THE RELATIONSHIP BETWEEN VERY HIGH GRAVITY FERMENTATIONS AND OXIDATIVE STRESS IN THE LAGER YEAST

Saccharomyces pastorianus

Alexander Mott, M.Res.

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ABSTRACT

Very High Gravity fermentations are an increasingly attractive proposition within the brewing industry as a means of energy saving and optimising process efficiency. However, the use of very high gravity (>20°P) wort is associated with a range of biological stress factors. Ethanoic and osmotic stresses have been widely analysed along with oxidative stress in relation to propagation and early stage fermentation. The aim of this research was to investigate the impact of wort gravity on oxidative stress, and understand how this affects the plasma membrane at VHG. Finally the effect altered ergosterol content was analysed. The characteristics of *Saccharomyces pastorianus* strains were assessed for their ability to withstand the increased pressures of VHG (22°P) fermentations. VHG fermentations showed increase ethanol production at the expense of fermentation length, and increased ethanol production during VHG fermentations was offset by an increase in fermentation length. All fermentations were observed to accumulate ROS and increased antioxidant levels, with levels being furtherelevated in the VHG environment. Further analysis of *S. pastorianus* strains indicated that the levels of oxidative stress observed in fermentation had a negative effect on membrane fluidity and increased damage of the plasma membrane was observed. Analysis of ergosterol enriched yeast indicated that although fermentation rate was increased, a trade off with alcohol and biomass production was observed. Furthermore in response to oxidative stress, ergosterol enriched yeast showed reduced tolerance, decreased membrane fluidity and increased membrane damage. This work will give further insight into the response of lager yeast to oxidative stress present during VHG fermentations.

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Abreviations

AU	Absorbance Units
Au	Arbitrary Units
°C	Degrees Celsius
°P	Degrees Plato
DHE	Di-hydroethidium
DCW	Dried cell weight
Gpx	Glutathione peroxidase
g	Gram
hL	Hectalitre
H_2O_2	Hydrogen Peroxide
$OH^{\bullet}$	Hydroxyl radical
Kb	Kilo-base pair
L	Litre
µMax=	Maximum exponential growth rate
Mg	Milligram
mL	Millilitre
mM	Millimolar
µL	Microlitre

µM	Micromolar
ng	Nanogram
O_2	Oxygen
psi	Pounds per square inch
ROS	Reactive oxygen species
RFR	Relative fluorescence ratio
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
SOD	Superoxide dismutase
v/v	Volume/volume concentration

CHAPTER 1: INTRODUCTION

1 Introduction

1.1 Brewing

Fermentations have been associated with human culture since as early as the 6th Century B.C. Studies have revealed evidence for the consumption of wine, based on the discovery of wine jars in ancient Egypt (Cavalieri *et al* 2003) and for beer, with records of barley domestication in the fertile crescent (Salamini *et al* 2002) and Sumerian tablets depicting brewing activity dating to 1800 BC (Katz & Mytag 1991). Brewing has arguably played a role in the development of cultures, helping civilisations to flourish and providing individuals with a beverage rich in nutrients and minerals. It is also widely believed that fermented beverages may have provided a microbiologically pure source of water, helping societies to thrive (McGovern 2009).The process of making beer-like beverages is believed to have evolved during the medieval era to yield a product more comparable to modern day ales, while lager style beverages began to be produced around the 15th Century A.D. (Libkind *et al* 2011).

1.1.1 **Brewing components**

There are four main constituents of beer: water, yeast, hops and a source of carbohydrates. The carbohydrates can be derived from one or more of a number of cereal crops. Although malted barley is most commonly used, grains such as wheat, rice and sorghum can also be employed, either directly or indirectly in the form of sugar supplements, or adjuncts (Briggs *et al* 1986; Delcour *et al* 1989; Agu 1995; Owuama 1997; Agu & Palmer 1998; Dechao & Yang 2002).

The individual character and flavour of beer is largely governed by its constituents. The broad characteristics are defined by the malt and hops, while the yeast lends desirable ester flavours to the final product (Engan 1974; Engan & Aubert 1977; Peddie 1990). Hops provide bitterness and floral notes (Sakamoto & Konings 2003), while the choice of malt can yield a variety of beer types providing a range of colours, and flavour characteristics with descriptors such as sweet, burnt, biscuit, smoke or malt (Kim *et al* 1998). The yeast culture functions to ferment available carbohydrates into alcohol, and in doing so produces flavour compounds which are derived from the other raw materials (Lodolo *et al* 2008).

Beer is typically made up of over 90% (v/v) water and as such this component plays a significant role, not just in the final product, but throughout the brewing process system (Lewis & Young 1995). The minerals present in water are able to affect the taste of beer (Godammer 1999; Warnakulasuriya *et al* 2002) and in the past different water types have traditionally led to certain geographical regions being better suited to different beer types. The most recognised is the water from Burton-on-Trent (UK) which contains high levels of gypsum, especially suited for the production of pale ales (Briggs *et al* 1981). As an established means of reproducing Burton water, a sulphate (often gypsum itself), can be added to the water supply in a process called Burtonisation (Boulton & Quain 2001). Current practice typically results in most large breweries closely controlling the nutritional components of their brewing water, rather than relying on natural sources (Lewis & Young 1995; Eumann and Schilback 2012).

1.1.2 **The brewing process**

The main steps in the brewing process involve the conversion of barley to malt (malting), the extraction of sugars and the addition of hops (wort production), and the conversion of sugars and nutrients to alcohol and flavours (fermentation). After fermentation the beer may be allowed a period of clarification (conditioning) which typically takes place within separate vessels prior to bottling or kegging (packaging), but can be conducted within the bottles themselves (Vanbenden *et al* 2006; Dekoninck *et al* 2013). These processes are illustrated in Figure 1.1.

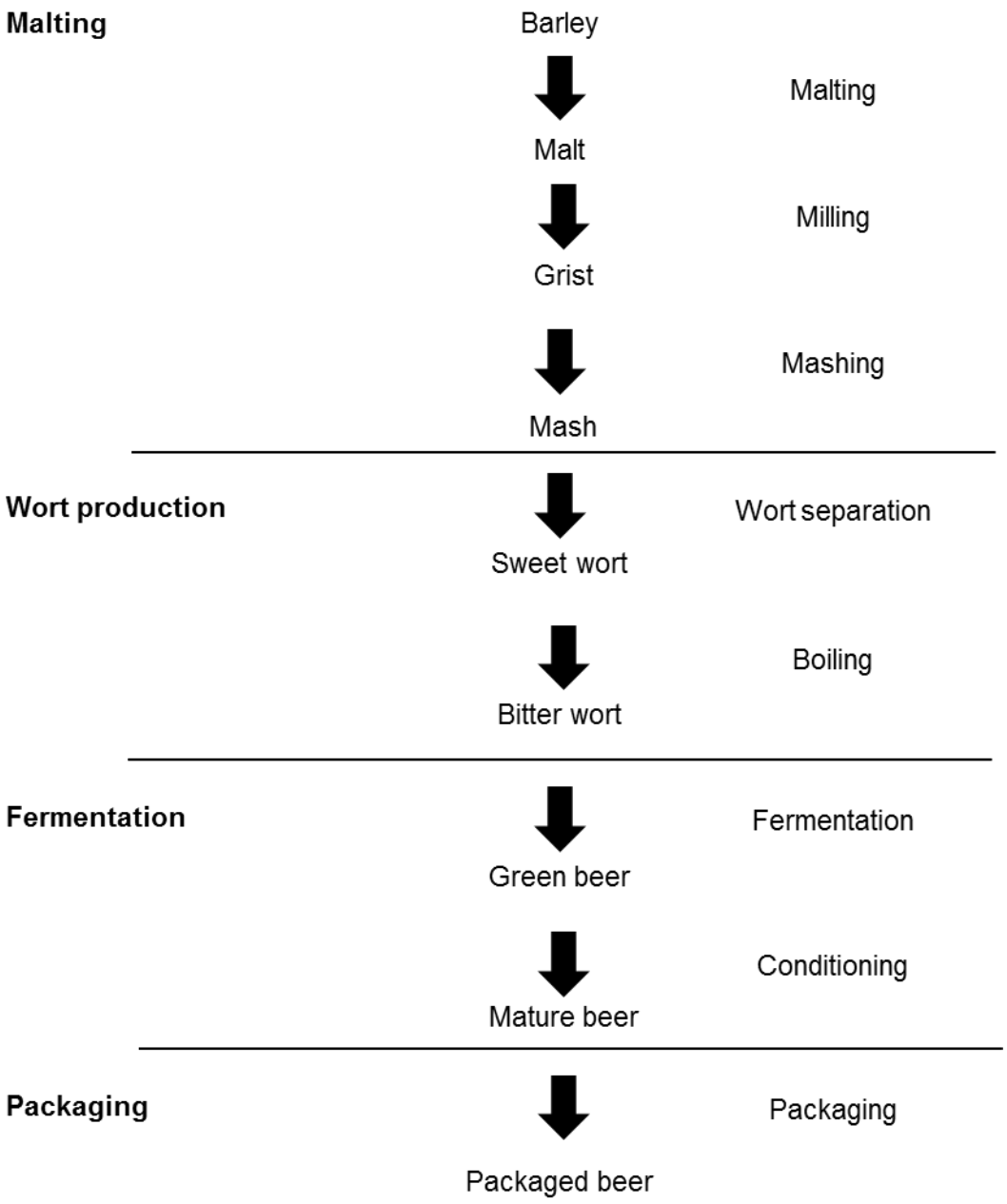


Figure 1.1 The brewing process, including the key production components: malting, wort production, fermentation and packaging.

1.1.3 **Malting**

During malting, steeping of barley grains is performed to stimulate enzymatic reactions which promote starch mobilisation for use by the embryo leading to germination (Hough *et al* 1982; Briggs 1998). Once the starch has been mobilised, germination is halted by heating the barley in a procedure known as kilning, resulting in malt. Kilning stabilises the grain and leads to enzymes and reserve materials becoming available for subsequent extraction and further degradation to release fermentable sugars during wort production (Taufel & Muller 1956). Varying the temperature and length of kilning can influence the release of sugars and nutrients which can have an impact on the colour and flavour of the finished beers (Blenkinsop 1991; Jupp 1994; Gruber 2001; Coghe *et al* 2004). During the mashing process, malt is converted into soluble sugars and amino acids, derived from grain proteins (Hargitt & Buckee 1977). At this stage the malt is crushed in a mill and the product (grist) is mixed with hot water to yield a product known as wort. The wort is then separated from the grain using a lauter tun and transferred into a 'kettle' or 'copper', leaving all non-soluble particles behind (Narziss 1986; Kuhbeck *et al* 2006).

1.1.4 **Wort**

1.1.4.1 Wort production

Sweet wort derived from the malting process is sterilized by boiling in the copper, which also helps trub (comprised of lipids and soluble proteins) to precipitate via coagulation (Ahvenainen *et al* 1979; Dufour *et al* 1986). During this stage of the process, alpha-acids derived from the hops are extracted into the wort and changed into iso-alpha-acids giving rise to the

bitter compounds in beer (Sakamoto & Konings 2003) and yielding hopped or 'bitter' wort.

Following production, the wort is clarified and the trub and spent hops are removed through filtration, often through hop cones. Hot bitter wort is then pumped into a whirlpool vessel and while trub collects at the centre of the vessel (Briggs *et al* 2004), clear wort is removed via an exit pipe, and passed through a heat exchanger to bring it to fermentation temperature.

1.1.4.2 Wort composition

Wort composition has a direct impact on beer characteristics such as colour, clarity and foam stability, as well as key aspects in flavour (Smart & Bamforth 2008 Egan 1979; Bamforth *et al* 1988). However, it can also influence beer character indirectly as it provides the starting materials by which yeast cells are able to create new and altered compounds (Dekoninck *et al* 2012; Van Mulders & Delvaux 2013). From the perspective of the yeast, wort is essentially a complex mixture of nutrients including sugars, lipids, amino acids, minerals, ions and nitrogen. Precise composition can be variable due to the use of vegetative sources which make up the wort (Bamforth 2008). Consequently, this can lead to batch to batch variation potentially resulting in sensorial differences in the final beer (Hudson 1973; Crab & Hudson 1975). In an effort to prevent such an occurrence, wort production is tightly regulated in an effort to standardise the starting materials prior to fermentation.

1.1.4.3 Carbohydrates

The principle nutritional component of wort is carbohydrate, in the form of sugars including maltose, maltotriose, glucose, fructose, sucrose and dextrins. Typically these sugars are derived from malt, although wort can be supplemented with sugar adjuncts (additional fermentable sugars, usually in the form of syrups) from a variety of sources (Rao & Narasimham 1975; Agu 2005; Piddocke *et al* 2009). The total carbohydrate content of wort is expressed in either specific gravity (SG) or degrees Plato ($^{\circ}$P). The former relates to the relative density of the wort compared to water, while the latter is a scale based on the percentage of sugar in solution.

Wort sugars are assimilated by yeast and used as an energy source as described previously (Section 1.1.4). Sucrose is broken down enzymatically into glucose and fructose and both of these sugars are taken up by the yeast via facilitated diffusion involving specific membrane transporters (Lagunas 1993). Uptake of maltose and maltotriose also occurs through specific permeases (Boulton & Quain 2001) while dextrins (longer chain sugars) are not typically utilised by brewing yeast strains (D'Amore *et al* 1988). Sugars are most commonly taken up in the following order: sucrose, glucose, fructose, maltose and maltotriose (Gjertsen 1953; Hargitt & Buckee 1977).

1.1.4.4 Nitrogen source

In order for yeast to function efficiently and for growth to occur, a source of nitrogen is required. Nitrogen is an essential component of proteins and enzymes and can also influence flavour production as a result of their use

in amino acid synthesis. Levels of free amino nitrogen (FAN) below 100mg/l cause cellular growth to be nitrogen dependent, however this dependence is reduced at higher concentrations and, above 220mg/L there is little effect (Boulton & Quain 2001; Lei *et al* 2013). According to Boulton & Quain (2001), a level of 150-200mg/L should be expected in a 10-12ºP all malt wort. A significant proportion of wort nitrogen is provided in the form of amino acids which are assimilated by yeast in a specific order allowing them to be divided into Groups A-D. Groups A and B are readily assimilated through specific permeases, while group C acids are taken up more slowly by the general amino acid permeases (GAP). In brewery fermentations group D amino acids, which includes the amino acid Proline alone are hardly absorbed at all (Ter Schure *et al* 2000; Chiva *et al* 2009).

1.1.4.5 Vitamins

Certain vitamins are needed by yeast as growth factors, although precise requirements are typically strain dependent (Amaha & Takeuchi 1961). Most strains require biotin, which is used by carboxylase enzymes and is necessary for lipid synthesis (Ohsugi & Imanishi 1985; Stolz *et al* 1999). Pantothenate is also widely required as it is a component of coenzyme A, a central molecule within a number of yeast metabolic pathways (Stuible *et al* 1998; Olzhausen *et al* 2009). Inositol is also an important vitamin which acts as an essential component of phospholipids (White *et al* 2001; White *et al* 2003)

1.1.4.6 Trace elements

Yeast require a number of trace elements for cell growth, function and maintenance. These elements include: zinc, iron, copper and magnesium, which function as enzyme cofactors (Maddox & Hough 1969; Walker 1998; Guerinot & David 1999; Udeh *et al* 2014); potassium, which is a component of the transport system for nutrient uptake (Muntz 1947; Rothstein & Bruce 1958); and calcium, which stimulates growth and has a role in yeast flocculation (Dombeck & Ingram 1986; Poreda *et al* 2009). Zinc is known to be particularly important since it is a component of enzymes involved in the final step of the ethanol production pathway (Chandresena & Walker 1997; Zhao *et al* 2009) and it has been shown that when present at concentrations below 0.1mg/L and above 0.6mg/L, fermentation can be restricted. Addition of zinc salts (when content is low) and manganese (when content is high) can help in counteracting this effect (Jacobsen *et al* 1977; Jacobsen & Volden 1981).

1.1.4.7 Lipids

Wort contains sterols and unsaturated fatty acids (UFA), although both of these lipids are normally only present in sub-optimal quaintities (Russell 1989). Sterols and UFA are utilised by yeast as a source of metabolic intermediates, as well as to make cellular structures (Briggs *et al* 2004). In particular, lipids are essential as a major component of the cell membrane and these can be either taken up from the medium or produced by the yeast internally during aerobic growth. Low concentrations of wort lipids are assimilated by yeast cells via facilitated diffusion, whereas at high concentrations they can enter via simple diffusion (Van der Rest *et al* 1995).

1.1.5 **Fermentation**

Fermentation occurs within specific vessels which are usually vertical and cylindro-conical in shape, but can vary in size and specific design (Boulton & Quain 2001). To initiate the fermentation, yeast (Section 1.2) is added or 'pitched', typically in the form of a ‘wet’ slurry, although dried yeast cultures can be employed (Debourg 1999). The type of yeast used defines the style of beer produced, with *Saccharomyces pastorianus* being used for lager beer and *S. cerevisiae* strains for ale products (Rank *et al* 1988; Legras *et al* 2007). Depending on company policy and brand specifications, ale fermentations are usually performed at temperatures of between 16-24°C, while lager fermentations are typically conducted at 12-18°C (Boulton & Quain 2001; Crumplen *et al* 1993). Typically the cooler the fermentation temperature is, the longer it takes to reach completion (Briggs *et al* 2004). Ale fermentations, conducted at warmer temperatures, tend to be shorter and yield more flavoursome products, due to greater production of esters and higher alcohols (Engan 1974; Engan 1977; Younis *et al* 1998).

Irrespective of the final beer classification (lager/ale), prior to inoculation with yeast, either sterile air or oxygen is passed through the wort. Aeration is essential for several metabolic pathways in yeast, including the creation of haem which can regulate specific genes, and more importantly, for the biosynthesis of sterols and fatty acids (Bard & Downing 1981; Bloch 1983; Basson *et al* 1986). Sterols are major components of the yeast cell membrane and are essential for cell division during the initial stages of fermentation (Kirsop 1982; Lodolo 1999). They are also important for maintaining cellular integrity under stressful conditions, such as osmotic

stress which has been suggested to occur at the start of fermentation due to the presence of sugars (Piper 1995).

Once yeast has been added to the fermentation medium, they rapidly take up oxygen from the environment, after which anaerobic conditions are established and maintained for the remainder of the process. Under aerobic conditions it might be expected that yeast would metabolise via the respiratory pathway to create the energy (ATP) required for cells to divide and produce biomass. In reality, yeast cultures often begin to use the fermentative pathway even under aerobic conditions due to the Crabtree effect (Crabtree 1929; De Deken 1966), whereby high concentrations of glucose suppress aerobic respiration. A comparison of the metabolic pathways associated with aerobic (respiratory) and anaerobic (fermentative) metabolism is displayed in Figures 1.2 and 1.3.

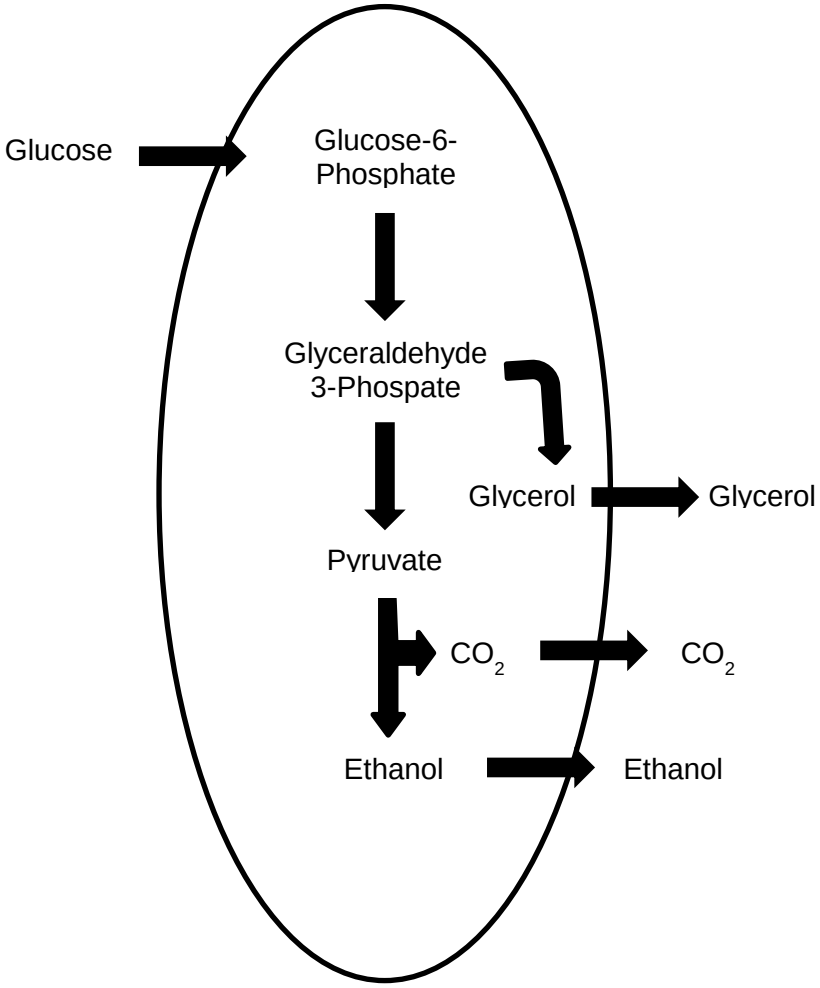


Figure 1.2 Yeast metabolism via the aerobic (respirative) pathway. Sugar (glucose) enters via glycolysis and energy is produced via the citric acid cycle and oxidative phosphorylation to produce carbon dioxide and water.

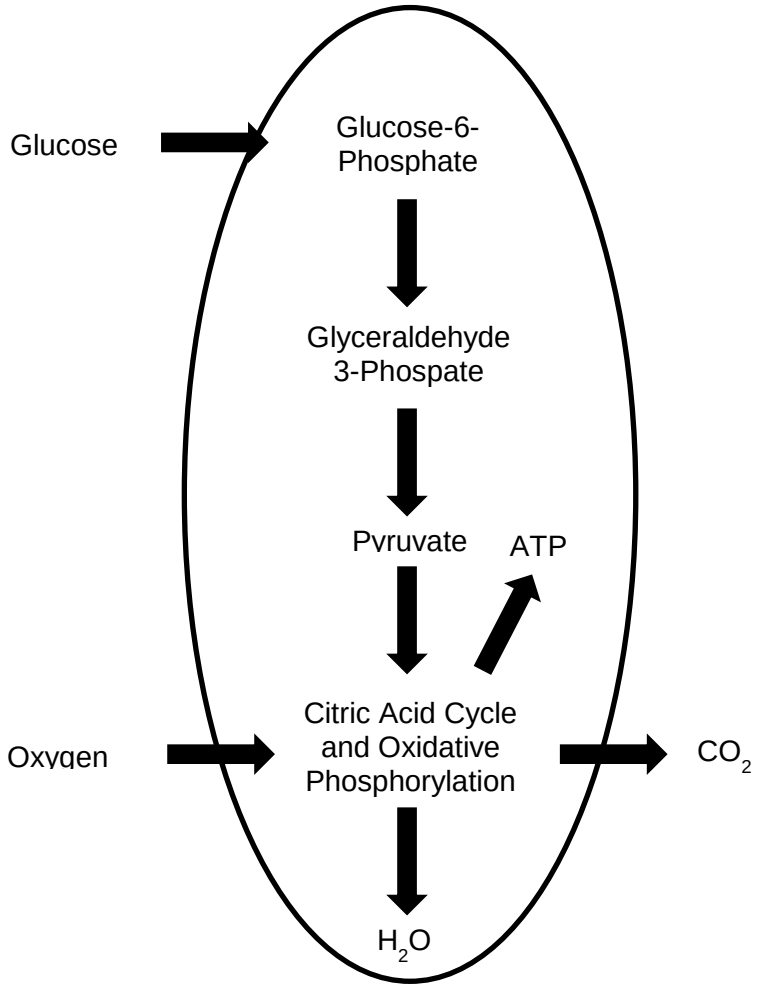


Figure 1.3 Yeast metabolism via the anaerobic (fementative) pathway. Sugar (glucose) enters via glycolysis which, due to the combination of a lack of oxygen and the Crabtree effect, is the only source of ATP production. To balance cellular redox potential, yeast produce ethanol and to a lesser extent glycerol, while carbon dioxide is also produced.

The fermentation pathway allows yeast to obtain ATP under anaerobic conditions by assimilating accessible sugars and converting them into ethanol and CO_2. As a result of yeast anaerobic metabolism, esters and higher alcohols are also produced, which give beer its characteristic flavour and aroma. Some compounds, such as the vicinal diketone (VDK) diacetyl, are first created, and then reabsorbed and broken down over time

during fermentation (Somalainen & Ronkainen 1968; Inoue & Yamamoto 1969; Cyr *et al* 2007; Duong *et al* 2011).

Towards the end of fermentation, yeast cells undergo a process called flocculation (Rhymes & Smart 1996; Vestrepen *et al* 2003; Soares 2011; Van Holle *et al* 2012), during which cells aggregate and clump together. Once flocculated, cells either rise to the surface (*S. cerevisiae*) or fall to the bottom (*S. pastorianus*) of a vessel, depending on the species of yeast used. The flocculation properties of the yeast can directly impact fermentation and beer quality; if flocculation occurs too early or too rapidly then ethanol yield may be lower than expected, with residual sugars present in the final beer (Van Nierop *et al* 2006). Poor flocculation can lead to beer which is hazy or requires further downstream processing to clarify the product (Van Neirop *et al* 2006).

Once flocculation has occurred the yeast is cropped from the vessel and can either be recycled, or discarded (Boulton 1991; Smart & Whisker 1995; Powell *et al* 2002; Powell *et al* 2004). Recycling or ‘re-pitching’ yeast is a common practice, although prolonged serial re-pitching can lead to changes in the genetic structure and quality of the yeast (Smart 1995; Jenkins *et al* 2003; Kobayashi *et al* 2007). Once the yeast has been removed from the vessel, the remaining product is termed 'green beer'.

1.1.6 **Conditioning**

Following fermentation, green beer is typically transferred to specific vessels for conditioning. During the conditioning process beer flavour matures, while the addition of finings agents such as isinglass finings can help remaining suspended solids to sediment and the beer to clarify

(Marchbanks 1986; Walker *et al* 2007). At this stage some carbonation may also take place and the interaction between ethanol and carbon dioxide can influence the foam and head character of the final beer (Vundla & Torline 2007). Once the desired product is achieved, the conditioning process is terminated, often by filtration to remove remaining yeast and other remaining particulate matter from the green beer, resulting in what is termed 'bright beer' (Fillaudeau & Carrere 2002).

For some types of beer, secondary conditioning in cask or bottles can also be performed, leading to the development of additional carbon dioxide and flavours (Fix 1989). The time required for secondary conditioning (sometimes referred to as re-fermentation) can range from a week to several months for complete flavour maturation. Beers produced in this fashion are typically not treated further and consequently a firm yeast sedimentation is desirable in order to prevent a hazy product at the point of sale (Vanbeneden *et al* 2006).

1.1.7 **Packaging**

Matured bright beer is typically transferred to a storage vessel and from there into final pack. Typical containers for the final product include casks and kegs (primarily for products which are sold within public houses or trade sales), glass or plastic bottle, and aluminium or steel cans (for home or off trade sales) (Brody 2001; Bamforth 2006; Browne 2006; Boulton & Quain 2001).

1.2 Yeast

1.2.1 **Industrial yeasts and laboratory strains**

Yeast are unicellular fungi which can reproduce sexually through sporulation, and asexually by a process known as budding (Kaeberlein, 2010; Section 1.3.2). At the cellular level yeast share similarities with many other eukaryotic organisms, including human cells (Drubin 1990; De Freitas *et al* 2003). This, combined with the fact that yeast are easy to grow, maintain and manipulate under laboratory conditions, has resulted in the use of laboratory strains of *S. cerevisiae* as model organisms in 'pure' scientific research. Although very similar to laboratory strains, industrial yeast strains are typically more robust. This is because laboratory strains are generally haploid or diploid, where they contain either a single or double compliment of DNA, while industrial strains are polyploid or aneuploid, implicated in improved survival capabilities (Pavelka *et al* 2010; Borneman *et al* 2011; Steensels *et al* 2014). In addition, most industrial strains are unable to produce spores (and divide sexually) due to their ploidy; they only divide mitotically by budding, producing theoretically identical 'copies' of the parent cell (Doffus 1971; Hammond *et al* 1993).

The majority of industrial yeast strains belong to the genus *Saccharomyces* and these organisms are widely found within the brewing, baking, wine and distilling sectors due to their particular properties. In brewing, *S. cerevisiae* yeast are used for the production of ale type beers, while lager beers are produced using strains belonging to the related species *S. pastorianus*. Interestingly, *S. pastorianus* lager yeast have an optimal growth temperature of around 28°C, and ferment best at lower temperatures of around 10-20ºC, while *S. cerevisiae* ale strains ferment best at higher

temperatures of between 20-35ºC (Torija *et al* 2003; Boulton & Quain 2001; Section 1.1.5). Ale strains were traditionally observed to flocculate by cells floating to the top of the vessel and can be referred to as 'top cropped yeast', while lager strains sank to the bottom and are sometimes referred to as 'bottom cropped yeast' (Vidgren & Londesborough 2011; Gibson & Liti 2014). This classification is less widely used at the current time since most large scale production of beer results in sedimentation and collection of yeast at the bottom of the vessel, irrespective of yeast type (Verstrepen *et al* 2003; Briggs *et al* 2004).

The divergence of *S. cerevisiae* and *S. pastorianus* has been mapped by Liti *et al* (2009), and it is currently thought that lager yeast strains are hybrid organisms derived from a mating event between a *S. cerevisiae* strain and another yeast, most likely closely related to *S. bayanus* (Tamai *et al* 1998), such as a *S. eubayanus* strain (Libkind 2011). Irrespective of the precise parental strains, lager yeast can be traced back to Europe where fermentations were first conducted under cooler temperatures (Lodolo *et al* 2008). In lager strains the mitochondrial DNA (mtDNA) and a significant proportion of genomic DNA (Rainieri *et al* 2008) is derived from the non-*cerevisiae* parent (*S. eubayanus* or *S. bayanus*, or another similar yeast), known to be more tolerant to cold temperatures. It is believed that the hybridisation event between the two species led to the selection of cold tolerant strains which were more suitable for the production of lager style beers (Libkind 2011; Pengelly & Wheals 2013).

1.2.2 **Yeast cell division**

In brewing yeast strains the predominant mode of reproduction is asexual and during this process yeast cells divide by mitosis to create offspring which are identical to the parental cell (Clancy 1998). In order to reproduce asexually, the cell and its environment must meet certain requirements before budding can occur. Such criteria include the attainment of a certain size and the availability of nutrients (Sherlock & Rosamond, 1993), conditions which must be met prior to entry into the cell cycle. Once the cell has passed through a cell cycle regulatory checkpoint known as 'START', the cell is committed to completing the budding process (Herskowitz 1988). During the cell cycle, the genetic material is synthesised and a bud forms, marked by a chitin ring, the position of which is dependent on whether the cell is diploid (bipolar) or haploid (axial) (Chant 1994). A simplified version of the cell cycle is illustrated in Figure 1.4 and includes the 4 main stages: G_1 or (gap phase 1), S (DNA synthesis), G_2 (gap phase 2) and M (Mitosis). The off-cycle stage known as G_0 or 'stationary phase' is also shown (Herkowitz 1988).

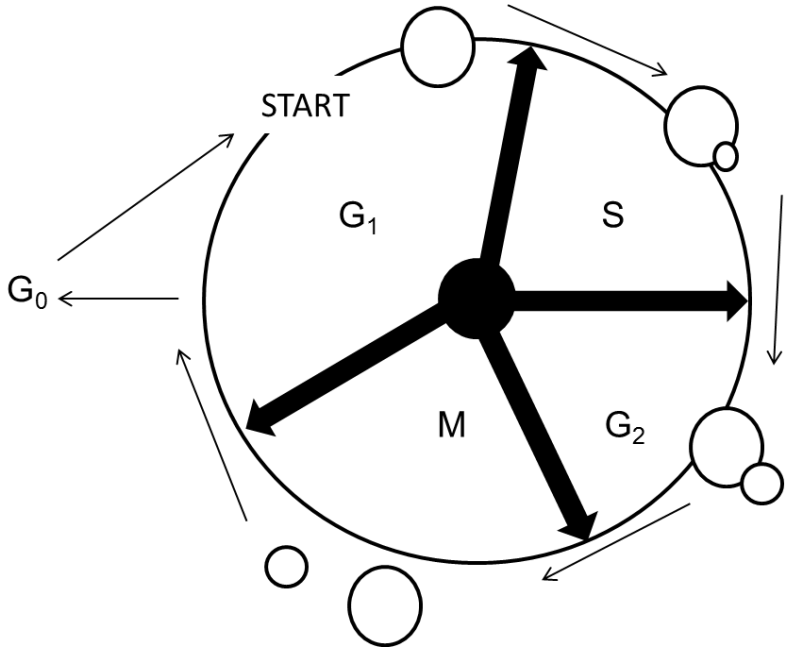


Figure 1.4 The yeast cell cycle. Key cell cycle stages are indicated: G_0, stationary phase; G_1, gap phase 1; S, DNA synthesis; G_2, gap phase 2; M, mitosis. An indication of cell budding state at each point is also represented. (Figure adapted from Hardin *et al* 2012)

1.3 Brewery yeast handling

Yeast activity is instrumental in achieving fermentations that are consistent in beer that is of the desired quality (Hough 1957; Kirsop 1974; Martens *et al* 1986; Pickerell *et al* 1991; Heggert *et al* 1999). The yeast strain employed, process factors, and the condition of the yeast prior to pitching also have concomitant effects on beer flavour (Quain *et al* 1981; Quain 1988; O'Connor-Cox 1996).

Pitching yeast typically originates from one of two sources: from a fresh propagation (Section 1.2.2.1) or from a previous fermentation. The latter involves a process known as serial re-pitching, whereby yeast is removed from a fermentation vessel, stored for a short period of time under

temperature controlled conditions (>4ºC) and then re-pitched into a fresh batch of wort (Boulton 1991; Smart & Whisker 1996). Regardless of the immediate origins of the culture, the yeast must be maintained efficiently to ensure consistent process and product quality. To ensure the long term quality of a yeast strain, stocks of production yeast are best maintained at -70ºC in freezers or cryopreserved in liquid nitrogen at -196ºC to minimise the risk of contamination and genetic mutation (Malik & Hoffmann 1993; Quain 1995; Hulse *et al* 2000).

1.3.1 **Yeast propagation**

The aim of propagation is to produce yeast which is in an optimal physiological state, so as to yield favourable and reproducible fermentations (Walker 1999; Novak *et al* 2007). Yeast propagation involves inoculation of yeast into successively greater volumes of wort until sufficient biomass is achieved to support a full scale fermentation (Quain 1995; Kennedy 2000).

Brewing yeasts were traditionally propagated using a series of aerated batch-type systems with relatively long cultivation times (Kunze 1996). However in recent times, oxygenated (rather than aerated) conditions are applied leading to a significantly increased biomass yield with yeast in an improved physiological state (Methner 1999; Cahill *et al* 2000; Wackerbauer *et al* 2004). Most brewery propagation systems currently utilise a scale up method between stages, such as 1L, 10L and 1hL, however very large capacity vessels can place time, space and energy demands on the propagation plant. If this demand cannot be met then there is a need for alternative systems. For example Maule *et al* (1979)

described an alternative propagation system to pitch into 800hL of wort, where small fermenters, or large part filled fermenters, were used to generate sufficient biomass (Figure 1.5). Irrespective of the propagation system, it is important to monitor cell growth (biomass production) while simultaneously maintaining hygiene at a very high level. The latter is important since the growth conditions during propagation are also favourable for microorganisms other than yeast, raising the risk of contamination which could ultimately lead to a complete loss of product (Jespersen & Jakobsen 1996).

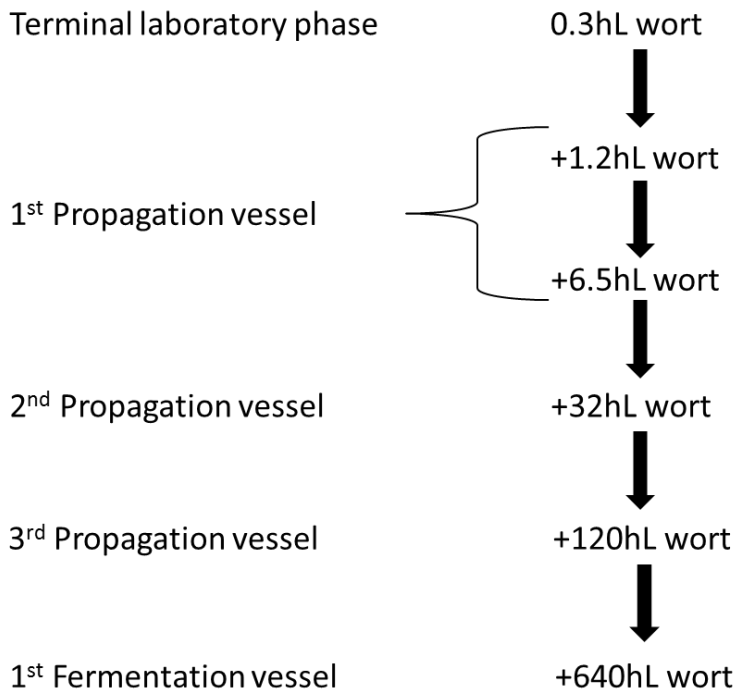


Figure 1.5 A typical brewery yeast propagation regime based on Maule (1979). Typical Quaintities of wort used at each stage of the process are indicated, although this is largely dictated by brewery size, fermenter size and the specific equipment in use.

1.3.1 **Yeast nutrition**

Wort contains a variety of nutrients, which include nitrogen, lipids, minerals and vitamins, each of which plays a part in determining fermentation performance and final beer quality (Casey *et al* 1984; Gardner *et al* 2005; Lododol *et al* 2008). Yeast utilise nitrogenous compounds from wort for protein formation, as well as an essential requirement for growth, metabolism and fermentation. The main form of nitrogen found in wort is Free Amino Nitrogen (FAN), which is made up of amino acids, ammonium ions and small di-and tri-peptides (Casey *et al* 1984; Pugh *et al* 2005; Stewart 2009).

Yeast also requires oxygen which is usually added to the wort as it is transferred to a fermentation vessel (Verbelen *et al* 2009). The oxygen is primarily utilised for the production of essential components necessary for cell division, such as lipid production (Section 1.2.3). In commercial brewing it is preferable to add oxygen rather than air to the fermentation due to the required level of oxygen being higher than air can provide (David & Kirsop 1973; Aries & Kirsop 1977; Jakobsen & Thorne 1980). Interestingly, the need for oxygen can be eliminated entirely by the addition of ergosterol and oleic acid, but this is costly and less effective than simple oxygen addition (Anderson & Stier 1952; Anderson & Stier 1954). Oxygen exerts its influence on yeast in fermentations primarily via the synthesis of sterols and latterly UFA synthesis. Sterols were first isolated by Gerard (1895), and constitute the majority of lipids present in yeast. 90% of the sterol composition of yeast is ergosterol, with the other 10% made up of other precursors within the ergosterol biosynthesis pathway (Figure 1.6).

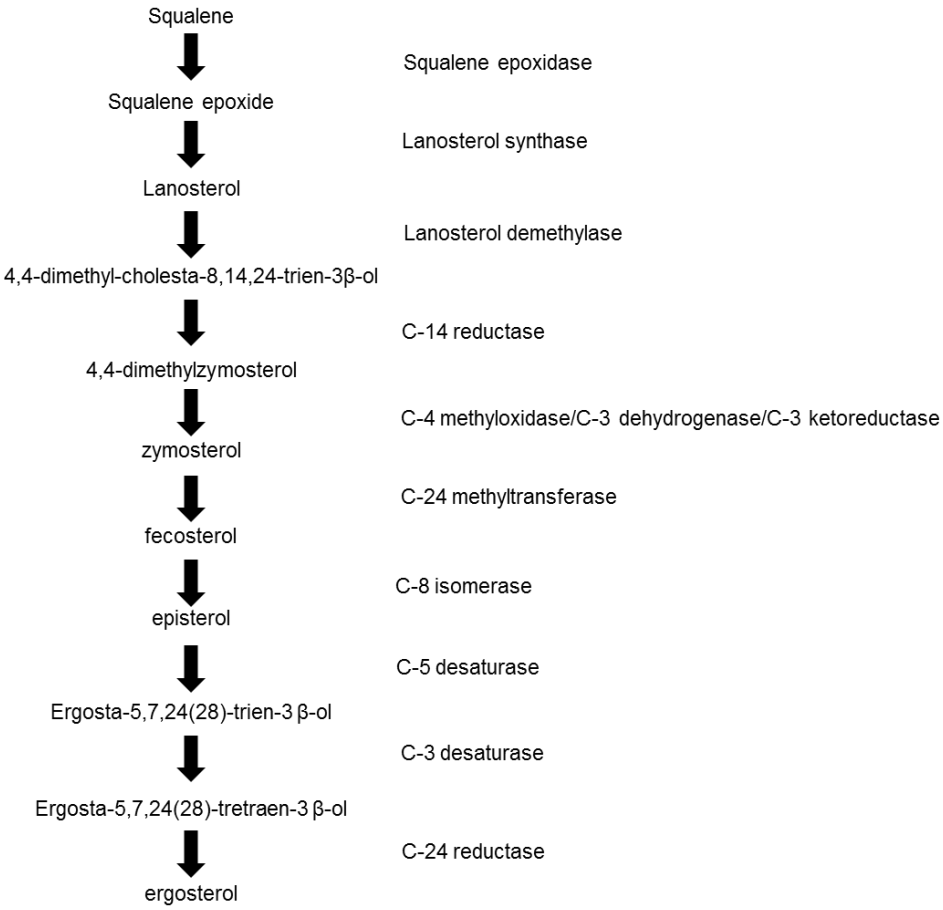


Figure 1.6 The ergosterol biosynthesis pathway in budding yeast showing the key metabolites and precursor compounds, alongside the enzymes responsible (Tiedje *et al* 2007).

1.3.2 **Serial re-pitching**

As an alternative to using freshly propagated yeast, it is common practice to reuse yeast from previous fermentations in a process known as serial re-pitching (Lawrence *et al* 2012; Miller *et al* 2013). Typically a yeast slurry can be used from between 5-20 fermentations, largely dictated by company policy and influenced by the health, quality and purity of the population (Briggs *et al* 2009). Several studies have shown that a reduction in quality is associated with serial re-pitching, with effects such

as a reduction in nitrogen utilization and altered carbohydrate assimilation, along with a variety of mutations to the genome being observed all of which can lead to a decrease in fermentation performance (Donnelly & Hurley 1996; Jenkins *et al* 2003; Lawrence *et al* 2012; Miller *et al* 2013). It should also be acknowledged that although most breweries reuse cultures between 5-20 re-pitchings, there are some that re-pitch indefinitely with no obvious defects, and as such it is likely that there is an element of strain dependency in the ability of yeast to remain stable (Powell *et al* 2003). It has also been noted that in relation to very high gravity fermentations the number of generations that can be achieved is typically significantly reduced (Gibson *et al* 2007; Stewart 2009).

1.3.3 **Yeast storage**

If yeast is to be used in serial re-pitching, there is inevitably a period of time during which yeast must be maintained before inoculation into another vessel. Once cropped, yeast is typically stored for a maximum of 48hours without any deterioration in physiological condition. Although yeast can be stored as pressed cake, within the brewing industry it is more commonly maintained as a yeast slurry (Knudsen 1985; Boulton 1991a). Slurried yeast is stored under specific conditions which minimise metabolic activity. Metabolic activity utilises endogenous reserves of intracellular glycogen levels, which are required during the initial stages of fermentation (Quain *et al* 1981). Severe depletion of glycogen causes a reduction in yeast vitality and reduces fermentation rate (Murray *et al* 1984; Knudsen 1985; Martens *et al* 1986;). The crop is typically maintained under anaerobic conditions and low temperatures at 2-4ºC. Even under these conditions metabolic activity proceeds at a slow rate, resulting in starvation (Knudsen 1985;

McCaig & Bendiak, 1985). Nutrient limitation, as well as stress caused by continued exposure to cold (McCaig & Bendiak 1985), low pH (Simpson & Hammond 1989) and ethanol (Odumeru *et al* 1992) all lead to a loss of vitality. Stored yeast is generally held under beer or water as this postpones nutrient stress (Kirsop 1974; Quain & Tubb 1982) and agitated intermittently to increase homogeneity (Gilliland, 1981) and to preserve vitality (McCaig & Bendiak 1985). It is seldom maintained for longer than 3-5 days and regularly used within 48 hours prior to intracellular glycogen levels becoming depleted (Quain & Tubb 1982; Murray *et al* 1984; Sall *et al* 1988).

1.4 Yeast and fermentation

1.4.1 **Standard and high gravity fermentations**

Depending on the product type and beer style, fermentations typically take place using worts which are of different initial sugar concentrations. However, broadly speaking, standard gravity lager fermentations are considered to be those conducted using worts in the range of 10-12ºP (Thomas *et al* 1996). Fermentations conducted using such worts are usually complete within 5 days and yield a product with 4-5% alcohol. However, within the brewing industry there is a growing trend to ferment at 'high' gravity (HG) and 'very high' gravity (VHG), terminology typically used to describe fermentations conducted at 13-17ºP and >20ºP respectively. In raising the starting gravity of the wort the ethanol yield is increased correspondingly, often to around 10% (Casey *et al* 1984; Baib *et al* 2008; Puligundala *et al* 2011), but sometimes to as high as 15% or greater (Laopaiboon *et al* 2009). HG and VHG beers are diluted to produce a

finished product with the desired alcohol content (Boulton and Quain 2001), and this process has a positive impact on yield while also offering savings in terms of time, efficiency and vessel capacity.

Whilst there are financial gains to be afforded from HG brewing, the composition of the wort and the resulting beer represent environments which are stressful to the yeast culture. It has been reported that HG brewing can lead to osmotic stress (Gasch *et al* 2000) as well as an increase in ethanol toxicity through carbohydrate conversion (Pratt-Marshall *et al* 2002). In addition, yeast populations may be starved of essential nutrients due to the necessity of using adjuncts in the manufacture of HG and VHG worts (Thomas *et al* 1995; Le Van *et al* 2001). The effects of yeast stress on fermentation are broad, but typically result in reduced performance in terms of speed and consistency (Section 1.5.1). In addition, there have also been reports of issues associated with reduced foam stability, which is related to protease release after cell death (Rees and Stewart 1997), as well as difficulty in matching flavour compared to beer brewed at normal gravities (Stewart 2009). However, it should be noted that some reports also indicate that beers brewed at HG exhibit increased flavour stability as well as a 'smoother' taste (Rees & Stewart 1997; Dragone *et al* 2004; Watanabe *et al* 2010), indicating that the process of increasing gravity is feasible from a product specification perspective as well as being commercially desirable.

1.4.2 **High gravity wort production**

In order to create worts with high gravities, adaptations to the production process are typically required. These include cycling of wort through the

mash filter back into the mash tun (Boulton and Quain 2001), or increasingly by the simple addition of sugar adjuncts to the wort boil (Gibson 2011; Section 1.1.4). This latter has a significant impact on wort composition since the majority of nutrients are derived from barley malt. Adjuncts are, by nature, nutritionally poor and therefore VHG worts produced with significant concentrations of sugar syrups may not adequately support yeast growth and metabolism. Due to the necessity of using adjuncts in the manufacture of worts with very high gravity, nutritional imbalance must therefore also be addressed, which can be a costly process (Casey *et al* 1984; Cunningham and Stewart 2000; Boulton and Quain 2001).

In addition, there are other associated effects of brewing at high gravity which necessitate close process control. With an increase in the carbohydrate concentration, oxygen solubility in wort is lowered (Baker & Morton 1977). This is of significance since oxygen is vital for yeast growth and lipid metabolism at the start of brewery fermentations (Ingledew *et al* 1987; D'Amore *et al* 1991). Lipids are an important component of the yeast cell membrane (Section 1.3.1.5.) and it has been shown that HG and VHG fermentations can lead to an increase in membrane permeability and leakage of cellular constituents, which contribute to a stale character in beer (Chi & Arneborg 1999; Gibson *et al* 2009).

1.5 Fermentation Stress Factors and Response

In order to survive and maintain an advantage over competing microorganisms, yeast are able to respond to environmental challenges, including fluctuating nutrient levels, variable temperatures and other stress

factors (Gibson *et al* 2007a). To deal with such challenges, yeast are able to utilise several stress response pathways including the STress-Responsive Element (STRE) (Grant 2000), the High Osmolarity Glycerol (HOG pathway) and the Heat Shock Response (HSR) (Trott & Morano 2004). Fortuitously, these pathways also allow yeast to adapt and deal with extracellular stresses that are associated with fermentation within the brewing industry. The HOG pathway allows yeast to deal with hyper-osmotic stresses (such as encountered within high gravity brewing (Section 1.4.2) by activating and releasing mitogen activated protein kinases which cascade and induce the release of increased cellular glycerol as a protectant (Dihazi *et al* 2004). The STRE and HSR pathways are considered to be global response mechanisms which act to counter a wide range of environmental challenges (Takemorit *et al* 2006; Akerfelt *et al* 2010; Morano *et al* 2011). These pathways function by regulating a number of different genes which are known to protect against or repair stress and are triggered by the STRE elements located in the promotor regions of the genes MSN2 and MSN4 (Schmitt & McEntee 1996; Hasan *et al* 2002; Gibson *et al* 2007; Erkina *et al* 2009).

1.5.1 **Osmotic stress**

Osmotic stress can be defined as the effect resulting from an imbalance of intracellular and extracellular osmotic concentrations, sufficient to cause a change in cellular physiology (Csonka & Hanson 1991). The presence or absence of solutes can lead to an environment for the yeast which is either hyper-osmotic or hypo-osmotic respectively. Osmotic stresses can affect plasma membrane, structure, permeability and mechanical properties (Wood 1999; Rep *et al* 2000; Montanes *et al* 2011).

During hypo-osmotic stress, the cell wall protects the yeast cell from bursting, and in doing so the cell can increase in size with the uptake of water through passive diffusion (Dihazi *et al* 2001). The removal of cytoplasmic solutes such as glycogen through aquaporins and electrolyte transporters is essential to balance the osmotic potential in these conditions (Berrier *et al* 1992, levina *et al* 1999; Mager *et al* 2000). Soveral *et al* (2006) suggested that the existence of aquaporins in yeast could be important for sustaining low-temperature water permeability, consistent with the fact that aquaporins facilitate the major part of water flow at low temperatures, whereas diffusion of water through the plasma lipid bi-layer dominates at high temperatures. Levels of mRNA coding for the *Saccharomyces* aquaporin genes AQY1 have been observed to be higher during fermentation of grape juice with strain UCD 713 (Karpel & Bisson 2006), suggesting an important role for ScAqy1 in the response to osmotic stress. However, Karpel & Bisson (2006) also found a functional Aqy1, (but not a functional Aqy2) in 5 wine and brewing strains, concluding that the yeast strains investigated were *not* absolutely dependent on aquaporins to adapt to stress under certain fermentation conditions. Furthermore, it was demonstrated that a low concentration of ethanol (4%) had an inhibitory effect on aquaporin activity (Madeira *et al* 2010), supporting the idea that aquaporins play a reduced role in osmotic regulation in wine and brewing yeast strains in the presence of high concentrations of ethanol.

Hyper-osmotic stress can be characterised by the loss of cellular water and subsequent turgor due to the requirement to balance osmotic pressure across the membrane (Wojda *et al* 2003; Wood 1999). This stress is particularly relevant to brewing fermentations as it is associated with the

practices of acid washing, employed to remove bacterial contaminants from pitching yeast slurries (Hammond *et al* 2001), and more importantly with yeast pitching (Gibson *et al* 2007; Section 1.3).

When yeast cells are pitched into wort, osmotic stress occurs due to the sudden contact of yeast with a complex and highly concentrated medium containing sugars (Briggs *et al* 2004). The use of VHG worts has been suggested to exacerbate osmotic stress leading to a decrease in cell viability (D'Amore 1992; Cahill *et al* 2000), as well as changes to vacuole size and cell surface ultrastructure (Pratt *et al* 2007; Stewart 2010). In particular, the cell membrane is also affected, with yeast cells able to actively decrease its fluidity in response to the environment (Beney and Gervais (2001) as described fully below (Section 1.5.1.1).

1.5.1.1 Osmotic stress response

Yeast are able to display a series of active processes by which cells are able to monitor and adjust osmotic pressure to repair cellular damage and regain turgor (Wodja *et al* 2003; Mager & Siderius 2002). When hyper-osmotic stress occurs, intracellular water is lost leading to cell shrinkage (Pratt *et al* 2003; Meikle *et al* 1988; Morris *et al* 1986; Section 1.5.1). The ability of yeast to respond to this pressure is largely dependent on the intrinsic cellular attributes of individual yeast, for example 'superior' membrane structure and vacuolar functioning, as well as increases in cellular protectants such as trehalose (Pratt *et al* 2007; Nass & Rao 1999; Sharma *et al* 1996; Latterich & Watson 1993; Wiemken 1990). These are largely artefacts of the high-osmolarity glycerol (HOG) pathway, and the yeast general stress response (GSR). The HOG pathway in yeast is the

central hyper-osmotic response (Hohmann 2002; DeNadal *et al* 2002; Zi *et al* 2010). This signalling pathway is responsible for sensing environmental changes through the induction of *Sho1* and *Sln1*, two plasma membrane proteins (Hohmann 2002). Signals from these proteins are transduced by independent components and initiate the conserved section of the HOG pathway (O'Rourke & Herskowitz 2004), leading to the phosphorylation of mitogen-activated protein kinase kinase (MAPKK) *Psb2* to mitogen-activated protein kinase (MAPK) *Hog1*. This is then followed by the translocation of *Hog1* to the nucleus and an increase in its kinase activity (Bilsland-Marchesan *et al* 2000; Proft *et al* 2001; Hohmann 2002; Klipp *et al* 2005; Zi *et al* 2010). Stimulation of glycerol biosynthesis is induced through genes such as *Gpd1* and *Gpp2* and mediation of sensitive glycerol channel activity occurs (Luyten *et al* 1994; Tamas *et al* 1999; Klipp 2005). This leads to the reduction of efflux of glycerol aiding in the accumulation of internal glycerol (Klipp 2005).

The general stress response (GSR) involves the induction of ~200 genes functioning in a diverse array of cellular behaviours (Ruis & Schuller 1995; Gasch *et al* 2000). It has been shown that these genes contain a general stress response element (STRE) (CCCCT or AGGGG) in their upstream regulatory region, and two zinc finger transcriptional activators Msn2p and Msn4p, which are responsible for the activation of STRE within the genes (Schmitt & McEntee 1996; Martinez-Pastor *et al* 1996; Watanabe *et al* 2011). STRE and STRE-like sequences have been identified in the promoter region of many stress-response inducing genes such as *Ctt1* and *Hsp12* and *Hsp 104 (*Marchler *et al* 1993; Varela *et al* 1995; Verbelen *et al* 2009).

1.5.2 **Ethanol stress**

As described previously, the amount of ethanol produced during fermentation can range from 5-15%, depending on the amount of fermentable sugars provided and the characteristics of the yeast strain (Laopaiboon *et al* 2009). Such concentrations of ethanol can lead to changes in the cells fermentative ability by influencing yeast gene expression (Alexandre *et al* 2001; Kaini & Takagi 2008), as well as affecting the physiological state of individual cells (Casey *et al* 1985; Gibreel *et al* 2009). Direct exposure to ethanol can cause an increase in cellular membrane fluidity (Ingram 1990; Weber & DeBont 1996; You *et al* 2003), resulting in a loss of amino acids, proteases and proteins (Piper *et al* 1995; Stanley *et al* 2009). The effect on the cell membrane is arguably the most important effect of ethanol toxicity; it has been suggested that the insertion of ethanol into the hydrophobic interior of the membrane results in changes to polarity, exchange of polar molecules and membrane protein positioning (Ingram & Buttke 1984; Lentini *et al* 2003; Jones & Greenfield 1987; Mizoguchi & Hara 1997). Ethanol can also have an effect on the mitochondrial membrane, disruption of which can lead to mitochondrial DNA damage and the generation of respiratory deficient, or petite, cells (Jiménez *et al* 1988; Chi & Arneborg 1999).

1.5.3 **Oxidative stress**

Oxygen is an essential component of the brewing process (Section 1.3.1.) and is required for the synthesis of unsaturated fatty acid and sterols (Lorenz & Parks 1991; Section 1.3.1). The presence of oxygen can however lead to the generation of reactive oxygen species (ROS), normal by-products of cellular metabolism under aerobic conditions (Halliwell &

Gutteridge 1999; Figure 1.7). The main source of ROS is through leakage of electrons passing down the respiratory chain leading to a reduction of oxygen (Grant *et al* 1997), although ROS can also be formed following exposure to radical generating compounds that are associated with metabolism under aerobic conditions (Halliwell 2006).

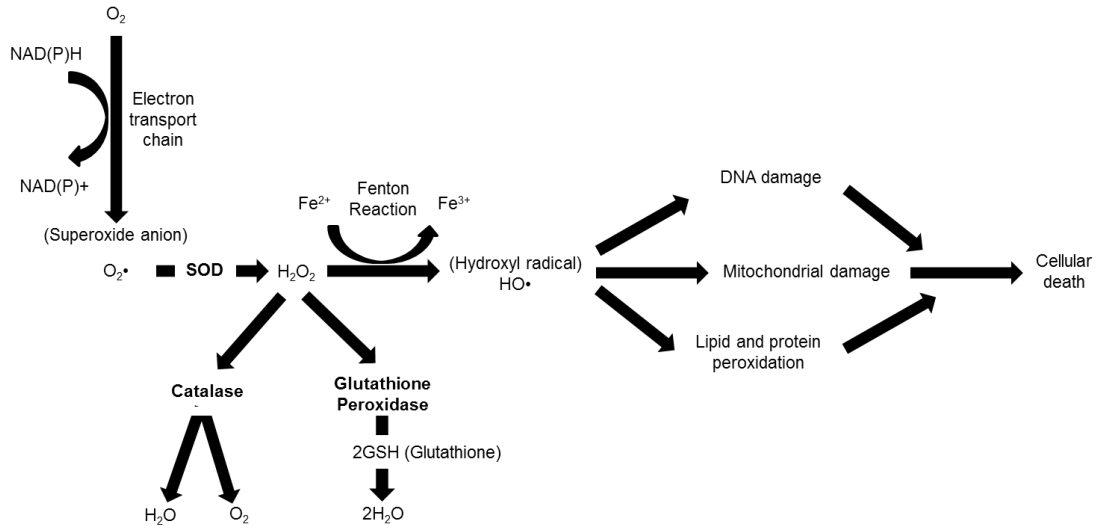


Figure 1.7 Overview of the mechanism of cellular damage by oxygen free radicals. The production of superoxide anions and their subsequent reduction by SOD to H_2O_2 is shown. H_2O_2 is converted to H_2O and O_2 by the action of catalase or $2H_2O$ with the addition of 2 glutathione (GSH) and the action of glutathione peroxide. H_2O_2 is converted to hydroxyl radicals ($OH^{\bullet}$) through Fenton reactions. The cellular targets for damage by the $OH^{\bullet}$ are also indicated.

1.5.4 **Oxidative stress and free radical formation**

1.5.4.1 Aerobic and anaerobic production of free radicals

Oxidative stress occurs as a result of the presence of free radicals released by oxygen related metabolism (Halliwell and Gutteridge 1989). These free radicals are generally referred to as Reactive Oxygen Species (ROS), and represent the different states of dioxygen (O_2) and include the singlet oxygen (also O_2 but referred to as singlet oxygen throughout this thesis), the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH•). These ROS are produced through Fenton and Haber-Weiss reactions (Equation 1.1 and 1.2), which start with the reduction of O_2 to O_2^-, through catalytic cycle reactions and the dismutation of two O_2^- molecules by two electrons reduction leads to the production of H_2O_2. The iron catalysed Fenton reactions, and the copper induced Haber-Weiss reactions can then form hydroxyl radicals from H_2O_2 as well as by the homolytic fission of water (Hohmann & Mager 2003; Zufall & Tyrell 2008). Fenton reactions are of specific relevance in brewing for analysis of beer aging, where Bamforth & Parsons (1985) have shown that hydroxyl radicals are important intermediates in the formation of aged flavour compounds in beer. As such, high levels of peroxides catalysed by copper ions through Haber Weiss reactions and heavy metals can lead to hydroxyl radicals and rapid development of stale flavour in beer (Bamforth 1986).

$Fe^{2+} + H_2O_2 = Fe^{3+} + OH\bullet + OH^-$

$Fe^{3+} + O_2^{\bullet-} = Fe^{2+} + O_2$

Net:

$2H_2O_2 + Fe = OH\bullet + OH^- + O_2^{\bullet-}$

Equation 1.1 Fenton reaction showing the role of iron (Fe) induced production of hydroxyl radicals.

$Cu^{2+} + O_2^- = Cu^+ + O_2$

$Cu^+ + H_2O_2 = Cu^{2+} + OH + OH^-$

Net:

$H_2O_2 + O_2^- + Cu = OH + OH^- + O_2$

Equation 1.2 Haber-Weiss reaction of copper (Cu) induced hydroxyl radical production.

1.5.4.2 Cytochrome P450 pathway

There have been many studies on the aerobic formation of free radicals (Halliwell 1987; Madeo *et al* 1999; Dawes 2004; Temple *et al* 2005; Halliwell & Gutteridge 2007), but reports have also indicated the occurrence of ROS accumulation throughout fermentation: an anaerobic system (Salmon *et al* 1998; Landolfo *et al* 2008). The main mechanism for ROS formation in an anaerobic system is through the cytochrome P450

pathway. The cytochrome P450 family of monooxygenase enzymes are a diverse collection of enzymes acting on various endogenous and xenobiotic molecules (Bogeart *et al* 2010). Monooxygenase enzymes are enzymes that incorporate one hydroxyl group into substrates in many metabolic pathways. In this reaction, the two atoms of dioxygen are reduced to one hydroxyl group and one H_2O molecule by the concomitant oxidation of NAD(P)H (Schenkman *et al* 1994). The majority of these catalysed hydroxylation reactions occur with substrates such as fatty acids and their related structures, which are involved in alkane conversion to ethyl alcohol (Funhoff *et al* 2006). This is of particular importance in brewing, where fatty acid degradation leads to the production of, p-cumaric acid and cinnamaic acid, which are enzymatically decarboxylated to form 4-vinylphenol and styrene. Incidentally, these compounds are particularly present in wheat beers, the later up to concentrations of 25µg/L, and are partly responsible for the 'sweet' and 'phenolic' flavours associated with these beer styles (Steele *et al* 1994; Fragnier *et al* 2003; Schwarz *et al* 2012).

The first stage of the P450 pathway is the binding of a substrate to a P450 enzyme, which causes a lowering of the redox potential by approximately 100mV (Ruckpaul *et al* 1989). This makes the transfer of an electron from NADH or NADPH much more favourable. It has also been suggested that the binding of the substrate brings about a conformational change in the enzyme triggering an interaction with the redox component (Veitch & Williams 1992). An Fe^{3+} ion is then reduced by an electron transferred from NAD(P)H via the electron transfer chain, followed by the binding of an O^2 molecule, that rapidly binds to the Fe^{2+} ion. There is evidence to suggest that this complex then undergoes a slow conversion to a more

stable complex, Fe^{3+} - O^{2-} (Archakov & Buchmanova 1990). The rate determining step is then the Fe^{3+} + $2O^{2-}$ complex, which is the most favourable starting point for the next stage of the reaction to occur. However, evidence from resonance Raman spectroscopy indicates the presence of a superoxide complex (Egawa *et al* 1991). The $2O^{2-}$ reacts with two protons from the surrounding solvent, breaking the O-O bond, forming water and leaving behind an $(Fe – O)^{3+}$ complex. This is then transferred to the substrate forming a hydroxylated form of the substrate, which is then released from the active site of the enzyme which returns to its initial state. Most importantly, throughout these reactions ROS can be formed at any stage where NAD(P)H is reduced (Veitch & Williams 1992).

1.5.4.3 Cellular defence of oxidative stress

As mentioned above, the two main forms of ROS in yeast cells are hydrogen peroxide (H_2O_2) and the superoxide anion (O_2^-), both of which can lead to the production of hydroxyl radicals which can cause cellular damage and death (Peronne *et al* 2008). Cellular damage is manifested in the form of lipid peroxidation, protein oxidation and DNA mutations (Girotti 1998). There are several ways in which *S. cerevisiae* cells can defend against oxygen free radicals: either by reducing the production of ROS, or by repairing any damage caused (Ruis & Hamilton 1992). These mechanisms can be triggered by the general stress pathways (Section 1.5.1) or as part of a ROS specific response mechanism (Santoro *et al* 1998; Jamieson 1998). For the latter, the primary response is to generate a series of antioxidant metabolites including superoxide dismutase (SOD) (Figure 1.8), glutathione (Figure 1.9) and catalase (Figure 1.10) which act to buffer ROS within the cell (Martins & English 2014; Miao *et al* 2006).

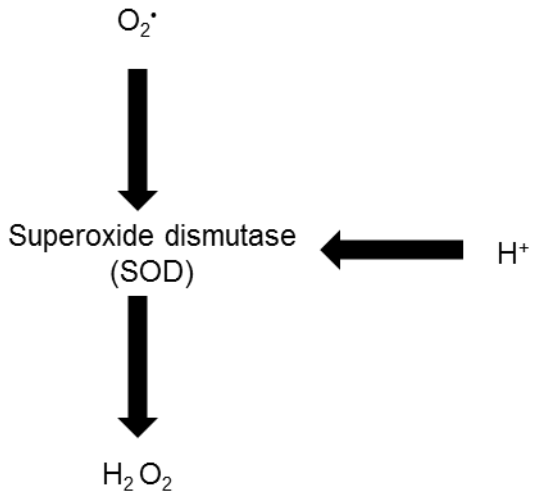


Figure 1.8 The reduction of superoxide anion by the enzyme superoxide dismutase (SOD) with the addition of a H^+ ion, to hydrogen peroxide (H_2O_2).

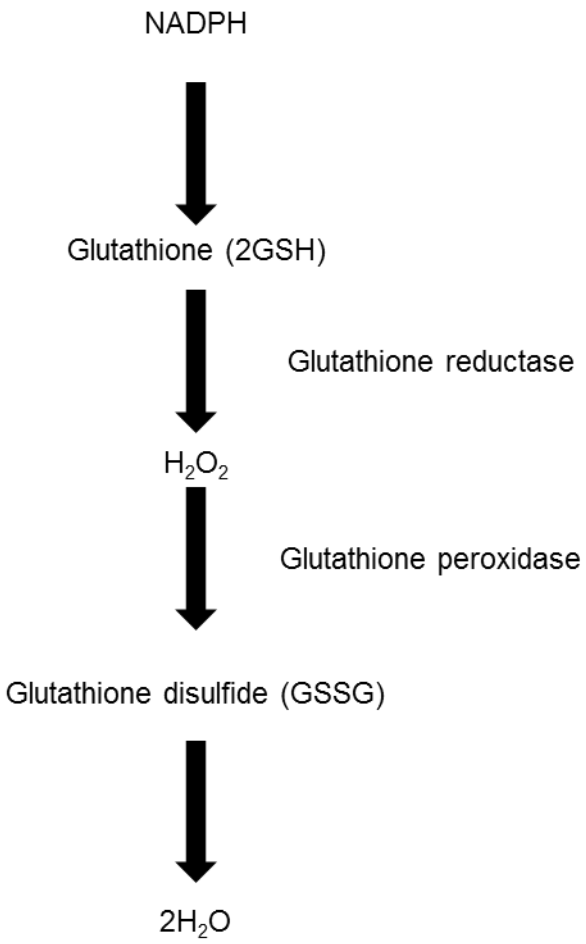


Figure 1.9 Glutathione pathway, indicating the reduction of NADPH by glutathione reductase to generate glutathione, which is combined with H_2O_2 and oxidised by glutathione peroxidase to generate glutathione disulphide and $2H_2O$ molecules (Hohmann and Mager 2003)

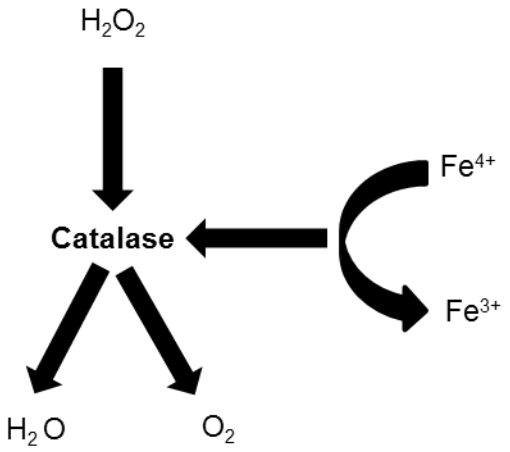


Figure 1.10 Catalase enzyme pathway. Catalase reduces hydrogen peroxide through the use of an Fe(4^+) heme group attached to the enzyme. This reduction then releases H_2O and O_2.

Glutathione (GSH) is a glutamate-cysteine-glycine tripeptide, synthesized by the action of γ-glutamaylcysteine synthetase (GSH1) and glutathione synthase (GSH2). GSH1 catalyses the condensation of cysteine onto the gamma carbon of glutamate, and GSH2 forms GSH by the addition of glycine (Penninck 2000; Grant and Dawes 1996). The cysteine residue is responsible for the GSH redox properties, because it has a very low redox potential (-240mV) that allows it to donate its electrons to other cysteine molecules (Hohmann and Mager 2003). Once oxidised, GSH dimerizes to the disulphide GSSG, which is reduced back to GSH by the FAD-bound NADPH dependent glutathione reductase (GLR1). This allows the cell to keep a cytoplasmic reduced GSH:GSSG ratio of 30:1 to 100:1 (Muller 1996). GSH is the most abundant cellular thiol with a concentration in the mM range. This, together with its high GSH:GSSG ratio and its low redox potential, gives it the properties of a redox buffer, and as such it plays a

crucial role in keeping the cytoplasmic thiol redox balance and in absorbing oxidizing equivalents (Figure 1.9)

Catalases are iron-containing enzymes with a haem reactive group buried inside the enzyme structure. Catalases reduce H_2O_2 by the dismutation mechanism shown in Equation 1.3.

$Fe(III) + H_2O_2 = Fe(V) + H_2O$

$Fe(V) + H_2O_2 = Fe(III) + H_2O + O_2$

Equation 1.3 Catalase detoxification of H_20_2.

Saccharomyces species contain two types of catalase: CTT1, the cytosolic form of catalase, and CTA1, the peroxisomal form of catalase (Ruis & Hamilton 1992). Both forms of catalase are bound by NADPH, which may prevent the formation of inactive Fe(IV), although NADPH is not essential for their catalytic cycle (Halliwell & Gutteridge 1998; Hillar *et al* 1994). Catalases have been shown to be most important during stationary phase rather than exponential phase, where knockout strains have been observed to have more sensitivity to H_2O_2 than wild type phenotypes (Grant *et al* 1998).

Yeast cells can also produce compounds such as superoxide dismutase (SOD) which functions to convert O_{2^-} into H_2O_2 and O_2 (Figure 1.4). The dismutation of O_{2^-} is catalysed by the transition-metal centre in SOD, which is iron, copper or manganese, and does not require the supply of an external source of reducing power, such as NADPH.

$O_{2^-} + M^{2+} = O_2 + M^+$

$O_{2^-} + M^+ + 2H^+ = H_2O_2 + M^{2+}$

Equation 1.4 SOD detoxification of the superoxide anion (O_{2^-}). M reflects the bound metal ion involved in the reaction.

There are two main forms of SOD within yeast: *Sod1*, which is a cytosolic Cu,Zn-superoxide dismutase that can be localized in the mitochondrial intermembrane space as well as within other cellular compartments, and *Sod2*, which is localized in the mitochondrial matrix. *Sod2* is the primary O_{2^-} scavenging enzyme giving protection for O_{2^-} generated during respiration. *Sod1* gives essential protection against O_2 toxicity, and knockout strains have been shown to exhibit an array of defects, such as reduced growth rate higher rates of spontaneous mutagenesis, sporulation defects and rapid loss of viability in stationary phase (Chang & Kosman 1990; Gralla & Valentine 1991; Gralla & Kosman 1992; Liu *et al* 1992; Longo *et al* 1996). Cells lacking in both *Sod1* and *Sod2* have more profound defects than single mutants, however they are still viable. This demonstrates that SOD activity although important, is not essential for survival, and that each SOD has a particular function (Liu *et al* 1992; Longo *et al* 1996).

Thioredoxin is a sulphydryl-rich protein that can be used by thioredoxin peroxidase and nucleotide reductase as a reductant (Jamieson 1998). Thioredoxins are heat-stable oxidoreductases containing two conserved cysteine residues in their active sites (Holmgren 1989). They are required

for a number of metabolic enzymes that form a disulphide as part of their catalytic cycle. These metabolic enzymes are necessary for many cellular processes, such as protein folding and regulation, the repair of oxidatively damaged proteins and sulphur metabolism (Grant 2001). The oxidized disulphide form of thioredoxin is reduced directly by NADPH and thioredoxin reductase. There are two main types of thiol-specific antioxidant (TSA). These are TSA1 which showsa a preference for H_2O_2, and TSA2, which shows a preference for alkyl hydroperoxides. The reaction mechanisms appears to involve the formation of an intermolecular disulphide bond between two protein molecules that can be reduced by thioredoxin (Chae *et al* 1993, Jeong *et al* 1999, Grant 2001)

In addition to cellular anti-oxidant defence mechanisms, metal binding proteins such as metalothioneines can be produced which bind to available ions, such as Cu, preventing free ions giving charge to molecular oxygen and the formation of more ROS (Peronne *et al* 2008). Finally transcriptional factors can also code for other binding proteins and essential components in antioxidant defence. Yap1p is an essential transcriptional binding site localising Yap1 into the nucleus. Along with the transcription factor SKn7p, Yap1 induces heavy metal resistance in response to oxidative stress in yeast. Activation of the phosphor-kinase pathway (PKA) has a major impact on Msn2 and Msn4, which mediate the transcription of genes controlling the STRE (stress response element) (Estruch & Carlson 1993; Martinez-Pastor *et al* 1996; Schmidt & Entee 1996). STRE-regulated genes are involved in carbohydrate metabolism and growth regulation, as well as in adaption to heat shock, DNA damage, and oxidative and osmotic stresses (Mai & Breeden 1997; Moskvina *et al* 1998; Smith *et al* 1998). PKA plays a very important role inhibiting nuclear

import of Msn2/Msn4, either through direct phosphorylation of their nuclear localization signal (Gorner *et al* 1998; Gorner *et al* 2002; Casado et al 2011). Metal binding is also activated by Mac1, which regulates the genes involved in Cu homeostasis and H_2O_2 induction of Ctt (Table 1.1).

Function	Gene(s)	Location	Role
Metal-binding proteins			
Metallothioneins	CUP1, CRS5	Cytochrome	Copper binding protein, multiple genes at locus, also binds Cd^{2+}
Transcription factors, regulators			
Yap1p	YAP1	Cytochrome/Nucleus	Oxidative stress, resistance to xenobiotics, cadmium
Skn7p	*SKN7*	Nucleus	Auxiliary transcription factor, functions with Yap1p for some oxidative stress, also acts in osmoregulatin
Msn2/4	*MSN2*, *MSN4*	Cytochrome/Nucleus	General stress responsive transcription factor responding to PKA pathway, responsive to heat, starvation, osmotic and oxidative stress
Yap1-binding protein	*YBP1*	Cytochrome	Protein involved in one mechanism for nuclear localisation of Yap1p
Haem activated protein	*HAP1,2,3,4,5*	Nucleus	Regulation of respiratory functions
Metal-binding activator	*MAC1*	Nucleus	Regulator of genes involved in copper ion homeostasis and H_2O_2-induction of *CTT1*
Cup2p	*ACE1/CUP2*	Nucleus	Cu-binding transcription factor activates *CUP1* at high copper

Table 1.1 Metal binding antioxidant defence systems and transcriptional antioxidant regulators. Their suspected roles in metal binding and in antioxidant response are indicated (Peronne *et al* 2008).

1.5.5 **DNA Damage as a result of oxidative stress**

Charged and relatively unstable superoxide anions are membrane-impermeable and, depending on the site of production, can be released into either the mitochondrial matrix or the intermembrane space. In these locations they can be converted into H_2O_2 the principal cellular mediator of oxidative stress. H_2O_2 is able to freely able to diffuse throughout the cell due to its stability and membrane permeability. Production of these ROS by mitochondria can lead to mtDNA damage and mutations, which can in

turn lead to progressive respiratory chain dysfunction and to a further increase in ROS production as a consequence of this dysfunction (Shokolenko *et al* 2009). As mentioned above, the relative stability and membrane permeability of H_2O_2 allows it to freely diffuse throughout the cell, where it can generate, through the Fenton reaction, the DNA damaging hydroxyl radical (Henle *et al* 1996a; Henle *et al* 1996b). It has been reported that the main products of mtDNA base damage are thymine glycol among pyrymidines and 7, 8-dihydro-8-oxo-20 -deoxyguanosine (8-oxoG) among purines (Wang *et al* 1998; De Bonet & Larebecke 2004). It has been shown that an increase in oxidative damage also causes damage to the plasma membrane when enzymatic antioxidant responses were reduced (Deryabina *et al* 2014).

1.5.6 **Stress factors associated with very high gravity fermentations**

Stress factors associated with VHG brewing are currently believed to be largely exacerbated versions of those which occur during standard fermentations (Gibson 2007; Figure 1.11). However, it is known that in VHG fermentations pitching rate and pre-oxygen levels should be increased (Fix 1989), potentially raising the precursors for oxidative stress at the start of the process. Another concern specifically related to VHG fermentations is the possible increase in metabolic activity due to the increased availability of sugars (Van Dijkenk & Scheffer 1986; Patkova *et al* 2000), as well as potential nutritional deficiencies exacerbated by the use of adjuncts in VHG wort production (Jones *et al* 1994; Gibson 2007; Puligundla *et al* 2011). Nutrition deficiency during fermentation progression may occur once the yeast have utilised available nutrients for

cell growth, and is also likely to coincide with an increase in ethanol. Hence stress under VHG conditions may well prove to be more complex than currently imagined, and is certainly likely to impose significantly different challenges to those at lower gravities.

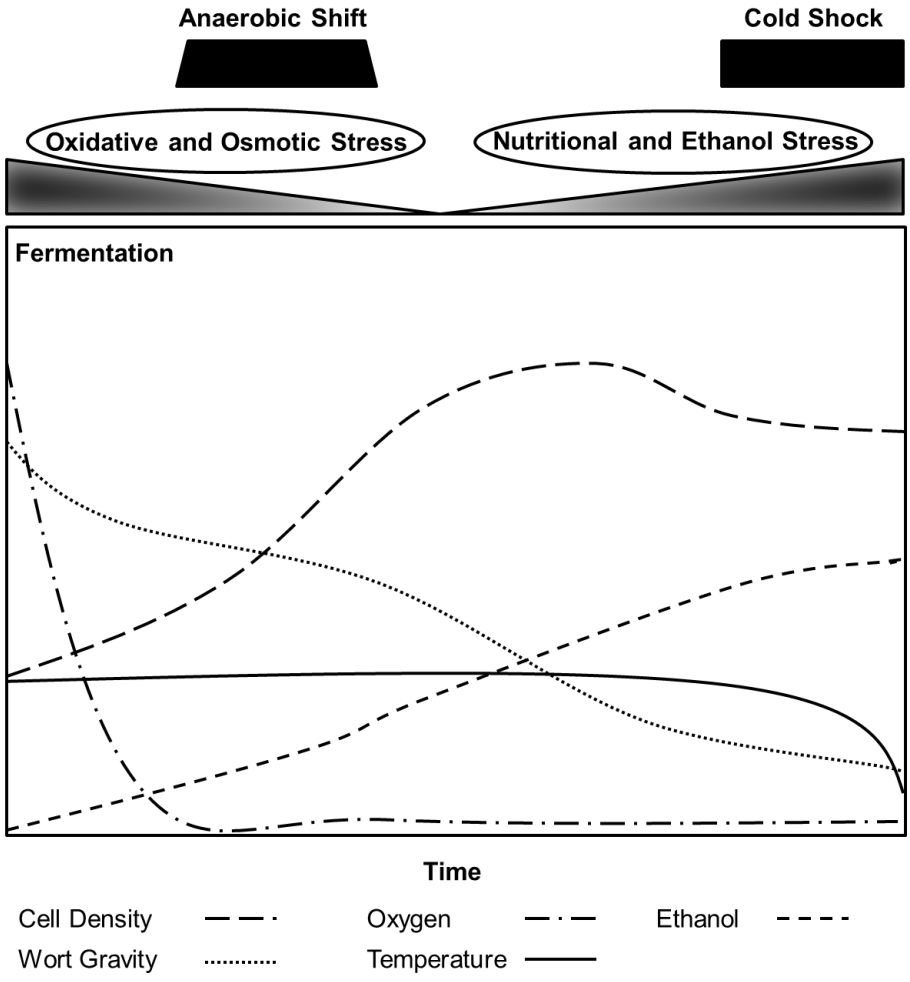


Figure 1.11 Traditional stress factors present during fermentation (Gibson *et al* 2007). These may be exacerbated during VHG fermentation, although little research has been conducted in this area. Oxidative and osmotic stress are present at the start of fermentation and nutritional and ethanol stress occur as the fermentation progresses. Cell density, oxygen levels, ethanol concentration, wort gravity and temperature at the start of fermentation are all indicated against fermentation time.

1.6 Aims and objectives

The brewing industry increasingly employs high gravity (HG) brewing as a means of increasing productivity without incurring additional plant costs. For continuing financial gains to be afforded, Very High Gravity (VHG) fermentation above 20°P is an increasingly attractive proposition. However the potential use of VHG wort creates a number of issues across the brewing process, from wort production through to fermentation, as well as in matching final product specifications.

From the perspective of the brewing yeast culture, the environment of VHG wort during fermentation is considerably different to that of standard gravity. Although previous studies have been performed to determine nutritional and environmental related stresses associated with VHG brewing, there are also additional responses that need to be understood. One example is the relationship between the extra demands placed on yeast metabolism and the generation of compounds which can be harmful to cells. In particular, during yeast metabolism, reactive oxygen species (ROS) are produced which can lead to oxidative stress (Herrero *et al* 2008*)*. ROS are normal by-products of cellular activity, and although they are more frequently associated with respiratory metabolism which occurs immediately after pitching, have been shown to be produced throughout fermentation *(*Mendes-Ferreira *et al* 2010*)*.

The aims of this work was to:

- analyse the tolerances of industrial lager yeast strains to stresses that occur during VHG fermentations;
- examine the fermentative abilities of industrial lager yeast strains under VHG conditions;

- investigate whether the production of ROS occurs throughout VHG lager fermentations and observe the generation of compounds which act as biomarkers for oxidative stress;
- evaluate the effect that oxidative stress from VHG fermentations has on yeast cellular membrane and DNA integrity;
- examine the possibility of effective enhancement of industrial lager yeast with sterols for better VHG fermentation;

The data presented in this thesis will provide an insight into the challenges faced by yeast in fermenting worts of particularly high sugar concentration, and indicate potential strategies for alleviating stress under VHG conditions.

CHAPTER 2: MATERIALS AND METHODS

2 Materials and Methods

2.1 Yeast strains

The lager yeast strains SMCC90, SMCC99 and SMCC100 were obtained from SABMiller plc. Strains were cultivated and stored on YPD media as described below (Section 2.2).

2.2 Yeast growth media

2.2.1 **YPD media composition**

Yeast strains were grown using YPD medium comprising 1% yeast extract (Oxoid, UK), 2% neutralised bacteriological peptone (Oxoid, UK) and 2% D-glucose (Fisher Scientific, UK), in reverse osmosis (RO) water (all w/v). YPD media was sterilised by autoclaving at 15 psi and 115°C for 15 minutes.

When solid media was required 1.5% w/v agar (Oxoid, UK) was added prior to autoclaving. After sterilisation, agar was dispensed into sterile petri-dishes, for YPD agar plates, or into 28 mL Universal tubes inclined at approximately 45°C, for YPD agar slopes. Agar was allowed to set at room temperature for 15 minutes inside a laminar flow hood.

2.2.2 **Wort**

All worts used were provided by SABMiller Plc from either a commercial facility or the SABMiller Research Brewery (SABRB) to proprietary specifications and represent medium used for commercial lager style fermentations. Sugar concentration is expressed in degrees Plato (°P),

representing wort density as an approximate percentage of sugar by weight (g extract/100 g wort). Commercially produced hopped wort, brewed to 15°P was employed for standard gravity fermentations. Very high gravity wort was prepared by the SABMiller research brewery, by supplementing a base hopped-wort with a glucose based corn syrup to achieve 22°P. In all instances gravity was confirmed by passing a sample through a DMA 4500 density meter (Anton Paar, UK) (Section 2.9.1). The VHG wort was supplemented with the commercially available supplement Yeast Life (ABVickers, UK) at a concentration of 50mg/L to balance nutritional defects and to ensure fermentation performance.

When solid wort media was required, 1.5% w/v agar (Oxoid, UK) was added to wort prior to autoclaving. After sterilisation, 23 mL of agar was dispensed into sterile petri-dishes. Agar was allowed to set at room temperature for 15 minutes inside a laminar flow hood.

2.3 Yeast storage

Long-term stocks of yeast were prepared by cultivating cells aerobically at 25°C in YPD (Section 2.1.1) media and storing samples in YPD supplemented with 25% (v/v) glycerol at -80°C until needed. Working cultures were prepared from frozen stocks by cultivation in 10 mL volumes of YPD medium at 25°C. From this culture, YPD agar slopes and YPD agar plates were prepared for medium- and short-term use respectively.

2.4 Yeast cell counting

2.4.1 **Total and viable cell counts**

Yeast cell numbers were primarily calculated by haemocytometer count, in conjunction with methylene blue staining for viability analysis. On occasion the fluorophore propidium iodide (PI) was also utilised to estimate cell number and viability, quantified using a Beckman Coulter flow cytometer as described in Section 2.11.2.

For yeast viability by methylene blue analysis, the method of Pierce (1970) was used to determine live cells based on membrane exclusion. Methylene blue (Sigma Aldrich, UK) was dissolved in a 2% w/v sodium citrate solution (Fisher scientific, UK) to a final working concentration of 0.01% (w/v). Yeast cells were diluted with sterile H_2O to reach a working concentration of approximately 1 x 10^7 cells/mL. 0.5 mL of the yeast suspension was combined with 0.5 mL of methylene blue solution and gently agitated. The combined solution was then incubated at room temperature for 5 minutes and a sample placed onto an improved Neubauer haemocytometer (depth 0.1mm, area 0.0025mm^2) (Fisher scientific, UK). Individual yeast cells were examined using a standard light microscope (Zeiss, Germany) at x400 magnification. Cells stained dark blue were considered dead and unstained cells were considered to be alive. The total cell count was calculated as shown in Equation 2.1, and cell viability was calculated as shown in Equation 2.2.

$$Cell\ concentration\ (cells/mL) = total\ cells\ in\ ruled\ area\ \times\ dilution\ factor\ (if\ any)\ \times 10^4\ \ (where\ 10^4\ represents\ the\ counting\ area\ of\ 10^{-4}\ cm^3)$$

Equation 2.1 Calculation of cell concentrations in yeast cell suspensions.

$$Cell\ viability\ (\%) = \frac{(total\ cells) - (dead\ cells)}{total\ cells}\ x\ 100$$

Equation 2.2 Calculation of cell viability.

2.5 Analysis of yeast growth

In order to investigate the growth characteristics of yeast under different parameters, yeast cell suspensions were analysed using a spot plate technique (Section 2.5.1) and by monitoring growth in 96 well plates (Section 2.5.2).

2.5.1 **Spot plate technique**

In order to investigate the tolerance of yeast to stress and/or changes in environment, a spot plate technique was employed. 10 µL of cell suspension at a concentration of $1x10^2$, $1x10^3$, $1x10^4$ or $1x10^5$ cells/mL were spotted onto a base media. Base media typically comprised a wort based agar (Section 2.2.2) supplemented with a defined concentration of one of a series of additional components, as specified below (Section 2.5.1.1-2.5.1.3). Cells were then incubated aerobically at 25°C for 72 hours and the growth of 'spots' of colonies on each plate was observed by visual examination.

2.5.1.1 Glucose tolerance with spot plates

Wort agar supplemented with glucose was used to determine the tolerance of yeast strains to stress factors associated with high levels of this sugar. A base wort agar at 15°P (Section 2.2.2) was supplemented to achieve a final concentration of 28, 30, 32, 34, 36, 38, and 40°P using dextrose sugar (Fisher scientific, UK). After inoculation, cells were incubated at 25°C for 72 hours and growth was observed visually for the size and shape of the resulting colonies.

2.5.1.2 Ethanol tolerance with spot plates

Wort agar supplemented with ethanol was used to determine the tolerance of yeast strains to high levels of alcohol. A base wort agar at 15°P (Section 2.2.2) was supplemented to achieve a final concentration of 5, 10, 15 and 20% v/v alcohol, using absolute ethanol (Fisher scientific, UK). After inoculation, cells were incubated at 25°C for 72 hours and growth was observed visually for the size and shape of the resulting colonies.

2.5.1.3 Oxidative tolerance with spot plates

Wort agar supplemented with either menadione or H_2O_2 were utilised to determine the tolerance of yeast strains to oxidative stress. A base wort agar at 15°P (Section 2.2.2) was supplemented to achieve a final concentration of 0.5, 1, 1.5, 2, 2.5 and 3mM Menadione (Sigma Aldrich, UK), or 1, 2, 3, 4, 5 and 6mM H_2O_2 (Fisher scientific, UK). After inoculation, cells were incubated at 25°C for 72 hours and growth on the plates was observed visually for the size and shape of the resulting colonies.

2.5.2 **Growth kinetics in 96 well plates**

In order to investigate the effect of increasing glucose, ethanol, and menadione and H_2O_2 on yeast growth kinetics, growth curves of each lager strain were obtained using a TECAN automated micro-plate reader (Infinite@ 200 PRO series, TECAN, UK). Wells within a 96 well plate were loaded with 95 µL of base media as described below (Section 2.5.2.1-2.5.2.3) along with 5 µL of culture. Cells were then incubated for 96 hours at 25°C and readings were generated by measuring optical density at 600nm every 25 minutes. All experiments were performed in triplicate. Data was collected and analysed by MagellanTM Data Analysis Software (TECAN, Switzerland).

2.5.2.1 Glucose tolerance

Wort supplemented with glucose was used to determine the tolerance of yeast strains to stress factors associated with high gravity. A base wort of 15°P (Section 2.2.1) was supplemented with dextrose sugar (Fisher scientific, UK) to achieve 28, 30, 32, 34, 36, 38, and 40°P media. An Alcolyzer-plus: beer system with incorporated DMA 4500 density meter (Anton Paar, UK) was used to measure and confirm the gravity of the wort prior to experimentation (Section 2.9.1.1). A VHG wort of 22°P (Section 2.2.1) was also included as an intermediate control to observe the capacity of strains to be cultivated under VHG concentrations. In each instance, 95µL of media was added to each well of a 96 well microwell plate (Nunc, US), and 5µL of a suspension containing 1.5 x 10^7 cells/mL of the relevant strain was added to the media. Cells were then incubated at 25°C for 96

hours in a TECAN Infinite 200 Pro microplate reader (TECAN, Switzerland). Population growth was monitored by observing changes in optical density over time at 600nm.

2.5.2.2 Ethanol tolerance

Wort supplemented with ethanol was used to determine the tolerance of yeast strains to alcohol. A base wort of 15°P (Section 2.2.1) was supplemented with absolute ethanol (Fisher scientific, UK) to achieve a final concentration of 5, 10, 15 and 20% v/v. An Alcolyzer-plus: beer system with incorporated DMA 4500 density meter (Anton Paar, UK) was used to measure and confirm the ethanol content of the wort prior to experimentation as described in section 2.9.1.1. In each instance, 95µL of media was added to each well of a 96 well microwell plate (Nunc, US), and 5µL of a suspension containing 1.5 x 10^7 cells/mL of the relevant strain was added to the media. Cells were then incubated at 25°C for 96 hours in a TECAN Infinite 200 Pro microplate reader (TECAN, Switzerland). Population growth was determined by observing changes in optical density over time at 600nm.

2.5.2.3 Oxidative stress tolerance

The oxidants menadione and H_2O_2 were utilised independently to observe the growth characteristics of yeast strains in the presence of oxidative stress. Menadione (Sigma Aldrich, UK) was added to 15°P wort (Section 2.2.1) to create final concentrations of 0.5, 1, 1.5, 2, 2.5 and 3mM menadione. H_2O_2 (Fisher scientific, UK) was added to 15°P wort (Section 2.2.1) to create final concentrations of 1, 2, 3, 4, 5, 6mM H_2O_2. 95 µL of

media was added to each well of a 96 well microwell plate (Nunc, USA), along with 5 µL of a suspension containing 1.5 x 10^7 cells/mL of the relevant strain. Cells were then incubated at 25°C for 96 hours in a TECAN Infinite 200 Pro microplate reader (TECAN, Switzerland). Population growth was determined by observing changes in optical density over time at 600nm.

2.5.3 **Analysis of population growth data using DMFIT software**

DMFit is an Excel add-in, fitting curves where a linear phase is preceded and followed by stationary phases. DMFit (DM: Dynamic Modelling) is based on the model proposed by Baranyi & Roberts (1994) which has been shown to be useful for analysis of yeast populations (Gerstgrasser *et al* 2016). The main difference between this model and other sigmoid curves like Gompertz or Logistic is that the mid-phase (exponential) is presumed to be close to linear, unlike classical sigmoid curves which exhibit a pronounced curvature at this point.

2.6 Genetic characterisation of yeast strains

2.6.1 **DNA extraction**

Extraction of genomic DNA was performed using a modified version of the method described by Powell and Diacetis (2007). A streak of cells was taken from an agar plate and transferred to an Eppendorf tube containing 660 µL of 50TE-SDS buffer comprising 200 mL of 50TE buffer (7.44 g/L EDTA, 6.06 g/L TRIS, adjusted to pH 7.5 with 1M HCl and sterilised by

autoclaving at 121°C at 15psi for 15minutes) along with 10% SDS solution (Sigma Aldrich, UK), pre-filtered through a 0.45 µM filter. Samples were then mixed by vortexing and incubated at 65°C for 10 minutes, with a further vortex mixing after 5 minutes. 340 µL of 5M potassium acetate was added and samples were placed at 4°C until proteins had coagulated. Once coagulated, the sample was centrifuged at 13,000rpm for 10minutes and 600 µL of supernatant was transferred to a fresh tube. 600 µL isopropanol was then added and the tube was mixed by inversion and incubated at room temperature for 10 minutes to isolate the DNA fraction. After incubation the sample was centrifuged at 10,000rpm for 10 minutes and the aqueous phase was discarded. The DNA pellet was then rinsed in 100 µL of 95% (v/v)) ethanol at -20°C and flash spun. The ethanol was then removed by pipette and the pellet was allowed to air-dry by placing the tube inverted onto absorbent paper. Once dry, the pellet was re-suspended in 60 µL of TE 1x by rubbing the tube across a rough surface, and incubated at 65°C for 5 minutes. Purified DNA was quantified using a NanoDrop 1000 UV-Vis Spectrophotometer DNA quantifier (NanoDrop Technologies Inc, US) at a wavelength of 600nm and stored at -20°C.

2.6.2 **Internal transcribed spacer analysis**

To identify yeast genera and species, the internal transcribed spacer (ITS) region of each organism was amplified using the universal primer pairs ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') as described by White *et al* 1990. PCR reactions were prepared using the components specified in Table 2.1 and amplification was conducted under the conditions shown in Table 2.2 using a Techne TC-512 thermal cycler (Bibby-Scientific, UK). The PCR products

were then digested in separate reactions using the restriction endonucleases *Hae*III and *Hinf*I (New England Biolabs, UK). Each digestion reaction consisted of 10 units of enzyme, 1X Buffer 4 (proprietary buffer), 1 µg of DNA and molecular grade water to a final volume of 25 µL. This was then incubated at 37°C for 90 minutes. The PCR products and restriction fragments were separated using a 3% (w/v) agarose gel pre-stained with ethidium bromide, at 100Mv for 2.5 hours. The separated fragments were visualized under UV light using a gel imaging system (Bio-Rad Laboratories, USA). A 100bp ladder (Promega, UK) was used as a reference for DNA samples to determine fragment size. The species and genus were confirmed by comparing DNA fragment sizes obtained to reference data previously published by Esteve-Zarzoso *et al* (1999) and Guillamon *et al* (1998).

Reagent	Final concentration
Forward primer (ITS1)	0.5µM
Reverse primer (ITS4)	0.5µM
Phusion Master Mix (New England Biolabs, UK)	1 x
DNA Template	~4 ng/µL
Molecular Grade H_2O (Fisher Scientific, UK)	to 25 µL

Table 2.1 PCR components for amplification of internal transcribed spacer regions of genomic DNA.

	Temperature	Duration	Number of cycles
Step 1	95°C	15 minutes	1
Step 2	94°C	10 seconds	35
	55.5°C	2 minutes	
	72°C	2 minutes	
Step 3	72°C	10 minutes	1
Step 4	4°C	Hold	n/a

Table 2.2 PCR cycling conditions for amplification of internal transcribed spacer regions of genomic DNA.

2.6.3 **Inter-delta region analysis**

Yeast strain differentiation was performed based on inter-delta sequence patterns created via PCR amplification with the primer pair inter-delta12 (5'-TCA ACA ATG GAA TCC CAA C-3') and inter-delta21 (5'-CAC TTA ACA CCG TAT ATG A-3') according to Legras and Karst (2003). Yeast genomic DNA was isolated as described above (Section 2.4.1). PCR amplification of desired regions was performed using a Techne TC-512 thermal cycler (Bibby-Scientific, UK) using Phusion Master Mix (New England Biolabs, UK). Reactions components (Table 2.3) were prepared on ice and DNA amplification was conducted by PCR according to the parameters detailed in Table 2.4. PCR products were separated on a 1.5% (w/v) agarose (Fisher Scientific, UK) gel pre-stained with 3 µL of 10 mg/mL ethidium bromide solution (Sigma Aldrich, UK). A 100-bp DNA ladder marker (Promega, UK) was used as a molecular weight DNA standard. DNA amplicons were visualized under UV light and photographed using a GelDoc XR+ imaging system (Bio-Rad, UK).

Reagent	Final concentration
Forward primer (delta 12)	1µM
Reverse primer (delta 21)	1µM
Phusion Master Mix (New England Biolabs, UK)	1 x
DNA Template	~4 ng/µL
Molecular Grade H_2O (Fisher Scientific, UK)	to 25 µL

Table 2.3 PCR components for amplification of inter-delta regions of yeast genomic DNA.

	Temperature	Duration	Number of cycles
Step 1	98°C	30 seconds	1
Step 2	98°C 48°C 78°C	10 seconds 30 seconds 3 minutes	35
Step 3	72°C	10 minutes	1
Step 4	4°C	Hold	n/a

Table 2.4 PCR cycling conditions for amplification of inter-delta regions of yeast genomic DNA

2.6.4 **Mitochondrial DNA extraction**

Mitochondrial DNA (MtDNA) was extracted according to the method of Defontaine *et al* (1991), with modifications as described by Nguyen *et al* (2000). MtDNA was extracted from cells cultured for 72 hours in 100 mL YPD at 25°C, with constant shaking at 120rpm. Cells were harvested by centrifugation at 4000rpm for 5 minutes. Cell pellets were washed in sterile water, re-suspended in washing buffer (1M sorbitol (Fisher Scientific, UK),

50mM KH_2PO_4 (Fisher Scientific, UK), pH 7.5) and transferred to 5 x 1.5 mL microfuge tubes. The cells were frozen for 30 minutes to disrupt the cell membrane, after which ice cold 50mM EDTA (Sigma-Aldrich, UK) was added and centrifuged at 4000rpm for 5 minutes. Cells were then re-suspended in 0.5 mL of cell wall lysis buffer (1.2M sorbitol, 50mM EDTA) supplemented with 2% β-mercoptoethanol (Sigma-Aldrich, UK) and incubated at 37°C for 10 minutes. Protoplasts were recovered by centrifugation at 1000rpm for 5 minutes and re-suspended in 0.5 mL of washing buffer (0.5M sorbitol, 10mM EDTA and 50mM Tris-HCl, pH 7.5) containing 200 µg/mL lyticase enzyme (from *Arthrobacter luteus*, Sigma-Aldrich, UK). Protoplasts were then lysed at 37°C for 1hour, inverting the tubes every 15 minutes. The suspension was sonicated for 15 secs at amplitude of 7µ using a Soniprep 150 (Sanyo, UK) to help break un-lysed proptoplasts within the suspension. Cell lysates were then centrifuged at 1,000rpm for 10 minutes and the supernatants containing the mitochondria were transferred to new microfuge tubes. Mitochondria were isolated by centrifugation at 15,000rpm for 10 minutes and the supernatant was discarded. The mitochondrial pellet was then re-suspended in 0.2 mL washing buffer (see above) plus 10 µL DNAse (Promega, UK) and 25 µL $MgCl_2$. Suspensions were incubated at 37°C for 15 minutes and centrifuged at 15,000rpm for 5 minutes to remove any possible genomic DNA contamination. The mitochondrial pellets were then washed twice with 1mL of washing buffer to remove any residual DNAse present.

Purified mitochondria were lysed by incubation in 0.5 mL lysis buffer (100mM NaCl, 10mM EDTA, 50mM Tris-HCl, pH 7.8 and 1% sarkosyl (Sigma-Aldrich, UK)) for 10minutes at room temperature. 0.5 mL of phenol-chloroform (Sigma-Aldrich, UK) was added and the tubes were

inverted to gently mix the solutions and separate the mtDNA from the lysate. Samples were centrifuged at 15,000rpm for 5 minutes and the aqueous phase was collected in a fresh tube. 0.6 mL of chloroform (Sigma-Aldrich, UK) was added to remove trace phenol in the samples. The solutions were inverted to gently mix and centrifuged at 15,000rpm for 5 minutes before the aqueous phase, containing the mitochondrial DNA, was collected. The mtDNA was then precipitated by adding 0.5mL of ice cold iso-propanol (Sigma-Aldrich, UK) and 125µM of NaCl (Sigma-Aldrich, UK) to each sample and incubating at room temp for 30 minutes. MtDNA was collected by centrifugation at 15,000rpm for 30 minutes at 4°C. The pellets containing purified mtDNA were then washed with 0.6 mL of 75% (v/v)) ethanol and air-dried for 30 minutes. The mtDNA was finally re-suspended in 40 µL of molecular grade water (Sigma-Aldrich, UK) and quantified using a NanoDrop 1000, Nanodrop, US) before being stored at -20°C.

2.6.5 **Mitcholdrial DNA RFLP**

Purified MtDNA was digested with *Hae*III, *Hinf*I and *Dde*I (New England Biolabs,UK) in separate reactions. Each digestion reaction consisted of 10 units of enzyme, 1X Buffer 4 (proprietary buffer), 1µg of DNA and molecular grade water up to a final volume of 25 µL. This was incubated at 37°C for 1 hour and restriction digests were separated on a 1.5% (w/v) agarose gel stained with ethidium bromide, at 100V for 1 hour. The separated fragments were visualized under UV light using a gel imaging system (Bio-Rad Laboratories, USA). A 1kb ladder (Promega, UK) was used as a reference for DNA samples and to determine fragment size.

2.7 Fermentations and Analysis

2.7.1 **Wort preparation**

Worts at 15°P and 22°P were obtained from SABMiller Plc (Section 2.2.2) and stored at -20°C in 2L aliquots until needed. Prior to fermentations, wort was defrosted at 4°C for a maximum of 24 hours and decanted into a Cornelius vessel. Wort was transferred to fermenters by applying a positive pressure of nitrogen to each Cornelius vessel. Wort was then oxygenated *in situ* by introducing oxygen at a rate of 5L/min until a yield of 15ppm dissolved oxygen was reached for 15°P worts and 22ppm dissolved oxygen for 22°P worts according to current practice applied throughout the industry (Fix *et al* 1999).

2.7.2 **Yeast propagation and pitching**

A representative sample of yeast cells was obtained from a YPD agar plate (Section 2.2.1) and inoculated into Universal tubes containing 10mL of standard gravity wort (15°P) (Section 2.2.2). Samples were incubated for 48 hours at 25°C in a shaking incubator at 120rpm (Certomat BS-1, Sartorius, Germany). Subsequently cultures were transferred to 500mL Erlenmeyer flasks containing 100mL of fresh wort and incubated under identical conditions for a further 48 hours. Samples were then transferred to a 2L Erlenmeyer flask containing 1L of fresh wort and incubated under identical conditions for a further 96 hours.

After growth, cells were harvested by centrifugation at 3000rpm for 10 minutes in a Beckman J2-21 centrifuge (Beckman Coulter, Germany), washed with sterile distilled water, and used to prepare a 50% yeast slurry (v/v) by dilution with an equal quantity of sterile RO water. Appropriate

volumes of propagated yeast cells (as determined by microscope count (Section 2.4), were added to wort to reach a final concentration (pitching rate) of $1.5x10^7$ viable cells/mL for 15°P standard gravity worts and $2.2x10^7$ viable cells for 22°P VHG worts. Pitching rate was based on the principle of 1 million cells per degree plato, originally selected as a trade-off between ensuring fermentation speed and loss of efficiency due to sugar utilisation in biomass formation (Briggs *et al* 2009).

2.8 Small scale fermentations (100mL)

Small scale fermentation vessels were constructed using 150mL glass hypovials (International Bottle Company, UK) containing a magnetic stirrer as described previously (Quain *et al* 1985). Each vessel was sterilised prior to use by autoclaving at 121°C and 15psi for 15minutes. Fermentations were conducted using either 15°P or 22°P worts in 100mL volumes, prepared as described previously (Section 2.2.2). Pre-aerated wort was transferred from oxygenation vessels (Section 2.7.1) to small scale fermentation vessels prior to inoculation with aerobically grown yeast (Section 2.1). Oxygenation and pitching rate were adjusted to 1ppm and $1x10^6$ cells/ml per degree plato as described previously (Section 2.7.2).

Irrespective of wort gravity, once yeast had been pitched, vessels were sealed using a rubber septa and metal crimps. A Bunsen valve was inserted through the rubber septa to allow carbon dioxide to escape (Figure 2.1). All vessels were incubated at 13°C and agitated at 120rpm using a magnetic stir plate (Fisher Scientific, UK) to maintain yeast in suspension. Vessels were weighed every 24 hours throughout the course of fermentation to observe weight loss over time. Weight loss corresponds to

the conversion of carbohydrates into CO_2, and as such can be used to give an indication of the rate of fermentation progression (Ayrapaa 1971). At the end of fermentation, yeast viability was determined by methylene blue staining (Section 2.4.1) and beer was analysed for final gravity and alcohol (Section 2.6.3).

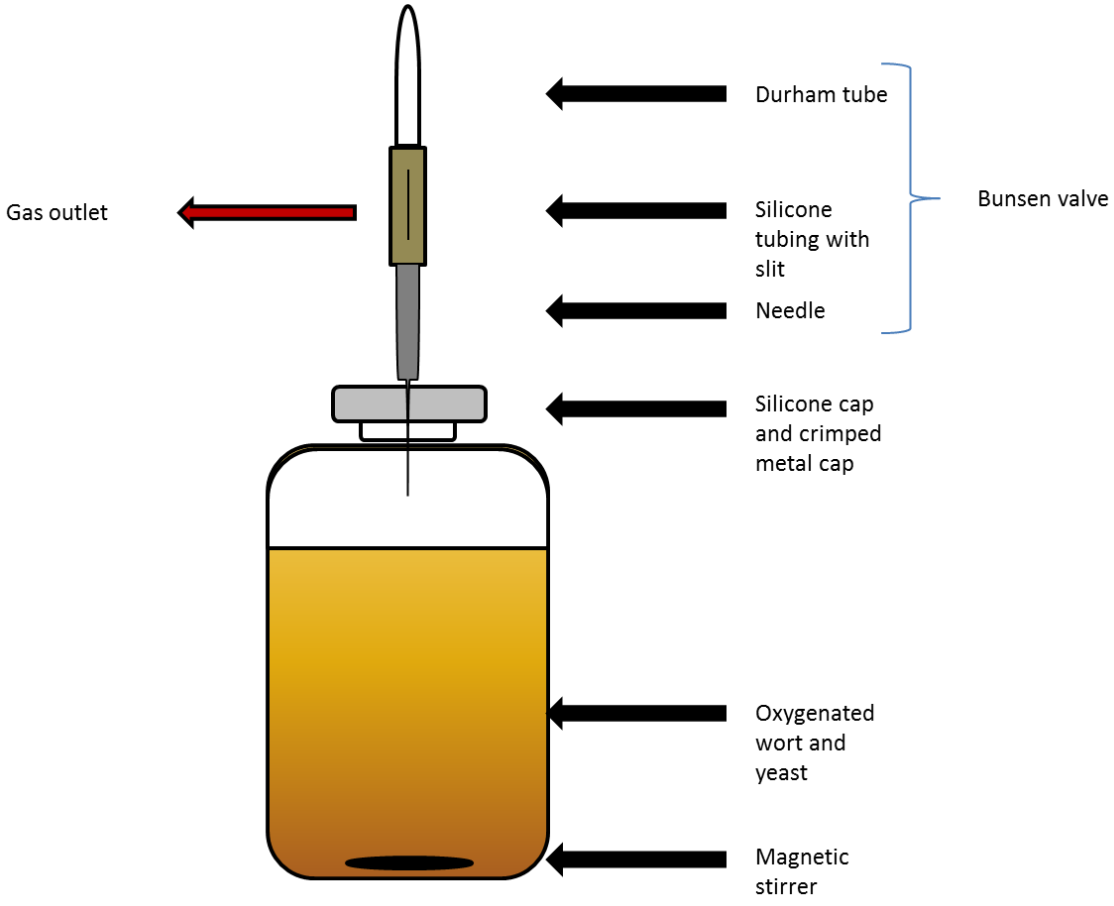


Figure 2.1 Small scale (100mL) fermenter design.

2.9 Small scale fermentations (10L)

Fermentations were conducted using *in situ* sterilisable, 12L stainless steel fermenters (Techfors-S, Infors-HT, Switzerland) with a working volume of 10 L. Fermenters comprised a cylindrical body of 200mm diameter and 505mm depth with a dish bottom. A central rotor from the top plate, equipped with impellers was used for agitation at a standard rate of 250rpm. Each vessel was oxygenated (Section 2.8.1) using a ring sparger located 30mm above the bottom. The control unit was utilised to monitor and control, pH, temperature, dissolved oxygen (DO) and stirring rate throughout fermentation.

All 10 L fermentations were conducted using temperature profiles based on standard SABMiller practice: fermentations were initiated at a temperature of 10°C, allowed to ramp up to 13°C for the first 96 hours at a rate of 0.13°C/hour and subsequently maintained at 13°C for the remainder of the fermentation. Oxygenation and pitching rate were adjusted to 1ppm and $1x10^6$ cells/ml per degree plato as described previously (Section 2.7.2). Samples were removed at regular intervals by withdrawing wort and yeast through a collection port at the bottom of the fermenter and immediately cooling on ice. Yeasts cells were separated from fermenting wort by centrifugation at 3000rpm for 10 minutes and stored at -20°C. The supernatant was also stored at -20°C until required for further analysis.

2.9.1 **Fermentation analysis**

2.9.1.1 Analysis of fermentation sample gravity and alcohol content

An Alcolyzer-plus: beer system with incorporated DMA 4500 density meter (Anton Paar, UK) was used to measure the gravity and alcohol content of wort and beer samples simultaneously. Fermentation samples were collected by removing yeast and other particulate matter by centrifugation at 3000rpm at 4°C for 10 minutes. Aliquots (25mL) of the filtered samples were passed through the sampling inlet. Approximately half of this volume was passed through without measurement in order to clean the glass U-tube inside the density meter, the second half was passed slowly to avoid introducing any air bubbles and the temperature of the sample was allowed to equilibrate to 20°C before measurements were recorded.

Gravity was determined by automated measurement of the oscillation of a glass U-tube, where more dense worts create a higher oscillation. The density was measured in specific gravity and was converted to degrees Plato using a conversion table formed by the polynomial equation in Equation 2.3 (Hough *et al* 1971). Alcohol by volume (ABV) was determined based on near infrared (NIR) absorption in the 1150 to 1200nm wavelength range.

$$Specific\ Gravity = \left\{ \frac{°Plato}{258.6 - \left(\left(\frac{°Plato}{258.2} \right) \times 227.1 \right)} \right\} + 1$$

Equation 2.3 Polynomial equation specifying the conversion of specific gravity to °Plato.

2.9.2 **Yeast oxidative stress analysis**

2.9.2.1 Cellular accumulation of reactive oxygen species

All reagents were obtained from Sigma Aldrich (UK) unless stated. Yeast populations comprising 1 x 10^7 cells/mL, derived from samples taken from fermentation vessels (Section 2.9), were washed in pH7.4 PBS (Fisher Scientific, UK) and incubated with 5µl of dihydroethidium (DHE) (10mg mL^{-1} in dimethyl sulphoxide (DMSO), at room temperature in the dark for 10minutes. DHE reacts with reactive oxygen species (ROS) present in the cytosol and is converted to ethidium which subsequently binds to cellular DNA (Figure 2.2). Bound DNA was visualised by fluorescent light emission at 605nm and observed using a FLUOstar OPTIMA fluorescence plate reader (BMGLabtech, Germany). A reference data set of 1x10^7 yeast were incubated in the presence of 10mM H_2O_2 for 1hr to cause maximum oxidative stress. These cells were then used a baseline for the ratiometric quantification, with a value of 1 indicating 100% ROS accumulation and 0 as no stress. Each sample set was also normalised to values obtained from 1x10^7 cells.

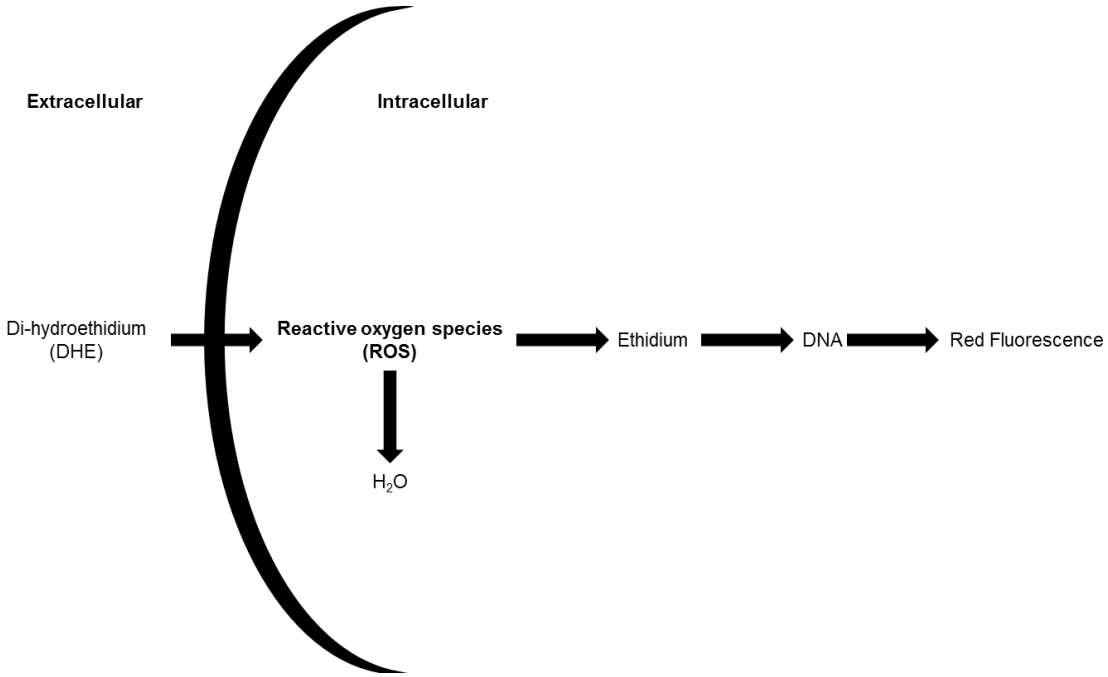


Figure 2.2 Analysis of reactive oxygen species (ROS) accumulation within yeast cells as determined by di-hydroethidium (DHE) staining. DHE is oxidised by ROS within the cell, releasing water and ethidium. The ethidium then binds with nuclear DNA and fluoresces red. The extent of red colouration is proportional to the amount of ROS present within yeast cells.

2.9.2.2 Analysis of Intracellular enzymatic anti-oxidants

2.9.2.2.1 Cell lysate extraction

Yeast populations comprising 1 x 10^7 cell/mL, taken from fermentation vessels (Section 2.9), were washed in PBS pH7.4 (Fisher Scientific, UK) and re-suspended in 0.5Mm Tris EDTA buffer. 300 µL of cell suspension was added to a 2 mL screw cap tube along with 100 µL of glass beads (Fisher Scientific, UK). Cells were lysed using a MagNAlyser (Roche, UK) by applying 6 x 60 second shakes to break open cell walls. The supernatant was separated from particulate matter by centrifugation at

13,000rpm using an Eppendorf cooling centrifuge (Eppendorf, Germany) at 4°C, transferred to a fresh tube, and stored at -80°C until needed.

2.9.2.2.2 Intracellular superoxide dismutase analysis

Cell lysates from fermentation samples were prepared as above (Section 2.9.2.2.1). The presence of superoxide dismutase (SOD) was determined using a SOD determination kit (19160-1KT-F, Sigma Aldrich, UK) based on the method of Lepock *et al* (1985). This method involves a coupled enzymatic reaction: in the presence of hydrogen peroxide, xanthine is reduced to water and uric acid. If SOD is present, the rate at which xanthine is reduced is lowered. This reaction is mirrored by a water soluble formazan dye which is released from WST-1-Formazan (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) upon reduction by hydrogen peroxide and manifests itself as a yellow colour (Figure 2.3). The presence of formazan dye was determined spectrophotometrically using a TECAN Infinite 200 Pro microplate reader (TECAN, Switzerland) at an absorbance wavelength of 450nm. Data was calculated using Equation 2.4, and SOD activity determined by comparison to a standard curve generated using Microsoft Excel 2010 (Figure 2.4). Assays were performed in triplicate and SOD concentrations expressed as Units equivalent SOD per 10^7 cells.

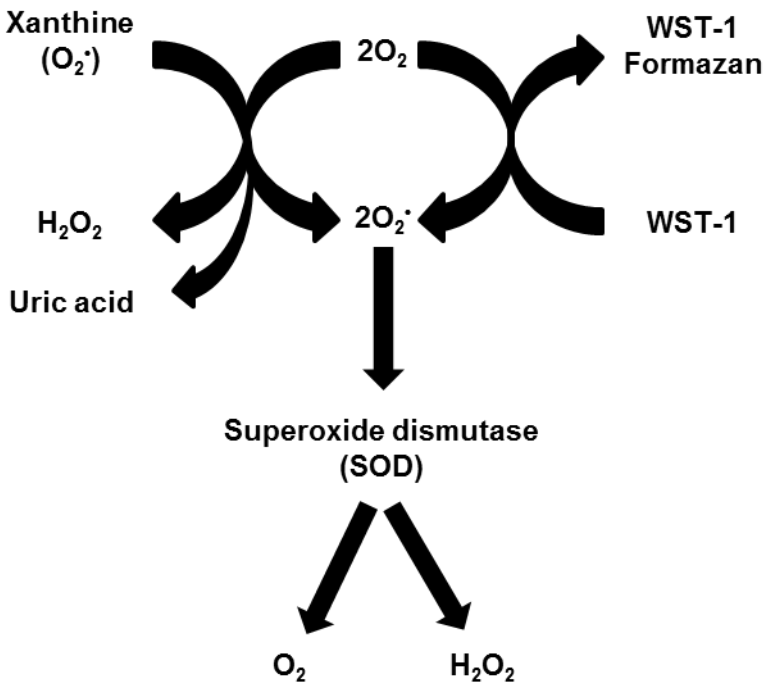


Figure 2.3 Analysis of SOD activity within yeast cells as determined by WST-1 formazan reduction. In the presence of hydrogen peroxide xanthine is reduced to water and uric acid. The presence of SOD reduces the rate at which xanthine is lowered. This reaction is mirrored by a water soluble formazan dye which is released from WST-1-Formazan (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) upon reduction by hydrogen peroxide and manifests itself as a yellow colour. The speed of colouration is proportional to the occurrence of SOD activity inside yeast cells. (Figure adapted from Sigma Aldrich, UK)

$$SOD\ activity\ (inhibition\ rate\ \%)$$

$$= \left\{ \frac{\left[(A_{blank1-}\ A_{blank3}) - (A_{sample-}\ A_{blank2}) \right]}{(A_{blank1-}\ A_{blank3})} \right\} \times 100$$

Equation 2.4 Equation calculating the % inhibition rate of SOD from a SOD determination kit (19160-1KT-F, Sigma Aldrich, UK).

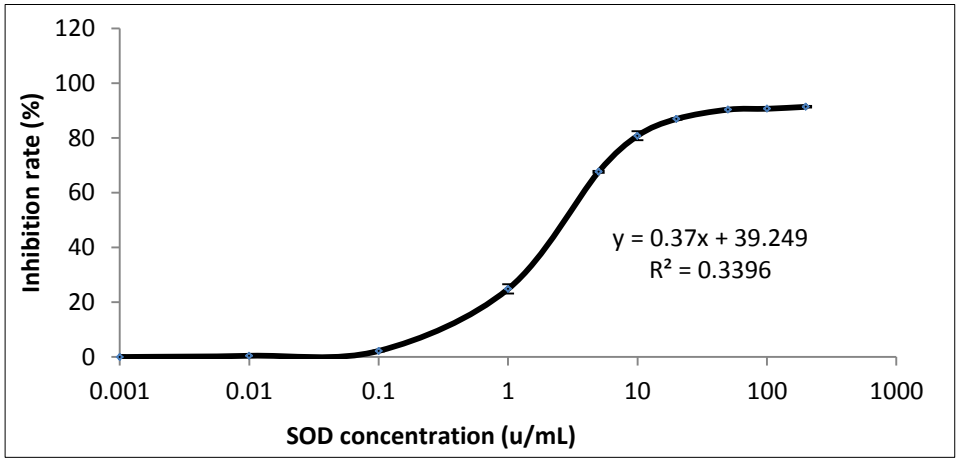


Figure 2.4 SOD inhibition standard curve. Data reflects the mean of 3 replicates ± the standard deviation.

2.9.2.2.3 Intracellular catalase analysis

Intracellular catalase was analysed using the Amplex® Red Catalase Assay Kit (A22180, Invitrogen, UK) based on the method of Mohanty *et al* (2002). This method involves a coupled enzymatic reaction: in the presence of catalase hydrogen peroxide is reduced to water and oxygen. Unreacted hydrogen peroxide reacts with the Amplex Red reagent with a

1:1 stoichometry in the presence of horseradish peroxidase (HRP) to produce resorufin, a fluorescent product. The resorufin signal is therefore inversely related to the amount of catalase present inside cells (Figure 2.5). The fluorescence of resorufin was measured using a TECAN Infinite 200 Pro microplate reader (TECAN, Switzerland) at an excitation of 540nm and an emission of 585nm. The concentration of catalase was determined by subtracting sample values from that of a negative (no-catalase) control and applying this figure to a standard curve prepared from known catalase concentrations provided with the kit. Assays were performed in triplicate and catalase concentrations expressed as units equivalent catalase per 10^7 cells/mL (Figure 2.6).

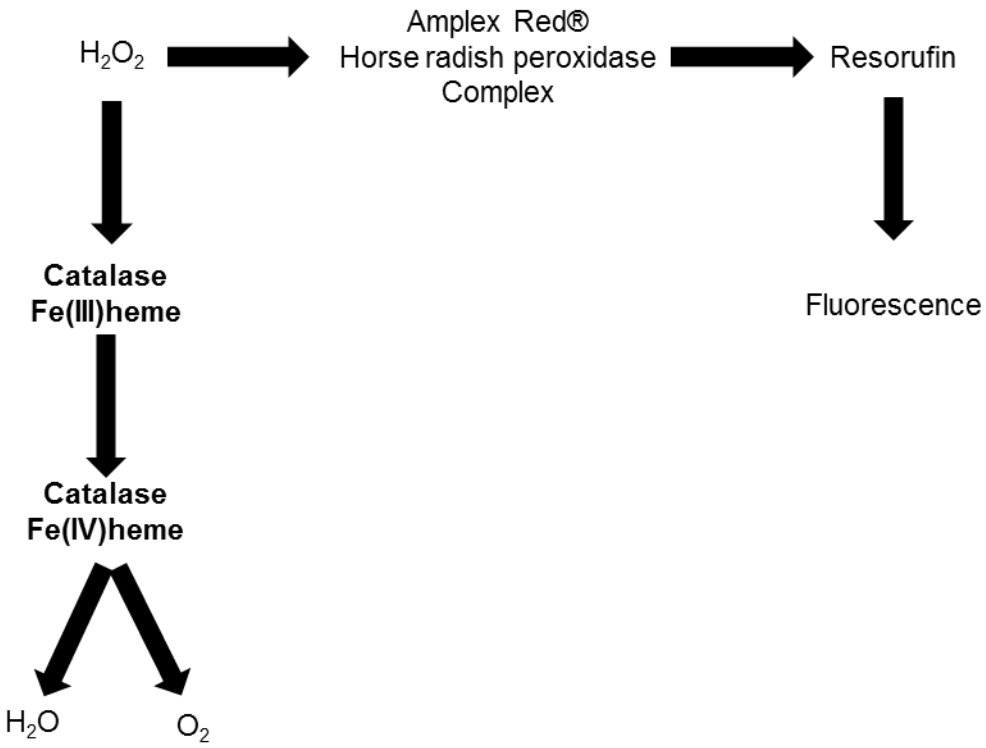


Figure 2.5 Analysis of available catalase levels within yeast cells as determined by the release of resorufin. The enzymatic reaction of catalase in the presence of hydrogen peroxide (H_2O_2) is reduced to water and oxygen. Unreacted H_2O_2 reacts with the Amplex Red reagent with a 1:1 stoichometry in the presence of horseradish peroxidase to produce resorufin, a fluorescent product. The level of fluorescence is proportional to the occurrence of available catalase inside yeast cells. (Figure adapted from Invitrogen, UK).

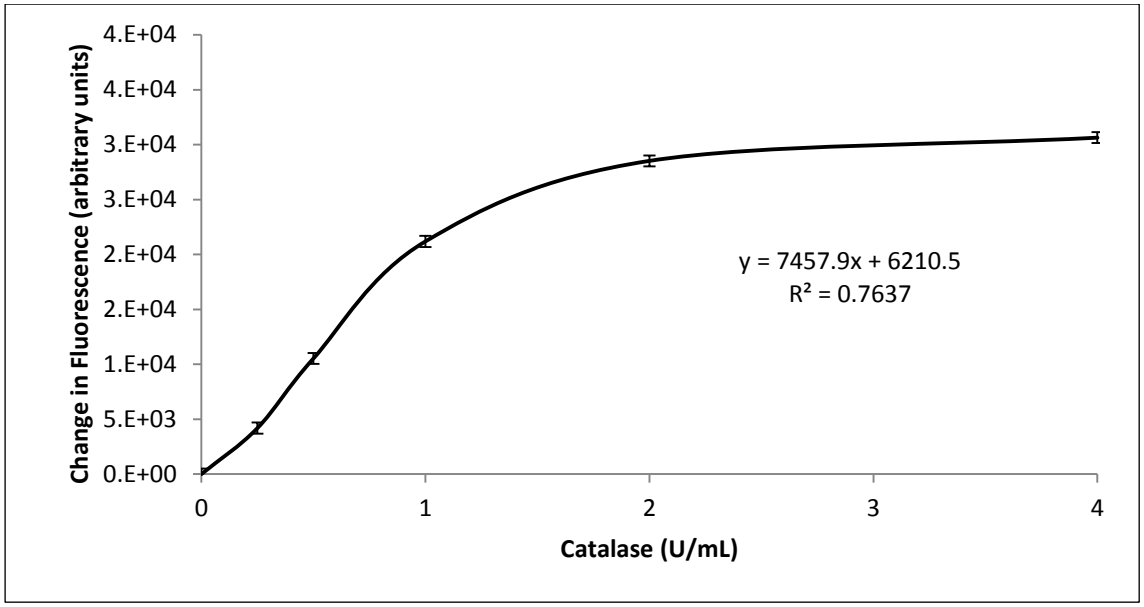


Figure 2.6 Catalase standard curve, data reflects the mean of 3 replicates ± the standard deviation.

2.9.2.2.4 Analysis of Intracellular Glutathione Peroxidase

The concentration of intracellular glutathione peroxidase (GPx) was analysed using a glutathione peroxidase assay kit (Abnova,UK), which indirectly measure GPx activity through a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG) is produced upon reduction of hydroperoxide (in this instance cumene hydroperoxide) by GPx, which is then recycled to its reduced state by GR and NADPH (Equations 2.5 and 2.6).

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340nm, which was measured using a TECAN automated micro-plate reader (Infinite@ 200 PRO series, TECAN, UK), Under conditions in which the GPx activity is rate limiting, the rate of the decrease in the A_{340} is related to the GPx activity of the sample (Figure 2.7). 10 µL of cell lysate ($1x10^7$ cells/mL) was added to a 96 well plate containing 90

µL of working buffer, (assay buffer, glutathione, 35mM NADPH, Glutathione Reductase enzyme). 100 µL of cumene hydroperoxide solution was added to each well and absorbance (340nm) monitored over 10 minutes. The concentration of GPx present in each of the cell lysate sample was quantified based on a standard curve prepared from known GPx standards (Figure 2.8). Assays were performed in triplicate and glutathione activity expressed as units equivalent GSH-GSSG conversion per minute per 10^7 cells

$$R - O - O - H + 2GSH \rightarrow (GPx) \rightarrow R - O - H + GSSG + H_2O$$

Equation 2.5 Oxidative action of glutathione peroxidase on reduced glutathione in the presence of hydroperoxide.

$$GSSG + NADPH + H \rightarrow GR \rightarrow 2GSH + NADP$$

Equation 2.6 Reductive action of glutathione reductase on oxidized glutathione in the presence of NADPH.

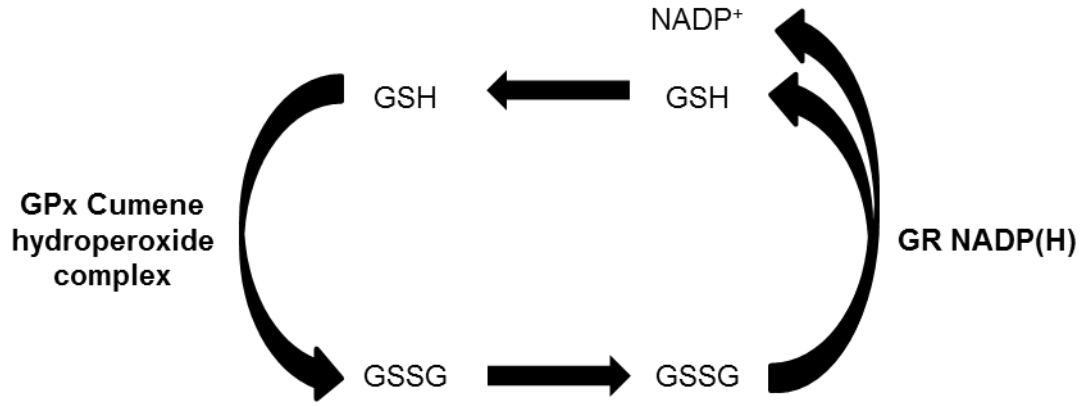


Figure 2.7 Assay principle for the analysis of glutathione peroxidase (GPx) activity. GPx activity is monitored through a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG) is produced upon reduction of cumene hydroperoxide by GPx, which is then recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340nm. The level of absorbance is linked to the level of glutathione peroxidase activity inside yeast cells. (Figure adapted from Abnova, UK)

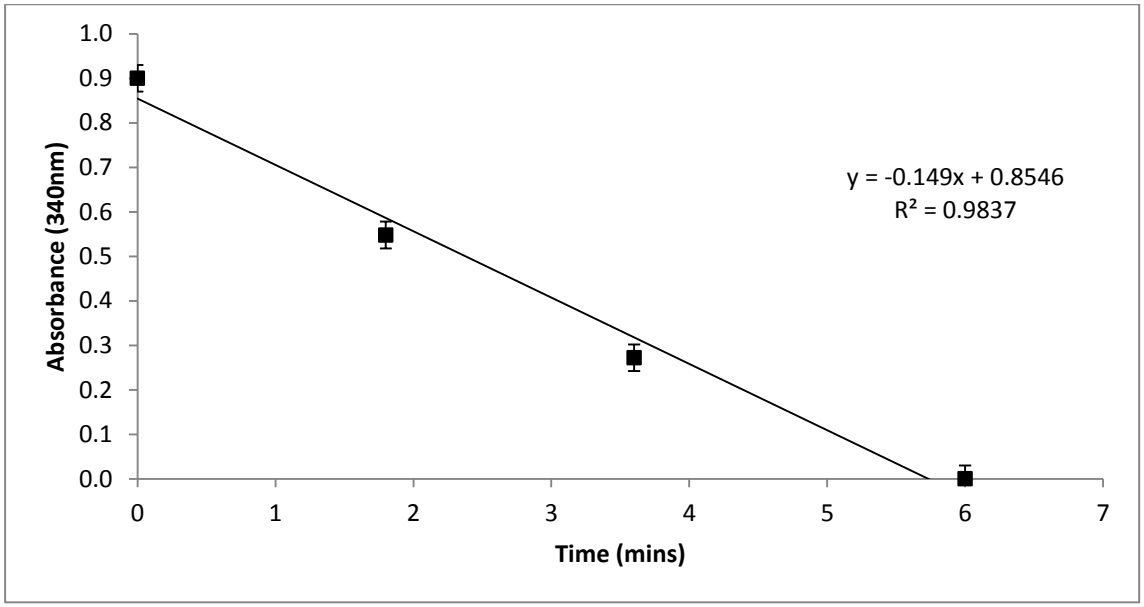


Figure 2.8 Glutathione peroxidase standard curve. Data reflects the mean of 3 replicates ± the standard deviation.

2.10 Analysis of oxidative stress and cell health

A single yeast colony from a YPD agar plate was transferred to 10 mL of YPD liquid broth and cultivated at 25°C for 48 hours. After this the culture was transferred firstly into 90 mL of YPD broth for 96 hours and then 900 mL of YPD broth for a further 2 days, all at 25°C continuously agitated at 120rpm. The cells were then centrifuged at 4000rpm for 10 minutes and the supernatant was discarded. Cells were resuspended in PBS pH7.4 (Fisher Scientific, UK) and enumerated as described previously (Section 2.4.1). 1x 10^7 cells/mL were transferred to a series of 50 mL conical flasks containing 10 mL of either 0 (water), 1, 5 or 10mM H_2O_2 to induce oxidative stress. Cells were then incubated at 25°C for 1 hour, shaking at 120rpm. After incubation cells were removed and collected by centrifugation for 5

minutes at 4000rpm. Cells were washed 3 times in PBS pH7.4 (Fisher Scientific, UK) and assessed for viability (Sections 2.4.1) before being evaluated for membrane damage and membrane fluidity (Section 2.10.1).

2.10.1 **Analysis of yeast viability and cell membrane damage by fluorescent staining**

Cells were analysed for membrane damage using a modified version of the protocol described by Simonin *et al* (2007). Propidium iodide (PI) was used to assess membrane integrity and cell viability due to its capacity to enter permeabilized cells and stain DNA structures (Deere *et al* 1998; Yamaguchi *et al* 1994; Schupp and Erlandsen 1987). Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (Bis-oxonol) was used to evaluate changes in membrane potential since this molecule can enter de-polarized cells and bind to intracellular proteins or membranes, exhibiting enhanced fluorescence and a red spectral shift (Simonin *et al* 2007; Attfield et al 2000; Epps *et al* 1994).

Stock solutions of the fluorescent stains were prepared at 200 µg/mL for propidium iodide (2 mg PI (Molecular probes, UK) in 10 mL of sterile distilled water), and 1 mg/mL for the Bis-oxonol (10mg Bis-oxonol (Sigma Aldrich, UK) in 10 mL of dimethyl sulfoxide (Sigma Aldrich, UK)). A 1 mL cell suspension containing $1x10^{10}$ cells/mL was labelled with 10 µL of propidium iodide and 3 µL of bis-oxonol, with the addition of 10 µL 4mM EDTA (148.9 mg of EDTA (Sigma Aldrich, UK) dissolved in 100 mL of sterile distilled water and pH to 7.0 with NaOH (Fisher Scientific, UK)) to facilitate the Bis-oxonol staining. Staining was conducted by incubating samples at room temperature in a dark environment for 10 minutes. The

stained suspension was then observed using a Beckman Coulter flow cytometer (Beckman Coulter, US), utilising a 15 mW argon laser to excite PI and Bis-oxonol. Discriminators were employed to reduce electronic background noise, and set to forward (FS) and side scatter (SS). The optical filters were set so that the PI fluorescence was measured at 610nm and the Bis-oxonol was measured at 525nm. In all cases there was an element of spectral overlap and the emitted fluorescence of the labels. In order to compensate for this the system compensation system was set to eliminate interference. Populations of unstressed high viability (>95%) 'live' and cells which had undergone 1hr heat shock at 45°C low viability (<1%) 'dead' cells were analysed as positive (Figure 2.9) and negative (Figure 2.10) controls to set compensation levels. Typical signals from $1x10^4$ cells were acquired and analysed using Weasel software version 3.0.2 (Walter and Elizabeth Hall Institute of medical research (WEHI), Australia).

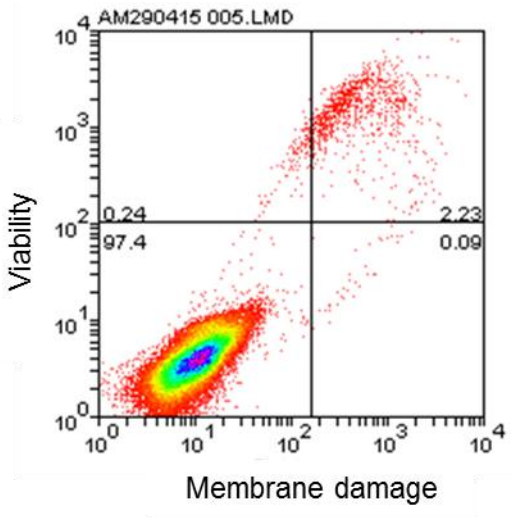


Figure 2.9 Live yeast cells dual stained with propidium iodide, indicating cell viability, and bis-oxonol, indicating membrane damage. Data points represent relative fluorescent units generated from analysis of $1x10^4$ cells. Lower relative fluorescent units indicate high levels of viability and low amounts of membrane damage (lower left quadrant).

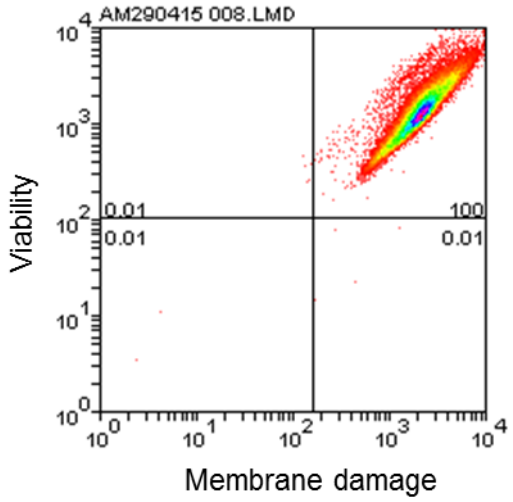


Figure 2.10 Dead yeast cells dual stained with propidium iodide, indicating cell viability, and bis-oxonol, indicating membrane damage. Data points represent relative fluorescent units generated from analysis of $1x10^4$ cells. Higher relative fluorescent units indicate low viability and high membrane damage (upper left quadrant).

2.10.2 **Analysis of yeast membrane fluidity**

Yeast membrane fluidity was determined by staining cells with the fluorescent probe laurdan (6-lauroyl-2-dimethylamino napthalene) according to previously published methodology (Learmonth and Gratton, 2002). Laurdan is known to be sensitive to environmental polarity and localises in the hydrophilic-hydrophobic interface of the cell membrane (Parasassi *et al* 1990; Chong and Kao 1990; Weber and Farris 1979). Whilst interacting with the liquid-crystaline and gel phases of the plasma membrane, laurdan emits differing maxima and emission spectra due to the differences in polarity (Parasassi and Gratton 1995). The generalized polarization (GP) of the membrane can be indicated by the amount of laurdan-generated fluorescence and so can be used as an indicator of the fluidity of the membrane.

Cells were washed twice in PBS pH 7.4 (Fisher Scientific, UK), diluted to an OD of 0.1 at 600nm (equating to a cell number of ~$1x10^6$ cells/mL) using a Genesys 10S vis spectrophotometer (Fisher Scientific, UK). The cell suspensions were then stained with 10 µL of laurdan solution (5mg of laurdan (Invitrogen, USA) in 2.83 mL of absolute ethanol (100% v/v; Fisher scientific, UK) at a final concentration of 5 µM. Cells were then incubated at room temperature in the dark for 1hour, after which 200 µL was aliquoted into a 96 well plate (Nunc, USA) for analysis.

Membrane fluidity was determined by measuring laurdan fluorescence at an excitation of 350nm, with 2 emission wavelengths of 440 and 490nm corresponding to the gel and liquid crystalline phases. A Varioskan Flash microplate reader (Thermo Fisher Scientific, UK) was used to determine

fluorescence at each wavelength, and the resulting data was used to calculate the GP value (Equation 2.7). The GP value was calculated as an index of membrane fluidity where the GP values range between -1 and +1, and are inversely related to membrane fluidity where a high GP indicates low membrane fluidity and *vice versa* (Learmonth and Gratton 2002; Parasassi *et al* 1990).

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

Equation 2.7 Calculation of GP value as an index of membrane fluidity, where I_{440} and I_{490} indicate relative fluorescence intensities at wavelengths representing gel (440nm) and liquid crystalline (490nm) phases of bilayer systems.

2.11 Yeast sterol analysis

A sample of SMCC100 yeast cropped from a VHG fermentation at 20°P (second generation yeast) was obtained from the SABMiller research brewery. The yeast slurry was transferred to a 10 L vessel and sparged with oxygen at 5 L/min for 6 hours at 4°C. Yeast was removed after each hour and yeast sterol content was analysed.

2.11.1 **Sterol extraction**

Sterol extractions were performed using a modified version of the sterol extraction method presented by Quail and Kelly (1996). Samples containing $1x10^7$ cells/mL were washed in PBS and a 1 mL aliquot was

centrifuged at 4000rpm for 5 minutes to harvest the cells. The cells were then transferred to a 20 mL Universal glass bottle with a screwcap lid (Fisher Scientific, UK). 3 mL of 100% v/v methanol (Fisher Scientific, UK) was added to the bottle, along with 2 mL of 0.5% v/v pyrogallol (100µL of pyrogallol (Sigma Aldrich, UK) in 20 mL of sterile distilled water), and 2 mL of 60% potassium hydroxide w/v (30g of potassium hydroxide (Sigma Aldrich, UK) dissolved in 50 mL sterile distilled water. The sample was gently vortexed to mix and incubated at 90°C for 2 hours in a Grant JB NOVA water bath (Grant Instruments, UK). Samples were then removed from the water bath and 5 mL hexane (Sigma Aldrich, UK) was added before centrifuging at 4000rpm for 5 minutes. The hexane layer, containing the dissolved sterol fraction, was then removed to a fresh glass Universal bottle and the hexane layer was evaporated by flushing the bottle nitrogen, leaving the sterol content within the bottle. An additional 5mL hexane was then added to the original extraction solution to remove any additional sterols present, and again transferred to the Universal bottle containing the extracted sterols. The hexane was again evaporated by flushing the bottle with nitrogen, leaving the extracted sterols behind. This method of hexane extractions of the sterol fractions were repeated 2 further times to ensure complete extraction of all the sterol fraction. Samples were then stored at -20°C until required for processing.

2.11.2 **Sterol analysis using gas-chromatography**

Sterol samples (2.11.1) were re-dissolved in 50 µL of 99.8% pure pyridine (Sigma Aldrich, UK). Once dissolved, 50 µL of N'O'-bis-(trimethylsilyl)-trifluoroacetamide ≤99.0% GC derivative (Sigma Aldrich, UK) was added and mixed by pipetting.

Samples were analysed using a Perkin Elmer Clarus 500 gas chromatography system (Perkin Elmer, UK). The column was a 30M ZB5 phenomenex column (Phenomenex, US), which was equilibrated to 260°C with the injector and detector being equilibrated to 300°C. The split ratio was set to 1:30 and hydrogen was used as the carrier gas, at 4mL/min. 1 µL of each prepared sample was then run for 40 minutes as described above. Ergosterol content (mg/gDCW) of triplicate samples was analysed and interpreted using Clarus 400 GC software (Perkin Elmer, UK) and Microsoft office Excel 10 (Microsoft, US). An internal standard of 50 µg of cholesterol was used to evaluate extraction efficiency.

2.12 Statistical analysis

All data sets were performed with a minimum of 3 biological replicates, with the mean and standard deviation of each data set calculated using Excel (Microsoft, USA). Statistical analysis was performed using SPSS version 20.0 for windows (IBM, USA). Data was subjected to paired sample T-tests to determine the significant difference between samples. The null hypothesis was that there was no significant difference between data sets, and differences were considered significant at $P \leq 0.05$. In this instance if the P value was less than 0.05, the null hypothesis of no significant difference was rejected. Specific statistical tests applied to raw data are indicated in the relevant sections.

CHAPTER 3: CHARACTERISATION OF LAGER BREWING YEAST

3 Characterisation of lager brewing yeast strains

3.1 Introduction

Yeast belonging to the genus *Saccharomyces* play an important role in brewing fermentations as they are responsible for the production of alcohol, carbon dioxide and a range of flavour compounds (Piškur *et al* 2006; Lodolo *et al* 2008; Pires *et al* 2014). It is possible to separate brewing yeast into two main groups depending on their final product: ale strains, belong to the species *Saccharomyces cerevisiae*; and lager yeast, which are classified as *Saccharomyces pastorianus* (Pedersen 1986; Vaughan-Martini & Martini 1998; Smart 2007). Lager strains have been shown to exhibit lower permissive growth temperatures than those of ale strains, and as such this has been used historically to differentiate between lager and ale strains (Guidici *et al* 1998). However, more recently, genomic analyses have developed which provide an alternative means of distinguishing between S. *cerevisiae* and S. *pastorianus* species (Pedersen 1986; Smart 2007). Lager yeast have been shown to be derived from a hybridisation event between a S. *cerevisiae* and a *S. bayanus*-like parental strain (potentially *S. eubayanus*) (Dunn & Sherlock 2008; Libkind *et al* 2011). However, physiological and molecular differences between S. *pastorianus* strains indicate the occurrence of at least 2 hybridisation events. Lager strains can thus be separated into two groups: group I / Saaz and group II / Frohberg (named after the locations in Germany where they were originally used) (Lindner 1909; Gibson *et al* 2013). Differences between these groups of yeast are relatively minor, but significant when considering brewing fermentations. Alterations in phenotypic characteristics include variations in growth preference, with Saaz yeast being better adapted for growth at lower temperatures (~10°C), and

Frohberg strains able to ferment better at a higher temperature (~22°C) (Gibson *et al* 2013). Nutritional requirements and differences in sugar utilisation have also been highlighted, with Saaz strains relatively unable to utilise maltotriose compared to Frohberg yeasts (Gibson *et al* 2013). Differences in production of flavour compounds have also been observed, with Saaz strains typically producing lower levels of esters such as isoamyl acetate (Gibson *et al* 2013; Walther *et al* 2014; Wendland 2014).

In addition to group specific variation, there are also broader differences between strains belonging to the *S. pastorianus* species, including changes to flocculation properties and tolerances to a range of stress factors (Hiralal *et al* 2014; Zhao & Bai 2009; Saerens *et al* 2008; Mortimer 2000). During brewing fermentations, yeast is subjected to a range of environmental stress factors (Csonka & Hanson 1991). These include challenges such as ethanol toxicity (Laopaiboon *et al* 2009), osmotic stress (Csonka & Hanson 1991) and oxidative stress (Halliwell & Gutteridge 1999), as well as nutritional stress and cold shock generally associated with yeast handling (Quain 1995) (Section 1.5). Under high gravity conditions many of the stress factors associated with fermentation are likely to be exacerbated given that both the starting materials and end products are present in elevated concentrations. While the effect of ethanol and osmotic stress factors on yeast is well documented (Jamieson 1998; Toledano *et al* 2003; Zhao & Bai 2009), there have been no previous studies investigating the physiological impact of the increased metabolic demands on lager brewing yeast, and particularly the potential for elevated oxidative stress.

In this study, yeast strains were initially characterised to determine species and strain identity. Subsequently, in order to gain an insight into the physiological properties of each strain in response to a range of conditions,

their capacity to grow in a range of wort-based media was examined. Finally, menadione-based media, known to elicit an oxidative stress reaction (Jamieson 1992; Collinson & Dawes 1992; Flattery-O'Brien e*t al* 1993; Jamieson *et al* 1994 Martin *et al* 2000), was used to examine the effect of oxygen free radicals on yeast strain growth parameters.

3.2 Results

3.2.1 **Genomic analysis of brewing yeast strains**

Prior to analysis of the physiological properties of brewing yeast, each strain was classified to the species and strain level. This was performed by analysing the ITS region of the genome to determine species and genus (Guillamon *et al* 1998; Section 2.3.2), and by analysing the frequency and distribution of inter-delta sequences using PCR fingerprinting to differentiate strains (Legras & Karst 2003; Section 2.3.3).

The ITS region of the yeast genome is a component of the ribosomal DNA and is known to be highly conserved within yeast species, but variable between different yeasts (White *et al* 1990). To determine the species of each yeast strain, the ITS region was amplified and consequently cut using restriction enzymes to produce a series of banding patterns (Section 2.3.2). Comparison of the size of the amplified ITS regions, and the size and number of the derived restriction fragments indicated that all of the lager yeast strains produced identical DNA profiles, as expected. In each instance the PCR fragment was observed to be 880bp in length (Figure 3.1) and, by cutting the amplified DNA using the restriction enzyme *Hae*III, it was observed that a number of smaller fragments were produced of 320, 230, 180 and 150bp in size (Figure 3.2). Comparison of these DNA

patterns to previous analysis in the literature (Guillamon *et al* 1998; Esteve-Zarzoso *et al* 1999), indicated that each strain was specific to *S. cerevisiae* yeast, rather than *S. pastorianus* as would be expected for lager strains. However it should be noted that these authors do not make a clear distinction between *S. cerevisiae* and *S. pastorianus*. Previous work conducted in this laboratory (Powell, personal communication) and elsewhere (Gibson *et al* 2013) have indicated that these restriction profiles match Saaz-type *S. pastorianus* strains. To clarify this discrepancy, each strain was also cultivated on solid YPD media at a series of temperatures: 25°C, 30°C and 37°C. This was performed since it is known that *S. cerevisiae* (ale) strains are able to reproduce at temperatures above 34°C, while *S. pastorianus* (lager) strains cannot (Boulton & Quain 2001). Growth analysis on YPD medium indicated that each strain was able to grow at 25°C, but was not cultivable at 37°C (Figure 3.3). This data, in conjunction with that obtained from analysis of the ITS region, indicates that each strain can be considered to belong to the Saaz-type sub-group of the species *S. pastorianus*.

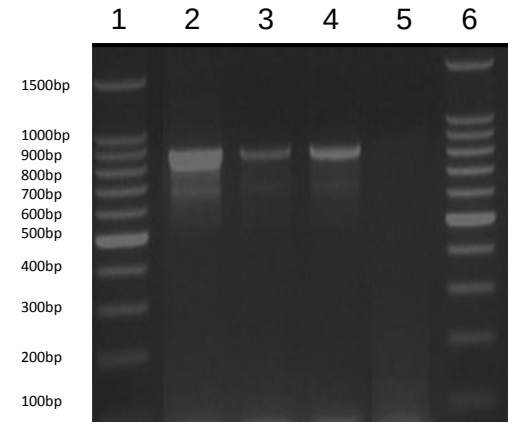


Figure 3.1 PCR amplification of the yeast ITS region. Strains SMCC100, SMCC90 and SMCC99 are shown in lanes 2-4 respectively. A blank sample is shown in Lane 5 and 100bp ladders can be seen in lanes 1 and 6.

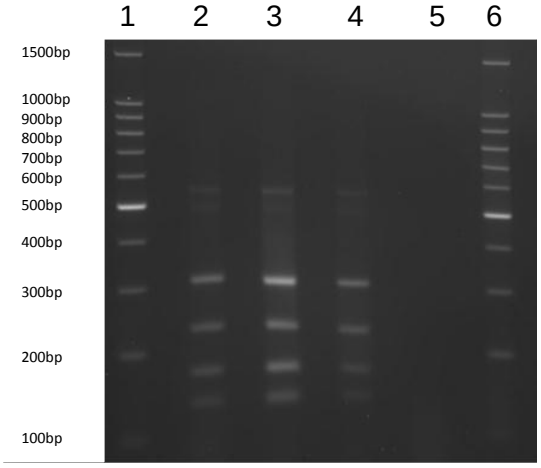


Figure 3.2 Restriction digest of ITS regions using the enzyme *Hae*III. Strains SMCC100, SMCC90 and SMCC99 are shown in lanes 2-4 respectively. A blank control sample is shown in lane 5. A 100bp ladder can be seen in lanes 1 and 6.

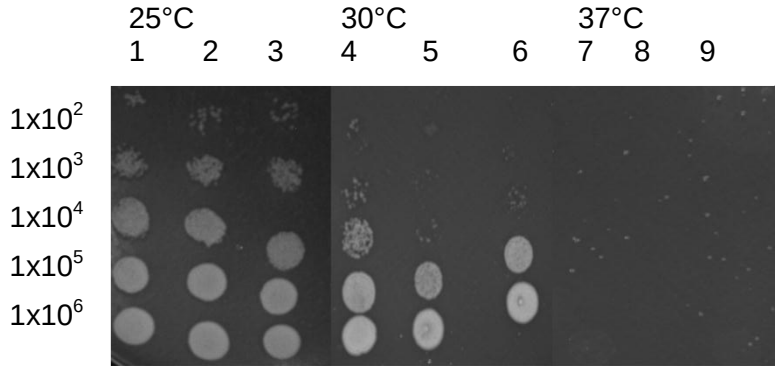


Figure 3.3 Determination of brewing yeast classification (lager/ale) based on temperature tolerance. Growth of yeast strains SMCC100 (lanes1,4,7), 90 (lanes 2,5,8) and 99 (3,6,9) was determined at 25°C , 30°C and 37°C to indicate permissive growth temperatures. Decreasing cell concentrations of 10^2-10^6 cells were spotted onto the surface of 15°P wort agar. No growth was observed at 37°C.

Once each strain had been classified to the species level, analysis of inter-delta sequences was performed to determine differences between strains (Legras & Karst 2003; Section 2.3.3). Inter-delta sequences are long terminal repeats that flank retrotransposons Ty1 and Ty2 within the yeast genome (Legras & Karst 2003) and can be used to differentiate *Saccharomyces* strains (Cameron *et al* 1979; Ness *et al* 1993; Legras & Karst 2003; Schuller *et al* 2004). This is because transposons are a major source of genetic variation, due to Ty-driven translocations, and as such are variable in terms of number and location between strains (Caretto 2008).

Analysis of the frequency and distribution of inter-delta sequences yielded PCR fingerprints which were similar but distinct for each strain (Figure 3.4).

It is known that lager yeast strains are closely related due to their recent genetic origins (Dunn & Sherlock 2008), and lager strains are notoriously difficult to differentiate (Van Zandyke *et al* 2007). Consequently these results were not unexpected. However, by analysis of specific amplicons it was possible to differentiate the 3 lager yeast strains (Figure 3.4). Strain SMCC100 could be differentiated by the absence of a band at 1250bp, while strain SMCC90 exhibited unique DNA fragments at 650 and 220bp. Strain SMCC99 was differentiated by the presence of amplicons of 400 and 200bp in size.

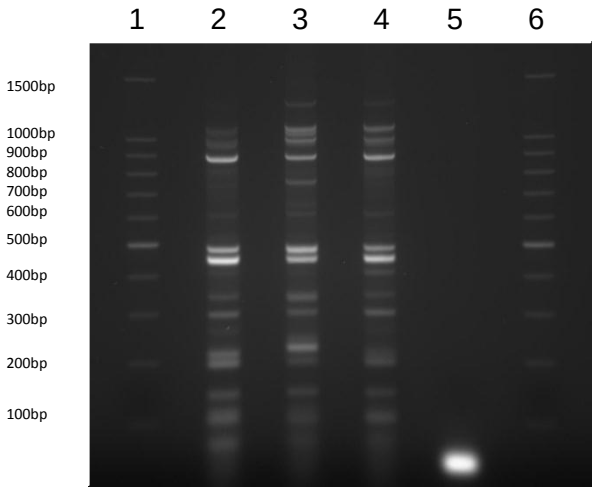


Figure 3.4 PCR analysis of yeast inter-delta regions. Strains SMCC100, SMCC90 and SMCC99 are shown in lanes 2-4 respectively. A blank (control) sample is shown in lane 5. A 100bp ladder can be seen in lanes 1 and 6.

3.2.2 **Analysis of lager brewing yeast mitochondrial DNA**

Although analysis of genomic DNA allowed strains to be differentiated, each yeast was also investigated for differences in mitochondrial DNA (MtDNA). Lee *et al* (1985) demonstrated that as MtDNA is less robust than genomic DNA, it is more prone to evolutionary changes (Brown 1981) and consequently can act as a more sensitive means of strain differentiation (Querol *et al* 1992). Previous analysis of brewing strains has shown that individuals can be differentiated based on the MtDNA restriction fragment length polymorphisms (RFLP) created using the enzymes HaeIII, HinfI and DdeI (Lee & Knudsen 1984; Aigle *et al* 1984; Vezinhet *et al* 1990; Su & Meyer 1991; Querol *et al* 1992; Guillamón *et al* 1994; Piškur *et al* 1998; Šoltésova *et al* 2000; Naumova *et al* 2010). Consequently, each strain was analysed using these enzymes to produce MtDNA fingerprints. Surprisingly it was observed that there were no detectable differences between strain mtDNA profiles when using either of the enzymes HinfI, HaeIII or DdeI (Figure 3.5). However, this may be due to mitchondrial inheritance patterns; Rainieri *et al* (2008) indicated that for *S.pastorianus*, the mtDNA is transferred from the non-*cerevisiae* strain. Hence as each of these three industrial brewing strains were previously identified as Frohberg strains (Section 3.2.1), they are likely to be derived from the same parental crossing, and share the same mtDNA.

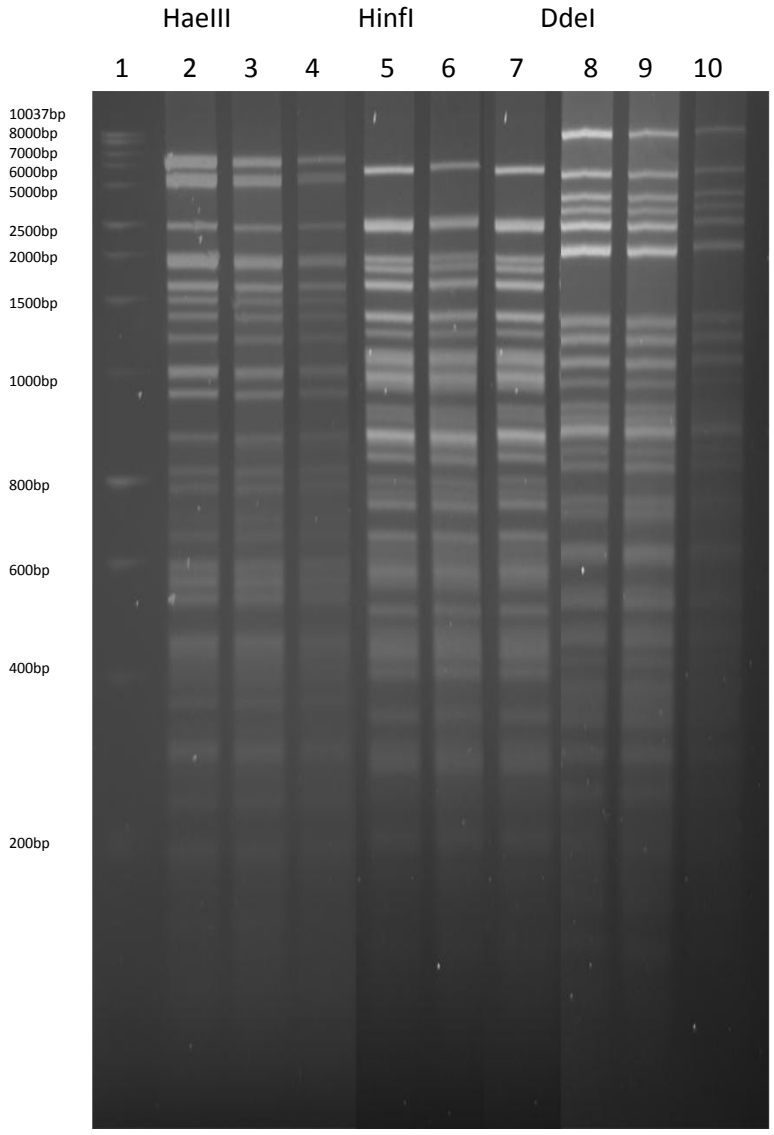


Figure 3.5 HaeIII, HinfI and DdeI RFLP of MtDNA extracted from samples of SMCC100 (lanes 2,5 and 8) SMCC90 (lanes 3,6 and 9) and SMCC99 (lanes 4, 7 and 10). HaeIII was represented in Lanes 2-4, HinfI was represented in lanes 5-7 and DdeI was represented in Lanes 8-10. A 1kb Hyperladder (Bioline, UK) can be seen in lane 1.

3.2.3 **Tolerance of lager yeast to high glucose concentrations**

Spot plate assays are a relatively simple method for screening yeast strains for tolerance to stress factors (Carrasco *et al* 2001; Miyazaki *et al* 2004; Serrano *et al* 2006; Lewis *et al* 2010) and other defined physiological conditions (Grosshans *et al* 2006; Avonce *et al* 2010). The assay relies on the capacity of a strain to grow and produce colonies on nutrient agar (Kumar & Snyder 2000). In order to determine the capacity of strains to tolerate conditions associated with elevated levels of fermentable sugars, each strain was cultivated by spot-plating onto wort agar comprising varying concentrations of glucose, from 15°P to 40°P. It can be seen from Figure 3.6 that although all strains had a high tolerance for increased glucose concentrations, with the same growth pattern being observed for all three strains up to 36°P, in each instance cell growth was diminished when glucose concentration was elevated above 22°P. SMCC99 appeared to have the highest tolerance for high glucose concentrations with growth observed at 36 and 38°P, in contrast to SMCC100 and SMCC90 which were not able to grow under such conditions. The most susceptible strain to increased glucose concentrations appeared to be SMCC90, where growth at 40°P was only observable with a starting inoculum of greater than $1x10^4$ cells/mL. At higher glucose concentrations, SMCC100 and SMCC99 appeared to have similar growth patterns, with both strains only exhibiting growth at inoculum rates of greater than $1x10^3$ cells/mL (Figure 3.6).

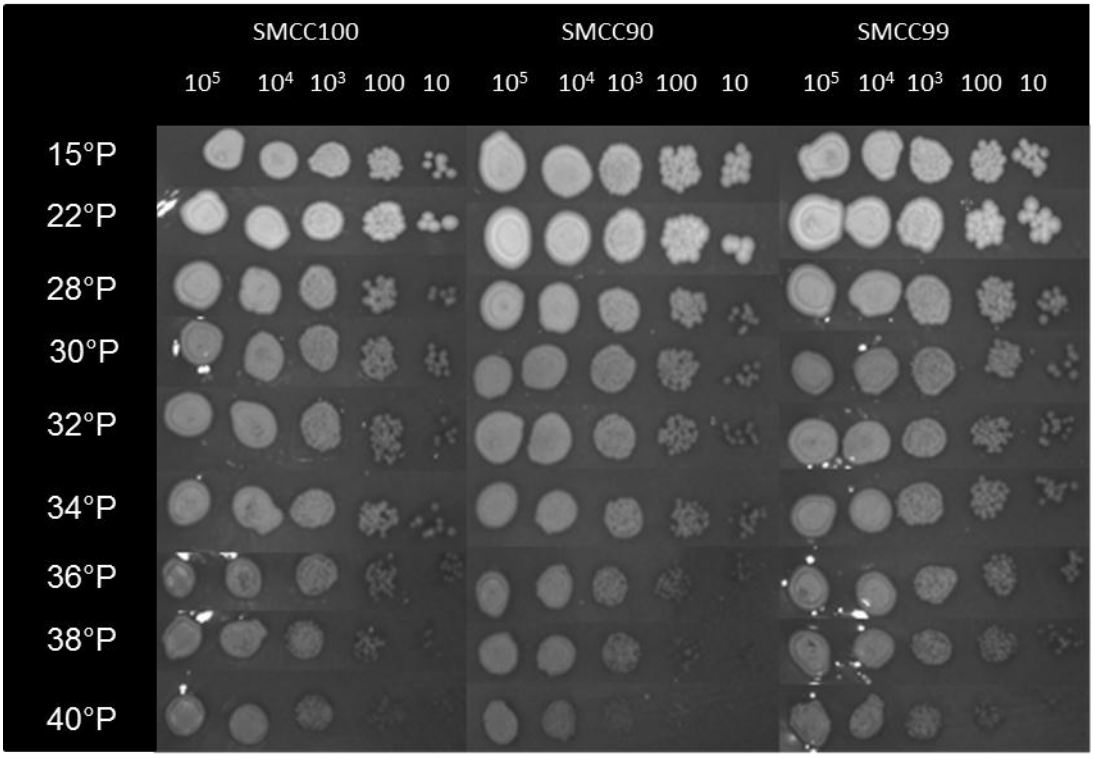


Figure 3.6 Growth of SMCC100, SMCC90 and SMCC99 on wort agar. A defined concentration of cells (10-1x10^5 cfu/mL) were spotted onto the surface of agar prepared at different gravities as indicated on the y axis (15-40°P).

Although trends can be observed via spot plate analysis, this method ultimately only provides limited information on the absolute growth limits of yeast under specific conditions. Consequently, a more comprehensive method of analysis was employed based on growth kinetics, where optical density was monitored over time to provide an indication of tolerance level under defined conditions via analysis of lag and exponential phases of growth in a 15°P wort supplemented with glucose at incremental amounts (Section 2.5.2.1). As indicated by monitoring absorbance at OD600, the growth rate of each strain was observed to decrease as wort gravity increased. This decrease occurred as a result of an extended lag phase,

and reduced growth rate during exponential phase leading to a reduction in final growth after 36 hours (Figures 3.7-3.9).

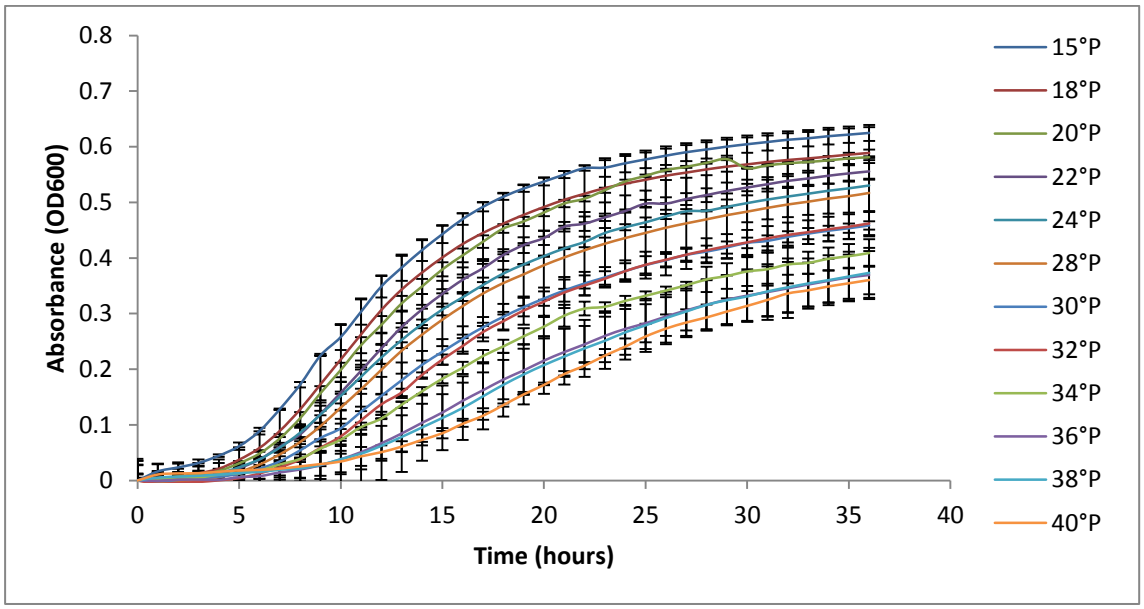


Figure 3.7 Growth of strain SMCC100 in wort, pitched at a cell concentration of $1x10^5$ cfu/mL into wort of various sugar concentrations (°P). Growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

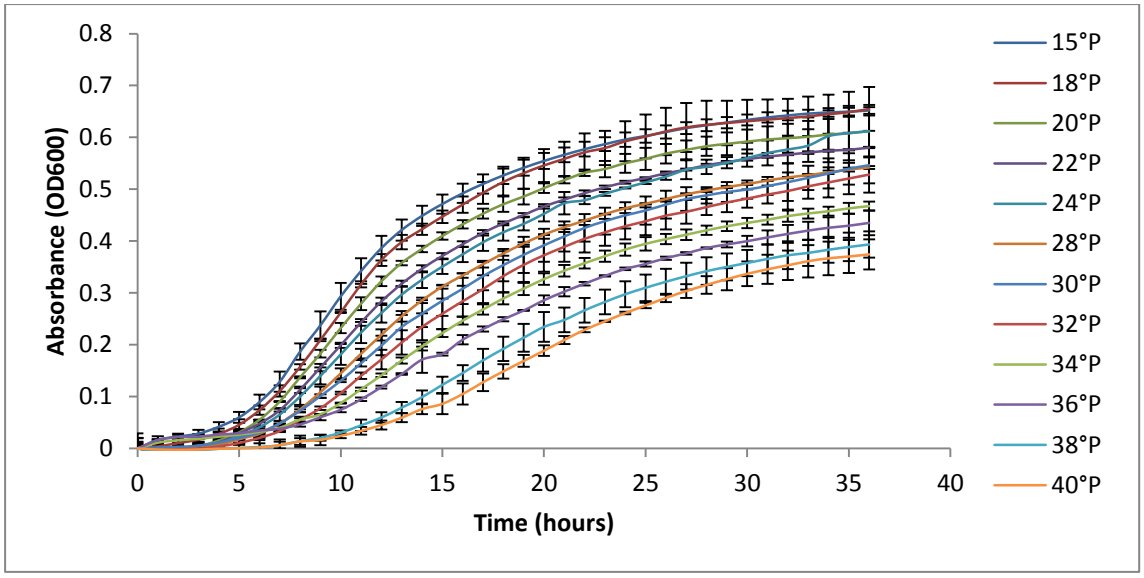


Figure 3.8 Growth of strain SMCC90 in wort, pitched at a cell concentration of $1x10^5$ cfu/mL into wort of various sugar concentrations (°P). Growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation

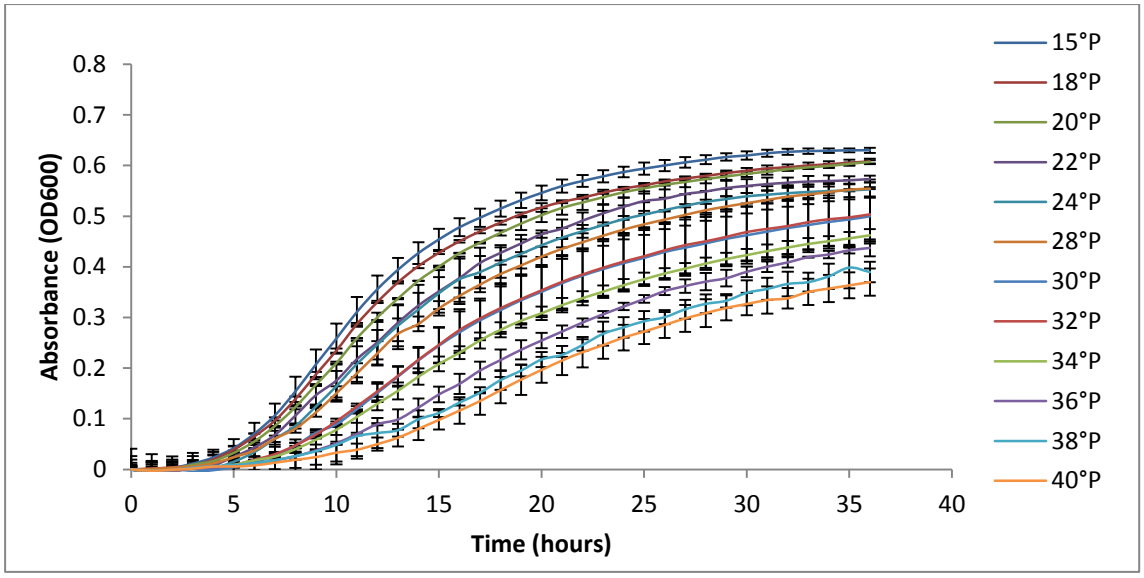


Figure 3.9 Growth of strain SMCC99 in wort, pitched at a cell concentration of $1x10^5$ cfu/mL into wort of various sugar concentrations (°P). Growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation

Growth curves were analysed using DMFit Software version 3.5 add-in for excel (Institute of food research (IFR), UK) (Koseki & Nonaka 2012; Section 2.5.2), to quantitatively analyse the length of each lag phase, as well as to determine the rates of the respective exponential phases (µmax). Based on this analysis, it can be seen that the length of the lag phase at 15°P (control) was 2.28 hours, 1.68 hours and 2.61 hours for SMCC100, SMCC90 and SMCC99 respectively. At 22°P this increased to 3.38 hours for SMCC100, 2.06 hours for SMCC90 and 3.05 hours for SMCC99. A similar trend was seen at 30°P (lag phase of 3.86 hours, 3.03 hours and 4.22 hours for SMCC100, SMCC90 and SMCC99 respectively) and at 40°P (lag phase of 10.56 hours for SMCC100, 9.81 hours for SMCC90 and

9.01 hours for SMCC99). When lag phase data was compared to wort gravity a positive correlation was observed (Figure 3.10). This was calculated with a fitness of >80% for all three strains (81%, 90%, 87% for SMCC 90, 99 and 100 respectively). It was also noted that although similar effects were observed between strains SMCC90 and SMCC99, the effect of wort gravity on lag phase was more pronounced for SMCC100, potentially indicating a greater overall impact (Figure 3.10). For this yeast, although lag phase was shorter than for the other strains at 15°P, at higher gravities there was little difference in lag phase duration.

The slope of the curve was also analysed during exponential phase to determine the rate of growth (μmax) (Section 2.5.2). This indicated a 51.4% decrease from μmax 0.035 to 0.017 for SMCC100 when comparing the slopes of the curves at 15°P to 40°P, with an R^2 value of 96%. SMCC90 showed less of a reduction in μmax with a 50% decrease from 0.036 at 15°P to 0.018 at 40°P with an R^2 value of 96%. SMCC99, at 55%, exhibited the lowest reduction in μmax from 0.038 at 15°P to 0.017 at 40°P with an R^2 value of 99%. Consequently it can be concluded that when exponential growth rate was related to wort gravity, a negative correlation was observed (Figure 3.11), indicating that as wort gravity increased the rate of exponential growth was reduced. It was also noted that although SMCC99 and SMCC90 showed similar patterns of growth, wort gravity was observed to have less of an impact on SMCC90 (Figure 3.11).

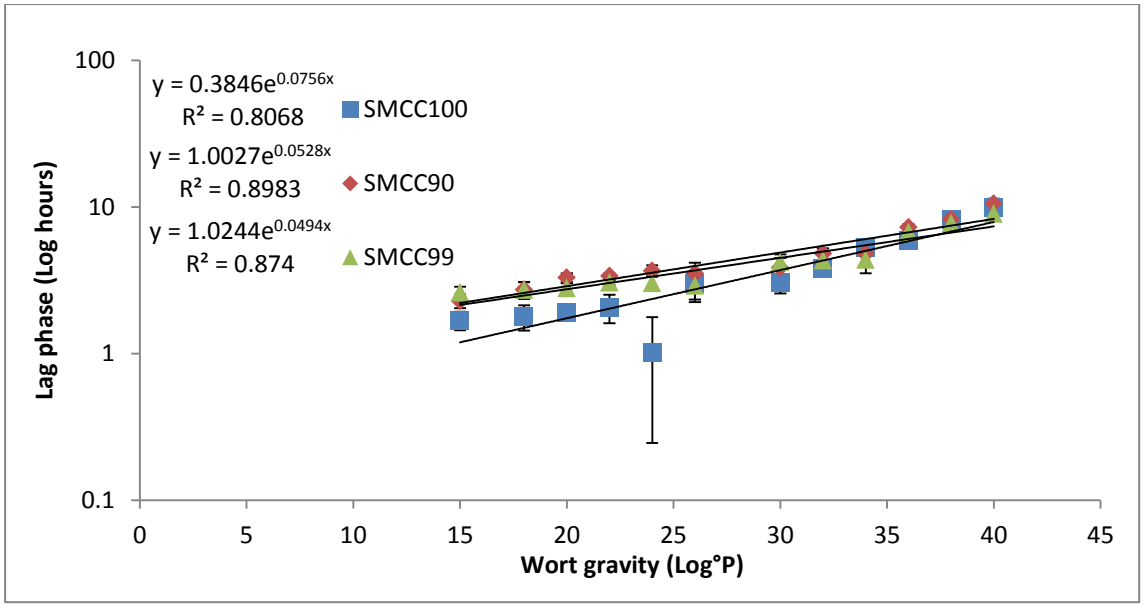


Figure 3.10 The relationship between lag phase (Log hours) and wort gravity (°P). Lag phase kinetics were calculated from the mean of triplicate samples with error bars indicating the standard deviation.

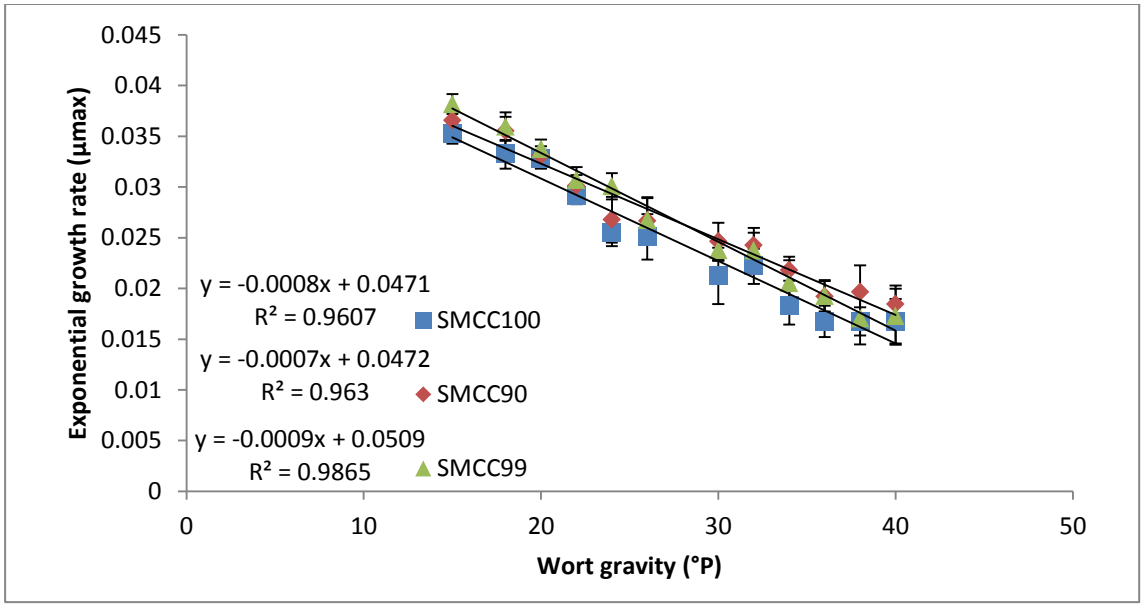


Figure 3.11 The relationship between exponential phase (µmax) and wort gravity (°P). µmax was calculated from the mean of triplicate samples with error bars indicating the standard deviation.

In addition to lag and exponential phase differences, there was also a marked decrease in final (maximum) growth as wort gravity increased. There was a reduction in AU (Absorbance Units) at 15°P from 0.73AU, 0.77AU and 0.77AU to 0.46AU, 0.50AU and 0.50AU at 40°P for SMCC 90, 100 and 99 respectively (Figure 3.7-9). It was interesting to note that the decrease in maximum yield was particularly marked for SMCC100 when compared to the other strains, indicating that SMCC900 and SMCC99 had higher glucose tolerance levels , but perhaps also a reflection of longer lag phase (Figure 3.10). This data suggests that for VHG fermentations where glucose is the major adjunct used, SMCC90 may be less well adapted to grow, divide and survive. The use of glucose as an adjunct for fermentation at very high gravity may therefore have an impact on each strains ability to ferment wort, influencing both final yields (as observed by the lowering of final growth levels when gravity is increased), and growth rate (where lag phase was extended and exponential phase rates were reduced).

3.2.4 **Yeast ethanol tolerance**

During fermentation brewing yeast must maintain viability under elevated ethanol concentrations, which is increasingly important when considering very high gravity (VHG) fermentations. Standard 10-12°P fermentations typically generate between 4 and 5% ethanol v/v, while increasing wort gravity to 20°P will lead to a corresponding increase in potential ethanol yield (Puligundla *et al* 2011). Understanding the tolerance levels of yeast under increasing ethanol concentrations can therefore indicate the ability of

a particular yeast strain to survive in the VHG environment. In order to achieve this, the response of each strain to ethanol was determined by assessing growth characteristics on solid and liquid media. As described in Section 2.5.1.2, wort agar plates (15°P) were infused with increasing concentrations of ethanol from 0-20% v/v. 10µL of culture containing a total of $1x10^5$, $1x10^4$, $1x10^3$, 100 and 10 cells were spotted onto plates, which were then incubated for 72hrs at 25°C. Each agar plate was then analysed for the presence of growth and, when present, for colony morphology.

It can be seen that after 72 hours all strains were able to tolerate levels up to 5% ethanol v/v with no change to colony morphology and no visible impact on growth when compared to control samples (0% ethanol) (Figure 3.12). At a concentration of 10% it was observed that the colony morphology appeared to alter, most obviously at a concentration of $1x10^5$ cells, where the colonies changed from having a round uniform smooth shape to being non-uniform colonies with uneven edges. The reason for this is unknown, although it is thought that a variety of stress factors can impact on colony morphology through activation of stress response genes. In particular it is known that starvation (particularly nitrogen deficiency) can induce pseudohyphal growth which can lead to differences in growth patterns on solid media (Gimeno et al 1992). Although colony morphology could be seen to be altered, it should be noted that all strains were visibly able to tolerate and grow at 20% ethanol v/v (Figure 3.12).

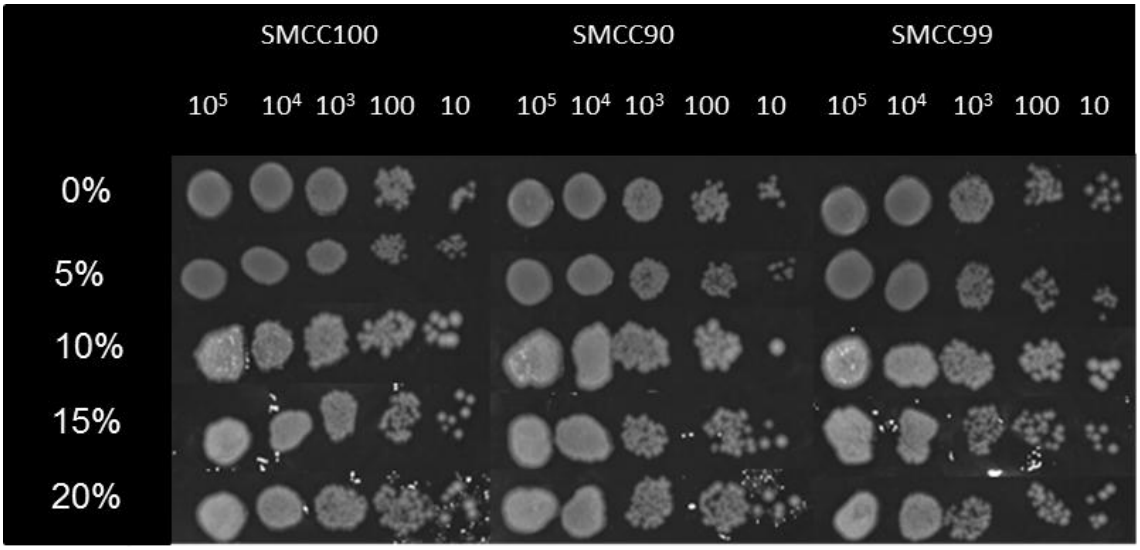


Figure 3.12 Growth of strains SMC100, SMCC90 and SMCC99 on wort agar supplemented with ethanol (0-20% v/v). Suspensions of 10-10^5 cells were spotted onto the surface of agar and observed visually for growth.

Following spot plate analysis, the growth kinetics of each strain were observed by inoculating 1x10^5 cells/mL into 15°P wort supplemented with ethanol at 0, 5, 10, 15 and 20% (v/v), and incubating at 25°C (Section 2.5.2). Absorbance at OD600 was then used to determine the growth kinetics of each strain using a 96 well plate reader (Section 2.5.2) and data was subsequently analysed using DMFit 3.5 software.

Control data, obtained by analysing the growth of each strain on identical media without ethanol (0% v/v), indicated that strain SMCC90 had the highest affinity for wort, based on growth curve kinetics. SMCC90 had a shorter lag phase (18.64 hours) than SMCC100 (24.15 hours) and SMCC99 (24.00 hours). The amount of total attainable growth was also

greater in SMCC90: an OD600 of 1.59AU compared to 1.44AU and 1.47AU for SMCC100 and SMCC99 respectively (Figure 3.13-3.15). The introduction of ethanol at 5% (v/v) to the media reduced the difference in final growth level between each strain with an OD600 of 1.35AU for SMCC99, 1.24AU for SMCC90 and 1.41AU for SMCC100. Although the amount of final growth for strain SMCC100 was higher than SMCC90, a reduction in comparative lag phases was observed with SMCC90. This strain exhibited a lag phase of 20.13 hours compared to 23.44 hours for SMCC99, and 22.76 hours for SMCC100. As ethanol levels in the media increased it was observed that the lag phase of all yeast cultures was extended until growth was restricted by the presence of 15% (v/v) and 20% ethanol (v/v). SMCC90 lag phase increased from 20.13 hours in the presence of 5% ethanol (v/v) to 26.67 hours in 10% ethanol (v/v). A similar pattern was observed in both SMCC100 and SMCC99 where, in the presence of 5% ethanol, the respective lag phases were 24.15 hours and 24.00 hours and in the presence of 10% ethanol (v/v) the lag phases increased to 34.11 hours and 37.95 hours. This data indicates that SMCC90 was the most tolerant to ethanol concentrations. Interestingly, both SMCC100 and SMCC99 (but not SMCC90) showed a similar lag time when ethanol was increased from 0% to 5%, indicating that these strains were not impacted by the presence of low levels of ethanol (Figure 3.13-3.15).

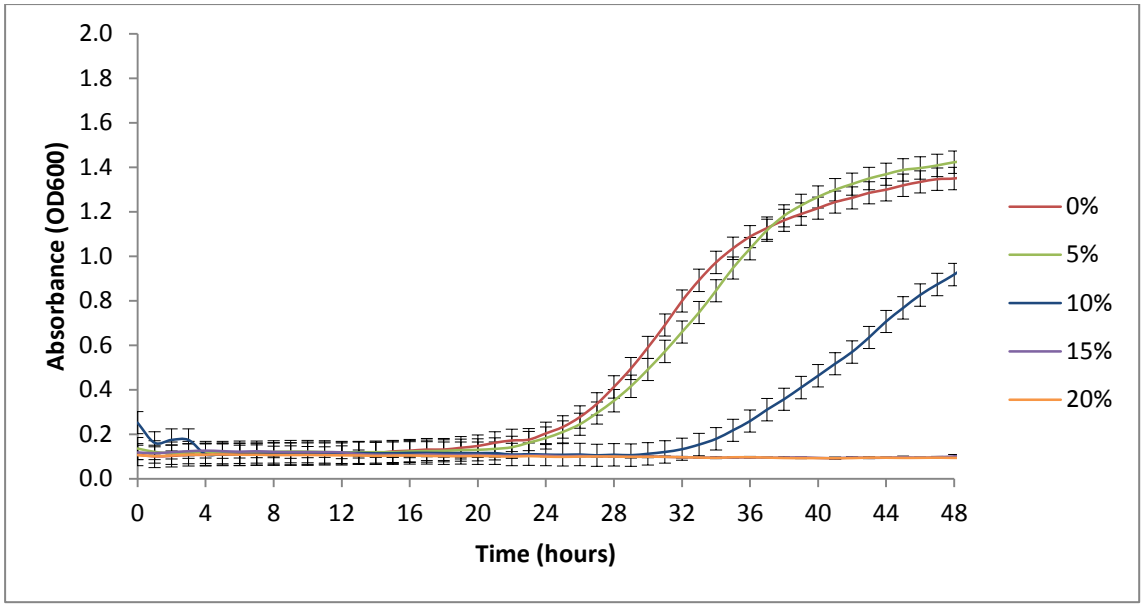


Figure 3.13 Growth of strain SMCC100 in 15°P wort supplemented with ethanol (0-20% v/v). Cells were inoculated at a concentration of $1x10^5$ cfu/mL and growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

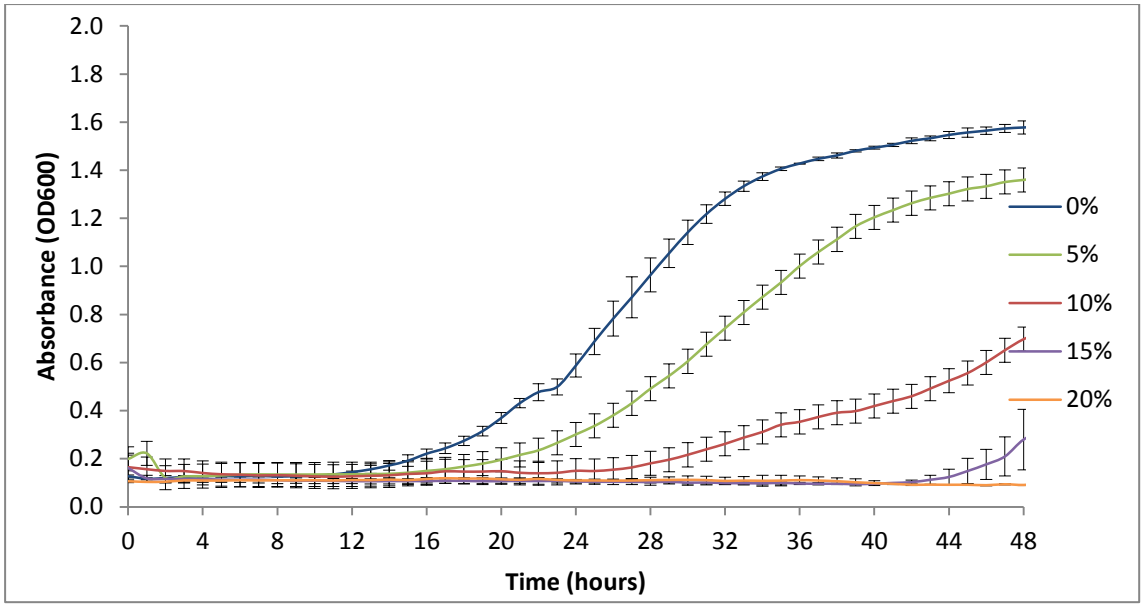


Figure 3.14 Growth of strain SMCC90 in 15°P wort supplemented with ethanol (0-20% v/v). Cells were inoculated at a concentration of $1x10^5$ cfu/mL and growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

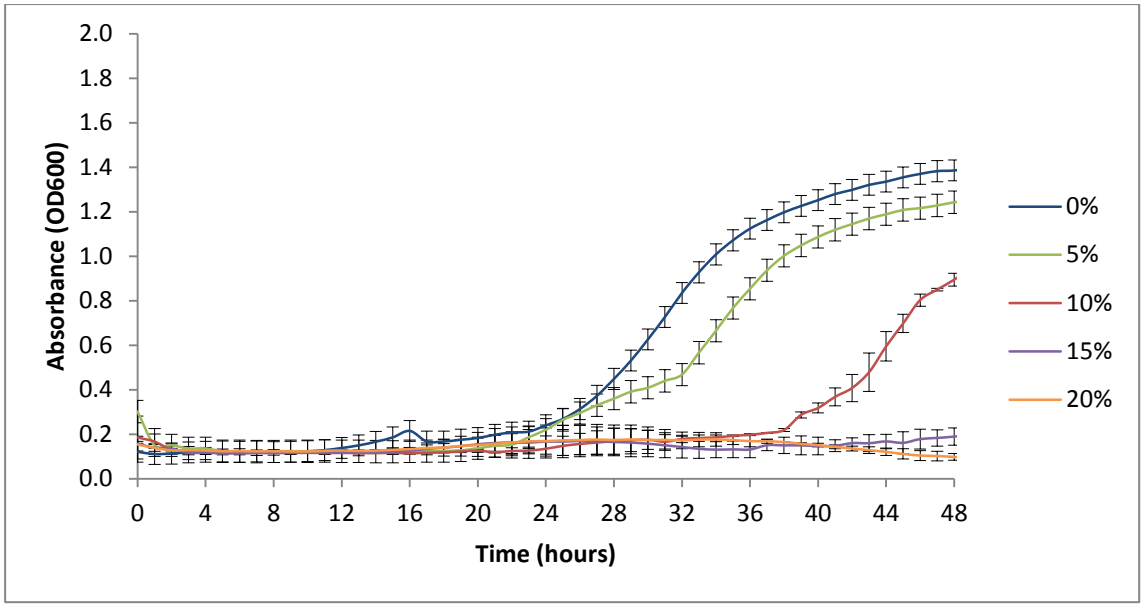


Figure 3.15 Growth of strain SMCC99 in 15°P wort supplemented with ethanol (0-20% v/v). Cells were inoculated at a concentration of $1x10^5$ cfu/mL and growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

To further analyse growth kinetics under ethanol stress, lag phase and exponential phase (μmax) data were assessed based on the growth conditions applied. Note that since growth was not observed at greater than 10% ethanol, only data obtained from analysis of 0, 5 and 10% ethanol were analysed. When lag phase data was compared to ethanol concentration a positive overall correlation was observed. This was calculated with >70% fitness for all three strains (72%, 84%, 88% respectively). It was also noted that although similar effects were observed for strains SMCC100 and SMCC99, the length of the lag phase for strain SMCC90 was less impacted by ethanol concentration, indicating a higher tolerance of SMCC90 to ethanol (Figure 3.16). Subsequently, the slope of

each curve was analysed during exponential phase (Section 2.5.2). This indicated a 42.6% decrease in μmax from 0.090 to 0.051 for SMCC90 between 0% and 10% ethanol, with an R^2 value of 88%. SMCC100 showed less of a reduction in μmax with a 36.9% decrease from 0.082 at 0% ethanol to 0.051at 10% ethanol with an R^2 value of 84%. SMCC99 exhibited the lowest reduction in of 26.3%, from 0.082 at 0% ethanol to0.060 at 10% ethanol (v/v) with an R^2 value of 72% (Figure 3.17).

It was also observed that there was an inverse correlation between ethanol concentration and growth rate. The confidence level of these correlations was calculated at >80% (81%, 100%, 87% for strains SMCC100, 90 and 99 respectively). It was noted that although SMCC99 and SMCC100 exhibited similar values, wort gravity was observed to have a greater impact on SMCC90 (Figure 3.17). This data indicates that SMCC90 was the strain most affected by ethanol concentration. When comparing growth kinetic data with the spot plate analysis, it can be seen that at lower concentrations of ethanol yeast growth in liquid media was impaired compared to that on solid media. On solid media comprising 20% ethanol (v/v) growth occurred readily for all strains, while growth was impaired in liquid media containing ethanol at a concentration of greater than 15% (v/v) (Figures 3.13-3.15). These data also indicated that for fermentations where ethanol is present in increasingly higher volumes, SMCC99 may arguably be the strain most capable of growing well. Increasing concentrations of ethanol during VHG fermentations (and perhaps storage under higher levels of ethanol during serial repitching) may therefore have an impact on the ability of each strain to ferment wort, based on growth kinetic analysis.

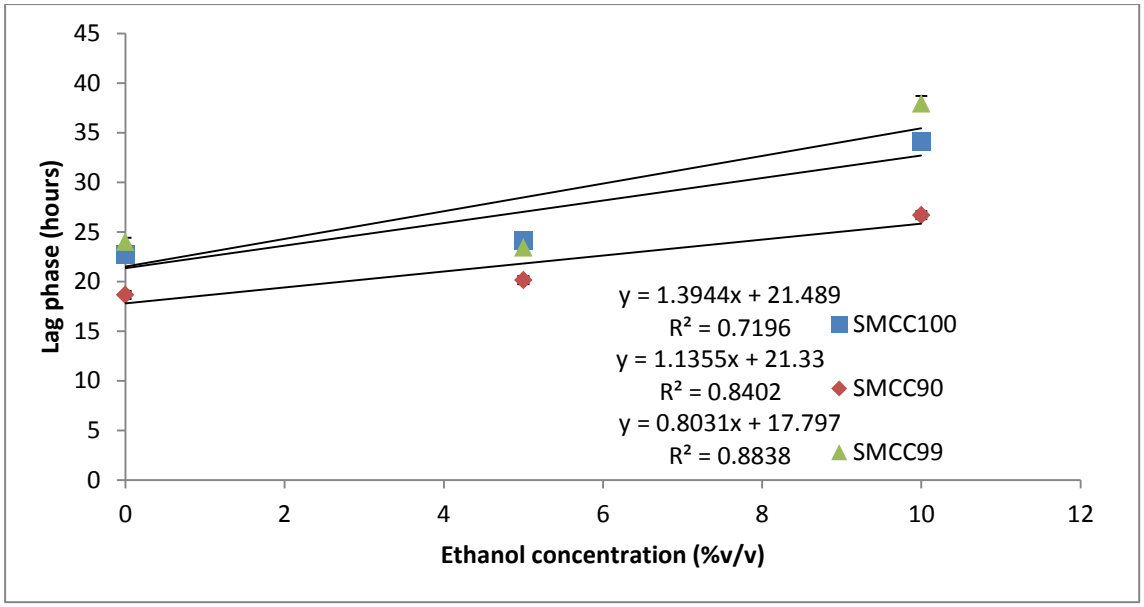


Figure 3.16 The relationship between lag phase (hours) and ethanol concentration (% v/v). Lag phase kinetics were calculated from the mean of triplicate samples with error bars indicating the standard deviation.

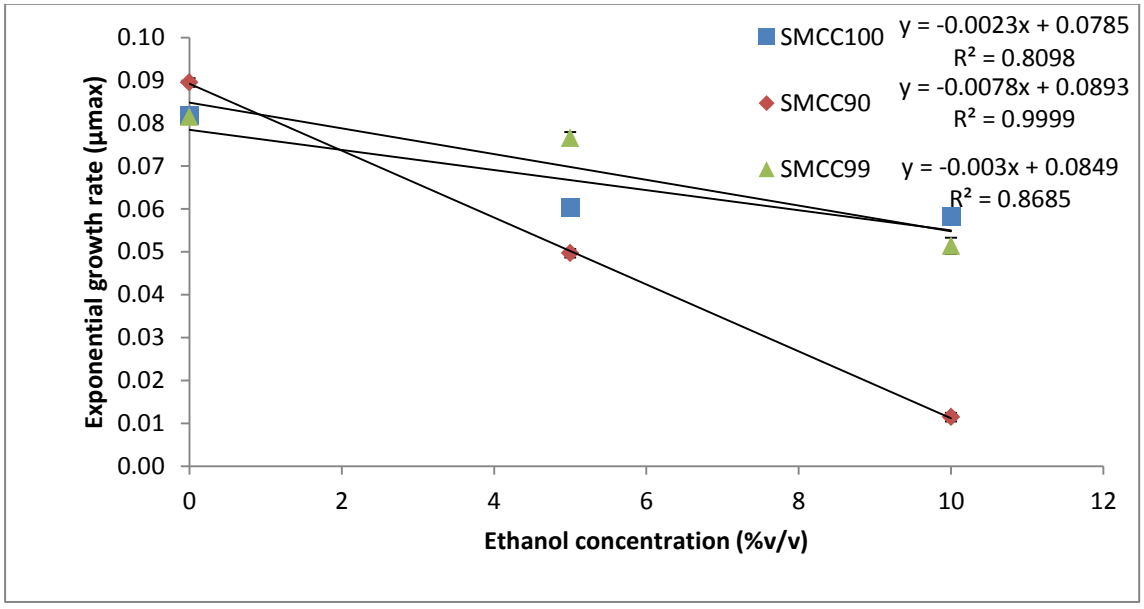


Figure 3.17 The relationship between exponential phase (µmax) and ethanol concentration (%v/v). µmax values were calculated using DMFit 3.5 software from the mean of triplicate samples with error bars indicating the standard deviation.

3.2.5 **Yeast Oxidative Stress Tolerance**

During brewery yeast handling there are several points at which oxidative stress may be encountered. This is primarily due to respiratory metabolism in the presence of oxygen which occurs during propagation, the initial stages of fermentation (although this is debatable due to the Crabtree effect (Section 1.1.5)), and also during storage (Gibson *et a*l 2007). However, studies have shown that reactive oxygen species (ROS) are also produced throughout fermentation (Landolfo *et al* 2008). Cells can generate ROS through the oxidation of NAD(P)H in metabolic pathways, including activity of the cytochrome P450 family involved in glycolysis, and in the conversion of alkanes to alcohols, and in fatty acid degradation (Steele *et al* 1994; Fragnier *et al* 2003; Funhoff *et al* 2006; Schwarz *et al* 2012; Section 1.5.4.2). Given that VHG fermentations comprise elevated sugar concentrations, it is possible that the overall 'metabolic activity' of a yeast culture is increased, which could lead to greater amounts of ROS being generated. In order to assess the effect of oxidative stress on yeast growth, cells were exposed to either menadione or hydrogen peroxide (H_2O_2). H_2O_2 induces oxidant defence mechanisms in yeast, such as glutathione peroxidase and catalase (Crawford and Davis 1994 and Davies *et al* 1999), while menadione leads to superoxide production. Irrespective of mechanism, Jamieson *et al* (1994) and Gasch *et al* (2000) have shown that the oxidative stress response of yeast to both menadione and H_2O_2 are very similar. Given that the tolerance level for yeast has been shown to be in the range of 0.5 - 3mM menadione and 2 - 5mM H_2O_2 (Jamieson *et al* 1992), similar conditions were applied in this particular study.

Solid wort media containing increasing concentrations of menadione or H_2O_2 were utilised for spot plate analysis. For each strain 10µL of culture

containing a total of $1x10^5$, $1x10^4$, $1x10^3$, 100 and 10 cells were added, incubated for 72hrs at 25°C and observed for growth and colony morphology. It can be seen from Figure 3.19 that with increasing menadione concentration, the ability to support growth decreased. Strain SMCC100 had a relatively high tolerance to menadione with observable growth at a concentration of $1x10^3$ cells. In comparison, for strains SMCC99 and SMCC90, a concentration of above 1mM menadione was sufficient to limit growth (Figure 3.18).

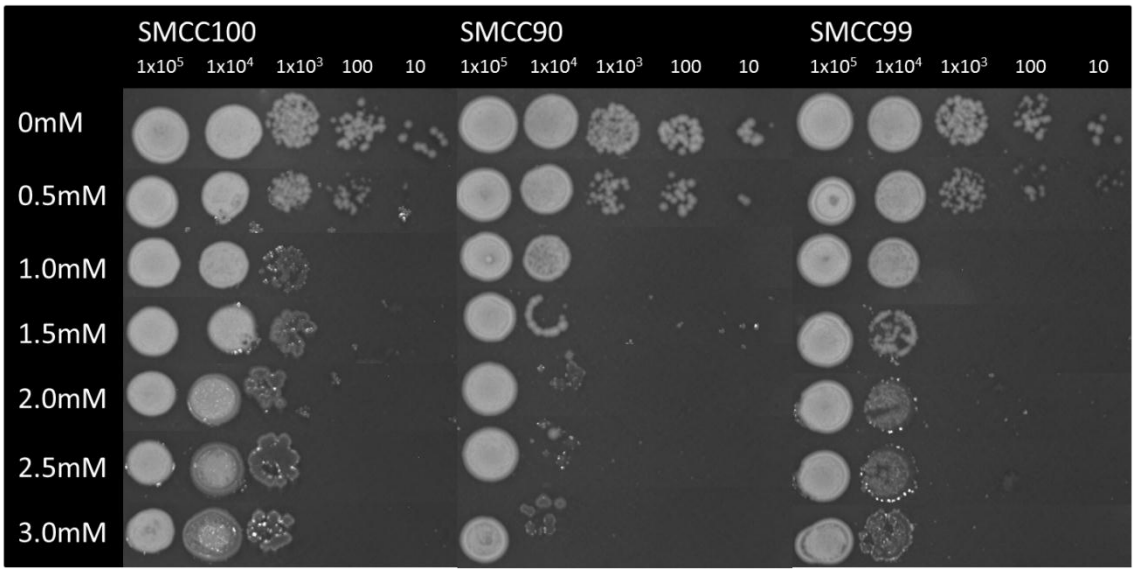


Figure 3.18 Growth of strains SMC100, SMCC90 and SMCC99 on wort agar containing 0-3mM menadione. Increasing cell concentrations of 10-10^5 cells were spotted onto the surface of agar prepared at different menadione concentrations as indicated and incubated at 25°C for 72 hours.

As described previously, spot plate analysis only provides limited information on the response of cells within a population. For a more

detailed indication of the effect of menadione levels on cell growth kinetics, 15°P wort was supplemented with menadione (0-3mM) and the growth kinetics of each strain were observed by measuring absorbance (OD600) over time (Section 2.5.2). Growth stages were determined and analysed using DMFit 3.5 software (Section 2.5.2.1). Increasing the concentration of menadione was observed to have a negative impact on growth for all strains (Figure 3.19-3.21), although the effect was relatively minor. Final growth levels were observed to be similar for all 3 strains (Figures 3.19-21). All strains were observed to have a similar lag phase at the start of experimentation under control conditions (0mM menadione) and under these conditions lag phase was approximately 7.80±0.02 hours. When compared to the other strains SMCC100 was observed to show the highest increase in lag phase in the presence of 3mM menadione, at 12.01 hours, compared to 10.09 hours for SMCC 90 and 8.83 hours for SMCC99. This indicated that SMCC99 may be more resistant to the oxidative stress caused by the presence of menadione in these conditions, with the lowest increase in lag phase of all of the strains analysed (Figure 3.19-20).

To further analyse growth kinetics under oxidative stress, lag phase and exponential phase data were compared against the concentration of menadione. As reported above, when the concentration of menadione was increased, the length of the lag phase was observed to become extended. For this analysis, control (growth on 0mM menadione) data was excluded since this was found to skew the results considerably. Based on analysis of growth in the presence of 0.5-3mM menadione it was calculated that lag phase exhibited >97% fitness for SMCC100, SMCC90 and SMCC99 (98.6%, 97% and 98% respectively). It was also noted that increasing menadione concentration had less of an impact on SMCC90 than the other

strains, indicating potentially higher tolerance (Figure 3.22). The slope of the curve was also analysed during exponential phase (μmax) (Section 2.5.2.1). This indicated a 12.9% decrease from μmax=0.071 to μmax=0.062 for SMCC100 when comparing the slopes of the curves 0mM menadione to 3mM menadione, with an R^2 value of 89%. SMCC90 showed a greater reduction in μmax (18.4%) from 0.071 in the presence of 0mM menadione to 0.058 in the presence of 3mM menadione, with an R^2 value of 85%. SMCC99 had the highest reduction in μmax (21.8%), from 0.071 in the presence of 0mM menadione to 0.055 in the presence of 3mM menadione, although a very low R^2 value of 34% was obtained, indicating a limited level of confidence in this data set (Figure 3.23).

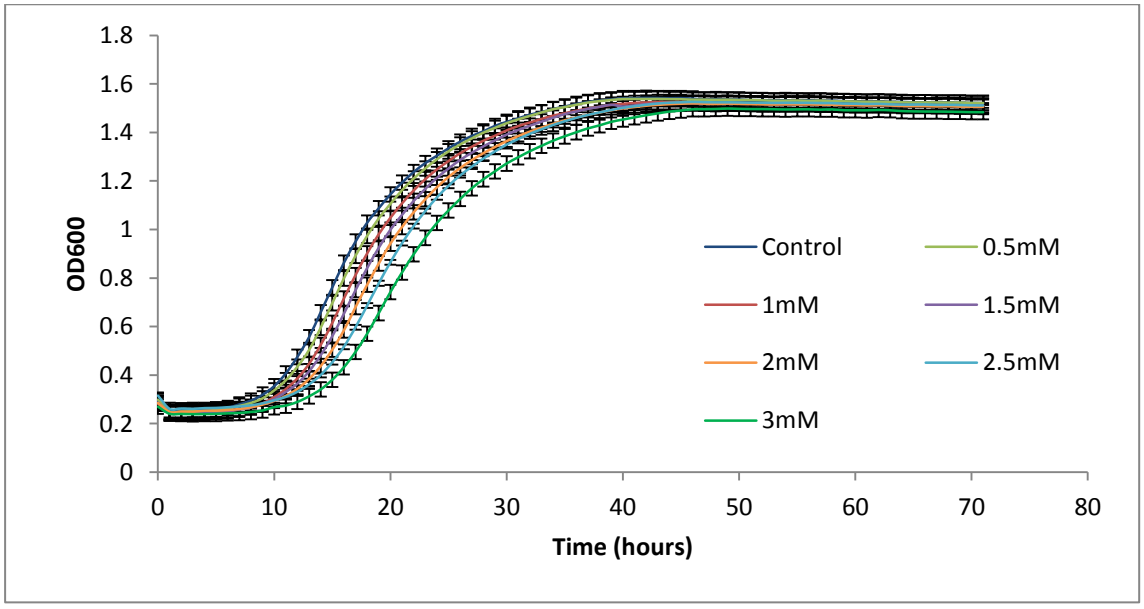


Figure 3.19 Growth of strain SMCC100 in 15°P wort supplemented with menadione (0-3mM). Cells were inoculated at a concentration of $1x10^5$ cfu/mL and growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

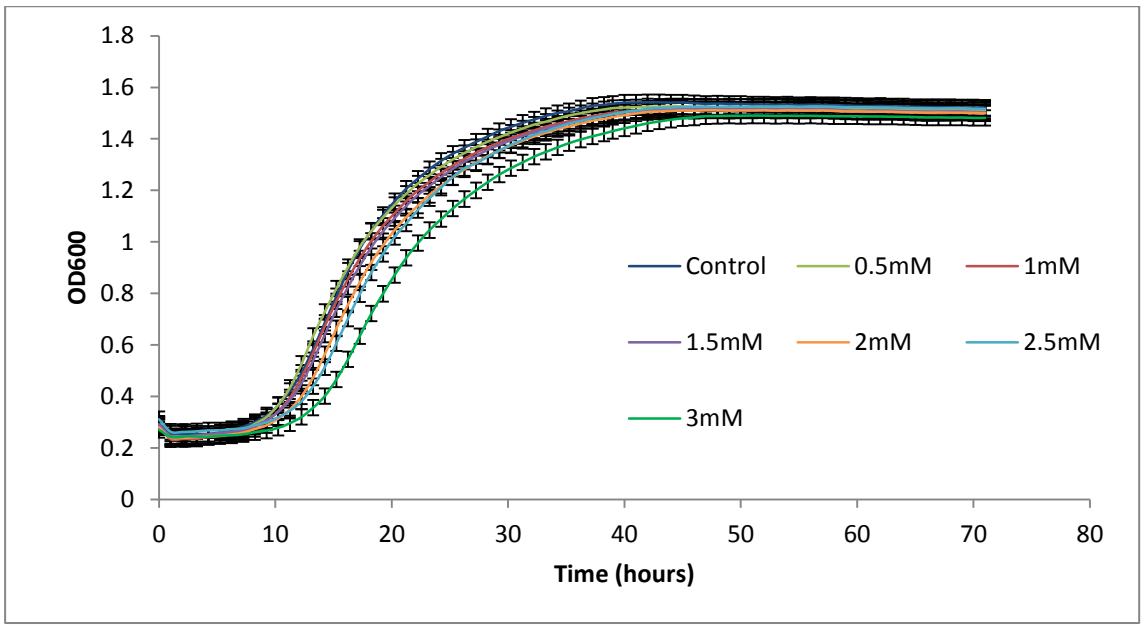


Figure 3.20 Growth of strain SMCC90 in 15°P wort supplemented with menadione (0-3mM). Cells were inoculated at a concentration of $1x10^5$ cfu/mL and growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

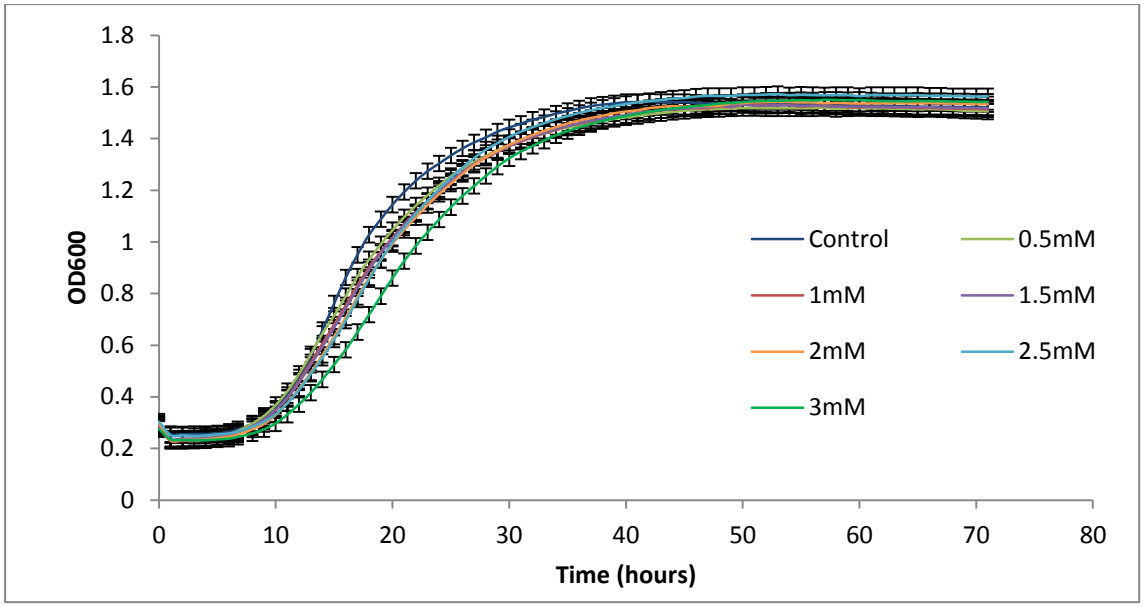


Figure 3.21 Growth of strain SMCC99 in 15°P wort supplemented with menadione (0-3mM). Cells were inoculated at a concentration of $1x10^5$ cfu/mL and growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

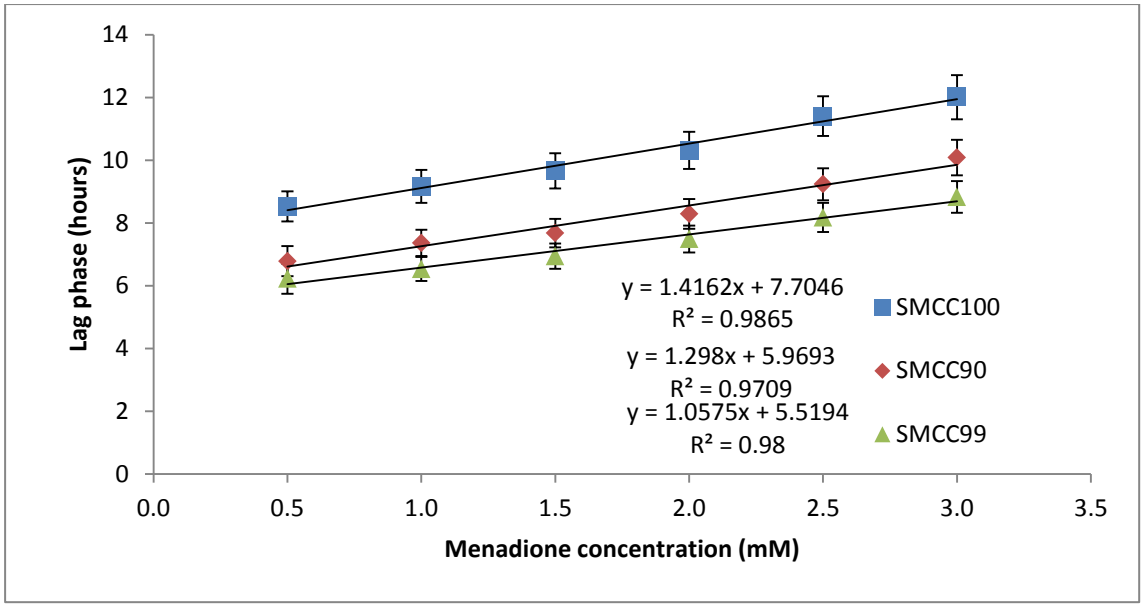


Figure 3.22 The relationship between menadione induced oxidative stress and lag phase. Lag phase data was processed using DMFit 3.5 software from the mean of triplicate samples with error bars indicating the standard deviation.

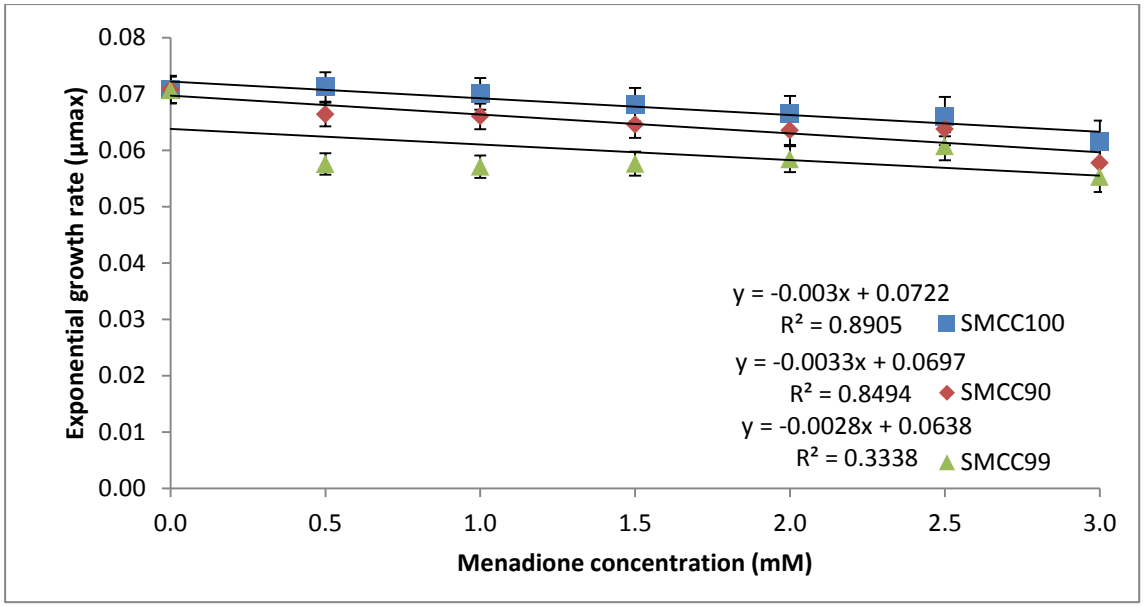


Figure 3.23 The relationship between menadione induced oxidative stress and exponential phase (µmax). µmax values were calculated using DMFit 3.5 software from the mean of triplicate samples with error bars indicating the standard deviation.

Although it was observed that menadione did lead to changes in visible growth on solid media and in growth kinetics in liquid media, in many instances these differences were marginal. Furthermore, previous work has indicated that there may be an inhibitory effect of wort on the efficacy of menadione (S. Nicholls, personal correspondence). Since wort and wort composition form a major part of this work, experiments were also undertaken to investigate an alternative means of inducing oxidative stress using hydrogen peroxide (H_2O_2) (Martin *et al* 2003). Consequently, solid wort media was supplemented with 0-6mM H_2O_2 and strains were analysed for growth analysis using the spot plate technique (Section 2.5.1). 10µL of culture containing a total of $1x10^5$, $1x10^4$, $1x10^3$, 100 and 10 cells were spotted onto plates, which were then incubated for 72hrs at 25°C and

observed for growth and colony morphology. At a H_2O_2 concentration of 2mM it was observed that both SMCC90 and SMCC99 showed a reduction in growth, which was not mirrored by SMCC100. However increasing the H_2O_2 concentration above 2mM impacted on all yeast strains, albeit strain SMCC100 appeared to be less affected, indicating a potential tolerance to H_2O_2 (Figure 3.24). This data supports the previous observation with regard to growth on menadione, providing further evidence that strain SMCC100 may display a high tolerance to oxidative stress.

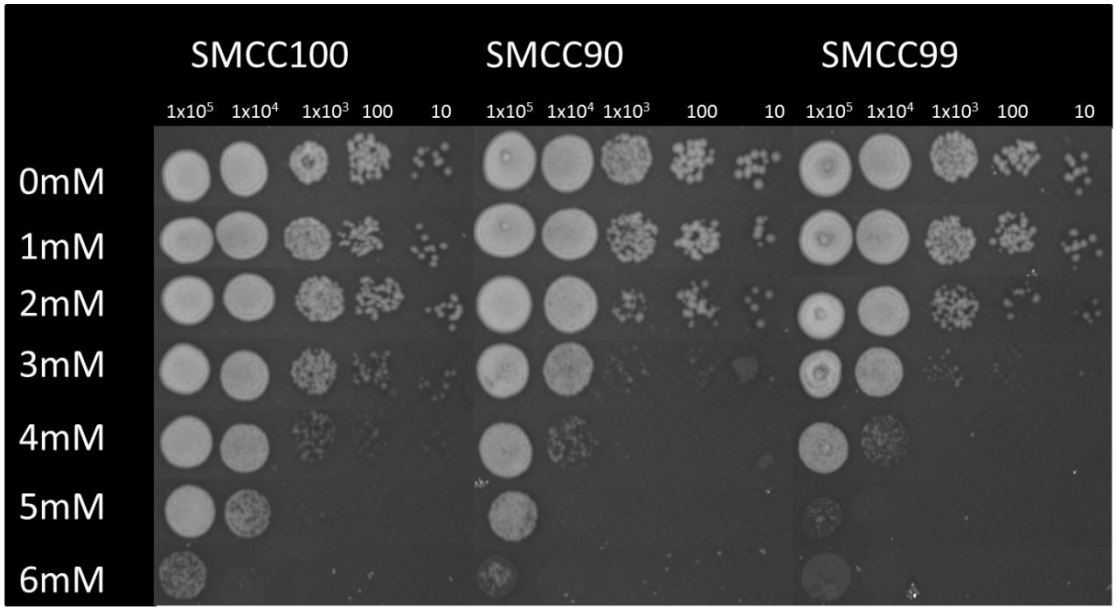


Figure 3.24 Growth of strains SMC100, SMCC90 and SMCC99 on wort agar containing 0-6mM H_2O_2. Cell concentrations of 10-10^5 cells were spotted onto the surface of agar prepared at different menadione concentrations as indicated and incubated at 25°C for 72 hours.

As discussed previously, although spot plate analysis is a useful tool for determining absolute limits of growth under specified conditions, it only

provides limited information on the response of the cells within a population. For a more detailed indication of the effect of menadione levels on cell growth kinetics, 15°P wort was supplemented with H_2O_2 (0-6mM) and the growth kinetics of each strain were observed by measuring absorbance (OD600) over time (Section 2.5.2), Growth stages were determined and analysed using DMFit 3.5 software (Section 2.5.2.1). As in the previous analysis describing oxidative stress induced by menadione, increasing the concentration of H_2O_2 caused an increase in the lag phase, as well as decreasing the rate of the exponential phase. This was observed to occur in all three strains. SMCC99 had the smallest increase in lag phase in the presence of 6mM H_2O_2 at 18.72 hours from 5.51 hours in the 0mM H_2O_2 control. Analysis of the lag phase of SMCC90 in the presence of 6mM H_2O_2 demonstrated a larger increase than SMCC99 at 23.38 hours compared to 6.33 hours for the control. Finally, growth of SMCC100 was most affected by the presence of H_2O_2, with an increase in lag phase from 7.79 hours to 25.76 hours in the presence of 0mM and 6mM H_2O_2 respectively. These data indicate that SMCC99 is most tolerant to the oxidative stress examined, followed by SMCC90 and finally SMCC100 is the least tolerant to H_2O_2 oxidative stress (Figure 3.25). Finally, it was observed that the final growth level was similar for all strains in the presence of all H_2O_2 concentrations based on absorbance at OD600 (Figures 3.25-27).

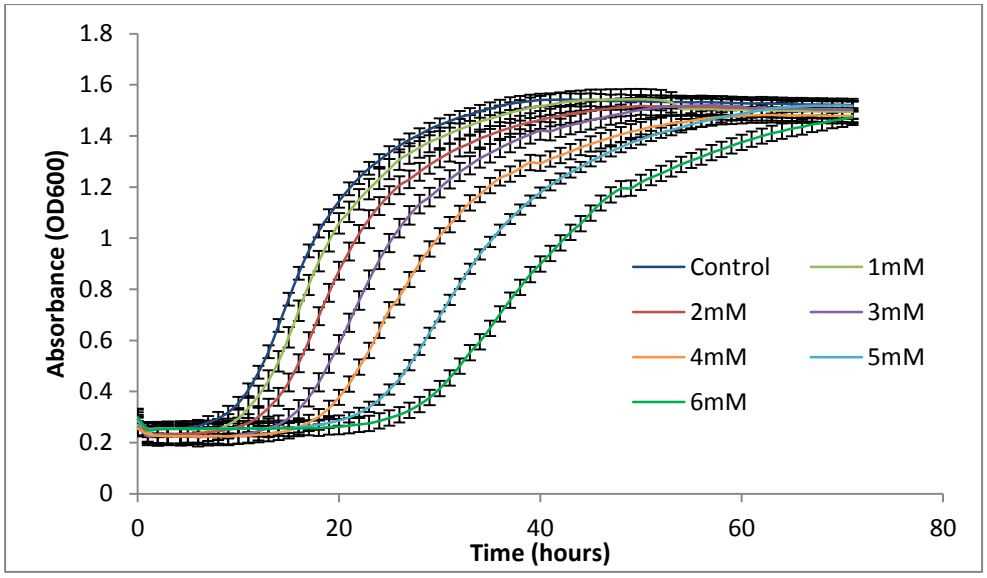


Figure 3.25 Growth of strain SMCC100 in 15°P wort supplemented with H_2O_2 (0-6mM). Cells were inoculated at a concentration of $1x10^5$ cfu/mL and growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

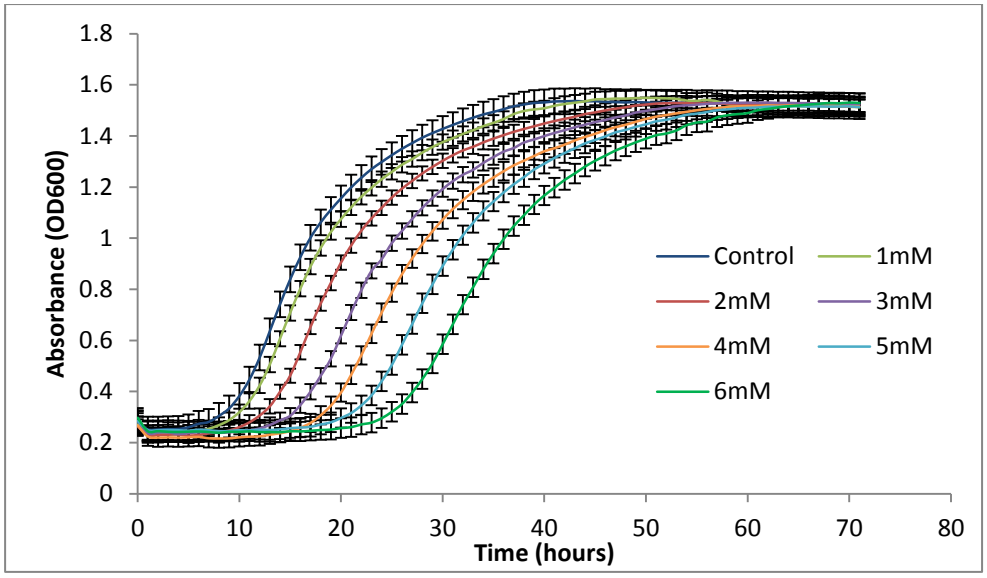


Figure 3.26 Growth of strain SMCC90 in 15°P wort supplemented with H_2O_2 (0-6mM). Cells were inoculated at a concentration of $1x10^5$ cfu/mL and growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

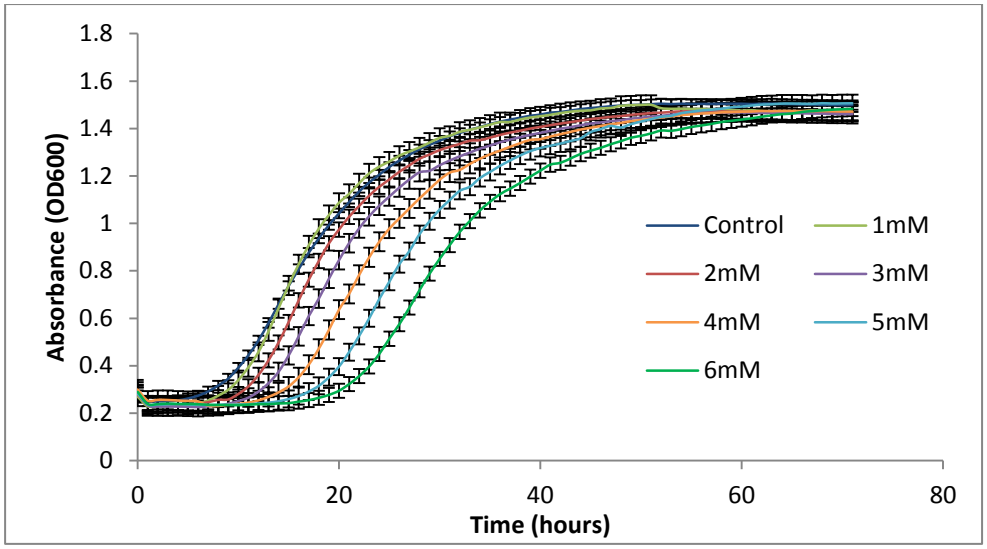


Figure 3.27 Growth of strain SMCC99 in 15°P wort supplemented with H_2O_2 (0-6mM). Cells were inoculated at a concentration of $1x10^5$ cfu/mL and growth was detected by measuring absorbance at OD600. Each data point represents the mean of triplicate samples with error bars indicating the standard deviation.

To further analyse the effect of H_2O_2 induced oxidative stress on lager yeast growth kinetics, lag phase and exponential phase data were analysed using DMFit 3.5 software (Section 2.5.2.1). When the lag phase data was compared to H_2O_2 concentration (mM), a positive correlation was observed. This was calculated with >97% fitness for all three strains (99%, 97%, 98% respectively). It was also noted that although a similar effect was observed for strains SMCC100 and SMCC99, the lag phase length of SMCC99 was less impacted by H_2O_2 concentration, indicating a higher tolerance of SMCC99 to H_2O_2 concentration (Figure 3.28). Analysis of growth rate (µmax) during exponential phase (Section 2.5.2.1) also revealed an inverse relationship with increasing H_2O_2 concentrations. It was observed that there was a 40% decrease in µmax from 0.071 to 0.043

in strain SMCC100 between 0mM and 6mM H_2O_2, with an R^2 value of 99%. SMCC90 showed a smaller reduction in μmax at 17.1%, from 0.066 to 0.055 between 0mM and 6mM H_2O_2, with an R^2 value of 74%. SMCC99 had the least reduction in μmax at 7%, from 0.054 to 0.050 between 0mM and 6mM H_2O_2. This yielded a low R^2 value of 40% indicating a lower level of confidence in this data set (Figure 3.29).

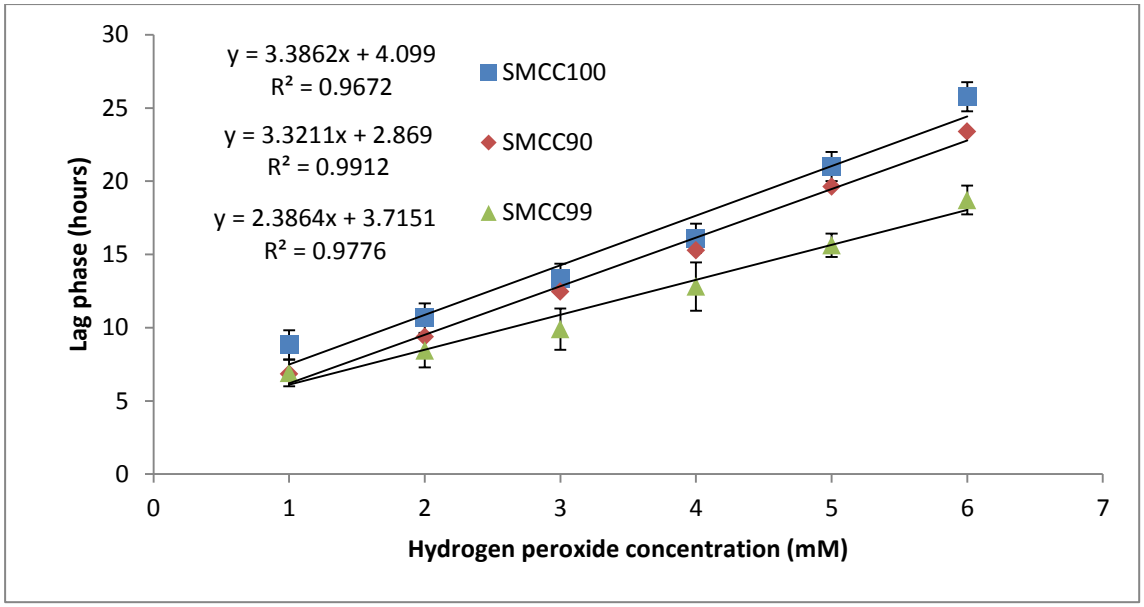


Figure 3.28 The relationship between H_2O_2 induced oxidative stress and lag phase. Lag phases were calculated using DMFit 3.5 software from the mean of triplicate samples with error bars indicating the standard deviation.

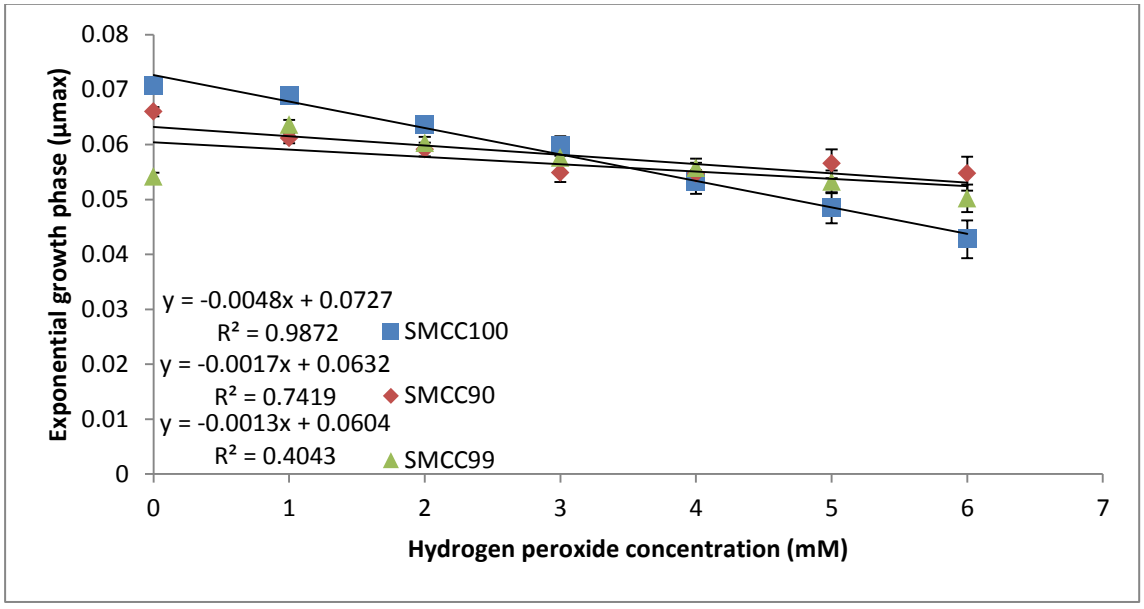


Figure 3.29 The relationship between H_2O_2 induced oxidative stress and exponential phase (µmax). µmax values were calculated using DMFit 3.5 software from the mean of triplicate samples with error bars indicating the standard deviation.

3.3 Discussion

The aim of this investigation was to characterise and differentiate the yeast strains utilised in this study to determine species and strain identity. Subsequently, in order to gain an insight into the physiological properties of each strain in response to glucose and ethanol tolerances, the capacity to grow in a range of wort-based media was examined. Finally, media known to elicit an oxidative stress reaction (Martin *et al* 2000) was utilised to examine the effect of oxygen free radicals on the growth kinetics of lager brewing yeast, to mirror free radical production potentially associated with the occurrence of increased metabolism of VHG fermentations. The data produced in this chapter indicates differences between each of the strains utilised, and gives an insight into the yeasts' ability to tolerate potential stresses which are known to occur in the brewery environment and are likely to be exacerbated in very high gravity fermentations.

In order to identify yeast utilised in this study to the genus level and therefore determine brewing classification (ale/lager), the internal transcribed spacer regions were analysed using a combination of PCR and RFLP. Although clear DNA fingerprints were generated, the results of the ITS analysis could not conclusively confirm the genus of each lager strain to be *S. pastorianus*. In contrast, the ITS patterns observed matched those normally associated with *S. cerevisiae* strains. The ITS PCR makes use of spacer regions of the conserved 18S, 5.8S and 28S rRNA genes to show a distinct genus, and in some cases the identification of specific species (White *et al* 1990). The inability to determine species in this case could be due to differing hybridisation events. The events meant that some *S.pastorianus* strains contain the ITS region of the *S.cerevisiae* parent, and some strains received the ITS region of the *S.eubayanus* parent.

Previous work has indicated that there are two separate patterns of the internal transcribed spacer regions for strains belonging to the *S. pastorianus* genus (Gibson and Liti 2014). These authors hypothesised that this is due to separate hybridisation events, leading to two 'types' of lager yeast, referenced as type 1 / Saaz and type 2 / Frohberg (Liti *et al* 2005, Dunn and Sherlock 2008). Analysis of these two types of lager yeast have indicated that the type 1 (Saaz) strains may be hybrids derived from a haploid *S. eubayanus* and a haploid *S. cerevisiae* strain, while type 2 (Frohberg) strains may be the result of hybridisation between a haploid *S. eubayanus* and a diploid *S. cerevisiae* (Dunn and Sherlock 2008, Gibson and Liti 2014). This understanding of multiple hybridisation events could explain the similar banding patterns between *S. pastorianus* and *S. cerevisiae* and the fact that the strains analysed here produced a 'cerevisiae' like profile. Indeed, previous work conducted has indicated that the restriction profiles obtained here match Saaz-type *S. pastorianus* strains (Gibson *et al* 2013). Lager classification was confirmed using alternative methodology based on a combination of permissive growth temperature (Casey *et al* 1994) and the ability to utilise the melibiose homolog X-α-Gal (Tubb & Liljestrom 1986; Box *et al* 2012). This latter technique allows identification of lager strains due to their ability to secrete α –galactosidase (melabiase), which ale strains do not, resulting in the development of a blue colouration derived from cleavage of X-α-Gal. Using a combination of these methods it was therefore possible to confirm that the strains SMCC100, SMCC90 and SMCC99 were lager yeasts belonging to the genus *S. pastorianus*. To further differentiate each yeast to the strain level, genomic DNA inter-delta regions and mtDNA RFLP analyses were conducted. mtDNA analysis did not reveal any differences between strains, likely due to the similar heritage and inheritance of

mitochondrial DNA by Saaz type yeasts. However, amplification patterns of inter-delta sequences indicated that each strain was different and could be clearly identified. The techniques utilised in this study are commonly used throughout the brewing industry. There are howewever, alternative methodologies that could be utilised to identify yeast strains. Techniques such as next generation sequencing (NGS) can, not only identify species but also strains. Characterisation of yeast strains for their tolerance to glucose and ethanol was conducted in order to provide an indication of their ability to cope in a VHG environment. This was attained through the use of solid and liquid media supplemented with various concentrations of glucose or ethanol. The data obtained indicates that each strain was able to reproduce and grow in the presence of high levels of sugar (≤40°P), but a reduction in final growth levels was noted as a cost impact in the presence of high glucose. Similarly, all strains were able to grow on solid media containing high concentrations of ethanol (20%v/v) but changes to colony morphology were observed. The reason for this is currently unclear, however it is known that certain stress factors can have a marked effect on colony growth, through activation of stress response genes. In particular it is known that deficiency in nutrients such as nitrogen can lead to cell morphological changes or pseudohyphal growth. This may lead to differences in growth patterns on solid media (Gimeno et al 1992; Boeckstaens et al 2007). Analysis of yeast strains in the presence of ethanol in liquid media showed growth to be impaired in the presence of ethanol concentrations over 10%, which was not always seen on solid media. Differences in gene expression have been observed in cells grown on solid compared to liquid media, for example IRR1 (involved in regulating mitotic cell division) has been shown to be necessary for growth on solid media but play no role in growth in liquid media (Kurlanzka *et al* 1999).

Another difference is the need for yeast on solid media to react to a complexity of nutrient and metabolite gradients and transfer nutrients from the media to cells far removed from the surface and passive capillary movement has been hypothesised to play a role in this (Boulton & Quain 2001; Meunier & Choder 1999). With multiple layers of cells between the surface and those above, the impact of compounds in the media is greatly reduced. Cells cultivated in liquid media are likely to be more homogeneous since they have an increased surface, are in contact with the media, and are more likely to be able to interact to nutritional compounds. The wort media base in these experiments was used to simulate how the yeast react at the start of fermentation, when they are introduced (pitched) into the media. Levels of ethanol can be present in stored yeast from previous fermentations, before they are re-pitched into further fermentations, but it should be noted that under fermentation conditions the yeast are only in contact with the highest levels of carbohydrates at the start of ferementation, as the levels reduce as the fermentation continues.Each yeast strain was also challenged with menadione and H_2O_2 to provide an indication of their tolerances to oxidative stress in order to provide a benchmark for subsequent analysis (Chapter 5.). Using spot plate analysis it was clear that both challenges created a negative effect on the growth of yeast, especially at high concentrations (3mM and 6mM for menadione and H_2O_2 respectively). In general it was found that SMCC100 was the most tolerant to stress on solid media, followed by SMCC99 and then SMCC90. When a more focused analysis of growth kinetics was conducted in liquid media supplemented with menadione it was noted that there was not a significant difference between strains irrespective of the level of stress applied. This was interesting since Flattery-O'Brien *et al* (1993) previously observed that

S. cerevisiae challenged with 0.5mM-1mM menadione amounted to a lethal dose. Our data contradicts this since growth was observed in wort containing 3mM menadione. It is likely that this may be a product of the robust nature of brewing yeast strains, potentially to their ploidy (Dunn & Sherlock 2008; Sanchez *et al* 2012).

Analysis of the impact of oxidative stress on growth kinetics for SMCC100 indicated that lag and exponential phases were the most severely affected. The effect of menadione on yeast growth kinetics is likely to be a direct function of the stress factor provided rather than any other growth related phenomenon. Under oxidative stress yeast must maintain the redox balance of the cell in order to survive. This can occur in several ways: a non-enzymatic response including implementation of glutathione and thioredoxin pathways; an enzymatic response such as production of catalase and glutathione peroxidase; and increasing NADPH reduction enzymes present in the pentose phosphate pathway. These stress responses occur as part of the general stress response in yeast, and the result is that they increase metabolic pressure for the reduction and removal of free radicals, which could explain the changes to growth kinetics observed. Differences between strains are more difficult to explain, but are likely to be due to differences in antioxidant capacity as investigated in Chapter 4. It should also be noted that concentrations of oxidants can convey a level of resistance to oxidative stress, effectively priming the oxidative responses in yeast (Jamieson 2008), which may lead to enhanced tolerance in certain yeast strains.

The ability to withstand stress can give an indication of a strains ability to cope with the taxing environment of VHG fermentations characterised by elevated sugar, ethanol and potentially oxidative stress factors. It should

be recognised that the glucose levels applied in this study were well above those used in potential VHG lager brewing (20°P). Some yeast strains are able to tolerate from 16% (Casey *et al* 1984) to 20-23% (Hayashida *et al* 1974), values which represent higher levels than were observed in this study. However, it has been shown that slowly increasing ethanol levels can lead to higher final tolerances (Benitez *et al* 1983; van Uden 1985), and as such yeast undergoing fermentation may be able to tolerate higher levels than those observed here. This also needs to be considered when rationalising the data obtained. Although there have been relatively few reports on oxidative stress during VHG lager fermentations, analysis of wine fermentations have shown that oxidative stress occurs throughout fermentation (Landolfo *et al* 2008). Oxidative stress during VHG forms the subject of Chapter 4. However, this preliminary characterisation indicates that SMCC99 may be the best equipped to thrive in an oxidative environment, while SMCC90 may be suitable for VHG due its relative tolerance to high glucose and ethanol. Strain SMCC100 appeared to be highly tolerant to stress on solid media, but was relatively sensitive when this was presented in a liquid form. The reason for the poor tolerance of this strain is currently unknown, however this strain is particularly important from a commercial perspective. Consequently, despite its physiological characteristics, this yeast was chosen for further analysis with the aim of gaining a greater insight into the challenges this yeast faces under VHG conditions.

CHAPTER 4: THE RELATIONSHIP BETWEEN WORT GRAVITY AND YEAST OXIDATIVE STRESS

4 The relationship between wort gravity and yeast oxidative stress

4.1 Introduction

During fermentation the primary role of the yeast culture is to convert sugars into alcohol, however the ability of the yeast to adapt to the wort environment is critical in creating a high quality and consistent product (Lagunas 1993; Gibson *et al* 2003). At the start of fermentation the culture is introduced to an aerobic environment; wort is oxygenated, typically at a rate of 1ppm per degree plato (Stewart 1999). Oxygen is an important factor in yeast health and consequently fermentation performance due to its role in the biosynthesis of sterols (Andreason & Stier 1953; David & Kirsop 1973; Section 1.3.1), which have an integral role in the structural integrity of cell membranes (Proudlock *et al* 1968).

Despite the importance of oxygen to yeast membrane health, oxygen is also known to have a toxic effect on yeast cells, primarily through the production of free radicals such as superoxide anions and hydroxyl radicals (Clarkson 1990). These reactive oxygen species (ROS) are normal by-products of cellular activity (Herrero *et al* 2008) and can cause lipid peroxidation (Girotti, 1998), protein inactivation (Cabiscol *et al* 2000), and damage to nucleic acids within the nucleus (Salmon *et al* 2004; Ribeiro *et al* 2006) and mitochondria (O'Rourke *et al* 2002; Doudican *et al* 2005; Gibson *et al* 2006). In terms of brewery yeast handling, ROS are frequently associated with respirative metabolism which occurs during propagation and potentially during the early stages of fermentation (Section 1.1.5). However, ROS have also been shown to be produced throughout fermentation through oxidation reactions as well as via the reduction of

oxygen containing molecules present in the cells. For example, this occurs during glycolysis and ethanol production, flavour generation and importantly via cytochrome P450 enzymatic events (Landolfo *et al* 2008; Herrero *et al* 2008; Mendes-Ferreira *et al* 2010; Section 1.5.4.2*)*. During the catalytic cycle of cytochrome P450 the reduction of oxygen containing molecules occurs (such as haem), potentially releasing ROS in the forms of superoxide anions and hydrogen peroxide through so-called 'leaky' branches into cell (Zangar *et al* 2004). The continuous production of ROS from this system is an inevitable result of NADPH consumption by the P450 pathway, although the level of 'leakiness' is dependent on wort composition, pH and other factors (Zhukov & Archakov 1982; Gorsky *et al* 1984; Blanck *et al* 1991).

The significance of free radical production to brewing is that, in the presence of elevated levels of ROS, the yeast stress response systems are promoted, glycolytic enzyme expression can be reduced, and the cells ability to produce ethanol may be compromised. (Mendes-Ferreira *et al* 2010). However, cells have mechanisms that help to detoxify ROS, allowing the cell to limit the occurrence of cellular damage. Antioxidant defence mechanisms include superoxide dismutase (SOD), glutathione peroxidise (GPx) and catalase. Yeast contain two forms of SOD: Mn-SOD, which is thought to provide protection from respiratory sources of oxygen radicals (Loon *et al* 1985; Quidot *et al* 1993); and Cu,Zn-SOD, which is reported to be cytosolic or peroxisomal (Kellar *et al* 1991; Slekar *et al* 1996). Both forms of SOD acts to convert the superoxide anion into hydrogen peroxide which can then be broken down by GPx and catalase into water and oxygen. SOD therefore represents the first line of defence

against ROS, although the presence of each of SOD, GPx and catalase can be used to indicate the cells ability to prevent redox imbalance.

Although a great deal is understood about the potential effects of oxygen on fermentation (David & Kirsop 1972; Clarkson *et al* 1990; Gibson *et al* 2006), very little is known about the links between free radical accumulation and oxidative stress, particularly at high gravity. The aim of this study was firstly to compare the fermentation characteristics of SMCC100 lager yeast in standard (15°P) and VHG (22°P) fermentations, and subsequently to determine the impact of wort gravity on ROS accumulation and cellular antioxidants.

4.2 Results

Lager strain SMC100 was chosen for further analysis due to its commercial value, despite the fact that it was not always the most tolerant to oxidative stress (Chapter 3). 10L fermentations were conducted to compare stress associated with standard (15ºP) and VHG (22ºP) wort (Sections 2.2.2.1 and 2.2.2.2). Three generations of yeast were used for comparative analysis: G0 freshly propagated yeast; G1 yeast cropped from the end of G0 fermentation; and G2 yeast cropped from the end of the G1 fermentation. This was performed to take into account differences associated with yeast generation number (Miller *et al* 2012; Smart and Whisker 1995). Standard (15°P) fermentations were pitched at $1.5x10^7$ cfu/mL and VHG (22°P) fermentations were pitched at $2.2x10^7$ cfu/mL. Prior to pitching, 15°P wort was oxygenated to 15ppm and 22°P wort was saturated to 22ppm oxygen.

4.2.1 **Analysis of fermentation, ROS and antioxidants during 15°P lager fermentations**

The fermentation characteristics of yeast strain SMC100 were determined by analysing fermentation progression, alcohol production, cell number and cell viability during standard (15°P) fermentations. In addition, ROS accumulation and the antioxidant response were also analysed to provide an understanding of yeast oxidative stress throughout fermentation. This allowed for a comparison between yeast generations to be made, as well as establishing a baseline for subsequent analysis of VHG (22°P) fermentations. In all instances, 10L small scale fermentations were conducted and samples were analysed for specific gravity and alcohol content (Section 2.9.1.1). Total cell count and viability were monitored using microscope count in conjunction with methylene blue staining (Section 2.4.1). In addition, the percentage of cells showing ROS accumulation was determined using the fluorescent stain Di-hydroethidium (DHE) (Section 2.9.2.1). In conjunction, cell lysates were analysed for glutathione peroxidase activity (Section 2.9.2.2.3), intracellular superoxide dismutase concentrations (Section 2.9.2.2.4), and intracellular catalase levels (Section 2.9.2.2.4).

4.2.1.1 Fermentation performance of SMCC100 lager yeast at standard gravity (15°P)

Analysis of the fermentation characteristics of SMCC100 at 15°P indicated that freshly propagated yeast (G0) took longer to reach attenuation (162 hours), than re-pitched yeast G1 and G2 yeast, which both required 138 hours to fully attenuate (Figure 4.1). Furthermore, G1 and G2

fermentations exhibited a lag phase of 6 hours compared to 24 hours for the G0 fermentation. This data is in line with previous reports (Miller *et al* 2012), indicating that serially re-pitched yeast displays a general reduction in lag phase when compared to G0 yeast.

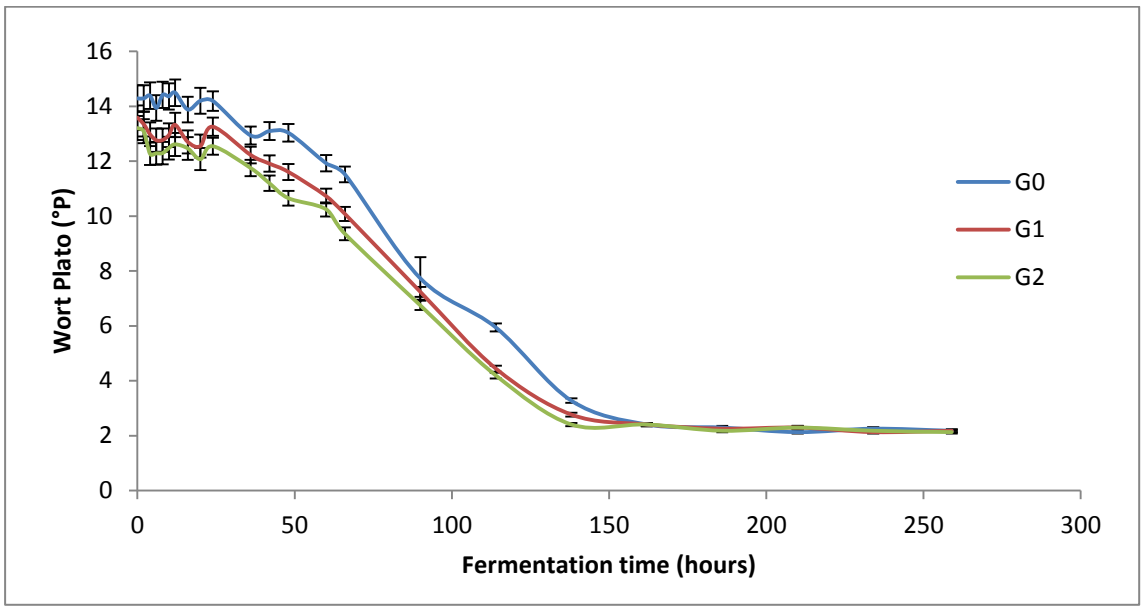


Figure 4.1 Fermentation of 15°P wort by strain SMCC100 in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

Analysis of the concentration of alcohol produced during standard (15°P) fermentations showed that although the same level of alcohol was achieved by completion (6.2 ±0.2%), G1 and G2 fermentations reached this at 140 hours, compared to the G0 fermentation which required 160 hours (Figure 4.2). Alcohol data corresponds to what might be expected from attenuation of a 15°P wort, where the maximum alcohol production according to Balling's formula would be 6.74% (data not shown). These

results indicated that in standard (15°P) fermentations, although G0 yeast performed slower than re-pitched yeast, ultimately the final alcohol yield was unaffected.

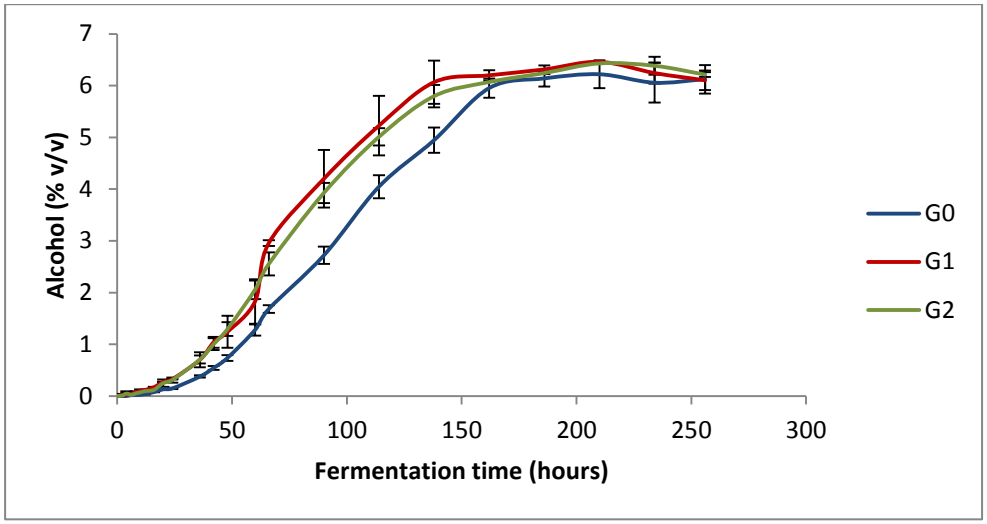


Figure 4.2 Alcohol production during fermentation of 15°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

Analysis of yeast health during fermentation indicated that in each instance the viability of the population remained above 98% (Figure 4.3). It should be noted that there was a decrease in viability for all fermentations at around 8 hours to ~96% viability. This may reflect the onset of division, since it is known that dividing cells may often appear dead with methylene blue staining leading to errors (O'Connor–Cox *et al* 1997). Irrespective, apart from this data point, all fermentations maintained a high viability throughout. Monitoring cell count indicated a 3 fold increase in the number of yeast cells present following pitching; an initial cell count of $1.5x10^7$

cells/ml was observed to increase to $1.23x10^8$ cells/mL in all instances (Figure 4.4). As indicated above, cell growth was observed to be slower for G0 fermentations in accordance with fermentation lag phase data.

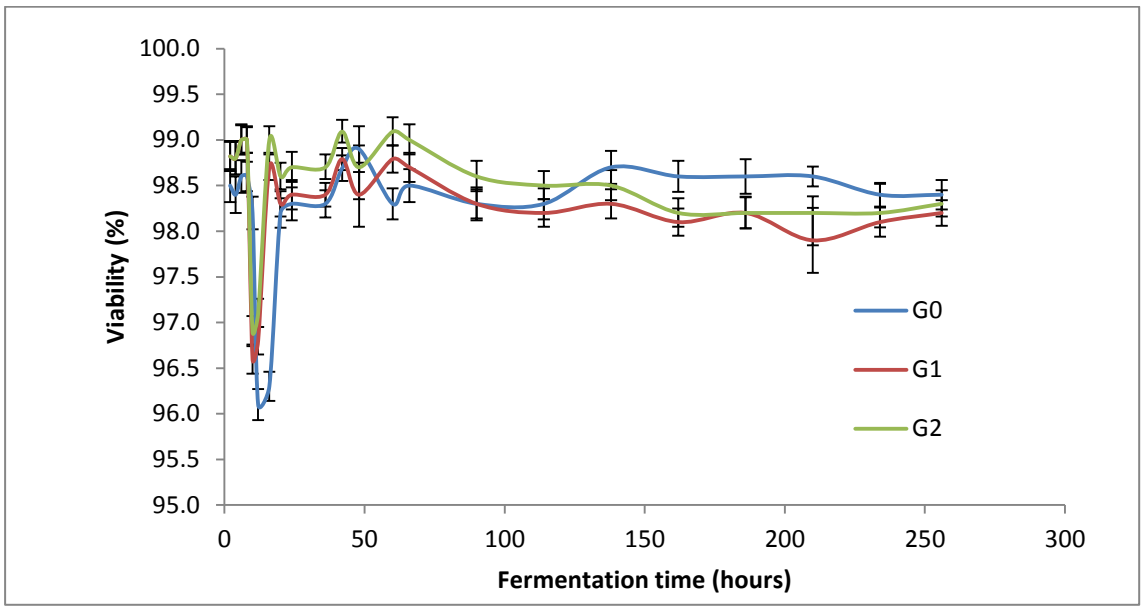


Figure 4.3 Viability of strain SMCC100 during fermentation of 15°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

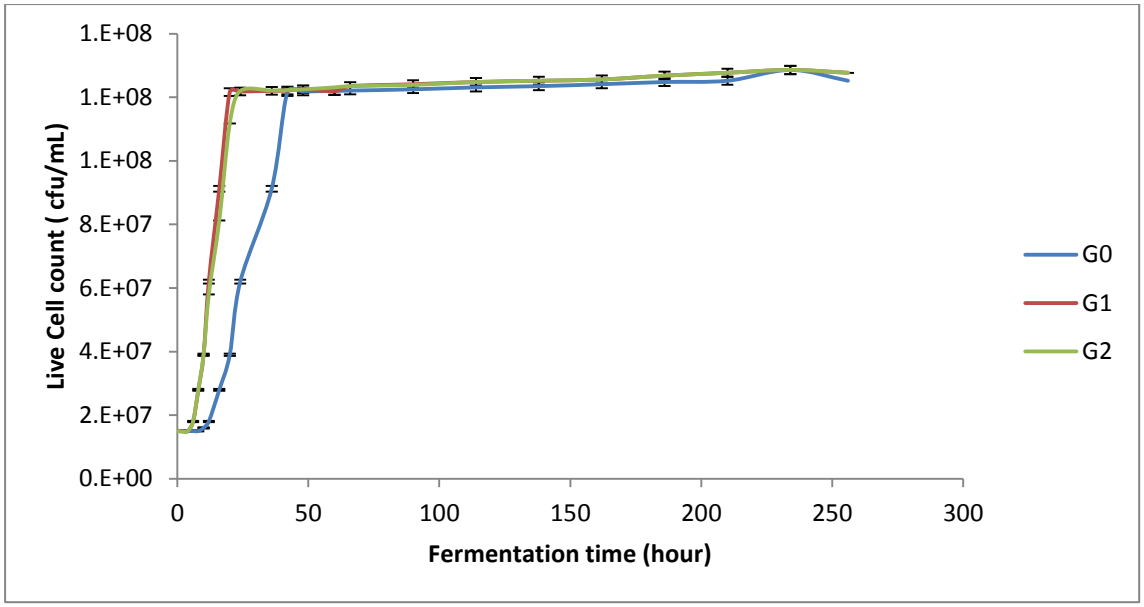


Figure 4.4 Analysis of total cells during fermentation of 15°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

Assessment of oxygen uptake showed that in G0 fermentations all oxygen was assimilated by 2.8 hours, while in G1 and G2 fermentations 3.6 and 5.3 hours were required, respectively (Figure 4.5). This pattern of results was perhaps surprising given that G0 yeast should already have been oxygen saturated, one hypothesis is that rapid uptake may be an artefact of the molecular condition of cells, with genes responsible for sterol production being upregulated. The difference in oxygen uptake between G1 and G2 populations is less easy to explain. However, for G2 yeast slow uptake may have been an artefact of the reduced physiological state of cells (vitality) associated with serial repitching, even though viability was good.

Analysis of pH during fermentation revealed a general reduction from pH 5.2 to a final pH of 3.8, in line with expectations. G1 and G2 fermentations displayed a faster decrease in pH closely matching the fermentation progression data shown above. It was observed that in G0 fermentations, a time of 162 hours was required for pH to stabilise, while for G1 and G2 fermentations a time of ~138 hours was required (Figure 4.6). Irrespective of generation number, the final pH was observed to be consistent at around 3.8.

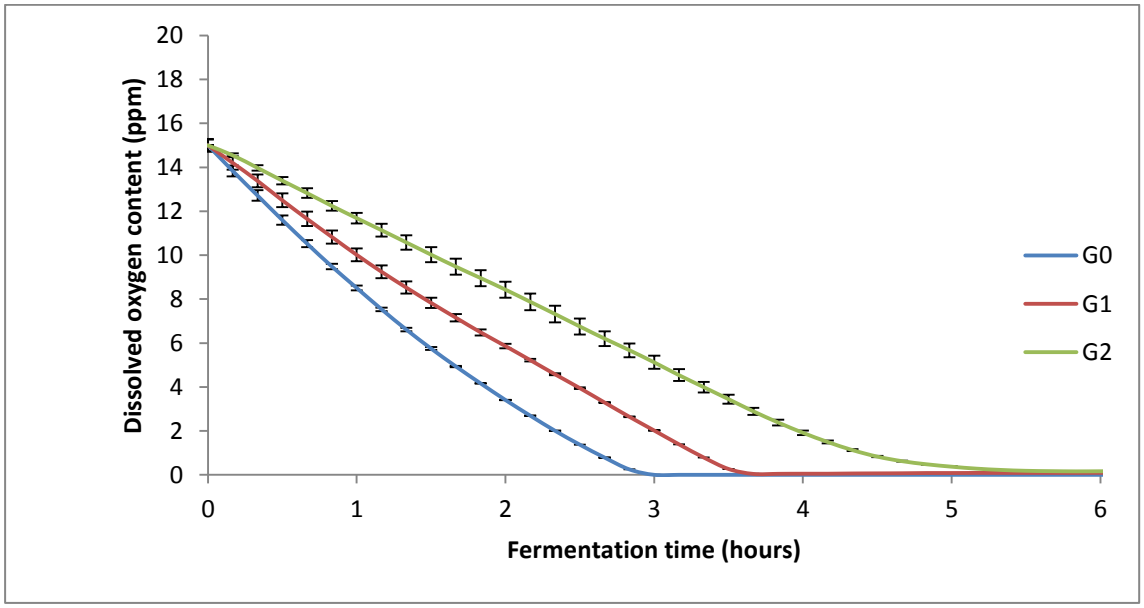


Figure 4.5 Dissolved oxygen (DO) content of wort during 15°P fermentations in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

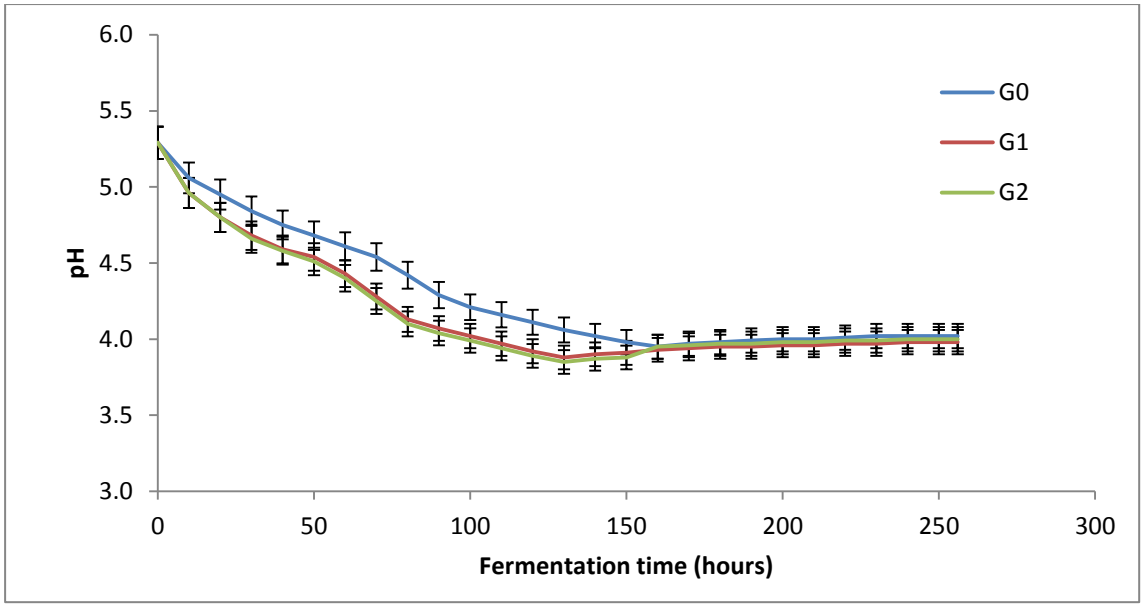


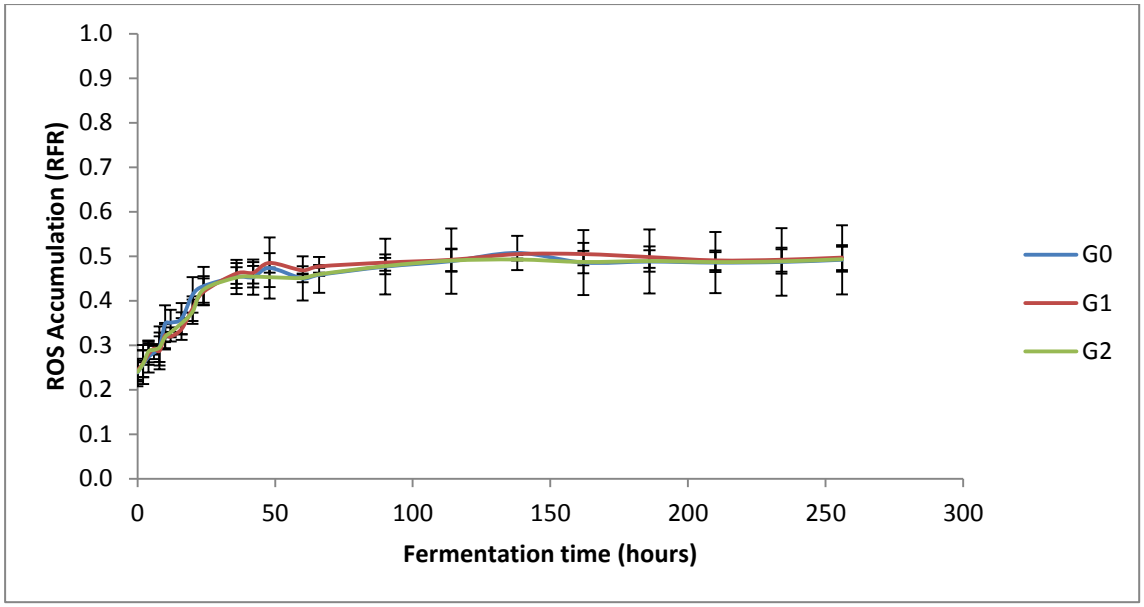
Figure 4.6 Analysis of pH during 15°P fermentations in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

4.2.1.2 Analysis of ROS and antioxidants during fermentation of 15°P wort using lager yeast SMCC100

In order to determine the level of ROS accumulation and the enzymatic cellular response of lager yeast SMCC100 to standard (15°P) gravity fermentations, the generation of compounds which act as biomarkers for oxidative stress were investigated, including ROS, glutathione, SOD and catalase. Each compound was determined by a series of fluorescent stains as described in Sections 2.9.2.2.2-2.9.2.2.2.4

4.2.1.2.1 Analysis of ROS during fermentation of 15°P wort

An indication of oxidative stress/damage was obtained by evaluating ROS accumulation using di-hydroethidium (DHE) staining and measuring fluorescence at 518/605nm. DHE is taken up by cells where the presence of ROS causes conversion of DHE to ethidium, which then binds to cellular DNA and fluoresces (Section 2.9.2.2.1) Samples were analysed using a fluorescence plate reader and a ratiometric quantification was employed to compare the levels of ROS accumulation of each sample. $1x10^7$ yeast were incubated in the presence of 10mM H_2O_2 for 1hr to cause a maximum oxidative stress. These cells were then used as a baseline for ratiometric quantification, with a value of 1 indicating 100% ROS accumulation by cells and 0 as an indication of the absence of stress. Analysis of DHE fluorescence during fermentations indicated that the ratio of cells displaying ROS accumulation increased during fermentation (Figure 4.7). This was despite the fact that viability remained at above 98%, as indicated previously (Figure 4.3), indicating the continual occurrence of low level cellular oxidative stress during fermentation. Accumulation of ROS within cells reached a maximum at 144hours (Figure 4.7), with an RFR (Relative Fluorescent Units) of 0.49AU irrespective of generation number. However, it should be noted that the occurrence of ROS generation was mainly observed within the first 50 hours at the start of fermentation (Figure 4.7). Considering that oxygen was depleted from the fermentation vessel within 5-6 hours (Figure 4.5), this indicated that the increase in ROS was not solely attributed to the 'aerobic' phase of fermentation, but that generation of ROS occurred throughout the anaerobic phase of fermentation as well as discussed in Section 1.5.4.



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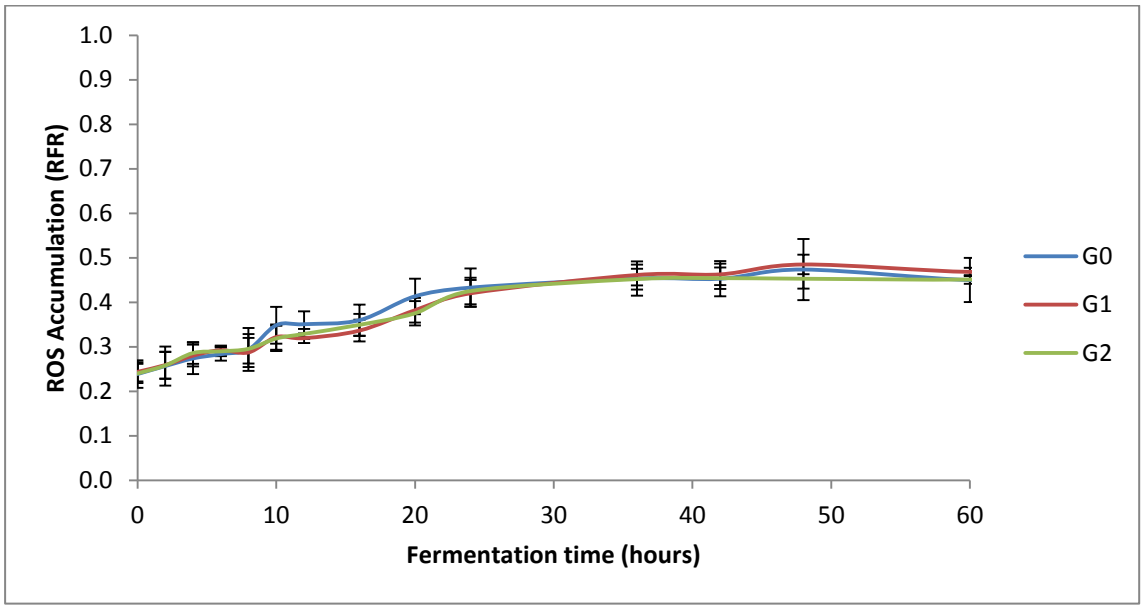

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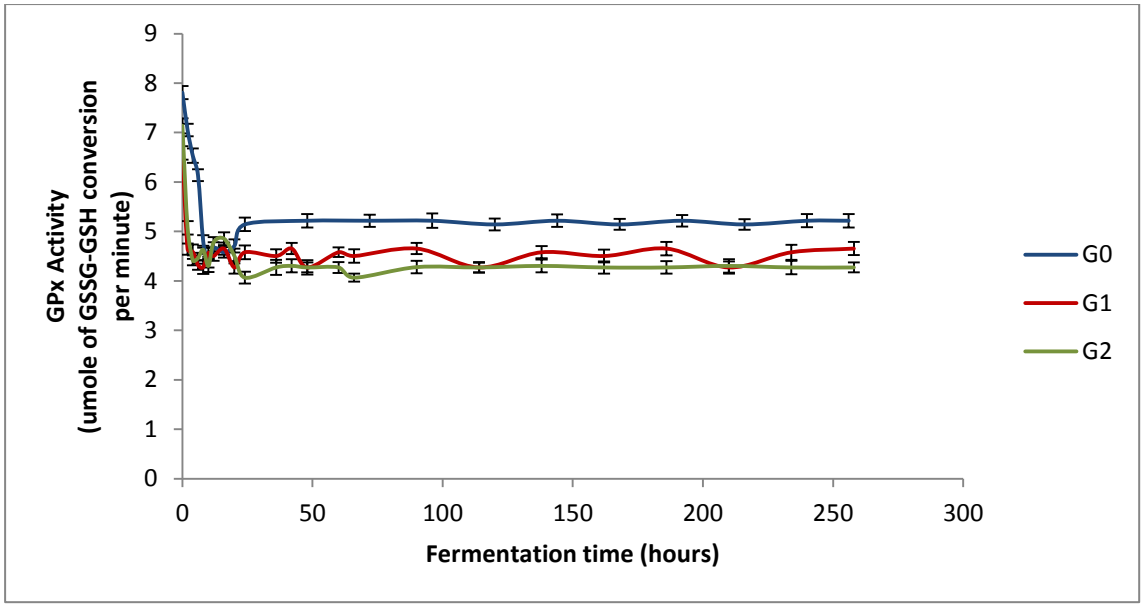
Figure 4.7a-b ROS accumulation during fermentation of 15°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Accumulation of ROS was determined over 256 hours (a) and data for the first 60 hours is also summarised (b). Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

4.2.1.2.2 Analysis of glutathione peroxidase activity during fermentation of 15°P wort

Glutathione peroxidase (GPx) is known to be produced in response to oxygen free radical generation and was consequently evaluated through fermentation. Concentrations of glutathione peroxidase activity were measured through a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG) is produced upon reduction of hydroperoxide (in this instance cumene hydroperoxide) by GPx, which is then recycled to its reduced state by GR and NADPH to glutathione (GSH) and monitored by measuring absorbance at 340nm (Section 2.9.2.2.4). Analysis of glutathione activity indicted that this compound was elevated at the start of fermentation with between 6.59±0.14 and 7.81±0.12 µmole of GSSG-GSH conversion per minute, depending on the generation number (Figure 4.8). When closer observation of the data was undertaken (Figure 4.8b), the activity level can be seen to reduce over the first 4 hours of G1 and G2 fermentations and over the first 10 hours of the G0 fermentation. Despite this, the activity of glutathione was observed to stabilise at approximately 4.5±0.31µmole of GSSG-GSH conversion per minute for G1 and G2 fermentations, and at a slightly higher value of 5.2±0.1 in G0 fermentation (Figure 4.8a). Although the percentage of cells demonstrating ROS accumulation was relatively consistent between generations, this indicates that the G0 fermentation responded in a different manner to the re-pitched G1 and G2 fermentations. The most likely reason for this difference is related to the stresses that the G1 and G2 yeast had already been subjected to previously. Unlike the G0 yeast, the re-pitched G1 and G2 yeast had undergone previous fermentations indicating that they may already have been exposed not only to the oxidative stress, but a combination of ethanol and nutritional related (starvation) stress factors.



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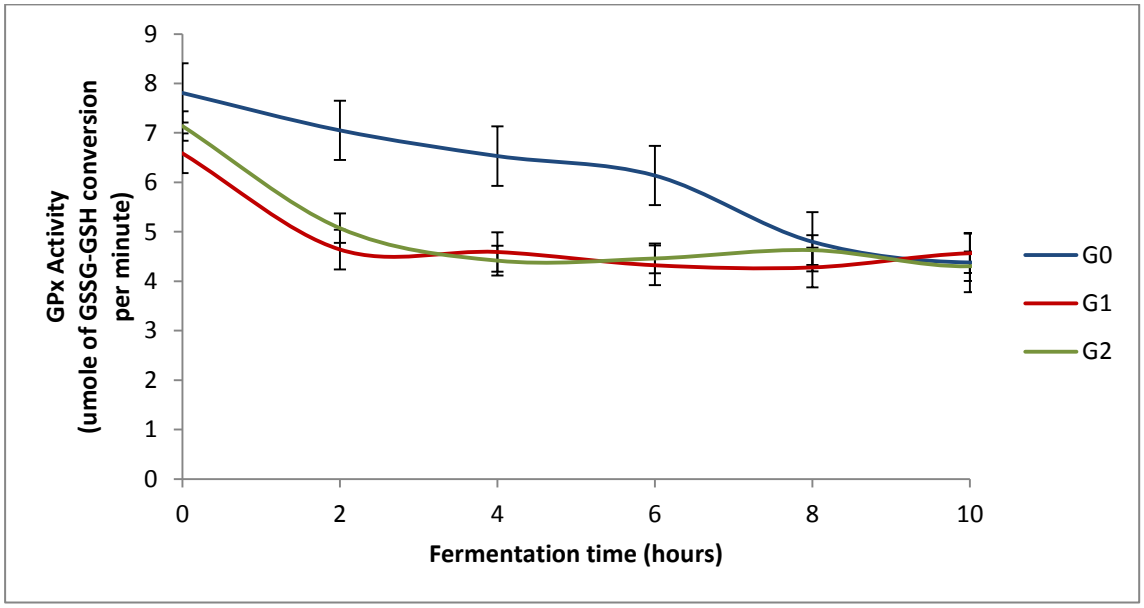


B

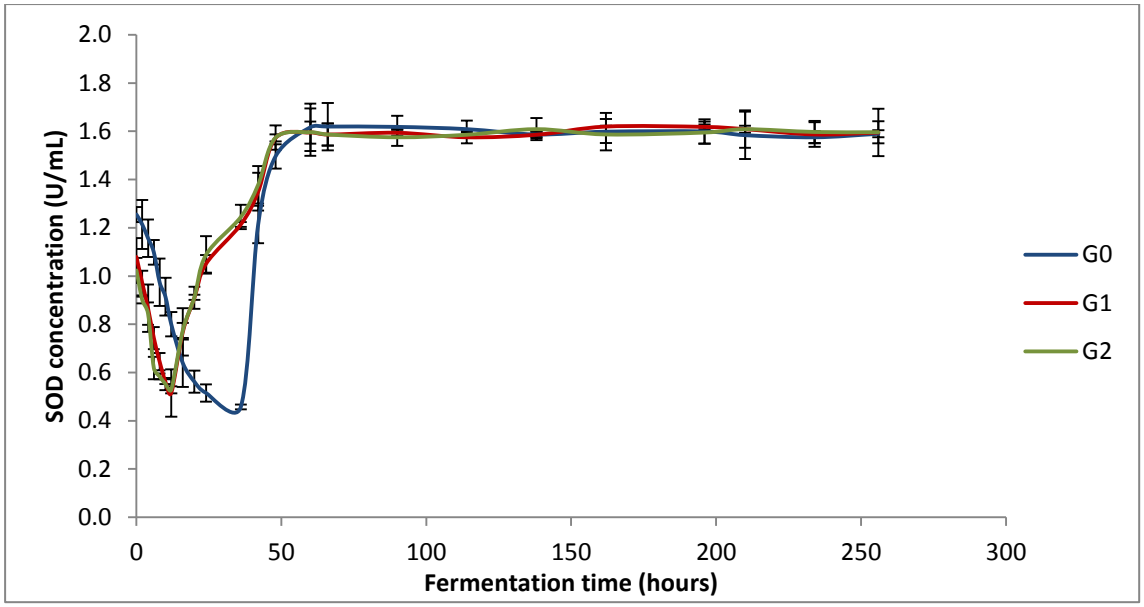
Figure 4.8a-b Glutathione peroxidase (GPx) (Umole of GS-SG per minute) activity during fermentation of 15°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Gpx activity was determined over 256 hours (a) and data for the first 10 hours is also summarised (b). Data Shown represents the mean of triplicate samples with error bars indicating the standard deviation.

4.2.1.2.3 Analysis of superoxide dismutase during fermentation of 15°P wort

Superoxide dismutase (SOD) is an enzymatic biomarker of oxidative stress since it is known to be produced in response to oxygen free radicals. For each fermentation, the availability of free SOD (i.e. not total SOD) was measured using a coupled enzymatic reaction. In the presence of hydrogen peroxide xanthine is reduced to water and uric acid. If SOD is present, the rate at which xanthine is reduced is lowered causing a colour change which was measured at 450nm. (Section 2.9.2.2.2).

The availability of intracellular SOD indicates the cells ability to prevent redox imbalance caused by ROS. Analysis of the available intracellular SOD throughout fermentation at 15°P indicated that initial levels were approximately 1 U/mL (G1 and G2 fermentations) and 1.25 U/mL (G0 fermentation) which was then rapidly reduced to 0.6 U/mL in all fermentations. After this point SOD production was stimulated to significantly higher concentrations >2.5 U/mL (Figure 4.9). The observed initial decrease in available SOD was not surprising given that this stage of fermentation reflects the period of oxygen utilisation metabolism, known to be closely associated with ROS formation. However, a stronger enzymatic response may have been expected in order to maintain the amount of free SOD within the cell. It is possible that the decrease in SOD observed could represent an initial mobilisation of resources, possibly as intracellular reserves were utilised in response to ROS generation as shown in Figure 4.7. Interestingly, previous studies have indicated that the activity of SOD genes may be highest at the start of fermentation, when SOD is required to detoxify the cell (Tan *et al* 2009 and Halyna *et al* 2012). Therefore, the initial drop in intracellular SOD observed at the start of fermentation (Figure

4.8b) could be a response to initial oxidative stress, whereby a high proportion of the available SOD is used by the yeast to degrade intracellular ROS. However, SOD concentration may also be influenced by cell division, since enzymes are distributed between mother and daughter cell prior to cytokinesis. Although similar patterns were seen in all populations, the profile of SOD availability was notably different in the G0 fermentation when compared to the G1 and G2 fermentations. The re-pitched fermentations had a swifter antioxidant response, responding in 12 hours whereas in the G0 fermentation it took 36 hours. However, it is interesting to note that the minimum concentrations of available SOD were similar: 0.53U-mL for G1 and G2 yeast and 0.46U/mL for G0 yeast. Consequently, it is possible that the G0 yeast was better equipped to defend against the presence of ROS due to the fact that it had transitioned from an aerobic environment. This is supported by the greater concentration of SOD at time 0 and indicates that the cellular response to ROS may only have been initiated when the concentration of available SOD reached a specific threshold. In addition, such an effect may be compounded by the fact that there may be a 4 hour lag in activation of SOD genes when moving from aerobic to anaerobic environments (Dirmeier *et al* 2002). As such this difference may be due to the time required for aerobically propagated yeast to adapt to the fermentation environment before being able to actively respond to fermentation related stress factors.



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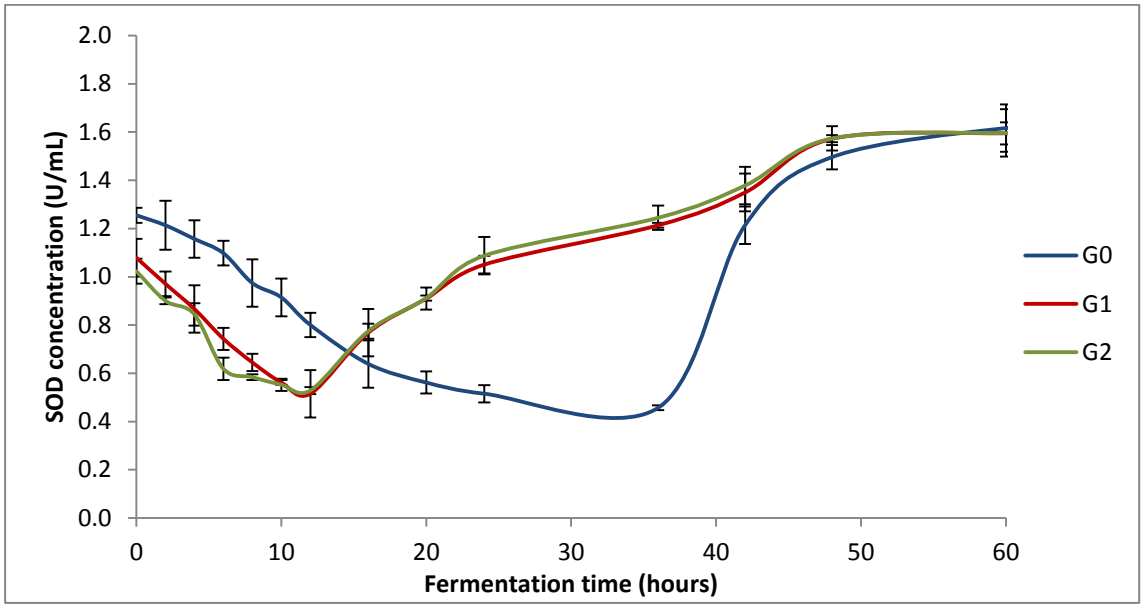


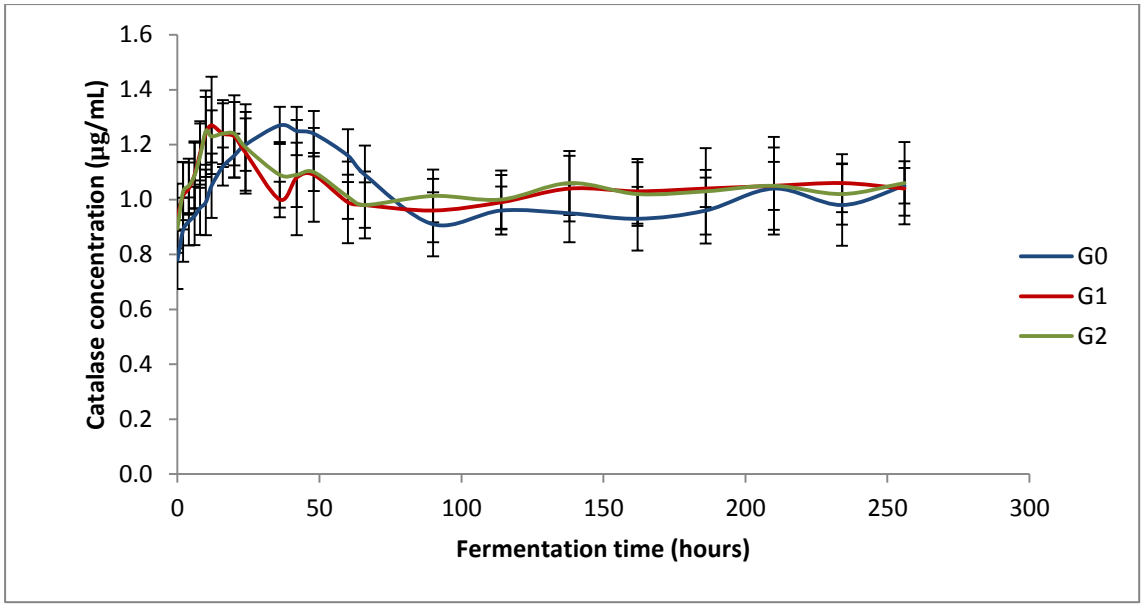
B

Figure 4.9a-b Intracellular superoxide dismutase (SOD) concentration (U/mL) during fermentation of 15°P in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. SOD concentration was determined over 256 hours (a) and data for the first 60 hours is also summarised (b). Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

4.2.1.2.4 Analysis of catalase during fermentation of 15°P wort

In a similar fashion to SOD, catalase acts as an enzymatic biomarker of oxidative stress, and functions to detoxify the cell by converting hydrogen peroxide into water and oxygen. Given its role in cell homeostasis, the intracellular catalase concentrations during standard fermentation (15°P) were also assessed. The concentrations of catalase were measured through a coupled enzymatic reaction where, in the presence of catalase, hydrogen peroxide is reduced to water and oxygen. Unreacted hydrogen peroxide reacts with Amplex Red reagent with a 1:1 stoichometry in the presence of horseradish peroxidase (HRP) to produce resorufin, a fluorescent product which can be measured at 540/585nm (Section 2.9.2.2.3).

Analysis of yeast intracellular catalase during standard fermentation revealed that there was only a small increase throughout the fermentation (Figure 4.8a). Levels of catalase varied between generation number, where G0 yeast reached a peak in catalase levels at 36 hours (1.27 µg/mL catalase), while G1 and G2 reached a peak at 12 hours. At this point, G1 yeast slurry exhibited 1.23 µg/mL catalase and G2 yielded 1.25 µg/mL catalase (Figure 4.8b). However, after this point the amount of catalase was observed to decrease to a similar level observed at the start of fermentation. This may indicate that the yeast culture acquired a transitional adaptive response to detoxify ROS generated at the start of fermentation before other adaptive mechanisms took over. Despite this, Fernades *et al* (2007) suggested that maintaining a base level of catalase may be a strategy allowing yeast cells to rapidly promote protection against ROS at a particular point in the future (Fernandes *et al* 2007).



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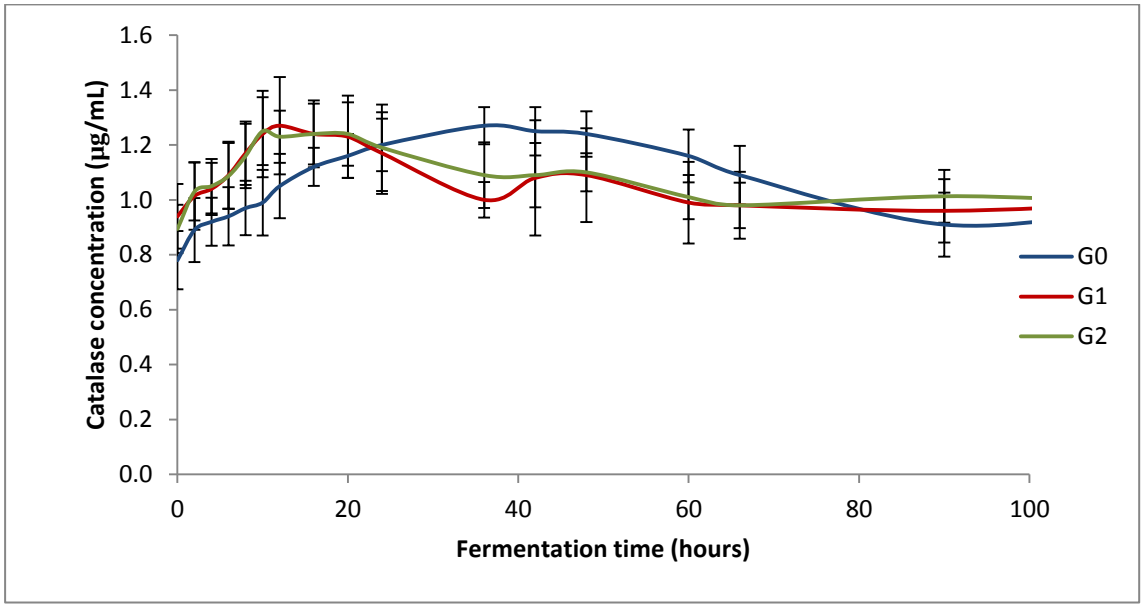


B

Figure 4.10 Intracellular catalase concentration (µg/mL) during fermentation of 15°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Catalase concentration was determined over 256 hours (a) and data for the first 100 hours is also summarised (b). Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

4.2.2 **Analysis of fermentation, ROS and antioxidants during 22°P lager fermentations**

In a similar fashion to the previous analyses above, the fermentation characteristics of SMC100 at high gravity (22°P) were determined by analysing fermentation progression, alcohol production, cell number and cell viability. In addition, ROS accumulation and the antioxidant response were analysed as a means of comparing to the previous data obtained at standard gravity (15°P). As in the previous trials, 10L small scale fermentations were conducted and samples were analysed for specific gravity and alcohol content (Section 2.9.1.1). Total cell count and viability were monitored using microscope count in conjunction with methylene blue staining (Section 2.4.1). In addition, the percentage of cells showing ROS accumulation was determined using the fluorescent stain Di-hydroethidium (DHE) (Section 2.9.2.1). In conjunction, cell lysates were analysed for glutathione peroxidase activity (Section 2.9.2.2.3), intracellular superoxide dismutase concentrations (Section 2.9.2.2.4), and intracellular catalase levels (Section 2.9.2.2.4).

4.2.2.1 Fermentation performance of SMCC100 lager yeast at very high gravity (22°P)

Analysis of the fermentation characteristics of SMCC100 at 22°P indicated that freshly propagated yeast (G0) took the same length of time to reach attenuation (330 hours) as re-pitched yeast G1 and G2 yeast (Figure 4.11). However, observation of sugar content over time indicated that although the fermentations started at the same gravity, the G0 fermentation failed to reach the same level of attenuation as the G1 and G2 fermentations. For

G0 fermentations 330 hours was required to reach 4.9°P, compared to the re-pitched yeast (G1 and G2), which took 330 hours to reach a final attenuation of 2.5°P. It should be noted that in all instances, the time required for attenuation was extremely long. This may represent an issue in terms of total residence time, but is a fair reflection on the performance of the yeast strain under VHG conditions (Katherine Smart, Personal Communication). In addition to extended total fermentation time, it was also observed that G1 and G2 fermentations displayed a shorter lag phase (8 hours) compared to 16 hours for the G0 fermentation (Figure 4.11). Corresponding to the difference in final attenuation, analysis of alcohol production indicated that although fermentations reached attenuation at approximately the same time-point, there was a decrease in alcohol production for the G0 fermentation. This yeast population was able to create 9.8% ethanol compared to 11.6% for the G1 and G2 fermentations (Figure 4.12).

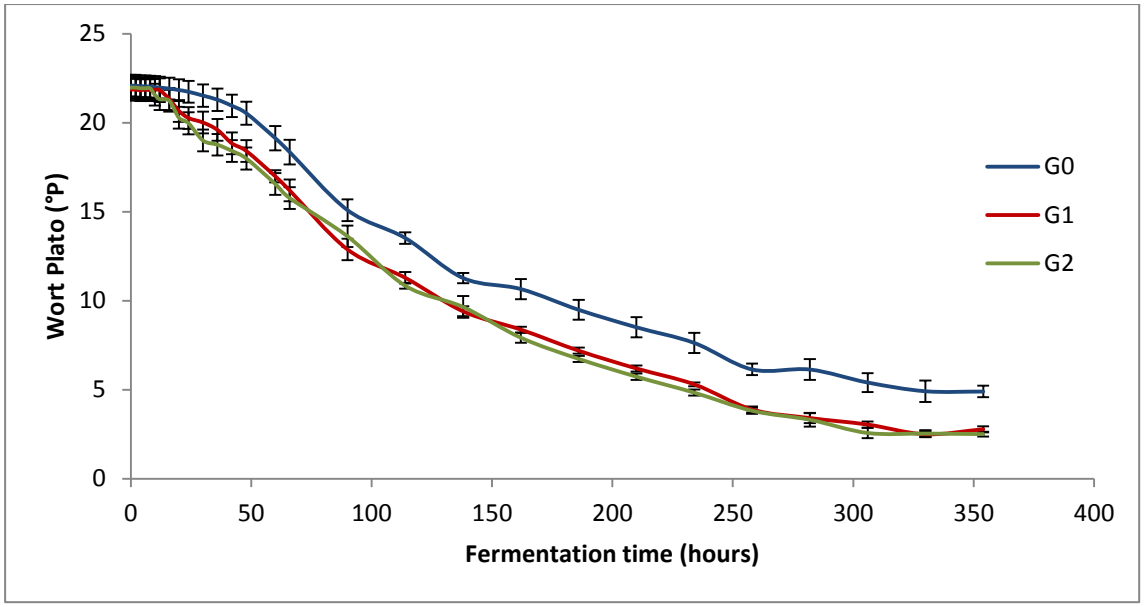


Figure 4.11 Fermentation of 22°P wort by strain SMCC100 using 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

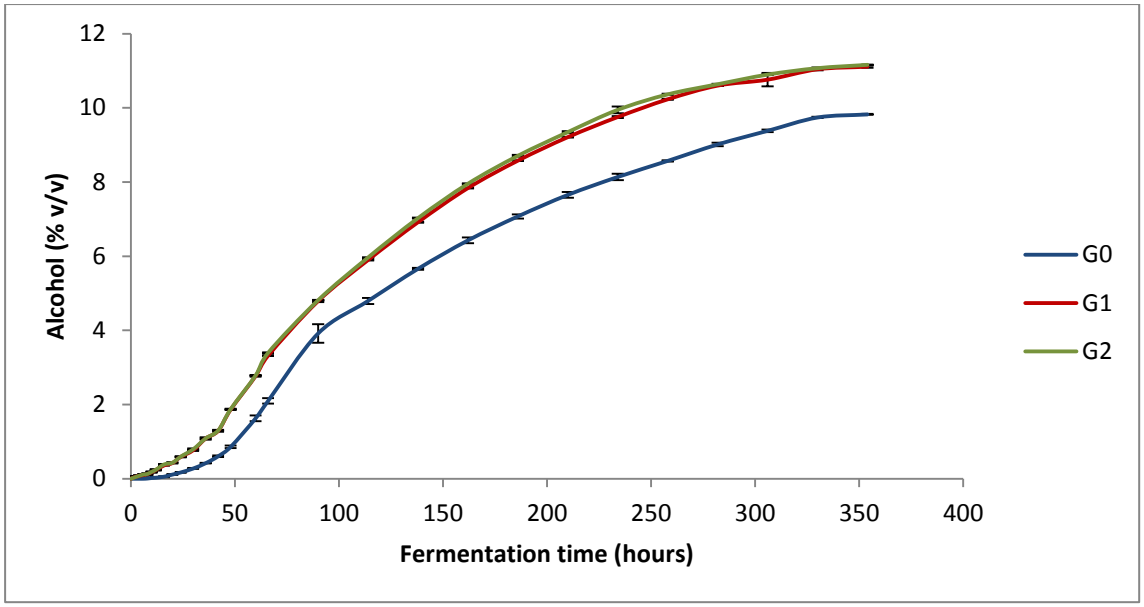


Figure 4.12 Alcohol production during fermentation of 22°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

The viability of each yeast population was observed to remain above 97% for the majority of each of the corresponding fermentations. As reported and discussed above (Section 4.2.1.1), there was a transient decrease in viability at the start of each fermentation, after which a high viability was established of around 98.5% (Figure 4.13). Towards the end of fermentation viability was observed to decline, resulting in final viabilities of approximately 96% (G0), 95% (G1) and 94% (G2). Interestingly, it can be seen that G2 yeast exhibited the greatest decline in viability, followed by G1 yeast, perhaps reflecting that damage or deterioration in quality had occurred over time, leading to a less 'fit' population.

Analysis of total yeast count indicated that there was a 4-fold increase in the number of yeast after the initial pitching of $2.2x10^7$ rising to $9x10^8$ cells/mL. This is slightly above the range normally expected to occur during fermentation, where 2-3 times the biomass is usually observed (Section 1.1.5). This may indicate that pitching rate optimisation may be required in the future for this yeast strain under VHG conditions. There was also a difference in the rate at which the G1 and G2 fermentations reached the maximal cell density, taking 12 hours, compared to 14 hours for the G0 fermentation. Despite this difference, all fermentations ultimately established the same population size (Figure 4.14).

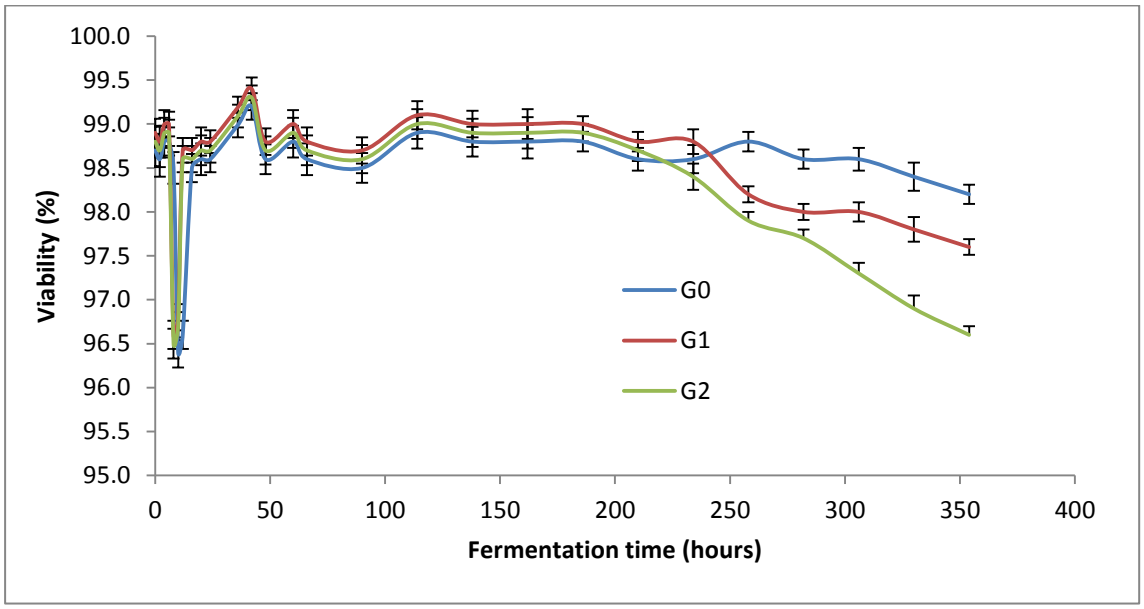


Figure 4.13 Viability of strain SMCC100 during fermentation of 22°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

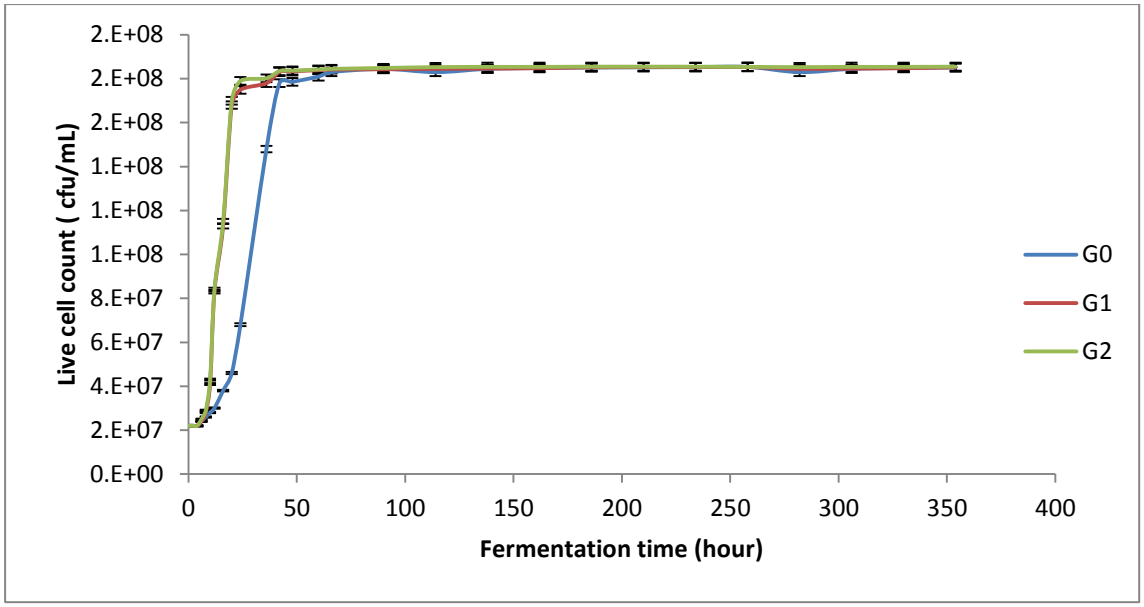


Figure 4.14 Analysis of total cells during fermentation of 22°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

Assessment of oxygen uptake showed that in G0 fermentations all oxygen was assimilated by 2.9 hours, while in G1 and G2 fermentations 3.5 and 3.8 hours were required respectively (Figure 4.13). pH was also monitored and a general reduction from pH 5.8 to a final pH of 3.8 was observed. G1 and G2 fermentations displayed a faster decrease in pH in line with fermentation progression data. It was observed that in all fermentations, a time of 90 hours was required for pH to stabilise (Figure 4.16) and that irrespective of generation number, final pH was consistent.

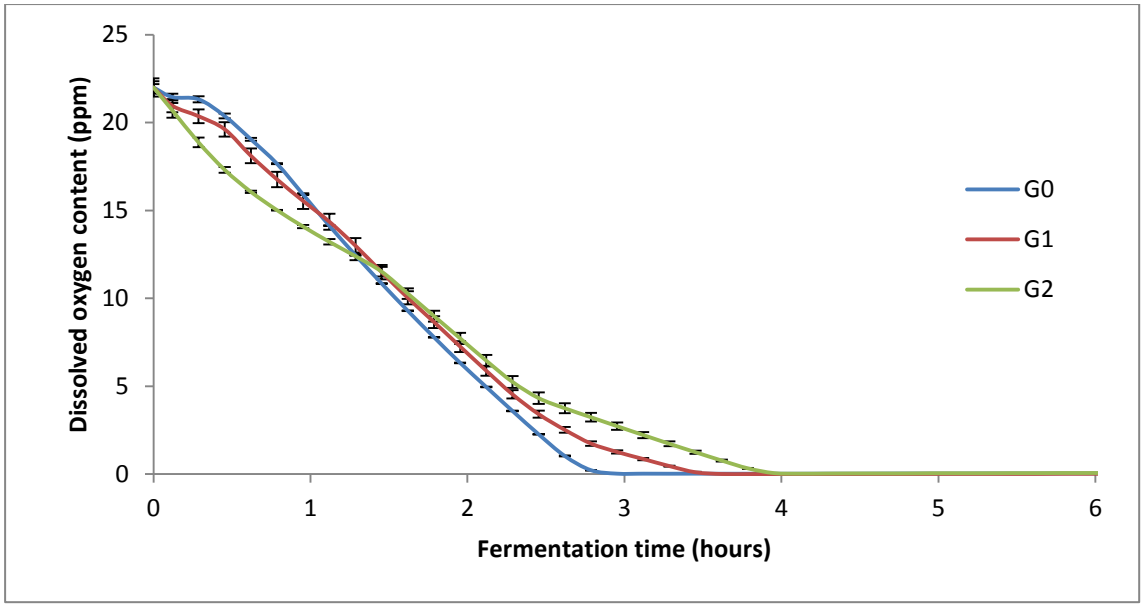


Figure 4.15 Dissolved oxygen (DO) content of wort during 22°P fermentation by SMCC100 using 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

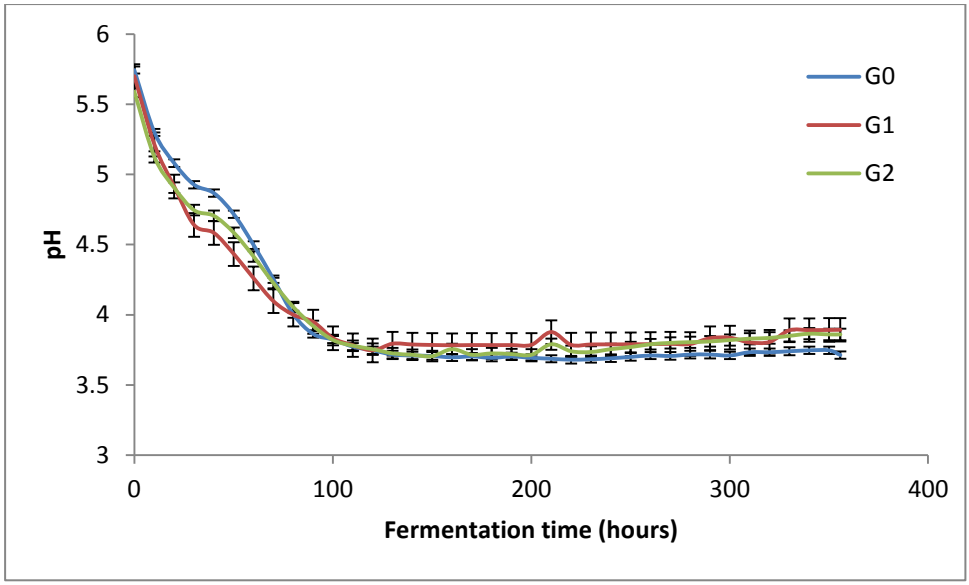


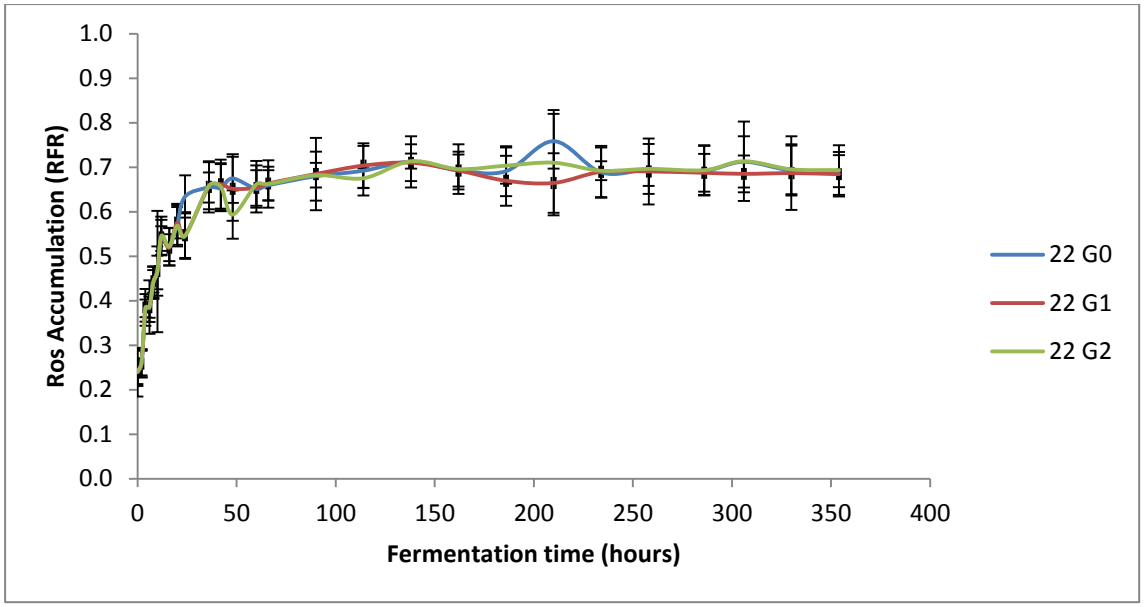
Figure 4.16 Analysis of pH during 22°P fermentation in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

4.2.2.2 Oxidative analysis of lager yeast SMCC100 in VHG wort

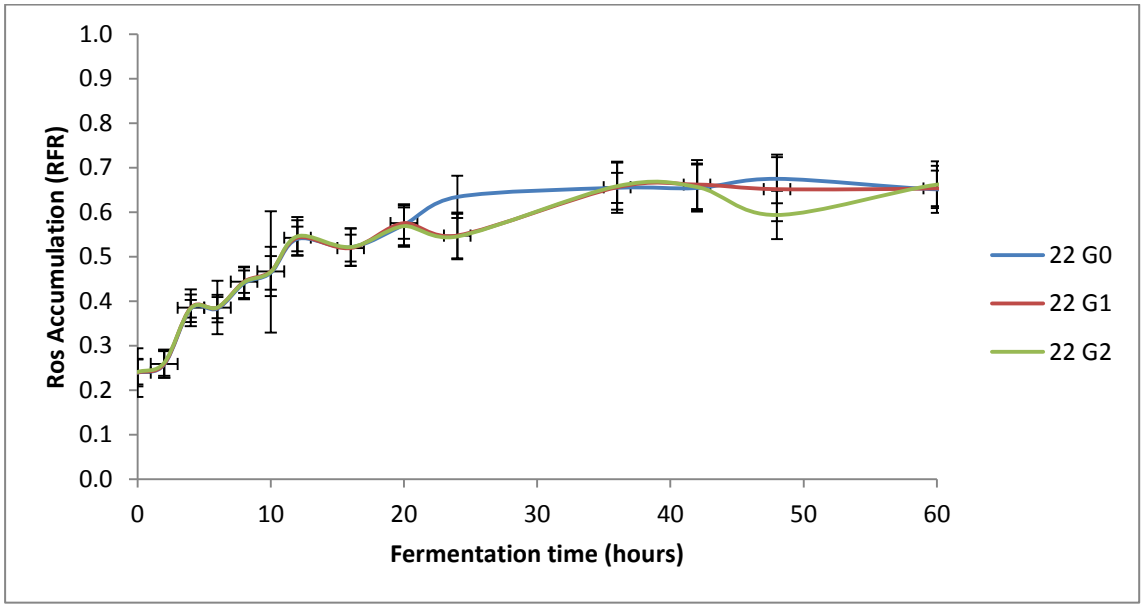
In order to determine the relationship between VHG lager fermentations, ROS production and cellular redox potential, the generation of enzymatic compounds which act as biomarkers for oxidative stress were investigated. In addition, the antioxidant response of cells during VHG fermentations was determined by analysing levels of glutathione, SOD and catalase.

4.2.2.2.1 Analysis of ROS accumulation levels in SMCC100 cells throughout VHG (22°P) fermentation

As previously stated (Section 4.2.1.2.1), ROS accumulation can be used as an indication of oxidative stress/damage, and can be analysed through the use of DHE staining. Analysis of DHE fluorescence during fermentations indicated that the number of cells displaying ROS accumulation, and as such indicating oxidative stress, occurred throughout all of the fermentations (Figure 4.17), despite viability remaining high throughout. This indicated that although cellular damage was occurring it was not causing total cell death. It was interesting to note that there was little difference between generations, with all generations reaching their peak levels of ROS accumulation at around 42 hours at an relative fluorescence ratio (RFR) of 0.69AU (Figure 4.17). As was noted with analysis of 15°P fermentations, the occurrence of ROS generation was mainly observed within the first 60 hours at the start of fermentation (Figure 4.17). As before, considering that oxygen was depleted from the fermentation vessel during the early stages of fermentation, this again indicated that the increase in ROS was not solely attributed to the 'aerobic' phase of fermentation, but that generation of ROS occurred throughout the anaerobic phase as well.



A

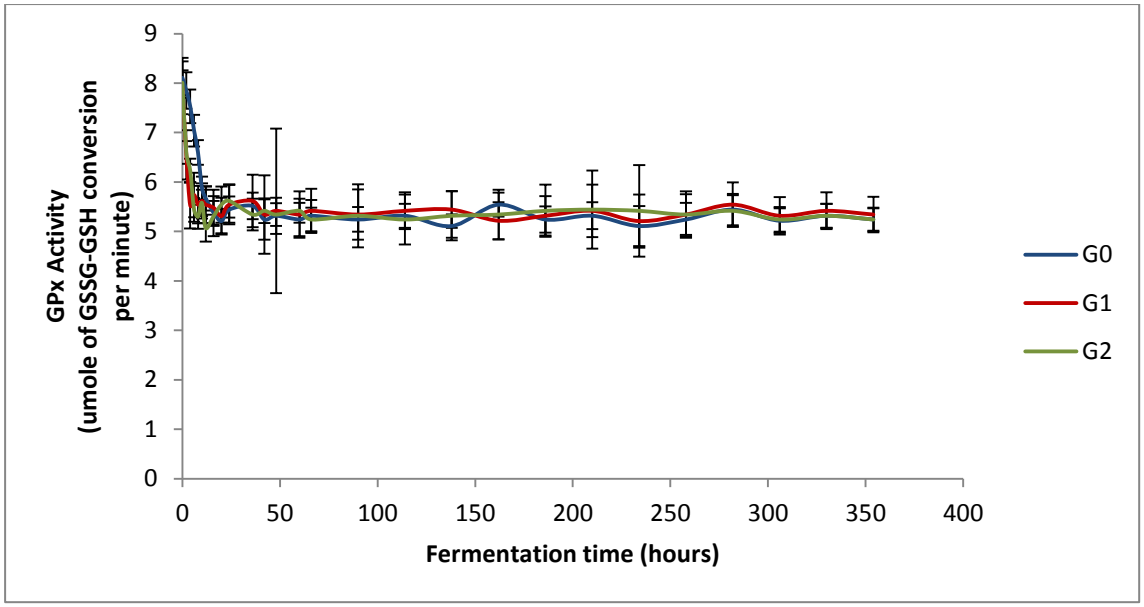


B

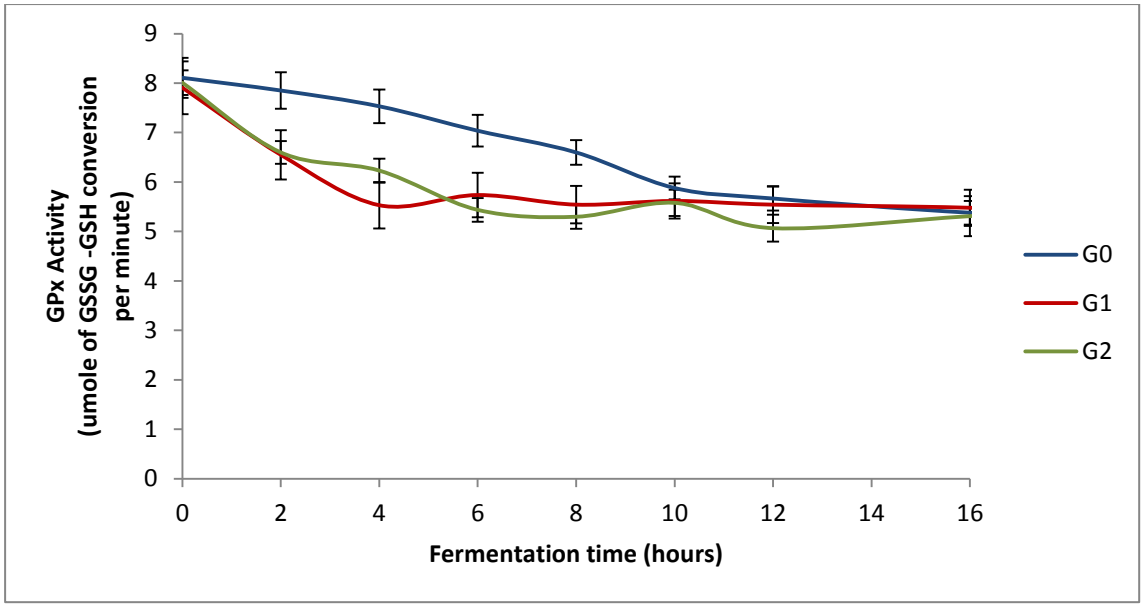
Figure 4.17a-b ROS accumulation during fermentation of 22°P in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Accumulation of ROS was determined over 256 hours (a) and data for the first 60 hours is also summarised (b). Samples were normalised to $1x10^7$ cells/mL and data represents the mean of triplicate samples with error bars indicating the standard deviation.

4.2.2.2.2 Analysis of glutathione peroxidase activity in SMCC100 cells throughout VHG (22°P) fermentation

Glutathione peroxidase (GPx) was evaluated through fermentation as a means of determining the cellular response to ROS at high gravity. It can be seen that there was a high level of activity at the immediate commencement of fermentation, after which glutathione activity decreased rapidly and then remained relatively constant (Figure 4.18). When data from the initial stages of fermentation was evaluated more closely, it can be seen that glutathione activity at the start of the VHG fermentations was approximately 7.8 µmole (GSSG-GSH conversion per minute) for G1 and G2 and 8.1 µmole for G0. In each instance after approximately 16 hours the activity of glutathione decreased to the constant level of 5.3 µmole (Figure 4.18). As discussed previously (Section 4.2.1.2.2) this indicates that glutathione peroxidase activity decreases after the initial stages of fermentation and thereafter remains consistent.


GPx Activity
(umole of GSSG-GSH conversion
per minute)
9
8
7
6
5
4
3
2
1
0
0
50
100
150
200
250
300
350
400
Fermentation time (hours)
G0
G1
G2

A.



B

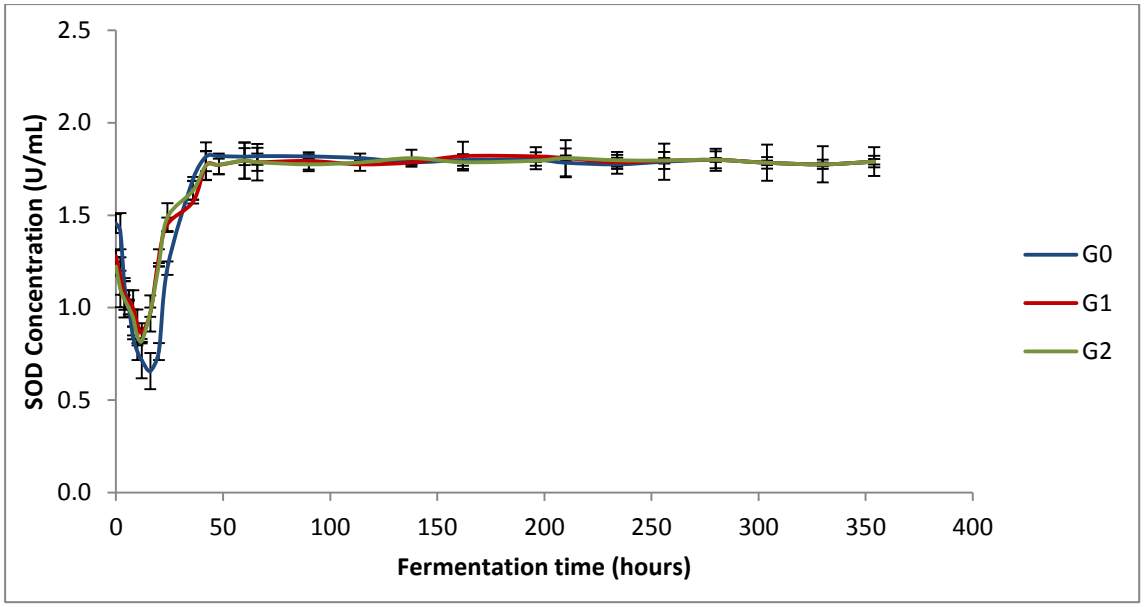
Figure 4.18a-b. Glutathione peroxidase (GPx) (Umole of GS-SG per minute) activity during fermentation of 22°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Gpx activity was determined over 360 hours (a) and data for the first 16 hours is also summarised (b). Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

4.2.2.2.3 Analysis of superoxide dismutase in SMCC100 cells throughout VHG (22°P) fermentation

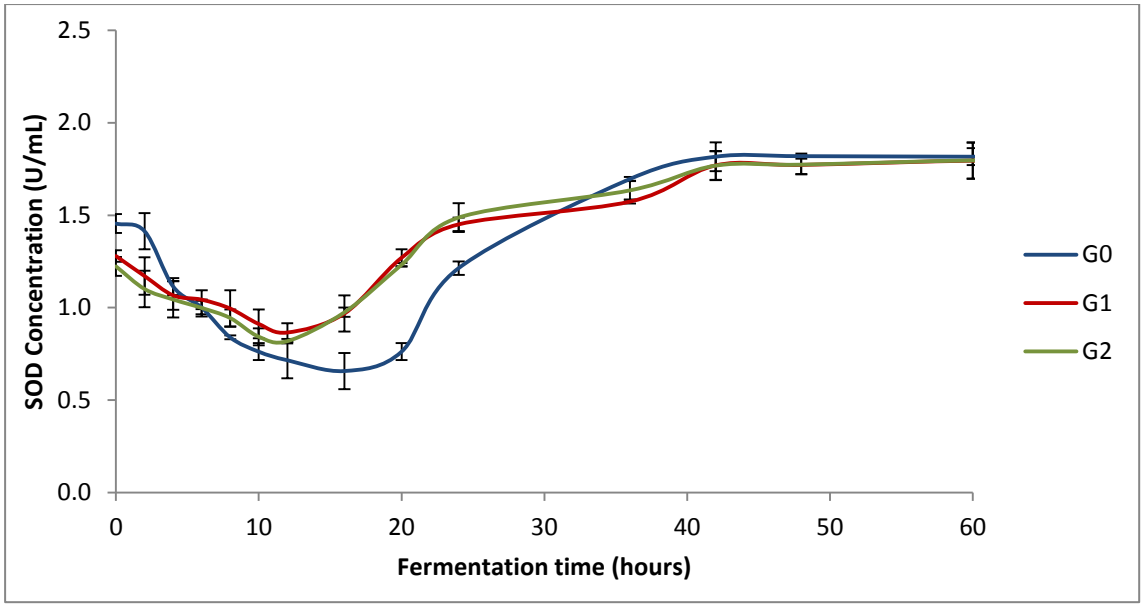
Cell samples obtained from fermentations were assessed for available SOD using the method described in Section 2.9.2.2.2 to indicate the cellular response to ROS generation at 22°P. Analysis of the intracellular concentrations of SOD throughout fermentation at 22°P indicated that the initial levels of intracellular SOD were approximately 1.2 U/mL (G1 and G2 fermentations) and 1.5 U/mL (G0 fermentation) which then rapidly reduced

to 0.82 U/mL and 0.76 U/mL for G1/G2 and G0 fermentations respectively (Figure 4.19). After this point SOD production was stimulated to significantly higher concentrations of 1.7 U/mL SOD for all yeast populations. It was also noted that the concentrations of available SOD did not return to the same as at the start of fermentation, but remained at an elevated level at around 1.7 U/mL (Figure 4.19).

As mentioned above (Section 4.2.1.2.3), the initial decrease in available SOD was unsurprising given that this stage of fermentation reflects a period of oxygen uptake and utilisation, known to be closely associated with ROS formation. It is possible that the decrease in SOD observed indicated an initial mobilisation of resources, possibly as intracellular reserves were utilised in response to ROS generation as described previously (Section 4.2.1.2.3). As in lower gravity wort, it was also observed that G0 yeast had similar minimum levels of available SOD, with a trough of 0.57U/mL compared to 0.82U/mL and 0.87U/mL for G1 and G2 yeast respectively. In this instance the minimum value observed for G0 yeast is lower than that observed in G1 and G2 yeast. The reason for this is unknown, although it is suggested that this may be an artefact of the delayed growth typically observed by G0 yeast during fermentation. It is possible that the extent of gene regulation associated with the transition from propagation to fermentation may also have resulted in an uncoordinated response and a disproportionate requirement for utilisation of available SOD.


SOD Concentration (U/mL)
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
250
300
350
400
Fermentation time (hours)
G0
G1
G2

A



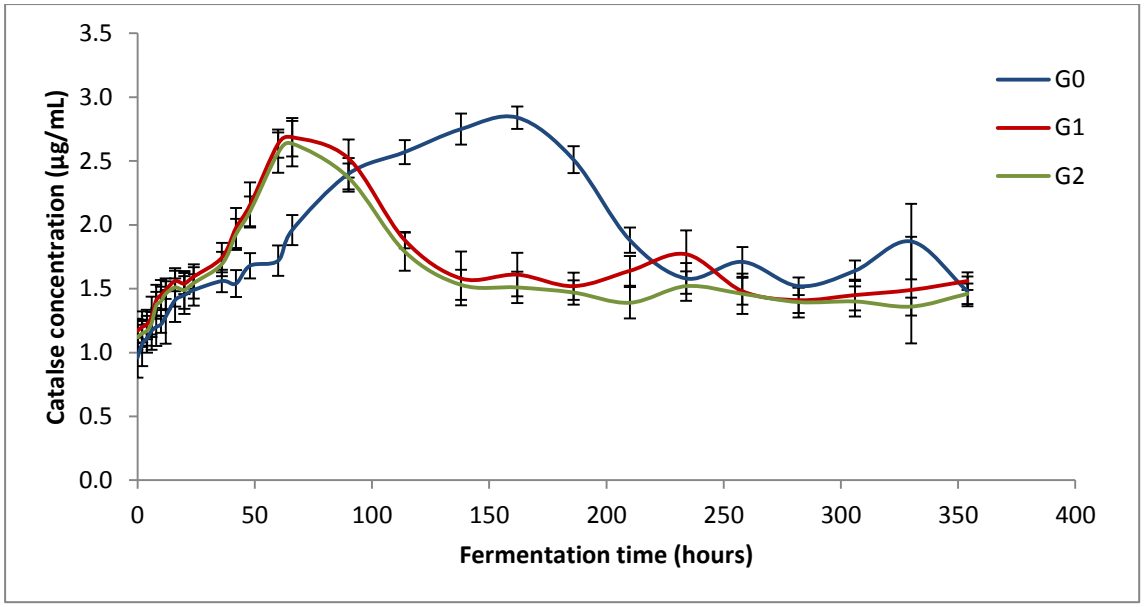
B

Figure 4.19a-b Intracellular superoxide dismutase (SOD) concentration (U/mL) during fermentation of 22°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. SOD concentration was determined over 360 hours (a) and data for the first 60 hours is also summarised (b). Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

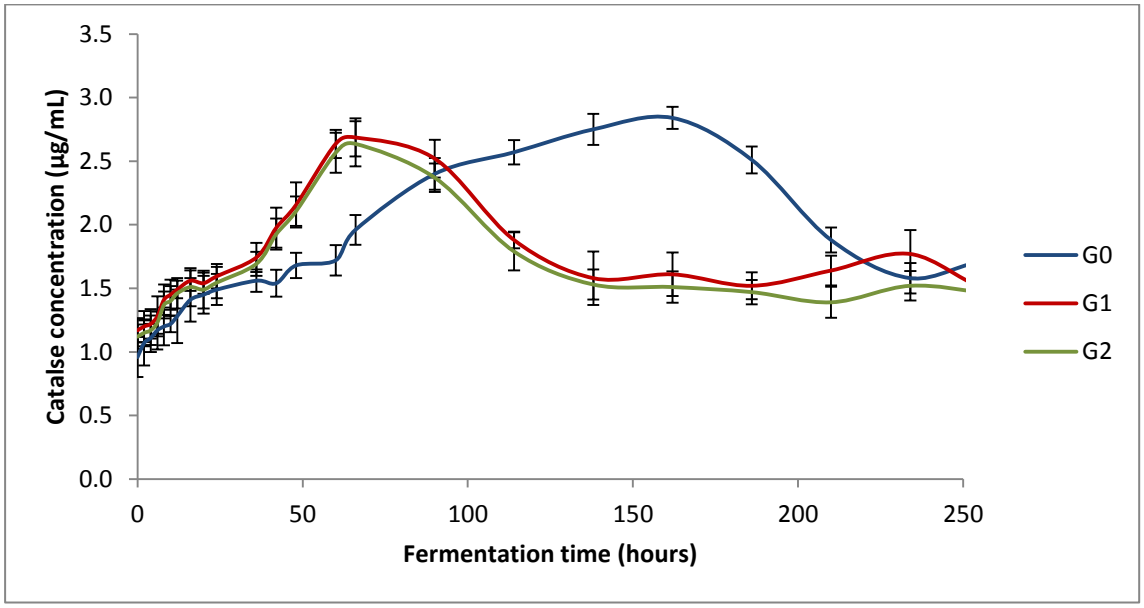
4.2.2.2.4 Analysis of catalase concentrations in SMCC100 cells throughout VHG (22°P) fermentation

As previously detailed (Section 4.2.2.1.4), catalase acts in a similar fashion to SOD as an enzymatic biomarker of oxidative stress, and functions to detoxify the cell by converting hydrogen peroxide into water and oxygen. Given its role in cell homeostasis, the intracellular catalase concentrations throughout VHG fermentation (22°P) were also assessed. Investigation

into the levels of intracellular catalase present in lager yeast during VHG fermentations showed that there was a marked increase in catalase level over time (Figure 4.20). The initial concentrations of catalase present in all fermentations was approximately 1.1 µg/mL, increasing to 2.84µg/mL at 162 hours for G0, and 2.6 µg/mL at 60 hours for both G1and G2 populations. The concentration of catalase then decreased to a plateau at 1.5 µg/mL, over 138 hours for the G1 and G2 fermentations, and subsequently at 234 hours for G0 (Figure 4.20). The initial increase and subsequent decrease in catalase response could be influenced by a downshift in pH through the fermentation. Chance (1951) showed that the relative activity of catalase increases when the pH falls below 5, but is restricted at below pH 4. This closely matches the peak observed for G1 and G2 fermentations, where pH was within this range between 20 and 80h (Figure 4.16). However, this does not reflect the peak observed for G0 yeast. Consequently, it is suggested that catalase may play an important role in cell detoxification during periods of sustained oxidative stress. It is possible that free SOD, and to a lesser extent glutathione, are utilised during the early stages of fermentation as an immediate response to stress, while catalase production is induced as a cell regulated mechanism for defence against ROS accumulation over extended periods of time. This would support the observation that catalase activity eventually decreases, since this corresponds to the end of fermentation where yeast activity is reduced.



A



B

Figure 4.20a-b Intracellular catalase concentration (µg/mL) during fermentation of 22°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Catalase concentration was determined over 360 hours (a) and data for the first 250 hours is also summarised (b). Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

4.2.3 **Comparative analysis of ROS and antioxidant production during fermentation of 15°P and 22°P worts**

Further analysis of the data presented above was performed to directly compare results obtained from analysis of standard (15°P) and very high gravity (22°P) worts. When comparing viability, it can be seen that populations typically maintained a live cell percentage of greater than 96% (Figure 4.21). This was perhaps surprising given that VHG fermentations are often associated with poor yeast health and a reduction in viability (Udeh et al 2013). However, it can be seen that in each of the VHG fermentations viability began to decrease towards the end of fermentation. It should be noted that each successive re-pitching of VHG wort resulted in a lower final viability: 98.2 for G0, 97.6 for G1 and 96.6 for G2 (Figure 4.21). This was in contrast to standard gravity fermentations where viability remained at approximately 98.2% irrespective of culture age. This suggests that it is likely that yeast is subject to greater stress related to VHG which manifests itself in terms of a reduction in cell health over time.

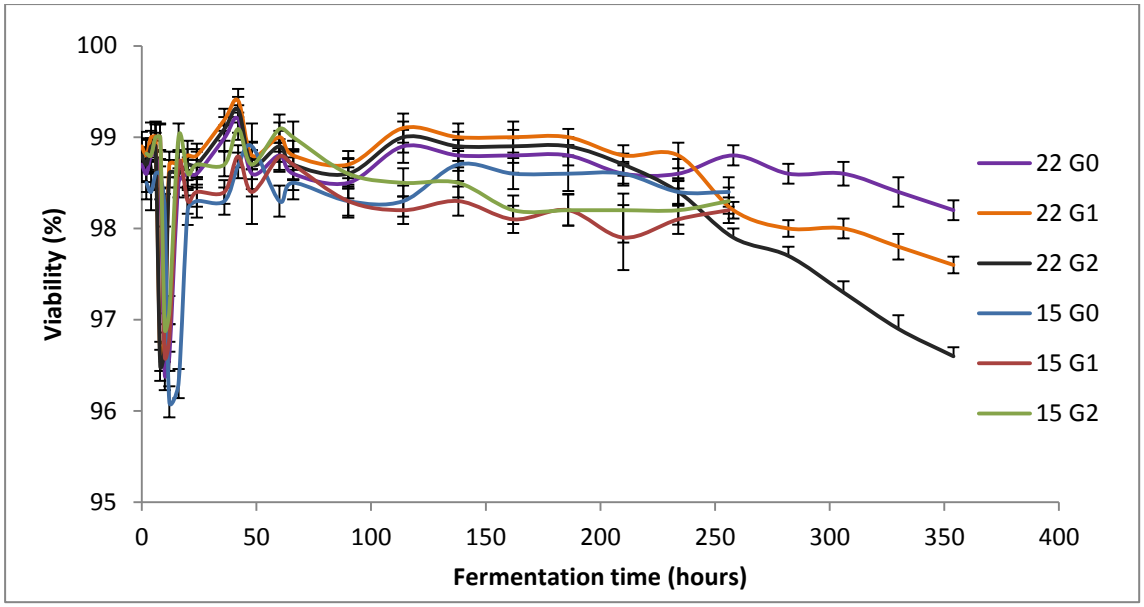


Figure 4.21 Comparison of viability during fermentation of 15°P and 22°P wort in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

Analysis of the production of ROS during fermentations indicated that there were significant variations between standard and VHG fermentations. It is important to note that data was normalised to 1x 10^7 cells to allow for direct comparison of results. Based on this, it can be seen that VHG fermentations showed elevated levels of ROS accumulation, indicating the occurrence of higher oxidative stress when compared to standard gravity (Figure 4.22). In addition, the maximum level of ROS also reached a peak at different points, 40 hours for VHG and 24 hours for standard fermentations. The increase in metabolic activity due to increased wort gravity throughout fermentation, as well as the increase in wort

oxygenation at the start of the VHG fermentations could lead to the increased rate at which ROS accumulation occurs within the yeast during VHG fermentation, reaching equilibrium with the elevated levels of antioxidant response earlier.

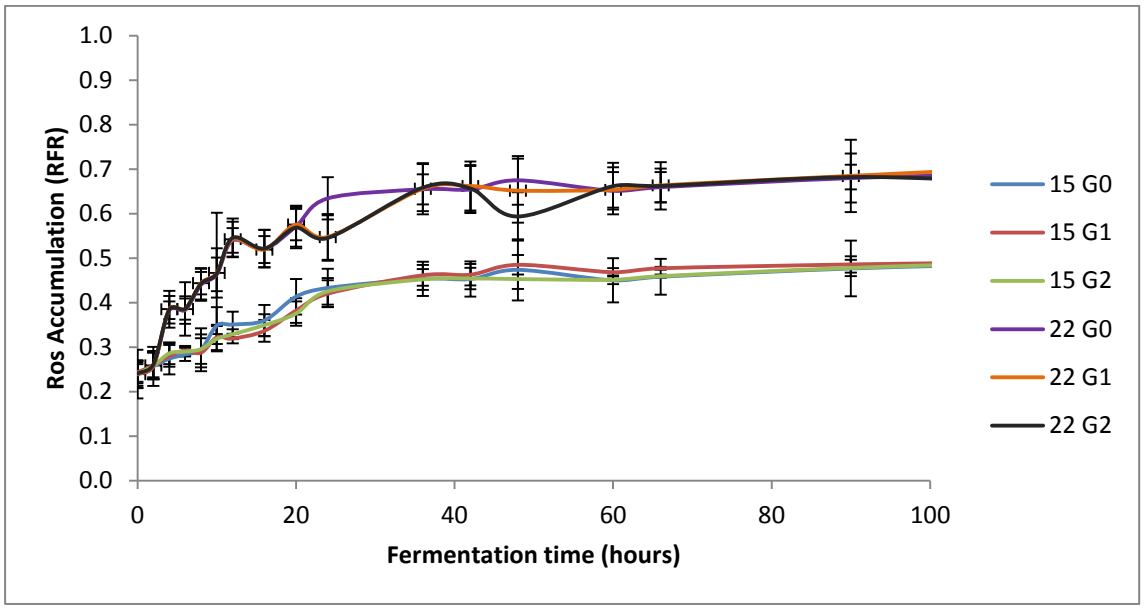


Figure 4.22 Comparison of ROS accumulation during fermentation of 15°P and 22°P worts in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Accumulation of ROS was determined over 100 hours. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

Analysis of the antioxidants glutathione, SOD and catalase indicated that concentrations during fermentation were influenced by initial wort gravity. For glutathione, a higher level of Gpx activity was observed during VHG fermentations compared to those conducted at standard gravity (Figure

4.23). This trend occurred irrespective of yeast generation number, although overall data patterns were similar. Gpx activity was observed to decrease over time, particularly during the first 10 hours of fermentation, and final Gpx values were not significantly different between wort type or generation number. A similar comparative relationship was also observed when SOD production was compared across wort types. In each instance the level of available SOD decreased rapidly at the start of fermentation before increasing again to a plateau (Figure 4.24). Despite this trend, available SOD remained higher during 22°P VHG fermentations than during standard 15°P fermentations throughout. This indicates that although being used, SOD was maintained at an elevated level in VHG fermentations when compared to those of standard worts. In both 15°P and 22°P fermentations levels of ROS accumulation were observed to occur throughout. This level was mirrored by an increase in available SOD. As lower levels of ROS were accumulated in 15°P fermentations, this may have resulted in reduced requirements for SOD. As the level of ROS accumulation was markedly increased in the 22°P fermentations so too was the amount of available SOD (1.8U/mL compared to 1.5 U/mL at 15°P). Although to some degree this implies that SOD was not being utilised during these time periods, it is possible that gene regulation in response to stress caused an excess of SOD to be produced and maintained within the cell. Alternatively, it is possible that SOD plays only a small role in oxidative stress associated with the latter stages of fermentation. Indeed, as described below, there is a significant mobilisation of catalase at this time (particularly in VHG fermentations) which may function to detoxify the cell, leaving excess SOD behind. While there was only a small increase in catalase at 15°P, at very high gravity (22°P) a significant increase in catalase was observed from 0.99 to 2.6

µg/mL. This peak occurred relatively late into fermentation, occurring at 60 hours for G1 and G2 yeast and at around 150 hours for G0 yeast. Concentrations of catalase also remained elevated during fermentations, supporting the previous suggestion that catalase may play a particularly important role in the antioxidant response at very high gravity.

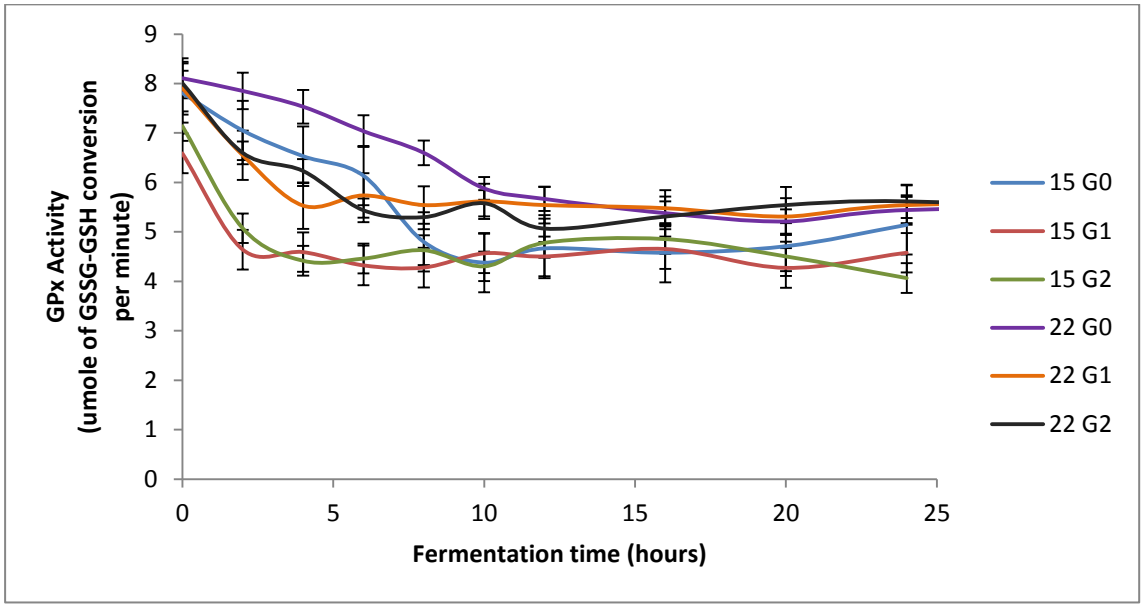


Figure 4.23 Glutathione peroxidase (GPx) activity (µmole of GSSG-GSH per minute) during fermentation of 15°P and 22°P worts in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. GPx activity was determined over 25 hours. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

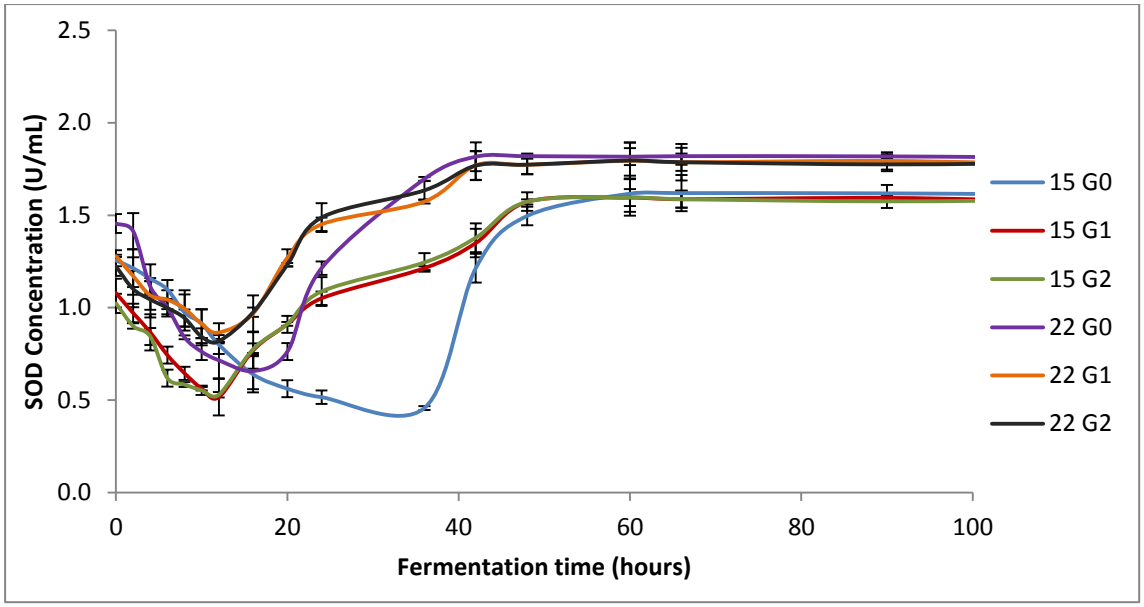


Figure 4.24 Comparison of intracellular SOD concentration (U/mL) during fermentation of 15°P and 22°P worts in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. SOD concentrations were determined over 100 hours. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

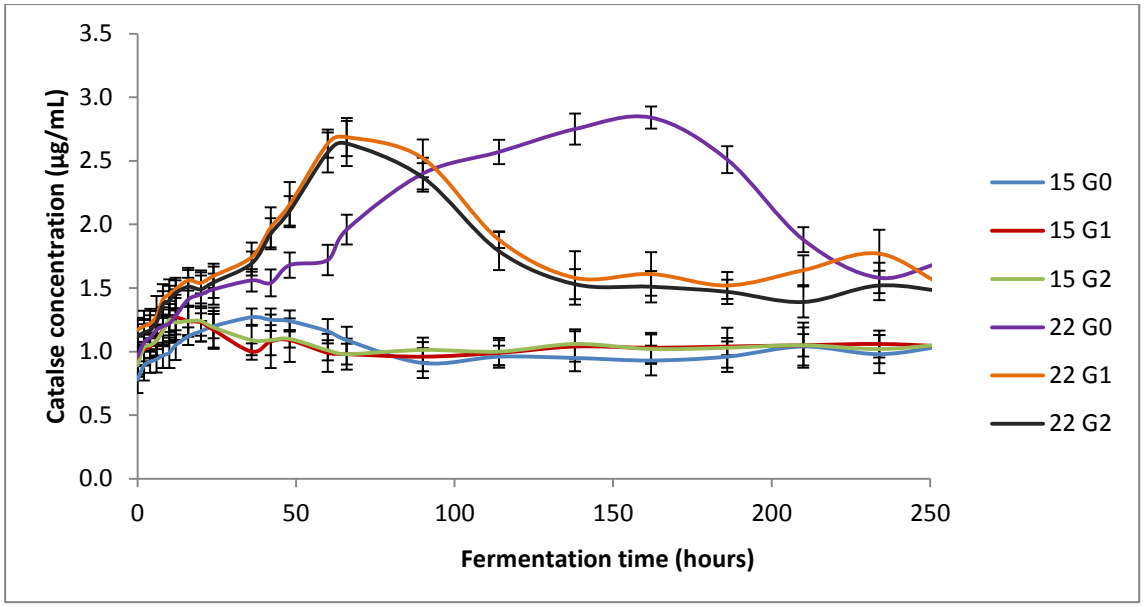


Figure 4.25 Catalase concentration (µg/mL) during fermentation of 15°P and 22°P worts in 10L stirred vessels. Populations derived from freshly propagated cells (G0) and serially re-pitched samples from generation 1 (G1) and generation 2 (G2) fermentations were employed. Catalase concentrations were determined over 250 hours. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

4.3 Discussion

This study aimed to compare the fermentation characteristics and cell physiology of populations of re-pitched yeast under standard (15°P) and VHG (22°P) conditions. Fermentation parameters including gravity, ethanol production, oxygen uptake and pH were monitored, and yeast cells were analysed for viability as well as for the presence of ROS and anti-oxidants.

It was observed that elevating wort gravity from 15°P to 22°P led to a consequential increase in overall fermentation time and ethanol yield. While 15°P fermentations were complete in approximately 150 hours, those conducted at 22°P required more than 350 hours. This is an extremely long fermentation time and hence raises question with regard to the suitability of the strain for VHG fermentations or the feasibility of employing 22°P. However, it should be noted that the total residence time observed under VHG conditions is not unusual for this particular yeast strain (Katherine Smart, Personal Communication), hence the reason why the type of analysis conducted here is pertinent. Despite extended fermentation time, alcohol data corresponded to theoretical values indicating that there was no major trade-off between ethanol production and other carbon derived end products, such as biomass, glycerol or storage sugars. The viability of all yeast populations was observed to remain above 97% for the majority of each of the corresponding fermentations. However under VHG conditions a decline was observed towards the end of fermentation. Although this did not reflect a major collapse of the population, it is reflective of the stressful conditions associated with high gravity fermentations. Interestingly, G2 yeast exhibited the greatest decline in viability, indicating that serial repitching may have led to the accumulation of damage over time. The reason for this reduction in viability is most likely due to the cumulative effect of stress factors during successive fermentations, which are known to elicit a negative effect on viability (Thomas 1979; Brown 1981; Van Unden 1984; D'Amore 1990).

Previous dogma has indicated that the majority of oxidative stress is likely to occur during the initial stages of fermentation, due to aerobic metabolism

(Halliwell and Gutteridge 1999). However, it should be noted that brewing yeast are subject to the Crabtreee effect and are therefore unlikely to utilise the respiratory pathway to a significant extent. In addition, oxygen is removed rapidly at the start of fermentation primarily for lipid synthesis, and as such there is a significant period of time where anaerobiosis is established. To determine the occurrence of oxidative stress during fermentation, cells showing damage by ROS were analysed. This indicated that cellular ROS accumulation occurred rapidly within the first 50 hours of the start of fermentation after which they remained constant. Considering that oxygen was assimilated within 5-6 hours, this indicated that the presence of ROS could not solely be attributed to the aerobic phase of fermentation, but that free radicals were produced continuously, including during the anaerobic phase. In addition, when comparing 15°P and 22°P worts, the latter were more associated with elevated levels of ROS accumulation, indicating the occurrence of higher oxidative stress when compared to standard gravity. This supports previous analysis conducted using wine fermentations, where it has been shown that ROS production is also continuous at high gravity (Lodolfo *et al* 2008). It is possible that the increase in metabolic activity due to the increased availability of sugar may be the cause of this, although it is also possible that increased ROS could simply be an artefact of the increase in wort oxygen provided at the start of the VHG fermentations. With regard to the suggestion of increased metabolic activity, in the absence of oxygen*, S. cerevisiae* activates NAD(P)H-dependent pathways, such as cytochrome P450 systems, which produce significant levels of H_2O_2, O_2^- and OH^- (Van Didjken & Scheffers 1986; Salmon *et al* 1998; Landolfo *et al* 2008). These systems are linked to the catalysed hydroxylation of substrates such as fatty acids, and their related structures, and are involved in alkane

conversion to ethyl alcohol (Funhoff *et al* 2006). As such, the increased metabolic output of VHG fermentation may lead to an increase in ROS through utilisation of these pathways. It should also be acknowledged that other stress factors linked to VHG fermentations and oxidative stress may also have had an influence at this time, most notably the increased ethanol concentrations. It has been well documented that ethanol stress increases ROS formation by altering the fatty acid membrane, which is of particular impact in VHG fermentations generating >10% ethanol (Gupta et al 1994; Perrone et al 2008; Perez-Gallardo et al 2013). However, it should also be recognised that the main phase of ROS generation occurred during the early stages of fermentation where ethanol content was low.

Analysis of the antioxidants glutathione peroxidase (GPx), SOD and catalase also indicated that concentrations of each were influenced by gravity. For glutathione, the trend was for a decline in GPx during the first 10 hours of fermentation, followed by stabilisation; final Gpx values were not significantly different between wort type or generation number. In addition, although a higher level of Gpx activity was observed during VHG fermentations compared to those conducted at standard gravity, there was no further change over time. This data was surprising since glutathione peroxidase (Gpx) has been shown to be one of the primary responses to oxidative stress and ROS accumulation within yeast (Ayer *et al* 2013; Winterbourn & Hampton 2008). The elevated levels of Gpx at the start of fermentation indicate that there was reaction of the cell to deal with the initial oxidative challenge presented by the wort oxygen levels, confirming other data indicating Gpx activity to be highest at the start of fermentation (Gibson *et al* 2006). Martins et al (2005), explain this due an increase in the affinity of glutathione in the presence of oxygen, and reported an 80%

increase in activity. As VHG fermentations require higher levels of wort oxygenation this may help to explain why the effect was more pronounced at 22°P.

Analysis of the available intracellular SOD throughout fermentations at 15°P and 22°P indicated that initial levels were reduced rapidly during the first 10 hours before increasing again to reach a peak at 40-50 hours. After this point the concentration of SOD remained relatively constant. Although similar patterns were seen in all populations, the profile of SOD availability was notably different in the G0 fermentation when compared to the G1 and G2 fermentations. The re-pitched fermentations had a swifter antioxidant response, responding in 12 hours whereas in the G0 fermentation it took 36 hours. However, it is interesting to note that the minimum concentrations of available SOD were similar. Consequently, it is possible that the G0 yeast was better equipped to defend against the presence of ROS due to the fact that it had transitioned from an aerobic environment. This is supported by the greater concentration of SOD at time 0 and indicates that the cellular response to ROS may only have been initiated when the concentration of available SOD reached a specific threshold. When analysing the profile of SOD over time, the initial reduction in intracellular SOD reflects the period of aerobic metabolism, known to be closely associated with ROS formation and it was anticipated that the cellular response at this time may have been greater. Interestingly, previous studies have indicated that the activity of SOD genes may be highest at the start of fermentation, when SOD is required to detoxify the cell (Tan *et al* 2009 and Halyna *et al* 2012). Therefore, the initial drop in intracellular SOD observed at the start of VHG fermentations could be a response to initial oxidative stress, whereby a high proportion of the

available SOD is used by the yeast to degrade intracellular ROS. This hypothesis is however, unlikely as the rate at which the SOD converts the O_2^- to H_2O_2 is 2 x $10^5 M^{-1}s^{-1}$ at pH 7 (Behar *et al* 1970), and a reaction as shown by the available SOD analysis would mean that the yeast were undergoing catastrophic oxidative stress. Another explanation could be that the half-life of SOD decreases as the concentration increases, so for a concentration of 0.05s at 0.1mM, compared to 14 hours for 0.1nM (Heinrich *et al* 2006). This hypothesis also seems unlikely due to the ~1U/mL concentrations of SOD present in the cytosolic samples. The most likely possibility examined, was that the period where the reduction occurred also coincided with the formation of biomass, and as such the occurrence of cell division. It is hypothesised that SOD distribution between mother and daughter cells is the most likely reasoning, confirming the work of Grzelak *et al* (2001) who observed a reduction in antioxidant levels due to cell division.

Analysis of intracellular catalase during standard fermentation revealed that there was a small increase within the first 10 hours after which the amount of catalase decreased starting levels. It is suggested that this may indicate that the yeast culture acquired a transitional adaptive response to detoxify ROS generated at the start of fermentation before other adaptive mechanisms took over. Alternatively, it may indicate that this enzyme had little function under the conditions applied. However, at high gravity there was a significant mobilisation of this enzyme at approximately 60 hours. Concentrations of catalase remained elevated during VHG fermentations, indicating that catalase may play a particularly important role in the antioxidant response at very high gravity. It has previously been suggested that catalase may play a particularly significant role in removing

oxygen free radicals generated by the cell under high gravity conditions (Gibson *et al* 2008). This data confirms this suggestion, with the observation that elevated levels of catalase were present in the 22°P fermentations compared to the 15°P. Unlike SOD, which mirrored ROS accumulation in its response, catalase levels were elevated further into fermentation. This may be linked to the pH of beer since the catalase profile fits the pattern stated by Chance (1952) where production of catalase is favoured within the pH range of 4-5. Analysis of pH during fermentations also revealed that a shorter shift was observed at 15°P fermentations, which may also have influence the amount of catalase present.

Levels of all antioxidants at the end of fermentation were not observed to match those at the start of the subsequent fermentations. Although this was not necessarily expected *per se*, it is interesting that some form of transitional response was present. It is possible that this was associated with storage of cells under cold temperatures between fermentations. This is known to elicit an effect as Zhang *et al* (2003) found that a reduction in temperature resulted in an antioxidant response involving the up-regulation of SOD1, CTT1 and GSH1. Gibson *et al* (2006) also analysed oxidative stress during brewery yeast handling, with a focus on storage and propagation and reported an upregulation of genes such as SOD1, SOD2 , CATT, CAA and GPX 4 at these times. While this indicates the presence of a potential osmotic stress, it is possible that this regulation occurred as part of the general stress response (STRE). Unfortunately, further analysis of these genes throughout fermentation, or serial repitching was not investigated at that time. It would be interesting to examine this in the future. Furthermore, analysis of protein targets of ROS could also be

included, such as the mitochondrial citric acid cycle proteins, α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and the iron-sulfur [4Fe-4S] cluster enzymes aconitase and succinate dehydrogenase, readily inactivated by H_2O_2 and by menadione-derived superoxide radicals (Cabiscol *et al* 2000; Cecarini *et al* 2007; Farrugia & Balzan 2012). A focussed analysis of these proteins throughout fermentation could give absolute confirmation that oxidative stress does occur giving rise to a specific oxidative stress response. Irrespective, the data shown here supports previous observations that ROS do occur throughout fermentation (Gibson *et al* 2016), with the additional provision that this is exacerbated at very high gravity. As a result, antioxidants were produced in enhanced concentrations. It is possible that free SOD, and to a lesser extent glutathione peroxidase, are utilised during the early stages of fermentation as an immediate response to stress, while catalase production is induced as a cell regulated mechanism for defence against ROS accumulation over extended periods of time. In Chapter 5 we will determine the potential effects of ROS production on cell physiology and health.

CHAPTER 5: THE EFFECT OF OXIDATIVE STRESS ON DNA INTEGRITY AND THE PLASMA MEMBRANE IN LAGER YEAST STRAINS

5 The effect of oxidative stress on DNA integrity and the plasma membrane in lager yeast strains

5.1 Introduction

Yeast cells require oxygen for the biosynthesis of sterols and unsaturated fatty acids, which are essential for biomass production (Herskowitz 1988; Lorenz & Parks 1991; Hammond *et al* 2000; Briggs *et al* 2004). However, exposure of yeast to oxygen in the fermentation vessel not only results in yeast growth, but can also lead to the occurrence of oxidative stress. As described in Section 1.5.4.1, oxidative stress refers to the effect of free radicals, or reactive oxygen species (ROS) on yeast, and typically results in a coordinated cellular response (Santoro *et al* 1998). The primary ROS encountered in yeast are the superoxide anion, hydrogen peroxide and hydroxyl radicals, all of which can have a range of impacts on yeast cells, including mutagenesis to DNA structures (Doudican *et al* 2005; Griffiths *et al* 2009) and damage to cellular components including lipids and proteins (Halliwell & Gutteridge 2007; Izawa *et al* 1995; Aebi 1984).

The data described in Chapter 4 indicated that ROS formation occurs during both standard and high gravity fermentations, but is elevated at high gravity. However, in response to oxidative stress, enzymatic antioxidants such as superoxide dismutase (SOD), catalase, glutathione peroxidase, as well as other non-enzymatic antioxidants including glutathione, metal ions, and vitamins C and E function to detoxify the cell and maintain redox balance (Grant 2001; Jamieson 1998; Moradas-Ferreira *et al* 1996; Section 1.5.4). Despite this, if stress occurs at a significant level, or for an extended period of time, the cell will inevitably suffer damage.

Mitochondrial DNA (MtDNA) has been shown to be particularly susceptible to mutations under oxidative stress, due to the close proximity of the electron transport chain, the main cellular source of reactive oxygen species (ROS) (Loschen *et al* 1973; Richter 1992, Shokolenko *et al* 2009). Within the mitochondria, superoxide anions are reduced to H_2O_2 which is able to diffuse freely through the cell due to its stability and membrane permeability. Reduction of H_2O_2 by glutathione can also lead to the production of the hydroxyl radical, which can subsequently cause damage to all cellular DNA structures and the plasma membrane, especially if the enzymatic antioxidant response is reduced (Henle et al 1996a; Henle et al 1996b; Deryabina *et al* 2004). While mtDNA damage may be particularly associated with aerobic metabolism (such as that characterised by conditions associated with yeast propagation), it is also known that free radicals are produced by cellular oxidation reactions during glycolysis, the main fermentation pathway (Salmon *et al* 1998; Section 1.1.5). Consequently, yeast are subjected to varying degrees of oxidative stress throughout brewery yeast handling. This Chapter attempts to evaluate the effect of oxidative stress on lager yeast cell physiology, focusing on the integrity and fluidity of the cell membrane and both genomic and mitochondrial DNA structures. Oxidative stress conditions similar to those observed during VHG fermentations were manufactured artificially using hydrogen peroxide as a free radical generator. Consequently, this system was used to determine the effects of ROS on cell viability, using a combination of propidium iodide and bis-oxonol staining in conjunction with flow cytometry. In addition, the impact of ROS on cell membrane fluidity was determined using laurdan staining to provide an indication of the generalised polarisation index, while DNA integrity was assessed using a variety of fingerprinting methods.

5.2 Results

5.2.1 Development of an artificial environment for the investigation of yeast and oxidative stress

To investigate the precise effect of oxidative stress on yeast cells, an artificial environment was created to mimic the conditions encountered during fermentation. In order to create a level of oxidative stress typically encountered during standard and very high gravity fermentations (as defined in Chapter 4), yeast cells were subjected to a range of H_2O_2 concentrations as described in Section 2.9. Cells were grown to exponential phase and then subjected to either 0mM (control), 1mM, 5mM or 10mM H_2O_2. For each concentration, cellular ROS accumulation was evaluated using di-hydroethidium (DHE) staining (Landolfo *et al* 2008; Section 4.4.1; Section 2.9.2.1). Due to the presence of ROS, DHE is oxidized to ethidium, which then binds to DNA and causes fluorescence to be generated (Section 2.8.4). Samples were analysed using a fluorescence plate reader and a ratiometric quantification was employed to compare the levels of ROS accumulation of each sample. The presence of ROS at a concentration of 10mM H_2O_2 was used to indicate 100% accumulation (relative fluorescence ratio of 1) (Section 2.8.5) and all other data was normalised against this value.

Analysis of the relationship between H_2O_2 and ROS within yeast cells revealed that 0mM H_2O_2 yielded a relative fluorescence ratio (RFR) of 0.23AU (Figure 5.1). Cells in the presence of 1mM showed an RFR of 0.45AU, while 5mM yielded 0.61AU and 10mM produced 1AU. These standards were then used to qualify cell samples taken from standard (15°P) and VHG (22°P) fermentations at 0, 20, and 100 hours, time periods

reflecting the minimum and maximum ROS values, as well as the period during which ROS accumulated the fastest. Yeast cells taken from the start (time 0) of both 15°P and 22°P yielded an RFR of approximately 0.22AU, comparable to that produced using control samples (0mM H_2O_2) (Figures 5.1-5.2). Cells taken from 15°P fermentations at 100 hours produced an RFR of 0.49AU, comparable to that observed in the presence of 1mM H_2O_2, while those from 20 hours displayed a slightly lower value at 0.38AU (Figure 5.1). For VHG worts, cells obtained after 20hours produced an RFR of 0.57, comparable to those produced using 1mM H_2O_2, while cells from 100 hours of fermentation yielded 0.69AU, similar to those seen with 5mM H_2O_2 (Figure 5.2). The values obtained using 10mM H_2O_2 were significantly greater than would be encountered during fermentation at either 15°P or 22°P, but were utilised to represent a severe stress threshold.

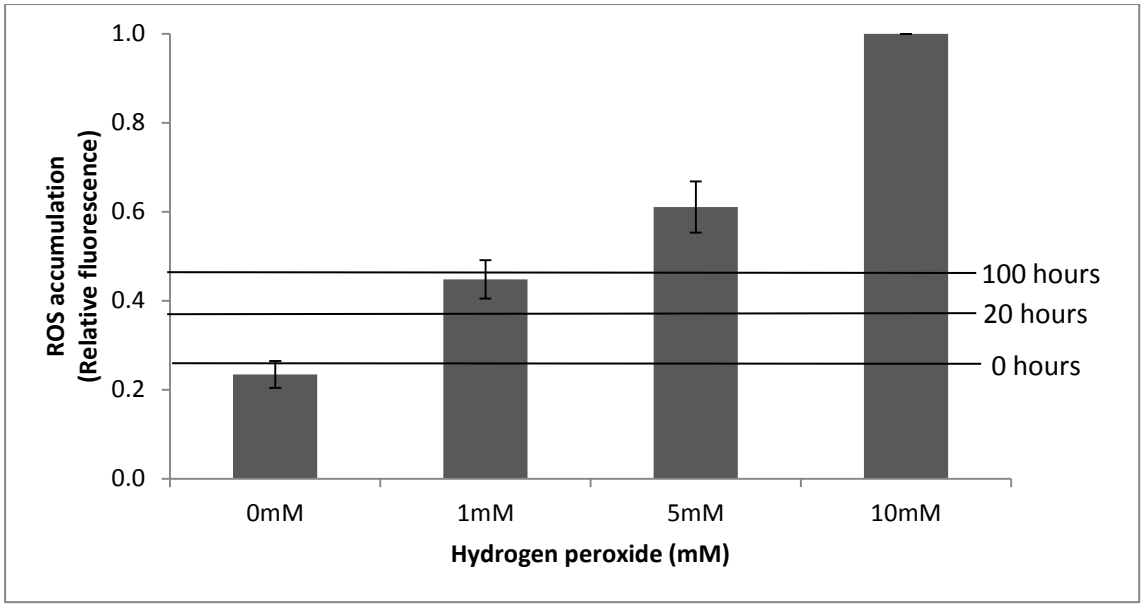


Figure 5.1 ROS generation induced by hydrogen peroxide. Cells were subjected to hydrogen peroxide stress and ROS accumulation was assessed by determining the relative fluorescence of DHE, normalized against $1x10^7$ cells in the presence of 10mM H_2O_2. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation. Horizontal lines indicate the relative concentrations of ROS at specific time points during standard (15°P) fermentations as determined from previous data (Chapter 4).

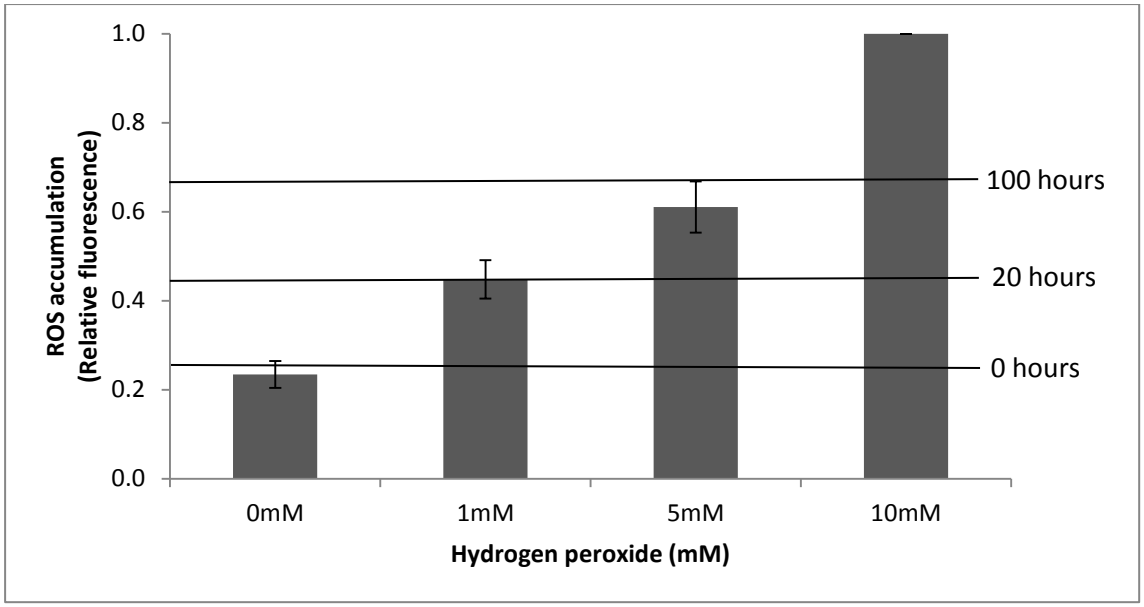


Figure 5.2 ROS generation induced by hydrogen peroxide. Cells were subjected to hydrogen peroxide stress and ROS accumulation was assessed by determining the relative fluorescence of DHE, normalized against $1x10^7$ cells in the presence of 10mM H_2O_2. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation. Horizontal lines indicate the relative concentrations of ROS at specific time points during VHG (22°P) fermentations as determined from previous data (Chapter 4).

5.2.2 The effect of oxidative stress on cell viability and DNA integrity in lager yeast strains

Previous analyses have demonstrated that oxidative stress can cause damage to membrane and DNA structures in a range of organisms (Barzilai & Yamamoto 2004; Apel & Hirt 2004; Salmon *et al* 2004; Jamieson 1998; Meneghini 1997; Izawa *et al* 1995). However these investigations typically involve subjecting cells to extreme levels of stress

and are often not representative of what might be encountered in natural or industrial environments. Given that brewing yeast strains are susceptible to ROS (Section 3.2.5; Section 4.2.4; Gibson *et al* 2008; Gibson *et al* 2007), the effects of H_2O_2 induced oxidative stress on cell health and physiology were investigated. In this study H_2O_2 was used to induce a level of oxidative stress typically encountered in 15°P and 22°P brewery fermentations (Section 5.2.1). This stress was applied to populations of lager yeast strains SMCC99, SMCC90 and SMCC100 over a 1 hour period and subsequently the impact on cell membrane health and DNA damage was determined.

To induce oxidative stress, cells were incubated in the presence of H_2O_2 at 0mM, 1mM, 5mM and 10mM H_2O_2 as described previously (Section 2.10). Subsequently the viability of strains SMCC100, SMCC90 and SMCC99 were analysed using the fluorescent stain propidium iodide (Section 2.10). Analysis of control samples indicated that in the presence of 0mM H_2O_2 (control samples) all strains maintained a viability of greater than 95% (Figure 5.3). At a concentration of 1mM H_2O_2, strains SMCC100 and SMCC99 exhibited 90% and 87% viability respectively, while strain SMCC90 was more adversely affected, with a viability of 60%. At 5mM H_2O_2 a similar pattern was observed. Strain SMCC100 exhibited a viability of 21%, with SMCC99 showing 10%, while SMCC90 was the most affected at 3%. At 10mM, representing an extreme level of stress, all cells were destroyed, as indicated by a viability of 0% (Figure 5.3). This data indicates that strain SMCC90 is the least tolerant to oxidative stress, in contrast to previous data (Chapter 3). However, it should be noted that the previous analyses were related to yeast growth, or stress over an extended time period, allowing for up-regulation of stress response pathways and

mobilisation of antioxidants. In the current work, the decrease in viability for this strain represents the intrinsic capacity to withstand oxidative stress over a short time period, perhaps reflecting the basal levels of antioxidants within the population, or the ability of cellular structures to withstand ROS.

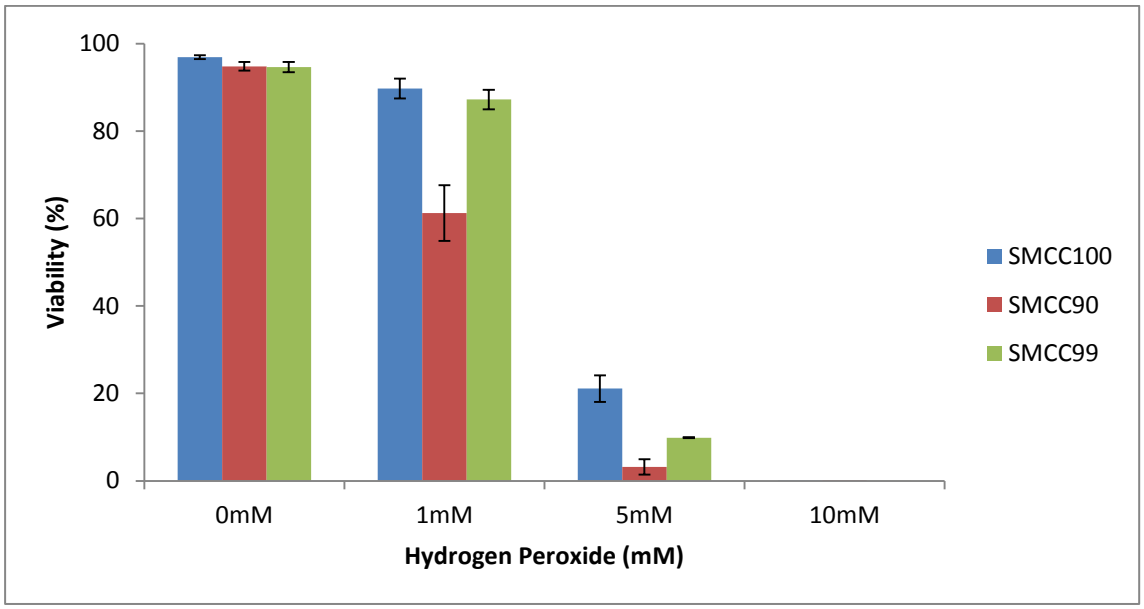


Figure 5.3 The effect of oxidative stress induced by H_2O_2 on the viability of lager yeast strains SMCC100, SMCC99 and SMCC90. Viability was determined using propidium iodide staining. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

Following analysis of the impact of oxidative stress on yeast viability, the effect on genomic DNA was determined. DNA was characterised by fingerprinting based on inter delta sequences, since these regions are known to be highly variable and have been shown to be useful tools in tracking genetic mutations (Xufre *et al* 2010). Analysis of the frequency and distribution of inter delta sequences indicated that no changes were induced by the presence of ROS under the concentrations and conditions applied. Irrespective of the concentration of H_2O_2, inter-delta sequence patterns for all 3 strains remained unchanged (Figure 5.6). This was surprising since it is well documented that ROS can lead to cell damage (Shigenga *et al* 1994; O'Rourke *et al* 2002; Doudican *et al* 2005). However, it is possible that this may be linked to periods of extended low levels of stress, rather than a sudden high level stress as was applied here.

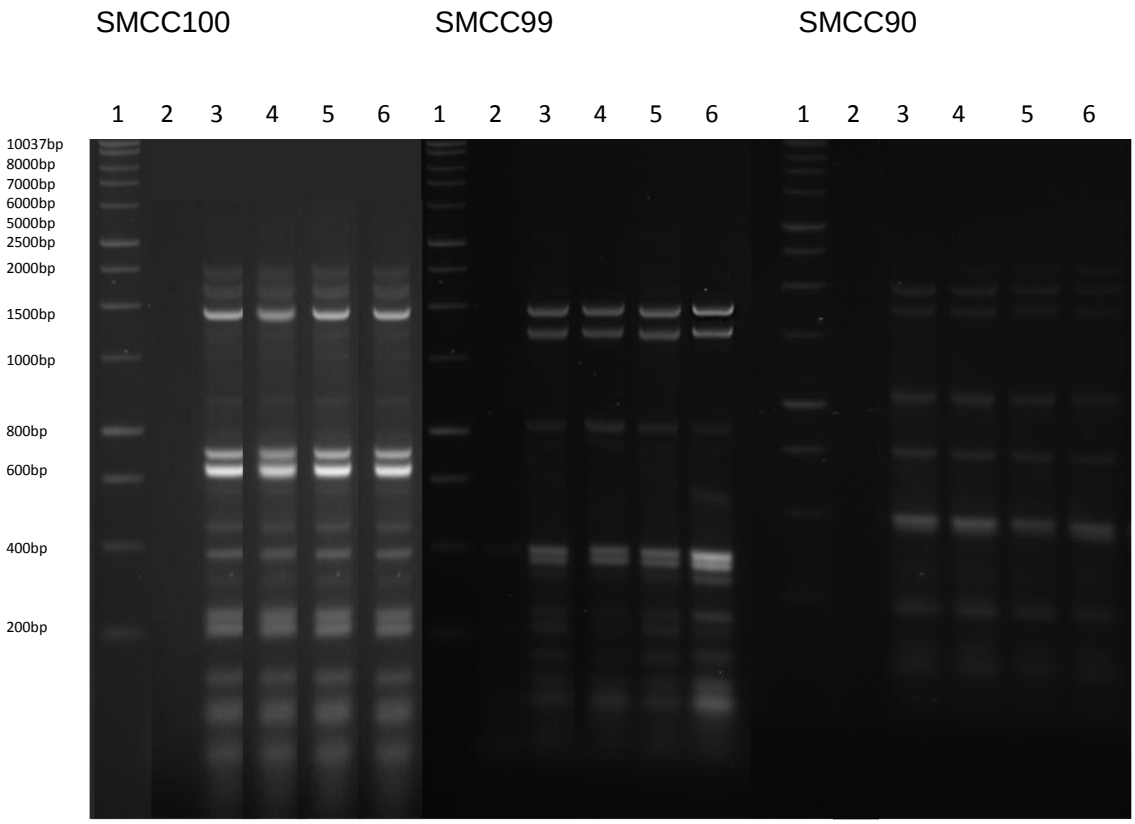


Figure 5.4 The effect of oxidative stress on DNA integrity in strains SMCC100, SMCC99 and SMCC90. DNA integrity was analysed by PCR amplification of inter-delta regions of the genome. In each instance, a 100bp DNA ladder is indicated in Lane 1, and lane 2 represents a blank sample. Yeast strains were subjected to oxidative stress induced by H_2O_2 at a concentration of 0mM (Control) (Lane 3), 1mM (Lane 4), 5mM (Lane 5) and 10mM (Lane 6).

Although analysis of genomic DNA did not show any change in response to oxidative stress, mtDNA was also analysed since it is known to be particularly sensitive to damage due to the absence of stabilising histones (Aiken Hobbs *et al* 2001). To assess mtDNA, mitochondria were isolated from populations of yeast cells prior to fingerprinting based on restriction

sequences as defined by enzymes HaeIII, HinfI and DdeI. These enzymes were applied since they were used previously for strain identification (Chapter 3.2.2), and are known to provide an indication of mtDNA integrity based on the location of restriction sites (Lewin *et al* 1978; Foury 1989; Sia *et al* 2000; Cheung *et al* 2012).

DNA fingerprinting by RFLP was performed on mtDNA extracted from all three strains after subjecting samples to stress with H_2O_2. As with genomic DNA, no changes were observed in the mtDNA fragments produced by RFLP of all three strains in the presence of 0mM (Figure 5.5), 1mM (Figure 5.6) or 5mM H_2O_2 (Figure 5.7). However, in contrast to the genomic DNA analysis, fingerprints were not able to be obtained from cells subjected to 10mM H_2O_2 (Data not shown). In this instance, no DNA was observed at all. This indicated that there was a severe impact on the mtDNA of cells, resulting in complete degradation of this structure. Several studies have shown that mtDNA stability can be affected by oxidative stress, and complete loss of mtDNA can occur (Doudican *et al* 2005; Shokolenko *et al* 2009). The failure to extract any mtDNA, not even damaged fragments, could also be linked to the complete loss of cell viability observed in all strains (Figure 5.3).

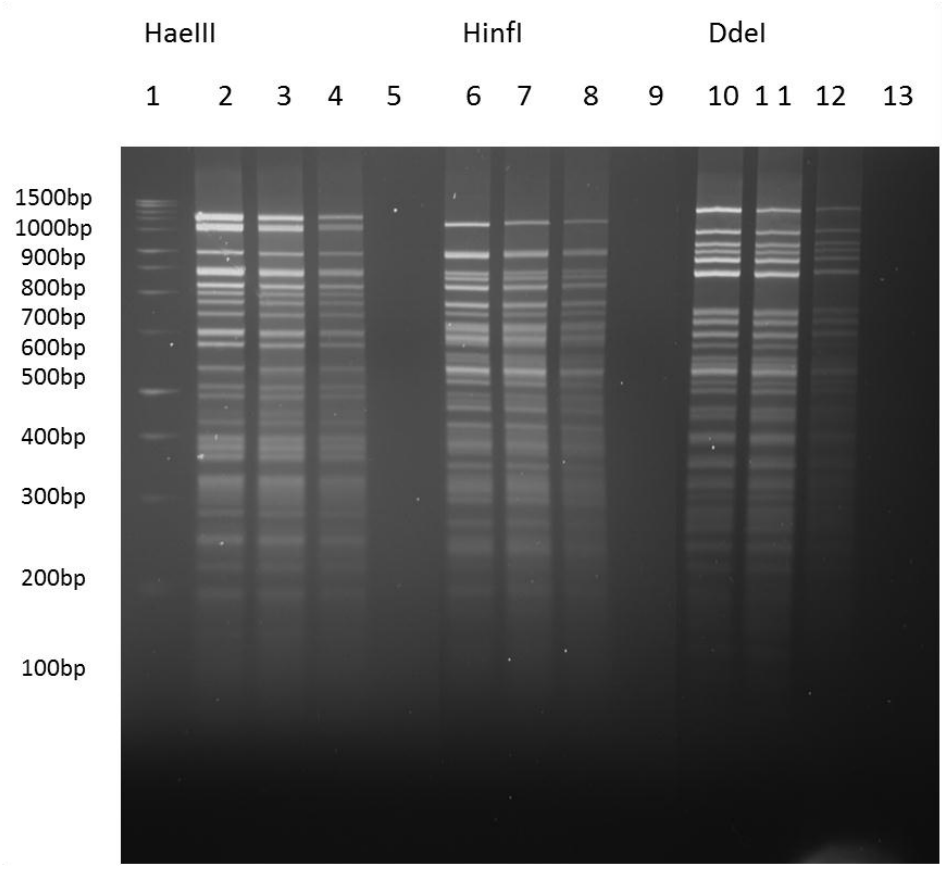


Figure 5.5 The effect of oxidative stress on mtDNA integrity in strain SMCC100. mtDNA integrity was analysed by RFLP of mtDNA extractions using HaeIII, HinfI and DdeI. Cell samples were subjected to oxidative stress induced by H_2O_2 at a concentration of 0mM (Control) (Lanes 2, 6 and 10), 1mM (Lanes 3, 7 and 11), 5mM (Lanes 4, 8 and 12) and 10mM (Lanes 5, 9 and 13). A 1kb DNA ladder is indicated in Lane 1.

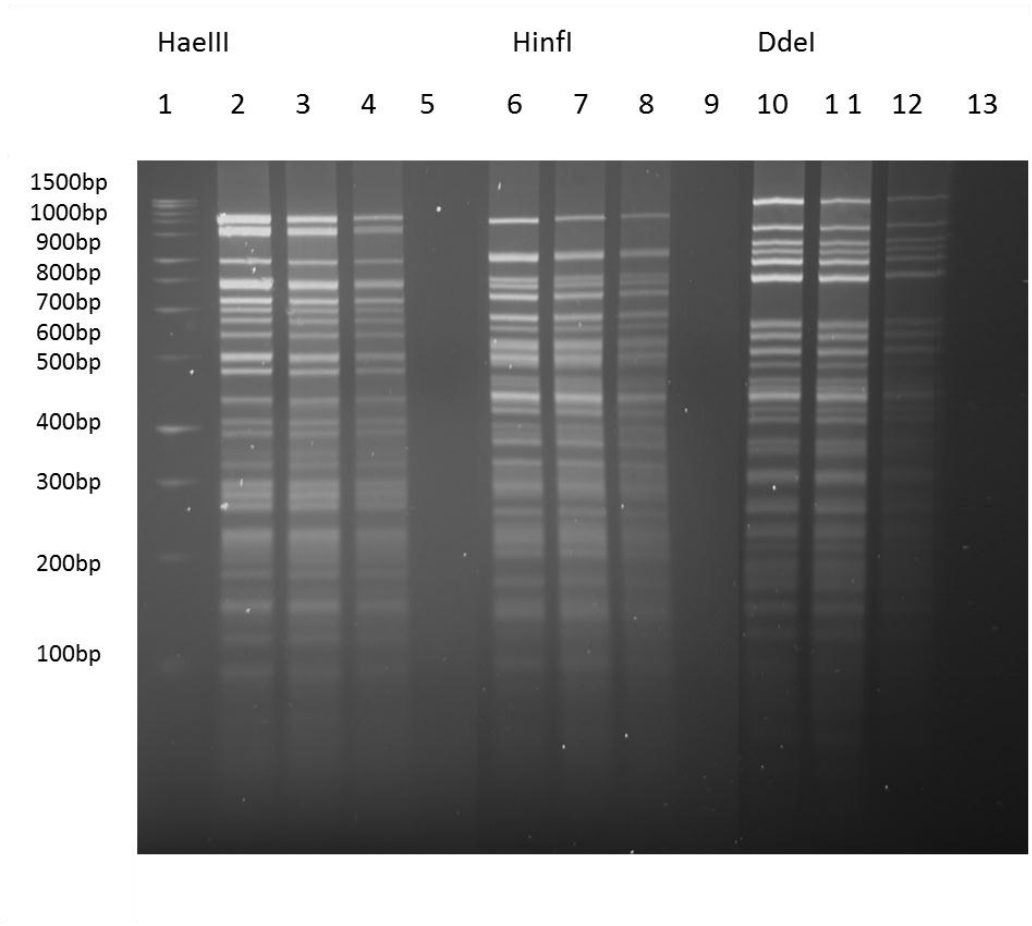


Figure 5.6 The effect of oxidative stress on mtDNA integrity in strain SMCC90. mtDNA integrity was analysed by RFLP of mtDNA extractions using HaeIII, HinfI and DdeI. Cell samples were subjected to oxidative stress induced by H_2O_2 at a concentration of 0mM (Control) (Lanes 2, 6 and 10), 1mM (Lanes 3, 7 and 11), 5mM (Lanes 4, 8 and 12) and 10mM (Lanes 5, 9 and 13). A 1kb DNA ladder is indicated in Lane 1.

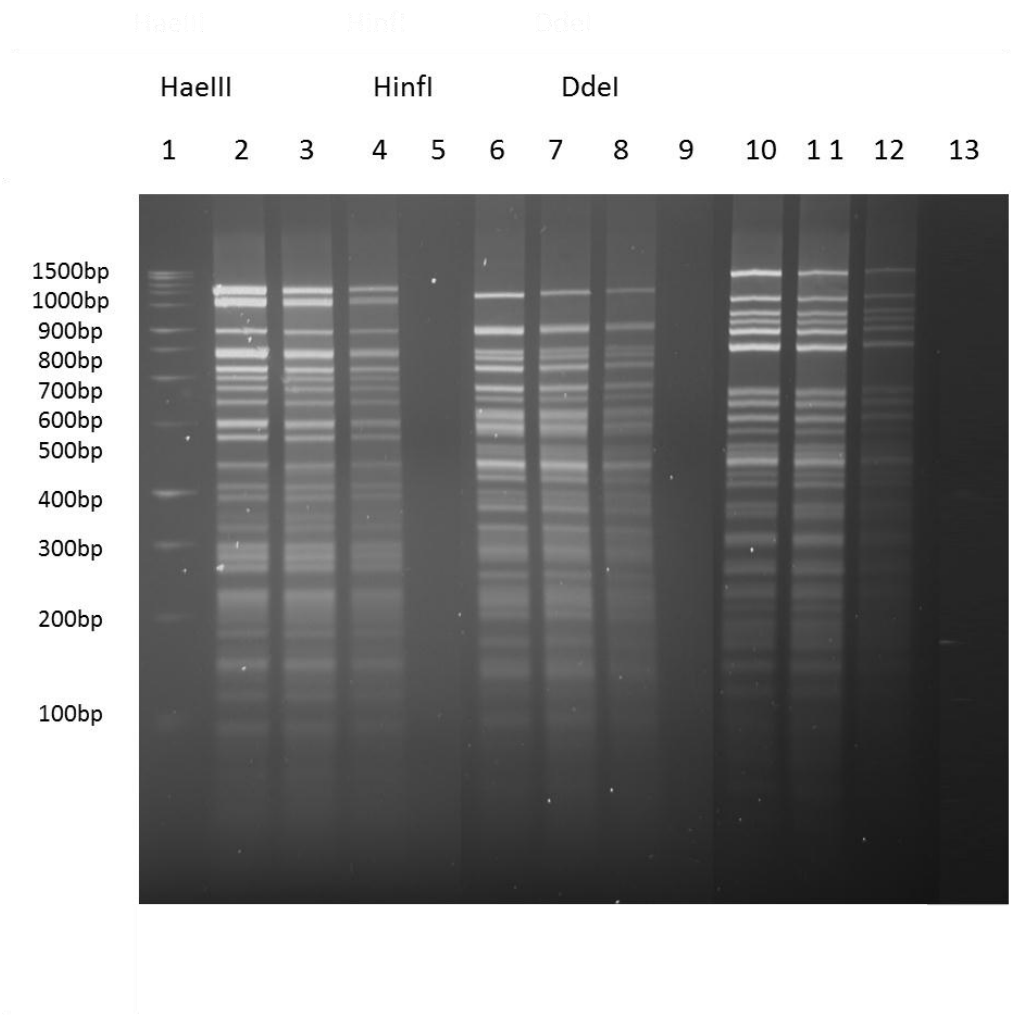


Figure 5.7 The effect of oxidative stress on mtDNA integrity in strain SMCC99. mtDNA integrity was analysed by RFLP of mtDNA extractions using HaeIII, HinfI and DdeI. Cell samples were subjected to oxidative stress induced by H_2O_2 at a concentration of 0mM (Control) (Lanes 2, 6 and 10), 1mM (Lanes 3, 7 and 11), 5mM (Lanes 4, 8 and 12) and 10mM (Lanes 5, 9 and 13). A 1kb DNA ladder is indicated in Lane 1.

5.2.3 The effect of oxidative stress on the plasma membrane of lager yeast strains

The plasma membrane of *Saccharomyces* yeasts acts as a semi-permeable barrier between the cell and its environment. Stresses, including oxidative, osmotic and ethanol challenges have all been shown to affect changes in the fluidity of the plasma membrane, which can therefore be used to indicate membrane damage (Learmonth & Gratton 2002; Turk *et al* 2011). A decrease in fluidity indicates that structural damage has occurred, resulting in membrane rigidity. This can lead to an increase in cell permeability, as well as affecting nutrient transport and cellular pH (Learmonth & Gratton 2002). Strains SMCC100, SMCC90 and SMCC99 were subject to oxidative stress by incubation in H_2O_2 at 0mM, 1mM, 5mM and 10mM H_2O_2 (Section 2.10.2). Subsequently, plasma membrane fluidity was assessed using the fluorescent stain Laurdan (Section 2.10.3) to indicate generalized polarisation (GP) where a high GP value indicates low membrane fluidity and *vice versa*.

In each instance, as the level of stress increased, so too did the GP value. Control samples (0mM) produced a GP of 0.12 units (U) for all strains. For strains SMCC100 and SMCC99 this increased to 0.14U and 0.17U respectively under 1mM H_2O_2 and to 0.27U and 0.29U respectively under 5mM H_2O_2. All strains yielded a similar GP of approximately 0.73U in the presence of 10mM H_2O_2, when all cells were dead (Figure 5.8). Although the response to H_2O_2 was similar for each yeast strain, data obtained for SMCC90 indicated that the membrane fluidity of this yeast was more impacted by the presence of H_2O_2. At 1mM and 5mM H_2O_2 membrane fluidity was observed to be 0.20U and 0.38U respectively. This correlates with the previous data indicating that viability in this strain was also more

impacted by osmotic stress (Section 5.2.2; Figure 5.8). Despite this correlation, it was not possible to determine whether changes in membrane fluidity were a cause of cell death or a result of it. Due to this an alternative means of assessing membrane health was developed to look specifically at additional biomarkers of membrane damage in conjunction with cell viability.

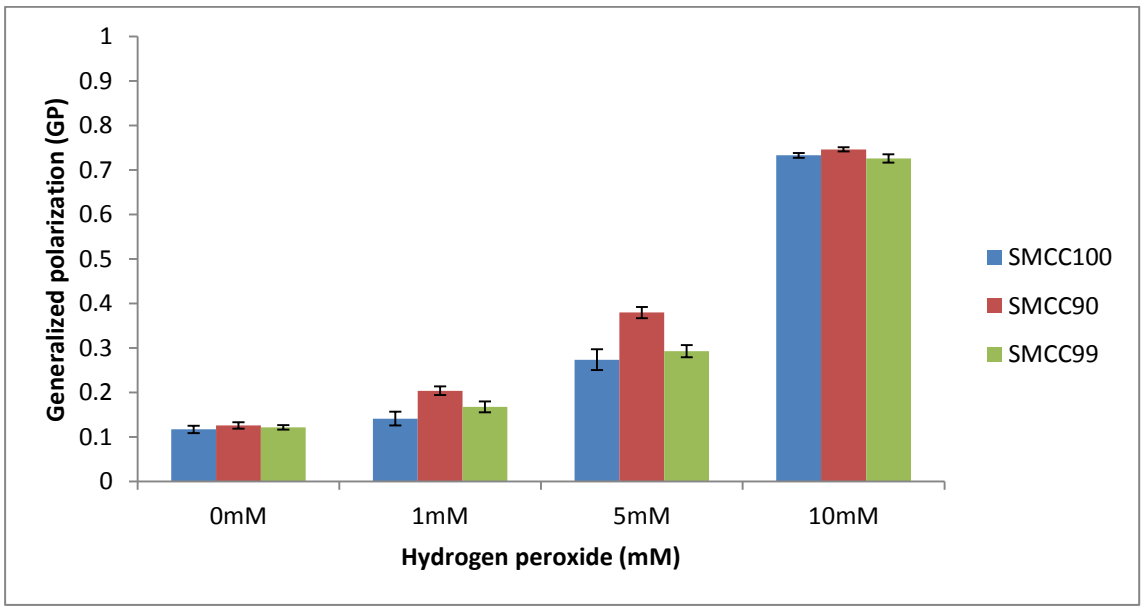
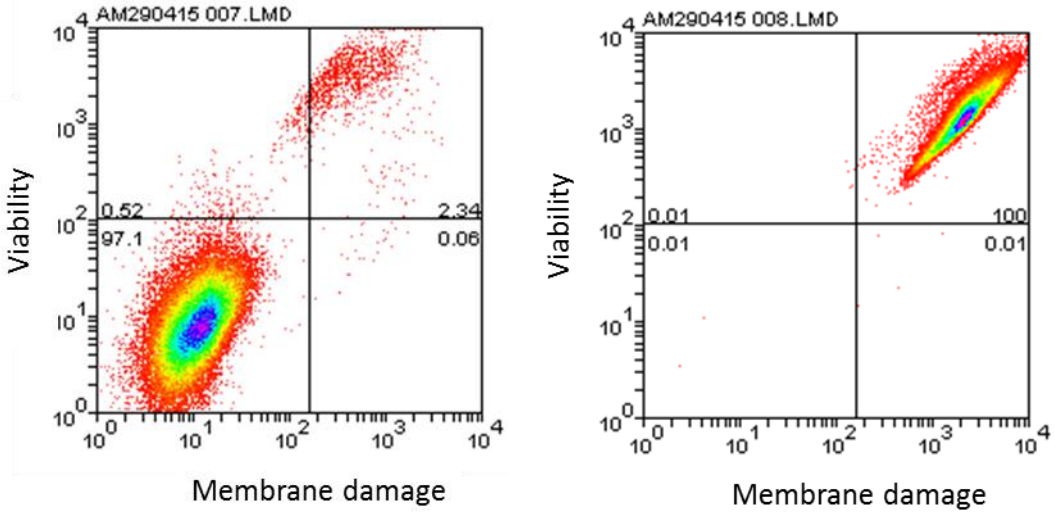


Figure 5.8 The effect of oxidative stress induced by H_2O_2 on the membrane fluidity of lager yeast strains SMCC100, SMCC99 and SMCC90. Membrane fluidity was determined using the general polarization ratio of Laurdan staining. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation. High GP values indicate low fluidity.

As a complement to membrane analysis using laurdan staining for fluidity analysis, membrane damage was also directly assessed using a combination of propidium iodide (for viability analysis) and Bis-oxonol (for membrane damage) (Section 2.10.2). Propidium iodide is able to enter dead cells and stains DNA structures, while Bis-oxonol can evaluate changes in membrane potential by entering de-polarized cells and binding to intracellular proteins or membranes (Simonin *et al* 2007; Attfield et al 2000; Epps *et al* 1994). Subsequently the relationship between ROS, cell viability and membrane damage in populations of oxidatively stressed lager yeast was investigated using flow cytometry (Section 2.10.1). This was conducted to facilitate analysis of the dual staining technique, but also to obtain a more accurate indication of the impact of ROS based on a significantly larger population size. Membrane damage was defined by cells that had assimilated bis-oxonol, and was reflected in increasing levels of relative fluorescence (RF) on the x-axis (Figure 5.9). Viability was defined as cells that were stained by propidium iodide and was reflected in increasing levels of relative fluorescence on the y-axis (Figure 5.9). To provide a benchmark, live and dead (heat killed; Section 2.10.1) cell populations of SMCC100 were stained using the protocol and analysed for cell viability and membrane damage (Figure 5.9a-b). In each instance, data is divided into 4 quadrants: lower left denotes viable cells with no membrane damage; lower right denotes viable cells with membrane damage; upper left denotes non-viable cells with no membrane damage; and upper right denotes non-viable cells with membrane damage (Figures 5.9). Sample points falling outside of the parameters for analysis, or that were unstained, were excluded as outliers, since there was a high possibility that they simply reflected debris within the samples.



A B

Figure 5.9a-b. The differentiation of cells by viability and membrane damage using dual staining of 100% viable (A) and 100% dead (B) SMCC100 cells. Membrane damage as determined by the fluorescence intensity of bis-oxonol are shown on the x-axis. Cell viability is indicated by the fluorescence intensity of propidium iodide on the y-axis. The concentration of cells is denoted by the coloured areas, where blue is the most densely populated area and red is the least densely populated area. The values stated in the corners of each quadrant denote the percentage of all cells observed in that area.

To determine the impact of oxidative stress on cell viability and membrane damage, strains SMCC100, SMCC90 and SMCC99 were subjected to oxidative stress by incubation in H_2O_2 at 0mM, 1mM, 5mM and 10mM H_2O_2 (Section 2.10.1) and analysed using the propidium iodide / bis-oxonol dual staining technique. It was observed that in the presence of 0mM H_2O_2, strain SMCC100 exhibited 96.8% viable cells with no membrane damage, while 0.1% of the population was viable with signs of membrane damage. The remainder of the population comprised dead cells with compromised membrane structures (Figure 5.10a, Figure 5.1). In the presence of 1mM a similar pattern of results was seen. However there was a movement towards the upper right quadrant reflecting dead cells with damaged membranes. In this instance, viable cells with healthy membranes comprised 89.3% of the population with 10.1% being dead with damaged membranes. Live cells with disrupted membranes continued to comprise a low percentage of the population (0.42%) (Figure 5.10a, Figure 5.11). This trend continued when 5mM H_2O_2 stressed cells were analysed with a continuing shift to the top right. Live cells with undamaged membranes comprised 21% of the population with 78.9% being dead and damaged. Interestingly, under these conditions, it can be seen that live cells also became affected; 5.2% of cells were viable but with signs of membrane disruption (Figure 5.10a, Figure 5.11). However it should also be noted that the majority of live cells in this instance, represented in the bottom left quadrant, exhibited an increase in the intensity of bis-oxonol, showing a shift towards the right and indicating low levels of membrane damage. At 10mM H_2O_2 all cells appeared to be dead, supporting the previous data indicating that cells were unable to tolerate these conditions.

Similar patterns were observed for strains SMCC90 (Figure 5.8b, 5.10) and SMCC99 (Figure 5.10c, 5.13) with a transition from bottom left (viable and healthy membranes) to top right (dead and disrupted membranes) with increasing concentrations of H_2O_2. However, some differences were observed between strains, with SMCC90 appearing to be the least tolerant to stress and SMCC99 exhibiting characteristics which were better than SMC90 but worse than SMCC100. For SMCC90 the poor stress tolerance can be seen by analysis of data relating to stress at 1mM H_2O_2. Under these conditions the number of viable cells with healthy membranes reduced to 60.5%, while the number of dead cells with disrupted membranes was 37.3% (Figure 5.10b, 5.112). Interestingly, for this strain there was no apparent shift to the right in live cell populations, indicating that membrane damage was not directly linked to cell death under these conditions. However, it should be acknowledged that this may be an artefact of the level of stress induced, since the data is otherwise comparable to that obtained using laurdan staining, indicating that this strain may be less tolerant to ROS in general. Indeed, if the data is examined, there is a downshift in the upper right quadrant which may indicate that a greater number of cells which were viable with damaged membranes (i.e. within the lower right quadrant) may have been observed if a slightly lower level of stress had been applied.

Analysis of strain SMCC99 showed results which were more similar to SMCC100. While control (0mM) samples were mainly healthy (94.4%), in the presence of 1mM H_2O_2, viable cells with healthy membranes comprised 86.7% of the population with 12.6% being dead with damaged membranes. At this stage live cells with disrupted membranes comprised just 0.5% of the population (Figure 5.10c, Figure 5.13). When cells

stressed with 5mM H_2O_2 were analysed, live cells with undamaged membranes comprised 5.98% of the population with 90.1% being dead and damaged. Similar to strain SMCC100, under these conditions 3.9% of cells were viable but with signs of membrane disruption (Figure 5.10c, Figure 5.13), while live cells were also noticed to shift towards the right, again indicating low levels of membrane damage as described for SMCC100.

Analysis of the combined data analysing cell membrane damage and membrane fluidity indicated that as oxidative stress increased, membrane fluidity decreased, becoming more gel like. This corresponded to an increase in membrane damage and ultimately in cell death. The change in membrane structure and integrity is likely to be a direct result of oxidative stress, where oxidation of lipids is known to cause a reduction in membrane fluidity and an increase in membrane damage (Gupta *et al* 1994). It is likely that this would lead to an increase in permeability, reducing the function of the plasma membrane as a boundary between the cell and its environment. In the context of this experiment, this would likely lead to increased ROS influx, resulting in lipid peroxidation, protein degradation and DNA damage resulting in cell death (Puligundla et al 2011; Landolfo *et al* 2008; Peronne *et al* 2008; Moradas-Ferreira *et al* 1996). However, in the context of beer production, this would allow the influx of wort and beer components, which would ultimately result in a similar effect.

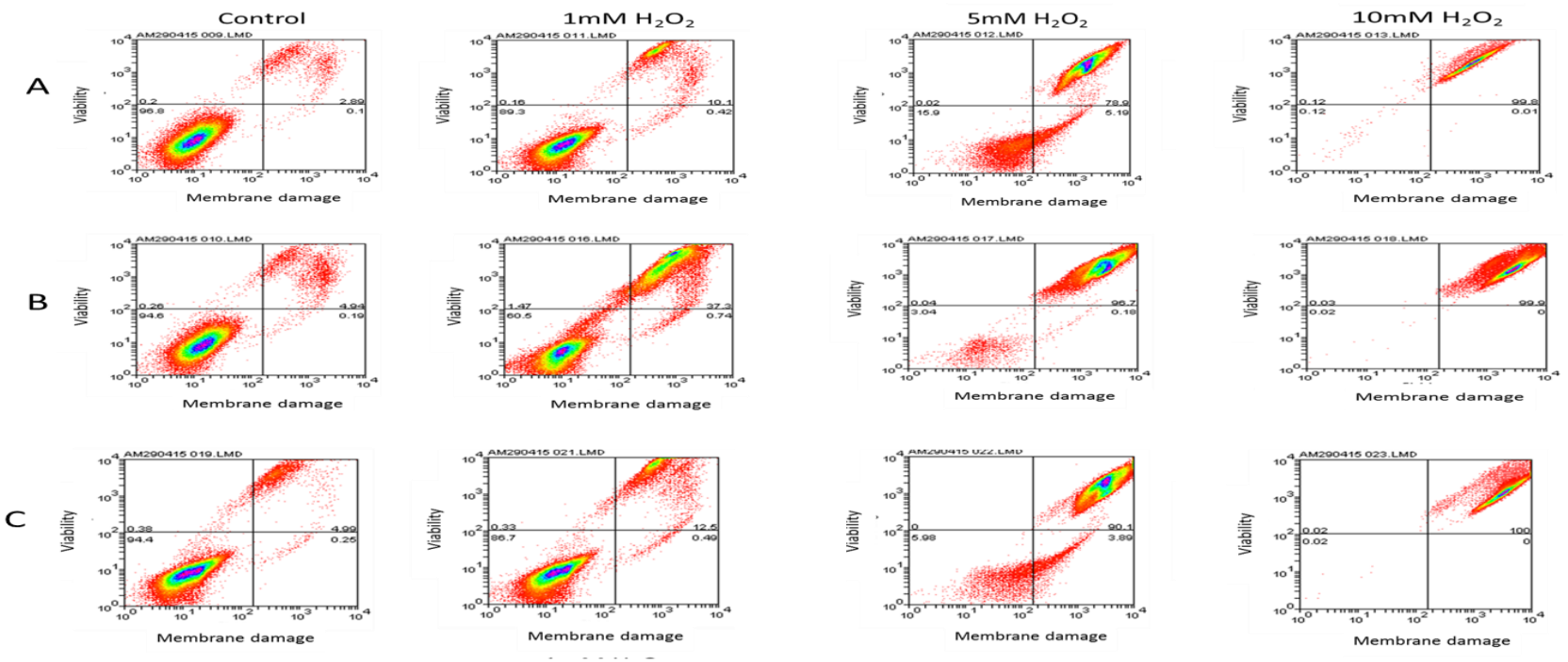


Figure 5.10a-c The differentiation of cells by viability and membrane damage using dual staining of SMCC100 (A), SMC90 (B) and SMCC 99 (C) after exposure to 0mM, 1mM, 5mM and 10mM H_2O_2 for 1hr. Membrane damage is indicated by the fluorescence intensity of bis-oxonol on the x-axis. Viability is indicated by the fluorescence intensity of propidium iodide on the y-axis. The concentration of cells is denoted by colour, where blue is the most densely populated area and red is the least densely populated area. The values in the corner of each quadrant denote the percentage of all cells observed within that specific area.

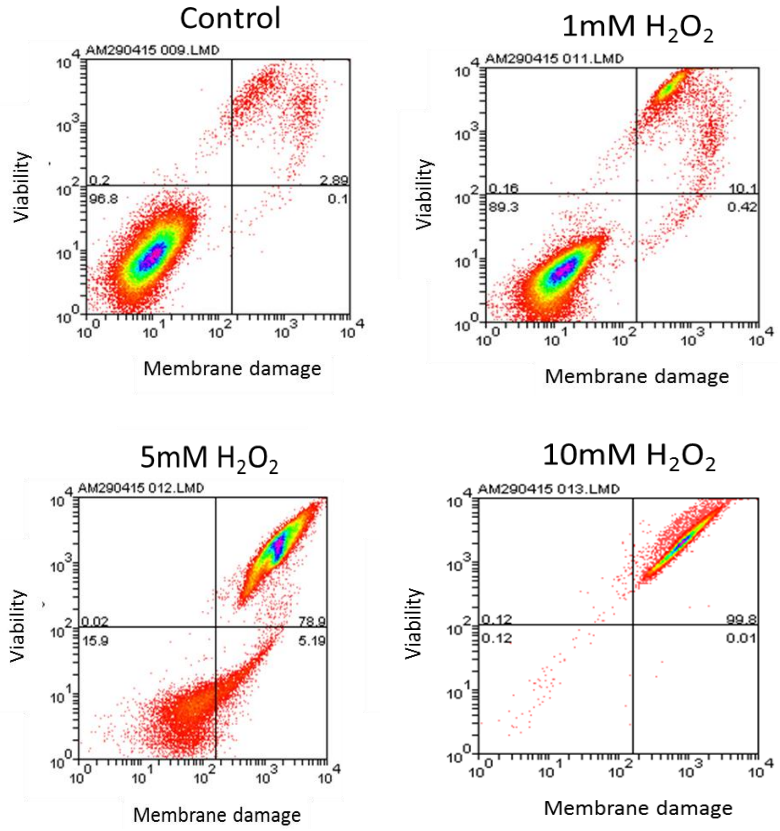


Figure 5.11 The differentiation of cells by viability and membrane damage using dual staining of SMCC100 after exposure to 0mM, 1mM, 5mM and 10mM H_2O_2 for 1hr. Membrane damage is indicated by the fluorescence intensity of bis-oxonol on the x-axis. Viability is indicated by the fluorescence intensity of propidium iodide on the y-axis. The concentration of cells is denoted by colour, where blue is the most densely populated area and red is the least densely populated area. The values in the corner of each quadrant denote the percentage of all cells observed within that specific area.

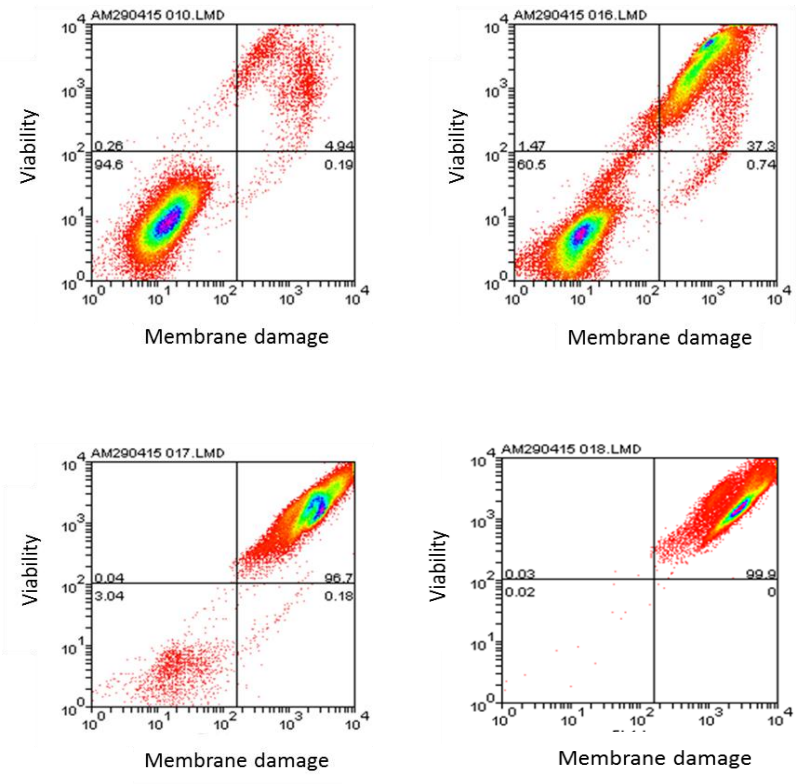


Figure 5.12 The differentiation of cells by viability and membrane damage using dual staining of SMC90 after exposure to 0mM, 1mM, 5mM and 10mM H_2O_2 for 1hr. Membrane damage is indicated by the fluorescence intensity of bis-oxonol on the x-axis. Viability is indicated by the fluorescence intensity of propidium iodide on the y-axis. The concentration of cells is denoted by colour, where blue is the most densely populated area and red is the least densely populated area. The values in the corner of each quadrant denote the percentage of all cells observed within that specific area.

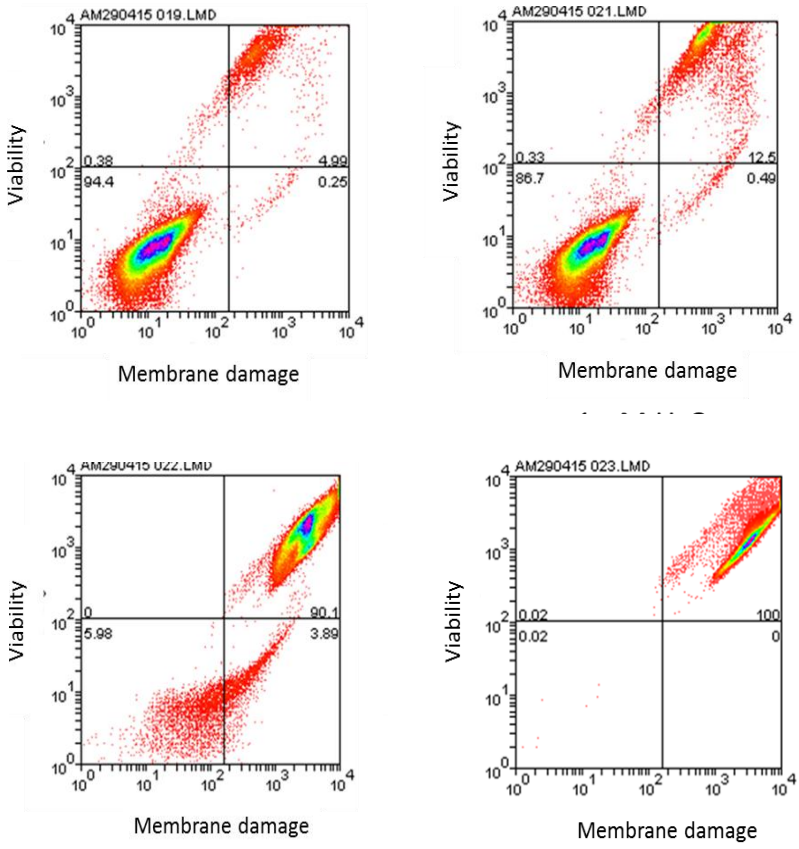


Figure 5.13 The differentiation of cells by viability and membrane damage using dual staining of SMCC 99 after exposure to 0mM, 1mM, 5mM and 10mM H_2O_2 for 1hr. Membrane damage is indicated by the fluorescence intensity of bis-oxonol on the x-axis. Viability is indicated by the fluorescence intensity of propidium iodide on the y-axis. The concentration of cells is denoted by colour, where blue is the most densely populated area and red is the least densely populated area. The values in the corner of each quadrant denote the percentage of all cells observed within that specific area.

5.3 Discussion

Yeast are exposed to oxidative stress throughout yeast handling, but particularly during propagation and fermentation (Quain & Boulton 1987; Boulton 1991; *Gibson et al 2008* Chapter 4). In this series of experiments the lager yeast strains SMCC100, SMCC90 and SMCC99 were investigated to determine the impact of oxidative stress on cell physiology, DNA integrity and membrane health. Initially a system to investigate oxidative stress was generated by correlating the amount of ROS generated by H_2O_2 to concentrations typically observed during fermentations at 15 and 22°P. It was observed that cells from fermentations typically exhibited a concentration of ROS equivalent to that generated by between 1 and 5mM H_2O_2. However, despite this relationship it is important to note that there are flaws within the developed system. Stress acquired over time and during active growth is known to lead to enhanced tolerance (Stewart 1988; D'Amore et al 1990). Consequently, while applying stress for a limited time period *in vitro* is useful to determine the intrinsic capacity to withstand an environmental challenge, applying this data to a real world scenario should be treated with caution. This can be seen based on viability analysis which indicated that populations began to exhibit cell death when 1mM H_2O_2 was applied for a period of 1hour. This concentration H_2O_2 corresponds to a level of ROS typically observed after approximately 100 hours during fermentation of 15°P wort and after just 20 hours in 22°P wort. Similarly, severe viability loss was observed for all strains in the presence of 5mM H_2O_2, corresponding to the mid-point of a 22°P fermentation. Although previous

data indicated that viability remained relatively high throughout fermentation (Chapter 4), a decline in viability was observed during 22°P fermentations; it is possible that this was linked to the presence of ROS, or a combination of ROS and other stress factors such as ethanol.

Analysis of the impact of ROS on DNA integrity revealed that there were no deleterious effects of H_2O_2 stress on genomic DNA used within the experimental parameters applied. This was surprising given that it has been reported that as little as 3mM H_2O_2 stress can lead to DNA damage (Madeo *et al* 1999). Other studies have indicated that DNA damage caused by up to 60 mins of 10mM H_2O_2 stress can be reversed due to a yeast cells ability to accomplish DNA repair (Azevedo *et al* 2011; Ribiero *et al* 2006), however this was unlikely to have occurred in the current series of experiments since analysis was performed directly after stress induction. Although there was no indication of oxidative stress induced DNA damage, it is possible that point mutations could have occurred throughout the genome which remained undetected. Further analysis could be performed in the future, employing shotgun sequencing methods such as those described by Nakao et al (2009) to examine DNA damage at the base level. This methodology could indicate point mutations, which would signify damage caused by the oxidative stress. It has also been reported that the length of lager yeast chromosomes can change during successive fermentations (Pedersen 1994), creating chromosome length polymorphisms (CLP's). Consequently, chromosome analysis techniques such as pulsed field gel electrophoresis (Schwartz *et al* 1984) could also be applied in an attempt to gain further confidence in the results.

A similar pattern of results was observed following analysis of mtDNA. Again, this was surprising given that this structure is known to be more

susceptible to oxidative damage than nuclear DNA, due to the absence of stabilising histones (Kang & Hamasaki 2002). It has been shown that MtDNA instability and damage through interaction with ROS can lead to mutation where respiratory deficient yeast or 'petites' are formed (Nagley & Linnane 1970; Goldring *et al* 1970). These cells exhibit a reduction in respirative capacity, atypical mitochondrial morphology and otherwise dysfunctional mitochondria (Shigenaga *et al* 1994) leading to poor fermentation performance (Gibson *et al* 2008; Ernandes *et al* 1993; Goode *et al* 1993). In this Chapter, experimental samples were analysed for petite generation using the tetrazolium triphenyl chloride (TTC) overlay technique (Ogur *et al* 1957), but <0.1% petites were observed in each instance (data not shown), supporting the analysis of mtDNA.

Plasma membrane integrity is essential for cell function. Not only does this structure form a barrier between the cell and its environment, but there are over 200 integral membrane proteins, many of which are involved in transmembrane solute transport. These include permeases for amino acid transport (Nelissen *et al* 1997; André 1995) and sugar uptake (Boles & Hollenberg 1997; André 1995), as well as proteins responsible for signal transduction (Özcan & Johnston 1996a; Özcan & Johnston 1996b). Alterations to membrane integrity will consequently have a detrimental impact on functionality. The data presented here indicated an increase in membrane damage as well as a reduction in membrane fluidity as oxidative stress was increased. This is likely to have occurred due to lipid peroxidation, which results in the accumulation of products such as lipid hydroperoxides within the hydrophobic core of the membrane (Kobayashi *et al* 1993; Girotti 1998). This can result in disturbances to the arrangement of phospholipid moieties and impairment of membrane

function manifesting in altered membrane fluidity and increased permeability (Howlett & Avery 1997; Van Ginkel & Sevanian 1997). One of the most important abilities of the plasma membrane is its distribution of lipids and proteins, which are arranged in several co-existing domains with different fluidity characteristics (Turk *et al* 2011; Simons & Toomre 2000; Strancar *et al* 2000). Any changes that occur to membrane fluidity can significantly affect membrane function (Hazel & Williams 1990), and as such the fermentative ability of yeast. Consequently, the fact that ROS caused a deterioration in cell membrane physiology in conjunction with cell viability indicates that ensuring membrane integrity may be an important consideration, especially when fermenting worts of very high gravity. This will be the subject of Chapter 6.

CHAPTER 6: THE EFFECT OF OXYGENATION ON STEROL CONTENT, PLASMA MEMBRANE, MEMBRANE FLUIDITY AND OXIDATIVE STRESS

6 The effect of oxygenation on sterol content, plasma membrane fluidity, and oxidative stress tolerance

6.1 Introduction

Although yeast are respiro-fermentative organisms, capable of ATP production in aerobic and anaerobic conditions, brewing yeast strains have an absolute requirement for oxygen. This is primarily due to its role in sterol biosynthesis, essential for the maintenance of membrane efficiency and for cell division in yeast (Landolfo *et al* 2010). Ergosterol forms the major sterol in brewing cells and plays an essential role in the stability of membranes by affecting rigidity, fluidity, and permeability (Abe & Hiraki 2009; Rog *et al* 2009; Folmer *et al* 2008). It has been observed that yeast mutants that are unable to produce ergosterol are hypersensitive to a range of environmental challenges, including oxidative stress (Higgins *et al* 2003). Furthermore, it is known that by increasing the unsaturation index and ergosterol content of yeast, cells acquire a greater tolerance to stress factors (Thomas *et al* 1978; You *et al* 2003; Shobayashi *et al* 2005).

Oxygen is a key requirement for sterol synthesis due to its importance in the biosynthetic pathway. The genes HMG1 and HMG2, involved in the conversion of Aetyl-CoA to mevalonic acid are oxygen regulated and determine access to the pathway (James *et al* 2003). Furthermore, oxygen has a direct role in the conversion of squalene to squalene epoxide (Section 1.3.1) to the extent that lack of availability results in a build-up of squalene and a failure to produce ergosterol. Hence, from a brewing perspective, oxygenation plays a key role in determining sterol content and the potential to resist stress factors, including, paradoxically, those associated with reactive oxygen species (ROS).

In order to ensure brewing yeast sterol content is adequate, wort is oxygenated throughout propagation and immediately prior to fermentation. Typically 1ppm oxygen per degree Plato is provided, which is sufficient to support the growth of yeast cells at standard gravities (Stewart 1999). However, the extent of oxygenation is known to be variable, since each strain has its own specific requirements (Gordon & Stewart 1972; Kirsop 1974; Jakobsen & Thorne 1980). Furthermore, there is no consensus with regard to the amount of oxygen which is required at high or very high gravities. The aim of this chapter was to investigate the effects of oxygenation on sterol synthesis, and subsequently the impact of this on tolerance to oxidative stress. While a similar study has been conducted by Verbelen *et al* (2008), these authors focused predominantly on the relationship between oxygenation and expression of genes involved in sterol synthesis and stress tolerance. In this study the aim was to assess the physiological relationship between oxygenation, sterol content and yeast fermentation performance, and subsequently the ability to withstand H_2O_2 induced oxidative stress.

6.2 Results

In order to determine the effect of oxygenation on plasma membrane health, fermentative ability and oxidative stress tolerance, the lager yeast strain SMCC100 was exposed to oxygen for specific lengths of time (Section 2.10.1). This was performed to create populations of cells exhibiting a range of sterol concentrations which were then evaluated for fermentation performance and stress tolerance.

6.2.1 The effect of oxygenation time on ergosterol formation in lager yeast strain SMCC100

The effect of oxygenation on the formation of ergosterol was analysed by oxygenating generation 5 (G5) serially re-pitched lager yeast SMCC100 yeast slurry for 6 hours, as described in Section 2.11. Samples were removed at hourly intervals and analysed for ergosterol using HPLC (Section 2.11.2). A dried cell weight (DCW) based on $1x10^7$ cells/mL was used for all samples, and an internal standard of 50µg cholesterol was used to evaluate extraction efficiency.

Results showed that there was an extraction efficiency of 95% ± 2.2%SD, as indicated by the concentration of cholesterol (internal standard) extracted from each sample (Figure 6.1). Measurements of ergosterol prior to oxygenation indicated the presence of 0.68mg/g DCW, indicating that even cells supposedly deplete in sterols still maintained a base cellular level of ergosterol. After 1 hour of oxygenation there was no significant increase in the concentration of ergosterol levels. However after 2 hours 0.88 mg/g DCW was observed, after which ergosterol concentration

continued to increase to 1.64 mg/g DCW after 6 hours (Figure 6.1). These values are within the typical range observed for brewing yeast strains as reported by Verbelen et al (2009). These authors observed between 0.7 and 1.8mg/g DCW ergosterol in brewing yeast cultures (Verbelen et al 2009).

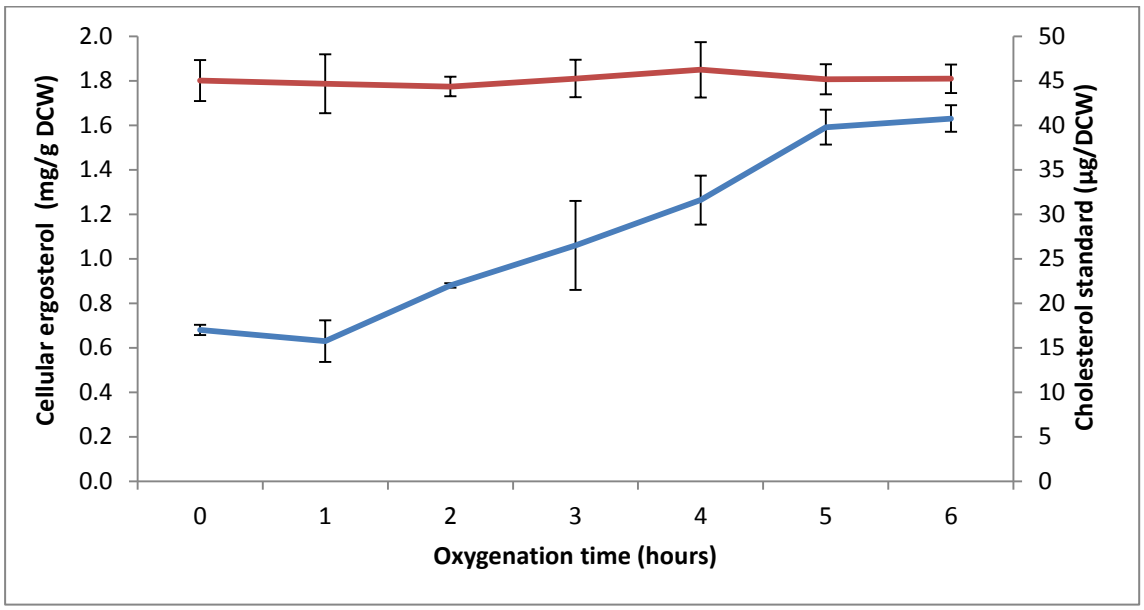


Figure 6.1 The effect of oxygenation time on cellular ergosterol (mg/g DCW) as shown on the primary axis (blue), and cholesterol standard (µg/g DCW) as indicated by the secondary axis (red). Dried cell weight (DCW) samples based on $1x10^7$ cfu/mL were taken from cells oxygenated at 15ppm/minute for up to 6 hours. Data shown represents the mean of triplicate samples with error bars representing the standard deviation.

6.2.2 The relationship between oxygenation, sterols and membrane fluidity in lager yeast strain SMCC100

In order to ascertain the effect of oxygenation on membrane fluidity in serially re-pitched lager yeast SMCC100, the fluorescent stain laurdan was used to show the level of generalised polarisation (GP) present in each sample (Section 2.10.2). GP was calculated using the relative intensities at 440nm, representing the gel phase, and 490nm, representing the liquid-crystalline phase where high resulting values indicate low fluidity and *vice versa* (Proszynski *et al* 2006; Learmonth & Gratton 2002; Yu *et al* 1996). Analysis of the data showed that the plasma membrane became less fluid as the length of oxygenation increased. On a 0-1 scale of GP, where 1 reflects a crystalline state and 0 is equal to a fluid membrane state, the un-oxygenated yeast had a GP of 0.3, which increased to 0.46 after 6 hours (Figure 6.2). Analysis of the data using Pearsons correlation coefficient indicated that there was an 85% (R=0.8492) correlation between membrane fluidity and ergosterol level (Figure 6.3). This result was surprising since it is generally considered that if the concentration of sterols increases, the membrane should become more fluid. However, it is known that the key factor in determining membrane fluidity is the association between sterols and phospholipids, which is a complex relationship. The steroid ring is attracted to the fatty acid component of phospholipids which provides rigour and helps immobilize the outer surface of the membrane. Hence although sterols function to assist in membrane health and fluidity, they do not cause fluidity *per se*. Oxygenated samples were consequently used for the remaining experiments within this Chapter where oxygenation at time 0-hour = 0.68 mg/g DCW, 1-hour = 0.63 mg/g DCW, 2-hour = 0.88 mg/g DCW, 3-hour = 1.06 mg/g DCW, 4-hour = 1.26 mg/g DCW, 5-hour = 1.59 mg/g DCW and 6-hour = 1.63 mg/g DCW.

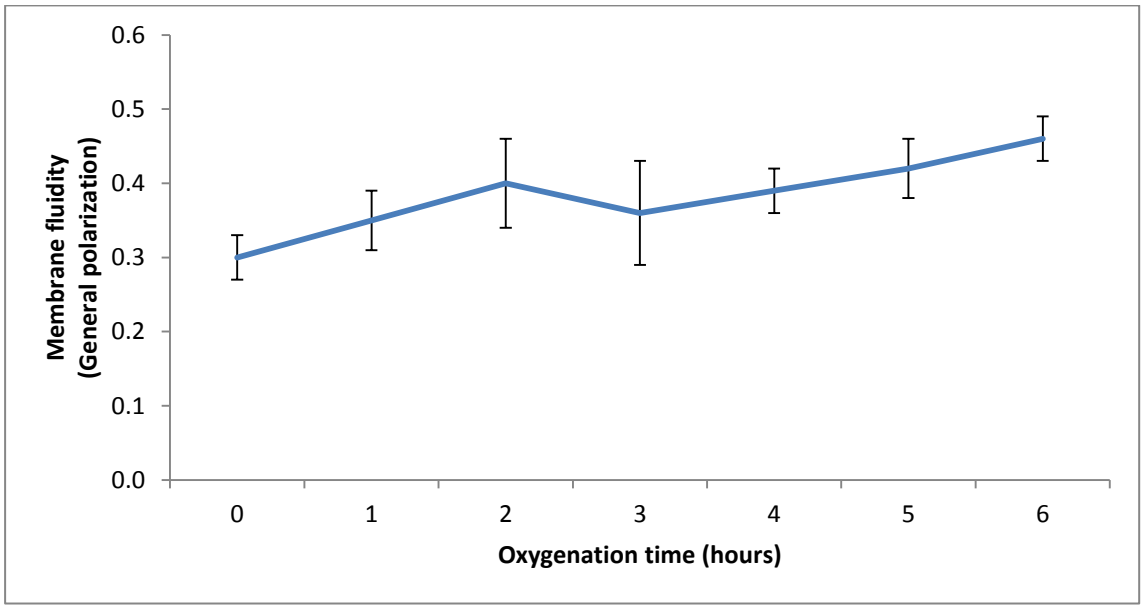


Figure 6.2 The effect of oxygenation on membrane fluidity. Membrane fluidity was determined by laurdan staining where high values indicate low fluidity and *vice versa*. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

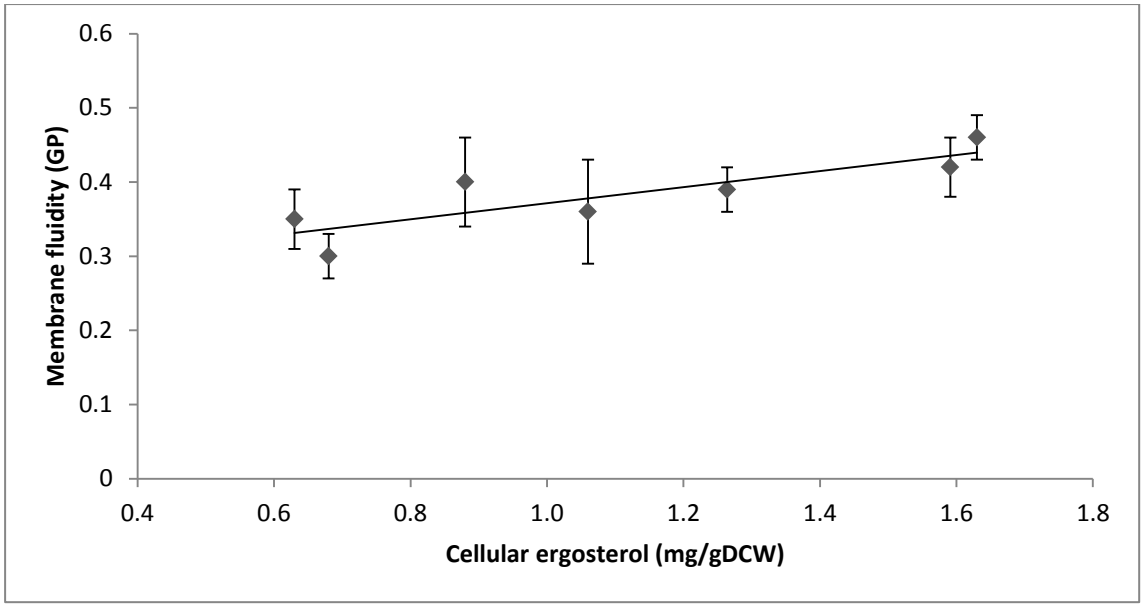


Figure 6.3 Correlation between ergosterol content and membrane fluidity. Membrane fluidity was determined by laurdan staining where high values indicate low fluidity and *vice versa*. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

6.2.3 The effect of sterol content on cell growth and ethanol production in lager yeast strain SMCC100

Once cell populations comprising different concentrations of sterols had been achieved these were analysed for fermentation performance at 15°P and 22°P using 100mL stir pots, pitched with $1.5x10^7$ cfu/mL and $2.2x10^7$ cfu/mL (Section 2.2; Section 2.7.1). Wort was de-aerated prior to use to prevent cellular production of sterols during fermentation. Each vessel was subsequently analysed for fermentation progression by weight loss (Quain *et al* 1985; Section 2.8) and the final products were analysed for attenuation, alcohol and cell biomass.

Analysis of standard (15°P) fermentations indicated that all samples reached the same level of attenuation (4.5%±0.2 weight loss), irrespective

of sterol content. However, the longer that yeast had been exposed to oxygen, the quicker attenuation was reached (Figure 6.4). Analysis of VHG (22°P) fermentations showed that the final level of attenuation was also the same for each of the oxygenated samples (7.4%±0.1 weight loss). However, the rate at which attenuation occurred was quicker when yeast had been oxygenated for longer. It was observed that the time needed for 0-hour oxygenated yeast to reach attenuation was 300 hours, which was significantly slower than 6-hour oxygenated yeast which only required 206 hours (Figure 6.5).

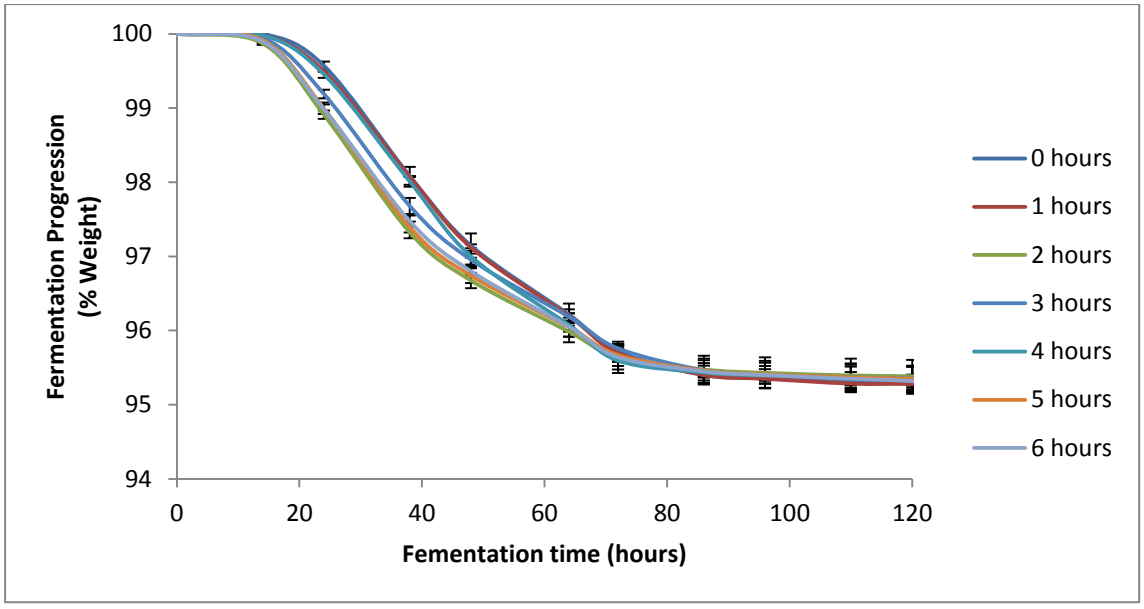


Figure 6.4 Fermentation of 15°P wort by strain SMCC100 in 100mL stirred vessels. Populations derived from cell samples oxygenated for 0-6 hours fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

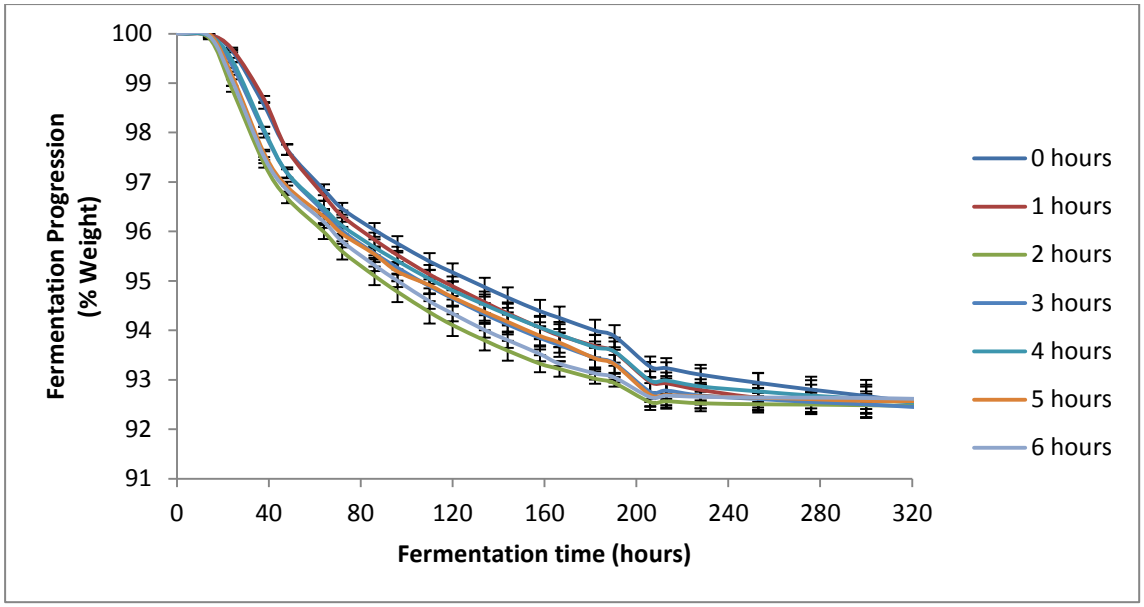


Figure 6.5 Fermentation of 22°P wort by strain SMCC100 in 100mL stirred vessels. Populations derived from cell samples oxygenated for 0-6 hours fermentations were employed. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation.

Fermentation progression was analysed using DMFit (DM: Dynamic Modelling) 2.1 software to analyse lag and exponential periods (Section 2.5.3). Comparison of the lag time in 15°P and 22°P fermentations for all oxygenated samples indicated that there was an exponential reduction in lag time with increasing oxygenation. For standard fermentations a reduction in lag time from 15.05 hours using the 0-hour oxygenated yeast was observed compared to 3.59 hours for 6-hour oxygenated yeast. For VHG fermentations a reduction in lag time from 21.58 hours using the 0-hour oxygenated yeast was observed compared to 1.38 hours for 6-hour oxygenated yeast. Consequently, initial lag time for 0-hour oxygenated yeast was greater in VHG worts than under standard conditions. However, when the rate of the exponential phase (μmax) was analysed it was

observed that there was no significant difference between fermentations, irrespective of oxygenation time or gravity of wort (P=<0.05) (Figure 6.7).

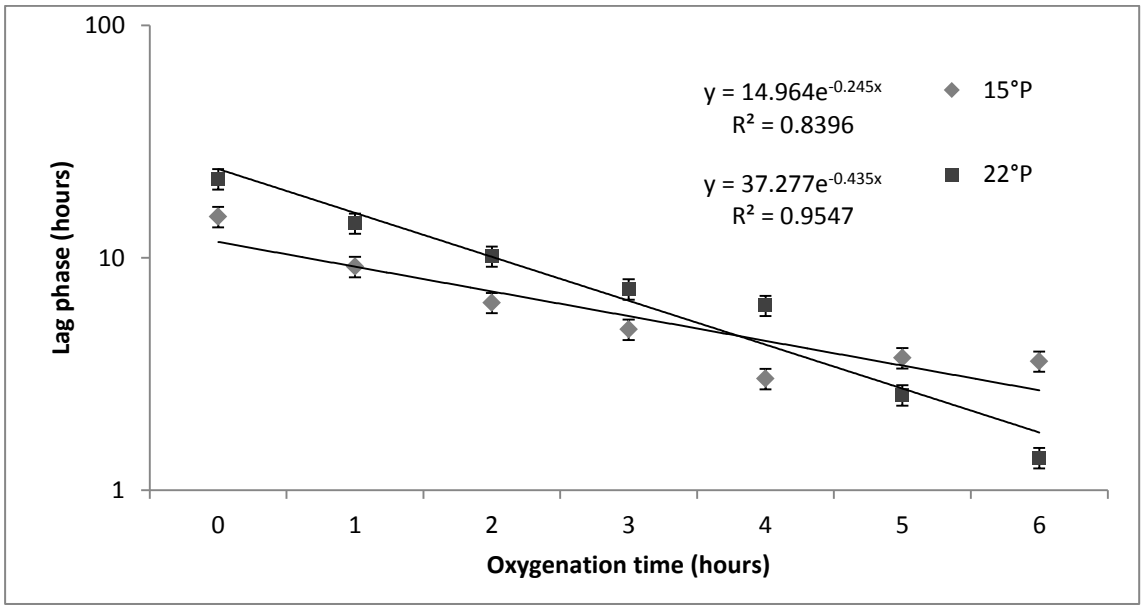


Figure 6.6 The relationship between oxygenation time and lag phase during lager fermentation. Lag phase kinetics were calculated from the mean of triplicate samples with error bars indicating the standard deviation

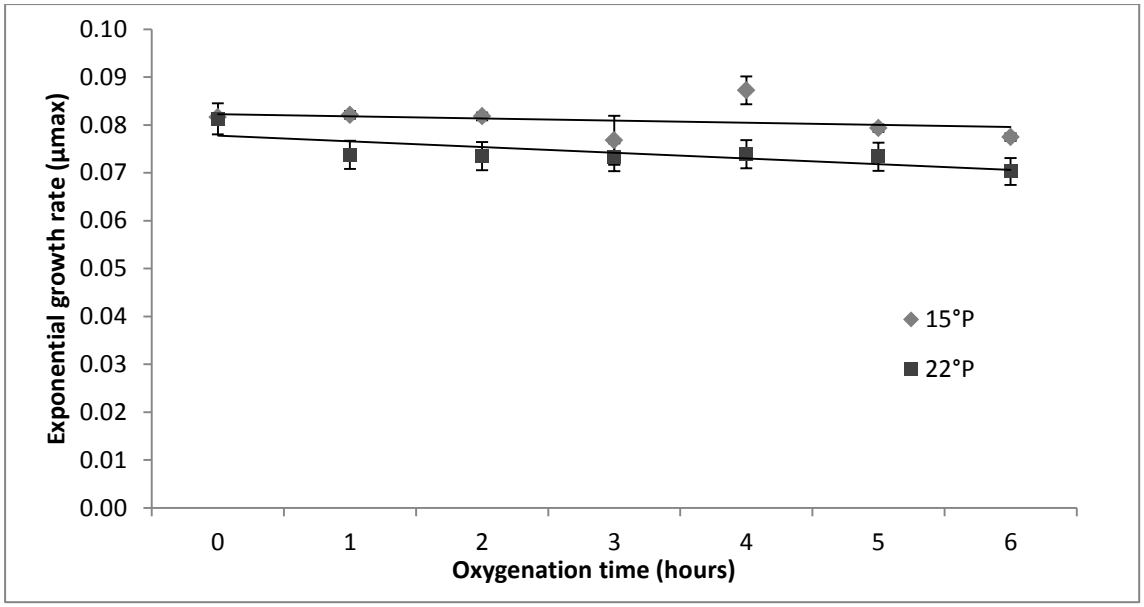


Figure 6.7 The relationship between oxygenation time and exponential phase during lager fermentation. µmax was calculated from the mean of triplicate samples with error bars indicating the standard deviation.

Analysis of beer produced during each fermentation indicated that all standard gravity (15°P) fermentations yielded a final gravity (°P) of 2.85± 0.05 (Figure 6.8) and produced 6.6% v/v alcohol regardless of oxygenation time (Figure 6.9). For VHG beer, the specific gravity was also similar for all samples (3.70 ± 0.13°P) (Figure 6.8). However, it was interesting to note that final alcohol level varied. It was observed that although the level of alcohol produced by the 0-hour, 1-hour and 2-hour was approximately 11.18% v/v, subsequent samples from the higher oxygenated yeast only reached 9.84% v/v (Figure 6.9). This is likely to have occurred due to a trade of with cell growth since final cell counts for VHG fermentations were observed to increase from 6.82×10^7 cfu/mL in 0-hour oxygenated yeast to 8.50×10^7 in 6-hour oxygenated yeast (Figure 10). Analysis of biomass and ethanol indicated that there was a direct correlation (R^2=0.896) between

these parameters (Figure 6.11). As one of the major goals of VHG fermentation is to increase alcohol production per batch, this outcome could potentially be unacceptable, although the decrease in fermentation time and potential positive impacts on yeast health may ultimately balance out this issue.

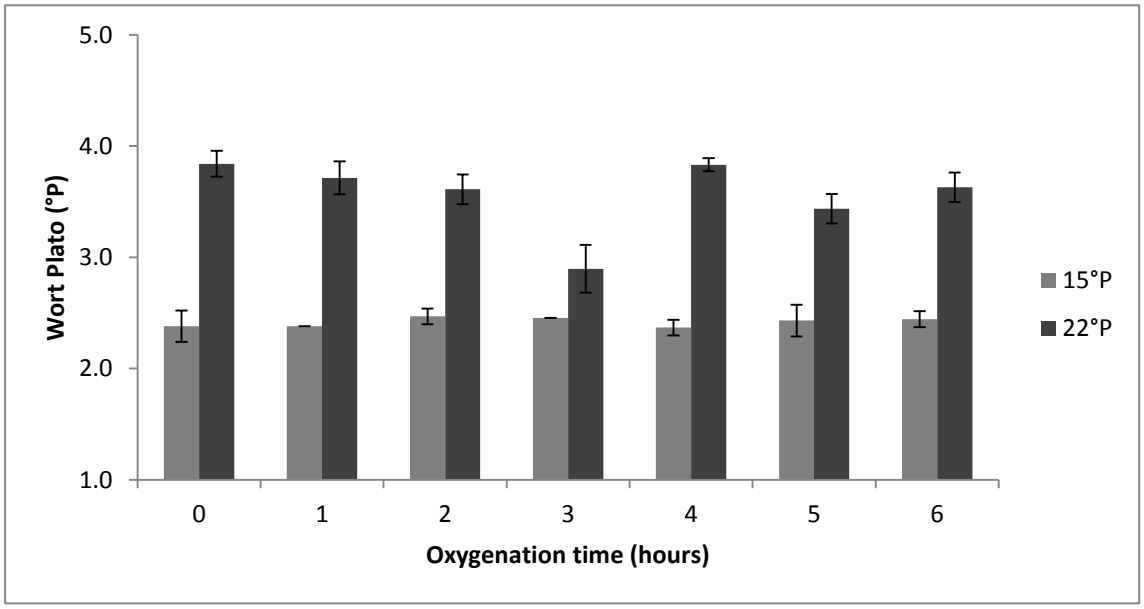


Figure 6.8 The effect of oxygenation time on final gravity in standard (15°P) and VHG (22°P) wort. Data shown represents the mean of triplicate samples with error bars representing the standard deviation.

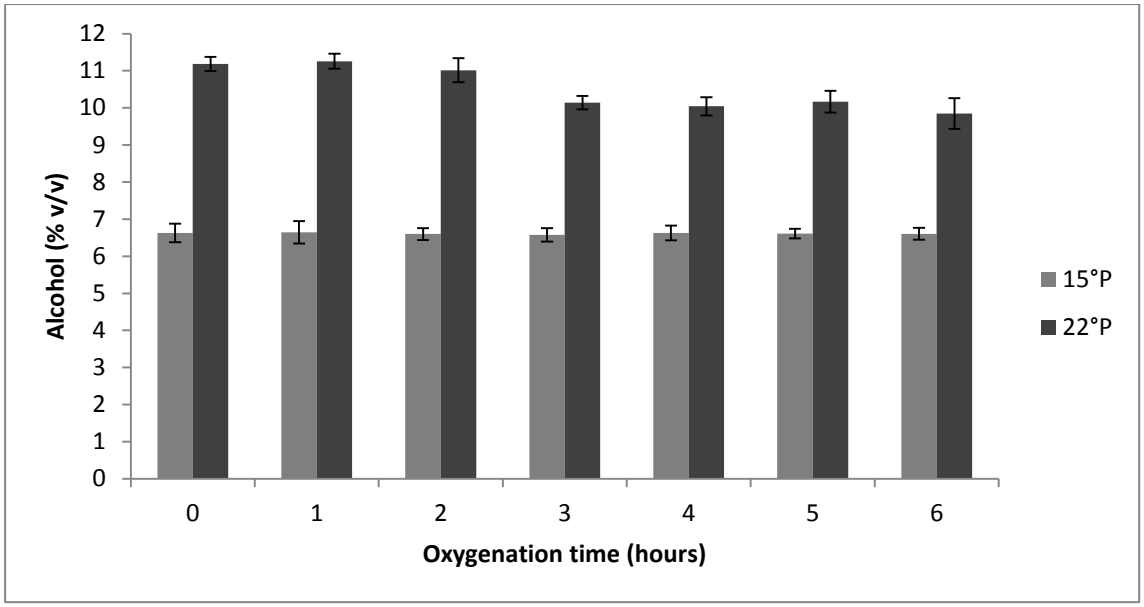


Figure 6.9 The effect of oxygenation time on final alcohol concentration in standard (15°P) and VHG (22°P) wort. Data shown represents the mean of triplicate samples with error bars representing the standard deviation.

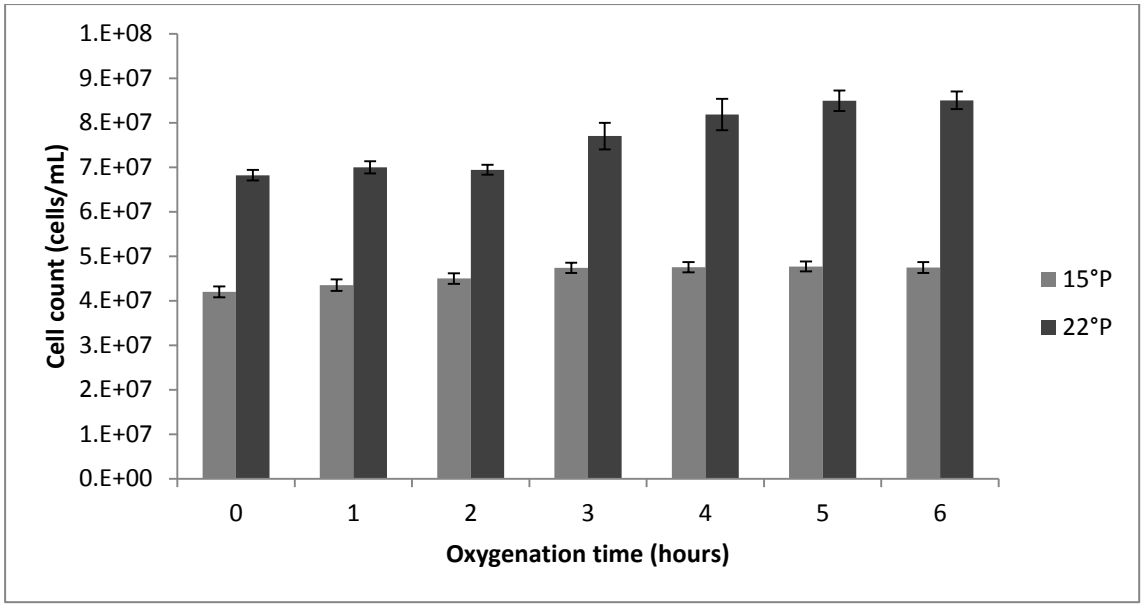


Figure 6.10 The effect of oxygenation time on yeast crop size, as determined by total cell number. Samples were obtained from standard (15°P) and VHG (22°P) fermentations. Data shown represents the mean of triplicate samples with error bars representing the standard deviation.

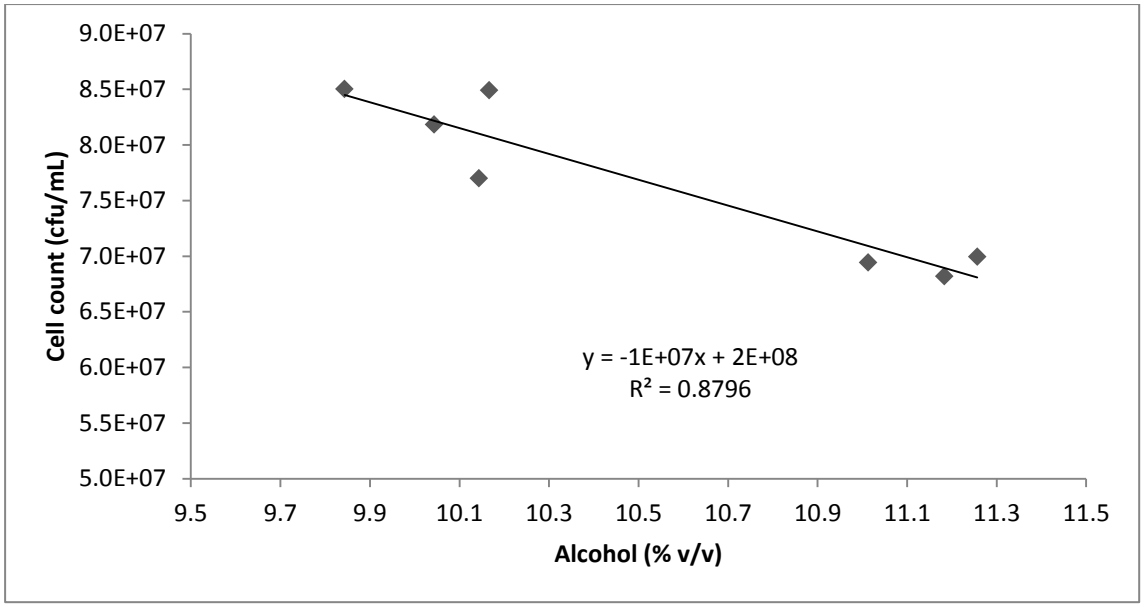


Figure 6.11 The correlation between alcohol content and total cell count at the end of fermentation. Data shown represents the mean of triplicate samples with error bars representing the standard deviation.

6.2.4 The relationship between sterol concentration, membrane fluidity and oxidative stress tolerance in lager yeast strain SMCC100

Once yeast cultures comprising cells with variable sterol content had been obtained, each population was subjected to oxidative stress (Section 2.8.7), designed to mimic levels of stress encountered in standard and VHG fermentations (Chapter 5). In response to this, yeast and membrane health were analysed by flow cytometry using propidium iodide and bis-oxonol fluorescent dual staining (Section 2.10.2).

Previous data indicated that increased oxidative stress can lead to decreased membrane fluidity and increased membrane damage. As a result of this, sub-optimal fermentation performance has been observed (Section 5.2; Section 5.3). It has also been noted that levels of oxidative

stress occur throughout standard (15°P) and VHG (22°P) fermentations (Section 4.3). In response to these challenges, the hypothesis that increasing the sterol content of yeast cells through oxygenation could lead to increased membrane stability and result in increased tolerance to the oxidative stresses that occur throughout fermentation was examined. In this analysis, oxygenated yeast samples of lager yeast SMCC100 were then subjected to H_2O_2 stress (as previously referred to in Chapter 5) being incubated at 25°C for 1 hour in the presence of 0, 1, 5 and 10mM H_2O_2 concentrations (Section 2.10). The samples were then analysed for viability and membrane damage using flow cytometry. The fluorescent stains propidium iodide (PI) and bis-oxonol were utilised to indicate viability (PI) and membrane damage (bis-oxonol) (Section 2.10.1).

Analysis of the viability of cell samples incubate in the presence of H_2O_2 showed that all oxygenated yeast maintained viabilities above 90%. This was slightly lower than freshly propagated yeast, normally >98%. As this yeast was recovered from several generations of VHG fermentation, the impact of the environmental stresses which are elevated when compared against standard fermentations. The viability in the presence of 1mM H_2O_2 fluctuated between 50 and 85%, with 6 hours oxygenation showing the lowest signs of viability at 53%, this was interesting as it indicated that sterol repletion was not producing a protective effect against the H_2O_2 stress. This was further indicated by the 5mM data, where a progressive drop from 0 hours, at 26% viability to 6 hours, where the viability had dropped to 1.2%, was observed. Once the strains were exposed to 10mM H_2O_2 stress, all strains showed more than 99% cell death. Unlike previous analysis of SMCC100 in the presence of 5mM H_2O_2, where the yeast had 21% viability, the re-pitched yeast used in this experiment was seen to

have an increased tolerance threshold, maintaining a viability of 26% (Figure 6.12).

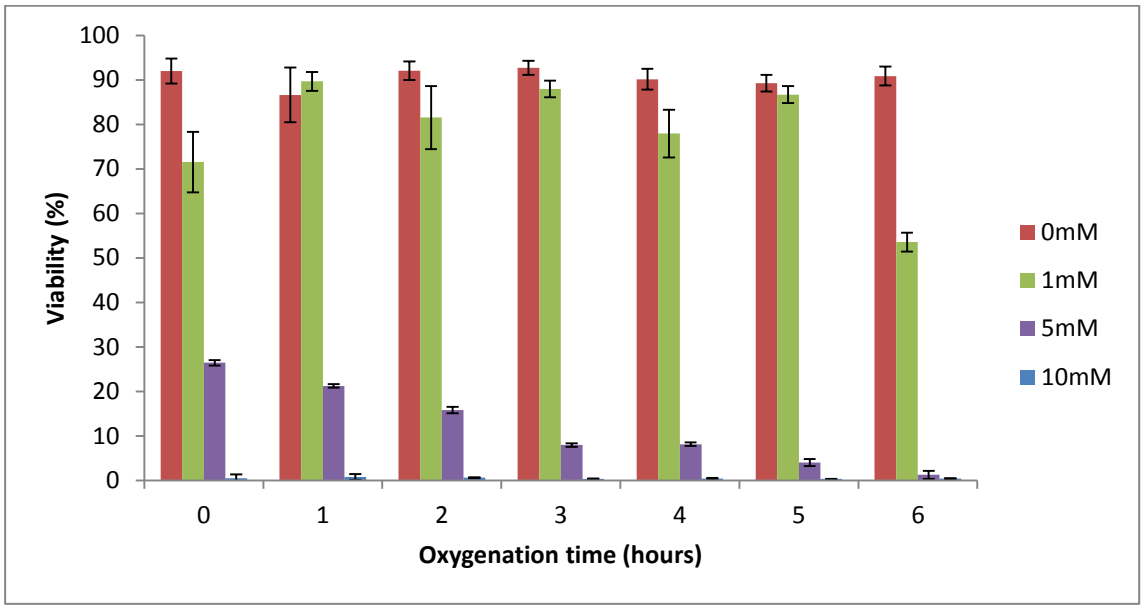


Figure 6.12 The effect of oxygenation time on resistance to oxidative stress. Cell fractions were subjected to H_2O_2 at concentrations of 0, 1, 5 and 10mM for 1 hour and analysed for viability. Data shown represents the mean of triplicate samples with error bars representing the standard deviation.

The cell samples from the oxygenated SMCC100 samples all indicated elevated levels of membrane damage, even the 0mM H_2O_2 control. The analysis of plasma membrane damage in the oxygenated yeast samples showed that membrane damage was present. It was interesting to note that all samples, even those from 0 hour oxygenation, showed membrane damage before any H_2O_2 stress was presented. There are several possible explanations for this result. The first explanation is that, as indicated by previous results, ROS accumulating throughout the previous

fermentations led to membrane damage occurring. The second possibility is that the heightened ethanol concentrations at the end of the VHG fermentations (around 12%v/v) could also lead to the membrane becoming compromised. Membrane damage was seen to be occurring in over 70% of the cells before any H_2O_2 stress was imposed on the yeast (Figure 6.13)

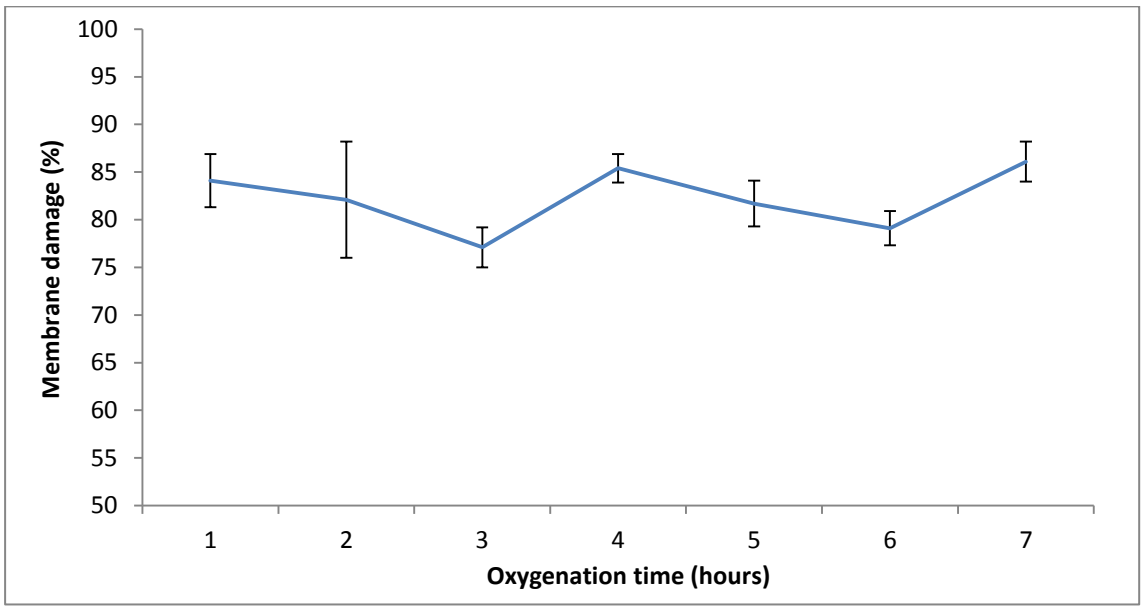


Figure 6.13. The effect of oxygenation on membrane damage. Cell fractions were subjected to H_2O_2 at concentrations of 0, 1, 5 and 10mM for 1 hour and analysed for membrane damage determined by bis-oxonol staining, Data shown represents the mean of triplicate samples with error bars representing the standard deviation.

Membrane damage was defined as cells that have taken up bis-oxonol, and was reflected in increasing levels of relative fluorescence (RF) on the x-axis. Viability was defined as cells that had taken up propidium iodide and was reflected in increasing levels of relative fluorescence on the y-

axis. The data was broken up into 4 quadrants; lower left, which denoted viable cells that had no membrane damage; lower right, which denoted viable cells that were undergoing membrane damage; upper left, which denoted non-viable cells with no membrane damage; and upper right which denoted non-viable cells that had undergone membrane damage.

When the data was analysed it could be seen that the viability of all oxygenated cells analysed was >90% ±7.14SD. It was also observed that all cells were undergoing a level of membrane damage from the start, with cells incubated in the presence of 0mM showing 84.1% membrane damage for 0hr oxygenation; 82.1% for 1hr; 77.1% for 2hr; 85.4% for 3hr; 81.7 for 4hr; 79.1% for 5hr; and 86.1% for 6hr (Figure 6.14). when the H_20_2 concentration was increased to 1mM there was an increase in dead cells for all of the oxygenated samples; with 0hr oxygenation showing 28.2% dead cells; 10.2% for 1hr oxygenation; 18.3% for 2hr oxygenation; 11.8% for 3hr oxygenation; 20.6% for 4hr oxygenation; 13.1% for 5hr oxygenation; and 45.7% for 6hr oxygenation. This meant that there was a 17.5% decrease in the viability of cells with the increased oxygenation time (Figure 6.15) In the presence of 5mM H_2O_2 a 43% drop in viability was observed for the yeast cells oxygenated for 0hr. Cell viability continued to drop for the other oxygenation levels; with the viability of 1hr oxygenated yeast dropping 67.3% to 25.7% viable cells; 2hr oxygenation dropped 65.7% to 15.1% viable cells; 3hr oxygenation dropped 92.2% to 7.32% viable cells; 4hr oxygenation dropped 91.0% to 7.59% viable cells; 5hr dropped 96.1% to 3.44% viable cells and 6hr dropped 98.3% to 0.86% viable cells (Figure 6.16). The observed data for cells incubated in the presence of 10mM H_2O_2 indicated that there was >99% cellular death for all oxygenated yeast (Figure 6.17).

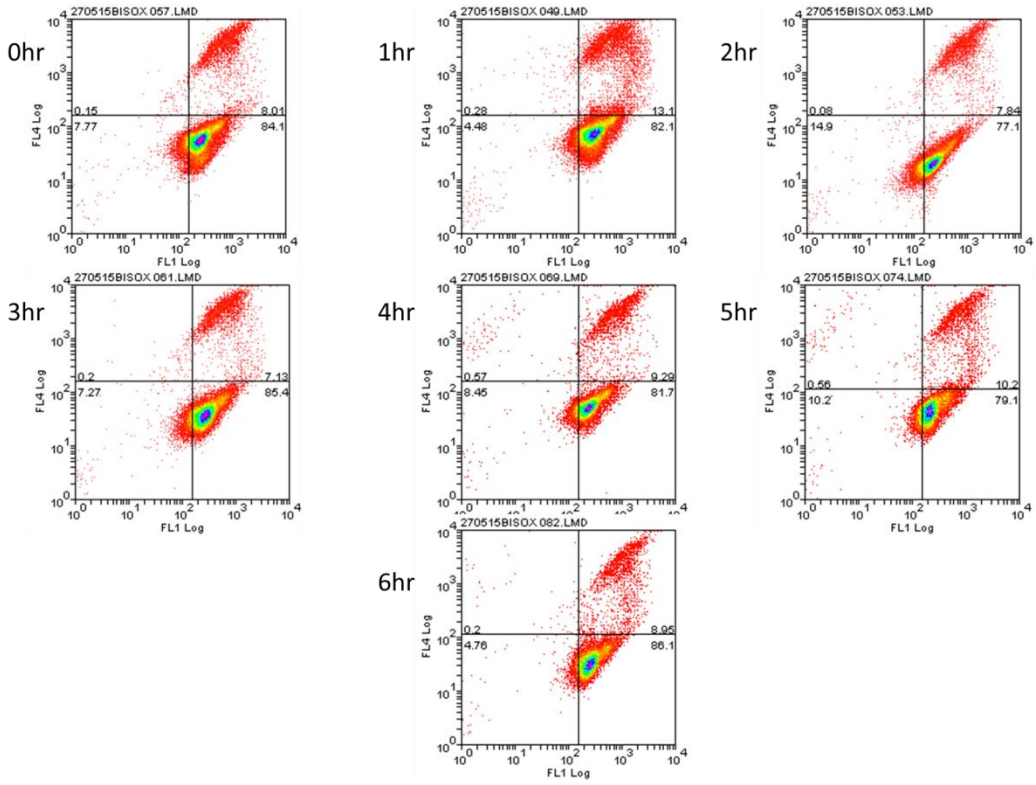


Figure 6.14 The differentiation of control cells (not oxygenated) by viability and membrane damage using dual staining. Membrane damage is indicated by the fluorescence intensity of bis-oxonol on the x-axis. Viability is indicated by the fluorescence intensity of propidium iodide on the y-axis. The concentration of cells is denoted by colour, where blue is the most densely populated area and red is the least densely populated area. The values in the corner of each quadrant denote the percentage of all cells observed within that specific area.

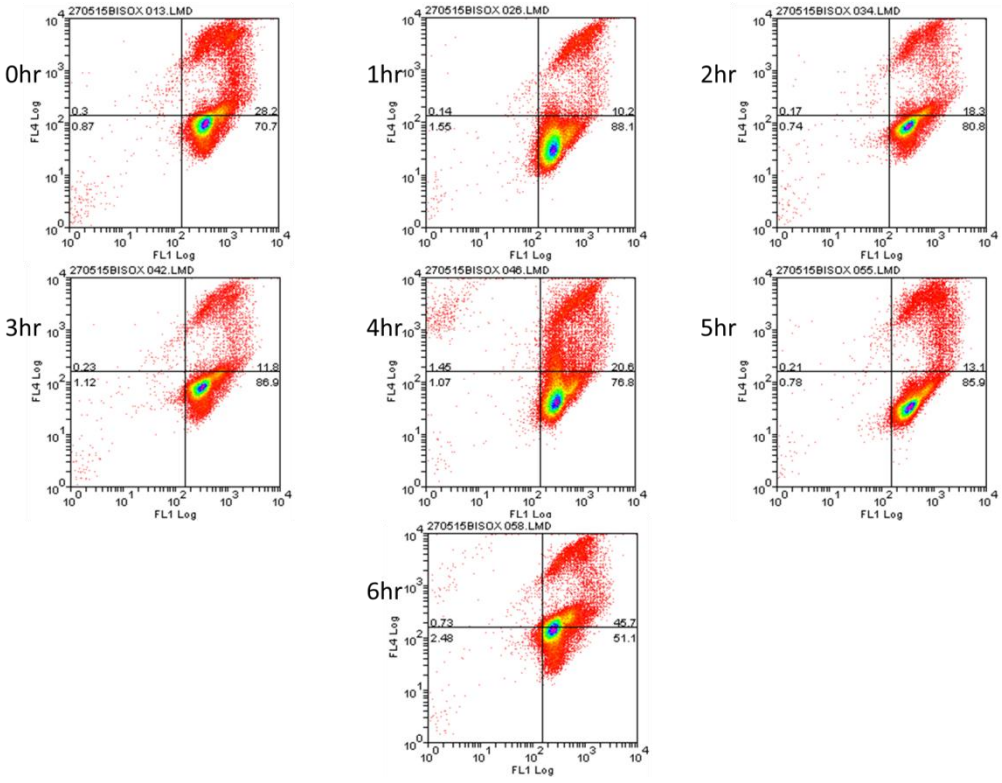


Figure 6.15 The differentiation of cells by viability and membrane damage using dual staining of oxygenated yeast (0-6hrs) after exposure to 1mM H_2O_2 for 1hr. Membrane damage is indicated by the fluorescence intensity of bis-oxonol on the x-axis. Viability is indicated by the fluorescence intensity of propidium iodide on the y-axis. The concentration of cells is denoted by colour, where blue is the most densely populated area and red is the least densely populated area. The values in the corner of each quadrant denote the percentage of all cells observed within that specific area.

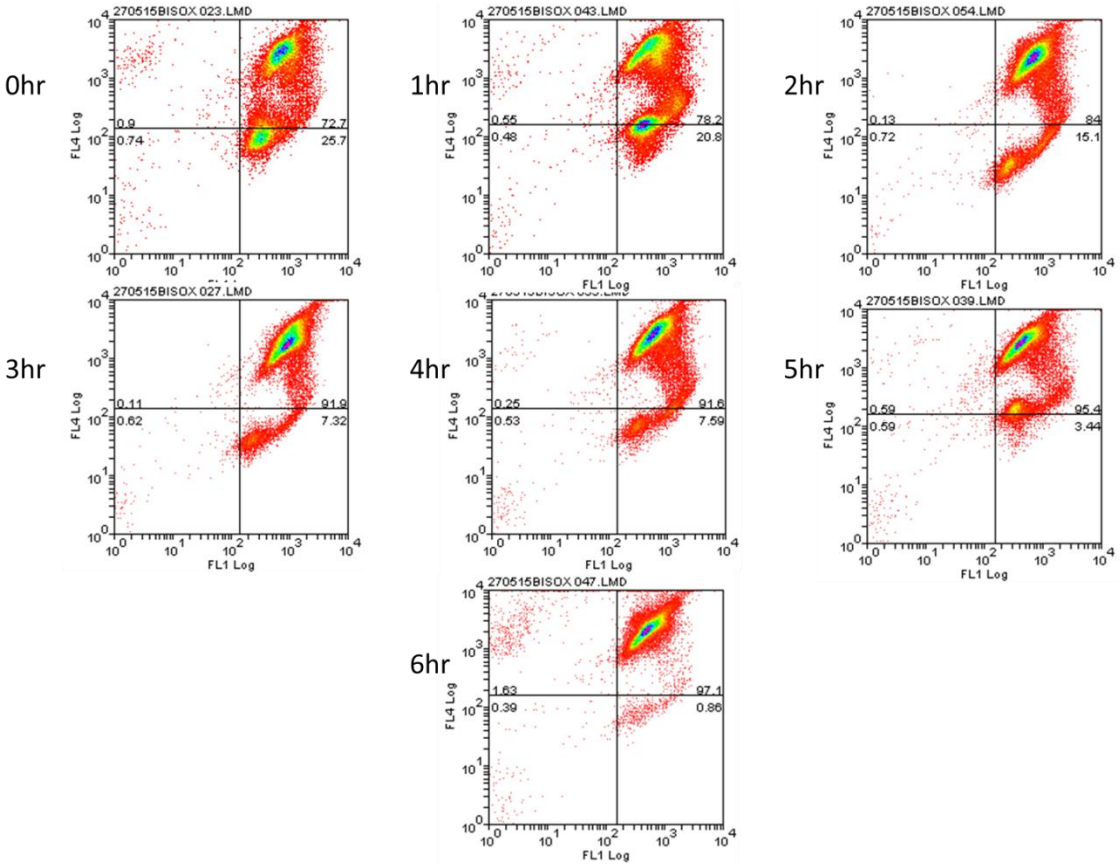


Figure 6.16 The differentiation of cells by viability and membrane damage using dual staining of oxygenated yeast (0-6hrs) after exposure to 5mM H_2O_2 for 1hr. Membrane damage is indicated by the fluorescence intensity of bis-oxonol on the x-axis. Viability is indicated by the fluorescence intensity of propidium iodide on the y-axis. The concentration of cells is denoted by colour, where blue is the most densely populated area and red is the least densely populated area. The values in the corner of each quadrant denote the percentage of all cells observed within that specific area.

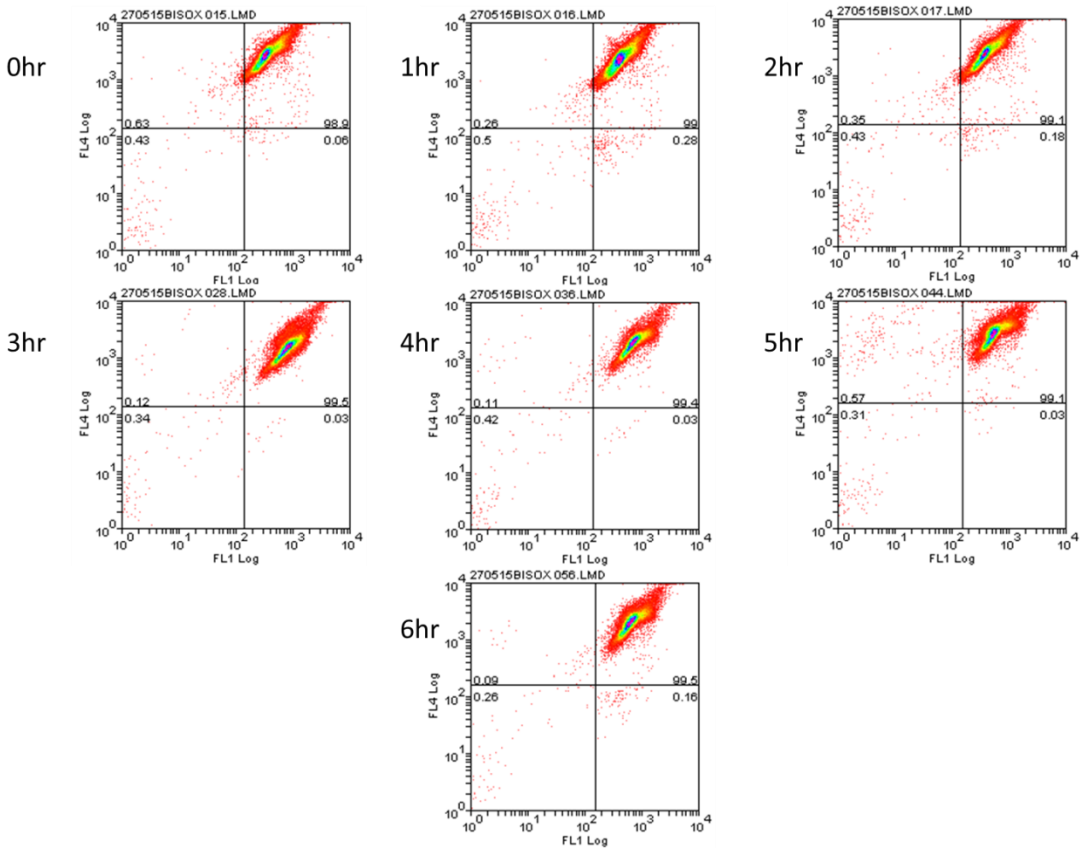


Figure 6.17 The differentiation of cells by viability and membrane damage using dual staining of oxygenated yeast (0-6hrs) after exposure to 1mM H_2O_2 for 1hr. Membrane damage is indicated by the fluorescence intensity of bis-oxonol on the x-axis. Viability is indicated by the fluorescence intensity of propidium iodide on the y-axis. The concentration of cells is denoted by colour, where blue is the most densely populated area and red is the least densely populated area. The values in the corner of each quadrant denote the percentage of all cells observed within that specific area.

This data indicated that there was no inference of protection from the levels of oxygenation used in this experiment on tolerance levels to H_2O_2 stress. Although an increase in ergosterol was observed in the extracted samples, this actually corresponded with a reduction in viability. As the ergosterol increased the reduction in viability from 26.4% to >1% viable cells was significant (P=<0.05). This indicated that rather than help the cells to tolerate the presence of H_2O_2, the oxygenation had a negative impact on the cells. (Figure 6.18). It was also observed that as the viability of the oxygenated yeast In the presence of 5mM H_2O_2 reduced, so did the membrane fluidity (Figure 6.19).

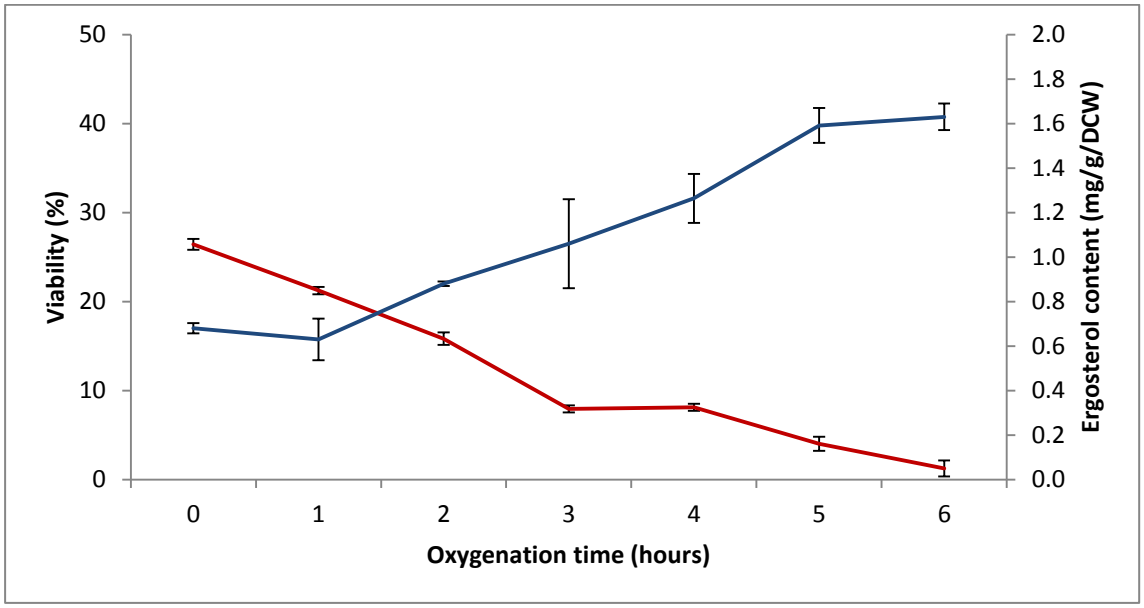


Figure 6.18 The effect of oxygenation time viability (%) as shown on the primary axis (red), and ergosterol content(mg/g DCW) as indicated by the secondary axis (red). Dried cell weight (DCW) samples based on $1x10^7$ cfu/mL were taken from cells oxygenated at 15ppm/minute for up to 6 hours. Data shown represents the mean of triplicate samples with error bars representing the standard deviation.

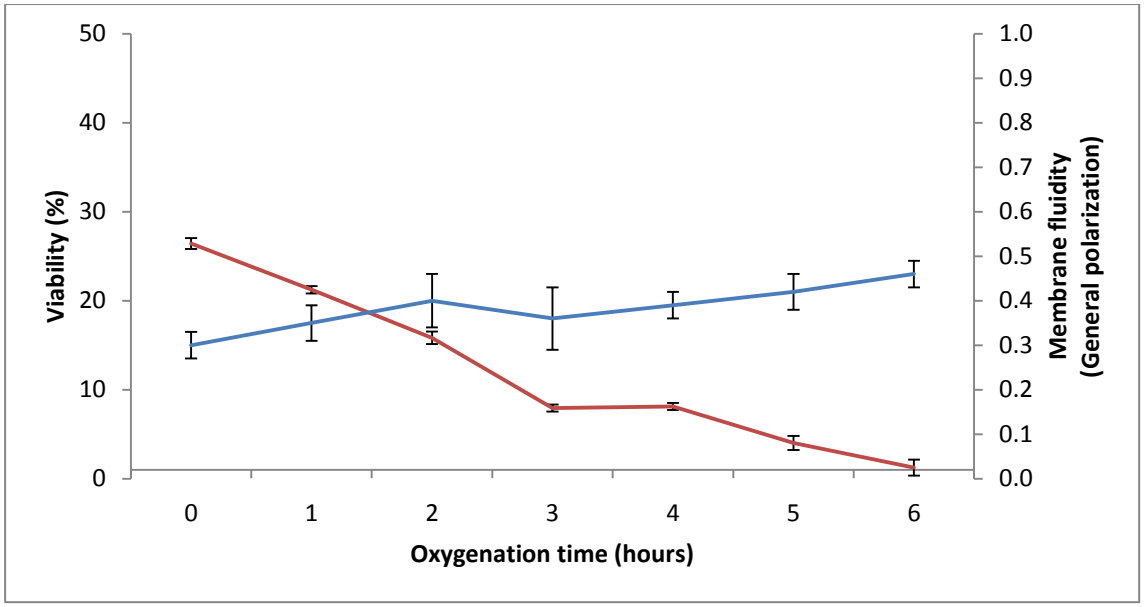


Figure 6.19 The effect of oxygenation time on viability (%) as shown on the primary axis (red), and membrane fluidity (general polarization) as indicated by the secondary axis (blue). Membrane fluidity was determined using the general polarization ratio of Laurdan staining. Data shown represents the mean of triplicate samples with error bars indicating the standard deviation. High GP values indicate low fluidity.

6.3 Discussion

The level of oxygen supplied to the yeast regulates the yeasts ability to synthesise sterols and UFAs, which are the primary lipid components of plasma membranes (Fornairon-Bonneford *et al* 2001; Andreasen & Stier 1954; Andreasen & Stier 1953). It has been stated that oxygenation of yeast prior to fermentation leads to improved fermentation performance (Boulton *et al* 2000). It has also been demonstrated that increased ergosterol leads to an increase in tolerance to a variety of fermentation related stress factors (Hossack and Rose 1977). However it is important to note that there is no clear consensus on the ratio of

ergosterol:phospholipid required for optimum function within the yeast plasma membrane (Patton and Lester 1991; Zinser *et al* 1991; Zinser *et al* 1993; Schneiter *et al* 1999). Lager yeast strain SMCC100 showed increased levels of ergosterol when oxygenated, over a 6 hour time period. Consequently, the hypothesis that an increase in ergosterol content through oxygenation could lead to improved oxidative stress tolerance was investigated. In this instance it was shown that increasing ergosterol does not confer increased tolerance to oxidative stress induced by H_2O_2, and furthermore, a negative impact was observed.

Although ergosterol content was investigated in this study, other sterols, particularly zymosterol, have also been observed to increase during oxygenation (Onyewu *et al* 2003; Agira et al 1977). As well as this, other studies have indicated that under brewery and cider-making conditions, only 20–30% (O'Connor Cox *et al* 1996; Kirsop, 1977) and 10–25% (Nogueira *et al* 2007; Le Quere *et al* 2004), respectively, of the available oxygen was used for sterol synthesis, a further 15% being utilised for unsaturated fatty acid production, with the rest being consumed by reactions such as the oxidation of wort components and CO_2 stripping (Kirsop 1982; Dufore *et al* 1986). Verbelen *et al* (2009) noted an increase in ergosterol content from 0.7 to 1.8mg/g corresponded to preoxygenation of yeast. This was very similar to the preoxygenated samples of this study, where the ergosterol content increased from 0.7-1.8mg/g after 6 hours oxygenation.

The fermentation analysis of these samples indicated that there was no increase in the overall rate of fermentations at standard gravity when increasing oxygenation in yeast. The data did indicate however, that the increased oxygenation could lead to an increase in fermentation rate of

VHG worts. It was observed that the overall fermentation length was reduced by 120 hours. Although a substantial decrease in fermentation time was observed, this also came with a loss of alcohol produced, as the faster fermentations produced 1.83% v/v less. As the standard fermentations produced 6.2% v/v in 120 hours, it could be suggested that the reduction in rate was the more important result in this case, as the slower fermentation took more than double the time of the standard fermentation, but failed to produce double the alcohol. The likely reason for the increased fermentation rate is in response to increased cell division, indicated by the higher cell density at the end of these fermentations. This appears to indicate an alternative pathway for the sugars within the fermentative media, through the diversion of sugars to the oxidative pathway to achieve increased ATP energy yield and biomass formation (Van Hoek *et al* 1998), rather than through the production of acetaldehyde and ethanol. It is generally known that the Crabtree effect however, should inhibit the conversion of glucose to biomass and instead produce ethanol especially under VHG fermentation, although it has been observed that in a high oxygen environment biomass production will occur (Akinyemi *et al* 2005). This is affected by the use of several different metabolic by-passes to channel carbon into the mitochondrion converting cytosolic pyruvate into either acetyl CoA or oxaloacetate (Frick & Wittmann 2005). With an increase in biomass, the rate of fermentation would increase, but the necessary sugars utilised for cell proliferation would lead to the reduction observed.. This indicates that the increased ergosterol content of the yeast led to an increase in cell division. Standard pitching rates for lager fermentations are between 1 and 1.5 x 10^7 cell/mL per °P, intimating that a VHG wort of 22°P would be pitched at 2.2 – 3.3 x 10^8 cell/mL (Edelen *et al* 1996). There have been several studies that looked at increased pitching

rates to improve the fermentability of VHG fermentations with differing levels of success (Kucharczyk & Tuszyński 2015; Verbelen *et al* 2009; Suikho et al 1992; Del Rosario et al 1979).

Unlike the data presented here Verben et al observed no effect on yeast viability through preoxygenation. Here, a reduction in viability and decreases in membrane fluidity were observed mirroring the results of Clarkson et al (1991), who saw a 5-7% decrease in viability. This was of special interest as it was also linked to the shift from an anaerobic (fermentation) environment to an aerobic system (preoxygenation (Clarkson *et al* 1991; Verbelen *et al* 2009). In this study, a correlation was also observed between ergosterol content and decreased membrane fluidity. This was suprising as ergosterol content is normally linked to increasing membrane fluidity (Alexandre et al 1994; Henderson et al 2011; Henderson & Block 2014). One explanation for this could be the upregulation of several important genes linked to lipid metabolism, namely ERG11 and ARE2. (Verbelen *et al* 2009). ERG11 is a member of the p450 pathway, which has been shown to produce ROS, and ARE2 contributes to the major esterification of sterols (Karst & Lacroute 1977; Ness *et al* 1998; Mora *et al* 2012) Fermentations performed utilising the preoxygenated yeast saw decreases in lag phase and overall fermentation length. A marked reduction was observed however, in the ethanoic output. This was found to be the result of a trade off with biomass production which was elevated in the preoxygenated yeast.

Analyisis of the effect of oxygenation indicated that over 90% of cells analysed were seen to have membrane damage, the impact on viability was less than 5%, with the membrane damage showed no observable impact on fermentative ability, as all samples pitched reached attenuation

and produced reasonable levels of alcohol. The use of this yeast in previous VHG fermentations could shed some light on the increased membrane damage. The previous VHG fermentations produced over 10% alcohol v/v which has been documented as a possible cause of the membrane damage observed (Lloyd *et al* 1993; Mishra & Prasad 1988; Jones & Greenfield 1987). Several additional studies have indicated ethanols effect on membrane permeability, can lead to membrane leakage and DNA damage (Gibson *et al* 2009; Jenkins *et al* 2009; Lentini *et al* 2003; Mizoguchi & Hara 1997; Jimenez *et al* 1988; Salgueiro *et al* 1988; Ingram & Buttke 1984; Leao & Van Uden 1984).

When investigating the impact of oxygenation on membrane damage in response to increased oxidative stress, it would be prudent to look at alternative ways in which the membrane can be damaged. Although this study has primarily looked at oxidative stress, increased ethanol levels occur during VHG fermentations and their impact on the plasma membrane must be considered. Ethanol has been shown to have a range of effects on biological membranes even at very low concentrations (Chin & Goldstein 1977), with one of its major effects being its ability to disorder or fluidize membranes (Littleton 1979), and has been observed in previous studies(Bangham and Mason, 1980; Pang *et al* 1979; Curran and Seeman 1979). However, the deleterious effects of ethanol represent one limitation on the productivity of yeast systems which are designed to generate potable or industrial ethanol (Jones & Greenfield 1987). When membrane become more fluid they aquire an enhanced passive permeability of which has been demonstrated by Inoue *et al* (1985), and previous studies confirm that increased membrane permeability can be directly related to increased

average membrane fluidity as ethanol concentration affect its physiological state (Jones & Greenfield 1987).

It has been shown that an increase in osmotic pressure can produce a concomitant decease in yeast viability, growth and fermentation (Pratt *et al* 2003; D'Amore *et al* 1988). Studies have shown that the occurrence of membrane permeabilization seems to be an important event in cell death, survival during dehydration or rehydration performance (Gervais et al. 1992; Marechal and Gervais 1994; Poirier et al. 1997, 1999).

Increases in the osmotic pressure of the medium especially prevalent to VHG fermentations have also been observed to cause damage to membranes, leading to structural modifications, although most were observed using model membrane systems (Swan & Watson 1997; Alexandre *et al* 1994; Chapman 1994; Webb *et al* 1993; Jones and Greenfield 1987).

The aim of this chapter was to analyse the sterol content in re-pitched yeast before, during and after oxygenation. Furthermore, challenging it with oxidative stress in the form of increasing H_2O_2 concentrations to observe if changes to the sterol content would elicit an increase in oxidative stress tolerance. Although an increase in sterol levels and membrane fluidity was observed during increased oxygenation timings, no increase in stress tolerance was observed. Finally the yeast were subjected to standard and VHG fermentations in order to observe the optimal levels of oxygenation for the yeast in both of these environments. It was shown that increasing the ergosterol content to its maximum greatly decreased fermentation length, but impacts overall ethanol yield.

7 Final discussion and future work

The primary role of the yeast culture during fermentation is to convert sugars into alcohol, however as the levels of sugar increase, the ability of the yeast to adapt to the wort environment is critical to creating a quality and consistent product (Lagunas 1993; Gibson *et al* 2006). Under conditions associated with standard gravity (12-15°P), the yeast are expected to produce 4-6% alcohol (v/v), and be re-pitched into multiple fermentations. The move to use very high gravity (VHG) worts (>20°P) where the sugar content of the wort is much higher than normal, is an attractive prospect for a modern brewery. VHG fermentations can lead to increased vessel capacity where output can be optimised and energy, waste, cleaning and labour costs can all be reduced.

In relation to the increase in wort sugars, the level of stress impacting yeast throughout fermentation also increases. In addition, the total metabolic output of the yeast population must increase to exploit the resources available and to maximise ATP production, thus also leading to elevated alcohol concentration (Rodrigues *et al* 1995; Guimarães & Londesborough 2007). With this enhanced metabolic activity, the potential to increase the production of reactive oxygen species (ROS) is also greater. This is likely to occur due to increased carbon flux through 'leaky' pathways where NADPH is utilised, leading to an increase in oxidative stress (Archakov & Buchmanova 1990; Zangar *et al* 2004; Landolfo et al 2008). The aim of this research was to understand the effect of increased wort gravity on lager yeast fermentation performance, physiology and health. This was achieved by investigating the relationship between wort gravity and the integrity of key cellular parameters, as well as intracellular ROS formation and the enzymatic (antioxidant) response. Furthermore, membrane health

and DNA damage were also observed, and the potential for enhancing membrane sterol content as a means of assuring yeast quality was assessed. In this thesis, three lager brewing strains were characterised and assessed for their ability to tolerate VHG conditions (Chapter 3), with the most relevant strain then taken forward and assessed in a series of lab scale fermentations, where fermentation characteristics and oxidative responses were analysed (Chapter 4). A series of oxidative stress experiments were performed on all three strains, observing effects on DNA integrity and the plasma membrane (Chapter 5). Finally an investigation into the effect of increased yeast sterol content on oxidative stress-induced plasma membrane damage was performed (Chapter 6).

Given that yeast are arguably the most important component of beer production, it was important to provide an insight into the ability of each yeast to tolerate a variety of stresses that would occur in a VHG fermentation. The initial investigation (Chapter 3), characterised three commercial yeast strains SMCC100, SMCC90 and SMCC99 as lager strains through permissive growth temperature, with the all strains unable to grow at 37°C. Furthermore it was confirmed with the use of ITS PCR, that all three strains were Saaz type lager yeast. As MtDNA is more susceptible to change than genomic DNA, MtDNA restriction profiles were utilised in an attempt to confirm that all three strains were individual strains. This was unsuccessful however, due to the proposed close relationship of all three strains. Inter-delta PCR of the genomic DNA, however, was able to confirm that all three strains were distinct. Each yeast strain was also assessed for its ability to tolerate stress factors associated with VHG fermentations including increasing glucose and ethanol concentrations, as well as their response to increasing oxidative challenges through H_2O_2 and

menadione. Increasing glucose levels led to an increase in lag phase and a decrease in growth rate, although growth still occurred in 40°P wort media. Yeast were able to tolerate and grow well in media containing ethanol up to 10% v/v, after which increasing the ethanol concentration in liquid media led to a complete inhibition of growth. The tolerance of each strain to oxidative stress was assessed in media containing increasing concentrations of menadione and H_2O_2. Although growth on solid media indicated a reduction in growth levels in the presence of 1mM menadione, growth in liquid media had a limited impact on lager yeast growth even at a concentration of 3mM menadione. H_2O_2 had a marked effect on the lager yeast, with lag phase increasing as H_2O_2 concentrations increased, although there was no lethal effect observed even in the presence of 6mM H_2O_2. These preliminary examinations provided an overview of the phenotypic characteristics of each strain, but were inconclusive in providing a candidate strain of interest to progress with. As such, strain SMCC100 was chosen to move forward with as it was the most commercially relevant of the strains available.

Further analysis of the fermentation characteristics and oxidative stress response of SMCC100 under standard and VHG conditions were then conducted (Chapter 4). Previous studies have looked at the relationship between fermentation media and oxidative stress during propagation and fermentation at standard gravity (Gibson 2008; Lodolo *et al* 2008; Udeh *et al* 2013), but no analysis of yeast at very high gravity has been performed. In order to investigate the effect of VHG fermentation on lager brewing yeast oxidative stress (Chapter 4), a series of 10L small scale fermentations were performed. These fermentations were conducted to analyse three generations of yeast, G0 (propagated), G1 (re-pitched G0)

and G2 (re-pitched G1), in both standard (15°P) and VHG (22°P) wort. G1 and G2 fermentations had almost identical fermentation performance irrespective of the wort gravity, with the G0 fermentations performed less well in all measures of analysis. As expected, VHG fermentations produced increased alcohol concentrations in accordance with starting gravity, however a compromise of almost double the fermentation length was observed. All fermentations were observed to accumulate ROS throughout fermentation, with VHG fermentations indicating higher levels and higher activity of antioxidant biomarkers. Previous studies have mainly focussed on the effects of the wort oxygenation as a potential source of oxidative stress (Gibson *et al* 2008, Verbelen *et al* 2009c), however this data indicates that oxidative stress occurs throughout fermentation and is exacerbated under VHG conditions. The data in Chapter 4 is supported by previous analysis of wine fermentations, indicating that although a distinctly anaerobic process, ROS can be generated and accumulated during fermentation (Landolfo *et al* 2008). Although wine and beer fermentations are distinct from one another, in terms of sugar content they both have a similarly high sugar content (>20°P) (Laopaiboon *et al* 2009; Landolfo *et al* 2008; Thomas & Ingledew 1992).

Based on the levels of ROS accumulation observed in Chapter 4, a system was developed to create a similar environment *in vitro*. Three lager strains were challenged with oxidative stress induced by H_2O_2 to determine the direct impact on cell physiology, specifically focused on membrane and DNA damage, and yeast viability (Chapter 5). There were no observable change to either genomic or MtDNA under oxidative challenge, however it is important to note that small changes to the genome may remain undetected. In addition, the artificial environment created does not

perfectly replicate a fermentation environment; stress was provided in a short burst rather than over an extended period of time. This has the benefit of allowing the absolute impact of ROS to be observed, but does not provide information on damage over time, or of the ability of cells to actively respond to the stress. Analysis of the impact of ROS on the plasma membrane revealed that damage occurred in conjunction with increasing oxidative stress. This trend was mirrored by a decrease in membrane fluidity, another important measure of membrane health. At high levels of oxidative stress (10mM H_2O_2) cell populations were killed with less than 1% of the population surviving. This was associated with mtDNA degradation and membrane collapse, although genomic DNA remained unchanged according to the method applied. These data indicate that there is a limit to the amount of oxidative stress that lager yeast are able to tolerate. While the analyses represent a single period of stress, the impacts of such stress can be extrapolated and it would be expected that over the course of serial repitching of yeast used for VHG fermentation, there may be gradual deterioration of the membrane over time, leading to lost energy for repair and maintenance, or ultimately in cell death. It should also be recognised that yeast do not encounter stress in isolation. Combining any affects with other stress factors may lead to the pressure on the plasma membrane being greatly increased. For example, it has been hypothesised that increasing ethanol stress can also act as a contributor towards membrane damage (Chi & Arneborg 1999; Sajbidor *et al* 1995; Alexandre *et al* 1994; Mishra & Prasad 1989; Ghareib *et al* 1988; Beaven *et al* 1982; Ingram 1976). Regardless, in this study we demonstrate that yeast cultures used in VHG fermentations may not always be able to perform at an optimum level due to a major contribution from oxidative stress. This results in a deterioration in quality, which is

potentially an important reason as to why VHG yeast cannot be repitched as many times as yeast used in standard gravity fermentations (Thomas *et al* 1994; Jones *et al* 2007; Puligundla *et al* 2011).

As alluded to above, the ability of a yeast population to withstand stress over time is related to a complex array of parameters. For example, it has been demonstrated that the number of repitchings that lager yeast cultures can tolerate is influenced by its replicative lifespan, which may in itself be determined by a strain's antioxidant potential and sensitivity to oxidative stress (Maskell *et al* 2003). In addition, there are a huge number of additional stress factors which may play an important role during brewing fermentations, and a similarly extensive range of stress response mechanisms will enable the cell to react to its environment. However, one of the major sites for oxidative stress damage is the cell membrane. This structure is integral to yeast function as the primary barrier between the cell and its environment and for uptake and regulation of molecules. The cell membrane is mainly composed of phospholipids interspersed with proteins and sterols (mainly ergosterol), which procure structural confirmation and fluidity. It has been observed that yeast mutants unable to produce ergosterol are hypersensitive to oxidative stress (Higgins *et al* 2003), and increasing ergosterol content can increase the ability of yeast to tolerate other fermentation stresses, namely ethanol stress (Thomas *et al* 1978; You *et al* 2003; Shobayashi *et al* 2005). Consequently, a further investigation of oxygenation, yeast sterol synthesis and oxidative stress tolerance was performed (Chapter 6). In order to achieve this a sterol-deficient yeast slurry was oxygenated over a 6 hour period to promote synthesis of ergosterol. It was observed that the sterol content of cells increased with oxygenation time, allowing samples to be taken

representing yeast with different sterol contents. These populations were pitched into small scale fermentations to observe the impact of sterols on fermentation characteristics. It was noted that although the lag phases decreased as the sterol content increased, there was a trade off with ethanol concentration and biomass production. The same populations were also challenged with increasing concentrations of H_2O_2 to determine the impact of sterols on tolerance to oxidative stress. The effect of increasing the sterol content led to a decrease in oxidative stress tolerance under the conditions applied. This was characterised by an increase in membrane damage as well as a decrease in membrane fluidity. The reasons for this are unknown however it is suggested that the ratio of ergosterol to the phospholipids varies within strains, and as such has been observed to be much lower in some strains (Zinser *et al* 1991; Patton & Lester 1991; Zinser *et al* 1993; Schneiter *et al* 1999). If this were the case here then it could help to explain that although ergosterol content rose, the level of phospholipids was much higher. Poly-unsaturated fatty acids are much more susceptible to oxidative stress, leading to lipid peroxidation and a decrease in membrane fluidity (Catala 2009; Catala 2012). Other possibilities include the increased ethanoic stress that occurred in the previous fermentations. Ethanoic stress has also been known to elicit a negative effect on the yeast membrane and cause decreases in membrane fluidity and membrane damage (Ingram 1976; Sajbidor *et al* 1995; Chi & Arneborg 1999). These fermentations were performed at >20°P, and as such multiple high level interactions between the yeast and high ethanol concentrations occurred. It is therefore possible that a better base yeast culture could be used before any fermentation stresses occur and confirm the results of Higgins *et al* (2003) inferring yeast with a higher tolerance to oxidative stress through elevation of ergosterol content.

Despite the advantages offered by VHG brewing, there are many issues associated with beer production using this system. These range from brewhouse efficiency demands through to fermentation related parameters. For the latter, yeast health remains a key aspect, both during fermentation and to ensure yeast quality during serial repitching. Consequently, ensuring yeast health is of paramount importance. However, as VHG brewing is a relatively recent concept, it is important to consider that current lager brewing strains may not be optimal for such environments, especially since they were originally selected for their ability at lower gravities. As such, other strains that are more tolerant to the VHG environment should be investigated. Despite this acknowledgment, the current study will give an insight into the effect of oxidative stress on yeast during fermentation, and lead to further optimisation of VHG brewing in the future.

7.1 Future Work

There are several areas of research which have the potential to both complement and expand on the data presented here:

(I)The expansion of the number and types of strains such as bio-ethanol and distilling strains, could be included in this study, and could potentially lead to a better overall understanding of the optimal yeast for VHG fermentations as a whole.

(II) High levels of glutathione (GSH) in yeast are important for surviving peroxide stress (Spector et al 2001). Glutathione has been shown to have a large effect on REDOX potential in cells and as such, analysis of glutathione (GSH) levels throughout fermentation could expand on the

understanding of the yeast antioxidant response during VHG lager fermentation.

(III) In order to look at changes in the transcription level, analysis using GeneChip hybridization and scanning (Affymetrix GeneChip) against the Yeast Genome 2.0 GeneChips array. In conjunction with the use of alternative quantitative gene methodologies further insight into the cells response to oxidative stress during VHG fermentations could be attained. The analysis of stress-responsive genes using quantitative real-time PCR could help to understand the links between gene expression and oxidative stress throughout fermentation, such as SOD1, SOD2 , MHR1, HAA1 and GPX1, GPX2, GPX6. Other genes linked with the stress response element (STRE) would also be of particular interest, such as HSP104, CTT1, MSN2/MSN4, and TFS1 The expression levels of these genes and the concentrations of their cellular compounds could lead to a better understanding of the stress response to VHG brewing

(IV) Protein targets of ROS could also be included, such as the mitochondrial citric acid cycle proteins, α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and the iron-sulfur [4Fe-4S] cluster enzymes aconitase and succinate dehydrogenase, readily inactivated by H_2O_2 and by menadione-derived superoxide radicals (Cabiscol *et al* 2000; Cecarini *et al* 2007; Farrugia & Balzan 2012). A focussed analysis of these proteins throughout fermentation could give a clear indication that oxidative stress does occur and a specific oxidative stress response is present.

(VI) Gibson et al (2008) have already looked at the oxidative stresses occurring during industrial propagation and the start of standard gravity fermentations (17°P). Landolfo et al (2008) have also sampled throughout

wine fermentations observing free radical accumulation and protein catabolism. Further study of large scale (>500hL) lager fermentations at higher gravities would increase our understanding of the effects of oxidative stress under conditions associated with concentrated worts, as well as giving an insight into how the oxidative stress affects the real world challenges of large scale fermentation.

(VII) Several studies have indicated that brewing yeast strains have an individual requirement for oxygen, potentially allowing for classification of strains based on this (David & Kirsop 1972; Kirsop 1974; Baker 1977; Jakobsen & Thorne 1980). Indeed, Jakobsen & Thorne (1980) were able to categorise a series of lager strains based on their oxygen requirements. It would be interesting to make a detailed comparison of strains based on their oxygen requirements and applying some of the techniques employed within this thesis to assess oxidative stress related phenomena. It is anticipated that further investigation of the oxygen requirements of a range of modern lager brewing yeast strains at VHG, in addition to other industrial strains, could further assist in understanding of yeasts interaction with oxygen.

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