Characterization of α-amylase in wheat and maize

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D7

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FOR MY LOVE, DR. MOHAMAD ISAM ALMADANI
AND
MY LOVELY DAUGHTERS: HANIN AND MARIAM
AND
MY SWEET SON NABIL

FOR MY MAJESTIC PARENTS
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AND
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Abbreviations

ABA: Abscisic Acid

α-amylase: alpha-amylase

β-amylase: Beta-amylase

BBCH: Bundesanstalt Bundessortenamt und CHemische industrie

BPNPG7: ρ-nitrophenyl maltoheptaoside

BSA: Bundessortenamt (Federal Office of Plant Varieties)

°C: degree Celius

cm: centimeter

CU: Ceralpha Unit

cv: cultivar

cvs: cultivars

DM: Dry Matter

dm: decimeter

DNA: Deoxyribonucleic Acid

DNS: 3,5-Dinitrosalicylic Acid

DWD: Deutsche Wetterdienst

e.g.: for example

Ed.: Editor

ELISA: Enzyme Linked Immunosorbent Assay

et al.: and others

etc.: and so on

EU: European Union

EC: Enzyme Commission

Exp: Experiment

FAEN: Forschungsverbund Agrar- und Ernährungswissenschaften Niedersachsen

FAO: Food and Agriculture Organization of the United Nations

FDA: FluoresceinDibutyrate Assay

FN: Falling Number

g: gram

GA:	Gibberellic Acid
ha:	hectare
HCl:	Hydrochloride Acid
HPLC:	High Performance Liquid Chromatography
hrs:	hours
ICC:	International Association for Cereal Science and Technology
i.e.:	that is to say
IEF:	Isoelectric focusing
JKI:	Institut für Pflanzenbau und Bodenkunde
KDa:	kilo dalton
Kg:	kilogram
k_m:	Michaelis-Menten constant
K_2O:	Potassium oxide
M:	Mole
mA:	miliamber
mbar:	millibar
mg:	milligram
µl:	microliter
min:	minute
ml:	milliliter
µmol:	micromole
MPa:	Mega Pascal
mRNA:	messenger ribonucleic acid
Mw:	Molecular weight
n:	number of observations
N:	Nitrogen
na:	not available
NaOH:	Sodium hydroxide
nm:	nanometer
No:	Number
P:	product
PAGE:	Polyacrylamide Gel Electrophoresis

GA:	Gibberellic Acid
pI:	isoelectric point
PPD:	Photoperiodic Response Genes
QTLs:	Quantitative Trait Loci
rpm:	revolution per minute
s:	seconds
SDS-PAGE:	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
UK:	United Kindom
USA:	United States of America
v:	volt
V_0:	initial velocity
V_{max}:	the maximum velocity
vs:	versus
VRN:	Vernalization genes
w/v:	weight/volume
%:	Percent
$:	Dollar

1. INTRODUCTION

Alpha-amylase belongs to a class of hydrolase enzymes that cleaves 0-glycosidic bonds in starch molecules and related polysaccharides to give diverse products including dextrin and smaller polymers composed of glucose units. It can be derived from several sources including plants, animals and micro-organisms (Hussain et al. 2013).

Cereal α-amylases are known as enzyme originates from cereals grain such as wheat, barley, maize, rye etc. (Muralikrishna and Nirmala 2005). It plays a dominant role in starch metabolism during grain development as well as germination. It is synthesized under the influence of plant growth hormones and exists in multiple forms (Cheng et al. 2014; Csiszar et al. 2010). Moreover, it is successfully used in the hydrolysis of starch to low weight products in the processing industry (Hussain et al. 2013). It is considered as an essential criterion in the technological processing of cereals (Csiszar et al. 2010), because of its overall effect on the quality of end-use products such as bread (Dencic et al. 2013; Goesaert et al. 2005), beer (Faltermaier et al. 2014), supplementary foods (Helland et al. 2002) and glucose syrup (Muralikrishna and Nirmala 2005). For example, in bread making, millers would prefer to have adequate activity of α-amylase in their wheat flour to sustain the production of sugars required in fermentation and consequent gas production (Goesaert et al. 2005). Additionally, low α-amylase activity from sorghum flour is desirable for stick porridge and couscous preparation (Dicko et al. 2006). While in the brewing industry, high α-amylase activity from malted cereals is desired to ensure the maximum production of fermentable sugars in malting process (Awoyinka and Adebawo 2008; Dicko et al. 2006).

However, an excess of α-amylase activity impairs grains quality since enzymatic hydrolysis of starch during food manufacture can lead to processing problems and unsatisfactory end-products (Dencic et al. 2013; Basinskiene et al. 2011). For instance, in baking industry, bread produced from high α-amylase activity (FN ≤ 180s to 62s, falling number (FN) is a special number calculated to quantify the level of α-amylase activity in the gelatinized flour/water suspension, see section 2.3.4 for details) in wheat flour has poor volume, deformed loaf with open holes, a compact, moist and sticky crumb structure that is difficult to slice and build up on slicer blades (Mohler et al. 2014; Dencic et al. 2013; Lunn et al. 2001b). In addition, grain flour with high enzyme activity loses its thickening power,

hence it can not be used in cream soup and gravy mixes. The noodles do not hold together because of increasing α-amylase activity in durum wheat. Consequently, significant financial losses affect both farmers and millers (Groos et al. 2002; Wahl and O'Rourke 1994).

From plant physiological point of view, the activity of α-amylase appears early during development of grains, culminates somewhere between 10 and 20 days after anthesis and then declines to low level as the grains mature (Dencic et al. 2013; Csiszar et al. 2010). However, the elevation of α-amylase activity germplasm at harvest-ripe grains has been identified in the European Union, South Africa, North America, Japan, Mexico and Australia (Barnard and Smith 2012). The major cause of high α-amylase production in cereal grains in these countries is pre-harvest sprouting. It is a phenomenon occurs occasionally in untimely rains prior to and during the harvest season in which enhances mature grains to germinate on the plant (Gao et al. 2013, see section 2.3.6. for details). It is determined by environmental condition (e.g. changes of temperature, precipitation etc.) and genotypes as well as by interactions between these factors (De laethauwer et al. 2013; Dencic et al. 2013; Gao et al. 2013; Kondhare et al. 2012). The main destructive feature in sprouted grains is increased α-amylase activity and other degrading enzymes, which affects significantly grains quality and utilization in bread making and other industrial products (Dencic 2013; Csiszar et al. 2010). This statement is supported by Singh et al. (2008) and Johansson (2002), who recognized alteration and/or breakdown of the grain biochemical composition in the sprouted grains results in decreasing starch, increasing sugars and protein, change in amino acid composition and loss of dry matter. Furthermore, Simsek et al. (2014) stated that the impact of pre-harvest sprouting on the end-products of wheat flour depends on enzyme activities present and breakdown of starch and protein. Direct annual losses caused by sprouting damage approach $1 billion dollars worldwide (Liu et al. 2013). This phenomenon has an intermittent nature, in which occurs approximately 1 in 4 years in many cereal-growing regions (Black et al. 2006; Biddulph et al. 2005). Selection for improved tolerance to pre-harvest sprouting is a goal of cereal breeding programs worldwide (Gao et al. 2013).

The activity of cereal α-amylases plays dual roles in the breeding program for pre-harvest sprouting. Firstly, it was found a significant difference in α-amylase activity between resistance and sensitive cereals cultivars to pre-harvest sprouting (Gao et al. 2013). The majority knowledge concerning the activity of α-amylase in sprouting grains is restricted to wheat. There is only little information on maize. This is due to the fact that wheat is more

susceptible to pre-harvest sprouting than maize (Bewley et al. 2013; Black et al. 2006). Secondly, the measurement of α-amylase activity in grains is an indicator of sprouting damage (Simsek et al. 2014; Khakimzhanov et al. 2011). The most popular method for detecting sprouting damage, particularly in bread making industry, is FN method (ICC Standard No. 107/1). However, several results have showed that FN can be influenced by other parameters than only the α-amylase activity (Wang et al. 2008; Lenartz et al. 2003). This can lead to underestimation or overestimation of actual activity of enzyme in which may either reject lots of grains from milling processing or get rid of cultivars from breeding programs. Instead of FN method, Ceralpha method (ICC Standard No. 303) is absolutely specific assay for α-amylase activity and has the major advantages of simplicity, accuracy and rapidity (Megazyme booklet 2012; Baks et al. 2006; Muralikrishna and Nirmala 2005). Hence, it is desirable to use method which gives a direct measure of α-amylase activity in order to screen cultivars to pre-harvest sprouting susceptibility.

From enzymological point of view, the characterization of cereal α-amylases have been of interest to researchers for many years till now, because of thier technological function in the end-products in different sectors ranging from food, fermentation and starch processing industry (Adefila et al. 2012; Biazus et al. 2009; Curvelo-Santana et al. 2008; Adewale et al. 2006; Dicko et al. 2006). The major focal point of such researches is to investigate the optimal conditions of using the amylases from cereals in starch hydrolysis processing. Detail knowledge about the properties of α-amylases from different cereals grain leads to more effective selection of appropriate cereal α-amylases that meet the prerequisites need for a specific application (El nour et al. 2013). In many developing countries, microbial amylases either fungal or bacterial sources are expensive because of importation. Hence, cereal amylases could be in principle a good substitute for microbial amylases if they are sufficiently stable and available in large quantities (Adefila et al. 2012). There are works support this idea. For example, studies carried out by Kumar et al. (2005) had found that malted sorghum cv. M-35-1 produced a thermostable α-amylase up to 60 °C, in which can be utilized in supplementary food that is reconstituted with hot milk or/and water before consumption. In addition, Adewale et al. (2006) recommended Nigerian maltsters to use α-amylase from malted sorghum over maize and millet as a brewing adjuncts in commercial malt production because they found among three α-amylases that sorghum α-amylase had the highest affinity, denotes as K_m , towards soluble starch (see

section 2.3.7 and 2.3.8 for details in kinetic properties of enzyme). This property of enzyme is efficient in the bioconversion of starch to simple sugars by low substrate concentration (Adefila et al. 2012).

Several studies showed that the properties of α-amylase varied somehow because of using different maturity stage of grain i.e. immature vs. malted grains (Muralikrishna and Nirmala 2005), different cultivars as well as different purification techniques (Daniel et al. 2008; Hunjan et al. 2006; Kumar et al. 2005). Considerable researches have been directed towards characterizing exogenous α-amylase from malted grains (e.g. El nour et al. 2013; Adefila et al. 2012; Biazus et al. 2009) which is *de novo* synthesis upon germination (Barreroet al.2013), while there are relatively few reports concerning the properties of endogenous α-amylase present in sound grains i.e. ungerminated grains (e.g. Rosell et al. 2001; Thevenot et al. 1992; Mares and Oettler 1991, Daussant and Renard 1987 and 1976; Marchylo et al. 1980 and 1976). In addition, the enzymatic hydrolysis by amylases is preferred to acid hydrolysis in starch processing industry e.g. glucose syrup, fructose syrup etc. (Sivaramakrishnan et al. 2006), but the industrial conditions in which enzyme are used are rather extreme, particularly with regards to temperature and pH (Prakash and Jaiswal 2010). Hence, there is enormous interest in biotechnology researches to find a particular cereal cultivar to be a good source of α-amylase in which the characterizations of enzyme meet the prerequisites for industrial applications (e.g. El nour et al. 2013 and 2010; Biazus et al. 2009). Furthermore, the low cost of cereals α-amylases from local cultivars in developing countries may enhance their utilization as a good alternative source for exporting microbials amylases (Adefila et al. 2012; Egwim and Oloyede 2006; Adewale et al. 2006). Such work needs to have thorough knowledge of the properties of amylase isolated from such source and also the industrial conditions under which it is going to be applied (El nour et al. 2013; Fernandes 2010; Biazus et al. 2009).

1.2. Objectives of the study

A key aspect of this work was to conduct systematic study on the basic characterization of cereal α-amylase with respect to its activity and properties. A major risk of α-amylase in cereal grains is the elevation of its activity in harvest-mature grains, making grains inappropriate for baking and other food industry. It was reported in many literatures that the activity of α-amylase is influenced by cultivars and environment conditions during

the development of the grains up to maturity. Therefore, a method able to quantify actual α-amylase activity is indispensable for a profound study of cereal α-amylases variability during different development stages. Using Ceralpha method (ICC Standard No. 303) is desirable because of its specific assay to α-amylase activity.

On the other hand, screening of a particular cereal cultivar to be a low-cost source of α-amylase, with characteristic suitable for diverse processing conditions has gained more attention in biotechnology in order to meet the increasing demand for that enzyme in various industries (El nour et al. 2013). The majority of works concerning characterization of α-amylase for food processing purpose was carried out on malted grains. Hardly any recent study presented data of endogenous α-amylase properties from sound grains i.e. in the normal physiological condition of grain without germination.

Therefore, our general goal was to give a complete picture of the nature of cereal α-amylase from its activity during grain developing and ripening in the field till its properties in sound harvest-mature grains.

Two specific objectives were drawn as following:

- In the first part of this study the objective was to assess the influence of cultivars and environments on α-amylase activity throughout grain development using Ceralpha method as a specific assay. In addition, biochemical compositions of grain (starch, protein and sugars) were determined in order to find correlation with enzyme activity.

- In the second part of the study the focus was to characterize the nature of α-amylase as present in sound grain from different cereal cultivars and environments.

To achieve this goal, two separate field experiments were conducted. In the first field, wheat plants were grown, whereas maize plants planted in the second one. Those two cereal crops were chosen as materials in this research because of the difference in their susceptibility to pre-harvest sprouting as one of essential reason of increasing α-amylase activity, and in their usage in food application as well. Wheat grains are more prone to pre-harvest sprouting rather than maize grains. In addition, they are used in baking industry, while maize grains are used in brewing and starch processing industry.

The present information from our study would be helpful to improve effective selection in pre-harvest sprouting breeding programs and to promote the utilization of

cereal α-amylase from particular cultivars as processing aids in hydrolysis of starch in many food applications.

2. LITERATURE REVIEW

2.1. Wheat and maize perspective

2.1.1. Description and classification

Botanically, bread wheat (*Triticum aestivum* L.) belongs to Pooideae subfamily of the monocot family Poaceae (Shewry 2009). However, maize (*Zea mays* L.) comes from Panicoideae subfamily in the family Poaceae (Fox and Manley 2009).

Agronomically, wheat is a temperate plant that grows best in a moderate climate (Diallo et al. 2012). The grain classification systems are based on grain color (white vs. red), grain hardness (hard vs. soft) and vernalization requirement (winter vs. spring) (Williams et al. 2008). Several workers (Diallo et al. 2012; Distelfeld et al. 2009) have identified the differences between winter and spring wheat. Winter cultivar requires vernalization. It is planted in the middle latitude area with autumn sowing and harvested in early summer. Spring cultivar does not have a vernalization requirement. It has planted in spring in the high latitude area and in the low latitude area with warm winter. The harvest time is in midsummer.

Maize is a tropical plant (Fox and Manley 2009) which requires warm growing temperatures. There are four main categories of commercial importance which are mentioned by McKevith 2004: (1) dent maize, is the most commonly grown for grain and silage and is identified by the dented distal end in the crown of the grain, (2) flint maize, is grown for food use and is characterized by an oval distal end, (3) sweet corn, is grown for green ears (~ 70 % moisture content) being used as a human food and is dent type with high sugar content and (4) popcorn, is consumed world-wide as a snack food and is characterized as a flint type maize which expands when heated.

2.1.2. Food utilization

Wheat and maize grains are important components of the daily diet, providing a major source of essential nutrients such as carbohydrates, proteins, fibers, vitamins and minerals (Gani et al. 2012) (Table 1). They contain also a range of phytochemicals which may provide a health promoting effects and have the potential to reduce risk of several chronic

diseases such as coronary heart disease, type 2 diabetes and certain type of cancer. These substances i.e. phytochemicals are phenolic compounds, phytosterols and carotenoids (Gani et al. 2012). They also contain a number of anti-nutrients such as phytate and tannin that inhibit micronutrient bioavailability (Gani et al. 2012).

Table 1: Chemical composition of cereals as average values

Chemical composition	Wheat	Maize
Moisture *	13.2	12.5
Protein	11.7	9.2
Lipid	2.2	3.8
Carbohydrate	59.6	64.2
Fiber	13.3	9.7
Minerals	1.5	1.3
Thiamin **	5.5	4.6
Niacin	63.6	26.6
Riboflavin	1.3	1.3
Pantothenic acid	13.6	5.9

Source: Belitz et al. 2009
(*)Units for the first group is weight % and (**) mg kg^{-1} for the second group

Wheat is widely consumed by humans because of its agronomic adaptability, ease of storage, nutritional values, and the ability of its flour to produce a wide range of foods (Wrigley et al. 2009). Wheat, as an important source of carbohydrate, provides on average one-fifth of the total calorific input of the world's population and half the total dietary calories in areas such as North Africa, Turkey and Central Asia (Chakraborty and Newton 2011). Dough produced from wheat flour has a unique visco-elastic property (Shewry 2009; Day et al. 2006) that is responsible for the universal use of wheat in a large range of ingredients and foods. They are fermented bread, flat breads, cookies, cakes, noodles, pasta, bulgur, couscous, semolina and wheat starch (Nadeem et al. 2010). For livestock nutrition, wheat feed cultivars are being supplemented by other protein-rich crops e.g. soybeans and oilseed residues. It is used to a limited extent in the making of beer, whisky, and industrial alcohol (Shewry 2009; Wrigley et al. 2009). Wheat straw is used for livestock bedding and as a fodder and manure (McKevith 2004).

Maize can be fed as green chop, dry forage, silage or grain in animal feed for meat, poultry, and milk production. Maize grains can be directly consumed in human diet or be processed for a range of uses such as corn starch, corn oil, food sweeteners and alcoholic beverages, but the latter usage is not widespread for maize (Adewale et al. 2006). They are

used as food ingredients in breakfast i.e. corn flakes, tortilla, porridge, steamed products, popcorn and snack (Gwirtz and Garcia-Casal 2013). Maize contributes also in many manufacturing sectors e.g. textile, insecticides, adhesive and paper industry (Balconi et al. 2007). It is also important substrate in biofuel (Wu and Gucl 2013). For a commercial production, there are two methods applied in maize, named as dry and wet milling, to produce specific end-products (Gwirtz and Garcia-Casal 2013).

2.1.3. Production

Worldwide

The total world of wheat production is annually over 600 million tons of grain from about 220 million ha, with an average yield of nearly 3 tons/ha (Wrigley et al. 2009; Shewry 2009). The consumption of wheat as food accounts for 53 % in the developed countries, and close to 85 % in the developing countries (Dencic et al. 2011). It provides more than 60 % of the total daily requirements of protein and calories. China is the largest wheat-growing area in the world, followed closely by USA and the Russian Federation. An extensive wheat growing regions occur in India, Australia, Canada, Pakistan, Argentina and some countries of EU (Nadeem et al. 2010). The major wheat-growing countries in EU are France, Germany, UK, Italy, Spain and Portugal (Curtis et al. 2002). Bread wheat occupies 95 % of the wheat grown worldwide, with the most of the remaining 5 % being durum wheat (T. *durum*). Small quantities of other wheat species, such as einkorn (T. *monococcum*), emmer (T. *dicoccum*), spelt (T. *spelta*), are still grown in some countries including Spain, Turkey, the Balkans, India and the Alpine areas (Shewry 2009).

At around 800 million tons, world maize production represents over one-third of world cereals output in 2011 (FAOSTAT 2013). The consumption of maize grain accounts for about 15 to 56 % of the total daily calories in human diets among 25 developing countries, particularly in Africa and Latin America (Cisse et al. 2013). The major maize-growing countries of the world are Mexico, Brazil, China, Argentina, USA and France (Wu and Gucl 2013). Maize as a crop has multiple uses but is chiefly grown for Livestock feed in which contributes to 60 - 70 % from the worldwide maize production. The remaining 30 - 40 % is used for human consumption and processing applications (Gwirtz and Garcia-Casal 2013).

Germany

Germany is ranked as the world seventh largest wheat producer, as well as the EU's second-largest wheat producer, with an output of 24 million tons in 2010 (FAOSTAT 2013). Most wheat in Germany is bread wheat either winter wheat (80 - 90 %) or spring wheat (10 - 20 %). Bavaria is the largest wheat producing state in Germany, followed by Lower Saxony. Spelt wheat and durum wheat are also cultivated in some regions (Mesdage and Donner 2000). In 2009/10, about 17 million tons of wheat were domestically utilized, 48.6 % for feed, 41 % for consumption and 10 % for industrial use (e.g. alcohol, malt, technological starch and energy) (BMELV 2011).

In 2011, the majority of maize-planted area was for silage with 2 million ha out 2.515 million ha. The production of maize as silage was about 96 million tons compared to 5 million tons as maize grain (Deutsches Maiskomitee 2014). The largest maize-cultivating federal states are Bavaria and Lower Saxony (Deutsches Maiskomitee 2014). Generally, the major maize-cultivated acreage is used for animal feed as silage (Bringezu et al. 2011) in which contributes to ~ 78 % (Frinke et al. 1999), while the remaining harvested area is used for foods industrial applications (Goertz et al. 2010). Currently, the biomass from maize is the dominant feedstock used in the biogas production in order to promote electricity production along with other renewable energies such as wind and solar (Britz and Delzeit 2013; Goertz et al. 2010). For example, the production maize area for biogas sector has exponentially increased during the past decade to more than 0.5 million ha in 2010. This corresponds to more than 20 % of the total maize acreage of 2.3 million ha in which is supplied 1 % of the total primary energy consumption in Germany (Grieder et al. 2012).

2.2. Characterization of the grain

2.2.1. Grain anatomy

Wheat and maize grains is a single-seeded fruit, called a caryopsis, in which that the pericarp is tightly fused with the seed. The seed itself consists of the testa, the endosperm and the embryo (Bewley et al. 2013). A longitudinal section of wheat and maize grains is shown in Figure 1.

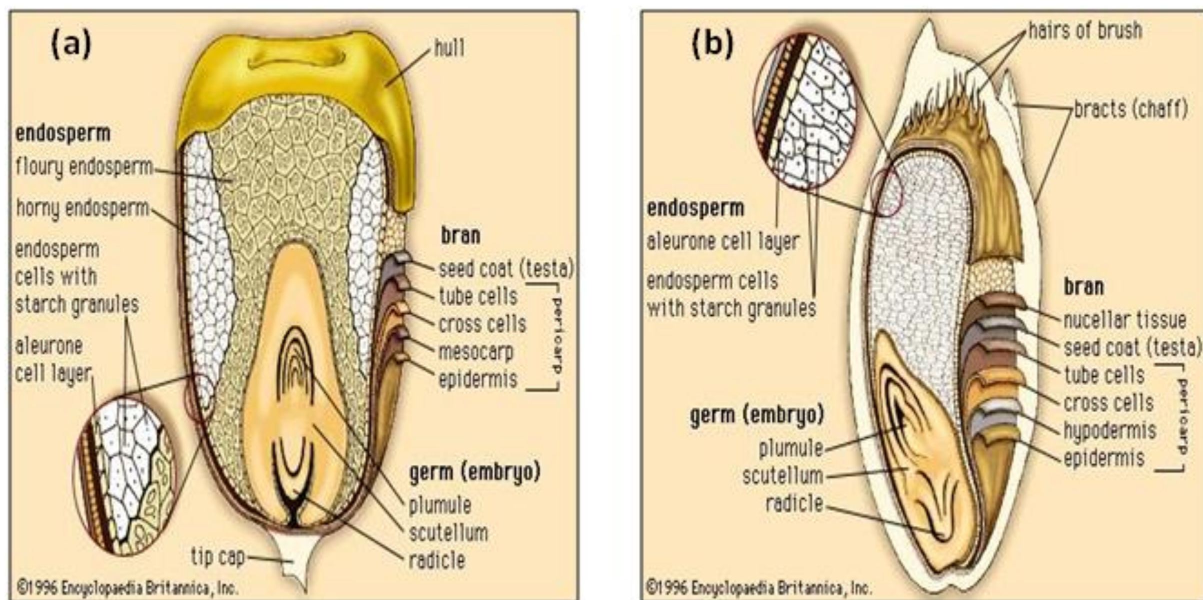

Figure 1: The structure of (a) maize (b) and wheat (b) grains

Source: Encyclopedia Britannica 2013

Pericarp and testa

Pericarp and testa are the external layers of the grains, which provide support and protect the interior parts of grains during development from the external environment (Bewley et al. 2013). They comprise more than 80 % fibres, mainly water-insoluble fibres, which serves as semi-permeable barrier (Sramkova et al. 2009). In addition, they have a cuticle layers, consisting fatty and waxy substances, which play a role in regulating water and gaseous exchange in growing grains, but become leaky on drying (Bewley et al. 2013).

Wheat grain has a unique morphological structure on the ventral side i.e. the opposite side to the embryo called “crease”, which is parallel to grain long axis. The role of crease is to facilitate the translocation of nutrients from the vascular strand to the developing grain. Furthermore, hairs, also named trichomes, are high silicon content that present on the ventral side of grain (Evers and Millar 2002). In addition, the chloroplasts are present in wheat’s pericarp in which gives the grain its green color in early stage of development (Evers and Millar 2002). Unlike wheat grain, maize grain does not have crease, hairs or chloroplast (Evers and Millar 2002), instead it has the tip cap area, which serves as the entry point for translocated nutrient from vascular strand into developing grain. As the grain matures, a dark layer of dense cells, named the black or hilar layer, is sealed the tip cap (Gwirtz and Garcia-Casal 2013) due to cessation of dry substance accumulation in harvest ripeness (Meade et al. 2013 and Sala et al. 2007).

Endosperm

Endosperm is the largest morphological proportion in all cereal grains, in which makes up 80 - 84 % in wheat grain (Stevenson et al. 2012) and 70 - 90 % of maize grain (Balconi et al. 2007). It consists of the outer aleurone layer and the inner starchy endosperm. Most of the aleurone layer is removed as part of the bran during roller milling (Stevenson et al. 2012). The aleurone layer is a uniform single layer of cells in both wheat (Sramkova et al. 2009) and maize (Balconi et al. 2007) grain and completely surrounds starchy endosperm and the embryo except for that adjacent to the scutellum (Evers and Millar 2002). It plays a vital role in both grain development and germination. It has the ability to divide to produce starchy endosperm cells in developing grain (Evers et al. 1999). A further function of some aleurone cells, which are called “transfer aleurone layer”, is to facilitate the uptake of nutrients from the maternal tissues into starchy endosperm and embryo during the grain maturation (Balconi et al. 2007). The inner endosperm is referred to as mealy or starchy endosperm (Sramkova et al. 2009). It consists of cells packed with nutrients, starch, protein and lipid storage, which can mobilize to support the growth of embryo on germination process (Stevenson et al. 2012). At maturity, endosperm becomes non-living, but aleurone cells remain alive for long period (Fox and Manley 2009).

Embryo

The embryo is the reproductive organ, in which contains the scutellum and an embryonic axis. The scutellum acts as a secretory and absorptive organ, leading to *de novo* synthesis and secretion of hormones and enzymes as well as absorption of solubilized nutrients during germination. The embryonic axis is the plant of the next generation. It is composed of the radical and plumule (Evers and Millar 2002).

2.2.2. Grain composition

Starch is the most abundant storage carbohydrate in all cereal grains, provides the energy for subsequent seedling metabolism during germination (Beckles and Thitisaksakul 2014). It is accounting for 65 % of the dry matter of the wheat grain and up to 72 % of the dry matter of the dent maize grain (Evers et al. 1999). Starch synthesis and storage take place mostly in the amyloplast in the growing endosperm, while some temporary starch reserves appear during development of the embryo and testa (Tetlow 2011; Keeling and

Myers 2010). Protein content is in a relatively narrow range in cereal grains with 10 - 15 % of the grain dry weight (She et al. 2011). Proteins of wheat and maize have primary deficiency in lysine and methionine, whereas the secondary deficiency is threonine in wheat and tryptophan in maize. Sugars occur in low content in mature cereals grains 1 - 2 % of the grain dry weight, while relatively high amount of them is accumulated in immature grains (Evers et al. 1999; Watson and Ramstad 1994). They are composed of monosaccharides, glucose and fructose, and disaccharides, sucrose and maltose (Halford et al. 2011). Enzymes present in cereals are relatively low in mature grain, but they are necessary for grain development and germination (Koehler and Wieser 2013). The majority of enzymes activity during developing period is concerned with synthesis, particularly the synthesis of storage components mainly starch and protein. However, some hydrolytic enzymes are found in the pericarp of the developing grain and may persist (Evers et al. 1999). Upon germination, the hydrolytic enzymes involved in the breakdown of starch and protein are predominant in order to provide the embryo with nutrients and energy (Koehler and Wieser 2013; Minic 2008). Furthermore, cereal grains contain various enzyme inhibitors such as α-amylase inhibitors (Juge and Svensson 2006) and protease inhibitors (Sharma and Gupta 2001). Maize grain is rich in lipids with 5 % of dry matter (Watson and Ramstad 1994), whereas wheat grain contains 0.8 - 1.5 % of its dry matter (Black et al. 2006). Cereal grain contains fibers, minerals and vitamins from E and B-group (Koehler and Wieser 2013).

2.2.3. Grain growth and maturation

Grain development starts after the process of a double fertilization is taken place inside the embryonic sac of the ovule. One pollen nucleus fuses with the egg nucleus to form a diploid zygote, which gives rise to the embryo. A second pollen nucleus fuses with the two polar nuclei forming the primary endosperm nucleus, which later produces a triploid endosperm (Bewley et al. 2013). Pericarp and testa originate as maternal tissues present in the carpel at the time of fertilization (Evers and Millart 2002).

The initial phase is characterized by cell division, histo-differentiation to form the embryo and endosperm tissues, followed by cell expansion due to the influx of water into the grain (Bewley et al. 2013; Bringezu et al. 2011). Thus the grain fresh weight increases due to the accumulation of solutes (Meade et al. 2013; Thakur et al. 2010). The phase is referred

to as “grain enlargement phase” (Balconi et al. 2007), “lag phase” (Meade et al. 2013; Thakur et al. 2010) or “pre-storage phase” (Bringezu et al. 2011).

Next is a “storage phase” in which cell division slowly ceases and deposition of storage reserves begins near linear (Meade et al. 2013; Bringezu et al. 2011). The sources of nutrients for storage reserve synthesis are provided by current photosynthesis and by remobilization reserve nutrients in vegetative tissues such as the leaves, stem and ear (Bewley et al. 2013). The grain is called milk grain as it appears to contain a thin liquid upon easily squashing (Edwards 2010; Dupont and Altenbach 2003). The grains of wheat have green color because of chloroplasts in pericarp layers, whereas no chloroplast appeared in the maize grain (Evers and Millar 2002). Cell expansion, which is related to endoreduplication (Balconi et al. 2007) and endomitosis of DNA inside the nuclei, continues during this phase in which influences the final grain size (Bringezu et al. 2011). After that, the grain dry weight rapidly increases, resulted in a reduction in water content gradually as the insoluble storage reserves displace water from the cytoplasm (Bewley et al. 2013), and the grain as a consequence has the consistency of dough (Edwards 2010; Dupont and Altenbach 2003). The color of dough grains in wheat changes from green to yellow (Dornez 2007), while in maize grain, a distinct horizontal line across the grain, called “milk line”, is appeared as boundary between the liquid and solid areas of the developing grain. This line slowly progresses to the tip of grain over next weeks (Sala et al. 2007; Santos et al. 2005). By the end of this phase, the grain reaches its maximum dry weight, commonly referred to as physiological maturity, even though it still contains approximately 30 - 35 % water (Meade et al. 2013; Thakur et al. 2010). Cell expansion and water accumulation stops first, followed by the accumulation of protein and starch ceases (Bewley et al. 2013). This prevention of reserve is caused due to the formation of a waxy layer at the placenta-chalazal region prevents the supply of nutrients into the grain (Dupont and Altenbach 2003). Around this time, the endosperm cells undergo a form of apoptosis i.e. programmed cell death, while the aleurone layer remains viable (Balconi et al. 2007). The plant has become completely yellow and the grain color in wheat turns to golden yellow (Dornez 2007). In maize grains, the “milk line” disappears (Sala et al. 2007 and Santos et al. 2005) and a “black layer” or “hilar” forms at the tip cap of the grains (Gwirtz and Garcia-Casal 2013) due to cessation of dry substance accumulation (Meade et al. 2013; Sala et al. 2007). The period from the beginning of grain growth until the grain reaches maximum dry weight at physiological maturity is called “grain

filling" period (Egli 2004), and grain quality is essentially formed during this period (Zhang et al. 2008).

Ripening (Edward 2010), the last phase, is associated with the loss of moisture, stable dry weight and entrance of grain into a quiescent state (Meade et al. 2013; Bewley et al. 2013; Thakur et al. 2010). The grain is usually harvested when it has dried to between 13 and 15 % moisture. This phase is sometimes termed as "harvest ripe", "grain ripe", "harvest maturity", "harvest ripeness" (Bringezu et al. 2011; Farrer et al. 2006).

2.3. Characterization of alpha-amylase

2.3.1. Classification and function

Alpha-amylase EC 3.2.1.1, denoted as α-amylase, belongs to glycoside hydrolysis enzyme that catalyzes the hydrolysis of internal α-1, 4-D-glucosidic linkage in a random manner of amylose and amylopectin chains with the retention of α-anomeric configuration in the products (Figure 2).

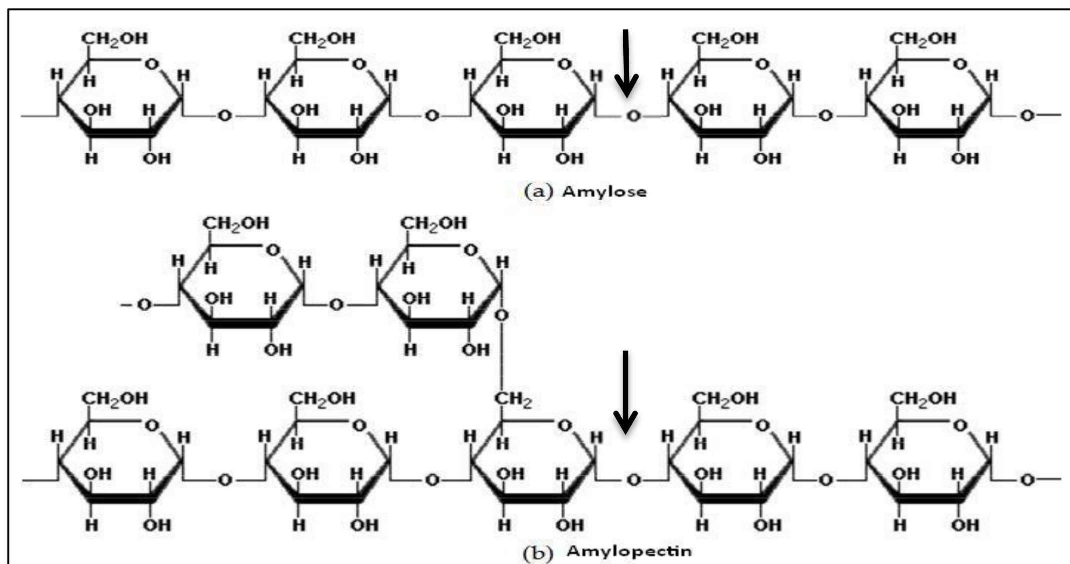

Figure 2: The action mode of α-amylase in (a) amylose and (b) amylopectin

Arrows indicate the linkage cleavage by α-amylase
Source: Sigma-Aldrich 2014

Alpha amylase differs from beta amylase with the mode of the action. It is endoamylase, whereas β-amylase is exoamylase (see Table 2). Initially, rapid breakdown of starch polymers by α-amylase to short chain dextrin is accompanied by a large decrease in viscosity and loss in iodine staining power. Further degradation of dextrin takes place on

longer incubation, reducing sugars then appear and finally α-maltose is formed (Belitz et al. 2009; Minic 2008; Muralikrishna and Nirmala 2005; Gupta et al. 2003).

Table 2: Characteristic features for α- and β-amylase

Characteristic features	α-amylase[1]	β-amylase[2]
Glycosidic bond specificity	α-1, 4-D-glucosyl of polymer at internal positions	α-1, 4-D-glucosyl from the nonreducing end of polymer
Mode of the action	Endo-attack	Exo-attack
Products of starch hydrolysis	α-dextrin, α-maltose and α-malto-oligoasaccharides	β-matose
Action at branch point α-1, 6-D-glucosyl	Can bypass	Can not bypass
Decrease in viscosity and Iodine staining	Rapid	Slow

Source: [1]Muralikrishna and Nirmala 2005, [2] Ziegler 1999

2.3.2. Structure

Alpha amylase exists in cereal grain such as wheat, maize, barely etc. in any development grain stage named cereal α-amylase to distinguish it from microbial α-amylase. Cereal α-amylase is small globular protein with molecular weights of 20 - 55 KDa. It is metalloenzyme that require calcium ions for their activity, structural integrity and stability. It is composed of a single polypeptide chain. The three dimensional structure, also named native structure, of α-amylases is based on $(\beta/\alpha)^8$ barrel fold as their main catalytic domain (Figure 3). It is four highly conserved regions in their primary structure containing all catalytic and most of the important substrate-binding sites. The catalytic sites of α-amylase consist of three invariant residues, two aspartates and one glutamate in beta-strands (Muralikrishna and Nirmala 2005).

Cereal α-amylase is heterogeneous and exists in multiple forms named as isoenzymes. The different isoenzymes can be separated into two major groups by isoelectric focusing on the basis of their isoelectric point (pI). They are encoded by two multigene families. Researchers have used different nomenclature for describing such groups. One of the groups is commonly referred to as the “green”, “pericarp” or “low pI group”. It is controlled by *α-AMY-2* genes located on the group 7 chromosomes in wheat. The other group is referred to as the “germination”, “malt” or “high pI group”. It is regulated by *α-AMY-1* genes on the long arm of the group 6 chromosomes in wheat (Cheng et al. 2014; Gao et al. 2013; Lunn et al. 2001a). Generally, low pI of α-amylase isoenzymes are appeared in

the early grain development and in late stage of germination, while high pI of α-amylase isoenzymes are mainly in germinating grains (Cheng et al. 2014).

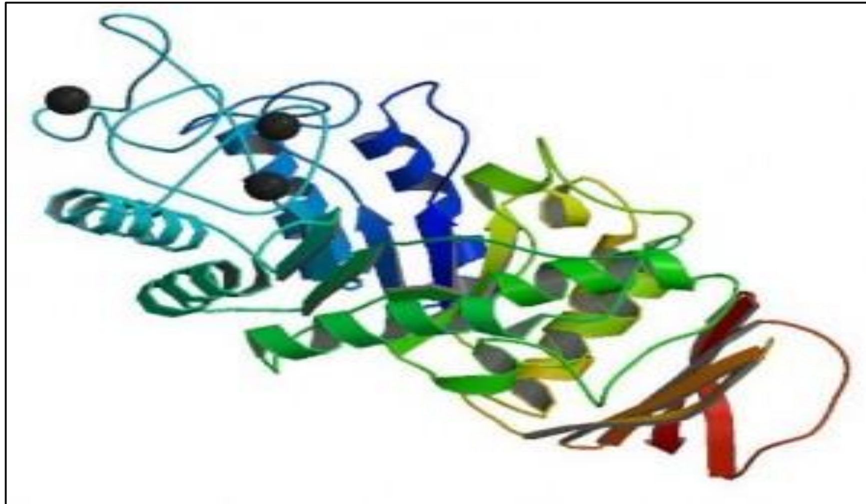

Figure 3: Ribbon diagram of cereal α-amylase structure

Source: Muralikrishna and Nirmala 2005

2.3.3. Synthesis

It was found that the pericarp tissue is the site of α-amylase synthesis during early grain development. The activity of α-amylase can be detected in immature grain with peak somewhere between 10 and 20 days after pollination and then enzyme activity decreased as grain matured and remains only trace at harvest ripeness. If pericarp α-amylase activity remains after the disappearance of the green color from grains in case of wheat, that could refer as "retained pericarp α-amylase activity" (De Laethauwer et al. 2013), however, it only exists in exceptional circumstances e.g. dehydration and rehydration in variable weather before harvest, presence of green grains at harvest. The activity α-amylase is associated with low pI isoenzymes (Lunn et al. 2001b).

Another source of enzyme is "late-maturity α-amylase activity", also known as "prematurity α-amylase activity", a genetic defect that is widespread in bread and durum wheat germplasm. It can be triggered in certain wheat cultivars during middle and late stage of ripening under certain environment e.g. cool temperature shock during the middle stages of grain development. It is characterized by synthesis high pI α-amylase throughout the entire aleurone layer in the endosperm cavity of the grain crease, independently of visible germination (Mohler et al. 2014; Barrero et al. 2013). Upon germination, the increase in α-amylase activity is the result of *de novo* synthesis of enzyme initially in the scutellum and

later by the adjacent aleurone (Barrero et al. 2013). If the germination process occurs in grains still on the ear during wet and cool harvest period, the process of that germination and induction of α-amylase activity is named “pre-harvest sprouting” (De Laethauwer et al. 2013; Gao et al. 2013). Two isoenzymes group of α-amylase have been identified in sprouted grains, high pI group in the early germination, and low pI in the later stages of germination (Lunn et al. 2001a). There is also another enzyme activity that is rarely recognized in literatures. This enzyme activity “pre-maturity sprouting” enhanced upon germination of developing grain that has high moisture content > 35 % (Lunn et al. 2001a).

The dominant role of α-amylase in grain development as well as germination grain is in starch metabolism (Cheng et al. 2014; Minic 2008). The regulatory mechanism that influence α-amylase gene expression and secretory processes during grain development, maturation and germination appeared to be controlled by several factors such as the growth hormones, intracellular calcium concentration and metabolic sugars (Muralikrishna and Nirmala 2005; Lovegrove and Hooley 2000). Typically, the secretion pathway of enzyme proceeds by means of transcription, translation of mRNA on rough endoplasmic reticulum, and intracellular transport of enzyme protein to the Golgi apparatus, and then to the plasma membrane (Mitsui and Iton 1997). Differences in the activity of α-amylase could be induced by genotypes, environmental conditions, quantitative trait loci (QTLs) and the interaction between these factors (De Laethauwer et al. 2013; Dencic et al. 2013).

2.3.4. Assay methods

A number of methods which are currently available for α-amylase assay are present in Table 3 (e.g. Megazyme booklet 2012; Xiao et al. 2006; Muralikrishna and Nirmala 2005; Gupta et al. 2003; ICC Standard booklet 1999). These are based mainly on four groups: (1) decrease in the blue color of starch-iodine complex. This procedure reflects the endocleavage of starch and can be used routinely to assay α-amylase. (2) Increase in the reducing sugars, 3, 5-dinitrosalicylic acid (DNS) is the most commonly method used for direct measure of starch hydrolysis because of its reliability and simplicity. (3) Decrease in the viscosity of starch solution, falling number (FN) method is accepted as international standard methods ICC Standard No. 107/1 in the bakery industry to assess the quality of the flour based on the determination of the rheological properties of the dough. The FN value above 220s is required for good bread quality (Dencic et al. 2013; Kreuzberger 2011; Johansson

2002). (4) Degradation of color-complexed substrate, ρ-nitrophenyl maltoheptaoside (BPNPG7) is an example of a chromogenic substrate which is sensitive for α-amylase determination in Ceralpha method (ICC Standard No. 303).

Table 3: Principle of some assay methods for α-amylase determination

Method	Substrate	Reaction principle	Unit
Starch-iodine[1]	Starch or amylose	Starch-iodine complex hydrolysis, reducing the complex and blue color	U ml^{-1}*
3,5dinitro-salicylic acid (DNS)[2]	Native or soluble starch	3,5-Dinitrosalicylicacid reduced to 3-amino-5-nitrosalicylic	µmol reducing sugar min^{-1}
Falling number (FN)[3]	Flour as native substrate	Measures the time required for a plunger to fall through heated suspension of flour in water at 100 °C in a large glass test tube	second (s)
Ceralpha [4]	ρ-nitrophenyl maltoheptaoside (BPNPG)	Endolytic attack followed by α-glucosidase/glucoamilase action on *p*-nitropphenol maltosaccharide fragment, releasing free *p*-nitropphenol	Ceralpha Unit (CU)

*U: One unit is definedas the disappearance of an average of 1 mg of iodine binding starch per min
Source: [1]Gupta et al. 2003; [2]Xiao et al. 2006; [3]ICC Standard booklet 1999; [4]Megazyme booklet 2012

Table 4 represents the α-amylase activity in two ICC Standard methods, Ceralpha and FN. FN method considers as indirect measurement of α-amylase activity in which the correlation between α-amylase activity and FN is negative. Different factors can affect FN as starch structure, the type of enzyme present and non-starch polysaccharides composition of flour (Lenartz et al. 2003). While Ceralpha method considers as direct measurement of α-amylase activity (Muralikrishna and Nirmala 2005) because it is used a short synthetic substrate in the assay which is more sensitive to any modification of α-amylase conformation (Megazyme booklet 2012).

Table 4: The activity of α-amylase in two ICC Standard methods, Ceralpha and FN

Ceralpha method ICC Standard No. 303 (CU)*	FN method ICC Standard No. 107/1 (s)*
0.15	373
0.25	290
0.28	258
0.33	249
0.45	224
0.62	189
1.2	162
2.6	120
4.3	73
4.6	62

Source: Kindred et al. 2005; Cato 2005; Bhandari 2002
*CU, Ceralpha Unit and s, second

2.3.5. Technological importance

Numerous studies in the past till now have been illustrated the significance of α-amylase in breadmaking (e.g. Belitz et al. 2009; Goesaert et al. 2005; Martinez-Anaya and Jimenez 1997). Their work can be summarized as follows, α-amylase has two primary functions in baking: (1) hydrolysis of native starch granules, damaged starch and gelatinized starch and subsequently reduction the dough viscosity resulting in improved dough handling and enhancement of crumb volume and texture, (2) the enhanced provision of fermentable sugars for yeast activity to produce carbon dioxide, thus improved the gas retention properties of dough and increased the loaf volume of bread. Additionally, bread flavor and crust color are improved both by sugars produced by α-amylase and Maillard reaction products.

Addition of α-amylase is required to optimize the α-amylase activity of the flour i.e. flour standardization and at retarding bread staling. Flour standardization is performed by malted barley and wheat or fungal α-amylase from *Aspergillus oryzae*.

However, the main issues with high α-amylase activity are softening of dough, sticky bread crumb, problems when slicing bread and bread crust overly colored as a result of sugar caramelization due to extreme amount of dextrin. These problems occur from flour of sprouted wheat grains as a result of unfavorable growing conditions of plant prior to harvest and it is discussed in details in section 2.3.6.

In addition to bread making, cereal α-amylases in barley, sorghum, maize and millet grains are necessary in the malt and beer production to ensure the maximum production of

fermentable sugars (Faltermaier et al. 2014; Taylor et al. 2013). They are also used in supplementary foods e.g. porridge in many African countries (Dicko et al. 2006; Helland et al. 2002).

2.3.6. Pre-harvest sprouting

The pre-harvest sprouting phenomenon is defined as the sprouting occurs on the ripe grains while still on the ear of the mother plant during wet harvest period due to the lack of dormancy (see Figure 4) (Gao et al. 2013). It is also termed as “precocious germination” (Bewley et al. 2013). Such phenomenon is a serious problem in cereal-growing regions, where cool damp conditions prior to harvest are possible, because it reduces grain quality due the presence of excessive α-amylase activity in sprout-damaged grains, and thus results in unsatisfactory end-products and significant economic losses (De Laethauwer et al. 2013).

Figure 4: Pre-harvest sprouting in (a) maize and (b) wheat

Source: Bewley et al. 2013

Physiological mechanism of pre-harvest sprouting

Sprouting of cereal grains on the ear prior to and during harvest period can be divided into a number of sequential physical and biochemical events. They are: imbibition of water, enzyme activation, metabolism of storage materials and subsequent mobilization to the growing embryonic axis, and finally rupture of the pericarp and testa tissues and the emergence of the seedling (Bewley et al. 2013).

Water uptake by grain via physical imbibition is an essential and initial step toward germination. It is influenced by factors such as availability of water, temperature and humidity. The water moves firstly to the vegetative parts of the ear, then to the grain surface i.e. pericarp and testa and finally to the embryo and endosperm tissues (Bewley et al. 2013). Therefore, the amount of wetting and water uptake by grains is affected by grain hardness, thickness of testa and other layers, grain size and surface-to-volume as well as the morphology of the ear such as the presence or absence of awns, husk structure, ear waxiness and ear-nodding angle (Black et al. 2006). Typically, the water uptake by grain is triphasic. In phase I " imbibition", the water absorption of the grain is rapid in the first three hours due to extremely low water potential (-100 MPa) of the grain, which attributed to its matric characteristics. The imbibed grain undergoes some metabolic events such as initiation of respiration, mitochondrial and DNA repair, the initial synthesis of enzyme and proteins due to transcription and translation of stored mRNA (Bewley et al. 2013). Phase II "plateau phase", this is the lag phase of water uptake and the grain has swollen to a maximum size. During this phase, the major metabolic events occur such as the synthesis of new DNA and mitochondria and synthesis protein and enzyme due to new mRNA (Black et al. 2006). The mobilization of the stored reserves is taken place via several enzymes in order to provide nutrients for seedling growth (Bewley et al. 2013). Finally, phase III "completion of the germination" is characterized by further increase of water uptake and is concurrent with the radical due to the cellular expansion and division (Bewley et al. 2013).

Economic losses of pre-harvest sprouting

Pre-harvest sprouting occurs in wet or humid weather during the harvest season of cereals grains in many regions of the world, including northern and Western Europe, North and South America, Australia, China, India and the southern parts of Africa. This phenomenon leads in some years to large losses to the agricultural industry. The average annual losses approach US $1 billion worldwide, mostly to wheat (Liu et al. 2013; Barnard and Smith 2012; Black et al. 2006). The extent of the problem is illustrated by a few examples in Table 5. Beside wheat grains, other cereal grains such as barley, rye, triticale and maize are prone to pre-harvest sprouting (Black et al. 2006).

Many scientists (Schramm et al. 2013; Csiszar et al. 2010; Black et al. 2006) have paid attention to the pre-harvest sprouting and its direct and indirect losses. The yield reduces

due to utilization of carbohydrates during respiration which, at the same time, creates an appropriate environment for fungi and bacteria infection. In addition, the test weight of grains decreases as a direct response to dry matter loss and irreversible swelling of the grain. Furthermore, the flour suitability of sprouted grain for food processing is considerably lower than those of non-sprouted grain. In severe case, sprouted grains may be downgraded to animal feed. All these effects together result in a reduction of the farmers' income since the sprouted grains will not be purchased for milling. The indirect loss of pre-harvest sprouting is resulted to prevent growing some high value crops in that area because of the hazard of sprouting damage. For example, red wheat cultivars, which are resistant to pre-harvest sprouting with low flour yield, are grown instead of white wheat cultivars in some regions in China (Xia et al. 2009), USA (Schramm et al. 2013) and Western Australian (Biddulph et al. 2008).

Regarding to wheat processing losses, breads baked from sprout-damaged wheat grains have collapsed loaves and sticky crumbs that lead to tearing when the breads are mechanically sliced (Mohler et al. 2014; Dencic et al. 2013; Csiszar et al. 2010). In durum wheat, sprout damaged-grains cause high cooking losses and poor color as well as low spaghetti stickiness values (Gelin et al. 2006). Sprouted wheat flour looses its thickening power, hence it can not be used in cream soup and gravy mixes (Groos et al. 2002).

Table 5: Summary of worldwide incidence and severity of pre-harvest sprouting and the effects on producer revenue

Country	Yearly rainfall distribution	Sprouting incidence (Years/10)	Magnitude of effect (%)	Percentage change in revenue due to sprout damage (%)			
				Food wheat	Feed wheat	Maize	Coarse grain
Australia-northern	Summer	Most	7	-9.40	6.45	0.04	-0.21
Canada	Winter/Spring	4	na	-8.50	2.49	-0.12	-0.41
USA	Winter/Spring	5	12	-3.86	-4.89	0.11	0.18
European Union:				-4.36	-4.92	0.05	0.09
Germany	Winter/Spring	3	23				
UK	Winter/Spring	5	69				
Poland	Winter/Spring	5	10				

na, not available
Source: Gatford 2004; Wahl and O'Rourke 1994

Tolerance to pre-harvest sprouting

Many factors can contribute to increase tolerance to sprouting damage such as a reduction in water absorption by the spike and the grain, increasing grain dormancy, the

presence of germination inhibitors, the alteration responses to phytohormones and the reduction in the activity of grain α-amylase activity (Gao et al. 2013; De Laethauwer et al. 2013).

Previous studies have been indicated that the presence of awns in wheat or husks in maize cob (Black et al. 2006), the strong glume tenacity (Liu et al. 2013) and high level of ear waxiness (King and von Wettstein-Knowles 2000) have a positive effect on the tolerance to pre-harvest sprouting by shedding water and decreasing grain imbibition.

Grain dormancy has been found as an important multigenic trait for effectively reducing grain-sprouting damage (Gao et al. 2013). The stronger grain dormancy of cereal cultivar has been, the more tolerant cultivar to pre-harvest sprouting is observed. Grain dormancy is strongly influenced by environmental factors and determined by seed coat and embryo factors (Schramm et al. 2013). Different dormant level could be determined by germination test or germination resistance index (Biddulph et al. 2008).

It was found by Weidner et al. (1999 and 2002) that wheat, rye and triticale grains with deep dormancy possessed a high concentration of phenolic compounds, particularly phenolic acids in the form of their soluble esters such as caffeic, p-coumaric, ferulic and sinapic acids. Theses phenolic compounds can act as germination inhibitors (Divashuk et al. 2012). Additionally, the pigmentation of testa has an influence on the tolerance to pre-harvest sprouting, where colored grain more tolerant to pre-harvest sprouting than white grain (Liu et al. 2013; Schramm et al. 2013).

Abscisic acid (ABA) and gibberellic acid (GA) signaling pathways and the tissue responses to these phytohormones affect cereal cultivars tolerance to pre-harvest sprouting through enhancing or delaying seed dormancy and germination (Kondhare et al. 2012; Finkelstein et al. 2008).

The activity of α-amylase differs significantly between resistant and sensitive wheat cultivars to pre-harvest sprouting (Gao et al. 2013). A number of factors, including genotypes and environmental factors, are suggested to induce or modify the α-amylase activity and synthesis (Dencic et al. 2013; Liatukas and Ruzgas 2009). Several studies have indicated that different environmental variables can affect amylase activity, either individually or in combination. They are high precipitation (Barnard and Smith 2009; Yanagisawa et al. 2005), changes of temperature (Farrell and Kettlewell 2009; Biddulph et al. 2008 and 2005; Osanai

et al. 2005) and the rate of grain desiccation (Kettlewell and Cashman 1997). It was also found that the grain size and weight (Farrell and Kettlewell 2009) and the application of fertilizers (Kindred et al. 2005) have been associated with differences in α-amylase activity. The FN is the most widely used test to screen wheat for pre-harvest sprouting, the higher the level of sprouting is detected, the lower of FN result will be (Barnard and Smith 2012; Liatukas and Ruzgas 2009). The FN value below 200s indicates grain damage by sprouting (Cato 2011). Therefore, the magnitude of effect of α-amylase has resulted in its being a focus for detecting pre-harvest sprouting damaged grains (Simsek et al. 2014; Khakimzhanov et al. 2011). Recently, many scientists have paid attention to the application of molecular marker technology and quantitative trait locus (QTL) analysis to achieve resistant cultivar breeding (e.g. Mohler et al. 2014 and 2012; Gao et al. 2013). It was reported that the infection by *Fusarium* spp. (Kreuzberger 2011; Wang et al. 2008) and orange wheat blossom midge (*Sitodiplosis mosellana*) larvae (Singh et al. 2008; Kindred et al. 2005) increased the α-amylase activity in grains. Therefore, both fungicide and insecticide treatments may have potential benefits in reduction α-amylase activity.

There are some steps can be taken to reduce the incidence of pre-harvest sprouting. Taking the weather conditions into account, sprouting susceptibility can somehow be predicted. Cereal crop may harvest earlier than usual at moisture content higher than > 15 %, with respect to grain viability and development of the fungal growth (Black et al. 2006; FAO 2002). Gains may be harvested, by reaping or swathing the grain and allowing it to dry in windrows, sheaves, shocks or stacked (FAO 2002). Additionally, the storage of harvested grain at low temperature ~ 10 - 13 °C (Black et al. 2006) until use is important to preserve dormancy (Knox et al. 2012).

2.3.7. Enzyme kinetics

Enzyme as a catalyst has the ability to accelerate the rate of biochemical reactions under normal cellular conditions. The overall reaction involving conversion of substrate (S) to product (P) with formation of enzyme-substrate complex (ES) can be written as:

$$E + S \rightarrow ES \rightarrow E + P \qquad (1)$$

Enzyme kinetics refers to the quantitative analysis of all factors influence the rate of enzyme catalyzed-reactions such as the concentration of enzyme and substrate, pH,

temperature, ionic strength, irradiation, inhibitors, activators etc. According to equation 1, the experimental determination of enzyme activity is based on the amount of product formed or substrate consumed under known set of conditions. This study provides information about the important aspects such as mechanism of enzyme action in the metabolic regulation as well as the parameters which characterize the properties of enzyme. The latter is important from a technological perspective, since it allows the utilization of enzyme in several foods and industrial processing (Panesar et al. 2010; Rogers and Gibon 2009; Illanes 2008).

2.3.8. Biochemical and kinetic properties of enzyme

The substrate concentration

In the cell, enzyme is exposed to change in the concentration of S, so the dependence of enzyme velocity on the concentration of S is the first line of metabolic regulation. The Michaelis-Menten equation is algebraic expression of enzyme kinetics, in which the velocity of enzyme reaction depends on the substrate concentration in the presence of constant enzyme concentration according to the following equation:

$$V_0 = \frac{V_{max}[S]}{K_m+[S]} \quad (2)$$

V_0 denotes the initial velocity, [S] denotes the concentration of substrate, V_{max} is the maximum velocity approached at substrate saturation of enzyme under a given set of conditions and K_m, Michaelis-Menten constant, is numerically equal to the concentration of specific substrate required to produce a velocity that one-half of V_{max}. The K_m parameter provides an indication of the binding strength of that enzyme to its substrate. A high K_m indicates a lower affinity for substrate and vice versa. The equation corresponds to a rectangular hyperbola. There are a number of graphical linearization methods to obtain V_{max} and K_m to avoid evaluating enzyme kinetics using a nonlinear plot, while today there are nonlinear curve-fitting programs which are preferred tools of determining the values of kinetic parameters (Panesar et al. 2010; Rogers and Gibon 2009; Illanes 2008; Copeland 2004; Ranaldi et al.1999).

The temperature effect

Temperature is the most relevant variable in any biological process. As the temperature is increased, the rate of reaction increases as well, but at the same time there is inactivation and/or denaturation of enzyme protein. Therefore, it has remarkable effect on enzyme reaction. Each enzyme shows so-called temperature optimum, which is maximum enzyme activity (Belitz et al. 2009; Daniel et al. 2008; Copeland 2004).

The pH effect

Because enzyme is polyionic polymer, the pH of the reaction medium affects enzyme activity. Each enzyme has a characteristic pH at which its activity is a maximum, named pH optimum of enzyme, while denaturation of enzyme may occurs at extremely low and high pH (Belitz et al. 2009; Copeland 2004).

Enzyme stability

Enzyme stability is not related to activity. It is regarded as the capacity of enzyme to retain its activity through the time. It is dependent on enzyme three-dimensional structure. Like all proteins, enzyme in its native structure is optimally stabilized by specific surrounding conditions of pH, temperature, ionic strength and so on. The alteration in the environment surrounding enzyme in which its native conformation is changed causes protein denaturation. This term denotes an event or a sequence of events leading to structural changes with subsequent aggregation or improper folding or even unfolding to the extent of altering the catalytic capacity of enzyme and loss its activity. This phenomenon may be reversible if the denaturing influence is removed, or be irreversible. It is worthwhile to mention that the solution conditions that are optimal for enzyme's protein stability may not necessarily the same as those for optimal enzymatic activity (Prakash and Jaiswal 2010; Belitz et al. 2009; Illanes 2008; Copeland 2004).

A summary of α-amylase characteristics from different sources is given in Table 6. Although broadly similar properties of cereal α-amylases are present, there are differences in the properties of α-amylases from different cereals grains and even individual isoenzymes of a particular cereal (Muralikrishna and Nirmala 2005). It has been concluded in earlier reports (Kruger 1989; Kruger and Lineback 1987) as well as recent reports (El Nour et al.

2013; Usha et al. 2011) that cereal α-amylases share common properties which distinguish them from other cereal enzymes. They are: (1) relative thermal stability and (2) acid lability.

Table 6: Cereal α-amylases and their biochemical properties

Cereal α-amylase	Temperature optimum(°C)	Temperature stability (°C)	pH optimum	pH stability	K_m * (mg ml^{-1})	V_{max} * (µmolmin^{-1})	Mw (KDa)	pI	References
Wheat *Triticum aestivum*	50	40 , 50	5.5-7	5 - 7.5 [a]	2.2-4	0.33 - 0.8	41.5-42.5[a]	6.05-6.2 [a]	Mohamed et al. 2009 [a] Muralikrishna and Nirmala 2005
Maize *Zea mays*	50	30 [b]	4-6.5	na	12.5 [b]	714 [+b]	67	4.1-5.7 [c]	Biazus et al. 2009 [b] Adwale et al. 2006 [C] Warner et al. 1991
Sorghum *Sorghum bicolor*	60	70 [d]	6.5	na	1.092	5248 [+]	47 [d]	4.4 - 5.4 [e]	Adefila et al. 2012 [d] Kumar et al. 2005 [e] Lecommandeur and Daussant 1989
Barley *Hordium vulgare*	45	40	5	5-8 [a]	5	1.56	45, 59 [f]	5.1, 6.2 - 6.4 [a]	Al-Bar 2009 [f] Brena et al. 1996
Rice *Oryzae sativa*	25, 55	na	na	na	1.1, 5	na	42, 45	5.8, 6	Abe et al. 2002
Pearl millet *Pennisetum typhoides*	70[g]	na	7	na	0.5	603	33	4.8, 5.2, 6.2 [a]	Kharkrang and Ambasht 2013 [g] EL nour et al. 2013
Finger millet *Eleusine coracana*	45 - 50	50 [b]	5-5.5	na	5.9-14.3	2381	45	na	Nirmala and Muralikrishna 2003
Tritical *Triticosecale*	54-56 [a]	45	4.5 - 6	6,6.5	0.025 - 0.029	1250 - 5000	40 - 41	na	Silvanovich and Hill 1977
Oat *Avena sativa*	47 [f]	40	4.7 [f]	5-7.5 [f]	na	na	45 [f]	4.9 [e]	Greenwood and Milne 1968
Rye *Secale cereale*	47 [f], 65	55 -70	5.5	5 -7.5 [f]	na	na	45 [f]	na	Gabor et al. 1991
Ryegrass *Lolium rigidum*	53	na	4 - 5	na	34.2	37.5	29 30-43	8-9 5-6	Goggin and Powles 2012

+ Unit: µg min^{-1}
na, not available
* Starch used as a substrate for kinetic parameters

2.4. Factors influencing the grain composition and enzyme activity

Environments and genetics greatly affect the composition of cereals grains, mainly starch (Beckles and Thitisaksakul 2014; Buresova et al. 2010), protein (Malik et al. 2011; Johansson et al. 2008) as well as α-amylase activity (Dencic et al. 2013; Barnard and Smith 2012). Furthermore, different growth and development stages of cereal crops before and after anthesis have an influence on grain number, size and composition due to different responses to environment (Bringezu et al. 2011; Farooq et al. 2011).

Dupont and Altenbach (2003) reported that prior to anthesis, the environmental conditions influence germination, photosynthesis, tiller formation, and ear development, whereas the grain composition and its quality attributes are influenced by weather post anthesis, which affects the rate and duration of grain maturation period (Farooq et al. 2011; Barnabas et al. 2008) and the leaf senescence as well (Guoth et al. 2009). It was revealed in many studies (e.g. Aslam et al. 2013; Farooq et al. 2011) that temperature and water availability between anthesis and maturity are the two factors that have a major influence on grain development, yield and quality in many cereal production areas. They have also a remarkable effect on the α-amylase activity (e.g. De Laethauwer et al. 2013; Barnard and Smith 2012; Biddulph et al. 2005).

On the other hand, the chemical compositions of grains (e.g. Buresova et al. 2010; Johansson et al. 2008; Johansson 2002) and the α-amylase activity differ between cereal cultivars (Dencic et al. 2013; Barnard et al. 2005). Furthermore, the differences in grain dormancy (Liatukas and Ruzgas 2009), grain filling duration (Egli 2004), plant responses to environmental stress (Guoth et al. 2009), grain size and weight (Farrell and Kettlewell 2009; Evers et al. 1995) exist due to differences between cultivars.

3. MATERIALS AND METHODS

3.1. Plant materials

Two field experiments were carried out with different cereal crops. The post-harvest treatments as well as the determination methods for different parameters and enzyme properties were the same in both experiments.

3.1.1. First experiment

Two bread wheat cultivars, winter wheat of cv. Cubus and summer wheat of cv. Amaretto, were provided from two separate trail locations belongs to project Forschungsverbund Agrar- und Ernährungswissenschaften Niedersachsen (FAEN) in the north of Göttingen, Germany. These locations termed as Gladebeck and Torland in the subsequent text. The grains collected ony from 2010, while in 2011 the crops were exposed to forest damage. Weather conditions during development period as well as geographical and cultivation information are shown in appendix Figure 18 and Table 16. Characteristic attributes of two cultivars are presented in appendix Table 18. The development stages of the cereals were identified using the extended BBCH-scale. The BBCH-scale defines the phenological growth stages with a standardized decimal code. The abbreviation BBCH derives from Bundesanstalt Bundessortenamt und CHemische industrie (Meier, 2001). Wheat grains were collected as milk (BBCH 75 - 77), dough (BBCH 87) and mature stage (BBCH 97) only from 2010, since in 2011 the plants were damaged by frost and now yield was obtained.

3.1.2. Second experiment

Seven cultivars of forage maize (Benicia, EsBombastic, Franki, Justina, Lukas LG 3226, Saludo, and PR39F58) were provided from one trail location belongs to Institut für Pflanzenbau und Bodenkunde, JKI, Braunschweig, Germany in 2010 and 2011. Further information about the weather data during development period and features of selected cultivars is shown in appendix Figure 19 and Table 18. Maize grains were harvested at corn mature stage (BBCH 97) in 2010, whereas in 2011 the samples were collected at three

development stages based on BBCH-scale as milk (BBCH 75 - 77), dough (BBCH 87) and corn mature stage (BBCH 97).

3.2. Post-harvest treatment

The grains were cleaned with an automatic cleaning apparatus (SLN model, Rationel Kornesrvice, Esbjerg, Denmark). Then the grains were immediately frozen at -20°C before freeze dried (CHRIST Epsilon 2-40, Osterode, Germany) to avoid decay of enzyme activities. The freeze dried grain were ground into whole grain flours by an automatic mill (Retsch Rotormill ZM 100, GmbH, Haan, Germany) with a sieve of 0.5 mm and stored in air-tight bottle at -20 °C until testing.

3.3. Determination of the chemical composition of grains

Freeze-dried whole flour was used to determine the chemical composition. Determination was repeated triplicate as technical measurements except protein content in duplicate. All values were based on dry matter of freeze dried samples.

3.3.1. Starch content

Starch was determined by the ICC Standard No. 123/1 (ICC Standard booklet 1999) using polarimeter type (V DrNa, Zeiss AG, Jena, Germany). Starch content was calculated using the following formula:

$$\text{Starch content}(\%\ \text{DM}) = \frac{\text{C}\times\text{V}}{\text{W}} \times 100 : \frac{\text{DM}}{100} \qquad (3)$$

$$\text{C} = \frac{\alpha}{\text{L}\times\text{D}} \qquad (4)$$

C = concentration of starch (g l^{-1})
V = extraction volume (ml)
W = weigh of sample (g)
DM = dry matter
α = measured optical rotation
L = length of polarimeter tube (1.901 dm)
D = specific rotation angle for wheat starch (182.7 °) and maize starch (184.6 °)

3.3.2. Protein content

Protein was determined by by a combustion method (ICC Standard No. 105/2) (ICC Standard booklet 1999) using elemental analyzer (Elementar Vario EL, Elementar Analysensysteme GmbH, Hanau, Germany). Protein content was calculated using the following formula:

$$\text{Protein content (\% DM)} = \text{N} \times \text{F}: \frac{\text{DM}}{100} \qquad (5)$$

N = measured nitrogen content (%)
F = protein conversion factor for wheat (5.7) and for maize (6.25)

3.3.3. Sugar content

Whole grain flour (1 g) was dissolved with distilled water (6.5, 2.5 ml for wheat and maize, respectively) and vortexed for 2 min to homogenize the solution. The extraction procedure was performed for 20 min at room temperature with magnetic stirring (Variomag Multipoint 15, H+P Labortechnik AG, Oberschleissheim, Germany). The sample was centrifuged at 20 °C for 20 min at 10 000 rpm (Du Pont Instrument, Sorvall, RC-5B, Germany).The extraction was repeated twice, then the supernatants were combined and filled up to 13 ml. Carrez solution No.1 (1 ml) and Carrez solution No.2 (1 ml) were added to the solution and shaken vigorously .The sample was subsequently centrifuged at 4 °C for 20 min at 11 000 rpm. The supernatant was applied to rotational vacuum concentrator (CHRIST, RVC 2-25 CD plus, Osterode am Harz, Germany) at 30 °C, vacuum 4.1mbar and vapour-pressure 15 mbar until reached 1 ml residue. The concentrated solution was washed twice with 0.5 ml distilled water and then centrifuged at 20 °C for 20 min at 14 000 rpm (Centrifuge 5804R, Eppendorf, Hamburg, Germany).

The HPLC system (Jasco LC-2000 Plus, Germany) was used to quantify fructose, glucose, sucrose and maltose concentrations in samples (modified Zörb et al. 2012). The column is LiChrospher, NH_2, 5 µm, 250 - 4. The detector was RI 2301. The eluent is ratio acetonitrile: water as 80 : 20. Twenty µl was used as injection volume, and the flow was 1 ml min^{-1}.

Different sugars concentrations were measured by comparing the peak of the samples to that of the standard. The following formula was used in calculation:

$$\text{Sugar content (\% DM)} = \frac{C \times V}{W \times 10} : \frac{DM}{100} \qquad (6)$$

C = measured concentration of sugar (g l^{-1})
V = extraction volume (l)
W = weigh of sample (g)
DM = dry matter

Total reducing sugars expressed as sum of fructose, glucose and maltose content in each sample. The total sugars were the sum of fructose, glucose, maltose and sucrose in each sample.

3.4. Ceralpha method

The α-amylase activity was assayed spectrophotometrically by using non-reducing-end blocked ρ-nitrophenyl maltoheptaoside (BPNPG7) as a substrate following Ceralpha method (ICC Standard No. 303) in Megazyme booklet (2012).

3.4.1. Enzyme extraction

Three gram of whole grain flour was extracted with 20 ml of buffer extraction [sodium malate (1 M) +sodium chloride (1 M) + calcium chloride (40 mM) + sodium azide (0.1 %), pH 5.4]. The mixture was incubated for 20 min at 40 °C, with occasional mixing (Thermomixer comfort, Eppendorf AG, Hamburg, Germany). At the end of 20 min, the mixture was centrifuged at 10000 rpm for 10 min at 20 °C (Centrifuge 5804R, Eppendorf, Hamburg, Germany). The modification in extraction and assay was done in grains of wheat because of excessive enzyme activity that its absorbance exceeded the range of spectrum of photometer. For enzyme extraction, 0.5 g of whole grain flour was mixed with 100 ml of buffer extraction for 15 min at room temperature, with occasional mixing and then centrifuged (10 000 rpm, 10 min at 20 °C). An aliquot of the supernatant was diluted 1: 100 and analyzed.

3.4.2. Enzyme assay

Enzyme solution and BPNPG7 were pre-incubated at 40 °C for 5 min before used. To 0.1 ml BPNPG7 solution, 0.1 ml of enzyme extraction was added and incubated at 40 °C for 20 min while the incubation time for enzyme extracted from wheat grains was 10 min. At the end of incubation, the reaction was stopped by adding 1.5 ml tris-sodium phosphate solution

(20 % (w/v), pH 11) and stirred vigorously. Subsequently the absorbance of mixture was measured by spectrophotometer (Hewlett Packard 8453, Palo Alto, USA). The blank was obtained by mixing 0.1 of BPNPG7 solution and 1.5 ml of the stopping solution. At the end of incubation, 0.1 ml of enzyme was added. One Ceralpha Unit (CU) of α-amylase was defined as the amount of enzyme producing one µmole ρ-nitrophenyl from BPNPG7 per one min at 40 °C and pH 5.4. The formula was used in calculation as following:

$$\text{Enzyme activity (CU g}^{-1}\text{ DM)} = \frac{\Delta \text{E400}}{10} \times \frac{\text{V1}}{\text{V2}} \times \frac{1}{\text{E}_{\text{mM}}} \times \frac{\text{V3}}{\text{W}} \times \text{Dilution} : \frac{\text{DM}}{100} \quad (7)$$

ΔE400 = absorbance of reaction – absorbance of blank
V1, V2, V3 = total volume, extraction volume and enzyme assay volume (ml), respectively
E_{mM} = absorbance of ρ-nitrophenyl in 1 % tris-sodium phosphate at 400 nm = 18.1

3.5. Properties of α-amylase

Enzyme properties were performed on whole grain flour from harvest-mature stage in wheat and maize samples. Each measurement was repeated triplicate as technical measurements.

3.5.1. Enzyme extraction and assay

The α-amylase activity was assayed by 3,5-dinitrosalicylic acid (DNS) procedure (Bernfeld 1955; Sigma-Aldrich protocol 2010) using 1 % soluble starch as substrate.

Enzyme extraction

Whole grain flour (0.5 g) was extracted with 10 ml of buffer solution [sodium acetate (0.1 M) + calcium chloride (10 mM), pH 6] for 30 min at 40 °C with gentle shaking using thermomixer. After centrifugation (10 000 rpm, 10 min, 20 °C), enzyme extract was used directly for further analyses.

DNS method

One ml of enzyme extract was incubated for 5 min at 40 °C with 1 ml of soluble starch as a substrate. The reaction was stopped by addition of 2 ml DNS .Reaction tube was placed in boiling water bath for 5 min and then cooled in an ice bath for 5 min. After the addition of 10 ml of distilled water, the absorbance of sample was read at 540 nm by spectrophotometer. A blank containing only a buffer extraction was run simultaneously with

the reaction mixture. Calibration curve was prepared from maltose and processed in the same way as the samples.

One unit of α-amylase activity was defined as the amount of enzyme required to produce reducing sugar equivalent to 1 µmol of maltose per min from soluble starch at 40 °C and pH 6 in 1 ml of enzyme. Enzyme activity was calculated as the following (Yaldagard et al. 2008):

$$\text{Enzyme activity } (\mu\text{mol min}^{-1}\text{ml}^{-1}) = \frac{\text{A}\times 1000\times \text{Dilution}}{\text{M}\times \text{T}\times \text{VE}} \qquad (8)$$

A = amount of maltose released (mg)
M = molecular weight of maltose (360.32 g mol^{-1})
T = incubation time (min)
VE = volume of enzyme used (ml)

3.5.2. Effect of temperature on enzyme activity and stability

The effect of temperature on α-amylase activity was determined by assaying enzyme extract at temperature from 30 to 90 °C. The relative activity at different temperatures was calculated, taking the maximum activity as 100 %. To determine temperature stability, enzyme extract was pre-incubated at various temperatures (30 - 90 °C) for 30 min prior to substrate addition. Then enzyme extract was cooled and the residual activity was assayed under the standard assay conditions, taking the maximum residual activity as 100 %.

3.5.3. Effect of pH on enzyme activity and stability

To determine the optimum pH of α-amylase activity, enzyme preparation and assay were done at different pH values (3 - 9) in the following buffers: 100 mM of sodium acetate buffer (3 - 6) and 100 mM Tris-HCl buffer (7 - 9). All buffers contained 10 mM calcium chloride. The relative activity at different pH values was calculated, taking the maximum activity as 100 %.

To estimate pH stability, enzyme extract was pre-incubated at various pH values (3 - 9) for 30 min. Then enzyme extract was adjusted to pH 6 by adding suitable amounts of HCl or NaOH. The assay was carried out in the same manner as described under enzyme assay and the residual activity was calculated, taking the maximum residual activity as 100 %.

3.5.4. Kinetic parameters

Kinetic parameters of α-amylase were obtained by using various soluble starch concentrations as substrate (S) of 0.005, 0.01, 0.015, 0.02 and 0.025 g ml^{-1} of reaction mixture under standard assay conditions. The estimation for Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) for enzyme were performed by computer-program software SigmaPlot 10 as well as plotted Michaelis-Menten curve.

3.5.5. Electrophoretic properties of enzyme

Preliminary test for isoenzymes detection was applied in mature-harvest stage of grains using partial purified α-amylase. The test failed to show any bands on gel. Then gel electrophoresis techniques were applied in milk-ripe stage of both wheat and maize because of the highest activity of α-amylase appeared in immature grains, therefore the bands on gels of target proteins can be obviously shown with partly enzyme purification applied. The samples were wheat collected only from Torland, while just two maize cultivars, Franki and Saludo, harvested in 2011 were used.

Extraction and purification of enzyme

Whole grain flour (0.5 g) was extracted with 5 ml of buffer solution [sodium acetate (0.1 M) + calcium chloride (10 mM), pH 6] for 2 hrs using magnetic stirring. After centrifugation (10 000 rpm , 10 min), the supernatant was heated at 70 °C for 15 min using thermomixer and rapidly cooled on ice bath in order to inactivate β-amylase. Enzyme was re-centrifuged to remove the pellet and the supernatant thus was treated as crude enzyme and was used for ammonium sulphate precipitation. The purification procedure was carried out stepwise at 4 °C. In the first step, the solid ammonium sulphate was added slowly to the crude enzyme extract to achieve 0 - 20 % saturation with gentle stirring for 3 hrs. The pellet was collected by centrifugation (10 000 rpm, 10 min) and re-solubilized in buffer extraction to use it in the second step. The percentage saturation of ammonium sulphate was brought to 20 - 60 %. Protein pellet then was re-collected by centrifugation and dissolved in appropriate sample buffer depends on further analyses eitherisoelectric focusing (IEF) or polyacrylamide gel electrophoresis(PAGE). The aliquot was dialysed for 10 hrs in a cellulose-membrane tubing (D977, Sigma, Germany) and then applied to rotational vacuum concentrator (30 °C, 4.1 mbar, and 10 hrs) in order to concentrate the solution. The purified

enzyme extract before applied to gels was monitored for enzyme activity by DNS method as well as for protein content by Bradford procedure using bovine serum albumin in calibration curve (Bio-Rad protein assay protocol). The purified enzyme extract kept at -80 °C until analyzed.

Isoelectric focusing (IEF)

Sample was run on IEF gels in duplicate, one to be stained for protein detection and the other to be used for enzyme detection, also named zymogram. Stained proteins on gel and zymogram were compared to determine the isoelectric point (pI) of target proteins of α-amylase.

IEF was carried out in a Multiphor II apparatus (GE Healthcare, Munich, Germany). Commercially purchased FocusGel (pH 3 - 7, size 250 x 115 x 0.65 mm, No. 43328 Serva Electrophoresis, Germany), IEF sample buffer (No. 42357 Serva Electrophoresis, Germany) and IEF standards (pH 3-10, No.39212 Serva Electrophoresis, Germany) were used. The loading sample was 20 µl. The setting conditions were according to manufacturer's instructions: 30 min 500 v, 1 hr and 30 min 850 v and 30 min 1000 v. The current was 20 mA and the run was performed in a cold room at 4 °C. For protein detection, the gel was fixed and stained in room temperature with Coomassie G-250 as instruction manual for FocusGels (Serva Electrophoresis, Germany).

A calibration curve of pI was made by plotting the migration distances from cathode of proteins in a set of standards versus their known pI. Then the pI of particular proteins bands were estimated based upon their positions relative to those of IEF standards.

For enzyme activity staining, the gel was immersed in soluble starch solution (1 %) for 30 min with gentle shaking. After that, the Lugol's solution was added. The zones of α-amylase activity appeared as clear bands against dark background.

Polyacrylamide gel electrophoresis (PAGE)

Two sets of experiments were performed in each sample: 1. sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to be stained for protein detection. 2. native-PAGE (nondenaturing) to be used for zymograms. Stained proteins and zymogram were compared to determine the molecular weight ofenzyme's protein.

SDS-PAGE was carried out using mini Protean II Cell (Bio-Rad Laboratories GmbH, Munich, Germany). The total monomer concentrations of polyacrylamide gel were 12.5 and 5 % for resolving gel and the stacking gel, respectively. The sample buffer (pH 6.8) had the following composition: 4 % SDS, 12 % glycerol, 0.61 % Tris-HCl, 5 % mercaptoethanol and 0.004 % bromophenol blue. The loading sample was 20 µl. The setting conditions were: 5 min 100 v and 40 min 200 v. The current was 10 mA and the run was performed at room temperature. The molecular weight standards from 10 to 250 kDa were used (Precision Plus Protein Stanadrds No. 161-0373, Bio-Rad, Germany). After the time was elapsed, the gel was fixed and stained with Coomassie brilliant blue R-250. The molecular weight of proteins bands were estimated based upon their positions relative to those of the molecular weight standards following the Bio-Rad protocol.

To visualize the activity of α-amylase, native PAGE was applied in the same manner of SDS-PAGE run but without using SDS and mercaptoethanol in the buffer sample (Walker 2002). The gel then was immersed in lugol's solution and soluble starch as substrate. The zones of α-amylase activity appeared as clear bands against dark background.

3.6. Statistics

The calculation of means, standard deviation, maximum and minimum values was performed with Microsoft Excel 2003 (Microsoft, USA). The data were analysed with SigmaStat (statistical software version 2.0, SPSS Inc., USA). The Kolmogorov-Smirnov's test was used to check the normal distribution of all data except protein data in which Shapiro-Wilk test was used. The means separation was performed using Student *t-test* in wheat data and one way analysis of variance (ANOVA) in maize data. Significant differences ($p \leq 0.05$) were represented by different letters. Correlation procedures between normally distributed parameters under our study were performed using Person correlation coefficients. The measure of significances were indicated with ***, ** and * which means $p \leq 0.001$, *0.01* and *0.05*, respectively, whereas ns showed no significant relationships. Kinetic parameters were calculated by SigmaPlot 10.0 (Systat software GmbH, Erkrath, Germany). The Michaelis-Menten curve and graphics were prepared by Sigmaplot 10.0 as well.

4. RESULTS

4.1. Influence of cultivars and environments on α-amylase activity and chemical composition throughout grain development

Experiment 1

The first experiment was carried out on two wheat cultivars, cv. Cubus and cv. Amaretto, planted on two field locations, Gladebeck and Torland, in 2010. The effect of two cultivars and locations on the activity of α-amylase at different level of maturity is presented in Figure 5.

Alpha-amylase had the highest activity in milk-ripe stage, while the lowest enzyme activity was detected in mature-harvest stage. Regarding to cultivars, cv. Amaretto had higher enzyme activity than cv. Cubus in both locations during grain growth and maturation with one exception in dough-ripe grains collected from Torland. Furthermore, differences in α-amylase activity between cultivars regardless of location was significantly different ($p \leq 0.05$) in mature-harvest stage. Regarding to locations, milk-ripe grains from Gladebeck in both cultivars displayed higher activity of α-amylase than those collected from Torland. In contrast, higher enzyme activity was detected in grains collected from Torland in dough-ripe and mature-harvest stages for both cultivars. Furthermore, the influence of location irrespective of cultivars was significantly different ($p \leq 0.05$) in milk-ripe and dough-ripe grains.

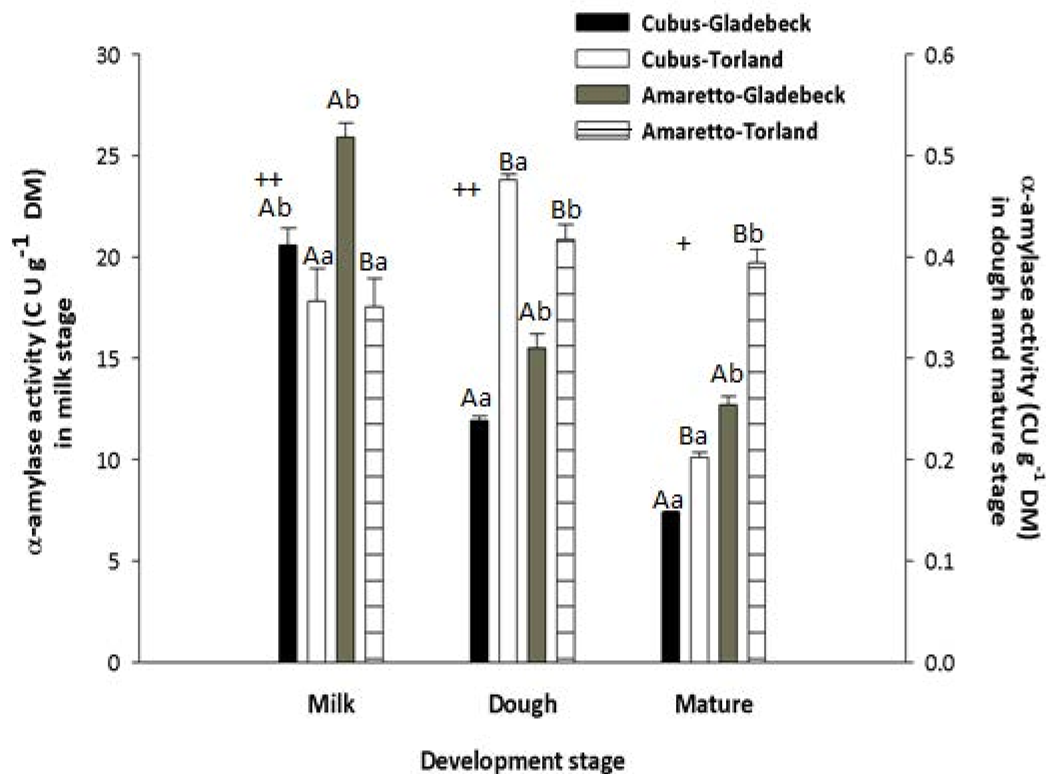

Figure 5: Activity of α-amylase in two wheat cultivars planted in two locations during three development stages

Data represents average ± standard deviation for triplicate measurements
a,b different letters indicate significant differences (*p ≤ 0.05*) among cultivars in the same location within a stage
A,B different letters indicate significant differences (*p ≤ 0.05*) among locations in the same cultivar within a stage
+ Indicate significant differences between cultivars, irrespective of locations by Kruskal-Wallis test, within a stage
++ Indicate significant differences between locations, irrespective of cultivars by Kruskal-Wallis test, within a stage

Changes in the chemical compositions, namely starch, protein and sugars content, during grain development period in both wheat cultivars differing in their planting locations are presented in Figure 6 and 7.

Generally, grains with differences in starch content showed compensatory changes in protein content. For instance, grains of cv. Cubus planted in Gladebeck had the highest starch content with 45.7 %DM (Figure 6a) and corresponded with 11.7 % DM protein content which was the lowest value (Figure 6b).

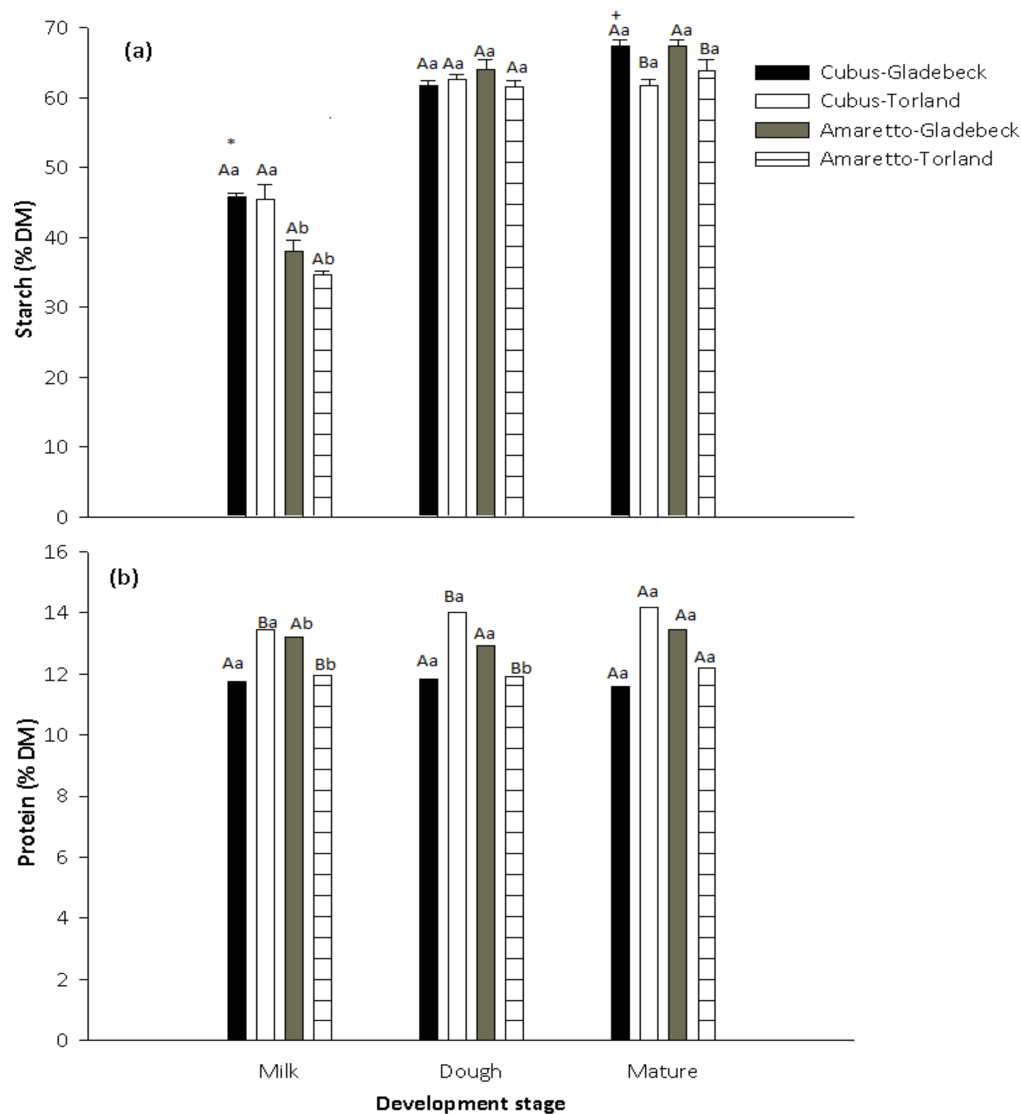

Figure 6: Starch (a) and protein (b) content in two wheat cultivars planted in two locations among three development stages

Data represents average ± standard deviation for triplicate measurements except protein with duplicate
a, b different letters indicate significant differences among cultivars (*p ≤ 0.05)* in the same location within a stage
A, B different letters indicate significant differences among locations (*p ≤ 0.05*) in the same cultivar within a stage
*indicates significant differences between cultivars (*p ≤ 0.05*) within single parameters within a stage, irrespective of loactions
+ indicates significant differences between locations (*p ≤ 0.05*) within single parameters within a stage, irrespective of cultivars

The accumulation of starch increased towards maturity. Milk-ripe wheat grains contained 40 %DM starch on average, while mature-harvest grains had 65.14 %DM starch (Figure 6a). Regarding to cultivars, starch content of grains in cv. Cubus was significantly higher than those in cv. Amaretto either in both locations or irrespective of locations. However, the influence of location on starch content was obvious in mature-harvest stage. Cv. Cubus harvested in Gladebeck contained more starch (67.39 %DM) than those in Torland (61.84 %DM). Similarly, cv. Amaretto had higher starch content in grains harvested in

Gladebeck (67.39 %DM) than those in Torland (63.95 %DM). Furthermore, mature grains harvested from Gladebeck contained significantly more starch compared with those harvested in Torland regardless of cultivars (Figure 6a).

Protein content was significantly influenced by the locations and cultivars in milk-ripe and dough-ripe stages (Figure 6b). For instance, milk-ripe grains of cv. Amaretto planted in Gladebeck accumulated more protein (13.9 %DM) compared to either the same cultivar planted in Torland (11.9 %DM) or cv. Cubus planted in the same location (11.7 %DM). On the other hand, grains from cv. Cubus-Torland in milk-ripe stage contained more protein (13.4 %DM) compared to those planted in Gladebeck (11.7 %DM) or cv. Amaretto-Torland (11.9 %DM). The similar interaction pattern between two locations and cultivars was also observed in protein content of dough-ripe grains.

The results of individual sugar monosaccharides, fructose and glucose, or disaccharides, sucrose and maltose, as well as the reducing sugars and total sugars are presented in Figure 7 and Table 7. The individual reducing sugar and its total sum values showed the maximum concentration at milk-ripe stage and then declined continuously towards maturity.

In order to follow the change in content of each of the four sugars, a collective data was obtained from particular stacked bars and a row for each sugar in Figure 7 and Table 7, respectively across cultivars and locations at different stages of maturity. Fructose declined sharply from a range within 12.2 - 34.3 %DM in milk-ripe grains of different cultivars to 0.4 - 0.91 %DM as wheat matured. Glucose followed a pattern similar to that fructose. Maltose concentration of grains decreased from a range within 8.4-21.6 %DM in milk-ripe stage to 5.1-7.5 %DM at dough-ripe stage, followed with an increase until the harvest time. On the other hand, the lowest concentration of the sucrose content was at milk-ripe stage with a range 0.74 - 1.3 %DM. Thereafter, sucrose content increased remarkably in dough-ripe grains with a range 2.6 - 4.6 %DM, followed with a slight increase in mature-harvest grains ,on a range 3.9 - 4.7 %DM. Fructose was predominant sugar in milk-ripe grains, whereas maltose was predominant in the dough-ripe and mature-harvest stages.

Regarding to cultivars, both cultivars did considerably differ in all sugars under investigation in the most development stages across both locations. Grains of cv. Amaretto in Torland accumulated more monosaccharides and less disaccharides compared with cv.

Cubus in the same location during grain development and maturation. Additionally, cv. Amaretto in Gladebeck accumulated more sugars i.e. mono-and di-saccharides compared to those of cv. Cubus in the same location at the most stages of ripening. However, the influence of cultivars irrespective of locations was statically detected at $p \leq 0.05$ in reducing sugars and total sugars at early stage as well as in both monosaccharides in dough-ripe stage. None of both disaccharides across three stages were influenced by cultivars irrespective of locations (Table 8).

Regarding to locations, cv. Amaretto collected from Gladebeck contained significantly more disaccharides ($p \leq 0.05$) than those in Torland at three different development stages. Moreover, more monosaccharides were accumulated in cv. Cubus in Gladebeck compared to those collected from Torland during grain development and maturation.

What is interesting that out of the four sugars assessed in this study, only maltose content varied significantly among locations irrespective of cultivars across three development stages. Additionally, the location of cultivation influenced the reducing sugars and total sugars in dough-ripe and mature-harvest stages (Table 7).

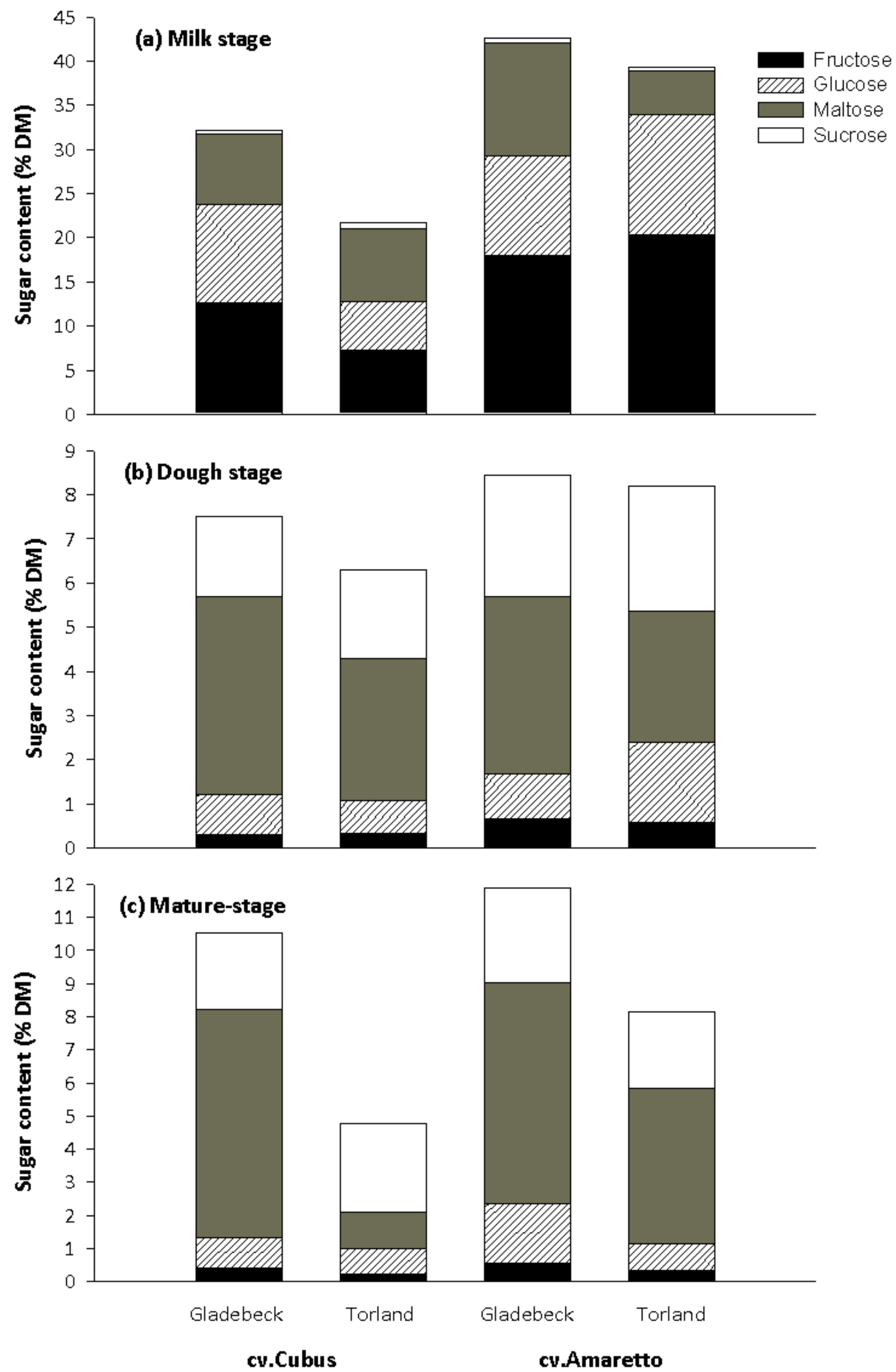

Figure 7: Sugar content in two wheat cultivars planted in two locations at (a) milk, (b) dough, and (c) mature stage.

Stacked bars represent average value for each sugar from triplicate measurements
Statistical evaluations among cultivars and locations within a stage are shown in Table 7

Table 7: Sugars content in two wheat cultivars planted in two locations among different development stages

Development stage	Sugar (%DM)	cv. Cubus		cv. Amaretto	
		Gladebeck	Torland	Gladebeck	Torland
Milk	Reducing sugars				
	Fructose**	12.60±2.30 Aa	7.20±0.40 Ba	17.90±2.00 Ab	20.30±0.40 Ab
	Glucose*	11.06±1.20 Aa	5.48±0.70 Ba	11.24±4.40 Aa	13.56±0.50 Ab
	Maltose**	7.97±0.40 Aa	8.22±0.49 Aa	12.84±1.01 Ab	4.99±1.07 Bb
	Sum*	31.60 ± 2.30 Aa	20.96±1.50 Ba	42.04±6.30 Aa	38.90±0.65 Ab
	Non-reducing sugar				
	Sucrose	0.52±0.07 Aa	0.75±0.05 Ba	0.57±0.08 Aa	0.44±0.04 Bb
	Total sum*	20.90±1.50 Aa	21.70±1.60 Ba	42.60±6.40 Aa	39.34±0.60 Ab
Dough	Reducing sugars				
	Fructose*	0.31±0.08 Aa	0.35±0.03 Aa	0.67±0.06 Ab	0.59±0.09 Ab
	Glucose*	0.91±0.20 Aa	0.72±0.09 Aa	1.00±0.27 Aa	1.82±0.47 Ab
	Maltose**	4.48±0.25 Aa	3.23±0.26 Ba	4.03±0.19 Aa	2.95±0.07 Ba
	Sum**	5.70±0.34 Aa	4.29± 0.35 Ba	5.70±0.15 Aa	5.35±0.35 Aa
	Non-reducing sugar				
	Sucrose	1.80±0.22 Aa	2.01±0.11 Aa	2.75±0.11 Ab	1.53±0.26 Bb
	Total sum**	7.50±0.50 Aa	6.30±0.44 Ba	8.40±0.15 Ab	6.80±0.18 Ba
Mature	Reducing sugras				
	Fructose**	0.40±0.03 Aa	0.24±0.03 Ba	0.54±0.20 Aa	0.35±0.04 Ab
	Glucose+	0.95±0.10 Aa	0.77±0.17 Aa	1.83±1.05 Aa	0.81±0.09 Aa
	Maltose+	6.87±0.26 Aa	1.07±0.03 Ba	6.65±0.57 Aa	4.66±0.78 Bb
	Sum**	8.21±0.35 Aa	2.08±0.20 Ba	9.01±1.60 Aa	5.80±1.70 Bb
	Non-reducing sugars				
	Sucrose	2.32±0.25 Aa	2.68±0.10 Aa	2.85±0.21 Ab	2.33±0.08 Bb
	Total sum**	10.54±0.50 Aa	4.76±0.23 Ba	11.80±1.80 Aa	8.15±0.70 Bb

Data represents average ± standard deviation for triplicate measurements
a,b different letters indicate significant differences among cultivars ($p \le 0.05$) in the same location within stage
A,B different upper case letters indicate significant differences among locations ($p \le 0.05$) in the same cultivar within a stage
* indicates significant differences between cultivars ($p \le 0.05$) within a single sugar within a stage, irrespective of locations
** indicates significant differences between locations ($p \le 0.05$) within a single sugar within a stage, irrespective of cultivars
+ indicates significant differences between locations within single sugar within stage ($p \le 0.05$) according to Kruskal-Wallis test, irrespective of cultivars

Correlation between α-amylase activity and chemical composition of wheat grains during development stages is displayed in three separate tables, based on the cultivars factor (Table 8), locations factor (Table 9) and both cultivars and locations (Table 10).

The activity of α-amylase was significantly correlated with protein and maltose content in all stage within each cultivar. Furthermore, there was a significant negative correlation between α-amylase activity and starch, reducing sugars and total sugars in mature-harvest stage in both cultivars. On the other hand, only enzyme activity in cv. Amaretto showed a high significant correlation with the sucrose content in three development stages (Table 8).

Table 8: Relation between α-amylase activity (CU g^{-1} DM) and chemical components (%DM) of wheat grains as Person Correlation Coefficient in different cultivars during three development stages (n=6)

Development Stage	Location factor	Chemical composition					
		Starch	Protein	Maltose	Reducing sugars[1]	Sucrose	Total sugars[2]
Milk	Gladebeck	-0.90*	0.99 **	0.92 *	0.79 ns	0.46 ns	0.79 ns
	Torland	0.24 ns	0.45 ns	0.29 ns	-0.06 ns	0.27 ns	-0.06 ns
Dough	Gladebeck	0.84 *	0.98 *	-0.75 ns	0.12 ns	0.95 **	0.86 *
	Torland	0.69 ns	0.92 ns	0.76 ns	-0.89 *	0.93 **	-0.67 ns
Mature	Gladebeck	-0.10 ns	0.97 *	0.83 *	0.43 ns	0.83 **	0.55 *
	Torland	0.70 ns	-0.97 *	0.94 **	-0.94 **	-0.94 **	-0.93 **

n, number of observations
[1] Sum of glucose, fructose and maltose content. [2]Sum of glucose, fructose, maltose and sucrose content
ns, not significantly different at $p \leq 0.05$
***, ** and * significance at $p \leq 0.001, 0.01$ *and* 0.05, respectively

In dough-ripe and mature-harvest grains harvested from both locations, α-amylase activity and sucrose showed a significant correlation. However, the correlation between enzyme activity and maltose and total sugars was only in mature-harvest grains from both locations significant (Table 9).

Table 9: Relation between α-amylase activity (CU g^{-1} DM) and chemical components (%DM) of wheat grains as Person Correlation Coefficient in different location during three development stages,(n=6)

Development Stage	Location factor	Chemical composition					
		Starch	Protein	Maltose	Reducing sugars[1]	Sucrose	Total sugars[2]
Milk	Gladebeck	-0.90*	0.99 **	0.92 *	0.79 ns	0.46 ns	0.79 ns
	Torland	0.24 ns	0.45 ns	0.29 ns	-0.06 ns	0.27 ns	-0.06 ns
Dough	Gladebeck	0.84 *	0.98 *	-0.75 ns	0.12 ns	0.95 **	0.86 *
	Torland	0.69 ns	0.92 ns	0.76 ns	-0.89 *	0.93 **	-0.67 ns
Mature	Gladebeck	-0.10 ns	0.97 *	0.83 *	0.43 ns	0.83 **	0.55 *
	Torland	0.70 ns	-0.97 *	0.94 **	-0.94 **	-0.94 **	-0.93 **

n, number of observations
[1] Sum of glucose, fructose and maltose content. [2]Sum of glucose, fructose, maltose and sucrose content
ns, not significantly different at $p \le 0.05$
***, ** and * significance at $p \le 0.001, 0.01$ *and* 0.05, respectively

In milk-ripe stage, α-amylase activity correlated positively with maltose, whereas a negative significant relationship between enzyme activity and maltose, reducing sugars and total sugars was detected in in dough grains (Table 10).

Table 10: Relation between α-amylase activity (CU g^{-1} DM) and chemical components (%DM) of wheat grains as Person Correlation Coefficient during three development stages in different cultivars and locations, (n=12)

Development Stage	Chemical composition					
	Starch	Protein	Maltose	Reducing sugars[1]	Sucrose	Total sugars[2]
Milk	-0.07 ns	0.40 ns	0.80 ***	0.54 ns	-0.02 ns	0.54 ns
Dough	0.01 ns	0.61 ns	-0.80 ***	-0.76 **	-0.19 ns	-0.68 *
Mature	-0.23 ns	-0.08 ns	-0.14 ns	-0.05 ns	-0.14 ns	-0.06 ns

n, number of observations
[1] Sum of glucose, fructose and maltose content. [2]Sum of glucose, fructose, maltose and sucrose content
ns, not significantly different at $p \le 0.05$
***, ** and * significance at $p \le 0.001, 0.01$ *and* 0.05, respectively

Experiment 2

The second experiment was carried out on seven maize cultivars (Benecia, EsBomabastic, Franki, Justina, Lukas, Saludo and PR38F58) planted in two years, 2010 at the mature stage and 2011 during the period of kernel development in the same field experiment.

The α-amylase showed the maximum activity at milk-ripe stage in all investigated maize cultivars with the range 3.17 - 6.61 %DM, and then enzyme activity declined continuously towards maturity until reached the minimum activity in range 0.09 - 0.45 %DM (Table 11).

The influence of cultivar on enzyme activity amongst maturation stages was detected in 2011. For instance, milk-ripe grains from cvs. Lukas, Justina and PR39F58 had high enzyme activity, which differed significantly (*p ≤ 0.05*) from other cultivars, while α-amylase from mature-harvest grains from cv. EsBombastic had the highest activity (*p ≤ 0.05*) across all samples. In 2010, cv. Lukas had the lowest activity of α-amylase (0.09 CU g^{-1} DM), which was significantly differentin enzyme activity from three cultivars, cv. Benecia, EsBombastic and PR38F58.

Table 11: Activity of α-amylase (CU g^{-1} DM) in grains of seven maize cultivars during three development stages in 2011 and in mature stage in 2010

Cultivar	Development stage			
	Milk	Dough	Mature - 2011	Mature - 2010 *
Benecia	3.17±0.10 b	0.32±0.03 d	0.20±0.01 b A	0.25±0.20 cd B
EsBombastic	3.84±0.70 bd	0.32±0.04 d	0.45±0.09 a A	0.21±0.06 c B
Franki	3.22±0.30 b	0.25±0.03 bcd	0.25±0.03 bc A	0.18±0.01 ac B
Justina	5.63±0.40 ad	0.35±0.03 ad	0.35±0.02 ac A	0.16±0.01 bc B
Lukas	6.61±0.50 ac	0.31±0.02 acd	0.24±0.00 bc A	0.09±0.01 b B
Saludo	3.61±0.80 b	0.28±0.02 cd	0.27±0.00 bc A	0.16±0.00 bd B
PR38F58	5.42±0.90 cd	0.23±0.02 bc	0.28±0.04 bc A	0.27±0.06 ac A

Data represents average ± standard deviation
a,b,c,d,e,f different letters indicate significant differences (*p ≤ 0.05*) among cultivars within a stage
A, B different letters indicate significant differences (*p ≤ 0.05*) among years in the same cultivar
*Indicates significant differences (*p ≤ 0.05*) among years, irrespective of cultivars

Regarding to years, α-amylase activity in mature-harvest maize grains cultivated in 2011 was significantly (*p ≤ 0.05*) higher than those in 2010, irrespective of cultivars. Additionally, there was a significant difference in enzyme activity across years within a cultivar of all studied maize grains except cv.PR39F58 (Table 11).

Changes of maize kernel constituents during development stages in 2011 were presented in Table 12 and Table 21 in appendix. Comparison of maize kernel composition between the two years, 2010 and 2011, was shown in Figure 8, 9 and Table 13, 14.

Again, the accumulation of starch content in maize kernels was accompanied with a relative decrease in protein content towards maturity. Starch and protein content were 56.2 and 11.5 %DM on average in milk-ripe maize kernels, while they represented 69 and 9.3 %DM on average in mature-harvest kernels, respectively (Table 12). Furthermore, similar compensatory pattern between starch and protein content was observed between cultivars in the same development stage. For example, the highest starch content, which was

detected in cv. PR39F58 at dough-ripe and mature-harvest stages, was associated with the lowest protein content in the same maize cultivar (Table 12).

Table 12: Chemical composition (%DM) of maize grains among three development stages planted in 2011

Chemical composition	Development stage	Average [(a)]	Maximum value		Cultivar	Minimum value		Cultivar
Starch	Milk	56.23±4.60	61.57	a	Benicia	47.42	b	Franki
	Dough	64.93±2.10	67.83	a	PR39F58	61.83	b	Justina
	Mature	69.04±1.70	71.30	a	PR39F58	67.00	b	Lukas
Protein	Milk	11.58±0.70	13.39	a	Franki	9.92	a	Benicia
	Dough	9.68±0.70	10.60	a	Franki	8.82	a	PR39F58
	Mature	9.34±0.80	10.20	a	Lukas	7.79	a	PR39F58
Fructose	Milk	13.71±3.40	16.75	a	Franki	9.77	a	Benicia
	Dough	8.54±2.70	11.47	a	Justina	4.89	b	Saludo
	Mature	1.76±1.10	3.37	a	Benicia	0.74	b	Franki
Glucose	Milk	15.70±4.10	20.91	a	Franki	11.23	a	Benicia
	Dough	9.54±3.00	12.63	a	Justina	5.42	b	Saludo
	Mature	1.73±0.89	3.10	a	Benicia	0.78	b	Franki
Maltose	Milk	0.07±0.00	0.18	a	Franki	0.02	b	Benicia
	Dough	0.12±0.00	0.19	a	EsBombtic	0.01	b	Benicia
	Mature	1.25±0.70	1.87	a	Justina	0.52	b	Benicia
Reducing sugars[1]	Milk	29.40±7.50	37.83	a	Franki	21.01	a	Benecia
	Dough	18.20±5.80	24.27	a	Justina	10.42	b	Saluda
	Mature	4.70±1.90	6.98	a	Benecia	2.05	b	Franki
Sucrose	Milk	0.75±0.40	4.44	a	PR39F58	0.99	b	Saludo
	Dough	0.88±0.60	2.17	a	PR39F58	0.30	b	Benicia
	Mature	0.02±0.00	0.07	a	Saludo	0.00	a	Benicia, R39F58, EsBombastic
Total sugars[2]	Milk	30.26±7.60	38.37	a	Franki	22.09	a	Benecia
	Dough	19.09±5.90	25.05	a	Justina	10.96	b	Saluda
	Mature	4.78±1.80	6.98	a	Benecia	2.08	b	Franki

[(a)]Average ± standard deviation within a single parameter within one development stage, irrespective of cultivars
a,b different letters indicate significant differences among cultivars within each stage ($p \leq 0.05$)
[1] Sum of glucose, fructose and maltose content. [2]Sum of glucose, fructose, maltose and sucrose content

Regarding to cultivars, maize cultivar with the maximum content of starch was significantly different from the cultivar with the minimum value across three growth phases (Table 12). In addition, Table 21 in appendix presents the influence of all studied cultivars on starch content at each development stage. Results in Figure 8a and Table 13 show that the cultivars harvested in 2011 contained more starch compared to those in 2010. Cultivars within a year and among years was significantly ($p \leq 0.05$) different in starch content as shown in Figure 8a. In 2010, cv. EsBomabstic differed significantly in starch content from cvs. Benecia and Justina, while in 2011 cvs. Saludo and PR39F58 had significantly higher starch content than cvs.Benecia, Franki and Lukas. Four cultivars out of seven were shown

significant differences ($p \leq 0.05$) in starch content across two years within a cultivar. They were EsBomabstic, Franki, Saludo and PR39F58.

No effect of cultivars on protein content was detected during development stages (Table 12 in context and Table 21 in appendix). There were no significant differences in protein content among years either irrespective of cultivars or within a cultivar (Figure 8b).

Table 13: Chemical composition (%DM) of mature maize grains in two years, 2010 and 2011, irrespective of cultivars

Year		Starch	Protein	Fructose	Glucose	Maltose	Reducing sugars[1]	Sucrose	Total sugars[2]
2010	Mean	66.00	9.32	1.65	2.83	1.27	5.75	8.54	14.29
	Median	65.50	9.20	1.46	2.52	1.07	5.18	8.85	14.47
2011	Mean	69.00	9.34	5.64	5.56	4.01	15.20	0.09	15.29
	Median	68.70	9.52	4.65	6.16	4.67	16.50	0.00	16.71
P		***	ns	***	***	***	***	***	ns

Mean and median value of calculated for all maize cultivars within each parameter in each year. Data represents in Figure 6 was used for starch and protein content and in data in Table 12 is ued for sugars content.
[1] Sum of glucose, fructose and maltose content. [2]Sum of glucose, fructose, maltose and sucrose content
P significant differences between years within a single parameter (***= $p \leq 0.001$) by Kruskal-Wallis test, irrespective of cultivars, ns= not significant.

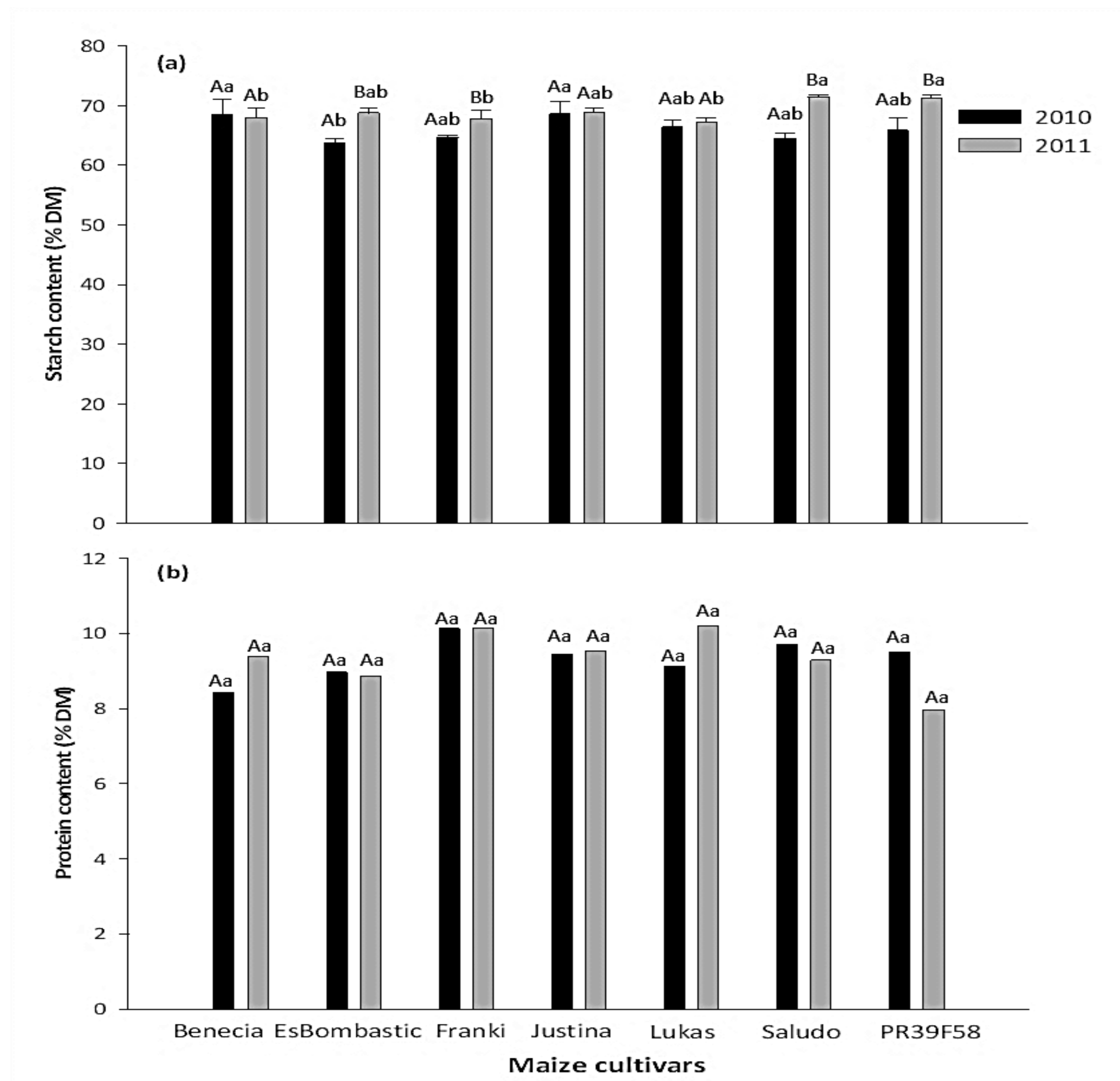

Figure 8: Starch (a) and protein (b) content in seven maize cultivars among two years, 2010 and 2011, respectively

Data represents average ± standard deviation for triplicate measurements except protein with duplicate
a,b different letters indicate significant differences among cultivars ($p \leq 0.05$) within a year
A, B different letters indicate significant differences among years ($p \leq 0.05$) within a cultivar

The kernels of all maize cultivars in 2011 showed a distinct decrease in both monosaccharides as well as sucrose content during the grain development and maturation period. On the other hand, the maltose content increased until reached its maximal level in mature-harvest stage (Table 14). The sugars content either individually or totally differed significantly ($p \leq 0.05$) among cultivars at any stage of grain maturity as is shown in Table 12 in context and Table 21 in appendix, however, one exception was revealed at milk-ripe stage for monosaccharides, reducing sugars and total sugars.

Maize cultivars harvested in 2011 accumulated more reducing sugars i.e. fructose, glucose and maltose than those in 2010 , whereas sucrose appeared as a trace with 0.09 %DM in maize kernels planted in 2011 compared to those in 2010 with considerable concentration of sucrose with 8.54 % DM (Figure 9 and Table 13 and 14).

Irrespective of cultivars, significant differences ($p \leq 0.05$) between years were revealed in each individual sugar as well as reducing sugars. The latter was not detected on the total sugars of maize kernels among two years (Table 13). Additionally, the influence of years within a cultivar was significantly different in all investigated cultivars for fructose, disaccharides and reducing sugars, while three out of seven cultivars, cvs. Benecia, EsBombastic and Lukas, varied in glucose and total sugars within each mentioned cultivar across years (Figure 9 and Table 14).

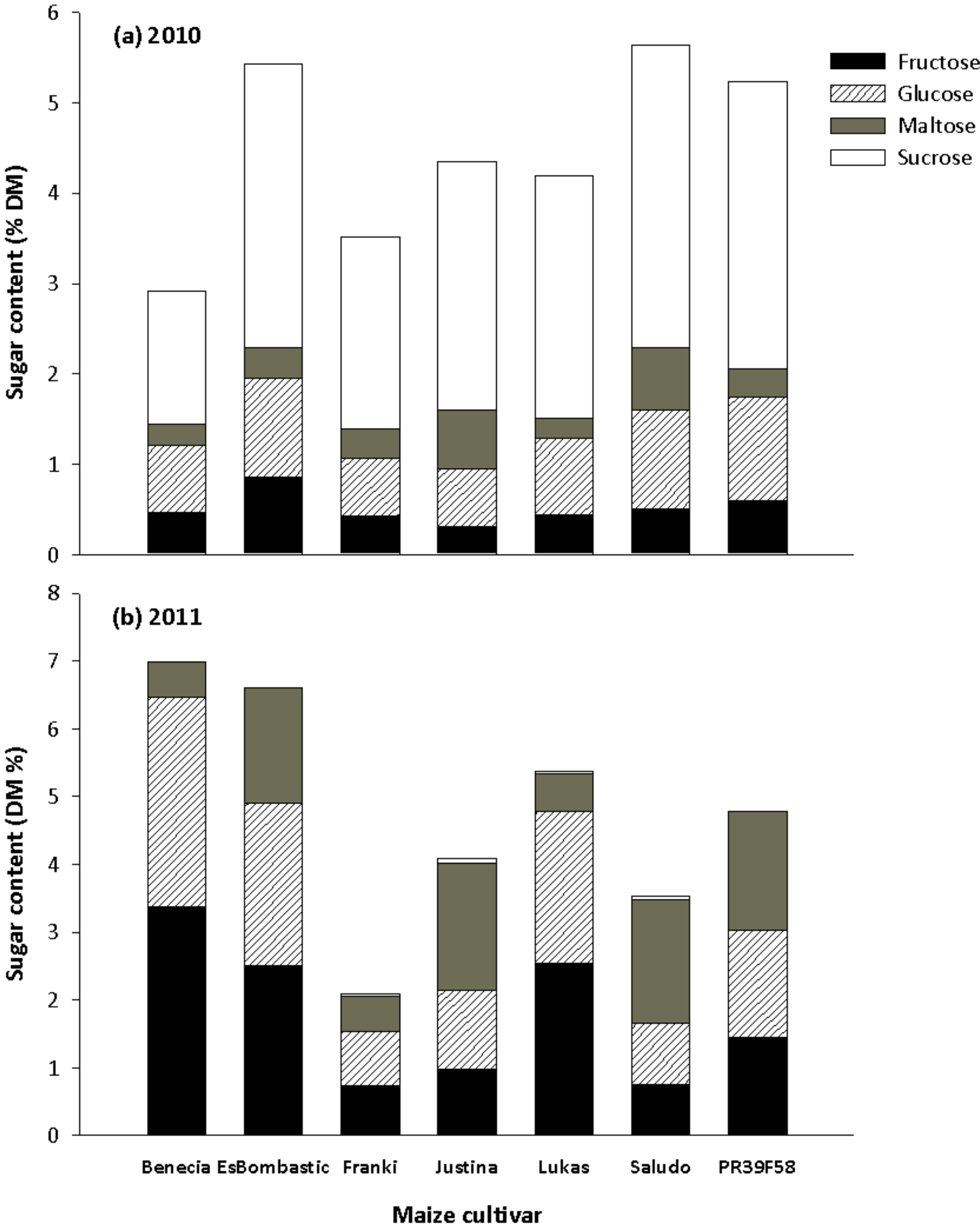

Figure 9: Sugar content in seven maize cultivars in (a) 2010 and (b) 2011

Stacked bars represent average value for each sugar from triplicate measurements
Statistical evaluations among cultivars and locations within a stage are shown in Table 14

Table 14: Sugars content (%DM) of mature grains in seven maize cultivars among two years, 2010 and 2011, respectively

Year	Sugar content	Benicia	EsBombastc	Cultivar Franki	Justina	Lukas	Saludo	PR38F58
2010	Reducing sugar							
	Fructose	0.47±0.00 Abd	0.86±0.00 Aa	0.42±0.00 Abc	0.31±0.00 Ac	0.44±0.00 Abc	2.54±0.00 Abd	0.60±0.00 Ad
	Glucose	0.47±0.00 Aabc	1.09±0.00 Acd	0.65±0.10 Ab	0.64±0.00 Abc	0.84±0.00 Aab	2.25±0.00 Aad	1.14±0.10 Aac
	Maltose	0.24±0.00 Ab	0.34±0.00 Ab	0.32±0.00 Ab	0.65±0.00 Aa	0.23±0.00 Ab	0.56±0.00 Aa	0.32±0.00 Ab
	Sum	1.40±0.10 Ab	2.20±0.00 Ac	1.39±0.20 Aabc	1.59±0.00 Aabc	1.51±0.00 Aabc	5.34±0.10 Aac	2.06 ±0.30 Aa
	Non reducing sugar Sucrose	1.47±0.30 A b	3.14±0.10 A a	2.12±0.40 A b	2.75±0.10A ab	2.68±0.50 A a	0.02±0.00 A	3.18±0.40 A ab
	Total sum	2.91±0.30 Ac	5.43±0.10 A ab	3.51±0.60 A bc	4.35±0.20A abc	4.19±0.20 A abc	5.36±0.15 A a	5.23±0.70 A abc
2011	Reducing sugar							
	Fructose	3.37±0.80 Ba	2.51±0.20 Ba	0.74±0.00 Bbc	0.99±0.20 Bbc	2.54±0.00 Ba	0.76±0.00 Bc	1.44±0.60 Abc
	Glucose	3.10±0.70 Bac	2.40±0.30 Bc	0.78±0.00 Ab	1.16±0.20 Abd	2.25±0.00 Bcd	0.89±0.00 Ab	1.58±0.60 Abc
	Maltose	0.52±0.20 Ab	1.7±0.20 Bc	0.52±0.00 Bb	1.87±0.50 Ba	0.56±0.00 Bbc	1.82±0.20 Bac	1.7±0.80 Ba
	Sum	6.98±1.70 Ba	6.60±0.60 Bab	2.05±0.20 Bb	4.01±1.00 Bab	5.34±0.10 Bab	3.47±0.20 Bab	4.78 ±20 Aab
	Non reducing sugar Sucrose	0.00±0.00 Ba	0.0±0.00 Ba	0.04±0.00 Ba	0.06±0.00 Ba	0.02±0.00 Ba	0.07±0.06 Ba	0.00±0.00 Ba
	Total sum	6.98±1.70 Ba	6.60±0.60 Bab	2.08±0.20 Ab	4.07±1.00 Aab	5.36±0.10 Bab	3.53±0.20 Aab	4.78±2.10 Aab

Data represents average ± standard deviation for triplicate measurements
a,b,c,d,e,fdifferent letters indicate significant differences among cultivars ($p \leq 0.05$) within a year
A,B different letters indicate significant differences among years ($p \leq 0.05$) within a cultivar

Correlation between α-amylase activity and chemical composition among seven maize cultivars in each kernel development stage is presented in Table 15. Correlation between α-amylase activity and chemical composition based on cultivar and year factor separately at mature stage is shown in Table 16.

A negative correlation between enzyme activity and sucrose content was detected in dough-ripe stage, while a positive correlation between enzyme activity and maltose content was detected in mature-harvest grains (Table 15).

Table 15: Relation between α-amylase activity (CU g^{-1} DM) and chemical composition in seven maize cultivars at three different development stages in 2011 as Person Correlation Coefficient, (n=21)

Development Stage	Chemical composition					
	Starch	Protein	Maltose	Reducing sugars[1]	Sucrose	Total sugars[2]
Milk	-0.13 ns	0.29 ns	-0.22 ns	0.25 ns	0.06 ns	0.25 ns
Dough	-0.66 ***	0.13 ns	0.02 ns	0.36 ns	-0.46 *	0.31 ns
Mature	0.07 ns	0.16 ns	0.51 *	0.15 ns	-0.03 ns	0.15 ns

n, number of observations. [1] Sum of glucose, fructose and maltose content. [2]Sum of glucose, fructose, maltose and sucrose content
ns, not significantly different at $p \leq 0.05$
***, ** and * significance at *p ≤ 0.001, 0.01 and 0.05*, respectively

The results showed that α-amylase activity was significantly correlated with the sucrose and maltose content in all maize cultivars except cv. PR38F58. Three out of seven maize cultivars showed a significant correlation between enzyme activity and starch, glucose, fructose and reducing sugars content. On the other hand, there was no correlation between enzyme activity and chemical composition of maize kernels within different years except maltose in maize grains harvested in 2011. Regardless of the cultivar and location effect, enzyme activity was significantly correlated to the contents of all analyzed carbohydrates (Table 16).

Table 16: Relation between α-amylase activity (CU g^{-1} DM) and chemical components (% DM) of mature maize kernels as Person Correlation Coefficient in seven maize cultivars and two years, 2010 and 2011

Factor	Factor level	Chemical composition					
		Starch	Protein	Maltose	Reducing sugars[1]	Sucrose	Total sugars[2]
Cultivar (n=6)	Benecia	0.34 *	-0.79 ns	-0.34 *	-0.69 ns	0.89 *	-0.59 ns
	EsBombastic	0.85 **	0.029 ns	0.91*	0.92 **	-0.86 **	0.95 **
	Franki	0.83 *	0.00 ns	0.65 ns	0.68 ns	-0.85 *	-0.80 ns
	Justina	0.06 ns	0.72 ns	0.94 ***	0.94 **	-0.97 ***	-0.06 ns
	Lukas	0.41 ns	0.83 ns	0.99 ***	0.94 ***	-0.98 **	0.79 ns
	Saludo	0.98 ***	-0.32 ns	0.94 ***	0.83 *	-0.93 ***	-0.77 ns
	PR38F58	0.44 ns	0.19 ns	-0.09 ns	-0.18 ns	-0.09 ns	-0.36 ns
Year (n=21)	2010	0.18 ns	-0.28 ns	-0.14 ns	0.20 ns	-0.08 ns	0.02 ns
	2011	0.07 ns	-0.16 ns	0.59 *	0.15 ns	-0.03 ns	0.15 ns
Cultivar * Year (n=42)		0.41***	0.02 ns	0.59 ***	0.49 ***	-0.53 ***	-0.15 ns

n, number of observations. [1] Sum of glucose, fructose and maltose content. [2]Sum of glucose, fructose, maltose and sucrose content.
ns, not significantly different at $p \leq 0.05$.
***, ** and * significance at *p ≤ 0.001, 0.01 and 0.05*, respectively

4.2. Characterization of α-amylase in wheat and maize

Experiment 1

For each wheat cultivar, the optimum temperature of α-amylase activity was 40 °C, while the optimum pH was 6 (Figure 10a and b). There was a remarkable loss of activity on the side of the optimum temperature and pH. For instance, an increase in temperature of 10 °C compared to the optimum resulted in 40 % decrease of activity. Around 30 - 70 % of hydrolysis activity was found to be in the temperature range of 30 - 90 °C, whereas 40 - 60 % of its activity was detected in alkaline condition. The cultivar factor had a major influence on the α-amylase activity in further temperatures beyond optimum as well as in acidic condition. The α-amylase enzyme originated from cv. Amaretto in both locations was more active than those in cv. Cubus with increasing temperatures as well as decreasing pH values. For example, at 70 °C, ~70 % of enzyme activity was measured in cv. Amaretto, whereas only 33 - 44 % of the activity was detected in cv. Cubus (Figure 10a). Furthermore, α-amylase originated from cv. Amaretto was more active (86 % of its maximal activity) than cv. Cubus (~ 15 % of its maximal activity) at pH 3. On the other hand, α-amylase from both cultivars exhibited a sharp decline in activity at pH 5 and 7 (Figure 10b).

The results in Figure 10c and 10d show that the favorable conditions to retain enzyme stability in both cultivars differing in their planting site were at 40 °C and pH 6. Only 10 - 40 % of residual activity of α-amylase was retained among various values of temperature and pH in both cultivars and locations. What is interesting that second peak of α-amylase residual activity was appeared at 70 °C in three cases (Figure 10c), α-amylase from cv. Amaretto planted in Gladebeck was the most heat stable enzyme because it retained about 60 % residual activity at 70 °C and 50 % at 80 °C (Figure 10c).

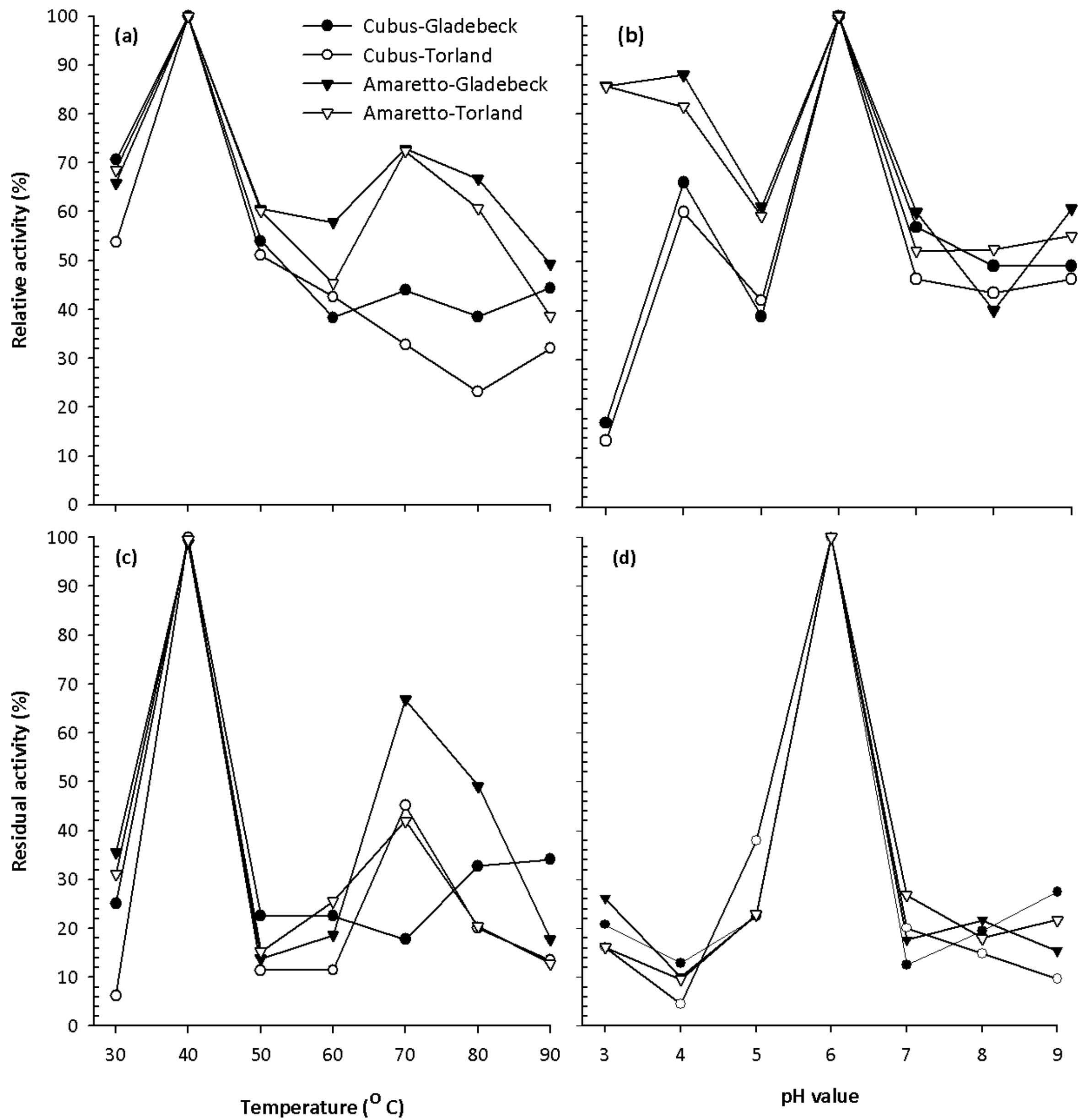

Figure 10: Biochemical characterization of α-amylase in two wheat cultivars planted in two locations, (a, b): Temperature and pH optimum (c, d): Temperature and pH stability

The maximum activity was taken as 100 %, (a, b): relative activity and (b, d): residual activity plotted against different temperature and pH values. Data represents average for triplicate measurements in each point

Enzyme kinetic data is presented in Michaelis-Menten curve in Figure 11, in which the initial rate of hydrolysis of soluble starch was plotted against starch concentration. The computer-estimated apparent K_m and V_{max}, from the nonlinear regression analysis, of the α-amylase activity is also shown in Figure 11. Their values were 2×10^{-3} g ml^{-1} and 0.8 µmol $min^{-1}ml^{-1}$, respectively. Enzyme activity from both cultivars in both locations had identical value in its kinetic properties.

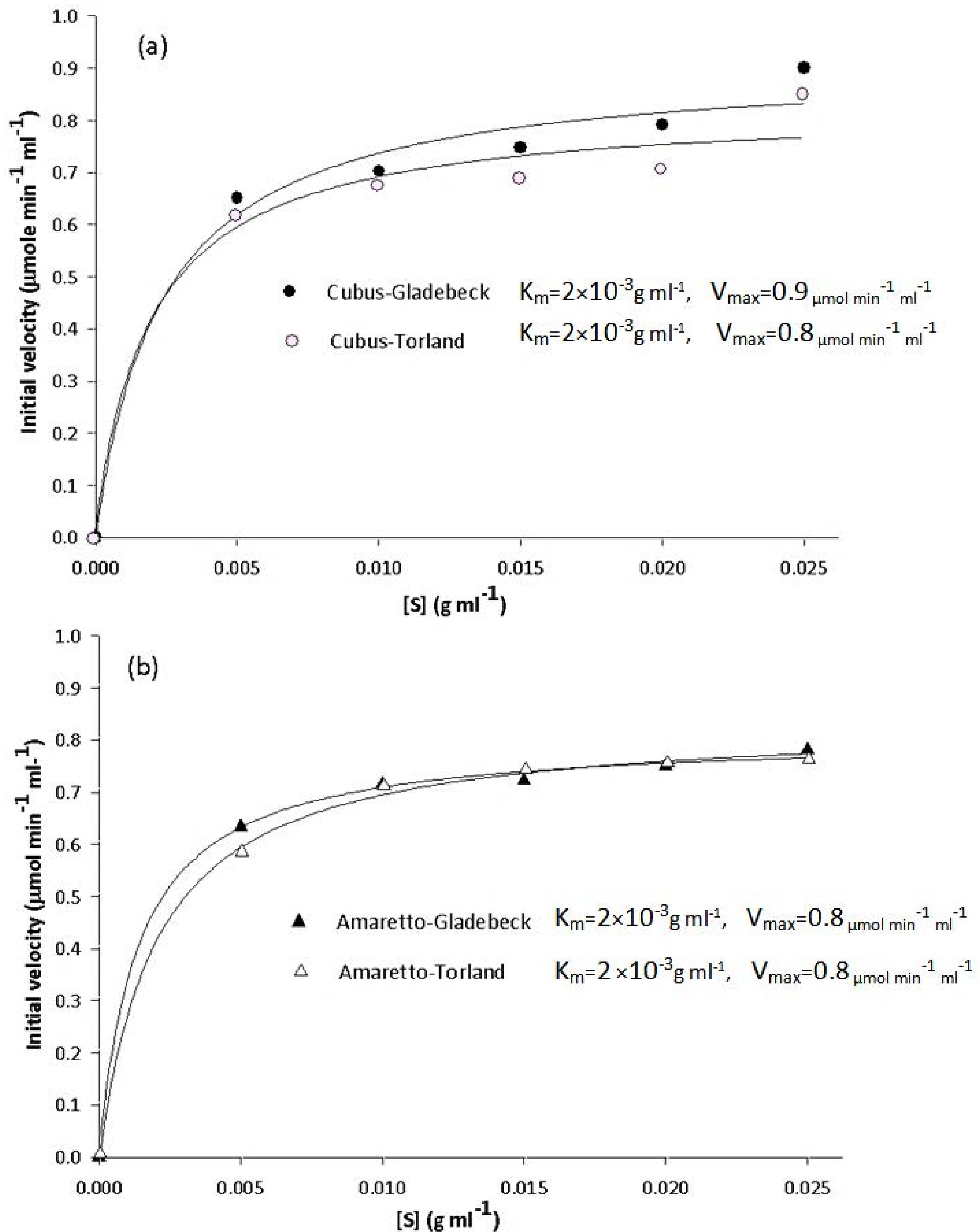

Figure 11: Michaelis-Menten curve for α-amylase activity in cv. Cubus (a) and cv. Amaretto (b) in two locations, Gladebeck and Torland

The results of milk-ripe grains from two wheat cultivars planted in Torland, with the highest enzyme activity, on IEF and electrophoresis experiments are presented in Figure 12 and 13. Three identical isoelectric components of α-amylase were obviously appeared across

two cultivars, their pIs were 4.4, 4.6 and 4.8 (Figure 12). The molecular weight value determined by SDS-PAGE was 37.2 kDa in both cultivars (Figure 13).

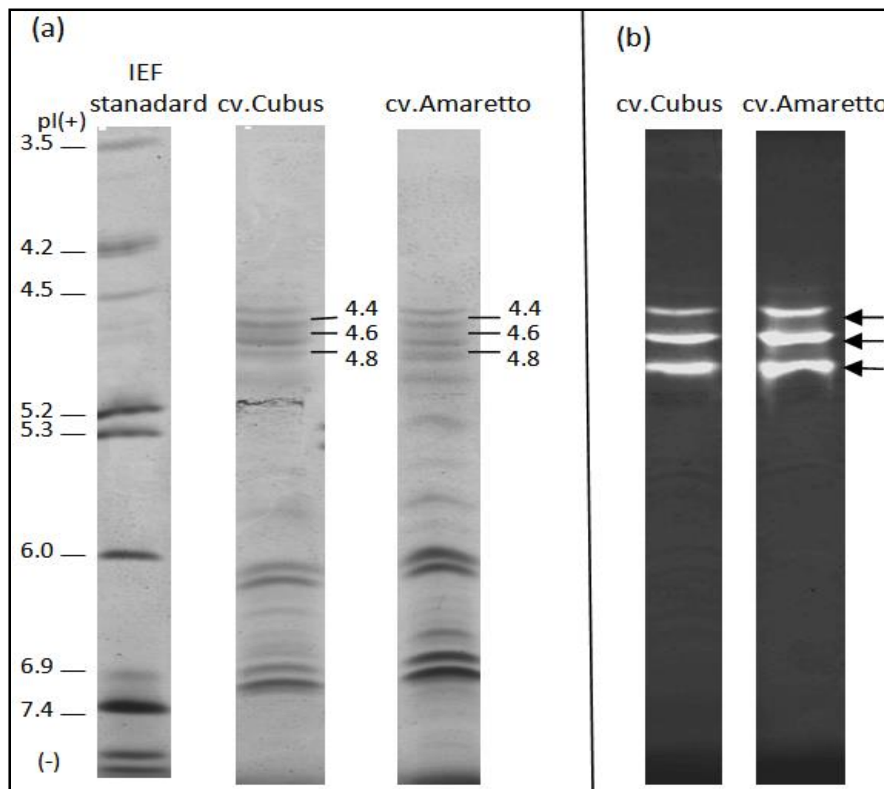

Figure 12: IEF (a) and activity staining (b) of α-amylase in two wheat cultivars at milk-ripe stage planted in Torland

The arrows indicate the zones of α-amylase activity appeared as clear bands against dark background. The amount of extract applied to the gel (Figure 12 and 13) corresponding to 1.16 - 1.61 µmol min^{-1} ml^{-1}. The protein concentration was 0.46 - 0.71 mg ml^{-1}

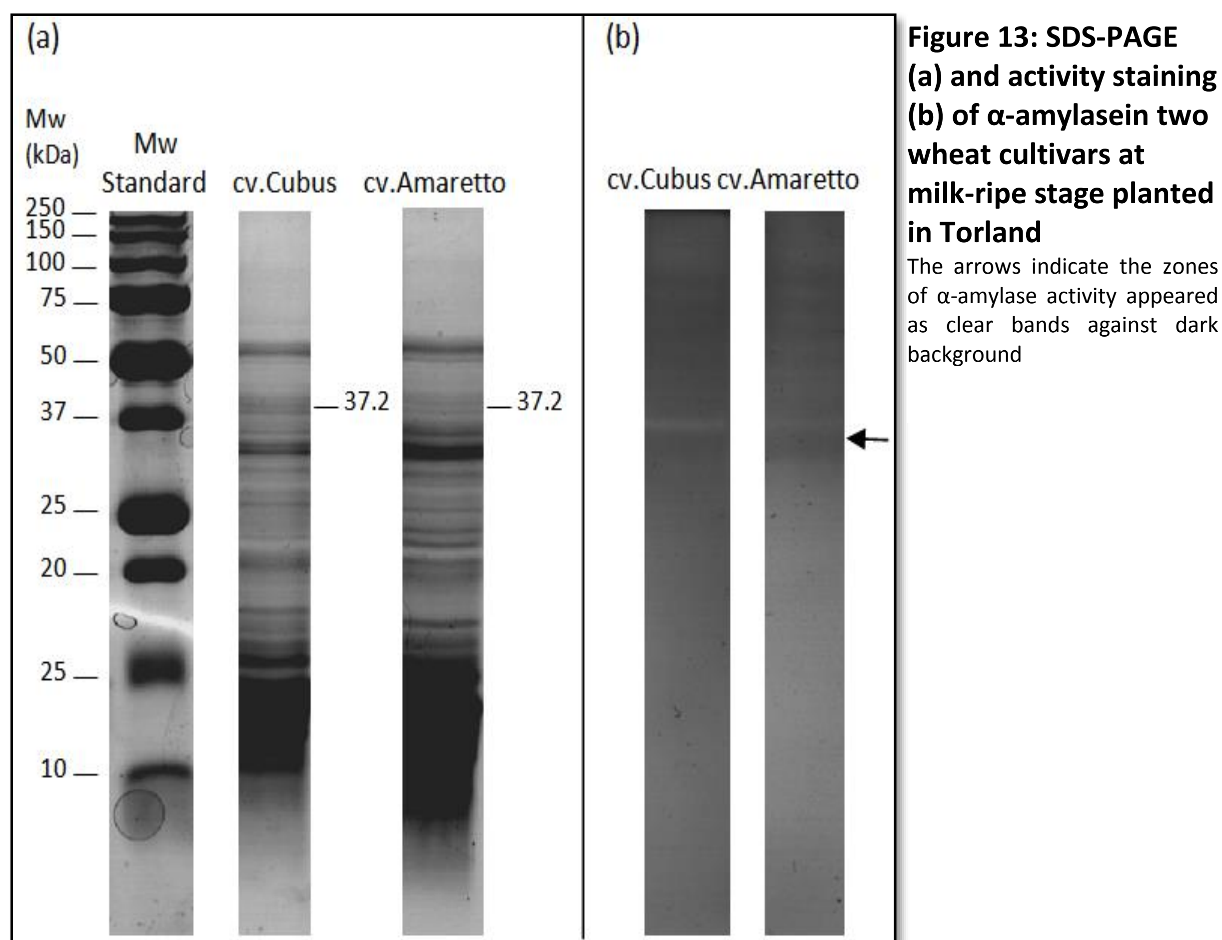

Figure 13: SDS-PAGE (a) and activity staining (b) of α-amylasein two wheat cultivars at milk-ripe stage planted in Torland

The arrows indicate the zones of α-amylase activity appeared as clear bands against dark background

Experiment 2

The characteristic of α-amylase originated from seven maize cultivars across two years is presented in Table 17. In order to highlight the trend of α-amylase attributes across different cultivars and years, the data of enzyme characterization in two cultivars, cvs. Franki and Saludo had been arbitrary chosen among other cultivars and then plotted among each year (Figure 14, 15, 16 and 17).

Table 17: Biochemical characterization and kinetic parameters of α-amylase in seven maize cultivars at mature stage in two years, 2010 and 2011, respectively

Cultivar	Temperature Optimum(°C)		Temperature stability(°C)		pH optimum		pH stability		K_m ($\times 10^{-3}$ g ml^{-1})		V_{max} (µmol min^{-1} ml^{-1})	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
Benecia	50	50	60	40	5	3	6	6	6	5	0.21	0.46
EsBombastic	40	40	60	40	4	6	5	6	6	5	0.21	0.44
Franki	50	50	50	50	5	6	5	6	6	4	0.24	0.26
Justina	40	40	40	40	4	3	6	7	8	2	0.13	0.28
Lukas	50	40	40	40	5	5-6	3	6	8	19	0.12	0.51
Saludo	40	40	40	40	5	6	6	6	7	3	0.29	0.27
PR39F58	70	40	70	40	5	6	6	7	6	2	0.13	0.31

Data represents average for triplicate measurements

The α-amylase was maximally active at 40 - 50 °C and pH 5 - 6 (Figure 14a and b). An increase in temperature of 10 °C compared to the optimum resulted in a 20 - 40 % decrease of activity but further increasing of the temperature to 70 °C resulted in further loss of enzyme activity. The activity of α-amylase differed among cultivars at elevated temperature above optimum temperature. For example, enzyme extracted from cv. Franki had higher activity (66 - 77 %) at 70 °C than those from cv. Saludo (45 - 57 %). Enzyme was active over a wide pH range with 20-80 % of hydrolyzing activity (Figure 14a). At pH lower or higher than the optimum pH, the activity of enzyme declined. At pH 4.0, a remarkable reduction in activity of α-amylase was observed in both cultivars (Figure 14b).

The maximal stability of α-amylase (100 % of residual activity) was observed at 40 - 50 °C and pH 6 in both cultivars planted in two years (Figure 14c and d). What is interesting that enzyme in both cultivars harvested in 2010 was more stable at 80 °C, in which recovered about 80 % of its original activity, compared with those harvested in 2011 with 20 % of residual activity (Figure 14c). Moreover, the pH stability for α-amylase produced in cultivars harvested in 2010 showed wider bell-shaped accompanied with higher residual activity (> 60 %), in comparison with those harvested in 2011 (Figure 14d).

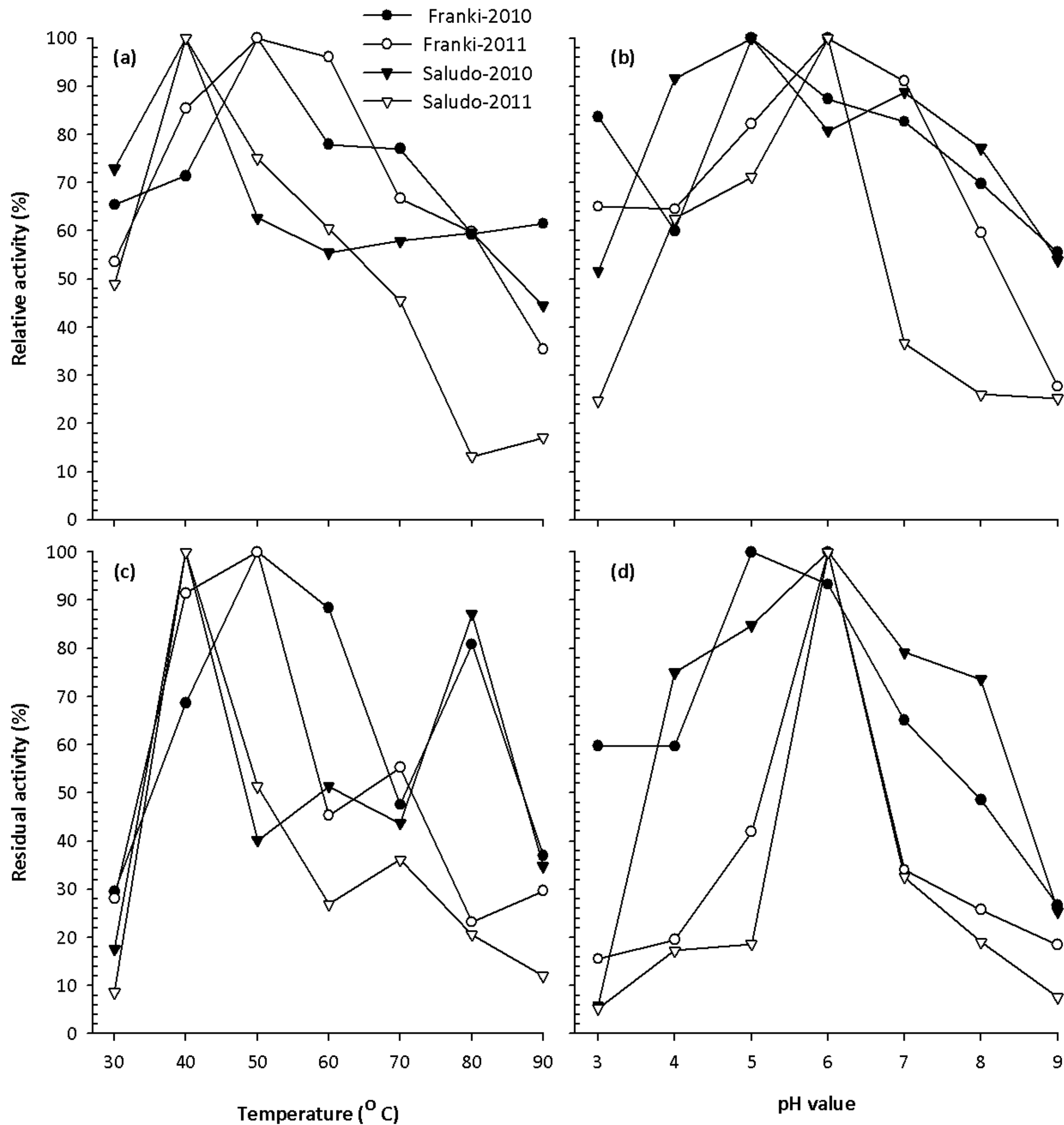

Figure 14: Biochemical characterization of α-amylase of mature grains in two maize cultivars among two years, (a, b): Temperature and pH optimum (c, d): Temperature and pH stability

The maximum activity was taken as 100 %, (a, b): relative activity and (c, d): residual activity plotted against different temperature and pH values. Data represents average for triplicate measurements in each point

Kinetic data of starch hydrolysis by α-amylase from two cultivars is showed in Figure 15. The K_m value for α-amylase from both cultivars was found to be in range 3-7×10^{-3} g ml^{-1} and its corresponding V_{max} was 0.2 µmol min^{-1} ml^{-1}.

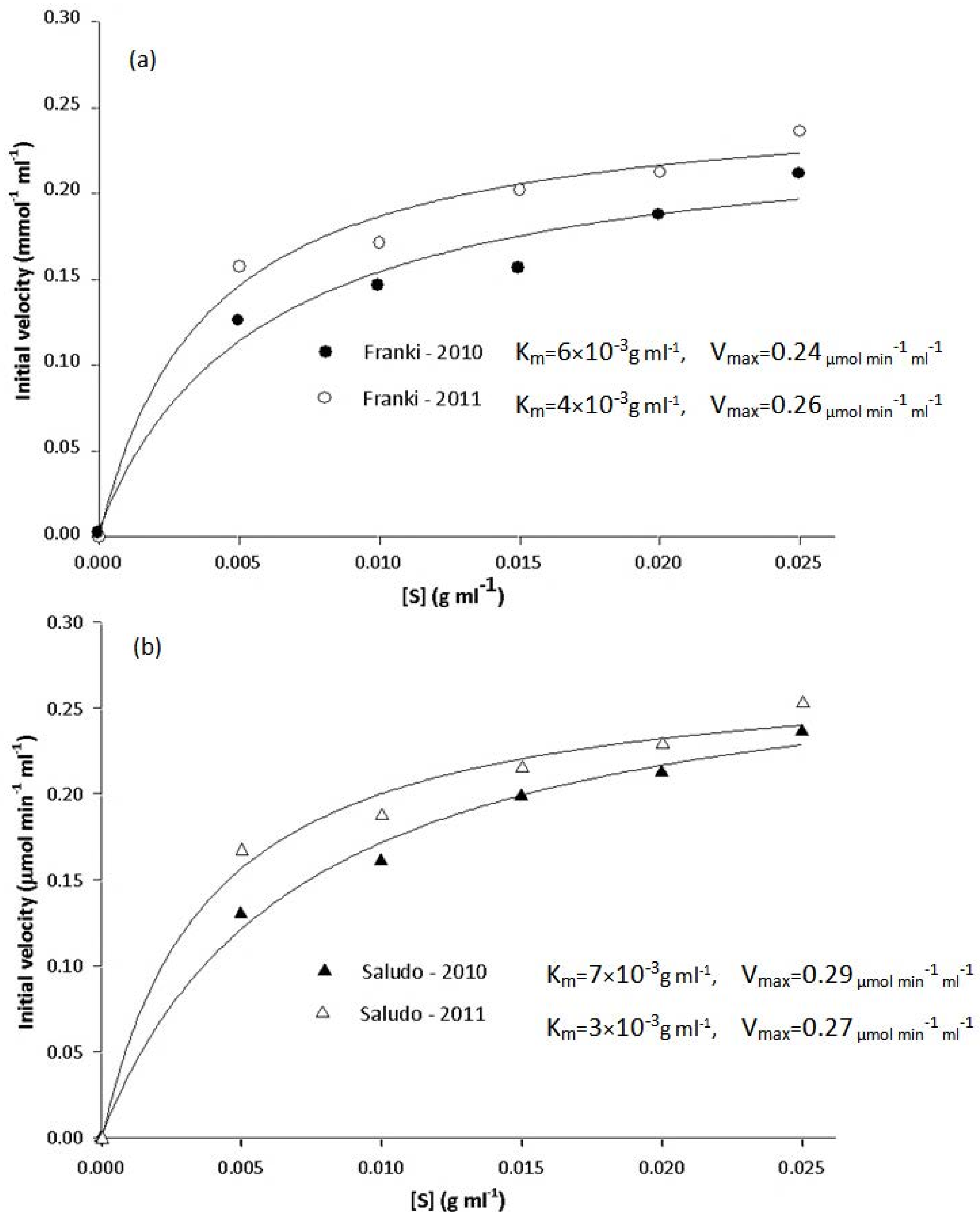

Figure 15: Michaelis-Menten curve for α-amylase activity in cv. Franki (a) and cv. Saludo (b) in two years, 2010 and 2011

Isoelectric focusing of α-amylase in milk-ripe grains resulted in one band with pI 4.1 for cv. Franki and pI 4.2 for cv. Saludo (Figure 16). The molecular weight value o was found to be 97 kDa for cv. Franki and 99 KDa for cv. Saludo (Figure 17).

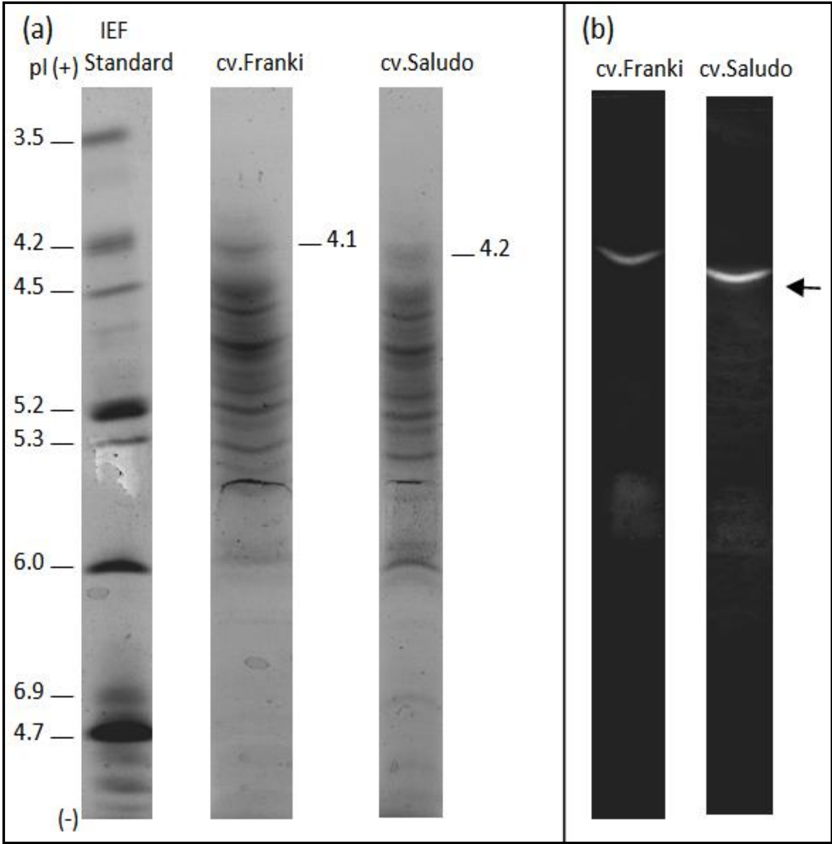

Figure 16: IEF (a) and activity staining (b) of α-amylase in two maize cultivars at milk-ripe stage planted in 2011

The arrows indicate the zones of α-amylase activity appeared as clear bands against dark background

The amount of extract applied to the gel (Figure 16 and 17) corresponding to 0.58 - 0.56 µmol $min^{-1}ml^{-1}$. The protein concentration was 0.38 - 0.47 mg ml^{-1}

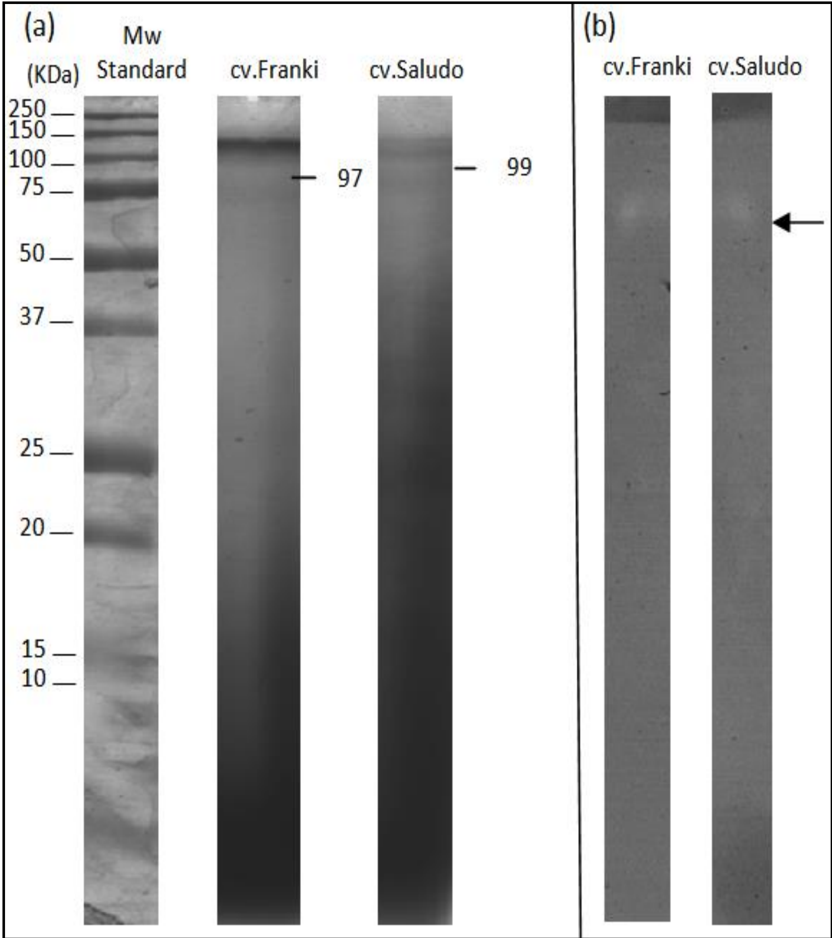

Figure 17: SDS-PAGE (a) and activity staining (b) of α-amylase in two maize cultivars at milk-ripe stage planted in 2011

The arrows indicate the zones of α-amylase activity appeared as clear bands against dark background

5. DISCUSSION

5.1. Influence of cultivars and environments on α-amylase activity and chemical composition throughout grain development

For convenience of discussion, the results from two experiments were combined to explain the influence of cultivars and environments on α-amylase activity and chemical composition of grains.

5.1.1. The activity of α-amylase

In the present study, the influence of the cultivars and environments (locations and years) on the α-amylase activity in cereals grains, wheat and maize, was investigated during grain growth and maturation.

Our results showed that the developmental pattern of α-amylase activity in grains from wheat (Figure 5) and maize cultivars (Table 11) was similar. Enzyme activity peaked at milk-ripe grains, followed by a rapid decline as the grains matured. This would be completely in agreement with a previous finding by De Laethauwer et al. (2013), who stated that the appearance of the highest α-amylase activity was detected during the first two weeks after pollination, and then the activity of enzyme declined during the latter stages of maturation. Furthermore, protein of α-amylase belongs to albumin and globulin fractions, in which the latter represent 90 % of the total proteins in the first two weeks of the grain growth, and then they decline until make up 20 - 30 % at mature-harvest stage (Emanuelsson et al. 2003; Evers et al. 1999), so it is reasonable to presume that α-amylase activity may somehow correlate with protein content as cited in several studies (Mikulikova et al. 2009; Barnard et al. 2005; Kindred et al. 2005; Every et al. 2002). In our study, a strong significant correlation between α-amylase activity and protein was observed in wheat across all grain development stages (Table 8 and 9).

Alpha-amylase is mainly located in the pericarp tissues (Barrero et al. 2013), although its presence has been reported in endosperm in immature wheat (Sidhu and Singh 2002) and maize (Mechin et al. 2004) grains at 14 days after anthesis. In the early grain development, pericarp of immature cereal grains represents a main part of the grain and contains a significant amount of transitory starch (Kalinga et al. 2013). Pericarp starch represents a

temporary energy store accumulated through active metabolism of the chloroplasts in the pericarp of immature wheat grains (Zeeman et al. 2010; Evers and Millar 2002). In maize, transitory starch is observed in the pedicel and pericarp tissues (Barnabas et al. 2008) before fertilization and remains after fertilization in immature kernels (Kalinga et al. 2013). Pericarp starch can be mobilized and translocated to other tissues during grain development in order to serve as source of short-primer for starch synthesis in the endosperm (Kalinga et al. 2013). Therefore, it was believed that the function of α-amylase activity at early development stage is involved in the breakdown of pericarp starch to produce maltose and malto-oligosaccharides for subsequent use as primer molecules and/or in respiration during starch synthesis in endosperm (e.g. Asthir et al. 2012; Piasecka-Kwiatkowska et al. 2007; Sidhu and Singh 2002). After that, α-amylase degrades progressively during grain maturation (Halford et al. 2011) and becomes undetectable at maturity-harvest stage (Faltermaier et al. 2014; lunn et al. 2001a). The degradation of enzyme coincides with disappearance of transitory starch (Sidhu and Singh 2002).

In the present study, the range of α-amylase activity at milk-ripe stage was higher in wheat grains (17.5 - 25.9 CU g^{-1} DM) than those in maize kernels (3.2 - 6.6 CU g^{-1} DM) (Figure 5 for wheat and Table 11 for maize). This is probably related to the chloroplast of pericarp tissues in wheat grains, whereas maize grains have no chloroplast in their pericarp (Evers and Millar 2002). It appears reasonable to assume that the more temporary starch in pericarp is present, the more enzyme induction is revealed as suggested by Sidhu and Singh (2002).

Regarding to the cultivars factor, the activity of α-amylase was significantly higher in grains of cv. Amaretto harvested from Gladebeck and Torland, 0.25 and 0.39 CU g^{-1} DM, respectively, than those from cv. Cubus in both locations, 0.14 and 0.20 CU g^{-1} DM, respectively, at mature-harvest stage. In addition, mature-harvest grains from cv. Amaretto showed significantly higher activity ($p \leq 0.05$) of α-amylase than those from cv. Cubus, irrespective of locations (Figure 5). This would be in agreement with Faltermaier et al. (2014), who reviewed that differences in α-amylase activity was observed in different cultivars and classes of wheat. Three reasons related to cultivar attributes are discussed for these differences.

The first reason is the differences in the flowering time between two wheat cultivars in our study. The flowering time is one of remarkable features in cereal grains, influencing duration and rate of grain filling as well as the harvest-maturity time (Egli 2004; Altenbach et al. 2003). It is controlled by photoperiodic response (PPD genes) and vernalization requirement (VRN genes) genes (Diallo et al. 2012; Distelfeld et al. 2009). The flowering time in spring wheat of cv. Amaretto occurred between 30th June and 5th July, which was late compared to winter wheat of cv. Cubus between 16th June and 20th June (Figure 18 in appendix). The difference in flowering time between spring and winter wheat is due to differences in VRN genes (Distelfeld et al. 2009), which leads to delay the mature-harvest stage of cv. Amaretto to 20th August compared to cv. Cubus on 3rd August. Johansson (2002) stated that wheat varieties flowering late were more vulnerable to pre-harvest sprouting. This is presumably true because they may expose to unfavorable weather conditions before harvest time (Farrer et al. 2006), resulting in an increase in α-amylase activity due to *de novo* synthesis of enzyme in sprouted grains (Barrero et al. 2013). Therefore, it can be assumed that cv. Amaretto grains are susceptible to pre-harvest spouting phenomenon, since they subjected to unfavorable weather condition, particularly high precipitation before harvest time (Figure 18 in appendix). Further details about the influence of weather condition on α-amylase activity will be discussed thereinafter within this subtitle 2.5.1.

Secondly, Biddulph et al. (2008) supposed that grain dormancy as genetic trait is involved in differences in α-amylase activity. The α-amylase activity is correlated with the degree of grain dormancy since α-amylase regards as one of factors that affect grain germination rate (Gao et al. 2013). Furthermore, Dencic et al. (2013) found that wheat cultivars could be classified based on their dormancy level and FN value. The first group was strongly dormant cultivars whose FN value above 300s. The second group was partially dormant cultivars that retain FN value not less than 250s. The third group was non-dormant cultivars that showed significantly low FN value below 250s under. It reasonable to assume that differences in α-amylase activity between two wheat cultivars in our study at mature-harvest stage may attribute to the differences in the degree of grain dormancy from those cultivars, although there was no information or preliminary germination test in both experiments to measure dormancy behaviour on grains from investigated cultivars in the

present study. The finding by Mohler et al. (2014) confirmed that there are many winter wheat varieties are characterized by non-deep physiological seed dormancy.

Besides the flowering time and grain dormancy, grain size and α-amylase activity are correlated with each other (Faltermaier et al. 2014). Evers et al. (1995) separated five UK wheat cultivars into different grain size categories, and measured FN for each group. They found that grains passing through 2.75 mm sieve had a higher FN value than the larger grains between 2.75 - 3.75 mm. Farrell and Kettlewell (2009) reported that the association between α-amylase activity and thousand grain weight as quantitative measure of grain size was not consistent enough to indicate a direct mechanistic link across all UK wheat varieties, although both genetic and environmental factors appeared to influence such association. Large grain generally has more endospermic cells, longer filling duration and higher filling rate than small grains (Dai et al. 2009). In both experiments, there was no grains size or thousand kernels weight measurement. The activity of α-amylase and other chemical components were calculated on a grain dry matter instead per grain. This calculation is supposed to eliminate grain size differences as suggested by Ahmadi and Baker (2001).

In the second experiment, differences in α-amylase activity were significantly detected ($p \leq 0.05$) among seven maize cultivars at different development stages (Table 11). The set of cultivars in our study was different in the hybrid type (one-way hybrid vs. three-way hybrid), maturity behaviour (fast vs. slow), the flowering date and the time to maturity (Table 20 in appendix). All mentioned factors may contribute to differences in α-amylase activity in Exp.2. In addition, the assumption of differences in grain dormancy and grain size in maize cultivars may cause differences in α-amylase activity, which have been discussed before for wheat cultivars.

Regarding to environments factor, both locations (Exp.1) and two years (Exp.2) had a major influence on differences in enzyme activity. These differences could be explained in term of weather conditions in the field. Precipitation and temperature are the two climatic factors that have a large effect on the plant growing periods (Barnabas et al. 2008) as well as the grain growth and maturation (Barnard and Smith 2009). The influence of the weather conditions on the cereals crops are distinguished between pre-anthesis impact and post-anthesis impact (Aslam et al. 2013). Since our study focused on the changes of α-amylase activity and chemical composition during grain filling and ripening, weather conditions

between anthesis and maturity are discussed in the following context. The climate data is presented in Figure 18 and 19 in appendix for both experiments.

Barnard and smith (2012 and 2009) found that the precipitation between the critical periods of anthesis and maturity is accounted for the most differences between year-to-year in both pre-harvest sprouting tolerance and the expression of dormancy, hence differences in α-amylase activity. The precipitation and subsequent high relative humidity during grain filling reduced the grain drying rate and dormancy that was initiated in mature grain. Such conditions can stimulate germination and associate with α-amylase formation and activation (Biddulph et al. 2005). It was found that the variation in precipitation at different locations accounted for almost 85 % of the variation in sprouting tolerance in wheat cultivars, particularly 20 day period before harvest (Barnard and Smith 2009). In addition, Barnard and Smith (2012) found in their study that the FN value of 7 out of 11 wheat cultivars was negatively affected by precipitation during the later stages of maturity. Furthermore, in a research conducted on 17 wheat cultivars in three sites in Virginia, USA, Barbeau et al. (2006), cited by Dencic et al. (2013), found that 6 cultivars had considerably lower FN than the other cultivars in the site with intense precipitation (> 3 inches) during the week before harvest. These cultivars did not differ from the others in the site with a dry period before harvest. Regarding to our results in Exp.1, dough-ripe and mature-harvest grains collected from Torland showed significantly higher ($p \leq 0.05$) enzyme activity than those in Gladebeck in each wheat cultivars (Figure 5). This difference seems to be related to the difference in amount of the precipitation in both locations (see Figure 18 in appendix). For instance, the average precipitation was 3.59 mm between 2nd July and 19th July in Torland, while it was 2.02 mm in Gladebeck. This difference in precipitation amount leads to double α-amylase activity in dough-ripe grain in cv. Cubus from Torland (0.48 CU g^{-1} DM) compared to those from Gladebeck (0.24 CU g^{-1} DM). Similarly, mature-harvest grains from cv. Amaretto in Torland had higher α-amylase activity (0.39 CU g^{-1} DM) than those in Gladebeck (0.25 CU g^{-1} DM). Again, the high precipitation in Torland caused high α-amylase activity. The average precipitation in Torland between 3rd August and 20th August was 5.5 mm, higher than in Gladebeck with 2.56 mm.

In addition, the intensive precipitation was above 8.8 mm in Torland before and at harvest date on 3rd August, which caused higher α-amylase activity in dough-ripe grains of cv. Amaretto from Torland (0.41 CU g^{-1} DM) compared to those from Gladebeck (0.31 CU g^{-1}

DM), where the precipitation was ~1 mm. Tillmann (2013) pointed out that the precipitation rate on August 2010 in Torland was higher than the period between 1960-1990.

Regarding to EXp.2, the significant difference in α-amylase activity between years was detected within a cultivar except PR38F58, and without consideration to cultivars (Table 11). Such difference appeared to be related to precipitation rate during grain maturity. In 2011, the average precipitation during kernel development was 4.41 mm, while in 2010 it amounted to 1.45 mm. The weather in 2011 was wetter than the period between 2000 and 2009, except 2007 (see Figure 20 in appendix) during the entire kernel development.

Beside the influence of precipitation on α-amylase activity by induction of pre-harvest sprouting, Joe et al. (2005) found that the early precipitation during grain growth modified α-amylase activity through its influence on gene expression. This may explain the significant higher enzyme activity in grains of cv. Amaretto from Gladebeck (25.91 CU g^{-1} DM) compared to those of cv. Cubus (20.59 CU g^{-1} DM) from the same location (Figure 5). In Gladebeck, the average daily precipitation at milk-ripe stage was higher for cv. Amaretto with 1.13 mm than and 0.28 mm for cv. Cubus. Furthermore, high temperature at milk stage of cv. Amaretto may also be reasonable for higher α-amylase activity than cv. Cubus, since high temperature increases enzyme activity and metabolic processes (Barnabas et al. 2008). The maximum, average, and minimum daily temperatures were 22, 19 and 14.5 °C at milk-ripe stage of cv. Amaretto, while they are 23, 17 and 9 °C at milk-ripe stage of cv. Cubus.

However, high ambient temperature in the summer is associated with high FN (Wang et al. 2008), and hence a reduction in α-amylase activity. There could be more than one explanation for this. On possible reason could be the gradual degradation of enzyme by the constant exposure to ambient high temperatures ≥ 30 °C in the absence of sprouting (Biddulph et al. 2008). The second reason could be shortened the period during which α-amylase could accumulate and become active (Joe et al. 2005). Furthermore, high temperature prior to harvest promotes the grain drying process and reduces the chance of wet condition around the mature grain and thus can favor timely harvest (Joe et al. 2005; Smith and Gooding et al. 1999). Barnard and Smith (2009) also found that high temperature during grain filling was associated with induced dormancy in certain cultivars. The findings mentioned above are consistent with our observation in differences in α-amylase activity with differences temperature patterns at dough-ripe and mature-harvest grains in both

cultivars and locations in Exp.1 (Figure 5). For example, dough-ripe grains of cv. Amaretto planted in Gladebeck was lower in their enzyme activity (0.31 CU g^{-1} DM) compared to those planted in Torland (0.41 CU g^{-1} DM) due to differences in the maximum daily temperature range at dough-ripe stage, 20 - 30 °C in Gladebeck and 15 - 25 °C in Torland. Furthermore, mature-harvest grain of cv. Cubus grains exhibited to the maximum daily temperature ranged 20 - 33 °C in Gladebeck, had lower activity than grains either from the same cultivars exhibited to the maximum daily temperature in range 16 - 21 °C in Torland, or from cv. Amaretto grains in the same location with the maximum daily temperature ranged 16 - 17 °C (Figure 18 in appendix).

The reduction in sunshine hours and temperature for a near constant precipitation reduces the ability of mature grain to dry by reducing evapotranspiration. This leads to delaying harvest and hence increasing susceptibility to pre-harvest sprouting damage (Hollins et al. 2004). This result is inconsistent to our results in Exp.2, where the weather in 2011 was wetter, hotter and more sunny hours in a day compared to 2010. The average of precipitation, the maximum daily temperature and daily sunshine hours in 2011 were 4.45 mm, 22°C and 6 hrs., whereas they were 1.45 mm, 19 °C and 4 hrs. (Figure 19 in appendix). The activity of α-amylase was significantly higher ($p \leq 0.05$) in maize grains harvested in 2011 than those in 2010 (Table 11). This difference may attribute to the dominant effect of precipitation on enzyme activity during mature-harvest stage and prior to harvest of mature grain as discussed before, in which leads to mitigate the positive effect of high temperature as well as the sunshine duration on reduction α-amylase activity of kernels harvested in 2011. Other possibility could be that the accumulated effect of low temperature at dough-ripe grains in 2010 was pronounced at mature-harvest stage (Beckles and Thitisaksakul 2014). It was found that low temperature 10 - 15 °C at dough-ripe stage (Osanai et al. 2005) induces deep grain dormancy compared to 25 °C (Biddulph et al. 2008). The maximum, average and minimum daily temperatures at dough stage in 2010 (18, 14 and 10 °C, respectively) were lower than those in 2011 (21, 17and 13 °C, respectively). Therefore, maize kernels from 2010 seem to have a deep dormancy in which prolonged to mature-harvest stage, and hence reduction in enzyme activity.

All together, the cultivars and environments affected clearly α-amylase activity in both experiments. The high α-amylase activity in mature-harvest stage in cv. Amaretto (see Figure 5 in Exp.1) and in almost all maize cultivars harvested in 2011 (see Table 11 in Exp.2)

was probably due to the induction of pre-harvest sprouting, although the grains in both experiments did not show any visible sprouting symptoms such as testa splitting or radicle emergence. Kindred et al. (2005) found that the sprouting might be incipient, although the grains were not visibly sprouted. They interpreted their statement that the grains with high α-amylase activity did not necessary germinate more quickly. Xing et al. (2009) provided further evidence that the activity of α-amylase may be sufficient to cause product quality degradation, although grains exhibit no visible symptoms, yet in the earliest stages of sprouting. It is worth mentioning that the involvement of late-maturity α-amylase activity can not be ruled out for two reasons. Firstly, under pre-harvest sprouting condition, late-maturity α-amylase activity may elevate (De Laethauwer et al. 2013). Secondly, Mohler et al. (2014) confirmed the presence of late-maturity α-amylase activity in some German bread wheat. Our study needs further methods to resolve the uncertainty in the presence of α-amylase activity in the absence of visual sprouting, where it was due to pre-harvest sprouting alone or in combination with late-maturity α-amylase activity. Enzyme linked immunosorbent (ELISA) assay for harvest-mature grains (Mohler et al. 2014, Zhang et al. 2014) has been used to screen for late-maturity α-amylase activity. The fluorescein dibutyrate assay (FDA), which used for lipase activity detection, could be used as further test to confirm the incipient of germination in the absence of visual sprouting observation (Kindred et al. 2005).

5.1.2. Chemical composition

In the present study, the influence of cultivars and environments (locations and years) on the chemical composition in wheat and maize was investigated during grain development stages.

Starch content increased remarkably toward maturity either in wheat (Figure 6a) or maize (Table 12) grains. It was 65.14 % on average of the dry matter of the mature-harvest wheat grains and up to 70 % of the dry matter of the mature-harvest maize kernels. These values are close to the boundary of starch content reported for cereals grains (Zörb et al. 2012). Such results are consistent with the facts that starch is the main component of the dry matter in cereal grains (Beckles and Thitisaksakul 2014) and increases with increasing maturity (Weichert et al. 2010; Sidhu and Singh 2002).

Starch content in cv. Amaretto was significantly lower (36.37 %DM) than that in cv. Cubus (45.47 %DM) at milk-ripe stage, irrespective of cultivation locations (Figure 6a). The influence of cultivar factor on differences in starch content could be explained by affecting starch accumulation as confirmed by many researches (e.g. Zhao et al. 2008; Beckles and Thitisaksakul 2014). This remarkable reduction in starch content in cv. Amaretto (9 % less) could be also explained by the effect of high temperature on starch content of grains. At milk-ripe stage, the maximum, average and minimum daily temperatures were 28, 22 and 16 °C, respectively for cv. Amaretto, while they revealed 23, 17 and 9 °C, respectively for cv. Cubus in both locations (Figure 18 in appendix).

The optimum temperature for wheat anthesis and grain filling ranges from 12 to 22 °C (Farooq et al. 2011). Temperatures above the optimum temperature enhance the evapotranspiration, respiration and leaf senescence, thereby limiting photoassimilates for grains (e.g. Farooqu et al. 2011; Barnabas et al. 2008; Plaut et al. 2004). In addition, high ambient temperature shortens the duration of grain filling, resulting in low starch content (Asthir et al. 2012). It is estimated that for every 1 °C rises in temperature above 15 - 20 °C, the duration of grain filling is reduced by ~3 day (Farooq et al. 2011). High temperatures above 25 °C during grain filling decline the key regulatory enzymes involved in starch synthesis (Hurkman et al. 2003) such as sucrose synthase (Zhao et al. 2008), ADPG-pyrophosphorylase and starch synthase (Barnabas et al. 2008) due to heat-induced denaturation (Farooq et.al. 2011). The reduction of the key starch-synthesizing enzymes was suggested to be due to a reduction in the sugar utilization in favor of starch rather than limitation in the supply of sugars to the ear or the availability of them within the endosperm (e.g. Lu et al. 2013; Zhao et al. 2008; Hurkman et al. 2003). This response might explain the low starch content, accompanied with the high sugars content in grains exposed to high temperatures in the early development stage. Regarding to our results in Table 7, the reducing sugars and total sugars were higher in grain of cv. Amaretto grains with 40.47 and 40.97 %DM, respectively, compared to those of cv. Cubus with 22.33 and 26.96 %DM, respectively, without consideration of locations.

Although the elevated temperature during grain growth may most often be accompanied by a decrease in starch content as mentioned before, in some circumstances, however, high temperature may positively influence starch content. At temperature less than 30 °C, the rate of grain filling has been reported to increase with increasing

temperature (Altenbach et al. 2003). The increase in the rate of grain filling reflects presumably the enhancement of enzyme activities and metabolic processes in which could compensate for the short grain-filling period, however, such increasing does not occur at temperature above 30 °C (Farooq et al. 2011; Barnabas et al. 2008). This finding may illustrate somehow the high starch with elevated temperature in mature-harvest grains in both experiments. For example, grains of cvs. Cubus and Amaretto in Gladebeck had higher starch content (67.39 and 67.30 %, respectively) than those in Torland (61.84 and 63.95 %, respectively) (Figure 6a). The maximum daily temperature in Gladebeck was higher 5 °C (on average) compared to Torland in mature-harvest stage of cv. Cubus (24 vs. 19 °C) and cv. Amaretto (22 vs. 17 °C) (Figure 18 in appendix). The higher starch content in mature grains collected from Gladebeck was associated with higher reducing sugars and total sugars content in both cultivars, compared to those from Torland. For example, the reducing sugars and total sugars content were 8.21 and 10.54 %, respectively, in mature grain of cv. Cubus in Gladebeck, whereas they were 2.08 and 4.70 % respectively, in those collected from Torland (Table 7). Additionally, a positive strong correlation between starch and fructose, maltose, reducing sugars and total sugars was also presented (Table 24 in appendix). It seems that starch content depends not only on the availability of carbon source in the grains, but there are other factors that may have an impact on starch synthesis and accumulation. The differences in the efficiency of biosynthetic enzymes in conversion the soluble carbohydrates to starch, the time of the onset and cessation of starch deposition and the time of onset of apoptosis of endosperm tissue are assumed to determine the differences in starch content (Asthir et al. 2012; Zhao et al. 2008; Altenbach et al. 2003).

In Exp.2, mature-harvest maize grains harvested from 2011 was higher in starch, reducing sugars and total sugars content than those in 2010, irrespective of cultivars (Table 11). Three temperature parameters, maximum, average and minimum, were higher in 2011 (22, 16 and 10 °C) than in 2010 (16, 12 and 9 °C) (Figure 19 in appendix). The average temperature in 2011 was representative to the period between 2000 and 2009 (see Figure 20 in appendix) during the entire kernel development. The positive correlation between starch and reducing sugars was observed in mature-harvest maize grains in some maize cultivars (see Table 25 and 26 in appendix).

Protein content varied considerably as a result of cultivars and environmental effect, such temperature, soil fertility and fertilizer application, on the accumulation of starch,

protein and both during grain development (William et al. 2008; Altenbach et al. 2003). In milk- and dough-ripe stage, protein content varied significantly (*$p \le 0.05$*) among two wheat cultivars (Figure 6b). This is consistent with previous finding by Malik et al. (2011), who found that the genetic background of wheat cultivars determined protein content.

High temperature during grain filling affects primarily starch and secondary the accumulation of protein (Triboi and Triboi-Blondel 2002). The low starch content with high protein content was detected in Exp1. For example, grains of cv. Amaretto planted in Gladebeck exposed to high temperature had lower starch content (38.11 %DM) and higher protein content (13.19 % DM) than those of cv. Cubus in the same location with 45.73 and 11.75 % DM for starch and protein, respectively (Figure 6a and b). The maximum daily temperature at milk-ripe stage in Gladebeck was 29 and 13 °C for cvs. Amaretto and Cubus, respectively. This result is supported by the finding of Farooq et al. (2011), who stated that grains subjected to high temperature during grain filling phase reduced starch content, while increased protein content. They interpreted such results by a reduction in starch deposition, which influenced protein content by allowing more nitrogen per unit of starch. In another study conducted by Zhao et al. (2008), it was declared that high temperature above 28 °C during grain filling may reduce the activity of glutamate pyruvic aminotransferase, which is key enzyme in regulation negatively protein deposition, and enhance protein content, when compared to 20 °C. Altenbach et al. (2003) found that the high temperature after anthesis altered the profile of both starch and protein accumulation. Lu et al. (2013) observed that high temperature (35/16 °C) resulted in higher protein content in maize kernels at 22 days after anthesis compared to controls (27/15 °C).

On the other hand, the high starch content with low protein content was detected in two experiments, where the wet weather was accompanied with high temperature. The two cases were:

(1) In mature-harvest grains of cv. Cubus planted in Gladebeck compared to those in Torland (Figure 6). The maximum daily temperature and precipitation were 24 °C and 4.59 mm in Gladebeck, respectively, while they were 19 °C and 2.56 mm in Torland, respectively.

(2) In mature-harvest of cvs. Saludo and PR39F58 planted in 2011 compared to those in 2010 (Figure 8).The maximum daily temperature and

precipitation were 22 °C and 4.41 mm in 2011, respectively, while they were 16 °C and 1.49 mm in 2010, respectively.

The first explanation was based on the negative relationship between starch and protein (Buresova et al. 2010; Csiszar et al. 2010), and the second explanation could be based on the finding by Smith and Gooding (1999). They found a negative relation between the precipitation and protein content because the precipitation enhanced dilution of nitrogen reserves by vegetative proliferation, encouraged leaching and other forms of soil nitrogen loss, and increased soil moisture reserves so that leaves life were extended during grain growth favoring carbohydrate assimilation and mobilization more than that of nitrogen. Weichert et al. (2010) provided further evidence that protein production was energetically more expensive because the required carbon cost per gram of grain yield was lower for starch than for protein.

However, Shewry (2006) found that different nutrition, particularly availability of nitrogen, in different locations and years caused a remarkable difference in protein content of grains. In Exp.1, Wheat grains collected from Torland had higher protein than those from Gladebeck in each development stage, irrespective of cultivars (Figure 6b). The total nitrogen available in Torland was 231.1 Kg/ha included with mineral nitrogen, whereas total nitrogen available in Gladebeck was 190 Kg/ha without mineral nitrogen. In addition, potassium fertilizer was applied only in Torland. It could assume then that the different soil nutrition in both locations may attribute to different protein content of grains in each location.

The reducing sugars and total sugars content had the maximum value at milk-ripe stage and then declined continuously in wheat (Figure 7 and Table 7) and maize grains (Table 12). This general trend of total sugars in cereal grains is comparable to a range of studies, which had shown that the depletion of the sugars content during grain filling was correlated to their rapid utilization for the synthesis of starch (e.g. Rosa et al .2009; Koch 2004; Helland et al. 2002). Sridhar et al. (2005) found that the total sugars content decreased from 15 days after pollination until harvest. In addition, Weshke et al. (2003) found that glucose and fructose were detected at pre-storage phase i.e. 1 day after anthesis in grain, particularly in pericarp, due to sucrose metabolism by invertase activity and later by sucrose synthase, in order to stimulate ongoing cell division and elongation processes. Afterward, those

monosaccharides and sucrose peaked at 10 - 12 day after anthesis when starch accumulation initiated.

The sugars content analyzed in our study differed significantly ($p \leq 0.05$) among wheat cultivars (Table 7) and maize cultivars (Table 12 and 14) with one exception in sucrose of maize grains harvest in 2011. This result is consistent with the finding by Rosa et al. (2009), who reviewed that the sugar content varies among cultivars.

Glucose predominated over other measurable sugars in all maize cultivars at all stages of grain development (Table 12), while maltose was the most abundant sugar in wheat cultivars in dough-ripe and mature-harvest stage (Figure 7 and Table 7). This is consistent with previous findings (Halford et al. 2011; Barnabas et al. 2008). The maltose content in milk-ripe grains was higher in wheat grains (Figure 7 and Table 7) than in maize kernels (Table 12). It could be assumed that higher α-amylase activity detected in wheat grains (Figure 5) than maize kernels (Table 11). This conclusion is based on the fact that maltose originated from the degradation of transitory starch by the action of α-amylase and other enzymes, such as β-amylase, α-D-glucosidase and debranching enzyme (Halford et al 2011; Minic 2008). The significant correlation between maltose and α-amylase activity was detected in wheat (Table 7, 8 and 10) and maize (Table 15 and 16). Furthermore, the significant correlation between α-amylase activity and starch content was detected across development stages in wheat (Table 8 and 9) and maize (Table 15 and 16), in which was in agreement with previous results of Mikulikova et al. (2009) and Every et al. (2002). The two relationships between α-amylase activity and both maltose and starch content imply that maltose produced by amylase, was utilized in starch accumulation. It was reported that soluble sugars formed via starch degradation via α-amylase provided energy and carbon skeleton to the growing grain (e.g. Gujjaiah and Kumari 2013; Halford et al. 2011).

Maize kernels harvested in wet and hot weather in 2011 (see chapter 5.1.1 and Figure 19 in appendix) had significantly higher ($p \leq 0.05$) glucose, fructose and maltose than those in 2010 among cultivars (Table 14) and irrespective of cultivars (Table 13). This result is in comparable with the statement of Halford et al. (2011), who reported that high temperature during grain filling increased reducing sugars and sugar phosphates. On the other hand, the sucrose content in kernels harvested in 2011 was significantly lower ($p \leq 0.05$) than in 2010 among cultivars (Table 14) and irrespective of cultivars (Table 13), while starch content was

significantly higher ($p \leq 0.05$) in 2011 than 2010 as discussed before. In addition, starch content was negatively correlated to sucrose content (Table 26 and 27 in appendix). It appeared that a rapid sucrose metabolism occurred in maize harvested in 2011 in favor of starch. Both sucrose synthase and invertase convert sucrose to UDP-glucose and fructose, and glucose and fructose, respectively (Halford et al. 2011).

5.2. Characterization α-amylase in wheat and maize

The main target of scientists with advances in biotechnology is to preserve the highest α-amylase activity (Bakes et al. 2005) and stability during industrial operating conditions (Buckow et al. 2007). Considerable efforts are underway to investigate the optimal conditions of application of cereal α-amylase isolated from new source with low cost in starch hydrolysis processing (El nour et al. 2013; Biazus et al. 2009; Kumar et al. 2008; Adewale et al. 2006). Therefore, the results of α-amylase properties are discussed from two viewpoints, enzymology and practical uses in the hydrolysis process of starch. We focused on characterization of α-amylase in sound mature-harvest grains except for the electrophetic analysis, since the focus of our study was on the attributes of endogenous α-amylase in cereals.

5.2.1. The temperature and pH optimum of α-amylase

The temperature optimum of α-amylase was 40 - 50 °C in all sets of wheat (Figure 10a) and maize cultivars (Table 17). These values are comparable with that of cereal amylases (Mohamed et al. 2009; Al-Bar 2009; Nirmala and Muralikrishna 2003). Alpha-amylase originated from wheat grain cv. Amaretto and maize grain cv. Franki was active with more than 60 % at 70 °C (Figure 10a for wheat, Figure 14a for maize). This result implies that in some cereals cultivars α-amylase may have a potential tolerance to high temperatures. This was confirmed by previous findings, which showed that α-amylase in some cereals grains, like Brazilian maize cultivars (Biazus et al. 2009) and Nigerian sorghum and maize cultivars (Egwim and Oloyede 2006), had temperature optimum at 60 - 70 °C. It was reported by Kumari et al. (2010) that the resistance of enzyme to high temperature indicates that enzyme-substrate complex is stable enough to protect enzyme from irreversible inactivation by heat.

From technological point of view, enzyme's temperature optimum considers an indicator for food processing application. In breadmaking, the temperature pattern during breadmaking process is divided into three stages (Drapron and Godon 1987): 1) dough-making, it includes mixing ingredients and fermentation. During this step the temperature does not exceed 25 °C. 2) Baking on the oven. Dough is subjected to a constantly increasing temperature from 25 °C up to a maximum of 100 °C. 3) Cooling and storage loaves, the bread is cooled to 25 °C. It is a common practice in breadmaking to use α-amylase from different sources as supplementation in flour to yiled fermentable sugars (Goesaert et al. 2005). Rosell et al. (2001) compared the temperature optimum of α-amylase from different origins (wheat, malted barley, fungi and bacteria) in order to optimize the use of α-amylase in flour standardization. They found that it is very difficult to satisfactorily control the level of bacterial α-amylase in bread making, because of its ability to survive at high temperatures found in the baking stage. On the other hand, cereal and fungal α-amylases had a different behavior. Fungal α-amylase is the most thermo labile, followed by those from cereal.

In another study conducted by Cato (2005), he stated that if α-amylase remains active until the temperature reaches around 70 °C throughout the bread baking period, further hydrolysis of gelatinized starch will occure in which release longer maltodextrins. Therefore, it is reasonable to presume that α-amylase originated from cv. Amaretto might be unsatisfactory as supplement in flour for breadmaking because it is more prone to negative effects on bread crumb upon overdosing.

On the other hand, the ability of maize α-amylase from cv. Franki to withstand high temperature about or below 70 °C is useful feature in dextrin production that run at 65 °C (Ba et al. 2013).

Our results also showed a remarkable reduction in the α-amylase activity in wheat grains cv. Cubus (Figure 10a) and maize grains cv. Saludo to 30 - 40 % after heating up to 70 °C (Figure 14a). Similar results were observed in cereal α-amylase by many authors (El Nour et al. 2013; Mohamed et al. 2009; Al-Bar 2009). From enzymological point of view, thermal inactivation of an enzyme is a result of the weakening of the intermolecular forces, i.e. covalent and non-covalent bonds, involved in the preservation of enzyme native structure leading to loss the activity (Illanes 2008). It is stated that use of cereal amylases in industrial processes is justified by enzyme's temperature (Biazus et al. 2009; Egwim et al. 2006). It

implies therefore that α-amylase originated from cvs. Cubus and Saludo may useful in starch-hydrolyzing industries that run at lower temperature than 70 °C because of their inactivation at high temperature 70 °C.

The pH range of the maximal activity of α-amylase was within 4 and 6 in both wheat (Figure 10b) and maize (Table 17 and Figure 14b). Similar results have been reported for α-amylase from wheat (Mohamed et al. 2009), maize, sorghum, (Egwim and Oloyede 2006) and barley (Al-Bar 2009). From food technological point of view, the pH in most of food applications can not be readily adjusted to the pH optimum of α-amylase. Therefore, the origin of enzyme has to be chosen on the basis of its activity at the natural food pH (Curvelo et al. 2008; Cato 2005). For instance, natural pH of starch surry is general around 4.5 (Sivaramakrish 2006).

Egwim and Oloyede (2006) tested the suitability of α-amylase from different sprouting Nigerian cereals (maize, acha, rice and sorghum) in starch hydrolysis processes based on the pH value. They found that the four cereals exhibited broad pH optima of α-amylase, ranging from 4 to 6, which was in agreement with the industrial practice. In addition, Curvelo et al. (2008) reported the optimal conditions to produce fermentable syrup from manioc starch by maize amylase. Their work showed that the maize amylase had the optimum pH values between 4.5 - 6.5 with optimum temperatures between 50 - 90 °C. From our present study, we may suggest to involve α-amylases from investigated maize cultivars in the hydrolysis process of starch by food industries because of thier broad pH optimum between 4 and 6.

Sometimes the activity of α-amylase that presents naturally in flour is not suffient to produce good bread volume and quality. Therefore, flour is routinely supplemented with exogenous amylases to optimize the amylase activity. Commercial amylase can be obtained from fungal, cereal or bacterial sources (Goesaert et al. 2005). Studies carried out by Rosell et al. (2001) have tested the activity of amylase from those commercial sources with the process condition in breadmaking. They found that the pH optimum of cereal α-amylase at 4.5 - 6.0 was appropriate to use this source of enzyme in flour standardization. We may suggest using them as source of amylases in flour standardization for breadmaking owing to the broad optimum pH of their amylases.

On the other hand, it was reported that irreversible inactivation of cereal α-amylase occurred below pH 4.5 (Brena et al. 1996) resulted in a sharp reduction in the activity (Al-Bar 2009; Nirmala and Muralikrishna 2003). Comparable results were also obtained in our study at pH 5 and 4 in wheat and maize amylases, respectively (Figure 10b for wheat and 14b for maize). It was stated that the pH profile and optimum of enzyme activity may differ from different cultivars (Mohamed et al. 2009; Kumar et al. 2005). Generally, an increase or decrease of the pH outside the optimal range declines the α-amylase activity (Usha et al. 2011). From enzymology point of view, this dependency may arise from several factors as mentioned by Panesar et al. (2010) and Belitz et al. (2009). First, a change in pH value may weaken the intermolecular forces stabilizing enzyme native structure leading to loss the activity. Second, it may influence the ionization degree in enzyme and its substrate in which alter the propensity of the two molecules to combine or decompose enzyme-substrate complex. Third, it may influence the the ionization of amino acid residue in the active site of enzyme which results in alteration of the breakage of enzyme-substrate complex to product (see enzyme rate equation No.1 in section 2.3.7).

Taking together, the optimum temperature and pH of α-amylase activity in both experiments indicate the favorable conditions in which the maximum numbers of the active site in enzyme are active (Egwim and Oloyede 2006). However, differences in the effect of temperature and pH on the same enzyme activity between several studies may contribute to differences in purification procedures, assay duration, different buffers and stabilizing agents used (e.g. Daniel et al. 2008; Buckow et al. 2007; Kumar et al. 2005).

5.2.2. The temperature and pH stability of α-amylase

The temperature stability of α-amylase, in which retained its native configuration as well the catalytic capacity, was at 40 - 50 °C in wheat (Figure 10c) and maize (Table 17 and Figure 14c) grains. This stability is in agreement with other studies which found that cereal α-amylase is generally stable up to 50 °C (Mohamed et al. 2009; Al-Bar 2009), but exceptions exist on both sides of the maximal temperature stability of enzyme. For instance, α-amylase was inactivated more or less completely at 70 - 90 °C in different cereals grains (Adefila et al. 2012; Mohamed et al. 2009). Sinha (2004) pointed out that beyond 45 °C enzyme get denatured and loose its activity due to change in three-dimension structure of protein. Daniel et al. (2008) reported that the temperature affects on the equilibrium position

between active and inactive forms of enzyme's protein. Evidence was supported by Gimbi and Kitabatake (2002), who found in their work that thermosatbility of α-amylase isolated from finger millet had an irregular pattern of changes, either increased, decreased and increased or remained constant, at 40-70 °C for 20 min. They interpreted to denaturation (inactivation) and renaturation (reactivation) of proteins during heating and cooling, respectively.Regarding to our study, we observed differences in enzyme response to temperature above the maximal temperature stability i.e. thermostable vs. thermolabile, among different cultivars within each cereal grains (Figure 10c for wheat and 14c for maize).

From industrial point of view, heat stability of α-amylase is a crucial factor for the use in several applications (Sivaramakrishnan et al. 2006). This is based on the fact that amylases are exposed to harsh conditions for purposes industrial such as high temperature and pressure to improve productivity and reduce the risk of microbial contamination (Kumar 2008; Buckow et al. 2007). Hence, it is necessary to have throughout knowledge of thermal stability of enzyme under which is going to be added (Fernandes 2010).

Study carried out by Kumar et al. (2005) had shown that the temperature stability of α-amylase from malted sorghum was higher compared to those from barley and finger millet at 70 °C. They concluded that sorghum's amylase from malted grains is a suitable source, among other cereal sources, for incorporation in supplementary foods that is reconstituted with hot milk or/and water for consumption. In brewing industry, three different temperatures are applied during adjunct mashing process as following (Young 2014): 1) The first hold is at 40 - 50 °C, 2) The second hold is at 63 - 68 °C and 3) the last hold of mash is raised at 70 - 90 °C. Adewale et al. (2006) reported that sorghum grains was better choice compared to maize and millet grains for maltsters in order to produce beer and other beverages in developing countries. This conclusion is most likely due to the superior of sorghum α-amylase properties compared to maize and millet amylases. One of these properties was the thermal stability of α-amylase during mashing process conducted at 50 °C. Among the three cereal amylases, sorghum amylase was found to be the most thermostable.

Regarding to our results, it seems that maize α-amylases isolated from cvs. Benecia, Esbomabstic, Franki and PR39F58 are suitable in starch hydrolysis industry because of thier thermal stability ≥ 50 °C. Mohamed et al. (2009) found that wheat amylase from cv. Balady

was stable up to 40 °C. They concluded that this property of wheat amylase with combination of others i.e. pH and temperature optimum, activators etc. meet the prerequisites need for food industry. Therefore, amylase of tested wheat cultivars has a good potential for the use in the food industry.

On the other hand, studies carried out by several authors (e.g. Goggin and Powles 2012; El nour et al. 2010; Muralikrishna and Nirmala 2005) claimed that the heating of crude extraction at 70 °C for 15 min was recommended for purification of α-amylase in order to inactivate other starch-hydrolyzing enzymes. Based on our observations, α-amylase from wheat cv. Amaretto and maize cv. Franki was more stable at 70 °C for 30 min (with 60 % of residual activity) compared to that from wheat cv. Cubus and maize cv. Saludo (with 20 - 35 % of residual activity) (Figure 10c for wheat and 14c for maize). Therefore, caution must be taken before applied heating at 70 °C for α-amylase purification procedures. We suggest conducting preliminarily experiments on residua activity of α-amylase at 70 °C in different time incubation before decide such step. In our study, this procedure was applied only in the extraction of α-amylase from milk-ripe grains used in electrophetic analysis (see section 3.5.2. and 3.5.5. for details).

The pH stability of α-amylase was 6 for both wheat (Figure 10d) and maize (Table 17 and Figure 14d). It conforms with the results published by Muralikrishna and Nirmala (2005), who found the maximal pH stability of cereal α-amylases, was between 5.5 and 8.0. It was reported that the α-amylase was inactive in extreme acidic condition, indicating acid labile nature of cereal α-amylases (Usha et al. 2011).

The presence of excessive α-amylase activity in flour from sprouted grains is an important problem in European countries, particularly in wheat grown regions (Dencic 2013). Several solutions for this problem were suggested. The acid treatment for flour containing high α-amylase activity was suggested by Kruger (1989) in order to ameliorate the deleterious effects of high α-amylase in bread quality. Regarding to wheat experiment, α-amylase from both wheat cultivars was the most labile at pH 4 (Figure 10d). These results imply that we may acidify flour from cvs. Amaretto and Cubus in case of a minor sprouting damage in those bread wheat cultivars.

Taking together, the optimal temperature and pH stability of α-amylase in both experiments indicate a specefic conditions to retain the original enzyme activity (Usha et al.

2011). This information should be considered in order to employ the α-amylase from different tested cultivars in starch-based industried (Egwim and Oloyede 2006). In addition, Goesaert et al. (2005) reported that the functionality of α-amylases used in breadmaking depends mainly on their thermostability and specify. Therefore, it is important to consider these conditions when applying α-amylase isolated from tested cultivars in industrial processes. On the other hand, different stability parameters of enzyme may differ between different α-amylase sources (Kumar et al. 2005). In addition, there are several factors playing a crucial role in the stability of enzyme. These factors include the presence of calcium, nature of substrate, stabilizers agents (Usha et al. 2011; Buckow et al. 2007), purity of enzyme extracted, protein content of the extract, the time of heating and pH value of extraction (Kumar et al. 2005; Muralikrishna and Nirmala 2005). Therefore, a major concern of modern biotechnology is to improve the stability of enzyme to justify the use of enzyme in diverse operational conditions (Fernandes 2010; Biazus et al. 2009). This can be attained by screening intrinsically stable enzyme, adding stabilizing agents, chemical modification, immobilization, protein engineering etc. (Sivaramakrishnan et al. 2006).

5.2.3. The kinetic properties of α-amylase

The kinetic parameters of wheat α-amylase were identical in both cultivars, the K_m and V_{max} values were 2×10^{-3} g ml^{-1} and 0.8 µmol min^{-1} ml^{-1}, respectively (Figure 11). Our observations in K_m value are similar to the results obtained in earlier studies in wheat (Mohamed et al. 2009), sorghum (Kumar et al. 2005), while the V_{max} values in our experiment are consistent with that reported by Mohamed et al. (2009). On the other hand, there were different kinetic properties of α-amylase from different maize cultivars in our investigation. The K_m and V_{max} values range of maize α-amylase in various cultivars was 2×10^{-3} – 8×10^{-3} g ml-1and 0.13 – 0.51 µmol min^{-1} ml^{-1}, respectively (Table 17 and Figure 15). There could be more than one explanation for this. One reason is suggested by Rogers and Gibon (2009), who believed that the use of partial purified enzyme to determine kinetic properties of enzymes can cause various interferences with other components such as specific or nonspecific inhibitors or activators. This can lead to underestimation or overestimation of the actual enzyme activity, particularly when non-saturating conditions were used. For instance, the presence of a competitive inhibitor, which competes with the substrate for binding to the active site of enzyme, increases the apparent K_m, while in the

presence of a noncompetitive inhibitor, which binds to enzyme at a site away from the active site, the value of V_{max} is reduced and K_m is unchanged (Guerra and Pastrana Castro 2012). Baks et al. (2006) studied the effect of carbohydrates such as soluble starch, glucose and malto-dextrin on α-amylase activity measurement, and they concluded that substrate inhibition and substrate competition can be a resource of errors during the activity measurement of any enzyme. Another possible reason is the presence of numerous enzymes in enzyme extract, as some of them may react with constituents of the assay leads to interference of actual activity of enzyme (Rogers and Gibon 2009). Furthermore, the presence of the *O*-linked glycosylation of maize α-amylases (Lecommandeur et al. 1990) as well as the *N*-linked carbohydrate of cereal α-amylases is also supposed to play an important role in the reaction kinetics of enzyme (Terashima et al. 1994). In addition, some researchers approved that the α-amylase isoenzymes had different affinities in binding to starch granules (Stanley et al. 2005; Mitsui and Iton 1997) and soluble starch as well (Reobertson et al. 2006). Regarding to miaze experiment, we may interpret the differences in kinetic properties of partial purified amylase due to the presences of different substances, either carbohydrates (Figure 9 and Table 14) or other enzyme, in a solution which interfered the actual measurement of activity of enzyme. In addition, the interference from the presence of different α-amylase isoenzymes, as suggestion by Reobertson et al. (2006), may exclude because there was only one isoenzymes detected in two maize cultivars Franki and Saludo (Figure 16).

From food technological point of view, it was reported by Curvelo-Santana et al. (2008) that the K_m and V_{max} values of cereal amylases are of paramount importance parameters that may influence the integration of enzymes in food processes. For example, Nirmala and Muralikrishna (2003) carried out a systamitic study on the basic aspect of finger millet as attempt to use it in breadmaking, weaning and infant food formulations in India. They have addressed many features, such as tested K_m of α-amylase from finger millet for various cereal starches (finger millet, rice and maize).

Regarding to our results, the low K_m value for α-amylases observed from both wheat cultivars as well as some maize cultivars cvs. Benecia, EsBombastic, Franki, PR39F58 indicates the high affinity of α-amylases for soluble starch as substrate (Rogers and Gibon 2009). Additionally, it implies that α-amylase would be easily saturated with substrate at a low concentration (Adefila et al. 2012). It seems thus that α-amylases from those culltivars

could be used efficiently in the bioconversion of starch to simple sugars by low substrate concentration in many industrial applications. There is good evidence in literatures about the utility of α-amylase that has the lowest K_m value among different cereals grains. For instance, Adewale et al. (2006) recommended Nigerian maltsters to use α-amylase from malted sorghum over maize and millet as brewing adjuncts in commercial malt production because they found among three α-amylases that the sorghum α-amylase had the lowest K_m value towards soluble starch. Similarly, Adefila et al. (2012) reported that Nigerian sorghum α-amylase had low K_m for starch in which could find applications as an additive in laundry detergents, as a fruit juice clarifier and in the paper industry.

Regarding to V_{max} value of enzyme, maize α-amylase originated from cvs. Benecia, EsBombastic and Lukas had higher V_{max}. This implies better enzymatic performance of amylase in those maize cultivars because a relatively higher V_{max} value means enzyme catalyzed-reaction can progress faster (Huestis 2008). This implication is supported by the work of Egwim and Oloyede (2006), who concluded that both sorghum and fonio α-amylases with the higher velocity compared to rice and maize are good sources employed in enzymatic saccharification of starch in most starch-based industries in Nigeria.

5.2.4. The electrophoretic properties of α-amylase

As mentioned above in section 3.5.5., the electrophoretic properties of amylase in our study were detected in milk-ripe grains. Three isoenzymes of α-amylase were detected on IEF from both wheat cultivars with similar pI values of 4.4, 4.6 and 4.8 (Figure 12). On the other hand, only one isoenzyme of α-amylase was detected in maize cultivars with a minor difference in the pI values between them. The pI values were 4.1 and 4.2 for cvs. Franki and Saludo, respectively (Figure 16). These values from wheat and maize amylases are comparable with that of cereal α-amylases (Muralikrishna and Nirmala 2005). Previous studies on cereal α-amylase divided the isoenzymes into two groups according to pI value: the high pI group (pI ≥ 5.8) and the low pI group (pI ≤ 5.5) (e.g. Cheng et al. 2014; Young et al. 1994; Warner et al. 1991). Regarding to our results, the isoenzymes of α-amylase in grains belong to low pI group. This result is consistent with Cheng et al. (2014), who found that low pI of α-amylase isoenzymes are appeared in the early grain development. The number of identified isoenzymes depends on the cultivars studied and the sensitivity of the resolving methods employed (Al-Bar et al. 2009; Mohamed et al. 2009). For instance, Al-

Maqtari et al. (2011) reported that the number of α-amylases detected in different cereal species varied from one to eight isoenzymes in finger millet, maize and wheat. Further evidence was reported by Hunjan et al. (2006), who stated that the number of barley α-amylase isoenzymes varied from 2 to 18 in literatures because of different purification techniques, different part of grains and different cultivars used. Genetic diversity is one of the main reasons suggested for multiplicity of cereal α-amylase isoenzymes. The high and low pI groups of α-amylase are encoded by the *α-AMY-1* and *α-AMY-2* genes, respectively (Cheng et al. 2014). For instance, *α-AMY-1* and *α-AMY-2* in wheat α-amylases localized on chromosomes 6 and 7, respectively (Gao et al. 2013). Although some of isoenzymes are derived from different genes, others are produced by post-translational modifications of enzyme (Young et al. 1994).

Interesting, the three α-amylase isoenzymes in wheat cultivars differed in their pI values (Figure 12) but not in molecular weight 37.2 kDa (Figure 13). It also conforms to the results published by other authors (e.g. Kharkrang and Ambasht 2013; Warner et al. 1991; San Segundo et al. 1990). A single band was observed in SDS-PAGE for α-amylase in maize with molecular weight exceeded 90 kDa (Figure 17). It was reported that the molecular weight determined of α-amylase varies between 50 - 120 kDa (Biazus et al. 2009) according to the isolation and purification methods used (Nirmala and Muralikrishna 2003), but usually the molecular weight of cereal α-amylase is in the range of 20 - 57 kDa (Usha et al. 2011; Muralikrishna and Nirmala 2005). Thus, the molecular weight of α-amylase from tested wheat cultivars was in the boundary of molecular weight reported for cereal α-amylase in grains. On the other hand, the high molecular weight of maize α-amylase > 90 kDa in our results was also stated by San Segundo et al. (1990). They interpreted such observation by the aggregation of α-amylase with other proteins. We suggested the same interpretation to our maize α-amylases results since we were used partial purified α-amylase enzyme (see section 3.5.5 for more details).

Isoelectric focusing technique is suggested by several authors (e.g. Zhang et al. 2014; De Laethauwer et al. 2013) to identify the origin of α-amylase in cereals grains. The retained pericarp α-amylase includes only low pI α-amylase isoenzymes, whereas late-maturity α-amylase includes only high pI α-amylase isoenzymes. However, pre-harvest sprouting grains contain both low and high pI α-amylase isoenzymes (For details see chapter 2.3.2 and 2.3.3). Regarding to our results, this technique was not used in harvest-ripe grains (see section

3.5.5.) and it did not give us any evidence to the origin of α-amylase activity measured by Ceralpha assay in mature grain at harvest time (Figure 5 for wheat and Table 11 for maize). Therefore, there is a clear need to use such method in mature-harvest stage of grain, in order to investigate the source of amylase activity, particularly in the presence of α-amylase activity in the absence of visual sprouting. Modern resolving techniques are also recommended to purify the α-amylase protein from grains at harvest stage to obtain clear results by IEF method. In addition, Kondhare et al. (2012) suggested the isoelectric separation of both α-amylase i.e. high vs. low pI α-amylase on agarose gel may beneficial tool in order to understand the effect of exogenous ABA and GA to particular α-amylase isoenzymes in regulating late-maturity α-amylase formation in wheat grains.

6. CONCLUSION

The present results contributed to the characterization of α-amylase from different cereals cultivars and environments (locations and years), and its relationship with the chemical composition of the grain.

In the first experiment, both cultivars and locations had significantly influence on α-amylase activity and all studied chemical components of wheat grains throughout the grain development period. Regarding to the cultivars factor, cv. Amaretto were characterized by higher α-amylase activity, starch and sugars content compared to cv. Cubus at dough and mature stage, regardless of the locations. On the other hand, grains in each cultivar collected from Torland showed higher α-amylase activity than those from Gladebeck at dough and mature stage. In the second experiment, α-amylase activity and all studied chemical components of mature maize kernels differed significantly ($p \le 0.05$) among two years, irrespective of cultivars. Mature kernels harvested in 2011 contained higher α-amylase activity, starch and sugars content except sucrose than those in 2010 among cultivars and regardless of the cultivars. It can be concluded that the weather conditions in both field experiments contributed to differences in α-amylase activity of cereals grains mainly between both locations and years, and partly between cultivars. For instance, the high precipitation rate and low temperature before and at harvest-mature time enhanced the α-amylase activity in wheat grains grown in Torland, which leads to suppose that pre-harvest sprouting may incipient in grains, although visible sprouting was absent. The involvement of late maturity α-amylase activity in mature sound grains cannot be ruled out, particularly this phenomenon has now been documented in German bread wheat germplasm.

Differences in α-amylase activity due to cultivars are important in breeding cereal cultivars tolerant to pre-harvest sprout damage as well as late maturity α-amylase since such differences in enzyme activity present as a valuable source of germplasm. From our results, the low enzyme activity of cv. Cubus promotes the utility of such cultivar as parent, and that characteristic in combination with other physiological mechanisms, e.g. insensitivity to GA and ABA sensitivity, can be selected for pre-harvest sprouting breeding program. On the other hand, the origin of high α-amylase activity detected in harvest-mature stage of wheat

cv. Amaretto and maize cv. EsBombastic was not definitely obvious, since there were no sprouting symptoms.

Difference in α-amylase activity of cereal grains in different locations and years was due to climate condition, mainly precipitation. Using cv. Cubus in Torland, where high precipitation during harvest period expected may suggest to avoid or reduce weathering damage. Furthermore, collecting wheat and maize grain earlier than harvest-mature stage and stored grains at low temperature ~10 °C to preserve dormancy are management options to avoid or reduce pre-harvest sprouting damage.

The significant correlation ($p \leq 0.05$) between α-amylase activity and starch, maltose and sucrose was detected in wheat and maize, while a strong significant correlation ($p \leq 0.05$) between α-amylase activity and protein was observed only in wheat across all grain development stages

In our study, although broadly similar properties of α-amylases from both cereals were detected, α-amylase of a particular cereal cultivar did differ. The characteristics of α-amylase from different cultivars differ and so many have different level of usefulness in food industires regarding to desired attributes of enzyme in a specific purpose.

Regarding to wheat experiment, α-amylase from cv. Amaretto was more active and stable at 70 °C than that of cv. Cubus. Thus, the use of cv. Amaretto as a source of α-amylase in breadbaking industry may lead to excess dexterinzation during baking process and a deterioration of bread quality upon overdosing. This is true in the case of using amylase of cv. Amaretto as processing aid in flour standardization. However, in the case of using tested wheat cvs. Amaretto and Cubus, as a flour from a minor sprouted damage, acid treatment of flour may suggest in order to ameliorate the deleterious effects of high α-amylase of wheat flour. This solution is based on the acid lability of amylase from wheat cultivars at pH 4.

Regarding to maize experiment, α-amylase from cvs. Benecia, EsBombastic, Franki and PR39F58 shows promoising characteristics for application in food industries such as beers, beverages, bakeries, syrups, formulation of paediatric food and others. This work shows optimal conditions of using maize amylase from mentioned cultivars depends on three superior properties as following: 1) broad pH optimum of amylase 4 - 6, 2) temperature stability of amylase ≥ 50 °C and 3) good kinetic capability of enzyme by either

low K_m and/or high V_{max} . Additionally, the thermal activity of amylase (~ 60 %) from cv. Fanki at 70 °C could be an added advantage.

7. Summary

Alpha-amylase is a starch-hydrolyzing enzyme and its characterization is an important quality parameter in cereal grains. In breadmaking, adequate α-amylase activity is required to produce bread of good quality. High α-amylase activity degrades the flour quality because it hydrolyses excessive amounts of flour starch and lowers flour viscosity which results in a sticky loaf and poor texture. The primary cause of high α-amylase activity is pre-harvest sprouting when the mature grains attached on the ear germinate in the field under wet and cold conditions. Moreover, some cereal cultivars produce unacceptable high α-amylase activity in the absence of pre-harvest sprouting as well. Therefore, α-amylase is influenced by both cultivar and environment factors. This study investigated the influence of cultivar and environment on α-amylase characterization during grain development stages in wheat and maize, and the relationship between α-amylase characterization and chemical composition of grains in both cereals.

The results in wheat and maize experiments showed that α-amylase activity changed considerably during grain development stages, with the highest activity was at milk-ripe grains. Regarding to wheat experiment, the grains of cv. Amaretto exhibited higher α-amylase activity than those of cv. Cubus at three grain development stages, irrespective of locations. The α-amylase activity of cultivars grown in Torland was significantly higher ($p \leq 0.05$) at dough-ripe and mature-harvest stages than those in Gladebeck, irrespective of cultivars. Regarding to maize experiment, α-amylase activity in mature-harvest grains cultivated in 2011 was significantly ($p \leq 0.05$) higher than those in 2010, irrespective of cultivars and within a cultivar except cv. PR39F58. Our results indicate that the difference in the activity of α-amylase was mainly attributed to environments, although the effect of different cultivars was also highly significant.

The results of biochemical and kinetic characterization of α-amylase from both wheat and maize have practical uses in the hydrolysis process of starch by food industries such as breadmaking, beers, beverages, syrups and others. This work shows the applicability of four investigated maize cultivars cvs. Benecia, EsBombastic, Franki and PR39F58 for use as a source of α-amylase in starch hydrolysis process. On other hand, thermal activity of amylase

isolated from cv. Amaretto is not a desirable feature in the case of using that cultivar as a source of exogenous amylase in order to optimize flour in breadmanking industry.

8. Bibliography

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9. Appendix

Table 18: Geographical features and cultivation information of field experiments

Geographical property	Wheat trail location		Maize trail location
	Gladebeck	Torland	Braunschweig
Altitude	165 m	140 m	75 m
Area	For Cubus ; 30 x 30 m^2 in 2 plots For Amaretto ; 2.4 x 30 m^2 in 4 plots		14.5 x 30 m^2 for each cv in 2 plots
Geographical coordinates	51.37°N 9.55°E	51.36° 9.52°E	52°17′ N, 10°26′ E
Soil type	Para-braunerde	Braunerde withlime stone	loamy sand
Valuation index of field	77	78	30-35
Cultivation information			
Soil cultivation	Rotary tiller , plough, rotary harrow		
Preceding crop	Wheat		Pea
Seeding rate	Cubus , Amaretto: 320 and 280 grains/m^2,respectively		10 grains/m^2
Planting date	Cubus, Amretto: 2nd October.2009 and 19th April2010, respectively		28th April.2010-2011
Irrigation	Non		3 times on July
Weed control	Baccara forte, Cadou and Pointer		Callisto and Milagro
Fungicide	2 times against fungal leaf pathogens		Non
Fertilization	190 kg N/ha as urea-ammonium nitrate	400 kg K_2O[1]/ha 190 kg N/ha as urea-ammonium nitrate	150 kg N/ha as calcium Ammonium nitrate
Mineral N[2] content of soil at 90 cm depth	-	46.1 kgN /ha	-

[1]K_2O, potassium oxide, [2]N, nitrogen

Table 19: Characteristic traits of two wheat cultivars

Characteristic trait *	cv. Cubus	cv. Amaretto
Type of wheat	Winter soft wheat	Spring soft wheat
Genotype	Line	Line
Quality group	A	A
Falling number	8	7
Crude protein	4	6
Sedimentation value	8	7
Baking volume	6	7
Dough elasticity	3	3
Breeder organisation	KWS Lochow GmbH	Saatzucht Bauer GmbH and Co.KG

Description is based on 1-9 scale, 1=very low degree, 5=medium degree and
9= very high degree
Source: BSA, 2012

Table 20: Characteristic attributes of seven maize cultivars

Maize cultivars	Type of hybrid	Type of corn	Flowering time*	Corn maturity days	Maturity behavior
Benecia	One way hybrid	Intermediate	29th July	250	Slow
EsBombastic	One way hybrid	Related to dent	2nd August	220	Slow
Franki	One way hybrid	Related to dent	1st August	260	Normal
Justina	One way hybrid	Intermediate	26th July	250	Fast
Lukas LG 3226	One way hybrid	Intermediate	26th July	240	Normal
Saludo	Three way hybrid	Related to dent	31st July	210	Normal
PR39F58	One way hybrid	Dent	2nd August	250	Normal

*Date of flowering in 2010, 50 % of female plants had reached EC 65
Source: BSA, 2012

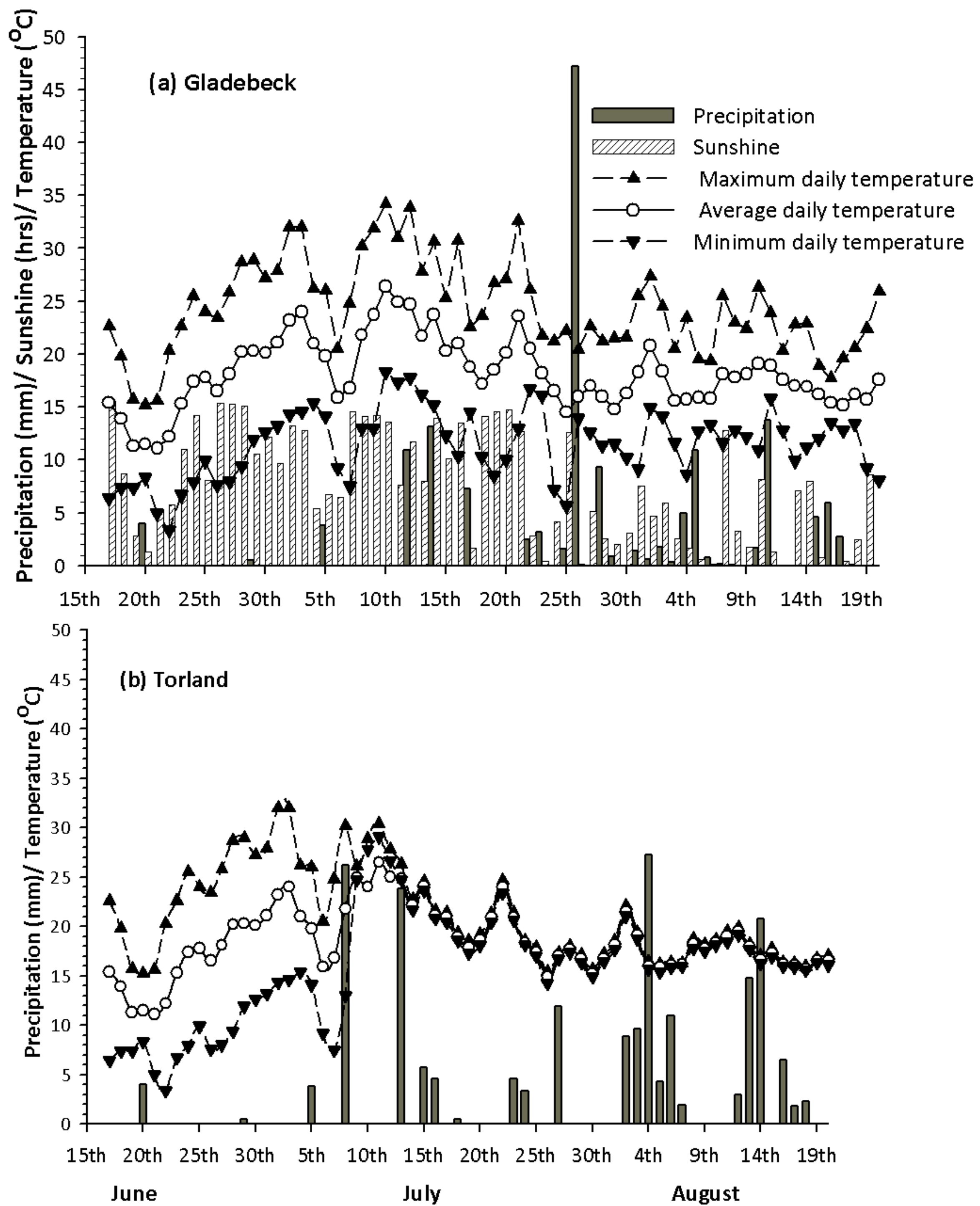

Figure 18: Weather conditions during wheat development period in two trail locations in 2010, (a) Gladebeck and (b) and Torland

A, B: flowering timefor cv. Cubus and cv. Amaretto, repectively
Sunshine data not available for Torland
Sources of data as following:

(a) Gladebeck, weather data and statistical express (WEST) of the German weather service (DWD,2010)
(b) Torland,Davis Vantage Pro2™-Weather station (Davis Instruments, Hayward, USA)in the field belongs to the project FAEN, George-August University

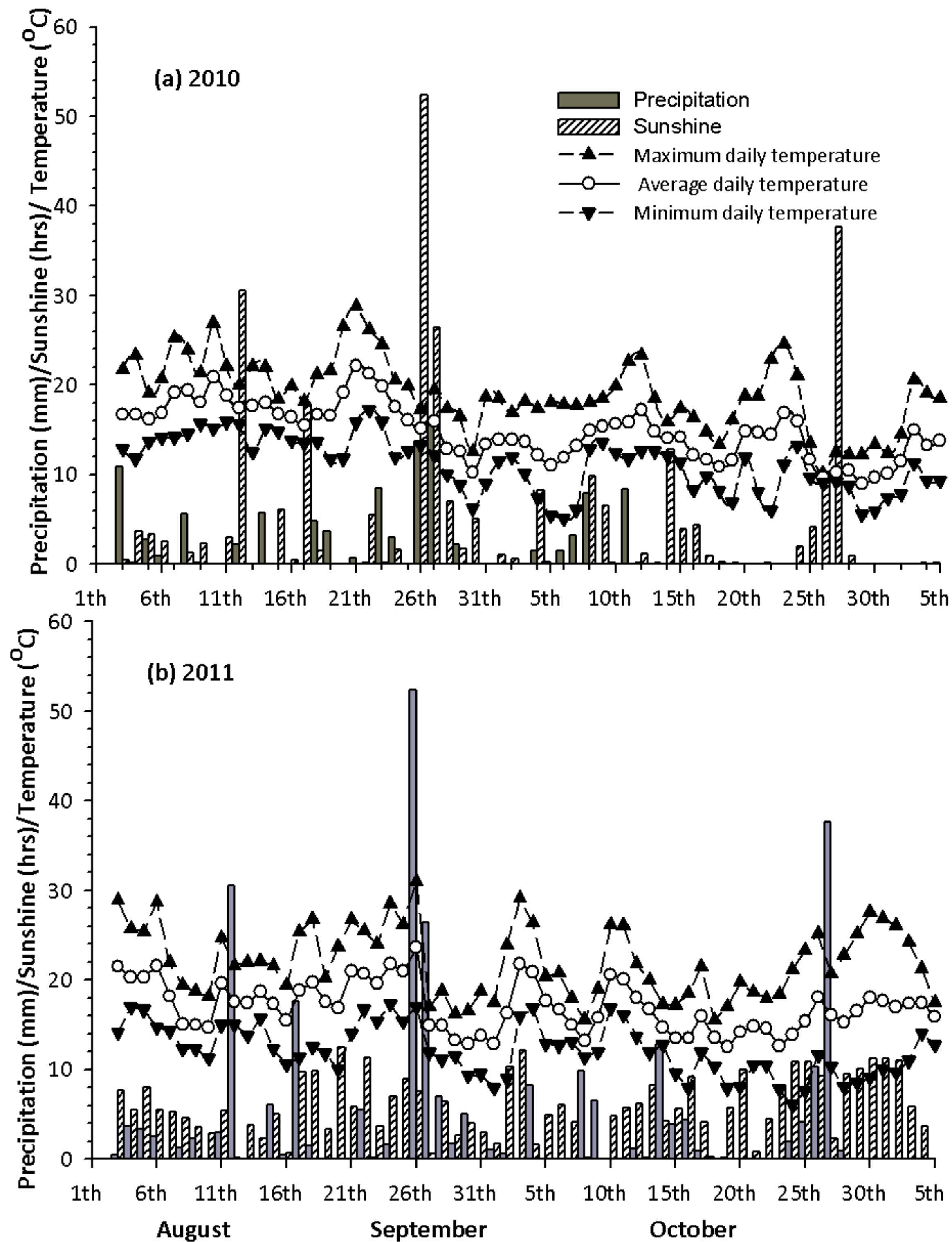

Figure 19: Weather conditions during maize development period in field experiment in Braunschweig among two years, (a) 2010 and (b) 2011

Source: Agricultural meteorological research Braunschweig (ZAMF) of the German weather service (DWD, 2011)

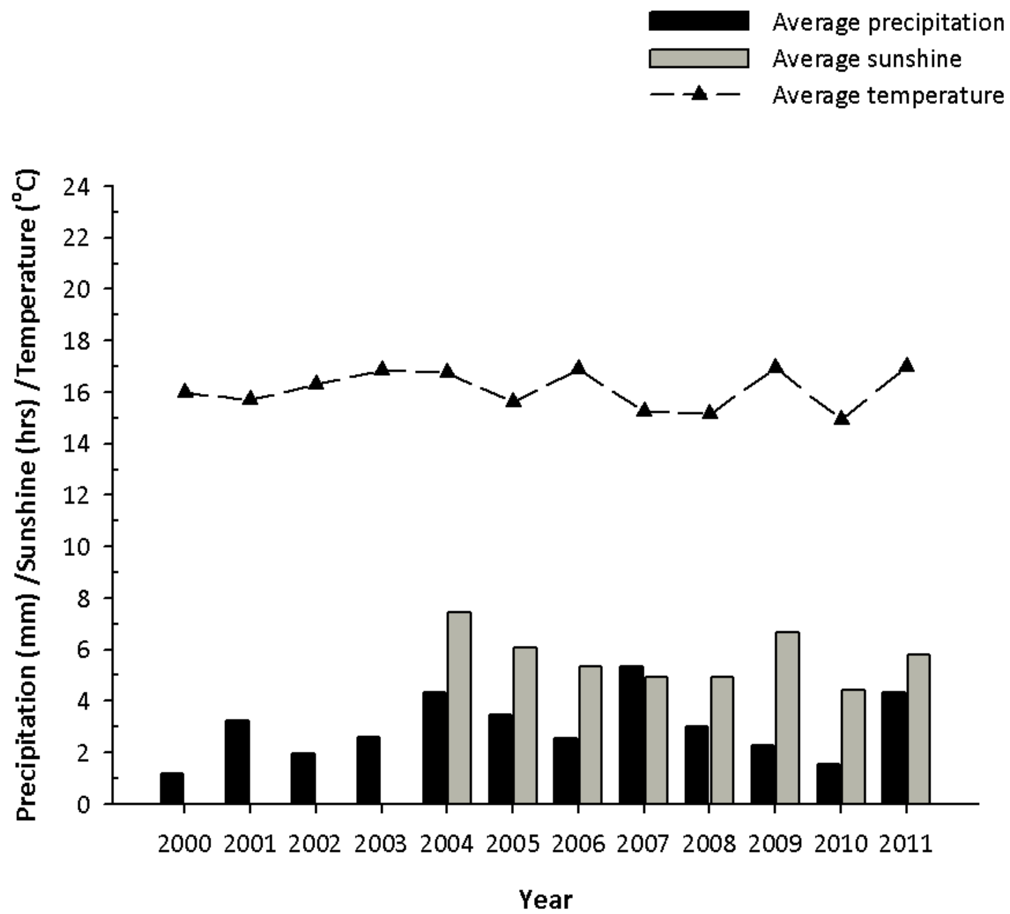

Figure 20: Weather conditions in field experiment in Braunschweig between 2000 and 2011.

The weather data parameters are average of three months, August, September and October

Source: Agricultural meteorological research Braunschweig (ZAMF) of the German weather service (DWD, 2011)

Table 21: Compositions of maize whole flour (% DM) and α-amylase activity (CU g^{-1} DM) during three development stages in seven cultivars planted in 2011

Development stage	Chemical composition	Benecia	EsBombastic	Franki	Justina	Lukas	Saludo	PR39F58
Mik	Starch	61.5±1.84 a	58.6±1.47 ac	47.4±0.98 bd	55.8±0.04 bc	52.5±0.59 b	59.4±0.87 a	58.2±1.65 ac
	Protein	9.92 abc	10.14 abc	13.39 a	11.41 abc	12.96 b	11.28 c	11.98 abc
	Fructose	31.3±17.5 a	44.7±15.5 a	53.6 ± 6.14 a	57.0± 4.97 a	46.3±8.44 a	40.7±8.80 a	41.3±3.07 a
	Glucose	36.0 ±19.9 a	50.7± 17.6 a	67.0 ± 7.97 a	66.0± 6.59 a	53.5±9.86 a	46.5± 9.98 a	48.0±3.90 a
	Maltose	3.47±2.23 ab	2.58 ±1.58 ab	1.72 ± 0.38 ab	1.87±0.20 b	1.92±0.43 ab	0.99±0.29 b	4.44±1.44 a
	Sucrose	0.07±0.12 b	0.46 ±0.10 ab	0.56 ± 0.02 a	0.24±0.04 b	0.21±0.03 b	0.10±0.00 b	0.11±0.10 b
Dough	Starch	65.1 ±1.14 ac	64. 2±1.06 bc	66.6 ± 1.21 ac	61.8±1.19 b	63.8±069 bc	65.1±1.26 ac	67.8±1.27 a
	Protein	8.97 a	9.63 a	10.58 a	10.60 a	9.02 a	10.11 a	8.82 a
	Fructose	33.5±3.64 ab	27.9 ± 4.93 ab	22.0±5.03 ab	36.7± 7.4 a	30.5±2.84 ab	15.6±13.7 b	25.2±2.4 ab
	Glucose	38.5±3.35 b	30.7 ± 5.20 ab	26.0±6.06 ab	40.4±7.98 b	33.4±3.08 ab	17.3±5.18 a	27. ±2.8 ab
	Maltose	0.95±0.45 b	3.25 ± 0.61 c	1.72±0.26 bc	2.49±0.53 bc	2.63±0.08 bc	1 .7 ±1.52 bc	6.96±0.61 a
	Sucrose	0.01±0.01 a	0.62 ± 0.16 b	0.55±0.25 ab	0.56±0.20 b	0.43±0.14 ab	0.35±0.34 ab	0.21±0.1 ab
Ripe	Starch	68.0±1.66 b	68.7±0.72 b	67.8 ±1.40 b	68.7±0.70 b	67.1±0.70 b	71.4±0.34 a	71.3±0.37 a
	Protein	9.29 a	8.85 a	10.13 a	9.40 a	9.52 a	10.20 a	7.97 a
	Fructose	10.8±2.61 a	8.02±0.73 a	2.38 ±0.2 bc	3.16±0.7 bc	8.12±0.28 a	2.42±0.14 c	4.61±2.08 c
	Glucose	9.91±2.33 ac	7.69±1.03 c	2.51±0.24 b	3.72±0.94 bd	7.2± 0.25 cd	2.86±0.23 b	5.05 ±2.2 bc
	Maltose	0.00 a	0.00 a	0.12±0.21 a	0.20±0.17 a	0.08±0.13 a	0.22±0.19 a	0.00 a
	Glucose	1.66±0.65 b	5.44±0.77 c	1.68±0.29 b	5.98±1.73 a	1.79±0.06 bc	5.82±0.64 ac	5.66±2.78 a

Data represent average ± standard deviation for triplicate measurements except protein with duplicate
a,b,c,d,e,f different letters indicate significant differences (*P ≤ 0.05*) between cultivars within a stage

Table 22: Correlation matrix between chemical components at milk-ripe stage in two wheat cultivars and two locations, as Person Correlation Coefficient

Effect level		Milk-ripe stage	Chemical composition						
Location	Cultivar		Protein	Fructose	Glucose	Maltose	Reducing sugar	Sucrose	Total sugars
Gladebeck and Torland	Cubus and Amaretto (n=12)	Starch	0.23ns	-0.86***	-0.52ns	0.13ns	-0.6*	0.58*	0.6*
		Protein		-0.44ns	-0.54ns	0.61ns	-0.28ns	0.77 *	-0.27ns
		Fructose			0.77***	-0.02ns	0.91***	-0.73***	0.94***
		Glucose				-0.09ns	0.84***	-0.74***	0.84***
		Maltose					0.28ns	0.33ns	0.29ns
		Reducing suagrs						-0.63*	1***
		Sucrose							-0.63*
	Cubus (n=6)	Starch	0.32ns	-0.05ns	0.16ns	-0.06ns	0.05 ns	-0.2 ns	0.04ns
		Protein		-0.99***	-0.97**	0.72ns	-0.99 ***	0.91ns	-0.99***
		Fructose			0.80ns	0.05ns	0.96**	-0.73ns	0.96**
		Glucose				-0.30ns	0.93**	-0.91ns	0.92**
		Maltose					0.55ns	-0.05ns	-0.04ns
		Reducing suagrs						-0.83*	1***
		Sucrose							-0.82*
	Amaretto (n=6)	Starch	0.87ns	-0.52ns	0.02ns	0.94**	0.74ns	0.90*	0.75ns
		Protein		-0.62ns	-0.34ns	0.99***	0.46ns	0.93ns	0.46ns
		Fructose			0.51ns	-0.67ns	0.09ns	-0.37ns	0.09ns
		Glucose				-0.28ns	0.63ns	-0.15ns	0.62ns
		Maltose					0.51ns	0.88*	0.52ns
		Reducing suagrs						0.625ns	1***
		Sucrose							0.63ns
Gladebeck	Cubus and Amaretto (n=6)	Starch	-0.9ns	-0.8*	0.19ns	-0.8*	-0.66ns	-0.36ns	-0.66ns
		Protein		0.97*	0.04ns	0.97*	0.75ns	0.48ns	0.75ns
		Fructose			0.07ns	0.87*	0.85*	0.67ns	0.866*
		Glucose				0.23ns	0.53ns	0.15ns	0.53ns
		Maltose					0.61ns	0.55ns	0.91*
		Reducing suagrs						0.60ns	1***
		Sucrose							0.61ns
Torland	Cubus and Amaretto (n=6)	Starch	0.98**	-0.97***	-0.96**	0.92**	-0.96**	0.93**	-0.94**
		Protein		-1***	-1***	0.95*	-0.99**	0.97*	-0.99***
		Fructose			0.99***	-0.92	0.99***	-0.96***	0.99***
		Glucose				-0.91*	0.99****	-0.95**	0.99***
		Maltose					-0.89*	0.95*	-0.89*
		Reducing suagrs						-0.95**	1***
		Sucrose							-0.9***

ns, not significantly different at p ≤.0.05, ***, ** and * significance at P ≤ 0.001, 0.01 and 0.05, respectively
n, number of observations

Table 23: Correlation matrix between chemical components at dough-ripe stage in two wheat cultivars and two locations, as Person Correlation Coefficient

Effect level: Location	Effect level: Cultivar	Dough-ripe stage	Protein	Fructose	Glucose	Maltose	Reducing sugars	Sucrsoe	Total sugars
Gladebeck and Torland	Cubusand Amaretto (n=12)	Starch	0.30 ns	0.24ns	-0.19ns	0.16ns	0.07ns	0.75**	0.48ns
		Protein		-0.007ns	-0.56ns	-0.24ns	-0.74*	0.43ns	-0.28*
		Fructose			0.32ns	-0.21ns	0.30ns	0.39ns	0.44ns
		Glucose				-0.44ns	0.40ns	-0.44ns	0.05ns
		Maltose					0.61*	0.39ns	0.67*
		Sucrose							0.81***
		Reducing suagrs							0.68*
	Cubus (n=6)	Starch	0.47 ns	-0.38ns	0.09ns	-0.52ns	0.87*	-0.46ns	-0.27ns
		Protein		0.53ns	-0.67ns	-0.90ns	0.43ns	-0.87ns	-0.76ns
		Fructose			-0.84*	-0.43ns	-0.43ns	-0.49ns	-0.61ns
		Glucose				0.74ns	0.29ns	0.79ns	0.90*
		Maltose					0.96***	-0.384	0.949**
		Reducing suagrs						-0.3ns	0.97***
		Sucrose							-0.07ns
	Amaretto (n=6)	Starch	0.65ns	0.32ns	-0.48ns	0.69ns	0.7ns	0.54ns	0.77ns
		Protein		0.92ns	-0.82ns	0.95*	0.99 **	0.34 ns	0.98 *
		Fructose			-0.79ns	0.67ns	0.08ns	0.63ns	0.56ns
		Glucose				-0.86*	-0.86*	-0.05ns	-0.7ns
		Maltose					0.55ns	0.93**	0.936**
		Reducing suagrs						0.42ns	0.69ns
		Sucrose							0.94**
Gladebeck	Cubus and Amaretto (n=6)	Starch	0.69 ns	0.64ns	0.74ns	-0.6ns	0.8ns	0.42ns	0.84*
		Protein		0.87ns	0.21ns	-0.63ns	0.99**	0.01ns	0.86ns
		Fructose			0.01ns	-0.77ns	0.83*	-0.16ns	0.64ns
		Glucose				-0.22ns	0.42ns	0.61ns	0.59ns
		Maltose					-0.96ns	0.44ns	-0.42ns
		Reducing suagrs						0.18ns	0.54ns
		Sucrose							0.92**
Torland	Cubus and Amaretto (n=6)	Starch	0.41 ns	-0.77ns	-0.63ns	0.53ns	0.54ns	-0.63ns	-0.56ns
		Protein		-0.99***	-0.93ns	0.62ns	0.92 ns	-0.9 ns	-0.6 ns
		Fructose			0.68ns	-0.54ns	-0.56ns	0.72ns	0.69ns
		Glucose				0.96**	-0.92**	0.96**	0.77ns
		Maltose					0.72ns	-0.39ns	-0.07ns
		Reducing suagrs						-0.81*	-0.5 ns
		Sucrose							0.51ns

For symbol explanation see legend of Table 22

Table 24: Correlation matrix between chemical components at mature-harvest stage in two wheat cultivars and two locations, as Person Correlation Coefficient

Effect level		Mature-harvest stage	Chemical composition						
Location	Cultivar		Protein	Fructose	Glucose	Maltose	Reducing sugars	Sucrose	Total sugars
Gladebeck and Torland	Cubus and Amaretto (n=12)	Starch	-0.5ns	0.63*	0.38ns	0.91***	0.8 ***	0.07 ns	0.8***
		Protein		-0.19ns	0.18ns	-0.63ns	-0.49 ns	0.66ns	-0.41ns
		Fructose			0.91***	0.71**	0.84***	0.43ns	0.87***
		Glucose				0.43ns	0.62*	0.64*	0.68*
		Maltose					0.97***	-0.08ns	0.95***
		Reducing suagrs							0.99***
		Sucrose							0.18 ns
	Cubus (n=6)	Starch	-0.87*	-0.52ns	0.02ns	0.94**	0.74ns	-0.90*	0.75ns
		Protein		-0.62ns	-0.3ns	0.99**	-0.46ns	0.93ns	0.46ns
		Fructose			0.51ns	-0.67ns	0.09ns	-0.37ns	0.09ns
		Glucose				-0.28ns	0.63ns	-0.15ns	0.62ns
		Maltose					0.51ns	0.88*	0.52ns
		Reducing suagrs						-0.6 ns	0.99***
		Sucrose							0.63ns
	Amaretto (n=6)	Starch	0.97 *	0.45ns	0.39ns	0.82*	0.69ns	0.77ns	0.70 ns
		Protein		0.44ns	0.57ns	0.91ns	0.81ns	0.89ns	0.82ns
		Fructose			0.96**	0.69ns	0.89*	0.84*	0.89*
		Glucose				0.67ns	0.89*	0.85*	0.89*
		Maltose					0.93***	0.95**	0.93**
		Reducing suagrs						0.98***	1***
		Sucrose							0.99*
Gladebeck	Cubus and Amaretto (n=6)	Starch	0.03ns	-0.37ns	-0.31ns	0.37ns	-0.13ns	0.21ns	-0.05ns
		Protein		0.41ns	0.58ns	-0.38ns	0.52 ns	0.82 ns	0.62 ns
		Fructose			0.97***	0.22ns	0.91*	0.73ns	0.91*
		Glucose				0.29ns	0.94**	0.78ns	0.95**
		Maltose					0.59ns	0.172ns	0.52ns
		Reducing suagrs						0.72ns	0.98 ***
		Sucrose							0.82*
Torland	Cubusand Amaretto (n=6)	Starch	0.98*	0.94**	0.26ns	0.74ns	0.73ns	-0.53ns	0.79ns
		Protein		-0.98*	0.54ns	-0.92ns	-0.93ns	0.87 ns	-0.9ns
		Fructose			0.14ns	0.91*	0.91**	-0.66ns	0.92*
		Glucose				0.06ns	0.12ns	-0.17ns	0.12ns
		Maltose					0.99***	-0.82*	0.99***
		Reducing suagrs						-0.83*	0.99 ***
		Sucrose							-0.79ns

For symbol explanation see legend of Table 22

Table 25: Correlation matrix between chemical components at three development stages across seven maize cultivars in 2011, as Person Correlation Coefficient (n=21)

Development stage	2011	Chemical composition						
		Protein	Fructose	Glucose	Sucrsoe	Maltose	Reducing sugars	Total sugars
Milk	Starch	-0.76**	-0.47*	-0.56**	0.28 ns	-0.66**	-0.53*	-0.51*
	Protein		0.29ns	0.38ns	-0.34ns	0.07ns	0.35ns	0.32ns
	Fructose			0.97***	0.14ns	0.53*	0.99***	0.99***
	Glucose				0.14ns	0.6**	0.99***	0.99***
	Sucrsoe					-0.11ns	0.14ns	0.2ns
	Maltose						0.57**	0.56**
	Reducing sugars							0.99***
Dough	Starch	0.23ns	-0.43ns	-0.41ns	0.37ns	-0.37ns	-0.43ns	-0.38 ns
	Protein		0.05ns	0.07ns	-0.16ns	-0.35ns	0.05 ns	0.04 ns
	Fructose			0.99***	0.12ns	0.11ns	0.99***	0.99***
	Glucose				0.09ns	0.09ns	0.99***	0.99***
	Sucrose					0.06ns	0.11ns	0.21ns
	Maltose						0.11ns	0.12ns
	Reducing suagrs							0.99***
Mature	Starch	0.63*	-0.49*	-0.44*	-0.05ns	0.55*	-0.27ns	-0.27ns
	Protein		-0.43ns	-0.44ns	0.29ns	0.17ns	-0.38ns	-0.37ns
	Fructose			0.98***	-0.43ns	-0.25ns	0.91***	0.91***
	Glucose				-0.43*	-0.16ns	0.94***	0.94***
	Sucrose					0.18ns	-0.37ns	-0.35ns
	Maltose						0.17ns	0.17ns
	Reducing suagrs							1***

For symbol explanation see legend of Table 22

Table 26: Correlation matrix between chemical components at mature-harvest stage across seven maize cultivars in two years, 2010 and 2011, as Person Correlation Coefficient

Year		Chemical composition						
		Protein	Fructose	Glucose	Sucrsoe	Maltose	Reducing sugars	Total sugars
2010 (n=21)	Starch	-0.71**	-0.5*	-0.38ns	-0.25ns	0.06ns	-0.39ns	-0.31ns
	Protein		0.24ns	0.19ns	0.33ns	0.05ns	0.025ns	0.32ns
	Fructose			0.73***	0.42ns	-0.2ns	0.73***	0.55**
	Glucose				0.75***	0.16ns	0.93***	0.85***
	Sucrsoe					0.53*	0.82***	0.98***
	Maltose						0.44*	0.52*
	Reducing sugars							0.92***
2011 (n=21)	Starch	0.63*	-0.49*	-0.44*	-0.05ns	0.55*	-0.27ns	-0.27ns
	Protein		-0.43ns	-0.44ns	0.29ns	0.17ns	-0.38ns	-0.37ns
	Fructose			0.98***	-0.43ns	-0.25ns	0.91***	0.91***
	Glucose				-0.43*	-0.16ns	0.94***	0.94***
	Sucrose					0.18ns	-0.37ns	-0.35ns
	Maltose						0.17ns	0.17ns
	Reducing suagrs							1***
Years *cultivars (n=42)	Starch	-0.2 ns	0.17 ns	0.10 *	-0.3***	0.59***	0.32 *	-0.15 ns
	Protein		-0.2 ns	-0.22 ns	0.07 ns	0.13 ns	-0.15 ns	0.15 ns
	Fructose			0.9 ***	-0.5***	0.26 ns	0.94 ***	0.6 ***
	Glucose				-0.4 **	0.26 ns	0.93 ***	0.7 ***
	Sucrose					-0.5***	-0.64 ***	0.08 ns
	Maltose						0.56 ***	0.22 ns
	Reducing suagrs							0.7 ***

For symbol explanation see legend of Table 22

Table 27: Correlation matrix between chemical components at mature stage in different maize cultivars across two years, 2010 and 2011, as Person Correlation Coefficient

Cultivar		Chemical composition						
		Protein	Fructose	Glucose	Sucrsoe	Maltose	Reducing sugars	Total sugars
Benecia	Starch	-0.53ns	-0.31ns	-0.32ns	0.37ns	-0.17ns	-0.31ns	09.27ns
	Protein		0.42ns	0.4ns	-0.53ns	-0.1ns	0.38ns	0.33ns
	Fructose			1***	-0.92*	0.88*	1***	0.99***
	Glucose				-0.91*	0.89*	1***	.99***
	Sucrsoe					-0.68ns	-0.9*	-0.83*
	Maltuse						0.9*	0.93**
	Reducing sugars							0.99***
Es	Starch	-0.55ns	-0.952**	0.89*	-0.98***	0.96**	0.95**	0.76ns
	Protein		-0.39ns	-0.44ns	0.55ns	-0.34ns	-0.38ns	0.11ns
	Fructose			0.98***	-0.99***	0.99***	1***	0.91*
	Glucose				-0.96**	0.95**	0.98***	0.92*
	Sucrsoe					-0.98***	-0.98***	-0.83*
	Maltose						0.99***	0.9*
	Reducing sugars							0.92*
Franki	Starch	-0.15ns	0.77ns	0.35ns	-0.87*	0.58ns	0.62ns	-0.86*
	Protein		0.26ns	0.69ns	0.28ns	0.39ns	0.44ns	0.56ns
	Fructose			0.81*	-0.89*	0.96**	0.97**	-0.71ns
	Glucose				-0.48ns	-0.88*	0.92*	-0.21ns
	Sucrsoe					-0.77ns	-0.77ns	0.96**
	Maltose						0.99***	-0.55ns
	Reducing sugars							-0.55ns
Justina	Starch	-0.25ns	0.01ns	0.07ns	-0.04ns	0.02ns	0.03ns	-0.02ns
	Protein		0.65ns	0.7ns	-0.84ns	0.61ns	0.64ns	-0.33ns
	Fructose			0.95**	-0.93**	0.99***	0.99***	0.13ns
	Glucose				-0.827*	0.98***	0.98***	0.33ns
	Sucrsoe					-0.88*	-0.89*	0.23ns
	Maltose						0.99***	0.25ns
	Reducing sugars							0.24ns
Lukas	Starch	0.13ns	0.48ns	0.5ns	-0.32ns	0.45ns	0.38ns	0.7ns
	Protein		0.78ns	0.75ns	-0.85ns	0.8ns	0.77ns	0.64ns

	Fructose			1***	-0.97**	0.99***	1***	0.84*
	Glucose				-0.95**	1***	1***	0.87*
	Sucrsoe					-0.95**	-0.96**	-0.68ns
	Maltuse						1***	0.86*
	Reducing sugars							0.85*
Sludo	Starch	-0.4ns	0.96**	-0.32ns	-0.9*	0.97**	0.92**	-0.69ns
	Protein		-0.53ns	-0.78ns	-0.02ns	-0.43ns	-0.72ns	-0.39ns
	Fructose			-0.12ns	-0.81ns	0.94**	0.96**	-0.55ns
	Glucose				0.67ns	-0.32ns	0.03ns	0.88*
	Sucrsoe					-0.89*	-0.70ns	0.93**
	Maltuse						0.93**	-0.67ns
	Reducing sugars							-0.4ns
PR	Starch	-0.59ns	0.63ns	0.32ns	-0.91*	0.71ns	0.61ns	-0.24ns
	Protein		-0.44ns	0.01ns	0.86ns	-0.61ns	-0.41ns	0.72ns
	Fructose			0.88*	-0.72ns	0.99***	1***	0.52ns
	Glucose				-0.43ns	0.83*	0.91*	0.75ns
	Sucrsoe					-0.81	-0.71ns	0.21ns
	Maltuse						0.99***	0.34ns
	Reducing sugars							0.54ns

For symbol explanation see legend of Table 22