2012

Characterization of normal and waxy corn starch for bioethanol production

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By

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study committee:

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Iowa State University

Ames, Iowa

2012

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ABSTRACT

The uncertainty of future oil supply and growing concerns of the energy security of the United States have boosted the investment in alternative energy carriers, including biofuels. Bioethanol, made from bio-renewable resources, has gained the mainstream usage in the USA. Ethanol is almost exclusively made from corn in the USA. The objectives of the study were: 1) To compare the differences of ethanol production between the normal and waxy corn using a cold-fermentation process; 2) To understand the effects of starch structure and properties on the ethanol production. Ethanol yields of the waxy corn ranged 33.1% (33.1g/ 100g dry grain) - 37.6% for 2009 grown corn, and ranged 34.8-37.9% for 2010 grown corn. Ethanol yields of the normal corn ranged 34.2-37.2% (2009) and 34.3-37.5% (2010). Ethanol yields positively correlated with the kernel starch contents of both normal and waxy corn. Average starch-ethanol conversion efficiency of the waxy corn (93.2%, 2009; 93.0%, 2010) was substantially greater than that of the normal corn (88.0%, 2009; 88.4%, 2010). This could be attributed to the greater starch hydrolysis rate of the waxy corn than that of the normal corn. Starch hydrolysis of uncooked dry-grind corn showed that > 90% of starch in the waxy corn was hydrolyzed, whereas < 80% of starch in the normal corn was hydrolyzed to glucose. It indicated that normal corn contained a significant portion of starch that was less readily hydrolyzed by the enzymes, which reduced the conversion efficiency. There were differences in starch physicochemical properties between the corn grown in 2009 and 2010 crop years. This was likely caused by the changes in the growing conditions (e.g. growing temperatures) between the two crop seasons.

GENERAL INTRODUCTION

Starch is naturally the second most abundant carbohydrate next to cellulose. Starch is the main source of carbohydrate in both food and feed, making up the most of our daily energy intake. Starch is bio-renewable, bio-degradable, environmentally friendly, and easy to process. Therefore, starch is an important material for a wide variety of food and non-food applications, including thickeners, stabilizers, binding agents, fat substitutes, texturizers, fillers, and feedstock for biofuels.

Starch consists of two types of polysaccharides, amylose and amylopectin. Amylose is primarily a linear molecule of D-glucopyranose units linked by α-1,4 glycosidic bonds with few branches. Amylopectin is highly-branched molecule that contains short chains of α-1,4 linked D-glucopyranose units. The short chains are linked by α-1,6 glycosidic bonds, which account for ~5% of the total glycosidic linkages. Amylose molecules are interdispersed among amylopectin molecules in the starch granule, and intertwine with the long branch-chains of amylopectin. Different types of starch, however, have different proportions of amylose and amylopectin. Waxy starch contains almost exclusively amylopectin and little amylose. Normal starch contains 15-30% amylose, depending on the botanical source of the starch. High-amylose starch usually has >50% amylose content, and also contains another molecule known as intermediate component (IC).

Chemical composition and structures of starch affect its physicochemical properties. For example, waxy corn starch shows higher digestibility than the normal corn starch. High-amylose corn starch, such as the starch of amylose-extender (***ae***) mutant corn, contains a significant amount of resistant starch (RS) that cannot be either digested or absorbed in the small intestine. It is known than amylose is more concentrated at the periphery of starch granule and intertwines with amylopectin, maintaining the integrity of starch granule and contributing to the resistance of starch

granule to enzyme hydrolysis. The digestibility of the ***ae wx*** double mutant corn starch, however, was reported to fall between the starch of the ***ae*** corn and wild type corn, displaying characteristics of slowly-digestible starch (SDS). Both RS and SDS have attracted interests for the exerted health benefits, such as lowering glycemic index and blood cholesterol level and reducing risks of colon cancer. On the other hand, starch that is more easily hydrolyzed to glucose is desirable in the bioethanol production.

With the depletion of fossil fuels and increased environmental concerns by burning gasoline, bioethanol has been used as one of the major gasoline substitutes in the United States. Bioethanol industry rapidly grew with recognition of the benefits of ethanol as a renewable fuel, such as increasing energy security by reducing dependence on foreign oil, producing less emissions for cleaner air, and boosting rural and farm economies. Corn is the most important starch crop in the United States, and is almost the exclusive feedstock for bioethanol production. Ethanol production in the United States underwent a rapid increase from 175 million gallons in 1980 to 13.9 billion gallons in 2011. Nevertheless, the expansion of production capacity of the corn-based ethanol industry is limited by availability of more farmland for growing corn. Therefore, it is important to maximize the ethanol yield by understanding how processing conditions and starch hydrolysis affect ethanol production.

In the United States, ethanol is mainly produced using a dry-grinding process. In a conventional fermentation process, starch in the dry-grind grain is liquefied by α-amylases at ~90℃ and then saccharified by amyloglucosidase s at ~60℃ to produce glucose that is fermented by yeast into ethanol. An industrial process of raw-starch fermentation was developed recently, in which starch saccharification and fermentation occur simultaneously. Raw-starch fermentation has been reported to reduce production costs compared with the conventional method. The process decreased the energy input and increased the ethanol yield by preventing the formation of amylose-lipid complex, starch retrogradation, and osmotic stresses to the yeast. Great efforts have been

devoted to investigate the effects of kernel composition, nitrogen sources, fermentation temperatures, and starch hydrolysis on the ethanol yield. The effects of starch structures on the starch conversion and ethanol yield in a raw-starch fermentation process, however, have not been fully understood.

The objectives of this study were:

- 1) To compare the ethanol yield between the waxy and normal corn lines using a cold-fermentation process;
- 2) To understand the impacts of starch structures and properties on the ethanol yield.

DISSERTATION ORGANIZATION

The dissertation consists of a general introduction, two chapters, one appendix, and acknowledgements. First chapter of the dissertation is a review of literature on the background knowledge and information relating to the research topics. Second chapter, “Characterization of normal and waxy starch for bioethanol production”, is organized with the format of a research paper for submission to *Journal of Agricultural and Food Chemistry*. A preliminary study, “Characterization of ***ae wx*** double mutant maize starch”, is included in the appendix. The literature cited in the Literature Review is listed in the alphabetical order of the first author’s last name.

CHAPTER 1. LITERATURE REVIEW

Bioethanol Overview

The United States is the largest petroleum consuming country in the world (18.8 million barrels per day of petroleum products during 2011), and about 45% of the petroleum is imported from foreign countries in 2011 (EIA 2012). In addition to the nation's great dependence on foreign oil supply, concerns have been raised for the depletion of fossil oil sources and rising demands for liquid fuels. Global reserve of liquid fuels today is estimated to meet just over half of the global demand by 2023, which indicates that the remaining 50% of the demand will have to be met with other sources (Owen et al. 2010). Environmental concerns have grown over the harmful tailpipe emissions of carbon monoxide, oxides of nitrogen, and other ozone-forming pollutants, by burning large volume of petroleum-based fuels (Sperling and Gordon 2007). To prepare for the uncertainty of future oil supply and reduce the air pollution, it is necessary to invest in alternative energy carriers that improve energy security (utilizing local resources) and decrease emissions (USGAO 2007, Owen et al. 2010).

Ethyl alcohol, also known as ethanol, is a colorless, flammable, volatile liquid that is widely used to produce beverages, solvents, and fuels. Ethanol is produced either synthetically through the hydration of ethylene (petrochemical) or biologically through yeast fermentation of simple carbohydrates (Mills and Ecklund 1987). Production of ethanol from fermentation, however, had been encouraged by the federal tax credit (which was discontinued in 2011) and low prices of corn in the United States (Keim 1983, Mills and Ecklund 1987). Therefore, ethanol is considered as bio-renewable source of energy because it can be produced from starchy crops or sugar-containing plants. Currently the feedstocks used for fuel ethanol production include corn, sugar cane, sugar beets and sorghum, but almost exclusively from corn in the United States (Gnansounou 2009, Sanchez and Cardona 2008).

Attempts to produce ethanol for transportation fuel started in early 1900s in the United States. At the beginning of 20th century, Henry Ford built a vehicle that could run either on gasoline or alcohol (Freudenberger 2009a, Kovarik et al. 1998). Nevertheless, ethanol failed to be used as a common fuel at that time because of the abundant and cheap supply of petroleum and natural gas. The disruption of oil supply from the Middle East in 1970s, however, re-boosted the production of ethanol (Bothast and Schlicher 2005). The subsequent policy support, including federal and state tax incentives, together with the legal restrictions of using oxygenated fuels further boosted the growth of ethanol industry (Bothast and Schlicher 2005). Ethanol production has undergone a sustained boom from 175 million gallons (662 million liters) in 1980 to 13.9 billion gallons (52.6 billion liters) in 2011 (**Figure 1**, RFA 2012). Energy Policy Act of 2005 and Energy Independence and Security Act of 2007 mandated the renewable fuels to increase to 36 billion gallons by 2022 (Urbanchuk 2010). From the industry perspective, ethanol production is expected to be at least 15 billion gallons by 2015 (Korves et al. 2008).

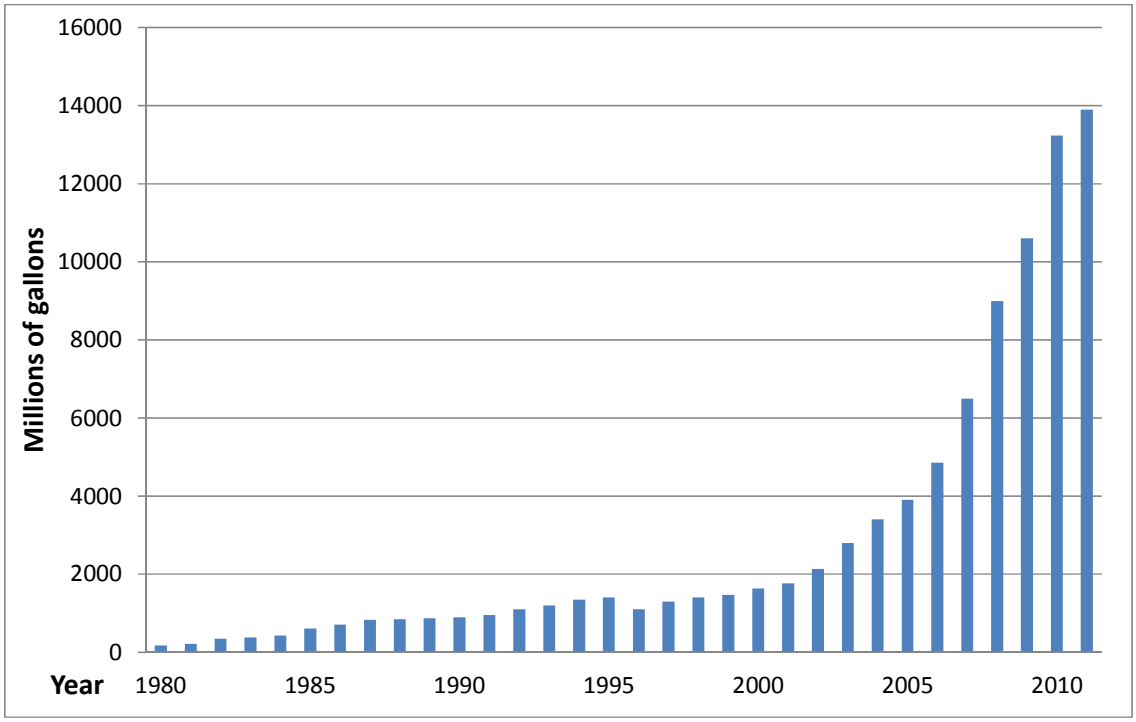

Figure 1. Historic U.S. ethanol production (RFA 2012)

Facts of ethanol fuel

Ethanol is widely used as a transportation fuel in the United States, which was responsible for 62.2% of the global ethanol fuel production in 2011 (RFA 2012). Ethanol represented 10% of the U.S. gasoline fuel supply in 2011, and the most commonly used form is E10 gasoline blend (ethanol blended with gasoline in a ratio of 1:9). There is another commonly used blend, E85, which consists of 85% ethanol and 15% gasoline. Engines of most cars need no modification when using E10, whereas E85 can only work in specially-designed flex-fuel vehicles to withstand high ethanol concentrations (Gnansounou 2009).

Ethanol as a fuel:

Octane rating is an important measure of the performance of a fuel. High octane rating is desired, because it means that more compression the fuel can withstand before self-ignition, which allows an increase in the engine's compression ratio for improved thermal efficiency (Freudenberger 2009b). Ethanol fuel has higher octane rating (106) than conventional gasoline fuel (usually 87-93). The heating value of ethanol, however, is only about 63% of the gasoline, which results from the presence of oxygen in the molecular structure of ethanol (Freudenberger 2009a).

Ethanol blend fuels have been blamed for several issues about the engine performance. Because ethanol has higher flash point and latent heat of vaporization, it is less volatile than gasoline, which raises concerns for starting difficulties of engine using blend gasoline in the cold weather. In effect, this is not a major issue as engine can be started on gasoline in the blends and generates enough heat to vaporize ethanol sufficiently (Freudenberger 2009b). Another issue challenging the use of ethanol blend is that ethanol could cause corrosion and degradation to metal parts, fuel lines, seal, and diaphragms in the engine. But the damage is related to the water content in the ethanol.

In the U.S., the ethanol blended into gasoline is anhydrous, and when the water content is below 5%, the corrosive effects are not significant (Freudenberger 2009b).

Economic impacts:

The ethanol industry is an important contributor to the employment, incomes of rural families, and development of rural economics. At the end of 2011, the ethanol industry comprised about 209 plants in 29 states with a gross nameplate production capacity of 14.7 billion gallons. The ethanol industry supported 90,200 direct jobs and 311,400 indirect jobs across the country in 2011, and contributed $42.4 billion to the national Gross Domestic Product (GDP) (Urbanchuk 2012). The ethanol industry increased $29.9 billion income to American families in 2011, mostly to corn growers who benefited from the demand of feedstocks. Increased use of biofuels contributes to the decline in foreign oil dependence, and the expansion of the ethanol industry will enable the country to break its dependence on fossil fuels. The production of 13.9 billion gallons of ethanol compensated for 485 million barrels of oil for refinery gasoline, which is equivalent of 13% total U.S. crude oil imports in 2011 (Urbanchuk 2012).

Total ethanol production cost of the whole industry was close to $40 billion during 2011, of which the expenditures on feedstocks (mostly corn) accounted for 83.3% ($33.3 billion) of the total costs. The cost of energy input including natural gas and electricity contributed to 6.5% of the total costs (Hostrand 2012). The most prominent studies showed that net energy balance of ethanol production ranged from 20,436 Btu/ga (British thermal unit per gallon) to 30,589 Btu/ga, which gives an energy return ranging from 1.29 to 1.65 (Shapouri et al. 2002, Shapouri et al. 2010).

Expansion of the corn-based ethanol industry, however, has been blamed to be the driving force behind higher agriculture commodity prices in recent years. Those concerns, however, were usually based on anecdotal evidences. Two independent studies by World Bank and OCED (Organization for Economic Co-operation and

Development) claimed that influences of biofuels on food/feed are much smaller than originally reported (Baffes and Haniotis 2010, OECD 2008). Actually, no single factor is the driver of food prices, but rather, food prices are influenced by a set of interrelated factors, such as increases in petroleum price, inflation pressures, and supply-demand balances. Ethanol demand is not the only factor that influences corn prices. And also, based on the analyses of historical price data (RFA 2011), corn prices did not show strong effects on the prices of livestock, poultry, egg and milk. Briefly, there is little statistic evidence supporting a conclusion that growth of the ethanol industry is the main driving force of steep rise of food prices.

Dry-grind ethanol production

There are two major traditional industrial processes for producing fuel ethanol in the United States: wet milling and dry-grinding. Wet milling formerly dominated as the method of ethanol production in the United States, but the dry-grind process is now the most widely used industrial method and represents >70% of the ethanol processing (Moseir and Ilelej 2006, Tiffany and Eidman 2005). In the wet milling process, corn kernels are soaked and softened before fractionation into germ, endosperm, fiber and gluten to produce a variety of products separately. In the dry-grind process, the whole grain is processed to produce ethanol and co-products (e.g. DDGS) (Bothast and Schlicher 2005). Dry-grind process produces ~2.8 gallons of ethanol and 7.7kg (17lbs) distiller's dry grains per bushel of corn (Mosier and Ileley 2006). Compared with the dry-grind process, the wet milling process is more versatile, which allows for a wet mill plant to better react with market conditions.

The large-scale and capital-intensive wet milling process, however, results in higher costs of constructions and operation. Therefore, with targeting at ethanol as the main product, a dry-grind process is preferred by producing ethanol more efficiently at a lower cost (Tiffany and Eidman 2005, Dale and Tyner 2006).

Conventional dry-grind process:

Production of ethanol from grains using the conventional dry-grind process includes several major steps: grinding, liquefaction, saccharification, fermentation, and recovery of ethanol and co-products (e.g. DDGS) (**Figure 2**).

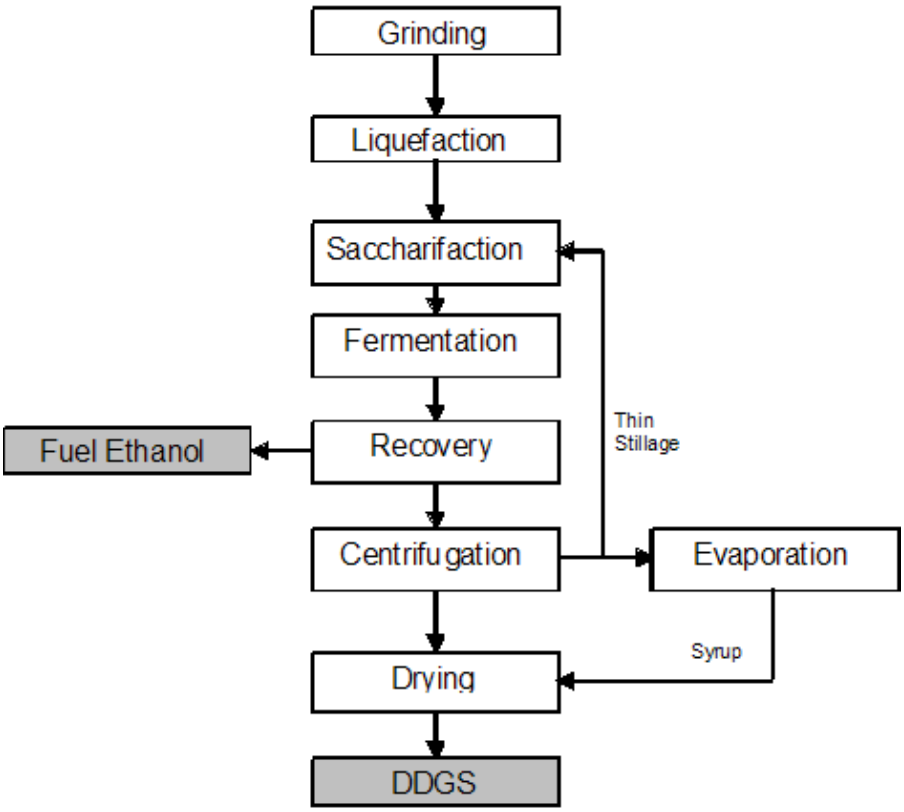

Figure 2. Overview of the conventional dry-grind ethanol fermentation process. Adapted from McAloon et al. (2000).

Grinding: Corn kernels are ground in hammer mills equipped with screens of pore size ranging between 3.2 and 4.8mm in diameter. After milling, more than 90% (w/w) of the ground corn has particle sizes between 0.5 and 2mm (Rausch et al. 2005).

Liquefaction: The dry-grind corn is mixed with process water (fresh or recycled water) to form slurry with ~30% solid content by weight (Kwiatkowski et al. 2006). The pH of the slurry is adjusted to 6.5, and thermostable α-amylase that hydrolyzes α-1,4 glycosidic

bonds at random points in the starch is added to the slurry to break down starch granules and produces dextrins with various sizes. The slurry is usually heated to above 90˚C through steam injection by a jet cooker to completely gelatinize the starch and make the starch more susceptible to enzyme hydrolysis. The liquefaction is continued for 60-90 minutes, and the slurry is then cooled down to around 60˚C, and mixed with the recycled water from the end of the ethanol distillation process that carries the critical nutrients for the yeast (McAloon et al. 2000).

Saccharification: Sulfuric acid is added to adjust the pH of the slurry to 4.5. Amyloglucosidase, an exo-enzyme, is then added to the slurry to hydrolyze α-1,4 and α-1,6 glycosidic bonds of the dextrins and releases glucose from the non-reducing ends. Saccharification usually continues for 6 hours at 60˚C to produce high concentrations of glucose as the substrate for fermentation. To save the processing time and reduce the energy requirement, saccharfication can take place in a fermentation tank where saccharification and fermentation occur simultaneously (SSF) (Lynd et al. 1999). SSF lowers the chance of microbial contamination and provides the yeast with a just-in-time supply of glucose, which reduces osmotic stress on the yeast (Bothast and Schlicher 2005).

Fermentation: The mash is cooled down to 30-32˚C and ammonium sulfate is added as the nitrogen source for the growth of yeast. Yeast is added to the slurry to ferment the glucose to produce ethanol and carbon dioxide:

$$C_6H_{12}O_6 \longrightarrow 2\ C_2H_5OH + 2\ CO_2$$

Saccharomyces cerevisiae yeast species are usually used for the ethanol fermentation because of its high production efficiency and stability to high glucose and alcohol concentrations (Butzen et al. 2003). Fermentation often continues for 48-72 hours to ensure the complete conversion of glucose to ethanol.

Distillation and dehydration: After fermentation, ethanol in the mash is separated from the water and fermentation residuals through several steps of distillation. The distilled and concentrated ethanol vapor is filtered through a molecular sieve system and reaches a purity higher than 99.6%. The remaining whole stillage is centrifuged, and the solid fraction (distiller's wet grains) is separated from the liquid (thin stillage). The thin stillage is condensed by evaporation to produce syrup containing more than 55% (w/w) solids (McAloon et al. 2000). The syrup is combined with the distiller's wet grains, and the mixture is dried to a moisture content of 9-10% in a rotary drum dryer. The resulted product is the distiller's dry grains with solubles (DDGS) that contains proteins, fibers and corn oils and is used as a feed ingredient for livestocks.

Cold-fermentation process:

Cold fermentation, also described as raw-starch, non-cooking, and non-conventional fermentation, is a simplified process compared with the conventional method. Production of ethanol using the cold-fermentation process consists of several major steps: grinding, simultaneous saccharification and fermentation (SSF), recovery of ethanol and co-products. The liquefaction step is eliminated in a cold-fermentation process, which means the starch in the dry-grind grain is not gelatinized or liquefied. Instead, a slurry containing the dry-grind grain and process water is directly mixed with hydrolyzing enzymes and yeasts, and the SSF occurs at 27-29˚C to produce ethanol. Therefore, a cold-fermentation technique requires an enzyme that is able to hydrolyze raw starch.

Compared with the conventional dry-grind process, cold-fermentation technique offers several benefits: decreasing the energy input and capital costs, minimizing the occurrence of Maillard reaction, amylose-lipid complex and retrograded-starch formation, reducing osmotic stresses of yeast, and producing more nutritious DDGS (Galvez 2005, Lewis et al. 2004, Robertson et al. 2006, Srichuwong and Jane et al. 2011).

Nevertheless, adoption of the cold-fermentation technique is impeded by the relatively low starch-ethanol conversion efficiency and increased chance of microbial contamination (Robertson et al. 2006).

Starch structures and properties

Starch, as the energy-reserving compound, is produced by green plants and can be found in different parts of the plant, including seeds, stems, leaves, fruits, tubers and roots. Starch granules for temporary storage are synthesized in chloroplasts, while starch granules for long-time storage are produced and store in amyloplasts (Robyt 1998). Starch granules vary in shapes (spherical, oval, polygonal, disk, elongated, etc.) and sizes (ranging between <1μm and >100μm), which depend on the botanical sources of the starch (Hoover 2001, Jane et al. 1994, Srichuwong et al. 2005, Tester et al. 2004). Native starch granules have semi-crystalline structures and are not soluble in water, which facilitates the isolation of starch granules by sedimentation, centrifugation and filtration. Molecular composition, organization and structures of the starch granule directly affect its physicochemical properties (Jane 2004).

Molecular composition and structure of starch granule:

Starch consists of two polysaccharides: amylose and amylopectin. Amylose is primarily a linear molecule composed of D-glucopyranose residues linked by α-1,4 glycosidic bonds. Amylopectin has a highly branched structure containing relatively short linear chains of D-glucopyranose units linked by α-1,4 glycosidic bonds. The short chains are linked by α-1,6 glycosidic bonds, which account for ~5% of the total glycosidic linkages (Banks and Greenwood 1975, Robyt 1998). Usually, normal starch contains 15-30% amylose, depending on the botanical origin, degree of maturity, and growing conditions of the plant. The amylose content in waxy starch is small (0-8% amylose), while high-amylose starch contains 50% or more amylose (Li et al. 2008, Perez and

Bertoft 2010). High-amylose starch also consists of another polysaccharide known as the intermediate component (IC), which has a similar molecular weight to that of amylose but contains relatively more branched structure (Baba and Arai 1984).

Amylose, amylopectin, and IC molecules have different characteristics of molecular weight, molecular structure, physical and chemical properties (e.g. crystallinity, complex formation). The composition, molecular structures and organization of these molecules in starch granules have significant effects on structures and properties of the starch.

Amylose in the starch of various botanical sources shows a broad range of molecular-weight distribution. The number average DP of amylose in maize and barley starch were found to be 960 and 1570 (Takeda et al. 1987, 1988, 1999), respectively, whereas that of potato and tapioca were found to be as large as 6360 and 6680 (Hizukuri and Takagi 1984), respectively. In general, amylose in cereal starch has smaller molecular sizes than that of tuber and root starch (Jane 2006, Takeda et al. 1987).

Percentage hydrolysis of purified amylose obtained from various sources was up to 70-90% by β-amylase (an exo-enzyme that hydrolyzes α-1,4 glycosidic linkage from the non-reducing end of polysaccharide chains) (Banks and Greenwood 1967, Hizukuri et al. 1981). The amylose, however, can be completely hydrolyzed by concurrent action of β-amylase and pullulanase, which suggested the presence of α-1,6 linked branches in amylose molecule (Hizukuri et al. 1981, Takeda et al. 1987). Amylose molecules of various origins have different characteristics of branched structures, including the number of branch-chains (5-21 per molecule) and average inner-chain-length (number average DP 50-160) (Shibanuma et al. 1994, Takeda et al. 1987, 1989).

Amylose has a strong tendency to complex with either a suitable complexing agent to form a single-helical inclusion complex (Katz and van Itallie 1930) or with another amylose to form a double helix (Miles et al. 1984). Amylose single-helical

inclusion complex is usually left-handed, and displays a V-type X-ray diffraction pattern (Takeo et al. 1973, Zobel 1988). The amylose single-helices usually have helical structures with 6-8 glucoses per turn, which depend on the size of the complexing agent, and the lamellar thickness of a single amylose V complex crystal is about 10nm (Yamashita 1965, Yamashita and Hirai 1966, Yamashita and Monobe 1971).

Amylopectin is generally a much larger molecule than amylose. Number average DP of amylopectin of various origins was reported to range from 9,600-15,900 (Takeda et al. 2003). Amylopectin is highly-branched molecule, and the branch-chains are designated into several categories, i.e. “A”, “B”, and “C” chains (Peat et al. 1952). A single amylopectin molecule contains only one C chain that carries the sole reducing end and other chains. The A chains are those connected to B or C chain through α-1,6 linkages and carry no other chains. The B chains carry one or several other A or B chains. The B chains are further grouped into B1, B2, B3, and even B4 chains. Classification of amylopectin branch-chains is shown in **Figure 3**.

A widely accepted model of amylopectin structure, the cluster model, was first proposed by French (1972) and Nikuni (1978), independently. As shown in **Figure 3**, adjacent amylopectin branch-chains (usually 4.22 chains to 34 chains) are closely packed (clustered) to form starch crystallites (Gallant et al. 1997). Clusters are linked to each other by long branch-chains. The average chain-lengths of the A and B1 chains are usually <24 (DP) and, thus, both the A and B1 chains can only extend within a single cluster. The B2 and B3 chains have average chain-lengths in the ranges of 42-48 and 69-75, extending through two and more clusters, respectively (Hizukuri 1986).

The intermediate component (IC) molecules possess molecular structures resembling that of both amylopectin and amylose. The IC molecules usually have larger ratios of long/ short branch-chains, and the proportion of long branch-chains has been reported to increase as decrease in molecular weight of the IC molecules (Takeda et al. 1993, Wang et al. 1993).

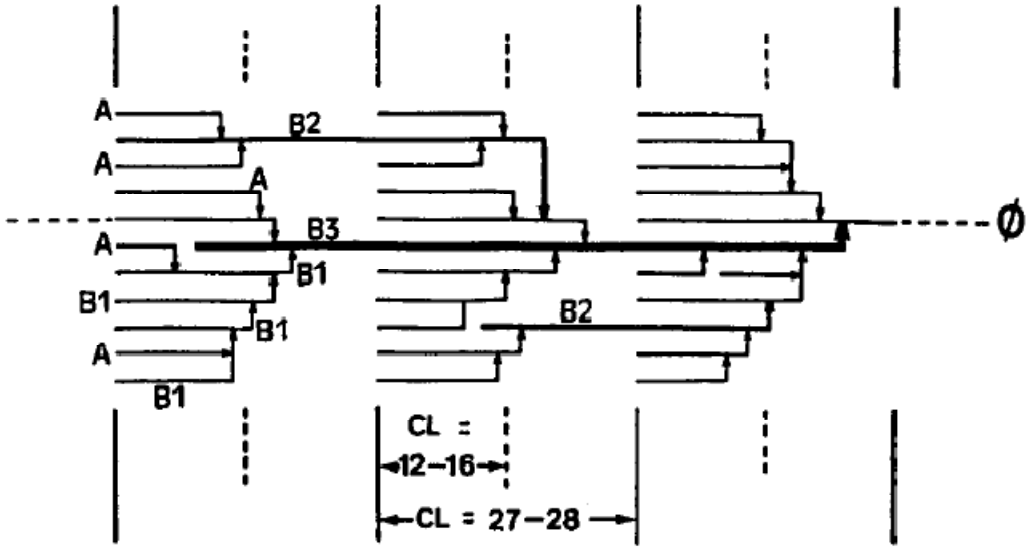

Figure 3. Cluster model and classification of amylopectin. (Adapted from Hizukuri 1986).

Organization of starch granule:

In a starch granule, amylopectin and amylose molecules are radially oriented side by side from the hilum to the periphery, which is reflected by Maltese cross of starch granules as viewed under a polarized-light microscope (Jane 2007). Amylopectin branch-chains form double helices and are clustered to form a crystalline lamella with a thickness of 9-10nm (Jenkins et al. 1993). It is proposed that branch-chains of amylopectin are organized in parallel to form clusters and crystalline regions (Gallant et al. 1997). The cluster structure is stabilized by hydrophobic interaction, hydrogen bonds and van de Waals forces between double helices of branch-chains (Imberty et al. 1988). Branch points of the branch-chains consist of the amorphous regions. The alternating crystalline and amorphous regions contribute to the semi-crystallinity of the starch granule. Amylose is in amorphous form and interdispersed among amylopectin molecules. Amylose molecules are more concentrated at the periphery of starch granule, and contribute to the integrity of the granular structure by intertwining with amylopectin (Jane et al. 1986).

There are three different crystalline polymorphs of starch semi-crystalline structure, A-, B-, and C-type. The unit cell of A-type polymorph is monoclinic (*a*=2.1224 nm, *b*=1.172nm, *c*=1.069 nm), whereas that of B-type is hexagonal (*a*=*b*=1.85 nm, *c*=1.04 nm) (Buleon et al. 1998, Wu and Sarko, 1978) (**Figure 4**). C-type polymorph is a mixture of the A- and B-type unit cells. Most cereal starch, like maize, barley, and wheat, has A-type polymorph, whereas the starch of potato and high-amylose corn displays the B-type polymorph. The differences are attributed to the amylopectin branch-chain-lengths of the starch from different origins (Jane 2004).

Properties of starch:

The semi-crystalline structure of starch is stabilized by the hydrophobic interaction, hydrogen bonds and van de Waals forces, as stated above. Application of heating to the starch granules with the presence of sufficient amount of plasticizer (i.e. water or glycerol) overcomes the molecular forces and results in melting of the crystalline structures. This process is called starch gelatinization, which is accompanied with the loss of crystallinity and briefringence, and even disruption of starch structures (Jane 2004). Structures and minor components of starch have significant effects on starch gelatinization properties. It was reported that starch onset gelatinization temperature negatively correlated with the proportion of short branch-chains (DP <12) of amylopectin (Jane et al. 1997, Jane et al. 1999, Srichuwong et al. 2005). The presence of negatively charged phosphate-monoester derivatives (i.e. potato starch), however, decreases starch gelatinization temperature by the charge-repulsion (Jane et al. 1999).

When the gelatinized starch is continuously heated in excess water, starch pasting occurs, which is accompanied with granular swelling, leaching of molecular components, development of viscosity, and complete disruption of the starch granule (Atwell et al. 1988). Starch containing larger amounts of amylose and phospholipids usually displays higher pasting temperature and lower peak viscosity (Jane et al. 1999,

Yoo and Jane 2002). Amylose and phospholipids restrict swelling of amylopectin and, therefore, reduce the pasting viscosity of starch (Craig et al. 1989).

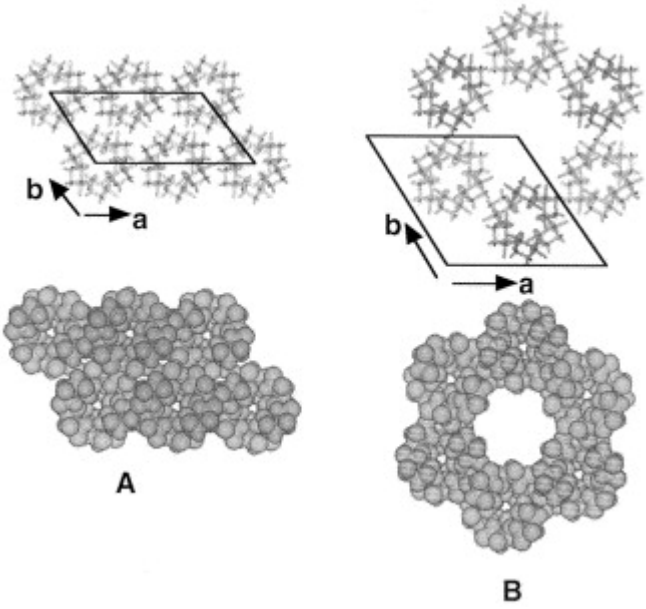

Figure 4. Crystalline packing of double helices in A-type and B-type polymorphs (Adapted from Buleon et al. 1998).

Gelatinized starches in the solution, paste, or gel tend to recrystallize upon cooling and during storage, with loss of water-binding capacity. The process is known as starch retrogradation. Starch retrogradation is significantly affected by the structures and minor components of starch. Amylopectin branch-chain-length positively correlated with its retrogradation rate (Jane et al. 1999, Perera et al. 2001). Amylose molecule with a chain-length of DP 80-100 displays maximal rate of retrogradation (Pfannermuller et al. 1971, Gidley and Bulpin 1987). The presence of lipids or phospholipids facilitates starch retrogrdation, which might be attributed to the restricting effects of the swelling of starch granule during cooking (Jane 2004).

Starch hydrolysis

Hydrolysis of starch to glucose is important for the maximal utilization of starch to provide energy for animals and plants, as well as to provide the substrate for ethanol fermentation by yeast. Digestibility of starch is impacted by a lot of factors, including the processing method, hydrolyzing enzymes, amylose/ amylopectin ratio, protein and lipid content, granular size and surface area, and starch granular structures (Rooney and Pflugfelder 1986, Svihus et al. 2005, Tester et al. 2004).

Effects of starch chemical composition and associated components:

Amylose/ amylopectin ratio of starch significantly affects the starch digestibility. Normal starch is usually less digestible than its waxy counterpart, and high-amylose starch has even poorer digestibility in both uncooked and cooked forms (Gallant et al. 1972, Perera et al. 2001, Rooney and Pflugfelder 1986). Amylose is known to be concentrated at the periphery of starch granule and intertwines with amylopectin, making the starch granule more resistant to enzyme hydrolysis (Jane 2007). Studies on high-amylose corn starch showed that the starch granules retained partially crystalline structures after cooking, and the resistant starch content positively correlated with the amylose content (Knutson et al. 1982, Li et al. 2008).

Several non-starch components (i.e. lipids and proteins) are associated with the starch granule, which may inhibit starch digestion (Svihus et al. 2005). Starch granules can be embedded in the protein matrix and cell-wall structures (i.e. corn and sorghum), which reduce the swelling of starch granule and the accessibility to enzymes and, thus, slow down starch hydrolysis (Rooney and Pflugfelder 1986). It has been reported that fatty acids, including palmitic and linoleic acid, form complex with amylose on the surface of starch granule (Baldwin et al. 1997, Crowe et al. 2000, Cui and Oates 1999, Tufvesson et al. 2001), which is associated with inhibited swelling of starch granule during cooking and reduced interaction between hydrolyzing enzymes and the starch (Svihus et al. 2005).

Effects of starch granular structures and surface area:

Studies showed that the B-type starch is less susceptible to enzyme hydrolysis than the A-type starch (Srichuwong et al. 2005), which is attributed to the differences in amylopectin branch-chain-length distribution. The B-type starch, like potato and high-amylose corn starch, has larger proportions of long branch-chains and longer average branch-chain-length, whereas the A-type starch (i.e. cereal starches) has less long branch-chains and shorter average branch-chain-length (Jane et al. 1999, Hizukuri 1986). The crystalline structure of the A-type starch is composed of mainly short A and B1 chains, which is susceptible to rearrangement and, thus, generates voids and channels inside of the granule, resulting in a loosely packed structure (Gray and BeMiller 2004, Huber and BeMiller 2000). On the other hand, the B-type starch contains larger amounts of long B-chains (B2 and B3) that extend through two or more clusters, stabilizing the crystalline structure, resulting in a solid granular structure (Jane 2006). In the contrary, the loosely packed structure of the A-type starch facilitates enzyme hydrolysis and, therefore, has greater starch digestibility.

Enzyme adsorption to starch granule and subsequent formation of enzyme-substrate complex is the prerequisite step for starch hydrolysis (Leloup et al. 1991). The amount of enzymes adsorbed to the granule surface is proportional to the surface area of the starch granule; as a result, the hydrolysis rate of starch is impacted by the granular surface area (Kong et al. 2003). Starch with small granules (i.e. cereal starch) usually has greater hydrolysis rate compared with that of large granules (i.e. potato starch), which is attributed to the relatively larger surface area of the small granules (Manelius and Bertoft 1996). In addition, it has been reported that small starch granules show less crystallinity and greater water affinity than that of large granules, which further enhance enzyme accessibility to the small granules (Svihus et al. 2005).

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CHAPTER 2. CHARACTERIZATION OF NORMAL AND WAXY CORN STARCH FOR BIOETHANOL PRODUCTION

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ABSTRACT

Objectives of this study were to: 1) Compare the differences of ethanol production between normal and waxy corn using a cold-fermentation process; 2) Understand the effects of starch structure/ properties on the ethanol production. Ethanol yields positively correlated with kernel starch contents of the normal and waxy corn. Average starch-ethanol conversion efficiency of the waxy corn (93.2%, 2009; 93.0%, 2010) was substantially greater than that of the normal corn (88.0%, 2009; 88.4%, 2010). Starches of the selected lines were isolated for characterization, including amylose content, amylopectin branch-chain-length distributions, thermal properties, and enzymatic hydrolysis of raw starch. Starch hydrolysis of uncooked dry-grind corn showed that > 90% of starch in the waxy corn was hydrolyzed, whereas < 80% of starch in the normal corn was hydrolyzed. It indicated that normal corn contained a significant portion of starch that was less readily hydrolyzed by the enzymes, which reduced the conversion efficiency.

Keywords: Bioethanol; cold fermentation; starch; enzymatic hydrolysis.

INTRODUCTION

To improve energy security by reducing the nation's dependence on foreign oil supply, substantial effort and research have been invested on biofuel production from renewable resources. Bioethanol is widely used as a transportation fuel in the United States, which was responsible for 62.2% of the global ethanol fuel production in 2011 (*1*). Ethanol is produced mainly from corn in the United States (*2,3*).

Production of ethanol from corn requires hydrolysis of starch to glucose, and glucose is then fermented by yeast to produce ethanol because yeast cannot utilize starch directly (*4,5*). A conventional process for ethanol production is to gelatinize the starch in dry-grind grain, and the gelatinized starch was hydrolyzed to dextrin using thermal-stable α-amylases (liquefaction). The resulting dextrin is then cooled to 60℃ and saccharified with amyloglucosidases to produce glucose that is the substrate for ethanol fermentation. Nevertheless, energy used to cook starch increases the production cost and decreases the energy return of bioethanol. In addition, formation of amylose-lipid complex and retrograded starch after starch gelatinization reduces the amount of fermentable starch and, thus, decreases the ethanol yield (*5,6*).

An industrial process of cold fermentation for ethanol production was developed by Lewis, et al. (*7*). The starch is hydrolyzed by raw starch hydrolyzing enzymes into fermentable sugars during simultaneous saccharification and fermentation (SSF) without cooking and liquefaction of the starch. Compared with conventional methods, this innovative technique for ethanol fermentation effectively reduces production costs by decreasing the energy input, simplifying the process, and minimizing the occurrence of the Maillard reaction, amylose-lipid complex and retrograded-starch formation during and after heating (*8*). Furthermore, raw starch fermentation does not require large capital investment and is more feasible for small-scale ethanol production (*4*).

Great efforts have been invested to improve the ethanol yield and starch-ethanol conversion efficiency of feedstocks. Many factors and processing conditions, which affect the ethanol production performance, have been investigated, including nitrogen sources, fermentation temperatures, starch hydrolysis, saccharification efficiencies, and chemical compositions of a wide variety of crops (*9*). The impacts of the starch structures and properties on the raw-starch ethanol fermentation, however, are not fully understood. Many studies have reported negative effects of the starch amylose content on the conversion efficiency in the conventional ethanol fermentation process (*9,10,11*). There are few studies, however, comparing the ethanol fermentation between waxy and normal corn in the raw-starch fermentation process (*12*).

In this study, ethanol production was conducted using a raw-starch fermentation process. The ethanol yields and starch-ethanol conversion efficiencies of waxy and normal corn lines were compared. Starch structures, thermal and pasting properties, and raw-starch digestibility of the corn starch were analyzed.

MATERIALS AND METHODS

Materials. Four normal corn lines (08GEM0**4701-4704**) and nine waxy corn lines (08GEM0**5036-5044**) were developed by the USDA-ARS Germplasm Enhancement of Maize (GEM) Project. Four of the nine waxy lines were released and assigned GEM codes, and all of the four normal lines were released. Additional information on the released lines can be found on the GEM website at www.public.iastate.edu/~usda-gem. For convenience the inventory numbers are used throughout the manuscript which indicates the seed source used. The corn lines were selected to represent a diverse group of racial diversity comprising germplasm from seven races, and three tropical hybrids which originated from eight countries. All the corn lines were grown at the North Central Regional Plant Introduction Station farm (Ames, IA), in both 2009 and 2010 crop seasons. Pedigree, racial background, and geographic origin of each line are shown in

Table 1. Ethanol Red™ dry yeast (>20×10^9 living cells/g) was obtained from Lesaffre yeast corporation (Milwaukee, WI). Lactrol (virginiamycin) was from Phibro Animal Health Co. (Ridgefield, NJ). Isotab (hop acid) was from Beta Tec Hop Products (Washington, DC). Raw-starch hydrolyzing enzymes containing a mixture of fungal α-amylase and amyloglucosidase were from Novozyme (Novozyme 5009, Novozyme, Franklinton, NC). Pseudomonas isoamylase (EC 3.2.1.68, 1000U/ml) and total starch kit were from Megazyme International Ireland Ltd. (Co. Wicklow, Ireland). All other chemicals were reagent grade and were purchased from either Sigma–Aldrich Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and used without further purification.

Dry grinding of corn kernels. Maize kernels of GEM lines were dried to approximately 12% moisture and were dry-ground using a Cyclone Mill (UDY Corp., CO), screening with a steel sieve of 0.5mm pore size.

Raw-starch ethanol fermentation. Dry-grind corn (35g, db) was placed in a polypropylene bottle (125ml, prior autoclave-sterilized). A mash of 100g total weight was made containing liquid urea (0.03%, w/w), lactrol (2ppm), isotab (40ppm) and acetate buffer (10mM, pH4.2). The mash was then mixed with 0.5g dry yeast and raw-starch hydrolyzing enzymes (0.46%, v/w of dry-grind corn, Novozyme 5009, Novozyme, Franklinton, NC). The fermentation samples were incubated in a shaker incubator (Lab-line Instruments Inc., Melrose Park, IL) at 29℃, 160r pm. Aliquots (8.0ml) were taken from the fermentation broth after 96h and centrifuged at 7,010*g* for 10min. The supernatant was filtered through a nylon membrane filter with 0.2μm pore size. The ethanol concentration was analyzed using a HPLC system consisting of a pump (Prostar 210, Varian, Walnut Creek, CA), an injection valve (model 7725i, Rheodyne), and a refractive index detector (Prostar 355, Varian, Walnut Creek, CA), following the procedures reported by Ai, et al. (*13*).

Ethanol conversion efficiency was calculated using the equation: conversion efficiency (%) = 100 × ethanol yield (w/w)/theoretical yield of ethanol. The theoretical

yield of ethanol is 56.73 g ethanol/100 g starch, which is calculated on the basis of 1 g of starch being hydrolyzed into 1.11 g of glucose, and 1 mol of glucose fermented to produce 2 mol of ethanol (*2*).

Starch content assay. The starch content of the corn grain was analyzed using a total starch kit (Megazyme International, Wicklow, Ireland; catalog no. K-TSTA), following the AACC 76.13 method (*14*).

Starch isolation by wet-milling. Starch was isolated from corn kernels using a wet-milling method reported by Li, et al. (*15*).

Amylose content of starch. Amylose contents of waxy and normal corn starch were analyzed using gel-permeation chromatography (GPC) followed by total carbohydrate analysis (*15*). Amylose contents of waxy and normal corn starch were also determined using iodine potentiometric titration following the method reported by Song and Jane (*16*).

Branch-chain-length distribution of amylopectin. Amylopection of normal corn starch was separated from amylose and collected using a GPC column packed with Sepharose CL-2B gel (Pharmacia Inc., Piscataway, NJ) (*5*). The amylopectin of normal corn starch and the waxy corn starch was debranched with isoamylase (Megazyme International Irelands. Ltd. Co., Wicklow, Ireland) following the method reported by Jane and Chen (*17*). The debranched sample was labeled with 8-amino-1,3,6-pyrenetrisulfonic acid (APTS), and analyzed using a fluorophore-assisted capillary electrophoresis (P/ACEDQ, Beckman Courter, Fullerton, CA) following the method reported by Jiang, et.al (*18*).

Thermal properties of starch. Thermal properties of the isolated starch were analyzed using a differential scanning calorimeter (Diamond DSC, Perkin-Elmer, Norwalk, CT) following the procedures of Song and Jane (*16*). Starch gelatinization onset (T_o), peak (T_p), and conclusion temperature (T_c), and enthalpy change (ΔH) were obtained using Pyris software (Perkin-Elmer). After seven-day storage at 4℃, the

samples were analyzed using the same parameters, and the percentage retrogradation was calculated using the equation: retrogradation (%) = 100 × Δ*H* of dissociation of retrograded starch/Δ*H* of starch gelatinization.

Starch hydrolysis of uncooked starch and dry-grind grain. Dry-grind corn containing 200mg starch (dsb) or isolated starch (200mg, dsb) suspended in a sodium acetate buffer (20ml, 10mM, pH4.2) was pre-incubated at 29℃ for 30min. Raw-starch hydrolyzing enzymes (0.67%, v/w of starch, Novozyme 5009, Novozyme, Franklinton, NC) was then added, and the incubation continued at 29℃ with constant stirring at 160rpm for 96h. Aliquots (0.1ml) of the hydrolysate were withdrawn at different time intervals and were mixed with 1ml 66% (v/v) ethanol. The mixture was centrifuged at 6,600g for 5min, and the supernatant was collected. The glucose content in the supernatant was determined using a glucose oxidase/peroxidase assay (Megazyme International Irelands. Ltd. Co., Wicklow, Ireland; catalog no. K-GLUC).

Pasting properties of starch. The pasting properties of isolated starch were analyzed using a Rapid Visco-Analyzer (RVA, Newport Scientific, Sydney, Australia). Starch aqueous suspension (28.0g, 8% w/w, dsb) was equilibrated at 50 ℃ for 1 min, heated up to 95 ℃ at a rate of 6 ℃/min, held at 9 5 ℃ for 5 min, and then cooled down to 50 ℃ at a rate of 6 ℃/min. The parameters, inclu ding the pasting temperature, and the peak, breakdown, and final viscosities were obtained from data analysis using Thermocline (Newport Scientific) software.

Statistical analysis. SAS (version 9.2, SAS Institute, Inc., Cary, NC) was used for statistical analysis. Correlations between the ethanol yield, total starch content, and physicochemical properties of the starch were analyzed using the Pearson correlation test. Statistical significance was evaluated using one-way ANOVA and multiple comparison using Tukey's adjustment with a 5% significance level.

RESULTS AND DISCUSSION

Ethanol yields of the dry-grind normal and waxy corn using a cold-fermentation process are shown in **Table 2**. The ethanol yields of the four normal lines grown in 2009 ranged from 34.2% (34.2 g / 100g dry grain, Line 4703) to 37.2% (Line 4701 and 4704), and those grown in 2010 ranged from 34.3% (Line 4703) to 37.5% (Line 4701). Ethanol yields of the nine waxy lines grown in 2009 ranged from 33.1% (Line 5041) to 37.6% (Line 5036), and those grown in 2010 ranged from 34.8% (Line 5042) to 37.9% (Line 5036). Data of Line 5041 (2010) was not available because of insufficient quantity of corn kernels for ethanol fermentation. Line 5036 gave the highest ethanol yield among the GEM waxy lines for both 2009 (37.5%) and 2010 (37.9%) crop seasons.

The average ethanol yields of the waxy and normal corn lines were 35.7% and 36.0%, respectively, for the 2009 samples, and were both 36.0% for the 2010 samples. To understand the mechanism of different ethanol yields between the normal and waxy corn, we analyzed the kernel starch contents of the corn lines (**Table 2**). The average starch contents of the waxy corn were 67.5% and 68.4% for 2009 and 2010 samples, respectively, which were lower than that of the normal lines (72.0% and 71.7% for 2009 and 2010 samples, respectively). Among the waxy corn lines, Line 5036 (2009) that produced the greatest ethanol yield, had the largest starch content (71.1%), whereas Line 5041 (2010) that gave the smallest ethanol yield, had the least starch content (62.3%). Starch contents of the waxy corn positively correlated with the ethanol yield of the 2009 (R^2=0.87, $p<0.001$) and 2010 samples (R^2=0.88, $p<0.001$). The starch content of normal corn also positively correlated with the ethanol yield of the 2009 (R^2=0.95, $p<0.05$) and 2010 samples (R^2=0.97, $p<0.05$).

The average conversion efficiencies of the waxy corn were 93.2% and 93.0% for 2009 and 2010 samples, respectively, whereas that of the normal lines were 88.0% (2009) and 88.4% (2010). The waxy corn lines showed significantly greater conversion efficiencies than that of the normal lines. The results were consistent with those reported

previously (*9-11*). The conversion efficiency from starch to ethanol cannot reach 100% because of consumption of sugars for yeast growth and proliferation, and losses in by-products formation, such as glycerol and succinate (*19, 20*). The lower conversion efficiency of normal corn, however, indicated a portion of the starch that was not utilized.

To reveal what structural features were responsible for the differences in the conversion efficiency from starch to ethanol, we isolated and characterized starches of selected lines. Starches of the four normal lines and six waxy lines (Line 5036, 5037, 5039, 5040, 5041, and5042) of both 2009 and 2010 crop seasons were isolated and characterized for their structures and properties, including amylose content, amylopectin branch chain length distribution, starch thermal properties, and starch digestibility.

Normal corn starch contained 28-30% amylose determined using iodine potentiometric titration and 31-35% amylose using GPC followed by total carbohydrate analysis (**Table 3, Figure 1**). The differences were attributed to the presence of low molecular-weight amylopectin (*21*). Amylose contents of waxy corn starch determined using iodine potentiometric titration ranged 0.9-2.2% (2009) and 1.6-4.6% (2010), and was not detectable using GPC analysis **(Table 3, Figure 2**). It was reported that long branch-chains of amylopectin can form single helical complex with iodine and develop blue color during potentiometric titration, which causes over-estimation of the amylose content of starch (*22*). In this study, the starch of two waxy corn lines (Line 5041 and 5042) grown in 2010 showed relatively greater amylose contents (4.5 and 4.6%, respectively) determined using iodine potentiometric tritration (**Table 3**). This could be attributed to the relatively larger portions of long branch-chains (21.5 and 20.2%, **Table 4**) of amylopectin of the two waxy lines.

Amylopectin branch-chain-length distributions of the selected waxy lines and the normal lines are shown in **Table 4**. Amylopectin of the waxy corn starch displayed shorter average branch-chain-length (DP 21.1-22.7, 2009; DP 21.4-23.5, 2010) than that of the normal lines (DP 22.4-24.4, 2009; DP 22.7-23.9, 2010). Specifically, the waxy corn

starch amylopectin consisted of smaller proportions of the long branch-chains of DP>37 (15.5-19.2%, 2009; 15.6-21.5%, 2010) than that of the normal corn starch (19.6-24.0%, 2009; 19.9-23.4%, 2010). These results agreed with that reported previously (*22*), and could be attributed to the lack of extra-long branch-chains of amylopectin in the waxy corn starch, which were synthesized by granule-bound starch synthase I (GBSS I). The enzyme is not produced in the waxy corn (*23*).

Starch thermal properties of the selected waxy lines and the normal lines are shown in **Table 5**. For the 2009 samples, the average gelatinization onset (64.2℃), peak (70.7℃), and conclusion (76.6℃) temperature of the w axy corn starch were significantly higher ($p<0.05$) than that of the normal corn starch (average T_o=60.7℃, T_p=67.8℃, and T_c=73.5℃, respectively). For the 2010 samples, average T_c (78.7 ℃) of the waxy corn starch was significantly higher ($p<0.01$) than that of the normal corn starch, whereas T_o and T_p of the waxy corn starch were not significantly different from that of the normal corn starch. Starch gelatinization enthalpy-changes for the waxy corn ranged from 14.8 to 16.4 J/g for the 2009 samples, and from 15.1 to 16.2 J/g for the 2010 samples, which were substantially greater than that of the normal corn (10.8-12.3 J/g, 2009; 11.4-12.3 J/g, 2010). These results were in agreement with the study of Sasaki et al. (*24*) who reported higher conclusion gelatinization temperature and larger enthalpy-change of waxy wheat starch than that of non-waxy wheat starch. These differences resulted from different crystalline structures of the starch. Waxy corn starch had close to 100% amylopectin and, thus, displayed larger gelatinization enthalpy-change (*22*). Percentages of retrogradation of the waxy corn starch (32.0-51.6%, 2009; 41.3-45.8%, 2010) were smaller than that of the normal corn starch (53.0-59.3%, 2009; 52.0-56.4%, 2010). The difference may be attributed to that amylose in normal corn starch intertwines with amylopectin molecule and restricts granule swelling and dispersion. Consequently, the gelatinized normal corn starch displayed expedite retrogradation (*25*). In addition, normal

corn starch had a larger proportion of long branch-chains (DP>37), which favored starch retrogradation.

Among the waxy lines, the percentages retrogradation positively correlated with the average branch-chain-length for 2009 (R^2=0.94, p<0.01) and 2010 samples (R^2=0.80, p<0.05). The results were in agreement with previous report that amylopectin with long branch-chains retrograded more easily (*22, 26, 27*).

Enzymatic hydrolysis of the starch in dry-grind corn using the raw-starch hydrolyzing enzymes (Novozyme 5009) is shown in **Figure 3** and summarized in **Table 6**. The dry-grind waxy corn samples displayed substantially greater hydrolysis rates than the normal counterparts. After 96h hydrolysis, more than 90% of starch in the dry-grind waxy corn was hydrolyzed; on the contrary, less than 80% of starch in the dry-grind normal corn was hydrolyzed to glucose (**Table 6**). The results suggested that the normal corn contained a portion of starch that was not readily hydrolyzed by the raw-starch hydrolyzing enzymes and, therefore, reduced the conversion efficiency (**Table 2**). These results agreed with previous report that waxy starches have greater digestibility than their normal counterparts, and the enzyme hydrolysis rate decreases as the amylose content of the starch increases (*11, 28-30*). The lower digestibility of normal corn starch may be attributed to the intertwining between amylose and amylopectin, which restricted swelling of starch granules and reduced the enzyme hydrolysis rate. Studies (*3, 25*) have shown that amylose is more concentrated at the periphery of starch granule, which further contributes to the resistance of starch granules to enzyme hydrolysis. On the other hand, the waxy corn starch granules had significantly less amylose than the normal corn starch (**Table 3, Figure 2**), and was reported to possess a relatively loosely packed peripheral structure, rendering the starch granule more susceptible to enzymatic hydrolysis (*25*).

Enzymatic hydrolysis of isolated starches using the raw-starch hydrolyzing enzymes is shown in **Figure 4**, and summarized in **Table 7**. As expected, normal corn

starch showed lower hydrolysis rate than waxy corn starch. Compared with that of the dry-grind grain, interestingly, the isolated starch displayed lower digestive rate during the first 3-6h hydrolysis. After 10h hydrolysis, however, the digestive rate of the isolated starch became greater than that of the dry-grind grain. The larger digestive rate of the starch in dry-grind corn at the initial hydrolyzing stage could be attributed to the presence of damaged starch granules that resulted from mechanical shearing during the dry-grinding process and the presence of endogenous amylases. Some starch granules in the dry-grind corn, however, were entrapped in the protein matrix and endosperm cell-wall structure as reported by Rooney et al. (*31*). The entrapment reduced the accessibility of those starch granules to the amylases and, therefore, decreased digestive rate of the starch in the dry-grind corn.

Pasting properties of the selected waxy and normal corn starch are shown in **Figure 5**, and summarized in **Table 8**. Starches of the waxy corn lines grown in both 2009 and 2010 displayed higher peak and break-down viscosities but lower set-back viscosities than that of the normal corn starch. The results were consistent with the previous reports (*17, 25*). It is known that amylopectin contributes to starch viscosity, whereas amylose restricts starch swelling (*32*). Therefore, starch pasting properties are affected by the amylose content and amylopectin branch-chain-length distribution (*22*). The waxy corn starch contained much less amylose (**Table 3**). Thus, the swelling of starch granules was not restricted and displayed a higher peak viscosity than that of the normal corn starch. The amylose of the normal corn starch contributed to the maintaining the integrity of swollen starch granule and decreased shear-thinning of starch paste, resulting in the lower break-down viscosity and higher set-back viscosity of the normal corn starch.

Physicochemical properties of the starch of 2010 samples showed differences from that of the 2009 counterparts. For example, T_o of Line 5040 starch was 64.3℃ for the 2009 sample, whereas it was 57.7℃ for the 2010 sam ple (**Table 5**). All the normal

corn starch from the 2010 crop season displayed higher T_o (61.7-67.1℃) than their 2009 counterparts (56.8-64.2℃). It is known that starch gr anular structures and properties are affected by the growing conditions, such as growing temperature and amount of rainfall. Badenhuizen et al. (*33*) reported that increasing proportions of small and abnormally shaped starch granules were present in waxy maize grown at a higher temperature. Lu et al. (*34*) also observed smaller starch granules and greater T_o in normal dent maize developed at 35℃ than those developed at 25℃. In t he current study, the accumulated growing degree units (GDU) from the day of pollination (week of July 25th) to the end of August in 2010 was 504.2℃, which was significantly high er than that of the 2009 season (378.8℃). There was also more rainfall in the August of 2010 (39.6cm) than that of the 2009 crop season (10.1cm). The differences in starch structures and properties shown in this study could result from the different growing temperatures and amount of rainfall between the two crop seasons.

Although the growing temperature could affect the starch physicochemical properties, the starch-ethanol conversion efficiencies of the GEM corn were similar between the two years' samples. This indicated that the weather pattern change did not have a significant impact on the efficiency of raw-starch ethanol fermentation.

CONCLUSIONS

Ethanol yields of GEM corn lines using a cold-fermentation technique positively correlated with starch contents in the dry-grind grain. The waxy corn displayed greater starch-ethanol conversion efficiencies than the normal corn using the cold-fermentation process. This could be attributed to the greater starch hydrolysis rate of the waxy corn than that of the normal corn. The results suggested that the waxy corn would give greater ethanol yield in a cold fermentation process, if the waxy corn has similar starch content to that of the normal corn. Starch in the dry-grind grain showed smaller hydrolysis rate than the isolated starch except for the first 3-6h. The difference was

attributed to the entrapment of starch granules in protein matrix and cell-wall structures in the dry-grind corn, making the starch granules less readily-accessible to amylases. Damaged starch present in the dry-grind grain resulted from the dry grinding process, and contributed to the faster hydrolysis rate during the first 3-6h hydrolysis. There were differences in starch physicochemical properties between the corn planted in 2009 and 2010 crop years. This was likely caused by the changes in the growing conditions (e.g. growing temperatures) between the two crop seasons. The growing conditions, however, did not significantly impact the efficiency of raw-starch ethanol fermentation.

ACKNOWLEDGEMENT

We thank the USDA-ARS Plant Introduction Research Unit and GEM Project for providing the nursery site in Ames, IA, and other support for this research.

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Table 1. Pedigree background, race, and country of origin of GEM normal and waxy corn lines

	Inventory #	Pedigree	Code[a]	Country	Race
Waxy	08GEM0**5036**	AR16035:S02-615-001-B wx	GEMS-0206	Arg	Cristalino Colorado
	08GEM0**5037**	CUBA164:S2012-444-001-B wx	GEMS-0223	Cuba	Mixed Creole
	08GEM0**5038**	CUBA164:S2012-966-001-B wx	GEMS-0185	Cuba	Mixed Creole
	08GEM0**5039**	DKB844:S1601-003-002-B wx	N/A[b]	Mexico	Tropical hybrid
	08GEM0**5040**	FS8A(S):S09-362-001-B wx	N/A	US	Mixed
	08GEM0**5041**	CH05015:N12-183-001-B wx	N/A	Chile	Camelia
	08GEM0**5042**	DKXL370:N11a20-036-002-B wx	GEMN-0186	Brazil	Tropical hybrid
	08GEM0**5043**	SCRO1:N1310-509-001-B wx	N/A	St. Croix	St. Croix
	08GEM0**5044**	UR13085:N0215-014-001-B wx	N/A	Uruguay	Cateto Sulino
Normal	08GEM0**4701**	DKB844:S1601-073-001-B-B-B-B-B-B	GEMS-0115	Mexico	Tropical hybrid
	08GEM0**4702**	GEMS-0002	GEMS-0002	US	Mixed
	08GEM0**4703**	BR52051(SE32):S17-B-023-001-B-B-Sib-B-B-B-B-B	GEMS-0003	Brazil	Dente Amarelo
	08GEM0**4704**	BR51721:N2012-098-002-B-B-SIB-B	GEMN-0156	Brazil	Dente Amarelo

[a] Codes of released GEM lines

[b] Not released or assigned

Table 2. Total starch content (%), ethanol yields, and conversion efficiencies of the normal and waxy corn

	Line	Starch content (%)		Ethanol yield (g/100g dry grain)		Conversion efficiency (%)	
		2009 crop year	2010 crop year	2009 crop year	2010 crop year	2009 crop year	2010 crop year
Waxy	5036	71.1±[b]0.3	72.3±0.2	37.6±0.5	37.9±0.2	93.1	92.5
	5037	71.0±0.6	72.8±0.0	36.3±0.1	37.0±0.3	90.1	89.6
	5038	67.5±0.5	67.3±0.7	36.3±0.1	35.9±0.1	94.6	94.0
	5039	68.0±0.6	69.2±0.0	35.7±0.8	36.0±0.4	92.5	91.5
	5040	66.6±0.3	66.4±0.2	35.4±0.2	35.3±0.6	93.7	93.8
	5041	62.3±0.2	N/A[a]	33.1±0.8	N/A[a]	93.5	N/A[a]
	5042	64.1±0.0	65.3±0.1	34.6±0.3	34.8±0.5	95.0	94.0
	5043	68.9±0.7	66.7±0.2	36.2±0.5	35.7±0.4	92.5	94.3
	5044	68.1±1.0	66.8±0.4	35.9±0.1	35.6±0.2	92.8	94.0
	Average	67.5	68.4	35.7	36.0	93.2	93.0
Normal	4701	74.3±0.7	74.1±0.5	37.2±0.5	37.5±0.4	88.3	89.2
	4702	71.5±0.8	70.5±0.4	35.2±0.8	35.1±0.4	86.8	87.6
	4703	68.2±0.5	68.1±0.1	34.2±0.4	34.3±1.3	88.5	88.8
	4704	74.1±0.7	74.1±0.6	37.2±0.7	37.0±1.1	88.4	87.9
	Average	72.0	71.7	36.0	36.0	88.0	88.4

[a] Not determined because of limited material.

[b] Standard deviation. Samples were analyzed in duplicates.

Table 3. Amylose content (%) of the normal and waxy corn starch

		Line	Amylose%	
			Iodine titration[a]	GPC[b]
2009 crop year	Waxy	5036	0.9±0.0	ND[d]
		5037	1.3±0.0	ND
		5039	1.5±0.1	ND
		5040	1.4±0.4	ND
		5041	NA[c]	ND
		5042	2.2±0.0	ND
	Normal	4701	27.9±0.1	34.9±1.4
		4702	28.3±0.1	34.9±0.1
		4703	28.2±0.2	32.4±1.3
		4704	28.0±0.6	31.0±0.3
2010 crop year	waxy	5036	1.6±0.0	ND
		5037	2.1±0.0	ND
		5039	2.5±0.2	ND
		5040	2.1±0.1	ND
		5041	4.5±0.1	ND
		5042	4.6±0.4	ND
	Normal	4701	28.3±0.2	34.3±0.8
		4702	29.0±0.0	34.6±0.0
		4703	30.4±0.5	31.0±0.4
		4704	28.5±0.1	33.4±1.3

[a] Determined using iodine potentiometric titration

[b] Determined using gel-permeation chromatography (GPC) followed by total carbohydrate analysis

[c] Not determined because of limited material

[d] Not detectable

Table 4. Amylopectin branch-chain-length distribution[a] of the normal and waxy corn starch

		Line	DP<12	DP13-24	DP25-36	DP>37	*ave.* CL[b]
2009 crop year	Waxy	5036	22.7±0.4	47.7±0.7	14.0±0.7	15.5±0.4	21.1±0.3
		5037	23.4±0.1	45.9±0.4	13.9±0.2	16.9±0.7	21.5±0.4
		5039	22.1±0.2	48.0±0.1	13.9±0.5	16.1±0.4	21.6±0.0
		5040	23.6±0.7	45.7±0.5	13.5±0.2	17.2±1.0	21.7±0.5
		5041	21.0±0.8	46.2±0.6	13.6±0.2	19.2±1.2	22.7±0.5
		5042	22.1±0.0	45.5±0.3	13.5±0.3	18.9±0.0	22.4±0.0
		Average	22.5	46.5	13.7	17.3	21.8
	Normal	4701	21.8±0.2	38.4±1.3	17.1±1.0	22.6±0.4	23.1±0.0
		4702	14.6±0.3	42.6±0.7	18.9±0.6	24.0±1.0	24.4±0.3
		4703	20.5±0.5	45.0±1.0	14.9±0.6	19.6±0.8	22.4±0.2
		4704	20.2±0.3	42.0±0.0	15.3±0.3	22.4±0.0	23.5±0.0
		Average	19.3	42.0	16.6	22.2	23.4
2010 crop year	waxy	5036	21.4±0.3	47.7±0.6	15.2±0.1	15.6±0.0	21.4±0.2
		5037	22.7±0.2	47.0±0.5	13.9±0.1	16.4±0.6	21.5±0.1
		5039	20.9±0.5	46.8±0.4	14.8±0.8	17.5±0.1	22.2±0.1
		5040	20.2±0.2	45.2±0.6	14.5±0.2	20.1±0.6	23.1±0.2
		5041	20.3±0.5	44.3±1.5	13.8±1.0	21.5±1.1	23.5±0.2
		5042	20.6±3.1	44.4±3.2	14.8±0.6	20.2±0.8	23.1±0.1
		Average	21.0	45.9	14.5	18.6	22.5
	Normal	4701	18.4±0.5	40.5±0.1	17.7±0.4	23.4±0.1	23.9±0.1
		4702	20.5±0.1	44.8±0.1	14.8±0.2	19.9±0.1	22.7±0.2
		4703	19.3±0.2	43.3±0.1	15.5±0.5	21.8±0.2	23.3±0.0
		4704	21.0±0.4	43.0±0.9	15.4±0.8	20.6±0.5	22.9±0.2
		Average	19.8	42.9	15.9	21.4	23.2

[a] Molar basis
[b] Average branch-chain-length of amylopectin

Table 5. Starch thermal properties of the normal and waxy corn

		Line	Native				Retrogradated				Retro.[b] (%)
			T_o(℃)[a]	T_p(℃)	T_c(℃)	ΔH(J/g)	T_o(℃)[a]	T_p(℃)	T_c(℃)	ΔH(J/g)	
2009 crop year	Waxy	5036	62.3±0.2	69.6±0.1	76.1±0.8	14.8±0.1	43.3±2.1	58.0±0.2	67.5±0.8	4.7±0.1	32.0±0.1
		5037	63.6±0.2	69.8±0.7	75.0±0.7	15.6±0.1	42.2±0.3	56.0±2.6	64.2±2.1	5.2±1.3	33.5±1.3
		5039	64.1±0.7	69.7±0.8	74.9±1.3	15.7±0.1	41.2±0.1	54.5±0.1	62.7±0.0	6.1±1.2	39.0±1.2
		5040	64.3±0.4	70.2±0.1	76.8±0.3	15.5±0.0	40.3±1.7	54.8±0.2	62.9±0.0	6.5±0.1	41.9±0.1
		5041	65.8±0.2	71.9±0.1	77.8±0.9	16.4±0.1	42.2±0.3	55.1±0.1	64.3±0.7	8.5±0.7	51.6±0.7
		5042	65.0±0.5	72.9±0.4	79.1±0.3	15.9±02	43.0±0.8	55.1±0.0	64.3±0.4	7.9±0.4	49.8±0.4
	Normal	4701	56.8±0.9	66.0±0.3	72.0±0.4	11.2±0.0	35.6±0.9	48.4±0.8	60.9±0.3	6.0±0.0	53.0±0.0
		4702	62.4±0.4	68.6±0.5	73.9±0.4	12.3±0.1	37.7±0.4	50.4±0.7	62.2±0.4	7.3±0.0	59.3±0.6
		4703	64.2±0.1	69.6±0.1	74.5±0.2	11.7±0.0	39.2±0.0	50.6±0.5	61.5±0.3	6.6±0.0	56.4±0.2
		4704	59.2±0.0	67.0±0.0	73.6±0.0	10.8±0.0	39.8±0.2	51.7±0.2	61.6±0.4	5.7±0.0	53.4±0.2
2010 crop year	waxy	5036	65.1±0.2	71.4±0.1	77.3±0.3	15.7±0.3	43.1±0.4	54.0±0.2	61.4±0.1	6.5±0.3	41.3±1.4
		5037	65.4±0.0	71.3±0.0	77.5±0.0	15.4±0.1	42.0±0.2	54.7±0.4	64.6±0.2	6.6±0.1	43.0±0.9
		5039	64.5±0.4	71.4±0.5	77.7±0.5	15.2±0.3	41.7±0.7	54.3±0.0	63.6±0.2	6.6±0.1	43.6±0.1
		5040	57.7±0.3	69.6±0.9	79.9±0.3	15.1±0.0	41.4±0.3	54.6±0.0	63.4±0.3	6.6±0.1	43.8±0.0
		5041	63.3±0.1	72.0±0.0	80.5±0.9	16.2±0.1	42.6±0.4	54.3±0.0	63.0±0.1	7.4±0.2	45.8±1.5
		5042	66.6±0.3	74.1±0.0	79.2±0.1	15.9±0.1	43.0±0.2	54.9±0.0	64.0±0.0	7.2±0.0	45.4±0.0
	Normal	4701	61.7±0.1	69.0±0.1	75.1±0.1	11.4±0.0	40.6±0.8	52.2±1.2	62.7±0.2	6.3±0.1	55.0±0.4
		4702	65.4±0.2	70.8±0.1	76.1±0.3	12.3±0.2	40.7±0.1	52.3±0.1	63.0±0.0	6.9±0.0	56.4±0.5
		4703	67.1±0.1	72.0±0.1	76.9±0.2	12.1±0.2	41.7±0.8	52.6±0.2	63.1±0.6	6.5±0.1	53.4±0.1
		4704	62.8±0.1	69.6±0.1	75.6±0.1	12.2±0.0	41.5±0.6	52.6±0.0	63.1±0.2	6.4±0.0	52.0±0.2

[a] T_o= onset gelatinization temperature, T_p= peak temperature, T_c= conclusion temperature, Δ*H*= enthalpy change.

[b] Retro. (%)=100 × Δ*H* of dissociation of retrograded starch/Δ*H* of starch gelatinization

Table 6. Percentage starch hydrolysis (%) of the dry-grind corn

		Line	3h	10h	24h	72h	96h
2009 crop year	Waxy	5036	26.5±0.3	47.2±0.1	75.3±0.4	96.8±0.6	97.3±0.3
		5037	22.4±0.0	37.7±0.3	67.1±0.5	94.1±0.8	94.7±0.0
		5039	23.7±0.3	40.0±0.4	67.2±0.5	91.2±0.3	93.6±0.1
		5040	20.9±0.1	36.0±0.1	64.3±0.4	94.9±0.5	95.2±0.3
		5041	24.4±0.1	39.9±0.1	66.6±0.4	93.5±0.9	93.8±0.5
		5042	23.0±0.1	40.0±0.0	65.4±0.5	93.5±0.3	93.5±0.8
		Average	23.5	40.1	67.7	94.0	94.7
	Normal	4701	19.2±0.2	29.8±0.2	40.7±0.1	68.8±1.1	79.2±0.4
		4702	17.3±0.1	25.2±0.6	36.0±0.3	64.0±0.4	72.7±0.6
		4703	26.7±0.1	35.5±0.1	48.0±0.4	71.0±0.1	78.8±0.8
		4704	19.9±0.1	28.5±0.2	40.8±0.0	69.0±0.8	78.6±0.2
		Average	20.8	29.8	41.4	68.2	77.3
2010 crop year	Waxy	5036	24.3±0.5	48.7±0.7	76.6±1.7	93.5±1.6	97.3±1.3
		5037	23.6±0.2	44.4±0.1	71.9±0.7	93.3±0.6	96.0±0.1
		5039	26.2±0.1	48.1±0.6	75.2±1.0	94.8±1.0	95.8±1.4
		5040	24.7±0.4	43.6±0.3	71.5±0.4	92.3±0.0	94.5±0.7
		5042	24.8±0.1	47.2±1.0	72.2±0.1	92.0±0.4	94.1±0.7
		Average	24.7	46.4	73.5	93.2	95.5
	Normal	4701	19.2±0.1	28.9±0.1	40.2±0.1	68.4±0.5	77.7±0.3
		4702	21.4±0.3	29.7±0.5	40.5±0.4	68.6±0.6	75.0±0.4
		4703	27.0±0.1	35.9±0.1	48.7±0.7	71.2±0.5	78.6±0.9
		4704	20.9±0.1	29.3±0.1	40.4±0.6	69.2±0.6	78.4±0.4
		Average	22.1	31.0	42.5	69.4	77.4

Table 7. Percentage starch hydrolysis (%) of the isolated starch

		Line	3h	10h	24h	48h	72h
2009 crop year	Waxy	5036	21.9±0.2	62.5±0.0	89.6±1.9	93.3±0.2	97.4±1.2
		5037	16.2±0.3	50.9±1.4	85.3±2.0	94.2±0.3	95.5±0.4
		5039	15.9±0.3	51.5±0.7	84.7±0.3	91.5±2.1	92.5±0.3
		5040	16.1±0.0	52.8±0.9	88.4±1.7	94.0±1.2	96.9±1.4
		5041	20.2±0.3	60.0±0.1	88.8±0.2	92.8±1.2	95.3±0.6
		5042	19.2±0.4	56.4±0.3	86.2±0.5	91.1±0.9	92.2±0.0
		Average	18.2	55.7	87.2	92.8	95.0
	Normal	4701	10.5±0.0	24.2±0.1	41.0±0.4	63.6±0.3	74.7±0.8
		4702	11.8±0.0	25.1±0.1	43.0±0.3	66.0±0.5	76.2±0.3
		4703	13.7±0.2	28.7±0.1	46.9±0.6	67.3±0.6	79.1±0.8
		4704	9.8±0.0	23.8±0.1	43.7±0.2	67.0±0.8	78.7±0.5
		Average	11.5	25.5	43.6	66.0	77.2
2010 crop year	Waxy	5036	19.1±0.3	59.3±0.7	90.7±0.2	96.5±0.2	96.2±0.2
		5037	17.6±0.2	49.9±1.2	86.1±1.6	94.8±0.8	97.2±0.1
		5039	19.6±0.7	54.9±0.2	89.0±0.4	96.9±0.8	97.7±2.2
		5040	30.9±0.1	64.1±1.1	91.0±0.9	95.7±0.1	95.9±1.3
		5041	24.3±0.4	61.9±1.1	91.5±0.7	96.8±0.9	96.8±0.7
		5042	20.5±1.4	59.8±0.2	91.9±0.4	97.4±0.3	97.0±1.4
		Average	22.0	58.3	90.0	96.4	96.8
	Normal	4701	9.3±0.1	21.5±0.1	39.5±0.3	62.6±0.1	78.5±0.2
		4702	8.0±0.1	19.0±0.2	32.8±0.6	54.2±0.1	65.4±0.8
		4703	11.8±0.1	26.2±0.1	47.7±0.3	68.6±1.0	79.1±0.6
		4704	12.1±0.0	26.5±0.1	47.9±0.1	69.2±0.2	79.9±0.8
		Average	10.3	23.3	42.0	63.7	75.7

Table 8. Starch pasting properties of the normal and waxy corn

		Line	PT(°C)[a]	Peak (RVU)[b]	Hold (RVU)	Final (RVU)	Set-back (RVU)
2009 crop year	Waxy	5036	68.5	213.9±4.4	66.8±7.6	92.7±0.5	25.9±8.1
		5037	69.8	253.3±6.5	72.5±8.2	103.9±6.9	31.4±1.3
		5039	68.8	254.0±8.7	78.5±1.3	105.3±4.7	26.8±3.4
		5040	71.0	235.4±1.6	76.0±1.4	106.1±1.7	30.2±3.1
		5041	71.7	222.3[c]	74.3	99.0	24.8
		5042	72.3	216.1±11.8	81.7±1.5	107.3±3.7	25.6±5.2
	Normal	4701	71.9	156.0±3.4	91.0±1.2	181.7±0.9	90.8±2.1
		4702	71.9	140.8[c]	73.3	144.6	71.3
		4703	72.5	181.5±1.1	81.7±1.6	162.4±1.2	80.7±2.8
		4704	71.6	173.3±0.1	100.6±4.2	188.9±4.1	88.3±0.1
2010 corp year	Waxy	5036	70.0	201.9±3.8	59.2±3.2	79.2±2.6	20.0±0.6
		5037	71.4	247.7±3.7	79.4±6.2	104.8±0.7	25.3±5.5
		5039	70.6	240.4±7.6	78.4±0.8	102.9±2.2	24.5±1.4
		5040	69.2	136.2±0.7	63.5±0.5	79.2±2.1	15.7±2.7
		5041	71.0	165.5±2.9	68.2±2.2	88.2±0.9	20.0±1.3
		5042	72.5	204.2±3.2	77.3±0.8	101.0±1.4	23.6±0.5
	Normal	4701	72.7	131.6±1.6	80.1±5.4	156.8±4.4	76.7±0.9
		4702	72.5	130.5±0.9	83.8±1.1	146.8±1.1	63.0±0.0
		4703	74.7	144.1±1.0	75.5±1.7	139.3±0.7	63.7±1.2
		4704	72.4	152.3±1.5	88.2±1.8	158.4±0.6	70.3±1.2

[a] PT: Pasting temperature
[b] RVU: Rapid Visco-units
[c] Values were analyzed one time because of limited material

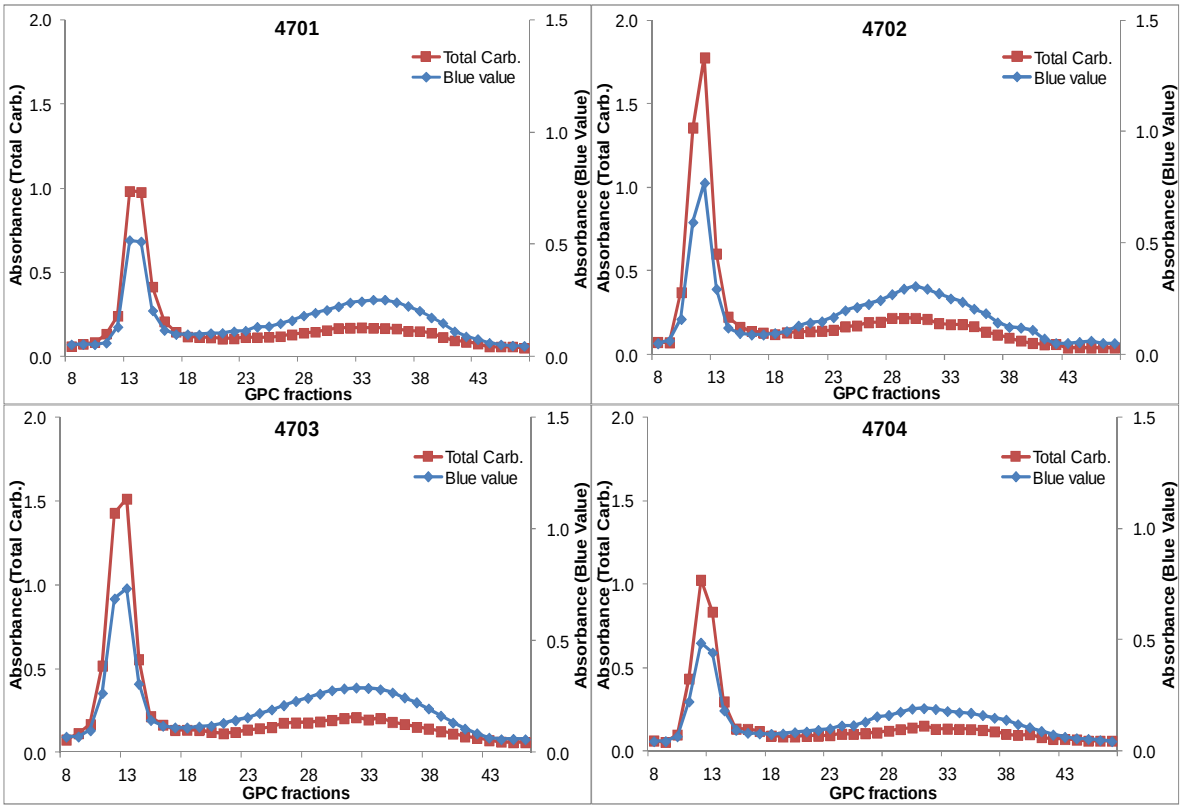

Figure 1. Gel-permeation chromatography profiles of the normal corn starch (2009 samples).

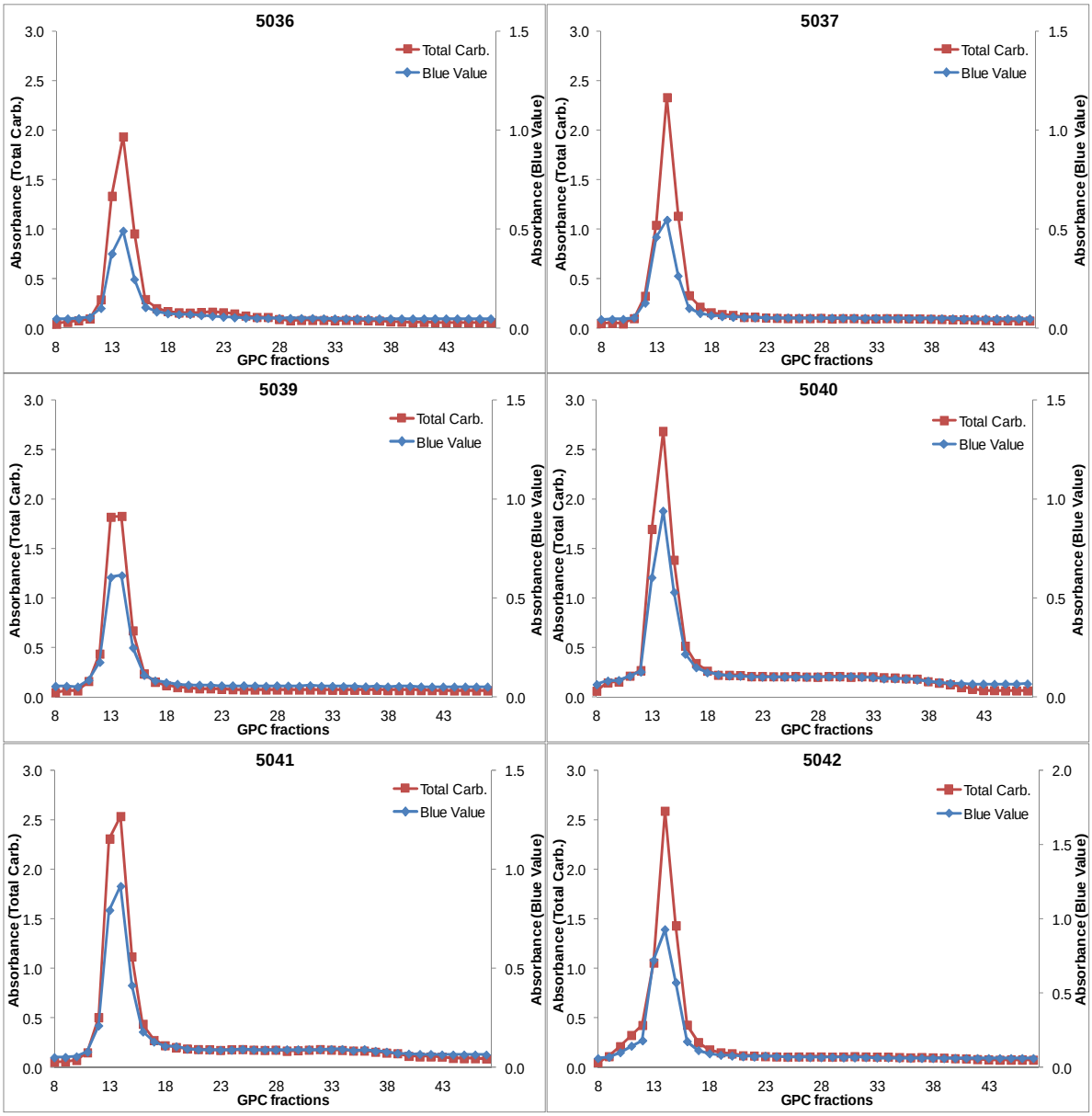

Figure 2. Gel-permeation chromatography profiles of the waxy corn starch (2009 samples).

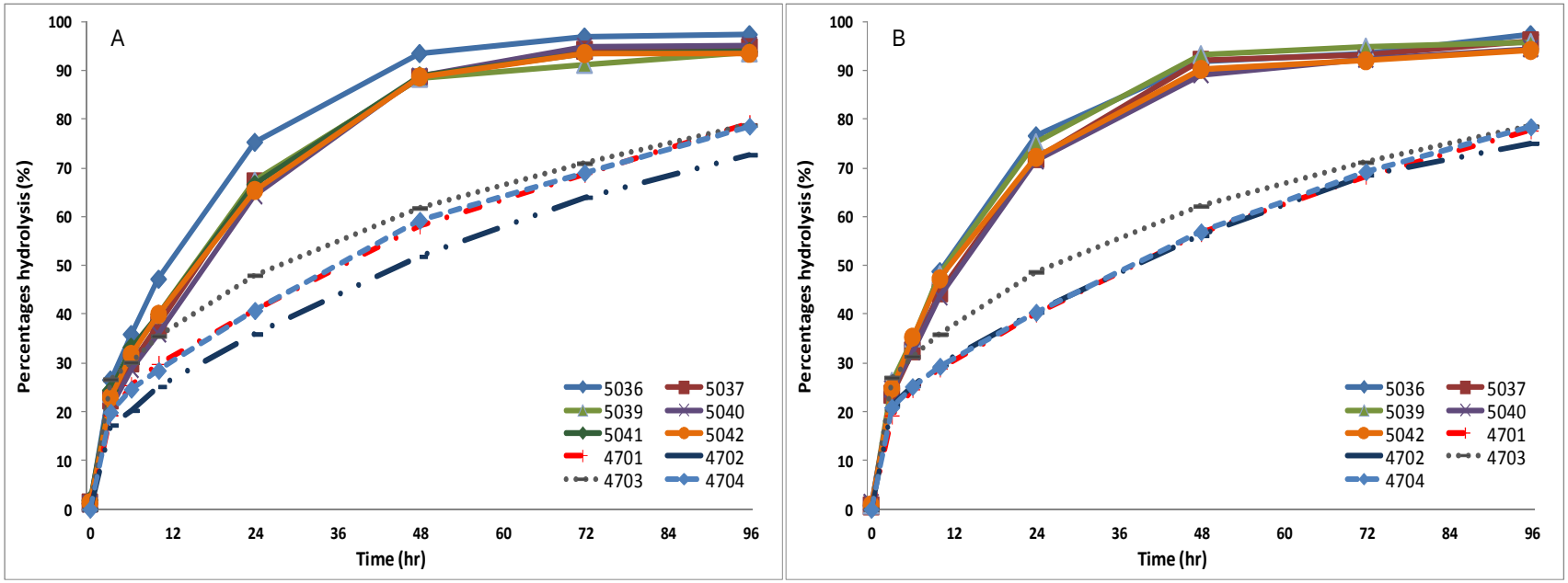

Figure 3. Enzymatic hydrolysis of the starch in the dry-grind grain. A: Dry-grind grain of 2009 crops; B: Dry-grind grain of 2010 crops.

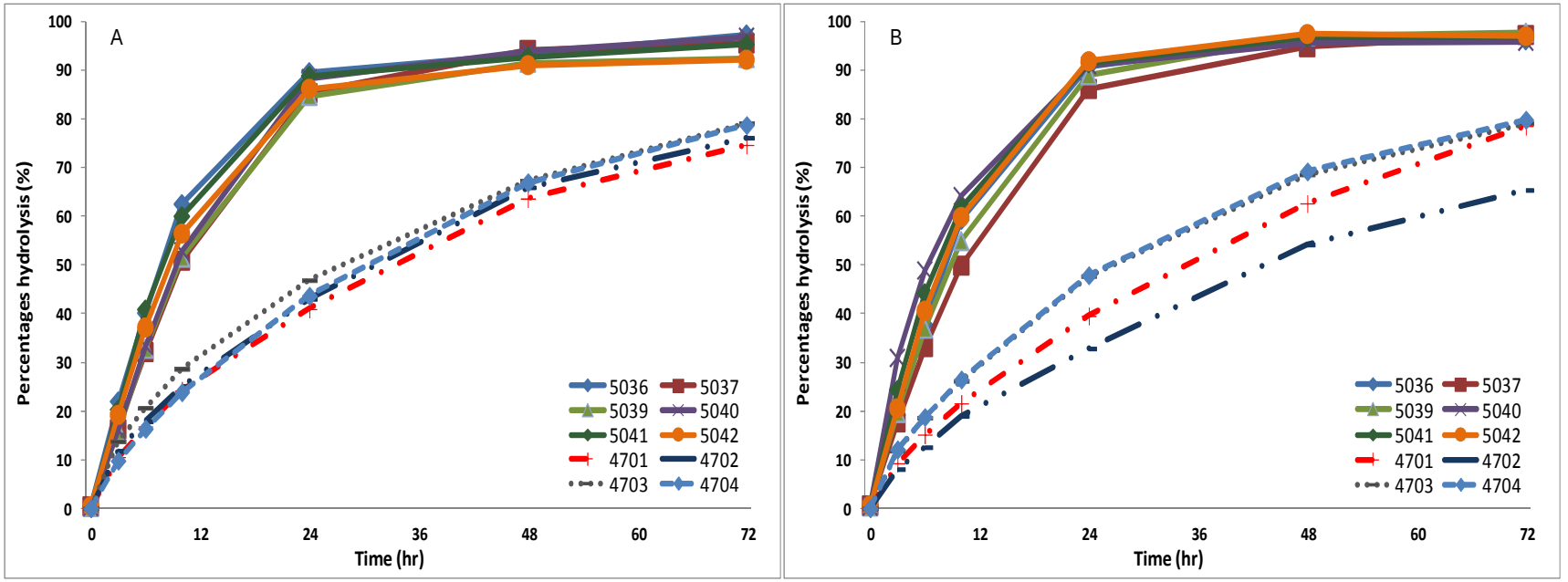

Figure 4. Enzymatic hydrolysis of the isolated starch. A: Isolated starches of 2009 crops; B: Isolated starches of 2010 crops.

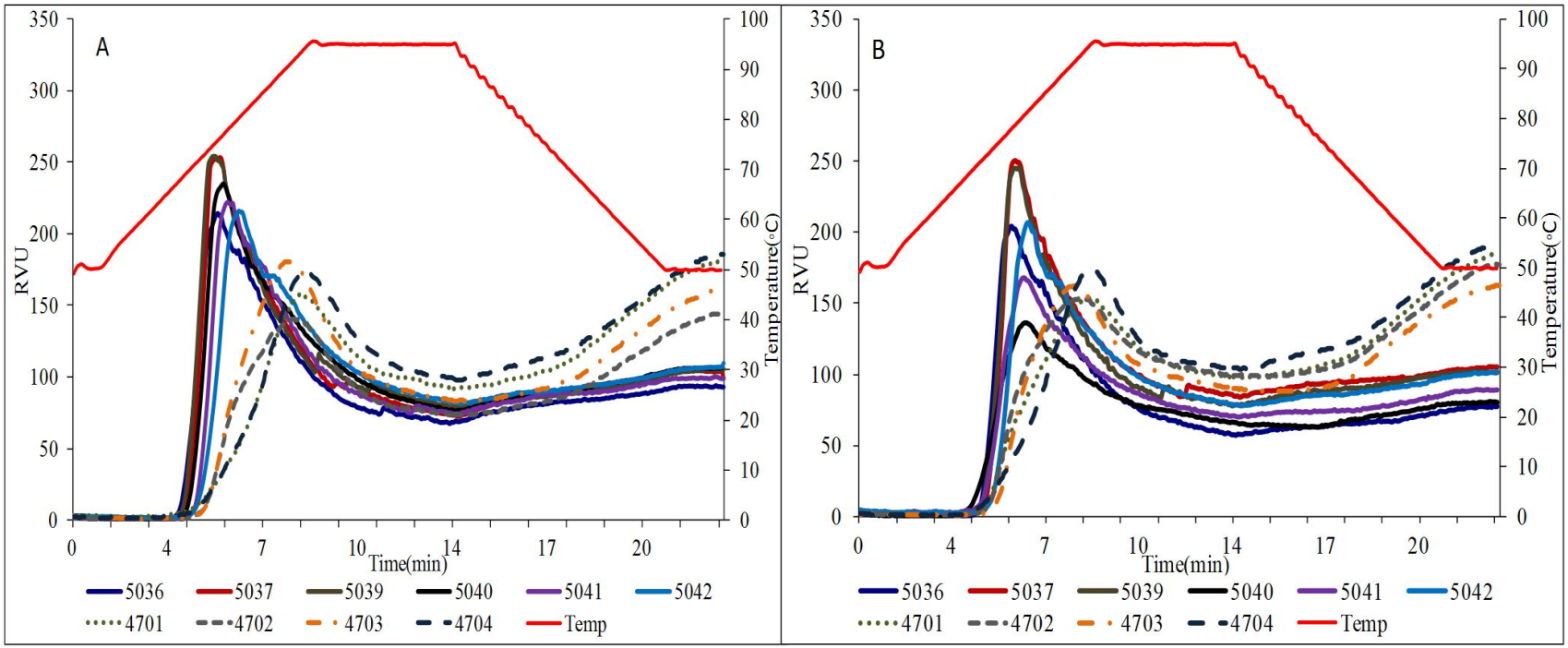

Figure 5. Starch pasting profiles of the normal and waxy corn. A: Isolated starch of 2009 samples; B: Isolated starch of 2010 samples.

APPENDIX-CHARACTERIZATION OF *ae wx* DOUBLE MUTANT MAIZE STARCH

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INTRODUCTION

The *amylose extender* (***ae***) mutant maize produces starch that contains amylose with elevated content and amylopectin with significantly elongated branch-chains compared with that of normal corn starch (Baba et al. 1982, Jane and Chen 1992, Li et al. 2008). The differences between the mutant and wild-type corn resulted from the defective functions or dysfunction of starch branching enzyme IIb in ***ae*** mutant maize (Hedman and Boyer 1982). Physicochemcial properties of ***ae*** maize starch are significantly impacted by the increased amylose/amylopectin ratio and the fine structures of amylopectin. It is known that ***ae*** maize starch has a B-type polymorph, higher peak and conclusion gelatinization temperatures, and reduced susceptibility to enzyme hydrolysis (Li et al. 2008, Jiang et al. 2010). The low digestibility of ***ae*** maize starch is attributed to its high amylose content, which restricts starch swelling during cooking and retains crystallinity (Li et al. 2008).

Starch is classified into rapidly-digestible starch (RDS), slowly-digestible starch (SDS), and resistant starch (RS), depending on its digestion rate and extent (Englyst et al. 1992). RDS causes rapid increase in plasma glucose and acute insulinemic response, while SDS releases glucose slowly and steadily, leading to a moderate increase in blood glucose and maintain the blood glucose level (Lehmann and Robin 2007). Consumption of SDS provides many health benefits: 1) Improve metabolic profile in obese, diebetes, and insulin-resistant subjects (Harbis et al. 2004, Wolever 2003); 2)

Improve cognition and mental performance (Benton et al. 2003); 3) Regulate satiety/ food intake (Lehmann and Robin 2007).

Several studies have been reported to produce SDS through physical and chemical modifications of starch, including heat-moisture treatment and enzyme digestion (Guraya et al. 2001, Shi et al. 2003). There are few studies, however, conducted on naturally occurring SDS. Amylopectin, instead of amylose, is the molecule associated with SDS (Zhang et al. 2008). SDS content is significantly impacted by the fine structures of amylopectin, such as branch density (Ao et al. 2007) and branch-chain-length (Zhang et al. 2008). Starch from ***ae wx*** double mutant rice (Kuob et al. 2010) and maize (Gerard et al. 2001) contained a significant portion of SDS, resulting from the long branch-chains of amylopectin.

The objectives of this study were to: 1) determine the content of slowly-digestible starch in several ***ae wx*** double mutant corn lines, and 2) understand the effects of starch structures on the SDS content.

MATERIALS AND METHODS

Materials. Four ***ae wx*** double mutant corn (09-**159-2**, 09-**167-1**, 09-**188-3**, and 09-**201-2**) and one ***ae*** single mutant corn (09-**148-3**) were obtained from Truman State University. Porcine pancreatic α-amylase (PPA) and amyloglucosidase from *Aspergillus niger* were obtained from Sigma Aldrich Corporation (St. Louis, MO). All other chemicals were reagent grade and were from either Sigma Aldrich Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and used without further purification.

Branch-chain-length distribution of amylopectin. Amylopection of ***ae*** mutant starch was separated from amylose and collected using a gel-permeation chromatography (GPC) column packed with Sepharose CL-2B gel (Pharmacia Inc., Piscataway, NJ). The amylopectin of the mutant corn starch was debranched with isoamylase (Megazyme International Irelands. Ltd. Co., Wicklow, Ireland) following the

method reported by Jane and Chen (1992). The debranched sample was labeled with 8-amino-1,3,6-pyrenetrisulfonic acid (APTS), and analyzed using a fluorophore-assisted capillary electrophoresis (P/ACEDQ, Beckman Courter, Fullerton, CA) following the method reported by Jiang et al. (2010).

Starch thermal properties. Thermal properties of the mutant corn starch were analyzed using a differential scanning calorimeter (Diamond DSC, Perkin-Elmer, Norwalk, CT) following the procedures of Song and Jane (2000). The starch sample (~7 mg, dsb) with excess water (3×) was heated at 10 ℃/min from 20 to 150 ℃ in a sealed stainless steal pan. An empty pan was used as the reference. Starch gelatinization onset (T_o), peak (T_p), and conclusion temperature (T_c), and enthalpy change (ΔH) were obtained using Pyris software (Perkin-Elmer).

Starch digestibility without cooking. 200mg starch (dsb) was suspended in 19mL of sodium phosphate buffer (0.1M, pH6.9) in a test tube. The suspension was equilibrated at 37 ºC for 1 hr. 1ml of the freshly prepared porcine pancrease α-amylase (PPA) solution (200units/ml) was added to the suspension. The test tube was vortexed and then incubated at 37ºC with shaking (100rpm). At each time interval (0hr, 3hr, 6hr, 12hr, 24hr, and 48hr), 0.3mL of the suspension was removed to a micro-centrifuge tube, and centrifuged at 6,600 *g* for 5 min. 0.1ml of the supernatant was transferred to 0.89ml sodium acetate buffer solution (0.1M, pH 4.5). 10µl of the amyloglucosidase (2-3 units of activity) was added into the transferred solution. The mixture was vortexed and then incubated at 50 ºC for at least 2 hr, 0.1mL of the solution was removed for GOPOD analysis.

Starch digestibility after cooking. The RDS, SDS, and RS contents were analyzed using *Englyst's method* (1992) with modifications. The starch samples (1.0g, dsb) in an acetate buffer solution (20 ml, 0.1 M, pH 5.2) were pre-cooked in a boiling-water bath for 20 min before the analysis. The RDS and SDS contents of the cooked starch samples were determined after 20 and 120 min hydrolysis, respectively.

RESULTS AND DISCUSSION

Amylopectin branch-chain-length distributions of the ***ae wx*** and ***ae*** mutant corn starch are shown in **Table 1**. The ***ae*** corn starch had smaller portion of short branch-chains (DP<12, 9.9%) and larger portion of long branch-chains (DP>37, 43.9%), compared with that of the ***ae wx*** samples (DP<12, 11.7-12.6%; DP>37, 29.8-31.0%). As a result, the average branch-chain-length of the ***ae*** starch (DP 34.7) was significantly longer than that of the ***ae wx*** samples (DP 29.1-29.4). The results were similar to the previous reports (Jane et al. 1999, Li et al. 2008). The ***ae*** gene-containing mutant corn starch showed significantly longer branch-chain-length than that of the normal maize starch (DP 24.4) (Jane et al. 1999).

Starch thermal properties of the mutant corn lines are shown in **Table 2**. Onset gelatinization temperatures (T_o) of the ***ae wx*** corn starch ranged from 64.3 to 69.7℃, whereas that of the ***ae*** corn starch was 64.6℃. Gelatinization conclusion tempe rature (T_c) of the ***ae*** corn starch (104.4℃), however, was significantly highe r than that of the ***ae wx*** corn (91.2-95.7℃). Gelatinization enthalpy change o f the ***ae*** corn starch (11.0 J/g) was smaller than that of the ***ae wx*** corn starch (18.6-20.2 J/g). The differences were attributed to the crystalline structures of the mutant corn starch. It is known that amylopectin contributes to the starch crystallinity, whereas amylose molecules are present in the amorphous form (Jane 2006). The ***ae*** corn starch contained mainly amylose and a small proportion of amylopectin and, thus, had smaller gelatinization enthalpy change than that of the ***ae wx*** starch.

Enzymatic hydrolysis of the uncooked starch using porcine pancrease α-amylases is shown in **Figure1** and summarized in **Table 3**. Starch digestive rate of B73, a normal corn line, was substantially greater than that of the ***ae***-containing mutant corn. After 48hr hydrolysis, 82.1% of the normal corn starch was hydrolyzed, whereas that of the mutant corn starch ranged from 29.8% to 34.7% (**Table 3**). Nonetheless, the ***ae wx*** corn starch displayed similar hydrolysis kinetics to that of the ***ae*** corn starch. The results

were in agreement with the previous reports that ***ae***-containing mutant starch was less susceptible to enzymatic hydrolysis than the wild-type counterparts (Gerard et al. 2001, Kubo et al. 2010).

RDS, SDS, and RS contents of the mutant corn and normal corn were analyzed using the *Englyst's method* (1992), and the data are summarized in **Table 4**. After cooking, the normal corn starch contained no RS, and the starch was almost all rapidly digestible (RDS 98.2%). In the contrary, ***ae*** corn starch contained a large portion of RS (25.3%), and a significant portion of SDS (5.2%). The ***ae wx*** samples had a RS content ranging from 4.2 to 8.1%, much less than that of the ***ae*** starch. The large content of RS in ***ae*** maize was associated with its thermal properties. T_c of the ***ae*** corn starch (104.4℃, **Table 2**) was above water-boiling temperature, suggesting that the ***ae*** starch granules retained partial crystalline structures after heating in boiling water, which contributed to the resistance of cooked ***ae*** starch to enzyme hydrolysis. ***ae wx*** corn starch contained larger portions of SDS (6.7-7.9%) than that of ***ae*** (5.2%) and normal corn starch (1.8%). A positive correlation between SDS content and the proportion of long B-chains of amylopectin was reported by Zhang et al. (2008). It suggested that the long B-chains facilitated molecular association and complex formation, contributing to the slowly digestive rates of the ***ae wx*** starch.

CONCLUSIONS

Starch of ***ae*** gene-containing mutant corn had large portion of long branch-chains and longer average branch-chain-length of amylopectin. The ***ae*** gene-containing mutant corn starch was less susceptible to enzyme hydrolysis compared with that of normal corn starch, in both uncooked and cooked form. The ***ae*** mutant corn starch contained large portions of RS, whereas the ***ae wx*** starch contained significant portions of SDS. The results suggested that ***ae wx*** double mutant maize is a potentially significant source of slowly-digestible starch.

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Table 1. Amylopectin branch-chain-length distribution[a] of the ***ae***-containing mutant corn starch

	Lines	DP<12	DP13-24	DP25-36	DP>37	*ave.* CL[b]
ae wx	201-2	11.9±0.0	42.1±0.2	15.1±0.1	30.9±0.1	29.4±0.3
	167-1	N/A[c]	N/A	N/A	N/A	N/A
	188-3	12.6±0.1	41.3±0.8	16.3±0.5	29.8±1.2	29.1±0.5
	159-2	11.7±0.1	41.6±0.6	15.7±0.4	31.0±1.0	29.4±0.0
ae	148-3	9.9±0.3	32.5±0.0	13.7±0.2	43.9±0.5	34.7±1.6

[a] Molar basis
[b] Average branch-chain-length of amylopectin
[c] Data is not available

Table 2. Starch thermal properties of the ***ae***-containing mutant corn lines

	Lines	Gelatinization			
		T_o(℃) [a]	T_p(℃)	T_c(℃)	△H(J/g)
ae wx	201-2	69.2±0.7	79.1±0.6	95.7±0.3	18.7±0.5
	167-1	69.7±0.6	79.6±0.9	95.2±1.8	18.6±0.1
	188-3	64.3±0.3	78.7±0.1	92.1±0.5	20.2±0.1
	159-2	65.0±0.4	78.4±0.0	91.2±0.1	18.9±0.2
ae	148-3	64.6±0.5	82.6±0.7	104.4±0.3	11.0±0.4

[a] T_o= onset gelatinization temperature, T_p= peak temperature, T_c= conclusion temperature, Δ*H*= enthalpy change.

Table 3. Percentage starch hydrolysis (%) of the ***ae***-containing mutant corn and a normal corn without cooking

	Lines	3h	6h	10h	24h	48h
ae wx	201-2	6.7±0.3	10.2±0.5	14.8±0.6	25.1±0.7	31.8±0.7
	167-1	6.7±0.1	10.3±0.3	14.8±0.3	23.4±0.1	29.8±0.2
	188-3	9.1±0.4	14.0±0.0	19.7±0.1	29.9±0.1	34.5±0.2
	159-2	7.1±0.2	11.2±0.2	15.9±0.3	26.9±0.5	31.9±0.6
ae	148-3	9.0±0.1	13.6±0.3	18.4±0.5	29.5±0.4	34.7±0.5
Wild type	B73	14.8±0.0	26.6±0.3	41.1±0.1	72.8±0.3	82.1±0.5

Table 4. RDS, SDS, and RS content of the mutant corn starch after cooking (*Englyst et al. 1992*)

	Lines	RDS%	SDS%	RS%
ae wx	201-2	87.5±0.7	7.9±0.6	4.6±1.4
	167-1	84.0±1.0	7.9±0.6	8.1±0.4
	188-3	88.2±1.1	7.6±2.0	4.2±0.9
	159-2	87.5±2.2	6.7±1.9	5.8±0.3
ae	148-3	69.5±0.1	5.2±1.0	25.3±1.0
Wild type	B73	98.2±1.5	1.8±0.8	0.0±0.6

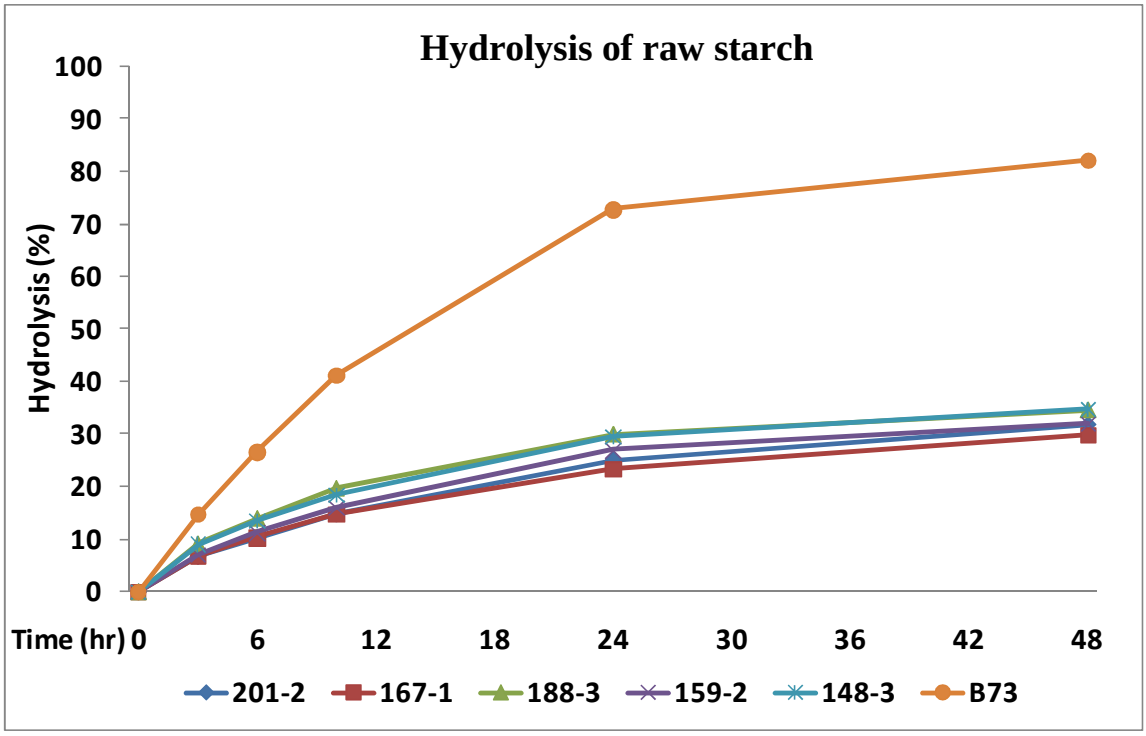

Figure 1. Enzymatic hydrolysis of the ***ae***-containing mutant corn starch and a normal corn starch (B73) without cooking.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and respect to the following:

To my major professor, Dr.Jay-lin Jane, for her guidance, encouragement, and providing me with insight that will help me to succeed in my future career;

To my committees, Dr.Michael Blanco and Dr.Zhiyou Wen, for their valuable instructions, advices, and help;

To Department of Food Science and Human Nutrition for the numerous assistance;

To my former and current colleagues, Dr.Hongxin Jiang, Dr.Jelena Medic, Dr.Jongtae Park, Dr.Seungwoo Cho, Dr. Changwoo Lee, Yongfeng Ai, Joshua Leutscher, Michael Reed, and Samuel Moore, for their generous time, kind help, and friendship;

To my friends in Ames, for all the great time and encouragement;

Finally, I want to give thanks to my family, for their understanding, encouragements and endless support. And I also want to thank my girlfriend, Yueyi Jiao, for her love and understanding.