SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF DRY-GRIND HIGHLY DIGESTIBLE GRAIN SORGHUM LINES FOR ETHANOL PRODUCTION

A Thesis

by

JOAN ROLLOG HERNANDEZ

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

MASTER OF SCIENCE

May 2009

Major Subject: Biological and Agricultural Engineering

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Approved by:

Chair of Committee,	Sergio C. Capareda
Committee Members,	Dirk B. Hays
	Raghupathy Karthikeyan
	Calvin B. Parnell, Jr.
	William L. Rooney
Head of Department,	Gerald L. Riskowsky

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ABSTRACT

Simultaneous Saccharification and Fermentation of Dry-grind Highly Digestible Grain Sorghum Lines for Ethanol Production. (May 2009)

Joan Rollog Hernandez, B.S.; M.S., University of the Philippines at Los Baños, Philippines

Chair of Advisory Committee: Dr. Sergio C. Capareda

The potential of high digestible grain sorghum (HDGS) with a modified starch protein endosperm matrix to replace corn in ethanol production was investigated using dry grind simultaneous saccharification and fermentation (SSF). Preliminary experiments showed that HDGS yielded higher amounts of glucose and ethanol than normal digestible grain sorghum (NDGS) and corn particularly in the first 48 hrs of fermentation. It was hypothesized that fast conversion of starch to glucose and ethanol during hydrolysis and fermentation are results of improved protein digestibility of HDGS.

The invagination of protein structures in HDGS produced a flourier endosperm texture, softer kernels and lower starch content than the normal digestible protein (ND) lines. Highly digestible protein (HD) lines have better pasting properties (significantly lower pasting temperature, faster rate of gelatinization and higher peak viscosity) than ND lines based on the RVA profile. Increasing protein digestibility of the HDGS improved starch digestibility (increased rate of glucose conversion and total glucose yield during saccharification), which is supported by highly significant correlation of turbidity with rate of glucose conversion and efficiency of enzymatic conversion.

The efficiency of ethanol conversion is significantly correlated with starch digestibility, pasting properties, and protein digestibility. Results also showed that HD sorghum lines had significantly faster rate of conversion and shorter reaction time needed to achieve completion than ND sorghum lines and corn. Increasing the dry solid concentration from 22% to 30% (w/v) increased the ethanol yield from 8% v/v to 13%v/v. This will allow considerable saving of water, reduced distillation cost and increased ethanol production for a given plant capacity and labor cost.

Fineness of grind influences the amount of sugar formed due to variation in surface area of the flour. The hypothesis that finer particles has faster and higher glucose yield, defined as g of glucose converted per g of theoretical glucose, is supported by highly significant correlation of mass fraction of 3 to 60 μm size range and mass median diameter (MMD) of 60 to 1000 μm size range with glucose conversion efficiency and glucose conversion rate during saccharification and fermentation.

DEDICATION

This thesis is dedicated to my father, Jose F. Hernandez, my mother, Julie R. Hernandez, and my only sister, Jonalyn R. Hernandez, who have always been supportive to me since the beginning of my studies. Their unconditional love and unwavering faith in me are my sources of strength and inspiration to dream more and achieve further.

Also, this thesis is dedicated to my fiancé, Amado L. Maglinao, Jr., who has been there always beside me through thick and thin. Having you in my life, every moment has been amazing, meaningful and exciting to live. Nothing is impossible to do having your love, kindness and care. With these, all I can do is to care and love you more than you do. And *"… all I wanna do, is grow old with you…"*.

Finally, this thesis is dedicated to all those who believe in the richness of learning, specifically to my future parents-in-law, Erlinda Maglinao and Dr. Amado Maglinao, Sr. Their guidance and advice, whether academic or personal, is a great source of direction, motivation and inspiration to move forward.

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I would like to extend my gratitude to Dr. Maglinao for devoting his precious time to edit my papers and manuscript. Thanks also to my current and former housemates (Madz, Froi, Noah, Rose, Bjorn, Sam and JC) and to the entire Filipino community (Roy & Faye, Kuya Pete & Ate Novie, Kuya Zenon, Ate Coco, *Tita* Nora and *Tito* Rey, Tita Lou and Tito John, Kuya Amor and a lot more) that I have met here in Bryan-College Station and all over the United States, for making my stay worthwhile. I really enjoy and appreciate all your company and help. May God bless you always in your daily lives.

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NOMENCLATURE

ANOVA- Analysis of Variance

BTU-British Thermal Unit

d.b-Dry basis

DDGS-Distiller Dried Grain Solubles

DG- Digestible Group

h- Hour

HD- Highly Digestible Protein

HDGS- High Digestible Grain Sorghum HPLC- High performance liquid chromatography

LSD- Fisher's Least Significant Difference Test

min- Minute

N- Normality

MTBE- metyl tert butyl ether

NIR- Near Infrared Reflectance Spectrophotometer

ND- Normal Digestible Protein

NDGS- Normal Digestible Grain Sorghum

PDA- Potato Dextrose Agar

RIL- Recombinant Inbred Line

RFS- Renewable Fuel Standard

RVA-Rapid Visco Analyzer

rpm- Revolutions Per Minute

SKHT- Single Kernel Hardness Test

SSF- Simultaneous Saccharification and Fermentation

TCA- Trichloro Acetic Acid

w.b.-Wet basis

WT- Wild Type

μL- Microliter

μm- Micrometer

ΔH^{o}_{c} – Heat of combustion

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CHAPTER I

INTRODUCTION: IMPORTANCE OF THE RESEARCH

The world's energy consumption of 462 quadrillion British thermal units (Btu) in 2005 is estimated to increase by 50% (695 quadrillion Btu) in 2030 as indicated in the International Energy Outlook 2008 projections of the Energy Information Administration (EIA, 2008a). This significant growth of the world energy consumption is expected despite the continuously increasing price of crude oil and natural gas in the global market as a result of strong economic development and expanding populations in the world's developing countries like China and India. But even at this time, the United States of America remains the overall world's largest energy producer, consumer, and net importer/exporter of energy. The U.S. ranks first in coal reserves, sixth in natural gas reserves, eleventh in oil reserves; but it is first in imports of oil and natural gas (EIA, 2008b).

The U.S. current consumption of crude oil is approximately 20 million barrels daily (about 7 billion barrel crude oil annually), almost 60% of which (12 million barrels) is imported according to the US Energy Profile 2008. Of the country's four primary sectors, the industrial sector has always been the largest energy user, consuming about 32% (32 quadrillion BTU) of the total (101 quadrillion BTU). It is followed by the transportation sector (29 quadrillion BTU), the residential (18 quadrillion BTU) and commercial sectors (EIA, 2008c).

This thesis follows the style of the *Transactions of the ASABE*.

As of 2007, the transportation sector consumes approximately 5 billion barrels of crude oil per year, currently accounting for 70% of the total crude oil consumption in the U.S. (EIA, 2008d). Since the oil crisis of the 1970's, ethanol as an octane enhancer to replace lead additives and as a gasoline extender was already considered a means of increasing the U.S. gasoline supply (Liu and Shen, 2007; and Coble et al., 1985). Therefore, to decrease our dependence on foreign oil, domestic oil production must be increased; and more fuel-efficient mode of transportation and/or new and existing forms of alternative energy like bioethanol and biodiesel must be developed.

Starch from corn, a polymer of glucose which is present as amylose (a linear molecule with alpha-1-4 glucosidic linkage) and amylopectin (contains alpha-1-6 glycosidic branch points in addition to alpha-1-4 linkages) (Shuller and Kargi, 2002) is the main feedstock for ethanol production in the US. Ethanol production in the U.S. reached 4.9 billion gallons in 2006, which corresponds to an increase of approximately 130% from 2002 (US RFA, 2007a and 2007b). In 2007, production was almost 7 billion gallons and by the end of 2008, an additional 6 billion gallons per year capacity is expected to be operational (EERE, 2007). With the ratification of the Energy Independence and Security Act of 2007, a nearly five-fold increase of the 2007 ethanol production in the year 2022 is anticipated.

Ethanol is produced from corn by either dry grind or wet milling process. These processes share common chemical and biological features associated to saccharification of starch to glucose and fermentation of glucose to ethanol. Producing soluble sugars from starch involves physical, thermal and biochemical treatments. Starch is initially

gelatinized by heating the starch suspension to make it accessible during enzymatic hydrolysis. Starch is a crystalline granule which is water insoluble. Cooking weakens the inter- and intra-molecular hydrogen, making the granules swell and absorb water resulting to increased viscosity of the starch solution. The starch suspension is thinned (liquefied) by alpha-amylase, an endohydrolase that breaks internal alpha-D-(1,4)-glucosidic linkages. The resulting short chain, referred to as dextrins, are subsequently saccharified to glucose and the dimer maltose by glucoamylase by hydrolyzing the alpha-D(1,6)-glucosidic linkages at branch points (Nichols *et al*., 2008).

The yeast *Saccharomyces cerevisiae* ferments glucose and maltose (a glucose dimer) derived from starch. Glucose, fructose and maltose can be transported into yeast cells and maltose is hydrolyzed intracellularly to glucose. Phosporylated glucose is metabolized through glycolysis to pyruvate, which is decarboxylated to acetaldehyde. Ethanol is formed by reduction of acetaldehyde. One glucose molecule is therefore converted to two ethanol and two CO_2 molecules. With the conversion of acetaldehyde to ethanol, NADH is oxidized to NAD^+, which serves to balance the reducing equivalents produced in glycolysis (Figure 1). However, yeast cells actually gain little energy benefit from fermenting glucose to ethanol, because most of the energy from glucose (heat of combustion [ΔH^o_c] of glucose is 2807 kJ/mol) is retained in the fermentation product (ΔH^o_c of ethanol is 1369 kJ/mol and 0 for CO_2). The thermodynamic yield for fermentation of glucose to ethanol is 97%. On a mass basis, the theoretical yield is 0.51g of ethanol and 0.49 g of CO_2 per g of fermented glucose. The actual yield is about 90 to 93% of the theoretical. Some loss is due to production of yeast

cell mass and side products such as glycerol, citric acid cycle intermediates and higher alcohols. Yield can be further reduced by contaminating microorganism, predominantly lactic acid bacteria, which divert a portion of the glucose to alternate fermentation products (Ingledew, 1999).

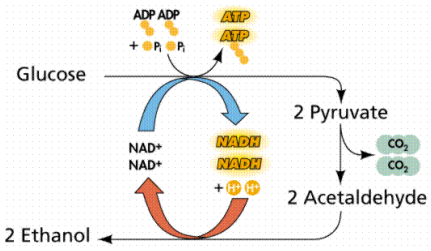

Figure 1. The glucose to ethanol fermentation pathway (Shuler and Kargi, 2002).

Industrial production of fuel ethanol commonly utilizes a dissolved content in the range of 20- 24 g of corn flour per 100 mL of mash, which is normally a grain-to-water ratio of 1:3. At the end of fermentation, the ethanol, 12 to 14% or more by volume, is separated from silage containing unfermented residuals (along with yeast cells and fermentation by products) in aqueous suspension and solution. Conventional distillation of the fermented mash (beer) yields near-azeotropic (96%) ethanol concentration. This is further dehydrated using molecular sieve to near-anhydrous (100%) ethanol and typically blended with 3 to 5% gasoline to denature for use in motor fuels and to exempt from beverage alcohol tax (Nichols et al., 2008).

Approximately 15-20% of the US corn crop was used to produce approximately 5.6 billion gallons of ethanol in 2007. According to the 2007 ethanol review prepared by

Brian Curtis Energies and Research Group (2008), the U.S. ethanol industry is characterized as a maturing corn ethanol industry with technology development well funded to accommodate cellulosic feedstock in the near future. Despite the remarkable increase in ethanol production using the current grain-starch-based technology, corn grain production for ethanol will become limited as it will compete with food and feed production. To be able to meet the enormous amount of corn or starch based material needed to produce the projected 15 billion gallon per year leveling capacity of corn ethanol, there is a need to develop alternative crops for bioenergy production for sustainable supply of sugar, starch and lignocellosic biomass.

There are several different species possible to be used as dedicated bioenergy crops. Sorghum (*Sorghum bicolor* L. Moench) is one of those species for several reasons. First, it is a drought tolerant crop because of its high water-use efficiency. Its production is usually associated with the hot and dry subtropical and tropical regions, like Sub Sahara Africa and India, where sorghum is used as a staple crop for food grain, feed grain and forage and is even used in industry as a fuel source via combustion. Second, it is a high yielding crop. When sorghum is cultivated under optimal conditions, it has a grain yield potential equal to or greater than the other cereal grains. And lastly, sorghum has a history of improvement in production of lignocelluloses, starch and sugar, which are all feedstock for ethanol production. Given the existing genetics improvement infrastructure available for the species, it is logical to expect that sorghum hybrids dedicated for bioenergy production can be developed in the near future and grown and used for ethanol production (Rooney et al., 2007).

The Sorghum Breeding Program at the Soil and Crop Sciences Department, Texas A&M University has developed and identified high digestible protein grain sorghum (HDGS) genotypes. This research intends to evaluate the potential of developed and existing high yielding HDGS genotypes as supplement to corn for a much economical starch based ethanol production. The objectives of this research are to:

1. investigate the production of ethanol using simultaneous saccharification and fermentation (SSF) of HDGS in comparison with normal digestible grain sorghum (NDGS) with low protein digestibility and corn; and
2. determine the best sorghum lines that have been developed by breeders that will require lower energy input during gelatinization and liquefaction and shorter SSF time for ethanol production.

CHAPTER II

SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF HIGH DIGESTIBLE VARIETY OF GRAIN SORGHUM FOR ETHANOL PRODUCTION

OVERVIEW

Ethanol presents a promising and fastest growing "clean" fuel substitutes for petroleum. Because of its high octane rating, it is currently used as the methyl *tert*-butyl ether (MTBE) substitute that can be mixed directly with gasoline and be used, at a certain blend, in existing internal combustion engine vehicles without any modifications. In the U.S, ethanol is mainly produced via fermentation of starch from corn grains. Because of the increasing demand for ethanol from corn and the competition it creates with food and feed production, the identification and development of additional sources of plant starch for conversion to ethanol become a priority.

The Sorghum Breeding Program at the Soil and Crop Sciences Department, Texas A&M University has developed and identified high digestible protein grain sorghum (HDGS) lines. These varieties have modified endosperm matrix that lack kafirin protein body structures surrounding the starch granules and restricting gelatinization. This study investigated the use of HDGS for dry grind ethanol fermentation. It also compared the starch digestibility and efficiency of starch conversion to glucose and ethanol of HDGS, normal digestible grain (NDGS) with low protein digestibility, and corn using simultaneous saccharification and fermentation (SSF).

INTRODUCTION

In recent years, world crude oil prices have risen dramatically because of dwindling petroleum supplies coupled with increasing demand (EIA, 2003). As of 2006, approximately 70% of the crude oil (about 840 million gallons) daily consumption in the U.S. accounted for the liquid transportation fuels such as gasoline, diesel, and jet fuel (Gray *et al*., 2006). In response, the U.S. government is constantly finding ways to reduce its dependence on non-renewable energy resources and also to minimize the environmental problems associated with fossil fuel combustion. Thus, more attention is now focused on the production of renewable and environmentally friendly fuels like bioethanol and biodiesel.

Ethanol presents one of the most promising and fastest growing "clean" fuel substitutes. It is already used as a substitute for methyl *tert*-butyl ether (MTBE) to increase the octane number of gasoline. It is also being mixed directly with gasoline in 10% (E10), 15% (E15) or even 95% (E95) ethanol blend which can be easily utilized by the current internal combustion engine vehicles (ICEVs) without any modifications (Hamelinck *et al*.,2005). According to Hill *et al*. (2006), the production and combustion of ethanol reduces 12% of the greenhouse gas emissions relative to the fossil fuels it has displaced.

Ethanol can be made synthetically from petroleum or biochemically through microbial fermentation of biomass materials (Badger, 2002). In the U.S, it is mainly produced via biochemical conversion of starch from corn grains (Gray *et al*., 2006; Mojovic *et al*., 2006). Based on the net energy balance of ethanol production, Hill *et al.*

(2006), estimated that ethanol yields 25% more energy than the energy invested in its production (including crop production, transportation, conversion, and purification). If all of the corn produced in the US were used for fermentation, about 13 billion gallons of ethanol per year could be realized (Gray *et al*., 2006). But because corn is also utilized for food and feeds, the use of a less expensive grain such as sorghum is advantageous.

Grain sorghum (*Sorghum bicolor* (L.) *Moench*) ranks third among the cereal crops in the US (Zhan *et al*., 2006). It is primarily used as a feed grain for livestock in the US, but in many semi-arid and tropical areas of the world, it serves as their staple food grain (Dicko *et al*., 2006). The feed value of grain sorghum is similar to corn in terms of its starch content (55% -75% of starch by kernel weight), but its protein and starch are less digestible (Serna-Saldivar and Rooney, 1995; Zhan *et al*., 2003). Due to poor wet-milling property and lower starch digestibility of normal sorghum, it has been underutilized for bio-based products and bio-energy production (Zhan *et al*., 2003; 2006). Several hypotheses have been suggested to explain this low digestibility and high energy requirements needed for gelatinization prior to liquefaction and saccharification. The predominant theory is that the starch being imbedded in the protein body (kafirin) matrices restricts gelatinization. During heating, the kafirins in the protein bodies form more highly networked matrices of kafirins bridged together via disulfide cysteine residues that surrounds the starch granules and restrict enzyme accessibility during liquefication and saccharification; however, this theory remains largely untested (Taylor *et al*., 1984; Chandrashekar and Kirlies, 1988).

The Sorghum Breeding Program at the Soil and Crop Sciences Department, Texas A&M University has developed and identified high digestible grain sorghum (HDGS) genotypes. These varieties with modified endosperm matrices lack kafirin protein body structures that surround the starch granules and restrict gelatinization. HDGS is hypothesized to have several added benefits in production of ethanol and distillers dried grain solubles (DDGS) for animal feed. First, the modified endosperm matrices lacking resistant protein body structures will reduce the temperature and duration at elevated temperatures needed to solubilize the grain starch for hydrolytic enzyme access and conversion to fermentable sugars. Second, the grain protein present has improved bioavailability (i.e. is more digestible) for food and feed uses, and the protein present has 60% higher lysine content, similar to high lysine corn lines (Weaver et al., 1998). Lysine, an essential amino acid, is present at very low levels in vegetable proteins and is frequently used as a nutrient supplement for herbivorous animals. This amino acid is commonly ingested as lysine or lysine-containing proteins in animal feed (Chen *et al*., 1996), therefore making HDGS more favorable feedstock for dry grind ethanol fermentation.

The development of HDGS genotype increases the potential of grain sorghum as feedstock in ethanol production as less time and energy will be required in the conversion process. In the end, using high digestible grain sorghum could result in a more positive net energy balance and more economically competitive ethanol production. It will also provide distillers an increased income and market share via the improved essential amino acid and nutrition quality of the DDGS feed product.

This study investigated the simultaneous saccharification and fermentation (SSF) of grain sorghum with improved protein digestibility (high digestible grain sorghum or HDGS) for ethanol production using commercially available α-amylase, glucoamylase and *Saccharomyces cerevisiae* yeast. Specifically, it compared HDGS starch digestibility with corn, and low protein digestibility grain sorghum (normal digestible grain sorghum or NDGS) using enzymatic hydrolysis and saccharification. It also evaluated the efficiency of starch conversion to glucose and ethanol during the simultaneous saccharification and fermentation of the NDGS, HDGS, and corn substrate.

MATERIALS AND METHODS

Substrates

Dry-milled samples of HDGS, NDGS, and corn grains obtained from the Sorghum Breeding Center, Soil and Crop Sciences Department, Texas A&M University, College Station, Texas were used. The sample grains were ground using a Jay Bee 1647 hammer mill to pass through a sieve of 1 mm opening diameter and oven dried at 105^{o}C to constant mass for moisture determination.

The grain sorghum samples used were a recombinant inbred line from the cross of BTx635 (high mold resistant grain sorghum cultivar) X P850029 (high lysine grain sorghum cultivar). The starch content of the samples was determined using a commercially available kit (Megazyme, Ireland) while in vitro protein digestibility was analyzed using the method modified by Mertz et al. (1984). The protein digestibility method involved three stages: (1) protein digestion; (2) protein extraction; and (3)

turbidity assay. The turbidity assay provided a measure of protein digestibility as absorbance is directly proportional to the protein concentration in the extraction buffer. The absorbance was measured using a spectrophotometer.

Microorganism and Culture Media

An industrial strain of *Saccharomyces cerevisiae* from the Home Brewery (Ozark, MO) was used for the fermentation. It was isolated from a commercially available Super Yeast® dry brewer's yeast which can produce and tolerate up to 20% ethanol. Stock culture was maintained in a 15 x 90 mm petri dish with Yeast Peptone Dextrose (YPD) medium containing 5 g/L yeast extract, 10 g/L peptone, 20 g/L glucose, and 20 g/L agar at pH 5.5 and stored at 4^{o}C. Pre-cultures were prepared by inoculating a loopful of yeast from an isolated colony of the stock culture into 400 ml of autoclaved Yeast Malt (YM) broth in a 500-ml Erlenmeyer flask closed with a cotton plug. The yeast cells were aerobically propagated in YM broth consisting of 3 g/L yeast extract, 2 g/L malt extract, 5 g/L peptone and 10 g/L glucose at pH 5.5 and 32 °C using a rotary shaker with a speed of 150 rpm for 48 h. An inoculum concentration of 10% v/v was used in the entire fermentation experiment.

Enzymes

The enzymes used in this study, namely SPEZYME® XTRA and G-ZYME® 480 Ethanol, were samples given by Genencor International, Incorporated (Rochester, NY). SPEZYME® XTRA enzyme, derived from a genetically modified strain of *Bacillus licheniformis,* was used to liquefy the grain samples. This thermostable starch-

hydrolyzing α-amylase can tolerate liquefaction temperatures greater than 85°C (185°F) and is very stable at liquefaction pH as low as 5.4. According to Genencor International's standard method for determination of α-amylase activity, one Alpha Amylase Units (AAU) of bacterial α-amylase was the amount of enzyme required to hydrolyze 10 mg of starch per minute under specified conditions. The typical enzyme activity of SPEZYME® XTRA was 14,000 AAU per gram and its typical density was 1.14 g/ml.

The G-ZYME® 480 Ethanol enzyme, which was an optimized blend of extracellular enzymes from selected strains of *Aspergillus niger*, *Rhizopus oryzae* and a genetically modified strain of *Bacillus licheniformis*, was used to produce glucose from the liquefied mash for ethanol fermentation. The typical density of the G-zyme® 480 Ethanol saccharifying enzyme was 1.13 g/ml to 1.15 g/ml and its minimum enzyme activity was 380 Glucoamylase Unit (GAU) per gram. One GAU is the amount of enzyme needed to release one gram of glucose per hour from soluble starch substrates under the conditions of the assay set by Genencor International. The optimal temperature range for G-ZYME® 480 Ethanol is 58 to 65 °C (137 to 149 °F), and it has excellent stability up to 65 °C.

Starch Hydrolysis and Saccharification

Hydrolysis was performed in four 2000-ml Erlenmeyer flasks heated on a the temperature-controlled hot plate (Fisher Scientific) with magnetic stirrer with agitation speed at 150 rpm. Split dosing of Spezyme® Xtra enzyme for liquefaction of dry-milled

grains of HDGS, NDGS and corn meal was used. The initial dose of enzyme (0.02% w/w of dry substrate) was added during the gelatinization stage of the starch to reduce the viscosity while cooking. Erlenmeyer flasks with 1000 ml mixture containing 220 g dry grain substrate, 3 g peptone, 1 g KH_2PO_4, and 1 g NH_4Cl at 5.6 pH were heated up to 100^{o}C for 1 h. The second dosing of Spezyme® Xtra enzyme (0.02% w/w of dry substrate) was done when the temperature reached 85°C and the pH was adjusted to 5.5 using 1 N H_2SO_4. Liquefaction was continued for 30 min at 80°C and then cooled for another 30 min until the temperature reached 65^{o}C. G-zyme® 480 Ethanol enzyme (0.1% w/w of dry solid) was added after adjusting the pH to 4.5 using 1 N H_2SO_4 at 65°C. Saccharification with G-zyme® 480 Ethanol was done for 30 min at 60°C and and the solution was then cooled for until the hydrolyzate attained 35°C.

Fermentation

When the hydrolyzates reached 35°C, they were transferred into a 2000-ml polyethylene bottle with screw cap that was sterilized using boiling water at 100^{o}C. For each type of substrate, three containers were inoculated with 48 h yeast culture (10% v/v) and the remaining container served as the control. All containers were incubated in a rotary shaker at 150 rpm and 32°C for 72 h. Samples were collected after the first 3 h of inoculation and then every 10 to 12 h thereafter. After sampling, about 1 ml was immediately plated for microbial analysis and approximately 15 ml was centrifuged at 3000 rpm for 10 min. The supernatant was placed in a 20 ml scintillation vial and stored at -4 ^{o}C until it was analyzed for sugar and ethanol content. Fermentation set up for each grain sample was done in triplicate while an additional set up that was not inoculated

with yeast served as a control for complete glucose conversion. Anaerobic condition was provided for the yeast to maximize the glucose to ethanol fermentation pathway during fermentation (Ingledew, 1999), except when the screw cap was being removed during sampling times and venting out of CO_2.

Analysis

For microbial analysis, samples were serially diluted using peptone saline diluent (1g/L peptone and 8.5g/L NaCl) and plated using Plate Count Agar (PCA) which contained 1g/L glucose, 2.5g/L yeast extract, 5g/L tryptone, and 15g/L agar. Sugar and ethanol concentrations were measured using HPLC (Consta Metric 3200 solvent delivery system from LCD Analytical) equipped with autosampler, Shodex SP0810 packed column and a Refractive Index (RI) detector. The column temperature was maintained at 78^{o}C. Each sample was analyzed for 20 min using HPLC water as the eluent at 0.8 ml/min flowrate.

Statistical software SPSS was used to analyze the data. One-way analysis of variance (ANOVA) was used to determine significant differences of the means. Least significant difference (LSD) was performed for multiple comparison of three replicates in each treatment at α=0.05.

RESULTS AND DISCUSSION

Protein Digestibility and Starch Content of Grain Sorghum

There was a rapid turbidity development observed after the addition of Trichloro Acetic Acid (TCA) extraction buffer (second stage extraction) to the washed NDGS

sample that was previously digested with pepsin solution. After 1 h of incubation, a significantly higher absorbance reading was measured for NDGS than for the HDGS sample (Table 1). It is to be noted that less protein were discarded from the NDGS during the protein digestion with pepsin (first stage extraction), leaving a higher amount of remaining protein in the digested grains and into the TCA extraction buffer (second stage extraction). Since absorbance is directly proportional to amount of protein in the TCA extraction buffer, turbidity assay showed a higher absorbance for NDGS than for HDGS sample. Calculating the % difference in absorbance showed that protein digestibility of HDGS was 34.15% higher than NDGS. The starch content of the NDGS and the HDGS samples did also differ significantly (P-value = 0.0000) and NDGS had higher starch content than the HDGS samples (Table 1).

Table 1. Absorbance reading of extraction buffer and starch content of the sorghum grain samples

Analysis	NDGS [(a)]	HDGS [(a)]	%Difference [(b)]
Absorbance reading of extraction buffer after 1 h incubation	0.41	0.27	34.15
Starch Content (%w.b.)	73.46	70.94	3.43

(a) Means of three replicates
(b) %Difference= (NDGS-HDGS) / NDGS *100

Starch Hydrolysis and Saccharification

Figure 2 shows the chromatograms of the NDGS, HDGS and corn substrates during the processes of gelatinization, liquefaction, and initial saccharification. After 1 h

of gelatinization at 100°C with 0.01% of SPEZYME®EXTRA α-amylase enzyme, there was a partial splitting of the large chains of carbon into various smaller units particularly dextrin and small amounts of glucose. The peak representing dextrin was lowest in the HDGS, followed by corn and NDGS. On the other hand, the glucose peak was observed to be highest in the HDGS, followed by corn and NDGS. Because starch grains are partially-crystalline form, gelatinization was needed to hydrolyze the starch granules and make it susceptible to enzyme action.

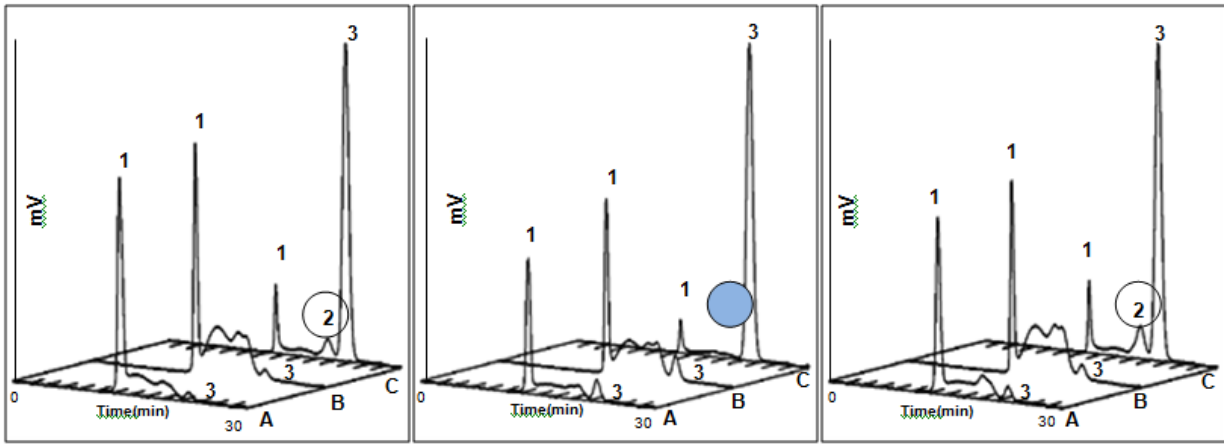

Figure 2. HPLC chromatograms of NDGS, HDGS and corn.
(A) Gelatinization at 100°C for 1 h; (B) Liquifaction at 80°C with SPEZYME®EXTRA at 60°C for 1 h and another 1 h while cooling down to 35°C with G-ZYME®480 Ethanol glucoamylase enzyme. Peaks: 1—dextrin; 2—maltose; 3—glucose.

Liquefaction of the starch was characterized by thinning of the gelatinized mixture due to further conversion of very long polymers of glucose monomer to shorter chains of glucose units such as dextrin, maltriose, and maltose. Extension of the second

enzyme dosing action for another 1 h at 80°C drastically reduced the viscosity of the gelatinized starch and increased in peak representing shorter monomer chains of glucose.

After 2 h of saccharification with G-ZYME®480, there was a drastic lowering of the dextrin peak and rise of the glucose peak in all three substrates. Also observed was the apprearance of maltose peak in both the NDGS and corn; whereas, maltose peak was absent in the HDGS chromatogram (Figure 2).

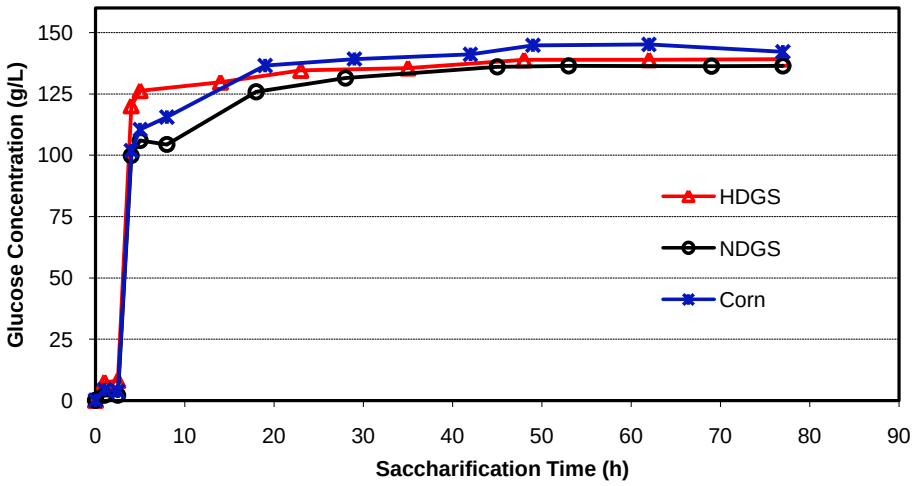

Figure 3. Glucose concentration of the control during starch hydrolysis and saccharification.
Conditions: Cooking time, 1hr; Hydrolysis time at 80°C, 1.5 hrs; Saccharification time at 60°C, 2.5 hrs; Saccharification time at 32°C, 72h.

The concentration of glucose converted in the corn, HDGS and NDGS control samples during hydrolysis and saccharification processes is shown in Figure 3. Of the three substrates used, the HDGS had the highest amount of glucose converted especially during the early stage of saccharification. This is further supported by Table 2 which shows the amount of glucose that can be converted from 22% (w/v) dry substance using

NDGS, HDGS and corn. Using the results from Table 1 for grain sorghum and the literature value of 73.7% (d.b.) starch content for commercial corn (Mojovic *et al*.,2006), with moisture content of 9.55% (w.b.), glucose yields of 126.3, 106.0, and 110.4 g/L was obtained after 1 h of saccharification of HDGS, NDGS, and corn, respectively. Correspondingly, 136.4, 138.9, and 145.1 g/L were produced 57 h after saccharification. Thus, improving the protein digestibility of grain sorghum also improves its starch digestibility. Zhan *et al.*(2006) and Hamaker (2004) confirmed that increasing the sorghum protein digestibility significantly increased its starch digestibility. The increase in protein digestibility must benefit the fermentation process.

Table 2. Glucose yield during starch hydrolysis and saccharification of the control samples of NDGS, HDGS and corn

Substrate	After 2.5 h [(b)]		After 5 h [(c)]		After 62 h [(d)]	
	g/L glucose	% Yield [(a)]	g/L glucose	% Yield [(a)]	g/L glucose	% Total Yield [(a)]
NDGS	2.1	1.4	106.0	72.8	136.4	93.6
HDGS	8.1	5.7	126.3	89.1	138.9	98.0
Corn	4.4	3.0	110.4	75.3	145.1	98.9

(a) %Glucose Yield = g/L glucose converted / g/L theoretical glucose*100
(b) 1 h of cooking at 100^{o}C and 1.5h hydrolysis at 80^{o}C with SPEZYME®EXTRA enzyme
(c) 1 h saccharification with G-ZYME®480 Ethanol enzyme at 80^{o}C and another 1.5 h while cooling until 35 ^{o}C
(d) 57 h saccharification with G-ZYME®480 Ethanol enzyme at 32^{o}C

Fermentation, Microbial Count and Ethanol Production

Figure 4 shows the microbial counts and glucose concentration in each set-up during 72 h of fermentation. Sample collection which was done every 10 to 12 h allowed a constant amount of oxygen supply into the mixture through the 1 L head space in the fermentation set-up. This small amount of oxygen supply was able to maintain the

number of viable cells throughout the experiment. An approximate ten-fold increase in microbial count was observed during the first 3 h of fermentation in all of the substrates used while loss of cell viability was observed after 40 h of fermentation.

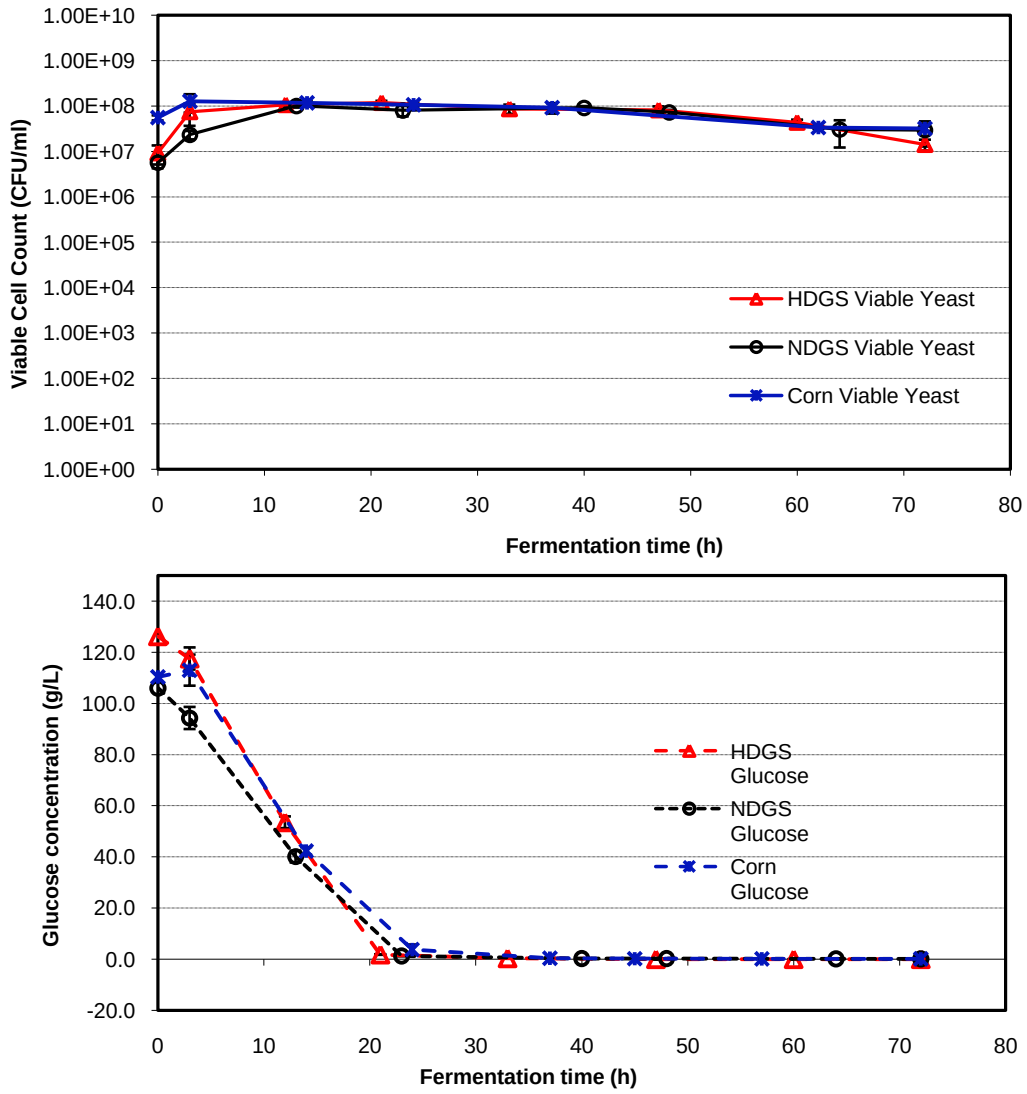

Figure 4. Change in viable yeast cells and glucose concentration during 72 hours of the grain hydrolyzates fermentation.

Hydrolysis conditions prior to inoculation: Cooking time, 1hr; Liquefaction and cooling time, 1.5 hrs; Saccharification and cooling time, 2.5 hrs.

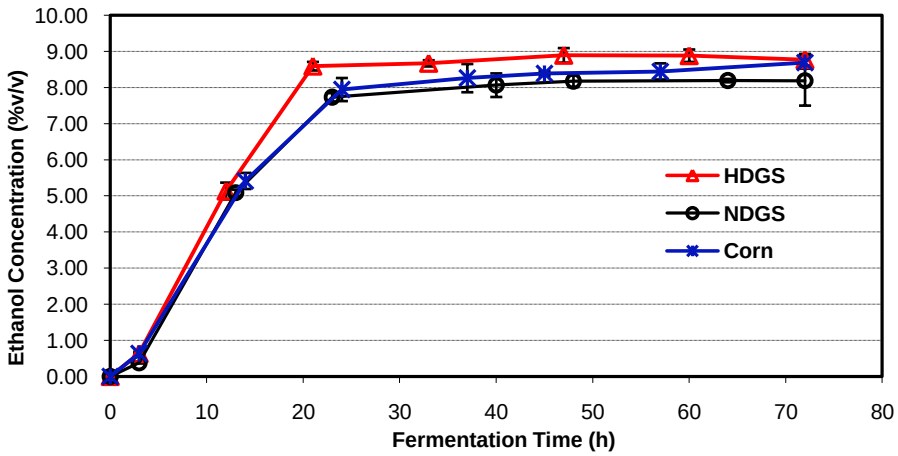

Figure 5. Ethanol concentration during 72 h of simultaneous hydrolysis and ethanol fermentation.

Ethanol production from dry milled HDGS, NDGS and corn using simultaneous saccharification and fermentation is shown in Figure 5. Among the three substrates, the HDGS gave the highest ethanol yield almost all throughout during the 72 h of fermentation. The difference was more noticeable after 20 h of fermentation when the calculated % ethanol yield was also highest in the HDGS (Table 3). After 21 to 24h of fermentation, ethanol yield of 94% was obtained from the HDGS compared to 81 and 84% for NDGS and corn, respectively. These results again support the hypothesis that the altered protein matrix in the genetically modified variety of grain sorghum improved the sorghum starch digestibility during enzymatic hydrolysis. This then contributed to the faster and higher starch conversion to glucose and ultimately to ethanol. It is to be noted that the enzymes, yeast and substrates were maintained under similar conditions in all tests during hydrolysis and fermentation so that the ethanol yield would just be dependent on the starch digestibility. Since sorghum has lower demand as food, the new variety of

highly digestible grain sorghum could very well serve as a viable substitute for corn in ethanol production.

Table 3. Ethanol yield during simultaneous saccharification and fermentation of grain substrates

Substrate	After 21to 24 h SSF			After 72 h of SSF			Overall[(c)]
	v/v %[(a)]	**g/L ethanol**	**% Ethanol Yield[(b)]**	**v/v%[(a)]**	**g/L ethanol**	**% Ethanol Yield[(b)]**	**$Y_{P/S}$ (g/g)**
HDGS	8.59	67.77	94.24	8.77	69.18	95.51	0.49
NDGS	7.73	60.98	81.31	8.19	64.58	86.72	0.44
Corn	7.94	62.67	83.60	8.69	68.55	91.45	0.47

(a) Means of three replicates
(b) % Ethanol Yield = g/L ethanol converted / g/L theoretical ethanol*100
(c) Ethanol yield based on amount of starch (g ethanol converted/ g theoretical glucose)

- No significant change in ethanol concentration was observed from 40 h to 72h of SSF using NDGS
- No significant change in ethanol concentration was observed from 21 h to 72h of SSF using HDGS
- No significant change in ethanol concentration was observed from 45 h to 72h of SSF using corn
- Efficiency of substrate for ethanol fermentation NDGS<CORN<HDGS

CONCLUSION

High digestible variety of grain sorghum (HDGS) yielded higher ethanol in shorter amount of time than the low digestible grain sorghum (NDGS) and corn by simultaneous saccharification and fermentation. The higher protein digestibility of HDGS resulted in its higher starch digestibility which brings to a faster and higher starch conversion to glucose and ethanol during hydrolysis and fermentation. The glucose yield

was highest in the HDGS particularly at the early part of saccharification. After 1 h of saccharification, a glucose yield of 126.3 g/L was obtained for the HDGS compared to 106.0 and 110.4 g/L for the NDGS and corn, respectively. The HDGS likewise had the highest ethanol production almost all throughout the 72 h of fermentation. Ethanol yield of 94% was obtained from the HDGS compared to 81 and 84% for NDGS and corn, respectively, after 21 to 24 h of fermentation. These results suggest that the altered protein matrix in the genetically modified variety of grain sorghum improved its protein digestibility. Consequently, it enhanced the sorghum starch digestibility during enzymatic hydrolysis and contributed to the faster and higher starch conversion to glucose and ultimately to ethanol.

Since sorghum has lower demand as food, the new variety of highly digestible grain sorghum could very well provide a viable substitute for corn in ethanol production. Aside from being a less expensive grain than corn, its enhanced starch digestibility may further reduce both material and processing cost. Possible process improvements include reducing enzyme dosages, shortening of liquefaction and fermentation times, and eliminating some of the unit processes through very high gravity SSF to lower energy needed during fermentation and distillation. Further cost reduction can also be achieved by optimizing the combination of substrates, enzymes and yeast during hydrolysis and fermentation. Moreover, using dry yeasts which are tolerant and viable at high ethanol concentration and enzymes with higher specific activities than the current commercial enzymes could provide more efficient starch conversion to glucose and ethanol at a shorter time.

CHAPTER III

FACTORS AFFECTING BIO-CONVERSION OF SORGHUM FLOUR IN DRY GRIND ETHANOL PROCESS

OVERVIEW

High world oil prices, supportive government policies, growing environmental and energy security concerns have provided favorable market conditions for ethanol. The need to generate a large and sustainable supply of biomass to make bioethanol will require the development of crops grown specifically for bioenergy production. Given the existing history of genetic improvement and infrastructure available for sorghum, it is expected that sorghum (*Sorghum bicolor* L. Moench) hybrids will be one of the several species dedicated as energy crop. Sorghum varieties with high protein digestibility and improved starch digestibility have already been reported although most of the previous research on grain sorghum was focused on the digestibility of sorghum protein from the nutritional point of view. The aim of the current study was to select best sorghum lines from a relatively large and diverse sorghum samples that breeders are currently working with for the development of new low energy input liquefaction, saccharification and fermentation methodologies to produce ethanol. Only few researches have been conducted on the performance of sorghum varieties in ethanol fermentation in relation to the protein and starch digestibility of sorghum.

INTRODUCTION

The worldwide production and consumption of ethanol as an alternative transportation fuel are dramatically increasing in response to growing environmental concerns and strengthening economic security. In the United States, ethanol is currently used in transportation fuel primarily as gasoline supplement to reduce automotive emissions and as oxygenate substitute for methyl-butyl ether (MTBE) because the use of MTBE in gasoline was already prohibited due to concerns regarding groundwater contamination. Since conventional cars produced from the late 1970's can run on gasoline with ethanol amounts of up to 10 percent by volume (E10), ethanol industry has grown at a phenomenal rate. Over eight million barrels of oil, accounting for two-thirds of the U.S. daily oil consumption, are required just to fuel over 225 million vehicles in America. So driven by this desire to reduce petroleum use as well as the benefits of bioethanol industry to farmers and rural economies, the U.S. annual production capacity drastically rose from about 2 billion gallons per year in 2000 to nearly 7 billion gallon per year in 2007, with an additional 6 billion gallon per year capacity expected to come in line by the end of 2008 (EERE, 2007).

Brazil and the United States are the world's largest ethanol consumers and producers mainly from sugarcane and corn starch, respectively. Ethanol is produced in the U.S from bioconversion of corn (70% starch, along with 9% protein, 4% lipids and 9% fiber on a dry weight basis) via two methods, dry grind and wet mill (Figure 6). In wet milling, kernels are separated into components and only the starch portion is fermented, whereas, in dry-grind process, whole kernels are ground to flour and fed

directly into the fermentation process. Though wet milling produces more co-products such as oil, gluten meal and gluten feed, it requires higher capital and operating cost than dry grind process (Nichols et al., 2008). Thus, most of the expansion of the ethanol industry is in new and large dry grind ethanol plants. In 2006, 82% of the US ethanol-producing capacity was in dry-grind facilities and 18% was in wet milling plants (RFA, 2008). Dry grind facilities produce 2.7 to 2.8 gallons (10.4 L) per bushel (25.5 kg) of corn, as well as a co-product animal feed called distillers dried grains with solubles (DDGS). DDGS is recovered at the end of the process and is mainly composed of yeast and non-fermentable parts of the corn like germ, fiber, and protein (Nichols et al., 2008).

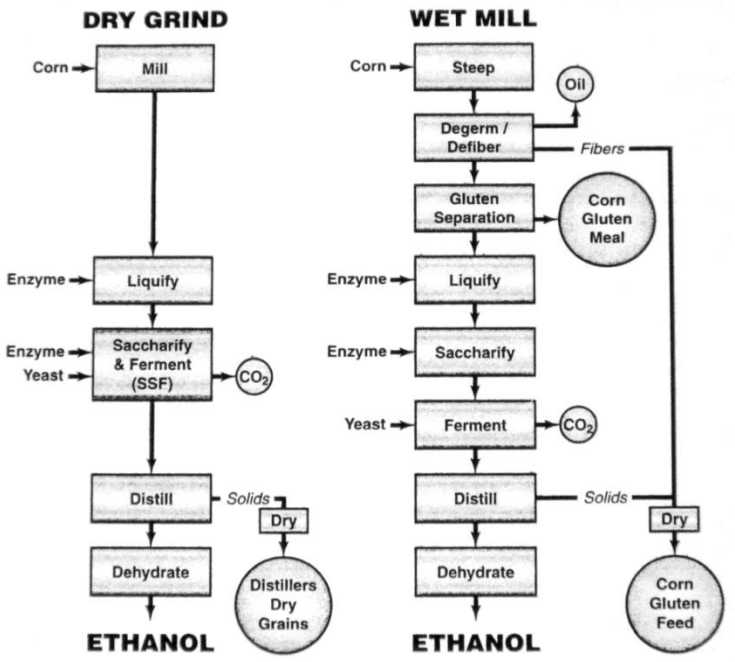

Figure 6. Comparison of the dry grind and wet mill processes for production of ethanol.

Courtesy of Corn to Ethanol Research Center. Reproduced from Nichols et al., 2006.

In a conventional dry-grind process, basic processes involve grinding, cooking, liquefaction, saccharification, fermentation, distillation and co-product recovery. Corn is ground and mixed with water to produce slurry. Slurry is cooked to breakdown the crystalline structure of starch granules and the resulting mash is further liquefied and saccharified by amylase enzymes to reduce viscosity and to produce sugars, respectively. And the fermentable sugars produced are then converted to ethanol by yeast during fermentation (Naidu et al., 2007 and Singh et al., 2006).

The combination of low corn prices, high energy prices, and strong ethanol demand encouraged by various government measures, stimulated the rapid expansion of corn-based ethanol production. This profitable growth in ethanol production substantially increased the demand for corn and is deemed to be the main factor that drove the price of corn up by approximately 90% from August 2006 to February 2007. The current price of ethanol is around $2.50 a gallon while the corn price has already reached a record of US $6 a bushel. Such increase is good news for corn growers, but a burden for ethanol producers. If the selling price of ethanol stays the same, and the cost of corn used in production increases, it is estimated that profit from ethanol processing would decrease from US$1.06 per gallon to a net average of US$0.03 per gallon.

Since corn is also used as food and feed source, there is a need to develop a dedicated bio-energy crop to generate a large, sustainable and low-cost feedstocks supply that will fit the existing fermentation infrastructure. Currently, interest in the utilization of sorghum in bio-industrial applications is growing in the U.S. Researchers

and ethanol producers have shown that grain sorghum is a reasonable feedstock for ethanol since it is cheaper, more drought-tolerant than corn and it can fit the existing fermentation infrastructure. On a well manage planting dates, grain sorghum offers exceptional rotation crop for corn and cotton because it provides ample residue for conservation-tillage system and potential yield for South Texas farmers (Smith, 2008). In conservation tillage system, previous crop's residues are purposely left on the soil surface to conserve soil and for more efficient water use to get higher yields for crops grown without irrigation in drought-prone soils like in semiarid regions (Sullivan, 2003).

Approximately 82 percent of the sorghum seed structure is the grain endosperm (Hoseney, 1994) which is comprised of cells containing protein bodies trapped in a protein matrix that surrounds the larger starch granules (Kulp and Ponte, 2000). Although grain sorghum has a chemical composition similar to corn, it has been underutilized for bio-based products and bio-energy production due to its poor wet-milling properties (Zhan et al., 2003) and low ethanol yield relative to corn. Compared with other cereals such as wheat and corn, sorghum is well known for its poor digestibility and low nutritional value, especially after wet-cooked. Although the cause of poor digestibility of sorghum proteins and starches is not yet fully understood, several plausible explanations have been proposed. Duodu et al. (2003) based their explanations on the interaction of the prolamins with polyphenolic tannins and starch and the protein cross-linking in response to pH or temperature changes in the surrounding environment. With the several proposed factors, the starch being imbedded in the protein body

(kafirin) matrices, which restrict gelatinization, is the predominant theory why normal grain sorghum has low protein and starch digestibility.

Sorghum breeders are working with thousands of sorghum genotypes with diverse genetic backgrounds, chemical, and physical properties. Most of the previous research on grain sorghum was focused on the digestibility of sorghum protein from the nutritional point of view (Beta et al., 2000, Duodu et al., 2003, Oria et al., 2000, Zhang and Hamaker 1998). Not much research has been conducted on the performance of sorghum varieties in ethanol fermentation in relation to the protein and starch digestibility of sorghum. By understanding and analyzing the relationship among the key factors impacting on the bio-processing of sorghum, the best sorghum genotypes with high conversion efficiency for ethanol production could be identified. This will assist the breeders in the development of new and improved sorghum hybrids for ethanol production and will increase the production and utilization of sorghum to meet the enormous feedstock demand of the future ethanol industry.

Recently, the Sorghum Breeding Program at the Soil and Crop Science Department, Texas A&M University has developed and identified high digestible grain sorghum (HDGS) genotypes with modified endosperm matrices that lack the kafirin protein body highways. HDGS genotypes are hypothesized to have several added benefits (low energy input during gelatinization prior and higher lysine protein content than corn), making these cultivars a suitable alternative for corn in the typical bio-ethanol-feed supplement system. The aim of the current study was to select the best sorghum lines from a relatively large and diverse sorghum samples that breeders are

currently working on for the development of new low energy input liquefaction, saccharification and fermentation methodologies to produce ethanol. The objectives of this research are: (1) to investigate the effect of grain sorghum protein digestibility on the temperature and time of starch gelatinization as well as on the rate and efficiency of enzymatic saccharification; (2) to evaluate the performance of the improved sorghum hybrids in ethanol fermentation via simultaneous saccharification and fermentation using dry yeast (Ethanol Red) from Fermentis and high concentration of dry solid (30%); and (3) to identify the key factors (chemical and physical properties, flour particle size, starch pasting property, yeast viability, enzyme and substrate concentration) affecting ethanol yield.

MATERIALS AND METHODS

Grain Sorghum Lines Used as Substrates

Eighteen recombinant inbred lines (RILs) of grain sorghum (parent lines and offsprings) were used in this study. These were grown and harvested from Welasco, Texas in 2006. Two parent lines of highly digestible protein (HD) grain sorghum (P850029 and P851171) with high lysine content were crossed with three parent lines of wild type (WT) grain sorghum (B.Tx635, R.Tx436 and 96GCPOB124) with high grain mold disease resistance to develop three families with four distinct RILs. The lines were phenotyped as highly digestible (HD), medium digestible (MD) and of normal digestible (ND) protein using the protease turbidity assay (Portillo, 2007). The resulting phenotype group defined as one or more RILs with absorbances (after 60 min of dilution in 72%

Trichloro Acetic Acid) not significantly different according to Tukey's HSD at a 0.05 level of significance is shown in Figure 7. Approximately 2 kg seeds of each RILs were collected, cleaned and ground (Cyclone Sample Mill, UDY Corp.) into flour with a particle size of less than 1 mm. They were used as the substrates for saccharification and ethanol fermentation.

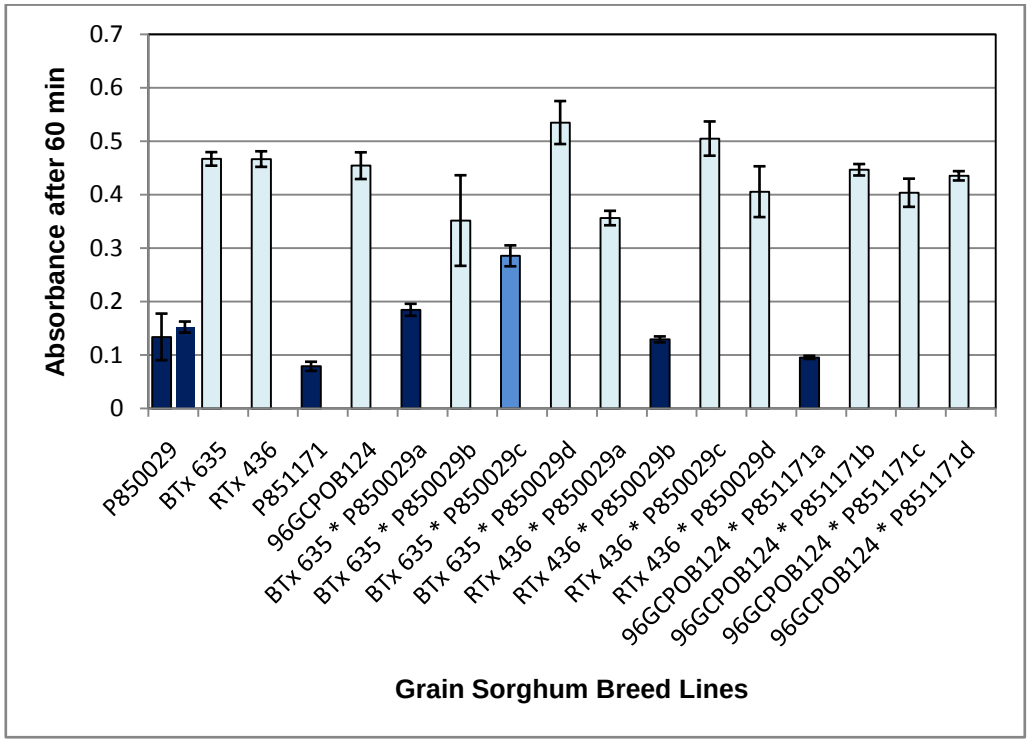

Figure 7. Digestible group based on 60 min turbidity assay. (Protein digestibility: HD; MD; and ND)

Physical Properties and Chemical Composition of the Sorghum Grains

The physical properties (endosperm texture index, seed hardness index, and kernel weight) and chemical compositions (starch, protein and moisture) of the sorghum

grains were determined by the Sorghum Breeding Laboratory at Soil and Crop Science Department, Texas A&M University (Portillo, 2007). Endosperm texture index was categorized as described by Rooney & Miller (1982) from 1 (flinty endosperm) to 5 (chalky endosperm) via visual examination of longitudinal half kernels while seed hardness index was characterized using the single kernel hardness test (SKHT) (Perten Single Kernel Characterization System SKCS 4100, Perten Instruments, Springfield IL). The average starch, protein and moisture content of the grains were determined using near-infrared reflectance (NIR) spectrophotometry (Perten PDA 7000 Dual Array with Grams Software, Perten Instruments, Springfield IL). Ash analysis was conducted according to ASTM standard E 1755-01, Standard Test Method for Ash in Biomass (ASTM, 2001a). The moisture content of flour was determined in accordance with Approved AACC Methods 44-15A (AACC, 2000).

Sorghum Flour Pasting Profile

The pasting properties of sorghum flours were evaluated using the Rapid-Visco-Analyzer (RVA, Newport Scientific PTY, Ltd., Warriewood, Australia) through the classic heat-hold-cool process. About 28 g mixture of sorghum flour (4.2 g flour d.b.) and water was prepared and equilibrated at 50 °C temperature. The suspension was then heated to and held at 95°C and then cooled again to 50°C. This caused the starch granule swelling, disruption and polymer re-association. The total test time was 13 min with viscosity and temperature readings taken every 2 sec. The peak viscosity [maximum paste viscosity achieved in the heating stage], the trough viscosity [minimum paste viscosity achieved after holding at the maximum temperature], the final viscosity

[viscosity at the end of run], the pasting temperature [starch granules begin to swell and gelatinize due to water uptake] and the peak time [when peak viscosity was recorded] were all measured and recorded. The breakdown and setback were calculated from the difference between the peak and trough viscosity and the final and trough viscosity, respectively. All runs were conducted in duplicate and average values were reported. The flour pasting profiles were described using the different values measured.

Particle Size Distribution

The particle size distribution (PSD) of the sorghum flour samples was analyzed in triplicate using the Malvern Instruments Mastersizer 2000 (Malvern Instruments Ltd., Mastersizer 2000, Worcestershire, UK). The instrument measures particle size based on light scattering (Mie) and is able to analyze both wet and dry samples in the range of 0.02 to 2000μm. Red light is used to produce forward, side, and back scattering while a blue light is used to produce wide angle forward and back scattering of light once it hits the particles. Thousands of light scatter patterns collected by the instrument were used to develop the relationship between percent volume and the equivalent spherical diameter (ESD). The ESD was then converted to AED (Aerodynamic equivalent diameter) using equation 1,

$$\text{AED} = \text{ESD}\sqrt{\frac{\rho_p}{\chi}} \qquad (1)$$

where

AED = aerodynamic equivalent diameter,
ESD = equivalent spherical diameter,
ρ_p = particle density (g/cm^3), and
χ = shape factor

The particle density of the samples was measured using the AccuPyc 1330 (Micromeritics, AccuPyc 1330 Pycnometer, Norcross, GA) pycnometer by the gas displacement method. The pycnometer releases helium of known volume into a container of fixed volume. The difference between the two volumes represents the volume of the particles inside the container. The mass was measured before inserting the sample into the pycnometer. The density was then calculated using equation 2,

$$\rho = \frac{m}{V} \tag{2}$$

where

ρ = particle density of sample (g/cm^3),
m = mass of sample (g), and
V = volume of material sample less open void space (cm^3).

Particle size distribution is a log-normal distribution that uses mass median diameter (MMD) as the geometric mean (calculated AED at $d_{50\%}$) and geometric standard deviation (GSD) in describing how spread out are the particle size. MMD is the particle size in which 50% of the overall particles by weight are smaller than the MMD and 50% are larger than half of the mass (Faulkner, 2004). The GSD is calculated using equation 3,

$$\text{GSD} = \sigma_g = \frac{d_{84.1\%}}{d_{50\%}} = \frac{d_{50\%}}{d_{15.9\%}} = \sqrt{\frac{d_{84.1\%}}{d_{15.9\%}}} \tag{3}$$

where $d_{n\%}$ is the particle size in which n percent of the mass is contributed by particles less than d.

Saccharification of Sorghum Starch Using Enzyme

The enzymatic conversion of starch to fermentable glucose units was based on the modified NREL LAP-016 (National Renewable Energy Laboratory Analytical Procedure, 2005). In this study, the enzymes used were alpha-amylase (SPEZYME® XTRA) and gluco-amylase (G-ZYME® 480 Ethanol) provided by Genencor International, Incorporated. One Alpha Amylase Unit (AAU) of bacterial α-amylase represents the amount of enzyme required to hydrolyze 10 mg starch per minute while one Gluco-amylase Unit (GAU) is the amount of enzyme needed to release one gram of glucose per hour from soluble starch substrates.

Two sets of incubation time and enzyme concentration were used to determine the conversion rate and conversion efficiency of starch to glucose. For conversion rate determination, enzyme solution of 0.2 mL of α-amylase and 0.2 ml glucoamylase each diluted to 50 ml using sodium acetate buffer were used while 2 ml of α-amylase and 2 ml glucoamylase diluted to 25 ml were prepared for conversion efficiency analysis. The samples were prepared in duplicate for conversion rate determination and triplicate for the conversion efficiency analysis.

Using a tared 15 ml plastic centrifuge with a tightly fitting screw cap, 100 mg of ground samples from each RIL was mixed with 0.2 mL 190 proof ethanol and 2 ml dimethyl sulfoxide (DMSO). The sample mixture was vortexed vigorously to aid starch dispersion and then incubated in a briskly boiling water bath for 5 min to gelatinize the starch. It is then acidified with 2.9 ml sodium acetate buffer (pH 4.5 containing 11.8 ml glacial acetic acid per liter solution) and 0.1 ml thermostable alpha-amylase solution

(6.38 AAU and 127.68 AAU) was added. To liquefy the starch, the mixture was then incubated at 80 ^{o}C using VWR digital water shaking at 300 strokes per min (for 6 min and 10 min). Another 4 ml sodium acetate buffer and 0.1 ml gluco-amylase (0.17 GAU and 3.46 GAU) were added and then incubated at 50 ^{o}C (for 30 min and 60 min) to saccharify the hydrolyzed starch. The samples that were tightly capped throughout the analysis had a final volume of 9.3 ml.

Sugar Analysis

Using 2 ml Eppendorf tubes, about 1.5 ml of the saccharified aliquot was heated for 2 min in a briskly boiling water to deactivate the enzyme and then centrifuged at 3000 rpm for 10 min. The samples were filtered through 0.22 μm membrane filter prior to HPLC analysis. The Waters Alliance® HPLC system with 2690 Separation Modules (integrates five 24-vial carrousel, solvent delivery system, onboard controller, compartment for column and column heater) and Waters 2410 RI detector were used for the analysis of glucose conversion. The Shodex SP0810 column (8.0 mm id x 300 mm) equipped with SP-G guard column (6.0 mm id x 50 mm) were used at column temperature of 60 ^{o}C using filtered and degassed deionized water as the eluent at 0.7 ml/min. Each sample was analyzed for 30 min and standards were run at the start, middle and end of sample analysis. For quality assurance, 20% of the samples were analyzed in duplicate and a blank was run every 10 sample injections.

Fermentation

Thirty five (35) g sorghum flour samples (equivalent to about 30 g dry mass) were mixed with 60 mL of sterilized and deionized water in sterilized 250 ml Erlenmeyer flask with cotton plug. The starch was gelatinized for 1 h in a boiling water bath while shaking at 150 strokes per minute using the VWR digital shaking water bath. First dosing of SPEZYME® XTRA (0.02% w/w of dry substrate) was added before the gelatinization stage of the starch to reduce the viscosity while cooking. The enzyme solutions for liquefaction and saccharification were prepared separately by diluting 1 ml of SPEZYME® XTRA and 2 mL G-ZYME ® 480 Ethanol to 100 ml using sodium acetate buffer. After cooking, the mixture was adjusted to pH 5.5 using 1 N NaOH and 1 N H_2SO_4 solution. Another 5 ml of sterilized and deionized water was added to reduce the temperature of the mixture to about 80°C before the second dosing of SPEZYME® XTRA (0.02% w/w of dry substrate). Liquefaction at 80°C was continued in the shaking water bath for 30 min. Before adding G-ZYME ® 480 Ethanol enzyme (0.1% w/w of dry solid), 5 ml of sterilized and deionized water was again added to reduce the temperature of the mixture to about 65^{o}C. Saccharification with G-ZYME ® 480 Ethanol was done for 30 min at 60°C, after which, flasks were removed from the water bath and cooled for another 30 min until the hydrolyzate attained 35°C.

After 30 min saccharification and cooling to room temperature, the fermentation medium was inoculated with 2.0 mL of activated dry yeast (Ethanol Red) from Fermentis. To activate the Ethanol Red, 5.00 g of dry yeast in 25 mL of sterilized peptone saline water (8.5 g of NaCl and 1 g of peptone per liter) was incubated in New

Brunswick shaker incubator (New Brunswick Scientific Inc., Edison, NJ) at 150 rpm and 32 °C for 25 min. The activated yeast suspension had a live cell concentration of about 1.0×10^9 cells/mL while the inoculated mash had a yeast cell concentration of approximately 1.0×10^7 cells/mL. Fermentation set up for each grain sample was done in duplicate and were incubated in the rotary shaker at 150 rpm and 32°C for 72 h.

Ethanol and Yeast Viability Analysis

Using a 3 ml sterile syringe, exactly 1 ml of the sample from each fermentation flasks was taken after 24, 48 and 72 h of yeast inoculation. The collected sample was diluted with 9 ml sterilized deionized water in a sterilized 15 ml test tube with cap and used for microbial and ethanol analysis.

For microbial analysis, 1 ml of the diluted sample was serially dilution in sterilized test tube with cap and peptone saline water. Using Plate Count Agar (PCA) containing 1 g/L glucose, 2.5 g/L yeast extract, 5 g/L tryptone, and 15 g/L agar, 100 µL aliquot from the 10^{-5} dilution was immediately plated in duplicate for yeast viability analysis. Spread plates were inverted and incubated at 35^oC for 48 h. Plates having a colony forming units (cfu) between 30 and 300 were used for the analysis.

The remaining 9 ml of the diluted sample was transferred into a15 ml plastic centrifuge with a tightly fitting screw cap. After centrifugation at 3000 rpm for 10 min, the supernatant was filtered through 0.22 µm membrane filter prior to HPLC analysis for ethanol. The HPLC condition, column and detector were the same as during sugar analysis. The fermentation efficiency was calculated from the theoretical yield of 56.72 g of ethanol produced from 100 g of dry starch assuming 1 g of starch could be

hydrolyzed into 1.11 g glucose, and each gram of glucose could produce 0.511 g of ethanol.

Statistical Analysis

Eighteen recombinant inbred lines (RILs) of grain sorghum were grouped as HD and ND for both parent lines and offsprings. Using SPSS 16, analysis of variance (ANOVA) for individual variables was performed for each digestible group. Using the least squares difference (LSD), the significance of variation among means of each digestible group was determined. Pearson correlation was also used to relate pasting parameters, particle size and physicochemical properties of the grain with glucose and ethanol yield. The level of significance was $P < 0.05$ for statistical methods, except as noted.

RESULTS AND DISCUSSION

Physical Properties and Chemical Composition of the Sorghum Grains

The average values of the physical properties and chemical composition of each digestible group (parent and offspring sorghum grain samples) are shown in Table 4. The analysis of variance (ANOVA) (Table 5) showed that there are significant differences in starch content, kernel hardness and kernel texture among the digestible groups. Least significant difference (LSD) test (Table 4) reveals that both parent and offspring from the same digestible group were not significantly different in both chemical and physical properties and only the starch content, kernel hardness and endosperm texture of the HD group were significantly different from those of the ND

group. The HD sorghum lines had significantly lower starch content and kernel hardness index than ND sorghum lines. In addition, the HD group exhibited chalky endosperm texture compared to the flinty texture of the ND group. Table 5 further indicates that there were no significant differences in the protein, moisture and ash content, kernel size and flour density between the digestible groups. However, the HD lines had more flour-like endosperm texture, softer kernels and lower starch content than the ND lines.

Table 4. Physical properties and chemical composition of the sorghum grain samples

Properties	Digestible Group			
	Parent		Offspring	
Chemical	**HD**	**ND**	**HD**	**ND**
Starch (%db)	80.21[a]	83.33[b]	80.10[a]	83.82[b]
Protein (%db)	15.09[a]	15.86[a]	15.08[a]	15.40[a]
Moisture (%wb flour)	11.49[a]	11.69[a]	11.63[a]	11.55[a]
Ash (%db)	2.16[a]	1.73[a]	1.74[a]	1.94[a]
Physical				
Kernel size (mm)	2.06[a]	2.13[a]	1.99[a]	2.28[a]
Kernel hardness (index)	34.86[a]	81.50[b]	33.16[a]	78.59[b]
Endosperm Texture	5[a] (Chalky)	2[b] (Flinty)	5[a] (Chalky)	2[b] (Flinty)
Flour Density (g/cm^3)	1.4319[a]	1.4278[a]	1.4315[a]	1.4274[a]

Note: Means with the same letter in the same row are not significantly different using LSD (α=0.05)

Table 5. Mean squares from ANOVA of physical and chemical properties among digestible group

	Sum of Squares	df	Mean Square	F	Sig.
Starch (%db)	151.3797	3	50.45989	15.88834	0.00000
Protein (%db)	3.683034	3	1.227678	0.887038	0.45429
Ash (%db)	1.117074	3	0.372358	1.583905	0.20492
Endosperm Texture	68.11111	3	22.7037	408.6667	0.00000
Kernel Hardness (index)	24701.61	3	8233.869	102.4416	0.00000
Flour Moisture (%wb)	0.218217	3	0.072739	0.757655	0.52318
Kernel Size(mm)	0.244467	3	0.081489	2.811809	0.07779

Sorghum Flour Pasting Profile

The average peak viscosity, breakdown, setback, peak time and pasting temperature of the sorghum groups measured during the 13-min heat-hold-cool process are shown in Table 6 while representative pasting curves of several HD and ND RILs (parents and offspring) from the 18 sorghum cultivars generated from the viscosity data are shown in Figure 8. The temperature profile during pasting is indicated by the solid straight line at the top of the RVA curves. While the RVA curves appeared to follow the same pattern, there were still observed differences between HD and ND groups. However, LSD test showed that pasting properties of both parent and offspring from the same digestible group were not significantly different.

The ANOVA showed significant differences at 95% confidence level between the HD and ND groups in peak time, pasting temperature, peak viscosity, trough, breakdown and setback but not in the final viscosity (Table 7). The initial swelling temperature of HD sorghum starches was significantly lower (77^{o}C) than that of ND cultivars (83^{o}C) and

HD lines also took less time (4.6min) to reach peak viscosity than ND lines (5.8min) (Table 6). There was no significant difference in the final viscosity among the digestible group, despite the significant difference in starch content. The HD lines had lower pasting temperature and faster rate of gelatinization compared to the ND lines. Thus, HDGS may require lower energy input during gelatinization and hydrolysis of starch granules and increase the bio-availability of starch during enzymatic hydrolysis prior to ethanol fermentation. This provides an advantage of the HD lines if used as a feedstock for ethanol production.

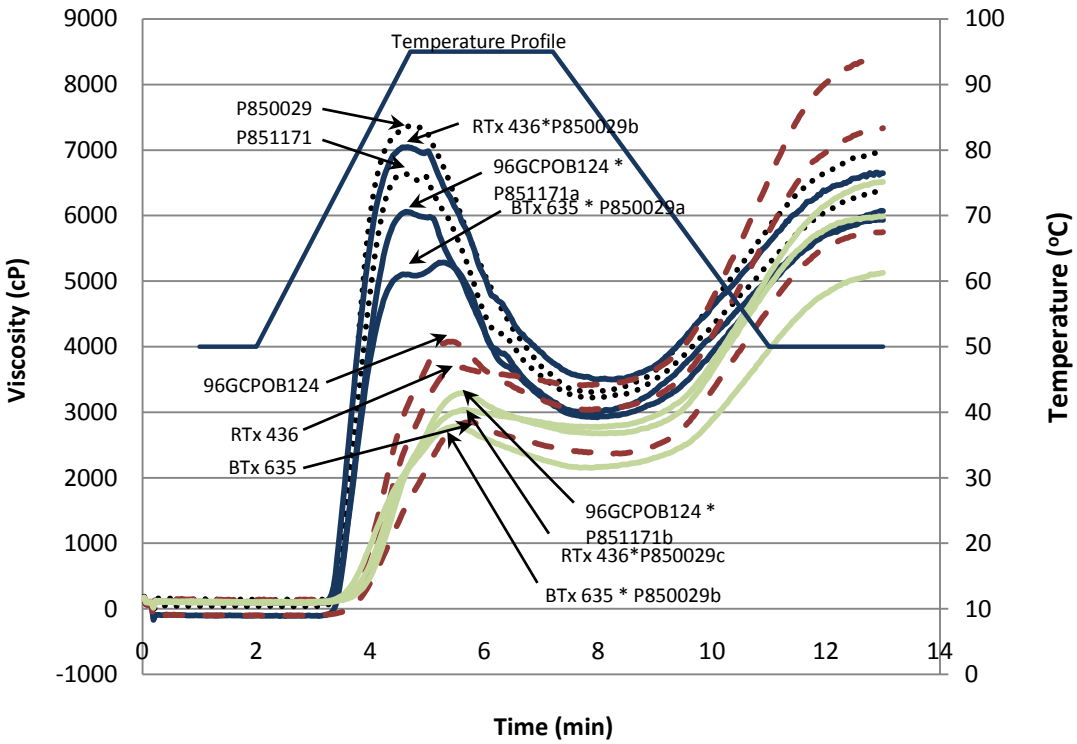

Figure 8. RVA curves of HD and ND sorghum samples selected from 18 cultivars using the 13-min temperature profile (•••••HD parent; ——HD offspring; – – ND parent; and —— ND offspring)

The HD RILs started to gelatinize and liquefy at lower temperatures and in shorter time than the normal endosperm RILs. The differences in swelling temperatures and gelatinization rate is possibly a result of genetic variation in varieties' kernel structures like more floury endosperm and increased protein digestibility attributed to the folded conformation of the endosperm protein bodies. The above results suggest that as protein digestibility increases, the pasting properties of flour also improve. According to Beta et al. (2000), grain floury endosperm texture was significantly and negatively correlated with starch amylose content. Turbidity level, which is inversely related to protein digestibility, was also significantly correlated with pasting temperature (.807**), peak viscosity (-.850**) and peak time (.858**) (see Table 10 on p. 46). This observation is thought to provide the easy access of the enzymes to the starch bodies which is advantageous during gelatinization and hydrolysis prior to ethanol fermentation.

Table 6. RVA parameters for ND and HD RILs using the 13-min temperature profile among digestible groups

RVA Properties	Digestible Group			
	Parent		Offspring	
	HD	ND	HD	ND
Peak Viscosity(cP)	6920^{a}	3540^{b}	6134^{c}	3194^{b}
Trough (cP)	3232^{a}	2935^{a,b}	3136^{a}	2656^{b}
Breakdown (cP)	3688^{a}	605^{b}	2998^{c}	537^{b}
Setback(cP)	3214^{a}	4222^{b}	3080^{a}	3528^{a,b}
Final Viscosity (cP)	6446^{a}	7157^{a}	6217^{a}	6185^{a}
Peak Time (min)	4.64^{a}	5.58^{b}	4.86^{c}	5.62^{b}
Pasting Temperature (^{o}C)	77.19^{a}	82.84^{b}	77.26^{a}	83.06^{b}

Note: Means with the same letter in the same row are not significantly different using LSD (α=0.05)

Table 7. Mean squares from ANOVA of starch pasting properties among digestible groups

	Sum of Squares	df	Mean Square	F	Sig.
Peak_viscosity	8.67E+07	3	2.89E+07	107.298	0.0000
Pasting_temp	12.382	3	4.127	15.928	0.0000
Peak_time	6.062	3	2.021	74.464	0.0000
Final_viscosity	4486622.668	3	1495541	2.79	0.0560
Trough	2054084.632	3	684694.9	7.445	0.0010
Breakdown	6.37E+07	3	2.12E+07	163.079	0.0000
Setback	4678934.658	3	1559645	6.773	0.0010

Saccharification

The conversion rate and conversion efficiency of sorghum starch to glucose were measured to evaluate its digestibility. These were done using two sets of gelatinization, liquefaction and saccharification time (5, 6, 30 min and 5, 10, 60 min) and SPEZYME® XTRA and G-ZYME® 480 Ethanol enzyme concentration (60 AAU with 1.7 GAU and 1270 AAU with 34.6 GAU per g of flour). The initial rate of conversion illustrates how fast the starch was being converted to glucose using a reduced enzyme concentration and reaction time while efficiency of conversion measures the overall accessibility and availability of starch when complete enzymatic hydrolysis and saccharification have been achieved.

Table 8 shows the rate and efficiency of enzymatic conversion to glucose of the starch from the different digestible groups of sorghum. The conversion rate of the HD lines was about 18% faster and around 15% more efficient than the ND lines under the conditions of the experiment. This implies that the HD lines has higher starch accessibility and digestibility during enzymatic conversion compared to ND lines.

Table 8. Rate and efficiency of starch enzymatic conversion to glucose among digestible groups

% Yield	Digestible Group			
	Parent		Offspring	
Glucose	HD	ND	HD	ND
Conversion (after 30min)	39.42[a]	32.38[b]	38.91[a]	33.36[b]
Conversion efficiency (after 1h)	84.65[a]	74.29[b]	82.11[a]	71.08[b]

Note: Means with the same letter in the same row are not significantly different using LSD (α=0.05)

Table 9. Mean squares from ANOVA of rate and efficiency of starch enzymatic conversion to glucose among digestible groups

	Sum of Squares	Df	Mean Square	F	Sig.
Glucose Conversion Rate	297.836	3	99.27868	11.25151	0.00003
Glucose Conversion Efficiency	1684.829	3	561.6098	7.401588	0.00034

The ANOVA showed that there was a significant difference in rate (P=0.000) and efficiency (P=0.003) of starch conversion to glucose among digestible groups (Table 9). However, the rate and efficiency of both the parent and offspring from the same digestible group did not significantly differ as shown by the LSD test in Table 8.

The increased protein digestibility of the HDGS most likely improved its starch digestibility and increased its rate of conversion and total glucose yield during saccharification. This is supported by the highly significant correlation between turbidity and rate of glucose conversion (-.708[**]) and between turbidity and efficiency of enzymatic conversion (-.405[**]) (see Table 10).

Table 10. Pearson correlation coefficients (r values) of sorghum starch properties, protein and starch digestibility and ethanol yield

	Peak Viscosity	Pasting Temperature	Peak time	Final viscosity	Starch (db)	Protein (db)	Turbidity	Glucose Conversion Efficiency	Glucose Conversion Rate	Ethanol %Yield (24h)	Ethanol %Yield (48)	Ethanol %Yield (72)
Peak Viscosity	1											
Pasting Temperature	-.870**	1										
Peak time	-.951**	.899**	1									
Final viscosity	0.163	0.16	-0.017	1								
Starch (db)	-.632**	.742**	.616**	.357*	1							
Protein (db)	-0.252	0.043	0.267	-.487**	-.502**	1						
Turbidity	-.850**	.807**	.858**	0.061	.589**	0.267	1					
Glucose Conversion Efficiency	.625**	-.562**	-.631**	0.209	-.356**	-0.239	-.405**	1				
Glucose Conversion Rate	.745**	-.634**	-.787**	0.091	-.366*	-.455**	-.708**	.669**	1			
Ethanol %Yield (24h)	.593**	-.688**	-.592**	-0.093	-.576**	0.034	-.617**	0.217	.402*	1		
Ethanol %Yield (48)	.484**	-.538**	-.412*	-0.159	-.546**	0.129	-.550**	0.016	.357*	.835**	1	
Ethanol %Yield (72)	.402*	-.376*	-.339*	-0.311	-.584**	.338*	-.426**	0.108	0.317	.453**	.749**	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Ethanol Fermentation

Using 100 ml fermentation volume with 30% dry sorghum flour, the ethanol concentration (%v/v) after 24, 48 and 72 h simultaneous saccharification and fermentation (SSF) of 18 sorghum cultivars are shown in Figure 9. Ethanol yields were in the range of 5.1 – 9.6 % , 9.2 – 13.1% and 11.2-13.2% (v/v) after 24, 48 and 72 h SSF, respectively. Assuming 1 g of starch could be hydrolyzed into 1.11 g glucose and each gram of glucose could produce 0.511 g of ethanol, the corresponding fermentation efficiency of each variety was calculated. The % ethanol yield is normally used to indicate the efficiency of ethanol production (Zhan et al., 2006).

Analysis of variance (ANOVA) at 95% confidence level confirms that there is a significant difference in fermentation efficiency between digestible groups after 24 and 48 h of fermentation (P= 0.000 and P=0.0033, respectively) but not after 72 h of SSF (P=0.1067) (Table 11). Least significant difference (LSD) test in Table 12 shows that % ethanol yield for both parent and offspring from the same digestible group are not significantly different throughout the 72 h of fermentation.

When HD lines were used, independent t-test at α=0.05 shows that there is a significant difference in % ethanol yield between 24 and 48 h (50 and 65% ethanol yield, respectively) and no significant difference between 48 and 72 h (65 and 65 % ethanol yield, respectively). However, when ND lines were utilized, significant increase in % ethanol yield can be observed until 72 h of SSF (38, 58 and 62 % ethanol yield at 24, 48 and 72 h SSF, respectively) (see Appendix). Results only show that HD sorghum lines have faster rate of conversion and shorter reaction time needed to achieve completion

than ND sorghum lines. Thus, HD lines have significantly higher % ethanol yield than the ND lines if fermentation time will be shortened to 48 h.

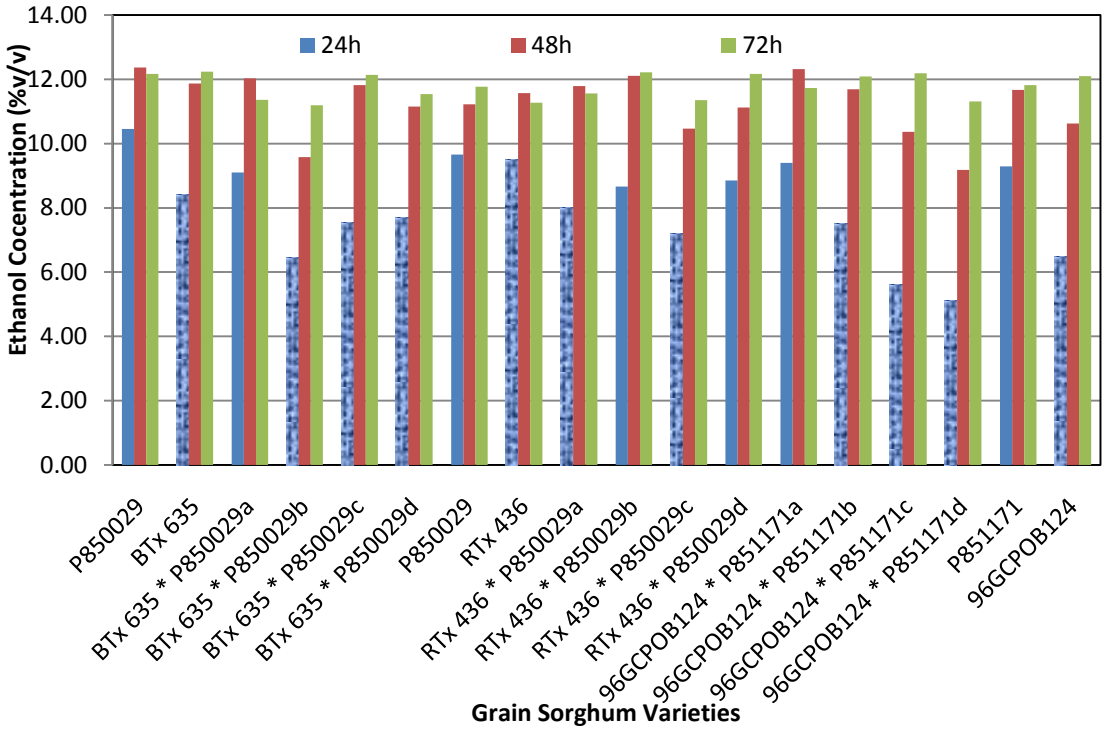

Figure 9. Ethanol concentrations (%v/v) at 24, 48 and 72 h SSF using the 18 sorghum cultivars. (HD lines and ND lines)

Table 11. Mean squares from ANOVA of % ethanol yield at 24, 48 and 72 h SSF among digestible groups

	Sum of Squares	df	Mean Square	F	Sig.
24h Fermentation	1127.83	3	375.9435	12.06638	0.00002
48h Fermentation	453.7155	3	151.2385	5.621919	0.00327
72h Fermentation	62.98735	3	20.99578	2.204339	0.10673

Table 12. Percent ethanol yield after 24, 48 and 72 h fermentation among digestible group

% Yield	Digestible Group			
	Parent		Offspring	
Ethanol	HD	ND	HD	ND
24h Fermentation	50.06[a]	42.59[b]	50.00[a]	37.51[b]
48h Fermentation	64.35[a]	59.59[b]	67.11[a]	58.11[b]
72h Fermentation	65.35[a]	62.29[a]	64.99[a]	62.41[a]

Note: Means with the same letter in the same row are not significantly different using LSD (α=0.05)

Higher fermentation efficiency means higher starch conversion rate. This hypothesis is supported by positive significant correlation (see Table 10) between rate of glucose conversion and % ethanol yield after 24 and 48 h SSF (0.402* and 0.357*, respectively). Also, the altered protein matrix in the genetically modified variety of grain sorghum, improved the sorghum starch digestibility during enzymatic hydrolysis and then contributed to the faster and higher starch conversion to glucose and ultimately to ethanol. This hypothesis is also supported by highly significant correlation between turbidity and % ethanol yield after 24, 48 and 72 h SSF (-0.617**,-0.550**, and -0.426**, respectively).

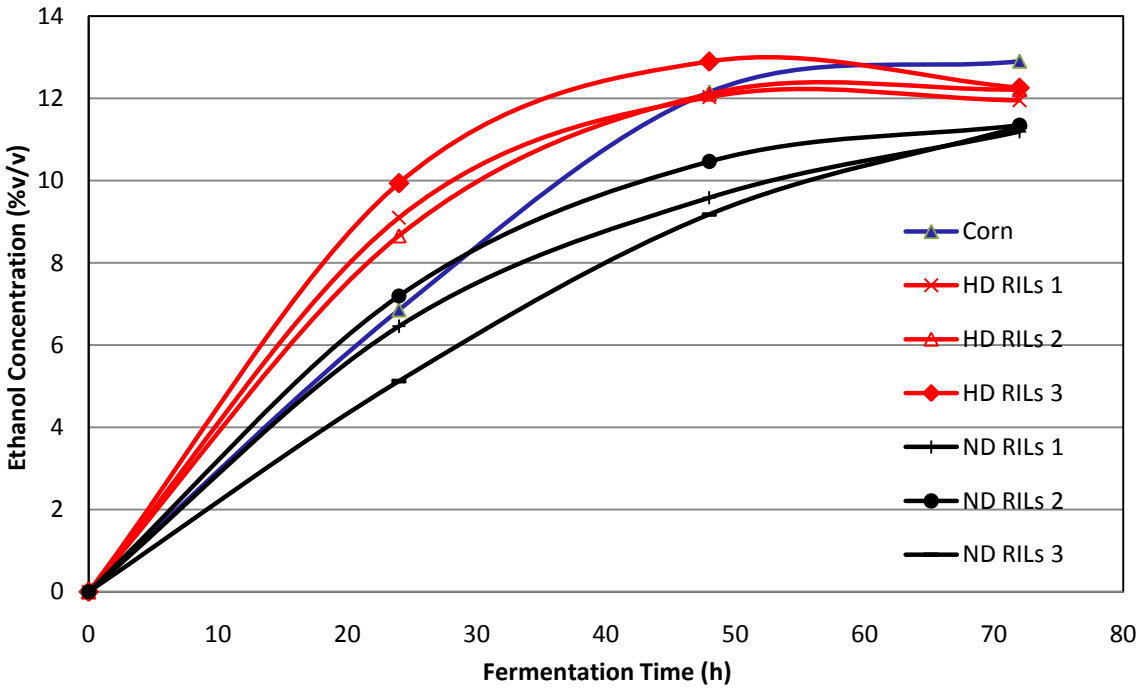

Figure 10. Comparison of ethanol concentration during 72 h SSF of HDGS, NDGS and corn at 30% dry solid.

Ethanol production from dry milled HDGS, NDGS and corn using simultaneous saccharification and fermentation at 30% dry solid is shown in Figure 10. Among the three substrates, the HDGS gave the highest ethanol yield all throughout the first 48 h of fermentation. Results suggest that HDGS starch is more digestible than corn starch and NDGS. HD lines also have shorter time of conversion which has been completed within 48 h of fermentation and faster and higher starch conversion to glucose and ultimately to ethanol than corn and NDGS. Since sorghum has lower demand as food, the new variety of highly digestible grain sorghum could very well serve as a viable substitute for corn in ethanol production.

Increasing the dry solid concentration from 22% to 30% (w/v) increases the ethanol yield by almost 60% (v/v) of the original (see Table 3). This will allow considerable saving of water, reduced distillation cost and increased ethanol production with given plant capacity and labor cost (Bvochora et al., 2000). However, fermentation efficiency generally decreases as substrate concentration increases (Zhan et al., 2006). Results showed that the overall % ethanol yield decreases by almost 30% when dry solid concentration is increased from 22% to 30% (w/v).

Since fermentation in this study was carried out in shake flasks, conditions may not be optimal to ensure complete fermentation. In this study, microbial counts in all fermentation were maintained with 10^7 cfu/ml though decrease in cell viability was observed after 48 and 72. Stressful environment for yeast include high solute and ethanol concentration, low pH and production of co products like acetic and lactic acid (Nichols et al., 2008). In order to maximize the benefit of high dry solid fermentation, further research is necessary to determine the optimum processing parameters and to improve the utilization of starch. High gravity fermentation under SSF has its potential of limited contamination and reduced osmotic stress to the yeast, because glucose is consumed as it formed.

Particle Size Distribution of Flour

Mass median diameter (MMD) was used as a means of expressing and comparing particle size on a statistical basis. The geometric standard deviation (GSD) on the other hand was used to show how spread out are the particle sizes. Generally, it is

assumed that attrition grinding or hammer milling of grains will result in sigmoidal particle size distribution (PSD) (Sahai et al., 2001). But, from the Malvern analysis, sorghum flour that was milled under 1 mm screen opening showed that it is a bimodal distribution (Figure11).

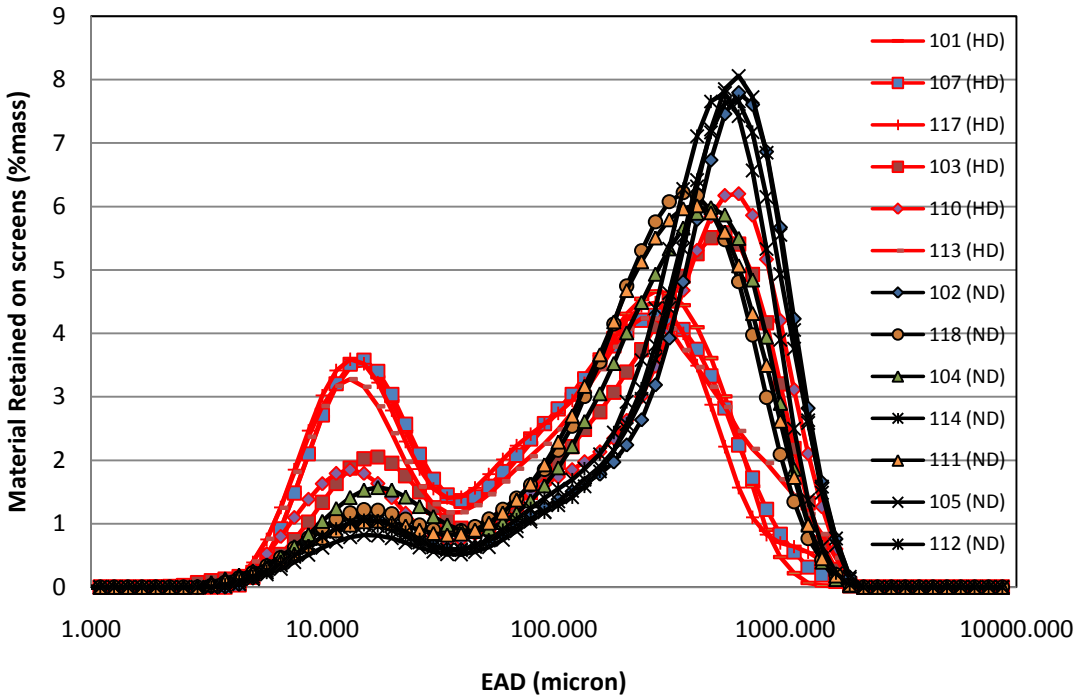

Figure 11. Particle size distribution of sorghum flour samples.

The proportions of each distribution which have a particle size range of 3 to 60 μm and 60 to 1000 μm, were calculated using the Malvern cumulative plot (Figure 12). Independent t-test for equality of means at 95% shows that there is a significant difference (P-value = 0.000) between HD and ND flour in the percentage of each size range. HD flour has a significantly higher portion of 0 to 60 μm (33 $\pm$ 7% w/w) and

significantly lower portions of 60 to 1000 μm (67 ± 6% w/w) than the ND flour (18 ± 7% w/w and 82 ± 6% w/w, respectively).

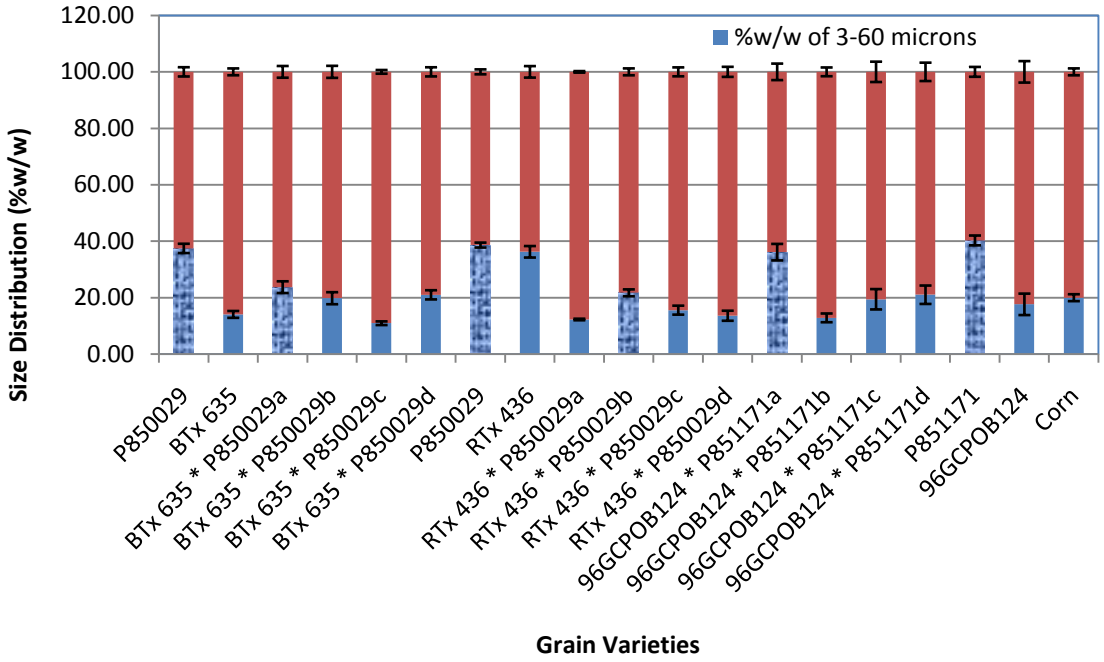

Figure 12. Percentage (%w/w) of the bimodal distribution which has a particle size range of 3 to 60 μm and 60 to 1000 μm. (HD lines and ND lines)

The MMD and the GSD for each size range were also analyzed. LSD test in Table 13 shows that MMD and GSD of parent and offspring from the same digestible group are significantly different for coarse particles but not with fines. Analysis of variance (ANOVA) at 95% confidence level confirms that there is no significant difference in MMD among digestible groups for 3 to 60 μm size range but significantly different for 60 to 1000 μm range (Table 14).

The PSD of sorghum flour samples shows that HD lines has greater portion of fine particles (about 3 to 60 μm) and lesser amount of coarse particles (about 60 to 1000 μm) than ND lines when hammer-milled using 1 mm screen openings. The physical characteristics of grain, such as kernel hardness and endosperm texture, have significant

Table 13. Average MMD and GSD among digestible group

Properties	Digestible Group			
	Parent		Offspring	
0 to 60 μm range	**HD**	**ND**	**HD**	**ND**
MMD	15.40[a]	15.21[a]	15.83[a]	15.28[a]
GSD	1.51[a]	1.71[b]	1.64[b]	1.76[c]
60 to 1000 μm range				
MMD	226.63[a]	406.68[b]	312.20[b]	365.97[c]
GSD	2.08[a]	2.05[b]	2.29[b]	2.07[c]

Note: Means with the same letter in the same row are not significantly different using LSD (α=0.05)

Table 14. Mean squares from ANOVA average MMD and GSD among digestible group

	Sum of Squares	df	Mean Square	F	Sig.
MMD (from 3-60μm)	1.522277	3	0.507426	0.713205	0.552066
GSD(from 3-60μm)	0.295016	3	0.098339	9.950877	0.000113
MMD (from 60-1000μm)	118453.2	3	39484.42	6.853663	0.00125
GSD(from 60-1000μm)	0.260656	3	0.086885	7.329964	0.000842

association with particle size distribution (0.424** and -0.439**) (Table 15). It could be deduced that HD grains breaks easily than the ND because HD RILs have soft and floury

endosperm matrix. This is also supported by the LSD results in Table 13. HD parent has the smallest MMD (15.40 and 226.63 for 0-60μm and 60-1000μm) while ND offspring has the largest MMD (447 μm). Though MMD of HD offspring is not significantly different in with ND parent, the HD offspring has the significantly highest GSD, meaning with most variation of particle size among the digestible groups.

Fineness of grind influences the amount of sugar formed due to variation in surface area of the flour. If particle is too large, starch granules are not easily gelatinized, forming fewer fermentable sugars (Naidu et al., 2000). The hypothesis that finer particles has faster and higher glucose yield is supported by highly significant correlation between MMD and glucose conversion efficiency (-0.742) and significant correlation between MMD and rate of glucose conversion during saccharification (-0.524) in Table 15. It was also reported that particle size has an effect on ethanol yield. Final ethanol concentration can increase by 22% if corn flour grinding screen size is decreased from 5 to 0.5 mm (Naidu et al., 2000). However, in this study, the difference in particle size of HD and ND has no significant effect on ethanol yield. We could therefore hypothesize that variation in particle size below 500 microns has no significant effect in ethanol yield.

Table 15. Pearson correlation coefficients (r values) of sorghum particle size, starch digestibility and ethanol yield

	Fine particles (%w/w)	Coarse particles (%w/w)	d50 (from 3-60μm range)	d50 (from 60-1000μm range)	Glucose Conversion Efficiency	Glucose Conversion Rate	Ethanol %Yield (24h)	Ethanol %Yield (48)	Ethanol %Yield (72)	Hardness (index)	Endosperm Texture	Ash (%db)
Fine particles (%w/w)	1											
Coarse particles (%w/w)	-1.000**	1										
d50 (from 3-60μm range)	-0.152	0.152	1									
d50 (from 60-1000μm range)	-.844**	.844**	0.145	1								
Glucose Conversion Efficiency	.648**	-.648**	-0.187	-.498**	1							
Glucose Conversion Rate	.665**	-.665**	-0.029	-.722**	.669**	1						
Ethanol %Yield (24h)	.534**	-.534**	.435*	-0.127	.381*	0.17	1					
Ethanol %Yield (48)	0.156	-0.156	.477*	0.199	0.313	-0.094	.782**	1				
Ethanol %Yield (72)	-0.197	0.197	0.155	0.288	0.173	-0.101	0.15	.602**	1			
Hardness	-.657**	.657**	-0.142	.339*	-.725**	-.506**	-.643**	-.447**	-0.015	1		
Endosperm Texture	.679**	-.679**	0.203	-.352*	.709**	.503**	.631**	.432**	0.008	-.943**	1	
Ash (db)	0.09	-0.09	0.319	-0.181	0.087	0.015	0.301	0.253	0.192	0.028	0.085	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

CONCLUSION

HD lines have more flour-like endosperm texture, softer kernels and lower starch content than the ND lines but are not significantly different in protein, moisture, ash content, kernel size and flour density. RVA results show that HD lines have lower pasting temperature and faster rate of gelatinization compared to ND lines, therefore it has an advantage over ND lines if used as a feedstock for ethanol production. The increased protein digestibility of the HDGS significantly improved its starch pasting property and starch digestibility (increased its rate of conversion and total glucose yield during saccharification) which further increases the ethanol yield. Results also show that HD sorghum lines have faster rate of conversion and shorter reaction time needed to achieve completion during fermentation than ND sorghum lines and corn.

Increasing the dry solid concentration from 22% to 30% (w/v) increases the ethanol yield from 8% v/v to 13%v/v. This will allow considerable saving of water, reduced distillation cost and increased ethanol production with given plant capacity and labor cost (Bvochora et al., 2000). However, fermentation efficiency generally decreases as substrate concentration increases (Zhan et al., 2006). Further research is necessary to determine the optimum processing parameters to attain maximum utilization of starch during fermentation.

Fineness of grind influences the amount of sugar formed due to variation in surface area of the flour. The hypothesis that finer particles has faster and higher glucose yield is supported by highly significant correlation between MMD and glucose conversion efficiency (-0.742^{**}) and significant correlation between MMD and rate of

glucose conversion during saccharification (-0.524**). It was also reported that particle reduction in particle size from 5 mm to 0.5 mm size has a significant increase on ethanol yield (Naidu et al., 2007). However, in this study, it was hypothesized that variation in particle size below 500 microns has no longer a significant effect in ethanol yield. Results showed that the difference in particle size of HD (156 to 339 microns) and ND (375 to 447microns) has no significant correlation on ethanol yield.

HDGS have lower energy input required during grinding, gelatinization and hydrolysis of starch granules. Improved protein digestibility also increased bio-availability of starch during enzymatic hydrolysis and ethanol yield. Since sorghum has lower demand as food, the new variety of highly digestible grain sorghum could very well serve as a viable substitute for corn in ethanol production.

HDGS requires lower energy inputs during grinding, gelatinization and hydrolysis of starch granules compared to NDGS. Since sorghum has lower demand as food, this new variety of grain sorghum could be a viable supplement for corn for a much economical production of ethanol.

CHAPTER IV

CONCLUSIONS

High digestible variety of grain sorghum (HDGS) yielded higher ethanol in shorter amount of time than the low digestible grain sorghum (NDGS) and corn by simultaneous saccharification and fermentation. The higher protein digestibility of HDGS resulted in its higher starch digestibility which brings to a faster and higher starch conversion to glucose and ethanol during hydrolysis and fermentation. The HDGS likewise had the highest ethanol production almost all throughout the 72 h of fermentation. These results suggest that the altered protein matrix in the genetically modified variety of grain sorghum improved its protein digestibility. Consequently, it enhanced the sorghum starch digestibility during enzymatic hydrolysis and contributed to the faster and higher starch conversion to glucose and ultimately to ethanol.

HD lines have more flour-like endosperm texture, softer kernels and lower starch content than the ND lines but are not significantly different in protein, moisture, ash content, kernel size and flour density. RVA results show that HD lines have lower pasting temperature and faster rate of gelatinization compared to ND lines, therefore it has an advantage over ND lines if used as a feedstock for ethanol production. The increased protein digestibility of the HDGS significantly improved its starch pasting property and starch digestibility (increased its rate of conversion and total glucose yield during saccharification) which further increases the ethanol yield. Results also show that HD sorghum lines have faster rate of conversion and shorter reaction time needed to achieve completion during fermentation than ND sorghum lines and corn.

Since sorghum has lower demand as food, the new variety of highly digestible grain sorghum could very well provide a viable substitute for corn in ethanol production, especially in areas with drier climates that won't support corn. The purpose of the paper is not to promote sorghum over corn: it is to document that HDGS sorghums are as good as corn and provide an alternative to corn using the same system. Aside from being a less expensive grain than corn, its enhanced starch digestibility may further reduce both material and processing cost. Possible process improvements include reducing enzyme dosages, shortening of liquefaction and fermentation times, and eliminating some of the unit processes through very high gravity SSF to lower energy needed during fermentation and distillation. Further cost reduction can also be achieved by optimizing the combination of substrates, enzymes and yeast during hydrolysis and fermentation. Moreover, using dry yeasts which are tolerant and viable at high ethanol concentration and enzymes with higher specific activities than the current commercial enzymes could provide more efficient starch conversion to glucose and ethanol at a shorter time.

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VITA

Name: **Joan Rollog Hernandez**

Office Address: **Biological and Agricultural Engineering Department**
324B Scoates Hall MS 2117
Texas A&M University
College Station, Texas 77843-2117

E-mail Address: joan_che98@tamu.edu, joan_che98@yahoo.com

Education: **M.S. Biological and Agricultural Engineering**, May 2009
Texas A&M University
College Station, Texas 77843

M.S. Chemical Engineering, March 2006
Minor in Food Science
University of the Philippines at Los Baños
College, Laguna. 4031. Philippines

B.S. Chemical Engineering *(cum laude)*, November 2002
Minor in Sugar Technology
University of the Philippines at Los Baños
College, Laguna. 4031. Philippines

Conference Paper and Presentation:

Hernandez, J.R, F.L. Aquino and S.C. Capareda. 2007. Activated carbon production from pyrolysis and steam activation of cotton gin trash. In the Proceedings of the 2007 Beltwide Cotton Conferences. New Orleans, LA; and Paper presented at ASABE Meeting Presentation. Paper Number: 076084. Minneapolis, MN.

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