of biomass-to-biofuel strategies and technologies. Exploratory economic analyses of the results in this study however, suggest that future work is necessary. Ultimately, because green processing represents a biosystems engineering approach, it can be adapted and applied to a multitude of disciplines and biofuel platforms.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIL	Acid insoluble lignin
ANOVA	Analysis of Variance
ASL	Acid soluble lignin
BGY	Billion gallons per year
BLY	Billion liters per year
CO_2	Carbon dioxide
COD	Chemical oxygen demand
DNS	3,5-dinitrosalicylic acid
EJ	Quintillion joules
GGE	Gallon of gasoline equivalent
GHG	Greenhouse gas
HPLC	High performance liquid chromatography
J	Juice
LGE	Liter of gasoline equivalent
M	Month
MC	Moisture content
MGD	Million gallons per day
MGY	Million gallons per year
MLD	Million liters per day
MLY	Million liters per year

NIST	National Institute of Standards and Technology
NREL	National Renewable Energy Laboratory
ODW	Oven dry weight
RCB	Randomized Complete Block
SSF	Simultaneous saccharification and fermentation
TKN	Total Kjeldahl nitrogen

CHAPTER 1

INTRODUCTION

The world is on the precipice of an energy crisis, and recent forecasts predict that global energy demands are likely to increase by 53% - from 533 quintillion joules (EJ) in 2008 to 812 EJ - by 2035 (U.S. Energy Information Administration 2011). Despite the anticipated growth of energy consumption over the next two decades, the transportation sector, which currently comprises over 70% of the United States' liquid fuel consumption, will continue to be largely dependent on non-renewable resources, namely petroleum (U.S. Energy Information Administration 2011). Among the many concerns of the U.S. and other developed nations is the impending threat to national security through oil restrictions and embargoes set by politically unstable, oil-exporting countries. Thus, establishing an uninterrupted supply of transportation fuel remains a critical priority in maintaining international trade and protecting our national security.

An additional concern of global importance is the unprecedented strain that the use of petroleum and other non-renewable resources have placed on the environment. In the U.S., about 34% (1,850 million metric tons) of the country's total greenhouse gas (GHG) emissions originate from the transportation sector alone (U.S. Energy Information Administration 2011), and with the rapidly growing economies of China and India, the worldwide situation is only likely to worsen. Presently, the U.S. is the leading biofuel-producing nation, generating 27 million gallons per day (MGD) or 102 million liters per day (MLD), followed by Brazil with 15 MGD (58 MLD) (International Energy Statistics 2010). As part of its Renewable Fuel Standards (RFS) program, the U.S. has set an aggressive goal to produce 36 billion gallons per year (BGY) (136 billion liters per year (BLY)) of renewable fuels by 2022 (*Renewable Fuel Standards* 2012). Much of the domestic biofuels, however, is in the form of ethanol and relies on the use of first generation feedstocks.

First generation biofuel feedstocks refer to starch (e.g., corn, cassava, and sorghum), sugar (sugarcane, sugar beet, and sweet sorghum), and edible oil seed crops (e.g., soybean, palm fruit, and rapeseed) which are grown for bioethanol and biodiesel production, respectively. (Although significant, biodiesel will not be discussed in further

detail as it falls outside the scope of this research.) The current large-scale use of agricultural feedstocks competes directly with food/feed industries, and has launched heated public debates on the issue of food/feed versus fuel.

In response to concerns over rising food prices, second generation feedstocks are being examined as alternative (non-edible) resources for biofuel production. Unlike first generation feedstocks, the fermentable sugars of second generation feedstocks are found predominantly within hemicellulose and cellulose structures; the former being a heterogeneous polysaccharide made primarily of a five carbon sugar backbone (e.g., xylose), and the latter being a homogeneous polysaccharide made entirely of six carbon glucose. The $\beta(1\rightarrow4)$ glycosidic bonds, which are characteristic of cellulose, make second generation feedstocks difficult to deconstruct into monomeric sugars (Liu et al. 2008); the precursor of most biofuels. Adding to the recalcitrant nature of these so-called lignocellulosic feedstocks is a non-carbohydrate polymer called lignin. Lignin acts effectively as an adhesive that stabilizes and reinforces (hemi)cellulosic structures. To access and hydrolyze the polysaccharides of holocellulose, the interactions of lignin, hemicellulose, and cellulose must first be disrupted through a process known as pretreatment.

Over the last few decades, many different strategies have been developed for the pretreatment of lignocellulosic biomass, but nearly all methods can be classified into one of four main categories: (1) biological, (2) physical, (3) chemical, and (4) hybrid (i.e., combinations of 1, 2, and/or 3). Biological pretreatments employ the use of microorganisms such as fungi to naturally digest lignin and expose structural carbohydrates for saccharification by commercial enzymes. An inherent disadvantage of biological pretreatments is the long residence time required for bioconversion processes (Shi et al. 2011). Physical pretreatments, as its name implies, rely on the physical destruction of lignocellulosic structures by unit operations such as milling, grinding, or ultrasonication. The intended goal of this pretreatment is to reduce the size of the incoming feedstock and facilitate the mass transfer of enzymes into (hemi)cellulose structures for improved hydrolysis. In modern laboratory and pilot scale practices, chemical pretreatments are the most common due to their consistently high release of fermentable sugars in short residence times. The mechanisms by which these

pretreatments operate vary significantly with the types of chemicals used, but the overall aim is to selectively remove/solubilize either lignin or hemicellulose from the feedstock to promote the deconstruction of recalcitrant plant structures.

Aqueous alkaline solutions such as sodium hydroxide and lime have been well known in pulp and paper industries (Sutermeister 1920) for specifically targeting and solubilizing lignin in plant biomass while leaving (hemi)cellulose relatively untouched in the solid fraction. Following pretreatment with dilute alkaline solutions, the sugars remaining in (hemi)cellulose fibers are saccharified into fermentable monomers by the addition of enzymes. The overall process requires the need for two enzymes: hemicellulases and cellulases (Sukumaran et al. 2009) which hydrolyze hemicellulose (xylan) and cellulose (glucan), respectively.

In contrast to alkaline solutions, dilute acid pretreatments selectively solubilize hemicellulose, leaving cellulose and lignin relatively untouched in the solid fraction. The five carbon backbone of hemicellulose and other amorphous carbohydrate structures of holocellulose are converted directly to monomeric sugars in the acid hydrolysate (Yu et al. 2011), and the use of hemicellulases are thus not necessary. In conventional downstream processes following dilute acid pretreatment, cellulases are added to the solid residue to saccharify the cellulose fibers. A multi-institutional techno-economic analysis of leading pretreatment strategies suggested that the dilute sulfuric acid pretreatment of lignocellulosic biomasses was perhaps the most economical (Eggeman and Elander 2005) and practical for large-scale biofuel applications in the U.S.

Crop availability and logistics also play a critical role in commercial biofuel production from second generation feedstocks, and many studies have identified perennial herbaceous crops as having potential on the continental U.S. (e.g., miscanthus, switch grass, energy cane, and sorghum) (Voigt et al. 2012). Very few studies however, have considered the performance of these and similar feedstocks in (sub)tropical climates of the world. *Pennisetum purpureum* (Napier grass) for example, is a lignocellulosic crop known to achieve extremely high yields of 42 dry U.S. tons per acre per year (94 dry metric tons per hectare per year) from its ratoons in Hawai‘i; nearly double the yield of sugarcane (Osgood et al. 1996). Despite high yields and the availability of modern

pretreatment techniques, the scale-up of second generation biomass-to-biofuel processes has not yet been proven to be economically viable at a commercial level in the U.S.

Better utilization of the whole feedstock and the generation of multiple products may be a simple approach for minimizing biofuel production costs and improving profitability. For example, innovations in biomass handling, such as green processing, can be applied to dedicated energy crops and combined with conventional dilute sulfuric acid pretreatments to enhance biofuel production. Green processing, defined as the upstream fractionation of lignocellulosic feedstocks into usable solid and liquid fractions through shredding and dewatering unit operations, has tremendous implications in Hawai‘i and other (sub)tropical climates where perennial crops like Napier grass experience rapid growth, high yields, and high moisture contents.

The upstream dewatering of energy crops by screw-pressing is an essential component of the innovative green processing approach which creates two distinct fractions: clean, solid cellulosic fibers for monomeric sugar production and nutrient-rich juice for microbial co-product production. Additionally, the screw-press is believed to produce shear stress on the biomass thereby promoting the disruption of structural components and improving structural sugar release during dilute acid pretreatment and enzymatic saccharification. Presently, green processing remains primarily at the conceptual phase and is relatively untested, but its potential advantages include the concurrent production of both biofuel and a suite of high-value co-products.

Green processing has significant implications when considered with the emerging biorefinery concept. Like many modern petroleum refineries, the biorefinery concept aims at producing a multitude of products from a single feedstock; and subsequently, multiple streams of revenue. Because green processing would be incorporated prior to biomass pretreatment in a biorefinery, it has the potential to generate an additional product stream (immediately after harvesting) without precluding high value co-products proposed by other studies further downstream (Ji et al. 2012; Chandel et al. 2012). The moisture and holocellulose contents of raw herbaceous crops, however, are intrinsically important characteristics of incoming feedstock, and play a pivotal role in determining the ultimate success of green processing. Biomass maturation in particular has been known to result in compositional changes of perennial grasses (Holmes 1980).

In this research, the overarching goal is to evaluate the green processing of a high yielding tropical grass, Napier grass (*Pennisetum purpureum*), and its potential in biofuel and co-product production in Hawai‘i and other (sub)tropical climates. Specifically, this work aims to fulfill the following objectives:

(1) To examine a front-end biomass fractionation technique, enabling the generation of high-value co-products and clean fiber for biofuel production;
(2) To investigate the efficacy of dilute acid pretreatment and enzyme hydrolysis on the overall sugar released from green/wet-processed clean fiber (compared to conventional biomass preprocessing strategies);
(3) To elucidate the constituents of Napier grass and compare biochemical changes with respect to feedstock maturation; and
(4) To conduct an exploratory economic analysis on the merits of adding green processing unit operations to biofuel facilities.

The outcomes of this project will guide future studies and decision-making in biofuels and green processing, as well as serve as a model for biorefineries on the continental U.S. Moreover, because green processing is a relatively unexplored fractionation approach, conclusions drawn from this study may serve as a foundation for future research on emerging biochemical platforms that aim to produce a slew of high value co-products and drop-in biofuels (Holtzapple and Granda 2009; Chang et al. 2010).

CHAPTER 2

LITERATURE REVIEW

2.1 The need for renewable biofuels

The United Nations (UN) predicts that by 2075, the world population will peak at 9.22 billion (Department of Economic and Social Affairs: Population Division 2004). With a 50% growth in less than a century, many have begun to question the sustainable capacity of the earth. Developed nations have prioritized and summarized their concerns into three overarching themes to guide future efforts in circumventing the following issues and maintaining our quality of life: national security, depleting world resources, and climate change.

2.1.1 National security

Beginning in the early 1970s, the United States (US) found itself in an unusual situation. The country's economy was being threatened by an oil embargo set by the Organization of Petroleum Exporting Countries (OPEC). America appeared to have undergone the transformation predicted nearly two decades earlier by M. King Hubbert, a Shell Development Company geologist, which forecasted a US transition from being an oil exporter to importer as a result of domestic oil peaking (Hubbert 1956). With the onset of gasoline shortages sweeping across the nation, the Nixon Administration had little alternative but to negotiate an end to the OPEC embargo and expose a critical weakness of the U.S.; oil dependency. In the aftermath of the Oil Crisis of 1973, the US government launched Project Independence with the goal to achieve energy self-sufficiency from imported resources (U.S. Department of State Office of the Historian). Decreasing demands of oil in the early 1980s however, coupled with an increase in production by OPEC resulted in a sharp drop of oil prices by 1986 (*Petroleum Chronology of Events 1970-2000*). The urgency of establishing energy security subsequently faded.

A little over a decade later, events resulting in a heightened public awareness over global activities, particularly in the Middle East, rekindled interest in renewable energy

production and also raised concerns about the near and long term energy security. Presently, the US continues to remain largely dependent on non-renewable resources, which accounted for 45% of the net petroleum imports in 2011 (U.S. Energy Information Administration 2012), and the threat of another embargo remains imminent with unstable political regimes in control of over half of the world's oil reserves as depicted in Figure 2.1. Without the advent of domestic transportation fuel, military and public vehicles will continue to be at the mercy of foreign nations.

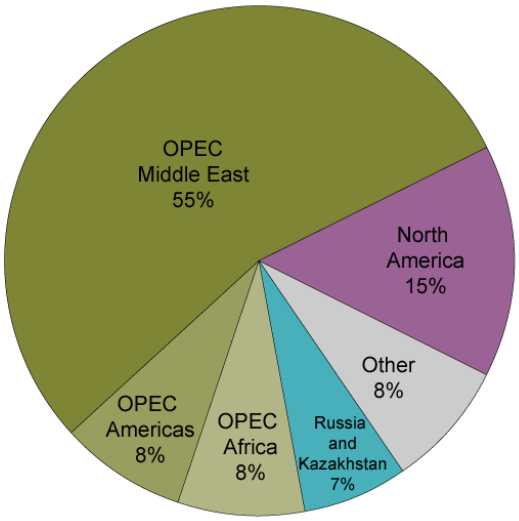

Figure 2.1. Shares of proven oil reserve holders/locations, 2010 (*Source: U.S. Energy Information Administration, International Energy Statistics, 2010*)

2.1.2 Depleting world resources

Paralleling the situation experienced four decades ago by the US, studies indicate that oil reserves of the world are likely to peak in the near future. As a result, rising demands will be met with a steady decrease in oil production, corresponding with smaller and fewer oil well discoveries each year. Accurate time estimates are difficult to predict due to incomplete and inaccurate data reported by government and private entities, but literature has suggested that oil peaking may occur as soon as 2020 (Sorrell et al. 2010).

Oil, along with other non-renewable resources like coal and natural gas, presently play a critical role in providing much of the world's energy. By 2035, it is expected that our global energy demands will increase by 53%, from 533 EJ (2008) to 812 EJ (U.S.

Energy Information Administration 2011); and the transportation sector will be responsible for about 20% of our total demands. Given the increasing globalization of the modern world, the onset of oil peaking can significantly and detrimentally impact the nation's economic growth and stability. There is thus an urgent need for the development of abundant and renewable fuels to meet our domestic and international transportation needs and maintain intercontinental commerce.

2.1.3 Climate change

In addition to the national security threats and diminishing oil supplies discussed previously, the effects of human activities on the environment have also gained significant attention, advocating for the replacement of fossil fuels with renewable alternatives to prevent climate change. The total greenhouse gas (GHG) emissions of the world were estimated to be about 44,153 million metric tons of carbon dioxide ($CO_{2\,eq}$) equivalent for 2005, representing a 13% increase from 2000 (Herzog 2009).

The transportation sectors of many developed and developing countries in particular, have been the focus of efforts to mitigate environmental detriment through advancements in automobile efficiency and renewable fuel production. This is due largely in part to the fact that developed countries emit some of the highest concentrations of GHG each year through their extensive use of petroleum. In the US for example, just over one-third (1,850 million metric tons) of the country's entire GHG emissions has been attributed to the transportation sector alone (U.S. Energy Information Administration 2011); and importantly, the US is the second largest contributor of CO_2 (from all economic sectors) after China. Biofuels (i.e., liquid fuels of biological origin) with little or no net contribution to GHG emissions are desperately needed (Scown et al. 2012) to mitigate the harmful environmental impacts of petroleum, and have been proposed to play an increasingly important role in the replacement of petroleum in the coming decades.

Other merits of biofuels, which are often overlooked, include the creation of domestic jobs and strengthening of the rural economy. Interestingly, the development of biofuels is not a new concept, and multiple generations of commercial feedstocks have and continue to be proposed in the US and other countries.

2.2 First generation feedstocks

The initial efforts for domestic biofuel production began largely in response to the Oil Crisis of 1973, and have centered on the generation of ethanol from starch- and sugar-based agricultural crops. Ethanol was introduced as a gasoline extender and octane enhancer to replace methyl tertiary butyl ether (MTBE); later satisfying the Clean Air Act Amendment (CAAA) of 1990 (Shapouri et al. 2003) by reducing the release of carbon monoxide (resulting from incomplete combustion). The US, Brazil, and Europe thus began exploring the prospects of growing and converting domestic feedstocks into renewable transportation fuel to protect national security interests and the environment.

First generation feedstocks typically refer to the three main classes of agricultural crops originally proposed for biofuel production: (1) starch-based, (2) sugar-based, and (3) edible, oil-based crops. The first two categories have been the focus for alcohol-type transportation fuel production, while the latter has been characterized primarily for biodiesel production. Within the scope of this project, only the first two groups will be reviewed and discussed in further detail.

Starch-based feedstocks primarily include corn, rye, rice, barley, sorghum, and cassava. Some of these crops are known to contain more than 70% starch on a dry weight basis (Bothast and Schlicher 2005) and can be easily converted to fermentable sugars: the precursor of most types of biofuels. Starch is a homogeneous polymer that consists entirely of glucose monomers bonded together in $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ glycosidic linkages to form amylose and amylopectin, respectively, (Figure 2.2).

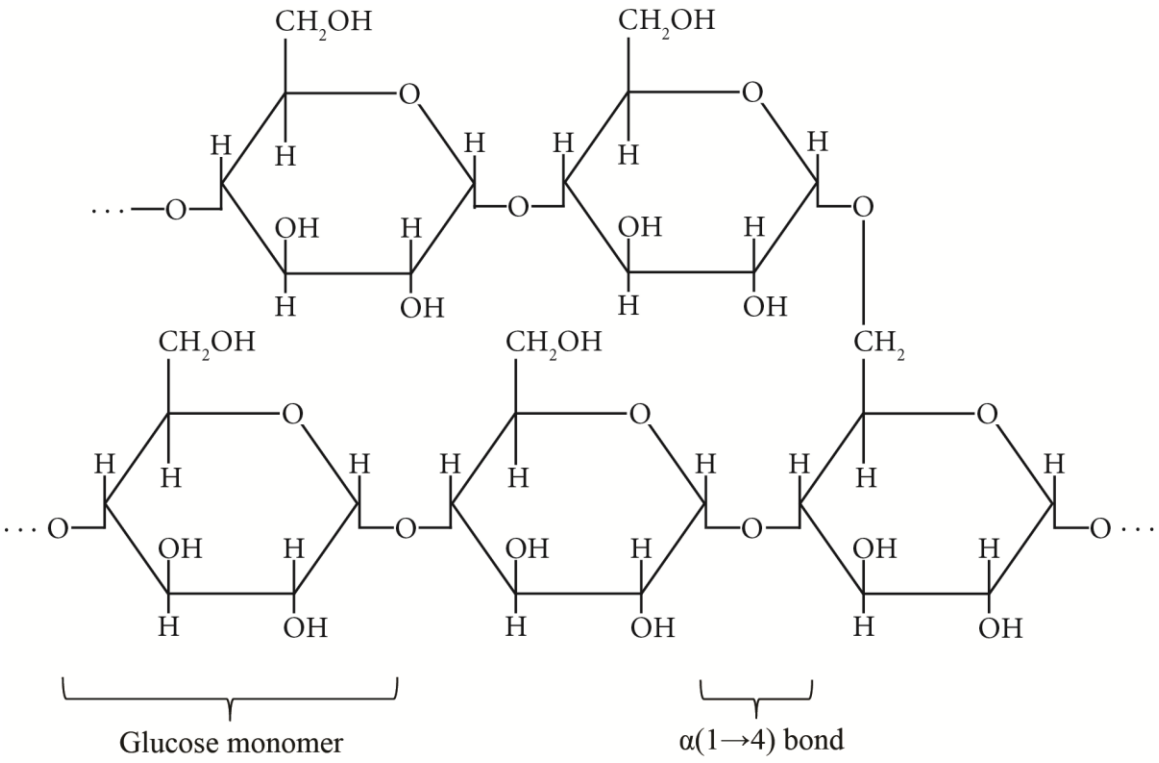

Figure 2.2. Chemical structure of amylose and amylopectin

During biofuel production processes, starch is first mixed with water and heated in a gelatinizing step (e.g., jet cooking) to interrupt the intermolecular bonds of amylose, and promote the release of short chain sugars (oligomers) into solution. A class of enzymes known as amylases is then added to the slurry mash to catalyze the release of glucose monomers by hydrolyzing the glycosidic bonds of the oligomers (Douglas et al. 1997). The resulting glucose solution is inoculated with distiller's yeast, *Saccharomyces cerevisiae*, and fermented to produce ethanol in a liquid fraction known as beer. During the final stages of processing, the beer is concentrated through several distillation (and dehydration) operations to generate the end product, anhydrous ethanol (200 proof). Similar approaches are used for converting sugar-based feedstocks such as sugarcane, sweet sorghum, and sugar beets into biofuels, however with these crops, the disaccharide, sucrose, is extracted from the biomass via physical pressing and fed directly to *S. cerevisiae*. Yeasts possess natural enzymes (e.g., sucrase) to catalyze the hydrolysis of sucrose into glucose and fructose prior to fermentation.

One of the pressing concerns of first generation feedstocks is the inherent competition created with food and feed crops for prime agricultural land and resources. In particular, the redirection of consumable products (such as corn or sugar) to biofuel production has resulted in much debate over the priorities of food/feed versus fuel. Although economic studies disagree about the extent to which domestic ethanol (from first generation feedstocks) contributes to hikes in consumer grocery prices, most agree that costs are steadily increasing (Mueller et al. 2011). As a result, the use of second generation feedstocks has gained considerable appeal in recent years.

2.3 Second generation feedstocks

Second generation feedstocks, in contrast to first generation feedstocks, do not compete directly with the food and feed industries, and consist mainly of agricultural residues, forest resources, and dedicated energy crops. These feedstocks are also known as lignocellulosic biomass due to the three primary constituents that make up their biochemical structure: lignin (10-25%), cellulose (35-50%), and hemicellulose (20-35%) on a dry weight basis (Liu et al. 2008). Analogous to starch, cellulose is a homogenous polymer comprised exclusively of glucose monomers; but the orientation of the chemical bonds, namely in $\beta(1\rightarrow4)$ glycosidic linkages (Figure 2.3), give cellulose a rigid, linear structure capable of forming strong crystalline orientations through hydrogen bonding. The interruption of crystalline cellulose bonds through gelatinization requires a temperature of 320°C compared to 60-70°C for starch (Deguchi et al. 2006). Hemicellulose, a heterogeneous polymer of five (e.g., xylose and arabinose) and six carbon sugars (e.g., glucose, mannose and galactose), is relatively amorphous and serves mostly as a cross-link between cellulose and lignin. Lastly lignin, the third main constituent, is a polymer of three alcohols (p-coumaryl, coniferyl, and sinapyl) derived originally from sugar compounds (Amthor 2003). In its native form, lignin does not contain any carbohydrates and functions primarily as a structural support as well as a barrier to protect plants from external physical and biological attacks, Figure 2.4.

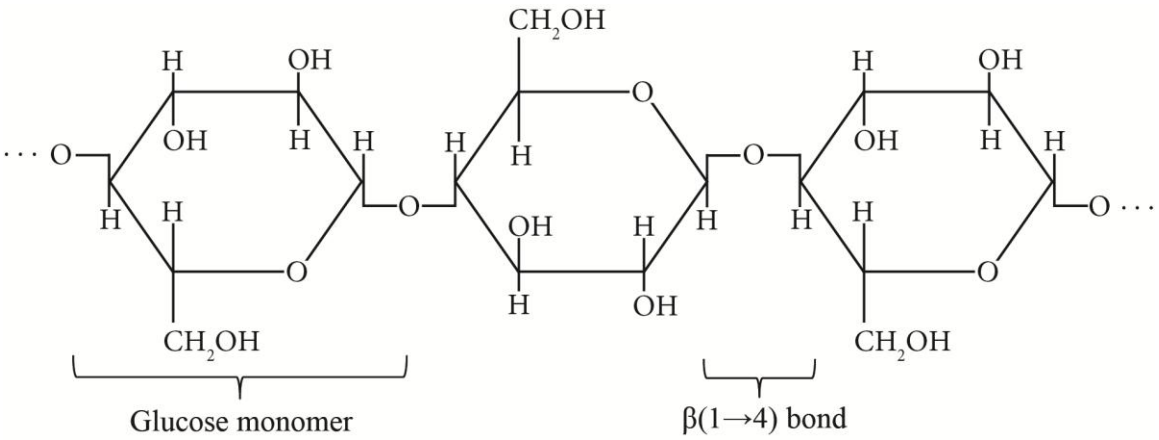

Figure 2.3. Chemical structure of cellulose

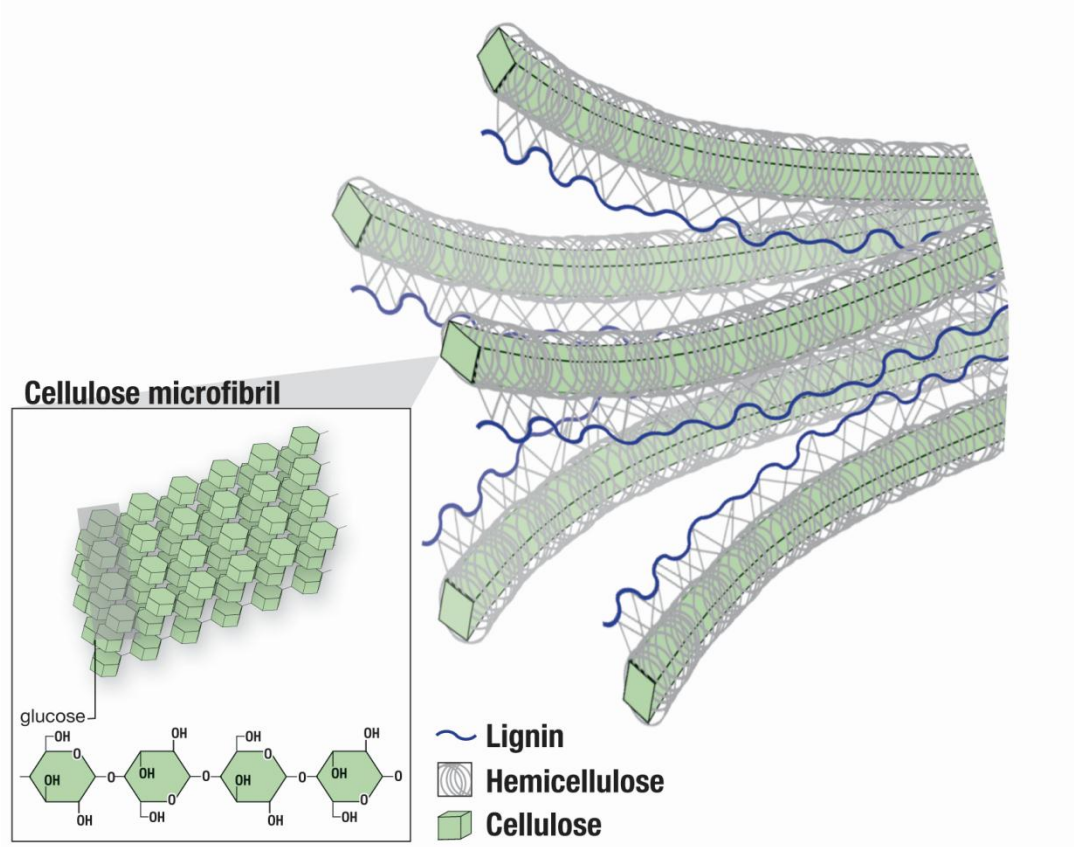

Figure 2.4. Interactions of hemicellulose, cellulose, and lignin

The interactions of lignin, hemicellulose, and cellulose collectively create a near impenetrable biomass and pose a significant challenge in the bioconversion of second generation feedstocks into biofuel. To mitigate this issue, processing strategies of biomass known as pretreatments are required to improve the deconstruction of structural carbohydrates into monomeric sugars for fermentation by yeast and other microorganisms. Although a plethora of pretreatment strategies have been developed to date, most conventional pretreatments fall into four main classifications: (1) biological, (2) physical, (3) chemical and (4) hybrid (i.e., combinations of the other three pretreatments). The merits and disadvantages of current leading pretreatment technologies are discussed further in the following sections.

2.4 Biological pretreatments

The biological degradation of lignocellulosic material may be the oldest form of pretreatment available for effectively accessing the five and six carbon sugars that comprise plant structures. Microorganisms like fungi excrete specific enzymes (e.g., ligninase) that dissolve the protective lignin layer of biomass, exposing the sugars of (hemi)cellulose for consumption. Conceptually, this type of pretreatment has many advantages inherent in its simplicity, but in practice, biological methods alone may be too slow and costly for large-scale industrial applications (Shrestha et al. 2008).

Ray et al. (2010) considered brown rot fungi for the biological pretreatment of softwood for improved glucose release from lignocellulose. The authors exposed pine sapwood to two species of fungi, *Coniophora puteana* and *Postia placenta*, for 20 and 25 days, respectively, before beginning saccharification (by commercial cellulolytic enzymes, cellulases) and ethanol fermentation (by *S. cerevisiae*). The glucose released after the addition of cellulase was reported to be about 70% of the theoretical yield; a moderate to low yield when compared to other pretreatments. Most significantly, the pretreatment process required a minimum of two weeks, not including the additional time needed for enzymatic saccharification and fermentation, suggesting significant limitations in the continuous production of biofuels.

Another study by Yang et al. (2009) reported the use of a thermophilic bacterium, *Anaerocellum thermophilum*, for ethanol production by direct growth on two common second generation feedstocks: poplar and switchgrass. The bacteria were cultivated at an elevated temperature of 75°C until a stationary growth phase was observed. Despite achieving high cell densities within 20 hours (much faster than fungal cultures), ethanol produced through the metabolic pathways of the microorganism was not detected in the product stream. Biological pretreatments with both fast turnover and high sugar yields have yet to be developed as a sole pretreatment strategy.

2.5 Physical pretreatments

Physical pretreatments describe the mechanical deconstruction of lignocellulosic biomass and often incorporate the use of cutting, ball, and hammer mills. Although physical pretreatments were originally proposed as single upstream unit operations to enhance enzymatic saccharification, it is now largely understood that all feedstocks (e.g., trees and perennial grasses) require some form of size reduction (i.e., physical pretreatment) to facilitate biomass handling in downstream processing. Innovative technologies such as ultrasonication also belong to this pretreatment category and in general, the term physical pretreatment has evolved to more broadly include non-chemical (and non-biological) strategies which disrupt the physico-chemical interactions of lignocellulose. Examples of such pretreatments include hot water washing and steam explosion which rely on the properties of water to cause physical disruption of lignocellulosic biomass.

2.5.1 Hot water washing

Hot water washing has received much attention due to its independence from the use of caustic chemicals and relatively milder environmental impacts. Hot water, superheated and pressurized to remain in the liquid phase, has been shown to promote the auto-hydrolysis of hemicellulose and amorphous cellulose in biomass structures. In a study by Mosier et al. (2005), corn stover was pretreated with water at 190°C for 15 minutes prior to saccharification by commercial cellulases. The authors reported a 90%

conversion of cellulose to monomeric glucose. A similar approach was applied to wheat straw, but lower yields were obtained. Hemicellulosic sugars from wheat straw were observed to be best removed at temperatures of 184°C (71% recovered), while cellulose was best removed at temperatures of 214°C, followed directly by enzymatic hydrolysis (91% of the theoretical glucose recovered); thus suggesting the implementation of a two-stage hot water approach (Pérez et al. 2008). For rice straw, yields of 85% glucose following enzymatic hydrolysis were reported at a temperature of 180°C (Yu et al. 2010).

More recently, hot water washing has been incorporated into flow-type reactors, improving hemicellulose breakdown from 60%-90% and lignin from 30%-75%. Pronyk and Mazza (2011) evaluated the use of high pressure (11 MPa) water in a custom-designed reactor on the cereal crop, triticale. Optimal conditions were determined by the maximum sugar released from hemicellulose, which occurred at a temperature of 165°C, 115 mL/min water flow rate, and a 60 mL/g liquid-to-solid ratio. The release of glucose from cellulose was reported to be highly temperature dependent, and 90% digestibility yields (following enzyme hydrolysis) were not achieved for temperatures below 170°C.

2.5.2 Steam explosion

Similar to hot water washing, steam explosion pretreatments employ the properties of water to facilitate the deconstruction of lignocellulosic feedstocks. Unlike the previously described methods however, steam explosion by itself produces comparatively low sugar yields; often around 70% under optimal conditions (Lu et al. 2010; Yang et al. 2010). Most steam explosion techniques are supplemented with acid catalysts (like sulfuric acid) or are conducted immediately following chemical pretreatments (Chen et al. 2011) to maximize the deconstruction of biomass. The fundamental concept behind steam explosion is analogous to a chemical pretreatment known as ammonia fiber expansion described later. High pressure is maintained in a reactor vessel to keep water in the liquid phase for a predetermined duration (varying by the types of crops and reactors). After the residence time has been achieved, the vessel is instantly decompressed to atmospheric conditions (Ramos 2003). The rapid change in pressure creates an explosion as the superheated liquid water is instantly vaporized. A

physical disruption of the biomass occurs, subsequently improving the accessibility of cellulases to cellulose fibers during saccharification.

2.6 Chemical pretreatments

A significant number of pretreatments today rely on the use of chemicals to solubilize and remove specific plant constituents, consequently destabilizing the interactions of lignin, hemicellulose, and cellulose. Despite the exhaustive list of chemicals and solvents tested and reported in literature, common practices often incorporate various dilute aqueous acid and alkaline solutions which have their roots in the pulp and paper industry (Sutermeister 1920).

2.6.1 Acid pretreatments

Dilute acid solutions are well known for their selectivity in solubilizing hemicellulose, thus enhancing the permeability and efficacy of commercial cellulases on cellulose fibers in downstream saccharification. Because hemicellulose is a heterogeneous polymer, the hydrolysate produced during dilute acid pretreatments cannot be fermented directly to biofuel by conventional *S. cerevisiae* since traditional (wild-type) distiller's yeasts are unable to metabolize pentose sugars. Research incorporating genetic manipulation of *S. cerevisiae* have been conducted in an attempt to produce a single hexose- and pentose-converting species (Kato et al. 2012), but these strains remain mostly in the testing and development stages. Other naturally occurring species like *Pichia stipitis* have been reported to successfully convert xylose to ethanol with yields as high as 0.44 g ethanol per gram substrate (Lin et al. 2012). Barring the advent of significant advancements in genetically engineered microorganisms in the near future, two stage and concurrent fermentations of xylose and glucose with *P. stipitis* and *S. cerevisiae*, respectively, have been proposed as a solution for increased ethanol production from lignocellulose (Li et al. 2011).

Hsu et al. (2010) examined the effects of dilute sulfuric acid on rice straw containing 36.6% glucose, 16.1% xylose, and 14.9% lignin on a dry weight basis at various acid concentrations of 0.5-1.0% (w/w) with residence times between 1-25

minutes. Following enzymatic hydrolysis, a maximum total sugar yield of 83% was achieved under optimal conditions: 1% (w/w) sulfuric acid, 1-5 minutes residence time, and a temperature of 160-180°C. Furfural and 5-hydroxymethylfurfural (HMF) are formed by the degradation (i.e., oxidation) of xylose and glucose, respectively, during pretreatment processes. Increases in the concentrations of these oxidized compounds correspond with decreases in available sugars for fermentation. Furfural and HMF are sometimes referred to as inhibitors as they are known to inactivate both cellulases and ethanol-producing yeast. The authors found that as the severity of the dilute acid pretreatment increased (with respect to acid concentration, temperature, and time), the concentration of inhibitors also increased. At 160°C, 1% (w/w) acid, and a residence time of 2.5 minutes, 0.1 and 0.3 g/L of HMF and furfural, respectively, were detected; while at 180°C, 1% (w/w) acid, and a residence time of 2 minutes, HMF and furfural concentrations were 0.2 and 1.9 g/L, respectively. The oxidation of xylose to furfural is likely more prominent than glucose to HMF with increasing pretreatment severity since hemicellulose is relatively amorphous in structure (i.e., not reinforced by hydrogen bonding) than cellulose and is easily and selectively hydrolyzed by dilute acid solutions.

Li et al. (2010) reported similar findings with the use of dilute sulfuric acid at a 1% (w/w) and 3% (w/w) solid loadings of switchgrass. Hemicellulose and other amorphous sugar polymers were solubilized from the solid fraction during pretreatment, and the authors compared their results to samples pretreated with an ionic liquid, 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]). The total time required to deconstruct switchgrass to monomeric sugars was observed to be significantly reduced from 72 hours (with dilute sulfuric acid pretreatment and saccharification) to 15 hours (with [C2mim][OAc] and saccharification). The costs and environmental impacts of using exotic chemicals in large scale reactors are relatively unknown, but further discussion of ionic liquids as a pretreating strategy has been included in a later section.

Several recent studies have sought to identify other acids for biomass pretreatments such as organic acids, suggesting that inorganic (mineral) acids may have caustic harmful effects on the microbial strains used in biofuel and biobased product generation. Most recently, Zhang et al. (2012) investigated the use of oxalic acid in the pretreatment of maple wood, a fast growing tree. The effects of the organic acid were

compared side by side with dilute sulfuric acid, dilute hydrochloric acid, and hot water. The authors found that oxalic and sulfuric acid outperformed the other two strategies by releasing greater than 84% of the hemicellulose-derived xylose at 160°C and 27.5 minutes. With respect to glucose, all of the pretreatments performed relatively equally following enzymatic hydrolysis, and released about 90% of the theoretical glucose. The use of organic acids is indeed a noteworthy approach, but the prevalence of sulfuric acid in modern industries (*Sulfur: Statistics and Information* 2012) and pilot-scale studies (Schell et al. 2003; Humbird et al. 2011) indicate its viability for commercial biofuel production.

2.6.2 Alkaline pretreatments

Unlike acids, aqueous alkaline solutions selectively solubilize and remove the lignin components of lignocellulosic biomass. After pretreatment, the remaining solid fibers, consisting primarily of cellulose and hemicellulose, can be saccharified to fermentable sugars via (hemi)cellulase enzymes.

A study by Chu et al. (2010) confirmed the specific solubilization of lignin (with 10% (w/w) sodium hydroxide (NaOH)) by Raman confocal microscopy. Control samples of *Miscanthus x giganteus*, a perennial grass feedstock, contained observable quantities of lignin and cellulose within the cell walls, but following pretreatment, only cellulose remained visible and intact. The authors were unable to detect hemicellulose before and after pretreatment due to its amorphous structure. Studies have shown that in some cases, hemicellulose can also be selectively removed by alkaline chemicals, but the efficiency of sugar release (< 80%) is often much less than that observed for dilute acid pretreatments and is contingent on the chemical used (Sun et al. 1995). Furthermore, aqueous alkaline pretreatments solubilize oligomers and require the addition of hemicellulases to catalyze hydrolysis to fermentable monomers.

Gupta and Lee (2010) compared the effects of ammonia (NH_3) and NaOH solutions on the pretreatment of switchgrass since both chemicals have been known to perform differently among various feedstocks. Aqueous ammonia, for example, has been reported to be more successful in targeting the lignin of non-woody crops (Kim et al. 2003), while sodium hydroxide has been particularly useful on woody crops. Gupta and

Lee (2010) demonstrated that when supplemented with hydrogen peroxide (H_2O_2), both ammonia and sodium hydroxide improved the digestibility of cellulose (by cellulases) releasing over 90% of the theoretical glucose. Interestingly, however, when hydrogen peroxide (H_2O_2) was applied to ammonia, sugar degradation (particularly from hemicellulose) was increased. The authors suggested that temperature may have played a crucial role in this occurrence in addition to the mechanism by which these chemicals react. Ammonia pretreatments occurred at 120°C, promoting the generation of free radicals from H_2O_2. The radicals were highly effective at breaking down lignin constituents, but also reacted with and degraded free carbohydrates. Pretreatments with sodium hydroxide at 85°C resulted in less loss of sugars.

Recently, NaOH pretreatments were optimized for wheat straw containing 36% cellulose and 26% hemicellulose on a dry weight basis. By varying NaOH concentrations (0.75%, 1.0%, and 2.0% (w/v)) and residence times (30, 60, and 90 minutes) at 121°C, McIntosh and Vancov (2011) determined an optimal sugar release of 87% of the total carbohydrates from holocellulose following enzyme saccharification. The authors reported the release of short chain oligomers from hemicellulose (i.e., oligoxylans and arabinoxylans) during the pretreatment process, but suggested that these compounds may have implications in high-value, non-biofuel products.

An insightful study comparing the efficacy of two leading chemical pretreatments, ammonia fiber expansion (AFEX) and dilute sulfuric acid was conducted by Lau et al. (2009) using corn stover as the feedstock. AFEX employs the low volatility and alkaline properties of anhydrous ammonia to facilitate the combined chemical and physical disruption of lignin, hemicellulose, and cellulose components in the lignocellulosic biomass. The setup of AFEX is similar to steam explosion (described in brevity previously), in which the substrate is steeped in superheated liquid ammonia for a predetermined residence time before being violently decompressed to (near) atmospheric conditions. The instantaneous pressure release results in the vaporization of ammonia and a physical disruption of biomass structures. The advantages of this approach (in comparison to steam explosion) include lower operating pressures, ammonia recovery, and faster pretreatment times (Teymouri et al. 2005). Lau et al. (2009) reported that while 10% of the sugars (particularly xylose) were degraded during dilute acid pretreatments,

all of the carbohydrates were preserved when AFEX pretreatment was applied. One of significant drawbacks worth restating for AFEX and all alkaline pretreatments is the inherent need for additional enzymes, hemicellulases, to solubilize sugar oligomers released from hemicellulose during pretreatment. In contrast to alkaline pretreatments, dilute acid pretreatments consistently hydrolyze hemicellulosic structures directly into fermentable monomeric sugars.

2.6.3 Ionic liquid pretreatments

An emerging field in the chemical pretreatment of biomass incorporates the use of ionic liquids (ILs) to promote the deconstruction of lignocellulosic structures. Various salts with low melting temperatures have been shown to selectively dissolve cellulose by interrupting the hydrogen bonds that reinforce fibrous structures (Swatloski et al. 2002). A significant limitation preventing the widespread application of ILs in biomass pretreatment, however, is its inactivation of the cellulases (Turner et al. 2003) required for hydrolyzing the solubilized cellulose into glucose. Also the heterogeneous polysaccharide, hemicellulose, remains in the solid fraction with lignin and can only be hydrolyzed for fermentation by the addition of hemicellulases.

Wang et al. (2011) considered the stability of commercial cellulases in 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]); an IL known to dissolve cellulose under relatively mild conditions. The authors mixed the enzymes into various concentrations of IL ranging from 5-30% (v/v) for time periods of 1.5, 3 and 24 hours. The activity of the enzymes, following exposure to [C2mim][OAc] was determined by digesting cellulose filter paper. Wang et al. (2011) reported a 70% retention of enzyme functionality after 24 hours in 30% (v/v) [C2mim][OAc] at 4°C, but at a typical saccharification temperature of 50°C, the enzymes only retained 31% of their activity in 30% (v/v) [C2mim][OAc]. Higher concentrations, temperatures, and residence times of IL pretreatment appeared to result in an increase in enzyme deactivation. Because commercial cellulases are an expensive component to all current biomass-to-biofuel processes (Klein-Marcuschamer et al. 2012), more research is needed to develop ILs which are less detrimental to enzymes. A summary of leading pretreatment strategies for pilot and commercial scale studies is presented in Table 2.1. IL pretreatments were not included as this approach continues to

remain in its infancy, and needs to overcome significant challenges with respect to its deactivation of enzymes and cost recovery of the exotic chemicals.

Table 2.1. Leading pretreatment strategies for scale-up to pilot and commercial scales

Pretreatment Method	Process	Feedstock	Yield Reported	Inhibitor Generation	Limitations	Reference
Physical	Hot water washing	Corn stover	90% glucose	Yes	Large water requirement, high temperature	(Mosier et al. 2005)
	Steam explosion	Corn stover	76% glucose	Not reported	Low sugar yield	(Yang et al. 2010)
Chemical	Dilute sulfuric acid	Corn stover	93% total sugars	Yes	Cost of equipment, need for neutralization	(Lloyd and Wyman 2005)
	Ammonia recycle percolation	Corn stover	92.5% glucose	Negligible	Requires two stages – increased cost/space	(Kim and Lee 2005)
Biological	*Irpex lacteus*	Corn stover	66% glucose	Not reported	Slow process and low sugar yield	(Xu et al. 2010)
Hybrid	Ammonia fiber expansion	Corn stover	100% glucose 80% xylose	Negligible	Ammonia cost and recovery	(Teymouri et al. 2005)
	Acid-steam explosion	Corn stover	85% glucose	Yes	Inhibitor generation, equipment cost	(Zimbardi et al. 2007)

2.7 Biomass to biofuel production

Conventional upstream handling of second generation feedstocks has been largely based on approaches that parallel first generation starch/sugar crops, Figure 2.5. Lignocellulosic feedstocks are harvested by mechanized processes and left in the field to dry prior to densification (i.e., baling) and transportation. Upon reaching the biofuel facility, the biomass is shredded by a cutting mill and sent for pretreatment. Solid fibers exiting pretreatment processes are hydrolyzed by enzymes to release monomeric sugars. Hemicellulose-derived monomers, released in dilute acid pretreatments, are mixed with the glucose-rich sugar stream exiting saccharification to produce a mixed sugar solution and are sent for fermentation by *S. cerevisiae*, *P. stipitis*, or both, into biofuel. (In alkali pretreated biomass, both hemicellulose and cellulose are hydrolyzed by a cocktail of hemicellulases and cellulases, subsequently releasing a mixed sugar stream directly after saccharification.) Ethanol is dehydrated and recovered through distillation processes, and the stillage/residue is remediated by chemical and/or biological methods. The effluent is applied to the land as fertilizer and irrigation (i.e., fertirrigation). Lignin and the remaining solid residues following saccharification presently have little commercial value and have been subsequently regarded as leftover material for burning to generate heat/electricity. There is potential, however, for these and other process residues to be redirected for use in other platforms in the production of high value co-products.

Technologies and innovations in renewable biofuels continue to expand rapidly, but to date, the challenges of converting lignocellulosic biomass to biofuel, by economically viable methods, remains to be developed. Feedstock production logistics (i.e., harvesting, transportation and storage), pretreatment, and saccharification are just a few examples of unit operations that contribute significantly to the overall costs of producing biofuels, in addition to downstream processes like fermentation and product separation and recovery. Careful planning and design of production facilities, however, have been shown to reduce costs with little or no technological advancements. For example, Kocoloski et al. (2011) reported that the strategic placement of infrastructure in locations that minimize the transportation of both feedstocks and products can result in a savings of up to $0.25 per gallon ($0.07 per liter) of biofuel produced.

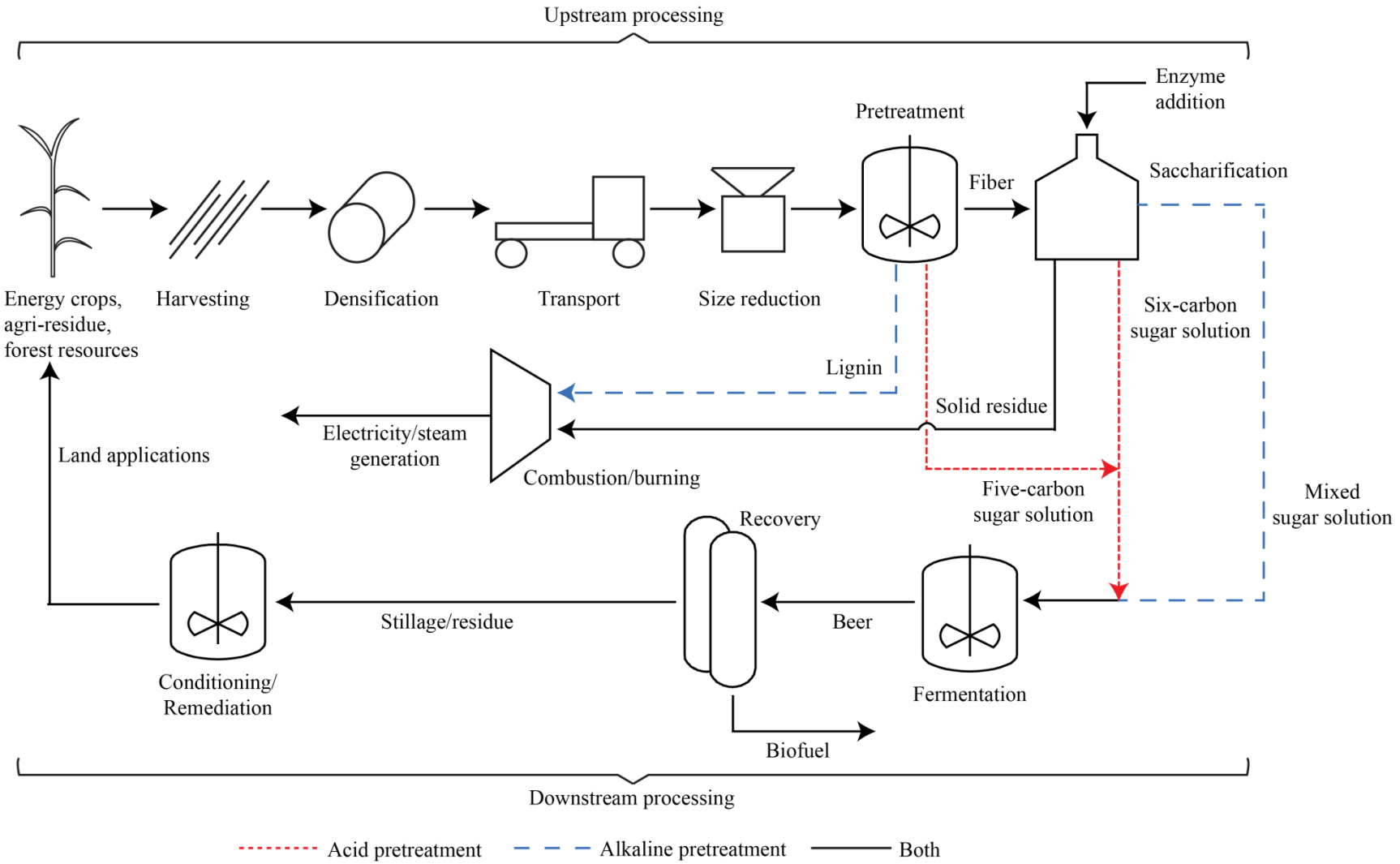

Figure 2.5. Flow diagram of second generation biomass to biofuel production

Higher solid loadings have also been considered to improve the overall economics of bioprocessing stages. By including solids at greater than 15% (w/w) in unit operations, the efficiency of biofuel production can be increased significantly. Starch processes currently accommodate a solids content of 30% (w/w) in contrast to second generation feedstocks, which have only been shown to incorporate 15-20% (w/w) in pilot scale studies (Modenbach and Nokes 2012). The advantages of implementing high-solids loadings include less water, higher titer and biofuel concentrations, and reduced energy inputs for heating and cooling (Modenbach and Nokes 2012). Zhang and Bao (2012) recently proposed a modified equation to predict the ethanol yield from the high-solids simultaneous saccharification and fermentation (SSF) of corn stover (after dilute acid and steam explosion pretreatments). The authors reported an unknown bias which underestimated their predictions, but the results from experimental and calculated data illustrate an important fact: ethanol production decreases with increasing solids content. The reasons for this occurrence are numerous and non-trivial from an engineering design perspective, and most often include mass/heat transfer limitations as well as the non-linear scale-up of laboratory methods. More research and development are thus required for improved bioprocessing at higher solids loadings.

As part of a larger, multi-institutional study conducted in 2005, a techno-economic analysis by Eggeman and Elander (2005) determined that the dilute sulfuric acid pretreatment of feedstocks was likely the most economically feasible for scaled up processing of lignocellulosic biomass. A more recent article by Kazi et al. (2010) reported a similar conclusion, and calculated a $8.72 per gallon of gasoline equivalent (GGE) or $2.30 per liter of gasoline equivalent (LGE) selling price for pioneer biofuel plants implementing dilute sulfuric acid pretreatments using corn stover. After the industry matured, the authors inferred that the n^{th} plant could sell ethanol for $5.13 GGE ($1.36 LGE); still higher than the current average prices of gasoline in the U.S. These cost projections are typically based on models established by the National Renewable Energy Laboratory (NREL) (Kazi et al. 2010; Leboreiro and Hilaly 2011; Kocoloski et al. 2011) and in many of the examined scenarios, the costs of producing biofuel are inherently tied to feedstock prices and availability, which depending on geography and resources (e.g., water), may be unpredictable and weather dependent. Attempts to

produce biofuel at lower costs (with government aid and subsidies) have in some cases met with success, but in general, the large scale production of biofuel at commercial volumes has not yet been proven to be competitive with gasoline. New perspectives on biomass handling may offer insight to more cost-effective approaches.

2.8 Green processing and biorefineries

Green processing is the innovative upstream fractionation of freshly harvested feedstock into solid and liquid streams, and may serve as one approach to improve the economics of large scale biomass-to-biofuel conversions. Combined with leading pretreatment strategies (such as dilute sulfuric acid pretreatments), green processing has the potential to produce biofuel and a suite of biobased co-products, thereby exemplifying a true biorefinery. The biorefinery concept is an emerging perspective that seeks to model biofuel industries after petroleum refineries, generating multiple products from a single feedstock (Yu et al. 2012; Moncada et al. 2012). Revenue generated from an array of high value co-products can be redirected to offset the cost of producing commodity fuels. In (sub)tropical regions of the world, green processing has the potential to be applied to perennial feedstocks of the sugarcane family (e.g., Napier grass, switchgrass, guinea grass, miscanthus) which have high moisture contents and fiber yields.

Dedicated energy crops can be harvested, fresh from the field, shredded and dewatered (via screw-pressing). The resulting clean solid fiber extruded from the screw-press can be pretreated with dilute sulfuric acid and subjected to conventional saccharification and fermentation unit operations. The liquid fraction, referred to as juice, can be collected and used for producing a suite of high value biobased products. Similar techniques have been proposed and applied to grasses in Europe (Schaffenberger et al. 2012).

The additional merits of implementing a green processing approach, particularly in (sub)tropical climates, include the obsolescence of biomass drying (an energy-intensive practice necessary in regions that experience winter seasons) and storage. Screw-pressing operations also improve the size reduction and disruption of biomass

structures (Hjorth et al. 2011), subsequently facilitating handling and the mass transfer of acid (during pretreatment) and enzymes (during saccharification) in downstream processes. To date, the potential for green processing has yet to be rigorously examined within the context of a biorefinery. The liquid fraction should ideally be recoverable in significant volumes and serve as a substrate for high value co-products generation, and the solid fiber should release significantly worthwhile concentrations of sugar for biofuel production. Figure 2.6 represents the potential for green processing within the context of a biorefinery.

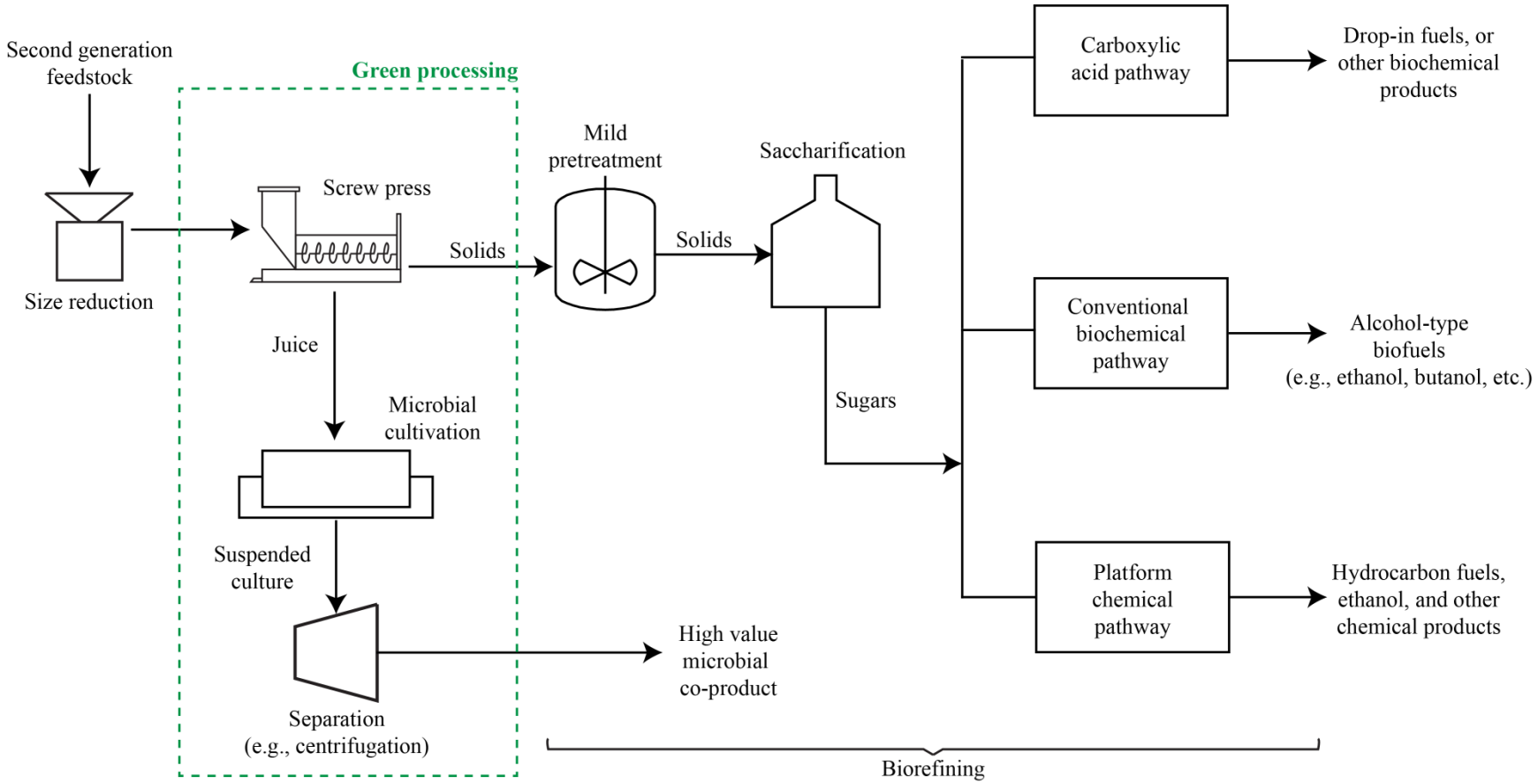

Figure 2.6. Green processing of second generation feedstocks within a biorefinery

Feedstock composition and age play a critical role in the applicability of green processing. As perennial herbaceous crops mature, physical and biochemical changes can occur which subsequently affect the cellulose, hemicellulose, lignin, and moisture content of the biomass (Holmes 1980). Research evaluating the biofuel and biobased product potential of green processing is necessary for illustrating the economic viability of scaling up and including this preprocessing strategy into future biorefineries.

2.9 Opportunities for Hawai‘i

Because of its geographical isolation, Hawai‘i can serve as the perfect testing site for green processing and the biorefinery concept, and function as a pilot scale for evaluating leading technologies prior to full size scale up. Presently, Hawai‘i is the most oil dependent state in the U.S., and meets over 90% of the energy demands through the importation of oil; more than 60% of the total demands are directed to the transportation sector (*About the Hawaii Clean Energy Initiative*). Thus there is already an urgent need for the development of renewable fuels on the islands to secure Hawaii's self-sufficient future and reduce negative impacts on its unique ecosystem.

The tropical climate of Hawai‘i affords the state with a year-round growing season and ideal conditions for producing high yields of dedicated energy crops for biofuel production. In particular, the perennial crop Napier grass (*Pennisetum purpureum*), also known as banagrass, has been reported to produce nearly 42 dry US tons of biomass per acre per year (94 dry metric tons per hectare per year) from its ratoons (Osgood et al. 1996); more than double the yields reported for many other crops grown on the continental US. The advantages of Napier grass in Hawai‘i, apart from its high yield, high moisture, and rapid growth, include its morphological similarity to sugarcane, the former staple crop of Hawai‘i. Consequently, much of the equipment, land, and knowhow of large-scale farming of sugarcane may be applied to Napier grass. Overall, the biofuel potential of Napier grass in Hawai‘i has been relatively unexplored, and there exists great opportunities for combining Napier grass with green processing for biofuel and biobased product generation in many (sub)tropical regions around the world.

CHAPTER 3

MATERIALS AND METHODS

3.1 Conventional and non-conventional preprocessing

Napier grass (*Pennisetum purpureum*) of 4 months old was hand harvested from the Waimanalo Research Station (Waimanalo, HI, USA) and sealed tightly in thick plastic bags (to minimize evaporative losses) during transport to the University of Hawai‘i at Mānoa campus. Samples were immediately shredded by a commercial cutting mill (Vincent Corporation, Tampa, FL, USA) and separated into two classifications designated as (1) unjuiced and (2) juiced. Napier grass belonging to the juiced category was fed through a dewatering screw-press (Vincent Corporation, Tampa, FL, USA) which exerted 40 psi of pneumatic backpressure down the length of the auger. The juice squeezed from the plant biomass was captured in a 5-gallon bucket at one end of the screw-press, and the extruded solid fiber was collected on the opposite end. The extruded solid fiber was separated into two more classifications designated as (1) dry and (2) wet.

Unjuiced samples were not fed into the dewatering screw-press and were also separated into a (1) dry and (2) wet category. Napier grass belonging to the dry category was placed in a convection oven at 105°C until constant weight was achieved. A total of four different preprocessing strategies were thus established: (1) wet/unjuiced, (2) dry/unjuiced, (3) wet/juiced, and (4) dry/juiced. A schematic diagram of upstream handling is presented in Figure 3.1.

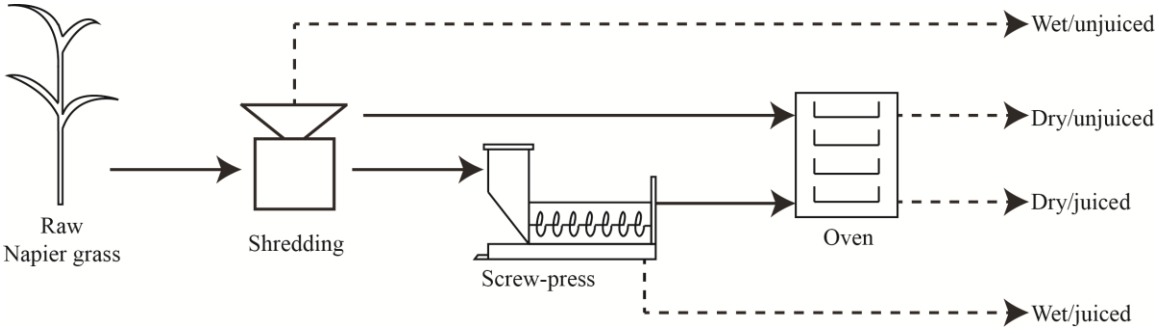

Figure 3.1. Schematic diagram of Napier grass preprocessing for comparison of conventional and non-conventional biomass handling

3.2 Moisture content analysis

Aluminum weigh dishes were dried for a minimum of four hours at 105°C or until a constant weight was achieved. Solid (or liquid) samples were carefully transferred to the respective dishes and were placed in a convection oven. Due to the high moisture content of Napier grass, samples were dried overnight until constant weight was achieved. A minimum of duplicate samples (Sluiter et al. 2008a) were used to determine the moisture content on a percent basis using the following equation:

$$\% MC = \left[1 - \frac{(DB - DP)}{(WB - DP)}\right] \times 100$$

where, MC represents moisture content in percent, DB represents the weight of the dry biomass, DP represents the weight of the empty dry pan, and WB represents the weight of the original wet biomass. Moisture content analyses were essential for establishing a dry weight basis for conducting and comparing other analytical experiments and data.

3.3 Dilute acid pretreatment

Three parameters were considered for the dilute sulfuric acid pretreatment of Napier grass: the concentration of acid, the temperature of pretreatment, and the residence time. Dilute sulfuric acid solutions were prepared at concentrations of 1.0%, 2.5% and 5.0% (w/w) and were added to Napier grass at a 1:6 (biomass-to-liquid) ratio. Pretreatment conditions were optimized on the basis of sugar release for each of the four preprocessing streams described in Section 3.1. Samples were placed in an autoclave (HVE 50 Hirayama, Japan) under varying temperatures (105°C, 120°C, and 135°C) and times (30, 45, 60 minutes). The effects of acid concentration, residence time, and temperature are known to be significant in improving the sugar release from lignocellulosic feedstocks (Canilha et al. 2011; Chum et al. 1990).

3.4 Reducing sugar analyses by spectrophotometry

The acid hydrolysate (also known as liquor) refers to the carbohydrate-rich supernatant that is produced following dilute sulfuric acid pretreatment. Under successful pretreatment conditions, the acid hydrolysate contains a high concentration of monomeric sugars derived from hemicellulose and amorphous cellulose. Because wet samples of Napier grass (e.g., wet/unjuiced and wet/juiced) were unstable beyond a period of two weeks at 4°C, spectrophotometric analyses of hydrolysate samples were used to expedite the detection of reducing sugar concentrations following pretreatment.

Aliquots of the acid hydrolysate were taken and analyzed for monomeric sugars using a modified dinitrosalicylic (DNS) acid colorimetric method (Miller 1959; Nitayavardhana et al. 2008). The DNS reagent consisted of 0.1% (w/v) 3,5-dinitrosalicylic acid, 30% (w/v) potassium sodium tartrate, and 20% (w/v) sodium hydroxide. Reagent (1 mL) was added to 100 μL of sample and heated for exactly 10 minutes, followed by immediate cooling in an ice bath for 5 minutes. The reacted sample was transferred to a cuvette and analyzed with a spectrophotometer at 570 nm (Hach Company, Loveland, CO, USA). Absorbances were correlated with a standard curve generated by known concentrations of D-glucose (and D-xylose) to determine the concentrations of reducing sugars in the acid hydrolysates.

3.5 Monomeric sugar detection by high performance liquid chromatography

Aqueous samples (5 mL) containing solubilized sugars were neutralized with calcium carbonate to a pH of 5.0 (Sluiter et al. 2006) prior to centrifugation at 10,000 rpm (12,857g) (F-34-6-38 Eppendorf, Hauppage, NY, USA) for 10 minutes. Aliquots of the supernatant (1 mL) were filtered through a 0.2 μm nylon membrane and injected into a Waters 2695 separations module equipped with a Waters 410 differential refractometer (Waters, Milford, MA, USA). Samples were run through a Rezex RPM Monosaccharide (Phenomenex, Torrance, CA, USA) column with degassed water (at 0.8 mL/min and 85°C) as the mobile phase. The run time was set to 20 minutes, followed by a 15-minute injection delay to reset the column between samples. Triplicate injections were performed for each analysis.

3.6 Enzyme hydrolysis of pretreated biomass

Solid fibers (post-pretreatment) were washed thoroughly with tap water, followed by a final rinsing with deionized water. The fibers were suspended in 20 mL glass scintillation vials with 0.1 M sodium citrate buffer (pH 4.8) and an equivalent of 0.15 g dry weight sample. Exactly, 100 μL of 2% (w/v) sodium azide was added as a biocide to prevent microbial contamination. The final volume was brought to 10 mL with deionized water (Selig et al. 2008). A cocktail of commercial cellulase (Accellerase 1000^{TM}, DuPont (formerly Genencor), USA) was added at 0.25 mL enzyme per gram cellulose as per the recommendations of the supplier. Because the optimization of enzymatic hydrolysis on the basis of sugar release was not the focus of any part of this study, the enzymes were deliberately loaded in excess such that the concentration of enzymes would not be a limiting factor in glucose release from cellulose. The enzyme-biomass slurry was kept for 72 hours at 50°C in an incubator-shaker (New Brunswick Scientific, Edison, NJ, USA) set at 200 rpm. After termination of the enzymatic reactions via ice bath, an aliquot (1 mL) was withdrawn and injected into the HPLC following the protocols presented in Section 3.5.

3.7 Age variation experiment

Napier grass was planted on April 18, 2011 in three rows at the Waimanalo Experiment Station, Oahu, HI. Elemental nitrogen (N) and potassium (K) were supplied at 60 lb. per acre, and the crops were lined with a drip irrigation system to maintain a total water delivery (from precipitation and irrigation) of 60 inches per year. The rows were numbered from 1 to 3 beginning with the western most row (nearest the mountain). Two month old planted Napier grass was collected from row 1 on June 18, 2011. Subsequent harvests occurred every two months to generate samples corresponding with four age categories: 2, 4, 6, and 8 month old Napier grass. A second series of harvests began on December 18, 2012 to provide replicate samples corresponding with the aforementioned age categories. Napier grass was not collected during or after rain to reduce the effects of weather biases on moisture content analyses.

3.8 Compositional analyses

Napier grass of varying ages was dried at 45°C until the moisture content was determined to be below 10%. The biomass was then ground in a commercial-grade 3 hp blender to a coarse powder in preparation of compositional analyses. Preliminary results suggested that sieving inadvertently fractionated the biomass samples and biased constituent values during compositional analyses (Hames et al. 2008). Subsequently, sieving was not incorporated in the methods reported here.

3.8.1 Extractives

Ground biomass samples were placed into cellulose thimbles (Whatman, USA); equivalent to approximately 4-5 g, and concurrently, a moisture content analysis was conducted to confirm that the biomass contained less than 10% moisture. (Samples were re-dried at 45°C if the moisture content exceeded 10%.) The extraction process essentially served to quantify the soluble, non-structural components of plant biomass such as inorganic compounds (soil and fertilizer) and other constituents such as chlorophyll and waxes (Sluiter et al. 2008c). Flat bottom boiling flasks (500 mL) were dried overnight in a convection oven at 105°C until constant weight was achieved. The dry weight of each flask was recorded, and 190 mL of deionized water was added to the flasks along with a magnetic stirrer (to prevent bumping). Biomass samples, prepared previously in cellulose thimbles, were placed in dry pre-weighed Soxhlet extraction tubes. The bottom of the Soxhlet tube was connected with the flat bottom flasks containing water, and the top of the tube was connected to a condenser with chilled water cycling through at 4°C. The setup was placed onto a magnetic-stir hotplate and brought to a boil. After exactly 24 hours, the flasks were switched with a new (pre-weighed) flat bottom flask containing 190 mL of 190-200 proof ethanol. The magnetic stirrer was rinsed and transferred to the ethanol flask. The system was reset on the hotplate and allowed to run for another 24 hours.

After the water and ethanol extractions, the solvents were evaporated with a rotary evaporator (Buchi, Flawil, Switzerland). The flat bottom flasks were placed in a

vacuum oven overnight at 40°C until constant weight was achieved. (To confirm NREL protocols, empty flat bottom flasks were dried overnight at 105°C in a convection oven and overnight at 40°C in a vacuum oven. The recorded weights between the two drying methods were determined to be identical.) The extractives were run in duplicates and were calculated by using the following equation (Sluiter et al. 2008c):

$$\%\ Extractives = \frac{W_{flask\ +\ extractives} - W_{flask}}{ODW_{sample}} \times \frac{V_{total}}{V_{total} - V_{removed}} \times 100$$

where, ($W_{flask\ +\ extractives}$) represents the weight of the dry flat bottom flask plus the extractives, W_{flask} represents the weight of the empty dry flat bottom flask, ODW_{sample} represents the weight of the dry weight of the biomass, V_{total} represents the volume of the water extractives brought to 200 mL, and $V_{removed}$ represents the aliquot taken (10 mL) for sucrose analysis. Note that for ethanol extractives, no aliquots were removed so the factor for volume correction was omitted.

Extracted solid fibers were removed from the thimble and rinsed with 100 mL of 190-200 proof ethanol in a Buchner funnel. The samples were allowed to dry under vacuum until the moisture content was determined to be less than 10% (approximately 2 days). The samples were subsequently used for carbohydrate analysis.

3.8.2 Carbohydrate analysis

The extracted solid fibers were digested with a two-stage sulfuric acid hydrolysis (Sluiter et al. 2008d). Biomass was added to glass pressure tubes, with a concurrent moisture content analysis of the stock biomass to confirm that moisture was less than 10%. Concentrated sulfuric acid (72% w/w) was added (3 mL), and the pressure tube was placed in a water bath at 30°C. The hydrolysis was allowed to occur for exactly one hour with mixing (by stir rod) every five to seven minutes. After one hour, the acid was diluted to a 4% (w/w) concentration with the addition of 84 mL of purified water to each pressure tube. The pressure tubes were capped and placed into an autoclave (HVE 50 Hirayama, Japan) for one hour at 121°C. Calibration standards containing known

concentrations of glucose, xylose, and arabinose (each at 5 g/L) were added (10 mL) in duplicates to pressure tubes with 4% (w/w) sulfuric acid, and placed in the autoclave concurrently with the biomass samples. The calibration standards known as sugar recovery standards (Sluiter et al. 2008d), or SRS, were essential in estimating the extent of degradation of monomeric sugars, namely xylose and arabinose, during the autoclaving process. The sample was passed through a filtering crucible (2 μm pores) and 40 mL of the liquid hydrolysate was collected for sugar analysis in an HPLC as per the methods described in Section 3.5. Aliquots of 5 mL were also taken for acid soluble lignin analyses via spectrophotometry. Structural carbohydrate constituents were calculated by the following equations (Sluiter et al. 2008d):

$$\%\, R_{sugar} = \frac{conc.\, detected\ by\ HPLC, mg/mL}{known\ conc.\, of\ sugar\ before\ hydrolysis, mg/mL} \times 100$$

$$C_x = \frac{C_{HPLC} \times dilution\ factor}{\%\, R_{ave.\ sugar}/100}$$

where, C_{HPLC} represents the concentration of sugar reported by the HPLC (mg/mL), $\%\, R_{ave.\ sugar}$ represents the average sugar recovery of specific sugars (averaged from duplicates), and C_x represents the concentration in mg/mL of sugar in hydrolysate after correcting for degradation during the autoclaving process.

Monomeric sugars were reported in polymeric form to mitigate inherent weight biases caused by the addition of water (during hydrolysis reactions). This was achieved by multiplying glucose concentrations by 0.90 and xylose by 0.88, representing the polymeric weight divided by the monomeric weight (e.g., 162/180 and 132/150 for glucose and xylose, respectively). Thus glucan and xylan were reported in lieu of glucose and xylose, respectively.

The following equations were used to convert the concentration of sugars into percent form:

$$\%\, Sugar_{ext\ free} = \frac{C_{anhydro} \times V_{filtrate}}{ODW_{sample}} \times 100$$

where, $V_{filtrate}$ represents 86.73 mL, calculated and presented in NREL protocols, ODW_{sample} represents the oven dry weight of the sample digested in the two stage acid hydrolysis.

$$\%\, Sugar_{as\ received} = \%\, Sugar_{ext\ free} \times \frac{(100 - \%\, Extractives)}{100}$$

where, % Extractives represents the extractives determined in Section 3.8.1. Note that the above equation was used to correct for the extractives removed from the biomass prior to carbohydrate analysis.

3.8.3 Lignin analysis

Lignin was determined as acid soluble and acid insoluble lignin (ASL and AIL, respectively) in two different processes in duplicate. It is important to note that true lignin analyses require time intensive, intricate chemical procedures which were not conducted in this research. Subsequently, it would be more appropriate to collectively treat this fraction of the biomass as lignin-like compounds; a more practical approach for biofuel characterization applications.

Acid soluble lignin was determined from hydrolysate aliquots collected in Section 3.8.2. The samples were diluted with water to produce an absorbance between 0.7-1.0 at 240 nm and the following equation was used to estimate the acid soluble lignin content (Sluiter et al. 2008d):

$$\%\, ASL = \frac{ABS \times V_{filtrate} \times dilution}{\varepsilon \times ODW_{sample} \times Path\ length} \times 100$$

where, ABS represents the ultraviolet visible range absorbance at 240 nm, $V_{filtrate}$ represents 86.73 mL (calculated and presented in the correlating NREL protocol), ε representing absorptivity of biomass (taken as 25 L/g cm), also determined and presented by Sluiter et al. (2008d), and ODW_{sample} represents the oven dry weight of the biomass digested in the two stage acid hydrolysis. The path length was 10 cm, and was an inherent property of the UV-spectrophotometer (Hach, Loveland, CO, USA).

Acid insoluble lignin was determined from the solid residue remaining in the filtering crucible. Note that the crucible were previously ashed (at 575°C) and brought to constant weight prior to beginning carbohydrate analyses. After separating the solid and liquid components of the two stage hydrolysis, the filtering crucibles were placed into a convection oven at 105°C for a minimum of four hours until constant weight was achieved. The values were recorded as the acid insoluble residue (AIR), and the filtering crucibles were transferred to a muffle furnace at 575°C. After 24 hours, the filtering crucibles were removed from the furnace and allowed to cool to room temperature in a dessicator for exactly 30 minutes. The weight of the samples were recorded and the following equation was used to estimate the AIL component of the biomass (Sluiter et al. 2008d):

$$\% AIL = \frac{(W_{crucible\ +AIR} - W_{crucible}) - (W_{crucible\ +\ ash} - W_{crucible}) - W_{protein}}{ODW_{sample}} \times 100$$

where, ($W_{crucible\ +\ AIR}$) represents the weight of the crucible and solid residue dried at 105°C, $W_{crucible}$ represents the weight of the empty crucible, ($W_{crucible\ +\ ash}$) represents the weight of the crucible plus the residue remaining after ignition at 575°C, $W_{protein}$ represents the protein content of the biomass, and ODW_{sample} represents the oven dry weight of the sample digested in the two stage acid hydrolysis. Note that $W_{protein}$ was not included for Napier grass fiber calculations as it constituted less than 1% of the dry weight of biomass for all samples.

3.8.4 Ash analysis

Duplicate ceramic crucibles were placed into a muffle furnace at 575°C. After 24 hours, the crucibles were removed and cooled in a dessicator for exactly 30 minutes. The crucibles were reheated at 575°C for one hour, and cooled for another 30 minutes. This process was continued until constant weight was obtained i.e., less than 0.3 mg change (Sluiter et al. 2008b). Upon achieving constant weight, a minimum of 0.5 g of ground (unextracted) biomass was put into the crucibles and weighed. A concurrent moisture content analysis was conducted to verify that the moisture in the biomass was less that 10%. The samples were placed in a muffle furnace for 24 hours and cooled for 30 minutes. When a constant weight of the ash was achieved, the following equation was used to determine the percent ash:

$$\%\ Ash = \frac{W_{crucible\ +\ ash} - W_{crucible}}{ODW_{sample}} \times 100$$

where, $W_{crucible\ +\ ash}$ represents the weight of the crucible plus the residue remaining after ignition at 575°C, $W_{crucible}$ represents the weight of the empty crucible, and ODW_{sample} represents the oven dry weight of the sample calculated from the moisture content determined previously.

3.8.5 Nitrogen analysis

The nitrogen content of Napier grass juice was determined as total Kjeldahl nitrogen (TKN) values. Samples were digested with concentrated sulfuric acid in a commercial digestion unit at 440°C (HACH, Loveland, CO, USA) as per the manufacturer's protocol (*Disgesdahl Digestion Apparatus: Instrument Manual*). The resulting digestate was brought to volume (100 mL) in the provided digestion flasks. Aliquots of 1 mL were transferred to 25 mL graduated cylinders and procedures for TKN determinations were followed using kit reagents (*Nitrogen, Total Kjeldahl: Nessler Method*). Nessler reagent was added to the graduated cylinders and reacted with the

samples for exactly two minutes at room temperature. The concentration of TKN was subsequently determined by a HACH DR 5000 UV-spectrophotometer (at 460 nm) with pre-installed software calibrated for the purchased reagents (HACH, Loveland, CO, USA). Ammonia standard solutions were made and analyzed periodically to verify the integrity of the commercial unit, kits, and spectrophotometer.

3.8.6 Chemical oxygen demand analysis

The organic content of Napier grass juice was quantified by chemical oxygen demand (COD) analyses and was determined by a commercial kit method (HACH, Loveland, CO, USA). Samples (0.3 mL) were pipetted into purchased vials containing a dichromate solution ($Cr_2O_7^{2-}$) which were heated for 2 hours. During this time, oxidizable organic compounds reacted specifically with dichromate ions to produce the spectrophotometrically quantifiable chromium (III) ion (Jirka and Carter 1975). The vials were placed into a HACH DR 5000 UV-spectrophotometer at 620 nm. Software installed in the equipment quantified and reported the concentration of COD in terms of mg/L.

3.9 Statistical analyses

Statistical analyses were conducted by computer software, Statistical Package for Social Sciences (SPSS) software version 17 (SPSS Inc., USA). To elucidate differences about mean values, an analysis of variance was determined followed by post-hoc Duncan's Multiple Range Tests for laboratory data. The SPSS software was also used to conduct randomized complete block analyses of variance (RCB ANOVA), trend analyses and orthogonal contrasts to report differences among Napier grass constituent data. RCB ANOVA is a commonly used agronomic statistical method that utilizes a technique known as blocking to minimize nuisance factors. Trend analyses identify patterns in data with respect to an independent variable, and where appropriate, fit a curve of significance. Orthogonal contrasts assign coefficients to group means (the sum of which equal to zero) to determine differences between the means.

Tables resulting from statistical analyses of data in this study have been included in Appendix B for reference. A single asterisk was used to denote values of significance where $p < 0.5 < 0.01$, and a double asterisk was used to denote significant values of $p < 0.01$.

3.10 Techno-economic analysis

Green processing can conceptually be considered as a standalone subsystem which fits into the larger, overarching biorefinery system. Subsequently, the economic merits of green processing can be considered and evaluated, independent of the entire biomass-to-biofuel process. In this analysis, the unit operations which function exclusively in green processing have been considered. Because the biorefinery concept and green processing, in particular, remain in the research and development phase, capital investments represent significant concerns and risks for pilot and commercial-scale application of technologies.

The four main unit operations considered to be inherently necessary and mutually exclusive to green processing were identified as the dewatering screw-press, bioreactor for microbial co-products, centrifuge, and co-product dryer. Shredding was determined to be required for both conventional and non-conventional upstream biomass handling and was thus not included in the analysis. Vendor quotations were obtained for two equipment, namely the screw-press and centrifuge, in 2012 U.S. dollars. Bioreactor costs, which consisted primarily of the reactor itself, an agitator, and air compressor, were estimated from literature describing the large-scale cultivation of the fungus *Trichoderma reesei* for cellulase production (Humbird et al. 2011). *T. reesei* serves as an appropriate surrogate for the model co-product, *R. oligosporus*, since both species are mesophilic, filamentous fungi. The co-product (spray) dryer was also estimated from literature (Li et al. 2011). *R. oligosporus* was assumed to have a market value of $428 per metric ton based on the average price of soybean meal for 2012. Because *R. oligosporus* and soybean meal have comparable protein contents, 45-50% crude protein on a dry weight basis (Nitayavardhana and Khanal 2010), *R. oligosporus* has the potential to replace soybean meal as an ingredient for fishmeal production in Hawai‘i.

The scale-up of industry quotations and literature values prove to be challenging in economic forecasting. For the four unit operations considered, costs were adjusted to reflect the capital investment required to produce biofuel at 1 million gallons per year (MGY) or about 3.8 million liters per year (MLY) from Napier grass grown in Hawai‘i. (Note that ethanol was used as a surrogate for biofuel production.) Both experimental and literature data were incorporated into calculations. Scale-up costs were estimated by the following equation reported by Humbird et al. (2011):

$$New\ cost = Base\ cost \left[\frac{New\ size}{Base\ size}\right]^{n}$$

The exponential factor, *n*, was reported to be between 0.6-0.7 for most unit operations.

Following scale-up estimations, values were adjusted to 2012 U.S. dollars by assuming a discount rate of 4.0%, determined by averaging the historical Federal Reserve discount rates from 1986-2012. A similar approach has been applied previously in literature (Tran et al. 2011). The annual income required to pay off the capital investment was calculated over a 25 year period with the 4.0% discount rate. The difference of the revenue generated by *R. oligosporus* for a 1 MGY (3.8 MLY) biofuel facility and the annual income required should be positive over a 25 year period to justify the capital investment in equipment. If the difference is negative, the addition of green processing unit operations could actually represent a financial burden to biorefineries.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Biochemical characterization of Napier grass

A number of studies to date have examined the chemical pretreatment of second generation, herbaceous feedstocks (e.g., miscanthus, sorghum, switch grass), but few have considered Napier grass and its potential for biofuel production. The biochemical constituents of Napier grass were determined for the first time in Hawai‘i with respect to glucan, xylan, lignin, and other minor components. Based on its availability at the start of this research, 4 month old Napier grass was selected as the substrate for subsequent experiments in Sections 4.1 to 4.4. Initially, samples of Napier grass were sent to a commercial laboratory (Microbac Laboratories, Boulder, CO, USA) employing standardized NREL protocols for compositional analyses. The results of the analyses are presented in Table 4.1.

Nearly 60% of the dry weight of Hawai‘i-grown Napier grass was found to consist of glucan and xylan, corresponding to cellulose and hemicellulose contents, respectively. (Note that all sugar data were presented in this chapter as anhydrous polysaccharides to permit comparison with other biomass constituents on a dry weight basis.) The mass closure determined by Microbac Laboratories was around 94 ± 6%. Losses deviating from a complete mass closure of 100% were believed to be a result of experimental and equipment errors, as well as natural variations inherent in biological samples. The % Others value in Table 4.1, consisting mainly of extractable soluble compounds, was much lower than the data reported later in Section 4.5. The difference may be attributed to oven drying as the outsourced samples were kept at 105°C until constant weight was achieved (following company recommendations) to prevent microbial degradation of the samples during overseas shipment from Hawai‘i. Drying Napier grass under such a condition was later found to volatilize some of the volatile organic compounds (VOCs) present in the biomass extractives and ash constituents. Samples analyzed in-house and reported in Section 4.5 were not dried at 105°C during any stage of analysis and subsequently contained higher extractives values.

Table 4.1. Major constituents of Napier grass

Napier grass (4 months old, planted crop)	
% Glucan	38.4
% Xylan	20.2
% Lignin	20.9
% Ash	7.8
% Others	6.7
Reported on % dry weight basis	

4.2 Preliminary green processing trials

Preliminary investigations with raw Napier grass in a laboratory-scale cutting mill (Retsch, Haan, Germany) resulted in immediate clogging of the equipment due to the high moisture content and fibrous nature of the feedstock. Napier grass appeared to have been crushed rather than shredded, and formed wet aggregates within the machine. The orientation of the rotor contained six disks fused together. On each disk, there were three cutting tips that were separated by 120 degrees as shown in Figure 4.1 (and Figure C.1). The maximum speed of the laboratory cutting mill was 1,500 revolutions per minute.

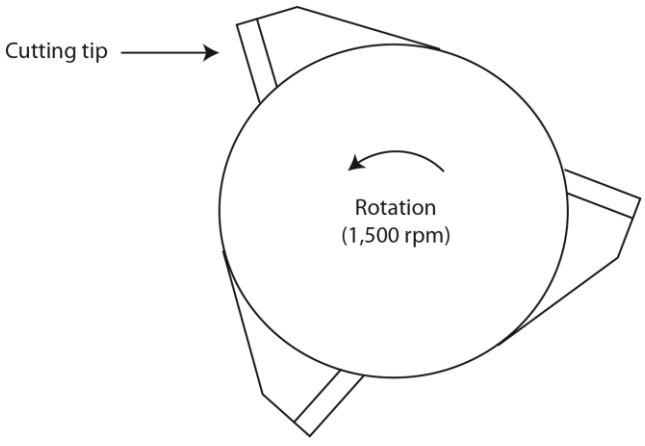

Figure 4.1. Side-view of one of six disks on laboratory cutting mill

An alternative cutting mill (Vincent Corporation, Tampa, FL, USA) with a total of eight swinging knife blades was tested on raw Napier grass, shown in Figure 4.2 (and

Figure C.2). The medium-scale commercial equipment had a higher operating speed (3,520 rpm) and successfully shredded the raw feedstock into sizes less than 5 cm in length. Because Napier grass is extremely fibrous with a high moisture content, blade orientation and speed were found to be critical properties of the shredder used in the upstream processing of wet biomass. In general, Napier grass was found to be more malleable than dry material, and subsequently experienced little or no physical changes/deformation when passed through a cutting mill at lower speeds (< 3,520 rpm). Moreover, the orientation and shape of the cutting tips on the laboratory-scale equipment may have precluded the severing of cellulosic fibers. Cutting edges with a 180° spacing is a relatively unique design which allows biomass to fall deeper into the shredder before being sliced by a high velocity blade (*Shredder Design*). Because the size reduction of wet feedstock is unconventional, blade design presents an important consideration for the application of green processing and/or similar upstream biomass handling techniques.

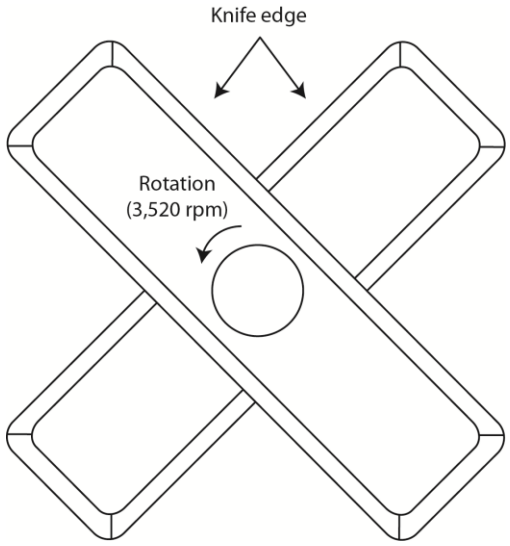

Figure 4.2. Side-view of two knife blades from the commercial cutting mill

Following shredding, the ability to fractionate Napier grass into solid and liquid fractions for green processing was evaluated. An initial attempt employed a custom-fabricated, stainless steel piston that exerted crushing forces (> 100 psi or >690 kPa) via a

hydraulic press (Figure C.3). No quantifiable volumes of juice were recovered, however, and pressure alone appeared to be insufficient in fractionating the shredded biomass.

Alternatively, shredded Napier grass was passed through a dewatering screw-press (Vincent Corporation, Tampa, FL, USA). (The equipment was procured shortly after several unsuccessful fractionation attempts with various pistons and hydraulic/pneumatic presses.) The screw-press created and maintained backpressure down the length of the stainless steel auger through a controllable pneumatic actuator at one end of the machine. Preliminary tests with the screw-press found that 40 psi (276 kPa) was the uppermost limit for the processing of Napier grass before clogging and mechanical failure became an issue. A schematic diagram of the screw-press setup is shown in Figure 4.3. Raw Napier grass was successfully fractionated in initial trials, and the blade-type cutting mill and dewatering screw-press (set at 40 psi backpressure) were used for the evaluation and demonstration of green processing in subsequent experiments.

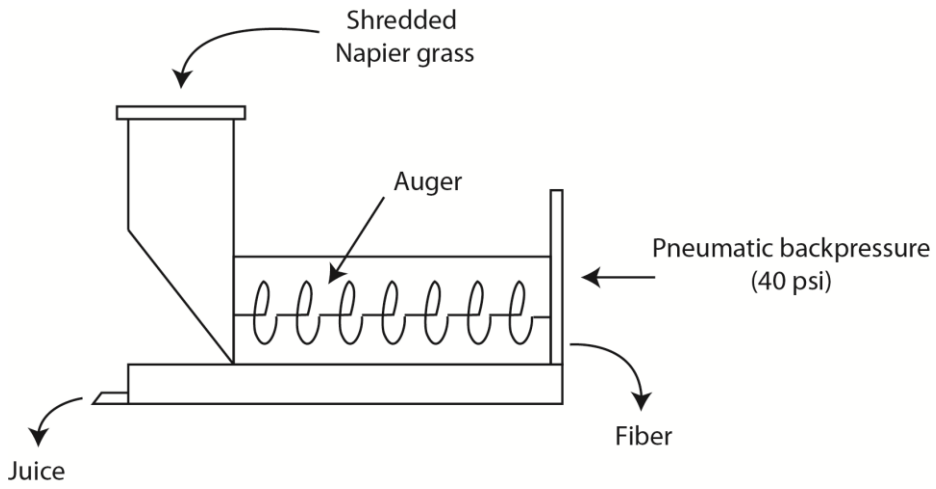

Figure 4.3. Schematic diagram of screw-press

4.3 Dilute sulfuric acid pretreatment optimization of Napier grass

The dilute sulfuric acid pretreatment of Napier grass has not been published in literature prior to this study. Therefore, a baseline optimization of pretreatment conditions was necessary to determine the typical concentrations of sugars solubilized during the pretreatment of Napier grass, and the overall effect that dilute sulfuric acid had

on deconstructing lignocellulosic structures. The pretreatment conditions for the four preprocessing categories described previously in Chapter 3 (namely, wet/unjuiced, dry/unjuiced, wet/juiced, and dry/juiced) were optimized on the bases of reducing sugars released with respect to acid concentrations, pretreatment temperatures, and residence times. Two parameters were held constant, while the third was varied, and the conditions which yielded the highest concentrations of structural sugars were taken as optimal. (See Table 4.2.)

Table 4.2. Optimal conditions for the dilute sulfuric acid pretreatment of Napier grass

	Acid Concentration (w/w)	**Temperature (°C)**	**Residence Time (min)**
Wet/Unjuiced (W/U)	5.0%	120°C	30 min
Dry/Unjuiced (D/U)	5.0%	120°C	30 min
Wet/Juiced (W/J)	5.0%	120°C	45 min
Dry/Juiced (W/J)	2.5%	105°C	30 min

Acid and enzyme hydrolysates collected from the optimal conditions (Table 4.2) were further elucidated by HPLC, seen in Figure 4.4. (The percent of theoretical glucose and xylose released from Napier grass under optimal conditions were included above the bars corresponding to each preprocessing method.) Among the four categories, wet/juiced Napier grass was found to release the highest concentration of monomeric sugars in the acid hydrolysate. This was confirmed statistically by an ANOVA ($p = 0.01$) and Duncan's comparison test of the raw data (Table B1 and B2). Following pretreatment with 5% (w/w) acid at 120°C for 45 minutes, and saccharification by Accellerase 1000^{TM} for 72 hours, near theoretical yields of xylose and ~85% of the glucose were obtained from the acid liquor and enzyme hydrolysate of wet/juiced Napier grass, respectively. Similar values were reported for the dilute acid pretreatment of other perennial grasses (Dien et al. 2006; Martin et al. 2007) and suggest that Napier grass may behave like conventional bioenergy crops. The underlying implications are worth noting

as an ideal biorefinery should be able to continuously incorporate a diversity of feedstocks while making little changes to operational protocols as possible.

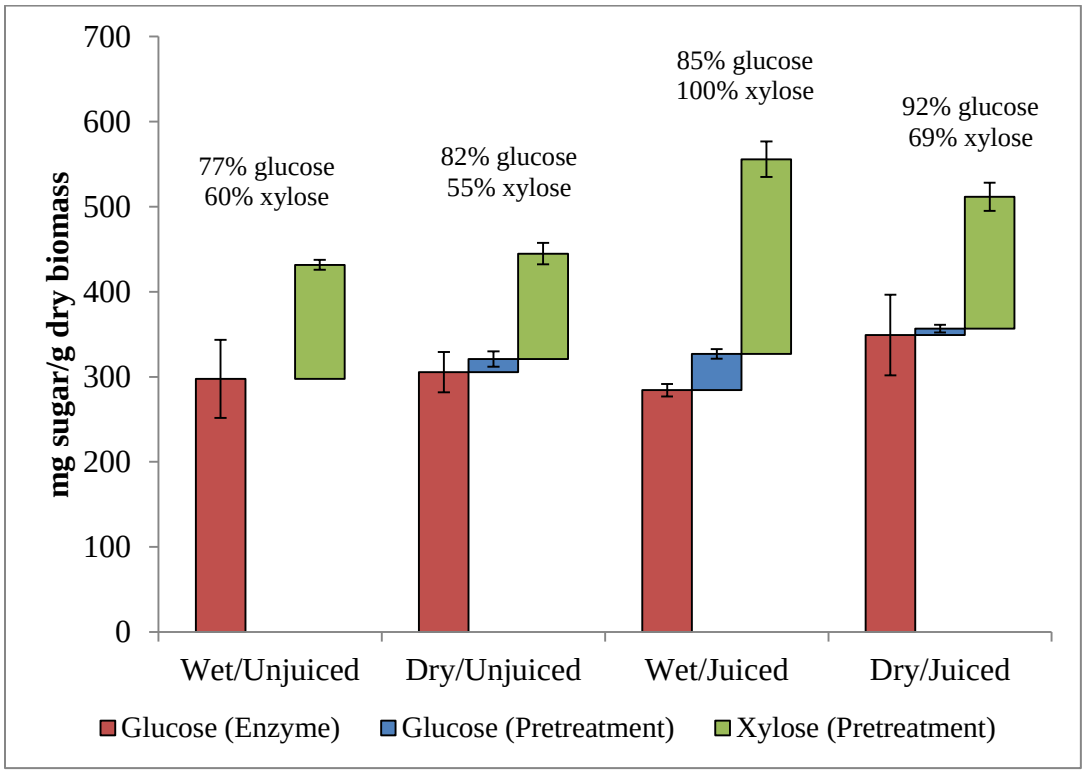

Figure 4.4. Structural sugars released under optimal dilute acid pretreatment conditions and enzyme saccharification as quantified by HPLC analyses for four preprocessing streams of raw Napier grass, (n = 3)

The screw-pressing unit operation appeared to significantly improve the sugar released during the pretreatment and saccharification of non-conventionally preprocessed Napier grass when compared to conventional handling of the incoming feedstock. It is believed that screw-pressing exerts shear stress along the length of feedstock fibers, resulting in localized increases in temperature that assist in the depolymerization and disruption of cellulose, hemicellulose, and lignin interactions (Hjorth et al. 2011).

Comparing the dewatered samples, dry/juiced Napier grass released statistically lower monomeric sugar yields than wet/juiced Napier grass during pretreatment as a result of biomass drying (Table B2). The drying process, in particular, have been shown to cause random structural collapse as fibers begin to shrink; consequently inhibiting the

diffusion of acid into the biomass irreproducibly (Selig et al. 2008). Lower xylose concentrations were thus observed during the pretreatment of dry/juiced samples, as well as larger standard deviations during saccharification (in comparison to wet/juiced samples).

Wet/unjuiced and dry/unjuiced samples were pretreated "as is" following shredding and drying (in the latter case). The samples were not further size reduced by screw-pressing, and remained in random lengths between 1-5 cm. (Note that prior to every experiment, stock Napier grass was mixed thoroughly for at least 10-15 minutes and sampled randomly.) The benefits of screw-pressing include additional size reduction and the production of uniform length cellulose fibers (less than 0.5 cm long); an important characteristic for the improved mass transfer of acid and enzymes in pretreatment and saccharification, respectively. Moreover, screw-pressing was also found to establish a uniform moisture content (~56%) within the extruded feedstock. For the case of wet/unjuiced Napier grass, it was believed that the high moisture content of the sample may have diluted sulfuric acid concentrations and precluded its full effect on hydrolyzing amorphous cellulose in the pretreatment liquor. Subsequently, no glucose was detected by HPLC in the acid liquor. A mass balance surrounding the unit operations of the four preprocessing schemes and the production of juice from raw fiber can be found in Figure 4.5. Juice released during the screw-pressing of raw fiber was collected and analyzed in the following experiment.

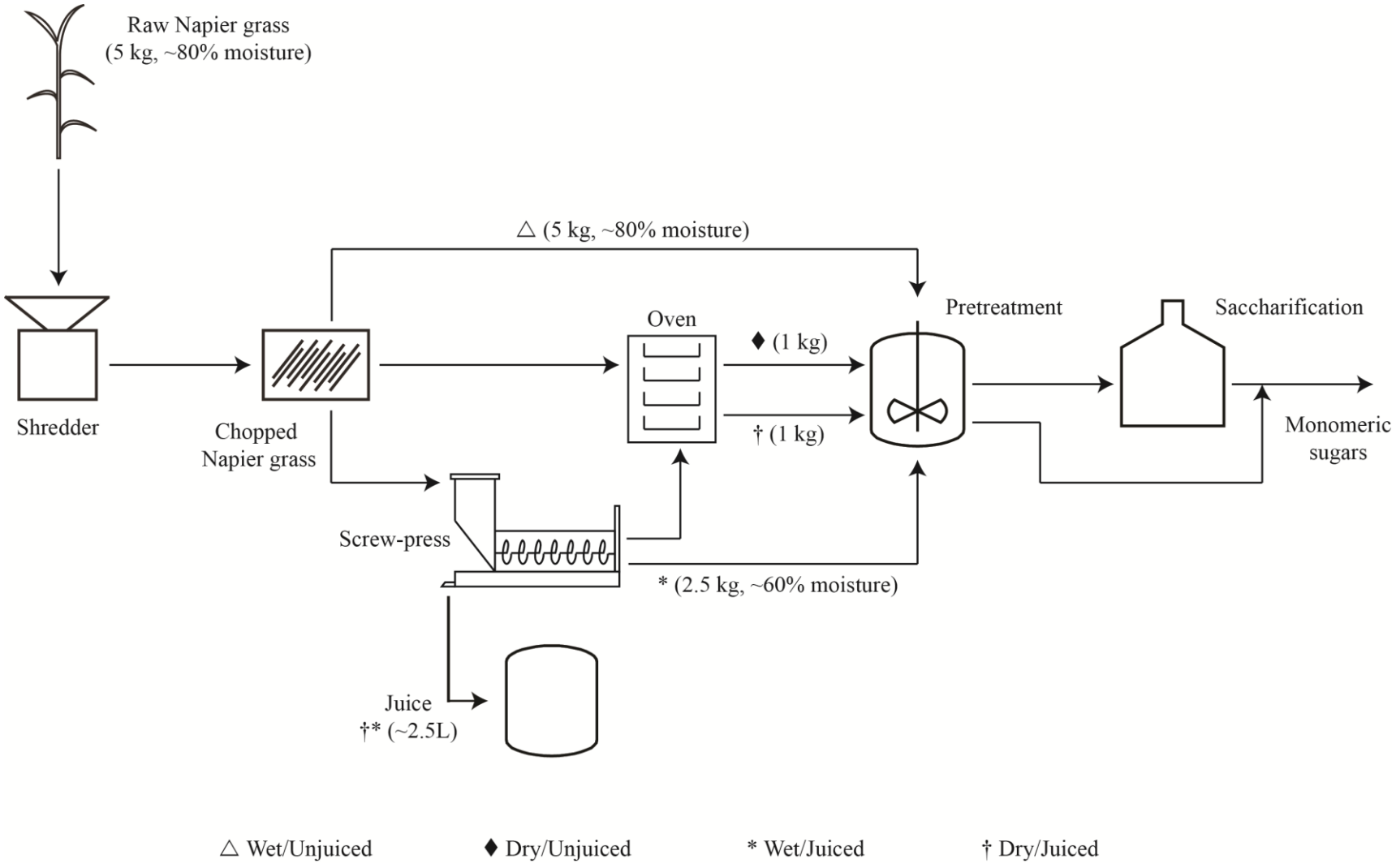

Figure 4.5. Mass balance of Napier grass during preprocessing

4.4 Co-product from Napier grass juice

Napier grass juice was examined for its potential in producing high value microbial co-products. One example of such a co-product is the edible fungi, *Rhizopus oligosporus*, which has significant implications in supplementing and reducing the production costs of aquaculture feed in Hawai‘i and the Pacific Region (Nitayavardhana and Khanal 2010). For the purposes of this study, *R. oligosporus* has been chosen to serve as a model co-product from green processing of Napier grass. It is important, however, to note that there remains a significant potential for research and development of other high value co-products from Napier grass juice other than *R. oligosporus*.

Napier grass juice was analyzed on the basis of chemical oxygen demand (COD) and total Kjeldahl nitrogen (TKN), as carbon and nitrogen sources, respectively, which are known to be vital for the cultivation of many microorganisms, particularly fungal species (van Leeuwen et al. 2012). Fungal inoculation of crude Napier grass juice (100 mL) was conducted at the laboratory-scale in 250 mL flasks to test the potential of fungal co-product generation. The juice was determined to have an initial COD and TKN concentration of 55.48 ± 3.97 and 1.67 ± 0.00 g/L, respectively, prior to inoculation. A biomass yield of 1.16 ± 0.34 g biomass increase/g initial biomass was obtained, correlating with a 60% removal of COD. The implementation of co-product-generating microbes was thus shown to provide opportunities for increased revenue while simultaneously reducing organic contents in the effluent. The concept also has implications in water reclamation. It is important to note that the goal of this study was not to optimize co-product generation from Napier grass, but rather to demonstrate its potential. The fungal biomass yield, however, was shown to be higher on Napier grass juice than on other substrates following optimization (Nitayavardhana and Khanal 2010). Yield data determined by this experiment were used in an economic analysis of green processing in the chapter. Rigorous optimizations of operational parameters (e.g., pH, temperature, residence time) could likely increase the observed yields of co-products, and more research is required to identify microbial species that have the highest value (for increased revenue of the biorefinery).

4.5 Compositional changes of Napier grass with respect to age

Field observations during the previous experiments, which evaluated green processing and the optimization of dilute acid pretreatments of Napier grass, made obvious the physical changes that occurred during the natural maturation of feedstocks. Because green processing was proposed as an upstream biomass handling technique immediately following harvests, the physical and biochemical characteristics of the raw feedstock has a large impact on the viability of green processing, and ultimately, the biorefinery. Moreover, the concept of green processing was proposed as a year-round strategy for continuous biofuel and biobased product generation in (sub)tropical regions of the world. Literature has suggested that some perennial grass species may undergo significant changes in holocellulose content during the course of maturation (Holmes 1980), thus affecting attainable biofuel and co-products yields.

Napier grass was planted at the Waimanalo Research Station (Waimanalo, HI, USA) on April 18, 2011 to monitor the effects of age on biomass fiber constituents (for biofuel production) and juice (for co-product production). This section presents the biochemical changes that were found to occur in Napier grass with respect to feedstock age. A complete list of the summative mass closure of constituents can be found in Appendix A, and all relevant statistical analyses for this chapter can be found in Appendix B.

4.5.1 Moisture content

One of the most significant properties of Napier grass (for co-product generation) is the moisture content. The moisture content of all biomass samples extruded during green processing under 40 psi of backpressure was determined to be about 56%, and higher moisture contents of the raw fiber produced higher volumes of juice for co-product generation. A graph illustrates moisture changes with respect to maturation in Figure 4.6. Error bars were used to represent the standard deviations of experimental data. A Duncan's Comparison Test of six measurements corresponding to each of the four age categories determined that the moisture contents in 2 and 6 month old Napier

grass (85% and 81%, respectively) were greater than the moisture contents of 4 and 8 month old feedstocks (74% and 70%, respectively).

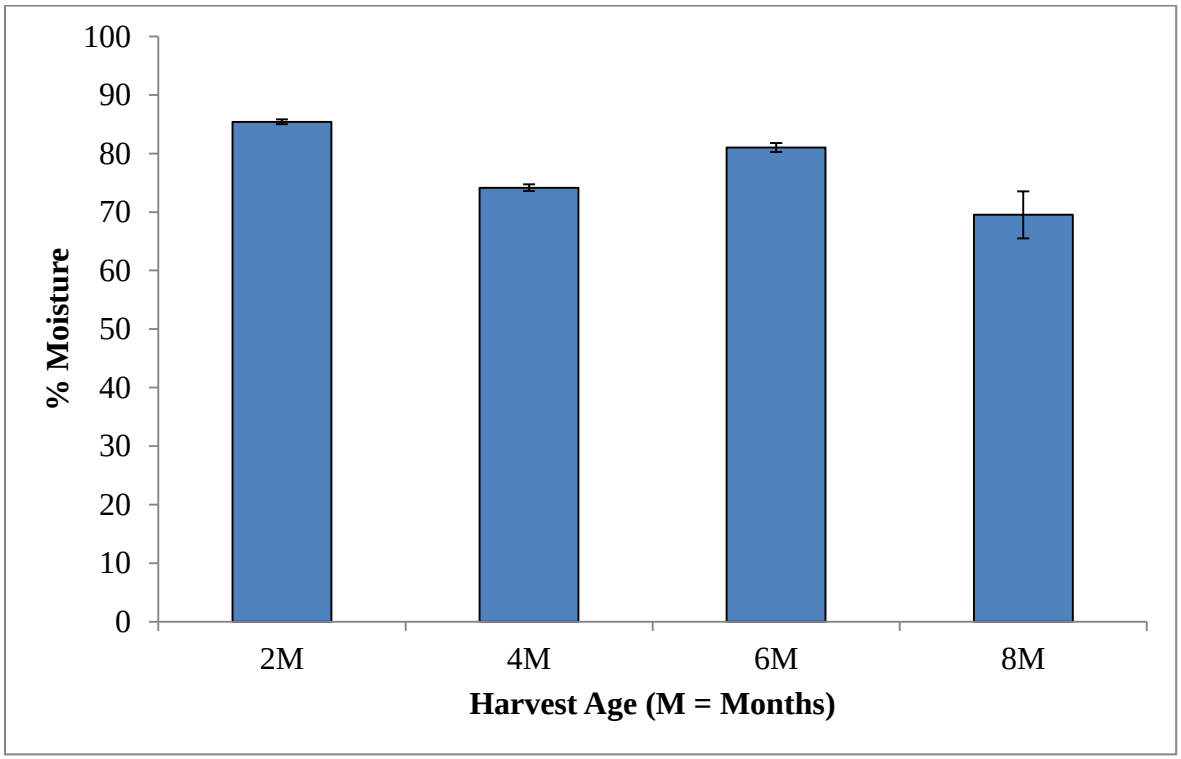

Figure 4.6. Moisture content of raw Napier grass with respect to harvest age, (n = 2)

Precipitation data from the Waimanalo Research Station were collected and compared with the harvesting ages of Napier grass to investigate the effects that weather may have had in influencing the overall moisture content of the feedstock (Figure 4.7). Regression analyses of the data found no significant relationships between the moisture content and cumulative precipitation ($p = 0.20$, Table B.5). Additionally, no relationships (e.g., linear or quadratic) were observed between the moisture content and Napier grass age ($p = 0.06$, Table B.6).

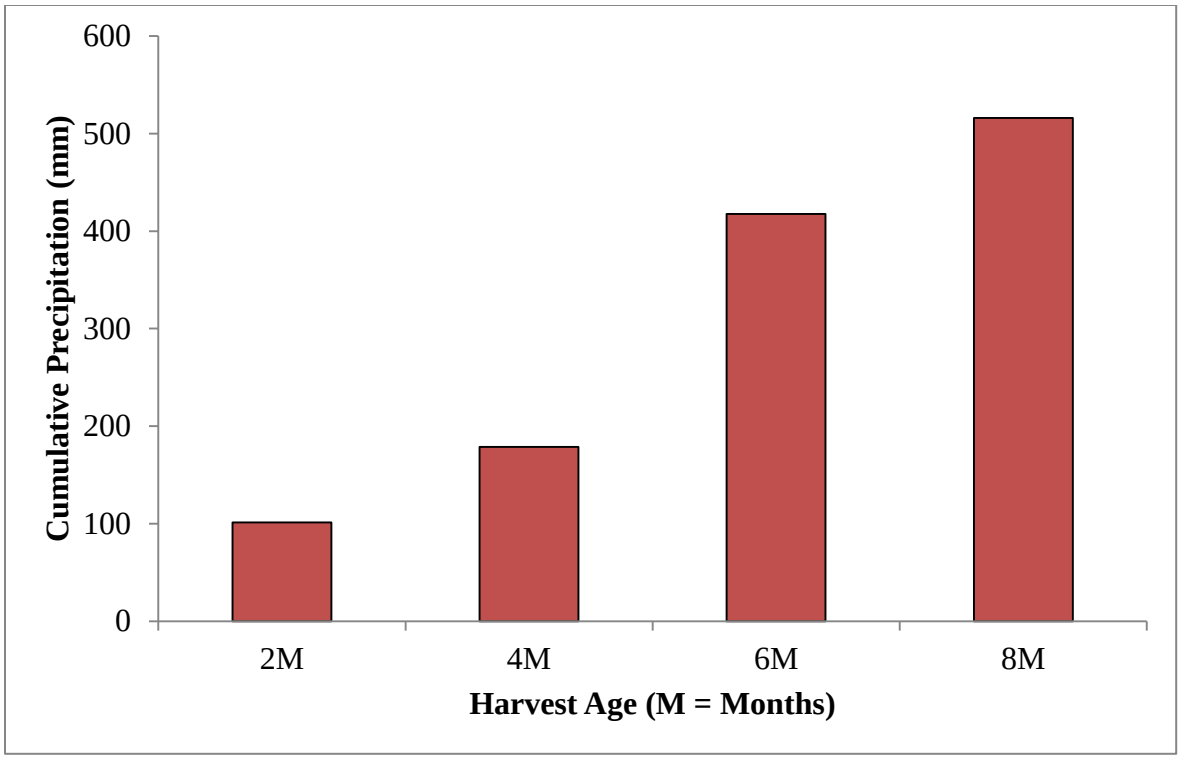

Figure 4.7. Cumulative precipitation versus harvest age

The relationship between moisture content and age was not obvious however, field images in Appendix C may provide an explanation for the observed trend. At 2 months old (Figure D.1), Napier grass was seen to develop a high density of leaves for photosynthesis and growth, but the increase in the surface area (of leaves) at this young age may have also contributed to water losses via transpiration (Bouman et al. 2005). At 4 months old, Napier grass more than doubled its height, and the leaf area was observed to decrease (Figure D.2); correlating with an increase in moisture seen through 6 months of age (in response to the reduced effects of transpiration). Upon achieving maturation, the lower fraction of the stalk began to dry and lignify (discussed later) for structural support, and another decrease in the overall moisture content occurred.

4.5.2 Extractives content

The extractives of biofuel feedstocks consist of water and ethanol soluble material that can interfere with the gravimetric analyses of biomass constituents in subsequent

experiments for compositional analyses. In herbaceous feedstocks, water-extracted components include soil, fertilizer, miscellaneous inorganic compounds, and non-structural carbohydrates, while ethanol-extracted components consist primarily of waxy materials (Sluiter and Sluiter 2010). If not removed from the biomass, plant extractives can precipitate out of solution during the acid hydrolysis of structural carbohydrates, and irreproducibly bias constituent values to be high; particularly in the case of acid insoluble lignin determination (Sluiter and Sluiter 2010). The quantified values for extractives removed from Napier grass with respect to age were represented on a dry weight basis in Figure 4.8.

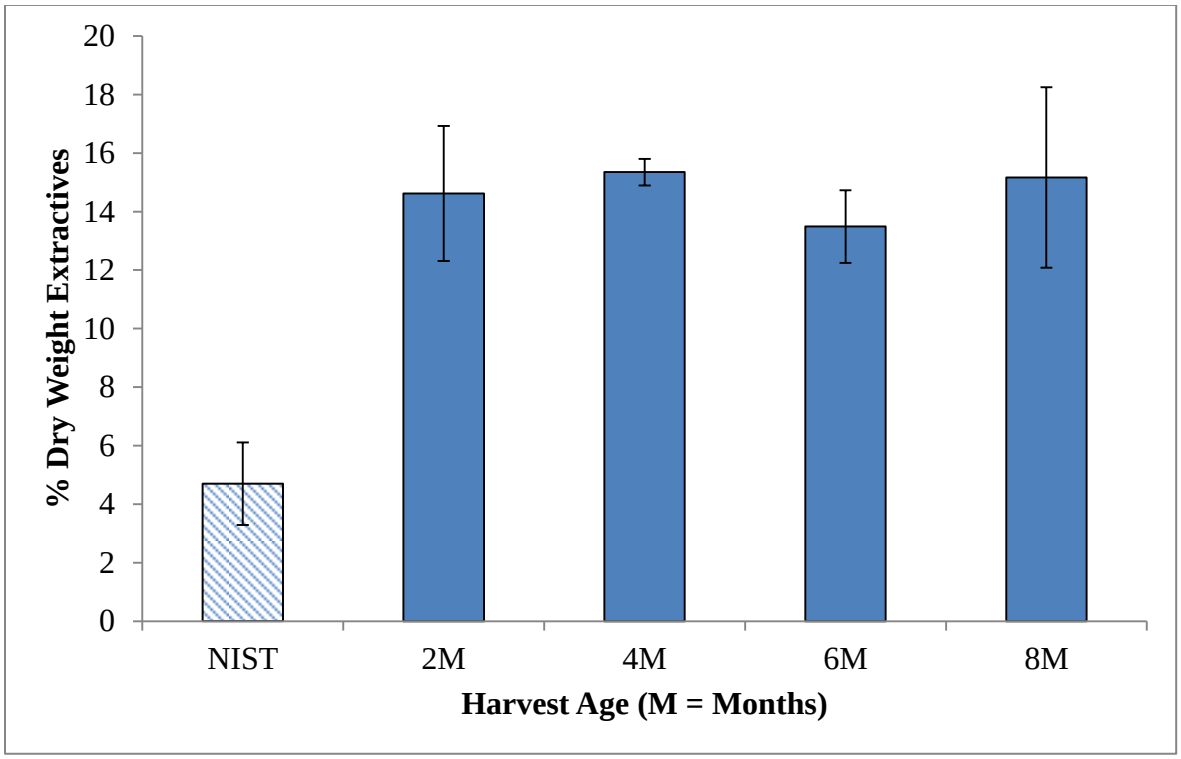

Figure 4.8. Extractives of Napier grass with respect to age, (n = 2)

An internal standard of known composition (bagasse #8491) from the National Institute of Science and Technology (NIST) served to validate analytical methods, and the extractives data of Napier grass were compared by a RCB ANOVA with respect age. (Note that the NIST standard is represented as the shaded bar in Figure 4.8.) At a confidence interval of 95%, changes in the extractives content of the feedstock appeared

to be unrelated to Napier grass maturation ($p = 0.84$, Table B.7). This was further supported by trend analyses (Table B.8) which showed no significant differences ($p = 0.81$). Further examination of the extractives component might provide additional insight into its relationship to external factors (e.g., solar radiation) affecting the Napier grass, however it is important to note that this fraction represents soluble non-structural constituents of the Napier grass and thus constitutes little value for improving biofuel yields from green processing.

4.5.3 Carbohydrate content

The structural carbohydrates of lignocellulosic feedstocks represent the most significant characteristic for biofuel production. Quantification of the holocellulose fractions in biofuel crops establishes a theoretical limit for sugar release during pretreatment and saccharification, and enables a determination of efficiency surrounding various unit operations.

Of particular interest in Napier grass were the effects that age had on the sugar constituents for biofuel production. As Napier grass matured, its (hemi)cellulose content per dry weight was believed to decrease as a result of feedstock lignification; observed in the field by the physical hardening of the stems. To increase the economic viability of biorefineries, feedstocks should ideally be harvested during ages that maximize the efficient utilization of most biomass components. Consequently, understanding the natural changes of holocellulose and other plant constituents with respect to age provides valuable insight for the true potential of green processing biorefineries. Figure 4.9 summarizes the key findings of the effects that age had on the Napier grass holocellulose content.

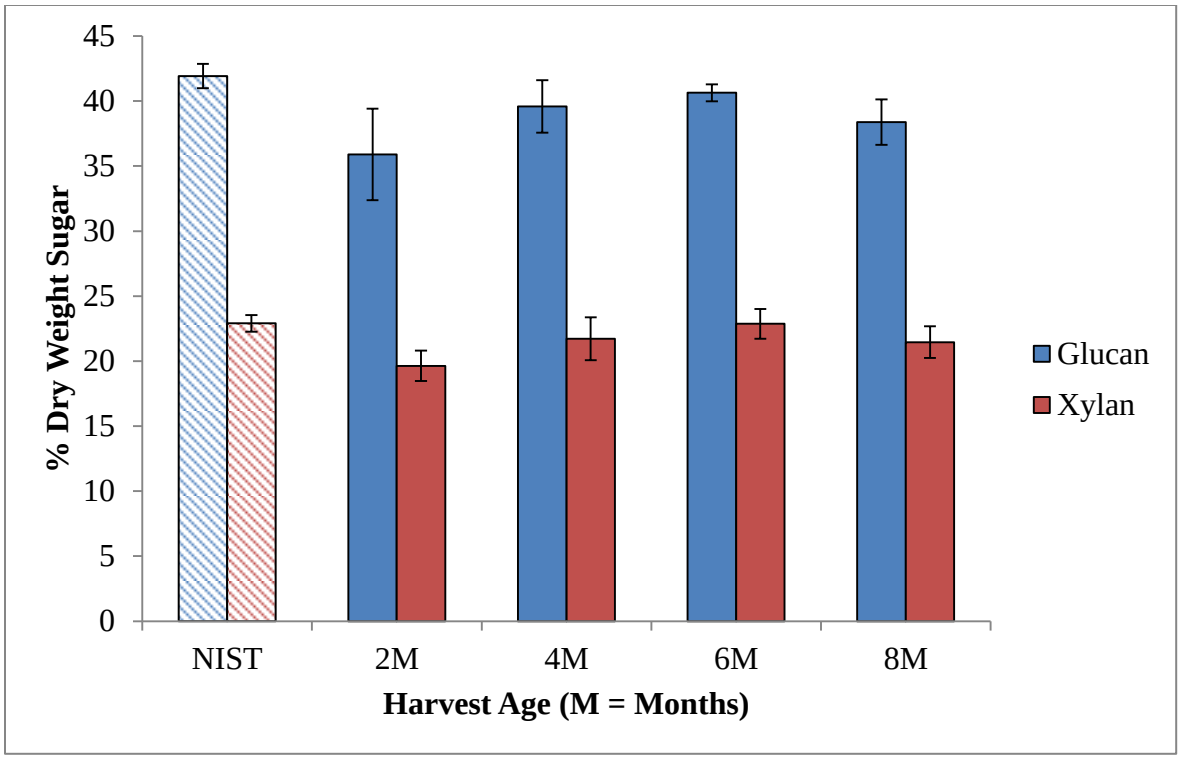

Figure 4.9. Carbohydrate content of Napier grass with respect to age, (n = 2)

Interestingly, statistical RCB ANOVA of the carbohydrate data for both glucan and xylan ($p = 0.21$ and $p = 0.27$, respectively) suggested that the structural sugar constituents of Napier grass were independent of age. (See Table B.9 and B.10 for details regarding statistical analyses.) Trend analyses of the data plotted against feedstock age further supported no significant relationships occurring between carbohydrate content and crop maturation ($p = 0.13$ and $p = 0.11$ for glucose and xylose in Table B.11 and B.12, respectively).

Comparing structural sugar data with a recent study of Napier grass cultivated in Thailand suggested that the carbohydrate content may have actually been a function of external, environmental factors. In particular, Napier grass in Thailand was found to have cellulose contents which were higher (at least 6% on a dry weight basis) than the biomass analyzed in this study and correspondingly, Rengsirikul et al. (2011) also reported lower lignin contents (by ~6%) than Hawai‘i-grown Napier grass. Although the intrinsic geographical differences of the two studies cannot not be neglected, it is quite possible that the growing conditions at the Waimanalo Research Station may have

favored the production of lignin over carbohydrates. Literature suggests that cellulosic plants may sometimes add lignin to its structures in response to non-injurious mechanical stress (Cipollini Jr., 1997). In comparing the climatic differences between Thailand and Hawai‘i in an international weather database, the average wind speed was found to be quite different between Thailand (1.7 m/s) and Hawai‘i (4.7 m/s) (RETScreen, 2012). Because Hawai‘i is known for its consistent trade winds, research investigating reductions in holocellulose in response to mechanical stress could play a significant role in siting land for large-scale Napier grass production.

4.5.4 Lignin content

Lignin is the third primary constituent of lignocellulosic feedstocks accounting for up to 25% of the dry weight (Liu et al. 2008), but does not include carbohydrates. As a result, lignin often represents the unusable fraction of second generation crops from the perspective of biofuel production through the biochemical pathway, and is presently regarded as a substrate for burning within the context of a biorefinery. Research has also been conducted to examine the conversion of lignin into a high-value co-product (Stewart 2008), but little success has been reported to date. An increase in lignin content often represents a decrease in percent dry weight of holocellulose and an increase in biomass rigidity, introducing complications in biomass conversion. Understanding and quantifying the lignin content of potential biofuel feedstocks prior their use in green processing is thus extremely critical.

Lignin determined by NREL standard laboratory analytical protocols was quantified by combined spectrophotometric and gravimetric analyses for acid soluble (ASL) and acid insoluble lignin (AIL), respectively (Sluiter and Sluiter 2010). The data reported here should thus be treated as lignin-acting or lignin-like compounds. Rigorous chemical elucidation of lignin is not relevant for biofuel production and is not reported by standard procedures (Sluiter et al. 2008d). Moreover, the constituent molecules of lignin are known to be highly variable by species, and extensive analytical methods must be optimized to elucidate native lignin from specific feedstocks. Because such analyses

represented detraction from the goal of this research, lignin, with respect to the age of Napier grass, was represented in Figure 4.10 as determined by standard protocols.

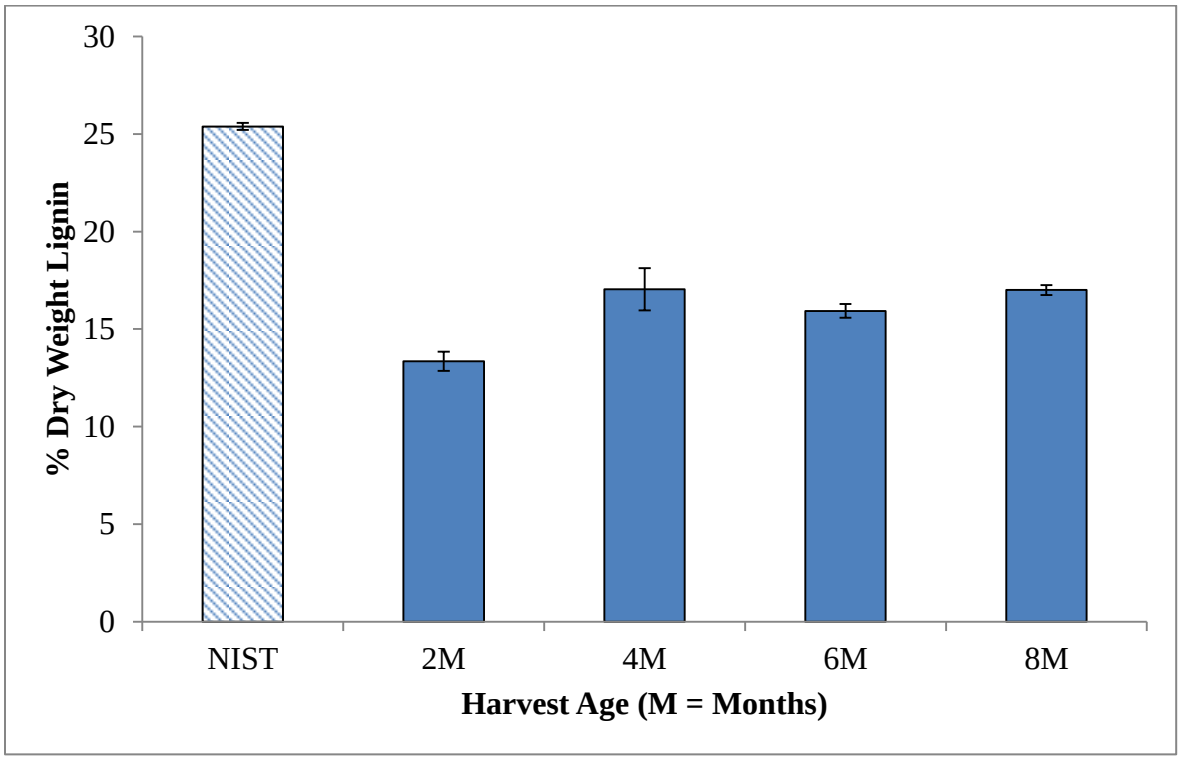

Figure 4.10. Lignin of Napier grass with respect to age, (n = 2)

The lignin content of Napier grass discussed previously in Section 4.5.3 was found to be greater in biomass grown in Hawai‘i compared to in Thailand. Because only two studies (including this study) are presently known to have characterized Napier grass in terms of constituents pertinent to liquid biofuel production, much uncertainty exists about how external factors may affect feedstock composition.

Statistical analyses of lignin by a RCB ANOVA found that the lignin content was significantly related to Napier grass age ($p = 0.04$, Table B.13). Orthogonal contrasts of the means were used to elucidate when the differences in lignin may have occurred with respect to maturation. Because four means were available, three orthogonal contrast sets were defined for analyses. In the first set, Napier grass of 2 months age was compared to a grouping of 4, 6, and 8 month old samples, and was found to be significantly different ($p = 0.02$). When compared to 4 month old feedstock alone however, 2 month old Napier

grass was similar ($p = 0.09$). In the third contrast, 4 month old feedstock was compared with 8 month old feedstock to determine if there were any advantages (with respect to lower lignin content) by harvesting Napier grass prior to achieving maturity. Lignin differences between 4 and 8 month old Napier grass were found to be insignificant ($p = 0.97$). Tables B.14-16 summarizes the results of statistical analyses.

Several conclusions and inferences can be made from the results discussed in the preceding paragraph. First, as expected, the lignin content of immature Napier grass (2 months old) was statistically less than the older, taller material. This occurrence correlated with observations of the younger material being closer to the ground (see Figure D.1), and is consistent with the role of lignin as a structural support. As the crop grew, no dramatic increases in lignin were determined beyond 4 months of age (confirmed by orthogonal contrasts of the means, Table B.16). It can be concluded that lignin production thus occurred consistently during the course of maturation rather than over a discrete period.

While it must be strongly emphasized that conclusions regarding wind cannot be drawn without additional data and/or biofuel literature (pertaining to Napier grass as a feedstock), the implications of mechanical stress influencing compositional changes as speculated is significant. In particular, many locations in Hawai‘i, like the Waimanalo Research Station for example, experience consistent gusts of wind year-round, and a shift from holocellulose production to lignin production in Napier grass directly results in lower theoretical biofuel yields. Across large areas of land, these changes could represent in significant losses in carbohydrate content.

4.5.5 Ash content

Similar to the extractives content described in Section 4.5.2, ash analyses quantify the inorganic material found in lignocellulosic feedstocks. These compounds consist primarily of soil, minerals, elements, as well as other components, and can exist within the plant structure or solubilized within vascular tissues. (Note that extractives, unlike ash, quantify only soluble components and not structural components of biomass.) One of the significant challenges of a complete compositional analysis of biomass is the

minimization of accidental double counting of feedstock constituents. The experiment used for the determination of Napier grass extractives in Section 4.5.2, for example, also quantifies an irreproducible fraction of solubilized non-structural material (Sluiter and Sluiter 2010) that is accounted for during ashing. The resultant double counting can often bias mass closure values over 100%. In this study, ash contents were determined both before and after extractives quantification. The difference obtained was subtracted from the extractives data, and ash was reported in a form consistent with literature. Although ash is not important for the production of biofuels, its presence in high quantities is significant for bioenergy production via thermo-chemical platforms. The percent dry weight of ash with respect to Napier grass age is presented in Figure 4.11.

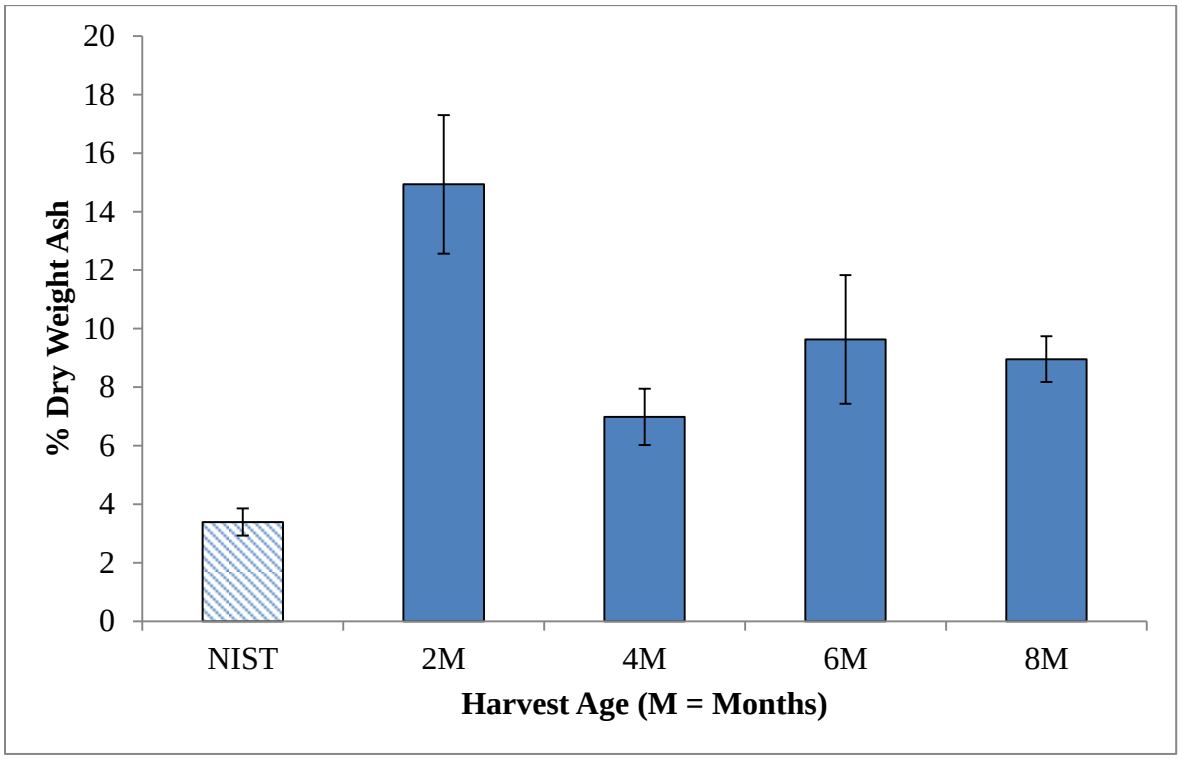

Figure 4.11. Ash content of Napier grass with respect to age, (n = 2)

Young Napier grass has been reported to contain higher ash contents than older biomass (Yoshida et al. 2008). Immature grasses are typically more leafy, and the leaves contribute significantly to the overall ash content; note that the leaves of most grasses are known to contain at least 3 times more ash than the stalks, often in the form of silica

(Cherney 2006). Statistical analyses of the data presented in Figure 4.11 above demonstrated that the ash content of Napier grass was related significantly to maturation ($p = 0.05$) however, similar to the case of lignin, orthogonal contrasts of the mean data did not identify a single age in which ash contents change significantly. Specifically, 2 month old Napier grass was not found to be different when compared to 4, 6, and 8 month old ($p = 0.13$) or to 4 months old feedstock alone ($p = 0.10$). The third contrast set considered was 4 month old Napier grass against 8 month old sample, and was also found to exhibit no notable differences ($p = 0.16$). Thus, it can be concluded that the ash content of Napier grass (like lignin) changed gradually with respect to maturation. Statistical analyses of the ash content have been summarized in Tables B.17-20.

4.5.6 Discussion

The preceding sections examined the compositional changes of Napier grass due to the effects of maturation, with an emphasis on constituents for biofuel production. Although variations superficially appeared to occur, statistical validation of the data pertaining to extractives and holocellulose did not support any significant relationships to Napier grass maturation. With respect to moisture content, lignin and ash, all three properties appeared to have been related to age. Changes in the lignin content however, were also suspected to be induced by external factors. Importantly, conditions which favor the production of lignin reduce the overall fraction and production of holocellulose, ultimately lowering biofuel yields in downstream processes.

4.6 Changes in Napier grass juice characteristics with respect to age

The constituents of Napier grass juice, represented collectively by the total Kjeldahl nitrogen (TKN) and total chemical oxygen demand (COD), are essential for determining the microbial co-product potential of green processing. Because the primary aim of green processing is to produce high value co-products, changes in the juice composition, which are detrimental to co-product formation significantly alter the economic viability of green processing biorefineries. Many fungal and bacterial species

have been shown to be capable of using TKN and COD as nitrogen and carbon sources, respectively, to generate high value products such as protein-rich fungal biomass (van Leeuwen et al. 2012) and bioplastics (Khardenavis et al. 2009). Changes in TKN and COD concentrations with respect to Napier grass age are illustrated in Figure 4.12 and Figure 4.13, respectively.

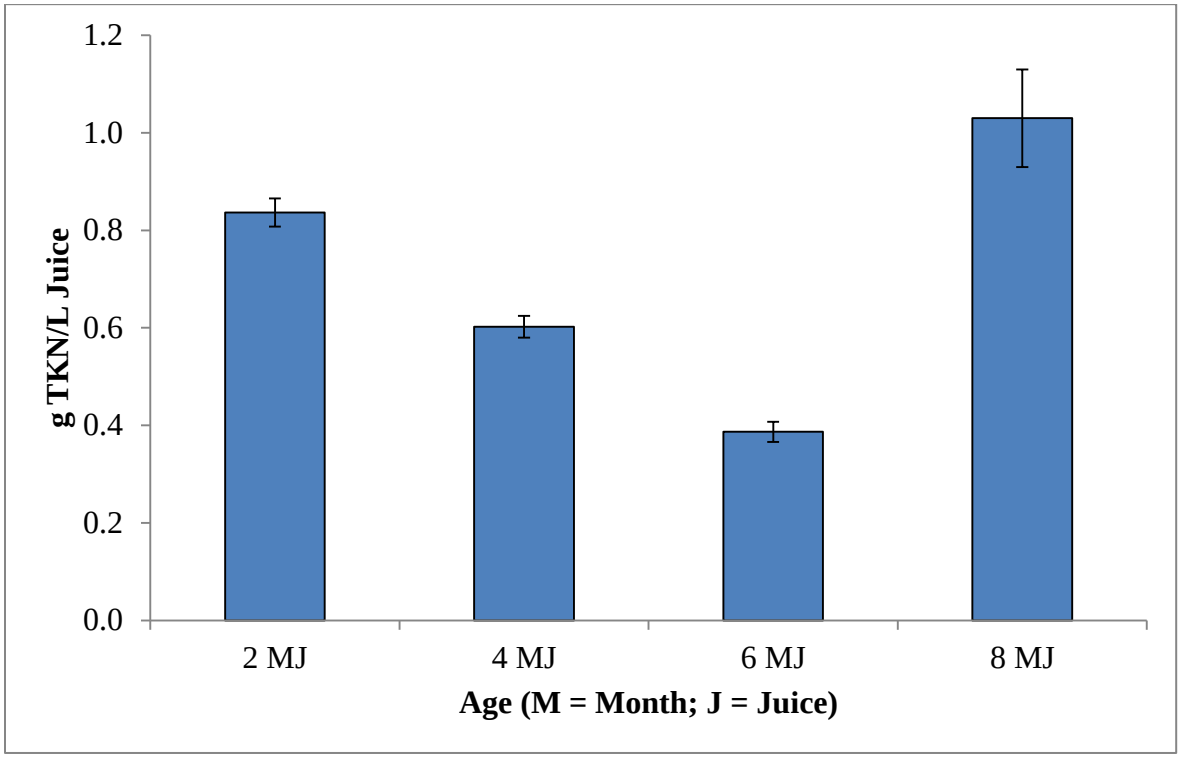

Figure 4.12. TKN of Napier grass juice with respect to age, (n = 2)

A RCB ANOVA of the data in Figure 4.12 found that the TKN concentration in Napier grass juice changed significantly with respect to age ($p < 0.01$; Table B.21). Orthogonal contrasts were used to further describe the observed differences between the four means illustrated in Figure 4.12. Three contrast comparisons were defined prior to statistical analyses: (i) 2 versus 4 month old juice, (ii) 2 versus 6 month old juice, and (iii) 2 versus 8 month old juice. From (i) and (ii), it was determined that 2 month old Napier grass juice had a higher TKN concentration than 4 and 6 month old juice ($p < 0.01$ for both cases), but was not significantly different from 8 month old juice ($p = 0.39$). In terms of TKN concentration and its implication for co-product generation, most

significantly, the data in Figure 4.12 indicated that Napier grass between the ages of 4 and 6 months old produced juice with low concentrations of soluble nitrogen; an element required by microbial co-product generating species. The results of statistical analyses can be found in Tables B.22-24.

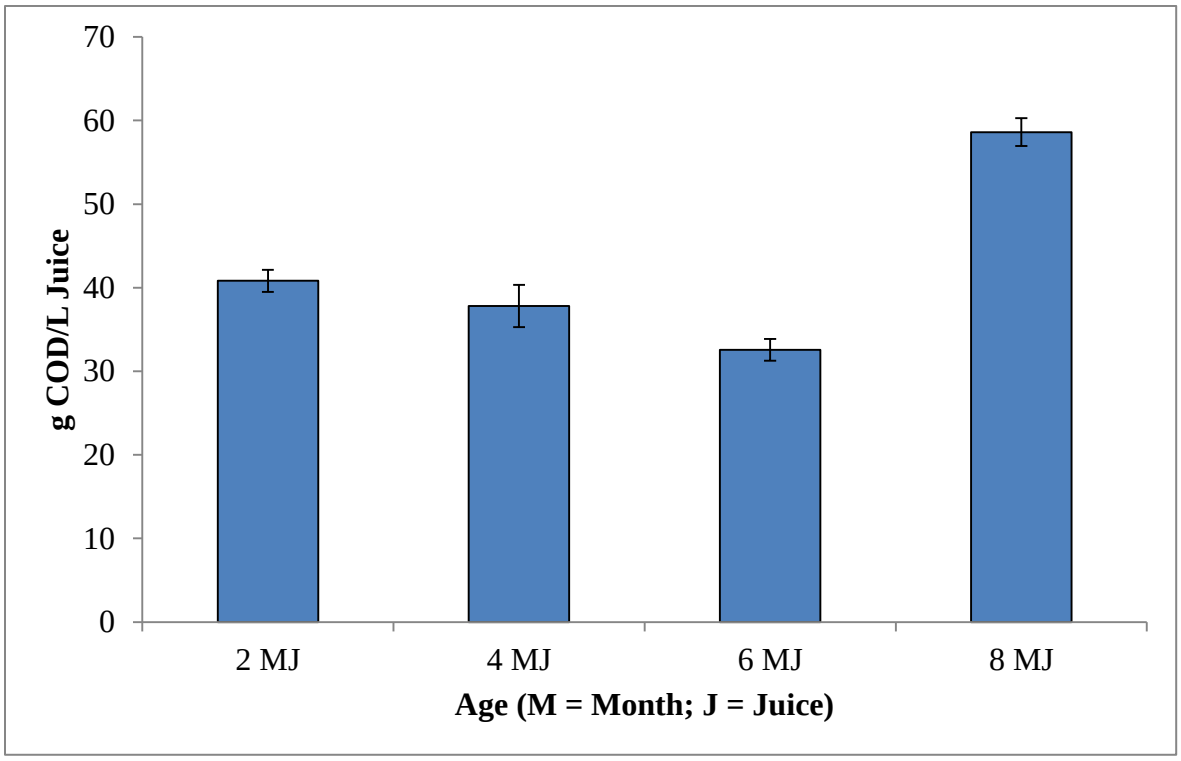

Figure 4.13. COD of Napier grass juice with respect to age, (n = 2)

A RCB ANOVA of the data in Figure 4.13 demonstrated that changes in the concentration of COD in the juice were related to Napier grass maturation ($p = 0.01$; Table B.25). Three orthogonal contrast sets were defined prior to analyses in a fashion similar to that of TKN concentration analyses: (i) 2 versus 4 month old juice, (ii) 4 versus 6 month old juice, and (iii) 6 versus 8 month old juice. Statistically relevant differences in COD concentration were not observed for (i) and (ii), $p = 0.55$ and 0.46, respectively, but was found to be of notable difference for 6 month old Napier grass juice versus 8 month old juice ($p = 0.01$). It was not clear why the COD content of Napier grass appeared to increase with respect to age. The moisture content was believed to be an influencing factor of the observed COD (and TKN) concentrations, whereby lower

moisture contents (as in the case of 8 month old Napier grass) would produce more concentrated juice, however, statistical analyses were unable to find significant relationships between both COD and TKN and feedstock moisture contents.

4.6.1 Discussion

As discussed previously, COD represents a complex substrate of various organic carbon-based compounds, and TKN quantifies organic nitrogen, ammonia, and ammonium. In microbial cultivations, the ratio of carbon to nitrogen (C:N) is particularly important in controlling the generation of products. For fungal biomass, C:N ratios of 10:1 have been reported to promote high protein contents, while quantities greater than 50:1 promote the production of alcohols, lipids, and secondary metabolites (van Leeuwen et al. 2012). In the case of bacteria, various concentrations of C:N have been reported, ranging from 33:1 to 48:1, to enhance the production of bioplastics from mixed culture systems (Johnson et al. 2010). Napier grass juice of all ages was found to have COD:TKN ratios greater than 36. Because *R. oligosporus* was indicated as the model co-product for this research, it is evident that additional nitrogen sources are required to promote the production of fungal protein. (Note that COD was used here only as a crude estimate. In C:N optimization studies, an ultimate analysis of the substrate is often conducted to quantify elemental constituents.)

Interestingly, both the TKN and the COD concentrations of Napier grass juice were found to be dependent on Napier grass age. In terms of co-product generation, Napier grass juice from either 2 or 8 month old feedstock appeared to have the most potential, however, 8 month old feedstock has been shown to contain more lignin and less moisture than 2 month old feedstock. For green processing, the physical properties of 8 month old Napier grass as observed in the context of this study present a concern in the fractionation unit operation that may result in increased mechanical failure of the screw-press. Trade-offs between producing fiber (for biofuel) and juice (for co-products) will need to be rigorously considered before applying green processing to Napier grass in biorefineries.

CHAPTER 5

TECHNO-ECONOMIC ANALYSIS OF GREEN PROCESSING

5.1 Anticipated revenue from green processing

The previous chapters have highlighted the technical capabilities of including green processing within the context of a biorefinery, but the economic viability of the upstream strategy remains to be examined. Green processing requires the purchase and inclusion of four additional equipment/unit operations not found in the conventional production of biofuel. These units, namely, a dewatering screw-press, bioreactor system, centrifuge, and fungal biomass dryer, represent additional capital investments and part of the risk involved from including the upstream approach for large-scale applications.

The overall economics of lignocellulosic biomass-to-biofuel conversion has been reported to be non-competitive with current gasoline prices (Eggeman and Elander 2005; Kazi et al. 2010). Thus, green processing should function as a profitable standalone subsystem within the larger biorefinery system if it is to offset the cost of biofuel production as previously proposed. Because green processing is a relatively unexplored approach to upstream biomass handling, much of the capital costs of equipment must be inferred from vendor quotations and present-day processes/products, serving as proxies for analyses.

Two main cost components, namely capital and operational costs, are essential in determining the economic viability of all biomass-to-biofuel processes. Capital costs represent the initial investment required to purchase equipment, and can be annualized and compared with annual revenue to determine the payback period of initial investments. Operational costs on the other hand represent the expenses required to produce a product, and include both variable and fixed costs. To minimize the introduction of uncertainty resulting from a multitude of assumptions, the operational costs in this section have been simplified to include only electricity and labor costs for green processing unit operations. Other expenses and utilities (i.e., overhead costs, etc.) were assumed to be maintained and included within the economics of the larger biorefinery.

Scaling adjustments for green processing equipment were calculated by assuming a 1 MGY (3.8 MLY) biofuel plant in Hawai‘i, with the generation of protein-rich fungal biomass as a high value co-product for feed applications. (Note that ethanol and *R. oligosporus* were chosen as model outputs for this facility, however, future biorefineries may produce other biofuels and co-products to improve its economic competitiveness or market demand.) In estimating the raw tonnage of Napier grass required for producing 1 MGY (3.8 MLY) of ethanol, both primary and secondary data were used. It was assumed that Napier grass in Hawai‘i had a glucan and xylan content of 38% and 21%, respectively, based on average compositional data from Chapter 4. A theoretical release of the entire holocellulose content, followed by theoretical production of 0.51 g ethanol/g monomeric sugar (for both glucose and xylose) resulted in a need of approximately 45,000 metric tons of raw feedstock. The moisture content of the incoming raw crop was taken as 78% based on the average of compositional data. Following screw-pressing, the extruded fiber had a moisture content of 56%, corresponding to 6 million gallons (23 million liters) of Napier grass juice captured during green processing. Primary fungal biomass production data from laboratory-scale experiments, suggested that 158 dry metric tons of protein-rich *R. oligoporus* could be generated from the juice.

Based on analyses of the *R. oligosporus*, it was found that approximately 45-50% of the dry weight of fungal biomass consisted of crude protein with amino acids essential for healthy fish/animal growth (Nitayavardhana and Khanal 2010). Similar nutritional qualities exist in soybean meal, and there is subsequently a potential for fungal biomass to replace soybean meal within current markets as an animal/fish feed ingredient. Using soybean meal as a proxy, the selling price of fungal biomass is approximately $512 per dry metric ton [$428 per dry metric ton, previously prior to US drought in 2012] (calculated from the average price of soybean meal from April to Oct 2012) (*Soybean Meal Monthly Price - U.S. Dollars per Metric Ton*). Note that soybean meal prices have increased recently in response to drought. The anticipated revenue generated by green processing co-product generation would be about $81,000 per year.

Table 5.1. Capital investment of green processing unit operations

Item	Original Cost	Adjusted Scaled Cost for 2012	Source
Screw-press	$438,000	$237,688	Vendor quotation
Bioreactor	$400,500	$365,620	(Humbird et al. 2011)
Agitator	$580,000	$529,487	
Air compressor	$175,000	$173,674	
Centrifuge	$200,000	$286,160	Vendor quotation
Spray dryer	$30,000	$31,765	(Li et al. 2011)
	Total	**$1,624,394**	

5.2 Payback period on capital investments

The capital investments for each unit operation exclusive to green processing are summarized in Table 5.1. The total capital cost was determined to be about $1.7M. The largest contributor to overall costs was the bioreactor system, estimated from the cultivation of *Trichoderma reseei*, a filamentous fungus with similar air and temperature requirements as the model co-product, *R. oligosporus*.

Considering capital costs alone for green processing unit operations and a discount rate of 4% (determined by averaging historic values (Tran et al. 2011)), it would take about 45 years to regain payback for the equipment, assuming that fungal biomass generates $81,000 per year of revenue. For the upstream strategy to regain payback within 25 years, an annual revenue of $107,000 must be achieved. Thus, from an economic standpoint, green processing does not seem viable at this scale, even when excluding the operational costs inherent in the facility. It is extremely important to note however, that the concept of green processing is a relatively novel and unexplored topic, and the projected values reported here may not truly reflect scale-up of primary data. Moreover, there exists significant potential to enhance the economics of green processing through improved engineering design (of equipment and processes) for both biomass fractionation and co-product generation.

5.3 Operational costs

The operational costs, representing electricity consumption and basic labor, were determined from commercial power rates on Oahu and average hourly wages in Hawai‘i

from the Bureau of Labor Statistics, respectively. For large commercial facilities, electricity expenses are the sum of three components (*Schedule P: Large Power Service*): customer charge ($350/month), demand charge ($24.34/month), and energy charge ($0.149103/kWhr). Basic labor costs were estimated to be approximately $13.50 per hour (not including benefits), and was determined by averaging the wages of occupational categories similar to green processing unit operations (*May 2011 State Occupational Employment and Wage Estimates: Hawaii*).

Assuming the same quantity of raw Napier grass and juice from Section 5.1, the total operating expenses for green processing would amount to $1.4M per year, including labor costs. Because of the large energy requirements and 72 hour residence time, the bioreactor system constitutes approximately 73% of the overall operational costs, and is also responsible for roughly 66% of the capital investment. Hawaii's electricity rates are among the highest in the nation, and simply relocating the biomass processing facility to another part of the country could lower electricity-related operating costs by about 50% (*Electric Power Monthly* 2012). Other approaches to increase the profitability and feasibility of green processing could include the implementation of renewable energy, such as electricity from photovoltaic panels or wind turbines, and/or the production of other higher value co-products. There are also inherent cost savings associated with green processing of Napier grass, (e.g., water reclamation and recycling), however, these values must be quantified in a complete and rigorous analysis of all influent and effluent streams of the entire biorefinery facility; similar to studies conducted by Humbird et al. (2011).

5.4 Potential for future innovation in co-products

It has been mentioned previously that rigorous optimization of fungal co-product generation was not the focus of this research. Consequently, although fungal biomass yields on Napier grass juice outperform the yields reported for other substrates, such as vinasse, there remains a great potential to significantly improve fungal biomass production through future optimization studies. A 40% increase in yield, for example, would make payback of capital investments achievable within 25 years or less.

Innovations can also be made in bioreactor design. Agitation and the bioreactor itself comprise nearly half of the total capital costs. Engineering designs implementing the use of airlift bioreactors, to supply both air and mixing (Nitayavardhana and Khanal 2010; van Leeuwen et al. 2012), may be one avenue for reducing initial equipment expenses and subsequent operational costs.

Alternatively, research for other co-products of higher value can be examined for Napier grass juice. For example, polyhydroxyalkanoates (PHA) or bioplastics, from the bacterium *Ralstonia eutropha* have been reported to have a market price greater than $2,000/dry metric ton (Yu and Chen 2006); approximately 3-4 folds greater than estimated fungal biomass prices. Its performance in terms of capital costs and PHA yield however, has yet to be evaluated on Napier grass juice and biorefinery process water. Other co-products include the possibility of in-house cellulase production for downstream biofuel processes, and/or cost savings from the reduction in nutrient-related costs for cultivating and maintaining fermentative microbial cultures (e.g., *S. cerevisiae* and *P. stipitis*).

CHAPTER 6

ENGINEERING IMPLICATIONS FOR GREEN PROCESSING

In Chapter 5, the implementation of green processing as a front-end, biomass handling technique was determined to be economically infeasible in its present state, particularly when incorporated under the same roof as biofuel production. Green processing, however, in its rudimentary form, is a general fractionation approach that can be adapted and modified to occur during any stage of biomass preprocessing.

6.1 Potential for mechanized infield processing

Transportation costs alone of biofuel feedstocks to biorefinery have been reported to comprise up to 65% of total biofuel production expenses (Judd et al. 2010). In the case of Napier grass, with an average moisture content of 78%, every 1,000 metric tons of raw feedstock harvested and transported, yields only 220 metric tons of dry fiber at the biofuel facility. Thus, a significant and obvious need exists for improved biomass fiber densities prior to transport to bioprocessing infrastructure.

One approach could couple the benefits of strategic plot design and mechanized harvesting with infield screw-pressing. Keffer et al. (2009) identified three regimes of slopes for the commercial production of bioenergy feedstocks in Hawai‘i. These areas were represented as low (< 10%), medium (10-20%), and high (> 20%) grades. The authors reported that historically, sugarcane (the morphological analogue of Napier grass) was successfully cultivated on slopes from the low to medium range, but that medium range slopes often introduced complications and erosion during mechanized harvests. Situating a biorefining facility at the bottom of a 10% or less graded slope, could allow for significant improvements for harvesting and green processing. In particular, raw Napier grass can be dewatered *in situ* to a moisture content of 56% before being transported to the primary processing facility. The juice during harvests can be dumped into irrigation-type concrete ditches (typically less than 4% slope (Gumiero et al. 2011)) and fed to co-product unit operations in the biorefinery via gravity (Figure 6.1). Future work must examine whether contamination presents an issue. In a laboratory study

conducted on bioprocess water from sugarcane-to-ethanol, sterilization did not appear to improve fungal co-product growth (Nitayavardhana and Khanal 2010).

It must be emphasized that the setup proposed here is not meant to suggest a simple solution to a non-trivial problem, but rather to illustrate that innovations in systems engineering has the potential to greatly improve the economics of green processing and biomass-to-biofuel with minor technological advancements. Transporting Napier grass with a moisture content of 56% for example, in comparison to 78%, doubles the dry fiber content entering the biorefinery. Rigorous techno-economic analyses and life-cycle assessments, however, must be conducted from cradle-to-grave to estimate the true cost savings of various systems engineering approaches on the bases of lower production costs, reduced GHG emissions, reduced environmental/ecological detriment, and social well-being.

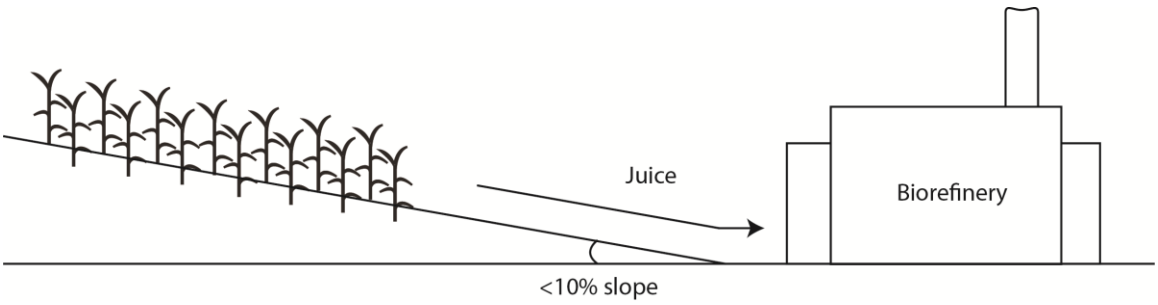

Figure 6.1. Conceptual layout of biorefinery incorporating infield green processing

6.2 Improvements in holocellulose hydrolysis

Proof-of-concept demonstrations of green processing highlighted the beneficial effects that screw-pressing imparted on Napier grass for reducing the high moisture content of the feedstock and facilitating the deconstruction of holocellulose into monomeric sugars, following dilute acid pretreatment and saccharification. The largest contribution to the economic infeasibility of green processing as determined in Chapter 5, extended primarily from fungal co-product generation. However, considered as an independent unit operation, rather than a co-product generating subsystem, the fractionation of Napier grass during green processing may indirectly benefit the overall

economic viability of biorefineries due to the resulting enhancements of biofuel production. Specifically, increased biomass densities for transport (discussed previously) and consistently high sugar yields from structural carbohydrates may justify the inclusion of large-scale screw-pressing of feedstock regardless of co-product generation.

6.3 Applicability of green processing in other platforms

Although not emphasized within the context of this study, green processing also has significant applications in emerging biorefinery approaches such as the carboxylic acid platform for biofuel production. In this setup, wet feedstock is anaerobically digested to generate carboxylic acid, which can be converted into drop-in biofuels (with properties similar to gasoline), methane, and various other co-products and biochemicals. Because the carboxylic acid platform uses robust anaerobes, the advantages in this downstream process include the conversion of nearly all non-lignin components into products (Holtzapple and Granda 2009; Agler et al. 2011).

The role of green processing in strategies like the aforementioned is to promote the size reduction and deconstruction of structural carbohydrates of raw feedstock through screw-pressing operations (Hjorth et al. 2011), which is believed to enhance the mass transfer of microbes and enzymes into biomass constituents. For mixed-culture methane production from perennial grasses, the addition of screw-pressing in upstream biomass handling was shown to significantly improve product generation by about 30% (Hjorth et al. 2011). The effects of screw-pressing on feedstocks for carboxylic acid platform products and biochemicals has not yet been demonstrated, but its inclusion could further enhance the reportedly high biofuel yields.

CHAPTER 7

CONCLUSIONS

Based on this research, several overarching conclusions can be drawn regarding the prospects of Napier grass and green processing in Hawai‘i and other (sub)tropical regions of the world. The important conclusions are summarized below.

- The shredding and fractionation of raw Napier grass are technically feasible, but required unique blade designs of the cutting mill and the incorporation of a screw-pressing unit operation, respectively. More specifically, high speeds (> 3,520 rpm) and blade orientations exhibiting 180° of gap between the cutting edges were shown to be the most effective for shredding wet biomass. With respect to fractionation, pressure alone was determined to be insufficient in promoting the separation of raw Napier grass into solid and liquid components. The combined effects of shear stress and backpressure (40 psi or 276 kPa) after shredding successfully produced clean lignocellulosic fiber and nutrient-rich juice.
- Green processing unit operations (particularly screw-pressing) were shown for the first time to significantly enhance monomeric sugar release from Napier grass fibers, following dilute acid pretreatment and enzymatic saccharification, when compared to conventional biomass handling. Near theoretical xylose and ~85% glucose per dry weight basis were achieved from wet/juiced Napier grass.
- Compositional analyses of Napier grass fibers with respect to age highlighted several unique trends not typically reported in biofuel literature. In particular, the structural carbohydrate and extractive contents of Napier grass were determined to be relatively independent of age. On the other hand, the moisture content lignin, and ash were determined to be linked to feedstock maturation. Moisture appeared to follow a cubic function which was unrelated to precipitation data. Additionally, lignin and holocellulose were speculated to be influenced by mechanical stressors (e.g., wind) however future research is necessary for validation.

- The juice of Napier grass was demonstrated to be a viable substrate for fungal co-product generation. However, analyses of the juice for TKN concentrations indicated that the nitrogen content was much lower than previously anticipated, suggesting that nutrient supplementation may be necessary for enhanced fungal protein production. Both the organic content (measured as COD) and TKN of the Napier grass juice were found to be related to the age of the feedstock.
- Finally, an exploratory economic analysis of green processing indicated that the upstream strategy may not be economically viable in its present form due largely in part to the production costs associated with fungal biomass generation. Hawaii's high electricity costs, in particular, were found to preclude the feasibility of high energy demanding equipment such as a bioreactor system. Potential improvements for the economics of green processing, however, were discussed in Chapter 5, and include the need for rigorous optimization studies of fungal co-product generation (to increase fungal biomass yields by at least 40%), and/or the identification of higher value co-products that require less capital input. Additionally, a thorough examination of green processing, within the context of a complete biorefinery system, may help to quantify the cost savings incurred from other unit operations to improve the overall economics of biomass-to-biofuel processes.

CHAPTER 8

FUTURE WORKS

Implicit in this research is the need for future studies to determine the true economic viability and potential of the green processing approach in a real biorefinery. The results reported within this study have exemplified the technical feasibility of fractionating Napier grass for biofuel and biobased product generation, indicating high monomeric sugar yields from the extruded fiber, and significant volumes of collectable Napier grass juice. However, much of the predictions made regarding the economic applicability of green processing within the context of a biorefinery have relied on model products, like ethanol and *R. oligosporus*, due to the availability of primary and secondary data. Significantly important to note is that these products may not represent the future for biorefineries as a result of high production costs and relatively low market value (when compared to other prospective products). Cultivating *R. oligosporus* in an agitated bioreactor, for example, was determined to comprise 66% of the capital investment of green processing equipment and 73% of the total operational costs. Fortunately, green processing represents innovation in concept and biosystems engineering, and is not limited to the production of just ethanol and *R. oligosporus*. Green processing can thus be adapted and accommodated into future biorefineries through further understanding of some of the key points summarized in brevity below.

- Green processing effectively reduces the moisture content of raw Napier grass from an average of 78% to 56%. Determining the techno-economic feasibility of biomass fractionation in the field is necessary for identifying and quantifying the cost savings incurred by increasing feedstock densities prior to transportation to the primary biorefining facility.
- Barring co-product generation, juice captured from the green processing of Napier grass can be quantified and analyzed for water reclamation and recycling within a biorefinery. Cost savings stemming from reductions in water consumption of biomass-to-biofuel processes must be identified and compared against the costs of producing microbial co-products.

- Improved co-product generation, either by increased microbial biomass yields or by the production of higher value co-products (e.g., bioplastics, biochemicals, etc.), must be examined for Napier grass juice, as it may be tied to the economic viability of the upstream preprocessing strategy.
- Emerging biorefinery approaches such as the carboxylic acid platform may replace conventional sugar platform approaches if proven successful at pilot-scale testing. Examining and quantifying the improvements in the carboxylic acid platform, from the incorporation of green processing, may have significant implications in the production of drop-in biofuels and biochemicals.
- With respect to compositional data determined in Section 4.5 of this study, correlating and quantifying the effects of environmental factors (such as wind and solar radiation) would have monumental implications for the commercialization of Napier grass as a biofuel feedstock. Having the ability to site land which can knowingly favor the production of holocellulose (over lignin and/or other constituents) can significantly improve the profitability of commercial biomass-to-biofuel ventures.
- Finally, an in-depth life cycle assessment (LCA) must be conducted to examine the effects of incorporating green processing into biorefineries and in particular, an emphasis should be made on sensitivity analyses with respect to economic viability and the production various types of biofuels and biobased products.

APPENDIX A

COMPOSITIONAL DATA OF NAPIER GRASS

Table A.1. Composition of planted Napier grass with respect to age

Sample	% Glucan	% Xylan	% Lignin	% Extractives	% Ash	% Total
NIST	41.9 ± 0.9	22.9 ± 0.6	25.4 ± 0.2	4.7 ± 1.4	3.4 ± 0.5	98.3
2M	35.9 ± 0.8	19.6 ± 1.0	13.4 ± 0.7	14.6 ± 3.5	14.9 ± 1.2	98.4
4M	39.6 ± 0.4	21.7 ± 0.9	17.0 ± 0.7	15.4 ± 2.0	7.0 ± 1.3	100.7
6M	40.6 ± 0.4	22.9 ± 0.6	15.9 ± 0.9	13.5 ± 2.3	9.6 ± 0.4	102.6
8M	38.4 ± 0.0	21.5 ± 0.0	17.0 ± 0.8	15.2 ± 0.9	9.0 ± 0.7	101.0

* NIST = National Institute of Standards and Technology bagasse standard #8491; M = Month

Table A.2. Napier grass juice characteristics with respect to age

Sample	TKN (g/L)	COD (g/L)
2 MJ	0.84 ± 0.03	40.82 ± 1.32
4 MJ	0.60 ± 0.02	37.80 ± 2.52
6 MJ	0.39 ± 0.02	32.55 ± 1.30
8 MJ	1.03 ± 0.01	58.61 ± 1.67

* M = Month; J = Juice

APPENDIX B

STATISTICAL ANALYSES OF EXPERIMENTAL DATA

Table B.1. ANOVA for combined glucose and xylose concentrations in acid hydrolysate with respect to preprocessing methods for Duncan's Multiple Range Test

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	90254.90	3	30084.97	146.40	0.00**
Within Groups	6576.14	32	205.50		
Total	96831.04	35			

Table B.2. Post-hoc Duncan's Multiple Range Test of combined glucose and xylose concentrations in acid hydrolysate with respect to preprocessing methods

Preprocess Method	N	Subset for alpha = 0.05		
		1	2	3
W/U	9	117.86		
D/U	9	120.09		
D/J	9		138.60	
W/J	9			239.65
Sig.		0.74	1.00	1.00

Means for groups in homogeneous subsets are displayed.
W/U = Wet/Unjuiced; D/U = Dry/Unjuiced; W/J = Wet/Juiced; D/J = Dry/Juiced

Table B.3. ANOVA of Napier grass moisture content with respect to age (in months) for Duncan's Multiple Range Test

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	901.05	3	300.35	44.50	0.00**
Within Groups	134.99	20	6.75		
Total	1036.04	23			

Table B.4. Post-hoc Duncan's Multiple Range Test of Napier grass moisture content with respect to age (in months)

Age	N	Subset for alpha = 0.05			
		1	2	3	4
8M	6	69.52			
4M	6		74.15		
6M	6			81.04	
2M	6				85.42
Sig.		1.00	1.00	1.00	1.00

Means for groups in homogeneous subsets are displayed.
M = Months (corresponding with Napier grass age)

Table B.5. Regression analysis of moisture content versus cumulative precipitation

Equation	Model Summary					Parameter Estimates			
	R Square	F	df1	df2	Sig.	Constant	b1	b2	b3
Quadratic	0.48	2.30	2	5	0.20	93.45	-0.11	0.00	

The independent variable was cumulative precipitation.
The dependent variable was the moisture content of Napier grass.

Table B.6. Regression analysis of moisture content versus Napier grass age (in months)

Equation	Model Summary					Parameter Estimates	
	R Square	F	df1	df2	Sig.	Constant	b1
Linear	0.48	5.53	1	6	0.06	86.75	-1.78

The independent variable was the Napier grass age (in months).
The dependent variable was the moisture content of Napier grass.

Table B.7. RCB ANOVA of Napier grass extractives with respect to age (in months)

Source	Sum of Squares	df	Mean Square	F	Sig.
Model	1723.89	5	344.78	68.42	0.00**
Rep	1.53	1	1.53	0.30	0.62
Age	4.20	3	1.40	0.28	0.84
Error	15.12	3	5.04		
Total	1739.01	8			

Rep = Replicates; Age = 2, 4, 6, 8 months old Napier grass

Table B.8. Regression analysis of extractives versus Napier grass age (in months)

Equation	Model Summary					Parameter Estimates			
	R Square	F	df1	df2	Sig.	Constant	b1	b2	b3
Cubic	0.202	0.337	3	4	0.801	5.16	7.93	-1.86	0.13

The independent variable was the Napier grass age (in months).
The dependent variable was the extractives content.

Table B.9. RCB ANOVA of Napier grass glucose with respect to age (in months)

Source	Sum of Squares	df	Mean Square	F	Sig.
Model	11971.93	5	2394.39	805.55	0.00**
Rep	11.02	1	11.02	3.71	0.15
Age	25.01	3	8.34	2.81	0.21
Error	8.92	3	2.97		
Total	11980.85	8			

Rep = Replicates; Age = 2, 4, 6, 8 months old Napier grass

Table B.10. RCB ANOVA of Napier grass xylose with respect to age (in months)

Source	Sum of Squares	df	Mean Square	F	Sig.
Model	3684.10	5	736.82	453.45	0.00**
Rep	2.04	1	2.04	1.26	0.34
Age	10.67	3	3.56	2.19	0.27
Error	4.88	3	1.63		
Total	3688.97	8			

Rep = Replicates; Age = 2, 4, 6, 8 months old Napier grass

Table B.11. Regression analysis of glucose versus Napier grass age (in months)

Equation	Model Summary					Parameter Estimates			
	R Square	F	df1	df2	Sig.	Constant	b1	b2	b3
Quadratic	0.56	3.12	2	5	0.13	29.06	4.17	-0.372	

The independent variable was the Napier grass age (in months).
The dependent variable was the glucose content.

Table B.12. Regression analysis of xylose versus Napier grass age (in months)

Equation	Model Summary					Parameter Estimates			
	R Square	F	df1	df2	Sig.	Constant	b1	b2	b3
Quadratic	0.59	3.63	2	5	0.11	15.42	2.51	-0.22	

The independent variable was the Napier grass age (in months).
The dependent variable was the xylose content.

Table B.13. RCB ANOVA of Napier grass lignin with respect to age (in months)

Source	Sum of Squares	df	Mean Square	F	Sig.
Model	2023.16	5	404.63	812.22	0.00**
Rep	0.12	1	0.12	0.25	0.65
Age	18.01	3	6.00	12.05	0.04*
Error	1.50	3	0.50		
Total	2024.66	8			

Rep = Replicates; Age = 2, 4, 6, 8 months old Napier grass

Table B.14. ANOVA of Napier grass lignin content changes with respect to age (for orthogonal contrasts)

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	18.01	3	6.00	14.85	0.01*
Within Groups	1.62	4	0.40		
Total	19.63	7			

Table B.15. Orthogonal contrast coefficients for changes in Napier grass lignin content with respect to age

Contrast	Age (Months)			
	2.00	**4.00**	**6.00**	**8.00**
1	3	-1	-1	-1
2	-1	1	0	0
3	0	1	0	-1

Table B.16. Orthogonal contrast comparison results of changes in lignin content with respect to Napier grass age

Sample	Contrast	Value of Contrast	Std. Error	t	df	Sig. (2-tailed)
Lignin	1	-9.93	1.34	-7.42	2.04	0.02*
	2	3.69	0.85	4.36	1.40	0.09
	3	0.04	0.79	0.04	1.10	0.97

Table B.17. RCB ANOVA of Napier grass ash with respect to age (in months)

Source	Sum of Squares	df	Mean Square	F	Sig.
Model	894.58	5	178.92	75.19	0.00**
Rep	4.84	1	4.84	2.03	0.25
Age	69.21	3	23.07	9.70	0.05*
Error	7.14	3	2.38		
Total	901.71	8			

Rep = Replicates; Age = 2, 4, 6, 8 months old Napier grass

Table B.18. ANOVA of Napier grass ash content changes with respect to age (for orthogonal contrasts)

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	69.21	3	23.07	7.71	0.04*
Within Groups	11.98	4	2.99		
Total	81.18	7			

Table B.19. Orthogonal contrast coefficients for changes in Napier grass ash content with respect to age

Contrast	Age (Months)			
	2.00	**4.00**	**6.00**	**8.00**
1	3	-1	-1	-1
2	1	-1	0	0
3	0	1	0	-1

Table B.20. Orthogonal contrast comparison results of changes in ash content with respect to Napier grass age

Sample	Contrast	Value of Contrast	Std. Error	t	df	Sig. (2-tailed)
Ash	1	19.23	5.33	3.61	1.26	0.13
	2	7.95	1.81	4.40	1.36	0.10
	3	-1.97	0.87	-2.25	1.93	0.16

Table B.21. RCB ANOVA of Napier grass juice TKN with respect to age (in months)

Source	Sum of Squares	df	Mean Square	F	Sig.
Model	13.60	5	2.72	35.29	0.00**
Rep	0.06	1	0.06	0.77	0.39
Age	1.41	3	0.47	6.08	0.00**
Error	1.46	19	0.08		
Total	15.06	24			

Rep = Replicates; Age = 2, 4, 6, 8 months old Napier grass

Table B.22. ANOVA of Napier grass juice TKN concentration changes with respect to age (for orthogonal contrasts)

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.41	3	0.47	6.16	0.00**
Within Groups	1.52	20	0.08		
Total	2.93	23			

Table B.23. Orthogonal contrast coefficients for changes in Napier grass juice TKN concentrations with respect to age

Contrast	Age (Months)			
	2.00	**4.00**	**6.00**	**8.00**
1	1	-1	0	0
2	1	0	-1	0
3	1	0	0	-1

Table B.24. Orthogonal contrast comparison results of changes in Napier grass juice TKN concentrations with respect to age

Sample	Contrast	Value of Contrast	Std. Error	t	df	Sig. (2-tailed)
TKN	1	0.21	0.03	6.44	6.91	.00**
	2	0.45	0.05	8.65	9.12	.00**
	3	-0.21	0.22	-0.93	5.19	0.39

Table B.25. RCB ANOVA of Napier grass juice COD with respect to age (in months)

Source	Sum of Squares	df	Mean Square	F	Sig.
Model	45555.00	5	9111.00	74.33	0.00**
Rep	16.20	1	16.20	0.13	0.72
Age	2300.92	3	766.97	6.26	0.00**
Error	2328.85	19	122.57		
Total	47883.85	24			

Rep = Replicates; Age = 2, 4, 6, 8 months old Napier grass

Table B.26. ANOVA of Napier grass juice COD concentration changes with respect to age (for orthogonal contrasts)

Source	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2300.92	3	766.97	6.54	0.00**
Within Groups	2345.05	20	117.25		
Total	4645.98	23			

Table B.27. Orthogonal contrast coefficients for changes in Napier grass juice TKN concentrations (with respect to age)

Contrast	Age (Months)			
	2.00	**4.00**	**6.00**	**8.00**
1	1	-1	0	0
2	0	1	-1	0
3	0	0	1	-1

Table B.28. Orthogonal contrast comparison results of changes in Napier grass juice COD concentrations with respect to age

Sample	Contrast	Value of Contrast	Std. Error	t	Df	Sig. (2-tailed)
COD	1	3.02	4.88	0.62	10.00	0.55
	2	5.25	6.72	0.78	8.20	0.46
	3	-26.06	7.38	-3.53	9.55	0.01*

APPENDIX C

SHREDDER AND SCREW-PRESS

Figure C.1. Retsch laboratory-scale cutting mill

Figure C.2. Vincent Corporation cutting mill for green processing of Napier grass

Figure C.3. Fabricated piston for Napier grass fractionation via hydraulic press

Figure C.4. Vincent Corporation screw-press for green processing of Napier grass

APPENDIX D

OBSERVED FIELD CHANGES IN NAPIER GRASS WITH RESPECT TO AGE

Figure D.1. 2 month old crop

Figure D.2. 4 month old crop

Figure D.3. 6 month old crop

Figure D.4. 8 month old crop

APPENDIX E

CALCULATIONS FOR TECHNO-ECONOMIC ANALYSES

Table E.1. Calculations for economic analyses

Incoming Napier grass	Raw fiber (wet)	49,738	U.S. ton
		45,113	metric ton
	Moisture content	78%	
	Eq. dry weight fiber	9.92E+09	g
	Glucose (% dry wt.)	38%	
	Glucose	3.77E+09	g
	Xylose (% dry wt.)	21%	
	Xylose	2.08E+09	g
Ethanol output	Glucose conversion	51%	
	Xylose conversion	51%	
	EtOH (volume)	3,785,000	L
		1,000,000	gal
Co-product output	Juice	22,565,232	L
		5,961,752	gal
	Juice-to-fungus conversion	0.007	g/mL
	Fungal yield	158	metric ton
		174	U.S. ton
	Protein yield	71	metric ton
		78	U.S. ton
	Soybean meal ($)	$512.00	per metric ton
	Estimated fungal value	$80,874	

Table E.2. Labor costs for Hawai‘i occupations analogous to green processing

Title	Average hourly	Average annual
Mixing and blending machine setters, operators, and tenders	$11.76	$24,450
Separating, filtering, clarifying, precipitating, and still machine setters, operators, and tenders	$12.34	$25,670
Crushing, grinding, and polishing machine setters, operators, and tenders	$16.41	$34,140
Estimated labor for green processing	$13.50	$28,087

(Source: http://www.bls.gov/oes/current/oes_hi.htm)

Table E.3. Estimated operational and labor costs for green processing

Process	Unit Operation	kWh	Operating costs* (not including labor)	Labor costs†
Fractionation	Screw-press	155,432	$23,161	$11,194
Bioreactor	Steam	3,979,440	$1,007,643	$90,566
	Air	1,049,256		
	Agitation	1,733,400		
Recovery	Centrifuge	41	$6.17	$1,215
	Drying	n/a	$157,957	$90,472

*Operating costs were based on electricity rates on Oahu and do not include the customer and demand charges (~$4,500 per year); †Labor costs do not include medical benefits

APPENDIX F

SONICATION OF NAPIER GRASS

Introduction

The purpose of Appendix F is to report the results obtained from experiments that examined the effects of sonication, as a physical pretreatment, for Napier grass. The implementation of sonication has been shown to successfully enhance the sugar released from structural carbohydrates during the saccharification of starch-based feedstocks like cassava (Nitayavardhana et al., 2008). The rapid compression and rarefaction of media created by sound waves establishes localized temperature increases and cavitation, and are believed to facilitate biomass deconstruction. When applied directly to lignocellulosic feedstocks however, preliminary data suggested that sonication alone was not enough to promote sugar release from Napier grass. Subsequently, sonication at 20 kHz was combined with dilute sulfuric acid, and was investigated following the pretreatment optimization experiments reported in Section 3.3.

Due to the inherent properties and electronic configurations of the sonicator (Branson 900 Series, Dansbury, CT), sonication was delivered in pulses of 10-second durations. A 2-second delay accompanied each burst as the machine reset prior to the deliverance of another 10-second burst. Triplicate samples of Napier grass were sonicated either before pretreatment (BP) or after pretreatment (AP) using the optimal dilute sulfuric acid conditions determined for wet/juiced Napier grass (5% (w/w) acid, 120°C, and 45 minutes). Sonication always occurred prior to saccharification, as it was determined in earlier exploratory experiments, that sonication resulted in decreased enzymatic activity, possibly due to denaturation. The resulting acid and enzyme hydrolysates from three sonication time regimes (10, 20, and 30 seconds) and two conditions (BP and AP) were quantified by HPLC and reported in Figure F.1.

Results

Statistical analyses of the data in Figure F.1 indicated no difference in the concentration of xylose and glucose released during pretreatment, with respect to sonication time and chronology (i.e., AP or BP), as determined by a post-hoc Duncan's Multiple Range Test at a 95% confidence interval. (See Table F.1.) The concentration of glucose released during saccharification was suggested to be slightly higher following sonication for 10 and 20 seconds AP.

Overall, the total sugars released from Napier grass following sonication, dilute acid pretreatment, and saccharification (for all conditions) were determined to be lower than the total sugars released following dilute acid pretreatment and saccharification alone. This occurrence was believed to be a consequence of the mechanism of sonication, which likely catalyzed the deconstruction of weaker chemical bonds found in hemicellulose and amorphous cellulose; also evidenced by the improved saccharification of glucose from starch. The effects of sonication on reinforced polysaccharide structures, like cellulose fibers stabilized by hydrogen bonds, however, were minimal. Lower sugar concentrations were released in the acid hydrolysate, and no improvements were seen in the enzyme hydrolysate of sonicated Napier grass. The use of sonication in conjunction with dilute sulfuric acid pretreatments was thus concluded to be unjustifiable within the context of this study.

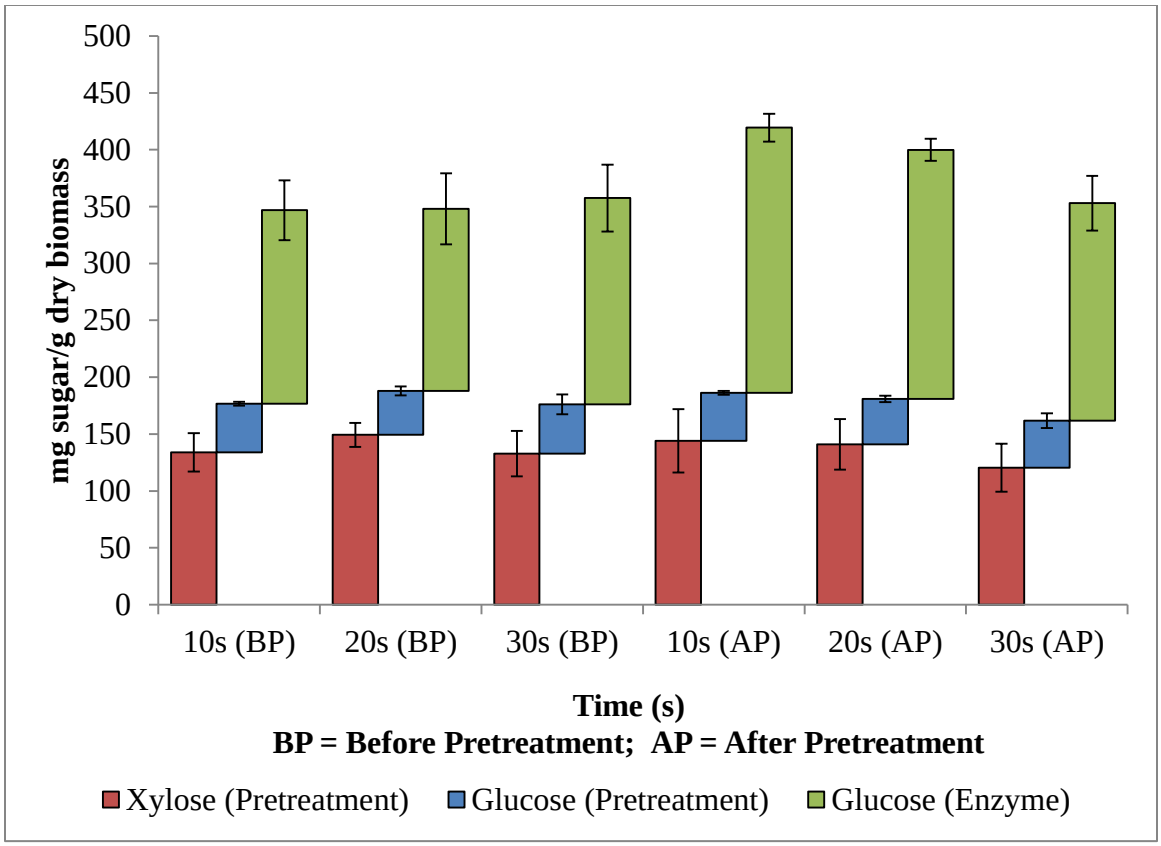

Figure F.1. Sugars released following the combined sonication and dilute sulfuric acid pretreatment of Napier grass, (n = 3)

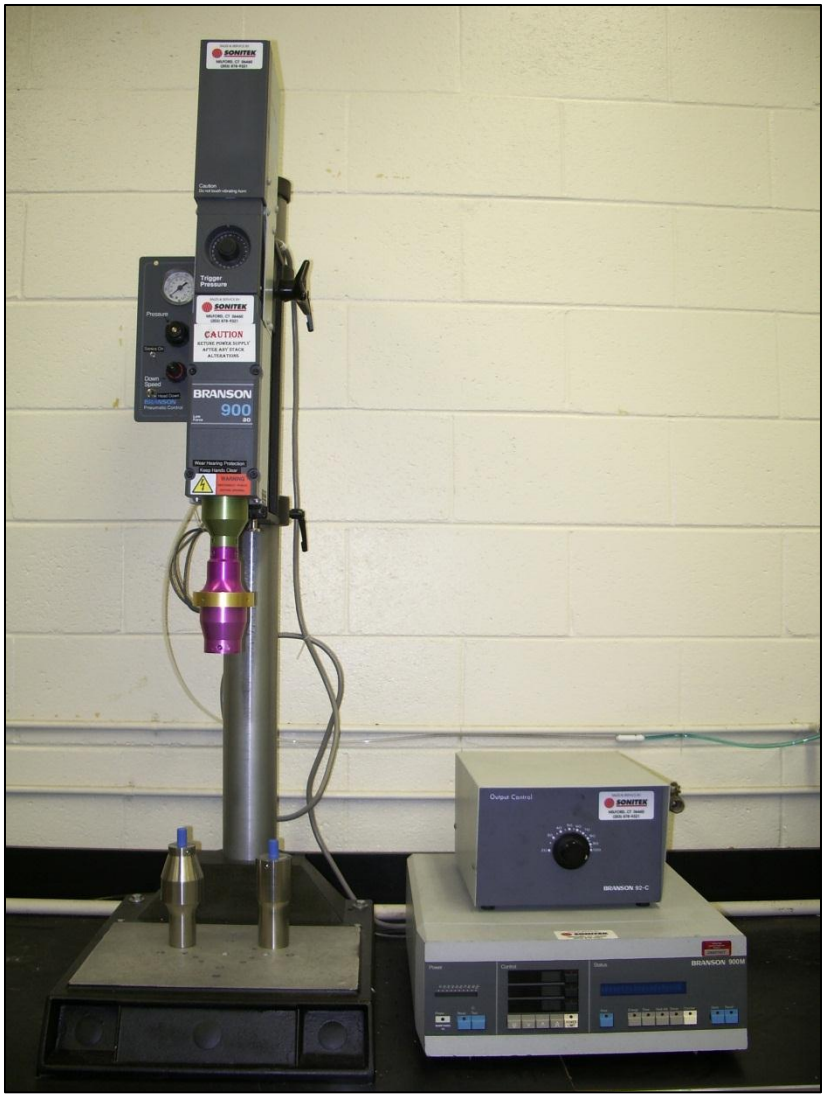

Figure F.2. Sonication system

Table F.1. ANOVA of sugars released in the acid hydrolysates for sonication BP and AP

		Sum of Squares	df	Mean Square	F	Sig.
Xylose (from Pretreatment)	Between Groups	4661.68	5	932.34	2.28	0.06
	Within Groups	19624.12	48	408.84		
	Total	24285.80	53			
Glucose (from Pretreatment)	Between Groups	146.24	5	29.25	1.18	0.33
	Within Groups	1186.95	48	24.73		
	Total	1333.19	53			

Table F.2. ANOVA of glucose released in the enzyme hydrolysates for sonication BP and AP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	35246.83	5	7049.37	16.46	0.00**
Within Groups	20563.48	48	428.41		
Total	55810.31	53			

Table F.3. Post-hoc Duncan's Multiple Range Test of glucose released from enzyme hydrolysate following sonication

Sonication Treatment	N	Subset for alpha = 0.05			
		1	2	3	4
20s BP	9	160.17			
10s BP	9	170.17	170.17		
30s BP	9		181.33	181.33	
30s AP	9			191.33	
20s AP	9				216.00
10s AP	9				233.67
Sig.		0.31	0.26	0.31	0.08

Means for groups in homogeneous subsets are displayed.
BP = Before pretreatment; AP = After pretreatment

APPENDIX G

PUBLICATIONS AND AWARDS

Peer-reviewed Publication:

Takara D., and Khanal, S. K. 2011. Green processing of tropical banagrass into biofuel and biobased products: An innovative biorefinery approach. *Bioresource Technology*. 102 (2): 1587-1592.

Other Publications:

Takara, D., Nitayavardhana, S., Munasinghe, P. C., K.C., S., and Khanal, S. K. 2012. Sustainable bioenergy from biofuel-derived residues. *Water Environment Research*. 84 (10):1568-1585.

Takara, D., and Khanal, S. K. 2012. Biomass pretreatment for biofuel production. In *Sustainable Bioenergy and Bioproducts. (eds.* K. Gopalakrishnan, H. van Leeuwen, and R. Brown). Springer-Verlag Inc., London, UK. pp 59-70.

Takara, D., Shrestha, P., and Khanal, S. K. 2010. Lignocellulosic biomass pretreatment. In *Biofuel and Bioenergy from Biowastes and Lignocellulosic Biomass*. (eds. Samir K. Khanal et al.). American Society of Civil Engineers. Reston, VA, USA. pp 158-171.

Takara, D., Nitayavardhana, S., Pinowska, A., and Khanal, S. K. 2010. Sustainable bioenergy from biofuel residues and wastes. *Water Environment Research*. 82 (10):1694-1719.

International Conferences:

Takara, D., and Khanal, S. K. Green processing of dedicated bioenergy crops for biofuel and biobased products. *Challenges in Environmental Science and Engineering (CESE)*, September 9-13, 2012, Melbourne, Australia

Takara, D., and Khanal, S. K. Enhanced sugar release and co-product generation of green banagrass. *Asian Congress on Biotechnology (ACB)*, May 11-15, 2011, Shanghai, China.

National Conferences:

Takara, D., and Khanal, S. K. Wet processing of banagrass: A biorefinery perspective. *American Society of Agricultural and Biological Engineers (ASABE) 2011 Annual International Meeting*, August 7-10, 2011, Louisville, KY, USA.

Takara, D., and Khanal, S. K. Enhanced sugar release and co-product generation of green banagrass. *Asian Congress on Biotechnology (ACB)*, May 11-15, 2011, Shanghai, China.

Takara, D., and Khanal, S. K. Green processing of banagrass (*Pennisetum purpureum*) for enhanced sugar release. *Pacific Rim Summit on Industrial Biotechnology and Bioenergy*, Dec. 11-14, 2010, Honolulu, HI, USA (Podium presentation).

Takara, D., and Khanal, S. K. Optimization of chemical pretreatment of banagrass (a variety of *Pennisetum purpureum*) for enhanced sugar release. *Pacific Rim Summit on Industrial Biotechnology and Bioenergy*, Nov. 8-11, 2009, Honolulu, HI, USA (Podium presentation).

Awards:

- MBBE Best Ph.D. Student Oral Presentation; College of Tropical Agriculture and Human Resources Symposium (2012)
- Ka Hana Po'okela Award; College of Tropical Agriculture and Human Resources (2012)
- Helen Jones Farrar Award in Bioengineering; Achievement Rewards for College Scientists (ARCS) Foundation (2010)
- Outstanding Poster Award in the Ph.D. category (1st place); College of Tropical Agriculture and Human Resources Symposium (2010)

REFERENCES

About the Hawaii Clean Energy Initiative. 2012. State of Hawaii [cited September 30 2012]. Available from http://www.hawaiicleanenergyinitiative.org/about/.

Agler, M. T., B. A. Wrenn, S. H. Zinder, and L. T. Angenent. 2011. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends in Biotechnology 29 (2):70-78.

Amthor, J. S. 2003. Efficiency of Lignin Biosynthesis: A Quantitative Analysis. *Annals of Botany* 91 (6):673-695.

Bothast, R. J., and M. A. Schlicher. 2005. Biotechnological processes for conversion of corn into ethanol. *Applied Microbiology and Biotechnology* 67:19-25.

Bouman, B. A. M., S. Peng, A. R. Castaneda, and R. M. Visperas. 2005. Yield and water use of irrigated tropical aerobic rice systems. *Agricultural Water Management* 74 (2):87-105.

Canilha, L., V. Santos, G. Rocha, J. A. Silva, M. Giulietti, S. Silva, M. Felipe, A. Ferraz, A. Milagres, and W. Carvalho. 2011. A study on the pretreatment of a sugarcane bagasse sample with dilute sulfuric acid. *Journal of Industrial Microbiology & Biotechnology* 38 (9):1467-1475.

Chandel, A. K., S. S. da Silva, W. Carvalho, and O. V. Singh. 2012. Sugarcane bagasse and leaves: foreseeable biomass of biofuel and bio-products. *Journal of Chemical Technology & Biotechnology* 87 (1):11-20.

Chang, H., N. J. Kim, J. Kang, and C. Jeong. 2010. Biomass-derived volatile fatty acid platform for fuels and chemicals. *Biotechnology and Bioprocess Engineering* 15 (1):1-10.

Chen, W. H., B. L. Pen, C. T. Yu, and W. S. Hwang. 2011. Pretreatment efficiency and structural characterization of rice straw by an integrated process of dilute-acid and steam explosion for bioethanol production. *Bioresource Technology* In Press, Accepted Manuscript.

Cherney, J. H. 2006. Ash Content of Grasses for Biofuel. Cornell University Cooperative Extension.

Chu, L. Q., R. Masyuko, J. V. Sweedler, and P. W. Bohn. 2010. Base-induced delignification of miscanthus x giganteus studied by three-dimensional confocal raman imaging. *Bioresource Technology* 101 (13):4919-4925.

Chum, H. L., D. K. Johnson, S. K. Black, and R. P. Overend. 1990. Pretreatment-Catalyst Effects and the Combined Severity Parameters. *Applied Biochemistry and Biotechnology* 24/25:1-14.

Cipollini Jr., D. F. 1997. Wind-induced mechanical stimulation increases pest resistance in common bean. Oecologia 111:84-90.

Crabb, W. D., and C. Mitchinson. 1997. Enzymes involved in the processing of starch to sugars. *Trends in Biotechnology* 15 (9):349-352.

Deguchi, S., K. Tsujii, and K. Horikoshi. 2006. Cooking cellulose in hot and compressed water. *Chemical Communications* (31):3293-3295.

Department of Economic and Social Affairs: Population Division. 2004. World Population to 2300. Department of Economic and Social Affairs: Population Division.

Dien, B. S., H. G. Jung, K. P. Vogel, M. D. Casler, J. F. S. Lamb, L. Itena, R. B. Mitchell, and G. Srath. 2006. Chemical Composition and Response to Dilute-acid Pretreatment and Enzymatic Saccharification of Alfalfa, Canarygrass, and Switchgrass. *Biomass and Bioenergy* 30:880-891.

Disgesdahl Digestion Apparatus: Instrument Manual. 2012. HACH [cited September 29 2012]. Available from http://www.hach.com/asset-get.download-en.jsa?id=7639982427.

Eggeman, T., and R. T. Elander. 2005. Process and economic analysis of pretreatment technologies. *Bioresource Technology* 96 (18):2019-2025.

Electric Power Monthly. 2012. U.S. Energy Information Administration 2012 [cited October 25 2012]. Available from http://www.eia.gov/electricity/monthly/epm_table_grapher.cfm?t=epmt_5_6_a.

Gumiero, B., B. Boz, P. Cornelio, and S. Casella. 2011. Shallow groundwater nitrogen and denitrification in a newly afforested, subirrigated riparian buffer. *Journal of Applied Ecology* 48 (5):1135-1144.

Gupta, R., and Y. Y. Lee. 2010. Investigation of biomass degradation mechanism in pretreatment of switchgrass by aqueous ammonia and sodium hydroxide. *Bioresource Technology* 101 (21):8185-8191.

Hames, B., R. Ruiz, C. Scarlata, A. Sluiter, J. Sluiter, and D. Templeton. 2008. Preparation of Samples for Compositional Analysis. National Renewable Energy Laboratory.

Herzog, T. 2009. *World Greenhouse Gas Emissions in 2005*. World Resources Institute 2009 [cited September 29 2012]. Available from http://www.wri.org/publication/world-greenhouse-gas-emissions-in-2005.

Hjorth, M., K. Gränitz, A. P. S. Adamsen, and H. B. Møller. 2011. Extrusion as a pretreatment to increase biogas production. *Bioresource Technology* 102 (8):4989-4994.

Holmes, W., ed. 1980. *Grass: Its Production and Utilization*. 2 ed: British Grassland Society.

Holtzapple, M., and C. Granda. 2009. Carboxylate Platform: The MixAlco Process Part 1: Comparison of Three Biomass Conversion Platforms. *Applied Biochemistry and Biotechnology* 156 (1):95-106.

Hsu, T. C., G. L. Guo, W. H. Chen, and W. S. Hwang. 2010. Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. *Bioresource Technology* 101 (13):4907-4913.

Hubbert, M. K. 1956. Nuclear Energy and the Fossil Fuels. Houston: Shell Development Company.

Humbird, D., R. Davis, L. Tao, C. Kinchin, D. Hsu, and A. Aden. 2011. Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover. National Renewable Energy Laboratory.

International Energy Statistics. 2010. U.S. Energy Information Administration.

Ji, X. J., H. Huang, Z. K. Nie, L. Qu, Q. Xu, and G. Tsao. 2012. Fuels and Chemicals from Hemicellulose Sugars Biotechnology in China III: Biofuels and Bioenergy, edited by F.-W. Bai, C.-G. Liu, H. Huang and G. T. Tsao: Springer Berlin / Heidelberg.

Jirka, A. M., and M. J. Carter. 1975. Micro semiautomated analysis of surface and waste waters for chemical oxygen demand. *Analytical Chemistry* 47 (8):1397-1402.

Johnson, K., R. Kleerebezem, and M. C. M. van Loosdrecht. 2010. Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs. *Water Research* 44 (7):2141-2152.

Judd, J., S. C. Sarin, J. S. Cundiff, and R. D. Grisso. 2010. An Optimal Storage and Transportation System for a Cellulosic Ethanol Bio-energy Plant. In *2010 ASABE Annual International Meeting*. Pittsburgh, Pennsylvania: American Society of Agricultural and Biological Engineers.

Kato, H., H. Suyama, R. Yamada, T. Hasunuma, and A. Kondo. 2012. Improvements in Ethanol Production from Xylose by Mating Recombinant Xylose-fermenting *Saccharomyces cerevisiae* strains. *Applied Microbiology and Biotechnology* 94:1585-1592.

Kazi, F. K., J. A. Fortman, R. P. Anex, D. D. Hsu, A. Aden, A. Dutta, and G. Kothandaraman. 2010. Techno-economic comparison of process technologies for biochemical ethanol production from corn stover. *Fuel* 89, Supplement 1 (0):S20-S28.

Keffer, V. I., S. Q. Turn, C. M. Kinoshita, and D. E. Evans. 2009. Ethanol technical potential in Hawaii based on sugarcane, banagrass, Eucalyptus, and Leucaena. *Biomass and Bioenergy* 33 (2):247-254.

Khardenavis, A. A., A. N. Vaidya, M. S. Kumar, and T. Chakrabarti. 2009. Utilization of molasses spentwash for production of bioplastics by waste activated sludge. *Waste Management* 29 (9):2558-2565.

Kim, T. H., and Y. Y. Lee. 2005. Pretreatment and fractionation of corn stover by ammonia recycle percolation process. *Bioresource Technology* 96 (18):2007-2013.

Kim, T. H., J. S. Kim, C. Sunwoo, and Y. Y. Lee. 2003. Pretreatment of corn stover by aqueous ammonia. *Bioresource Technology* 90:39-47.

Klein-Marcuschamer, D., P. Oleskowicz-Popiel, B. A. Simmons, and H. W. Blanch. 2012. The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnology and Bioengineering* 109 (4):1083-1087.

Kocoloski, M., W. M. Griffin, and H. S. Matthews. 2011. Impacts of facility size and location decisions on ethanol production cost. *Energy Policy* 39 (1):47-56.

Lau, M. W., C. Gunawan, and B. E. Dale. 2009. The impacts of pretreatment on the fermentability of pretreated lignocellulosic biomass: a comparative evaluation between ammonia fiber expansion and dilute acid pretreatment. *Biotechnology for Biofuels* 2 (1):30.

Leboreiro, J., and A. K. Hilaly. 2011. Biomass transportation model and optimum plant size for the production of ethanol. *Bioresource Technology* 102 (3):2712-2723.

Li, C., B. Knierim, C. Manisseri, R. Arora, H. V. Scheller, M. Auer, K. P. Vogel, B. A. Simmons, and S. Singh. 2010. Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification and enzymatic saccharification. *Bioresource Technology* 101 (13):4900-4906.

Li, J., D. Zhu, J. Niu, S. Shen, and G. Wang. 2011. An Economic Assessment of Astaxanthin Production by Large Scale Cultivation of *Haematococcus pluvialis*. *Biotechnology Advances* 29:568-574.

Li, Y., J. Park, R. Shiroma, and K. Tokuyasu. 2011. Bioethanol production from rice straw by a sequential use of Saccharomyces cerevisiae and Pichia stipitis with heat inactivation of Saccharomyces cerevisiae cells prior to xylose fermentation. *Journal of Bioscience and Bioengineering* 111 (6):682-686.

Lin, T. H., C. F. Huang, G. L. Guo, W. S. Hwang, and S. L. Huang. 2012. Pilot-scale ethanol production from rice straw hydrolysates using xylose-fermenting Pichia stipitis. *Bioresource Technology* 116 (0):314-319.

Liu, Z., B. C. Saha, and P. J. Slininger. 2008. Lignocellulosic Biomass Conversion to Ethanol by *Saccharomyces*. In *Bioenergy*, edited by J. Wall, C. Harwood and A. Demain. Washington, D.C.: ASM Press.

Lloyd, T. A., and C. E. Wyman. 2005. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. *Bioresource Technology* 96 (18):1967-1977.

Lu, Y., Y. Wang, G. Xu, J. Chu, Y. Zhuang, and S. Zhang. 2010. Influence of High Solid Concentration on Enzymatic Hydrolysis and Fermentation of Steam-Exploded Corn Stover Biomass. *Applied Biochemistry and Biotechnology* 160 (2):360-369.

Martin, G. L., B. Alriksson, A. Sjode, N. Nilvebrant, and L. J. Jonsson. 2007. Dilute Sulfuric Acid Pretreatment of Agricultural and Agro-industrial Residues for Ethanol Production. *Applied Biochemistry and Biotechnology* 137:339-352.

May 2011 State Occupational Employment and Wage Estimates: Hawaii. 2012. Bureau of Labor Statistics [cited Oct 25 2012]. Available from http://www.bls.gov/oes/current/oes_hi.htm.

McIntosh, S., and T. Vancov. 2011. Optimisation of dilute alkaline pretreatment for enzymatic saccharification of wheat straw. *Biomass and Bioenergy* 35 (7):3094-3103.

Miller, G. L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry* 31:426.

Modenbach, A. A., and S. E. Nokes. 2012. The Use of High-Solids Loadings in Biomass Pretreatment - A Review. *Biotechnology and Bioengineering* 109:1430-1442.

Moncada, J., M. M. El-Halwagi, and C. A. Cardona. 2012. Techno-economic analysis for a sugarcane biorefinery: Colombian case. *Bioresource Technology* Corrected Proof.

Mosier, N., R. Hendrickson, N. Ho, M. Sedlak, and M. R. Ladisch. 2005. Optimization of pH controlled liquid hot water pretreatment of corn stover. *Bioresource Technology* 96 (18):1986-1993.

Mueller, S. A., J. E. Anderson, and T. J. Wallington. 2011. Impact of biofuel production and other supply and demand factors on food price increases in 2008. *Biomass and Bioenergy* 35 (5):1623-1632.

Nitayavardhana, S., and S. K. Khanal. 2010. Innovative biorefinery concept for sugar-based ethanol industries: production of protein-rich fungal biomass on vinasse as an aquaculture feed ingredient. *Bioresource Technology* 101 (23):9078-9085.

Nitayavardhana, S., S. K. Rakshit, D. Grewell, J. H. van Leeuwen, and S. K. Khanal. 2008. Ultrasound Pretreatment of Cassava Chip Slurry to Enhance Sugar Release for Subsequent Ethanol Production. *Biotechnology and Bioengineering* 101 (3):487-496.

Nitrogen, Total Kjeldahl: Nessler Method. 2012. HACH [cited September 29 2012]. Available from www.hach.com/asset-get.download-en.jsa?id=7639983809.

Osgood, R. V., N. S. Dudley, and L. A. Jakeway. 1996. A Demonstration of Grass Biomass Production on Molokai.

Pérez, J. A., I. Ballesteros, M. Ballesteros, F. Sáez, M. J. Negro, and P. Manzanares. 2008. Optimizing Liquid Hot Water pretreatment conditions to enhance sugar recovery from wheat straw for fuel-ethanol production. *Fuel* 87 (17-18):3640-3647.

Petroleum Chronology of Events 1970-2000. 2012. U.S. Energy Information Administration [cited September 29 2012]. Available from http://www.eia.gov/pub/oil_gas/petroleum/analysis_publications/chronology/petroleumchronology2000.htm.

Pronyk, C., and G. Mazza. 2011. Fractionation of triticale, wheat, barley, oats, canola, and mustard straws for the production of carbohydrates and lignins. *Bioresource Technology* 106:117-124.

Ramos, L. P. 2003. The Chemistry Involved in the Steam Treatment of Lignocellulosic Biomass. *Quimica Nova* 26 (6):863-871.

Ray, M. J., D. J. Leak, P. D. Spanu, and R. J. Murphy. 2010. Brown rot fungal early stage decay mechanism as a biological pretreatment for softwood biomass in biofuel production. *Biomass and Bioenergy* 34 (8):1257-1262.

Renewable Fuel Standards. 2012. U.S. Environmental Protection Agency 2012 [cited September 29 2012]. Available from http://www.epa.gov/otaq/fuels/renewablefuels/index.htm.

Rengsirikul, K., Y. Ishii, K. Kangvansaichol, P. Pripanapong, P. Sripichitt, V. Punsuvon, P. Vaithanomsat, G. Nakamanee, and S. Tudsri. 2011. Effects of Inter-cutting Interval on Biomass Yield, Growth Components and Chemical Composition of Napiergrass (*Pennisetum purpureum* Schumach) Cultivars as Bioenergy Crops in Thailand. *Grassland Science* 57:135-141.

RETScreen 4. 2012. CanmetENERGY, Natural Resources Canada.

Schaffenberger, M., J. Ecker, W. Koschuh, R. Essl, M. Mandl, H. G. Boechzelt, H. Steinmueller, and H. Schnitzer. 2012. Green Biorefinery - Production of Amino Acids from Grass Silage Juice Using an Ion Exchanger Device at Pilot Scale. *Chemical Engineering Transactions* 29:505-510.

Schedule P: Large Power Service. 2012. Hawaiian Electric Company [cited Oct 25 2012]. Available from http://www.heco.com/vcmcontent/StaticFiles/FileScan/PDF/EnergyServices/Tarrifs/HECO/HECORatesSchP.pdf.

Schell, D. J., J. Farmer, M. Newman, and J. D. McMillan. 2003. Dilute-Sulfuric Acid Pretreatment of Corn Stover in Pilot-Scale Reactor. *Applied Biochemistry and Biotechnology* 105-108:69-85.

Scown, C. D., W. W. Nazaroff, U. Mishra, B. Strogen, A. B. Lobscheid, E. Masanet, N. J. Santero, A. Horvath, and T. E. McKone. 2012. Lifecycle Greenhouse Gas Implications of U.S. National Scenarios for Cellulosic Ethanol Production. *Environmental Research Letters* 7 (1):1-9.

Selig, M., N. Weiss, and Y. Ji. 2008. Enzymatic Saccharification of Lignocellulosic Biomass. National Renewable Energy Laboratory.

Shapouri, H., J. A. Duffield, and M. Wang. 2003. The Energy Balance of Corn Ethanol Revisited. *Transactions of the Amercian Society of Biological Engineers* 46 (4):959-968.

Shi, J., Q. Qing, T. Zhang, C. E. Wyman, and T. A. Lloyd. 2011. Biofuels from cellulosic biomass via aqueous processing. In *Fundamentals of Materials for Energy and Environmental Sustainability*, edited by D. S. Ginley and D. Cahen: Cambridge University Press.

Shredder Design. 2012. Vincent Corporation [cited September 22 2012]. Available from http://www.vincentcorp.com/Shredders_Description.

Shrestha, P., M. Rasmussen, S. K. Khanal, A. L. Pometto III, and J. H. van Leeuwen. 2008. Solid-Substrate Fermentation of Corn Fiber by *Phanerochaete chrysosporium* and Subsequent Fermentation of Hydrolysate into Ethanol. *Journal of Agricultural and Food Chemistry* 56:3918-3924.

Sluiter, A., B. Hames, D. Hyman, C. Payne, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and J. Wolfe. 2008a. Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples. National Renewable Energy Laboratory (NREL).

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, and D. Templeton. 2006. Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples. National Renewable Energy Laboratory (NREL).

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, and D. Templeton. 2008b. Determination of Ash in Biomass. National Renewable Energy Laboratory.

Sluiter, A., R. Ruiz, C. Scarlata, J. Sluiter, and D. Templeton. 2008c. Determination of Extractives in Biomass. National Renewable Energy Laboratory.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker. 2008d. Determination of Structural Carbohydrates and Lignin in Biomass. National Renewable Energy Laboratory.

Sluiter, J., and A. Sluiter. 2010. Summative Mass Closure. National Renewable Energy Laboratory.

Sorrell, S., J. Speirs, R. Bentley, A. Brandt, and R. Miller. 2010. Global oil depletion: A review of the evidence. *Energy Policy* 38 (9):5290-5295.

Soybean Meal Monthly Price - U.S. Dollars per Metric Ton. 2012. Indexmundi [cited Sept 29 2012]. Available from http://www.indexmundi.com/commodities/?commodity=soybean-meal.

Stewart, D. 2008. Lignin as a base material for materials applications: Chemistry, application and economics. *Industrial Crops and Products* 27 (2):202-207.

Sukumaran, R. K., R. R. Singhania, G. M. Mathew, and A. Pandey. 2009. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. *Renewable Energy* 34 (2):421-424.

Sulfur: Statistics and Information. 2012. U.S. Geological Survey 2012 [cited Oct 1 2012]. Available from http://minerals.usgs.gov/minerals/pubs/commodity/sulfur/index.html

Sun, R., J. M. Lawther, and W. B. Banks. 1995. Influence of alkaline pre-treatments on the cell wall components of wheat straw. *Industrial Crops and Products* 4 (2):127-145.

Sutermeister, E. 1920. *Chemistry of Pulp and Paper Making*. New York: John Wiley & Sons, Inc.

Swatloski, R. P., S. K. Spear, J. D. Holbrey, and R. D. Rogers. 2002. Dissolution of Cellulose with Ionic Liquids. *Journal of the American Chemical Society* 124:4974-4975.

Teymouri, F., L. Laureano-Perez, H. Alizadeh, and B. E. Dale. 2005. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. *Bioresource Technology* 96:2014-2018.

Tran, N., P. Illukpitiya, J. F. Yanagida, and R. Ogoshi. 2011. Optimizing biofuel production: An economic analysis for selected biofuel feedstock production in Hawaii. *Biomass and Bioenergy* 35:1756-1764.

Turner, M. B., S. K. Spear, J. G. Huddleston, J. D. Holbrey, and R. D. Rogers. 2003. Ionic liquid salt-induced inactivation and unfolding of cellulase from Trichoderma reesei. *Green Chemistry* 5 (4):443-447.

U.S. Department of State Office of the Historian. 2012. *OPEC Oil Embargo, 1973-1974*. U.S. Department of State Office of the Historian [cited September 26 2012]. Available from http://history.state.gov/milestones/1969-1976/OPEC.

U.S. Energy Information Administration. 2011. Annual Energy Outlook 2011 with Projections to 2035.

U.S. Energy Information Administration. 2011. Emissions of Greenhouse Gases in the United States 2009.

U.S. Energy Information Administration. 2011. International Energy Outlook 2011. U.S. Energy Information Administration.

U.S. Energy Information Administration. 2012. *How dependent are we on foreign oil?* U.S. Energy Information Administration, July 13, 2012 2012 [cited September 29 2012]. Available from http://www.eia.gov/energy_in_brief/foreign_oil_dependence.cfm.

van Leeuwen, J. H., M. L. Rasmussen, S. Sankaran, C. R. Koza, D. T. Erickson, D. Mitra, and B. Jin. 2012. Fungal Treatment of Crop Processing Wastewaters with Value-Added Co-Products Sustainable Bioenergy and Bioproducts, edited by K. Gopalakrishnan, J. van Leeuwen and R. C. Brown: Springer London.

Voigt, T. B., D. K. Lee, and G. J. Kling. 2012. Perennial Herbaceous Crops with Potential for Biofuel Production in the Temperate Regions of the USA. *CAB Reviews* 7 (15):1-13.

Wang, Y., M. Radosevich, D. Hayes, and N. Labbé. 2011. Compatible Ionic liquid-cellulases system for hydrolysis of lignocellulosic biomass. *Biotechnology and Bioengineering* 108 (5):1042-1048.

Xu, C., F. Ma, X. Zhang, and S. Chen. 2010. Biological Pretreatment of Corn Stover by *Irpex lacteus* for Enzymatic Hydrolysis. *Journal of Agricultural and Food Chemistry* 58 (20):10893-10898.

Yang, J., X. Zhang, Q. Yong, and S. Yu. 2010. Three-stage hydrolysis to enhance enzymatic saccharification of steam-exploded corn stover. *Bioresource Technology* 101 (13):4930-4935.

Yang, S. J., I. Kataeva, S. D. Hamilton-Brehm, N. L. Engle, T. J. Tschaplinski, C. Doeppke, M. Davis, J. Westpheling, and M. W. W. Adams. 2009. Efficient Degradation of Lignocellulosic Plant Biomass, without Pretreatment, by the Thermophilic Anaerobe "*Anaerocellum thermophilum*" DSM 6725. *Applied and Environmental Microbiology* 79:4762-4769.

Yoshida, T., S. Q. Turn, R. S. Yost, and M. J. Antal. 2008. Banagrass vs Eucalyptus Wood as Feedstocks for Metallurgical Biocarbon Production†. *Industrial & Engineering Chemistry Research* 47 (24):9882-9888.

Yu, G., S. Yano, H. Inoue, S. Inoue, T. Endo, and S. Sawayama. 2010. Pretreatment of Rice Straw by a Hot-Compressed Water Process for Enzymatic Hydrolysis. *Applied Biochemistry and Biotechnology* 160 (2):539-551.

Yu, J., and L. X. L. Chen. 2006. Cost-Effective Recovery and Purification of Polyhydroxyalkanoates by Selective Dissolution of Cell Mass. *Biotechnology Progress* 22 (2):547-553.

Yu, J., T. Zhang, J. Zhong, X. Zhang, and T. Tan. 2012. Biorefinery of sweet sorghum stem. *Biotechnology Advances* 30 (4):811-816.

Yu, X., Y. Zheng, K. M. Dorgan, and S. Chen. 2011. Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. *Bioresource Technology* 102 (10):6134-6140.

Zhang, J., and J. Bao. 2012. A modified method for calculating practical ethanol yield at high lignocellulosic solids content and high ethanol titer. *Bioresource Technology* 116 (0):74-79.

Zhang, T., R. Kumar, and C. E. Wyman. Comparison of glucose and Xylose yields from dilute oxalic acid pretreatment followed by enzymatic hydrolysis of red maple to results with dilute sulfuric and hydrochloric acids and just hot water. *Carbohydrate Polymers* (accepted manuscript).

Zimbardi, F., E. Viola, F. Nanna, E. Larocca, M. Cardinale, and D. Barisano. 2007. Acid impregnation and steam explosion of corn stover in batch processes. *Industrial Crops and Products* 26 (2):195-206.