A COMPARATIVE STUDY ON NORMAL AND HIGH SUGARY CORN GENOTYPES FOR EVALUATING RAW MATERIAL QUALITY AND ENZYME CONSUMPTION DURING DRY-GRIND ETHANOL PRODUCTION

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ABSTRACT

Agronomic and biochemical characteristics of four high sugary corn genotypes (HSGs) and four parent field corn lines (PFCs) were determined to evaluate raw material quality. Subsequently, the effects of kernel sugars on the enzyme requirements, fermentable sugar and ethanol yield, and co-product quality were investigated. Major agronomic characteristics differed among the corn genotypes. Sugar accumulation in the kernels showed a negative correlation with flowering time (FT), grain filling period (GFP) and black layer maturity (BLM). These findings showed that the genotypes exhibiting lower FT, GFP and BLM would have higher amounts of sugars. HSGs contained higher amounts of total soluble sugars (TSS) and lower amounts of starch than the respective PFCs. As a result, a significant negative correlation was observed between kernel starch and sugar content. TSS content in HSGs ranged between 4.43-6.72% in 2012 and 4.64-7.47% in 2013, while it varied in PFCs between 0.76%-1.36% in 2012 and 0.85-1.27% in 2013. Kernel starch ranged between 66.34-69.85% in HSGs and 67.37-72.08% in PFCs in 2012, and 65.89-70.41% in HSGs and 68.69-73.61% in PFCs in 2013. Conventional hydrolysis under four enzyme loads showed that HSGs produced optimum concentration of reducing sugars (RS) while consuming an enzyme load of 3.0 kg/MT, whereas PFCs required 4.0 kg/MT for maximum sugar yield. Conventional fermentation was conducted by simultaneous saccharification and fermentation (CSSF) technique using an initial solid load 250 g/L. Ethanol concentration varied between 98.7-112.5 g/L in HSGs and 80.8-86.8 g/L in PFCs when enzyme load was 3.0 kg/MT. As the enzyme load increased to 4.0 kg/MT, ethanol concentration reached 102.3-113.1 g/L in HSGs and 85.1-99.5 g/L in PFCs. During granular starch hydrolysis (GSH), RS yield in HSGs did not vary significantly above the enzyme load 1.5 kg/MT, while for the PFCs it did not show a significant increase above a higher enzyme load, ranging between 2.0 and 2.5 kg/MT. The final ethanol concentration after granular starch hydrolysis and simultaneous fermentation (GSHSF) with an initial solid load 300 g/L, ranged from 15.25% to 17.5% (v/v) in HSGs and 11.66% to 13.65% in PFCs at the enzyme load 1.5 kg/MT. Ethanol concentration increased to 16.49–17.94% in HSGs and 14.32–16.85% in PFCs as the enzyme load increased to 2.0 kg/MT. Ethanol concentration showed a negative correlation with kernel starch, whereas, a positive correlation was observed between kernel sugars and ethanol yield. The average yield of distiller's dried grains with soluble (DDGS) among the corn genotypes ranged from 25.07% to 32.44% for CSSF and 26.97% to 31.69% for GSHSF. Among the biochemical components in DDGS, starch content varied significantly between PFCs and HSGs, and the two enzyme doses used for fermentation. Other components in DDGS for both HSGs and PFCs were well within the values reported in the literature. In conclusion, the study has shown that higher kernel sugar in the corn genotypes is able to improve raw material quality for dry-grind ethanol production as it has the potential to reduce enzyme consumption and produce enhanced amounts of ethanol.

ABSTRAK

Ciri-ciri agronomi dan biokimia empat genotip jagung manis (HSGs) dan empat '*parent field corn lines*' (PFCs) dari dua musim penanaman pada tahun 2012 dan 2013, telah ditentukan dalam kajian ini untuk menilai kualiti keseluruhan bahan mentah. Seterusnya, kesan gula kernel dan variasi genotip terhadap keperluan enzim ke atas gula fermentasi, hasil etanol, dan kualiti produk sampingan juga telah dikaji. Ciri-ciri utama agronomi berbeza sangat ketara antara lapan genotip jagung, dan purata kumpulan untuk sifat-sifat ini juga berbeza-beza antara HSG dan PFC. Pengumpulan gula di dalam kernel menunjukkan hubung kait yg negative dengan masa berbunga (FT), tempoh pengisian bijirin (GFP), dan kematangan lapisan hitam (BLM). Keputusan ini menunjukkan genotip yang menunjukkan FT, GFP dan BLM rendah, mempunyai lebih banyak gula. HSGs mengandungi jumlah gula larut yang lebih tinggi dan kurang jumlah kanji berbanding PFCs. Oleh yang demikian, hubungan negatif yang signifikan diperhatikan antara kuantiti kanji dan TSS. Kandungan TSS dalam HSGs adalah di antara 4.43% ke 6.72% pada tahun 2012, dan 4.64% ke 7.47% pada tahun 2013, manakala bagi PFCs adalah di antara 0.76% hingga 1.36% dan 0.85-1.27%. Sebaliknya, kandungan kanji yang dicatitkan adalah lebih rendah bagi HSGs jika dibandingkan dengan PFCs, dengan catitan 66.34-69.85% bagi HSGs dan 67.37-72.08% bagi PFCs pada 2012, dan 65.89-70.41% bagi HSGs dan 68.69-73.61% dalam PFCs pada tahun 2013 . Hidrolisis enzim konvensional genotip jagung menunjukkan jumlah RS yang cukup dihasilkan oleh HSGs dengan menggunakan beban enzim 3.0 kg/MT, sedangkan, PFCs menggunakan 4.0 kg/MT untuk penghasilan maksimum. Semasa *saccharification* serentak konvensional dan fermentasi (CSSF), didapati bahawa kepekatan etanol dalam cecair penapaian kedua-dua HSGs dan PFCs meningkat seiring dengan peningkatan beban enzim dan tempoh fermentasi. Walau bagaimanapun, HSGs menghasilkan kepekatan etanol yang lebih tinggi dengan menggunakan jumlah enzim yang lebih rendah. Fermentasi tepung jahung selama 72 jam dengan 250 g/L beban pepejal awal menghasilkan 98.7-112.5 g/L dalam HSGs dan 80.8-86.8 g/L dalam PFCs apabila beban enzim adalah 3.0 kg/MT, dan mencapai ke 102.3-113.1 g/L dalam HSGs dan 85.1-99.5 g/L sekiranya beban enzim di-tingkatkan kepada 3.0 kg/MT. Semasa hydrolysis granul kanji, hasil gula penurun dalam HSGs tidak berbeza jika beban enzim melebihi 1.5 kg/MT jagung kering, manakala PFCs menunjukkan tidak menunjukkan peningkatan pada beban enzim yang melebihi di antara 2.0 dan 2.5 kg/MT. Purata kepekatan etanol dalam HSGs dan PFCs selepas 96 jam hidrolisis kanji berbutir dan penapaian serentak (GSHSF) dengan 300 g/L beban pepejal awal, adalah di antara 15.25% ke 17.5% (v/v) dan 11.66% ke 13.65%, pada beban enzim 1.5 kg/MT jagung kering, dan meningkat kepada ke 16.49-17.94% bagi HSGs dan 14.32-16.85% bagi PFCs jika beban enzim di-tingkatkan kepada 2.0 kg/MT. Hasil etanol telah menunjukkan hubungan negatif dengan kanji kernel, sedangkan, terdapat hubungan yang positif di antara hasil etanol dan gula kernel. Purata hasil DDGS antara genotip jagung adalah di antara 205.07% hingga 32.44% bagi CSSF dan 26.97% hingga 31.69% bagi GSHSF. Antara komponen biokimia di dalam DDGS, kandungan kanji ketara berbeza antara PFCs dan HSGs, dan dua dos enzim yang digunakan semasa penapaian. Komponen lain di dalam DDGS untuk kedua-dua HSGs dan PFCs adalah baik dalam nilai yang dilaporkan didalam kajian sebelumnya. Kesimpulannya, kandungan gula yang tinggi dalam bijirin jagung dapat meningkatkan kualiti bahan mentah, penghasilkan lebih etanol, dan mengurangkan penggunaan enzim dan juga kos pengeluaran semasa pengeluaran etanol *dry-grind* tanpa menjejaskan kualiti produk.

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LIST OF SYMBOLS AND ABBREVIATIONS

ANPR	:	Advance Notice of Proposed Rulemaking
AT	:	Anthesis time
BC	:	Before Christ
BLM	:	Black layer maturity
bu	:	Bushel
CDS	:	Condensed distiller's solubles
CHN	:	Carbon, hydrogen and nitrogen
cm	:	Centimeter
CO	:	Carbon monoxide
CO_2	:	Carbon dioxide
CSSF	:	Conventional simultaneous saccharification and fermentation
CV	:	Coefficient of variation
DDGS	:	Distiller's dried grains with solubles
df	:	Dilution factor
DG	:	Dry-grind
DM	:	Dry matter
DNS	:	Di-nitro salicylic acid
EL	:	Ear length
ELD	:	Eight leaves development
ENP	:	Ear number per plant
EtOH	:	Ethanol
EU	:	European Union
EW	:	Ear weight
FLD	:	Four leaves development

g/L	:	Gram per liter
g/L/h	:	Gram per liter per hour
ga	:	Gallon
GAU/g	:	Glucoamylase unit per gram
GBSS	:	Granule bound starch synthase
GFP	:	Grain filling period
GSH	:	Granular starch hydrolysis
GSHE	:	Granular starch hydrolyzing enzyme
GSHSF	:	Granular starch hydrolysis and simultaneous fermentation
GY	:	Grain yield
h	:	Hour
HSG	:	High sugary genotypes
K	:	Kelvin/Potassium
KD	:	Kernel depth
kg	:	Kilogram
kg N/ha	:	Kilogram nitrogen per hectare
kg/ha	:	Kilogram per hectare
kg/MT	:	Kilogram per metric ton
kJ/kg	:	Kilojoules per kilogram
KNE	:	Kernels number per ear
KNP	:	Kernel number per plant
kPa	:	Kilo pascal
L/ha	:	Liter per hectare
L/kg	:	Liter per kilogram
lb	:	Pound
LL	:	Leaf length

LNP	:	Leaf number per plant
LU	:	Liquefon unit
m	:	Meter
m^2	:	Meter square
mg	:	Milligram
min	:	Minutes
ml	:	Milliliter
MTBE	:	Methyl tertiary butyl ether
N	:	Nitrogen
P	:	Phosphorous
PFC	:	Parent Field corn
PH	:	Plants height
ppm	:	Parts per million
PY	:	Potential yield
RCBD	:	Randomized complete block design
rpm	:	Rotation per minute
RS	:	Reducing sugars
s	:	Second
SAPU	:	Spectrophotometric acid protease unit
SEM	:	Standard error mean
SET	:	Seed emergence time
SHF	:	Separate hydrolysis and fermentation
SSF	:	Simultaneous saccharification and fermentation
ST	:	Silking time
t/ha	:	Ton per hectare
TCA	:	Trichloroacetic acid

TKW	:	Thousand kernel weight
TOC	:	Total organic carbon
TOM	:	Total organic matter
TRS	:	Total reducing sugars
TS	:	Thin stillage
TSCA	:	Toxic Substance Control Act
TSS	:	Total soluble sugars
USA	:	United States of America
w/v	:	Weight per volume
w/w	:	Weight per weight
WDG	:	Wet distiller's grains
WM	:	Wet mill
YPD	:	Yeast extract peptone dextrose
α	:	Alpha
μL	:	Microliter

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CHAPTER 1: INTRODUCTION

1.1 Research Background

Currently the world is mostly dependent on fossil fuels for meeting its energy demand, and more than 80% of the total global energy is obtained by burning fossil fuels, of which 58% alone is consumed by the transport sector (Escobar et al., 2009). In particular, petroleum based fuels are mainly used for energy that include gasoline, diesel, liquefied petroleum gas, and compressed natural gas (Demirbas, 2009). The rapid increase in the consumption rate of all kinds of fossil fuels due to the growing industrialization and motorization of the world has resulted in the fast depletion of these limited and non-renewable energy sources (Agarwal, 2007), which has been anticipated to be exhausted by next 40-50 years (Vohra et al., 2014). The usage of fossil fuels have also raised another concern due to its contribution to the emission of greenhouse gases and global warming that causes climate change, rise in sea level, loss of biodiversity and urban pollution (Farrell et al., 2006). Furthermore, political crisis, particularly in the Middle East, which resulted in the incidence of oil supply disruption by the major oil producer countries in the 1970s, has also led to a re-think our dependence on fossil fuels, since such crises are unsettling to the energy sector of both the developed and developing nations (Ogbonna et al., 2001). Taking into consideration the above facts, it is necessary to find out an alternative source of energy for our industrial economies and consumer societies by using renewable, sustainable, efficient and cost-effective feedstocks with lower emissions of greenhouse gases (Dhaliwal et al., 2011; Herrera, 2004).

Four strategically important and sustainable options have been considered either on a small or large scale, as attempts to find out alternative energy sources, and these include biofuels, hydrogen, natural gas and syngas (synthesis gas) (Nigam & Singh, 2011). Among these four categories, biofuels have been proven to be the most eco-friendly,

sustainable and is being vigorously explored to replace fossil fuels because of their renewability, biodegradability and production of exhaust gases with acceptable quality (Bhatti et al., 2008). Biofuels include mainly bioethanol, biomethanol, vegetable oils, biodiesel, biogas, bio-synthetic gas (bio-syngas), bio-oil, bio-char, Fischer-Tropsch liquids, and biohydrogen (Demirbas, 2008).

Ethanol (C_2H_5OH) is a liquid biofuel and is referred to as bioethanol when it is produced from renewable feedstocks (Nigam & Singh, 2011). Compared to other biofuels, bioethanol is known to be an attractive alternative to fossil fuel due to its ease of production and lack of toxicity (Carere et al., 2008; Lu et al., 2012; Tomas-Pejo et al., 2008). Many countries such as USA, Brazil, China, and several EU member states have already proclaimed commitments to bioethanol programs as a renewable source of energy (Balat & Balat, 2009), where the former two countries have shown the largest committments thus far (Larson, 2008).

Bioethanol has been earmarked as a promising fuel over gasoline (C_7H_{17}) due to it having several advantageous properties. Even though one liter of ethanol affords 66% of the energy provided by the same amount of gasoline, the former has a higher octane number (106-110) than the latter (91-96), which enhances the performance of gasoline when blended with ethanol (Nigam & Singh, 2011). The higher octane level of ethanol also allows it to be burnt at a higher compression ratio with shorter burning time, resulting in lower engine knock (Balat, 2007; Kar & Deveci, 2006). In addition, ethanol has a higher evaporation enthalpy (1177 kJ/kg at 60^{o}C) than gasoline (348k kJ/kg at 60^{o}C) and a higher laminar flame speed (around 33 and 39 cms^{-1} at 100 kPa, 325 K for gasoline and ethanol, respectively) (Al-Hasan, 2003; Bayraktar, 2005; Hara & Tanoue, 2006; Naik et al., 2010). The higher heat of vaporization for ethanol (840 kJ/kg) than that of gasoline

(305 kJ/kg) ensures that the volumetric efficiency of ethanol blends is higher than that of pure gasoline, improving power output (Lynd, 1996).

With regard to environmental impact, bioethanol is an eco-friendly oxygenated fuel containing 34.7% of oxygen, whereas, gasoline contains no oxygen at all and this results in about 15% higher combustion efficiency of ethanol than that of gasoline (Kar & Deveci, 2006), thereby keeping down the emission of particulate and nitrogen oxides as well as other greenhouse gases (Malça & Freire, 2006; Searchinger et al., 2008). Compared to gasoline, ethanol contains negligible amount of sulfur, and mixing of these two fuels helps to decrease sulfur content in the fuel as well as emission of sulfur oxide, which is a carcinogen and can contribute to acid rain (Pickett et al., 2008). Moreover, due to ethanol's lower ambient photochemical reactivity, there is reduced interference on the ozone layer (Lynd et al., 1991). The by-products of incomplete oxidation of ethanol are acetic acid and acetaldehyde, which are less toxic than those of other fuels (Vohra et al., 2014). Bioethanol is also a safer substitute to methyl tertiary butyl ether (MTBE), which is a commonly used as an octane enhancer for gasoline and is added to the latter for its clean combustion so that production of carbon monoxide (CO) and carbon dioxide (CO_2) can be reduced (McCarthy & Tiemann, 1998). MTBE has been reported to make its way into ground water that contaminates drinking water causing severe detrimental effect on health (Green & Lowenbach, 2001). The US Energy Policy Act released an ANPR (Advance Notice of Proposed Rulemaking) in 2000 under the TSCA (Toxic Substance Control Act) to limit the use of MTBE as a gasoline extender (Yao et al., 2009).

Ethanol can be combined and blended with gasoline or used in its pure form in modified spark ignition engines as a fuel additive, octane enhancer and an oxygenate (Nigam & Singh, 2011). When ethanol is blended with gasoline it is called ‘‘gasohol”. The blended ethanol E-5 (5% ethanol and 95% gasoline by volume) is used as an

oxygenate under the EU quality standard-EN/228 (Demirbas, 2008). The most commonly used blend ethanol in USA is E-10 (10% ethanol and 90% gasoline by volume) and is known as a fuel extender (Balat & Balat, 2009; Demirbas, 2008). Brazil, on the other hand, uses either pure or blended ethanol, in a combination of 24% ethanol with 76% gasoline by volume (De Oliveira et al., 2005). Ethanol can also be used as E-85 (85% ethanol and 15% gasoline by volume) in flexible fuel vehicles (Demirbas, 2008). Another term used for bioethanol as fuel, is "Diesohol" that comprises a blend of diesel, hydrated ethanol and an emulsifier (84.5%, 15% and 0.5% by volume, respectively) (Demirbas, 2008).

Bioethanol is produced from biomass containing considerable amount of free sugars or carbohydrate polymers capable of being converted into soluble sugars (Aggarwal et al., 2001). These feedstocks can be divided into three major groups such as (1) sugar crops (sugar cane, sugar beet, sweet sorghum) and sugar materials (cane molasses, beet molasses, cheese whey), (2) starchy cereals (corn, wheat, triticale) and root crops (potato, cassava), and (3) lignocellulosic biomass (agricultural wastes, municipal and industrial solid waste, forest biomass and waste, perinnial grass and energy crops), which differ considerably from each other with regard to the obtainment of sugar solutions (Mussatto et al., 2010; Sanchez & Cardona, 2008; Vohra et al., 2014). Sugar crops require only an extraction process to get fermentable sugars, while starchy crops need to under the hydrolysis step to convert starch into glucose using amylolytic enzymes. Lignocellulosic biomass has to be pretreated before hydrolysis in order to alter cellulose structures for enzyme accessiblity, which is more difficult and complicated (Mussatto et al., 2010; Szymanowska-Powałowska et al., 2014). Consequently, lignocellulosic materials are still considered ecnomically non competitive for bioethanol production, despite the fact that they are abundant, inexpensive and substantial research has been done on lignocellulosic ethanol in recent years (Szymanowska-Powałowska et al., 2014). As a result, almost all

of the commercial ethanol is produced from sugar and starchy feedstocks (Pickett et al., 2008; Szymanowska-Powałowska et al., 2014), and not surprisingly, the former produces cheaper ethanol than the latter (Quintero et al., 2008). However, sugar crops cannot be grown globally due to their requirement for selective climatic conditions and soil types (Barcelos et al., 2011). Nonethless, starch based bioethanol is relatively well established and produces about 60% of the total ethanol, compared to nearly 40% as produced from sugar sources (Johnston & McAloon, 2014; Mussatto et al., 2010). Therefore, the major portion of the world relies on starch based feedstocks for bioethanol production (Balat & Balat, 2009; Mojović et al., 2006).

Corn is a major feedstock in the starch based bioethanol industry and its use has been increased dramatically in recent years (Johnston & McAloon, 2014). United States is the dominant producer of corn ethanol, and it produced a record amount of ethanol from this feedstock (14.3 billion gallons) in 2014, and at the same time it exported roughly 825 million gallons of ethanol to 51 countries across the world (RFA, 2015). It has also made its goal to produce 36 billion gallons of ethanol by 2022 using corn and corn stover (Schnepf & Yacobucci, 2010). Recently, certain European countries, for example, Serbia that produces nearly 40% excess corn over its domestic need has shown interest for producing ethanol from corn (Nikolić et al., 2010). Therefore, corn ethanol has gradually become a global biofuel with a great economic importance (Plumier et al., 2015). Parallel with the increasing global interests in corn ethanol, there has also quality improvement for its sustainability and economic viability (Johnston & McAloon, 2014).

The quality of raw material is an important attribute for any commercial product, which can be determined by considering several essential parameters. Corn quality attributes includes agronomic traits, physicochemical properties and characteristics of the starch that can have a direct or an indirect effects on ethanol yield (Reicks et al., 2009;

Singh, 2012; Torney et al., 2007; Yangcheng et al., 2013). These quality parameters may be affected by environmental conditions, seasonal variations, crop management practices, soil quality, geographical locations, and plant density (Miao et al., 2006a; Miao et al., 2006b). For this reason, the quality of any hybrid is normally evaluated for at least two successive seasons under suitable conditions as optimized by preliminary trials for the selected location (Reicks et al., 2009; Yangcheng et al., 2013).

Among the agronomic properties of maize, kernel yield is most important, from both an economic point of view and its sustainability as an ethanol feedstock (Reicks et al., 2009). To the producers and farmers, high kernel yielding hybrids are most popular (Tokatlidis & Koutroubas, 2004). The success of breeding among maize hybrids is primarily dependent on the grain yield (D'Andrea et al., 2009). However, overall kernel yield always vary in response to the hybrids, geological locations and environmental conditions (Farnham, 2001; Graybill et al., 1991). Grain yield is primarily associated with the number of kernel per ear or plant (Cantarero et al., 1999; Sangoi et al., 2001). Several other agronomic factors and yield related components also greatly affect kernel yield, such as duration of different growth stages, ear size and weight, kernel number and weight, kernel depth, and potential yield (Cirilo et al., 2009; D'Andrea et al., 2009; Wong et al., 1994).

Dry matter in normal corn (also known as field or yellow dent corn) kernels consists of starch, protein, fat, fiber, ash and poor amount of free sugars (Belyea et al., 2004; Manikandan & Viruthagiri, 2010). These biochemical components significantly affect the conversion process of corn into fermentable sugars and ethanol (Wu et al., 2006). Starch is the major carbohydrate and fermentable component in kernels, comprising roughly 70-72% of the total dry weight (Bothast & Schlicher, 2005). The bioavailability of starch during enzymatic hydrolysis varies with regard to the cultivars, affecting the conversion

efficiency and final ethanol yield (Moorthy, 2002). However, it is a fact that ethanol yield or conversion efficiency has not been reported to be exclusively dependent on the amount of kernel starch (Reicks et al., 2009; Singh & Graeber, 2005). Rather, the chemical structure of starch and the starch-protein matrix may affect the starch to ethanol conversion ability (Dien et al., 2002). A starch granule consists of two glucan polymers of α-D-glucose, which are amylose and amylopectin (Copeland et al., 2009). Amylose is a straight chain, helical polymer formed by α-1→4 linked glucose residues containing about 1000 glucose units, while amylopectin is highly branched, with chains made up of α-1→ 4 linked and α-1, 6 linked glucose residues (Copeland et al., 2009). Typically, normal corn starch contain about 20-30% amylose, while amylopectin constitutes the remaining 70-80% (Torney et al., 2007). The physicochemical properties of starch are often determined by its amylose and amylopectin ratio that can significantly affect final ethanol yield (Singh & Graeber, 2005; Yangcheng et al., 2013). Protein, fiber, ash and fat are the non-fermentable components and are recovered as co-products after fermentation (Wu et al., 2006).

Currently, most of the corn ethanol is produced either by the dry grind (DG) or the wet mill (WM) method, obtaining 9.5 L (WM) to 10.6 L (DG) of ethanol per bushel (56 lbs; 25.4 kg) of corn (Bothast & Schlicher, 2005). The basic difference between DG and WM processes is the use of whole ground corn in the former, while different components are separated and only starch is used for producing ethanol in the latter method (Singh et al., 2001). Although both processes are now being employed in ethanol production from corn, the majority of commercial plants employ the DG process (Bothast, 2005; Mueller, 2010). The WM method requires extensive equipment and high capital investment, and produces large amounts of ethanol and a variety of co-products, whereas, the DG process is suitable for producing ethanol on a smaller scale requiring less equipment and capital investment that produces two major products such as ethanol and distiller's dried grains with solubles

(DDGS) (Singh et al., 2001). Furthermore, higher ethanol yield has been reported in DG process (0.395 L/kg) than that of WM method (0.372 L/kg) (Shapouri et al., 2002). As a result, the DG process is the most attractive and widely used for generating bioethanol (Bothast, 2005; Singh et al., 2001). Moreover, recent growths in the corn ethanol industry has been made mainly on DG plants, which produces 70-86 % of total ethanol in USA with this method (Bothast, 2005; Mosier & Ileleji, 2014; Mueller, 2010).

The conventional DG method involves preparation of slurry by mixing corn flour with water, which is then cooked at a higher temperature (90-105^{o}C in the laboratory or up to 165^{o}C in a commercial plant) and liquefied with thermostable α-amylase to breakdown starch into dextrin. The liquefied slurry is usually saccharified at a relatively lower temperature to convert dextrin into glucose using glucoamylase, and subsequently subjected to yeast fermentation to produce ethanol from glucose (Lamsal et al., 2011; Plumier et al., 2015). After fermentation, ethanol is recovered through distillation of the beer, leaving whole stillage, which is subsequently separated into thin stillage and a solid portion. One portion of the thin stillage is recycled as backset in the process to reduce water consumption and another portion is condensed into thick stillage (syrup). The solid portion is mixed with thick stillage to obtain a final co-product, distiller's dried grains with solubles (DDGS), which is sold and used as animal feed (Branca & Di Blasi, 2015).

A conventional DG process can be accomplished either by separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) (Mojović et al., 2006). In SHF, starch in the corn slurry is initially hydrolyzed by the action of an α-amylase (liquefaction) and a glucoamylase (saccharification). After completion of the two-step hydrolysis, the obtained hydrolysate is then subjected to fermentation separately. However, enzymatic action can be inhibited by the accumulation of sugars in the solutions that results from the incomplete hydrolysis of starch (Mojović et al., 2006). In addition, a

higher concentration of sugars in the hydrolysate may cause osmotic stress on the yeast cells (Nikolić et al., 2010), and as a consequence, ethanol productivity and final yields are affected (Borzani & Jurkiewicz, 1998). On the other hand, SSF is carried out using liquefied slurry obtained after liquefaction by adding glucoamylase and yeast simultaneously during fermentation (Nikolić et al., 2010). In comparison, the SSF process can be used both on laboratory and industrial scales over SHF, in order to avoid osmotic stress on the yeast cells, end product inhibition on the enzyme and microbial contamination during fermentation (Srichuwong et al., 2009).

In spite of industrial maturity, corn ethanol is still facing some challenges for its long-term economic survival as well as expansion of the industry. One of them is the requirement of amylolytic enzymes for converting starch into glucose before fermentation which is considered a cost increasing factor for corn ethanol (Shigechi et al., 2004b). The cost of enzymes required for this process has been reported to be nearly 10-20% of the total production cost (Gregg et al., 1998). Another challenge for corn ethanol is the cost of raw materials (Mojović et al., 2006; O'Brien et al., 2000), which is supposed to be more apparent in the near future, when it is expected that lignocellulosic ethanol will come to light commercially. An improvement in the production process of fuel ethanol, to reduce its cost by even 2-5 cents per gallon will be significant for this industry (O'Brien et al., 2000). High energy consumption during conventional ethanol production causes another concern (Chu-Ky et al., 2016), as energy requirement for cooking and liquefaction has been estimated to be 30-40% of the ethanol produced (Lim et al., 2003). Furthermore, thermal processes employed during enzymatic hydrolysis may produce unwanted by products through Maillard reactions (Szymanowska & Grajek, 2011).

Recently, a non-cooking method has been introduced to decrease energy consumption during ethanol production that involves hydrolysis of granular starch at sub-gelatinization

temperature using a granular starch hydrolyzing enzyme (GSHE) (Białas et al., 2014; Szymanowska-Powałowska et al., 2014), which has been named as granular starch hydrolysis and simultaneous fermentation technique (GSHSF) (Lamsal et al., 2011). Ethanol production by this process does not require high temperature and thereby reducing energy consumption (Robertson et al., 2006), as well as decreasing the production of undesirable by products of the Maillard reaction (Wang et al., 2010). Additionally, the overall operational process could be simplified during GSHSF due to the lower viscosity of the slurries and requiring only a single step (Li et al., 2014; Robertson et al., 2006).

However, low hydrolysis rate and incomplete hydrolysis of starch at sub-gelatinized temperature due to structural heterogeneity and crystallinity of native starch have made the process challenging (Li et al., 2012). To overcome these shortcomings and increase the hydrolysis efficiency as well as ethanol yield, enzyme manufacturers have recommended to conduct a mild heat treatment (*e.g.* at 60^{o}C) prior to fermentation and to supplement the media with urea and protease (Genencor, 2009). Urea has been reported to increase hydrolysis rate and yield (Li et al., 2012), and protease increase ethanol productivity and yields during ethanol production from starch based feedstocks (Duan et al., 2009; Gohel et al., 2013).

In spite of these process modifications, it is still necessary to use large quantities of enzymes for converting starch to fermentable sugar (Białas et al., 2014). Although several efforts have been made for fermenting starch directly without adding any exogenous enzyme, by developing recombinant yeast capable of expressing amylolytic enzymes (Altıntaş et al., 2002; Shigechi et al., 2004b), all of these efforts are still confined to the laboratory and have not reached a satisfactory level for industrial usage (Aydemir et al., 2014). Although, utilization of higher amounts of dry solid in a batch is always of

commercial interest, the starch concentration in the slurries is an important factor for its efficient conversion, since starch above a certain concentration level may cause substrate inhibition of the enzymes, resulting in an incomplete conversion of starch, lower ethanol yield and increased production costs (Mojović et al., 2006).

A typical dry-grind ethanol process produces 11.8 L ethanol and 7.7 kg DDGS from one bushel (25.4 kg) of corn, where the latter is an important co-product of this industry and is popularly used around the world for, particularly, ruminant animal feed as a low cost alternative (USGC, 2012). Even though the market value of DDGS primarily depends on its protein content (Kwiatkowski et al., 2006), it also contains significant amounts of fat and fiber (Kim et al., 2008). The marketing and prices of DDGS is affected by its quality and one of the major quality indicator is the physicochemical properties (Liu, 2009). DDGS composition can differ considerably based on a good number of factors including raw material quality, production process, plant conditions, and process parameters (Belyea et al., 2004; Liu, 2008; Spiehs et al., 2002). There has been substantial interest to the researchers for conducting studies on the composition of DDGS not only among the ethanol producers, but also people of animal science and feed industry (Kim et al., 2008b).

Over last several years, substantial modifications have been made on technical and technological aspects of ethanol production process to produce enhanced and cost effective ethanol from corn (Johnston & McAloon, 2014). Nevertheless, attempts to improve corn quality are rather limited, even though quality parameters such as corn composition affect the dry-grind ethanol yield (Singh, 2012). One of the possible ways to improve the quality of corn is to alter kernel composition, particularly the fermentable components (Torney et al., 2007), and high starchy hybrids have been suggested for this purpose (Sanchez & Cardona, 2008). However, increased kernel starch will not

necessarily produce enhanced ethanol for some reasons. Firstly, ethanol yield from starch is affected by several factors such as its amylose content and physicochemical properties (Yangcheng et al., 2013), and consequently, no significant correlation has been reported between quantity of starch and ethanol yield (Dien et al., 2002; Reicks et al., 2009; Singh & Graeber, 2005). Secondly, starch to ethanol conversion in the current dry-grind ethanol process is incomplete, as a result of the inefficiency of the process as well as representing an economic loss of the desired product (Plumier et al., 2015). Thirdly, a higher amount of starch will eventually increase enzyme consumption, in addition to the fact that excess starch can act as substrate inhibitor above a certain level, decreasing hydrolysis efficiency (Mojović et al., 2006). Alternatively, increased amount of free sugars in the corn kernels could be more effective to reduce enzyme requirements since kernel sugars will be fermented simultaneously with the product of starch hydrolysis and produce an additional amount of ethanol without consuming any enzyme, thereby enhancing ethanol yield as well as improving the quality of corn (Chen et al., 2014a).

1.2 Problem Statement

Even though corn kernel with higher amounts of free sugar is expected to decrease enzyme consumption during dry-grind ethanol production, the current corn feedstock (field corn) contains low amounts of free sugars. On the other hand, sweet corn contains a higher amount of sugars as its kernels are enriched with sucrose (Lertrat & Pulam, 2007), glucose and fructose (Wong et al., 1994). Nevertheless, it is not economical and ethical to use sweet corn as ethanol feedstock due to the fact that it has been exclusively used as human food, requires special care during growth, and more importantly, its grain yield is lower than that of the normal corn (Rajablarijani et al., 2014; Santos et al., 2014). In light of the above situation, research efforts have been made recently to increase sugar accumulation in the corn kernels by crossing temperate with tropical maize (Chen et al.,

2014a). Nevertheless, there has been no report on developing high sugary genotype (HSG) via crossing sweet corn with field corn. It will be interesting to evaluate normal and HSG comparatively for selected agronomic and biochemical properties, fermentable sugar yield, ethanol yield and quality of DDGS.

1.3 Hypothesis and Research Questions

High sugary corn genotypes (HSGs), with increased sugar content, can be developed by crossing sweet corn with field corn genotypes. It is hypothesized that the developed HSGs will be a promising raw material and have the potential to decrease enzyme consumption and produce higher amounts of fermentable sugars as well as ethanol yield, in addition to providing good quality co-products (DDGS), showing expected agronomic and biochemical properties. It is hoped the present study will provide the answers to the following questions:

a. How will HSGs differ from PFCs in their agronomic and biochemical traits?
b. Will HSGs produce higher amounts of fermentable sugars and ethanol than PFCs while consuming lower amounts of enzymes during conventional hydrolysis and fermentation?
c. Will HSGs produce higher amount of fermentable sugars and ethanol than PFCs while consuming lower amounts of enzymes during granular starch hydrolysis and fermentation?
d. How will the DDGS quality vary between HSGs and PFCs?

1.4 Objectives

The main aim of this research work is to conduct a comparative study on four high sugary corn genotypes (HSGs) and their parent field corn lines (PFCs), by investigating the effect of kernel sugars on raw material quality, enzyme consumption, fermentable

sugars and ethanol yield, and co-product quality during dry grind ethanol production. The four major objectives of the study are as follows:

a. To determine the agronomic and biochemical traits of the HSG and PFC genotypes.
b. To evaluate enzyme consumption and product yields in HSG and PFC genotypes during conventional hydrolysis and fermentation.
c. To investigate enzyme consumption and product yields in HSG and PFC genotypes during granular starch hydrolysis and fermentation.
d. To determine the composition and evaluate the quality of DDGS.

1.5 Scope of the Study

Apart from lignocellulosic biomass which is still not competitive for commercial ethanol production (Szymanowska-Powałowska et al., 2014), current feedstocks are derived from sugar and starch sources (Jonker et al., 2015; Prajapati et al., 2015). This has resulted in research outcomes that show only on a single pattern for carbohydrate utilization by yeast cells. Since HSGs contain free sugars and starch in the kernels, fermentation of these genotypes will provide a new approach in bioethanol research, which will include the consumption patterns for both sugars and starch.

Current bioethanol research is broadly confined to two main areas, namely, the search for promising new raw materials and improvement in current conversion technologies. Research and development of new crop varieties for improved quality of raw materials has shown much interest in recent years (Gumienna et al., 2016; Murthy et al., 2009; Ratnavathi et al., 2011).

The present study deals with eight corn genotypes belonging to two distinct groups, namely HSG and PFC. It has been reported that corn hybrids with higher amounts of

fermentable sugars are promising not only for the enhanced ethanol yield (Chen et al., 2014; Gumienna et al., 2016), but also for having the capability to reduce enzyme consumption during dry-grind ethanol production (Zabed et al., 2016b). Therefore, a comprehensive and comparative study on HSGs and PFCs is expected to occupy a major place in hybrid based bioethanol research.

Many earlier studies have reported that the suitability of any corn material or hybrid as ethanol feedstock often associated with its quality and performance in the overall process and this includes its agronomic properties (Lacerenza et al., 2008; Reicks et al., 2009), kernel composition (Mojović et al., 2013; Singh, 2012; Wu et al., 2006; Yangcheng et al., 2013), starch structure (Wu et al., 2006; Yangcheng et al., 2013), ability to produce high fermentable sugar during hydrolysis (Tester et al., 2006; Uthumporn et al., 2010), ability to produce high ethanol during fermentation (Lemuz et al., 2009; Murthy et al., 2009), and the quality of the co-product (Belyea et al., 2004; Bothast & Schlicher, 2005; Han & Liu, 2010). In the present study the above parameters were investigated and characterized. The agronomic traits considered for characterizing a corn hybrid includes the duration required for reaching the different growth stages, ear size and weight, kernel number per plant, thousand grain weight, kernel depth, and potential yield kernel number per plant (Cirilo et al., 2009; D'Andrea et al., 2009; Sangoi et al., 2001). The major biochemical components in corn kernels of any hybrid are starch, protein, fat, fiber, ash and free sugars (Gumienna et al., 2016; Wu et al., 2006). Kernel starch consists of two main components, amylose and amylopectin in different ratios among the genotypes (Wu et al., 2006; Yangcheng et al., 2013). A comprehensive study on these essential parameters will establish a relationship between corn characteristics and fermentable sugar yield, ethanol yield and co-product quality.

Hydrolysis and fermentation constitute two key steps in the dry-grind ethanol production from corn and other starch based materials (Duvernay et al., 2013; Mojović et al., 2006). Hydrolysis of corn starch produces fermentable sugars but the yield is often affected by many factors. Therefore, hydrolysis experiments conducted under different conditions will generate a clear picture on the degree of effects of these factors on sugar yield. Moreover, the optimum conditions obtained from the hydrolysis step will usually mimic the optimum conditions for fermentation. However, hydrolysis and fermentation of the corn materials differ significantly in conventional (CSSF) and non-cooking (GSHSF) techniques (Wang et al., 2005). A comparative study of these techniques using HSGs and PFCs will enlighten us on the sugar and ethanol yields from the different corn genotypes.

1.6 Thesis Organization

The thesis is organized into seven chapters, which includes a general introduction, literature review, four articles from the respective four objectives of the study and a general conclusion. A general introduction is included in the first chapter which describes the importance of the present work in the light of current literature. It also covers the problem statement, objectives, scope and thesis structure. The second chapter covers literature review which includes a comprehensive description on the fossil fuels, bioethanol, ethanol production methods with a special emphasis on the dry-grind process, feedstocks for bioethanol, characteristics of corn, hydrolysis, fermentation, factors affecting ethanol yield, and co-product quality. Two review papers were published from this Chapter in the "*Scientific World Journal*" and "*Renewable & Sustainable Energy Reviews*".

The first article is described in the third chapter entitled "Characterization of normal and high sugary genotypes for predicting the effect of different traits on carbohydrate

accumulation in kernels". This chapter presents a comparative description of the agronomic and biochemical properties of HSGs and PFCs. It also describes the relationship between the accumulation of kernel carbohydrates and the different characteristics of the genotypes. The results of this Chapter has been published as a research paper in the journal "*Industrial crops and Products*". The fourth and fifth chapters cover the main thrust of the present study as hydrolysis and fermentation of the corn genotypes are described in these chapters. Both sugar and ethanol production were conducted using two different dry-grind techniques so that enzyme consumption and product yield from the corn genotypes can be evaluated with respect to the current techniques. The results in Chapters 4 and 5 have already been published in the *Chemical Engineering Journal* and *Journal of the Taiwan Institute of Chemical Engineers*, respectively, in 2016. Both are Tier 1 journals in chemical engineering. Chapter 6 describes the outcomes of the last objective and discusses the quality of distiller's dried grains with solubles (DDGS), obtained from the two dry-grind techniques (CSSF and GSSF), under the two enzymatic conditions.

Finally, a general conclusion is included in Chapter 7, which summarizes the findings of the study described in Chapters 3 to 6. This Chapter also provides a brief discussion on the implications of the outcomes and suggestions for future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Fossil Fuels and Global Challenges

Although fossil fuels are exclusive sources of the global energy, three major challenges have been realized over the last several decades. Firstly, the consumption rate of conventional fuels has increased with the growing increase in world population, industrialization and motorization (Agarwal, 2007). As shown in Figure 2.1, the consumption rate of three major fossil fuels have increased enormously from zero in the 1700s, to more than 7500 million tons for natural gas, nearly 6000 million tons for oils and more than 2000 million tons for coal, in 1900s. However, the reserves for these fuels are limited and have been anticipated to be exhausted within the next 40-50 years (Vohra et al., 2014). A recent study has estimated that world oil reserves will meet only half of the global energy demand by 2023, and the remaining 50% of the energy will have to be managed from other sources (Owen et al., 2010).

Secondly, burning of fossil fuels has been shown to be responsible for global warming as it emits greenhouse gases into the atmospheres (Singh et al., 2010). CO_2 is the major greenhouse gas, which has increased dramatically in the atmosphere over the last three centuries, from almost zero in 1850 to nearly 10,000 million tons in 2012 (Figure 2.2).

Thirdly, global politics on the marketing and reserves of fossil fuels has unsettled the energy sector during last several decades. A major incidence recorded so far was the disruption of oil supply by the oil producing countries in the 1970s that resulted in a sudden rise in oil prices and a shortage of this essential fuel in the world market (Ogbonna et al., 2001). However, today many oil producing countries are facing economic crises due to a sudden drop in oil prices.

To prepare for the uncertainty of future fuel supply and reduce air pollution, it is necessary to find out an alternative source of energy. Bioethanol, in this regard, is the most promising source for renewable energy having the potential to be an attractive alternative to the conventional petroleum-based fuels (Tomas-Pejo et al., 2008).

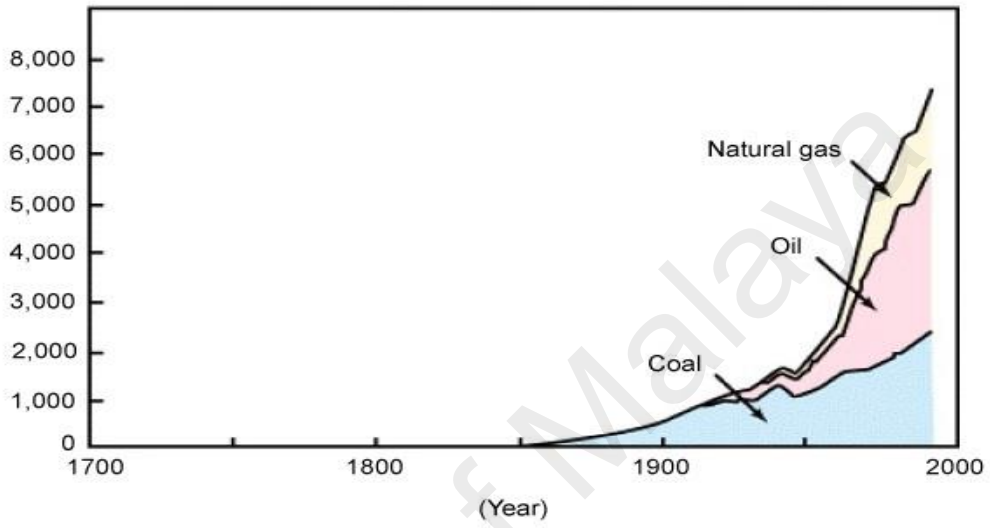

Figure 2.1: Trends in Global Fossil Fuel Consumptions (Million Tons Oil Equivalent) During Last Four Centuries (1700-2000s) (Source: EA, 1998)

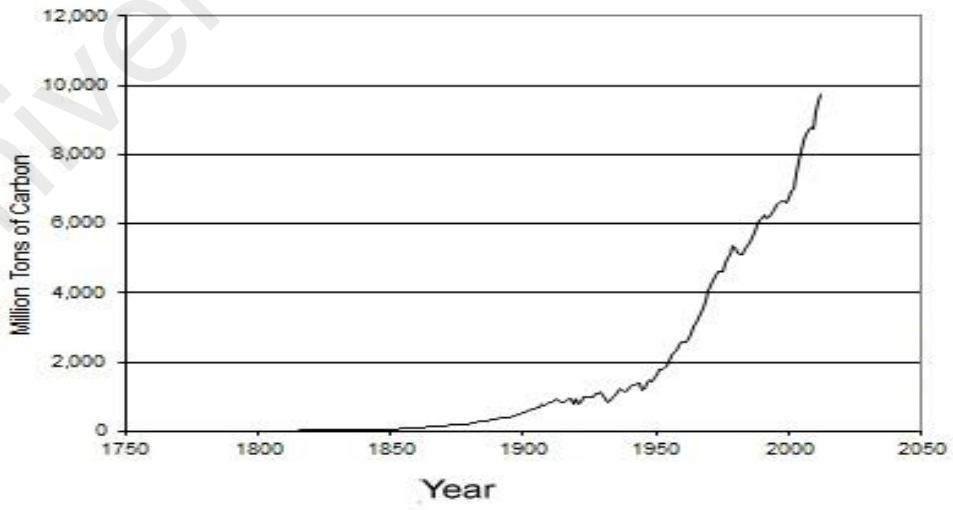

Figure 2.2: Trends in Global Carbon Dioxide Emissions from Fossil Fuel Burning (1751-2012) (Source: Adams, 2013)

2.2 Bioethanol: An Overview

Ethanol (C_2H_5OH) is a colorless, clear liquid that looks like water and is completely miscible with water. It has a sweet flavor if it is mixed and diluted with water. However it gives a more pungent and burning taste with an ether-like odor when it is concentrated. Fuel ethanol, which is often referred to as bioethanol and obtained from the fermentation of soluble sugars by yeasts, has been proven to be an ideal alternative to fossil fuels due to its ease of production and lack of toxicity (Lu et al., 2012; Nigam & Singh, 2011). Being used as a gasoline extender, it increases the oxygen content in gasoline and provides better oxidation of hydrocarbons that decreases the emissions of aromatic compounds and carbon monoxide into the atmosphere (Quintero et al., 2008). The emission of CO_2 from the burning of ethanol is compensated by the absorption of CO_2 during the growth of crops, from which ethanol is generated. As a result, there is a balance in the net emissions of this greenhouse gas (McMillan, 1997).

The use of bioethanol as transportation fuel is not a new concept and has a long history. During the nineteenth century, in 1826 and 1876 respectively, Samuel Morey developed an engine and Nicholas Otto made a combustion engine , both of which could run on ethanol (Demirbas et al., 2009). The first automobile made by Henry Ford in 1896 ran on pure ethanol, which was later on designated as Ford Model-T in 1908. The Ford Model-T had a carburetor adjustment that allowed vehicles running on either gasoline or ethanol or any mixture of both (Bothast & Schlicher, 2005; Solomon et al., 2007). Bioethanol was used for transportation fuel in Europe and USA until the early 1930s, which then showed a gradual decrease, particularly after the World War II. The reasons behind this decrease was mostly the higher production costs of ethanol than that of petroleum based fuels, even though there was still an interest in bioethanol as an antiknock agent and a prospective substitute for fossil fuels (Bothast & Schlicher, 2005; Solomon et al., 2007). Around three

decades later, a renewed interest in fuel ethanol was revived in the 1970s, due to the oil supply disruption by the Middle East countries. Today, production and use of bioethanol has increased dramatically in many countries, particularly in the USA and Brazil (Balat & Balat, 2009; Quintero et al., 2008).

Ethanol, as a clean and renewable fuel, is considered a good alternative to replace petroleum oil (Mussatto et al., 2010). As a whole, fuel ethanol provides multiple economic, social and environmental benefits to the regions and countries that produce it. Domestic production and use of bioethanol can obviously reduce the dependence on foreign oil and trade deficits, create jobs in rural areas, and decrease air pollution, climate change and CO_2 build up (Ibeto et al., 2011). Burning of ethanol rather than gasoline can decrease carbon emissions by more than 80%, and eliminate entirely the release of sulfur dioxide that causes acid rain (Lashinky & Schwartz, 2006; Mussatto et al., 2010). In addition, bioethanol can be used as a replacement for methyl tertiary butyl ether (MTBE), which is used as an octane enhancer for gasoline but contaminates ground water used for drinking purposes (Green & Lowenbach, 2001; McCarthy & Tiemann, 1998).

2.3 Raw Materials for Bioethanol Production

Bioethanol can be produced from a variety of renewable sources, which are broadly classified into sugars, starch and cellulosic biomass (Mussatto et al., 2010). In general, carbohydrate rich plant biomasses are widely used for ethanol production and these include corn, wheat, cassava, sugarcane, sugar beet, sweet sorghum, barley, potatoes, bagasse, straws, wood, paper, grasses and agricultural residues (Ibeto et al., 2011). Among these feedstocks, sugarcane is a major raw material used in the tropical countries like Brazil and India (Quintero et al., 2008), while starchy biomass are used in North America and Europe (Balat & Balat, 2009; Sanchez & Cardona, 2008; Yangcheng et al., 2013). In recent years, extensive research efforts have been made on the conversion of

lignocellulosic biomass into ethanol, as attempts to meet the growing demand for ethanol and reduce the use of food crops (Szymanowska-Powałowska et al., 2014). However, tproduction of ethanol from lignocellulosic biomass is more difficult, complicated and costly when compared to sugar and starch based ethanol production. For this reason, almost all commercial ethanol is produced from sugar and starch, where the latter is relatively well established and used globally more than sugar based ethanol production (Quintero et al., 2008). Among the starch based raw materials, corn is predominantly used for producing ethanol and its use has increased dramatically during the last two decades, particularly in the USA, Canada, China and some European countries (Balat & Balat, 2009; Johnston & McAloon, 2014).

2.4 Corn: An Overview

Corn (*Zea mays* L.; Family Poaceae) has been ranked as the third most important cereal crops in the world, after rice and wheat (Zilic et al., 2011). It is a warm seasonal and monoecious grass plant, having staminate type of male flowers, known as tassel, and pistillate type of female flowers, known as silk. The former is borne on an apical inflorescence and the latter on one or more lateral branches (Borrás et al., 2007). The anthesis or development of tassel in corn plants during its growth is commonly known as tasseling, while the same physiological appearance for female flower is referred to as silking. Upon fertilization by means of wind or gravitational forces, the silks of corn are converted into rachises, often called ears, which bear the kernels. It has been reported that the state of silking in corn plant is associated either with the biomass allocation to the ear (N'Guettia et al., 1991) or the expansion of ear growth (Cárcova et al., 2003).

With regard to the origin of maize, historians and researchers are divided in their opinion, because different regions and geographical locations have been suggested as the origin of maize. However, based on some evidence found in New Mexico, researchers

believe that corn originated from the Tehuacán Valley in West and Central Mexico (Hemeyer, 2010) in 5000 BC (Mangelsdorf, 1974) or 3600 BC (Long et al., 1989). After domestication in Mexico, this cereal crop was disseminated to North America (Canada) and South America (Argentina) (Hemeyer, 2010). The movement route of Native Americans was discovered in 1941 from Carl Sauer's map, which included northward to Mexico and the state of Arizona of today's USA before heading to the east (Hemeyer, 2010). It has been reported that two crew members of Christopher Columbus's ship collected corn seeds in 1492 while Columbus was exploring the interior of Cuba (Walden, 1966). After the discovery of the Americas by Columbus, corn was established and subsequently moved rapidly to Europe, Africa, and Asia (Hemeyer, 2010).

Corn, being a natural source of carbohydrate for producing energy and various food and feed products is one of the most important cereal crops (Radosavljević et al., 2012; Semenčenko et al., 2015). Over many years, corn starch and its hydrolysis-derived sugars have been used in food and other related industries for manufacturing different products such as corn gluten meal, corn syrup (glucose syrup), high fructose corn syrup and high maltose corn syrup. It has also been used traditionally as a thickener, gelling agent, bulking agent and water retention agent in the food industry (Singh et al., 2003). In more recent times, this cereal crop has drawn much attention for fuel ethanol production as attempts to reduce the dependence on fossil fuels gathers pace globally.

2.5 Types of Corn

Corn is one of the most versatile crops found in nature and has been divided into several types, which differ considerably with regard to kernel characteristics and mode of utilization. The major types of corn include dent corn (normal or field corn), sweet corn, popcorn, high oil corn, waxy corn, high lysine corn, blue corn, ornamental corn and corncob corn (Dickerson, 2003; Knott et al., 1995). Of these, field corn and sweet corn

are well known and most important from an economic point of view as these corns are used as food, feed and raw materials for many industrial products.

Today's field corn was originally developed from crosses between the late-flowering Southern dent corn (Gourd seed) and the early-flowering Northern flints (Dickerson, 2003). It is a primary source of starch. Field corn with an adequate amount of protein is also used for animal feed as a cheap protein source (Chen et al., 2014b).

Sweet corn (*Zea mays* L. var. *saccharata*) probably originated from a mutation of a Peruvian corn *Chuspillo* or *Chullpi* (Dickerson, 2003). It is cultivated exclusively for human consumption, and is widely used as a raw or processed material for the food industry (Özlem et al., 2014). Sweet corn primarily varies from field corn with regard to the expression of genes that determine carbohydrate contents in the endosperm (Özlem et al., 2014). In comparison with the field corn, sweet corn contains higher amounts of sugars and lower amounts of starch in the endosperm (Kwiatkowski et al., 2011), which is an excellent component for producing bioethanol (Chen et al., 2014a; Gumienna et al., 2016). However, sweet corn has not been considered for bioethanol production as it has been grown specifically for human consumption and requires special care during growth, and more importantly, its grain yield is lower than that of field corn (Rajablarijani et al., 2014; Santos et al., 2014).

Sugar content in the corn kernels can be increased for producing enhanced ethanol through conventional breeding between sweet corn and other suitable corn such as field corn or tropical corn. This will produce new genotypes with higher amounts of sugars as a result of transferring genetic characters from sweet corn into the low sugary corn (Zabed et al., 2016a). In recent studies, high sugary corn genotypes (HSGs) have been developed from the breeding between sweet corn and field corn (Zabed et al., 2016a; Zabed et al., 2016b). These HSGs produced higher amounts of fermentable sugars and ethanol using

lower quantity of amylolytc enzymes than the parent field corn genotypes (PFCs). In another study, Chen et al. (2014a) also reported enhanced amounts of ethanol was produced by new high sugar containing genotypes developed from the crossing between sweet corn and tropical corn.

2.6 Characteristics of Corn

2.6.1 Agronomic Properties

Evaluation of agronomic characters for any corn hybrid, either new or old, is a traditional way to determine its sustainability, economic viability and popularity to the producers (Zilic et al., 2011). Crop phenology, in this aspect, is considered the primary agronomic trait, which is the time required for a corn plant to reach different stages of growth. It has been reported that crop phenology can affect kernel development, grain yield and kernel composition (Borrás et al., 2007; Severini et al., 2011). The major crop phenology parameters often evaluated for different corn hybrids are, the time for seedling emergence, leaf developments, tasseling, silking, grain filling period and maturity (Couto et al., 2013; Ning et al., 2013). Among other agronomic traits, plant heights, leaf number per plant and leaf length are important for any hybrid to get the base line data. These traits have significant effects on the physiological and biochemical well as other agronomic properties (Lucchin et al., 2003; Ning et al., 2013). The most important agronomic trait for a corn hybrid is the kernel yield (Tsimba et al., 2013), which is associated with various yield related agronomic traits, including ear number per plant, ear length, ear weight, kernel depth, kernel weight, ear radius, cob radius and potential yield (Tsimba et al., 2013; Wong et al., 1994).

2.6.2 Physical Structure of Corn Kernel

Each corn kernel consists of four major parts, such as embryo, endosperm, pericarp and tip cap (Figure 2.3). Among these four parts, endosperm alone occupies 82% of the total dry weight of the kernel (Hemeyer, 2010), whereas, small portions are occupied by the rest, estimated roughly as, 10% for embryo, 5% for pericarp and only 1% for tip cap (Earle et al., 1946). Most of the carbohydrates, both starch and sugars, are found in the endosperm that is surrounded by a protein matrix (Hemeyer, 2010). Endosperms are physically of two types, namely vitreous (hard) and floury (soft) endosperms. The soft endosperm is found in the central core of the kernel, while the hard endosperm present on the outer part of the kernel and surrounds the central core of the soft endosperm (Hemeyer, 2010). The ratio of hard to soft endosperms usually varies, based on the location of the kernels on the ear. The endosperm contains almost all the starch (98%), and 74% of the total protein of the kernels (Earle et al., 1946), while the embryo contains most of the lipids (around 80%) (Murthy et al., 2006).

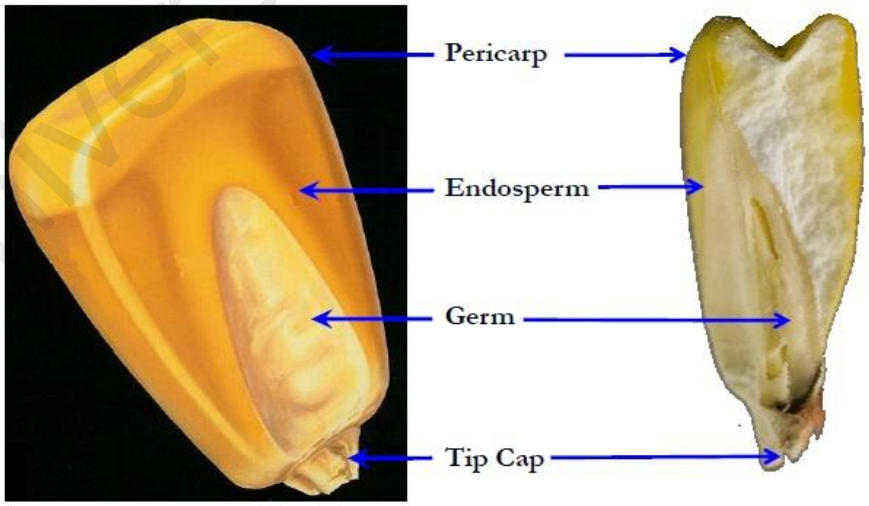

Figure 2.3: Different Parts of a Typical Corn Kernel (Reprint from Singh, 2009)

2.6.3 Biochemical Composition of Kernels

The biochemical composition of corn kernels differs significantly with regard to the types of corn and hybrids, particularly between normal and sugary corn hybrids. Irrespective of the corn types, the major biochemical components in corn kernels include starch, sucrose, reducing sugars (glucose, fructose, maltose), protein, lipids, fiber and ash (Semenčenko et al., 2015; Zilic et al., 2011).

Starch is the major fermentable component in corn and it is converted into ethanol during dry-grind ethanol production. However, research findings have established that ethanol yield does not significantly correlate with the quantity of starch (Reicks et al., 2009; Singh & Graeber, 2005). Rather, the structure of starch and the starch-protein complex affect the overall conversion process (Dien et al., 2002). On the other hand, free sugars in the kernels have been found to be more promising than starch because of their easy availability as fermentable sugars without any enzymatic conversion (Zabed et al., 2016b). Moreover, recently it has been reported that kernel sugars are positively correlated with ethanol yield (Chen et al., 2014a; Gumienna et al., 2016).

Protein, lipid, fiber and ash are the non-fermentable components but play important roles in the overall process and economy of the process (Hemeyer, 2010; Wu et al., 2006). In general, the dry matter of kernel contains around 10 % of protein, where roughly 47% of the protein is zein (a protein with low lysine and tryptophan) (Perry, 1988). Even though a variation in protein does not necessarily affect the fermentation efficiency (Wu et al., 2007), it has an influence on final ethanol yield (Lacerenza et al., 2008). The kernel protein is recovered in distiller's dried grains with solubles (DDGS) after fermentation, which improves the quality of this co-product (Liu, 2011). Kernel lipids has been reported to help to maintain the integrity of yeast cell membrane during fermentation (Murthy et

al., 2006) and is an important ingredient for DDGS, where it contributes to the energy content of DDGS (Hemeyer, 2010).

2.6.4 Composition and Structure of Starch

Starch is the major storage carbohydrate in plants and acts as an energy reservoir for higher plants, such as cereals, legumes and tubers (Miao et al., 2015). Like other cereal crops, starch is deposited as insoluble, semi-crystalline granules in corn kernels storage tissues (Copeland et al., 2009). It is composed of two polymers containing, namely, amylose and amylopectin, which are made of α-D-glucose units. Amylose is linked by α-1→4 glyosidic bond, while both α-1→4 and α-1→6 linkages are found in amylopectin (Copeland et al., 2009). Amylose is a linear polymer made up of around 1000 glucose units (Figure 2.4), while amylopectin possess a highly branched configuration, having one linked branch with α-1→6 bonds in every 20 linkages (Figure 2.5) (Copeland et al., 2009). Amylose content in starch can vary among the corn types and hybrids, and can affect the conversion of starch into fermentable sugars and ethanol (Karlsson et al., 2007; Yangcheng et al., 2013).

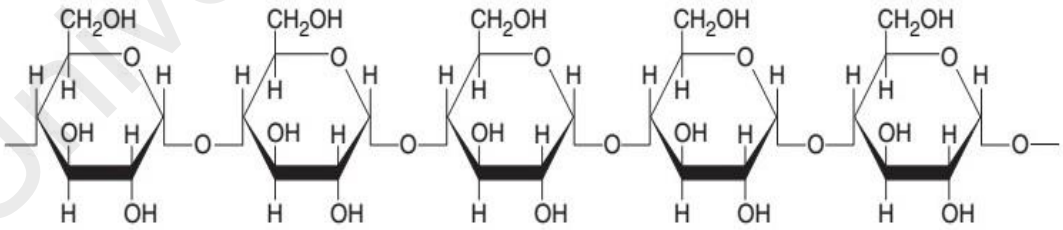

Figure 2.4: Structure of an Amylose Molecule (Reprint from Tester et al., 2004)

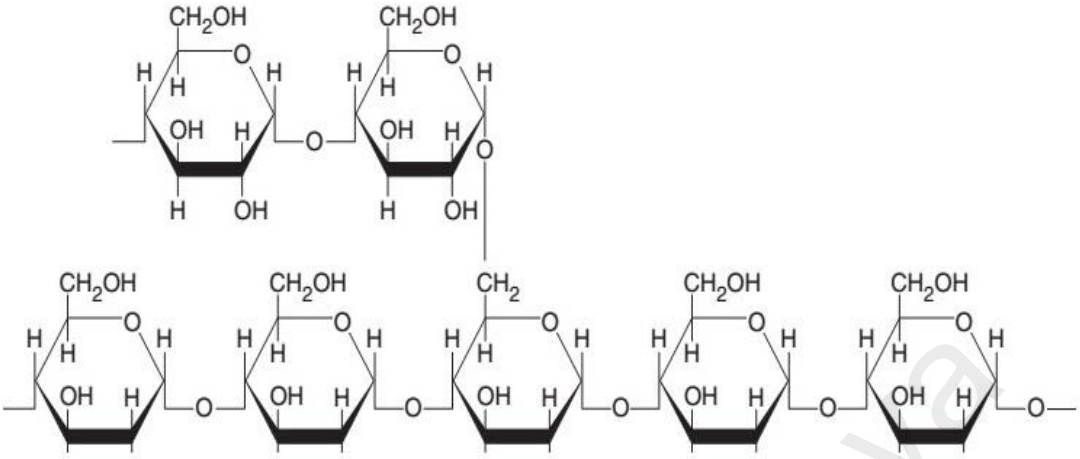

Figure 2.5: Structure of an Amylopectin Molecule (Reprint from Tester et al., 2004)

2.7 Effect of Hybrid Variability of Corn on Ethanol Production

Corn has been proven to be a promising feedstock for bioethanol production because of its high kernel and ethanol yields, accounting for 8.0 t/ha and 417 L/ha, respectively (Gumienna et al., 2016). Corn kernel yield has increased progressively since the introduction of hybrids, primarily in the USA and subsequently in the rest of the world (Duvick, 2005). However, the quality and availability of corn is important to ensure sustainability of the corn ethanol industry, and this can partially be done by developing new and promising hybrids (Bothast & Schlicher, 2005; Ramchandran et al., 2015). Nevertheless, ethanol yield varies significantly among the hybrids due mostly to variations in kernel properties (Figure 2.6). Both kernel composition and starch structure of the hybrids are influenced by their genetic make-up (Medic, 2011; Semenčenko et al., 2015). Numerous research efforts in recent years have focused on the development and cultivation of high quality corn hybrids with capability to produce higher amounts of ethanol (Chen et al., 2014a; Gumienna et al., 2016; Murthy et al., 2009; Zabed et al.,

2016b). Although high starch containing corn hybrids have been suggested for enhanced ethanol yield (Sanchez & Cardona, 2008), high fermentable sugar containing hybrids are considered more promising over the former from both economic and technical points of views (Chen et al., 2014a; Chen et al., 2013; Zabed et al., 2016a).

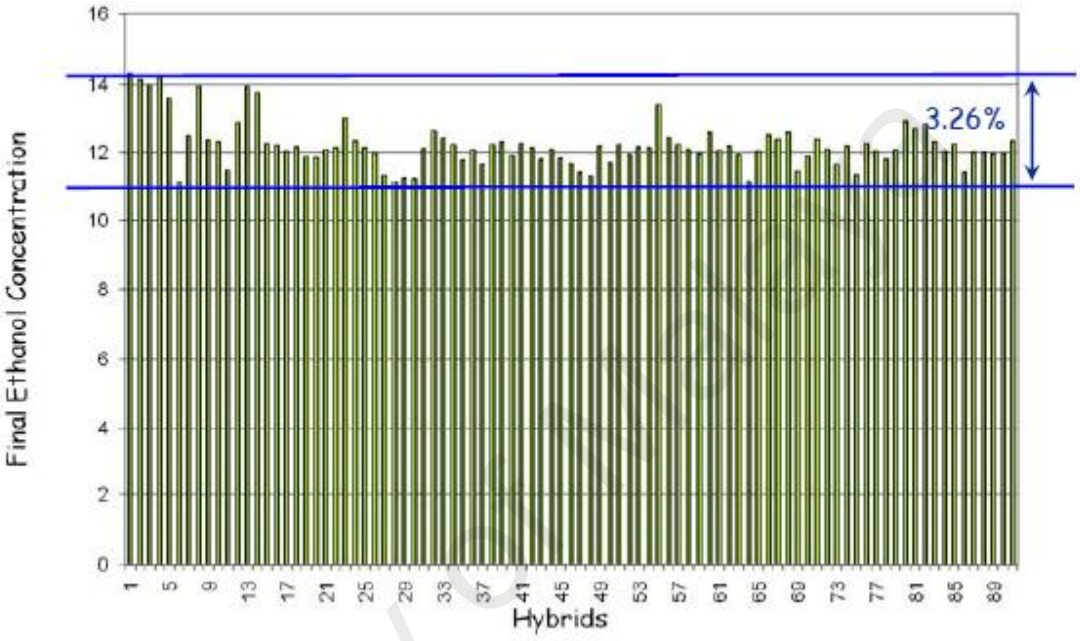

Figure 2.6: Effect of Hybrid Variability of Corn on Final Ethanol Concentration (%, v/v) (Adapted from Singh, 2009; Singh & Graeber, 2005)

2.8 Methods for Producing Ethanol from Corn

At present, ethanol is produced from corn either by the dry grind (DG) or the wet mill (WM) method, accounting for around 67% and 33% respectively of the ethanol produced (Bothast & Schlicher, 2005). Both methods differ primarily with regard to how the raw corn is processed prior to subjecting it to hydrolysis and fermentation. In a DG method, whole ground corn is used as feedstock, while different components are separated and only starch is used for producing ethanol in a WM method (Singh et al., 2001).

As was described earlier (Chapter 1; Section 1.1), the WM method requires higher capital investments and energy as the corn kernels are first separated into its components,

such as starch, fiber, gluten, and germ (Figure 2.7). Among these components, starch is subjected to hydrolysis and fermentation for generating ethanol. The remaining components are processed separately to produce a variety of co-products. One of the major co-products in the WM technique is corn oil, which is extracted from germ. Corn germ meal is produced by mixing germ with fiber and hull. Gluten is used to make corn gluten meal and a high-protein animal feed named as corn gluten feed.

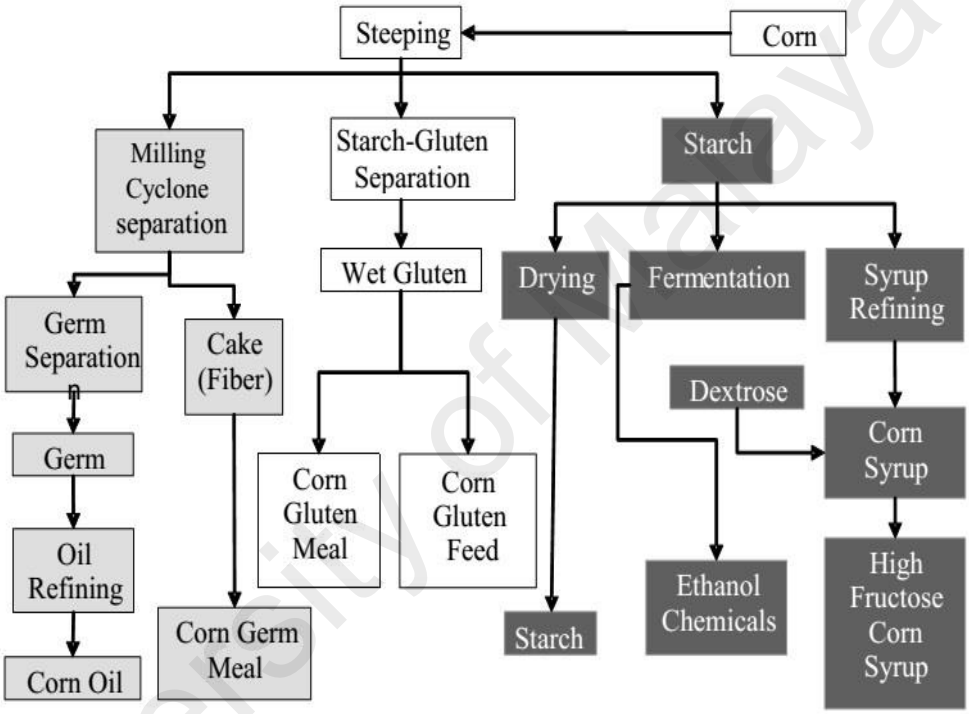

Figure 2.7: Schematic Diagram of a Conventional Wet Mill Method for Ethanol Production (Reprinted from Erickson et al., 2005).

Ethanol production in a conventional DG method is accomplished in six steps as illustrated in Figure 2.8: (1) grinding or milling of the dried kernels to reduce particle size, (2) preparation of slurry, (3) cooking and liquefaction, (4) saccharification (5) fermentation, and (6) product recovery. In brief, ground corn is mixed with water to prepare slurry, which is then cooked, liquefied and saccharified with amylolytic enzymes to produce soluble sugars. The hydrolysate is then subjected to a typical yeast

fermentation for producing ethanol. The ethanol dissolved in the fermented broth is then distilled and dehydrated to create fuel-grade ethanol. The remaining broth with solid is termed whole stillage, which is centrifuged to separate a coarse solid fraction (wet distillers' grains, WDG) and a liquid fraction (thin stillage, TS) (Kim et al., 2008b). Subsequently, TS is either recycled as backset or concentrated in evaporators to produce condensed distiller's solubles (CDS) (Ganesan et al., 2006). The CDS and WDG can be sold locally to cattle feeders without further processing or alternatively, both are combined and dried to produce DDGS.

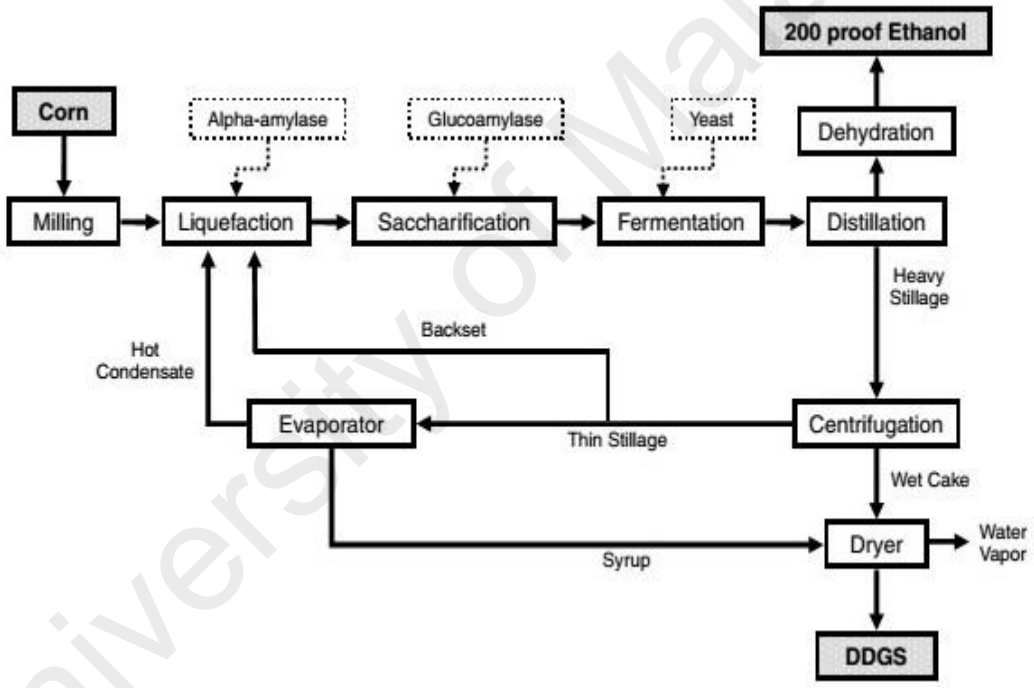

Figure 2.8: Schematic Diagram of a Conventional Dry-Grind Method for Ethanol Production (Reprinted from Kim et al., 2008a).

2.9 Enzymatic Hydrolysis of Corn

Enzymatic hydrolysis is conducted to convert starch into soluble sugars before fermentation, because yeast cells can not use starch directly (Kunz, 2008). Industrially conventional hydrolysis is usually completed in three steps, which includes cooking,

liuefaction and saccharification (Duvernay et al., 2013; Singh et al., 2001; Wu & Miao, 2008). The slurry is first pre-heated at 40-60°C in a pre-mixing tank, followed by cooking it at 90-165°C, and finally, liquefied at 60°C using a thermostable α-amylase (Kelsall & Lyons, 1999). However, cooking and liquefaction are often combined in a laboratory practice and carried out at 85-105^{o}C (Lamsal et al., 2011; Plumier et al., 2015).

During liquefaction, starch is first gelatinized at a high temperature (between 50 and 70^{o}C). The completeness in starch gelatinization is important as it will influence the conversion of starch into fermentable sugars in the subsequent steps (Lin & Tanaka, 2006). Almost all the amylose is solubilized and leached out from the starch granules during gelatinization and results in an increased viscosity of the slurry as it swells up at this time (Xu et al., 2016). The α-amylase used is an endoenzyme and randomly cleaves α-1→4 glycosidic linkages in starch molecules (O'Brien & Wang, 2008). Gelatinization and liquefaction of starch polymer usually convert the starch polymer into shorter chains, namely dextrins, maltose and maltotriose (Pietrzak & Kawa-Rygielska, 2015).

Saccharification is carried out at a relatively lower temperature (50-60^{o}C or even at fermenting temperature (30^{o}C) using glucoamylase (Mojović et al., 2006; Plumier et al., 2015). Glucoamylase works on the dextrin and converts it to soluble sugars, mostly glucose and maltose (Chu-Ky et al., 2016a).

As discussed above, hydrolysis of corn starch in the conventional method requires high energy for cooking and liquefaction. In an earlier study, it was estimated that energy consumption during cooking and liquefaction is equivalent to 30–40% of the fuel value of the ethanol produced (Lim et al., 2003). Recently, a new technology has been introduced in corn ethanol research for hydrolyzing native starch at the sub-gelatinized temperature (<48°C) using a granular starch hydrolyzing enzyme (GSHE) (Uthumporn

et al., 2010; Wang et al., 2007). GSHE contains both α-amylase and glucoamylase activities (Genencor, 2009).

2.10 Fermentation

2.10.1 Basic Concept on Ethanol Fermentation

Fermentation is a metabolic process by which microorganisms convert soluble sugars into alcohol. Some bacteria and yeasts can metabolize monosaccharaides (*e.g.*, glucose) and disaccharides (*e.g.*, sucrose) in the absence of oxygen, which results in the production of ethanol and carbon dioxide (Sarris & Papanikolaou, 2016). In a typical yeast fermentation with glucose, microbial conversion of glucose into ethanol occurs according to the reaction shown in Equation (2.1). From this reaction, the theoretical ethanol yield can be calculated. As per Equation (2.1), 100 kg glucose will produce 51.1 kg ethanol and 48.8 kg CO_2 (Singh et al., 2001).

$$C_6H_{12}O_6 + H_2O + \text{Yeast} \rightarrow 2CO_2 + 2C_2H_5OH + H_2O + \text{Heat} \quad \text{.....(2.1)}$$

Glucose	Water	Carbon dioxide	Ethanol	
(100kg)		(48.8kg)	(51.1kg)	(1700BTU)

In an ethanol fermentation process, roughly 95% soluble sugars are converted into ethanol and CO_2, 1% into cellular matter of the yeast cells, and 4% into other soluble byproducts such as glycerol (Boulton et al., 1999). The cost for yeast in an ethanol production process accounts for nearly 10% of the total production costs (Wingren et al., 2003).

2.10.2 Microorganisms

Both bacteria and yeast can be used for ethanol fermentation, having potential to meet the necessary requirements. The major requirements for an ethanol producing

microorganism are: (1) good growth in simple and inexpensive media, (2) high ethanol yield (>90.0% of theoretical), (3) tolerance to high ethanol concentration (>40.0 g/L), (4) good ethanol productivity (>1.0 g/L/h), and (5) ability to retard contaminants from growth condition (*e.g*., acidic pH and high temperature) (Dien et al., 2003). Several yeast species such as *Saccharomyces cerevisiae*, *S. diastaticus*, *Kluyveromyces marxianus* and one bacterial species, *Zymomonas mobilis* are well known in ethanol fermentation (Zabed et al., 2014).

The most frequently used microorganism in ethanol fermentation is the yeast, *S. cerevisiae* (Chen et al., 2016). Fermentation of sugars using this yeast is one of the oldest practices in biotechnology, which has been used for producing drinking alcohol since time immemorial. Today, yeast fermentation is widely used for generating fuel ethanol from renewable sources. Several unique properties of *S. cerevisiae* have made it most attractive for ethanol fermentation. Firstly, it has shown greater efficiency in the conversion of sugar into alcohol and tolerance to high concentrations of ethanol (Snoek et al., 2016). Secondly, it has the capability for producing flocs during its growth in the fermentation media, making it easier to settle down or suspend when required (Kosaric & Velikonja, 1995). Moreover, it is a generally recognized as a safe (GRAS) microorganism (Lin & Tanaka, 2006).

2.10.3 Mode of Fermentation

Bioethanol is produced mainly by three modes of fermentation, such as batch, fed-batch and continuous (Oliveira et al., 2016). The choice of the most suitable mode depends on the kinetic properties of fermenting microorganisms, type of feedstock used and costs (Thomas et al., 1996). Batch mode is the simplest fermentation technique and is usually done in a closed culture system. The feedstock is added to the fermentation vessel along with the yeast inoculum, nutrients and other ingredients at the beginning of

fermentation, for whole batch. The fermentation is continued until the nutrients are exhausted, and does not require any further supplementation with the ingredients, except for the possible addition of acid or alkali to control the pH of the media. The microorganism used in a batch fermentation works in high substrate concentration initially and a high product concentration finally (Olsson & Hahn-Hägerdal, 1996).

The fed–batch system combines batch and continuous modes and is typically used in the commercial production of ethanol. In a fed-batch mode, one or more ingredients are added to the vessel as fermentation continues (Gnansounou & Dauriat, 2005). While the fermentation progresses, microorganism works at low substrate concentration but increased ethanol concentration over time. Compared to the batch system, fed-batch mode often provides better ethanol yield and productivity (Balat, 2011). However, the ethanol produced in fed–batch fermentation is affected by the feed rate and cell mass in the media (Chandel et al., 2013).

Continuous fermentation is conducted through a sequential input of the required ingredients and removal of the products from the fermentation vessel continuously. In other words, the feed containing substrate, culture medium and nutrients, is pumped continuously into an agitated vessel (Balat, 2011). It can be performed in two kinds of bioreactors, such as stirred tank reactors (single or series) or plug flow reactors. Continuous fermentation often gives a higher productivity than batch or fed-batch fermentations. It produces higher amounts of ethanol and requires less down time for vessel cleaning and filling (Brethauer & Wyman, 2010). However, it is a more complex mode of fermentation and is suitable for fermentation on a large scale rather than on a laboratory scale.

2.11 Factors Affecting Hydrolysis and Fermentation

Various factors have been reported to affect the efficiency of hydrolysis and fermentation in ethanol production, which are related to substrate, enzyme, microorganism, and process parameters (Li et al., 2015; Ramchandran et al., 2015; Ramírez et al., 2016). Substrate related factors are ground corn particle size and initial solid load. Enzymes and microorganisms related factors include types and concentrations of enzymes and microorganisms. Process parameters include pH, temperature, incubation time, contaminating bacteria and soluble byproducts. These are discussed below.

2.11.1 Particle Size of Ground Corn

Particle size of ground corn has a significant effect on the sugar yield during enzymatic hydrolysis and ethanol yield during fermentation (Khullar et al., 2013). For this reason, dried kernels are ground to reduce particle size prior to enable a more efficient hydrolysis and fermentation. It has been suggested that, large particle sized kernels decreases the surface area available, for the action of enzymes molecules, to bind and catalyze effectively during starch hydrolysis and/or fermentation (Barcelos et al., 2011). Small particle size has often been shown to favor the effective conversion of starch into glucose and the release kernel sugars in the hydrolysates (Zabed et al., 2016). However, to produce smaller particle size kernels requires a higher energy input during grinding and this in turn increases overall production costs and creates difficulties in the downstream process after fermentation (Wang et al., 2008). As a result, current dry–grind ethanol industries use particle size less than 1.0 mm, taking into account the overall influence of particle size on the processes mentioned (Szymanowska-Powałowska et al., 2014).

2.11.2 Initial Solid Load

Initial solid concentration in the slurry is an important factor as it has a direct effect on the rates of starch hydrolysis and fermentation and on the growth of microbial cells (Li et al., 2015; Modenbach & Nokes, 2013; Mojović et al., 2006). High solid load is often desired in a commercial plant since it generates enhanced amounts of ethanol and decreases the downstream processing costs. However, a high solid load in the slurry reduces the efficiency of the starch conversion process, particularly during conventional dry-grind ethanol production. Gelatinization of starch at a high temperature is the major part of a conventional method that increases viscosity of the slurry and causes difficulties in mixing and pumping of the contents (Robertson et al., 2006; Uthumporn et al., 2010). Furthermore, the increased viscosity creates problem in the proper dispersion of the starch and enzymes and results in the incomplete conversion of starch (Mojović et al., 2006; Uthumporn et al., 2010). Although this viscosity problem can be overcome during granular starch hydrolysis, the best operation procedures limits solid load to 12-38% of initial solid (Foerster, 2010; Szymanowska-Powalowska et al., 2012).

The actual relationship between the initial substrate concentration and the fermentation rate is rather more complex. In general, the fermentation rate will increase with an increase in sugar concentration, but up to a certain level (Lemuz et al., 2009). Excessively high sugar concentrations will exceed the uptake capacity of the microbial cells leading to a steady rate of fermentation. In a batch fermentation, high ethanol productivity and yield can be obtained at high initial sugar concentration, but it takes longer fermentation time and subsequently increases recovery costs (Zabed et al., 2014).

2.11.3 Enzyme Load

An appropriate enzyme dose is required for the efficient conversion of starch into glucose. When the enzyme load is lower than the optimum dosage significant amounts of starch remains unreacted and unconverted (Devantier et al., 2005). On the other hand, the conversion efficiency and sugar yield do not necessarily increase above a certain level of enzyme concentration (Mojović et al., 2006). One of the reasons for the insignificant changes in starch conversion above a certain level of enzyme dosage, is the unavailability of starch molecules for increased enzyme loads (Apar & Özbek, 2004; Hagenimana et al., 1992). Another reason is the possibility of some sort of enzyme–enzyme inhibition that decreases the efficiency for binding to starch molecules (Textor et al., 1998). The efficiency or rate of an enzyme reaction will not increase even though more enzymes are added if the substrate concentration in the solution is limited. During hydrolysis of starch for dry-grind ethanol production, initial solid load as well as starch (substrate) concentrations are limited to a certain level, which also explains the reason for insignificant changes in sugar production above a certain level of enzymes.

2.11.4 Incubation Time

Incubation time has a significant effect on the enzymatic hydrolysis and the fermentation of corn (Szymanowska-Powałowska et al., 2014). In general, extremely short or longer incubation time affect overall yield. During enzymatic hydrolysis, enzymes are usually diffused into the slurry and are able to have access to the starch molecules. The enzymes then bring about the conversion of starch into fermentable sugars. The overall conversion process requires an appropriate time, whereby a short incubation time would interrupt the hydrolysis process and result in the incomplete hydrolysis of starch. However, the sugar yield shows a plateau after a certain time of

hydrolysis. It has been suggested that this happens due to an end product inhibition of the enzymes, after a prolonged period of time (Hill et al., 1997; Mojović et al., 2006).

Furthermore a short incubation time during fermentation, causes an inadequate growth of yeast cells as well as an inefficient conversion of sugar into ethanol. On the other hand, a longer fermentation time can have a toxic effect on microbial growth, particularly in the batch mode due to the high concentration of ethanol produced (Srichuwong et al., 2009). It has been reported that the ideal fermentation period for ethanol production is 48-72 h (Ingledew, 1998). However, a longer fermentation period such as 96 h has also been reported even though the productivity of ethanol significantly decreases as the fermentation time increases from 72 to 96 h (Johnston & McAloon, 2014).

2.11.5 pH

The efficiency of starch hydrolysis is always affected by the pH of the slurry as it is an enzyme catalyzed reaction. Likewise, ethanol yield during fermentation depends on the pH of the broth as it has a direct effect on the growth of the yeast cells and their cellular processes (Kasemets et al., 2007; Masiero et al., 2014). In particular, the concentration of proton (H+) in the fermentation media can change the total charge of plasma membrane, and eventually affect the permeability of some essential nutrients into the cells (Zabed et al., 2014). The optimum pH differs considerably between hydrolysis and fermentation. The optimum pH for a conventional liquefaction is 6.0-6.5 (Apar & Özbek, 2004; Mojović et al., 2006), while the optimum pH for conventional saccharification, GSH and fermentation with *S. cerevisiae* range between 4.0 and 5.0 (Lin et al., 2012; Treebupachatsakul et al., 2016; Uthumporn et al., 2010).

2.11.6 Temperature

Temperature is another important factor carefully regulated during hydrolysis and fermentation as it has vital impact on these processes as well as the product yields. During conventional hydrolysis of starch, the optimum temperature for liquefaction has been reported to be between 85-105^{o}C (Lamsal et al., 2011; Plumier et al., 2015), while a separate saccharification is usually done at 50-60^{o}C (Mojović et al., 2006; Plumier et al., 2015). On the other hand GSH is done at 30-35^{o}C (Li et al., 2012; Uthumporn et al., 2010).

Efficient fermentation primarily relies on the proper growth of the yeast cells in the fermentation media and the overall metabolic activities of yeast cells are influenced by the temperature of the fermentation system. It is generally believed that the ideal fermentation temperature range is between 20 and 35^{o}C and higher temperatures in almost all fermentation processes creates problem (Ballesteros et al., 2004; Phisalaphong et al., 2006). However, the optimum temperature range has been reported to be between 28°C and 32°C for ethanol fermentation with *S. cerevisiae* (Torija et al., 2003). At higher temperatures above 35°C, the fermentation efficiency of *S. cerevisiae* is low (Banat et al., 1998) as a high temperature is considered as a stress factor for yeast cells. Yeast cells produce heat-shock proteins in response to stressful conditions as well as the inactivation of its ribosomes. In addition, microbial activity and fermentation are carried out by different enzymes which are also sensitive to high temperatures since it can denature the tertiary structure and eventually inactivate enzymes (Phisalaphong et al., 2006).

2.11.7 Contaminating Microorganisms

One of the major challenges for a controlled fermentation is the contamination of media by bacteria, in particular, lactic acid bacteria (Szymanowska-Powałowska et al.,

2014). Contaminant bacteria can affect final ethanol yield and productivity by competing with yeast cells for fermentable sugars, nutrients (trace minerals, vitamins, and free amino nitrogen) and producing inhibitory byproducts, such as lactic acid (Skinner & Leathers, 2004; Thomas et al., 2001). In order to decrease bacterial contamination during fermentation, antibiotics are often used in the hydrolysates in ethanol plants (Narendranath et al., 1997; Narendranath & Power, 2005).

2.11.8 Soluble Byproducts

During ethanol fermentation, some soluble by-products (*e.g.*, lactic acid, glycerol etc.) are produced as a result of metabolic activities of yeasts and contaminating bacteria (Graves et al., 2006). Lactic acid is usually produced as a result of carbohydrate metabolism by contaminating lactic acid bacteria. Production of lactic acid during fermentation is not desirable as it affects yeast growth and ethanol yield (Białas et al., 2010). Glycerol is another byproduct produced by yeast cells and even by contaminating bacteria during fermentation (Sarris & Papanikolaou, 2016a). Even though glycerol production is part of sugar metabolism by yeasts, high glycerol concentration in the fermentation broth is undesirable. The production of glycerol is an energy intensive process and will affect the final ethanol yield (Murthy et al., 2006).

2.12 Conventional Simultaneous Saccharification and Fermentation (CSSF)

The conventional DG ethanol production can be carried out by two different techniques, such as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Ethanol production by SHF includes a separate hydrolysis of corn starch through liquefaction and saccharification as described in Section 2.9. Subsequently, the soluble sugars are fermented in a separate step by yeasts to produce ethanol (Ratnavathi et al., 2011). A slight modification of SHF, the SSF, is conducted by

adding yeast and glucoamylase to the liquefied slurry, followed by both saccharification and fermentation. (Manikandan & Viruthagiri, 2010).

Although both SHF and SSF are used for conventional dry-grind ethanol production, the latter is often chosen in laboratory and industrial practices because it has attractive advantages. Firstly, conventional simultaneous saccharification and fermentation (CSSF) has been reported to show higher ethanol productivity (25-40%) than that of SHF (Öhgren et al., 2007). Secondly, SHF involves liquefaction and saccharification of the slurry before subjecting it to fermentation, requiring different temperatures for each step, which eventually increases overall process time and production costs (Sánchez & Cardona, 2008). Thirdly, in a SHF, soluble sugars are accumulated in the hydrolysates as a result of saccharification, which may causes inhibition of enzyme activity as well as an incomplete hydrolysis of starch when sugar concentrations reaches to a certain level (Mojović et al., 2006). This kind of inhibition can be avoided in CSSF as it allows simultaneous sugar production and consumption (Srichuwong et al., 2009). Fourthly, as the starch hydrolysates containing high concentration of sugars are used in SHF, it may exert osmotic stress on the yeast cells, affecting ethanol productivity and yield during SHF (Nikolić et al., 2010). However the CSSF process is suitable to overcome the unwanted effect of sugars (Srichuwong et al., 2009). Finally, SHF is more vulnerable to microbial contamination than CSSF (Szymanowska-Powałowska et al., 2014).

2.13 Granular Starch Hydrolysis and Simultaneous Fermentation (GSHSF)

GSHSF is a non-cooking method that does not require any gelatinization and liquefaction of starch prior to fermentation unlike in CSSF. Both hydrolysis and fermentation can be completed in a single step using a granular starch hydrolyzing enzyme (GSHE) (Chu-Ky et al., 2016; Szymanowska-Powałowska et al., 2012). GSHSF has been considered more promising for dry–grind ethanol production as it offers some

advantages over the conventional method. Firstly, GSHE can catalyze and hydrolyze starch polymers at sub-gelatinized temperature due to having both α–amylase and glucoamylase activities (Szymanowska & Grajek, 2011; Wang et al., 2009). It does not require any activators, such as Ca^{2+}, as is frequently used in a conventional enzyme system (Mojović et al., 2006; Szymanowska & Grajek, 2011). Therefore, GSHSF simplifies the overall ethanol production process and reduces energy consumption by 10–20% (Robertson et al., 2006).

Secondly, gelatinization of starch at high temperatures in a conventional method results in the increase in viscosity of the slurry by 20 fold, thereby causing difficulties in mixing and pumping of the hydrolysates (Robertson et al., 2006; Uthumporn et al., 2010). Furthermore the increased viscosity may hinder the dispersion of starch and enzyme molecules throughout the mixture, particularly under high dry solid conditions and result in the incomplete conversion of starch (Mojović et al., 2006; Uthumporn et al., 2010). Alternatively, viscosity problems can be overcome using GSHSF as it does not require any gelatinization of starch (Robertson et al., 2006; Szymanowska & Grajek, 2011). Moreover, GSHSF is suitable for using high solid load in the slurries (12–38%) without encountering any mixing problems with enzymes and substrates (Foerster, 2010; Szymanowska-Powalowska et al., 2012).

Compared to the conventional dry–grind ethanol process, GSHSF produces higher amounts of ethanol, because less sugar is loss during hydrolysis as often occurs in the conventional process through the Maillard reaction (Galvez, 2005; Robertson et al., 2006; Srichuwong & Jane, 2011; Szymanowska & Grajek, 2011). Additionally, this method decreases osmotic stress on yeast cells during fermentation (Szymanowska & Grajek, 2011), and produces more nutritious distiller's dried grains with solubles (DDGS) (Srichuwong & Jane, 2011). It has been reported previously that higher amounts of

ethanol was produced with higher fermentation rates during dry–grind ethanol production from raw corn using GSHSF compared to the conventional method (Wang et al., 2005).

2.14 Co-Product Quality

The solid residues and stillage obtained after fermentation, when mixed and dried, are known as DDGS (Singh et al., 2001). A dry–grind ethanol method typically produces roughly 10.6 liter of ethanol and 7.7 kg of DDGS from one bushel of corn (25.4 kg) (Mosier & Ileleji, 2014; USGC, 2012). DDGS is a major co-product in the DG process which is widely used for animal feed as a source of protein and energy (Branca & Di Blasi, 2015). In parallel to the yield of ethanol, the quality of DDGS is also important for a DG ethanol plant because it has a significant role on the overall economy of the plant (Belyea et al., 2004; Liu, 2008). It has been suggested that any raw material for ethanol production should meet certain criteria in place, to ensure it produces good quality DDGS (Corredor et al., 2006). The quality of DDGS is critical as it is marketed as animal feed (Belyea et al., 2004). The nutritious composition of DDGS includes protein, fat, fiber, ash and starch (Liu, 2011). The source of these components is primarily the composition of the kernels (Liu, 2008). However, the source of protein can be from yeast itself, in addition to that of kernel (Belyea et al., 2004; Liu, 2009).

2.15 Conclusion

Although production of ethanol from corn has been studied extensively and different corn hybrids also considered, a conclusive work yet to be done by including the determination of agronomic performance, biochemical composition and DDGS quality. The effect of kernel sugars on fermentable sugar and ethanol yield as well as quality of DDGS have also not been studied conclusively. Moreover, development of high sugary

corn genotypes and potential of these hybrids for ethanol production have not been reported, which necessitate a further and conclusive study of high sugary genotypes.

CHAPTER 3: CHARACTERIZATION OF NORMAL AND HIGH SUGARY GENOTYPES FOR PREDICTING THE EFFECT OF DIFFERENT TRAITS ON CARBOHYDRATE ACCUMULATION IN KERNELS

3.1 Introduction

Corn is one of the most important cereal crops, being a natural source for carbohydrates that can be used to produce various food, feed and industrial products (Radosavljević et al., 2012; Semenčenko et al., 2015). In more recent times, this cereal crop has drawn much attention for fuel ethanol production as attempts to reduce the dependence on fossil fuels gathers pace globally. Numerous studies have shown that agronomic and biochemical properties of corn hybrids affect final ethanol yield (Dien et al., 2002; Lacerenza et al., 2008; Lorenz et al., 2009; Pollak & Scott, 2005; Wu et al., 2006; Yangcheng et al., 2013), suggesting that these characteristics should be taken into consideration prior to selecting any hybrid as ethanol feedstock.

Among the agronomic traits, grain yield is the most important parameter, which can vary with variety even if all other factors are supposed to be similar (MiJa et al., 2012). Moreover, it has been well documented that grain yield has a significant effect on ethanol productivity (Obuchowski et al., 2010). Other agronomic parameters are interlinked either with the grain yield or carbohydrate accumulation in the kernels (D'Andrea et al., 2009), thereby affecting ethanol yield indirectly. Furthermore, several agronomic traits, such as thousand kernel weight (TKW) has been reported to affect final ethanol yield directly (Swanston et al., 2007). TKW also showed a positive correlation with the amount of kernel starch (Obuchowski et al., 2010). It has been shown that the time duration required for any crop plant to reach its different growing stages (crop phenology) has a significant influence on its agronomic properties and physicochemical composition (Wang et al., 1999; Yang et al., 2008). Kernel number per plant (KNP), ear length and

weight, and thousand kernel weight (TKW) are directly correlated with kernel yield (D'Andrea et al., 2008).

Kernel composition significantly affect ethanol yield (Singh 2012), and quality of the co-product (Belyea et al., 2004; Osorno & Carena, 2008) during dry-grind ethanol production. It has been reported that an average of 3% ethanol loss could occur in an ethanol plant with 100 million gallon capacity owing to variation in grain quality, which is equivalent to a loss of 3 million gallons ethanol per year (Ramchandran et al., 2015). The biochemical composition of kernels can be divided into two groups, namely, fermentable and non-fermentable components. In all types of corn, starch is the major fermentable component, while varying quantities of sugars may also present based on the types of corn (Manikandan & Viruthagiri, 2010; Yangcheng et al., 2013). The non-fermentable components are mainly protein, fat, fiber and ash (Singh, 2012; Wu et al., 2006). However, the kernel compositions of corn as well as any other crop vary in different hybrids (MiJa et al., 2012).

Although starch is the major carbohydrate in corn, there is controversy with regard to the relationship between quantity starch and ethanol yield. Several researchers have reported an insignificant but positive correlation between starch content and ethanol yield (Dien et al., 2002; Reicks et al., 2009; Singh & Graeber, 2005). However, a significant positive correlation has been reported elsewhere (Yangcheng et al., 2013). More recently, a negative correlation has been reported between starch and ethanol yield by studying 258 corn hybrids (Gumienna et al., 2016). Therefore, even though starch is the major fermentable component in cereal grains, it is not reliable to predict ethanol yield directly from the quantity of starch (Riffkin et al., 1990; Swanston et al., 2005). Alternatively, determining the protein content has been suggested as an indirect but effective way of predicting ethanol yield (Kindred et al., 2008), which is positively correlated with kernel

protein, whereas, a negative correlation was reported between starch and protein (Lacerenza et al., 2008; Liu et al., 2013). However, evaluation of starch and protein in relation to ethanol yield has been performed without considering kernel sugars. A recent study on the production of bioethanol from different corn hybrids has shown that kernel sugars significantly correlated with ethanol yield, where high sugar containing hybrids produced enhanced amount of ethanol (Gumienna et al., 2016).

Amylose content in starch is another important biochemical component that influences the physicochemical properties of starch (Karlsson et al., 2007) and can eventually affect the conversion of starch to ethanol (Yangcheng et al., 2013). It was reported that a lower and even a lack of amylose in waxy corn causes easy gelatinization (Jobling, 2004), resulting in the easy conversion of starch to fermentable sugar during hydrolysis. Amylose content in starch significantly differs in normal, waxy, high amylose and sugary corns (Singh et al., 2005a). The efficiency and sugar yield from the hydrolysis of native starch has been shown to decrease with increasing amylose content in starch (Tester et al., 2006). With regard to amylose content, corn has been classified into three major groups, such as (1) normal or dent corn that contains 20-30% amylose, (2) waxy corn containing almost 0% amylose and (3) high amylose corn containing 40-70% amylose (Nelson & Pan, 1995; Singh et al., 2006; Torney et al., 2007). Studies on several waxy and normal corn varieties, have shown that ethanol yield were significantly lower in normal corn than in the waxy corn varieties (Yangcheng et al., 2013).

Taking into account the above facts, a comparative study was carried out on four high sugary corn genotypes (HSGs) and four parent field corn genotypes (PFCs) for their agronomic and biochemical traits in light of ethanol production, and the results are presented in this chapter. The main aim of characterizing the corn genotypes is to evaluate the effects of agronomic traits on the biochemical composition of the kernels, particularly

carbohydrate content (both sugars and starch). Carbohydrates in the kernels are the major fermentable components for corn during ethanol production, as will be discussed in Chapters 4 and 5. Furthermore, composition of corn kernels are also important for the co-product quality in a typical dry grind ethanol production process as will be described in Chapter 6. As a result, a comprehensive study on the characteristics of HSGs and PFCs and its relationship to ethanol yield and co-product quality will be more effective and meaningful towards increasing ethanol yield.

3.2 Materials and Methods

3.2.1 Location of Field and Laboratory Experiments

Field experiments and data collection were done in two successive cropping years (September to December 2012 and August to November 2013) at the experimental field site near the department of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia (3° 7' 1" N and 101° 39' 12" E). Laboratory studies were carried out at the Functional Food Laboratory on level 4, Block B, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia from August 2012 to July 2015.

3.2.2 Collection of the Materials

Seeds of four high sugary corn genotypes (HSGs), namely, UM.NF-1, UM.NF-4, UM.NF-6 and UM.NF-11, and their parent field corn lines (PFCs), PFC-1, PFC-4, PFC-6 and PFC-11 respectively, were collected from Dr. Golam Faruq, Institute of Biological Sciences, Faculty of Science, University of Malaya. The enzymes used in the analytical methods are (1) SPECZYME® FRED (an acid-stable and thermostable α-amylase) with a minimum activity of 17400 LU (liquefon unit) per gram; one LU is the time needed to produce a color change with iodine solution, representing a distinct phase of starch

dextrinization under a specified condition (temperature, pH and starch concentration); (2) OPTIDEX® L-400, a glucoamylase with the minimum activity of 350 GAU/g; one GAU is the quantity of enzyme needed to produce 1 g of glucose from starch under a specified condition; and (3) FERMGEN, an acid fungal protease with an activity of ≥1000 SAPU/g (Spectrophotometric Acid Protease Units), as defined by the manufacturer (Genencor, Palo Alto, CA). The enzymes were kind gifts from DuPont Industrial Biosciences (DuPont Genencor Science, Palo Alto, CA). All the chemicals, reagents and standards used in this study were reagent grade and purchased either from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Waltham, MA, USA).

3.2.3 Determination of Seed Quality

Before sowing in the field, collected seeds were sampled and tested for important quality attributes, such as moisture, seed weight (thousand seed weight), seed viability, and purity. Moisture content was determined gravimetrically in duplicate for each genotype by drying 3 g of sample at 105°C for 6 h as described in the next Section (3.10.3) (AOAC, 1990). Thousand seed weight was determined in duplicate by weighing 100 seed and multiplying the result by 10 (Buffo et al., 1998). Viability of the seeds was determined in duplicate by the top paper method (Wang & Hampton, 1989) using tissue paper soaked in water and placed in Petri dishes. Ten randomly selected seeds were placed on the top of the tissue paper in each Petri dish and were allowed to germinate under room temperature for 10 days. The percent viability of seeds was counted from the ratio of number of seed germinated to the total number of seeds inoculated in the dish (Equation 3.1). The purity of seeds was determined by a reference method described by Karrfalt (2008). Ten grams of seed sample was taken from each genotype in duplicate and checked for any abnormalities, followed by taking the weight of the pure seeds. The percent purity

was calculated from the ratio of the weight of the pure seeds to the weight of the initial sample (Equation 3.2).

$$\text{Seed viability } (\%) = \frac{\text{No. of seeds germinated}}{\text{No. of seeds used for viability test}} \times 100 \quad \text{.....(3.1)}$$

$$\text{Seed purity } (\%) = \frac{\text{Weight of pure seeds}}{\text{Weight of sample seeds}} \times 100 \quad \text{.....(3.2)}$$

3.2.4 Soil Analysis at the experimental site

Soil samples were collected in 2012 and 2013 from three random sampling points from each plot at 0 to 0.4 m depth one week before sowing the seeds, and mixed thoroughly in a large bucket to form one composite soil sample for each plot (Cirilo et al., 2009). A suspension of fresh soil and water was prepared at the ratio of 1:2 and the pH measured using a pH meter (Sheldrick, 1984). Moisture content was determined gravimetrically by drying 5 g of sample at 105^{o}C until constant weight (AOAC, 1990). The remaining portions of the samples were dried at 65^{o}C in an oven for 6 h, homogenized and gently crushed in a mortar and pestle and sieved through a standard sieve of 2.0 mm mesh size (Bhattacharjee et al., 2013; Brown, 1998). Total organic matter (TOM) was determined using the method described by Azlan et al. (2013). The total organic carbon (TOC) in the soil samples was estimated by the chromic acid wet oxidation method (Walkley & Black, 1934), as modified by Sheldrick (1984). Briefly, 0.5 g of soil was dissolved in 10 ml of 1 N $K_2Cr_2O_7$ and 20 ml of conc. H2SO4. Then 500 ml of distilled water, 10 ml of phosphoric acid and 1 ml of Barium diphenylamine Sulphonate (indicator) were added to it. The titration of the solution was then conducted using 0.5M of Ferrous Sulfate until purple or blue color. Total phosphorus was determined by the perchloric acid digestion method (Sheldrick, 1984). The quantity of available phosphorus in soil was estimated

using the sodium bicarbonate extraction method (Olsen, 1954), as modified by Sheldrick (1984). Nitrogen content in the soil sample was determined in a CHN analyzer (CHNS/O 2400 Series II, Perkin Elmer). Potassium content in soil was determined by the flame photometric method after ammonium acetate-acetic acid digestion of soil samples (AOAC, 1990).

3.2.5 Environmental Data

Data for climatic conditions were collected from the Malaysian Metrological Department (MetMalaysia), which is under the Ministry of Science, Technology and Innovation (MOSTI). Monthly average temperature, humidity and rainfall were considered for environmental conditions during the study.

3.2.6 Corn Genotype Growth

The corn genotypes were grown in the field during 2012-2013 (September-December in 2012 and August-November in 2013) under rain fed condition following randomized complete block design (RCBD) with two replications. An individual experimental plot consisted of 5 rows, 2.5 m long and 0.75 m apart, with hill to hill distance maintained at 0.5 m giving the gross area of each plot was 7.5 m^2. The plots were planted with four seeds in each hill on September 3 (2012) and August 1 (2013). The plots were thinned to 8 plants/m^2 (80,000 plants/ha) by removing extra plants around 10 days after sowing at the three-leaf stage (V3) (Cirilo et al., 2009; Gambín et al., 2007). Similar amounts of N, P and K were applied to all plots of each genotype (HSGs and PFCs) in the form of 15-15-15 at a rate of 125 kg/ha prior to sowing the seeds and only nitrogen as urea was applied twice during growth of the genotypes, 15 days after sowing (around the four-leaf stage, V4) and on 40 days after planting (15–20 days before anthesis) with 86 and 62 kg N/ha respectively, based on the optimization work of a previous study on the same field.

Insects and weeds were adequately controlled throughout the growing cycle of the corn genotypes. Hand weeding was done thrice during the growing season at four and eight weeks after planting.

3.2.7 Crop Phenology

The duration for seedling emergence (50% of the total plants were visible on the soil surface), 4 leaves development (in 50% plants), 8 leaves development (in 50% plants), anthesis or tasseling (50% of plants showed visible tassel), silking (50% of plants showed visible silk), and maturity (when 50% of the plants showed black layer formation in the kernels) were recorded in each plot during the experimental period (Cirilo et al., 2009).

3.2.8 Harvesting and Sampling

Corn plants were harvested when moisture level reached to around 24-26% (Reicks et al., 2009). During harvesting, samples were collected from the middle rows of each plot with an area of 2 m^2 (total 16 plants) in order to study agronomic and biochemical traits. Middle rows were sampled as this is the standard practice in the agronomic studies to avoid contamination by other surrounding plots.

3.2.9 Measurement of Agronomic Traits

Ten plants were taken randomly from each plot and plants height (PH), leaf number per plant (LNP) and leaf length (LL) estimated. All the ears were removed from the sampled plants and counted to estimate the ear number per plant (ENP) for each plot. Thereafter, 10 ears were randomly selected from each plot to determine length, radius and weight of the ears. Three randomly selected ears were broken in half, and kernel depth was measured with a ruler (Wong et al., 1994). Subsequently, kernels were separated from three randomly selected ears from each plot and counted to determine the kernels

number per ear (KNE), which was then converted into kernel number per plant (KNP) multiplying ENP with KNE. Thousand kernel weight (TKW) was determined by randomly selecting and weighing 100 kernels, followed by multiplying with 10 (Buffo et al., 1998). Potential yield for each genotype was estimated by multiplying average ear numbers per plant, average ear weight and assumed population density of 45000 plants/ha as per the Equation (3.3) (Wong et al., 1994). Kernel yield was estimated using the Equation (3.4) (Wong et al., 1994). Thereafter, 3 ears were dried in an oven at 105^{o}C until a constant weight was reached, and final moisture content was determined gravimetrically by subtracting final weight from initial weight (AOAC, 1990). All the weights were then adjusted to a 15% moisture level (Lauer, 2002; Mekuria et al., 2014). Subsequently, all ears (sampled and harvested) were dried in an oven at 40^{o}C until the moisture level reached 15% (Reicks et al., 2009). After drying, kernels were removed from ears, ground in a laboratory grinder and preserved at 4^{o}C until further analysis.

$$\text{Potential yield (t/ha)} = \frac{\text{ENP} \times \text{EW} \times 45000}{907.18} \quad \text{.....(3.3)}$$

where, ENP is the ear number per plant; EW is the ear weight (kg); 45000 is the assumed standard plant density per hectare (ha) (Wong et al., 1994); 907.18 is the conversion factor from kg to ton

$$\text{Kernel yield (t/ha)} = \frac{\left[\pi(r_{ear})^2 \times l_{ear}\right] - \left[\pi(r_{cob})^2 \times l_{ear}\right]}{\pi(r_{ear})^2 \times l_{ear}} \times \text{PY} \quad \text{.....(3.4)}$$

where, r_{ear} is the ear radius (cm); $_{lear}$ is the ear length (cm); r_{cob} is the radius of cob as determined by subtracting the kernel depth (cm) from the ear radius (cm); PY is the potential yield (t/ha) (Wong et al., 1994).

3.2.10 Biochemical Composition of corn kernels

3.2.10.1 Samples

The dried and ground kernels of the eight corn genotypes (Section 3.2.9) were used as samples for biochemical analysis. Prior to analysis, all samples were sieved in a 0.5 mm laboratory sieve.

3.2.10.2 Preparation of Reagents and Standard Solutions

The preparations of different reagents required to analyze biochemical components are described in Appendix A. Likewise, preparation of standard solutions for different spectrophotometric methods are described in Appendix B.

3.2.10.3 Determination of Moisture Content

The moisture content in corn samples was determined by the standard method described in AOAC (1990). A crucible was labelled with porcelain marker and placed in an oven at 105^{o}C for at least 5 h. The crucible was then removed from the oven and cooled in a desiccator. Subsequently, the weight of the crucible was recorded (W1). Thereafter, 10 g of corn sample was taken in the same crucible and placed in the oven at 105^{o}C and dried to a constant weight. The sample plus crucible was then removed from the oven and cooled in a desiccator and the weight was recorded (W2). Moisture content in the sample was calculated from the initial and final weight using the Equation (3.5).

$$\text{Moisture } (\%, \text{w/w}) = \frac{(\text{W2} - \text{W1})}{\text{W}} \times 100 \quad \text{.......................................(3.5)}$$

where, W is the weight of the sample (g); W1 is the weight of the empty crucible (g); W2 is the weight of the crucible plus sample (g)

3.2.10.4 Determination of Total Soluble Sugars (TSS)

The amount of TSS in the kernel was estimated by the anthrone method as described in Rose et al. (1991). Prior to analysis, a sugar solution was prepared from corn meal by hot alcohol extraction, followed by de-proteinization of the extract as described in the subsequent sections.

(a) ***Extraction of Kernel Sugars***

The corn meal was extracted with hot alcohol using a slightly modified method of Cerning-Beroard (1975). In a 50 ml falcon tube, 5 g of corn flour was taken and mixed with 20 ml of 80% ethanol. The tubes were then placed in a boiling water bath and heated at 95^{o}C for 30 min with occasional stirring. Thereafter, tubes were removed from the water bath, cooled and centrifuged at 3500 rpm for 10 min and the supernatant collected in a 100 ml volumetric flask. The extraction procedure was repeated twice with 20 ml of 80% ethanol each time, and supernatants collected and combined. Finally, the volume of the extract was adjusted to 100 ml with 80% ethanol.

(b) ***De-Proteinization***

The sugar solution obtained after hot alcohol extraction was de-proteinized by treating it with trichloroacetic acid (TCA) using the slightly modified method of Dubowski (1962). Briefly, 10 ml of the sugar extract was mixed with 90 ml of 3% TCA and left for 10 min at room temperature. The solution was then filtered through a Whatman filter paper No. 1 and the filtrate collected for further analysis. De-proteinization of the sugar extract resulted in a further 1:10 dilution of the extract.

(c) ***Colorimetric Determination***

In separate test tubes, 0.5 ml of sample and different concentrations of glucose (0-0.25 mg/ml; Appendix B) solutions was taken and 5 ml of anthrone reagent (Appendix A) added. The tubes were loosely capped and mixed briefly in a vortex mixer, followed by heating in a boiling water bath for 10 min. After cooling, absorbance of the reaction mixture was read at 625 nm in a UV-visible spectrophotometer.

(d) ***Calculation***

A calibration curve was prepared and regression equation was determined by plotting absorbance against glucose concentrations. The regression equation followed the following format (Equation 3.6):

$$Y = a + bX \quad \text{.....(3.6)}$$

where, Y is the absorbance units; a is the intercept of the calibration curve; b is the slope of the calibration curve; X is the concentration of standard

The values of 'a' and 'b' were found to be –0.013 and 4.4, respectively (Appendix C). Therefore, the concentration of TSS was calculated using the Equation (3.7).

$$X_{TSS} = \frac{Y + 0.013}{4.4} \quad \text{.....(3.7)}$$

where, X_{TSS} is the concentration of TSS as glucose equivalent (mg/ml); Y is the absorbance of the sample

The percentage of TSS in the corn kernel was calculated by Equation (3.8), and the results were expressed on a dry weight basis.

$$\text{TSS (\%, w/w)} = \frac{X_{TSS} \times V \times df}{dw} \times 100 \quad \text{.....(3.8)}$$

where, X_{TSS} is the concentration of TSS as glucose equivalent (mg/ml); V is the final volume of the sugar solution after extraction (ml); df is the dilution factor after de-proteinization of the sugar solution; dw is the initial dry weight of the corn sample (mg)

3.2.10.5 Determination of total reducing sugars (TRS)

The TRS was determined by the 3′, 5′-dinitrosalicylic acid (DNS) method (Miller, 1959). Sugar solution was prepared by hot alcohol extraction of the corn samples, followed by de-proteinization of the extract as described previously (Sections 3.2.10.4).

(a) ***Colorimetric assay***

In separate test tubes, 0.5 ml of sample and different concentrations of glucose (0-1.0 mg/ml; Appendix B) solutions were taken and mixed with 0.5 ml of DNS reagent (Appendix A). The tubes were heated in a boiling water bath for 10 min, followed by cooling in an ice water bath to room temperature. Thereafter, 5 ml of water was added to each tube including blank, and absorbance was read at 540 nm in a UV-visible spectrophotometer.

(b) ***Calculation***

A calibration curve was prepared by plotting absorbance against glucose concentrations to get a regression equation (Equation 3.6).

The values of 'a' and 'b' were found to be –0.012 and 1.27, respectively (Appendix C). Therefore, concentration of TRS was calculated using the Equation (3.9).

$$X_{TRS} = \frac{Y + 0.012}{1.27} \quad \text{.....(3.9)}$$

where, X_{TRS} is the concentration of TRS as glucose equivalent (mg/ml); Y is the absorbance of the sample.

The percentage of TRS was calculated by Equation (3.10), and results were expressed on a dry weight basis.

$$\text{TRS (\%, w/w)} = \frac{X_{TRS} \times V \times df}{dw} \times 100 \quad \text{.....(3.10)}$$

where, TRS is the total reducing sugars; X_{TRS} is the concentration of TRS as glucose equivalent (mg/ml); V is the final volume of the sugar solution after extraction (ml); df is the dilution factor after de-proteinization of the sugar solution; dw is the initial dry weight of the corn sample (mg).

3.2.10.6 Determination of Sucrose

The amount of sucrose in kernels was determined by the slightly modified method of Finley & Fellers (1973). The sugar solution was prepared by hot alcohol extraction of the corn samples, followed by de-proteinization of the extract as described earlier (Sections 3.2.10.4), taking 10 g of corn flour instead of 5 g and making the volume of the extract 50 ml instead of 100 ml.

(a) ***Colorimetric Assay***

One ml of each sample and standard (0-2.5 mg/ml; Appendix B) were taken in separate test tubes. In each tube, 9 ml of Fehling solution was added, loosely capped and mixed briefly in a vortex mixer. The tubes were heated in a boiling water bath for 15 min, followed by cooling in an ice water bath to room temperature. Thereafter, 0.5 ml of the aforementioned solution was transferred to a fresh tube and 5 ml of anthrone reagent (Appendix A) was added to it, and mixed briefly in a vortex mixer. The solution was heated in a boiling water bath for 10 min, and then cooled to room temperature in an ice water bath. Absorbance was read at 610 nm in a UV-visible spectrophotometer.

(b) ***Calculation***

A calibration curve was prepared by plotting absorbance against sucrose concentrations to get a regression equation (Equation 3.6).

The values of 'a' and 'b' were found to be 0.002 and 0.328, respectively (Appendix C). Therefore, concentration of sucrose was calculated using the Equation (3.11).

$$X_{SUC} = \frac{Y - 0.002}{0.328} \qquad (3.11)$$

where, X_{SUC} is the concentration of sucrose in the sample (mg/ml); Y is the absorbance of the sample.

The percentage of kernel sucrose was calculated by Equation (3.12), and the results were expressed on a dry weight basis.

$$\text{Sucrose (\%, w/w)} = \frac{X_{SUC} \times V \times df}{dw} \times 100 \qquad (3.12)$$

where, X_{SUC} is the concentration of sucrose in the sample (mg/ml); V is the final volume of the sugar solution after extraction (ml); df is the dilution factor after de-proteinization of the sugar solution; dw is the initial dry weight of the corn sample (mg).

3.2.10.7 Determination of Glucose

The glucose content in the kernels was determined by the o-Toluidine method (Dubowski, 1962). The sugar solution was prepared by hot alcohol extraction of the corn samples, followed by de-proteinization of the extract as described earlier (Sections 3.2.10.4), taking 10 g of corn flour instead of 5 g.

(a) ***Colorimetric Assay***

One ml of sample and standard (0-3.0 mg/ml; Appendix B) were taken in test tubes and 3.0 ml of o-Toluidine reagent was added to each tube (Appendix A). The solutions were mixed properly and heated in a boiling water bath for 10 min. Thereafter, tubes were removed from the water bath and cooled to room temperature in an ice water bath. Absorbance of the greenish color solution was then read at 630 nm in a UV-visible spectrophotometer.

(b) ***Calculation***

A calibration curve was prepared by plotting absorbance against glucose concentrations to get a regression equation (Equation 3.6).

The values of ‘a’ and ‘b’ were found to be –0.012 and 0.45, respectively (Appendix C). Therefore, concentration of glucose was calculated using the Equation (3.13).

$$X_{GLU} = \frac{Y + 0.012}{0.45} \quad \text{.....(3.13)}$$

where, X_{GLU} is the concentration of glucose in the sample (mg/ml); Y is the absorbance of the sample

The percentage of kernel glucose was calculated by Equation (3.14), and the results were expressed on a dry weight basis.

$$\text{Glucose (\%, w/w)} = \frac{X_{GLU} \times V \times df}{dw} \times 100 \quad \text{.....(3.14)}$$

where, X_{GLU} is the concentration of glucose in the sample (mg/ml); Y is the absorbance of the sample; df is the dilution factor after de-proteinization of the sugar solution; dw is the initial dry weight of the corn sample (mg).

3.2.10.8 Determination of Fructose

The amount of kernel fructose was determined by the anthrone method (Johnson et al., 1964). The sugar solution was prepared by hot alcohol extraction of the corn samples, followed by de-proteinization of the extract as described earlier (Sections 3.2.10.4), taking 10 g of corn flour instead of 5 g.

(a) ***Colorimetric Assay***

In separate test tubes, 0.5 ml of sample and standard (0-50 µg/ml; Appendix B) were taken and 5 ml of anthrone reagent (Appendix A) added to each tube. The contents of the tubes were thoroughly mixed and heated in a water bath for 20 min set at 50°C. The tubes were removed from the water bath and cooled to room temperature, wrapped in aluminium foil. Subsequently, absorbance was read at 620 nm in a UV-visible spectrophotometer.

(b) ***Calculation***

A calibration curve was prepared by plotting absorbance against fructose concentrations to get a regression equation (Equation 3.6).

The values of 'a' and 'b' were found to be 0.003 and 0.008, respectively (Appendix C). Therefore, concentration of fructose was calculated using the Equation (3.15).

$$X_{FRU} = \frac{Y - 0.003}{0.008} \quad \text{.....(3.15)}$$

where, X_{FRU} is the concentration of fructose (µg/ml); Y is the absorbance of the sample.

The percentage of kernel fructose was calculated by Equation (3.16), and the results were expressed on a dry weight.

$$\text{Fructose (\%, w/w)} = \frac{X_{FRU} \times V \times df}{dw} \times 100 \quad \text{.....(3.16)}$$

where, X_{FRU} is the concentration of fructose in the sample (µg/ml); V is the final volume of the sugar solution after extraction (ml); df is the dilution factor after de-proteinization of the sugar solution; dw is the initial dry weight of the corn sample (µg).

3.2.10.9 Determination of Starch

The starch content in the kernel was determined using the perchloric acid method as described by Rose et al. (1991).

(a) ***Starch Extraction***

Five grams of corn flour was mixed thoroughly with 20 ml of 80% ethanol and then heated in a boiling water bath for 30 min with occasional stirring. Thereafter, tubes were removed from the water bath, cooled and centrifuged at 3500 rpm for 10 min, and the supernatant discarded. To the remaining residue, 20 ml of 80% ethanol was added and the extraction process repeated discarding the supernatant each time.

(b) ***Perchloric Acid Hydrolysis of Starch***

To the residue obtained from sugar extraction described above, 7.5 ml of 35% perchloric acid was added and stirred with a glass rod for 5 min and thereafter, occasionally for 30 min. Then the aqueous starch solution was poured into a 100 ml volumetric flask. This step was repeated again, and starch solutions combined with the previously collected solution. Two additional rinses were done with 5 ml of 35% perchloric acid, each time collecting the liquid fraction. Therefore, the total volume of perchloric acid in the starch solution was 25 ml. The solution was filtered through Whatman No. 1 filter, rinsing the flasks and filter paper with distilled water at least two times. The filtrate was transferred to a 100 ml volumetric flask and the volume was adjusted to 100 ml with distilled water. From this solution, 10 ml was transferred to a fresh 100 ml volumetric flask and 90 ml distilled water was added to make a final volume of 100 ml and a dilution of 1:100.

(c) ***Colorimetric Assay for Glucose in the Hydrolysates***

In separate test tubes, 0.5 ml of sample and different concentrations of glucose (0-0.25 mg/ml; Appendix B) solutions was taken and 5 ml of anthrone reagent (Appendix A) added. The tubes were loosely capped and mixed briefly in a vortex mixer, followed by

heating in a boiling water bath for 10 min. After cooling, absorbance of the reaction mixture was read at 625 nm in a UV-visible spectrophotometer.

(a) ***Calculation***

A calibration curve was prepared by plotting absorbance against glucose concentrations to get a regression equation (Equation 3.6).

The values of 'a' and 'b' were found to be –0.013 and 4.4, respectively (Appendix C). Therefore, the concentration of TSS was calculated using the Equation (3.17).

$$X_{GLU} = \frac{Y + 0.013}{4.4} \quad \text{.....(3.17)}$$

where, X_{GLU} is the concentration of glucose in the sample (mg/ml); Y is the absorbance of the sample.

The amount of starch was calculated by Equation (3.18), and the results were expressed on a dry weight basis.

$$\text{Starch (\%, w/w)} = \frac{X_{GLU} \times V \times df \times hf}{dw} \times 100 \quad \text{.....(3.18)}$$

where, X_{GLU} is the concentration of glucose in the sample (mg/ml); V is the final volume of the sugar solution after extraction; df is the dilution factor after de-proteinization of the sugar solution (10); dw is the initial dry weight of the corn sample (mg); hf is the hydrolysis factor of starch = 0.9 (Volenec, 1986).

3.2.10.10 Protein Determination

(a) ***Extraction of Proteins***

Cereal grains contain mainly four groups of protein, such as albumin, globulin, prolamin and glutelin (Kumamaru et al., 1988). These proteins were extracted from corn kernels using the method of (Kumamaru et al., 1988). In a 15 ml centrifuge tube, 100 mg of corn meal was mixed with 1 ml of solvent−A (Appendix A) for extracting albumin and globulin. The suspension was then centrifuged at 3000 rpm for 10 min, and the supernatant collected. To the remaining residue, 1 ml of Solvent-B (Appendix A) was added and extraction was carried out following the same way to get prolamin protein. To the remaining residue, 1 ml of Solvent-C (Appendix A) was added and extraction was repeated as described above to get glutelin protein. After extraction centrifugation was carried out at 3000 rpm for 10 min, and the supernatants collected and combined. The volume of this solution was adjusted to 8 ml with distilled water and 66 µl of 2% Deoxycholic acid (DCA) was added to it. After 15 min, 2.6 ml of 24% Trichloroacetic acid (TCA) was added to it and left for overnight at 4^{o}C. Thereafter, it was centrifuged at 3000 rpm for 15 min and the supernatant discarded. To the precipitate, 2 ml of 1.0 M NaOH was added to solubilize protein and diluted up to 100 ml with distilled water, followed by the determination of total protein by the spectrophotometric method of Lowry et al. (1951).

(b) ***Colorimetric Determination of Protein***

In separate test tubes, 0.1 ml of the protein sample and different concentrations of standard (0-1.0 mg/ml, Appendix B) was taken and volume was made to 1.0 ml with distilled water. To each tube, 5.0 ml of reagent C (Appendix A) was taken and the volume adjusted to 1.0 ml with distilled water. To each tube, 5.0 ml of reagent C (Appendix A)

was added, thoroughly mixed and allowed to react for 10 minutes at room temperature. Subsequently 0.5 ml of reagent D (Appendix A) was added to each tube and mixed immediately in a vortex mixer. After an interval of 30 minutes at room temperature, absorbance was measured at 750 nm in a UV-visible spectrophotometer.

(c) ***Calculation***

A calibration curve was prepared by plotting absorbance against protein concentrations to get a regression equation (Equation 3.6).

The values of 'a' and 'b' were found to be 0.0 and 0.43, respectively (Appendix C). Therefore, concentration of protein in the extract was calculated using the Equation (3.19)

$$X_{PRO} = \frac{Y}{0.43} \quad \text{.....(3.19)}$$

where, X_{PRO} is the concentration of protein (mg/ml); Y is the absorbance of the sample.

The percentage of kernel protein was calculated by Equation (3.20), and the results were expressed on a dry weight basis.

$$\text{Protein (\%, w/w)} = \frac{X_{PRO} \times V \times df}{dw} \times 100 \quad \text{.....(3.20)}$$

where, X_{PRO} is the concentration of protein in the sample as BSA equivalent (mg/ml); V is the final volume of the protein solution (ml); df is the dilution factor after de-proteinization of the sugar solution; dw is the initial dry weight of the corn sample (mg).

3.2.10.11 Determination of Fat

Fat content in the kernels was determined gravimetrically using the method described by Phillips et al. (1997).

(a) ***Extraction and Fat Estimation***

Ten grams of corn flour was taken in a 250 ml borosilicate glass bottle. To the corn meal, 0.5 M sodium acetate (Appendix A), 80 ml methanol and 40 ml chloroform were added, and shaken in an orbital shaker for 2 h at 325 rpm. Thereafter, 40 ml of chloroform was added to it and shaken in an orbital shaker for 30 min at 300 rpm. Subsequently, 40 ml of distilled water was added to the suspension and shaken in an orbital shaker for 30 min at 275 rpm. It was then left at room temperature to equilibrate into different the solvent layers. In a 50 ml centrifuge tube, 30 ml of the chloroform layer (bottom most) was taken, centrifuged at 2500 rpm for 10 min. The centrifuge tube was then placed in a water bath at 25^{o}C to equilibrate for 15 min. A corex glass centrifuge tube (30 ml) was taken, placed in an oven at 105^{o}C for 30 min, cooled in a desiccator and weighed (W1). Thereafter, 15 ml of the extract (chloroform layer) was poured into it and evaporated to dryness in a water bath at 60^{o}C. The tube was then placed in an oven at 105^{o}C for 30 min, cooled in a desiccator, and weighed (W2).

(b) ***Calculation***

Total fat was calculated using the Equation (3.21).

$$\text{Total fat } (\%, \text{w/w}) = \frac{(W2 - W1) \times Vc \times 100}{Va \times SW} \quad \text{.....(3.21)}$$

where, W1 is the weight of the empty glass tube (g); W2 is the weight of the glass tube and dried extract (g); Vc is the total volume of the chloroform (ml) = 80 ml; W2 is the volume of the extract dried (ml) = 15 ml; SW is the weight of corn flour (g).

3.2.10.12 Determination of Fiber

The fiber content in corn kernels was determined by the enzymatic-gravimetric method (Asp et al., 1983).

(a) ***Methodology***

In a 500 ml Erlenmeyer flask, 15 g of corn meal was mixed with 200 ml of petroleum ether to remove fat from the sample. The suspension was agitated with a magnetic stirrer at room temperature for 15 min, and then allowed to settle down. The solvent was withdrawn with a pipette and the remaining sample residue air dried at room temperature. Thereafter, 10 g of air dried sample was mixed thoroughly with 50 ml of 0.1 M sodium phosphate buffer (Appendix A) in a 250 ml Erlenmeyer flask, and the pH adjusted to 6.0 with 2.0 M HCl or 5.0 M NaOH. To this suspension, 20 µl of SPECZYME FRED was added and heated in water bath at 90^{o}C for 90 min with occasional shaking. Then the pH of the suspension was adjusted to 4.0 and 20 µl of OPTDEX L 400 was added to it, followed by incubation in a water bath at 60^{o}C for 4 h with occasional shaking. Afterwards, the flask was removed from the water bath and 20 ml of distilled water was added to it. The pH of the suspension was adjusted to 4.0 and 10 µl of FERMGEN was added to it prior to incubating in a water bath at 40^{o}C for 1 h with an agitation rate of 150 rpm. The flask was removed from the water bath and 20 ml of distilled water added to it, and pH adjusted to 6.8. Next, 100 mg of PANCREATIN 4×NF was added to the suspension and incubated in a water bath at 40^{o}C for 1 h with an agitation rate of 150 rpm. The flask was removed from the bath and the contents transferred to a 500 ml Erlenmeyer flask. The pH of the suspension was adjusted to 4.5 and fiber was precipitated by adding 300 ml of warm (60^{o}C) ethanol (95%). The suspension was then filtered through a dry and weighed crucible (porosity 2) containing 0.5 g dry Celite as the filter aid. The filter was washed two times with 10 ml of 78% ethanol, two times with 10 ml of

95% ethanol and two times with 10 ml of acetone. Thereafter, the crucible plus sample was dried in an oven at 105^{o}C overnight and then cooled in a desiccator, and weighed (W1). Subsequently, the crucible plus sample was incinerated at 550^{o}C for 5 h and then cooled in a desiccator, followed by taking the weight (W2) again. A blank was run by taking 10 ml distilled water instead of the air dried and fat removed sample and treated in similar manner as described above.

(b) ***Calculation***

Kernel fiber was determined using the Equation (3.22).

$$\text{Fiber}(\%, \text{w/w}) = \frac{(\text{W1} - \text{W2} - \text{B}) \times 100}{\text{W}} \quad \text{.....(3.22)}$$

where, W is the weight of Sample taken (g); W1 is the weight of oven dried crucible plus sample (g); W2 is the weight of incinerated crucible plus sample (g); B is the weight of the ash free blank (g).

3.2.10.13 Determination of Ash

The ash content in corn kernels was determined gravimetrically using the method established in National Renewable Energy Laboratory (NREL), USA (Sluiter et al., 2005).

(a) ***Methodology***

Empty crucibles were placed in a muffled furnace and heated at 550^{o}C for 5 h. The crucibles were then removed from the furnace and cooled in a desiccator, followed by taking its weight (W1). Thereafter, 10 grams of the oven dried sample (dried at 105^{o}C for 5 h) was taken in the same crucible and heated to 550^{o}C for another 5 h. The sample plus

crucible was then removed from the furnace and cooled in a desiccator. Thereafter, weight was taken and recorded (W2).

(b) ***Calculation***

The ash content in the kernels was determined using the Equation (3.23).

$$\text{Ash } (\%, \text{w/w}) = \frac{(\text{W2} - \text{W1}) \times 100}{\text{W}} \quad \text{.....(3.23)}$$

where, W is the weight of the oven dried ample (g); W1 is the weight of the empty crucible (g); W2 is the weight of the crucible plus sample (g).

3.2.10.14 Isolation of Starch

Starch was isolated from the corn kernels using the methods described by Sandhu et al. (2004). To 100 g kernels was added 600 ml of water containing 0.16% sodium hydrogen sulphite ($NaHSO_3$) and kept for 12 h at 50 °C. The steep water was drained off, and the grains ground in a laboratory blender in equal volume of distilled water. The ground slurry was filtered through nylon cloth (100 mesh) and the filtrate allowed to stand for 2 h at room temperature. The cloudy supernatant was drained off, and the sediment was then steeped in 5–6 volumes of NaOH solution (0.2%) at room temperature for 12 h. The slurry was then passed through a 325-mesh sieve. The material left over on the sieve was discarded and the filtrate wasre-suspended in distilled water. The supernatant was then removed by suction. Starch was repeatedly washed with distilled water to remove all the alkali until the pH of the filtrate was between 6.0 and 6.5. The starch was then collected and dried in an oven at 40 °C for 24 h.

3.2.10.15 Determination of Amylose and Amylopectin in Starch

The amylose content in starch was determined by the spectrophotometric described in Williams et al. (1970). Amylopectin content was estimated by subtracting the percentage of amylose from 100.

(a) ***Colorimetric Determination of Amylose***

In separate 50 ml beakers, 20 mg of starch and different amounts of standard amylose (0-12 mg) were dissolved in 10 ml of 0.5 N KOH solution (Appendix A) by stirring with a magnetic stirrer for 5-10 min. The content of the beaker was then transferred to a 100 ml volumetric flask and diluted with distilled water up to 100 ml. From this solution, 10 ml was taken in a 50 ml volumetric flask and 5 ml of 0.1 N HCl added and mixed, followed by adding 0.5 ml of iodine reagent B (Appendix A) and adjusting the volume to 50 ml with water. The absorbance was then determined at 625 nm after 5 min.

(b) ***Calculation***

A calibration curve was prepared by plotting absorbance against different weights of amylose initially taken to get a regression equation (Equation 3.6).

The values of 'a' and 'b' were found to be 0.01 and 0.042, respectively (Appendix C). Therefore, the amylose content in the starch was calculated using the Equation (3.24):

$$X_{AMY} = \frac{Y - 0.01}{0.042} \quad \text{.....(3.24)}$$

where, X_{AMY} is the amylose content (mg); Y is the absorbance of the sample.

The percentage of kernel protein was calculated by the Equation (3.25), and the results were expressed on a dry weight basis.

$$\text{Amylose (\%, w/w)} = \frac{X_{AMY}}{dw} \times 100 \quad (3.25)$$

where, X_{AMY} is the amylose content in the starch sample (mg); dw is the initial dry weight of the starch sample (mg).

3.2.11 Statistical Analysis

Data were analyzed using a Minitab statistical software, version 16 (State college, PA, USA) to calculate the mean, standard deviation (SD), coefficient of variation (CV) for each attribute using the replications of the genotypes. Data were also tested for one way ANOVA (analysis of variance) to determine significant effects of genotypes on different attributes. Fisher's least significant differences (LSD) test was done for comparing the parameters within the corn genotypes if main effects were found significant at $P \leq 0.05$ after ANOVA test. To compare single genotypes between two cropping years for a single trait, student's t-test was done at 95% confidence level. Correlations among the variables were determined using Pearson's correlation coefficient. Minitab statistical software, version 16 (State college, PA, USA) was used for these analyses considering 5% level of significance ($P \leq 0.05$).

3.3 Results

3.3.1 Seed Quality

The major seed quality attributes of the corn genotypes are presented in Table 3.1. Seeds were mainly of three different visible colors, such as orange, yellow and white. The seeds of a corn genotype were similar in color over the two cropping years. Almost all of the seeds of HSGs appeared similar in color to their PFCs. The moisture content of the seeds was found to be very similar for each genotype, ranging from 12.21 to 12.76% in 2012 and 12.43 to 13.09% in 2013. The thousand seed weight of the corn genotypes

varied between 220.80 and 361.33 g in 2012, while the range was between 225.18 and 350.19 g in 2013. The maximum and minimum seed viability was found to be 75% and 95% in 2012, and 80% and 85% in 2013. Seed purity was recorded above 90% in all genotypes in both cropping years.

Table 3.1: Seed quality of the Corn Genotypes Determined before Plantation (Mean Value)

Genotypes	Year	Seed color	Moisture (%)	1000 seed weight (g)	Viability (%)	Purity (%)
UM.NF-1	2012	Moderate orange	12.34	257.01	75	95.87
	2013	Moderate orange	12.76	287.20	80	98.42
UM.NF-4	2012	Light orange	12.62	333.20	95	96.55
	2013	Light orange	13.09	283.51	85	95.47
UM.NF-6	2012	Dark orange	12.36	220.80	85	94.46
	2013	Dark orange	12.72	225.18	85	100.0
UM.NF-11	2012	White	12.76	361.33	95	93.98
	2013	White	12.73	350.19	85	97.32
PFC-1	2012	Yellow	12.30	285.23	85	98.19
	2013	yellow	12.75	276.17	80	96.84
PFC-4	2012	Yellow	12.58	275.96	80	93.92
	2013	Yellow	12.81	291.26	85	89.56
PFC-6	2012	Moderate orange	12.39	255.95	85	96.58
	2013	Moderate orange	12.51	235.25	80	96.31
PFC-11	2012	White	12.21	329.37	75	97.01
	2013	White	12.43	339.70	85	98.29

3.3.2 Soil Analysis

Soil samples from the experimental field were analyzed in both cropping years prior to sowing the seeds. The results for the different soil parameters denoting the quality of soil are summarized in Table 3.2. The low pH of the soil samples revealed the slight acidic nature of the field. The average moisture content in the soil samples were found to be 16.36% in 2012 and 14.52% in 2013. Small amounts of TOM and TOC were found in

both years, ranging from 2.86 to 3.15% and 1.04 to 1.29%, for 2012 and 2013 respectively. Good amounts of NPK were present in the soil samples (Table 3.2).

Table 3.2: Physicochemical Properties of Soil (Mean Values)

Parameter	2012	2013
Moisture (%, w/w)	16.36	14.52
pH	5.42	5.83
TOM (%, w/w)	2.86	3.15
TOC (%, w/w)	1.04	1.29
Total P (mg/kg)	96.48	87.48
Available P (mg/kg)	56.39	61.48
Total N (mg/kg)	856.26	976.43
Total K (mg/kg)	574.28	546.62

3.3.3 Environmental Conditions

The monthly data collected from the Malaysian Metrological Department (MetMalaysis) over the experimental period, are presented in Table 3.3. An almost similar temperature was recorded throughout the experimental period in both years. There was a good amount rainfall in each month that resulted in a higher humidity for the environment.

Table 3.3: Climatic Condition during Experimental Period (Monthly ean)

Months	Year	Temperature (^{o}C)	Humidity (%)	Rainfall (mm)
September	2012	28.4	75.3	141.0
October	2012	27.7	79.9	459.0
November	2012	27.2	83.7	684.0
December	2012	27.1	83.5	455.2
August	2013	28.5	71.3	189.8
September	2013	27.7	74.7	249.4
October	2013	27.9	75.5	341.2
November	2013	27.1	81.7	289.8

Source: Malaysian Metrological Department (MetMalaysia), Ministry of Science, Technology and Innovation (MOSTI)

3.3.4 Agronomic Traits

The plant growth was found to be optimal throughout the study without any significant incidents, such as diseases or attack by pests, until harvesting time (Figure 3.1 and 3.2).

A good numbers of kernels were observed in each ear, in different sizes and colors (Figure 3.3). However, the color of the kernels were shown here only to present the difference in kernel colors and it does not indicate any effect on ethanol yield. Likewise, the ground corn kernels showed good physical appearances under different particle sizes (Figure 3.4). The agronomic characters recorded over the two years are summarized in Table 3.4 and 3.5. Although the agronomic traits of the corn genotypes varied slightly between the two cropping years (2012 and 2013), the F-test analysis revealed that the effect of year on these parameters was insignificant in most cases as the *P* values were greater than 0.05. However, individual genotypes showed significant variation in different traits between the two cropping years ($P < 0.05$).

Figure 3.1: A portion of the Corn Plants in the Experimental Field Grown in 2012 (up) and 2013 (down)

Figure 3.2: Pictorial Illustration of Harvesting and Data Collection of Corn Genotypes

Figure 3.3: Corn Kernels with Three Different Colors

Figure 3.4: Ground Corn with Different Particle Sizes

As shown in Table 3.4, there were no significant variation in the average time required for seed emergence time (SET) among the corn genotypes in 2012, which ranged between 6.5 and 7.0 d. However, the duration was found to be slightly longer in the 2013 cropping year and varied between 6.25 and 8.75 d on average (Table 3.5). The duration needed to develop 4 and 8 leaves in the plants (V4 and V8, respectively) varied among the corn genotypes.

Table 3.4: Mean Values of Different Agronomic Traits of the Corn Genotypes Grown in 2012

Parameters	UM.NF-1	UM.NF-4	UM.NF-6	UM.NF-11	PFC-1	PFC-4	PFC-6	PFC-11
SET (d)	6.50 ^{a}	6.75 ^{a}	7.00 ^{a}	6.75 ^{a}	7.00 ^{a}	6.75 ^{a}	6.25 ^{a}	6.50 ^{a}
FLD (d)	17.50 ^{d}	20.25 ^{abcd}	21.50 ^{abc}	19.25 ^{bcd}	22.25 ^{ab}	21.00 ^{abcd}	23.75 ^{a}	18.25 ^{cd}
ELD (d)	28.75 ^{e}	34.75 ^{bc}	36.25 ^{abc}	33.75 ^{cd}	37.00 ^{ab}	35.75 ^{bc}	38.75 ^{a}	31.25 ^{de}
AT (d)	54.00 ^{d}	57.75 ^{bc}	60.50 ^{a}	56.25 ^{cd}	61.50 ^{a}	59.75 ^{ab}	62.00 ^{a}	55.50 ^{cd}
ST (d)	59.75 ^{e}	62.50 ^{cd}	64.25 ^{bc}	60.25 ^{de}	66.25 ^{ab}	65.50 ^{ab}	67.25 ^{a}	59.25 ^{e}
GFP (d)	21.75 ^{e}	29.75 ^{bc}	30.25 ^{b}	27.00 ^{cd}	31.50 ^{ab}	32.50 ^{ab}	34.25 ^{a}	24.25 ^{de}
BLM (d)	81.50 ^{e}	92.25 ^{c}	94.50 ^{bc}	87.25 ^{d}	97.75 ^{ab}	98.00 ^{ab}	101.50 ^{a}	83.50 ^{de}
PH (cm)	119.11 ^{d}	132.98 ^{cd}	142.17 ^{c}	125.84 ^{d}	163.22 ^{b}	165.02 ^{b}	181.61 ^{a}	123.14 ^{d}
LNP	18.49 ^{a}	13.16 ^{cd}	15.75 ^{abc}	16.62 ^{ab}	14.11 ^{bcd}	12.71 ^{d}	13.66 ^{cd}	17.60 ^{a}
LL (cm)	82.69 ^{cd}	77.79 ^{e}	80.50 ^{de}	93.29 ^{a}	89.49 ^{ab}	79.63 ^{de}	92.88 ^{a}	85.98 ^{bc}
ENP	1.13 ^{ab}	1.28 ^{ab}	1.20 ^{ab}	1.30 ^{a}	1.08 ^{ab}	0.95 ^{b}	1.35 ^{a}	1.08 ^{ab}
EW (g)	241.07 ^{d}	261.19 ^{c}	279.42 ^{bc}	277.22 ^{bc}	317.11 ^{a}	280.94 ^{b}	294.07 ^{b}	230.81 ^{d}
EL (cm)	24.39 ^{a}	19.41 ^{b}	19.54 ^{b}	22.59 ^{ab}	21.43 ^{ab}	23.92 ^{a}	23.47 ^{ab}	24.71 ^{bc}
KD (cm)	0.81 ^{bc}	0.83 ^{bc}	0.84 ^{abc}	1.01 ^{a}	0.88 ^{ab}	0.68 ^{c}	0.80 ^{bc}	0.72 ^{a}
KNP	437.89 ^{ab}	376.51 ^{bc}	507.84 ^{a}	334.32 ^{c}	366.69 ^{bc}	301.34 ^{c}	503.69 ^{a}	287.65 ^{c}
TKW (g)	259.85 ^{cd}	306.70 ^{b}	221.41 ^{e}	336.04 ^{a}	259.77 ^{cd}	267.72 ^{c}	252.40 ^{d}	310.16 ^{b}
PY (t/ha)	8.04 ^{bc}	9.96 ^{abc}	9.97 ^{abc}	10.47 ^{ab}	10.05 ^{ab}	7.94 ^{bc}	11.67 ^{a}	7.43 ^{c}
GY	7.17 ^{cd}	9.43 ^{abc}	8.74 ^{bcd}	10.20 ^{ab}	9.14 ^{abc}	7.31 ^{cd}	11.28 ^{a}	6.36 ^{d}

Legends: AT, Anthesis time; BLM, black layer maturity; EL, ear length; ELD, eight leaves development; ENP, ear number per plant; EW, ear weight; FLD, four leaves development; GFP, grain filling period; GY, grain yield; KD, kernel depth; KNP, kernel number per plant; LL, leaf length; LNP, leaves number per plant; PH, plant height; PY, potential yield; SET, Seed emergence time ; ST, silking time; TKW, thousand kernel weight

Values followed by the same small letters in a row denotes insignificant difference in mean (P>0.05)

Table 3.5: Mean Values of Different Agronomic Traits of the Corn Genotypes Grown in 2013

Parameters	UM.NF-1	UM.NF-4	UM.NF-6	UM.NF-11	PFC-1	PFC-4	PFC-6	PFC-11
SET (d)	7.25 ^{abc}	8.00 ^{ab}	6.25 ^{c}	8.75 ^{a}	6.50 ^{bc}	7.50 ^{abc}	7.75 ^{abc}	7.00 ^{bc}
FLD (d)	19.25 ^{cd}	23.25 ^{ab}	18.25 ^{d}	23.50 ^{ab}	19.25 ^{cd}	23.00 ^{ab}	25.25 ^{a}	21.00 ^{bc}
ELD (d)	32.75 ^{de}	37.25 ^{bc}	31.75 ^{e}	38.75 ^{ab}	35.25 ^{cd}	39.00 ^{ab}	40.75 ^{a}	35.25 ^{cd}
AT (d)	57.25 ^{d}	60.75 ^{bc}	57.25 ^{d}	62.50 ^{ab}	56.75 ^{d}	62.75 ^{ab}	64.25 ^{a}	58.25 ^{cd}
ST (d)	63.50 ^{bc}	65.50 ^{ab}	59.50 ^{d}	65.00 ^{ab}	61.50 ^{cd}	65.50 ^{ab}	67.00 ^{a}	62.50 ^{c}
GFP (d)	25.50 ^{d}	32.00 ^{a}	26.50 ^{d}	30.75 ^{cd}	28.50 ^{bc}	34.75 ^{a}	33.25 ^{bc}	27.75 ^{cd}
BLM (d)	89.00 ^{bc}	97.50 ^{a}	86.00 ^{c}	95.75 ^{a}	90.00 ^{b}	97.25 ^{a}	96.00 ^{a}	90.25 ^{b}
PH (cm)	126.46 ^{d}	139.34 ^{c}	131.91 ^{cd}	129.21 ^{cd}	154.51 ^{ab}	149.91 ^{b}	164.47 ^{a}	135.33 ^{cd}
LNP	15.89 ^{bc}	17.42 ^{ab}	13.92 ^{cd}	19.78 ^{a}	12.59 ^{d}	16.39 ^{bc}	15.39 ^{bcd}	14.36 ^{cd}
LL (cm)	87.90 ^{abc}	72.68 ^{e}	76.99 ^{de}	86.49 ^{abc}	90.79 ^{ab}	94.94 ^{a}	80.21 ^{cde}	83.37 ^{a}
ENP	1.05 ^{a}	1.18 ^{a}	1.15 ^{a}	1.05 ^{a}	1.10 ^{a}	1.25 ^{a}	1.10 ^{a}	1.23 ^{a}
EW (g)	241.11 ^{e}	305.81 ^{a}	268.61 ^{cd}	313.95 ^{a}	288.34^{abc}	295.50 ^{ab}	258.46 ^{de}	272.36^{bcd}
EL (cm)	19.57 ^{bc}	25.92 ^{abc}	22.39 ^{bc}	27.15 ^{ab}	18.68 ^{bc}	18.32 ^{c}	27.50 ^{ab}	31.62 ^{a}
KD (cm)	0.82 ^{a}	0.77 ^{ab}	0.85 ^{a}	0.82 ^{a}	0.81 ^{ab}	0.67 ^{b}	0.72 ^{ab}	0.73 ^{ab}
KNP	291.35 ^{b}	358.36 ^{b}	338.16 ^{ab}	332.53 ^{ab}	288.64 ^{ab}	340.41 ^{ab}	317.40 ^{ab}	380.31 ^{a}
TKW (g)	261.60 ^{b}	310.40 ^{b}	247.95 ^{e}	346.67 ^{a}	286.99 ^{c}	268.19 ^{d}	221.12 ^{f}	306.88 ^{b}
PY (t/ha)	7.70 ^{b}	10.86 ^{a}	9.13 ^{ab}	9.65 ^{ab}	9.22 ^{ab}	10.90 ^{a}	8.62 ^{ab}	9.80 ^{ab}
GY (t/ha)	6.82 ^{c}	10.40 ^{a}	8.44 ^{abc}	9.02 ^{abc}	8.61 ^{abc}	9.49 ^{ab}	7.37 ^{bc}	8.76 ^{abc}

Legends: AT, Anthesis time; BLM, black layer maturity; EL, ear length; ELD, eight leaves development; ENP, ear number per plant; EW, ear weight; FLD, four leaves development; GFP, grain filling period; GY, grain yield; KD, kernel depth; KNP, kernel number per plant; LL, leaf length; LNP, leaves number per plant; PH, plant height; PY, potential yield; SET, Seed emergence time ; ST, silking time; TKW, thousand kernel weight

Values followed by the same small letters in a row denotes insignificant difference in mean (P>0.05)

The average anthesis time (AT) ranged from 54.0 to 60.5 d in 2012 and 57.25 to 62.5 d in 2013 among HSGs (Table 3.4 and 3.5), which did not vary significantly between the cropping years (P>0.05). Likewise, AT in PFCs did not vary significantly in the two years of planting, even though the ranges of AT in this group of genotypes were slightly higher than those in the HSGs. However, there was significant difference in AT between 2012 and 2013 cropping years when a single genotype was considered ($P < 0.05$). As expected, silking time (ST) was found to be higher than AT in all genotypes over the cropping years (Table 3.4 and 3.5).

In 2012, the minimum and maximum mean values for grain filling periods (GFP) were found to be 21.75 d in HSGs and 34.25 d in PFCs, respectively. Likewise, the lowest GFP was observed among HSGs in 2013, whilst the highest GFP was recorded among PFCs in the same year. The highest and lowest black layer maturity (BLM) was observed in PFCs and HSGs, respectively in both 2012 and 2013 (Table 3.4 and 3.5).

Plant height (PH), leaf number per plant (LNP) and leaf length (LL) are the major morphological traits of the corn plants, and these varied significantly among the corn genotypes over the cropping years. In both years, the lowest plant height (PH) was found in UM.NF-1, accounting for 119.11 cm in 2012 and 126.46 cm in 2013. On the other hand, PFC-6 showed the highest PH in the two years. In 2012, the average leaf number per plant (LNP) varied between 13.16 in UM.NF-4 and 18.49 in UM.NF-1 among the eight genotypes, whilst PFC-1 and UM.NF-11 recorded the lowest (12.59) and highest (19.78) LNP in 2013. The average leaf length (LL) ranged from 72.68 cm in UM.NF-4 to 94.94 cm in PFC-4 among the genotypes over two years.

With regard to kernel yield and yield related components, ear numbers per plant (ENP) was found to be almost similar in all genotypes and there was no significant difference in ENP among the genotypes (P>0.05) (Tables 3.4 and 3.5). The maximum ear weight (EW) was found to be 317.11 g in PFC-1 in 2012 and 313.95 g in UM.NF-11 in 2013. Like other agronomic traits, the yield related parameters, such as ear length (EL), kernel depth (KD), kernel number per plant (KNP) and thousand kernel weight (TKW) did not show any exclusive increase or decrease between two groups of genotypes. Similarly, grain yield (GY) did not differ significantly in most of the genotypes, regardless of the group of genotypes. In 2012, the average grain yields varied from 7.17 in UM.NF-1 to 10.20 t/ha in UM.NF-11 among HSGs, while amongst the PFCs it ranged from 6.36 t/ha in PFC-11 to 11.28 t/ha in PFC-6.

The group mean, standard error mean (SE), coefficient of variation (CV) and F-test analysis for the agronomic traits of two groups of genotypes are summarized in Table 3.6. It was observed that all the attributes varied with regard to the traits and genotypic groups. In most cases, the F-test analysis revealed that the group means differed significantly between HSG and PFC (P < 0.05). However, SET, ENP, PY and GY did not differ significantly between two groups of genotypes (P>0.05). The highest CV value observed was 32.58 among the PFCs for EL in 2013, while the lowest CV recorded was 4.17 among HSGs for ST in 2012.

Table 3.6: Statistical Comparison of the Corn Genotypes on the Agronomic Traits

Traits	Groups	2012				2013			
		Mean	SE	CV (%)	F-test	Mean	SE	CV (%)	F-test
SET (d)	Among HSGs	6.88	0.18	10.46	1.64	7.56	0.39	20.47	2.46
	Among PFCs	6.50	0.20	12.56	1.33	7.19	0.25	13.65	1.37
	Between groups	6.69	0.14	11.67	1.90	7.38	0.23	17.48	0.67
FLD (d)	Among HSGs	19.63	0.53	10.83	4.09*	21.06	0.77	14.55	6.59*
	Among PFCs	21.31	0.65	12.20	7.19*	22.13	0.66	11.89	13.66**
	Between groups	20.46	0.44	12.16	4.04	21.59	0.51	13.25	1.11
ELD (d)	Among HSGs	33.38	0.86	10.26	10.35**	35.13	0.88	10.07	11.33**
	Among PFCs	35.69	0.80	8.84	18.77**	37.56	0.78	8.36	6.52*
	Between groups	34.53	0.61	9.98	3.94	36.34	0.62	9.67	4.25*
TT (d)	Among HSGs	57.13	0.75	5.27	7.68**	59.44	0.78	5.25	5.23*
	Among PFCs	59.69	0.76	5.11	12.05**	60.50	0.95	6.27	9.71**
	Between groups	58.41	0.57	5.57	5.73*	59.97	0.61	5.77	0.75
ST (d)	Among HSGs	61.69	0.64	4.17	4.42*	63.38	0.76	4.78	7.24**
	Among PFCs	64.56	0.88	5.48	20.06**	64.13	0.65	4.02	15.00**
	Between groups	63.13	0.60	5.35	6.90*	63.75	0.49	4.38	0.57
GFP (d)	Among HSGs	27.19	0.98	14.48	14.50**	28.69	0.81	11.22	13.89**
	Among PFCs	30.63	1.07	13.95	22.39**	29.25	0.56	7.64	3.79*
	Between groups	28.91	0.78	15.23	5.60	28.97	0.48	9.46	0.33
BLM (d)	Among HSGs	88.88	1.50	6.75	11.48**	92.06	1.40	6.09	12.56**
	Among PFCs	95.19	1.87	7.87	38.79**	95.19	1.46	6.15	13.38**
	Between groups	92.03	1.31	8.05	6.92*	92.71	0.85	5.16	2.38
PH (cm)	Among HSGs	130.02	3.15	9.70	3.87*	131.73	1.94	5.89	2.75
	Among PFCs	158.25	5.91	14.94	30.00**	151.05	3.20	8.48	10.04**
	Between groups	144.03	4.16	16.31	17.76**	141.39	2.53	10.13	26.62**
LNP	Among HSGs	16.01	0.69	17.23	4.27*	16.75	0.69	16.41	7.42**
	Among PFCs	14.52	0.62	17.19	5.65*	14.68	0.62	16.94	2.06
	Between groups	15.26	0.48	17.66	2.56	15.71	0.49	17.72	4.99*
LL (cm)	Among HSGs	83.57	1.66	7.93	20.66**	81.01	1.98	9.76	9.13**
	Among PFCs	87.00	1.41	6.47	16.79**	87.33	2.12	9.71	4.09*
	Between groups	85.28	1.11	7.38	2.49	84.17	1.53	10.31	4.75*
ENP	Among HSGs	1.23	0.06	18.50	0.43	1.11	0.05	17.92	0.39
	Among PFCs	1.11	0.05	21.49	2.68	1.17	0.05	15.53	0.74
	Between groups	1.17	0.04	20.21	1.87	1.14	0.03	16.67	0.87
EW (g)	Among HSGs	264.72	5.01	7.57	6.72*	282.37	8.34	11.81	18.74**
	Among PFCs	280.73	8.56	12.19	39.96**	278.66	5.80	8.33	2.76
	Between groups	272.73	5.09	10.55	2.61	280.52	5.01	10.10	0.13
EL (cm)	Among HSGs	21.48	0.94	17.45	2.03	23.76	1.61	27.13	1.19
	Among PFCs	23.38	0.67	10.71	1.33	24.03	1.96	32.58	5.29*
	Between groups	22.43	0.58	14.62	2.84	23.89	1.25	29.53	0.01
KD (cm)	Among HSGs	0.87	0.04	19.47	1.34	0.82	0.03	12.98	0.27
	Among PFCs	0.77	0.02	12.11	11.01**	0.73	0.02	12.63	1.57
	Between groups	0.82	0.03	17.55	4.32*	0.77	0.02	13.76	5.60*

Table 3.6 (Continued)

KNP	Among HSGs	414.10	24.50	23.63	3.85*	330.10	15.50	18.74	0.79
	Among PFCs	364.80	26.00	28.47	10.48**	331.70	15.70	18.88	1.76
	Between groups	389.50	18.10	26.28	1.91	330.90	10.80	18.50	0.01
TKW (g)	Among HSGs	281.00	11.50	16.32	154.86**	301.86	9.43	12.50	56.95**
	Among PFCs	272.51	5.92	8.68	85.94**	270.79	8.46	12.50	61.99**
	Between groups	276.75	6.39	13.06	0.432	286.33	6.83	13.49	6.01*
PY (t/ha)	Among HSGs	9.61	0.46	19.04	1.52	9.33	0.56	25.38	1.79
	Among PFCs	9.27	0.59	25.64	4.73*	9.64	0.46	0.38	1.18
	Between groups	9.44	0.37	22.18	0.20	9.49	0.34	20.48	0.19
GY (t/ha)	Among HSGs	8.89	0.45	20.44	2.70	8.67	0.55	25.38	2.26
	Among PFCs	8.52	0.62	28.90	6.56*	8.56	0.38	17.61	1.50
	Between groups	8.70	0.38	24.55	0.23	8.61	0.33	21.55	0.03

Legends: AT, Anthesis time; BLM, black layer maturity; CV, coefficient of variation; EL, ear length; ELD, eight leaves development; ENP, ear number per plant; EW, ear weight; FLD, four leaves development; GRF, grain filling period; GY, grain yield; KD, kernel depth; KNP, kernel number per plant; LL, leaf length; LNP, leaves number per plant; PH, plant height ; PY, potential yield; SE, standard error; SET, Seed emergence time; ST, silking time; TKW, thousand kernel weight

*, significant at 5% probability level (P<0.05)

**, significant at 1% probability level (P<0.01)

3.3.5 Biochemical Composition of the Kernels

The dry matter content did not vary significantly among the genotypes, which were almost consistent over the cropping years (Table 3.7 and 3.8). Irrespective of the cropping years, significantly higher amounts of sugars were obtained in HSGs, which had lower amounts of starch. On the contrary, PFCs contained lower amounts of sugars and higher amounts of starch in the kernels. .

Table 3.7: Mean Values of the Biochemical Composition of Corn Kernels from Eight Genotypes grown in 2012

Parameters	UM.NF-1	UM.NF-4	UM.NF-6	UM.NF-11	PFC-1	PFC-4	PFC-6	PFC-11
Dry matter	84.66 ^{ab}	84.41 ^{ab}	84.16 ^{b}	85.23 ^{a}	84.96 ^{ab}	85.14 ^{a}	85.03 ^{ab}	84.86 ^{ab}
Sucrose	3.86 ^{a}	4.05 ^{a}	4.59 ^{a}	2.84 ^{b}	0.79 ^{c}	0.57 ^{c}	0.44 ^{c}	0.91 ^{c}
Glucose	0.61 ^{ab}	0.80 ^{a}	0.54 ^{b}	0.48 ^{b}	0.17 ^{c}	0.10 ^{c}	0.06 ^{c}	0.07 ^{c}
Fructose	0.48 ^{ab}	0.28 ^{bc}	0.66 ^{a}	0.26 ^{bc}	0.10 ^{c}	0.08 ^{c}	0.07 ^{c}	0.10 ^{c}
TRS	1.53 ^{ab}	1.66 ^{ab}	1.77 ^{a}	1.39 ^{b}	0.38 ^{cd}	0.25 ^{d}	0.24 ^{d}	0.55 ^{c}
TSS	5.15 ^{bc}	5.74 ^{b}	6.72 ^{a}	4.43 ^{c}	1.36 ^{d}	0.84 ^{d}	0.76 ^{d}	1.14 ^{d}
Starch	69.39 ^{abc}	63.14 ^{d}	65.40 ^{cd}	69.06 ^{abc}	70.26 ^{ab}	67.61 ^{bc}	71.45 ^{ab}	72.08 ^{a}
Protein	9.75 ^{bc}	13.09 ^{a}	11.62 ^{ab}	12.02 ^{a}	8.41 ^{c}	11.29 ^{ab}	7.73 ^{c}	9.58 ^{bc}
Fat	5.33 ^{cd}	7.16 ^{a}	6.08 ^{bc}	4.95 ^{de}	5.60 ^{bcd}	6.53 ^{ab}	5.07 ^{cde}	4.14 ^{e}
Fiber	11.16 ^{ab}	8.71 ^{c}	11.69 ^{a}	8.07 ^{c}	9.62 ^{bc}	10.72 ^{ab}	11.41 ^{a}	8.47 ^{c}
Ash	2.19 ^{ab}	3.15 ^{a}	1.82 ^{b}	1.29 ^{b}	1.34 ^{b}	1.48 ^{b}	1.31 ^{b}	1.30 ^{b}

Values followed by the same small letters in a row denotes insignificant difference in mean P>0.05)

Table 3.8: Mean Values of the Biochemical Composition of Corn Kernels from Eight Genotypes grown in 2013

Parameters	UM.NF-1	UM.NF-4	UM.NF-6	UM.NF-11	PFC-1	PFC-4	PFC-6	PFC-11
Dry matter	85.20 ^{ab}	85.30 ^{ab}	85.09 ^{ab}	85.14 ^{ab}	85.65 ^{a}	84.90 ^{b}	84.89 ^{b}	84.8 ^{b}
Sucrose	4.08 ^{a}	4.87 ^{a}	5.64 ^{a}	3.22 ^{b}	0.89 ^{c}	0.65 ^{c}	0.47 ^{c}	0.96 ^{c}
Glucose	0.54 ^{b}	0.73 ^{ab}	0.87 ^{a}	0.50 ^{b}	0.18 ^{c}	0.12 ^{c}	0.13 ^{c}	0.12 ^{c}
Fructose	0.38 ^{b}	0.61 ^{a}	0.67 ^{a}	0.53 ^{ab}	0.11 ^{c}	0.08 ^{c}	0.11 ^{c}	0.06 ^{c}
TRS	1.49 ^{a}	1.32 ^{ab}	1.21 ^{ab}	1.15 ^{b}	0.53 ^{c}	0.42 ^{c}	0.57 ^{c}	0.66 ^{c}
TSS	5.51 ^{c}	6.24 ^{b}	7.38 ^{a}	4.73 ^{d}	1.27 ^{e}	0.97 ^{e}	0.84 ^{e}	1.28 ^{e}
Starch	68.50 ^{de}	65.51 ^{f}	67.10 ^{ef}	70.36 ^{cd}	72.0 ^{bc}	68.51^{de}	73.12^{ab}	74.06 ^{a}
Protein	10.26 ^{bc}	12.22 ^{a}	9.71 ^{bcd}	8.59 ^{d}	9.32 ^{cd}	12.23 ^{a}	10.3 ^{bc}	10.63 ^{b}
Fat	4.25 ^{e}	4.90 ^{de}	5.04 ^{cde}	5.47 ^{bcd}	6.25 ^{ab}	7.22 ^{a}	5.3 ^{bcde}	6.22 ^{abc}
Fiber	10.20 ^{a}	9.14 ^{ab}	8.82 ^{ab}	9.15 ^{ab}	10.28 a	9.28 ^{ab}	8.87 ^{ab}	8.17 ^{b}
Ash	1.73 ^{bc}	2.39 ^{ab}	2.76 ^{a}	1.35 ^{c}	1.11 ^{c}	1.48 ^{c}	1.45 ^{c}	1.26 ^{c}

Values followed by the same small letters in a row denotes insignificant difference in mean P>0.05)

In 2012, the average TSS content in HSGs varied between 4.43% in UM.NF-11 to 6.72% in UM.NF-6, while in PFCs it ranged from 0.76% to 1.36% (Table 3.7). The ratio of total sugar content between HSG and the respective PFC was found to be 3.79 in UM.NF-1, 6.83

in UM.NF-4, 8.84 in UM.NF-6 and 3.88 in UM.NF-11. Similarly, higher amounts of individual sugars, such as sucrose, glucose and fructose, and total reducing sugars (TRS) were recorded in HSGs compared to the PFCs. Among the individual sugars, sucrose was much higher than the other sugars (glucose and fructose) in all the genotypes, particularly the HSGs, accounting for 3.86% in UM.NF-1, 4.05% in UM.NF-4, 4.59% in UM.NF-6 and 2.84% in UM.NF-11 (Table 3.7). Interestingly, TRS in HSGs was found to be higher than the sum of their glucose and fructose (two reducing sugars) content.

The F-test analysis of variance revealed significant variations in TSS, TRS, individual sugars between the HSGs and the respective PFCs ($P < 0.001$). Furthermore, HSGs showed significant variation in all the sugar content themselves ($P < 0.05$), while the PFCs contained not only smaller amounts of sugars but also showed insignificant variations in their kernel sugars ($P > 0.05$), indicating the fact that they could not be distinguished based on sugar content alone. Unlike the kernel sugars, HSGs contained significantly lower amounts of starch compared to their respective PFCs ($P < 0.05$). The average starch content in HSGs ranged from 63.14% to 69.39%, whilst in the PFCs it varied between 67.61% and 72.08% (Table 3.7). The starch contents of the four HSGs were very similar to each other, showing no significant variations among them ($p > 0.05$). Likewise, the PFCs also did not show any significant variation in their starch content, with the exception of PFC-4 and PFC-11. The protein, fat, fiber and ash contents in the kernels varied among the HSGs or PFCs genotypes (Table 3.7).

As in 2012, the biochemical traits showed a similar trend in 2013 (Table 3.8). However, there were slight increases in all the sugars during this time. More importantly, the order of HSGs, based on their sugar content, was found to be similar for both cropping years. The

highest sugar containing genotype was UM.NF-6, while UM.NF-11 contained the lowest amount of sugar among the HSGs. With regard to the sugar content in HSGs, the starch content in PFCs increased during 2013 (Table 3.8). The highest starch containing genotype was PFC-11, which contained 72.08% in 2012 and 74.06% in 2013, while UM.NF-4 had the lowest starch in both years (Table 3.7 and 3.8).

The amylose and amylopectin content in the starch were determined in all the genotypes and the results are presented in Table 3.9. The HSGs did not differ significantly from their PFCs in amylose content as well as amylose to amylopectin ratios, except for UM.NF-4 which contained significantly lower amounts of amylose. Compared to the respective PFCs, a slight increase was observed in amylose content in UM.NF-1, while three other HSGs showed small decreases in 2012. Likewise, amylose content in UM.NF-4 was lower than that of its PFC in 2013. Since amylopectin and the ratio of amylose to amylopectin are directly related to the amylose content in starch, they showed a similar trend with regard to the latter in both years.

Table 3.9: Starch Composition of the Corn Genotypes

Genotypes	Amylose (AL)		Amylopectin (AM)		AL to AM ratio	
	2012	2013	2012	2013	2012	2013
UM.NF-1	24.76 ^{a}	27.28 ^{a}	75.25 ^{c}	72.72 ^{c}	0.33 ^{a}	0.38 ^{a}
UM.NF-4	19.92 ^{c}	21.40 ^{c}	80.09 ^{a}	78.60 ^{a}	0.25 ^{c}	0.27 ^{c}
UM.NF-6	23.56 ^{ab}	20.59 ^{c}	76.44 ^{bc}	79.41 ^{a}	0.31 ^{ab}	0.26 ^{c}
UM.NF-11	24.79 ^{a}	26.16 ^{ab}	75.22 ^{c}	73.84 ^{bc}	0.33 ^{a}	0.36 ^{ab}
PFC-1	24.28 ^{ab}	23.17 ^{bc}	75.72 ^{bc}	76.84 ^{ab}	0.32 ^{ab}	0.30 ^{bc}
PFC-4	26.49 ^{a}	24.10 ^{abc}	73.52 ^{c}	75.90 ^{abc}	0.36 ^{a}	0.32 ^{abc}
PFC-6	21.06 ^{bc}	22.40 ^{bc}	78.95 ^{ab}	77.61 ^{ab}	0.27 ^{bc}	0.29 ^{bc}
PFC-11	23.82 ^{ab}	25.56 ^{ab}	76.19 ^{bc}	74.44 ^{bc}	0.31 ^{ab}	0.34 ^{ab}

Notes: Values followed by the same small letters in a row denotes insignificant difference in mean P>0.05)

Two groups of genotypes were compared statistically with regard to their biochemical composition among the genotypes of each group and between the groups of genotypes as summarized in Table 3.10, showing mean, standard error mean (SEM), coefficient of variation (CV) and F-value with significance level. It is noteworthy that significant F-values were obtained for all the sugars between the two groups of genotypes, which were much higher than those of individual group (HSG or PFC). This revealed that there were significant variations between HSGs and PFCs regarding their sugar content. Similarly, higher values of coefficient of variations for sugar content between the two groups rather than within the individual group were observed. On the other hand, an insignificant and lower F-value was found for amylose between the two groups of genotypes.

Table 3.10: Statistical Comparison of the Corn Genotypes Based on the Biochemical Traits

Traits	Groups	2012				2013			
		Mean	SE	CV (%)	F-test	Mean	SE	CV (%)	F-test
Dry matter (%)	Among HSGs	84.62	0.21	0.97	1.33	85.18	0.11	0.53	0.13
	Among PFCs	85.00	0.08	0.38	0.46	85.07	0.13	0.59	3.71*
	Between groups	84.81	0.11	0.76	3.01	85.13	0.08	0.56	0.41
Sucrose (%)	Among HSGs	3.84	0.24	24.89	3.53*	4.45	0.27	24.33	11.08**
	Among PFCs	0.68	0.08	35.26	6.55*	0.74	0.06	34.88	6.38*
	Between groups	2.25	0.31	77.35	165.04**	2.6	0.36	78.55	177.9**
Glucose (%)	Among HSGs	0.61	0.05	33.9	2.28	0.66	0.06	38.86	2.16
	Among PFCs	0.10	0.02	63.94	4.16*	0.14	0.01	34.1	1.43
	Between groups	0.35	0.05	84.15	88.25**	0.4	0.06	80.85	64.56**
Fructose (%)	Among HSGs	0.42	0.06	60.08	3.17	0.55	0.05	35.79	1.94
	Among PFCs	0.08	0.01	32.95	1.32	0.09	0.01	42.16	2.46
	Between groups	0.25	0.04	97.21	27.90**	0.32	0.05	85.07	84.31**
TRS (%)	Among HSGs	1.59	0.06	16	2.14	1.29	0.07	21.16	1.22
	Among PFCs	0.35	0.04	49.65	4.60*	0.54	0.03	23.18	3.95*
	Between groups	0.97	0.12	68.36	256.49**	0.92	0.08	47.25	98.48**
TSS (%)	Among HSGs	5.51	0.3	21.51	4.57*	5.96	0.27	18.23	24.32**
	Among PFCs	1.03	0.08	29.21	9.01**	1.09	0.07	25.09	4.28*
	Between groups	3.27	0.43	74.37	215.08**	3.53	0.46	73.59	302.8**
Starch (%)	Among HSGs	66.82	1.01	5.74	4.33*	67.84	0.59	3.5	5.98*
	Among PFCs	70.34	0.75	4.24	2.16	71.92	0.59	3.28	21.43**
	Between groups	68.58	0.68	5.58	8.43*	69.88	0.55	4.46	23.80**
Protein (%)	Among HSGs	11.61	0.49	16.77	2.76	10.2	0.4	15.55	10.97**
	Among PFCs	9.25	0.43	18.43	8.06**	10.61	0.32	12.19	9.44**
	Between groups	10.43	0.38	20.76	13.38**	10.4	0.25	13.83	0.66
Fat (%)	Among HSGs	5.88	0.26	17.87	8.80**	4.91	0.25	20.67	0.98
	Among PFCs	5.34	0.28	20.94	7.19*	6.25	0.21	13.72	7.70**
	Between groups	5.61	0.2	19.65	2.01	5.58	0.2	20.55	16.17**
Fiber (%)	Among HSGs	9.91	0.49	19.87	7.71**	9.33	0.26	11.37	1.40
	Among PFCs	10.05	0.37	14.71	6.11*	9.14	0.31	13.33	2.86
	Between groups	9.98	0.3	17.18	0.06	9.23	0.2	12.21	0.20
Ash (%)	Among HSGs	2.11	0.26	49.85	3.29	2.05	0.21	40.32	3.52*
	Among PFCs	1.35	0.11	33.23	0.11	1.32	0.11	31.94	0.62
	Between groups	1.73	0.16	50.6	7.20*	1.69	0.13	44.17	9.90*
Amylose (%, w/w)	Among HSGs	23.25	0.75	12.85	3.59*	23.86	0.96	16.06	6.32*
	Among PFCs	23.91	0.71	11.91	3.86*	23.81	0.68	11.45	1.00
	Between groups	23.58	0.51	12.26	0.41	23.83	0.58	13.72	0.02

*, significant at 5% probability level (P<0.05); **, significant at 1% probability level (P<0.01)

3.3.6 Correlation among the Agronomic and Biochemical Traits

As shown in Table 3.11, significant correlations were observed between the duration of the different growth stages. These crop phenological parameters included seed emergence time (SET), Anthesis time (AT), silking time (ST), grain filling period (GFP) and black layer maturity (BLM). The correlation coefficients among these parameters ranged between 0.26 and 0.88 which are significant at the probability level of either 1% or 5%. Furthermore, crop phenology was also found to be strongly correlated with plant height (PH). The AT, ST, GFP and BLM significantly correlated with ear weight (EW), which is a major yield related trait.

It was observed that GFP and BLM had a significant influence on GY as the correlation coefficients were found to be significant. Furthermore, kernel number per plant (KNP) significantly and positively correlated with GY ($r = -0.61$; $P < 0.01$). The important biochemical attributes in this study are sugar and starch. The total soluble sugar (TSS) showed negative correlations with crop phenology such as AT, ST, GFP, and BLM. On the other hand, kernel starch did not correlate significantly with crop phenology. The TSS and starch content in the kernels were found to be negatively correlated with each other ($r = 0.61$; $P < 0.01$). However kernel protein content showed positive correlation with TSS ($r = 0.28$; $P < 0.05$), but a negative correlation was observed between protein and starch ($r = 0.50$; $P < 0.01$).

Table 3.11: Correlation Coefficients between the Different Agronomic Traits and Biochemical Composition of the Corn Genotypes

	SET	AT	TS	GFP	BLM	PH	LNP	LL	ENP	EW	EL	KNP	TKW	GY	TSS	STC	PRO	FAT
AT	0.36[a]																	
ST	0.29[b]	0.84[a]																
GFP	0.15	0.61[a]	0.69[a]															
BLM	0.26[b]	0.81[a]	0.91[a]	0.88[a]														
PH	0.00	0.50[a]	0.62[a]	0.634[a]	0.69[a]													
LNP	0.19	0.01	–0.09	–0.16	–0.09	–0.38[a]												
LL	–0.18	0.10	0.06	0.08	0.09	0.16	0.09											
ENP	0.04	0.01	0.02	0.19	0.12	0.03	0.00	0.11										
EW	0.13	0.44[a]	0.42[a]	0.64[a]	0.56[a]	0.39[a]	–0.03	0.14	0.03									
EL	0.18	–0.03	–0.00	0.04	0.02	–0.00	0.18	–0.21	–0.08	0.04								
KNP	0.01	0.06	0.14	0.21	0.15	0.15	–0.01	–0.04	0.74[a]	0.06	–0.05							
TKW	0.19	–0.26[b]	–0.26[b]	–0.14	–0.27[b]	–0.50[a]	0.28[b]	0.14	0.00	0.07	0.10	–0.31[b]						
GY	0.09	0.21	0.21	0.47[a]	0.35[a]	0.20	–0.05	0.13	0.83[a]	0.48[a]	–0.05	0.61[a]	0.08					
TSS	0.10	–0.24	–0.28[b]	–0.25[b]	–0.33[a]	–0.56[a]	0.23	–0.44[a]	0.07	–0.12	–0.12	0.17	0.12	0.04				
STC	–0.01	–0.01	–0.06	–0.18	–0.07	0.15	–0.03	0.33[a]	–0.03	–0.01	0.22	–0.08	0.01	–0.08	–0.61[a]			
PRO	0.04	–0.10	–0.07	0.02	–0.00	–0.21	0.09	–0.24	0.17	–0.07	–0.04	0.01	0.12	0.12	0.28[b]	–0.50[a]		
FAT	0.09	0.23	0.22	0.30[b]	0.30[b]	0.28[b]	–0.35[a]	0.10	0.12	0.18	–0.19	0.10	–0.12	0.16	–0.14	–0.22	0.21	
FIB	–0.14	0.03	0.16	0.21	0.15	0.28[b]	0.01	0.06	–0.15	0.12	–0.14	0.27[b]	–0.40[a]	–0.12	0.02	–0.16	–0.10	–0.04

Legends: AT, anthesis time; BLM, black layer maturity; EL, ear length; ENP, ear number per plant; EW, ear weight; GRF, grain filling period; GY, grain yield; KNP, kernel number per plant; LL, leaf length; LNP, leaves number per plant; PH, plant height; PRO, protein; SET, Seed emergence time; ST, silking time; STC, starch; TKW, thousand kernel weight; TSS, total soluble sugars;

a, significant at 5% probability level ($P<0.05$)

b, significant at 1% probability level ($P<0.01$)

3.4 Discussion

3.4.1 Agronomic Traits

Most of the agronomic properties were found to be significantly different among the genotypes, irrespective of the groups of genotypes. Furthermore, high CV values within and between groups obtained for agronomic traits (Table 3.6), indicated that there was great variability in the replications due to lower uniformity (Couto et al., 2013). Among the agronomic traits, crop phenology has often been given priority for characterizing any hybrid (Sacks et al., 2010). Crop phenology includes, seed emergence time (SET), four leaf development (FLD), eight leaf development (ELD), anthesis time (AT), silking time (ST), grain filling period (GFP), and black layer maturity (BLM) (Couto et al., 2013; Ritchie et al., 1997).

Flowering time (AT and ST) is associated with the kernel development, kernel number per plant (KNP) and grain yield (GY) (Andrade et al., 1999; Tollenaar et al., 1992). In this study, some of the corn genotypes showed significant variations in AT and ST, without showing any consistent variation between the two groups of genotypes, which indicated that such variations were simply genotypic and had no additional effects on HSGs. The average AT among the genotypes ranged from 54 to 62.5 days in 2012 and 56.75 to 64.25 days in 2013, while ST ranged from 59.25-67.25 and 59.5-67 days, respectively. However, lower ST values (53-58 days) were reported by (Wang et al., 1999) in corn, whilst Couto et al. (2013) reported a longer time for ST (81-85 days). The flowering time is usually affected by the interactions between the genotypes and the environment (Borrás et al., 2007; Echarte et al., 2004), which probably explains the reason for observation of different ST among the genotypes in this study.

The post-silking time to maturity is important for dry matter (DM) accumulation in corn and its kernels, and it has been reported that around 50% of DM is accumulated

during this time (Lee & Tollenaar, 2007; Rajcan & Tollenaar, 1999). The post-silking time includes the grain filling period (GFP) and black layer maturity (BLM), which were found to be significantly lower in HSGs than PFCs (Table 3.6). This could partially be due to the fact that the new hybrids are able to accumulate more DM during post-silking time than the old hybrids, and hence require a relatively lower time to reach maturity for the new genotypes (HSGs) (Echarte et al., 2008).

Although the corn genotypes can differ in plant height (PH), leaf number per plant (LNP), and leaf length (LL), the overall PH, LNP and LL were not found to be consistently different between two groups of genotypes in this study. Significantly lower PH was recorded in HSGs than in PFCs (Table 3.6). Similarly, significantly lower LL was observed in HSGs. However, LNP was found to be higher in HSGs than PFCs. Numerous earlier studies have reported that new corn varieties may develop higher number of leaves and show a longer duration of photosynthesis during the grain filling period (Borrell et al., 2001; Duvick, 2005; Echarte et al., 2008).

Among the yield related traits, ear number per plant (ENP) was found to be almost similar in the eight HSGs and PFCs genotypes. On the other hand, ear weight (EW) differed significantly among the genotypes, particularly between HSGs and PFCs. Similar observations were also reported by Wong et al. (1994), during their study with various sweet corn hybrids. Ear length (EL) differed significantly among the corn genotypes as was also reported in a previous study by Szymanek (2009). Likewise, kernel depth (KD) also varied among the genotypes, which was in agreement with the findings of Wong et al. (1994). The kernel number per plant (KNP) and thousand kernel weight (TKW), which primarily determine grain yield in corn genotypes (Poneleit & Egli, 1979; Severini et al., 2011), In this study, KNP did not vary significantly among the genotypes, which was

contrary to what observed for TKW. Variations in KNP and TKW in different corn hybrids were also reported elsewhere (D'Andrea et al., 2008).

The mean grain yield (GY) of the corn genotypes varied between 6.82 and 10.40 t/ha in HSGs and 6.36 to 11.28 t/ha in PFCs. It has been well documented that grain yield can vary, based on different management practices, planting locations, environmental conditions and hybrids (Reicks et al., 2009). Reicks et al. (2009) reported that the mean grain yield of several field corn hybrids ranged between 5.1 and 11.9 t/ha based on management practices, locations and seasons. In another study, Gao et al. (2011) reported GY between 6.3 to 12.1 t/ha during their study with seven corn hybrids. Although it is difficult to give a satisfactory explanation for the variation in GY among the corn hybrids, the high GY in any hybrid could be due to its genetic makeup and the absence of limiting factors, such as climatic condition, crop management practices, fertilization and irrigation (Couto et al., 2013).

3.4.2 Biochemical Composition

All the HSGs contained higher amounts of free sugars and lower amounts of starch in their kernels, while reverse was true in PFCs. The higher sugar content in HSGs can be attributed to the crossing of field corn with sweet corn and the transfer of genetic material from the latter to the former (Zabed et al., 2016a; Zabed et al., 2016b).

With regard to the individual sugars, HSGs varied from 2.84 to 4.59% in sucrose, 0.48 to 0.8% in glucose and 0.26 to 0.66% in fructose during 2012 (Table 3.7). With a slight increase in sugar content during 2013, HSGs ranged from 3.22-5.64% in sucrose, 0.5-0.87% in glucose and 0.38-0.67% in fructose (Table 3.8). It has been reported previously that glucose, fructose and sucrose content in sugary corn differ significantly in response to the plant growth stages after pollination, with an estimation of around 0.4-0.5%

glucose, 0.2-0.5% fructose and 3.8-7.5% sucrose in dried kernels (Ferguson et al., 1979). It was also observed that TRS content in HSGs were higher than the sum of glucose and fructose together (Table 3.7 and 3.8). An earlier investigation, has reported that sweet corn kernels may contain significant amounts of other reducing sugars such as maltose (up to 3.28% after 40 days of pollination) resulting in the overall increase in TRS of the dried kernels (Ferguson et al., 1979). High maltose content in the kernels also result in incomplete conversion of sugars into starch (Ferguson et al., 1979), and it probably explains the reason for obtaining lower amounts of starch in HSGs than PFCs. The TSS in HSGs ranged from 4.43 to 6.72% in 2012 and 4.73 to 7.38% in 2013, whilst in PFCs it ranged from 0.76 to 1.36% and 0.84 to 1.27% respectively (Table 3.7 and 3.8). The results are comparable with the findings of earlier studies such as those reported by Ferguson et al. (1979) who observed/recorded 4-11% TSS in dry sweet corn kernels. On the other hand, <2% of TSS was reported for normal corn by Manikandan & Viruthagiri (2010).

Unlike sugar content in the corn genotypes, all HSGs contained significantly lower amounts of starch than their respective PFCs, which could possibly be attributed to the higher sugar content in the kernels and incomplete conversion of these sugars into starch (Ferguson et al., 1979; Zabed et al., 2016b). In addition, variation in starch content among the corn genotypes could have occurred due to the complexity in the regulation of starch biosynthesis in the endosperm, where different genes and several enzymes are involved (Hannah & James, 2008). The expression levels of these genes in different genotypes could have varied in response to certain intrinsic and extrinsic factors. The HSGs did not show any consistent variation in protein, fat, fiber and ash content compared to their respective PFCs, possibly due to the use of similar management practice for both groups of genotypes (Reicks et al., 2009). It has been reported that cereal grains with as much as

60–75% starch are considered as ideal candidates for ethanol production (Singh et al., 2002).

The highest and lowest amylose content among the genotypes (both PFCs and HSGs) varied between 19.92 to 26.49% in 2012 and 20.59 to 27.28% in 2013, which were close to the findings of Torney et al. (2007), who reported between 20 to 30 % amylose in normal corn starch. According to Jobling (2004), amylose synthesis in starch is brought about by the action of a granule bound starch synthase (GBSS), and the different expression levels of the GBSS genes result in the varying amylose content in starch.

3.4.3 Correlation between the Different Traits

It is well known that agronomic and biochemical traits can be significantly associated with each other in either corn or other crops. In this study, similar correlations were also observed among the traits as summarized in (Table 3.11). A significant positive correlation was observed between KNP and GY ($r = 0.61$; $P < 0.01$) and an almost similar correlation coefficient value (0.67) was reported by (D'Andrea et al., 2008). Flowering time was found to be significantly correlated with GFP and BLM as was reported by Wang et al. (1999). Furthermore, GFP showed a positive and significant correlation with GY, which was in agreement with the findings of several previous studies (Jobling, 2004; Wang et al., 1999; Wych et al., 1982). The kernel TSS showed negative correlation with BLM, as also reported earlier (Szymanek, 2009). The aforementioned authors have reported that higher BLM results in lower sweetness of the kernels. The TSS did not significantly correlate with TKW as also reported in other study (Kumari et al., 2007).

Among the biochemical traits, a significant negative correlation was observed between starch and TSS. A similar correlation was reported recently in a study on various corn genotypes (Gumienna et al., 2016). Likewise, negative correlations were found between

starch and protein, starch and fiber, and starch and fat. Similar findings were also reported in earlier studies with corn (Lacerenza et al., 2008; Obuchowski et al., 2010; Russell et al., 1992). However, some of the correlations obtained among these traits were different from previous findings reported in literature. For example, it has been reported that starch content in triticale grains positively correlated with TKW (Obuchowski et al., 2010). However, no significant correlation was observed between starch and TKW in this study.

From the correlations observed in this study, it can be seen that both agronomic and biochemical (non-carbohydrate) characteristic significantly affect carbohydrate accumulation in the kernels of the corn genotypes. ST, GFP, BM, PH and LL are the agronomic traits that negatively influence sugar accumulation in kernels, whilst LL positively associated with starch accumulation in kernels. Protein content negatively influence starch accumulation in the kernels, although there was a positive effect of protein on TSS. More importantly, sugar and starch are negatively related to each other and high sugar content will consequently reduce starch accumulation in the kernels.

3.5 Conclusion

Although HSGs differed in the agronomic characteristics, these variations were not unusual when compared to PFCs. However, it is noteworthy that all the HSGs contained unusually higher amounts of sugars and lower proportion of starch than PFCs, which makes them most attractive for dry-grind ethanol production. The variations in sugars and starch between the two groups of genotypes (HSG and PFC) were further confirmed through statistical analysis, obtaining much higher coefficient of variation and significant F-values. Moreover, a negative correlation between starch and sugars indicated that normal corn contains higher amounts of starch than sugary corn genotypes, and as a consequence, the former will require theoretically higher quantity of enzymes for ethanol production. The average grain yields for the two groups of genotypes were very similar,

which strengthens the case for the use of HSGs as ethanol feedstocks. Furthermore, it can be noted that ST, GFP and BLM negatively correlated with sugar accumulation in the kernels. Relatively lower ST, GFP and BLM in HSGs than in PFCs would make HSGs more promising for accumulating higher amounts of sugar in their kernels, in addition to reducing total cropping time. The study has shown that HSGs has the potential to be an attractive feedstock for dry-grind ethanol production.

CHAPTER 4: TWO–STEP ENZYMATIC HYDROLYSIS AND SIMULTANEOUS FERMENTATION OF CORN GENOTYPES TO EVALUATE ENZYME CONSUMPTION AND PRODUCT YIELD IN A CONVENTIONAL DRY–GRIND PROCESS

4.1 Introduction

As was discussed in Literature review (Chapter 2; Section 2.8), conventional dry-grind ethanol production includes two major steps, namely hydrolysis and fermentation. Hydrolysis is required to convert starch into soluble sugars before fermentation, since yeast can not metabolize starch directly (Kunz, 2008). During hydrolysis, amylose and amylopectin molecules in the starch undergo biochemical changes and are converted into glucose. However, the conversion efficiency as well as the yield of fermentable sugar differs significantly with regard to the process parameters and other factors (Mojović et al., 2006; Tester et al., 2006).

Hydrolysis of starch can be accomplished via three ways, *viz*, acid hydrolysis, temperature and pressure extrusion, and enzymatic hydrolysis (Duvernay et al., 2013). Although acid hydrolysis is an effective way of generating fermentable sugars from starch, the necessity for acid recovery after hydrolysis and prior to fermentation has made it unattractive (Farone & Cuzens, 1996). Moreover, acid hydrolysis is often associated with the production of undesirable by-products, such as furfurals and hydroxymethylfurfurals (HMF), which are toxic to microbial cells and inhibit the growth of yeast cells during fermentation (Kim & Hamdy, 1985). Likewise, the inhibitory effect of high pressure on α-amylase and the slow conversion rate during hydrolysis by the temperature and pressure extrusion method, result in an inefficient starch conversion with low yield of fermentable sugar (Buckow et al., 2007). On the other hand, enzymatic

hydrolysis is of biological origin, eco-friendly, simple, specific, and an efficient method to convert starch into fermentable sugars (Duvernay et al., 2013).

There are two basic steps in the conventional enzymatic hydrolysis of starch, which are liquefaction and saccharification (Duvernay et al., 2013; Wu & Miao, 2008). During liquefaction, corn starch is gelatinized, cooked and liquefied at high temperatures (85-105^{o}C in laboratory or up to 165^{o}C in commercial plant) using a thermostable α-amylase (Lamsal et al., 2011). Subsequently, saccharification is carried out at a relatively lower temperature (50-60^{o}C or even at the fermenting temperature, such as 30^{o}C) using glucoamulase (Mojović et al., 2006; Plumier et al., 2015). The heat-stable α-amylase is obtained from thermophilic bacteria, such as *Bacillus licheniformis* or from recombinant strains of *Escherichia coli* and other *Bacillus* strains (Rakin et al., 2009; Sánchez & Cardona, 2008). Glucoamylase can be produced from *Aspergillus niger* or *Rhizopus* species (Rakin et al., 2009; Shigechi et al., 2004a).

The conventional dry-grind ethanol production is usually done either by the separate hydrolysis and fermentation (SHF) or the simultaneous saccharification and fermentation (SSF) technique. In SHF, a two-step enzymatic hydrolysis is carried out separately prior to subjecting the hydrolysate into yeast fermentation (Ratnavathi et al., 2011). On the other hand, liquefied slurries obtained from the liquefaction are saccharified and fermented concurrently during SSF (Cot et al., 2007; Manikandan & Viruthagiri, 2010). The SSF technique has been considered more advantageous, technically feasible and produces higher amounts of ethanol than SHF, as was discussed previously (Chapter 2; Section 2.12). For this reason, SSF is widely used on both laboratory and industrial scales during dry-grind ethanol production (Srichuwong et al., 2009). Moreover, numerous studies in recent years have shown that SSF is more effective to produce ethanol from

either single corn material or different hybrids (Lemuz et al., 2009; Murthy et al., 2009; Reicks et al., 2009; Singh & Graeber, 2005; Yangcheng et al., 2013)

The aim of the present work was to study high sugary corn genotypes (HSGs) and their respective parent field corn lines (PFCs) for fermentable sugar yield during two-step enzymatic hydrolysis under different enzymatic and process conditions. Subsequently, a conventional simultaneous saccharification and fermentation (CSSF) process was carried out using the optimum conditions obtained from enzymatic hydrolysis. Finally, enzyme consumption during hydrolysis and fermentation along with sugar and ethanol yield were compared between HSG and PFC genotypes.

4.2 Materials and Methods

4.2.1 Corn Materials

Corn genotypes, including four HSGs (UM.NF–1, UM.NF–4, UM.NF–6 and UM.NF–11) and four PFCs (PFC–1, PFC–4, PFC–6 and PFC–11), grown in the cropping year 2012 (Chapter 3; Section 3.2.9) were used as raw materials for the two-step enzymatic hydrolysis and CSSF. Harvested and dried kernels were ground in a laboratory grinder, and sieved manually using test sieves (PRADA, Scientific Jaya, Malaysia) to four different particle sizes (PS), such as PS≤0.5, 0.5<PS≤1.0, 1.0<PS≤2.0, and 2.0<PS≤3.0 mm that were expressed as 0.5, 1.0, 2.0 and 3.0 respectively. The corn samples were preserved at 4^{o}C until further analyses were carried out.

4.2.2 Chemical and Reagents

All the chemicals, reagents and standards used in this study were reagent grade and purchased either from Sigma–Aldrich (St. Louis, MO, USA) or Fisher Scientific (Waltham, MA, USA). The ingredients used for microbial media preparation were purchased from BD Biosciences (Franklin Lakes, NJ, USA) or OXOID (Basingstoke,

Hampshire, UK). IsoStab (hop acid) used to inhibit bacterial contamination during fermentation was a kind gift from Beta Tec Hop Products GmbH (Bahnhofstr, Schwabach, Germany). Chloramphenicol used during hydrolysis and fermentation were purchased from Sigma-Aldrich (Product code, C0378).

4.2.3 Enzymes

Two enzymes used for liquefaction and saccharification of the corn slurry. These were SPEZYME® FRED, and OPTIDEX® L-400, SPEZYME® FRED is an acid-stable and thermostable α-amylase, with a minimum activity of 17400 LU (liquefon unit) per gram. One LU is the time needed to produce a color change with iodine solution, representing a distinct phase of starch dextrinization under a specified condition. OPTIDEX® L-400 is a glucoamylase with a minimum activity of 350 GAU/g. One GAU is the quantity of enzyme needed to produce 1 g of glucose from starch under specified conditions. FERMGEN is an acid fungal protease and used during CSSF. It had an activity of ≥1000 SAPU/g (Spectrophotometric Acid Protease Units), as defined by the manufacturer (Genencor, Palo Alto, CA). The enzymes were kind gifts from DuPont Industrial Biosciences (DuPont Genencor Science, Palo Alto, CA).

4.2.4 Yeast and Inoculum Preparation

Saccharomyces cerevisae (ATCC 96581) was obtained from ATCC, Manassas, VA, USA. The yeast was maintained on YPD agar slant consisting of yeast extract (10.0 g/L), peptone (20.0 g/L), dextrose (20.0 g/L), agar (15.0 g/L) and distilled water (up to 1000 ml). Prior to use as inoculum for fermentation, yeast cells were aerobically grown in a 250 ml Erlenmeyer flask in a shaking incubator (200 rpm) at 30^{o}C for 48 h in YPD broth supplemented with KH_2PO_4 (2.0 g/L), $(NH_4)_2SO_4$ (1.0 g/L) and $MgSO_4.7H_2O$ (0.4 g/L). Subsequently, the growth medium was centrifuged at 5000 rpm for 15 min to separate the

cells. Finally, a yeast suspension (cell density ~1×10^8 CFU/ml) was prepared in fresh YPD broth.

4.2.5 Two–Step Enzymatic Hydrolysis

4.2.5.1 Liquefaction

Unless otherwise stated, 20 ml of corn slurry was prepared in a 50 ml falcon tube with a solid concentration 250 g/L by mixing around 5.0 g corn meal (taking into account its moisture content) with distilled water containing 0.01% (w/v) $CaCl_2$ (Mojović et al., 2006). The pH of the slurry was adjusted to 6.0 with 2.0 M HCl or 5.0 M NaOH. Thereafter, a dose of SPEZYME ® FRED was added to the slurry. The tubes were placed in a water bath at 90^{o}C for 2 h with vigorous shaking in the first 5 min and then every 15 min (Duvernay et al., 2013). Samples were withdrawn periodically and centrifuged at 5000 rpm for 15 min to collect supernatant for determination of reducing sugars (RS) in the hydrolysates.

The effect of enzyme load on RS yield was studied using four SPEZYME ® FRED doses, such as 0.025, 0.05, 0.075 and 0.1% (v/v), which is equivalent to 1.0, 2.0, 3.0 and 4.0 kg/MT of dry corn respectively. The effect of reaction time on the liquefaction was studied using a SPEZYME ® FRED load 3.0 kg/MT and conducting the liquefaction for 3 h along with sample collection after 0, 0.5, 1, 2 and 3 h. The effect of particle size of the corn meal on RS yield was studied by preparing slurries with four different particle sizes (0.5, 1.0, 2.0 and 3.0 mm), followed by conducting liquefaction for 2 h using a SPEZYME ® FRED load 3.0 kg/MT. The effect of initial solid load on RS yield was studied by preparing slurries with four different solid loads (150, 200, 250 and 300 g/L) and carrying out the liquefaction for 2 h using a SPEZYME ® FRED load 3.0 kg/MT. All experiments were done in duplicate for each plot and the results averaged.

4.2.5.2 Saccharification

As described above, corn slurries were prepared in fresh falcon tubes and liquefied for 2 h adding a SPEZYME ® FRED load 3.0 kg/MT. Unless otherwise stated, the liquefied slurry was then cooled to room temperature in an ice water bath and the pH adjusted to 4.2 with 2.0 M HCl. The slurry was supplemented with sodium azide (0.15%, w/v) to prevent microbial contamination during saccharification, which was carried out at 30ºC for 48 h in a shaking incubator set at 200 rpm using an OPTIDEX ® L–400 dose 3.0 kg/MT. Samples were withdrawn periodically and centrifuged at 5000 rpm for 15 min to collect supernatant for determination of reducing sugars (RS) in the hydrolysates.

The effect of enzyme load on RS yield was studied using four OPTIDEX ® L–400 doses, such as 0.025, 0.05, 0.075 and 0.1% (v/v), which was equivalent to 1.0, 2.0, 3.0 and 4.0 kg/MT of dry corn respectively. The effect of reaction time on liquefaction was studied using an OPTIDEX ® L–400 load 3.0 kg/MT and conducting the liquefaction for 3 h and collecting samples after 0, 0.5, 1, 2 and 3 h. The effect of particle size of the corn meal on RS yield was studied by preparing slurries with four different particle sizes (0.5, 1.0, 2.0 and 3.0 mm), followed by conducting liquefaction for 2 h using a OPTIDEX ® L–400 load 3.0 kg/MT. The effect of initial solid load on RS yield was studied by preparing slurries with four different solid loads (150, 200, 250 and 300 g/L) and carrying out the liquefaction for 2 h using an OPTIDEX ® L–400 load 3.0 kg/MT. All experiments were done in duplicate for each plot and results averaged.

4.2.5.3 CSSF

Based on the optimum condition obtained from the two-step hydrolysis described above, 100 ml of corn slurry was prepared in a 250 ml Erlenmeyer flask containing 250 g/L initial solid through mixing approximately 25 g of corn flour (1.0 mm) with 75 ml of

distilled water containing 0.01% (w/v) $CaCl_2$ (Mojović et al., 2006). The pH of the slurry was adjusted to 6.0 with 2.0 M HCl or 5.0 M NaOH. A dose of SPEZYME ® FRED (1.0, 2.0, 3.0 and 4.0 kg/MT of dry corn) was added to the slurry. Samples were then subjected to liquefaction at 90°C for 2 h with vigorous shaking in the first 5 min and then every 15 min. The resulting hydrolysate was cooled and the pH adjusted to 4.2 with 2.0 M HCl. The slurry was supplemented with urea (400 ppm), FERMGEN (0.2 kg/MT of dry corn), IsoStab (40 ppm) and chloramphenicol (50 μg/ml). CSSF was carried out by adding 2 ml inoculum of *S. cerevisiae* and a single enzyme load (OPTIDEX ® L–400; 1.0, 2.0, 3.0 and 4.0 kg/MT of dry corn) to flasks containing 1.0, 2.0, 3.0 and 4.0 kg/MT SPEZYME ® FRED respectively. Fermentation was conducted for 72 h at 30°C in a shaking incubator at 200 rpm. It was assumed that pasteurization of the slurry during liquefaction at 90°C for 2 h was adequate to control microbial contamination, and so no further sterilization was carried out before conducting SSF (Mojović et al., 2006). Samples were withdrawn every 24 h, and one part was analyzed for microbial count (total viable yeast and bacterial counts) after making proper dilutions (10^{-3} after 24 h, 10^{-4} after 48 h 10^{-5} after 72 h). The other part of the sample was centrifuged at 5000 rpm for 15 min to analyze the supernatant for ethanol, glycerol and lactic acid. Samples collected after 72 h were also analyzed for residual starch, glucose, sucrose, fructose and total soluble sugars (TSS).

4.2.6 Analytical Methods

4.2.6.1 Determination of Moisture Content

Moisture content in the sample was determined by drying it at 105°C in a hot air oven until the mass was constant with time as was described in Chapter 3 (Section 3.2.10.3).

4.2.6.2 Determination of RS

The concentration of RS in the hydrolysate was determined by the 3', 5'–dinitrosalicylic acid (DNS) method (Miller, 1959). Prior to the analysis, supernatant of the collected sample was treated with 2.0 M HCl at pH 1.0 at 85^{o}C, followed by neutralization with 5.0 M NaOH to hydrolyze sucrose into glucose and fructose (Mazaheri et al., 2012). Absorbance was measured at 540 nm and RS calculated as glucose equivalent using a calibration curve. Detail of the method was described in Chapter 3 (Section 3.2.10.5).

4.2.6.3 Determination of Ethanol

Ethanol concentration was estimated by the dichromate oxidation method (Seo et al., 2009). The collected sample was diluted accordingly in distilled water based on the preliminary trials and reacted with potassium dichromate solution.

(a) ***Colorimetric Assay***

To 5 ml of fermentation broth (after centrifugation at 5000 rpm for 15 min), 5 ml of tributyl phosphate (TBP) was mixed on a vortex mixer for 30 min, and the upper layer collected for $K_2Cr_2O_7$ oxidation. In a fresh tube, 2.0 ml of sample was mixed with 2.0 ml $K_2Cr_2O_7$ solution (Appendix A) and 5 ml distilled water. The sample was left for 30 min at room temperature for oxidation. Thereafter, absorbance was read at 595 nm in a UV-visible spectrophotometer.

(b) ***Calculation***

A calibration curve was prepared to obtain the regression equation by plotting absorbance against different ethanol concentrations. The regression equation was in the format as described in Chapter 3 (Equation 3.6).

The values of 'a' and 'b' were found to be 0 and 0.092, respectively (Appendix C). Therefore, the concentration of ethanol was calculated using the Equation (4.1).

$$X_{EtOH} = \frac{Y}{0.092} \quad \text{.....(4.1)}$$

where, X_{EtOH} is the concentration of ethanol (%, v/v); Y is the absorbance of the sample.

The kinetic parameters for ethanol fermentation were calculated using the Equations (4.2) to (4.4).

$$P_{EtOH} = X_{EtOH} \times 0.789 \times 10 \quad \text{.....(4.2)}$$

where, P_{EtOH} is the Ethanol titer (g/L); X_{EtOH} is the concentration of ethanol (%, v/v); 0.789 is the specific gravity of ethanol; 10 is the conversion factor from 100 ml to 1.0 L.

$$Q_{EtOH} = \frac{P_{EtOH}}{t} \quad \text{.....(4.3)}$$

where, Q_{EtOH} is the volumetric ethanol productivity (g/L/h); P_{EtOH} is the ethanol titer (g/L); t is the fermentation time.

$$Y_{EtOH} = \frac{P_{EtOH}}{S} \quad \text{.....(4.4)}$$

where, Y_{EtOH} is the ethanol yield (g/g of dry corn); P_{EtOH} is the ethanol titer (g/L); S is the initial solid concentration (g/L).

4.2.6.4 Determination Carbohydrate Concentrations

The concentration of starch, TSS, sucrose, glucose and fructose were determined using the methods described in Chapter 3 (Section 3.2.10).

4.2.6.5 Total Viable Yeast Count

The concentration of viable yeast cells during CSSF was determined by inoculating 0.1 ml sample from each dilution on YPD agar plate containing chloramphenicol (50 μg/ml). The plates were incubated at 30^{o}C for at least 72 h.

4.2.6.6 Total Viable Bacterial Count

The concentration of viable bacteria in the fermentation media was determined indirectly using the pour plate technique. Sample from each dilution (0.1 ml) was inoculated into nutrient agar plate (BD, 297801) containing cycloheximide (4 μg/ml), and incubated at 35^{o}C for 48 h.

4.2.6.7 Determination of Glycerol

Glycerol concentration in the fermentation broth was determined by the spectrophotometric method through subtracting total carbohydrate concentration from glycerol plus total carbohydrate concentration (Pons et al., 1981). Prior to conducting colorimetric assay samples were de-proteinized following the method of Dubowski (1962), as described in the previous chapter (Section 3.2.10.4).

(a) ***Colorimetric Assay***

In separate test tubes, 0.5 ml of sample and different concentrations of standard, containing a mixture of glucose and glycerol (Appendix B) were mixed with 5 ml anthrone reagent (Appendix A). The contents of the tubes were mixed thoroughly in a

vortex mixer, and then heated in a boiling water bath for 10 min. After cooling, absorbance of the reaction mixture was read in a UV-visible spectrophotometer at 510 nm for glycerol + total carbohydrate and 590 nm for total carbohydrate.

(b) ***Calculation***

A calibration curve was prepared to obtain the regression equation by plotting absorbance against different concentrations of standard. The regression equation was as described in the previous chapter (Chapter 3; Equation 3.6).

For total carbohydrate, the values of 'a' and 'b' were found to be 0.002 and 3.362, respectively (Appendix C). Therefore, the concentration of total carbohydrate was calculated using the Equation (4.5).

$$X_{TC} = \frac{Y - 0.002}{3.362} \quad \text{.....(4.5)}$$

where, X_{TC} is the concentration of total carbohydrate (mg/ml); Y is the absorbance of the sample.

For glycerol + total carbohydrate, the values of 'a' and 'b' were found to be 0 and 14.711, respectively (Appendix C). Therefore, the concentration of glycerol + total carbohydrate was calculated using the Equation (4.6).

$$X_{Gly+TC} = \frac{Y}{14.711} \quad \text{.....(4.6)}$$

where, X_{Gly+TC} is the concentration of glycerol + total carbohydrate (mg/ml); Y is the absorbance of the sample

Finally, the concentration of glycerol was determined by the Equation (4.7), as follows:

$$X_{Gly} = X_{Gly+TC} - X_{TC} \quad \text{.....(4.7)}$$

Where, X_{Gly} is the concentration of glycerol (mg/ml); $X_{Gly + TC}$ is the concentration of glycerol + total carbohydrate (mg/ml); X_{TC} is the concentration of total carbohydrate (mg/ml)

4.2.6.8 Determination of Lactic Acid

Lactic acid in the fermentation media was determined using the colorimetric method described by Taylor (1996).

(a) ***Colorimetric Assay***

In separate test tubes, 0.5 ml of sample and different concentrations of lactic acid (0-0.05 mg/ml; Appendix B) were mixed with 3 ml concentrated H_2SO_4. The tubes were then heated in a boiling water bath for 10 min, and subsequently cooled to room temperature in an ice water bath. Thereafter, 50 µl of 4% $CuSO_4.5H_2O$ reagent and 100 µl p-phenylphenol solution (1.5% p-phenylphenol in 95% ethanol) were added to each tube. The contents were mixed thoroughly in a vortex mixture and left for 30 min at room temperature. Absorbance of the reaction mixture was read at 570 nm in a UV-visible spectrophotometer.

(b) ***Calculation***

A calibration curve was prepared to obtain the regression equation by plotting absorbance against different concentrations of lactic acid. The regression equation was as the format as described in the previous chapter (Chapter 3; Equation 3.6).

The values of 'a' and 'b' were found to be 0.018 and 16.371, respectively (Appendix C). Therefore, the concentration of total carbohydrate was calculated using the Equation (4.8).

$$X_{Lac} = \frac{Y - 0.018}{16.371} \quad \text{.....(4.8)}$$

where, X_{Lac} is the concentration of lactic acid (mg/ml); Y is the absorbance of the sample.

4.2.7 Statistical Analysis

Data were tested for one way ANOVA (analysis of variance) to determine the significant effects of genotypes and different factors on the yield of hydrolysis and CSSF. Fisher's least significant differences (LSD) test was done for comparing the parameters to see if the main effects were significant at $P \leq 0.05$ after ANOVA test. Pearson's product moment correlation coefficient was determined to evaluate the relationship between the parameters. Minitab statistical software, version 16 (State college, PA, USA) was used for all analyses considering a 5% level of significance ($P \leq 0.05$).

4.3 Results

4.3.1 Two–Step Enzymatic Hydrolysis under Different Condition

4.3.1.1 Effect of Enzyme Load on RS Yield

During liquefaction, RS concentration in the liquefied slurries increased with increase in enzyme dose (Figure 4.1). However, the analysis of variance for RS under four enzyme loads revealed that HSGs did not differ significantly in the concentration of RS between the enzyme loads 3.0 and 4.0 kg/MT. Liquefaction with the lowest enzyme load (1.0 kg/MT) generated minimum amounts of RS in all genotypes, which varied between 6.19

g/L in PFC–1 and 26.41 g/L in UM.NF–6 (HSG). Under maximum enzyme load (4.0 kg/MT), PFC–6 produced the lowest amounts of RS among the eight corn genotypes studied, whilst, the highest concentration of RS was generated by UM.NF–6 (Figure 4.1).

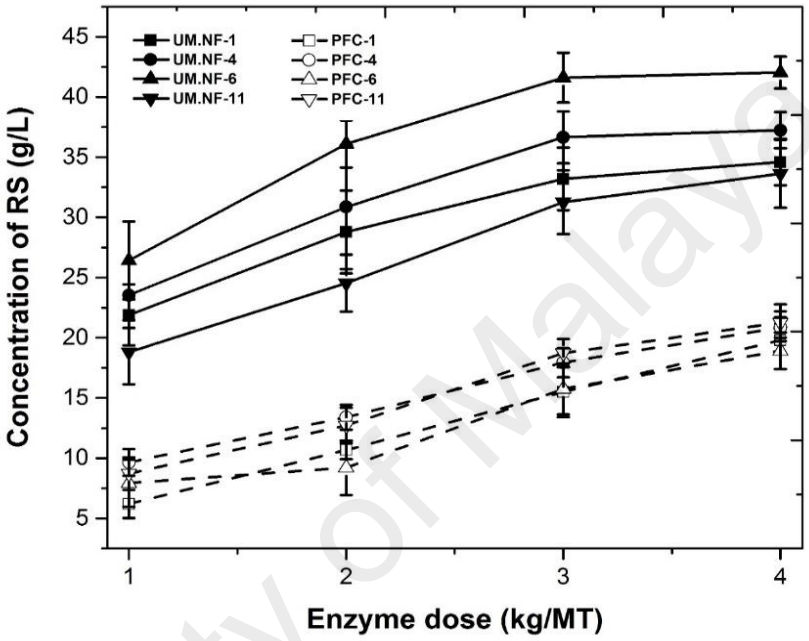

Figure 4.1: Effect Of Enzyme Load on Reducing Sugar Yield during Liquefaction of Corn Genotypes. Process Conditions: Enzyme, SPECZYME ® FRED (α−Amylase); Initial Solid Load 250 G/L; Particle Size of Ground Corn 1.0 mm; Temperature, 90°C; pH, 6.0; Reaction Time, 2 h. Values are Expressed as Mean ± SD.

As was observed in the liquefaction experiments, enzyme load brought about almost the same effects on RS yield during saccharification, presumably because HSGs produced significantly higher amounts of RS under all enzymatic conditions compared to the PFCs (Figure 4.2). Under the lowest enzyme load (1.0 kg/MT), the concentration of RS among HSGs varied from 117.08 g/L in UM.NF–11 to 131.65 g/L in UM.NF–6, while in the PFCs it ranged between 89.23 g/L in PFC-6 and 98.14 g/L in PFC-4. The concentration

of RS increased significantly in all the genotypes as the enzyme load increased to 3.0 kg/MT and it continued in the PFCs until the enzyme dose reached the highest amount (4.0 kg/MT). However, although HSGs showed a slight increase in RS at maximum enzyme load, the mean differences could be considered insignificant as the confidence level was found to be lower than 95% ($P > 0.05$).

Table 4.1 shows RS yield of the corn genotypes during liquefaction and saccharification under four enzymatic conditions. It can be observed that sugar yield was much lower in all genotypes under the lowest enzyme dose, and apart from showing an increase, PFCs still produced lower amounts of RS as the enzyme dose increased to 2.0 kg/MT. Although HSGs showed considerably higher yield under this enzymatic condition (2.0 kg/MT), a significant increase continued in these genotypes until the enzyme load reached to 3.0 kg/MT, followed by insignificant variations at a higher enzyme load. However, PFCs consumed the highest amounts of enzyme (4.0 kg/MT) for maximum RS yield, even though still they produced lower concentrations of RS compared to that of HSGs utilizing an enzyme load 3.0 kg/MT.

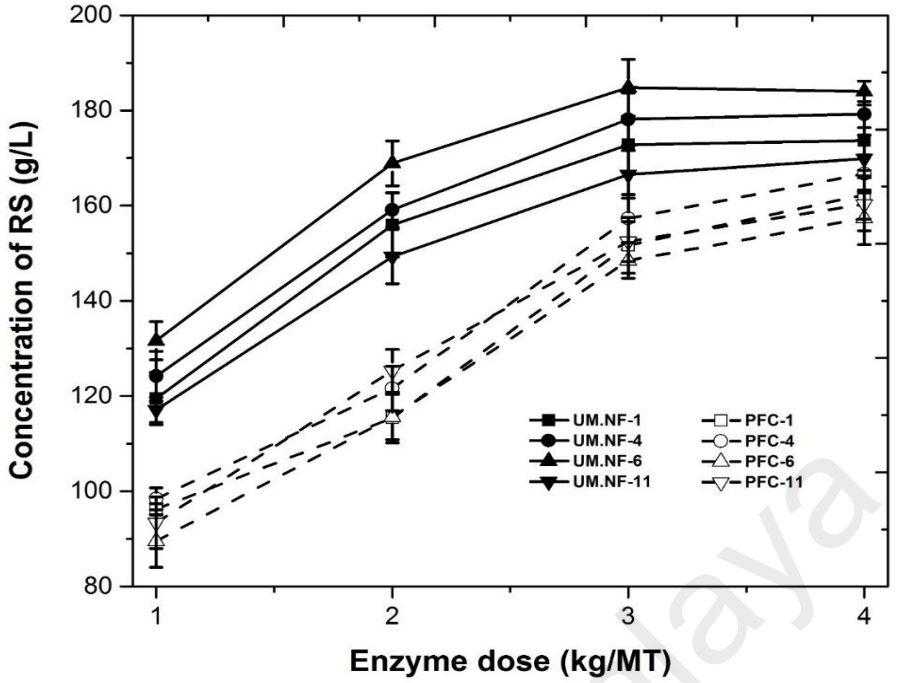

Figure 4. 2: Effect of Enzyme Load on Reducing Sugar Yield during Saccharification of Corn Genotypes. Process Conditions for Saccharification: Enzyme, OPTIDEX ® L 400 (glucoamylase); Initial Solid Load 250 g/L; Particle Size of Ground Corn 1.0 mm; Temperature, 30ºC; pH, 4.2; Reaction Time, 48 h; Shaking Rate, 200 rpm. Values are Expressed as Mean ± SD.

Table 4.1: Average Sugar Yield during Two–Step Enzymatic Hydrolysis Under Four Different Enzymatic Conditions

Genotypes	Hydrolysis step	Reducing sugar yield (g/g of corn)			
		Enzyme dose (kg/MT of dry corn)			
		1.0	2.0	3.0	4.0
UM.NF–1	Liquefaction	0.09 ^{c}	0.12 ^{b}	0.13^{ab}	0.14 ^{a}
	Saccharification	0.48 ^{c}	0.62 ^{b}	0.69 ^{a}	0.69 ^{a}
UM.NF–4	Liquefaction	0.09 ^{c}	0.12 ^{b}	0.15 ^{a}	0.15 ^{a}
	Saccharification	0.50 ^{c}	0.64 ^{b}	0.71^{a}	0.72 ^{a}
UM.NF–6	Liquefaction	0.11 ^{c}	0.14 ^{b}	0.17 ^{a}	0.17 ^{a}
	Saccharification	0.53 ^{c}	0.68 ^{b}	0.74 ^{a}	0.74 ^{a}
UM.NF–11	Liquefaction	0.08 ^{b}	010 ^{b}	013 ^{a}	013 ^{a}
	Saccharification	0.47 ^{c}	0.60 ^{b}	0.67 ^{a}	0.68 ^{a}
PFC–1	Liquefaction	0.02 ^{d}	0.04 ^{c}	0.06 ^{b}	0.08 ^{a}
	Saccharification	0.38 ^{d}	0.46 ^{c}	0.61 ^{b}	0.65 ^{a}
PFC–4	Liquefaction	0.04 ^{d}	0.05 ^{c}	0.07 ^{b}	0.08 ^{a}
	Saccharification	0.39 ^{d}	0.49 ^{c}	0.63 ^{b}	0.67 ^{a}
PFC–6	Liquefaction	0.03 ^{d}	0.04 ^{c}	0.06 ^{b}	0.08 ^{a}
	Saccharification	0.36 ^{d}	0.46 ^{c}	0.59 ^{b}	0.63 ^{a}
PFC–11	Liquefaction	0.03 ^{d}	0.05 ^{c}	0.07 ^{b}	0.09 ^{a}
	Saccharification	0.37 ^{d}	0.50 ^{c}	0.61 ^{b}	0.64 ^{a}

Notes: Values followed by the same small letter in a row denote insignificant variations among the enzyme doses.

4.3.1.2 Effect of Reaction Time on RS Yield

During liquefaction, it was observed that the concentration of RS increased over time, irrespective of the genotypes (Figure 4.3). At the initial stage, the slurries of HSGs received considerable amounts of RS from kernel sugars, which ranged from 2.39 to 5.88 g/L. As the liquefaction time increased from 0 to 2 h, RS concentration in the hydrolysates of HSGs increased, ranging between 31.26 and 41.61 g/L, but subsequently it showed no further significant increase or decrease in RS with increasing time (33.38 to 42.47 g/L). On the other hand, PFCs contained much lower amounts of RS in the slurries at the initial stage compared to HSGs. As a results, significantly lower amounts of RS was produced by PFCs over time during liquefaction that ranged from 15.75 to 18.73 g/L after 2 h, which reached 17.83 to 20.45 g/L after 3 h. The F–test analysis of variance for RS among

the different time periods revealed that both HSGs and PFCs did not vary significantly in their RS concentrations after 2 h ($P > 0.05$).

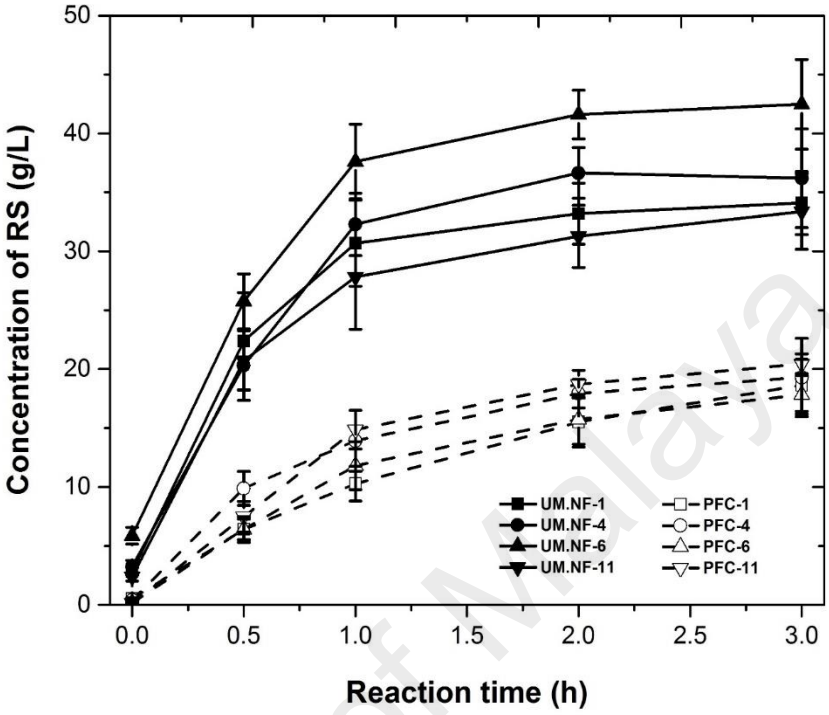

Figure 4. 3: Effect of Reaction Time on Reducing Sugar Yield during Liquefaction of Corn Genotypes. Process conditions: enzyme, SPECZYME ® FRED (α–amylase); enzyme load 3.0 kg/MT of dry corn; initial solid load 250 g/L; particle size of ground corn 1.0 mm; temperature, 90ºC; pH, 6.0. Values are expressed as mean ± SD.

As observed in liquefaction experiments, the concentration of RS increased significantly over time during saccharification (Figure 4.4). Both groups of the corn genotypes (PFCs and HSGs) produced their RS maxima after 72 h, ranging from 169.05 to 184.48 g/L in the HSGs and 151.84 to 162.56 g/L in PFCs. However, the concentration of RS in the hydrolysates after 72 h were not found to be statistically different from the RS obtained after 48 h ($P < 0.05$).

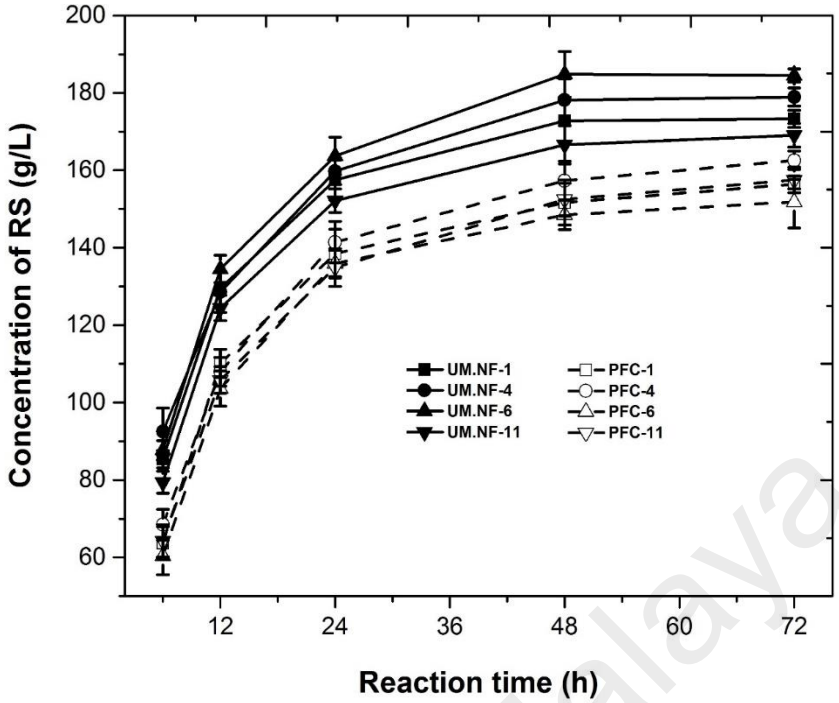

Figure 4. 4: Effect of Incubation Time on Reducing Sugar Yield during Saccharification of the Corn Genotypes. Process conditions for saccharification: enzyme, OPTIDEX ® L 400 (glucoamylase); enzyme load 3.0 kg/MT of dry corn; initial solid load 250 g/L; particle size of ground corn 1.0 mm; temperature, 30°C; pH, 4.2; shaking rate, 200 rpm. Values are expressed as mean ± SD.

4.3.1.3 Effect of Particle Size on RS Yield

During liquefaction of the slurries containing ground corn with different particle sizes, the concentration of RS in the hydrolysates decreased significantly with increase in particle size ($P < 0.05$), irrespective of the genotypes (Figure 4.5). As a result, maximum amount of RS was obtained from the particle size 0.5 mm, which dropped moderately as the particle size increased to 1.0 mm. The production of RS reduced drastically in all corn genotypes with a further increase in particle size. Compared to PFCs, all the HSGs produced higher amounts of RS at all particle sizes, indicating that particle size had no extra effect on the sugar releasing capacity of HSGs under the conditions of starch

hydrolysis. Liquefaction was conducted for 2 h using an enzyme dose 3.0 kg/MT, and the final concentration of RS in HSGs ranged from 36.48 to 47.29 g/L for 0.5 mm, 31.26 to 41.61 g/L for 1.0 mm, 18.54 to 26.77 g/L for 2.0 mm and 12.49 to 15.16 g/L for 3.0 mm, while for the PFCs it ranged from 18.83 to 22.71 g/L, 15.50 to 18.73 g/L, 9.73 to 13.53 g/L and 6.76 to 8.24 g/L respectively (Figure 4.5). Among the HSGs, the lowest and highest amounts of RS were produced by UM.NF–11 and UM.NF–6, respectively, while no consistent increase or decrease was observed among the PFCs.

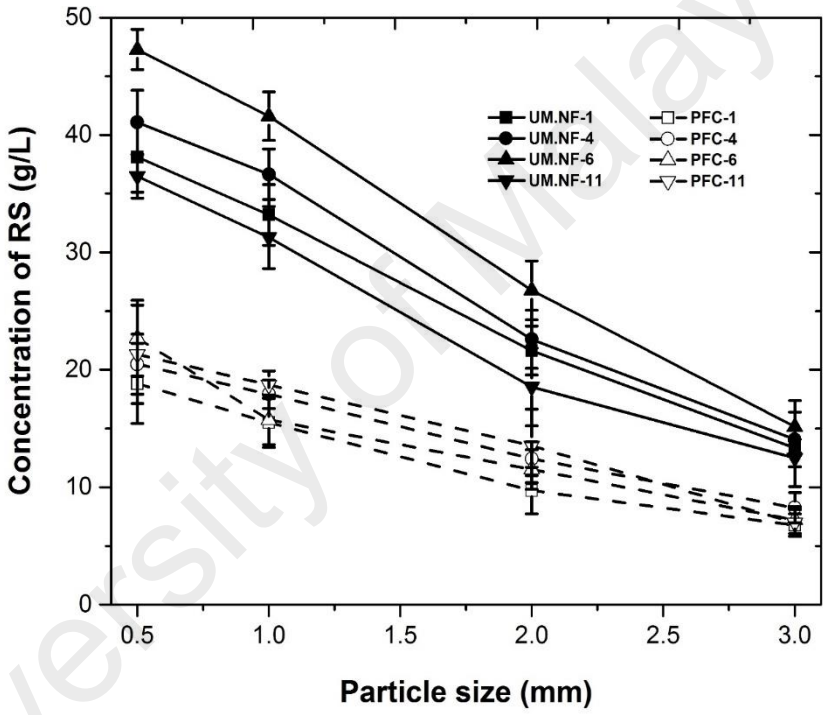

Figure 4.5: Effect of Particle Size of Ground Corn on Reducing Sugar Yield During Liquefaction of Corn Genotypes. Process conditions: enzyme, SPECZYME ® FRED (α–amylase); enzyme load 3.0 kg/MT of dry corn; initial solid load 250 g/L; temperature, 90°C; pH, 6.0; reaction time 2 h. Values are expressed as mean ± SD.

The effect of particle size on RS was also apparent during saccharification, where a similar trend to liquefaction was recorded. It was observed that 0.5 mm particle size

produced the highest amounts of RS in all genotypes, while 3.0 mm produced the lowest concentration of RS (Figure 4.6). Compared to PFCs, all the HSGs generated higher amounts of RS after two–step hydrolysis, ranging from 171.05 to 188.47 g/L, compared to 157.81 to 166.60 g/L in the PFCs with the smallest particle size (0.5 mm). This was followed by a gradual decrease in RS as the particle size increased. Nevertheless the HSGs still produced higher concentrations of RS than the PFCs.

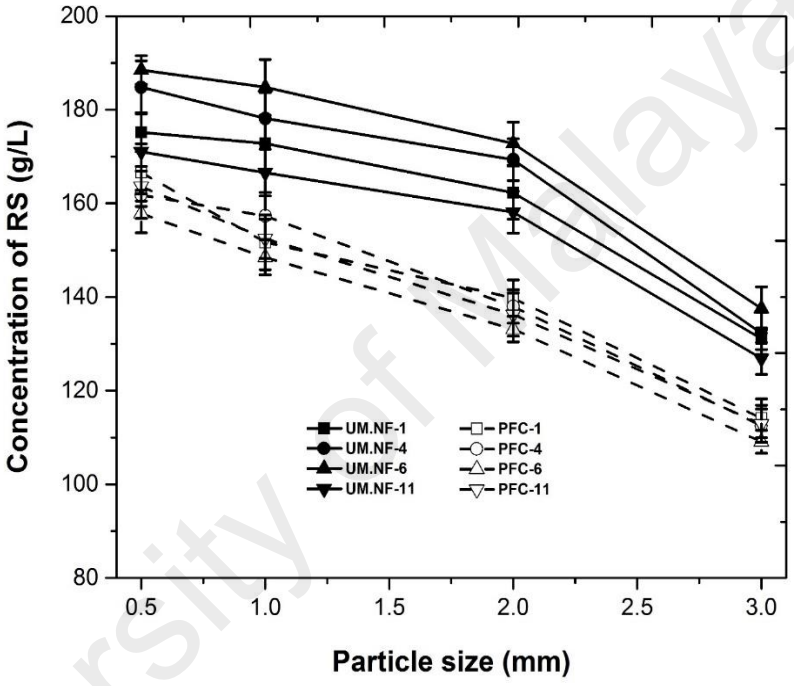

Figure 4.6: Effect Of Particle Size of Ground Corn on Reducing Sugar Yield During Saccharification of Corn Genotypes. Process conditions for saccharification: enzyme, OPTIDEX ® L 400 (glucoamylase); enzyme load 3.0 kg/MT of dry corn; initial solid load 250 g/L; particle size of ground corn 1.0 mm; temperature, 30°C; pH, 4.2; incubation time, 48 h; shaking rate, 200 rpm. Values are expressed as mean ± SD.

As shown in Table 4.2, RS yield during two–step hydrolysis was significantly different under the four particle sizes, irrespective of the genotypes. However, some genotypes did not show any significant variation in RS yield between the particle sizes 0.5 and 1.0 mm.

In all genotypes, RS yield was significantly lower at 2.0 and 3.0 mm particle sizes than those of 0.5 and 1.0 mm.

Table 4.2: Average Sugar Yield During Two–Step Enzymatic Hydrolysis (Liquefaction and Saccharification) under Four Different Particle Sizes

Genotypes	Hydrolysis step	Reducing sugar yield (g/g of corn)			
		Particle size (mm)			
		0.5	1.0	2.0	3.0
UM.NF–1	Liquefaction	0.15[a]	0.13[b]	0.09[c]	0.05[d]
	Saccharification	0.70[a]	0.69[a]	0.65[b]	0.52[c]
UM.NF–4	Liquefaction	0.16[a]	0.15[a]	0.09[b]	0.06[c]
	Saccharification	0.74[a]	0.71[b]	0.68[c]	0.53[d]
UM.NF–6	Liquefaction	0.19[a]	0.17[b]	0.11[c]	0.06[d]
	Saccharification	0.75[a]	0.74[a]	0.69[b]	0.55[c]
UM.NF–11	Liquefaction	0.15[a]	0.13[b]	0.07[c]	0.05[d]
	Saccharification	0.68[a]	0.67[a]	0.63[b]	0.51c
PFC–1	Liquefaction	0.08[a]	0.06[b]	0.04[c]	0.03[d]
	Saccharification	0.67[a]	0.61[b]	0.56[c]	0.46[d]
PFC–4	Liquefaction	0.08[a]	0.0[b]	0.05[c]	0.03[d]
	Saccharification	0.65[a]	0.63[b]	0.55[c]	0.45[d]
PFC–6	Liquefaction	0.09[a]	0.06[b]	0.05[c]	0.03[d]
	Saccharification	0.63[a]	0.59[b]	0.53[c]	0.44[d]
PFC–11	Liquefaction	0.09[a]	0.07[b]	0.05[c]	0.03[d]
	Saccharification	0.65[a]	0.61[b]	0.54[c]	0.45[d]

Notes: Values followed by the same small letter in a row denote insignificant variations among the particle sizes.

4.3.1.4 Effect of Initial Solid Load on RS Yield

The production of RS during liquefaction was found to be significantly affected by the initial solid load of the slurries, showing a gradual increase in RS with increasing solid load until 250 g/L. There was a slight increase or plateau or even decrease in RS concentration when it increased to 300 g/L (Figure 4.7). However, it can be noted that RS concentration in PFCs increased when the initial solid load decreased from 200 g/L to 150 g/L, except for PFC–4 which showed a plateau under this condition. PFCs showed a

significant drop in RS concentration as the solid load increased from 250 g/L to 300 g/L, with the exception of PFC–4. On the other hand, HSGs showed a continuous increase in their RS concentration when the solid load increased from 150 g/L to 300 g/L, with the exception of UM.NF–11 which produced lower amounts of RS under the solid load 300 g/L than under 250 g/L.

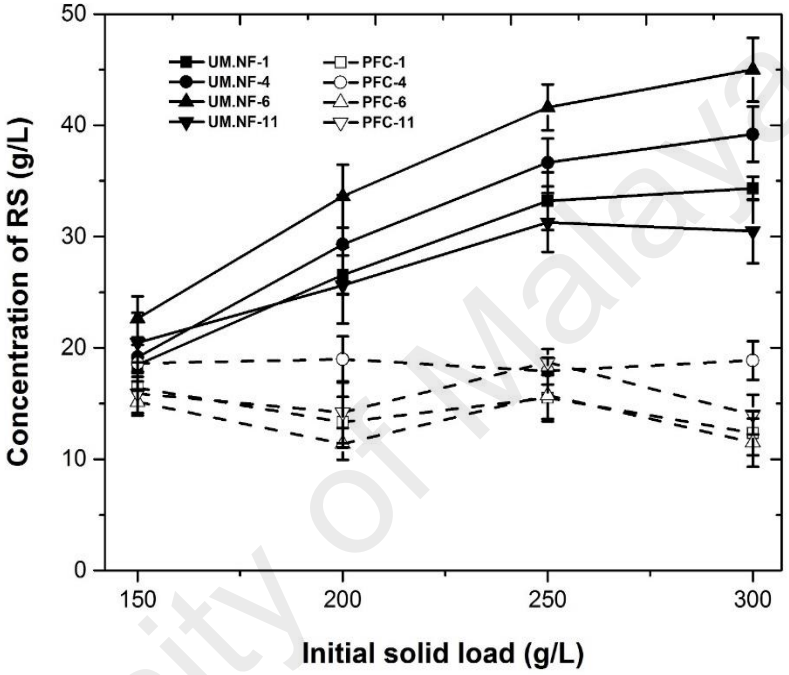

Figure 4.7: Effect of Initial Solid Load on Reducing Sugar Yield During Liquefaction of Corn Genotypes. Process conditions: enzyme, SPECZYME ® FRED (α–amylase); enzyme load, 3.0 kg/MT of dry corn; particle size of ground corn 1.0 mm; temperature, 90ºC; pH, 6.0; reaction time, 2 h. Values are expressed as mean ± SD.

During saccharification, the concentration of RS in HSGs significantly increased when the solid load increased from 150 g/L to 300 g/L (Figure 4.8). On the other hand, PFCs showed a significant drop in RS as the solid load increased from 250 g/L to 300 g/L, with the exception of PFC-4, which showed an increase (Figure 4.8). Under all the conditions of initial solid load, HSGs produced significantly higher amounts of RS than PFCs.

However, it was interesting to note that the variations in RS between HSGs and the respective PFCs, under the lowest solid load (150 g/L), were not found to be significant (P>0.05).

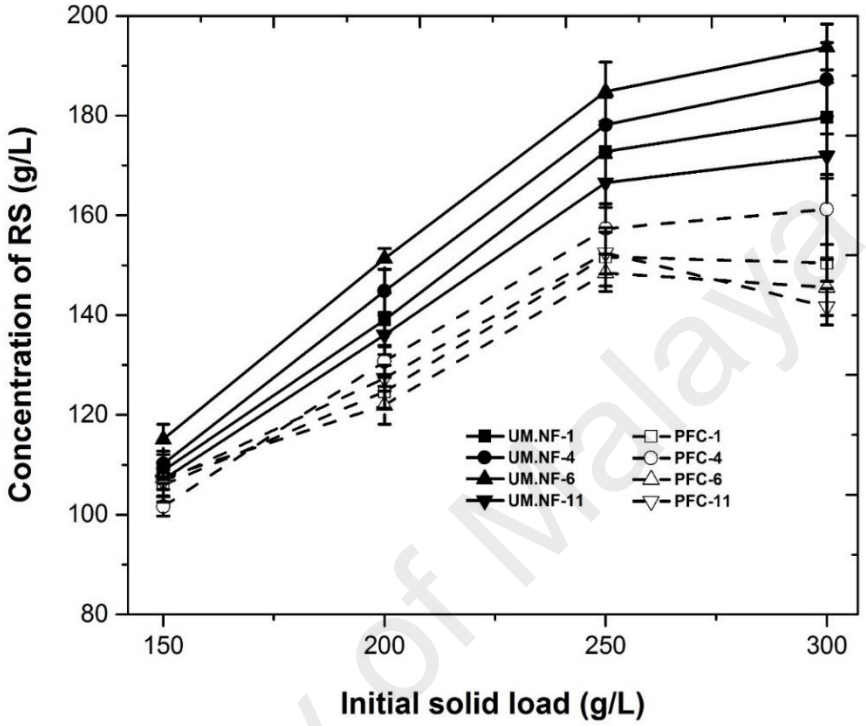

Figure 4.8: Effect of Initial Solid Load on Reducing Sugar Yield During Saccharification of Corn Genotypes. Process conditions for saccharification: enzyme, OPTIDEX ® L 400 (glucoamylase); enzyme load, 3.0 kg/MT of dry corn; particle size of ground corn 1.0 mm; temperature, 30ºC; pH, 4.2; reaction time, 48 h; shaking rate, 200 rpm. Values are expressed as mean ± SD.

The RS yield for the eight corn genotypes during liquefaction and saccharification under four different initial solid loads are summarized in Table 4.3. It can be noted that although RS yield increased slightly in HSGs during liquefaction as the solid load increased from 250 g/L to 300 g/L, all genotypes (PFCs and HSGs) showed a significant drop in RS yield during saccharification at this solid load.

Table 4.3: Average Sugar Yield During Two–Step Enzymatic Hydrolysis (Liquefaction and Saccharification) under Four Different Initial Solid Loads

Genotypes	Hydrolysis step	Reducing sugar yield (g/g of corn)			
		Initial solid load (g/L)			
		150	200	250	300
UM.NF–1	Liquefaction	0.12^{c}	0.18^{b}	0.22^{a}	0.23^{a}
	Saccharification	0.73^{a}	0.70^{b}	0.69^{b}	0.60^{c}
UM.NF–4	Liquefaction	0.13^{c}	0.20^{b}	0.24^{a}	0.26^{a}
	Saccharification	0.73^{a}	0.72^{ab}	0.71^{b}	0.62^{c}
UM.NF–6	Liquefaction	0.15^{c}	0.22^{b}	0.28^{a}	0.30^{a}
	Saccharification	0.77^{a}	0.76^{ab}	0.74^{b}	0.65^{c}
UM.NF–11	Liquefaction	0.14^{b}	0.017^{b}	0.21^{a}	0.20^{a}
	Saccharification	0.71^{a}	0.68^{b}	0.67^{b}	0.57^{c}
PFC–1	Liquefaction	0.11^{a}	0.09^{b}	0.10^{b}	0.08^{c}
	Saccharification	0.71^{a}	0.62^{b}	0.61^{b}	0.50^{c}
PFC–4	Liquefaction	0.12^{a}	0.13^{a}	0.12^{a}	0.13^{a}
	Saccharification	0.68^{a}	0.65^{b}	0.63^{c}	0.54^{d}
PFC–6	Liquefaction	0.10^{a}	0.08^{b}	0.11^{a}	0.08^{b}
	Saccharification	0.72^{a}	0.61^{b}	0.59^{c}	0.49^{d}
PFC–11	Liquefaction	0.11^{a}	0.09^{b}	0.12^{a}	0.09^{b}
	Saccharification	0.71^{a}	0.64^{b}	0.61^{c}	0.47^{d}

Notes: values followed by the same small letter in a row denote insignificant variations among the initial solid loads.

4.3.2 CSSF of the Corn Genotypes

4.3.2.1 Ethanol Profile

It was observed that ethanol concentration in the fermentation broth increased with the increase in enzyme load (Figure 4.9–4.11) and fermentation period (Figure 4.12–4.15) in both HSGs and PFCs. However, the corn genotypes, particularly among the HSGs did not vary significantly in ethanol production after 24 h of CSSF, apart from a few exceptions (Figure 4.9). It was also observed that HSGs produced significantly higher proportions of ethanol under all enzymatic conditions than PFCs ($P < 0.05$). Likewise, higher amounts of ethanol were produced by HSGs in each fermentation period, when

compared to those of PFCs. However, the degree of variation in ethanol concentration between HSGs and PFCs decreased with the increase in enzyme quantity.

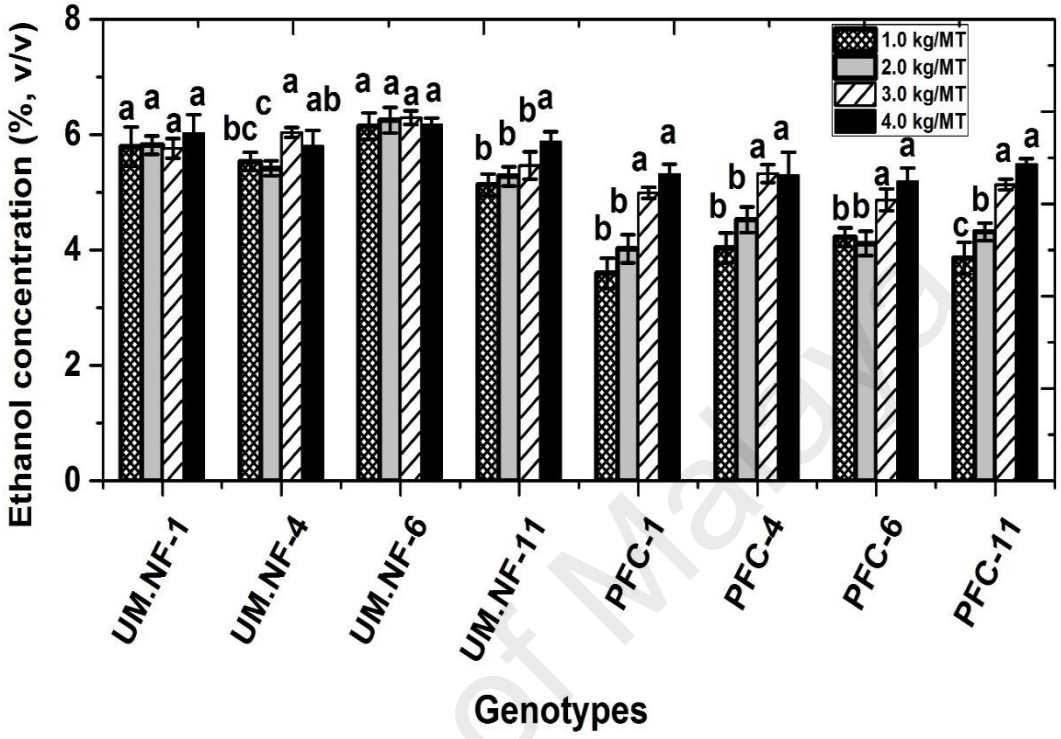

Figure 4.9: Ethanol Production by the Corn Genotypes After 24 H Under Four Different Enzymatic Conditions. Similar letters on different bars in a single genotype denotes insignificant variations in ethanol among the enzyme loads (*P* > 0.05).

After 24 h of CSSF, PFCs produced lower amounts of ethanol than HSGs under the lowest enzyme load, which gradually increased with increasing enzyme concentration No significant variations were observed in ethanol concentration between PFCs. HSGs under the highest enzyme dose (4.0 kg/MT) (Figure 4.9). Similarly, significantly lower amounts of ethanol were produced by PFCs than HSGs under the enzyme doses 1.0, 2.0 and 3.0 kg/MT after 48 and 72 h (Figure 4.10 and 4.11). At the highest enzyme dose, PFCs showed its maximum ethanol profile both after 48 and 72 h, even though HSGs was still producing slightly higher amounts of ethanol.

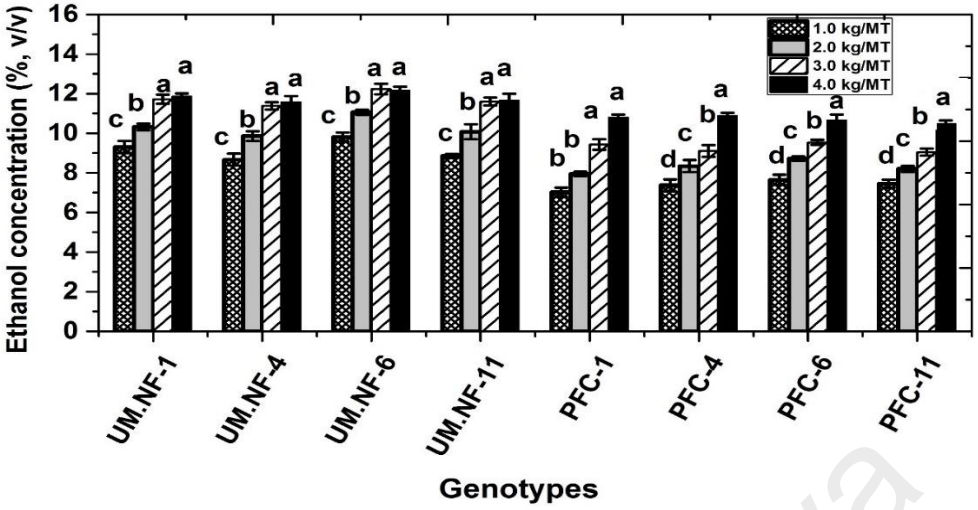

Figure 4.10: Ethanol Production by the Corn Genotypes After 48 h Under Four Different Enzymatic Conditions. Similar letters on different bars in a single genotype denotes insignificant variation in ethanol among the enzyme loads (P > 0.05).

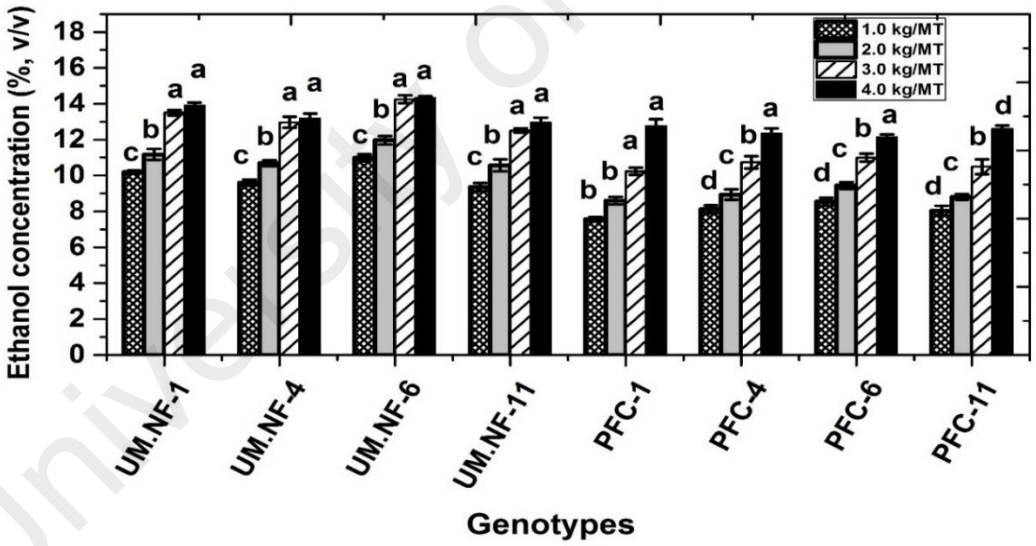

Figure 4.11: Ethanol Production by the Corn Genotypes after 72 h under Four Different Enzymatic Conditions. Similar letters on different bars in a single genotype denotes insignificant variations in ethanol among the enzyme loads (*P* > 0.05).

With the enzyme dose 1.0 kg/MT, HSGs produced roughly 1.5 times more ethanol than PFCs after 24 h., Almost the same ratio was observed even after 48 and 72 h (Figure

4.12). The average final ethanol concentration (after 72 h) ranged from 10.57% (v/v) to 11.97% in HSGs and 8.60% to 9.44% in PFCs under the enzyme load 2.0 kg/MT (Figure 4.13), which reached 12.51% to 14.25% in HSGs and 10.24 to 11.0% in PFCs as the enzyme load increased to 3.0 kg /MT (Figure 4.14). At the highest enzyme load of 4.0 kg /MT, all the PFCs produced significantly higher amounts of ethanol than under the other three enzyme concentrations (Figure 4.15). However, although the HSGs produced slightly higher amounts of ethanol at the highest enzyme concentration, the analysis of variance for ethanol revealed that the variations in ethanol concentrations between the enzyme load 3.0 and 4.0 kg /MT were insignificant ($P > 0.05$).

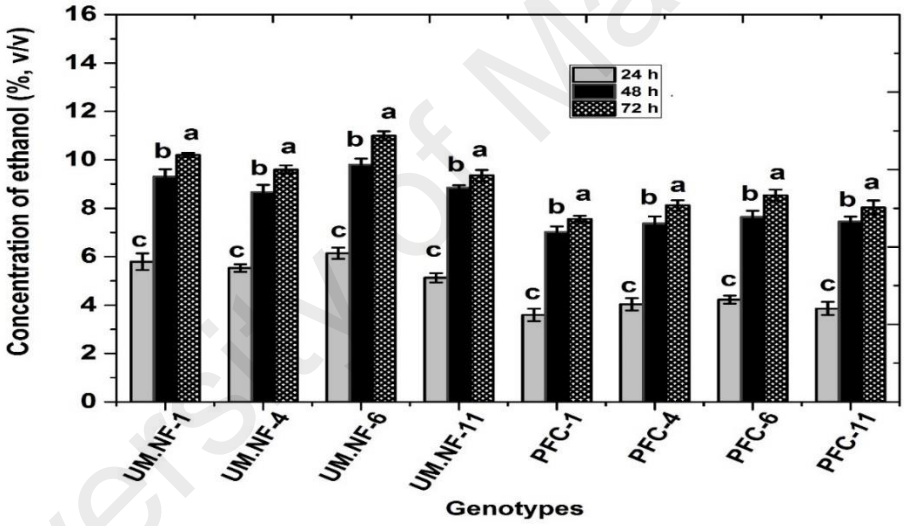

Figure 4.12: Ethanol Production by the Corn Genotypes over Time Under Enzyme Load 1.0 Kg/MT of Dry Corn. Different letters on the bars of a single genotype denote significant variations in ethanol among the fermentation periods ($P < 0.05$).

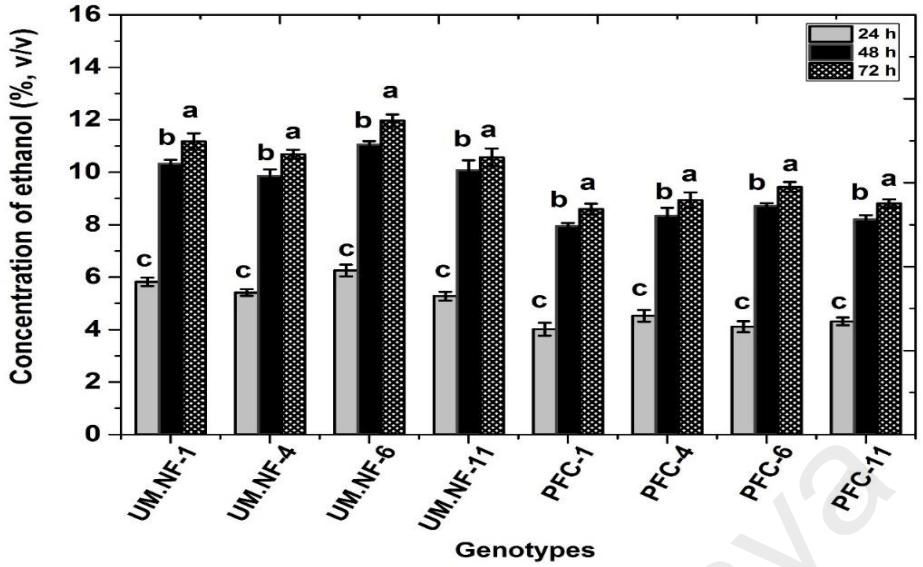

Figure 4.13: Ethanol Production by the Corn Genotypes over Time under Enzyme Load 2.0 kg/MT of Dry Corn. Different letters on the bars of a single genotype denote significant variations in ethanol among the fermentation periods (P < 0.05).

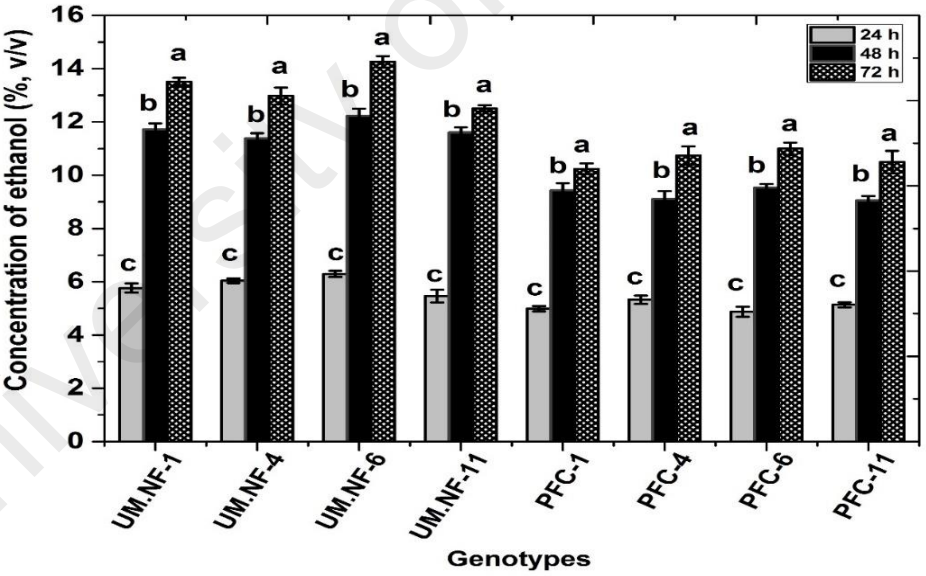

Figure 4.14: Ethanol Production by the Corn Genotypes over Time under Enzyme Load 3.0 kg/MT of Dry Corn. Different letters on the bars of a single genotype denote significant variations in ethanol among the fermentation periods (P < 0.05).

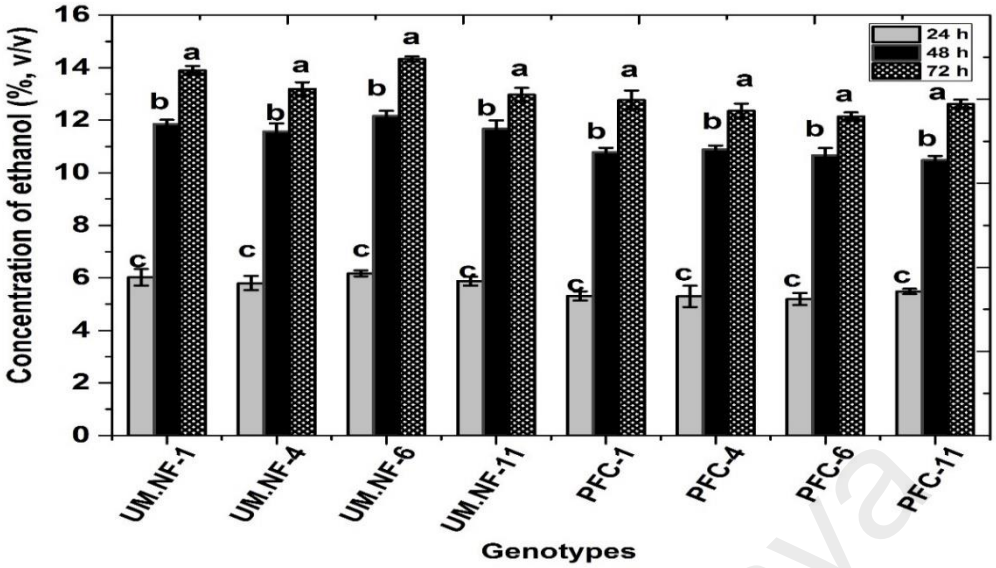

Figure 4.15: Ethanol Production by the Corn Genotypes over Time under the Enzyme Load 4.0 kg/MT of Dry Corn. Different letters on the bars of a single genotype denote significant variations in ethanol among the fermentation periods ($P < 0.05$).

Compared to the respective PFCs, the final ethanol concentration in HSGs were found to have increased by 24.16% in UM.NF–1, 17.20% in UM.NF–4, 22.85% in UM.NF–6 and 16.11% in UM.NF–11, when enzyme concentration was 3.0 kg /MT. It ranged from 2.77 % in UM.NF–11 to 15.24% in UM.NF–6 under the highest enzyme load (4.0 kg/MT). Among the eight corn genotypes, UM.NF–6 produced the highest amounts of ethanol under all conditions that corresponded to its maximum kernel sugars (Chapter 3, Table 3.7).

The kinetics of ethanol production during CSSF is summarized in Table 4.4. In general, ethanol titer and ethanol yield increased over time, while the volumetric ethanol productivity decreased as the fermentation period increased. However, it can be noted that these kinetic parameters were found to have increased with increasing enzyme load. All the HSGs showed higher ethanol productivity than PFCs under all conditions,

particularly, at lower fermentation time and with lower enzyme load. HSGs showed no significant variation in productivity between the enzyme load 3.0 and 4.0 kg/MT or even had similar values under these two conditions. After 24 h of CSSF with an enzyme dose of 1.0 kg/MT, the volumetric ethanol productivity ranged from 1.69 to 2.02 g/L/h in HSGs and 1.18 to 1.69 g/L/h in PFCs. The final ethanol productivity in both HSGs and PFCs were almost similar under all enzyme loads. The final ethanol yield increased with increasing enzyme concentration (Table 4.4). However, the variations in ethanol yield among the HSGs between enzyme load 3.0 and 4.0 kg/MT were not significant, except for UM.NF–11. The average final ethanol yield of HSGs under the enzyme load 1.0, 2.0, 3.0 and 4.0 kg/MT ranged from 0.30 to 0.35, 0.33 to 0.38, 0.39 to 0.45 and 0.41 to 0.45 g/g of dry corn respectively, in which the lowest yield was recorded in UM.NF–11 and the highest in UM.NF–6 under all conditions. On the other hand, PFCs exhibited highest ethanol yield at the highest enzyme concentration and the yield was significantly different from the other three enzyme loads. The average final ethanol yield in PFCs varied between 0.38 g/g in PFC–6 and 0.40 g/g in PFC–1 under the enzyme load 4.0 kg/MT (Table 4.4).

Table 4.4: Kinetics of Ethanol Fermentation from High Sugary Corn Genotypes and Their Parent Field Corn Lines during CSSF under Four Enzymatic Conditions

Genotypes	Time course	*P* (g/L)				*Q* (g/L/h)				*Y* (g/g of dry corn)			
		Enzyme concentrations (kg/MT of dry corn)											
		1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0
UM.NF–1	24	45.70	45.90	45.47	47.53	1.90	1.91	1.89	1.98	0.18	0.18	0.18	0.19
	48	73.42	81.43	92.47	93.60	1.53	1.70	1.93	1.95	0.29	0.33	0.37	0.37
	72	80.43	88.17	106.53	109.70	1.12	1.22	1.48	1.52	0.32	0.35	0.43	0.44
UM.NF–4	24	43.67	42.73	47.63	45.80	1.82	1.78	1.98	1.91	0.17	0.17	0.19	0.18
	48	68.37	77.77	89.80	91.33	1.42	1.62	1.87	1.90	0.27	0.31	0.36	0.37
	72	75.77	84.30	102.33	104.10	1.05	1.17	1.42	1.45	0.30	0.34	0.41	0.42
UM.NF–6	24	48.50	49.33	49.67	48.70	2.02	2.06	2.07	2.03	0.19	0.20	0.20	0.19
	48	77.33	87.23	96.53	96.03	1.61	1.82	2.01	2.00	0.31	0.35	0.39	0.38
	72	86.73	94.43	112.47	113.07	1.20	1.31	1.56	1.57	0.35	0.38	0.45	0.45
UM.NF–11	24	40.47	41.63	43.10	46.37	1.69	1.73	1.80	1.93	0.16	0.17	0.17	0.19
	48	69.83	79.47	91.53	92.13	1.45	1.66	1.91	1.92	0.28	0.32	0.37	0.37
	72	73.77	83.37	98.70	102.33	1.02	1.16	1.37	1.42	0.30	0.33	0.39	0.41
PFC–1	24	28.37	31.70	39.37	41.93	1.18	1.32	1.64	1.75	0.11	0.13	0.16	0.17
	48	55.40	62.70	74.47	85.13	1.15	1.31	1.55	1.77	0.22	0.25	0.30	0.34
	72	59.67	67.83	80.80	100.77	0.83	0.94	1.12	1.40	0.24	0.27	0.32	0.40
PFC–4	24	31.83	35.70	42.00	41.77	1.33	1.49	1.75	1.74	0.13	0.14	0.17	0.17
	48	58.13	65.80	71.87	85.90	1.21	1.37	1.50	1.79	0.23	0.26	0.29	0.34
	72	64.03	70.50	84.73	97.47	0.89	0.98	1.18	1.35	0.26	0.28	0.34	0.39
PFC–6	24	33.30	32.43	38.40	40.97	1.39	1.45	1.60	1.71	0.13	0.13	0.15	0.16
	48	60.33	68.77	75.30	84.17	1.26	1.43	1.57	1.75	0.24	0.28	0.30	0.34
	72	67.37	74.50	86.77	95.83	0.94	1.03	1.21	1.33	0.27	0.30	0.35	0.38
PFC–11	24	30.43	34.03	40.50	43.27	1.27	1.42	1.69	1.80	0.12	0.14	0.16	0.17
	48	58.80	64.70	71.37	82.73	1.23	1.35	1.49	1.72	0.24	0.26	0.29	0.33
	72	63.37	69.50	82.80	99.50	0.88	0.97	1.15	1.38	0.25	0.28	0.33	0.40

Legends: *P*, Ethanol titer; *Q*, Volumetric ethanol productivity; *Y*, ethanol yield

4.3.2.2 Residual Sugars and Starch Concentrations

The starch, glucose, sucrose, fructose and total soluble sugar (TSS) were monitored in each fermented mash at the end of CSSF to determine residual carbohydrates. However, sucrose and fructose were not detected in the fermentation broth at the end of CSSF. Rather, based on a preliminary trial, it was found that most of the sucrose and fructose were used up by 24 h and was almost exhausted after 48 h, resulting in the presence of glucose and other sugar molecules derived from the incomplete hydrolysis of starch in the residual sugars.

At the end of CSSF, considerable amounts of starch remained unutilized, and although the quantity of residual starch increased in both group of genotypes with the decrease in enzyme load, as a whole, the amount of residual starch were relatively higher in PFCs than in HSGs (Figure 4.16). The concentration of residual starch recorded for the lowest enzyme load ranged from 42.76 to 53.28 g/L in HSGs and 56.29 to 64.74 g/L in PFCs, but it showed a significant drop under the highest enzyme load, accounting for 5.29 to 9.56 g/L residual starch in HSGs and 10.81 to 14.79 g/L in PFCs. However, the analysis of variance revealed that the amount of residual starch did not vary significantly between the enzyme loads of 3.0 and 4.0 kg/MT in HSGs, except for UM.NF–11. In PFCs it showed significant variations in residual starch quantity. Accordingly, the utilization of starch during CSSF increased as the enzyme load increased, and thereby HSGs showed higher utilization than the PFCs (Figure 4.17). The starch conversion rate recorded for HSGs varied between 94.47% and 96.77%, whereas, for PFCs it ranged between 91.79% and 93.58% when the enzyme load was 4.0 kg/MT.

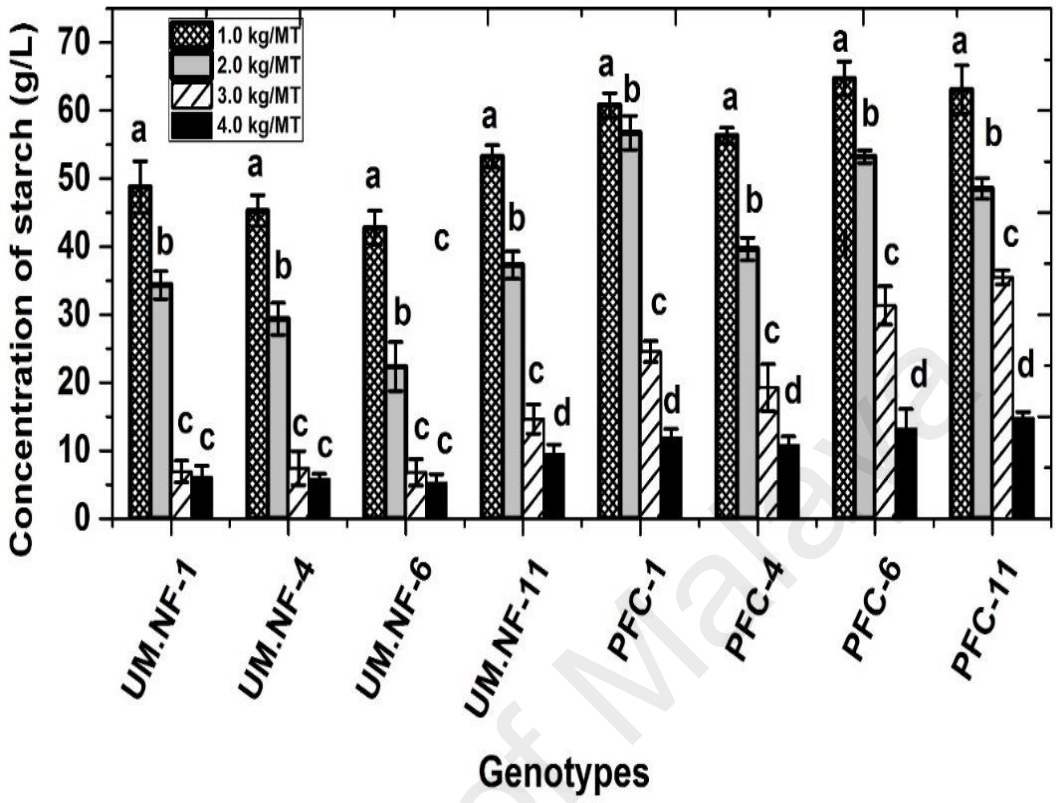

Figure 4.16: Concentration of Starch at the End of CSSF under Enzymatic Conditions. Different letters on the bars of a single genotype denote significant variations in residual starch among the enzyme loads (P < 0.05).

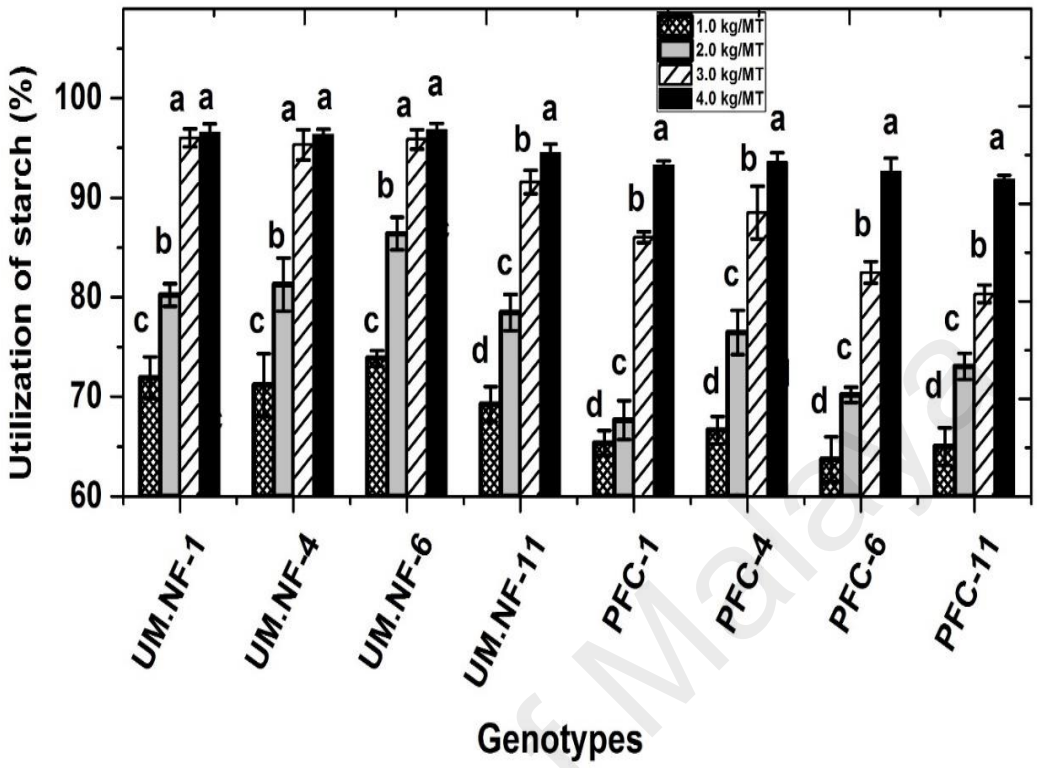

Figure 4.17: Utilization of Starch during CSSF under Four Enzymatic Conditions as Determined at the end of CSSF. Different letters on the bars of a single genotype denote significant variations in utilization of starch among the enzyme loads (P < 0.05).

In contrast to residual starch, small amounts of glucose remained unutilized and the concentration of residual glucose increased with increasing enzyme load, particularly in PFCs (Figure 4.18). In seven of the eight genotypes, with the exception of UM.NF-1, the amount of residual glucose was found to be less than 1.0 g/L during CSSF when the first three doses of enzyme were used. A similar range of residual glucose was recorded in three HSGs, even under the highest enzyme load (4.0 kg/MT), while the remaining HSG (UM.NF–1) and all the PFCs left behind slightly higher amounts of residual glucose under the same enzyme load. However, PFCs left lower amounts of residual glucose than HSGs under lower

enzyme concentrations but showed a significant increase with the increase in enzyme concentration ($P < 0.05$). Similar to residual glucose, small amounts of TSS were recorded after CSSF, even though the values were higher than those of glucose (Figure 4.19).

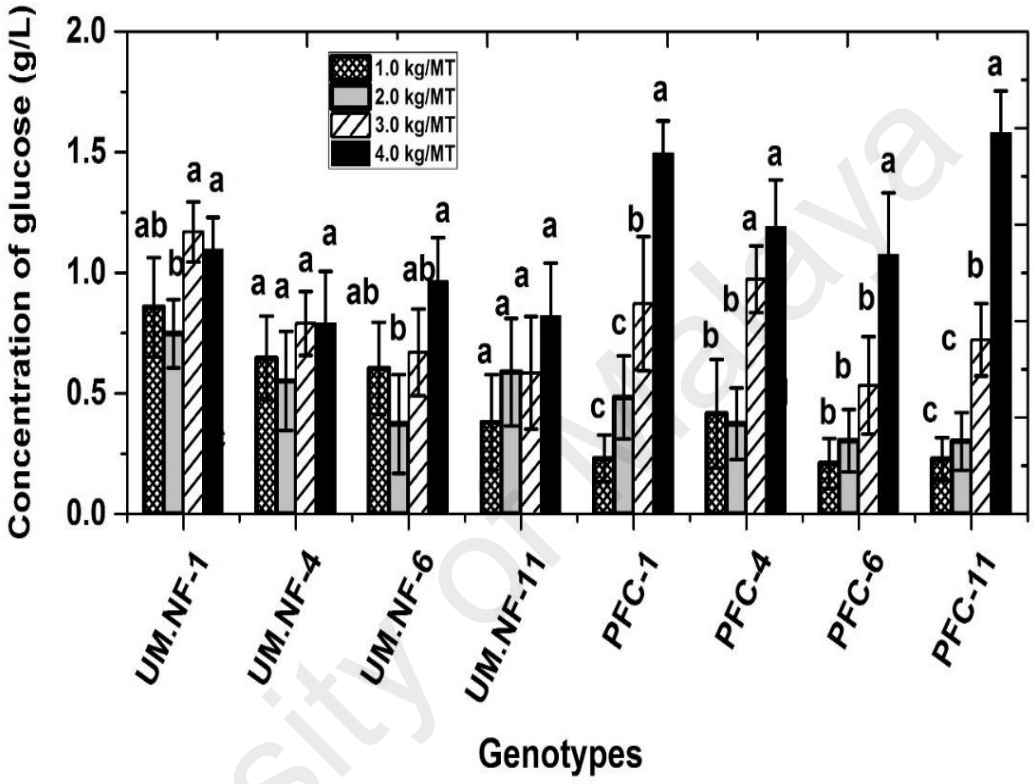

Figure 4.18: Concentration of Glucose at the end of CSSF with Four Different Enzyme Loads. Different letters on the bars of a single genotype denote significant variations in residual glucose among the enzyme loads ($P < 0.05$).

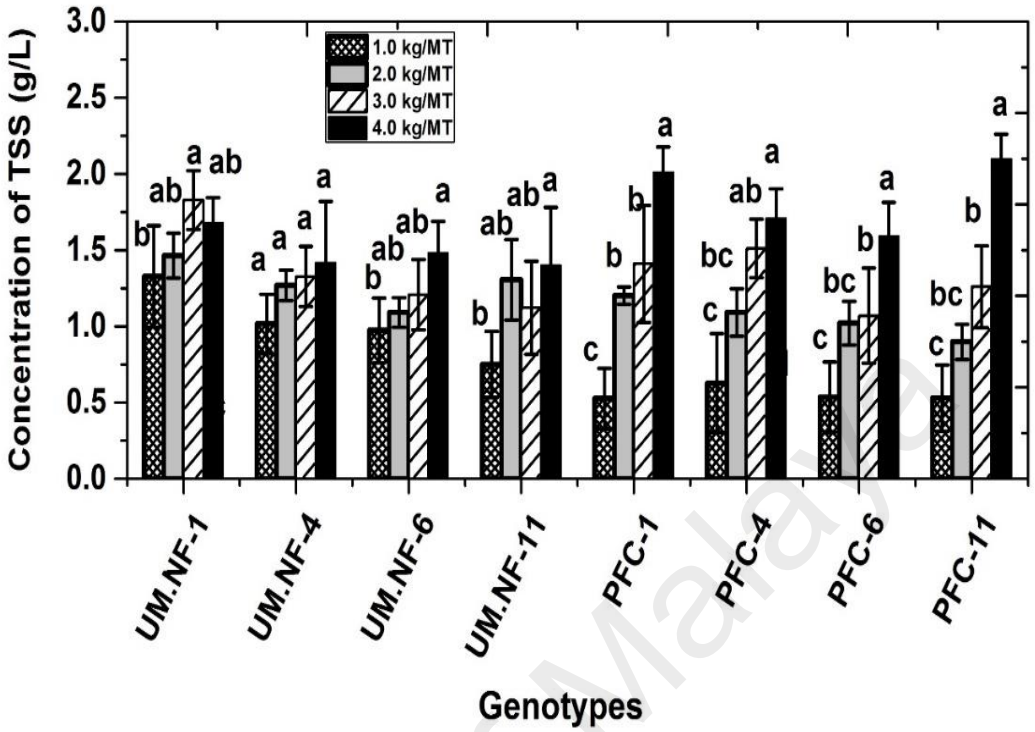

Figure 4.19: Concentration of TSS at the End of CSSF with Four Enzyme Loads. Different letters on the bars of a single genotype denote significant variations in residual TSS among the enzyme loads ($P < 0.05$).

4.3.2.3 Viable Counts during CSSF

During CSSF, yeast cells grew exponentially until 48 h and subsequently underwent a stationary phase, irrespective of enzyme load and corn genotypes (Figure 4.20). The viable yeast counts did not vary significantly among the different corn genotypes and enzyme doses for a specific period of fermentation ($P > 0.05$). The viable bacterial counts in the fermentation broth were found to be relatively lower after 24 h of CSSF, which ranged from 7.43×10^3 to 1.73×10^4 CFU/ml (Figure 4.21). But, it increased moderately after 48 h. The viable bacterial counts showed a sharp increase at the end of CSSF under all conditions.

However, the overall counts among the genotypes and under different enzymatic conditions were almost similar for a specific period of CSSF.

4.3.2.4 Production of Glycerol and Lactic Acid during CSSF

During the course of CSSF, the accumulation of glycerol and lactic acid in the fermentation broth increased significantly over time (Figure 4.22 and 4.23), as shown by analysis of variance ($P < 0.05$). Glycerol accumulation in the fermentation broth showed a sharp increase over time, with low values of around 2.0 g/L after 24 h and reaching a peak after 72 h with 14.0g/L (Figure 4.22). On the other hand, lactic acid concentration in the broth was below 1.0 g/L after 24 and 48 h, but increased significantly afterwards and finally reached 2.0 g/L after 72 h, as lactic acid accumulation reached its peak (Figure 4.23).

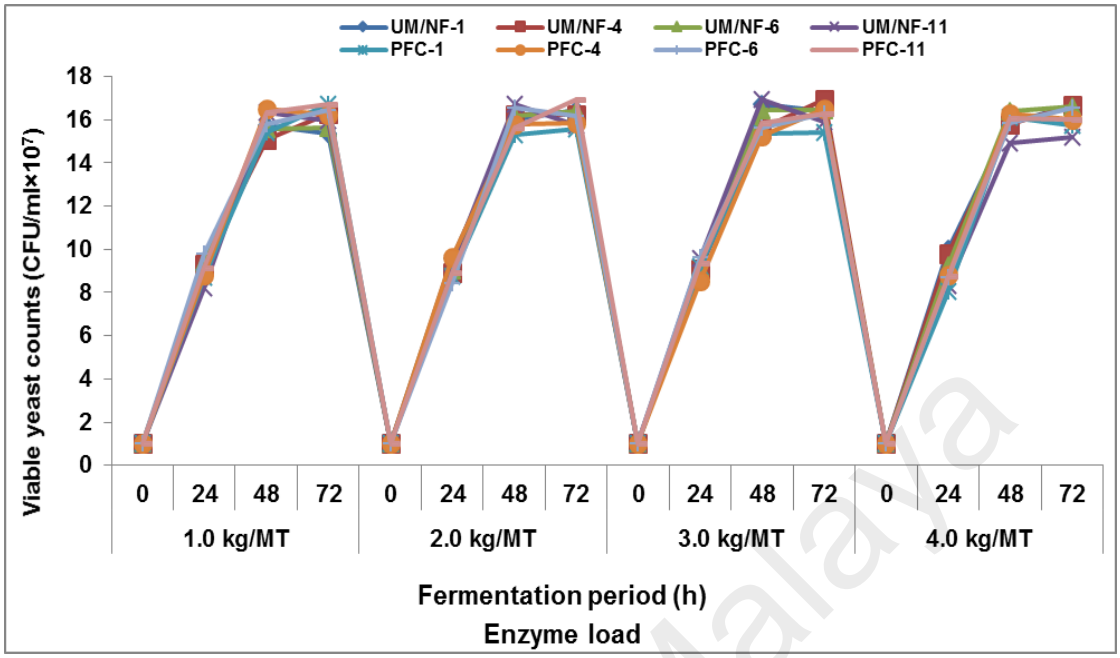

Figure 4.20: Changes in the Viable Yeast Counts over Time during CSSF under four enzymatic conditions.

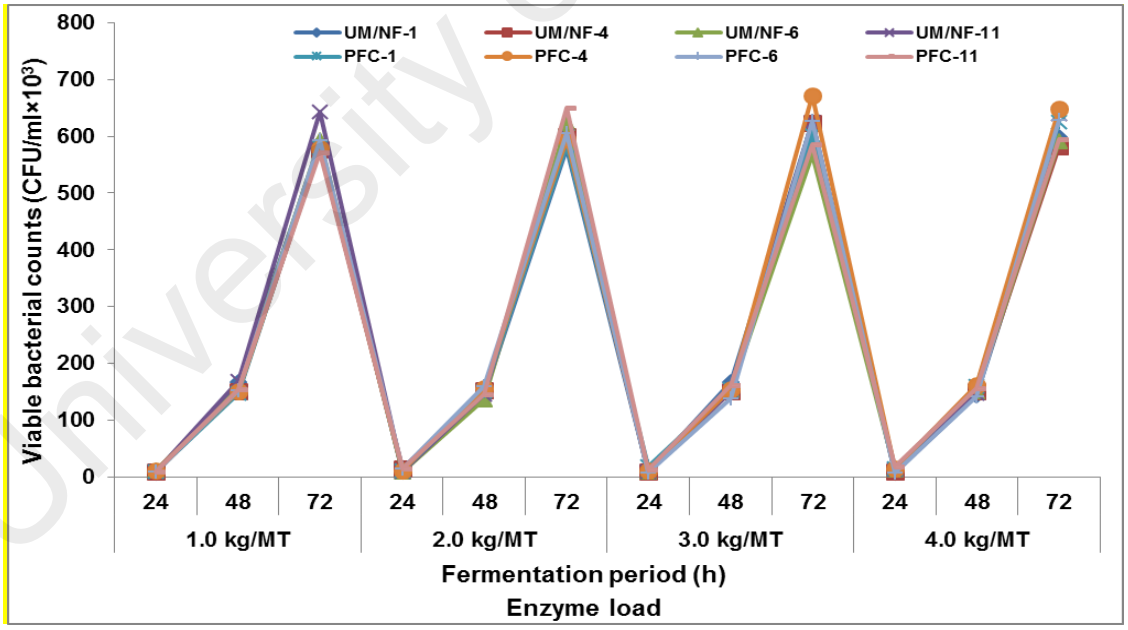

Figure 4.21: Changes in the Viable Bacterial Counts over Time during CSSF under Four Enzymatic Conditions.

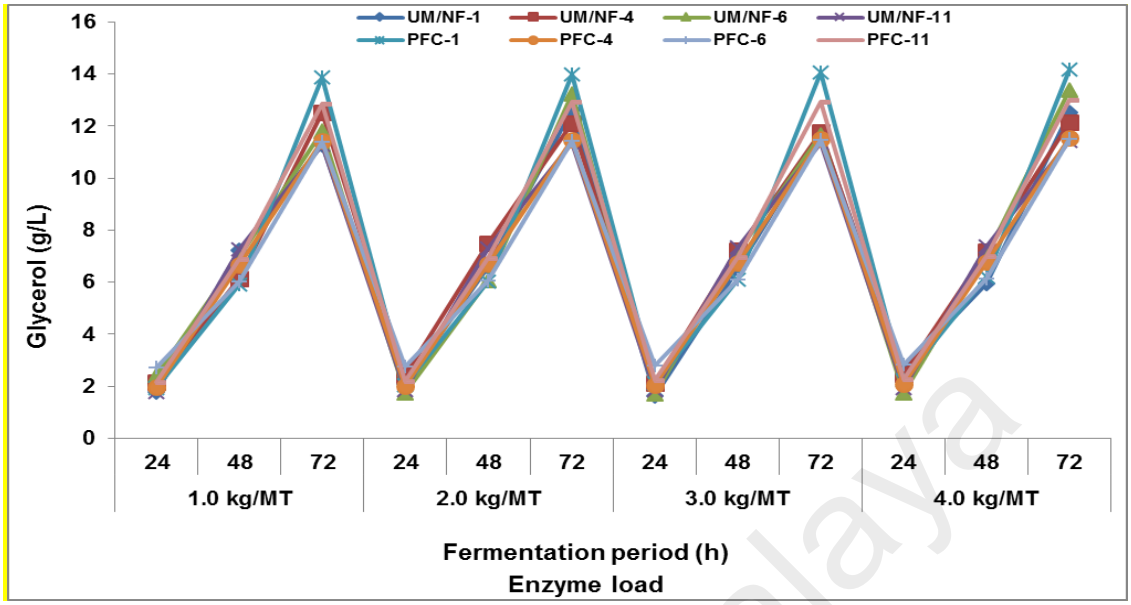

Figure 4.22: Accumulation of Glycerol in the Fermentation Broth over Time During CSSF Under the Four Enzymatic Conditions.

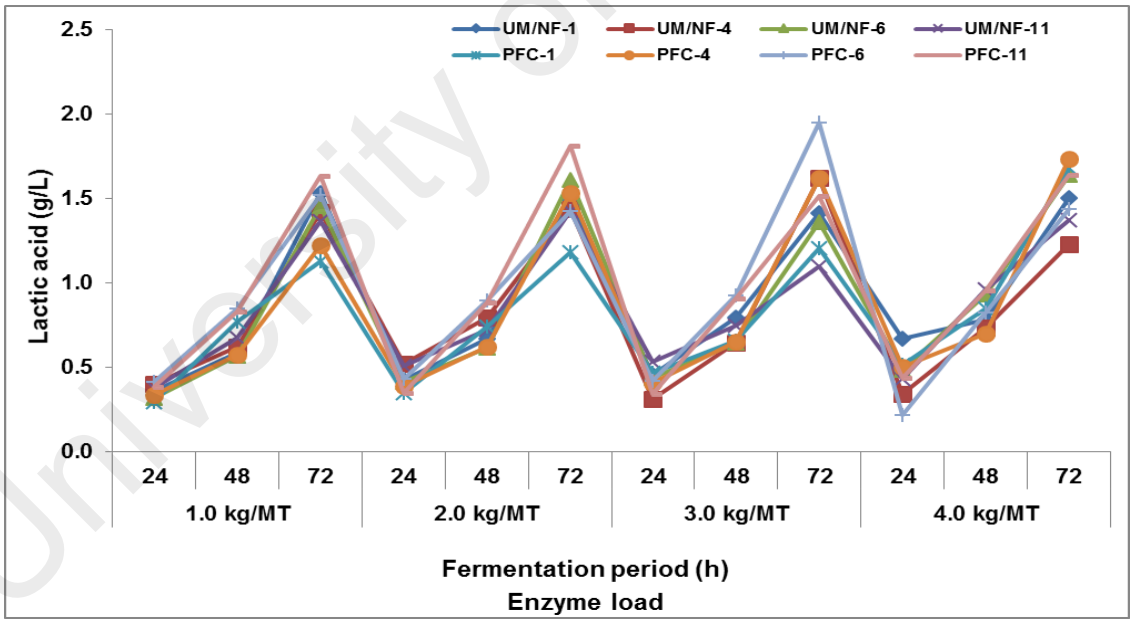

Figure 4.23: Accumulation of Lactic Acid in the Fermentation Broth over Time during CSSF under the Four Enzymatic Conditions.

4.3.2.5 Correlation among Different Parameters

The relation among fermentable sugar yield after two-step enzymatic hydrolysis, ethanol yield and residual starch after CSSF with agronomic and agronomic traits were evaluated through determination of correlation of coefficients using data obtained from Chapter 3 for 2012. As shown in Table 4.5, fermentable sugar yield, ethanol concentration and residual starch showed significant correlation with, particularly, biochemical traits. All the types of kernel sugars were found to be significantly and positively correlated with fermentable sugars and ethanol yields, whereas, kernel starch showed negative correlation with fermentable sugars and final ethanol yields. On the contrary, the quantity of residual starch of the corn genotypes highly correlated with kernel starch ($r = 0.638$; $P < 0.01$). A few agronomic traits also showed significant correlations with ethanol concentration.

Table 4.5: Correlations between Yields of Hydrolysis and CSSF, Agronomic and Biochemical Properties

Parameters	Fermentable sugars	Ethanol	Residual starch
Kernel sucrose	0.734**	0.809**	–0.843**
Kernel glucose	0.636**	0.624**	–0.806**
Kernel fructose	0.621**	0.807**	–0.710**
Kernel TRS	0.793**	0.810**	–0.789**
Kernel TSS	0.757**	0.811**	–0.854**
Kernel starch	–0.609**	–0.299	0.638**
Kernel protein	0.542**	0.297	–0.551**
Kernel fat	0.511**	0.135	–0.488**
Kernel fiber	0.144	0.221	–0.252
Amylose	–0.374*	–0.293	0.158
Seed emergence time	0.293	0.421*	–0.202
Anthesis time	–0.210	–0.287	0.118
Silking time	–0.230	–0.353*	0.056
Grain filling period	–0.165	–0.416*	0.163
Black layer maturity	–0.203	–0.408*	0.122
Plant height	–0.287	–0.526**	0.324
Leaf number per plant	0.028	0.322	–0.149
Leaf length	–0.507**	–0.397*	0.530**
Ear weight	–0.126	–0.234	0.132
Ear length	–0.352*	–0.341	0.225
Kernel number per plant	0.183	0.314	–0.363*
Thousand kernel weight	–0.168	–0.342	0.200
Grain yield	–0.040	–0.174	–0.085
Kernel ash	0.342	0.298	–0.517**
Fermentable sugar		0.735**	–0.784**
Ethanol			–0.721**

*, significant at 5% probability level ($P < 0.05$)

**, significant at 1% probability level ($P < 0.01$)

Agronomic and biochemical data were collected from Chapter 3

4.4 Discussion

4.4.1 Two–Step Enzymatic Hydrolysis

Enzymatic hydrolysis of starch relies on some factors, such as particle size, initial solid load in the slurry, pH, temperature, enzyme load and incubation time (Mojović et al., 2006). Optimum conditions of these parameters have been well established by either the enzyme manufacturers or other researchers in their studies (Apar & Özbek, 2004; Zheng et al., 2009). However, the present study was conducted with different particle sizes, enzyme concentrations, solid loads and reaction times, investigating whether these parameters had any effects on the sugar releasing capacity of HSGs. Under all conditions, it was found that HSGs produced significantly higher amounts of RS than PFCs, and the differences in RS concentration between HSGs and PFCs were more apparent during liquefaction than what was observed in saccharification, which indicated that most of the kernel sugars were released during liquefaction (Zabed et al., 2016a).

4.4.1.1 Effect of Enzyme Load on RS Yield

The enzyme loads used for both liquefaction and saccharification varied between 1.0 and 4.0 Kg/MT of dry corn. The highest enzyme load (4.0 Kg/MT) used in this study was also the optimum dose for many earlier studies during bioethanol production from corn (Lemuz et al., 2009; Murthy et al., 2009), and also recommended as the optimum dose by an enzyme manufacturer (http://www.genencor.com). Although production of RS increased with the increase in enzyme load, corn genotypes produced significantly lower amounts of RS under the enzyme loads 1.0 and 2.0 Kg/MT during both liquefaction and saccharification (Figure 4.1 and 4.2; Table 4.1), which can be considered insufficient for producing the desired amounts of ethanol in the subsequent fermentation step. However, HSGs produced

substantial amounts of RS using the enzyme load 3.0 Kg/MT, and it did not vary significantly with a further increase in enzyme concentration. This might be due to the unavailability of starch molecules for the increased amount of enzyme added (Hagenimana et al., 1992). In addition, the phenomenon of the end product inhibition of the enzymes involved may also result it (Hill et al., 1997; Mojović et al., 2006), since HSGs have contributed considerable amounts of kernel sugars in the hydrolysates. The PFCs, in contrast, produced their highest amount of RS in the presence of 4.0 Kg/MT enzyme load. This could be due to the fact that PFCs contained higher amounts of starch in the kernels as well as in the slurries, in addition to their capability of releasing small amounts of sugars from kernels (Zabed et al., 2016a).

4.4.1.2 Effect of Reaction Time on RS Yield

The RS yield was evaluated at different reaction times during both liquefaction and saccharification to determine the optimum time to produce sufficient amounts of soluble sugars through hydrolysis of kernel starch. At the same time HSGs releases its kernel sugars into the hydrolysates. It can be noted that there was an apparent and significant difference in the concentrations of RS between HSGs and PFCs during liquefaction, which was found to be minimized during saccharification, probably due to the favorable conditions for starch hydrolysis in PFCs. Although liquefaction was carried out for 3 h, RS production did not vary significantly after 2 h in both groups of corn genotypes (Figure 4.3), which was also reported elsewhere (Mojović et al., 2006). In the same way, the corn genotypes did not show any significant variations in their RS production after 48 h during saccharification (Figure 4.4), which was also similar to the findings of (Mojović et al., 2006). The incapability of enzymes to exert significant changes in RS production above a certain period of time during

hydrolysis could be due to the inhibition of the hydrolytic enzymes by the soluble sugars produced (Hill et al., 1997; Noda et al., 1992).

4.4.1.3 Effect of Particle Size on RS Yield

Particle size of ground corn has significant effects on RS yield during enzymatic hydrolysis (Al-Rabadi et al., 2009). Hence, liquefaction and saccharification were conducted using four different particle sizes (0.5, 1.0, 2.0 and 3.0 mm) with an enzyme dose of 3.0 kg/MT. Another purpose for studying hydrolysis with different particle sizes, was to investigate whether the release of kernel sugars in HSGs are affected by changes in particle size. It was observed that the concentration of RS in the hydrolysates of PFCs and HSGs increased with the decrease in particle size (Figure 4.5 and 4.6). Particle size of the ground grains subjected to enzymatic hydrolysis usually influence the available surface area for the chemical and biochemical reactions during starch hydrolysis, and smaller particle size with larger surface area favor efficient enzymatic catalysis (Al-Rabadi et al., 2009; Barcelos et al., 2011a), as well as the release of available soluble sugars into the hydrolysates (Zabed et al., 2016a). For this reason, 0.5 mm produced maximum RS during both liquefaction and saccharification in all genotypes. Nevertheless, a smaller particle size requires higher energy during grinding that ultimately increases production cost, in addition to the difficulties in downstream processing (Wang et al., 2008). Furthermore, current dry–grind ethanol industries use corn meal with a particle size of <1.0 mm (Szymanowska-Powałowska et al., 2014). Considering the aforementioned facts, corn meal with 1.0 mm particle was selected for all further experiments.

4.4.1.4 Effect of Initial Solid Load on RS Yield

During liquefaction, the concentration of RS increased proportionally to the initial solid load (Figure 4.7 and 4.8). However, the production of RS in PFCs interestingly showed a slight drop when the initial solid load increased from 150 g/L to 200 g/L, except for PFC–4 that showed a plateau in RS concentration under this condition. The reason for these findings could possibly be due to the fact that significantly lower amounts of starch were present in the slurries of PFCs at the initial solid load 150 g/L. The initial solid load of 150g/L, is favorable for enzyme–substrate reaction, avoiding the substrate–substrate collision and substrate–enzyme competition (Mojović et al., 2006). A similar phenomenon might have occurred in PFC–4 when the initial solid load was 200 g/L, as it contained the lowest starch in the kernels among PFCs (Chapter 3, Table 3.7).

The concentration of RS increased exponentially during saccharification in all genotypes until an initial solid load of 250 g/L. However, although RS concentration increased slightly in HSGs as the initial solid load increased from 250 g/L to 300 g/L, PFCs showed a plateau or even decreased under this condition. A decrease in sugar production above a certain solid load was also reported in an earlier study during ethanol production from normal corn (Lemuz et al., 2009), where the authors reported that ethanol yield had increased with the increase in initial solid concentration from 20 to 30%, followed by a decrease when solid concentration was further increased. Lower solid content in the slurries are usually suitable for effective agitation and mixing of the substrate and enzymes during hydrolysis, and results in a higher efficiency of the process (Lemuz et al., 2009). However, even though HSGs produced higher amounts of RS under all conditions, production of RS under an initial solid load 150 g/L did not differ significantly from that of PFCs. This could be due to the fact that the slurries of

PFCs received lower amounts of starch at lower initial solid load, and as a result, starch could have been hydrolyzed more efficiently under proper enzyme–substrate conditions as well as minimum substrate inhibition of the enzyme (Mojović et al., 2006). At the same time, the presence of free sugars in the slurries in HSGs also decreased, as the amounts of corn meal decreased under lower solid concentration.

4.4.1.5 Sugar Yield of the Corn Genotypes

Different amounts of RS were produced as a result of the enzymatic conversion of starch during liquefaction and saccharification, and the higher proportion of RS indicated that higher amounts of smaller sugar molecules in the hydrolysate could be easily fermented by yeast in the subsequent step. The results obtained from hydrolysis under the optimum conditions clearly shows that HSGs produced relatively higher amount of RS than PFCs under all conditions due to having higher proportions of kernel sugars. In a previous study, (Mojović et al., 2006) reported a DE value 68.1 (equivalent to 170.25 g/L of RS) after two–step hydrolysis of normal corn meal during dry–grind ethanol production. In this study, although PFCs were found to be almost similar to the findings of (Mojović et al., 2006) under the optimum conditions, HSGs produced higher amounts RS than the reported values.

4.4.2 Ethanol Production during CSSF

Ethanol fermentation of the corn genotypes was carried out by SSF at 30^{o}C, adding glucoamylase and yeast simultaneously, since it reduces feedback inhibition on the enzyme and osmotic stress on yeast cells (Srichuwong et al., 2009). Prior to SSF, starch in the slurries was liquefied using four different SPECZYME ® FRED concentrations. Subsequently, SSF was conducted by adding different concentrations of OPTIDEX ® L–400, which were added

such a way that one flask got similar concentration of both enzymes. During hydrolysis, effects of both α–amylase and glucoamylase loads on fermentable sugar yield were studied separately, whereas during SSF similar dose of both enzymes in a single flask was tested to investigate their combined effect on ethanol yield. Corresponding to the results obtained from hydrolysis, HSGs showed higher ethanol titer, volumetric productivity and ethanol yield under all enzymatic conditions (Table 4.4) as they contained higher amount of kernel sugars.

As was shown in Figures 4.14 and 4.15 in the results section, the final ethanol concentrations produced by HSGs and PFCs are quite comparable with those of several previous investigations conducted with different corn hybrids. In a study with eleven corn hybrids, Murthy et al. (2009) reported that ethanol concentration varied between 12.7% and 13.7% among the hybrids. Investigating five corn hybrids (high amylose, high oil, white, waxy and dent hybrids), Lemuz et al. (2009) reported the ethanol concentration between 1.06 ga/bu (*i.e.*, 39.5 g/L) in high amylose hybrids and 2.92 ga/bu (*i.e.*, 39.5 g/L) in dent hybrid. Studying eight waxy and four normal corn lines, Yangcheng et al. (2013) reported that the formers produced higher amounts of ethanol, ranging from 34.6 to 37.9 g/100 g corn (equivalent to 86.0 to 94.8 g/L), while the latter produced between 34.2 and 37.5 g/100 g of corn (equivalent to 85.5 to 93.8 g/L).

4.4.3 Utilization of Carbohydrates during CSSF

The quantity of residual starch increased with decrease in enzyme load, and as a whole, PFCs left relatively higher amounts of residual starch than HSGs (Figure 4.16), due to the fact that the latter contained lower concentrations of starch in the slurries at the initial stage, corresponding to lower amounts of kernel starch (Chapter 3; Table 3.7), which enabled starch to react efficiently with the same amount of enzyme used in both groups of genotypes

(Mojović et al., 2006). Moreover, SSF with lower enzyme load left higher amounts of starch unutilized, because there is insufficient enzyme to catalyze the hydrolysis under these conditions (Hagenimana et al., 1992). As a result, the utilization of the starch during CSSF increased as the enzyme load increased, where HSGs showed higher rate of starch utilization than the PFCs (Figure 4.17). Plumier et al. (2015) reported that more than 5% of starch remained unutilized during the conventional dry–grind ethanol production. In the present study, utilization of starch by HSGs varied between 94.47% and 96.77%, whereas, for PFCs it ranged from 91.79% to 93.58% in the utilization of starch during CSSF under the highest enzyme load.

Compared to starch, small amount of residual glucose was recorded at the end of fermentation in all genotypes. It has been documented in the literature that glucose is sometimes not fully utilized partially due to the limited ethanol tolerance of yeast over time (Srichuwong et al., 2009). Most of the sucrose and fructose in the mash were used up by 24 h, and a complete exhaustion was observed after 48 h (based on a preliminary study of fermentation broth every 24 h). A similar observation was reported for ethanol production from tropical maize containing a mixture of sucrose, glucose and fructose (Chen et al., 2013). Consequently, the total soluble sugar (TSS) present in the fermentation broth after 24 h and subsequent courses contained only glucose and/or other soluble sugars obtained from the incomplete hydrolysis of starch, such as saccharides containing four or more glucose units (DP_{4+}), maltotriose (DP_3), and maltose (DP_2) (Devantier et al., 2005).

4.4.4 Viable Yeast and Bacterial Counts during CSSF

The growth of yeast cells should be monitored during fermentation to determine whether it is affected by the presence of any inhibitory substances in the media. Furthermore,

proliferation and survival of yeast cell may also be affected by osmotic stress and an ethanol tolerance limit. On the other hand, bacterial contamination in the fermentation broth is undesirable, since they affect final ethanol yield by consuming soluble sugars and producing different byproducts (Thomas et al., 2001). As an attempt to reduce bacterial contamination during fermentation, antibiotics are often used in commercial plants, even though bacterial contamination still exist (Narendranath et al., 1997).

During CSSF, yeast cells grew exponentially in all samples until 48 h and subsequently underwent a stationary phase (Figure 4.20). The exponential growth of yeast cells until 48 h was also reported by (Szymanowska-Powałowska et al., 2014) during ethanol production from corn. However, the stationary phase was reported after 10–12 h three yeast strains in a different study, even though more than half of the ethanol was produced during this stationary phase (Devantier et al., 2005). The growth of yeast cells in this study did not show significant variation with the changes in enzyme load, which is in excellent agreement with the findings of Devantier et al. (2005). The total viable bacterial counts showed a gradual increase over time, and finally, after 72 h, ranged between 5.68×10^5 and 6.72×10^5 CFU/ml (Figure 4.21). In a previous study, total bacterial concentration in corn mash has been reported to be between 2.0×10^9 and 3.3×10^9 CFU/ml after 72 h, without having any significant effect on the final ethanol yield (Szymanowska-Powałowska et al., 2014).

4.4.5 Production of Soluble Byproducts during CSSF

In the present study, the average concentration of glycerol varied between 1.61 g/L after 24 h to 14.18 g/L after 72 h (Figure 4.22). These findings are well with the range of what has been reported for conventional dry-grind ethanol production. It was reported that typically 12–15 g/L glycerol is produced during fermentation (Russell, 2003). The lactic acid

concentration in the broth was found to be below 1.0 g/L until 48 hours, which reached to nearly 2.0 g/L after 72 h (Figure 4.23). This was slightly higher than what has been reported earlier (< 0.3 g/L) by Murthy et al. (2009). On the other hand, a higher concentration of lactic acid (0.5–4 g/L) has been reported during fermentation of corn mash, which did not significantly affect the final ethanol yield (Białas et al., 2010).

4.4.6 Correlation among different parameters

In the present study, the quantity of RS as well as ethanol showed positive correlations with all kinds of kernel sugars, but negatively correlated with kernel starch and amylose (Table 4.5), which were also reported in several earlier studies (Gumienna et al., 2016; Li et al., 2004; Tester et al., 2006).

4.5 Conclusion

This study has shown that HSGs produced a higher concentration of fermentable sugars and ethanol than PFCs during conventional hydrolysis and fermentation, consuming a lower dose of enzyme. It can also be noted that HSGs showed higher volumetric ethanol productivity and left lower residual starch after fermentation, which makes them a promising feedstock for bioethanol production. Furthermore, HSGs were able to produce appropriate amounts of ethanol at a relatively lower enzyme load, which will result in lowering the production costs. We therefore conclude that HSGs are promising candidates for improving ethanol yield and reducing enzyme consumption during dry–grind ethanol production.

CHAPTER 5: GRANULAR STARCH HYDROLYSIS AND SIMULTANEOUS FERMENTATION OF CORN GENOTYPES TO EVALUATE ENZYME CONSUMPTION AND PRODUCT YIELD IN A NON COOKING DRY–GRIND PROCESS

5.1 Introduction

As was described previously (Chapter 2, Section 2.8), the conventional method for dry-grind ethanol production requires high energy during cooking and liquefaction of the corn slurries. To minimize the energy consumption, a granular starch hydrolyzing enzyme (GSHE) has been developed by Genencor (Genencor, 2009), which hydrolyzes raw starch at sub-gelatinized temperature (<48°C), and the whole process has been termed as granular starch hydrolysis (GSH) or raw starch hydrolysis (RSH) (Uthumporn et al., 2010; Wang et al., 2007). However, simultaneous hydrolysis and fermentation using GSHE is referred to as granular starch hydrolysis and simultaneous fermentation (GSHSF), in which raw starch is hydrolyzed by GSHE to generate soluble sugars and fermented simultaneously by yeast, and finally ethanol produced (Yangcheng et al., 2013).

GSHSF has some exclusive advantages over the conventional method as described in Chapter 2 (Section 2.13). However, a low hydrolysis rate at sub–gelatinized temperatures, due to the structural heterogeneity and crystallinity of native starch, poses an additional challenge for overall conversion process and yields (Li et al., 2012). To overcome the shortcomings, it has been suggested to modify the process that includes a mild heat treatment of the corn slurries at around 60^{o}C, supplemented with urea and protease before subjecting to GSHSF (Genencor, 2009; Li et al., 2012; Gohel et al., 2013).

Research efforts have been made recently to compare the conventional and GSHSF based dry-grind ethanol production with regard to ethanol yield and DDGS yield. It concluded that GSHSF has the potential to be an attractive technique for producing ethanol in a cost effective and eco-friendly way (Wang et al., 2005; Wang et al., 2007). As a result, GSHSF has been used extensively to produce ethanol from corn, on both large and small scale operations (Białas et al., 2014; Białas et al., 2010; Lamsal et al., 2011; Szymanowska-Powałowska et al., 2014; Wang et al., 2007).

It has been well documented that several factors have a significant influence on the enzymatic conversion of starch (Apar & Özbek, 2004; Mojović et al., 2006), and its optimization have been reported by earlier investigators and enzyme manufacturers. However, most of these optimization studies were confined to the conventional ethanol production process and scarce information available for GSH of corn, despite the fact that this kind of hydrolysis has been investigated on other feedstocks such as Indian broken rice and pearl millet (Gohel & Duan, 2012b).

In the present study, the grain sugars, which are higher in HSGs must be released in the hydrolysates during GSH to increase the fermentable sugar concentration that might be affected by the process conditions of starch hydrolysis. Therefore, several relevant and possible interfering factors were studied to investigate whether they had significant effect on the sugar releasing capacity of HSGs, which included with and without prior heat treatment, enzyme dosage, reaction time, particle size and initial solid load. This is the similar work done during conventional hydrolysis (Chapter 4). However, the optimization results obtained from a conventional starch hydrolysis may not be appropriate for GSH since the latter is usually conducted under granular stages without employing any liquefaction or cooking at a

high temperature to gelatinize and modify starch granules. Moreover, a range of different factors was modified during GSH from those of conventional hydrolysis taking into account the process condition.

The aim of the present work was to evaluate enzyme consumption, fermentable sugar yield and ethanol yield during GSH and GSHSF using four high sugary corn genotypes (HSGs) and their respective parent field corn lines (PFCs). Sugar production was investigated under different process conditions during GSH. Finally, GSHSF was carried out under the optimum conditions obtained from GSH for investigating the ethanol production capability of the corn genotypes.

5.2 Materials and Methods

5.2.1 Corn Materials

The corn genotypes including HSGs (UM.NF–1, UM.NF–4, UM.NF–6 and UM.NF–11) and PFCs (PFC–1, PFC–4, PFC–6 and PFC–11), grown in the cropping year 2013 (Chapter 3; Section 3.2.9) were used as raw material for conducting GSH and GSHSF. The harvested and dried kernels were ground in a laboratory grinder, and sieved manually using test sieves (PRADA, Scientific Jaya, Malaysia) of five different particle sizes (PS), such as PS≤0.2, 0.2<PS≤0.5, 0.5<PS≤1.0, 1.0<PS≤1.5 and 1.5<PS≤2.0 mm that were expressed as 0.2, 0.5, 1.0, 1.5 and 2.0, respectively. The corn samples were preserved at 4^{o}C until further analyses were carried out.

5.2.2 Chemicals, Enzymes and Microorganism

The chemicals, reagents and standards used in this study are analytical grade as described previously (Chapter 4; Section 4.2.2). The granular starch hydrolyzing enzyme (GSHE) used

in this study was STARGEN 002, which contains a mixture of α–amylase from *Aspergillus kawachi* and glucoamylase from *Trichoderma reesei* (Genencor, Palo Alto, CA). The activity, specific gravity and optimum pH range as declared by the manufacturer are ≥570 GSHU/g (Granular Starch Hydrolyzing Units), 1.13–1.16 g/ml and 4.0–4.5, respectively. FERMGEN, an acid fungal protease, was obtained and used as described in chapter 4 (4.2.3). The collection, maintenance and inoculum preparation of yeast (*Saccharomyces cerevisae*) was described earlier (Chapter 4; Section 4.2.4).

5.2.3 Granular Starch Hydrolysis (GSH)

Unless otherwise stated, around 15 g of corn flour was mixed with distilled water in a 150 ml Erlenmeyer flask to get a 300 g/L of initial solid concentration, taking into account the moisture content of the samples that ranged between 14.73 and 15.18%. The slurry was stirred at room temperature for 20 min and incubated in a water bath at 60^{o}C for 1 h as per the manufacturer's recommendation (Genencor, 2009). Subsequently, pH of the slurry was adjusted to 4.2 with 2.0 M HCl or 5.0 M NaOH, and supplemented with urea (400 ppm) and chloramphenicol (50 μg/ml) (Genencor, 2009; Szymanowska-Powałowska et al., 2014). GSH was started by adding an enzyme dose and incubating at 30^{o}C for 72 h in a shaking incubator set at 200 rpm (Genencor, 2009). Samples were withdrawn periodically and hydrolysis was stopped by adding 2.0 M HCl until the pH of the hydrolysate reached 1.5±0.1 (Uthumporn et al., 2010). Samples were then centrifuged at 5000 rpm for 15 min and supernatants collected and analyzed for reducing sugars, while, residues were analyzed for starch.

GSH was studied under different conditions such as with and without prior heat treatment, under five enzymatic conditions (0.5, 1.0, 1.5, 2.0 and 2.5 kg/MT of dry corn), and under

slurries with different particle sizes of ground corn (0.2, 0.5, 1.0, 1.5 and 2.0 mm) and with different initial solid loads (150, 200, 250, 300 and 350 g/L).

The kinetics of RS production during GSH were determined under all conditions using the Equations (5.1) and (5.2).

$$Q_{RS} = \frac{P_{RS}}{t} \quad (5.1)$$

where, Q_{RS} is the volumetric productivity of reducing sugars (g/L/h); P_{RS} is the concentration of reducing sugars (g/L); t is the hydrolysis time

$$Y_{RS} = \frac{P_{RS}}{S} \quad (5.2)$$

Where, Y_{RS} is the yield of reducing sugars (g/g of dry corn); P_{RS} is the concentration of reducing sugars (g/L); S is the initial solid concentration (g/L)

5.2.4 Granular Starch Hydrolysis and Simultaneous Fermentation (GSHSF)

Based on the results of GSH, 100 ml of slurry was prepared by mixing around 30 g of corn flour with distilled water in a 250 ml Erlenmeyer flask to get 300 g/L solid concentration. The slurry was stirred at room temperature for 20 min and incubated in a water bath at 60^{o}C for 1 h (Genencor, 2009). Samples were cooled down to room temperature and pH of the slurry adjusted to 4.2 with either 2.0 M HCl or 5.0 M NaOH (Genencor, 2009). The slurry was supplemented with urea (400 ppm), FERMGEN (0.2 kg/MT of dry corn), IsoStab (40 ppm) and chloramphenicol (50 μg/ml) (Genencor, 2009; Szymanowska-Powałowska et al., 2014). GSHSF was started by adding two concentrations of STARGEN 002 separately (1.5

and 2.0 kg/MT of dry corn) and 2 ml inoculum of yeast, and finally, incubated at 30^{o}C for 96 h in a shaking incubator set at 200 rpm (Johnston & McAloon, 2014; Lamsal et al., 2011).

Samples were withdrawn every 24 h, and one part was analyzed for microbial counts (viable yeast and total bacteria). The other part of the sample was centrifuged at 5000 rpm for 15 min, and supernatants analyzed for ethanol, total soluble sugars (TSS), individual sugars (glucose, fructose and sucrose) and soluble byproducts (glycerol and lactic acid). Residues were analyzed for starch.

5.2.5 Analytical Methods

5.2.5.1 Determination of Moisture

Moisture content in the corn sample was determined as described previously in Chapter 3 (Section 3.2.10.3).

5.2.5.2 Determination of RS

The RS concentration in hydrolysate was determined as described earlier in Chapter 3 (Section 3.2.10.5).

5.2.5.3 Determination of Ethanol

Ethanol concentration and kinetics of ethanol fermentation was studied during GSHSF as described in earlier in Chapter 4 (Section 4.2.7.3).

5.2.5.4 Determination of Carbohydrate Concentration

The concentration of starch, TSS, sucrose, glucose and fructose were determined using the methods described in Chapter 3 (Section 3.2.10).

5.2.5.5 Total Viable Yeast & Bacterial Counts

Total yeast count and total bacterial count were carried out as described previously in Chapter 4 (Section 4.2.7.5 and 4.2.7.6).

5.2.5.6 Determination of Glycerol and Lactic Acid

Glycerol and lactic acid concentration in the fermentation broth were determined as described in Chapter 4 (Section 4.2.7.11 and 4.2.7.12).

5.2.6 Statistical Analysis

Data were analyzed with Minitab statistical software, version 16 (State college, PA, USA) to calculate mean and standard deviation (SD) for each attribute as studied at least in triplicate. Data were also tested for one way ANOVA as described in Chapter 4 (Section 4.2.8). The comparison between the outcomes of two samples or two groups was performed by two sample t–test. Pearson's correlation coefficient was calculated to determine the relationship among different components. Minitab statistical software, version 16 (State college, PA, USA) was used for these analyses considering 5% level of significance ($P \leq 0.05$).

5.3 Results

5.3.1 Granular Starch Hydrolysis (GSH)

5.3.1.1 GSH Under With and Without Pre–Heat Treatment Conditions

During GSH, it was observed that the amount of starch decreased (*i.e.*, converted into glucose or smaller carbohydrate molecules) exponentially until 48 h in all genotypes, irrespective of the pre–treatment conditions (with or without prior heat treatment of the

slurry) (Figure 5.1). Subsequently the decrease in starch concentration was minimal. However, not surprisingly, the conversion rate of starch into sugar increased over time, which showed significant differences until 60 h (Figure 5.2). Corresponding to the conversion rate of starch in the slurry, the concentration of RS (P_{RS}) increased, and the variation in RS production was found to be significant until 72 h, which was followed by either a small decrease or plateau in the subsequent course of hydrolysis (Figure 5.3). The F–test analysis of variance revealed that the changes in RS and starch concentration after 72 h were statistically insignificant ($p > 0.05$). The volumetric productivity of RS (Q_{RS}) decreased significantly over time (Figure 5.4), particularly, after 72 h. Therefore, the effective time for GSH was taken to be 72 h and the final sugar yield (Y_{RS}) calculated for this course of hydrolysis is as presented in Figure 5.5.

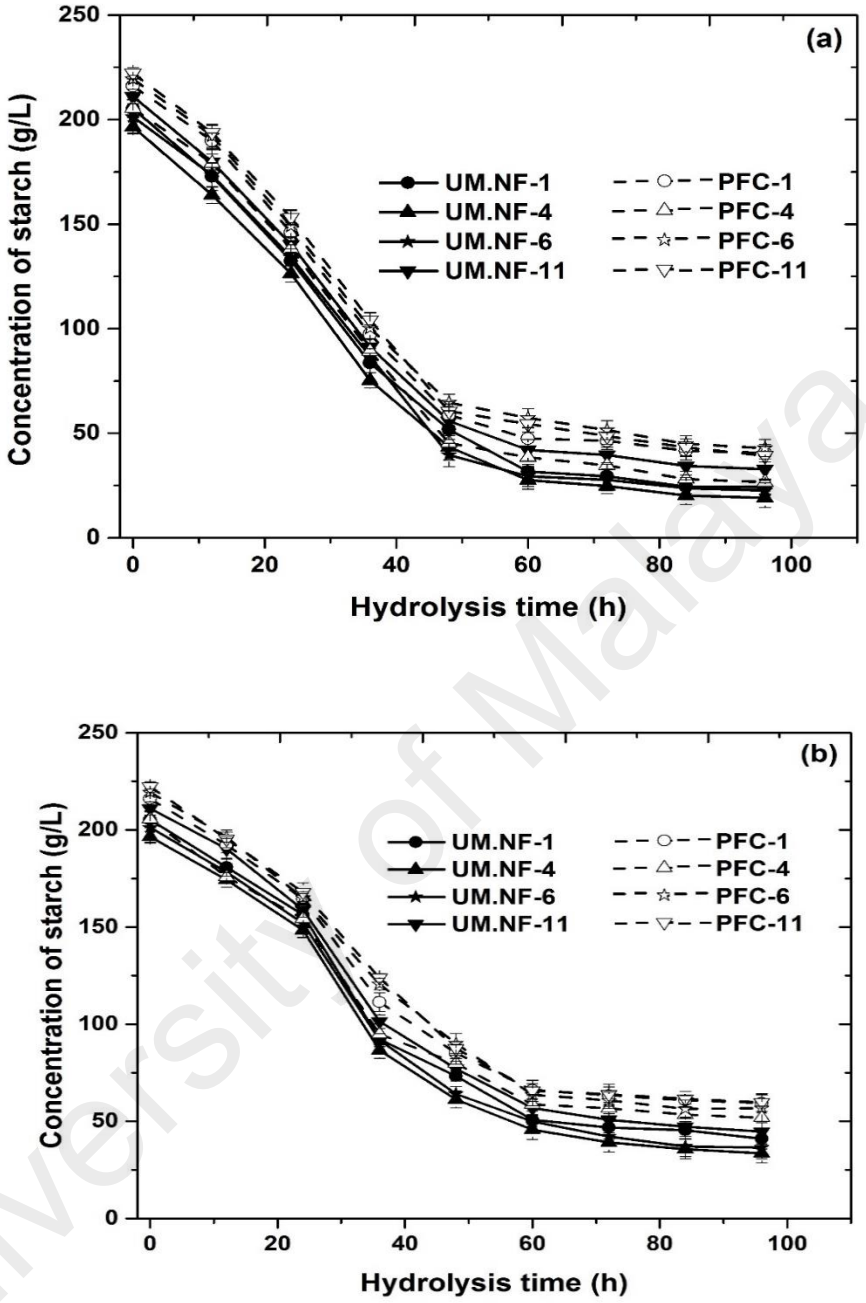

Figure 5.1: Changes in Starch Concentration in the Hydrolysates during Granular Starch Hydrolysis (GSH) (a) With and (b) Without Prior Heat Treatment Conditions

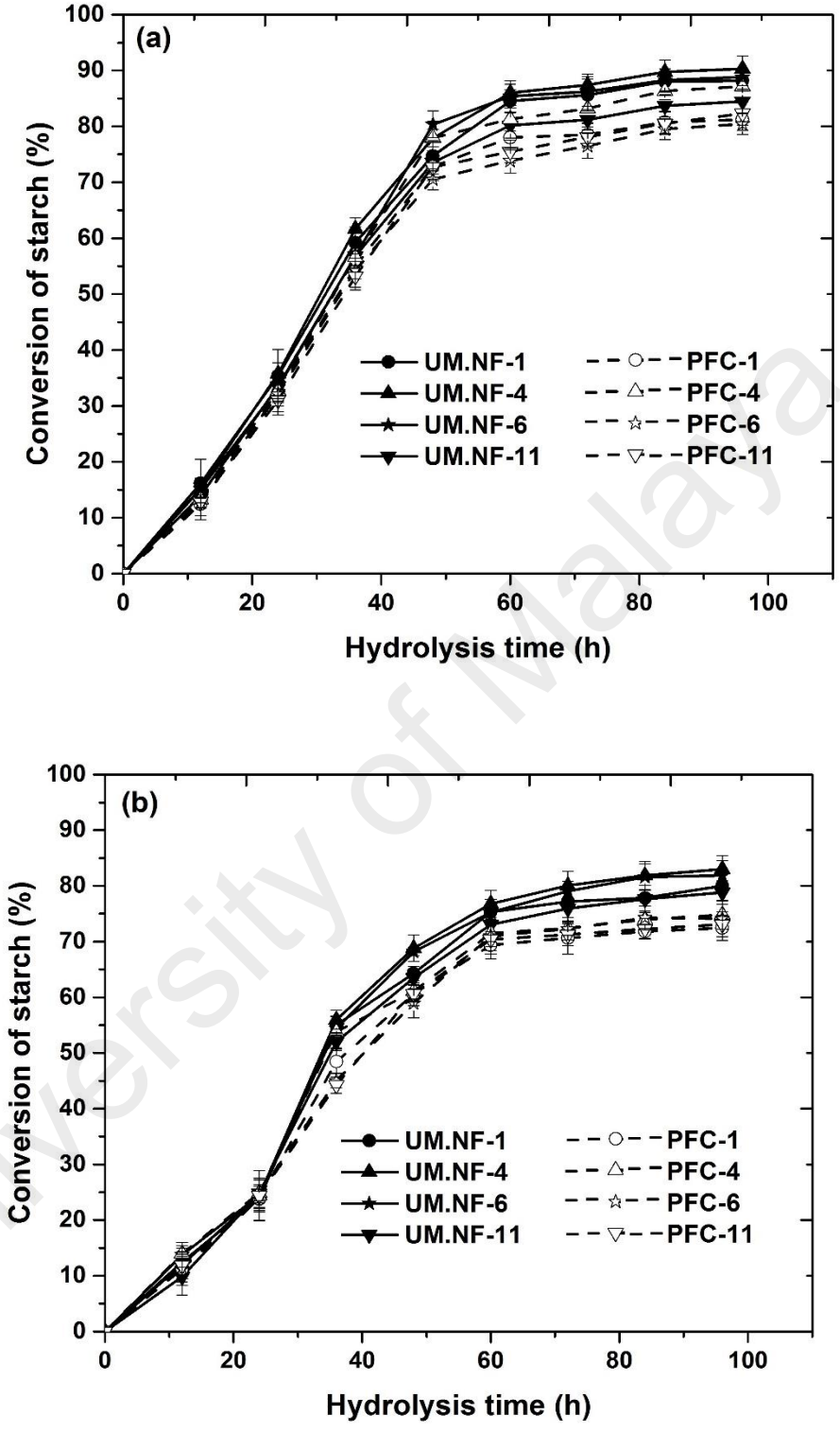

Figure 5.2: Percent Conversion of Starch During Granular Starch Hydrolysis (GSH)

(a) With and (b) Without Prior Heat Treatment Conditions

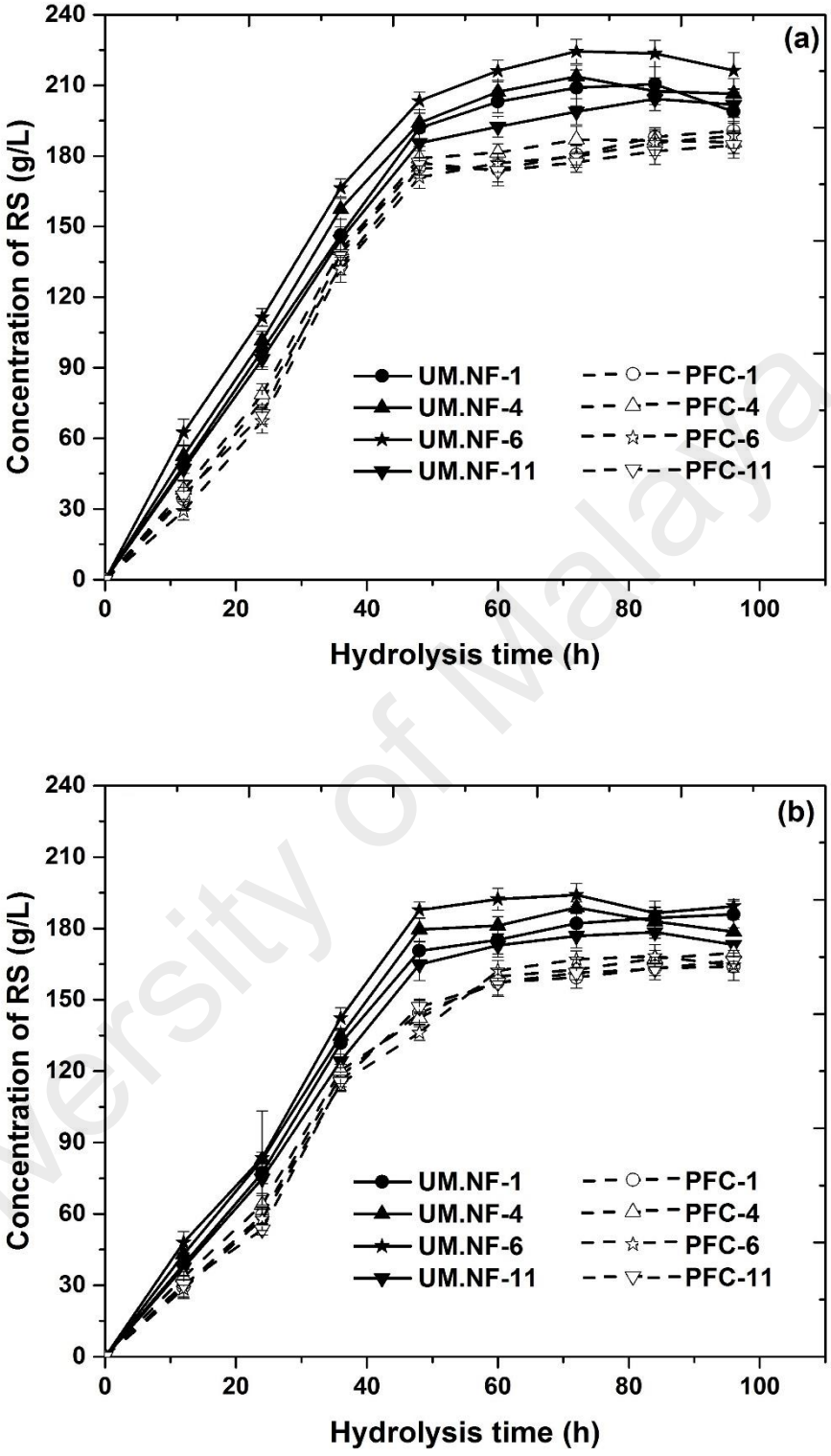

Figure 5.3: Changes in Reducing Sugar Concentration During Granular Starch Hydrolysis (GSH) (a) With and (b) Without Prior Heat Treatment Conditions

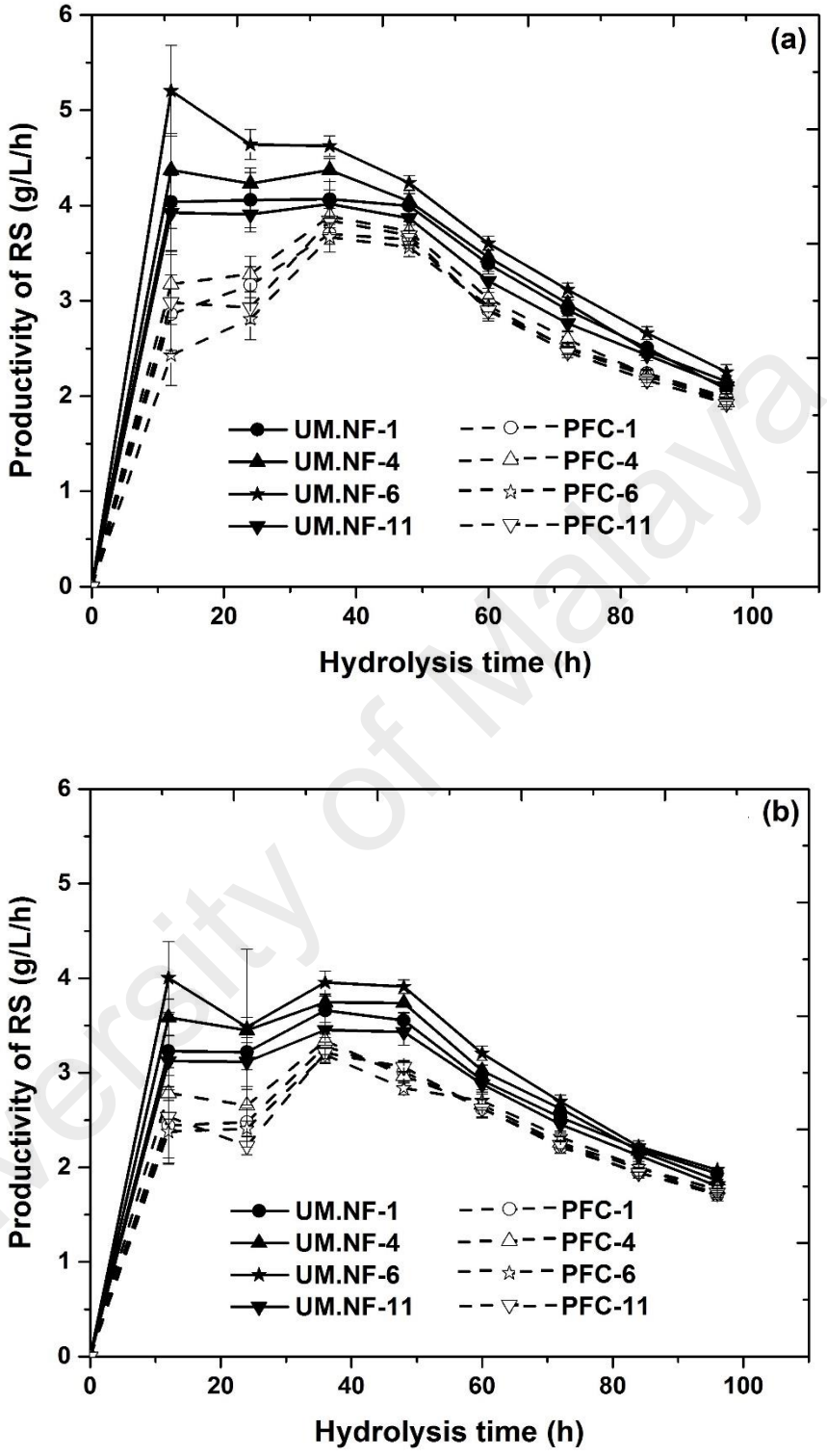

Figure 5.4: Changes in Reducing Sugar Productivity During Granular Starch Hydrolysis (GSH) (a) With and (b) Without Prior Heat Treatment Conditions

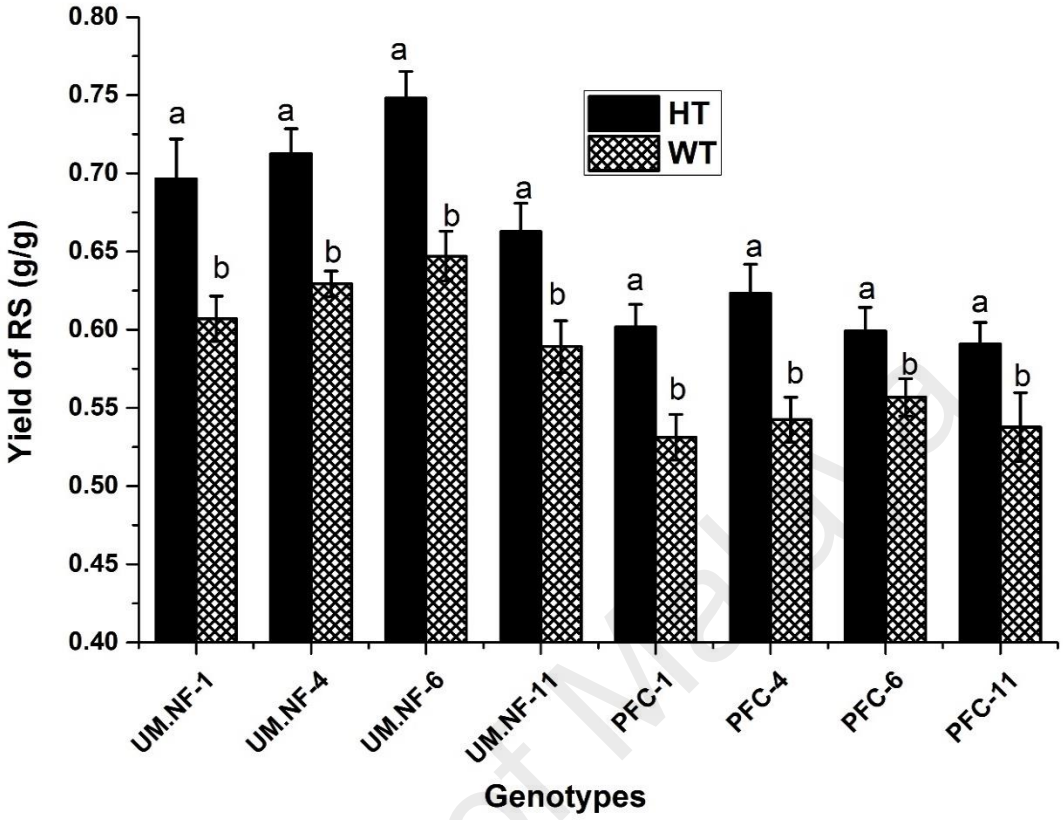

Figure 5.5: Final Reducing Sugar Yield After 72 h Of Granular Starch Hydrolysis (GSH) With and Without Prior Heat Treatment Conditions

Overall, it can be seen that the starch conversion rate into soluble sugars, sugar yield and productivity were significantly higher in all genotypes when slurries were heated at 60^{o}C before GSH. When PFCs were compared with HSGs, it was observed that all the HSGs produced higher amounts of RS with higher productivity under both conditions.

5.3.1.2 GSH under Different Enzymatic Conditions

It was observed that RS concentration in the hydrolysates increased with the increase in enzyme load, whilst starch content of the slurry decreased as the enzyme dose increased (Figure 5.6). However, a good amount of starch remained unreacted in both groups of

genotypes at the end of GSH even at the highest enzyme load. The analysis of variance for RS and starch revealed that GSHE with an enzyme load above 1.5 kg/MT did not bring about significant changes in RS as well as starch utilization in HSGs, since the *P* values were greater than 0.05, with the exception for UM.NF–11, which along with PFCs showed significant changes in RS and starch until the enzyme load reached 2.0 kg/MT.

Corresponding to the accumulation of RS in the hydrolysates, the percentage of utilization of starch also increased as the enzyme load increased (Figure 5.7). Likewise, volumetric productivity of RS increased in all genotypes with the increase in enzyme load, where HSGs showed higher productivities than those of PFCs (Figure 5.8). Although the production of RS increased proportionally with the enzyme load, yield was significantly lower with enzyme loads 0.5 and 1.0 kg/MT in both HSGs and PFCs (Figure 5.9), which can be considered insufficient for producing appropriate amounts of ethanol during fermentation.

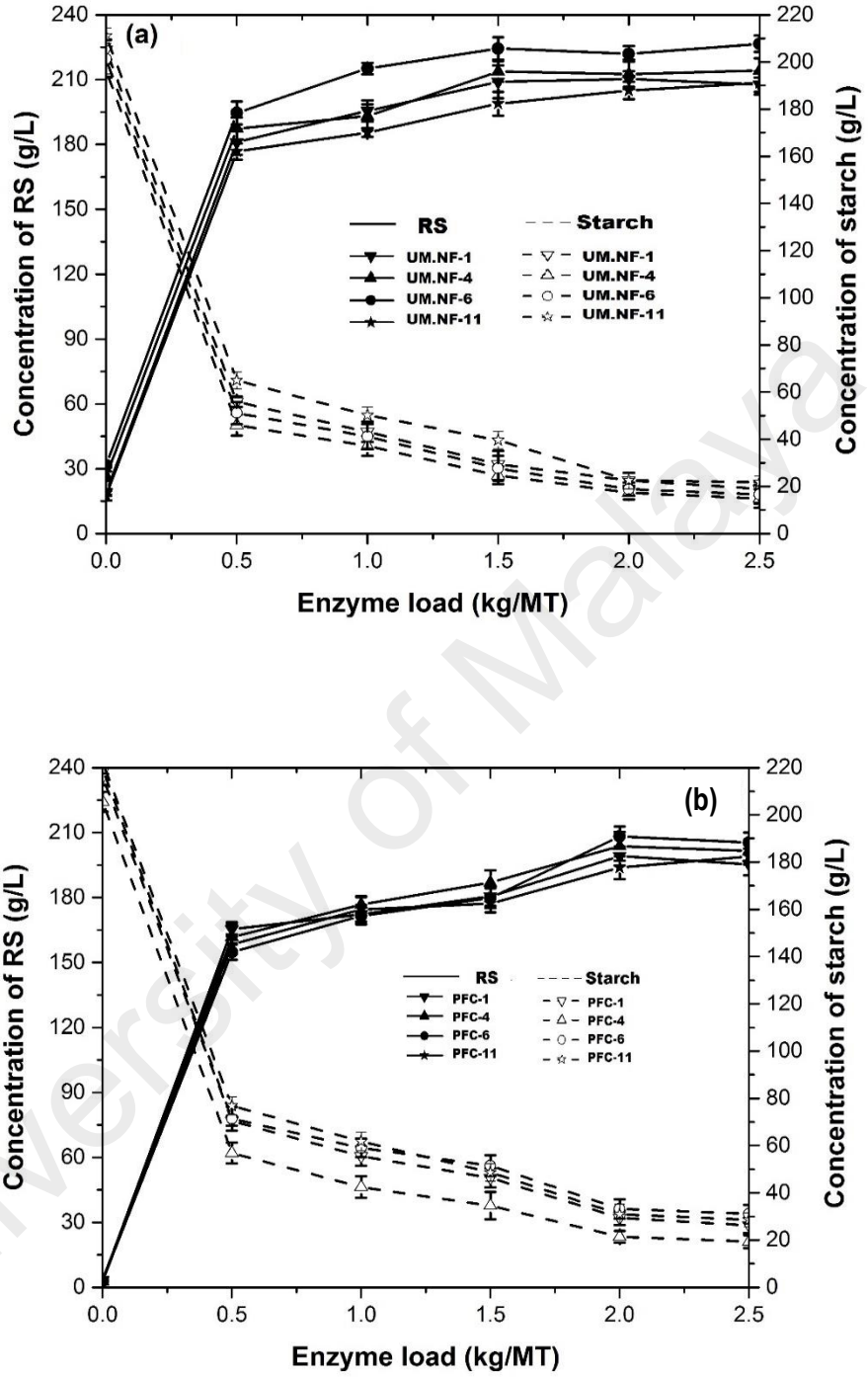

Figure 5.6: Changes in the Final Reducing Sugars and Starch Concentrations During Granular Starch Hydrolysis (GSH) Under Different Enzymatic Conditions: (a) HSGs and (b) PFCs.

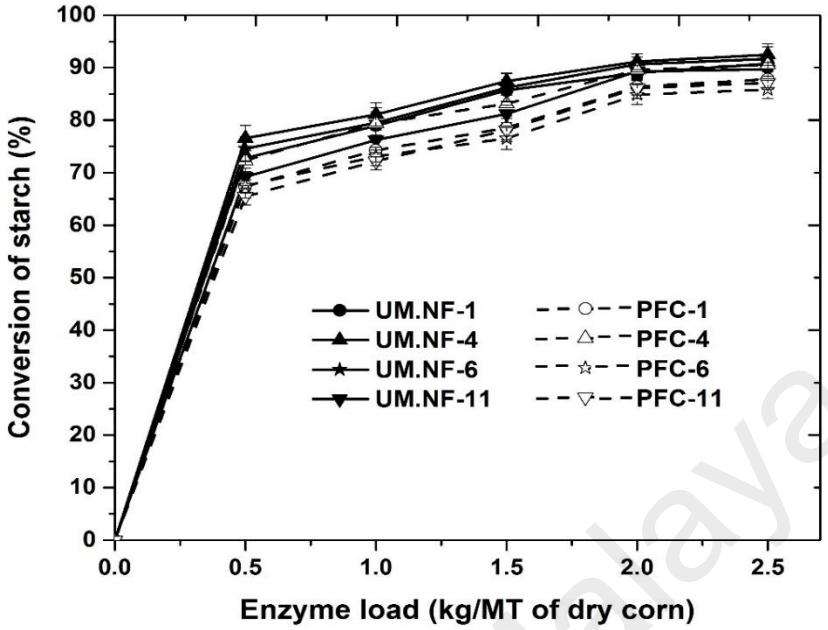

Figure 5.7: Percent Conversion of Starch during Granular Starch Hydrolysis (GSH) Under Different Enzymatic Conditions

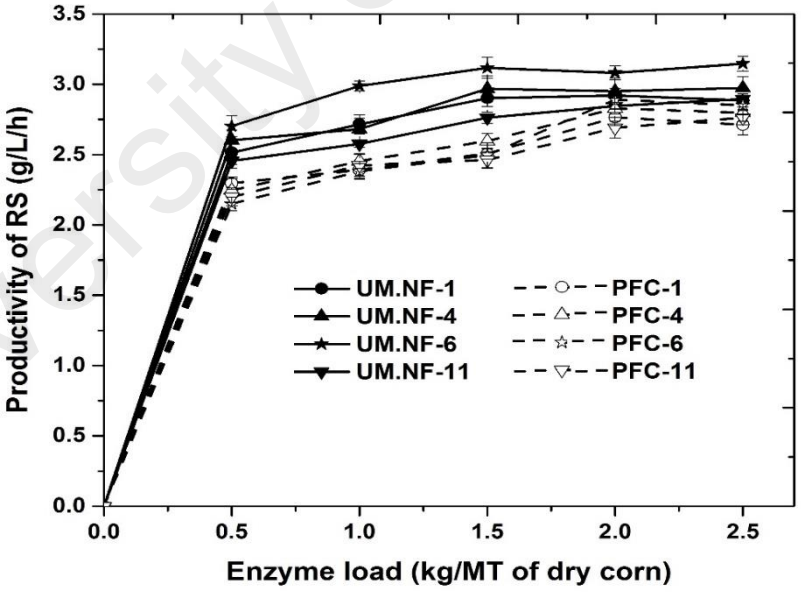

Figure 5.8: Changes in Final Productivity of Reducing Sugar During Granular Starch Hydrolysis (GSH) of Corn Genotypes Under Different Enzymatic Conditions.

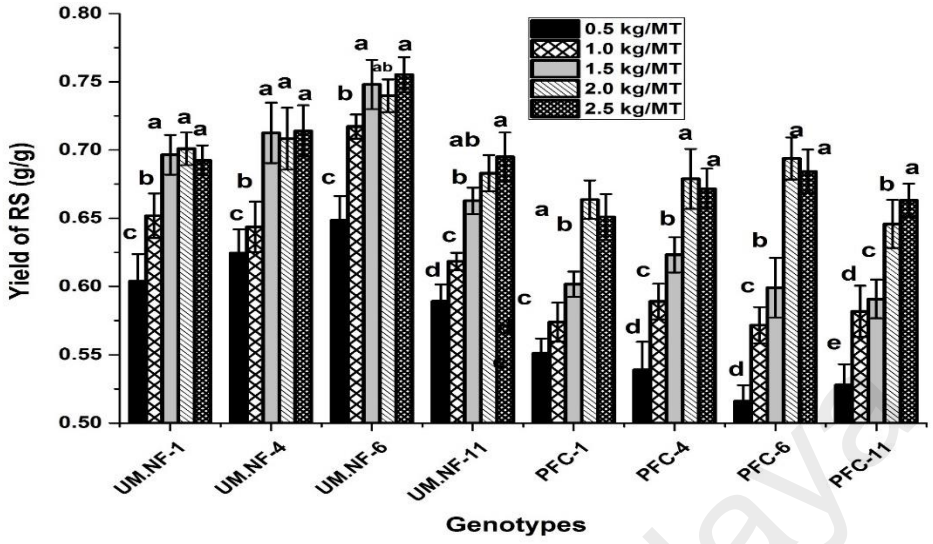

Figure 5.9: Final Reducing Sugar Yield During Granular Starch Hydrolysis (GSH) Under Different Enzymatic Conditions

5.3.1.3 GSH Using Different Particle Sizes of Ground Corn

Both groups of corn genotypes showed an increase in the final concentration of RS inversely related to corn particle size, while starch content in the hydrolysates increased with increase in particle size (Figure 5.10). As a result, maximum conversion of starch into RS was achieved in both PFCs and HSGs during GSH with the smallest particle of corn (0.2 mm) (Figure 5.11). Compared to PFCs, all the HSGs produced significantly higher amounts of RS with all particle sizes, which resulted in more unreacted starch in PFCs than in HSGs.

As the production of RS increased with the decrease in particle size, volumetric productivity increased in the lowest particle size of ground corn (Figure 5.12). When particle size was 0.2 mm, the Q_{RS} in HSGs ranged between 2.9 and 3.3 g/L/h, which dropped sharply as particle size reached to 2.0 mm, where it ranged from 2.1 to 2.6 g/L/h. A similar trend was observed in PFCs, even though the Q_{RS} values were much lower than those of HSGs under

all conditions. The final sugar yield also varied significantly due to the changes in particle size of corn (Figure 5.13).

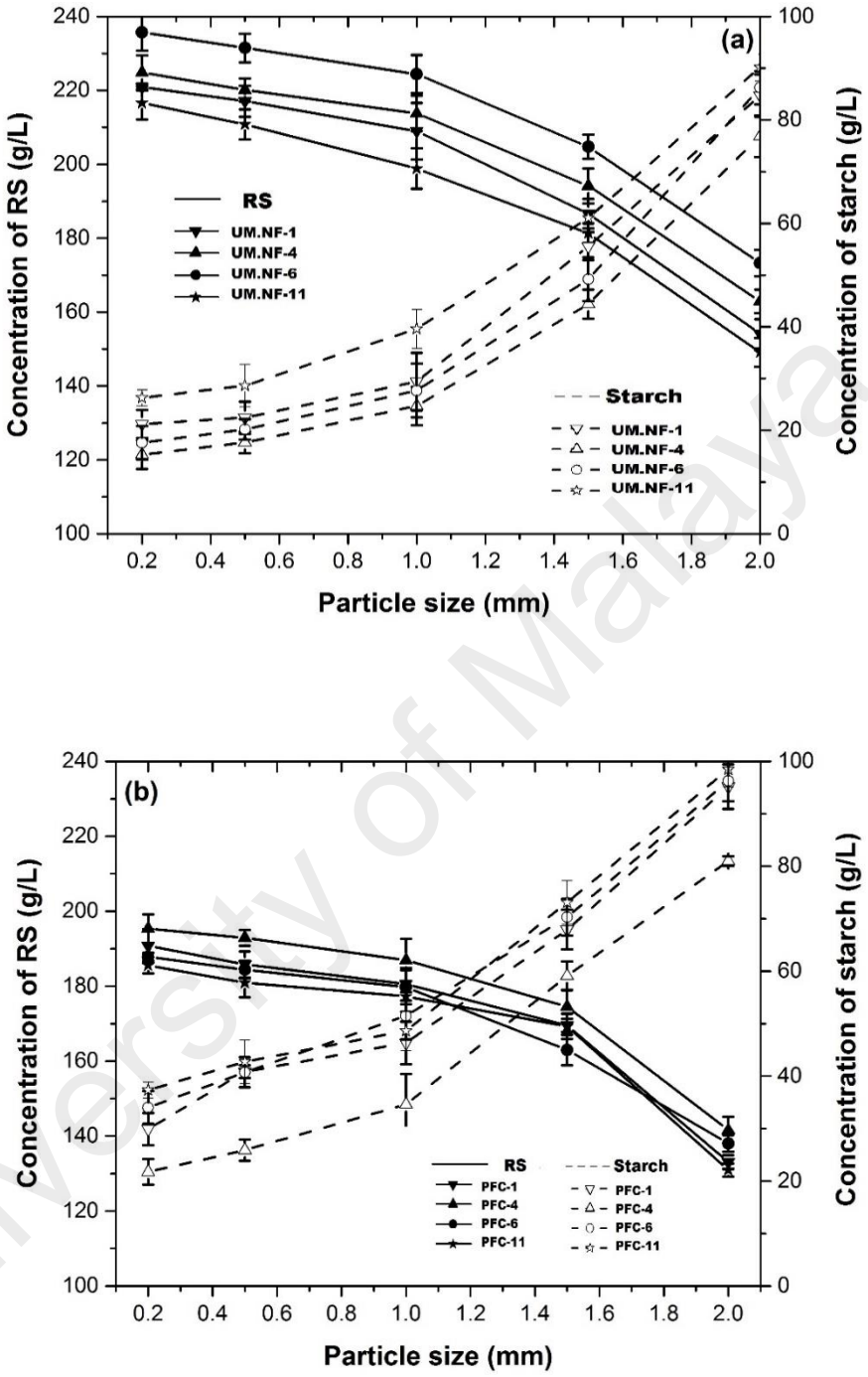

Figure 5.10: Changes in the Final Reducing Sugar and Starch Concentrations During Granular Starch Hydrolysis (GSH) Under Different Particle Sizes of Corn Meal: (a) HSGs and (b) PFCs.

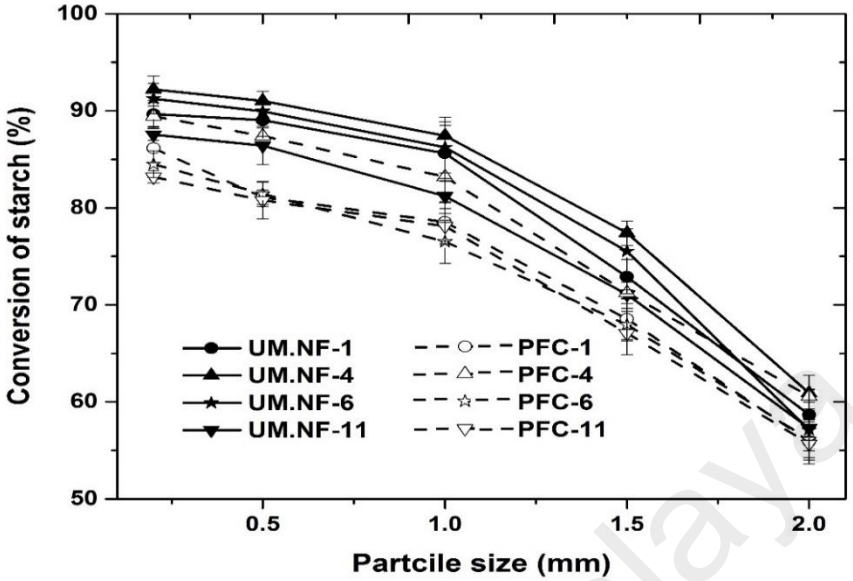

Figure 5.11: Percent Conversion of Starch During Granular Starch Hydrolysis (GSH) Under Different Particle Sizes of Corn Meal

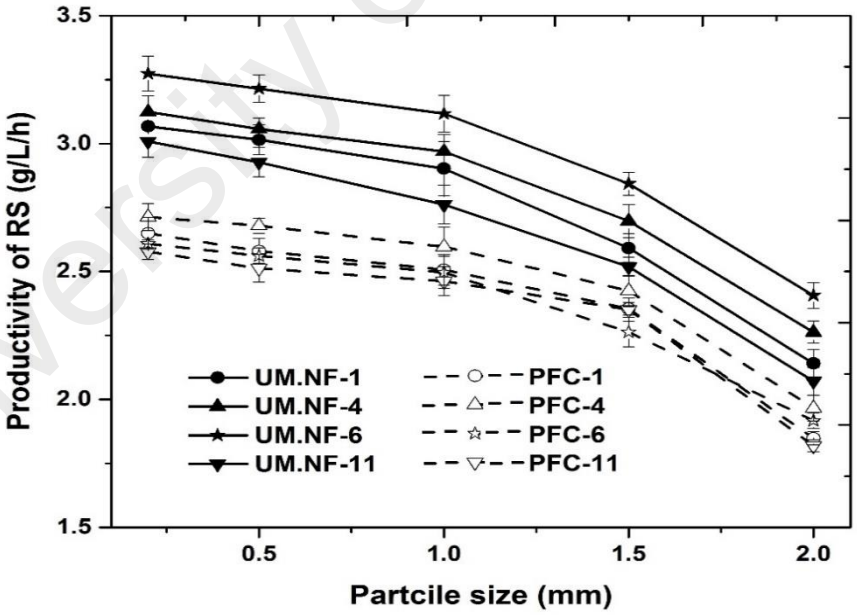

Figure 5.12: Changes in the Final Productivity of Reducing Sugar During Granular Starch Hydrolysis (GSH) Under Different Particle Sizes of Corn Meal.

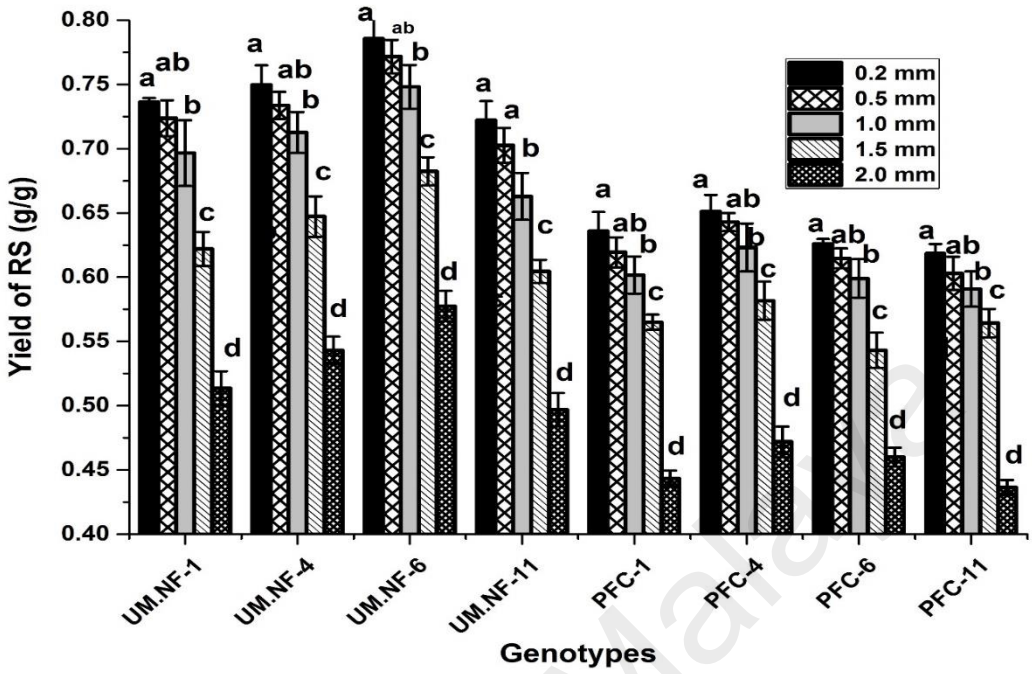

Figure 5.13: Final Reducing Sugar Yield during Granular Starch Hydrolysis (GSH) Under Different Particle Sizes of Corn Meal.

5.3.1.4 GSH under Different Solid Loads

It was observed that the concentration of RS in the hydrolysates of both HSGs and PFCs increased with the increase in initial solid load until 300 g/L (Figure 5.14). However, subsequently the concentration of RS showed a plateau or even decreased when initial solid load reached to 350 g/L, and considerable amounts of starch remained unreacted at this point. Moreover, the conversion rate of starch into soluble sugars was found to decrease significantly as the initial solid load increased, which was much lower at the highest initial solid load (Figure 5.15). The volumetric productivity of RS showed a gradual increase in both PFCs and HSGs until 72 h, which then plateaued when the solid load increased to 350 g/L (Figure 5.16). The final sugar yield was also found to be significantly higher under lower initial solid loads (Figure 5.17). However, it should be noted that although HSGs produced a

higher amount of RS under all conditions than PFCs, differences in RS yield between the two groups of genotypes were less significant under the lower initial solid load concentrations, particularly, when initial solid loads were between 15 and 20% in the slurry.

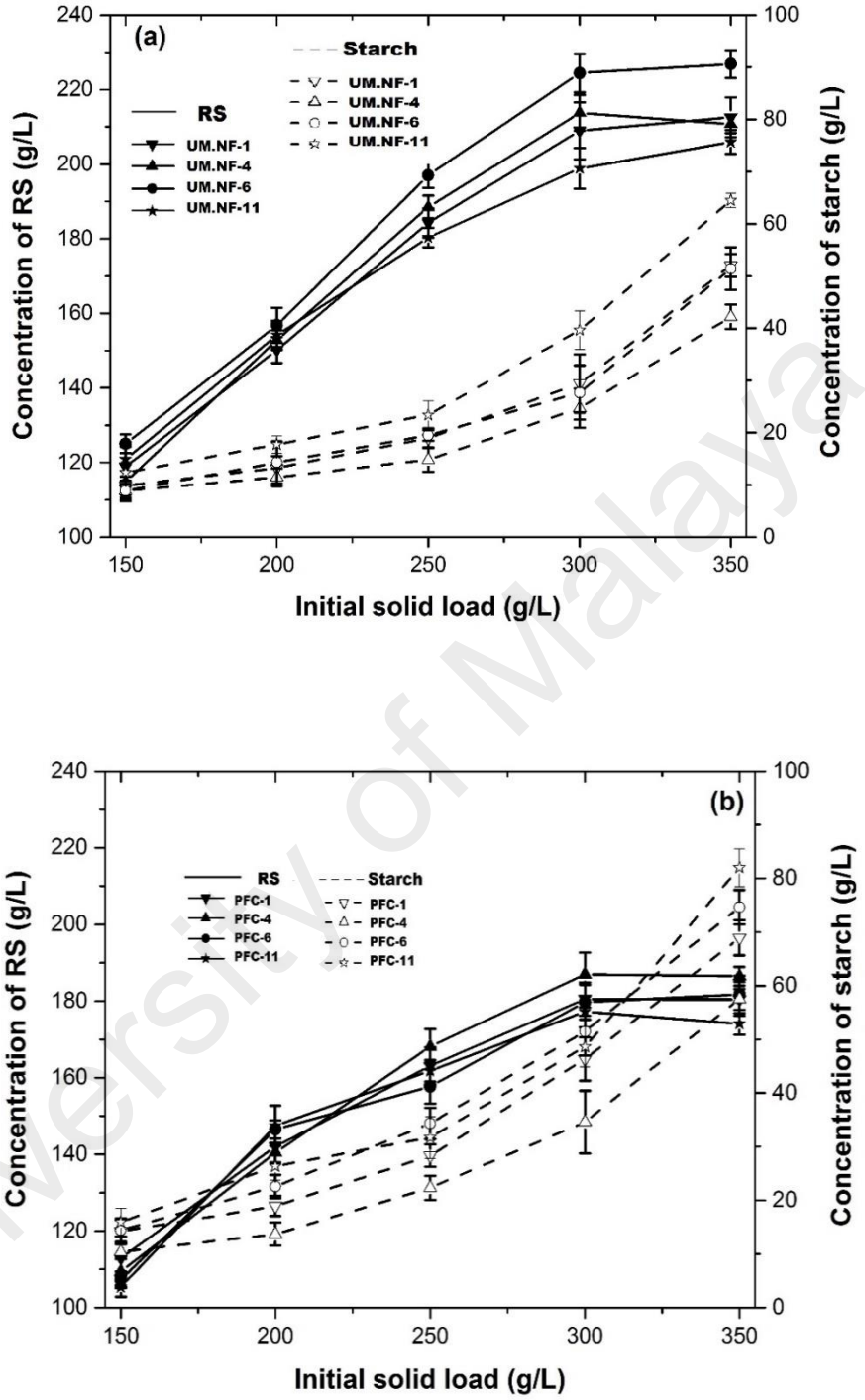

Figure 5.14: Changes in the Final Reducing Sugar and Starch Concentrations During Granular Starch Hydrolysis (GSH) Under Different Initial Solid Loads: (a) HSGs and (b) PFCs.

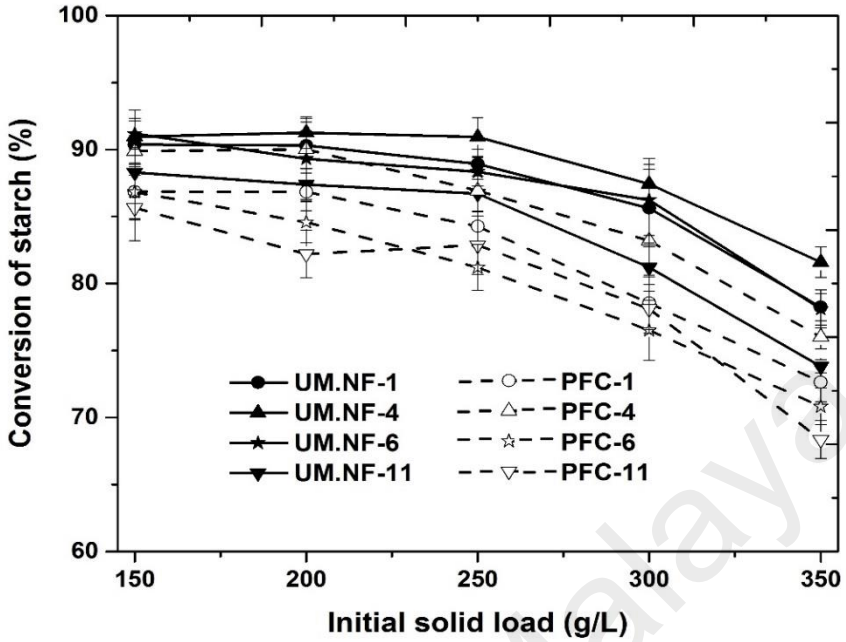

Figure 5.15: Percent Conversion of Starch during Granular Starch Hydrolysis (GSH) Under Different Initial Solid Loads

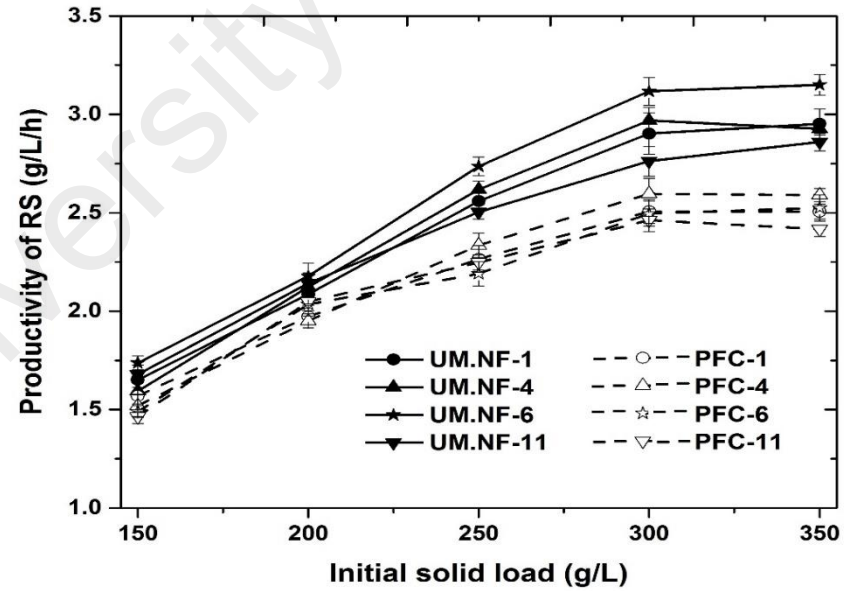

Figure 5.16: Changes in Final Productivity of Reducing Sugar During Granular Starch Hydrolysis (GSH) Under Different Initial Solid Loads.

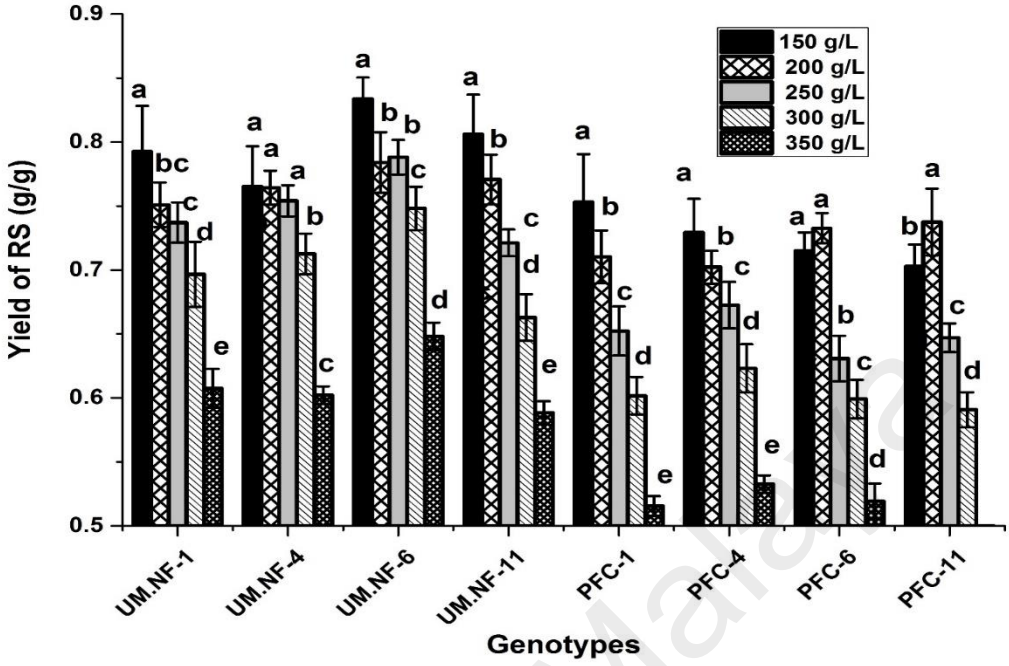

Figure 5.17: Final Reducing Sugar Yield During Granular Starch Hydrolysis (GSH) under Different Initial Solid Loads

5.3.1.5 GSHSF

5.3.2 Ethanol Production during GSHSF

Based on the results of GSH, two enzyme doses (1.5 and 2.0 kg/MT) were used during GSHSF taking into account the optimal enzymatic condition for the two groups of corn genotypes. In general, ethanol production increased over time with increasing in enzyme load (Figure 5.18). Initially ethanol production increased exponentially with time until 72 h, after which the increments was very small. Under the lowest enzyme concentration (1.5 kg/MT), all the HSGs produced higher amounts of ethanol in all courses of fermentation and the average final ethanol concentration ranged from 15.25 % (v/v) in UM.NF–11 to 17.5% in UMNF–6, compared to those of PFCs which varied between 11.66 and 13.65% (Figure 5.18a). When the enzyme load increased to 2.0 kg/MT, the final ethanol concentration

increased significantly in PFCs, and varied between 14.32 and 16.85% with the exception of PFC–4 which showed no significant change in ethanol concentration between two enzyme loads and produced the lowest ethanol at maximum enzyme dosage (Figure 5.18 b). Even though HSGs showed a slight increase in ethanol concentration at the highest enzyme dosage, the difference in ethanol concentration between the enzyme dosages of 1.5 and 2.0 kg/MT can be regarded as insignificant since the *P* values were greater than 0.05. Among the eight corn genotypes, UM.NF–6 produced the highest ethanol under both enzymatic conditions, which corresponded to the maximum sugar content in its kernels (Chapter 3, Table 3.8).

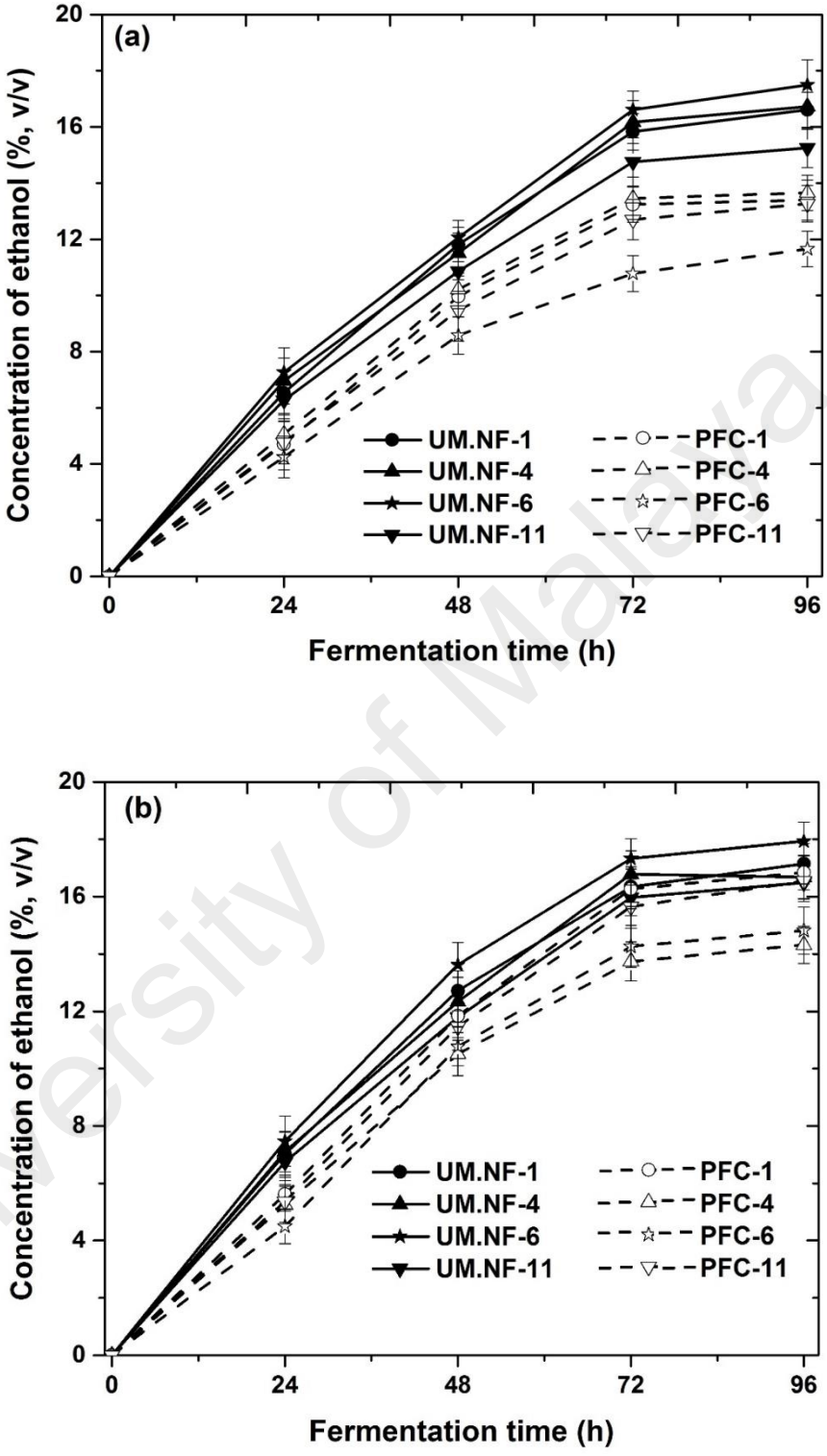

Figure 5.18: Time Course of Ethanol Concentration During GSHSF under Two Enzymatic Conditions; (a) 1.5 kg/MT and (b) 2.0 kg/MT.

The kinetic parameters estimated during the course of GSHSF under two enzyme loads are summarized in Table 5.1. The ethanol titer (g/L) and ethanol yield (g/g of dry con) increased with the increase in fermentation period in all genotypes and produced more than half of the total ethanol by 48 h, whereas, the volumetric ethanol productivity (g/L/h) decreased over time. Corresponding to the ethanol production (Figure 5.18), ethanol titers, ethanol yield and volumetric productivity were also found to be higher in HSGs under the lowest enzyme load. Regardless of the slight increase in these kinetic parameters at the highest enzyme load (2.0 kg/MT), ANOVA results revealed that the differences were not significant ($p > 0.05$). Under the lowest enzyme load (1.5 kg/MT of dry corn), the average final ethanol yield in HSGs varied between 0.4 and 0.46 g/g, while in PFCs it ranged between 0.31 and 0.35 g/g. When the enzyme dosage was increased to 2.0 kg/MT of dry corn, ethanol yield increased slightly in HSGs and significantly in PFCs, which ranged from 0.43 to 0.47 g/g and 0.38 to 0.44, respectively (Table 5.1).

Table 5.1 Kinetics of Ethanol Fermentation during Granular Starch Hydrolysis and Simultaneous Fermentation (GSHSF) of the Corn Genotypes under Two Enzymatic Conditions (All Attributes are Expressed as Mean Values).

Genotypes	Course of GSHSF (h)	Ethanol titer, P_{EtOH} (g/L)		Volumetric ethanol productivity, Q_{EtOH} (g/L/h)		Ethanol yield, Y_{EtOH} (g/g of dry corn)	
		Enzyme doses (kg/MT of dry corn)					
		1.5	2.0	1.5	2.0	1.5	2.0
UM.NF–1	24	52.15	55.47	2.17	2.31	0.17	0.18
	48	93.21	100.33	1.94	2.09	0.31	0.33
	72	124.87	128.98	1.73	1.79	0.42	0.43
	96	131.05	135.31	1.37	1.41	0.44	0.45
UM.NF–4	24	54.86	56.02	2.29	2.33	0.18	0.19
	48	90.79	97.31	1.89	2.03	0.3	0.32
	72	127.63	132.45	1.77	1.84	0.43	0.44
	96	132.00	131.61	1.37	1.37	0.44	0.44
UM.NF–6	24	57.33	58.91	2.39	2.45	0.19	0.20
	48	95.21	107.46	1.98	2.24	0.32	0.36
	72	131.11	136.71	1.82	1.9	0.44	0.46
	96	138.05	141.52	1.44	1.47	0.46	0.47
UM.NF–11	24	49.50	53.23	2.06	2.22	0.16	0.18
	48	85.74	93.02	1.79	1.94	0.29	0.31
	72	116.46	126.03	1.62	1.75	0.39	0.42
	96	120.35	130.13	1.25	1.36	0.40	0.43
PFC–1	24	37.11	44.29	1.55	1.85	0.12	0.15
	48	78.61	93.42	1.64	1.95	0.26	0.31
	72	104.44	128.34	1.45	1.78	0.35	0.43
	96	105.73	132.95	1.1	1.38	0.35	0.44
PFC–4	24	39.92	41.40	1.66	1.72	0.13	0.14
	48	80.66	82.90	1.68	1.73	0.27	0.28
	72	106.20	108.36	1.47	1.50	0.35	0.36
	96	107.49	112.98	1.12	1.18	0.36	0.38
PFC–6	24	33.51	35.51	1.40	1.48	0.11	0.12
	48	67.70	84.98	1.41	1.77	0.23	0.28
	72	85.05	112.54	1.18	1.56	0.28	0.38
	96	91.92	116.88	0.96	1.22	0.31	0.39
PFC–11	24	37.61	42.34	1.57	1.76	0.13	0.14
	48	74.69	90.47	1.56	1.88	0.25	0.30
	72	100.20	123.48	1.39	1.71	0.33	0.41
	96	104.67	130.53	1.09	1.36	0.35	0.44

5.3.2.1 Utilization of Carbohydrates by the Corn Genotypes during GSHSF

Changes in carbohydrate concentration was monitored every 24 h during GSHSF. Unlike GSH (Section 5.3.4), individual sugars were studied here to observe their consumption patterns by yeast cells. It can be seen that starch concentration decreased as the enzyme load increased, although a good amount of starch remained unutilized in all genotypes after fermentation (Figure 5.19). Even though the quantity of residual starch increased in both group of genotypes with the decrease in enzyme load, as a whole, the amounts of residual starch were relatively higher in PFCs than in HSGs. However, a F–test analysis revealed that the amount of residual starch was not significantly different between the two enzyme loads of 1.5 and 2.0 kg/MT in HSGs. Compared to GSH (Figure 5.1), the amounts of unutilized starch was found to be lower during GSHSF.

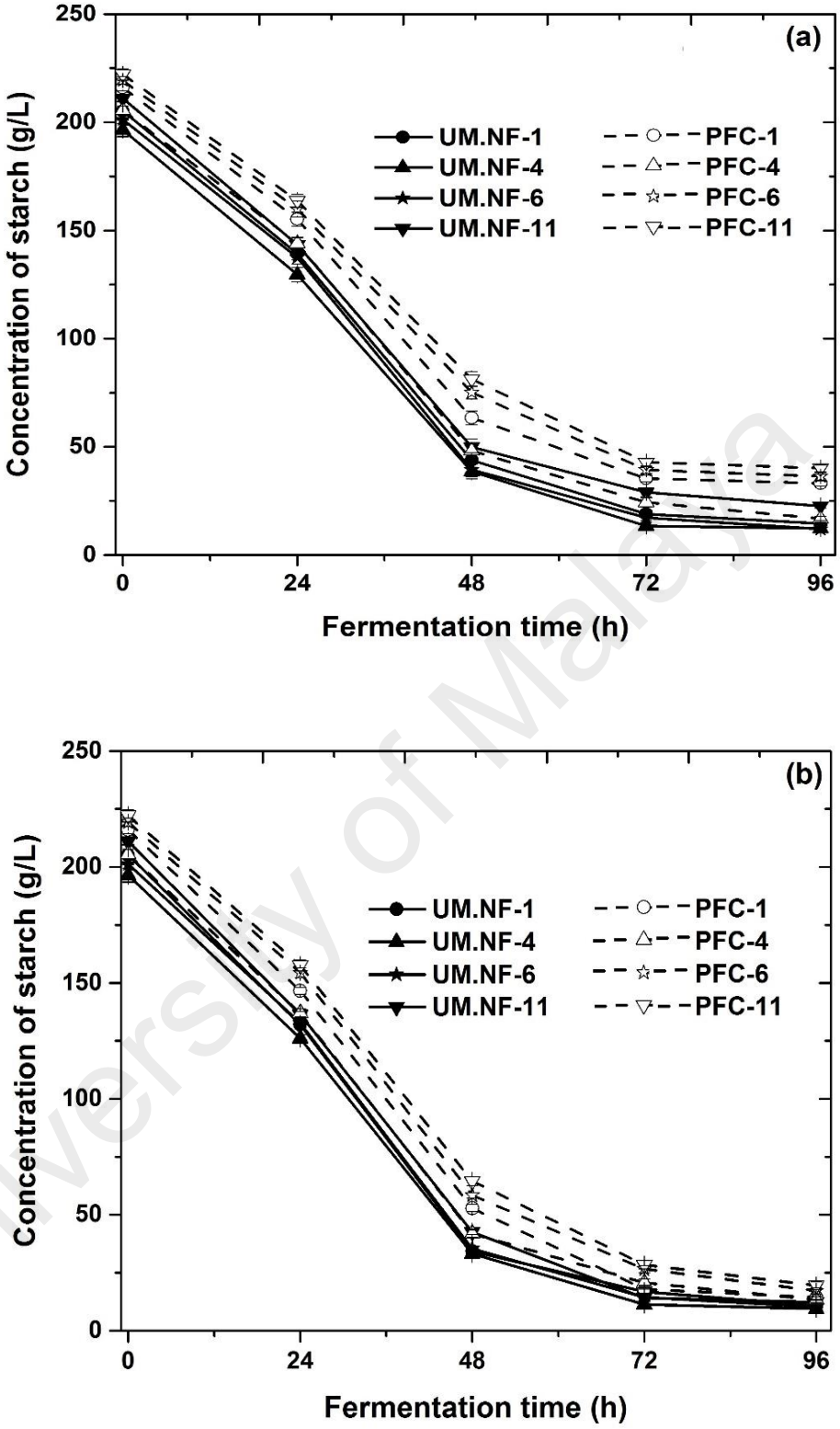

Figure 5.19: Changes in Starch Concentration Over Time During GSHSF under Two Enzymatic Conditions; (a) 1.5 kg/MT and (b) 2.0 kg/MT.

Most of the sucrose and fructose in the mash were used up by 24 h, particularly, in PFCs, and a complete exhaustion was observed in all genotypes after 48 h (Figure 5.20, 5.21). Therefore, most of the soluble sugars in the mash after 48 h consisted of mainly glucose and products of the incomplete hydrolysis of starch. The concentration of glucose as well as TSS was found to increase significantly after 24 h, followed by either a small increase or decrease after 48 h, and subsequently both types of sugars dropped significantly after 72 h, which continued until the end of fermentation (Figure 5.22 and 5.23). However, PFCs showed a decrease in glucose and TSS concentrations in the fermentation broth after 24 h when the enzyme load was 1.5 kg/MT (Figure 5.22a and 5.23a). It dropped sharply after 48 h under the higher enzyme load of 2.0 kg/MT (Figure 5.22b and 5.23b).

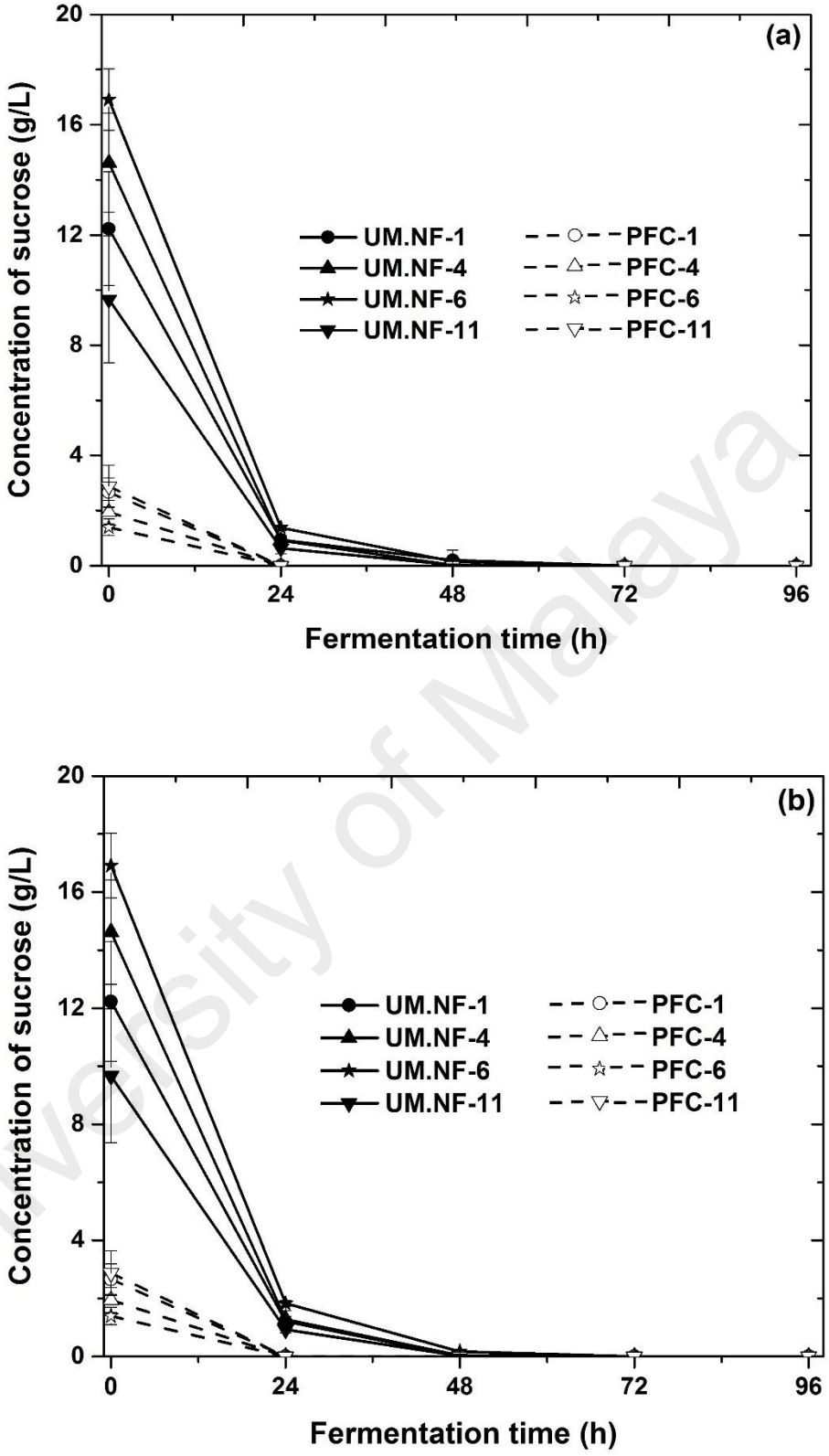

Figure 5.20: Changes in Sucrose Concentration Over Time During GSHSF under Two Enzymatic Conditions; (a) 1.5 kg/MT and (b) 2.0 kg/MT; (a) 1.5 kg/MT and (b) 2.0 kg/MT.

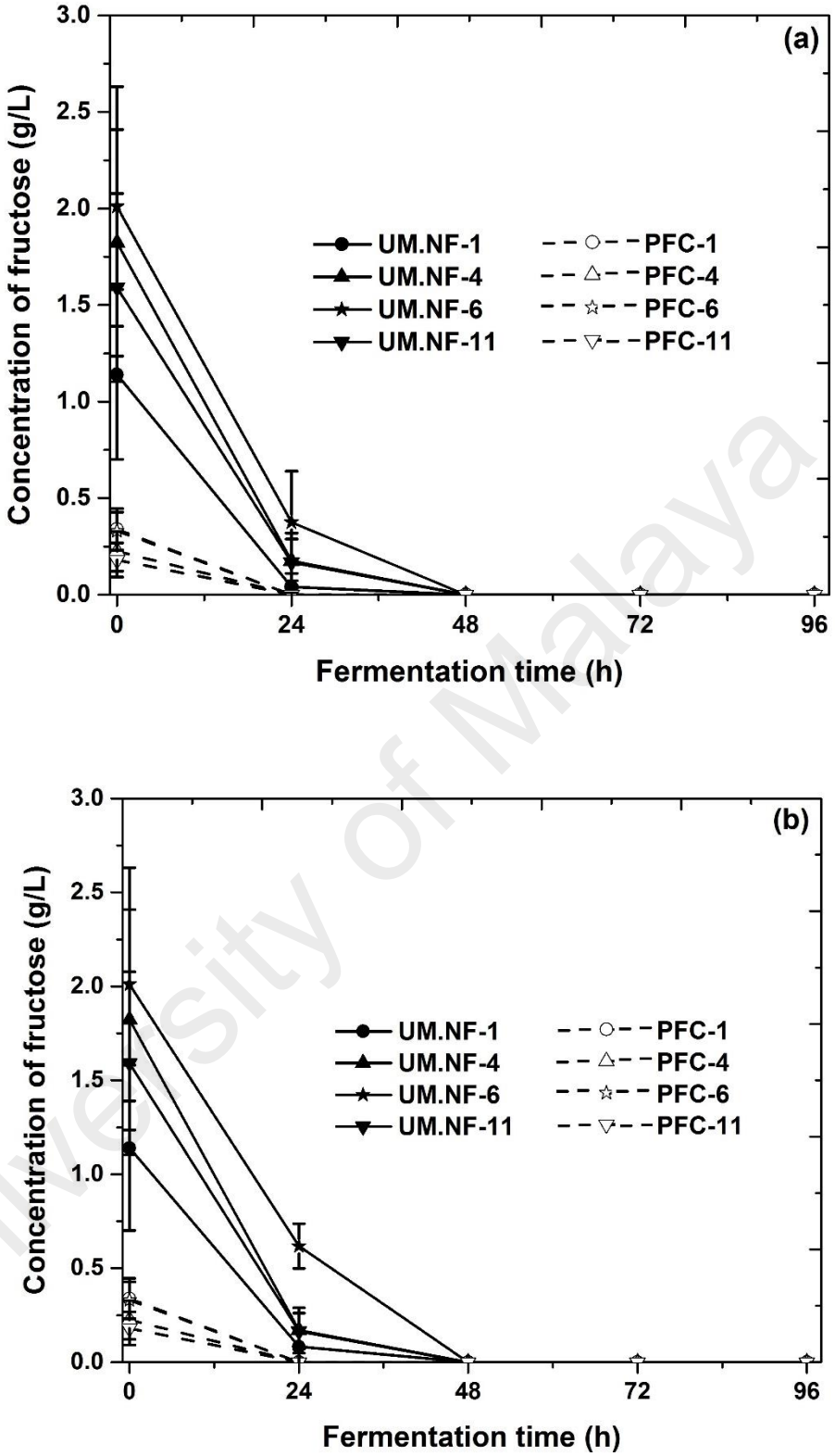

Figure 5.21: Changes in Fructose Concentration Over Time During GSHSF under Two Enzymatic Conditions; (a) 1.5 kg/MT and (b) 2.0 kg/MT.

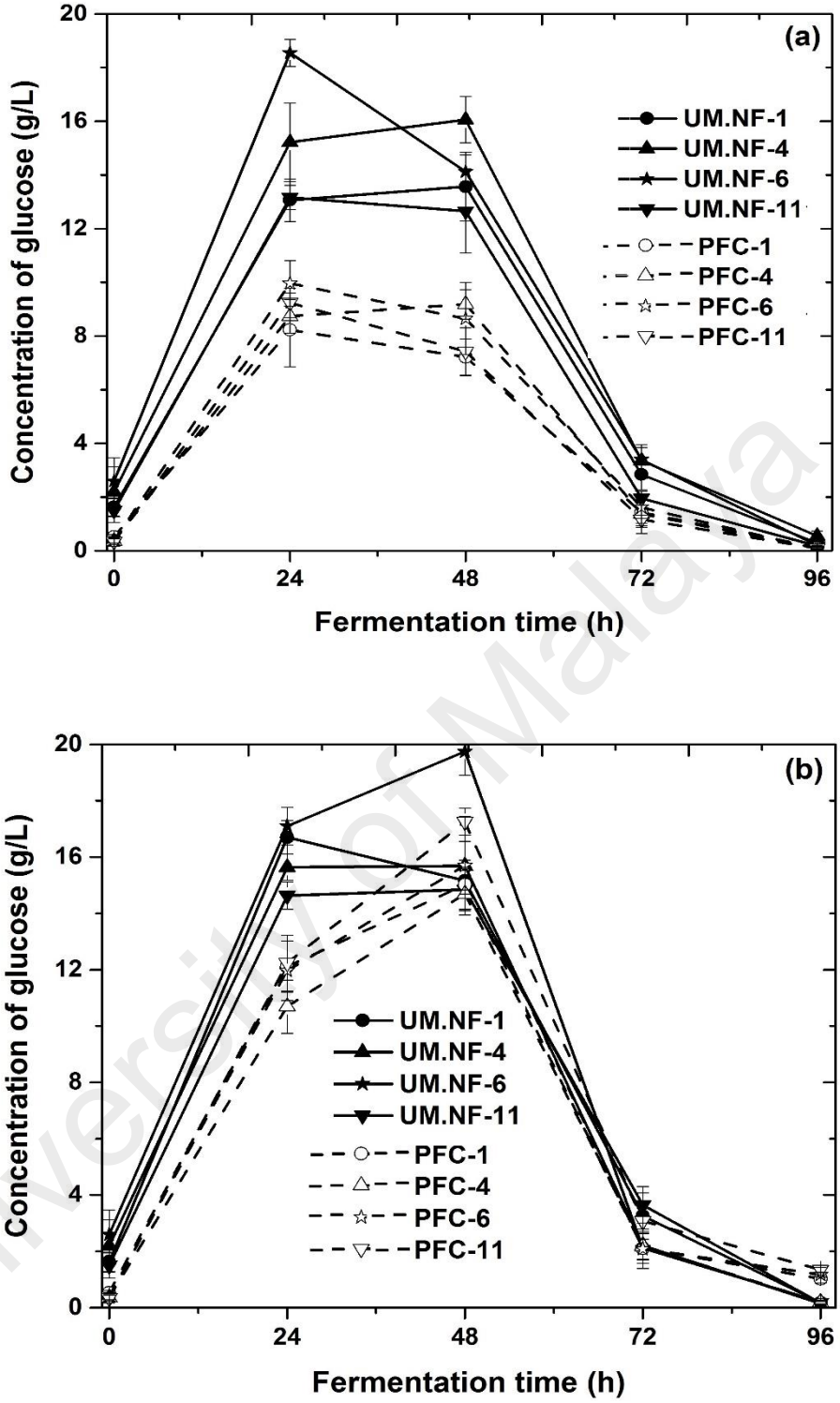

Figure 5.22: Changes in Glucose Concentration Over Time During GSHSF under Two Enzymatic Conditions; (a) 1.5 kg/MT and (b) 2.0 kg/MT.

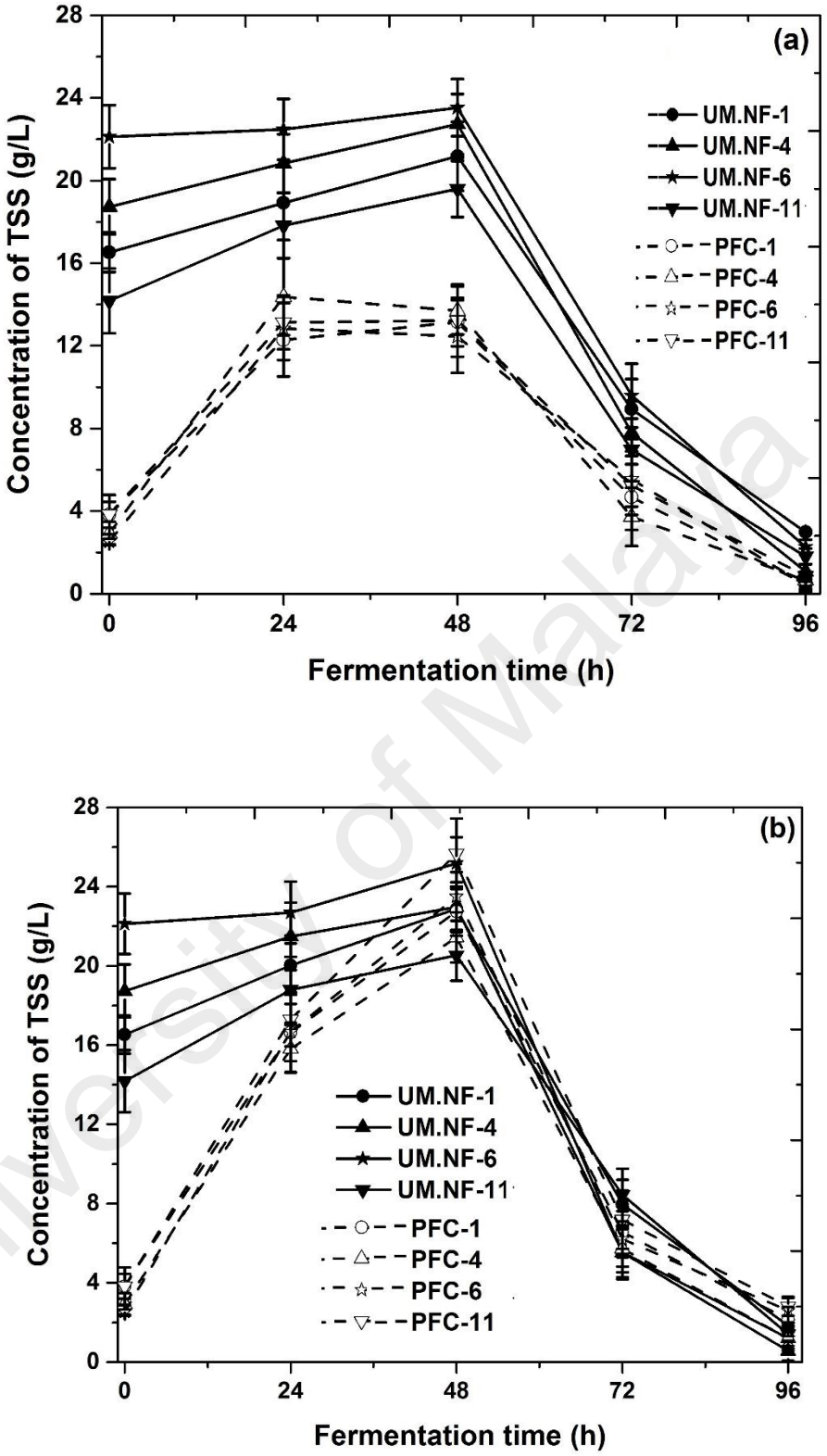

Figure 5.23: Changes in TSS Concentration Over Time During GSHSF under Two Enzymatic Conditions; (a) 1.5 kg/MT and (b) 2.0 kg/MT.

5.3.2.2 Viable Microbial Loads during GSHSF

During GSHSF, yeast cells grew exponentially in all samples until 48 h and subsequently underwent a stationary phase (Figure 5.24), showing a significant difference in viable yeast counts between the initial, after 24 h and 48 h ($P < 0.05$). However, the growth of yeast cells as well as the total viable yeast count did not change significantly with the changes in enzyme load during GSHSF. Total bacterial count showed a decrease initially followed by a gradual increase over time (Figure 5.25). The increase in bacterial count peaked between 72 and 96 hours. However, the bacterial count in the eight corn genotypes samples almost similar for a specific period of GSHSF. Overall, both yeast and bacterial counts did not show any significant variations between the different enzyme loads.

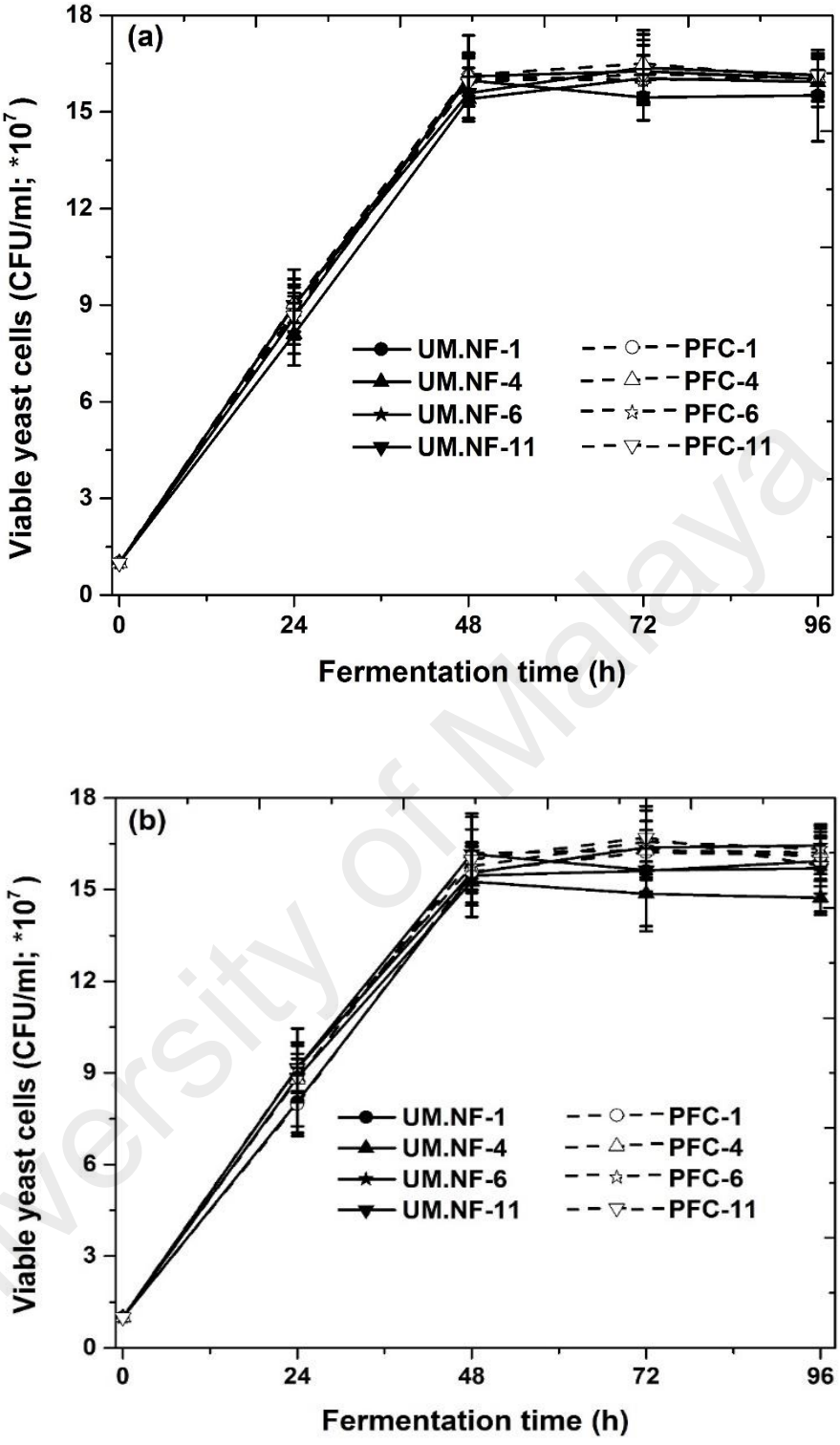

Figure 5.24: Changes in Viable Yeast Count Over Time During GSHSF under Two Enzymatic Conditions; (a) 1.5 kg/MT and (b) 2.0 kg/MT.

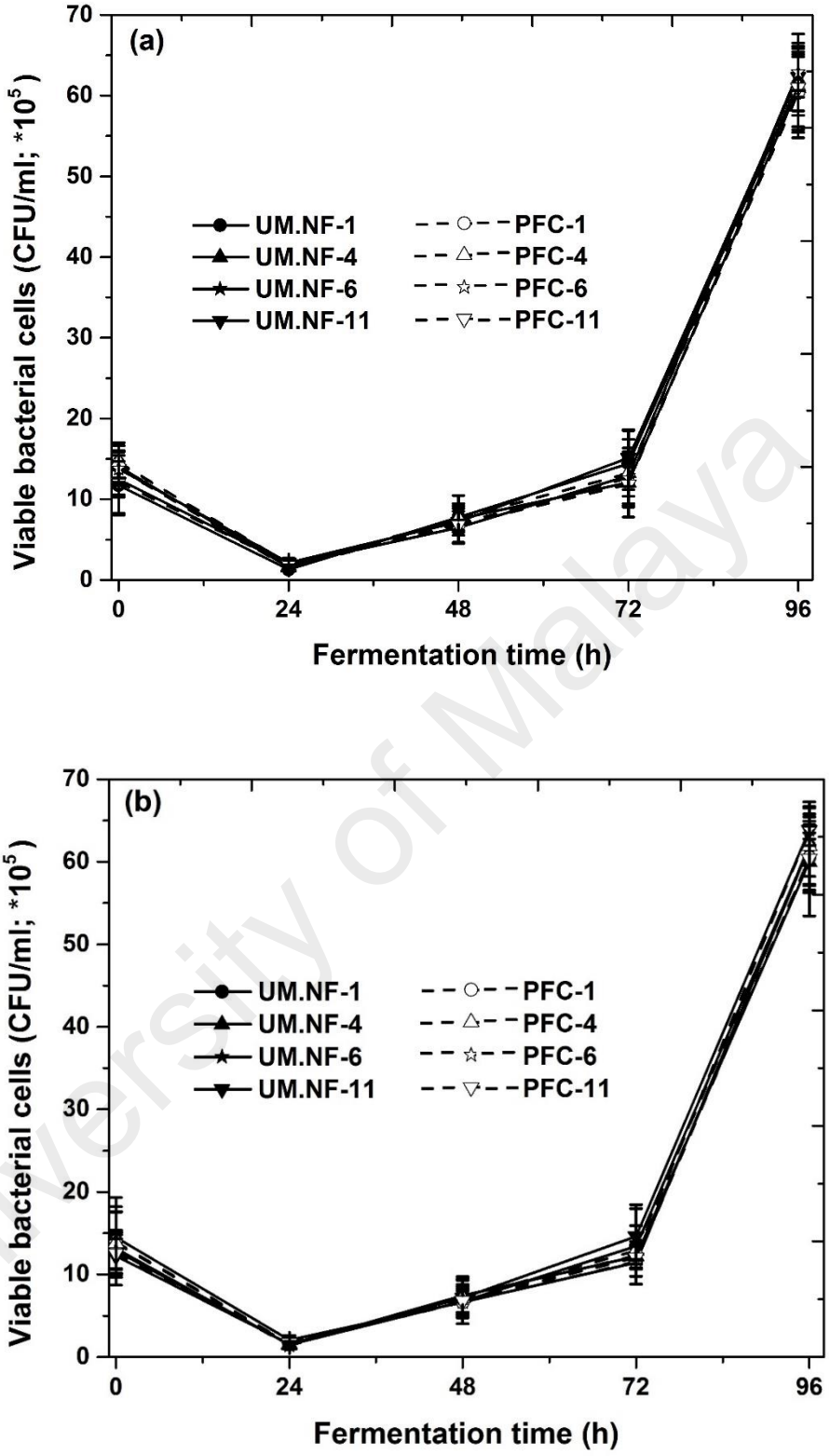

Figure 5.25: Changes in Viable Bacterial Count Over Time During GSHSF Under Two Enzymatic Conditions; (a) 1.5 kg/MT and (b) 2.0 kg/MT.

5.3.2.3 Accumulation of the Soluble Byproducts during GSHSF

The accumulation of glycerol in the fermentation broth increased significantly and exponentially over time in all genotypes and under both enzymatic loads (Figure 5.26). Likewise, the production and accumulation of lactic acid in the fermentation media was found to increase with time and showed a dramatic increase from 72 to 96 h (Figure 5.27). Although microbial counts and soluble byproducts varied slightly among the corn genotypes, these variations could be considered statistically insignificant since the *P* values were greater than 0.05.

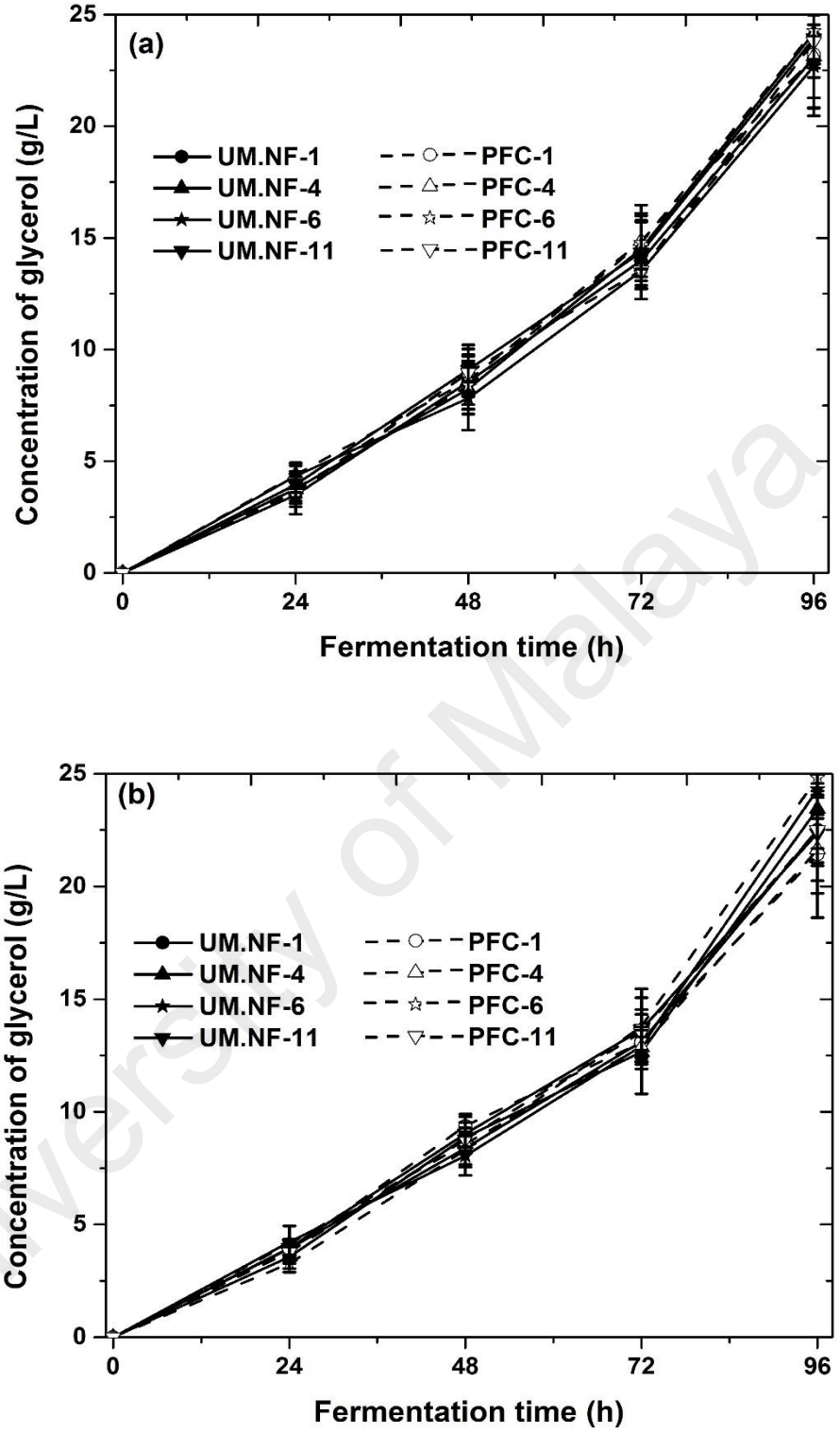

Figure 5.26: Changes in Glycerol Concentration Over Time During GSHSF under Two Enzymatic Conditions; (a) 1.5 kg/MT and (b) 2.0 kg/MT.

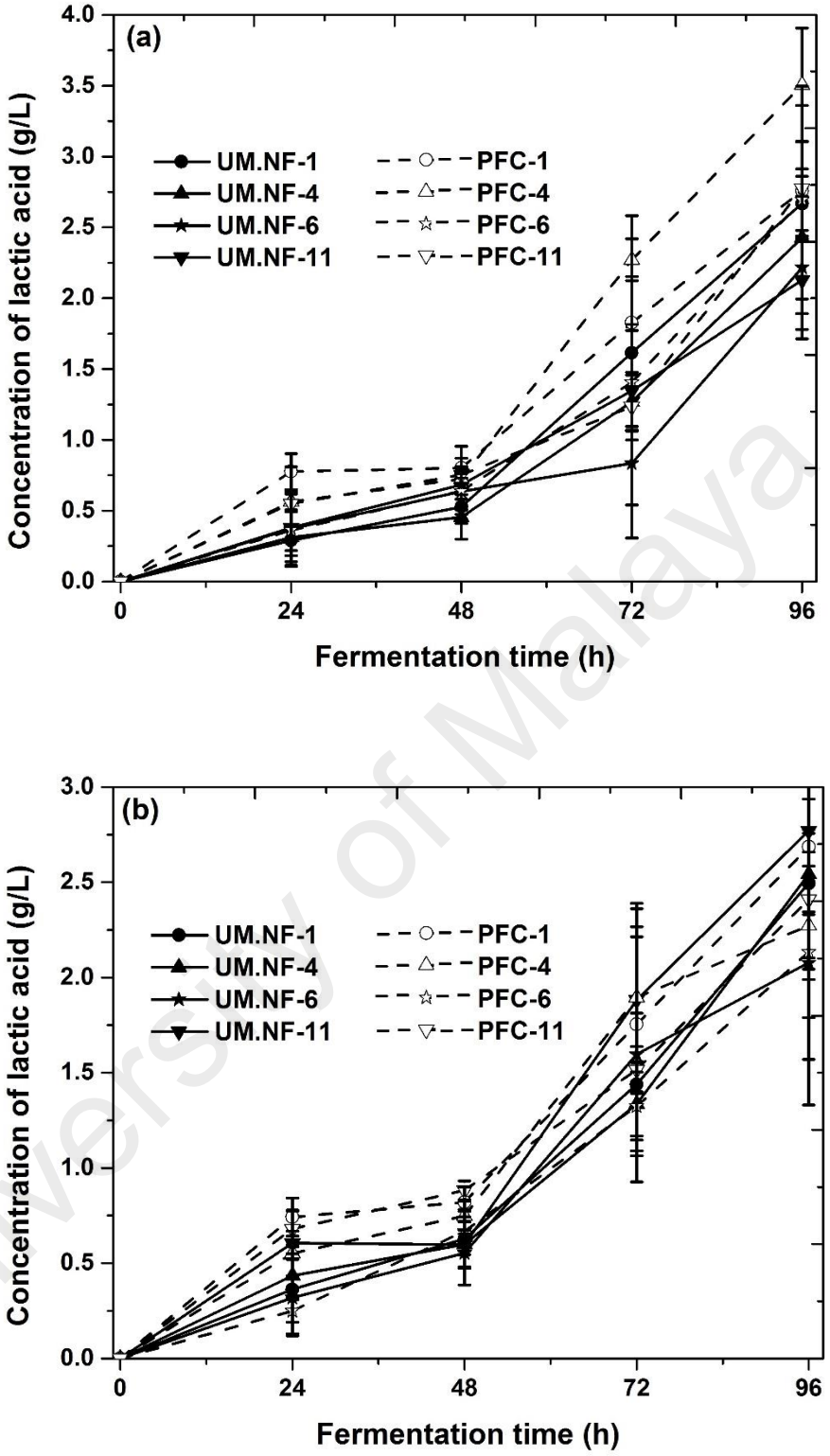

Figure 5.27: Changes in Lactic Acid Concentration Over Time During GSHSF Under Two Enzymatic Conditions; (a) 1.5 kg/MT and (b) 2.0 kg/MT.

5.3.2.4 Correlations among Different Parameters

In order to determine the relationship among the different parameters studied, Pearson's product moment correlation coefficients were determined and summarized in Table 5.2. Most of the agronomic parameters did not show any significant correlation with yield and attributes of GSH and GSHSF. However, anthesis and silking time, grain filling period and maturity of the corn genotypes were found to be negatively correlated with ethanol concentration. From Table 5.2, it can be seen that RS and ethanol yield negatively correlated with kernel starch, whereas, there were positive correlations between RS, ethanol yield and kernel sugars. On the other hand, kernel composition did not influence yeast growth, bacterial contamination and soluble byproduct accumulation in the fermentation broth during GSHSF, since they were poorly correlated with kernel components.

Table 5.2: Correlation-Coefficient of Different Parameters Obtained from Kernel Composition, Hydrolysis (GSH) and Fermentation (GSHSF) of Corn Genotypes

Kernel parameters	Sugar conc.	Ethanol conc.	Residual Starch	Residual TSS	Yeast	Bacteria	Glycerol	Lactic acid
Seed emergence	–0.007	–0.250	–0.023	–0.110	–0.179	0.473**	–0.016	0.156
Anthesis time	–0.126	–0.635**	0.203	0.010	–0.129	0.342	0.122	–0.034
Silking time	–0.149	–0.607**	0.150	0.004	–0.232	0.360*	0.114	0.117
Grain filling period	–0.074	–0.406*	–0.090	–0.380*	–0.257	0.073	–0.221	0.165
Black layer maturity	–0.130	–0.588**	0.036	–0.215	–0.283	0.253	–0.060	0.163
Plant height	–0.172	–0.545**	0.407*	0.211	0.161	0.147	0.004	–0.011
Leaf number	0.130	–0.196	–0.228	–0.291	–0.056	0.123	0.048	0.326
Leaf length	–0.392*	–0.345	0.223	0.251	0.306	0.069	–0.370*	0.136
Ear number	–0.131	–0.093	0.061	–0.047	0.015	–0.071	–0.066	0.078
Ear weight	–0.112	–0.040	–0.200	–0.420*	–0.032	–0.109	–0.115	0.288
Ear length	0.003	0.156	0.196	–0.031	–0.057	0.032	–0.010	0.007
Kernel depth	0.145	0.359*	–0.200	–0.029	–0.178	0.071	–0.117	–0.048
Grain yield	–0.096	0.019	–0.178	–0.350*	–0.138	–0.100	–0.172	0.100
Kernel number	–0.073	–0.044	0.085	–0.041	–0.018	–0.031	0.087	0.144
1000 kernel weight	–0.320	0.289	–0.207	–0.180	–0.323	–0.063	–0.306	0.425*
Kernel sucrose	0.679**	0.627**	–0.763**	–0.532**	–0.191	–0.024	0.197	–0.035
Kernel glucose	0.682**	0.563**	–0.711**	–0.522**	–0.257	0.121	0.220	0.015
Kernel fructose	0.662**	0.496**	–0.681**	–0.528**	–0.104	0.110	0.266	0.047
Kernel TRS	0.497**	0.550**	–0.645**	–0.404*	–0.184	0.157	0.193	0.185
Kernel TSS	0.698**	0.626**	–0.773**	–0.538**	–0.209	0.003	0.207	0.007
Kernel starch	–0.589	–0.212	0.792**	0.680**	0.120	–0.029	–0.078	–0.006
Kernel amylose	–0.439	–0.342	0.434*	0.172	–0.035	0.158	–0.241	0.127
Kernel protein	0.015	–0.355*	–0.060	–0.277	–0.071	–0.226	0.058	–0.188
Kernel fat	–.410*	–0.407*	0.379*	0.120	0.181	–0.014	–0.270	0.061
Kernel fiber	0.088	–0.030	–0.176	0.111	0.211	–0.041	0.010	0.324
Kernel ash	0.595**	0.356*	–0.517**	–0.451**	0.059	0.025	0.406*	–0.030
Sugar conc.		0.297	–0.628**	–0.458**	0.169	0.188	0.429*	–0.023
Ethanol conc			–0.506**	–0.352*	–0.051	–0.146	–0.032	0.087
Residual starch				0.844**	0.160	0.064	0.050	–0.044
Residual TSS					0.203	0.074	0.076	–0.011
Yeast						0.083	0.379*	0.162

**Correlation is significant at $p<0.01$(2–tailed)

*Correlation is significant at $p<0.01$ (2–tailed)

Data used for determining correlation–coefficient include all corn genotypes and most optimal conditions

5.4 Discussion

5.4.1 Granular Starch Hydrolysis (GSH)

5.4.1.1 Effect of Pre-Heat Treatment of the Slurry on GSH

During GSH, a significant effect of prior heat treatment was observed on the bioconversion of starch into RS. The RS concentration increased exponentially until 48, gradually increased until 72 h, after which, there was either a small decrease or plateau in sugar yield (Figure 5.3). The reason for the decrease in the accumulation of RS in the hydrolysates after a certain time could be due to microbial contamination that might use soluble sugars for their metabolism. Furthermore, the concentration of RS plateaued after 72 h during GSH possibly as a result of end product inhibition to the amylase by RS (Mojović et al., 2006). A similar pattern of changes in sugars as well as ethanol yield was also reported in an earlier study with corn meals (Lamsal et al., 2011). The sugar yield pattern observed in this study versus time is, however, not similar to that of the conventional hydrolysis as described in Chapter 4, and also as have been reported in literature (Mojović et al., 2006; Murthy et al., 2009; Nikolić et al., 2010), where a plateau in sugar production was observed after 48 h. The reason of this variation might be due to the gelatinization and liquefaction of the slurries at high temperature in the conventional process, which made starch molecules more accessible to enzymes at the early stages of hydrolysis (Mojović et al., 2006; Zabed et al., 2016b).

Overall, sugar yield, productivity of RS and the conversion rate of starch were significantly higher in all the genotypes when slurries were pretreated. Lower sugar yield in the absence of prior heat treatment could be due to inefficient hydrolysis of starch, because heat treatment has been shown to have a significant effect on amylase activity on raw starch granules (Shariffa et al., 2009). Compared to PFCs, it can be seen that all HSGs produced higher amounts of RS with higher productivity under both conditions, due to

the fact that it contained a higher amount of kernel sugars (Chapter 3, Table 3.8). More importantly, the enzyme load was not optimum for the PFCs, since GSH was carried out using an enzyme load of 1.5 kg/MT of dry corn (Section 5.4.1.2).

5.4.1.2 Effect of Enzyme Load on GSH

It was observed that RS concentration and percentages of starch conversion increased proportionally to the enzyme dosage (Figure 5.6 and 5.7). The insignificant variations in RS yield and starch utilization during GSH above a certain level of enzyme load, could be attributed to the limited supply of substrates (Apar & Özbek, 2004; Hagenimana et al., 1992). The other possibility is that enzyme inhibition could have happened that resulted in the reduction of enzyme efficiency for binding to the substrate starch molecules (Textor et al., 1998). The lower enzyme requirement for HSGs with higher RS yields could be explained by the fact that they contained higher amounts of free sugars, which would add to the total RS yield. Another possible reason could be the relatively lower amounts of starch in HSGs than in PFCs, which needed lower amounts of enzyme for completion of hydrolysis (Mojović et al., 2006). However, a reasonable amount of starch remained unreacted in both group of genotypes at the end of GSH under all enzymatic conditions (Figure 5.6), possibly as a result of the inhibition of the enzyme by its end product (Mojović et al., 2006). It could also possibily be due to the fact that the hydrolysis efficiency of the starch is also based on their amylose/amylopectin content ratios and other physicochemical properties (Karlsson et al., 2007; Yangcheng et al., 2013).

5.4.1.3 Effect of Particle Size of Ground Corn on GSH

It was observed that the final RS concentrations increased inversely in both groups of corn genotypes with particle size. Maximum amounts of RS were achieved in PFCs and HSGs with the smallest particle size (0.2 mm). Compared to PFCs, all the HSGs produced

significantly higher amounts of RS with all particle sizes, presumably due to the fact that the enzyme dosage (1.5 kg/MT) used was optimum for HSGs and not for PFCs (section 5.4.1.2). As a result, the starches in PFCs were incompletely hydrolyzed. From these findings, it can be assumed that particle size had no effect on the sugar releasing capacity of HSGs under the condition of GSH.

An earlier study with different particle sizes of ground corn reported that particle size had a direct effect on ethanol as well as sugar yields, with smaller particle size more favorable for the efficient conversion of starch into sugars (Naidu et al., 2007). The authors concluded that yield, capital costs and operating costs of a dry–grind ethanol plant would be affected by particle size distribution. However the effect of particle size on the change in starch concentration was, opposite to the production of RS during GSH (Figure 5.6). Particle size of ground cereals usually influence the available surface area for reaction during starch hydrolysis, where smaller size increases surface area and favors enzyme binding and efficient catalysis (Al-Rabadi et al., 2009; Barcelos et al., 2011a). Furthermore, smaller particles have been observed to favor the release free sugar in HSGs in the hydrolysates (Zabed et al., 2016b). However, grinding of corn into smaller particle size requires higher energy that ultimately increases production cost, in addition to the difficulties in downstream processing (Wang et al., 2008). In the current dry–grind ethanol industries, corn meal with a particle size <1.0 mm is usually used as ethanol feedstock (Szymanowska-Powałowska et al., 2014). Considering the aforementioned facts, corn flour with 1.0 mm particle was selected for all further investigations in this study.

5.4.1.4 Effect of Initial Solid Load on GSH

As has been reported in the Results section above, RS concentrations in the hydrolysates of both HSGs and PFCs increased with increase in solid contents until 300

g/L. However, the concentration of RS plateaued and even decreased when the initial solid load reached to 350 g/L, and considerable amounts of starch remained unreacted under this condition (Figure 5.10). Similar findings were also reported in an earlier investigation during ethanol production from corn meal (Lemuz et al., 2009), where the authors observed that ethanol yield increased with an increase in initial solid concentration from 20 to 30%, followed by a decrease when solid concentration was further increased.

Lower amounts of dissolved solid in the slurries are usually suitable for effective agitation and mixing of the contents (substrate and enzymes) during hydrolysis and should result in higher efficiency of the process (Lemuz et al., 2009). However, although HSGs produced higher amounts of RS under all conditions than PFCs, the degree of variations in RS yield between the two groups of genotypes were lower when the initial solid load used was between 15 and 20%. This could partially be due to the fact that slurries of PFCs received lower amounts of starch at lower initial solid load, and as a result, starch could be hydrolyzed more efficiently with the proper enzyme–substrate ratios, as well as with minimum substrate inhibition of the enzyme (Mojović et al., 2006; Zabed et al., 2016b). At the same time, the amount of free sugar in the slurries of HSGs decreased as the amount of corn meal decreased under lower solid concentrations. High initial solid concentration is often desired on a commercial scale for producing cost effective ethanol. In this perspective, HSGs showed potential due to producing higher amounts of soluble sugars at higher solid concentration. As a result, 30% solid load was chosen for subsequent experiments, which is also the typical amount for industrial and many laboratory practices (Kelsall & Lyons, 1999; Lemuz et al., 2009).

5.4.2 GSHSF

5.4.2.1 Ethanol Production during GSHSF

Similar to the RS yield during GSH, ethanol concentration also increased significantly with the higher enzyme load among PFCs (Figure 5.18). However, PFC–4 showed no significant variation in ethanol concentration between the two enzyme loads used and produced the lowest amount of ethanol among the PFCs, at maximum enzyme dosage. PFC–4 also recorded the lowest amount of starch among the PFCs (Chapter 3, Table 3.8), in which meant a more efficient conversion of starch to RS due to minimum substrate inhibition of the enzyme.

Even though HSGs showed a slight increase in ethanol concentration under the higher enzymatic load, the difference in ethanol concentrations between the two enzymatic load (1.5 and 2.0 kg/MT of dry corn) can be regarded as insignificant since the *P* values were greater than 0.05. The higher ethanol production by HSGs could be due to the presence of higher amounts of kernel sugars that would release into the mash, unlike sugar derived from starch hydrolysis (Zabed et al., 2016b). Another explanation for HSGs producing higher amounts of ethanol was the fact that they produced higher amounts of fermentable sugars as revealed by GSH (Section 5.3.1.) and conventional hydrolysis (Chapter 3, Section 4.3.1). On the other hand, all genotypes differed significantly in ethanol production during GSHSF over time until the end of fermentation (96 h), even though the yield was much higher during the earlier periods of GSHSF.

Under the lower enzymatic dosage the average final ethanol yield in HSGs varied between 0.4 and 0.46 g/g, while in PFCs it ranged between 0.31 and 0.35 g/g. As the enzyme dose increased to 2.0 kg/MT of dry corn, ethanol yield increased slightly in HSGs but significantly in PFCs, which ranged from 0.43 to 0.47 g/g and 0.38 to 0.44, respectively. These findings can be compared with the ethanol yield from other cereals

as well as corn materials. For example, the average final ethanol yield has been reported to be 0.36 g/g (458.84 L/MT) for Indian broken rice and 0.32 g/g (404.03 L/MT) for pearl millet under similar conditions of this study (30% dry solid and supplemented with protease), with the exception that the fermentation was done via the conventional method (Gohel & Duan, 2012a). The ethanol yield from corn was reported between 0.31 and 0.33 in a previous study (Wang et al., 2005). Similar ethanol yield has been reported in another study during ethanol conversion from corn in USDA (Shapouri et al., 2002).

5.4.2.2 Utilization of Carbohydrates during GSHSF

As was shown in Figure 5.19, the starch concentration decreased as the enzyme load increased, although significant amounts of starch remained unutilized after fermentation in all genotypes. However, the amount of final residual starch were lower than those observed during GSH (Figure 5.1), probably due to the simultaneous production and utilization of fermentable sugars during GSHSF minimizing end product inhibition (Mojović et al., 2006). It has been reported previously that more than 5% of starch remained unreacted after dry–grind ethanol production, using the current practice (Plumier et al., 2015). The quantity of residual starch showed strong correlations with kernel starch (Table 5.2). Among HSGs, UM.NF–11 contained the highest amount of initial starch in the slurry, corresponding to its high kernel starch for the HSGs. UM.NF–1 was next highest. Not surprisingly, UM.NF–11 left a significantly higher amount of starch unutilized under the lower enzymatic dosage, compared to UM.NF–1 (Figure 5.19). Similarly, the lowest starch containing PFC (PFC–4) left the lowest amount of residual starch in the slurry. For the same reasons, the other three PFCs left higher amounts of residual starch under both enzymatic conditions, particularly when the enzyme load was lower. These results have shown that higher amounts of residual starch

could be obtained under inappropriate enzyme load if the starch concentration in the mash exceeded a certain threshold, even a small change in initial starch concentration.

Most of the sucrose and fructose in the mash were used up in 24 h, and a complete exhaustion was observed after 48 h (Figure 5.20 and 5.21). A similar observation was also reported earlier for ethanol production from tropical maize containing a mixture of sucrose, glucose and fructose (Chen et al., 2013). Consequently, total soluble sugars (TSS) present in the mash after 24 h and the subsequent courses of fermentation contained only glucose and/or other soluble sugars obtained from the incomplete hydrolysis of the starch such as saccharides containing four or more glucose units (DP_{4+}), maltotriose (DP_3), and maltose (DP_2) (Devantier et al., 2005). Under both enzymatic conditions, HSGs contained significantly higher concentrations of sugars in the mash than those of PFCs at initial stages of GSHSF, since they got sugars not only from starch hydrolysis but also from their kernel sources that could have resulted in higher production rate than consumption rate. In contrast, PFCs failed to produce considerable amount of sugars in the mash from their kernel source, and as a result, they could only accumulate smaller amount of TSS until 24 h, even though the enzyme load was an effective dosage for PFCs (2.0 kg/MT). However, as the course of GSHSF increased, sugar production by PFCs also increased resulting in an overall increase of TSS concentration after 48 h due to the higher production than consumption rate. After 48 h, the sugar concentration dropped suddenly, in all the genotypes under both enzymatic conditions that continued until the end of fermentation.

5.4.2.3 Viable Microbial Loads

The growth of yeast cells should be monitored during fermentation to determine whether it is affected by the presence of any inhibitory substances in the media. This is particularly important when an antibiotic is used to inhibit bacterial growth. Furthermore,

proliferation and survival of yeast cells may also be affected by osmotic stress and the ethanol tolerance limit of yeast cells. Just as important, bacterial contamination in the fermentation beer is undesirable, since it can affect the final ethanol yield by consuming soluble sugars and producing different byproducts (Thomas et al., 2001). As an attempt to reduce bacterial contamination during fermentation, antibiotics are often used in commercial plants, even though bacterial contamination can still exist in the process (Narendranath et al., 1997).

The growth of yeast cells in this study did not show any significant variation with change in enzyme load, which is in agreement with the findings of the aforementioned investigation by Devantier et al. (2005). Total bacterial counts showed an initial decrease after 24 h, which followed a gradual increase over time. The final viable bacterial count, after 96 h, varied between 6.0×10^6 and 6.3×10^6, irrespective of the enzyme dosage and corn genotypes. In a previous study, the total bacterial concentration in the corn mash was reported to be $2.0–3.3 \times 10^9$ CFU/ml after 72 h, without showing any adverse effect on the final ethanol yield (Szymanowska-Powałowska et al., 2014).

5.4.2.4 Accumulation of Soluble Byproducts

As the course of GSHSF increased, accumulation of glycerol and lactic acid in the fermentation media also increased (Figure 5.26 and 5.27). The average glycerol concentrations ranged between 3.76 g/L after 24 h and 24.31 g/L after 96 h (Figure 5.26) (Russell, 2003) reported that conventional dry grind corn ethanol fermentation typically produces 12–15 g/L glycerol, but values as high as 41 g/L has been observed elsewhere during GSHSF (Białas et al., 2010). In this study, the maximum final lactic acid concentration in the mash was found to be 3.51 g/L among the corn genotypes after 96 h. Accumulation of lactic acid observed in this study was, however, slightly higher than what has been reported earlier (<0.3 g/L) during conventional fermentation of the corn

mash liquefied at high temperature (90^{o}C) prior to fermentation (Murthy et al., 2009). Białas et al. (2010) reported higher concentrations of lactic acid (0.5–4 g/L) produced during GSHSF of corn, which did not have any adverse effects on the final ethanol yield

5.4.3 Correlation among Parameters

In order to determine the relationship among the different parameters studied, Pearson's product moment correlation coefficients were determined and summarized in Table 5.2. It can be seen that both individual sugars and TSS of the corn kernels showed strong negative correlations with starch. Similarly, both RS and ethanol yield negatively correlated with kernel starch, whereas, there were positive correlations among RS, ethanol yield and kernel sugars. However the relationship between ethanol yield and kernel starch found in this study was contrary to the findings of several other workers (Reicks et al., 2009; Singh & Graeber, 2005), who reported a positive but insignificant correlation between these two parameters. In this study, the sugar contents of HSGs as well as PFCs (although amounts were lower in PFCs) were taken into consideration. Since HSGs produced higher amounts of ethanol in relation to their corresponding higher sugar contents but not starch, it is not surprising to get a negative correlation between starch and ethanol yield. Moreover, a recent study also reported a negative correlation between kernel starch and ethanol after investigating 257 corn genotypes (Gumienna et al., 2016). However, this does not necessarily mean that lower starch content in corn would enhance ethanol yield. It will be only true if both starch and sugars are taken into consideration. Ethanol concentration did not show any significant correlation with kernel protein, which was also reported elsewhere (Singh & Graeber, 2005).

5.5 Conclusion

This study has shown that the free sugar content in corn kernels have substantial effects on enzyme consumption and ethanol yield during granular starch hydrolysis and simultaneous fermentation. High sugary corn genotypes (HSGs) produced considerable amounts of ethanol consuming lower quantities of enzyme (1.5 kg/MT of dry corn) compared to normal (field) corn (PFCs), which required an enzyme load of 2.0 kg/MT to produce even lower amounts of ethanol. It was also observed that when starch content exceeded a certain level, a significant amount of starch remained unreacted, even though enzyme load was optimum. A strong positive correlation was observed between kernel sugars and ethanol yield, and hence, the former could be a useful indicator for assessing raw material quality of corn as ethanol feedstock. In conclusion, high sugar content in corn grains was able to improve raw material quality, produce higher amounts of ethanol, and reduce enzyme consumption and production costs during dry–grind ethanol production.

CHAPTER 6: EVALUATION OF THE CO–PRODUCT QUALITY FOR NORMAL AND HIGH SUGARY CORN GENOTYPES DURING DRY–GRIND ETHANOL PRODUCTION

6.1 Introduction

The increasing demand for ethanol as an alternative energy source and attempts to reduce the dependence on fossil fuels have led to a dramatic increase in the use of corn as ethanol feedstock (Liu, 2009). The dry–grind ethanol production process is a widely used method for generating ethanol from corn (Bothast & Schlicher, 2005; Singh et al., 2001). A typical dry–grind process produces primarily two co–products, namely, distiller's dried grains with solubles (DDGS) and carbon dioxide, of which DDGS play an important sustainable economic role (Belyea et al., 2004; Liu, 2008). DDGS comprises primarily of water, protein, fat, fiber and residual starch that has remained after fermentation (Belyea et al., 1998; Belyea et al., 2004; Kim et al., 2008). The price and marketing of DDGS is often affected by its quality, particularly its physicochemical composition (Belyea et al., 2004; Liu, 2009). During dry–grind ethanol production, the fermentable components of corn kernels (starch and sugars) are converted into ethanol, while the non–fermentable ingredients (protein, fat, fiber and ash) remain unconverted and concentrated in the fermented broth that finally accumulate in DDGS (Liu, 2011).

A typical dry–grind ethanol process produces around 2.8 gallons ethanol and 7.7 kg DDGS from one bushel of corn (25.4 kg) (Mosier & Ileleji, 2014; USGC, 2012). Generally, the solid residues mixed with thin stillage from the dry–grind ethanol production process are known as DDGS (Singh et al., 2001). In a commercial plant, DDGS is usually produced from the whole stillage that contains water and solid that remains after distillation of fermented broth (Kim et al., 2010b; USGC, 2012). First of all, the whole stillage is centrifuged to separate a coarse solid fraction (containing about

35% dry matter) and a liquid fraction, which are called wet distillers' grains (WDG) or wet cake and thin stillage (TS), respectively (Kim et al., 2008). Subsequently, a good amount of TS (≥15%) is recycled as backset to be used as process water for preparing slurry from the corn meal (Kwiatkowski et al., 2006). The remaining TS is concentrated in evaporators and the resultant liquid is referred to as condensed distiller's solubles (CDS) or syrup that contains around 30% dry matter (Ganesan et al., 2006). The CDS and WDG can be sold locally to the cattle feeders without further processing or alternatively, both are combined and dried to produce DDGS (containing about 88% dry matter) in order to lengthen its shelf life (McAloon et al., 2000).

DDGS is popularly used across the world as a source of protein and energy in livestock feed (Branca & Di Blasi, 2015; USGC, 2012). The usage of DDGS has been basically limited to ruminant diets due to its high fiber content and nutrient variability (Rausch & Belyea, 2006). In recent years, research efforts have been made to remove the fiber portion from DDGS to make it suitable for use as feed for non–ruminant animals (Kim et al., 2010a). The fiber portion, upon removal, can be used for various purposes, which include the production of corn fiber oil and corn fiber gum (Singh et al., 2002). Furthermore, additional amounts of ethanol can be produced from the corn fiber or DDGS (Elander & Russo, 1993; Gáspár et al., 2007; Mosier et al., 2005; Saha et al., 1998; Singh & Eckhoff, 1997). On the other hand, separation of fiber from DDGS ultimately increases the concentration of protein and fat in DDGS, making it more desirable as feed for poultry and swine (Singh et al., 2005; Srinivasan, 2006; Srinivasan et al., 2005).

The nutritional composition of DDGS is primarily the non–fermentable components of raw corn, and can vary greatly with regard to raw material quality, production process, plant conditions, and process parameters (Belyea et al., 2004; Liu, 2008; Spiehs et al., 2002). As a result, it has been recommended that a complete chemical analysis should be

done on a regular basis for each source of DDGS (Liu, 2011). It is expected that there might be a significant relationship between the composition of raw corn and DDGS. However, there have been contradictory findings on the relationship between the composition of corn kernels and DDGS. Belyea et al. (2004) reported that variations in the chemical composition of corn kernels does not significantly relate to the nutritional quality of DDGS. On the contrary, Liu (2009) reported a significant relationship between the components of DDGS and that of kernels.

Among the nutritional components, the most important is protein, which can vary significantly with sources of DDGS. A variation in protein in animal diets may cause mis–formulation, which can affect animal productivity (Belyea et al., 2004). Generally, DDGS derived from a dry–grind ethanol plant consists of 26–35% protein (Belyea et al., 1989; Cromwell et al., 1993). The source of protein in DDGS is not only the protein content of raw corn but also the dry biomass of yeast cells grown during the fermentation period (Belyea et al., 2004; Liu, 2009). The starch content in DDGS is the residual starch remaining after fermentation, which may vary between 3.2% and 5.9% (Belyea et al., 2004; Liu, 2008).

In the present investigation, dry grind ethanol production was carried by two different methods (CSSF and GSHSF) using two groups of corn genotypes (HSGs and PFCs) as was described in Chapters 4 and 5. The main variation observed between HSGs and PFCs was in enzyme consumption, with HSGs requiring lower amounts of enzymes than the PFCs, in both methods. During CSSF, the optimum enzyme dosage was 3.0 kg/MT for HSGs and 4.0 kg/MT for PFCs (Chapter 4; Zabed et al., 2016a), while the respective enzyme loads for GSHSF were found to be 1.5 kg/MT and 2.0 kg/MT (Chapter 5; Zabed et al., 2016b). Taking into account the above facts, the quality of DDGS (protein, fat, fiber, ash and starch) was determined for all the genotypes (four HSGs and four PFCs)

by analyzing the samples collected from CSSF and GSHSF conducted under two enzymatic conditions.

6.2 Materials and Methods

6.2.1 Sample Preparation

The fermentation broths were collected from CSSF under the enzymatic loads 3.0 and 4.0 kg/MT of dry corn (Chapter 4; Section 4.2.6), and from GSHSF under the enzymatic loads 1.5 and 2.0 kg/MT (Chapter 5; Section 5.2.6). DDGS was prepared from the collected samples by the modified method of Corredor et al. (2006) and Wang et al. (2005), so that it mimicked the process followed in a commercial plant. Firstly, ethanol was evaporated from the collected broths at 90°C for 3 h in a water bath (Wang et al., 2005). The broths were then centrifuged at 5000 rpm for 15 min to separate WDG and TS, which were the residues and supernatant respectively. Thereafter, TS was concentrated in a rotary evaporator to a moisture level of around 30–35%, for getting condensed distiller's solubles (CDS) (Bothast & Schlicher, 2005). The WDG and CDS were mixed thoroughly and dried in an oven at 49^{o}C until the moisture level reached 12–14%. Finally, four groups of DDGS samples were obtained and labeled as DDGS-1 (obtained from CSSF under the enzyme load 3.0 kg/MT), DDGS-2 (obtained from CSSF under the enzyme load 4.0 kg/MT), DDGS-3 (obtained from GSHSF under the enzyme load 1.5 kg/MT) and DDGS-4 (obtained from GSHSF under the enzyme load 2.0 kg/MT).DDGS yield for each sample was determined using the Equation (6.1).

$$Y_{DDGS}\ (\%) = \frac{W_{DDGS}}{W_{Corn}} \times 100 \qquad (6.1)$$

where, Y_{DDGS} is the yield of DDGS (%, w/w); W_{DDGS} is the dry weight of DDGS (g); W_{Corn} is the dry weight of the corn sample initially used for fermentation (g).

6.2.2 Chemical and Reagents

The reagents, chemicals and enzymes required for this were purchased from Sigma–Aldrich (St. Louis, MO, USA), Fisher Scientific (Waltham, MA, USA), and DuPont Industrial Biosciences (DuPont Genencor Science, Palo Alto, CA) as described in Chapter 3 (Section 3.2.2).

6.2.3 Determination of Moisture Content

Moisture content in DDGS was determined by drying it at 105^{o}C in a hot air oven as described in Chapter 3 (Section 3.2.10.3).

6.2.4 Determination of Starch

The starch content in DDGS was determined using the perchloric acid method as described in Chapter 3 (Section 3.2.10.9).

6.2.5 Determination of Protein

The protein content in DDGS was determined using the spectrophotometric method as described in Chapter 3 (Section 3.2.10.10).

6.2.6 Determination of Fat

The fat content in DDGS was determined by the gravimetric method as described in Chapter 3 (Section 3.2.10.11).

6.2.7 Determination of Fiber

The fiber content in DDGS was determined by the enzymatic–gravimetric method as described in Chapter 3 (Section 3.2.10.12).

6.2.8 Determination of Ash

The ash content in DDGS was determined gravimetrically using the method established in National Renewable Energy Laboratory (NREL), USA as described in Chapter 3 (Section 3.2.10.13).

6.2.9 Statistical Analysis

Data were analyzed with Minitab statistical software, version 16 (State college, PA, USA) to calculate mean, standard deviation (SD) and coefficient of variation (CV) for each attribute. Data were also tested for one-way ANOVA (analysis of variance) to determine significant effects of genotypes on different components of DDGS. Fisher's least significant differences (LSD) test was done to compare the attributes among the DDGS samples of corn genotypes when main effects found significant at $P \leq 0.05$ after ANOVA test. Three-way ANOVA was performed to determine the effects of genotypes, dry-grind methods and enzyme loads used during fermentation on the biochemical components of DDGS. Pearson's correlation coefficient was calculated to determine the relationship among different components. Minitab statistical software, version 16 (State college, PA, USA) was used for these analyses considering 5% level of significance ($P \leq 0.05$).

6.3 Results

6.3.1 DDGS Yield

Irrespective of the enzymatic conditions applied during fermentation, the average yield of DDGS among the corn genotypes ranged from 25.07% to 32.44% for CSSF and 26.97% to 31.69% for GSHSF (Figure 6.1). In all cases, UM.NF–4 produced the highest amount of DDGS among HSGs, while in PFCs, PFC–4 produced the highest proportion of DDGS. It can be noted that the changes in DDGS yield under the two enzymatic

conditions and in both methods, were not consistently higher or lower between the genotypes.

To evaluate the variations in DDGS yield several statistical parameters were determined for the two groups of genotypes, and these are summarized in Table 6.1. The average yields for a single group (either PFC or HSG) as well as between the two groups of genotypes were very close to each other for both methods and enzymatic conditions. Almost similar standard errors (SE) were found for the group means, which recorded below 1.0. The coefficient of variations ranged from 5.84% to 11.31%. The F–test analysis of variance for DDGS yield among the eight corn genotypes during CSSF and GSHSF further revealed that DDGS yield of the corn genotypes for each group had varied significantly between the two enzymatic conditions and the two dry-grind methods ($P<0.05$). However, the variations in DDGS yield between the two groups of genotypes were not statistically significant ($P > 0.05$), with the exception for the yield of DDGS-2 that differed significantly between the two groups of genotypes ($P<0.001$).

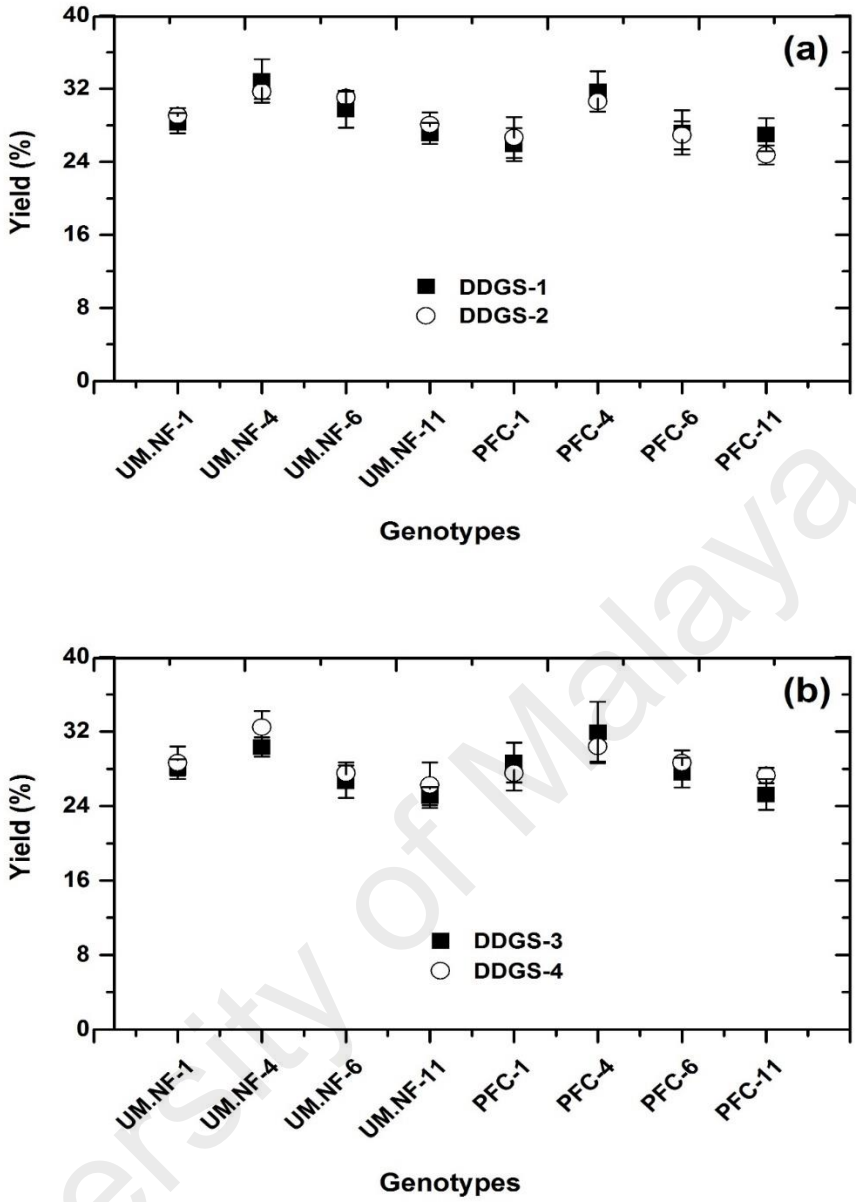

Figure 6.1: DDGS Yield; (a) DDGS Obtained from CSSF under Two Enzymatic Conditions (DDGS-1 and 2) and (b) DDGS Obtained from GSHSF under Two Enzymatic Conditions (DDGS-3 and 4).

Table 6.1: Comparison of Corn Genotypes for Yield of DDGS after CSSF and GSHSF

Type of samples	Group of genotypes	Mean (%)	SE	CV (%)	df	R^2	F–test	*P*–value
DDGS-1	Among HSGs	29.48	0.68	9.25	3	0.67	8.13	0.00
	Among PFCs	27.94	0.74	10.60	3	0.61	6.18	0.01
	Between groups	28.71	0.51	10.12	1	0.07	2.33	0.14
DDGS-2	Among HSGs	29.96	0.44	5.84	3	0.75	12.23	0.00
	Among PFCs	27.22	0.65	9.50	3	0.71	9.92	0.00
	Between groups	28.59	0.46	9.02	1	0.29	12.30	0.00
DDGS-3	Among HSGs	27.55	0.57	8.29	3	0.78	14.08	0.00
	Among PFCs	28.35	0.80	11.31	3	0.60	5.87	0.01
	Between groups	27.95	0.49	9.90	1	0.02	0.67	0.42
DDGS-4	Among HSGs	28.72	0.73	10.10	3	0.68	8.49	0.00
	Among PFCs	28.46	0.45	6.39	3	0.48	3.62	0.05
	Between groups	28.58	0.42	8.34	1	0.00	0.09	0.77

Notes: CSSF, conventional simultaneous saccharification and fermentation; CV, coefficient of variation; df, degree of freedom; GSHSF, granular starch hydrolysis and simultaneous fermentation; SE, standard error

6.3.2 Dry Matter Content in DDGS

The dry matter (DM) content in DDGS varied from 87.22% in PFC–4 to 88.59 % in PFC–6 among the eight corn genotypes for DDGS-1, which gave almost similar values for DDGS-2, ranging from 86.36 in PFC–4 to 88.45% in UM.NF–11 (Figure 6.2 a). Despite the slight variation among the DM of the DDGS of the corn genotypes, the F–test analysis of variance revealed that the variations were not statistically significant, since the probability level exceeded 0.05. Likewise, the DM content in DDGS-3 and DDGS-4 did not differ significantly among the corn genotypes and between the two enzymatic conditions, apart from containing slightly higher amounts of DM than those of DDGS-1 and DDGS-2 (Figure 6.2 b).

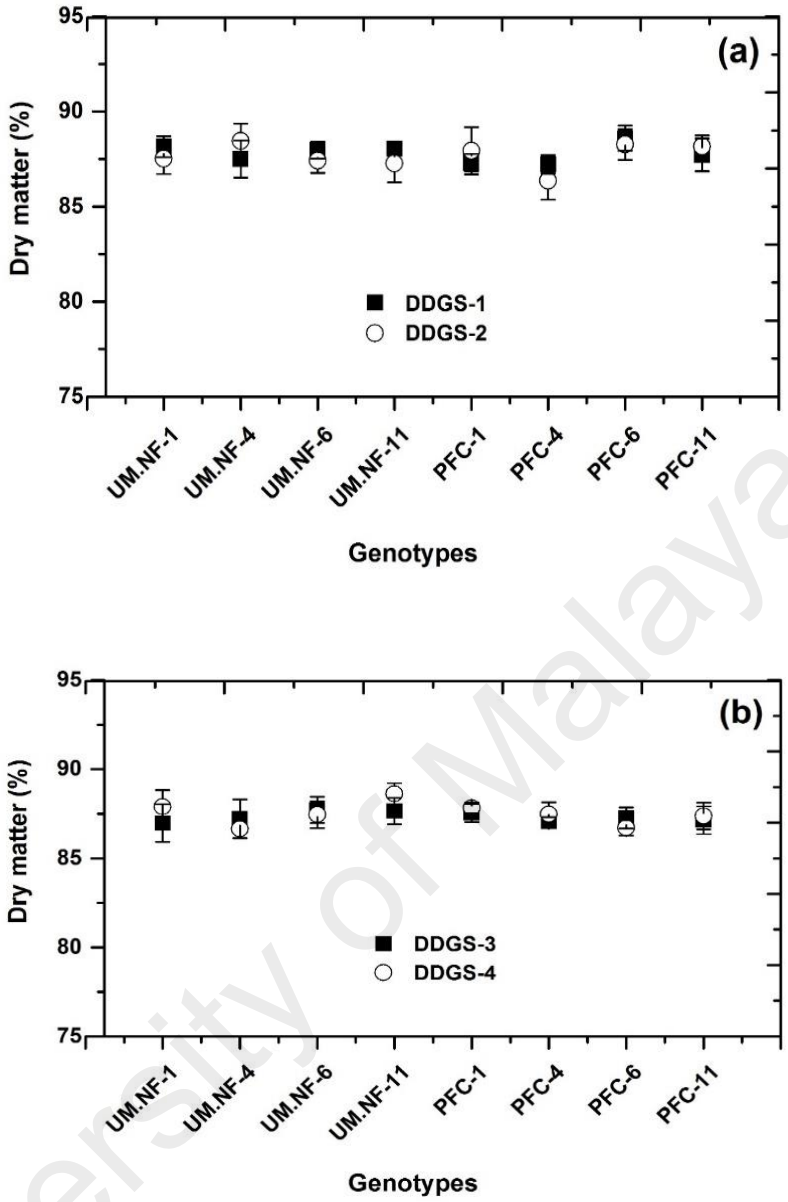

Figure 6.2: Dry Matter Content in DDGS; (a) DDGS Obtained from CSSF under Two Enzymatic Conditions (DDGS-1 and 2) and (b) DDGS Obtained from GSHSF under Two Enzymatic Conditions (DDGS-3 and 4).

6.3.3 Biochemical Composition of DDGS

The biochemical composition of DDGS-1 (obtained from CSSF under the enzyme load 3.0 kg/MT), DDGS-2 (obtained from CSSF under the enzyme load 4.0 kg/MT), DDGS-3 (obtained from GSHSF under the enzyme load 1.5 kg/MT) and DDGS-4 (obtained from GSHSF under the enzyme load 2.0 kg/MT) for the eight corn genotypes are shown in

Figures 6.3 to 6.7, along with the composition of raw corn kernels for each genotype as was mentioned in Chapter 3 (Table 3.7 for kernel-1 and Table 3.8 for kernel-2). The biochemical components included starch, protein, fat, total fiber and ash.

The starch content of DDGS was the residual starch that remained unfermented after CSSF and GSHSF. Overall, starch in DDGS of PFCs for both dry–grind methods were found to have decreased with the increase in enzyme load, resulting in significantly lower amounts of starch in DDGS-2 and DDGS-4 than those of DDGS-1 and DDGS-3 (Figure 6.3). However, DDGS of HSGs did not vary significantly in starch content under the two enzymatic conditions in both methods (P>0.05). Among the HSGs, DDGS starch content ranged from 3.08% to 5.98% for DDGS-1 and 4.06% to 8.52% for DDGS-3 on a dry matter basis, while in PFCs it ranged from 7.83–13.74% for DDGS-1 and 6.30–14.81% for DDGS-3. When the enzyme load was maximum, starch content in DDGS-3 for HSGs and PFCs ranged from 2.98% to 4.23% and 4.73% to 6.32% respectively, while the starch content in DDGS-4 was found to be 3.55–5.22% for HSGs and 4.87–7.20% for PFCs.

Compared with the raw corn, the average starch content in DDGS-1 for HSGs decreased 20.77 times in UM.NF–1, 20.73 times in UM.NF–4, 18.97 times in UM.NF–6 and 11.73 times in UM.NF–11 (Figure 6.3a). On the other hand, higher amounts of starch were recorded in DDGS-1 for PFCs as it decreased by 7.13–fold in PFC–1, 8.89–fold in PFC–4, 6.12–fold in PFC–6, and 5.28–fold in PFC–11 compared to the original kernel starch. The decreases in starch in DDGS-2 were 13.71, 14.88, 13.29, 11.47–folds respectively for PFCs. As observed in the DDGS-1 and DDGS-2, a similar decrease pattern for starch was recorded in DDGS-3 and DDGS-4 for HSGs and PFCs, although the degree of decrease were lower than those observed in DDGS1 and DDGS2 (Figure 6.3b).

Unlike starch, the protein content in DDGS increased compared to the starting kernel protein in all the conditions (Figure 6.4). Compared to the kernel composition, the average protein content in DDGS samples were found to have increased by 2.42–3.84 times for CSSF (DDGS-1 and DDGS-2) and 2.38–3.42 times for GSHSF (DDGS-3 and DDGS-4). Among the DDGS samples of the eight corn genotypes, the average protein content varied from 26.12% in PFC-1 to 35.63% in PFC-4 for DDGS-1, 24.96% in PFC-1 to 34.65% in UM.NF-4 for DDGS-2, 24.48% in PFC-6 to 36.85% in UM.NF-4 for DDGS-3, and 24.66% in PFC-6 to 38.07% in UM.NF-4 for DDGS-4 (Figure 6.4).

As was observed for protein content in DDGS, other biochemical components, such as fat, total fiber and ash also increased when compared to the respective kernel components of the corn genotypes (Figure 6.5-6.7). However, the average values for fat and ash were much lower than those of protein in all DDGS samples, whereas, total fiber was found to be very close to that of protein content in DDGS. On average, the increases in fat, fiber and ash in DDGS samples from the respective components of original kernel samples ranged from 1.52-fold in DDGS-1 to 2.63-fold in DDGS-4 for fat (Figure 6.5), 2.74-fold in DDGS-1 and 3.99-fold in DDGS-4 for total fiber (Figure 6.6), and 2.46-fold in DDGS-3 to 4.82-fold in DDGS-2 for ash among all DDGS samples (Figure 6.7).

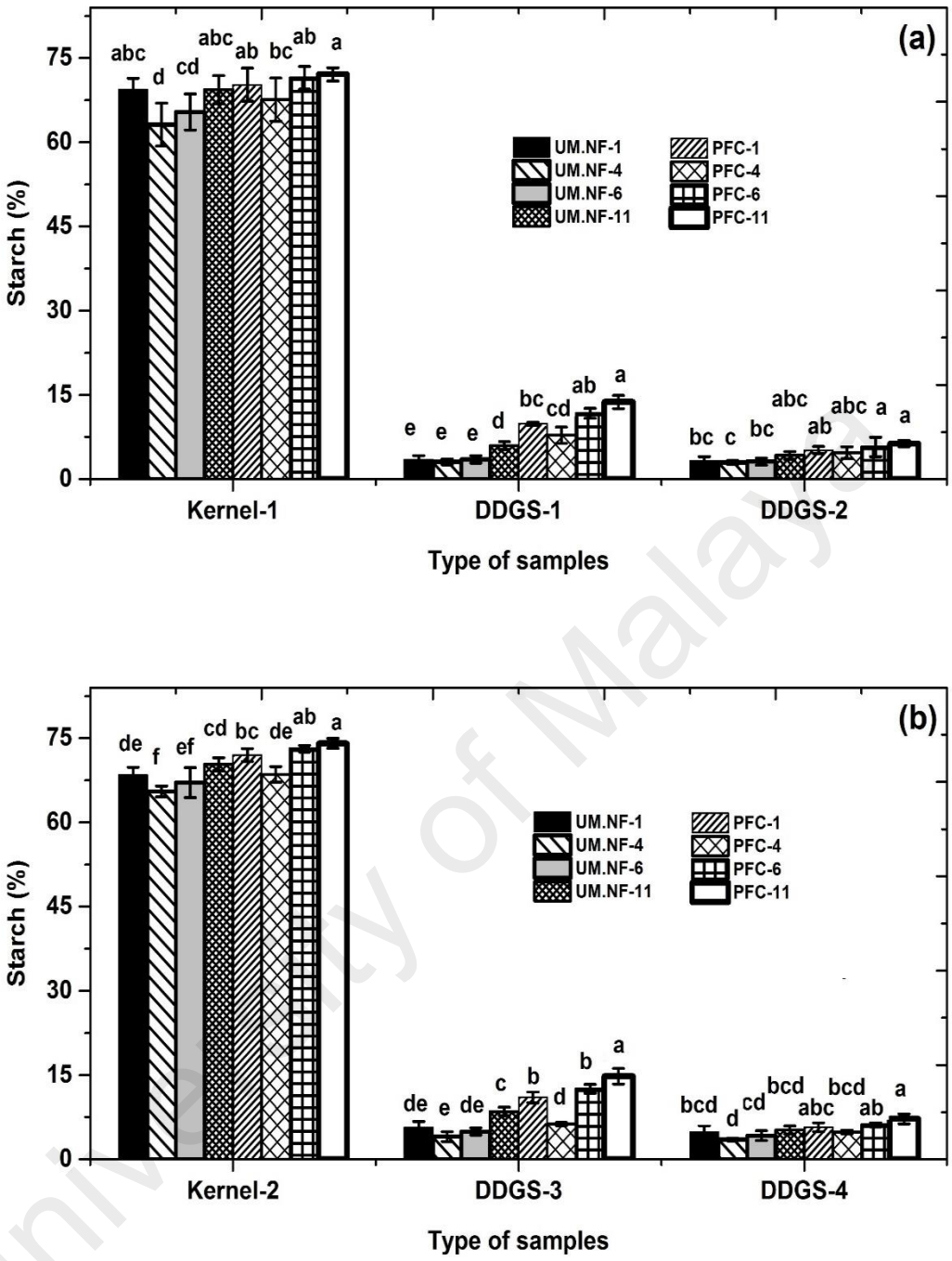

Figure 6.3: Starch Content in DDGS; (a) DDGS Obtained from CSSF under Two Enzymatic Conditions (DDGS-1 and 2) and (b) DDGS Obtained from GSHSF under Two Enzymatic Conditions (DDGS-3 and 4). The Same Letter on the Bars Denote Insignificant Variations among Eight Corn Genotypes (*P*>0.05).

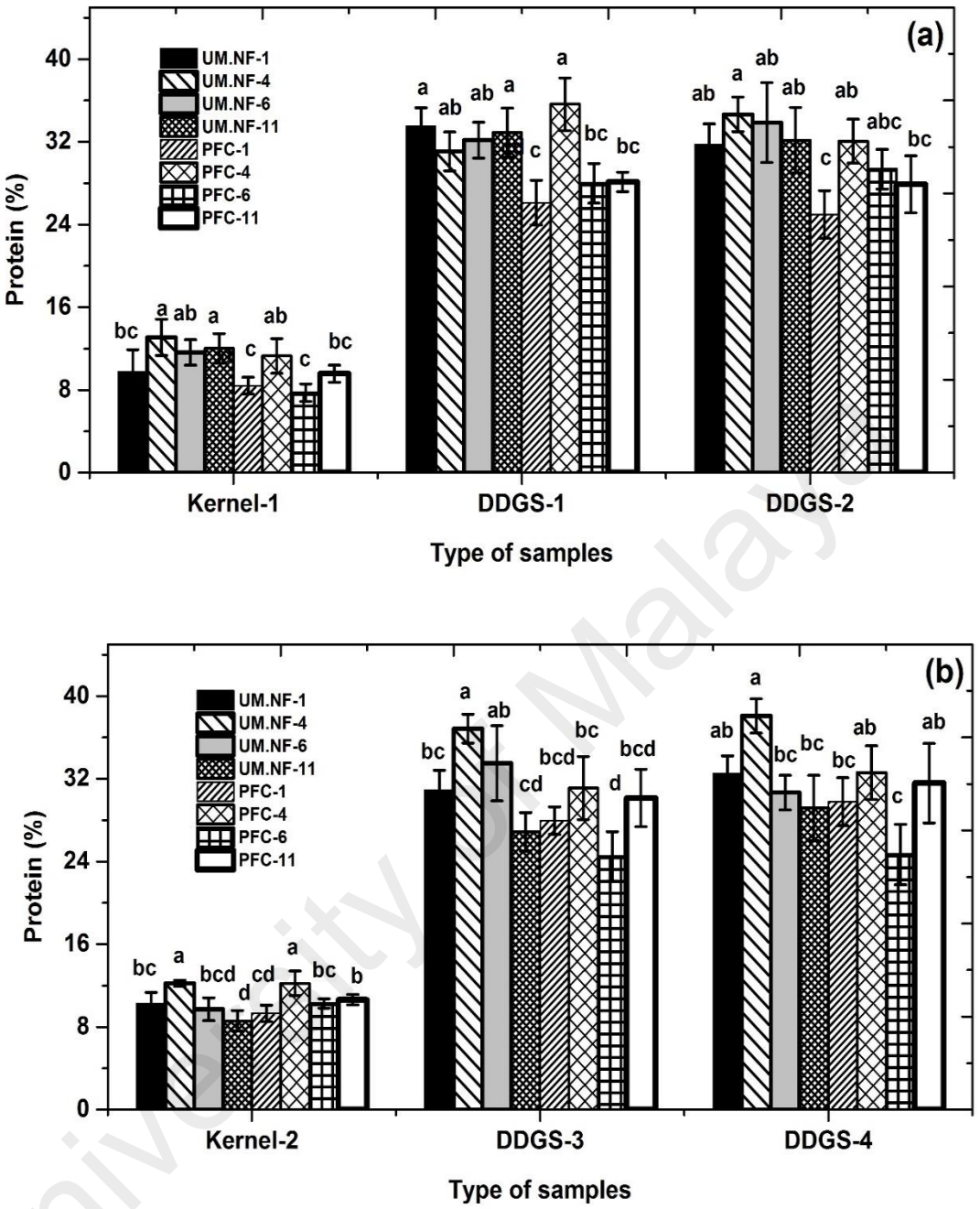

Figure 6.4: Protein Content in DDGS; (a) DDGS Obtained from CSSF under Two Enzymatic Conditions (DDGS-1 and 2) and (b) DDGS Obtained from GSHSF under Two Enzymatic Conditions (DDGS-3 and 4). The Same Letter on the Bars Denote Insignificant Variations among Eight Corn Genotypes (*P*>0.05).

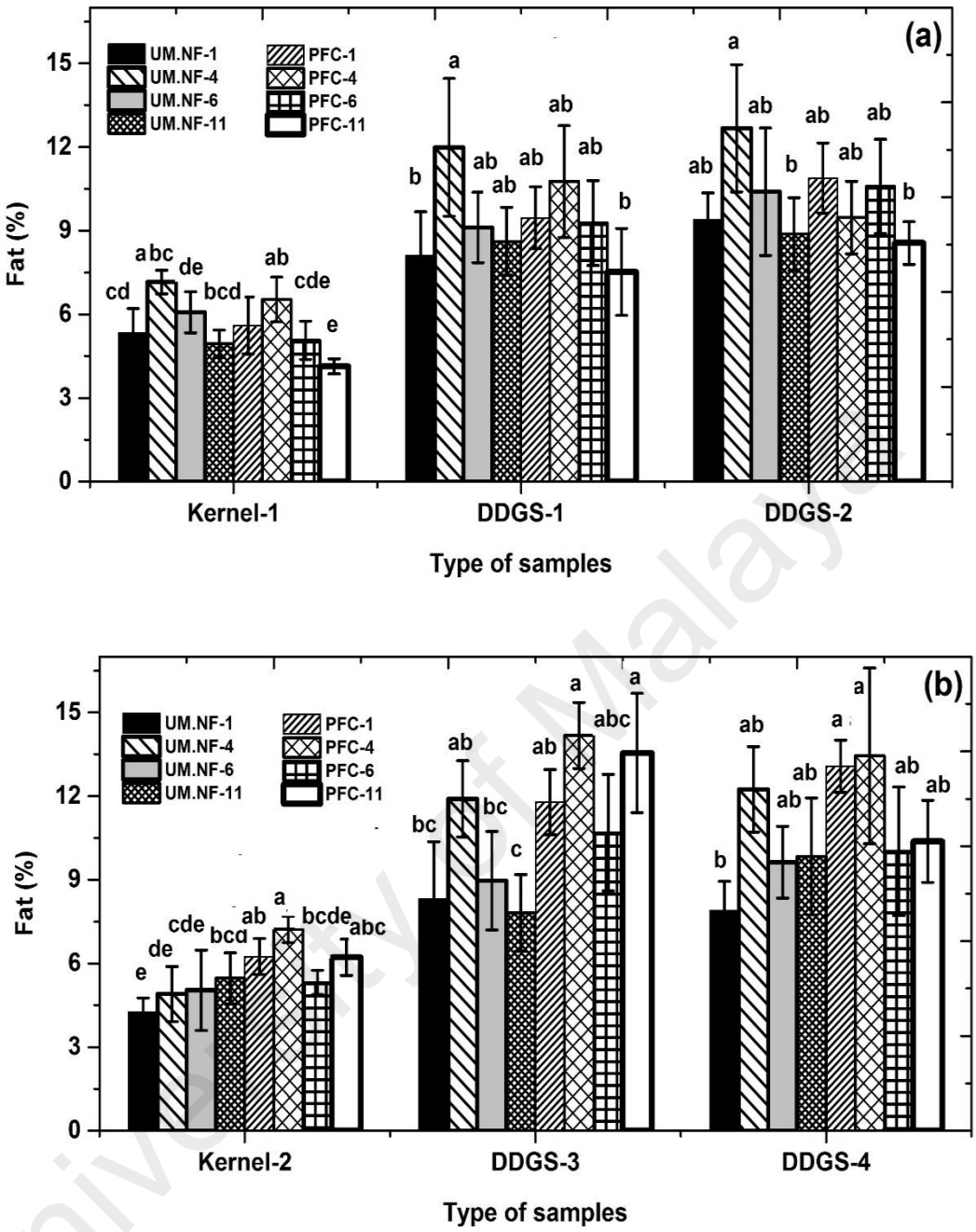

Figure 6.5: Fat Content in DDGS; (a) DDGS Obtained from CSSF under Two Enzymatic Conditions (DDGS-1 and 2) and (b) DDGS Obtained from GSHSF under Two Enzymatic Conditions (DDGS-3 and 4). The Same Letter on the Bars Denote Insignificant Variations among Eight Corn Genotypes (*P*>0.05).

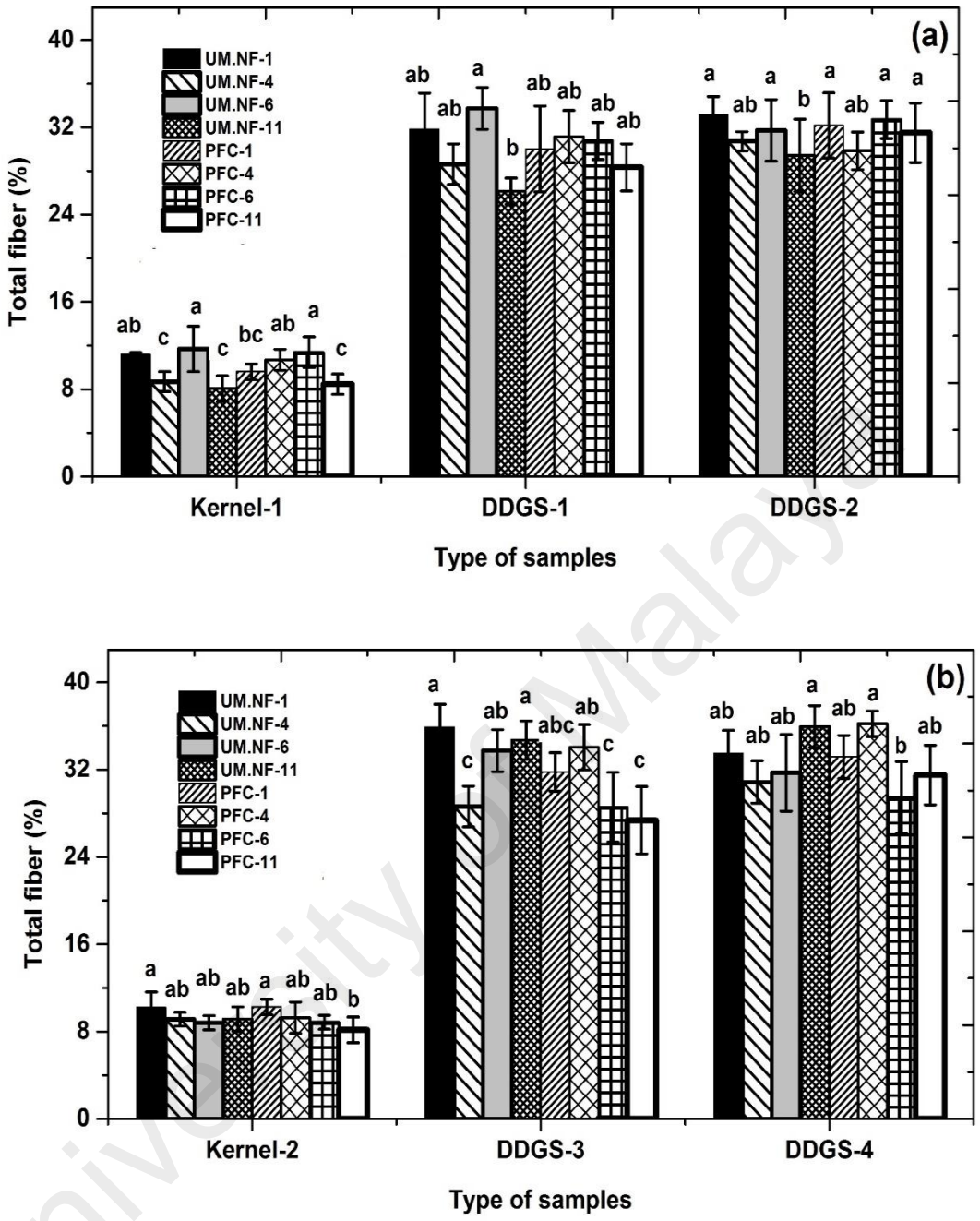

Figure 6.6: Fiber Content in DDGS; (a) DDGS Obtained from CSSF under Two Enzymatic Conditions (DDGS-1 and 2) and (b) DDGS Obtained from GSHSF under Two Enzymatic Conditions (DDGS-3 and 4). The Same Letter on the Bars Denote Insignificant Variations among Eight Corn Genotypes (*P*>0.05).

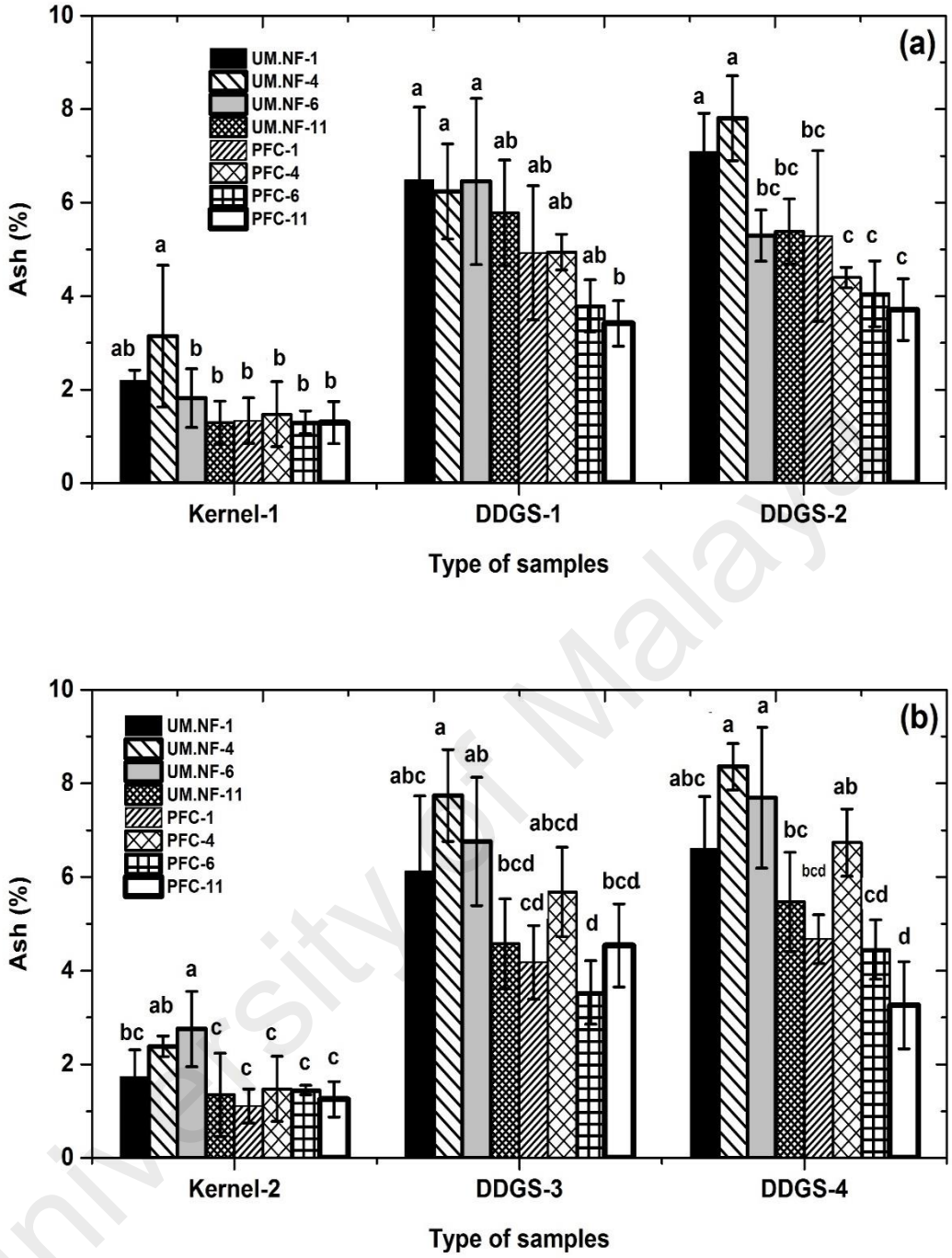

Figure 6.7: Ash Content in DDGS; (a) DDGS Obtained from CSSF under Two Enzymatic Conditions (DDGS-1 and 2) and (b) DDGS Obtained from GSHSF under Two Enzymatic Conditions (DDGS-3 and 4). The Same Letter on the Bars Denote Insignificant Variations among Eight Corn Genotypes (*P*>0.05).

The group mean for the two groups of genotypes was determined along with standard error and coefficient of variation (CV) through statistical analysis to determine the degree of variation in each component of the DDGS samples. Similarly, F–test analysis was conducted to determine whether the variations between groups and among the genotypes were significant. The results are summarized in Tables 6.1 and 6.2. It can be seen that the variations in starch content of the four types of DDGS samples were highly significant for the genotypic variations in each group as well as between the two groups of genotypes ($P<0.05$), with a few exceptions. High CV values for starch were also observed, which revealed that there were high variations between DDGS of the corn genotypes. Likewise, other biochemical parameters, such as protein, fat, fiber and ash also varied considerably among the DDGS samples and groups of corn genotypes. However, insignificant F–values were found in some cases.

Table 6.2: Comparison of Corn Genotypes Composition of DDGS Obtained from CSSF

Parameters	Type of samples	Group of genotypes	Mean (%)	SE	CV (%)	df	R^2	F–test	*P*–value
Starch	DDGS-1	Among HSGs	4.01	0.33	32.99	3	0.80	16.42	0.00
		Among PFCs	10.79	0.61	22.70	3	0.85	23.16	0.00
		Between groups	7.40	0.70	53.38	1	0.76	94.99	0.00
	DDGS-2	Among HSGs	3.41	0.19	22.41	3	0.43	3.05	0.07
		Among PFCs	5.49	0.29	21.19	3	0.28	1.56	0.25
		Between groups	4.45	0.25	32.21	1	0.54	35.75	0.00
Protein	DDGS-1	Among HSGs	32.41	0.50	6.14	3	0.22	1.13	0.38
		Among PFCs	29.46	1.04	14.14	3	0.82	18.02	0.00
		Between groups	30.93	0.63	11.45	1	0.18	6.52	0.02
	DDGS-2	Among HSGs	33.09	0.70	8.50	3	0.19	0.97	0.44
		Among PFCs	28.56	0.84	11.72	3	0.62	6.61	0.01
		Between groups	30.82	0.67	12.37	1	0.36	17.16	0.00
Fat	DDGS-1	Among HSGs	9.46	0.55	23.04	3	0.51	4.14	0.03
		Among PFCs	9.25	0.46	20.00	3	0.42	2.86	0.81
		Between groups	9.36	0.35	21.29	1	0.00	0.08	0.78
	DDGS-2	Among HSGs	10.34	0.55	21.33	3	0.46	3.45	0.05
		Among PFCs	9.87	0.38	15.22	3	0.41	2.72	0.09
		Between groups	10.10	0.33	18.52	1	0.02	0.48	0.49
Total fiber	DDGS-1	Among HSGs	30.09	0.90	11.98	3	0.70	9.27	0.00
		Among PFCs	30.08	0.66	8.79	3	0.18	0.86	0.49
		Between groups	30.08	0.55	10.34	1	0.00	0.00	1.00
	DDGS-2	Among HSGs	31.26	0.64	8.16	3	0.31	1.76	0.21
		Among PFCs	31.58	0.60	7.56	3	0.22	1.11	0.39
		Between groups	31.42	0.43	7.75	1	0.00	0.13	0.72
Ash	DDGS-1	Among HSGs	6.24	0.32	20.68	3	0.05	0.21	0.89
		Among PFCs	4.27	0.26	23.87	3	0.47	0.36	0.05
		Between groups	5.26	0.27	28.90	1	0.43	22.94	0.00
	DDGS-2	Among HSGs	6.39	0.33	20.47	3	0.73	10.86	0.00
		Among PFCs	4.36	0.28	25.39	3	0.30	1.70	0.22
		Between groups	5.38	0.28	29.31	1	0.43	22.38	0.00

Notes: CSSF, conventional simultaneous saccharification and fermentation; CV, coefficient of variation; df, degree of freedom; SE, standard error.

Table 6.3: Comparison of Corn Genotypes Composition of DDGS Obtained from GSHSF

Parameters	Type of samples	Group of genotypes	Mean (%)	SE	CV (%)	df	R^2	F–test	*P*–value
Starch	DDGS-3	Among HSGs	5.80	0.47	32.51	3	0.84	20.71	0.00
		Among PFCs	11.17	0.83	29.81	3	0.94	57.15	0.00
		Between groups	8.48	0.67	44.95	1	0.51	31.59	0.00
	DDGS-4	Among HSGs	4.46	0.25	22.25	3	0.44	3.09	0.07
		Among PFCs	5.98	0.26	17.33	3	0.71	9.57	0.00
		Between groups	5.21	0.22	24.19	1	0.38	17.99	0.00
Protein	DDGS-3	Among HSGs	32.03	1.08	13.49	3	0.76	12.68	0.00
		Among PFCs	28.42	0.86	12.09	3	0.58	5.63	0.01
		Between groups	30.22	0.75	14.08	1	0.19	6.84	0.01
	DDGS-4	Among HSGs	32.60	0.99	12.19	3	0.77	13.17	0.00
		Among PFCs	29.66	1.03	13.92	3	0.58	5.61	0.01
		Between groups	31.13	0.75	13.68	1	0.12	4.22	0.05
Fat	DDGS-3	Among HSGs	9.25	0.55	23.94	3	0.55	4.84	0.02
		Among PFCs	12.55	0.52	16.71	3	0.47	3.50	0.50
		Between groups	10.90	0.48	24.81	1	0.39	18.75	0.00
	DDGS-4	Among HSGs	9.90	0.53	21.32	3	0.58	5.40	0.01
		Among PFCs	11.73	0.62	21.21	3	0.41	2.76	0.88
		Between groups	10.81	0.43	22.68	1	0.14	5.03	0.03
Total fiber	DDGS-3	Among HSGs	33.24	0.84	10.04	3	0.74	11.15	0.00
		Among PFCs	30.16	0.96	12.78	3	0.60	5.97	0.01
		Between groups	31.70	0.69	12.23	1	0.16	5.85	0.02
	DDGS-4	Among HSGs	32.88	0.79	9.60	3	0.34	2.04	0.16
		Among PFCs	32.59	0.84	10.32	3	0.58	5.55	0.01
		Between groups	32.74	0.59	9.81	1	0.00	0.06	0.80
Ash	DDGS-3	Among HSGs	6.30	0.41	26.03	3	0.53	4.44	0.03
		Among PFCs	4.48	0.27	24.47	3	0.54	4.67	0.02
		Between groups	5.39	0.29	30.66	1	0.31	13.53	0.00
	DDGS-4	Among HSGs	7.03	0.38	21.37	3	0.57	5.28	0.02
		Among PFCs	4.78	0.36	30.14	3	0.80	16.26	0.00
		Between groups	5.90	0.33	31.25	1	0.38	18.71	0.00

Notes: GSHSF, granular starch hydrolysis and simultaneous fermentation; CV, coefficient of variation; df, degree of freedom; SE, standard error

6.3.4 Effect of Corn Genotypes, Enzyme Load and Dry–Grind Method on DDGS Composition

A three-way ANOVA was performed for all the attributes determined in this study to evaluate the effects of genotypes, enzyme dosage and the dry-grind methods on the different parameters, either individually or combined. In general, all the parameters of DDGS varied significantly with corn genotypes (P<0.001), except for DM content of DDGS (Table 6.4). On the other hand, DM varied significantly between the two dry-grind methods (P<0.01). Similarly, the dry-grind methods had significant effects on fat and total fiber content. It can be noted that enzyme dosage had a significant effect on the starch content of DDGS (F = 422.23; P<0.001). However, surprisingly, the combined effects of the dry-grind methods and enzyme loads, as well as genotypes, dry-grind methods and enzyme loads on the different attributes were found to be insignificant (P>0.05). Protein content in DDGS samples did not differ significantly between the two enzyme loads and the dry-grind methods individually, but the combined effects of genotypes and dry-grind methods had significant effects on the protein content of DDGS.

6.3.5 Correlation between Kernel and DDGS Components

Correlations between the components of corn and components of DDGS were evaluated separately for all conditions by determining Pearson's product moment correlation coefficients. It can be seen that all components of corn significantly correlated with the respective components of DDGS under all conditions, with the exception of fiber in DDGS-2, DDGS-3 and DDGS-4, as well as ash in DDGS-1. Both did not correlate significantly with the corresponding kernel components (Table 6.5).

Table 6.4: Effects of Genotype, Enzyme Load during Fermentation and Dry–Grind Methods on Composition of DDGS

Parameters	R^2	F–test (*P*–value)						
		G	M	E	G×M	G×E	M×E	G ×M×E
Yield	0.692	**24.138**	1.629	0.751	**3.768**	0.884	1.631	1.502
		(0.000)	(0.205)	(0.388)	**(0.001)**	(0.522)	(0.205)	(0.176)
Dry matter	0.384	1.388	**10.287**	0.228	**2.799**	1.100	0.301	1.717
		(0.219)	**(0.002)**	(0.634)	**(0.011)**	(0.369)	(0.585)	(0.114)
Starch	0.949	**142.849**	**37.21**	**422.233**	**3.503**	**42.66**	1.066	1.107
		(0.000)	**(0.000)**	**(0.000)**	**(0.002)**	**(0.000)**	(0.305)	(0.365)
Protein	0.719	**23.978**	0.233	0.881	**8.171**	0.750	1.411	1.917
		(0.000)	(0.630)	(0.350)	**(0.000)**	(0.631)	(0.238)	(0.075)
Fat	0.602	**11.043**	**14.083**	1.230	**4.946**	1.174	1.928	1.138
		(0.000)	**(0.000)**	(0.270)	**(0.000)**	(0.325)	(0.168)	(0.346)
Total fiber	0.578	**5.301**	**11.307**	**7.415**	**7.224**	**2.551**	0.121	1.045
		(0.000)	**(0.001)**	**(0.008)**	**(0.000)**	**(0.019)**	(0.729)	(0.405)
Ash	0.712	**27.383**	3.394	3.132	**2.979**	0.87	1.189	1.539
		(0.000)	(0.069)	(0.080)	**(0.007)**	(0.533)	(0.278)	(0.163)

Notes: E, Enzyme load used during dry-grind ethanol production (maximum and minimum doses); G, corn genotypes (Eight genotypes, including HSGs and PFCs); M, dry-grind methods (CSSF and GSHSF)

Table 6.5: Correlation-Coefficients between Kernel and DDGS Compositions

Kernel components	Types of sample	DDGS components				
		Starch	Protein	Fat	Fiber	Ash
Starch	DDGS-1	0.572**	–0.355*	–0.473**	–0.204	–0.518**
	DDGS-2	0.496**	–0.471**	–0.187	0.250	–0.525**
	DDGS-3	0.865**	–0.640**	0.248	–0.285	–0.719**
	DDGS-4	0.675**	–0.562**	–0.072	–0.239	–0.748**
Protein	DDGS-1	–0.597**	0.500**	0.240	0.050	0.519**
	DDGS-2	–0.440*	0.518**	0.051	–0.281	0.375*
	DDGS-3	–0.227	0.456**	0.397*	–0.192	0.450**
	DDGS-4	–0.117	0.501**	0.317	0.057	0.294
Fat	DDGS-1	–0.515**	0.382*	0.597**	0.050	0.389*
	DDGS-2	–0.427*	0.350*	0.362*	–0.302	0.473**
	DDGS-3	0.326	–0.214	0.369*	–0.094	–0.163
	DDGS-4	0.218	–0.020	0.393*	0.249	–0.346
Fiber	DDGS-1	–0.134	0.197	0.061	0.652**	0.242
	DDGS-2	–0.211	0.108	–0.1460	0.340	–0.011
	DDGS-3	–0.217	–0.203	–0.031	0.343	–0.048
	DDGS-4	–0.309	0.076	0.049	0.237	0.062
Ash	DDGS-1	–0.511**	0.203	0.438*	0.116	0.321
	DDGS-2	–0.491**	0.479**	0.344	0.085	0.483**
	DDGS-3	–0.546**	0.476**	–0.124	0.086	0.460**
	DDGS-4	–0.441*	0.227	–0.218	0.097	0.526**

* Significant at $P<0.05$

** Significant at $P<0.01$

6.4 Discussion

6.4.1 DDGS Yield

It has been recently reported that a typical dry-grind ethanol process produces roughly 30% of DDGS (Mosier & Ileleji, 2014). In the present study, the yield of DDGS varied between 25.07 to 32.44%, irrespective of the corn genotypes, enzymatic conditions and dry-grind methods used (Figure 6.1). The DDGS yield did not vary between the two enzymatic conditions and the two dry grind methods used during ethanol fermentation. These findings were not in agreement with those of an earlier study by Wang et al., (2005), where it was reported that DDGS yield for GSHSF was significantly lower (9.8%) than that of conventional method (28.3%). However, the authors separated the fibers from

germ, endosperm and pericarp of the corn prior to conducting GSHSF, whereas, the conventional method was carried out using the whole ground corn without such separation, which was how it was done in the present study. It is not surprising that the yield of DDGS would be lower when fibers are separated from the germ. This probably explains the reason for the insignificant variation in DDGS yield between two methods used in the present study.

6.4.2 DDGS Composition

The biochemical components in DDGS can vary significantly among the samples, as has been reported in numerous studies previously, conducted by collecting and analyzing the samples from different commercial plants (Belyea et al., 2004; Kim et al., 2008; Liu, 2008). The variations in the composition of DDGS are primarily associated with fermentation batches, changes in corn characteristics and processing conditions (Belyea et al., 2010). The present study also showed that all the components of DDGS, such as starch, protein, fat, fiber and ash varied significantly among the DDGS samples of the corn genotypes, even though DDGS was produced in this study on a laboratory scale. The reason for such variations among the genotypes could be primarily due to the variations in the composition of raw corn (Liu, 2009), which differed among the corn genotypes as was described in Chapter 3 (Table 3.7 for kernel-1 and Table 3.8 for kernel-2).

Although the biochemical components in DDGS samples varied among the genotypes and under different enzyme loads during fermentation, the range in variation of the components found here (Figures 6.3 to 6.7) were well within the range reported in the literature. Cromwell et al. (1993) reported 26.0-31.7% protein, 9.1-14.1% fat, 3.7-8.1% ash, 11.4-20.8% acid detergent fiber (ADF), and 33.1-43.9% neutral detergent fiber (NDF). Spiehs et al. (2002) assessed the nutrient content and variability of DDGS in a total of 118 samples from 10 fuel ethanol plants over three successive years, and found

on average, 30.2% protein, 10.9% fat, 5.8% ash and 53.1% fiber. In another study, it was reported that DDGS contained 7.9–15.1% fat, 28–30% protein, 38–49% NDF, 14–19% ADF, and 3.7–4.6% ash (Singh et al., 2002).

During the dry-grind ethanol production, unlike other biochemical components that are concentrated and increased, the starch content decreased as it is converted to sugar and ethanol (Liu, 2008). However, since the conversion of starch is usually incomplete, there is still some residual starch present in DDGS (Liu, 2009; Liu, 2008). According to Plumier et al. (2015), roughly 5% of starch remained unreacted or unfermented during dry-grind ethanol production. Belyea et al. (2004) reported the range for starch in DDGS varied between 4.7 and 5.9%. On the other hand, much higher amounts of starch was observed in another study conducted with 11 DDGS samples, which reported 11.1 to 17.6% starch (Liu, 2008). The authors explained that the reasons for the high starch content in DDGS might be due to unconventional processes. In the present study, four groups of DDGS samples obtained from CSSF and GSHSF of eight corn genotypes (four HSGs and four PFCs) under two enzymatic conditions were studied. As was shown in Figure 6.3, it was observed that DDGS-1 (from CSSF with an enzyme load of 3.0 kg/MT) and DDGS-3 (from GSHSF with an enzyme load of 1.5 kg/MT) of PFCs contained higher amounts of starch than those observed in DDGS-2 (from CSSF with enzyme load of 4.0 kg/MT) and DDGS-4 (from GSHSF with enzyme load of 2.0 kg/MT). In contrast, all the DDGS samples of HSGs contained relatively lower amounts of starch than those of PFCs, and there were no significant variation between the two enzymatic loads in either method, except for DDGS of UM.NF-11.

Compared to the DDGS from CSSF, the starch content in DDGS from GSHSF (DDGS-3 and DDGS-4) were found to be higher in all genotypes. This could be due to the fact that GSHSF was conducted under sub–gelatinized temperature without

undergoing liquefaction at 90^{o}C as was the case in CSSF (Chapter 4; Chapter 5; Zabed et al., 2016a; Zabed et al., 2016b). The other possible reason would be that GSHSF was conducted using 300 g/L initial solid load compared to 250 g/L for CSSF resulting in a relatively lower conversion of starch into ethanol due to inappropriate mixing of enzyme and substrate (Mojović et al., 2006), and as a consequence, higher amounts of starch was recovered in DDGS.

Protein content in DDGS is important for its overall quality, and unusual variation in protein may cause misformulation, which can affect animal productivity, and obviously the market value for DDGS as animal feed (Corredor et al., 2006). Among the DDGS samples of the eight corn genotypes, average protein content varied from 24.96% to 35.63% for CSSF and 24.48% to 38.07% for GSHSF (Figure 6.4). During dry–grind ethanol production, the biochemical components fluctuate considerably from ground corn kernels to DDGS (Han & Liu, 2010). However, variations in protein content of DDGS may occur due to two major factors, in addition to the protein content of the kernels. Firstly, DDGS is produced from two processing streams, which includes the mixing of wet distiller's grains (WDG) and condensed distiller's solubles (CDS) (Singh et al., 2001) and it has been reported that the composition of CDS can vary significantly from batch to batch (Belyea et al., 1998). Secondly, protein in DDGS is derived from two main sources, yeast and corn composition (Corredor et al., 2006). As yeasts multiply, they ferment soluble sugars and produce cell mass, much of which is yeast protein (60 g/100 g) (Belyea et al., 2004). Therefore, a proportion of the protein in DDGS is of yeast origin, and contributes around 20-50% to the total protein in DDGS (Belyea et al., 2004; Han & Liu, 2010).

Fat in DDGS is another important component providing energy for animals, and like the protein, it also determines the market value for DDGS (Belyea et al., 2004). According

to Liu (2009), the fat content of different DDGS samples can vary between 11.0% and 12.15% which were well within the findings of this study (Figure 6.5). Ash and fiber also contribute a significant portion to the composition of DDGS. Like protein and fat of the DDGS samples, these two components also varied significantly with the dry-grind methods and corn genotypes.

6.4.3 Correlation

Correlations between the components of corn kernels and the components of DDGS were determined for both dry-grind methods and enzymatic conditions. In this case, only positive coefficient value (r) would be meaningfully interpreted, and correlations were considered non-existing or hard to define when r values were found to be negative or near zero, which was suggested and followed elsewhere during the nutritional analysis of DDGS (Liu, 2009; Liu, 2008).

It was observed that most of the components of DDGS significantly correlated with the respective kernel components (Table 6.5). The correlations found in this study were in agreement with the findings of an earlier study by Liu (2009). However, Belyea et al. (2004) reported an insignificant correlation between corn kernel composition and the components of DDGS, and concluded that there was no scientific basis for the assumption that variation in the biochemical components of DDGS results from the changes in kernel composition. However Liu (2009), suggested the variations in DDGS composition is due to the kernel composition of corn. Liu (2009) explained that all the components of DDGS are derived from the varying concentrations of the respective kernel components (2.89-3.59 times concentrated in DDGS than those of kernel composition), which -indicates a relationship between kernel and DDGS composition. This would probably explain the reason for the variations in the different DDGS components observed in the present study.

6.5 Conclusion

It has been shown that all the biochemical components of DDGS obtained from both CSSF and GSHSF vary significantly among the corn genotypes. However, the DDGS of both groups of genotypes (PFCs and HSGs) were well within the reported values for these components. Apart from the significant differences in starch content of DDGS for PFCs under two enzymatic conditions, other parameters such as protein, fat, fiber and ash were quite similar under both conditions and dry-grind methods in all genotypes. DDGS derived from HSGs under both enzymatic conditions were found to be well within the findings of literature. This showed that the quality of DDGS will not be affected in HSGs under lower, but optimum enzyme load. Overall, HSGs produced DDGS with higher amounts of protein and ash and lower amounts of starch than those of PFCs. In conclusion, HSGs can be used as ethanol feedstock using lower amounts of enzymes without affecting the overall quality of the major co-product of a dry-grind method.

CHAPTER 7: CONCLUSION

7.1 General Conclusion

The present study was conducted to evaluate four high sugary corn genotypes (HSGs) as ethanol feedstocks, on the assumption that they have the potential to reduce enzyme consumption during dry-grind ethanol production with the desired ethanol yield and co-product quality. A comparison with the current practice that uses normal corn for ethanol production, four parent field corn genotypes (PFCs), which had been bred with sweet corn lines to develop HSGs, were also studied.

Although HSGs varied in their agronomic and biochemical characteristics, all HSGs contained higher amounts of sugars and lower proportion of starch than PFCs, which made them an attractive proposition for dry-grind ethanol production. A significant negative correlation was observed between the kernel starch and sugars, which indicated that normal corn genotypes contained higher amounts of starch than the sugary corn genotypes. As a consequence, the former required a higher quantity of enzymes for ethanol production. The average grain yields for the two groups of genotypes were found to be similar, which minimized the concern on grain yield trait for HSGs. Among the agronomic traits of the corn genotypes, silking time (ST), grain filling period (GFP) and black layer maturity (BLM) negatively correlated with sugar accumulation in the kernels, and relatively lower ST, GFP and BLM were observed in HSGs than in PFCs. which made HSGs more promising for accumulating higher amounts of sugar in their kernels, in addition to reducing total cropping time.

Both the conventional and granular starch hydrolysis of HSGs under different conditions showed that HSGs can produce higher amounts of sugars in all the conditions studied through hydrolyzing the starch as well as releasing the kernel sugars into the hydrolysates. It was observed that even though sugar yield varied significantly with

different time periods, various enzyme loads, particle sizes of the ground corn and initial solid loads in both HSGs and PFCs, there were no additional effects on the sugar production capability of HSGs as seen in PFCs. Moreover, the optimum conditions obtained for the hydrolysis of HSGs were similar to that of the PFCs, as well as the optimum conditions that has been established for both industry and laboratory practices, with the desirable exception that HSGs required lower amounts of enzyme than PFCs.

The results of two dry-grind methods revealed that sugar contents in corn kernels had substantial effects on enzyme consumption during hydrolysis and fermentation. HSGs produced higher amounts of ethanol than PFCs during both CSSF and GSHSF consuming lower quantity of enzymes due to their higher kernel sugars, a result which indicated that HSGs have the potential to reduce the ethanol production costs. A strong positive correlation was observed between kernel sugars and ethanol yield in contrast to the negative correlation between kernel starch and ethanol yield. Hence, the former could be a useful indicator for assessing corn raw material quality as ethanol feedstock. It was also observed that HSGs showed higher volumetric ethanol productivity and left lower residual starch after fermentation, which makes them a promising feedstock for bioethanol production.

With regard to the co-product quality, all the biochemical components of DDGS obtained from both CSSF and GSHSF varied significantly among the corn genotypes. However, the DDGS values of both groups of genotypes were well within the reported values in the literature for these components. Apart from the significant differences in starch content in the DDGS for PFCs under the two enzymatic conditions, other parameters such as protein, fat, fiber and ash were found to be almost the same for the individual genotypes under both conditions and dry-grind methods. DDGS values derived from HSGs under both enzymatic conditions were found to be similar to that previously

reported for corn in the literature. This showed that the quality of DDGS was not affected in HSGs under the lower but the optimum enzyme load.

In conclusion, a high sugar content in corn grains would be able to improve raw material quality, produce enhanced amounts of ethanol, and reduce enzyme consumption without affecting the co-product quality during dry–grind ethanol production. This makes HSGs promising candidates for bioethanol production in a dry-grind method, via either conventional or recently introduced (non-cooking) techniques.

7.2 Recommendations for Future Work

The present study was conducted on small scale, both field and laboratory experiments, suggesting that future work on a pilot or large scale would be the appropriate next thing to do. The HSGs used in this study were in their fifth and sixth generations, previously developed by conventional breeding. Since sugar content in the kernels is the desired trait for HSGs, the genetic basis for sugar accumulation in the endosperm of the kernels will be a suitable area for future studies on these genotypes, to increase their sugar content. It will be interesting to investigate the changes in sugar and starch content in the endosperm at different growth stages of HSGs, starting from 20 days after pollination, so that they can be harvested and used for ethanol production at the appropriate stage of growth. Sugar and starch content in the endosperm as well as the ethanol production capability of any corn hybrid usually fluctuates during storage, recommending a comprehensive research effort to determine the appropriate storage conditions and suitable storage period for HSGs for ethanol production.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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6. Zabed, H., Faruq, G., Boyce, A. N., Sahu, J. N. 2014. Effect of high sugar content in corn grains on enzyme consumption during dry-grind ethanol production, 20th Biological Sciences Graduate Congress (BSGC), Chulalongkorn University, Thailand, 9-11 December 2015.

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