

**EFFECT OF SORGHUM GENOTYPE, GERMINATION, AND PRETREATMENT ON
BIOETHANOL YIELD AND FERMENTATION**

by

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B.S., Zhengzhou Grain College, China, 1988
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Biological and Agricultural Engineering
College of Engineering

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Manhattan, Kansas

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Abstract

Grain sorghum is the second major starch-rich raw material (after corn) for bioethanol production in the United States. Most sorghum feedstock for bioethanol production is normal non-tannin sorghum. Waxy sorghum and tannin sorghum are rarely used due to lack of scientific information about waxy sorghum fermentation performance and the way to increase fermentation efficiency of tannin sorghum. The main objectives of this study were to investigate the fermentation performance of waxy sorghum and to improve fermentation efficiency of tannin sorghum using techniques such as germination and ozonation treatments. The ethanol fermentation performance on both waxy sorghum and tannin sorghum were evaluated using a dry grind ethanol fermentation procedure. Fermentation efficiencies of tested waxy sorghum varieties ranged from 86 to 93%, which was higher than normal (non-waxy) sorghum varieties. The advantages of using waxy sorghums for ethanol production include less energy consumption, higher starch and protein digestibility, shorter fermentation time, and less residual starch in distillers dried grains with solubles (DDGS). Results from germination study showed germination significantly increased fermentation efficiency of tannin sorghum. The laboratory results were further confirmed by those from five field-sprouted grain sorghum samples. Significantly increased free amino nitrogen (FAN) contents in sprouted sorghum samples accelerated the ethanol fermentation process. Results from both laboratory-germinated and field-sprouted samples demonstrated that germination not only increased fermentation efficiency (higher than 90%) but also reduced fermentation time by about 50%, which could result in energy saving and increased production capacity without additional investment. The excellent performance of sprouted sorghums may provide farmers a new market for field-sprouted sorghum (poor quality as food or feed) in a bad year. A previous study showed ozone had a strong connection to degradation of lignin macromolecules. The hypothesis was that ozone treatment may also reduce tannin activity and increase fermentation efficiency of tannin sorghum. Results showed that the ethanol production performance (ethanol yield, fermentation efficiency, and fermentation kinetics) of the ozone-treated, tannin sorghum flours was significantly improved compared with the untreated control. The other effects of ozonation on sorghum flour include pH value decrease, discoloration, and inactivation of tannin. In summary,

these studies showed sorghum, no matter it was waxy, field-sprouted, or tannin sorghum, can be an excellent feedstock for ethanol production.

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Approved by:

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Dedication

This work is dedicated to my beloved family and the people who have helped me during my study in USA.

Chapter 1 - Introduction

Problem Statement

The fuel ethanol production from plant-based materials has become dominant since 1970s and currently shows no sign of slowing down. Ethanol has many advantages over gasoline in reducing green house gas emission and creating a renewable energy base. Ethanol fuel as a gasoline alternative has been experiencing a significant jump in production in the United States in recent years. The availability of ethanol at the fuel pump is becoming more prevalent because of U.S. mandates for mixing ethanol into gasoline, which is creating strong demand and rapid growth in the ethanol industry. For example, The Energy Independence and Security Act of 2007 expand the Renewable Fuels Standard (RFS2), which predicts the annual ethanol production will grow to 15 billion gallons by 2012 and 36 billion gallons by 2022. In the U.S., corn is the dominant starch-based feedstock for bioethanol. However, to meet the high demand for ethanol, to sustain the environment, and to revive nation's economy, other alternative feedstocks must be sought for ethanol production.

Grain sorghum not only has similarity to corn composition in starch content, but also has advantages over corn on drought and heat stress tolerance, low fertilizer and pesticide input, high yield ability, and established production systems in the Great Plains region from South Dakota to Texas. Grain sorghum could be a reasonable feedstock for ethanol production and could make a larger contribution to the nation's fuel ethanol requirement.

Historically, sorghum is used mainly for animal feed in the U.S. with limited amounts for human food. Some characteristics of grain sorghum such as high tannin content and sprouting tendency limit its food and feed applications. Along with these considerations, the use of tannin and damaged grains and/or low food/feed value grains provide additional feedstock sources for ethanol production.

Tannin grain sorghum is desired for its resistance to birds, insects, weathering and high yield potential. From a sorghum producer's perspective, tannin sorghum is favored for planting and storage. However, tannin sorghum for ethanol has been reported to have difficulty in liquefaction with abnormally high viscosity when compared with non-tannin samples during the pretreatment for ethanol production (Wu et al 2007). Mullins and NeSmith (1986) studied the

ethanol fermentation from bird-resistant and non-bird-resistant grain sorghum and reported high tannin levels greatly reduce the rate of ethanol production.

Sprouting caused by unpredicted rainy weather prior to harvest and/or improper postharvest storage conditions is another common problem for grain sorghum, which decreases the quality of grain for food and feed applications. However, the production of fermentation ethanol requires applying and seeking new approaches and technology for conversion of agricultural byproducts and wastes which do not conflict with current food supplies.

Waxy or glutinous grain sorghum is a special genetic cultivar for grain sorghum with zero or low amylose content, and is reported to have higher starch digestibility than normal grain sorghum. However, there is little information available on yield and efficiency for waxy sorghum compared with normal sorghum.

To target these three traits of grain sorghum as feedstocks for bioethanol production, the goal of this study was to study the effect of genotype, sprouting, and pretreatment on ethanol yield and fermentation efficiency. In addition, ozonation was used to treat tannin sorghum to determine sorghum ethanol fermentation efficiency and yields.

Objectives

The goal of this research was to study the effect of genotype (tannin and waxy sorghum), field sprouting, and pretreatment (laboratory germination and ozone treatments) of grain sorghum on ethanol yield and fermentation efficiency. The ultimate goal of this study was achieved through the following specific objectives.

- 1) to investigate the fermentation performance of waxy grain sorghum for ethanol production as well as the relationship among physical properties, chemical composition, and thermal properties on ethanol yield and fermentation efficiency;
- 2) to investigate the effect of germination of tannin grain sorghum on ethanol yield and fermentation efficiency;
- 3) to investigate physicochemical and biochemical characteristics of field-sprouted grain sorghum and its fermentation performance on ethanol yield;
- 4) to investigate the effect of ozonation on physicochemical properties of whole tannin sorghum flours and their ethanol fermentation performance.

Related Current and Previous Research

Grain Sorghum as Feedstock for Fuel

Grain sorghum (*Sorghum bicolor* (L.) Moench) is the third leading cereal crop in the United States just behind corn and wheat in planted acres ([USDA-NASS](#)). In the United States, sorghum is mainly produced in the Great Plains region from South Dakota to Texas (other states include Kansas, Nebraska, Oklahoma, and Missouri). Sorghum is mainly used as a livestock feed in the U.S. with only a small portion for human consumption. However, sorghum has been used in ethanol fermentation for a long time (e.g. beer production in Africa and Mexico, and spirit production in China). Sorghum can produce almost the same amount ethanol per bushel as corn ([Rooney and Waniska 2000](#)) because sorghum is high in starch. Only in recent years has it been used in large scale bioethanol production in the U.S. In 2009, more than 30% of the grain sorghum production in the United States was used in ethanol production ([Jessen 2010](#)). Its ethanol by-product, distillers grains, is a value-added, high-protein feed for animals.

Sorghum is a cereal of remarkable genetic variability. More than two hundred genes characterize specific genotypic, phenotypic, and cytogenetic traits in sorghum. For instance, the color, appearance, and quality of grain sorghum are influenced by genes controlling pericarp color (R and Y) and thickness (Z), the presence (B_1 and B_2) and or absence (b_1 and b_2) of a testa, and endosperm color and structure (wx) ([Rooney and Miller 1982](#)). Two epicarp color genes, R and Y , interact to produce a red ($RRYY$), yellow ($rrYY$), or colorless or white ($RRyy$ and $rryy$) sorghum. ZZ or Zz will produce thin pericarp, whereas zz produces thick pericarp of sorghum; B_1B_2 produces a pigmented testa layer, in which tannin is located; whereas B_1b_2 , b_1B_2 , or b_1b_2 will cause a pigmented testa to be absent in sorghum. Because the appearance and quality of sorghum are affected by its genetically controlled characters along with other environmental factors, ethanol production was reported to be related to both the chemical composition and physical properties of grain sorghum ([Zhan et al 2003](#)).

It is difficult to classify sorghum due to its wide diversity. However, the U.S. Federal Grain Inspection Service ([USDA-FGIS 1993](#)) has classified grain sorghum into white, sorghum (yellow), brown, and mixed classes based on grain color and pigmented testa. White sorghum has a white or colorless pericarp without a pigmented testa. Nearly all of the sorghum marketed in the United States is yellow sorghum, which can contain red, yellow, white, pink, and many

other variations in pericarp color and not more than 10% kernels with a pigmented testa. Mixed sorghum contains mixtures of sorghum with and without pigmented testa. All sorghum kernels with a pigmented testa are classified as brown sorghums, or tannin sorghums (Hahn et al 1984). Grain sorghum cultivars have been classified into Type I, II, and III based on the tannin content and the genes which control it (Price and Butler 1977). Type I sorghum does not contain tannins; Type II and III sorghum contain low and high tannins, respectively. Tannin is the primary nutrient-limiting component in grain sorghum. High levels of condensed tannins can reduce starch and protein digestibility up to 10% (Leeson and Summers 1997). Mullins and NeSmith (1986) studied the ethanol fermentation from bird-resistant and non-bird-resistant grain sorghum and reported that high tannin levels greatly reduce the rate of ethanol production.

The waxy gene, *wx*, causes the production of a waxy endosperm in sorghum. The normal endosperm type of sorghum is approximately 75% amylopectin and 25% amylose, while waxy endosperm mutants contain nearly 100% amylopectin. Heterowaxy sorghum contains less than 20% of amylose. Waxy and heterowaxy varieties generally have higher fermentation efficiencies than non-waxy varieties, because amylose is likely to form amylose-lipid complexes which are resistant to enzymatic hydrolysis in seeds or during mashing (Wu et al 2006). Waxy sorghum is reported to have higher starch digestibility than normal grain sorghum in food applications. However, there is little information available on ethanol yield and fermentation efficiency of waxy sorghum compared with normal sorghum.

Starch is the major component followed by protein in grain sorghum. Starch content in sorghum flour was a good predictor for ethanol yield (Lacerenza et al 2008; Zhao et al 2009). Protein degradation from enzymes or other treatment could provide nitrogen for yeast growth during fermentation. Yeast only uptakes free amino nitrogen and short peptides not proteins. Research has been conducted on protein and protein digestibility for ethanol fermentation from grain sorghum (Lacerenza et al 2008; Pérez-Carrillo and Serna-Saldívar 2007; Pérez-Carrillo et al 2008; Zhao et al 2008). Little research has been conducted on effect of free amino nitrogen on the conversion efficiency of sorghum varieties in ethanol fermentation.

Germination, or sprouting, is a common problem for grain sorghum when weather is moist during harvest or the environment is humid during storage. Germination promotes the development of cytolytic, proteolytic, and amylolytic enzymes that are not active in dry kernels (Bamforth 2006; Dewar et al 1997b; Klose et al 2009) and could cause significant changes in

kernel composition and physical properties (Agu and Palmer 1996; Beta et al 2000; Elmaki et al 1999; Iwuoha and Aina 1995; Lasekan et al 1995; Muria and Bechtel 1998; Murty et al 1984; Osuntogun et al 1989; Palmer 1991; Singh and Bains 1984; Swanston et al 1994). Germination not only causes compositional changes in the sorghum grain, but also initiates a series of biochemical and physiological changes. Intrinsic enzymes such as amylases, proteases, lipases, fiber-degrading enzymes, and phytases are activated; this disrupts protein bodies and degrades proteins, carbohydrates, and lipids to simpler molecules, which increases digestibility of proteins and carbohydrates in the kernel and makes nutrients available and accessible for enzymes (Chavan and Kadan 1989; Dicko et al 2006; Ratnavathi and Ravi 1991; Subramanian et al 1992; Taylor 1983). Therefore, field-sprouted grain sorghum might benefit bioethanol production. However, there is no information on ethanol fermentation from sprouted grain sorghum.

Sorghum germination and malting are not the same. Sorghum has been malted and used for production of traditional alcoholic and nonalcoholic beverages for centuries (Dufour et al 1992). Malting conditions have to be controlled to achieve uniform and high quality sorghum malts and ensure quality required for food products (Dewar et al 1997b). However, biofuel ethanol production does not have the same requirements. The most important issues in industrial ethanol production are yield, efficiency, and energy consumption. Using germination-damaged sorghum for industrial ethanol production might be beneficial to the producer and end user in expanding market uses for what has been historically considered a low value commodity (Suresh et al 1999). However, there is little information available for germinated or sprouted grains for bioethanol production.

Using grain sorghum for bioethanol production has been proceeding in our laboratory. Decortication has been used to remove sorghum bran to increase starch loading to improve sorghum fermentation performance (Corredor et al 2006). Factors impacting ethanol production from grain sorghum in the dry-grind process were investigated by Wu et al. (2007).

Physical and enzymatic treatment sorghum for ethanol fermentation has been reported (Pérez-Carrillo et al 2008; Pérez-Carrillo and Serna-Saldívar 2007). However, chemical treatment on sorghum grain for ethanol fermentation is scarce. Ozone is a strong oxidant with oxidation potential 2.07 eV and is advantageous since it can easily degrade ingredients and kill microorganisms. Ozone has been used in waste-water treatment and corn and wheat steeping prior to milling (Dhillon et al 2009; Ruan et al 2004). Previous studies have shown that ozone is

able to degrade macromolecules such as lignin, protein and carbohydrates (Wang et al 1999; Wang et al 2008; Yosef et al 1994). Ozone treatment could be a good alternative to chemical treatment because it has been used in very low dosage ppm and does not leave residue in the treated product. However, there is little information about ozone treatment of sorghum feedstock for ethanol production.

Tannins in Sorghum

Sorghum is unique among cereals because of relatively large amounts of polyphenols in the grain and plant (Hoseney et al 1987). Polyphenols widely distributed in plants are not directly involved in any metabolic process and are considered secondary metabolites. They serve as defense chemicals, protecting the plant from predatory attacks of herbivores, pathogenic fungi, and parasitic weeds.

Polyphenols are named by the presence of more than one phenol unit as their molecular building block. Polyphenols have been divided into three categories: phenolic acids, flavonoids, and condensed polymeric phenols (Chung et al 1998).

Polyphenols are well-known to have specific attributes in sorghum: to protect sorghum seedlings from insect attack, to prevent sorghum losses from premature germination and damage due to fungal attack, and to resist birds (Butler and Roger 1985; Harris and Burns 1973; Waniska et al 1989). Phenolic acids are present in sorghum either free or bound as esters and concentrated in the outer layers of the grain. They inhibit growth of microorganisms (Hahn et al 1983). In fact, tannic acid is one of phenolic acids but it is not present in grain sorghum. Flavonoids (e.g. lignin and catechin) in sorghum are called anthocyanidins, which are derivatives of the monomeric polyphenol flavan-4-ol. Flavonoids are reported to resist grain molds (Jambunathan et al 1986) and to resist birds (Subramanian et al 1983; Tipton et al 1970). Flavonoid monomers are synthesized and then condensed to form oligomeric proanthocyanidins of five to seven units during grain development. Gupta and Haslam (1980) referred to sorghum tannins as procyanidins (proanthocyanidins) that result from condensation of flavan-3-ols and/or flavan-4-ols. Total phenols can be measured using the Folin-Ciocalteu reaction. Results are typically expressed as gallic acid equivalents (GAE).

The term *tannin* was first introduced in 1796 to describe the chemical constituents of various plant extracts which were responsible for transforming fresh animal hides into leather

(Hulse et al 1980). Later, Bate-Smith and Swain (1962) defined tannins as water soluble, polyphenolic compounds with molecular weights ranging from 500 to over 3,000. Serrano et al. (2009) defined tannins as a unique group of phenolic metabolites with molecular weights between 500 and 30,000. Some very large condensed tannins are insoluble in water. Thus, all plant phenols are not tannins but all sorghums contain phenols.

Tannins are present in sorghums having a pigmented testa layer, which is controlled by two complementary dominant genes designated B_1 and B_2 (Blakeley et al 1979). Based on the distribution and location of tannins in sorghum, sorghums are classified as Type I (no pigmented testa layer and no tannins), Type II (tannins in pigmented testa), and Type III (tannins in pigmented testa and pericarp) (Waniska and Rooney 2000). Therefore, not all the sorghums contain tannins. Only Type III includes the well-known “bird-resistant” sorghums or tannin sorghums.

There are two types of tannins in plants: hydrolysable and condensed tannins. The hydrolysable tannins can be hydrolyzed by enzymes and acids to release sugar molecules and phenolcarboxylic acid; whereas the condensed tannins cannot be hydrolyzed by enzymes. However, the condensed tannins can be decomposed by acids to release small amounts of anthocyanidins other than sugar molecules. Sorghum does not contain hydrolysable tannins but some sorghum cultivars contain condensed tannins. Tannin sorghums have a wide range of seed color, with light-colored varieties having potentially high tannin content (Waniska et al 1992).

A number of methods have been used to determine tannins in sorghums qualitatively and quantitatively. Qualitatively, the scratch test along with visual observations is a fast and direct method for tannin sorghum classification (Waniska et al 1992; Xiang 2009). Bleaching is another relatively accurate, inexpensive, and rapid test method that has been used for sorghum kernel grading and classification.

Burns (1971) developed the vanillin hydrochloric acid (V-HCl) method for sorghum tannins content determination. Maxson and Rooney (1972) evaluated ten methods and modified the V-HCl method (MV-HCl) by adding 1% HCl in extraction solvent for sorghum tannin determination and summarized another eight methods unsuitable for analysis of sorghum tannins. These eight methods were: Snell’s colorimetric method (1953), the Folin-Denis two methods by Burns (1963), the AOAC tannin in tea (1965), the ferric ammonium citrate by Burns (1963), the ferric ammonium sulphate by Mejbaum-Katzenellenbogen and Kudrewicz-Hubica (1966), the

Bate-Smith and Rasper's methanolic-HCl method (1969), the protein precipitation method by Hagerman and Butler (1978) and Schanderl (1970). A few years later, Price et al. (1978) studied MV-HCl assay procedures in more detail and claimed a new modified MV-HCl giving excellent reproducibility for sorghum tannins. However, due to the complexity of tannins in sorghum and the specificity of each of the quantitative methods, tannins in sorghums were reported arbitrarily (varied quantitatively and qualitatively). Catechin equivalent (CE) of tannin content mg/100mg by the MV-HCl method has been used most for sorghum tannin measurement as a reasonable reference standard. In summary, the colorimetric method is the major method for tannin quantitative test in sorghum.

Recently, sorghum tannins have been reported having antioxidant activity (Awika and Rooney 2004; Hagerman et al 1998; Sikwese and Duodu 2007). Rooney (2008) reported tannin sorghum had promise to lower cholesterol in animals and had anti-cancer compounds. Also, as mentioned early, the presence of tannins is associated with decreased bird preference, resistance to preharvest germination, insects and molding, increased storage stability, and mass reduction. The agronomic importance and advantages of tannins will sustain sorghum production. Effect of tannins on sorghum proteins and carbohydrates

Clearly and evidently, tannins react with proteins. Tannins have been used for over a thousand years in the hide industry for conversion of raw animal skins to durable and impermeable leather. Using gelatin to form precipitates with tannins has been one of the methods for determination of tannins in sorghum (Hagerman and Butler 1978; Maxson and Rooney 1972). There are at least four modes of reaction possible between tannins and proteins: 1) hydrogen bonds between OH groups in the tannins and receptor groups (e.g. NH, SH, and OH) in the proteins (Van Buren and Robinson 1969); 2) ionic bonds between anionic groups in the tannins and cationic groups in the proteins; 3) hydrophobic interaction; 4) covalent linkages between tannins and proteins (Butler et al 1984). A familiar characteristic of tannins is their oral stringency, a trait that probably results from their reaction with the glycoproteins in saliva (Loomis 1969; Haslam 1974).

Tannins bind to both exogenous and endogenous proteins including enzymes of the digestive tract, affecting the utilization of proteins (Eggum et al 1983; Hagerman and Butler 1980). Van Buren and Robinson (1969) pointed out that tannins interact with proteins to form soluble and insoluble complexes and act as enzyme inactivators, thus affecting the growth of

animals when fed tannin sorghum. Watson (1975) reported sorghum tannins inhibit enzymic reactions and microbial activities which are required during the brewing of beer. Daiber (1975a) reported that the amylase solubility of bird-resistant sorghum cultivars was the lowest compared with those from non-bird-resistant sorghum cultivars and other sorghum cultivars without a dark testa, a correlation of $r = 0.981$ between enzyme inhibition and total tannin content. Gomez-Cordoves et al. (2001) studied sorghum tannins on tyrosinase activity and growth of melanoma cells and revealed that sorghum tannins have a greater ability to interact with proteins causing a consequential decrease in enzymatic activities.

Harris et al. (1970) determined the tannin content and *in vitro* protein digestibility of 43 varieties of sorghum and reported there was a highly significant and negative correlation between tannin content and *in vitro* protein digestibility. Arora and Luthra (1974) reported that there was a significant negative correlation between tannin content and *in vitro* protein digestibility of 17 varieties of sorghum. They also found a significantly larger amount of nitrogen in the residue from *in vitro* protein digestibility of the tannin varieties than in the residue from the low-tannin varieties suggesting that the protein had become bound in the tannin varieties. Recently, Elkin et al. (1996) reported that condensed tannins were only partially responsible for variations in nutrient digestibility of sorghum grain cultivars.

Because tannins are present in a pigmented testa layer and pericarp of sorghum, some physical methods (e.g. decortication) have been applied to remove them to reduce their effect on protein digestibility for food applications. Youssef (1998) studied protein digestibility and extractability from tannin and low-tannin varieties of sorghum and reported that dehulling of sorghum led to increased protein extractability and digestibility. The protein digestibility is not the only component affected by tannins. Digestibility of carbohydrates is affected by tannins as well. Davis and Hosney (1979) studied the biological activity of condensed tannins of some varieties of sorghum on starch and reported that tannins isolated from bird-resistant sorghum were shown not only to inhibit α -amylase but also to bind grain starch to varying degrees. Dreher et al. (1984) reported that the presence of tannins in the grain contributed to the poor digestibility of starch in some varieties of sorghum. Little information is available on the effect of tannins on digestibility of amylose and amylopectin and the mechanism of interaction between starch and tannins in sorghum. Rebole (1994) reported that condensed tannins cause a decline fiber digestibility and organic matter digestibility in ruminant nutrition.

Effect of Tannins on Grain Sorghum Fermentation

Tannins' astringent taste, inhibition enzymes, and negative effects on protein and starch digestibility limit their applications. Tannin is the primary nutrient-limiting component in grain sorghum. High levels of condensed tannins can reduce starch and protein digestibility up to 10% (Leeson and Summers 1997). The activities of intrinsic enzymes in tannin genotypes were lower than those in low-tannin genotypes (Ratnavathi and Sashidhar 2000).

Since tannins are located in the testa layer of the pericarp, milling processes can remove them easily. Decortication was used to remove tannins from tannin sorghum in order to improve fermentation performance (Perez-Carrillo et al 2008). Dehulling also was used to reduce tannins inhibition on proteases and increase protein digestibility by Chibber et al. (1980). Wood ash is used in traditional treatment in Africa to reduce the level of tannin in brown sorghums and improve the nutritional quality (Muindi and Thomke 1981). Hassan and El Tinay (1995) reported that fermentation improved starch and protein digestibilities of tannin sorghum and decreased tannin content. Waichungo and Holt (1995) studied the use of ammonium hydroxide to treat tannin grain sorghum and found the level of assayable tannin in tannin sorghum decreased. Germination was also found to decrease tannin content in sorghum (Osuntogun et al 1989).

Because tannins bind with proteins and inactivate malt enzymes, tannin sorghum is not preferred and screened for sorghum malt production in most of Africa (Taylor and Dewar 2000; Waniska et al 1992). However, Daiber (1975a) revealed that it was essential to inactivate tannins to prevent the subsequent inhibitory effects of tannins during brewing. Daiber (1975b) patented a process of inactivating tannins by soaking sorghum grain in a very dilute solution of formaldehyde for 4 to 6 hr at the beginning of steeping for tannin sorghum malting. Actually, alkaline, and lime water, dilute aqueous ammonia have been used to inactivate tannins in different regions where sorghum is used for staple cereal (Dewar et al 1997a; Okolo and Ezeogu 1996; Price and Butler 1979; Rizley and Suter 1977; Waichungo and Holt 1995). Mullins and NeSmith (1986) studied the ethanol fermentation from bird-resistant and non-bird-resistant grain sorghum and reported high tannin levels greatly reduce the rate of ethanol production. Tannins affect the availability of sufficient nitrogen for yeast and reduce the ethanol fermentation rate.

Rationale and Significance

With the growth of population and the development of industry, needs for energy grow and other sources of energy are sought. Today, society looks at the sustainability of the present model or resource consumption, and the use of renewable sources appears as a feasible alternative. Ethanol fuel as a gasoline alternative has been experiencing a significant increase in production and production capacity in the United States in recent years. The availability of ethanol at the fuel pump is becoming more prevalent because of U.S. mandates for ethanol, which create strong demand and rapid growth in the ethanol industry. The Energy Independence and Security Act of 2007 expanded the Renewable Fuels Standard (RFS2). The expected annual ethanol production will grow to 15 billion gallons by 2012 and 36 billion gallons by 2022. In the U.S., currently corn is the dominant starch-based feedstock for bioethanol. However, to revive the economy, to sustain the environment, and to meet the high demand for ethanol in the U.S., alternative feedstocks for bioethanol have to be sought.

It is very important to select the feedstock for ethanol production from economic and agronomic points of view. Currently, in the United States, ethanol is mainly produced from starch-based crops — corn. In 2009, approximately 96% of the 10.75 billion gallons ethanol was produced from corn ([USDA-NASS](#)). However, to meet the high demand of renewable ethanol in the U.S., the door is opened for other starch-based feedstocks as alternatives for bioethanol production.

Sorghum is an annual plant that can grow at harsh climate conditions, requires few fertilizers and pesticides, and has minimal water requirement. In addition, it has a high photosynthetic efficiency (2-3%) and high productivity. Sorghum as an ethanol feedstock is a new and growing market in the U.S. Both producers of sorghum and ethanol need to take advantage of this tremendous opportunity in using grain sorghum as an ethanol feedstock to boost local and state economies and meet the national ethanol requirement. The variability of grain sorghum is large because the crop is grown under diverse climate conditions which affect the grain composition. Also, the varieties are many in grain sorghum (e.g. normal sorghum, heterowaxy, waxy, tannin-free sorghum, low tannin sorghum, and tannin sorghum).

Biological production of ethanol is accomplished by yeast through fermentation of glucose. All agricultural crops and crop residues containing carbohydrates can be used in the production of ethanol. Therefore, three types of agricultural feedstocks are available for ethanol

fermentation: sugar crops (e.g. sugarcane, sugar beets, sweet sorghum); starch crops (e.g. corn, wheat, barley, rye); and lignocellulosic residues (e.g. crop residues, forage crops, grass and trees). Grain sorghum is one of starch crops that could be used for ethanol production.

Sorghum can produce almost the same amount of ethanol per bushel as corn using the same production process – dry grind. Current corn ethanol plants with dry grind process could use grain sorghum as feedstock without any modification in the ethanol production and could decrease their input cost because sorghum is cheaper than corn. Also, the states of major sorghum production could establish sorghum ethanol plants to support local farmers, to sustain the local economy, and to boost the nation's ethanol demand.

Theoretically, germination and natural sprouting could provide plenty of free amino nitrogen for ethanol fermentation. Yeast is a workhorse for ethanol production. Yeast needs nutrients to keep it working for ethanol conversion. Currently, almost 100% of industrial scale ethanol production uses yeast to convert sugars from starch-rich or sugar-rich biomass to ethanol. The availability of yeast food is vital to yeast growth and working efficiency during fermentation. Free α -amino nitrogen (FAN) is an essential nutrient for yeast growth during fermentation (Pickerell 1986; Taylor and Boyd 1986). During fermentation, yeast takes up fermentable sugars for ethanol production as well as nutrients (amino acids, mineral and vitamins) for its own invertase and permeases, which are responsible for sugar transportation and conversion. In this case, the fermentation efficiency of germinated/sprouted grain sorghum will be much higher than normal sorghum grain feedstock. Therefore, the fermentation process could be shortened to save energy input, which provides scientific information for ethanol industry.

Sorghum has a large variable genus with many cultivars. A large number of varieties of sorghum exist and more are being developed through plant breeding for selecting and concentrating desired characteristics in new varieties for food and feed applications (Rooney and Serna-Saldivar 2000). New sorghum cultivars could be developed for ethanol fermentation when the scientific information is correct. Therefore, more research has to be done on different cultivars of grain sorghum as ethanol feedstocks.

More studies should be conducted to 1) investigate the fermentation performance of waxy grain sorghum for ethanol production as well as the relationship among the physical properties, chemical composition, and thermal properties on ethanol yield and fermentation efficiency; 2) to investigate the effect of germination on tannin grain sorghum ethanol yield and fermentation

efficiency; 3) to investigate physicochemical and biochemical characteristics of field-sprouted grain sorghum and its fermentation performance on ethanol yield; and 4) to evaluate the performance of pretreatment on improvement of grain sorghum as feedstock for ethanol fermentation. The proposed research would provide scientific information and knowledge which will benefit both sorghum breeders and the sorghum bio-industry. Results from this research would lead to capabilities to improve the efficiency of sorghum bioconversion processes; increase sorghum bioconversion yield to biofuels; and enhancement of the economy and rural development, especially across the many sorghum-growing states.

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Chapter 2 - Evaluation of Waxy Grain Sorghum for Ethanol Production¹

Abstract

The objective of this research was to investigate the fermentation performance of waxy grain sorghum for ethanol production. Twenty-five waxy grain sorghum varieties were evaluated using a laboratory dry-grind procedure. Total starch and amylose contents were measured using colorimetric procedures. Total starch and amylose content ranged from 65.4 to 76.3% and 5.5 to 7.3%, respectively. Fermentation efficiencies were in the range of 86-93%, corresponding to ethanol yields of 2.61-3.03 gallons/bushel. The advantages of using waxy sorghums for ethanol production include less energy consumption during the cooking process, higher starch and protein digestibility, higher free amino nitrogen content, and shorter fermentation times. The results showed a strong linear relationship between free amino nitrogen content and fermentation rate. Fermentation rate increased as free amino nitrogen content increased, especially during the first 30 hr of fermentation ($R^2 = 0.90$). Total starch content in distillers dried grains with solubles (DDGS) was less than 1% for all waxy varieties.

Introduction

Unlike wheat, corn, and rice, grain sorghum is a starch-rich cereal that can be grown economically in the semi-arid regions of the world. In the United States, sorghum is the second-ranking feed grain and is cultivated primarily in the Great Plains, including the Midwest and the Southwest. Although it is primarily used as feed in the United States, grain sorghum has been reported in wide uses such as wall board, fermented beverages, traditional foods (porridges and flat breads), conventional pan bread for gluten-free markets ([Owuama1997](#); [Rooney and Serna-Saldivar 2000](#); [Schober et al 2005](#); [Taylor et al 2006](#)). Sorghum utilization by the ethanol industry has been growing in the United States in recent years ([RFA 2007](#); [Sarath et al 2008](#)). Currently, about 95% of the US fuel ethanol is produced from corn and ~4% is from sorghum grain, which uses 30-35% of the total sorghum production in the United States ([Kubecka 2011](#);

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USDA-NASS, 2011). Sorghum could make a larger contribution to the nation's fuel ethanol requirements (Farrell et al 2006; Rooney et al 2007; Wu et al 2007).

Overall, sorghum composition is similar to corn. Starch is the major grain component followed by protein. Most sorghum starches contain 20-30% amylose and 70-80% amylopectin, but waxy and heterowaxy sorghums contain 0-15% amylose and 85-100% amylopectin (Rooney and Serna-Saldivar 2000). Starch content in grains is a good predictor for ethanol yield (Lacerenza et al 2008; Zhao et al 2009). The presence or absence of amylose may influence ethanol yield and conversion efficiency. Wu et al (2007) reported that low amylose content in sorghum grain may be associated with increased ethanol conversion efficiency. One of the aims for this study, which was conducted on 25 varieties of waxy grain sorghum, was to investigate further whether ethanol yield and fermentation efficiency were influenced by the ratio of amylose and amylopectin in waxy grain sorghums.

Both ethanol yield and fermentation efficiency have been studied to evaluate the performance of grain sorghum in ethanol production (Wu et al 2007). Recent research has shown that the key factors affecting the ethanol yield from grain sorghum include grain hardness, starch content, starch digestibility, level of extractable proteins, protein and starch interaction, mash viscosity, amount and types of phenolic compounds present in sorghum, amount of amylose, and formation of amylose-lipid complexes during mashing, (Wu et al 2007; Wang et al 2008; Yan et al 2009; Zhao et al 2008). Sorghum as a raw material can be converted to ethanol with a wide range of efficiency (Wu et al 2007).

Currently, almost 100% of industrial ethanol is produced by yeast from starch-rich or sugar-rich biomass. The availability of yeast food is vital to yeast growth and working efficiency during fermentation. As such, most yeast fermentation systems need nutrient supplementation. Yeast uptakes not only fermentable sugars for ethanol production, but also nutrients (amino

acids, mineral and vitamins) for its own growth and functional maintenance (e.g. levels of invertase and permeases), which are responsible for sugars transportation and conversion. Free α -amino nitrogen (FAN) is an essential nutrient for yeast growth during fermentation (Pickerell 1986; Taylor and Boyd 1986). Protein is the second major component in grain sorghum. Protein degradation could provide nitrogen for yeast growth during fermentation. Recent research has found that ethanol yield and conversion efficiency significantly increased as free α -amino nitrogen increased in laboratory-germinated and field-sprouted grain sorghum (Yan et al 2009; Yan et al 2010). Yeast can only utilize free amino nitrogen and short peptides, not large intact proteins. Much research has been conducted on protein and protein digestibility for ethanol fermentation from grain (Lacerenza et al 2008; Pérez-Carrillo and Serna-Saldívar 2007; Perez-Carrillo et al 2008; Wang et al 2005; Wu 1989; Zhao et al 2008), but little research has been conducted on the effect of free amino nitrogen on the conversion efficiency of sorghum varieties in ethanol fermentation.

Sorghum is a large, variable genus with many cultivars. A large number of varieties of sorghum exist and more are being developed through plant breeding to select and concentrate desired characteristics in new varieties for food and feed applications (Rooney and Serna-Saldívar 2000; Mace and Jordan 2010). We believe genetically improving the quality of grain sorghum for ethanol production could increase the utilization of sorghum for ethanol production in the near future.

The main objective of this research was to investigate the fermentation performance of waxy grain sorghum for ethanol production

Materials and Methods

Grain Sorghum

Twenty-five waxy grain sorghum varieties were from the USDA-ARS-NPA, Grain, Forage, and Bioenergy Research Unit (Lincoln, NE). The origin of these waxy varieties was collected from ten different countries around the world and the seeds of these accessions were increased by the USDA-ARS in Nebraska (Pedersen et al 2007). Detail sample information is listed in Table 2.1. The samples were manually cleaned by removing plant debris and foreign materials, and then were ground to flour using an Udy cyclone sample mill (Udy, Fort Collins, CO) with 1.0 mm screen.

Preparation of Mash and Inoculation of Yeast

Liquozyme SC DC, a heat-stable α -amylase from *Bacillus licheniformis* was used for liquefaction (Novozyme, Franklinton, NC). The listed enzyme activity was 240 KNU/g (one Kilo Novo Unit, or KNU, is the amount of enzyme that breaks down 5.26 g of starch per hr at Novozyme's standard method for determination of α -amylase). Spirizyme Fuel (Novozyme, Franklinton, NC), an amyloglucosidase from *Aspergillus niger*, was used for saccharification. Its listed enzyme activity was 750 AGU/g (one AGU is the amount of enzyme that hydrolyzes 1 μ mol of maltose per minute under specified conditions). Ethanol red active dry yeast (*S. cerevisiae*), a gift from Fermentis (Milwaukee, WI), was used for simultaneous saccharification and fermentation (SSF). Before inoculation, dry yeast was activated by adding 1.0 g of dry yeast cells into 19 mL of preculture broth (containing 20 g glucose, 5.0 g peptone, 3.0 g yeast extracts, 1.0g KH_2PO_4 , 0.5 g $\text{MgSO}_4\cdot\text{H}_2\text{O}$ per liter) and shaking at 200 rpm in a 38°C incubator for 30 min. The activated yeast culture had a cell concentration of roughly 1×10^9 cells/mL.

Thirty grams (db) of sorghum flour for each sample was dispersed in 100 mL of water (containing 0.1 g KH_2PO_4 and preheated to about 60°C) in a 250-mL Erlenmeyer flask. Twenty- μ L of high-temperature α -amylase (Liquozyme, 240KNU/g) was added into the sorghum flour slurry. The flasks were transferred to a 70°C water-bath shaker operating at 170 rpm. The water-bath temperature was gradually increased from 70°C to 85°C over a 30 min period. The liquefaction process continued at 85°C for another 60 min. The flasks were then removed from the water-bath shaker and cooled to room temperature. Materials sticking to the inner surface of the flasks were scraped back into the mash with a spatula, then the inner surface was rinsed with

2-3 ml of distilled water using a fine-tipped polyethylene transfer pipette. The pH of the mashes was adjusted to 4.2-4.3 with 2N HCl. After pH adjustment, 100 μ L amyloglucosidase (Spirizyme Fuel), 1 mL of activated yeast broth, and 0.3 g of yeast extract (1 mL of fresh prepared 30% yeast extract solution) were added to each flask. The inoculated flasks were then sealed with S-shaped airlocks and transferred to an incubator shaker for ethanol fermentation (SSF). All samples were run in duplicate.

Fermentation and Distillation

Ethanol fermentation was conducted at 30°C in an incubator shaker (Model I2400, New Brunswick Scientific, Edison, NJ) operating at 150 rpm for 72 hr. The fermentation process was monitored by measuring the weight loss from evolution of carbon dioxide (CO₂) during fermentation. The weight loss was related to ethanol yield during fermentation ($C_6H_{12}O_6 \rightarrow 2C_2H_6O + 2CO_2^{\uparrow}$). The ratio of ethanol to carbon dioxide is theoretically 51:49.

After 72 hr fermentation, finished mash in each 250 mL flask was entirely transferred to a 500-mL distillation flask and the Erlenmeyer flask was washed 4 times with 100 mL (25 mL \times 4) of distilled water. Two drops of antifoam agent 204 was added into distillation mash to prevent foaming during distillation. The contents were distilled on a distillation unit and the distillates were collected into a 100-mL volumetric flask that was immersed in ice water. When the distillates in the volumetric flask approaching the 100mL mark (<0.5 mL to the mark), the volumetric flask was removed from the distillation unit and the distillation process was stopped. The distillates in the volumetric flask were equilibrated for a few hrs in a 25°C water bath, then brought to the 100 mL mark with distilled water. Ethanol concentrations in the distillates were analyzed by HPLC with a Rezex RCM column (Phenomenex, Torrance, CA) and refractive index detector (Wu et al 2006).

Morphological Structure of Waxy Grain Sorghum

The microstructures of waxy sorghum kernels were examined using a scanning electron microscope (SEM) with an accelerating voltage of 5.0 kV (Hitachi S-3500N, Hitachi Science Systems, Ltd., Japan). Samples were vacuum-coated with a mixture of 60% gold and 40% palladium particles using sputter coater-Desk II sputter/etch unit (Denton Vacuum, LLC, NJ).

Single Kernel Characterization and Particle Size Analysis

Kernel hardness, kernel weight, and kernel size of waxy sorghum samples were analyzed by using the single kernel characterization system 4100 (SKCS) (Perten Instruments, Springfield, IL) controlled by Microsoft Windows software SK4100. The reported data were the means of 300 kernels.

The particle size of ground sorghum flour were measured by an LS 13 320 single wavelength Laser diffraction particle size analyzer (PSA) with Tornado dry powder system (Beckman Coulter Inc., Miami, FL). Samples were run in duplicate.

Pasting Properties

Pasting properties of the sorghum flour samples were measured using a Rapid-Visco-Analyzer (model RVA-3D, Newport Scientific Ltd., Australia). For sample preparation, 4 g of sorghum flour (14% moisture basis) and distilled water (25 mL) were added to an aluminum canister at room temperature. A plastic paddle was inserted into the canister, jogged and rotated manually for about 30 sec to break up any lumps. The paddle (with the sample canister) then was attached to the electric motor in the head of RVA. The sample was premixed by initially running the motor at 960 rpm for 10 sec, then, the motor was slowed to a speed of 160 rpm for the rest of the test. The standard 23-min profile of AACC Method 76-21 ([AACC International 2009](#)) was followed for sample testing. Each sample was analyzed in duplicate.

Thermal Properties

Differential scanning calorimetry (DSC-Pyris 1, Perkin-Elmer, Norwalk, CT) measurement was conducted and calibrated with indium. Sorghum samples were weighed accurately (~10 mg) into stainless steel pans using a microbalance. Deionized distilled water was added carefully with a micropipette into the sample pan. The weight ratio of water to dry flour was 3:1. The pans were sealed and allowed to rest for about 1 hr. Samples were analyzed at heating and cooling rates of 10°C/min. The temperature regime consisted of heating from 25°C to 150°C with an initial 1-min hold. Data from the DSC scans were analyzed using Pyris software for Windows (v.7.0). Enthalpies are reported on a dry flour weight basis. Each sample was analyzed at least in duplicate.

Protein Digestibility

Protein digestibility was determined by following the method of Mertz et al (1984) with modification: 200 mg of sorghum samples were suspended in 35 ml of pepsin solution (1.5g of enzyme/L of 0.1 M potassium phosphate buffer, pH 2.0) and incubated with vigorous shaking at 37°C. Pepsin (Sigma P-7000; activity 924 units per mg of protein) digestion was stopped by addition of 2 mL of 2 M NaOH at the end of 2-hr digestion course. After centrifugation at 4,000 ×g for 15 min, the supernatant was discarded, and the residue was washed in 10 mL of 0.1 M phosphate buffer (pH = 2.0) and centrifuged as before. After the second washing and centrifugation, the residue was frozen, then lyophilized. The freeze-dried residue was then weighed and analyzed for nitrogen content.

Analytical Methods

AOAC Official Methods were used to analyze sorghum flour samples for dry matter/moisture (930.15), crude protein (990.03), ash (942.05), crude fiber (962.09), and crude fat (920.39) (AOAC International 2000). Total starch and amylose contents were measured using colorimetric assay procedures (Megazyme total starch and amylose/amylopectin kits, procedures are available at URLs <http://secure.megazyme.com/downloads/en/data/K-TSTA.pdf> and <http://secure.megazyme.com/downloads/en/data/K-AMYL.pdf>). The presence of amylose in the waxy sorghum kernels was qualitatively examined using the iodine staining techniques (Pedersen et al 2004). Free amino nitrogen (FAN) was analyzed by using the European Brewery Convention (EBC) method (EBC 1987) with modification. One hundred fifty mg grain sorghum flour was mixed with 1.5 mL deionized distilled water in a 2.5-mL microcentrifuge tube and vortexed 5 times in 10 min, then centrifuged at 12,000 rpm for 20 min. The supernatant was then ready for FAN analysis. A tannin bleach test followed the Xiang method (2009). Glucose, glycerol, and ethanol in samples were determined by HPLC (Shimadzu Scientific Instruments, Columbia, MD) according to the method described by McGinley and Mott (2008). The column used was a Rezex ROA column (Phenomenex, Torrance, CA) and the detector was refractive index detector (model RID-10A, Shimadzu) maintained at 40°C. The mobile phase was 5 mM sulfuric acid at a flow rate of 0.6 mL/min and the oven temperature was 65°C. HPLC data were analyzed using Shimadzu EZStart 7.4 software. Fermentation efficiency was calculated as a ratio

of the actual ethanol yield (determined by HPLC) to the theoretical ethanol yield (calculated from the total starch content in the sample) (Yan et al 2009).

Statistical Analyses

All experiments were performed at least in duplicate. The tabular results presented were the mean values of repeated experimental data.

Results and Discussion

As clearly indicated by the major components of the samples from proximate analyses, the waxy sorghum samples used in this project had very diverse genetic background and physical and chemical properties and such. Normal cultivars on the market have starch content of 72-76% (db) and protein content of around 12% (db). The starch content of these samples ranged from 65% to 76% (db), their protein content was from 12% to 15.8% (db). Details of the proximate analysis results are listed in Table 2.1.

Effect of Starch on Ethanol Production

Figure 2.1 shows correlation between total starch content and ethanol yield from fermentation of 25 waxy grain sorghum samples. Ethanol yield (gallons/bushel) was linearly correlated with total starch content ($R^2=0.7946$). This result is in agreement with those reported by Wu et al (2007) and Lacerenza et al (2008). Sorghum cultivars with high starch and low protein contents are cultivars of choice for fuel ethanol production. Wu et al (2008) reported that higher starch content means higher ethanol yield, better processing efficiency, and less residues after fermentation, therefore, total starch content of waxy grain sorghum can be a predictor for ethanol yield. Average ethanol yield from waxy grain sorghum is similar to corn (Lemuz et al 2009). Although the sorghum samples tested in this study have diverse genetic backgrounds, which translate into different starch and protein contents, the ethanol yields ranged from 2.6 to 3.0 gallons per bushel, with an average of 2.8 gallons per bushel.

Endosperm of waxy grain sorghum contains little or no amylose when tested by rapid iodine staining techniques (Pedersen et al 2004). If enough amylose is present in the grain, iodine will bind with amylose in the endosperm of a grain kernel and turn its color into dark blue; while waxy grains contain no or little amylose, it will turn reddish brown (Pedersen et al 2004). The iodine test results showed that kernels of most waxy sorghum samples do not have enough

amylose, a few samples had a little higher amylose contents that the staining method barely detected. The Megazyme amylose assay and DSC analysis results further confirmed the iodine staining results. All 25 tested cultivars of waxy grain sorghum had small amount of amylose, ranging from 5.5% to 7.3%. Fortunately, amylose content in waxy grain sorghum had no significant effects on ethanol yield ($R^2 = 0.1341$, Figure 2.1). This is probably because amylose contents in the tested samples were all very low (<7.3%) and within a narrow range (from 5.5% to 7.3%, Table 2.1). The chances for such small amount of amylose to complex with lipids in waxy grain sorghum were lower than in normal grain sorghum.

DSC results confirmed that only four sorghum samples (PI220636, PI217897, PI548008, and PI562758) out of the 25 tested waxy cultivars showed an amylose-lipid complex enthalpy peak at temperatures around 100°C (Table 2.2). Fermentation efficiencies of those four cultivars (with amylose-lipid complex peaks) were lower than those of the other cultivars (without amylose-lipid complex). Actually, two of these four samples (PI562758 and PI548008) had the lowest fermentation efficiencies of 86.0% and 87.7% among all the 25 tested samples. The average efficiency of all the 25 samples was 89.6%. Previous research conducted on different ratios of commercial amylose and amylopectin for ethanol production showed that high amylose content led to low ethanol yield (Wu et al 2006). In normal wheat, corn and sorghum, amylose is located in amorphous region of starch granules and amylopectin is in crystalline region of starch granules. When amylose content is high in starch granules, amylose readily can leach out of starch granules when the granules are absorbing water. On the other hand, higher amylose content also provides increased amylose-lipid complex formation that inhibits swelling of starch granules. In waxy varieties, amylose content is low, and there is little amylose leaching out from starch granules when they are absorbing water. Thus, amylose in waxy grain sorghum flour does not significantly affect starch granules among waxy varieties. RVA pasting profiles of waxy grain sorghum flour shows a lower pasting temperature and higher peak viscosity than normal sorghum starch. Hence, the small amount of amylose in waxy grain sorghum does not have significant effect on the dry grind ethanol process and final ethanol yield.

Two common phenomena were observed when waxy grain sorghum kernel was scanned with SEM (Figure 2.2). One was the feature of kernel texture (low magnification, 500x) and the other was features of starch granules (high magnification, 5000x). Figure 2.2A shows there are a lot of cracks on the kernel, the cracks may render waxy grain kernels easier to be ground and

generate more damaged starches in the flour. The SKCS and PSA data showed that the hardness index (average of 88.6) of waxy sorghum was similar to that of normal sorghum (87.7) (Pedersen et al 1996) and significantly lower than that of corn (Abdelrahman and Hoseneey 1984), and can be more easily ground into fine particles, which implies less energy consumption for grinding and higher conversion rate in mashing and ethanol yield in fermentation. In previous research, Abdel-Aal et al (2002) reported there were more damaged starches in waxy wheat and waxy corn flour than those from normal wheat and corn. Waxy sorghum starches probably share the same properties with waxy wheat and waxy corn. Figure 2.2B shows many holes in waxy starch granules, which obviously make waxy starch granules more susceptible to enzymatic digestion because water and enzymes can more easily enter starch granules through these pores. The results are in agreement with the conclusion drawn by Sullins and Rooney (1975). That is, waxy starch granules were more susceptible to enzymes degradation than non-waxy starch granules. Sullins and Rooney (1975) also found waxy grain sorghum had a less dense peripheral endosperm than non-waxy grain sorghum. The waxy sorghum flours, however, absorbed significantly more water than did normal sorghum flours, which could be explained by the presence of pores on the waxy starch granules. Wu et al (2007) reported that waxy sorghum cultivars had higher conversion efficiency in the laboratory dry-grind ethanol process than non-waxy cultivars because waxy starches were more easily hydrolyzed and gelatinized during mashing process. Data from this study came to the same conclusion as Wu et al did (2007).

Effect of Free Amino Nitrogen on Ethanol Production

Researchers have found one of the factors limiting the production of high levels of ethanol by brewing yeast to be nutritional deficiency (Casey and Ingledeew 1986). When a nitrogen source is supplemented in the fermentation system, the nutritional supplement can promote the rapid fermentation to higher ethanol level without the need to genetically improve yeast. Therefore, free amino nitrogen in the original sample is crucial to yeast performance. A strong positive linear relationship between fermentation efficiency at the 30th hr of fermentation and free amino nitrogen content in the original samples was observed in this study (Figure 2.3), but by the end of fermentation, no linear correlation was found between free amino nitrogen contents in the original samples and the final fermentation efficiency. Sufficient free amino nitrogen in the fermentation mash is critical to yeast cell growth and proliferation during the

early stage of fermentation. The higher the free amino nitrogen levels in finished mash, the faster the fermentation process. Because almost all the sugars in the tested samples were converted into ethanol, the final fermentation efficiencies among the samples at the end of fermentation were very close. Our previous research strongly supports the effects of free amino nitrogen on ethanol fermentation efficiency from field-sprouted sorghum (Yan et al 2010). Casey et al (1984) made the same conclusion about the effect of free amino nitrogen on fermentation efficiency of high-gravity brewing from wheat. Therefore, free amino nitrogen content in a sample could be a useful indicator of a sample's performance in ethanol fermentation. Data from our previous studies (Yan et al 2009, 2010) showed similar results, which agree with results reported by several other investigators (Lekkas et al 2005; Casey et al 1984). Mullins and NeSmith (1987) studied ethanol fermentation using tannin sorghum and revealed that the addition of nitrogen accelerated ethanol fermentation rate.

Effect of Protein Digestibility on Ethanol Production

Protein digestibility has been used as a quality indicator for human foods and animal feeds. A protein with high digestibility potentially has better nutritional value than those with low digestibility. The protein digestibility of sorghum has been studied extensively *in vitro* using pepsin because the *in vitro* pepsin digestibility results correlate well with *in vivo* digestibility results (Maclean et al 1981), which make sense because human and animal produce pepsin in their digestion tracts. In contrast, yeast does not produce any exoprotease for ethanol fermentation. However, Wang et al (2008) reported a strong linear correlation between protein digestibility of some normal grain sorghum samples and their fermentation efficiency in ethanol production. The same protein digestibility methodology was applied in this study for waxy grain sorghums. The ethanol fermentation data on waxy sorghum samples showed that fermentation efficiency in the laboratory dry-grind process did not show any linear correlation with protein digestibility ($R^2 = 0.0093$) (Figure 2.4). The presence of tannins in some of the sorghum cultivars used in this study could be the main cause for such a divergence. Tannins have been related with lowering starch digestibility by inactivating amylases (Davis and Hosenev 1979). The same phenomenon of enzyme inactivation may be applied here to pepsin in protein digestibility test. Protein hydrolyzing activity of pepsin in the digestibility test could have been inhibited by the tannins in the tested samples. A qualitative tannin test (bleach test) revealed that 16 of the 25

tested waxy cultivars contained tannins. Although yeast itself does not produce exoproteases during normal fermentation process, addition of proteinases during mashing or the SSF process nevertheless can generate favorable results (increasing fermentation rate, cell tolerance to ethanol, and final ethanol yield) because hydrolysis of proteins in the raw materials might help with the release of more starch granules from the protein matrix and increase free amino nitrogen content in the mash (Perez-Carrillo et al 2008; Thomas and Ingledew 1990), which will facilitate yeast growth, increase ethanol fermentation rate and ethanol fermentation efficiency.

Effect of Tannins on Ethanol Production

Sorghum tannins have attracted great attention from a number of researchers because of their effects on product yield and quality, processing properties, starch and protein digestibility, and health (Beta et al 2000; Dlamini et al 2009; Serrano et al 2009; Wang et al 2008). Wu et al (2007) claimed that sorghum tannins retarded the liquefaction process during mashing and resulted in high viscosity mash, slow starch-to-glucose conversion, and lower conversion efficiency. The data showed that ethanol yields and fermentation efficiencies of tannin waxy sorghums were 2-3% lower than those of waxy sorghums without tannins. Mullins and NeSmith (1986) studied ethanol fermentation from bird-resistant and non-bird-resistant grain sorghum and reported that high tannin levels greatly reduced the rate of ethanol production. Evidently the rate of ethanol production was much slower from bird-resistant grain sorghum than from non-bird-resistant grain sorghum because tannins partially inhibited the activities of amylases and glucose was generated at a much slower rate in the bird-resistant grain sorghum mash. On the other hand, tannins could cause sorghum protein crosslinking during heating or cooking and prevent starch granules from absorbing water and enzymatic degradation (Duodu et al 2003).

Chemical Composition of Distillers Dried Grains with Solubles (DDGS)

DDGS is a by-product from ethanol production and is typically used as animal feed. The nutritional composition is critical to buyers. Table 2.3 shows the major components of DDGS from waxy and non-waxy grain sorghum varieties (Saunders and Rosentrater 2009; Stein and Shurson 2009; Urriola et al 2009; Wu and Sexson 1984). Residual starch contents in industrial corn or sorghum DDGS are around 5%. DDGS from normal grain sorghums using a laboratory dry-grind ethanol process had 1-2% starch. The residual starch contents in DDGS from waxy grain sorghum samples in this study were much lower, only around 0.5%, which means that

starch in waxy grain sorghum was more efficiently used for ethanol production than that in normal grain sorghum. Researchers have shown that waxy cereals contain elevated lipid and ash contents compared with their normal counterparts. Because waxy sorghum generally has higher protein content than corn and normal sorghum and starch in waxy sorghum is more efficiently utilized in ethanol production, DDGS from waxy sorghum will have higher crude protein, crude fat, and ash content than DDGS from normal grains (Saunders and Rosentrater 2009). Because the major market for DDGS right now is the animal feed industry, higher protein content means better quality, broader application, and possibly better market price.

Conclusion

This ethanol production study on waxy grain sorghum varieties demonstrated that ethanol yields from waxy sorghums were essentially proportional to their starch contents. Amylose contents in the tested waxy sorghum samples were very low and had little effect on ethanol yield and fermentation efficiency. Ethanol yields from the tested waxy grain sorghums were around 2.8 gallons/bushel, which is similar to that reported for corn. The fermentation efficiency was greatly affected by free amino nitrogen content in waxy sorghums, which had a strong positive linear correlation with early stage (the first 30-36 hr) fermentation efficiency. Tannins were found in most of the tested waxy sorghums and had negative effects on ethanol yield and fermentation efficiency. DDGS from waxy sorghums had higher protein but lower starch contents, which implies better quality and makes the products more attractive to the animal feed industry.

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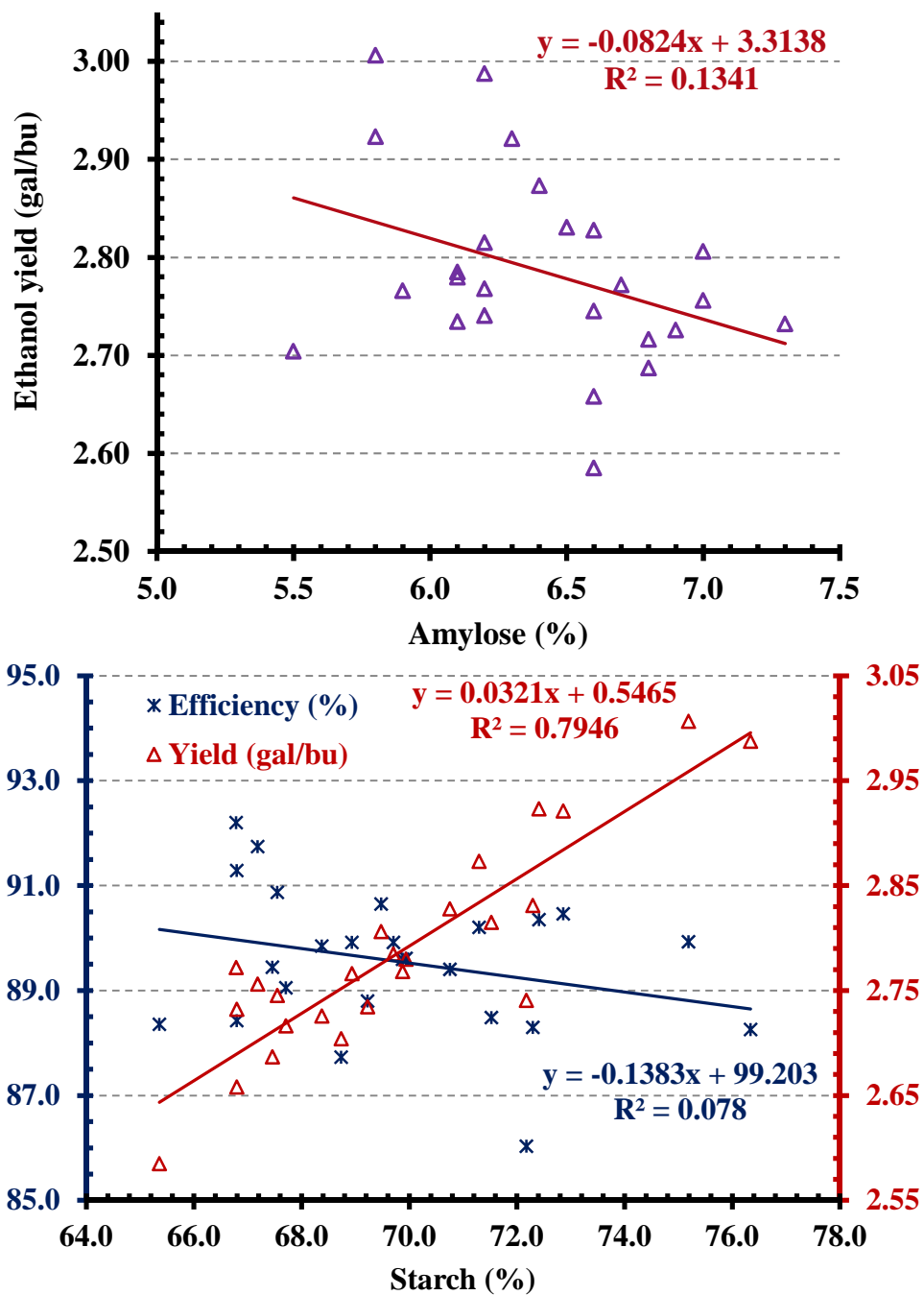
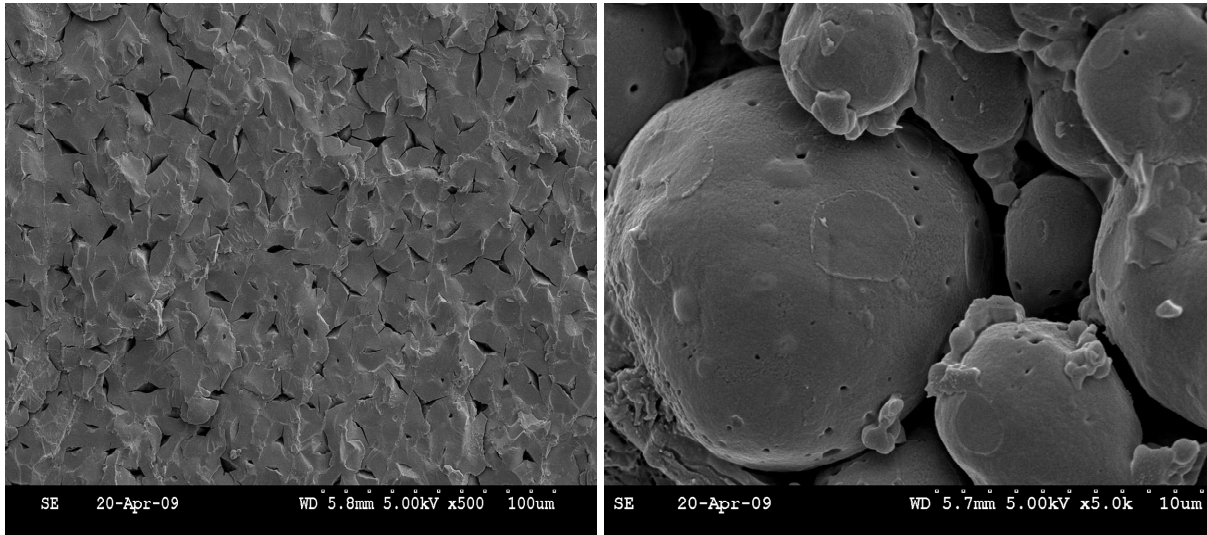


Figure 2.1. Relationship between amylose contents and ethanol yield (top); starch contents of waxy grain sorghum and fermentation efficiency and ethanol yields (bottom).



A: Cracks in endosperm starch granules

B: Many fine pores in starch granules

Figure 2.2. SEM images of waxy grain sorghum endosperm (A) showing cracks and starch granules (B) with many fine pores.

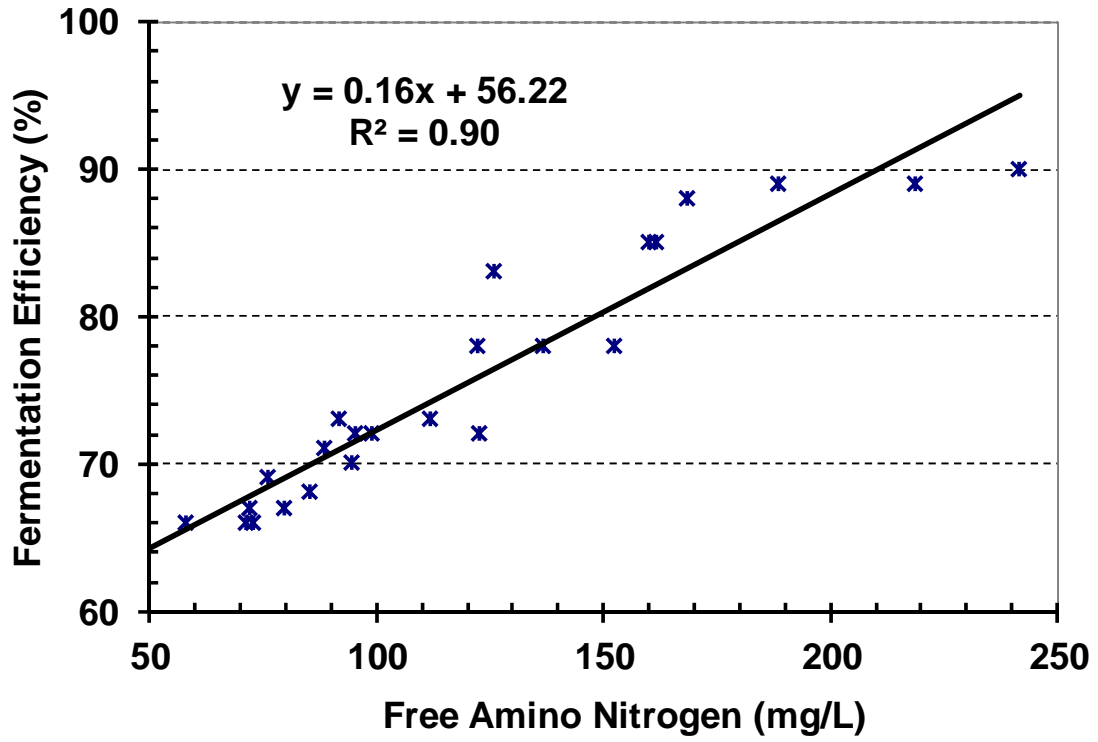


Figure 2.3. Linear correlation between free amino nitrogen contents (mg/L) in original sorghum samples and fermentation efficiency of waxy grain sorghum at 30th hr of fermentation.

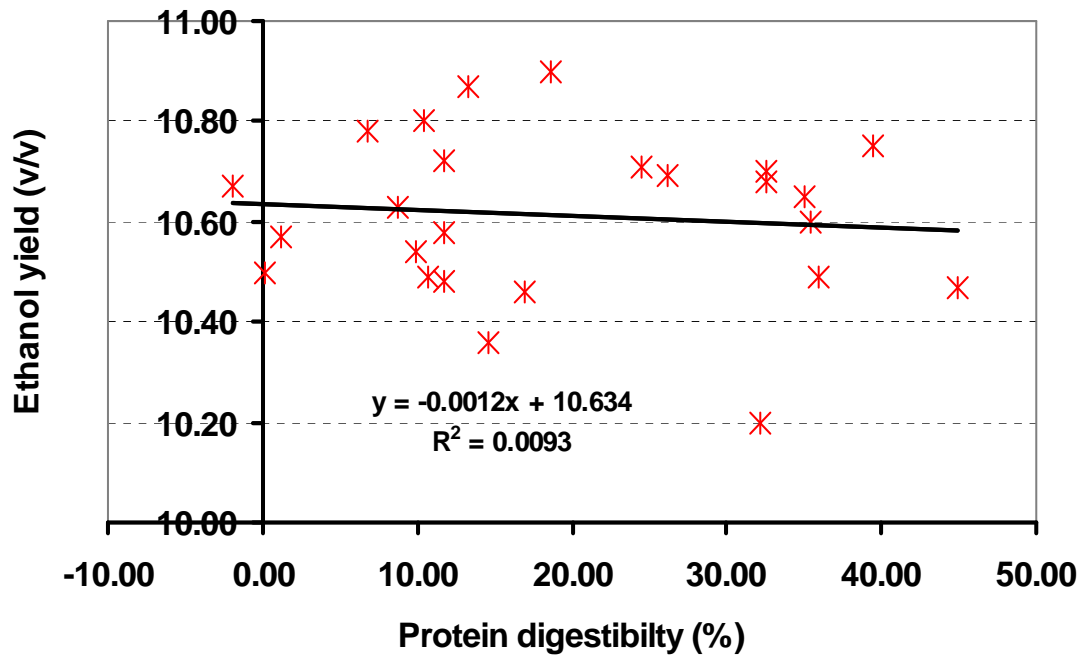


Figure 2.4. Relationship between ethanol fermentation yield and protein digestibility of tested sorghum samples.

Table 2.1. Sample information and contents of major components (% , db) from proximate analysis

Accession #	Local name	Origin	GBSS	Allele	Amylose	Starch	Protein*	Fat	Fiber	Ash	Effic.	Tannin
PI 220636	Nai-Shaker	Afghanistan	no	wx ^a	6.6±0.57	66.80	14.46	6.56	1.56	2.64	88.4	+
PI 23231	Brown Kaoliang	China	yes	wx ^b	6.8±0.38	67.46	13.75	5.02	2.22	2.01	89.4	+
PI 548008	Huang Ke Jiao	China	no	wx ^a	5.5±0.70	68.74	15.09	4.98	1.52	2.54	87.7	+
PI 563576	LV 129	China	no	wx ^a	6.9±0.87	68.38	15.80	5.70	1.59	2.38	89.8	-
PI 563670	L 1999B-17	China	yes	wx ^b	6.2±0.20	76.34	11.22	3.78	1.75	1.85	88.3	-
PI 563671	L 1999B-18	China	yes	wx ^b	5.8±0.90	72.41	12.40	3.60	1.98	1.94	90.3	-
PI 586524	IS 27929	China	no	wx ^a	6.2±0.35	69.88	13.53	5.21	1.86	2.16	89.6	+
PI 586526	IS 27931	China	no	wx ^a	7.0±0.15	69.48	12.40	5.37	1.75	1.89	90.6	+
PI 586529	IS 27935	China	no	wx ^a	6.7±0.45	66.79	14.29	5.69	1.55	1.72	92.2	+
PI 455543	ETS 3634	Ethiopia	no	wx ^a	6.6±0.15	70.76	13.60	5.35	1.53	2.01	89.4	-
PI 586448	Cody	Hungary	no	wx ^a	5.8±0.64	75.19	12.02	4.81	1.77	1.96	89.9	-
PI 586454	Leoti	Hungary	no	wx ^a	6.8±0.72	67.71	14.17	5.11	1.80	2.12	89.1	+
PI 217897	305	Indonesia	yes	wx ^b	5.9±0.20	68.94	12.02	5.18	1.61	1.71	89.9	+
PI 234456	Unknown	Japan	no	wx ^a	6.4±1.12	71.30	12.15	4.94	1.58	1.75	90.2	+
PI 82340	Kaoliang-WX	Korea	no	wx ^a	6.5±0.30	72.30	12.34	4.23	1.89	2.09	88.3	+
PI 87355	Bomususu	Korea	no	wx ^a	6.1±0.40	69.71	14.40	5.17	1.88	2.14	89.9	+
PI 88004	Susu zairai shu	Korea	no	wx ^a	6.1±0.59	69.23	13.68	5.04	1.63	2.16	88.8	+
PI 563015	Kaura Mai Faran Kona	Nigeria	no	wx ^a	6.6±0.51	65.36	15.46	5.64	1.75	1.93	88.4	+
PI 567803	Yungju	South Korea	no	wx ^a	7.0±0.35	67.19	13.51	5.46	1.57	2.06	91.7	+
PI 567809	Unknown	South Korea	no	wx ^a	6.6±0.38	67.55	14.29	5.27	1.51	1.82	90.9	+
PI 567811	Unknown	South Korea	no	wx ^a	7.3±0.20	66.80	13.43	5.09	1.70	1.97	91.3	+
PI 562758	Basuto Red Q2-1-29	USA	no	wx ^a	6.2±1.02	72.18	16.75	4.28	2.03	2.28	86.0	-
PI 563068	IS 8303	USA	no	wx ^a	6.2±0.83	71.53	14.30	3.00	2.25	2.06	88.5	-
PI 563402	IS 10497	USA	no	wx ^a	6.1±0.10	69.94	15.04	3.78	1.94	2.49	89.6	-
	Ellis	USA, wild	no	wx	6.3±0.10	72.86	13.28	4.46	1.91	1.71	90.5	-

*: Protein contents were calculated by 6.25x N contents from the Leco method (AOAC method 990.03); Effic.= efficiency; + indicates presence of tannins; - indicates absence of tannins.

Table 2.2. Differential scanning calorimetry properties of waxy grain sorghum flour

	Onset Temp (°C)	Peak Temp (°C)	Conclusion Temp (°C)	2nd Peak Temp (°C)	Enthalpy of gelatinization (ΔH ,J/g)
Min	69.91	75.59	81.47	99.00	8.19
Max	73.51	78.13	94.38	102.6	11.88
Mean	71.58	76.54	84.27	100.1	9.62

Values are average of two measurements.

Among 25 varieties, only 4 had the 2nd peak--- amylose-lipid complex.

Table 2.3. Chemical composition of distillers dried grains with solubles from waxy and non-waxy grain sorghum varieties (% , db)

Sample ID	Starch	Protein	Lipids	Fiber	Ash
Waxy	0.44-0.72	33.3-42.0	9.5-16.3	3.2-6.0	5.8-7.9
Non-waxy	2.0-5.7	30.3-36.6	10.8-12.5	7.5-11.6	2.1-5.3

Chapter 3 - Germination-Improved Ethanol Fermentation Performance of Tannin Sorghum in a Laboratory Dry-Grind Process²

Abstract

A tannin sorghum cultivar with 3.96% tannin content was used to study the effects of germination on its ethanol fermentation performance in a laboratory dry-grind process. Tannin sorghum sample was germinated for 3 and 4 days. Original and germinated samples were analyzed for tannin, starch, protein, free amino nitrogen (FAN), and glucose content. Endosperm structures and flour pasting properties of germinated and non-germinated sorghum samples were examined using a scanning electron microscope (SEM) and rapid visco analyzer (RVA). Germination reduced tannin content from 3.96% to negligible levels. The free fermentable sugars (glucose, maltose, and maltotriose) in the germinated samples were significantly higher than those in the non-germinated control. Judged by the starch (starch plus dextrin) and free amino nitrogen contents in the mashed samples, germination improved degree of hydrolysis for starch by 13–20% and for protein by 5- to 10-fold during mashing. Germination significantly shortened the required fermentation time for ethanol production by 24–36 hr, increased ethanol fermentation efficiency by 2.6–4.0%, and reduced the residual starch content in the distillers dried grains with solubles (DDGS) compared with the non-germinated control. Ethanol yield for the 3-day germinated samples was 2.75 gallons/bushel, which was 3.1% higher than the 2.67 gallons for the non-germinated control. Ethanol yield for the 4-day germinated sorghum was 2.63 gallons/bushel due to excessive loss of starch during germination.

Introduction

In recent years, there has been increasing interest in producing ethanol from grain sorghum to meet the massive demand for renewable fuel. Sorghum is one of the most important crops in the United States; it is the third most important cereal crop after corn and wheat based on production and it is more drought-tolerant than wheat and corn ([U.S. Grains Council, <http://www.grains.org/sorghum>](http://www.grains.org/sorghum)). Because of climate diversity and continuing decline of water

² This chapter has been published in 2009 in *Cereal Chemistry* 86:597-600.

resources, the use of our dry land for sorghum cultivation is becoming increasingly important and can help ensure sustainable economic development and rational economic distribution.

Tannin is the primary nutrient-limiting component in grain sorghum. High levels of condensed tannin can reduce starch and protein digestibility up to 10% (Leeson and Summers 1997). Ratnavathi and Sashidhar (2000) reported that activities of intrinsic enzymes in tannin genotypes were lower than those in low tannin genotypes. Zhan et al (2003) reported that extrusion reduced tannin content and increased sorghum digestibility, ethanol yield, and fermentation efficiency. Germination has been used to increase starch and protein digestibility for food applications (Evans and Taylor 1990). Germination of sorghum could activate intrinsic enzymes in sorghum seeds, facilitating the breakdown of starch and protein matrices, and resulting in increased levels of monosaccharides, oligosaccharides, and free amino acids. Free amino acids are essential nutrients for yeast growth and are beneficial to ethanol fermentation (Ratnavathi and Ravi 1991; Taylor 1983; Thomas and Ingledew 1990). Using germinated sorghum to produce ethanol may have advantages over the normal process using sorghum meals and could achieve higher ethanol yield and fermentation efficiency because of activated intrinsic enzymes, decreased tannin content, and increased starch digestibility in the germinated sorghum. A negative effect of germination is loss of fermentable sugars in respiration of the seeds during germination.

The objective of this research was to investigate effects of germination on sorghum fermentation efficiency and ethanol yield.

Materials and Methods

Grain Sorghum and Germination

A sorghum cultivar with 3.96% tannin was used. Germination was achieved by rinsing sorghum seeds with tap water. Seeds, covered with four layers of wet gauze, were rinsed with tap water for 3 min then germinated at room temperature for 3 or 4 days. During germination, samples were rinsed at 2-hr intervals with tap water for 1 min during daytime. After germination, sorghum samples were dried in an oven at 50°C for 48 hr to achieve a final moisture content of 10% (wb). Samples for chemical analysis were milled through a 0.25-mm screen in a cyclone mill (Udy, Ft. Collins, CO). Samples for ethanol fermentation were ground into flour in a grain

mill (Magic Mill III plus, Magic Mill Products & Appliances, Monsey, NY) with particle size of <1 mm.

Microorganism and Preparation of Mash

Yeast strain. The *Saccharomyces cerevisiae* strain ATCC 24860 was used for ethanol fermentation and was maintained on yeast extract/peptone/dextrose (YPD) agar slants sealed with sterile mineral oil at room temperature. The strain was subcultured to YPD agar slants and incubated at 25°C for 3 days before being cultured in a preculture broth (containing 20 g/L of glucose; 5.0 g/L of peptone; 3.0 g/L of yeast extracts; 1.0 g/L of KH₂PO₄, and 0.5 g/L of MgSO₄·7H₂O) for final inoculation. The 48-hr yeast culture had a cell concentration of $\approx 2.5 \times 10^8$ cell/mL.

Liquefaction. Sorghum samples (31.0–34.0 g containing 21.00 g of starch) mixed with distilled water (100 mL) in 250-mL Erlenmeyer flasks were liquefied using a thermostable α -amylase, Liquozyme (240 KNU/g) from *Bacillus Licheniformis* (Novozymes, Franklinton, NC). Mash liquefaction occurred in two phases. First, mashes were combined with Liquozyme (10 μ L, ≈ 3 KNU) and held at 95°C in a water bath shaker (Labline microprocessor, Melrose Park, IL) for 45 min with a rotation rate at 160 rpm. Subsequently, mash temperature was reduced to 80°C at which liquefaction was continued for another 30 min after the addition of more Liquozyme (10 μ L, ≈ 3 KNU) to each flask.

Saccharification. After temperature of the liquefied mash was reduced to 60°C, glucoamylase (Spirizyme, 750 AGU/g, from *A. niger*, Novozymes) was added to each flask at 150 AGU/g of starch. Flasks were kept at 60°C for 30 min in a water bath shaker rotating at 160 rpm. Flasks with finished sorghum mashes were removed from the water bath and cooled to $\approx 30^\circ\text{C}$. Mashes were adjusted to pH 4.2–4.3 with 2N HCl before inoculation with yeast.

Fermentation

Sorghum mashes were inoculated with 5 mL of yeast preculture (cell concentration of $\approx 1.5 \times 10^7$ cells/mL), which was prepared as Wu et al (2006) described. Ethanol fermentation was performed at 30°C for 72 hr in an incubator shaker (model I2400, New Brunswick Scientific, Edison, NJ) operating at 150 rpm. Flasks were sealed with S-bubblers filled with ≈ 2 mL of mineral oil. Fermentation was conducted in duplicate. Ethanol concentrations in fermentation broths were determined at different time intervals and were also monitored by measuring the

total weights of the fermentation flasks because the weight loss by CO₂ evolution is proportional to the amount of ethanol produced during ethanol fermentation following the method of Wu et al (2006).

Pasting Properties of Sorghum Flour by RVA

A rapid visco analyzer (model RVA-3c, Newport Scientific Ltd., Warriewood, Australia) was used to determine pasting properties of sorghum flours. Sorghum flour (4.0 g, 14% mc) and water (25 mL) were mixed at 50°C; the slurry was held at that temperature for 1 min then heated from 50 to 95°C. The hot paste was held at 95°C for 2.5 min, cooled to 50°C, and held at 50°C for 2 min. The total process was 13 min.

Morphological Structure of Sorghum Endosperms

Endosperm microstructures of germinated and normal sorghum seeds were examined using a scanning electron microscope (SEM) (Hitachi S-3500N, Hitachi Science System, Japan) with an accelerating voltage of 5.0 kV. Samples were vacuum-coated with a mixture of 60% gold and 40% palladium particles.

Analytical Methods

Sample moisture, starch, and crude protein contents were analyzed by using AOAC official methods 925.10, 996.11, and 990.03 (AOAC International 2000), respectively. A factor of $N \times 6.25$ was used to calculate crude protein content. Free amino nitrogen (FAN) was analyzed by the European Brewery Convention (EBC 1987) method. Tannin was assayed using the method of Price et al (1978). Glucose, maltose, maltotriose, glycerin, and ethanol in the samples were determined by HPLC according to the method described by Wu et al (2006). Analysis of variance (ANOVA) and least significant difference (LSD) at $P < 0.05$ were conducted using statistical software (SAS Institute, Cary, NC).

Results and Discussion

Ethanol Production from Germinated Grain Sorghum

Germination had a significant effect on ethanol yield and conversion efficiency when tannin sorghum samples were used for ethanol production. Germinated sorghum produced higher ethanol concentrations than non-germinated sorghum (8.43 and 8.27%, w/v, for 4-day and 8.12%

for 3-day germinated samples in the finish beers with volumes of 120–125 mL, which were equivalent to 13.16, 13.11, and 12.56%, v/v, standardized per 100 mL, respectively) and required less time to reach the highest concentrations (36 hr for germinated sorghum vs. 72 hr for non-germinated). The conversion efficiency was calculated by dividing the actual ethanol yields measured using HPLC by the theoretical ethanol yield (assuming 1 g of starch could be hydrolyzed into 1.11 g of glucose, and each gram of glucose could generate 0.511 g of ethanol). Conversion efficiencies of germinated sorghums were 86.81% for the 3-day germinated sorghum and 87.14% for 4-day germinated sorghum, which were 2.6–4.0% higher than the 83.20% for the non-germinated sorghum (Figure 3.1). Increases in fermentation rate may be due to enzyme activity. Germination increased enzyme activities in sorghum seeds. Enzymes such as α -amylase, β -amylase, and other glucanases are important for starch hydrolysis. Among these malting enzymes, α -amylase is the most important in sorghum (Ratnavathi and Ravi 1991). Proteinase and carboxypeptidase activities may also be activated during germination, which could have contributed to the favorable results (Evans and Taylor 1990). Actions of proteinases break down protein matrices, which not only releases more starches and leads to more available starch and higher ethanol yield but also generates FAN, resulting in high fermentation rate (Evans and Taylor 1990; Taylor 1983). Results of chemical analysis (Tables 3.1 and 3.2) and SEM images also confirmed these results. Ethanol yields per bushel would be 2.67 gallons for non-germinated sorghum, 2.75 gallons for 3-day germinated, which was a 3.1% improvement. Although the fermentation efficiency for the 4-day germinated sorghum was higher than both the 3-day germinated and non-germinated sorghum, the ethanol yield for the 4-day germinated sorghum was 2.63 gallons/bushel. The reason for the lower ethanol yield for the 4-day germinated sorghum could be the excessive loss of starch during germination (Table 3.1).

Effect of Germination on Chemical Composition and Physical Properties of Sorghum

Chemical composition change. Table 3.1 shows changes in starch, glucose, maltose, maltotriose, tannin, FAN, and total nitrogen contents during germination. Tannin content decreased significantly in germinated sorghum grain. Increases in glucose, maltose, maltotriose, and FAN in germinated samples may be due to activities of activated enzymes during germination, mainly amylases and proteases, which break down starches and proteins into smaller molecules during germination.

Total starch content decreased as germination time increased from 3 to 4 days. This could result from development and growth of embryos during germination that consumed some generated glucose and released carbon dioxide and water. Although free amino acid content in the germinated samples was significantly higher than that in the original non-germinated sample, total nitrogen content essentially did not change, which implies the proteins converted between different forms (structural, functional, or degraded forms) but was not lost during germination.

Pasting properties of sorghum flour. The effect of germination on viscosity is shown in Figure 3.2. Germinated sorghum had a higher peak viscosity than the non-germinated sorghum, and germinated samples took less time to reach peak viscosity. In contrast, the non-germinated sorghum did not show a trough viscosity during heating and holding periods but did show a gradual increase during the course of heating, holding, and cooling because non-damaged starch granules continue to swell as the water moves through the granule slowly, inhibited by the structure, which has no damage by the intrinsic enzymes. The final viscosity of non-germinated sorghum flour was much higher than that of germinated sorghum flour. Results indicated that starch in germinated sorghum flour is much easier to swell and breakdown than that in non-germinated sorghum flour. Also, starches in germinated sorghum may undergo some hydrolysis during pasting because of the activities of activated amylases. Setback viscosities of non-germinated sorghum were much higher than those of germinated sorghum. The higher setback viscosity of the non-germinated sorghum was most likely caused by the leached amylose, and the lower setback viscosity of germinated samples could be the result of partial hydrolysis of starches by the activated intrinsic amylases.

Morphological structure of sorghum endosperms. Figure 3.3A shows the endosperm of non-germinated sorghum seed, where intact starch granules are wrapped in relatively thick cell wall, whereas Figure 3.3B and C shows endosperm of germinated sorghum seeds, where starch granules are wrapped in relatively thin cell wall. Many tiny holes on starch granules of germinated seeds were observed. Starch granules in endosperm of 3-day germinated sorghum were attacked less severely than those of 4-day germinated sorghum. Starch granules around the germ (Figure 3.3D) of the germinated seed were attacked more significantly than those in the endosperm. These results agree with the results of chemical composition change in Table 3.1. The 4-day germinated sorghum had lower starch and FAN contents and relatively higher content of simple carbohydrates from hydrolyzed starch compared with 3-day germinated seed.

Therefore, germinating sorghum seeds could be a good way to activate intrinsic enzymes to help degrade starches and proteins and to increase ethanol yield and conversion efficiency. However, longer germination time might have a negative effect on ethanol production because of excessive loss of starch. Probably 3-day or less germination is the optimal option for the conversion from starch to ethanol.

Compositional changes during mashing and after fermentation. Table 3.2 shows differences in starch, glucose, maltose, maltotriose, FAN, and total nitrogen contents in sorghum mashes and distillers dried grains with solubles (DDGS). Total starch in the non-germinated sorghum mash was higher than that in germinated sorghum mashes, and starch content in mash from 3-day germinated sorghum was higher than from 4-day germinated sorghum. Although total starch content in germinated sorghum were lower than that of non-germinated sorghum, ethanol concentration from the same amount of 4-day and 3-day germinated sorghums was greater (8.43 and 8.27% in the finish beer, w/v) than that (8.12%, w/v) from non-germinated sorghum. Germinated sorghum must have some mechanism to compensate for starch lost during germination and make more efficient use of resources during the follow-up mashing and fermentation processes. Activation of the intrinsic enzymes could be the main cause for this. Higher fermentable sugars and FAN contents in the germinated sorghums indicate the actions of such enzymes during germination (Table 3.1). The more fermentable sugars (glucose, maltose, and maltotriose) in mashes and less residual starch in DDGS of germinated sorghums (Table 3.2) suggested that germination helped release more starch granules from germinated sorghum and improved digestibility of sorghum starch.

In addition to improvement in starch digestibility, germination also increased protein digestibility of sorghum. Free amino nitrogen content in mashes from germinated sorghums was much higher than that in mash from non-germinated sorghum (Table 3.2). This indicates that more proteins were hydrolyzed by the activated intrinsic proteases in the germinated sorghum (during germination and mashing) than in the non-germinated sorghum. This damaged protein matrices, released more formerly embedded starch granules, and resulted in better digestibility of both starch and protein in germinated sorghum. The higher contents of fermentable sugars and free amino acids in mashes of germinated sorghum significantly improved performance of the yeast and contributed to the faster fermentation rate, higher ethanol concentration, and fermentation efficiency (Figure 3.1). This agrees with results of a previous study by Pickerell

(1986), the higher the initial content of FAN, the greater the rate of ethanol production, but the rate is greatly affected by amount of sugar in the mash.

In summary, germination is a possible way to treat tannin sorghum to improve its performance in ethanol production. This treatment decreased tannin content, activated intrinsic enzymes to break down the protein matrix and release formerly embedded starches, increased ethanol yield, enhanced efficiency of fermentation conversion, and shortened fermentation time. Actions of activated intrinsic enzymes in the germinated sorghum improved digestibility of starch and protein and compensated for the starch loss due to respiration of seeds during germination.

Acknowledgments

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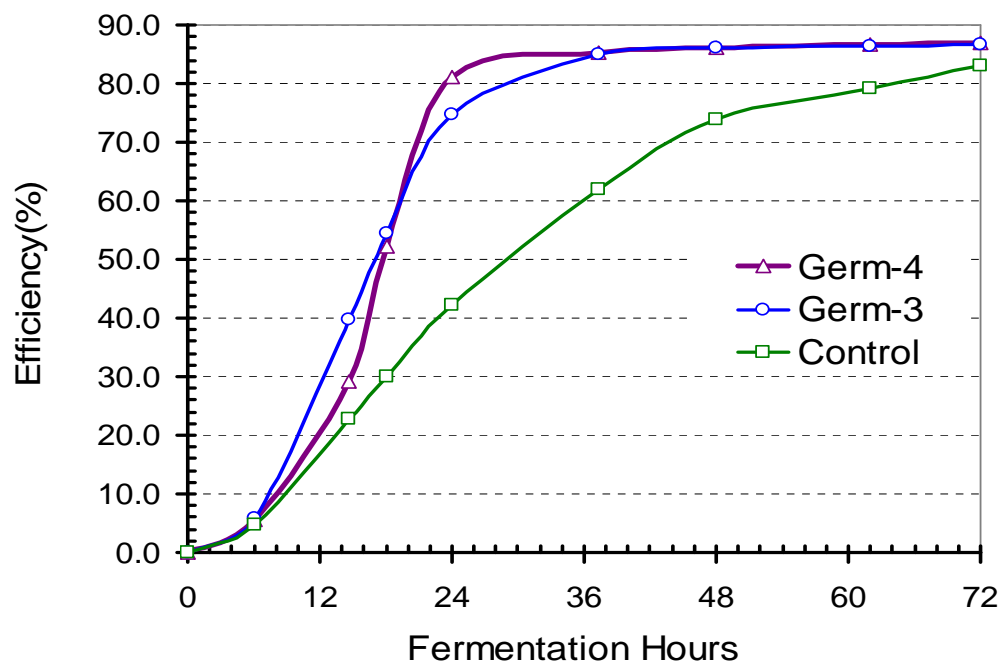


Figure 3.1. Fermentation efficiency as a function of fermentation time; Germ-3 and Germ-4 indicate 3-day and 4-day germinated grain sorghum, respectively.

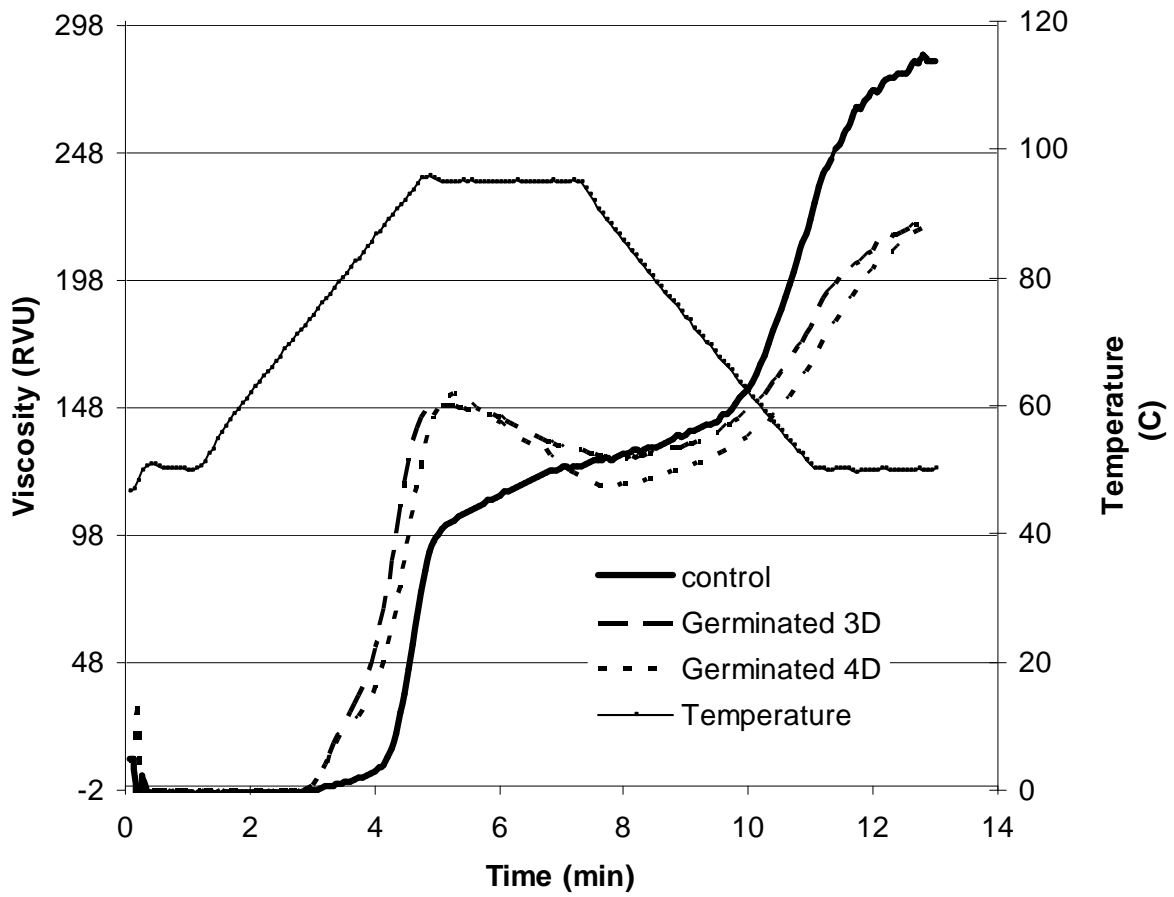


Figure 3.2. Pasting properties of non-germinated (control), 3-day and 4-day germinated sorghum flours as tested on an RVA using the 13 min standard procedure.

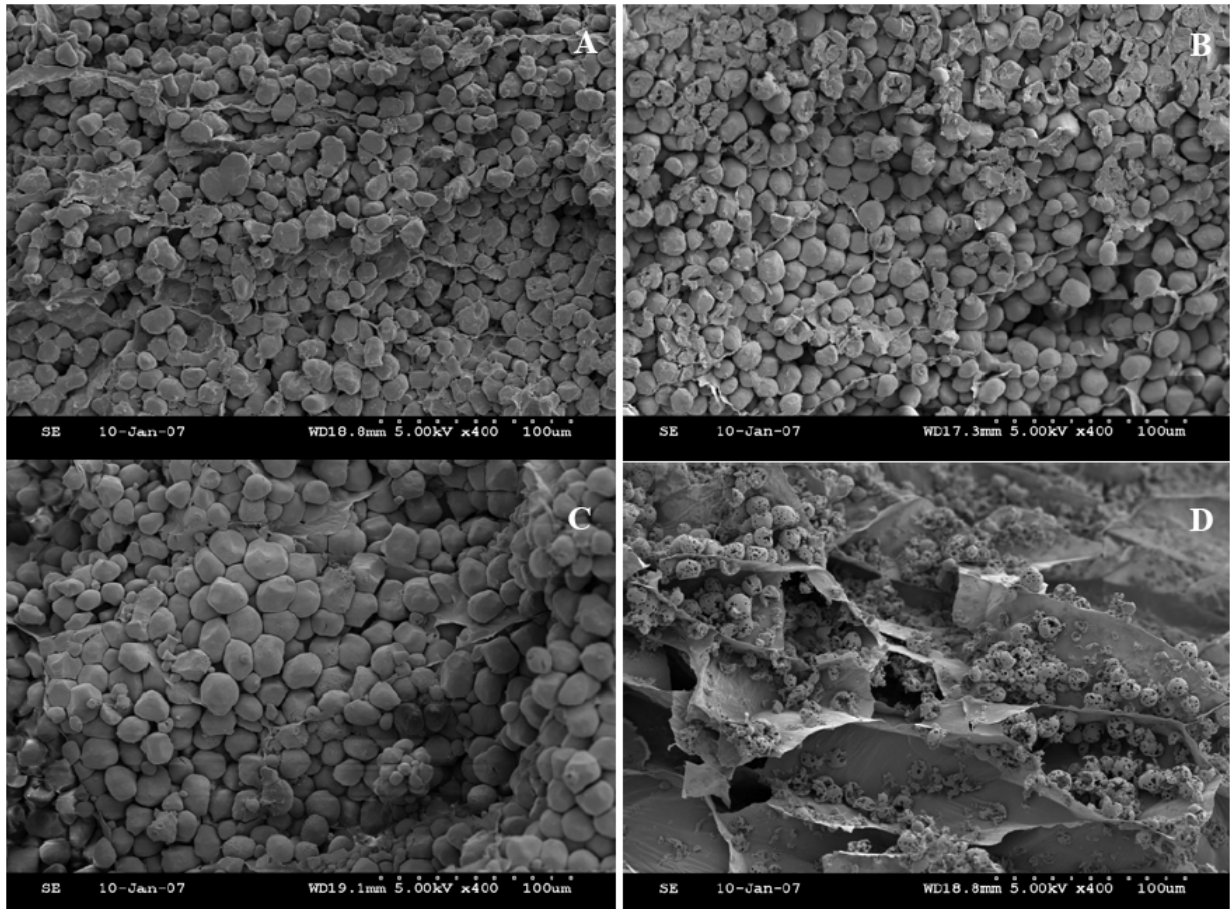


Figure 3.3. SEM images of sorghum seeds. A, Endosperm of non-germinated sorghum. B, Endosperm of 3-day germinated sorghum. C, Endosperm of 4-day germinated sorghum. D, Endosperm close to germ of 4-day germinated sorghum.

Table 3.1. Chemical composition changes during germination

Sample	Starch ^a (%)	Glucose (%)	Maltose (%)	Maltotriose (%)	Tannin (%)	FAN (mg/L)	Total nitrogen (%, Protein)	Ethanol (%, v/v)
Control	66.43a	0.36	0.07	0.57	3.96a	86.65c	11.24	10.56
Germinated 3D	65.61b	1.10	0.53	0.64	0.0113b	252.7a	11.89	13.11
Germinated 4D	62.47c	2.19	1.25	0.84	0.0121b	114.7b	12.11	13.16

^a Total starch includes glucose, maltose and oligosaccharides;

Data in the same column followed by different letters are different at the 0.05 level.

Table 3.2. Chemical composition changes during mashing and after fermentation

Composition	Mashed Samples*			DDGS		
	Non-germ	3day-germ	4day-germ	Non-germ	3day-germ	4day-germ
Starch ^a (%)	10.16a	6.85b	3.81c	2.31a	1.40b	0.94c
Glucose (%)	27.15	31.12	34.46	0.08	0.07	0.10
Maltose (%)	10.18	4.49	4.21	1.63	1.49	1.61
Maltotriose (%)	2.93	1.37	1.37	0.58	0.48	0.54
FAN (mg/L)	100.7	648.6	1,113	147.6	297.1	834.9
Total nitrogen ^b (%, Protein)	11.68	11.85	12.23	31.29a	31.74ab	30.69b
Ethanol concentration (%, v/v)	10.56	13.11	13.16			

^a Starch data in mashed samples were measured after washing twice with 10 mL of water and centrifuging at 20,000 g for 10 min.

^b Results of 6.25*nitrogen from Leco analysis. Data in the same row under the same category (mashed samples or DDGS) followed by different letters are significantly different at the 0.05 level.

* mashed samples were collected for analysis at the end of mashing.

Chapter 4 - Properties of Field-Sprouted Sorghum and Its Performance in Ethanol Production³

Abstract

The objective of this research was to investigate physicochemical and biochemical characteristics of field-sprouted grain sorghum and its fermentation performance in ethanol production. Five field-sprouted grain sorghum varieties, which received abnormally high rainfall during harvest, were used in this study. Enzyme activities, microstructure, flour pasting properties, kernel hardness, kernel weight, kernel size, flour size and particle distribution of field-sprouted grain sorghum were analyzed. The effect of germination (i.e., sprouting) on conversion of grain sorghum to ethanol was determined by using a laboratory dry-grind ethanol fermentation procedure. Sprouted sorghum had increased α -amylase activity; degraded starch granules and endosperm cell walls; decreased kernel hardness, kernel weight, kernel size, and particle size; and decreased pasting temperature and peak and final viscosities compared with non-sprouted grain sorghum. The major finding is that time required for sprouted sorghum to complete fermentation was only about half that of non-sprouted sorghum. Also, ethanol yield from sprouted sorghum were higher (416-423 L/ton) than that from non-sprouted sorghum (409 L/ton) on a 14% moisture basis.

Introduction

The U.S. demand for ethanol has increased sharply in recent years. Currently, feedstock for fuel ethanol production is ~95% corn grain and ~4% sorghum grain (RFA 2007). Researchers and ethanol producers consider grain sorghum a viable and renewable feedstock (i.e., technically acceptable, fits the infrastructure, and can be economically viable) for ethanol, and sorghum could make a larger contribution to the nation's fuel ethanol requirements (Farrell et al 2006; Rooney et al 2007; Wu et al 2006, 2007).

Both ethanol yield and fermentation efficiency have been used to evaluate the performance of feedstocks in ethanol production. Recent research shows that key factors affecting ethanol yield and ethanol fermentation efficiency of sorghum include starch content,

³ This chapter has been published in 2010 in Journal of Cereal Science 51:374-380.

starch digestibility, level of extractable proteins, protein and starch interaction, mash viscosity, amount of phenolic compounds, ratio of amylose to amylopectin, and formation of amylose-lipid complexes in the mash (Wang et al 2008; Wu et al 2007; Zhao et al 2008). In addition to chemical and physical properties of grain sorghum, Yan et al. (2009) studied the effect of germinated sorghum on ethanol fermentation and fermentation efficiency. Results from laboratory-germinated, tannin-containing grain sorghum (i.e. sorghum with a pigmented testa) showed that germination not only decreased tannin content and improved sorghum fermentation performance, but also activated intrinsic enzymes and shortened fermentation time. To a certain degree, germination of feedstocks may not be negative for ethanol fermentation.

Germination, or sprouting, is a common problem for grain when weather is moist during harvest or the environment is humid during storage. When kernels absorb moisture from their surroundings to a sufficient level, the embryo and endosperm are hydrated. Hydration switches on the metabolism of the embryo, which sends hormonal signals to the aleurone layer, triggering the synthesis of enzymes responsible for digesting components of the starchy endosperm. Germination promotes the development of cytolytic, proteolytic, and amylolytic enzymes that are not active in dry kernels (Bamforth 2006; Dewar et al 1997; Klose et al 2009) and could cause significant changes in kernel composition and physical properties (Agu and Palmer 1996; Bamforth 2006; Beta et al 2000; Dewar et al 1997; Elmaki et al 1999; Iwuoha and Aina 1995; Lasekan et al 1995; Muria and Bechtel 1998; Murty et al 1984; Osuntogun et al 1989; Palmer 1991; Singh and Bains 1984; Swanston et al 1994). Germination not only causes compositional changes in the sorghum grain but also initiates a series of biochemical and physiological changes. Intrinsic enzymes such as amylases, proteases, lipases, fiber-degrading enzymes, and phytases are activated; this disrupts protein bodies and degrades proteins, carbohydrates, and lipids to simpler molecules, which increases digestibility of proteins and carbohydrates in the kernel and makes nutrients available and accessible for enzymes (Chavan and Kadan 1989; Dicko et al 2006; Ratnavathi and Ravi 1991; Subramanian et al 1992; Taylor 1983; Yan et al 2009). Balogun et al. (2006) reported that *in vitro* fermentability of sorghum grain was significantly higher when grain was germinated. Research on baby food also showed that germination can activate enzymes, decrease the level of antinutritional factors (tannins, phytic acid), and increase digestibility of macronutrients, bioavailability of minerals, and content of

essential amino acids (Correia et al 2008; Dicko et al 2006; Subramanian et al 1992; Taylor 1983; Taylor et al 1985).

Sorghum has been malted and used for production of traditional alcoholic and nonalcoholic beverages for centuries (Dufour et al 1992). Malting conditions must be controlled to achieve uniform, high-quality sorghum malts and ensure quality required for food products (Dewar et al 1997). Biofuel ethanol production does not have the same requirements. The most important issues in industrial ethanol production are yield, efficiency, and energy consumption. Our laboratory results in terms of ethanol yield and ethanol fermentation efficiency from artificially germinated tannin sorghum suggest that huge potential energy savings exist in production of ethanol from germinated sorghum grain. Using germination-damaged sorghum for industrial ethanol production might benefit the producer and end user by expanding market uses of what has been historically considered a low-value commodity (Suresh et al 1999; Yan et al 2009).

The objective of this research was to investigate physicochemical and biochemical characteristics of field-sprouted grain sorghum and its fermentation performance in ethanol production.

Materials and Methods

Grain Sorghum

Five field-sprouted sorghum varieties (DK5400, DK5311, Asgrow567, Pio8313, and Pio82G10) from south central Texas, which received abnormally high rainfall during harvest, were used in this study. The received dry samples had visible shoots but no visible mold-contamination. Non-sprouted DK5400 was used as a control. Samples were carefully cleaned, and foreign materials were removed manually. Samples were ground to flour with a Magic Mill III plus grain mill (Magic Mill Products & Appliances, Monsey, NY.) set at the level IV for fermentation. Samples for chemical composition analysis were ground with a Udy cyclone sample mill (Udy, Fort Collins, CO) with a 0.5-mm screen.

Particle Size Analysis

Size distributions of sorghum flour were measured with an LS 13 320 single wavelength laser diffraction particle size analyzer using the Tornado dry powder system (Beckman Coulter Inc., Miami, FL). Samples were run in duplicate.

Morphological Structure of Field-Sprouted Grain Sorghum

Microstructures of field-sprouted sorghum kernels and control grain sorghum were examined with a scanning electron microscope (SEM) with an accelerating voltage of 5.0 kV (Hitachi S-3500N, Hitachi Science Systems, Ltd., Japan). Samples were vacuum coated with a mixture of 60% gold and 40% palladium particles by using a Sputter Coater-Desk II SPUTTER/ETCH UNIT (Denton Vacuum, LLC, NJ).

Measurement by the Single Kernel Characterization System

Kernel hardness, kernel weight, and kernel size of field-sprouted sorghum samples and the control were measured with a single kernel characterization system (SKCS) 4100 (Perten Instruments, Springfield, IL) controlled by Microsoft Windows Software SK4100 as optimized for sorghum (Bean et al 2006). Data presented are the mean values of 300 kernels.

Analysis of Enzyme Activity and Flour Pasting Properties

A Megazyme alpha-amylase assay kit was used to measure α -amylase activity (CU/g). Flour pasting properties were determined with a Brabender Visco-Amylo-graph (VAG, C. W. Brabender Instruments Inc., NJ). For VAG sample preparation, 14 g of sorghum flour with 14% moisture content and distilled water (100 mL) was added to the amylograph bowl at room temperature. A 20-min measurement profile with a heat/cool rate of 7.5°C/min was used as follows: increase the slurry temperature from room temperature to 95°C in the first 6 min, hold at 95°C for 5 min, decrease from 95 to 50°C in 5 min, and hold at 50°C for 2 min. Each sample was analyzed in duplicate.

Microorganism, Preparation of Mash and Inoculation

Dry alcohol yeast (*Saccharomyces cerevisiae*, Red Star Ethanol Red) provided by Fermentis (Milwaukee, WI), was used for simultaneous saccharification and fermentation. Before inoculation, dry yeast was activated by adding 1.0 g of cells into 19 mL of preculture

broth (containing 20 g glucose, 5.0 g peptone, 3.0 g yeast extracts, 1.0g KH₂PO₄, and 0.5 g MgSO₄·H₂O per liter) and incubated at 38°C for 30 min in an incubator operating at 200 rpm. The activated yeast culture had a cell concentration of roughly 1×10⁹ cells/mL.

Liquozyme SC DC (Novozyme, Franklinton, NC), a heat-stable α-amylase from *Bacillus licheniformis* was used for liquefaction. Enzyme activity was 240 KNU/g (One Kilo Novo Unit, KNU, is the amount of enzyme that breaks down 5.26 g of starch per hr at Novozyme's standard method for determination of α-amylase). Spirizyme Fuel (Novozyme, Franklinton, NC), an amyloglucosidase from *Aspergillus niger*, was used for saccharification. Enzyme activity was 750 AGU/g (One AGU is the amount of enzyme that hydrolyzes 1 μmol of maltose per minute under specified conditions).

Whole sorghum flour (30 g, db) was dispersed in a 250 mL Erlenmeyer flask with 100 mL of fermentation broth containing 0.1 g KH₂PO₄ (preheated to about 60°C), and 20 μL Liquozyme (240 KNU/g) were added to the flask. The flasks were transferred to a 70°C water bath shaker operating at 170 rpm. The water bath temperature gradually increased from 70°C to 85°C over about 30 min. After 60 min at 85°C, flasks removed from the water bath shaker and cooled to room temperature. Materials sticking on the inner surface of the flasks were scraped back into the mash with a spatula, and then the inner surface was rinsed with 2–3 mL of distilled water by using a fine-tipped polyethylene transfer pipette. The pH of the mashes was adjusted to around 4.2–4.3 with 2N HCl. After pH of each mash was adjusted, 100 μL amyloglucosidase, 0.3 g yeast extract, and 1 mL activated yeast broth (1×10⁹ cells/mL) were added to each flask. Inoculated flasks were sealed with S-bubblers/airlocks and transferred to an incubator shaker for ethanol fermentation. Each sample was run in duplicate.

Fermentation and Distillation

Ethanol fermentation was conducted at 30°C in an incubator shaker (Model I2400, New Brunswick Scientific, Edison, NJ) operating at 150 rpm for 72 h. The fermentation process was monitored by measuring the weight loss of evolved carbon dioxide (CO₂) during fermentation.

At the end of fermentation, all fermented mash in each 250-mL flask was transferred to a 500-mL distillation flask. Each Erlenmeyer flask was washed with distilled water four times (4×25 mL). The washing water was pooled in the distillation flask, and then the distillation flask was distilled on a distillation unit. Distillates were collected in a 100-mL volumetric flask

immersed in ice water. When distillates in the volumetric flask was approaching the 100-mL mark (~0.5 mL below the mark), the distillation process was stopped. Distillates in the volumetric flask were equilibrated to 25°C and adjusted to 100 mL with distilled water if necessary. Distillates were analyzed for ethanol by a Shimadzu HPLC with a Rezex RCM column (Phenomenex, Torrance, CA) and refractive index detector (Wu et al 2006).

Analytical Methods

Methods for the analyses of dry matter/moisture, starch, crude protein, ash, crude fiber, and crude fat of samples were AOAC Official Methods 930.15, 996.11, 990.03, 942.05, 962.09, and 920.39 (AOAC International 2000), respectively. Free amino nitrogen (FAN) was analyzed by the European Brewery Convention method (EBC 1987) with modification. Grain sorghum flour (150 mg) was mixed with 1.5 mL deionized distilled water in a 2.5-mL microcentrifuge tube, vortexed five times within 10 min, then centrifuged at 12,000 rpm for 20 min. At this point, the supernatant was ready for FAN analysis. Glucose, glycerol, and ethanol in the finished beers were determined by HPLC (Shimadzu Scientific Instruments, Columbia, MD) according to the method described by McGinley and Mott (2008). The column used was a Rezex ROA column (Phenomenex, Torrance, CA), and the detector for HPLC was a refractive index detector (model RID-10A, Shimadzu) with the detection unit maintained at 40°C. The mobile phase was 5 mM sulfuric acid at a flow rate of 0.6 mL/min and the oven temperature was 65°C. HPLC data were analyzed with Shimadzu EZStart 7.4 software. Fermentation efficiency was calculated as a ratio of actual ethanol yield (determined by HPLC) to theoretical ethanol yield (calculated from the total starch content in the sample).

Statistical Analyses

All experiments were performed at least in duplicate. Tabular results presented are mean values of repeated experimental data. An ANOVA was conducted to determine the significant differences at a 5% significance level ($P < 0.05$).

Results and Discussion

Chemical Composition of Field-Sprouted Grain Sorghum and Control Grain Sorghum

Table 4.1 shows chemical composition of the five field-sprouted samples and the control (non-sprouted, DK5400C). The FAN in the non-sprouted sample was lower than that in the sprouted samples even though the non-sprouted sorghum sample had the highest protein content. Enzymatic degradation of protein by activated intrinsic proteases during sprouting resulted in an increase in FAN contents and short peptides, which accounted for the significant increase in FAN levels of field-sprouted sorghum samples (Agu and Palmer 1996; Evans and Taylor 1990; Ogbonna et al 2003; Taylor 1983). These activated intrinsic proteases have optimal temperatures of around 50°C and retain much activity at 70°C for some time (Ogbonna et al 2004). FAN contents in the mashes of sprouted sorghum samples will further increase during the slurry and liquefaction process. Also, α -amylase activity in the non-sprouted control was lower than that in sprouted grain sorghum, which agrees with results reported by Murty et al. (1984). The diverse values of FAN and α -amylase activity also revealed that samples had experienced different degrees or durations of field sprouting. All field-sprouted samples had high starch content (> 66% wb).

Results from SKCS and Particle Size Analyzer

The SKCS originally was designed to analyze wheat kernels but has been modified to measure grain hardness, kernel size, and kernel weight for sorghum (Bean et al 2006). The SKCS can provide rapid measurements of sorghum grain information based on the variability present in the samples. Non-sprouted sorghum had higher values for kernel hardness, kernel weight, and kernel diameter than field-sprouted sorghum (Table 4.1). Hardness is one of the most important traits for grain milling; it affects grain milling quality and parameters such as particle size, damaged starch, and flour water absorption. The hardness index (HI) obtained from the SKCS is inversely related to particle size less than 200 μm . Grains with higher HI values had a lower percentage of small particle size. The field-sprouted Pio sorghum varieties had higher HI than other samples. With the same setting on the mill, the sample with high HI had a larger portion of particles with diameters bigger than 200 μm . In contrast, the portions of smaller particles (<200 μm) in sprouted DK sorghum samples was higher than that in the Pio samples. The non-sprouted DK5400C had the highest HI, highest amount of large particles (>200 μm), and lowest amount

of small particles (<200 μm). The particle size distribution curve (Figure 4.1) of the control sample had two pronounced peaks around 18 and 450 μm , whereas those of the field-sprouted samples had 3 peaks-- an extra peak at about $125 \pm 5 \mu\text{m}$ beside the 18 and 450 μm peaks. The samples with higher HI also had larger particles, whereas samples with a low HI had smaller particles. These results are in agreement with those reported by Beta et al. (1995) and Lee et al. (2002). In addition, Lasekan et al. (1995) reported that sorghum variety affected germination and sugar production from sorghum malts. Our HPLC data agree with the above trends (data not shown). One purpose of this study was to evaluate fermentation performance of sprouted sorghum for ethanol production. Naidu et al. (2007) reported that particle size significantly affects ethanol yield. Our results showed that ethanol yield was inversely related to kernel HI (a linear regression equation with $R^2 = 0.855$). This is probably because sorghum with higher HI had a higher percentage of large particles. Previous research has shown a negative relationship between particle size and ethanol yield (Naidu et al 2007; Kelsall and Lyons 2003).

Morphological Structure of Field-Sprouted Grain Sorghum

Figure 4.2 shows SEM images of endosperm close to germ, endosperm, and whole kernels of field-sprouted and non-sprouted sorghum. Starch granules in endosperm close to germ (Figure 4.2A) had many more holes than those in endosperm (Figure 4.2B). These holes indicate that starch granules were degraded or attacked by activated enzymes during field sprouting. Grain contains abundant enzymes in the germ. While grain kernels are dry, enzymes are inactive (because of enzyme inhibitors) and will remain so until moisture content of the kernels is high enough to trigger germination. The new shoot and root will emerge from the kernel when the embryo begins to germinate. As the intrinsic enzymes (e.g., proteases, amylases, and lipases) in sorghum kernels are activated (Correia et al 2008), the reservoir chemical constituents (e.g., proteins, starch, and lipids) are degraded by these enzymes into simple compounds that are used to make new compounds (i.e., shoot and root). Because of water intake rate and germination, macromolecules in and around the germ are broken down by enzymes more rapidly than those in the endosperm (Figure 4.2A, 4.2B, and 4.2C). Moss (1977) studied the rate of moisture movement into the kernel using autoradiography. There was an initial rapid movement of water into the germ and along the edge of the endosperm region. Because of rapid movement, the effect of germination/sprouting is more pronounced on the germ than on the endosperm.

Enzymes are working not only on starch granules but also on protein and cell walls (Correia et al 2008; Glennie et al. 1983). Figure 4.2 shows starch granules and cell walls of sprouted sorghum kernels (Figure 4.2A and 4.2B); relative position of endosperm, germ and root of sprouted kernels (Figure 4.2C); and starch granules and cell walls of non-sprouted grain sorghum kernels (Figure 4.2D). These SEM images clearly indicate that various degrees of damages occurred to starch granules both around the germ and in the endosperm of sprouted sorghum kernels (Figure 4.2A, 4.2B and 4.2C). Cell walls of the sprouted sorghum kernels also were degraded by the activated intrinsic cell-wall-degrading enzymes and apparently were thinner than cell walls of non-sprouted sorghum kernels. These intrinsic enzymes mainly convert part of the insoluble polymers in sorghum kernels into soluble smaller molecules, which makes the sprouted sorghum a better feedstock for ethanol production. After field-sprouted grain sorghum is harvested, shoots and roots of some field-sprouted kernels might not be noticeable if they have shrunk during drying. Therefore, total mass of field-sprouted grain sorghum kernels might not change. In a laboratory germination test, the significant decrease in mass was due to the loss of solubles during rinsing and loss of shoots or roots during drying (Yan et al 2009). In industrial biofuel production, field-sprouted grain sorghum may be a better feedstock because of its easy digestibility of enzymatically damaged starch granules, thin cell walls, and higher content of readily available sugars.

Pasting Properties of Field-Sprouted Sorghum Flour

The effect of sprouting on viscosity was analyzed with a Brabender Visco-Amylo-graph. The visco-amylo-graph curves of field-sprouted sorghum were significantly different from those of non-sprouted sorghum in terms of peak viscosity, holding strength, final viscosity, peak temperature, and peak time (Figure 4.3). In general, field-sprouted sorghum flour had a short peak time (took less time to reach the peak viscosity), clear holding strength, and low final viscosity (low setback). In addition, field-sprouted sorghum required less time to begin pasting than non-sprouted sorghum, indicating that starch in the sprouted flour swelled easily and consumed less energy during the cooking process. Compared with field-sprouted samples, non-sprouted sorghum had no peak viscosity but a significantly higher final viscosity. This is due to the difference in α -amylase activity and high content of damaged starch granules in the sprouted sorghum compared with the non-sprouted sorghum. Compared with intact starch granules in

non-sprouted sorghum, enzyme-damaged starch granules swell readily and easily break down into small fragments, resulting in low peak and final viscosities in the field-sprouted sorghum pasting profile.

On the other hand, differences in peak viscosity, holding strength, and final viscosity also were observed among the field-sprouted sorghum varieties, which could be due to degree of sprouting and differences in kernel hardness that resulted in different particle sizes and degrees of damaged starch. Obviously, HI of the non-sprouted kernels was significantly higher than that of all sprouted samples. There was an inverse correlation between peak viscosity and kernel HI. Among the five sprouted samples, DK5400 had the lowest HI and highest peak viscosity and Pio 82G10 had the highest HI and lowest peak viscosity. Among all samples, DK5400C had the highest final viscosity and lowest α -amylase activity, whereas Asgrow567 had the lowest final viscosity and highest α -amylase activity (Table 4.1). This indicates that sprouted sorghum samples originally had very different hardness and/or were at very different stages of the germination process because differences in time and duration of exposure to high moisture conditions before harvest (e.g., unfavorable weather in the field) would result in sprouted kernels with different enzyme activities and related degraded products (Evans and Taylor 1990; Ogbonna et al 2003).

Ethanol Production from Field-Sprouted Grain Sorghum

Figure 4.4A shows ethanol yield of five field-sprouted sorghum varieties and the non-sprouted control. The ethanol fermentation process essentially was completed within 36 h for the sprouted sorghum, and ethanol yield did not increase significantly after the 36th h, indicating the fermentation process using field-sprouted sorghum could be stopped at the 36th h after yeast inoculation in the beginning of fermentation. This result agrees with results from a study on laboratory-germinated tannin sorghum (Yan et al 2009) and further confirms that using sprouted grain sorghum for ethanol production could shorten the fermentation time without significantly decreasing ethanol yield. Grain damaged by sprouting may lose value for food applications but may not affect ethanol production and final ethanol yield. In this study, ethanol yield from field-sprouted sorghum actually was slightly higher than that from the non-sprouted control sorghum (Figure 4.4A). The actions of cell-wall-degrading enzymes in the field-sprouted sorghum might

have contributed to this high yield. The fermentation process for sprouted grain could be much shorter than that required for normal grain (Wu 1989; Yan et al 2009).

Fermentation course varied among sorghum varieties in the 18th to 30th h (Figure 4.4A). The two Pio varieties had lower ethanol yield than the other three varieties within the same fermentation period (at 24 h). This might be due to kernel hardness, particle size, and availability of nutrients for yeast in the mash. The harder sorghum had larger particles, which might prevent nutrients from being released rapidly to the mash during this period. However, as the fermentation process proceeds in the mash and water continues penetrating into larger particles, the structures of larger particles eventually would be disrupted and nutrients would be released into the mash. On the other hand, availability of FAN for yeast might affect fermentation course and rate. Pio82G10 had the lowest FAN, highest HI, and lowest ethanol yield during the 18th to 30th h (Table 4.1 & Figure 4.4A).

One of the most important physicochemical changes that occurs during germination is degradation of the proteinaceous matrix that holds starch granules within the cells in the endosperm and conversion of these substances into soluble peptides and amino acids, which contribute to the increased FAN and provides nutrients for yeast growth. The effect of FAN on the fermentation process was further confirmed by the presence of yeast extracts during fermentation (Figure 4.4B). Sorghum mashes with added yeast extract (solid lines in Figure 4.4B) had a much faster fermentation rate and took less time (36 h vs. 72 h) to complete fermentation than sorghum mashes without yeast extract (dashed lines in Figure 4.4B). These results support previous findings that FAN is a positive factor for the fermentation process (Pérez-Carrillo and Serna-Saldívar 2007; Pierce 1982, 1987). *Saccharomyces cerevisiae* can assimilate amino acids and low-molecular-weight peptides but not proteins. The non-sprouted control sample, DK5400C, had the lowest FAN among the samples (Table 4.1) and the lowest ethanol yield at the end of the 72h fermentation both with and without added yeast extract. Without the addition of yeast extract, fermentation rates depended on the availability of FAN in the mashes. Sprouted DK5400 had the highest FAN content among three samples and the fastest fermentation rate and highest ethanol yield both with and without added yeast extract. This further supports the idea that FAN is important for yeast growth and fermentation rate, especially for yeast proliferation. Sprouted sorghum with high FAN content benefits ethanol fermentation efficiency and reduces fermentation time.

The HPLC analysis of finished beer showed a significant amount of sugar left in the finished beer (72 h fermentation) when fermentation was conducted using sorghum mashes without added yeast extract (Figure 4.5). There was little sugar left in the finished beer when yeast extract was added to the sorghum mashes. In addition, the amount of sugar remaining varied among the three samples (peak area is proportional to the sugar concentration). Among three samples without added yeast extract, the non-sprouted control sample (DK5400C) had the most amount of residual sugar left and the field-sprouted sample of the same sorghum variety (DK5400) had the least residual sugar. The three samples appear in the same order when ranked in terms of FAN content and ethanol level: DK5400 > Pio82G10 > DK5400C (Table 4.1 and Figure 4.4). This finding is in agreement with previous observations (Pierce 1982, 1987).

Conclusion

Field sprouting damaged starch granules, protein matrices, and cell walls in sorghum kernels, consequently decreasing kernel hardness, kernel weight, and kernel size. Field sprouting also changed the chemical composition and pasting properties of field-sprouted grain sorghum, which could shorten fermentation time without decreasing ethanol yield. Field-sprouted grain sorghum had relatively high FAN content. The FAN provided efficient buffering capacity and optimal yeast performance, and field-sprouted sorghum had a more rapid fermentation rate than non-sprouted sorghum. FAN played a key role in increasing conversion efficiency for ethanol production. Using weathered and/or sprouted sorghum from regions affected by unusually high moisture events during grain fill and harvest may provide an opportunity for ethanol producers to maintain ethanol production efficiency, while shortening processing time. This could offer sorghum producers an opportunity to receive a premium, or at least a fair market, value for sorghum when such environmental events occur.

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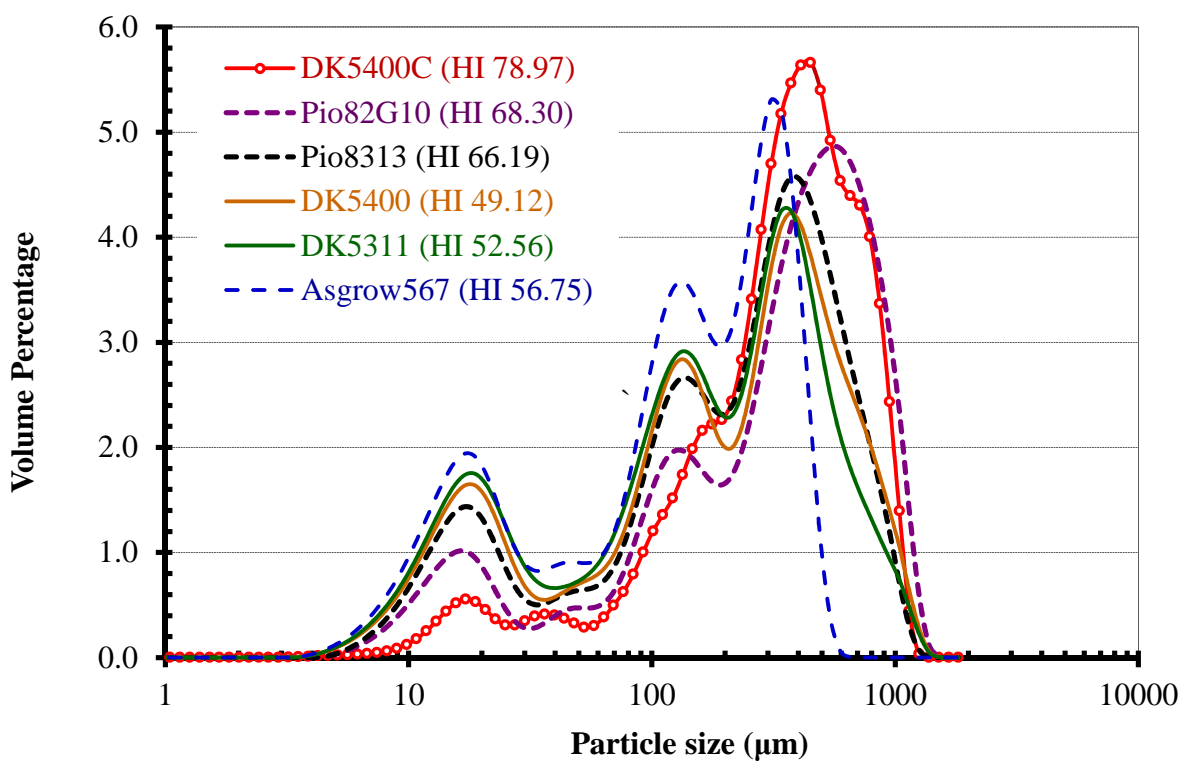
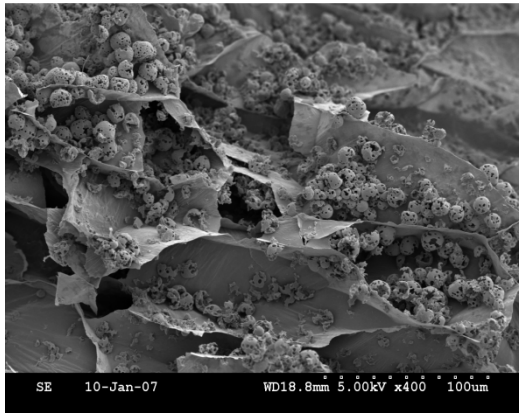
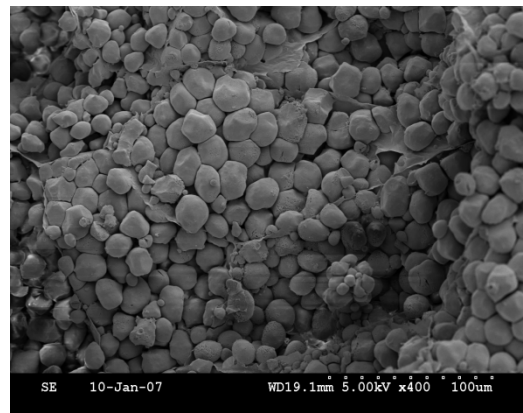


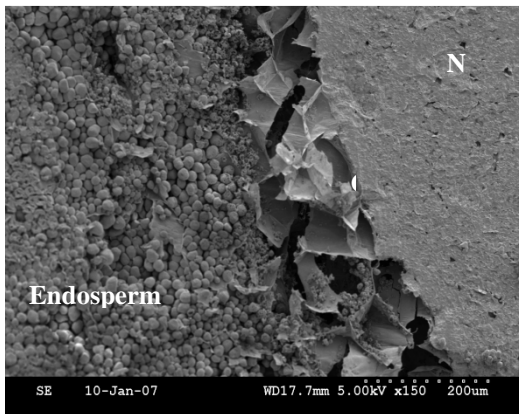
Figure 4.1. Average particle size distribution for field-sprouted sorghum and control sorghum flours. Average hardness index is listed after each sample ID in the legend. Note that the control sample has two pronounced peaks at about 18 and 450 µm, whereas the field-sprouted samples have three peaks, one of which is at around 125 ± 5 µm. Also, note that the samples with higher hardness index values have larger particle sizes.



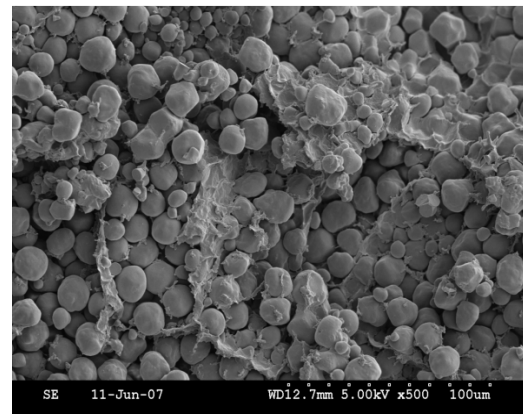
A: Endosperm close to germ



B: Endosperm



C: Whole Kernel



D: Non-sprouted endosperm

Figure 4.2. Scanning electron microscope images of starch granules: A: Endosperm close to germ of field-sprouted sorghum. B: Endosperm of field-sprouted sorghum. C: Cell walls of field-sprouted grain sorghums. D: Non-sprouted sorghum.

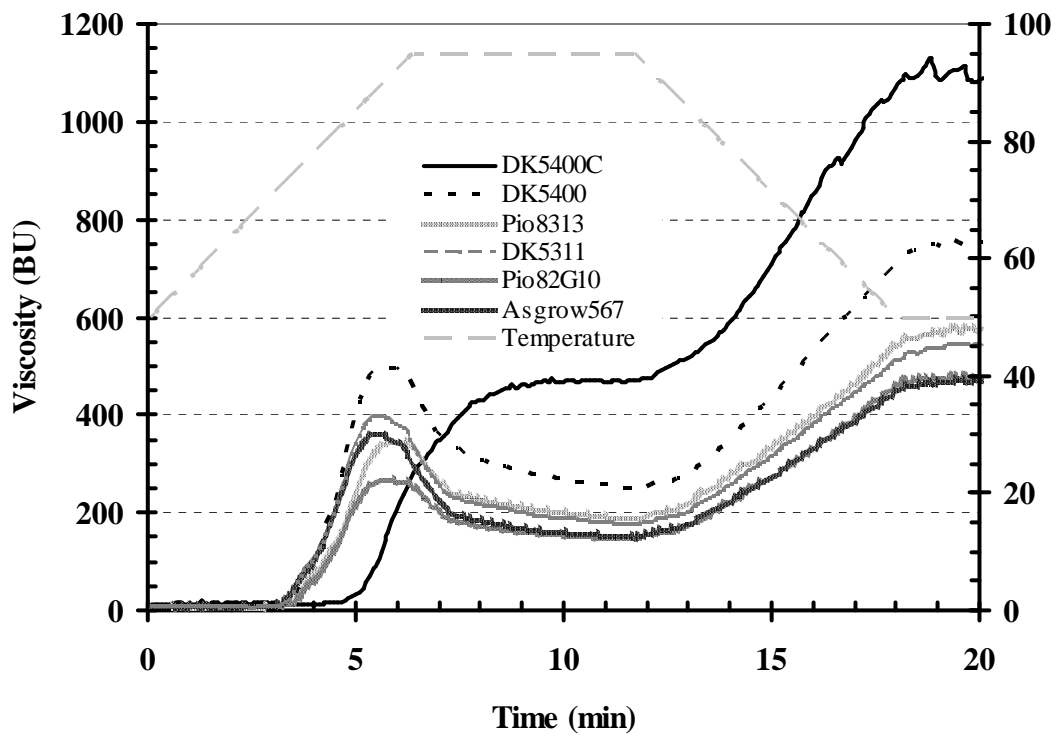


Figure 4.3. Pasting properties of flours from five field-sprouted sorghum varieties and a non-sprouted control (DK5400) on a Brabender Visco-Amylo-graph using a 20 min standard procedure.

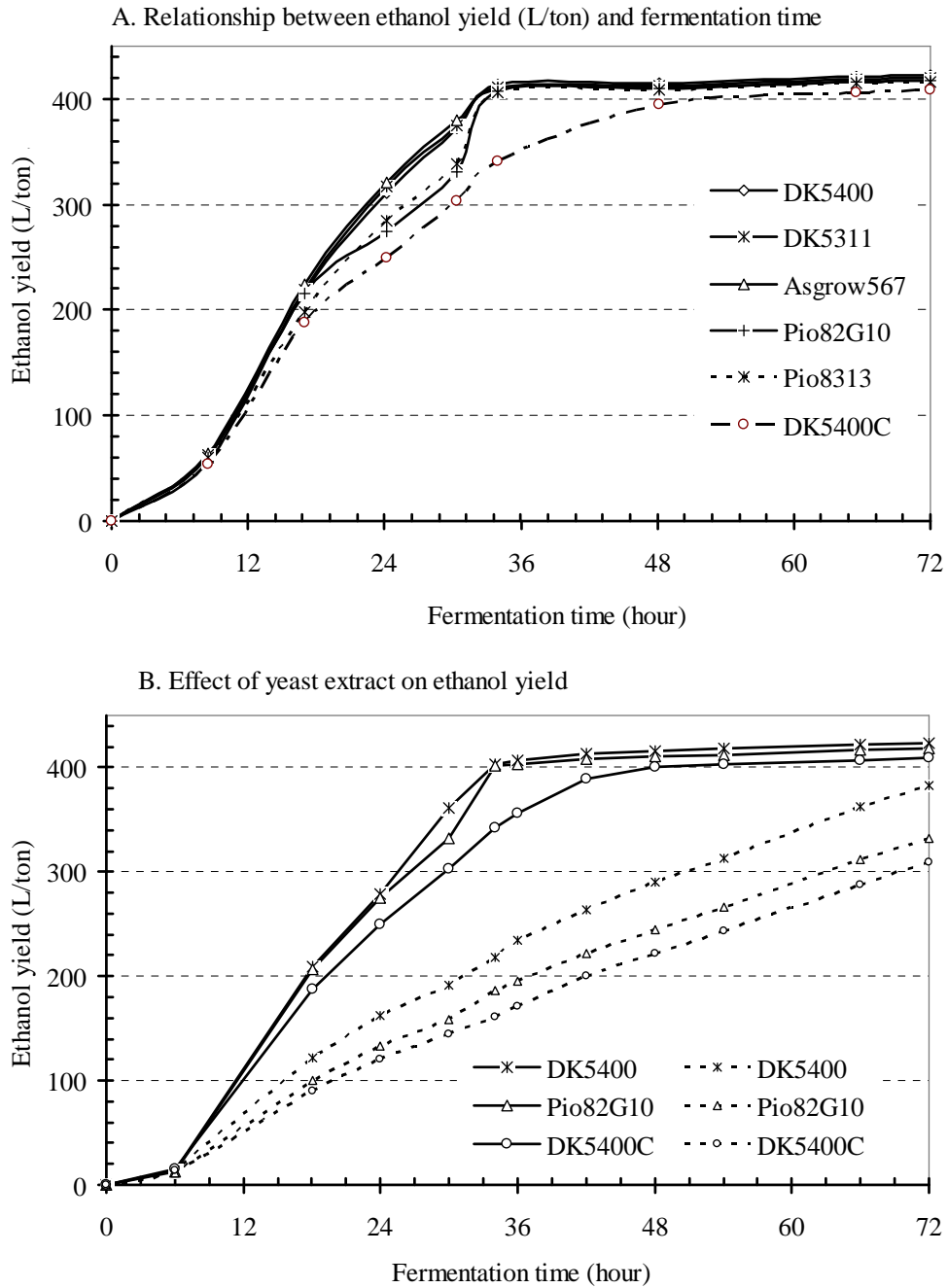


Figure 4.4. Ethanol yields from five field-sprouted sorghum variety samples and a non-sprouted sorghum sample (DK5400C) (A) and Effect of yeast extract on ethanol yields (B).

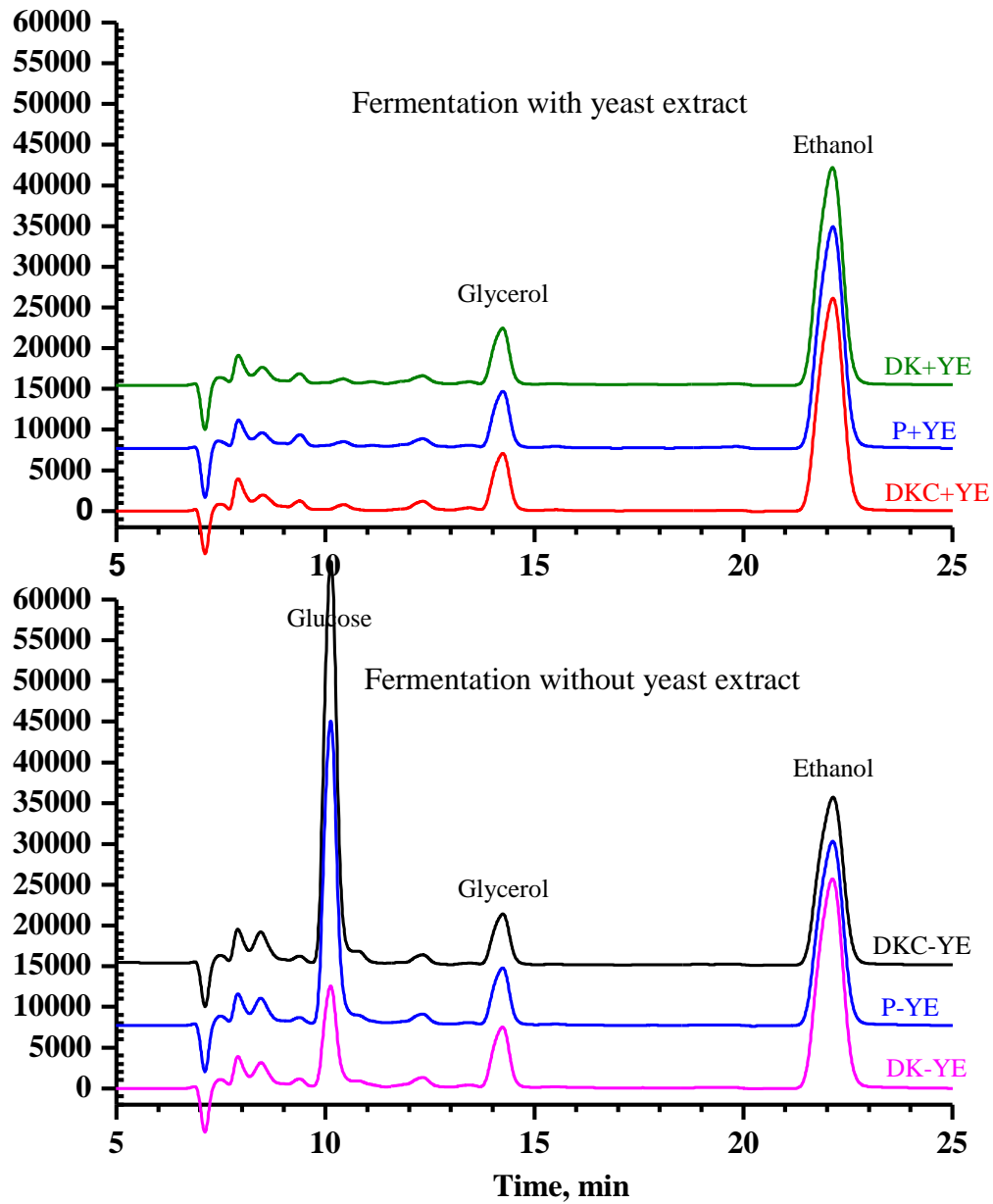


Figure 4.5. Ethanol yield and residual glucose contents in the finished beers (DKC: non-sprouted DK5400C; P: field-sprouted Pio82G10; DK: field-sprouted DK5400; +YE: with yeast extract; -YE: without yeast extract).

Table 4.1. Chemical composition, kernel hardness and kernel size of grain sorghum samples

Sorghum samples	Chemical composition (% wb)						FAN ¹ (mg/L)	α -amylase activity (CU/g)	Hardness index	Kernel weight (mg)	Kernel diameter (mm)
	MC ²	Ash	Protein	Fiber	Lipids	Starch					
DK5400 C	10.38 ^f	1.62 ^a	11.59 ^a	1.15 ^e	3.22 ^a	64.50 ^d	162.0 ^e	5.60 ^c	78.97 ^a	30.00 ^a	1.98 ^a
DK5400	12.28 ^d	1.18 ^c	6.66 ^e	2.12 ^{ab}	2.27 ^c	67.30 ^c	221.9 ^c	12.03 ^b	49.12 ^e	24.76 ^c	1.68 ^c
DK5311	12.97 ^a	1.19 ^c	7.02 ^d	2.24 ^a	2.29 ^c	66.78 ^c	234.8 ^{bc}	12.60 ^b	52.56 ^d	25.42 ^{bc}	1.73 ^{bc}
Asg567	11.92 ^e	1.26 ^b	7.60 ^b	2.07 ^{bc}	2.34 ^c	67.80 ^{bc}	284.3 ^a	15.79 ^a	56.75 ^c	25.53 ^b	1.76 ^b
Pio82G10	12.71 ^b	1.10 ^e	7.27 ^c	1.95 ^{cd}	2.40 ^{bc}	69.28 ^{ab}	189.5 ^d	13.35 ^b	68.30 ^b	25.98 ^b	2.01 ^a
Pio8313	12.52 ^c	1.15 ^d	6.96 ^d	1.91 ^d	2.49 ^b	69.65 ^a	258.4 ^{ab}	13.18 ^b	66.19 ^b	26.00 ^b	1.78 ^b

¹ FAN = Free amino nitrogen.

² MC = Moisture content (wb).

Different superscript letters in the same column indicate significant difference ($P < 0.05$).

Chapter 5 - Ozone Treatment on Tannin Grain Sorghum Flour and Its Ethanol Production Performance

Abstract

Grain sorghum lines containing tannins have been reported to have increased resistance to drought, birds, mold, preharvest germination, and higher grain yield than non-tannin grain sorghum lines. However, tannins have been considered an adverse factor in the utilization of sorghum as a feedstock for bio-ethanol production. Ozone is a strong oxidant and is capable of degrading macromolecules such as lignins. Thus we hypothesized that ozone treatment may also reduce tannin activity and increase fermentation efficiency of tannin sorghum lines. Therefore, the objective of this research was to study the physicochemical properties of ozone-treated whole tannin grain sorghum flour and its fermentation performance in ethanol production. Results showed that the ethanol yields from ozone-treated tannin grain sorghums were significantly higher than that from the untreated flour. The fermentation efficiency of ozone-treated tannin grain sorghum was approximately 90%, which was 8-14% higher than that of untreated samples at the 36th hour of fermentation. At the end of 72 hour fermentation, the efficiencies of ozone-treated sorghum flour were 2-5% higher than that of untreated samples. Measured tannin levels of ozone treated samples decreased significantly from 3.8% to 2.7%. Gel permeation chromatography indicated that starch in ozone-treated flours was degraded. Rapid visco analyzer data show that the setback of viscosity of ozone-treated flour was lower than that of untreated flours. Therefore, ozonation could be a novel and useful method to improve ethanol yield and fermentation efficiency of tannin grain sorghum.

Introduction

The use of ethanol as a gasoline alternative has been experiencing a significant increase and ethanol production and production capacity in the United States are still growing. The availability of ethanol is increasing because of US federal government mandates mixing of ethanol in gasoline, which has created a large demand for ethanol and a rapid growth of the ethanol industry. As required by the Energy Independence and Security Act of 2007 and the expanded Renewable Fuels Standard (RFS), annual ethanol production will grow to 15 billion gallons by 2012 and to 36 billion gallons by 2022. Currently, ethanol is mainly produced from

crop-based starch-rich grains in the United States. Grain sorghum has been one of the primary feedstocks for ethanol production. In 2009, more than 30% of the US grain sorghum crop was used for ethanol production. As a major sorghum grower, the state of Kansas used approximately 50% of its 2010 sorghum crop for ethanol production ([Agri-Energy Solutions 2009](#); [Jessen 2010](#)).

Grain sorghum is a viable feedstock for ethanol production ([Wang et al 2008a](#)) and the performance of grain sorghum in ethanol production has been studied and evaluated recently ([Monk et al 1984](#); [Wu et al 2007, 2010](#)). Ethanol conversion efficiency has been intensively studied because of the importance and public concern of net energy gain in ethanol production. Due to the fact that virtually all of the current commercial sorghum lines in the US are tannin free, most of the previous research involving sorghum has been focused on normal grain sorghum types. Little research has been conducted on ethanol production from tannin sorghum lines. Interest in tannin sorghum utilization has increased with the recent health benefits associated with tannins ([Awika and Rooney 2004](#)) and tannin grain sorghum lines have agronomic benefits such as resistance to drought, birds, mold, insects, disease, and higher grain yield than normal, non-tannin grain sorghum ([Hahn and Rooney 1986](#); [Reichert et al 1980](#)). Though growing tannin sorghum has the advantages of less production and storage cost, and higher grain yields, the production of tannin sorghum and its use for ethanol production and animal feed are not desired. This is largely due to the adverse effects of tannin on enzyme activity, starch and protein digestion, and ethanol fermentation efficiency. For identity preserved and specialty markets however, the use of tannin sorghum is very attractive.

Tannins are a group of highly hydroxylated phenolic compounds and are very common in plants. Sorghum tannins have been actively studied for some time now with regards to food or feed uses. Tannins may impact the processing, product quality, and nutritional values of sorghum ([Kobue-Lekalake et al 2009](#); [Awika and Rooney 2004](#); [Elkin et al 1996](#)). Sorghum tannins are located in the outer layers of the kernel, beneath the pericarp in the pigmented testa layer of sorghum grain. Tannins protect sorghum grains from attacks by birds, insects, mold and preharvest germination ([Hahn and Rooney 1986](#); [Reichert et al 1980](#)). Because of their negative nutritional effects in animal nutrition, many studies have been conducted to remove or deactivate tannins from sorghum. [Chibber et al \(1978\)](#) reported that mechanical abrasion/decortication of sorghum coat layers could reduce tannin content of sorghum flour for food uses by physically

removing the tannins. Reichert et al (1980) reported that anaerobic storage of grain sorghum treated with water or HCl solution decreased tannin content in grain sorghum. Daiber and Taylor (1982) steeped sorghum seeds in dilute formaldehyde solution and dilute NaOH to decrease tannin levels. These methods likely degraded or cross-linked the tannins making them inactive. Germination of grain sorghum is another way to reduce tannin content (Reichert et al 1980; Yan et al 2009). Deactivation of tannins during germination was similar to the reduction reported during anaerobic storage of water-treated sorghum. The deactivation mechanism in both processes may be the same, i. e., enzymatic degradation (Yan et al 2009).

Mullins and NeSmith (1986) studied ethanol fermentation from bird-resistant (i.e. tannin containing) and non-bird-resistant (i.e. non-tannin) grain sorghum and reported that high tannin levels greatly reduced the ethanol production rate. Wu et al (2007) reported that tannin content was one of the major factors affecting ethanol conversion efficiency of grain sorghum in lines containing tannins. Ethanol yield and fermentation efficiency increased when tannins were removed or inactivated (Yan et al 2009).

Ozone is a strong oxidant with oxidation potential of 2.07 eV. Ozone has the power of quick degrading vital components in living cells and killing microorganisms. It works at low dosage levels and leaves no residues in the treated product. Because of these many advantages, ozone has been used in water treatment, food processing (Kim et al 1999), and in corn and wheat steeping prior to milling (Dhillon et al 2009; Ruan et al 2004). Previous studies have shown that ozone is able to degrade macromolecules such as lignin (Sugimoto et al 2009), protein and carbohydrates (Wang et al 1999; Wang et al 2008b; Yosef et al 1994). Seo et al (2007) reported chitosan could be depolymerized by ozone treatment. Thus the hypothesis of the current project was that ozone treatment may degrade/inactivate sorghum tannins and reduce its adverse activity during fermentation, thereby increasing the fermentation efficiency of tannin sorghum. Deactivating sorghum tannins prior to ethanol fermentation would provide additional uses for tannin containing sorghum lines grown for special uses, i.e. nutraceuticals, or in areas of the world where tannin containing sorghums are still widely grown.

Materials and Methods

Grain Sorghum

Tannin grain sorghum used in this study was cleaned manually and ground to flour with a Magic Mill III plus grain mill (Magic Mill Products & Appliances, Monsey, NY) set at the level V for fermentation use. Samples for chemical composition analysis were ground with a Udy cyclone sample mill (Udy, Fort Collins, CO) with a 1.0 mm screen.

Experimental Design and Ozonation Treatment

A factorial design was used in this study. Flow rate and treatment time were two factors and were investigated to determine the main effect and the interaction between the two factors during ozonation. Each factor was run at two levels at room temperature. Ozonation was conducted using a randomized design and each treatment was run in replicate. Flour samples (500 g each treatment) were tumbled in a metal drum (MIAG, Braunschweig) equipped with motor-rotation. Ozone gas was generated by a pilot scale ozone generator (Clear Water Tech, Inc., San Luis Obispo, CA, USA; donated by Dr. Joseph Montecalvo, California Polytechnic State University) using oxygen from a SeQual oxygen concentrator (SeQual Technologies, Inc., San Diego, CA, USA) at the set flow rates. The rate of ozone production as a function of time (10, 20, 30, 36 and 40 min) was measured using an iodometric method (Rakness et al 1996). Ozone concentration (y in ppm) at the oxygen input flow rate of 0.06 L/min is linearly correlated with ozonation time (x in min): $y = 0.08x - 0.45$ ($R^2 = 0.95$) (Chittrakorn 2008). Ozone was fed into a rotary metal drum containing 500 grams of tannin sorghum flour. The residual ozone from the exit of the rotary metal drum was entrapped in 2% potassium iodide solution plus starch.

Starch Isolation from Tannin Grain Sorghum Flour

Twenty-five gram of whole tannin grain sorghum flour (ozone-treated and untreated) was dispersed into 200 ml distilled water in a flask. The pH was adjusted to 4.0-4.2 with hydrochloride acid, and 0.4% (v/v) protease GC106 (Genencor International, Inc. NY) was added to hydrolyze protein and facilitate starch extraction. To prevent microbial contamination, 100 μ L of 10% NaN_3 was added to each flask. The flasks with mixed flour suspension were

incubated in a 45°C water-bath shaker for 72 hr with constant agitation. The content from the flask was sieved through a No. 200 wire sieve (opening 75 µm) and the retained overs were washed twice with 200 mL (2×200 mL) distilled water to recover starch. The washed overs were discarded, whereas the throughs were collected and passed through a No. 200 wire sieve (opening 75 µm). Again, the overs were discarded, and the throughs were centrifuge at 3000 × g for 30 min. Each time after centrifugation, the supernatant and tailings were removed and discarded. The prime starch was washed with distilled water and centrifuged at 3000 × g for 30 min for a total of 10 times to obtain clean prime starch. The prime starch was freeze dried for gel permeation chromatography test.

Gel Permeation Chromatography (GPC)

Four milligrams of whole grain sorghum flour was mixed with 4 mL dimethyl sulfoxide (DMSO) and stirred in a boiling water bath for 24 hr. The sample was filtered through a 2 µm filter and then 200 µL was injected into a PL-GPC 220 instrument (Polymer Laboratories, Inc., Amherst, MA, USA) equipped with three Phenogel columns of different pore sizes (100Å, 10-3Å, & 10-5Å, Phenogel™ GPC, 10 µm, 300 × 7.8 mm), a guard column (Phenomenex, Inc., Torrance, CA, USA), and a differential refractive index detector. The eluent system was DMSO containing 5.0 mM NaNO₃ at a flow rate of 0.8 mL/ min. The column oven temperature was controlled at 80°C. Standard dextrans (American Polymer Standards Co., Mentor, OH, USA) with different molecular weights (MW) were used for MW calibration.

pH Value Measurement

The pH of whole sorghum flour samples was measured following the AACC method 02-52.01 ([AACC International 2009](#)). Ten gram flour was added to 100 mL of distilled water. The flour suspension was stirred on a stirring plate for 15 min. Flour samples were allowed to stand for 10 min after removed from the stirring plate, then, the supernatant was decanted and used for pH measurement.

Pasting Properties of Sorghum Flour by RVA

A rapid visco analyzer (model RVA-3D, Newport Scientific Ltd., Warriewood, Australia) was used to test pasting properties of the sorghum flours. Sorghum flour (4.0 g of 14% mc, 3.44 g dry mass) and water (25 mL including water from the sample flour) were mixed at 50°C; the slurry was held at 50°C for 1 min then heated from 50 to 95°C. The hot paste was held at 95°C for 2.5 min, cooled to 50°C, and held at 50°C for 2 min. The total process was 13 min.

Microorganism, Preparation of Mash and Inoculation

The dry yeast (*Saccharomyces cerevisiae*, Red Star Ethanol Red) was provided by Fermentis (Milwaukee, WI), and was used for simultaneous saccharification and fermentation (SSF). Before inoculation, dry yeast was activated by adding 1.0 g of cells into 19 mL of preculture broth (containing 20 g glucose, 5.0 g peptone, 3.0 g yeast extracts, 1.0g KH₂PO₄, and 0.5 g MgSO₄·H₂O per liter) and incubated at 38°C for 30 min in an incubator operating at 200 rpm. The activated yeast culture had a cell concentration of approximately 1×10⁹ cells/mL.

Liquozyme SC DC (Novozyme, Franklinton, NC), a heat-stable α-amylase from *Bacillus licheniformis*, was used for liquefaction. Enzyme activity of the Liquozyme SC DC was 240 KNU/g (One Kilo Novo Unit, KNU, is the amount of enzyme that breaks down 5.26 g of starch per hr at Novozyme's standard method for determination of α-amylase). Spirizyme Fuel (Novozyme, Franklinton, NC), an amyloglucosidase from *Aspergillus niger*, was used for saccharification. Enzyme activity of the Spirizyme Fuel was 750 AGU/g (One AGU is the amount of enzyme that hydrolyzes 1 μmol of maltose per min under specified conditions).

Whole sorghum flour (34 g, as is) was dispersed in a 250-mL Erlenmeyer flask with 100 mL of fermentation broth containing 0.1 g KH₂PO₄ and 20 μL Liquozyme (240 KNU/g). The flasks were transferred to a 70°C water bath shaker operating at 170 rpm. The water bath temperature gradually increased from 70°C to 95°C over a period of 90 min. After 90 min, flasks were removed from the water bath shaker and cooled to room temperature. Materials sticking to the inner surface of the flasks were scraped back into the mash with a spatula, and the inner surface was rinsed with 2–3 mL of distilled water using a fine-tipped polyethylene transfer pipette. One hundred μL amyloglucosidase, 0.3 g yeast extract, and 1 mL activated yeast culture (1×10⁹ cells/mL) were added to each flask. Inoculated flasks were sealed with S-

bubblers/airlocks and transferred to an incubator shaker for SSF ethanol fermentation. Each sample was run in duplicate.

Fermentation and Distillation

Ethanol fermentation was conducted at 30°C in an incubator shaker (Model I2400, New Brunswick Scientific, Edison, NJ) operating at 150 rpm for 72 h. The fermentation process was monitored by measuring the weight loss of each flask from evolved carbon dioxide (CO₂) during fermentation.

At the end of fermentation, the finished mash in each 250-mL flask was transferred to a 500-mL distillation flask. Each Erlenmeyer flask was washed with distilled water four times (4×25 mL). The washing water was pooled in the distillation flask, and then the distillation flask was distilled on a distillation unit. Distillates were collected in a 100 mL volumetric flask immersed in ice water. When distillates in the volumetric flask approaching the 100 mL mark (~0.5 mL below the mark), the distillation process was stopped. Distillates in the volumetric flask were equilibrated in a 25°C water bath for at least two hr before adjusting the total volume to 100-mL with distilled water. Distillates were analyzed for ethanol using a Shimadzu HPLC with a Rezex RCM column (Phenomenex, Torrance, CA) and refractive index detector (Wu et al 2006).

Tannin Measurement

Tannin contents in the control whole sorghum flour and ozone-treated whole sorghum flours were determined by following the modified vanillin assay procedures for measurement of condensed tannin (Price et al 1978). Pure catechin (Sigma, St. Louis) was used as a standard for calibration curve. The whole sorghum flours for tannin test were freshly ground using a Udy cyclone sample mill (Udy, Fort Collins, CO) with a 0.5-mm screen on the day of assay.

Free Amino Nitrogen Determination

Free amino nitrogen (FAN) was analyzed using the European Brewery Convention method (EBC 1987) with modification. Grain sorghum flour (150 mg) was mixed with 1.5 mL deionized water in a 2.5-mL microcentrifuge tube. The mixture was vortexed five times within 10 min, then centrifuged at 12,000 rpm for 20 min. A portion of the supernatant was sampled for FAN analysis.

Color Measurement

The L^* , a^* , b^* color spaces system was developed in 1976 and adopted by the International Commission on Illumination (CIE), which became a joint ISO and CIE standard ([ISO 11664-4:2008\(E\)/CIE S 014-4/E:2007](#) and [ISO 11664-5:2009\(E\)/CIE S 014-5/E:2009](#)). L^* is a measure of the lightness with values of 0 for black and 100 for white; a^* describes red-green color. Positive a^* values indicate redness and negative a^* values indicate greenness; b^* describes yellow-blue color, Positive b^* values indicate yellowness and negative b^* values indicate blueness. A Minolta chroma meter (model CR-210, Entest, Inc., Carrollton, TX) was used for color determination. The instrument was calibrated with a white calibration tile. The colorimeter was set to an illuminant condition C and a 2° standard observer. Each sample was placed in the standard sample holder for color measurement. Test was done in replicate. In this study, the effects of ozonation on sorghum color were measured against the untreated control sample.

Analytical Methods

Moisture content was determined using the AACC approved method 44-15A ([AACC International 2009](#)). Total starch content was measured using Megazyme total starch test kits and the DMSO procedures according to AACC approved method 76-13 ([AACC International 2009](#)).

Results and Discussion

Effect of Ozonation on pH Values of Sorghum Flours

Figure 5.1 shows pH-value changes of the tannin sorghum flours after ozone-treatment. Compared with the pH of the control sorghum flour, pH-values of all the ozone-treated sorghum flours were lower following treatment. The pH-values of the same sorghum flour decreased as ozonation doses increased (higher ozone flow rates and/or longer treatment time). Statistically, the pH-values were significantly different among treatments with ozone flow rates at 0, 0.02 and 0.06 L/min ($P < 0.05$). pH-values of ozone-treated sorghum flour were significantly different from the control. However, No significant difference was found between pH values of the 15 min and 30 min ozonated flours ($P < 0.05$).

Decreasing in pH-values after treatment means more $[H^+]$ ions in the water slurry systems from treated flours. It has been reported that the increases of carboxyl and carbonyl contents in ozone treated starches were proportional to the doses of ozone ([Chan et al 2009](#)). The

increase in carboxyl groups in the oxidized starches were the results of oxidative break down of starch polymeric molecules. In whole sorghum flour, additional carbohydrate polymers such as hemicelluloses and cellulose beside starches may contribute to the formation of carboxyl groups during ozone treatment. This could be one explanation for the behavior of pH decrease in ozonated sorghum flours depicted in Figure 5.1.

Effect of Ozonation on Sorghum Tannins

Measured tannin levels in ozone treated sorghum flours decreased by more than 20% compared to that of the untreated control (Figure 5.2). The tannin content decreased as ozone levels increased. At either treatment time (15 min or 30 min), the tannin contents in sorghum flours treated at higher ozone flow rate (0.06 L/min) were significantly lower than those treated at lower flow rate (0.02 L/min). However, no significant difference in tannin contents was found between treating time of 15 min and 30 min at both ozone flow rates. The combined effect of flow and time dose might be the reason for such different results. At either treating time, the combined ozone doses tripled when the flow rate was raised from 0.02 L/min to 0.06 L/min; whereas, the combined ozone dose only doubles at either flow rate when the treatment time increased from 15 min to 30 min. If triple dosage change is the minimum required to cause significant changes in tannin content, then double combined ozone doses might show the decreasing trend in tannin content, but not enough to cause significant changes in tannin content.

As previously reported, ozone can degrade macromolecules such as lignin, starches etc. similar degradation could happened to tannins in our ozone treated sorghum flours. SEC (HPLC) was used to test the tannins in the treated sorghum flour samples. Unfortunately, the SEC (HPLC) analysis could not differentiate the differences in tannins in the ozone treated and non-treated control (data not shown). One possible reason could be that the changes in tannin molecules were too small for SEC HPLC to detect, or the SEC conditions used for normal tannin analysis did not suit for ozonated tannin analysis. Ozone treatment could change the structures of some functional groups in tannin molecules and therefore affect their enzyme inhibition and protein precipitating activity. However, such changes (e.g. formation of carboxyl, carbonyl groups, or break of limited amount of short branches) were not dramatic enough for SEC (HPLC) to detect and differentiate.

Effect of Ozonation on Starch Molecule Distribution

Figure 5.3 shows the molecular weight distribution of starches from ozone-treated tannin sorghum flour and control sorghum flour as determined with GPC. Compared with the molecular weight distribution of starch in the control sorghum flour, the molecular weight distribution curves of starches from ozone-treated tannin sorghum flours were either shifted toward the low molecular weight side or the proportion of the low molecular weight fraction increased. Several previous investigations demonstrated that ozone treatment could change structural, physicochemical and functional properties of starches (An and King 2009; Chan et al 2009; Kuakpetoon and Wang 2006). These studies speculated that the low final viscosity of ozone-treated starch could be attributed to degraded starch molecules and weakened starch granules during ozonation. However, there was no data to directly support their assumptions. Our GPC data showed two different types of changes occurred to ozone treated sorghum starches. First, it confirmed that ozone caused different degrees of degradation to starch molecules. The shift of GPC curves of the ozone treated starches toward the low molecule weight side (left) is direct evidence of such degradation (Figure 5.3). Significant amount of lower molecular weight starch molecules have been generated during ozone treatment. However, the shift of starch molecules toward the low molecular weight direction was not proportional to the ozone dosage as we normally expected. In contrast, more low-molecular-weight starches were found in samples treated at lower flow rate than at higher flow rate. On the other hand, the GPC curves showed that some crosslinking among starch molecules might have occurred too, because the high molecular weight portions of the GPC curves were obviously larger than that of the non-treated control. The GPC curves of starches from low flow rate treatment had larger middle portions (LogMW from 5.5 to 7.5) than the control; whereas, the GPC curves of starches from higher flow rate treated samples had larger peaks in the high molecular weight end (LogMW from 9 to 10). Our data show that both oxidative degradation and crosslinking could have happened to sorghum starches during ozone treatment. The reason for less low-molecular-weight starch molecules in higher ozone dose treated samples was probably due to the formation of larger molecules from crosslinking of oxidative degraded starch molecules (starches with carboxyl and carbonyl groups). At present, there is no information regarding the types of crosslink reactions and structural features of cross-linked molecules. Further studies are needed to elucidate the actual mechanism behind such changes. To obtain a treated product with appropriate amount of

oxidized starch molecules and suitable physical and chemical properties for ethanol production, ozone treating doses (control of flow rate and treatment time) need to be further optimized.

Effect of Ozonation on Pasting Properties of Sorghum Flour

Pasting properties of ozone-treated tannin grain sorghum flours and control flour analyzed with a Rapid Visco Analyzer are shown in Figure 5.4. All the curves did not show clear peak viscosity and break down viscosity. This could be caused by two factors. One was high tannin content (~ 4%) in the flour. Tannins have the ability to bind, coagulate, and precipitate proteins. This conclusion has been reviewed by Butler et al. (1984), who summarized that under optimal conditions, sorghum tannin is capable of binding and precipitating at least 12 times its own weight of protein by means of hydrogen bonding, hydrophobic interaction, electrostatic attraction and covalent bonding associated with oxidation. Because tannins interact with proteins during mashing or during the RVA analysis, the tannin-protein complexes will inhibit the water absorbing rate of starch granules and prevent starch granules from swelling rapidly. Another factor could be particle size of sorghum flours. Whole sorghum flour samples used in this study were prepared using a Magic Mill III plus grain mill set at the level V. The particle sizes of such prepared flours were relatively larger than those from cyclone mill with 1.0 mm screen or industrial flours. Normally, the sizes of cereal starch granules are in the range of 0-50 μm . If a 1.0 mm (1000 μm) screen was used, some large particles in the ground sorghum flours could contain more than a hundred starch granules. As a result, water absorbing rates by starch granules in larger particles will be inhibited. Sudden increase in mash viscosities of tannin sorghum samples during mashing confirmed this.

The setback viscosity indicates degrees of retrogradation of starch molecules during cooling. A high setback value indicates a high tendency of starch molecules to retrograde. Pasting curves in Figure 5.4 shows that the setback viscosities of sorghum flours treated with lower ozone flow rates were ~ 200cp lower than those of sorghum flours ozonated at the higher flow rates. This result agrees with Figure 5.3, which shows that amylopectin degraded more in low flow rate treated samples but some crosslinking occurred in the higher flow rate treated samples. Usually, the lower setback viscosity is a sign of higher α -amylase activity. Lower setback viscosity is a good trait for ethanol production from grain sorghum because low setback

viscosity means easy disruption of starch granules and less tendency to form retrograded starch molecules during mashing, which are more resistant to enzyme hydrolysis (Yan et al 2010).

Effect of Ozonation on Ethanol Yield and Fermentation Efficiency

Ethanol yields from ozone-treated whole grain sorghum flour and control sorghum flour are listed in Table 5.1. Ethanol yields from all the ozone-treated sorghum flour were significantly higher than that from the non-treated control sorghum flour. Ethanol yields from samples treated at lower flow rates were significantly higher than those from both higher ozone flow rate treated sorghums and non-treated control ($p < 0.05$). This indicates that the ozone flow rate had a significant effect on ethanol yields in the treated flow rate range. The favorable effects of ozonation on ethanol yields were found not proportional to ozone doses as measured by flow rates (0.02 vs 0.06 L/min). In fact, some favorable effects might have been offset partially as ozone flow rate increased from 0.02 to 0.06 L/min. On the other hand, when we examined the effect of ozonation time on ethanol yield, no significant difference in ethanol yield was found between 15 min and 30 min of ozonation ($p < 0.05$). Nevertheless, ethanol yields from ozone treated sorghum samples showed a decrease trends as treatment time increased at both flow rates. The reason for the no significant changes in ethanol yields could be the duration of 30 min was not long enough. Interactions between the ozone flow rate and treatment duration on ethanol yield were not significant ($p > 0.05$).

Fermentation efficiency is a very important parameter to evaluate the performance of a material for ethanol production. Ethanol fermentation efficiencies from ozone-treated whole grain sorghum flours and control sorghum flour are shown in Figure 5.5. By the end of the 72 hr fermentation process, the efficiencies of ozone-treated grain sorghum flours were 2-5% higher than that of the control flour. The efficiencies of the low flow rate treated samples were ~3% higher than those of higher flow rate treated samples. When we examined the fermentation efficiencies at the 36th hr, the efficiencies of ozone-treated sorghum samples ranged from 86% to 92%, whereas the efficiency of the non-treated control sorghum was 78%. Efficiencies of samples treated with lower ozone flow rate (0.02 L/min) were 12.9 to 13.8% higher than that of the non-treated control; whereas the fermentation efficiencies of higher ozone flow rate treated samples were 8-10% higher than that of the non-treated control. Figure 5.5 shows that the fermentation efficiency in the end of the fermentation process (72 hr) did not increase very much

after the 36th hr point except for that of the control. Therefore, fermentation time could be shortened to 36 hr to reduce energy consumption without obvious loss in ethanol yield if ozone-treated grain sorghum flour is used for ethanol production. This indicates that ozone treatment could be a novel way to shorten fermentation time and increase production capacity of ethanol plants.

Effect of Ozonation on Sorghum Flour Color

Figure 5.6 shows results from color measurement using a colorimeter. Compared with the non-treated control grain sorghum flour, ozone-treated tannin sorghum flours had higher L^* values indicating they became light colored. As ozonation time and ozone flow rate increased, the whiteness of sorghum flour increased; whereas a^* values (redness) showed a decline trend as ozone dosage increased. Yellowness (b^* values) varied among the treatments. Xiang (2009) reported that sorghum colors are determined and affected by many factors such as pericarp and the presence of pigment testa layer.

Beside tannins, many other naturally occurred compounds such as lignin, carotenoids, anthocyanins etc. may give plant origin materials dark colors. Degradation of these compounds usually leads to a lighter colored material (Henry et al 2000; Miki 1994; Tiwari et al 2009). Ozone as a powerful oxidant definitely has the potential to degrade such pigment compounds including tannins and turn sorghum flour into lighter color, which could have contributed to the color changes (lighter and brighter, reduction in redness and variable in yellowness) of ozone-treated sorghum.

Effect of Ozonation on DDGS

DDGS is a by-product from ethanol production. Its composition and quality are critical for its market value as animal feed, which accounts for a major portion of revenues in ethanol plants. Protein and fat contents are the two major nutrients affect the nutritional values and thus market prices of DDGS. Table 5.2 shows the chemical composition of DDGS from ozone-treated and non-treated samples. Normal DDGS has a crude protein contents of 25-29% and crude fat contents of 7-11% (Saunders and Rosentrater 2009). Data in Table 5.2 show that DDGS from sorghum has much higher protein contents than normal DDGSs in the ethanol industry and has a comparable crude fat contents, which gives sorghum DDGS a label of better quality at least from a nutritional point of view. Comparing among DDGSs from different treatments within this

study, protein contents were higher in DDGS from ozone-treated samples than that in DDGS from the non-treated control. Residual starch contents in normal industrial DDGSs are around 5% (Belyea et al 2004), starch residues in DDGS from ozone-treated samples were all less than 1%, and were lower than that in DDGS from the non-treated control (1.69%), which is reasonable due to the higher fermentation efficiencies and higher ethanol yields of the ozone-treated sorghum flours. Overall, ozone treatment not only enhanced fermentation efficiencies and ethanol yields, but also generated a high quality DDGS.

Conclusion

Ozonation not only decreased tannin content and pH value of tannin sorghum, but also had effect on sorghum flour, starch granules, and starch molecular distribution. Fermentation efficiency is a very important parameter to evaluate the performance of a material for ethanol production. Ethanol fermentation efficiency from ozone-treated sorghum increased greatly over 10% compared to that from the control. This indicates that ozonation has great impact on ethanol yield and fermentation efficiency and is an effective way to increase ethanol yield and shorten the fermentation process without decreasing ethanol yield.

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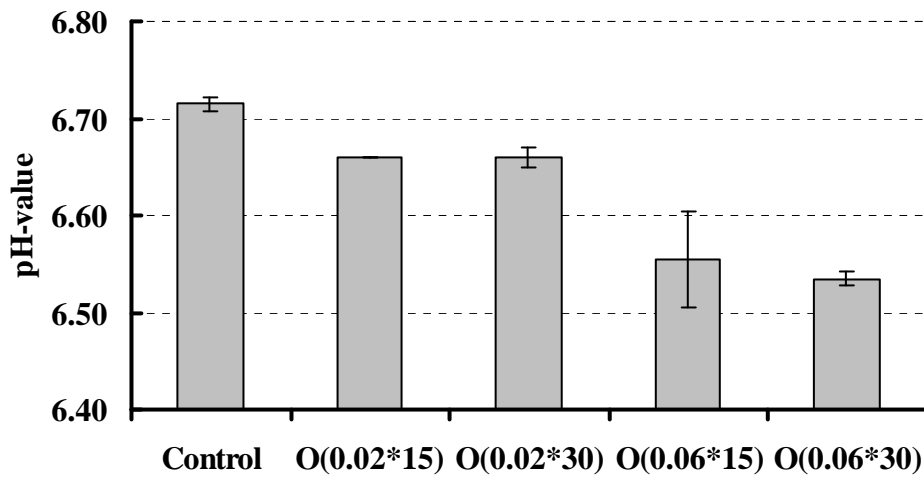


Figure 5.1. Effect of ozone treatments on pH-value of grain sorghum flour. Numbers in the parentheses after the letter O are ozone doses designated by ozone flow rate (L/min)*treatment duration (min).

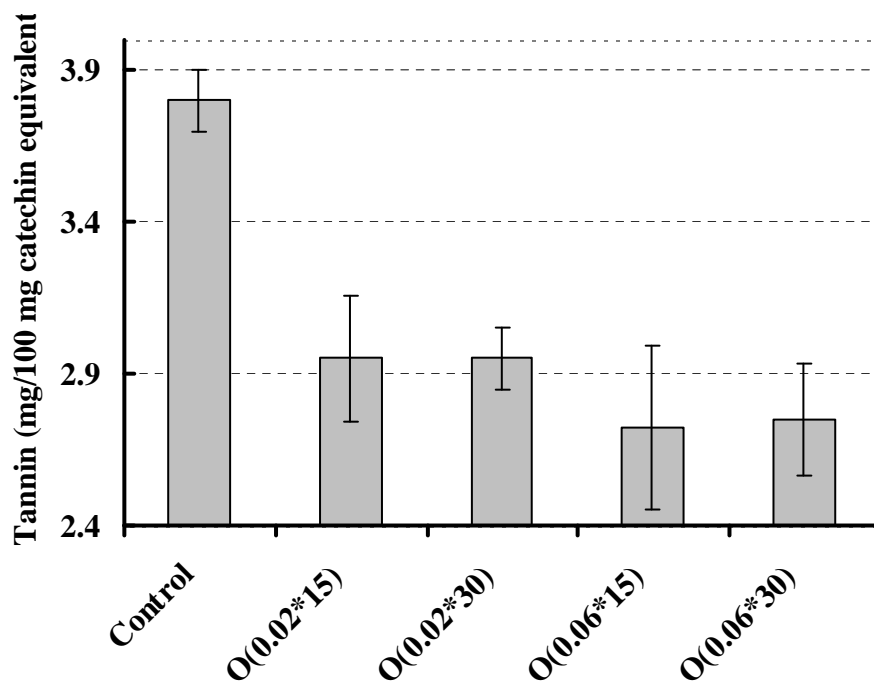


Figure 5.2. Effect of ozone treatments on tannin content change in grain sorghum flours. Numbers in the parentheses after the letter O are ozone doses designated by ozone flow rate (L/min)*treatment duration (min).

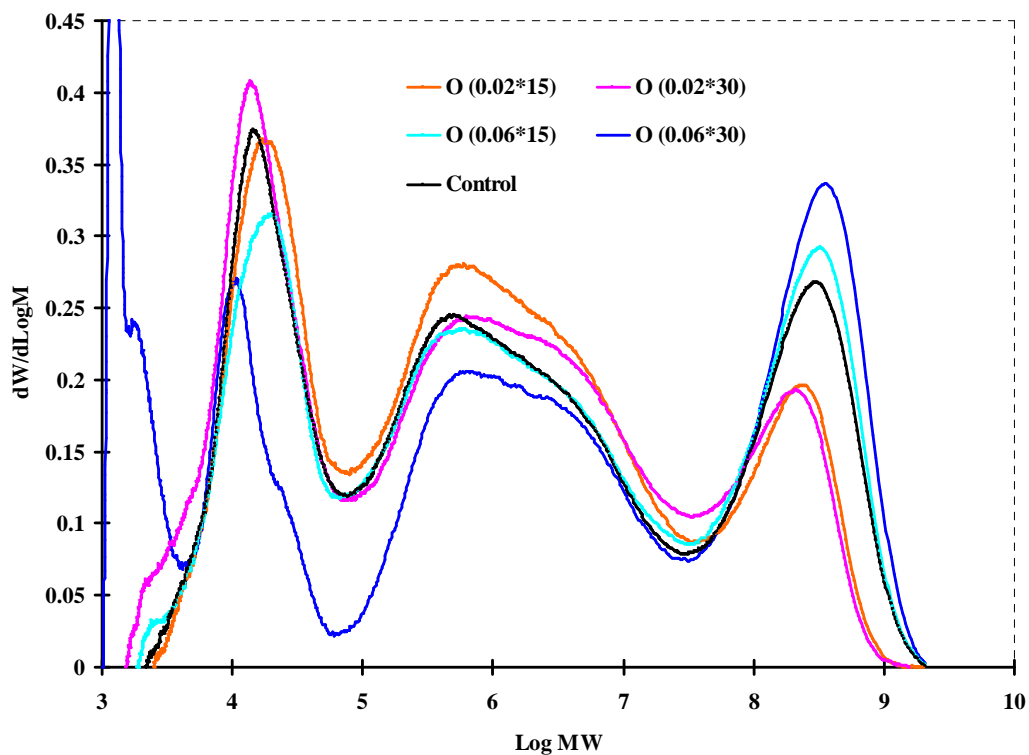


Figure 5.3. Effect of ozone treatment on molecular weight distributions of sorghum starches. GPC was performed on a PL-GPC 220 with three 300x7.8 mm Phenogel columns (100Å, 10-3Å, & 10-5Å) and a RI detector. The eluent system was DMSO containing 5.0 mM NaNO₃ at 0.8 mL/ min. The oven temperature was 80°C. Numbers in the parentheses after the letter O are ozone doses designated by ozone flow rate (L/min)*treatment duration (min).

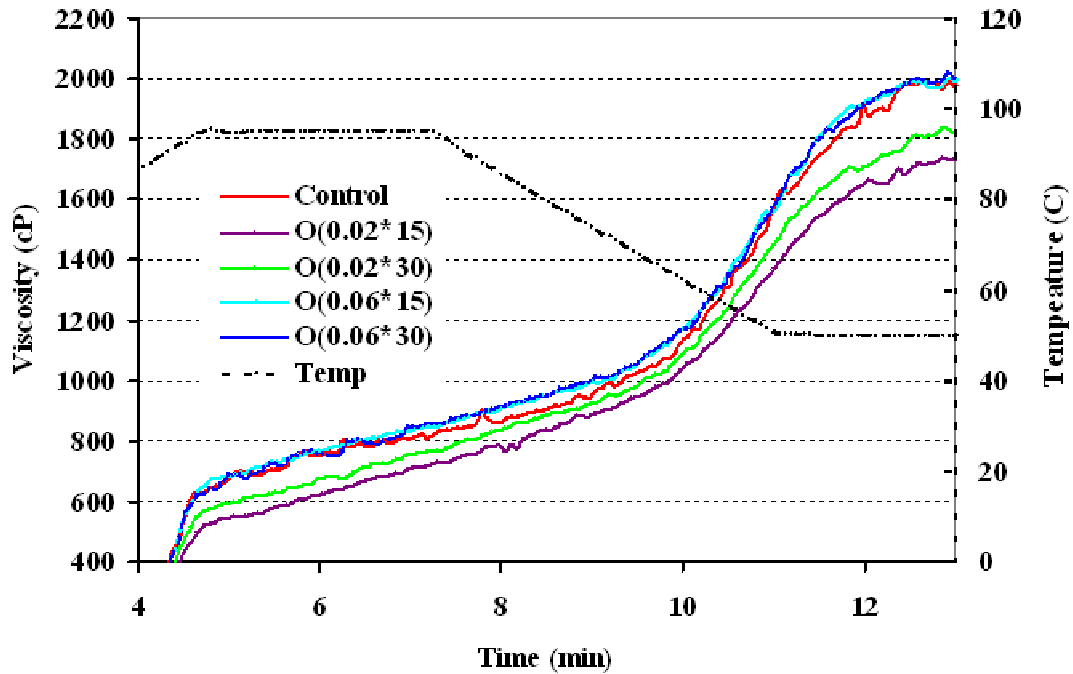


Figure 5.4. Pasting properties of ozonated tannin sorghum flours and non-treated control flour using RVA with a standard 13 min procedure. Numbers in the parentheses after the letter O are ozone doses designated by ozone flow rate (L/min)*treatment duration (min).

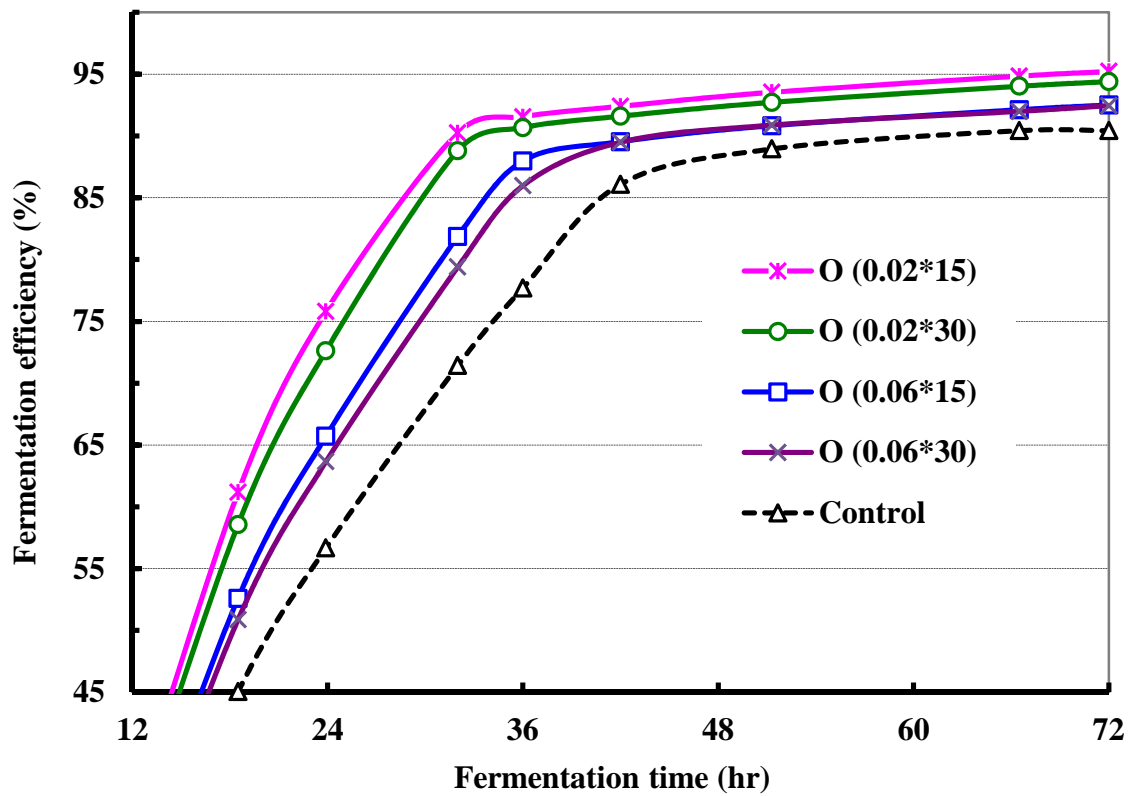


Figure 5.5. Fermentation efficiencies of ozonated tannin sorghum and non-treated control using a laboratory dry-grind process. Numbers in the parentheses after the letter O are ozone doses designated by ozone flow rate (L/min)*treatment duration (min).

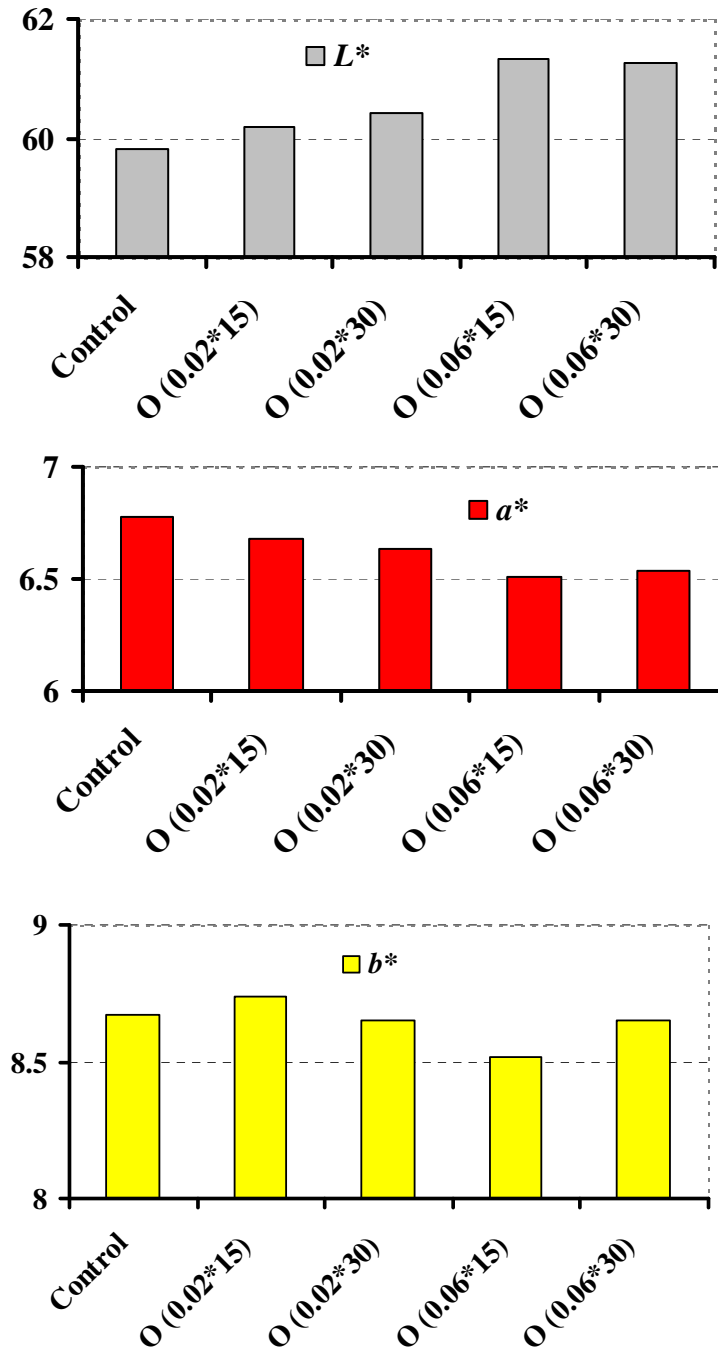


Figure 5.6. Effect of ozone treatment on sorghum flour color measured in L^* , a^* , b^* color space. Numbers in the parentheses after the letter O are ozone doses designated by ozone flow rate (L/min)*treatment duration (min).

Table 5.1. Ethanol yields from ozonated whole grain sorghum flours and control sorghum flour

Sample	Ethanol yield (L/Ton)	Ethanol yield (Gal/bu)
Control	356.47 ± 5.76	2.68±0.04
O (0.02*15) [#]	375.54 ± 5.40	2.82±0.04
O (0.02*30)	373.02 ± 1.44	2.80±0.01
O (0.06*15)	364.39 ± 1.80	2.74±0.01
O (0.06*30)	362.95 ± 3.96	2.73±0.03

[#]: Numbers in the parentheses after the letter O are ozone doses designated by ozone flow rate (L/min)*treatment duration (min).

Table 5.2. Proximate analysis results on major components of DDGSs (% , db) from ozonated sorghum samples and non-treated control

Sample	Starch	Crude protein	Crude fats	Crude fiber	Ash
Control	1.69 ± 0.01	33.82 ± 0.01	8.56 ± 0.01	4.99 ± 0.01	5.66 ± 0.01
O (0.02*15) [#]	0.95 ± 0.01	35.34 ± 0.01	8.14 ± 0.02	4.74 ± 0.03	5.87 ± 0.11
O (0.02*30)	0.94 ± 0.00	35.14 ± 0.88	8.63 ± 0.03	4.99 ± 0.15	5.76 ± 0.00
O (0.06*15)	1.03 ± 0.15	35.08 ± 1.22	8.51 ± 0.02	4.86 ± 0.05	5.84 ± 0.35
O (0.06*30)	0.99 ± 0.08	34.79 ± 0.26	8.48 ± 0.16	4.88 ± 0.48	5.74 ± 0.16

[#]: Numbers in the parentheses after the letter O are ozone doses designated by ozone flow rate (L/min)*treatment duration (min).

Chapter 6 - Conclusions and Recommendations

Conclusions

After studying the physical, chemical, and ethanol fermentation performance of waxy sorghum, laboratory germinated tannin sorghum, field sprouted sorghum samples, and ozone treated tannin sorghum flours, we come to the following conclusions.

Beside their common low amylose contents, waxy grain sorghum cultivars varied greatly in other chemical and physical features. Ethanol yields from waxy sorghums were essentially proportional to their starch contents. The narrow-ranged and very low amylose contents in the tested waxy sorghum samples showed little effect on ethanol yield and fermentation efficiency. Ethanol yields from the tested waxy grain sorghums were around 2.8 gallon/bushel, which is similar to that reported for corn. The fermentation efficiency was greatly affected by FAN content in waxy sorghum, which showed a strong positive linear correlation ($R^2=0.90$) with early stage (the first 30-36 hr) fermentation efficiency. Tannins were found in most of the tested waxy sorghums and had negative effects on ethanol yield and fermentation efficiency. DDGS from waxy sorghums had higher protein contents than those from normal sorghum and corn, which implies better quality as animal feed.

Short period (~3 days) of germination treatment could activate many intrinsic enzymes in sorghum kernels and cause dramatic changes to physical and chemical properties of sorghum kernels and its performance in ethanol production. These changes include significantly decreased tannin content, break down of protein matrix and release of formerly embedded starch granules, improvement in protein and starch digestibility, which worked favorably together in the dry-grind ethanol process with significantly higher fermentation rate (essentially completed in 36 hr instead of 72 hr), enhanced fermentation efficiency, and increased ethanol yield. Activated intrinsic enzymes in the germinated sorghum kernels definitely played an important role in improving digestibility of starches and proteins (possibly the hydrolysis of other components) and compensated for the loss of starch due to respiration during germination.

The study on field-sprouted sorghum samples further confirmed the results from the laboratory germination study in every aspect. Sprouted sorghum samples had significantly different features and properties compared to those of the sound control. Sprouted samples had smaller kernel sizes, weight and lower hardness index values, damaged physical structures

(starch granules, protein matrices, and cell walls etc.), changed chemical composition and pasting properties. Those property changes rendered sprouted sorghum not good for food or animal feed use anymore. Fortunately, many of these changes make it a better feedstock for ethanol production. Less hard makes it easier (less power consumption and equipment wear) to be ground into fine particles; damaged starches and protein matrix plus activated intrinsic enzymes improved starch and protein hydrolysis and pasting characteristics (lower viscosity during mashing, less residual starch in DDGS, and higher FAN in finished mash). Together, these features made sprouted sorghum even a better feedstock for ethanol production with more rapid fermentation rate, improved fermentation efficiency and ethanol yield. This could offer an alternative market outlet for field-sprouted sorghum when unfavorable weather caused field sprouting occurs.

Ozonation not only decreased tannin contents and pH values of tannin sorghum flours, but also had effects on sorghum flour, starch granules, starch molecular size distribution as well as the ethanol production performance as evaluated with the laboratory dry-grind process. Ethanol fermentation efficiency from ozone treated sorghum flour increased significantly by more than 10% when compared with that of the untreated control flour. Therefore, ozonation could be an effective way to reduce tannin contents in tannin flour, overcome tannin's adverse effects on starch hydrolysis, and eventually lead to improved ethanol fermentation efficiency and higher ethanol yield.

Recommendations

Tannins have been reported responsible for the low digestibility of starches and proteins and considered anti-nutritional factors in many plant foods. On the other hand, tannins and many phenolic compounds have been related with antioxidant activities of many foods and vegetables. Both germination and ozone treatment demonstrated effective in reducing tannin contents or lessening their adverse effects on starches and proteins hydrolysis. No research has been conducted on the molecular mechanism of the changes during germination or ozone treatment, nor has any investigation on the antioxidant activities of treated samples either. Information from such studies may not only be very useful for optimizing feedstock treatment for ethanol production, but also be used in food processing to make use of the antioxidant properties of tannin and its degraded products.

Germination and field sprouted sorghum both demonstrated improved performance in ethanol production. The significantly increased FAN contents in mashes were directly correlated with fermentation rate. Laboratory germinated sorghum had a more uniform and controllable FAN levels. However, the field sprouted samples had very diverse and unpredictable FAN contents, which make it difficult for an ethanol plant to adjust its processing strategies if sprouted sorghums do become a feedstock for ethanol production. Fast and reliable method to predicate FAN in finished mashes from various kinds of sprouted sorghum would be very helpful for ethanol plants to handle such feedstocks. The widely used instant FT-IR and quick SKCS methods may play a role. Nevertheless, no research on this area has been conducted.

Ethanol fermentation results showed that ozonation significantly improved the performance of tannin sorghum in ethanol production (higher fermentation rate, shorter fermentation time, higher efficiency and yield). However, the improvement did not proportional to the applied ozone dosage. GPC analysis on starch molecular weight distribution revealed that both degradation and crosslinking occurred to starch molecules during ozone treatment. Further investigating the molecular mechanisms under such changes in molecular weight distribution could help to understand the relationship of each type of change with performance in ethanol production, therefore, optimize ozone treating dosage.

A. Supplement Materials

Abbreviations Used

The following is a list of abbreviations used in the dissertation.

AACC	American Association of Cereal Chemists
AGU	Amyloglucosidase unit
AOAC	Association of Official Analytical Chemists
CE	Catechin equivalent
CIE	International Commission on Illumination
db	Dry basis
DDGS	Distillers dried grains with solubles
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
EBC	European Brewery Convention
Effic.	Efficiency
FAN	Free amino nitrogen
GAE	Gallic acid equivalent
GBSS	Granule-bound starch synthase
GPC	Gel permeation chromatography
HI	Hardness index
KNU	Kilo Novo unit
MV-HCl	Modified V-HCl method for sorghum tannin determination
RFA	Renewable fuels association
RFS	Renewable Fuels Standard
RVA	Rapid visco analyzer
SEC	Size exclusion chromatography
SEM	Scanning electron microscope
SSF	Simultaneous saccharification and fermentation
Temp.	Temperature
VAG	Visco Amylo Graph
V-HCl	Vanillin hydrochloric acid method for sorghum tannin determination

Figures and Tables Within Appendix A

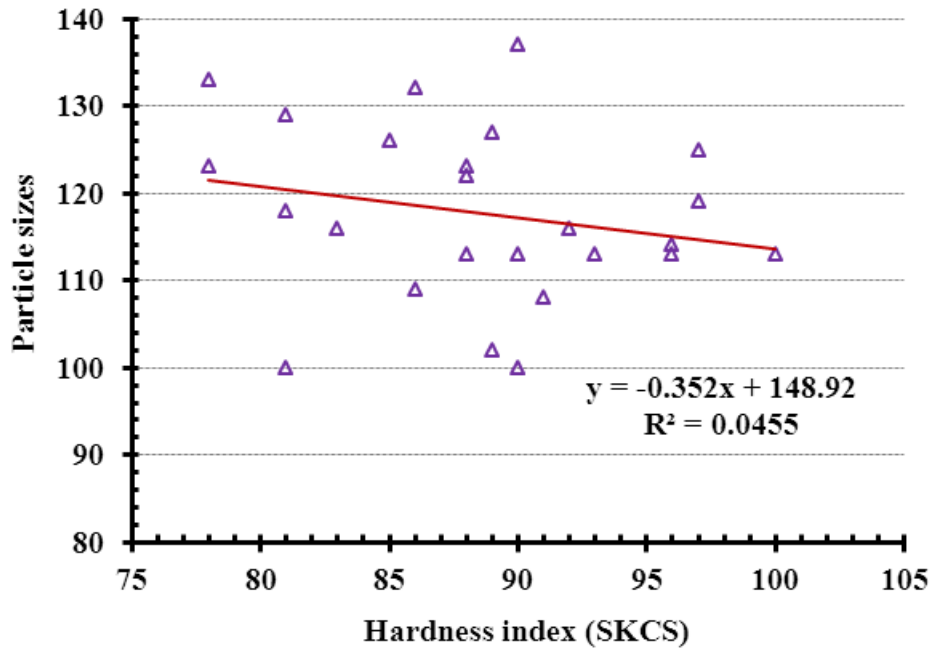


Figure A.1. Relationship between kernel hardness index measured with an SKCS and average particle sizes of ground sorghum samples.

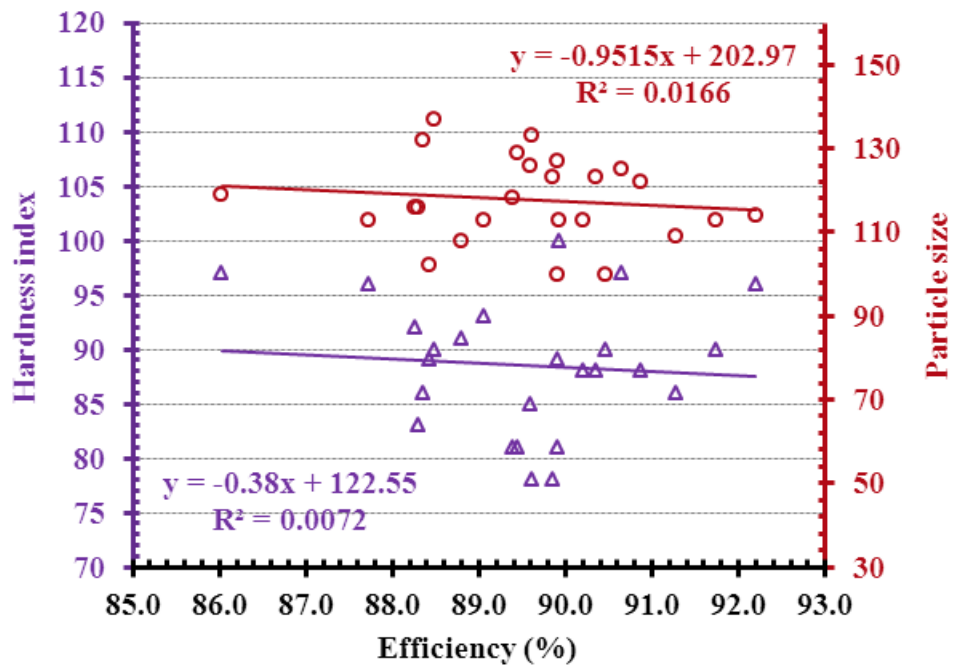


Figure A.2 Relationship between hardness index of sorghum kernels, particle sizes of ground samples and fermentation efficiencies.

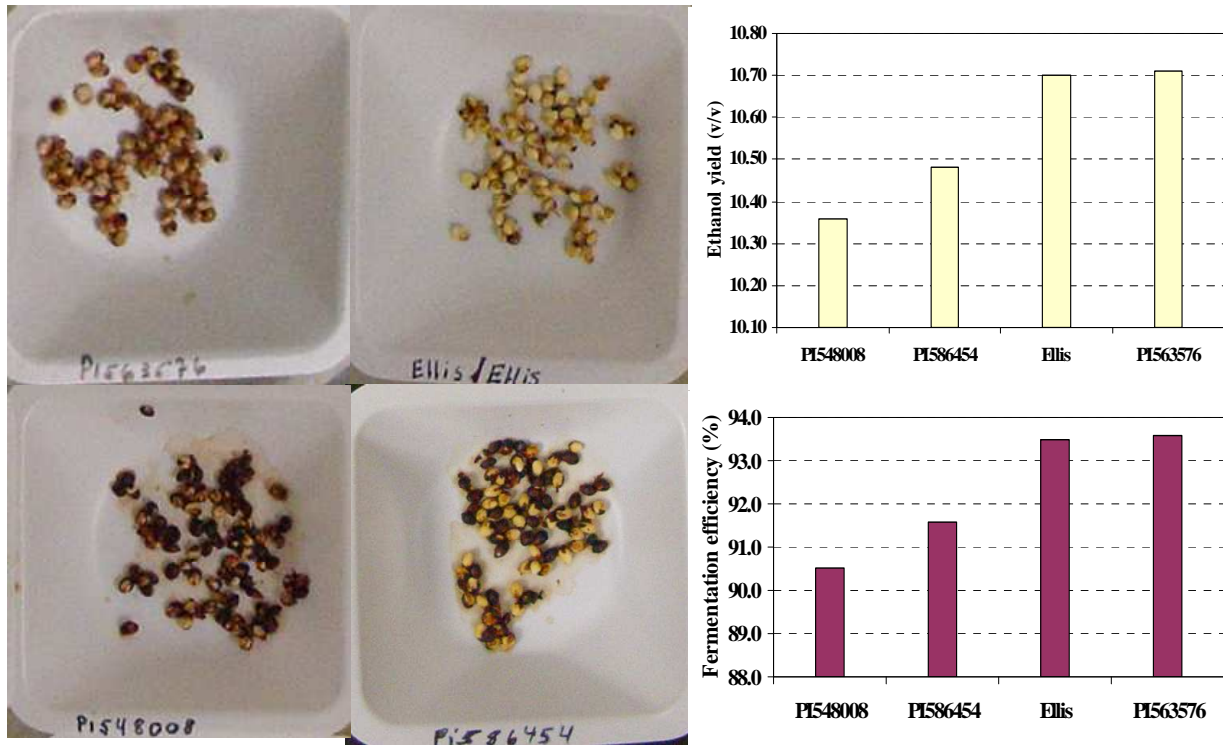


Figure A.3 Selected samples of tannin bleach test results and its relation with ethanol yield.



**Oxygen
Concentrator**

**Ozone
Generator**

**Metal Drum for
Sample Treatment**

Figure A.4 Equipment used for ozone treatment of sorghum flour samples in this study.

Table A.1 SKCS data on the 25 tested waxy sorghum samples

Accession #	Origin	Hardness index	Weight (mg)	Moisture (%)	Diameter (mm)
PI220636	Afghanistan	80.84	22.22	6.76	2.26
PI23231	China	88.31	19.78	6.71	1.77
PI548008	China	97.25	18.45	7.23	1.67
PI563576	China	92.03	21.58	7.33	1.90
PI563670	China	87.72	25.37	7.25	2.08
PI563671	China	89.83	21.67	7.17	1.81
PI586524	China	96.69	18.75	6.95	1.71
PI586526	China	95.88	17.47	7.35	1.63
PI586529	China	82.65	23.52	7.30	1.84
PI455543	Ethiopia	96.18	21.16	7.48	1.83
PI586448	Hungary	93.28	16.01	6.78	1.64
PI586454	Hungary	85.33	19.14	7.36	1.91
PI217897	Indonesia	89.04	19.78	7.18	1.83
PI234456	Japan	81.16	18.59	7.21	1.69
PI82340	Korea	81.08	20.41	7.01	1.95
PI87355	Korea	91.48	21.19	6.80	1.78
PI88004	Korea	89.70	17.99	7.18	1.79
PI562758	Nigeria	86.49	27.74	7.63	2.21
PI567803	South Korea	87.53	21.25	7.28	1.72
PI567809	South Korea	86.27	23.20	7.21	1.84
PI567811	South Korea	100.1	17.48	7.34	1.45
PI5623402	USA	77.74	23.73	6.94	2.06
PI563015	USA	89.68	17.28	7.13	1.62
PI563068	USA	78.37	32.80	7.26	2.71
Ellis	USA	89.19	21.62	7.05	1.92