DESIGNER SORGHUM COMBINING THE HIGH DIGESTIBILITY AND WAXY GRAIN TRAITS IN SORGHUM FOR IMPROVED NUTRITION BIOETHANOL BEER FEED AND FOOD PRODUCTS

A Dissertation

by

BABITHA JAMPALA

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

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Plant Breeding

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ABSTRACT

Designer Sorghum Combining the High Digestibility and Waxy Grain Traits in Sorghum for Improved Nutrition Bioethanol Beer Feed and Food Products.

(May 2012)

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Sorghum (*Sorghum bicolor* (L). Moench) is used for human consumption in parts of Africa and Asia and as an animal feed mainly in the U.S. Though sorghum grain contains higher amounts of protein than other cereal grains such as wheat and corn, it is not as readily available for enzyme degradation in humans and animals. Protein body matrices called kafirins surround the starch granules in sorghum. Because the protein is less digestible, the starch is also less digestible for biofuel production. However variation for this trait exists and the line P850029 has a higher protein digestibility compared to other normal grain sorghum lines. This increase in digestibility of protein is due to the rearrangement of the kafirins in the prolamin protein bodies where, the γ-kafirins are rearranged in the seed endosperm and the amount of γ-kafirin in the grain is also reduced. The assay to phenotype the HD trait is time consuming and unpredictable. So identifying a quantitative trait loci (QTL) controlling the protein digestibility trait in sorghum would be beneficial in breeding. A recombinant

inbred lines (RILs) population derived from P850029 x ‘Sureno’, were developed and used to map QTL regulating the protein digestibility trait. A single QTL was identified on chromosome 1 between Xtxp43 and Xtxp329. Validation of the identified QTL was done on heterogeneous inbred families (HIFs). The results validate the same QTL identified on the RIL population on chromosome 1.

Later the high digestibility trait (HD) was integrated with the Waxy trait in sorghum. The Waxy (WX) sorghums have starch completely in the form of amylopectin. The effect of endosperm type on ethanol yield and fermentation efficiencies was studied among HD, WX and HD-WX lines. The HD-WX lines fermented in a shorter time i.e. completed fermentation in 48 h and their fermentation efficiencies were also higher around 90%. The DDGS of the HD-WX lines also had lower residual starch content and 50% higher amino acid lysine content when compared to wildtype sorghum.

Moreover, the relation between endosperm traits and grain yield in sorghum has not been fully explored. In this study, we compared the yield and yield components of four unique endosperm phenotypes, HD, WX, HD-WX and wildtype lines. A total of 100 $F_{2:4}$ derived recombinant inbred lines population from a cross between Tx2907/P850029 were selected with 25 lines from each HD, WX, HD-WX and wild-type line were included in the study. These lines were grown in three replications in College Station and Halfway, Texas in a randomized complete block design. The results show that there are no significant differences in the grain yield.

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CHAPTER I

INTRODUCTION AND OBJECTIVES

Sorghum (*Sorghum bicolor* L. Moench) is an important crop grown in arid and semi-arid regions of the world. The grain is used for human consumption in Asian and African countries. In the developed nations, sorghum is used as animal feed, for ethanol production, beer and celiac food applications. In the U.S. the major producers of sorghum are Texas, Oklahoma, Kansas, and Nebraska. Sorghum can be grown in arid, semiarid climates and marginal soils. In recent years the usage of sorghum for bioethanol production has been increasing. Sorghum is a source of protein in human and animal diets in most parts of the world. Though sorghum grain contains higher amounts of protein than other cereal grains such as wheat and corn (Dowling et al., 2002; Gualtieri and Rapaccini, 1990), it is not as readily available for enzyme degradation in humans, animals and also for biofuel production. Indeed, both protein and starch digestion are reduced in the human and animal consumption of sorghum grain.

The proteins in sorghum grain are alcohol soluble prolamines called kafirins, which make up about 50% of the endosperm matrix (Paulis and Wall, 1979).

This dissertation follows the style of Field Crop Research.

There are three types of kafirins, of which, α-kafirins are highly soluble, whereas, β-kafirins and γ-kafirins are not easily digested because they form enzyme resistant structures.

Oria et al. (2000) suggested that the lower digestibility of proteins in the sorghum endosperm is due to strong disulphide bonds formed by β and γ-kafirins, which result in enzyme resistant structure on the periphery of the protein body. The disulphide bonds in the protein matrix also limit the expansion of starch granules and access of amylases (Ezeogu et al., 2008) during starch hydrolysis. Since the highly digestible α-kafirins are located in the interior, the peripheral enzyme resistant layer negatively influences protein hydrolysis. The γ-kafirins are the most hydrophobic of the kafirins (Belton et al., 2006). This likely results in the slower digestion of sorghum starch during ethanol conversion. β- and γ-kafirins, which are rich in cysteine form extended, web like structures or sheet like structures with starch embedded within, after cooking. Formation of these extended structures reduces digestion of both protein and starch.

Sorghum line P850029 has higher protein digestibility compared to other normal grain sorghum lines, which might be due to rearrangement of kafirins. In the high digestible parent, the β- and γ-kafirins are rearranged in the seed endosperm and the amount of γ-kafirin in the grain is also reduced (Tesso et al., 2006). Due to this rearrangement and reduction, the grain with the high digestible trait can be easily digested during ethanol conversion (Fig1).

Moreover, the distillers dried grain solubles (DDGS) a by-product from the ethanol production is used as a feed ingredient. The amino acid content of the DDGS is an important quality parameter. Interestingly, the DDGS produced from the high digestible lines had higher lysine content than the normal sorghum lines (Wu et al., 2010). This would be an added advantage because it will help ethanol refineries to sell its by-product at a premium.

Another trait of interest, the waxy endosperm in sorghum was identified in 1933 (Karper, 1933). The starch in waxy sorghum is completely in the form of amylopectin that gives a candle wax type appearance, hence the name waxy. The advantage of starch in the form of amylopectin is that it gelatinizes at lower temperatures and to completion than do normal sorghum lines containing high amylose content (Wu et al., 2010). The waxy sorghum lines are also easily digested improving the feed efficiency in animals. Fig.1 shows the relative distribution of kafirins within a protein body for wildtype and high digestible sorghum.

Fig.1: Protein bodies in the endosperm of sorghum seed. a. Wildtype b. High Digestible parent. The black is β and δ-kafirins and grey is the α-kafirin.

Unfortunately, the protein digestibility assay by Mertz et al., 1984 developed to phenotype high digestible lines is time consuming and there is a lot of variation in the results. Identification of molecular markers linked to the high digestible trait would be beneficial in sorghum breeding. Wu et al., 2010 reported that high digestible lines take shorter time to ferment and waxy lines have higher fermentation efficiency. There is little information on how the ethanol yield and fermentation efficiency are affected by combining the two traits. Additionally, earlier research also suggests that waxy lines yield lower than the wildtype sorghum. But there is no information on how high digestible trait affects the yield components. This gap of knowledge is a critical impediment to the development of sorghum cultivars with an optimal endosperm matrix for bioethanol conversion (i.e. waxy and high digestible).

Based on these observations the *objectives of this proposal* are to define the phenotypic and genotypic expression of the high digestible trait, to identify and validate the quantitative trait loci (QTL) regulating protein digestibility in

sorghum grain. The *central hypothesis* of this proposal is by combining high digestible and waxy traits both the ethanol yield and fermentation efficiencies would be improved and the late expression of the high digestible, waxy or high digestible and waxy endosperm trait during grain development will also not confer a detrimental impact on yield. The following specific objectives will be used to test my hypotheses:

Objective 1. Identify QTL regulating the high digestibility trait in the recombinant inbred lines (RIL) and validate QTL identified in the heterogeneous inbred lines (HIFs). The hypothesis for this objective is that protein digestibility is a quantitatively inherited trait, yet the HD trait is controlled by one gene. A population of RILs developed by crossing two parents; P850029 and Sureno, where P850029 is an high digestible parental line and Sureno is an elite sorghum wildtype will be used. RILs were phenotyped for their expression of the high digestible trait. The high digestible trait in sorghum population is regulated by a discrete set of QTLs regulating the expression of γ-kafirins. Winn et al. (2009), identified two major QTLs on chromosome 1 that regulate protein digestibility in sorghum. Between the two QTLs, one QTL positively regulates the digestibility and the other negatively. By using some of the same SSR markers used by Winn et al. (2009) and some other additional SSR markers on the chromosome 1, validation of the identified protein digestibility QTL was done on HIF population.

Objective 2. Compare the ethanol yields and fermentation efficiencies of HD, Waxy and HD/Waxy lines. This study included lines from two crosses. Out of 29 lines included in this study, 14 lines were from the cross of TxARG-1/P850029 and 12 lines from a cross of Tx2907/P850029 and three parental lines were also included. Ethanol yields and fermentation efficiencies at 24 h and 72 h were calculated and compared.

Objective 3. Determine the differences in yield components among the HD, waxy, HD/waxy, and wildtype lines in multi-location yield trials. The effect of the endosperm traits on yield components was elucidated in this objective. Rooney et al. (2005) studied the relationship between endosperm type and grain yield potential in sorghum waxy and wildtype lines. Extending this study, we analyzed the effect of endosperm type on yield with HD, waxy, HD/waxy, and wildtype lines. 24 lines from each category (HD, WX, HD-WX, and Wildtype) were grown in two locations and in 2 replications at each location and the measurements of yield components recorded were grain yield, plant height, test weight, 100 kernel weight and seed number /panicle.

CHAPTER II

IDENTIFICATION AND VALIDATION OF THE QTL ASSOCIATED WITH THE PROTEIN DIGESTIBILITY TRAIT IN SORGHUM

2.1. Introduction and Literature Review

Sorghum is an important crop in the arid and semiarid regions of the world. In the United States, sorghum is grown in many states such as Texas, Oklahoma, Kansas, and Nebraska. According to United Nations Food and Agriculture Organization (FAO), 2009 statistics, United States ranked second in sorghum production with 9.7 million metric tons (http://faostat.fao.org). Sorghum is used for human consumption in most parts of Asia and Africa, but in the U.S. it is mainly used as animal feed. Approximately 45% of the sorghum produced within the U.S. is used as animal feed. Of the remainder, 30-35% is currently being used for bioethanol production (http://www.sorghum-growers.com). Sorghum production could significantly increase if demand for its use in ethanol production rises. After fermentation to ethanol, the by-product dried distillers grain solubles (DDGS) is sold as animal feed.

Like other cereal grains, sorghum also has a starch-rich endosperm. Starch ranges of 60-77% and 64-78% have been reported for sorghum and maize respectively (Dowling et al., 2002; Gualtieri and Rapaccini, 1990). However, the limitations for the use of sorghum in the ethanol industry is its potentially lower protein digestibility and like maize the poor amino acid composition of the DDGS. Hypotheses have been suggested by different

researchers to explain the low digestibility of protein and starch in sorghum compared to other cereals. The most commonly proposed theory is that the starch is surrounded by protein body (kafirin) matrices in the endosperm. These kafirin body matrices are hydrophobic in nature and form an enzyme resistant layer around starch, which restricts gelatinization and enzyme accessibility during hydrolysis into fermentable sugars (Taylor et al., 1984; Chandrashekar and Kirlies, 1988).

In sorghum endosperm, the primary protein storage molecules are prolamins. These prolamins, called kafirins, are aqueous alcohol-soluble, and are characterized into three distinct categories: α-, β-, and γ-kafirins based on molecular weight, solubility, and structure (Shull et al., 1991). α-Kafirins are the predominant type of kafirins, which make up to 80% of the kafirins or 60-70% of the total proteins found in sorghum grain (Hamaker et al., 1995). The α-kafirins comprise the central portion of the protein body, with γ- and β-kafirins residing on the periphery. Both β- and γ-kafirins have high concentrations of the amino acid cysteine. β-kafirins have 5% cysteine and γ-kafirins have 7% cysteine in total amino acid content, respectively (Shull et al., 1992). While the nutrient content of sorghum is comparable to other cereals, studies have shown that the availability of nutrients in sorghum is low in terms of starch and protein digestibility (MacLean et al., 1981, Hamaker et al., 1986). The reason is thought to be due to the high cysteine concentration in β- and γ-kafirins, which causes extensive disulphide cross linking and causes the normally highly digestible α-

kafirins to be inaccessible to proteolytic enzymes (Duodu, 2003). The presence of the kafirin protein body matrix surrounding starch granules has also been attributed to the poor starch granule digestibility of cooked and uncooked sorghum. The kafirin protein body matrix forms a hydrophobic enzymatic barrier to starch hydrolysis, which requires higher gelatinization temperatures prior to enzymatic hydrolysis or saccharification.

The high digestible sorghum mutant was discovered in a population of sorghum lines derived from crosses of P721Q (high lysine mutant) and hard endosperm, food grade sorghums (Weaver et al., 1998; Mohan, 1975). The high digestible trait confers approximately 10-15% higher protein digestibility when uncooked and 25% higher digestibility when cooked. The digestibility of α-kafirins also increased to 90-95% following pepsin digestion in the high digestible mutants, as compared with 45-60% digestibility in normal lines (Weaver et al., 1998). Oria et al., (2000) suggested that the higher digestibility of these lines is due to rearrangement of the kafirins, particularly the γ-kafirins, located at the periphery of the protein body. Unlike wildtype sorghum with the γ-kafirins located at the exterior of protein bodies, high digestible mutants possess γ-kafirins that are found only in the pockets of folds (Oria et al., 2000).

The changes in the protein body structure lead to the exposure of the interior α-kafirins, making them susceptible to proteolytic enzymes. It has been reported that the protein body matrix in the high digestible mutant is also often not surrounding the starch granule as in the wildtypes, but rather coalesced into

protein body pockets (Hamaker et al., 1986). The high digestible trait would also improve the exposure of starch granules to gelatinization, saccharification and ethanol fermentation (Wu et al., 2010). The amino acid content of the DDGS is an important quality parameter. Not surprisingly, like the mutant itself, which is reported to have 60% high lysine content (Hamaker et al., 1986). The results show that the DDGS produced from these high digestible lines also has higher lysine content than the normal sorghum lines (Wu et al., 2010). Given these results the high digestible trait, like the waxy trait, represents an ideal trait for introgression into new sorghum hybrids specifically designed for the bioethanol industry. Unfortunately, the protein digestibility assay developed by Mertz et al., 1984, which is used to phenotype the high digestible trait is time consuming and expensive. The reliability and sensitivity of this assay is also inconsistent. The identification of molecular markers linked to the high digestible trait would be highly beneficial in sorghum breeding.

Based on these observations the objective of this study is to identify the QTL regulating the protein digestibility trait in Recombinant Inbred Lines (RILs) and to validate the identified QTL in HIFs.

2.2. Material and Methods

2.2.1. Parental Lines and RIL Population Development

A set of 191 F_4 RILs was derived from a cross between P850029/Sureno grown in College Station, TX. P850029 is a high digestible protein sorghum line developed at Purdue University from a population derived from P721Q (Weaver

et al., 1998; Mohan, 1975). Sureno is a white sorghum with a wildtype/normal endosperm, low digestible protein and good mold resistance (Meckenstock et al., 1993).

2.2.2. Phenotypic Data

Phenotyping of the high digestible trait was done by visual examination. 25 seed of each RIL were cut into halves and observed on a light box. Fig. 2 shows the endosperms of wildtype and high digestible parent. The wild-type parent (Sureno) has two types of endosperms in the seed. The central endosperm is the floury type and the outer waxy layer called the corneous endosperm. The P850029 mutant has only one type of endosperm in the seed, which is floury endosperm. The corneous endosperm is absent in the mutant. Based on the observation of seed on the light box, the RIL population was scored into wild-type or high digestible.

a b

Fig.2: Seeds of the sorghum cut into halves a. Sureno b. P850029 (high digestible parent).

2.2.3. Genotypic Data

DNA was isolated by harvesting fresh leaf tissue (~0.33g) as described by (Dellaporta et al., 1983). The primers that were on chromosome 1 of sorghum were preselected based on the results of Winn et al., 2009. A set of 89 sorghum simple sequence repeats (SSR) selected primers were used for the genotypic analysis. Out of the 89 SSRs, 68 SSRs were obtained from Agricultural Research Station, Lubbock from Dr Gloria Burow and they are designated as Xsbarslbk and the rest were Xtxp markers. The PCR solutions used were: 2μl buffer (5X), 0.2μl dNTPs (10mM), 0.2μl each if forward and reverse primer (20μM), 0.5μl MgCl2 (25mM), 0.02μl BSA, 2μl DNA (10ng/μl), 0.1μl Taq Polymerase, and 4.88μl water to which 2μl of DNA at the concentration of 24ng/ul was added to make the final volume to 10ul. PCR reactions were set up using the conditions: 94C for 5min, 40 cycles at 1min, annealing temperature for 1min, and 72C for 1min, followed by 10min at 72C. SFR agarose gels (Amresco) were used for band separation. One of the marker was not showing polymorphism on the agarose gel, so these samples were analyzed on QIAxcel using QIAxcel DNA high resolution kit.

2.2.4. Development of Heterogeneous Inbred Family

Heterogeneous inbred family (HIF) were developed using the method by Tuinstra et al., 1997. The RILs segregating for the markers Xtxp43, Xtxp325 and Xtxp329 were selected. Eight segregating RILs from the population of 191 were selected. A hundred seed from each of these eight lines were selected and

planted at the Texas A&M University field in College Station, TX. During the growing season, leaf tissue was collected and at the end of the plant growth, seed were collected. The leaf tissue collected was used for DNA extraction and later genotyping. The heads were bagged to prevent cross pollination. The seed collected were dried and phenotyped on a light box and the lines were scored as high digestible or wild-type. For the genotyping of the HIF, the primers that were significant on the initial analysis i.e. Xtxp43, Xtxp325 and Xtxp329 were rerun on the HIF population. The data collected from both phenotyping and genotyping was used to validate the protein digestibility QTL. The genotypic data for the three markers of interest were run on a QIAxcel using QIAxcel DNA high resolution kit.

2.2.5. Molecular Mapping and QTL Analysis

A genetic linkage map was generated for the RIL population using Mapmaker/Exp v3. Two or three markers with known chromosomal locations will be used as an initial framework for chromosome 1 of sorghum genome. Markers were then added to this framework using the assign and try commands, with final marker order confirmed using the ripple command.

The QTL cartographer (V2.5) was used for the identification of the QTL. Composite interval mapping (CIM) was used to determine the QTL position and effects. For the identification of QTL in RILs, a log of likelihood score (LOD) of 1.5 and Kosambi's map function were used to establish linkage. A 1000 permutation test was used to determine the LOD threshold at a significance level

of P=0.05. A forward and backward regression method (p=0.05) with a 10cm window was used to identify QTL in CIM. For the fine mapping, a LOD score of 10 was used.

2.3. Results and Discussion

2.3.1. QTL Identification

Out of 89 SSR primers, only 16 markers showed polymorphism on the parents. These 16 markers were run on the population of 191 RILs. Though all the markers selected were from chromosome 1 of sorghum, only 10 markers among the 16 polymorphic markers were linked into one group by Mapmaker 3.0. A linkage map of the 10 markers is shown in Fig. 3. The distance between the markers is in centimorgans (cM). The alignment of the markers on the linkage map developed is similar with the sequence mentioned in the map developed by Bhattramakki et al., 2000.

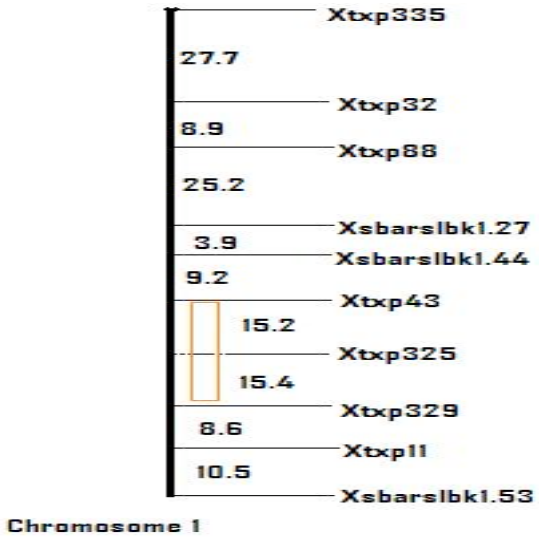


Fig.3: Linkage map of primers segregating with high digestibility trait. The red box shows the significant QTL.

Results from QTL cartographer show a single significant QTL for the protein digestibility trait on the chromosome 1 of sorghum at a LOD>1.5. The markers Xtxp43, Xtxp325 and Xtxp329 are significantly associated with the QTL identified (Fig. 4). The QTL at this locus displays only additive effect (Fig. 5). The percent of phenotypic variation (R^2) explained by the alleles at this locus accounts for approximately 8% of the total phenotypic variation seen (Fig. 6).

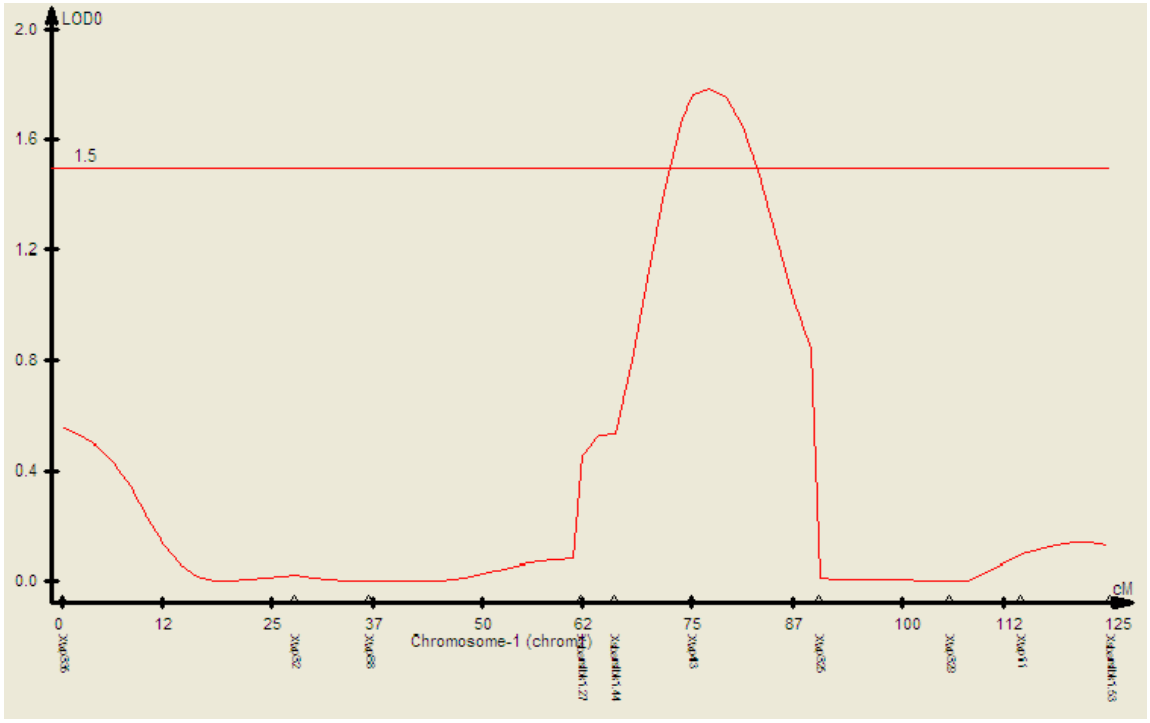


Fig.4: Significant QTL identified and markers associated with the QTL.

Fig.5: Additive effect of the protein digestibility QTL identified.

Fig.6: R^2 value for the protein digestibility QTL identified.

The markers Xtxp43 was significantly linked to the protein digestibility QTL identified at p=0.01, while markers Xtxp325 and Xtxp329 were significantly linked to the QTL at p=0.05 (Table 1).

Table1: Summary of marker segregation (Chromosome 1). The marker name along with the LOD score according to the QTL distribution and the probability that each marker segregates independently of the protein digestibility trait (using Mapmaker 3.0/QTL Cartographer Single Marker Analysis).

Name of marker	LOD	pr(F)
Xtxp 335	0.63	0.071
Xtxp32	0.08	0.746
Xtxp88	0.02	0.168
Xsbarslbk1.27	0.06	0.147
Xsbarslbk1.44	0.51	0.117
Xtxp43	1.87	0.005**
Xtxp325	0.75	0.012*
Xtxp329	0.03	0.022*
Xtxp11	0.03	0.256
Xsbarslbk1.53	0.10	0.736

2.3.2. QTL Validation Results

The results of QTL validation show that the primers Xtxp43 and Xtxp325 are highly associated with the high digestible trait and also identified a highly significant QTL on the two markers associated (Fig. 7). This QTL also has an additive effect as mentioned earlier (Fig. 8). The percent of phenotypic variation (R^2) explained by the alleles at this locus in HIF population is 45% of the total variation seen (Fig. 9). After the primer Xtxp325, the phenotypic variation increases to 91% and later reduces sharply before reaching primer Xtxp329.

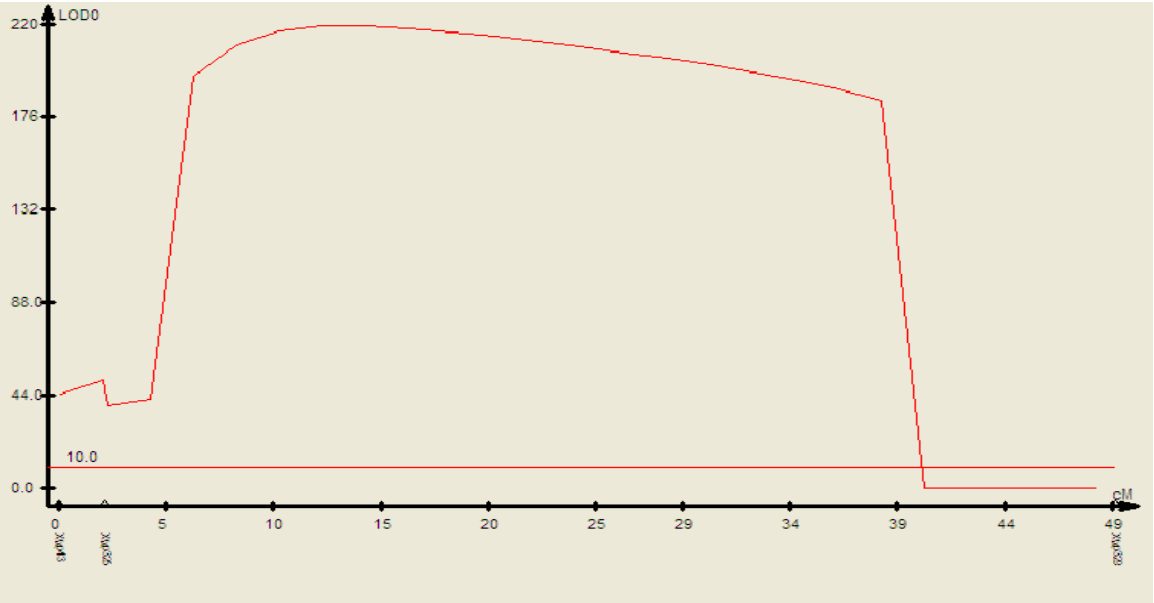


Fig.7: QTL identified on chromosome 1 associated with the protein digestibility trait on the HIF population of 800 lines.

Fig.8: Additive effect of the QTL identified on HIF population.

Fig.9: R^2 value for the protein digestibility QTL identified on HIF population.

The markers Xtxp43 and Xtxp325 were significantly associated with the identified QTL of protein digestibility trait at p=0.01 (Table 2).

Table 2: Summary of the marker segregation. The marker name along with LOD scores according to the QTL distribution and the probability that each marker segregates independently of the protein digestibility trait (using Mapmaker/QTL Cartographer Single Marker Analysis) for the HIF.

Name of the marker	LOD score	pr(F)
Xtxp43	44.0	0.003***
Xtxp325	40.1	0.004***
Xtxp329	0.5	0.175

2.4. Conclusion

There are two markers Xtxp43 and Xtxp325, which are closely associated with the protein digestibility trait in sorghum. Earlier study by Winn et al., 2009 suggests that there are two QTLs that work against each other for the protein digestibility trait in sorghum. Our study identified only one QTL for the protein digestibility trait on marker Xtxp43, Xtxp325 and Xtxp329 in the RIL population, which is the QTL 2 identified by Winn et al., 2009. This QTL has only additive effect and explains less percentage of phenotypic variation in the RILs. The validation results validate the same QTL identified in the RIL population, but the percentage of phenotypic variation explained by the QTL in HIF population is around 45%. There is a kafirin gene cluster (22kD) mapped on marker Xtxp 325 region of the chromosome 1 of sorghum. The position of the marker Xtxp325 is 7020894 to 7020916 bp on chromosome 1 and the kafirin cluster is located at 7020894 to 7021141 bp on the same chromosome (Phytozome search). This

suggests that Xtxp325 is at the beginning of the kafirin cluster. The LOD score of the QTL increases many times just after marker Xtxp325 (Figure 6) which can be explained as there is a kafirin gene located at that position on the chromosome. All these results strongly suggest that kafirin rearrangement in the high digestible mutants may indeed be the reason for the increase in the digestibility of proteins and in turn starch digestion.

CHAPTER III

COMPARING THE ETHANOL YIELDS AND FERMENTATION EFFICIENCIES OF HIGH DIGESTIBLE (HD), WAXY (WX) AND HIGH DIGESTIBLE-WAXY (HD-WX) LINES

3.1. Introduction and Literature Review

Sorghum is an important crop grown in the arid and semiarid regions of the world. Sorghum grain is used for various purposes such as food, animal feed, beer production and also biofuel production. In the U.S. the highest sorghum acreage is in Texas, Oklahoma, Kansas, and Nebraska. Corn is the major crop used for bioethanol production in the U.S. but many factors such as high production costs, and water demand limit its usage for biofuel. Compared to corn, sorghum use for biofuel production is of particular benefit because sorghum is well adapted to drought, marginal soils and has lower fertilizer requirement (Rosenow and Clark, 1981). In 2009, sorghum production in the U.S. was 9.7 million metric tons (http:faostat.fao.org), of which 10-20% was used for ethanol production (http://www.sorghum-growers.com). In 2011, approximately 30-35% of the grain sorghum grown in the U.S. is being used for ethanol production. The demand for the grain sorghum in the biofuel industry has been increasing rapidly. Like other cereal grains, sorghum also has a starch rich endosperm. Starch ranges of 60-77% and 64-78% have been reported for sorghum and corn respectively (Dowling et al., 2002).

However, the barriers for the use of sorghum grain in ethanol production are its potentially lower starch digestibility and requirement of higher energy for gelatinization, enzyme hydrolysis and fermentation. In wildtype sorghum, the higher energy for starch gelatinization is thought to result from the fact that the starch granules in the endosperm are surrounded by a hydrophobic protein body matrices (Taylor et al., 1984; Chandrashekar and Kirlies, 1988).

Several mutants of sorghum with modified endosperm matrices have been developed and identified by many researchers. Among them, the most important are the high protein digestible and waxy sorghum lines. Oria et al., 2000 studied the structure of protein bodies in both the wildtype and an high digestible mutant and found rearrangement of γ-kafirin bodies in the mutant. They suggested that the rearrangement may be the reason for the increase in the digestibility of the mutant. Among the three types of kafirins α-, β- and γ, in the high digestible mutants the digestibility of α-kafirins increased to 90-95% following pepsin digestion, as compared with 45-60% digestibility in wildtype lines (Weaver et al., 1998). The changes in the protein body structure lead to the exposure of the interior α-kafirins, making them susceptible to proteolytic enzymes. This may in turn improve the exposure of the starch granules for gelatinization and enzyme degradation. The starch swells and pastes at lower temperatures and the dry distiller's grain solubles (DDGS), derived as a by-product of fermentation has a higher lysine content.

Sorghum starch requires much higher temperatures to gelatinize compared to many other cereals (Lineback, 1984) due to a higher amylose to amylopectin ratio. Both the fermentation efficiency and ethanol yield decrease as amylose content increases in the sorghum lines (Wu et al., 2010). Karper (1933) was the first to report a sorghum genotype with 100% amylopectin, designating it as a waxy sorghum. Waxy endosperm sorghum lines are generally more digestible, leading to improved feed efficiency, and are easier to process, which is useful both industrial and food uses (Del Pozo-Insfran et al., 2004). Waxy sorghums also gelatinize more rapidly, at lower temperatures and are more susceptible to enzyme hydrolysis and hence are better for biofuel conversion (Taylor et al., 2006).

Combining the high digestible modified endosperm and waxy trait into one cultivar will solve some limitations for using sorghum for biofuel production. The combination will remove the inhibitory kafirin protein matrices surrounding the starch granules, reduce the temperatures required for starch gelatinization and enzymatic hydrolysis, and increase the bioavailability of nutrients and essential amino acids such a lysine in the DDGS. Wu et al., 2010 has reported that HD lines with modified endosperm protein matrix have good fermentation characteristics such as high ethanol yield, high fermentation efficiency and fast fermentation. They also reported that ideal sorghum lines for biofuel production would be lines with both the high digestible and waxy endosperm traits. Their study included 13 sorghum lines from Texas, which included wildtype, HD and

Waxy lines. The objective of this study is to compare the ethanol yield and fermentation efficiencies of HD, Waxy and HD/Waxy lines developed from a cross of HD and waxy parents. Residual starch and amino acid composition in the DDGS were also analyzed to check the quality of the DDGS.

3.2. Materials and Methods

3.2.1. Plant Material

Twenty nine sorghum lines with different endosperm matrices (HD, WX or HD-WX) were planted in the normal cropping season of 2009 at Texas Agrilife Research Farm, College Station, TX. The entries included the 14 lines, which are progeny from cross TxARG-1/P850029, 12 lines which are progeny from cross RTx2907/P850029 and three parental lines: TxARG-1, Tx2907 and P850029. Among the three parents, TxARG-1 (Miller et al., 1992) and RTx2907 (Miller et al., 1996) are the waxy lines and P850029 is a high digestible protein, which was developed at Purdue University from a population derived from P721Q (Weaver et al., 1998; Mohan, 1975). The plots were combine harvested and the grain was used for the remainder of the experiments. The sorghum seed were ground for analysis and fermentation using a Udy cyclone sample mill with a 0.5 mm screen (UDY Corp., Fort Collins, CO). All the experiments were conducted in the lab of Dr. Donghai Wang in Kansas Agricultural Experiment Station, Manhattan, KS 66506.

3.2.2. Chemicals Used for Analytical Methods

Analysis was completed using he methodology described in Wu et al., 2010. Potassium phosphate monobasic, magnesium sulfate, dextrose, sodium acetate, hydrochloric acid, sodium hydroxide, acetic acid, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Fairlawn, NJ). Difco yeast extract and Difco peptone were from Becton-Dickinson (Sparks, MD). Maltose, maltotriose, 4-morpholinepropanesulfonic acid (MOPS), and analytical standard glucose were from Supelco (Bellefonte, PA). Standard reference ethanol (SRM 2899a) was purchased from NIST (Gaithersburg, MD). All other chemicals were reagent grade or better.

The hydrolyzing enzymes, Liquozyme (a high-temperature α-amylase produced by *Bacillus licheniformis*) and Spirizyme (a glucoamylase produced by *Aspergillus niger*), were provided by Novozymes (Novozymes North America, Inc., Franklinton, NC). The dry alcohol yeast, Ethanol Red, was provided by Fermentis in vacuum-packed aluminium foil bags (Lesaffre Yeast Corp., Milwaukee, WI).

3.2.3. Starch and Amino Acid Analysis

3.2.3.1. Total Starch in the Original Sorghum Samples and DDGS

Total starches in sorghum samples and corresponding freeze-dried DDGS were determined by using Megazyme K-TSTA kits with modified DMSO procedures (http://www.megazyme.com/downloads/en/data/K-TSTA.pdf.). Starches in the samples were completely solubilized in DMSO and hydrolyzed in

two steps in glucose by using thermostable α-amylase (100 °C, pH 6-6.5) and amyloglucosidase (50 °C, pH 4.5).

3.2.3.2. Amino Acid Composition of DDGS

Samples were weighed and then placed in about 0.5 mL of 6 N HCl along with the internal standard and hydrolyzed at 100 °C for 20h. An aliquot, usually 10 or 20 μL, of that HCl was diluted up to 250 μL with 0.4 M borate buffer to dilute the sample and raise the pH. After precolumn derivatization with o-phthalaldehyde (OPA) and 9-fluorenylmethylchloroformate (FMOC), 1 μL of this diluent was injected into an HPLC system with a C18 column (Hypersil AA-ODS, 2.1 X 200 mm, 5 μm). Mobile phase A was 20 mM sodium acetate buffer with 0.018% (v/v) triethylamine, 0.05 mM EDTA, and 0.3% tetrahydrofuran, pH adjusted to 7.2 using acetic acid. Mobile phase B was 100mM sodium acetate:acetonitrile:methanol (20:40:40, v/v). The elution conditions were from 100% A to 60% B in 17 min at 0.45 ml/min. Amino acid derivatives were detected with a fluorescent detector at 340/450 nm (excitation/emission) for primary amino acids and 266/305 nm for secondary amino acids. Human serum albumin was used as control, and norvaline and sarcosine were used as internal standards.

3.2.3.3. Ethanol Fermentation

Ground samples containing 30 g of dry mass were mixed with 100 mL of preheated (≅60-70 °C) enzyme solution (containing 1.0 g/L KH_2PO_4 and 200 μL/L Liquozyme) in a clean 250 mL Erlenmeyer flask to form an evenly

suspended slurry. The temperature program for mashing and the procedures and conditions for simultaneous saccharification and fermentation (SSF) were the same as described by Wu et al., 2008.

Ethanol concentration in the finished product was determined by HPLC with a Rezex RCM column and RI detector after distillation as described by Wu et al., 2006. The fermentation efficiency was calculated on the basis of the theoretical ethanol yield of 56.72 g from 100.0 g of dry starch.

3.2.4. Statistical Analysis

The differences among the sorghum samples for each trait were determined using the LSD Line option of PROC GLM. The relation between starch content, ethanol yield and fermentation efficiency were determined using PROC CORR using SAS (SAS v9.2, SAS Institute Inc., Cary, NC, USA).

3.3. Results and Discussion

3.3.1. Physical and Chemical Composition

There were differences in chemical components among all the sorghum samples included in the study. Previous study by Wu et al., 2010 had HD, WX and wildtype sorghum samples, hence our study did not include wildtype sorghum lines. Our study includes HD, WX and HD-WX sorghum lines. Moisture content of the sorghum flours differed significantly among the samples though all the sorghum lines were harvested and dried for the same amount of time. Sample 20, which is HD-WX and sample 1, HD parent P850029 had the lowest moisture content and sample 8 and 16 both HD-WX lines had the highest

moisture content. On an average, moisture content of HD was 7.78%, WX was 8.89% and HD-WX was 8.26% (Table 3). Starch content of all the samples was slightly lower than the normal sorghum and corn starch contents. Two HD-WX lines had significantly higher starch contents of 67.38% and 66.90% and two other HD-WX lines had significantly lower starch contents of 56.54% and 57.64%. Mean starch contents of HD, WX and HD-WX samples were 63.99, 62.61 and 62.16%.

3.3.2. Fermentation Efficiencies and Ethanol Yields

The fermentation efficiencies of the sorghum samples were good, with an average of 91.3%. But there were significant differences observed among the different samples at final fermentation efficiencies. Both the WX parents TxARG-1 and Tx2907 had the lowest fermentation efficiencies of 89.8% and 89.4% respectively. HD parent P850029 and sample # 18, a HD-WX line had the higher fermentation efficiencies of 93.01% and 92.60% respectively. The fermentation efficiency of HD-WX lines ranged from 90.30 to 92.60%. Waxy lines had lower fermentation efficiency and HD lines had higher fermentation efficiency at 24 h after the initiation of fermentation. The average fermentation efficiency of the HD, WX and HD-WX at 24 h was 89.45, 87.46 and 91.96%.

Table 3. Means of the moisture, starch content and fermentation efficiencies at 24 and 72 h for the endosperm types HD, WX and HD-WX sorghum samples.

Entry	Endosperm Type	Moisture	Total Starch	Efficiency at 24 h	Efficiency at 72 h
1	HD	7.78	63.99c	89.45b	92.10c
2	Waxy	8.89	62.61b	87.46a	90.70a
3	HD/Waxy	8.26	62.16a	91.96c	91.32b

Means with different alphabets are significantly different.

The correlation between starch content and ethanol yield was positive and strong (R^2 = 0.9671) (Fig.10). The R^2 value was higher that reported in previous studies by Wu et al. (2007 and 2010). Our study along with previous studies suggests that starch content in one of the most important factor affecting ethanol production. The correlation between starch content and fermentation efficiency is also positive but weak with R^2 value of 0.1476.

The tested samples showed diverse fermentation kinetics in the laboratory SSF dry-grind process. The samples were classified into one of the three categories based on their fermentation rate and ethanol yields: fast, medium or slow. Fig. 11 shows the fermentation rates and ethanol yields of parental lines and population from a cross of TxARG-1 and P850029. The mutant parent (sample 1) along with some HD-WX lines (samples 10, 13, 14 and 16) are in the fast fermentation group. These lines are yielding about 375 L/ton of ethanol in 24 h of fermentation. The fermentation of these lines was nearly completed in 36 h. Waxy parents (samples 2 and 3) along with some HD-WX lines (samples 5 and 11) belong to slow fermentation group. These lines ethanol yield is approximately 330 L/ton at 24 h of fermentation but their ethanol yields

are approximately 440 L/ton by the end time of fermentation (72 h). All other lines (samples 4, 6, 7, 8, 9, 12, 15, and 17) belong to the intermediate group. These lines have ethanol yields higher than the fast group but, less than the slow group. The lines in the intermediate group can finish fermentation in 48 h.

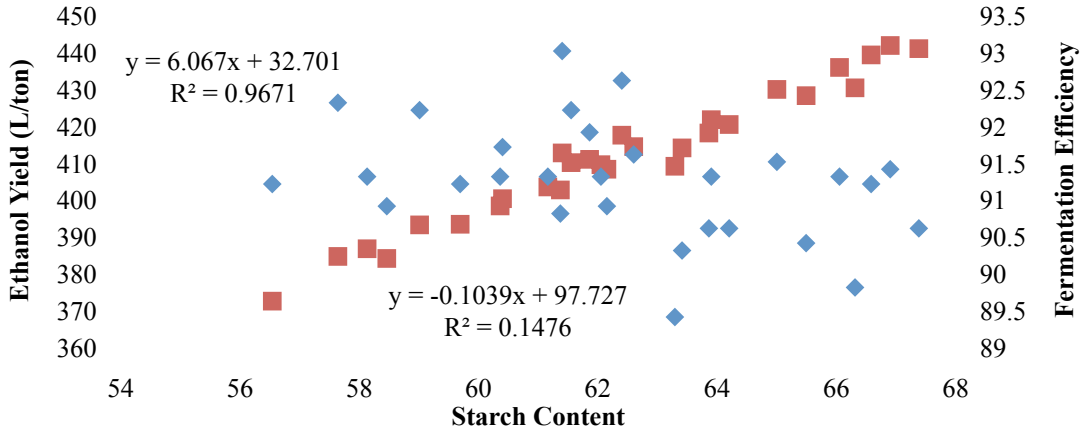


Fig.10: Relationship between starch content (%), ethanol yield (L/ton) and fermentation efficiency (%).

Fig.12 shows the fermentation rates and ethanol yields of parental lines and a population from a cross of Tx2907 and P850029. These lines could not be distinctly separated in to three categories. Even the separation of the parental lines into distinct groups is not possible. Samples 2, 3 and 24, which consist of WX and HD-WX samples were in the fast fermentation group. Samples 27, 29 and 33 consisting of HD-WX and WX endosperm types belonged to slow fermentation rates. All other samples belonged to medium fermentation types.

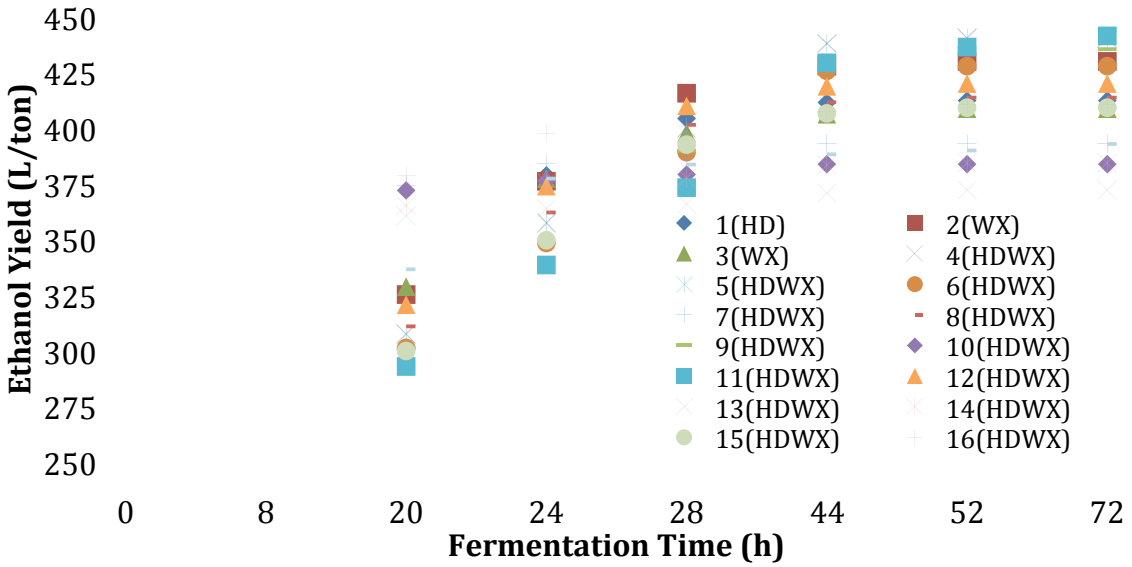


Fig.11: Kinetics of ethanol fermentation process for high digestible parent (P850029), Waxy parents (TxARG-1 and Tx2907) and population from a cross TxARG-1/P850029.

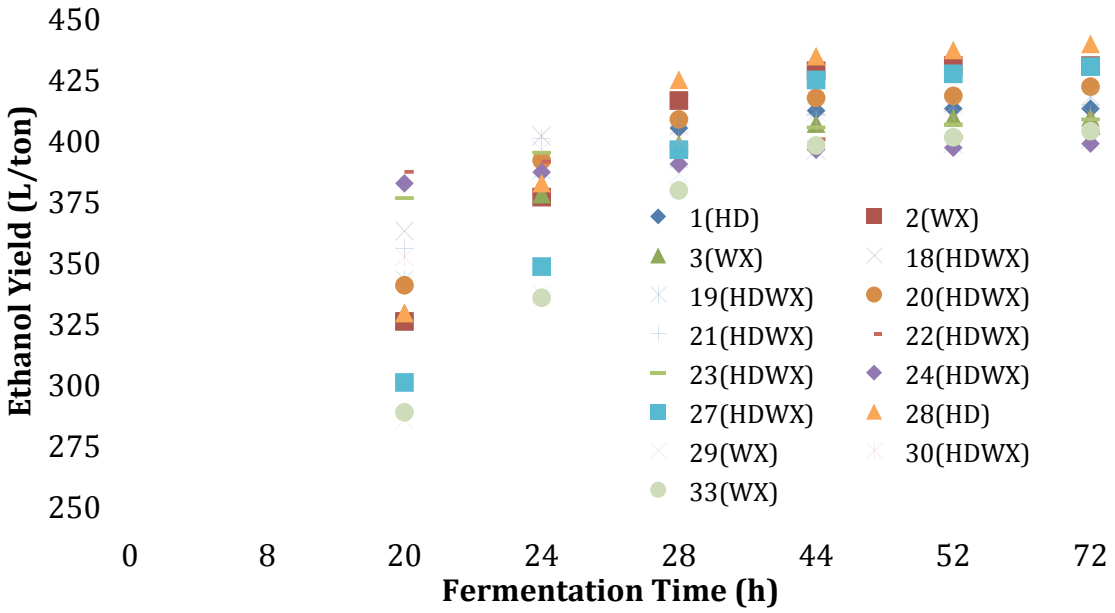


Fig.12: Kinetics of ethanol fermentation process for high digestible parent (P850029), Waxy parents (TxARG-1 and Tx2907) and population from a cross Tx2907/P850029.

3.3.3. Analysis of the DDGS

DDGS is the important byproduct of the ethanol production systems and if its feed quality is higher it can be marketed at profit margin. The two quality parameters that will improve the feed value of the DDGS are the residual starch and amino acid composition especially lysine content. The lower the residual starch and higher the lysine content, the higher will be the feed value of the DDGS (Wu et al., 2010). The residual starch contents of the DDGS samples tested are listed in Table 4. Earlier research suggests that wild-type sorghum samples after fermentation would have approximately 1% residual starch content. All the samples in our study had a residual starch content of less than 1% with an average of 0.58%. The highest residual starch contents among all the samples tested were 0.85 and 0.96% for samples 2 and 3 respectively. These two lines were the waxy parents TxARG-1 and Tx2907 respectively. The higher residual starch in the above mentioned samples suggest that starch was less efficiently utilized. All other samples had the high digestible trait, which suggests that the inhibitory protein body matrix was removed. Hence starch was readily available for gelatinization and enzymatic degradation. In the waxy parents, starch was completely in the form of amylopectin, but the starch was surrounded by enzyme resistant kafirin body matrix. Hence the starch in waxy lines was not efficiently utilized. The mean residual starch content of HD, WX and HD-WX samples are 0.62, 0.75 and 0.52%.

Table 4: Mean residual starch percentages for HD, WX and HD-WX sorghum samples.

Endosperm Type	Residual Starch (%)
HD	0.615b
WX	0.752c
HD-WX	0.519a

Numbers with different alphabets indicate significant difference.

Table 5: Mean amino acid lysine content (%) for HD, WX and HD-WX sorghum samples.

Endosperm Type	Amino acid Lysine (%)
HD	3.0c
WX	2.1a
HD-WX	2.4b

Numbers with different alphabets indicate significant difference.

Amino acid compositions of the DDGS proteins are shown in Table 5. Normal wild-type grain sorghum has a lysine content of 0.59% (Vendemiatti et L., 2008), wild-type corn DDGS has a lysine content of 0.85% (Stein et al., 2006) and high lysine sorghum varieties could have 1.5 – 2.5% lysine (Reddy et al., 2002). In our study the HD parent had a lysine content of 3.2% and waxy parents had a lysine content of 2.3%. The HDWX lines had an average lysine content of 2.4%, though this percentage is not higher than the HD parent, it is higher when compared to wild-type sorghum samples (Wu et al., 2010).

3.4. Conclusion

Though all sorghum samples were dried for the same period of time, there were differences among them in moisture content, which could not be explained. The sorghum samples of HD-WX endosperm type analyzed had a wide range of variation in the traits measured in the study. Some of the HD-WX lines showed starch content as high as the parental lines. Also some of the HD-WX lines reached 76 - 96% of their fermentation efficiencies in 24 h and essentially completed their fermentation process in 48 h. The final ethanol yields of HD-WX lines were in the range of 372 to 440 L/ton. In the DDGS analysis of HD-WX samples, the residual starch content were from 0.31 to 0.73% and the amino acid lysine content ranged from 1.7 to 2.8% of net protein weight. The traits that are desirable for the production of ethanol are high starch content, high fermentation speed and high fermentation efficiency. The study concludes that there are some HD-WX lines like sample 13 and 18 that are performing better than the parents. The high performing HD-WX lines can be selected and improved further for the traits of interest such as high starch content, high ethanol yield and lower residual starch and high lysine amino acid contents in the DDGS.

CHAPTER IV

ESTIMATING THE RELATIVE EFFECTS OF THE ENDOSPERM TRAITS OF WAXY AND HIGH PROTEIN DIGESTBILITY ON YIELD IN GRAIN SORGHUM

4.1. Introduction and Literature Review

Sorghum (*Sorghum bicolor* L. Moench) is an important crop grown in arid and semi-arid regions of the world. The grain is used primarily for human consumption in Asian and African countries and in the developed nations, it is traditionally used as animal feed and more recently, ethanol production. Compared to corn, sorghum grain contains lower fat and higher protein concentrations, and is similar in starch content (Dowling et al., 2002; Gualtieri and Rapaccini, 1990). Another primary difference between corn and sorghum is that the protein and starch in sorghum is not as readily available for enzyme degradation in animal, human or industrial processing of the grain (Spicer et al., 1982, 1983).

The reduced digestibility of sorghum endosperm is thought to be due to specific endosperm storage proteins in grain sorghum. These proteins are in the form of alcohol soluble prolamines called kafirins, which make up about 50% of the endosperm matrix (Paulis and Wall, 1979). Kafirin storage proteins come in three types: α-, β- and γ-kafirins. The α-kafirins are highly soluble and easily digested while the latter two are much less soluble and are not easily digested because they form enzyme resistant structures. Oria et al. (2000) suggested that the lower digestibility of proteins in the sorghum endosperm is due to strong

disulphide bonds formed by β and γ-kafirins which produce an enzyme resistant structure on the periphery of the protein body. During starch hydrolysis, the disulphide bonds in the protein matrix also limit both the access of amylases to and the expansion of starch granules (Ezeogu et al., 2008). Since the highly digestible α-kafirins are located in the interior, the peripheral enzyme resistant layer of β and γ-kafirins negatively influences protein hydrolysis. Finally, the γ-kafirins are the most hydrophobic of the kafirins (Belton et al., 2006) and these likely results in the slower digestion of sorghum starch during ethanol conversion.

Genetic variation for starch and protein digestibility is known to exists in sorghum. The genotype P850029 is reported to have higher protein digestibility compared to other normal grain sorghum lines (Weaver et al., 1998). The increased digestibility is due to structural rearrangement of β- and γ-kafirins in the endosperm and a reduction in the total amount of γ-kafirin in the endosperm (Tesso et al., 2006). Thus the genotypes that possess these modifications produce grain that is easier to digest in any application, ranging from animal feeding to ethanol production (Nyannor et al., 2007).

In addition to the effect of protein on endosperm, starch content and composition can also influence processing characteristics. Normal sorghum genotypes produce both amylopectin and amylose starches in a 3:1 ratio in the endosperm, but variants that adjust the proportion of these compounds exist. For example, “waxy” endosperm sorghum types do not produce amylose

resulting in an endosperm in which all the starch is amylopectin (Karper, 1933). The waxy phenotype in sorghum is conditioned by a single gene in the recessive form designated as *wx* (Melvin and Sieglinger, 1952) which results in the absence or inactivation of granule-bound starch synthase (GBSS). Two distinctly different naturally occurring waxy alleles have been identified in sorghum. The waxy GBSS- allele also designated as wx^a has no GBSS present while the other allele waxy GBSS+ also designated as wx^b has inactive GBSS present (Pedersen et al., 2005). From a processing and utilization standpoint, the advantage of amylopectin starch is a lower gelatinization temperatures which means that processing and hydrolysis requires less energy and time to complete (Wu et al., 2010). There are no negative effects on growth and development on animals by feeding waxy sorghums in animal feeding operations (Shelton et. al., 2004).

In theory, combining the high digestible and waxy traits should make it possible to develop a grain sorghum endosperm with reduced energy input gelatinization requirements and improved enzymatic hydrolysis. These traits are valued in the ethanol industry as they result in reduced energy requirement and faster conversion and turnover in the production. However, improved digestibility traits in cereal grain are commonly associated with lower grain yield potential and increased susceptibility to grain weathering and both of these traits would limit the potential value of this combination. In sorghum Rooney et al., (2005) reported a 17% reduction in yield between waxy and non-waxy groups derived

from the same population but that several high yielding waxy lines were present in the trial. Thus, there was the potential with breeding to enhance and improve the yield of the waxy endosperm sorghums.

There are no reports on the effect of the high digestible trait on agronomic potential or in the combination with waxy endosperm. Therefore, the objective of this study is to determine the relative effect on agronomic performance of the high digestible trait per se and in combination with waxy endosperm.

4.2. Materials and Methods

4.2.1. Parental Lines and Population Development

A set of 100 $F_{2:4}$ derived recombinant inbred lines (RIL) was developed from an F_2 population of the cross between Tx2907/P850029. Tx2907 is a waxy endosperm sorghum parental line which was released from the Texas Agrilife Research sorghum breeding program (Miller et al., 1996). This waxy line has normal protein digestibility. P850029 is a sorghum line with normal (non-waxy) endosperm and high digestible protein, which was developed at Purdue University from a population derived from P721Q (Weaver et al., 1998; Mohan, 1975).

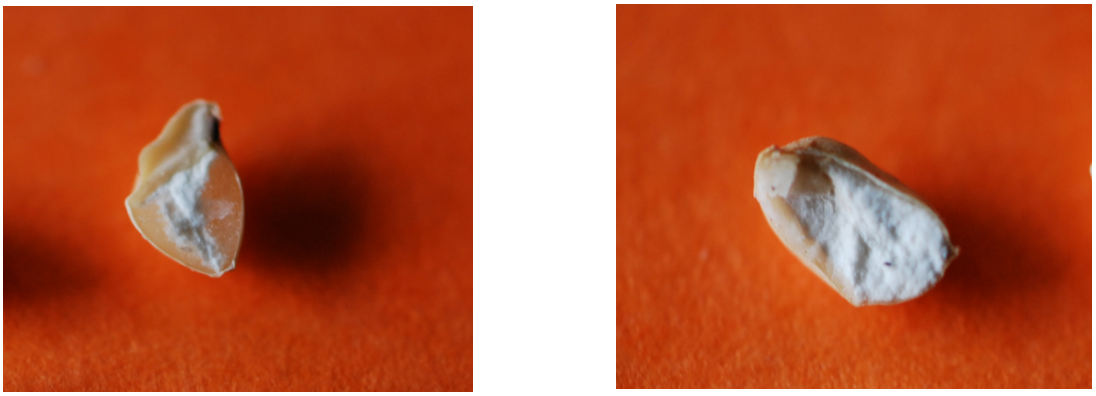
To develop the RILs, 200 randomly selected F_2 panicles were self pollinated. From these, $F_{2:3}$ progeny were self pollinated to produce the $F_{2:4}$ seed. Because the two parental lines do not segregate for major height (*Dw*) and maturity (*Ma*) genes, differences in maturity and/or height are limited to segregation in smaller effect genes. At the F_4 generation, all of the RILs were

phenotyped for the high digestible and waxy traits. Lines uniform for the high digestible trait were identified by visual observation and chemical analysis. For visual observation, 25 seed from each RIL were halved and observed on a light box. Seed from lines with wildtype endosperm have a small oval floury center and chalky outer layer surrounding the waxy center while seed from lines with the high digestible endosperm have a completely floury endosperm (Fig.13). Chemical analysis used a modified version of the protein digestibility assay from Mertz et al. (1984). In this modifications, the seed samples are ground to fine flour, freeze dried and protein digestibility was calculated using the formula: 1-(digested flour protein/total flour protein) x 100. All other methods were as described by Mertz et al. (1984).

Lines were screened for waxy endosperm using the iodine staining technique described by Pedersen et al., (2004). The seed from each entry were crushed and placed in each well of 96 well plate. Water was added into each well and the mixture was heated to 95°C for 1h. Later after the plates are cooled, iodine stain solution was added to each well and the wells were color scored after 10 to 60 s. The wild-type seed stained purple due to the presence of amylose, while the waxy lines stained reddish brown due to the presence of amylopectin.

Any lines that were still segregating for either trait were eliminated and, all remaining RILs were placed into one of four categories; (i) highly digestible protein, and wild-type endosperm, referred to herein as HD, (ii) highly digestible

protein and waxy endosperm, referred to herein as HD-WX, (iii) normal digestibility and wild-type endosperm, referred to herein as Wildtype, and (iv) normal digestibility and waxy endosperm, referred to herein as WX. Based on the phenotypes, 24 lines of each combination were randomly selected for evaluation.



(a) (b)

Fig.13: Visual examination of the seed. a. Wild-type b. High Digestible mutant seed.

4.2.2. Field Study

The lines were evaluated in College Station and Halfway, Texas in 2010. In each environment, the experimental design was a randomized complete block with three replications, in addition to the 96 experimental lines, the parental lines and two check lines Tx631 (Miller, 1986) and Tx2928 (Rooney et al., 2010) were included for a total of 100 entries. The soil type at the College Station and

Halfway were Ships Clay loam and Pullman Clay loam respectively. The field plots were managed using agronomic practices typical for production of sorghum at each location and supplemental irrigation was available as needed. Plant height (cm) was measured just prior to harvest. Grain yield (MT ha^{-1}) was measured by hand harvesting the plots at maturity and threshing them using an Almaco Plot Thresher. At harvest, panicle number was also recorded. From each location, the grain from all the replications for each entry was bulked and test weight (Kg hl^{-1}), and 100 kernel weight (g) were recorded. Test weight measurements were only recorded in Halfway. Test weight and 100 kernel weight were recorded twice on the same replication.

4.2.3. Statistical Analysis

Data from each environment was analyzed separately, partitioning the sources of variation to replication, endosperm type and entries nested within the endosperm. Error mean squares in the analysis of the variation across environments were not heterogeneous, so the data was combined for all the environments and analyzed using PROC MIXED model with replications, environments and entries as random effects and endosperm type as a fixed effect. To identify best performing genotypes regardless of endosperm type, the data set was analyzed using the same model except that endosperm type was removed as a source of variation.

All data were analyzed using general linear model in SAS (SAS v9.2, SAS Institute Inc., Cary, NC, USA) procedure. Means within individual and

combined environments were calculated and the differences in the means were identified using least significant difference (LSD) test at $P < 0.05$.

Because the lines were randomly derived from a single population, the broad sense heritability (H^2) of yield components was estimated from the variance components derived from PROC MIXED:

$H^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_{ge}/e + \sigma^2_{error}/re)$

Where σ^2_g , σ^2_{ge} ,σ^2_{error} , r and e represent the genotype, genotype X environment, error variances, number of replications per location and number of environments respectively.

4.3. Results and Discussion

4.3.1. Individual Location Analysis

In Halfway, variation among endosperm types was detected only for plant height at $P<0.05$ (Table 6). In College Station, variation among endosperm types was detected only for Plant height at $P<0.01$ (Table 7). No variation was detected for grain yield, test weight, 100 kernel weight and seed number per panicle in the individual analysis at each location.

Table 6. Mean squares for Halfway, TX of F_4 HD, HD-WX, Wildtype and WX lines from Tx2907/P850029 cross in 2010.

Source	Grain Yield	Plant Height	Test Weight	100 Kernel Weight	Seed number / Panicle
Replication	408960.7	1914.6			
Endosperm type	175855.8	2346.8*	130.2	0.33	521516.9
Entry (Endosperm type)	231610.5	702.6	19.8	0.23	168943.7
Error	331516.1	817.9	2.0	0.005	273675.5

* Significant at P=0.05

Table 7. Mean squares for College Station, TX of F_4 HD, HD-WX, Wildtype and WX lines from Tx2907/P850029 cross in 2010.

Source	Grain Yield	Plant Height	100 Kernel Weight	Seed number / Panicle
Replication	3657954.4	1466.1	-	-
Endosperm type	1337544.3	6093.9**	0.03	366078.3
Entry (Endosperm type)	1100096.3	1750.8	0.28	675494.5
Error	1559917.7	1522.1	0.007	348853.2

** Significant at P=0.01

4.3.2. Combined Analysis

In combined analysis, endosperm type did not affect any measured variables except plant height (Table 8). Variation due to entry within endosperm type for grain yield was the only significant source of variation. Environment had an affect on 100 kernel weight only. Furthermore, no endosperm type X environment effect was detected, indicating that the response of the endosperm type was consistent across the tested environments.

Table 8. Mean squares from the combined analysis of F_4 HD, HD-WX, Wildtype and WX lines from Tx2907/P850029 cross in evaluation in two environments across Texas in 2010.

Source	DF	Grain yield	Height	100 kernel weight
Env	1	280624.2	12411.4**	3.2*
Rep(Env)	4	2033367.0	1690.4	-
Endosperm type	3	1047599.7	7292.9**	0.15
Entry(Endosperm type)	97	1131675.8*	1889.1**	0.27
Endosperm type*Env	3	1131151.1	779.4	0.14
Entry(Endosperm type)*Env	97	990613.9	560.5	0.24
Error	386	867405.5	1167.3	0.005

* Significant at P=0.05
** Significant at P=0.01

Endosperm types did consistently differ for plant height; wildtypes group was the tallest while WX endosperm type shortest (Table 9). The absence of a genotype x environment interaction indicates that these results were consistent across environments. The results imply that may be there is sampling error or the genes controlling these endosperm types are either pleiotrophic with plant height or at least one of them is linked to a dwarfing gene. The reason for the differences in plant height in our study can be confirmed by using large population sizes in future. However, the waxy gene is mapped to chromosome 10 of sorghum genome, but on this chromosome no dwarfing genes are identified yet. Also earlier research suggests that sorghum chromosome 10 has synteny with chromosome 9 of maize genome (Mcintyre et al., 2008). The chromosome 9 of maize genome has dwarfing genes mapped. So there may be a dwarfing gene close to the waxy gene, which still needs to be identified in sorghum.

Table 9. Means by endosperm type for grain yield, plant height, test weight, 100 kernel weight and seed number per panicle for HD, HD-WX, Wildtype and WX F_4 lines from (Tx2907/P850029) population that were evaluated in two environments across Texas in 2010.

		HD	HD-WX	Wildtype	WX	LSD
Grain Yield (MT ha^{-1})	Halfway	2.3	2.2	2.3	2.5	0.35
	College Station	2.2	2.3	2.1	2.4	0.33
	COMBINED	2.3	2.3	2.2	2.4	0.30
Plant Height (cm)	Halfway	146.5	137.6	151.4	145.1	4.10
	College Station	159.8	140.4	160.3	152.7	6.05
	COMBINED	153.2	139.0	155.9	148.9	2.63
Test weight (Kg hl^{-1})	Halfway	71.8	68.1	72.9	75.3	5.50
100 Kernel Weight (g)	Halfway	2.9	2.8	2.9	2.9	0.23
	College Station	2.7	2.7	2.7	2.6	0.20
	COMBINED	2.8	2.7	2.8	2.8	0.13
Seed number / Panicle	Halfway	1441	1479	1394	1489	97
	College Station	1425	1492	1378	1496	120
	COMBINED	1433	1485	1386	1492	110

Rooney et al. 2005 concluded that waxy sorghum lines have lower test weights when compared to wildtype sorghum. Our study results show that there are no significant differences in test weights among the different endosperm types (Table 9). However, the test weights were recorded only in one location. The 100 kernel weights for all the endosperm types were lower in College Station compared to Halfway. These differences in kernel weights in two locations may be attributed to the environment difference as cultural practices were similar in both the locations.

All the endosperm types included in the study had no significant differences in grain yield, test weight, 100 kernel weight and seed number per panicle on both Halfway and College Station.

4.3.3. Performance of Individual Lines

The data presented thus far clearly imply that endosperm per se does not affect average yield potential of a group. However, breeding and development is truly interested in advancing only the most elite genotypes. Therefore, it is important to evaluate all genotypes to determine if specific entries of each genotype are elite and among the highest yielding lines. Since endosperm type was not significant source of variation, an analysis was completed to evaluate genotypes independent of endosperm.

As expected, significant variation for grain yield was detected in Halfway and the combined analysis. Of the ten highest yielding lines in Halfway, four were HD which ranked first, third, seventh and eighth, two were WX which ranked fifth and sixth, only one was HD-WX endosperm type which ranked ninth and none of these were statistically different from the top yielding line. In the combined analysis, in the top ten high yielding genotypes, two were HD, three were WX, two were HD-WX and three were wild-type (Table 10). The top 10 yielding lines in both Halfway and in combined analysis were not significantly different from each other. These results show that elite HD-WX lines could be identified given larger population size.

Table 10. Top 10 performing lines among the 100 lines based on average grain yield in the combined analysis in 2010 with LSD value 0.6.

Rank	Pedigree	Average Yield (MT ha^{-1})	Endosperm Type
1	Tx2907/P850029-WFF2-CS49	3.8	WX
2	Tx2907/P850029-WFF2-CS114	3.7	HD-WX
3	Tx2907/P850029-WFF2-CS35	3.6	Wild-type
4	Tx2907/P850029-WFF2-CS32	3.5	WX
5	Tx2907/P850029-WFF2-CS25	3.5	Wild-type
6	Tx2907/P850029-WFF2-CS84	3.5	WX
7	Tx2907/P850029-WFF2-CS89	3.4	HD
8	Tx2907/P850029-WFF2-WE51	3.3	HD-WX
9	Tx2907/P850029-WFF2-CS21	3.3	Wild-type
10	Tx2907/P850029-WFF2-CS9	3.3	HD

4.3.4. Heritability Estimate

Depending on the trait, the estimates for heritability were variable (Table 11). The test weight had the highest heritability of 98.4% where as the seed number /panicle had the lowest estimated heritability of 4.8%. Heritability estimate for the test weight was calculated only for the Halfway as the data was not available for the College Station.

Table 11. Variance components and broad sense heritability estimates for yield components of F_4 lines from (Tx2907/ P850029) populations that were evaluated in Halfway and College Station, TX in 2010.

Traits	σ^2_g	σ^2_{ge}	σ^2_{error}	H^2 (%)
Grain Yield	56985.8	41069.5	867405.5	25.6
Plant Height	1122.1	202.3	1167.3	79.1
Test Weight	130.6	-	2.0	98.4
100 Kernel Weight	0.015	0.078	0.005	27.4
Seed number / Panicle	3814.9	50569.6	303532.5	4.8

4.4. Conclusion

The results presented herein indicate that there is no yield penalty when the high digestibility and Waxy endosperm traits are combined. Furthermore, it appears possible to produce specific genotypes that are HD and waxy that are comparable to normal lines from the same cross. In prior work, Rooney et al., (2005) reported in hybrids that the endosperm types were not different but that the best genotypes were always normal endosperm types. It is important to test our observation in hybrid combination to determine if the trends are consistent. If so, this implies that it will be possible to produce high yielding grain sorghums with improved processing and utilization characteristics.

CHAPTER V

SUMMARY

One QTL was identified for the protein digestibility trait in both RIL and HIF populations. The two markers closely associated with the protein digestibility trait are Xtxp43 and Xtxp325. Though the LOD score of the identified QTL was low in the RIL population, the LOD score for the same QTL was high in the HIF population. The markers closely linked to the protein digestibility trait can be used in marker-based selection off high digestible sorghum genotypes effectively. The sorghum lines with both high digestibility and waxy traits have high range of variation for the starch content, moisture and fermentation efficiencies at 24 and 72 h. This variation can be used in the breeding sorghum for the ethanol industry. The ethanol fermentation study concludes that there are some HD-WX lines like sample 13 and 18 that are performing better than the parents. The high performing HD-WX lines can be selected and improved further for the traits of interest such as high starch content, high ethanol yield and lower residual starch and high lysine amino acid contents in the DDGS. The results from the yield trials concluded that there is no effect of on grain yield by combining the high digestible and waxy traits in sorghum. The wide variation present within the genotypes with high protein digestibility and waxy traits can be effectively used in breeding better sorghums for the bioethanol industry.

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APPENDIX A

CHEMICAL COMPOSITION AND FERMENTATION EFFICIENCIES AT 24 AND 72 HOURS OF FERMENTATION OF HD, WX AND HD-WX SORGHUM SAMPLES

Entry	Endosperm phenotype	Moisture (%)	Starch	Efficiency at 24h	Efficiency at 72h
1	HD	6.81	61.40hi	91.9l	93.0l
28	HD	8.76	66.58s	87.0i	91.2defg
4	HD/Waxy	8.96	67.38u	81.1e	90.6cd
5	HD/Waxy	9.27	65.49q	81.4f	90.4bc
6	HD/Waxy	8.94	59.69f	97.7x	91.2defg
7	HD/Waxy	8.97	63.41m	87.5j	90.3bc
8	HD/Waxy	9.22	66.05r	80.1b	91.3efgh
9	HD/Waxy	8.20	58.46d	98.0y	90.9cdef
10	HD/Waxy	8.90	66.90t	76.6a	91.4efgh
11	HD/Waxy	9.12	64.20o	89.0k	90.6cd
12	HD/Waxy	8.96	56.54a	97.7x	91.2defg
13	HD/Waxy	9.05	57.64b	97.7x	92.3jk
15	HD/Waxy	8.07	58.13c	97.5w	91.3efgh
16	HD/Waxy	9.16	61.86j	96.8u	91.9hij
18	HD/Waxy	9.04	62.40l	96.2p	92.6kl
19	HD/Waxy	8.49	60.40g	96.3q	91.7ghij
20	HD/Waxy	6.53	63.90n	92.8n	91.3efgh
21	HD/Waxy	8.24	62.60l	96.6s	91.6ghi
22	HD/Waxy	7.04	61.37hi	97.1v	90.8cde
23	HD/Waxy	8.85	62.15kl	96.7t	90.9cdef
24	HD/Waxy	8.99	60.36g	97.1v	91.3efgh
27	HD/Waxy	9.20	65.00p	80.9d	91.5fgh
30	HD/Waxy	8.57	61.55i	96.5r	92.2ijk
2	Waxy	8.84	66.31r	87.5j	89.8ab
3	Waxy	9.18	63.29m	92.3m	89.4a
14	Waxy	8.82	62.05jk	85.4h	91.3efgh
17	Waxy	8.92	59.01e	96.0o	92.2ijk
29	Waxy	9.09	63.86n	80.5c	90.6cd
33	Waxy	8.52	61.16h	83.1g	91.3efgh

APPENDIX B

RESIDUAL STARCH CONTENT (%) OF DDGS FOR HD, WX AND HD-WX SORGHUM SAMPLES

Sample	Endosperm type	Residual Starch (%)
1	HD	0.57
28	HD	0.66
4	HDWX	0.48
5	HDWX	0.70
6	HDWX	0.43
7	HDWX	0.62
8	HDWX	0.55
9	HDWX	0.42
10	HDWX	0.34
11	HDWX	0.60
12	HDWX	0.37
13	HDWX	0.51
14	HDWX	0.73
15	HDWX	-
16	HDWX	0.57
18	HDWX	0.53
19	HDWX	0.31
20	HDWX	0.57
21	HDWX	0.69
22	HDWX	0.49
23	HDWX	0.53
24	HDWX	0.50
27	HDWX	0.57
30	HDWX	0.40
2	WX	0.85
3	WX	0.96
17	WX	0.66
29	WX	0.61
33	WX	0.68

APPENDIX C

AMINO ACID COMPOSITION (AS WEIGHT PERCENTAGE OF NET PROTEIN) OF DDGS FOR HD, WX, AND HD-WX SORGHUM SAMPLES

	Essential Amino acids								Non Essential Amino acids							
Sample	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Arg	Ala	Asx	Glx	Gly	Pro	Ser	Tyr
1	2.6	4.6	12.2	3.2	2.2	6.4	4.6	5.7	5.3	8.6	9.6	14.1	3.3	7.8	5.2	4.3
2	2.6	4.7	13.4	2.3	2.1	6.6	4.4	5.6	4.1	9.2	9.1	15.3	2.8	8.3	5.0	4.4
3	2.6	4.9	13.3	2.3	1.8	7.0	4.3	5.5	4.1	9.2	9.0	15.6	2.7	8.5	5.1	4.1
4	2.4	5.0	13.8	2.2	1.9	6.9	4.3	5.6	4.0	9.3	8.7	15.0	2.6	9.0	5.0	4.3
5	2.3	4.9	13.3	2.6	2.0	7.0	4.6	5.8	4.3	9.1	9.0	15.0	2.8	8.0	5.0	4.3
6	2.3	4.7	12.8	2.7	2.2	6.3	4.6	5.7	5.1	8.6	9.7	15.3	2.9	7.7	5.3	4.1
7	2.3	4.7	14.1	1.7	2.1	6.6	4.2	5.4	3.9	9.3	8.6	16.5	2.5	8.6	5.1	4.3
8	2.3	4.7	14.0	2.0	2.1	6.7	4.3	5.4	4.3	8.9	8.9	15.9	2.4	8.6	5.0	4.6
9	2.2	4.8	12.6	2.8	1.9	6.3	4.6	5.7	5.0	8.6	10.4	15.5	2.9	7.5	5.2	4.0
10	2.2	4.8	13.8	2.1	2.1	6.5	4.4	5.6	4.6	9.1	9.2	14.8	2.7	8.5	5.1	4.4
11	2.2	4.8	13.3	2.5	2.0	6.8	4.5	5.6	5.0	8.1	9.7	15.3	2.5	8.2	4.9	4.6
12	2.1	4.8	12.2	2.7	1.9	6.2	4.7	5.7	5.2	8.5	10.6	15.4	3.1	7.5	5.2	4.0
13	2.7	4.9	12.4	3.0	2.5	6.6	4.8	5.9	5.4	8.2	9.6	14.4	2.9	7.4	5.2	4.2
14	2.2	4.7	14.2	1.7	2.2	6.6	4.2	5.4	4.0	9.6	8.7	16.3	2.5	8.6	5.0	4.2
15	2.2	4.7	12.8	2.5	2.0	6.3	4.6	5.7	4.9	8.8	10.1	15.9	2.9	7.6	5.2	4.0
16	2.2	4.9	12.5	2.7	2.0	6.3	4.9	5.8	5.1	8.8	10.2	14.1	3.2	7.9	5.3	4.0
17	2.1	4.8	12.8	2.5	2.0	6.5	4.7	5.8	4.9	8.7	9.9	15.3	2.9	8.0	5.1	4.0
18	2.3	5.1	12.3	2.8	2.0	6.4	5.0	6.0	5.7	8.4	10.3	14.0	3.2	7.5	5.1	3.9
19	2.3	4.8	13.0	2.7	2.0	6.6	4.7	5.7	5.3	8.3	10.2	14.9	2.8	7.5	5.0	4.3
20	2.1	5.1	12.7	2.7	2.1	6.4	4.7	6.2	5.3	8.7	10.4	14.3	3.1	6.9	5.1	3.9

Continued Appendix C

	Essential Amino Acids								Non Essential Amino Acids							
Sample	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Arg	Ala	Asx	Glx	Gly	Pro	Ser	Tyr
21	2.8	4.9	12.4	2.7	1.9	6.4	5.1	5.8	5.4	8.4	10.4	14.1	3.1	7.2	5.4	4.0
22	2.1	5.1	12.4	2.8	1.9	6.4	4.8	6.2	5.2	8.5	10.7	14.5	3.1	7.2	5.2	4.0
23	2.0	5.2	12.5	2.7	1.9	6.5	4.8	6.2	5.0	8.6	10.3	14.0	3.1	8.0	5.2	3.9
24	2.1	5.1	12.6	2.7	2.0	6.4	4.7	6.2	5.3	8.6	10.7	14.6	3.0	7.0	5.1	3.9
27	1.9	5.2	13.8	1.9	1.9	6.6	4.3	5.9	4.4	9.2	9.3	15.6	2.6	8.4	4.9	4.2
28	2.3	5.2	12.8	2.9	1.8	6.8	4.6	6.1	5.7	7.8	10.5	14.2	2.7	7.4	4.7	4.4
29	2.0	4.8	13.8	1.9	2.0	6.6	4.3	5.6	4.4	9.1	9.8	16.0	2.6	8.0	4.9	4.3
30	1.9	5.2	12.7	2.5	1.9	6.4	4.6	6.1	4.9	8.7	10.5	14.5	3.0	8.1	5.1	4.2
33	2.5	5.0	14.5	1.9	2.3	6.4	4.2	5.8	4.1	9.0	8.5	15.7	2.5	8.4	5.1	4.1

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