

**EVALUATION OF THE POTENTIAL MALTING, BREWING AND ANTIYEAST  
CHARACTERISTICS OF SELECTED ZAMBIAN SORGHUM CULTIVARS**

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Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

## Abstract

The potential malting, brewing and antiyeast characteristics of twelve selected sorghum cultivars from Zambia (MMSH 375, 625, 707, 1038, 1077, 1194, 1257, 1356, 1363, 1365, 1376 and 1401) was evaluated. This was done through the determination of tannin content, proximate composition, %germinative energy (%GE), micro-structure, diastatic power (DP) and free amino nitrogen (FAN); as well as the antiyeast activity of N-containing compounds for each cultivar.

All cultivars were tannin-free and thus suitable for brewing potential exploration. Seven cultivars (MMSH-375, 625, 1038, 1194, 1257, 1401 and 1376) had a %GE considered appropriate for malting and brewing. A maximum of 25% of the DP level recommended for commercial malting was achieved in this study. In spite of the low DP observed in all cultivars after malting, high extract levels ranging between 18.7 and 25.5°P were achieved. In unmalted grains a significantly FAN level ( $p \leq 0.05$ ) was observed in three cultivars (MMSH-375, 1194 and 1077). These FAN values, however, represented less than 10% of the recommended FAN level necessary to support proper yeast functioning during wort fermentation. The 150 mg/L threshold which is necessary to support proper yeast function and is employed in modern day brewing was achieved in malts of sorghum cultivars MMSH-375 (intermediate cultivar) and 1401 (intermediate cultivar). A FAN level between 100 and 140 mg/L, which is considered enough to support optimal fermentation, was achieved in cultivars MMSH-625 (intermediate cultivar), 1077 (hard cultivar), 1194 (intermediate cultivar), 1356 (soft cultivar) and 1376 (intermediate cultivar) after malting. Mashing did not significantly increase FAN levels in all sorghum cultivars. The addition of potassium metabisulphite (KMS) in the mash did, however, lead to a further increase of FAN levels in all cultivars. Scanning electron micrographs revealed that the corneous endosperm was inaccessible to degradation by enzymes and only the soft endosperm was modified in all cultivars during malting.

The N-containing compounds from selected malts of sorghum cultivars with good malting and brewing potential (MMSH-375, 625, 1401 and 1194) were isolated and their antiyeast activity was determined. It was found that the N-containing compounds were playing an essential role during fermentation by contributing positively towards yeast growth in all four cultivars. However, a 20-30% reduced growth at the lower concentrations of the putative %yeast growth promoting N-containing compounds was observed in all cultivars. Irrespective of the reduced %yeast growth observed in all four cultivars at low concentrations of the putative yeast growth N-containing compounds, it could not be concluded that the sorghum cultivars utilised in this study possess antimicrobial peptides and proteins because only small compounds (<1 kDa) were detected with UPLC-MS. The dose-response data showed that extracts of MMSH-375 and MMSH-625 resulted in generally a higher %yeast growth when compared to other cultivars. The UPLC-MS also confirmed the dose response data that MMSH-375 has a better qualitative yeast-feeding value. MMSH-375 is thus rated as the best cultivar out of the four that were screened and is recommended to be used in the commercial production of sorghum lager beer.

## Opsomming

Die potensiële mout, brou en teen-gis eienskappe van twaalf geselekteerde Zambiese sorghum kultivars (MMSH 375, 625, 707, 1038, 1077, 1194, 1257, 1356, 1363, 1365, 1376 en 1401) was geëvalueer. Dit is gedoen deur die bepaling van tannien inhoud, proksimale samestelling, % ontkiemings energie (%OE), mikrostruktuur, diastatiese krag (DK) en vrye amino stikstof (VAS) inhoud; sowel as die teen-gis aktiwiteit van stikstofbevattende verbindings vir elke kultivar.

Alle kultivars was tannien vry en dus geskik om te ondersoek vir brou potensiaal. Sewe kultivars (MMSH 375, 625, 1038, 1194, 1257, 1401 en 1376) het 'n %OE geskik vir mouting en brouery gehad. 'n Maksimum van 25% van die voorgestelde DK vlak vir kommersiële brouery was gevind in hierdie studie. Hoë ekstrak vlakke van tussen 18.7 en 25.5°P is gevind, ten spyte van die lae DK wat gevind is in alle kultivars na mouting. 'n Betekenisvolle VAS vlak ( $p \leq 0.05$ ) was gevind in die ongemoute graankorrels van drie kultivars (MMSH 375, 1194 en 1077). Hierdie VAS vlakke was egter minder as 10% van die voorgestelde VAS vlakke benodig om gis funksie te ondersteun tydens fermentasie. Die 150 mg/L drumpelwaarde benodig vir korrekte gis funksie en gebruik in moderne brouery is bereik in gemoute sorghum kultivars MMSH 375 (intermediêre kultivar) en 1401 (intermediêre kultivar). 'n VAS vlak van tussen 100 en 140 mg/L, gereken as genoegsaam vir optimal fermentasie, was bereik in kultivar MMSH 625 (intermediêre kultivar), 1077 (harde kultivar), 1194 (intermediêre kultivar), 1356 (sagte kultivar) en 1376 (intermediêre kultivar) na mouting. Warm water weking het nie die VAS vlakke in die sorghum kultivars betekenisvol verhoog nie. Die byvoeging van kalium metabisulfiet (KMS) tot die weekvloeistof het egter gelei tot 'n verdere verhoging van VAS vlakke in alle kultivars. Skandering elektronmikrograwe het aangedui dat die growwe endosperm ontoeganklik is vir ensiematiese afbraak en dat slegs die sagte endosperm van alle kultivars veranderinge ondergaan het tydens mouting.

Die stikstofbevattende verbindings van die gemoute sorghum kultivars geselekteer op grond van hul goeie mout en brou potensiaal (MMSH 375, 625, 1401 en 1194) was geïsoleer en hul teen-gis aktiwiteit was bepaal. Daar was bevind dat die stikstofbevattende verbindings 'n noodsaaklike rol speel tydens fermentasie deur 'n positiewe bydrae tot gisgroei te maak in al vier kultivars. Verminderde groei (20-30%) was egter opgelet by laer konsentrasies van die vermeende groei-bevorderende stikstofbevattende verbindings in alle kultivars. Ten spyte van hierdie verminderde gisgroei by lae konsentrasies kon daar nie afgelei word dat die sorghum kultivars gebruik in hierdie studie wel antimikrobiële peptiede en proteïene bevat nie aangesien slegs klein verbindings (<1 kDa) waargeneem word met UPLC-MS. Die dosis-respons data het aangedui dat ekstrakte van MMSH 375 en 625 oor die algemeen gelei het tot hoër gisgroei in vergelyking met ander kultivars. UPLC-MS het ook bevestig dat MMSH 375 'n beter kwalitatiewe gisvoedingswaarde het. MMSH 375 word dus gereken as die beste kultivar uit die vier gekeurde kultivars en word aanbeveel vir gebruik in die kommersiële produksie van sorghum lager bier.

**Trust in the Lord and He will give you the desires of your heart**  
**Psalm 37:34**

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**List of abbreviations**

ATP	Adenosine triphosphate
Ala	Alanine
$\alpha$	Alpha
AACC	American Association Cereal Chemists
Anon	Anonymous
AMPs	Antimicrobial peptides
Arg	Arginine
Asp	Asparagine
BSSP1	Barley seed peroxidase 1
BP1	Barley-seed specific peroxidase
$\beta$	Beta
$\beta$ Glu	$\beta$ -glucanases
$^{\circ}$ C	Degrees celcius
DNA	Deoxyribonucleic acid
DP	Diastatic power
<i>et al.</i>	<i>et alibi</i> (and co-workers)
EBC	European Brewery Convention
Fig.	Figure
FHB	<i>Fusarium</i> head blight
FAN	Free amino nitrogen
$\gamma$	Gamma
GE	Germinative energy
GM	Grain mould
GW	Grain weathering
HP-TLC	High performance thin layer chromatography
h	Hour
i.e.	<i>idest</i> (that is)
Ile	Isoleucine
IOB	Institute of Brewing
ICC	International Association for Cereal Science and Technology
pI	Isoelectric point
kDa	Kilodalton
LSD	Least significant difference
Leu	Leucine
LC-MS	Liquid chromatography-mass spectrometry

<i>m/z</i>	Mass over charge
MIC	Minimum inhibitory concentration
Mr	Relative molecular mass
MMSH	Mount Makulu Sorghum Hybrid
N-containing	Nitrogen containing
Ns-LTPs	Non-specific lipid-transfer proteins
OD	Optical density
PR	Pathogenesis-related
PHD	Postharvest deterioration
KMS	Potassium metabisulphite
$K_2S_2O_5$	Potassium metabisulphite
PYF	Premature yeast flocculation
PIs	Proteinase inhibitors
$R_f$	Retardation factor
RNA	Ribonucleic acid
RIPs	Ribosome inactivating proteins
<i>S.cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SEM	Scanning electron microscopy
SDU/g	Sorghum Diastatic Unit/g
NaOH	Sodium hydroxide
NaCl	Sodium Chloride
NaClO	Sodium Hypochlorite
TOF-ESMS	Time of flight electrospray mass spectrometry
TLPs	Thaumatococcus-like proteins
TEA	Triethanolamine
TFA	Trifluoroacetic acid
Tyr	Tyrosine
UPLC	Ultra performance liquid chromatography
UPLC-MS	Ultra performance liquid chromatography linked to mass spectrometry
USA	United States of America
ZARI	Zambia Agricultural Research Institute
Val	Valine

**Chapter 1**  
Introduction

## Chapter 1

### Introduction

Sorghum grain has traditionally been used in the production of porridges, alcoholic beverages (opaque beer) and for bread making in certain parts of Africa and India for many centuries (Serna-Saldivar *et al.*, 1988). Sorghum was first considered a brewing adjunct in lager beer production during the second world war when the conventional brewing material (barley) was scarce (Obido *et al.*, 2002). Despite the initial early considerations, the most notable development took place in 1988 when a shift in Nigeria's food import policy banned the importation of wheat, barley malt and other temperate cereals (Palmer, 1992). This was due to a shortfall of foreign exchange and the food policy at the time dictated that local raw materials should be used when manufacturing cakes, biscuits, non-alcoholic beverages and beer (Palmer, 1992). This policy shift caused breweries in Nigeria to seek alternative local grains, and sorghum malt was identified as a suitable substitute for barley malt in commercial lager beer production (Nzelibe & Nwasike, 1995). Subsequent research conducted on the extract levels, protein and free amino nitrogen content of sorghum worts reaffirmed sorghum as a suitable alternative to barley in lager beer production (Owuama, 1999).

There has been a growing interest in the use of sorghum for the production of lager beer since the initial work done in Nigeria (Dewar *et al.*, 1997). Sorghum lager beer is brewed commercially in Uganda, Tanzania, Zambia and Zimbabwe (INTSORMIL, 2008). The utilisation of a locally grown crop as a brewing material has provided a boost to local economies in Africa, where it has benefited sorghum farmers with guaranteed income and thus reducing unemployment (INTSORMIL, 2008). In addition, the use of a local crop reduces logistical costs for manufacturers resulting in reduced beer retail prices (Mackintosh & Higgins, 2004). Manufacturers have also been able to negotiate lower taxes with government on sorghum-based beer which has also contributed to another significant cost reduction of particular benefit to the consumer (Mackintosh & Higgins, 2004; Taylor *et al.*, 2013). It has also provided an affordable lager beer to consumers for whom this type of beer was previously unaffordable (INTSORMIL, 2008), which has led to an increased growth in the brewing industry (Mackintosh & Higgins, 2004; Taylor *et al.*, 2013). In developed countries, where sorghum was previously primarily used for animal feed, the demand for sorghum in the production of gluten-free foods and beverages is increasing (Dicko *et al.*, 2006; Veith, 2009). In the United States of America (USA) alone, there are about 3 million coeliacs (Taylor *et al.*, 2006) requiring gluten-free foods and beverages. Commercially produced lager beer (e.g. Bard's Tale) using malted sorghum is currently available on the market in the USA as a gluten free beer (Veith, 2009).

In spite of sorghum lager brewing being successful, particularly in Africa, the use of sorghum in the brewing industry is linked to malting, brewing and fermentation problems (Taylor *et al.*, 2006). Sorghum grain has high malting losses, and poor wort filtration characteristics (Dale *et al.*, 1990; Bajomo & Young, 1993). Malting losses are attributable to sorghum grains losing up to 25%

starch because of respiration which takes place during malting (Aisien & Muts, 1987). Sorghum malting losses can be reduced by increasing the length of air-rest and incorporating dilute alkaline (0.1% NaOH) liquor into the steep regime (Ezeogu & Okolo, 1994; Okolo & Ezeogu, 1996). When brewing with barley malt, the husk functions as a filter bed during lautering. The absence of a husk in sorghum has led to difficult wort filtration in the past, and this was considered a major problem when brewing sorghum lager beer. This problem was solved in the 1990s by developing tangential-flow mash filters with automatic discharge of spent material (Hermia & Rahier, 1992).

Compared to barley, sorghum malt has lower levels of  $\beta$ -amylase and the starch has a higher gelatinisation temperature (Taylor & Robbins, 1993; Agu & Palmer, 1998; Taylor *et al.*, 2006). These differences cause decreased breakdown of starch and the production of less fermentable sugars in sorghum wort when compared to barley wort (Taylor *et al.*, 2006). Sorghum malt  $\beta$ -amylase activity can be beneficially manipulated by germination time (7 days), temperature (24-28°C) and moisture conditions (Abiodun, 2002). The development of a decantation mashing method has resolved the differences in gelatinisation and saccharification temperatures of sorghum malt starch. In this process mashing is carried out at 45°C for 30 min. A portion of the mash the 'active enzymic liquid' is then separated from the grist prior to gelatinisation. Thereafter, the grist is immediately heated to 80°C or 100°C in order to gelatinise the starch. The mash is then cooled and added to the previously separated (active enzymic liquid) to achieve a conversion temperature of 65°C (Etokakpan, 1988). This mashing method produces worts with extracts similar to those of barley malts (Ogbonna, 2011).

Despite the work that has been done to improve the efficacy of sorghum lager beer production, there are still challenges experienced during fermentation (INTSORMIL, 2008; Ng'andwe, 2008). When brewing with barley malt, fermentation problems such as incomplete fermentations and premature yeast flocculation (PYF) are occasionally encountered (Van Nierop, 2005). This negatively affects fermentation because it leads to poor attenuation of sugars and consequently a low final concentration of alcohol (Porter *et al.*, 2010). Malts which are associated with PYF showed a high level of antimicrobial activity in barley grains (Van Nierop, 2005). The high level of antimicrobial activity in grains is believed to be because of microbial contamination of barley (while still in the field or during malting) which leads to increased production of antimicrobial peptides (AMPs) and proteins collectively known as Pathogenesis-related (PR) proteins in the grains (Gorjanović *et al.*, 2004).

PR proteins were originally defined as proteins induced only in pathological or related situations (Antoniw & White, 1980; Van Loon *et al.*, 1994). However, subsequent studies have confirmed the presence of PR proteins in normal (uninfected) plant tissues and cereal grains, their concentration is increased during fungal infection (Van Loon & Van Strien, 1999; Chandrashekar & Satyanarayana, 2006). PR proteins form part of the innate defense system which plants developed in order to resist a wide range of pathogenic microorganisms and insects (Gorjanović, 2007). These proteins have been isolated and studied in a number of plant species including

cereals such as wheat (Molano *et al.*, 1979), rice (Muthukrishnan *et al.*, 2001), maize (Wu *et al.*, 1994) barley (Gorjanović, 2007) and sorghum (Chandrashekar *et al.*, 2000). However, in comparison to other cereal grains, little is known about sorghum PR proteins (Muthukrishnan *et al.*, 2001).

AMPs in barley malt are thought to be one of the factors leading to fermentation problems (Van Nierop *et al.*, 2008). These peptides are very stable and are able to withstand the harsh conditions of the malting and brewing processes (Gorjanović, 2007). Their robust structures are attributed to multiple disulphide bridges which are known to confer thermal stability (Van Nierop *et al.*, 2008). AMPs also have powerful fungicide activity (Castro & Fontes, 2005) and consequently disturb normal yeast metabolism patterns during fermentation (Van Nierop *et al.*, 2008). One of the most notable AMPs in the brewing industry are thionins, which have been found to have toxic effects against yeast and bacteria (Castro & Fontes, 2005).

PR proteins have been isolated from sorghum and also found to have antifungal activity (Chandrashekar & Satyanarayana, 2006). In contrast to AMPs in barley, the effect of sorghum PR proteins on brewer's yeast is unknown. It has been reported that during small scale laboratory sorghum wort fermentation the same yeast crop was used for over five consecutive fermentations without negatively affecting yeast growth (Bajomo & Young, 1994). However in the commercial production of sorghum lager beer only up to three consecutive fermentations can be achieved using the same yeast crop (Ng'andwe, 2008). Despite the fact that laboratory fermentation systems and conditions differ significantly from those obtained in commercial practice (Bajomo & Young, 1994) there could be other factors playing a role in these observed differences. It is believed that sorghum PRs could be adversely affecting the physiological status of brewer's yeast by decreasing its viability and vitality. This could possibly partially explain the fermentation problems (PYF, incomplete fermentation and re-pitching problems) often encountered in the sorghum brewing industry.

The aim of this project was thus to:

- determine the malting and brewing potential of 12 selected sorghum cultivars and;
- isolate N-containing compounds from selected malts of interest (sorghum cultivars with good malting and brewing potential) and determine their antiyeast activity.

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**Chapter 2**  
Literature review

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## 1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is a cereal crop which belongs to the grass family *Poaceae* (Agu & Palmer, 1998b). It originated in about 3000 B.C. in North Africa and is reported to have been cultivated in Egypt as early as 2200 B.C. (Kent & Evers, 1994). Sorghum is a gluten-free cereal that can be consumed by coeliacs (Taylor *et al.*, 2006; Veith, 2009). A unique feature of this cereal is that it can be grown under both drought and water logged conditions (Obido *et al.*, 2002; Taylor *et al.*, 2006). This makes sorghum an important cereal crop, particularly in tropical countries. In these countries the cultivation of temperate cereals such as barley is not feasible (Dufour & Mélotte, 1992), and sorghum is a lucrative alternative for the production of lager beer.

Sorghum is Africa's fourth most important crop in terms of tonnage after maize, rice and wheat (FAO, 2012). In 2012 the total world production of sorghum was estimated at about 57.1 million tons, of which 40% was cultivated in Africa (FAO, 2012). The United States of America (USA), Australia and other developed countries cultivate sorghum primarily for animal feed while developing countries utilise it both for animal feed and human nutrition (Dicko *et al.*, 2006). To this end, more than 300 million people in developing countries rely on sorghum as an energy source (Bajomo & Young, 1993; Taylor & Robbins, 1993). In certain parts of Africa and India sorghum grain has traditionally been utilised in the production of porridges, alcoholic beverages and for bread making (Agu & Palmer, 1998b). In developed countries, where sorghum was previously primarily used for animal feed, the demand for sorghum in the production of gluten-free foods and beverages is increasing (Veith, 2009). In USA alone, there are about 3 million coeliacs (Taylor *et al.*, 2006) requiring gluten-free foods and beverages. Commercially produced lager beer (e.g. Bard's Tale) using malted sorghum is currently available on the market in the USA as a gluten-free beer (Veith, 2009).

Sorghum was first considered a brewing adjunct in lager beer production during the second world war when the conventional brewing material (barley) was scarce (Obido *et al.*, 2002). In 1988 a significant shift took place in Nigeria's food policy when the importation of wheat, barley malt and other temperate cereals were banned (Nzelibe & Nwasike, 1995; Agu & Palmer, 1998a). This was due to a shortfall of foreign exchange and a new food policy which determined that local raw materials should be used when manufacturing cakes, biscuits non-alcoholic beverages and beer (Palmer, 1992). This policy caused the breweries in Nigeria to seek alternatives and sorghum malt was identified as a suitable substitute for barley malt in commercial lager beer production (Nzelibe & Nwasike, 1995).

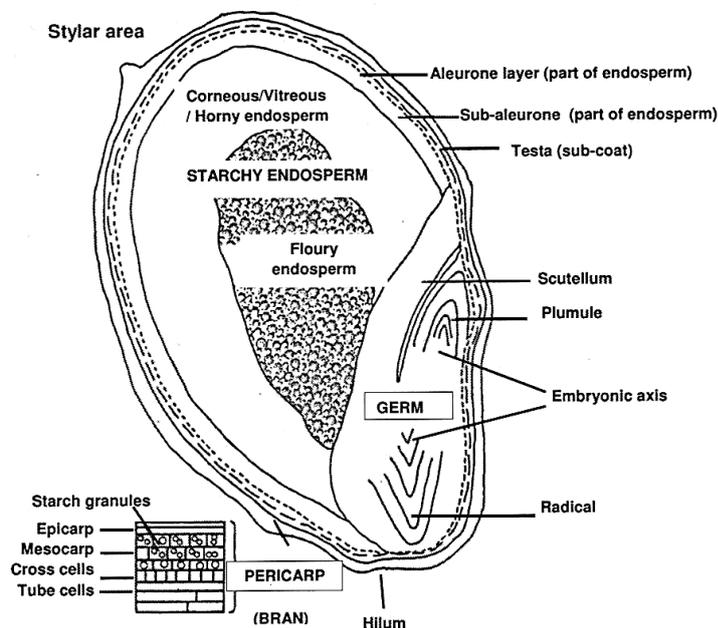
There has been a growing interest in the use of sorghum in the production of clear lager style beer since the initial work done in Nigeria (Dewar *et al.*, 1997). Sorghum lager beer is brewed commercially in Uganda, Tanzania, Zambia and Zimbabwe (INTSORMIL, 2008).

The utilisation of this local crop as a brewing material in Africa has provided a boost to the local economies where it has benefited farmers with guaranteed income (INTSORMIL, 2008). Furthermore, the lowered beer production costs associated with the use of a local crop has been augmented by reduced government-imposed taxes on locally produced products. These benefits in turn have resulted in a reduction of the beer's retail selling price, leading to increased growth in the sorghum lager beer brewing industry (Mackintosh & Higgins, 2004).

This literature review will focus on sorghum grain and malt as an alternative cereal to barley malt in lager beer production. Sorghum structure, the malting and brewing process as well as challenges experienced during wort fermentation will be discussed in detail.

## 2. Sorghum grain structure

Sorghum grains (Fig. 2.1) are described as round caryopses which lack a husk (Palmer, 1992). The grains can either be red, white, yellow or brown in colour (Hoseney, 1994) and weigh between 3 and 80 mg (Waniska, 2000). There are three major anatomical parts in the grain: the pericarp (7.9%), germ (9.8%), and starchy endosperm (82.3%) (Hoseney, 1994; Wrigley, 2004). Sorghum grains are composed of starch (75-79%), protein (9.0-14.1%) and lipid (1.5-5.0%) (Wrigley, 2004).



**Figure 2.1** Longitudinal section through a sorghum grain (Taylor & Belton, 2002).

### 2.1 Pericarp

The pericarp is a coat fused to the periphery of the sorghum grain (Palmer, 1992). It is made up of three layers, the endocarp, the mesocarp and the epicarp (Wrigley, 2004) (Fig. 2.1). The endocarp is the innermost layer of the pericarp (Rooney & Miller, 1982) and is comprised of cross and tube cells (Wrigley, 2004). The tube cells are responsible for the transportation of water during grain germination and the cross cells form a layer which prevents the loss of moisture (Waniska, 2000).

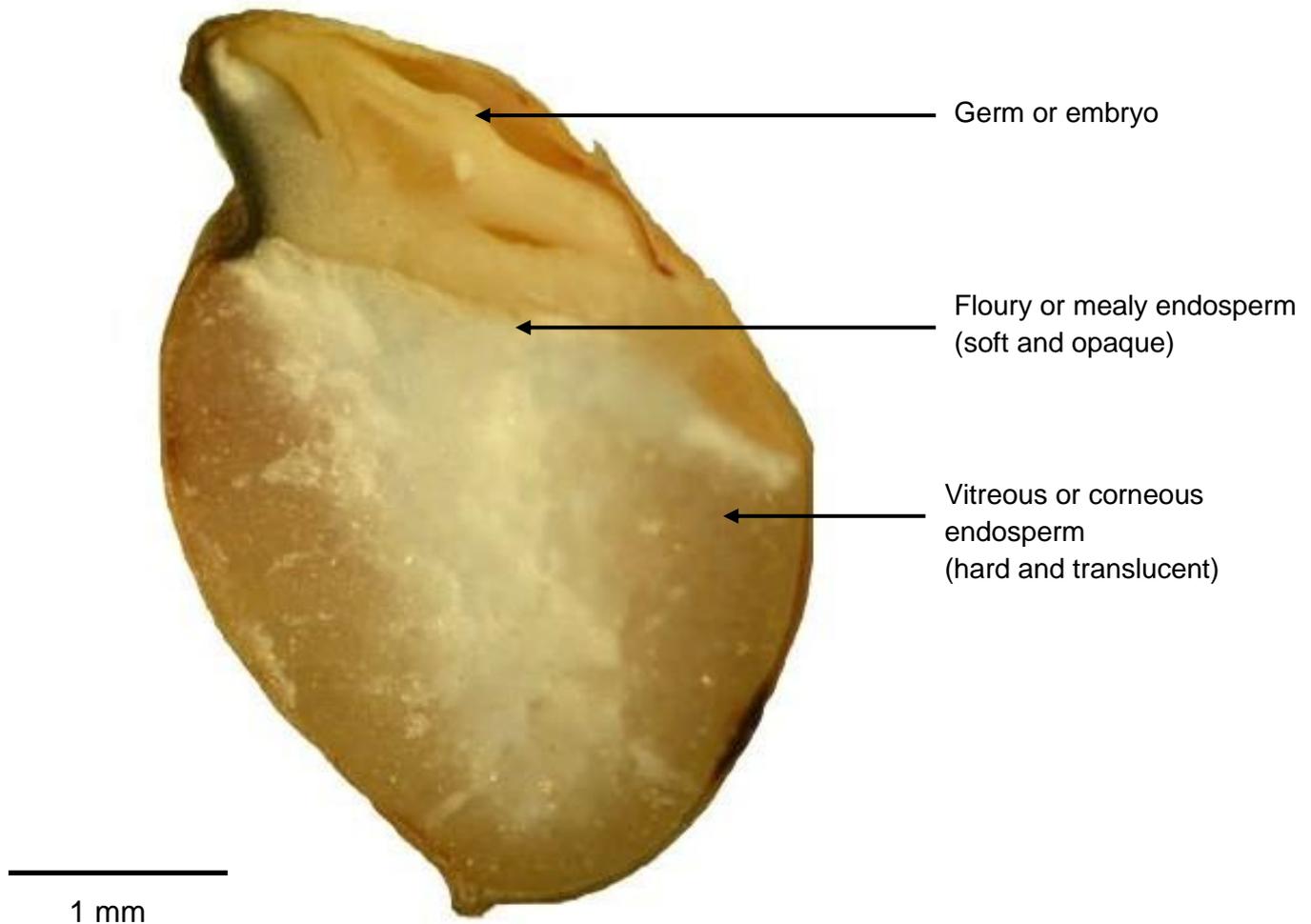
When compared to other cereals, sorghum is unique because it contains starch granules, arranged in layers in the mesocarp, which determines the thickness of the pericarp (Wrigley, 2004). A thick pericarp contains three or four layers of mesocarp which are filled with starch granules (Waniska, 2000). The thickness of the mesocarp layer is controlled by the Z-gene and varies in different sorghum cultivars (Rooney & Miller, 1982). The thickness of this layer also plays an important role in disease resistance. Sorghum grains with a thin mesocarp contain less starch and are therefore less susceptible to microbial attack (Rooney & Miller, 1982). The mesocarp is also important for the transportation of moisture and nutrients during germination and grain development (Wrigley, 2004). The pericarp plays a major role in sorghum processing because of its variable content of polyphenols and its thickness (Taylor & Schüssler, 1986).

### 2.2 Germ

The germ is made up of two major parts, the embryonic axis and the scutellum (Rooney & Miller, 1982) (Fig. 2.1). The embryonic axis contains the new plant, which forms during germination. The embryonic axis can be divided into the radicle and the plumule (Waniska, 2000). The scutellum cells contain lipid, protein bodies, enzymes, minerals and a few starch granules (Rooney & Miller, 1982; Waniska, 2000).

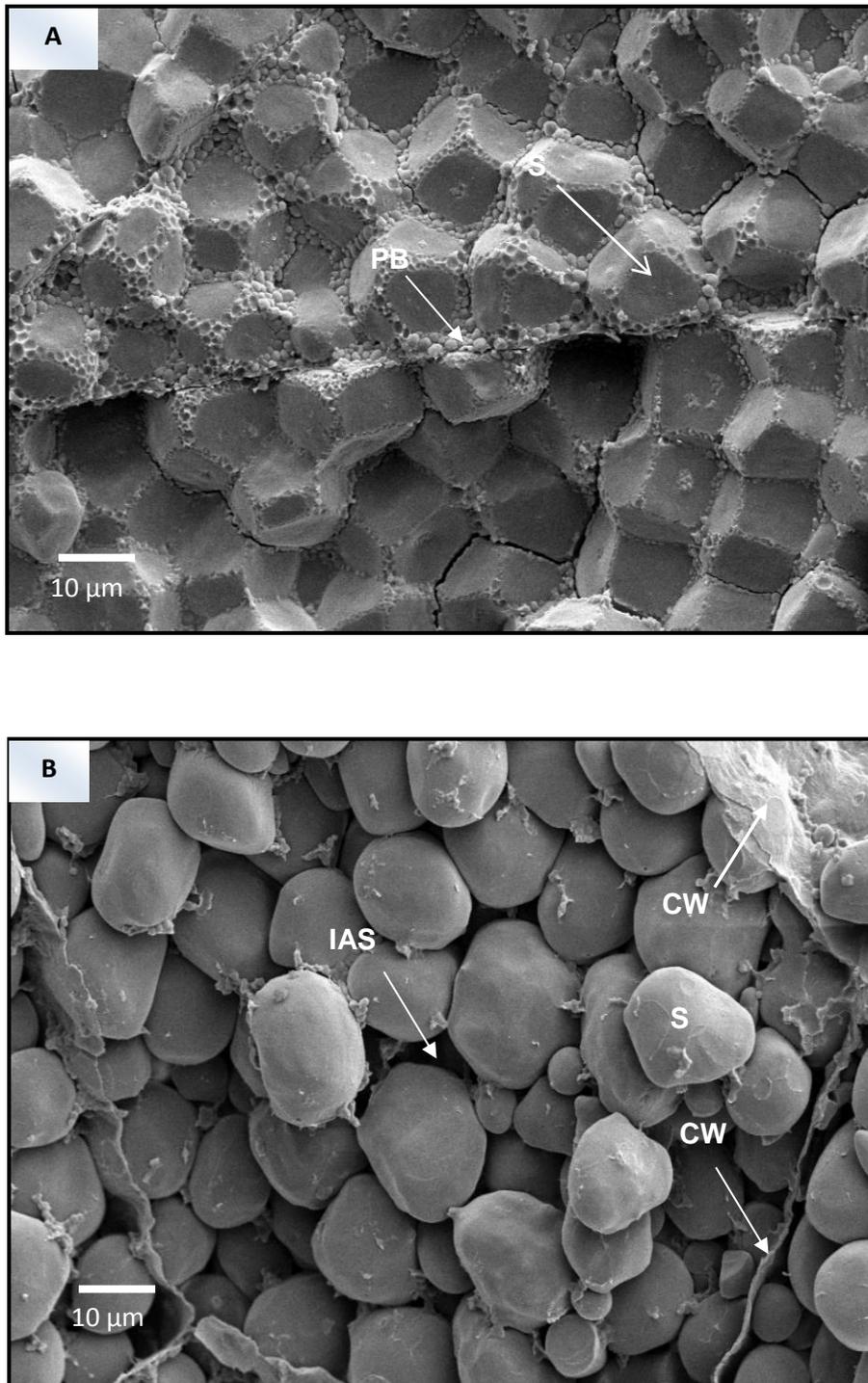
### 2.3 Endosperm

Sorghum endosperm is the largest tissue in the grain and mainly contains starch granules, storage proteins and cell wall material (Dicko *et al.*, 2006). It is located below the pericarp and is covered by the aleurone layer, which is a single layer of cells. Sorghum endosperm is divided into two types (Fig. 2.2), i.e. flourey (soft and opaque) and vitreous (hard and translucent) (Chandrashekar & Satyanarayana, 2006). The vitreous endosperm encloses the inner flourey endosperm (Palmer, 1992) as can be seen in Fig. 2.2.



**Figure 2.2** Stereo micrograph of a longitudinal cross-section through a sorghum grain.

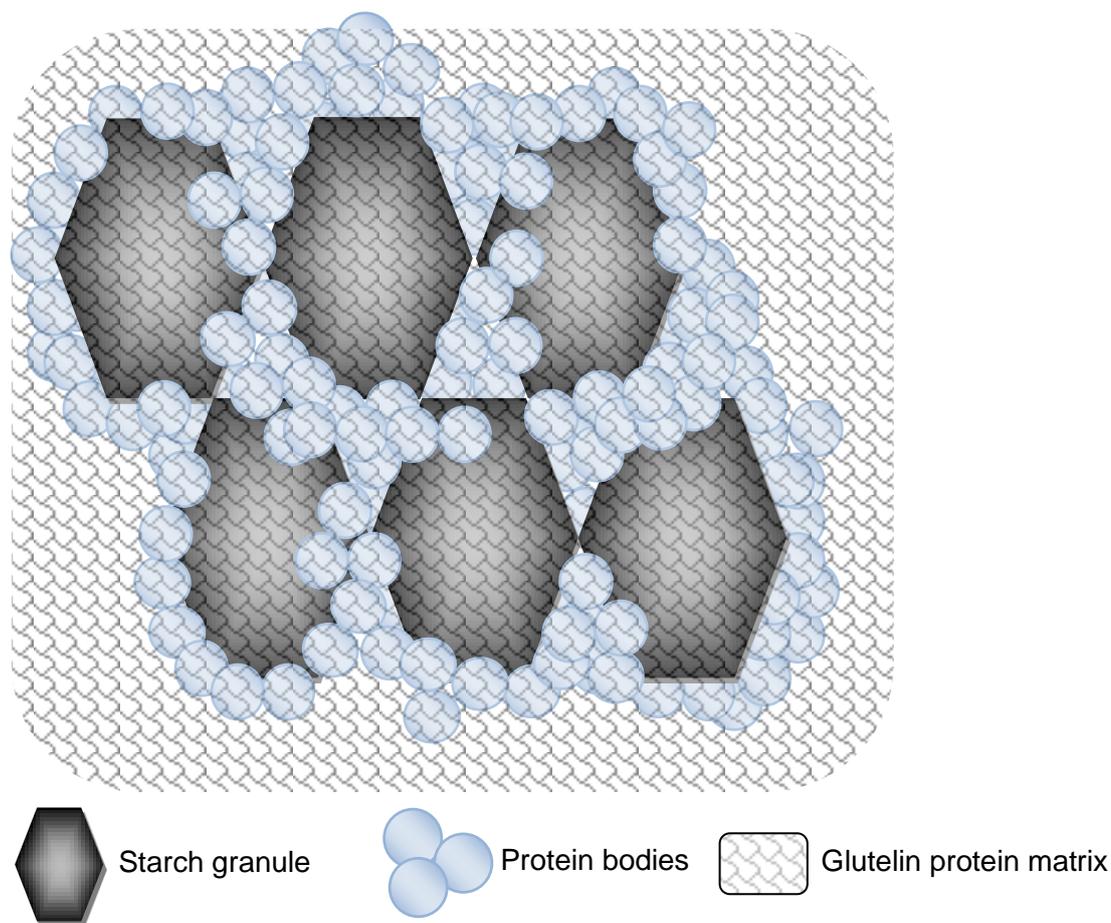
The vitreous endosperm (Fig. 2.3a) is a tightly packed structure which has no intergranular air spaces whereas the floury endosperm (Fig. 2.3b) has large intergranular air spaces (Hoseney *et al.*, 1974). Apart from these air spaces, the floury endosperm (Fig. 2.3b) is relatively free of protein bodies and predominately contains starch (Rooney & Miller, 1982; Chandrashekar & Kirleis, 1988). In contrast, the vitreous endosperm contains a number of protein bodies (Fig. 2.3a) with a diameter between 0.4 and 2  $\mu\text{m}$  (Rooney & Miller, 1982; Chandrashekar & Kirleis, 1988).



**Figure 2.3** Scanning electron micrograph of vitreous (A) and floury (B) sorghum endosperms; PB = protein bodies, S = starch granule, IAS = intergranular air space, CW = cell wall.

In the endosperm, protein bodies surround starch granules and are embedded in a glutelin protein matrix which binds the protein bodies and starch granules together (Taylor *et al.*, 1985; Mazhar & Chandrashekar, 1995) (Fig. 2.4). The cell wall material of sorghum (Fig. 2.3b) contains about 30% (w/w)  $\beta$ -D-glucan, 5% (w/w) pentosan (arabinoxylans) and 60% (w/w) protein (Etokakpan & Palmer, 1994). During sorghum malting the cell wall remains

intact and modification of the endosperm is mainly due to starch degradation in the soft endosperm (Glennie *et al.*, 1983).



**Figure 2.4** Starch granules and protein bodies in the vitreous endosperm, embedded in a glutelin protein matrix (adapted from de Mesa-Stonestreet *et al.*, 2010).

The proportion of the two endosperm types (floury and vitreous) determines the hardness of the grain. Grain hardness is a major deterrent to microbial infection in low tannin grains (Palmer, 1992). During storage, insects also attack sorghum grains with a large proportion of the soft, mealy endosperm rather than sorghums with hard, vitreous endosperms (Rooney & Miller, 1982). It is proposed that protein bodies in the hard endosperm cause starch granules to be inaccessible to degradation by pathogens, while in contrast starch granules in the soft endosperm which are relatively free of protein bodies and are more exposed. This is attributable to the presence of kafirins (sorghum prolamin storage proteins) in the hard endosperm of the sorghum grain at higher concentrations than in grains with soft endosperm (Mazhar & Chandrashekar, 1995).

### 3. Sorghum storage proteins

Seed storage proteins are classified by Osborne into four groups based on their solubility in a series of solvents; prolamins (alcohol/water mixtures), glutelins (dilute acid or alkali), albumins (water) and globulins (dilute saline) (Shewry *et al.*, 1995). Approximately 80% of sorghum grain protein is located in the endosperm, 16% in the germ and 3% in the pericarp (Taylor & Schüssler, 1986).

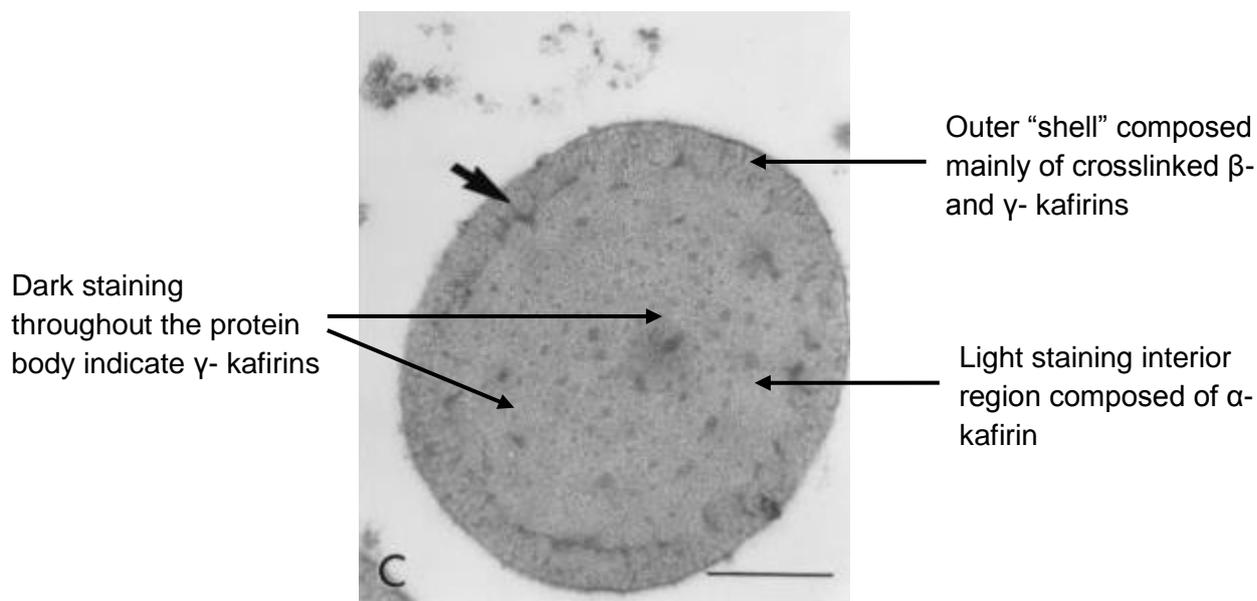
#### 3.1 Kafirins

Kafirins are also characteristically highly hydrophobic in comparison to the other cereal prolamins (Belton *et al.*, 2006). They account for approximately 70-80% of the total protein in sorghum grains and are mainly located in the hard endosperm inside protein bodies (Oria *et al.*, 2000). Kafirins are divided into four groups based on molecular weight (Mr), electrophoretic mobility and alcohol solubility i.e.  $\beta$  (16 000, 18 000 and 20 000),  $\gamma$  (28 000),  $\alpha$  (23 000 and 25 000) and  $\delta$  (14 400 and 21 000) (Shull *et al.*, 1991). Alpha-kafirins make up approximately 80% of the total kafirin,  $\beta$ -kafirin comprises approximately 5% and  $\gamma$ -kafirin approximately 15% (Hamaker & Bugusu, 2003). Delta-kafirins have only been identified at gene and transcript level and not at protein level (Belton *et al.*, 2006).

The outer “shell” of protein bodies is mainly composed of  $\beta$ - and  $\gamma$ -kafirins and the inside predominately consists of  $\alpha$ -kafirins (Fig. 2.5) (Oria *et al.*, 2000; de Mesa-Stonestreet *et al.*, 2010). The disulphide-linked polymeric nature of the  $\beta$ - and  $\gamma$ -kafirins found in the outer shell maintains the rigid structure of protein bodies. This structural role was observed in a study on high digestibility sorghum mutants where a change in the location of the proteins from the outer shell of the protein bodies resulted in altered structure (Hamaker & Bugusu, 2003).

During germination and mashing the protein in sorghum grains is broken down by enzymes (exogenous, or those produced during malting if sorghum malt is utilised) into free amino nitrogen (FAN) which is required as a nutrient for yeast growth (Taylor & Boyd, 1986). FAN is produced by proteolysis of mainly kafirins during malting and mashing of sorghum grains (Taylor *et al.*, 1985). The  $\gamma$ - and  $\beta$ -kafirins, which contain high proportions of the sulphur-containing amino acid cysteine (Shull *et al.*, 1992), are the first to be digested (Mazhar & Chandrashekar, 1993). The disulphide bonding of  $\gamma$ - and  $\beta$ -kafirins, and possibly other proteins located in the outer “shell” of protein body, result in the formation of enzymatically resistant protein polymers which retard the digestion of  $\alpha$ -kafirin (Oria *et al.*, 1995). The difficulty experienced in digesting the unreduced  $\alpha$ -kafirin could therefore be due to its location inside protein bodies (Oria *et al.*, 1995). The addition of reducing agents such as 2-mercaptoethanol (Hamaker *et al.*, 1987), sodium bisulphite (Ng'andwe *et al.*, 2008) and ascorbic acid (Arbab & El Tinay, 1997) have been found to decrease molecular disulphide

bridges present in kafirins. Reducing agents therefore increase sorghum protein digestibility and consequently promote higher FAN production.



**Figure 2.5** Transmission electron micrograph of a normal sorghum protein body (Bar = 0.5  $\mu$ m) (Oria *et al.*, 2000).

### 3.2 Glutelins

After kafirins, glutelins are the second largest individual protein fraction in sorghum grains (Taylor & Schüssler, 1986). Glutelins account for approximately 13.6 to 17.3% of the total protein in sorghum grains (Taylor & Schüssler, 1986) and are located mainly within the endosperm and the pericarp (Taylor *et al.*, 1984). They constitute the protein matrix which surrounds the protein bodies in the endosperm (Taylor *et al.*, 1984) (Fig. 2.4). The glutelin matrix (Fig. 2.4) which binds protein bodies and starch granules has an adverse effect on beer production (Wong *et al.*, 2009). During sorghum malting, the hard endosperm remains completely unmodified due to its tightly packed starch-protein matrix which is inaccessible to degrading enzymes (Aisien & Muts, 1987). The strength of the protein-starch adhesion is however cultivar dependent (Hoseney *et al.*, 1974).

### 3.3 Albumins and globulins

Albumins and globulins are located mainly in the germ. They account for approximately 16% of the grain's total protein (Taylor & Schüssler, 1986). These proteins include biologically important proteins such as enzymes, nucleoproteins, membrane and glycoproteins (Wall & Paulis, 1978).

## 4. Sorghum beer production

The production of beer involves three processes namely malting, brewing and fermentation and two important ingredients, the malted or unmalted grains and yeast (Owuama, 1999).

### 4.1 Malting

Malting involves steeping, germination and kilning (Owuama, 1997). The main aim of malting sorghum grains is for it to develop enzymes which break down the cell wall material and starchy endosperm to fermentable sugars as well as proteins to amino acids and peptides which are required for yeast nutrition in fermentation ( Taylor, 1982; Palmer, 1992). Enzymes which are produced during malting include  $\alpha$ -amylases,  $\beta$ -amylases,  $\alpha$ -glucosidase (maltase), peroxidases, lipases, carboxypeptidases and proteinases (Owuama, 1999).

Alpha-amylases catalyse the hydrolysis of starch available in the grain to dextrans (O'Rourke, 2002). The production of  $\alpha$ -amylases requires oxygen and can be inhibited in the presence of excess carbon dioxide (Owuama, 1999). Beta-amylases catalyse the hydrolysis of starch and dextrans to give a mixture of glucose, maltose, maltotriose (O'Rourke, 2002). Sorghum grains have no  $\beta$ -amylase activity (Owuama, 1997). A significant amount of  $\beta$ -amylase is produced in the germ during malting (Palmer *et al.*, 1989). During malting  $\beta$ -amylase is also activated from the starchy endosperm (Palmer *et al.*, 1989) and depends on germination time and temperature (Owuama, 1997). Approximately 18% to 39% of the saccharifying activity of sorghum malt is because of  $\beta$ -amylase (Owuama, 1997). Within two days of germination,  $\beta$ -amylase activity increases rapidly and this rate of increase declines at about 6.5 days (Owuama, 1999). Diastatic power is a measurement of the combined activity of  $\alpha$ - and  $\beta$ -amylases and is more essential in sorghum malt than wort (Owuama, 1997). The activity of amylase in sorghum is 11 to 41 SDU/g (Sorghum Diastatic Unit/g) (Owuama, 1999). Alpha-glucosidase is an enzyme which is also involved in starch degradation during sorghum grain germination to yield glucose (Manners, 1974). It is however not the dominant glucose producing enzyme in sorghum malt because it is possible for malt with high levels of  $\alpha$ -glucosidase to produce wort with low glucose levels (Owuama, 1999).

Lipases catalyse the hydrolysis of triglycerides to glycerol and fatty acids (Jaeger & Eggert, 2002). Sorghum has higher levels of lipids than barley which are further converted to hydroperoxides and aldehydes by lipoxygenases present in malt. This has a negative effect on beer because it leads to poor beer acceptability and reduces the shelf-life (Osagie, 1987; Kobayashi *et al.*, 1993). However, kilning results in a decrease in sorghum malt lipase activity to between 24 and 66% of the total lipase activity in green malt (Owuama, 1999). Moreover, mashing at 65°C results in wort without lipase activity (Nwanguma *et al.*, 1996).

Carboxypeptidases catalyse the hydrolysis of proteins to FAN which is important for the yeast during wort fermentation (Owuama, 1999).

#### 4.1.1 Steeping

When steeping, sorghum grains are soaked in water at 25°C for 24 h (Palmer, 1992). The purpose of steeping sorghum grains is to primarily increase their moisture content to about 33-36% (Agu & Palmer, 1998b) and to also initiate the metabolism of the living tissues which are dormant when the grains are dry (Briggs, 1998). It also removes some pigments, microorganisms and bitter substances from the grains (Owuama, 1997). During the first 4 h grains can be steeped in a dilute alkali solution in order to increase the rate of water uptake (Dewar *et al.*, 1997). Increase in moisture to above optimal content destroys the germ and results in a decrease in extract and malt diastatic power (Owuama, 1997; Owuama, 1999). It is therefore important for the steeping process to be regulated so that a suitable moisture content of the grains can be achieved (Owuama, 1999). To prevent microbial infection of steeped sorghum grains, 0.1% formaldehyde may be added during the steeping process (Palmer, 1992).

#### 4.1.2 Germination

When steeping is complete, the grains are allowed to germinate for a maximum of 7 days (Palmer, 1992). Germination involves the development and growth of the plumule and radicle in sorghum grains and consequently the production of hydrolytic enzymes (Owuama, 1999). It is conducted at 25°C and at a relative humidity of between 90% and 100% or just above 90% (Abiodun, 2002). While the grains are germinating their moisture content is kept above 40% by sprinkling water daily and therefore hydrating the grains. Some enzymes produced during germination break down cell walls and some proteins (FAN production) present in the starchy endosperm (endosperm modification), causing grains to be friable and therefore easier to mill (Bamforth, 2006). The enzymes in malt degrade starch, proteins, adjuncts and other reserves in malt into a fermentable extract during mashing (Taylor & Boyd, 1986).

#### 4.1.3 Kilning

To terminate germination the malt is placed in a kiln or oven at 50°C for 24 hours (Owuama, 1999). During kilning the moisture content of the malt is decreased to a level which arrests endosperm modification (Bamforth, 2006). This process prevents denaturing of hydrolytic enzymes present in malt and contributes to the development of the colour and flavour of sorghum malt. The duration of kilning, temperature and moisture content of the malt influences amylase activity of sorghum malt (Owuama, 1999). If temperatures higher than

50°C are used, the already low amylase activity will be significantly reduced (Dewar *et al.*, 1995).

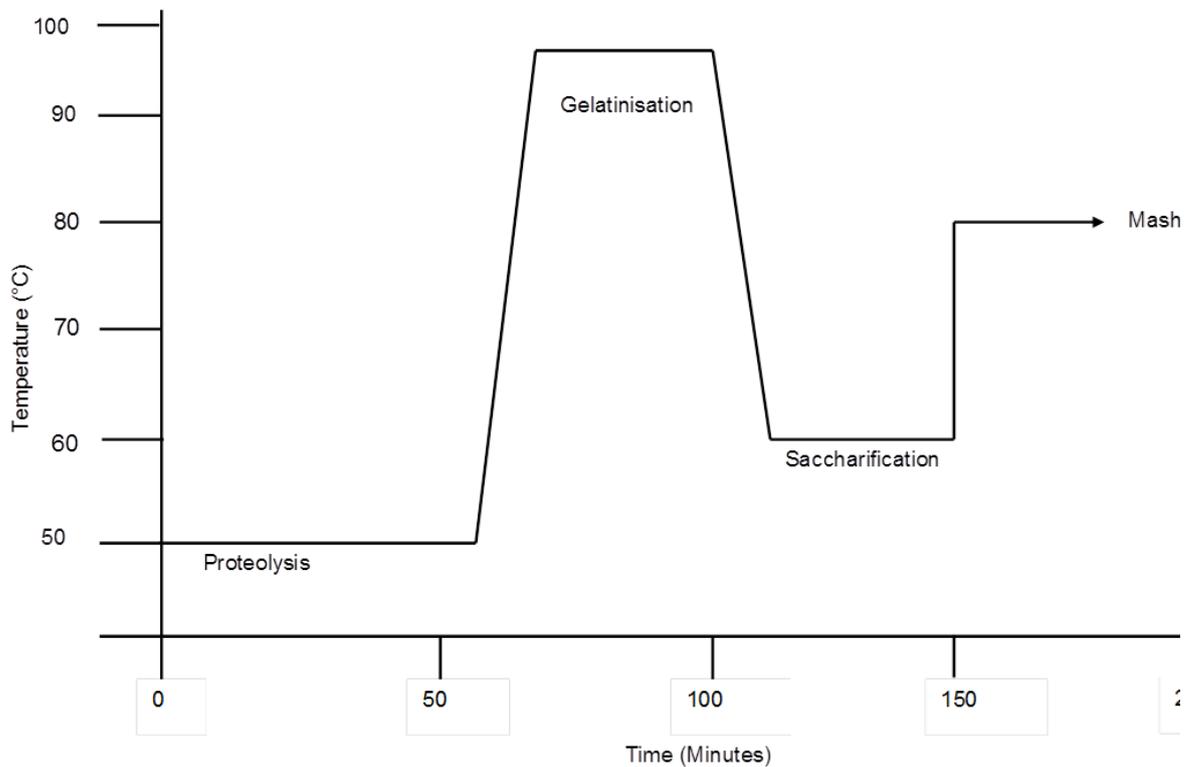
#### 4.2 Brewing

In the brewing industry sorghum can be used for three purposes. Firstly, it can be used as brewing adjunct. Adjuncts are materials which serve as sources of starch in order to increase the specific gravity of wort (Owuama, 1999). When adjunct brewing is used, malted sorghum or malted barley serves as a source of hydrolytic enzymes (Owuama, 1999). Secondly, malted sorghum can be used instead of malted barley. In this scenario sorghum malt provides both hydrolytic enzymes and starch. Thirdly, unmalted sorghum grains, together with exogenous enzymes, can be utilised in order to produce lager beer. It is important for exogenous enzymes to be added in this scenario because enzyme activity of unmalted sorghum grain is very low (Bajomo & Young, 1992).

Before brewing, malted or unmalted grains are finely milled then mixed with mashing water with a correct mix of salts in order to prepare a mash (Bamforth, 2006). This process is called mashing and it is an important step in the brewing industry because it yields wort which serves as a yeast growth and fermentation medium (Olatunji *et al.*, 1993). During mashing water soluble substances dissolve in water and enzymes then hydrolyse proteins and carbohydrates (Owuama, 1997).

##### 4.2.1 Brewing with unmalted sorghum grain

For the industrial production of sorghum lager beer a single infusion method is recommended with temperature stands at 50°C, 80-95°C and finally 60°C (Fig. 2.6) (Little, 1994; Lamidi, 1995). In this method, unmalted sorghum grains are milled finely and mixed with mash water at 50°C (sorghum ratio of 3:1). Thereafter,  $\text{Ca}(\text{OH})_2$  is added to give  $\text{Ca}^{2+}$  ions a concentration of 100 mg/L and consequently adjustment of the pH of the mash from 6.5 to 7.0. A mixture of enzymes consisting of neutral proteases, thermostable  $\alpha$ -amylase and  $\beta$ -glucanase is added to the mixture after pH adjustment. The mixture is then allowed to stand for 30 min. After this period more thermostable bacterial  $\alpha$ -amylases are added and the mash is heated to 80°C at the rate of 1°C/min. After a 10 min rest the mash is further heated to 90°C and kept at this temperature for 50 min. The mash is then cooled to 60°C and HCl is added to adjust the pH to 5.5. A mixture of neutral proteases, thermostable and normal  $\alpha$ -amylases, amyloglucosidase and  $\beta$ -glucanases is added to the mash to ensure proper attenuation limit. The mash is then kept at 60°C for 30 min and then heated to 75°C for 20 min. When this is complete the mash gets transferred into mash filters where spent grains are separated from wort.



**Figure 2.6** Mashing profile of unmalted sorghum grain (adapted from Ogbonna, 2011).

#### 4.2.2 Brewing with sorghum malt

Compared to barley, sorghum malt has lower levels of  $\beta$ -amylase and the starch has a higher gelatinisation temperature (Taylor & Robbins, 1993; Agu & Palmer, 1998a; Taylor *et al.*, 2006). The mashing procedure utilised in the production of barley larger beer is not suitable for sorghum malt due to differences in starch gelatinisation temperatures of sorghum (67-81°C) and barley (51-60°C) (Aisien & Muts, 1987). These differences cause the breakdown of starch and the production of fermentable sugars to be limited in sorghum wort, when compared to barley wort (Taylor *et al.*, 2006). Sorghum malt  $\beta$ -amylase activity can be manipulated by germination time (7 days), temperature (24-28°C) and moisture conditions (relative humidity above 90%). The highest malt  $\beta$ -amylase activity was obtained when malting was conducted under high moisture conditions (Palmer, 1992; Taylor & Robbins, 1993; Abiodun, 2002).

The development of a decantation mashing method has resolved the differences in gelatinisation and saccharification temperatures of sorghum malt starch. In this process mashing is carried out at 45°C for 30 min. A portion of the mash, the 'active enzymic liquid', is separated from the grist prior to gelatinisation. Thereafter, the grist is immediately heated to 80°C or 100°C in order to gelatinise the starch. The mash is then cooled and added to the previously separated active enzymic liquid to achieve a conversion temperature of 65°C (Etokakpan, 1988). Decantation at 100°C gives better results in terms of wort composition

and development when compared to decantation at 80°C because boiling mash at 100°C ensures adequate sorghum malt starch gelatinisation (Igyor *et al.*, 2001). Decantation mashing method produces worts with starch extracts similar to those of barley malts (Ogbonna, 2011).

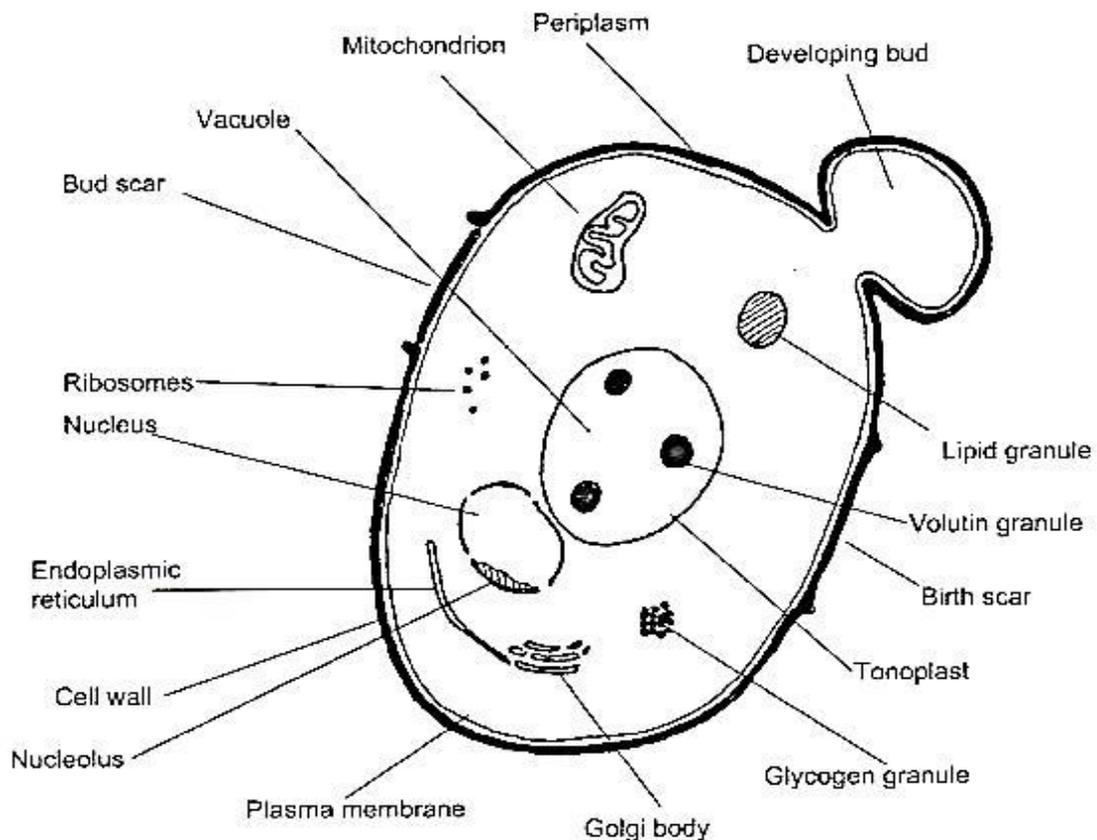
## 5. Brewing yeast

Yeasts (Fig. 2.7) are facultative anaerobic fungi of which a number of species are extensively used in brewing. The shape of yeast cells is either spherical or ellipsoidal and they have a diameter of 5-13 µm (Bamfroth, 2006). Yeast cells have a cell wall (Fig. 2.7) which determines their shape, the degree to which the yeast cells will interact with one another and other materials, and it also provides protection (Bamforth, 2006). The cell wall is primarily comprised of carbohydrates (80-90%) and proteins (5-10%) (Boulton & Quain, 2001). It has two layers; the inner layer consists mainly of β-1-3-glucan linked covalently to chitin and is mainly responsible for the yeast cell's stability and resilience (Eßlinger, 2009).

Glucans account for about 30 to 60% of the cell wall and therefore they are the major polymers (Boulton & Quain, 2001). The outer layer is made up of densely packed glycosylated mannoproteins which are responsible for decreased yeast cell wall permeability and acts as a barrier for dissolved substances (Eßlinger, 2009). Mannoproteins account for about 25 to 50% of the cell wall and they are essential in brewing as receptors in the flocculation process (Boulton & Quain, 2001).

### 5.1 *Saccharomyces cerevisiae*

The genus of yeast used in the brewing industry is *Saccharomyces* and the species of interest is *S. cerevisiae* (Eßlinger, 2009), commonly known as brewer's yeast. There are two types of *Saccharomyces* which are used in the production of beer, namely the top fermenting and bottom fermenting yeast strains (Eßlinger, 2009). There are many different types of beers which are available and they are divided into two major types, i.e. ale and lager. Lager beers are produced by utilisation of bottom fermenting yeast strains, while the production of ales utilises top fermenting yeast strains (Hohmann, 2006). Top and bottom fermenting yeasts differ in their fermentative ability, rate of sugar usage, temperature tolerance, flocculation characteristics and profile of volatiles (Eßlinger, 2009). When producing an ale, fermentation is carried out at temperatures between 20 to 25°C followed by a short period of aging or no aging. During the production of a lager, lower fermenting temperatures (8 to 15°C) are used followed by a long period of aging (Hohmann, 2006).



**Figure 2.7** A cross section through a yeast cell (Boulton & Quain, 2001).

*S. cerevisiae* (top or bottom fermenting) ferments sugars available in wort such as glucose, fructose, maltose and maltotriose in order to produce alcohol and carbon dioxide (Hodžić *et al.*, 2008). These fermentable sugars are taken up in a specific order by the yeast during fermentation. The monosaccharides, glucose and fructose are used up first together with sucrose. Although sucrose is a disaccharide it behaves like a monosaccharide because it is broken down to glucose and fructose outside the yeast cell by yeast enzyme invertase. As soon as the level of monosaccharides in wort decreases, the yeast starts using the disaccharide maltose, which is the most abundant sugar in wort. Maltose is transported into yeast cells and broken down to glucose. Then lastly the trisaccharide, maltotriose is broken down at a very slow rate (O'Rourke, 2002).

### 5.2 Nutrients essential for yeast growth

Apart from sugars, the yeast also requires other nutrients for optimum function during fermentation. These nutrients include free amino nitrogen (FAN), trace elements (copper and zinc ions) and sulphur, calcium and magnesium (Bamforth, 2006). FAN serves as a source of nitrogen for the yeast (Taylor *et al.*, 1985). It plays a role in the growth of the yeast and consequently alcohol and flavour compound production (Agu & Palmer, 1998a). The

level and quality of FAN in wort affects both yeast growth and the final alcohol content of beer (Taylor *et al.*, 1985). A minimum FAN level of 100 to 140 mg/L is considered necessary to support optimal sorghum wort fermentation (Bajomo & Young, 1993).

During fermentation the amino acids which contribute to FAN are not taken up at the same rate by the yeast (Jones & Pierce, 1967). Amino acids are divided into four groups based on their rate of absorption from wort (Jones & Pierce, 1967). Table 2.1 shows sorghum wort free amino acid composition in order of importance to the yeast. Amino acids in group A are taken up by the yeast rapidly. Group B amino acids are absorbed slowly and those which are in group C are only absorbed after a considerable lag. Proline in group D is not taken up at all by the yeast under anaerobic fermentation conditions. More than 50% of the total free amino acids in sorghum malt wort belong to group A, while proline accounts for only 25% of the total (Table 2.1). In barley wort, proline accounts for about one-third of the total free amino acids which make up FAN (Jones & Pierce, 1967). The low level of proline in sorghum wort suggests that sorghum worts have a better qualitative yeast-feeding value than barley worts (Taylor *et al.*, 1985).

During fermentation, sugars and other nutrients present in wort enter the yeast cells and are metabolised by enzymes in order to provide energy (ATP) for the yeast while alcohol, heat, carbon dioxide and flavour compounds are produced as by-products and diffuse out of the yeast cells (Pilkington *et al.*, 1998).

**Table 2.1** Free amino acid composition in sorghum wort (Taylor *et al.*, 1985).

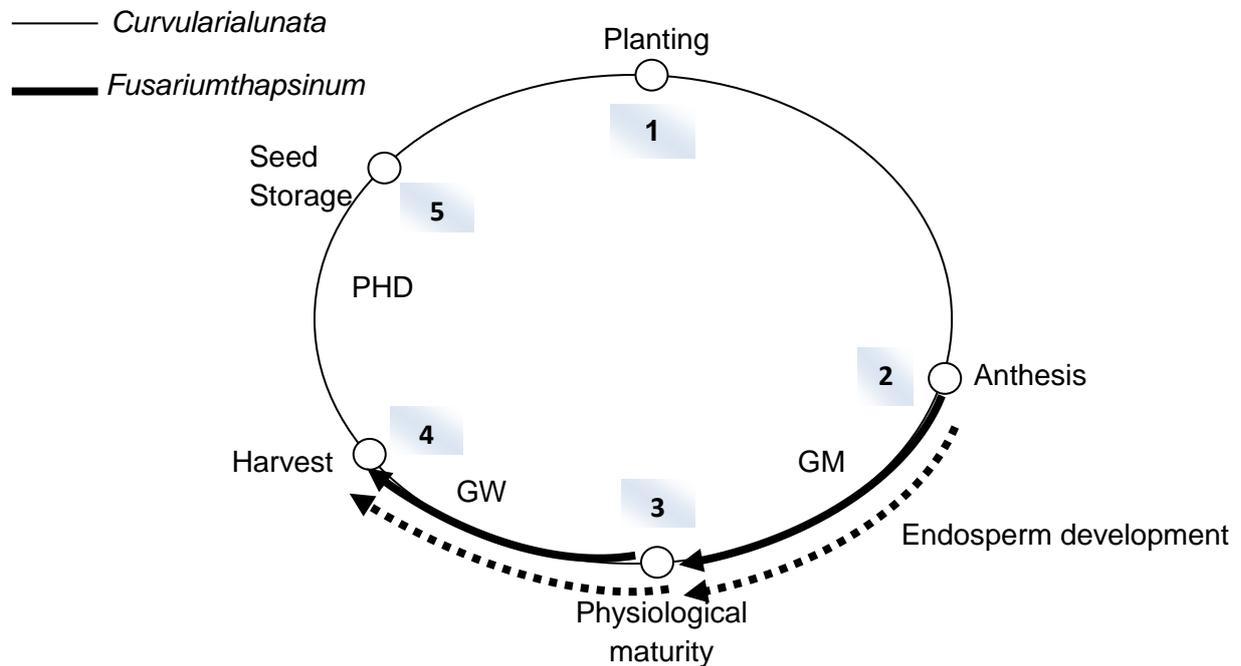
Amino acid	Group	g amino acid/100 g
Aspartic acid		3.5
Threonine		2.3
Serine		3.1
Asparagine		22.5
Glutamic acid	A	4.5
Glutamine		9.5
Lysine		2.5
Arginine		3.5
<b>Subtotal</b>		<b>51.4</b>
Valine		4.1
Methionine		1.3
Isoleucine	B	1.2
Leucine		6.3
Histidine		3.5
<b>Subtotal</b>		<b>16.4</b>
Glycine		1.3
Alanine		5.8
Tyrosine	C	3.9
Phenylalanine		3.6
Tryptophan		1.3
<b>Subtotal</b>		<b>15.9</b>
Proline	D	12.5
<b>Subtotal</b>		<b>12.5</b>
Cystine		0.7
Ornithine	Not	2.8
$\gamma$ -amino butyric acid	classified	0.2
<b>Subtotal</b>		<b>3.7</b>

## 6. Resistance of sorghum grains against diseases and pests

While still in the field and during storage, plants are constantly exposed to a wide range of pathogenic organisms such as fungi, viruses, bacteria, protozoa, mycoplasma and nematodes (Castro & Fontes, 2005). Early infestation of sorghum grains by pathogenic microorganisms results in the arrest of kernel development, discolouration, increased endosperm and germ colonisation and breakdown, decrease in mass and density of grains, decrease in seedling vigor and possible mycotoxin contamination (Little, 2000). This leads to great financial losses due to the reduction in both quantity and quality of the grains (Frederiksen, 1982; Rodríguez-Herrera *et al.*, 1999).

Grain mould is the most significant disease in sorghum grains. It results from the infection of developing grains by parasitic fungi while still in the field (Frederiksen, 1982) and can occur as early as the anthesis phase (Fig. 2.8), a critical point during development where the sorghum plant is most susceptible to fungal infestation. Grain mould is the single greatest impediment for optimum grain yield where anthesis occurs during humid, warm and

rainy seasons. *Fusariumthapsinum* and *Curvularialunata* are the most important fungal species which cause grain mould in sorghum worldwide (Little, 2000).



**Figure 2.8** Life cycle stages at which the sorghum plant is susceptible to deterioration by parasitic fungi while still in the field and during storage (GM=grain mould, GW=grain weathering, PHD=postharvest deterioration) (adapted from Little, 2000).

In contrast to animals, plants do not have an immune system (Vigers *et al.*, 1991; Kitajima & Sato, 1999). They protect themselves from pathogenic microorganisms by hardening their cell walls, producing antibiotic compounds (phenolic compounds and phytoalexins) and antimicrobial proteins called pathogenesis related (PR) proteins (Kitajima & Sato, 1999). This review will focus primarily on PR proteins, which have been found to play a significant role during barley beer production.

### 6.1 Defence molecules in sorghum

Phenolic compounds have been implicated in plant defence (Chandrashekar & Satyanarayana, 2006). Phenolic compounds are present in all sorghums and are divided into three categories, i.e. phenolic acids, flavonoids and condensed tannins (Dicko *et al.*, 2006; Dykes & Rooney, 2006). These compounds are primarily present in the pericarp, testa (seed coat) and aleurone layer. Phenolic properties of the grain are important when brewing with sorghum because they cause astringency, inhibit enzymatic action and lead to the development of unwanted colour taints in beer (Palmer, 1992). Higher levels of phenolic

compounds in the grains, however, also lead to an increase in grain mould resistance (Waniska, 2000).

In most sorghum varieties condensed tannins are absent. However, all sorghums contain phenolic acids and most of them contain flavonoids (Dykes & Rooney, 2006). There are three types of sorghums, namely; type I (tannins absent), type II (tannins are present and located in the pigmented testa) and type III (tannins are present in both the testa and pericarp) (Waniska, 2000). The presence of tannins in the grain are beneficial as they protect the grain against insects, birds and microbial attack (Duodu *et al.*, 2003). Unfortunately, they also reduce the digestibility of proteins, minerals and carbohydrates by binding to them and rendering them insoluble (Duodu *et al.*, 2003; Dicko *et al.*, 2006). Type I sorghum varieties are typically used for food and feed (Chandrashekar & Satyanarayana, 2006).

### 6.2 Pathogenesis related proteins (PRs)

Pathogenesis related proteins (PRs) are a group of antimicrobial proteins and peptides (AMPs) which were originally defined as proteins induced only during pathological or related situations, and were assumed to be involved in plant defence (Antoniw & White, 1980; Leslie *et al.*, 1993; Van Loon *et al.*, 1994; Van Loon & Van Strien 1999). However, subsequent studies have confirmed the presence of PR proteins in normal (uninfected) plant tissues and cereal grains (Muthukrishnan *et al.*, 2001; Chandrashekar & Satyanarayana, 2006).

PR proteins form part of the innate defence system which plants have evolved in order to resist a wide range of pathogenic microorganisms and insects (Gorjanović, 2009). They are located in various parts of the grain and in protein bodies (Chandrashekar & Satyanarayana, 2006). PR proteins have compact structures which are stabilised by a number of disulphide bridges and are either acidic or basic (Gorjanović, 2009). Due to their compact structures PR proteins are able to withstand extremes of pH and temperature, and are also resistant to proteolysis. They are, therefore, able to withstand food processing and are present in processed foods and beverages (Van Loon *et al.*, 2006; Gorjanović, 2009; Porter *et al.*, 2010).

PR proteins are divided into 17 families (Table 2.2) and are numbered in the order of discovery. PR proteins belonging to different families differ in their isoelectric point (pI), molecular weight (Mr) and immunological cross-reactivity (Muthukrishnan *et al.*, 2001; Gorjanovic, 2009). Within each family of PR proteins there are a number of classes which are either basic or acidic (Gorjanović, 2009), as discussed under section 4.2.1. The 17 PR protein families are not all represented in all plant species (Van Loon *et al.*, 2006).

**Table 2.2** Families of pathogenesis-related proteins, their properties and mechanism of action (Van Loon & Van Strien, 1999; Chandrashekar & Satyanarayana, 2006).

Family	Properties	Mechanism of action	Reference
PR protein-1	Antifungal	Unknown	Van Loon & Van Strien, 1999; Van Loon <i>et al.</i> , 2006
PR protein-2	$\beta$ -1,3-glucanase	Fungal cell wall digestion	Roberts & Selitrennikoff, 1986; Leslie <i>et al.</i> , 1993; Leubner-Metzger & Meins, 1999; Muthukrishnan <i>et al.</i> , 2001; Selitrennikoff, 2001; Gorjanović, 2009
PR protein-3	Chitinase I-II, IV, V, VI, VII	Fungal cell wall digestion	Roberts & Selitrennikoff, 1988; Punja & Zhang, 1993; Neuhaus <i>et al.</i> , 1996; Neuhaus 1999; Gorjanović, 2009
PR protein-4	Chitinase type I, II	Fungal cell wall digestion	Neuhaus <i>et al.</i> , 1996; Neuhaus 1999; Gorjanović, 2009
PR protein-5	Thaumatin-like protein (TLP)	Alteration of membrane permeability	Hejgaard <i>et al.</i> , 1991; Seetharaman, 1996; Cvetković <i>et al.</i> , 1997; Gorjanović, 2007; Gorjanović, 2009; Kontogiorgos <i>et al.</i> , 2007
PR protein-6	Proteaseinhibitor (PI)	Inhibition of proteases	Richardson, 1977; Gorjanović, 2009
PR protein-7	Endoproteinase	Protein digestion	Richardson, 1977; Jordá <i>et al.</i> , 2000; Madala <i>et al.</i> , 2010
PR protein-8	Chitinase type III	Fungal cell wall digestion	Neuhaus <i>et al.</i> , 1996; Neuhaus 1999; Gorjanović, 2009
PR protein-9	Peroxidase	Detoxification of H <sub>2</sub> O <sub>2</sub> , lignin synthesis	Johansson <i>et al.</i> , 1992; Hiraga <i>et al.</i> , 2001; Laugesen <i>et al.</i> , 2007; Almagro <i>et al.</i> , 2009
PR protein-10	'Ribonuclease-like'	Activates ribonuclease, transport of sterols and cytokines	Roberts & Selitrennikoff, 1986; Leslie <i>et al.</i> , 1993; Leslie <i>et al.</i> , 2000; Little, 2000; Selitrennikoff, 2001
PR protein-11	Chitinase, type I	Fungal cell wall digestion	Neuhaus <i>et al.</i> , 1996; Neuhaus 1999; Gorjanović, 2009
PR protein-12	Defensin	Antimicrobial, membrane permeabilization	(Broekaert <i>et al.</i> , 1995; Castro & Fontes, 2005; Lay & Anderson, 2005; Gorjanović, 2009
PR protein-13	Thionin	Affects membrane properties	Stec <i>et al.</i> , 2004; Castro & Fontes, 2005; Gorjanović, 2009
PR protein-14	Lipid transfer protein (ns-LTP)	Lipid transport, membrane	Lindorff-Larsen & Winther, 2001; Gorjanović <i>et al.</i> , 2005; Gorjanović, 2007; Gorjanović, 2009
PR protein-15	Oxalate oxidase	Produces hydrogen peroxidase	Zhang <i>et al.</i> , 1995; Wei <i>et al.</i> , 1998; Christensen <i>et al.</i> , 2002
PR protein-16	'Oxalate oxidase-like'	Produces hydrogen peroxidase	Zhang <i>et al.</i> , 1995; Wei <i>et al.</i> , 1998; Christensen <i>et al.</i> , 2002
PR protein-17	Unknown	Protein digestion	Christensen <i>et al.</i> , 2002; Gorjanović, 2009

### 6.2.1 PR protein-1

All members of the PR-1 family are similar in structure, possessing four  $\alpha$ -helices, four  $\beta$ -strands and share several conserved residues (Van Loon *et al.*, 2006). PR-1 proteins are

about 135 amino acids long with six cysteine residues which form three disulphide bridges (Van Loon & Van Strien, 1999; Van Loon *et al.*, 2006). They have been found present in all species investigated to date (Van Loon & Van Strien, 1999). Of all families of PR proteins, the function of PR-1 proteins is least understood (Van Loon & Van Strien, 1999).

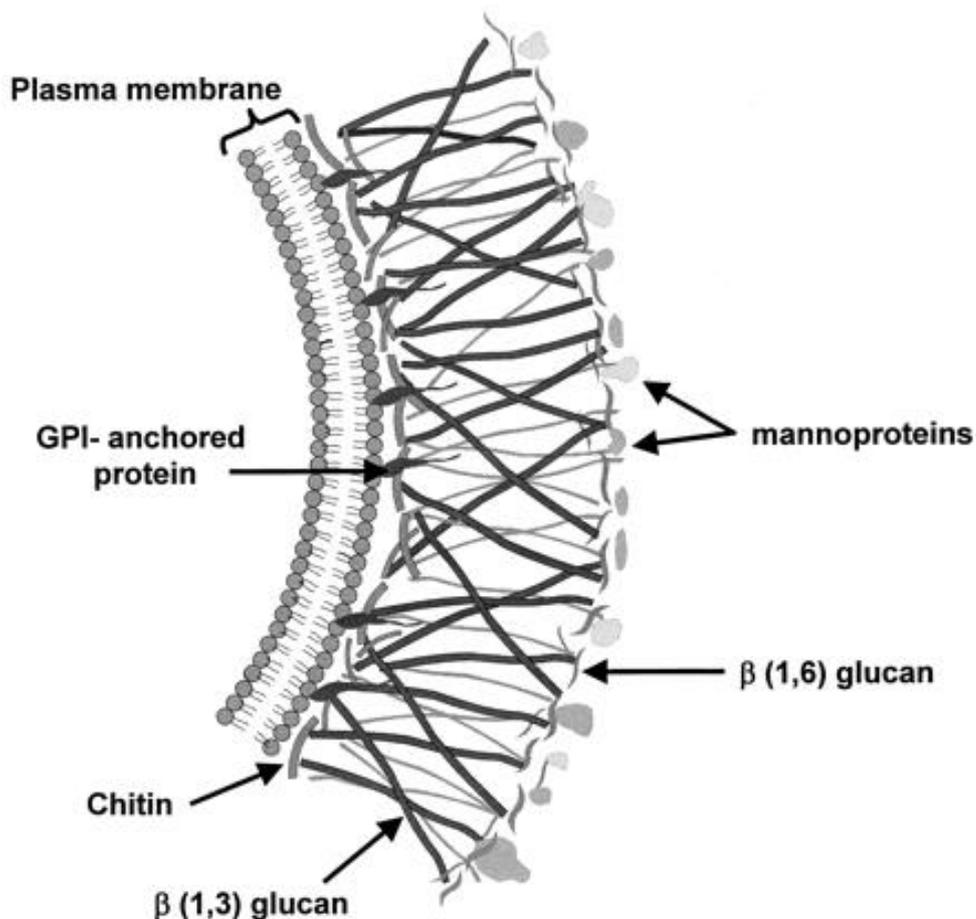
### 6.2.2 PR protein-2

The PR proteins-2 are known as (1,3)- $\beta$ -glucanases ( $\beta$ Glu) (Leubner-Metzger & Meins, 1999; Gorjanović, 2009). These enzymes are highly regulated and widely distributed in seeds (Leubner-Metzger & Meins, 1999). The (1,3)- $\beta$ -glucanases are classified into a number of structurally distinct isoforms, with basic and acidic counterparts. The  $\beta$ Glu isoforms also differ significantly in enzymatic and antifungal activity. The basic isoforms have shown inhibitory effects against a wide range of fungi and they act synergistically with chitinases (Leah *et al.*, 1991; Gorjanović, 2009). Class I glucanases are basic proteins found in the vacuole with Mr around 33 kDa. Classes II and III are extracellular proteins with a Mr of about 36 kDa (Selitrennikoff, 2001).

Fungal cell walls are mainly composed of (1-3)- $\beta$ -glucan and to a lesser extent they also contain some (1-6)- $\beta$ -glucan (Fig. 2.7) (Leslie *et al.*, 1993). Beta-1-3-glucanases catalyse cleavage of 1,3- $\beta$ -D-glucosidic linkages in (1,3)- $\beta$ -glucan (Roberts & Selitrennikoff, 1986; Leslie *et al.*, 1993; Muthukrishnan *et al.*, 2001). This weakening of the fungal cell wall leads to cell lysis and eventual death (Gorjanović, 2009).

### 6.2.3 PR protein-3, PR protein-4, PR protein-8 and PR protein-11

These PR proteins are known as chitinases (Mr 26-43 kDa). They are enzymes which hydrolyse chitin (Fig. 2.9); an insoluble linear  $\beta$ -1,4-linked polymer of N-acetylglucosamine (Roberts & Selitrennikoff, 1988; Punja & Zhang, 1993). The four families of chitinases are further classified into classes (Table 2.3) which are distinguished by their primary structure (Neuhaus *et al.*, 1996; Neuhaus 1999). Seed chitinases belong to classes I, II, III, IV and VII (Gorjanović, 2009).



**Figure2.9** Fungus cell wall (Selitrennikoff, 2001).

Chitin is present as the main structural component in the cell walls of invertebrate animals, crustaceans, fungi and certain algae (Leslie *et al.*, 1993; Waniska, 2000). Plants do not contain chitin in their cell walls and produce chitinases to protect themselves from chitin-containing parasites (Roberts & Selitrennikoff, 1988). In plants, chitinases are expressed constitutively at low levels, but their concentration is increased by both abiotic agents (ethylene, salicylic acid, salt solutions, ozone and UV light) and biotic factors (fungi, bacteria, viruses, viroids, fungal cell wall components and oligosaccharides) (Punja & Zhang, 1993). When  $\beta$ -1,3-glucanases (PR protein-2) and chitinases (PR protein-3, PR protein-4, PR protein-8 and PR protein-11) were tested for antifungal activity it was found that they both inhibit the growth of a variety of pathogenic fungi (Muthukrishnan *et al.*, 2001). Sorghum acidic chitinases are primarily involved in active defence responses whereas the basic fraction are part of the pre-formed defence mechanism (Huang & Backhouse, 2006).

**Table 2.3** Nomenclature for chitinase genes (Neuhaus *et al.*, 1996).

PR protein	Class	Gene name
PR-3	I	<i>Chia1</i>
	II	<i>Chia2</i>
	IV	<i>Chia4</i>
	V	<i>Chia5</i>
	VI	<i>Chia6</i>
	VII	<i>Chia7</i>
	PR-8	III
PR-11	VI	<i>Chic1</i>
PR-4	I	<i>Chid1</i>
	II	<i>Chid2</i>

#### 6.2.4 PR protein-5

Proteins which belong to the PR protein-5 family are known as thaumatin-like proteins (TLPs) because of their structural similarities to thaumatin (Velazhahan *et al.*, 1999; Gorjanović *et al.*, 2007). They are divided into three subclasses based on their acidic, basic and neutral members with either an extracellular or vacuolar localisation (Gorjanović, 2009). Based on Mr they are classified into two groups: one group with proteins in the size range of 22 to 26 kDa (201 to 229 amino acids), and the second group proteins with sizes of approximately 16 kDa (148 to 151 amino acids). The pI values of TLPs ranges from 3.4 to 12 (very acidic to very basic). Acidic TLPs are extracellular in origin, whereas basic TLPs are located in the vacuole (Velazhahan *et al.*, 1999).

In sorghum seeds a high concentration of TLPs has been reported (Seetharaman *et al.*, 1996). While several isoforms of TLPs have also been isolated from barley leaves, only two isoforms of LTPs were isolated in barley seeds (Hejgaard *et al.*, 1991). Barley seed TLPs isoforms (TLP-S and TLP-R) are highly basic, have a pI around 9 and Mr of 22 kDa. They mainly have a  $\beta$ - sheet structure and a small helix stabilised by eight disulphide bridges (Hejgaard *et al.*, 1991; Gorjanović, 2009). Barley seed TLPs isoforms act synergistically to inhibit the metabolic activities of yeast and other pathogenic fungi (Cvetković *et al.*, 1997). When compared to wheat grass TLPs, which denature at temperatures above 75°C (Kontogiorgos *et al.*, 2007), barley seed TLPs have a low denaturing temperature (58°C). Therefore, barley seed TLPs are denatured during mashing and do not inhibit yeast during brewing or influence beer quality (Gorjanović, 2009).

### 6.2.5 PR protein-6

The members of the PR protein-6 family are known as proteinase inhibitors (PIs). PIs account for up to 10% of the total proteins present in cereal seeds (Gorjanović, 2009). They are responsible for protecting the seeds against proteinases of foreign invaders (Richardson, 1977). Barley seed PIs (Table 2.4) are resistant against *Fusarium* species responsible for the most devastating disease of barley, i.e. *Fusarium* head blight (FHB, scab) (Gorjanović, 2009). Most plant proteinase inhibitors are very resistant to denaturation by heat (Richardson, 1977).

**Table 2.4** Barley seed proteinase inhibitors (Gorjanović, 2009)

Inhibitor family	Relative molecular mass (M <sub>r</sub> )	Isoelectric point (pI)	Number of disulfidebridges
Bifunctional α-amylase/subtilisin inhibitor (BASI) Kunitz type	19.9 kDa	7.2	2
Chymotrypsin/subtilisininhibitors (CI-1 A, B, C and CI-2A) form dimers or trimers	9 kDa	4.5-5.5; 6-8	No cysteines
Barley Browman-Birk type trypsin inhibitor (BBBI)	16 kDa	4.2	10
Trypsin/α-amylase inhibitor (CMA-e) form dimers or multimers	12-16 kDa	4-7	5
Serine protease inhibitors (Serpins or Protein Z4, Z7, Zx)	40-45 kDa	4-7	None

### 6.2.6 PR protein-7

Based on their active sites and reaction mechanisms proteolytic enzymes found in nature are subdivided into four groups. These are serine proteases (e.g. trypsin, chymotrypsin, thrombin, plasmin and elastase); the sulphhydryl proteinases (papain, bromelain and ficin); the metalloproteinases (carboxypeptidases A and B, and amino peptidases) and the acidic proteinases (pepsin and rennin) (Richardson, 1977). Members of the PR protein-7 family are serine proteinases (Jordá *et al.*, 2000). Serine proteinases, based on their substrate specificity, are divided into two families, i.e. trypsin-like (also known as chymotrypsin-like) and subtilisin-like (Madala *et al.*, 2010). Subtilisin-like enzymes are the largest of serine proteinase. Over 200 subtilisin-like enzymes are currently known, however, their role in plants is not well understood (Jordá *et al.*, 2000).

### 6.2.7 PR protein-9

A pathogen attack and other different stress conditions lead to the production of reactive oxygen species, which can damage DNA and proteins as well as disrupt the normal functioning of the membrane in plants (Hiraga *et al.*, 2001; Almagro *et al.*, 2009). As a result of increased production of oxygen radicals, plants activate an antioxidative system in order to scavenge these species (Hiraga *et al.*, 2001; Almagro *et al.*, 2009). The main plant enzymes involved in this process are peroxidases (PR protein-9) (Hiraga *et al.*, 2001; Almagro *et al.*, 2009). In barley, three products of peroxidase genes have been isolated: barley-seed specific peroxidase (BP1); barley seed peroxidase 1 (BSSP1); and putative barley peroxidase (Laugesen *et al.*, 2007). BP1 is the major peroxidase in barley and is a basic protein with an isoelectric point around 9, has 309 amino acids and four disulphide bridges (Johansson *et al.*, 1992).

### 6.2.8 PR protein-10

Ribosome inactivating proteins (RIPs) are basic (pI ranging from 8 to 10), very stable proteins which are found in seeds, roots, leaves and sap of many plants (Stirpe *et al.*, 1983). They are RNA N-glycosidases that cleave N-glycosidic bonds of adenines in ribosomal RNA and result in the arrest of protein synthesis in target cells due to ribosome damage (Leslie *et al.*, 1993; Selitrennikoff, 2001). In sorghum grains RIPs hinder the growth of pathogenic fungi by inhibiting the protein translation of the invading fungi and thus play a very essential role in defense against parasites (Roberts & Selitrennikoff, 1986; Little, 2000).

### 6.2.9 PR protein-12

Plant defensins have been isolated in radishes, sorghum, barley, wheat and other species of *Brassicaceae* (Castro & Fontes, 2005). They are small highly basic peptides ( $M_r \sim 5$  kDa). They have the same molecular weight as thionins (PR protein-13 members) but the number of disulphide bridges and their structures are very different (Broekaert *et al.*, 1995). Members of the PR protein-12 family share a common three dimensional structure but differ in amino acid composition and biological activity (Lay & Anderson, 2005). They inhibit the growth of pathogenic fungi (Gorjanović, 2009).

### 6.2.10 PR protein-13

The members of PR protein-13 are known as thionins. They are small (45-47 amino acids) basic peptides (pI>8) and have a low  $M_r$  (~5 kDa) (Castro & Fontes, 2005; Gorjanović, 2009). They are peptides which are very rich in basic (arginine and lysine) and sulphur-containing amino acid residues (cysteine) (Castro & Fontes, 2005). There are four major types of thionins in plants. The highly basic type 1 is abundant in the endosperm of seeds belonging

to the family *Poaceae* and has antifungal activity (Gorjanović, 2009). Thionins form dimers which upon binding to the membrane dissociate and cause membrane solubilisation and lysis (Stec *et al.*, 2004).

#### 6.2.11 PR protein-14

Non-specific lipid-transfer proteins (ns-LTPs) are divided into two subfamilies based on their  $M_r$ : ns-LTP1 (9kDa) and ns-LTP2 (7kDa) (Gorjanović *et al.*, 2007). Barley seed ns-LTP1 is a basic protein with a pI around 9 and comprises 91 amino acids whereas barley ns-LTP2 has a pI around 8 and comprises 69 amino acids (Gorjanović, 2009). The three dimensional structure of ns-LTPs is stabilised by four disulphide bridges (Douliez *et al.*, 2000) and their high resistance to proteases and denaturation by heat is attributable to this structure. The denaturing temperature of ns-LTP1 is 100°C and even higher temperatures are required to denature ns-LTP2 (Lindorff-Larsen & Winther, 2001; Gorjanović, 2009). In barley, ns-LTPs inhibit the growth of pathogenic fungi (Gorjanović *et al.*, 2005; Van Nierop, 2005). In this grain, ns-LTP1 is the most abundant of the ns-LTPs (Gorjanović *et al.*, 2007).

#### 6.2.12 PR protein-15, PR protein-16 and PR protein-17

Oxalate oxidase or germins (PR protein-15), oxalate oxidase-like or germin-like (PR protein-16) and PR-17 have been recently isolated in barley leaves and added to the families of PRs (Zhang *et al.*, 1995; Wei *et al.*, 1998; Christensen *et al.*, 2002). Members of the PR-17 family are known as Hv-PRs and in barley HvPR-17a and HvPR-17b have been isolated. The pI of HvPR-17a ( $M_r$  26 kDa) is 4.7 whereas the pI of and HvPR-17b ( $M_r$  24 kDa) is 8.0 (Christensen *et al.*, 2002; Gorjanović, 2009).

### 7. Grain antiyeast activity during brewing

Of the 17 families, thionins, plant defensins and ns-LTPs are three key groups of antimicrobial peptides (AMPs) that have been purified in barley (Castro & Fontes, 2005). The majority of proteins which are present in barley lager beer are AMPs (Van Nierop *et al.*, 2008). They have compact structures due to multiple disulphide bridges and are therefore able to withstand harsh mashing and wort boiling conditions while the majority of barley seed proteins are denatured or broken down by proteases (Van Nierop *et al.*, 2008). Some AMPs are important in the brewing industry for formation, stabilisation and maintenance of foam (Gorjanović, 2010).

Barley AMPs, however, are one of the factors contributing to problematic fermentations during brewing by inhibiting yeast growth (Van Nierop, 2005). During wort fermentation they lead to problems such as premature yeast flocculation (PYF), incomplete fermentations and gushing of the final beer (Van Nierop *et al.*, 2008). PYF occurs when yeast cells flocculate

early while fermentable sugars are still available in wort (Porter *et al*, 2010). Incomplete fermentations refer to a scenario where yeast cells are viable but cannot ferment the sugars and they also do not flocculate (Van Nierop *et al.*, 2008). Gushing is when an excessive flow-out of bubbles occurs immediately after opening the beer bottle (Gorjanović, 2010). Barley malts associated with PYF have a high level of antimicrobial peptides in barley grains (Van Nierop, 2005). Both PYF and incomplete fermentations lead to poor attenuation of sugars and consequently a low final concentration of alcohol. They negatively affect beer quality and lead to financial problems in the malting and brewing industry (Porter *et al*, 2010).

In sorghum grains PR proteins have been isolated (Leslie *et al.*, 1993; Chandrashekar & Satyanarayana, 2006). Sorghum PR proteins have antifungal activity and play an essential role in grain mould resistance (Waniska, 2000; Chandrashekar & Satyanarayana, 2006). Most of these antifungal proteins were observed from grain mould resistant sorghums in the hard endosperm (Kumari & Chandrashekar, 1994). Despite their known antifungal activity, the effect of sorghum PR proteins on brewer's yeast is unknown (Chandrashekar & Satyanarayana, 2006). Like barley AMPs, sorghum PR proteins could also inhibit the growth of yeast during wort fermentation; leading to fermentation problems (PYF and incomplete fermentation). PR proteins could also be responsible for re-pitching problems experienced during the commercial production of sorghum lager beer. It has been reported that during barley wort fermentation the same yeast crop can be re-pitched over a number of consecutive fermentations without negatively affecting yeast growth (Bajomo & Young, 1994). In commercial production, however, far less consecutive fermentations can be achieved using the same yeast crop (Ng'andwe, 2008). It is believed that sorghum PRs could adversely affect the physiological status of brewer's yeast by decreasing its viability and vitality. This could possibly partially explain the fermentation problems (PYF, incomplete fermentation and re-pitching problems) often encountered in the sorghum brewing industry.

## 8. Conclusions

The corneous nature of the hard endosperm, low solubility of cell wall material, the presence of disulphide bonds in  $\gamma$ - and  $\beta$ -kafirins all contribute to poor sorghum protein digestibility which consequently results in low FAN and extract levels. The strong adhesion of the starch-glutelin protein matrix, particularly in the hard endosperm, causes starch granules to be inaccessible to degrading enzymes and consequently leads to low FAN and extract levels. The development of a decantation mashing method has resolved the differences in gelatinisation and saccharification temperatures of sorghum malt starch. Although condensed tannins play an important role in protecting the grains against insects, birds and microbial attack leading to increased quantity and quality of sorghum grains, their presence

has a negative effect on brewing. These tannins reduce the digestibility of proteins, minerals and carbohydrates by binding to them and rendering them insoluble. Since they are however not present in all sorghum cultivars, only condensed tannin-free sorghums should be utilised in the production of lager beer.

While still in the field or during storage sorghum grains are exposed to a wide range of pathogenic organisms. Pathogenesis related proteins (PRs) play a role in protecting sorghum grains against microbial attack. This defence system benefits farmers because it eliminates disease problems and leads to higher yields. The presence of sorghum PRs in wort, however, potentially affects brewers negatively during fermentation because PR proteins have antifungal activity. Sorghum PRs might inhibit the growth of yeast which is used when producing beer and lead to fermentation problems (PYF and incomplete fermentation), as well as re-pitching problems. These problems cause great financial losses in the brewing industry during the production of barley lager beer, and therefore could be having similar effect in sorghum lager beer production.

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### **Chapter 3**

Malting and brewing potential of 12 sorghum cultivars and their malts

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#### Abstract

The malting and brewing potential of 12 selected Zambian sorghum cultivars was investigated with regard to tannin content, proximate composition, germinative energy (GE), micro-structure, diastatic power and free amino nitrogen (FAN). All cultivars were tannin free and thus suitable for brewing potential exploration. Moisture content of all unmalted grains and corresponding malts ranged from 7.0 to 7.4% and 1.6 to 2.3% respectively. The ash content of unmalted grains ranged from 1.3 to 1.4%. A significant decrease in ash content was observed in all cultivars after malting due to the removal of roots and shoots after kilning. The GE ranged from 80 to 98%. During malting protein content decrease in high GE cultivars (MMSH-375, 625, 1401, 1194, 1038, 1257 and 1376) and low GE (MMSH-707, 1077, 1356, 1363, and 1365) ranged between 0.1-1.6% and 0.2-1.0% respectively. It was observed that the cultivar with highest protein content (MMSH-1194) in both malt and grain of 9.7% and 10.2% respectively did not necessarily provide the highest FAN content. Protein content negatively correlated ( $r = -0.13$ ) with level of FAN produced in all cultivars. A FAN level of more than 200 mg/L was achieved in MMSH-375 and MMSH-1401 during malting. Mashing did not significantly increase FAN levels in any of the cultivars. The addition of potassium metabisulphite significantly increased FAN in all cultivars except for MMSH-625 and MMSH-1376. Despite the fact that only 25% of the DP level recommended suitable for commercial malting was achieved, high extracts levels ranging between 18.7 and 25.5°P were observed in all cultivars. Overall sorghum cultivars MMSH-375, 1401 and 1194 possess beneficial qualities for potential malting and production of clear lager style beer commercially.

#### Introduction

Sorghum is Africa's fourth most important cereal after maize, rice and wheat in terms of tonnage (FAO, 2012). In certain parts of Africa and India sorghum grain was only utilised in the production of porridges, traditional (opaque) alcoholic beverages and for bread making (Agu & Palmer, 1998). Sorghum was first considered a brewing adjunct in lager beer production during the second world war when the conventional brewing material (barley) was scarce (Obido *et al.*, 2002). In the late 1980s a significant policy shift took place in Nigeria when the importation of temperate cereals was banned due to shortage of foreign exchange (Nzelibe & Nwasike, 1995; Agu & Palmer, 1998). This change in policy caused the breweries in Nigeria to seek alternatives and sorghum was identified as the most economically and technically viable alternative to barley for industrial brewing of clear lager style beer (Nzelibe & Nwasike, 1995; Owuama, 1997; Abiodun, 2002). In Africa, research on the industrial utilisation of sorghum for malting and brewing of lager beer started in the mid-to-late 1980s (Owuama, 1999; Taylor *et al.*, 2006). The chemical composition of sorghum

reassures it as an alternative cereal to barley in lager style beer production particularly in the tropical countries (Owuama, 1999). Sorghum has a distinct advantage over barley of being a drought resistant crop which can be cultivated under hot and dry, as well as water logged conditions usually without the application of fertilisers (Obido *et al.*, 2002; Taylor *et al.*, 2006).

Brewing with sorghum malt is however linked to problems of high malting losses, poor wort filtration characteristic (Hermia & Rahier, 1992), low levels of  $\beta$ -amylases attained during malting (Taylor & Robbins, 1993; Abiodun, 2002), high gelatinisation temperature of starch (Etokakpan, 1988) and low free amino nitrogen (FAN) in wort (Taylor & Robbins, 1993; Oria *et al.*, 1995; Taylor *et al.*, 2006). Efforts have been made to improve sorghum for lager beer brewing. Sorghum malting losses have been reduced by increasing the length of the air-rest and incorporating dilute alkaline (0.1% NaOH) liquor into the steep regime (Ezeogu & Okolo, 1994; Okolo & Ezeogu, 1996). The absence of a husk in sorghum leads to poor wort filtration compared to barley malt where the husk acts as a natural filter bed. This was considered a major problem until the 1990s when this problem was addressed by the development of tangential-flow mash filters with automatic spent grain discharge (Hermia & Rahier, 1992). The low sorghum malt  $\beta$ -amylase activity can be increased by germination time (7 days), temperature (24-28°C) (Abiodun, 2002) and high relative humidity conditions (above 90%) (Palmer, 1992; Taylor & Robbins, 1993; Abiodun, 2002). The adoption of a decantation mashing method has resolved the differences in gelatinisation and saccharification temperatures of sorghum malt starch (Etokakpan, 1988). This mashing method produces worts with extract levels similar to those of barley malts (Ogbonna, 2011).

The low solubility of endosperm cell wall material, corneous nature of the hard endosperm, the strength of the protein-starch adhesion (Hoseney *et al.*, 1974), the presence of disulphide bonds and hydrophobic nature of the kafirins (Oria *et al.*, 1995; Ogbonna *et al.*, 2004) contribute to poor sorghum protein digestibility which consequently results in low FAN and extracts levels (Aisien & Muts, 1987; Palmer *et al.*, 1989; Taylor & Belton, 2002; Duodu *et al.*, 2003). During barley germination, endosperm degrading enzymes (endo-glucanases and pentosanases) slowly perfuse into and degrade endosperm cell wall material (rich in water-extractable  $\beta$ -glucans) and thus expose starch granules and proteins to extensive degradation by endoproteases and amylases (Etokakpan & Palmer, 1990; Verbruggen, 1996; Boulton & Quain, 2001). About 75% of  $\beta$ -glucans, 40% of the barley proteins and 10% of starch are degraded during malting (Anderson & Reinbergs, 1985; Boulton & Quain, 2001). Further, barley endosperm degradation occurs during mashing and high extract yields are obtained (Boulton & Quain, 2001). In contrast to barley endosperm cell wall material which is rich in water-extractable  $\beta$ -glucans, sorghum cell wall material is rich in water-unextractable glucuronarabinoxylans (Verbruggen, 1996; Taylor *et al.*, 2013). During sorghum malting the endosperm cell wall thus remains intact and starch hydrolysis occurs mainly in the soft endosperm during malting (Glennie *et al.*, 1983). The hard endosperm also remains completely unmodified due to its tightly packed starch-protein matrix which is inaccessible to degrading enzymes (Aisien & Muts, 1987). This negatively affects the production of free amino nitrogen and

leads to low extract levels (Ogbonna *et al.*, 2004). The strength of the protein-starch adhesion is however cultivar dependent (Hoseney *et al.*, 1974). The selection and breeding of cultivars with increased beneficial properties for brewing is also improving sorghum for lager beer brewing. Annually through plant breeding research, several more cultivars with improved quality are developed and released by breeders (Haln, 1966; Dicko *et al.*, 2006). More than 10 000 sorghum cultivars exist and some of their malts possess beneficial qualities for lager brewing such as good diastatic power and extract recovery (Owuama & Adeyemo, 2009).

The aim of this study was to determine the malting and brewing potential of 12 Zambian sorghum cultivars.

## **Materials and Methods**

### *Sorghum samples*

Twelve sorghum [*sorghum bicolor* (L.) Moench] cultivars Mount Makulu Sorghum Hybrid (MMSH)-375, 625, 707, 1038, 1077, 1194, 1257, 1356, 1363, 1365, 1376 and 1401) used in this study were cultivated in and obtained (from the same harvest) from the Mt Makulu Central Research Station of the Zambia Agricultural Research Institute (ZARI), Chilanga, Zambia.

### *Malting and sample preparation*

Broken kernels were removed by hand and 100 g of grain was placed in nylon bags and steeped in a glass beaker containing tap water at a temperature between 25 and 30°C for 24 h. For the first 4 h they were steeped in 2000 ppm NaOH. Every 4 h the grains were allowed to air rest for 15-20 min and placed back into a beaker containing fresh water for the next 4 h steep period. This procedure was done for the first 12 h after which the grains were left in water for twelve more hours to complete the 24 h cycle. The grains were transferred into a baking proofer (Macadams convecta 8, Cape Town, South Africa) where they were allowed to germinate at temperatures between 25 and 30°C and relative humidity above 90% for a period of 4 days. Green malt was kilned at 50°C using a baking oven (Macadams convecta 8, Cape Town, South Africa) for 24 h, after which the shoots and roots were removed by pouring dried malt in nylon bags and rubbing. Unmaltered sorghum grains and malt were then milled using a laboratory mill (Retsch model ZMI, Haan Germany) fitted with a 0.5 mm mesh size ring sieve. The ground sample was transferred into a polyethylene container fitted with a lid, mixed with a spatula and stored at 5°C until analysis.

### *Tannins*

The presence of tannins in the sorghum varieties was determined using method 177 of the International Association for Cereal Science and Technology (ICC, 2012). One hundred sorghum kernels were placed in a 50 mL beaker with a sodium hypochlorite (NaClO) solution containig 5% NaOH. For each sample the test was performed in duplicate.

### *Moisture content*

Moisture content was determined using an adapted method of the American Association Cereal Chemists 44-15.02 (AACC, 1999). Moisture dishes were dried in a vacuum oven (Heraeus Model RVT 360, Henau, Germany) at 130°C for 30 min and cooled in a desiccator for 40 min. The mass of the pre-dried moisture dishes with lids was recorded ( $W_1$ ). The sample to be dried ( $5 \pm 0.001$  g) was poured into tared moisture dishes. Moisture dishes containing samples were placed in the vacuum oven and samples were dried at 130°C for 1 h. The moisture dishes were then removed from the oven and stored in a desiccator for 45 min to cool. The new mass of the covered moisture dishes with dried sample was recorded to the nearest 0.001 g ( $W_3$ ). Moisture content was calculated as the loss in weight, expressed as a percentage of the weight of the original sample using the following equation:

$$\% \text{ moisture} = [W_2 - W_3 / W_2 - W_1] \times 100.$$

For each sample three replicates were performed to determine moisture content.

### *Ash content*

Ash content of the sorghum grains and malt was determined by AACC International method 08-03.01 (AACC, 1999). Two grams of sample was poured into previously ignited, cooled and tared crucibles. The crucibles were then inserted in a muffle furnace at 600°C for 2 h. The residue was weighed and percentage ash was calculated using the following equation:

$$\% \text{ Ash} = (\text{weight of residue} / \text{sample weight}) \times 100.$$

For each sample three replicates were performed to determine ash content.

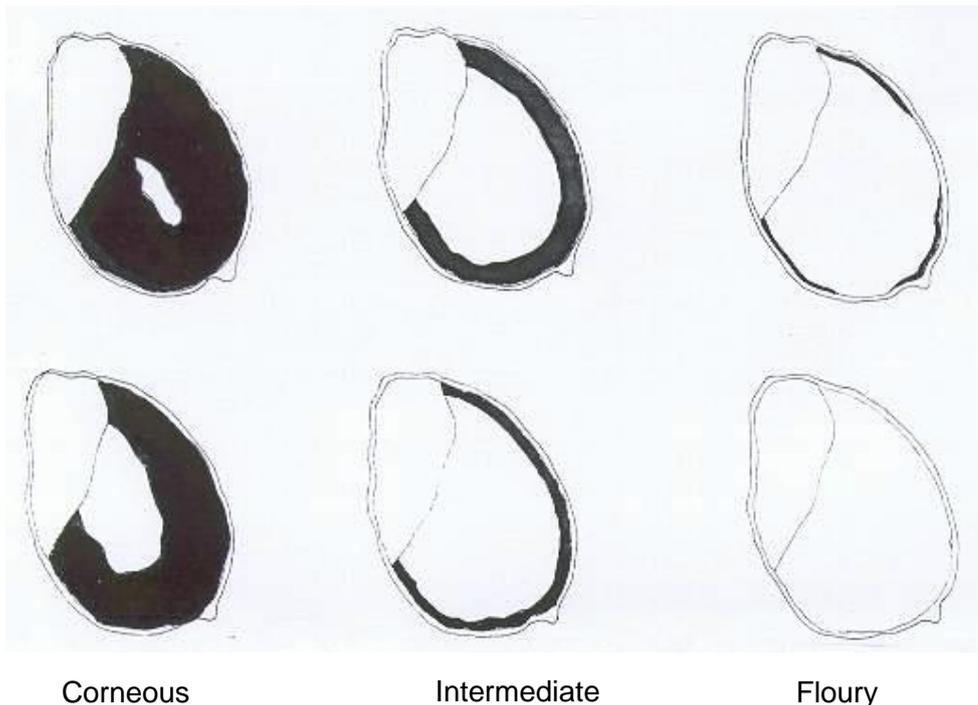
### *Germinative energy (GE)*

Percentage germinative energy was determined by method 174 of the International Association for Cereal Science and Technology (ICC, 2011). Germinated grains were those with a root penetrating the pericarp. 100 sorghum grains were placed in a petri dish containing a damp filter paper and incubated at 25°C and 100% relative humidity for 24, 48 and 72 h in a baking proofer (Macadams convecta 8, Cape Town, South Africa). The percentage of germinated grains at specific time periods (24, 48 and 72 h) were calculated. For each sample and time period the analysis was performed in triplicate.

### *Visual hardness*

To estimate grain endosperm texture method 176 of the International Association for Cereal Science and Technology (ICC, 2012) was used. 20 sorghum kernels per cultivar were cut in half longitudinally and compared with the illustration (Fig 3.1). Based on the ratio of hard to soft endosperm in each kernel, cultivars were classed as either soft (endosperm is mainly floury, corneous endosperm is narrow), intermediate (less than 50% of the endosperm is corneous) or hard (more than 50% of the endosperm is corneous). Micrographs of one kernel per cultivar were

taken using a stereomicroscope (Nikon eclipse E600, Tokyo, Japan) fitted with a digital camera (Nikon DXM1200, Tokyo, Japan).



**Figure 3.1** Sorghum grain texture illustrations (ICC, 2012). Corneous cultivars are those whose total endosperm is corneous or more than 50% of it is corneous, intermediate cultivars comprise less than 50% of the corneous endosperm having the inner part of the endosperm being floury and floury cultivars are those whose endosperm is totally floury with very narrow corneous endosperm.

#### *Scanning Electron Microscopy (SEM)*

Micrographs of the unmalted and malted kernels were taken with a Leo® 1430VP Scanning Electron Microscope (Zeiss, Germany). A single kernel was randomly selected for each cultivar and cut in half longitudinally. The kernels were then sputter-coated with gold. Beam conditions during imaging were 7 KV and approximately 1.5 nA, with a working distance of 13 mm and a spot size of 60 dp. Micrographs were taken at the center (soft endosperm) and edge (hard endosperm) of the same kernel at a magnification of 1000x.

#### *Diastatic Power (DP)*

Diastatic power of sorghum malts was determined by the Institute of Brewing (IOB) method as described by the American Society of Brewing Chemists (Anon, 1958). 25 g of sample was mixed with 500 ml of 0.5% NaCl and allowed to stand for 2.5 h at 20°C with shaking at 20 minutes interval. At the end of 2.5 h the mixture was filtered using Whatman No1 filter paper and analysed.

#### *Hot water extract*

To obtain wort, ground malt sample (50 g) was mixed with 75 mL tap water and mashed in a shaking water bath at 100°C for 90 min. At the end of the 90 min all samples were cooled by

diluting it to 125 g with cold tap water. The mash was centrifuged (TJ-25 centrifuge, California, USA) at 10 000 g for 10 min. A refractometer (Extech RF10, Massachusetts, USA) was used to determine the °Brix of wort. This was done in duplicate for all 12 cultivars. The Braukaier refractometer Brix reading to wort Plato and SG conversion table (Anon., 2013) was used to convert extract values from °Brix to °Plato.

### *Protein*

A Dumas combustion analyser (Model Truspec® N elemental Determinator, Michigan, USA) was used to determine the protein content of samples. Alfalfa with a known nitrogen content of 3.28% - 3.36% was analysed prior to protein determination in order to calibrate the instrument. Alfalfa was weighed ( $0.10 \pm 0.001$  g) into a tin foil sample cup, twisted and rolled into an egg shape and placed on the carousel loading head of the instrument. The same procedure was followed for the samples. A 6.25 conversion factor was used to convert from nitrogen to protein content. Protein analyses were performed in duplicate.

### *Free amino nitrogen (FAN)*

#### Grain

Ground sample (10 g) was mixed with tap water (15 g). One mL of the sample was taken in duplicate and diluted in a 500 ml volumetric flask. Ten mL of the diluted samples was centrifuged at 5 000 rpm for 10 min. The clear supernatant was analysed for FAN using the ninhydrin assay, method 8.8.1 prescribed by the European Brewery Convention (EBC, 1987). Glycine was used as a reference amino acid.

#### Malt

Ground sample (10 g) was mixed with tap water (15 g) and mashed in a shaking water bath at 55°C for 45 min. One mL of the sample was taken at the beginning and end of mashing in duplicate and diluted in a 500 mL volumetric flask. Ten mL of the diluted samples was centrifuged at 5 000 rpm for 10 min. The supernatant was analysed for FAN using the ninhydrin assay, method 8.8.1 of the European Brewery Convention (EBC, 1987). Glycine was used as a reference amino acid. The experiment was repeated; in this case grain and malt were treated with potassium metabisulphite ( $K_2S_2O_5$ ) (0.1% (w/w) of sample) at the beginning of mashing.

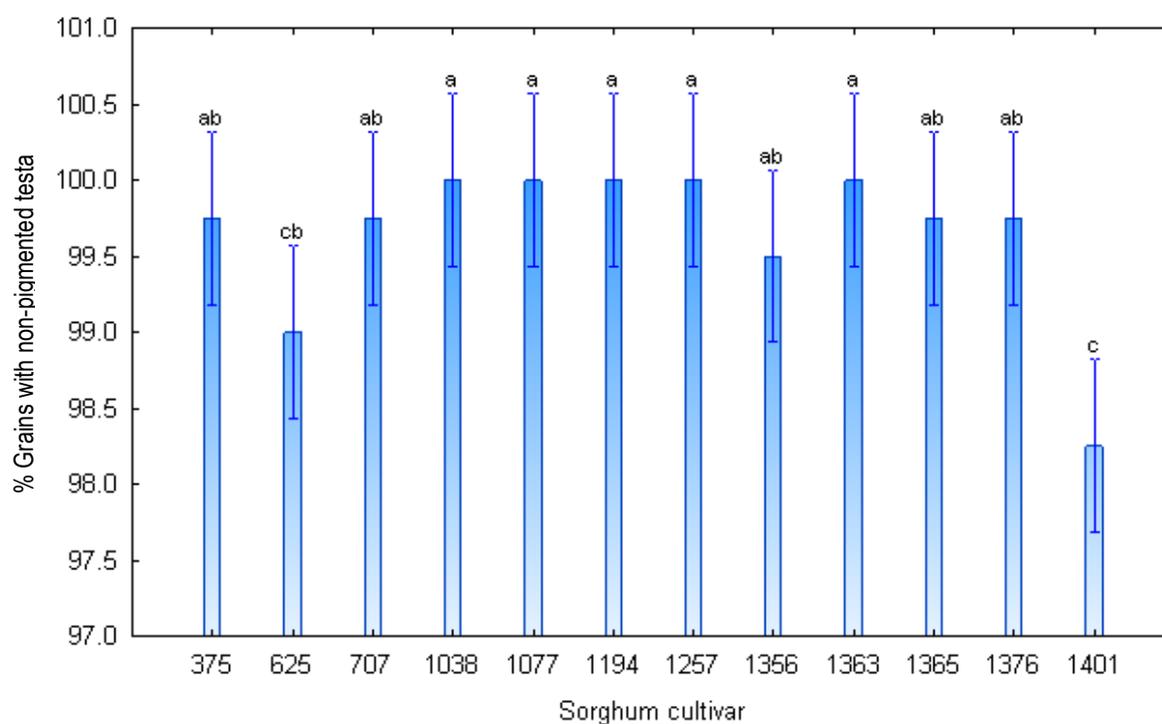
### *Statistical analysis*

Statistical analysis was performed and graphs were generated with Statistica version 10.0 (StatSoft, Inc., Tulsa, OK, 74104, USA). The vertical bar represents 95% confidence interval for the averages of the analyses. The least significant difference (LSD) post-hoc testing was performed to determine any significant differences amongst the means.

## Results and Discussion

### Tannins

Cultivars used in this study were all non-tannin sorghums (Fig. 3.2) and could therefore all be further analysed to ascertain their potential use in the production of lager beer. Batches containing  $\geq 95\%$  grains with non-pigmented testa are considered non-tannin sorghums (Taylor & Taylor, 2008). Tannins play in protecting the grain against insects, birds and microbial attack (Duodu *et al.*, 2003), however sorghum cultivars utilised for the production of beer must be tannin-free. Tannins affect fermentation negatively by reducing the digestibility of proteins and carbohydrates by binding to them and rendering them insoluble (Dicko *et al.*, 2006; Duodu *et al.*, 2003). This consequently negatively affects growth and metabolism of the yeast, alcohol and flavour compounds production during fermentation (Agu & Palmer, 1998). Tannins also bind amylases and thus inhibit their activity and consequently hinder starch hydrolysis (Daiber, 1975).

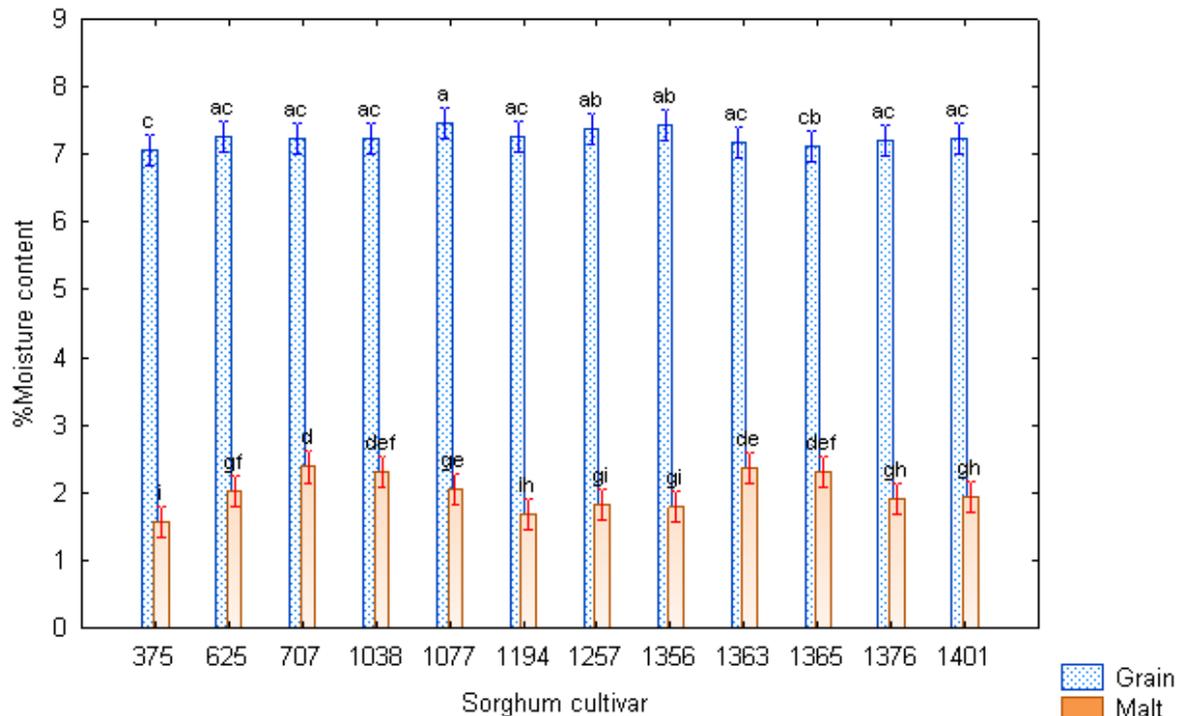


**Figure 3.2** Percentage grains with non-pigmented testa in batches of twelve Zambian sorghum cultivars. All results are means of at least two replicates. Cultivars with different letter super scripts differ significantly ( $p \leq 0.05$ ) from each other.

### Moisture content

Moisture content of all unmalted grains and malt ranged from 7.0 to 7.4% and 1.6 to 2.3% respectively (Fig. 3.3). It has been reported that grains at a moisture content of up to 11.7% will keep safe without deterioration during storage (Agu & Palmer, 1998). During malting the moisture content is increased to about 33-36% in order to initiate the metabolism of the living tissues which are dormant when the grains are unmalted (Agu & Palmer, 1998; Briggs, 1998). When germination is complete, endosperm modification is inhibited by kilning the green malt to moisture

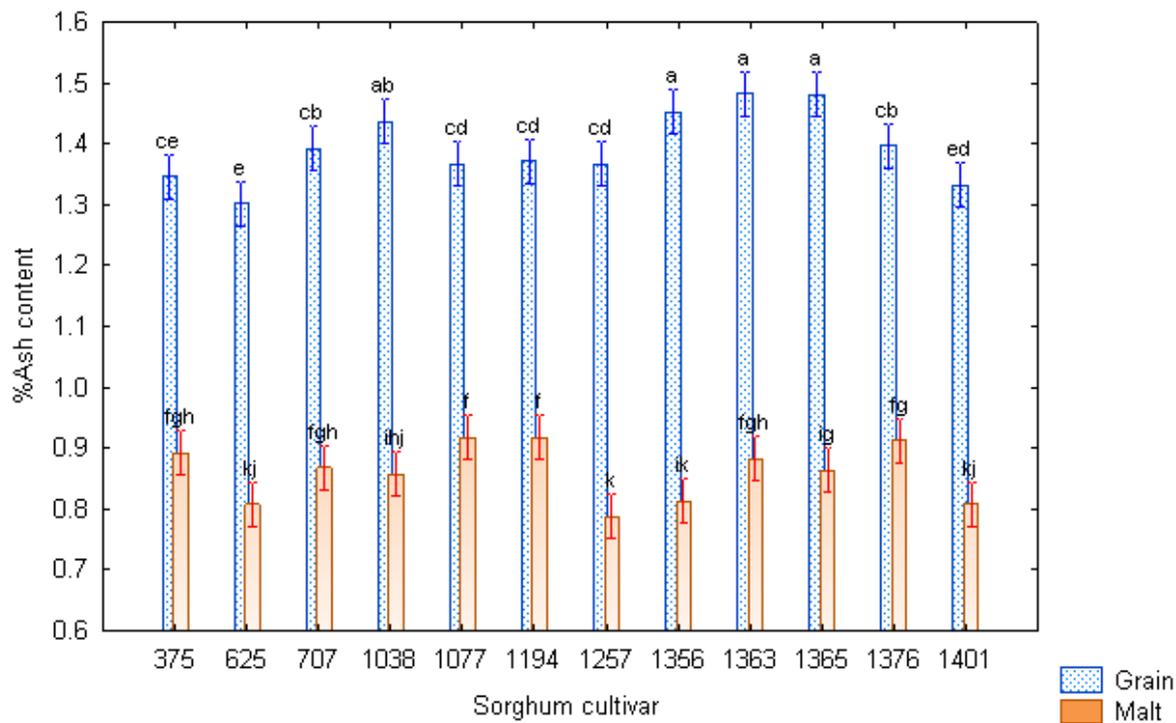
content between 5.2 and 5.7% at a specific time and temperature (Bamforth, 2006; Ogu *et al.*, 2006). Green malt is also kilned for the purpose of producing a shelf stable product (Taylor & Belton, 2002). The low moisture content (1.6 to 2.3%) in malts used in this study could be a result of difficulties encountered in controlling temperatures at which the green malt was kilned and thus leading to high moisture loss.



**Figure 3.3** Effect of malting on percentage moisture content of 12 Zambian cultivars. All results are means of at least three replicates. Cultivars with different letter super scripts differ significantly ( $p \leq 0.05$ ) from each other.

#### Ash content

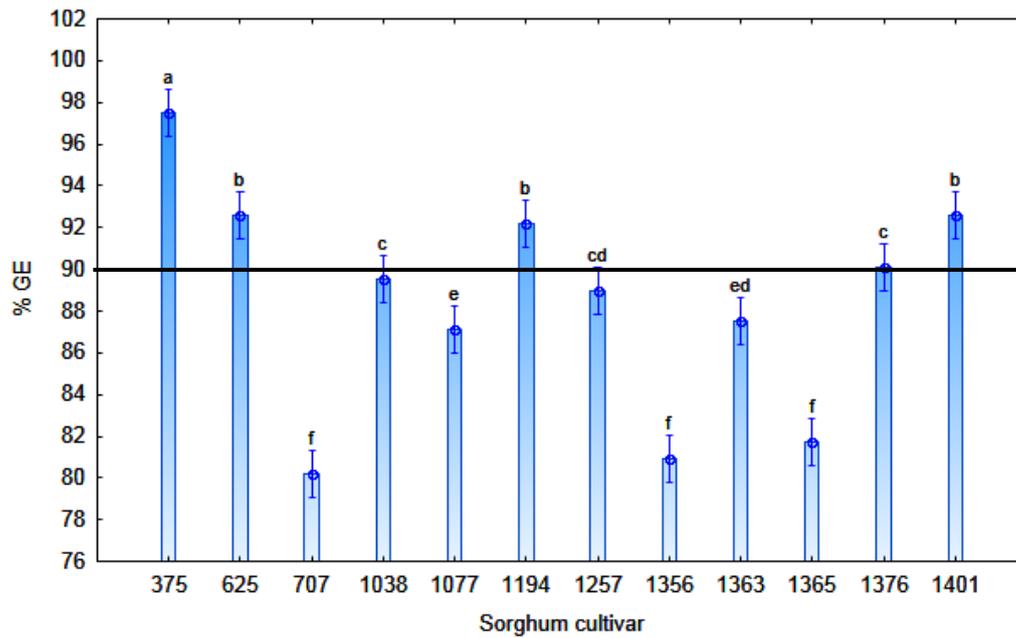
The ash content of unmalting grains ranged from 1.3 to 1.4% (Fig.3.4). The observed ash content falls within the range reported in literature (0.3 and 1.7%) (Cagampang & Kriels, 1984; Pontieri *et al.*, 2011). This is beneficial for lager brewing as the yeast needs minerals for optimum function during wort fermentation (Bamforth, 2006). A significant decrease in ash content was observed in all cultivars after malting, this was due to the removal of roots and shoots after kilning (Fig 3.4). Despite the loss in ash content all cultivars were still within the 0.3 and 1.7% range reported in literature (Cagampang & Kirleis, 1984; Pontieri *et al.*, 2011).



**Figure 3.4** Effect of malting on percentage ash content of 12 Zambian sorghum cultivars. All results are means of at least three replicates. Cultivars with different letter super scripts differ significantly ( $p \leq 0.05$ ) from each other.

#### *Germinative energy (GE)*

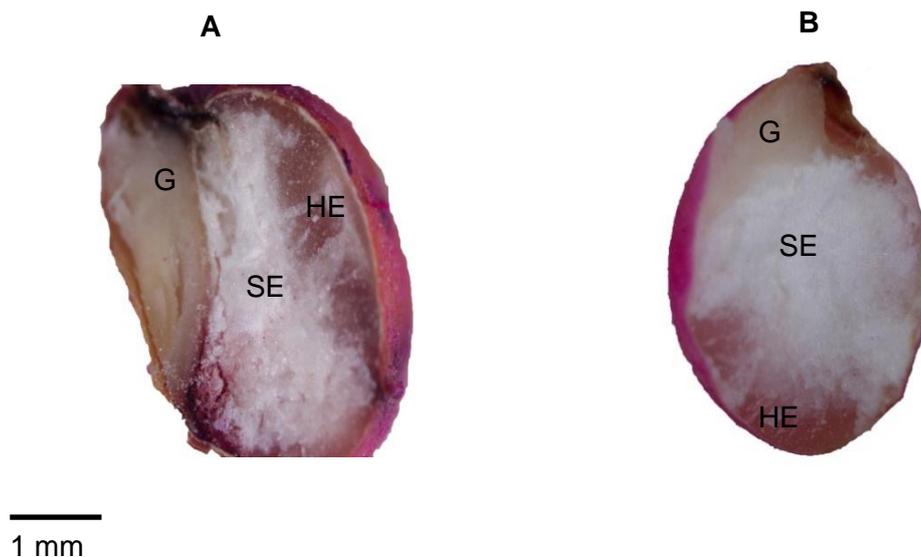
Cultivar MMSH-375 had the highest GE of 97% followed by MMSH-625, 1401 and 1194 which did not differ significantly ( $p \geq 0.05$ ) (Fig. 3.5). Cultivar MMSH-1376 had a GE of 90% and did not differ significantly with MMSH-1038 and 1257. Low GE ( $< 90\%$ ) was observed in cultivars MMSH-707, 1376, 1077, 1356, 1363 and 1365 (Fig. 3.5). A cultivar with  $GE \geq 90\%$  is considered appropriate for malting and brewing (Taylor & Taylor, 2008). Cultivars with GE values below 90% (MMSH-707, 1376, 1077, 1356, 1363 and 1365) (Fig. 3.5) are not suitable for malting as they are likely to be associated with insufficient enzyme development and consequently low extract levels due to inadequate endosperm modification (Agu & Palmer, 1998). Low GE indicates the inability of viable grains to germinate under conditions suitable for germination (dormancy). The expression of dormancy is influenced by both genetic and environmental factors (Ramagosa *et al.*, 2001). The peripheral tissues of the kernel rather than the embryo itself may induce or maintain dormancy in cereal seeds by limiting oxygen supply to the embryo (Van Beckum *et al.*, 1993). Storage conditions and duration of storage are main environmental factors inducing dormancy in barley seeds (Koornneef *et al.*, 2002).



**Figure 3.5** Percentage germinative energy (GE) of 12 Zambian sorghum cultivars. All results are means of at least three replicates. Cultivars with different letter super scripts differ significantly ( $p \leq 0.05$ ) from each other. The horizontal line is used to indicate cultivars whose GE is 90% or above and these are cultivars considered appropriate for malting.

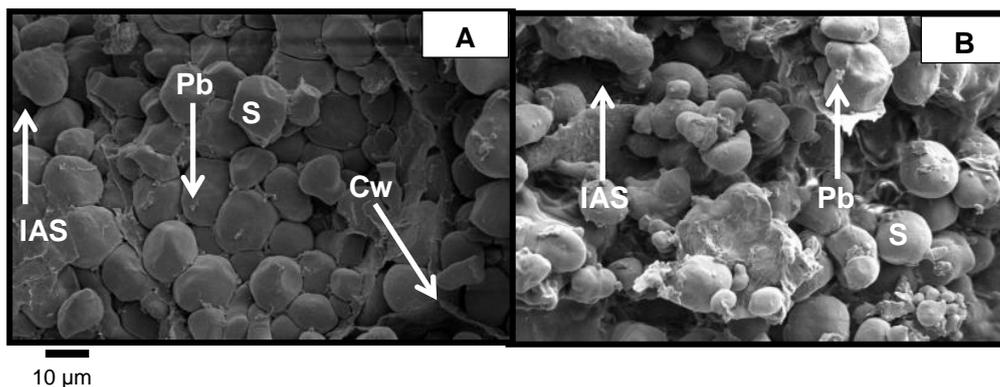
#### *Visual hardness and Scanning Electron Microscopy (SEM)*

Sorghum cultivars MMSH-1356 and 707 were classified as soft grains due to the high proportion of soft endosperm (Figs. 3.6 and 3.7). Sorghum cultivars MMSH-1401, 375, 1376, 1194, 1365 and 625 were classified as intermediate grains (Figs. 3.8 and 3.9) because less than 50% of their endosperm was corneous. Sorghum cultivars MMSH-1038, 1363, 1257 and 1077 had a high proportion of the hard endosperm fraction and were thus classified as hard grains (Fig. 3.10).



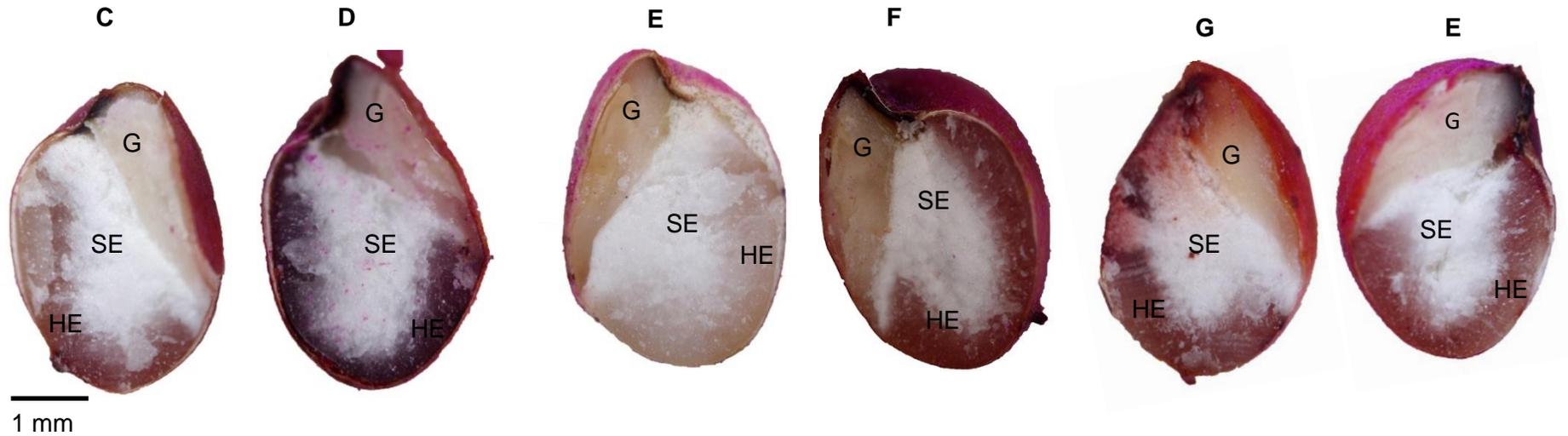
**Figure 3.6** Micrographs of soft sorghum cultivars taken under a stereomicroscope (A = MMSH-1356, B = MMSH-707) where G= germ, SE = soft endosperm and HE = hard endosperm.

The soft grains could be suitable for malting provided their GE is  $\geq 90\%$ . This is due to the loosely packed starch granules with larger intergranular spaces clearly visible in Fig. 3.7 which consequently results in exposure of starch granules and proteins to degradation by hydrolytic enzymes during malting (Hoseney *et al.*, 1974; Palmer, 1992). When compared to the corneous endosperm (Fig. 3.11), the floursy endosperm (Fig. 3.7) is however relatively free of protein bodies. This could therefore result in production of low FAN levels and negatively affect yeast growth during fermentation. The breeding of cultivars with higher protein content in the soft endosperm protein matrix could possibly be ideal for lager style beer production.

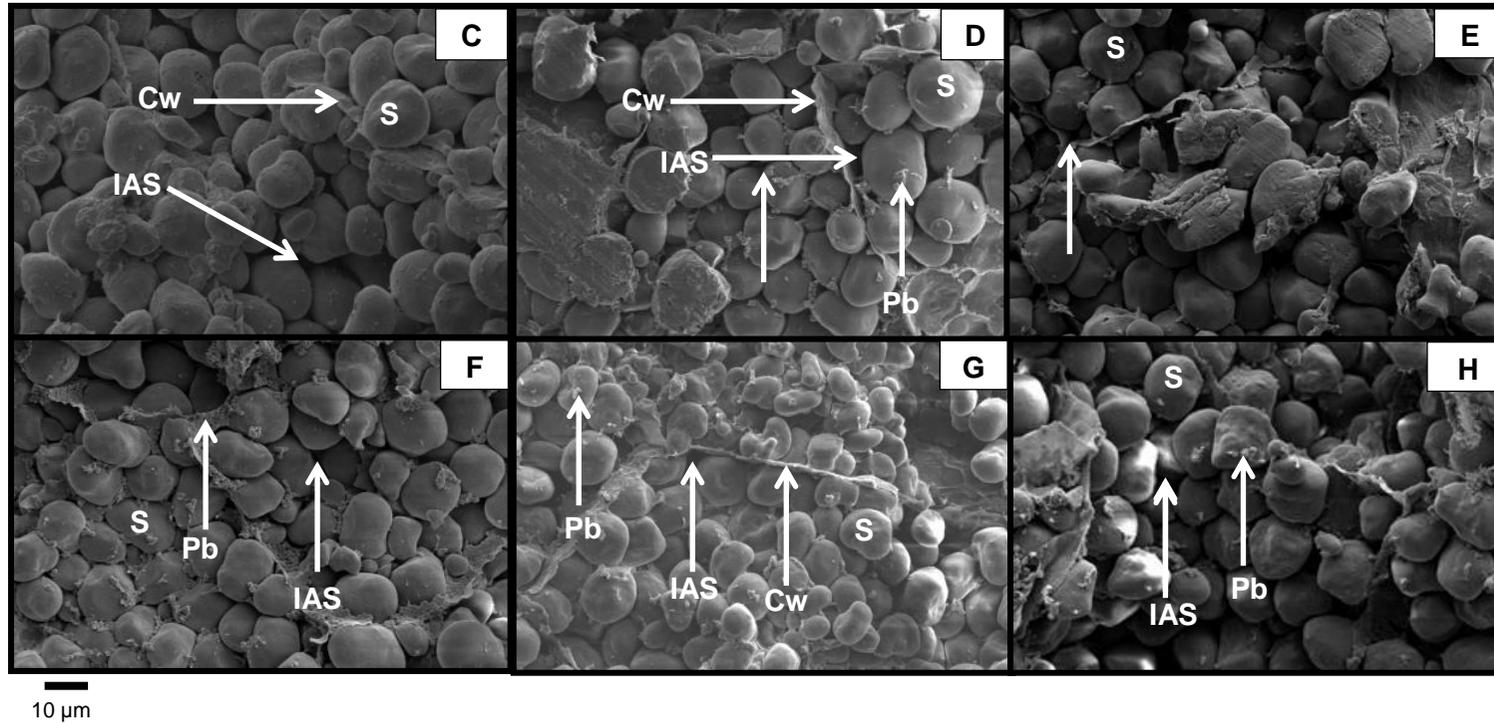


**Figure 3.7** Scanning electron micrographs of the soft endosperm of 2 Zambian sorghum cultivars with soft endosperm texture (A = MMSH-1356 and B = MMSH-707) where S = starch, IAS = intergranular air space, Pb = protein bodies and Cw = cell wall material.

Intermediate cultivars (MMSH-1401, 375, 1376, 1194, 1365 and 625) have a relatively equal proportion of soft and hard endosperm (Fig. 3.8). All these intermediate cultivars except for MMSH-1365 had a GE considered suitable for potential malting and brewing. In a study on the effect of kernel size and texture on the malting properties of 12 sorghum cultivars, intermediate sorghum cultivars were found to be more suitable for malting than those with mainly floursy endosperm (Abiodun, 2002). This could possibly be as a result of the generally few protein bodies observed in scanning electron micrographs of the soft endosperm (Fig. 3.7) and the GE of each cultivar which plays an important role during malting.

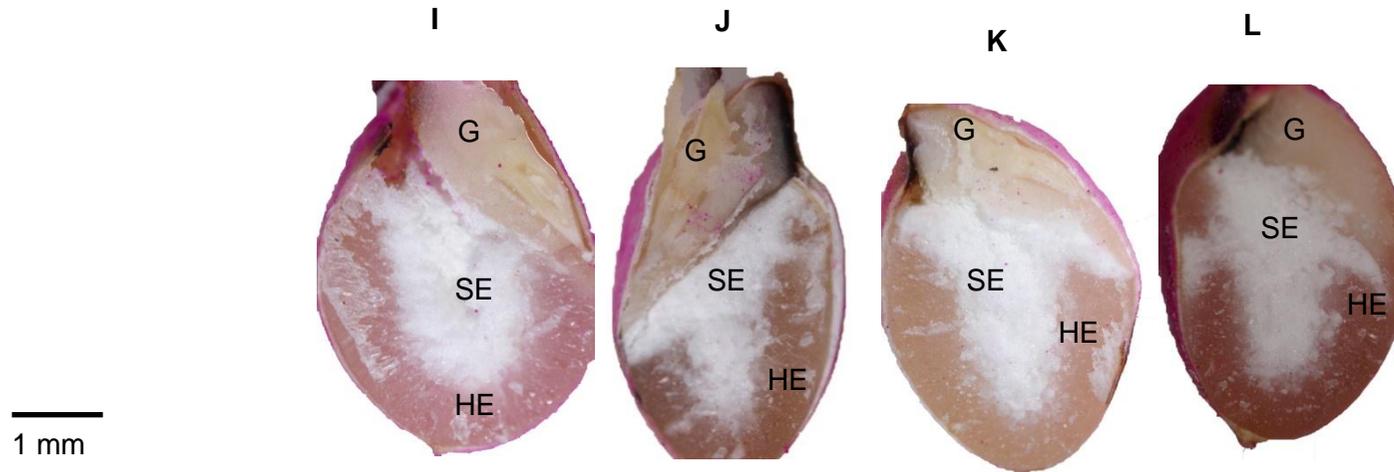


**Figure 3.8** Micrographs of Zambian sorghum cultivars with intermediate endosperm texture taken under a stereomicroscope (C = MMSH-1401, D = MMSH-375 and E = MMSH-1376; F = MMSH-1194; G= MMSH-1365 and H= MMSH-625) where G = germ, SE = soft endosperm and HE = hard endosperm.

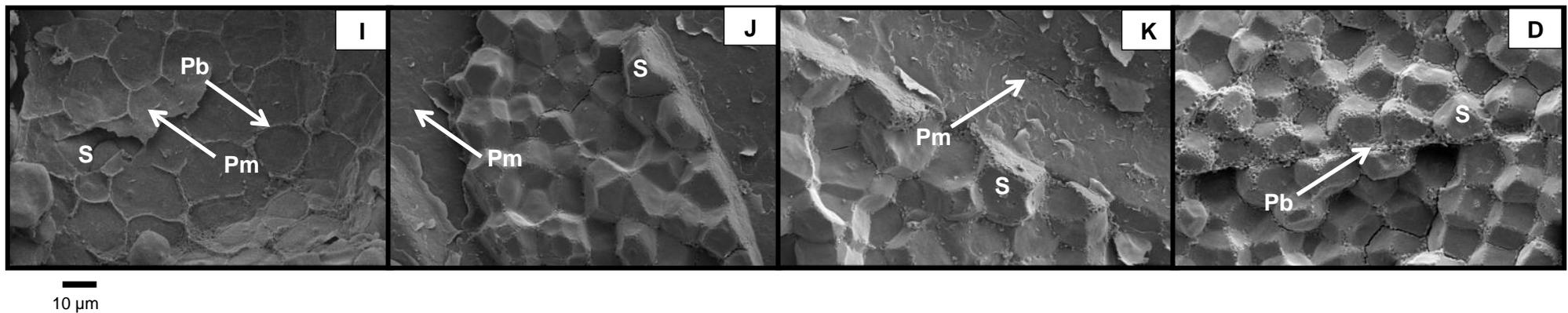


**Figure 3.9** Scanning electron micrographs of the soft endosperm of 6 Zambian sorghum cultivars with intermediate endosperm texture (C = MMSH-1401, D = 375, E= 1376, F = 1194, G = 1365 and H = 625), where S = starch, Cw = cell wall material, Pb = protein bodies, IAS = intergranular air space.

The hard sorghum cultivars MMSH-1038, 1363, 1257 and 1077 (Fig. 3.10) had a high proportion of the hard endosperm fraction. Scanning electron micrographs of all cultivars revealed the tightly packed structure of the hard endosperm which had no intergranular air spaces (Fig. 3.11). The protein bodies which are clearly revealed in MMSH-1038 surround starch granules and are embedded in a glutelin protein matrix which binds them together (Fig. 3.11). The corneous nature of the hard endosperm, the strength of the protein-starch adhesion and low solubility of cell wall material negatively affects the degradation of proteins and starch granules (Palmer *et al.*, 1989; Ogonna *et al.*, 2004). It would be likely that sorghum cultivars with high proportion of the hard endosperm will have lower extracts and free amino nitrogen (FAN) when compared with other sorghum types.

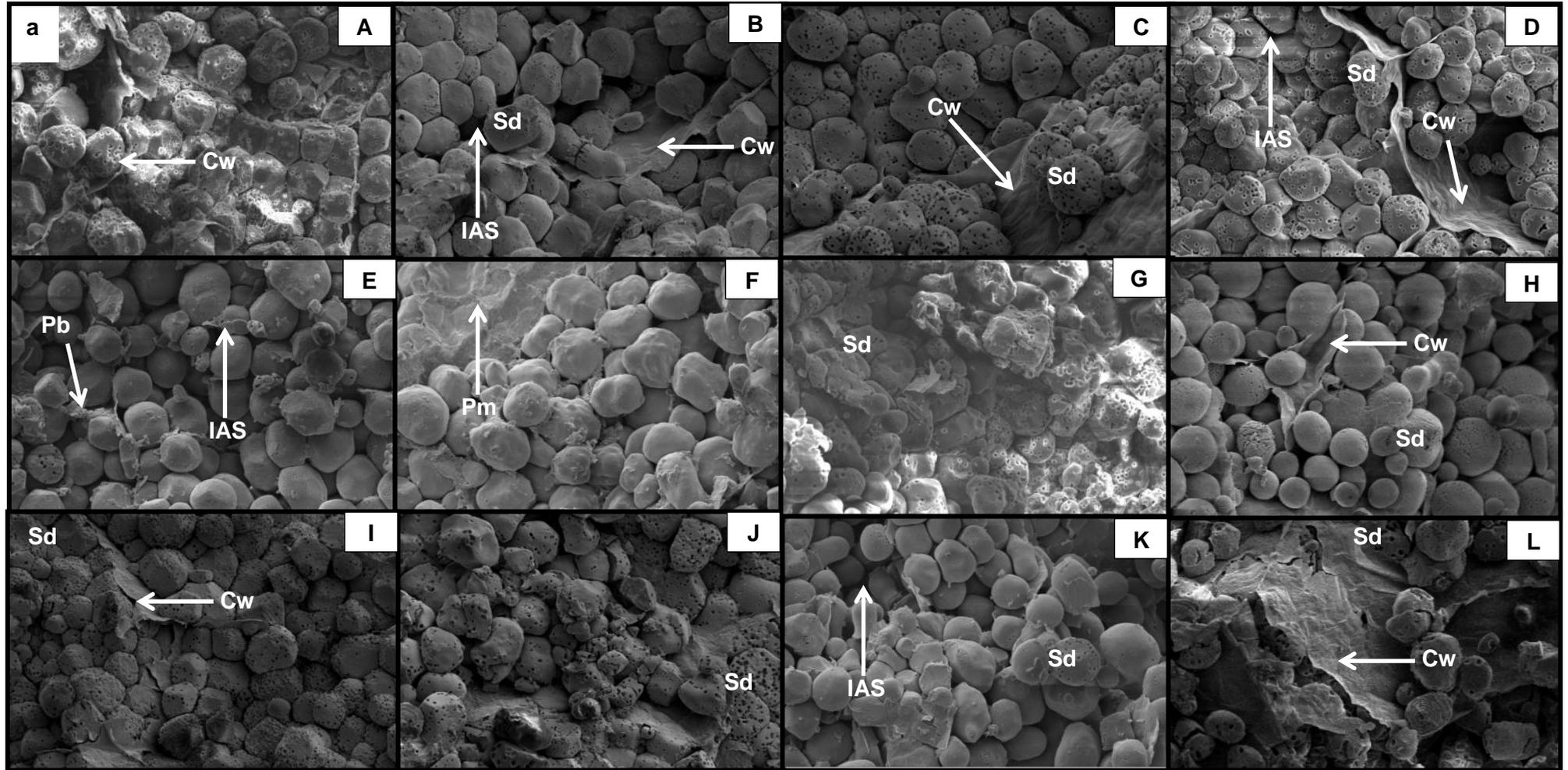


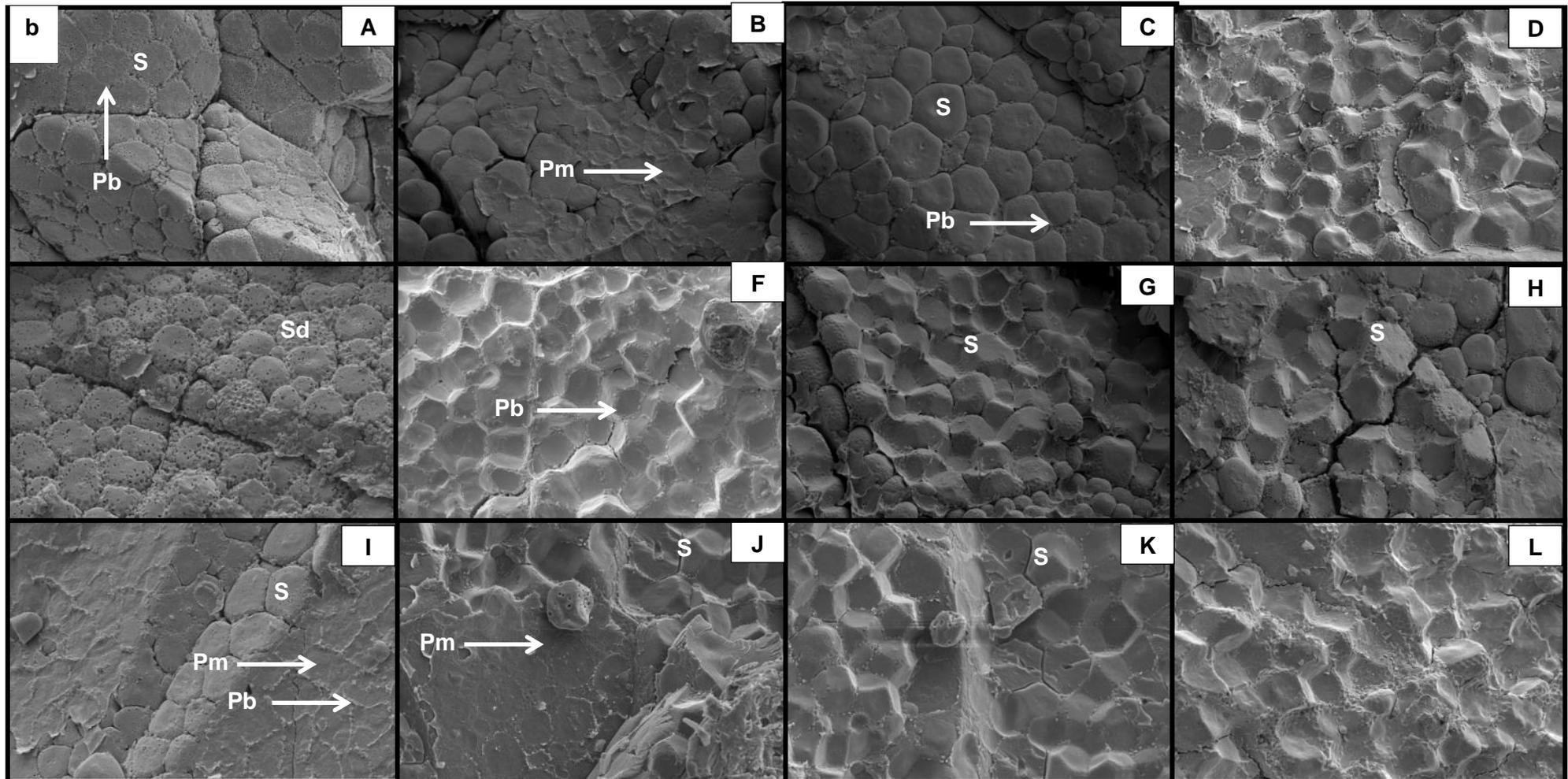
**Figure 3.10** Micrographs of Zambian sorghum cultivars with hard endosperm texture taken under a stereomicroscope (I = MMSH-1038, J = MMSH-1363, K = MMSH-1257 and L = MMSH-1077, where G = germ, SE = soft endosperm and HE = hard endosperm).



**Figure 3.11** Scanning electron micrographs of the hard endosperm of 4 Zambian sorghum cultivars with hard endosperm texture (I = MMSH-1038, J = 1363, K= 1257 and L = 1077, where S = starch granule, Pb = protein bodies and Pm = protein matrix).

After malting sorghum cell wall remained intact which is visible in Fig. 3.12 (a) and modification of the endosperm was restricted to starch degradation in the soft endosperm (Fig. 3.12 (a)). The development of holes (portals) was evident only in the soft endosperm (Fig. 3.12 (a)). Amylolytic enzymes produced degraded starch granules (resulting in development portals) and protein leading to production of fermentable sugars and FAN. The portals were most noticeable in soft cultivars MMSH-1356 (low GE) and MMSH-707 (low GE), intermediate cultivars MMSH-375 (high GE), 1401 (high GE) and 1365 (low GE) as well as in a hard cultivar MMSH-1038 (high GE) (Fig 3.12 (a)). The area at which the scanning electron micrographs were taken within each cultivar might have influenced the obtained results. Enzymes are produced in the germ; therefore most degradation might have probably occurred in parts of the soft endosperm close to the germ. It is possible that the micrographs of cultivars with low GE were taken close to the germ and therefore appears as if high starch degradation occurred in these whereas that could not be the actual case. The hard endosperm remained completely unmodified in all cultivars due to their tightly packed starch-protein matrix which was inaccessible to degrading enzymes (Fig. 3.12 (b)). This negatively affects the production of free amino nitrogen and leads to low extract levels (Ogbonna *et al.*, 2004). Therefore the utilisation of cultivars with mainly corneous endosperms in the production of sorghum lager beer might not be suitable because these cultivars may not yield levels of fermentable sugars which are considered enough to support optimal yeast function.



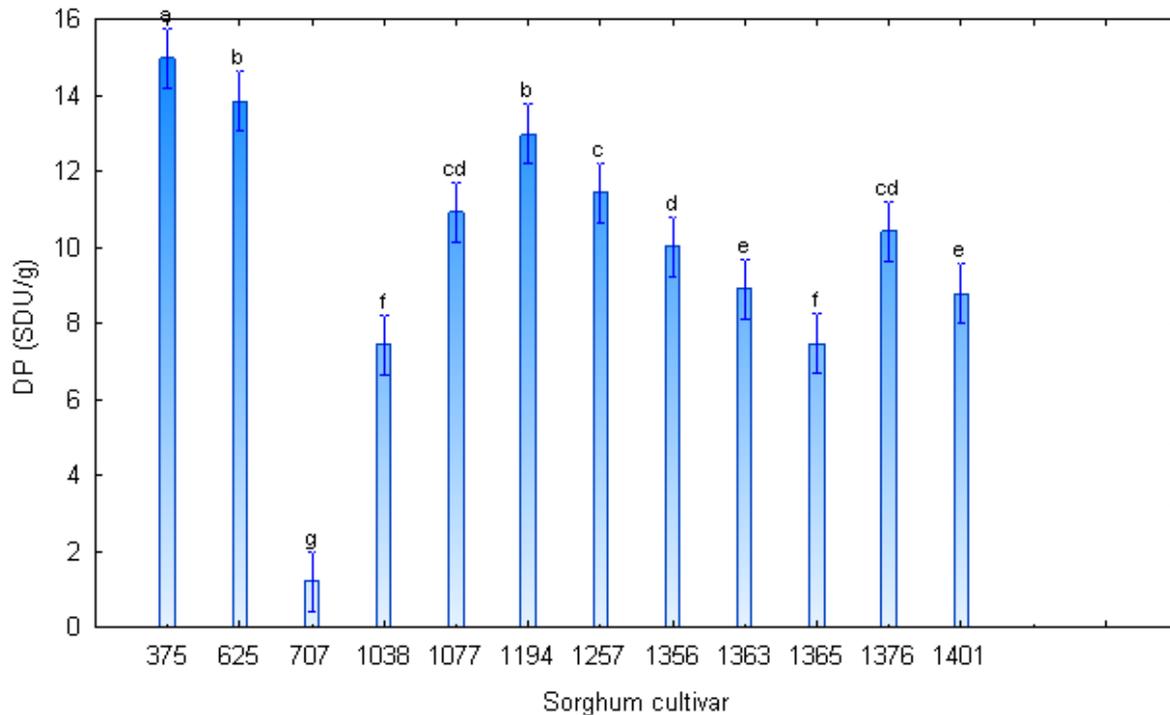


10 µm

**Figure 3.12** Scanning electron micrographs of the soft (a) and hard (b) endosperm of malts of 12 Zambian sorghum cultivars (A = 375, B = 625, C=1038, D = 1077, E = 1194, F = 1257, G = 1356, H = 1363, I = 707, J = 1401, K = 1376 and L = 1365 where S = starch granule, Cw = cell wall material, Pb = protein bodies, Pm = protein matrix.

*Diastatic power (DP)*

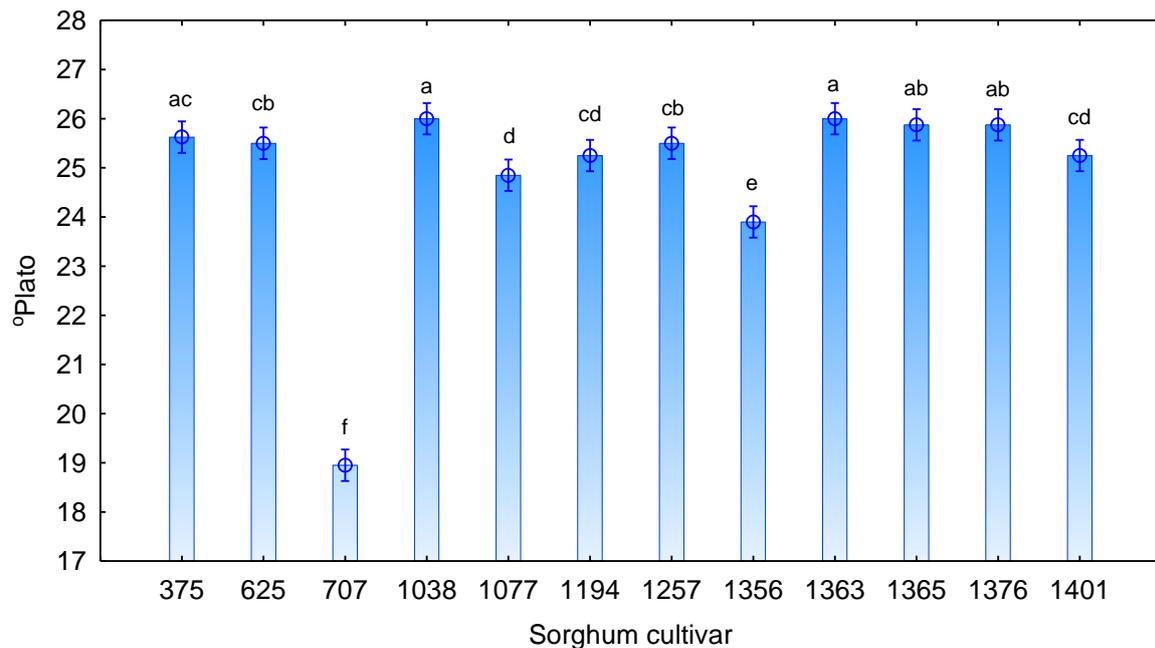
Cultivar MMSH-375 had a significantly higher ( $p \leq 0.05$ ) DP than all other cultivars (Fig. 3.13). This was, however, only 25% of the amount recommended suitable for commercial malting. Cultivars not reaching a diastatic power between 60 and 80 SDU/g are not suitable for commercial malting (Novellie, 1962).



**Figure 3.13** Diastatic power of malts of twelve Zambian sorghum cultivars. All results are means of at least three replicates. Cultivars with different letter super scripts differ significantly ( $p \leq 0.05$ ) from each other.

Diastatic power is a measurement of combined activity of  $\alpha$ - and  $\beta$ -amylases and plays an essential role in sorghum malt than wort (Owuama, 1997).  $\alpha$ -Amylase catalyses the hydrolysis of starch available in the grain to dextrins (O'Rourke, 2002) whereas  $\beta$ -amylase catalyses the hydrolysis of both starch and dextrins to give a mixture of glucose, maltose and maltotriose (O'Rourke, 2002; Owuama, 1999).  $\beta$ -amylase shows a significant correlation with malt DP accounting for 18-39% of saccharifying activity of sorghum malt (Owuama, 1997; Owuama, 1999). It has been reported that  $\alpha$ -amylase is more resistant to heat denaturation when compared to  $\beta$ -amylase (Botes *et al.*, 1967). The optimal temperature for  $\beta$ - and  $\alpha$ -amylase is  $60 \pm 0.5^\circ\text{C}$  and  $70^\circ\text{C}$  (Botes *et al.*, 1967) respectively, this is however cultivar dependant (Novellie, 1962; Owuama & Okafor 1990). Therefore duration of kilning, kilning temperature and moisture content of the malt influences amylase activity of sorghum malt (Owuama, 1999). Kilning temperatures above  $50^\circ\text{C}$  significantly reduce amylase activity in malted grain (Dewar *et al.*, 1997). Obeta *et al.*, (2000) reported that kilning at  $40^\circ\text{C}$  gave the highest  $\alpha$ -amylase activity when compared to kilning at  $50^\circ\text{C}$ ,

however this was cultivar dependant.  $\alpha$ -Glucosidase (maltase) is an enzyme which is also involved in starch degradation during sorghum grain germination to yield glucose (Sun & Henson, 1992). It is however not the dominant glucose producing enzyme in sorghum malt because it is possible for malt with high levels of  $\alpha$ -glucosidase to produce wort with low glucose levels (Agu & Palmer, 1997). In this study, despite the fact that the diastatic power of all cultivars did not reach the 60 to 80 SDU/g considered suitable for commercial malting and brewing the extract levels (sugars) were high (Fig. 3.14).

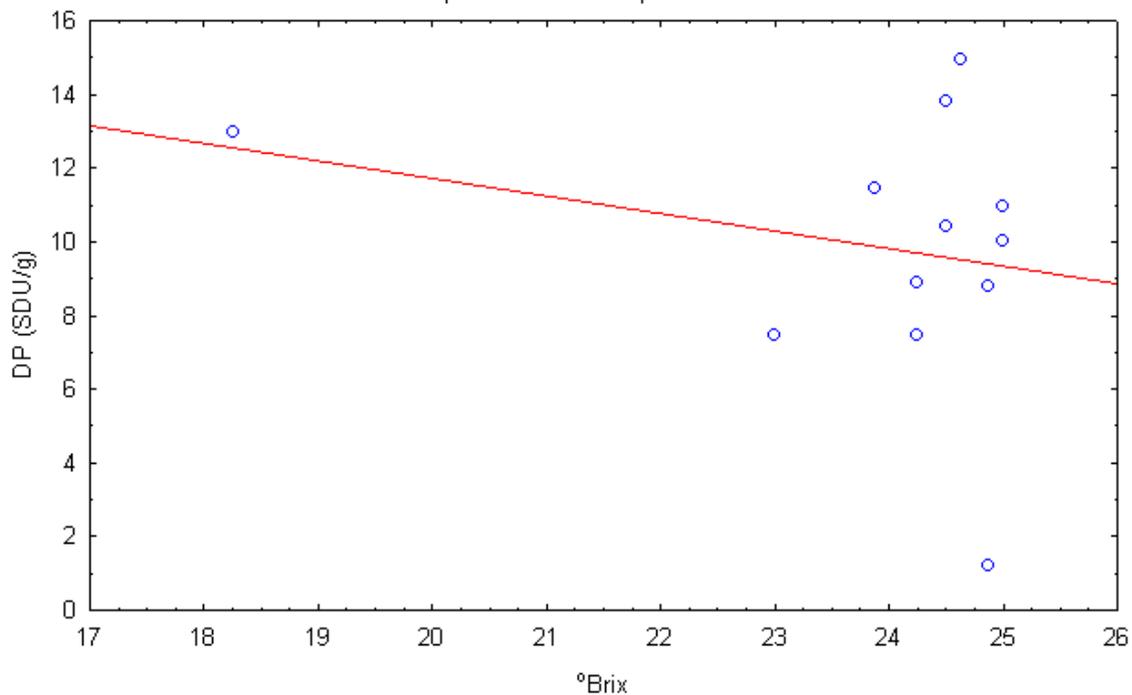


**Figure 3.14** Extract levels of malts of twelve Zambian sorghum cultivars. All results are means of at least three replicates. Cultivars with different letter super scripts differ significantly ( $p \leq 0.05$ ) from each other.

The extract levels ( $^{\circ}$ P) in all malts ranged between 19 and 26 $^{\circ}$ P and was negatively correlated ( $r = -0.08$ ) to DP (Fig. 3.15). This was contrary to the positive correlation which was expected between DP and extract levels. This meant that the level of DP achieved in each cultivar did not influence the extract levels in the wort as would have been expected. It was evident that DP levels illustrated in Fig. 3.13 were not the true amylase levels produced. Sufficient enzymes were actually produced during malting which consequently resulted in the high extracts in all cultivars (Fig. 3.14).

The negative correlation observed between DP and extracts could be due to the fact that during malting difficulties were encountered with maintaining kilning temperature constant at 50 $^{\circ}$ C. Due to an inability of the proofer thermostat to effectively maintain a temperature of 50 $^{\circ}$ C, green malt was kilned in a baking oven at temperatures between 55 $^{\circ}$ C and 65 $^{\circ}$ C. During kilning  $\beta$ -amylase might have been inactivated which could be the reason for the negative correlation observed between extract levels and DP. It is believed that

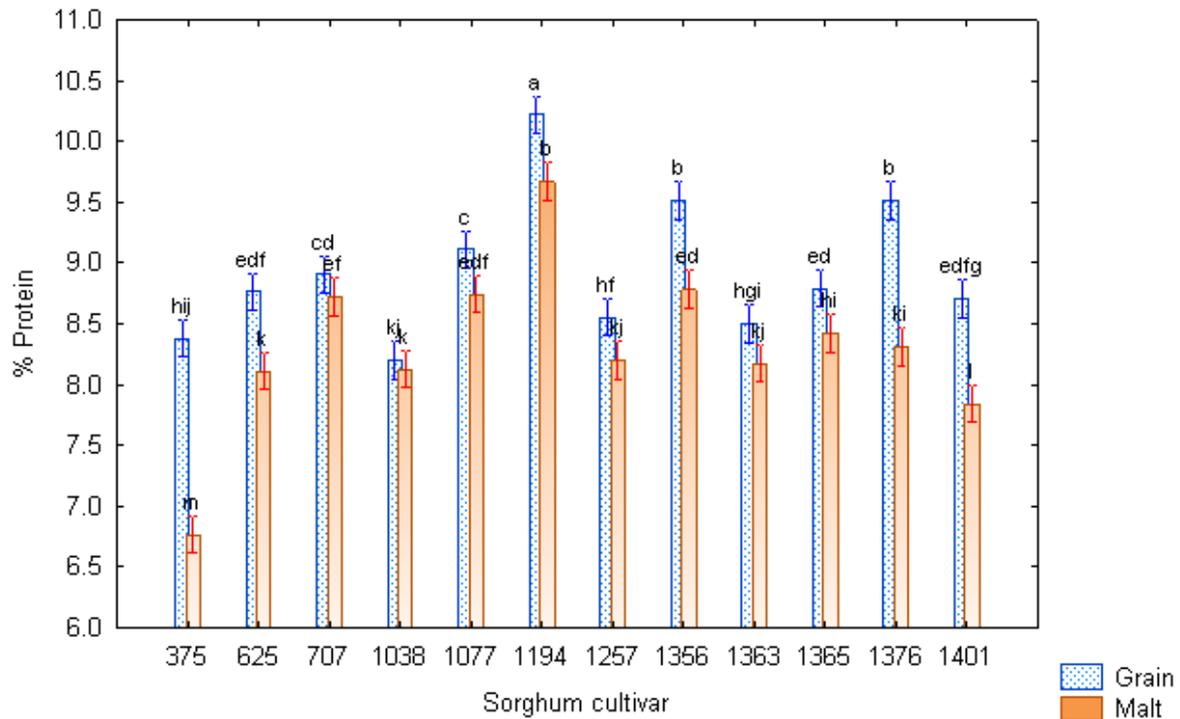
determining DP of green malt could have presented true amylase levels which were produced during malting. It is also possible that an error in the methodology could have also occurred during the determination of DP in malts.



**Figure 3.15** Correlation between diastatic power (DP) and extract of malts of twelve Zambian sorghum cultivars.

#### *Protein content*

A significantly high ( $p \leq 0.05$ ) protein content in both unmalted and malted grains was observed in MMSH-1194 (Fig 3.16). Malting decreased the protein content of all cultivars significantly ( $p \leq 0.05$ ) except for MMSH-1038. During malting, percentage decrease in protein content in high GE cultivars (MMSH-375, 625, 1401, 1194, 1038, 1257 and 1376) and low GE (MMSH-707, 1077, 1356, 1363, and 1365) ranged between 0.1-1.6% and 0.2-1.0% respectively (Fig. 3.16). The generally high percentage decrease in protein content in high GE cultivars was due to the removal of roots and shoots after kilning (Fig. 3.16).



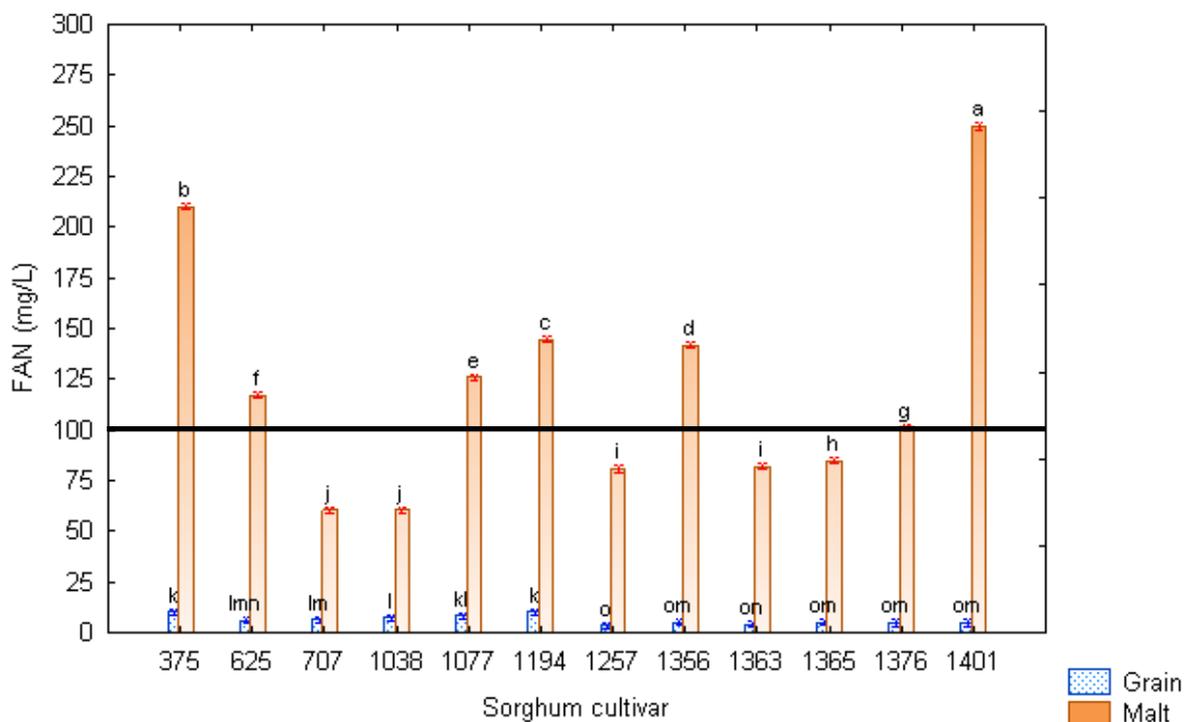
**Figure 3.16** Effect of malting on percentage protein content of 12 Zambian sorghum cultivars. All results are means of at least three replicates. Cultivars with different letter super scripts differ significantly ( $p \leq 0.05$ ) from each other.

MMSH-1194 had a high GE and protein content and thus, it was expected that after malting the protein content will decrease significantly (as with MMSH-375) when compared to other cultivars (Fig. 3.16). The high protein content observed in both unmalted and malted MMSH-1194 confirms that sorghum's protein digestibility is also influenced by other factors apart from high GE and protein content. These include exogenous (organisational structure) and endogenous factors (disulphide cross-linking, hydrophobicity of kafirins and changes in protein secondary structure) (Duodu *et al.*, 2003). The insignificant decrease in protein content in MMSH-1038 (hard grain and high GE) is due to the fact that protein in the hard endosperm was inaccessible to proteolytic enzymes during malting as a result of the corneous nature of the endosperm (Fig. 3.12b). The observed slight decrease in protein was mainly due to degradation of protein present in the soft endosperm (Fig. 3.12a). The production of proteolytic enzymes which differs from cultivar to cultivar could have also been low in MMSH-1038 and thus leading to the observed insignificant decrease in protein content after malting (Fig. 3.16).

#### *Free Amino Nitrogen (FAN)*

In unmalted grains a significantly high ( $p \leq 0.05$ ) FAN level was observed in cultivars MMSH-375, 1194 and 1077 (Fig. 3.17). These FAN values were however less than 10% of the recommended FAN level necessary to support proper yeast (Bajomo & Young, 1992)

functioning during wort fermentation. This suggests that very high levels of proteolytic enzymes would be required to produce FAN levels sufficient to support yeast when mashing with unmalted sorghum grain of these specific sorghum cultivars (Bajomo & Young, 1992; Agu & Palmer, 1998; Goode *et al.*, 2002). FAN level of 100 to 140 mg/L is considered enough to support optimal fermentation (Bajomo & Young, 1993). It is however generally believed that for high gravity brewing which is employed in modern day brewing a minimum of 150 mg/L of FAN is necessary to support proper yeast function (Beckerich & Denault, 1987).

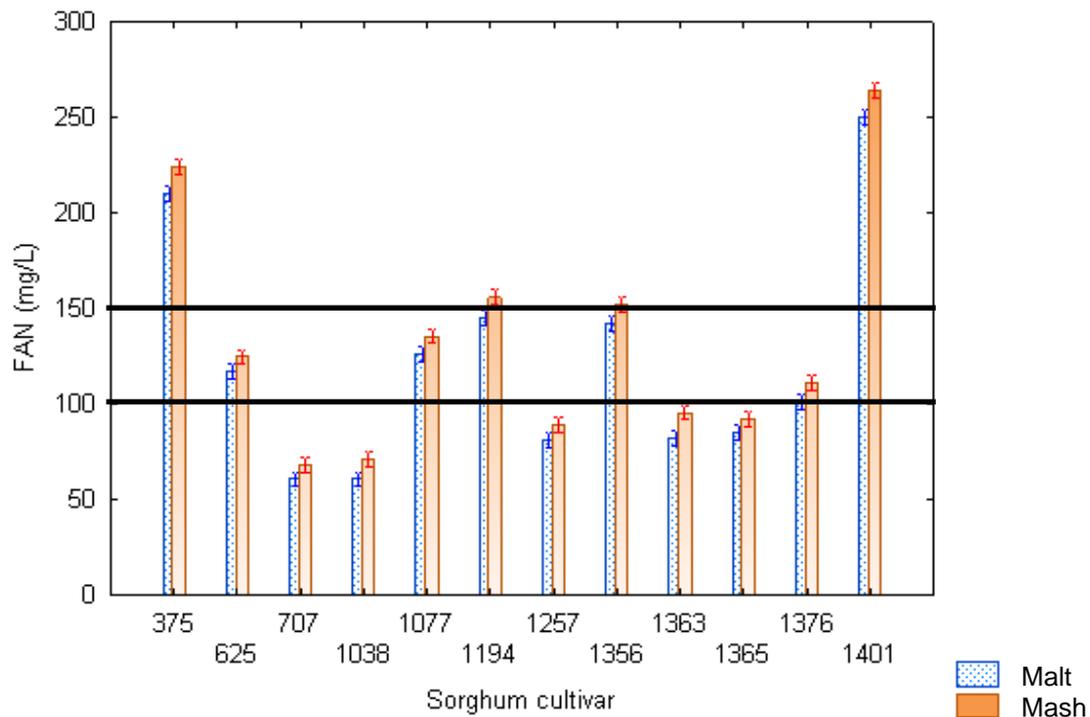


**Figure 3.17** Effect of malting on free amino nitrogen (FAN) levels of 12 Zambian sorghum cultivars. All results are means of at least three replicates. Cultivars with different letter superscripts differ significantly ( $p \leq 0.05$ ) from each other. The horizontal line is used indicate cultivars whose FAN levels were above 100 mg/L which is considered enough to support optimal fermentation and those above the 150 mg/L threshold recommended for high gravity brewing.

Malting increased FAN level in all cultivars significantly ( $p \leq 0.05$ ) (Fig. 3.17). Percentage increase in FAN was cultivar-dependent and ranged from 89 to 98%. During malting the 150 mg/L threshold recommended for high gravity brewing was only achieved in two intermediate hardness cultivars MMSH-375 and 1401 with high GE (Fig. 3.17). FAN level between 100 and 140 mg/L which is considered enough to support optimal fermentation was achieved in cultivars MMSH-625 (intermediate, GE >90%), MMSH-1077 (hard, GE <90%), MMSH-1194 (intermediate, GE >90%), MMSH-1356 (soft, <90%) and MMSH-1376 (intermediate, GE >90%) (Fig. 3.16). Low FAN levels (below 100 mg/L) were observed in cultivars MMSH-707 (soft, GE <90%), MMSH-1038 (hard, GE 90%), MMSH-1257 (hard, GE 90%), MMSH-1363

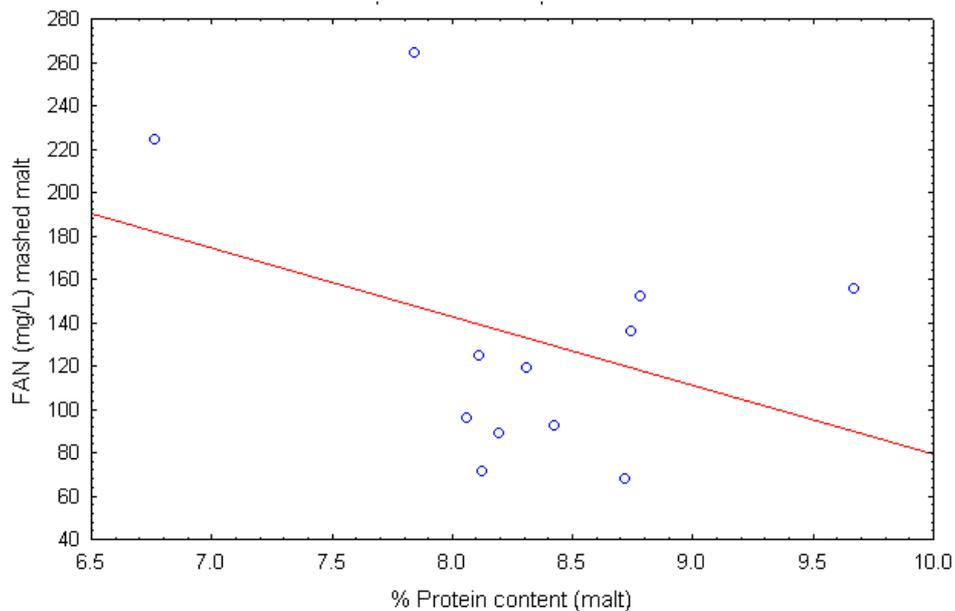
(hard, GE <90%) and MMSH-1365 (intermediate, GE <90%) (Fig.3.17). FAN levels below the 100 mg/L were expected only in the soft and hard sorghum cultivars with low GE due to the high proportion of the soft (relatively free of protein bodies) and hard endosperms (tightly packed starch-protein matrix which is inaccessible to degrading enzymes during malting). This was observed in all cultivars except for MMSH-1365 (intermediate, GE <90%), MMSH-1077 (hard, GE <90%) and MMSH-1356 (soft, GE <90%). MMSH-1365 had a low FAN level due to its GE of 81.5%. The reason for high FAN levels observed in cultivars MMSH-1077 and 1356 despite their low GE and nature of endosperms is not clear, they could be intermediate cultivars. It is therefore recommended that the endosperm texture of MMSH-1077 and 1356 be verified using other methods which have been used in literature to determine grain hardness. These include the Brabender microhardness tester (BHMT), Stenvert hardness tester (SHT), Particle Size Index (PSI) and Near infrared (NIR) (Pomeranz, 1986; Anglani, 1998).

Mashing malt increased FAN level in all cultivars (Fig. 3.18). FAN increase was cultivar dependant and it ranged from 5.5 to 10%. However mashing did not increase FAN level significantly in all cultivars ( $p \geq 0.05$ ) (Fig. 3.18). After mashing the 150 mg/L threshold necessary to support proper yeast functioning was achieved only in MMSH-1194 (intermediate, GE >90%) and MMSH-1356 (soft, GE <90%). Despite mashing, low FAN levels (below 100 mg/L) were still observed in cultivars MMSH-707 (soft, GE <90%), MMSH-1038 (hard, GE 90%), MMSH-1257 (hard, GE 90%), MMSH-1363 (hard, GE <90%) and MMSH-1365 (intermediate, GE <90%) (Fig. 3.18).



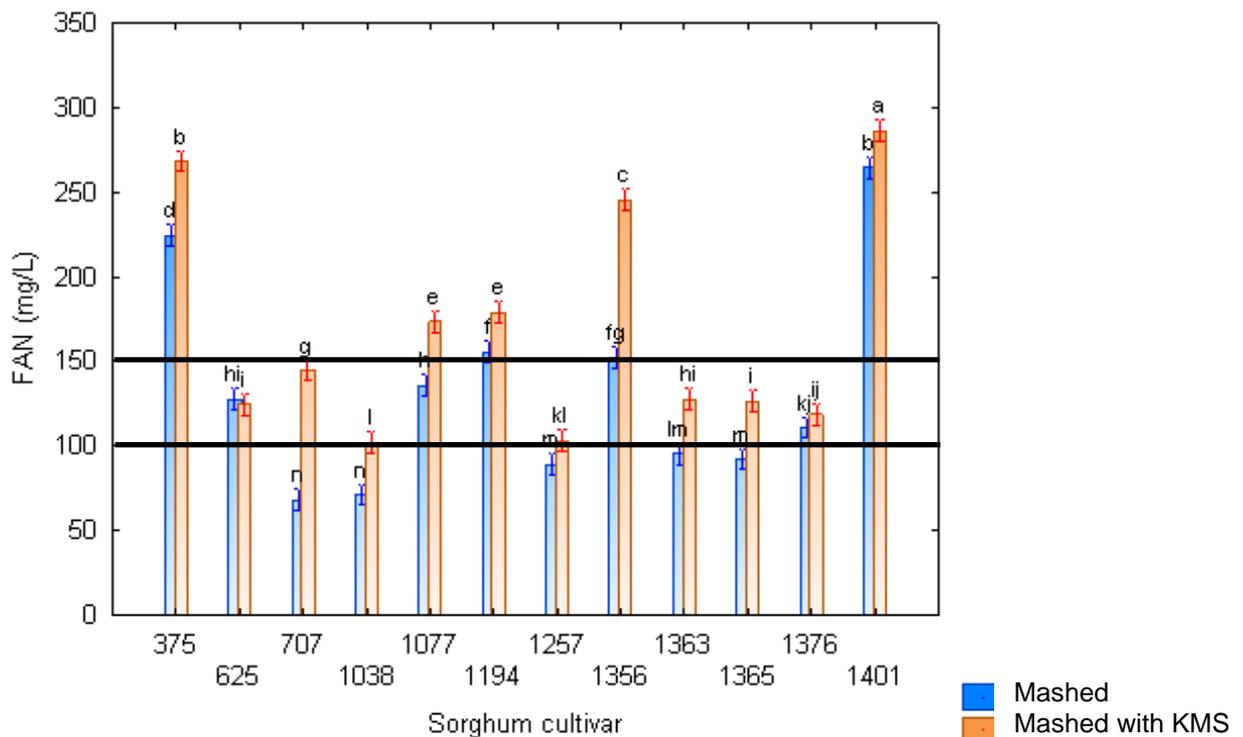
**Figure 3.18** Effect of malting on free amino nitrogen (FAN) levels of 12 Zambian sorghum cultivars. All results are means of at least three replicates. The horizontal lines are used to indicate cultivars whose FAN levels were above 100 mg/L which is considered enough to support optimal fermentation and those above the 150 mg/L threshold recommended for high gravity brewing. The letter superscripts did not differ significantly in all cultivars ( $p \geq 0.05$ ) from each other and we not inserted in the graph.

It was also observed that the cultivar with highest protein content (MMSH-1194) in both malted and unmalted grain of 9.7 and 10.2% respectively (Fig. 3.18) did not necessarily provide the highest FAN level content even after mashing. Also in all other cultivars, protein content negatively correlated ( $r = -0.13$ ) with level of FAN produced in all cultivars (Fig. 3.19). This thus confirms that there are a number of factors which affect proteolysis. These include the corneous nature of the hard endosperm, low solubility of cell wall material, decrease in protein digestibility as a result of wet cooking, the presence of disulphide bonds in proteins, hydrophobic nature of the kafirins and most likely varying proteolytic activity in the cultivars (Palmer *et al.*, 1989; Oria *et al.*, 1995; Taylor & Belton, 2002; Duodu *et al.*, 2003; Ogonna *et al.*, 2004).



**Figure 3.19** Correlation between free amino nitrogen (FAN) levels of mashed malts and protein content of malts of twelve Zambian sorghum cultivars.

The addition of a reducing agent, potassium metabisulphite (KMS) led to a further increase in FAN levels in all cultivars (Fig. 3.20). The FAN levels in all cultivars were above 100 mg/L which is considered enough to support optimal fermentation (Bajomo & Young, 1993). This was in agreement with studies which showed that reducing agents such as 2-mercaptoethanol (Hamaker et al., 1987), sodium bisulphite (Oria *et al.*, 1995), potassium metabisulphite (Ng'andwe *et al.*, 2008) and ascorbic acid (Arbab & El Tinay, 1997) increase sorghum protein digestibility. They reduce molecular disulphide bridges present in sorghum proteins (kafirins). The percentage increase in FAN when mash was treated with KMS was cultivar dependant and ranged from 2 to 53%. KMS increased FAN level in MMSH-1077 to above 150 mg/L. Cultivars MMSH-625 (intermediate), 707 (soft), 1038 (hard), 1257 (hard), 1363 (hard) and 1376 (intermediate) did not furnish the 150 mg/L FAN level necessary to support proper yeast function in high gravity brewing employed in modern day brewing even when mashed KMS. The reason for the insignificant increase in FAN levels when mashed with KMS observed in cultivars MMSH-625 and MMSH-1376 could be linked to the levels of proteolytic enzymes produced in the cultivars.



**Figure 3.20** Effect of addition of potassium metabisulphite (KMS) during mashing on free amino nitrogen (FAN) levels in mashed malts of 12 Zambian sorghum cultivars. All results are means of at least three replicates. Cultivars with different letter super scripts differ significantly ( $p \leq 0.05$ ) from each other. The horizontal lines are used to indicate cultivars whose FAN levels were above 100 mg/L which is considered enough to support optimal fermentation and those above the 150 mg/L threshold recommended for high gravity brewing.

## Conclusions

Although all 12 sorghum cultivars used in this study were tannin-free, they differ in their malting and brewing potential. Sorghum cultivars MMSH-375, 625, 1038, 1194, 1257, 1401 and 1376 had a GE considered appropriate for malting and brewing. Although the highest DP achieved in this study was only 25% of the level recommended suitable for commercial malting, high extract levels (between 18.7 and 25.5°P) in all cultivars were observed. The negative correlation between DP and extract levels obtained suggests that the obtained DP levels are not a true reflection of the amylase levels produced during malting. Although there is likelihood that saccharifying enzyme activity may have been reduced as a result of poor control of kilning temperatures, it is also possible that error in the methodology could have also occurred during the determination of DP in malts leading to lower DP results.

After malting the FAN levels above the minimum threshold generally considered enough to support high gravity brewing and support optimal yeast function during the production of sorghum lager beer were achieved only in MMSH-375 and 1401. FAN levels more than 200

mg/L were achieved in cultivars MMSH-375 and 1401. Mashing did not significantly increase FAN levels in all sorghum cultivars; however the addition of potassium metabisulphite (KMS) led to a further significant increase of FAN levels in all cultivars except for MMSH-625 and MMSH-1376. The reason for the insignificant increase in FAN levels when mashing with KMS observed in cultivars MMSH-625 and MMSH-1376 could be due to low solubility of cell wall material, decrease in protein digestibility as a result of wet cooking and most likely low proteolytic activity in the cultivars (Hoseney *et al.*, 1974; Glennie *et al.*, 1983; Aisien & Muts, 1987; Oria *et al.*, 1995; Ogbonna *et al.*, 2004).

Scanning electron micrographs revealed that the corneous endosperm was essentially unmodified during malting, indicating that it was inaccessible to hydrolytic enzymes. It was only the soft endosperm which was modified in all cultivars during malting. The breeding of cultivars with intermediate endosperm texture, weak strength of the protein-starch adhesion and high solubility cell wall material is a necessity. It will highly be beneficial for the malting and consequently brewing industry. Also the addition of KMS during mashing reduces disulphide bonds present in kafirins leading to increased FAN production and thus should be employed during the production of clear sorghum lager style beer. Overall it was observed that intermediate cultivars with high GE gave the most desirable results for potential malting and brewing of sorghum lager beer. MMSH-375, MMSH-1401, MMSH-1194 and MMSH-625 on basis of GE, endosperm texture and FAN levels furnished during malting are recommended to be utilised in the commercial production of sorghum lager beer.

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## **Chapter 4**

Determination of antiyeast activity in selected sorghum cultivars with malting and brewing potential

## Chapter 4

### Determination of antiyeast activity in selected sorghum cultivars with malting and brewing potential

#### Abstract

Nitrogen containing compounds from selected malts of interest (MMSH-375, MMSH-625, MMSH-1194 and MMSH-1401) were isolated and their effect on yeast growth was determined. Thin layer chromatography (TLC) and ultra performance liquid chromatography-linked mass spectrometry (UPLC-MS) fingerprinting was carried out in order to characterise N-containing factors (amino acids and peptides) in the extract. Differences were observed in all four cultivars with the HP-TLC data in terms of total ninhydrin response. UPLC-MS showed both similarities and differences between the cultivars and some compounds present in the extracts were characterised. In all four cultivars N-containing compounds had a positive effect on fermentation by promoting yeast growth. In general MMSH-375 and MMSH-625 had the highest %yeast growth when compared to other cultivars; this indicated that they have higher concentrations of group A, B and C amino acids and possibly small peptides (di-, tri- and tetra peptides) consisting of these amino acids and UPLC-MS results confirmed this. Overall MMSH-375 is recommended as the best overall choice to be used in the commercial production of sorghum clear lager beer.

#### Introduction

During lager beer production, *S. cerevisiae* ferments sugars available in wort (glucose, fructose, maltose and maltotriose) producing alcohol, heat, carbon dioxide and flavour compounds as by-products which diffuse out of the yeast cells (Pilkington *et al.*, 1998; Hodžić *et al.*, 2008). Apart from sugars, the yeast also requires other nutrients for optimum function during fermentation. These nutrients include free amino nitrogen (FAN), vitamins, trace elements (copper and zinc ions) and sulphur, calcium and magnesium as well as minor growth factors (Bamforth, 2006; Eßlinger, 2009). FAN is primarily produced as a result of protein hydrolysis during malting of sorghum, with small amounts being produced during the mashing stage of the brewing process (Lekkas *et al.*, 2007). The constituents of FAN include individual amino acids (Jones & Pierce, 1967), ammonium ions and small peptides (Lekkas *et al.*, 2007). FAN serves as a source of nitrogen for the yeast (Taylor *et al.*, 1985), and plays a role in the growth of the yeast and consequently alcohol and flavour compound production (Agu & Palmer, 1998). The level and quality of FAN in wort affects both yeast growth and the final alcohol content of beer (Taylor *et al.*, 1985). During yeast growth

nitrogen is required for the synthesis of amino acids, purines, pyrimidines, some carbohydrates, lipids, enzyme cofactors and other substances (Willey *et al.*, 2007). Purines and pyrimidines are utilised for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis (Eßlinger, 2009). Insufficient FAN in wort leads to inefficient yeast cells growth and consequently inefficient fermentation performance (Lekkas *et al.*, 2007). A minimum FAN level of 100 to 140 mg/L is considered necessary to support optimal sorghum wort fermentation (Bajomo & Young, 1993).

There is however a group of antimicrobial proteins and peptides known as pathogenesis related (PR) proteins which are located in various parts of the sorghum grain and in protein bodies (Chandrashekar & Satyanarayana, 2006). PR proteins have compact structures which are stabilised by a number of disulphide bridges and are either acidic or basic (Gorjanović, 2009). Due to their compact structures PR proteins are able to withstand extremes of pH and temperature, and are also resistant to proteolysis. They are therefore, able to withstand food processing and are present in processed foods and beverages (Van Loon *et al.*, 2006; Gorjanović, 2009; Porter *et al.*, 2010). PR proteins were originally defined as proteins induced only during pathological or related situations and assumed to be involved in plant defense (Antoniw *et al.*, 1980; Leslie *et al.*, 1993; Van Loon *et al.*, 1994; Van Loon & Van Strien 1999). However, subsequent studies confirmed the presence of PR proteins in normal (uninfected) plant tissues and cereal grains (Muthukrishnan *et al.*, 2001; Chandrashekar & Satyanarayana, 2006). PR proteins are divided into 17 families and are numbered in the order of discovery. PR proteins belonging to different families differ in their isoelectric point (pI), molecular weight (Mr) and immunological cross-reactivity (Muthukrishnan *et al.*, 2001; Gorjanovic, 2009). The 17 PR protein families are not all represented in all plant species (Van Loon *et al.*, 2006). In sorghum grains some PR proteins have been isolated and characterized (Leslie *et al.*, 1993; Chandrashekar & Satyanarayana, 2006). Examples of PR proteins which have been isolated and characterised in sorghum grains are given in Table 4.1. The presence and function of some PR protein families in sorghum grains remain to be explored (Chandrashekar & Satyanarayana, 2006).

**Table 4.1** PR protein families which have been isolated in sorghum grains

Antimicrobial peptide or protein	Molecular mass	Reference
Chitinases	A band of 29 kDa and three additional bands ranging from 21 to 24 kDa were identified	Leslie <i>et al.</i> , 1993
(1→3)- $\beta$ -glucan hydrolases	One band of 30 kDa was identified	Leslie <i>et al.</i> , 1993
Sormatins (thaumatin-like proteins, TLPs)	Two bands of 22 and 29 kDa were identified	Vigers <i>et al.</i> , 1991; Leslie <i>et al.</i> , 1993
Proteinase inhibitors	5 kDa	Bloch & Richardson, 1991
Ribosome inactivating proteins (RIPs)	32 kDa	Seetharaman <i>et al.</i> , 1996
Thionins	5 kDa	Bloch <i>et al.</i> , 1998

PR proteins form part of the innate defence system which plants have evolved in order to resist a wide range of pathogenic microorganisms and insects (Gorjanović *et al.*, 2007). Grain mould is the most significant disease in sorghum grains. It results from the infection of developing grains by parasitic fungi while still in the field (Frederiksen, 1982) and can occur as early as the anthesis phase, which is a critical point during development where the sorghum plant is most susceptible to fungal infestation (Little, 2000). Grain mould is the single greatest impediment for optimum grain yield where anthesis occurs during humid, warm and rainy seasons. *Fusariumthapsinum* and *Curvularialunata* are the most important fungal species which cause grain mould in sorghum worldwide (Little, 2000).

Sorghum PR proteins have antifungal activity and play an essential role in grain mould resistance (Waniska, 2000; Chandrashekar & Satyanarayana, 2006). Most of these antifungal proteins were observed from grain mould resistant sorghums in the hard endosperm (Kumari & Chandrashekar, 1994). Despite their known antifungal activity, the effect of sorghum PR proteins on brewer's yeast is unknown (Chandrashekar & Satyanarayana, 2006). It has been reported that during small scale laboratory sorghum wort fermentation, the same yeast crop was used for over five consecutive fermentations without negatively affecting yeast growth (Bajamo & Young, 1994). However in the commercial production of sorghum lager beer only up to three consecutive fermentations can be achieved using the same yeast crop (Ng'andwe, 2008). Despite the fact that laboratory fermentation systems conditions differ significantly from those obtained in commercial practice (Bajamo & Young, 1994) there could be other factors playing a role in these

observed differences. Sorghum PR proteins could inhibit yeast growth during wort fermentation due to their antifungal activity. This could lead to fermentation problems (PYF and incomplete fermentation) which sorghum brewers are not yet aware of and also cause re-pitching problems experienced during the production of sorghum lager beer. This could cause great financial losses in the brewing industry.

In order to determine the effect of sorghum PR proteins on brewer's yeast a reliable and sensitive antimicrobial assay which uses brewing yeast as an indicator organism needs to be utilised. Several methods have already been developed to determine antiyeast activity. Some of these methods specifically focus on the assessment of microbial membrane permeabilisation, e.g. measuring cellular leakage of ions such as potassium (Terras *et al.*, 1993; Thevissen *et al.*, 1999), and cellular uptake of a particular dye (Thevissen *et al.*, 1999). Other methods assess membrane lysis; this is measured by detecting intracellular enzymes such as  $\alpha$ -glucosidase extracellularly (Jewell *et al.*, 2002) or by monitoring the uptake of glucose by the microorganism (Wetter *et al.*, 2003). Microbial growth inhibition was initially determined by using culture plates. The disadvantage of this method is that it is semi-quantitative (Hadacek & Greger, 2000). This method was therefore adapted to microtiter plates (Broekaert *et al.*, 1990) which offers the greatest potential of all other methods (Hadacek & Greger, 2000). In comparison to the method which uses culture plates to determine growth inhibition, the microtiter plate method requires less test samples and allows simultaneous testing of many different samples at different concentrations by micro-dilutions (Van Nierop *et al.*, 2008).

The aim of this research chapter was to obtain basic (cationic) crude extracts containing most of the nitrogen containing compounds (i.e. amino acids, peptides, proteins, amino sugars etc) contributing to FAN from the four worts of sorghum malts selected in chapter 3 namely; (Mount Makulu Sorghum Hybrid (MMSH)-375, MMSH-625, MMSH-1401 and MMSH-1194. Activity on yeast was determined using micro-broth antiyeast assay by Van Nierop *et al.*, 2008. Thin layer chromatography (TLC) and liquid chromatography-mass spectrometry (LC-MS) fingerprinting was also carried out in preliminary characterisation of the N-containing crude extracts, as well as determining if these methods are viable for "fingerprinting" of wort extracts.

## **Materials and methods**

### *Sorghum samples*

Four sorghum [*Sorghum bicolor* (L.) Moench] cultivars MMSH-375, MMSH-625, MMSH-1401 and MMSH-1194 used in this study were cultivated at and obtained from the Mt Makulu Central Research Station of the Zambia Agricultural Research Institute (ZARI), Chilanga, Zambia.

### *Malting and sample preparation*

Broken kernels were removed by hand and 100 g of grain was placed in nylon bags and steeped in a glass beaker containing tap water at between 25 and 30°C for 24 h. For the first 4 h they were steeped in 2000 ppm NaOH. Every 4 h the grains were allowed to air rest for 15-20 minutes and placed back into a beaker containing fresh water for the next 4 h steep period. This procedure was done for the first 12 h after which the grains were left in water for another 12 h to complete the 24 h cycle. The grains were transferred into a baking proofer (Macadams convecta 8, Cape Town, South Africa) where they were allowed to germinate at temperatures between 25 and 30°C and relative humidity above 90% for a period of 98 h. Green malt was kilned at 50°C using a baking oven (Macadams convecta 8, Cape Town, South Africa) for 24 h, after which the shoots and roots were removed by pouring dried malt in nylon bags and rubbing.

### *Mashing*

To obtain wort, a ground malt sample (50 g) was mixed with 75 mL tap water and mashed in a shaking water bath at 100°C for 90 minutes. At the end of the 90 minutes all samples were cooled by adding 125 mL cold tap water. The mash was centrifuged (TJ-25 centrifuge, California, USA) at 10 000 revolutions per minute (RPM) for 10 minutes and stored at -20 C until analysed.

### *Crude basic extract (N-containing factors)*

#### Cation exchange

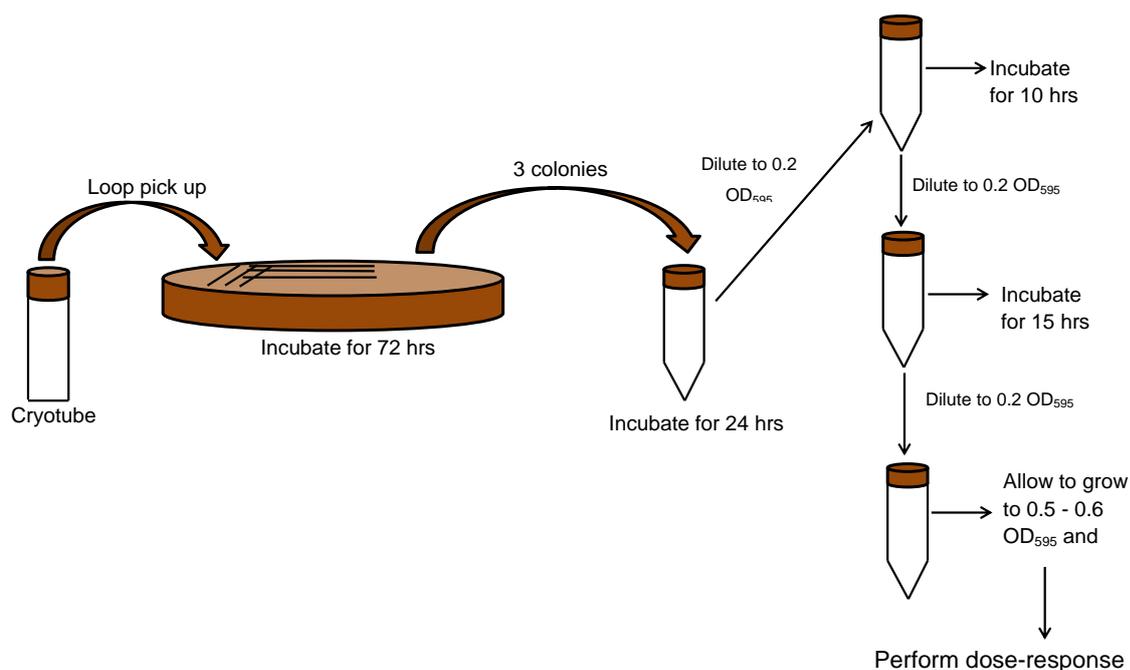
Peptides and amino acids were extracted from wort using cation exchange chromatography on a carboxymethyl Sepharose™ stationary phase. Four separate columns were packed (5 mL) and each initially washed with 5 bed volumes (25 mL) of analytical quality water (prepared through a Milli Q system), followed by washing with 5 bed volumes of 0.1% trifluoroacetic acid (TFA). Three mL of wort and 2 mL 0.1%TFA were mixed in a syringe and loaded on the column and left to interact for one hour. This was done for all four cultivars. Compounds with a net negative charge (same charge as stationary phase) were washed out of each column with 0.1% TFA into 50 mL sterile Falcon™ tubes. This acidic fraction was stored at -20°C. The remaining unbound compounds in the columns were washed from the column matrix with 50 mL analytical quality water (prepared through a Milli Q system). Triethanolamine (TEA) at 1% was used in all four columns to raise the pH to above 9, resulting in the cationic compounds no longer being able to form ionic interactions with the negatively charged stationary phase. This consequently caused the cationic compounds (the basic fraction) to elute from the column. The basic fraction from all columns was then

collected into 50 mL sterile Falcon™ tubes and transferred into round bottom flasks which were freeze dried and stored dry at -18°C until analysed.

### *Yeast growth and inhibitory activity*

#### Sub-culturing

To prepare the liquid growth medium, 0.3% malt extract, yeast extract, 1% glucose and 0.5% peptone (MYGP) were dissolved in 1L of water and autoclaved for 20 minutes under pressure to attain 121°C. For the preparation of agar plates, 2% agar was added to the growth medium and transferred to petri dishes after autoclaving. Cryopreserved yeast (*Saccharomyces cerevisiae*) was transferred to an agar plate and incubated at 30°C for 72 h. Three viable colonies were selected from the MYGP plates and inoculated into 25 mL of liquid growth medium for 24 h (Fig. 4.1). Yeast growth was monitored with a spectrophotometer, and used for the assay at optical density ( $OD_{595}$ ) between 0.5 and 0.6 (yeast's mid-log growth phase).



**Figure 4.1** Yeast sub-culturing procedure for dose-response assays.

#### Antiyeast assay

The micro-dilution broth anti-yeast assay described by Van Nierop *et al.*, 2008 was utilised in order to determine the dose-response of sorghum's N-containing compounds on brewer's yeast. In this method a 96 well microtiter plate (flat bottomed non-binding, BibbySterilin, Staffordshire, England) was utilised (Fig. 4.2). Only inner block of wells (C3-C10, D3-D10 and E3-E10) were utilised for the antiyeast activity assay (Fig. 4.2). Each of these wells

contained 10 µL of extract and 90 µL of liquid growth medium in yeast. In a separate dilution plate, dilution solution (50 µL of analytical quality water) was dispensed into the second well up to the eighth well of the row which was used for diluting the extract. A 100 µL of 2 mg/mL extract stock solution was dispensed into the first well of the row which was used for the dilution series. The extract was then diluted two-fold across the row (eight wells) by transferring 50 µL of the 100 µL to the next well and discarding the final 50 µL.

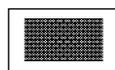
The wells in rows B3-B10 and F3-F10 containing 100 µL of liquid growth medium with yeast were used as growth controls (Fig. 4.2). Wells in row G3-G10 (Fig. 4.2) were used as a positive control and contained 10 µL of gramicidin S doubling dilution series (1.56-100 µg/mL) from *Bacillus brevis* (Sigma, St. Louis, USA) in 90 µL of liquid growth medium with yeast. In the remaining wells 100 µL of yeast in liquid growth medium was also added except for C1, C12, D1, D12, E1, E12, F1 and F12 which were utilised as sterility control and only contained 100 µL liquid growth medium (Fig. 4.2). The experiment was conducted in triplicate for each cultivar. After filling the wells, the plates were wrapped tightly in foil and incubated at 30°C for 24 h. Yeast growth was then monitored by measuring light dispersion at 595 nm using a BioRad microtiter plate reader (Model 680, Hercules, CA, USA). The equations below were used to calculate the % growth and % inhibition to construct for dose-reponse curves. Data was further analysed using GraphPad Prism 4.03 (GraphPad Software Incorporated).

$$\% \text{ Inhibition} = 100 - \left( \frac{100 \times (\text{OD}_{595}\text{E} - \text{Average SC})}{\text{Average GC} - \text{Average SC}} \right)$$

$$\% \text{ Growth} = 100 - \% \text{ Inhibition}$$

Where E = Extracts, SC = Sterility control and GC = Growth control

	1	2	3	4	5	6	7	8	9	10	11	12
A	#	#	#	#	#	#	#	#	#	#	#	#
B	#	#									#	#
C		#									#	
D		#									#	
E		#									#	
F		#									#	
G	#	#									#	#
H	#	#	#	#	#	#	#	#	#	#	#	#



Sterility control (SC)

Extracts (E)

Growth control (GC)

Positive control (PC)

Yeast and medium (YM)

**Figure 4.2** Diagrammatic representation of the 96 well microtiter plate layout utilised for the antiyeast assay.

### *High performance thin layer chromatography*

High performance thin layer chromatography (HP-TLC) glass plates (20 x 10 cm) precoated with silica gel as stationary phase were utilised. The mobile phase consisted of 90 parts of butanol, 80 parts of pyridine, 60 parts of glacial acetic acid and 72 parts of analytical quality water. The stock solution was prepared by analytically weighing 2.00 mg samples (in triplicate) of extract for each cultivar into 15X45 mm clear 1 dram glass vials and dissolving it in 100  $\mu$ L analytical quality water to attain 20 mg/mL stock solution. Stock solution was withdrawn and 2  $\mu$ L accurately sprayed in the form of bands 2 cm above the bottom of the HP-TLC plate using Linomat 5 (CAMAG Linomat 5, Muttenz, Switzerland). A 2  $\mu$ L sample of wort (2-fold dilution) was also sprayed into the HP-TLC plate. The HP-TLC plate was then placed in an equilibrated developing chamber (Aldrich rectangular TLC developing chamber, 29.6 x 26.8 x 9.85 cm) containing mobile phase until the desired running distance was reached (90 mm). The HP-TLC plate was then dried using a current of warm air (hair dryer) and sprayed with 2% ninhydrin in 95% ethanol and incubated at 110°C for 10 min. The HP-TLC plate was allowed to cool down and the image of the HP-TLC plate was captured under UV 254, UV 366 and visible light using a TLC Visualizer with an integrated 12 bit camera (CAMAG TLC Visualizer, Muttenz, Switzerland). UN-SCAN-IT *gel*<sup>TM</sup>, version 6.1 (Silk Scientific Corporation) was utilised to digitise the scanned plates (per lane) and to generate TLC chromatograms. The equation below was used to calculate  $R_f$  values of the visually identified bands on the TLC plate:

$$R_f = \frac{\text{distance of band}}{\text{total distance of eluent}}$$

### *Ultra performance Liquid chromatography-mass spectrometry*

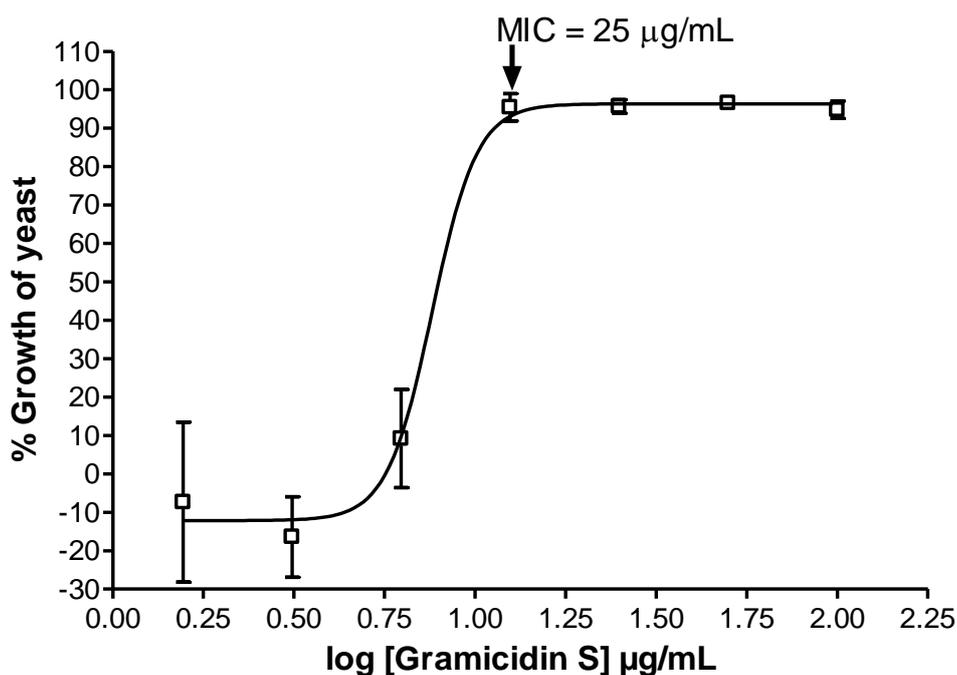
Ultra performance liquid chromatography-mass spectrometry (UPLC-MS) of sorghum N-containing extracts was conducted using ultra performance liquid chromatography (UPLC) and time of flight electrospray mass spectrometry (TOF-ESMS) on a Waters Acquity autosampler attached to a Waters QTOFUltima mass spectrometer. A Waters UPLC BEHC<sub>18</sub> column (2.1 x 50 mm, 1.7  $\mu$ m spherical particles) was utilised with gradient created at a flow rate of 0.450  $\mu$ L/mL using 1% formic acid (solvent A) and acetonitrile containing 1% formic acid (solvent B) in order to separate the compounds present in the extracts. The gradient was generated as follows: 100% A for the first 0.5 min, 0.5-12 min gradient from 0-58% B; 12-13 min gradient from 58-90% B; 13 -13.5 min 100% B (wash); 13.5-14 min gradient from 0-100% B 14-17 min 100% A (column regeneration). A 3  $\mu$ L of 2.0 mg/mL in water sample of each extract was injected into the ESMS and subjected to capillary voltage

of 3.0 kV and a source voltage was 15 V at 120 °C. The data was then collected scanning over an  $m/z$  range of 300-2000 in the positive mode.

## Results and discussion

### *Yeast growth and inhibitory activity*

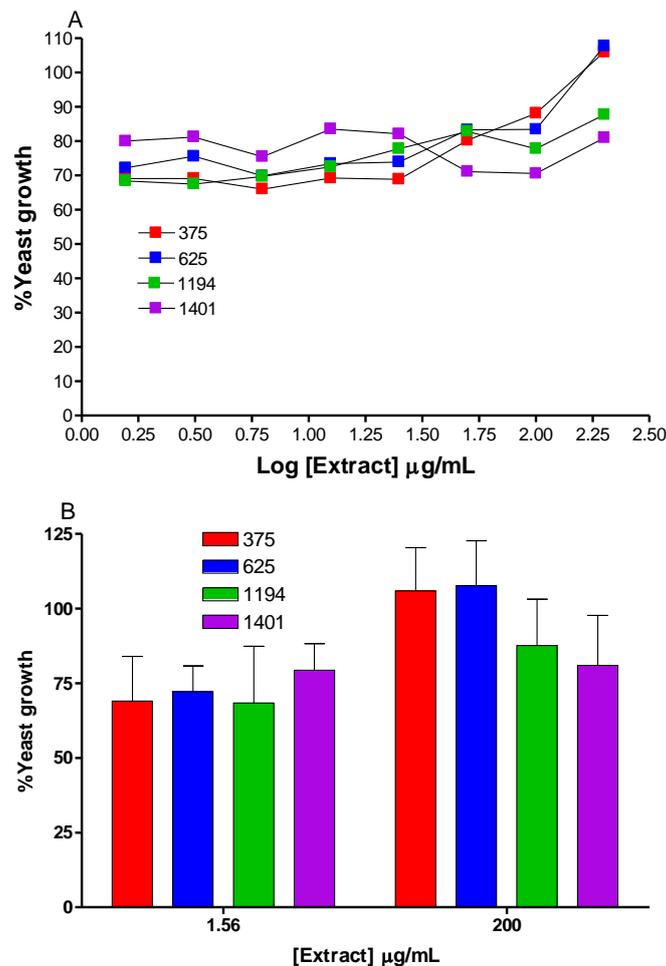
In essence anti-yeast assay was used to assess both growth and inhibition of brewer's yeast in the presence of the four sorghum extracts. The assay was regarded as contamination free for there was no observed increase in the absorbance for the sterility control wells. The growth control contained solvent and media in the yeast culture and represented the 100% growth of the culture in the assay. The positive anti-yeast control, the highly lytic peptide gramicidin S was found to be active against *Saccharomyces cerevisiae*. The minimum inhibitory concentration (MIC) of gramicidin S in this assay was found to be 25  $\mu\text{g/mL}$  (Fig. 4.3). This was in agreement with work done by Troskie *et al.* (2012) and Nieuwoudt *et al.* (2014).



**Figure 4.3** Representative dose-response of positive control antimicrobial peptide, gramicidin S, toward *Saccharomyces cerevisiae*. Each data point is the average of triplicate repeats with standard error of the mean (SEM). The minimum inhibitory concentration (MIC) is indicated.

The cationic N-containing compounds from sorghum malts with good malting and brewing qualities were analysed with the antiyeast assay developed by Van Nierop *et al.* (2008) The addition of N-containing compounds promoted yeast growth in all four cultivars (Fig 4.4). The increase in % yeast growth (Fig 4.4A) observed in all four cultivars at high

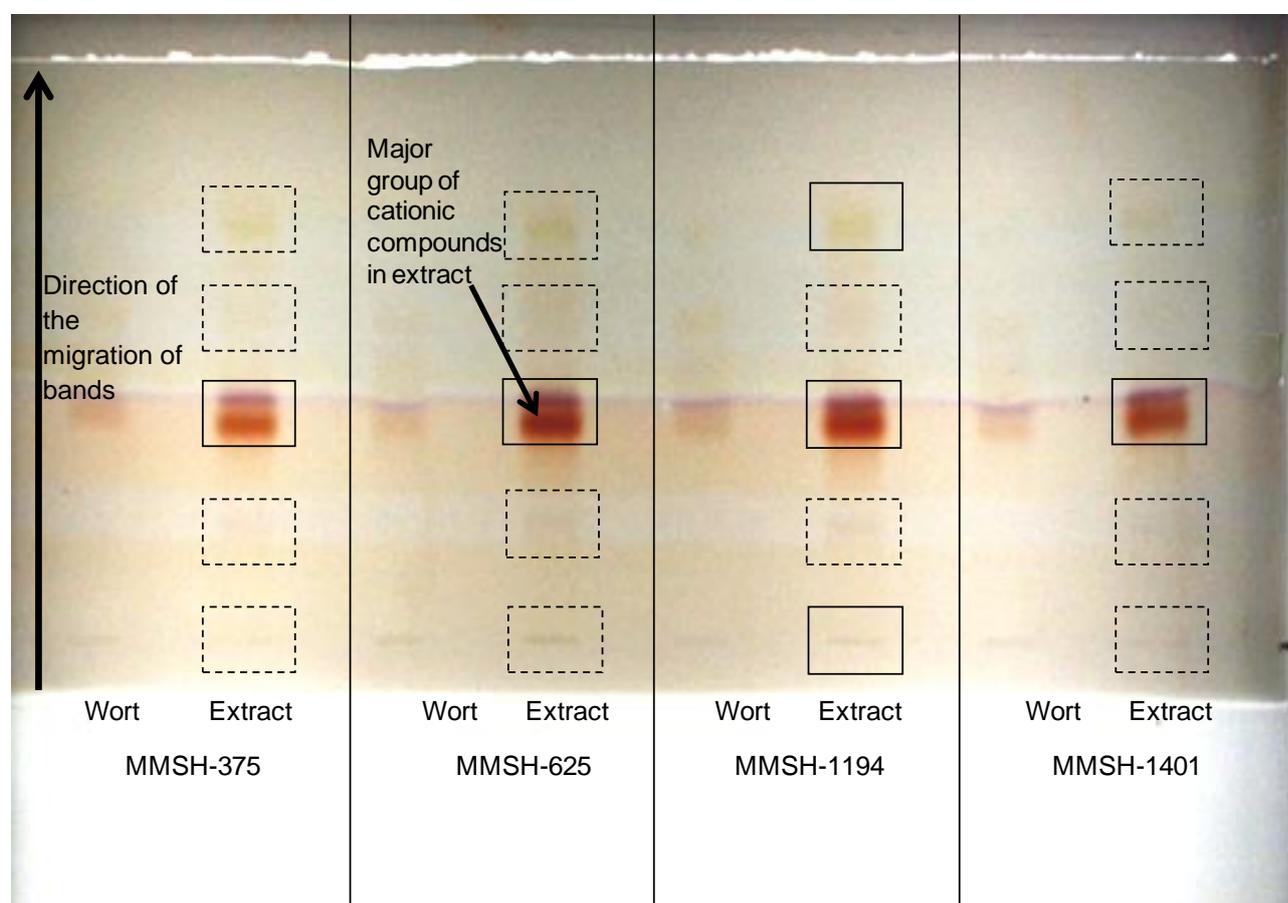
concentrations (200 and 100  $\mu\text{g/mL}$ ) of N-containing compounds suggests that the additional nutrients in the extracts could be overshadowing the effect of the antimicrobial compounds that were indicated by the 20-30% reduced growth at the lower concentrations, correlating with results reported by Van Nierop (2005). It is thus still possible the antimicrobial proteins/peptides or compounds may be present inhibiting yeast growth at the lower extract concentrations. Alternatively, the high concentrations of extracts may contribute to the aggregation of antimicrobial peptides (Rautenbach *et al.*, 2006) or antagonise their activity leading to loss of antiyeast activity. The generally higher % yeast growth observed in MMSH-375 and MMSH-625 suggests that these cultivars may have a better qualitative yeast-nutritional value i.e. high level of group A, B and C amino acids and possibly small peptides (di-, tri- and tetrapeptides) consisting of these amino acids. Such improved growth may therefore promote yeast fermentation and lead to higher levels of final alcohol content of beer.



**Figure 4.4** Combined results showing dose-response of the anti-yeast activity of sorghum N-containing factors toward *Saccharomyces cerevisiae* over the concentration range on 1.56-200  $\mu\text{g/mL}$  (A) and the comparison of the yeast growth at the lowest and highest extract concentrations (B).

### High performance thin layer chromatography

Compounds present in worts and extracts of the four cultivars were successfully separated with high performance thin layer chromatography (HP-TLC) (Fig 4.5) in order to preliminarily characterise the N-containing compounds enhancing %yeast growth during fermentation. The purple- and dark red-brown coloured bands on the HP-TLC plates indicated that a reaction between N-containing compounds in the worts and extracts with ninhydrin occurred. This therefore suggests that the cation exchange chromatography method utilised for extraction of N-containing compounds in sorghum wort was successful in order to isolate a range of N-containing compounds. In general, similarities were observed between the four cultivars and distinct bands were visible on the HP-TLC plate for both worts and extracts (Fig. 4.5).



**Figure 4.5** High performance thin layer chromatography of four sorghum worts and their extracts developed with 2% ninhydrin. The most prominent ninhydrin active bands are shown in solid line squares and the less stained bands in dashed line squares.

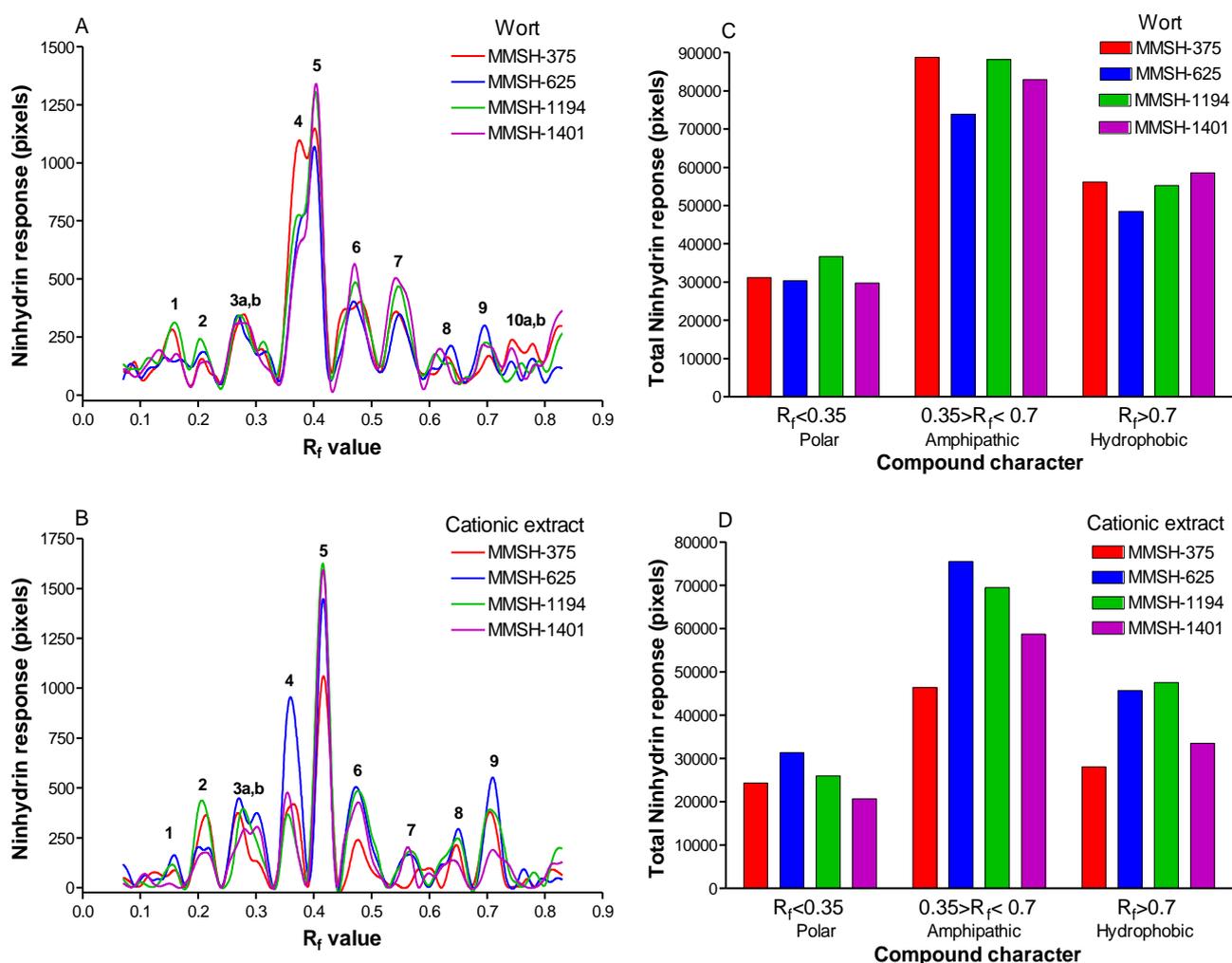
The development of the HP-TLC plate with ninhydrin allowed the quantification of the majority of N-containing compounds, relating directly to the ninhydrin dependent FAN determination. The character of the detected compounds on the HP-TLC plate could be discerned from their  $R_f$  values with the most polar associating strongly with the silica oxide in

the HP-TLC plate yielding low  $R_f$  values while hydrophobic compounds will associate with the mobile phase and move much further on the plate yielding high  $R_f$  values. The compounds with intermediate polarity and hydrophobicity (amphipathic character), such as the antimicrobial peptides will migrate to the middle of the plate with appropriate solvent systems. Five bands were visually identified in the extracts and highlighted in squares (Fig. 4.5). The  $R_f$  values of the first two bands from the bottom of the HP-TLC plate were  $<0.35$  (arbitrary cut-off) and could possibly be attributed to polar free amino acids, peptides or other polar N-containing compounds. The two major bands in the middle of the plate can be attributed to N-containing compounds with amphipathic character for their  $R_f$  values were  $>0.35$ . The last band had  $R_f$  value  $>0.7$  (arbitrary cut-off for non-polar compounds) thus indicating the presence of hydrophobic amino acids or peptides in the cultivars.

To further elucidate the visual differences between the four worts and cationic extracts the HP-TLC results were further analysed by quantifying the ninhydrin response via pixilation to obtain TLC chromatograms (Fig. 4.6). Similarities and differences between worts and extracts of the four cultivars were clearly observed on the chromatograms (Fig. 4.6A, B). With the refined analyses ten major peaks were observed in worts of the four cultivars (Fig. 4.6A). The total ninhydrin response of the worts was  $\text{MMSH-375} = \text{MMSH-1194} > \text{MMSH-1401} > \text{MMSH-625}$ . In the worts peaks 3-7 were the most prominent and also represent the amphipathic portion of the extract in which antimicrobial peptides could be expected (Fig 4.6 A). MMSH-1401 with the highest FAN level (250 mg/L) had the highest overall intensity in major peaks 5, 6 and 7, although it did not show the overall highest ninhydrin response as expected. The profile of MMSH-1194 closely correlated with that of MMSH-1401 for peaks 4-8, while MMSH-375 showed a quite different profile for peaks 4-8. The profile of MMSH-625 showed some correlation with that of MMSH-375, particularly peaks 6 and 7. In general it was observed that the N-containing compounds with amphipathic character were the most abundant, followed by the hydrophobic compounds (Fig 4.6 C). There was also very little difference between the distribution in terms of character between the four worts (Fig 4.6A, C).

Nine major peaks were observed in extracts of the four cultivars (Fig. 4.6B). The total ninhydrin response of the extracts was  $\text{MMSH-625} > \text{MMSH-1194} > \text{MMSH-1401} > \text{MMSH-375}$ . Like with the worts, peaks 3-6 which represent the amphipathic portion where antimicrobial peptides can be expected were also prominent in the extracts (Fig. 4.6 B). The profile of MMSH-1194, MMSH-1401 and MMSH-375 closely correlated with each other at peak 4 while MMSH-625 showed a quite different profile. The profile of MMSH-625, MMSH-1194 and MMSH-1401 showed some correlation particularly peaks 5 and 6 while MMSH-375 showed a different profile. The N-containing compounds with amphipathic character were also the most abundant in the extracts, followed by the hydrophobic compounds (Fig 4.6 B,

D). There was also very little difference between the distribution in terms of character between the four worts (Fig 4.6D). The compounds with amphipathic character were also the most abundant in both the worts and extracts of all cultivars followed by the hydrophobic compounds (Fig. 4.6C, D). It was observed that some compounds with amphipathic and hydrophobic character in the wort of MMSH-375 were non cationic because of the observed decrease in the total ninhydrin response in the extract when compared to the wort (Fig. 4.6C, D). In general there was no major difference between the character of worts and extracts in all four cultivars (Fig.4.6C, D) and the HP-TLC results indicated that the extracts contain similar compounds and consequently could have the same brewing potential.

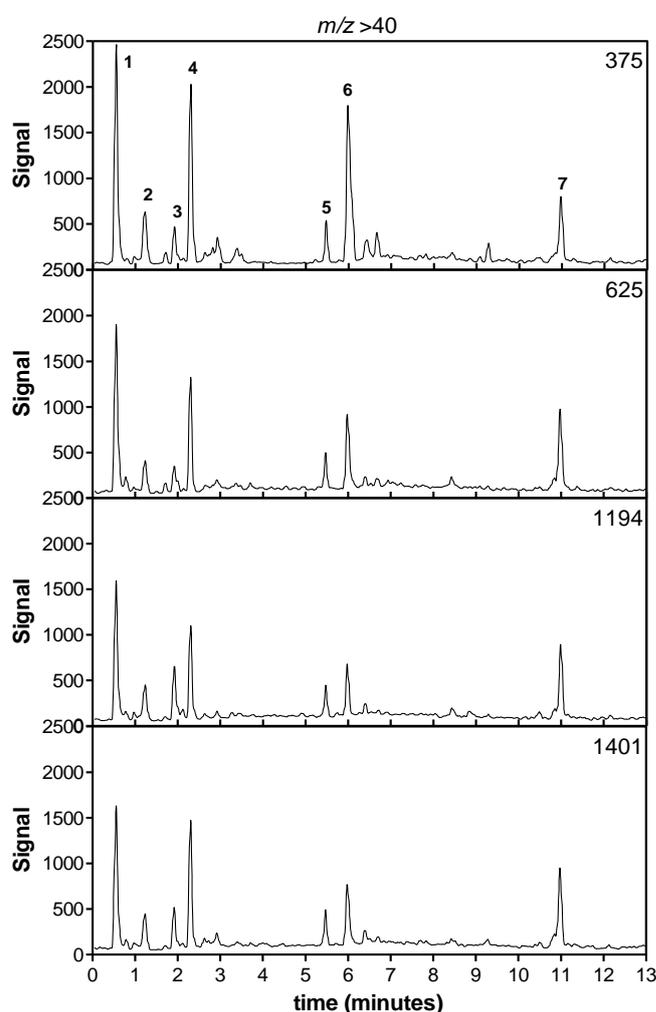


**Figure 4.6** HP-TLC chromatograms of worts (A) and cationic wort extracts (B). The bar graphs show the comparison between the polar, amphipathic and hydrophobic N-containing compounds in the worts (C) and cationic wort extracts (D).

#### *Ultra performance liquid chromatography-mass spectrometry*

The cationic extracts were further analysed with UPLC-MS in order to characterise the extracts and consequently obtain more detailed analyses which could not be achieved with

HP-TLC. When the detection was done at  $m/z > 40$  to include all the small compounds (including amino acids) more than 15 peaks were found with seven prominent peaks in all four cultivars (Fig. 4.7, chromatograms on left). When the detection was done at  $m/z > 300$  to exclude all the small compounds and limit their interference with ionisation of larger compounds, much more complex profiles emerged for the four worts. (results not shown). However, with close inspection similar compounds were found for the two types of analyses, and most of the differences could be attributed to complex mixtures of compounds at low signal intensity (result not shown). It was therefore decided to focus on only the major peaks in the UPLC-MS analysis with detection at  $m/z 40-2000$  atomic mass units. This broad ESMS detection would include the free amino acids and amino sugars, as well as small N-containing compounds.

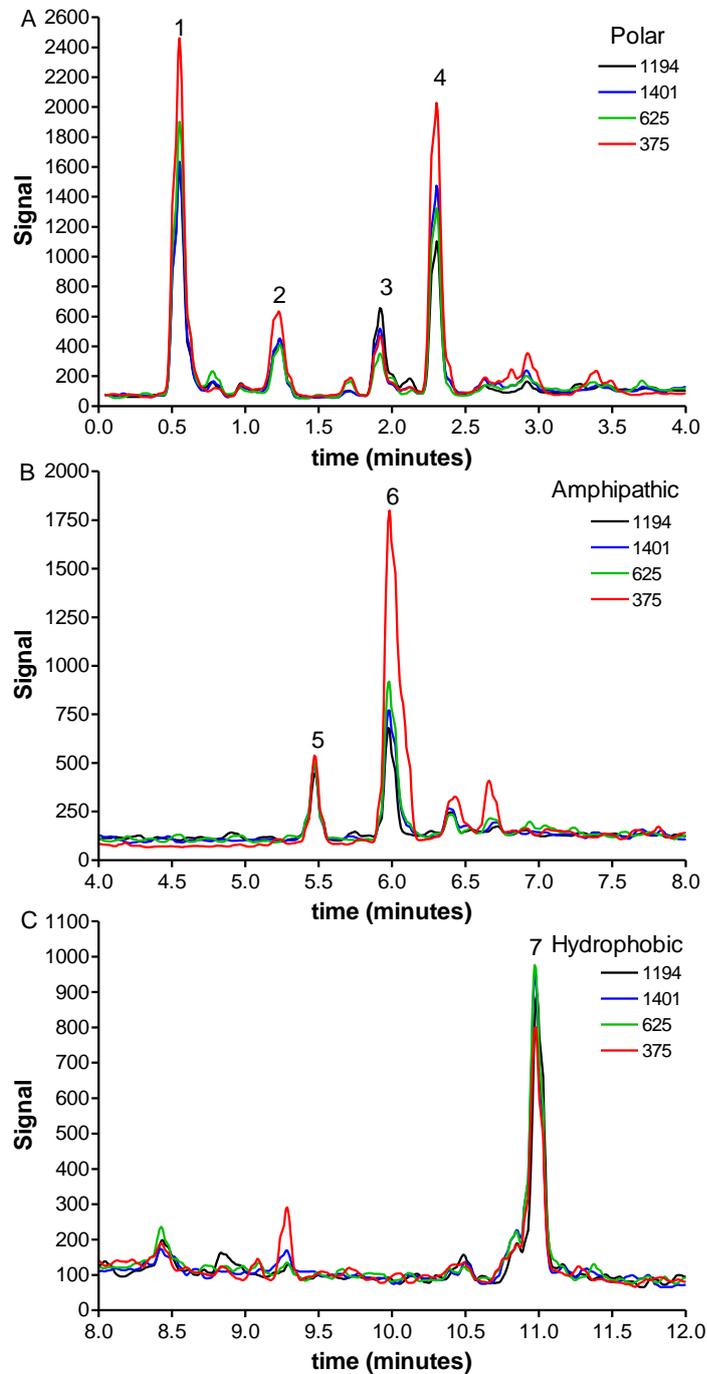


**Figure 4.7** UPLC-MS chromatograms showing differences and similarities between basic extracts obtained from wort of four sorghum cultivars (625 = MMSH-625, 375 = MMSH-375, 1401 = MMSH-1401 and 1194 = MMSH-1194). The chromatograms depict all the compounds with  $m/z > 40$ , which include amino acids. The seven most prominent peaks or peak clusters are numbered in the top chromatogram.

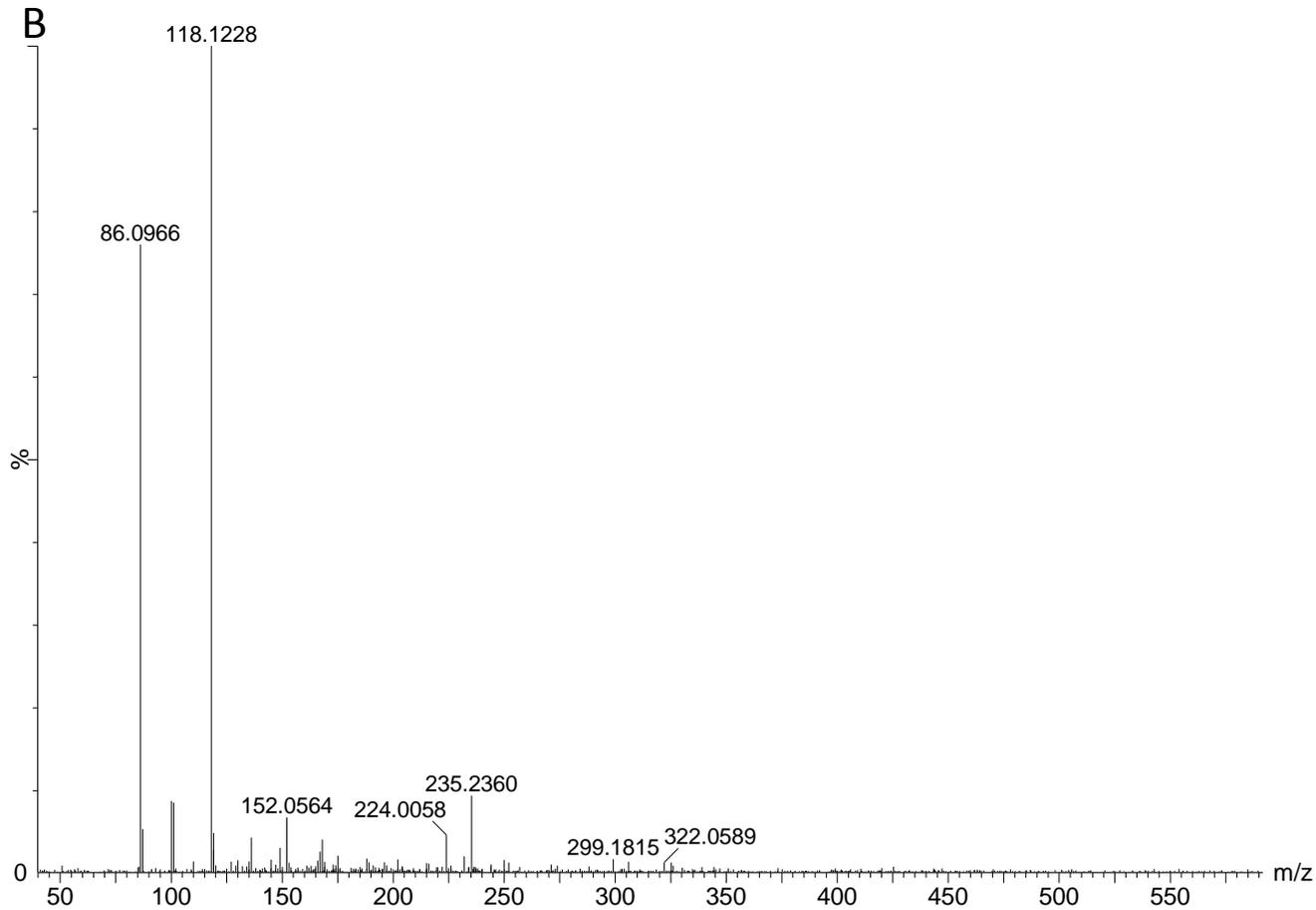
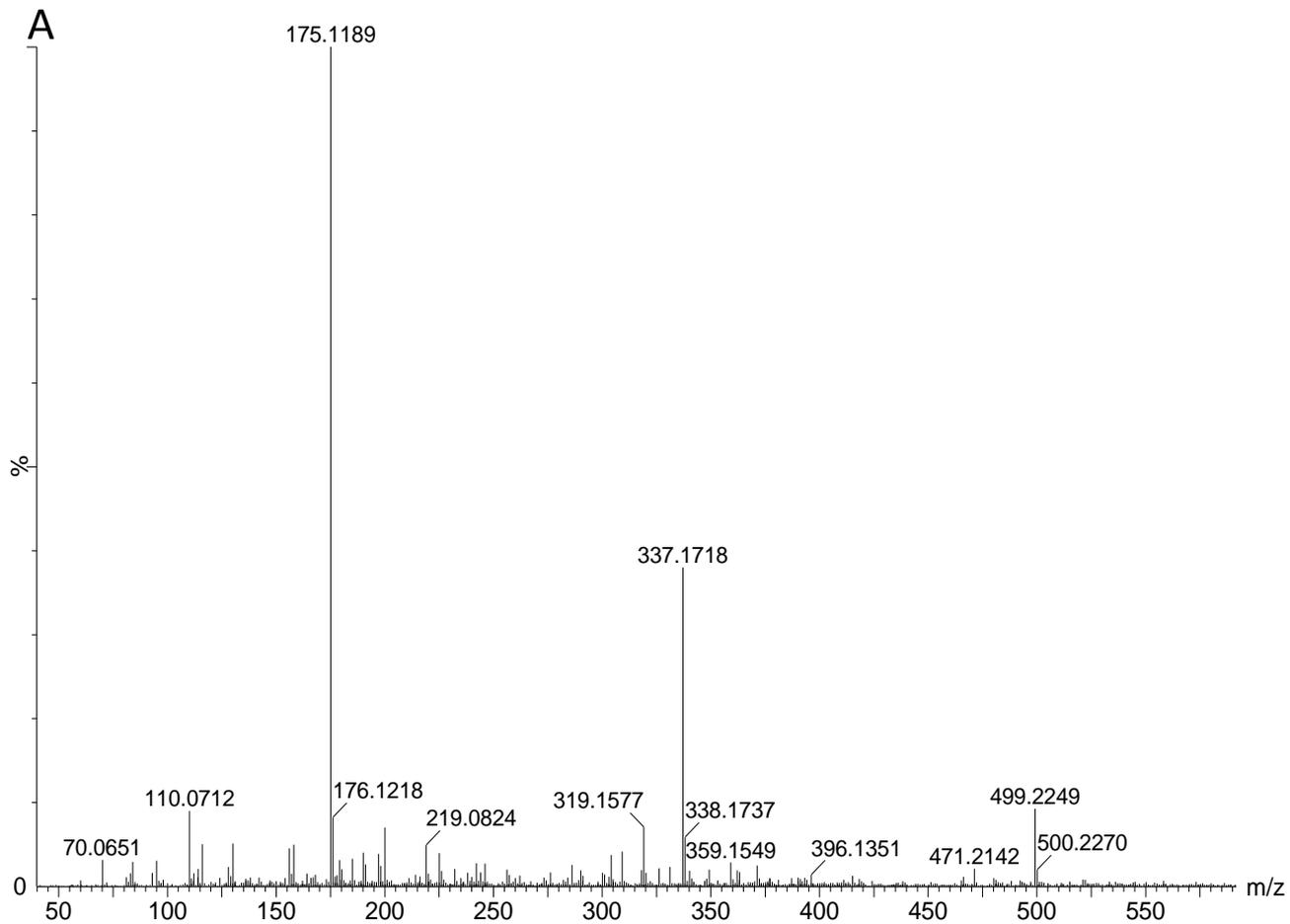
With the UPLC-MS method the compounds were separated on a hydrophobic matrix ( $C_{18}$ ) and the most polar compounds eluted first, followed by the amphipathic and hydrophobic compounds. The detection was limited to compounds that ionised under the ESMS positive mode conditions and ionisation varies from compound to compound. The intensity of the peaks can therefore not be used as a measure of concentration and compared with other eluting peaks. Only peaks eluting at the same time with same compounds in the peak fraction were therefore directly compared. From the UPLC-MS chromatograms (Fig. 4.8) it could be seen that the extract separated into the three groups again namely the early eluting polar compounds (0-4 minutes, peaks 1-4; Fig. 4.8C), amphipathic compounds (4-8 minutes, peaks 5, 6; Fig. 4.8B) and hydrophobic compounds at (8-12 minutes, peak 7; Fig. 4.8C).

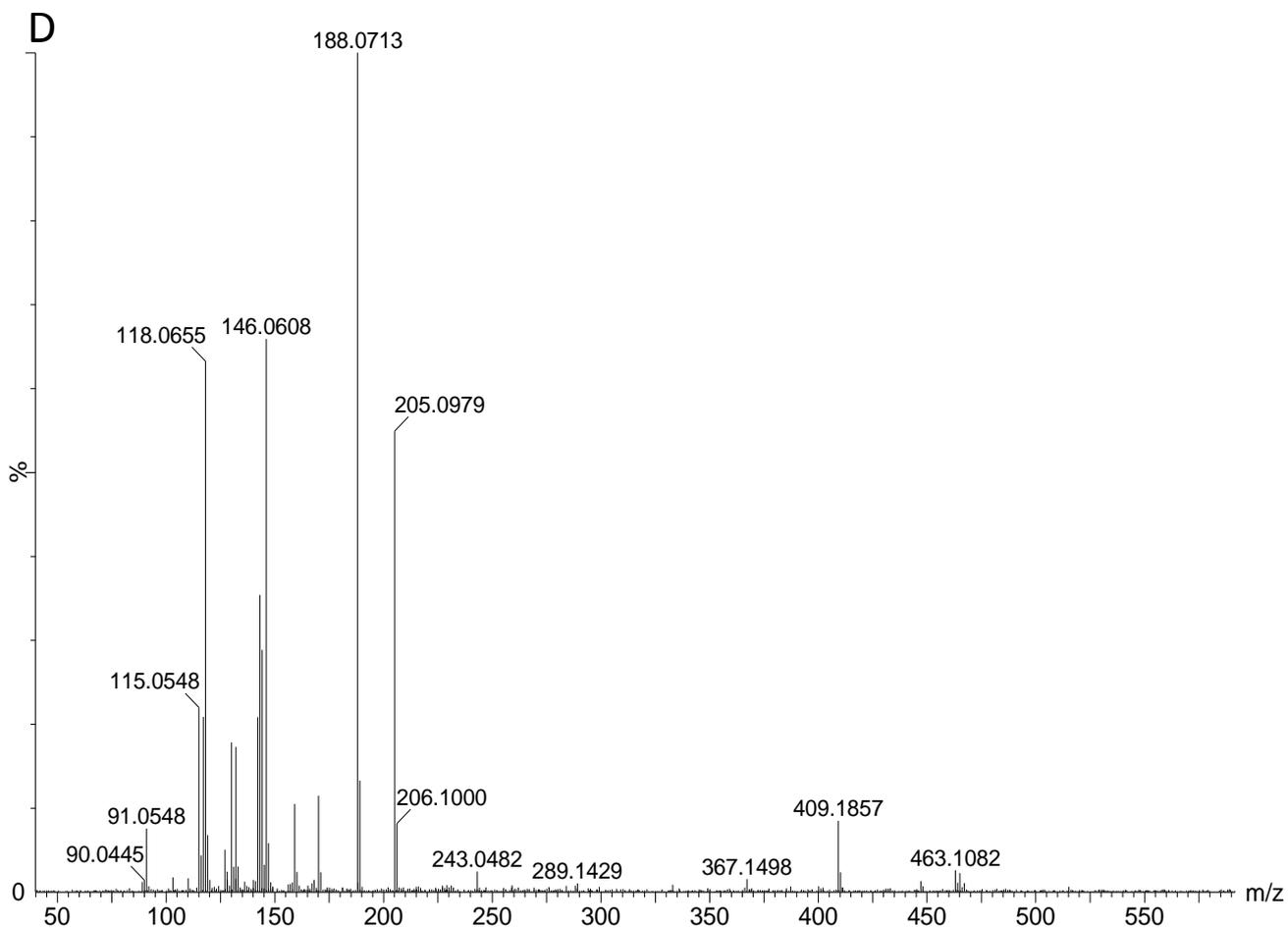
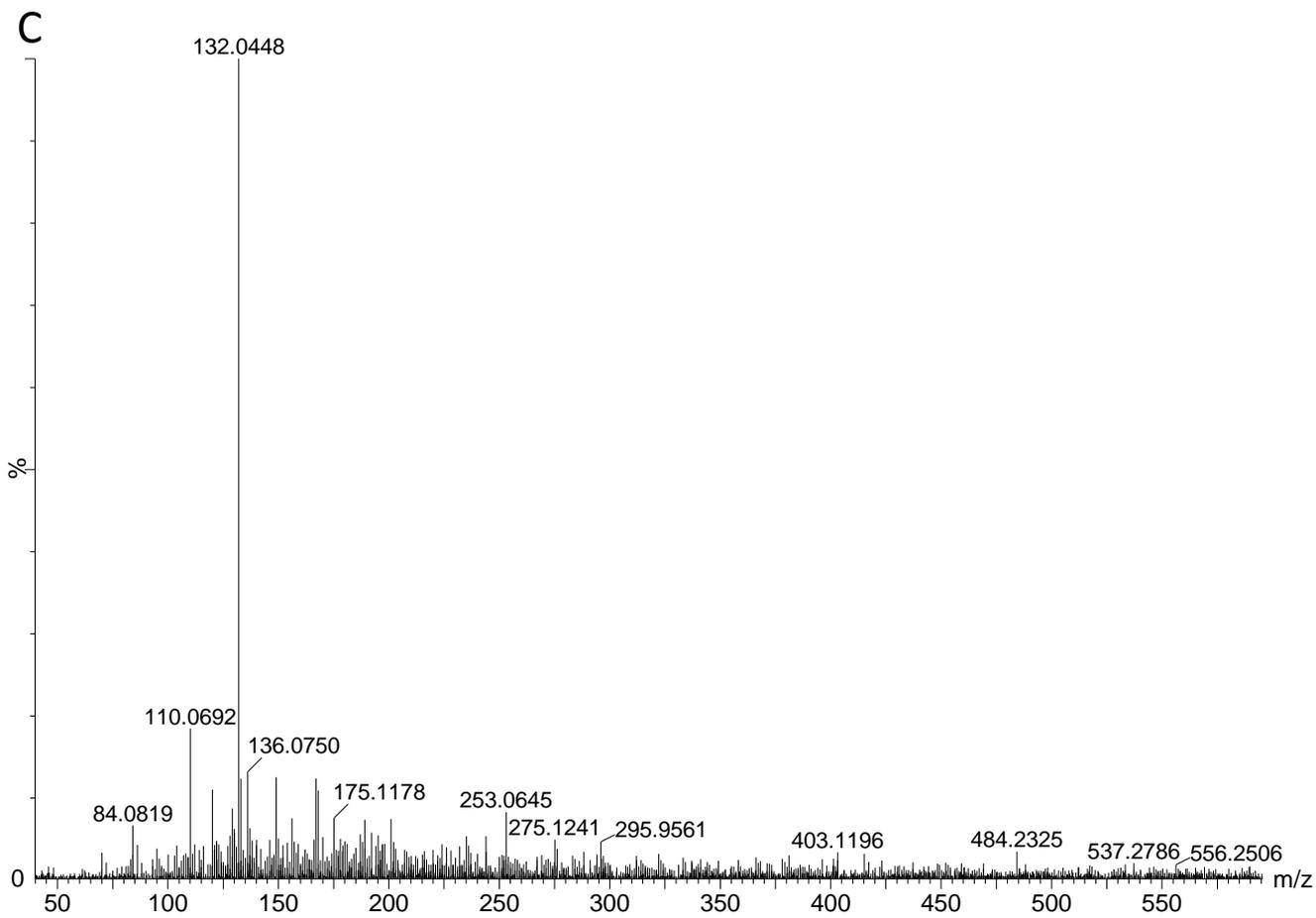
The mass spectra of seven prominent peaks observed when detection was done at  $m/z > 40$  were examined in all four cultivars and only that of MMSH-375 is given below because a similar trend was observed in the four cultivars (Fig. 4.9). It was observed that these molecules are singly charged and therefore their  $m/z$  values represent their actual masses in Daltons (Da) plus 1.007825 (atomic mass of hydrogen as the molecular ions are protonated). After close scrutiny of all the UPLC-MS data, we were unable to detect possible antimicrobial peptides or proteins in the extract, only small compounds (<1 kDa) were detected and as expected, peaks with the same retention time contained the same compounds in all four cultivars (Table 4.2). Only a few of the compounds in the peaks could be tentatively identified by using the determined accurate masses. The 20 free amino acids, hydroxy-lysine, hydroxy-proline, 400 possible dipeptides, 5 N-bases, 10 (deoxy) nucleosides and selected amino sugars were considered. The major compound in Peak 1 was identified as the polar amino acid, Arginine (Arg) ( $m/z=175.1190$ , expected  $m/z=175.1185$ ), containing four nitrogens in its structure (Table 4.2). A putative dipeptide containing Arg and Tyrosine (Tyr) also eluted in this fraction. This was in agreement with the HP-TLC data that early eluting peaks represent polar compounds, as well as our cation exchange column extraction protocol. The major compounds in peaks 2 and 3 could not be identified in the group of N-containing compounds that were considered. Mass extraction of Peak 4 revealed a number of molecular ions, possibly three compounds namely, free valine (Val), a dipeptide containing Alanine (Ala) and Asparagine (Asp) and an unknown compound (Table 4.2). Both Val and the dipeptide are less polar than Arg correlating with their later elution. However, an Ala-Asp containing dipeptide would be anionic and Val neutral at pH 7. This indicated that the cation exchange column washing steps in the extraction protocol had been insufficient to remove some of the neutral and anionic compounds. The compounds in peak 5 were identified as possibly dipeptides containing Ala and Isoleucine (Ile) or Ala and Leucine (Leu) as well as an unknown compound. Similarly these are neutral peptides which

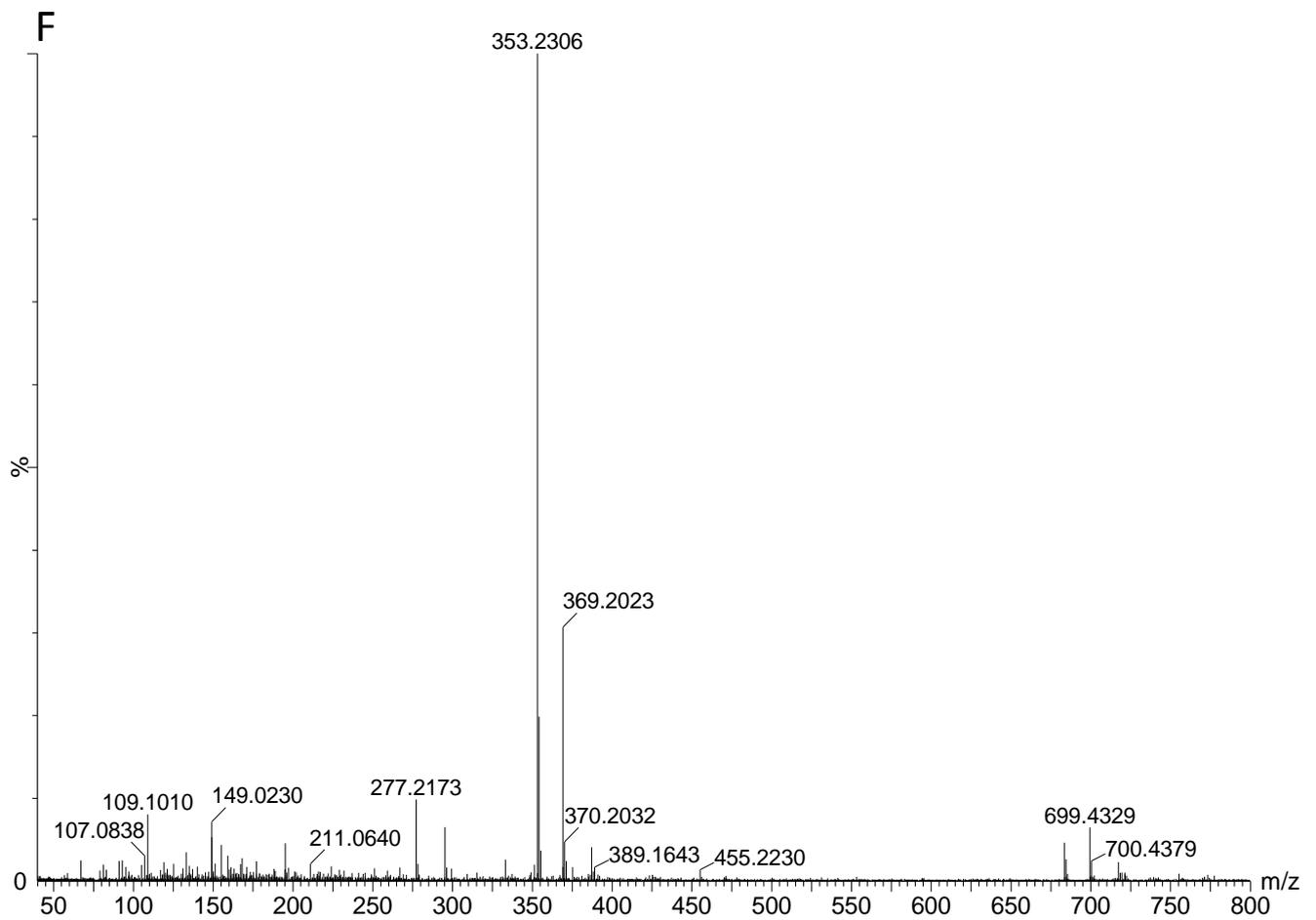
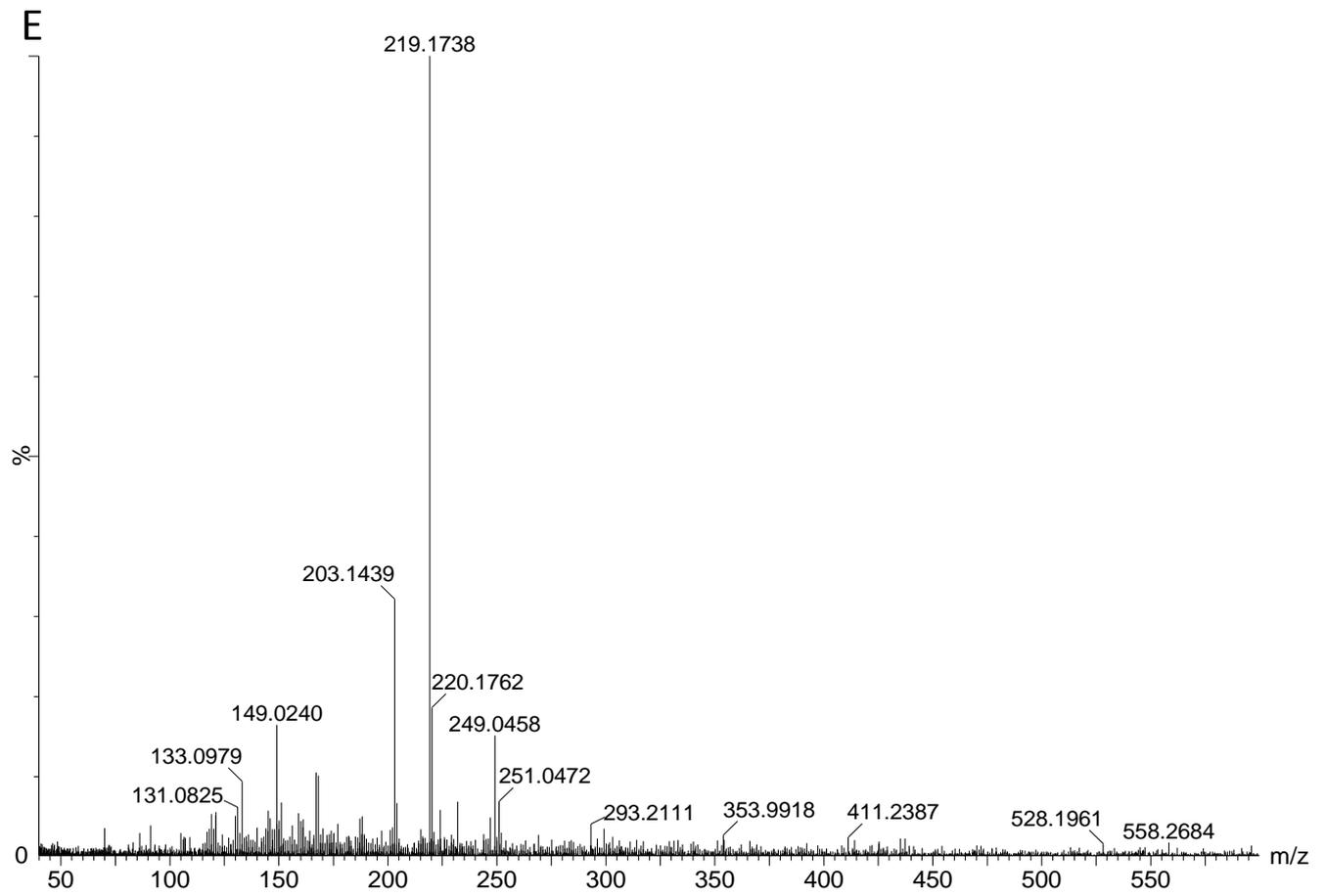
still remained in our “cationic fraction”, co-eluted with the highly cationic Arg from the cation exchanger column. However, these dipeptides are hydrophobic and thus correlate with later elution from the hydrophobic UPLC column. Peak 6 contained an unknown compound, possibly with a hydroxyl group attached to it. The last peak contained a background contaminant and an unknown compound (Table 4.2).

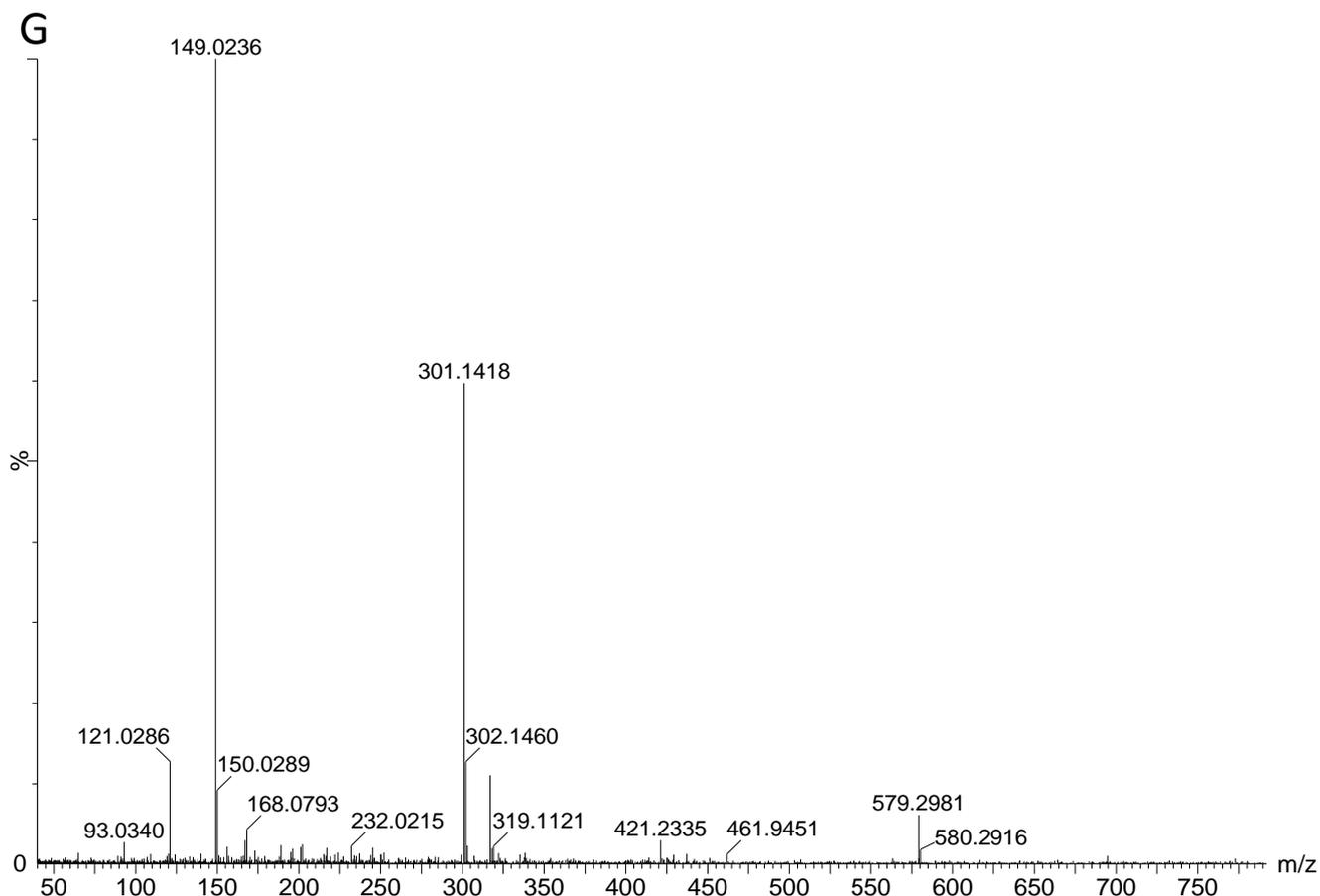


**Figure 4.8** UPLC-MS chromatograms showing differences and similarities between extracts obtained from wort of four sorghum cultivars (625 = MMSH-625, 375 = MMSH-375, 1401 = MMSH-1401 and 1194 = MMSH-1194). The chromatograms show the overlays of the four wort extracts with A over 0-4 minutes; B over 4-8 minutes and C over 8-12 minutes.









**Figure 4.9** Extracted positive mode ESMS spectra of the seven major peaks detected with  $m/z > 40$  for MMSH-375 (A = peak 1, B = peak 2, C = peak 3, D = peak 4, E = peak 5, F = peak 6 and G = peak 7).

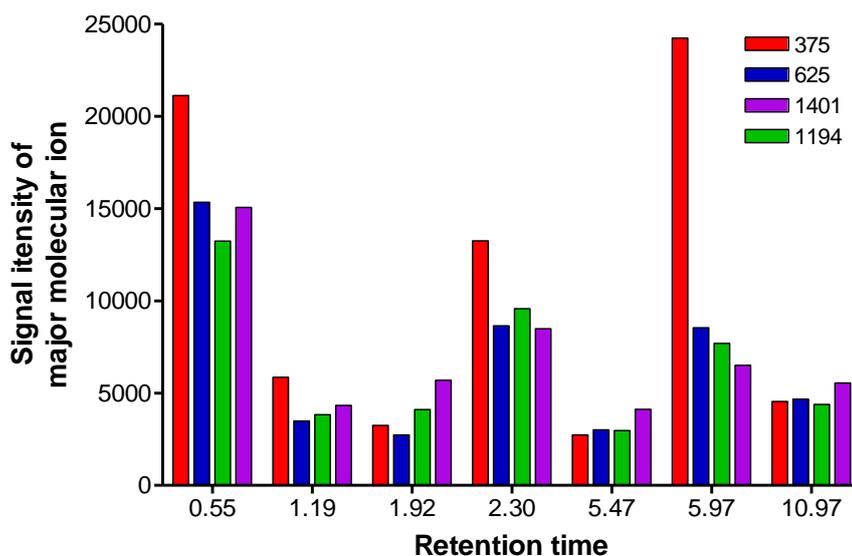
From the data in Table 4.2, UPLC MS-profiles, as well as the HP-TLC profiles it is clear that all four cultivars contained the same compounds (Table 4.2). They were however not in the same concentration (Fig. 4.10). In peaks 1, 2, 4 and 6 MMSH-375 contained the highest concentration of the putative yeast growth promoting N-containing compounds when compared to other cultivars (Fig. 4.10). This explains the results obtained in Fig. 4.4, namely the overall high %yeast growth observed in MMSH-375 that was probably due to the higher level of N-containing compounds, in particular Arg and some dipeptides, when compared to other cultivars. By comparison with the dose response results (Fig. 4.4), UPLC MS-profiles showed that MMSH-625 is the second cultivar with a higher concentration of the putative yeast growth promoting N-containing compounds followed by MMSH-1401 and MMSH-1194 (Fig. 4.10). The HP-TLC results were however dissimilar to the above observed correlation between dose response results and UPLC-MS profiles. The HP-TLC total ninhydrin response of the extracts was MMSH-625 > MMSH-1194 > MMSH-1401 > MMSH-375. It is possible that some compounds in each cultivar did not react with ninhydrin and thus yielding different results. UPLC-MS is thus the most viable method of the two for “fingerprinting” wort extracts for yeast growth promoting character.

**Table 4.2** Predictions of possible identities of seven prominent peaks found in all four cultivars when the detection was done at  $m/z > 40$ 

Peak number	Retention time (Rt)	MMSH-375	MMSH-625	MMSH-1194	MMSH-1401	Possible identity (expected m/z)
<i>m/z value of major molecular specie</i>						
1	0.55	175.1189	175.1191	175.1188	175.1190	Arg (175.1195)
		<i>337.1718</i>	<i>337.1710</i>	<i>337.1727</i>	<i>337.1576</i>	Arg, Tyr dipeptide - H (337.1723)
2	1.23	118.1205	118.1227	118.1223	118.1232	Unknown
		<i>86.0966</i>	<i>86.0996</i>	<i>86.0962</i>	<i>86.0968</i>	Unknown
3	1.92	132.0448	132.0434	132.0447	132.0447	Unknown
4	2.30	188.0684	188.0708	188.0690	188.0708	Asp;Ala dipeptide - OH (188.0770)
		<i>118.0639</i>	<i>118.0640</i>	<i>118.0639</i>	<i>118.0653</i>	Val (118.0868)
		<i>146.0585</i>	<i>146.0605</i>	<i>146.0605</i>	<i>146.0601</i>	Unknown
		<i>205.0955</i>	<i>205.0974</i>	<i>205.0967</i>	<i>205.0974</i>	Asp;Ala dipeptide (205.0797)
5	5.47	219.1703	219.1743	219.1752	219.1751	Unknown
		<i>203.1388</i>	<i>203.1391</i>	<i>203.1382</i>	<i>203.1431</i>	Ala;Ile/Leu dipeptide (203.1396)
6	5.97	353.2271	353.2288	353.2271	353.2308	Unknown
		<i>369.1984</i>	<i>369.2002</i>	<i>369.1999</i>	<i>369.2016</i>	Unknown +OH
7	10.99	149.0192	149.0227	149.0241	149.0232	Background contaminant
		301.1417	301.1400	301.1433	301.1419	Unknown

Values in italics depicts  $m/z$  values of lesser species, refer to above spectra;

Unknown refers to molecular species that could not be identified from in the general groups of free amino acids, dipeptides and amino sugars



**Figure 4.10** Comparison of the signal intensities of the major molecular species in the different peaks showing differences and similarities between basic extracts obtained from wort of four sorghum cultivars (625 = MMSH-625, 375 = MMSH-375, 1401 = MMSH-1401 and 1194 = MMSH-1194).

## Conclusions

A proportional relationship between the concentration of the extract added in each well and yeast growth was observed in all four cultivars. This indicated that N-containing compounds extracted in all cultivars play an essential role during fermentation by contributing positively towards yeast growth. The antiyeast dose-response data showed that extracts of MMSH-375 and MMSH-625 resulted in generally a higher %yeast growth when compared to other cultivars. This indicated that MMSH-375 and MMSH-625 are best suitable for utilisation in the commercial production of sorghum lager beer.

HP-TLC and UPLC-MS fingerprinting was also carried out in preliminary characterisation of the N-containing crude extracts. HP-TLC data did not show major differences between the worts of the cultivars. The UPLC-MS results, however, confirmed the dose response data on the extracts that MMSH-375 may have a better qualitative yeast-feeding value i.e. high level of group A, B and C amino acids and dipeptides consisting of these amino acids. From these results MMSH-375 could be rated as the best cultivar out of the four that were screened and is recommended for use in the commercial production of sorghum lager beer.

It could not be determined at this stage if the cultivars utilised in this study possess antimicrobial proteins, peptides or like compounds, possibly because of very low concentrations or losses during the extraction process. Furthermore, the nutrients in the extracts could have overshadowed the antimicrobial activity that may be present by enhancing yeast growth. It is therefore very important for sorghum wort to have a high nutritional value during fermentation in order to support yeast growth even in the presence of anti-yeast compounds.

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## **Chapter 5**

General discussions, conclusions and recommendations

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### General discussions, conclusions and recommendations

The utilisation of a local crop (sorghum), as a brewing material in Africa has provided a boost to the local economies where it has benefited farmers with guaranteed income (INTSORMIL, 2008). Furthermore, the lowered beer production costs associated with the use of a local crop has been augmented by reduced government-imposed taxes on locally produced products. These benefits in turn have resulted in a reduction of the retail price of sorghum beer, leading to increased growth in the sorghum lager beer brewing industry (Mackintosh & Higgins, 2004). The selection and breeding of sorghum cultivars with increased beneficial properties for brewing is improving sorghum for lager beer brewing. Through plant breeding research, several more cultivars with improved quality have been developed and released by breeders annually (Haln, 1966; Dicko *et al.*, 2006). More than 10 000 sorghum cultivars already exist and some of their malts possess beneficial qualities for clear lager style brewing such as good diastatic power and extract recovery (Owuama & Adeyemo, 2009).

The first brewing issue examined in this work (Chapter 3) was to evaluate the malting and brewing potential of 12 sorghum cultivars (MMSH-375, 625, 707, 1038, 1077, 1194, 1257, 1356, 1363, 1365, 1376 and 1401). It was found that all cultivars were tannin-free and thus suitable for human consumption however, they differed in their malting and brewing potential. Only sorghum cultivars MMSH-375, 625, 1038, 1194, 1257, 1401 and 1376 had a %GE considered appropriate for malting and brewing. Only up to 25% of the diastatic power (DP) level recommended suitable for commercial malting was achieved in this study. In spite of the low DP observed in all cultivars after malting, high extract levels ranging between 18.7 and 25.5°P were achieved. This negative correlation between DP and extract levels obtained suggested that the obtained DP levels were not the true amylase levels produced during malting. There is likelihood that saccharifying enzyme activity may have been reduced as a result of poor control of kilning temperatures. Alternatively, the methodology used for determination of DP in malts, may not have been fully optimised leading to lower DP results. Future studies must entail optimisation and determining the DP under tightly controlled conditions to ensure that better results will be achieved.

It is generally believed that for high gravity brewing which is employed in modern day brewing a minimum of 150 mg/L of FAN is necessary to support proper yeast function (Beckerich & Denault, 1987). In unmalted grains a significantly high ( $p \leq 0.05$ ) FAN level was observed in cultivars MMSH-375, 1194 and 1077. These FAN values were, however, less than 10% of the recommended FAN level necessary to support proper yeast functioning during wort fermentation. This suggests that very high levels of proteolytic enzymes will be required to produce FAN levels sufficient to support yeast when mashing with unmalted

sorghum (Bajomo & Young, 1992; Agu & Palmer, 1998; Goode *et al.*, 2002). This will consequently lead to increased production costs and thus increase in the retail price of the beer. After malting the FAN levels above the minimum threshold (150 mg/L) generally considered enough to support high gravity brewing and support optimal yeast function during the production of sorghum clear lager beer were achieved only in MMSH-375 (intermediate cultivar) and 1401 (intermediate cultivar). FAN level between 100 and 140 mg/L considered enough to support optimal fermentation (Bajomo & Young, 1993) was achieved in cultivars MMSH-625 (intermediate cultivar), 1077 (hard cultivar), 1194 (intermediate cultivar), 1356 (soft cultivar) and 1376 (intermediate cultivar) after malting.

Mashing did not significantly increase FAN levels in all sorghum cultivars, however, the addition of potassium metabisulphite (KMS) led to a further increase of FAN levels in all cultivars. KMS could have reduced the stabilising disulphide bridges present in proteins particularly in the kafirins. This was in agreement with studies which showed that reducing agents such as 2-mercaptoethanol (Hamaker *et al.*, 1987), sodium bisulphite (Oria *et al.*, 1995), potassium metabisulphite (Ng'andwe *et al.*, 2008) and ascorbic acid (Arbab & El Tinay, 1997) increase sorghum protein digestibility. The level of increase in FAN when mash was treated with KMS was cultivar-dependant and ranged from 2 to 53%.

Scanning electron micrographs revealed that during malting, the corneous endosperm was essentially unmodified. This suggests that it was inaccessible to hydrolytic enzymes. It was only the soft endosperm which was modified in all cultivars during malting. It was observed that intermediate cultivars with high %GE provided the most desirable results for potential malting and brewing of clear sorghum lager style beer. The breeding of cultivars with intermediate endosperm texture, weak strength of the protein-starch adhesion (Hoseney *et al.*, 1974; Aisien & Muts, 1987) and high solubility cell wall material (Taylor & Belton, 2002; Duodu *et al.*, 2003; Ogbonna *et al.*, 2004) is a necessity. Such cultivars will be highly beneficial for the malting and consequently brewing industry. Also the addition of KMS during mashing reduces disulphide bonds present in kafirins leading to increased FAN production and thus should be employed during the production of clear sorghum lager style beer. Overall on basis of high %GE (>90%), endosperm texture (intermediate cultivars), total soluble sugars and FAN levels furnished during malting, sorghum cultivars MMSH-375, MMSH-1401, MMSH-625 and MMSH-1194 are recommended to be utilised in the commercial production of sorghum clear lager style beer.

Despite the work that has been done to improve the efficacy of sorghum lager beer production, there are still challenges experienced during fermentation (Ng'andwe, 2008; INTSORMIL, 2008). During small scale laboratory sorghum wort fermentation it was shown that yeast could be repitched over five consecutive fermentations without negatively affecting its growth (Bajomo & Young, 1994). However, in the commercial production of sorghum

lager beer only up to three consecutive fermentations can be achieved using the same yeast crop (Ng'andwe, 2008). Despite the fact that laboratory fermentation systems and conditions differ significantly from those obtained in commercial practice (Bajomo & Young, 1994) there could be other factors playing a role in these observed differences. Sorghum antimicrobial peptides and proteins could inhibit yeast growth during wort fermentation due to their antifungal activity. This could be leading to fermentation problems (PYF and incomplete fermentation) and also causing re-pitching problems experienced during the production of sorghum lager beer and consequently resulting in great financial losses in the brewing industry.

The second brewing issue examined in this work (Chapter 4) was thus to isolate N-containing compounds from selected malts of interest (sorghum cultivars with good malting and brewing potential) and determine their antiyeast activity.

Antiyeast activity was determined using an established 96 well microtiter plate antiyeast method (Van Nierop *et al.* 2008). It was found that there was a proportional relationship between N-containing compounds added in each well and yeast growth. This meant that the N-containing compounds were playing an essential role during fermentation by contributing positively towards yeast growth. It is possible that the additional nutrients in the extracts overshadowed the effect of the antimicrobial compounds (Van Nierop, 2005) that was indicated by the 20-30% reduced growth at the lower concentrations of the N-containing compounds. Alternatively, the concentrations of extracts may have contributed to the aggregation of antimicrobial peptides (Rautenbach *et al.*, 2006) and antimicrobial proteins leading to loss of antiyeast activity.

High performance thin layer chromatography (HP-TLC) and ultra performance liquid chromatography-mass spectrometry (UPLC-MS) fingerprinting was conducted in order to characterise N-containing compounds present in the extracts. In the HP-TLC chromatography, compounds separated into the three putative groups namely polar, amphipathic and hydrophobic compounds in all four cultivars with the amphipathic group showing the highest concentrations. Only minor differences were observed between the four cultivars with the HP-TLC data in terms of total ninhydrin response of the different groups. More detailed characterisation were obtained with UPLC-MS and certain amino acids such as Arg and Val and dipeptides containing Asp+Ala, Tyr+Arg and Ala+Ile/Leu compounds were tentatively identified. The fact that neutral and anionic dipeptides were also found in the extract indicated that the cation exchange was not fully optimised to yield only the cationic fraction. This could also indicate that the antimicrobial peptide may not have been fully recovered from the cation exchange column. However the reduced % yeast growth observed in all four sorghum cultivars at low concentrations of the extracts, indicated that some antimicrobial compounds (possibly peptides) was present, albeit at low concentrations

as only small compounds (<1 kDa) were clearly detected with UPLC-MS. Overall, the proportional relationship between N-containing compounds and %yeast growth suggests that it is very important for sorghum wort to have a high nutritional value during fermentation in order to effectively support yeast growth even in the presence of anti-yeast compounds.

The dose-response data showed that extracts of MMSH-375 and MMSH-625 resulted in generally a higher %yeast growth when compared to other cultivars. The UPLC-MS also confirmed the dose response data that MMSH-375 has a better qualitative yeast-feeding value i.e. high level of group A, B and C amino acids and dipeptides consisting of these amino acids. Overall, out of the twelve sorghum cultivars whose malting, brewing and antiyeast characteristics were evaluated MMSH-375 is rated as the best cultivar and is recommended to be used in the commercial production of sorghum lager beer.

For future work it is recommended N-containing compounds which contribute negatively towards yeast growth (antimicrobial peptides and proteins) should be isolated individually (per type of compound) with optimised protocols in all for sorghum cultivars (MMSH-375, 625, 1194 and 1401) and studied separately. This will eliminate the role played by additional nutrients present in the extracts and will precisely indicate which antimicrobial compounds are responsible for the observed 20-30% decrease in yeast growth at low concentrations of the putative yeast growth N-containing compounds. Antimicrobial peptides and proteins should be isolated from sorghum cultivars (MMSH-375, 625, 1194 and 1401) cultivated at different locations and during different seasons since their production within grains is elevated during humid, warm and rainy seasons, as well as when infections in the field occurs (Van Nierop *et al.* 2008). Their isolation should be conducted before and after wort boiling in order to determine whether temperature has any effect on their structure and thus their effect on yeast during fermentation.

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