Starch properties, endogenous amylase activity, and ethanol production of corn kernels with different planting dates and drying conditions

by

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ABSTRACT

This study was conducted with aim to understand how planting dates and drying conditions affected starch properties and dry-grind ethanol production of corn kernels. Three corn varieties with planting dates between 4 April and 11 June during 2007-2009 growing seasons as the treatments were used in this study. The maximum grain yield and kernel starch content were obtained with corn planted between late April and mid-May; both decreased when planting was delayed to late May and June. Later planting dates resulted in larger proportions of short amylopectin branch-chains for one of the studied varieties, less amylose contents of starch, lower starch gelatinization and pasting temperatures, and greater peak viscosity of starch paste for both corn varieties. While these changes may impact the quality and yield of starch, they might not be of sufficient magnitude to impose major problems in processing of products containing starch.

*S*tarches isolated from corn kernels planted on a late date (11 June) were hydrolyzed at comparable rates as those planted on early dates (late April and mid-May) using a raw starch hydrolyzing enzyme. Consequently, kernels planted in late May and in June gave similar ethanol yields (on kernel dry weight basis) as those planted on earlier dates. The results showed that the planting date of corn did not affect the ethanol yield on the basis of kernel weight. The grain yield, however, decreased with late planting dates, and thus, reduced the ethanol yield on the basis of unit planting area (g ethanol/hectare of planted area).

Freshly harvested kernels of three commercial corn hybrids were dried at low (10ºC), ambient (25ºC), intermediate (45 and 65ºC), and high (85ºC) air temperatures to 14%

moisture content to assess how kernel drying temperatures impacted functional properties of starch and ethanol yield of the kernels. The air drying temperature at 10˚C increased starch gelatinization temperature and enthalpy change and reduced the swelling power of starch compared with the control (25˚C). This can be attributed to that the drying temperature at 10˚C was close to the optimum temperature of starch crystallization (4˚C) and, thus, enhanced starch crystallinity. The intermediate air drying temperatures (45˚C and 65˚C) increased starch gelatinization temperature and enthalpy change and narrowed the gelatinization temperature range of starch compared with the control. These results suggested that starch molecules annealed during the drying at 45˚C and 65˚C and perfected the crystalline structure of starch. The drying temperature of 85˚C partially gelatinized the starch granules during the drying and reduced their crystallinity. Consequently, starch isolated from kernels dried at 85˚C exhibited higher gelatinization temperature and reduced swelling power of starch compared with the control.

Changes in the starch structure reduced the granule susceptibility to the enzyme hydrolysis and, thus, decreased the ethanol yield of ground kernels dried at the intermediate (45˚C and 65˚C) and high (85˚C) air temperatures. Kernels dried at 85˚C produced the least yield of ethanol, which might be resulted from the most severe reduction in the starch swelling power that inhibited enzyme penetration into the starch granule in addition to the loss of the endogenous enzyme activity of kernels during drying at 85˚C.

Kernels dried at temperatures up to 65˚C air temperatures displayed similar levels of endogenous amylase activity, whereas those dried at ≥85˚C contained partially reduced amylase activity as indicated by the reduced amount of reducing sugars produced in the ground kernel suspensions incubated at 40 ˚C for 20h. Among the endogenous amylases, β-

amylase was most heat-labile and showed reduced activity after the kernel was dried at 45°C. Pullulanase and isoamylase showed reduced enzyme activity in kernels after the drying at 85°C. The α-amylase was relatively stable up to 85°C but significantly lost its activity after drying at 105 and 125°C air temperatures.

CHAPTER 1. GENERAL INTRODUCTION AND RESEARCH JUSTIFICATION

With the depletion of oil resources and negative environmental impact associated with the use of fossil fuels, there is a significant interest in alternative energy sources (Singhania et al 2009). So far, grain-based ethanol and biodiesel have been the only commercial-scale renewable transportation fuels available in the marketplace. The annual U.S. ethanol production reached record 50.1 billion liters (13.23 billion gallons) in 2010 (RFA 2010). Nevertheless, with the limited area of fertile land available for corn growth, the corn-based ethanol industry does not have a perspective to increase more farmland for corn production and thereby expand its capacity. For this reason, significant resources have been invested in 1) breeding programs to increase corn yields and develop corn varieties suitable for the ethanol production and 2) the improvement of ethanol production efficiency to maximize the ethanol yield.

The production of ethanol from dry-grind corn involves several steps: grinding and suspending kernels in water, adding enzymes (capable to produce glucose from starch) and yeast (that converts glucose to ethanol), and separating ethanol from the fermentation broth. A recent breakthrough in the ethanol industry is the invention of the cold-fermentation process, which does not require heating of corn slurry to gelatinize starch because it utilizes raw-starch hydrolyzing enzymes. This technology consumes 10-20% less energy to produce ethanol than the conventional process and produces greater ethanol yields because starch can be more fully hydrolyzed to glucose without the formation of retrograded starch and amylose-lipid complex (Lewis et al 2005, Robertson et al 2006). Even though the ethanol production using cold fermentation process has advanced in the efficiency, there is still room

for improvement. Choosing proper crop management practices, such as selection of desirable hybrids, dates of planting, kernel drying and storage conditions, can lead to further improvement in the ethanol production and ethanol yield maximization.

Previous studies have shown that large ethanol yields can be produced from kernels containing more starch and less protein and lipid contents (Wu et al 2006, Srichwong et al 2009). Additionally, corn kernels containing starch with larger proportions of short amylopectin chains have shown to be more completely hydrolyzed to glucose during the ethanol fermentation (Srichuwong et al 2009). Thus, the kernel composition and starch structure have shown to be critical factors determining the ethanol yield from corn.

The kernel composition and starch structure of corn are determined by genetic background of the corn, but can also be influenced by environmental conditions (e.g. growing temperature and soil moisture) (Asaoka et al 1984, Asaoka et al 1985, Asaoka et al 1987, Shi et al 1994, Tester et al 1995, Lu et al 1996). Research studies reporting the impact of planting date on starch structures exist in the literature, but results are inconclusive. The amylose content of starch has been reported not to change with planting dates in rice (Williams et al 1958), normal (common) corn (Campbell et al 1994), and sweet potato (Noda et al 1997). In high-amylose corn, the amylose content of starch has been reported to increase with delayed planting (Helm et al 1968), whereas in wheat it decreased with a delay in the planting date (Singh et al 2010). There was no significant effect of the planting date found on the branch-chain length of sweet potato amylopectin (Noda et al 1997).

Postharvest handling of corn kernels, such as kernel drying and storage conditions, has been shown to affect the quality and storage stability of the kernel. Corn kernels dried at elevated temperatures (>70˚C) have shown to display increased breakage during handling

(Hooseney 1986, Peplinski et al 1994), altered starch properties (Haros et al 2003, Altay and Gunasekaran 2006, Malumba et al 2009), and decreased corn protein solubility, protein moisture-binding capacity, and enzymatic activity (Wall et al 1975, Eckoff and Tso, 1991). These changes resulted in lower flaking grit yield in dry milling and poor starch-protein separation in wet milling process (Singh et al 1998), but it is not well understood how they affect the ethanol yield from dry-grind corn.

The objectives of this study were to:

- understand effects of the planting date on 1) the grain yield and kernel composition of corn, 2) the structure and functional properties of the corn starch, and 3) ethanol yield produced using the cold-fermentation process with uncooked dry-ground corn kernels as the substrate.
- determine how kernel drying temperatures affect 1) functional properties of starch, 2) endogenous amylase activity within the kernel, and 3) ethanol yield of ground corn kernels using the cold-fermentation process.

CHAPTER 2. DISSERTATION ORGANIZATION

The present dissertation consists of a general introduction, a literature review, four papers, general conclusions, an appendix, and acknowledgements. The first paper, "Effect of planting date on properties of corn. Part I: Grain yield, kernel composition, and structure and properties of starch", and the second paper, "Effect of planting date on properties of corn. Part II: Enzyme hydrolysis of starch and ethanol yield of ground kernels", are prepared for submission to the *Journal of Agricultural and Food Chemistry*. The third paper entitled "Starch properties and ethanol production of corn affected by kernel drying conditions" follows the format of the *Journal of Cereal Science* for submission to the aforementioned journal. The fourth paper "Endogenous amylase activity of corn affected by kernel drying temperature" follows the format of the journal of *Cereal Chemistry*.

CHAPTER 3. LITERATURE REVIEW

Ethanol overview

Ethanol (C_2H_5OH) is a clear, colorless, volatile liquid, and is widely used in food, pharmaceutical, and fuel industries (Lee 2007). Ethanol is considered a renewable source of energy because it is derived from starch- or other sugar-containing plant materials that are produced from carbon dioxide and sunlight, which cannot be depleted. During photosynthesis, plants sequester carbon-dioxide using sunlight, and produce glucose and starch for the energy of the growth. The plants are later used as feedstocks for the production of this alcohol. The most common feedstocks for the ethanol production are corn, sugar cane, sugar beet, sweet sorghum, etc. (Gnansounou 2009).

The history of fuel ethanol started as early as 1826, when Samuel Morey built an engine that ran on ethanol and turpentine (Gnansounou 2009). The first car "Model T Ford", designed to run on either gasoline or pure alcohol, was constructed by "Ford Motor Automobile" company in 1908. Henry Ford designed this famous car to run on alcohol saying that it was "the fuel of the future" (Freudenberger 2009a). The first U. S. fuel ethanol plant was built in 1940's to provide fuel for the U.S. army. Although these early efforts to introduce ethanol as a fuel source failed due to low gasoline prices, oil supply disruptions in the Middle East and environmental concerns over the use of lead as a gasoline octane booster renewed interest in ethanol in the late 1970's (Freudenberger 2009a). Ethanol production in the United States grew from 662 million liters (175 million gallons) in 1980 to 50.1 billion liters (13.23 billion gallons) in 2010 (**Figure 1**) (RFA, 2011). In the U.S., ethanol is currently blended with gasoline in the ratio of 1:9 to make E10 gasoline blend, which can be used to

power standard cars without any modifications to the engine. E85 gasoline blend, which contains 85% ethanol and 15% gasoline, is used to power flex-fuel vehicles that are designed to withstand high ethanol concentrations (Gnansounou 2009).

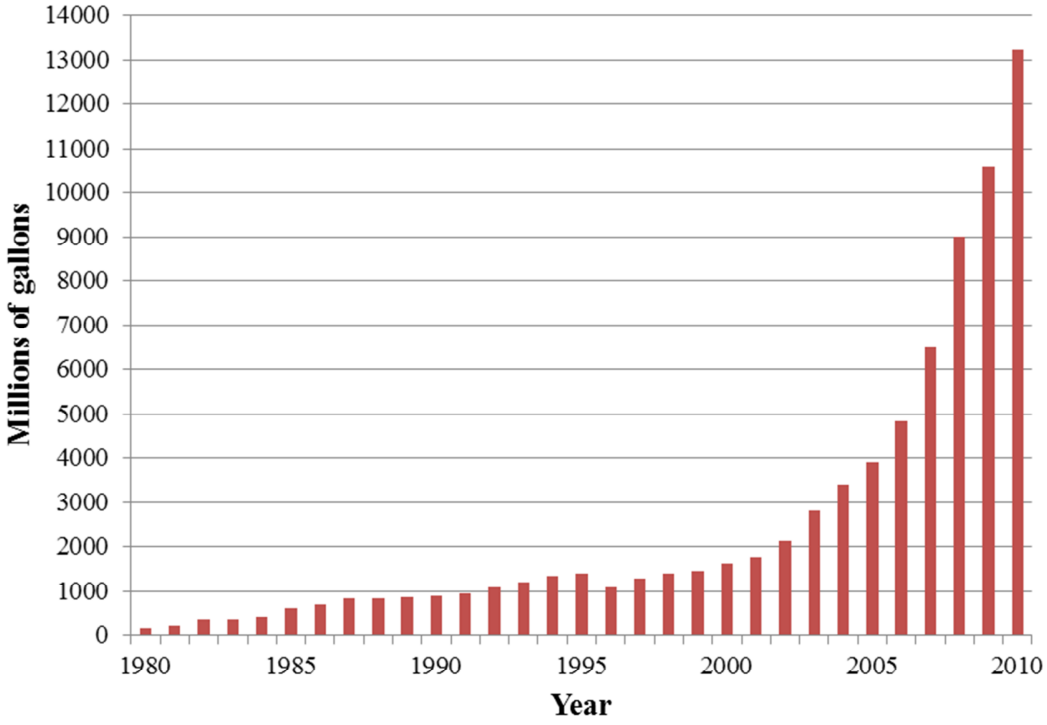

Figure 1. Historic U. S. ethanol production (RFA 2011)

Positive impacts of ethanol

The expansion of ethanol fuel industry can be attributed to its positive impact on the economy, national/energy security, and the environment. In 2010, the fuel ethanol industry secured more than 400,677 jobs in the U.S., added $53.6 in spending to the nation's Gross Domestic Product, and provided an additional $36,004 in income for American households (Urbanchuk 2011). Additionally, ethanol fuel industry has increased crop market

opportunities for farmers and thereby, advanced rural development. Ethanol has replaced 10% of the U.S. gasoline supply, and reduced its dependence on foreign oil.

The combustion of fuel in an engine generates by-products know as exhaust emissions. The four main automobile emissions are hydrocarbons, carbon monoxide, nitrogen oxides, and carbon-dioxide (Freudenberger 2009b). Ethanol is considered as a clean form of energy that releases significantly lower concentrations of pollutants and greenhouse gasses from vehicle exhausts than the gasoline (Liska et al 2009). It is made from biorenewable, plant-based feedstocks. Thus, the carbon-dioxide released during the ethanol production and combustion is absorbed during photosynthesis for the growth of future feedstock crops, resulting in the lower net carbon-dioxide emissions in the atmosphere (Rassmusen 2009). Ethanol emits less carbon-monoxide and nitrogen oxides than the gasoline as well. On contrary to petroleum based fuels, ethanol contains oxygen in its molecular structure, which results in more complete combustion in automobiles, resulting in less emission of these pollutants (Singhania et al 2009). Due to more complete combustion of ethanol, ethanol-gasoline blends have reduced aromatic hydrocarbons (e.g. benzene) emissions. All this contributes to 48-59% less greenhouse gas emissions and positive net impact on the environment (Liska et al 2009).

Challenges in the ethanol fuel use

Corn based ethanol has been blamed to be the main reason for increases in the food prices in the last few years. The increased demand for grains, driven by the ethanol production, has been thought to cause the increase in the prices of meat, egg, dairy and other products. A detailed analysis of Hofstand (2008) has shown, however, that several other

reasons besides grain-based biofuels influenced the sudden food price rise: dwindling grain reserves, adverse crop events, rapid population and economic growth in developing countries, bans of export, strong (oil) petroleum prices, and weak U.S. dollar. The onset of increase in the grain prices has started long before biofuels expanded. According to Hofstand (2008), we had entered a period when grain usage outstripped its production. The deficit was covered by drawing down grain reserves; the unstable grain supply resulted in high grain prices.

The major contributor to the increased food prices has been thought to be a sudden increase in the petroleum price. Food industry is heavily dependent on petroleum, both for processing and transportation purposes. Also, petroleum powers machinery used for plowing, planting, fertilization, harvesting and transportation of crops and thus, directly impacts grain prices and indirectly, meat, dairy and egg production (where grains are used as animal feed). What is certain, nevertheless, is the fact that the demand for biofuels will continue to grow. Unfortunately, fertile land, available for corn growth, is limited. To meet the increased demand for corn, farmers would need to divert lands designated for other crops and thus, upset agricultural markets. The competition between food and biofuel production if not now, will certainly arise in the next few years. To prevent this, significant resources have been invested in breeding programs to increase corn yields and develop corn varieties suitable for the ethanol production.

Researchers like Pimentel (2001) and Searchinger et al. (2008) have criticized grain-based ethanol for being energy inefficient, and are mainly responsible for its negative publicity. Pimentel (2001) claimed that 70% more energy is required to produce ethanol than the energy that actually is in ethanol. His study published in 2001 initiated series of articles,

in which corn ethanol was proclaimed as “crime against humanity”. Pimentel’s calculation, however, was based on outdated results. A more recent study has shown that ethanol yields 130% more energy than it takes to produce it (Shapouri et al. 2010). With new advancements in the corn ethanol production, such as cold fermentation technology, energy burden has been reduced even further as it does not include heating of corn slurry and mixing of highly viscous gelatinized starch. More details about the dry-grind cold fermentation process will be discussed later in the text.

Properties of ethanol fuel

Octane Rating. Ethanol fuel has higher octane rating than regular gasoline’s octane rating of 87. The octane number is a measure of fuel’s detonation resistance or its ability to withstand auto-ignition, measured against octane (the component of gasoline) as a standard. Auto-ignition is undesirable because it creates substantial pressures and localized heat in the engine, which puts considerable stress on engine components and can even burn holes in the piston (Freudenberger 2009b).

Cold-Weather Starting**.** Engines that run on ethanol have difficulties starting at temperatures below 1˚C. Ethanol’s flash point and latent heat of vaporization are significantly higher than those of gasoline, so alcohol fuel is less volatile, which can induce starting difficulties in cold conditions. The E-85 ethanol fuel blend has enough gasoline in it to start an engine in cold weather without the help of cold-starting aids (Freudenberger 2009b).

Corrosion and Degradation**.** Ethanol has ability to corrode metal parts and degrade soft components of automobile engines, which is related to ethanol’s water content. The

movement of ions in water carries a current that is capable of slowly dissolving metals such as aluminum alloys or zinc (Freudenberger 2009b). These problems can be eliminated if the water content of ethanol is reduced to ≤ 5%.

Dry-grind ethanol production process

The major feedstock for the U.S. ethanol production is corn, accounting for 97% of produced ethanol (USDA 2007). Small amounts of ethanol are produced from sorghum, wheat, and processing waste. The dry-grind ethanol process is the most widely used industrial method to produce ethanol, accounting for more than 70% of the production (Mosier and Ilelej 2006, RFA 2007). This is mainly due to the simplicity of the dry-grind process and low capital investments compared with the wet-milling process.

Conventional dry-grind process

The conventional dry-grind ethanol process produces 10.2-10.6 liters (2.7-2.8 gallons) ethanol, 7.7 kg (17 lbs) of carbon-dioxide, and 7.7 kg (17 lbs) of distiller's dry grains per bushel (56 lbs; 25kg) of corn (Mosier and Ileley 2006, RFA 2009). Each of the products accounts for approximately one third of the initial corn kernel weight (Rassmusen 2009). Conventional dry-grind ethanol production process steps are displayed in **Figure 2**.

Milling. Whole corn kernels are ground using a hammer mill to reduce the particle size and make starch more accessible to enzyme hydrolysis. The ground corn particles usually have a mean diameter of 0.9-1 mm (Rausch et al 2005). In the following step, ground corn is mixed with fresh and recycled water (e.g. thin stillage and evaporator distillate) in the slurry tank to make a mash containing 20-40% solids (Dale and Tyner 2006).

Liquefaction. Thermostable α-amylase (an endo-enzyme that hydrolyzes internal α-1, 4 bonds in starch) is subsequently added to the mash to initiate the hydrolysis of starch and produce dextrins (glucose chains) of various sizes. Raw starch has a semicrystalline structure and thus, is not efficiently hydrolyzed by thermostable α-amylase in the native form. The mash needs to be heated to temperatures above the gelatinization temperature of starch in order to melt the crystallites and make starch more susceptible to enzyme hydrolysis. That is achieved using jet-cookers that inject steam to heat the mash above 100˚C. Besides maximizing the activity of thermostable α-amylase, high-temperature heating is used to inactivate microbial contaminants present in the mash. The mash is subsequently cooled to 90˚C and more thermostable α-amylase is added to continue the liquefaction process for another 60-90 minutes.

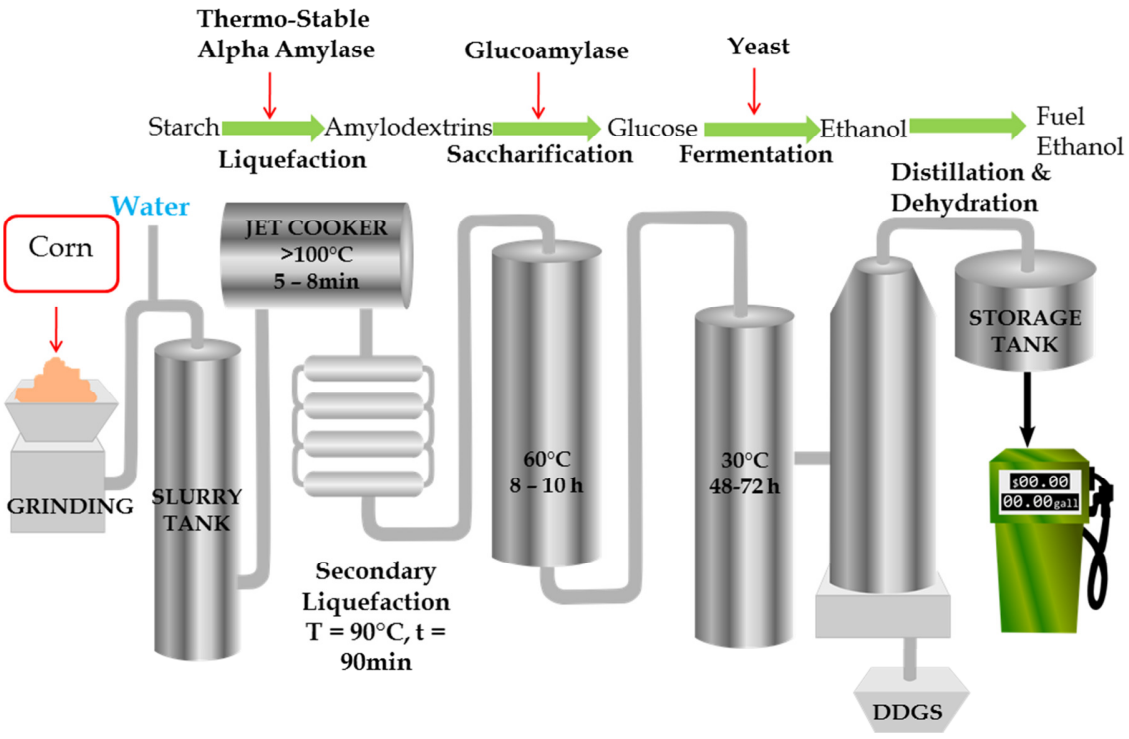

Figure 2. Conventional dry-grind ethanol production steps

Saccharification. After the liquefaction step, the mash is cooled to 60˚C and glucoamylase (an exo-enzyme that cleaves α-1, 4 and α-1, 6 linkages of starch) is added to hydrolyze dextrins to glucose. This step can take place in a saccharification tank or in a fermenter if the saccharification and fermentation steps take place simultaneously. The simultaneous saccharification and fermentation method is most often used in practice because it reduces time and energy required for the process, lowers the chance of microbial contamination and osmotic stress on the yeast (Bothast and Schlicher 2005).

Fermentation. During the fermentation, yeast metabolizes glucose and other simple sugars (fructose, maltose and maltotriose at slower rates) to produce ethanol and carbon-dioxide. *Saccharomyces cerevisiae* strain of yeast is most often used in the ethanol production process because it is highly productive and can withstand high ethanol concentrations and osmotic stress (Butzen et al 2003). Fermentation usually takes place at 30-32˚C and pH 4.0-4.2 for 48-72h.

Distillation and dehydration. After fermentation, ethanol needs to be separated from the residual mash in several distillation steps. Lastly, the distilled ethanol passes through a molecular sieve to remove residual water. Gasoline is added (up to 5%) to render ethanol undrinkable and make it fuel grade. The residual mash, remained after ethanol separation, is called whole stillage. The whole stillage is centrifuged to separate the solid fraction (distiller's wet grains) from the liquid (thin stillage). A portion of thin stillage (30-50%) is recycled back to the ethanol production process to provide nutrients for yeast and for pH adjustment of mash (Rausch 2007). The remaining thin stillage is condensed to about 30% solids in evaporators to produce syrup (also called distiller's solubles). Water (the distillate), removed during the evaporation step, is recycled back to the process and mixed with fresh

corn. The syrup is combined (mixed) with the distiller's wet grains and dried to 8-10% moisture content to produce distiller's dry grains with solubles (DDGS). DDGS is used as an animal feed ingredient for dairy, cattle, swine, and poultry.

Cold-fermentation (BPX^{TM}) process

The dry-grind ethanol production has advanced in efficiency with the development of cold fermentation process. Poet (Sioux Falls, SD) patented this technology by the name BPX^{TM} (Lewis et al 2005). In its basics, cold fermentation process is very similar to the conventional process - entire kernels are ground in a hammer mill and mixed with water, enzymes, and yeast (**Figure 3**). The fundamental difference between the two dry-grind processes lies in the fact that the cold fermentation employs an enzyme being able to hydrolyze raw starch, which eliminates need for heating (cooking) the mash. Thus, the jet-cooking and liquefaction steps are eliminated in the cold fermentation process. Instead, a mash, which contains ground corn kernels and fresh and recycled water, is directly fed to fermenters and raw-starch hydrolyzing enzyme and yeast are added at the same time. The raw-starch hydrolyzing enzyme hydrolyzes starch to produce glucose, which is subsequently utilized by yeast to produce ethanol. The simultaneous saccharification and fermentation process takes place at 26-27˚C and pH 4.2 for 96 hours. All subsequent steps (distillation and dehydration) are identical to those in the conventional process.

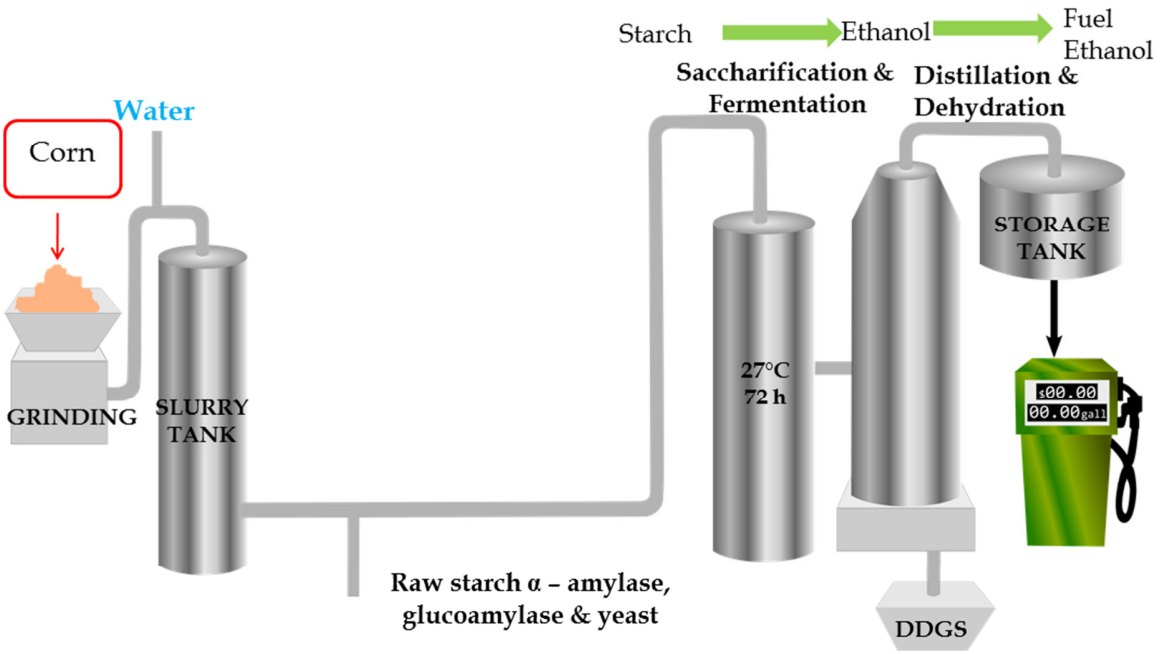

Figure 3. Cold-fermentation ethanol production steps

The cold-fermentation process offers several benefits in comparison with the conventional process (Lewis et al. 2010, Robertson et al. 2006):

- 10-20% less energy consumed during the production due to elimination of the heating step
- produces large ethanol yields (up to 23%, v/v) because starch can be more completely hydrolyzed to glucose without formation of retrograded starch and amylose-lipid complex
- utilizes endogenous enzymes of corn to aid starch hydrolysis and, thus, requires less exogenous enzymes for the process
- require lower capital costs
- produces more nutritious DDGS because proteins are preserved in the native form due to elimination of the heating step

Corn kernel properties

Corn (*Zea mays L.*) is one of the major cereal crops grown in the world. It belongs to the grass family (Gramineae) (Farnham et al. 2003). Fruits of corn, usually called kernels, are used as feedstock in food, feed, and ethanol industries. Corn kernel consists of the pericarp (seed coat), endosperm, and germ. The pericarp is a thin outer layer that protects the enclosed endosperm and embryo (Farnham 2003), and comprises approximately 5.3% of dry kernel weight (Watson 2003). The endosperm constitutes 82-84% of the kernel dry weight and is composed mainly of starch (86-89% dry basis) (Watson 2003). Starch granules are tightly packed within the protein matrix of endosperm. The germ, composed of embryo and scutellum, makes up 10-12% of the kernel dry weight (Watson 2003). The germ stores nutrients and hormones, which are mobilized by enzymes during the initial stages of germination for the growth of seedlings (Logan et al. 2001).

Amylolytic activity of corn kernels

Amylases are class of hydrolases widely distributed in microorganisms, plants and animals, which specifically cleave O-glycosidic bonds (Muralikrishna and Nirmala 2005). Amylases involved in degradation of starch can be categorized into three groups, depending on their mode of action: endoamylases, exoamylases, and debranching enzymes. Endoamylases hydrolyze α-1, 4 glycosidic bonds in the inner regions of starch, which results in rapid decrease in the starch molecular weight. α-Amylase (EC 3.2.1.1) belongs to this group of amylases and produces glucose chains of various sizes. This enzyme is indispensable for transient starch degradation in leaves to provide energy for plants during the night, but it also plays a significant role in maturation and germination of seeds.

Exoamylases cleave glycosidic bonds from the non-reducing end of starch chains by a successive removal of maltose or glucose in a stepwise manner (Muralikrishna and Nirmala 2005). Glucoamylase (also known as amyloglucosidase, α-glucosidase; EC 3.2.1.20) cleaves mainly α-1, 4 bonds of starch or glycogen to produce glucose, but it is capable to cleave α-1, 6 bonds at a slower rate. β-Amylase (EC 3.2.1.2) is an exoamylase that cleaves specifically α-1, 4 bonds of starch or glycogen to produce maltose.

Debranching enzymes play an important role in biosynthesis and degradation of starch in plants. Two classes of debranching enzymes exist in plants: pullulanase- (also known as R-enzyme and limit dextrinase; EC 3.2.1.41) and isoamylase-type debranching enzymes (EC 3.2.1.68). Pullulanase-type debranching enzymes cleave α-1, 6 linkages of pullulan and amylopectin, but have little activity toward glycogen. Isoamylase-type debranching enzymes readily hydolyze α-1, 6 bonds of amylopectin and glycogen, but are not able to cleave α-1, 6 bonds of pullulan. The main function of pullulanase is hydrolysis of α-1, 6 bonds in starch molecules of germinating seeds, nevertheless substantial pullulanase activity has been found in developing kernels of rice (Nakamura et al. 1996) and corn (Beatty et al. 1999), suggesting its role in starch synthesis as well. In contrast, isoamylase is indispensable for normal starch synthesis, but some studies have suggested its role in degradation of starch in *Arabidopsis* leaves as well (Smith et al. 2005).

During germination, endogenous amylases hydrolyze starch granules to produce sugars, such as glucose and maltose. These sugars are subsequently utilized as energy and carbon sources in the seedlings. The mechanism of starch degradation in corn kernels is not entirely elucidated, but it is believed that α-amylase is a key enzyme to initiate the hydrolysis (Sun and Henson 1991, Subbarao et al. 1998). Subsequent steps in the hydrolysis of starch

involve actions of β-amylase, branching enzymes and α-glucosidase, which hydrolyze fragments released by α-amylase (Beck and Ziegler 1989, MacGregor, 1987, Sanwo and DeMason 1992, Smith et al. 2005).

Molecular structure of starch

Starch is the primary energy reserve in corn kernels and constitutes up to 75% of kernel dry weight. It is synthesized in the granular form and composed of two polymers of glucose: amylose and amylopectin. The amounts of amylose and amylopectin vary with species. For example, normal corn starch contains between 15-30% amylose. Waxy corn mutant of starch is devoid of amylose, whereas high-amylose mutants of corn contain more than 50% of amylose.

Amylose. Amylose is a linear polymer made up of D-glucose monomers linked mainly by α-(1→4) bonds, but also contains a few branches (α-(1→6) bonds) (**Figure 4a**). The average molecular weight of amylose molecules is about 10^6 with a degree of polymerization (DP) between 500-5000 glucose units (Spence 1998, Greenwood 1970, Gailliard and Bowler 1987). Cereal starches contain amylose of smaller molecular sizes than tuber and root starches (Jane et al. 2006). For example, a number average DP for corn and potato have been found to be 990 and 2110 glucose units, respectively (Takeda et al. 1988, Suzuki et al. 1994).

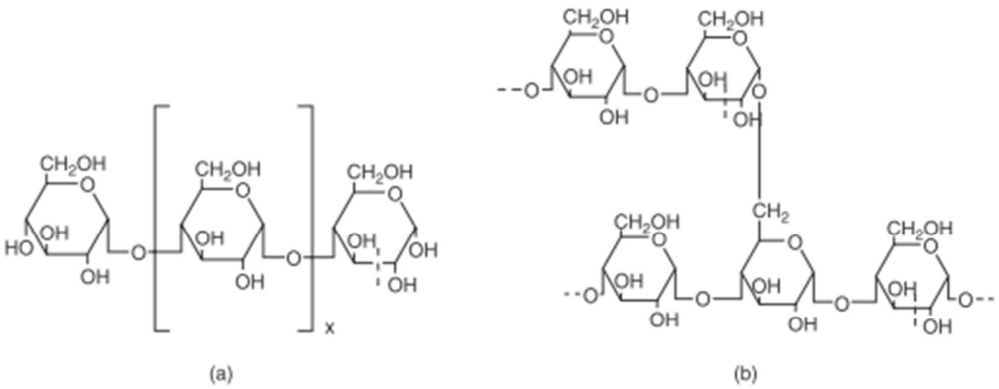

Figure 4. Schematic diagram of a) amylose and b) amylopectin with a branch point at the O6 position (Perez et al. 2009)

Amylose exists in unstable random coil conformation in aqueous solution, but gradually complexes with itself or amylopectin molecules to form double-helical conformation (French and Murphy 1977, Zobel 1988). This process of amylose-amylose and amylose-amylopectin molecules reassociation is called retrogradation. Amylose instantaneously forms single-helical complexes with complexing agents, such as iodine, various alcohols, dimethyl sulfoxide, and lipids. Single helices have 6-8 glucose units per turn, depending on the size of complexing agent that enters the central, hydrophobic cavity of the amylose helix (Rundle and French 1943, Banks et al. 1971, Yamashita and Monobe 1971, French and Murphy 1977, Billiaderis and Galloway 1989). The complexing agents can be used to separate amylose from amylopectin and/or prevent amylose retrogradation (Schoch 1942, Lansky et al. 1949, Kuge and Takeo 1968, Gudmundson and Eliasson 1990, Eerlinger et al. 1994).

Amylopectin. Amylopectin is highly branched polymer composed of relatively short chains of α-(1→4) linked D-glucose units, which are connected by α-(1→6) glycosidic bonds (**Figure 4b**). The average molecular weight and branch-chain length of amylopectin are

about 10^8 (Yoo and Jane 2002) and 19-29 glucose units (Jane et al. 1999), respectively, and vary with the botanical origin of starch.

The amylopectin chains can be categorized as A, B, and C chains (**Figure 5**). A-chains are linked to other amylopectin chains with α-(1→6) bonds, but do not carry any other chains themselves (Jane et al. 2009). B-chains are attached to other B- or C- chains, and are branched by A- or B-chains (Jane et al. 2009). B-chains can be further categorized as B1, B2, B3 and B4 chains that span through, 1, 2, 3, and 4 or more amylopectin clusters, respectively (Hizukuri 1986). Every amylopectin molecule contains only one C chain that carries a reducing end (Jane et al. 2009).

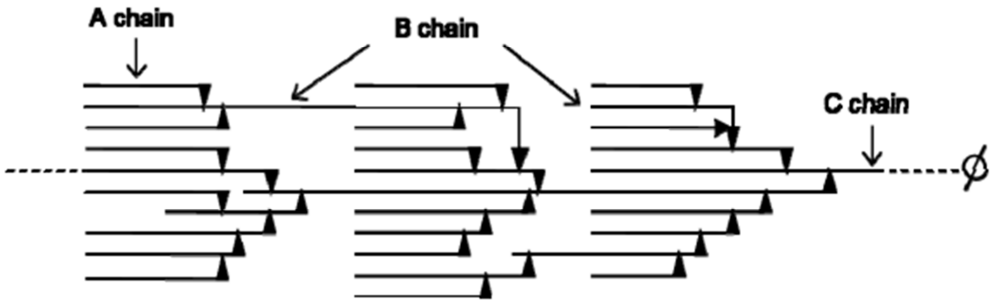

Figure 5. Model of the amylopectin cluster structure

The branch-chains of amylopectin can also be classified based on their degree of polymerization (DP). The length of amylopectin branch-chains can range from 6 to 140-150 glucose units (Hizukuri et al. 1989). Hanashiro et al. (1996) termed A, B1, B2, and B3 chains as having DP 6-12, 13-24, 25-36, and ≥37, respectively. The proportion of these branch-chains in amylopectin is genetically determined, but can also vary with the environmental conditions during plant growth and the stage of plant (starch) development. Most of cereal starches, including normal and waxy varieties of corn, wheat, rice, etc., have greater

proportion of short and smaller proportion of long amylopectin chains than potato, green leaf canna, and high-amylose corn starches (Jane et al. 1999). Corn ears grown at 35ºC have been reported to display lower molecular size and content of amylose, larger proportion of medium and smaller proportion of short amylopectin branch-chains in starch granules than the corresponding ones developed at 25ºC (Lu et al. 1996). Similar results were reported for rice, potato and wheat starch grown at different environmental temperatures (Asaoka et al. 1984, Asaoka et al. 1985, Asaoka et al. 1989, Tester et al. 1995, Shi et al. 1994). These starch structural differences were attributed to up- and down-regulation of starch-synthesizing enzymes (starch-branching enzymes), which were affected by elevated temperature during development (Jiang et al. 2003). The structure of starch from the same cocoyam genotypes have been reported to vary with different growing seasons (Lu et al. 2005), which can be ascribed to differential climates.

Starch granules are arranged in a semi-crystalline form, which gives starch granules characteristic Maltese cross when viewed under a light microscope. The central part of the Maltese cross is called hilum that represents a location where starch biosynthesis is initiated. Starch molecules are synthesized from the hilum to the surface of the granule in a radial arrangement. Amylopectin and amylose are synthesized side by side by starch synthase and granular bound starch synthase enzymes, respectively (Nakamura 2002). It is believed that the branch-chains of amylopectin are organized in clusters (**Figure 6a**) and form alternating crystalline and amorphous regions (**Figure 6b**). The crystalline regions are composed of parallel amylopectin chains packed closely, which interact with each other by hydrophobic interaction, hydrogen and van der Waals bonds to form double helices (Imberty et al. 1988). The amorphous regions are composed mainly of the branch points. Amylose is present in an

amorphous form and is intertwined and interspersed with the amylopectin molecules (Jane et al. 1992).

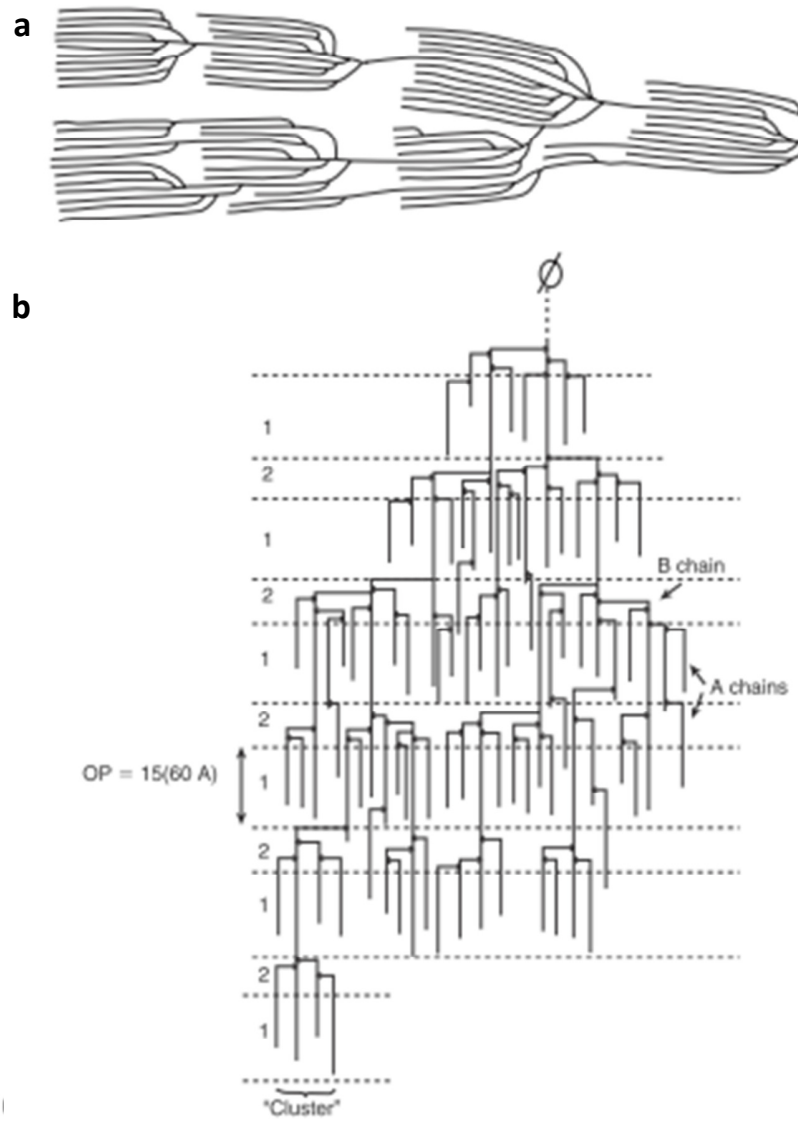

Figure 6. Structural cluster models for amylopectin. 1 and 2 in the model represent crystalline and amorphous regions, respectively (adopted from Jane et al. 2009).

Double-helices of amylopectin can be arranged in A-, B-, and C- type crystalline polymorphs, as shown in **Figure 7**. The double helices of A-type polymorphs are packed into a monoclinic lattice, whereas those of B-type polymorphs in a hexagonal assembly (Buleon et al. 1998). The C-type polymorphs are mixtures of A- and B-type polymorphs.

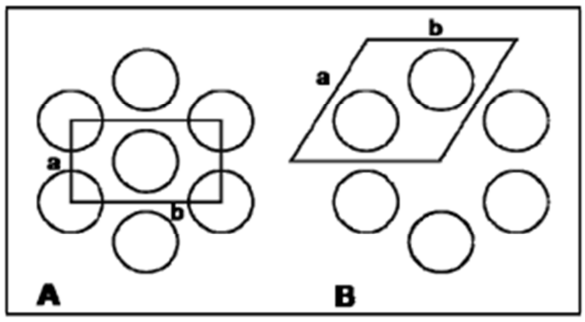

Figure 7. The arrangement of the double helices in starch crystalline structure into A-type (A) and B-type (B) polymorphs

Normal and waxy varieties of corn contain A-type starches, whereas high-amylose corn contains the B-type starch. In general, the A-type polymorphs are composed of shorter amylopectin chains than the B-type polymorphs (Hizukuri 1985). The branching points of amylopectin chains in A-type starches are scattered in both the amorphous and crystalline regions, whereas in B-type starches the branching points are located mostly in the amorphous regions (**Figure 8**) (Jane et al. 1997).

Amylose and amylopectin are not uniformly distributed within the starch granule. Amylose is synthesized in later stages of granule development and thus, is more concentrated in the periphery of the granule (Jane and Shen 1993, Pan and Jane 2000, Li et al. 2007).

Amylopectin present in the core of the granule contains more long branch-chains than that located at the periphery of the granule (Jane and Shen 1993, Pan and Jane 2000).

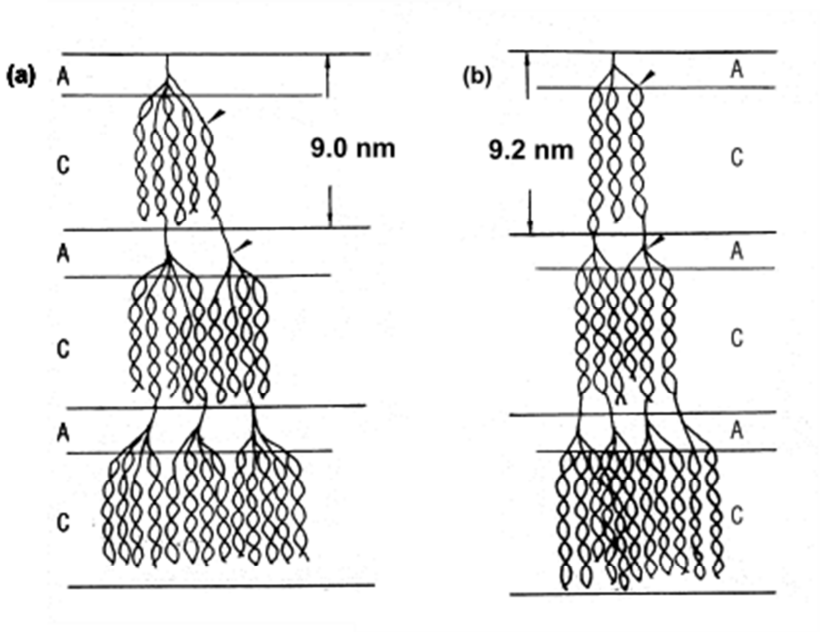

Figure 8. Proposed models for branching patterns of (a) waxy corn starch and (b) potato starch. "A" and "C" stand for the amorphous and crystalline regions, respectively. (Jane et al. 1997).

Properties of starch

Thermal properties

At ambient temperatures, starch granules are not soluble in water and exhibit limited swelling. During heating in a sufficient amount of water, the thermal energy overcomes the hydrophobic interactions, intra- and inter-molecular hydrogen and van der Walls bonds in starch granules, which results in the disruption of double helices and loss of starch crystalline

structure (McPherson 1999). This irreversible process is called gelatinization. The transition from the ordered crystalline structure to amorphous state of starch occurs over a temperature range. A temperature at which starch crystallites begin to melt is termed the onset gelatinization temperature, whereas a temperature at the end of the crystallites melting is called the completion gelatinization temperature of starch. The gelatinization temperature of starch is influenced by the starch structure: amylopectin branch-chain length and the type of crystallite packing. Starches containing amylopectin with larger proportion of short branch-chains (DP 6-12) have been shown to display lower gelatinization temperatures (Jane et al. 1999, Srichuwong et al. 2005). Short amylopectin chains cannot form stable double helices, contribute to destabilization of starch crystallites, and decrease the gelatinization temperature of starch (Jane et al. 1999, Srichuwong et al. 2005, and Srichuwong et al. 2009).
B-type polymorphs exhibit lower gelatinization temperatures than A-type polymorphs, when they have the same amylopectin branch-chain length (Whittan e al. 1990); this can be ascribed to the number of water molecules present in the unit cell of crystallites. The unit cell of B-type polymorph contains 36 molecules of water, whereas that of A-type polymorph is more tightly packed containing 8 molecules of water (Sarko and Wu 1978, Imberty et al. 1991).

Hydrothermal treatments, such as annealing and heat-moisture treatment, alter thermal properties of starch without destroying the granule integrity. Annealing is a process of incubation of starch in excess water at temperatures above glass transition but below the gelatinization temperature of starch (O'Brien and Wang 2007). Annealed starches exhibit higher gelatinization temperature, narrower gelatinization temperature range, and larger or unchanged enthalpy change than the native (untreated) starches (as reviewed by Jayakody

and Hoover 2008). This can be explained by the enhanced crystallinity of annealed starches due to the rearrangement and alignment of starch molecules to form more perfect crystallites that gelatinize at higher temperatures. The heat-moisture treatment can be defined as incubation of starch at relatively low water contents (10-30%) and elevated temperatures (e. g. 90-120˚C) (Maache-Rezzoug et al. 2008, Chung et al. 2009). Heat-moisture treated starches display higher gelatinization temperatures than the native counterparts, and change their crystallite arrangement from B- type to A-type polymorphic packing.

Gelatinized starch molecules tend to recrystallize during storage and form retrograded starch. This process was explained earlier in the text and termed retrogradation.

Pasting properties

Atwell et al. (1988) defined pasting as "the phenomenon following gelatinization in the dissolution of starch. It involves the granular swelling, exudation of molecular components from the granule, and eventually, total disruption of the granules". This process is depicted in **Figure 9**. During early stages of heating, starch granules absorb water and increase in the size, which results in a significant increase in the viscosity of starch. At a certain point, swollen granules rupture, and amylose and smaller portion of amylopectin molecules leach out of from the granules to form starch paste. The property of starch to form pastes has been extensively used in the food industry, for example in the manufacture of thickeners. Upon cooling, the leached molecules tend to associate to form a network called gel. The gel is composed of swollen, gelatinized granules that are imbedded in a continuous amylose gel matrix. This phenomenon is used in the production of puddings.

The pasting properties of starch are affected by the amylose content of starch, the amylopectin branch-chain length, and presence of minor starch components, such as lipids and phosphate monoesters. Amylose does not contribute to the swelling of starch and consequently, starches with large amylose contents (e.g. high-amylose corn starch) display high pasting temperatures and low peak viscosity values (Srichuwong et al. 2005). Conversely, waxy corn starch that is devoid of amylose has lower pasting temperatures and higher peak viscosity than the normal corn counterpart (Jane et al. 1999). Amylose strongly interacts with amylopectin molecules and holds the integrity of starch granule. Consequently, starch granules containing small amylose contents have ability to swell more and absorb water to larger extent than the high-amylose counterparts. Previous studies have shown that starch containing amylopectin with large amounts of short amylopectin chains exhibit a low pasting temperature (Edwards et al. 1999, Han et al. 2001, Noda et al. 2001, Franco et al. 2002, Vanderputte et al. 2003, Wong et al. 2003, Srichuwong et al. 2005). Short amylopectin chains (DP 6-12) do not form stabile double helices, contribute to destabilization of starch crystalline structure, and have less ability to hold the integrity of swollen granules.

Cereal starches display higher pasting temperature and lower peak viscosities than root and tuber starches, which could be explained by the higher content of lipids in the cereal starches. Lipids readily complex with amylose molecules and reduce the swelling power of starch granules in water (Tester and Morrison 1990). Certain tuber starches (e.g. potato) contain large amounts of phosphate monoesters, which further reduce the pasting temperature and increase the peak viscosity of starch through repulsion forces of negatively charged phosphate groups.

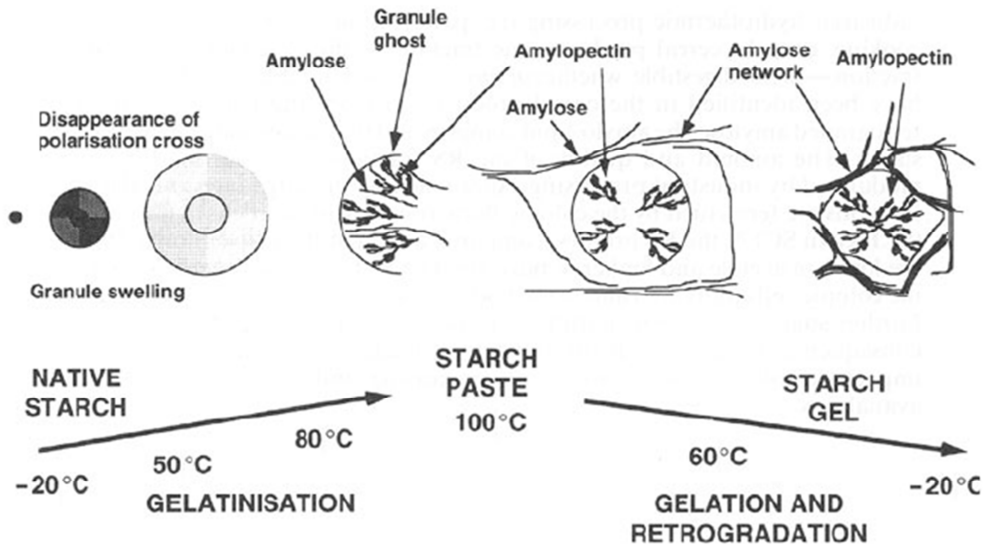

Figure 9. Diagram depicting pasting and gelling of starch in water (Bornet 1993)

Enzyme hydrolysis

Native (raw) starch granules are digested by enzymes at slower rates than gelatinized starch owing it to their semicrystalline structure. Molecules of native starch are not easily accessible to enzymes because they are buried in hard to breach crystallites. The rate of raw-starch hydrolysis is affected by the starch granule structure (Jane et al. 2003), granule surface area (Franco et al. 1992, Yook and Robyt 2002, Kong et al. 2003, Kim et al. 2008), presence of protein and lipids on the granule surface (Greenwell et al. 1985, Oates 1997), and enzyme types (Planchot et al. 1995, Blazek and Copeland 2010).

Starches containing large amylose contents are not susceptible to enzyme hydrolysis because amylose interacts with amylopectin molecules, holds the granule integrity and thus, prevents granule swelling and enzyme penetration to the granule interior (Jane et al. 2003, Jane 2006). A-type starches are hydrolyzed faster than B-type starches because they contain

larger amounts of short amylopectin chains that form weak points in the starch crystallites (Jane et al. 2003). A large number of branching points present in the crystalline regions of A-type starches further weakens the crystallites and increases granule susceptibility to enzyme hydrolysis (Jane et al. 1997). A type starches such as waxy and normal corn starches, also contain internal voids and channels in the granules whereas B-type starches have more homogenous granule structure (Jane 2006). Enzymes use those voids to burrow to the granule interior more easily.

Larger starch granules are hydrolyzed at slower rate than small granules because they have smaller relative surface area (Kong et al. 2003, Kim et al. 2008). Proteins that surround starch granules in the ground corn flour or that remained on the granule surface after isolation of starch reduce the granule surface area and hinder binding of enzymes to the starch molecules. Lipids interact with amylose to form enzyme resistant amylose-lipid complex (Jane and Robyt 1984).

Upon gelatinization, starch granules lose their crystalline structure and can be rapidly digested by enzymes. Retrograded starch, however, is less susceptible to enzyme hydrolysis because it regains certain level of ordered structure and form amylose-lipid and amylose-amylose complexes, which are resistant to enzyme hydrolysis (Jane and Robyt 1984).

Reference cited

Altay, F., and Gunasekaran, S. 2006. Influence of drying temperature, water content, and heating rate on gelatinization of corn starches. J. Agric. Food Chem. 54: 4235–4245.

Asaoka, M., Okuno, K., and Fuwa, H. 1985. Effect of environmental temperature at the milky stage on amylose content and fine structure of amylopectin of waxy and nonwaxy endosperm starches of rice (*Oryza sativa* L.). Agr. Biol. Chem. 49: 373-379.

Asaoka, M., Okuno, K., Konishi, Y., and Fuwa, H. 1987. The effects of endosperm mutations and environmental temperature during development on the distribution of molecular weight of amylose in rice endosperms. Agr. Biol. Chem. 51: 3451- 3453.

Asaoka, M., Okuno, K., Sugimoto, Y., Kawakami, K., and Fuwa, H. 1984. Effect of environmental temperature during development of rice plants on some properties of endosperm starch. Starch/Stärke 36: 189-193.

Atwell, W. A., Hood, L. F., Lineback, D. R., Varriano-Marston, E., and Zobel, H. F. 1988. The terminology and methodology associated with basic starch phenomena. Cereal Food. World 33:306-310.

Banks, W., Greenwood, C. T., and Khan, K. M. 1971. The interaction of linear amylose oligomers with iodine. Carbohydr. Res. 17:25-33.

Beatty, M. K., Rahman, A., Cao, H., Woodman, W., Lee, M., Myers, A. M., and James, M. G. 1999. Purification and Molecular Genetic Characterization of ZPU1, a Pullulanase-Type Starch-Debranching Enzyme from Corn. Plant Physiol. 119 (1):255-266.

Beck, E., and Ziegler, P. 1989. Biosynthesis and degradation of starch in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:95-117.

Billiaderis, C. G., and Galloway, G. 1989. Crystallization behavior of amylose-V complexes: Structure-property relationships. Carbohydr. Res. 189:31-48.

Blazek, J., and Copeland, L. 2010. Amylolysis of wheat starches. II. Degradation patterns of native starch granules with varying functional properties. J. Cereal Sci. 52(2):295-302.

Bornet, F. 1993. Technological treatments of cereals. Repercussions on the physiological properties of starch. Carnohydr. Polym. 21(2-3):195-203.

Bothast, R. J., and Schlicher, M. A. 2005. Biotechnological processes for conversion of corn into ethanol. Appl Microbiol Biotechnol. 67:19-25.

Buléon, A. Colonna, P., Planchot, V., and Ball, S. 1998. Starch granules: structure and biosynthesis. Int. J. Biol. Macromol. 23:85-112.

Butzen, S., Haefele, D., and Hillard, P. 2003. Corn processing II: Dry-grind ethanol production. Crop Insights. 13(3):1-4.

Campbell, M. R., Pollak, L. M., and White, P. J. 1994. Effect of planting date on corn starch thermal properties, Cereal Chem. 71: 556–559.

Chung, H.J., Liu, Q., and Hoover, R. 2009. Impact of annealing and heat-moisture treatment on rapidly digestible, slowly digestible and resistant starch levels in native and gelatinized corn, pea and lentil starches, Carbohydr. Polym. 75:436–447.

Dale, R. T., and Tyner, W. E. 2006. Economical and technical analysis of ethanol dry milling: model description. Department of Agricultural Economics, Purdue University. http://ageconsearch.umn.edu/bitstream/28674/1/sp060004.pdf. Accessed April 14, 2011.

Eckoff, S. R., and Tso, C. C., 1991. Starch recovery from steeped corn grits as affected by drying temperature and added commercial protease. Cereal Chem. 68 (3): 319-320.

Edwards, A, Fulton, D. C, Hylton, C. M, Jobling, S. A, Gidley, M, Rossner, U., Martin, C., and Smith, A. M. 1999. A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. Plant J. 17: 251-261.

Eerlingen, R. C.., Jacobs, H., and Delcour, J. A. 1994. Enzyme resistant starch VI. Effect of endogenous lipids and added sodium dodecyl sulfate on formation of resistant starch. Cereal Chem. 61:351-355.

Farnham, D. E., Benson, G. O., and Pearce, R. B. 2003. Corn Perspective and Culture. pp 1-33. In: Corn Chemistry and technology, 2nd edition. P. J. White and L. A. Johnson, eds. American Association of Cereal Chemists, St. Paul, MN.

Franco, C. M. L, Wong, K-S, Yoo, S-H, and Jane, J. 2002. Structural and functional characteristics of selected soft wheat starches. Cereal Chem. 79: 243-248.

Franco, C. M. L., Preto, S. J. R., and Ciacco, C. F. 1992. Factors that affect the enzymatic degradation of natural starch granules- effect of the size of the granules. Starch/Stärke 44:422–426.

French, D., and Murphy, V. G. 1977. Computer modeling in study of starch. Cereal Food World 22:61-70.

Freudenberger, R. 2009a. Chapter 1: About alcohol fuel, pp. 3-21. In: Alcohol fuel. New society publishers. Gabriola Islands, Canada.

Freudenberger, R. 2009b. Chapter 9: Alcohol as an engine fuel, pp. 155-205. In: Alcohol fuel. New society publishers. Gabriola Islands, Canada.

Galliard, T., and Bowler, P. 1987. In: Starch: Properties and Potential. Editor T. Galliard, Society of Chemical Industry, UK. pp. 55-78.

Gnansounou, E. 2009. Fuel ethanol Current Status and Outlook. pp 57-70. In: Handbook of plant-based biofuels. A. Pandey, eds. CRC Press, Boca Raton, FL.

Greenwell, P., Evers, A.D., Gough, B.M., and Russell, P.L. 1985. Amyloglucosidase-catalysed erosion of native, surface-modified and chlorine-treated wheat starch granules. The influence of surface protein. J. Cereal Sci. 3:279–293.

Greenwood, C. T. 1970. In: The Carbohydrates, Vol. IIB, W. Pigman and D. Hallberg, L. M. & Chinachoti, P. (1992) J. Food Sci. 57, 1201.

Gudmundsson, M., and Eliasson, A. C. 1990. Retrogradation of amylopectin and the effects of amylose and added surfactants/emulsifier. Carbohydr. Polym. 13:295-315.

Han, X-Z, and Hamaker, B. R. 2001. Amylopectin fine structure and rice starch paste breakdown. J. Cereal Sci. 34: 279-284.

Hanashiro, I., Abe, J., and Hizukuri, S. 1996. A periodic distribution of the chain length of amylopectin as revealed by high-performance anion-exchange chromatography. Carbohydr. Res. 283:151-159.

Haros, M., Tolaba, M-P., and Suarez, C. 2003. Influence of corn drying on its quality for the wet-milling process. J. Food Eng. 60: 177–184.

Helm, J.L., Fergesan, V.L., and Zuber, M.S. 1968. Effect of planting date on high amylose corn (*Zea Mays* L.). Agron. J. 60: 530–531

Hizukuri, S. 1985. Relationship between the distribution of the chain length of amylopectin and the crystalline structure of starch granules. Carbohydr. Res. 141:295-306.

Hizukuri, S. 1986. Polymodal distribution of the chain lengths of amylopectins, and its significance. Carbohydr. Res. 147:342-347.

Hizukuri, S., Takeda, Y., Maruta, N., Juliano, B. 0. 1989. Molecular structures of rice starch. Carbohydr. Res. 189: 227-235.

Hofstand, D. 2008. International perspectives on food and fuel. AgMRC Renewable Energy Newsletter.

Hooseney, R. C., 1986. Principles of Cereal Science and Technology, first ed. American Association of Cereal Chemists, St. Paul, MN.

http://www.agmrc.org/renewable_energy/biofuelsbiorefining_general/international_perspectives_on_food_and_fuel.cfm. Accessed April 13, 2011.

Imberty, A., Chanzy, H., Perez, S., Buleon, A., and Tran, V. 1988. The double-helical nature of the crystalline part of A-starch. J. Mol. Biol. 201:365-378.

Jane, J. 2006. Current understanding on starch granule structure. J. Appl. Glycosci. 53:205-213.

Jane, J. 2009. Structural Features of Starch Granules II. pp 193-236. In: Starch Chemistry and Technology, 3rd edition. Editors J. BeMiller and R. Whistler. Academic Press, London.

Jane, J. and Robyt, J. F. 1984. Structure studies of amylose-V complexes and retrograded amylose by action of alpha amylases, and a new method for preparing amylodextrins. Carbohydr. Res. 132:105-118.

Jane, J. and Shen, J. J. 1993. Internal structure of the potato starch granule revealed by chemical gelatinization. Carbohydr. Res. 247:279-290.

Jane, J., Ao, Z., Duvick, S. A., Wiklund, M., Yoo, S., Wong, K. and Gardner, C. 2003. Structures of Amylopectin and Starch Granules: How Are They Synthesized? J. Appl. Glycosci. 50(2):167-172.

Jane, J., Chen Y.Y., Lee, L.F., McPherson, A.E., Wong, K.S., Radosavljevic, M., and Kasemsuwan, T. 1999. Effects of Amylopectin Branch Chain Length and Amylose Content on the Gelatinization and Pasting Properties of Starch. Cereal Chem. 76:629-637.

Jane, J., Wong, K., and McPherson, A. E. 1997. Branch-structure difference in starches of A- and B-type X-ray patterns revealed by their Naegeli dextrins. Carbohydr. Res. 300:219-227.

Jane, J., Xu, A., Radosavljevic, M., and Seib, P. A. 1992. Location of amylose in normal starch granules I. Susceptibility of amylose and amylopectin to cross-linking reagents. Cereal Chem. 69:405-409.

Jayakody, L., and Hoover, R. 2008. Effect of annealing on the molecular structure and physicochemical properties of starches from different botanical origins- A review. Carbohydr. Polym. 74(3*)*:691-703.

Jiang, H., Dian, W., and Wu, P. 2003. Effect of high temperature on fine structure of amylopectin in rice endosperm by reducing the activity of the starch branching enzyme. Phytochemistry 63:53–59.

Kim, J., Kong, B., Kim, M., and Lee, S., 2008. Amylolytic Hydrolysis of Native Starch Granules Affected by Granule Surface Area. J. Food Sci.73:621–624.

Kong, B. W., Kim, J. I., Kim, M. J., and Kim, J. C. 2003. Porcine-pancreatic α-amylase hydrolysis of native starch granules as a function of granule surface area. Biotechnol. Prog. 19:1162-1166.

Kuge, T., and Takeo, K. 1968. Complexes of starchy materials with organic compounds Part II. Complex formation in aqueous solution and fractionation of starch by 1-methanone. Ag. Biol. Chem. 32:1230-1238.

Lansky, S., Kooi, M., and Schoch, T. J. 1949. Properties of the fractions and linear subfractions from various starches. J. Am. Chem. Soc. 71:4065-4075.

Lee, S. 2007. Ethanol from corn. pp: 323-341. In: Handbook of alternative fuel technologies. Sunggyu Lee , James G. Speight , and Sudarshan K. Loyalka, eds. CRC Press, Boca Raton, FL.

Lewis, S. M., Van Hulzen, S.E., & Roth, D. L. 2005. Continuous process for producing ethanol using raw starch. U.S. Patent number 20050239181.

Lewis, S. M., Van Hulzen, S.E., & Roth, D. L. 2010. Continuous process for producing ethanol using raw starch. U.S. Patent number 20100041116.

Li, L., Blanco, M., and Jane, J. 2007. Physicochemical properties of endosperm and pericarp starches during corn development. Carbohydr. Polym. 67:630-639.

Liska, A. J., Yang, H. S., Bremer, V. R., Klopfenstein, T. J., Walters, D. T., Erickson, G. E., Cassman, K. G. 2009. Imporvements in life cycle energy efficiency and greenhouse gas emissions of corn ethanol. J. Ind. Ecol. 13:58-74.

Logan, D. C., Millar, A. H., Sweetlove, L. J., Hill, S. A., and Leaver, C. J. 2001. Mitochondrial biogenesis during germination of corn embryos. Plant Physiol. 125:662-672.

Lu, T., Chen, J., Lin, C., and Chang, Y. 2005. Properties of starches from cocoyam (*Xanthosoma sagittifolium*) tubers planted in different seasons. Food Chem. 91 (1):69-77.

Lu, T., Jane, J., Keeling, P. L., and Singletary, G. W. 1996. Corn fine structures affected by ear developmental temperature. Carbohydr. Res. 282: 157-170.

Maache-Rezzoug, Z., Zarguili, I., Loisel, C., Queveau, D. and Buléon, A. 2008. Structural modifications and thermal transitions of standard corn starch after DIC hydrothermal treatment, Carbohydr. Polym. 74:802–812.

MacGregor, A. W. 1987. α- Amylase, limit dextrinase, and α-glucosidase enzymes in barley and malt. CRC Crit. Rev. Biotech. 5:117-128.

Malumba, P., Massaux, C., Deroanne, C., Masimango, T., and Béra, F., 2009. Influence of drying temperature on functional properties of wet-milled starch granules. Carbohydr. Polym. 75: 299-306.

McPherson, A. E. 1999. Physicochemical properties of selected root and tuber starches and characterization of extruded, chemically modified corn starches. Thesis (Ph.D.)--Iowa State University.

Mosier, N. S., and Ilelej, K. 2006. How fuel ethanol is made from corn. Purdue Extension BioEnergy. ID-328. http://www.extension.purdue.edu/extmedia/ID/ID-328.pdf. Accessed April 14, 2011.

Muralikrishna, G., and Nirmala, M. 2005. Cereal α-amylases- an overview. Carbohydr. Polym. 60: 163-173.

Nakamura, Y. 1996. Some properties of starch debranching enzymes and their possible role in amylopectin biosynthesis. Plant Sci. 121:1-18.

Nakamura, Y. 2002. Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. Plant Cell Physiol. 43:718-725.

Noda, T, Tohnooka, T, Taya, S, and Suda, I. 2001. Relationship between physicochemical properties of starches and white salted noodle quality in Japanese wheat flours. Cereal Chem. 78: 395-399.

Noda, T., Takahata, Y., Sato, T., Ikoma, H., and Mochida, H. 1997. Combined effects of planting and harvesting dates on starch properties of sweet potato roots. *Carbohydr. Polym.*, 33**:**169–176.

O'Brien, S., and Wang, Y. 2007. Susceptibility of annealed starches to hydrolysis by α-amylase and glucoamylase. Carbohydr. Polym. 72 (4):597-607.

Oates, CG., 1997. Towards an understanding of starch granule structure and hydrolysis. Trends Food Sci. Technol. 8:375-382.

Pan, D. D., and Jane, J. 2000. Internal structure of normal corn starch granules revealed by chemical surface gelatinization. Biomacromolecules 1:126-132.

Peplinski, A. J., Paulis, J. W., Bietz, J. A., and Pratt, 1994. Drying of high-moisture corn: changes in properties and physical quality. Cereal Chem. 71(2): 129-133.

Perez, S., Baldwin, P. M., and Gallant, D. J. 2009. Structural features of starch granules I. In Starch : Chemistry and Technology. Edited by J. BeMiller, and R.Whistler, 3rd edition, Academic Press, Burlington, MA.

Pimentel, D. 2001. Ethanol fuel from corn faulted as unsustainable unsubsidized food burning in analysis of Cornell scientist. http://www.news.cornell.edu/releases/Aug01/corn-basedethanol.hrs.html.

Planchot, V., Colonna, P., Gallant, D.J, and Bouchet, B. 1995. Extensive degradation of native starch granules by alpha-amylase from *Aspergillus fumigatus*. J. Cereal Sci. 21:163–171.

Rasmussen, M. 2009. Enhancing dry-grind corn ethanol production with fungal cultivation and ozonation. Thesis (Ph.D.)--Iowa State University.

Rausch, K. D., Belyea, R. K., Ellersieck, M. R., Singh, W., Johnston, D. B., and Tumbelson, M. E. 2005. Particle size distributions of ground corn and DDGS from dry grind processing. Trans. ASAE. 48, 273-277.

RFA 2007. Ethanol Industry Outlook 2007: Building New Horizons. http://www.ethanolrfa.org/page/-/objects/pdf/outlook/RFA_Outlook_2007.pdf?nocdn=1 Accessed April 14, 2011.

RFA 2009. Industry resources: Coproducts. http://www.ethanolrfa.org/pages/industry-resources-coproducts. Accessed April 14, 2011.

RFA 2011. http://www.ethanolrfa.org/pages/statistics. Accessed on March 27, 2011.

Robertson, G. H., Wong, D. W. S., Lee, C. C., Wagschal, K., Smith, M. R., and Orts, W. J. 2006. Native or raw starch digestion: A key step in energy efficient biorefining of grain. J. Agric. Food Chem. 54:353-365.

Rundle, R. E., and French, D. 1943. The configuration of starch in the starch-iodine complex. III X-ray diffraction studies of the starch-iodine complex. J. Am. Chem. Soc. 65:1707-1710.

Sanwo, M. M., and DeMason, D.A. 1992. Characteristics of α-amylases during germination of two high-sugar sweet corn cultivars of zea mays L. Plant Physiol. 99:1184-1192.

Sarko, A., and Wu, H.-C. H. 1978. The crystal structures of A-, B-, and C-polymorphs of amylose and starch. Starch/Staerke 30:73-78.

Schoch, T. J. 1942. Fractionation of starch by selective precipitation with butanol. J. Am. Chem. Soc. 63:2957-2961.

Searchinger, T., Heimlich, R., Houghton, R. A., Dong, F., Elobeid, A., Fabiosa, J.,Tokgoz, S., Hayes, D.,and Yu, T. 2008. Use of U.S. Croplands for Biofuels Increases Greenhouse Gases Through Emissions from Land-Use Change. Science: 319 (5867):1238 – 1240.

Shapouri, H., Gallagher, P. W., Nefstead, W., Schwartz, R., Noe, S., and Conway, R. 2010. 2008 energy balance for the corn-ethanol industry. United States Department of Agriculture. Agricultural Economic Report Number 846. http://www.usda.gov/oce/reports/energy/2008Ethanol_June_final.pdf. Accessed April 14, 2010.

Shi, Y.-C., Seib, P.A., and Bernardin, J.E. 1994. Effects of temperature during grain filling on starches from six wheat cultivars. Cereal Chem. 71:369–383.

Singh, S., Gupta, A. K., Gupta, S. K., and Kaur, N. 2010. Effect of sowing time on protein quality and starch pasting characteristics in wheat (*Triticum aestivum* L.) genotypes grown under irrigated and rain-fed conditions. Food Chem. 122: 559-565.

Singh, V., Haken, A. E, Paulsen, M. R., and Eckhoff, S. R. 1998. Starch yield sensitivity of corn hybrids to drying temperature and harvest moisture content. Starch/Stärke 50: 181-183.

Singhania, R. R., Parameswaran, B., and Pandey, A. (2009). Plant-based biofuels, An introduction. In: Handbook of plant-based biofuels. Edited by A. Pandey. CRC Press Taylor & Francis Group., Boca Raton, Florida.

Smith, A. M., Zeeman, S. C., and Smith, S. M. 2005. Starch degradation. Annu. Rev. Plant Biol. 56: 73-98.

Spence, K. E. 1998. Effects of starch structure on the enzymatic resistance of starch, viscoelasticity of starch gels, and biodegradability of starch in plastics. Thesis (Ph.D.)--Iowa State University.

Srichuwong, S., Gutesa, J., Blanco, M., Duvick, S. A., Gardner, C., and Jane, J. 2009. Characterization of corn grains for dry-grind ethanol production. J. ASTM Int. 7(2):1-10.

Srichuwong, S., Sunarti, T.C., Mishima, T., Isono, N., and Hisamatsu, M. 2005. Starches from different botanical sources I: Contribution of amylopectin fine structure to thermal properties and enzyme digestibility. Carbohydr. Polym. 60:529-538.

Subbarao, K. V., Datta, R., and Sharma, R. 1998. Amylases synthesis in scutellum and aleurone layer of corn seeds. Phytochemistry 49 (3):657-66.

Sun, Z., and Henson, C. A. 1991. A quantitative assessment of the importance of barley seed α-amylase, β-amylase, debranching enzyme and α-glucosidase in starch degradation. Arch. Biochem. Biophys. 284:298.

Suzuki, A., Shibanuma, K., Takeda, Y., Abe, J., and Hizukuri, S. 1994. Structures and pasting properties of potato starches from Jaga Kids Purple ’90 and Red ’90. Oyo Toshitsu Kagaku 41:425-436.

Takeda, Y., Shitaozono, and Hizukuri, S. 1988. Molecular structure of corn starch. Starch/Staerke. 40:51-59.

Tester, R.F. and Morrison, W.R. 1990. Swelling and gelatinisation of cereal starches. I. Effects of amylopectin, amylose and lipids. Cereal Chem. 67:551-557.

Tester, R.F, South, J.B., Morrison, W.R., Ellis, R.P., Piggott, J.R., Batts, G.R., Wheeler, T.R., Morison, J.I.L., Hadley, P., Ledward, D.A. **1995.** Effects of elevated growth temperature and carbon dioxide levels on some physicochemical properties of wheat starch. J. Cereal Sci. 22:65–71.

Urbanchuk, J. M. 2011. Contribution of the ethanol industry to the economy of the United States. Report prepared for the Renewable Fuel Association. http://ethanolrfa.org/page/-/Ethanol%20Economic%20Contribution%202010%20Final%20Revised%20010411.pdf?nocdn=1. Accessed March 27, 2010.

USDA 2007. Crop production 2006 summary. United State Department of Agriculture, National Agricultural Statistics Service.

Vandeputte, G. E, Derycke, V, Geeroms, J, and Delcour, J. A. 2003. Rice starches. II. Structural aspects provide insight into swelling and pasting properties. J. Cereal Sci. 38:53-59.

Wall, J. S., James, C., and Donaldson, G. L. 1975. Corn proteins: Chemical and physical changes during drying of grain. Cereal Chem. 52: 779-790.

Watson, S. A. 2003. Description, development, structure, and composition of the corn kernel. pp 69-106. In: Corn Chemistry and technology, 2nd edition. P. J. White and L. A. Johnson, eds. American Association of Cereal Chemists, St. Paul, MN.

Whittam, M. A., Noel, T. R., and Ring, S, G. 1990. Melting behavior of A- and B-type crystalline starch. Int. J. Biol. Macromol. 12:359-362.

Williams, V. R., Wu, W. T., Ysai, H. Y., and Bates, H. G. 1958. Varietal differences in amylose content of rice starch. J. Agric. Food Chem. 6: 47-48.

Wong, K-S., Kubo, A., Jane, J., Harada, K., Satoh, H., Nakamura, Y. 2003. Structures and properties of amylopectin and phytoglycogen in the endosperm of sugary-1 mutants of rice. J. Cereal Sci. 37: 139-149.

Wu, X., Zhao, R., Wang, D., Bean, S. R., Seib, P. A., Tunistra, M. R., Campbell, M., and O'Brien, A. 2006. Effects of amylose, corn protein, and corn fiber contents on production of ethanol from starch-rich media. Cereal Chem. 83 (5): 569-575.

Yamashita, Y., and Monobe, K. 1971. Single crystals of amylose V complexes. III Crystals with a helical configuration. J. Polymer Sci. A-2:1471-1481.

Yoo, S., and Jane, J. 2002. Molecular weights and gyration radii of amylopectins determined by high-performance size-exclusion chromatography equipped with multi-angle laser-light scattering and refractive index detectors. Carbohydr. Polym. 49 (3):307-314.

Yook, C., and Robyt, J. F. 2002. Reactions of alpha amylases with starch granules in aqueous suspension giving products in solution and in a minimum amount of water giving products inside the granule. Carbohydr. Res. 337:1113–1117.

Zobel, H. F. 1988. Molecules to granules: A comprehensive starch review. Starch/Staerke 40:44-67.

CHAPTER 4. EFFECT OF PLANTING DATE ON PROPERTIES OF CORN. PART I: GRAIN YIELD, KERNEL COMPOSITION, AND STRUCTURE AND PROPERTIES OF STARCH

A paper prepared for submission to the Journal of Agricultural and Food Chemistry

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ABSTRACT

Objectives of this study were to understand how grain yield and characteristics of kernels and starch were affected by different planting dates of corn. Three corn germplasm sources grown in Iowa with planting dates between 4 April and 11 June during 2007-2009 growing seasons were used in this study. The maximum grain yield and starch content of kernels were obtained with corn planted between late April and mid-May; both decreased when planting was delayed to late May and June. Later planting dates resulted in smaller amylose contents of starch and larger proportions of short amylopectin chains for some varieties, whereas the amylopectin branch-chain length of other varieties was not affected by planting dates. Gelatinization and pasting temperatures of starch decreased, whereas the peak viscosity of starch paste increased with the delay in planting date of corn. Corn planted very

early (4 April 2009 and 16 April 2008) produced starch with more amylose contents and short amylopectin chains.

KEYWORDS: corn, starch structure, properties, planting date, grain yield, kernel composition

INTRODUCTION

Planting corn within a window of optimum time is recommended for producers as a method to optimize grain yield in the central U.S. The optimal planting dates for corn vary with the location, environmental conditions, and the corn genotype (*1*). The optimal window of planting dates for Iowa is 20 April-10 May (*2*), Kansas is 1 April-5 May (*3*), Wisconsin is 1 May-7 May (*4*), whereas in the eastern Nebraska an optimal planting date of 10 May has been reported (*5*).

Producers are not always able to plant corn within these optimum dates because of adverse weather or soil conditions. Soils need to be adequately dry with the soil temperature at ≥ 10ºC before farmers can plant corn seeds (*6*). When unfavorable weather conditions occur, such as long winter seasons (cold temperatures) or excessive rainfall, corn planting can be delayed beyond the optimum planting dates. Delayed planting can reduce corn grain yield because the length of growing season is shortened, and the growing temperatures are lower and solar radiation is reduced during the grain-filling period (*7, 8*). In Wisconsin, the average grain yield for corn declined 31% when the planting date was delayed from May 1 to May 30 (*4*).

Corn starch is a common ingredient widely used in food and non-food applications. Changes in the properties of starch require adjustments of processing parameters and reformulation of products, and can lead to inferior product quality. Thus, it is of crucial importance to understand factors influencing the structure and functional properties of starch. Starch properties are determined by amylose and amylopectin contents and their structures, which change with different environmental conditions (e.g. growing temperature and soil moisture) (*9-14*) and plant maturation stages (*15*). Impacts of planting dates on starch structures have been reported, but results are inconclusive. The amylose content of starch has been reported not to change with late planting dates in rice (*16*), normal (common) corn (*17*), and sweet potato (*18*). In high-amylose corn, the amylose content of starch has been reported to increase with delayed planting (*19*), whereas in wheat it decreased with a delay in the planting date (*20*). No significant effect of the planting date on the branch-chain length of sweet potato amylopectin has been reported (*18*).

Objectives of this study were to understand effects of the planting date on 1) the grain yield and kernel composition of three corn germplasm sources grown in Iowa, and 2) the structure and functional properties of starches isolated from those kernels. Results obtained from this study provide data on the importance of corn planting date on kernel compositions and starch properties.

MATERIALS AND METHODS

Materials

Three corn germplasm sources, B73 (a public inbred), Pioneer 37Y14, and Pioneer 34A20 (commercial hybrids, Pioneer Hi-Bred International, Inc., Johnston, IA) with 115, 98,

and 109 day relative maturities, respectively, were used in this study. Multi-year (2007, 2008, and 2009) and multi-location (Iowa State University Research and Demonstration Farms) field trials were conducted for a total of five location-years (Table 1). Research was located near Kanawha (north central Iowa), Nashua (northeast Iowa), and Ames (central Iowa). B73 inbred was planted in a completely random design, whereas hybrids 37Y14 and 34A20 were planted in a randomized complete block design with four replications and three to five planting dates as the treatment. Experimental units measured approximately 15 m (length) by 3 m (width), with 76-cm row spacing. Kernels of B73 inbred corn were harvested on 65 days after pollination. Kernels of hybrids 37Y14 and 34A20 were harvested at a single date in each planting year regardless of planting date (**Table 1**). Grain yield was measured from the center two rows of each four-row plot with a grain sample collected from the combine. Kernels were oven dried and stored at room temperature until analysis.

Kernel Composition

Corn kernels were ground using a Cyclone Mill (UDY Corp., Fort Collins, CO, USA) to pass through a sieve with a size of 0.5mm. The starch content of the ground corn sample was determined following the AACC Method 76-13 (*21*) using the Megazyme total starch assay kit. The nitrogen content of the corn sample was determined using a Vario MAX CN Analyzer (Elementar Analysensysteme, Hanau, Germany) and multiplied by 6.25 to obtain the protein content of kernels.

Isolation of Starch

Starch was isolated from corn kernels using a laboratory wet-milling method (*22*).

Morphology of Starch Granules

Starch specimens were prepared and mounted on a brass-disc according to the procedure of Jane et al (*23*) and examined using a scanning electron microscope (JEOL model 1850, Tokyo, Japan).

Apparent Amylose Content of Starch

The apparent amylose content of the isolated starch was determined by measuring the iodine affinity of defatted starch. Starch was defatted with 85% methanol in a Soxhlet extractor. Iodine affinity was determined using a potentiometric autotitrator with Metrodata recording software (702 SM Titrino, Brinkman Instrument, Westbury, NY, USA) (*24*).

Branch-Chain Length Distribution of Starch Amylopectin

Amylopectin was separated from amylose using *1*-butanol (*25*) and debranched using isoamylase (*26*). The debranched chains were labeled with 8-amino-1,3,6-pyrenetrisulphonic acid (APTS), and the branch-chain length distribution was analyzed using a capillary electrophoresis (P/ACE MDQ, Beckman Courter, Fullerton, CA) (*22*). Maltohexaose was used as a reference standard.

Thermal Properties of Starch

Thermal properties of starch samples were determined using a differential scanning calorimeter (DSC) (Diamond DSC, Perkin-Elmer, Norwalk, CT, USA) following the procedure of Jane et al (*27*).

Starch Pasting Properties

Starch pasting properties were determined by using a Rapid ViscoAnalyser (RVA) (Newport Scientific, Sydney, Australia) according to the procedure of Jane et al (*27*).

Statistical Analysis

Data obtained in this study were analyzed using analysis of variance (ANOVA) with a PROC general linear model procedure in SAS 9.2 (SAS Inc.). For each variety in varying growing season and planting location a separate ANOVA was performed. For corn hybrids, the ANOVA for a randomized complete block design was used with field blocks and the planting date treatment as fixed effects. For the inbred corn, the ANOVA for a completely randomized design was used. Variables in the analysis were the grain yield, kernel composition, starch structure and properties. Tukey's adjustments were used for comparison between planting date means for a particular variety, location, and growing season. The level of significance was set at $\alpha=0.05$.

RESULTS AND DISCUSSION

The optimum planting dates for the selected hybrids ranged from 2 May to 15 May during the 2007-2009 growing seasons (**Figure 1**), which is within the recommended planting period for corn crops grown in Iowa (*2*). Planting corn 2-4 weeks after the observed optimum dates reduced the grain yield from 5% (37Y14 hybrid planted on 29 May 2009, $p=0.08$) to 23% (37Y14 planted on 10 June 2008, $p<0.05$). Early planting dates, 2-4 weeks prior to the optimum dates, resulted in the grain yield similar to that of the optimum dates. Although the corn grain yield was reduced with the planting date delayed into late May and June in all three years, the reduction was less severe in 2009.

Maximum starch contents of kernels were obtained with planting dates in late April and early May in 2007 and 2008 seasons (2 May 2007 for hybrid 34A20, and 30 April 2008 for hybrid 37Y14) (**Figure 2A**). Later planting dates produced kernels with starch contents reduced from 3% (hybrid 34A20 planted on 29 May 2007; $p<0.05$) to 6% (hybrid 37Y14 planted on

10 June 2008; $p<0.05$) compared with the corresponding optimum dates. In 2009, however, late planting dates had little effect on the starch content of 37Y14 kernels, similar to the little change in the grain yield. The results of B73 inbred corn confirmed that late planting dates in 2008 were associated with reduced starch contents of kernels, whereas late plantings in 2009 produced kernels with larger starch contents than those of early dates (**Figure 2B**). Protein contents of 34A20, 37Y14, and B73 kernels did not change significantly with planting dates, although slightly greater contents were observed with plantings in late May and June (**Figure 3**). The exception was samples of B73 planted in 2009 that resulted in reduced protein contents of kernels with later planting dates (May 20).

In general, significantly reduced grain yields and kernel starch contents were observed with planting dates delayed into late May and June of 2007 and 2008 compared with the optimum dates. The reduction could be attributed to the shorter effective growing season and reduced grain-filling period of kernels planted on late days (*7*, *8*), resulting in less starch deposition in the kernels. Contrary to those of 2007 and 2008, the late planting dates in 2009 appeared to have less severe effect on the grain yield and starch contents of kernels. The difference could be attributed to an unusual weather pattern of 2009 (**Table 2**). The growing season of 2009 had unusually low temperatures during June, July, August, and October; average temperatures for these months were from 1.1° to 3.9° C below normal compared with the 30-year average (**Table 2**). USDA National Agricultural Statistics Service reported that despite lower acreage of planting, productivity of corn in 2009 was the highest on record (13.2 billion bushels), which was 1% and 9% greater than that of the 2007 and 2008, respectively (*28*). This could be in part explained by the cool weather conditions that

were favorable for corn production (less stress), which could result in less yield loss of corn planted on later dates in 2009.

Starch was isolated from kernels of hybrid 37Y14 and inbred B73 planted in 2008 and 2009 to reveal effects of planting date on the starch structure and properties. Starch samples of hybrid 34A20 grown in 2007 were not obtained because of the insufficient quantity of kernels. Scanning electron micrographs of starches isolated from kernels of hybrid 37Y14 and inbred B73 planted on different dates in 2008 are shown in **Figures 4 and 5**. A significant number of very large and polygonal-shaped starch granules with the diameter >20 μm were found in samples planted early, 6.8% for hybrid 37Y14 on 16 April and 5.7% for B73 inbred planted on 6 May (**Figures 4A** and **5A**). The starch isolated from corn planted in June, however, had spherical granules with more uniform granule size distribution and a smaller number of very large polygonal-shaped granules (2.1% for hybrid 37Y14 planted on June 11 and 1.7% for inbred B73 planted on June 10) (**Figures 4C** and **5C**). The morphology of starch granules suggested that starch granules isolated from corn planted early had more time to develop and grow to larger sizes than the granules from corn planted on a late date. In addition, starch from corn planted on early contained larger number of granules with dimple-like indentations on the surface (**Figures 4D** and **5D**). These indentations on the surface of the granules originated from protein bodies surrounding starch granules (*29, 30*). The dimple-like indentations on the surface of starch granules resulted from the large content of starch in corn kernels planted early (71.9% kernel db for hybrid 37Y14 on 16 April and 70.5% for B73 inbred on 6 May) where starch granules were tightly packed in the limited space of endosperm. Kernels of corn planted in June contained less starch (68.5% for hybrid 37Y14 on 16 April and 67% for B73 inbred on 6 May). Thus, starch granules were loosely

packed in the endosperm and fewer starch granules displayed dimple-like indentations (**Figures 4F** and **5F**). It was not possible, however, to determine with certainty if delay in planting date had any effect on a number of pinholes on the granule surface, which originate from endogenous amylase hydrolysis of starch.

The apparent amylose content of starch decreased with delayed planting date of corn (**Figure 6**). In 2008, the apparent amylose content of hybrid 37Y14 decreased from 27.4% (16 April) to 26.6% (11 June) ($p<0.05$). In 2009, the amylose content of hybrid 37Y14 starch decreased from 27.4% (4 April) to 25.6% (15 May) ($p=0.07$) and then increased to 26.0% (29 May). Similar results were observed for B73 inbred starch that displayed a decrease in the amylose contents with late planting dates (10 June 2008 and 20 May 2009).

Kernels of corn planted on late dates (10 and 11 June of 2008) contained smaller starch granules and less amylose, which is in agreement with previous reports that small starch granules contain less amylose (*32-34*). Li et al (*15*) found that the amylose content of starch granules increased with corn kernel maturation because amylose was synthesized at a faster rate in later stages of development. Thus, the reduction in the granule size and amylose content implied that with late planting dates some starch granules might not be fully developed (mature) before harvesting.

Branch-chain length distributions of isolated amylopectin obtained from kernels with different planting dates are shown in **Table 3**. The proportion of short branch-chains of DP 6-12 increased with the delay in planting date of hybrid 37Y14 in 2008 (from 24.8% on 30 April to 26.1% on 11 June). It increased even more in 2009, from 21.7% (17 April) to 25.7% (29 May). The percentage of long branch-chains of DP≥37 decreased with delayed planting date of hybrid 37Y14 in 2008 (from 13.8% to 11.1%) and 2009 (from 9.9% to 8.3%). It is

known that the amylopectin branch-chain length of corn starch changes with growing temperature (*9*) and kernel development stages (*15*), which could be attributed to the different expression levels of starch branching enzymes I and IIb (*35*). Corn planted on late dates has shorter effective growing and grain-filling periods, which could affect synthesis of amylopectin. The amylopectin of corn planted on the earliest dates showed a very large proportion of short chains of DP 6-12 (26.4% on 16 April 2008, and 25.7% on 4 April 2009) and a small proportion of chains of DP≥ 37 (10.4% and 8.4%, respectively). The molecular weight of these samples was slightly reduced compared with that of samples planted on later dates (data not shown), indicating a possible degradation of amylopectin molecules by β-amylase. Unusually high levels of the rainfall in October of 2008 and 2009 (**Table 2**) might have stimulated the activity of endogenous amylases in mature corn kernels.

The amylopectin branch-chain length distribution of B73 starch was not significantly affected by postponed planting dates of corn and varied slightly in both years. The effect of planting dates on the amylopectin branch-chain length also varied with different varieties of sweet potato planted on the same location (*17*). Thus, the genetic background of plants might have an important role in determining the impact of delayed planting on the amylopectin structure. Another factor that might have contributed to the weak response of B73 corn starch to planting dates could be the location where it was grown. The corn line 37Y14 was grown in the north-central and northeast region of Iowa, which are more sensitive to delayed planting date than the central Iowa where B73 was grown (*36*).

Thermal properties of the starch with different planting dates are shown in **Table 4**. The onset and peak gelatinization temperature of 37Y14 hybrid and B73 inbred corn gradually decreased when planting date was delayed from mid-April and early May to late

May and June, with an exception of 37Y14 hybrid planted on 4 April and B73 inbred planted on 11 May of 2009. This can be attributed to the decreased amount of long chains of DP≥ 37 and larger proportion of short amylopectin chains of DP 6-12 for 37Y14 starch isolated from kernels planted on later dates. Short amylopectin chains cannot form stabile double helices, contribute to destabilization of starch crystallites, and decrease the gelatinization temperature of starch (*22*, *27, 37*). The completion gelatinization temperature of starch was reduced with the delay in the planting date in 2008, but did not significantly change in 2009.

The pasting temperature of 37Y14 starch did not significantly change with plantings between 16 April-25 May in 2008 (70.2°C -70.4°C) and 4 April-15 May in 2009 (69.8°C-70.4°C), but was decreased for the latest planting dates (69.1°C for 11 June of 2008 and 29 May of 2009) (**Table 5**). The pasting temperature of B73 starch was also decreased with late planting dates in 2009, but did not show any defined trend of change in 2008. The decline in the pasting temperature of starch planted in late May and June was due to the low apparent amylose content of starch. Amylose restricts swelling of starch granules and consequently, starches with large amylose contents display high pasting temperatures and low peak viscosity values (*27*).

The peak viscosity of 37Y14 starch did not significantly change with planting dates between 30 April to 25 May in 2008 (174.3 RVU to 177.3 RVU), but was decreased for the earlier (155.8 RVU, 16 April) and later planting dates (161.9 RVU, 11 June) (**Table 5**). In 2009 the largest peak viscosity of starch was obtained with planting on 17 April (160 RVU), and it slightly declined with earlier (155.8 RVU, 4 April) and later planting dates (156.4 RVU, 29 May). It is known that amylopectin is primarily responsible for granule swelling (*38*) and that long amylopectin chains contribute to starch paste viscosity (*26*). Thus, large

proportions of short amylopectin chains of DP 6-12 and small proportions of longer chains contributed to the low peak viscosity values of samples planted on earliest and latest dates. B73 starch did not show any defined trend of change in the peak viscosity with the planting dates, possibly due to the limited change in the amylopectin branch-chain length distribution.

In summary, the grain yield and starch content of kernels decreased with the delay in planting of corn hybrids in 2007 and 2008; possibly due to shorter growing season and grain-filling period that resulted in less than optimum starch deposition in the kernels. In 2009, however, late planting dates had a moderate effect on the corn grain yield and starch content of kernels. This may partially be due to the cooler growing season in 2009. The protein content of kernels was not significantly affected by planting dates of corn. Corn starches showed decreased granule size and contents of amylose with delayed planting dates. Effect on the amylopectin branch-chain length varied with corn varieties: B73 inbred corn was less sensitive to the planting date and showed little change, whereas 37Y14 hybrid displayed a decrease in the proportion of long, but an increase in the proportion of short amylopectin branch-chains with the delay in planting date. Samples of 37Y14 hybrid planted very early (early April) contained a large proportion of short amylopectin chains of DP 6-12, but large amylose contents of starch, possibly due to the slight endogenous amylase hydrolysis. Thermal and pasting properties of starch were in a good agreement with amylose contents and amylopectin branch-chain length distributions of starches and varied with planting dates. The results of this study indicated that the structure and properties of starch were affected by planting dates, but the magnitude of the change varied with corn varieties and/or their planting locations. While these changes may impact quality and yield of starch, they might

not be of sufficient magnitude to impose major problems in processing of products containing starch.

REFERENCE CITED

1. Sacks, W. J.; Deryng, D.; Foley, J. A.; Ramankutty, N. Crop planting dates: an analysis of global patterns. *Global Ecol. Biogeogr*. **2010**, 19, 607-620.
2. Farnham, Dale. Corn Planting Guide, PM1885, 2001. Iowa State Extension Online Store. http://www.extension.iastate.edu/Publications/PM1885.pdf (accessed December 08, 2010)
3. Sindelar, A. J; Roozeboom, K. L.; Gordon, W. B.; Heer, W. F. Corn response to delayed planting in the Central Great Plains. *Agron. J.* **2010**, 102, 530-536.
4. Lauer, J. G.; Carter, P. R.; Wood, T. M.; Diezel, G.; Wiersma, D. W.; Rand, R. E.; Mlynarek, M. J. Corn hybrid response to planting date in the Northern Corn Belt. *Agron. J.* **1999**, 91, 834-839.
5. Swanson, S. P.; Wilhelm, W. W. Planting date and residue effects on growth, partitioning, and yield of corn. *Agron. J*. **1996**, 88, 205-210.
6. Abendroth, L.J., R.W. Elmore, M.J. Boyer, and S.K. Marlay. 2011. Corn growth and development. PMR 1009. Iowa State University Extension, Ames, IA.
7. Cirilo, A. G.; Andrade, F. H. Sowing date and corn productivity: I. Crop growth and dry matter partitioning. *Crop Sci.* **1994**, 34, 1039-1043.
8. Nielsen, R. L.; Thomison, P. R.; Brown, G. A.; Halter, A. L.; Wells, J.; Wuethrich, K. L. Delayed planting effects on flowering and grain maturation of dent corn. *Agron. J.* **2002**, 94, 549-558.

9. Lu, T.; Jane, J.; Keeling, P. L.; Singletary, G. W. Corn fine structures affected by ear developmental temperature. *Carbohydr. Res.* **1996**, 282, 157-170.

10. Asaoka, M.; Okuno, K.; Sugimoto, Y.; Kawakami, K.; Fuwa, H. Effect of environmental temperature during development of rice plants on some properties of endosperm starch. *Starch/Stärke*. **1984**, 36, 189-193.

11. Asaoka, M.; Okuno, K.; Fuwa, H. Effect of environmental temperature at the milky stage on amylose content and fine structure of amylopectin of waxy and nonwaxy endosperm starches of rice (*Oryza sativa* L.). *Agr. Biol. Chem.* **1985**, 49, 373-379.

12. Asaoka, M.; Okuno, K.; Konishi, Y.; Fuwa, H. The effects of endosperm mutations and environmental temperature during development on the distribution of molecular weight of amylose in rice endosperms. *Agr. Biol. Chem.* **1987**, 51, 3451- 3453.

13. Shi, Y.-C.; Seib, P.A.; Bernardin, J.E. Effects of temperature during grain filling on starches from six wheat cultivars. *Cereal Chem.* **1994**, 71, 369–383.

14. Tester, R.F; South, J.B.; Morrison, W.R.; Ellis, R.P.; Piggott, J.R.; Batts, G.R.; Wheeler, T.R.; Morison, J.I.L.; Hadley, P.; Ledward, D.A. Effects of elevated growth temperature and carbon dioxide levels on some physicochemical properties of wheat starch. *J. Cereal Sci.* **1995**, 22, 65–71.

15. Li, L.; Blanco, M.; Jane, J. Physicochemical properties of endosperm and pericarp starches during corn development. *Carbohydr. Polym.* **2007**, 67, 630-639.

16. Williams, V. R.; Wu, W. T.; Ysai, H. Y.; Bates, H. G. Varietal differences in amylose content of rice starch. *J. Agric. Food Chem*. **1958**, 6, 47-48

17. Campbell, M. R.; Pollak, L. M.; White, P. J. Effect of planting date on corn starch thermal properties, *Cereal Chem.* **1994,** 71, 556–559.

18. Noda, T.; Takahata, Y.; Sato, T.; Ikoma, H.; Mochida, H. Combined effects of planting and harvesting dates on starch properties of sweet potato roots. *Carbohydr. Polym.* **1997**, 33**,** 169–176.

19. Helm, J.L.; Fergesan, V.L.; Zuber, M.S. Effect of planting date on high amylose corn (*Zea Mays* L.). *Agron. J.* **1968**, 60, 530–531

20. Singh, S.; Gupta, A. K.; Gupta, S. K.; Kaur, N. Effect of sowing time on protein quality and starch pasting characteristics in wheat (*Triticum aestivum* L.) genotypes grown under irrigated and rain-fed conditions. *Food Chem.* **2010**, 122, 559-565.

21. AACC. Approved Methods of the AACC, 10th ed.; American Association of Cereal Chemists: St. Paul, MN, 2000.

22. Srichuwong, S.; Gutesa, J.; Blanco, M.; Duvick, S. A.; Gardner, C.; Jane, J. Characterization of corn grains for dry-grind ethanol production. *J. ASTM Int*. **2009**, 7(2), 1-10.

23. Jane, J.; Kasemsuwan, T.; Leas, S.; Zobel, H.; Robyt, J. F. Anthology of starch granule morphology by scanning electron microscopy. *Starch/Stärke*, **1994**, 46, 121-129.

24. Kasemsuwan, T., Jane, J., Schnable, P., Stinard, P., and Robertson, D. 1995. Characterization of dominant mutant amylose-extender (Ae-5180) corn starch. *Cereal Chem*., **1995**, 72, 457-464.

25. Schoch, T. J. Fractionation of starch by selective precipitation with butanol. *J. Am. Chem. Soc*. **1942**, 64, 2957-2961.

26. Jane, J.; Chen, J. F. Effect of amylose molecular-size and amylopectin branch chain-length on paste properties of starch. *Cereal Chem.* **1992**, 69, 60-65.

27. Jane, J.; Chen Y.Y.; Lee, L.F.; McPherson, A.E.; Wong, K.S.; Radosavljevic, M.; Kasemsuwan, T. Effects of Amylopectin Branch Chain Length and Amylose Content on the Gelatinization and Pasting Properties of Starch. *Cereal Chem.* **1999**, 76, 629-637.

28. U.S. Department of Agriculture's National Agricultural Statistics Service (NASS), 2010. Newsroom. http://www.nass.usda.gov/Newsroom/2010/01_12_2010.asp (accessed March 10, 2011).

29. Hasjim, J.; Srichuwong, S.; Scott, P. M.; Jane, J. Kernel composition, starch structure, and enzyme digestibility of *opaque-2* corn and quality protein corn. *J. Agric. Food Chem.* 2009, 57, 2049-2055.

30. Wassom, C. E.; Hooseney, R.C. Selection of high lysine corn using a scanning electron microscope. *Crop Sci.* **1973**, 13, 462-463.

31. Jane, J. Current understanding on starch granule structures. *J. Appl. Glycosci*. **2006**, 53, 205-213.

32. Yoshida, H.; Nozaki, K.; Hanashiro, I.; Yagi, F.; Ito, H.; Honma, M. Structure and physicochemical properties of starches from kidney bean seeds at immature, premature and mature stages of development. *Carbohydr. Res.* **2003**, 338**,** 463–469

33. Jane, J.; Shen, J.J. Internal structure of the potato starch granule revealed by chemical gelatinization, *Carbohydr. Res.* **1993**, 247, 279–290

34. Pan, D. D.; Jane, J. Internal structure of normal corn starch granules revealed by chemical surface gelatinization. *Biomacromolecules* **2000**, 1, 126–132.

35. Gao, M.; Fisher, D.K.; Kim, K; Shannon, .J.C.; Guiltinan, M.J. Evolutionary conservation and expression patterns of corn starch branching enzyme I and IIb genes suggests isoforms specialization, *Plant Molecular Biology* **30** (1996), pp. 1223–1232.
36. Abendroth, L.; Elmore, R. Updated planting date recommendations for Iowa, 2010. Iowa State University Agronomy Extension. http://www.agronext.iastate.edu/corn/production/management/planting/recommendations.html (accessed January 27, 2011)
37. Srichuwong, S.; Sunarti, T.C.; Mishima, T.; Isono, N.; Hisamatsu, M. Starches from different botanical sources I: Contribution of amylopectin fine structure to thermal properties and enzyme digestibility. *Carbohydr. Polym*. **2005**, 60, 529-538.
38. Tester, R. F.; Morrison, W. R. Swelling and gelatinization of cereal starches. Effect of amylopectin, amylose, and lipids. *Cereal chem*. **1990**, 67 (6), 551-557
39. Perera, C.; Lu, Z.; Sell, J.; Jane, J. Comparison of physico-chemical properties and structures of sugary-2 corn starch with normal and waxy cultivars. *Cereal Chem.* **2001**, 78, 249-256.

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Table 1. Summary of corn lines, planting and harvesting dates

Corn line	Growing season	Planting locations	Planting dates	Harvest dates
34A20	2007	Ames (central Iowa)	2-May, 11-May, 21-May, 29-May	2-Nov
37Y14	2008	Nashua (northeast Iowa)	16-Apr, 30-Apr, 13-May, 25-May, 11-June	18-Oct
37Y14	2009	Kanawha (north central Iowa)	4-Apr, 17-Apr, 4-May, 15-May, 29-May	12-Nov
B73	2008	Ames (central Iowa)	6-May, 15-May, 10-June	65 DAP[1]
B73	2009	Ames (central Iowa)	4-May, 11-May, 20-May	65 DAP

[1]DAP- days after pollination

Table 2. Average air temperature and monthly precipitation at each research location for May through October of 2007-2009, and the 30 year average.

	Average air temperature (°C)								Average Monthly Total Precipitation (mm)							
	Ames				Nashua		Kanawha		Ames				Nashua		Kanawha	
	2007	2008	2009	30-yr	2008	30-yr	2009	30-yr	2007	2008	2009	30-yr	2008	30-yr	2009	30-yr
May	18.3	15.6	15.6	16.4	13.6	15.6	14.9	15.0	146.3	215.6	102.1	115.6	110.0	109.2	129.6	106.7
June	21.7	21.1	21.1	21.4	20.2	20.6	19.5	20.6	46.2	271.3	104.4	118.3	238.3	127.0	80.5	127.0
July	23.6	23.3	20.6	23.4	22.4	22.8	19.3	22.8	66.8	234.2	69.9	117.6	151.4	114.3	114.3	109.2
Aug	23.8	21.1	21.1	22.1	20.5	21.7	19.3	21.1	168.8	53.3	122.9	119.2	36.1	106.7	34.8	101.6
Sep	18.3	17.8	17.8	18.2	17.2	16.7	17.4	16.7	35.4	78	24.4	81.7	62.7	86.4	61.0	78.7
Oct	12.8	11.7	7.8	11.2	9.8	10.6	6.1	10.0	98.0	91.7	186.2	61.7	67.3	58.4	180.8	55.9

Table 3. Amylopectin branch-chain length distribution of starch isolated from 37Y14 hybrid and B73 inbred corn planted on different dates

Corn line/year (growing location)	Planting date	Percentage distribution				Average chain length
		DP 6-12	DP 13-24	DP 25-36	DP ≥ 37	
37Y14/2008 (Nashua)	16-April	26.4[a*]	52.2[a]	11.0[a]	10.4[b]	19.8[a]
	30-April	24.8[b]	50.2[a]	11.2[a]	13.8[a]	21.1[a]
	13-May	25.2[ab]	50.9[a]	11.2[a]	12.7[ab]	20.5[a]
	25-May	24.9[b]	51.0[a]	11.6[a]	12.5[ab]	20.7[a]
	11-June	26.5[a]	51.8[a]	10.8[a]	10.9[b]	20.0[a]
	Standard error	0.5	0.6	0.2	0.6	0.4
37Y14/2009 (Kanawha)	4-April	25.7[a]	55.1[a]	10.9[a]	8.3[bc]	20.0[a]
	17-April	21.7[b]	56.6[a]	11.8[a]	9.9[ab]	20.1[a]
	4-May	21.8[b]	56.4[a]	11.8[a]	10.0[a]	20.6[a]
	15-May	22.4[b]	56.2[a]	11.7[a]	9.7[a]	20.5[a]
	29-May	25.7[a]	54.9[a]	11.1[a]	8.3[c]	20.3[a]
	Standard error	0.5	0.7	0.5	0.2	0.4
B73/2008 (Ames)	6-May	26.2[a*]	51.0[a]	11.5[a]	11.3[a]	20.2[a]
	15-May	25.1[a]	51.2[a]	11.2[a]	12.5[a]	20.6[a]
	10-June	25.2[a]	51.9[a]	11.2[a]	11.7[a]	20.3[a]
	Standard error	0.6	0.7	0.3	0.6	0.3
B73/2009 (Ames)	4-May	22.5[a]	56.5[b]	11.4[a]	9.6[b]	20.0[a]
	11-May	22.0[a]	57.7[a]	11.1[b]	9.2[b]	19.8[a]
	20-May	22.3[a]	56.2[b]	11.6[c]	9.9[a]	20.0[a]
	Standard error	0.2	0.2	0.1	0.1	0.1

Means for each corn variety and growing season in the same column with the same letter subscript are not significantly different

Table 4. Thermal properties of starch isolated from 37Y14 hybrid and B73 inbred corn planted on different dates

Corn line/year (growing location)	Planting date	Thermal properties			
		To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
37Y14/2008 (Nashua)	16-April	62.8[a]	68.1[a]	72.3[a]	12.0[a]
	30-April	62.5[a]	67.8[a]	72.2[a]	11.5[a]
	13-May	61.3[b]	66.8[b]	72.0[a]	11.4[a]
	25-May	60.9[b]	67.5[ab]	72.2[a]	11.9[a]
	11-June	60.7[c]	65.5[c]	71.1[b]	12.0[a]
	Standard error	0.1	0.2	0.2	0.2
37Y14/2009 (Kanawha)	4-April	63.3[b]	68.8[a]	74.7[a]	11.8[a]
	17-April	65.6[a]	70.2[a]	74.9[a]	11.9[a]
	4-May	64.2[ab]	70.0[a]	76.3[a]	12.1[a]
	15-May	63.6[b]	69.0[a]	75.1[a]	12.0[a]
	29-May	63.7[b]	68.8[a]	75.4[a]	12.4[a]
	Standard error	0.2	0.3	0.4	0.3
B73/ 2008 (Ames)	6-May	62.5[b]	69.8[a]	74.7[a]	11.0[a]
	15-May	62.3[a]	68.7[b]	73.6[b]	11.3[a]
	10-June	61.8[ab]	66.9[c]	72.3[c]	11.0[a]
	Standard error	0.4	0.1	0.2	0.1
B73/ 2009 (Ames)	4-May	67.9[a]	72.8[a]	78.2[a]	12.7[a]
	11-May	68.0[a]	73.0[a]	78.3[a]	13.1[a]
	20-May	64.4[b]	69.8[a]	75.7[b]	11.7[a]
	Standard error	0.6	0.8	0.7	0.4

Means for each corn variety and growing season in the same column with the same letter subscript are not significantly different

Table 5. Pasting properties of starch isolated from 37Y14 and B73 corn lines planted on different dates

Corn line/ year (growing location)	Planting date	Pasting temperature (°C)	Peak viscosity (RVU)	Breakdown viscosity (RVU)	Setback viscosity (RVU)	Final viscosity (RVU)
37Y14/ 2008 (Nashua)	16-April	70.2^{a}	155.8^{b}	64.7^{a}	91.5^{a}	182.5^{a}
	30-April	70.2^{a}	174.3^{a}	75.9^{a}	96.0^{a}	194.5^{a}
	13-May	70.2^{a}	177.3^{a}	71.4^{a}	91.3^{a}	197.2^{a}
	25-May	70.4^{a}	174.3^{a}	66.4^{a}	92.7^{a}	200.6^{a}
	11-June	69.1^{a}	161.9^{b}	69.2^{a}	91.8^{a}	187.4^{a}
	Standard error	0.8	5.2	3.0	1.5	4.2
37Y14/ 2009 (Kanawha)	4-April	70.4^{a}	155.8^{a}	56.2^{a}	77.6^{a}	176.1^{a}
	17-April	69.1^{b}	160.0^{a}	59.8^{a}	78.4^{a}	178.7^{a}
	4-May	70.0^{ab}	155.3^{a}	58.1^{a}	78.8^{a}	176.1^{a}
	15-May	70.4^{a}	157.9^{a}	61.3^{a}	75.7^{a}	172.2^{a}
	29-May	69.8^{ab}	156.4^{a}	60.1^{a}	81.2^{a}	177.5^{a}
	Standard error	0.2	2.4	1.7	2.0	3.8
B73/ 2008 (Ames)	6-May	72.0^{b}	162.2^{c}	57.3^{b}	99.6^{a}	204.4^{b}
	15-May	73.6^{a}	166.1^{b}	54.7^{ab}	100.4^{a}	211.8^{a}
	10-June	73.1^{a}	171.1^{a}	58.3^{a}	98.7^{a}	211.4^{a}
	Standard error	0.1	0.2	0.5	0.6	0.5
B73/ 2009 (Ames)	4-May	72.7^{a}	135.1^{b}	35.5^{b}	92.6^{a}	192.3^{a}
	11-May	72.7^{a}	130.9^{b}	43.7^{b}	75.5^{a}	162.7^{b}
	20-May	71.0^{b}	163.8^{a}	62.9^{a}	88.8^{a}	189.7^{a}
	Standard error	0.2	1.8	3.0	4.0	3.9

Means for each corn variety and growing season in the same column with the same letter subscript are not significantly different

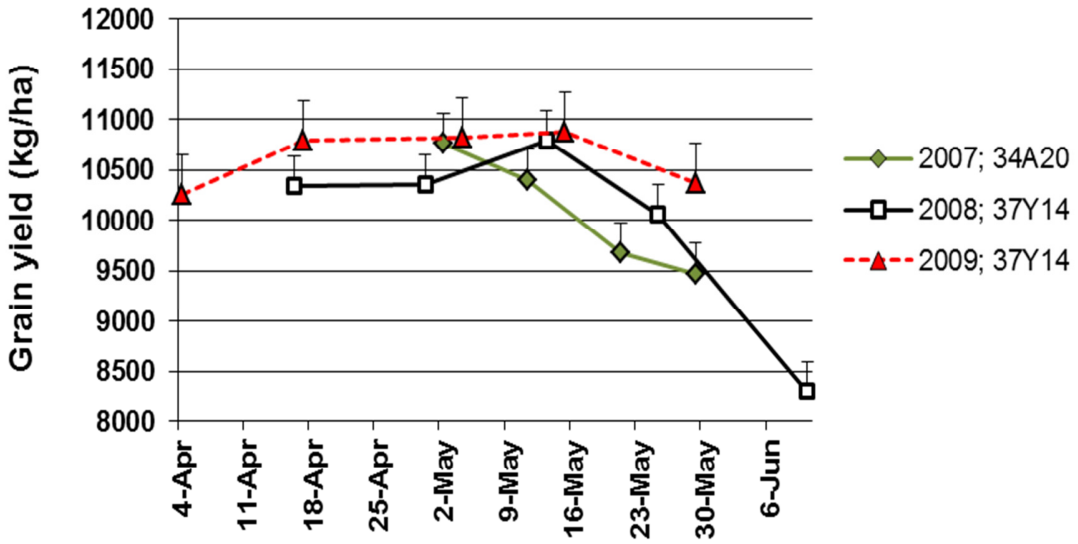

Figure 1. Grain yield (15% moisture basis) of 34A20 and 37Y14 corn hybrid kernels planted on different dates. Data points are means of four replications + standard errors.

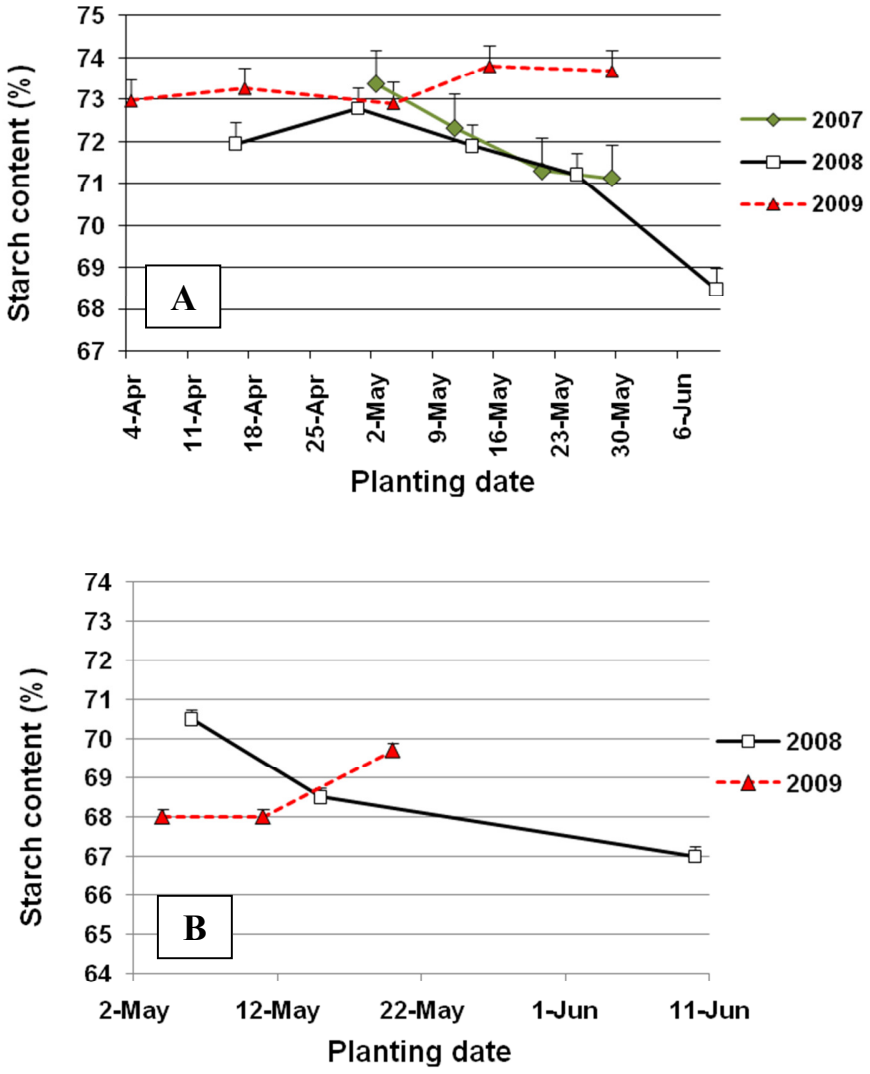

Figure 2. Starch content (% db) of corn kernels planted on different dates. A: 37Y14 hybrid, B: B73 inbred corn. Data points are means of four replications + standard errors.

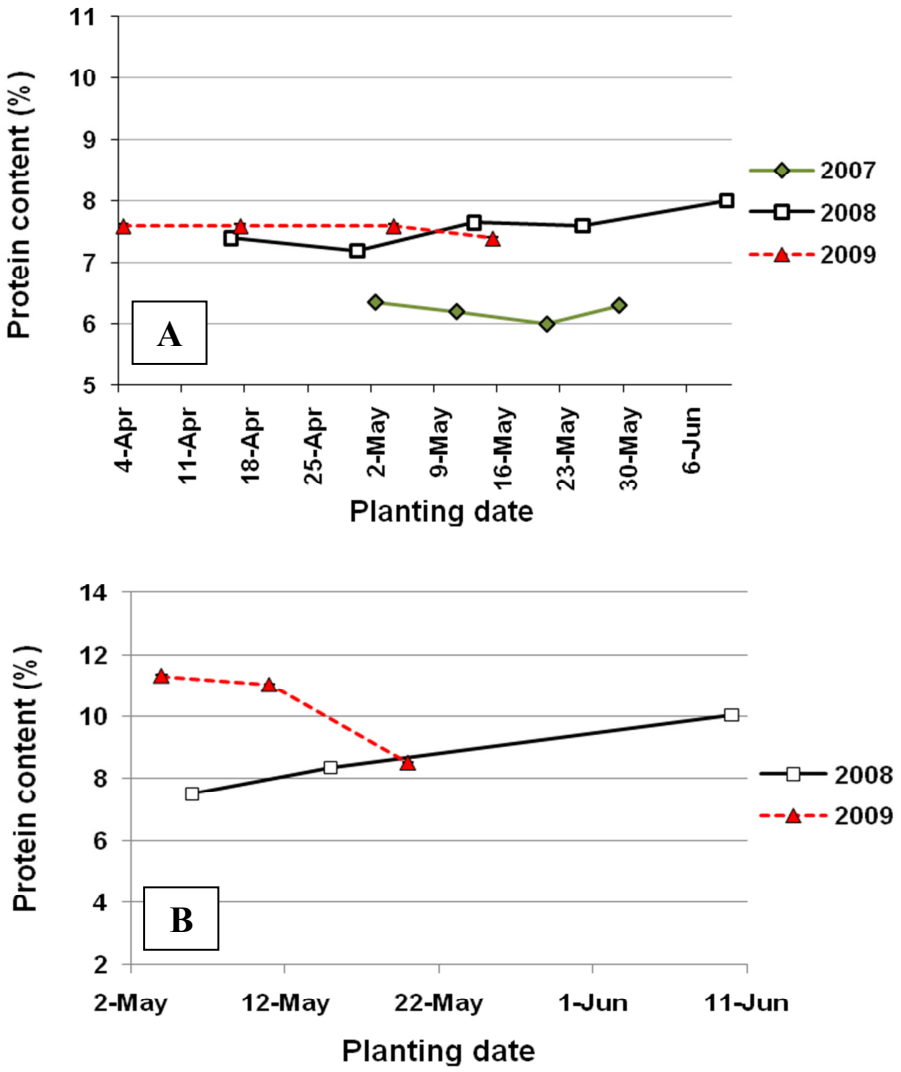

Figure 3. Protein content (% db) of corn kernels planted on different dates. A: 37Y14 hybrid, B: B73 inbred corn. Data points are means of four replications + standard errors. The protein content of 37Y14 kernels planted on 29 May 2009 was not determined because of the insufficient sample quantity.

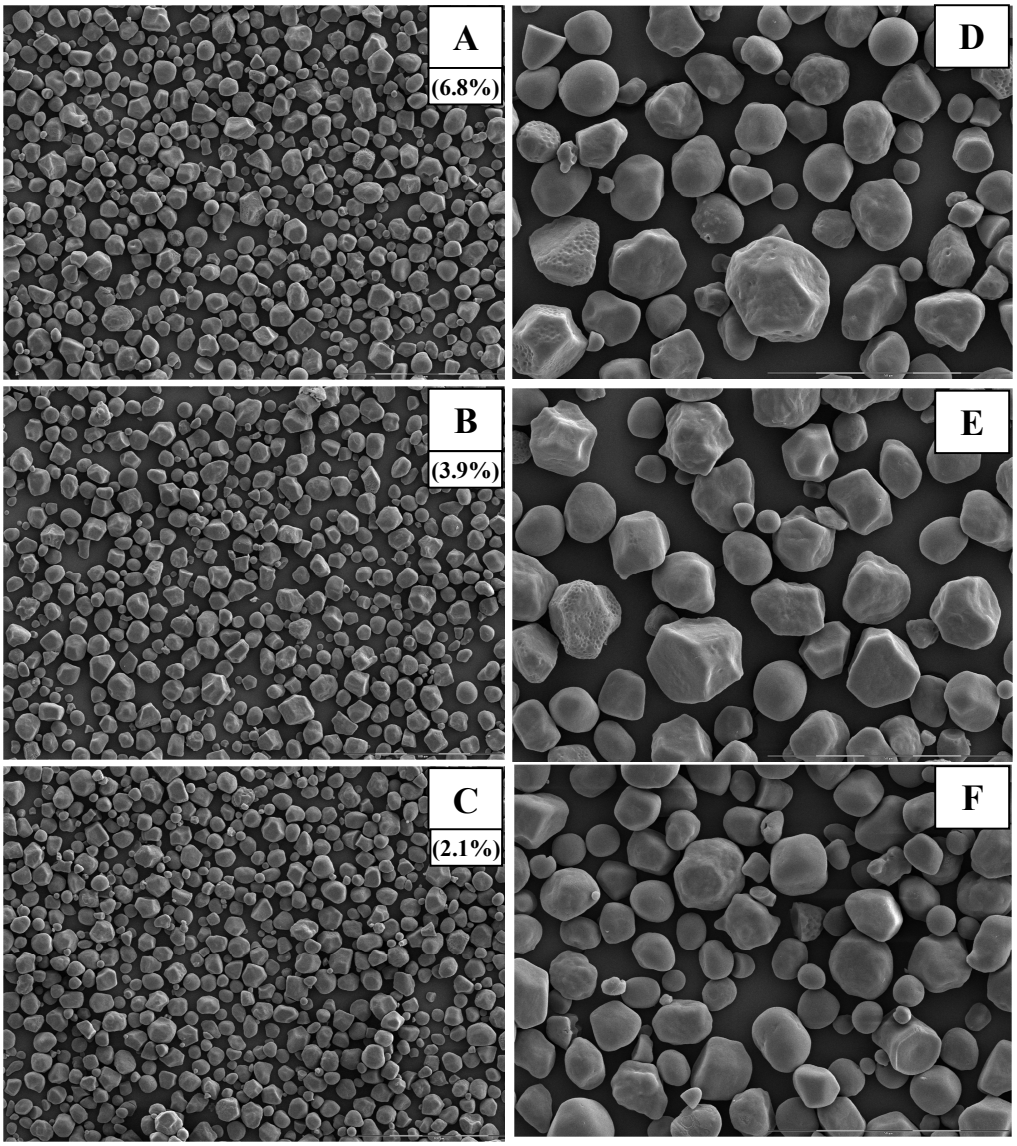

Figure 4. Scanning electron micrographs of starches isolated from 37Y14 corn planted on different dates in 2008. **A**- 16 April, 500X; **B**- 30 April, 500X; **C**- 10 June, 500X; **D**- 16 April, 1500X; **E**-30 April, 1500X; **F**-11 June, 1500X. Numbers in parentheses are the percentages of granules with size ≥ 20 μm.

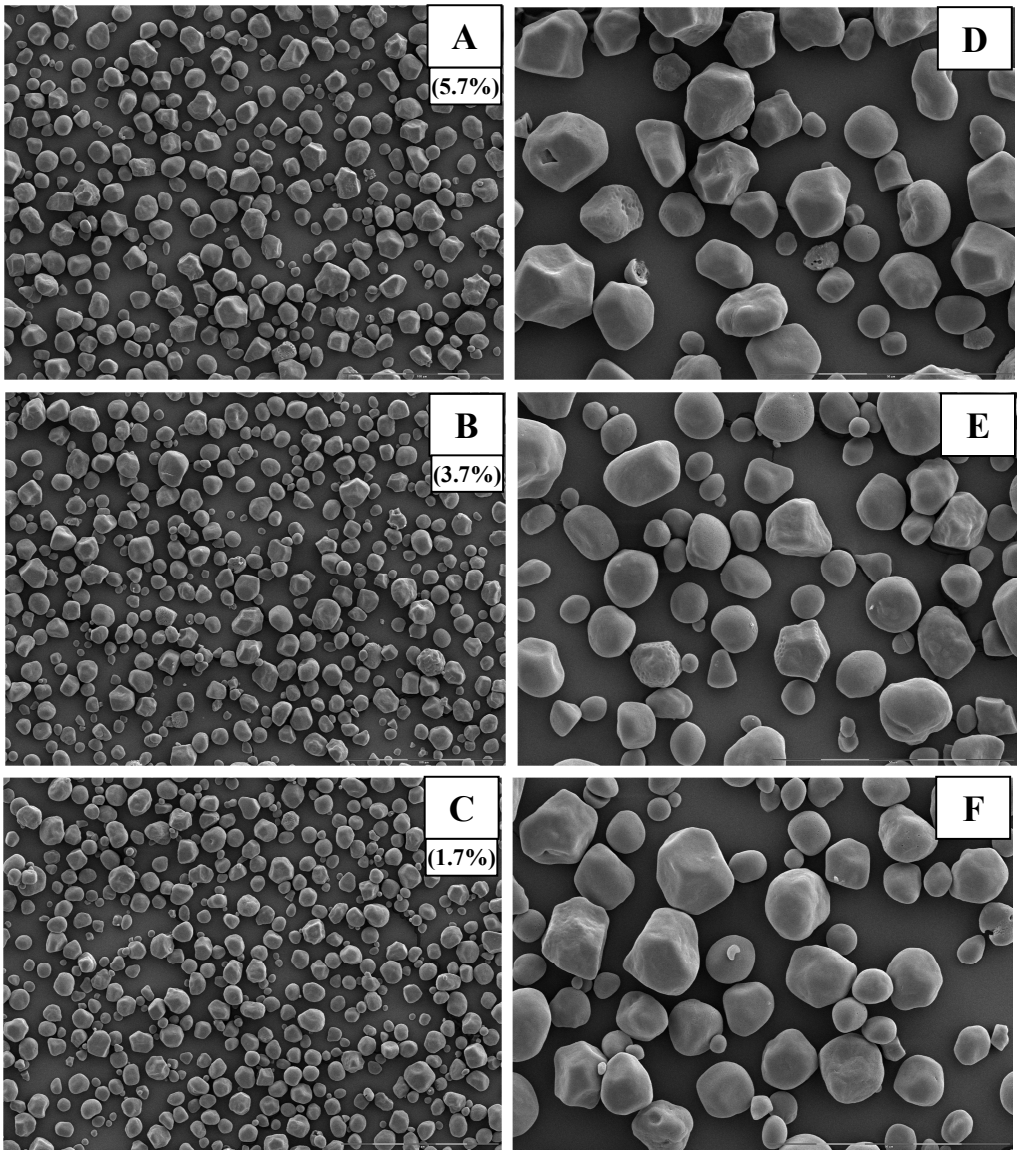

Figure 5. Scanning electron micrographs of starch isolated from B73 corn planted on different dates in 2008. **A**- 6 May, 500X; **B**- 15 May, 500X; **C**- 11 June, 500X; **D**- 6 May, 1500X; **E**-15 May, 1500X; **F**-10 June, 1500X. Numbers in parentheses are the percentages of granules with size ≥ 20 μm.

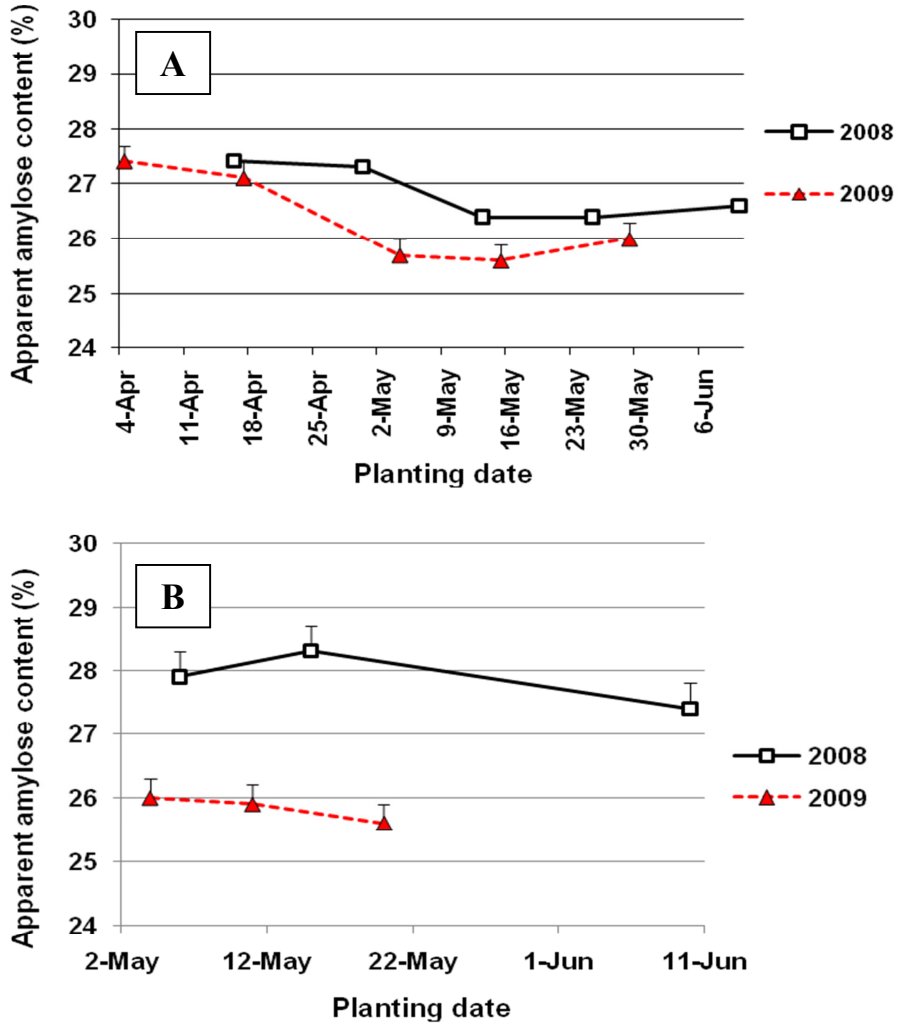

Figure 6. Apparent amylose (% db) of starch isolated from corn kernels planted on different dates. A: 37Y14 hybrid, B: B73 inbred. Data points are means of four replications + standard errors.

CHAPTER 5. EFFECT OF PLANTING DATE ON PROPERTIES OF CORN. PART II: ENZYME HYDROLYSIS OF STARCH AND ETHANOL YIELD OF GROUND KERNELS

A paper prepared for submission to the Journal of Agricultural and Food Chemistry

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ABSTRACT

The objective of this study was to understand how planting date of corn impacts the ethanol yield produced from ground corn kernels. Kernels of three corn germplasm sources grown at selected locations in Iowa in three consecutive years with planting dates between 4 April and 11 June as the treatments were subjected to the cold fermentation process. The ethanol yield calculated on the basis of dry kernel mass was not significantly affected by dates of planting corn (p>0.05). Per unit of corn planting area, the ethanol yield was significantly affected by the planting date of corn (p<0.05). Compared with planting dates between early April and mid-May (3998-4413 L/ha), plantings made in late May and June resulted in significantly reduced ethanol yields (3396–4108 L/ha).

KEYWORDS: planting date, corn kernel, enzyme digestibility of starch, ethanol yield

INTRODUCTION

The depletion of fossil reserves and escalation in fuel prices has stimulated development of alternative fuels to meet the global energy demand. Currently, ethanol produced from corn kernels is the most prevalent alternative fuel in the USA with the annual production of 50.1 billion liters in 2010 (*1*).

In the conventional dry-grind ethanol process, ground corn kernels (containing up to 75% starch db) are suspended in water and heated to gelatinize and make starch more susceptible to enzyme hydrolysis. In the subsequent steps, starch is hydrolyzed to glucose using thermostabile α-amylase and amyloglucosidase, and glucose is utilized by yeast (*Saccharomyces cerevisiae*) to produce ethanol. More advanced technologies, such as producing ethanol from raw starch, have been introduced in the recent years (*2, 3*). This "cold fermentation" process does not necessitate heating of corn slurry because it utilizes enzymes being able to hydrolyze raw starch. Such an advanced technology consumes 10-20% less energy to produce ethanol than the conventional process (*2, 4*). The cold fermentation process also produces a higher ethanol yield than the conventional process because starch can be fully hydrolyzed to glucose without formation of enzyme-resistant retrograded starch and amylose-lipid complex. Even though the ethanol production using cold fermentation process has improved the efficiency, there is still room for further improvement, such as selection of desirable hybrids, date of planting, kernel drying and storage conditions, to maximize the ethanol yield.

In the conventional dry-grind ethanol process, large ethanol yields have been produced from kernels containing large starch and small protein and lipid contents (*5, 6*). Additionally, Srichwong et al. (2009) reported that corn kernels containing starch with larger

proportions of short amylopectin chains were more promptly hydrolyzed to glucose during fermentation (*5*). Thus, the kernel composition and starch structure were important factors determining the ethanol yield.

The part I of our study has shown that the composition and starch structure of kernels changed with different dates of corn planting in Iowa (*7*). Early planting dates (early April) produced kernels with large starch contents, large amylose contents of starch, and amylopectin with large proportions of short chains. In contrast, corn kernels planted in late May and June contained small starch contents, small amylose contents of starch, and large proportions of short amylopectin chains. It is not known how these changes would affect the enzyme hydrolysis of starch and the ethanol production yield. Thus, kernels of three corn germplasm sources grown in Iowa in three consecutive years with planting dates between 4 April and 11 June were subjected to the cold fermentation process to determine if the date of planting corn could have any impact on the ethanol yield.

MATERIALS AND METHODS

Materials. Three corn germplasm sources, B73 (a public inbred), Pioneer 37Y14, and Pioneer 34A20 (commercial hybrids, Pioneer Hi-Bred International, Inc., Johnston, IA) with 115, 98, and 109 day relative maturities, respectively, were used in this study. Multi-year (2007, 2008, and 2009) and multi-location (Iowa State University Research and Demonstration Farms near Kanawha (north central Iowa), Nashua (northeast Iowa), and Ames (central Iowa)) field trials were conducted for a total of five location-years (Table 1). B73 inbred was planted in a completely random design, whereas hybrids 37Y14 and 34A20 were planted in a randomized complete block design with four replications and three to five

planting dates as the treatment. Experimental units measured approximately 15 m (length) by 3 m (width), with 76-cm row spacing. Corn plants were naturally open pollinated, harvested at physiological maturity (after black layer formation), dried and shelled by hand to obtain about 1 kg of kernels for analysis.

Glucose assay kit (GOPOD kit) was purchased from Megazyme International (Wicklow, Ireland). Raw-starch hydrolysing enzyme (Novozyme 50009) is a product of Novozyme (Franklinton, NC), IsoStab^{TM} of BetaTech hop products (Washington, DC), and Lactrol® of PhibroChem (Ridgefield Park, NJ). Ethanol Red ™ dry yeast (>2 X 10^9 living cells/g) was contributed by Lesaffre yeast corporation (Milwaukee, WI). All chemicals used in the study were purchased from Fisher Chemicals (Waltham, MA) and Sigma-Aldrich (St. Louis, MO) and used as received.

Dry-grind Ethanol Production Using Cold Fermentation Process

Corn kernels (35 g db) were ground to pass a 0.5mm screen and suspended in an aqueous solution to make 100 g of mash. The aqueous solution contained liquid urea (0.03% w/w, mash) as a nitrogen source for yeast, Lactrol® (2 ppm) and IsoStab^{TM} (40 ppm) as antimicrobial reagents, and acetate buffer (200 mM, pH 4.2, 5ml) for pH adjustment. The mash was mixed using a magnetic stirrer for 30 min, and dry yeast (0.5g) and raw-starch hydrolyzing enzyme (0.16 mL/100g mash) were added to the slurry. The fermentation was carried out at 27˚C and 160 rpm shaking speed for 4 days. Aliquots (4 ml) were removed from the fermentation broth after 96h and centrifuged at 7233 x g for 10 min to remove the solid. The supernatant was filtered through a nylon membrane filter (0.45 μm) and analyzed to obtain ethanol yields. The analysis was performed using a Waters High Performance Liquid Chromatography system (Millipore Corporation, Milford, MA, USA) equipped with a

Waters Model 401 refractive index detector. An Aminex HPX-87H anion-exchange column (Bio-Rad, Richmond, CA, USA) was used for ethanol separation, and a diluted aqueous sulphuric acid solution (0.012N) was used as the eluent. The system was maintained at 65°C and a flow rate of 0.8 ml/min. The ethanol yield was expressed as: ethanol yield (% kernel db) = total mass of ethanol produced/ initial mass of ground kernels (db) x 100; ethanol yield (liters / hectare) = total volume of ethanol produced (L/kg db grain) x grain yield (kg db /ha). The ethanol conversion efficiency was calculated as: ethanol conversion efficiency (%) = actual (measured) yield of ethanol / theoretical yield of ethanol x 100, where the theoretical yield of ethanol is 56.73 g ethanol / 100 g starch (1g starch is hydrolyzed to 11.1 g glucose, and 1 mol glucose is fermented to yield 2 mols of ethanol). Values for the grain yield were obtained from part I of the study (*7*).

Isolation of Starch

Corn kernels were steeped in a 0.23 % sodium metabisulfite solution containing 0.1 M NaCl at 4ºC for 12h. The germ was separated manually from the endosperm. Endosperm was mixed with the sodium metabisulfite solution and ground in a micro-blender for 2 min. The ground endosperm was filtered through a nylon screen with a pore size of 53 μm. Crude starch sediment was separated from the metabisulfite solution by centrifugation (7233 x *g*, 20 min) and washed several times with distilled water. The isolated starch was resuspended in 0.1M aqueous NaCl solution containing 10% toluene and stirred for 1 h using a magnetic stirrer. The toluene layer was siphoned off along with extracted proteins. This step was repeated until the toluene layer became clear, and contained no protein. The purified starch was washed three times with water and twice with ethanol and dried at 30°C for 48h.

Enzymatic Hydrolysis of Starch

The rate of starch hydrolysis was determined using the raw-starch hydrolyzing enzyme following the procedure of Srichuwong et al (2009) (*5*). Starch (100 mg db) was suspended in a sodium acetate buffer (200 mM, pH 4.2) containing 0.02% sodium azide and 0.5% v/v enzyme mixture and incubated in a shaker water-bath at 27°C for 96 h. Aliquots were taken at different incubation times. The glucose content of aliquots was determined using a Glucose Oxidase/Peroxidase (GOPOD) assay. Percentage starch hydrolysis (%) = glucose content/initial starch content (db) x 162/180 x 100.

Statistical Analysis

Data obtained for the ethanol yield and ethanol conversion efficiency were analyzed using analysis of variance with a PROC general linear model procedure in SAS 9.2 (SAS Inc., Cary, NC). For each corn variety in varying growing season a separate ANOVA was performed. For corn hybrids, the ANOVA for a randomized complete block design was used with field blocks and the planting date treatment as fixed effects. For the inbred corn, the ANOVA for a completely randomized design was used. Significant differences between means for a particular variety and growing season were determined using Tukey's adjustments. The level of significance was set at α=0.05.

The statistical analysis for enzyme hydrolysis rate was done using a two-stage approach. It was assumed that enzyme hydrolysis rate follows Gompertz curve in time. Through this assumption we were able to characterize trends in the enzyme hydrolysis rate through two interpretable parameters c and d, where:

Gompertz curve: $f(t) = 100e^{-e^{c(d-t)}}$

t = time in hours

c = the relative growth rate at the inflection point (first derivative at time d)

d = the time at which concavity of the curve changes (concave up to concave down)

Using the estimates of c and d we analyzed whether parameters differed by planting date. We used ANOVA to determine whether c and d were different.

RESULTS

Ethanol production from dry-grind corn kernels using cold fermentation process

Kernels of corn crops planted on earlier dates (early April to mid-May) produced ethanol yields comparable to those of crops planted on later dates (late May and June). Kernels of hybrid 34A20 planted in a period from 2 May-21 May of 2007 produced ethanol at 37.0-37.2 % kernel db (2.66-2.67 US gallon/bushel), whereas those planted on 29 May yielded 37.4% (2.69 US gallon/bushel) ethanol (**Figure 1A**). The ethanol yield of hybrid 37Y14 planted in a period from 16 April - 10 June, 2008 was between 37.5% and 38.0% (2.69-2.73 US gallon/bushel), whereas in 2009 from 38.2% (2.74 US gallon/bushel) on 4 April to 38.8% on 29 May (2.79 US gallon/bushel) (**Figure 1A**). Similarly, the ethanol yield of B73 inbred corn kernels was not significantly affected by the planting date and ranged from 36.2-37.0% (2.60-2.66 US gallon/bushel) for the period 6 May-10 June of 2008, and 35.8-36.2% (2.57-2.60 US gallon/bushel) for the period 4 May-20 May of 2009 (**Figure 1B**).

Ethanol yields calculated on the basis of unit planting area (g ethanol/hectare of corn planting area), are displayed in **Figure 2**. The ethanol yield was found to decrease significantly with delayed planting dates of corn hybrids 34A20 and 37Y14 (the grain yield of B73 was not measured). Planting dates between 4 April and 15 May resulted in the ethanol yields ranging from 3998 - 4413 L/ha (428 - 472 US gallons/acre), and the yield decreased to

3396 – 4108 L/ha (363 – 439 US gallons/acre) with plantings delayed into late May and June (p<0.05).

Ethanol conversion efficiency

The ethanol conversion efficiency of corn kernels is shown in **Figure 3**. The results indicated the conversion of starch to ethanol was more efficient with the kernels planted in a period from mid-May to June (91.1-93.8% starch converted to ethanol) than those planted in early April-mid May (89.2-91.85%). These results suggested that the larger ethanol production rates of corn kernels planted on later dates could be attributed to more complete hydrolysis of starch to glucose and subsequent fermentation to ethanol.

Hydrolysis of native starch using raw-starch hydrolyzing enzyme

The hydrolysis rate of isolated starch granules using the raw-starch hydrolyzing enzyme did not statistically differ across different planting dates for the given corn varieties as indicated by the insignificant difference between the estimated parameters of Gompertz curve d (p=0.2) and c (p=0.6). This means that starch hydrolysis curves followed the same trend and were not found structurally different with different planting dates. Comparison between means of percentage starch hydrolysis after 96h hydrolysis reaction, however, showed that starch samples isolated from corn crops planted on earlier dates (early to mid-May) were less digestible than those planted on later dates (late May and June) (**Table 2**). Corn samples planted very early (hybrid 37Y144 planted on 4 April 2009 and 16 April 2008) reached levels of starch hydrolysis comparable to those of samples planted on late dates (late May and June) after 96 hour reaction. Differences in the yield of hydrolyzed starch after 96h

of reaction could be attributed to their differential starch structures that were previously reported in the part I of our study (*7*).

DISCUSSION

The optimum window of planting dates in Iowa has been considered to be 20 April-10 May because it produces the maximum grain yield of corn crops (*8*). Planting date of corn, however, can be delayed beyond the optimum planting window when unfavorable weather conditions occur, such as low temperatures (≤10˚C) and wet soils (*8*). Late planting dates (late May and June) have been shown to produce corn kernels with altered properties and chemical compositions (*7*), which in turn might affect processing parameters and the yield of ethanol fermentation. The results of the current study demonstrated that kernels of crops planted in the period from early April to mid-May produced ethanol yields similar to those of crops planted in late May and June (**Figure 2**).

Large ethanol yields are typically achieved with corn kernels containing large starch contents (*5, 6*). The part I of our study showed decreased starch contents of corn kernels with planting dates delayed into late May and June in Iowa (*7*). Despite small contents of starch, corn kernels planted on later dates (late May and June) gave ethanol yields comparable to those of kernels planted on early dates (early April to mid-May). This was due to the larger ethanol conversion efficiency of samples planted in late May and June (**Figure 3**), which indicated that starch was more completely converted to ethanol during the fermentation and yielded more ethanol.

The large ethanol conversion efficiency of kernels planted in late May and June was obtained because the raw-starch hydrolysing enzyme achieved larger percentage of starch

hydrolysis after 96h reaction with samples planted on late dates than those planted early in May (**Table 2**). The large percentage of starch hydrolysis after 96h of hydrolysis could be attributed to small amylose contents and large proportions of short amylopectin branch-chains of starch samples planted on later dates, as reported in the part I of our study (*7*). The amylose content of starch is inversely related to the enzyme digestibility of native starch (*9*) because it interacts with amylopectin molecules and holds the granule integrity, which restricts granule swelling in water and reduces the accessibility of enzyme to hydrolyze the starch (*10-13*). Starch granules containing amylopectin with large proportions of short branch-chains are more susceptible to the enzyme hydrolysis (*13*). Short amylopectin branch-chains do not form stable double helices and create defects in the crystalline regions of starch, which are more susceptible to enzyme hydrolysis (*5*, *14-15*).

When calculated on the basis of unit planting area of corn, the ethanol yield has been found to significantly decrease with delayed planting date of corn crops (**Figure 2**). The ethanol yield, expressed as a quantity of ethanol produced from kernels harvested from a hectare of planted area, is closely associated with the grain yield; reduced grain yields obtained with later planting dates of corn (reported in Part I of the study (*7*)) resulted in smaller ethanol outputs. Thus, corn planted on late days would require larger planting area to produce the same amount of ethanol as that planted early. This is not easily achieved presently because the corn planting area in the USA has already trended upwards to meet the increased corn grain market demand driven by the growing ethanol industry. The corn planting area in 2010 was about 36 million hectares (88 million acres), which is a 7% increase from that in 2005 (*18*). Further increases in the planting area of corn would need to be offset by a reduced planting area of soybeans, cotton, and other crops in the USA (*19*).

Thus, planting of corn within the optimum planting dates is essential to maximize the grain yield, improve the ethanol production rates, and meet the current energy demands.

CONCLUSIONS

On the dry basis of ground corn kernels, the ground corn kernels gave similar ethanol yields regardless of planting date. This indicated that delayed planting of corn did not affect the ethanol production from a processing perspective. On the basis of corn planting area, however, the ethanol yield (L/ha) was found to decrease significantly with planting dates delayed into late May and June. This could be attributed to the reduced grain yields of corn crops planted on late dates, which resulted in small ethanol yields.

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REFERENCE CITED

1. Renewable Fuels Association 2011. http://www.ethanolrfa.org/news/entry/2010-annual-ethanol-production-13.23-billion-gallons/. Accessed on April 26, 2011.
2. Lewis, S. M., Van Hulzen, S.E., & Roth, D. L. 2005. Continuous process for producing ethanol using raw starch. U.S. Patent number 2005/0239181 A1

3. Wang, P., Singh, V., Xue, H., Johnston, D. B., Rausch, K. D., and Tumbleson, M. E. 2007. Comparison of Raw Starch Hydrolyzing Enzyme with Conventional Liquefaction and Saccharification Enzymes in Dry-Grind Corn Processing. Cereal Chem. 84 (1), 10-14.
4. Robertson, G. H., Wong, D. W. S., Lee, C. C., Wagschal, K., Smith, M. R., and Orts, W. J. 2006. Native or raw starch digestion: A key step in energy efficient biorefining of grain. J. Agric. Food Chem. 54:353-365.
5. Srichuwong, S.; Gutesa, J.; Blanco, M.; Duvick, S. A.; Gardner, C.; Jane, J. Characterization of corn grains for dry-grind ethanol production. *J. ASTM Int*. 2009, 7(2), 1-10.
6. Wu, X., Zhao, R., Wang, D., Bean, S. R., Seib, P. A., Tunistra, M. R., Campbell, M., and O'Brien, A. 2006. Effects of amylose, corn protein, and corn fiber contents on production of ethanol from starch-rich media. Cereal Chem. 83 (5): 569-575.
7. Medić**,** J., Abendroth, L. J., Elmore, R. W., and Jane, J. unpublished. Effect of planting date on properties of corn. Part I: Grain yield, kernel composition, and structure and properties of starch.
8. Farnham, Dale. Corn Planting Guide, PM1885, 2001. Iowa State Extension Online Store. http://www.extension.iastate.edu/Publications/PM1885.pdf (accessed December 08, 2010)
9. Jane, J. Current understanding on starch granule structures. *J. Appl. Glycosci*. 2006, 53, 205-213.
10. Case, S. E, Capitani, T., Whaley, J. K., Shi, Y. C., Trzasko, P., Jeffcoat, R., and Goldfarb, H. B. (1998). Physical properties and gelation behaviour of a low-

amylopectin starch and other high-amylose corn starches. Journal of Cereal Science, 27, 301-314.

11. Jiang, H., Campbell, M., Blanco, M., and Jane, J. 2010. Characterization of corn amylose-extender (ae) mutant starches. Part II: Structures and properties of starch residues remaining after enzymatic hydrolysis at boiling-water temperature.
12. Shi, J. –C., Capitani. T., Trzasko, P., and Jeffcoat, R. 1998. Molecular structure of a low-amylopectin starch and other high-amylose corn starches. Journal of Cereal Science, 27, 289-299.
13. Perera, C.; Lu, Z.; Sell, J.; Jane, J. Comparison of physico-chemical properties and structures of sugary-2 corn starch with normal and waxy cultivars. *Cereal Chem.* 2001, 78, 249-256.
14. Jane, J.; Chen Y.Y.; Lee, L.F.; McPherson, A.E.; Wong, K.S.; Radosavljevic, M.; Kasemsuwan, T. Effects of Amylopectin Branch Chain Length and Amylose Content on the Gelatinization and Pasting Properties of Starch. *Cereal Chem.* 1999, 76, 629-637.
15. Srichuwong, S.; Sunarti, T.C.; Mishima, T.; Isono, N.; Hisamatsu, M. Starches from different botanical sources I: Contribution of amylopectin fine structure to thermal properties and enzyme digestibility. *Carbohydr. Polym*. 2005, 60, 529-538.
16. Li, L.; Blanco, M.; Jane, J. Physicochemical properties of endosperm and pericarp starches during corn development. *Carbohydr. Polym.* 2007, 67, 630-639.
17. Pan, D. D.; Jane, J. Internal structure of normal corn starch granules revealed by chemical surface gelatinization. *Biomacromolecules* 2000, 1, 126–132.

18. USDA National Agricultural Statistics Service. http://quickstats.nass.usda.gov/results/1223E1AB-B912-3889-ACE1-DF46731C00AB. Accessed: March 22, 2011.

19. USDA, 2007. http://www.nass.usda.gov/Newsroom/2007/03_30_2007.asp. Accessed: March 22, 2011.

Table 1. Summary of corn lines and planting dates used in the study

Corn line	Growing year	Planting locations	Planting dates
34A20	2007	Ames (central Iowa)	2-May, 11-May, 21-May, 29-May
37Y14	2008	Nashua (northeast Iowa)	16-Apr, 30-Apr, 13-May, 25-May, 11-June
37Y14	2009	Kanawha (north central Iowa)	4-Apr, 17-Apr, 4-May, 15-May, 29-May
B73	2008	Ames (central Iowa)	6-May, 15-May, 10-June
B73	2009	Ames (central Iowa)	4-May, 11-May, 20-May

Table 2. Enzyme hydrolysis rate of starch isolated from 34A20, 37Y14 and B73 corn kernels planted on different dates in 2007-2009 growing seasons

Corn line/growing season (Planting location)	Planting date	Percentage starch hydrolysis (%)[1]						
		3h	6h	12h	24h	48h	72h	96h
34A20/ 2007 (Ames)	2-May	14.0	19.7	29.1	45.7	69.5	84.4	95.8[a]
	11-May	14.4	20.1	29.5	45.7	71.8	87.0	98.1[b]
	21-May	14.8	20.3	29.9	47.1	73.6	88.3	98.8[b]
	29-May	15.3	20.4	31.9	48.3	77.4	91.7	100.0[c]
	Standard error							0.6
37Y14/ 2008 (Nashua)	16-April	16.2	25.5	37.2	62.8	97.6	100.0	100.0[b]
	30-April	19.2	28.1	41.7	64.4	92.3	95.0	97.7[a]
	13-May	18.0	25.9	41.9	69.2	96.4	94.8	98.0[a]
	25-May	20.4	28.2	42.8	73.4	93.8	95.8	98.3[a]
	11-June	19.3	28.6	43.8	77.7	100.0	100.0	100.0[b]
	Standard error							0.4
37Y14/ 2009 (Kanawha)	4-April	16.9	22.5	36.6	57.9	82.2	93.9	96.9[b]
	17-April	17.2	20.4	33.8	55.1	79.4	90.1	94.9[a]
	4-May	16.5	22.8	35.3	56.9	80.4	93.5	94.6[a]
	15-May	16.0	20.4	31.8	56.6	79.8	89.1	96.7[b]
	29-May	17.2	21.0	35.5	58.0	81.5	92.9	96.9[b]
	Standard error							0.3
B73/ 2008 (Ames)	6-May	21.0	28.5	37.9	60.5	86.2	94.1	96.4[ab]
	15-May	20.0	28.1	39.2	64.2	88.8	94.6	95.7[a]
	10-June	22.2	31.8	44.1	69.7	90.2	94.6	97.6[c]
	Standard error							0.3
B73/ 2009 (Ames)	4-May	16.7	22.0	37.2	54.2	82.0	94.0	96.0[a]
	11-May	22.6	29.9	45.5	66.1	88.2	98.6	97.9[b]
	20-May	24.9	31.8	52.3	75.1	90.0	100.0	100.0[c]
	Standard error							0.5

[1] Percentage starch hydrolysis (%) = glucose content/initial starch content (db) x 162/180 x 100

Means for each corn variety and growing season in the same column with the same letter subscript are not significantly different

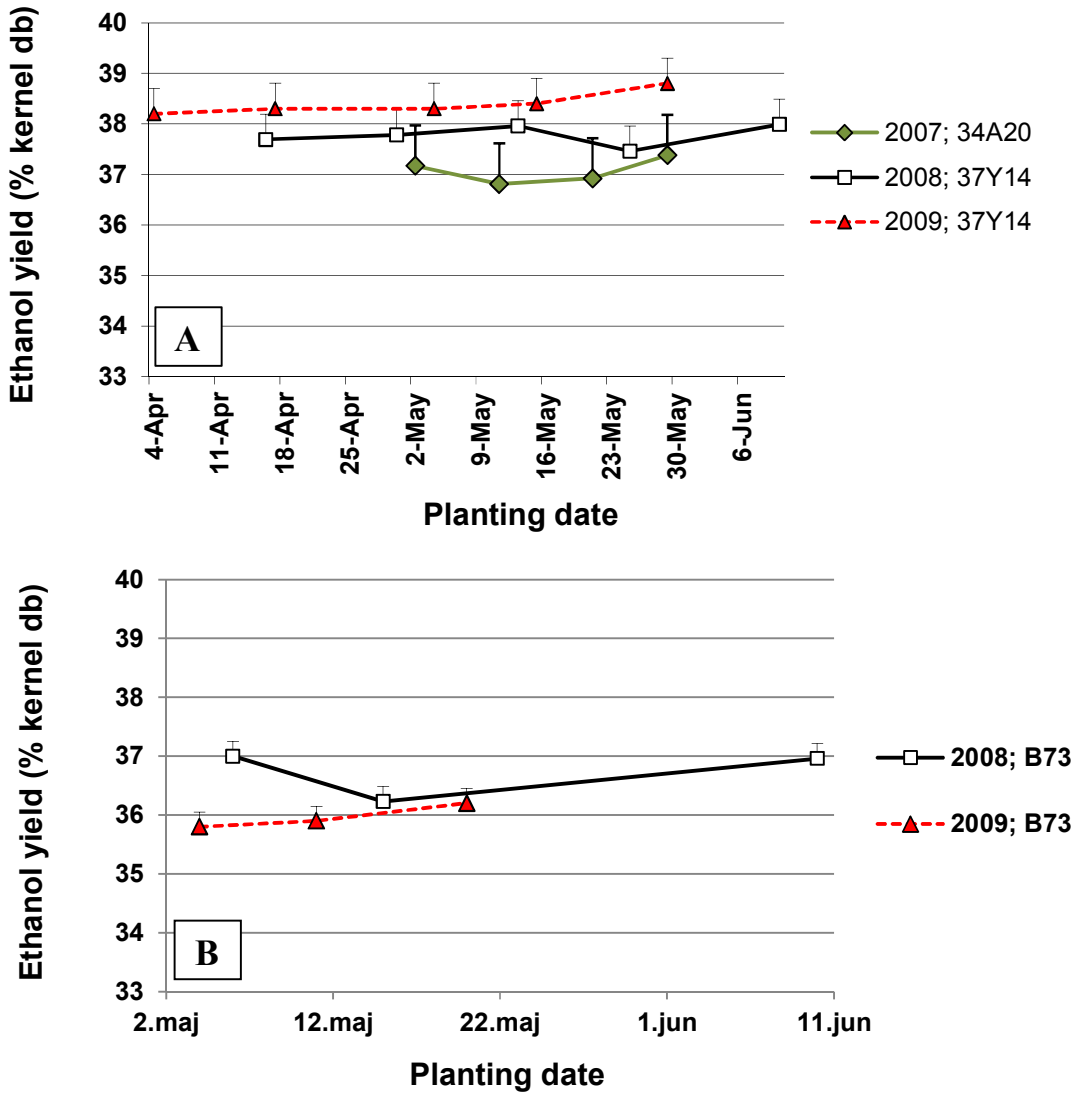

Figure 1. Ethanol yields of ground kernels planted on different dates in 2008 and 2009 using cold fermentation. **A**-hybrids 34A20 and 37Y14, **B**-B73 inbred corn. Ethanol yield (% kernel db) = total mass of ethanol produced/ initial mass of ground kernels x 100. Data points on the figure represent means of four replications + standard errors.

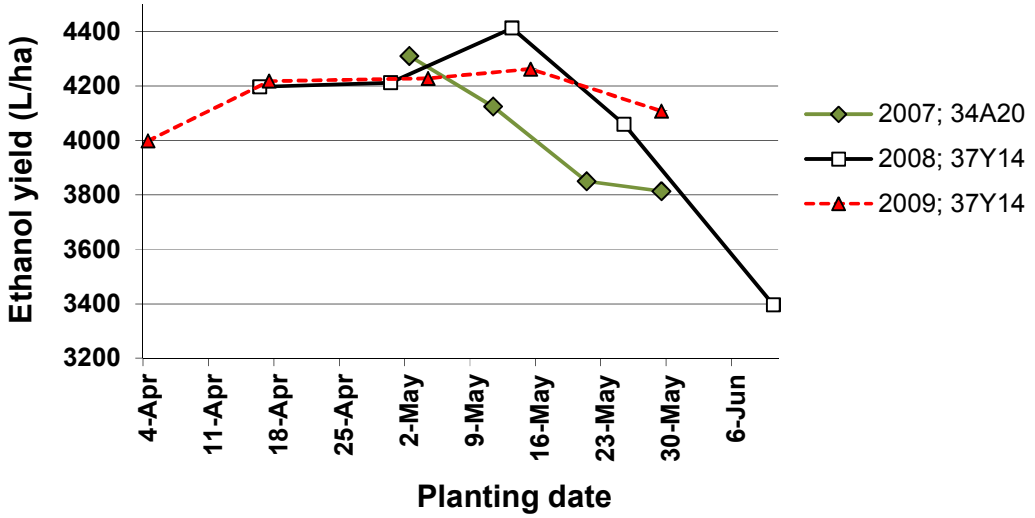

Figure 2. Ethanol yields of hybrids' 34A20 and 37Y14 ground kernels harvested from a hectare of planted land in 2007-2009 growing seasons using cold fermentation. Ethanol yield (liters/ hectare) = total volume of ethanol produced (L/kg grain, db) x grain yield (kg/ha, db). Values for the grain yield were obtained from part I of the study. Data points on the figure represent means of four replications + standard errors.

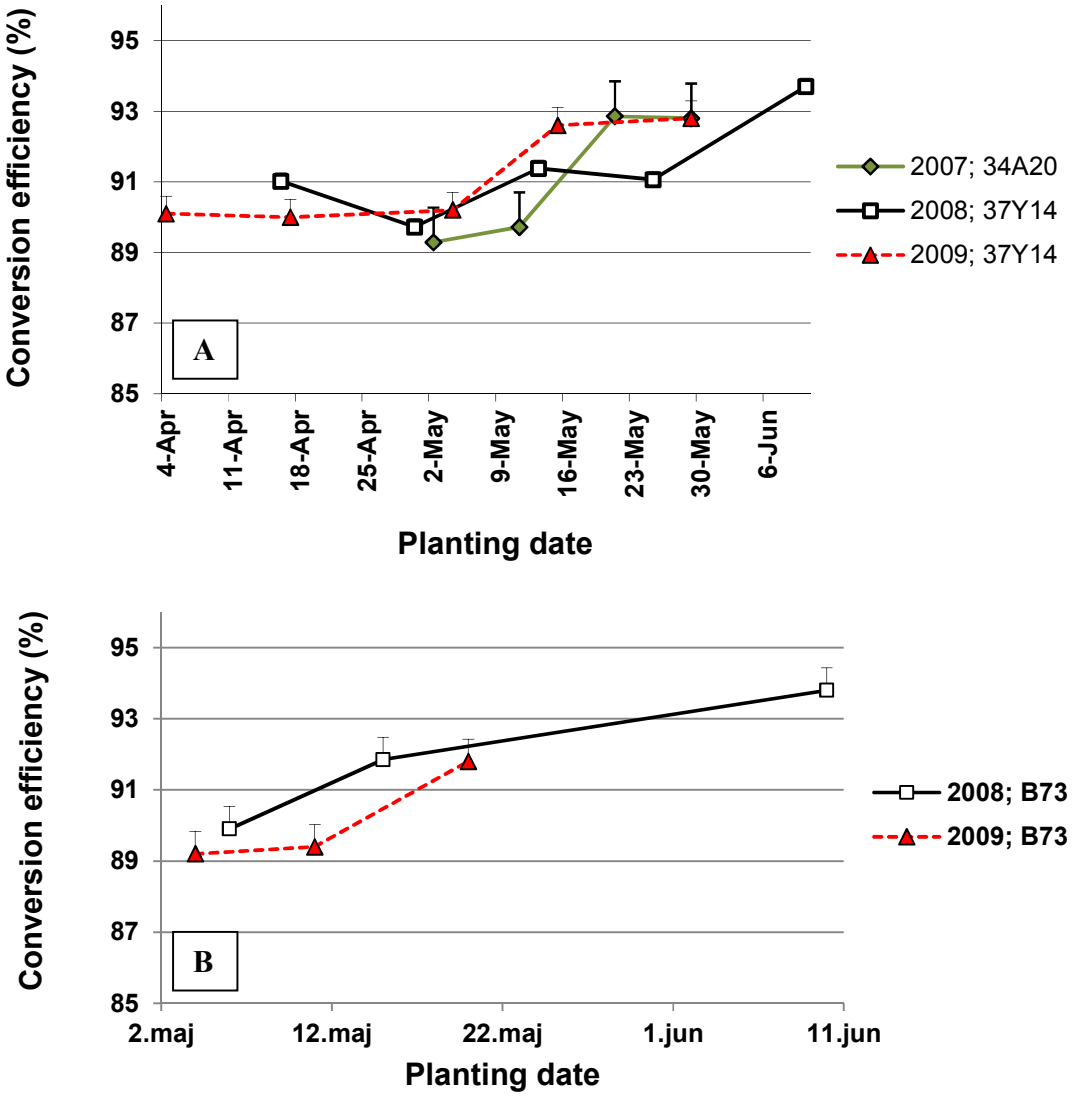

Figure 3. Ethanol conversion efficiency of ground kernels planted on different dates in 2007-2009 growing seasons using cold fermentation. **A**-corn hybrids 34A20 and 37Y14, **B**-B73 inbred corn. Ethanol conversion efficiency (%) = actual (measured) yield of ethanol / theoretical yield of ethanol x 100, where the theoretical yield of ethanol is 56.73 g ethanol / 100 g starch. Data points on the figure represent means of four replications + standard errors.

CHAPTER 6. STARCH PROPERTIES AND ETHANOL PRODUCTION OF CORN AS AFFECTED BY KERNEL DRYING CONDITIONS

A paper prepared for submission to the Journal of Cereal Science

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ABSTRACT

The objective of this study was to determine how drying of freshly harvested corn kernels at low (10°C), moderate (45°C and 65°C), and high (85°C) air temperatures affected the functional properties of starch and ethanol yield of ground kernels compared with the control (25°C). Starches isolated from kernels dried at 10°C, 45°C and 65°C displayed increased, whereas those dried at 85°C a reduced enthalpy change of starch compared with the control. Some granules lost birefringence after the drying of kernels at 85°C. Corn kernels dried at all selected temperatures exhibited reduced swelling power and increased gelatinization temperatures of starch and resulted in the slower enzyme hydrolysis of starch

and smaller ethanol yields than the control. The severity of changes in starch properties and the ethanol yield loss increased with the drying temperature and moisture content of freshly harvested kernels.

KEYWORDS: drying temperature, corn kernels, ethanol yield, starch properties

INTRODUCTION

Corn is one of the major crops globally and is used as a feedstock in food, feed, and biofuel industries. Freshly harvested corn kernels have moisture contents more than 20% and thus, need to be dried to prevent microbial growth and ensure safe storage. The drying of kernels can be achieved with ambient air (or that heated to 5°C above ambient temperature) using low-temperature drying systems (for review see Sharp, 1982) or with heated air using modern drying systems. Elevated drying temperatures accelerate the rate of drying (Barrier-Guillot et al, 1993), but do not produce excellent grain quality if the drying systems are not constructed well. For example, kernels dried at elevated temperatures have shown increased breakage during handling ([Hooseney, 1986] and [Peplinski et al., 1994]), altered starch properties ([Altay and Gunasekaran, 2006], [Haros et al., 2003], and [Malumba et al, 2009]), and decreased corn protein solubility, protein moisture-binding capacity, and enzymatic activity ([Eckoff, and Tso, 1991] and [Wall et al., 1975]). These changes result in the lower flaking grit yield in dry milling and poor starch-protein separation in wet milling process (Singh et al., 1998). It is not well understood, however, how the drying temperature affects the ethanol yield produced from dry-grind corn.

In order to produce ethanol from corn kernels, ground corn kernels need to be suspended in water and treated with α-amylase and amyloglucosidase to hydrolyze starch to glucose. Glucose is subsequently utilized by yeast to produce ethanol. The yield of ethanol is determined by the content of starch in kernels and the efficiency of starch hydrolysis during fermentation (Srichuwong et al., 2009). The rate of starch hydrolysis is affected by the starch granule structure (Jane et al., 2003), granule surface area ([Franco et al., 1992], [Kim et al., 2008], [Kong et al., 2003], and [Yook and Robyt, 2002]), presence of protein and lipids on the granule surface ([Greenwell et al., 1985] and [Oates, 1997]), and enzyme types ([Blazek and Copeland, 2010] and [Planchot et al., 1995]). Starch isolated from corn kernels dried at ≥60˚C air temperatures have been reported to show higher gelatinization and pasting temperatures, and lower peak viscosity and swelling power than the controls dried at ambient temperature (20˚C) ([Altay and Gunasekaran, 2006], [Haros et al., 2003], and [Malumba et al, 2009]). The findings suggest that starch undergoes structural changes during the drying of kernels, which might affect the enzyme hydrolysis kinetics of starch and ethanol yield of corn kernels.

This study was conducted with aim to determine how low (10˚C), moderate (45˚C and 65˚C) and elevated (85˚C) air drying temperatures affect ethanol yield of corn kernels in cold (raw-starch) fermentation process compared with the control (25˚C). Changes in the enzymatic hydrolysis, thermal properties, and swelling power of native starches were also discussed in relation to different drying temperatures of kernels.

MATERIALS AND METHODS

Materials

Kernels of three corn hybrids grown in the North Central Iowa in 2007 year were harvested, shelled and immediately dried. The hybrids were Dekalb 61-58, Agrigold, and Dekalb 61-66, designated with names 49, 50, and 51 respectively. For each hybrid, 15 kg of kernels was split into 15 samples of 1kg each. Samples of each hybrid were dried at 10ºC, 25ºC, 45ºC, 65ºC, and 85ºC air temperatures in triplicates. The corresponding kernel temperatures were approximately 12ºC, 22ºC, 38ºC, 58ºC, and 77ºC. Initial moisture contents of kernels were 22.7%, 23.6%, and 27.9% for hybrids 49, 50, and 51, respectively. Drying was stopped at a predetermined final weight based on the initial weight and moisture of samples to insure all samples had kernel moisture content of 14% after the drying. The low-temperature (10ºC) and 25ºC drying was conducted in a barrel dryer, whereas the drying at higher temperatures was performed in a forced convection oven. The order of temperatures was randomized. The oven had a digital temperature setting that was verified with a NIST-traceable glass thermometer. Prior to drying, kernels were placed in plastic perforated bags, and spread into 1-2 cm layers to allow uniform drying of kernels in the oven. Kernels were equilibrated at room temperature after drying, and starch was isolated from them using a laboratory wet-milling procedure described by Srichuwong et al. (2009). Samples dried at 25ºC were used as controls.

Glucose Oxidase/Peroxidase (GOPOD) assay kit was purchased from Megazyme International (Wicklow, Ireland). Raw-starch hydrolyzing enzyme (Novozyme 50009) was a product of Novozyme (Franklinton, NC), Isostab^{TM} of BetaTech hop products (Washington, DC), and Lactrol® of PhibroChem (Ridgefield Park, NJ). Ethanol Red ™ dry yeast (>2 X 10^9 living cells/g) was obtained from Lesaffre yeast corporation (Milwaukee, WI). All

chemicals used in the study were purchased from Fisher Chemicals (Waltham, MA) and Sigma-Aldrich (St. Louis, MO) and used without further purification.

Morphology of Starch Granules

Morphology of isolated starch was examined using a light microscope (Labophot, Nikon, Japan) equipped with Infinity 2-series digital imaging system (Lumenera Coorp., Ottawa, Canada). A starch sample was mounted onto a microscope slide, dispersed in water, and covered with a cover slip. The presence of birefringence of starch granules was examined under polarized light. The micrographs were taken at 40X magnification.

Thermal properties of starch

Thermal properties of starch were analyzed using a differential scanning calorimeter (DSC) (Diamond DSC, Perkin-Elmer Corp., Norwalk, CT, USA) equipped with Pyris thermal analysis software (Perkin–Elmer Corp., Norwalk, CT, USA) (Jane et al., 1999).

Solubility and swelling power of starch

The solubility and swelling power of starch samples heated in water at 80˚C for 20 minutes were determined by following the method of Hasjim et al. (2009).

Residual-protein content of starch granules

The nitrogen content of the isolated starch sample was determined using a Vario MAX CN Analyzer (Elementar Analysensysteme, Hanau, Germany) and multiplied by 6.25 to obtain the protein content of starch.

Starch hydrolysis using raw-starch hydrolyzing enzyme

Starch (100 mg, dry basis) was suspended in a sodium acetate buffer (200 mM, pH 4.2, 10 ml) containing 0.02% sodium azide and 0.5% v/v of raw-starch hydrolyzing enzyme. The mixture was vortex-mixed and incubated in a water bath set at 26°C with constant

shaking for total of 96h. Aliquots (0.5 ml) were taken from the mixture at different time intervals and transferred into ethanol solution (70% v/v, 5 ml) to inactivate the enzyme. The supernatant containing glucose was separated from the insoluble portion of starch by centrifugation at 6600 x *g* for 15 min. The glucose content of supernatant was determined using a Glucose Oxidase/Peroxidase (GOPOD) assay. Percentage starch hydrolysis (%) = glucose content/initial starch content x 162/180 x100.

Dry-grind ethanol production using raw-starch hydrolyzing enzyme

Corn kernels (35 g db), ground to pass a 0.5mm screen, were suspended in water containing urea (0.03% w/w, mash) and microbial reagents (Lactrol®, 2 ppm; and Isostab^{TM}, 40 ppm) to make 100 g of mash. The mash was mixed using a magnetic stirrer for 30 min, and acetate buffer (200 mM, pH 4.2) was added to adjust pH to 4.2. After the pH adjustment, dry yeast (0.5g) and raw-starch hydrolyzing enzyme (0.16 mL/100g mash) were added to the slurry. A polypropylene bottle containing the mash was placed in a shaker incubator adjusted at 27˚C and shaken at 160 rpm. Aliquots (4 ml) were taken from the fermentation broth after 2 and 4 days fermentation time and centrifuged at 7233 x g for 10 min to remove solids. Clear supernatant was filtered through a nylon membrane filter (0.45 μm) and analyzed to obtain ethanol yields. The analysis was performed using Waters High Performance Liquid Chromatography system (Millipore Corporation, Milford, MA, USA) equipped with a Waters Model 401 refractive index detector. An Aminex HPX-87H anion-exchange column (Bio-Rad, Richmond, CA, USA) was used for ethanol separation, and a diluted aqueous sulphuric acid solution (0.012N) was used as the eluent. The system was maintained at 65°C and a flow rate of 0.8 ml/min. The ethanol yield was expressed as: ethanol yield (% kernel db)= total mass of ethanol produced/ initial mass of ground kernels X 100.

Statistical analysis.

Data obtained for thermal properties, swelling power, percentage solubility, and protein content of starch for each hybrid were analyzed using one-way ANOVA. The significant difference between means was determined using Tukey's adjustment.

The statistical analysis for the enzyme hydrolysis rate was done using a two-stage approach through the assumption that enzyme hydrolysis rate follows Gompertz curve in time. Trends in the enzyme hydrolysis rate were characterized through two interpretable parameters c and d, where:

Gompertz curve: $f(t) = 100e^{-e^{c(d-t)}}$

t = time in hours

c = the relative growth rate at the inflection point (first derivative at time d)

d = the time at which concavity of the curve changes (concave up to concave down)

Using the estimates of c and d we analyzed whether parameters differed across drying temperatures. ANOVA was used to determine whether c and d were different.

For the ethanol yield analysis, split-split plot experimental design was used. Different size experimental units were used in the analysis: replications, hybrids, and temperatures. Comparison between mean difference combinations of temperature and hybrids were performed using Bonferonni corrections. Analysis was done using SAS proc mixed in SAS 9.2 (SAS Inc.). The level of significance was set at α=0.05.

RESULTS AND DISCUSSION

Starch granules viewed under a polarized-light microscope showed Maltese cross, indicating the presence of ordered crystalline structure of all starch specimens (**Figures 1**

and 2). Nevertheless, some granules isolated from kernels dried at 85˚C air temperatures (77˚C kernel temperature) partially lost birefringence in the regions close to hilum and at the periphery of granules (**Figure 2**). This suggested that a partial gelatinization of starch granules took place during the drying of kernels at 85˚C air temperature.

Thermal properties of starches isolated from kernels dried at different air temperatures are summarized in **Table 1**. The low-temperature drying of kernels at 10°C air temperature (12°C kernel temperature) increased the gelatinization temperature and the enthalpy change of starch compared with the control (25°C air temperature). This could be attributed to that the drying temperature of 10˚C was close to the optimal temperature (4-5˚C) for starch crystallization (Slade and Levine, 1987), and thus, increased starch crystallinity. Starches isolated from kernels dried at 45˚C (38˚C kernel temperature), 65˚C (58˚C kernel temperature), and 85˚C air temperatures displayed higher gelatinization temperature and narrower gelatinization temperature range than the control samples (25°C). The increase in the gelatinization temperature became more severe as the drying temperature of kernels increased from 45˚C to 85°C. The onset gelatinization temperature of hybrid 49 starches increased between 1.6 and 1.9°C compared with the control (for 45 and 85°C air temperatures, respectively), whereas that of hybrids 50 and 51 increased 1.5-2.8°C and 1.9-2.8°C, respectively. Thus, the increase in the onset gelatinization temperature was least evident for hybrid 49 starch. A similar trend was observed for the peak and completion gelatinization temperatures of starch. The results are in agreement with previously reported increased gelatinization temperatures of starch samples dried at 70°C, 90°C, 100°C, and 110°C compared with those dried at 20°C ([Altay and Gunasekaran, 2006] and [Haros et al.,

2003]). Starch samples dried at 45°C and 65°C air temperatures displayed increased enthalpy change of starch compared with the control sample dried at 25°C. This indicated an enhancement of starch crystalline structure during the drying at those temperatures. Samples dried at 85˚C air temperature showed a slightly lower enthalpy change of starch than the controls, indicating a partial loss of starch crystalline structure after the drying at the high temperature. The result is in line with the lost birefringence of some granules observed under a microscope.

The swelling power and percentage solubility of isolated starch were also affected by the kernel drying temperature (**Table 2**). Starches isolated from kernels dried at 10˚C exhibited reduced swelling power and percentages solubility compared with the controls. This could be explained by the increased crystallinity of starches, which restricted water penetration and limited swelling of granules in water. Starch samples dried at 45°C, 65°C, and 85°C temperatures also exhibited reduced swelling power and percentages solubility compared with the controls. Haros and Suarez (1997) and Malumba et al. (2009) observed similar phenomenon with starch samples dried at 70°C, 90°C, 100°C, and 110°C.

Changes in the starch functional properties, with the exception of the swelling power, were most prominent in kernels of hybrid 51, followed by those of hybrids 50 and 49, respectively. Different sensitivity to the drying temperature of kernels might be attributed to the different genetic background of the hybrids and/or moisture content of freshly harvested kernels. The initial moisture content of kernels prior to drying was 27.9%, 23.6%, and 22.7% for hybrids 51, 50, and 49, respectively. This indicated that the effect of drying temperature on starch properties was more pronounced in kernels with higher harvest moisture contents.

The low moisture content of hybrid 49 kernels allowed for little changes in the starch structure to take place during the drying process. Singh et al (1998) have reported that the high-temperature drying (110˚C) reduced the starch yield in a wet-milling process, and the reduction was more severe as the harvest moisture of corn kernels increased..

Altay and Gunasekaran (2006) proposed that changes in the thermal properties and swelling power of starch, caused by increased air drying temperatures, could be ascribed to the internal structural changes of starch and/or to the increased contents of residual protein of starch remaining after isolation process. Isolated starch samples used in the current study contained similar residual protein contents regardless of the drying temperature (**Table 2**). Thus, the changes in the thermal properties and swelling power of the starches dried at elevated temperatures could be attributed solely to the internal structural changes of starch that occurred during the drying process. Hydrothermally treated starch (annealed and heat-moisture treated), prepared by incubation in water at temperatures above the glass transition temperature of starch, has been reported to have increased onset gelatinization temperature, a narrower gelatinization temperature range, increased or unchanged enthalpy change, and reduced swelling power of starch (as reviewed by Jayakody, and Hoover, 2008) than native starch. These changes have shown to be initiated in starch at as low as 35°C and the water content ≥20% (Tester et al., 1998). After the drying at 45°C and 65°C air temperatures, samples of hybrids 49, 50, and 51 showed changes in starch properties similar to those of hydrothermally treated starch. Thus, it is plausible to believe that the drying of kernels at the intermediate air temperatures was analogous to the hydrothermal treatment of starch, and that starch molecules underwent a molecular rearrangement and aligned in a more perfect way to

form crystallites with improved crystallinity during drying. This process, however, would be limited only to early stages of drying when kernels still contained sufficient water content for this process to take place. Findings of Whistler et al (1958, 1959) indicated formation of internal cavities and porous structure inside of starch granules during the drying process. Huber and BeMiller (1997) later found the internal cavities in granules of wet starch as well, but Whistler's studies undoubtedly showed that the number of these cavities increased as the kernel moisture decreased and as the drying temperature increased during the drying. Nakazawa and Wang (2003) have proposed that annealing also produces void spaces that lead to formation of porous starch structures, which is an another example of analogy between kernel drying process and the hydrothermal treatments of starch.

Starch hydrolysis rates using raw-starch hydrolyzing enzyme are shown in **Figure 3**. The enzyme hydrolyzed native starches isolated from kernels dried at 10°C, 65˚C, and 85˚C air temperatures slower than the control starch, as indicated by the significantly different d (time at which 50% of starch hydrolysis occurs) and c (rate of change at time d) parameters of Gompertz curve for the given temperatures. That can be ascribed to the increased crystallinity and reduced swelling power of starches dried at 10°C and 65˚C air temperatures, which impeded hydration of starch granules and penetration of the enzyme to their interior. On the other side, partially gelatinized starch granules in kernels dried at 85˚C most likely retrograded upon cooling of kernels to the ambient temperature and became resistant to enzyme hydrolysis. Starch isolated from kernels dried at 45˚C was hydrolyzed at similar rate as that of the control. For all three corn hybrids, the lowest percentage hydrolysis of starch

after 96h was obtained for the corn samples dried at the 10˚C (81.3-84.7%) and 85˚C (82.1-84.6%) air temperatures.

Ethanol yields produced from ground corn kernels dried at different air temperatures are displayed in **Figure 4**. The production of ethanol from kernels of hybrids 49, 50, and 51 became gradually reduced as the drying temperature of kernels was successively increased from 25˚C to 85˚C. Kernels dried at 45˚C and 65˚C produced less ethanol than the control after 96h fermentation, although the reduction was not significant for kernels of hybrid 49 (p>0.05). The most severe reduction in the ethanol yield was observed for kernels dried at 85˚C compared with the control (p<0.05 for all three hybrids), which was in agreement with the decreased rate of starch hydrolysis using raw-starch hydrolyzing enzyme described earlier. Despite reduced enzymatic hydrolysis of starch, kernels dried at 10˚C air temperature did not show a statistically significant difference in the ethanol yield compared with the control (p<0.05). Less severe reduction in the ethanol yield can be attributed to the presence of endogenous amylases that survived the drying process (our unpublished result) and aided starch hydrolysis during fermentation (along with exogenously added raw-starch hydrolyzing enzyme), which resulted in higher than expected ethanol yield.

In summary, drying of corn kernels at different air temperatures changed functional properties of the isolated starches. The air drying temperature of 10˚C increased the gelatinization temperature and enthalpy change, and reduced the swelling power of starch compared with the control (25˚C). This can be attributed to that the drying temperature of 10˚C was close to the optimum temperature of starch crystallization (4˚C) and thus, enhanced starch crystallinity. Drying temperatures of 45˚C and 65˚C increased the gelatinization

temperature and enthalpy change of starch, and narrowed the gelatinization temperature range of starch compared with the control. The results suggested starch molecules annealed during the drying at the corresponding temperatures to form more perfect crystallites. The temperature of 85˚C partially gelatinized some starch granules during the drying and reduced their crystallinity. Consequently, starch isolated from kernels dried at 85˚C exhibited higher gelatinization temperature and reduced swelling power of starch compared with the control. Changes in the starch properties reduced the rate of starch hydrolysis and consequently, decreased the ethanol yield of ground kernels dried at 45˚C, 65˚C, and 85˚C air temperatures. The most severe reduction in the ethanol yield was observed for kernels dried at 85˚C. The information obtained in this study could be used as guidance for the ethanol industry to adjust the drying conditions of corn and maximize the ethanol yield.

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REFERENCES

Altay, F., Gunasekaran, S., 2006. Influence of drying temperature, water content, and heating rate on gelatinization of corn starches. Journal of Agricultural and Food Chemistry 54, 4235–4245.

Barrier-Guillot, B., Zuprizal, Jonderville, C., Chagneau, A.M., Larbier, M., Leuillet, M., 1993. Effect of heat drying temperature on the nutritive value of corn in chickens and pigs. Animal Feed Science and Technology, 41, 149-159.

Blazek, J., Copeland, L., 2010. Amylolysis of wheat starches. II. Degradation patterns of native starch granules with varying functional properties. Journal of Cereal Science 52(2), 295-302.

Eckoff, S. R., Tso, C. C., 1991. Starch recovery from steeped corn grits as affected by drying temperature and added commercial protease. Cereal Chemistry 68 (3), 319-320.

Franco, C. M. L., Preto, S. J. R., Ciacco, C. F., 1992. Factors that affect the enzymatic degradation of natural starch granules- effect of the size of the granules. Starch/Stärke 44, 422–426.

Greenwell, P., Evers, A.D., Gough, B.M., Russell, P.L., 1985. Amyloglucosidase-catalysed erosion of native, surface-modified and chlorine-treated wheat starch granules. The influence of surface protein. Journal of Cereal Science 3, 279–293.

Haros, M., Suarez, C., 1997. Effect of drying, initial moisture and variety in corn wet milling. Journal of Engineering 34, 473-481.

Haros, M., Tolaba, M-P., Suarez, C., 2003. Influence of corn drying on its quality for the wet-milling process. Journal of Food Engineering 60, 177–184.

Hasjim, J., Srichuwong, S., Scott, P. M., Jane, J., 2009. Kernel composition, starch structure, and enzyme digestibility of *opaque-2* corn and quality protein corn. Journal of Agricultural and Food Chemistry 57, 2049-2055.

Hooseney, R. C., 1986. Principles of Cereal Science and Technology, first ed. American Association of Cereal Chemists, St. Paul, MN.

Huber, K.C., Bemiller, J.N., 1997. Visualization of Channels and Cavities of Corn and Sorghum Starch Granules. Cereal Chemistry 74 (5), 537-541.

Jane, J., Ao, Z., Duvick, S. A., Wiklund, M., Yoo, S., Wong, K., Gardner, C., 2003. Structures of amylopectin and starch granules: how are they synthesized? Journal of Applied Glycoscience 50, 167-172.

Jane, J., Chen Y. Y., Lee, L. F., McPherson, A. E., Wong, K. S., Radosavljevic, M., Kasemsuwan, T, 1999. Effects of Amylopectin Branch Chain Length and Amylose Content on the Gelatinization and Pasting Properties of Starch. Cereal Chemistry 76, 629-637.

Jayakody, L., & Hoover, R., 2008. Effect of annealing on the molecular structure and physicochemical properties of starches from different botanical origins- A review. Carbohydrate polymers 74(3*)*, 691-703.

Kim, J., Kong, B., Kim, M., Lee, S., 2008. Amylolytic Hydrolysis of Native Starch Granules Affected by Granule Surface Area. Journal of Food Science, 73, 621–624.

Kong, B. W., Kim, J. I., Kim, M. J., Kim, J. C., 2003. Porcine-pancreatic α-amylase hydrolysis of native starch granules as a function of granule surface area. Biotechnology Progress 19, 1162-1166.

Malumba, P., Massaux, C., Deroanne, C., Masimango, T., Béra, F., 2009. Influence of drying temperature on functional properties of wet-milled starch granules. Carbohydrate polymers 75, 299-306.

Nakazawa, Y., Wang, Y.–J., 2003. Acid hydrolysis of native and annealed starches and branch-structure of their Naegeli dextrins. Carbohydrate Research 338(24), 2871-2882.

Oates, CG., 1997. Towards an understanding of starch granule structure and hydrolysis. Trends in Food Science and Technology 8, 375-382.

Peplinski, A. J., Paulis, J. W., Bietz, J. A., Pratt, 1994. Drying of high-moisture corn: changes in properties and physical quality. Cereal chemistry 71(2), 129-133.

Planchot, V., Colonna, P., Gallant, D.J, Bouchet, B., 1995. Extensive degradation of native starch granules by alpha-amylase from *Aspergillus fumigatus*. Journal of Cereal Science 21, 163–171.

Sharp, J. R., 1982. A review of low temperature drying simulation models. Journal of Agricultural Engineering Research, 27, 169-190.

Singh, V., Haken, A. E, Paulsen, M. R., Eckhoff, S. R., 1998. Starch yield sensitivity of corn hybrids to drying temperature and harvest moisture content. Starch/Stärke 50, 181-183.

Slade, L. and Levine H. 1987. Recent advances in starch retrogradation. In: Industrial Polysaccharides- The Impact of Biotechnology and Advanced Methodologies. Stivala S. S., Crescenzi V., and Dea I. C. M. (Eds). New York, NY: Gordon and Breach, pp 387-430.

Srichuwong, S., Gutesa, J., Blanco, M., Duvick, S. A., Gardner, C., Jane, J., 2009. Characterization of corn grains for dry-grind ethanol production. Journal of ASTM International 7(2), 1-10.

Tester, R. F, Debon, S. J. J., Karkalas, J., 1998. Annealing of wheat starch. Journal of Cereal Science 28, 259-272.

Wall, J. S., James, C., Donaldson, G. L., 1975. Corn proteins: Chemical and physical changes during drying of grain. Cereal Chemistry, 52, 779-790.

Whistler, R. L., Goatley, J. L., Spencer, W. W., 1959. Effect of drying on the physical properties and chemical reactivity of corn starch granules. Cereal Chemistry 36, 84-90.

Whistler, R. L., Spencer, W. W., Goatley, J. L., Nikuni, Z., 1958. Effect of drying on the presence of cavities in corn starch granules. Cereal Chemistry 35, 331-336.

Yook, C., Robyt, J. F., 2002. Reactions of alpha amylases with starch granules in aqueous suspension giving products in solution and in a minimum amount of water giving products inside the granule. Carbohydrate Research 337, 1113–1117.

Table 1. Thermal properties of starches isolated from kernels of corn hybrids 49, 50, and 51 dried at different air temperatures

Corn hybrid	Air drying temperature[1] (˚C)	Thermal properties				
		To (°C)	Tp (°C)	Tc (°C)	Tc-To (°C)[2]	ΔH (J/g)
	10	68.9^{a}	74.3^{a}	78.4^{a}	9.5^{b}	12.8^{b}
	25 (control)	**67.1^{b}**	**72.9^{b}**	**77.3^{a}**	**10.2^{a}**	**11.9^{ab}**
49	45	68.7^{a}	74.3^{a}	78.4^{a}	9.7^{b}	12.8^{ab}
	65	68.6^{a}	74.0^{a}	78.4^{a}	9.8^{ab}	14.0^{a}
	85	69.0^{a}	74.1^{a}	78.3^{a}	9.3^{b}	11.4^{a}
Standard error		0.3	0.2	0.3	0.2	0.5
	10	68.1^{ab}	74.5^{a}	78.6^{a}	10.5^{a}	12.6^{a}
	25 (control)	**66.7^{b}**	**72.8^{b}**	**77.2^{a}**	**10.5^{a}**	**11.5^{a}**
50	45	68.2^{ab}	74.2^{ab}	78.2^{a}	10.0^{ab}	12.2^{a}
	65	68.7^{ab}	74.3^{a}	78.3^{a}	9.6^{ab}	12.2^{a}
	85	69.5^{a}	74.6^{a}	78.8^{a}	9.3^{b}	10.9^{a}
Standard error		0.4	0.3	0.4	0.3	0.4
	10	67.1^{a}	74.0^{a}	78.7^{a}	11.6^{ab}	12.9^{a}
	25 (control)	**64.5^{b}**	**72.3^{a}**	**76.1^{b}**	**11.6^{ab}**	**12.5^{a}**
51	45	66.4^{a}	74.1^{a}	78.8^{a}	12.4^{a}	12.9^{a}
	65	67.2^{a}	74.1^{a}	78.7^{a}	11.5^{ab}	12.8^{a}
	85	67.3^{a}	73.6^{a}	78.1^{a}	10.8^{b}	11.2^{a}
Standard error		0.4	0.6	0.3	0.3	0.3

[1]Approximate kernel temperatures during drying were 12ºC, 22ºC, 38ºC, 58ºC, and 77ºC, respectively

[2]Gelatinization temperature range of starch

Table 2. Percentage solubility, swelling power, and residual-protein content of starches isolated from kernels of corn hybrids 49, 50, and 51 dried at different air temperatures

Sample	Air drying temperature[1] (ºC)	% Solubility[2]	Swelling power[3]	Protein content (% starch db)[4]
	10	6.9[a]	11.9[a]	0.1[b]
	25 (control)	**7.0[a]**	**12.6[a]**	**0.2[a]**
49	45	6.9[a]	12.2[a]	0.1[b]
	65	6.9[a]	11.8[a]	0.2[a]
	85	6.6[a]	11.6[a]	0.1[b]
Standard error		0.2	0.3	0.0
	10	6.4[a]	12.3[a]	0.1[a]
	25 (control)	**6.6[a]**	**13.3[a]**	**0.1[a]**
50	45	6.6[a]	13.4[a]	0.1[a]
	65	5.9[a]	11.6[a]	0.1[a]
	85	6.3[a]	11.1[a]	0.1[a]
Standard error		0.2	0.6	0.0
	10	8.0[b]	11.0[b]	0.2[a]
	25 (control)	**8.8[a]**	**11.5[a]**	**0.3[a]**
51	45	7.9[b]	10.5[c]	0.3[a]
	65	7.9[bc]	10.2[d]	0.3[a]
	85	7.8[c]	10.1[d]	0.2[a]
Standard error		0.0	0.1	0.0

[1]Approximate kernel temperatures during drying were 12ºC, 22ºC, 38ºC, 58ºC, and 77ºC, respectively

[2]% Solubility= (total carbohydrate in supernatant after heating (g) × 0.9)/ initial starch mass (db) × 100

[3]Swelling power = (weight of solid residue after heating × 100) / [initial starch mass (db) × (100- % solubility)]

[4]Protein content (% starch db)= nitrogen content of isolated starch granules (% db) X 6.25

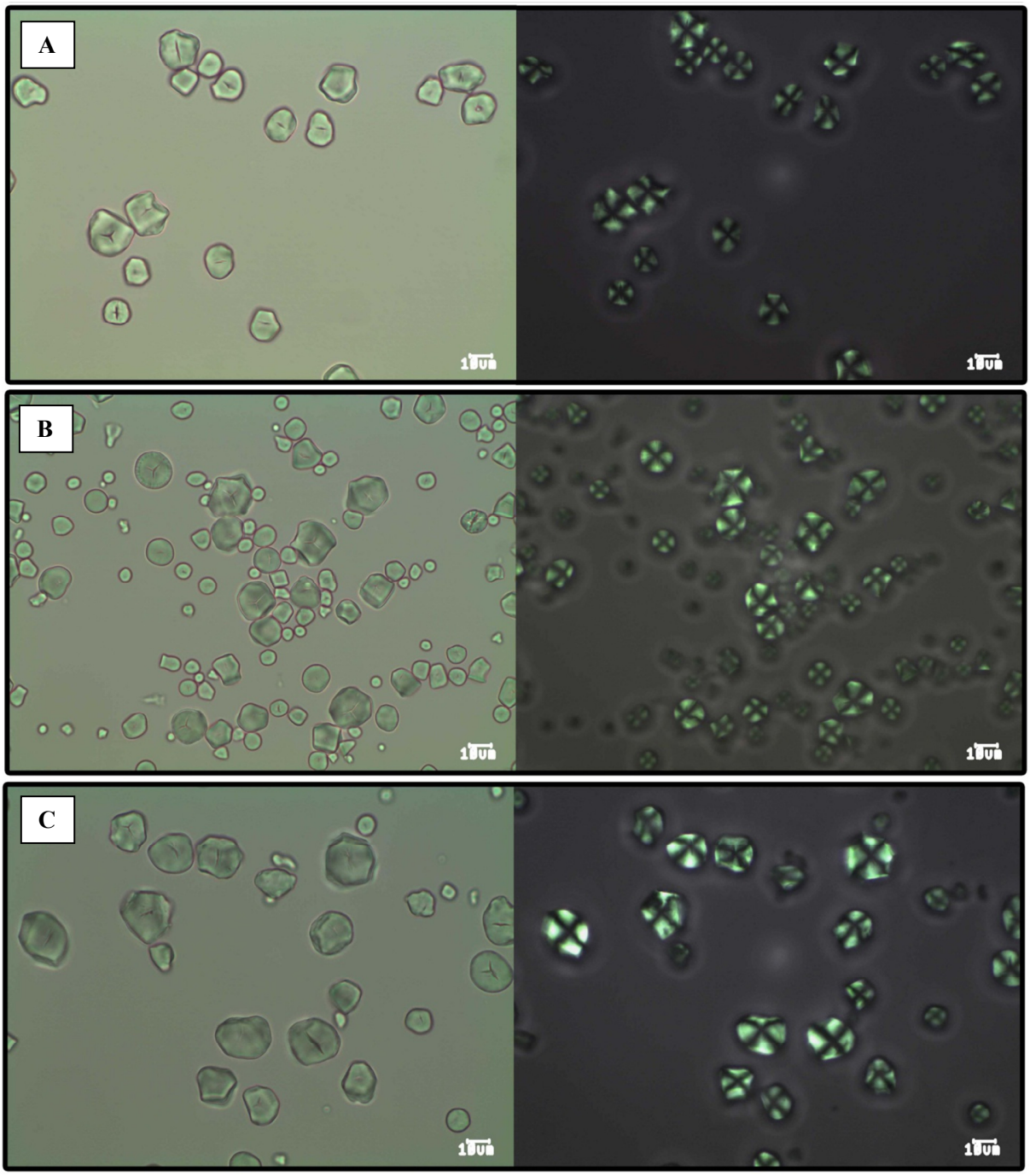

Figure 1. Light and polarized-light micrographs of starch granules isolated from corn kernels dried at 25˚C air temperatures. **A**- hybrid 49, **B**- hybrid 50, **C**-hybrid 51.

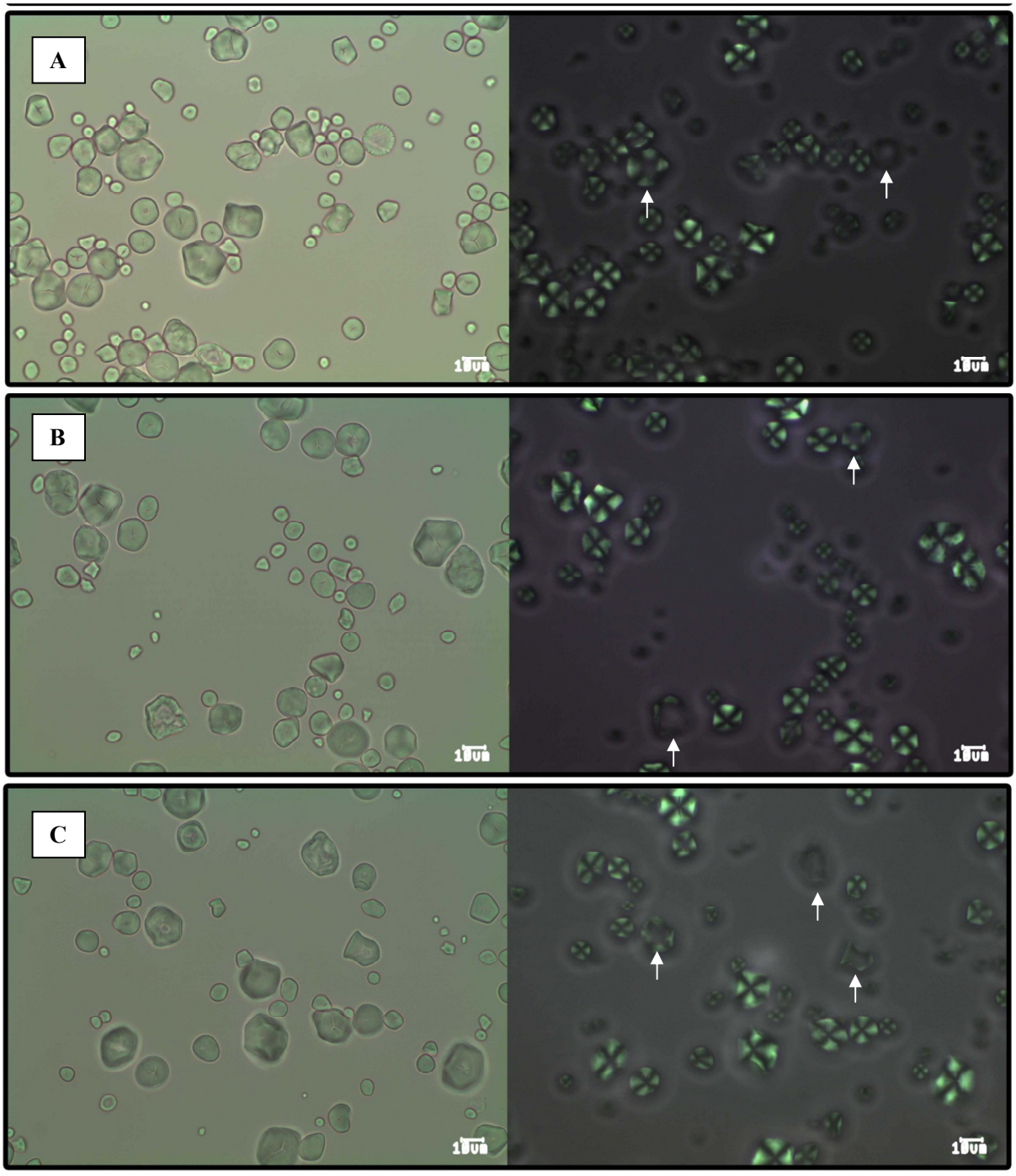

Figure 2. Light and polarized-light micrographs of starch granules isolated from corn kernels dried at 85˚C air temperatures. **A**- hybrid 49, **B**- hybrid 50, **C**-hybrid 51. Arrows indicate granules with partially lost birefringence.

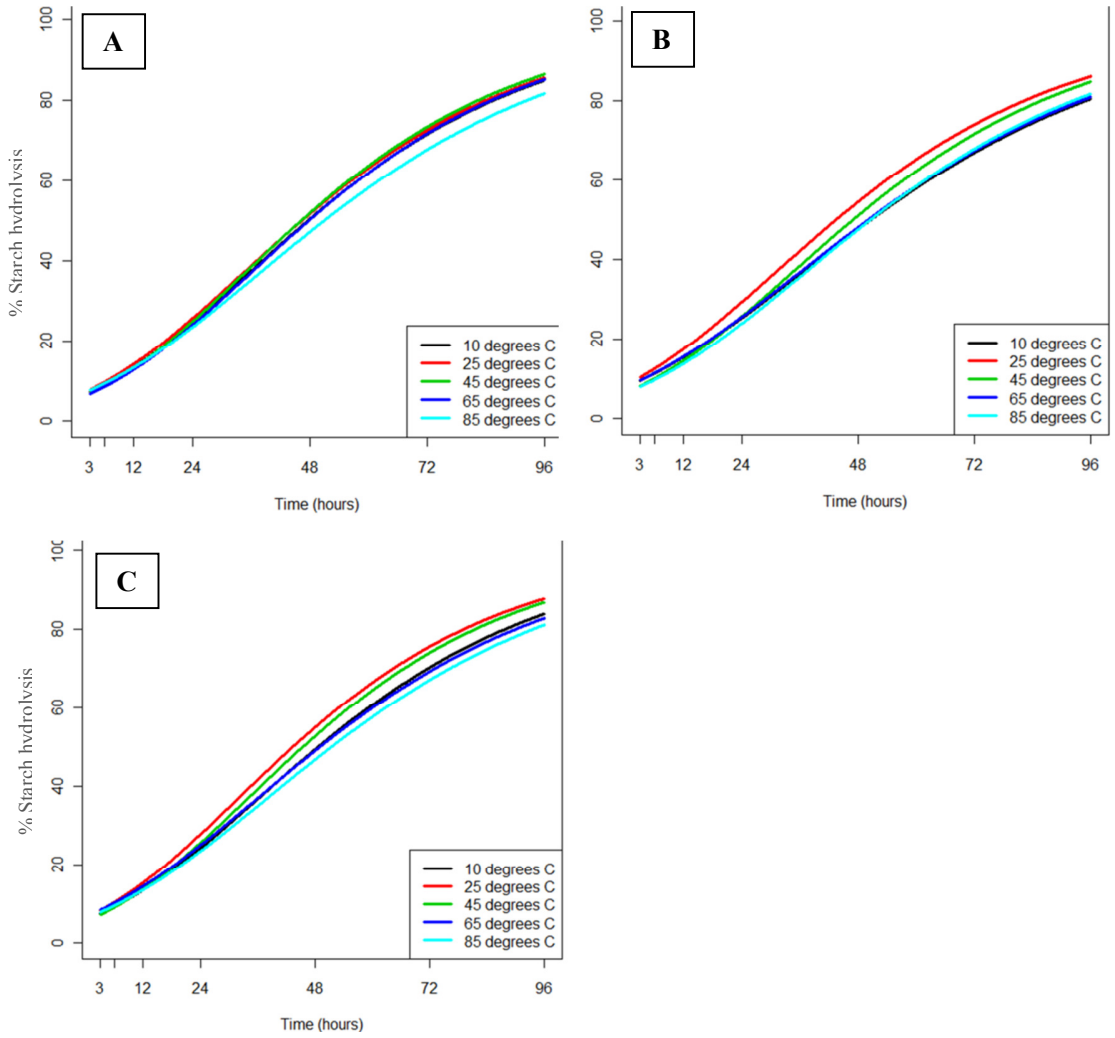

Figure 3. Hydrolysis rate of native starches isolated from kernels of three corn hybrids dried at different air temperatures using raw-starch hydrolyzing enzyme. **A**-hybrid 49, **B**-hybrid 50, and **C**-hybrid 51. Percentage starch hydrolysis (%) = glucose content/initial starch content x 162/180 x100.

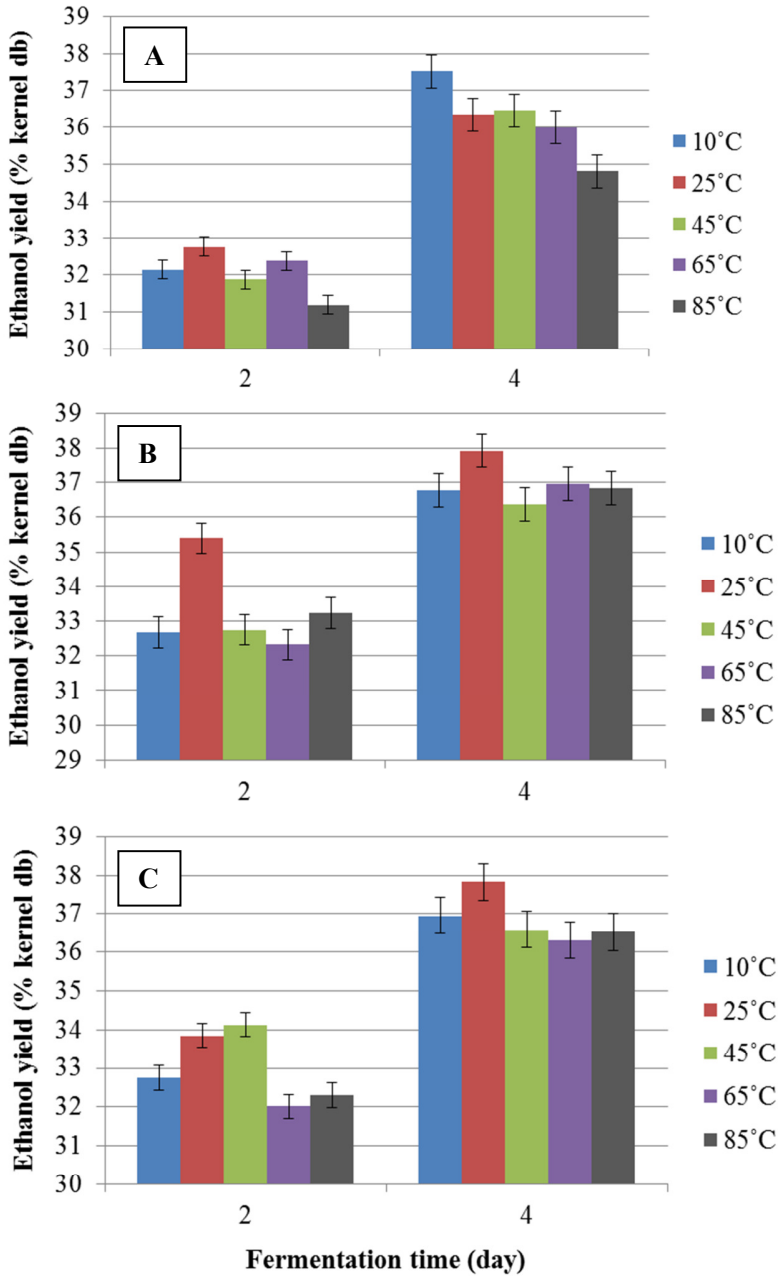

Figure 4. Ethanol yields of ground kernels dried at different temperatures using cold fermentation. **A**-hybrid 49, **B**-hybrid 50, and **C**-hybrid 51. Ethanol yield (% kernel db)= total mass of ethanol produced/ initial mass of ground kernels X 100. Bars represent means of three replications ± standard error.

CHAPTER 7. THE ENDOGENOUS AMYLASE ACTIVITY OF CORN AS AFFECTED BY KERNEL DRYING TEMPERATURE

A paper prepared for publication to the Cereal Chemistry Journal

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ABSTRACT

Objectives of this study were to understand the effect of kernel drying temperature on the activity of endogenous amylases of corn. Freshly harvested kernels of three commercial corn hybrids were dried at 10ºC, 25ºC, 45ºC, 65ºC, 85ºC, 105ºC, and 125ºC air temperatures to 14% moisture content. The results showed no significant difference in the activity of endogenous amylases in kernels dried at 10˚C, 25˚C, 45˚C and 65˚C, which hydrolyzed 6.4-8.1 % starch (kernel db) in ground corn suspensions after 20h incubation. Elevated air temperatures of ≥85˚C partially damaged the endogenous amylases as indicated by the reduced starch hydrolysis rate (2.6-6.4% kernel db after 20h incubation) in the ground kernel suspensions. β-Amylase showed reduced activity in kernels after drying at ≥45˚C, whereas decreased activity of starch debranching enzymes (pullulanase and isoamylase) was observed at ≥85˚C. The activity of α-amylase in kernels remained relatively unchanged with the drying temperatures up to 85˚C, but was significantly reduced after the drying at 105˚C and 125˚C.

KEYWORDS: corn kernel, drying temperature, amylase activity, α-amylase, β-amylase, starch debranching enzymes

INTRODUCTION

Starch is the major storage carbohydrate in seeds and tubers of higher plants. Cereal seeds (e.g. corn kernels) can contain up to 75% starch (db). During germination, endogenous amylases hydrolyze starch granules to produce sugars, such as glucose and maltose. These sugars are subsequently utilized as energy and carbon sources in the seedlings. The mechanism of starch degradation in corn is not entirely elucidated, but it is believed that α-amylase (EC 3.2.1.1) is a key enzyme to initiate the hydrolysis (Subbarao et al 1998; Sun and Henson 1991;). Subsequent steps in the hydrolysis of starch involve actions of β-amylase (EC 3.2.1.2), branching enzymes (pullulanase, EC 3.2.1.41; and isoamylase, EC 3.2.1.68) and α-glucosidase (maltase, EC 3.2.1.20), which hydrolyze fragments released by α-amylase (Beck and Ziegler 1989; MacGregor 1987; Sanwo and DeMason 1992; Smith et al 2005).

Even though the activity of amylases in dormant corn seeds is generally low, it may have an important role in some fermentation processes. For example, novel fermentation processes to produce ethanol from dry-grind corn are undertaken at ambient temperature (e.g. 27 ºC) and rely on endogenous amylases to aid starch hydrolysis along with exogenously added raw-starch hydrolyzing enzymes (Lewis et al 2005). Thus, preservation of the endogenous enzyme activity is crucial for corn kernels intended for the seed and ethanol industries.

Freshly harvested corn kernels are usually dried artificially to reduce the moisture content and prevent microbial growth during storage. Elevated drying temperatures have been reported to change quality characteristics of corn kernels, such as protein solubility and protein moisture-binding capacity, among others (Malumba et al 2009; McGuire and Earle 1957; Peplinski et al 1994; Wall et al 1975). Peplinski et al. (1994) reported that little if any changes in kernel physical properties occurred when corn was subjected to drying temperatures between 25˚C and 50˚C; kernels dried at 55˚C had significantly reduced germination potential, whereas those dried at temperature above 55 ˚C did not germinate. The effect of kernel drying temperature on endogenous amylase activity of corn is well not understood.

In the present study, freshly harvested kernels of three corn hybrids were subjected to drying at selected air temperatures between 10˚C and 125˚C and assayed for the total amylolytic activity and the activity of individual amylases. The results of this study provide a better understanding of how kernel drying temperature affects endogenous amylase activity of corn kernels.

MATERIALS AND METHODS

Materials

Commercial corn hybrids Dekalb 61-58, Agrigold, and Dekalb 61-66 designated as 49, 50, and 51, respectively, were grown in the North Central Iowa in 2007 growing seasons. Freshly harvested kernels of each hybrid (15kg) were divided into 15 parts (1kg each) and immediately dried to 14% moisture content. Initial moisture contents of kernels were 22.7%,

23.6%, and 27.9% for hybrids 49, 50, and 51, respectively. Drying was stopped at a predetermined final weight on the basis of the initial weight and the moisture content of each sample. The air drying temperatures were 10ºC, 25ºC, 45ºC, 65ºC, and 85ºC, whereas the approximate kernel temperatures were 12ºC, 22ºC, 38ºC, 58ºC, and 77ºC, respectively. The drying at each temperature was done in triplicate for each corn hybrid. The drying with air temperatures of 10ºC and 25ºC was conducted in a barrel dryer, whereas the drying at higher temperatures was conducted in a forced convection oven. The order of temperatures was randomized. The oven had a digital temperature setting that was verified with a NIST-traceable glass thermometer. Prior to drying, kernels were placed in plastic perforated bags, and stretched into 1-2 cm layers to allow their uniform drying in the oven. The study was repeated in the following year using crops grown in 2008 season. Kernels of the same three hybrids grown at the same location were dried at 25ºC, 105ºC, and 125ºC using the conditions explained earlier. Kernels of each hybrid were dried at each of these temperatures in triplicate. Antimicrobial agents, IsoStab^{TM} was a product of BetaTech Hop Products (Washington, DC), and Lactrol® was from PhibroChem (Ridgefield Park, NJ). All chemicals used in the study were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher-Scientific (Waltham, MA) unless otherwise noted.

Endogenous Amylolytic Activity in Corn Kernels

Corn kernels were surface cleaned with 3% hydrogen peroxide and ground to pass 0.5mm screen prior to analysis. The glassware and accessories used for the analysis were sterilized by autoclaving. The ground corn sample (15 mg) was suspended in a sodium acetate buffer solution (pH 4.8, 0.1 M, 5ml) containing calcium chloride (3 mM) and antimicrobial reagents (2 ppm Lactrol®, and 40 ppm IsoStab^{TM}). The suspension was vortex-

mixed and incubated at 40˚C with shaking (120 rpm) for 0, 3, 6, 12 and 20 hours. After each time interval, the samples were removed, heated in a boiling-water bath for 15 minutes with stirring, and centrifuged at 3222 x *g* for 10 minutes. The reducing sugar concentration of the supernatant was determined using the Somogyi- Nelson method (Nelson 1944; Somogyi M 1945). A standard curve was made with glucose of selected concentrations. The amylolytic enzyme activities were presented as the percentage of reducing sugars generated from the initial dry starch weight of corn sample.

Gel Zymogram Analysis

The activity analysis of amylolytic enzymes using zymogram was performed following the procedure of Dinges et al (2001) with slight modifications. Surface cleaned corn kernels were ground in liquid nitrogen using mortar and pestle. The ground corn sample (0.75g) was suspended in Tris HCl buffer (pH 7.5, 50 mM, 2 ml) containing 10 mM dithiothreitol (DTT) and incubated at 25ºC with shaking (100 rpm) for 1 hour. The suspension was centrifuged at 9165 x *g* to obtain a clear enzyme extract. The enzyme extract (7 µl for α-amylase and 9.3 µl for debranching enzymes detection) was applied to a 4-15% native gradient polyacrylamide gel (Sigma, St. Louis, MO) and separated at 4°C, 9 mA for 4 h using a Protean II cell (Bio-Rad Laboratories, Hercules, CA) in an electrode buffer (25 mM Tris, 192 mM Gly, pH 8.8, and 2 mM dithiothreitol). After the separation, the gel was electroblotted to a polyacrylamide gel containing 7% (w/v) acrylamide, 0.3% (w/v) normal corn starch (Cargill, Wayzata, MN), and 375 mM Tris-HCl (pH 8.8) overnight at 4ºC and 20 V in the electrode buffer. Amylase activities were detected by staining the gel with I_2/KI solution. The amylolytic enzymes were identified on the zymogram gels following the method of Dinges et al. (2001) and Dinges et al. (2003).

β-Amylase Activity of Kernels

β-Amylase activity present in corn kernels was determined using a BETA AMYLASE (Betamyl-3 method) assay kit (Megazyme Intl., Wicklow, Ireland; catalog no. K-BETA3). One unit of β-amylase activity was defined as the amount of enzyme, in the presence of excess thermostabile β-glucosidase, able to release 1 μmol of *p*-nitrophenol from *p*-nitrophenyl- β-D-maltotrioside per min under defined assay conditions.

RESULTS AND DISCUSSION

The starch hydrolysis rates of hybrid 49, 50 and 51 ground corn kernels incubated in a sodium acetate buffer with endogenous amylases are displayed in **Figures 1** and **2**. No significant difference ($p>0.05$) in the rate of starch hydrolysis in ground corn suspensions was observed between samples dried at 10˚C, 25˚C, 45˚C, and 65˚C air temperatures (6.5-7.4%, 6.6-8.0%, 6.4-8.1%, 6.5-7.6% starch hydrolysis after 20h incubation, respectively). The corresponding kernel temperatures during drying were 12ºC, 22ºC, 38ºC, and 58ºC, respectively. In suspensions of corn kernels dried at 85˚C air temperatures (77˚C kernel temperature), however, the starch hydrolysis rates were significantly reduced (6.4, 5.0, and 6.2% after 20h incubation for hybrids 49, 50, and 51, respectively) compared with the controls dried at 25˚C ($p<0.05$) (**Figures 1A**, **1B**, and **1C**). The results indicated that air temperatures below 85ºC were not sufficiently high to cause severe changes in the enzyme activity of kernels. Thus, the study was repeated in the following year with crops grown in 2008 season using 25˚C, 105˚C and 125˚C air temperatures (**Figures 2A**, **2B**, and **2C**). It was found that the reduction in the endogenous amylase activities became more severe as the air

drying temperature increased to 100˚C and 125˚C (3.7-5.1% and 2.6-3.1% starch hydrolysis after 20h incubation for temperatures 100˚C and 125˚C, respectively).

It must be noted that enzymes other than amylases are capable of producing reducing sugars in the ground corn suspension, such as cellulases, hemicellulases, and invertase, and might overestimate values obtained for the percentage of hydrolyzed starch. Soluble sugars in ground corn suspensions before the incubation were maltose and maltoheptaose, and those sugars along with starch molecules were hydrolyzed to glucose after 20 hours of incubation (results not shown). Thus, the contribution of invertase and pentosanases to the production of reducing sugars was ruled out. The activity of β-glucosidase in dormant corn kernels has not been reported in the literature. While the possibility of β-glucosidase activity during the reaction could not be eliminated, it is very unlikely that it interfered with assaying of amylases to any large extent. First, starch can be readily hydrolyzed to provide energy required for plant metabolism, whereas cellulose serves as a structural component and to prevent pest and microorganism attack and thus, cannot be easily degraded. Second, starch is a major component of corn kernels comprising 61-78%, whereas cellulose and lignin together comprise only 3.3-4.3% of kernel dry basis (Watson 2003). Consequently, even if β-glucosidase was present in the corn suspension, its activity would be very low due to small concentration of the substrate and thus, would not significantly interfere with assaying of amylases.

Individual amylases were separated and detected on zymogram gels, and their activity was assessed by determining the intensity of the corresponding bands. The zymograms showed that the activity of α-amylase in corn kernels was not significantly affected by drying temperatures up to 85˚C. Kernels dried at 105˚C and 125˚C, however, displayed significantly

reduced α-amylase activity, as shown by diminished intensity of the corresponding bands on the zymogram gels (**Figures 3A** and **3B**). Some residual activity of α-amylase was observed in the kernels even after drying at 125˚C air temperature. This might be explained by: 1) a very fast rate of drying at such high temperatures and consequently, short drying time that did not allow prolonged heating of the kernel interior, and 2) the low moisture content of kernels before drying (22.7-27.9%) that allowed limited enzyme denaturation within the kernel during the drying. Pullulanase- (also known as R-enzyme and limit dextrinase) and isoamylase-type debranching enzymes, which play an important role in both biosynthesis and degradation of starch in plants (Beatty et al 1999; Nakamura et al 1996; Smith et al 2005), were found to be more heat labile than α-amylase (**Figure 3C**). Kernels dried at 65˚C and 85˚C air temperatures displayed a reduced activity of isoamylase- and pullulanase-type branching enzymes as shown by the low intensity of their corresponding bands, whereas those dried at 105˚C and 125˚C did not show bands on the zymogram gel. Even though this result might indicate a complete loss of pullulanase and isoamylase activity at such high temperatures, some residual activity might still be remained but was bellow the detection level. β-Amylase band did not appear on the zymogram gels, demonstrating low or no activity in corn kernels. This was also confirmed by an alternative analysis that employed preparation of crude amylase extract from ground corn and its reaction with p-nitrophenyl-β-D-maltotrioside (**Figure 4**), which indicated very low activity of β-amylase in the corn kernels. The activity of β-amylase in kernels was found to vary significantly with different corn hybrids. Kernels dried at 25˚C (control samples) contained β-amylase activity at levels of 0.37, 0.39, and 0.70 U/g kernel db for hybrids 49, 50, and 51, respectively. Despite differences in the initial β-amylase activity of kernels, all three hybrids responded in a similar

fashion to the drying temperature treatment. The corn kernels dried at 10˚C and 25˚C had very similar levels of β-amylase activity (0.38-0.77 and 0.37-0.70 U/g kernel db, respectively), and the activity in kernels was significantly reduced after drying at temperatures of 45˚C and higher (0.07-0.43 and 0.00-0.15U/g kernel db, for temperatures 45˚C and 125˚C, respectively). The activity of α-glucosidase was not analyzed in this study.

The results of this study are in agreement with previous studies that reported a negligible level of β-amylase activity in mature corn kernels (Laurière et al 1992). Also, β-amylase from corn kernel has shown to be more heat labile than α-amylase. Wang et al. (1992) has reported that the enzyme lost its activity within 5min of heating at 65˚C, whereas α-amylase retained 80% of its activity after heating at 70˚C for 15min.

In summary, no significant difference in the total amylase activity was observed between samples dried at 10˚C, 25˚C, 45˚C and 65˚C. Air temperatures of ≥85˚C reduced the activity of endogenous amylases, but the activity in corn kernels was not completely lost even after the drying at 125˚C. The least stable amylase was β-amylase that showed reduced activity in kernels after drying at ≥45˚C, followed by the starch debranching enzymes (pullulanase and isoamylase) at ≥85˚C. The activity of α-amylase remained relatively unchanged in kernels with drying temperatures up to 85˚C, but it was significantly reduced after drying at 105˚C and 125˚C.

CONCLUSIONS

The results of this study indicate that the drying temperature of kernels has a profound effect on the activity of endogenous amylases present in the kernel. The endogenous starch hydrolysis in ground corn suspensions was significantly reduced after ≥

85ºC drying of kernels. Different classes of amylases were found to have different susceptibility to the drying temperature treatment.

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REFERENCE CITED

Beatty, M. K., Rahman, A., Cao, H., Woodman, W., Lee, M., Myers, A. M., James, M. G. 1999. Purification and Molecular Genetic Characterization of ZPU1, a Pullulanase-Type Starch-Debranching Enzyme from Corn. Plant Physiol. 119 (1): 255-266.

Beck, E., Ziegler, P. 1989. Biosynthesis and degradation of starch in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:95-117.

Brown, R. B., Fulford, G. N., Daynard, T. B., Meiering, A. G., Otten, L. 1979. Effect of drying method on grain corn quality. Cereal Chem. 56(6): 529-532.

Dinges, J. R., Colleoni, C., Myers, A. M., James, M. 2001. Molecular structure of three mutations at the corn sugary1 locus and their allele-specific phenotypic effects. Plant Physiol. 125: 1406-1418.

Dinges, J. R., Colleoni, C., Myers, A. M., James, M. 2003. Mutational analysis of the pullulanase-type debranching enzyme of corn indicates multiple functions in starch metabolism. The Plant Cell 15:666-680.

Estrada, J. A., Litchfield, J. B. 1993. High humidity drying of corn: Effect on drying rate and product quality. Dry. Technol. 11(1): 65-84.

Laurière, C., Doyen, C., Thèvenot, C., Daussant, J. 1992. β-Amylases in cereals. A study of the corn β-amylase system. Plant Physiol. 100: 887-893.

Lewis, S. M., Van Hulzen, S.E., & Roth, D. L. 2005. Continuous process for producing ethanol using raw starch. U.S. Patent number 2005/0239181 A1

MacGregor, A. W. 1987. α- Amylase, limit dextrinase, and α-glucosidase enzymes in barley and malt. CRC Crit. Rev. Biotech. 5: 117-128.

Malumba, P., Janas, S., Masimango, T., Sindic, M., Deroanne, C., Béra, F. 2009. Influence of drying temperature on the wet-milling performance and the protein solubility indexes of corn kernels. J. Food Eng. 95: 393-399.

McGuire, T. A., Earle, F. R. 1957. Changes in the solubility of corn protein resulting from the artificial drying of high-moisture corn. Cereal Chem. 35: 179-188.

Nakamura, Y. 1996. Some properties of starch debranching enzymes and their possible role in amylopectin biosynthesis. Plant Sci. 121: 1-18.

Nelson, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375 – 380.

Peplinski, A. J., Paulis, J. W., Bietz, J. A., Pratt, R. C. 1994. Drying of high-moisture corn: Changes in properties and physical quality. Cereal Chem. 71(2): 129-133.

Smith, A. M., Zeeman, S. C., Smith, S. M. 2005. Starch degradation. Annu. Rev. Plant Biol. 56: 73-98.

Sanwo, M. M., DeMason, D.A. 1992. Characteristics of α-amylases during germination of two high-sugar sweet corn cultivars of Zea mays L. Plant Physiol. 99: 1184-1192.

Somogyi, M. 1945. A new reagent for the determination of sugars. J. Biol. Chem. 160: 61 – 68.

Subbarao, K. V., Datta, R., Sharma, R. 1998. Amylases synthesis in scutellum and aleurone layer of corn seeds. Phytochem. 49 (3): 657-66.

Sun, Z., and Henson, C. A. 1991. A quantitative assessment of the importance of barley seed α-amylase, β-amylase, debranching enzyme and α-glucosidase in starch degradation. Arch. Biochem. Biophys. 284: 298-305.

Wall, J. S., James, C., Donaldson, G. L. 1975. Corn proteins: Chemical and physical changes during drying of grain. Cereal Chem. 52: 779-790.

Wang, S-M., Wu, S-Y., and Chen, J. 1992. Purification and characterization of beta-amylase in corn kernel. Bot. Bull. Acad. Sin. 33: 359-367.

Watson, S. Description, development, structure, and composition of the corn kernel. Pages 69-106 in: Corn chemistry and technology. P. J. White and L. A. Johnson, eds. American Association of Cereal Chemists, Inc.: St. Paul, MN.

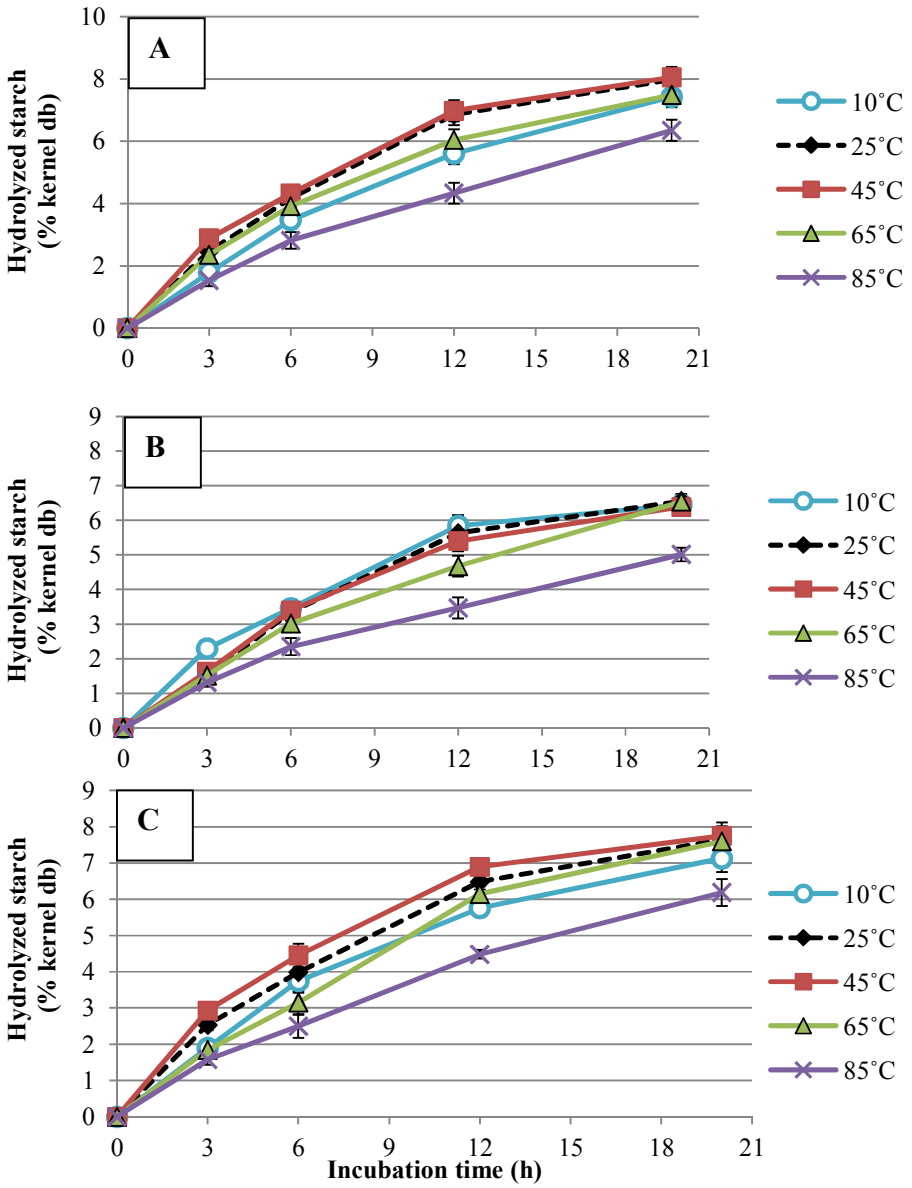


Figure 1. Starch hydrolysis rates of corn kernels dried at different air temperatures with endogenous amylases. **A**- hybrid 49, **B**- hybrid 50, **C**- hybrid 51. Percentage hydrolyzed starch (% ground corn db) = total mass of produced reducing sugars expressed as glucose/ initial dry mass of starch present in ground corn x162/180x100. Data points represent means of three replicates ± standard errors.

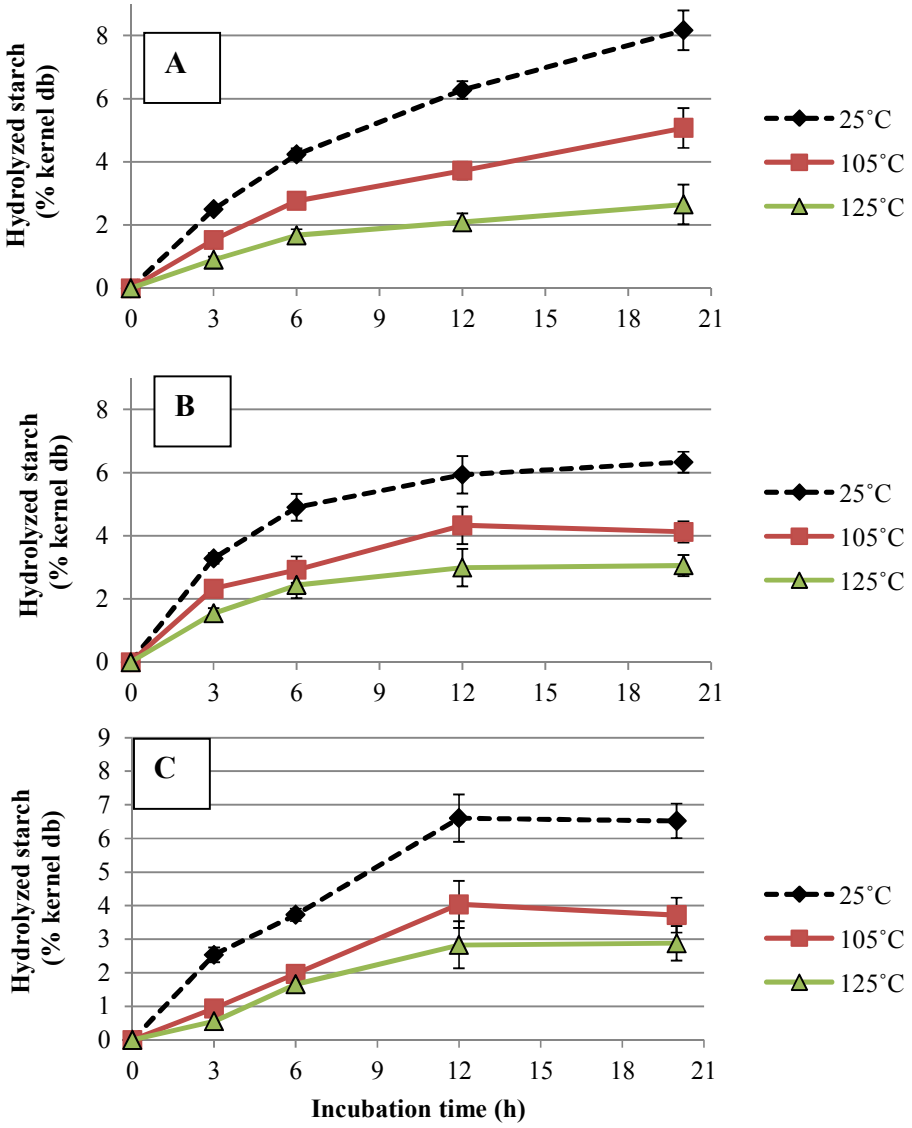


Figure 2. Starch hydrolysis rates of corn kernels dried at different air temperatures with endogenous amylases. **A**- hybrid 49, **B**- hybrid 50, **C**- hybrid 51. Percentage hydrolyzed starch (% ground corn db) = total mass of produced reducing sugars expressed as glucose/ initial dry mass of starch present in ground corn x162/180x100. Data points represent means of three replicates ± standard error.

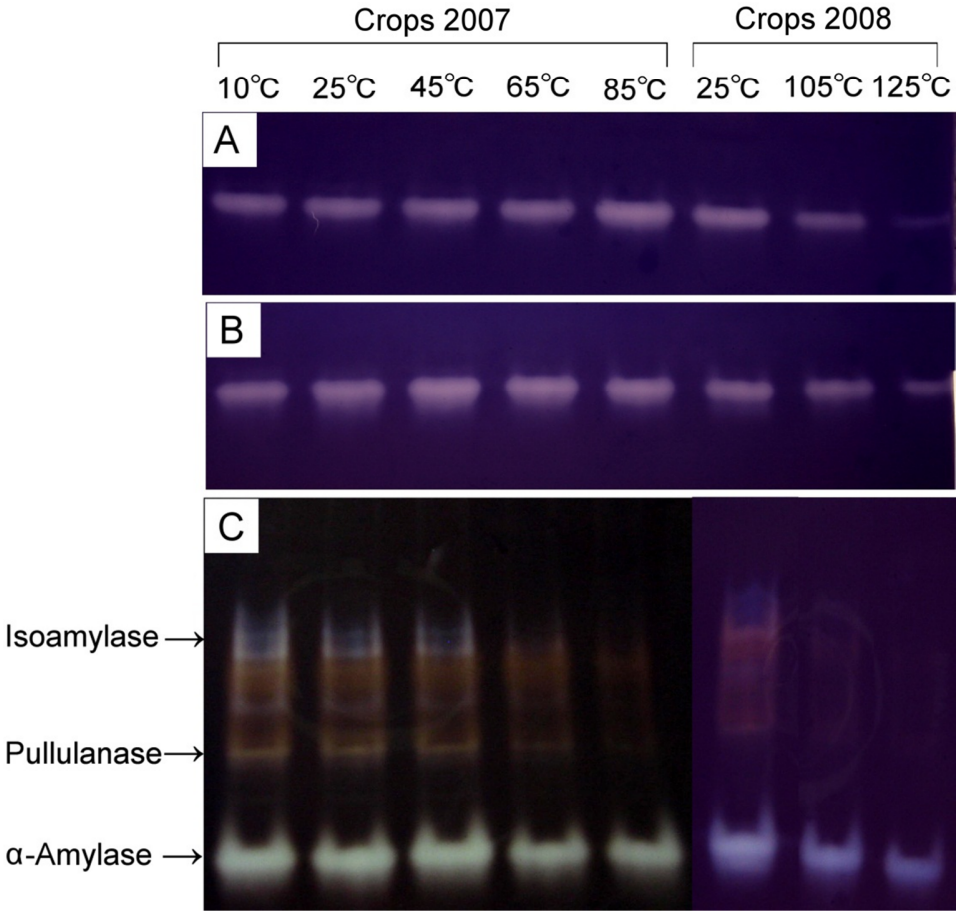


Figure 3. Gel zymogram analysis of individual amylases in corn kernels dried at different temperatures. **A**- α-Amylase activity in kernels of hybrid 49, **B**- α-Amylase activity in kernels of hybrid 50, **C**- individual amylases in corn kernels of hybrid 51. The amount of crude enzyme extract applied to a native PAGE gel was 7 µl (**A** and **B**) and 9.3 µl (**C**).

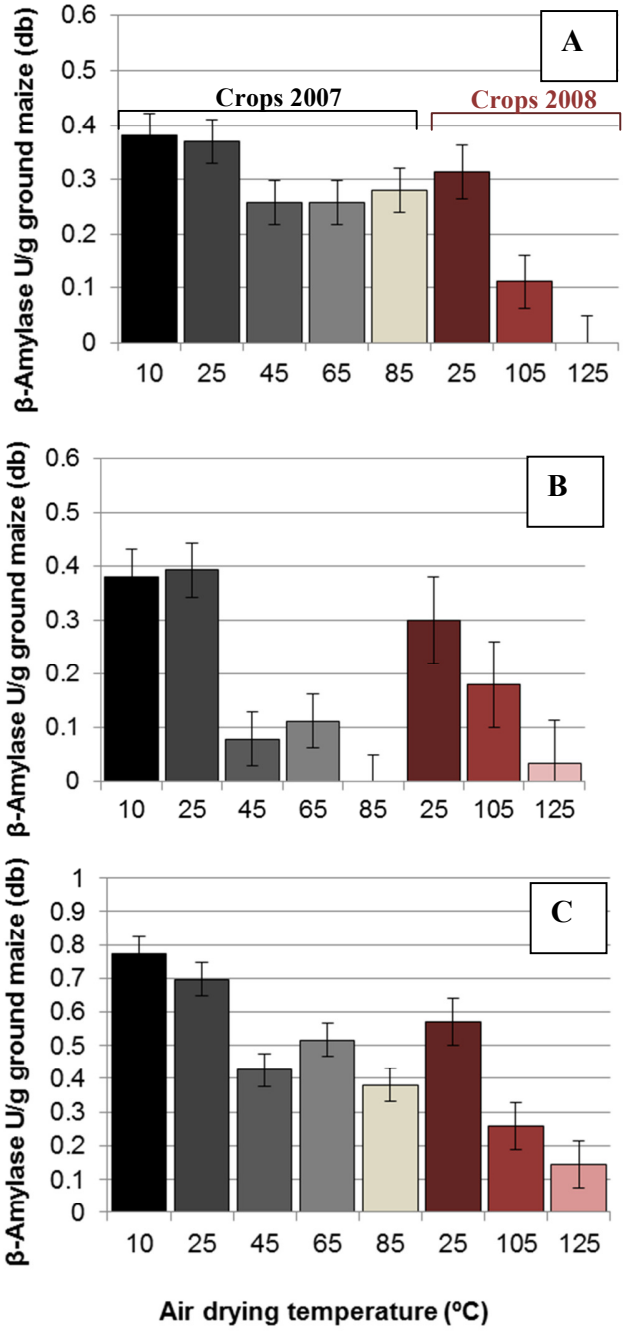


Figure 4. Endogenous β-amylase activity in corn kernels of **A**- hybrid 49, **B**- hybrid 50, and **C**- hybrid 51dried at different air temperatures. Bars represent means of three replicates ± standard errors. One unit of β-amylase activity is defined as the amount of enzyme able to release 1 µmol of *p*-nitrophenol from *p*-nitrophenyl- β-D-maltotrioside per min.

CHAPTER 8. GENERAL CONCLUSIONS

The results of this study demonstrated that the optimum planting dates for the studied corn hybrids ranged between 2 May and 15 May during the 2007-2009 growing seasons. Planting corn 2-4 weeks after the optimum dates reduced the grain yield and starch contents of kernels, but did not affect the protein content of kernels.

Starch isolated from kernels planted in mid-April and early May contained larger number of granules with the diameter >20 μm and granules with dimple-like indentations on the surface, and larger contents of amylose than those planted in June. The results suggested that starch granules isolated from corn planted early had more time to develop and grow to very large sizes than the granules from corn planted late. The effect of planting date of corn crops on the amylopectin branch-chain length varied with corn varieties and/or planting locations.

The alterations in the starch structures, caused by delayed planting date of corn, affected thermal and pasting properties of starch, but this change might not be of sufficient magnitude to impose major problems in processing of products containing starch.

Delayed planting date of corn did not affect the ethanol production from a processing perspective as indicated by the similar ethanol yields (calculated on the dry basis of ground corn kernels) of kernels planted on various planting dates. On the basis of corn planting area, however, the ethanol yield (L/ha) was found to decrease significantly with planting dates delayed into late May and June. This could be attributed to the reduced grain yields of corn crops planted on late dates, which resulted in small ethanol yields.

The results of the Chapter 3 showed that the drying temperature of kernels altered functional properties of starch, such as gelatinization temperature, enthalpy change, swelling power, and percent solubility of starch. Changes in the starch properties negatively affected the rate of starch hydrolysis and consequently, reduced the ethanol yield of ground kernels dried at 45˚C, 65˚C, and 85˚C. The most severe reduction in the ethanol yield was observed for kernels dried at 85˚C, which might be explained by the largest reduction in the starch swelling power that inhibited enzyme penetration into the starch granule interior in addition to the reduced endogenous enzyme activity of kernels.

Ground kernels dried at 10˚C, 25˚C, 45˚C and 65˚C displayed similar levels of amylolytic activity, as shown in the Chapter 4. Elevated air temperatures of ≥85˚C partially damaged the endogenous amylases as indicated by the reduced endogenous starch hydrolysis rate. Among the endogenous amylases, β-amylase was most heat-labile and showed reduced activity after the kernel was dried at 45°C. Pullulanase and isoamylase showed reduced enzyme activity at 85°C air drying temperature. The α-amylase was relatively stable up to 85°C but significantly lost its activity after drying at 105 and 125°C air temperature.

The results indicated that drying of kernels at elevated temperatures ≥85˚C is not desirable for corn lots intended for the ethanol industry.

APPENDIX A. EFFECT OF CORN DEVELOPMENTAL STAGE ON ACTIVITY OF ENDOGENOUS STARCH HYDROLYZING ENZYMES IN CORN SEEDS AND ETHANOL YIELD OBTAINED FROM THE COLD FERMENTATION PROCESS

Objectives

The activity of endogenous enzymes changes during corn kernel development and increases significantly during germination. In the conventional dry-grind ethanol production process, endogenous enzymes are inactivated during a jet-cooking process and lose the ability to hydrolyze starch. In the cold-fermentation process, simultaneous starch hydrolysis and fermentation take place at the ambient temperature, and consequently, the endogenous enzyme activity of corn is preserved. Thus, the endogenous enzymes contribute to starch hydrolysis during the fermentation process, which reduces the need for addition of the exogenous starch hydrolysing enzymes to the process.

The objective of this study was to understand changes in hydrolytic enzyme activity of corn during maturation. Kernels of five corn GEM lines grown in 2007 were harvested on 37 days after pollination (DAP), 45 DAP, and 55 DAP and were subjected to tests of hydrolytic enzyme activities and analysis of the hydrolytic products. In addition, the effect of corn maturity on the starch content and ethanol yield produced from corn using the cold-fermentation process was investigated.

Materials and Methods

Materials

GEM corn lines were provided by Dr. Michael Blanco of USDA-ARS Plant Introduction Station (Ames, IA). Five GEM lines were harvested at different stages of maturity. Corn 05GEM02989, 05GEM03094, 05GEM02683, 05GEM03099 and 05GEM06031 lines were harvested on 37, 45 and 55 DAP. Samples were ground using a Cyclone Mill (UDY cor., CO, USA) with 0.5 mm screen. Moisture contents of the dry-ground corn samples were measured to determine the dry weight of each sample. All the chemicals were purchased from Fisher Chemicals (Waltham, MA). The total starch and glucose diagnostic kits were purchased from Megazyme International (Wicklow, Ireland; catalog no. K-TSTA and K-GLUC, respectively).

Methods

Total Starch Content. The total starch content of GEM line ground corn was determined following the AOAC 996.11 method. The analysis was done using the Megazyme total starch assay kit. The GEM line ground corn sample (100mg) was placed in a plastic centrifuge tube, and 0.2 mL of aqueous ethanol (80 % v/v) was added to aid dispersion. The tube was vortex-mixed, and 3 mL of thermostable α-amylase (300 U) in a MOPS buffer (50mM, pH 7.0) was added. The tube was heated in a boiling-water bath for 6 minutes, and then transferred into a water bath at 50 ˚C. After the temperature was equilibrated, 4 mL of 200 mM sodium acetate buffer (pH 4.5) was added and mixed, followed by the addition of 0.1 mL amyloglucosidase (20 U). The mixture was incubated for 30 minutes. The entire content of the tube was transferred into a 100 mL-volumetric flask, and volume was made to 100 mL with deionized water and thoroughly mixed. An aliquot of this diluted solution was centrifuged at 1704 x *g* for 10 minutes. The supernatant (0.1 mL) was mixed with 3 mL of GOPOD reagent and incubated at 50 ˚C for 20 minutes. The

absorbance was read at 510 nm against the reagent blank. The total starch content (TSC) was calculated following the equation:

TSC (%) = ΔA x F x 100/0.1 x 1/1000 x 100/W x 162/180 x 100/(100 – moisture content (% w/w))

ΔA: absorbance read against the reagent blank

F: 100 divided by absorbance for 100 µg of glucose

W: sample weight

Determination of starch degrading enzyme activities. The ground corn sample (15 mg) was suspended in 3 ml of 0.1 M sodium acetate buffer (pH 5), 3 mM calcium chloride, 2 ppm lactrol, and 40 ppm isostab . The glassware and supplies used in the preparation of suspension were sterilized. The suspension was vortex-mixed and placed in a water bath at 40 ˚C and shaken at 120 rpm. The samples were incubated for 0, 2, 4, 6, 12 and 20 hours. After each time interval, the samples were removed and heated in a boiling-water bath for 15 minutes to solubilise released sugars. After heating, the samples were centrifuged at 3222 x *g* for 20 minutes. The reducing sugar content in the supernatant was determined using Somogyi- Nelson method (Somogyi M., 1945; Nelson N., 1944). Proper dilutions of the supernatants were made and 1 ml aliquots were mixed with 1 ml reagent D that contained cupric sulphate. The concentration of reducing sugar was determined by measuring the amount of Cu_2O formed. The cuprous oxide reacted with an arsenomolybdate reagent (reagent C) that gave a blue-green color measured at 520 nm. The absorbance value was converted into grams of reducing sugars by using a standard curve made with glucose of selected concentrations. The starch degrading enzyme activities were presented as the

percentage of reducing sugars generated from the initial dry starch weight in the flour (% hydrolyzed starch).

Glucose and reducing sugar yield of ground corn samples incubated for 20h. The reducing sugar content in ground corn samples, initially and after 20 h incubation time, was determined using Somogyi-Nelson method described above. Reducing sugar yield was calculated as the percentage of reducing sugars in the ground corn (dry basis) subtracted by percent initial reducing sugar content in the ground corn (dry basis).

The glucose yield was calculated in the same way as reducing sugar yield. Glucose contents in the initial corn sample weights and after 20 hour incubation time were determined using the GOPOD reagent.

Molecular weight distribution of starch present in ground corn. The molecular-weight distributions of starches in the ground corn before and after endogenous enzyme hydrolysis were determined following the method of Jane and Chen (1992). Corn flour (100 mg) was dispersed in 90% dimethysulfoxide (DMSO) (10 ml). The suspension was mechanically stirred while heating in a boiling-water bath for 1 h and then stirred at 25ºC for 12 h. An aliquot (2 ml) was mixed with 5 volume of ethanol (10 ml) to precipitate starch. The precipitate was separated by centrifugation at 6,750 x *g* for 20 min. The starch pellet was then redissolved in boiling water (10 ml) and mechanically stirred for 30 min in a boiling-water bath. The sample dispersion was filtered through a nylon membrane filter (5.0 μm). The filtered sample (2 ml) containing 4-5 mg of starch was injected into a column (0.5 × 50 cm) packed with Sepharose CL-2B gel. Deionized water containing 10 mM NaOH and 50 mM NaCl was used as the eluent. Fractions of 2 ml each were collected and analyzed for

total carbohydrate content (phenol-sulfuric acid method) and blue value (iodine staining) at 490 and 630 nm, respectively.

Qualitative analysis of simple sugars by thin layer chromatography. Soluble sugars, present in the ground corn suspension, were determined and identified using thin layer chromatography. The analysis was done following the procedure described by Atichokudomchai et al. (2006).

Summary of results

Kernels of 2007 GEM lines harvested on 37 DAP had a lower starch and higher reducing sugar content than the kernels harvested at later stages of maturity (45 and 55 DAP) (**Tables 1 and 2**). Endogenous enzyme activity of 2007 crops varied with different genetic background and maturity stages. Corn lines 05GEM02989, 05GEM03094, and 05GEM06031 did not show significant differences in the endogenous enzyme activity between different maturation stages, whereas 05GEM02683 and 05GEM03099 lines showed the largest enzyme activities in the early stage of development (37 DAP) and declined in the activity during maturation (**Figure 1**).

The starch molecular weight distribution of the ground corn after 0h and 20h incubation did not show detectable reduction in the molecular weight, suggesting that the endogenous enzymes did not show liquefying effect, but rather hydrolyzed starch to produce a large number of small molecules (**Figure 3**). Maltose and maltoheptaose, initially present in the ground corn, were completely hydrolyzed to glucose after 20 hours of incubation (**Figures 4 and 5**). These results suggested presence of glucoamylase in the ground corn samples.

Because of limited quantities of samples, 2007 crops of GEM corn samples were not fermented to determine their potential for ethanol production. Instead, 2008 crops of the GEM corn were obtained and subjected to fermentation reaction.

Starch contents of the 2008 GEM line corn samples varied between lines and maturity as shown in **Table 3**. The starch content analysis of five corn lines showed the lowest contents on 37 DAP, and continued to increase up to 55 DAP. For all the corn lines, except 05GEM06031, the starch yield dropped on 60 DAP. The 05GEM06031 line showed a steady increase in the starch content over the whole maturation period and reached the starch yield of 74.59% (db) on 60 DAP. The yield was the highest among the corn lines studied. Other corn lines, 05GEM02683, 05GEM02989, 05GEM03099, and 05GEM03094 had the starch contents of 72.97%, 72.07%, 67.81%, and 64.91% on 55 DAP. Samples harvested in 2008 had starch contents comparable to those harvested in 2007. It was concluded that in terms of starch content, optimal harvesting time for corn was on 55 DAP. Keeping corn longer in the field resulted in a loss of starch that was either utilized for corn respiration (since leaves are already withered at this stage) or hydrolyzed by endogenous enzymes in a process of germination.

In average, ethanol yields of 2008 GEM corn varied slightly after four days of fermentation (**Figure 6A**); samples harvested on 55 DAP had the highest yield (20.35 %v/v), followed by those harvested on 60 DAP (20.32 %v/v), 45 DAP (20.29 %v/v), and 37 DAP (20.24 %v/v), respectively. Rate of fermentation, however, varied significantly with different maturation stages. Samples harvested on 37 DAP had the slowest rate of fermentation, followed by those of 55 DAP and 60 DAP, and the highest was for that of 45 DAP.

Fermentation reaction plateaued after 3 days of fermentation for samples harvested on 45 DAP.

Ethanol yields varied with different genetic background (**Figures 6B, 6C, 6D, 6E, and 6F**). Lines 05GEM02989, 05GEM02683, and 05GEM06031 gave large ethanol yields, 20.93% (v/v), 20.91% (v/v), and 20.76% (v/v), respectively. The corn lines with low starch content (05GEM03099 and 05GEM03094) gave small ethanol yields of 20.47% (v/v) and 19.56% (v/v), respectively.

Conclusions

Starch contents of corn samples harvested on different maturation dates varied between lines and maturity stages. Corn harvested in early stages of maturation (37 DAP) gave the lowest starch and largest reducing sugar contents. The starch content of corn increased up to 55 DAP and dropped again on 60 DAP (except for one corn line). Endogenous enzyme activity in corn varied with different genetic background and maturity stages. Some corn lines did not show significant differences in the endogenous enzyme activity between different maturation stages, whereas others showed the largest enzyme activities in the early stage of development (37 DAP) and declined in the activity during maturation.

Products of endogenous enzyme hydrolysis were mainly small molecules. Maltose and maltoheptaose, initially present in the ground corn, were completely hydrolyzed to glucose after 20 hours of incubation. The results suggested presence of glucose-producing enzyme in the ground corn samples.

Ethanol yields of corn samples varied slightly at the end of four days of fermentation; samples harvested on 55 DAP had the largest ethanol yield, followed by those harvested on

60 DAP, 45 DAP, and 37 DAP, respectively. Samples harvested on 37 DAP had the slowest rate of fermentation, followed by those of 55 DAP and 65 DAP, and the highest was for that of 45 DAP. A modest positive correlation between the starch content and ethanol yield was found in these samples.

Reference cited

Atichokudomchai N., Jane J., and Hazlewood G. 2006. Reaction pattern of a novel thermostable α-amylase. Carbohydr. Polym. 64 (4):582-588

Nelson, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. J. Biol. Chem. 153**:**375 – 380.

Somogyi, M. 1945. A new reagent for the determination of sugars. J. Biol. Chem. 160:61 – 6

Table 1. Total starch contents (%) of 2007 corn GEM line and B73 inbred corn ground kernels harvested on selected days after pollination (DAP)

Stage of development	**Corn lines**					
(DAP)	**05GEM02989**	**05GEM02683**	**05GEM03099**	**05GEM03094**	**05GEM06031**	**B73**
37	74.24± 0.5	67.88± 0.5	68.12± 0.2	66.47± 0.0	68.00± 0.0	
45	73.94± 0.08	68.12± 0.0	68.53± 0.25	67.08± 0.25	70.00± 0.58	
55	73.06± 0.33	69.12± 0.08	68.12± 0.17	67.47± 0.5	70.76± 0.50	67.60 ± 1.98
60	-	-	-	-	-	69.75 ± 3.42
65	-	-	-	-	-	70.59 ± 1.52

Table 2. The initial content of reducing sugars of the GEM corn line kernels

Corn line	% Reducing sugars		
	37 DAP	**45 DAP**	**55 DAP**
05GEM02989	2.13 ± 0.05	1.47 ± 0.06	1.56 ± 0.07
05GEM03099	2.44 ± 0.03	1.5 ± 0.12	1.37 ± 0.09
05GEM06031	1.65 ± 0.03	1.69 ± 0.02	1.37 ± 0.02
05GEM03094	1.25 ± 0.1	0.73 ± 0.41	1.11 ± 0.05
05GEM02683	1.27 ± 0.04	1.23 ± 0.03	1.02 ± 0.01
Mean	1.75	1.32	1.29

Table 3. Total starch contents (% db) of ground corn kernels harvested on different days after pollination (DAP) in 2008

Stage of development	**Corn lines**				
(DAP)	**05GEM02989**	**05GEM02683**	**05GEM03099**	**05GEM03094**	**05GEM06031**
37	68.73±1.6	67.00±1.2	65.76±1.5	63.81±1.7	69.66±1.5
45	71.65±2.0	69.14±1.0	63.62±1.4	65.64±1.4	70.58±1.6
55	72.07±1.1	72.97±1.2	67.81±1.2	64.91±1.0	73.46±1.8
60	70.20±0.6	70.73±1.1	66.04±1.2	64.30±1.3	74.59±1.7

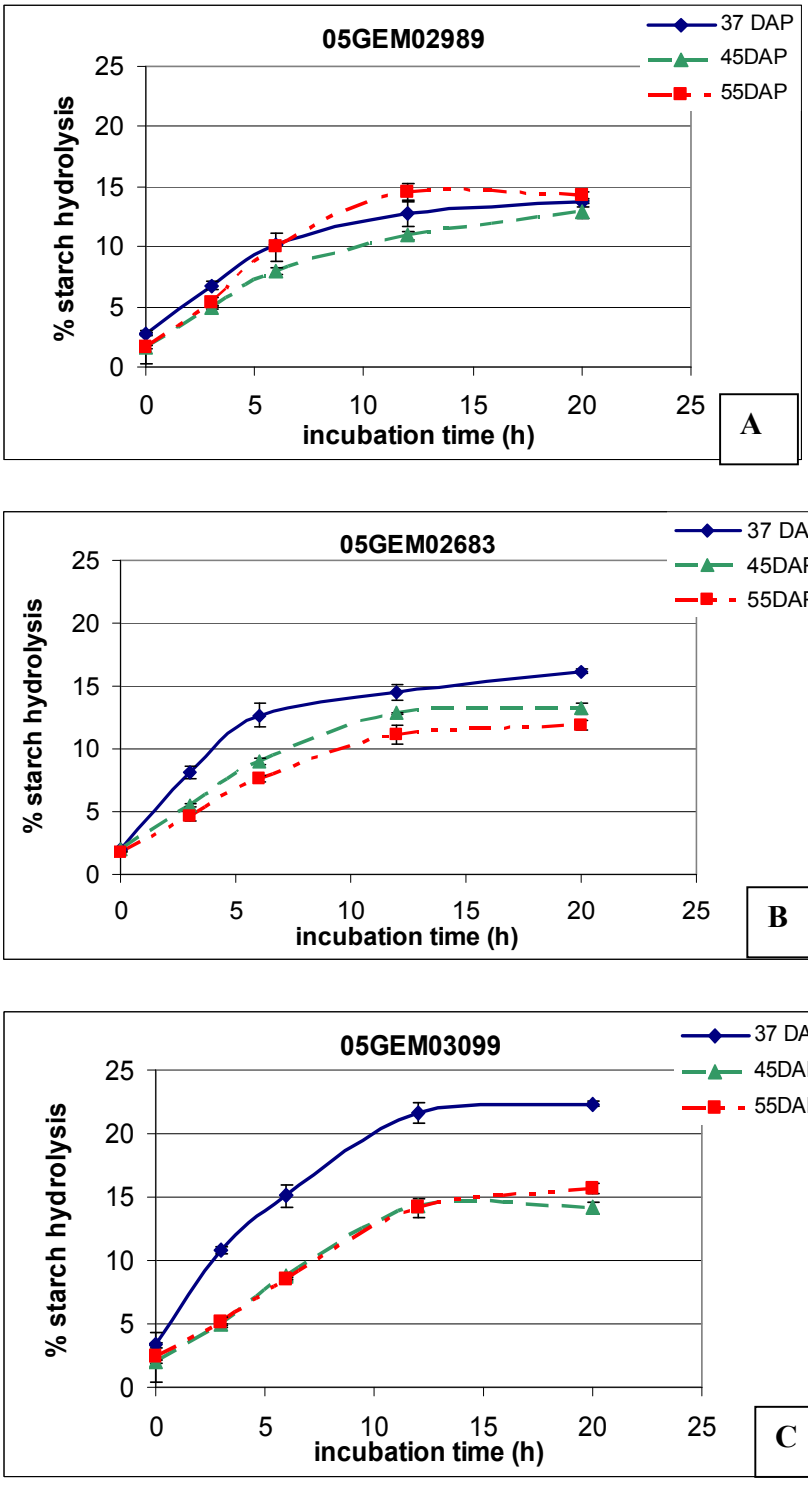


Figure 1. continued

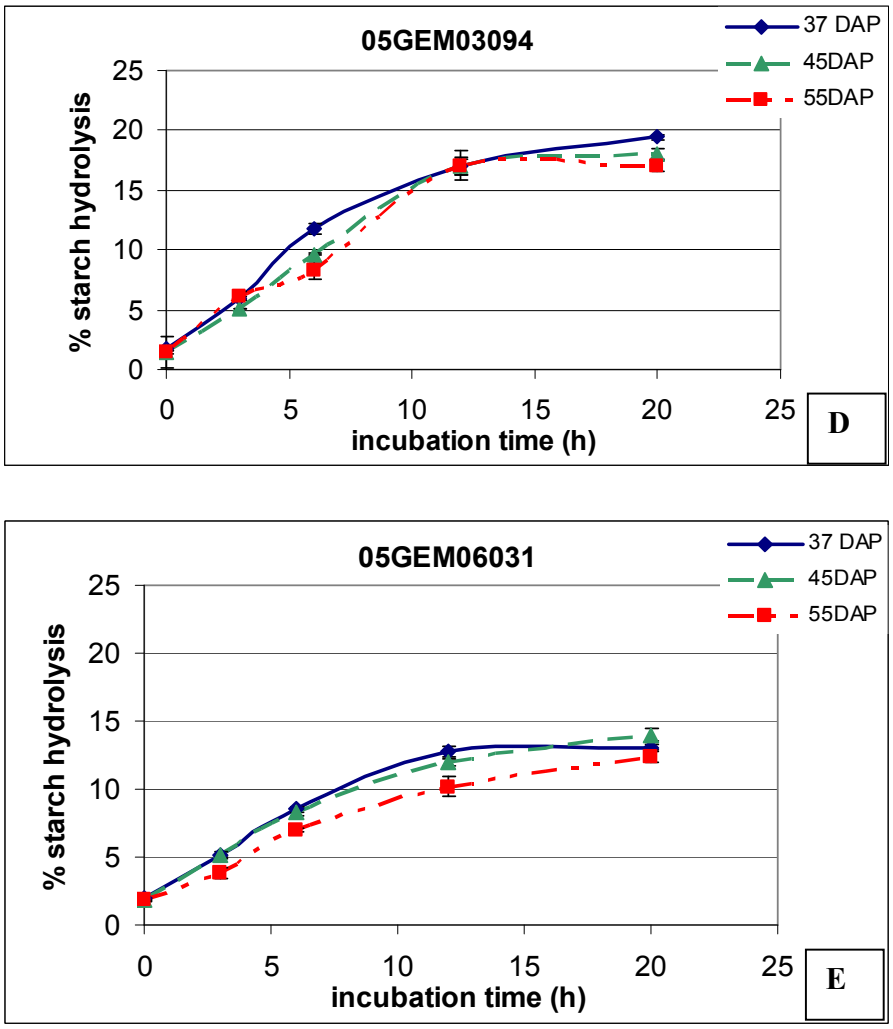


Figure 1. Percentage starch hydrolysis (*100xg reducing sugar/g dry starch basis*) in five ground GEM line corn samples at 0, 2, 4, 6, 12 and 20 h of incubation in 0.1M acetate buffer, 3mM $CaCl_2$, 2 ppm lactrol, and 40 ppm isostab**. A**: 05GEM02989, **B**: 05GEM02683, **C**: 05GEM03099, **D**: 05GEM03094, **E**: 05GEM06031

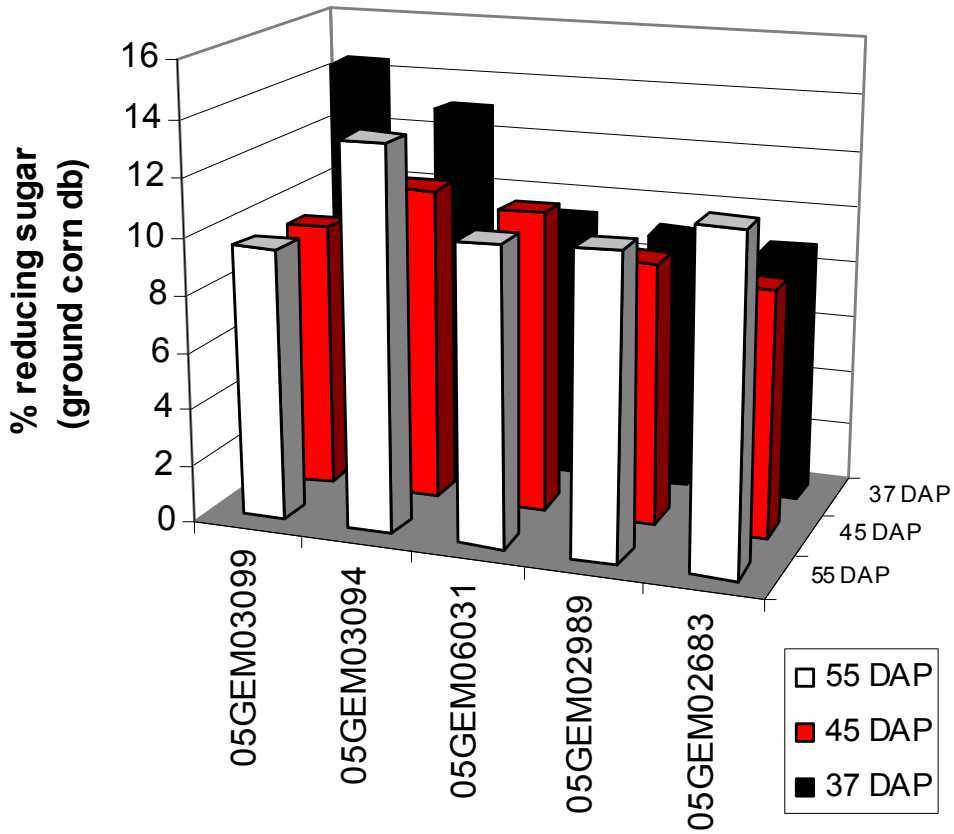


Figure 2. Reducing sugar yield *(100x(g reducing sugar 20h -g reducing sugar 0h)/ g dry corn)* in the five ground GEM line corn samples after 20h incubation in 0.1M acetate buffer, 3mM $CaCl_2$, 2 ppm lactrol, and 40 ppm isostab

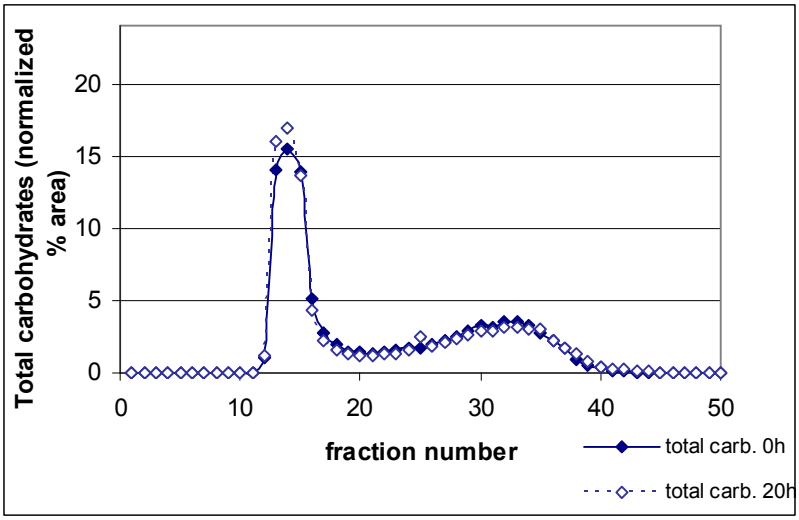


Figure 3. Gel permeation chromatogram (Sepharose CL-2B) of 05GEM02989 corn line flour incubated in 1M acetate buffer and 3mM $CaCl_2$ for 0 h and 20 h

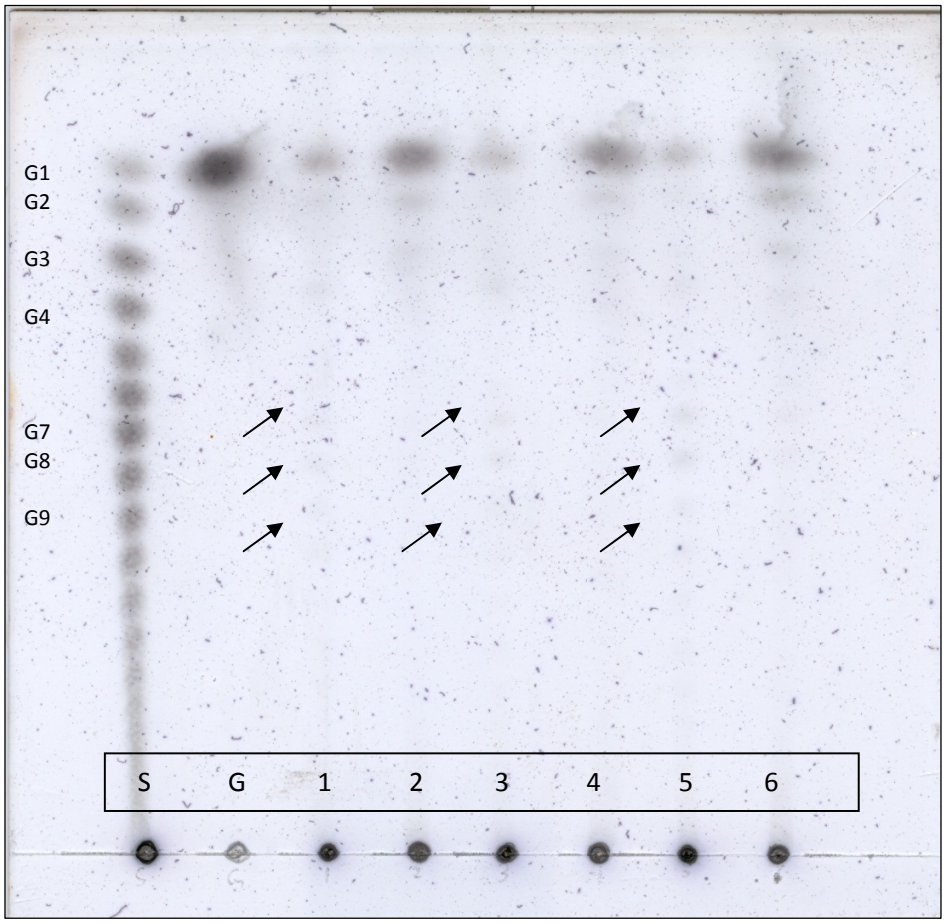


Figure 4. Thin layer chromatogram. 05GEM02989 corn line:

1- 37 DAP, 0h incubation

2- 37 DAP, 20h incubation

3- 45 DAP, 0h incubation

4- 45 DAP, 20h incubation

5- 55 DAP, 0h incubation

6- 55 DAP, 20h incubation

S- linear maltodextin

G- glucose

Arrows indicate less obvious spots on the chromatogram that represent G7, G8, and G9

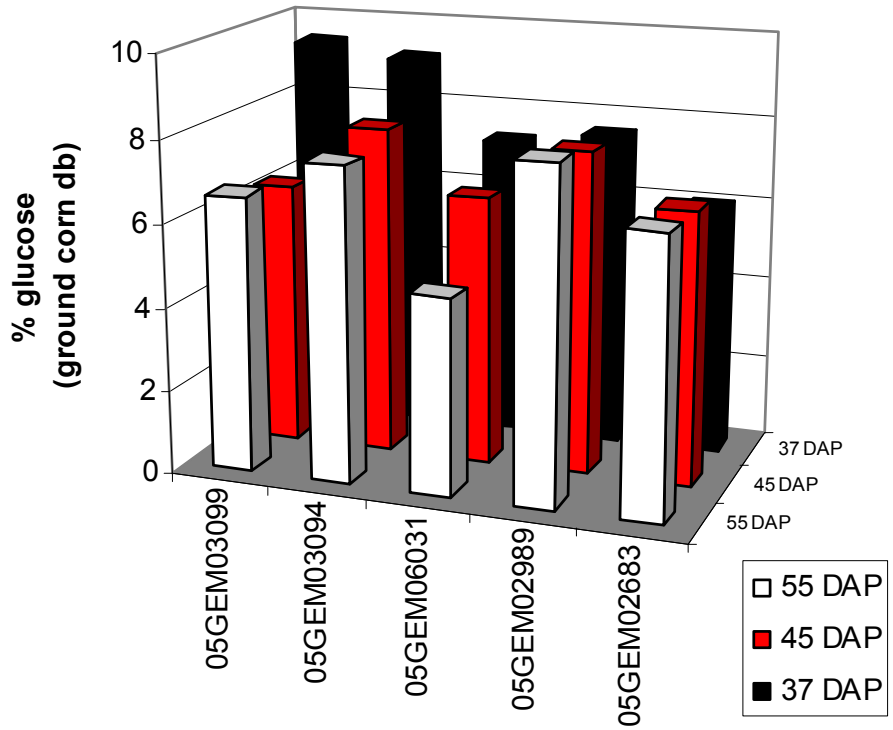
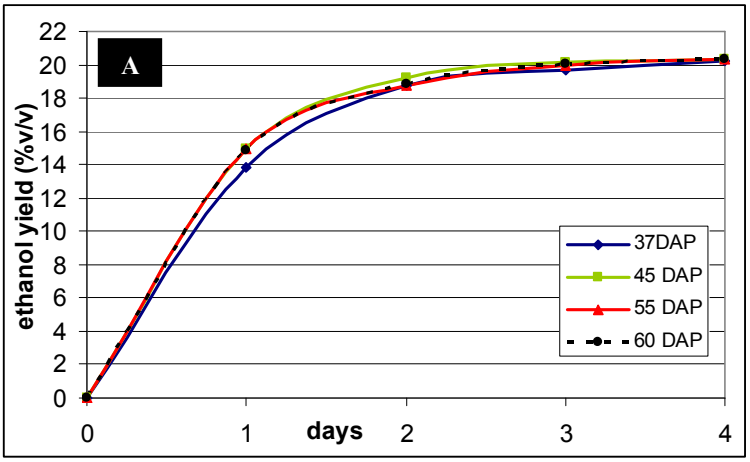
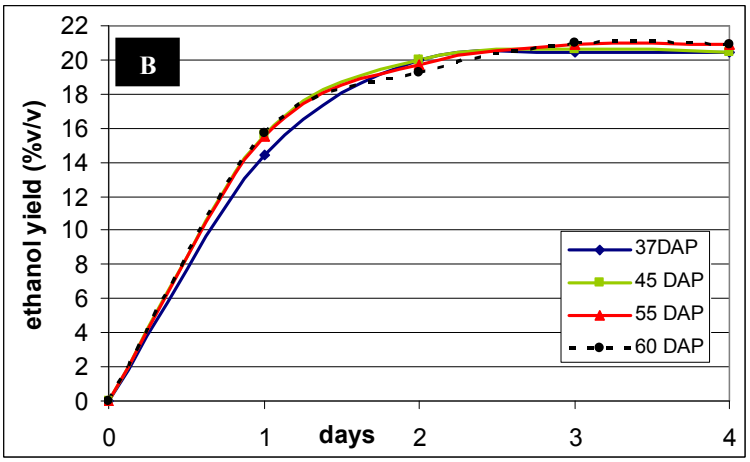
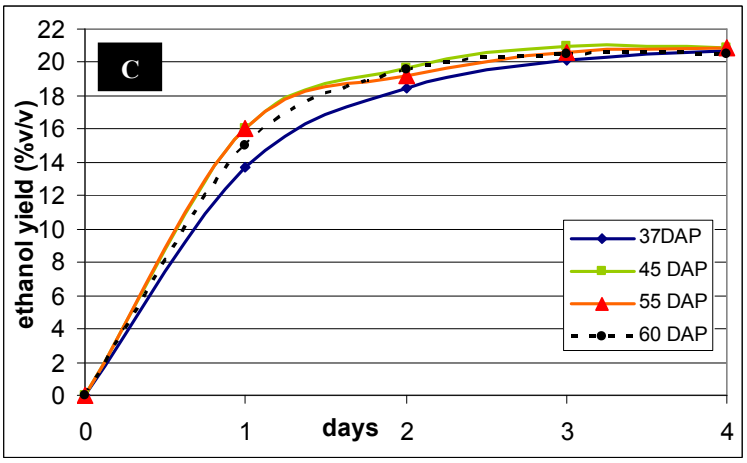


Figure 5. Glucose *(100x(g glucose 20h –g glucose 0h)/ g dry ground corn db)* yield in the five ground GEM line corn samples after 20h incubation in 0.1M acetate buffer, 3mM $CaCl_2$, 2 ppm lactrol, and 40 ppm isostab







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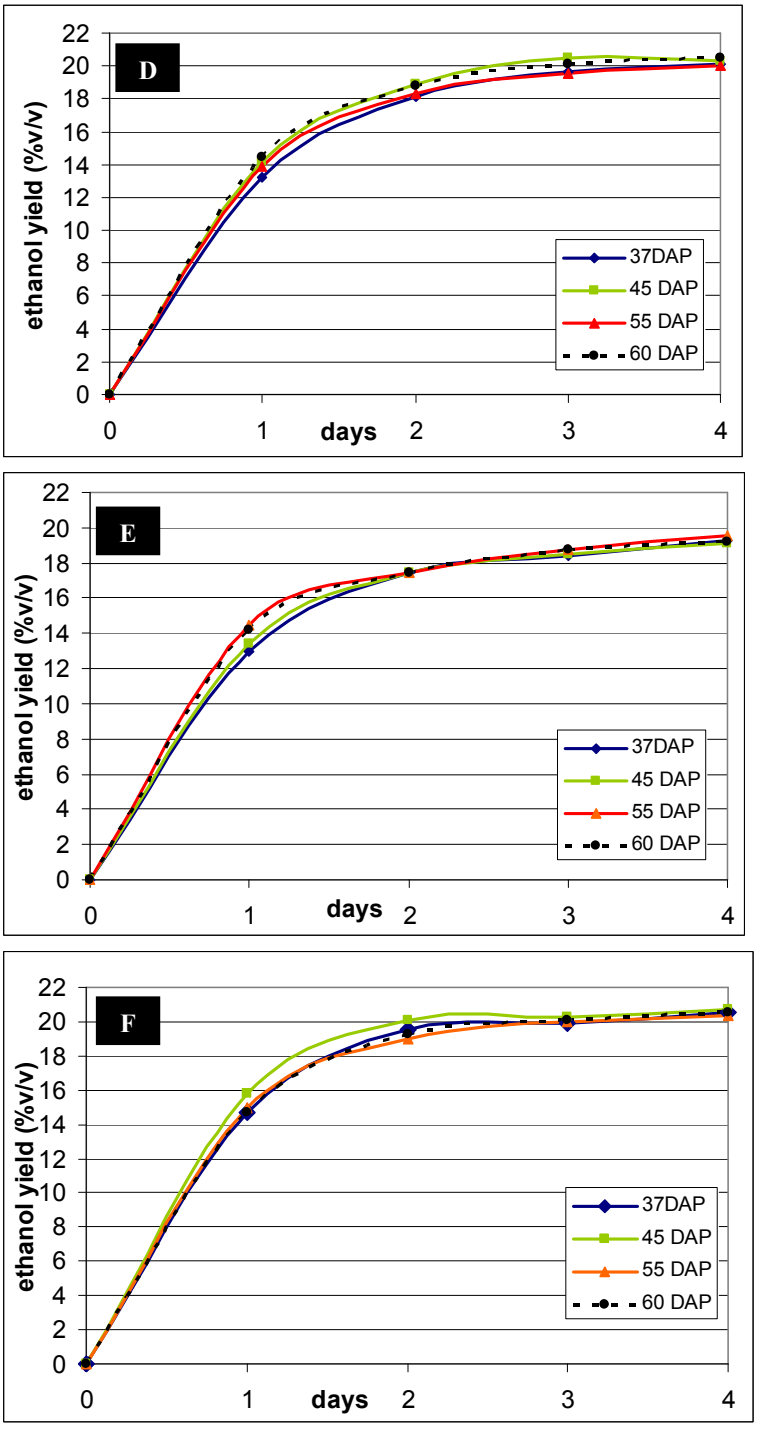


Figure 6. Ethanol yields of 2008 GEM corn lines. A. Average values for five corn lines; B. 05GEM02989; C. 05GEM02683; D. 05GEM03099; E. 05GEM03094; .F. 05GEM06031.

APPENDIX B. EFFECT OF EXOGENOUS GIBBERELLIC ACID (GA_3) ON ACTIVITY OF ENDOGENOUS STARCH HYDROLYZING ENZYMES IN CORN SEEDS

Objectives

Gibberellic acid (GA_3) is a naturally occurring plant growth hormone that has various effects on plant growth and development. It has been shown that GA_3, among other functions, can stimulate the synthesis of new proteins such as enzymes (Palmer, 1974). Studies have shown that α-amylases in the scutellum of barley and rice are not synthesized to the significant extent in the presence of exogenous GA_3, whereas the synthesis of aleurone layer α-amylases are greatly enhanced by exogenous GA_3 (MacGregor and Marchylo, 1986; Panabieres et al., 1989). There is limited understanding of the effect of exogenous GA_3 on the α-amylase synthesis in corn kernels during germination.

The objective of this study was to understand the effect of exogenous GA_3 on the starch hydrolysis rate of corn kernels, and identify the location in the kernel where the most extensive starch degradation occurs during germination.

Materials and Methods

Materials

Kernels of the GEM corn line 05GEM06031 harvested at 37 and 45 days after pollination (DAP) were provided by Dr. Michael Blanco of USDA-ARS Plant Introduction Station (Ames, IA). Samples were ground using a Cyclone Mill (UDY cor., CO, USA) using 0.5 mm screen. The moisture contents of the dry-ground corn samples were measured to

determine the dry weight of each sample. All the chemicals were purchased from Fisher Chemicals (Waltham, MA).

Methods

The effect of exogenous gibberellic acid (GA_3) on starch degrading enzyme activity. Corn kernels were surface cleaned using 3% hydrogen peroxide prior to analysis. Whole kernels, kernel halves and ground corn samples (~1g) were suspended in 5ml of 0.1 M sodium acetate buffer (pH 5), 3 mM calcium chloride, 2 ppm lactrol, and 40 ppm isostab. In the treated samples, 100 μM GA_3 was added. The samples were incubated for four days. After each time interval, the samples were removed and heated in a boiling-water bath for 15 minutes to solubilize released sugars. After heating, the samples were centrifuged at 3222 x *g* for 20 minutes. The reducing sugar content in the supernatant was determined using Somogyi-Nelson method (Somogyi M., 1945; Nelson N., 1944).

Procedure was slightly modified for the samples in which antifungal agents were added. Corn kernels were surface sterilized with 3% hydrogen peroxide. Whole kernels and ground corn samples (~1 g) were suspended in 5 ml of 0.1 M sodium acetate buffer (pH 5), 3 mM calcium chloride, 2 ppm lactrol, and 40 ppm isostab. To suppress yeast and fungi growth, saturated benzoic acid or 0.02% sodium azide were used. To the treated samples, GA_3 (100 μM) was added. The samples were incubated for four days. After each time interval, aliquots were taken from the suspension, filtered through filter paper of 5 μm pore size, and subsequently heated in a boiling-water bath for 15 minutes. The glucose and reducing sugar contents in the aliquots were determined following the Somogyi-Nelson method (Somogyi M., 1945; Nelson N., 1944) and GOPOD analysis, respectively.

Glucose content (GOPOD analysis). The glucose content of corn suspensions was determined using Megazyme glucose diagnostic kit (GOPOD). Ground corn sample (30 mg) was dissolved in 3 ml water. The suspension was vortex-mixed, heated in a boiling-water bath for 15 min, and subsequently centrifuged at 3222 x *g*. An aliquot of 0.1 ml was taken and 3 ml of GOPOD solution was added. The mixture was incubated in a shaker water-bath set at 50 ˚C for 30 min. The absorbance was measured at 520 nm against the reagent blank. The glucose content was calculated following the equation:

Glucose (% of ground corn db)= $\Delta A/\Delta A_g$ x100 x3/0.1x100/Wx 100/(100- ground corn moisture content (%w/w))

ΔA: absorbance read against the reagent blank

ΔA_g: glucose standard absorbance read against the reagent blank

W: sample weight

Morphology of starch granules. Mature corn kernels were submerged in 0.1 M sodium acetate buffer (pH 5) containing saturated benzoic acid, 2 ppm lactrol and 40 ppm isostab for four days. Dry (untreated) and four days treated kernels were frozen in liquid nitrogen and cracked open with a razor blade and a hammer. Scanning electron micrographs (SEM) of kernel specimens were taken using a scanning electron microscope (JEOL JSM-35, Tokyo, Japan) at the Bessey Microscopy Facility, Iowa State University. The corn kernel specimens were coated with gold–palladium (60:40), and the SEM images were taken at 40 kV (Jane et al, 1994)

Germination test. Whole kernels were surface cleaned using 3% hydrogen peroxide prior to analysis. Kernels were submerged in deionized water or GA_3 solution (100 μM) for 6 hours. Kernels were subsequently transferred to a cheese cloth soaked in deionized water or

GA_3 solution (100 μM) and incubated at room temperature for 72 or 84h in the dark. Shoot lengths of 50 corn kernels were measured using a ruler and averaged to obtain a mean shoot length.

Summary of results

Three different methods of application of GA_3 were used:, (1) suspension of ground corn in a GA_3 solution for four days, (2) suspension of whole kernels in a GA_3 solution for four days, and (3) germination of corn kernels previously soaked in GA_3 solution for 6h (total germination time 84h). In all three treatments, the concentration of GA_3 solution was 100 μM.

For the first application method, exogenous GA_3 did not have any effect on the endogenous enzyme activity of ground immature kernels (harvested on 37 DAP). The ground immature kernels suspended in a GA_3 solution had identical starch hydrolysis rate as that of untreated corn sample (29.1% starch hydrolysis after four days of incubation) (**Figure 1**). Ground mature corn showed a slightly faster starch hydrolysis rate after the addition of GA_3, but it reached only 23.2% starch hydrolysis after four days of incubation. The regions around the hilum of the crushed starch granules (exposed during grinding of kernels) were eroded after enzyme hydrolysis, suggesting that enzymes preferentially hydrolyzed loosely packed starch molecules around the hilum of the starch granule. Granules with pinholes on the surface, debris and broken granules were also observed (**Figures 2 and 3**).

In the second experiment using whole kernels as the substrate, GA_3 did not significantly affect the endogenous enzyme activity, regardless of corn maturity stage. After four days of hydrolysis, immature and mature corn reached 4.31% and 2.94% starch hydrolysis, respectively (**Figure 4**). SEM images suggested that during the hydrolysis, starch

was hydrolyzed predominantly in the embryo region of the kernel. The aleurone layer, which is also known to synthesize starch degradation enzymes, did not show starch hydrolysis in the adjacent area (**Figures 7 and 8**).

In the third method, the addition of GA_3 increased a germination rate of mature corn seeds (harvested on 55 DAP). A mean shoot length in the GA_3 treated mature corn (16.25 mm) was longer than that of untreated corn (14.75mm) after 72h of germination (**Figure 9**). No further analysis was conducted to determine an extent of starch hydrolysis of this sample, nor images taken to determine in which region of the kernel starch was most extensively hydrolyzed.

Conclusions

Suspension of ground corn and whole kernels in a 100 μM GA_3 solution for four days failed to have any significant effect on the endogenous starch hydrolyzing enzyme activity in corn. The application of GA_3 during controlled germination of kernels increased the enzyme activity of corn as indicated by the increased shoot length of germinated kernels in the presence of GA_3. Further studies are necessary to understand the mechanism by which the exogenous GA_3 increase the corn germination rate and to find a potential commercial use of the germinated corn with increased enzyme activity.

Reference cited

MacGregor, A.W., and Marchylo, B.A. 1986. α-Amylase components in excised, incubated barley embryos. Journal of the Institute of Brewing 92: 159-161.

Nelson, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. J. Biol. Chem. 153**:**375 – 380.

Palmer, G.H. 1974. The industrial use of gibberellic acid and its scientific basis-a review. Journal of Institute of Brewing 80:13-27

Panabieres, F., Kerhardy, F., Montembault A., Daussant J., and Delseny, M. 1989. Induction of α-amylase isoenzymes by gibberellic acid in imbibed rice half-seeds. Plant Science 64:15-23

Somogyi, M. 1945. A new reagent for the determination of sugars. J. Biol. Chem. 160:61 – 68.

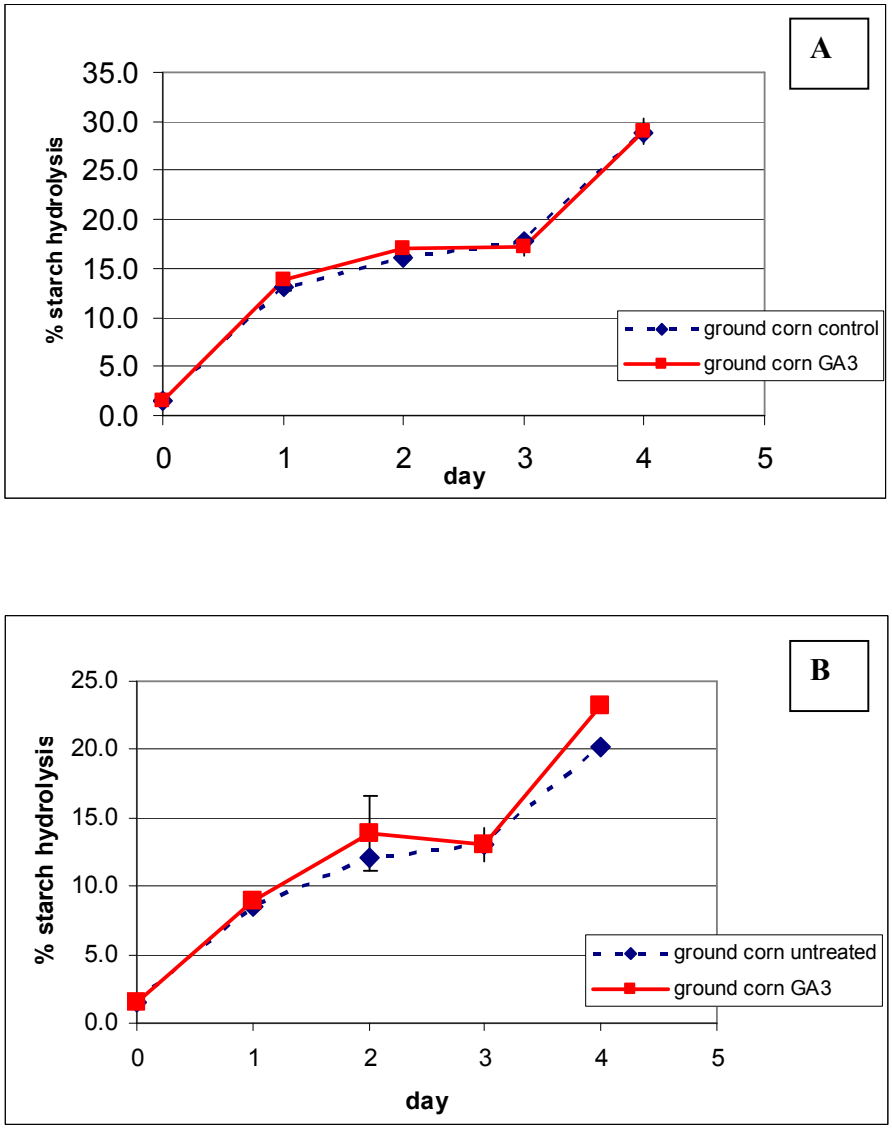


Figure 1. Effect of 100 µM GA_3 on the starch hydrolysis rate in the 05GEM06031 ground corn samples harvested on **A**: 37 DAP and **B**: 55 DAP

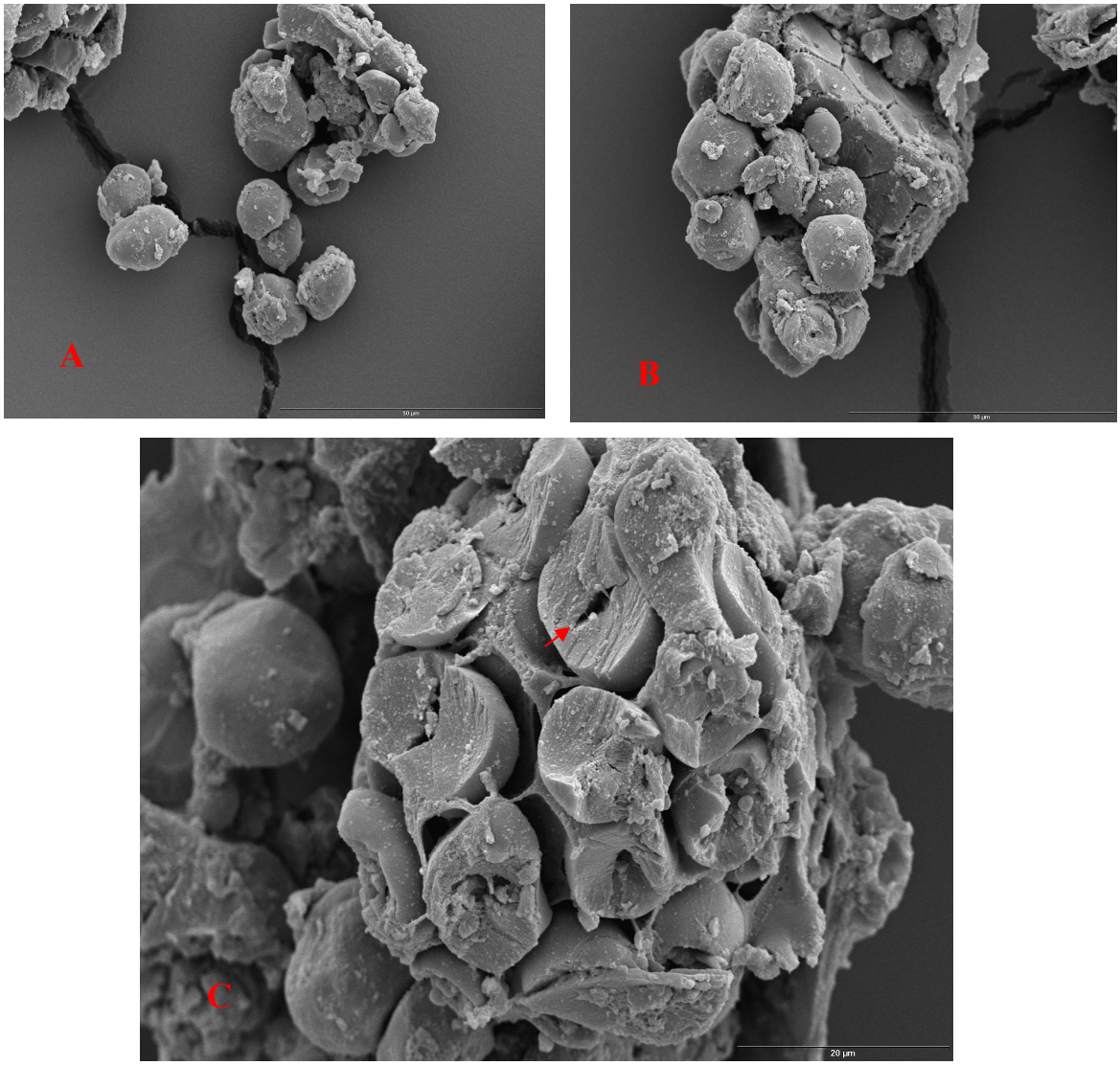


Figure 2. Scanning electron micrographs of starch granules in the ground corn (05GEM06031 corn line). **A** and **B**: overview of the ground corn, **C**: damaged granules with hilum regions exposed

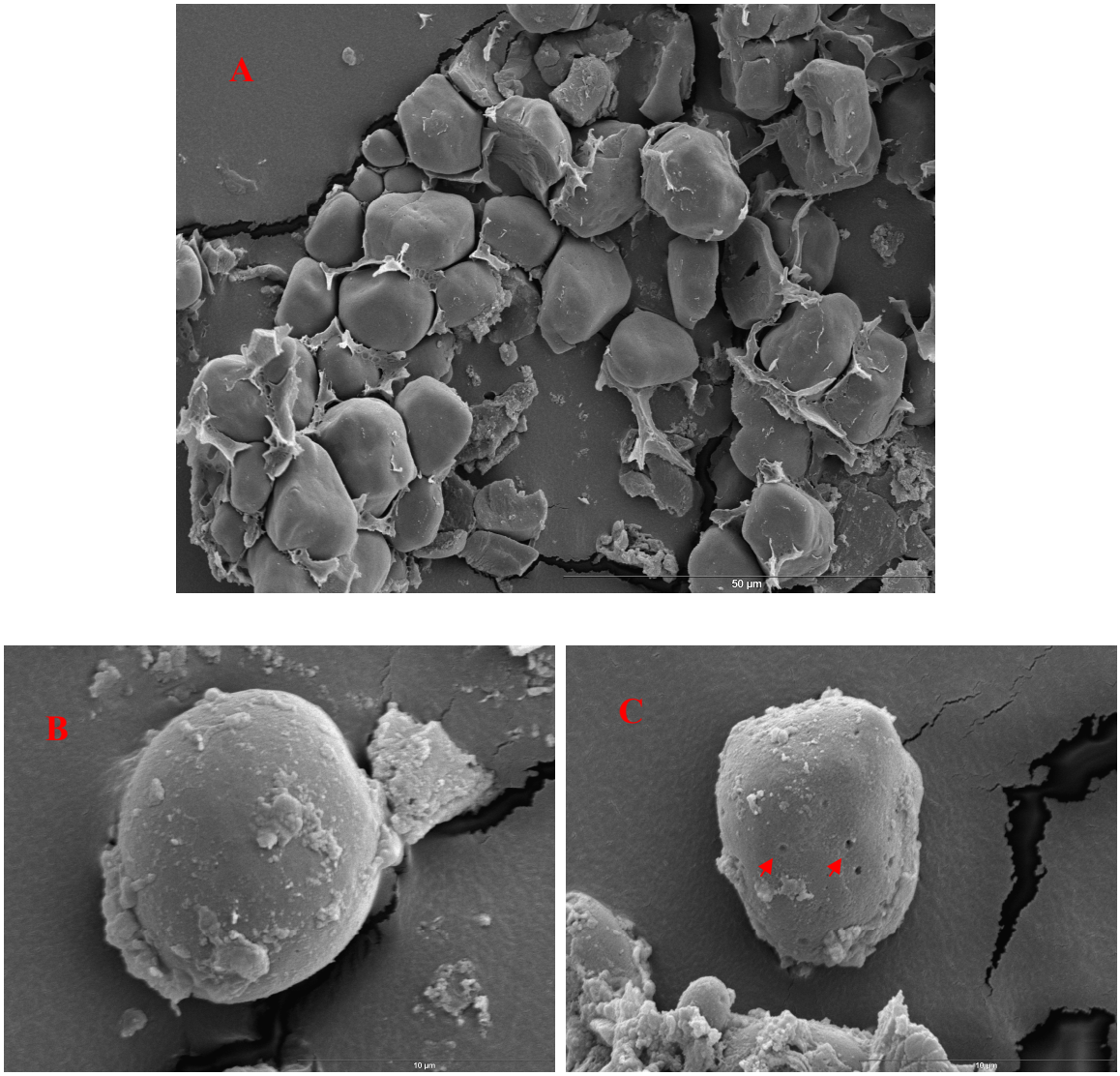


Figure 3.

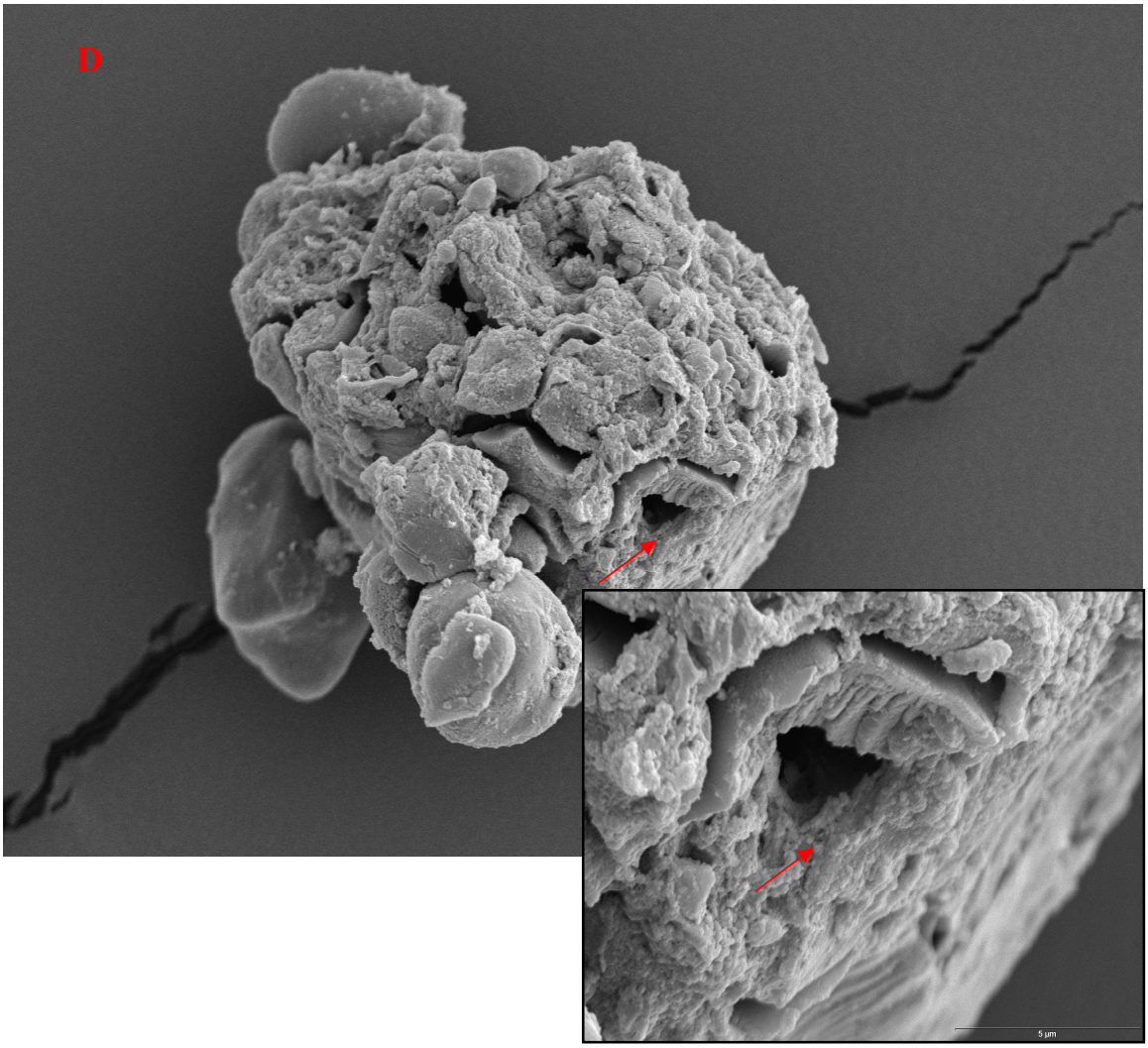


Figure 3. Cont.

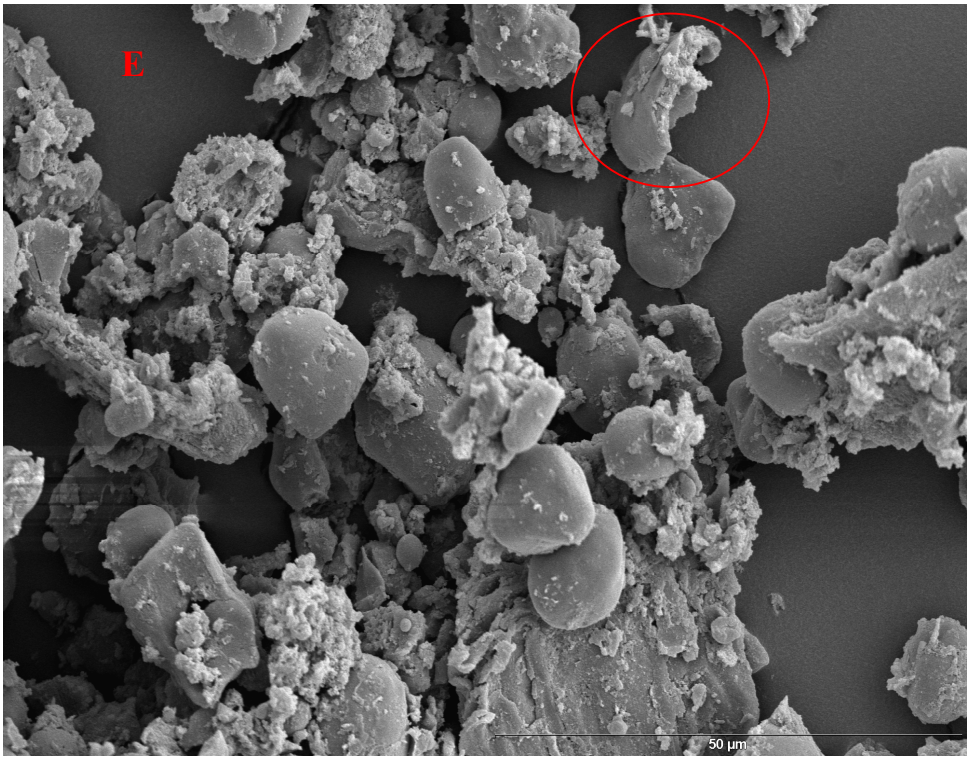
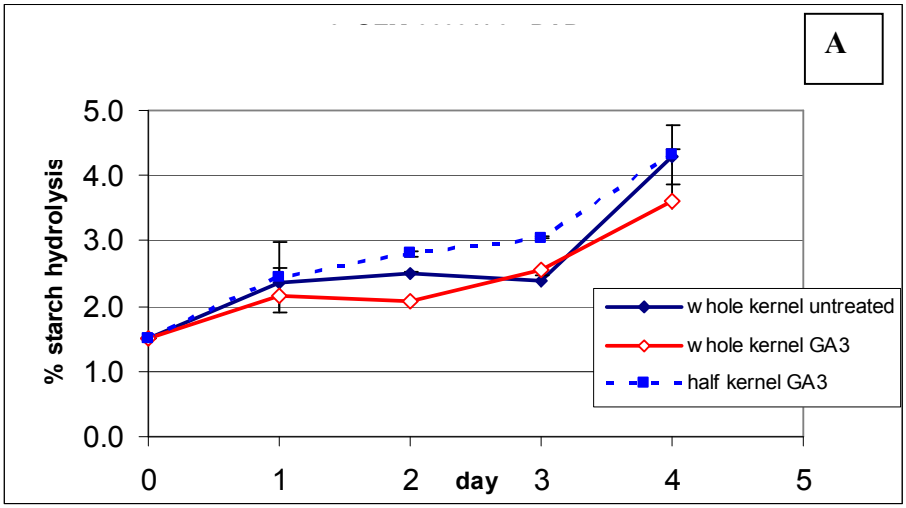


Figure 3. Scanning electron micrographs of starch granules in the ground corn incubated in water for four days (05GEM06031 corn line). **A**: overview of ground corn, **B**: a granule showed less damage, **C**: a granule with a lot pinholes on the surface, **D**: highly eroded region at the hilum, **E**: debris and broken granules



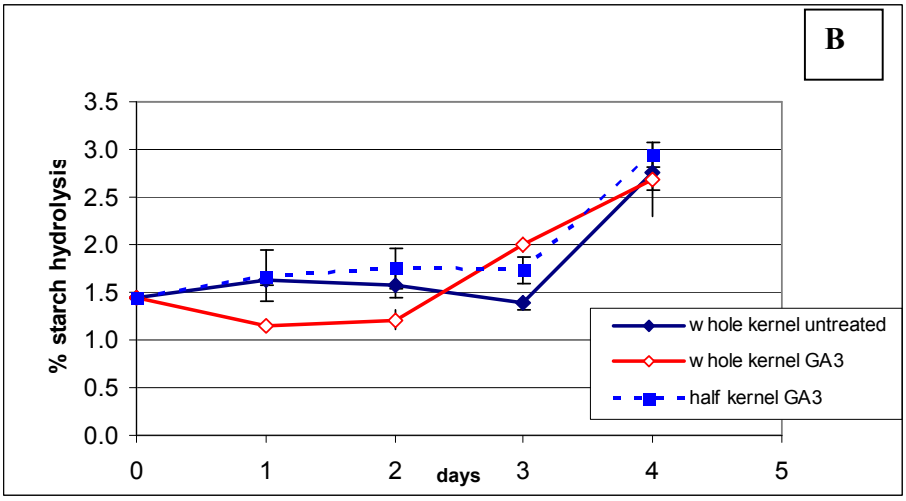


Figure 4. Effect of 100 μM GA_3 on the starch hydrolysis rate in the 05GEM06031 corn line kernels harvested on **A**: 37 DAP and **B**: 55 DAP

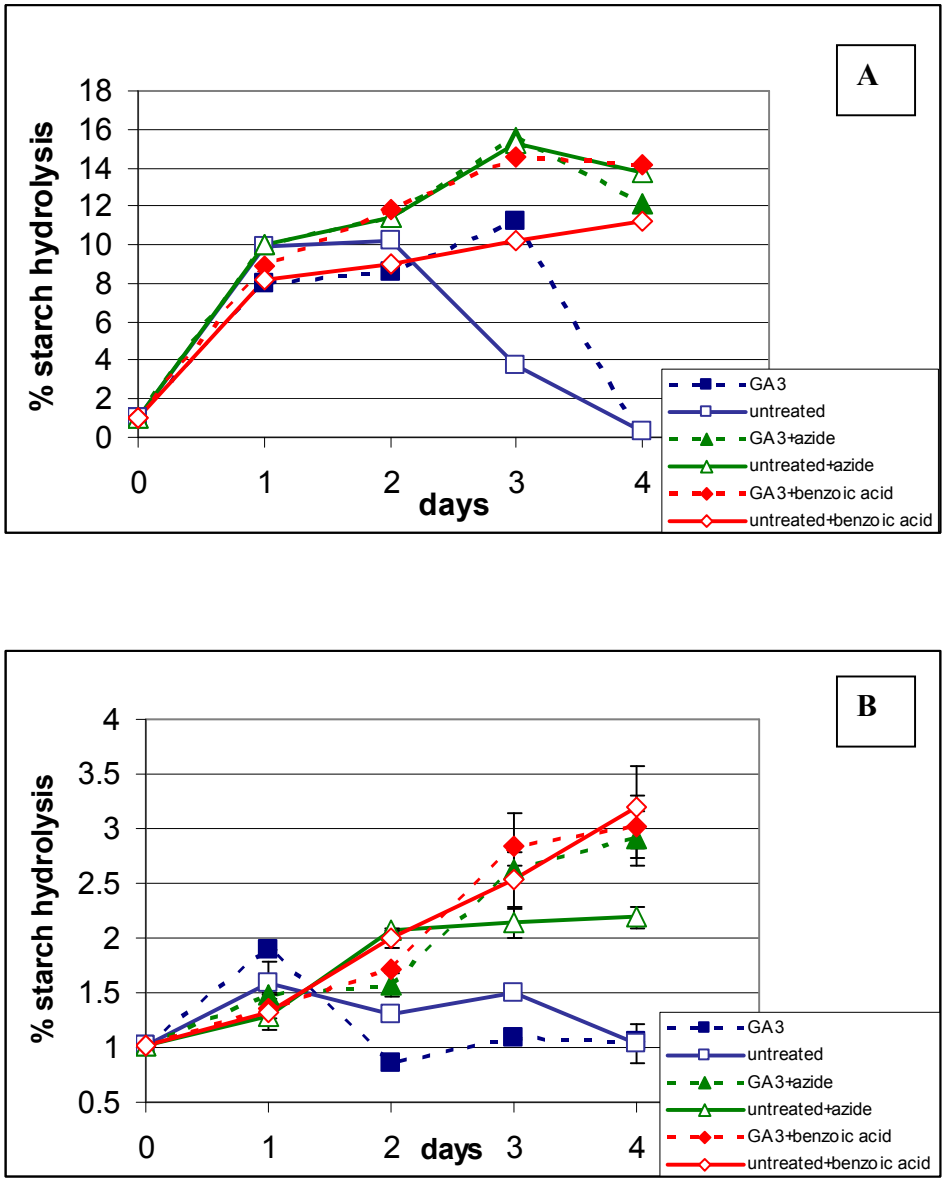


Figure 5. Effect of 100 µM GA_3 on the starch hydrolysis rate in the 05GEM06031corn line kernels and ground samples harvested on 55 DAP and incubated in different antifungal mediums. **A**: ground corn, **B**: whole kernel

GA_3, and untreated => 2 ppm lactrol, and 40 ppm isostab

GA_3+ azide and untreated+azide => 0.02% azide, 2 ppm lactrol, and 40 ppm isostab*GA_3+ benzoic acid and untreated+benzoic acid* => saturated benzoic acid, 2 ppm lactrol, and 40 ppm isostab

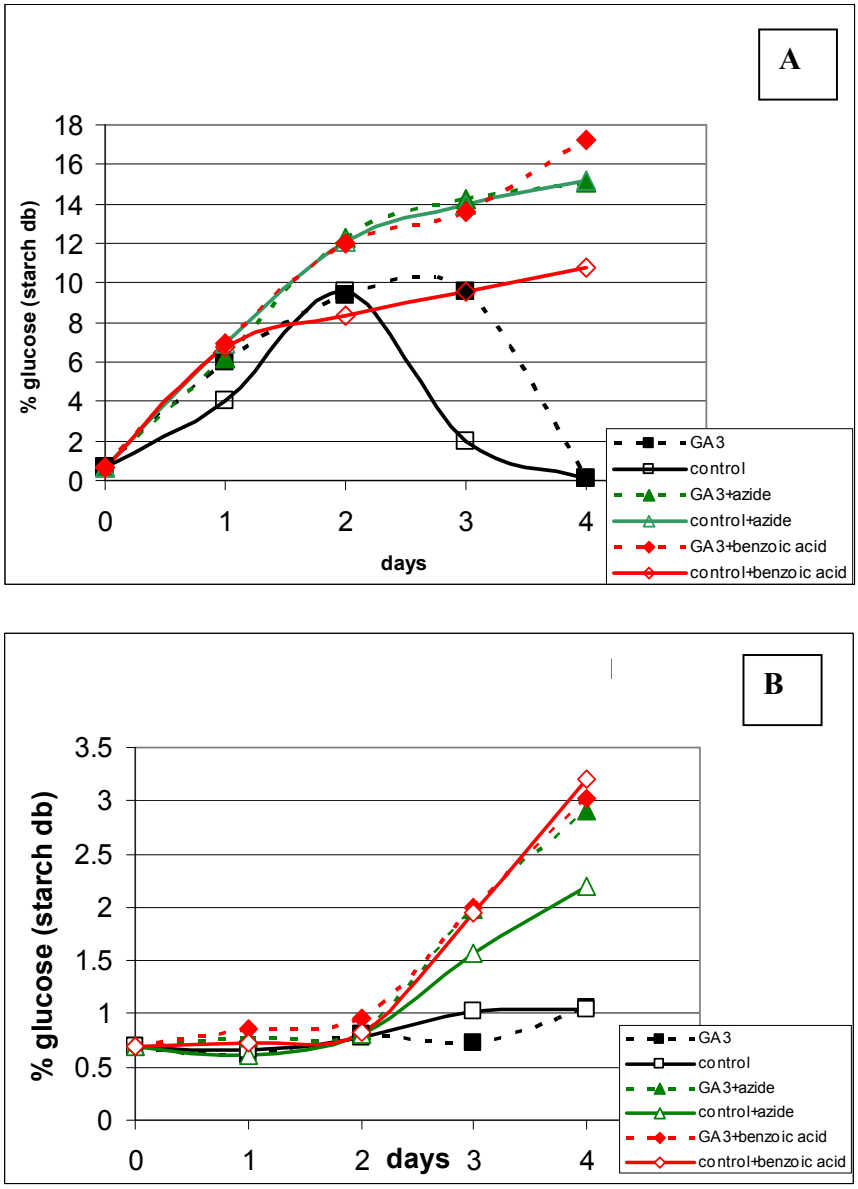


Figure 6. Effect of 100 µM GA_3 on the glucose yield in the 05GEM06031 corn line kernels and ground samples harvested on 55 DAP and incubated in different antifungal mediums. **A**: ground corn, **B**: whole kernel

GA_3, and untreated => 2 ppm lactrol, and 40 ppm isostab

GA_3+ azide and untreated+azide => 0.02% azide, 2 ppm lactrol, and 40 ppm isostab

GA_3+ benzoic acid and untreated+benzoic acid => saturated benzoic acid, 2 ppm lactrol, and 40 ppm isostab

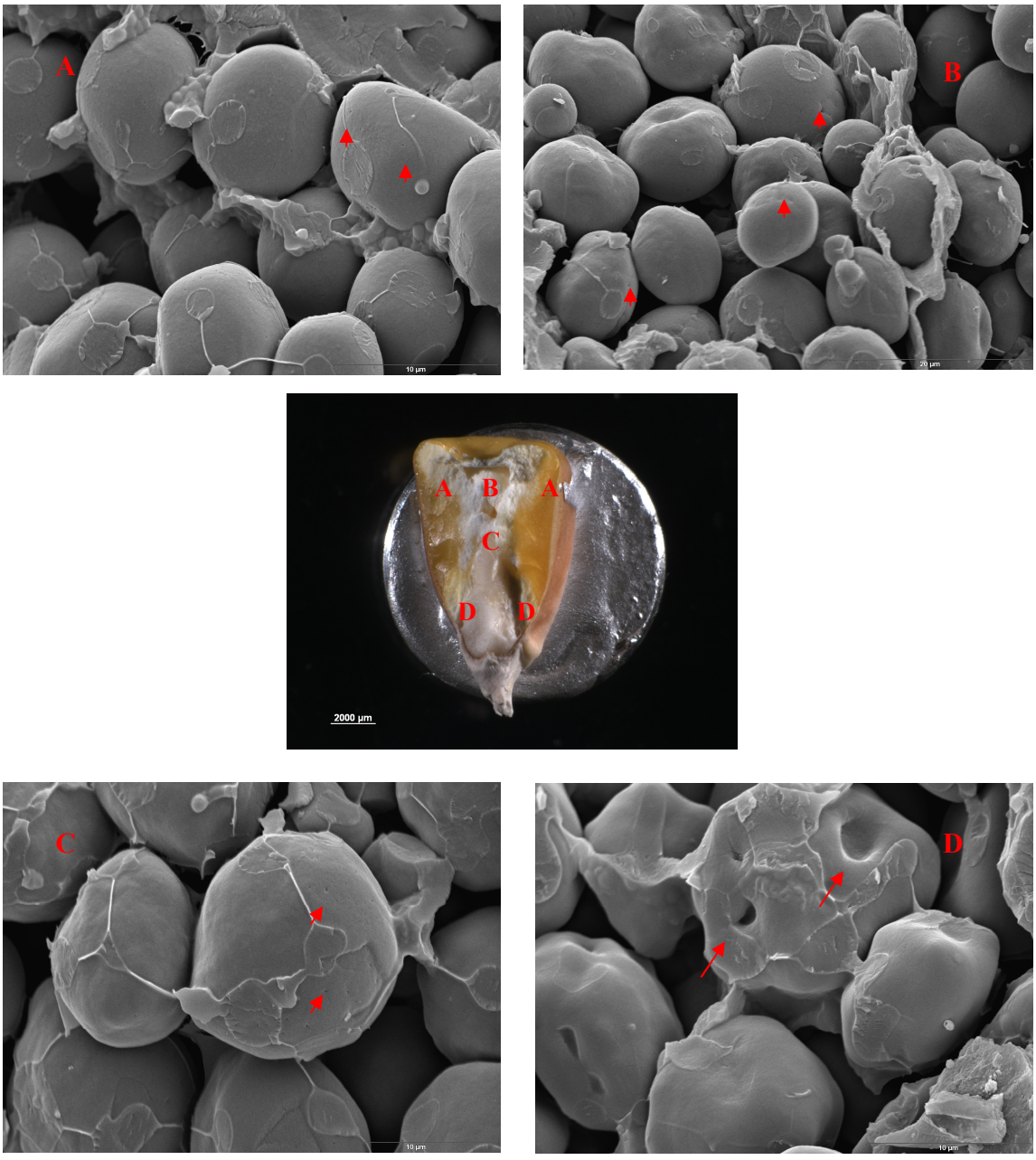


Figure 7. Scanning electron micrographs of native starch granules located in different regions of 05GEM06031 mature, untreated kernels. **A**: endosperm area close to the aleurone layer, **B**: central part of the endosperm, **C** and **D**: area in endosperm close to the embryo

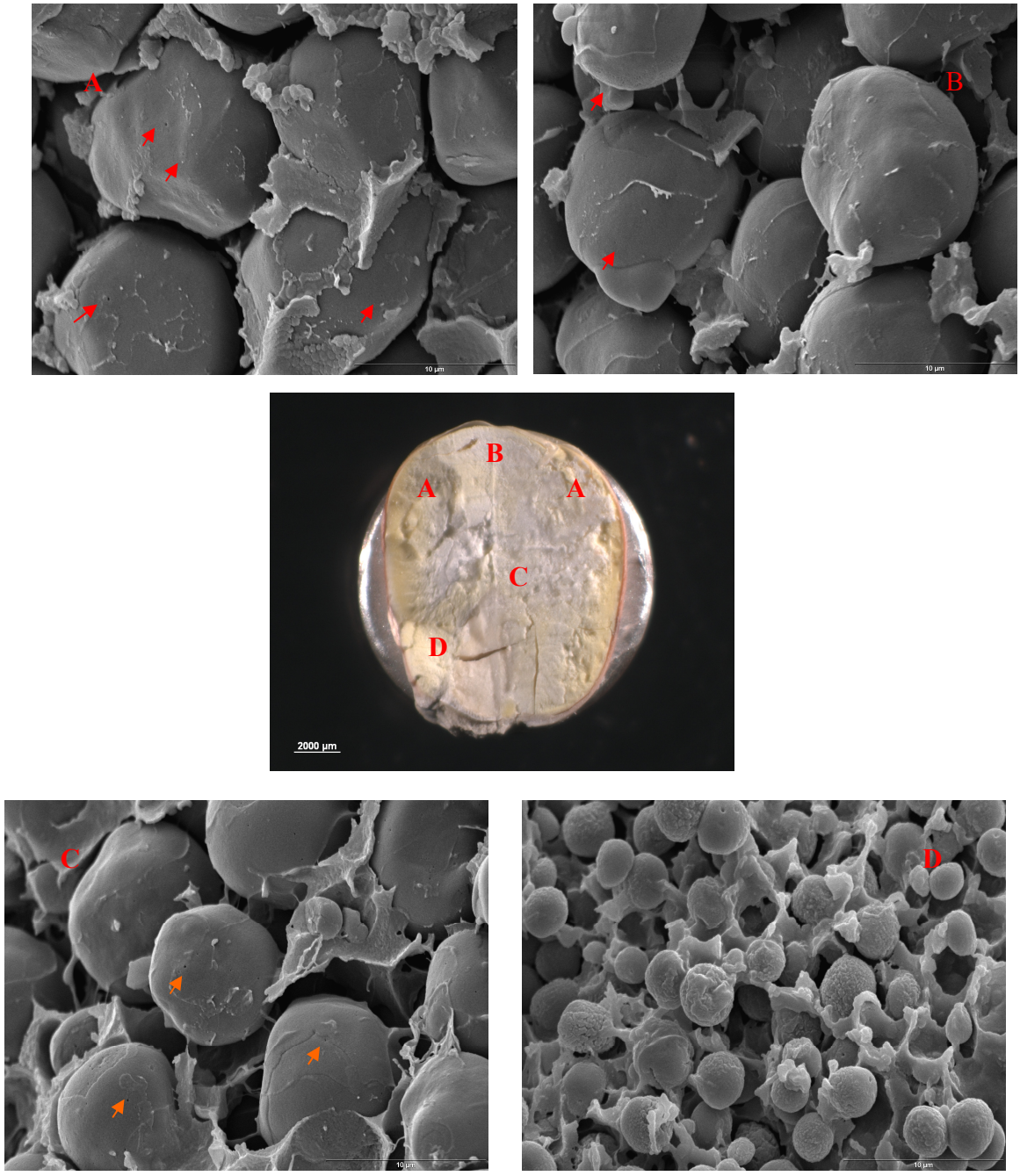


Figure 8. Scanning electron micrographs of starch granules located in different regions of 05GEM06031 mature kernels submerged in water for four days. **A**: endosperm area close to the aleurone layer, **B**: central part of the endosperm, **C** and **D**: area of endosperm close to the embryo

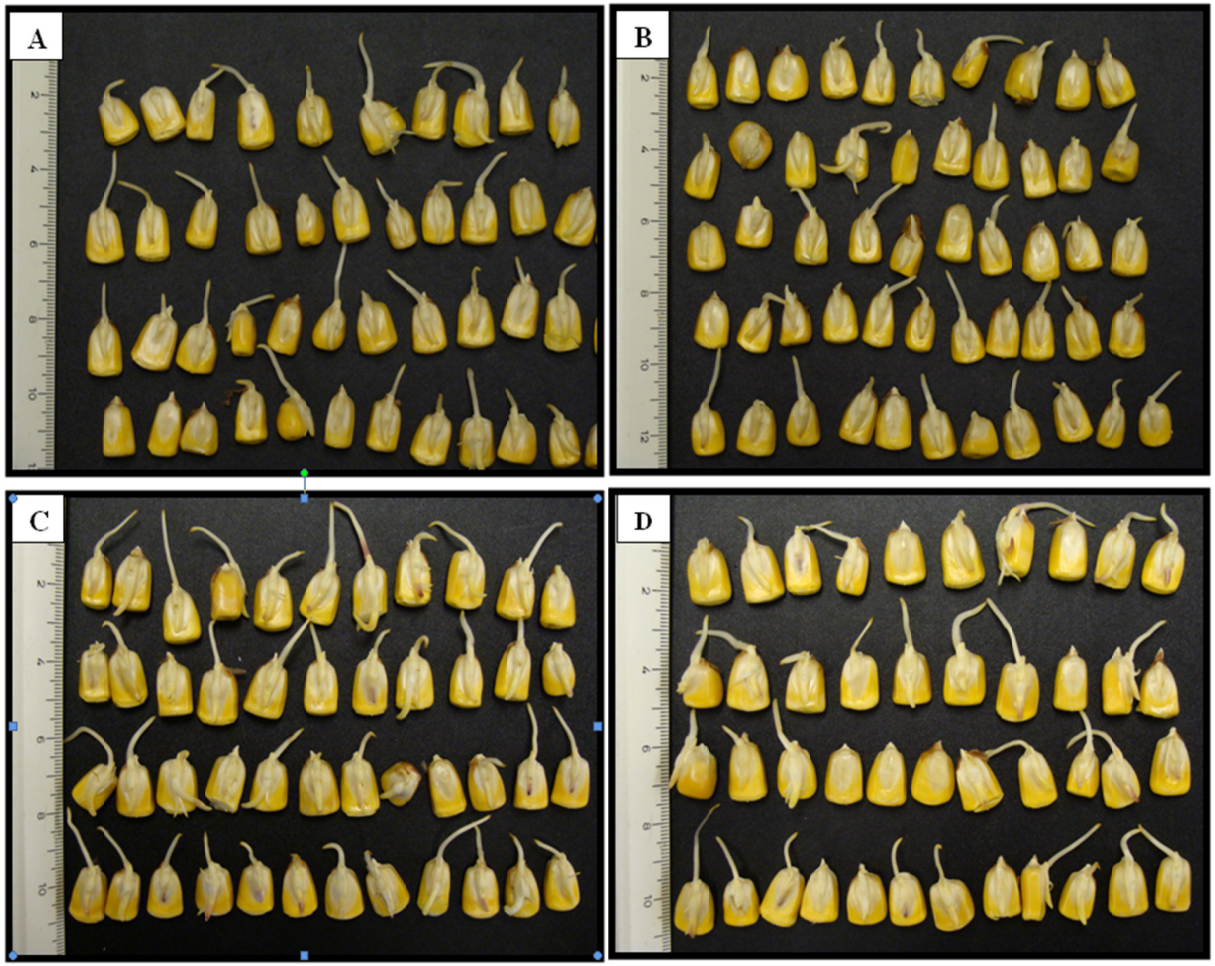


Figure 9. Kernels of 05GEM06031 mature kernels (**A**) treated with 100 µM GA_3 and incubated for 72h, (**B**) untreated and incubated for 72h, (**C**) treated with 100 µM GA_3 and incubated for 84h, (**D**) untreated and incubated for 84h

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This dissertation is dedicated to my parents who have provided me with unconditional love and encouragement throughout my life. My deepest gratitude goes to two of them who have worked tirelessly to make sure I have every opportunity to succeed personally and professionally. Finally, I wish to thank my husband Djordje who left the familiarity of home to join me at Iowa State University to pursue our dreams. He is my endless support, my soulmate, and my best friend.