Yeast fermentation of sorghum worts: Influence of nitrogen sources

by

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DECLARATION

I Bhekisisa Chushuta Dlamini declare that the thesis, which I hereby submit for the degree PhD Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: ……..

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Abstract

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Prof. E.M. Buys

Nitrogen limitation is one of the main causes of incomplete or protracted fermentations during brewing. In this study, the effects of sorghum type, exogenous protease enzymes and malting on the production of FAN were investigated. Further, the effects of different nitrogen sources on yeast fermentation performance, as well as the resulting changes at the phenotypic and genotypic level were studied.

With unmalted grain in the absence of exogenous proteases, white non-tannin sorghum produced substantially higher levels of FAN than white type II tannin sorghum. Similarly, in the presence of exogenous neutral proteinase enzyme, unmalted white non-tannin sorghum yielded more wort FAN than white type II tannin sorghum. Incubating unmalted W sorghum grain with neutral proteinase and amino-peptidase enzymes in combination, improved FAN production with white non-tannin sorghum. The two sorghum types produced similar FAN levels when malted, apparently as a result of the reduction in assayable tannins during malting. Malting did not influence the wort free amino acid profile. Group B amino acids which are utilized more slowly after group A amino acids have been utilized, constituted the highest percentage (42-47%).

Ammonia supplementation, in the form of diammonium phosphate, improved fermentation performance in terms of improved maltose and maltotriose uptake and more rapid reduction in specific gravity. Glycine supplementation negatively affected yeast fermentation performance when compared to the control. Lysine supplementation increased ethanol yield (up to 7.4% v/v) when compared to the control (7.1% v/v). However, it negatively affected yeast counts at the end of fermentation. Sorghum malt supplementation improved ethanol levels compared to the 100% raw sorghum control.
Serial repitching negatively influenced maltose and maltotriose uptake. Without nitrogen

supplementation and with lysine supplementation, yeast cells from serially repitched sorghum worts were distorted and irregular. In contrast, yeast cells supplemented with ammonia were less affected by serial repitching.

The extent of growth supported by ammonia or lysine on brewing yeast cells was studied using phenotypic microarray. With regard to nitrogen utilization, ammonia supplementation supported more active growth under aerobic conditions than the control and lysine supplementation. Lysine did not support growth when it was used as a sole nitrogen supplement. Concerning carbon utilization, nitrogen supplementation did not affect sugar utilization except for maltotriose and xylose. Maltotriose did not support growth with the control and lysine supplementation, while xylose supported growth and this was probably an artefact. There was no genome change with serial respitching as shown by PCR fingerprinting with the $(GTG)_5$ primer. However, relationships of the sequenced data indicated that mutation in the yeast genome occurred with lysine supplementation.

This study shows that nitrogen limitation negatively affects fermentation performance when brewing with unmalted sorghum. This is because nitrogen is required for the growth and metabolic activity of yeast cells during fermentation. Mashing white non-tannin sorghum grain and supplementing the resultant wort with ammonia in the form of diammonium phosphate are effective methods of improving assimilable nitrogen to ensure fast and complete fermentations.

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1: INTRODUCTION

Sorghum lager beer is becoming very popular across sub-Saharan Africa. Sorghum is now the grain of choice for brewing because of its ability to withstand harsh environmental conditions found in most parts of the sub-continent (Owuama, 1997). Further, since sorghum is gluten-free, sorghum beverages can be consumed by people suffering from coeliac disease, mainly in developed countries (Taylor, Schober and Bean, 2006). Large-scale production of sorghum lager beer is already in place in some African countries like Nigeria and Uganda. However, not all the challenges associated with brewing with sorghum have been resolved (Ogbonna, 2011).

Malting is normally the key step in the production of beer. Malting provides hydrolytic enzymes to ferment sugars and provides yeast nutrients (Ogbonna, Abuajah, Ide and Udofia, 2012) and may also control beer flavour and colour (Briggs, Boulton, Brooks and Stevens, 2004). Despite providing adequate free amino nitrogen (FAN) levels for proper yeast growth, sorghum malts are associated with problems such as high malting losses and high wort viscosities that cause filtration problems (Ogbonna, 2011). Further, to improve extract yield, exogenous enzymes are added during mashing of malted sorghum. Since exogenous enzymes can be used to mash unmalted sorghum, it has been suggested that brewing with unmalted sorghum is more economical than brewing with malt (Bajomo and Young, 1994).

A major problem of brewing with unmalted sorghum is that it produces low levels of wort FAN. This is attributed to the poor digestibility of sorghum proteins (Taylor and Belton, 2002; Duodu, Taylor, Belton and Hamaker, 2003), particularly kafirin proteins which are the major (up to 80%) proteins in sorghum grain (Taylor and Belton, 2002). To support proper yeast growth, FAN levels of about 120 mg/ L are adequate. However, with the advent of high gravity worts, FAN levels up to 150 mg/ L have become common (Beckerich and Denault, 1987). Brewing with low FAN levels results in stuck or incomplete fermentations, mainly due to nutritional stress (Gibson, Lawrence, Leclaire, Powell and Smart, 2007).

Several methods have been employed to improve FAN levels when mashing with unmalted grain. These range from the use of reducing agents such as potassium

metabisulphite (Ng'andwe, Hall and Taylor, 2008), development of sorghum varieties that will produce high soluble proteins during mashing (Okolo and Ezeogu, 1996a; Agu and Palmer, 1998), supplementation of sorghum with soybean protein concentrates (Awadalkareem, Mustafa and El Tinay, 2008), to the use of exogenous proteolytic enzymes (Ng'andwe et al., 2008; Lei, Zhao and Zhao, 2013a;). Supplementation of worts with different nitrogen sources has also been applied to improve FAN, particularly during high gravity brewing. The type of nitrogen source supplement as well as the amount of supplement is mainly dependent on the yeast strain (Torrea, Valera, Ugliano, Ancin-Azpilicueta, Leigh Francis and Henschke, 2011; Gutiérrez, Chiva, Sancho, Beltran, Arroyo-Lopez and Guillamon, 2012).

Serial repitching, which is the re-use of cropped yeast in subsequent fermentations, is a normal practice in brewing. Yeasts can be repitched between 8–15 generations (Powell and Diacetis, 2007). The number of times that yeast can be repitched depends on the yeast strain, the quality of the cropped slurry and company policies (Powell, Quain and Smart, 2003; Powell and Diacetis, 2007). According to Jenkins, Kennedy, Hodgson, Thurston and Smart, (2001) lager yeast strains begin to deteriorate after 10 serial repitchings. This is probably due to the fact that serial repitching causes modification in yeast physiology and fermentation performance (Jenkins, Kennedy, Hodgson, Thurston and Smart, 2003). In addition, serial repitching has been associated with production of higher quantities of flavour compounds that negatively affect beer quality (Quilter, Hurley, Lynch and Murphy, 2003). Since mashing unmalted sorghum grain produces insufficient FAN for a complete fermentation, it would seem that serial repitchings of more than three generations cannot be achieved, mainly because of nutritional limitation.

Apart from nutritional stress, brewing yeasts are exposed to a repertoire of other stresses such as oxidative stress, osmotic stress, and pH fluctuations during industrial fermentation (Powell, Van Zandycke, Quain and Smart, 2000; Briggs et al., 2004). Therefore the ability of yeast to survive or adapt to these fluctuations is crucial, not only for the production of good quality beer but also for maintaining good fermentative capacity and viability in subsequent fermentations (Powell et al., 2000; Gibson et al., 2007). To survive these challenges, yeast cells have the ability to re-organize their genomic expression and hence change the patterns of cellular proteins and metabolites (Gasch and Werner-Washburne,

2002). Therefore, modifications in yeast populations do occur due to changes in environmental conditions.

The objective of this research was to gain a deeper understanding of yeast fermentation performance and the factors affecting it when brewing with sorghum. The effect of sorghum type, exogenous enzymes and malting on the production of FAN was investigated. Further, the effects of different nitrogen sources on yeast fermentation performance, as well as the resulting changes at the phenotypic and genotypic level were studied.

2: LITERATURE REVIEW

This review will first give an overview of the brewing process from malting to fermentation with a focus on sorghum. The challenges of brewing with sorghum in relation to sorghum grain structure and chemistry will be discussed. In addition, the different ways of improving FAN when brewing with unmalted sorghum grain are reviewed. Brewing yeast nutrition with respect to nitrogen and carbon utilization will then be discussed, including the effects of high gravity brewing and serial repitching on the yeast fermentation performance. Yeast metabolism with respect to ammonia and lysine degradation, as well as tools for the assessment of brewing yeast physiology and genotype will be reviewed.

2.1 The Brewing Process

Sorghum has been used for centuries to produce traditional type of beers in Africa. Of interest though, is the current use of sorghum in the production of lager beer (Owuama, 1997; Mackintosh and Higgins, 2004). This practice started in Nigeria after the government banned the importation of barley in 1988 (Taylor and Dewar, 2000). In Uganda, a white sorghum variety that produces lager beer with a quality nearly equivalent to that of European beers has been successfully developed (Mackintosh and Higgins, 2004). This has led to reduction in input costs of manufacturing, reduction in retail selling price of beers and growth in the industry. Many different methods exist for the production of beers. Generally, the process first involves enzymic hydrolyses of starch, protein and other constituents of the malt or unmalted cereal adjuncts and then fermentation of the wort using yeast (Taylor and Dewar, 2000). The basic steps involved in brewing lager beer are shown in Figure 2.1. In this review, emphasis will be on malting, mashing and fermentation.

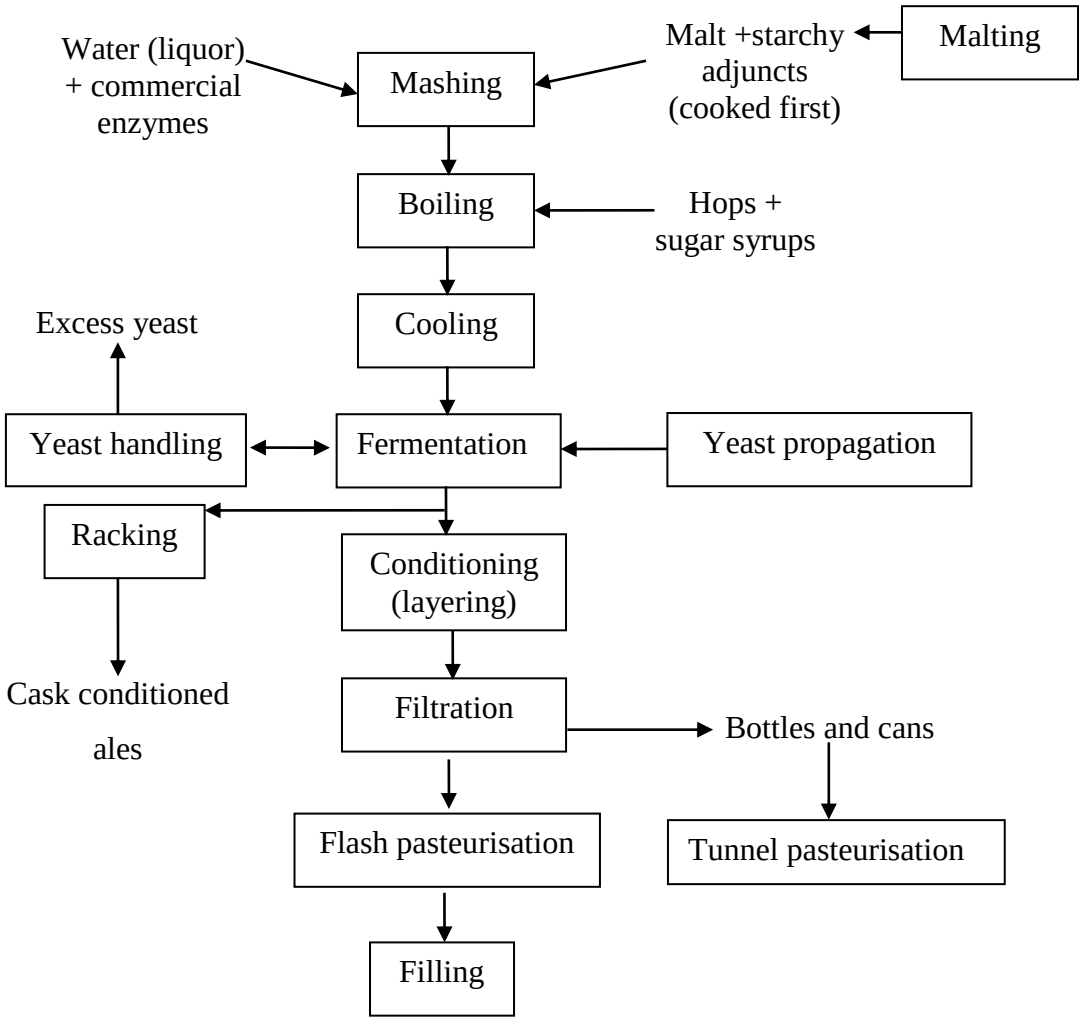

Figure 2.1. Lager beer brewing process (adapted from Adams and Moss, 2004)

2.1.1 Malting

Malting is the process by which cereal grains are germinated in moist air under controlled conditions (Dewar, Taylor and Berjak, 1997). The main objective of malting is to activate the grain's endogenous hydrolytic enzymes, mainly the amylases which break down starch into fermentable sugars (Taylor and Dewar, 2000). Barley is the cereal grain that is most widely malted. However, sorghum malting is gaining popularity in Africa for lager brewing (Ogbonna, 2011). The quality of the malt is affected by factors such as the type of grain, the grain cultivar and different malting conditions. The malting process can be divided into three operations namely, steeping, germination and drying (Dewar et al., 1997).

Steeping

Steeping involves immersing the grain in water for a certain period under controlled conditions such as temperature and time (Novellie, 1962; Owuama and Abdullahi, 1994). As the grain absorbs water, it swells and the process of germination commences (Owuama, 1997). Steeping is reportedly the most critical stage of the malting process (French and MRuer, 1990) since it initiates germination and affects the resultant malt quality.

However, this step has in the past been considered unimportant in sorghum brewing (Novellie, 1962). This was because in sorghum malting, the grain is watered during germination while in barley malts, the grain must receive all the water needed for germination during steeping. Sorghum is steeped commercially for between 4-6 hours to a maximum of 24 hours under non-controlled conditions (Taylor and Dewar, 2000). The notion that the steeping step was regarded not important in brewing with sorghum is evidenced by the lack of standard prescribed steeping procedure in South Africa (Taylor and Dewar, 2000). Contrary to this, other studies have indicated that steeping time and temperature significantly affects the sorghum malt quality in terms of diastatic power (DP) (amylase activity), FAN and hot water extract (HWE) (Dewar et al., 1997).

During steeping, some pigments, microorganisms and bitter substances are removed from sorghum grain (Novellie, 1962). Controlling the steep moisture is also important since it affects the extract, reducing sugar, diastatic power of the malt and the level of amino acids in the wort (Pathirana, Sivayogasundaram and Jayatissa, 1983; Dufour, Melotte and Srebnik, 1992; Dewar et al., 1997). A steep moisture of 35% achieved after steeping sorghum for 18 to 22 h at 30 °C is optimal for enzyme activity (Pathirana et al., 1983; Morrall, Boyd, Taylor and Van der Walt, 1986). Owuama and Asheno (1994) reported that increasing steeping moisture with steeping time from 12 to 20 h at 30 °C resulted in a similar increase in reducing sugars and cold and hot-water extracts. The latter seem to be directly proportional to the DP of the malt (Pathirana et al., 1983).

Furthermore, aeration during steeping, which is a common practice in barley malting, has been found to improve the quality of the sorghum malt produced (Ezeogu and Okolo, 1995; Dewar et al., 1997). Aeration provides grain with oxygen, removes growth-impeding carbon dioxide and removes some of the heat produced by the metabolising grain (Briggs et al., 2004). Steeping sorghum grain in dilute NaOH has been reported to improve the DP

(Okolo and Ezeogu, 1996a) and FAN content (Okolo and Ezeogu, 1996b) of sorghum malt. Sodium hydroxide presumably achieves this by disrupting the sorghum pericarp cell wall structure (Dewar, Orovan and Taylor, 1997). This allows faster hydration rate during steeping but does not cause oversteeping.

Germination

The onset of germination is indicated by the appearance of the small, white "chit" root sheath (coleorhiza) that comes from the base of the grain (Briggs et al., 2004). Germination is terminated before exhaustion of seed nutrients but after enough enzymes have been produced for the malt (Ogbonna and Egunwu, 1994; Owuama, 1997). During this process storage proteins in the sorghum endosperm are broken down to provide nitrogenous compounds for grain growth (Glennie, Harris and Liebenberg, 1983; Agu and Palmer, 1996). This is followed by translocation of small peptides and products of partial protein hydrolysis across the scutellum to the embryo. A number of enzymes including amylases, proteases and lipases are involved in germination. Alpha-amylase hydrolyses starch chains at α-1,4 glucosidic linkages at random (Hough, Briggs, Stevens and Young, 1982). Sorghum malt has high α-amylase activity, 25 to 183 U/ g, but this is influenced by the sorghum variety (Beta, Rooney and Waniska, 1995).

In sorghum, β-amylase is probably hydrolysed de novo during germination (Agu and Palmer, 1998). It catalyses the hydrolyses of α-1,4 glucosidic bonds at the non-reducing end of the starch molecule releasing maltose (Taylor and Robbins, 1993). Beta-amylase activity in sorghum malt is low and ranges between 11 to 41 SDU/ g (Sorghum Diastatic Units/ g) (Beta et al., 1995). Beta-amylase is more heat sensitive than α-amylase and hence it is influenced by temperature and germination time. Higher levels of β-amylase are achieved in malts made at 25 °C and 30 °C than malts made at 20 °C (Agu and Palmer, 1996). The use of a final warm water steam together with air rests during malting have been found to increase levels of β-amylase in sorghum malts (Ezeogu and Okolo, 1995).

Another important enzyme in germination is carboxypeptidase. This hydrolyses peptide bonds at the carboxylic end of the peptide to release free amino nitrogen (Evans and Taylor, 1990). FAN provides nutrients for yeast growth during fermentation. Germinating sorghum grains require an optimal temperature of between 25 and 30 ºC depending on the

grain variety (Palmer, Etokakpan and Igyor, 1989). At this temperature, sorghum malts of DP, good HWE, sugar contents and FAN are achieved.

Kilning (Drying)

Kilning involves drying the malt in an oven or kiln at a relatively high temperature until the rootlets become friable or brittle (Owuama, 1999). This arrests the development from seed to plant and reduces the moisture content of the malt to 10% or less (Adams and Moss, 2004). However, kilning sorghum malt at higher temperatures of more than 50°C may reduce the activity of heat labile enzymes such as β-amylase (Taylor and Robbins, 1993). Lower kilning temperatures, on the other hand, may impart unacceptable grainy, green malt flavour to the beer (Agu and Palmer, 1996). The moisture content of the green malt and the duration of kilning also influence the amylase activity of the sorghum malts. Kilning (high temperature drying) green malt with high moisture content at elevated temperatures accelerates enzyme inactivation while kilning green malt with moisture content less than 10% at 100 ºC for 3-4 h has less negative effect on hydrolytic enzymes and diastatic power (Briggs, Hough, Stevens and Young, 1981).

2.1.2 Mashing

Mashing is the production of sweet wort using either a mixture of ground malt, grist materials (adjuncts) and supplementary enzymes or entirely unmalted preparations of cereals mixed with industrial enzymes (Briggs et al., 2004). During mashing, water soluble substances are dissolved and enzymic hydrolysis occurs after which the dissolved substances are separated (Owuama, 1997). Several enzymes are involved during mashing and these include amylases, proteases, peptidases, phosphorylases, beta-glucanases and pentosanases. The activities of these enzymes are regulated by factors such as temperature, pH, time, and concentration of the wort (Buglass, 2011). The main objective of mashing is to produce a wort with the correct composition, flavour and colour in the shortest time with a reasonable yield (Briggs et al., 2004).

During mashing, enzymes act simultaneously with some acting out of their optimal conditions in terms of substrate concentration, pH and enzyme stability (Briggs et al., 2004). As mashing continues, enzymes are inactivated at different rates depending on, for instance, temperature and pH. This is accompanied by a change in relative enzyme concentration as substances such as starch are nearly totally degraded.

Mashing is normally done at temperatures that exceed 60 °C in order to enable the saccharification enzymes (amylases and limit dextrinase) to hydrolyse starch to soluble sugars (Buglass, 2011). The most common mashing regimes include stepwise infusion, infusion, decoction and double infusion (Buglass, 2011). The stepwise infusion method involves taking the mash through a series of temperature stages, with ramping and rest periods that are aimed at optimizing the conditions for different enzymes. The infusion method was traditionally used for ales but nowadays it is also used in the brewing of lagers where low protein malts are used instead of high protein malts. It involves holding the mash at a fixed temperature of about 65 °C for 1-2 h to allow optimal starch hydrolysis.

The decoction mashing method was traditionally used for brewing lager beers (Briggs et al., 2004). In this method, a proportion of the mash termed "the decoction" is withdrawn, boiled and re-introduced into the main mash while still hot. This step is repeated to increase the temperature of the mash in steps. Increasing the temperature in steps avoids the risk of overheating and premature total enzyme destruction. Double infusion mashing is mainly used for the production of high adjunct beers (Buglass, 2011). It involves the use of unmalted adjuncts such as rice, maize and sorghum to provide a cheaper and lower flavour extract. However, the adjuncts must be cooked in a cereal cooker to gelatinize the starch before use.

2.1.3 Fermentation

During fermentation, brewing yeast ferments the available wort sugars into ethanol and carbon dioxide as the major products of metabolism (Adams and Moss, 2004). A number of other minor metabolites are produced and these include esters, higher alcohols and acids (Briggs et al., 2004). Minor metabolites contribute to the beer flavour and it is through them that the drinker differentiates and characterises a beer brand. Sorghum worts are associated with high glucose levels when compared to other worts (Dufour et al., 1992). This is probably due to the enzyme α-glucosidase (Taylor and Dewar, 1994) which hydrolyses maltotriose and maltose to glucose. High glucose levels may adversely affect yeast enzymic composition and cell structure, consequently affecting fermentation (Hough et al., 1982).

Theoretically, about 85% of sugars are converted into ethanol and the remainder is used for yeast biomass formation and other metabolites (Briggs et al., 2004). To achieve these

levels, yeast needs a good supply of nutrients such as nitrogen. Low FAN levels may result in poor fermentations during brewing. Two main factors affect the process of fermentation namely, the condition of the yeast strain and process control parameters (Lodolo, Kock, Axcell and Brooks, 2008). The former includes yeast viability, vitality and purity, while the latter includes sufficient nutrient supply, correct pitching rate, optimised dissolved oxygen addition and temperature control. These factors affect the rate and extent of yeast growth and hence the quality of the end product.

After fermentation, the beer may be subjected to a variety of downstream processes depending on the type of beer produced (Adams and Moss, 2004). These may include a secondary fermentation (conditioning/ layering) which influences the beer flavour, filtration and pasteurization. Clarification of beer is also important since it prevents defects like haze formation (Hough et al., 1982). Packing and pasteurization of beer is normally done after a combination of filtration and centrifugation for bottled and canned beers (Adams and Moss, 2004).

2.2 Brewing with sorghum

Compared to brewing with barley, brewing with sorghum comes with challenges and brewing with raw (unmalted) sorghum is even more challenging. These include insufficient amylase activity to produce sufficient fermentable sugars for brewing purposes (Agu and Palmer, 1998) and low FAN levels. Sorghum starch also has a higher gelatinization temperature compared to that of barley and this may affect starch hydrolysis and the production of fermentable sugars (Taylor et al., 2006).

To achieve the desired wort FAN levels, brewing with raw or unmalted sorghum may require commercially unacceptable levels of exogenous enzymes (Bajomo and Young, 1993) and this has been associated with mashing and fermentation difficulties. Slow and incomplete saccharification and poor wort and beer filtration are some of the difficulties reported (Ogbonna, 2011). Furthermore, maintaining a consistent beer quality seems not to be easy when brewing with raw sorghum. Firstly, because of the lack of specific sorghum varieties for brewing and secondly because variations in the specifications of commercial enzymes may cause differing products after hydrolyses of substrate (Albini, Briggs and Wadeson, 1987; Agu, Okenchi, Aneke and Onwumelu, 1995).

Bajomo and Young (1993) reported that brewing with raw sorghum may produce fair levels of hot water extract when α-amylase and proteolytic enzymes are used. However, the FAN levels achieved are very low. As a result, high levels of proteolytic enzymes are needed (Albini et al., 1987; MacFadden and Clayton, 1989) to improve the FAN levels.

Despite all the afore-mentioned challenges, brewing lager beer with sorghum has shown a huge potential in Africa. Several attempts have been made to improve FAN levels, either through optimizing mashing or through addition of reducing agents (Dewar et al., 1997; Ng'andwe et al., 2008). In addition, breeding of sorghum varieties with beneficial qualities for beer brewing has also been investigated (Agu and Palmer, 1997; Mackintosh and Higgins, 2004). As explained, the use of exogenous enzymes when brewing with raw sorghum has also been proposed since enzymes are also used when brewing with malted grains (Bajomo and Young, 1993).

2.3 Sorghum grain structure and chemistry

2.3.1 Structure

The sorghum grain is similar to that of maize grain in that both grains have corneous (horny) and floury endosperms and a germ that is not only relatively large but is also rich in fat (Taylor and Dewar, 2000). In contrast to barley, both sorghum and maize grains are also devoid of a true husk (hull). The sorghum grain structure is, however, unique in that its pericarp is more friable than that of many other cereals. This means that sorghum flour can be contaminated with bran during dry milling. The lack of a true husk in sorghum grain was initially seen as a major disadvantage in using sorghum for brewing lager and stout beers since the husk in barley malt acts as a filter bed in lautering (the process by which the wort is separated from spent grain) (Little, 1994).

2.3.2 Chemistry

Sorghum is also unique in that some sorghum varieties (called variously tannin, bird-proof, brown, bird-resistant) contain condensed tannins, also known as proanthocyanidins (Serna-Saldivar and Rooney, 1995). These are located mainly in the seed coat (testa). Though tannins are agronomically advantageous, their presence in sorghum grain is not ideal for processing. Research has revealed that tannins can bind with grain proteins (Emmambux and Taylor, 2003) and with enzymes of the digestive tract (Price, Hagerman and Butler, 1980), hence negatively affecting the nutritional value of the grain.

The protein content of the sorghum grain ranges from 6 to 15% (Serna-Saldivar and Rooney, 1995). Sorghum proteins can be divided into prolamins (kafirins) and non-prolamins (albumins, globulins and glutelins). The sorghum grain contains up to 77–82% kafirin proteins in the endosperm, while non-prolamins make up to 18% of the proteins (Belton, Delgadillo, Halford and Shewry, 2006). Kafirins contain high levels of proline and glutamine and are soluble in non-polar solvents such as aqueous alcohols (Shewry and Tatham, 1990). Kafirins are further divided into α-, β-, γ- or δ- sub-classes based on molecular weight and solubility. Apart from the problem posed by tannins, sorghum grain protein is also deficient in the essential amino acid lysine (Taylor and Dewar, 2000). This deficiency is also prevalent in many other cereals.

In brewing, yeast utilizes products of protein hydrolysis, in particular amino acids as a source of nitrogen. This highlights the importance of protein digestibility in the brewing process. However, a major challenge with sorghum protein is that its digestibility is significantly reduced upon wet cooking (Mertz, Hassen, Cairns-Whitten, and Kirleis, 1984; Zhang and Hamaker, 1998; Duodu, Nunes, Delgadillo, Parker, Mills, Belton and Taylor, 2002). This has been attributed to both exogenous or endogenous factors (Duodu, et al., 2003). Exogenous factors include interactions of proteins with non-protein components such as polyphenols, phytates, lipids, starch, and cell wall components while endogenous factors involves protein-protein interactions.

The non-protein components are believed to form complexes with kafirins and cooking enhances these interactions (Duodu et al., 2003). The resultant complexes are resistant to digestion, hence the reduction in protein digestibility. Starch, in gelatinised form, has been reported to reduce protein digestion through reducing the access of proteolytic ezymes to protein bodies (Duodu et al., 2002). Therefore, the addition of α-amylase prior to proteolytic enzymes has been reported to improve the digestion of sorghum proteins (Duodu et al., 2002).

Furthermore, cooking sorghum causes disulphide bonding of β- and γ-kafirins, which forms enzymatically resistant protein polymers (Rom, Shull, Chandrashekar, and Kirleis, 1992; Hamaker, Mertz and Axtell, 1994). Since α-kafirin, which is more digestible, is located centrally within the protein body, its digestion is impeded by the surrounding disulphide cross-linked proteins (Rom et al., 1992; Hamaker et al., 1994). Taylor, Bean,

Ioerger and Taylor (2007) reported that complexations of kafirin and condensed tannins can also result in a 50% reduction in protein digestibility. Similarly to tannins, phytic acid also complexes with kafirins (Ravindran, Cabahug, Ravindran, Bryden, 1997). However, the role of phytic acid in protein digestibility is ameliorated since phytic acid content is reduced upon cooking.

2.4 Improving free amino nitrogen in sorghum wort

As stated, several attempts have been made to improve the level of FAN in sorghum grain worts and these include addition of exogenous enzymes (Bajomo and Young, 1993; Agu and Palmer, 1998; Ng'andwe et al., 2008), addition of soy protein concentrate (Awadalkareem, 2008) and reducing agents such as 2-mercaptoethanol (Hamaker, Kirleis, Butler, Axtell and Mertz, 1987), sodium bisulphite (Rom et al., 1992; Oria, Hamaker and Schull, 1995; Arbab and El Tinay, 1997), ascorbic acid (Arbab and El Tinay, 1997) and potassium metabisulphite (Ng'andwe et al., 2008).

Compared to chemical methods, enzymic hydrolysis of proteins has the advantage of being rapid and the enzymes are highly specific (Mesa-Stonestreet, Alavi and Bean, 2010). The latter, however, demands the use of more than one enzyme to accomplish a task that could be done by a single chemical process. Proteases, in particular, have been used to improve protein digestibility and functionality (Kunst, 2003). Until recently, sorghum proteolysis had been mainly used to isolate sorghum starch in order to improve starch digestibilty (Pérez-Carrillo, Serna-Saldivar, Alvarez and Cortes-Callejas, 2008; Xu, 2008). There is, therefore, a paucity of information on the impact of proteases on the digestibility or functionality of sorghum proteins (Mesa-Stonestreet et al., 2010).

Exogenous protease enzymes can hydrolyse the glutelin protein matrix surrounding the starch granules (Ng'andwe et al., 2008). This was observed with both raw and cooked sorghum flours using confocal scanning and transmission electron micrography. Furthermore, the use of exogenous protease enzymes in combination with potassium metabisulphite not only hydrolysed the glutelin matrix but also the exterior parts of the kafirin protein bodies.

Soy peptides are believed to improve fermentation and yeast growth by providing amino acids such as phenylalanine which are absorbed faster in peptide form than when presented

as a free amino acid (Kitagawa et al., 2008). Reducing agents improve hydrolysis of sorghum protein into FAN by reducing the stabilizing inter- and intra-molecular disulphide bonds (Hamaker et al., 1987; Oria et al., 1995; Ng'andwe et al., 2008). Consequently, the structure of the protein body opens up and allows access of proteolytic enzymes to the more digestible α-kafirin (Oria et al., 1995; Ng'andwe et al., 2008).

The digestibility of sorghum proteins can be influenced by several factors such as the presence of tannins and the reduction of protein digestibility during wet cooking (Duodu et al. 2003). The former will be addressed by investigating of the low tannin and tannin-free sorghum varieties, while protein digestibility can be addressed by investigating the use of exogenous proteolytic and malting enzymes and exogenous nitrogen sources.

2.5 Brewing yeasts

Brewing yeasts are typically members of the *Saccharomyces cerevisiae* species (Briggs et al., 2004). Brewing yeasts are heterotrophic, facultative anaerobes. They have an adaptable metabolism which is influenced by the availability of oxygen and the concentration and source of carbohydrates. Brewing yeast metabolism may be fully aerobic and oxidative or fermentative. Beer quality is influenced by the genotype of the yeast strain and its phenotype. The latter is further influenced by the composition of the wort and the conditions established in the fermentation vessel.

Generally, all *Saccharomyces* species will produce ethanol as a fermentation end-product. However, based on flocculation behaviour, two types of yeast were originally classified, namely "top-fermenting" ale yeast (*S. cerevisiae*) and "bottom-fermenting" lager (*S. pastorianus*, formerly called *S. carlsbergensis*) strains (Jentsch, 2007). Ale yeast strains have been used in beer brewing for thousands of years, while lager strains are believed to have emerged a few hundred years ago with the advent of the low temperature fermentation (Hornsey, 2003). Ale strains constitute a wide variety with most of them closely related to *S. cerevisiae* (Tornai-Lehoczki and Dlauchy, 2000; Kobi, Zugmeyer, Potier and Jaquet-Gutfreund, 2004). Lager strains have recently been confirmed to be hybrids of *S. cerevisiae* and *S. bayanus* (Naumova, Naumov, Masneuf-Pomarede, Aigle and Dubourdieu, 2005; Caesar, Palmfeldt, Gustafsson, Pettersson, Hashemi and Blomberg, 2007; Dunn and Sherlock, 2008). Ale yeast have a higher fermenting temperature, approx. 20°C, while lager strains perform optimally between 6-14°C (Bamforth, 2009).

2.5.1 Yeast nutrition

Yeast requires a repertoire of nutrients which are necessary for survival and growth. These are mainly provided by the wort (Briggs et al., 2004). Yeast nutrient demand includes sources of carbon, nitrogen, minerals and growth factors such as vitamins, purines and certain lipids. This review will focus on yeast nutrition with respect to carbon and nitrogen utilization.

2.5.1.1 Utilization of nitrogen sources

In beer wort, most of the nitrogen is provided by ammonium ions, amino acids, peptides, purines and pyrimidines (Briggs et al., 2004). A large portion of the wort FAN is used by yeast for protein formation (structural and enzymic) which is required for yeast growth (Pierce, 1987), while the remainder is utilized for other functions such as osmoregulation (Hohmann, 2002). Brewing yeast prefers asparagine, glutamine and glutamate as nitrogen sources (Batistote, da Cruz and Ernandes, 2006). Although ammonia is preferred by yeasts in preference to the other amino acids, brewing yeast will utilize these amino acids before utilizing ammonium ions. In the absence of the preferred amino acids, including ammonium ions, yeast will use secondary nitrogen sources such as other amino acids, smaller peptides and amides. Brewer's yeast does not utilize higher molecular weight peptides and proteins since it does not produce exogenous proteases and it is not capable of transporting larger peptides inside the cell (Briggs et al., 2004).

The utilization of secondary nitrogen sources is a more intricate process since it requires the synthesis of specific catabolite enzymes and permeases, the expression of which is highly regulated by nitrogen catabolite repression (NCR) (Batistote et al., 2006). Through NCR, readily assimilable nitrogen sources suppress the synthesis of uptake systems and catabolic enzymes of other less readily used sources of nitrogen (Magasanik and Kaiser, 2002). As a result, the utilization of amino acids during alcoholic fermentation has been grouped according to their assimilation by Pierce (1987). Group A amino acids are utilized first and rapidly, followed by group B amino acids which are utilized more slowly (Table 2.1). Group A and B amino acids are required for anabolic metabolism mainly protein synthesis and their permeases are not subject to nitrogen catabolite repression. Group C amino acids are only taken up when group A amino acids have been exhausted and nitrogen catabolite repression is relieved. The group D amino acid proline is not

utilized during fermentation since its oxidation requires a mitochondrial oxidase which is repressed during fermentation (Wang and Brandriss, 1987).

This classification of amino acids is not absolute and their repressive effects may vary significantly between different yeast strains. For example, ammonia appears to be the preferred nitrogen source for ∑1278b-derived strains but not for S288c-derived strains when the two strains are compared on the basis of whether pathways for alternative sources are derepressed (Magasanik and Kaiser, 2002). This is despite the fact that both strains grow well on ammonia as a source of nitrogen.

The quality of a nitrogen source can be referred to as being preferred (good) or non-preferred (poor) using two different criteria (Ljungdahl and Daignan-Fornier, 2012). The first criterion is based on the yeast growth rate that can be supported by a particular compound when present as a sole nitrogen source. The second criterion is based on the yeast control systems for the uptake of other nitrogenous sources during growth on a particular nitrogen source. If the nitrogen source of interest allows the activation of permeases and enzymes for the uptake of other nitrogen sources, then it is not a preferred source of nitrogen. Therefore nitrogen sources that do not allow for utilization of other nitrogen sources are termed the preferred nitrogen sources.

Table 2.1. Classification of amino acids based on the utilization order from wort during fermentation (Pierce, 1987), as cited by Briggs et al. (2004).

Group A	Group B	Group C	Group D
Arginine	Histidine	Alanine	Proline
Asparagine	Isoleucine	Ammonia	
Aspartate	Leucine	Glycine	
Glutamate	Valine	Phenylalanine	
Glutamine	Methionine	Tyrosine	
Lysine			
Serine			
Threonine			

Good nitrogen sources are characterized by relatively higher yeast growth rates compared to poor nitrogen sources (Schure, van Riel and Verrips, 2000). These include ammonia, glutamine and asparagine, while proline and urea are categorised as poor nitrogen sources. Within the cell, yeast cells convert the nitrogen source molecule into glutamate and glutamine (Ljungdahl and Daignan-Fornier, 2012). These two amino acids serve as a nitrogen donor for all other nitrogen containing compounds in the cell. Ammonia can also provide the amino group in the direct synthesis of glutamate and glutamine. NADPH-dependent glutamate dehydrogenase NADPH-GDH converts ammonia and α-ketoglutarate into glutamate, while glutamine synthetase produces glutamine out of ammonia and glutamate (Ljungdahl and Daignan-Fornier, 2012).

In the presence of an appropriate source of carbon and ammonium ions, yeast cells can synthesize all L-amino acids that are used in protein synthesis. As explained above, this is because ammonia is incorporated during the formation of glutamate from α-ketoglutarate by NADPH-dependent glutamate dehydrogenase (*GDH1*), and glutamine from glutamate by glutamine synthetase (*GLN1*) (Ljungdahl and Daignan-Fornier, 2012).

Nitrogen limitation results in poor yeast viability and stuck or sluggish fermentations, especially in high gravity worts (Casey, Magnus and Ingledew, 1984). This condition can be prevented by providing sufficient amounts of nitrogen in the wort. Wu, Jampala, Robbins, Hays, Yan, Xu, Rooney, Peterson, Shi and Wang (2010) reported that sorghum high gravity worts with higher initial FAN (more than 100 mg/L) fermented faster than

those with lower initial FAN content (less than 50 mg/L). The faster fermentation performance was attributed to increases in yeast cell numbers and better tolerance to higher ethanol concentration that are associated with high FAN levels (Kłosowski, Mikulski, Czuprynski and Kotarska, 2010). This shows that the initial nitrogen level of the wort has a significant impact on the yeast growth rate and yeast viability as was confirmed by Lei, Zhao, Yu and Zhao (2012).

Yeast fermentation performance is not only affected by the concentration of the nitrogen source, but also by its structural complexity (Cruz, Cilli and Ernandes, 2002). For example, supplementation with a nitrogen source in the peptide form (peptone) was found to be more positive for yeast metabolism, inducing higher biomass and ethanol production and preserving yeast viability when compared to supplementing with other nitrogen sources such as ammonium salts and free amino acids (Cruz et al., 2002). However, the study was done using artificial media (yeast nitrogen base) which has a different composition to that of a normal brewing wort like sorghum wort. In another study, lysine inhibited cell growth and fermentation performance when used as a single nitrogen source during wheat mash fermentation, while various other amino acids improved fermentation performance (Thomas and Ingledew, 1992). The reason why lysine inhibited yeast growth was not clear. In contrast, Lekkas, Stewart, Hill, Taidi and Hodgson (2007) found that lysine supported faster fermentation rates possibly due to increases in the suspended yeast cell concentration in lysine supplemented worts compared to the control.

Ammonia has been used to improve fermentation performance, mainly in wine fermentations (Deed, van Vuuren and Gardner, 2011; Ugliano, Kolouchova and Henschke, 2011). This is not only through its NCR effect but also through its other roles such as regulating enzyme activities, permease activities and transcription of nitrogen regulated genes (Schure et al., 2000). It has also been reported that ammonia does not induce full NCR but regulates some of the NCR genes during fermentation (Deed et al., 2011). Jiménez-Martí and Del Olmo (2008) investigated the effect of ammonia or amino acid addition to a nitrogen depleted medium on global yeast expression patterns during alcoholic fermentation. These workers found that ammonia addition resulted in a higher expression of amino acid biosynthesis genes, while addition of amino acids prepared the cell for protein synthesis. Further, they reported that most of the NCR-regulated genes were more expressed after adding ammonia than with amino acids. Genes related to stress

responses such as oxidative stress were more highly expressed when ammonia was added than with amino acids.

Supplementation with ammonia has also been found to induce poor yeast performance in terms of lower biomass accumulation and poor ethanol production (Cruz et al., 2002). This was attributed to the yeast using the carbohydrate source as both a carbon and an energy source. Therefore, additional energy and carbon were needed by the cell for the synthesis of amino acids derived from both the carbon source and ammonium sulphate in the media. Consequently, excess amounts of protons were generated in the cell. These had to be pumped out to maintain the internal pH, resulting in acidification of the medium, which affected ammonium ions uptake and growth.

2.5.1.2 Utilization of carbon sources

A standard barley malt brewer's wort contains approx. 90% carbohydrates, comprising the fermentable sugars glucose, fructose, maltose and maltotriose (Gibson, Boulton, Box, Graham, Lawrence, Linforth and Smart, 2008). Maltose and maltotriose are the most abundant sugars, followed by glucose and fructose. Similar sugars, glucose, maltose and matotriose, were also found in sorghum wort (Espinosa-Ramírez, Perez-Carrillo and Serna-Saldívar, 2014). If sucrose is present, it normally occurs in low concentrations and is first hydrolysed by the enzyme invertase (β-D-fructofuranosidase) into glucose and fructose before being taken up into the cell (Heggart, Margaritis, Pilkington, Stewart, Dowhanick and Russel, 1999). Sucrose utilization is followed by the monosaccharides glucose and fructose and then maltose and maltotriose (Gibson et al., 2008). In addition to these sugars, wort also contains dextrins which are non-fermentable carbohydrates (Briggs et al., 2004).

The uptake of sugars by yeast is subject to the sugar catabolite repression which ensures an ordered sequence of sugar utilization (Cruz, Batistole and Ernandes, 2003). For example, the presence of a preferred carbon source such as glucose, represses the expression of genes and enzymes for the utilization of alternative carbon sources. Consequently, maltose and maltotriose are utilized only after the depletion of the monosaccharides (Lagunas, 1993). Further, the ability to utilize maltose and maltotriose varies widely with brewing yeast strain, with maltotriose showing the widest variation (Meneses, Henschke and Jiranek, 2002; Dietvorst, Londesborough and Steensma, 2005). Brewing yeast can also

utilize alternative sources of carbon such as galactose, mannose and the pentose sugar D-xylose (Walker, 1998). Native *S. cerevisiae* strains do not metabolize xylose, however, strains capable of fermenting xylose have been genetically engineered (Madhavan, Tamalampudi, Srivastava, Fukuda, Bisaria and Kondo, 2009).

Slow sugar uptake appears to be one of the causes of sluggish fermentations in a nitrogen limiting medium. According to Varela, Pizarro and Agosin (2004) normal wine must fermentations (380 mg/L FAN) showed a significantly higher sugar uptake (up to 10-fold higher) than fermentations with low FAN contents (65 mg/L). This was thought to be due to the weaker transporter activity in sluggish fermentation cells or to fewer transporters per cell. In support of the latter, Bisson (1999) found that nitrogen limitation had an impact on the transporter turnover and on the expression of at least one transporter, HXT1. Varela et al. (2004) also reported that in spite of lower sugar uptake, adding biomass to sluggish fermentations reversed the effects of nitrogen limitation. It increased the fermentation rate and it was thought that it would less likely affect the quality of resulting wine since it did not change the chemistry of the wort.

Lei et al. (2012) investigated the effect of wort gravity and nitrogen level on the fermentation performance of brewer's yeast. They found that rate of sugar utilization in high gravity worts (HGW) increased with increasing nitrogen level. High nitrogen levels also induced higher expression of *TDH* genes which may correlate with higher glycolytic flux activity and could explain the increased sugar uptake. The *TDH* genes encode related isozymes of glyceraldehyde-3-phosphate dehydrogenase, which catalyze the reaction of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate in glycolysis (McAlister and Holland, 1985). The content of carbohydrates and their composition in the wort, including the manner in which they are taken up by yeast, has a direct influence on yeast metabolism and the organoleptic profile and ethanol concentration of the final product (Boulton and Quain, 2001).

2.5.1.3 Importance of glycogen in brewer's yeast

Glycogen, a polymer of glucose, is a major carbohydrate reserve in brewer's yeast and is considered crucial in rapid adaptation of yeast cells to environmental-changing conditions (François and Parrou, 2001). For example, increasing ethanol concentration from 2 to 8% (v/v) in a culture medium resulted in increased levels of glycogen (Dake, Jadhv and Patil,

2010). Other stresses that trigger the accumulation of glycogen in yeast cells include nutrient deprivation, high temperature and weak organic acids (François and Parrou, 2001; Somani, Bealin-Kelly, Axcell and Smart, 2012). High glycogen levels in yeast cells are associated with high yeast vitality, while low glycogen levels indicate an exhausted state of the yeast cells and such yeast is not suitable to be used for a new fermentation (Hutter, 2002). Therefore to study the effect of pitching yeast physiology on fermentation performance, yeast cells are normally aged to reduce the glycogen content (Lodolo, O'Connor-Cox and Axcell, 1999).

Pitching wort with a yeast crop consisting mainly of aged yeast may result in an extended lag phase and subsequently longer fermentation periods (Powell et al., 2000). This is thought to be due to low DNA formation at the early stages of fermentation when compared to fresh yeast (Lodolo and Cantrell, 2007). Low DNA formation in aged cells is attributed to a loss in growth capacity due to the low glycogen levels. The artificial ageing process of constantly stirring yeast at 25 °C for up 18 h may result in approx. 6.7% less glycogen in the yeast at the onset of the experiment (Lodolo et al., 1999).

Accumulation of glycogen in brewing yeast occurs during the late logarithmic phase (François and Parrou, 2001). This is followed by a partial reduction in glycogen levels upon entry into the stationary phase (Wang, Wilson, Fujino and Roach, 2001). The fall in glycogen levels is thought to be due to changes in metabolic profile as cells adjust to the depletion of glucose and other limiting nutrients. It has also been reported that glycogen levels may increase sharply after the onset of the stationary phase and may continue to increase even when the yeast viability drops (Pereira, Guimarães, Teixeira and Domingues, 2011). The latter was thought to be due to the fact that glycogen is a poorly soluble polymer, hence it cannot easily escape the cell. A rapid procedure to measure glycogen levels in yeast cells using flow cytometry has been developed (Hutter, 2002). Using this procedure, it was observed that yeasts achieve high proliferation rates after a reduction in glycogen content and that storing cropped yeast at elevated temperatures of up to 22°C may result in low glycogen accumulation at the end of fermentation. Determination of glycogen content is therefore used as an indicator of stressed yeast.

When pitching yeast, the level of glycogen can influence fermentation performance as glycogen provides yeast with a survival and reproductive advantage (François and Parrou,

2001). However, this depends on the pitching rate used and the extent to which glycogen reserves are depleted during storage of yeast under brewery conditions (Wang et al., 2001). The role that glycogen plays in chronological cell ageing is still debatable. However, reports strongly indicate that periodic replenishment of glycogen reserves can substantially extend yeast chronological lifespan (Samokhvalov, Ignatov and Kondrashova, 2004; Nagarajan, Kruckeberg, Schmidt, Kroll, Hamilton, McInnery, Summers, Taylor and Rosenweig, 2014).

2.5.1.4 High gravity brewing and its effects on brewing yeast

High gravity brewing (HGB) is a technology that involves brewing with worts of higher sugar concentrations than that of normal gravity (12–14 °P) brewing (Lei et al., 2012). With HGB brewing, increased productivity is achieved through higher final ethanol concentrations without major additional equipment investment. Compared with other approaches, HGB brewing benefits include considerable savings in water, labour and energy and also a reduction in bacterial contamination (Thomas, Dhas, Rossnagel and Ingledew, 1995). Moreover, costs associated with the treatment of effluent are reduced (Puligundla, Smogrovicova, Obulam and Ko, 2011). The benefits of HGB brewing are reviewed in detail by Puligundla et al. (2011). However, HGB has several drawbacks that can lead to sluggish and incomplete fermentations (Lei et al., 2012). These are related to yeast physiology, where HGB exposes yeast to high osmotic stress and ethanol toxicity due to high initial sugar concentrations and later high ethanol production, respectively (Rautio, Huuskonen, Vuokko, Vidgren and Londesborough, 2007). This results in low yeast viability, vitality and subsequently poor yeast fermentation performance. High gravity brewing technology has been widely adopted by the brewing and bioethanol industries, hence its use in this study.

Nutritional supplementation, mainly with nitrogen sources, has been found to improve fermentation performance during HGB brewing. Ethanol yields of up to 8.4% (w/v) have been obtained when 24 °P wort was supplemented with yeast extract compared to about 5.5% (w/v) levels with unsupplemented worts (O'Connor-Cox, Paik and Ingledew, 1991). Chang, Lin, Huang and Duan (2011) reported that FAN supplementation under HGB conditions not only results in high ethanol yields but also substantially shortens fermentation time. Similar results of increased fermentation rate and decreased fermentation time were reported by Ugliano, Fedrizzi, Siebert, Travis, Magno, Versini and

Henschke (2009) on 24° Brix grape musts supplemented with diammonium phosphate. The effect of ammonium salts on yeast growth has been explained.

The capacity to withstand the high osmotic stress that is associated with HGB is dependent on the yeast strain. Some *S. cereviasie* strains may show a marked decrease (from above 85% to less than 65%) in viability upon inoculation in high gravity worts, while others may resist the osmotic shock better (less than 10% drop in viability) (Pereira et al., 2011). The ability to survive high osmotic shock by the latter strain was attributed to its rapid accumulation of intracellular glycerol. Glycerol plays an essential role as a compatible solute to counteract hyperosmotic stress (Kaino and Takagi, 2008).

HGB is also associated with oxygen deficiencies since less oxygen can be dissolved in high gravity worts (Casey et al., 1984). Correction of these oxygen deficient worts with the right oxygen concentration allows rapid and complete fermentations (Lodolo et al., 1999; Boulton and Quain, 2001). Oxygen in brewery fermentations promotes the biosynthesis of unsaturated fatty acids and sterols by yeast which are required for adequate yeast growth (Boulton and Quain, 2001). These lipids also regulate membrane fluidity and permeability and influence the yeast's response to stress (Jones, Petty, Hoyle, Hayes, Oliver, Riba-Garcia, Gaskell and Stateva, 2004). Lin, Chien, Duan and Chang (2011) showed that aeration of high gravity worts results in high yeast viability and high ethanol productivity. Lodolo et al. (1999) studied the effects of oxygen limitation on different worts using European Brewery Convention fermentation tubes and suggested that worts need an oxygen level of approx. 16 mg/L dissolved oxygen for optimal yeast performance.

2.5.2 Serial-repitching of brewing yeast

Serial repitching involves cropping the yeast after fermentation, storing it for a short period, and then re-pitching into a fresh batch of wort (Powell and Diacetis, 2007). Depending on the yeast strain, the quality of the cropped slurry and company policies, brewer's yeast can be repitched between 8–15 generations (Powell et al., 2003; Powell and Diacetis, 2007). When brewing yeasts are serially repitched, their viability progressively decreases (Smart and Whisker, 1996; Jenkins et al., 2003) and this affects the quality of the beer produced. According to Jenkins et al. (2001) lager yeast strains begin to deteriorate after 10 serial repitchings.

Kobayashi, Shimizu and Shioya (2007) investigated the fermentation performance of yeast cells during serial repitching in yeast extract-dextrose medium with FAN levels the same as that of a low-malt beer. They reported that serial repitching resulted in a decrease in specific growth rate. In addition, increases in the levels of isoamyl alcohol (isopentyl alcohol), which causes undesirable flavour, and residual FAN were reported after multiple repitching. These workers further reported that serial repitching strongly affected the production of volatile compounds but not the fermentation performance in terms of sugar uptake and ethanol production.

Some ale strains have also been reported to be stable in the utilization of sugars when serially repitched over a long period of up to 98 serial repitchings (Powell and Diacetis, 2007). This was despite changes in the macro-morphological characteristics of the yeast occurring over the course of the 98 serial repitchings. Another study showed the net effect of wort osmolarity on brewing yeast and showed that serial repitching substantially reduced yeast sedimentation ability and also delayed the onset of the sedimentation (Sigler, Matoulková, Dienstbier and Gabriel, 2009). Serial repitching also resulted in a decrease in the maximum suspended cell count and slowed down fermentation. These serial-repitching effects were aggravated with increased osmotic pressure of the wort, the greatest effects occurring with 20° P wort.

It has been reported that yeast viability drops drastically as soon as fermentation stops and this affects the quality of repitching yeast (Pereira et al., 2011). To avoid the drop in yeast viability, it has been proposed that harvesting yeast cells for repitching must be done as soon as fermentation stops (Guimarães and Londesborough, 2008). The drop in yeast viability was found related to a critical ATP decline that occurred mainly at the late stages of fermentation. At this point, ethanol stress increased and due to low ATP production, the yeast cell failed to maintain reactions that are necessary to keep it viable.

Successive cropping and repitching of yeast can select for an undesirable sub-population enriched with elderly cells (Deans, Pinder, Catley and Hodgson, 1997; Powell et al., 2003). This is because older cells are naturally larger in size and thus they settle more rapidly and are more abundant in the lower region of the fermenter cone (Deans et al., 1997). To avoid cropping a population that consists mainly of older cells, specific cropping mechanisms have been proposed. For example, cropping can be done in two stages. The first crop

consisting of elderly cells may be discarded with the second crop which consists of middle aged and virgin cells used for subsequent fermentation (Powell et al., 2000). Alternatively, early cropping may be done where older yeast is removed from the cone prior to cooling so that some yeast remains in suspension (Loveridge, Ruddlesden, Noble and Quain, 1999).

Yeast storage is probably the most crucial factor in serial repitching (Briggs et al., 2004). The length of the storage period and conditions thereof, influence the physiological state, overall fermentation performance and flocculation behaviour (Rhymes and Smart, 2001). In order to maintain strain integrity, serial-repitched yeast is subjected to a number of quality assurance targets. Failure to meet these targets may result in scrapping of a certain percentage of the yeast or the whole crop being discarded (Lentini, Rogers, Higgins, Dawes, Chandler, Stanley and Chambers, 2003; Thiele and Back, 2007).

2.5.3 Yeast stress responses during brewing

Brewing yeast are exposed to a wide range of stresses during commercial fermentations (Gibson et al., 2007). These include changes in oxygen concentrations, osmotic potential, pH, ethanol concentration, nutrient availability and temperature (Briggs et al., 2004; Gibson et al., 2007). Therefore the ability of the yeast to survive or adapt to these fluctuations is crucial, not only for production of good quality beer but also for maintaining good fermentative capacity and viability in subsequent fermentations (Powell et al., 2000; Gibson et al., 2007). To survive these challenges, yeast cells have the ability to re-organize their genomic expression and hence change the patterns of cellular proteins and metabolites (Gasch and Werner-Washburne, 2002). Some survival genes are expressed under a variety of stresses and they are aptly termed “general response genes”, while others appear to be expressed under specific environmental conditions (Mager and De Kruijff, 1995; Ruis and Schüller, 1995).

The exposure to one type of stress may induce tolerance to other subsequent stresses that the yeast cell has not been exposed to (Briggs et al., 2004). Furthermore, yeast cells posses a global stress response (GSR) which is activated by a number of environmental stresses such as oxidative, pH, heat, osmotic stresses and nitrogen starvation (Ruis and Schüller, 1995; Martinez-Pastor, Marchler, Schüller, Marchler-Bauer, Ruis and Estruch, 1996). GSR allows survival in a non-specific manner and maintains cellular fecundity

(reproduction) while specific responses are activated (Ruis and Schüller, 1995; Martinez-Pastor et al., 1996).

Apart from the yeast's intrinsic survival mechanisms, other techniques such as genetic modification have been shown to improve yeast stress tolerance and ultimately fermentation performance (Dequin, 2001; Saerens, Duong and Nevoigt, 2010). In addition, more 'natural' approaches such as adaptive evolution have been introduced (Blieck, Toye, Dumortier, Verstrepen, Delvaux, Thevelein and Van Dijck, 2007). Adaptive evolution involves selection for brewing yeast variants with improved fermentation capacity under stressful conditions such as high osmotic strength. Ekberg, Rautio, Mattinen, Vidgren, Londesborough and Gibson (2013) reported that shorter fermentation times and beer with similar organoleptic properties were achieved when fermenting with a selected osmo-tolerant variant of an ethanol-tolerant strain.

2.5.4 Yeast metabolism

2.5.4.1 Ammonia degradation

As indicated, all nitrogen sources are converted into glutamate and glutamine before being used by yeast cells. These serve as nitrogen donors for all other nitrogen containing compounds in the cell (Schure et al., 2000). Both glutamate and glutamine can be synthesized directly using ammonia as the amino group, in two ways (Figure 2.2) (Schure et al., 2000). In the first pathway, glutamate is generated by coupling ammonia to α-ketoglutarate catalysed by the NADPH-dependent glutamate dehydrogenase. Subsequently, glutamate and ammonia are converted into glutamine by glutamine sythetase (GS). In the second pathway, glutamine is produced by GS and glutamate is generated via glutamate synthase (GOGAT) which converts one molecule of glutamine and one molecule of α-ketoglutarate into two molecules of glutamate.

2.5.4.2 Lysine degradation

The degradation of lysine is more complex than that of ammonia. Its degradation involves acetylation of the 6-amino group to the initial product N^6-acetyl-L-lysine (Figure 2.3) (Large and Robertson, 1991; Bode, Thurau and Schmidt, 1993). This is followed by transamination of the N^6-acetyl-L-lysine and oxidative decarboxylation of 2-keto-acetamidocaproate to 5-acetamidovalerate. The latter is deacetylated to 5-

acetamidovalerate followed by transamination to glutarate semialdehyde with a loss of the second amino group and subsequent oxidation to glutarate.

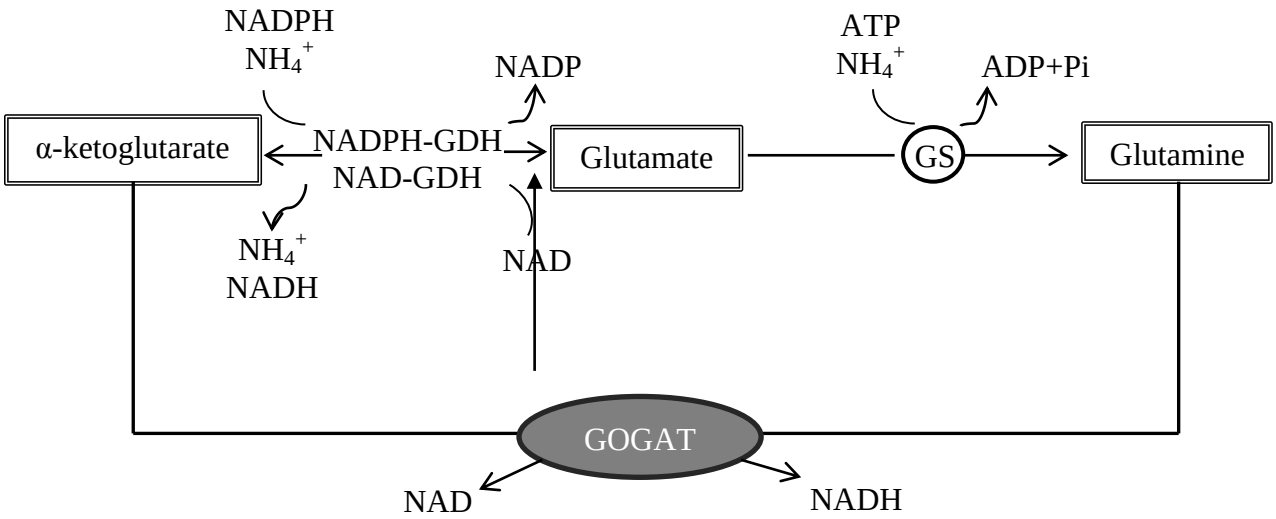

Figure 2.2. Interconversion of ammonia, α-ketoglutarate, glutamate and glutamine in yeast cells (adapted from Schure et al., 2000). GS: glutamine synthetase

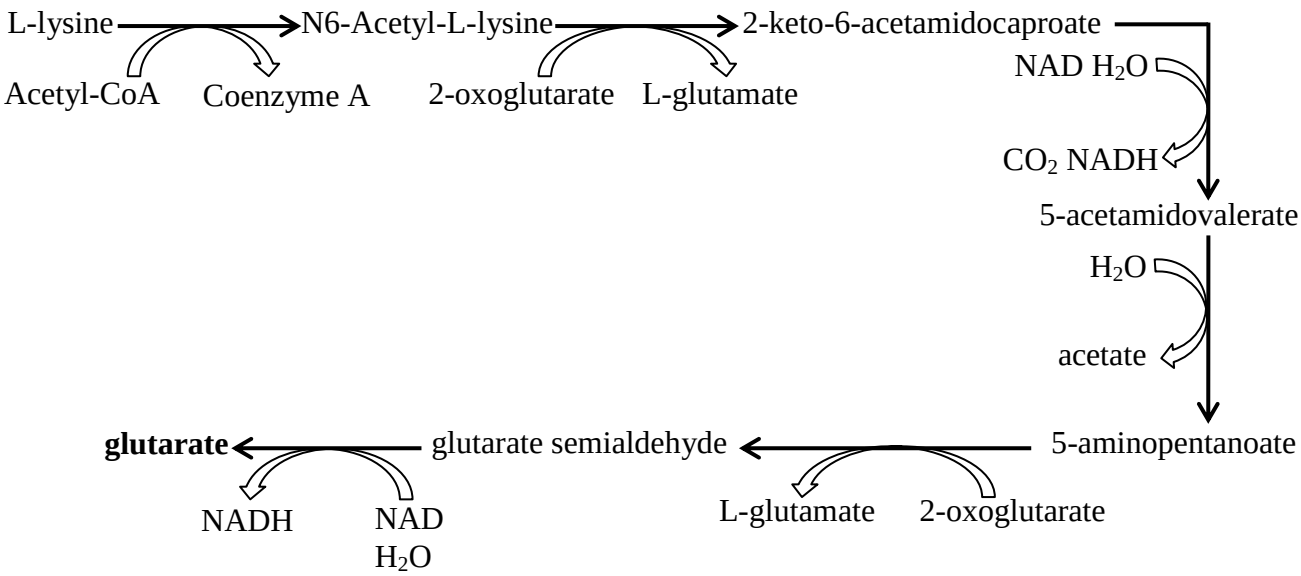

Figure 2.3. Pathway for the degradation of lysine in *Saccharomyces cerevisiae* (adapted from YeastCyc)

2.5.4.3 Respiration and fermentation

In this study, two metabolic processes will be investigated in yeast - respiration using Phenotype Microarray and fermentation. During both processes, yeast cells metabolise glucose molecules to yield pyruvate, either under aerobic or anaerobic conditions (Figure 2.4) (Faria-Oliveira, Puga and Ferreira, 2013). In alcoholic fermentations, pyruvate is decarboxylated to give acetaldehyde and carbon dioxide by pyruvate decarboxylase (Pdc1p). Acetaldehyde is then reduced to yield ethanol and carbon dioxide in a reaction catalysed by alcohol dehydrogenase (Adhp). In aerobic respiration, pyruvate is converted to Acetyl-CoA catalysed by the pyruvate dehydrogenase multi enzyme complex. The Tricarboxylic acid cycle (TCA) is initiated and it supplies the respiratory chain with reducing equivalents in the form of Nicotinamide Adenine Dinucleotide Hydrogen (NADH) and Flavin Adenine Dinucleotide ($FADH_2$) obtained from the oxidative decarboxylation of Acetyl-CoA. In addition to the generated energy, the final products of respiration are carbon dioxide and water.

Although brewing yeast is able to both respire and ferment, it generally does not respire in wort due to the Crabtree effect (Faria-Oliveira et al., 2013). This phenomenon occurs in the abundance of fermentable sugars and it causes aerobically grown yeast to suppress the respiratory pathway in favour of fermentation. Generally, all fermentable sugars induce the Crabtree effect, but glucose exhibits the strongest effect.

Instead of anaerobic respiration, aerobic fermentation occurs in the early stages of beer fermentation. This is because aerobic fermentation can occur in the abundance of sugar and oxygen (Faria-Oliveira et al., 2013). The importance of oxygen for a healthy fermentation has been highlighted (Verbelen, Saerens, Van Mulders, Delvaux and Delvaux, 2009). Growing cells under aerobic conditions is, therefore, crucial for their subsequent survival under anaerobic fermentation. This is because yeast uses dissolved oxygen in the biosynthesis of unsaturated fatty acids and sterols (Boulton and Quain, 2001). These are essential components of the cell membrane and the yeast cell ceases to bud when their levels are too low. The shortage of sterols and fatty acids may result in weak cells and may subsequently cause stuck fermentations.

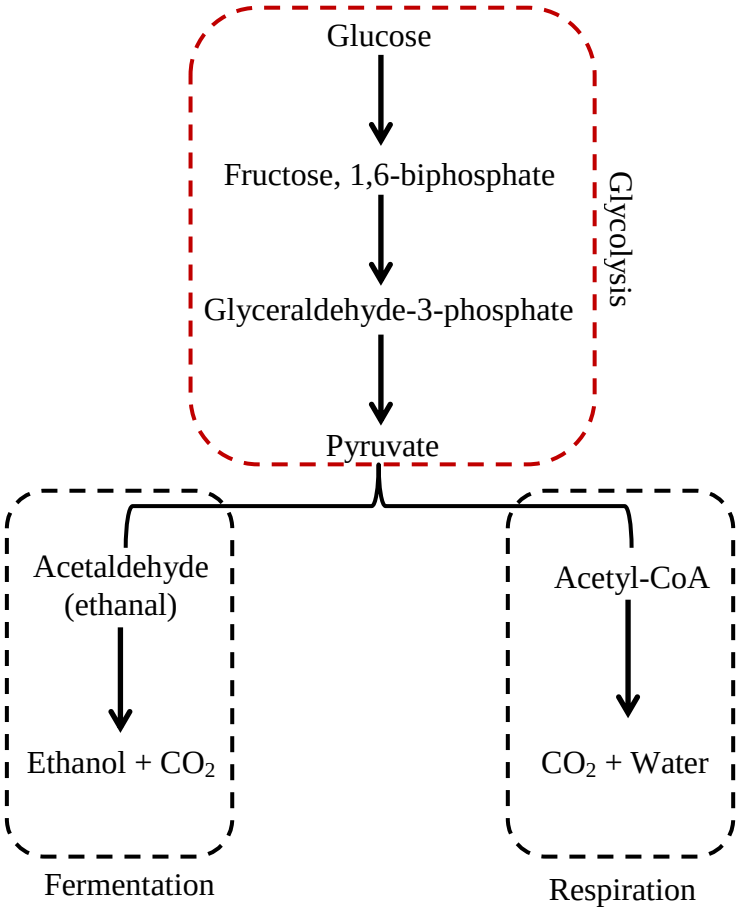

Figure 2.4. Metabolism of glucose during fermentation and respiration (adapted from Faria-Oliveira et al., 2013)

2.6 Tools for assessment of brewing yeast physiology and genotype

2.6.1 Scanning electron microscopy

Scanning electron microscope (SEM) is a powerful tool that is used to study surface morphology and topography on samples (Börjesson, 2006). The SEM obtains an image by scanning an electron beam accross a surface thereby generating different signals which are emitted from the area of the specimen. The signals are then amplified and used to form an image. The spacial resolution of the SEM is better than that of the optical microscope, hence SEM can generate high resolution images.

During fermentation, yeast cells are subjected to different environmental conditions that are constantly changing such as temperature, osmolarity, oxygen, pH, ethanol and nutrients (Pratt, Bryce and Stewart, 2007). Yeast cells must cope with these changes in order to ensure continued growth and metabolic activity. To achieve this, yeast cells respond to the

different stress situations through a variety of cellular responses. One of them, which can be observed using scanning electron microscopy (SEM), is morphological alterations. *S. cerevisiae* yeast cells are described to be spheroidal, subglobose, ovoid, ellipsoidal or cylindrical to elongate (Briggs et al., 2004). They occur singly or in pairs and are occasionally in short chains or clusters.

Using SEM, Shimada, Andou, Naito, Yamada, Osumi and Hayashi (1993) investigated the effect of hydrostatic pressure on the cell surface of *S. cerevisiae* at room temperature. These workers reported that pressures above 500 MPa caused damages to the bud area in at least 20-30% of the cells. In other SEM work, Bonin, Wzorek and Koper (2006) investigated the effect of long-term continuous wine fermentation of high-sugar fruit must on yeast immobilized on foam glass. These authors reported that yeast cells at the end of the fermentation were of various shapes. Some cells were elongated, others were in the form of few connected cells while others were "pear" shaped. Cell elongation, which results from cells increasing their surface area to volume ratio, is thought to be a mechanism of the cell to find nutrients, particularly glucose (Hill and Robinson, 1988; Bonin and Skwira, 2008). Further, Bonin et al. (2006) observed wrinkled or folded cells with irregular rough surfaces. This phenomenon was also reported by Martınez-Rodrıguez, Polo and Carrascosa (2001) after a few months of aging yeast in wine. They attributed the wrinkling and folding of the cells to autolysis which destroys the cell membrane. Since the cell membrane is the osmotic barrier of the cell, turgor is lost leading to a decrease in cell diameter. Autolysis is, however, affected by the physiological conditions of the cell, culture and age.

Fermentation by-products have also been reported to cause changes on yeast morphology. For example, when yeast cells are exposed to acetic acid they become elongated, irregular and daughter cells fail to separate from mother cells after cell division (Maiorella, Blanch and Wilke, 1983). These changes can be monitored with SEM. Acetaldehyde reportedly increases the yeast cell size to over twice their normal size and also interferes directly with cellular transport processes (Barber, Vriesekoop and Pamment, 2002).

2.6.2 Phenotype Microarray

Phenotype Microarray (PM) technology is a high throughput system that allows analysis of cellular phenotypes using the Biolog system (Bochner, Gadzinski and Panomitros, 2001).

Phenotypes are observable characteristics of a cell. The PM system can test nearly 2000 distinct traits simultaneously by using twenty 96-well microplates, with each well testing a different cellular phenotype (Bochner, 2003). It gives a comprehensive scan of the physiology of the cell and allows phenotypes to be recorded and stored electronically to facilitate comparison over time. The PM system uses tetrazolium redox chemistry to colorimetrically detect the respiration of cells (Bochner et al., 2001). Respiring cells reduce the tetrazolium dye, resulting in the formation of a purple colour that accumulates in the well over time. Non-respiring cells do not produce any colour change and this implies that the cells are not active and the specific substrate is not metabolized.

PM can be used to directly assay the effects of genetic changes on cells, mainly gene knock-outs (Bochner et al., 2001), characterization of microorganisms (Greetham, Wimalasena, Kerruish, Brindley, Ibbett, Linforth, Tucker, Phister and Smart, 2014a), testing catabolic pathways in the cell for carbon, nitrogen, phosphorus and sulphur, as well as biosynthetic pathways (Bochner, 2003). Praphailong, Van Gestel, Fleet and Heard (1997) evaluated the potential of the Biolog system for the identification of food-associated yeasts. The Biolog system correctly identified 49 of 72 yeast strains.

Identification of microorganisms with the PM system is normally through differences in the utilization of carbon sources (Konopka, Oliver and Turco Jr., 1998). However, the PM system may fail to correctly identify strains that fail to grow or cells that show abnormal substrate utilization. Bolat, Walsh and Turtoi (2008) used the PM system to compare the catabolism of carbon sources and nitrogen sources of two new lager yeast strains. These workers reported that the metabolic profiles of the two lager strains were similar on both carbon and nitrogen source. Salinas, Mandaković, Urzua, Massera, Miras, Combina, Angelica Ganga and Martínez (2010) investigated the metabolism of carbon compounds and a number of kinetic and fermentative parameters by three yeast strains using PM. The strains showed very similar phenotypes for the fermentation profiles with only one strain showing the ability to ferment galactose.

In other studies, Greetham (2014) and Greetham, Takagi and Phister (2014) used the PM system to investigate the protective effect of proline on weak acid stressed *S. cerevisiae*. These workers reported that cells that had high intracellular proline levels were more tolerant to weak acids than cells that were unable to synthesize proline. The tolerance to

weak acids was proline specific and also dependent on concentration. Greetham et al. (2014a) modified the Biolog PM plate to test the sensitivity of yeast strains to a number of inhibitory products present in lignocellulosic material. The modified PM assay showed that some inhibitors were more toxic when used in combination than on individual challenge.

2.6.3 Microsatellite genotyping

The high genetic diversity that occurs with *S. cerevisiae* strains has been monitored through multiple analyses at the molecular level (Schuller , Valero, Dequin and Casal, 2004). Different methods are used for the analysis of polymorphism, including karyotype analysis, δ sequence typing, mtDNA restriction analysis and microsatellite genotyping (Schuller et al., 2004). In this review, microsatellite genotyping will be discussed. Microsatellites, also known as "short tandem repeats", are simple tandemly repetitive DNA sequences that are found in abundance in eukaryotic genomes (Richard, Hennequin, Thierry and Dujon, 1999; Walczak, Czaplinska, Barszczewski, Wilgosz, Wojtatowicz and Robak, 2007). The PCR-fingerprinting method based on the microsatellite primer $(GTG)_5$ has been mainly used to characterise yeast population dynamics over the fermentation period (Esteve-Zarzoso, Peris-Torán, Garcia-Maiquez, Uruburu and Querol, 2001; da Silva-Filho et al., 2005). This is because modification in yeast populations occurs in response to changes in environmental conditions or by the input of wild yeast cells that may come from the main substrate (da Silva-Filho et al., 2005).

The $(GTG)_5$ primer triggers site-specific annealing and initiates PCR amplifications of genomic segments which are flanked by inversely oriented and closely spaced repeat sequences (Lieckfeldt, Meyer and Börner, 1993). Schuller et al. (2004) investigated the genetic polymorphism by different molecular methods in 23 commercial wine yeast strains. These workers concluded that microsatelite typing has similar discriminatory power to other methods such as mtDNA restriction analysis and karyotyping. In another study using *Escherichia coli*, the robustness of five rep-PCR methods such as REP-RCR, ERIC-PCR, ERIC2-PCR, BOX-PCR, and the $(GTG)^5$-PCR were evaluated to discriminate between 271 strains (Mohapatra and Mazumder, 2008). The $(GTG)_5$-PCR method was found to be the most robust molecular tool for differention of the *E. coli* population.

Differences in the rRNA internal transcribed spacer (ITS) has also been used to identify yeast species (Heras-Vazquez, Mingorance-Cazorla, Clemente-Jimenez and Rodriguez-Vico, 2003). This region is where the 5.8S rRNA gene is located. It is flanked by two variable and non-coding regions ITS1 and ITS2, which show low intraspecific variability and high interspecific polymophism (Esteve-Zarzoso, Belloch, Uruburu and Querol, 1999). The ITS1 and ITS4 primers are used to amplify this region of the rRNA gene repeat unit (Guillamón, Sabaté, Barrio, Cano and Querol, 1998). To achieve greater polymorphism, the amplified PCR products are treated with restriction enzymes such as C*fo*I, *Hin*fI and *Hae* III for identification of yeasts at species level (Esteve-Zarzoso et al., 1999; Granchi, Bosco, Messini and Vincenzini, 1999). Further, the ITS1 and ITS4 primers can be sequenced to either confirm or identify isolates which gave ambiguous results (Clemente-Jimenez, Mingorance-Cazorla, Martínez-Rodríguez, Heras-Vázquez and Rodríguez-Vico, 2004; Combina, Elía, Mercado, Catania, Ganga and Martinez, 2005).

2.7 Conclusions

Considerable work has been carried out to improve brewing yeast health in high gravity brewing. One such method is supplementation of wort with various nitrogen sources. These improve yeast growth and yeast fermentation performance. Most studies have, however, been done using synthetic media or all-malt barley malt wort and little work has been done using sorghum wort. The potential of sorghum as a material for lager beer brewing has been proven. However, brewing with sorghum comes with challenges, particularly the low FAN levels associated with brewing with unmalted sorghum grain. Yeast fermentation performance is affected by the type and complexity of nitrogen source, its concentration and yeast strain. Identification of nitrogen sources that support good yeast fermentation performance in the correct medium is required. This can be achieved through assessing the morphological, phenotypic and genotypic changes that occur in yeast during fermentation on sorghum supplemented with different nitrogen sources.

3: HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Mashing white tan-plant (non-tannin) sorghum with commercial protease enzymes will produce more FAN than mashing white Type II tannin sorghum with commercial protease enzymes. This is because the latter type of sorghum contains condensed tannins which are associated with reduced protein digestibility in sorghum (Duodu et al., 2002) due to irreversible binding to proteins, probably through hydrogen bonding (Murray, Williamson, Lilley and Haslam, 1994).

Supplementing sorghum grain wort with different nitrogen sources will improve FAN levels and yeast fermentation performance in terms of yeast viability, yeast growth, sugar utilization and ethanol production. This is because nitrogen supply strongly influences yeast growth and fermentation improvement depends on the correct type of nitrogen source in the fermenting medium (Cruz et al., 2003). For example, some amino acids such as glutamine and also ammonia are more preferred by yeast and their presence in wort prevents the uptake and utilization of other less preferred amino acids such as proline (Deed et al., 2011).

If brewing yeast cells in their sixth brewing cycle are serially repitched up to nine brewing cycles using sorghum wort, morphological and genotypic alterations will occur in the yeast cells. This is because serial repitching will induce nutritional stresses and the yeast will adapt by changing its morphology, for example cell elongation (Bonin et al., 2006) and DNA copy number (Infante, Dombek, Rebordinos, Cantoral and Young, 2003). Also, mutations such as aneuploidy can increase the number of beneficial genes to protect cells against lethal or deleterious alleles (Puig, Querol, Barrio and Pérez-Ortin, 2000).

3.2 Objectives

To determine the effect of sorghum type and commercial protease addition on the production of FAN from sorghum grain and malt during mashing.

To determine the effect of adding different nitrogen sources to sorghum grain worts on yeast fermentation performance

To determine the effect of serial repitching on the morphological and genotypic constitution of the yeast cells when brewing with sorghum grain

4: RESEARCH

4.1 Effects of Sorghum Type, Malting and Soya addition on Production of Free Amino Nitrogen in conjunction with Exogenous Protease Enzymes

4.1.1 Abstract

Sorghum types suitable for brewing and bioethanol are required. The effects of sorghum type (white non-tannin versus white type II tannin) and defatted soya flour on free amino nitrogen (FAN) production from sorghum grain and malt using exogenous protease enzymes was investigated over extended incubation at moderate temperature (45 $^{\circ}$C).

In the absence of exogenous proteases, unmalted white non-tannin sorghum grain produced substantially higher levels of FAN than unmalted white type II tannin sorghum grain. Incubating sorghum grain with neutral proteinase and amino-peptidase in combination, improved FAN production. The two sorghum types (non-tannin and type II tannin) produced similar FAN levels when malted and incubated in the absence of the exogenous proteases. When both sorghums were malted and incubated with neutral proteinase alone substantially more FAN resulted (124-126 mg/100 g sorghum) than with unmalted grains (61-84 mg/100 g sorghum). The combination of amino-peptidase and proteinase did not improve FAN further. Neither, did malting influence wort free amino acid profile. Group B amino acids constituted the highest percentage (42-47%). Incubating defatted soya flour with neutral proteinase substantially increased FAN production by up to 376 mg/100 g soya.

For incubations with unmalted grain, white non-tannin sorghum with proteinase and amino-peptidase treatment yielded the highest FAN. For incubations with malted grain, both white non-tannin and white type II tannin sorghums with proteinase treatment yield the highest FAN.

4.1.2 Introduction

The use of sorghum either as malt and/or raw (unmalted) grain for the large-scale brewing of lager and stout beers or malt non-alcoholic beverages is a major industry in several African countries (Mackintosh and Higgins, 2004; Ogbonna, 2011; Taylor, Dlamini and Kruger, 2013). In other countries, such as the USA, the market for sorghum beers is rising due to its "gluten-free" advantage (Taylor et al., 2013) and sorghum grain is a major feedstock in bioethanol production (Zhao, Bean, Wu and Wang, 2008). The use of sorghum, however, comes with challenges. Notable among these is insufficient free amino nitrogen (FAN), particularly when raw (unmalted) grain is used (Bajomo and Young, 1993). For example, FAN levels of up to 150 mg/ L are required during high gravity brewing (Beckerich and Denault, 1987). Low levels of FAN during fermentation may lead to incomplete or protracted fermentations since nitrogen is utilized by yeast to synthesize new cellular and enzymic proteins (Pierce, 1987). Peptides are also consumed by yeast during fermentation depending on the peptide type but generally at a slower rate than amino acids (Lekkas, Hill, Taidi, Hodgson and Stewart, 2009).

The low FAN levels with sorghum grain are attributable to the low digestibility of the sorghum prolamin protein (kafirin) upon cooking. This is thought to be due to the cross-linking of kafirin by disulphide bonding (Ng'andwe et al., 2008). This phenomenon is varietal as genetically modified sorghum types with reduced levels of γ-kafirin yield higher levels of FAN than normal sorghums (Kruger, Oelofse, Taylor and Taylor, 2012). A number of approaches have been applied to improve the digestibility of sorghum protein (Makokha, Oniang'o, Njoroge and Kinyanjui, 2002; Ng'andwe et al., 2008; Mugode, Portillo, Hays, Rooney and Taylor, 2011) and hence improve wort FAN. Supplementation of sorghum flour with soy protein concentrate improves protein content (Awadalkareem et al., 2008), and soy protein hydrolysates reportedly improve yeast fermentation performance (Zhao et al., 2014).

Cultivar differences in barley malt brewing are a major cause of variability in beer wort quality (Bamforth, 2008) and the same has been reported for sorghum (Owuama, 1999). In different parts of Africa, various types of sorghum are used for the production of lager beers. For example, in Nigeria malted white Type II tannin sorghum is often used (Taylor, 2003), while non-tannin white tan-plant sorghum is used in Eastern and Southern Africa in the form of raw grain (Mackintosh and Higgins, 2004). A recent study in our laboratory

reported wide variations (31–139 mg/ L) in wort FAN from grain of different sorghum cultivars (Adetunji, Khoza, de Kock and Taylor, 2013). Similarly, malt FAN levels were found to be substantially influenced by variety in 11 Botswana sorghum cultivars (Mokhawa, Kerapeletswe-Kruger and Ezeogu, 2013). White tan-plant sorghum is notably low in phenolics, whereas white type II tannin sorghum contains some condensed tannins (proanthocyanidins), which are beneficial by being protective against biotic stresses during cultivation (Butler, Riedl, Lebryk and Blytt, 1984).

The objective of this study was to compare how these sorghum types influence FAN production in raw grain and malt in conjunction with the addition of exogenous proteases, with the aim of supporting rapid fermentation.

4.1.3 Materials and Methods

4.1.3.1 Grains and Malts

Raw grain and malt (with external roots and shoots removed) of white Type II tannin (WT) sorghum (cultivar Gadam el Hamam type) and of white-tan plant sorghum (W) (cultivar Macia) were sourced from Zimbabwe and kindly provided by the South African Breweries. The malt was produced commercially in a pneumatic malting, under standard sorghum malting conditions. The moisture content of the sorghums was 12.5 g/ 100 g. Toasted defatted soy flour was obtained from Nedan Oil Mills, Mokopane, South Africa. The grains and malts were hammer milled in a laboratory mill (Falling Number, Huddinge, Sweden) fitted with a 1.2 mm opening screen and the flour was stored in zip-lock type polythene bags at 6-8 °C.

4.1.3.2 Enzymes

Two types of commercial proteolytic enzymes: an amino-peptidase (Flavourzyme 500 MG) (Novozymes, 2007a) and a neutral proteinase (Neutrase 1.5 MG,) (Novozymes, 2007b) were kindly provided by Novozymes SA, Benmore, Johannesburg, South Africa. The amino-peptidase had an enzyme activity of 500 Leucine Amino Peptidase Units/gram (LAPU/g), while that of the neutral proteinase was 1.5 Anson Units/gram (AU/g).

4.1.3.3 Incubation of sorghum

Milled malted or unmalted sorghum or soya flour (37.7 g, dry basis) was mixed with distilled water (360 mL) pre-heated to 45 °C contained in stirred stainless-steel beakers in a BRF Mashing Bath (Brewing Research Foundation, Nutfield, UK). The contents of the beakers were stirred continuously by magnetic stirring at a speed which maintained the grain/malt in suspension. The pH was measured and adjusted with phosphoric acid to ensure that it was between pH 5.5-6.0 for all the treatments. This range was within the pH optimum of the neutral proteinase. Freshly prepared neutral proteinase (1 mg/ kg sorghum, 5 mg/ kg sorghum and 10 mg/ kg sorghum) or amino-peptidase enzyme (1 mg/ kg sorghum) solutions were added. The enzymes were prepared in water, as would be done in brewing, and the beakers were closed with watch glasses during the incubation period. The samples were incubated isothermally at 45 °C which is within the optimum temperature range (45-55°C) for the neutral proteinase. Temperature was monitored during mashing. Beakers were covered with watch glasses during incubation. At intervals, 0, 1, 2, 4, 6 and 24 h aliquots (20 mL) were removed, centrifuged at 6400 g for 10 min (4 °C) to obtain a clear supernatant for FAN determination. In another experiment, a mixture (1:1) of the two enzymes was added (1 mg/ kg sorghum, in total) to determine if there was any synergistic effect since neutral proteinase is an endoprotease enzyme while amino-peptidase is an exopeptidase enzyme.

4.1.3.4 DP, moisture and protein content

Malt diastatic power with peptone and water extractions, was performed according to the South African Bureau of Standards Method 235 (SABS, 1970). Protein content was determined by combustion analysis according to AACC Method 46-30 and moisture content was determined according to AACC Method 44-15A (AACC, 2000).

4.1.3.5 Free amino nitrogen

Free amino nitrogen was determined using the European Brewery Convention ninhydrin colorimetric assay (EBC, 1998) with glycine as standard. The results were expressed as mg FAN /100 g sorghum (dry basis).

4.1.3.6 Free amino acids

Wort free amino acids were determined on freeze dried samples by using the PICO.TAG-method (Bidlingmeyer, Cohen and Tarvin, 1984). The wort samples were not hydrolysed

with HCl. Amino acids were analysed using a PICO.TAG C18 (3.9 mm x 300 mm) column for free amino acids (Waters, Millipore Corp., Milford, MA). Wort free amino acid analyses were carried out on the white tan-plant sorghum grain and malt because of its reported potential to produce high FAN levels (Adetunji et al., 2013).

4.1.3.7 Total phenols and condensed tannins

Total phenolic content was determined using a modified (extraction with 1% v/v HCl in methanol) Folin-Ciocalteu method (Waterman and Mole, 1994). Condensed tannins were determined using the modified Vanillin HCl method (extraction with 1% v/v HCl in methanol) of Price, Van Scoyoc and Butler (1978).

4.1.3.8 Statistical analyses

All experiments were repeated at least once and the means of closely agreeing replicates are reported. Single factor analysis of variance (ANOVA) was used to determine the effect of proteolytic enzymes on FAN production. Multifactor ANOVA was used to determine the effect of the malting, temperature and cultivar on FAN. ANOVA was performed using Statistica software for Windows, version 10 (Tulsa, OK).

4.1.4 Results and discussion

4.1.4.1 Effect of neutral proteinase enzyme on production of FAN from soya flour

The effects of neutral proteinase concentration on FAN production were investigated using defatted toasted soya flour. Defatted soya flour was chosen for its high protein content (53% w/w db) and relatively high lysine levels. Lysine, a group A amino acid is important for yeast growth (Lekkas et al., 2007).

The addition of neutral proteinase to soya flour substantially increased wort FAN levels with the highest increase being observed at 10 mg/ kg of the enzyme to flour (Figure 4.1). The highest FAN production with neutral proteinase addition was observed within the first 4 h of incubation for all the levels. Thereafter, FAN levels rose more slowly. There was no significant increase in FAN levels of the control sample as no enzyme had been added. Compared to the control, incubating soy flour with the neutral proteinase at 1 mg/ kg increased FAN levels by approximately 2-fold (269 mg/100 g) after 24 h.

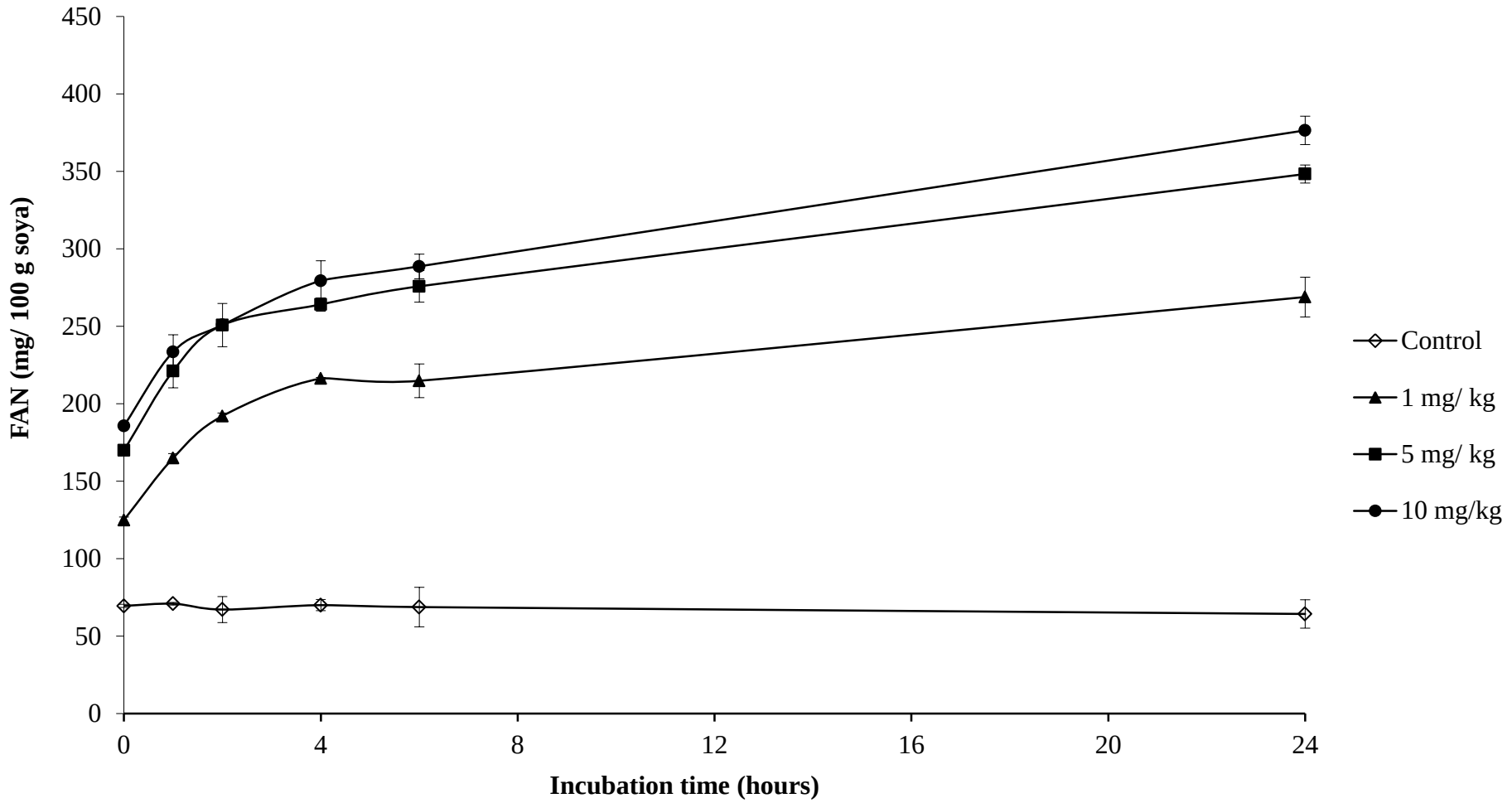

Figure 4.1. Effect of neutral proteinase enzyme concentration on production of FAN from defatted soya flour incubated at 45 °C for up to 24 h. Error bars indicate standard deviations. (n = 2)

An increase in neutral proteinase concentration to 5 mg/ kg soya produced 348 mg FAN/100 g soya, an approximately 3-fold increase. A further increase of the enzyme concentration to 10 mg/ kg soay produced 376 mg FAN/100 g soya, and only a minor further increase. The high concentration of FAN can be attributed to the high level of digestible protein in soya flour (Awadalkareem et al., 2008). The high initial FAN levels with the neutral proteinase treatment could be due to the enzyme preparation as enzymes are proteins. Incubating with the highest concentration of enzyme did not produce proportionally higher levels of FAN compared to 5 mg/ kg enzyme concentration. This could be due to substrate becoming limiting.

4.1.4.2 Sorghum type composition

Both sorghum types had similar low protein contents (6.0-7.0 g/ 100 g) and were thus comparable for studying FAN production (Table 4.1). A higher protein content of up to 12.4 g/ 100 g has been reported for regular white sorghum (Pérez-Carrillo and Serna-Saldívar, 2007). The W and WT malts both had lower protein contents than their corresponding raw grains. The level of total phenols in WT raw grain and malt was 3-4 times that in W because of the tannins in the former. Further, as expected there were no detectable tannins in W raw grain but significant levels (approx. 2.0 g/ 100 g) in WT. However, this level is substantially lower than that in high tannin (Type III) sorghum (Chiremba, Taylor and Duodu, 2009). As has been reported elsewhere (Beta, Rooney, Marovatsanga, and Taylor, 2000), malting caused a substantial reduction in the assayable tannins in WT. The reduction in tannins during malting was thought to be due to the reduction in assayable tannins during steeping in water (Beta et al., 2000). The peptone extract DP (potential amylase activity) of the WT malt was far less (13 SDU/ 100 g less) than that of the water extract DP (actual amylase activity). The low water extract DP shows that the tannins remaining still substantially inhibited the amylase activity of the malt. However, the water extract DPs of the WT and W were similar, also showing that the malts of the two sorghum types were comparable.

Table 4.1. Protein, total phenols, diastatic power and tannin contents of the sorghum types

Sorghum type	Protein (N X 6.25) (g/ 100 g dwb*)	Total phenols (g catechin equiv. /100 g dwb)	Tannin (g catechin equiv. /100 g dwb)	Diastatic Power
White tan-plant grain	6.58[c] (0.4)	0.28[a] (0.6)	ND	NA
White tan-plant malt	6.27[b] (0.6)	0.22[a] (0.2)	ND	11P, 10W
White type II tannin grain	7.04[d] (0.3)	0.95[c] (0.1)	1.98[c] (0.12)	NA
White type II tannin malt	5.97[a] (0.1)	0.70[b] (0.1)	0.79[b] (0.16)	21P, 8W

Figures in parentheses indicate standard deviations; ND – not detected; NA – not applicable; *dry weight basis; P- peptone extract; W – water extract; values with different letter superscripts in a column are significantly different (n=2)

4.1.4.3 Effect of raw grain sorghum type on FAN production with neutral proteinase

Neutral proteinase was selected based on its pH optimum range of between 6 and 8 and its activity at relatively low temperature (optimum at 50 °C) (Adetunji et al., 2013), enabling it to be active in mashing (Dale, Young and Omole, 1990; Agu and Palmer, 1997) and in fermentation applications (Kawa-Rygielska and Pietrzak, 2014). In general, increasing the concentration of neutral protease resulted in a corresponding increase in FAN production with both the W and WT raw grain (Figure 4.2). Free amino nitrogen production from W was significantly higher than that from WT sorghum grain for all three enzyme concentrations investigated.

Increasing the enzyme concentration from 1 mg/ kg sorghum to 10 mg/ kg resulted in 3- and 4-fold increases in FAN levels with W and WT sorghum grains, respectively. Overall, total wort FAN levels for all the WT sorghum grain enzyme treatments were substantially lower by approx. 35, 14 and 19% at 1 mg/ kg, 5 mg/ kg and 10 mg/ kg, respectively, when compared to corresponding enzyme treatment for the W grain.

The low FAN levels that occurred with WT sorghum grain in the absence of the proteinase enzyme indicates that the WT sorghum grain had inherently low levels of available free amino acids, dipeptides and tripeptides when compared to the W sorghum. In addition, the presence of tannins in the WT sorghum (Table 4.1) may have contributed to the low FAN levels by inhibiting endogenous enzymes. The WT sorghum without the addition of neutral proteinase produced substantially higher FAN levels. This means that the proteins in the WT sorghum were somewhat digestible despite the presence of tannins. Also, during incubation, some of the tannins may have leached into the incubation medium and hence partly released bound proteins. The reduction of total phenols, total flavonoids and tannins in sorghum after soaking has been reported by Afify, El-Beltagi, El-Salam and Omran (2012).

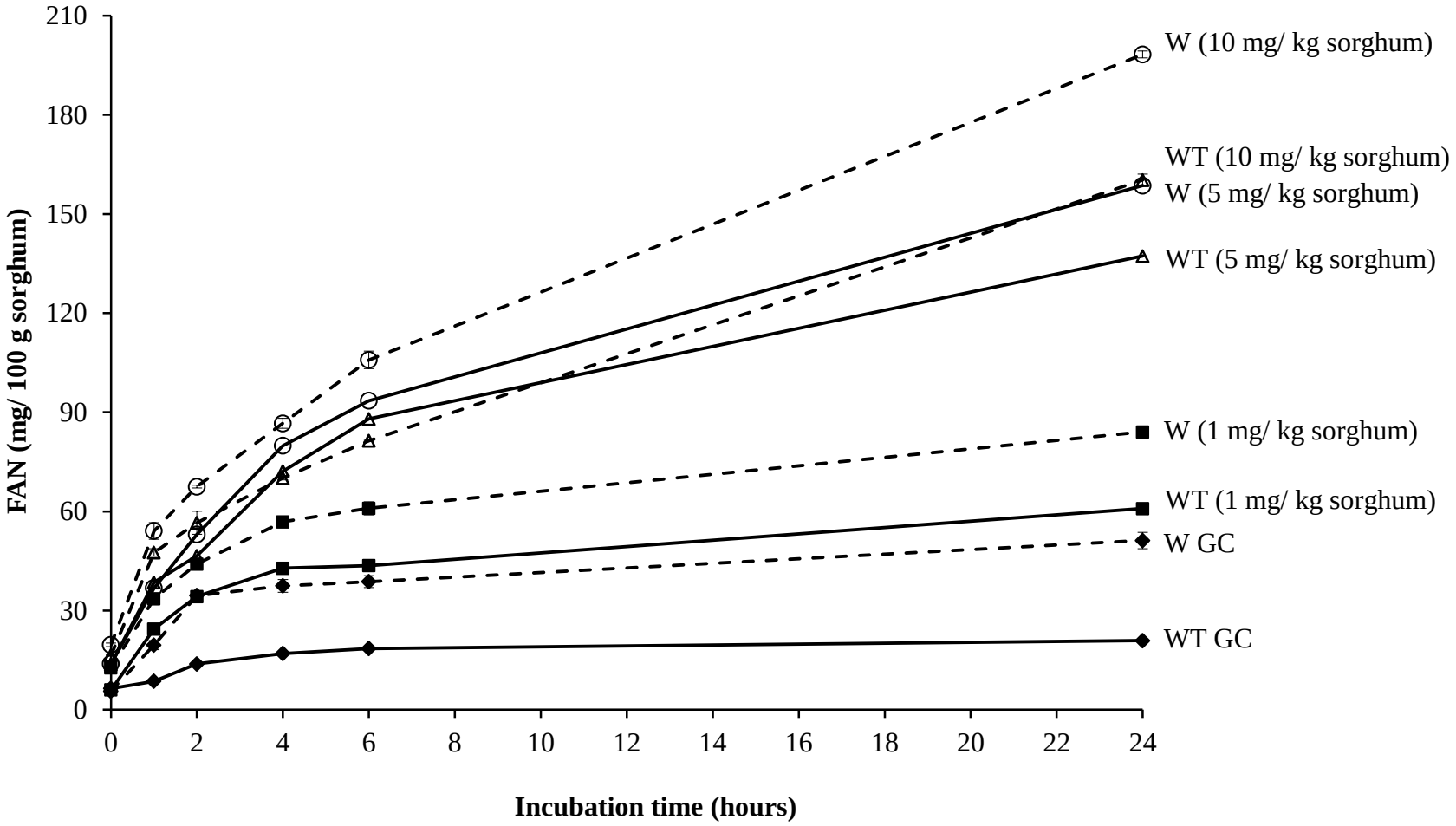

Figure 4.2. Effect of sorghum type on FAN production when incubating raw sorghum grain with neutral proteinase enzyme for up to 24 h at 45 °C. W - white tan-plant sorghum; WT - white type II tannin sorghum; GC – grain control. Error bars indicate standard deviations (n = 2).

The lower FAN levels from WT sorghum when compared to W sorghum in the presence of the proteinase can be attributed to the inherently low levels of available free amino acids and small molecular weight peptides in the WT grain. It is also probable that the proteolytic profile of the WT sorghum was lower than that of the W grain. Differences in the proteolytic profiles among sorghum grains has been reported by Mokhawa, Ralefala, Bulawayo and Ezeogu (2009). Also, the protein digestibility of WT sorghum may have been lower than that of the W sorghum grain due to the presence of tannins. Tannins are associated with reduced protein digestibility in sorghum, owing to their irreversible binding to proteins (Murray et al., 1994; Scalbert, Déprez, Mila, Albrecht, Huneau and Rabot, 2000). Such bonding will take place in the aqueous environment of incubation. Wu, Zhao, Bean, Seib, McLaren, Madl, Tuinstra, Lenz and Wang (2007) reported that the protein digestibility of tannin sorghum was approx. 28% while a digestibility of more than 50% was measured with normal (non-tannin) sorghum. In addition, as shown in Figure 4.2 the tannins also inhibited enzymes (Pérez-Carrillo and Serna-Saldívar, 2007) and thus impeded sorghum protein hydrolysis. In fact, this work shows that even a very high concentration of exogenous protease addition (10 mg/ kg) did not completely overcome the tannin inhibition.

4.1.4.4 Effect of malted sorghum type on FAN production

Sorghum malt produced substantially more FAN (up to 59 mg/100 g sorghum) than sorghum grain (up to 46 mg/100 g) when incubated in the absence of neutral proteinase (Figure 4.3). This was due to the action of the endogenous proteinase and peptidase enzymes in the malt on the protein reserves of the sorghum malt (Dewar et al., 1997). For example, there is substantial carboxypeptidase activity in sorghum malt but not in the grain (Evans and Taylor, 1990). This enzyme is important in the hydrolysis of peptide products of endoprotease cleavage of prolamins into free amino acids (Simpson, 2001). Malting sorghum has also been reported to increase the nitrogen solubility index (Elkhalifa and Bernhardt, 2010). This is thought to be due to the gradual hydrolysis of reserve proteins into amino acids and small peptides, which is triggered by an increase in the level of protease enzymes during malting. In addition, malting sorghum has been reported to increase other protein quality attributes such as the protein content. According to Dewar (2003), the germination phase of the malting process can increase the total protein content by about 2.8-4.7% after 5 days. However, in the present study the protein content did not improve after malting, hence it did not contribute to the increase in FAN production.

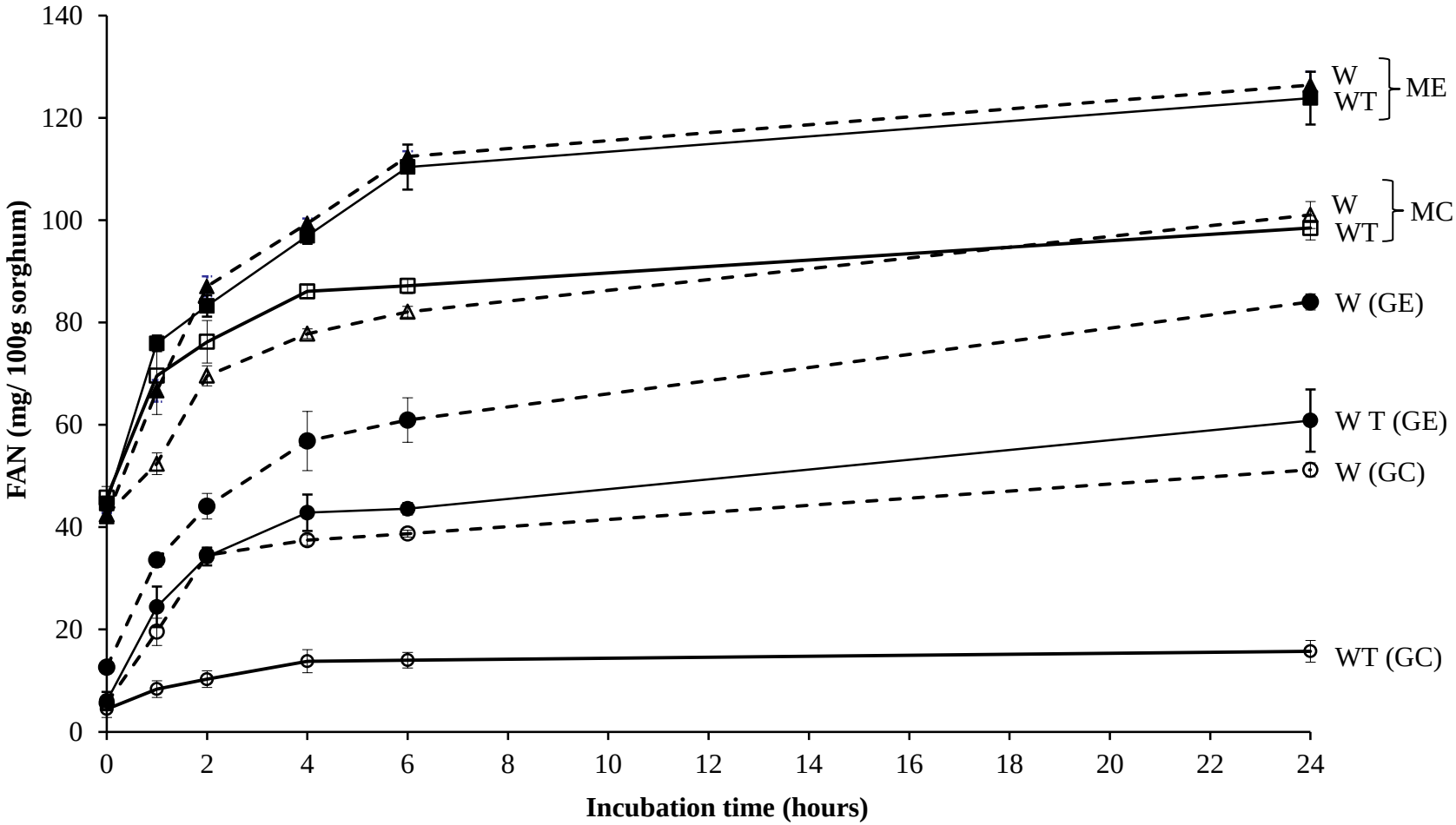

Figure 4.3. Effect of sorghum type and malting on FAN production when incubated with neutral proteinase enzyme (1 mg/ kg) for up to 24 h at 45 °C. W – white tan-plant sorghum; WT – white type II tannin sorghum; GC – grain control; GE – grain enzyme; MC – malt control; ME – malt enzyme. Error bars indicate standard deviations (n = 2).

Incubating W and WT sorghum malt with the neutral proteinase increased FAN levels to approx. 84 and 79 mg/100 g sorghum, while smaller increases in FAN, 71 and 55 mg/100 g occurred when W and WT sorghum grain was incubated with the enzyme, respectively (Figure 4.3). The higher FAN production that occurred with the malt compared to the raw grain is presumably because some of the available proteins had already been partially hydrolysed in the malt due to the action of endogenous proteolytic enzymes. In research into traditional sorghum opaque beer, it was found that mashing (2 h incubation at 60°C) accounted for about 30% of the FAN in the wort with the remainder pre-formed in the malt (Taylor and Boyd, 1986).

Incubating W and WT sorghum malts with the neutral proteinase resulted in only small increases (by 25 and 26 mg/100 g sorghum, respectively) in total FAN produced when compared to incubating the malts without the enzyme (Figure 4.3). This indicates that the use of the enzyme on malts did not substantially improve FAN production, because the malt endogenous proteases were active.

4.1.4.5 Effect of malted sorghum type on the free amino acid profile

Although malting significantly increased sorghum wort FAN content (Figure 4.3), it did not substantially influence the free amino acid profile of the worts after incubation with neutral proteinase (Table 4.2). This is probably because the malt protein substrate did not differ substantially in protein composition from the grain because the external roots and shoots had been removed from the malt. Group B amino acids constituted the highest percentage of the total amino acids (42-47%), followed by Group A amino acids (28-29%) in both sorghum grain and malt worts. The proportion of proline (Group D) in the sorghum malt wort was similar (8%) to that of the sorghum grain wort (10%).

It has been reported that sorghum worts that were not hydrolyses with HCl, had a rather higher proportion of Group A amino acids than other groups when mashed with and without an exogenous proteinase (Bajomo and Young, 1993). The different findings in the present work may be due to differences in sorghum type (the sorghum type used in the Bajomo and Young (1993) study was not indicated) and the fact that a different proteinase was used, from *Bacillus subtilis* as opposed to from *B. amyloliquefaciens* in this study.

Table 4.2. Free amino acid composition (g amino acid/ kg amino acid analysed) of white tan-plant sorghum (Macia) grain and malt worts incubated with neutral proteinase (1 mg/ kg sorghum)

Amino acid	**Raw grain**	**Malt**
***Group A**		
Glutamic acid/ Glutamine	160	183
Aspartic acid/ Asparagine	65	40
Serine	29	32
Threonine	15	23
Lysine	12	7
Total	***281 (28)***	***285 (29)***
Group B		
Valine	90	91
Methionine	34	30
Leucine	235	210
Isoleucine	111	92
Histidine	4	ND
Total	***474 (47)***	***422 (42)***
Group C		
Glycine	19	21
Tyrosine	ND	7
Tryptophan	ND	7
Alanine	150	159
Total	***169 (17)***	***194 (19)***
Group D		
Proline	**76 (8)**	**99 (10)**

Figures in parentheses represent relative percentages of each group; ND – not detected; *Pierce (1987).

In this study, the most abundant amino acids in both sorghum grain and malt worts were leucine followed by glutamic acid/glutamine, alanine and isoleucine (Table 4.2). This is probably related to the amino acid composition of the sorghum kafirin proteins, which are rich in glutamine and non-polar amino acids including proline, leucine and alanine (Belton et al., 2006). The high proportions of leucine (210-235 g/ kg amino acid), isoleucine (92-111 g/ kg amino acid) and particularly valine (90-91 g/ kg amino acid) in both sorghum grain and malt suggest that the wort produced could result in a beer with good flavour stability. It has been reported that the production of diacetyl (butane-2, 3-dione) during fermentation decreases with an increase in the uptake of valine (Krogerus and Gibson, 2013). Diacetyl produces a strong "butterscotch" or "toffee" aroma or taste that causes objectionable flavours when present above its threshold of around 0.15 ppm (Briggs et al., 2004). The effect of leucine and isoleucine on diacetyl production is not clear. Barton and Slaughter (1992) reported that leucine slightly influences production of diacetyl, while opposite results showing isoleucine to have a slight influence and leucine not influencing diacetyl production, have been reported by Pang and Duggleby (2001).

4.1.4.6 Effect of sorghum type on FAN production when incubated with neutral proteinase and amino-peptidase enzymes

The effect of sorghum type on FAN production when incubated with neutral proteinase and amino-peptidase enzymes, in combination was investigated to determine whether there was any synergistic effect since these two enzymes should work collaboratively to hydrolyse proteins into free amino acids (Rao, Tanksale, Ghatge and Deshpande, 1998). The neutral proteinase is an endo-protease and preferably cleaves peptide bonds in the middle of the protein molecule and produces two peptides of substantial molecular size (Rengel, Ospelt and Gay, 2007). In contrast, the amino-peptidase is an exopeptidase, which hydrolyses only the peptide bonds located at or near the ends (amino-terminal or carboxyl-terminal) of the polypeptide chains and produces a large molecular weight peptide and a small molecular weight peptide or an amino acid (Rao et al., 1998).

Incubating W sorghum grain with the combination of neutral proteinase and amino-peptidase increased wort FAN levels to 87 mg/100 g sorghum (Figure 4.4). When compared to incubating with neutral proteinase alone, FAN production was increased by approx. 28 mg/100 g sorghum (Figure 4.3). However, incubating WT sorghum with the

combination of neutral proteinase and amino-peptidase only increased FAN production by approx. 7 mg/100 g sorghum when compared to incubating with neutral proteinase alone. This indicates that incubating W sorghum with the combination of the two enzymes had an additive effect on FAN production, while the enzyme combinations with WT sorghum did not substantially improve FAN production. The lack of an additive effect with the WT sorghum could be due to the tannins inhibiting the enzymes and the intrinsic poor protein digestibility of tannin sorghums (Wu et al., 2007). Incubating sorghum malt with the two enzymes did not increase FAN production with either W or WT (Figure 4.4), when compared to incubating with neutral proteinase alone (Figure 4.2). In fact, FAN levels decreased by approx. 15 and 18 mg/100 g in total, with both W and WT malts, respectively, when incubated with both enzymes. The lack of an additive effect with the malts was probably due to the 50% reduction in concentration of each enzyme in the mixture. This would have affected the enzyme to substrate ratio and subsequently the hydrolysis rate. Another possibility is that the enzymes hydrolysed each other since enzyme are proteins. Delaying the addition of the exopeptidase enzymes might have helped since its action is more important after the large peptides have been hydrolysed.

4.1.5 Conclusions

It has been proposed that FAN levels of about 150 mg/ L (Beckerich and Denault, 1987) are required during high gravity fermentation to avoid incomplete or protracted fermentations. Based on this, unmalted W and WT sorghum grain worts without exogenous enzymes have inadequate FAN levels for rapid and complete fermentation. Similarly, W and WT sorghum malt worts without exogenous enzymes produce insufficient FAN levels.

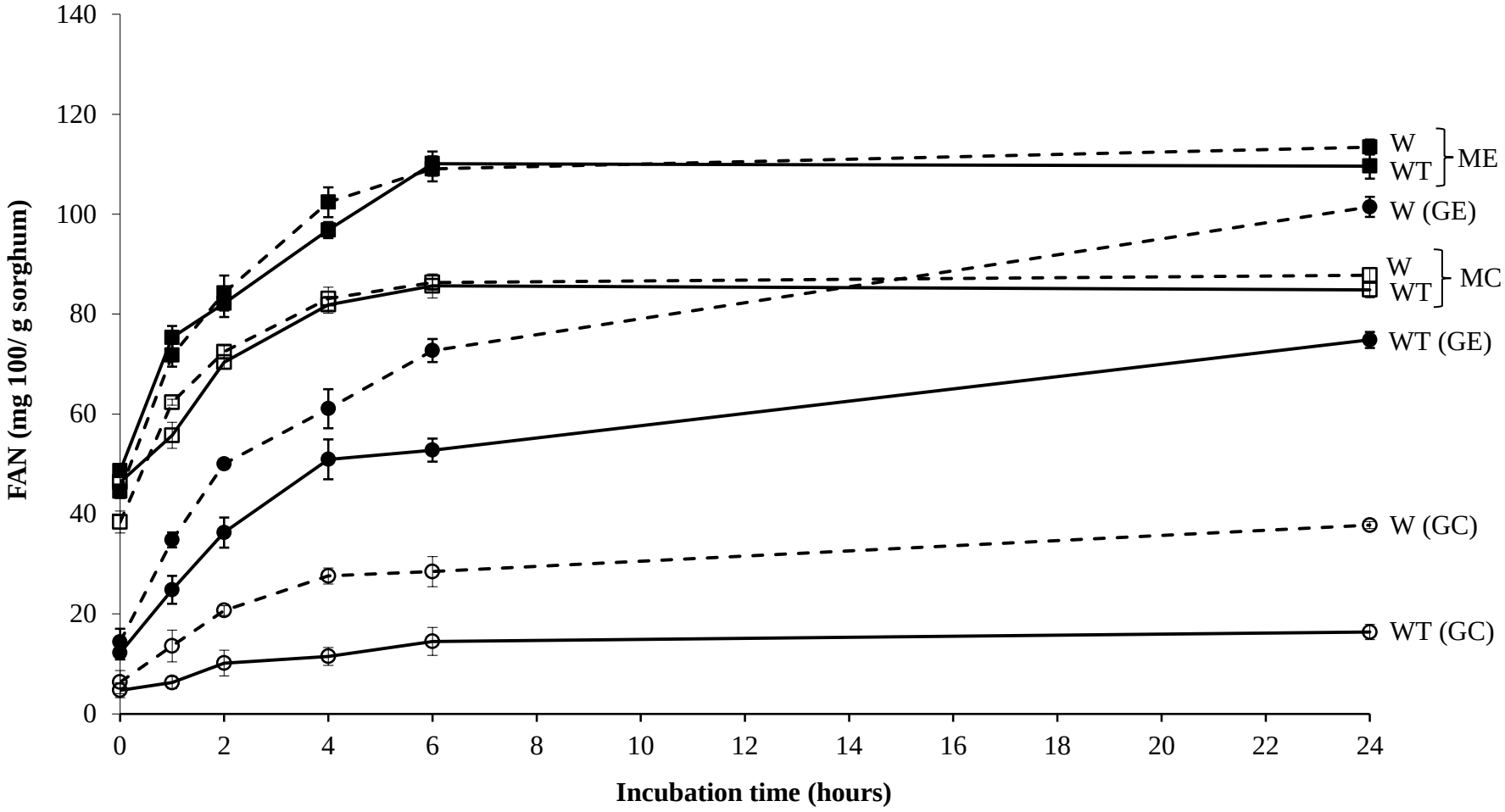

Figure 4.4. Effects of sorghum type and malting on FAN production when incubated with neutral proteinase and amino-peptidase (1 mg/ kg, in total) in combination for up to 24 h at 45 °C. W – white tan-plant sorghum; WT – white type II tannin sorghum; GC – grain control; GE – grain enzyme; MC – malt control; ME – malt enzyme. Error bars indicate standard deviations (n = 2).

In the absence of exogenous proteases, unmalted W sorghum produces substantially higher levels of FAN than unmalted WT sorghum grain. Incubating unmalted sorghum grain with neutral proteinase and amino-peptidase enzymes in combination, improves FAN production with W sorghum but does not substantially improve FAN with WT sorghum. When both sorghums were malted and incubated with neutral proteinase alone substantially more FAN resulted (124-126 mg/100 g sorghum) than with unmalted grains (61-84 mg/100 g sorghum). The combination of amino-peptidase and proteinase did not improve FAN further. Neither did malting influence wort free amino acid profile. Group B amino acids which are utilized more slowly after group A amino acids have been utilized, constituted the highest percentage (42-47%).

Concerning sorghum type, unmalted white non-tannin sorghum with proteinase and amino peptidase treatment yielded the highest FAN than unmalted white type II tannin grain. This is because of low levels of digestible proteins and partly the presence of tannins in the latter sorghum. However, white type II tannin malt produce similar FAN levels to that of white tan-plant malt, apparently as a result of the reduction in assayable tannins during malting.

4.2 Effect of nitrogen supplementation on yeast fermentation performance

4.2.1 Abstract

Nitrogen sources that improve yeast fermentation performance when brewing with sorghum grain are required. The effects of supplementing sorghum grain wort with glycine, ammonia (in the form of diammonium phosphate) and lysine on yeast fermentation performance were investigated. In addition, the effect of pitching with aged yeast and the effect of serial repitching on yeast fermentation performance were studied. Further, the effect of supplementing sorghum grain with different proportions of sorghum malt (10% and 20%) on yeast fermentation performance was investigated.

Diammonium phosphate supplementation substantially improved yeast fermentation rate in terms of improved maltose and maltotriose uptake and reduction in specific gravity. These effects were probably due to the fact that ammonia regulates enzyme activities, permease activities and transcription of nitrogen regulated genes in the yeast cell. Glycine supplementation inhibited yeast fermentation performance, reducing the rate of total reducing sugar uptake. Lysine supplementation increased ethanol yields (up to 7.4% (v/v)) when compared to the control (7.1% (v/v)) but without any increases in yeast numbers.

Pitching sorghum grain wort with aged yeast cells negatively affected yeast fermentation performance rate, probably due to low glycogen levels at the start of fermentation. Serial repitching negatively affected maltose and maltotriose uptake. With serial repitching, supplementing sorghum grain wort with sorghum malt did not improve maltose and maltotriose uptake but resulted in improved ethanol levels. These results suggest that DAP is a better supplement than lysine, glycine and sorghum malt since it improves yeast fermentation rate.

4.2.2 Introduction

Nitrogen limitation is one of the main causes of incomplete or protracted fermentations during sorghum lager beer brewing. This is because sorghum wort provides insufficient FAN for proper yeast growth, particularly when mashing with unmalted grain (Bajomo and Young, 1993). A FAN content of 130-150 mg/ L is required for adequate yeast metabolism (O'Connor-Cox and Ingledew, 1989). Low FAN levels negatively affect fermentation rate and increase the time needed to complete an alcoholic fermentation (Mendes-Ferreira, Mendes-Faia and Leao, 2004). To improve FAN levels and subsequently fermentation performance, nitrogen supplementation has become the common practice.

Yeast is able to utilize different nitrogen sources but not all sources support growth equally (Lekkas et al., 2007). Good nitrogen sources such as glutamine, asparagine or ammonium ions decrease the level of enzymes required for the utilization of poor nitrogen sources through a mechanism known as nitrogen catabolite repression (Beltran, Novo, Rozes, Mas and Guillamón, 2004). Other nitrogen sources such as lysine and histidine have been reported to increase the number of suspended yeast cells, and ethanol production (Lei, Li, Mo, Zheng, Zhao, Zhao, 2013b). Ammonia supplementation has also been reported to improve yeast fermentation performance (Mendes-Ferreira et al., 2004; Gutiérrez, Chiva, Beltran, Mas and Guillamon, 2013). Most studies on nitrogen supplementation have been done using grape must or barley wort. There has, however, been little research with sorghum grain, particularly for sorghum lager beer brewing.

The need for nitrogen supplementation has been intensified by the advent of high gravity brewing (Pham and Wright, 2008). During high gravity brewing, yeast cells are exposed to severe conditions such as high osmotic stress and high ethanol levels (Pátková, Šmogrovičová, Dömény, Bafrncová, 2000; Erten, Tanguler and Cakiroz, 2007). For example, brewing with 16°P wort has been found to trigger a rapid reduction in yeast glycogen levels compared to brewing with a 10°P wort (Kordialik-Bogacka and Diowksz, 2013). Glycogen is the major energy reserve in yeast cells and adequate levels of glycogen are required during pitching to avoid incomplete fermentations (Kordialik-Bogacka and Diowksz, 2013).

At the end of the primary fermentation, yeast is cropped and stored at low temperatures for use in subsequent fermentations. This minimizes capital and revenue costs that are associated with yeast propagation. Yeast can be repitched 7-20 fermentations depending on the yeast strain, production demands and company policy (Jenkins et al., 2003; Powell et al., 2003; Stewart, 2009). It has also been reported that the quality of cropped yeast starts to degenerate after 10 serial repitchings, probably due to the repetitive stress injury associated with serial repitching (Jenkins et al., 2001).

The amount of nitrogen supplement required is also dependent on the type of yeast strain (Gutiérrez et al., 2012). Some strains have high nitrogen requirements, while others require low nitrogen levels for optimal performance (Brice, Sanchez, Tesnière and Blondin, 2014). The objectives of this work were to determine the effect of nitrogenous supplements (glycine, lysine, ammonia), and serial repitching on yeast fermentation performance in sorghum grain brewing. In addition, the effect of different proportions of sorghum malt supplementation on raw sorghum grain wort were investigated.

4.2.3 Materials and Methods

4.2.3.1 Materials

Grains

White-tan plant sorghum grain, Macia variety and sorghum malt from this variety were sourced from Zimbabwe and provided by the South African Breweries. They were milled in a laboratory hammer mill 3100 (Falling Number, Huddinge, Sweden) to $\leq$ 1.2 mm and the flour was stored in zip-lock type polythene bags at 6-8 °C.

Enzymes

Commercial brewing enzymes: Cerezyme 2X Sorghum and Fungamyl Brew Q were kindly provided by Novozymes SA, Benmore, Johannesburg, South Africa.

Yeast

A commercial *S. cerevisiae* lager yeast strain was kindly provided by South African Breweries (Rosslyn, South Africa). The yeast, in form of a slurry, was transported on ice and stored at 4°C until use within 24 h after collection.

4.2.3.2 Small-scale raw sorghum grain lager beer type mashing

Mashing was conducted in stirred stainless-steel beakers using a BRF mashing bath (Brewing Research Foundation, Nutfield, UK). During all mashing operations, stirring was carried out by magnetic stirring and the mashing beakers were covered with watch glasses. Distilled water (approx. 318 mL), pre-heated to 50ºC, containing 365 mg/Litre calcium chloride (approx. 130 ppm of calcium with respect to water) was measured into a stainless steel mashing beaker. A stirrer bar was put in and stirring commenced. A representative sample of sorghum flour (106 g) was weighed and added into the mashing beaker, to give a grist/liquor ratio of 1:3. The contents were then thoroughly mixed and the pH of the mash was monitored to ensure it stayed between pH 5.6-5.8. If otherwise (pH 5.9 or above), the pH was adjusted using a few drops of orthophosphoric acid. Five mL distilled water containing 0.0795 g Cerezyme 2X Sorghum enzyme (0.80 g/ 50 mL distilled water) was added and the mash allowed to rest at 50°C for 30 min.

The temperature was then raised to 94°C at a rate of 1ºC/min and held at 94°C for 45 min. The mash was cooled to 70ºC and Cerezyme 2X Sorghum enzyme (0.80 g/50 mL distilled water) was added. After a 15 min stand at 70ºC, the temperature was further reduced to 58 ºC. Fungamyl Brew Q enzyme (1.80 g/50 mL distilled water) was added at this point. After a 60 min stand at 58 ºC, the temperature was raised to 72 ºC followed by a 15 min rest at the same temperature or until the mash was starch negative as tested by iodine.

The temperature was then raised to 78ºC and the mash was filtered through one layer of cheese cloth. The spent grain was washed with 100 mL hot distilled water at approx. 78 ºC. This sparging water was added to the sweet wort and the wort transferred to a 500 mL measuring cylinder. The wort was diluted to 16° Brix using distilled water. The wort was then clarified by centrifugation at 6420 g for 10 min at 4ºC. The clarified wort was stored at 4ºC for not more than 24 h before analysis. The complete mashing regime is shown in Figure 4.5.

4.2.3.3 Fermentation

Fermentation was performed in EBC tubes (EBC, 1977) capped at the open end with cotton wool. All fermentations were conducted using high gravity worts (15° Plato or 16° Plato). Fermentation was at 15 °C and monitored by assessing specific gravity (° Plato) on

a daily basis until the attenuation point (all fermentable sugars taken up) was achieved. The sorghum wort was oxygenated by shaking in a flask 35 times. The flask was uncapped after 10, 10, and 15 shakes. The wort was then allowed to stand for 2 h in the flask at 15 °C. The wort was shaken another 35 times uncapped to allow the carbon dioxide escape. This resulted in a total oxygen charge of 14 mg/L of dissolved oxygen.

Before pitching, a yeast viability test using citrate methylene blue (see 4.2.3.12 below) and consistency measurements were conducted. Using the theoretical maxima of 100% viability and 60% consistency, the wort was pitched at 14 g yeast slurry/ L wort corrected for the actual viability and consistency. This is equivalent to a pitching yeast count of 23–25 x 10^6 viable cells per mL wort. During fermentation, wort samples (50 mL) were removed at intervals and cooled immediately on ice to be processed for analysis. The yeast and resulting wort were separated by centrifugation (5,000 g) for 10 min at 4 °C. In some cases, at the end of fermentation yeast cells were collected and immediately re-pitched following the procedure stated above, for up to three times. The fermentation graphs are presented in a format that shows comparison of the treatments with the control.

4.2.3.4 Fermentation of sorghum grain wort supplemented with glycine

Glycine was added at 100 mg/L to 15 °P sorghum grain wort containing approximately 50 mg/L FAN, resulting to a total FAN of approximately 150 mg/L. The fermentation was carried out for 4 days with 5th generation yeast obtained from South African Breweries.

4.2.3.5 Fermentation of sorghum grain wort supplemented with lysine

Lysine was added at a high concentration of 100 mg/L (HL-FAN) and at a medium concentration of 50 mg/L (ML-FAN) to sorghum grain wort, which contained approximately 50 mg/L FAN. In total, the HL-FAN wort contained approximately 150 mg/L FAN, while the ML-FAN contained approximately 100 mg/L FAN. The initial wort gravity was 16 °P and the fermentation was carried out for 7 days with 6th generation yeast.

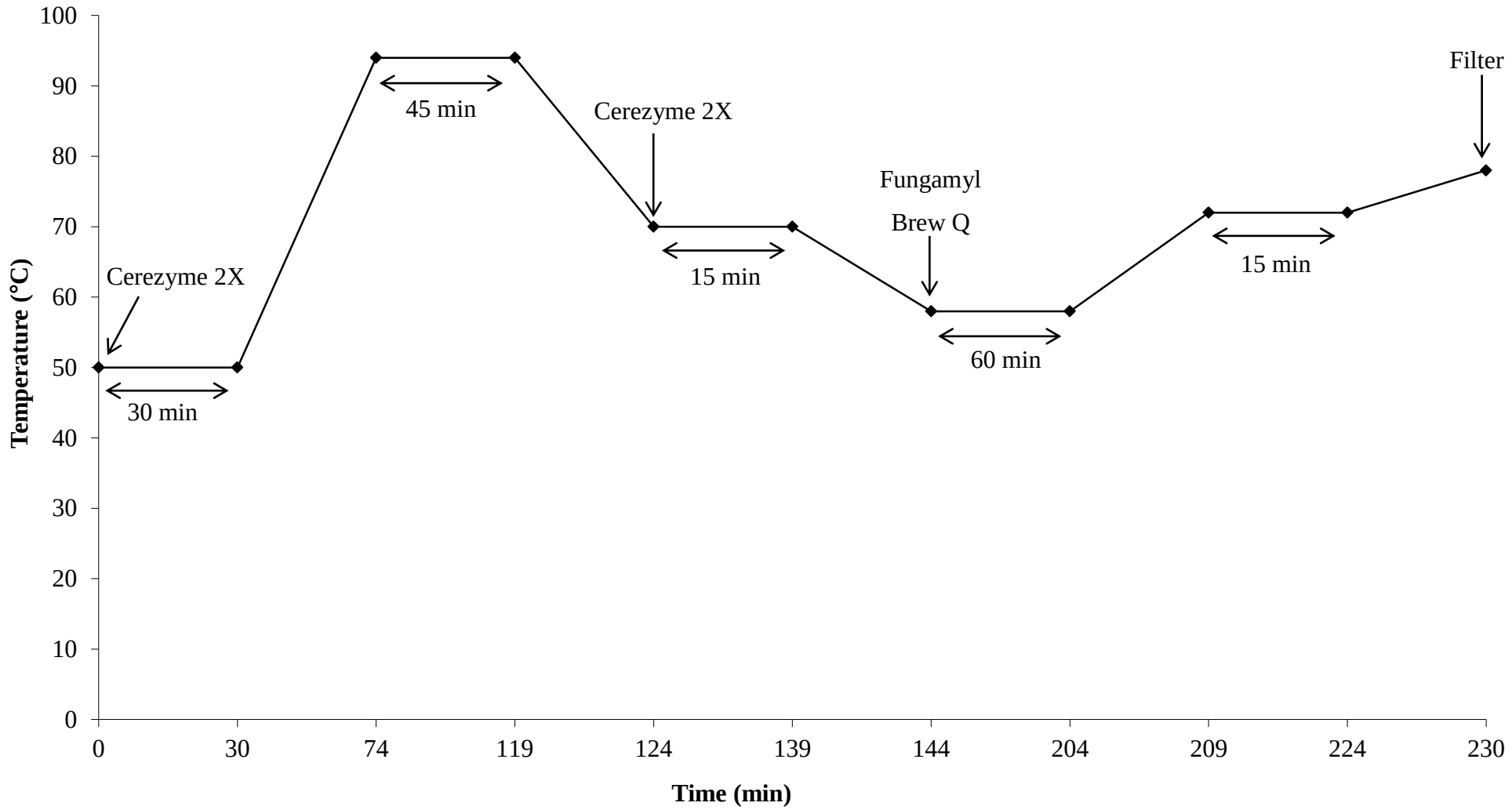

Figure 4.5. Mashing regime used in the study

4.2.3.6 Fermentation of sorghum grain wort supplemented with diammonium phosphate (DAP)

Diammonium phosphate was added at 100 mg/ L, with respect to nitrogen, to 15 °P sorghum grain wort containing approximately 50 mg/L FAN, resulting to a total FAN of approximately 150 mg/L. Fermentation was carried out for 4 days with 5th generation yeast. Ammonia uptake was estimated following the consumption trend reported by Lekkas et al. (2007; 2009). Variability in ammonia consumption was modelled using the lognormal probability distribution in an Excel (Microsoft, Rendmond, WA) spreadsheet add-in programme, @Risk (version 6.3.0, Pallisade, Newfield, NY). The model was simulated automatically until convergence. It was assumed that yeast ammonia consumption in the current study was similar to that of the lager yeast *S. pastorianus* used by Lekkas et al. (2007; 2009); and that the ammonia consumption pattern in sorghum wort was similar to that of all barley malt wort; and ammonia consumption at 15 °C was similar to that of 18 °C (Lekkas et al., 2007) and the difference in the initial FAN and other wort composition parameters did not influence ammonia consumption.

4.2.3.7 Fermentation of sorghum grain wort with aged yeast cells

Sorghum grain wort of 16 °P was pitched with aged yeast cells. Yeast was aged by constant stirring at 5400 rpm of slurry at 25°C for 18 h. Fermentation was carried out for 10 days with 5th generation yeast obtained from South African Breweries. The control was yeast cells without ageing, but stored on ice.

4.2.3.8 Fermentation of sorghum grain wort supplemented with sorghum malt

Sorghum malt was incubated for 8 h prior to mashing to increase its FAN level. Sorghum grain wort (16 °P) was then supplemented with the sorghum malt at proportions of 90:10 or 80:20, respectively. The yeast was serially repitched for up to three times immediately after cropping. Fermentation was carried out for 7 days with 6th generation yeast obtained from South African Breweries.

4.2.3.9 Free amino nitrogen

Free amino nitrogen was determined using the European Brewery Convention Method 8.10 ninhydrin colorimetric assay (European Brewery Convention, 1998).

4.2.3.10 Sugar spectrum

Sorghum wort filtering for HPLC

Kieselguhr (Merck: Cat. # 107910) (25 g) was spread evenly over a double layer of cellulose filter paper (Whatman # 4, 125 mm diameter) on a funnel. The filtering unit was conditioned with 50 mL of wort, which was discarded. Filtering was aided by vacuum and the filtrate was filtered through a 0.45 µm membrane filter (Microsep, Johannesburg, South Africa). The filtered wort was stored in McCartney bottles at 4 °C for not more than 24 h before analysis.

Sugars were determined according to Phaweni, O'Connor-Cox, Pickerell and Axcell (1992). In brief, an Ultra Fast Liquid Chromatography (Shimadzu, Kyoto, Japan) fitted with a Refractive Index detector (RID-10A), solvent delivery pump (LC-20AB), autosampler (SIL-20A) and column heater (CTO-10AS) was used. A Phenomenex Rezex Monosaccharide column (300 x 7.8 mm) (Phenomenex, Torrance, CA) was used. The column temperature was at 45 °C, the flow rate was 0.5 mL/min and the injection volume was 10 µl. Maltotriose (Sigma), maltose monohydrate (Merck), glucose monohydrate (Merck), fructose (Merck) and glycerol (Merck) were used as calibration standards.

4.2.3.11 Specific gravity

Yeast performance was determined by measuring the specific gravity of the wort using refractometry, pycnometry or a DMA 4500 density meter (Anton Paar, Graz, Austria).

4.2.3.12 Methylene blue staining for determining yeast viability

Methylene blue stain was prepared by mixing 0.01% methylene blue and 2% sodium citrate dihydrate in distilled water according to Boyd, Gunasekera, Attfield, Simic, Vincent and Veal (2003). Yeast slurry (1 mL) was diluted in 9 mL buffered peptone water (0.1%), after which equal amounts of methylene blue and diluted yeast cells were wet-mounted on a glass slide with a coverslip. Cells which stained dark blue were considered to be dead, while cells which stained light blue were considered alive. Budding yeast cells were counted as one cell if the bud was less than one half the size of the mother cell (Kuřec, Baszczyński, Lehnert, Mota, Teixeira and Brányik, 2009). A light microscope was used to view the cells and 400 cells in total were counted. The percentage of viable cells was calculated as the number of live cells divided by the total number of cells.

4.2.3.13 Yeast cell numbers

Yeast counts were enumerated on plate count agar (Merck) acidified to pH 3.7 with 2M lactic acid and incubated at 25 °C for 5 days. Ten-fold serial dilutions were made and 1 mL was spread plated in duplicate, as described by Harrigan and McCance (1998). Duplicate plates containing 30–150 colony-forming units (cfu) were counted after incubation and the yeast cell counts were expressed in cfu/ mL. Yeast cell numbers were also counted using an improved Neubauer haemocytometer (Weber Scientific Int., Middlesex, U.K) at x40 magnification using a light microscope. About 4 sets of 16 corner squares were counted and the results were expressed as million cells/ mL.

4.2.3.14 Reducing sugars

Total reducing sugars (TRS) were determined by a colorimetric ferricyanide method (Hoffman, 1937). Maltose was used as a standard.

4.2.3.15 Ethanol

Ethanol production was measured using an Alcolyzerplus (Anton Paar, Graz, Austria). The instrument evaluates absorption bands occurring in the Near Infrared region related to overtones and combinations of fundamental vibrations of the –OH functional group (Reich, 2005).

4.2.3.16 Statistical analyses

Multifactor analysis of variance (ANOVA) was used to determine the effect of specific gravity, yeast counts, FAN, pH, TRS, sugar spectrum and ethanol production on yeast fermentation performance. ANOVA was performed using Statistica software for Windows, version 10 (Tulsa, OK).

4.2.4 Results and discussion

4.2.4.1 Effect of Diammonium Phosphate Supplementation on Yeast Fermentation Performance

The effect of supplementing sorghum grain wort with diammonium phosphate (DAP) on yeast fermentation performance was studied. Diammonium phosphate is widely used in the wine industry to overcome nitrogen limitations (Manginot, Roustan and Sablayrolles, 1998; Mendes-Ferreira et al., 2004). It became the nitrogen supplement of choice because it is a powerful modulator of sulphur dioxide and other fermentation-derived volatiles. Ammonia is classed as a Group C amino acid and is taken up once Group A amino acids have been exhausted (Pierce, 1987).

The addition of DAP resulted in increased FAN levels (Figure 4.6). However, the ninhydrin assay does not distinguish between free amino acids and ammonia. In the ninhydrin assay reduced ninhydrin and ammonia are required for colour development (Sun Wang, 2007). Therefore the presence of additional ammonia enhanced colour development. Sorghum wort supplemented with DAP showed a high uptake of ammonia (55.6 mg/100 g sorghum, in total) compared to the control wort (16.7 mg FAN/100 g sorghum, in total). Most of the ammonia was taken up after 24 h of fermentation in both the ammonia supplemented wort (59%) and the control wort (26%). Thereafter, ammonia levels on DAP supplemented and control worts decreased to 7.4 mg/100 g sorghum and 15.2 mg/100 g sorghum after 4 days of fermentation. The observed high residual ammonia in DAP supplemented wort either indicates that not all the ammonia was taken up by the yeast or ammonia repressed the uptake systems of other nitrogen sources through nitrogen catabolite repression.

When the ammonia levels were estimated separately from FAN, most of the ammonia uptake (approx. 91%) took place in the first 2 days with both the control and DAP supplemented worts (Figure 4.6). Again, not all the ammonia was taken up at the end of fermentation in both the control and DAP supplemented wort.

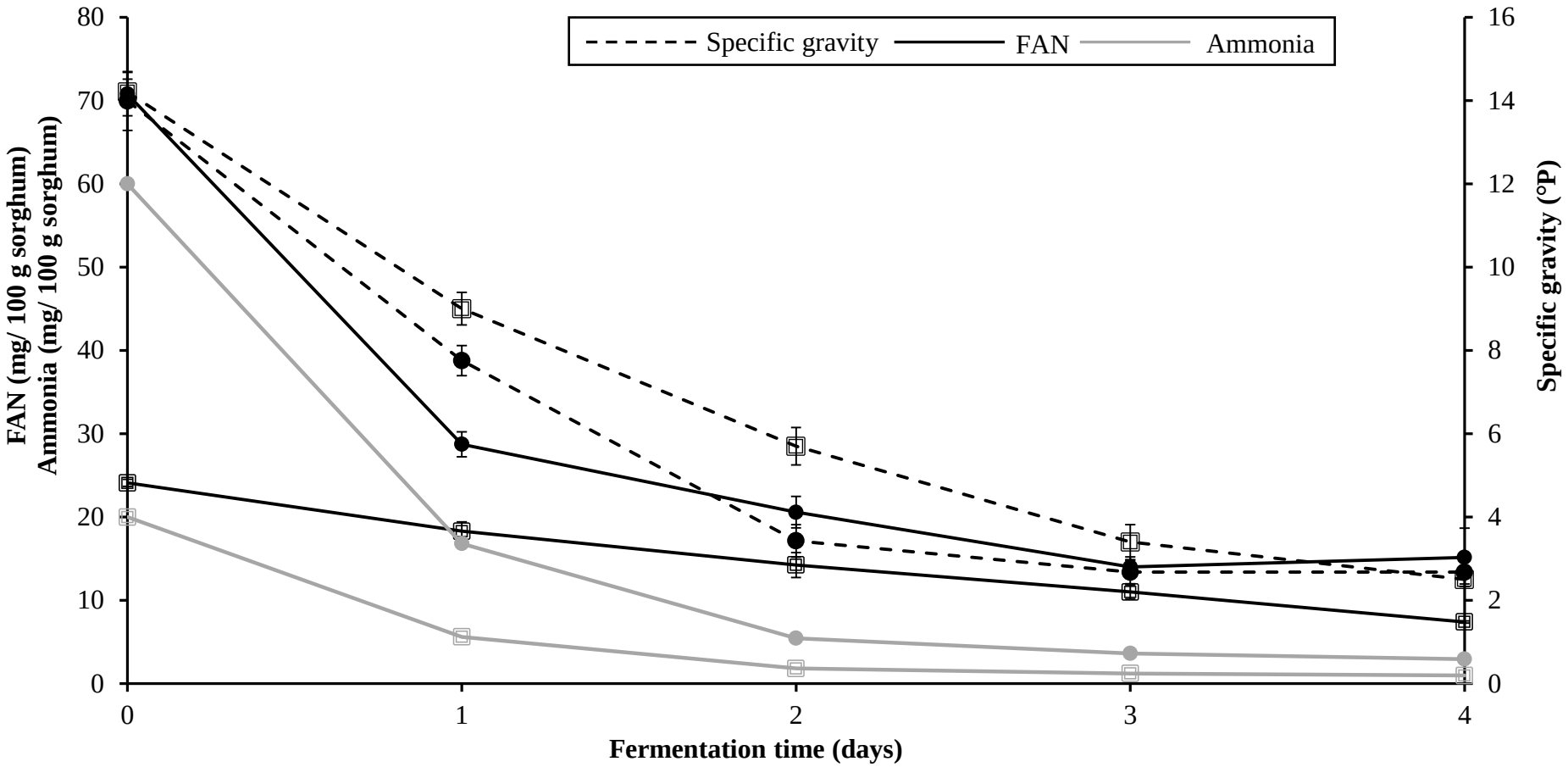

Figure 4.6 Effect of DAP supplementation on FAN and ammonia uptake by yeast and specific gravity during fermentation of sorghum grain wort at 15°C for up to 4 days. Control – squares; DAP supplemented – closed circles. Error bars indicate standard deviations. (n=2)

Lekkas et al. (2007) found a rapid utilization of ammonia, approximately 90% and 95% within 24 h and 48 h in both ale and lager fermentations, respectively. However, not all the ammonia was taken up as residual levels were detected at the end of fermentation.

Specific gravity indicates the dissolved solids content of the fermentation medium (Thomas, Hynes and Ingledew, 1994). In brewing, it is used as a crude measure of sugar uptake to monitor fermentation progress. Sorghum grain wort supplemented with DAP showed a significantly more rapid reduction rate in specific gravity than the control wort (Figure 4.6). However, both the control and the DAP supplemented worts showed a similar reduction in specific gravity after 4 days of fermentation. With the control wort, a steady reduction in specific gravity, from 14.2 to 3.4 °P, took place in the first 3 days followed by slight reduction to 2.5 °P. Diammonium phosphate supplemented wort showed a more rapid reduction in the first 2 days, from 14.0 to 3.4 °P and then slow reduction to 2.7 °P by 4 days of fermentation.

A significantly more rapid rate of maltose and maltotriose uptake took place with ammonia supplementation than with the control, despite the latter showing a higher residual maltotriose level after 4 days (Figure 4.7). The most rapid uptake of maltose and maltotriose took place in the first 3 days of fermentation with both the control DAP supplemented worts. The rapid uptake of maltose and maltotriose with DAP supplementation is similar to what occurred with the specific gravity. The rate of glucose and fructose uptake was similar in both DAP supplemented wort and control wort (Figure 4.7). Glucose and fructose levels decreased sharply in the 1st day with 95% of glucose and fructose taken up after this period with both the control and DAP supplemented worts.

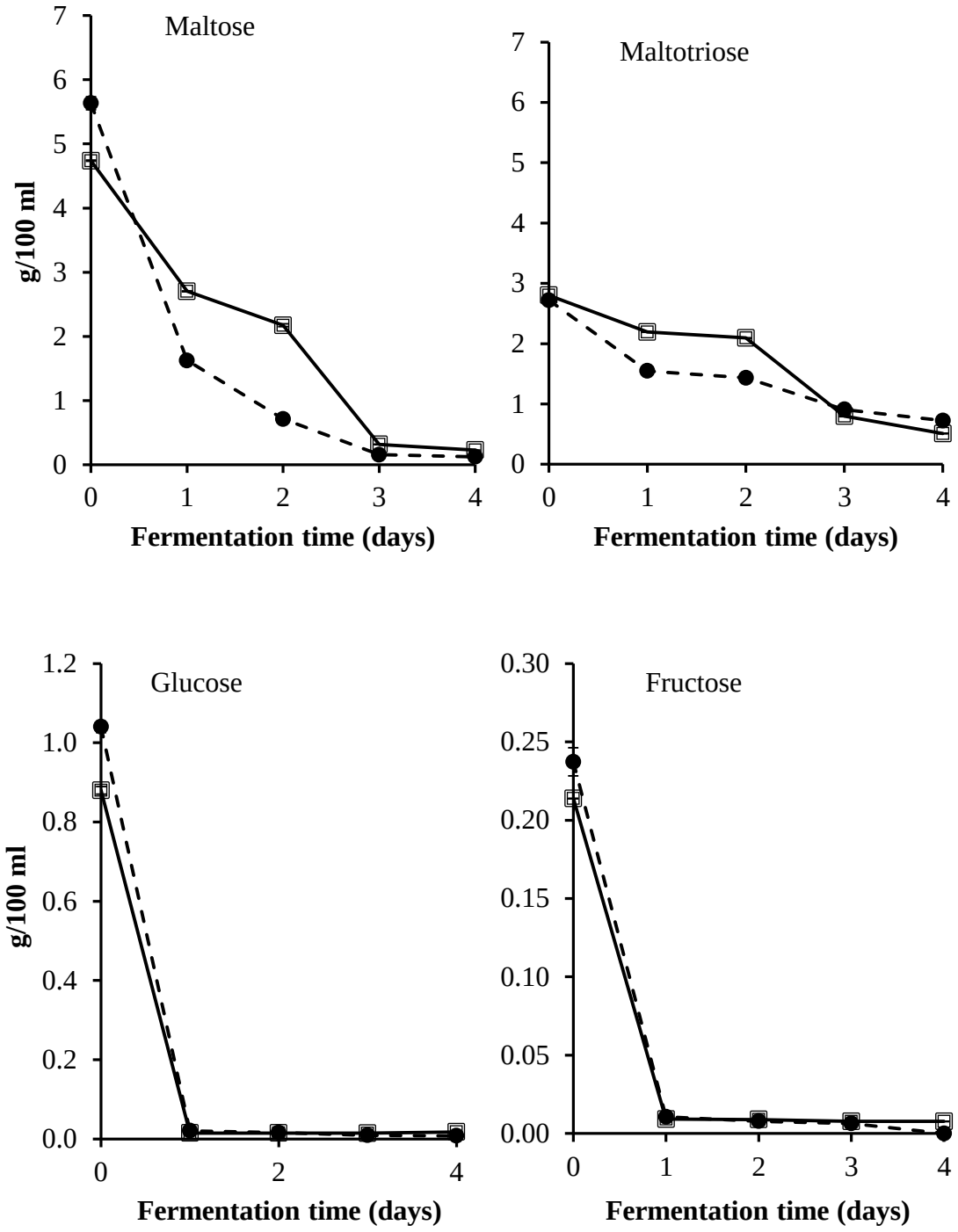

Figure 4.7. Effect of DAP supplementation on sugar spectrum during fermentation of sorghum grain wort at 15 °C for up to 4 days. Control – squares; DAP supplement – closed circles. Error bars indicate standard deviations. (n=2)

Yeast counts in the control and DAP supplemented worts did not differ substantially ($p<0.05$) after 4 days of fermentation (Table 4.3). However, a slight increase in yeast counts, 0.8 $\log_{10}$ cfu/ mL, occurred with DAP supplementation after 4 days. As the increase was less than 1 $\log_{10}$ cfu/ mL it may be considered as not microbiologically significant. An increase in yeast count with DAP supplementation at 66 mg/ L nitrogen was reported by Mendes-Ferreira et al. (2004). These workers reported that supplementing a synthetic grape juice medium after 24h with DAP increased cell populations to 8.0 log cfu/ mL after 120h compared to the unsupplemented control (7.8 log cfu/ mL). According to Magasanik and Kaiser (2002), ammonia is preferentially used by yeast, leading to high yeast growth rates. Schure et al. (2000) classified ammonia as a good nitrogen source and reported that good nitrogen sources yield relatively higher growth rates.

A more rapid reduction in pH took place with the DAP supplemented sorghum wort than the control wort, with the former showing a lower final pH (Figure 4.8). This is in line with what occurred with specific gravity and the uptake of maltose and maltotriose. The pH of the DAP supplemented wort decreased from 4.9 to 3.3 after 1 day and maintained the same pH after 4 days of fermentation. A steady reduction in pH from 4.8 to 3.7 took place in the first 3 days for control wort and the same pH was maintained after 4 days of fermentation. These findings are similar to those of Ugliano, Henschke, Herderich and Pretorius (2007) with a grape must fermentation. These workers attributed the low pH with ammonia supplementation to the notable high level of phosphate anion that remains when yeast utilizes ammonium cations. According to Rowe, Simpson and Hammond (1994), wort pH during a typical lager fermentation reduces from 5.5 to approximately 4.1. This change is, however, dependent on factors such as the buffering capacity of the wort, initial wort pH and the extent of yeast growth (Heggart et al., 1999). The reduction in pH is attributed to the production of carbonic acids from carbon dioxide, secretion of organic acids (notably lactic, citric, pyruvic, malic, formic and butyric acid) and the consumption of buffering compounds (basic amino acids and primary phosphates) in the wort (Coote and Kirsop, 1976).

Table 4.3. Effect of DAP supplementation on yeast counts after 4 days of sorghum grain wort fermentation at 15°C

Treatment	**Day 0**	**Day 4**
	(Log_{10} cfu/mL)	
Control	6.90^{a} (0.06)	7.11^{a} (0.03)
DAP	6.94^{a} (0.02)	7.70^{b} (0.09)

Figures in parentheses represent standard deviations; values with different letter superscripts in a column or row are significantly different (n=2) ($p<0.05$)

In the current study, the addition of DAP improved fermentation rate in terms of improved maltose and maltotriose utilization and specific gravity reduction. Du Preez, de Jong, Botes and Lategan (1985) reported that ammonium chloride improved the rate of sorghum fermentation. Their study investigated ethanol production from tannin sorghum grain using a dual enzyme process. They reported that the time to attain maximal ethanol production decreased by up to 50% when 2 g/ L ammonium chloride (equivalent to 524 mg/ L N) was added to the hydrolysate. Ammonia was thought to have stimulated fermentation through its function as a substrate for protein synthesis, rather than by directly activating the glycolytic enzymes. Jones and Ingledew (1994) reported a rapid fermentation rate during fermentation of very high gravity wheat mashes supplemented with ammonium ion. In their work, mashes supplemented with a mixture of diammonium phosphate and ammonium sulphate (equivalent to 432 mg/ L N, in total) completed fermentation in 5 days compared to unsupplemented mashes which completed fermentation after 9 days.

However, contrary to the current findings, Bajomo and Young (1993) reported that supplementing raw sorghum grain wort with ammonium sulphate (0.5 g/ L) did not improve fermentability. These workers suggested that sorghum wort contains sufficient nutrients in terms of assimilable nitrogen, trace elements and vitamins to attain full attenuation. Importantly, however, their study was done with normal gravity wort, while high gravity wort was used in this study which subjected yeast to greater stress levels.

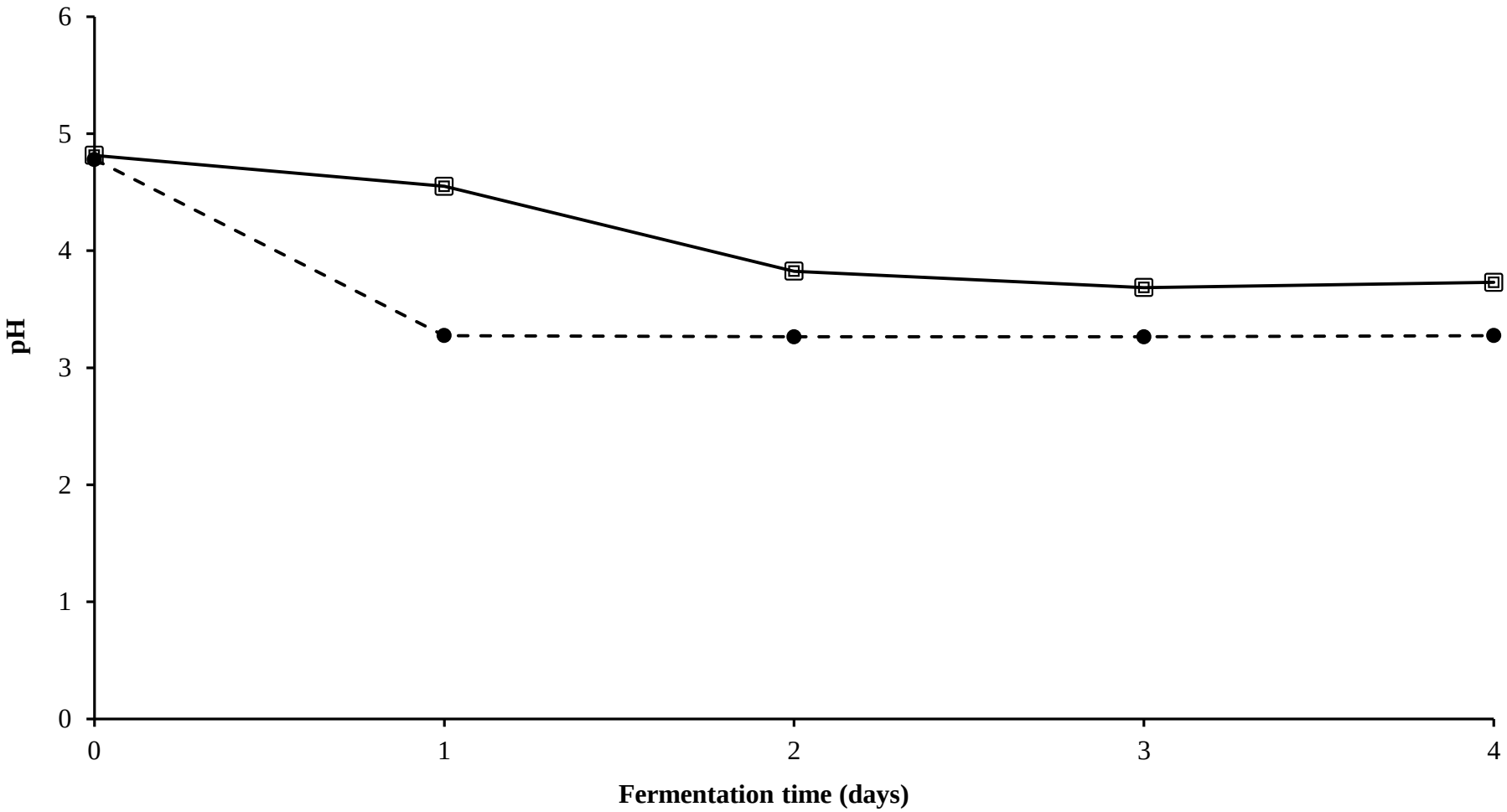

Figure 4.8**.** Effect of DAP supplementation on pH during fermentation of sorghum grain wort at 15°C for up to 4 days. Control – squares; DAP supplement – closed circles. Error bars indicate standard deviations. (n=2)

4.2.4.2 Effect of Glycine Supplementation on Yeast Fermentation Performance

The effect of supplementing sorghum grain wort with glycine as a single supplement on yeast fermentation performance was investigated. Glycine is a Group C amino acid according to the order of assimilation by yeast (Pierce, 1987). It is only taken up when Group A amino acids become limiting and when the nitrogen catabolite repression is relieved (Briggs et al., 2004).

Glycine supplemented yeast cells assimilated more FAN, in total, (34 mg/100 g sorghum) than control cells (15 mg/100 g sorghum) over the 4 day fermentation period (Figure 4.9). The highest uptake of FAN took place during the first day of fermentation with both control and glycine supplemented cells assimilating about 31% of the available FAN. Thereafter, FAN reduced gradually to about 8.6 and 18.8 mg/100 g sorghum, respectively, after 4 days for the control and glycine supplemented worts.

A more rapid reduction in specific gravity took place in the control wort than glycine supplemented wort (Figure 4.9). However, both the control and glycine supplemented worts showed a similar pattern of a gradual reduction in specific gravity over the 4 days of fermentation, with the glycine supplemented wort showing a substantially higher end point than the control.

Similar to the changes in specific gravity, a more rapid reduction in TRS took place in the control wort than the glycine supplemented wort (Figure 4.10). The most rapid reduction took place in the first 2 days in the control wort, with TRS decreasing from 9.9 to 4.4 maltose equiv./ 100 mL. Thereafter, TRS decreased gradually to 1.8 maltose equiv./ 100 mL after 4 days of fermentation. The higher final TRS levels in the glycine supplemented fermentation indicates that glycine reduced the fermentation performance.

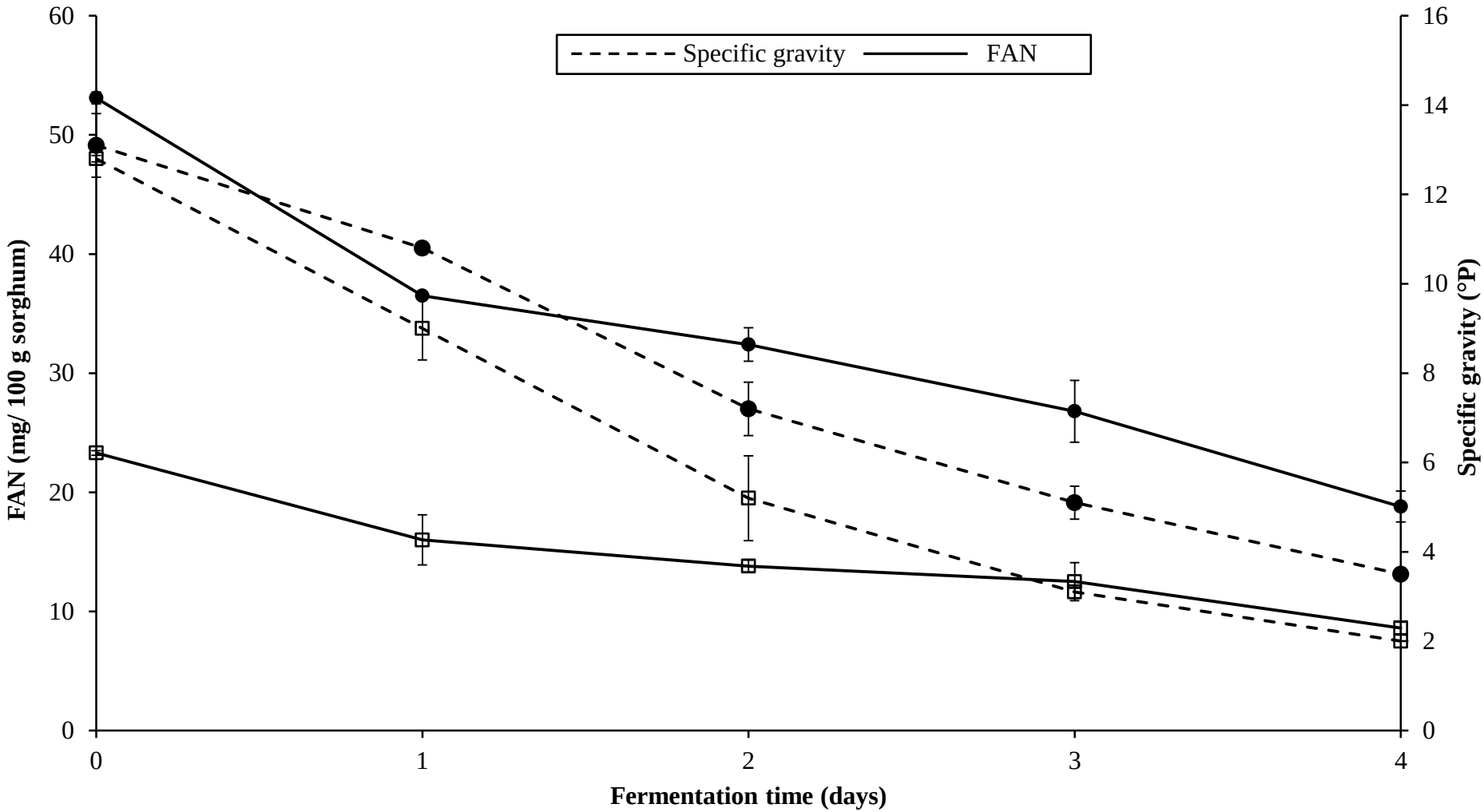

Figure 4.9. Effect of glycine supplementation on FAN uptake and specific gravity during fermentation of sorghum grain wort at 15°C for up to 4 days. Control – squares; glycine supplemented – closed circles. Error bars indicate standard deviations. (n=2)

Yeast counts did not change substantially in either the control or glycine supplemented worts over the 4 day fermentation period (Table 4.4). The largest pH change occurred in the first day of fermentation with both the control wort and the glycine supplemented wort (Figure 4.10). The latter, however, showed a slower reduction in pH in the 1st day of fermentation than the control and this paralled the change in specific gravity after the same time period.

The findings of this study indicate that supplementing sorghum grain wort with glycine negatively affected yeast fermentation performance despite being taken up by the yeast cells. This is probably due to the inability of the yeast to utilize the two-carbon skeletons of glycine (Thomas and Ingledew, 1990). The assimilation of two-carbon compounds by *Saccharomyces cerevisiae* requires an active glyoxylate cycle which occurs only under aerobic and non-repressing conditions (Lee, Jang, Kim and Maeng, 2010). Thus, the choice of an amino acid to be used as a nitrogen source must take into account the nature of the carbon skeleton derived from that amino acid. This should help ensure effective utilization of the amino acid by the yeast.

Table 4.4. Effect of glycine supplementation on yeast counts after 4 days of sorghum grain wort fermentation at 15°C

Treatment	**Day 0**	**Day 4**
	(Log_{10} cfu/ mL)	
Control	6.79^{a} (0.04)	7.14^{a} (0.06)
Glycine	6.84^{a} (0.06)	7.15^{a} (0.10)

Figures in parentheses represent standard deviations. Values with different letter superscripts in a column or row are significantly different (n=2) ($p<0.05$)

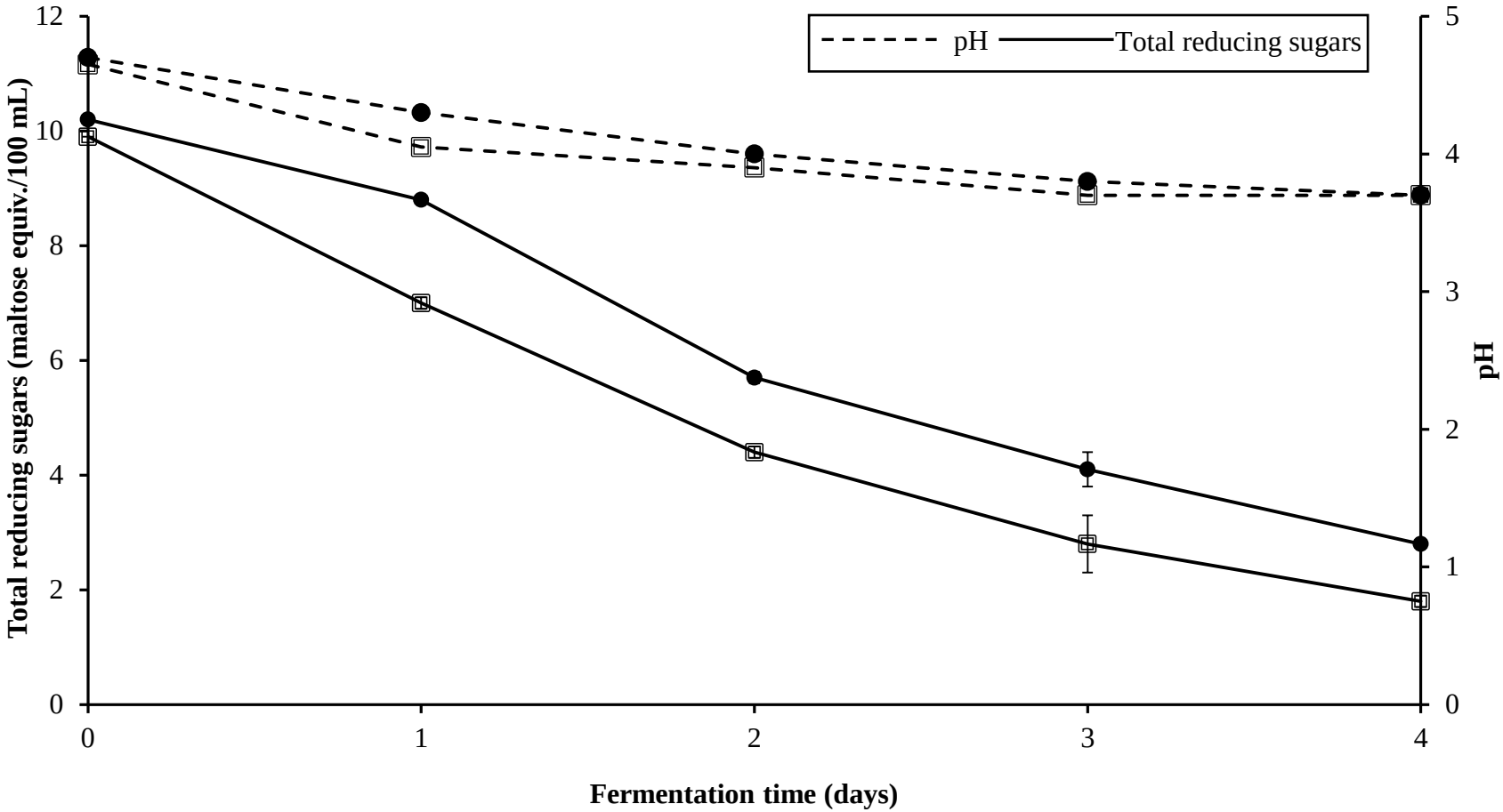

Figure 4.10. Effect of glycine supplementation on total reducing sugar uptake by yeast and pH during fermentation of sorghum grain wort at 15°C for up to 4 days. Control – squares; glycine supplemented – closed circles. Error bars indicate standard deviations. (n=2)

Contrary to these findings, a study by Thomas et al. (1994) found glycine to be the best single supplement to stimulate yeast growth and increase yeast viability. These workers studied the effects of particulate materials and osmoprotectants (such as glycine betaine and proline) on the fermentation of very high concentrations of glucose by the brewing strain *S. cerevisiae (uvarum)* NCYC 1324. Glycine was included in their study because it is a precursor of glycine betaine (Trüper and Galinski, 1990). Thomas et al. (1994) found that glycine, as a single supplement, was more effective than proline and glycine betaine. These workers suggested that glycine could have other biological roles in yeast cells such as acting directly or indirectly as osmoprotectants rather than being a nitrogen source. The yeast used in the current study would have been subjected to stresses during serial repitching at the brewery. Hence, it could have been more tolerant to osmotic stresses.

4.2.4.3 Effect of Lysine Supplementation on Yeast Fermentation Performance

The effect of supplementing sorghum wort with lysine as a single supplement on yeast fermentation performance was investigated. Lysine was added at a high concentration of 100 mg/ L (HL-FAN) and at a medium concentration of 50 mg/ L (ML-FAN). This amino acid was chosen because it is a Group A amino acid (Pierce, 1987), meaning that it is among the first amino acids assimilated by yeast (Briggs et al., 2004). Lysine has also been reported to be important for yeast growth (Lekkas et al., 2007).

A significantly higher FAN uptake (approx. 50 mg/100 g sorghum) occurred with the HL-FAN supplemented wort than with the ML-FAN wort (approx. 32.1 mg/100 g sorghum) and the control wort (approx. 18.5 mg/100 g sorghum) (Figure 4.11). The highest FAN uptake occurred during the first day of fermentation where about 43%, 47% and 49% of available FAN was taken up by the control, ML-FAN and HL-FAN yeast cells, respectively. Increasing the initial FAN level resulted in a corresponding increase in residual FAN.

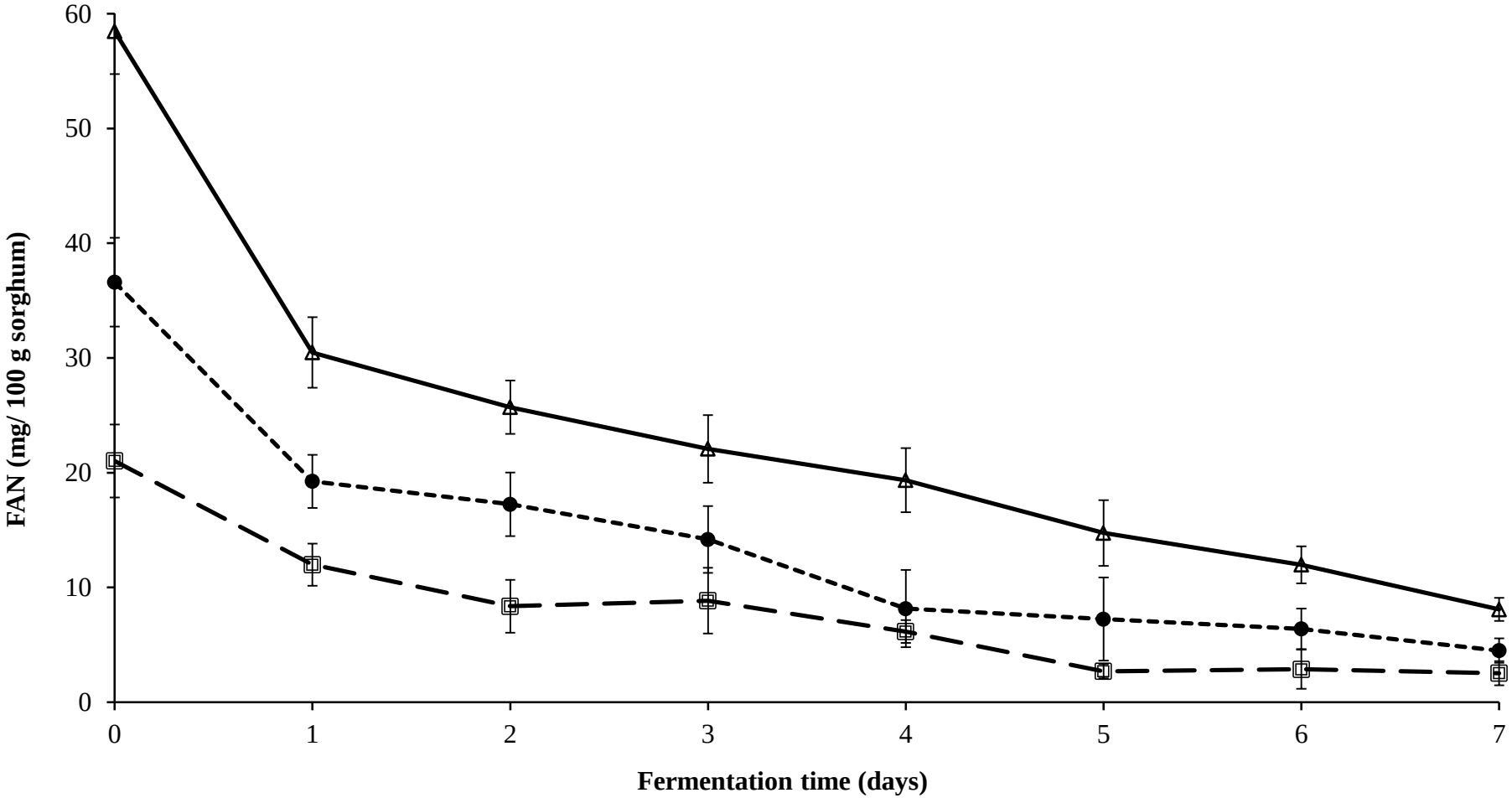

Figure 4.11. Effect of lysine supplementation on FAN uptake during fermentation of sorghum grain wort at 15°C for up to 7 days. Control – squares; ML-FAN – circles; HL-FAN - triangle. Error bars indicate standard deviations. (n=3)

The high residual FAN with the HL-FAN treatment indicates that not all the lysine was utilized by the yeast or that lysine inhibited the uptake of other amino acids through the Nitrogen Catabolite Repression. The former is, however, unlikely when considering the findings of Lekkas et al. (2007). These workers reported that lysine was readily removed from the wort and could not be detected after 43 h of fermentation. According to Thomas and Ingledew (1990), FAN remaining at the end of fermentation could be non-utilizable forms such as higher peptides ($\geq$ 4 amino acid residues) or soluble proteins.

Lekkas et al. (2005) reported that fermentations supplemented with lysine were more rapid than unsupplemented fermentations. They attributed this to the increase in suspended cell concentration on lysine supplemented yeast. They, however, stated that the reason why lysine stimulated cell growth was unclear and worthy of further investigation. According to Lei et al. (2013b), lysine supplementation substantially up-regulates Ssy1p-Ptr3p-SSy5p (SPS)-regulated genes compared to nitrogen catabolite repression genes. The SPS genes control the expression of specific permeases that are responsible for the transport of early consumed amino acids at the beginning of fermentation (Ljundahl, 2009).

A similar reduction in specific gravity took place with the control, ML-FAN wort and HL-FAN wort in the first 3 days of fermentation (Figure 4.12). Thereafter, ML-FAN wort and HL-FAN wort showed a significantly faster reduction in specific gravity than the control wort. The higher final gravity that occurred with the control wort indicates that not all the assimilable sugars were fermented by the yeast. Importantly, lysine supplementation increased the final ethanol levels with the highest increase occurring in the HL-FAN supplemented wort. There was no significant difference ($p \geq 0.05$) in the amount of ethanol produced between day 0 and day 3 between the control, ML-FAN and HL-FAN worts. The control wort ethanol production reached 7.1 g/ 100 g, while ML-FAN and HL-FAN were 2.0% and 2.2% higher, respectively, after 7 days of fermentation.

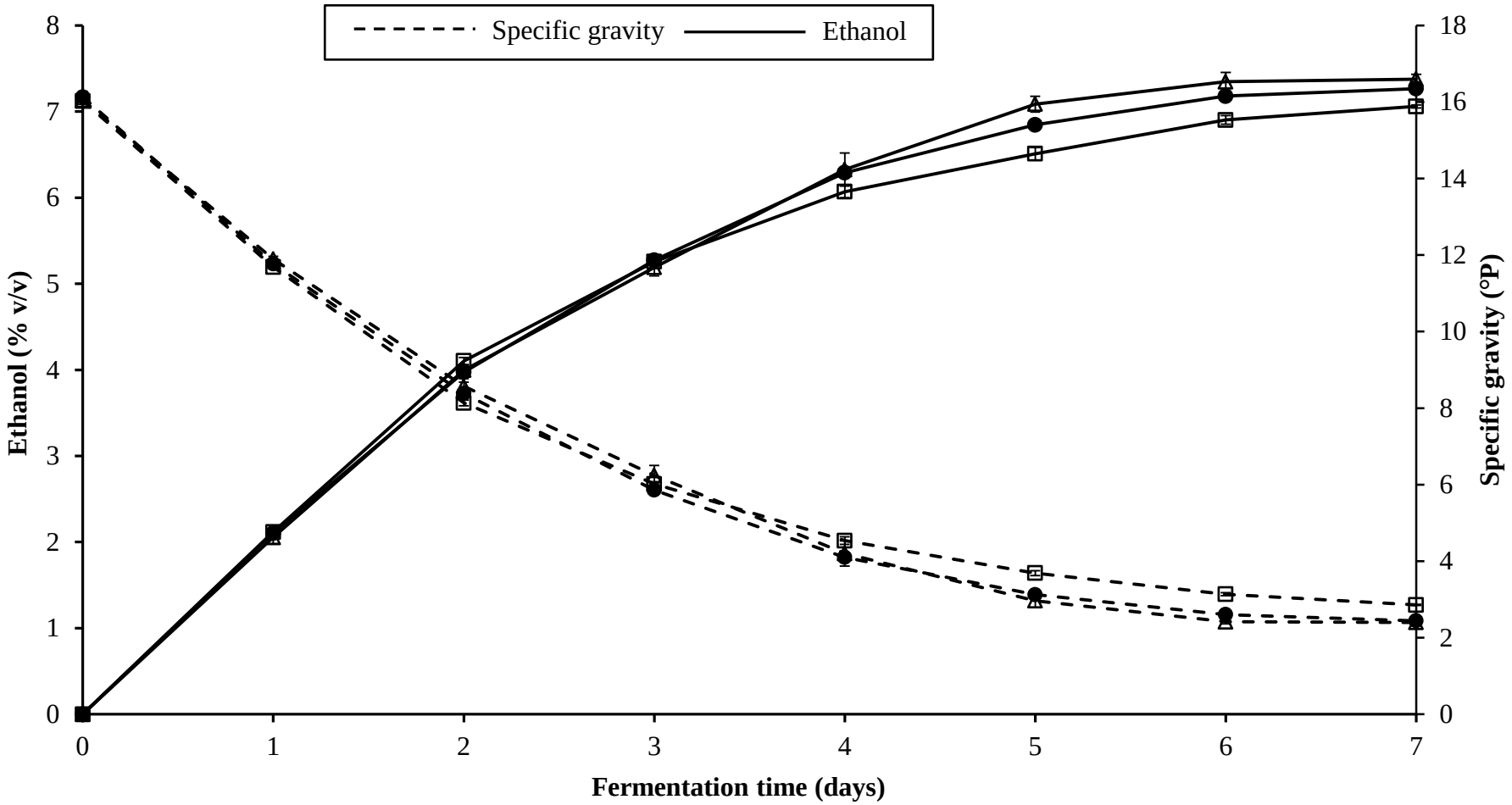

Figure 4.12. Effect of lysine supplementation on specific gravity and ethanol production during fermentation of sorghum grain wort at 15°C for up to 7 days. Control –squares; ML-FAN – filled circles; HL-FAN - triangles. Error bars indicate standard deviations. (n=3)

Lysine supplementation resulted to a more rapid uptake of maltose and maltotriose than in the control wort (Figure 4.13). Increasing the lysine supplementation resulted in a further increase in maltose and maltotriose uptake from the wort. The rapid uptake of maltose and maltotriose could be the cause of improved ethanol levels with lysine supplementation (Figure 4.12). A more rapid uptake of maltose and maltotriose took place between day 1 and 4 of fermentation in the lysine supplemented worts. A generally slow glucose uptake took place with all treatments after 5 days fermentation, while more than 94% fructose was taken up after 1 day. The slow uptake of glucose was not expected since yeast utilizes glucose at the start of fermentation (Briggs et al., 2004).

The control wort and HL-FAN wort yeast counts slightly decreased from 29.5 x 10^6 cells/ mL to approx. 20.5 x 10^6 cells/ mL after 7 days, while ML-FAN wort yeast counts decreased more substantially to 16.5 x 10^6 cell/ mL after the same period (Table 4.5). This indicates that the availability of excess amounts of assimilable nitrogen did not promote increased number of cells at the end of fermentation. This finding is similar to that reported by O'Connor-Cox et al. (1991) when supplementing very high gravity worts with different concentrations of yeast extract. These workers attributed the high ethanol production with supplemented worts to increased cell mass rather than cell number. They assumed that this increased the actual rate of ethanol production on per cell basis.

According to O'Connor-Cox and Ingledew (1989), FAN stimulates active yeast growth as nitrogen is used in the synthesis of cellular proteins and other cell compounds. As mentioned, Lekkas et al. (2005) reported that fermentations supplemented with lysine are more rapid and attributed this to an increase in suspended yeast cell concentration. As also stated, lysine supplementation has been associated with up-regulation of genes that control the expression of specific permeases (Ljundahl, 2009).

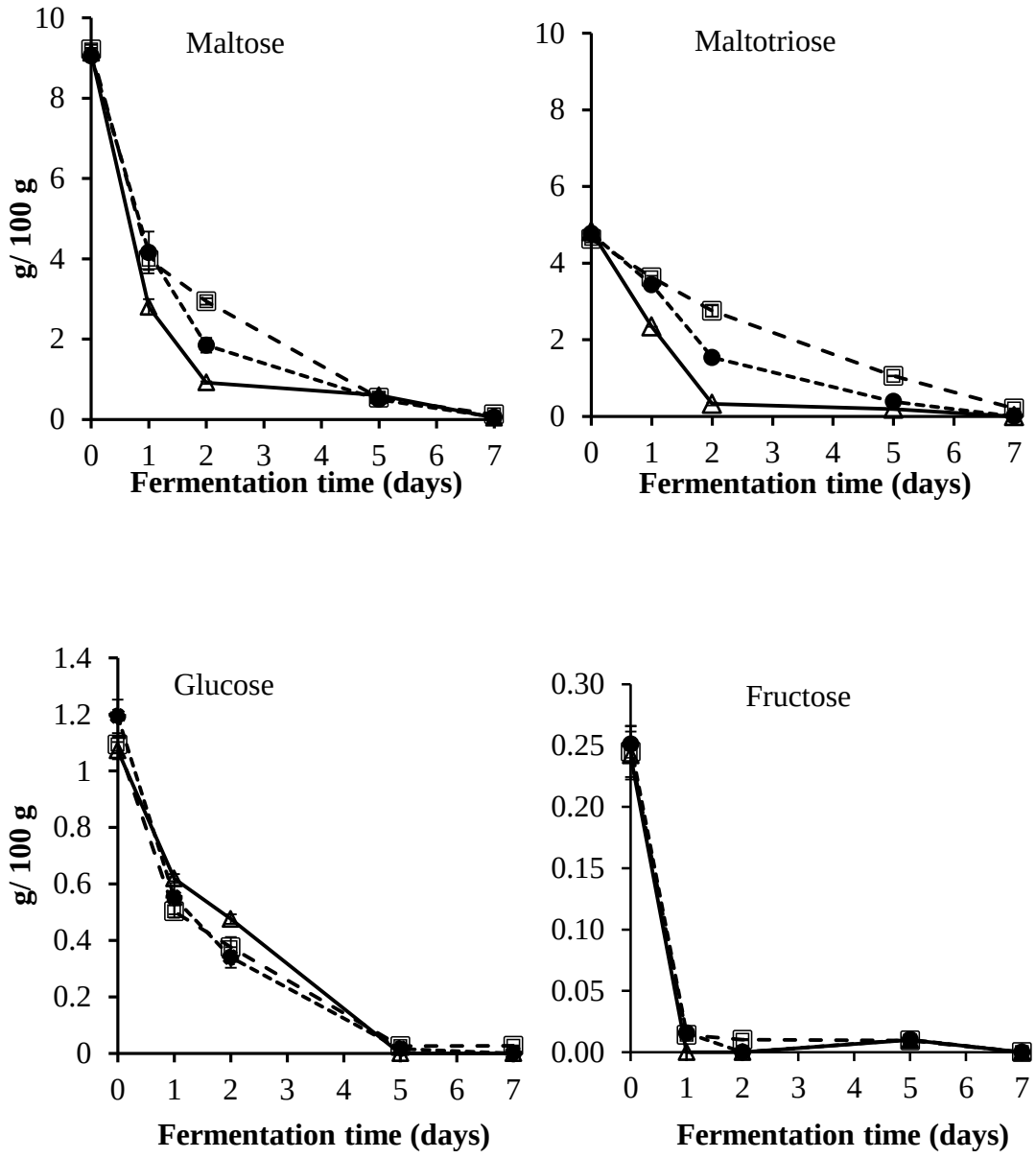

Figure 4.13. The effect of lysine supplementation on sugar spectrum during fermentation of sorghum grain wort at 15 °C for up to 4 days. Control – squares; ML-FAN – closed circles; HL-FAN - triangles. Error bars indicate standard deviations. (n=3)

Table 4.5. Effect of lysine supplementation on yeast count after 7 days of sorghum grain wort fermentation at 15°C

Treatment	Day 0	Day 7
	($x10^6$ cells/ mL)	
Control	29.48^{c} (2.28)	20.50^{b} (2.54)
ML - FAN	30.33^{c} (2.01)	16.50^{a} (3.12)
HL - FAN	28.90^{c} (1.21)	19.16^{b} (3.51)

Figures in parentheses represent standard deviations. ML – medium lysine; HL – high lysine; values with different letter superscripts in a column or row are significantly different (n=2) ($p<0.05$)

However, Thomas and Ingledew (1992) reported that lysine inhibited yeast growth when added as a single nitrogen supplement. Upon adding a number of other nitrogen sources in combination with lysine, they found out that this inhibition was partially or completely reversed and lysine stimulated yeast growth and fermentation. They concluded that the inhibitory effect of lysine occurs when assimilable nitrogen in the medium is in growth-limiting concentrations. In another study by Monteiro, Trousdale and Bisson (1989), lysine was considered a poor nitrogenous source since the yeast *S. cerevisiae* does not have the necessary enzymatic system for its complete degradation. Agreeing with this, Ayestarán, Garrido and Ancín (1998) reported that lysine was more poorly consumed than other basic amino acids. These workers studied the influence of sedimentation on viura (white variety of wine grape) must's concentration of free amino acids compared to an unclarified sample.

Effect of lysine supplementation on the free amino acid profile

The amino acid profile of the sorghum wort during fermentation of wort supplemented with lysine (50 mg/ L) was determined in order to relate lysine uptake with yeast fermentation performance. Group A amino acids constituted the highest initial amount (46 mg/ L) of amino acids, followed by group C (21.4 mg/ L) and group B (8.2 mg/ L) amino acids (Table 4.6). The high proportion of group A amino acids was due to the added lysine. A high proportion of group A amino acids at the beginning of a fermentation has been associated with increased yeast growth, FAN uptake rate, sugar utilization rate and ethanol production rate (Beltran et al., 2004; Perpète, Santos, Bodart and Collin, 2005; Lekkas et al., 2007).

Proline, the sole member of group D constituted the lowest proportion which was substantially lower than the total of group B amino acids.

Apart from the lysine which was added, the sorghum wort contained a high proportion of tyrosine which was totally consumed by day 7. This was followed by alanine and glutamic acid, both with an uptake of more than 89% at the end of fermentation. A typical sorghum grain wort is rich in glutamic acid, leucine and alanine (Gassem and Osman, 2003). The amino acid composition of sorghum wort seems to be affected by the type of enzymes used during mashing (Goode and Arendt, 2003). Lei et al. (2013a) reported that mashing with different enzymes also affects the total amount of amino acids produced. In their study, treating all barley malt high gravity (20°P) wort with Neutrase (a neutral proteinase) produced 2930 mL/ L amino acid in total, while about 2768 mg/ L was produced with Protamex (a serine endoprotease).

Generally, the yeast utilized more than 80% of all the available amino acids, except for isoleucine and proline. The poor utilization of proline was expected as its utilization requires a mitochondrial oxidase, which is repressed during fermentation (Wang and Brandriss, 1987). However, the small consumption of proline (about 35%) suggests that the yeast was at some stage short of nutrients. Research has indicated that proline has other functions in the cell, such as acting as a stress protectant (Morita, Nakamori and Takagi, 2003; Sekine, Kawaguchi, Hamano and Takagi, 2007). This means that utilization of proline confers an adaptive advantage, particularly under high gravity brewing.

About 83% of the lysine was assimilated by the yeast after 7 days of fermentation. The residual lysine suggests that the yeast required a certain amount of the amino acid for a complete fermentation. Similarly, residual FAN occurred when the amino acid was supplemented at the same level (Figure 4.11). The amounts of lysine and histidine assimilated in high gravity fermentation has been reported to correlate well with fermentability, while that of alanine, tyrosine and asparagine negatively correlated with fermentability (Lei et al., 2013b). This explains the improved ethanol levels that occurred with lysine supplementation.

Table 4.6. Free amino acid composition of sorghum grain wort supplemented with 50 mg/L lysine and fermented 15°C for up to 7 days

Amino acid	Day 0 (mg/ L wort)	Day 7 (mg/ L beer)	Consumption%
***Group A**			
Glutamic acid	7.5 (0.1)	0.8 (0.0)	89.3
Aspartic acid	4.6 (0.1)	0.2 (0.0)	95.7
Serine	1.0 (0.0)	0.2 (0.0)	80.0
Threonine	1.1 (0.1)	0.0 (0.0)	100.0
Lysine	26.5 (3.4)	4.4 (0.2)	83.4
Arginine	5.3 (0.3)	0.0 (0.0)	100.0
Total	46.0	5.6	91.4
Group B			
Valine	2.3 (0.3)	0.2 (0.0)	91.3
Methionine	0.5 (0.0)	0.1 (0.0)	80.0
Leucine	2.9 (0.0)	0.3 (0.0)	89.7
Isoleucine	2.5 (0.1)	1.1 (0.1)	56.0
Total	8.2	1.7	79.2
Group C			
Glycine	1.7 (0.0)	0.1 (0.0)	94.1
Tyrosine	12.0 (0.7)	0.0 (0.0)	100.0
Alanine	7.7 (0.6)	0.3 (0.0)	96.1
Total	21.4	0.4	96.7
Group D			
Proline	6.9 (0.2)	4.5 (0.0)	34.8

Figures in parentheses represent standard deviations. *Pierce (1987)

4.2.4.4 Effect of Pitching with Aged Yeast on Yeast Fermentation Performance

The effect of pitching sorghum grain wort with aged yeast on fermentation performance was studied to mimic the stress associated with serial repitching. Figure 4.14 shows the effect of pitching with aged yeast on FAN uptake and specific gravity during fermentation of sorghum wort at 15 °C for up to 10 days. There was no significant difference ($p \geq 0.05$) in the total amount of FAN (26.9 mg/100 g sorghum) taken up by yeast in the control and yeast aged worts. This means that ageing pitching yeast did not influence FAN uptake. The largest uptake of FAN occurred in the first day of fermentation, with both the control wort and aged yeast worts taking up about 52% of the available FAN.

A similar reduction pattern in specific gravity was observed over the 10 day fermentation period with both the control and aged yeast worts (Figure 4.14). Aged yeast showed a significantly slower reduction in specific gravity between day 3 and 7. Thereafter, there was no significant difference ($p \geq 0.05$) between the aged yeast wort and control wort. Phaweni et al. (1992) reported that brewery aged yeast showed a slower attenuation rate than fresh yeast. However, in their study brewery aged yeast attenuated at a higher end level. The differences may be due to the differences in substrates since they used a barley malt wort supplemented with various types of adjuncts.

Similar to what took place with specific gravity between day 3 and 7, a significantly slower reduction in TRS was observed with aged yeast wort than control wort after 5 days of fermentation (Figure 4.15). Thereafter, both control and aged yeast worts showed the same end point, 2.2 g (maltose) equivalent/ 100 mL, after 10 days. Maltose uptake in the aged yeast wort showed a slower uptake than control between day 3 and 5 of fermentation, while a generally slower uptake of maltotriose in the aged yeast wort took place after 3 days of fermentation compared to the control (Figure 4.16). Glucose and fructose uptake showed a similar pattern with more than 90% of glucose and fructose taken up after 1 day with both the aged yeast and control worts (Figure 4.16).

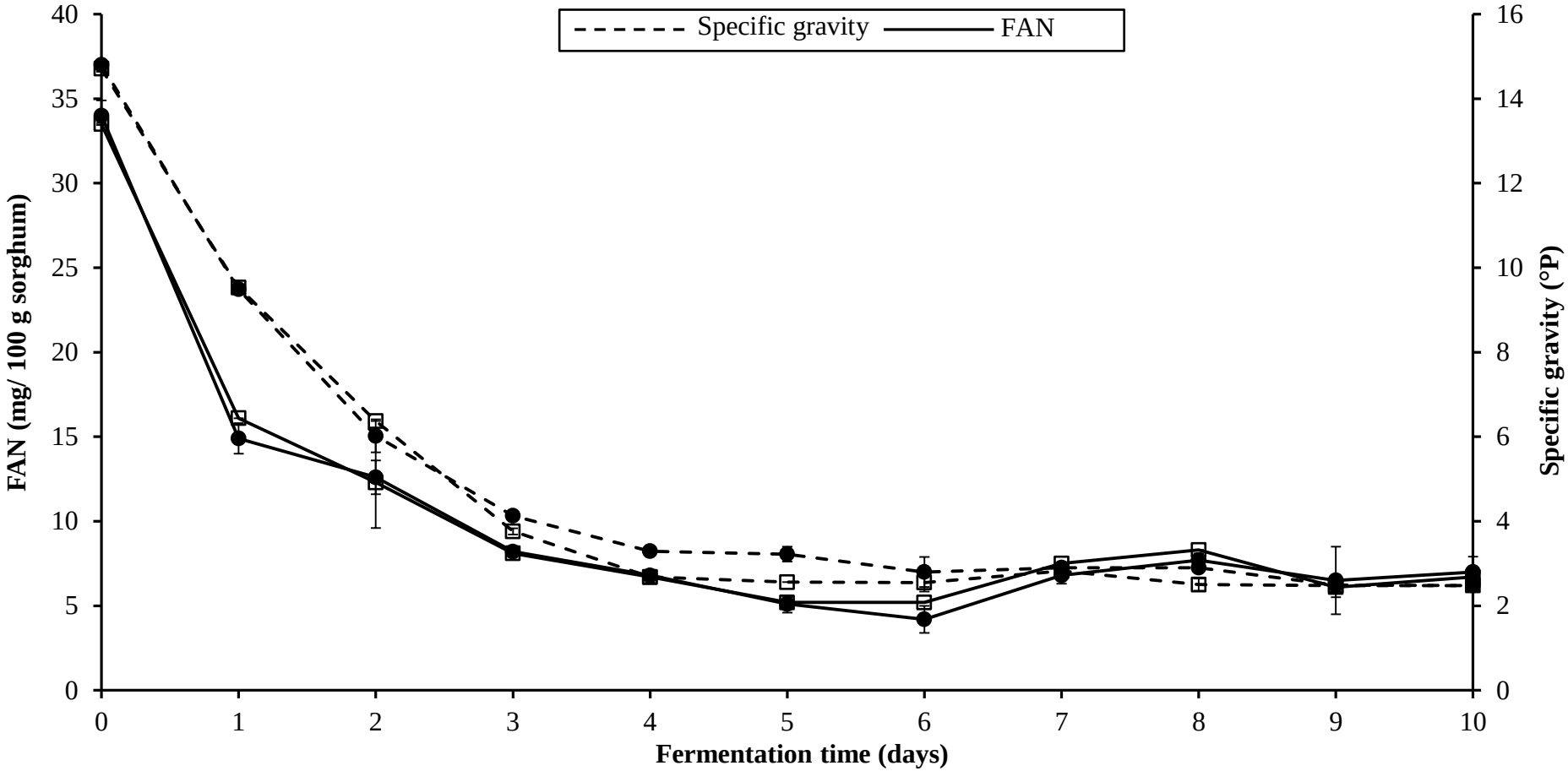

Figure 4.14. Effect of pitching with aged yeast on FAN uptake and specific gravity during fermentation of sorghum grain wort at 15°C for up to 10 days. Control – squares; Aged yeast – closed circles. Error bars indicate standard deviations. (n=2)

A slight but significant ($p<0.05$) greater decrease (1.1 $\log_{10}$ cfu/ mL) in yeast counts occurred with the aged wort compared to the control wort (0.6 $\log_{10}$ cfu/ mL) after 10 days of fermentation (Table 4.7). The greater reduction in yeast counts with aged yeast wort can be attributed to aged yeasts having low glycogen levels (Quain and Tubb, 1982) and the fact that older cells are more flocculent than younger cells. In a study to determine the impact of yeast cell age on fermentation performance, Powell et al. (2003) reported that older cells have rough cell surfaces that favour cell to cell adhesion during the onset of flocculation. A similar reduction in pH took place with both the control and aged yeast worts with both of them having a final pH of 3.9 after 10 days of fermentation (Figure 4.15).

In this study, fifth generation yeast was aged to reduce the glycogen levels and hence mimic the stress associated with serial repitching. The process of ageing yeast by constant stirring reportedly results in approximately 7% less glycogen and 3-7% g.d.w less trehalose at the onset of fermentation (Lodolo et al., 1998). Glycogen provides the sole source of metabolic energy for the synthesis of essential lipids (unsaturated fatty acids and sterols) during the first 2 h following pitching (Quain and Tubb, 1982). These lipids are essential for the normal growth of the yeast population and overall fermentation performance (Verbelen et al., 2009). Trehalose is important during periods of starvation and its role is more of a stress protectant rather than as a main source of metabolic energy (Heggart et al., 1999). Pickerell, Hwang and Axcell (1991) reported that low glycogen levels in pitching yeast correlated with low cell viability, extended fermentation times and high diacetyl, acetaldehyde and sulphur dioxide levels at the end of fermentation. Aged yeast show impaired physiological states which may affect fermentation performance (Powell et al., 2000). This was attributed to restricted lipid synthesis and subsequently low glycogen levels in pitching yeast.

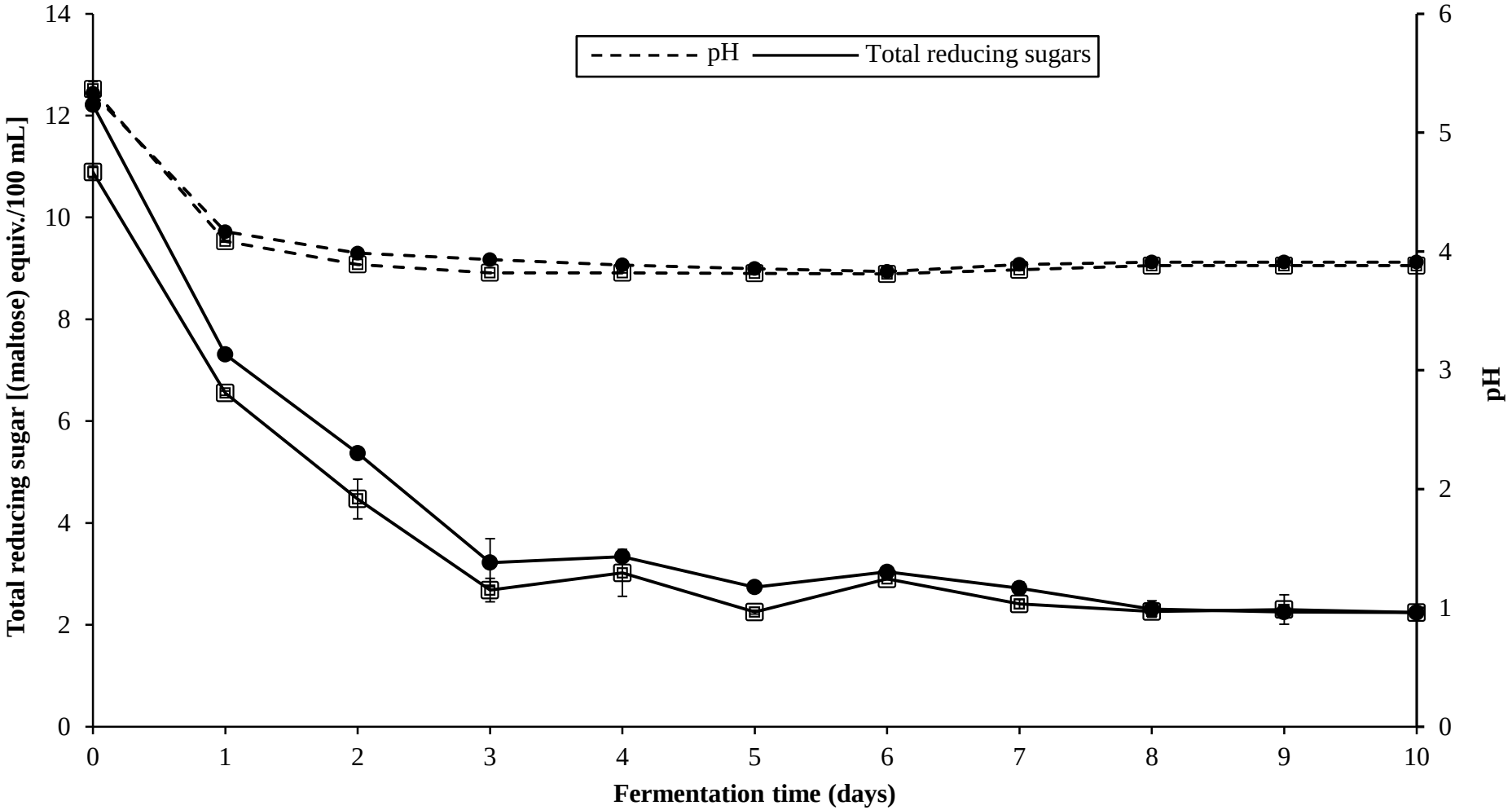

Figure 4.15. Effect of pitching with aged yeast on total reducing sugar and pH during fermentation of sorghum grain wort at 15°C for up to 10 days. Control – squares; Aged yeast – closed circles. Error bars indicate standard deviations. (n=2)

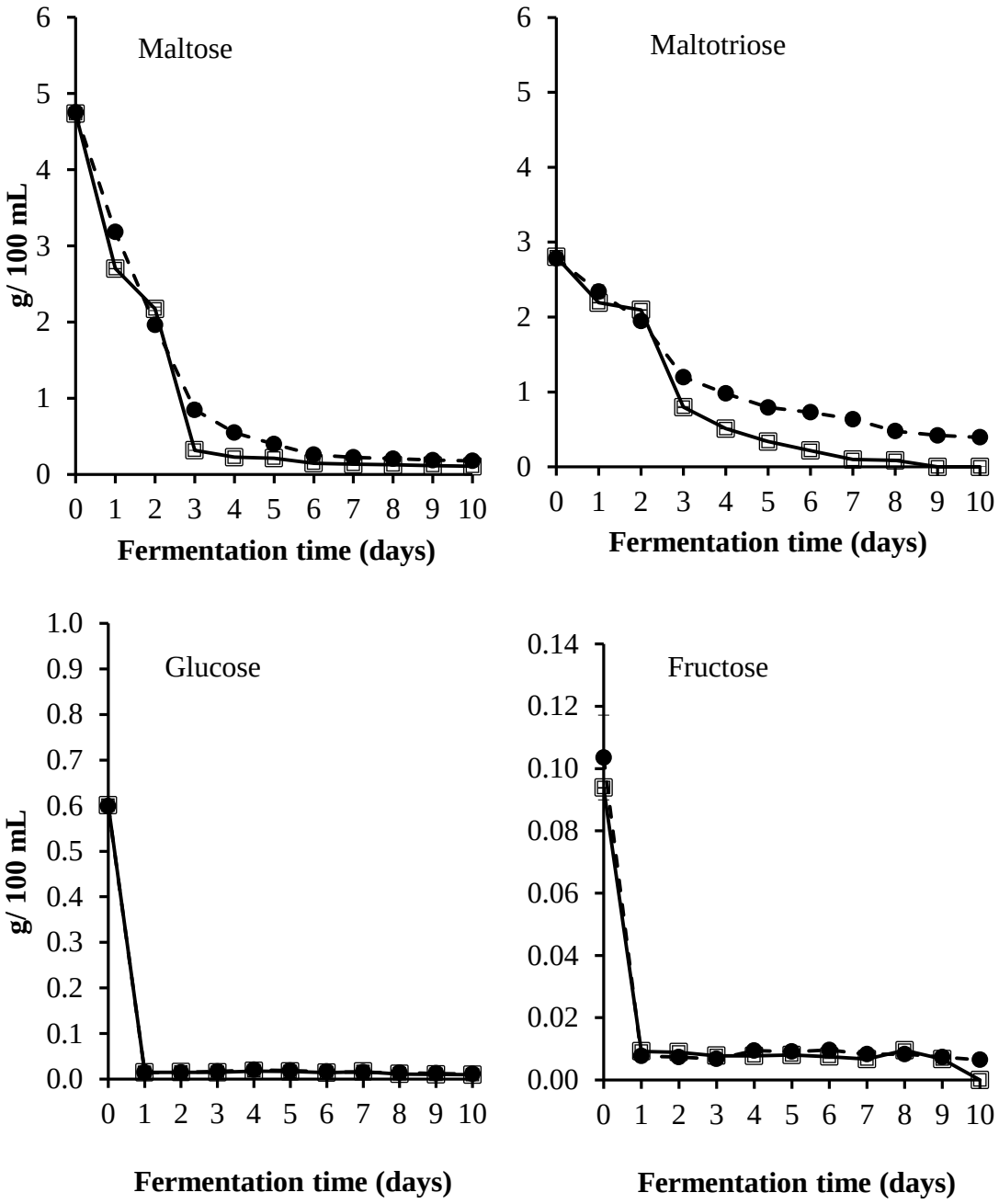

Figure 4.16. The effect of pitching with aged yeast on sugar spectrum during fermentation of sorghum grain wort at 15 °C for up to10 days. Control – squares; Aged yeast – closed circles. Error bars indicate standard deviations. (n=2)

Table 4.7. Effect of pitching with aged yeast on yeast counts after 10 days of sorghum grain wort fermentation at 15°C

Treatment	Day 0	Day 10
	(Log_{10} cfu/ mL)	
Control	6.60^{c} (0.06)	5.96^{b} (0.04)
Aged yeast	6.67^{c} (0.03)	5.57^{a} (0.08)

Figures in parentheses represent standard deviations; values with different letter superscripts in a column or row are significantly different (n=2) ($p<0.05$)

It has been shown that yeast physiology and viability change with serial repitching (Smart and Whisker, 1996). In line with the current findings, Lodolo and Cantrell (2007) reported that an artificially aged yeast crop showed a significantly decreased DNA percent formation (synthesis) after 6 h of inoculation into growth medium when compared to fresh yeast. These workers studied the sensitivity of DNA replication as an indicator of yeast vitality. They attributed the low DNA synthesis to a loss in growth capacity and potential in the aged yeast since it had low glycogen reserves at pitching. Based on the above, it is concluded that the observed poor fermentation performance that occurred with the aged yeast wort was probably due to low glycogen levels.

4.2.4.5 Effects of Serial Repitching and Supplementation with Sorghum Malt on Yeast Fermentation Performance of Sorghum Grain Wort

The effect of serial repitching on yeast fermentation performance of raw sorghum grain wort supplemented at different proportions (10% or 20%) with sorghum malt was studied. One of the reasons for using malted sorghum in lager beer brewing is that it should provide sufficient FAN for optimal yeast performance (Palmer et al., 1989; Bajomo and Young, 1993).

With the control wort (100% sorghum grain), serial repitching did not affect FAN uptake in the first three fermentations, while a reduction in total FAN uptake occurred in the 4th fermentation (Figure 4.17a). In the 1st three fermentations, a total of about 24.9 mg/100 g sorghum FAN was taken up by yeast cells in the control worts after 7 days of fermentation. This indicates that serial repitching did not influence FAN uptake from the control wort up to this point.

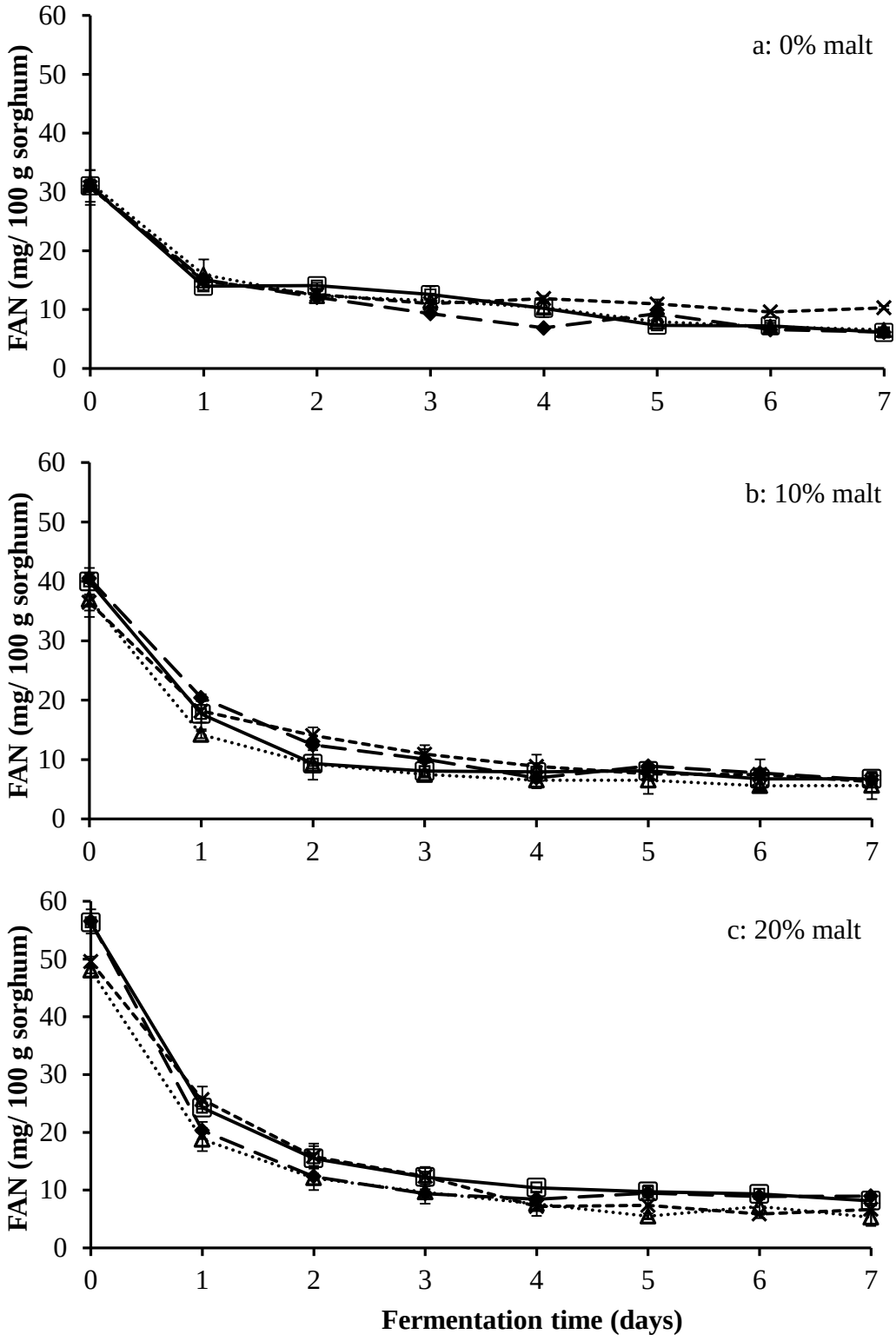

Figure 4.17. The effects of serial repitching yeast and sorghum malt supplementation on FAN uptake during fermentation of sorghum grain wort fermented at 15 °C for up to 7 days. Fermentation 1 – closed diamonds; Fermentation 2 – squares; Fermentation 3 – triangles; fermentation 4 – crosses. Error bars indicate standard deviations. (n=2).

However, the 4th fermentation showed a significantly slower reduction in FAN after 4 days and had the highest residual FAN level of 10.3 mg/100 g sorghum after 7 days of fermentation. This was approximately 40% more residual FAN than with the other fermentations. The higher residual FAN level with the 4th fermentation suggests that yeast quality was affected as the yeast was serially repitched. This can be attributed to ageing of the yeast or to the stress associated with fermenting with sorghum grain wort.

According to Powell et al. (2003), ageing may result in changes in yeast cell physiology, morphology and gene expression. This may in turn influence yeast fermentation performance. The inadequate amounts of FAN associated with brewing with sorghum grain wort may impact some nutrient stress to the yeast over time. Insufficient FAN may limit development of reserve carbohydrates during fermentation and this may affect yeast vitality and viability during subsequent fermentations. Similar to these findings, Kobayashi et al. (2007) reported increases in residual FAN after multiple repitchings.

Increasing the wort FAN level by supplementing sorghum grain wort with 10% sorghum malt resulted in an increase in total FAN uptake by the yeast when compared to the control (Figure 4.17b). A total of 33.9, 33.2, 31.4 and 30.2 mg FAN/100 g sorghum was taken up by the yeast in the 1st, 2nd, 3rd and 4th fermentations, respectively. This indicates that the total amount of FAN taken up by the yeast was slightly reduced with serial repitching as the 4th fermentation took up 11% less FAN than the 1st fermentation. Fermentations 2 and 3 showed a significantly more rapid reduction in FAN levels in the first 3 days of fermentation than fermentation 1. This can be attributed to the yeast adjusting to the new growth conditions brought about by the sorghum malt in the 1st fermentation.

Increasing the initial FAN level further by supplementing with 20% sorghum malt resulted in a further increase in FAN uptake by the yeast when compared to the 10% sorghum malt wort and the control (Figure 4.17c). Upon serial repitching of yeast, a similar pattern of FAN reduction to that of the 1st fermentation took place in all the subsequent fermentations. The total amount of FAN taken up increased slightly with serial repitching from 47.6 mg/100 g sorghum (1st fermentation) to 50.3 mg/100 g sorghum (4th fermentations).

Serial repitching caused a substantially slower reduction in specific gravity and an increase in the final specific gravity in the wort (Figure 4.18a). With 100% raw sorghum grain (0% sorghum malt) a rapid reduction in specific gravity took place after 1 day with fermentation 1, with 46% of the reduction occurring during this period. When the yeast was serially repitched, there was a slower reduction in specific gravity. The slower reduction in specific gravity with the control wort was similar to what was observed with FAN uptake. The final gravity increased with an increase in the number of serial fermentations from 2.0 °P with the 1st fermentation to 4.5 °P with fermentation 4. This indicates that serial repitching negatively influenced the fermentation of sugars to ethanol.

The slower reduction in specific gravity with serial repitching is in contrast to the findings of Bajomo and Young (1993) who, with 100% sorghum wort found a similar pattern of decrease in specific gravity for all their five pitches. Their contrasting results could in part be due to the fact that their study was carried out at normal gravity (12 °P), with yeast not being subjected to as high an osmotic stress as was used in the current study (16°P). Also, their yeast was serially repitched up to five times, compared to the current study, where sixth generation yeast was repitched up to three times, making the total repitchings to be 9. This means that the yeast used in the current study was probably subjected to more nutritional and ageing stresses.

Increasing the amount of FAN in the wort with 10% sorghum malt supplementation resulted in a slower reduction in the specific gravity with serial repitching (Figure 4.18b). An increase in the final gravity also occurred when compared to 100% raw sorghum grain. Most (43%) of the specific gravity changes occurred in the 1st day of fermentation. When serially repitched, fermentations 3 and 4 showed a similar reduction in specific gravity but significantly slower than that for fermentations 1 and 2. The slower reduction in specific gravity and high final gravity with fermentations 3 and 4 indicates that yeast quality was adversely affected by serial repitching despite the higher wort FAN. It also suggests that the sorghum malt might have introduced some unfermentable sugars. In addition, the sorghum malt may have introduced inhibitory compounds such as anti-yeast compouds (thionins and defensins) that may have affected yeast growth (van Nierop, Axcell, Cantrell and Rautenbach, 2008).

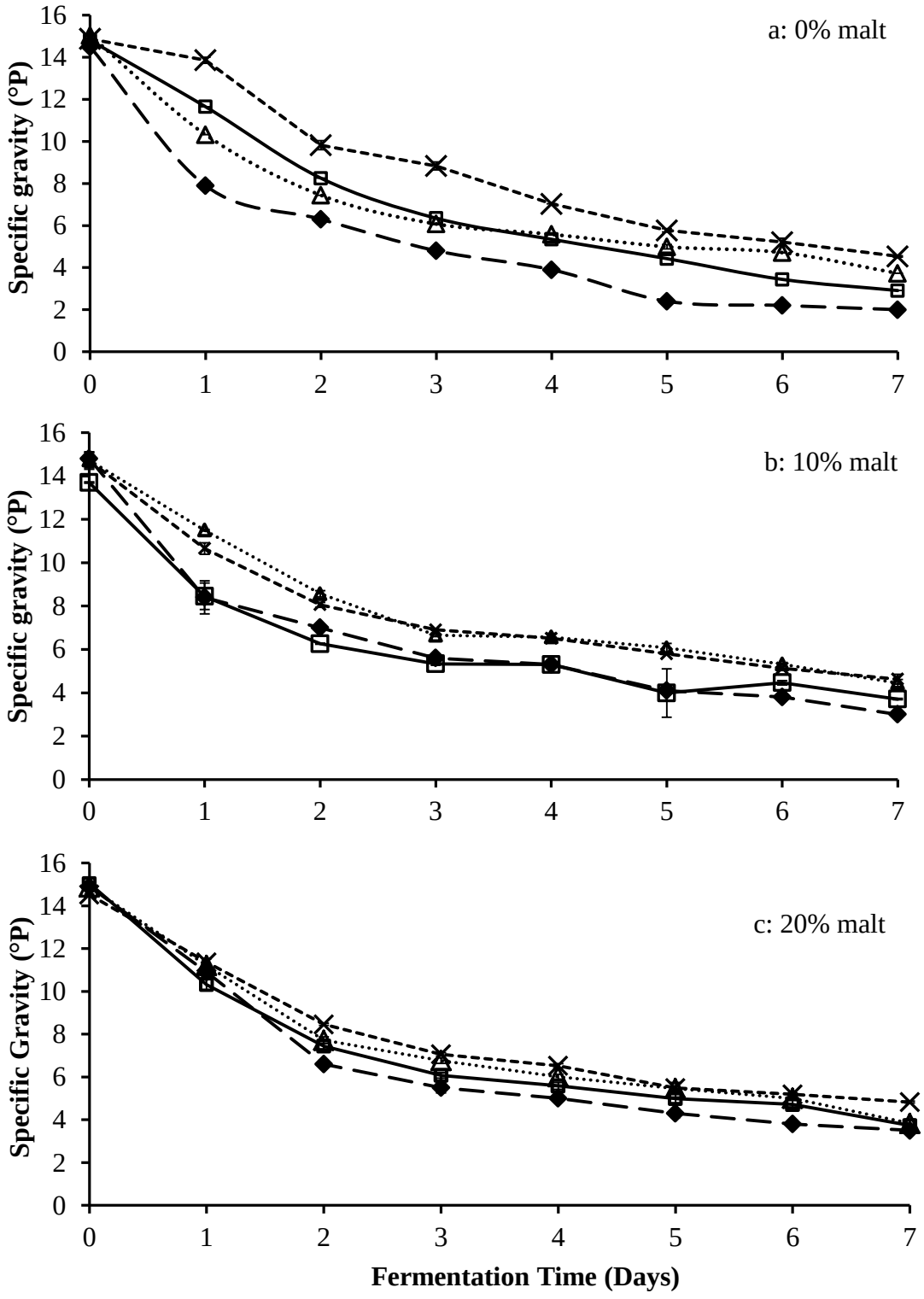

Figure 4.18. The effects of serial repitching yeast and sorghum malt supplementation on specific gravity during fermentation of sorghum grain wort fermented at 15 °C for up to 7 days. Fermentation 1 – closed diamonds; Fermentation 2 – squares; Fermentation 3 – triangles; fermentation 4 – crosses. Error bars indicate standard deviations. (n=2)

Increasing the wort FAN further by supplementing with 20% sorghum malt resulted in a further increase on the final gravity (Figure 4.18c). Most (56%) of the specific gravity reduction took place in the first 2 days of fermentation in all the fermentations. Upon serial repitching, a slower decrease in specific gravity took place compared to fermentation 1 and the gravity end point increased with serial repitching. This could be attributed to the effect of serial repitching.

With the control wort, serial repitching negatively influenced the rate of maltose and maltotriose uptake (Figure 4.19). A faster uptake of maltose and maltotriose took place in the 1st fermentation over the 7 days compared to the subsequent fermentations. Most of the maltose (69%) and maltotriose (51%) uptake, occurred over the first 2 days of fermentation. Upon serial repitching, the rate of maltose and maltotriose uptake was substantial reduced to approx. 16% and 18%, respectively, after 2 days of fermentation in the 2nd fermentation. Maltose and maltotriose uptake in the 3rd and 4th fermentations after 2 days was better than that of the 2nd fermentation with the 4th fermentation showing a substantially higher residual maltose and maltotriose than the other fermentations. Nearly all (99%) the control wort glucose was taken up after 2 days in all fermentations and fructose was not detected.

Serial repitching negatively influenced maltose and maltotriose uptake in the wort supplemented with 10% sorghum malt (Figure 4.20). The rate of maltose and maltotriose uptake decreased with serial repitching, with fermentations 3 and 4 showing the slowest uptake and higher residual maltose and maltotriose levels after 7 days than the other fermentations. Supplementing sorghum grain wort with 10% sorghum malt resulted in an 18% increase in the initial glucose levels. Nearly all the glucose was taken up after 2 days of fermentation, while fructose levels were not detected.

Increasing the supplementation of sorghum malt to 20% resulted in a slight increase in the total glucose levels, compared with the 10% malt (Figure 4.21). Similar to the 10% sorghum malt supplement, all the glucose was consumed after 2 days in all fermentations. Fructose was not detected in any of the treatments.

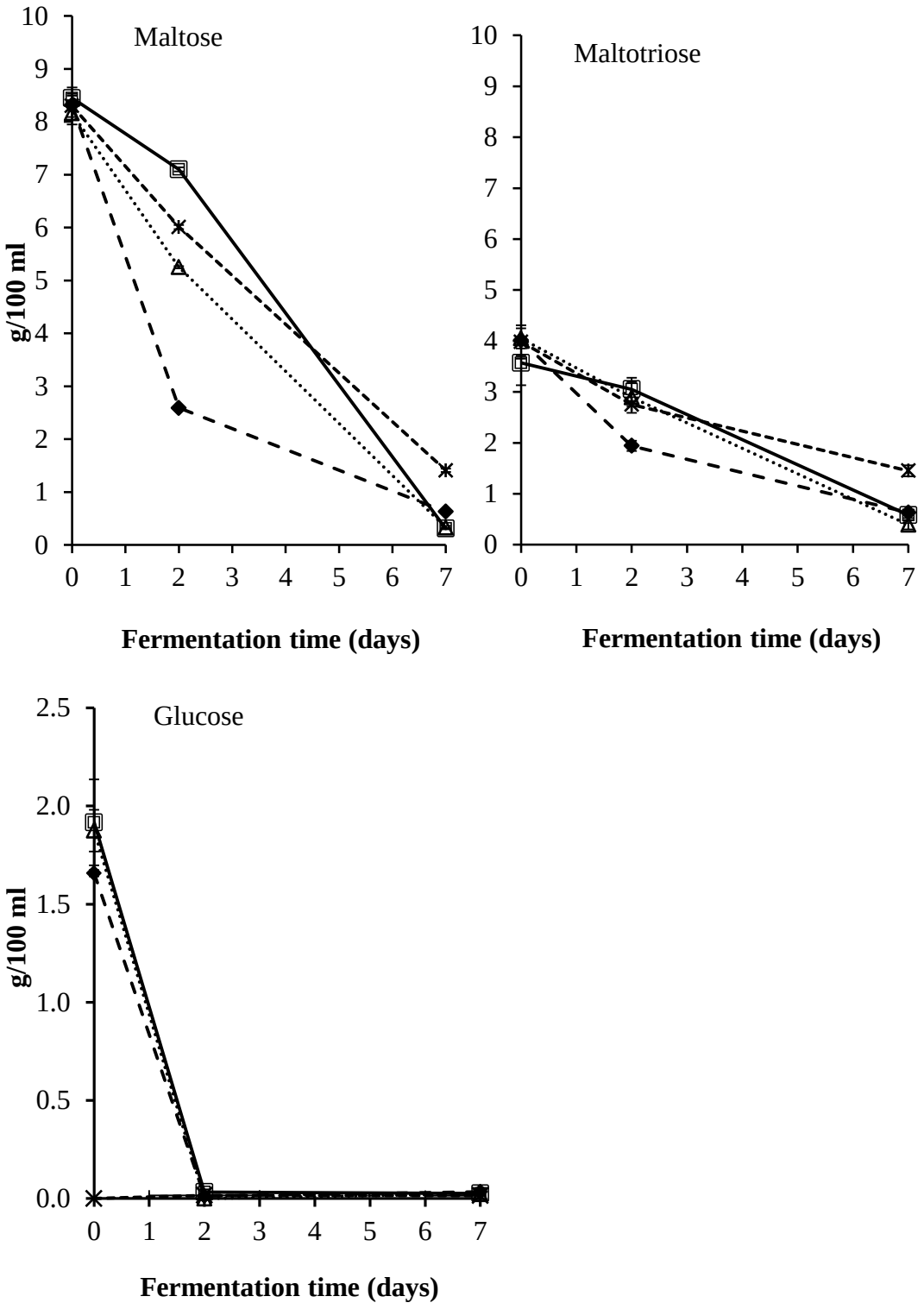

Figure 4.19. The effect of serial repitching yeast on sugar spectrum during fermentation of sorghum grain wort (0% sorghum malt) at 15 °C for up to 7 days. Fermentation 1 – diamonds; Fermentation 2 – squares; Fermentation 3 – triangles; fermentation 4 – asterisk. Error bars indicate standard deviations. (n=2)

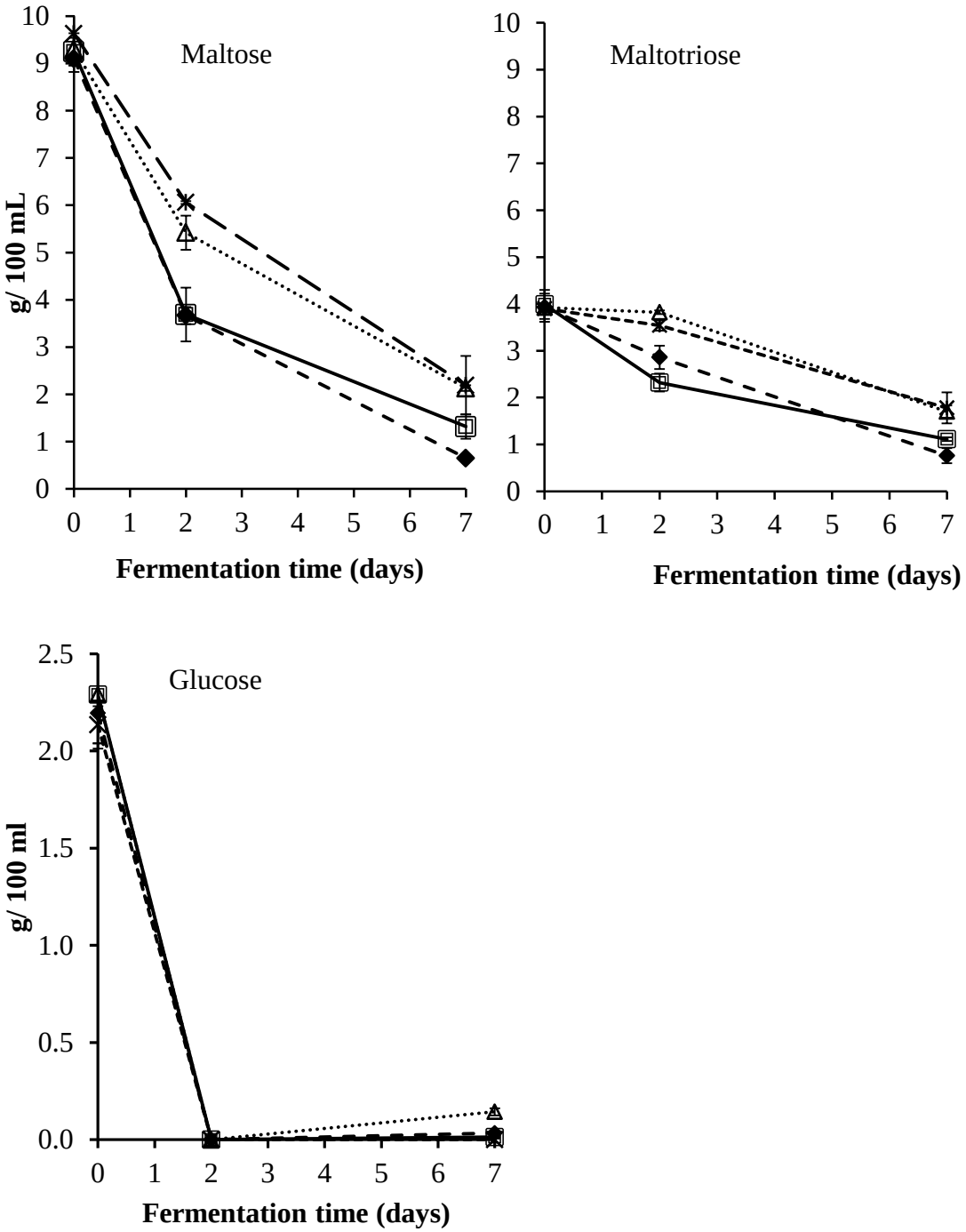

Figure 4.20. The effect of serial repitching yeast on sugar spectrum during fermentation of sorghum grain wort supplemented with 10% sorghum malt and fermented at 15 °C for up to 7 days. Fermentation 1 – diamonds; Fermentation 2 – squares; Fermentation 3 – triangles; fermentation 4 – Asterisk . Error bars indicate standard deviations. (n=2)

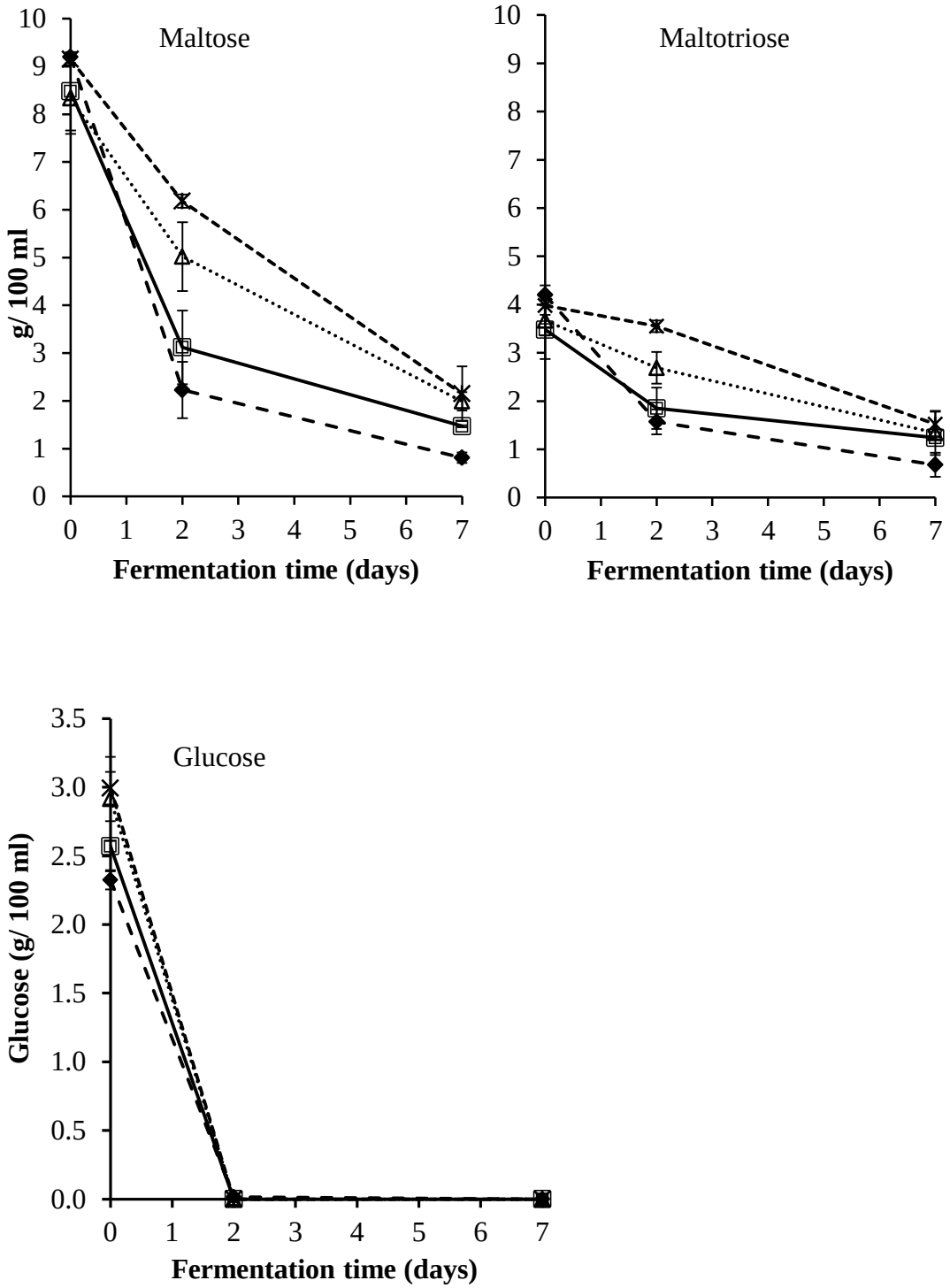

Figure 4.21. The effect of serial repitching yeast on sugar spectrum during fermentation of sorghum grain wort supplemented with 20% sorghum malt and fermented at 15 °C for up to 7 days. Fermentation 1 – diamonds; Fermentation 2 – squares; Fermentation 3 – triangles; fermentation 4 – Asterisk . Error bars indicate standard deviations. (n=3)

In general, lower residual levels of maltose and maltotriose were found in this study than those reported by Casey et al. (1984). These workers reported high residual maltose, 2.7 g/100 mL, after eight days of fermentation on unsupplemented corn (maize) grits adjunct commercial wort. Further, they reported final maltotriose concentrations of less than 1.4 g/ 100 mL after repitching a 28% maltose wort supplemented with yeast extract, ergosterol and oleic acid for up to five times. They also concluded that these levels indicate normal attenuation of maltotriose. Since the final maltotriose levels of the current study are below 1.4 g/ 100 mL, complete attenuation of maltotriose for all fermentations can be assumed, except for the last fermentation of the 20% malt supplement.

Figure 4.22 shows the effects of serial repitching and sorghum malt supplementation on ethanol production after 7 days of fermentation. In general, serial repitching caused a reduction in total ethanol production levels, except with the 20% malt supplementation in the second fermentation (Figure 4.22). For example, 5.3% (v/v) ethanol was produced by 10% malt supplementation in the fourth fermentation. This was 22% less than with the 1st fermentation. Supplementing with sorghum malt improved ethanol levels when compared to the 100% raw grain control but not in the first fermentation. The latter can be attributed to the yeast adjusting to growth on new nutrients from the sorghum malt, as observed by the significantly lower ethanol levels with a high (20% malt) supplement in the first fermentation. The improved ethanol levels with sorghum malt supplementation could be due to the increase in available micronutrients that are associated with malting. For example, substantial increases in magnesium and zinc during germination have been reported (Irakoze, Zhou, Zhang, Zhu, Li, Murekatete, 2001)

The reduction in total ethanol produced with serial repitching could have been due to nutritional limitation, particularly nitrogen. As stated, Casey et al. (1984) demonstrated that production of high levels of ethanol by brewing yeast is limited by nutritional deficiencies. These workers found that addition of nitrogenous sources such as yeast extract and lipids (ergosterol and oleic acids) may improve ethanol yields by up to 15%. They attributed the increase to prolonged and increased production of yeast cell mass that comes with supplementation. In another study, O'Connor-Cox et al. (1991) reported that supplementing wort with yeast extract enhanced production of ethanol by up to 1.3%.

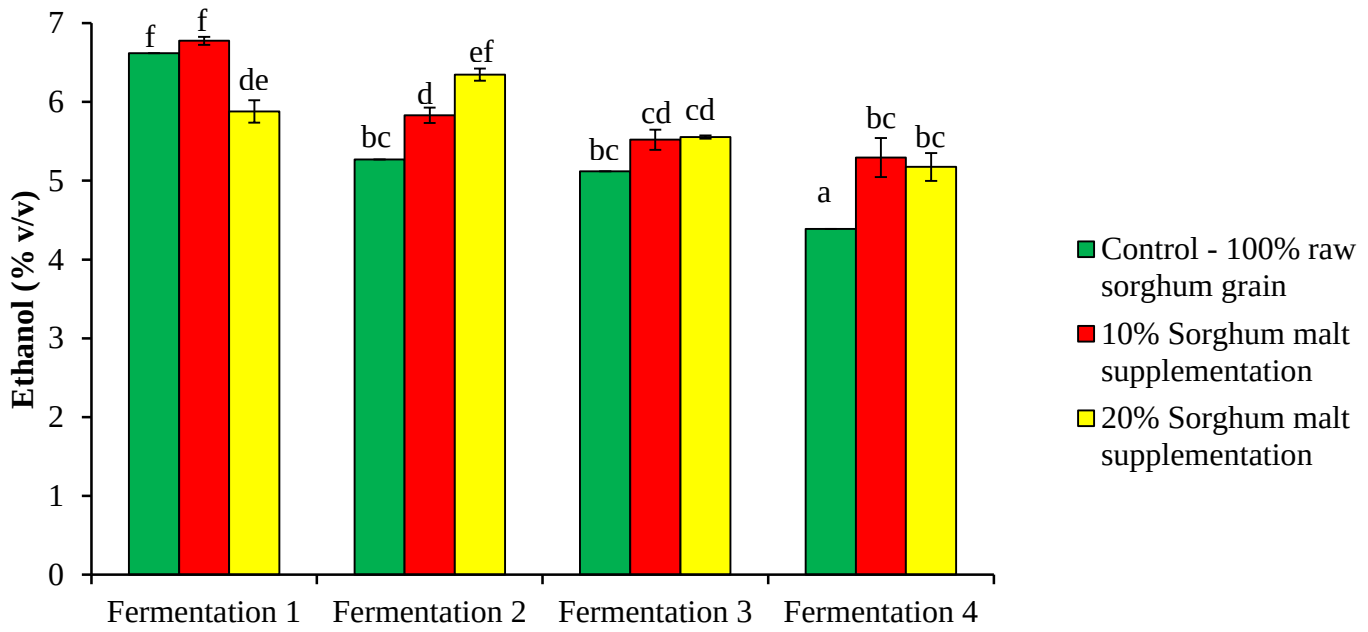

Figure 4.22. The effects of serial repitching yeast and sorghum malt supplementation on total ethanol production during fermentation of sorghum grain wort fermented at 15 °C for up to 7 days. Error bars indicate standard deviations. Letters that are different within fermentation are significant (n=2).

Contrary to these findings, Vilanova, Ugliano, Valera, Siebert, Pretorius and Henschke (2007) reported that supplementing grape wine musts with different concentration of ammonia did not have a significant effect on ethanol production but only increased the fermentation rate.

Table 4.8 shows the effects of serial repitching and sorghum malt supplementation on yeast counts during fermentation of sorghum grain wort. There was a significant interaction between serial fermentation period, treatment (malt addition) and the day of fermentation (Table 4.9). Yeast counts in the second fermentation were low in all treatments after 7 days of fermentation when compared to the other fermentations. Supplementing sorghum grain wort with 20% sorghum malt resulted in lower yeast counts after 7 days in the first 3 fermentations than the control (100% raw sorghum grain) and 10 % malt supplementation. This means that supplementing with 20% malt hindered yeast growth when compared to the control and 10% malt supplement. This was probably due to inhibitory substances that could be introduced into the wort by the sorghum malt. Kil, Seong, Ghimire, Chung, Kwon, Goh, Heo, Kim, Lim, Lee, Yu (2009) reported that crude organic extracts from sorghum malt have antimicrobial activities against yeast such as *Candida albicans*. In their study, methanol extracts gave higher levels of antimicrobial activity than other fractions.

Serial repitching did not show a clear effect on pH (Figure 4.23). With the control wort, a slow reduction in pH took place after 3 days in the fourth fermentation. The second fermentation of the 10% malt supplementation showed a rapid reduction in pH after 7 days when compared to the other fermentations. A similar pH reduction took place in all the fermentations with the 20% sorghum malt supplementation. The final pH values for all the fermentations ranged from 3.8 to 4.2. These final pH's are similar to what was observed by Bajomo and Young (1994). These workers reported similar pH values for all serial pitches with a final pH value of 4.0 with 100% raw sorghum wort.

Table 4.8. Effects of serial repitching and sorghum malt supplementation on yeast counts during fermentation of sorghum grain wort at 15 °C for up to 7 days

Fermentation	**Treatment**	**Day 0**	**Day 7**
		(Log_{10} cfu/ mL)	
1	Control[1]	7.13 (0.02)	6.56 (0.08)
1	10% Malt	7.23 (0.04)	6.64 (0.08)
1	20% Malt	7.22 (0.03)	6.21 (0.08)
2	Control[1]	7.02 (0.01)	5.83 (0.02)
2	10% Malt	6.99 (0.01)	5.93 (0.05)
2	20% Malt	7.12 (0.21)	5.77 (0.04)
3	Control[1]	6.98 (0.01)	6.80 (0.19)
3	10% Malt	6.92 (0.02)	6.35 (0.07)
3	20% Malt	6.96 (0.01)	5.95 (0.02)
4	Control[1]	6.59 (0.05)	7.28 (0.01)
4	10% Malt	6.50 (0.03)	6.61 (0.13)
4	20% Malt	6.50 (0.04)	6.22 (0.56)

[1]100% raw sorghum grain; figures in parentheses represent standard deviations (n=2) ($p<0.05$)

Table 4.9. Statistical analyses of the effects of serial repitching and sorghum malt supplementation on yeast counts during fermentation of sorghum grain wort at 15 °C for up to 7 days

Source	*Degrees of Freedom*	*P value*
Fermentations (1 - 4)	3	0.00
Treatment (control (100% raw sorghum grain), 10% sorghum malt, 20% sorghum malt)	2	0.00
Day (Day 0, Day 7)	1	0.00
Fermentation* Treatment* Day	6	0.15

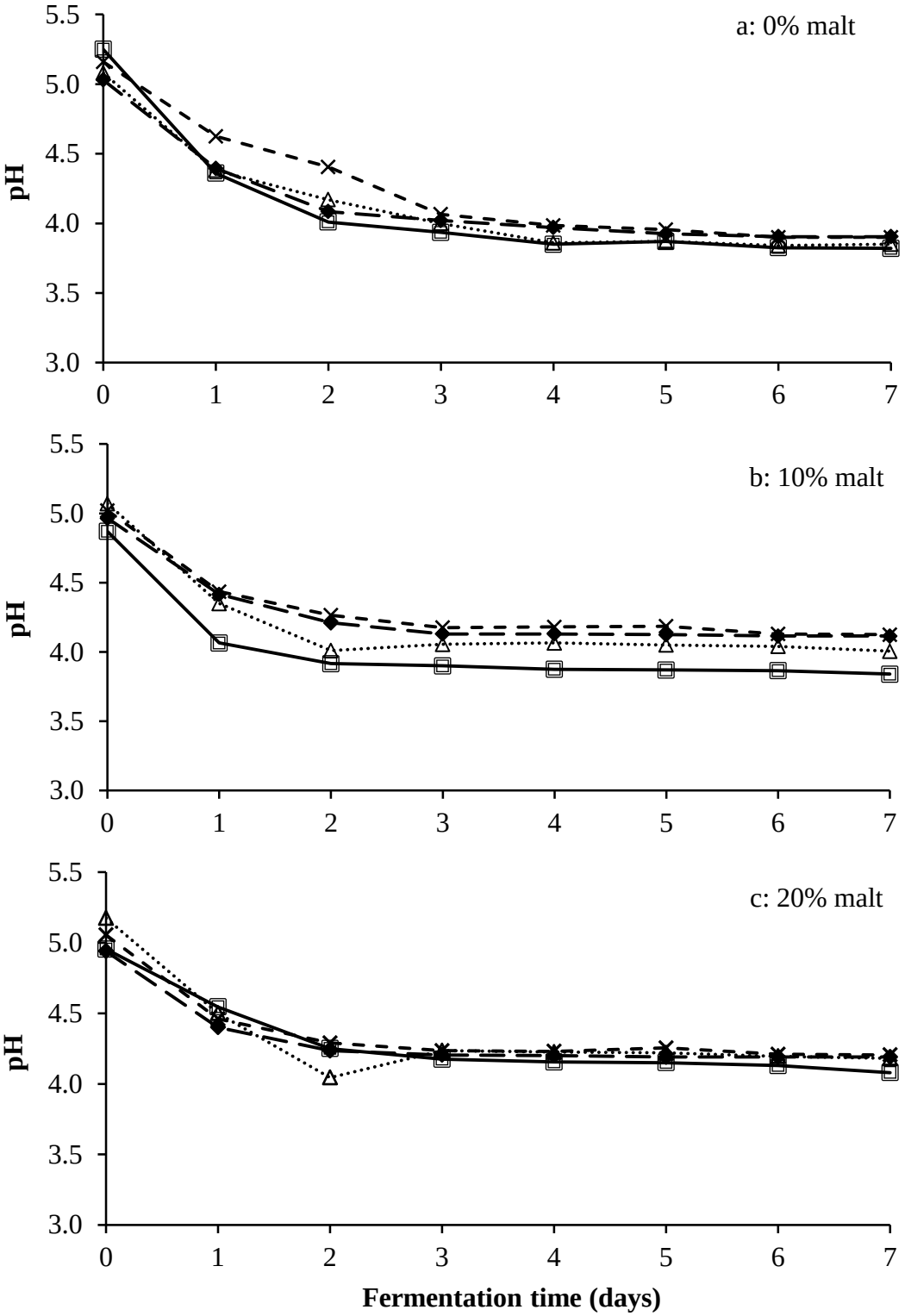

Figure 4.23. The effect of serial repitching yeast on pH during fermentation of sorghum grain wort supplemented with different proportions of sorghum malt at 15 °C for up to 7 days. Fermentation 1 – closed diamonds; Fermentation 2 – squares; Fermentation 3 – triangles; fermentation 4 – Crosses . Error bars indicate standard deviations. (n=3)

4.2.5 Conclusions

Diammonium phosphate supplementation substantially improves yeast fermentation rate in terms of improved maltose and maltotriose uptake and reduction in specific gravity. These effects were probably due to the fact that ammonia regulates enzyme activities, permease activities and transcription of nitrogen regulated genes in the yeast cell. Glycine supplementation inhibits yeast fermentation performance, reducing the rate of total reducing sugar uptake. Lysine supplementation improves final ethanol levels (up to 7.4% (v/v)) when compared to the control (7.1% (v/v)) but results in low yeast counts at the end of fermentation.

Pitching sorghum grain wort with aged yeast cells negatively affects yeast fermentation performance rate, probably due to low glycogen levels at the start of fermentation. This highlights the importance of pitching with yeast of good quality, particularly when serial repitching is practiced. Serial repitching negatively influences yeast fermentation performance in terms of maltose and maltotriose uptake. Sorghum malt supplementation did not improve the uptake of maltose and maltotriose, but resulted in improved ethanol levels compared to the 100% raw sorghum control. Supplementing sorghum grain wort with 20% sorghum malts results in lower yeast counts at the end of fermentation. This is probably caused by inhibitory substances introduced into the wort by the sorghum malt. These results suggest that DAP is a better supplement than lysine, glycine and sorghum malt since it improves yeast fermentation rate.

4.3 Determination of utilization of amino acids by yeast using Phenotypic Microarray and the effect of serial repitching on yeast morphological and genotypic characteristics

4.3.1 Abstract

Nitrogen and carbon are the major nutrients required for a complete brewing yeast fermentation. Their deficiency in wort, particularly that of nitrogen, may lead to undesirable situations such as stuck and sluggish fermentations. In this work, the extent of growth under aerobic conditions supported by ammonia (as diammonium phosphate) and lysine on brewing yeast cells was studied with Phenotypic Microarray. Further, morphology alterations on the yeast cells after serial repitching were investigated using scanning electron microscopy. The effect of serial repitching on the genotypic integrity of brewing yeast cells was studied with molecular methods based on the $(GTG)_5$ and internal transcribed spacer (ITS1 and ITS4) primers.

Ammonia supported more active yeast growth under aerobic conditions than the control and lysine supplementation when used as a sole nitrogen source or in combination with other amino acids. This is probably due to the role it plays in regulating enzyme activities, permease activities and transcription of nitrogen regulated genes. Lysine supplementation did not support any growth in all treatments when it was used as a sole nitrogen supplement, but it supported some growth when it was in combination with other amino acids. With regard to the utilization of carbon sources, nitrogen supplementation did not affect sugar utilization except for maltotriose which did not support growth with the control and lysine supplementation. Xylose supported active growth despite the fact that native *S. cerevisiae* strains are not known to metabolize xylose. Thus, this was probably an artefact. Scanning electron microscopy of the serially repitched yeast showed distorted and irregular sized cells with lysine supplementation and in the raw sorghum grain wort control. In contrast, yeast cells supplemented with ammonia were less affected by serial repitching.

PCR fingerprinting with the $(GTG)_5$ primer does not show any genome change with serial repitching. Genomic relationships determined with the ITS primers indicated that yeast cells supplemented with ammonia were not affected by serial repitching, while mutation

occurred with lysine supplementation. The latter is probably due to adaptive evolution of the yeast strain.

These findings indicate that ammonia supports more active growth than lysine and maintains yeast morphology during serial repitching and the yeast is less subject to mutation. Therefore, ammonia in the form of diammonium phosphate is a better supplement for sorghum grain wort.

4.3.2 Introduction

Brewing wort is a source of carbon, nitrogen, minerals and growth factors such as vitamins, purines and certain lipids required for proper yeast growth (Gorinstein, Zemser, Vargas-Albores, Ochoa, Paredes-Lopez, Scheler, Salnikow, Martin-Belloso and Trakhtenberg, 1999; Briggs et al., 2004). Most of the nitrogen is provided by ammonium ions, amino acids, peptides, purines and pyrimidines (Briggs et al., 2004). Maltose and maltotriose are the most abundant sugars in barley malt brewer's wort, followed by glucose and fructose (Gibson et al., 2008). Similar sugars occur in sorghum wort (Figure 4.9).

The utilization of both nitrogen and carbon sources during fermentation is an ordered process. Through nitrogen catabolite repression, readily assimilable nitrogen sources such as asparagine, glutamine, glutamine and ammonia (Batistote et al., 2006) suppress the synthesis of uptake systems and catabolic enzymes of other less readily used sources of nitrogen (Magasanik and Kaiser, 2002). Nitrogen requirement during fermentation varies with yeast strain. Interestingly, there is no correlation between strain fermentation capacity and its ability to store nitrogen (Brice et al., 2014). The effect of nitrogen supplementation is normally investigated during fermentation which limits the studies to a few amino acids at a time. There is little work on the utilization of all amino acids at the same time and over a short period of time (48 h). Investigating the utilization of all amino acids at the same time is more economical than fermentations that investigate the effectiveness of individual amino acids.

Similar to the utilization of nitrogen sources, the uptake of sugars by yeast is subject to the sugar catabolite repression, which ensures an ordered sequence of sugar utilization (Cruz et al., 2003). The presence of a preferred carbon source such as glucose, represses the expression of genes and enzymes for the utilization of alternative carbon sources. Consequently, maltose and maltotriose are utilized only after the depletion of the monosaccharides (Lagunas, 1993). Sluggish fermentations with limiting nitrogen levels have also been associated with decreased rate of sugar uptake (Salmon, 1989).

During fermentation, yeast cells are subjected to different environmental conditions that are constantly changing, such as temperature, osmolarity, oxygen, pH, ethanol and

nutrients (Pratt et al., 2007). Yeast cells must cope with these changes in order to ensure continued growth and metabolic activity. To achieve this, yeast cells respond to the different stress situations through a variety of responses (Pratt, Bryce and Stewart, 2003). These include alterations in cell morphology such as cell elongation (Bonin et al., 2006) and genetic modification to improve yeast stress tolerance and ultimately fermentation performance (Dequin, 2001; Saerens et al., 2010). Further, yeast cells have the ability to re-organize their genomic expression and hence change patterns of cellular proteins and metabolites (Gasch and Werner-Washburne, 2002).

Several methods can be used for the analysis of yeast polymorphism, including karyotype analysis, δ sequence typing, mtDNA restriction analysis and microsatellite genotyping (Schuller et al., 2004). The PCR-fingerprinting method based on the microsatellite primer has been mainly used to characterise yeast population dynamics during fermentation (Esteve-Zarzoso et al., 2001; da Silva-Filho et al., 2005). Microsatellite typing with the $(GTG)_5$ primer has been reported to have similar discriminatory power to other methods such as mtDNA restriction analysis and karyotyping (Schuller et al., 2004). To directly assay the effects of genetic changes on cells, mainly gene knock-outs (Bochner et al., 2001), Phenotypic Microarray (PM) technology is used (Biolog, 2011). This system involves testing catabolic pathways in the cell for carbon, nitrogen, phosphorus and sulphur, as well as biosynthetic pathways under different growth conditions (Bochner, 2003).

Understanding how brewing yeasts respond to the changing environment during beer fermentation is critical in improving yeast health and ultimately yeast fermentation performance. In this work, the utilization of nitrogen sources by the brewery yeast used in Research Chapter 4.2 following supplementation with ammonia in the form of diammonium phosphate or lysine was studied with the PM technology. This was done to determine the nitrogen source that supports optimal fermentation performance, particularly when brewing with sorghum grain. It was shown in Research Chapter 4.2 that nitrogen supplementation improved the utilization of fermentable sugars and that this depended on the type of nitrogen source. Since brewing yeast which had been used to ferment barley wort was used on sorghum wort, the $(GTG)_5$ microsatellite typing method was used to ensure that no contaminating yeast are involved in the study. Sequencing of PCR products

amplified with the internal transcribed spacer (ITS) region primers was also performed to determine any changes in the gene sequences caused by serial repitching.

4.3.3 Materials and Methods

4.3.3.1 Yeast

As reported in Research Chapter 4.2, a commercial *S. cerevisiae* lager yeast strain was kindly provided by the South African Breweries (Rosslyn, South Africa). The yeast, which was previously used to ferment barley malt wort, was in its sixth fermentation cycle and in this study it was used further for three fermentation cycles. During collection, the yeast, in form of slurry, was transported on ice and stored at 4°C until use, within 24 h.

4.3.3.2 Fermentations

Fermentations were conducted in European Brewery Convention (EBC) tubes (EBC Analytica Microbiologica, Method 2.5.4) (EBC, 1977) capped at the open end with cotton wool. All fermentations were conducted on high gravity sterile sorghum wort (16 °Plato) with a working volume of 2 L. Sorghum worts which contained approximately 50 mg/L free amino nitrogen (FAN) were supplemented with 50 mg/L lysine or diammonium phosphate (DAP), giving a total FAN level of approximately 100 mg/L for each treatment. Wort was inoculated with 14 g/L of yeast slurry and this was estimated to give an initial yeast count of 23 – 25 x 10^6 viable cells/ mL. Fermentations were performed at 15 °C and samples were removed daily from day 0 – 7 and cooled immediately on ice. The yeast and resulting wort were separated by centrifugation (8,000 g, 10 min, 4°C).

4.3.3.3 Specific gravity and suspended yeast cell measurements

The specific gravity (°P) of the fermenting wort was measured by pycnometry, EBC method 4.5.1 Extract of Malt: Congress Mash AM (EBC, 1998). The number of suspended yeast cells were counted by haemocytometry and the results expressed as cells/mL.

4.3.3.4 Yeast morphology

Yeast cells were fixed with 2.5% (v/v) glutaraldehyde in 0.075 M phosphate buffer (pH 7.4) for 30 min. This was followed by rinsing three times in buffer. Rinsed cells were then dehydrated in graded ethanol series (50, 70, 90 and 100%) and dried to the critical-

point. The cells were then mounted onto scanning electron microscopy (SEM) specimen stubs and coated with carbon using an Emitech K550X sputter coater (Quorum Technologies, London) and observed using a Zeiss Ultra Plus 55 FEG scanning electron microscope (Oberkochen, Germany).

4.3.3.5 Phenotypic microarray

Yeast cells, freshly obtained from the brewery, were sub-cultured onto plate count agar plates and incubated at 25 °C for 4 days. Individual colonies were picked up from the surface of the plate using a cotton swab. These cells were suspended in inoculation fluid to a concentration of 10^6 – 10^7 cfu/mL using a Biolog turbidimeter 21907 (Biolog, Hayward, CA). The final concentration of ingredients in the inoculation fluid after the addition of the cell suspension were IFY-0 = 1x, Dye mix D = 1x, glucose = 100 mM, potassium phosphate buffer (pH 6.0) = 5 mM and sodium sulphate = 2 mM. The suspensions were then inoculated into nitrogen (PM3) or carbon source (PM1) microplates (Biolog, Hayward, CA) at a volume of 100 µL/well. Substrates on the plate were combined with lysine or DAP at a concentration of 50 mg/L per supplement. Data were recorded as Omnilog values (area under the curve), which is the measure of growth that is relative to the amount of colour that develops in each well in response to irreversible reduction of a tetrazolium dye. The negative control area was subtracted from each area of the other wells. Kinetic response data was collected every 15 min but absorbance data after 24 h and 48 h are presented in this study.

4.3.3.6 DNA Extraction

Genomic DNA was extracted from individual pure yeast cultures of day 0 and 7 for fermentation 7, and day 7 for both fermentation 8 and 9. DNA extraction was done with the PureLink™ Genomic DNA Mini kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The quantity and quality of the genomic DNA samples were analysed using a nanodrop spectrophotometer (Genway Biotech, San Diego, CA).

4.3.3.7 Rep-PCR amplification

The microsatellite oligonucleotide primer $(GTG)_5$ was used. Each reaction mixture contained 10 µL Mastermix, 2 µL CoraLoad (Qiagen, Hilden, Germany), 2 µL DNA template, 0.4 µL dimethyl sulphoxide (DMSO) (4%), 0.28 µL $(GTG)_5$ primer (Integrated DNA Technologies, Coralville, Iowa) and made up to a final volume of 20 µL with RNase

free water. DNA was amplified in DNA Engine peltier thermal cycler (Bio-Rad, Hercules, CA) under the following conditions: initial denaturation at 95 °C for 5 min; 30 cycles: denaturation at 95 °C for 30 s, annealing at 40 °C for 1 min, polymerisation at 65 °C for 3 min and a single final polymerisation at 65 °C for 8 min. The mixture was subsequently cooled to 4 °C. The PCR amplified DNA was separated by electrophoresis at 100 V for 100 min on a 1% agarose gel with 1-kb DNA ladder (Life Technologies, Carlsbad, CA). The amplified DNA bands were visualized following ethidium bromide staining under UV light and the banding patterns were captured in a Gel Doc XR molecular imager system (Bio-Rad, Hercules, CA). The rep-PCR fingerprints were analysed using the BioNumerics software, version 7.10 (Applied Maths, East Flanders, Belgium).

4.3.3.8 DNA amplification for sequencing

To confirm the affiliation of the brewing yeast strain used in this study to *S. cerevisiae* species, DNA amplifications were done with ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) primers as described by White, Bruns, Lee, and Taylor (1990). The thermal cycling parameters were an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. Amplification was performed in a DNA Engine peltier thermal cycler (Bio-Rad, Hercules, CA). Alignment of the resulting sequences was performed and a phylogenetic tree was constructed using the Neighbour-joining method. *Saccharomyces cariocanus* was used as the rooting strain because it contains the ITS1 region (Huelsenbeck, Bollback and Levine, 2002).

4.3.3.9 Statistical analyses

One-way ANOVA was used to determine the effects of specific gravity, yeast counts and pH on yeast fermentation performance. ANOVA was performed using Statistica software, version 12 (Tulsa, OK).

4.3.4 Results and Discussion

4.3.4.1 Fermentation performance of serially repitched yeast

To generate yeast cells for this work, fermentations similar to those in Research Chapter 4.2 were carried out but with ammonia and lysine supplementations, both at 50 mg/ L nitrogen. The fermentations were done with yeast which had been used for six barley malt lager brewing cycles. It was then repitched up to fermentation cycle 9 in sorghum wort. The mean of the three fermentation cycles was used since the fermentation parameters did not vary with serial repitching. As found previously, a more rapid reduction in specific gravity occurred with the ammonia supplemented worts than with the lysine supplemented wort (Figure 4.24). However, both the ammonia and lysine supplemented worts gave the same final gravity (2.1 °P) after 7 days of fermentation. The control wort showed the slowest reduction in specific gravity and had a higher final gravity (2.6 °P). A more rapid reduction in pH occurred in the ammonia supplemented wort compared to the lysine supplemented and control wort (Figure 4.24). Most pH change occurred in the first 3 days of fermentation with all the treatments.

Yeast counts for the control and lysine supplemented wort showed a substantial decrease after 7 days fermentation compared to the DAP supplemented wort yeast counts which decreased slightly after the same time period (Table 4.10). The low yeast count at the end of fermentation with lysine supplementation is similar to what occurred previously (Table 4.5). It also confirms that lysine supplementation inhibited yeast growth.

Table 4.10. Effects of lysine and DAP supplementation on yeast counts after 7 days of sorghum grain wort fermentation at 15°C

Treatment	**Day 0**	**Day 7**
	($x10^6$ cell/ mL)	
Control	25.5^{de} (2.8)	16.8^{b} (1.8)
DAP	24.0^{d} (2.1)	21.3^{c} (1.1)
Lysine	26.5^{de} (2.8)	15.0^{a} (3.5)

Figures in parentheses represent standard deviations; values with different letter superscripts are significantly different (n=2) ($p<0.05$)

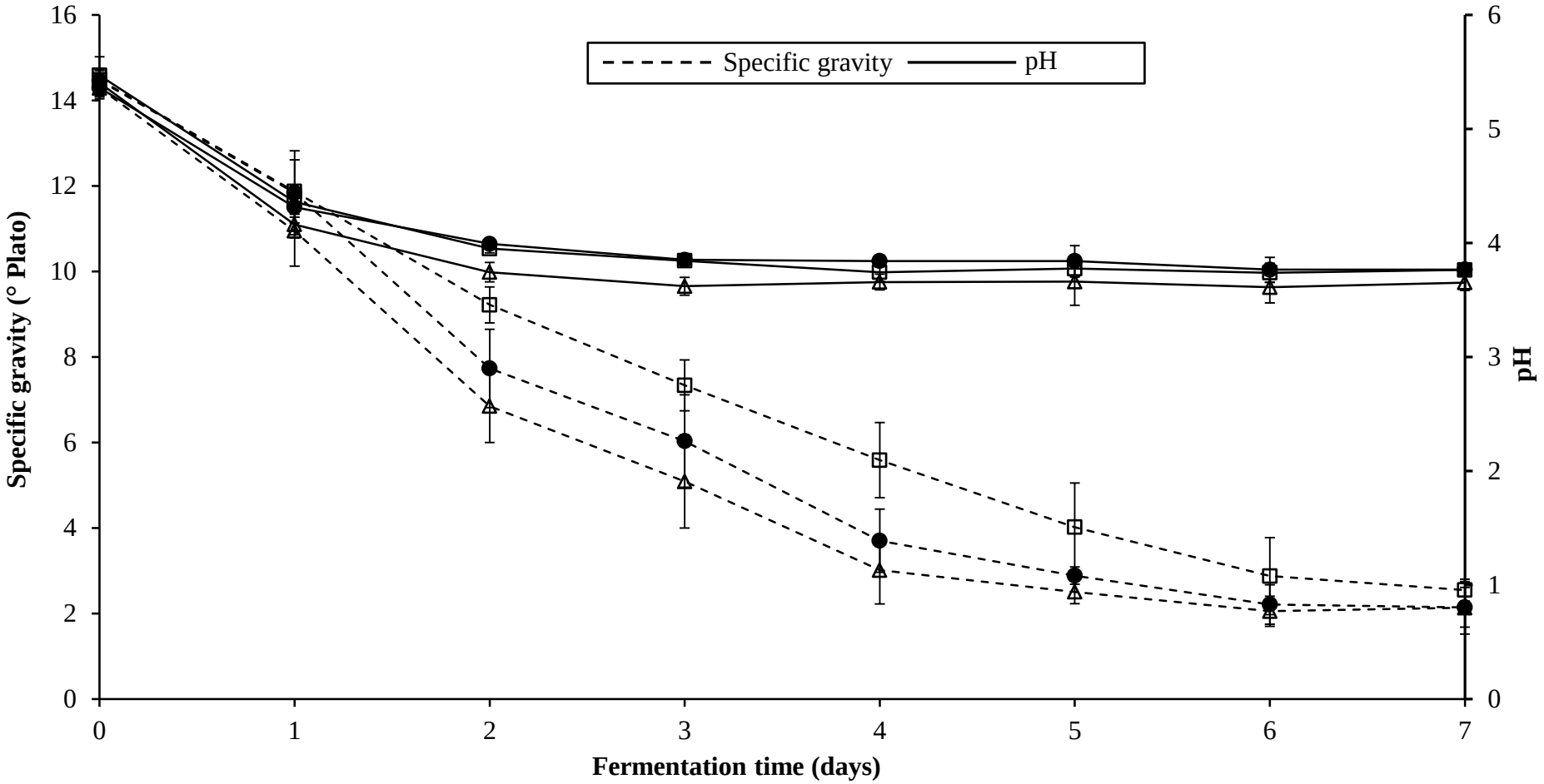

Figure 4.24. Effects of nitrogen supplementation on specific gravity and pH during fermentation of sorghum grain wort at 15°C for up to 7 days. Control: squares; Lysine: closed circles; DAP: triangles. Error bars indicate standard deviations. (n=2)

4.3.4.2 Morphology of serially repitched yeast

Yeast morphology was observed using SEM to determine whether there were any stress related morphological alterations to the yeast cells due to the serial fermentation on sorghum wort and possible nitrogen limitation. In general, the control and lysine treatment yeast cells showed substantial changes in morphology, while only minor changes were observed with ammonia treatment yeast cells (Figure 4.25). The yeast cells in the control worts (without nitrogen supplementation) of fermentation cycles 8 and 9 were of different sizes compared to Day 0 cells of fermentation cycle 7. The small sized cells which notably had polar bud scars appeared uniformly rough because of a well-defined wrinkling of the cell wall. The buds indicate that they are not daughter cells but cells going through autolysis (Martınez-Rodrıguez et al., 2001). During autolysis the cell membrane is destroyed resulting in the loss of cell turgor. A decrease in cell size follows resulting in wrinkled or folded yeast cells (Martınez-Rodrıguez et al., 2001). The observed autolysis in the control treatment yeast cells could be due to nitrogen limitation. According to Moench, Krueger and Stahl (1995), yeast cell autolysis can occur due to exposure to excess stress or repeated exposure to low-level stress. Some of the cells had also collapsed at the end of fermentation cycle 9.

Similar to what occurred with the control wort, the yeast cells in the lysine supplemented wort comprised different sized cells after fermentation cycles 8 and 9. They were also distorted and wrinkled. Fermentation cycle 9 yeast cells also showed a number of distorted and collapsed yeast cells. This indicates that lysine was affecting the viability of the yeast cells, which is in agreement with the decrease in yeast counts that occurred with the lysine supplemented wort (Table 4.10). In general, fermentation cycle 9, Day 7, yeast cells in the ammonia supplemented wort did not differ much from the Day 0 ammonia supplemented yeast cells of fermentation cycle 7. Therefore, a few wrinkled cells were observed after cycles 8 and 9 but the majority of the cells showed a normal oval-shaped slightly smooth surface similar to that of the fermentation cycle 7.

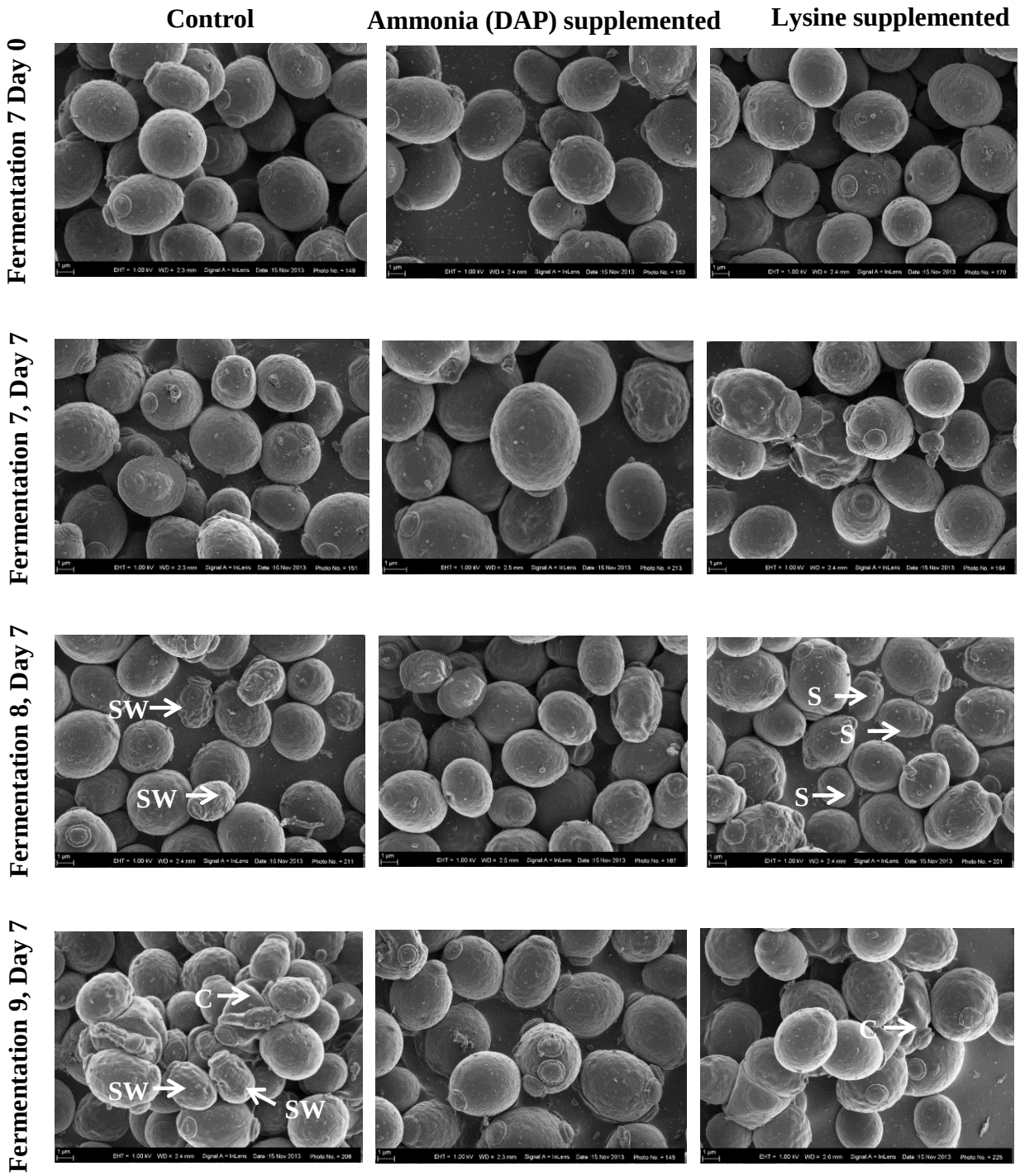

Figure 4.25. Effect of serial repitching on yeast morphology during fermentation of sorghum grain wort supplemented with ammonia (DAP) and lysine at 15°C for up to 7 days. S: small sized cells; SW: small wrinkled cells; C: collapsed cells

4.3.4.3 Phenotypic microarray of serially repitched yeast

Phenotypic microarray (PM) technique was used to assess the extent of growth supported by ammonia or lysine on brewing yeast cells when supplied as a single supplement and/or in combination with other nitrogen or carbon sources. The PM system is generally used in an aerobic respiratory environment and this was done here as during brewing, the aerobic phase of fermentation is important in the synthesis of sterols and unsaturated fatty acids (Briggs et al., 2004). These lipids play a major role in yeast multiplication and they influence the rate and extent of fermentation.

Yeast utilization of nitrogen sources

Here, only amino acids essential to yeast that are present in brewer's wort are discussed. The amino acids were grouped into four according to their assimilation during fermentation (Pierce, 1987). Group A is assimilated immediately after the yeast come in contact with the wort. Group B is taken up slowly but gradually throughout the fermentation. Group C is not utilized until Group A amino acids have disappeared from the wort while Group D or proline is the least preferred amino acid. Analysis of the PM data revealed that the control, DAP and lysine supplementation gave different growth characteristics after 24 h and 48 h incubation (Figure 4.26). Generally, yeast cells supplemented with DAP appeared to support better growth either as a sole nitrogen source or in combination with other amino acids than the control and lysine supplemented yeast cells.

Among the group A amino acids, arginine, asparagine, glutamine and serine supported most active respiration in all treatments (control, DAP and lysine supplementation) after 24 h compared to the other Group A amino acids. Interestingly, no growth occurred for all treatments when lysine was used as sole nitrogen source but supported growth when it was in combination with other amino acids. The inhibitory effect of lysine on yeast growth is similar to that reported by Thomas and Ingledew (1992) on wheat mashes. As found here, these workers further reported that this phenomenon is partially or completely reversed when lysine is used with other nitrogen sources. The mechanism involved is not well understood.

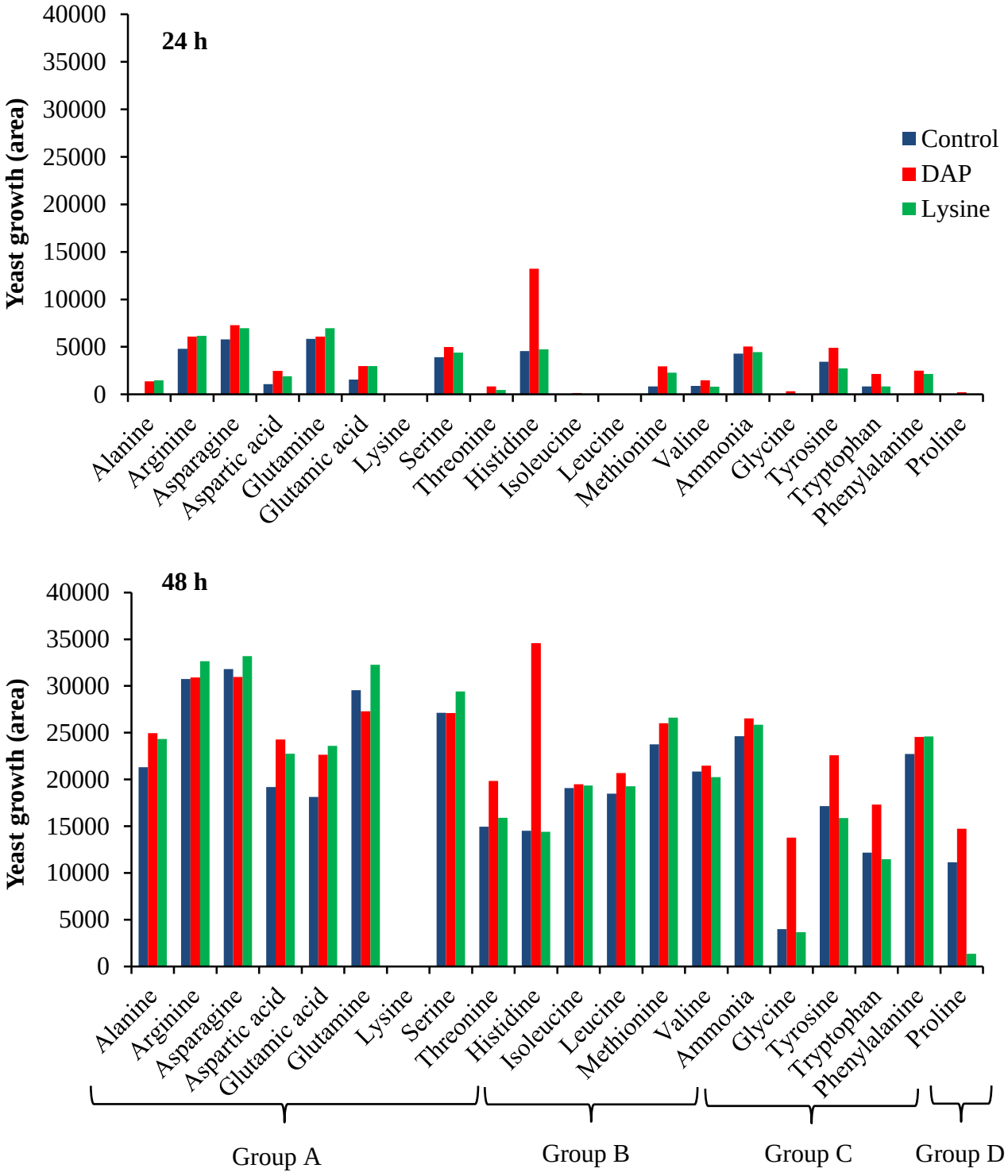

Figure 4.26. Extent of growth of yeast cells on nitrogen source phenotypic microarray plate supplemented with diammonium phosphate (DAP) or lysine and incubated for up to 48 h at 25 °C

Karthikeyan, Polasa, Sastry and Reddy (2008) reported that complexation of lysine with chromium positively influences lysine metabolism in yeast in terms of yeast growth and rapid uptake of lysine. This was thought to be due the lysine-chromium complex allowing retention of lysine in the cytosol rather than in the vacuole. In *S. cerevisiae* cytosolic amino acids are metabolizable, but the vacuolar pools of amino acids are not (Messenguy, Colin and Ten Have, 1980). It is possible that lysine deposition in the vacuoles could have adversely affected its utilization in this current study.

It has also been reported that lysine causes a feedback inhibition of the homocitrate synthase enzyme (regulated by LYS20 or LYS21) in the α-aminoadipate pathway for lysine biosynthesis in yeast cells (Ljungdahl and Daignan-Fornier, 2012). This reduces the production of α-aminoadipate semialdehyde (AAS) and subsequently causes apparent repression of the lysine genes (Andi, West and Cook, 2005). AAS is the co-inducer that is important for the Lys14p stimulated expression of the lysine genes. In another study, the addition of 50 mM lysine inhibited the α-aminoadipate reductase enzyme in yeast (Zabriskie and Jackson, 1999). The α-aminoadipate reductase enzyme catalyses the conversion of α-aminoadipic acid to α-aminodipic semialdehyde in the α-aminoadipate pathway for lysine biosynthesis. Suvarna, Seah, Bhattacherjee and Bhattacherjee, J.K. (1998) reported that lysine supplementation repressed the transcription of the *LYS2* gene. This gene is one of the genes that encode the aminoadipate reductase enzyme in *S. cerevisiae*. Their study reported that *LYS2* mRNA levels from cells grown in minimal medium supplemented with lysine was significantly lower compared to cells grown in minimal medium without lysine supplementation.

Histidine supported better growth than the other amino acids in Group B after 24 h incubation. When in combination with DAP, histidine showed a greater extent of growth, even more than occurred with the Group A amino acids. A recent study by Lei et al. (2013b) reported that histidine is one of the key amino acids for lager yeast during beer brewing. In their study, histidine stimulated yeast cell growth, increased fermentation rate and ethanol production, possibly through inhibiting the nitrogen catabolite repression system (NCR). The NCR allows yeast to select preferred nitrogen sources for growth, whilst preventing the uptake of poorer nitrogen sources in the presence of better sources (Beltran et al., 2004).

With Group C amino acids, ammonia, either as a sole nitrogen source or in combination with lysine, supported better growth after 24 h than the other Group C amino acids. This is because ammonia is preferentially used by yeast (Magasanik and Kaiser, 2002). Amino acids preferentially utilized by yeast include asparagine, glutamine and glutamate. As explained, utilization of any nitrogen source by yeast cells first involves converting it to glutamate and glutamine (Schure et al., 2000). These two amino acids can be synthesized directly using ammonia as the amino group donor (Briggs et al., 2004). The effectiveness of ammonia in increasing cell population and fermentation performance has also been reported by other workers (Mendes-Ferreira et al., 2004; Deed et al., 2011; Ugliano et al., 2011). In the yeast cell, ammonia regulates enzyme activities, permease activities and transcription of nitrogen regulated genes (Schure et al., 2000). Incubation of proline, the sole member of Group D did not result in growth in any of the treatments after 24 h.

Several fold increases in growth were observed after 48 h in nearly all the treatments compared to that at 24 h (Figure 4.26). This indicates that utilization of most of the amino acids required synthesis of specific catabolic enzymes and permeases by the yeast. The permeases are required for the transport of nitrogen-containing compounds (Magasanik and Kaiser, 2002). Similar to what occurred after 24 h, in all treatments arginine, asparagine, glutamine and serine supported better growth than other Group A amino acids. Again, after 48 h lysine did not support growth in any of the treatments when used as a sole nitrogen source. Methionine supported best growth in all treatments among Group B amino acids, followed by valine, leucine and isoleucine. In contrast, Lekkas et al. (2007) reported that methionine supplementation inhibited growth and produced lower yeast numbers compared to lysine supplemented wort under fermentative conditions. This difference can be attributed to differences in experimental conditions, such as amino acid concentrations and the specificity of yeast strains. Unlike what occurred after 24 h, histidine supported the least growth in control and lysine treatments when compared to the other Group B amino acids. However, when histidine was in combination with ammonia, it resulted in more than two fold growth compared to the control and lysine treatments.

Among Group C amino acids, phenylalanine and ammonia supported more growth in all treatments than the other amino acids, while glycine supported the least growth. The inhibitory effect of lysine on yeast growth has been explained. Proline did not support growth when it was in combination with lysine. However, it supported some growth in the

control and DAP supplemented yeast cells. The utilization of proline was expected since its metabolism requires the presence of a mitochondrial oxidase, which is only active under aerobic conditions (Wang and Brandriss, 1987). However, its poor utilization after 24 h suggests that it is the least preferred amino acid even under aerobic conditions.

In general ammonia supplementation supported more growth than the control and lysine supplementation when in combination with the other amino acids, except for lysine. The effect of ammonia on yeast growth has been explained above. In most cases, lysine supplementation did not support more growth than the control. However, positive effects of lysine supplementation under fermentative conditions have been reported. For example, Lekkas et al. (2005, 2007) reported that lysine improved fermentation performance possibly due to the increase in the number of suspended yeast cells with lysine supplementation. Lei et al. (2013) reported that lysine improved fermentation performance under high gravity conditions. These workers attributed the positive effect of lysine to improved expression of the Ssy1p-Ptr3p-Ssy5p (SPS) gene. The SPS mechanism controls the uptake of early consumed amino acids by specific permeases at the onset of fermentation (Ljungdahl, 2009).

Yeast utilization of carbon sources

The objective of this part of the study was to determine the effect of ammonia and lysine supplementation on the utilization of carbon sources under aerobic respiratory conditions. Previous research has reported that depletion of a nitrogen source from an external media may cause a substantial decrease in the sugar transport activity (Salmon, Vincent, Mauricio, Bely and Barre, 1993). In general, the utilization of sugars was not substantially affected by nitrogen supplementation (Figure 4.27). After 24 h incubation, glucose, xylose, ribose, mannose supported more yeast growth with the control, DAP and lysine supplementation when compared to the other carbon sources. Fructose and galactose also supported some growth. There was no growth with maltose, maltotriose and sucrose. This indicates that the yeast did not utilize these carbon sources during this period. The sorghum wort contained the fermentable sugars glucose, fructose, maltose, and maltotriose (Figure 4.7), plus with some dextrins. Brewing yeasts are capable of utilizing all these sugars in this particular sequence during fermentation, but not the dextrins (Gibson et al., 2008). Glucose and sucrose, if present, are normally the preferred sugars since they enter the glycolytic pathway directly (Carlson, 1987).

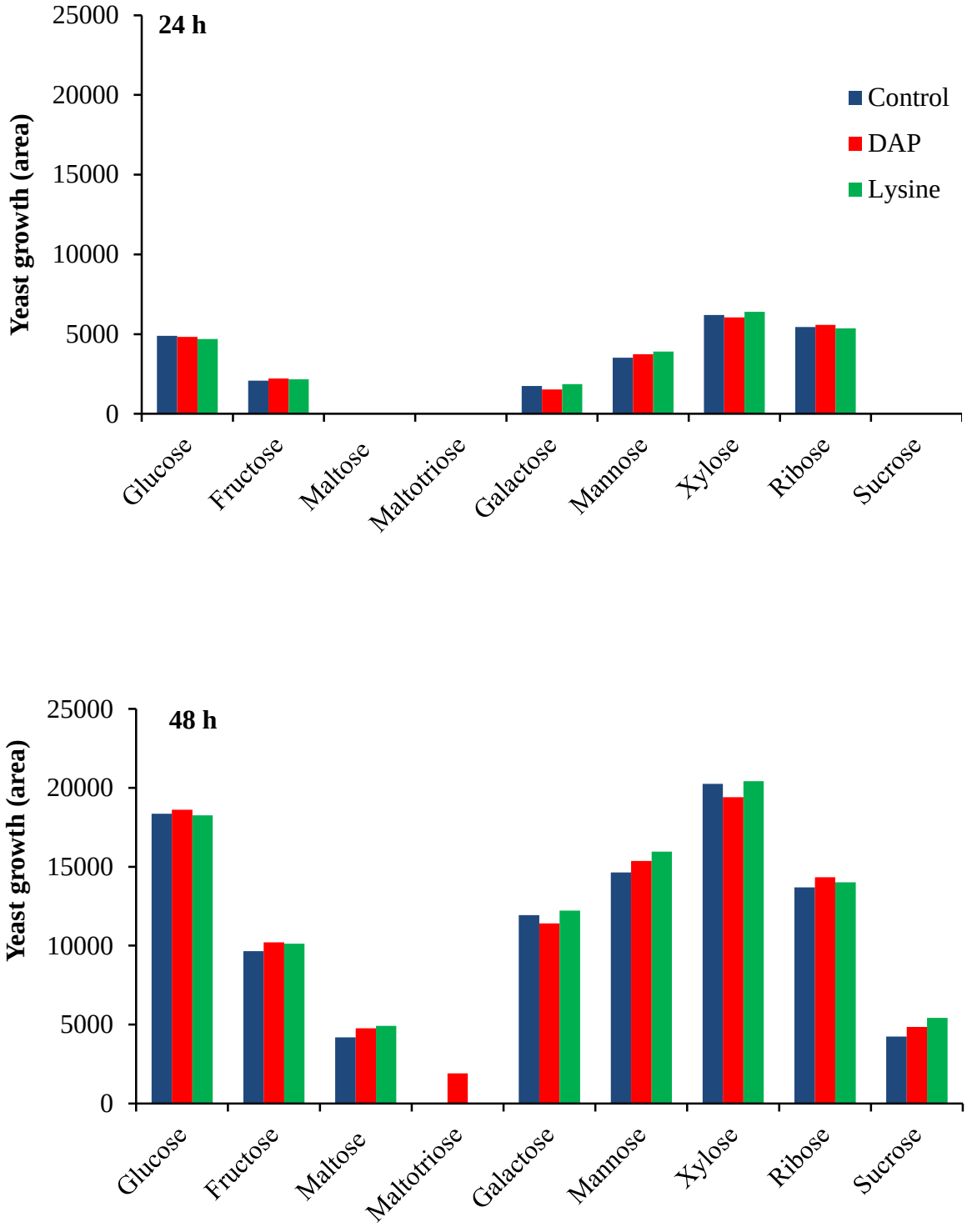

Figure 4.27. Extent of growth of yeast cells on carbon source phenotypic microarray plate supplemented with diammonium phosphate (DAP) or lysine and incubated for up to 48 h at 25°C.

Although sucrose is utilized first during fermentation, it has to be hydrolysed externally by the enzyme invertase (β-D-fructofuranosidase) (Heggart et al., 1999). The resulting products, glucose and fructose, are then taken up into the cell by facilitated diffusion. This might be the reason why sucrose did not support respiration after 24 h of incubation when it was used as a sole carbon source. The utilization of less preferred carbon sources such as maltose and maltotriose requires the synthesis of α-glucosidase enzyme, which is responsible for the intracellular hydrolysis of both maltose and maltotriose (Londesborough, 2001). This may result in an extended lag phase when maltose and maltotriose are used as sole carbon sources, as was observed after 24 h.

After 48 h of incubation, substantially more yeast growth had occurred with most of the carbon sources compared to after 24 h. Glucose and xylose supported more active growth in all the treatments. These were followed by mannose, ribose, galactose and fructose. The reason why glucose supported more growth has been explained above. However, native *S. cerevisiae* strains are not known to metabolize xylose (Jeffries and Jin, 2004). *Saccharomyces cerevisiae* lack the enzyme xylose isomerase despite containing all necessary enzymes for the conversion of xylose to ethanol (van Maris, Abbott, Bellissimi, van den Brink, Kuyper, Luttik, Wisselink, Scheffes, van Dijken and Pronk, 2006). It is therefore not known why xylose supported active yeast growth with in this study. It is possible that the PM plate did not contain the correct sugar, xylose or it might have been contaminated with microorganisms that utilize xylose.

Research has demonstrated the existence of natural *Saccharomyces* isolates that are capable of xylose metabolism (Attfield and Bell, 2006; Wenger, Schwartz and Sherlock, 2010). Genetic characterization of the trait by Wenger et al. (2010) identified a gene (*XDH1*) that is found in many wine yeast strains but not found in the reference wine yeast S288c genome. This gene encodes a putative xylitol dehydrogenase sufficient to allow wild-type laboratory strains of *S. cerevisiae* to grow slowly in xylose. There is, however, no evidence that these strains can grow anaerobically with xylose or that they produce ethanol. Since an ethanol producing *S. cerevisiae* strain was used in this study, the existence of such yeast could be indicative of contamination. Researchers have also engineered xylose metabolism into yeast by introducing genes for xylose reductase and xylitol dehydrogenase from xylose-fermenting species by recombinant DNA technology (Madhavan et al., 2009). Alternatively, exogenous xylulose isomerase is added to convert

xylose to xylulose, hence allowing *S. cerevisiae* to metabolize xylulose into ethanol during fermentation (Miller, Gowtham, Henson and Harcum, 2012).

The utilization of galactose, mannose and ribose was expected as these sugars are respired by brewing yeast (Walker, 1998). In contrast to what occurred after 24 h, maltose and sucrose supported some growth after 48 h but not to the same extent as the other carbon sources that supported growth under aerobic conditions. It has been reported that the uptake of maltose from the medium is facilitated by both high- and low-affinity transport activities, while only the low-affinity transport activity is active in the case of maltotriose (Alves, Herberts, Hollatz, Trichez, Miletti, de Araujo and Stambuk, 2008). This explains the better utilization of maltose by the yeast when compared to maltotriose. In addition, maltotriose is less preferred than maltose (Day, Rogers, Dawes and Higgins, 2002) and some strains utilize it with difficulty (Meneses et al., 2002).

Interestingly, maltotriose, which was the second most abundant sugar in sorghum wort, did not support active yeast growth under aerobic conditions with the control and lysine supplementation but partly supported growth with ammonia supplementation. This is probably due to ammonia being preferentially used by yeast and supporting active growth (Bell and Henschke, 2005) (Figure 4.26). It also confirms that nitrogen supplementation enhances sugar transport through derepression of the hexose transport systems, as suggested by Salmon (1989). Further, the stimulation of protein turnover after nitrogen starvation is thought to be responsible for the inactivation of sugar transporters in brewing yeast (Lucero, Moreno and Lagunas, 2002). The observation that lysine did not support yeast growth under aerobic conditions with maltotriose is probably due the combined effect of having a carbon source that was not easily utilizable and a supplement that does not support respiration when present as a sole nitrogen source.

4.3.4.4 Microsatellite analysis during serial fermentation

A PCR-fingerprinting method based on the microsatellite primer $(GTG)_5$ was used confirm that the yeast that was used in the present study did not change genetically and that no contaminating yeast were present. This means that the phenotype differences that occurred during fermentation could only be due to the treatment conditions and not to contamination or genetic changes. Sixth generation yeast was used to ferment sorghum grain wort instead of barley malt wort, which the yeast was previously exposed to.

The $(GTG)_5$ analysis showed five different bands, ranging from 400–2500 bp for all the treatments (Figure 4.28). Cluster analysis of the genomic fingerprints, based on the presence or absence of major bands showed two main groups, with a similarity value of 63%. The first group consisted of fermentation 7 treatments (control, DAP and lysine supplementation), while the second group showed a 100% homology of the fermentations 8 and 9 treatments. Fermentation 8 lysine supplemented cells after 7 days fermentation showed a shift in one of the bands. However, this was not a significant change since the BioNumerics software showed a 100% homology within the groups.

The $(GTG)_5$ microsatellite primer has been widely used for the discrimination of *Saccharomyces* yeast species and for differentiation between strains within species (Xufre, Simões, Gírio, Clemente and Amaral-Collaço, 2000; Capece, Salzano and Romano, 2003). Da Silva-Filho et al. (2005) used this primer to characterize yeast succession of the industrial fuel-ethanol fermentation process. They also showed that indigenous strains present in the crude sugar cane substrate can be more adapted to the industrial process than commercial strains. Capece et al. (2003) used the primer to indicate the evolution of yeast species throughout wine fermentation and for the differentiation of non-*S. cerevisiae* yeast species associated with the winery process. The $(GTG)_5$ primer has also been used to investigate genetic relationships of 24 phenotypically different strains isolated from sorghum beer in West Africa (Naumova, Korshunova, Jespersen and Naumov, 2003). However, PCR profiles generated by the $(GTG)_5$ primer in the latter study did not show significant heterogeneity among sorghum beer yeasts.

In order to identify ITS polymorphism probably caused by serial fermentation or nutrient limitation, the PCR products amplified with the ITS primers were then sequenced. The amplification of the ITS region is commonly used in the identification of yeast species because it has sequences that are highly conserved and sequences which show a high degree of genetic variability between strains of different species (Bhima, Marrivada, Devi, Reddy, Rao, 2010; Bzducha-Wróbel et al., 2013). The products had a molecular mass of approx. 850 bp (Figure 4.29), confirming that the yeast strain used in this study belonged to the *S. cerevisiae* species. Bzducha-Wróbel, Kieliszek and Błażejak (2013) obtained a *S. cerevisiae* PCR product of approx. 860 bp, while Mirhendi, Diba, Rezaei, Jalalizand, Hosseinpur and Khodadadi (2007) obtained PCR fragments of about 850 bp using the same primers.

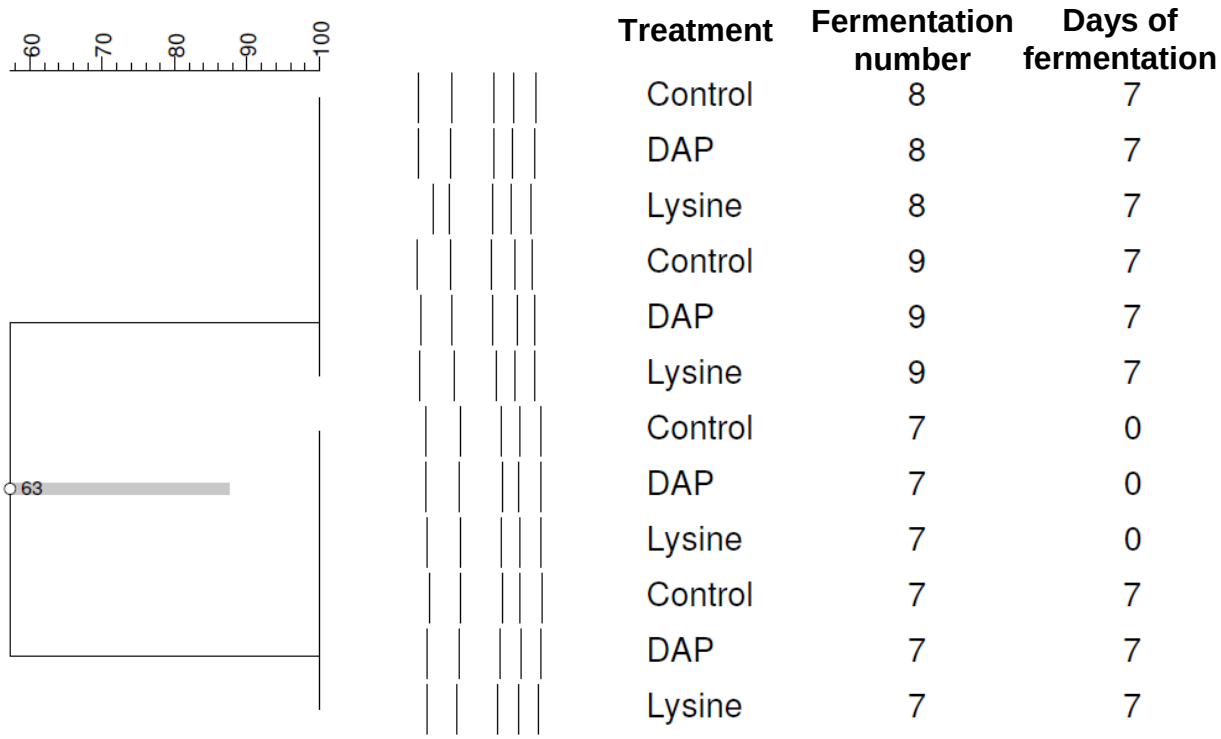

Figure 4.28. Tree plot and diagram representation of the $(GTG)_5$ amplification patterns as produced after fermentation of sorghum wort supplemented with lysine or ammonia (DAP)

Sequencing revealed that fermentation 9 control yeast cells at Day 7 (CD7F9) were more than 55% related to all the other treatments including Day 0 control yeast cells of fermentation 7 (CD0F7) (Figure 4.30). However, DAP supplemented yeast cells of Fermentation 7, at Day 0 (DD0F7), were 55% related to Fermentation 9 DAP supplemented yeast cells at Day 7 (DD7F9). This indicates that there was little change on the DNA sequence of the DAP supplemented yeast cells after serial repitching. A bootstrap value of more than 50% indicates very close similarity (Kurtzman and Robnett, 2003).

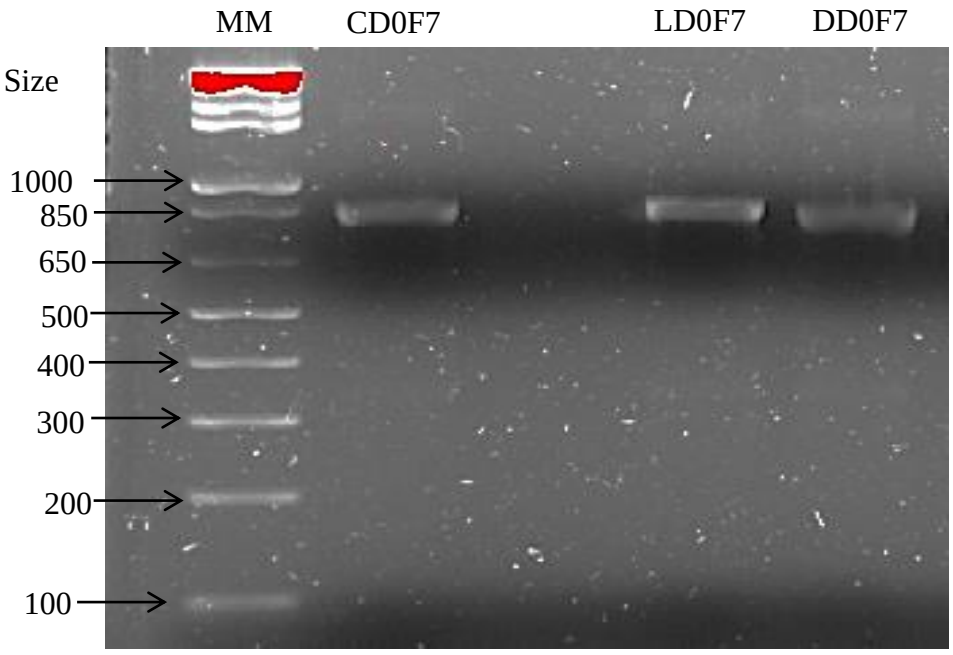

Figure 4.29. PCR fingerprinting of brewing yeast amplified with ITS1 and ITS4 primers. MM: 1 kb DNA ladder; CD0F7: Control day 0, fermentation 7; LD0F7: lysine supplementation day 0, fermentation 7; DD0F7: DAP supplementation day 0, fermentation 7.

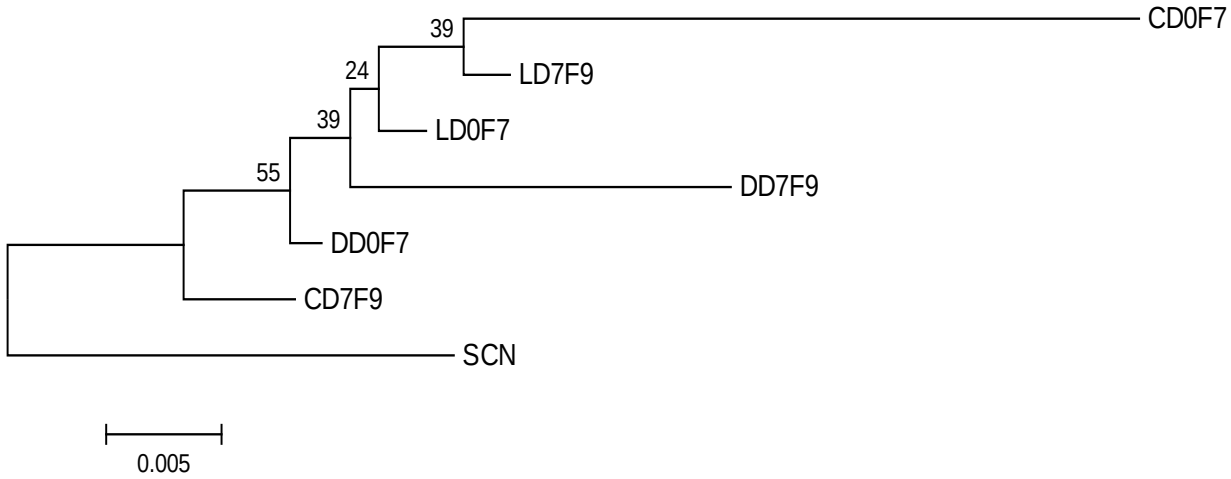

Figure 4.30. Relationships of serially repitched brewing yeast supplemented with DAP or lysine at a concentration of 50 mg/L and fermented for up to 7 days. The optimal tree with the sum of branch length = 0.0951 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. CD0F7: control day 0, fermentation 7; LD0F7: lysine supplementation day 0, fermentation 7; DD0F7: DAP supplementation day 0, fermentation 7; CD7F9: control day 7, fermentation 9; LD7F9: lysine supplementation day 0, fermentation 9; DD7F9: DAP supplementation day 0, fermentation 9; SCN: *S. cariocanus* Contrary to the

findings with the DAP supplementation, lysine supplemented Day 0 yeast cells of Fermentation 7 were related in 24% of the bootstrap replications to Fermentation 9 lysine supplemented Day 7 yeast cells. This indicates that a mutation occurred with lysine supplementation after serial repitching. Genomic rearrangement may confer an adaptive advantage to different environmental conditions (Bakalinsky and Snow, 1990). According to Ibáñez, Pérez-Torrado, Chiva, Guillamón, Barrio and Querol (2014), *S. cerevisiae* strains isolated from fermentations where proline was more abundant contained a higher number of *PUT1* genes and were more efficient in assimilating proline as a nitrogen source. Similarly, a strain isolated from sugarcane juice in which arginine was a limiting amino acid, contained less copies of *CAR2* genes that are involved in arginine catabolism. It is therefore, suggested that the mutation that occurred with lysine supplementation could be due to adaptive evolution of the *S cerevisiae* strain. This genome change did not, however, appear to occur at the $(GTG)_5$ site, as previously indicated.

4.3.5 Conclusions

Ammonia in the form of diammonium phosphate supports better yeast growth under aerobic conditions than lysine either as a sole nitrogen source or in combination with other amino acids. This is probably due to the role it plays in regulating enzyme activities, permease activities and transcription of nitrogen regulated genes. Lysine supplementation supports growth when in combination with other nitrogen sources but not when used as a sole nitrogen supplement. With regard to the utilization of carbon sources, nitrogen supplementation did not affect sugar utilization except for maltotriose which did not support growth with the control and lysine supplementation. Xylose supported active growth despite the fact that native *S. cerevisiae* strains are not known to metabolize xylose. Thus, this was probably an artefact. Scanning electron microscopy of the serially repitched yeast showed distorted and irregular sized cells with lysine supplementation and in the raw sorghum grain wort control. In contrast, yeast cells supplemented with ammonia were less affected by serial repitching.

PCR fingerprinting with the $(GTG)_5$ primer does not show any genome change with serial repitching. Relationships of the sequenced data indicate that the genome of yeast cells supplemented with ammonia is not affected by serial repitching, while mutation in the

yeast genome occur with lysine supplementation. The latter is probably due to adaptive evolution of the yeast strain.

These findings indicate that ammonia supports more active growth than lysine and maintains yeast morphology during serial repitching and the yeast is less subject to mutation. Therefore, ammonia in the form of diammonium phosphate is a better supplement supplement for sorghum grain wort.

5: GENERAL DISCUSSION

5.1 Methodological considerations

For this work, two sorghum types that are used for the production of lager beers in different parts of Africa were investigated. Malted white Type II tannin sorghum is used in Nigeria (Taylor, 2003), while white tan-plant sorghum is used in Eastern and Southern Africa in the form of raw grain (Mackintosh and Higgins, 2004). The white Type II tannin sorghum grain contains condensed tannins which impede protein digestibility and lead to worts with low free amino nitrogen (FAN). However, when malted, the tannins are inactivated resulting in FAN levels that are similar to non-tannin grains (Research Chapter 4.1). Tannin sorghum has an agronomical advantage since the tannins protect the crop against biotic stresses (Waniska, Poe and Bandyopadhyay,1989). The white tan-plant sorghum grain does not contain tannins and it produces worts with substantially higher levels of FAN than the white Type II tannin grain (Research Chapter 4.1). The two sorghum types, either as raw grain or malt, contain insufficient FAN levels for a complete fermentation (Research Chapter 4.1). Exogenous enzymes are added to improve FAN production during mashing of sorghum (Mackintosh and Higgins, 2004). Since enzymes need to be used for both malt and grain, it has been suggested that brewing with sorghum grain is more economical (Bajomo and Young, 1993).

Two exogenous proteolytic enzymes, a neutral proteinase and an exopeptidase, were investigated in Research Chapter 4.1. The neutral proteinase was an endo-protease and preferably cleaves polypeptide chains within the chain and away from the nitrogen and carbon termini (Simpson, 2001). According to Agu and Palmer (1998) neutral proteinases are more effective than acid proteases in producing free amino acids, peptides and soluble proteins. They are also reported to help improve starch yield (Mezo-Villanueva and Serna-Saldívar, 2004) and ethanol yields (Lei et al., 2013a). The amino-peptidase was an exopeptidase, which hydrolyses only the nitrogen terminal endo-polypeptides and in doing so, cleaves off either single amino acids or very short di- and tripeptides (Simpson, 2001). The effectiveness of the exopeptidase enzyme in improving FAN production has been previously reported by our laboratory (Ng'andwe et al., 2008). This was found to be due to the reduction of the glutelin protein matrix surrounding the protein bodies.

The incubation study was done isothermally at 45°C, which is within the temperature optima (45-55°C) of the neutral proteinase (Novozymes, 2007). The incubation was done for 24 h, which may be too long for a commercial brewery cycle unless a separate incubation vessel is used. The pH was adjusted with phosphoric acid to a pH range of 5.5-6.0. This pH range was also within the pH optima of the neutral proteinase (Novozymes, 2007). A low grist to liquor ratio of <10% was used to avoid the potential problem of product inhibition of the enzyme. An enzyme concentration of 1 mg/kg was used for most of the incubations and this approximated the dose of enzyme to substrate for neutral proteinase (Novozymes, 2007). This enzyme dosage is, however, not economical in industry since it is approx. 10 times more than the required enzyme to substrate ratio.

A liquor to grist ratio of 1:3 was used to produce sorghum grain wort (Research Chapter 4.2 and 4.3). This liquor/grist ratio imitates high gravity brewing, which reportedly improves yield, saves energy and equipment. The mashing regime included a mashing in temperature of 50°C. One half of Cerezyme sorghum 2X enzyme (mixture of proteases, peptidases, amylases, xylanases and beta-glucanases) was addded at the mashing in step followed by a protein rest of 30 min at the same temperature. This protein rest temperature is recommended by the enzyme supplier (Novozymes, 2008). After the protein rest, the mash temperature was raised to 94°C and held for 45 min to ensure complete gelatinization of starch. This gelatinization temperature was necessitated by the higher gelatinisation temperature of sorghum starch (78°C), (Taylor and Emmambux, 2010) compared with that of barley (60-62°C) (Briggs et al., 2004). A second dose of the enzyme preparation was added after cooling the mash to 70°C, followed by a 15 min rest. This step allowed for more liquefaction of the gelatinized starch. Lastly, a fungal alpha-amylase enzyme (Fungamyl BrewQ) was added at 58°C followed by a saccharification rest of 60 min. This enzyme was used to hydrolyse down dextrins to maltotriose and maltose (Novozymes, 2008).

The ninhydrin method was used to determine FAN. This assay is suitable for determining the available nitrogen for proper yeast growth. It measures the amount of free amino acids and small peptides (dipeptides and tripeptides) in the wort (Lekkas et al., 2005). The limitation of the assay is that it is unable to quantify other important forms of FAN such as ammonia (Lekkas et al., 2007). Although ammonia was used as a supplement in this study, its utilization was not measured separately but was estimated following the consumption

trend reported by Lekkas et al. (2007; 2009). Variability in ammonia consumption was modelled using the lognormal probability distribution in an Excel (Microsoft, Rendmond, WA) spreadsheet add-in programme, @Risk (version 6.3.0, Pallisade, Newfield, NY). The measurement of ammonia in our laboratory using an ammonia assay kit (Catalogue #: AA0100, Sigma) has previously resulted in incorrect results. Other assays such as the 2,4,6-trinitrobenzene sulphonic acid (TNBS) assay that measures both FAN and peptides during fermentation have shown that sorghum produces higher levels of FAN products than when measured using the ninhydrin assay (Agu, 2002). Despite these limitations, the ninhydrin assay is a routine method for the determination of FAN and it is a standard European Brewery Convention (EBC) method (EBC method 8.10 Free Amino Nitrogen in Wort by Spectrophotometry (IM)) (European Brewery Convention, 1998).

Laboratory scale fermentations were carried out in EBC glass tubes (height: 200 cm; diameter: 5.6 cm) constructed according to the design of the EBC (European Brewery Convention, 1977). The EBC tubes have the right geometry when compared to large scale industrial cylindrical conical fermentation vessels. However, they may not be a good model due to differences in hydrostatic pressures. Cylindroconical fermenters have increased hydrostatic pressure, carbon dioxide levels and decreased dissolved oxygen levels (Briggs et al., 2004). It is, however, difficult to perform hydrostatic experiments on a laboratory scale. Hence, the EBC tubes were used to compare results to published research.

Specific gravity, which was used as a crude method to measure sugar uptake, was measured using a pycnometry method. This is an accurate assay for the determination of sugar uptake during fermentation. However, it also measures dextrins which are not fermented by yeast (Briggs et al., 2004). The results were reported in degrees Plato (°P) which, for historical reasons, is widely used in the brewing industry (Lei et al., 2012). The pycnometry method is a standard brewing assay (EBC Method 8.2.1 Specific Gravity of Wort using a Pycnometer) (European Brewery Convention, 1998).

Yeast viability was determined by staining cells with methylene blue dissolved in 2% sodium citrate dihydrate solution to a final concentration of 0.01% (w/v). Dissolving methylene blue in sodium citrate to a final concentration of 0.01% (w/v) gives an accurate estimate of healthy and starved yeast cells (Smart et al., 1999). However, it may be

inaccurate for viabilities lower than 90%. Therefore, the method was used to estimate the viability of fresh yeast before inoculation. Viable cells either exclude the dye or reduce it to the colourless "leuco" form while dead cells stain blue (Sami, Ikeda and Yabuuchi, 1994). The method is based on the fact that methylene blue is an autoxidable dye, which upon entry into the cytoplasm of the living cells, is oxidized to its colourless leuco-form. The inability of a cell to retain the stain is an indication that the rate of influx of the dye through the membrane is balanced with the rate of oxidation of the stain (Heggart, Margaritis, Pilkington, Stewart, Sobczak and Russell, 2000). Although inexpensive and easy to perform, concerns have been raised about the reproducibility and accuracy of the methylene blue method mainly with apparent yeast viabilities below 90% (Lodolo et al., 2008). It is also reported that the method may overestimate (Greetham, et al., 2014b) or underestimate yeast viability (Willetts, Seward, Dinsdale, Suller, Hill and Lloyd, 1997). In addition, living but damaged cell membranes may cause variable cell shading which is difficult to interpret (White, Richardson, Schiewe, White and 2003). However, staining cells with methylene blue solution containing 0.01% methylene blue and 2% sodium citrate gives results that are consistent with staining cells with more specific commercial kits such as the LIVE/DEAD *Bac*Light (*Bac*Light Molecular Probes, Oregon, USA) (Zhang and Fang, 2004). Also, despite the above challenges, the methylene blue staining method continues to be used in research for the determination of yeast viability. For example, recently it has been used to determine viability of heat shocked yeast (Pillet, Lemonier, Schiavove, Formosa, Martin-Yken, Francois and Dague, 2014), viability of yeast grown on soy protein hydrolysates (Zhao et al., 2014) and the protective effect of proline on yeast cells subjected to weak acids (Greetham et al., 2014b).

Yeast counts were enumerated on plate count agar acidified to pH 3.7 with 2M lactic acid and expressed in cfu/ mL. After acquiring an improved Neubauer haemocytometer, yeast cell numbers were expressed in million cells/ mL. The haemocytometry method is a standard brewing method (EBC Method 2.2.2.1 Determination of Concentration of Cells in Suspension) (European Brewery Convention, 1998).

Yeast glycogen levels were not measured in this study when determining the effect of pitching with aged yeast on the yeast fermentation performance. This was because previous research had shown that the aging of yeast by constant stirring results in less glycogen levels at the onset of fermentation (Lodolo et al., 1998). The purpose of pitching

with the aged yeast was to show the importance of pitching wort with good quality yeast on the yeast fermentation performance, particularly when serial repitching is practiced.

The PICO-TAG method of Bidlingmeyer et al. (1984) was used to measure the amounts of individual free amino acids in the wort and beer. The method involves pre-column derivatization with the derivatives separated by reverse-phase high performance chromatography and detected by their ultra violet absorbance. As in this work, the samples were not hydrolysed, to measure the available amino acids for yeast growth, the procedure did not quantify peptides.

A PCR-fingerprinting method based on the microsatellite primer $(GTG)_5$ was used to ensure that the fermentation yeast was not contaminated and that the treatments did not cause any genetic changes. Microsatellite analysis was chosen because it is accurate and expresses results as base pair number or as a number of repeats (Schuller et al., 2004).

The phenotype microarray (PM) technology was used to assess the extent of growth supported by ammonia or lysine on brewing yeast cells when supplied as a single supplement and/or in combination with other nitrogen or carbon sources. The PM technology was used in this study because it is a high throughput system that allows analysis of a wide range of cellular phenotypes. Further, it gives a comprehensive scan of the physiology of a growing cell over time (Bochner, 2003). The challenge with this technique was that the concentration of the nitrogen and carbon sources on the PM plates was not revealed by the manufacturer. This made it difficult to calculate the exact concentration of each substrate responsible for the observed differences in growth. Despite this, differences in the utilization of either the carbon or nitrogen source were successfully determined using the area under the growth curve. Also, importantly, the PM study was done under aerobic conditions. Although aerobic respiration is crucial and occurs during the early stages of alcoholic fermentation (Gibson et al., 2007), it would have been valuable to also run the tests anaerobically since yeast is subjected to severe stresses during the later stage of fermentation. Oxygen absorbing packs and carbon dioxide producing packs have been used by other workers to create anaerobic conditions (Greetham, 2014). Also, it would have been valuable to assess the yeast strains at the beginning of the study and after serial repitching. However, this was not done due to the high costs associated with using the PM technique.

5.2 The impact of sorghum and nitrogen sources on yeast health in lager beer fermentation

In this study the effect of sorghum type (white non-tannin versus white Type II tannin) was first investigated and it was found that white non-tannin grain yielded the highest FAN, while both sorghum types produced similar FAN levels when malted. The latter was attributed to the malting process which causes a substantial reduction in assayable tannins (Beta et al., 2000) and also activates endogenous proteolytic enzymes (Dewar et al., 1997). Considering the amount of FAN required for a complete fermentation, it was concluded that sorghum malt produces sufficient FAN.

The above findings then prompted an investigation into the effect of supplementing sorghum grain with different proportions (10% and 20%) of sorghum malt to improve FAN of the grain. The results revealed that supplementing with sorghum malt negatively affected fermentation performance when compared to the 100% raw sorghum grain control. The effect was aggravated with an increase in malt supplementation (20%). Briefly, sorghum malt supplementation at the highest concentration caused a substantial reduction in yeast counts and ethanol production in the first fermentation. Although malt supplementation improved ethanol levels with subsequent fermentations compared to the control, the total ethanol production was decreasing, as a results of serial repitching. These results suggest that the yeast needed time to acclimatize to the new substrate (sorghum malt) since it had been used for barley malt fermentation for a long period. Secondly, the loss in yeast counts suggests that sorghum malt may contain inhibitory compounds that affect yeast growth.

Identifying the inhibitory compound or factor that could be more prevalent in the malt than in the grain is not easy. More so, because it can be a factor that is not measured during the assessment of malt quality. However, crude organic extracts from sorghum have been reported by Kil et al. (2009) to have antimicrobial activities against yeast such as *Candida albicans*. In their study, methanol extracts showed higher levels of antimicrobial activity than other fractions. It is unknown whether the malting process could enhance the antimicrobial activity of some sorghum cultivars. It is, however, reported that malting may substantially increase the levels of lactic acid bacteria (LAB) compared to unmalted grain (O'Sullivan, Walsh, O'Mahony, Fitzgerald and van Sinderen, 1999). These bacteria are

present as part of the natural microbiota in cereals and they reportedly persist during malting and mashing of barley (O'Sullivan et al., 1999). Although LAB can produce a variety of antimicrobial substances which are known to inhibit undesirable bacteria and fungi in wort and beer (Vaughan, O'Sullivan and van Sinderen, 2005), LAB can also compete with *S. cerevesiae* for micro and macro-nutrients and produce inhibitory end products such as acetic acid and/or lactic acid (Narendranath, Thomas and Ingledew, 2001; Thomas, Hynes and Ingledew, 2001). These end products can decrease yeast growth rates, reduce the biomass and ethanol yields of the target yeast.

Another possible reason for the inhibitory activity of the sorghum malt could be the presence of anti-yeast compounds. These are widely prevalent in barley malts and methods for their quantification have been optimized (van Nierop et al. 2008). Anti-yeast compounds such as antimicrobial peptides (thionins and defensins) are naturally found in plants to protect them against pathogenic microbial attack or other forms of stress such as drought or chemical exposure (Castro and Fontes, 2005). They act on the microbial plasma causing leakages of ions, metabolites and in some cases even proteins and other macromolecules (Bechinger and Lohner, 2006). They can also cause total membrane disruption resulting in cell lysis (Bechinger and Lohner, 2006).

In a study to determine the sensitivity of lager brewing yeast strains towards anti-yeast compounds, van Nierop et al. (2008) differentiated barley malt samples according to their antimicrobial activity. Malts that had high anti-yeast activity were associated with premature yeast flocculation and gushing. Similar compounds with antifungal activity such as a γ-thionin protein Siα1 have been reported in sorghum (Bloch, Patel, Baud, Zvelebil, Carr, Sadler and Thornton, 1998). Further, proteins of 18, 26 and 30 kDa isolated from sorghum endosperm have been shown to affect hyphal growth of *Fusarium moniliforme* (Kumari and Chandrashekar, 1994). Characterization of sorghum malt anti-yeast compounds is therefore required to determine if they play a role in inhibiting yeast growth during fermentation.

Of the nitrogen sources that were investigated in this study, ammonia supplementation, in the form of diammonium phosphate, proved to be the most effective. A rapid fermentation rate in terms of specific gravity and sugar uptake was found (Research Chapter 4.2). Similar results of ammonia supporting better growth than other nitrogen sources occurred

when using the PM technology under aerobic conditions (Research Chapter 4.3). In addition, SEM indicated that more minor morphological alterations occurred with ammonia supplementation compared with supplementing with lysine.

There are a number of reasons why ammonia supported better fermentation than lysine. These include that it is one of the most preferred nitrogen sources by yeast and that it regulates enzyme activities, permease activities and also functions as a substrate for protein syntheses (Schure et al., 2000). There is, however, paucity of work in the literature on the application of ammonia in lager beer brewing. Most of its application has been in the wine industry where supplementation of grape must with ammonium phosphate/sulphate is a common practice (Salmon, 1989). Despite being highly preferred for supplementing must, contrary reports of ammonium salts inducing poor fermentation performance have been reported. For example, Cruz et al. (2002; 2003) reported that ammonium salts induced poor performance in lager yeast strains in terms of biomass accumulation and ethanol yield compared to other nitrogen supplements such as peptone. Their fermentation study was, however, done in yeast nitrogen base media supplemented with different nitrogen sources and incubated at a higher temperature of 30 °C. In a similar study, but using different carbon sources (glucose and fructose), Júrnior, Batistole and Ernandes (2008) reported that casamino acids (a mixture of amino acids and small peptides) and ammonium sulphate supplementation induced poor fermentation performance with lower biomass, ethanol production and loss of yeast viability. Again, their study, which investigated the fermentation performance of baking, ale and lager brewing yeast strains, was done with yeast nitrogen base at 30 °C. The differences in experimental conditions and yeast strain could explain the discrepancies in the findings.

The addition of ammonia in alcoholic fermentation is, however, regarded in some quarters as uneconomical (Kawa-Rygielska and Pietrzak, 2014). In this work, the effect of other nitrogen sources such as lysine was investigated. Lysine is a group A amino acid, making it to be among the amino acids that are consumed early during fermentation. In this study, lysine supplementation at 50 mg/ L nitrogen supported a similar fermentation performance compared with high lysine supplementation of about 100 mg/ L nitrogen. The former gave final ethanol levels of about 7.5% (v/v), while the latter produced about 7.6% (v/v) ethanol but with high residual FAN. This suggests that there is a level above which lysine supplementation fails to further improve yeast fermentation performance. Therefore, lysine

supplementing at 50 mg/ L nitrogen seems to be more economically viable. As reported (Table 4.10), lysine resulted in low yeast counts at the end of fermentation. The inhibitory effect of lysine on yeast growth was also shown by PM where lysine did not support growth when used as a single supplement. Phenotypic Microarray further revealed that the utilization of L-cysteine, glycine, L-histidine, L-proline, L-tryptophan, L-tyrosine, adenine, and small peptides (Gly-Asn, Gly-Gln, Gly-met) by the lysine supplemented yeast was slower than the one supplemented with ammonia. These effects occurred with the 50 mg/ L nitrogen supplementation and suggest that they may be aggravated at higher concentrations.

As reported, the negative effects of lysine supplementation on yeast growth are either fully or partially relieved when lysine is in combination with other amino acids. Since sorghum wort contains a mixture of amino acids (Table 4.2), the inhibitory activity of lysine was not expected. It is also worth mentioning that the ethanol levels that were produced with lysine supplementation were higher than those produced with supplementing sorghum grain wort with either 10% or 20% sorghum malt (max. 6.8% (v/v) for 10% malt). This indicates that lysine is a better supplement when compared with the sorghum malt.

The exact mechanism that is responsible for the improved fermentation performance with lysine supplementation is not clear. According to Lei et al. (2013b), lysine supplementation substantially up-regulates Ssy1p-Ptr3p-SSy5p (SPS)-regulated genes compared to nitrogen catabolite repression genes. The SPS genes control the expression of specific permeases that are responsible for the transport of early consumed amino acids at the beginning of fermentation (Ljundahl, 2009). Consequently, the consumption of amino acids such as Glu, Met, Ile, Phe, and Lys is significantly increased with lysine supplementation compared to the control and other supplements such as histidine (Lei et al., 2013b).

As explained, serial repitching is known to negatively affect yeast fermentation performance since it leads to decreased yeast growth rate, increases in residual FAN and undesirable flavours such as 3-methyl-1-butanol (Kobayashi et al., 2007). For this work, 6^{th} generation yeast was used, meaning that the yeast had been used for about six barley malt brewing cycles. This yeast was then further used for three more fermentation cycles in a new substrate, sorghum wort. Therefore, it was hypothesized that the sudden change to

ferment a new substrate was going to cause more stress on the yeast, leading to stuck fermentations. As shown in Research Chapter 4.3, serial repitching with either diammonium phosphate or lysine as supplements did not affect fermentation parameters such as FAN uptake, sugar consumption, yeast counts and pH change. This indicates that the yeast was not affected much by the new substrate, particularly because ethanol levels of up to 7.5% (v/v) were attained in the first fermentation on sorghum wort.

The capacity of yeast to remain stable over several serial repitchings seems to be strain dependent. Some lager strains can be repitched up to 98 times without any changes in the rate of attenuation and production of flavour compounds (Powel and Diacetis, 2007). Similarly, Bühligen, Rüdinger, Fetzer, Stahl, Scheper, Harms and Müller (2013) reported that serial repitching of up to 20 successive times does not cause major physiological changes in lager brewing yeast. Kobayashi et al. (2007), however, reported that serial repitching of up to eight brewing cycles mainly affects the production of volatile compounds and not fermentation performance such as sugar uptake and ethanol production. The latter study was, however, done with yeast extract-dextrose medium with FAN levels similar to industrial low-malt wort. Since the beer maturation step was not done in the current study, the effect of serial repitching on the volatile compounds could not be determined.

5.3 Future Research

The current work investigated the fermentability of sorghum grain wort supplemented with various nitrogen sources. Further research is needed to determine the impact of supplementing sorghum grain wort on the overall flavour and aroma profile of sorghum lager beer. This is because sorghum wort will have a different amino acid profile to that of barley wort and the addition of nitrogen supplements such as lysine and DAP will further change the amino acid profile of the wort.

Although the EBC fermentation tube method is mainly used for laboratory scale fermentations and also allows comparison of results to literature, studies need to be carried out at pilot brewing scale. Additional stresses such as hydrostatic stress may be encountered during industrial brewing and these may be influenced by nitrogen supplementation.

In the brewing industry, the yeast is stored at cold temperatures after cropping and also undergoes acid washing (Lodolo et al., 2008). The storage period and the acid washing step induce some extra stress to the yeast cell, which may influence its performance in subsequent fermentations. These processes need to be included enable better extrapolation to industrial brewery practice.

It was found that supplementing sorghum wort with lysine improves yeast fermentation levels when the right concentrations are applied (Research Chapter 4.2). According to Lei et al. (2013b) this is attributed to the up-regulation of genes responsible for the uptake of early consumed amino acids. Their study was, however, done with all malt barley wort, hence gene expression levels in sorghum grain wort supplemented with lysine need to be studied in sorghum lager beer fermentation. This will provide further insight on the mechanisms involved in improving yeast health.

6: CONCLUSIONS AND RECOMMENDATIONS

With unmalted grain in the absence of exogenous proteases, white non-tannin sorghum produces substantially higher levels of FAN than white type II tannin sorghum. When incubated with exogenous neutral proteinase, unmalted white non-tannin sorghum grain yields more wort FAN (up to 84 mg/ 100 g sorghum, in total), than white type II tannin grain (61 mg/ 100 g sorghum). Incubating unmalted W sorghum grain with neutral proteinase and amino-peptidase enzymes in combination, improve FAN production with W sorghum. The two sorghum types produce similar FAN levels when malted, apparently as a result of the reduction in assayable tannins during malting.

Ammonia supplementation, in the form of diammonium phosphate, improves fermentation performance in terms of improved maltose and maltotriose uptake and more rapid reduction in specific gravity. Glycine supplementation negatively affects yeast fermentation performance when compared to the control. This is probably due to the inability of yeast to utilize the two-carbon skeletons of glycine. Lysine supplementation increases ethanol yield (up to 7.4% v/v) when compared to the control (7.1% v/v). However, it negatively affects yeast counts at the end of fermentation. Serial repitching negatively influences yeast fermentation performance in terms of maltose and maltotriose uptake. Sorghum malt supplementation did not improve maltose and maltotriose uptake, but resulted in improved ethanol levels compared to the 100% raw sorghum control.

Without nitrogen supplementation and with lysine supplementation, yeast cells from serially repitched sorghum were distorted and irregular. In contrast, yeast cells supplemented with ammonia are less affected by serial repitching.

With regard to nitrogen utilization, ammonia supplementation supports more active growth under aerobic conditions than the control and lysine supplementation, either as a sole nitrogen source or in combination with other amino acids. Lysine supplementation supports growth when in combination with other nitrogen sources but not when used as a sole nitrogen supplement. Concerning carbon utilization, nitrogen supplementation did not affect sugar utilization except for maltotriose and xylose. The former does not support growth with the control and lysine supplementation, while xylose supported growth and this was probably an artefact. There was no genome change with serial respitching as

shown by PCR fingerprinting with the $(GTG)_5$ primer. However, relationships of the sequenced data indicates that mutation in the yeast genome occurs with lysine supplementation.

This study shows that nitrogen limitation negatively affects fermentation performance, particularly when brewing with unmalted sorghum. This is because nitrogen is required for the growth and metabolic activity of yeast cells during fermentation. Mashing white non-tannin sorghum grain and supplementing the resultant wort with ammonia in the form of diammonium phosphate are effective methods of improving assimilable nitrogen to ensure fast and complete fermentations.

This study provides valuable information on alleviating nitrogen limitations with respect to yeast nutrition and fermentation during sorghum grain lager beer brewing. However, further work needs to be carried out at pilot fermentation scale to determine the effect of the ammonia or lysine supplementation when additional stresses, such as hydrostatic stress, are encountered. The use of ammonium salts in brewing may be regarded as not "natural". Further work is needed to understand how lysine supplementation improves yeast fermentation performance. Understanding the mechanisms involved with lysine supplementation may address the challenges that occur at higher lysine supplementation because the addition of nitrogen supplements change the amino acid profile of the wort. Research is needed on the impact of supplementing sorghum grain wort with ammonia or lysine on the overall flavour and aroma profile.

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8: Publications and conference presentations based on this research

Dlamini, B.C., Buys, E.M., Taylor, J.R.N., 2014. Effect of sorghum type and malting on production of free amino nitrogen in conjunction with exogenous protease enzymes. Journal of the Science of Food and Agriculture 95, 417-422.

Taylor, J.R.N., Dlamini, B.C., Kruger, J., 2013. 125th anniversary review: The science of the tropical cereals sorghum, maize and rice in relation to lager beer brewing. Journal of the Institute of Brewing 119, 1-14.

Oral Presentation: SAAFoST (South African Association for Food Science and Technology) 2013 Congress. 20th Biennial International Congress and Exhibition, 7-9 October 2013, CSIR International Convention Centre, Pretoria. “Challenges of Low FAN in Sorghum Lager Beer Brewing and Possible Solutions.

Poster Presentation: FoodMicro 2014 Conference, Nantes, France, 1-4 September 2014. Poster “Yeast Utilization of Amino Acids with Phenotypic Microarray and Morphological alterations after Serial Repitching in Sorghum Wort”.