Free amino nitrogen improvement in sorghum malt brewing

By

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DECLARATION

I declare that the dissertation herewith submitted for the degree MSc (Agric) Food Science and Technology at the University of Pretoria, has not previously been submitted by me for a degree at any other University or Institution of higher education.

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ABSTRACT

Free amino nitrogen improvement in sorghum malt brewing

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Although sorghum malt is relatively rich in free amino nitrogen (FAN), the 150 mg FAN/L threshold recommended for brewing is difficult to obtain. The vitreous nature of the sorghum endosperm hinders proteolysis during brewing. Hence, exogenous proteolytic enzymes are often required to increase hydrolysis of sorghum malt protein to produce sufficient FAN in order to support rapid yeast growth during fermentation.

Ten exogenous proteases were examined for their production of FAN in sorghum malt mashing. Mashing was done at 55^0C for 45 minutes. Levels of FAN, as determined by the ninhydrin method, showed great variation among the proteolytic enzymes, ranging from 96 in control to 182 mg/100 g malt with possibly of most effective proteolytic enzyme. The variation in FAN level was possibly due to different optimal mashing conditions of exogenous proteases used and perhaps due to low ratios of exopeptidase/endopeptidase in the enzyme preparations.

Low temperature (40^0C) and long duration mashing for (7 hours) gave good FAN production during mashing to a total of 113 and 138 mg/100 g malt in control and the treatment with exogenous proteolytic enzyme Flavourzyme plus malt, respectively.

The exogenous enzyme (Flavourzyme) plus potassium metabisulphite (PMB) increased FAN production during mashing in the ratio of 2 to 1 in a treatment where PMB was added compared to one without. Similarly, hot wort extract (HWE) increased by 8% during mashing with exogenous enzyme plus PMB compared to one without PMB, respectively. PMB was involved in destabilizing the disulphide bonds in the sorghum protein

polypeptide chains allowing proteolytic enzymes better accessibility to proteins. The increase in HWE was possibly due to the starch being freed from the sorghum protein matrix.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showed some oligomeric and polymeric kafirins after mashing. With transmission electron microcopy (TEM), protein bodies of varying sizes with partially degraded peripheral edges and some holes were seen after mashing. SDS-PAGE and TEM results suggest insufficient proteolysis.

High protein digestibility sorghum's potential for brewing was examined with reference to FAN production. Although during mashing FAN increased by approx. 82 and 115% for unmalted normal and high digestibility sorghums, respectively, the 150 mg FAN/L threshold, recommended for brewing was not achieved.

FAN production to levels above 150 mg/L may only be realized if normal sorghum malt or high protein digestibility sorghum malt is mashed with exogenous enzymes containing sufficient exopeptidases coupled with appropriate mashing conditions.

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1.   INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is a cereal that has significant genetic variability with more than 30,000 selections present in the world collections in India and elsewhere (Serna-Saldivar and Rooney, 1995). It is believed that sorghum plant is native to equatorial Africa (Serna-Saldivar and Rooney, 1995). It is now distributed throughout the semi-arid equatorial regions and other semi tropics of the world. The sorghum plant is uniquely well adapted to these conditions in that it is drought-resistant and will withstand periods of flooding. In fact, sorghum can produce a crop in these regions, where other cereals such as barley and maize cannot be economically cultivated (Doggett, 1988).

Sorghum is the fifth most important crop in the world after wheat, maize, rice and barley International Crops Research Institute for the Semi-Arid Tropics/ Food and Agriculture Organization (ICRISAT/FAO, 1996). The leading producers during 1999-2003 periods include the USA 10.4 million metric tons (MT), India 8.5 million MT, Nigeria 8.1 million MT, Mexico 6.5 million MT and Sudan 4.4 million MT (ICRISAT/FAO, 1996).

In Africa, the major sorghum growing areas run across West Africa south of the Sahara to the coast and eastward into Sudan, Ethiopia, and Somalia (House, 1995). Sorghum is a staple food crop of millions of poor people in semi-arid tropics of Africa and Asia. Moreover, it has gained increasing importance as a fodder (green/dry) and feed crop in the last decade (ICRISAT, 1996).

In some countries, the trend of per capita agricultural production index for cereals has progressively been increasing during the past 25 years (1980-2005) (FAO, 1996). Although both area and production of sorghum in Eastern and Southern Africa has increased from the early 1970's to 2005, there has not been an increase in yield (ICRISAT, 1996). However, the use of sorghum in commercial production of lager and stout beers (Taylor et al., 2006), may motivate producers of sorghum and boost production and income to smallholder farmers as the case is in Uganda and elsewhere where sorghum is being utilized for lager beer (Mackintosh and Higgins, 2004).

The potential of sorghum as an alternative substrate for lager beer brewing was recognized over five decades ago (Owuama, 1997). Success in replacing barley malt using sorghum malt in lager beer brewing has been cited in several parts of Africa, particularly in Nigeria and elsewhere (Agu and Palmer 1998; Owuama, 1997).

In sub-Saharan African countries, Nigeria has been the leading country in using sorghum for lager beer production. This development came about due to government ban on importation

of barley as a raw material in brewing which then forced brewers to find an alternative local source of raw material (Owuama and Okafor, 1987; Agu and Palmer, 1998).

Recently in East and Central African countries for example in Uganda and Zambia, sorghum lager beers brewed from raw sorghum combined with commercial enzymes was pioneered by SABMiller and is receiving great support by farmers and governments concerned (Mackintosh and Higgins, 2004). The reason for such a flourishing interest in the use of sorghum as opposed to barley is that sorghum will not only provide African farmers with a reliable market but also save foreign exchange, which would otherwise be required to import barley which cannot grow well in tropical conditions (Mackintosh and Higgins, 2004; Taylor et al., 2006) etc.

The overall aim of this investigation was to determine ways of improving the brewing technology of sorghum lager beer using malted sorghum.

1.1 Problem statement

When brewing with sorghum, insufficient proteolysis due to the corneous nature of the endosperm in sorghum grain has a negative effect on production of free amino nitrogen (FAN) and extracts levels (Ogbonna et al., 2004). FAN, the product of protein degradation of grain and malt proteins, comprises free amino acids and short chain peptides (Taylor and Boyd, 1986). It is an important nutrient for yeast growth during fermentation. In the sorghum grain, the starch granules are embedded in the endosperm protein matrix (Klopfenstein and Hoseney, 1995). Hydrolysis of the protein matrix would improve wort quantity as it is to a large extent related to the degree of proteolysis in sorghum malt (Ogbonna et al., 2004). In addition, the degree of proteolysis affects wort filtration, colour and flavour development as well as foam and head retention of the resulting beer (Ogbonna et al., 2004). Addition of exogenous enzymes can complement malt endogenous enzymes to improve proteolysis, filtration and the level of wort extract (Agu and Palmer, 1998; Bajomo and Young, 1994).

This investigation was conducted to determine the effect of exogenous proteases on efficiency in brewing with sorghum malt, with emphasis on the development of FAN and wort extract.

2.   LITERATURE REVIEW

This section will present a brief overview of sorghum grain structure and its chemical composition with particular reference to proteins. The principles and practice of sorghum malting and brewing will be discussed. Mashing science with particular reference to proteolysis and, improving FAN when mashing with sorghum malt will be reviewed. Lastly, the various types of exogenous proteases with particular reference to sorghum lager beer brewing and some aspects of brewing analysis will be reviewed.

2.1   Sorghum grain structure and chemical composition with particular reference to proteins

Sorghum is a naked kernel (threshed free from the hull) (Serna-Saldivar and Rooney, 1995). In terms of size and shape sorghum varieties differ widely. The general size of sorghum is 4 mm long, 2 mm wide and the test weight is (25-35 mg), with a density usually in the range of 1.15-1.38 g/cm^3 (Serna-Saldivar and Rooney, 1995). Figure 2.1 shows a section of a sorghum kernel.

The proximate composition of sorghum grain varies significantly partly because of wide varietal differences but mainly due to the diversity of environmental conditions (such as water availability, soil fertility and temperature) under which sorghum is grown (Klopfenstein and Hoseney, 1995). The chemical composition of the components of the kernel by weight are pericarp (outer bran part) 7.9%, endosperm (storage organ) 82.3% and germ also referred to as (embryo) 9.8% (Klopfenstein and Hoseney, 1995). The embryo is the heart of the seed and from embryonic axis emerges a shoot and root during germination to produce a new sorghum plant.
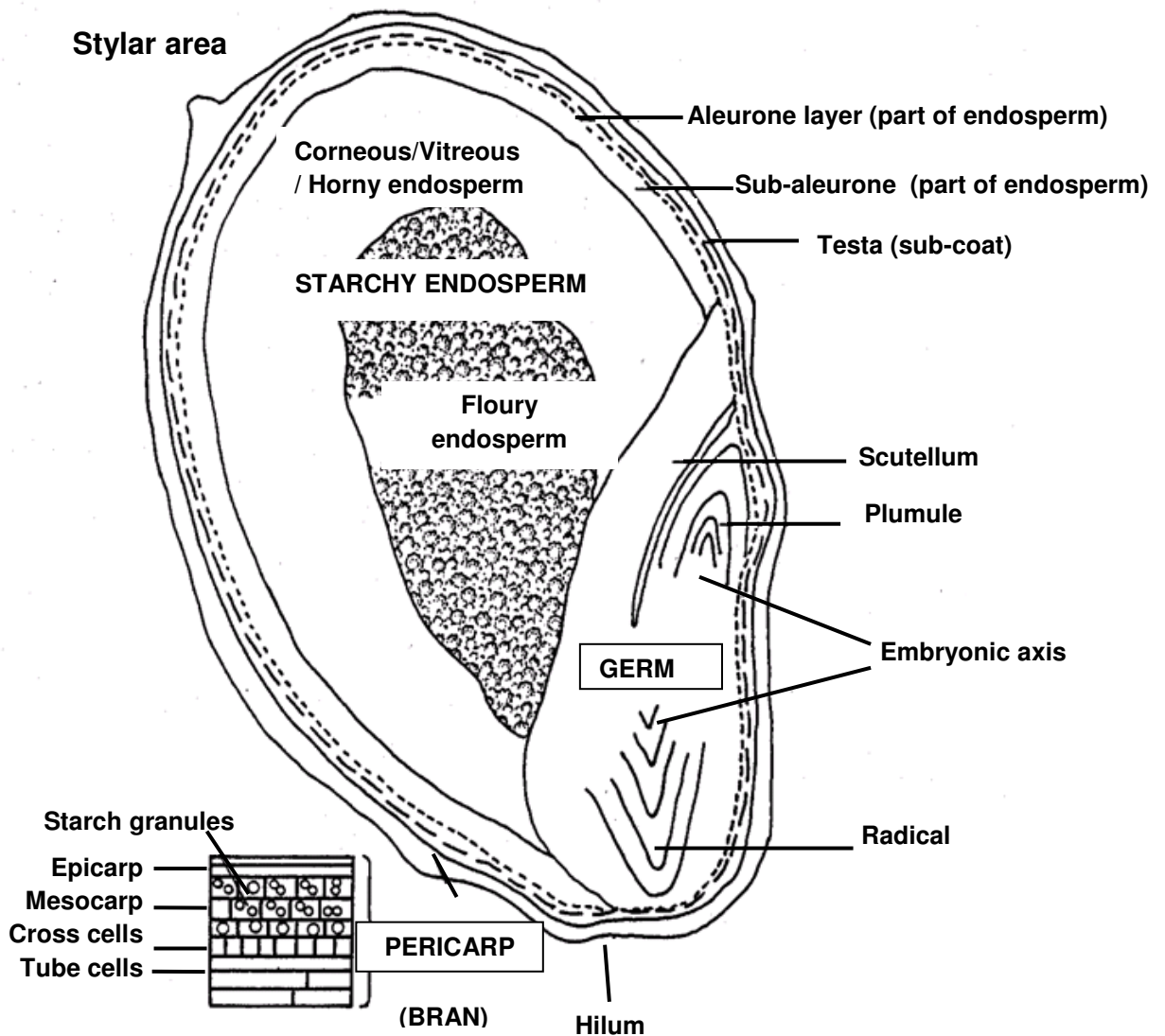
Figure 2.1: A section of a sorghum kernel (from Taylor, 2007)

The protein content of sorghum varies considerably from 7.3 to 15.6% (Serna-Saldivar and Rooney, 1995). Approximately 80% of the kernel protein is located in the endosperm, the majority being in the form of the prolamin protein called kafirin (Taylor and Schüssler, 1986). The kafirins are located in the organelles called protein bodies. The size of the protein bodies is about 0.4 and 2 µm (Taylor, Novellie and Liebenberg, 1984a). Kafirin is deficient in the essential amino acid lysine (Taylor and Schüssler, 1986). The second largest protein fraction in the endosperm is G_3-glutelin. This protein is somewhat richer in lysine and most likely forms the gluten matrix around protein bodies (Serna-Saldivar and Rooney, 1995). The germ contains almost 16% of the kernel protein, the majority being low molecular weight

nitrogen, albumins and globulins (Taylor and Schűssler, 1986). The pericarp has some 3% of the kernel protein. Table 2.1 shows the amino acid composition of protein fractions from the endosperm, germ, and pericarp of Barnard Red sorghum variety (Taylor and Schűssler, 1986). The amino acid composition of this fraction G_3 in (Table 2.1) showed that it is poor in glutamic acid and richer in amino acid Lysine. The significance of this aspect is associated with low proteolysis.

Table 2.1: Amino acid composition of the protein fractions from the endosperm, germ and pericarp of Barnard Red sorghum (g/100 g protein)

	Albumin+globulins			Prolamins			G_2-glutelins			G_3 glutelins			Residue		
Amino acid	E[a]	G[b]	P[c]	E	G	P	E	G	P	E	G	P	E	G	P
As	9.2	8.1	10.1	5.2	6.1	8.5	10.3	9.9	11.6	8.0	8.7	9.4	6.4	7.1	10.0
Thr	5.1	4.5	4.9	2.8	3.9	4.2	3.7	4.3	4.1	4.6	4.4	4.3	3.8	4.4	5.1
Ser	4.8	5.1	7.4	3.9	4.5	5.1	2.4	4.4	3.6	4.4	4.6	4.7	4.0	5.1	4.9
Gl	11.3	13.0	10.4	22.0	17.9	14.6	17.3	11.1	11.8	11.2	12.6	13.3	17.5	17.2	11.2
Pro	5.5	4.2	4.7	9.8	8.6	6.4	11.0	6.0	7.1	7.3	5.2	6.0	13.0	10.8	5.9
Gly	6.7	6.2	6.9	1.7	4.2	5.3	6.7	6.9	7.9	4.7	6.1	6.0	5.8	6.6	7.8
Ala	7.2	6.8	7.7	10.6	9.8	9.1	5.2	5.6	6.5	6.6	6.7	7.3	6.2	5.4	7.1
Cys	3.0	2.1	2.6	0.5	0	0.5	1.0	1.0	1.3	0.5	0.6	0.4	0.3	0.5	0.3
Val	6.0	6.7	6.6	4.8	5.2	6.5	5.7	6.7	6.5	5.4	6.9	6.6	6.3	5.2	7.0
Met	1.9	2.0	0.7	2.4	2.5	1.8	1.0	0.4	0	2.2	1.6	1.4	0.9	1.1	1.7
Ile	4.1	3.7	4.2	4.3	4.2	4.6	3.0	3.8	3.8	5.8	4.7	4.9	3.9	4.1	5.0
Leu	7.5	7.6	6.5	14.7	12.7	11.6	7.3	6.6	6.9	8.3	9.0	10.1	9.7	7.6	9.2
Tyr	4.2	3.1	3.7	4.9	4.6	3.8	2.8	3.9	3.4	5.4	3.8	3.8	1.7	3.4	3.9
Phe	5.4	4.9	3.9	5.8	5.1	5.3	3.6	4.9	4.5	6.6	5.6	5.9	4.8	5.6	5.9
His	2.1	3.7	2.3	2.2	2.7	1.9	6.9	4.8	3.4	3.9	3.4	2.7	5.4	3.5	2.6
Lys	5.5	7.0	5.6	0.2	2.0	3.7	4.2	7.9	5.2	6.1	6.6	4.7	3.1	4.3	4.6
NH_3	2.0	1.5	4.1	2.4	2.1	2.1	2.7	4.3	4.0	1.2	1.1	1.7	3.1	1.7	1.8
Arg	8.9	10.2	7.7	1.8	3.9	5.0	5.3	11.1	8.2	7.8	8.4	6.8	4.1	6.4	6.3

[a] Endosperm; [b] germ; [c] pericarp(Taylor and Schűssler, 1986)

2.2 Principles and practice of malting and brewing

2.2.1 Principles and practice of malting

Malting, believed to be the oldest biotechnology dating back to 10,000 BC can be defined as the limited germination of cereal grains under controlled moist conditions (Briggs, 1998). According to Taylor et al. (2005) there are three main objectives of sorghum malting for beer brewing:

a) To mobilize the endogenous hydrolytic enzymes of the grain,

b) By means of these enzymes to modify the constituents of the grain during malting so that they are readily solubilised during the souring process (pH of the fermenting material decreases) of sorghum beer brewing in order to produce fermentable medium and

c) By means of the enzymes to solubilise the unmalted cereal grain during the mashing process.

Figure 2.2 shows the various steps involved in the malting process.
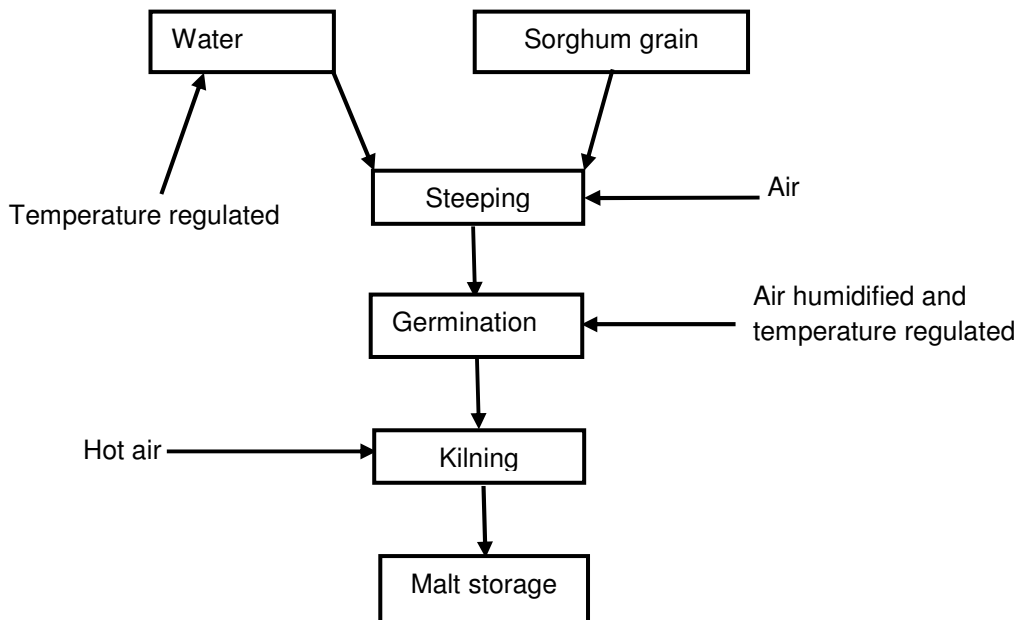
Figure 2.2: Simplified flow diagram of the malting process (adapted from Briggs, 1998)

Steeping

Steeping, involves soaking sorghum grains in water. The fundamental reason is to hydrated the grain and initiate metabolism of living tissues, which are habitually dormant when the grains are dry (Briggs, 1998). For efficient production of good quality sorghum malt, a steep out moisture level of 33-35% (wet/weight basis) is recommended (Dewar, Taylor and Berjak 1997a). Essentially, steeping in water at 23-25^{0}C, with a cycle of 3 hours wet, followed by 1 hour dry period for 24 hours can suffice to obtain the level of moisture required (Morrall, Boyd, Taylor and Van der Walt, 1986). A secondary objective of steeping is to clean the grains in order to prevent microbial contamination during germination (Lefyedi and Taylor, 2006). Several steeping regimes have been studied and developed for the purpose of enhancing the quality of sorghum malt. For example, steeping sorghum in dilute alkali solution gave faster increase in water uptake (Dewar, Orovan and Taylor, 1997b). This was attributed to disruption of the sorghum pericarp cell wall structure.

Germination

Germination is the process of seedling growth and involves the development of the plumule and radicle of the seedling (Briggs, 1998). At the biochemical level, the fundamental reason for germination is to develop amylases, proteases and other endogenous hydrolytic enzymes. During germination the storage proteins in the grain are hydrolyzed by protease enzymes (proteinases and peptidases) (Taylor, 1993). The proteinases catalyse the hydrolysis of internal peptide bonds within protein molecule to free short chain peptides. The most important peptidases in malting and brewing are the carboxypeptidases that hydrolyse the terminal peptide bond at the carboxylic acid end of the peptide to release free amino acids, which are essential for yeast growth and rapid fermentation (Evans and Taylor, 1990). At the same time, alpha-amylase (E.C. 3.2.1.1), also called 1, 4-α-D-glucan glucanohydrolase) develops. Alpha-amylase hydrolyzes (α -1, 4 glucosidic bond of starch randomly (Briggs, 1998). Beta-amylase (E.C. 3.2.1.2), also chemically known as (α-1, 4)-glucan maltohydrolase) develop during sorghum malting. Its role in mashing is to hydrolyse penultimate alpha (1->4) glucosidic bond at the non reducing end of the starch molecules, releasing maltose (Taylor and Von Benecke, 1990). Generally, sorghum malt beta-amylase activity is low (Taylor, 1992) and in fact in the ungerminated state, sorghum grain does not exhibit beta-amylase activity (Taylor and Robbins, 1993). It has been found that the overall diastatic power (alpha- amylase and beta-amylase activity) of sorghum increases with germination time (Morrall, Boyd, Taylor and Van der Walt, 1986). The germination step is terminated prior to the exhaustion of the seed nutrients (Owuama, 1997).

Kilning (drying)

The principal objective of drying is to produce shelf-stable malt with a good level of amylase activity. The kilning temperature suitable for retention of high amylase activity and other endogenous hydrolytic enzymes is approximately 50^0C (Hough et al., 1971). A moisture content of approx. <11% is considered safe and guarantees longer shelf life. Drying at temperatures higher than 50^0C may lower amylase activity particularly beta-amylase, which is temperature labile (Taylor and Robbins, 1993). However, lower temperatures than recommended can be detrimental to the quality of malt as it may impart unacceptable grainy, green malt flavour to the beer (Agu and Palmer., 1996).

The practice (technology) of malting

There are two systems of malting that are practiced to obtain malt: Floor malting and pneumatic malting (Briggs et al., 2004). In the two systems the steeping process is common and utilizes the same equipment. The differences between the two processes occur in germination and drying (Taylor, Dewar and Joustra, 2005). With reference to steeping, a self-emptying stainless vessel shown in Figure 2.3, cylindrical or rectangular in cross-section with a bottom at an angle of 45° or a flat-bed (Nordon type) steeping tank in Figure 2.4 can be used.

In outdoor floor malting, the steeped sorghum is germinated on a flat but slightly sloped concrete floor outside as shown in Figure 2.5 (Taylor et al., 2005). Usually a layer of grain 10-30 cm thick is covered with shade cloth or sack to prevent excessive loss of moisture and also to protect against predators (birds and rodents). Temperature is controlled by the level thickness, e.g thicker beds retain more metabolic heat than thinner beds and allow sorghum to be malted when it is cold in winter. The grain is watered at intervals by a hose-pipe and watering is stopped when sufficient germination is achieved. Good malt can be made in this way, but only in small quantities about 10 t/batch) and with considerable man power (Briggs et al., 2004).
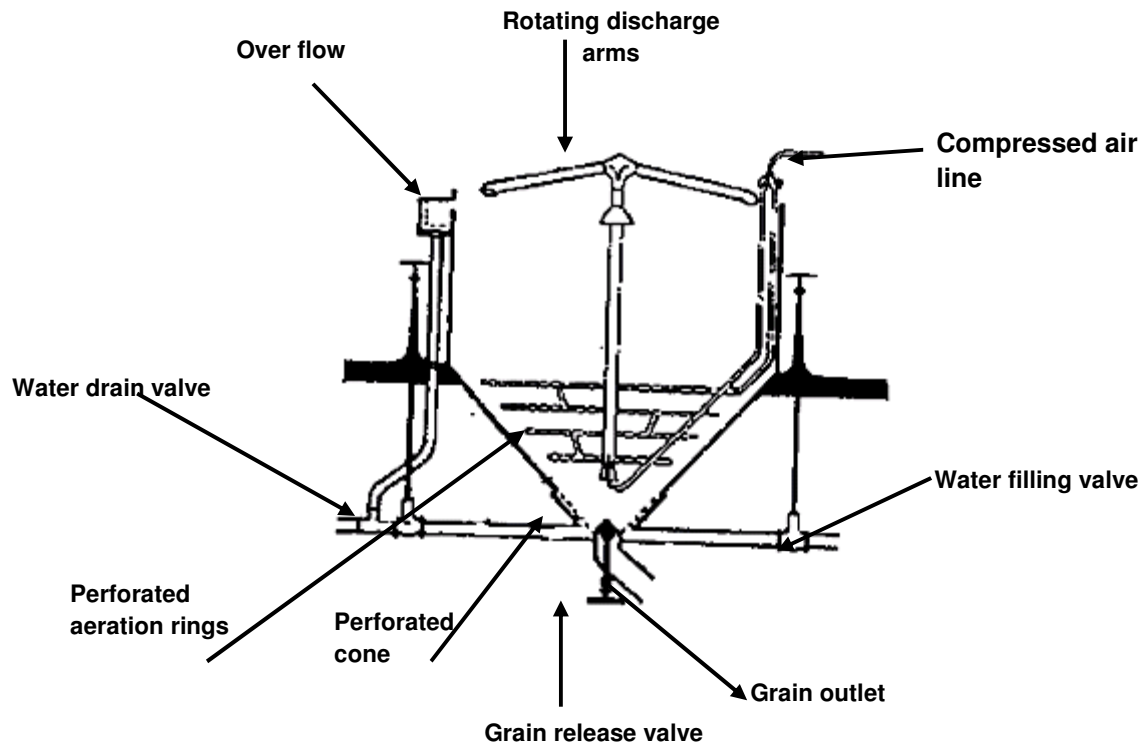
Figure 2.3: Self-emptying conical steep tank with a mixing "geyser" with rotating hollow arms and aeration rings (adapted from Taylor et al., 2005)
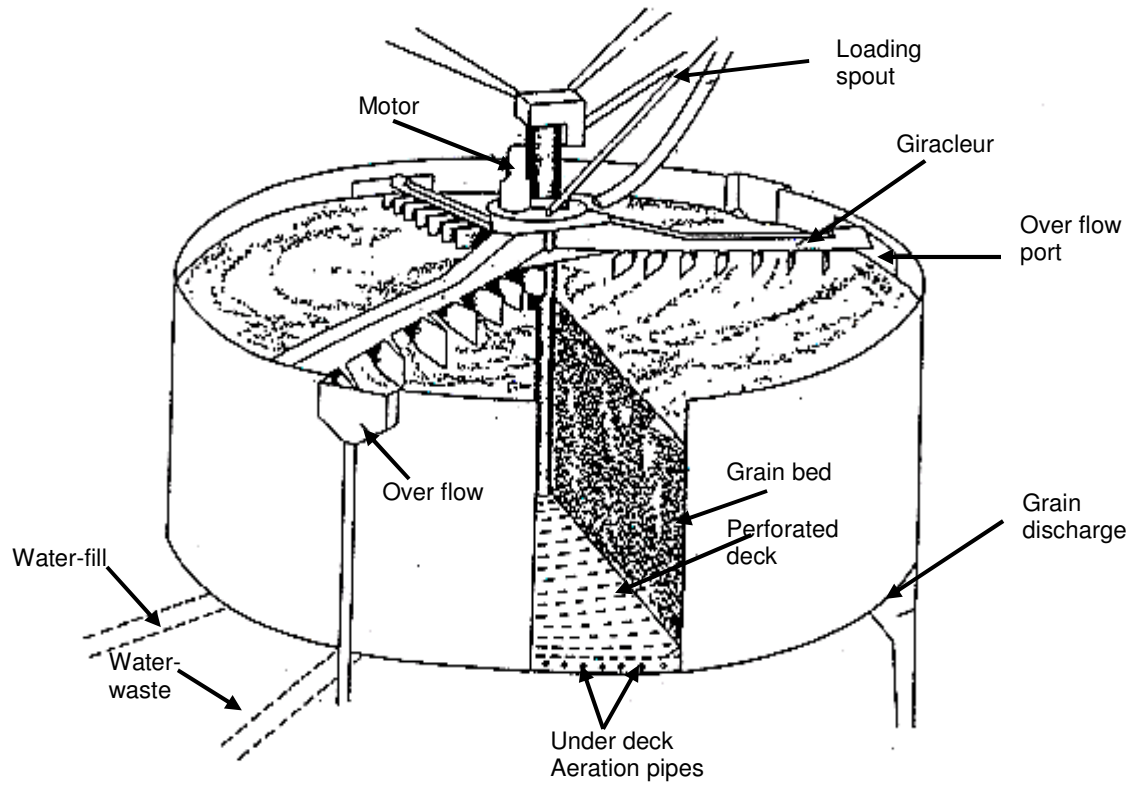
Figure 2.4: Flat-bed (Nordon type) steeping tank (adapted from Taylor et al., 2005)
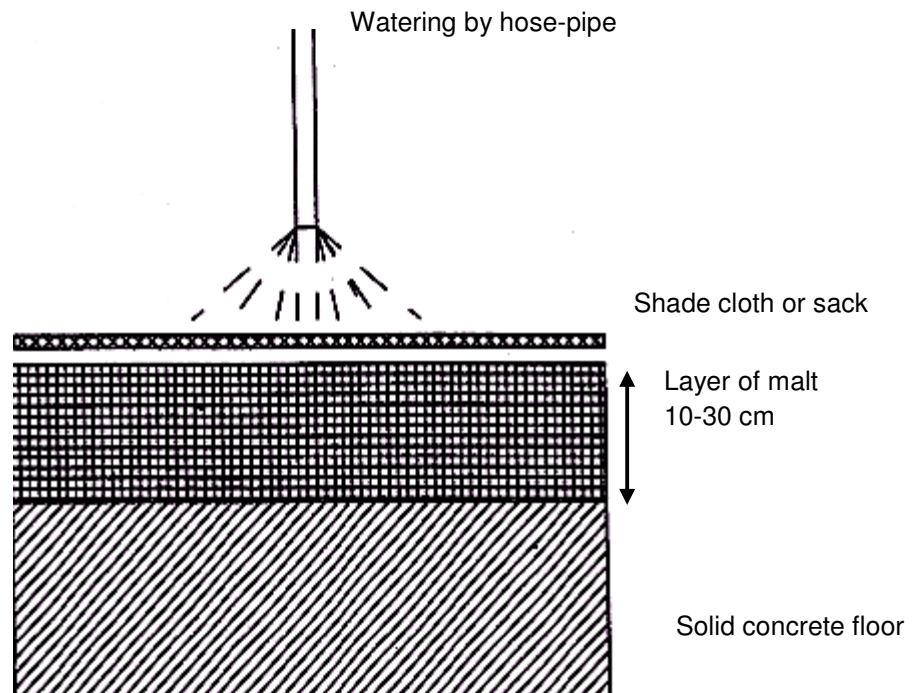
Figure 2.5: Outdoor floor malting of sorghum (adapted from Taylor et al., 2005)

In pneumatic malting, the grain is ventilated with a stream of temperature-adjusted, humidified air. Air removes excess heat and carbon dioxide and supplies oxygen (Briggs et al., 2004). Pneumatic malting of sorghum is similar to that of barley, except that the germination temperature in sorghum malting is 25–30°C, approximately 10 degrees higher than barley malting temperature. Figure 2.6 shows a Saladin box, representing a typical example of pneumatic system used is Southern Africa for sorghum malting (Taylor et al., 2005). The grains are turned mechanically by a screw and the temperature is controlled by forcing a stream of attemperated and water-saturated air through a bed of grain (Briggs et al., 2004).

After germination, the floor malted grain can either be sun dried or dried by using modern kilns through which a current of air is fan-driven from below to dry the malt to a safe moisture level (Briggs et al., 2004).
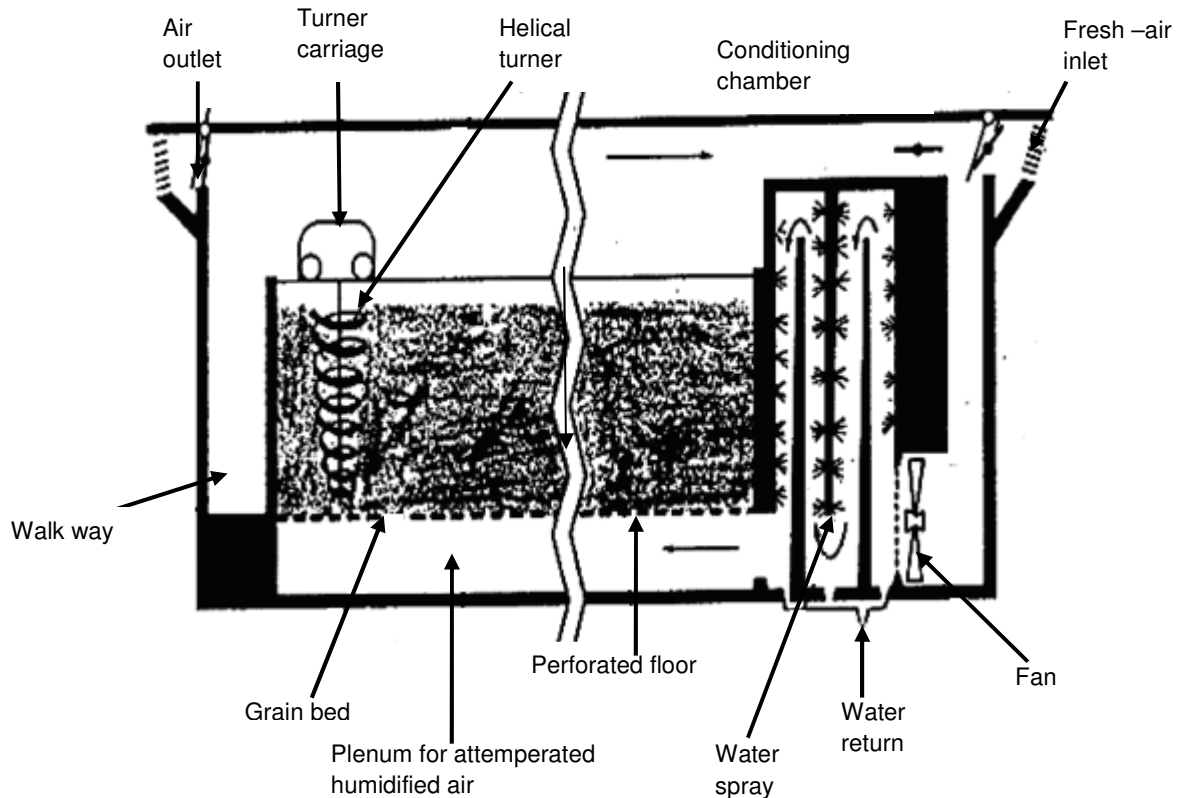
Figure 2.6: Saladin box compartment type (a general view of germination vessel, fan and conditioning chamber) (adapted from Taylor et al., 2005)

Drying pneumatic malted sorghum

Warm dry air is blown through the green malt, temperature not exceeding 50°C, to preserve the amylase activity of the malt. The process reduces the moisture content of sorghum malt to around 10% to produce a shelf-stable product. Sometimes the malt is sun-dried by spreading the grain out in thin layer and turning it periodically (Taylor et al. 2005).

2.2.2   Principles and practice of brewing

Brewing can be defined as the production of malt beverages (lager beer or ale) from malt and hops by grinding and boiling them and fermenting the resulting wort with yeast. The objective of the brewing process is to physically and enzymatically solubilise starch, protein and other constituents of the cereal adjunct and malt, then to ferment the wort using yeast to produce ethanol and carbon dioxide (Briggs et al., 2004). Figure 2.7 shows the major steps in the process to brew lager beer. Raw materials (water, malt and exogenous enzymes are measured (weighed) in appropriate ratios prior to the mashing stage.

```
┌──────────┐   ┌───────────────────────────┐   ┌──────────────┐
│  Water   │   │ Malt and other unmalted   │   │  Exogenous   │
│          │   │        adjuncts           │   │   enzymes    │
└──────────┘   └───────────────────────────┘   └──────────────┘
                  Mashing (conversion)

                  Wort separation and          Spent grain
                        sparging

   Hops              Hop boiling                  Trub

  Yeast             Fermentation              Carbon dioxide
  pitching

 Surplus yeast       Green beer

              Polishing final treatments
                     and bottling
```
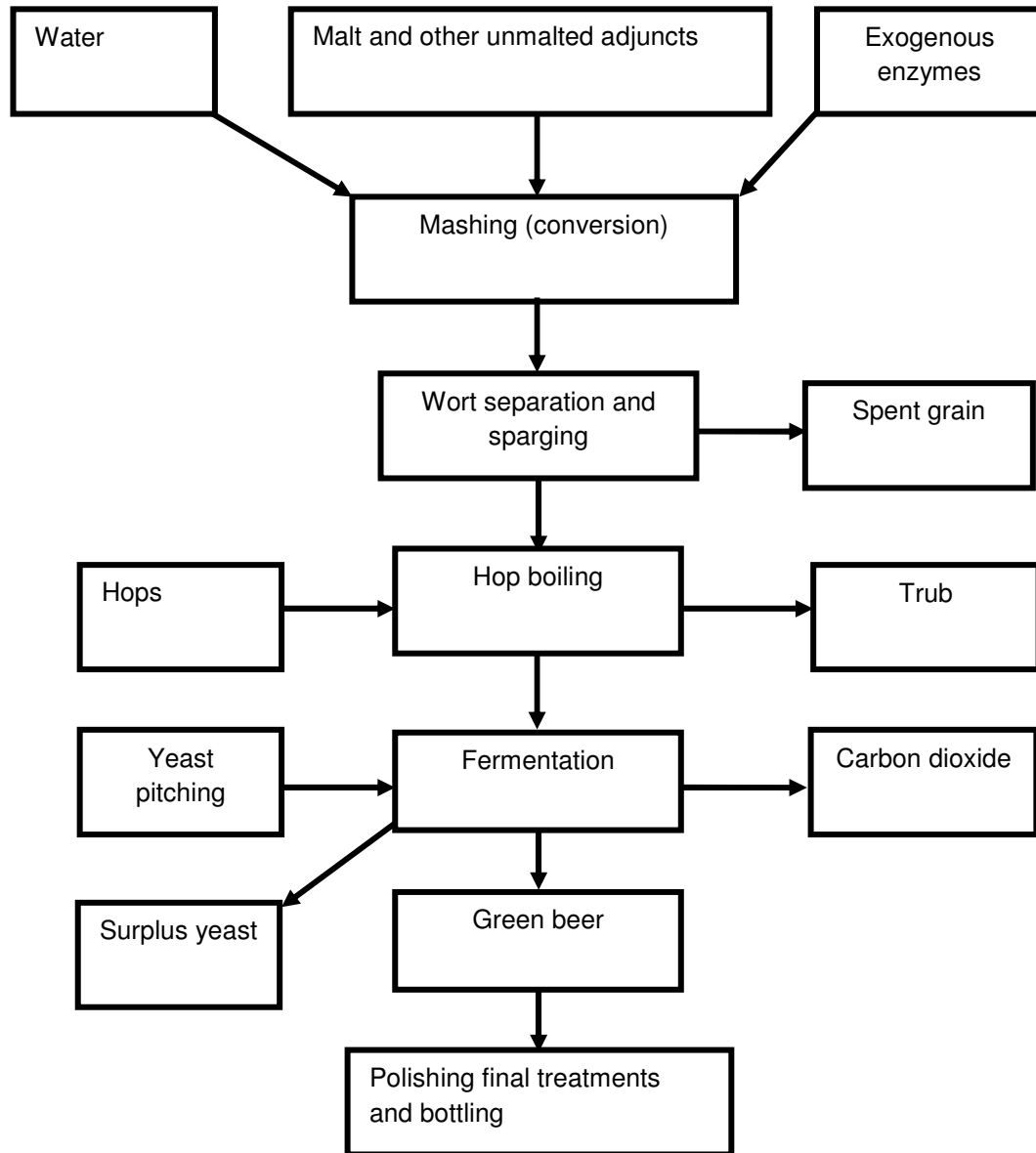
Figure 2.7: Simplified diagram of the lager beer brewing process (adapted from Briggs, 1998)

Mashing

Mashing, also referred to as wort production, is a process that involves mixing crushed malt and hot water (Briggs et al., 2004). The primary aim of mashing is to produce optimal substrate for yeast fermentation (Hallgren, 1995). Traditionally, barley malt was the main ingredient, but today other starchy cereals such as maize, sorghum, rice are being used to

brew lager beer, particularly in sub-Saharan African countries (Owuama and Okafor, 1987; Taylor et al., 2006).

There are a number of different mashing methods: infusion mashing, double decoction mashing and temperature programmed mashing (Briggs et al., 2004. In all these mashing methods, the objective is degradation of starch, proteins, lipids, beta-glucans, pentosans and xylans to produce a fermentable wort (Briggs et al., 2004). The starch degrading enzymes alpha-amylase and beta-amylase developed during malting are responsible for hydrolysis of starch into fermentable sugars. The optimum temperature for alpha-amylase during mashing is in the range of 55 and 60°C, while the beta-amylase is temperature labile, thus effective between 50 and 55°C (Sivaramakrishnan, Gangadharan, Nampoothiri, Soccol, and Pandey, 2006). In the case of protein hydrolysis, proteases are responsible for degradation of storage protein to produce FAN. As stated, FAN is essential for yeast growth and fermentation.

After mashing, sweet wort is produced and spent grain as by product. The wort is then cooled and hops are added and the wort boiled for approximately 60-90 minutes. The importance of adding hops is allowing alpha- and beta- acids present in hops, provide bitterness and aroma to the final product (Briggs et al., 2004).

Fermentation

The process involves yeast pitching to the freshly produced wort. The objective is to enhance fermentation and conversion of fermentable carbohydrates (glucose, fructose, maltotriose and sucrose) of the wort into ethanol and carbon dioxide (Hough et al., 1971). The FAN present in the wort is vital for yeast growth and rapid fermentation (Taylor and Boyd 1986). After fermentation, the so-called green beer is conditioned (lagered) (stored at around 0ºC) to improve its flavour and reduce haze formation (Briggs et al., 2004).

Filtration stage

The residual yeast and suspended solids are removed during filtration. Diatomaceous earth (bentonite) may be added to improve clarity of beer by removing haze leaving the beer sparkling clear (Hough et al., 1971). Finally pasteurisation is generally conducted to improve the microbiological stability of beer.

2.3 The science of sorghum malting with particular reference to proteolysis

During the process of malting, sorghum endosperm protein break down (proteolysis) to free amino acids and short chain peptides is brought about by proteinase enzymes (Taylor and Evans, 1989). It was reported by Agu and Plamer (1996) that enzymic breakdown of endosperm proteins of sorghum was more effective at 20°C than at 25 and 30°C with regard to total protein solubilization, amino acid and peptide production. Work done by Taylor and Evans (1989) showed that protein bodies (the organelles of protein storage in the grain starchy endosperm) were degraded mainly from the periphery. Glutelin (matrix protein) was first hydrolysed, followed by the prolamin protein body. Proteinase extracts from both the germ and endosperm of germinated sorghum were capable of degrading the protein bodies. These findings led to the conclusion that the proteinases responsible for sorghum endosperm proteolysis were synthesized in the germ and then secreted into the starchy endosperm during germination.

2.4 Mashing science with particular reference to proteolysis

Proteolysis during sorghum beer mashing is described by Briggs et al. (2004) as a process of breakdown of protein into free amino acids and other low molecular protein compounds. It is said to be most rapid at low temperature, or so-called `conventional' protein rest temperatures (45-55°C), but it does not cease immediately the temperature rises. In mashing, the proteinases and the carboxypeptidases are the most important enzymes in generating soluble nitrogenous substances (Briggs et al., 2004). Between 30 and 50% of FAN is formed by enzyme action during mashing while about 50% of the total soluble nitrogen is produced when mashes are made at 65°C. By encouraging proteolysis the colour of wort is increased, probably because the elevated levels of nitrogen-containing substances favour melanoidin formation during the copper boil (Briggs et al., 2004). Figure 2.8 shows the temperature region during mashing in which proteolysis is mostly active, and saccharification and enzyme inactivation regions.
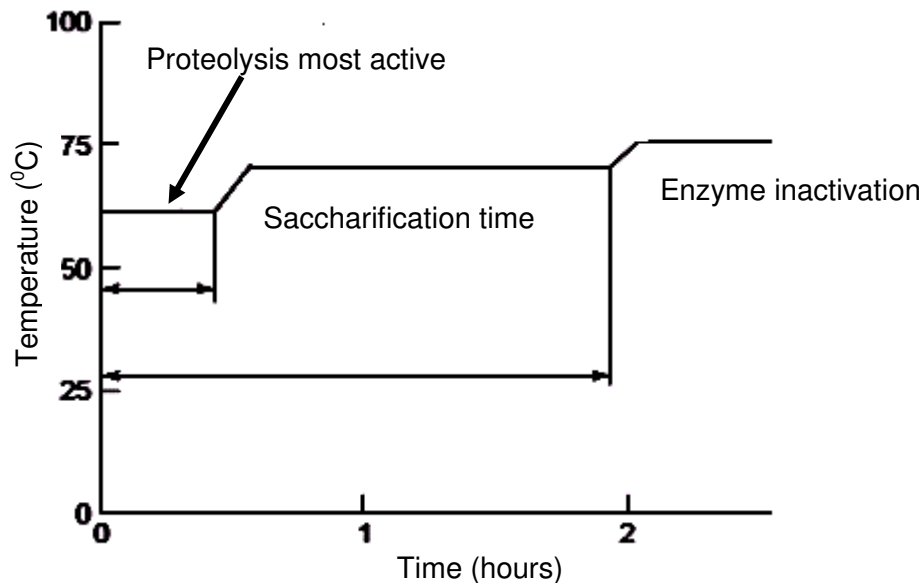
Figure 2.8: A shortened double decoction mashing process lasting for 2 ½ hours (adapted from Briggs et al., 2004).

2.5   Improving FAN when mashing with sorghum malt

Several options or strategies to improve levels of FAN have been proposed by various researchers. Taylor and Boyd (1986) summarized some of the options as follows: increasing the ratio of malt to adjunct, reducing the mashing temperature or adjusting mashing pH to 4.6 and by using microbial proteolytic enzymes. Some of these methods have some limitations, such as cost of enzymes or low activity of enzymes due to wrong temperature application. However, ensuring that the initial malt has sufficient levels of FAN plus careful choice of exogenous proteolytic enzymes and temperature programmes may increase FAN levels and improve extract levels (Briggs et al., 2004).

2.5   Use of exogenous proteases with particular reference to sorghum lager beer brewing

Agu and Palmer (1998) found that the use of microbial exogenous enzymes during mashing with sorghum malt complemented malt endogenous enzymes by improving proteolysis, filtration and the level of wort extract. Similarly Bajomo and Young (1994), Mackintosh and Higgins (2004) and others have reported various success in brewing lager beer using a combination of exogenous enzyme and raw sorghum.

<u>Exopeptidases</u>

In general, exopeptidases cleave the peptide bond proximal to the amino or carboxyl termini of the substrate (Rao et al., 1998). They are further classified as either aminopeptidase or carboxypeptidase, respectively. Aminopeptidases are intracellular enzymes that occur in a wide variety of microbial species including bacteria and fungi. They cleave at a free N terminus of the polypeptide chain and liberate a single amino acid residue. On the other hand, carboxypeptidases act on the C terminal end of the polypeptide chain and can liberate a single amino acid or dipeptides and operate effectively in the pH range 4 to 6.

<u>Endopeptidases</u>

The endopeptidases are characterized by their preferential action at the peptide bond in the inner regions of polypeptide chain away from the N and C termini of the substrate (Rao et al., 1998). They are subdivided into subgroups such as serine protease, cystein protease, aspartic protease, metalloprotease and endopetidase of unknown origin.

Serine proteases characterized by their substrate specificity, prefer to hydrolyze a peptide bond which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. Serine proteases are produced by several bacteria such as *Arthrobacter*, *Streptomyces* and *Flavobacterium* spp. The optimum pH for alkaline serine proteases is around pH 10. Aspartic proteases, also known as aspartic acid proteases, depend on the aspartic acid residues for their catalytic activity hence its name (aspartic acid protease).. Similarly, metalloproteases class of enzymes are named based on their preferential requirement for divalent metal ions for their activity. The last but not the least in this group is the cysteine protease. Generally cysteine protease is active only in presence of a reducing agent such as HCN or cysteine.

2.7   Conclusions

Malted sorghum has been successfully used for brewing clear beer, especially at laboratory level. However, insufficient proteolysis which affects production of FAN due to the vitreous nature of the sorghum endosperm structure has been a major challenge at the commercial level. Hence, attempts to use exogenous proteolytic enzymes to hydrolyze sorghum malt protein have been directed towards improving production of adequate FAN to support rapid yeast growth during fermentation. However, this option too has been a challenge particularly

to identify effective proteases and their optimal mashing conditions. Sorghum malt brewing is still prospective in tropical countries despite these challenges.

3. HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Exogenous proteases will improve protein hydrolysis during brewing with sorghum malt due to augmentation of the action of endogenous proteases on the sorghum protein matrix enveloping the starch granules in malted sorghum.

Increased protein hydrolysis caused by exogenous proteases will produce more peptides and free amino acids (FAN) in the wort.

As a result of hydrolysis of the endosperm matrix protein, the quantity of fermentable wort will be increased due to increased freed starch granules being available for hydrolysis by amylases during mashing.

FAN production will be greater with high protein digestibility sorghum compared to the normal protein digestibility sorghum because the protein bodies of high protein digestibility sorghum are invaginated. Therefore the gamma-kafirin will not inhibit digestion of the protein bodies (Oria, Hamaker, Axtell and Huang., 2000).

FAN production will be greater in the malt from high protein digestibility sorghum compared to the malt from normal protein digestibility sorghum because the protein will be more available for hydrolysis by the malt endogenous and exogenous protease enzymes.

The protein digestibility of sorghum malt from high protein digestibility sorghum will be higher than that in the malt of normal protein (control) due to the protein bodies being invaginated (Oria et al., 2000) and therefore the gamma- kafirin will not inhibit digestion.

3.2 Objectives

To determine the effect of different exogenous proteases on FAN and wort extract when mashing with malted sorghum.

To determine the protein digestibilities of malts produced from normal and high protein digestibility sorghum cultivars.

To determine the effect of commercial proteolytic enzymes on FAN production during mashing with malt produced from normal and high protein digestibility sorghum cultivars.

To determine the effect of commercial proteolytic enzymes on FAN production during mashing with grain of normal and high protein digestibility sorghum cultivars.

4.   RESEARCH

The following sub-sections are written essentially in the style of scientific papers, for Cereal Chemistry. Flow diagrams of the experimental design used in this research are shown in Figures 4.1.1 and Fig 4.1.2

```
┌─────────────────────┐      ┌──────────────────────┐      ┌─────────────────────┐
│ Whole sorghum grain │ ───▶ │ Steeping in 25°C     │ ───▶ │ Malting at 25°C     │
│ (tannin free)       │      │ water                │      │                     │
└─────────────────────┘      └──────────────────────┘      └─────────────────────┘
                                                                      │
                             ┌──────────────────────┐      ┌─────────────────────┐
                             │        Malt          │ ◀─── │ Kilning at 50°C     │
                             └──────────────────────┘      └─────────────────────┘
                                        │
                             ┌──────────────────────┐
                             │  Removal of shoots   │
                             └──────────────────────┘
                                        │
                             ┌──────────────────────┐
                             │ Sorghum malt hammer  │
                             │ milled               │
                             └──────────────────────┘
```

Mashing regime 1 — Different commercial proteases — Mash at 55°C 45 minutes — Analysis • FAN — Cool to room temperature by adding tap water making up to 250 g initial weight to replace water loss due to evaporation — Analyses • Hot wort extract • Starch test • FAN

Mashing regime 2 — Flavourzyme + Ceramix+ PMB — Mash at 55°C 25 minutes — Increase to 90°C hold for 45 minutes — Cool to room temperature by adding tap water making up to 250 g initial weight to replace water loss due to evaporation — Analyses • SDS-PAGE • Amino acids • TEM

Mashing regime 3 — Flavourzyme + Ceramix+ PMB — Mash at 40°C for 420 minutes — Increase to 90°C hold for 45 minutes — Cool to 60°C keep for 90 minutes — Cool to room temperature by adding tap water making up to 250 g initial weight to replace water loss due to evaporation — Freeze dry

Figure 4.1.1: Flow chart of experimental design for sorghum malting, then mashing with commercial proteolytic enzymes (section 4.1).

SORGHUM CULTIVARS

- High protein digestibility parent
- High protein digestibility progeny
- Normal protein digestibility sorghum
- Normal protein digestibility (control)

Cleaning		Steeping in $25^{o}C$ water

Sorghum grain		Malting at $25^{0}C$

Malt

Kilning at $50^{0}C$

Sorghum grain or malt
(hammer milling)

Removal of shoots

Milled unmalted
sorghum

Flavourzyme or
Bioprotease P.conc

Milled malted
sorghum

Mash at $55^{0}C$

45 minutes

Mash at $55^{0}C$

45 minutes

Analysis

- Protein digestibility

Analysis

- FAN

Analysis

- Protein digestibility

Figure 4.1.2: Flow chart of experimental design for grains, malting and mashing with commercial proteolytic enzymes (section 4.2).

4.1 Effects of different commercial proteases on free amino nitrogen production and their impact on hot water extract when mashing with malted sorghum

ABSTRACT

The effects of ten exogenous proteases and mashing conditions on free amino nitrogen (FAN) production and wort properties were investigated. Mashing was done at 55^0C for 45 min and also with long protein rest at 40^oC (low temperature) for 420 min. FAN as determined by the ninhydrin method showed that addition of exogenous proteases improved total wort FAN levels to a maximum of 182 mg FAN/100 g malt compared to 96 mg FAN/100 g malt in control. Low temperature mashing with Flavourzyme plus potassium metabisulphite (PMB) increased FAN by twice as much in a treatment with PMB compared to the one without PMB. Hot wort extract increased by 8% more in the treatment with exogenous enzyme plus PMB compared to the one without PMB. SDS-PAGE under reducing conditions of the sorghum protein after mashing showed an intense band of monomeric kafirins whereas no monomeric kafirins were seen under non-reducing conditions, possibly suggesting that residual undigested proteins in the form of polymeric and oligomeric kafirin were present. When transmission electron microscopy was performed, it appeared that slight hydrolyses of endosperm protein had occurred as evidenced by partial degradation of protein bodies, mostly in the treatment where Flavourzyme was used. Addition of exogenous proteases plus PMB in conjunction with low temperature mashing for a long period can be used to improve wort FAN somewhat.

4.1.1 INTRODUCTION

Malts are defined as grains that have been steeped, germinated and dried under controlled conditions (Briggs et al., 2004). Traditionally, barley has been the grain of choice for producing malt in lager beer brewing. However, in tropical countries barley does not grow successfully. Thus, lager beer production from sorghum grain or malt is an economically viable option. According to Owuama (1997), the potential of sorghum as an alternative substrate for lager beer brewing was recognized over five decades ago. Success in replacing barley malt using sorghum malt in lager beer brewing has been reported in several parts of Africa (Agu and Palmer, 1998; Owuama, 1997).

When brewing with sorghum, insufficient proteolysis due to the corneous nature of the endosperm sorghum grain (Ogbonna et al., 2004) and starch granules being embedded in the endosperm protein matrix Klopfenstein and Hoseney (1995), negatively effects production of free amino nitrogen (FAN) and extract levels. FAN is comprised of free amino acids and short chain peptides (Taylor and Boyd, 1986). It is an important nutrient for yeast growth during fermentation. Therefore, better hydrolysis of the grain protein matrix should improve levels of FAN and hot water extract (Ogbonna et al., 2004). The objectives of this work were to determine the effect of different exogenous proteases on FAN and wort extract when mashing with malted sorghum.

4.1.2 MATERIALS AND METHODS

4.1.2.1 Materials

Sorghum malt

A white tan plant sorghum cultivar, NK8828, with 95% germinative energy (GE) and 32.6 g thousand kernel weight was malted using the method described by Taylor et al. (2005). Malt was dried at 50^0C, after which the external roots and shoots were removed by putting whole malt grains in a coarse mesh nylon bag and rubbing so that roots and shoots were broken off and fell through the mesh. The sorghum malt was then milled using a laboratory scale hammer mill type (Falling Number AB, Huddinge, Sweden) fitted with a 1.6 mm opening screen. The malt flour was stored in Ziploc type polyethylene bags and stored at approximately 6°C until required.

Commercial enzymes used in the study

In this study different proteolytic enzymes listed below, donated by Novozymes SA (Pty) Ltd (Benmore, Johannesburg, South Africa) and Kerry Biosciences (Johannesburg, South Africa) were studied.

Novozymes enzymes*

1. Flavourzyme 500 MG. An aminopeptidase, from *Aspergillus oryzae,* granular in form with an activity of 500 LAPU/g, information on optimum pH and temperature was not provided.

2. Neutrease 1.5 MG. A neutral protease from *Bacillus amyloliquefaciens,* granular in form with an activity of 1.5 AU/g, operates at optima of pH 5.5-7.5 and temperature 45-$55^{o}C$.

3. NS26001. A mixture of exopetidase and endopeptidase, granular in form. Details of the source, activity, optimum pH and temperature were not provided.

4. Papain. The source is papaya, liquid in form, with an activity of 6100 NFu/ mg^{3}, optima of pH 4.0-5.0, temperature 55-$60^{o}C$.

5. NS26009. An exo- and endoprotease, liquid in form. Details of the source, activity, optimum pH and temperature were not provided.

6. NS26023. An acid protease is liquid in form. Details of the source, activity, optimum pH and temperature were not provided.

7. Alcalase. An alkaline protease, from *Bacillus* source, granular in form with an activity of 2.5 AU/g, optimum pH 8.5 and temperature $50^{o}C$.

8. NS26068. A protease with α-amylase, glucanases and xylanase. Granular in form. Details of source, activity, optimal pH and temperature were not provided.

9. Ceramix plus MG. Granular, from *Bacillus spp, Bacillus stearothermophillus and Humicola spp.* A mixture of cellulases, α-amylase, β-glucanases, neutral protease, xylanase and pentosanases. Granular in form with activity of 115 KNU_{B}/g. Information on optimal of pH and temperature were not provided.

Kerry-Biosciences enzymes*

1. Bioprotease P.conc. A protease from *Aspergillus oryzae*. Granular in form with an activity 400,000 HUT u/g, optima of pH 4.0-7.0 and temperature $55^{o}C$.

2. Bioprotease FV. A neutral / acidic protease from Aspergillus oryzae. Granular in form with an activity 1000 LAP/u/g, optima of pH 5.0-7.0 and temperature 45-$50^{o}C$.

3. Bioptotease NL 100. From *Aspergillus subtillis.* Granular in form with an activity 100,000 NPU/ml, optimal of pH 6.0-8.0 and temperature 55-60°C.

4. Bioprotease A conc. An acidic protease from *Aspergillus niger.* Granular in form with an activity 570,000 U/g, optimal of pH 3.0-5.0 and temperature 50-60°C.

----

* HUT = Haemoglobin units on a tyrosine basis KNU = Kilo Novo units, α-amylase units; LAPU = Leucine amino peptidase units; AU = Anson units; NFU = NF papain units

4.1.2.2 Methods

Three different mashing regimes were investigated as shown on the experimental design (Figure 4.1.1).

Mashing regime (1)

An enzyme to malt flour ratio of 1: 100 was prepared and mashed as described below. Ten grams of milled malt was weighed into a 250 ml pre-weighed Erlenmeyer flask. Thirteen ml of tap water pre-heated to approximately 55°C and 1 ml calcium chloride solution (200 ppm) were added and the flask mixed thoroughly. The temperature of the mixture was kept at 55°C in a shaking water bath. One ml of an enzyme solution prepared to give a ratio of 1: 100 (enzyme to flour) was added. The contents were let to mash at temperature for 45 min. At the end of mashing, one ml was taken in duplicate and diluted to 500 ml. Approximately 10 ml was removed from the flask and centrifuged at 5,000 g for 10 min. Two ml clear supernatant was subjected to the European Brewery Convention ninhydrin assay, method 8.8.1 (European Brewery Convention, 1987).

Mashing regime (2)

Mashing was carried out in a BRF mashing bath (Brewing Research Foundation, Nutfield, UK). Beakers were weighed on a top pan balance. One hundred grams malt flour was weighed into a mashing beaker and mixed with 150 ml tap water at a mashing in temperature of 55^{0}C. Calcium chloride at 200 ppm and protease Flavourzyme at initial dosage of 1% (w/w) enzymes to the malt flour and 275 mg of Ceramix ® (a cocktail of enzyme containing heat-stable bacterial alpha-amylase, neutral proteases, beta-glucanases, xylanases and cellulases) were added. In one treatment potassium metabisulphite (PMB) 12.5 mg (equiv. to 125 g/ton sorghum) was added. Mashing was carried out for 25 minutes at 55°C. Temperature was increased to 90°C at 1°C over 35 min period, held for 45 min.

Beakers were removed and cooled to room temperature with stirring in sink of room temperature water. The beakers were weighed and the weight made up to 250 g with tap water to replace the water lost due to evaporation. Contents were transferred to centrifuge tubes and centrifuged at 14,000 g for 10 minutes. Clear supernatant was taken for analysis of hot water extract and FAN, and for starch test.

Low temperature mashing regime (3)

Milled malt was mashed using the BRF mashing bath. Malt flour (100 g) was mixed with 150 ml tap water at a mashing in temperature of 40^0C. Calcium chloride at 200 ppm and a proteolytic enzyme Flavourzyme at an initial dosage of 1% (w/w) of enzyme to malt flour were added. Figure 4.1.3 shows this particular mashing profile.
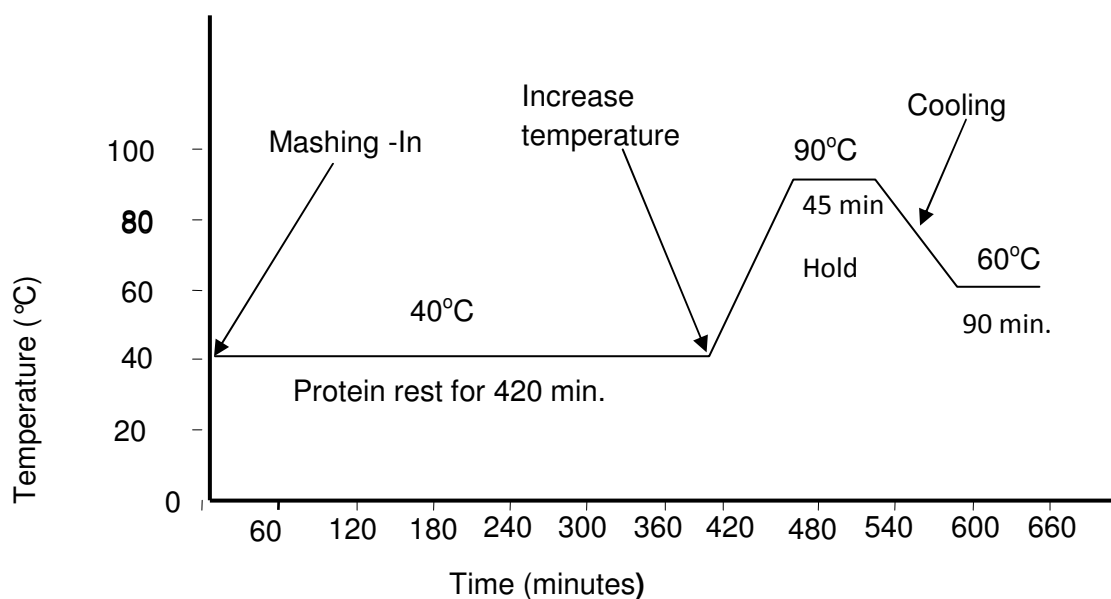
Figure 4.1.3: Mashing profile for sorghum malt

Protein rest was carried out at 40°C for 420 min followed by an increase in temperature to 90^0C in 35 min. The temperature was then held at 90^0C for 45 min to disrupt starch granules, after which the mash was cooled to 60°C. Then Ceramix® was added and mashed for 90 min. At the end of the mashing period cold water equivalent to water loss due to evaporation was added (i.e. making up to 250 g). The other treatment (control)

contained 100 g sorghum malt flour without Flavourzyme. Calcium at a dosage of 30 mg (200 ppm) of chloride and potassium metabisulphite (PMB) at a dosage of 12.5 mg equivalent to 125g /ton sorghum was added. Mashing was performed as in (Figure 4.1.3).

4.1.2.3   Analyses

Germinative Energy (GE)

Germinative energy, a measure of the percentage of grains which can likely germinate during malting, was performed using the method of Dewar, Taylor and Joustra (1995).

Free amino nitrogen (FAN)

One ml samples were taken at the beginning and at the end of the mashing regimes. Samples were diluted to a 500 ml flask. Ten ml was removed from the diluted samples and centrifuged at 1,900 g for ten minutes. FAN was determined by the European Brewery Convention ninhydrin assay, method 8.8.1 (European Brewery Convention, 1987) using glycine as a reference amino acid.

Hot water extract

Hot water extract was measured by the ASBC Beer 2 method (American Society of Brewing Chemists (1976). In this method, the quantity of malt as solubilised was measured by determining the specific gravity of clear wort using a Reischauer pycnometer. ^{O}Brix of the wort and refractive index (RI) were determined using a refractometer. Wort extract (^{O}Plato) was calculated using the DeClerk, 1957) conversion formula:

Extract (^{o}P) = (-463.37) + (668.72 * SG) – (205.35 *SG^2)

Where: SG = specific gravity of the wort sample.

Starch test

Qualitative analysis of starch was done on hot wort extract and spent grains using the iodine colour complex reaction. The principle in this test is that iodine forms a complex with the starch to form a blue black-colour, indicating the presence of starch.

Amino acid analysis

Amino acids were determined using the PICO.TAG-Method (Bidlingmeyer et al., 1984). Wort samples for amino acid analysis were freeze dried. The samples were subjected to reversed phase chromatography with pre-column derivatisation using a Waters high performance liquid chromatograph model 440 (Waters, Milford, USA). The column used was a PICO.TAG column for hydrolysate amino acid analysis, 150 x 3.9 mm internal diameter.

Transmission electron microscopy (TEM).

Transmission electron microscopy (TEM) was performed as described by Taylor, Schussler and Liebenberg (1984b), with some modifications. The samples were infused with glutaraldehyde 2.5 % (v/v) in 0.1 M sodium phosphate buffer pH 7.4 and left to stand for one hour. The specimens were then rinsed in phosphate buffer three times with a 15 minutes interval each. Osmium tetroxide was added to the specimens to fix lipid and left for 1 hour to stand. This was followed by rinsing in phosphate buffer three times. The specimens were then dehydrated in a series of alcohols (50%, 70% and 90% ethanol). A sequence of Quetol epoxy resins was added and 33%, 66%, pure Quetol epoxy resin (100%) for 18 hour and later pure Quetol resin for 4 hours to fill in spaces originally occupied by water and ethanol. After embedding in Epon-Aradite, the specimens were sectioned using an ultramicrotome. Specimens were then double-stained with uranyl acetate and lead acetate. After sectioning, the specimens were examined using a Philips EM301 TEM (Eindhoven, Netherlands).

Sodium dodecyl sulphate-polyacrylamides gel electrophoresis (SDS-PAGE)

A vertical electrophoresis system, using (protean II xi Cell, Bio-Rad Laboratory, Hercules, CA, USA) equipment was used for SDS-PAGE under non-reducing conditions. Protein samples were prepared using a method similar to that used by Byaruhanga et al. (2005), with modification on loading pattern. A Novex® Tris-Acetate Mini gradient gel (4-12%) acrylamide, of length 140 mm and 1.5 mm thick was used for separating proteins. Samples with varying weights were dissolved in (0.125% (w/w) Tris/HCl, 20% (v/v) glycerol, 2% (w/v) SDS, and 5% (w/v) bromophenol blue. For experiments under reducing conditions 0.1% (v/v) 2-mercaptoethanol was added to the sample buffer. Two glass beads were added to facilitate mixing. Samples were vortexed mixed and placed in boiling water bath for 15 minutes. Every 5 minutes throughout the period, samples were mixed by vortexing before replacing in the boiling water bath. Samples without mercaptoethanol were vigorously mixed for a further 15 minutes to ensure complete protein dissolution. Glass beads were then

removed before centrifuging at 6800 g for 10 minutes. Molecular weight marker solution ready to use, low range (Invitrogen Corporation, Mowbray, South Africa) consisting of 12 proteins in the range of M_r 2.5–200 kDa was used. For molecular marker standard, 5 µL was loaded without diluting. Samples were loaded in tracks of 15 µL with 15 µg protein for constant protein, while other treatments were loaded according to proportion of insoluble protein at the end of mashing. The electrophoresis system was set at constant voltage of 200 V and current at 13 mA per gel for 80 minutes. Proteins were stained with 0.03% (w/v) Coomassie Brilliant Blue R 250 in 7% (v/v) acetic acid and 20% (v/v) methanol and 3.2% trichloroacetic acid (TCA). For destaining, 4% acetic acid and 29% (v/v) methanol and 3% TCA was used. After destaining, gels were scanned on a flat bed scanner.

Statistical analysis

All experiments were replicated at least once. One way analysis of variance with the least significant difference test was performed to ascertain whether there were any significant differences amongst the treatment means.

4.1.3 RESULTS AND DISCUSSION

Table 4.1.1 shows that of the Novozymes proteases, Flavourzyme and NS-26009 increased FAN the most, by 43 and 40%, respectively. Neutrase produced the least FAN, a 16.5% increase.

Table 4.1.1: Effect of various exogenous proteases supplied by Novozymes on FAN production when mashing with sorghum malt for 45 min at 55^0C

Enzyme	Wort FAN (mg/100 g malt)	FAN produced by exogenous enzyme (mg/100 g malt)	FAN increase (%)
Flavourzyme	138.3[e] (1.4)	41.7	43.2
NS-26009	135.5[e] (1.0)	38.9	40.3
Papain	120.6[d] (2.1)	24.0	24.8
Alcalase	117.3[c] (3.6)	20.7	21.4
NS-26001	113.0[b] (0.5)	16.4	17.0
Neutrase	112.5[b] (2.1)	15.9	16.5
Control (endogenous protease)	96.6[a] (1.0)		

All results are means of two experiments with standard deviation in brackets. Means with different letter superscripts within the column differ significantly ($p < 0.05$)

The different exogenous proteases produced different levels of FAN. In all cases the minimum level of FAN was lower than that required for yeast growth and effective fermentation as reported by Taylor, Boyd, and Pickerell (1985). The authors, in a study of the role of FAN in the brewing of sorghum beer, established that a level of FAN of approximately 80 and 100 mg FAN /L was able to produce a higher level of alcohol, whereas poorer yeast growth in worts containing 40-60 mg FAN /L was observed, and resulted in beers with low alcohol content. According to Beckerich and Denault (1987), it is generally accepted that 150 mg/L of FAN is required in wort for normal fermentation. To achieve the levels of FAN shown in Table 4.1.1 very high levels of enzymes were used. In fact, even

after excluding FAN contributed by endogenous protease which could have been generated during the malting process, less than one third of the proposed minimum FAN was produced by the most effective proteases (Flavourzyme and NS-26009).

The findings of this study appear to agree with work done by other workers (Goode et al., 2003; Bajomo and Young, 1994; Macfadden, 1989), on optimization of mashing of unmalted sorghum with commercial enzymes. They pointed out that effective proteolysis can be achieved when high levels of commercial enzymes are used but with inevitably increased level of production cost. Therefore, they proposed that a balance of levels of enzyme dosage must be established to maximize the output while keeping the cost of production at a minimum.

Table 4.1.2: Effect of exogenous proteases supplied by Kerry-Biosciences on FAN production when mashing with sorghum malt for 45 min at $55^{0}C$

Enzyme	Wort FAN (mg/100 g malt)	FAN produced by exogenous enzyme (mg/100 g malt)	FAN increase (%)
Bioprotease P. Conc	181.8^{d} (4.1)	85.3	85.0
Bioprotease NL100	141.1^{c} (3.3)	44.6	46.2
Bioprotease FV	137.6^{c} (2.6)	41.1	42.6
Bioprotease A conc	120.7^{b} (2.6)	24.2	25.1
Control (endogenous protease)	96.5^{a} (1.3)		

All results are means of two experiments with standard deviation in brackets. Means with different letter superscripts within the column differ significantly ($p<0.05$)

Table 4.1.2 shows that addition of the exogenous proteases from Kerry-Biosciences resulted in substantial FAN improvement. Bioprotease P conc. increased FAN by approximately 85%. The least increase in FAN production was with Bioprotease A conc., which increased FAN by approximately 25%. Bioprotease P Conc produced twice as much FAN compared to Flavourzyme and NS-26009. This could possibly be due to high concentration of the enzyme compared to the other proteases used in this study.

Tables 4.1.1 and 4.1.2 clearly show that different proteolytic enzymes produced different amounts of FAN, indicating that the degree of proteolysis caused by each enzyme varied enormously. Agu and Palmer (1998) found that a neutral protease from *Bacillus subtilis* was more active in proteolysis than an acid protease from *Aspergillus niger*. The neutral protease produced more α-amino nitrogen, peptides and soluble proteins than the acid protease. Similarly, Goode et al. (2003) in a study of optimization of mashing conditions of unmalted sorghum with Bioprotease P.Conc and Bioprotease N-100 L reported that activity of the enzyme, temperature and pH were critical for efficient use of the enzymes.

In this research, pH was not regulated and temperature was fixed at 55^{0}C. As such, it is most likely that these conditions together with the difference in activities of the different proteolytic enzyme preparation used were the causes of the considerable variation in FAN production.

The type of protease determines the level of FAN in wort. According to Rao et al. (1998), proteases are subdivided into two major groups: exopeptidases and endopeptidases. Furthermore, exopeptidases, depending on their site of action, at either the N or C terminus, are classified as either amino-or carboxypeptidases, respectively. Aminopeptidases act at the free N-terminus of the polypeptide chain and liberate a single amino acid, a dipeptide, or a tripeptide, whereas carboxypeptidases act at the C-terminal of the polypeptide chain and liberate a single amino acid or a dipeptide (Hamada, 2000; Rao et al, 1998). On the other hand, endopeptidases are basically characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N or the C termini. The differences in levels of FAN produced by the different proteolytic enzymes in this research could additionally have been as a result of differences in proportions of exopeptidase versus the endopetidase components in the enzyme preparation used.

According to Hamada (2000) characterized the functional properties of rice bran proteins modified by Flavourzyme (a mixture of endopeptidase and exopeptidase) and Alcalase (an endopeptidase). They found that peptides with molecular weights smaller than 1 kDa increased more than 2-fold with Flavourzyme compared to Alcalase. The reason Flavourzyme produced more of the smaller short chain peptides was because of its mixture of endopeptidase and exopeptidase components and the pH condition of pH 5, which most likely favoured its activity. In contrast, Alcalase produced less short chain peptides with molecular weights smaller than 1 kDa. These findings are similar to the results in Table 4.1.1 where Flavourzyme and Alcalase produced 43 and 21 mg FAN / 100 g malt, respectively, representing a ratio of ratio of 2 to 1.

The presence of metal ions promotes the activity of certain proteolytic enzymes (Rao et al., 1998). Metalloproteases were reported to operate optimally in the presence of Zn^{2+} ion (Rao et al., 1998). In this study, Ca^{2+} ions (from calcium chloride at a concentration of 200 ppm) was used similar to Taylor and Daiber (1988). The authors reported this level coupled with mashing at pH 4 gave a higher amount of reducing sugars. Based on the limited information provided by the suppliers (Novozymes, South Africa and Kerry Biosciences, South Africa) it was not possible to classify the enzymes as metalloproteases.

Table 4.1.3: Effects of exogenous protease (Flavourzyme) and potassium metabisulphite (PMB) on mashing with malted sorghum for 7 hours at 40°C, followed by 90°C for 45 min with added Ceramix at 60^{0}C for 90 min on wort free amino nitrogen (FAN) and hot water extract (HWE)

Treatment	Wort FAN (mg/100 g malt)	FAN Increase (%)	HWE (ºBrix)	HWE (ºBrix) Increase (%)	HWE (ºPlato)	HWE (ºPlato) Increase (%)
Malt + Ceramix only	113.2[a] (3.7)		32.4[a] (0.8)		32.5[a]	
Malt + Ceramix + Flavourzyme	126.0[b] (1.6)	10.3	34.0[b] (0.7)	4.9	35.1[b]	8.0
Malt+ Ceramix +Flavourzyme + KMS	137.6[c] (1.5)	21.6	35.3[c] (0.3)	9.0	35.1[b]	8.0

All results are means of two experiments with standard deviations in brackets. Means with different letter super scripts within the columns differ significantly ($p<0.05$)

Addition of Flavourzyme resulted in an increase of FAN of approximately 10%, whereas in the treatment which had Flavourzyme plus PMB, FAN increased by approximately 22% (Table 4.1.3). An increase of 8% in HWE was achieved with Flavourzyme only and with the Flavourzyme plus KMS treatment. Briggs et al. (2004) reported that a careful choice of enzymes and temperature programmes may improve HWE and FAN levels.

The fact that the ratio of FAN increased by twice as much in the treatment where PMB was added suggests that reducing the disulphide bonds present in sorghum protein (kafirin) improves proteases action leading to increased FAN production.

Hydrolysis of the endosperm protein matrix by endogenous and exogenous proteases should increase the fermentable wort because of increased access to starch granules by the thermal stable α-amylase present in Ceramix ®. As stated HWE increased by approx. 8% with exogenous enzymes treatment. However, unlike in FAN improvement, the role of KMS in hot water extract improvement was not significant.

According to Agu and Palmer (1998) and Goode et al. (2003) exogenous enzymes when used in sorghum mashing can bring about improvement in FAN and extract levels. This work (Table 4.1.3) agrees with the above mentioned actions.
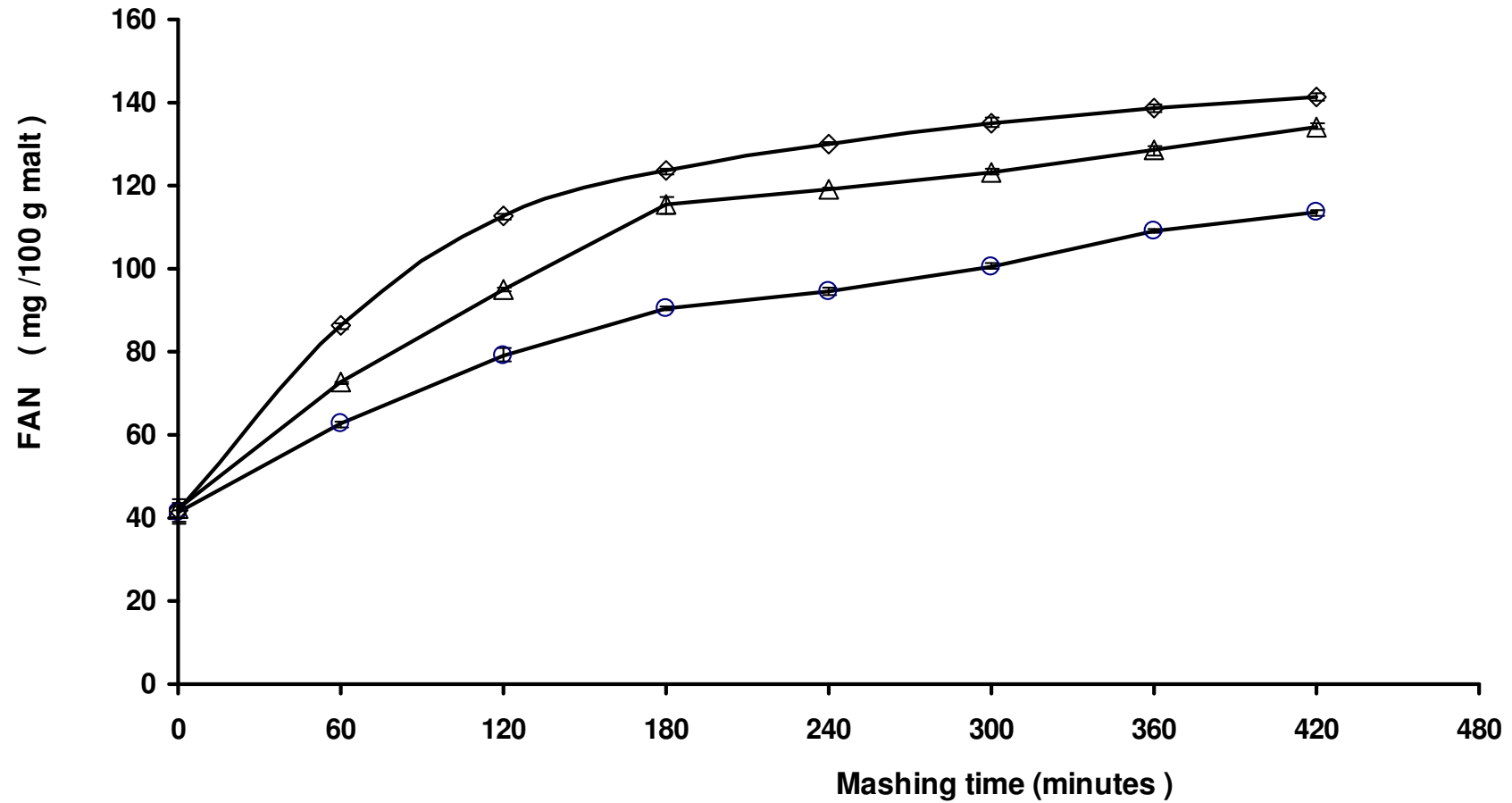
Figure 4.1.4: Effects of Flavourzyme and potassium metabisulphite (PMB) on FAN production during mashing at 40^oC for 7 hours. Circles =control (malt only), Triangles =malt plus Flavourzyme, Diamonds =malt plus Flavourzyme and PMB. Bars are $\pm$ one standard deviation. All treatments were significantly different ($p<0.05$)

When mashing over an extended time (7 hours) there was a progressive production of FAN with time (Fig. 4.1.4). By the seventh hour of mashing, the treatment with Flavourzyme and PMB was 25.5% higher in FAN than the control. The treatment with Flavourzyme increased FAN by 18.5% compared to control. The substantial increases in FAN observed when mashing over a 7 hour period indicate that the enzyme had an adequately long time to hydrolyse protein and release FAN.

Table 4.1.4: Effects of Flavourzyme and potassium metabisulphite (PMB) on the wort amino acid profile of sorghum malt protein after mashing at 40°C for 7 hours, then at 90°C for 45 minutes, and finally with Ceramix at 60^{0}C for 90 minutes (Moles percent of sorghum malt protein)

Group	Amino acid	Enzyme treatment		
		Ceramix	Ceramix + Flavourzyme	Ceramix + Flavourzyme + PMB
A	Aspartic acid	7.4	7.0	6.7
	Glutamic acid	11.3	11.5	12.0
	Serine	5.6	5.6	5.3
	Arginine	3.4	3.4	3.3
	Threonine	4.9	4.9	4.7
	Lysine	5.3	3.3	4.8
	Sub- total A	37.9%	35.7%	36.8%
B	Histidine	0.0	0.0	0.9
	Valine	8.0	8.3	7.7
	Methionine	1.1	0.0	2.3
	Isoleucine	5.0	5.2	4.9
	Leucine	12.4%	13.2	11.4
	Sub-total B	26.5%	26.7%	27.2%
C	Glycine	8.2	8.5	7.9
	Alanine	10.2	13.2	11.8
	Tyrosine	3.4	3.4	3.2
	Phenylalanine	4.5	4.3	4.1
	Sub-total C	26.3%	29.4%	27.0%
D	Proline	9.3	8.9	9.1
	Cysteine	0.0	0.0	0.0
	Sub-total D	9.3%	8.9%	9.1%

[1]Classification of amino acids is based on their rate of uptake by yeast during fermentation (Jones and Pierce, 1964).

The enzyme treatments did not produce significant effects on wort amino acid composition Table 4.1.4. The amino acid composition of FAN is important as it affects yeast nutrition and growth during fermentation. Jones and Pierce (1964) classified amino acids in A, B, C and D groups based on their rate of uptake. Group A are rapidly taken up by yeast, while group D are the slowest or not taken up at all by yeast. In this study, the amounts of amino acids produced according to Jones and Pierce (1964) classification had approx. ratios of 37: 27: 27: 9, respectively. Work done by Taylor et al. (1985) on role of FAN in the brewing of sorghum beer reported similar ratios of 51: 16: 16: 13: 4, representing A: B: C: D: NC (not classified). A large percentage of amino acids produced in this present work were those in group A, approx. 37%, similar to the findings reported by Taylor and Boyd (1986) on FAN production during sorghum beer mashing. Group A amino acids include; aspartic acid and glutamic acid, serine, arginine, threonine and lysine. Jones and Pierce (1964) described these amino acids as the most rapidly absorbed by yeast during fermentation. The fact that these results generally agree with Taylor and Boyd (1986), showing a relatively low level of proline, only approx 9%, indicate that sorghum beer mashes have a better yeast-feeding value than those of barley malts, in which proline is usually the predominant amino acid (Taylor and Boyd, 1986).

Concerning the proteins remaining after mashing, with SDS-PAGE under reducing conditions, all tracks showed a similar pattern of bands (Fig. 4.1.5). The intensities of the monomeric kafirins around 21-25 kDa (El Nour et al., 1998), were high compared to the dimers between 36-50 kDa, also similar to those reported by El Nour et al. (1998). Very weak polymeric kafirin bands appeared in the region 96-116 kDa in all treatments.

Under non-reducing conditions, the monomeric kafirins of molecular weights between 21-25 kDa did not appear in tracks 1, 2 and 3, which had Ceramix only. However, in tracks 4, 5, and 6 treatments with Flavourzme, and tracks 7 and 9 the treatments with Flavourzyme plus PMB showed some weak monomeric kafirin bands. The faint monomeric kafirins were as a result of the action of Flavourzme and possibly PMB. Probably PMB was involved in reducing the stabilising S-S bonds such that the proteases had access to polymeric kafirins and reduced them to monomeric kafirins. Most likely the appearance and intensity of the monomeric kafirins under reducing conditions was due to the reducing agent 2-mercaptoethanol's ability to reduce the inter-and intramolecular disulphide bonds that were formed in kafirin during the cooking process during brewing.

Under non-reducing conditions, oligomeric and polymeric kafirins appeared in all treatments. This indicates that possibly the S-S cross-linking reduced the hydrolysis of the kafirin polymers to smaller molecular weight monomeric kafirins. A distinct band of molecular weight of approximately 55 kDa was observed in reducing and non- reducing conditions. This band was most likely an amylase enzyme residue which could have remained bound to protein during the sorghum protein purification process using the Megazyme Total Starch Method (Megazyme., 2007). In this method (AA/AMG), in phase one, starch was partially hydrolyzed and totally solubilised by cooking in the presence of thermostable α-amylase. In phase two, the dextrins were quantitatively hydrolyzed to glucose by amyloglucosidase. Lastly, samples were centrifuged leaving proteins for SDS-PAGE analysis. Incomplete removal of amylase may have occurred at the last stage.
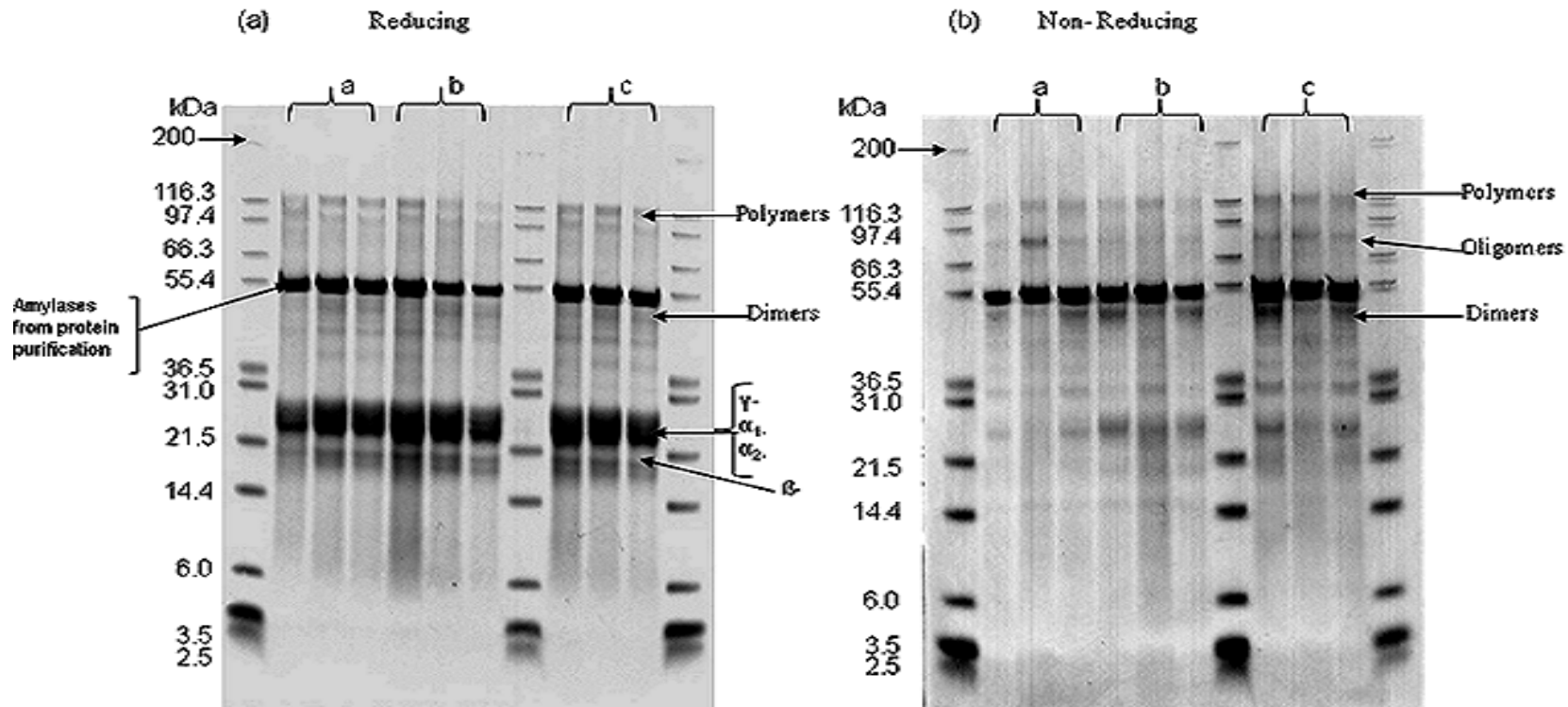
Figure 4.1.5: SDS-PAGE of malted sorghum malt proteins after mashing at low temperature (40°C) for 7 h, then at 90°C for 45 minutes and finally at 60°C for a 90 min with Ceramix. a=control. b= Flavourzyme , c= Flavourzyme plus Ceramix plus PMB, M_r = molecular weight (kDa). Tracks 1=malt at t=7 h 2=malt at t=0, 3=malt at t=7 h low protein loading, 4=malt +Flavourzyme at t=7 h, 5=malt at t=0, 6=malt +Flavourzyme at t=7 h, 7=malt +Flavourzyme +KMS at t=7h, 8=malt +Flavourzyme + PMB at t=0, and 9=malt + Flavourzyme +PMB at t=7 h at low protein loading.

TEM of samples that were mashed with Ceramix only showed varying sizes of protein bodies apparently with partially degraded peripheral edges (Fig 4.1.6 **a** and **b**). This pattern of protein body degradation is similar to that reported by Taylor et al. (1985). They observed that protein body degradation took place mainly by progressive reduction in size of the protein bodies starting from the surface of the protein body (PB). This seemed to occur in the treatment which had Ceramix only (Fig.4.1.6 **a** and **b**). The appearance of holes in the protein body centre also (Fig 4.1.6 **c** and **d**) indicates that hydrolysis of prolamin proteins took place within the organelles. Taylor et al. (1985b) observed protein bodies with holes and proposed that the holes provided access of proteinase enzymes to degrade protein bodies internally. The treatment involving Ceramix, Flavourzyme plus KMS (Fig 4.1.6 **e** and **f)**. seemed to show more of the protein body degradation compared to the other treatments. Flavourzyme plus PMB possibly complemented the activity of the endogenous protease. PMB in particular may have complemented that activity of the protease by reducing the stabilizing bonds (disulphide bonds) that are formed primarily at the periphery of the protein bodies by β- and γ-kafirin (Duodu et al., 2002; Oria et al., 1995). This would have allowed proteolytic enzymes to break the peptide bonds with the polypetides bonds and release low molecular weight peptides.
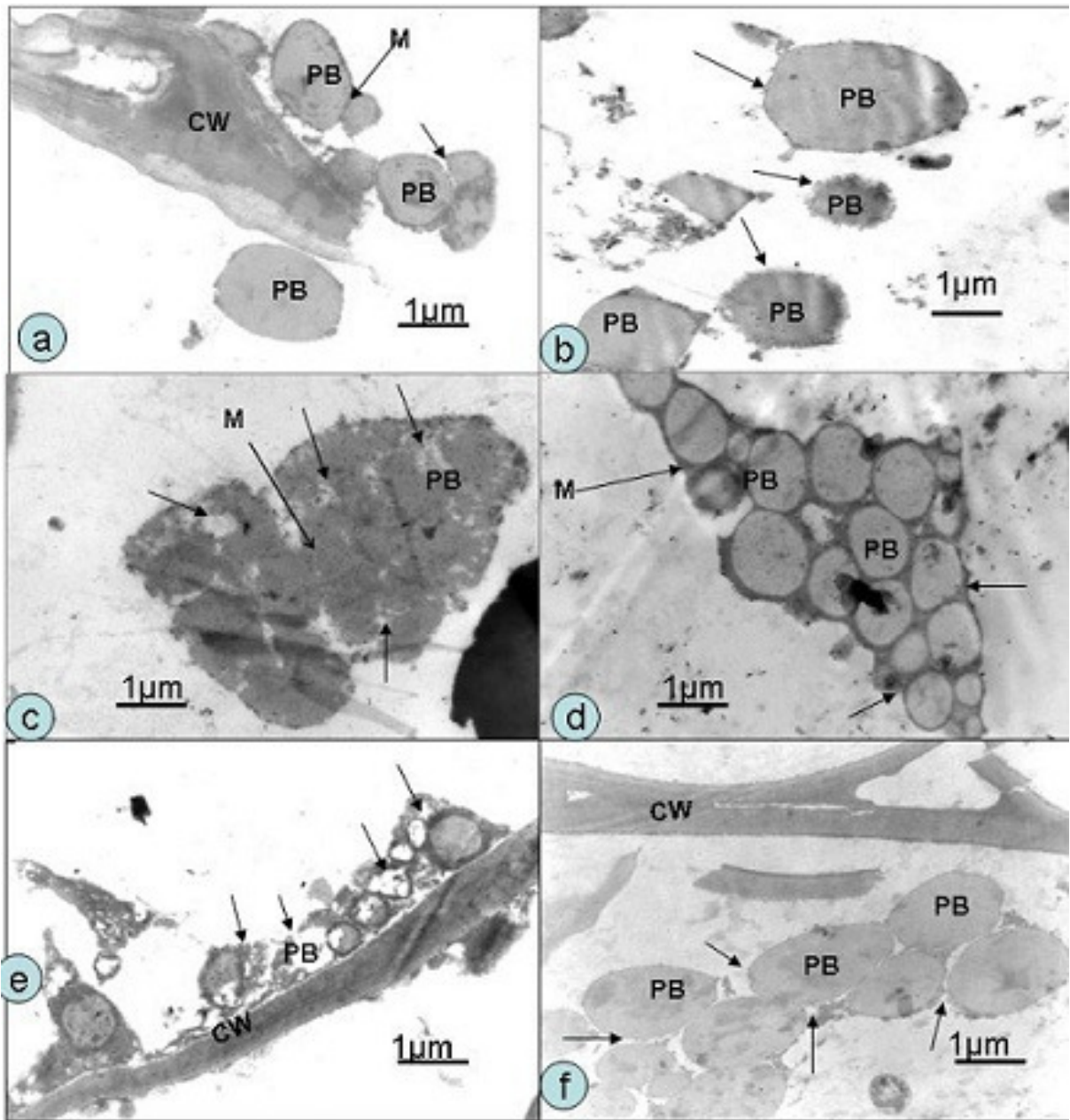
Figure 4.1.6: Effects of Flavourzyme and potassium metabisulphite (PMB) on malt protein after low temperature mashing for 7 hours at 40°C, then 90°C for 45 minutes and finally with Ceramix at 60^{0}C for 90 minutes. (a) and (b) are protein bodies from the treatment which had Ceramix only, (c) and (d) clumps of protein bodies from the treatment with Flavourzyme and Ceramix, (e) and (f) are protein bodies from the treatment with Flavourzyme and PMB plus Ceramix. PB=protein bodies, M= with solid arrow indicates position of protein matrix, CW=cell wall, solid arrows indicate position of partial degradation protein bodies

4.1.4   CONCLUSIONS

Addition of different exogenous proteolytic enzymes in sorghum malt mashing results in great variation in FAN production. The variation in FAN increase shown by different enzymes is attributed to several factors, i.e. the difference in specific activity of the enzymes, the optimum temperature and pH, which appear specific for each proteolytic enzyme. Mashing at low temperature (40^0C) and long duration is proposed for sorghum malt brewing as it provides longer contact time for the proteolytic enzymes to act on the protein substrate and the low temperature retards enzyme denaturation rate.

4.1.5 REFERENCES

Agu, R. C., and Palmer, H. G., 1996. Enzymic breakdown of endosperm proteins of sorghum at different malting temperatures. Journal of the Institute of Brewing. 66: 415-418.

Agu, R. C., and Palmer, G. H., 1998. Effect of mashing with commercial enzymes on the properties of sorghum worts. World Journal of Microbiology and Biotechnology.14: 43-48.

American Society of Brewing Chemists. 1976. Method WORT- 2. Specific gravity by Pycnometer. Methods of Analysis of the American Society of Brewing Chemists. ASBC. St Paul, MN. Pp. 2-3.

Bajomo, M.F., and Young, T. W., 1993. The properties, Composition and fermentabilities of worts made from 100% raw sorghum and commercial enzymes Journal of Institute of Brewing. 99: 153-158.

Bajomo, M. F., and Young, T. W., 1994. Fermentation of worts made from 100% raw sorghum and enzymes. Journal of the Institute of Brewing. 100: 79-84.

Beckerich, R. P., and Denault, L. J., 1987. Enzymes in the preparation of beer and fuel alcohol. In: Kruger, J.E., Lineback, D., and Stauffer, C.E. (Eds.). Enzymes and their Role in Cereal Technology. American Association of Cereal Chemists, St Paul, MN, Pp.335-354.

Bidlingmeyer, B. A., Cohen, S. A., and Tarvin, T. L., 1984. Rapid analysis of amino acids using pre-column derivatization. Journal of Chromatography. 336: 93-104.

Briggs, D. E., 1998. Malts and Malting. Blackie and Academic Professional, London. Pp. 269-685.

Briggs, D. E., Boulton, C. A., Brookes, P.A., and Stevens, R., 2004. Brewing Science and Practice. Woodhead Publishing. Cambridge. Pp. 10-606.

Byaruhanga, Y. B., Erasmus, C., Emmambux, M. N., and Taylor, J. R. N., 2005. Effect of heating cast kafirin films on their functional properties. Journal of the Science of Food and Agriculture. 87: 167-175.

DeClerck, J., 1957. A Text of Brewing. Vol. 1. Chapman and Hall, London, Pp. 424-460.

Dewar, J., Orovan, E., Taylor, J. R. N., 1997b. Effect of alkaline steeping on water uptake and malt quality in Sorghum. Journal of the Institute of Brewing. 103. 283-285.

Dewar, J., Taylor, J. R. N., and Berjak, P., 1997a. Determination of improved steeping conditions for sorghum malting. Journal of Cereal Science. 26: 129–136.

Dewar, J., Taylor, J. R. N., and Joustra, S. M. 1995. Accepted Methods of Sorghum Malting and Brewing Analyses. CSIR Bio/Chemtek, Pretoria.

Doggett, H., 1988. Sorghum. 2nd ed. Longman Scientific and Technical, London, Pp. 1-6.

Duodu, K. G., Nunes, A., Delgadillo, I., Parker, M. L., Mills, E. N. C., Belton, P S., and Taylor, J. R. N., 2002. Effect of grain structure and cooking on sorghum and maize in vitro protein digestibility. Journal of Cereal Science. 35: 161-174.

El Nour, I. N. A., Peruffo, A. D. B., and Curioni, A., 1998. Characterization of sorghum kafirins in relation to their cross-linking behaviour. Journal of Cereal Science. 28: 197–208.

European Brewery Convention. 1987. Method 8.8.1. Ninhydrin colorimetric method. (International method). Analytica-EBC, 4th ed. Braueri-und Getranke-Rundschau, Zurich, p. E 141.

Evans, D.J., and Taylor, J. R. N., 1990. Influence of cultivar and germination conditions on proteolytic activities in sorghum malt. Journal of the Institute of Brewing. 96: 399-402.

Food and Agriculture Organization (FAO). 1996. The world sorghum and millets: Facts and, trends and outlook. Viale delle Terme di Canracalla, 00100 Rome, Italy. Pp 1-27.

Goode, D. L., Halbert, C., and Arendt, E. K., 2003. Optimization of mashing conditions when mashing with unmalted sorghum and commercial enzymes. Journal of the American Society of Brewing Chemists. 61: 69-78.

Hallgren, L., 1995. Lager beers from sorghum. In: Dendy, D. A. V (Ed.). Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St Paul, MN. Pp 69-124.

Hamada, J. S., 2000. Characterization and functional properties of rice bran proteins modified by commercial exoproteases and endoproteases. Journal of Food Science: 65: 305-310.

Hough, J. S., Briggs, D. E. and Stevens, R., 1971. Malting and Brewing Science. Chapman and Hall, London. Pp. 104.

House, L. R., 1995. Sorghum and millets: History, taxonomy, and distribution. In: Dendy, D. A. V (Ed.). Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St Paul, MN. Pp . 1-10.

International Crop Research Institute for Semi –Arid Tropics (ICRISAT). 1996. The world sorghum and millets: Facts and, trends and outlook. Parancheru 502 324, Andhra Pradesh, India. Pp 1-25.

Jones , M., and Pierce, J. S., 1964. Absorption of amino acids from wort by yeasts. Journal of the Institute of Brewing. 70: 307-315.

Klopfenstein, C. F. and Hoseney, R. C., 1995. Nutritional properties of sorghum and the millets. In: Dendy, D. A. V (Ed.). Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St Paul, MN. Pp 125-168.

Lefyedi, M. L., and Taylor, J. R. N., 2006. Effect of dilute alkaline steeping on the microbial contamination, toxicity and diastatic power of sorghum malt. Journal of the Institute of Brewing. 112: 108–116.

Macfadden, D. P., 1989. Use of exogenous enzyme in sorghum brewing. In: Proceedings of the 2nd Scientific and Technical Convention, Institute of Brewing. Pp. 306.

Mackintosh, I., and Higgins, B., 2004. The development of a sorghum-based lager beer in Uganda: a model of co-operation between industry and government in the development of local ingredients for the production of quality lager beer and consequential benefits for the parties involved. Aspects of Applied Biology. 72: 235-245.

Megazyme., 2007. Total Starch Assay Procedure α-amylase method. http//www.megazyme.com/downloads/en/data/K-TSTA.pdf. Accessed : 23 May 2007.

Morrall, P., Boyd, H. K., Taylor, J. R. N., and Van der Walt, W. H. 1986. Effect of germination time, temperature and moisture on malting of sorghum. Journal of the Institute of Brewing. 92: 439-445.

Ogbonna, A. C, Obi, S. K. C., and Okolo, B. N., 2004. Optimization of proteolytic activities in malting sorghum. Process Biochemistry. 39: 713-718.

Oria, M. P., Hamaker, B. R., and Shull, J. M., 1995. In vitro protein digestibility of developing and mature sorghum in relation to α-, γ-, and β- kafirin disulphide crosslinking. Journal of Cereal Science. 22: 85-93.

Oria, M. P., Hamaker, B. R., Axtell, J. D., and Huang, C. P., 2000. A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. Proceedings of the National Academy of Sciences of the United States of America. 97: 5065–5070.

Owuama, C. I., 1997. Sorghum: a cereal with lager beer brewing potential. World Journal of Microbiology and Biotechnology. 13: 253-260.

Owuama, C. I. and Okafor, N., 1987. Studies on mashing methods for brewing with sorghum. MIRCERN Journal. 3: 243-253.

Rao, M. B, Tanskale, A. M., Ghatge, M. S., and Deshpande, V. V., 1998. Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Reviews. 62: 597-635.

Serna-Saldivar, S.O., and Rooney, L.W., 1995. Structure and chemistry of sorghum and millets. In: Dendy, D. A. V (Ed.). Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St Paul, MN. Pp 69-124.

Sivaramakrishnan, S., Gangadharan, D., Madhavan, N., Soccol. R. C. and Pandey. A., 2006. α-Amylases from microbial sources- an overview on recent developments in food. Food Technol. Biotechnol. 44:173-184.

Taylor, J. R. N., 1983. Effect of malting on the protein and free amino nitrogen composition of sorghum. Journal of the Science of Food and Agriculture. 34: 885-92.

Taylor, J. R. N., 1992. Mashing with malted grain sorghum. Journal of the American Society of Brewing Chemists. 50: 13-18.

Taylor, J. R. N., 1993. Sorghum malt: Its current use and future potential for brewing in Southern Africa. In: Taylor J. R. N., Randall P.G., and Viljoen J.H. (Eds.). Cereal Science and Technology, Impact on a Changing Africa. CSIR. Pretoria, South Africa. Pp. 413.

Talyor J. R. N 2007. Revised sorghum grain structure. In Taylor, J. R. N., and Belton, P. S. 2002. Sorghum. In: Belton, P. S. and Taylor, J. R. N. (Eds.) Pseudocereals and Less Common Cereals, Grain Properties and Utilization Potential. Springer-Verlag, Berlin. pp. 25-81.

Taylor, J. R. N., and Daiber, K. H., 1988. Effect of calcium ions in sorghum beer mashing. Journal of the institute of Brewing. 94: 68-70.

Taylor, J. R. N., and Evans, D. J., 1989. Action of sorghum proteinase on the protein bodies of sorghum starchy endosperm. Journal of Experimental Botany. 40:763-768.

Taylor, J. R. N., and Schüssler, L., 1986. The protein compositions of the different anatomical parts of sorghum grain. Journal of Cereal Science. 4: 361-369.

Taylor, J. R. N., and Boyd, H. K., 1986. Free α-amino nitrogen production in sorghum beer mashing. Journal of Science Food and Agriculture. 37: 1109-1117

Taylor, J. R. N., Boyd, H. K., and Pickerell, A. T. W., 1985. The role of free alpha amino nitrogen (FAN) in the brewing of sorghum beer. Food Review. August/September: 82-83.

Taylor J. R. N., Dewar, J., Joustra, S. J., 2005. Sorghum Malting Technology Course and Methods of Sorghum Grain and Malt Quality Analysis. University of Pretoria. Pretoria. Pp 30-66.

Taylor, J. R .N., and Robbins, D. J., 1993. Factors influencing beta-amylase activity in sorghum malt. Journal of the Institute of Brewing. 99: 413-416.

Taylor, J. R. N., Novellie, L., and Liebenberg, N. v. d. W., 1984a.Sorghum protein body composition and ultrastructure. Cereal Chemistry. 61: 69-73.

Taylor, J. R. N., Novellie, L., and Liebenberg, N. v. d. W., 1985b. Protein body degradation in the starchy endosperm of germinating sorghum. Journal of Experimental Botany. 36: 1287-1295.

Taylor, J. R. N., Schober, T. J., and Bean, S. R., 2006. Novel food and non-food uses for sorghum and millets. Journal of Cereal Science. 44: 252-271.

Taylor, J. R. N.,Schüssler., and Liebenberg, N. V. D. W., 1984b. Protein body formation in the starchy endosperm of developing *Sorghum bicolor* (L.) Moench seeds. South African Journal of Botany. 51: 35-40.

Taylor, J. R. N.,and Von Benecke, R.,1990. Development of the simple assay for the determination of β-amylase in sorghum malt. In: Taylor J. R. N. Proceedings of the Third Aviemore Conference on Malting, Distilling and Brewing, 1. Campbell (Ed.) Institute of Brewing, London. Pp. 344-347.

4.2 Effect of high digestibility sorghum cultivars on free amino nitrogen (FAN) production during mashing

ABSTRACT

High digestibility sorghum mutants have been developed. Increased protein body surface area appears responsible for their improved digestibility. The effect of these high digestibility sorghums on free amino nitrogen (FAN) production during mashing was investigated. Grain and malt of two high protein digestibility and two normal sorghums were mashed at 55^0C for 45 min with and without the exogenous proteolytic enzymes Flavourzyme and Bioprotease P. Conc. FAN production was measured by the ninhydrin method. In general, high protein digestibility sorghum grain and malt had a higher FAN than normal sorghum control. However, the increase in FAN during mashing was the same for unmalted high protein digestibility as for the normal sorghum.   In contrast, mashing with malted high protein digestibility sorghum produced approx. 15% more FAN than respective normal sorghum controls.   However, unmalted and malted high protein digestibility sorghum produced somewhat more FAN when mashed with exogenous proteases than their respective normal controls.

4.2.1 INTRODUCTION

Sorghum is considered as a suitable alternative to barley in lager beer production, particularly in tropical countries where barley cannot grow due to unfavourable climatic conditions (Agu and Palmer, 1998a; Owuama, 1997). However, one of its major limitations is poor protein for degradation by the proteolytic enzymes to produce free amino nitrogen (FAN) (Taylor and Belton, 2002). FAN is a vital source of nitrogen for optimal yeast growth and efficient fermentation (Taylor et al., 1985). According to Duodu et al. (2003), sorghum's protein digestibility is influenced by several factors: exogenous factors such as grain organizational structure, polyphenols, phytic acid, starch and non-starch polysaccharides and endogenous factors e.g disulphide and non-disulphide crosslinking, kafirin hydrophobicity and changes in protein secondary structure. In brewing, low protein digestibility of sorghum storage proteins (kafirin) consequently affects proteolysis negatively, often causing insufficient FAN production.

High protein digestibility sorghum mutants have been developed which have a unique characteristic (Oria et al., 2000). The protein bodies are irregular in shape with numerous invaginations, a feature linked to easy accessibility of digestive enzymes to α-kafirin, the major storage protein. The increased surface area of the protein bodies appear responsible for high *in vitro* protein digestibility (Oria et al., 2000). Therefore this investigation was conducted in order to determine whether high protein digestibility sorghums will yield more wort FAN.

4.2.2 Materials and methods

4.2.2.1 Materials

Sorghum cultivars

Normal protein digestibility parent (LD parent 96GCPOB124), high digestibility parent (HD parent PI851171) and high digestibility progeny (HD progeny 04CS11278X 851171/96GCP0124) were supplied by Dr Dirk Hays, Texas A & M, University, College Station, Texas, USA. A normal control white tan plant cultivar (NK8828) from the Agricultural Research Council, Potchefstroom, South Africa was included.

Commercial proteolytic enzymes

Flavourzyme was donated by Novozymes SA (Pty) Ltd (Benmore, Johannesburg, South Africa) and Bioprotease P.Conc. by Kerry Biosciences (Johannesburg, South Africa).

4.2.2.2 Methods

Germinative Energy (GE)

Germinative energy, a measure of the percentage of grains which can likely germinate during malting, was performed using the method of Dewar, Taylor and Joustra (1995).

Malting

The cultivars were malted using the method of Taylor et al. (2005). Malt was dried at 50^0C, after which the external roots and shoots were removed as described in chapter 4.1. The sorghum malt and raw grains were milled using a laboratory scale hammer mill type (Falling Number AB, Huddinge, Sweden) fitted with a 1.6 mm opening screen. The flour was stored in Ziploc type polyethylene bags at approximately 6°C until required.

Mashing

An enzyme (Flavourzyme or Bioprotease (P. conc.) grain or malt flour ratio of 1: 100 (w/w) was prepared and mashed as described below. Ten grams of milled malt was weighed into a 250 ml pre-weighed Erlenmeyer flask. Thirteen ml of tap water pre-heated at approximately 55°C and 1 ml calcium chloride solution (200 ppm) was added and the flask mixed thoroughly. The temperature of the mixture was kept at 55°C in a shaking water bath. One ml enzyme solution prepared to give a ratio of 1: 100 (enzyme to flour) was added and then mixed. The contents were mashed at 55^{0}C for 45 min. At the end of mashing, 1 ml was taken in duplicate and diluted to 500 ml in a volumetric flask. Approximately 10 ml was removed from the flask and centrifuged at 5,000 g for 10 min. Two ml clear supernatant was subjected to the European Brewery Convention (1987) ninyhdrin assay for determination of FAN.

4.2.2.3 Methods

In vitro protein digestibility

In vitro protein digestibility of sorghum was determined using a modification of the method of Hamaker et al. (1987). Two hundred mg of each sample were weighed into 100 ml plastic tubes with caps. Each sample was analyzed in triplicate. Thirty five ml of citrate buffer (pH 2.0) containing pepsin (Sigma P-700: activity 1200 units per mg of protein) was added to each sample and shaken to ensure adequate mixing. The samples were placed in a water bath (37°C) for 2 hours. The samples were vortex mixed at 15 minutes intervals. After the 2 hour period the pepsin reaction was stopped by addition of 2 ml of 2 M NaOH. The tubes were centrifuged at 5,000 g at 4°C for 20 minutes. A firm pellet was formed at the bottom of the tube. The supernatant was carefully decanted off. Fifteen ml of distilled water was added to each sample and shaken. Samples were centrifuged and distilled water decanted. Each sample was washed with distilled water three times. The samples were placed in an oven at 100°C over night to dry. The dry material was scraped from the centrifuge tube and analysed by the Dumas combustion method. Protein content was calculated using N X 6.25.

% Protein digestibility = $\underline{(X - Y)}$ x   100

X

Where: X = Mean total protein content (mg) before digestion

Y = Mean residual protein content (mg)

Free amino nitrogen (FAN)

One ml samples were taken at the beginning and at the end of the mashing regimes. Samples were diluted to a 500 ml flask. Ten ml was removed from the diluted samples and centrifuged at 1,900 g for ten minutes. FAN was determined by the European Brewery Convention ninhydrin assay, method 8.8.1 (European Brewery Convention, 1987) using glycine as a reference amino acid.

Statistical analysis

All experiments were replicated at least once. A one way analysis of variance with the least significant difference test was performed to ascertain whether there were any significant differences amongst the treatment means.

4.2.3   RESULTS AND DISCUSSION

Table 4.2.1: Germinative energy (GE) (%) for normal and high protein digestibility sorghum cultivars

Sorghum Cultivar	48 hours germination	72 hours germination
Normal digestibility parent	70	75
High digestibility parent	92	92
High digestibility progeny	86	90
Normal digestibility control (NK 8828)	80	89

The high protein digestibility parent and progeny had the highest GE of 92 and 90%, respectively, Table 4.2.1. The lowest GE was observed in normal digestibility parent 75%. A GE > 90% is considered appropriate for malting and brewing (Hough et al., 1971) These high protein digestibility sorghum types could be suitable for malting. A lower value suggests dormancy of the grain, which could either be linked to cold storage or immaturity of the grains. Microbes also reduce the percentage germination and vigour of seedlings (Briggs et al., 2004).   The steep-out moistures for high protein digestibility parent and progeny were approximately 36 and 35%, respectively, while those for normal digestibility parent and control were 30 and 32%, respectively. The values found in this study, fall within the range of results reported by Morrall, Boyd, Taylor and Van der Walt (1986).   The authors studied the effect of germination time, temperature and moisture on malting conditions of sorghums. A steep-out moisture of sorghum ranging from 33 to 36% was reported. In the case of barley, steep-out moisture of 44 to 46% has been reported (Hough et al., 1971).
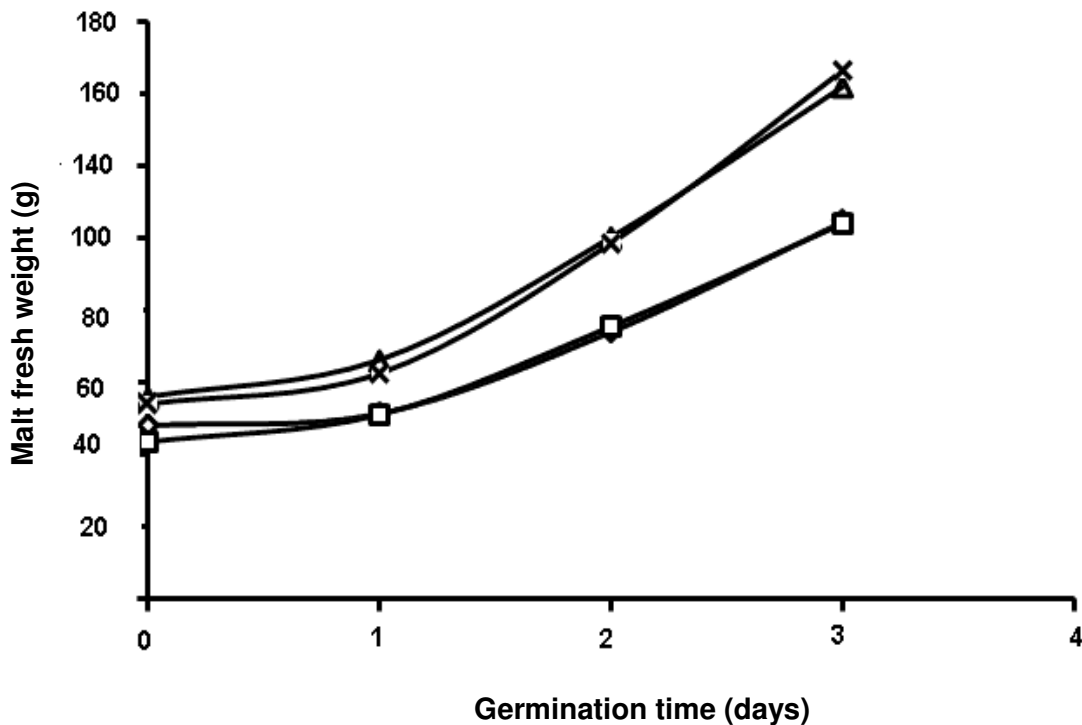
Figure 4.2.1: Effect of malting normal and high protein digestibility sorghums on malt fresh weight. Squares = normal digestibility sorghum (parent), diamonds = normal protein digestibility control (NK8828), Triangles = high digestibility parent and crosses = high digestibility progeny. Control had 32.6 g/ thousand kernel weight. The high digestibility sorghum cultivar's physical characteristics were not determined

When the cultivars were malted there was a progressive increase in malt fresh weight by all sorghum cultivars with time of germination (Fig. 4.2.1). The high digestibility parent and progeny's malt fresh weights increased by approximately 3 fold compared to the normal protein digestibility parent and the control, which increased their malt fresh weight by approximately 2 fold, respectively. In the case of high protein digestibility parent and progeny probably took up more water during germination due to their high germinative energies.

Table 4.2.2 shows that normal protein digestibility sorghum grain cultivars had the lowest protein digestibilities 51% compared to high protein digestibility sorghum cultivars which had approximately 69%. Protein digestibility of sorghum in general has been cited as low (Hamaker et al., 1986). After malting, the high protein digestibility sorghum cultivars had higher protein digestibilities (79 and 83%), but recorded the lowest increase in protein digestibility compared to the normal protein digestibility cultivars. The increase in the protein digestibility in normal protein digestibility cultivars was approximately 18% greater. The higher percentage of protein digestibility in high digestibility cultivars was probably because the protein bodies are invaginated Oria et al. (2000) and therefore the γ-kafirin did not inhibit digestion.

In a study of the effect of malting on protein digestibility of low- and high-tannin sorghum varieties, Makokha, Oniang'o, Njoroge and Kinyanjui (2002) reported that malting improved protein digestibility. In raw unmalted low-and high tannin sorghum, the protein digestibility ranged from 45.5 to 66%. When the same varieties were malted, protein digestibility ranged from 45.5 to 88.7%. The authors reported that the malting process improved protein digestibility but did not explain the mechanism that influenced protein digestibility. Bhise, Chavan and Kadam (1988) proposed that during sorghum malting, the storage proteins undergo partial hydrolysis by endogenous proteases to produce soluble proteins and free amino acids which are more susceptible to pepsin attack. The increase in protein digestibility in this study is probably as a result of the same reasoning of Bhise et al. (1988) and Taylor (1983) whom in a study of the effect of malting on the protein and free amino nitrogen reported similar findings that during malting the storage proteins (prolamins) of the grain undergo partial hydrolysis by endogenous proteases to produce soluble proteins and free amino acids. The former are more susceptible to pepsin attack.

Table 4.2.2. Effects of malting on protein digestibilities of normal and high protein digestibility sorghum cultivars

Sorghum cultivar	Grain		Malt		
	Protein (% dry basis)	Protein digestibility (%)	Protein (% dry basis)	Protein digestibility (%)	Increase in protein digestibility with malting (%)
Normal digestibility parent	13.8	51.5[a] (1.5)	12.1	67.5[a] (1.6)	31.1
High digestibility parent	10.1	65.8[b](4.1)	8.7	78.8[b] (0.6)	19.8
High digestibility progeny	10.9	73.7[b] (3.1)	9.0	82.9[b] (1.6)	12.4
Normal digestibility control (NK8828)	7.7	52.2[a] (2.3)	6.9	71.8[a] (1.0)	37.5
Mean	**10.6**	**60.8 (2.8)**	**9.2**	**75.3 (1.2)**	**25.2**

All results are means of two experiments with standard deviations in brackets. Means with different letter superscripts within the columns differ significantly ($p < 0.05$)

Table 4.2.3 shows that unmalted sorghum showed a slight increase in FAN at the end of mashing. The increase during mashing was not significantly different between the cultivars. In contrast, malted grains (Table 4.2.4) showed a substantial increase in FAN during mashing. The increase in FAN was due to the action of endogenous proteinase and peptidase enzymes on the protein reserves of the grain, similar to the findings of Taylor and Boyd (1986). The authors reported that endogenous protease development during sorghum malting usually brings about production of FAN. Malted high protein digestibility progeny produced the highest amount of FAN compared to the normal protein digestibility control, which produced the least FAN. The increase in FAN in the high digestibility progeny was more than twice that of the normal digestibility control. The FAN produced by the high protein digestibility parent and the normal protein digestibility parent was intermediate and approximately the same. The higher FAN in the high protein digestibility cultivars was probably because the protein bodies were invaginated (Oria et al., 2000). As explained, this probably resulted in the greater surface area and change in the location of γ-kafirin there by exposing the α-kafirin to digestion. Consequently, the protein bodies were more readily available for hydrolysis by endogenous proteases.

In addition to the above reason for variation in FAN, proteinase activity varies among sorghum cultivars. A study by Evans and Taylor (1990) on the influence of cultivar and germination conditions on proteolytic activities in sorghum malt reported that different cultivars exhibited different levels of proteinase activity. Other workers, Ogbonna et al. (2003), in a study of optimization of proteolytic activities in malting sorghum, demonstrated that the carboxypeptidase enzyme activities in sorghum grains were related to the variety of sorghum.

The normal digestibility control (NK8828) in this study had a lower FAN level of 57.6 mg /100 g malt compared to approximately 96.6 mg /100 g malt reported in Chapter 4.1. An explanation for the difference is based on the duration of malting. In this present work, malting was done for 3 days compared to 4 days malting reported in chapter 4.1. A study by Evans and Taylor (1990) on the influence of cultivar and germination conditions on proteolytic activity of sorghum malt, reported that unlike proteinase, carboxypeptidase activity increased substantially with time of germination. Malting at 24^0C resulted in the highest activity being obtained after 4 days germination. Therefore, it is most likely that the same interpretation could explain the variation in FAN in this study.

Table 4.2.3: Effects of mashing with unmalted grains of normal and high protein digestibility sorghum cultivars for 45 minutes at 55°C on FAN production

Sorghum cultivar	FAN at start of mashing (mg / 100 g sorghum)	FAN at end of mashing (mg/100 g sorghum)	FAN production (mg/100 g grain)	FAN increase (%)
Normal digestibility parent	10.6^a (0.8)	13.8^a (1.3)	3.2	30.2
High digestibility parent	12.1^{ab} (0.4)	16.0^a (1.7)	3.9	32.2
High digestibility progeny	15.5^b (0.7)	19.3^a (0.9)	3.8	24.5
Normal digestibility control (NK8828)	14.2^{ab} (0.7)	20.2^a (3.2)	6.0	42.3
Mean	**13.1 (0.7)**	**17.3 (1.8)**	**4.2**	**32.3**

All results are means of two experiments with standard deviations in brackects. Means with different letter superscripts within the columns differ significantly ($p < 0.05$)

Table 4.2.4: Effects of mashing with malted normal and high protein digestibility sorghum cultivars for 45 minutes at 55°C on FAN production

Sorghum cultivar	FAN at start of mashing (mg/100 g malt)	FAN at end of mashing (mg/100 g malt)	FAN produced (mg/100 g malt)	FAN increase (%)
Normal digestibility parent	73.1[b] (2.5)	83.8[b] (2.2)	10.8	14.6
High digestibility parent	83.3[c] (2.4)	96.1[c] (3.3)	12.8	15.4
High digestibility progeny	103.4[d] (2.6)	120.1[d] (2.4)	16.7	16.2
Normal digestibility control (NK8828)	50.4[a] (1.5)	57.6[a] (0.8)	7.2	14.3
Mean	**77.6 (2.3)**	**89.4 (2.2)**	**11.9**	**15.1**

All results are means of two experiments with standard deviations in brackets. Means with different letter superscripts within the columns differ significantly (p<0.05)

Addition of Flavourzyme resulted in 25% higher FAN production in high digestibility sorghum cultivars than normal digestibility sorghums cultivars (Table 4.2.5). The increase in FAN due to Flavourzyme was in the range of approx. 82 to 115% for the normal and high protein digestibility sorghums, respectively. Although the increase appears to be significant, a FAN level in the range 80 and 100 mg FAN /L reported by Taylor et al. (1985) was not achieved by Flavourzyme. The authors, in a study of the role of FAN in the brewing of sorghum beer, established that the stated level of FAN gave high level of alcohol production during fermentation. In contrast, addition of Bioprotease P.Conc. resulted in considerablly greater FAN production, 30.4% higher in high protein digestibility sorghum cultivars than in the normal digestibility sorghum cultivars (Table 4.2.6). The increase in FAN due to Bioprotease P.Conc. was in the range of approx. 132 to 259% for the normal and high digestibility cultivars, respectively. Overall, FAN production was greater in the unmalted high protein digestibility sorghum compared to the unmalted normal digestibility sorghum probably because protein bodies of the high protein digestibility cultivars are invaginated (Oria et al., 2000).

The difference in FAN produced by the two proteolytic enzymes was probably due to differences in concentration and activity of the respective proteolytic enzymes. The Bioprotease P. Conc. produced the higher degree of proteolysis. This is evidenced by the increase in FAN of about 1.6 to 2.3 times for the normal and high digestibility, respectively. This can be attributed to the high concentration of active protease present in the Bioprotease P Conc. preparation i.e. 400,000 haemoglobin units of tyrosine (HUT) u/g compared to Flavourzyme with 500 Leucine amino peptidase units (LAPU) /g. Another factor could be linked to the ratio of exopeptidase/endopetidase composition of the proteolytic enzymes. The lower the exopetidase, the less FAN likely to be produced. This is because exopeptidases such as carboxypeptidases hydrolyse bonds at the C termini of polypeptide chains to free a single amino acid (Rao et al., 1998), whereas aminopeptidases another exopeptidase type hydrolyses bonds at the free N termini of polypeptide chains to release amino acids and other smaller molecular weight products such as dipeptides or tripeptides. It is therefore vital that a commercial protease preparation for brewing should contain a larger proportion of exopeptidases in order to maximize the release of free amino acids. Of relevance is that according to Adler-Nissen (1993) reported that most commercial enzyme preparations especially those from the fungal source possess substantial amounts of carboxypeptidase enzymes.

Table 4.2.5: Effect of exogenous protease (Flavourzyme) on FAN production when mashing with unmalted normal and high protein digestibility sorghum cultivars for 45 minutes at 55°C

Sorghum cultivar	FAN at start of mashing (mg / 100 g sorghum)	FAN at end of mashing (mg /100 g sorghum)	FAN produced (mg/100 g sorghum)	FAN increase (%)
Normal digestibility parent	22.6^a (1.1)	45.0^a (2.2)	22.4	99.1
High digestibility parent	22.7^a (2.5)	51.2^b (1.3)	28.5	125.6
High digestibility progeny	24.9^a (1.7)	53.4^b (2.4)	28.5	114.5
Normal digestibility control (NK8828)	24.3^a (1.0)	44.3^a (1.0)	20.0	82.3
Mean	**23.6 (1.6)**	**48.5 (1.7)**	**24.9**	**105.4**

All results are means of two experiments with standard deviations in brackets. Means with different letter superscripts within the columns differ significantly ($p<0.05$)

Table 4.2.6: Effects of exogenous protease (Bioprotease P. Conc.) on FAN production when mashing with unmalted normal and high protein digestibility sorghum cultivars for 45 minutes at 55°C

Sorghum cultivar	FAN at start of mashing (mg / 100 g grain)	FAN at end of mashing (mg /100 g grain)	FAN produced (mg/100 g grain)	FAN increase (%)
Normal digestibility (parent)	19.1^a (1.1)	63.5^a (2.1)	44.4	232.5
High digestibility parent	22.4^b (0.5)	80.6^c (1.7)	58.2	259.8
High digestibility progeny	26.1^c (1.3)	89.9^d (1.1)	63.8	244.4
Normal digestibility control (NK8828)	29.2^d (2.1)	67.8^b (2.5)	38.6	132.2
Mean	**24.2 (1.3)**	**75.5 (1.9)**	**51.3**	**217.2**

All results are means of two experiments with standard deviations in brackets. Means with different letter superscripts within the columns differ significantly ($p < 0.05$)

Table 4.2.7 shows that during mashing malted high protein digestibility progeny produced approx.30.7% more FAN than the normal digestibility control, which produced the least. The high digestibility parent and normal digestibility parent produced comparatively the same amount of FAN. As stated, the increase in FAN due to Flavourzyme was not significantly different in all treatments. The increase in FAN in high protein digestibility cultivars was probably due to their proteins being invaginated (Oria et al., 2000) and therefore the gamma-kafirin probably did not inhibited digestion of the protein bodies by the exogenous proteolytic enzyme and endogenous proteolytic enzymes. According to Oria et al. (2000) numerous studies have suggested that it is the nature of the proteins within the protein bodies that is mostly responsible for low-protein digestibility of sorghum grain. The authors in a study involving a highly protein digestibility mutant sorghum cultivar proposed that a change in location of γ-kafirin, and exposure of α-kafirin to digestive proteases plus structural alteration of the protein bodies resulted in greater surface area compared to normal protein bodies. These factors cause the rapid digestion of sorghum α-kafirin. Hence, this probably explains the increase in FAN observed in high digestibility cultivars. Table 4.2.8 further shows that malted high digestibility progeny produced the highest amount of FAN when mashed with enzyme Bioprotease P.conc., approximately 29.0% more than normal digestibility control which produced the least. The FAN produced by the high digestibility parent and the normal digestibility parent was intermediate and approximately the same. The increase in FAN due to Bioprotease P. Conc. was not significantly different in all treatments.

Table 4.2.7: Effect of exogenous protease (Flavourzyme) on FAN production when mashing with malted normal and high protein digestibility sorghum cultivars for 45 minutes at 55°

Sorghum cultivar	FAN at start of mashing (mg / 100 g malt)	FAN at end of mashing (mg/100 g malt)	FAN produced (mg/100 g malt)	FAN increase (%)
Normal digestibility (parent)	68.3[b] (1.8)	91.9[b] (1.8)	23.6	34.6
High digestibility parent	89.3[c] (1.7)	112.7[c] (1.9)	23.4	26.2
High digestibility progeny	103.6[d] (2.0)	129.0[d] (1.2)	25.4	24.5
Normal digestibility control (NK8828)	51.9[a] (1.3)	69.5[a] (2.4)	17.6	33.9
Mean	**78.3 (1.7)**	**100.8 (1.8)**	**22.5**	**29.8**

All results are means of two experiments with standard deviations in brackets. Means with different letter superscripts within the columns differ significantly (p<0.05)

Table 4.2.8: Effects of exogenous protease (Bioprotease P. conc.) on FAN production when mashing with malted normal and high digestibility sorghum cultivars for 45 minutes at 55°C

Sorghum cultivar	FAN at start of mashing (mg / 100 g malt)	FAN at end of mashing (mg/100 g malt)	FAN produced (mg/100 g malt)	FAN increase (%)
Normal digestibility parent	82.1^b (1.8)	135.1^b (4.1)	53.0	64.6
High digestibility parent	88.5^b (1.1)	148.1^b (4.1)	59.6	67.3
High digestibility progeny	111.2^c (3.5)	174.4^c (2.6)	63.2	56.8
Normal digestibility control (NK8828)	52.7^a (0.6)	97.6^a (2.1)	44.9	85.2
Mean	**83.6 (1.8)**	**138.8 (3.2)**	**55.2**	**68.5**

All results are means of two experiments with standard deviations in brackets. Means with different letter superscript within the columns differ significantly ($p < 0.05$)

4.2.4 CONCLUSIONS

High protein digestibility sorghum grain and malts have higher protein digestibility compared to normal sorghum. The FAN production in grain and malts when high protein digestibility are mashed did not differ significantly. Addition of proteolytic enzyme to high protein digestibility sorghum mutants results into increase in FAN compared to their respective controls. However, FAN production during mashing of unmalted and malted high protein digestibility sorghum is much higher than from their respective normal sorghum controls.

4.2.5 REFERENCES

Agu, R. C., and Palmer, G. H., 1998. Effect of mashing with commercial enzymes on the properties of sorghum worts. World Journal of Microbiology and Biotechnology.14: 43-48.

Alder-Nissen, J., 1993. Proteases, In: Nagodawitha, T. W., and Reed, G. E. 3rd ed. Enzymes in Food Processing. Academic Press, San Diego, CA, Pp. 159-204.

Bhise, V. J. Chavan, J. K., and Kadam, S. S., 1988. Effect of malting on proximate composition and in vitro protein and starch digestibility of grain sorghum. Journal of Food Science and Technology. 25: 327-329.

Briggs, D. E., Boulton, C. A., Brookes, P.A., and Stevens, R., 2004. Brewing Science and Practice. Woodhead Publishing. Cambridge. Pp. 10-606.

Dewar, J., Taylor, J. R. N., and Joustra, S. M. 1995. Accepted Methods of Sorghum Malting and Brewing Analyses. CSIR Bio/Chemtek, Pretoria.

Duodu, K. G., Taylor, J. R. N., Belton, P. S., and Hamaker. B. R, 2003. Factors affecting sorghum protein digestibility. Journal of Cereal Science. 38: 117-131.

El Nour, I. N. A., Peruffo, A. D. B., and Curioni, A., 1998. Characterization of sorghum kafirins in relation to their cross-linking behaviour. Journal of Cereal Science. 28: 197–208.

European Brewery Convention. 1987. Method 8.8.1. Ninhydrin colorimetric method. (International method). Analytica-EBC, 4th ed. Braueri-und Getranke-Rundschau, Zurich, p. E 141.

Evans, D.J., and Taylor, J. R. N., 1990. Influence of cultivar and germination conditions on proteolytic activities in sorghum malt. Journal of the Institute of Brewing. 96: 399-402.

Hamaker, B. R., Kirleis, A. W., Butler, L. G., Mertz, E. T., and Axtell, J. D., 1986. Effect of cooking on the protein profiles and in vitro digestibility of sorghum and maize. Journal of Agricultural and Food Chemistry. 34: 647-649.

Hamaker, B. R., Kirleis, A. W., Butler, L. G., Axtell, J. D., and Mertz, E. T., 1987. Improving the in vitro protein digestibility of sorghum with reducing agents. Proceedings of the National Academy of Sciences of the United States of America. 84: 626-628.

Hough, J. S., Briggs, D. E. and Stevens, R., 1971. Malting and Brewing Science. Chapman and Hall, London. Pp. 104.

Makokha, A. O., Oniang'o, R., Njoroge, S. M., Kinyanjui, P. K., 2002. Effect of malting on protein digestibility of some sorghum (*Sorghum bicolor*) varieties grown in Kenya. African Journal of Food, Agriculture, Nutrition and Development. 2: 67-77.

Morrall, P., Boyd, H. K., Taylor, J. R. N., and Van der Walt, W. H. 1986. Effect of germination time, temperature and moisture on malting of sorghum. Journal of the Institute of Brewing. 92: 439-445.

Ogbonna, A. C., Obi, S. K. C, Okolo, B. N., and Odibo, F. J. C., 2003. Purification and some properties of a protease from sorghum malt variety KSV8-11. Journal of the Institute of Brewing. 109: 179-186.

Oria, M. P., Hamaker, B. R., Axtell, J. D., and Huang, C. P., 2000. A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. Proceedings of the National Academy of Sciences of the United States of America. 97: 5065–5070.

Owuama, C. I., 1997. Sorghum: a cereal with lager beer brewing potential. World Journal of Microbiology and Biotechnology. 13: 253-260.

Rao, M. B, Tanskale, A. M., Ghatge, M. S., and Deshpande, V. V., 1998. Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Reviews. 62: 597-635.

Taylor, J. R. N., 1983. Effect of malting on the protein and free amino nitrogen composition of sorghum. Journal of the Science of Food and Agriculture. 34: 885-92.

Taylor, J. R. N., and Belton, P. S. 2002. Sorghum. In: Belton, P. S. and Taylor, J. R. N. (Eds.) Pseudocereals and Less Common Cereals, Grain Properties and Utilization Potential. Springer-Verlag, Berlin. pp. 25-81.

Taylor, J. R. N., and Boyd, H. K., 1986. Free α-amino nitrogen production in sorghum beer mashing. Journal of Science Food and Agriculture. 37: 1109-1117.

Taylor, J. R.N., Boyd, H. K., and Pickerell, A. T. W., 1985. The role of free alpha amino nitrogen (FAN) in the brewing of sorghum beer. South African Food Review. August/September. 82-83.

Taylor J. R. N., Dewar, J., and Joustra, S. J., 2005. Sorghum Malting Technology Course and Methods of Sorghum Grain and Malt Quality Analysis. University of Pretoria. Pretoria. Pp 30-66.

Taylor, J. R. N., and Boyd, H. K., 1986. Free α-amino nitrogen production in sorghum beer mashing. Journal of Science Food and Agriculture. 37: 1109-1117.

Taylor, J. R .N., Boyd, H. K., and Pickerell, A. T. W., 1985. The role of free alpha amino nitrogen (FAN) in the brewing of sorghum beer. South African Food Review. August/September. 82-83.

Taylor J. R. N., Dewar, J., and Joustra, S. J., 2005. Sorghum Malting Technology Course and Methods of Sorghum Grain and Malt Quality Analysis. University of Pretoria. Pretoria. Pp 30-66.

5. GENERAL DISCUSSION

In this chapter the following will be discussed: the experimental methods applied: namely the choice of the sorghum variety, the malting process, the mashing regimes with reference to FAN production; FAN determination using ninhydrin assay, the analysis of the protein microstructure using TEM; SDS-PAGE and lastly amino acid analysis using PICO-TAG method. The efficiency of exogenous proteolytic enzymes in sorghum protein matrix degradation and the practicality of employing a long protein rest at low temperature will be evaluated. The discussion will also draw attention to the prospects of using high protein digestibility sorghums in brewing.

5.1 Methodological considerations

A white tan plant sorghum cultivar (NK8828) was selected based on the fact that it was tannin-free and contained very low levels of other phenolic pigments. Many studies to evaluate the possibility of using unmalted or malted sorghum in lager beer brewing have either focused attention on low- or tannin-free sorghum varieties. According to Palmer, Etokakpan and Igyor (1989), tannin-free sorghums are good and therefore recommended their use for lager beer production, probably for the reason that they have not been implicated with kafirin-tannin complex formation. Furthermore, NK8828 had a high germinative energy (GE) of >90%, a level considered suitable for grain intended for malting and brewing purposes (Hough et al.,1971). Since the process of malting is rather expensive and time-consuming, most brewers favour sorghums with a high germination percentage, which is an indication that good grain modification during malting would result.

During malting the grains were steeped using the procedure of Taylor et al. (2005). Sorghum (100 g) was steeped in dilute (0.2%) NaOH at 25^0C, using a steeping cycle of 3 hour in water and 1 hour air dry, for a period of 24 hours. Different steeping systems have been studied by many researchers (Owuama, 1997; Dewar et al., 1997b). The objective of steeping is to hydrate the grain (Taylor et al., 2005) and enables grains to absorb water to levels of approx 33-35% (Morrall et al.,1986) and in some cases up to 40% w/w (fresh weight basis) depending on sorghum variety (Palmer, Etokakpan and Igyor, 1989). A similar steeping regime with barley gives a range of 42-46% (Hough et al., 1971), which is an optimal moisture for enzyme activity.

Steeping time and temperatures have a significant influence on the overall malt quality particularly with respect to FAN development and diastatic power (Dewar, et al., 1997b; Taylor and Boyd, 1986; Ezeogu and Okolo, 1994; Taylor, 1992; Agu et al., 1995; Agu and Palmer, 1997). Steeping in dilute alkali reduces mould and coliforms growth to levels considered harmless and unable to pose health risk (Leyfedi et al., 2006).

Germination during malting was performed using the method of Taylor et al. (2005). The primary biochemical reason for germinating grains is the mobilization of hydrolytic enzymes (amylases, proteases and other endogenous hydrolytic enzymes) (Briggs, 1998). For this work, malting was intended for the development of proteases which were key to proteolysis in which sorghum storage proteins of the grain are hydrolyzed by protease enzymes (proteinases and peptidases) (Taylor, 1993).

A number of different mashing methods have been proposed and used to produce hot wort extract. These include infusion mashing, double decoction mashing and temperature programmed mashing (Briggs et al., 2004). In all these methods, the common feature is degradation of starch, proteins, lipids, beta-glucans, pentosans and xylans to produce fermentable wort.

The first mashing regime (Chapter 4.1) had an enzyme to malt flour ratio of 1: 100. The procedure involved mashing in temperature of $55^{o}C$ in a shaking water bath for 45 minutes protein rest (the first step or portion in mashing process). In this regime proteins in the malt are broken down by proteolytic enzymes into peptides, and some free amino acids for 45 minutes. The regime is different from the other two types which are classified as infusion mashing types. The later methods involved a temperature programmed mashing in a BRF (Brewing Research Foundation) mashing bath.

Mashing regime 2 had 1:1.5 malt to mashing liquor plus 1% w/w exogenous enzyme (Flavourzme) to malt flour. A temperature of $55^{0}C$ was held for 25 minutes for the protein rest, then temperature was increased to $90^{o}C$ at $1^{o}C$ over 35 minutes period, then held for 45 minutes followed by cooling to room temperature while stirring in room temperature water.

The last mashing regime defined as low temperature mashing regime 3, was similarly performed in a BRF mashing bath using 1:1.5 malt to mashing liquor. Mashing in temperature was set at $40°C$ for the protein rest for 420 minutes, followed by an increase to $90°C$ with an increase of $1^{o}C$ over 35 minutes then held for 45 minutes for starch gelatinisation, and $60°C$ for 90 minutes to allow amylase action. The total mashing cycle was 570 minutes. Literature suggests that an infusion or modified mashing procedure

which involves gradually rising temperatures with a series of programmed temperature rest is superior to all other mashing types because it makes possible the action of the different enzymes (i.e proteolytic enzymes) to hydrolyses protein optimally (Beckerich and Denault, 1987).

Several authors have used different grist to liquor ratios in the mash to produce wort. For example, Odibo et al. (2002) used 50 g to 500 ml water and mashed at 55^0C with Neutral protease. The protein rest was conducted for 30 minutes. Temperature was increased to 100^0C, cooled to 65^0C held for another 30 minutes. Consequently, it can be suggested that the temperatures used in this study were not damaging to proteases. The foregoing step (protein-rest) was followed by starch gelatinisation at around 90-100^0C. Such a temperature ensures that sorghum starch is fully cooked (above gelatinisation temperature). Therefore, the thermal stable α-amylase was able to effectively saccharify the sorghum starch.

The EBC ninhydrin method (European Brewery Convention, 1987) an official method was used to determine the FAN in the wort. In summary, the ninhydrin test is used frequently for the detection of glycine in the absence of other interfering substances such as Fe^{3+} (Aniello et al., 1985). It provides an idea of the presence of amino acids in wort. The mechanism in which the formation of the purple colour occurs is dependent on the reaction between α-amino acids and ninhydrin reagent as illustrated by the following reactions according to Sun-Wang (2007).

- Alpha-amino acid + ninhydrin $\longrightarrow$ Reduced ninhydrin + alpha-amino acid + H_2O. An oxidative deamination reaction that removes two hydrogen from the alpha-amino acid to yield an alpha-imino acid. Simultaneously, the original ninhydrin is reduced and loses an oxygen atom with the formation of a water molecule.

- Alpha-amino acid + H_2O $\longrightarrow$ Alpha-keto acid +NH_3
  The NH group in the alpha-imino acid is rapidly hydrolyzed to form an alpha-keto acid with the production of an ammonia molecule. This alpha-keto acid further undergoes decarboxylation reaction

- Alpha-keto acid + NH_3 $\longrightarrow$ Aldehyde + CO_2
  In a heated condition an aldehyde is formed that has one less carbon atom than the original amino acid. A carbon dioxide molecule is also produced here.

- Alpha-amino acid + 2 ninhydrin $\longrightarrow$ CO_2 + aldehyde + final complex (blue) + $3H_2O$.

The first three steps produce the reduced ninhydrin and ammonia that is required for the production of the colour in the last step. In theory only amino acids will lead to colour development (Sun-Wang, 2007). Chemical compounds such as Fe^{3+} ions can interfere with colour development. Very low concentrations of these ions can significantly inhibit colour development. The limitation of the ninhydrin test is that although the test is quick and sensitive for the presence of alpha-amino acids, it cannot be used to analyze the individual amino acids in a mixture, because of non selectivity. Furthermore, the colour intensity developed is dependent on the type of amino acid.

From TEM, there was some evidence of sorghum protein matrix degradation (Chapter 4.1 Fig. 4.1.3). The principle involved in TEM is that high energy electron beam is transmitted through a very thin sample and an image with atomic scale resolution is observed (Kutz et al., 1985). The extent of protein matrix degradation was not as much as expected. The appearance of holes at the protein body centre indicate that some hydrolysis of prolamin proteins took place. The exogenous enzymes plus KMS augmented the activity of the endogenous protease thereby improving proteolysis. KMS in particular may have improved accessibility of the exogenous and endogenous enzymes by reducing disulphide bonds that could have formed primarily at the periphery of the protein bodies, involving β- and γ-kafirins (Oria et al., 1995; Duodu et al., 2003).

The use of osmium in sample preparation was to improve structural details of the images and to ensure clarity of image resolution at the stage of viewing on the screen. Heavy metals such as uranium and lead can be used in a similar way (Kutz et al., 1985). However, sample preparation prior to conducting the test was challenging and required maximum attention. This being said, structural images of proteins bodies obtained by the treatment which had exogenous proteolytic enzyme and KMS during mashing revealed varying sizes of protein bodies with possible degraded peripheral edges, a pattern similar to the pattern of protein degradation reported by Taylor et al. (1985).

SDS-PAGE was carried out with the view to separate the proteins remaining after mashing according to their sizes. In this study SDS-PAGE offered the means to indicate the presence of kafirins with molecular weights between 21-25 kDa (El Nour et al.,1998). The study further revealed that oligomeric and polymeric kafirins, which were not reduced during proteolysis, were present. It was speculated that addition of proteolytic enzyme and KMS enhanced the weakening of disulphide bonds of kafirin polymers resulting in an

increase in monomeric kafirins. On the contrary, oligomers and polymeric kafirins were produced and appeared in all treatments under non reducing conditions, indicative of insufficient proteolysis (Chapter 4.1). Under reducing conditions, some intense bands of the monomeric kafirin appeared which suggests that 2-mercaptoethanol was able to reduce the inter- and intramolecular disulphide bonds that are formed during the cooking process.

In this work, the residual kafirins not hydrolysed by the exogenous and endogenous proteolytic enzymes were not quantified. A further difficulty encountered was the protein purification process. This was revealed by the presence of a distinct band of what was speculated to be a residue of an amylase enzyme, appearing in the range of 55 kDa under both reducing and non-reducing conditions.

The PICO-TAG method of Bidlingmeyer et al. (1984), was used to determine the quantities of different amino acids in wort. The method uses the reversed phase chromatography principle with pre-column derivatisation. The wort amino acids were classified using Jones and Pierce (1964) classification in groups based on the rate at which they are taken up by yeast during fermentation. However, Cohen 2003 reported that the PICO-TAG method has some limitations, which includes poor derivatisative stability, interference from reagents peak and lack of reactivity with secondary amino acids. Despite these limitations, the method gave results similar to findings reported by others, as stated in (Chapter 4.1).

5.2 Effectiveness of proteolytic enzymes in sorghum endosperm protein matrix degradation

Different exogenous proteolytic enzymes were used in sorghum malt mashing at 55^0C for 45 minutes to assess their efficacy in FAN production. As stated in the problem statement in section 1.1, insufficient proteolysis due to the corneous nature of the endosperm in sorghum grain has a negative effect on production of FAN and extract.

The use of microbial proteolytic enzymes as proposed by Taylor and Boyd (1986) to improve FAN levels is practised in modern sorghum lager beer brewing. Several workers have used microbial proteolytic enzymes during mashing with sorghum grain or sorghum malt (Agu and Palmer, 1998; Bajomo and Young, 1994; and Mackintosh and Higgins, 2004), to improve hydrolysis of sorghum endosperm protein matrix and hence to improve extract levels, and FAN in particular. However, the application of exogenous proteolytic enzymes remains a challenge particularly in establishing suitable enzymes with maximum activity and threshold levels of application which would be cost effective (McFadden, 1989).

This work (Chapter 4.1 Tables 4.1.1 and 4.1.2) agrees that, addition of exogenous proteases during mashing improved FAN levels. However, some of the exogenous proteases examined were not effective at degrading endosperm protein in sorghum malt (Fig. 4.1.6).

The variation in amount of FAN produced by the different enzymes was due to several factors such as the pH, mashing temperature, activity and duration of mashing which are influential to effective proteolysis. This study agrees with work done by Goode at al. (2003), who reported that Bioprotease P.Concentrate with activity of 400,000 U/mL at pH 5.5-6.0, temperature 50-55°C and Bioprotease N-100 L with an activity 100,000 U/mL pH 6, temperature 55°C differed in their efficiency in FAN production.

5.3 Practicality of employing a long protein rest at low temperature for production of free amino nitrogen

The mashing regime used in this study produced significant improvement in FAN production. However, it is essential to consider a number of factors prior to recommending the method commercially. The mashing temperature of 40^0C is within the less detrimental temperature limit for both endogenous and exogenous proteases activity. The benefit for low temperature mashing and long protein rest is twofold, i.e. the long duration allows sufficient time for endosperm protein hydrolysis as evidenced by FAN increase (Chapter 4.1 Table 4.1.3). The other is that proteases remain active without being denatured.

However, commercially the regime may have additional cost implications to a brewer. For example, restructuring the brewhouse by building extra steep tanks jacketed around capable of holding or allowing steam to circulate around the tank for proteolysis. This is a major capital investment that would be of concern. Interestingly though, according to Briggs et al. (2004) in modern brew houses some heat savings, for example, using heat-recovery systems occurs. Heat generated from processes like wort boiling at 90^0C in order to gelatinise starch, can produce excess thermal energy which could be channelled to operate the protein rest for hydrolysis of proteins into peptides and amino acids. However, such a process (long extended protein rest at low temperature (40^oC) can reduce the number of brews in a day which may not be profitable.

Assuming that this is the case, continuous brewing for 24 hours a day would yield approximately three protein rest durations of 420 minutes each. Thus a reduction by 3 over 24 hours, if the number of brews anticipated are 8 times with each lasting 8 hours from protein rest to final wort production as the case is in most brewing practices (Mr. B. Higgins, Brewery Consultant, Novozymes, personal communication). Possibly another problem is if the protein rest is too long, too much protein breakdown could occur of producing large and medium-sized protein molecules in the wort. This would ultimately harm the final beer composition (body) and eventually affect foam retention (Miller, 1984).

The addition of KMS to the sorghum malt mashing at a low temperature 40^0C improved FAN twofold (Chapter 4.1 Table 4.1.3). The KMS enhanced reduction of disulphide bonds present in the sorghum kafirin, thus making the kafirin more digestible. According to Dufour, Melotte and Srebrnik (1992) cultivar selection, and choosing suitable proteases as reported by Taylor and Boyd (1986) are effective ways that can improve FAN production.

5.4 Prospects of using high protein digestibility sorghums in lager beer brewing

In normal sorghum, protein digestibility is generally low (Hamaker et al., 1987). Consequently, mashing with normal sorghum gives insufficient production of FAN required for yeast growth. Thus, the prospects of using high protein digestibility sorghum in lager beer brewing were investigated. High protein digestibility sorghum has protein bodies with increased surface area, a feature reported to be responsible for improved their protein digestibility (Oria et al., 2000). From the brewer's point of view the prospect for using high digestibility sorghum in brewing should be considered on the basis of how much FAN such cultivars can produce other than looking at the pepsin digestibility.

Despite their higher protein digestibility, the high protein digestibility cultivars being reported here appeared to produce more FAN during mashing only when its malts were mashed with commercial proteases compared to their respective controls. Hence, identification of a suitable cultivar, with a trait such as those reported by Oria et al. (2000), i.e high protein digestibility, not negatively affected by cooking or environment and above all with improved proteolysis (FAN production) needs to be carefully done, otherwise the issue still remains a challenge.

6.   CONCLUSIONS AND RECOMMENDATIONS

In this study some of the exogenous proteases examined were not effective at degrading the endosperm protein in the sorghum malt. The variation in FAN shown by different enzymes is attributed to the differences in specific activity, the endopeptidase/exopeptidase ratio of enzyme preparation and optimum temperature and pH specific for each proteolytic enzymes. Mixtures of proteases should contain a higher percentage of exopeptidases to maximize the release of FAN. Hence, an increase in FAN could be achieved when suitable conditions of temperature and duration of mashing are used.

Potassium metabisulphite (PMB) as a result of it destabilizing the disulphide bonds in the protein polypeptide chains, consequently provides better accessibility for protease action and improved proteolysis as observed by a 22 to 10% increases in FAN in treatments with PMB compared to one without PMB, respectively. Hence in brewing it could be successfully used to boost up proteolysis..

Using a low temperature, long time mashing is an effective way of improving FAN. The reasons are twofold. There is sufficient long time for proteolysis and low temperature most likely reduces the risk of proteolytic enzyme denaturation thus it is recommended for use industrially.

FAN production is slightly greater when malt of high protein digestibility sorghum was mashed with exogenous proteases compared to normal sorghum. The reason is probably associated with increased protein body surface area. Therefore, malted high protein digestibility sorghum could be used commercially in brewing if mashing is done with its malt plus commercial proteolytic enzymes.

Selecting suitable sorghum cultivars, temperature and pH, with inclusion of PMB plus addition of effective commercial proteases can improve FAN production during sorghum mashing. However, given that high doses of exogenous proteolytic enzymes are required for efficient FAN production, it would be costly to brew with exogenous enzymes though the venture still remains a better option than importing barley.

7 REFERENCES

Agu, R. C., and Palmer, H. G., 1996. Enzymic breakdown of endosperm proteins of sorghum at different malting temperatures. Journal of the Institute of Brewing. 66: 415-418.

Agu, R. C., and Palmer, H.G., 1997. Effect of mashing procedures on some sorghum varieties germinated at different temperatures. Process Biochemistry. 32: 147-158.

Agu, R. C., and Palmer, G. H., 1998a. Effect of mashing with commercial enzymes on the properties of sorghum worts. World Journal of Microbiology and Biotechnology.14: 43-48.

Agu. R. C., and Palmer, G. H., 1998b. A reassessment of sorghum for lager-beer brewing. Bioresource Technology. 66: 253-261.

American Society of Brewing Chemists. 1976. Method WORT- 2. Specific gravity by Pycnometer. Methods of Analysis of the American Society of Brewing Chemists. ASBC. St Paul, MN. Pp. 2-3.

Alder-Nissen, J., 1993. Proteases, In: Nagodawitha, T. W., and Reed, G. E. 3rd ed. Enzymes in Food Processing. Academic Press, San Diego, CA, Pp. 159-204.

Aniello, A, Onofrio, G, Pischetola, M., and Strazzullo, L., 1985. Effect of various substances on the colorimetric amino acid-ninhydrin reaction. Analytical Biochemistry. Review. 144: 610-1.

Bajomo, M. F., and Young, T. W., 1993. The properties, composition and fermentabilities of worts made from 100% raw sorghum and commercial enzymes. Journal of the Institute of Brewing. 99: 153-158.

Bajomo, M. F., and Young, T.W., 1994. Fermentation of worts made from 100% raw sorghum and enzymes. Journal of the Institute of Brewing. 100: 79-84.

Beckerich, R. P., and Denault, L. J., 1987. Enzymes in the preparation of beer and fuel alcohol. In: Kruger, J.E., Lineback, D., and Stauffer, C.E. (Eds.). Enzymes and their Role in Cereal Technology. American Association of Cereal Chemists, St Paul, MN, Pp.335-354.

Bhise, V. J. Chavan, J. K., and Kadam, S. S., 1988. Effect of malting on proximate composition and in vitro protein and starch digestibility of grain sorghum. Journal of Food Science and Technology. 25: 327-329.

Bidlingmeyer, B. A., Cohen, S. A., and Tarvin, T. L., 1984. Rapid analysis of amino acids using pre-column derivatization. Journal of Chromatography. 336: 93-104.

Briggs, D. E., 1998. Malts and Malting. Blackie and Academic Professional, London. Pp. 269-685.

Briggs, D. E., Boulton, C. A., Brookes, P.A., and Stevens, R., 2004. Brewing Science and Practice. Woodhead Publishing. Cambridge. Pp. 10-606.

Byaruhanga, Y. B., Erasmus, C., Emmambux, M. N., and Taylor, J. R. N., 2005. Effect of heating cast kafirin films on their functional properties. Journal of the Science of Food and Agriculture. 87: 167-175.

Cohen, S. A., 2003. Amino Acid Analysis Using pre-Column derivatization with 6-aminoquinolyl *N* -hydroxysuccinimidyl carbamate Analysis of hydrolyzed proteins and electroblotted samples; In Methods in molecular biology vo.211 Protein sequencing Edited by J. Smith Humana Press, Totowa. Pp. 143-154

DeClerck, J., 1957. A Text of Brewing. Vol. 1. Chapman and Hall, London, Pp. 424-460.

Dewar,J., Orovan, E.,and Taylor, J. R. N., 1997a Effect of alkaline steeping on water uptake and malt quality in Sorghum. Journal of the Institute of Brewing. 103{. 283-285.

Dewar, J., Taylor, J. R. N., and Berjak, P., 1997b. Determination of improved steeping conditions for sorghum malting. Journal of Cereal Science. 26: 129–136.

Dewar, J., Taylor, J. R. N., and Joustra, S. M. 1995. Accepted Methods of Sorghum Malting and Brewing Analyses. CSIR Bio/Chemtek, Pretoria.

Doggett, H., 1988. Sorghum. 2nd ed. Longman Scientific and Technical, London, Pp. 1-6.

Dufour, J. P., Melotte, L., and Srebrnik, S., 1992. Sorghum malts for the production of lager beer. Journal of the American Society of Brewing Chemists. 50: 110-119.

Food and Agriculture Organization (FAO). 1996. The world sorghum and millets: Facts and, trends and outlook. Viale delle Terme di Canracalla, 00100 Rome, Italy. Pp 1-27.

Duodu, K. G., Nunes, A., Delgadillo, I., Parker, M. L., Mills, E. N. C., Belton, P S., and Taylor, J. R. N., 2002. Effect of grain structure and cooking on sorghum and maize in vitro protein digestibility. Journal of Cereal Science. 35: 161-174.

Duodu, K. G., Taylor, J. R. N., Belton, P. S., and Hamaker. B. R, 2003. Factors affecting sorghum protein digestibility. Journal of Cereal Science. 38: 117-131.

El Nour, I. N. A., Peruffo, A. D. B., and Curioni, A., 1998. Characterization of sorghum kafirins in relation to their cross-linking behaviour. Journal of Cereal Science. 28: 197–208.

European Brewery Convention. 1987. Method 8.8.1. Ninhydrin colorimetric method. (International method). Analytica-EBC, 4th ed. Braueri-und Getranke-Rundschau, Zurich, p. E 141.

Evans, D.J., and Taylor, J. R. N., 1990. Influence of cultivar and germination conditions on proteolytic activities in sorghum malt. Journal of the Institute of Brewing. 96: 399-402.

Ezeogu, L I., and Okolo, B. N., 1995. Effect of air rest periods on malting sorghum response to final warm water steep. Journal of Institute of Brewing. 101: 39-45.

Goode, D. L., Halbert, C., and Arendt, E. K., 2003. Optimization of mashing conditions when mashing with unmalted sorghum and commercial enzymes. Journal of the American Society of Brewing Chemists. 61: 69-78.

Hallgren, L., 1995. Lager beers from sorghum. In: Dendy, D. A. V (Ed.). Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St Paul, MN. Pp 69-124.

Hamada, J. S., 2000. Characterization and functional properties of rice bran proteins modified by commercial exoproteases and endoproteases. Journal of Food Science. 65: 305-310.

Hamaker, B. R., Kirleis, A. W., Butler, L. G., Mertz, E. T., and Axtell, J. D., 1986. Effect of cooking on the protein profiles and in vitro digestibility of sorghum and maize. Journal of Agricultural and Food Chemistry. 34: 647-649.

Hamaker, B. R., Kirleis, A. W., Butler, L. G., Axtell, J. D., and Mertz, E. T., 1987. Improving the in vitro protein digestibility of sorghum with reducing agents. Proceedings of the National Academy of Sciences of the United States of America. 84: 626-628.

Hough, J. S., Briggs, D. E. and Stevens, R., 1971. Malting and Brewing Science. Chapman and Hall, London. Pp. 104.

House, L. R., 1995. Sorghum and millets: History, taxonomy, and distribution. In: Dendy, D. A. V (Ed.). Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St Paul, MN. Pp . 1-10.

International Crop Research Institute for Semi –Arid Tropics (ICRISAT). 1996. The world sorghum and millets: Facts and, trends and outlook. Parancheru 502 324, Andhra Pradesh, India. Pp 1-25.

Jones , M., and Pierce, J. S., 1964. Absorption of amino acids from wort by yeasts. Journal of the Institute of Brewing. 70: 307-315.

Klopfenstein, C. F. and Hoseney, R. C., 1995. Nutritional properties of sorghum and the millets. In: Dendy, D. A. V (Ed.). Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St Paul, MN. Pp 125-168.

Kutz, S. M., Bentley, D. L., and Sinclair, N. A., 1985. Improved fixation of cellulose-acetate reverse-osmosis membrane for scanning electron microscopy. Applied and Environmental Microbiology. 49: 446-450.

Lefyedi, M. L., and Taylor, J. R. N., 2006. Effect of dilute alkaline steeping on the microbial contamination, toxicity and diastatic power of sorghum malt. Journal of the Institute of Brewing. 112: 108–116.

Macfadden, D. P., 1989. Use of exogenous enzyme in sorghum brewing. In: Proceedings of the 2nd Scientific and Technical Convention, Institute of Brewing. Johannes burg.Pp. 306.

Mackintosh, I., and Higgins, B., 2004. The development of a sorghum-based lager beer in Uganda: a model of co-operation between industry and government in the development of local ingredients for the production of quality lager beer and consequential benefits for the parties involved. Aspects of Applied Biology. 72: 235-245.

Makokha, A. O., Oniang'o, R., Njoroge, S. M., Kinyanjui, P. K., 2002. Effect of malting on protein digestibility of some sorghum (*Sorghum bicolor*) varieties grown in Kenya. African Journal of Food, Agriculture, Nutrition and Development. 2: 67-77.

Megazyme., 2007. Total Starch Assay Procedure α-amylase method. http//www.megazyme.com/downloads/en/data/K-TSTA.pdf. Accessed : 23 May 2007.

Miller, D., 1984. Mashing and protein rests, dry hopping, false bottom materials, and Single-Infusion vs. step and decoction mashing. Brewing Techniques. http/www.franklinbrew.org/brewinfo/bt.html. Accessed 17 August, 2008.

Morrall, P., Boyd, H. K., Taylor, J. R. N., and Van der Walt, W. H. 1986. Effect of germination time, temperature and moisture on malting of sorghum. Journal of the Institute of Brewing. 92: 439-445.

Odibo, F. J. C., Nwankwo, L. N., and Agu, R. C., 2002. Production of malt extract and beer from Nigerian sorghum varieties. Process Biochemistry. 37: 851-855.

Ogbonna, A. C., Obi, S. K. C, Okolo, B. N., and Odibo, F. J. C., 2003. Purification and some properties of a protease from sorghum malt variety KSV8-11. Journal of the Institute of Brewing. 109: 179-186.

Ogbonna, A. C, Obi, S. K. C., and Okolo, B. N., 2004. Optimization of proteolytic activities in malting sorghum. Process Biochemistry. 39: 713-718.

Oria, M. P., Hamaker, B. R., Axtell, J. D., and Huang, C. P., 2000. A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. Proceedings of the National Academy of Sciences of the United States of America. 97: 5065–5070.

Oria, M. P., Hamaker, B. R., and Shull, J. M., 1995a. In vitro protein digestibility of developing and mature sorghum in relation to α-, γ-, and β- kafirin disulphide crosslinking. Journal of Cereal Science. 22: 85-93.

Owuama, C. I., 1997. Sorghum: a cereal with lager beer brewing potential. World Journal of Microbiology and Biotechnology. 13: 253-260.

Owuama, C. I., and Okafor, N., 1987. Studies on mashing methods for brewing with sorghum. MIRCEN Journal. 3: 243-253.

Palmer, G. H., Etokakpan, U. O., and Igyor, A. M., 1989. Sorghum as brewing material. World Journal of Microbiology. 5: 265-275.

Rao, M. B, Tanskale, A. M., Ghatge, M. S., and Deshpande, V. V., 1998. Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Reviews. 62: 597-635.

Serna-Saldivar, S.O., and Rooney, L.W., 1995. Structure and chemistry of sorghum and millets. In: Dendy, D. A. V (Ed.). Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St Paul, MN. Pp 69-124.

Sivaramakrishnan, S., Gangadharan, D., Madhavan, N., Soccol. R. C. and Pandey. A., 2006. α-Amylases from microbial sources- an overview on recent developments in food. Food Technology Biotechnology. 44:173-184.

Sun Wang, N., 2007. Amino acid assay by ninhydrin colorimetric assay. http://www.eng.umd.edu/~nsw/ench485/lab3a.htm. Accessed: 1 July 2008.

Taylor, J. R. N., 1983. Effect of malting on the protein and free amino nitrogen composition of sorghum. Journal of the Science of Food and Agriculture. 34: 885-92.

Taylor, J. R. N., 1992. Mashing with malted grain sorghum. Journal of the American Society of Brewing Chemists. 50: 13-18.

Taylor, J. R. N., 1993. Sorghum malt: Its current use and future potential for brewing in Southern Africa. In: Taylor J.R.N., Randall P.G., and Viljoen J.H. (Eds.). Cereal Science and Technology, Impact on a Changing Africa. CSIR. Pretoria, South Africa. Pp. 413.

Taylor, J. R. N., and Belton, P. S. 2002. Sorghum. In: Belton, P. S. and Taylor, J. R. N. (Eds.) Pseudocereals and Less Common Cereals, Grain Properties and Utilization Potential. Springer-Verlag, Berlin. Pp. 25-81.

Taylor, J. R. N., and Boyd, H. K., 1986. Free α-amino nitrogen production in sorghum beer mashing. Journal of the Science of Food and Agriculture. 37: 1109-1117.

Taylor, J. R. N., and Daiber, K. H., 1988. Effect of calcium ions in sorghum beer mashing. Journal of the institute of Brewing. 94: 68-70.

Taylor, J. R. N., and Evans, D. J., 1989. Action of sorghum proteinase on the protein bodies of sorghum starchy endosperm. Journal of Experimental Botany. 40:763-768.

Taylor, J.R.N., and Robbins, D.J., 1993.   Factors influencing beta-amylase activity in sorghum malt. Journal of the Institute of Brewing. 99: 413-416.

Taylor, J. R. N., and Schüssler, L., 1986. The protein compositions of the different anatomical parts of sorghum grain. Journal of Cereal Science. 4: 361-369.

Taylor, J. R. N., Boyd, H. K., and Pickerell, A. T. W., 1985. The role of free alpha amino nitrogen (FAN) in the brewing of sorghum beer. South African Food Review. August/September. 82-83.

Taylor J. R. N., Dewar, J., Joustra, S. J., 2005. Sorghum Malting Technology Course and Methods of Sorghum Grain and Malt Quality Analysis. University of Pretoria**.** Pretoria. Pp 30-66.

Taylor, J. R. N., Novellie, L., and Liebenberg, N. v. d. W., 1984a. Sorghum protein body composition and ultrastructure. Cereal Chemistry. 61: 69-73.

Taylor, J. R. N., Novellie, L., and Liebenberg, N. v. d. W., 1985. Protein body degradation in the starchy endosperm of germinating sorghum. Journal of Experimental Botany. 36: 1287-1295.

Taylor, J. R. N., Schober, T. J., and Bean, S. R., 2006. Novel food and non-food uses for sorghum and millets. Journal of Cereal Science. 44: 252-271.

Taylor, J. R. N., and Schüssler, and Liebenberg, N. v. d. W., 1984b. Location of zein-2 and Crosslinked Kafirin in maize and sorghum protein bodies. Journal of Cereal Science. 240-255.

Taylor, J. R. N.,and Von Benecke, R.,1990. Development of the simple assay for the determination of β-amylase in sorghum malt. In: Taylor J.R.N. Proceedings of the Third Aviemore Conference on Malting, Distilling and Brewing, 1. Campbell (Ed.) Institute of Brewing, London. Pp. 344-347.

Taylor, J R N. 2007. Revised sorghum grain structure . In: Belton, P. S. and Taylor, J. R. N. (Eds.) Pseudocereals and less common cereals, grain properties and utilization potential. Springer-Verlag, Berlin. pp. 25-81.